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Inflammation-Associated Autophagy-Related Programmed Necrotic Death of Human Neutrophils Characterized by Organelle Fusion Events

Cristina C. Mihalache,* Shida Yousefi,* Sébastien Conus,* Peter M. Villiger,† E. Marion Schneider,‡ and Hans-Uwe Simon*†

The most common form of neutrophil death, under both physiological and inflammatory conditions, is apoptosis. In this study, we report a novel form of programmed necrotic cell death, associated with cytoplasmic organelle fusion events, that occurs in neutrophils exposed to GM-CSF and other inflammatory cytokines upon ligation of CD44. Strikingly, this type of neutrophil death requires PI3K activation, a signaling event usually involved in cellular survival pathways. In the death pathway reported in this study, PI3K is required for the generation of reactive oxygen species, which somehow trigger the generation of large cytoplasmic vacuoles, generated by the fusion of CD44-containing endosomes with autophagosomes and secondary, but not primary, granules. Neutrophils demonstrating vacuolization undergo rapid cell death that depends on receptor-interacting protein 1 kinase activity and papain family protease(s), but not caspases, that are most likely activated and released, respectively, during or as a consequence of organelle fusion. Vacuolated neutrophils are present in infectious and autoimmune diseases under in vivo conditions. Moreover, isolated neutrophils from such patients are highly sensitive toward CD44-mediated PI3K activation, reactive oxygen species production, and cell death, suggesting that the newly described autophagy-related form of programmed neutrophil necrosis plays an important role in inflammatory responses. The Journal of Immunology, 2011, 186: 000–000.

Neutrophils represent the most abundant leukocytes in human blood and are essential in innate immune responses against pathogens (1). Apoptosis is the most common physiological cell death of neutrophils both in vitro and in vivo and prevents the release of histotoxic contents from the dying cell and therefore limits tissue damage (2). However, nonapoptotic types of neutrophil death have also been described. For instance, Siglec-9 mediates death signals that are caspase-independent when neutrophils are concurrently exposed to certain proinflammatory cytokines (3). This cell death is associated with cytoplasmic vacuolization (3). Moreover, neutrophils have been shown to extracellularly bind and kill bacteria by generating so-called extracellular neutrophil traps (NETs) (4, 5). One mechanism, by which DNA might be released, seems also to be dependent on a nonapoptotic form of cell death (6). Together, these recent reports suggest that neutrophils can undergo both apoptotic and nonapoptotic types of cell death; the latter seems to be related to inflammatory conditions.

CD44 is a transmembrane cell-surface protein involved in cell–cell and cell–matrix interactions. Early reports have implicated CD44 as a regulator of hematopoietic progenitor cells (7–9). Chronic granulomatous disease (CGD), a disease that results in impaired intracellular killing and microbial burden, varies from mild forms to severe conditions with life-threatening infections, which can be controlled by GM-CSF treatment (10). CD44 is constitutively expressed on many other cell types, including mature leukocytes, macrophages, and endothelial cells (10). CD44 has a large number of isoforms generated by alternative RNA splicing (10). The most common standard isoform is highly expressed on neutrophils (11). The glycosaminoglycan disaccharide hyaluronan (HA) is the main ligand of CD44, although other extracellular matrix proteins seem also able to interact with CD44 (10). In neutrophils, CD44 promotes adhesion and emigration, is required for neutrophil sequestration in inflamed liver sinusoids (13), supports bacterial phagocytosis (11), and acts as a physiologic ligand of E-selectin (14).

The induction of neutrophil apoptosis during the resolution of an innate immune response can be mimicked in vitro by culturing the cells in the absence of sufficient amounts of survival factors, a process that is called spontaneous neutrophil apoptosis (2). Moreover, FAS-mediated neutrophil apoptosis might play a role in the limitation of inflammatory responses, as shown in an experimental model of rheumatoid arthritis (15). Interestingly, caspase-8 is not only activated by activation of death receptors, such as FAS. Caspase-8 has also been shown to be cleaved and activated by cathepsin D, a cysteine protease released from primary (azurophilic) granules, suggesting an important role of this organelle in the induction of spontaneous neutrophil apoptosis (16).

In this report, we describe a novel caspase-independent death pathway in neutrophils that involves several organelles, but no primary granules. It is triggered by ligation of CD44 in the presence of GM-CSF or FMLF and depends on the striking activation of PI3K and the subsequent generation of large amounts of re-
active oxygen species (ROS). Under these conditions, endosomes, autophagosomes, secondary granules, and possibly secretory vesicles fuse, resulting in the generation of large vacuoles, which appear to release or activate noncaspase proteases, leading to cell death of neutrophils. Receptor-interacting protein 1 ( RIP1) kinase activity was also required for death induction, indicating that the type of death could be considered as programmed necrosis (17, 18). Although neutrophil apoptosis is blocked during inflammatory responses, the newly identified necrotic death pathway operates particularly in the presence of large amounts of survival factors, and, therefore, might contribute to cell number regulation under such conditions.

