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Phagocytosis of Apoptotic Cells by Neutrophil Granulocytes: Diminished Proinflammatory Neutrophil Functions in the Presence of Apoptotic Cells

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Neutrophil granulocytes are rapidly recruited from the bloodstream to the site of acute inflammation where they die in large numbers. Because release of toxic substances from dead neutrophils can propagate the inflammatory response leading to tissue destruction, clearance of dying inflammatory neutrophils has a critical function in the resolution of the inflammatory response. Apoptotic neutrophils are phagocytosed primarily by macrophages, provided these cells are present in adequate numbers. However, macrophages are rare at sites of acute inflammation, whereas the number of neutrophils can be extremely high. In the current study, *in vitro* experiments with human neutrophils were carried out to investigate whether neutrophils can ingest apoptotic neutrophils. We show that naïve granulocytes isolated from venous blood have a limited capacity to phagocytose apoptotic cells. However, exposure to activating stimuli such as LPS, GM-CSF and/or IFN- γ results in enhanced phagocytosis of apoptotic cells. The efficient uptake of apoptotic cells by neutrophils was found to depend on the presence of heat labile serum factors. Importantly, the contact to or uptake of apoptotic cells inhibited neutrophil functions such as respiratory burst and the release of the proinflammatory cytokines TNF- α and interferon-inducible protein-10. Contact to apoptotic cells, however, induced the secretion of IL-8 and growth-related oncogene- α , which was independent of NF- κ B and p38 MAPK but involved C5a and the ERK1/2 pathway. The data suggest that activated neutrophils participate in the clearance of apoptotic cells. In addition, because apoptotic cells inhibit proinflammatory functions of neutrophils, uptake of apoptotic cells by neutrophils contributes to the resolution of inflammation. *The Journal of Immunology*, 2010, 184: 391–400.

Large numbers of polymorphonuclear neutrophil (PMN) granulocytes are rapidly recruited from the bloodstream to the site of infection or injury via transmigration through the vascular endothelium. Neutrophils constitute the “first line of defense” and are considered as primary effector cells in infection-induced acute inflammatory reactions where they serve to destroy invading pathogens (1). Neutrophils are inherently short-lived cells with a half life of only \sim 6–10 h in the circulation and rapidly undergo spontaneous apoptosis (2). In infected tissues their apoptosis

can be delayed both by microbial constituents and by proinflammatory stimuli (3, 4). Finally, however, tissue neutrophils die in large numbers. Because uncontrolled release of toxic substances from dead neutrophils can propagate the inflammatory response leading to tissue destruction, recognition of dying inflammatory neutrophils has a critical function for the resolution of the inflammatory response (5, 6). It leads not only to the removal of the inflammatory cells themselves, along with anything they have ingested, but also to the generation of anti-inflammatory mediators that shut down the ongoing inflammation (7).

Although it is known that apoptotic inflammatory neutrophils can be phagocytosed by macrophages (MFs) (5, 8), MFs are rare at sites of acute inflammation where the number of neutrophils within the tissue can be extremely high. Therefore, it is quite reasonable to hypothesize that nonapoptotic neutrophils are able to phagocytose apoptotic cells. In a recent study, neutrophils with ingested apoptotic neutrophils were seen in inflamed tissue after administration of LPS suggesting that neutrophils themselves, as a form of cellular “cannibalism,” participate in the clearance of aged neutrophils (9). In our own laboratory, in cultures of *Chlamydia*-infected neutrophils, we observed that neutrophils can phagocytose apoptotic neutrophils *in vitro*. Based on these findings, we investigated whether neutrophils can efficiently phagocytose apoptotic neutrophils. The data obtained suggest that human neutrophils exposed to proinflammatory stimuli, such as bacterial LPS and/or proinflammatory cytokines, become potent effector cells for the phagocytosis of apoptotic neutrophils. This process requires the presence of heat-labile serum factors. Moreover, we show that apoptotic cells inhibit the oxidative burst and the release of proinflammatory cytokines TNF- α and interferon-inducible protein-10 (IP-10), whereas the release of IL-8 and growth-related oncogene- α (GRO- α) is enhanced on contact to apoptotic cells.

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Abbreviations used in this paper: C5aRA, C5a receptor antagonist; DC, dendritic cells; DHR, dihydrorhodamine; iDC, immature dendritic cells; GRO- α , growth-related oncogene- α ; IP-10, interferon-inducible protein-10; NHS, normal human serum; MF, macrophage; PI, propidium iodide; PMN, polymorphonuclear neutrophil; ROS, reactive oxygen species.

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Materials and Methods

Isolation and culture of human peripheral blood neutrophil granulocytes

Peripheral blood was collected by venipuncture from healthy adult volunteers using lithium-heparin and neutrophils were isolated as described previously (10). Blood was layered on a two-layer density gradient consisting of lymphocyte separation medium 1077 (upper layer, PAA, Pasching, Austria) and Histopaque 1119 (bottom layer, Sigma-Aldrich, Deisenhofen, Germany) and centrifuged for 5 min at $300 \times g$, followed by 20 min at $800 \times g$. PBMCs from the upper layer consisting mainly of lymphocytes and monocytes were discarded. The granulocyte-rich lower layer was collected, leaving the erythrocyte pellet at the bottom of the tube. Granulocytes were washed once in PBS, resuspended in complete medium (RPMI 1640 medium) (Sigma-Aldrich) supplemented with $50 \mu\text{M}$ 2-mercaptoethanol, 2 mM L-glutamine, 10 mM HEPES (all from Biochrom, Berlin, Germany), and 10% heat-inactivated FCS (Sigma-Aldrich) and were further fractionated on a discontinuous Percoll (Amersham Biosciences, Uppsala, Sweden) gradient consisting of layers with densities of 1.105 g/ml (85%), 1.100 g/ml (80%), 1.087 g/ml (70%), and 1.081 g/ml (65%). After centrifugation for 20 min at $800 \times g$, the interface between the 80% and 70% Percoll layers was collected, washed once in PBS, and resuspended in complete medium. All procedures were conducted at room temperature. The cell preparations contained $>99.9\%$ granulocytes as determined by morphological examination of >1000 cells on Giemsa-stained cytocentrifuge (Shandon, Pittsburgh, PA) slides (11). Cell viability was $>99\%$, as determined by trypan blue exclusion. Infection of neutrophils with *Chlamydia pneumoniae* was carried out as described previously (12).

Generation of MFs and monocyte-derived immature dendritic cells

Monocytes were isolated from PBMCs by using the CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were allowed to mature into MFs over a 7-d period in RPMI 1640 containing 10% heat-inactivated FCS, supplemented with 5 ng/ml M-CSF (PeproTech, Rocky Hill, NJ). Monocyte-derived immature dendritic cells (iDC) were generated by culturing monocytes in RPMI 1640 with 10% heat-inactivated FCS, 5 ng/ml GM-CSF, and 5 ng/ml IL-4 (PeproTech) for 7 d. At day 7, $>90\%$ of the cells were CD14⁺CD83⁻HLA-DR^{low} DC.

Generation of apoptotic cells

To facilitate neutrophil apoptosis, $350 \mu\text{l}$ of the freshly isolated neutrophils were irradiated with 256 nm wavelength UV light ($200\text{--}1000 \text{ mJ/cm}^2$) using a Stratalinker (Stratagene, Heidelberg, Germany). Subsequently, irradiated cells were incubated in complete medium for 4 h at 37°C . Double staining with annexin A5-Fluos (Roche Molecular Biologicals, Mannheim, Germany) and propidium iodide (PI, Sigma-Aldrich) was used to assess apoptosis and necrosis of the irradiated cells, respectively. Irradiation with 200 mJ/cm^2 UV light led to the generation of cells in early stage of apoptosis, $>80\%$ of these cells were annexin A5-positive and $<5\%$ were PI-positive necrotic (Fig. 1). This irradiation protocol was used in all subsequent experiments to generate UV-induced early apoptotic neutrophils.

To obtain apoptotic thymocytes, single-cell suspension of thymus cells from 8-wk-old BALB/c mice were cultured at the concentration of $1 \times 10^6/\text{ml}$ in RPMI 1640 medium containing 10% FCS and $1 \mu\text{M}$ dexamethasone (Sigma-Aldrich) overnight.

