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

Lars Esmann,*1 Christian Idel,*1 Arup Sarkar,* Lars Hellberg,* Martina Behnen,* Sonja Möller,* Ger van Zandbergen,*2 Matthias Klinger,† Jörg Köhl,‡ Uta Bussmeyer,* Werner Solbach,* and Tamás Laskay*

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 naive 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-κB 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 ~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.

*Institute for Medical Microbiology and Hygiene, †Institute for Anatomy, and ‡Institute for Systemic Inflammation Research, University of Lübeck, Lübeck, Germany; and 1Division of Molecular Immunology, Cincinnati Children’s Hospital Medical Center and University of Cincinnati College of Medicine, Cincinnati, OH 45229

L.E. and C.I. contributed equally to this work.

1Current address: Institute for Medical Microbiology and Hygiene, University Hospital Ulm, Ulm, Germany.

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Address correspondence and reprint requests to Tamás Laskay, Ph.D., Institute for Medical Microbiology and Hygiene, University of Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany. E-mail address: Tamas.Laskay@uk-sh.de

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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 × g, followed by 20 min at 800 × 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 μM 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 × 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 were centrifuged for 10 min at 300 × g and washed once in PBS, resuspended in complete medium, 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 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 μl medium were added to 100 μl heparinized whole blood and incubated for 90 min at 37˚C in a humidified atmosphere containing 5% CO₂. 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. Neutrophopetis 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 effectors. 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 μg/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 cytospin slides (Shandon). 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 acidic compartments. To obtain apoptotic thymocytes, single-cell suspension of thymus cells from 8-wk-old BALB/c mice were cultured at the concentration of 1 × 10^6/ml in RPMI 1640 medium containing 10% FCS and 1 μM 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 × 10^−5 M PKH-26 or in 5 × 10^−5 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.

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 apo-

 ptotic cells by MFs (13). Apoptotic neutrophils (25 × 10⁶) 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. Cytocen-

 trifice slides were prepared immediately after mixing the two cell pop-

 ulations 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 × 10⁵ PKH-67–labeled MFs or iDC were coincubated with 2 × 10⁶

 autologous apoptotic neutrophils for 90 min at 37°C in RPMI 1640 me-

 dium, 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 × 10⁶/ml) were coincubated

 with 2 × 10⁶/ml UV-irradiated autologous apoptotic neutrophils for 18 h in

 RPMI 1640 medium containing either 10% FCS or 30% NHS. For stim-

 ulation of cytokine release, the cells were exposed to various combina-

 tions 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 Bio-

 sciences), 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

 15 min in the presence of the inhibitors of certain signaling pathways.

 **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 × 10⁶) in 100 μl com-

 plete medium were pretreated with 100 ng/ml LPS (Sigma-Aldrich) for 1 h at

 37°C in the presence or absence of 2.5 × 10⁶ autologous apoptotic cells.

 Subsequently, 1 μM DHR was added for 5 min, followed by stimulation with

 1 μM MIP-2 (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 FACS Calibur 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 ± 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 apo-

 ptosis 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 non-

 apoptotic 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 apo-

 ptotic 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 to simultaneously assess the 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 phagocyte apoptotic

 neutrophils (Fig. 3B). A population of monocytes acquired red

 fluorescence indicating that these cells ingested apoptotic neutro-

 phils (Fig. 3C). Importantly, not only monocytes but also a signif-

 icant 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 lack

 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 pres-

 ence 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 ingest, phagocytosis was inhibited in the

 coculture experiments with cytochalasin D. Cytochalasin D treat-

 ment 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, neu-

 trophils were coincubated with apoptotic thymocytes. Neutrophils

 ingested apoptotic thymocytes as detected by microscopical ex-

 amination of apoptotic nuclei inside neutrophils (Fig. 2B).

 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 ap-

 omtotic cells by neutrophils was investigated in culture medium

 containing 30–50% fresh autologous serum.
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. 4D, 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. 2A and the uptake of apoptotic thymocytes (Fig. 2J) suggested that neutrophils are able to phagocytose whole apoptotic cells, investigations by applying fluorescence microscopy showed that neutrophils possibly also phagocytosed 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. 6A, 6B), MFS phagocytosed apoptotic neutrophils in culture medium...
supplemented with heat-inactivated FCS (Fig. 6D), the presence of fresh serum in the culture medium did not enhance the uptake of apoptotic neutrophils by MFs (Fig. 6E). The iDC were also able to phagocytose apoptotic neutrophils in the absence of fresh serum (Fig. 6G). However, adding fresh serum to the culture medium enhanced the phagocytosis of apoptotic neutrophils by iDC (Fig. 6H). Stimulation of neutrophils with LPS further increased their capacity to ingest apoptotic cells (Fig. 6C). However, LPS did not enhance the phagocytic capacity of MFs and iDC (Fig. 6F, 6I).

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. 6D–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. 3A–H). After 90 min of coculture, however, green-labeled cells containing bright red particles were seen (Fig. 2E–H, Supplemental Fig. 3I–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. 2H, Supplemental Fig. 3L, 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.
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 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 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.

**FIGURE 5.** Proinflammatory stimuli enhance the capacity of neutrophil granulocytes to ingest apoptotic cells. Neutrophils (5 × 10⁵) 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).

**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.
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).

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 antagonist A871–73 (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 coincubated with apoptotic cells in the presence of wortmannin (inhibitor of PI3K phosphorylation), used to inhibit the NF-κB 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-κB had no effect on the enhanced IL-8 release (Fig. 9).

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 antagonist A871–73 (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.

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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...
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 TGFβ 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 TGFβ 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-epithelialization (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 CsA 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-κB 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-κB 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.

References
212: 363–370.
Supplementary material

Fig. S1
Isolated neutrophils phagocytose *E. coli*
PKH-26-labeled freshly isolated neutrophils (A) were co-incubated with FITC-labeled *E. coli* for 30 min in culture medium containing 10% heat-inactivated FCS at 37°C (C) or on ice (B). Phagocytosis was assessed by flow cytometry. The data are from one experiments and are representative for two experiments performed.
Supplementary material

Fig. S2
*Transmission electronmicrographs* of A) a freshly isolated human neutrophil, B-C) apoptotic neutrophils, and D) an apoptotic (ap) and a non-apoptotic (n-ap) neutrophil in a co-culture. In contrast to the nuclear morphology of fresh neutrophils (A, D), condensed chromatin and loss of bridges between the nuclear fragments can be seen in apoptotic neutrophils (B-D).
Supplementary material

Fig. S3
Co-culture of PKH67-labeled freshly isolated neutrophils and pHrodo SE-labeled apoptotic neutrophils.
A-H) PKH67-labeled freshly isolated neutrophils and pHrodo SE-labeled apoptotic neutrophils were mixed and immediately photographed under a fluorescent microscope.
I-P) PKH67-labeled freshly isolated neutrophils and pHrodo SE-labeled apoptotic neutrophils were co-incubated for 90 min in medium containing fresh human serum and photographed under a fluorescent microscope.
A,E,I,M) green fluorescence (excitation 488 nm, emission detected through a 515-585 nm bandpassfilter); B,F,J,N) red fluorescence (excitation 546 nm, emission detected through a 590 nm longpassfilter); C,G,K,O) overlay of green and red fluorescence; D,H,L,P) overlay of green and red fluorescence with the brightfield images.
In a pH neutral environment, the light emission of pHrodo-SE is almost undetectable under the fluorescent microscope. It means that the pHrodo-SE-labeled apoptotic cells that are not ingested by neutrophils (white arrows in panels D,H, L,P) are not fluorescent or very dim.
Supplementary material

Fig. S4.

Neutrophils phagocytose apoptotic neutrophils. Confocal microscopy image stacks of a neutrophil containing apoptotic material.
PKH 67-labeled (green) neutrophils were co-incubated with PKH-26 (red) labelled apoptotic PMN for 90 min in culture medium containing 50 % fresh autologous serum and 100 ng/ml LPS. Cytospin preparate was fixed with 1 % paraformaldehyde and analysed with a Zeiss confocal laser scanning microscope. Scan Mode: Stack; Scaling: X: 0,09 μm, Y: 0,09 μm, Z: 0,44 μm; Stack Size X:26,38 μm, Y: 26,38 μm, Z: 5,27 μM; Filters: Ch2: BP 505-530, Ch3: BP 560-615; Wavelength: 488 nm T1 20,8%, 543 nm T2 74,3 %, 514 nm T2 86,3%.
Supplementary material

Fig. S5
Uptake of an apoptotic cell by a neutrophil granulocyte. PKH67-labeled freshly isolated neutrophils were co-incubated for 90 minutes with pHrodo™-labeled apoptotic neutrophils. Three dimensional orthogonal slice projection analysis (xy, xz, yz) of a confocal microscopy (Zeiss LSM5 Meta microscope with 63x/1.2 water immersion objective) section is shown. The large central panel shows a single optical slice through which an x axis (green line) and a y axis (red line) were defined for sliced z-axis reconstruction. The corresponding results for the horizontal xz slice (top) and the vertical yz slice (right) are shown. The blue line represents the position of the central panel image in the z stack. The red colour of the cell inside a green-labeled fresh neutrophil indicates that the pHrodo SE-labeled apoptotic neutrophil is located in an acidified environment inside a viable neutrophil which has fully engulfed the apoptotic cell.
Supplementary material Video S1. Uptake of an apoptotic cell by a neutrophil granulocyte. PKH67-labeled freshly isolated neutrophils were co-incubated for 90 minutes with pHrodo™-labeled apoptotic neutrophils. 3D animation of a viable neutrophil granulocyte (green) after phagocytosis of a pHrodo™-labeled apoptotic neutrophil (red). The 3D image was reconstructed from a confocal z-stack of 31 optical sections (stack size: x= 27,72 µm, y = 25,19 µM) acquired using Zeiss LSM5 Meta microscope (63x/1.2 water immersion objective). The animation shows, that the apoptotic cell is engulfed and surrounded by the viable granulocyte. The red colour of the cell inside a fresh neutrophil indicates that the pHrodo™-labeled apoptotic neutrophil is located in an acidified environment inside a viable neutrophil.