Fluorescent labeling of cells

The fluorescent membrane stains PKH-26 (red) and PKH-67 (green) (both Sigma-Aldrich) were used to label freshly isolated and apoptotic neutrophils, respectively. Cells (15×10^6) were washed in serum-free RPMI 1640 medium, the pellet was resuspended in 0.5 ml $2.3 \times 10^{-6} \text{ M}$ PKH-26 or in $5 \times 10^{-7} \text{ M}$ PKH-67 staining solution and incubated for 5 min at room temperature. After stopping the labeling by adding 0.5 ml FCS, cells were washed three times with complete medium.

Assays for phagocytosis of apoptotic cells

Phagocytosis in whole blood. PKH-26-labeled autologous apoptotic cells (2×10^6) in $100 \mu\text{l}$ medium were added to $100 \mu\text{l}$ heparinized whole blood and incubated for 90 min at 37°C in a humidified atmosphere containing 5% CO_2 . Subsequently, erythrocytes were lysed by using FACS-Lyse solution (BD Biosciences, Heidelberg, Germany), the leukocytes stained with FITC-conjugated anti-CD14 mAb (Dako, Hamburg, Germany), and analyzed by flow cytometry using a FACS-Calibur with CellQuest Pro software (BD Biosciences). In some experiments, instead of apoptotic neutrophils, 2×10^6 PKH-26-labeled freshly isolated nonapoptotic neutrophils were used as target cells in phagocytosis assays (Fig. 3E).

Phagocytosis assay using isolated neutrophils. Apoptotic neutrophils were labeled with PKH-26 and used as target cells in the phagocytosis assay. Because apoptotic cells were obtained after 4 h of incubation, the autologous effector neutrophils were freshly isolated from the same donor to avoid using senescent effector cells. Blood was taken twice from the blood donor, once to obtain apoptotic neutrophils, and, 4 h later, to isolate fresh effector neutrophils. Freshly isolated neutrophils were labeled with the green fluorescent dye PKH-67 and used as effector cells in the phagocytosis assay. For phagocytosis, 5×10^5 effector neutrophils were coincubated with 2×10^6 apoptotic neutrophils at 37°C in RPMI 1640 medium without serum or supplemented with 10% FCS, various concentrations of fresh autologous serum (normal human serum [NHS]), or heat inactivated (56°C , 30 min) autologous serum (hi-serum). Phagocytosis was assessed by fluorescence microscopy (Axioskop 40, Zeiss, Jena, Germany), confocal laser scanning microscopy (LSM, Zeiss), and by flow cytometry. In some experiments, before adding to the apoptotic cells, neutrophils were cultured for 60 min with various combinations of 100 ng/ml rhM-CSF (PeproTech), 100 ng/ml rhGM-CSF (PeproTech), 100 ng/ml LPS (*Escherichia coli* 0111:B4, Sigma-Aldrich), and 100 U/ml IFN- γ (PeproTech) or in medium alone as control. To inhibit phagocytosis, the coculture of apoptotic and nonapoptotic neutrophils was carried out in the presence of $10 \mu\text{g/ml}$ cytochalasin D (Sigma-Aldrich).

For phagocytosis of apoptotic thymocytes, 5×10^5 effector neutrophils were coincubated with 2×10^6 apoptotic neutrophils at 37°C in RPMI 1640 medium, supplemented with 30% NHS. Phagocytosis was assessed by light microscopy in Giemsa-stained cytocentrifuge (Shandon) slides. **Phagocytosis of apoptotic neutrophils labeled with a pH sensitive dye.** Apoptotic neutrophils were labeled with the pH sensitive stain pHrodo-SE (Invitrogen, Paisley, U.K.), which emits strong red (532 nm) fluorescence in

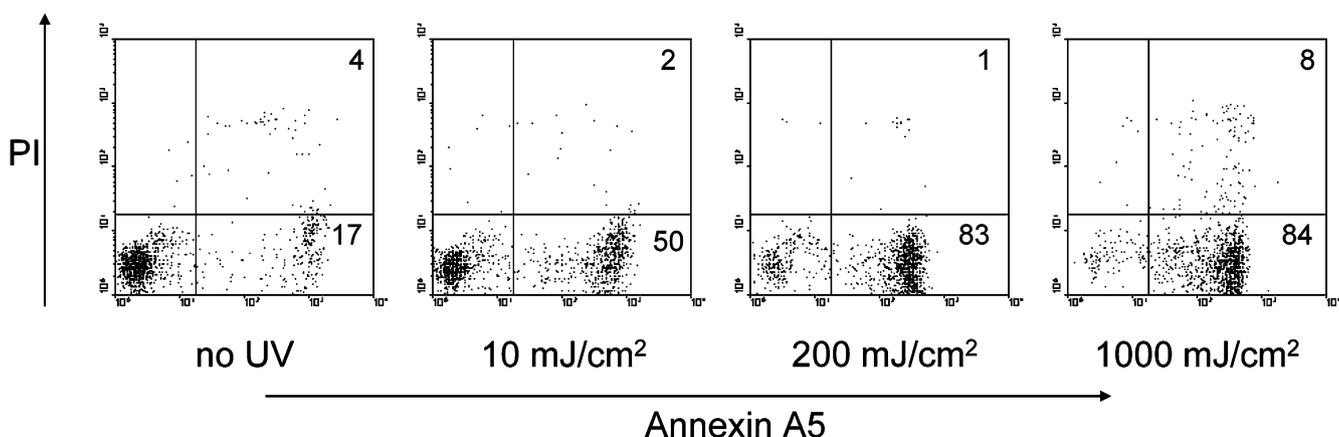


FIGURE 1. Generation of early apoptotic neutrophils by using UV irradiation. Freshly isolated neutrophils were exposed to different doses of UV irradiation and incubated in complete medium for 4 h at 37°C . Double staining with annexin A5-Fluos and PI was used to assess apoptosis and necrosis, respectively, and analyzed by flow cytometry. The numbers indicate the ratio (%) of cells in the given quadrant.

an acidic (pH 4–6) environment and is nonfluorescent at neutral pH. This technique was applied in a recent study to show the engulfment of apoptotic cells by MFs (13). Apoptotic neutrophils (25×10^6) in 25 ml PBS were incubated with 20 ng/ml pHrodo-SE at room temperature for 30 min. After washing, labeled apoptotic cells were cocultured with autologous nonapoptotic neutrophils at a ratio of 4:1 in culture medium containing 30% autologous serum, 100 ng/ml LPS, and 100 U/ml IFN- γ . Cyto-centrifuge slides were prepared immediately after mixing the two cell populations or after 90 min of coculture, mounted, and photographed under a fluorescent microscope or with a laser scanning microscope.

Phagocytosis of apoptotic neutrophils by MFs and iDC. For phagocytosis, 5×10^5 PKH-67–labeled MFs or iDC were coincubated with 2×10^6 autologous apoptotic neutrophils for 90 min at 37°C in RPMI 1640 medium, supplemented either with 10% FCS or with 30% NHS with or without 100 ng/ml LPS (*E. coli* 0111:B4, Sigma-Aldrich).

Assessment of neutrophil cytokine release

To investigate the effect of apoptotic cells on the cytokine secretion of neutrophils, freshly isolated neutrophils (1×10^6 /ml) were coincubated with 2×10^6 /ml UV-irradiated autologous apoptotic neutrophils for 18 h in RPMI 1640 medium containing either 10% FCS or 30% NHS. For stimulation of cytokine release, the cells were exposed to various combinations of 100 ng/ml LPS, 500 U/ml IFN- γ , and 100 U/ml GM-CSF. Cell-free supernatants from neutrophil cultures were collected and stored at -20°C until cytokine determination and were measured using ELISA according to the manufacturer's instructions, TNF- α (OptEIA ELISA set, BD Biosciences), IP-10 and IL-8 (ELISA, CytoSets, Biosource, Camarillo, CA), and GRO- α (R&D Systems, Wiesbaden, Germany).

In some experiments, prior to coculture with UV-irradiated autologous apoptotic neutrophils, freshly isolated neutrophils were preincubated for 15 min in the presence of the inhibitors of certain signaling pathways. Wedelolactone (Calbiochem, San Diego, CA) was used at a concentration of 25 μM , SB203580 (Calbiochem), and U0126 (Cell Signaling Technology, Beverly MA) at a concentration of 10 μM . The C5a receptor antagonist (C5aRA, A8 Δ^{71-73}) (14) was used at a concentration of 10 μM . The inhibitors and C5aRA were present in the culture medium (RPMI 1640 medium containing 30% NHS) during the subsequent 5 h coincubation. The inhibitors at the applied concentrations did not affect neutrophil apoptosis as detected by annexin-V binding and had no toxic effect as measured by trypan blue and PI exclusion (not shown).

Oxidative burst assay

The intracellular production of reactive oxygen species (ROS) was assayed by using the substrate dihydrorhodamine 123 (DHR; Invitrogen), which is fluorescent on interaction with ROS. Neutrophils (2×10^5) in 100 μl complete medium were pretreated with 100 ng/ml LPS (Sigma-Aldrich) for 1 h at 37°C in the presence or absence of 2.5×10^5 autologous apoptotic cells. Subsequently, 1 μM DHR was added for 5 min, followed by stimulation with 1 μM fMLP (Sigma-Aldrich) for 5 min at 37°C. The reaction was stopped on ice, and the fluorescence intensity of the cells was analyzed immediately by flow cytometry using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences).

Electron microscopy

Freshly isolated and apoptotic neutrophils as well as neutrophils from *C. pneumoniae*-infected cultures (12) were fixed with 5% glutaraldehyde for 1 h, treated with 1% OsO₄ for 2 h, and dehydrated in ethanol. The samples were embedded in Araldite (Fluka, Buchs, Switzerland), ultra-thin sections were contrasted with uranyl acetate and lead citrate, and were examined with a Philips EM 400 (Eindhoven, The Netherlands) electron microscope.

Statistical analysis

Data are presented as mean \pm SD and analyzed by using the Student *t* test. Differences were considered statistically significant at $p < 0.05$ and are indicated with an asterisk.

Results

Neutrophils phagocytose apoptotic cells

In a previous study, we observed that infection with the Gram-negative bacterium *C. pneumoniae* inhibits the spontaneous apoptosis of neutrophil granulocytes. In infected cultures, $>50\%$ of the neutrophils were nonapoptotic, whereas in the noninfected cultures nearly all neutrophils ($>95\%$) were apoptotic after 48 h of culture

(12). Electron-microscopical study of neutrophil cultures 48 h after *C. pneumoniae*-infection resulted in an unexpected observation. In an electron micrograph, a nonapoptotic neutrophil was seen in the process of ingesting an apoptotic cell (Fig. 2A). In this micrograph both the intact morphology and segmented nucleus of the nonapoptotic neutrophil as well as the condensed chromatin and aberrant morphology of the apoptotic or already dead cells are clearly visible.

This observation indicated that neutrophils have the capacity to ingest apoptotic neutrophils. This finding led us to investigate, in more general term, the ability of neutrophils to phagocytose apoptotic cells.

Experiments using whole blood and autologous UV-irradiated neutrophils were carried out to investigate the uptake of apoptotic cells by human neutrophils. By applying flow cytometry, this assay enabled us the simultaneous assessment of phagocytic capacity of various leukocyte populations such as lymphocytes (region *R1* in Fig. 3A), monocytes (region *R2* in Fig. 3A), and granulocytes (region *R3* in Fig. 3A). Staining with FITC-conjugated anti-CD14 mAb provided additional proof that cells in *R2* region are monocytes because they express CD14 at a high level (Fig. 3C), cells in region *R3* are granulocytes with markedly lower level of CD14 expression (Fig. 3D, 3E), whereas lymphocytes in region *R1* are CD14 negative (Fig. 3B). As expected, lymphocytes did not phagocytose apoptotic neutrophils (Fig. 3B). A population of monocytes acquired red fluorescence indicating that these cells ingested apoptotic neutrophils (Fig. 3C). Importantly, not only monocytes but also a significant ratio of neutrophils phagocytosed apoptotic neutrophils (Fig. 3D). This finding supports the electron microscopic finding (Fig. 2A) that neutrophils are able to phagocytose apoptotic cells. We could also show that only apoptotic, but not nonapoptotic neutrophils, were ingested by blood neutrophils (Fig. 3E).

In subsequent experiments, highly purified human neutrophils were used as effector cells in phagocytosis assays. The ultrastructure of fresh neutrophils and early apoptotic neutrophils in the coculture is shown in Supplemental Fig. 2. Freshly isolated neutrophils showed only low phagocytic capacity (Fig. 4A), which was markedly lower than the phagocytosis rate observed in whole blood (Fig. 3D). This low phagocytic capacity was, however, not caused by a general low function of the purified neutrophils because these cells readily phagocytosed bacteria (Supplemental Fig. 1). However, there was a major difference in the culture conditions as compared with the whole blood assay, where the phagocytosis took place in the presence of 50% plasma. The experiments with isolated neutrophils (Fig. 4A) were carried out in cell culture medium with 10% heat-inactivated FCS. After adding fresh serum into the culture medium, the phagocytosis rate increased to the level observed in whole blood (Fig. 4B). However, adding heat-inactivated serum did not enhance the phagocytosis rate (Fig. 4C).

To discriminate the cells phagocytosing apoptotic cells from those just adherent to but not ingesting, phagocytosis was inhibited in the coculture experiments with cytochalasin D. Cytochalasin D treatment reduced the ratio of fresh neutrophils with red fluorescence (double positive cells) by $>80\%$ (Fig. 4E, 4F) indicating that the red fluorescence is a result of phagocytosis rather than just adherence.

To investigate whether neutrophils, in addition to apoptotic neutrophils, can phagocytose other types of apoptotic cells, neutrophils were coincubated with apoptotic thymocytes. Neutrophils ingested apoptotic thymocytes as detected by microscopical examination of apoptotic nuclei inside neutrophils (Fig. 2I, 2J).

These observations suggest that human neutrophils are able to ingest apoptotic cells more efficiently in the presence of heat labile serum factors. In subsequent experiments phagocytosis of apoptotic cells by neutrophils was investigated in culture medium containing 30–50% fresh autologous serum.

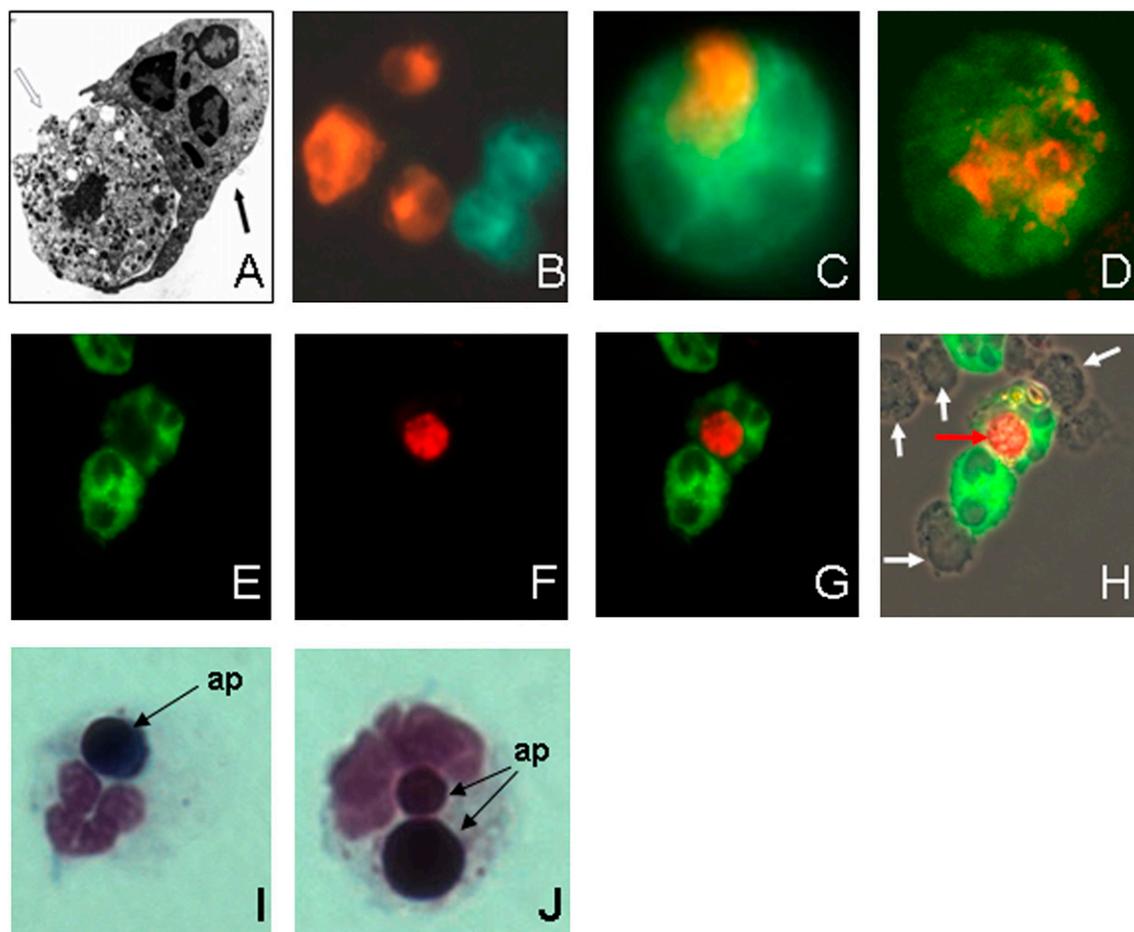


FIGURE 2. Neutrophils phagocytose apoptotic neutrophils. *A*, Electronmicrograph of cells from *in vitro* culture of neutrophils 48 h postinfection with *C. pneumoniae*. An intact neutrophil with segmented nucleus (black arrow) and an apoptotic/dead cell with condensed chromatin and aberrant morphology (white arrow) are visible. *B–D*, Uptake of PKH-26–labeled (red) apoptotic neutrophils by PKH-67–labeled (green) fresh neutrophils. *B*, PKH-67–labeled (green) fresh neutrophils with PKH-26–labeled (red) apoptotic neutrophils at the start of coculture. *C–D*, Neutrophils (green) containing apoptotic material (red) after 90 min coculture with red-labeled apoptotic neutrophils in culture medium containing 50% fresh autologous serum and 100 ng/ml LPS. *E–H*, Uptake of a pHrodo-SE–labeled apoptotic neutrophil by a neutrophil granulocyte. PKH-67–labeled freshly isolated neutrophils were cocultured for 90 min with pHrodo-SE–labeled apoptotic neutrophils in culture medium containing 30% fresh human serum and 100 ng/ml LPS; (*E*) green fluorescence (excitation 488 nm, emission detected through a 515–585-nm bandpass filter); (*F*) red fluorescence (excitation 546 nm, emission detected through a 590-nm longpass filter), (*G*) overlay of green and red fluorescence, (*H*) overlay of green and red fluorescence with the bright field images. The red color of the cell inside of a fresh neutrophil (red arrow) indicates that the pHrodo-SE–labeled apoptotic neutrophil is located in an acidified environment. Noningested pHrodo-SE–labeled apoptotic neutrophils are non-fluorescent (black arrows). *I–J*, Uptake of apoptotic thymocytes by fresh neutrophils. Freshly isolated neutrophils were cocultured for 90 min with apoptotic thymocytes in culture medium containing 30% fresh human serum. The arrows show apoptotic nuclei (ap) inside Giemsa-stained neutrophils. Photographs taken with a fluorescence microscope (*B, C, E–H*) or confocal laser scanning microscope (*D*) or by brightfield microscopy (*I, J*). Magnification $\times 630$ (*B–J*).

LPS, IFN- γ , and GM-CSF enhance the capacity of neutrophils to phagocytose apoptotic cells

According to a previous finding (9), neutrophils phagocytose apoptotic cells in infected tissues where they are exposed to microbial constituents as well as to proinflammatory cytokines. Moreover, neutrophils from GM-CSF–treated renal carcinoma patients were also shown to be able to phagocytose apoptotic cells (15). Therefore, we assessed the ability of isolated human neutrophils to ingest apoptotic cells after exposure to the proinflammatory stimuli LPS, IFN- γ , and GM-CSF. All of these treatments enhanced significantly the capacity of freshly isolated neutrophils to ingest apoptotic cells (Figs. 4*D, 5*). Although the combination of LPS and IFN- γ as well as IFN- γ and GM-CSF had a tendency to further increase the phagocytic capacity (Fig. 5), these differences, as compared with LPS or IFN- γ alone, were not statistically significant. M-CSF, a cytokine lacking activating effects on neutrophils, however, did not enhance their capacity to internalize apoptotic cells (Fig. 5).

Although the electron micrograph in Fig. 2*A* and the uptake of apoptotic thymocytes (Fig. 2*I, 2J*) suggested that neutrophils are

able to phagocytose whole apoptotic cells, investigations by applying fluorescence microscopy showed that neutrophils possibly also phagocytosed apoptotic material smaller than a whole cell (Fig. 2*C, 2D*, Supplemental Fig. 4). Flow cytometry analysis of neutrophil-apoptotic cell cocultures also showed that the fluorescence intensity of most neutrophils that ingested apoptotic cells (upper right quadrants in Fig. 4) did not reach the fluorescence intensity equivalent to whole apoptotic cells. The reason for that may be that parts of the engulfed apoptotic cells have been digested during the 90-min duration of the phagocytosis assay. However, neutrophils may ingest also apoptotic bleb-like material.

Because it is well established that MFs and dendritic cells (DC) ingest apoptotic cells, in subsequent experiments the phagocytosis of apoptotic neutrophils by neutrophils was compared with that of by MFs and DC. Although all three cell types ingested apoptotic neutrophils (Fig. 6), some major differences could be observed. Whereas neutrophils required the presence of fresh serum (Fig. 6*A, 6B*), MFs phagocytosed apoptotic neutrophils in culture medium

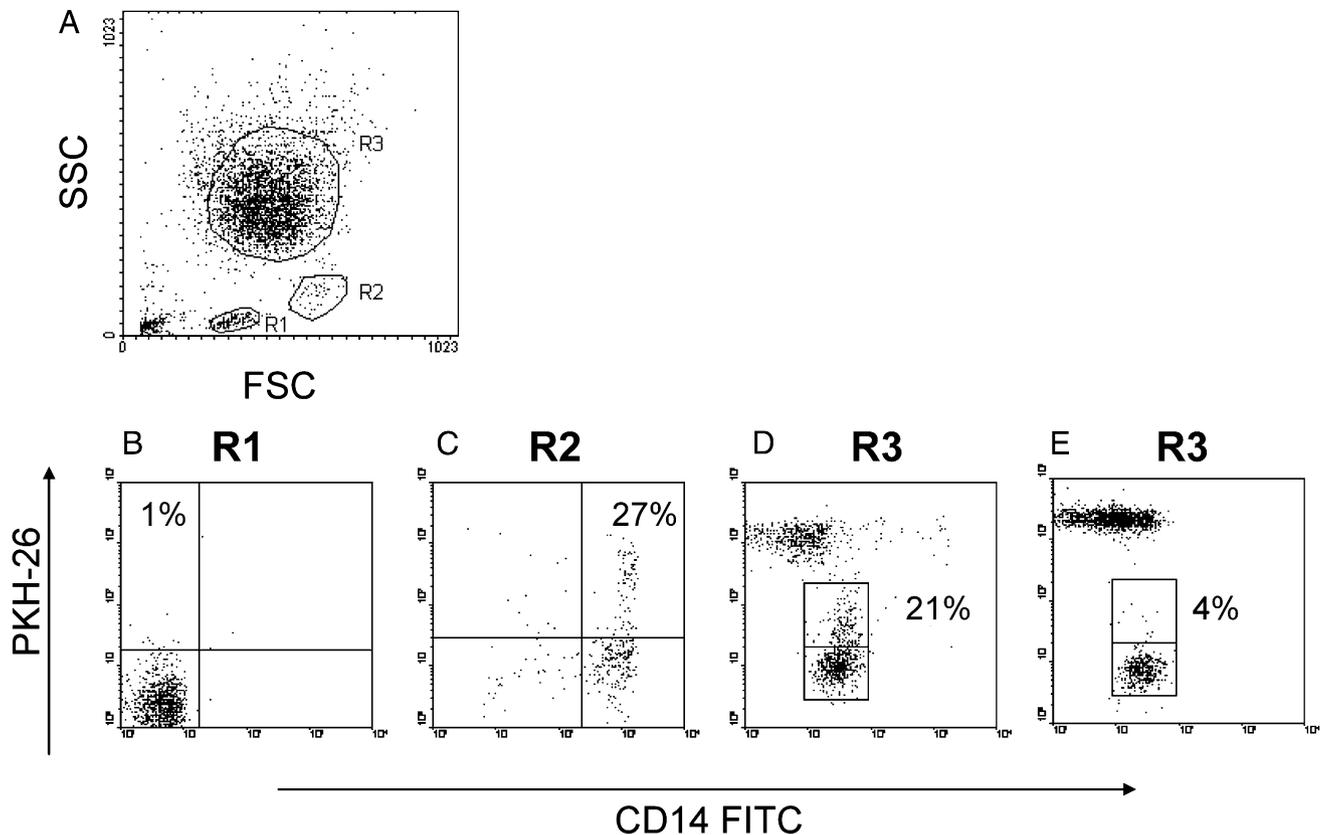


FIGURE 3. Neutrophils in whole blood ingest apoptotic but not viable neutrophils. PKH-26-labeled apoptotic (*B, C, D*) or nonapoptotic (*E*) neutrophils were added into heparinized whole blood and incubated at 37°C for 90 min. Subsequently, erythrocytes were lysed, the leukocytes stained with FITC-conjugated anti-CD14 mAb and analyzed by flow cytometry. *A*, Forward light scatter-side scatter analysis of leukocytes. The regions R1, R2, and R3 show the gated populations of lymphocytes (R1), monocytes (R2), and neutrophils (R3). *B*, Uptake of apoptotic neutrophils by lymphocytes. *C*, Uptake of apoptotic neutrophils by monocytes. *D*, Uptake of apoptotic neutrophils by neutrophils. *E*, Uptake of nonapoptotic neutrophils by neutrophils. In *D* and *E* the strongly red fluorescent cells in the upper left corner are the PKH-26-labeled apoptotic neutrophils that were added to the whole blood whereas the double-squares inside *D* and *E* contain the blood neutrophils. The lower part of the double-squares include the neutrophils without phagocytosing apoptotic cells as determined in the control tube sets without adding apoptotic cells (not shown). The upper parts of the double-squares show the neutrophils with increased red fluorescence indicating the ratio of whole blood neutrophils that phagocytosed PKH-26-labeled apoptotic (*D*) or viable (*E*) neutrophils. The data are representative of three experiments.

supplemented with heat-inactivated FCS (Fig. 6*D*), the presence of fresh serum in the culture medium did not enhance the uptake of apoptotic neutrophils by MFs (Fig. 6*E*). The iDC were also able to phagocytose apoptotic neutrophils in the absence of fresh serum (Fig. 6*G*). However, adding fresh serum to the culture medium enhanced the phagocytosis of apoptotic neutrophils by iDC (Fig. 6*H*). Stimulation of neutrophils with LPS further increased their capacity to ingest apoptotic cells (Fig. 6*C*). However, LPS did not enhance the phagocytic capacity of MFs and iDC (Fig. 6*F, I*).

Similar to neutrophils, most DC phagocytosed apoptotic material smaller than a whole cell. In contrary, the fluorescence intensity of most MFs that ingested apoptotic cells (Fig. 6*D–F*) reached the fluorescence intensity equivalent to whole apoptotic cells suggesting that MFs ingested whole apoptotic neutrophils, whereas neutrophils and iDC may prefer to take up smaller apoptotic material.

Phagosomes with ingested apoptotic material mature to phagolysosomes in neutrophils

In phagocytic cells, ingested material is digested in phagolysosomes, which arise after the fusion of phagosomes with lysosomes containing degrading acid hydrolases (16). To analyze whether the engulfed material is digested in neutrophils, we investigated whether the ingested apoptotic material is located in an acidic environment, which is a clear indication that the phagosome is matured to phagolysosomes. The postphagocytic fusion of phagosomes and lysosomes leads to

a drop in pH within the phagolysosome compartment because of the acidic environment of lysosomes. This pH drop can be detected by the pH sensitive dye pHrodo-SE that emits red fluorescent light at an increased intensity with decreasing environmental pH (13). In a pH neutral environment, the light emission of pHrodo-SE is almost undetectable under the fluorescence microscope. It means that non-phagocytosed apoptotic cells are not fluorescent or are very dim. The signal, however, becomes intensive after phagocytosis and phagosome-lysosome fusion (13).

Apoptotic neutrophils were labeled with pHrodo-SE prior to coculture with PKH-67-labeled fresh neutrophils. Immediately after the start of the coculture the two cell populations could be easily distinguished in the culture medium with a neutral pH. The pHrodo-SE-labeled apoptotic cells did not emit fluorescence, whereas the PKH-67-labeled neutrophils were green (Supplemental Fig. 3*A–H*). After 90 min of coculture, however, green-labeled cells containing bright red particles were seen (Fig. 2*E–H*, Supplemental Fig. 3*I–P*). These red particles represent pHrodo-SE-labeled apoptotic cells in an acidic environment. A pH drop in the culture medium can be excluded, because the nongreen-cell-associated apoptotic cells after 90 min of coculture still did not emit light (Fig. 2*H*, Supplemental Fig. 3*L, 3P*). The bright red particles were always associated with the green-labeled neutrophils. Confocal laser microscopy clearly showed (Supplemental Fig. 5, Video 1) that the red particles were located inside the green-labeled fresh neutrophils.

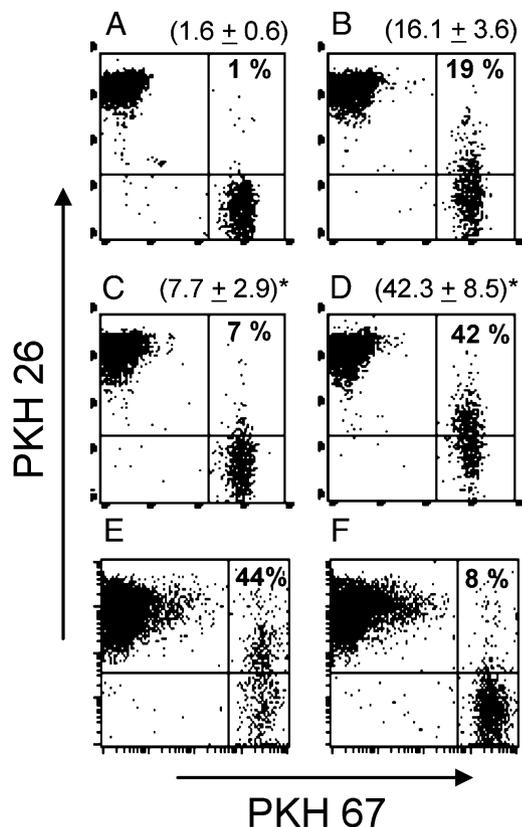


FIGURE 4. Activated neutrophils phagocytose apoptotic neutrophils in the presence of fresh serum. PKH-67–labeled freshly isolated neutrophils were coincubated with PKH-26–labeled apoptotic neutrophils at a ratio of 1:4 for 90 min at 37°C in culture medium containing 10% heat-inactivated FCS (A), 50% fresh autologous serum (B), 50% heat-inactivated autologous serum (C), or in the presence of 50% fresh autologous serum after 60 min preincubation with 100 ng/ml LPS and 100 U/ml IFN-γ (D). Phagocytosis was assessed by flow cytometry. The numbers in the upper right quadrants indicate the ratio of PKH-67–labeled neutrophils with ingested apoptotic cells/material. The data are from one experiment and are representative of three experiments performed. Mean ± SD from the three experiments are shown above the panels. *Indicates significant differences between (C) and (B) and between (D) and (B). E and F, PKH-67–labeled freshly isolated neutrophils were coincubated with PKH-26–labeled apoptotic neutrophils at a ratio of 1:4 for 90 min at 37°C in culture medium containing 50% fresh autologous serum (100 ng/ml LPS and 100 U/ml IFN-γ) in the absence (E) or in the presence (F) of 10 μg/ml cytochalasin D.

These observations clearly show that the ingested apoptotic material is located in an acidified environment. These data suggest that the phagosome containing the ingested apoptotic cells fused with lysosomes to form a phagolysosome. Because the content of phagolysosomes is destined for degradation, digestion may be an explanation for the presence of small sized apoptotic materials in neutrophils. However, although fluorescent microscopy showed the presence of larger apoptotic material inside neutrophils (Fig. 2E–H; Supplemental Figs. 3L, 3P, and 5; and Video 1), flow cytometry analyses (Figs. 4, 6) revealed that only few neutrophils ingested whole apoptotic cells, most neutrophils contained apoptotic material smaller than a whole cell. Therefore, neutrophils, in contrast to MFs, appear to prefer small-sized apoptotic material.

Apoptotic cells inhibit the ability of neutrophils to mount an oxidative burst

In previous studies, apoptotic cells have been shown to exert an inhibitory effect on effector functions of MFs and DC (7, 17–19). Because the oxidative burst is one of the most important effector

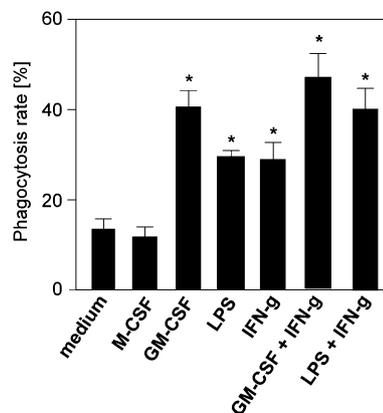


FIGURE 5. Proinflammatory stimuli enhance the capacity of neutrophil granulocytes to ingest apoptotic cells. Neutrophils (5×10^5) were preincubated for 60 min in medium alone or in the presence of 100 ng/ml M-CSF, 100 ng/ml GM-CSF, 100 ng/ml LPS, 100 U/ml IFN-γ, or combinations of IFN-γ with LPS or GM-CSF, labeled with PKH-67 and coincubated with PKH-26–labeled apoptotic neutrophils at 37°C for 90 min in the presence of 50% fresh autologous serum. The ratio of neutrophils that ingested apoptotic cells was assessed by flow cytometry. The data show mean ± SD from three independent experiments. *Indicates significant difference ($p < 0.05$) as compared with the phagocytosis rate of neutrophils without stimulation (medium).

functions of neutrophils, we assessed the production of ROS in the presence of apoptotic cells. The intracellular production of ROS was quantified by using flow cytometry. As shown in Fig. 7, the presence of apoptotic cells led to a reduced ability of neutrophils to mount an oxidative burst on exposure to fMLP + LPS. The inhibitory effect of apoptotic cells on the ROS-production of neutrophils was observed both in the presence and absence of fresh serum (not shown).

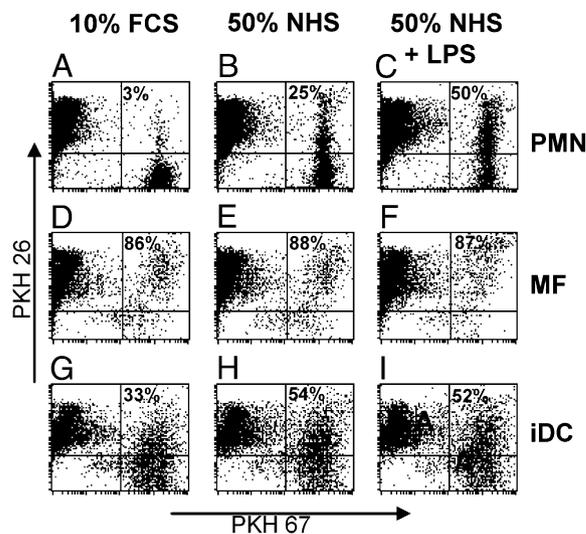


FIGURE 6. Phagocytosis of apoptotic neutrophils by neutrophils, MFs, and iDC. Freshly isolated neutrophils (PMN; A–C), MFs (D–F), or iDC (G–I) were labeled with PKH-67 and coincubated with PKH-26–labeled apoptotic neutrophils at a ratio of 1:4 for 90 min at 37°C in culture medium containing 10% heat-inactivated FCS (A, D, G), 50% fresh autologous serum (B, E, H), or 50% heat-inactivated autologous serum in the presence of 100 ng/ml LPS. Phagocytosis was assessed by flow cytometry. The numbers in the upper right quadrants indicate the ratio of PKH-67–labeled neutrophils (A–C), MFs (D–F), or iDC (G–I) with ingested apoptotic cells/material. The data are from one experiment and are representative of three experiments performed.

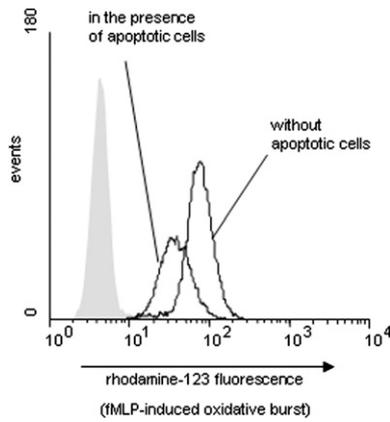


FIGURE 7. Apoptotic cells inhibit the ROS production by neutrophils. Neutrophils (5×10^5) were pretreated with 100 ng/ml LPS in the presence or absence of 5×10^5 autologous apoptotic cells, followed by stimulation with 1 μ M fMLP. The intracellular ROS production was analyzed by flow cytometry by using the substrate DHR-123. The shaded area shows the fluorescence intensity of DHR-123-loaded fresh neutrophils without exposure to fMLP.

Apoptotic cells inhibit the secretion of the proinflammatory cytokines TNF- α and IP-10 by neutrophils

Ingestion of apoptotic cells is generally believed to be anti-inflammatory. Both MFs and DC have been shown to produce less proinflammatory cytokines in the presence of apoptotic cells (7, 17–19). Neutrophils are an important source of cytokines that can modulate the inflammatory reaction (20). We assessed the effect of apoptotic cells on the production of the proinflammatory cytokines TNF- α and CXCL10/IP-10 by human neutrophils. After exposure to LPS, freshly isolated neutrophils secreted a significant amount of TNF- α (Fig. 8A). The TNF- α secretion of neutrophils, however, was markedly inhibited in the presence of apoptotic neutrophils (Fig. 8A).

CXCL10/IP-10 is a proinflammatory cytokine that has been shown to be released by activated neutrophils (21). After exposure to LPS and IFN- γ freshly isolated neutrophils secreted high levels of this chemokine (Fig. 8B). Apoptotic neutrophils secreted very little CXCL10/IP-10. The presence of apoptotic neutrophils resulted in a significant reduction of CXCL10 secretion by neutrophils (Fig. 8B).

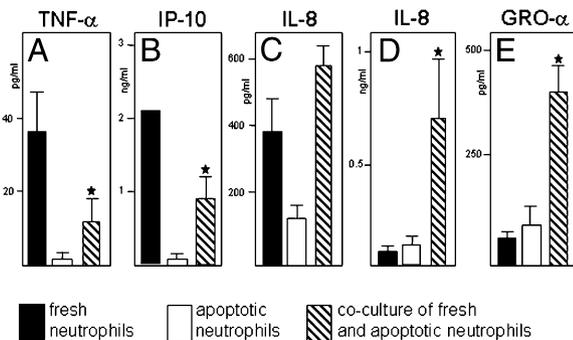


FIGURE 8. Effect of apoptotic cells on cytokine secretion by neutrophils. Freshly isolated nonapoptotic neutrophils (1×10^6 /ml), apoptotic neutrophils (4×10^6 /ml), or cocultures of the two cell populations were incubated in culture medium containing fresh human serum in the presence of LPS (A, C, E), LPS + IFN- γ (B), or without additional stimulus (D). Culture supernatants were collected after 18 h and assayed for cytokines using ELISA. *Indicates significant difference ($p < 0.05$) as compared with cytokine release by fresh neutrophils in the absence of apoptotic cells. The data show mean \pm SD from three independent experiments.

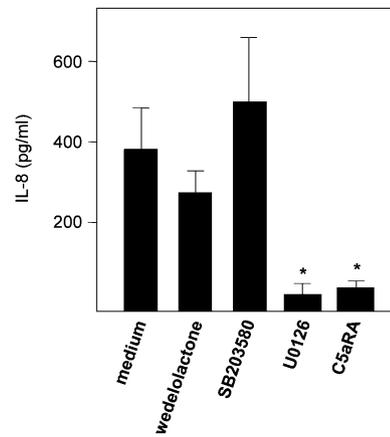


FIGURE 9. Effect of pharmacological inhibitors on the apoptotic cell-induced IL-8 secretion by neutrophils. Freshly isolated neutrophils (1×10^6 /ml) were preincubated for 15 min in the presence of 25 μ M wedelolactone (inhibitor of I κ B phosphorylation), 10 μ M SB203580 (p38 MAPK-inhibitor), or 10 μ M U0126 (MEK1/2-inhibitor used to inhibit the ERK1/2 pathway), or 10 μ M C5aRA, or in medium alone prior to coculture with UV-irradiated autologous apoptotic neutrophils in culture medium containing 30% fresh human serum. Culture supernatants were collected after 5 h and assayed for IL-8 using ELISA. *Indicates significant difference ($p < 0.05$) as compared with IL-8 release in the coculture in the absence of inhibitors (medium). The data show mean \pm SD from three independent experiments.

Enhanced release of IL-8 and GRO- α by neutrophils on contact to apoptotic cells

IL-8 (CXCL8) is a major chemotactic factor for neutrophils. However, neutrophils not only respond to but also secrete IL-8 (20). This autocrine mechanism is thought to serve as positive feedback for neutrophil recruitment (22). Because neutrophil recruitment is generally considered to be proinflammatory, we tested the effect of apoptotic cells on the IL-8 release by human neutrophils. As positive control, neutrophils were exposed to LPS. LPS-treated fresh neutrophils released significant amounts of IL-8 (Fig. 8C). However, in contrast to the observations with TNF- α and IP-10, the LPS-induced IL-8 release was not affected by the presence of apoptotic neutrophils (Fig. 8C). Unexpectedly, contact to apoptotic cells, in the absence of LPS, induced the release of IL-8 by neutrophils (Fig. 8D).

Because the apoptotic cell-induced enhanced IL-8 release was observed only in the presence of fresh serum, it is reasonable to assume that complement cleavage products could activate neutrophils for enhanced production of this cytokine. One such cleavage product with known strong neutrophil activating capacity is C5a. To investigate the potential role of C5a for the IL-8 release, the C5a receptor was blocked by using the C5aRA A8^{A71-73} (14). Blocking the C5a-receptor inhibited almost completely the IL-8 release (Fig. 9) indicating that in cocultures of fresh and apoptotic neutrophils complement cleavage products can activate neutrophils for enhanced IL-8 production.

In a previous study signaling through the ERK1/2 pathway was found to be involved in the C5a-induced IL-8 release (23). To explore the signaling pathways involved in the apoptotic cell-induced and C5a-dependent IL-8 production, neutrophils were cocultured with apoptotic cells in the presence of wedelolactone (inhibitor of I κ B phosphorylation, used to inhibit the NF- κ B pathway), SB203580 (p38 MAPK-inhibitor), and U0126 (MEK1/2-inhibitor, used to inhibit the Erk1/2 pathway). Inhibition of the Erk1/2 pathway resulted in a diminished IL-8 release (Fig. 9). However, inhibition of p38 MAPK or NF- κ B had no effect on the enhanced IL-8 release (Fig. 9).

Because IL-8 is a chemokine acting primarily on neutrophils, we tested whether the presence of apoptotic cells leads to enhanced secretion of another neutrophil-targeting chemokine GRO- α (CXCL1). Similarly to IL-8, coincubation with apoptotic cells resulted in the enhanced secretion of GRO- α by neutrophils (Fig. 8E). These data show that the secretion of neutrophil-targeting chemokines is induced in cultures of neutrophils on contact to apoptotic neutrophils.

Discussion

Inflammation is a beneficial host response to foreign challenge involving numerous soluble factors and cell types, including PMN granulocytes. Although the life span of neutrophils is extended in inflamed tissue, apoptosis of infiltrating neutrophils has been documented *in vitro* as well as *in vivo*. As long as phagocytic clearance of an apoptotic cell occurs before breakdown of its cell membrane, none of its cytosolic contents will be released into the extracellular space. In this way, despite the billions of cells (primarily neutrophils) that die in inflamed tissues by apoptosis, tissues are protected from an otherwise harmful exposure to the contents of dying cells.

Engulfment of apoptotic neutrophils by MFs is believed to be a crucial component in the resolution of inflammation (6, 7). In addition, DC can ingest apoptotic neutrophils as well (24). However, in the early phase of an acute inflammation, the inflammatory infiltrate consists primarily of tissue neutrophils. The number of inflammatory neutrophils can be extremely high. At the same time, in the early phase of inflammation, the number of MFs and DC is low and not likely to cope with the billions of dying neutrophils. In a recent study, neutrophils with ingested apoptotic neutrophils were seen in inflamed tissue after administration of LPS suggesting that neutrophils themselves, as a form of cellular "cannibalism", can participate in the clearance of aged neutrophils (9). In another previous study, circulating neutrophils in patients after GM-CSF therapy were shown to have the ability to ingest apoptotic material (15). Because neutrophils are professional phagocytes, it is likely that they evolved mechanisms not only for the ingestion of microorganisms but also for essential physiological function such as the clearance of apoptotic cells.

In the current study, *in vitro* studies were carried out to investigate the capacity of human neutrophils to phagocytose apoptotic cells. We presented evidence that neutrophils possess the ability for efficient ingestion of apoptotic cells. We described that this event depends on activatory stimuli and on the presence of heat labile serum factors. Moreover, we showed for the first time that apoptotic neutrophils exert an inhibitory effect on proinflammatory neutrophil functions.

It was observed that neutrophils ingested apoptotic cells at a low level in the absence of serum. However, their capacity to phagocytose apoptotic neutrophils was markedly enhanced in the presence of fresh serum. The serum-dependent uptake of apoptotic cells is not unique for neutrophils. Murine glomerular mesangial cells can phagocytose apoptotic cells also in a serum dependent manner (25). In addition, in a previous study supplementation of the culture medium with fresh serum increased the uptake of apoptotic thymocytes by blood-derived MFs (26). However, fresh serum was reported to play a role for the phagocytosis of late but not early apoptotic neutrophils by MFs (27). In the current study we also observed that MFs readily ingested early apoptotic neutrophils in the absence of fresh serum. The presence of fresh serum was, however, required for the ingestion of early apoptotic neutrophils by neutrophils. DC showed an intermediate phenotype, although apoptotic neutrophils were ingested by DC in the absence of fresh serum, the level of phagocytosis was further enhanced in the presence of fresh serum.

The requirement for fresh serum suggests a major role of heat labile serum components, especially complement factors in the uptake of apoptotic cells by neutrophils. Indeed, in addition to their role in facilitating phagocytosis of invading pathogens, molecules of the complement system were implicated in the uptake of apoptotic cells in several previous studies (28, 29). Splenic marginal zone DC were shown to ingest apoptotic leukocytes in a manner dependent on complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) and in hypocomplementemic animals the clearance of apoptotic cells was diminished (24). C1q was shown to opsonize apoptotic cells for the uptake by MFs and by DC and regarded as a multiligand-bridging molecule in apoptotic cell recognition (30, 31). Because in our studies heat labile serum factors were required for the enhanced uptake by neutrophils, it is quite reasonable to speculate that complement factors are involved in the uptake of apoptotic cells by neutrophils.

Human neutrophils were assessed for their capacity to phagocytose apoptotic cells also in whole blood. Without stimulation, these cells had only a limited phagocytic capacity. In the whole blood experiments monocytes were also not highly active to phagocytose apoptotic cells. It is in line with previous findings that circulating monocytes have a limited capacity to ingest apoptotic cells (7). These findings suggest that circulating leukocytes have generally a low capacity to ingest apoptotic cells. However, after exposure to bacterial constituents and proinflammatory mediators in infected/inflamed tissues, they acquire the ability for efficient clearance of apoptotic cells.

MFs and DC used in the current study were not in a resting but in an activated state after the 7 d *in vitro* culture. Therefore, it is not surprising that their phagocytic capacity was higher than that of freshly isolated neutrophils. In this study we showed that exposure of resting neutrophils to activating stimuli such as GM-CSF, LPS, or IFN- γ enhanced significantly their capacity to ingest apoptotic cells. This is in line with a previous study showing that after 40 d therapy with GM-CSF neutrophils from renal carcinoma patients acquired the capacity to phagocytose apoptotic Jurkat cells in a whole blood assay (15). In our current *in vitro* study, a short-term incubation with GM-CSF enhanced markedly the uptake of apoptotic neutrophils by neutrophils. By using highly purified neutrophils, our results clearly show that GM-CSF exerts a direct stimulatory effect on neutrophils. Previously, GM-CSF was shown to rapidly enhance neutrophil functions such as phagocytosis, respiratory burst, as well as the expression of CD11b/CD18, Fc-, and complement receptors (32). Because GM-CSF is released by a variety of cells, including endothelial cells after exposure to endotoxins (33), GM-CSF is one of the most potent neutrophil-activating cytokines produced at sites of acute inflammation or infection. In our study, both LPS and GM-CSF enhanced the phagocytosis of apoptotic neutrophils by neutrophils. Although no detailed screening of activating cytokines was carried out, we could show that IFN- γ also enhanced the capacity of neutrophils to phagocytose apoptotic cells. In the early phase of an infection, IFN- γ is rapidly produced by cells of the innate immune system such as NK and NKT cells (34). Therefore, at sites of acute infection, microbial constituents and locally produced cytokines are likely to act together to boost the uptake of apoptotic cells by neutrophils.

The finding that exposure to proinflammatory cytokines did not enhance the phagocytosis of apoptotic neutrophils by MFs is in line with the previous observation that a short (2 h) exposure to such stimuli does not potentiate the clearance of apoptotic neutrophils by MFs (35).

Phagocytosis of apoptotic cells by MFs and DC usually results in an anti-inflammatory state (7). LPS-stimulated human monocytes and MFs were shown to produce much less TNF- α and IL-1- α in

the presence of apoptotic neutrophils (17). After uptake of apoptotic cells, the gene-expression and secretion of proinflammatory mediators such as IL-12 was diminished also by DC (18). Phagocytosis is, however, not necessary to achieve this effect, cell to cell contact with apoptotic cells is sufficient to induce profound inhibition of proinflammatory cytokine gene expression and secretion by both DC and MFs (19). Similar to these previous findings, we showed that apoptotic cells had an inhibitory effect on the release of proinflammatory cytokines also by neutrophils. Moreover, ROS production was also markedly reduced in the presence of apoptotic cells. These data show for the first time the negative regulation of proinflammatory functions of neutrophils by apoptotic cells.

In a previous study (36), early apoptotic neutrophils were reported to inhibit zymosan-induced IL-8 release by human MFs. In our current study, LPS-induced IL-8 release by neutrophils was not inhibited by early apoptotic neutrophils. The reason for this discrepancy is not clear. However, in contrary to MFs, a significant amount of preformed IL-8 can be found in neutrophils (37). The regulation of IL-8 secretion differs in MFs and neutrophils because the release of preformed IL-8 does not require de novo gene expression.

In addition to the reduced production of proinflammatory cytokines, contact to apoptotic cells was shown to induce the production of anti-inflammatory cytokines as well. IL-10 and TGF β are believed to play a major role for dampening inflammatory reactions by apoptotic cells (15, 38–40). In our studies, however, we were not able to demonstrate neutrophil-mediated release of TGF β or IL-10 on contact to apoptotic cells (data not shown). This finding may simply be the result of a low capacity of neutrophils to secrete these cytokines. However, as reported previously, the anti-inflammatory effect of apoptotic cells does not necessarily involve autocrine or paracrine actions of anti-inflammatory cytokines (24).

In contrast to the inhibitory effect on the secretion of TNF- α and IP-10, coculture with apoptotic cells led to an enhanced release of the neutrophil-targeting chemokines IL-8 and GRO- α . After exposure to inflammatory stimuli, IL-8 is produced in the tissues primarily by epithelial cells, keratinocytes, fibroblasts, and endothelial cells. Once sufficient numbers of neutrophils are present in the infected tissue, neutrophils themselves are the most abundant source of this chemokine (41). Therefore, because neutrophils are also the primary cellular target of IL-8, neutrophil-derived IL-8 production acts as an amplifying loop (20, 22). Although the secretion of the proinflammatory cytokines TNF- α and IP-10 were negatively regulated by apoptotic cells in our experiments, the presence of apoptotic neutrophils led to enhanced release of IL-8 by neutrophils. It is not unique for neutrophils because apoptotic cells enhanced IL-8 secretion also by MFs in previous studies (42). Because IL-8 recruits neutrophils, IL-8 is often regarded as a proinflammatory cytokine. However, IL-8 release and the recruitment of neutrophils does not necessarily mean inflammation-associated tissue damage. The presence of neutrophils that rapidly undergo apoptosis, via their anti-inflammatory effect on MFs, are considered crucial for the downregulation of inflammation. Moreover, IL-8 and GRO- α stimulate keratinocyte migration and proliferation and are associated with rapid wound healing (43). IL-8 and neutrophils were reported to promote re-epithelization (44) and to induce angiogenesis, which in turn helps tissue repair (45). In addition, apoptotic neutrophils express CCR5 and sequester CCL3 and CCL5 and therefore act as terminators of chemokine signaling during resolution of inflammation (46).

In a previous study phagocytosis of apoptotic cells by rat vascular smooth muscle cells led to the release of CINC, the rat

functional homolog of IL-8 (47). In another study MF-mediated digestion of apoptotic thymocytes was accelerated in the presence of neutrophils both in vivo and in vitro suggesting that recruitment of neutrophils to the site of massive apoptosis does not induce inflammation but rather accelerates complete digestion of apoptotic cells (48). In light of our results, neutrophils likely contribute to the disappearance of apoptotic cells by participating actively in the clearance of apoptotic cells.

The finding that blocking the C5aRA inhibited the apoptotic cell-induced IL-8 release suggests that apoptotic cells activate complement in the presence of fresh serum. Indeed apoptotic cells were shown to activate complement on binding C1q and mannose binding lectin (49). In addition, apoptotic cells may activate the alternative pathway directly (49). All activation pathways may result in C3 deposition and activation of the terminal pathway involving the generation of cleavage products such as C5a. C5a is a strong inducer of IL-8 production. IL-8, in addition to its effects on neutrophils, can promote proliferation and structural reorganization of epithelial cells leading to tissue repair (50). In addition, binding of apoptotic cells to CR3 also signals the phagocyte to downregulate the secretion of IL-12 and IFN- γ and therefore has an anti-inflammatory effect (51). Similarly, C5a has been shown to suppress the release of IL-12 family cytokines in MFs demonstrating that C5a, in addition to its proinflammatory function, exerts immunoregulatory properties (52).

Inhibition of the ERK1/2 pathway resulted in a diminished apoptotic cell-induced IL-8 release by neutrophils. However, inhibition of p38 MAPK or NF- κ B had no effect on the enhanced IL-8 release. These data are in line with the previous observation that recognition of apoptotic cells by MFs activated ERK1/2 rapidly but did not activate NF- κ B or p38 MAPK (53). The anti-inflammatory effect of apoptotic cells and of C5a on MFs has also been shown to depend on ERK1/2 signaling (54, 52). The potential involvement of C5a in the apoptotic cell-induced effects fits to these finding because the antiapoptotic effect of C5a effect was shown to be mediated via the ERK1/2 pathway (55) and the C5a-mediated neutrophil priming was reported to be mediated by ERK1/2 rather than the p38 pathway (56).

In summary, our data show that neutrophils after contact to apoptotic neutrophils secrete neutrophil-targeting chemokines resulting in the local accumulation of neutrophils. Accumulated neutrophils would help to clear masses of apoptotic cells at early inflammatory sites. The results suggest that at sites of acute infection/inflammation, neutrophils are important effector cells for the clearance of apoptotic cells. Overall, this process is clearly noninflammatory and likely contributes to the termination of inflammatory reaction.

Disclosures

The authors have no financial conflicts of interests.

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