Materials and Methods

Reagents

GM-CSF was provided by Novartis Pharma (Nürnberg, Germany) and G-CSF from Aventis Pharma (Zurich, Switzerland). IL-1, IL-8, IFN-γ, and HA (high m.w.) were from R&D Systems Europe (Abingdon, U.K.) and IFN-α from PBL Biomedical Laboratories (distributed by Alexis, Lausen, Switzerland). The caspase inhibitor z-Val-Ala-Asp (zVAD)-fluoromethylketone was from BD Biosciences (Erembodegem, Belgium). Anti-CD44 mAb (clone A3D8), pepstatin A (PepA), CA-074-ME (CA), E64d, 3-mercaptoethanol (3-MA), dithiob bisulphite (DHB), and LMF were purchased from Sigma-Aldrich (Buchs, Switzerland). Anti-FAS agonistic mAb (CH11) and rabbit anti-Ag7 Ab were from MBL International (Woburn, MA). Rabbit anti-cathepsin G Ab, PA, diphenylene iodonium (DPI) chloride, LY294002, and Wortmannin were from Calbiochem-Novabiochem (La Jolla, CA). Rabbit anti-microtubule-associated protein-1 (LAMP-1), rabbit anti-phospho-Akt (Ser473), and rabbit anti-Akt Abs were from Cell Signaling Technology (Danvers, MA). Rabbit anti-myceloperoxidase (MPO) Ab was from Dako-Cytomation (Baar, Switzerland) and rabbit anti-lactoferrin from Santa Cruz Biotechnology (distributed by Labforce, Nunningen, Switzerland). Anti-GPDPH mAb was from Chemicont International (Chandlers Ford, U.K.). HRP-conjugated secondary Abs were obtained from Amersham Pharmacia Biotech (Dübendorf, Switzerland). Control IgG1 and the F(ab′)2 fragments of the secondary goat anti-mouse (GaM) Ab were from Jackson ImmunoResearch Laboratories (Milan Analytica; Roche Diagnostics, Roche, Switzerland). Anti-CD44 mAb was from Serotec (distributed by Morpholins AbD, Duesseldorf, Germany). Anti-CD16 microbeads for eosinophil isolation were from Miltenyi Biotec (Bergisch-Gladbach, Switzerland).

Cells

Peripheral blood neutrophils were purified from healthy normal individuals as well as patients suffering from septic shock and chronic granulomatous disease (CGD), respectively, by Ficoll-Hypaque centrifugation (3, 16). We also isolated neutrophils from joint fluids of rheumatoid arthritis (RA) patients (3). Septic shock patients fulfilled the following inclusion criteria: 1) documented or suspected infection; 2) signs of systemic inflammation in response to infection; and 3) systemic arterial blood pressure <70 mm Hg, despite adequate fluid resuscitation, in the absence of other causes of hypotension (19). We obtained Institutional Review Board approval for the study (Kantonalale Ethikkommission Bern). The purity of the isolated human neutrophil populations was always >95% as assessed by staining with Diff-Quik (Baxter, Dudingen, Switzerland) and light microscopy analysis. Blood eosinophils were isolated as previously described (20).

Cell cultures

Human blood neutrophils were cultured at 1 × 10⁶/ml. Neutrophils were cultured in complete culture medium (RPMI 1640 containing 10% FCS) in the presence and absence of GM-CSF (25 ng/ml), G-CSF (25 ng/ml), IMLF (1 μM), IL-1 (25 ng/ml), IL-6 (25 ng/ml), IL-8 (25 ng/ml), IFN-α (500 U/ml), IFN-γ (85 ng/ml), anti-Fas (1 μg/ml), CA (10 μM), PepA (100 μM), E64d (10 μM), LY294002 (25 μM), Wortmannin (100 nM), 3-MA (20 μM), Nec-1 (25 μM), DPI (1 μM), and zVAD (20 μM) for the indicated times. Cytokine stimulation before adding anti-CD44 mAb and preincubation with inhibitors were performed for 30 min (except for DPI, 5 min only). Anti-CD44 and anti-CD84 Abs were used at 6 μg/ml or as indicated. HA was used at 1.5 μM.

Determination of cell death and apoptosis

Cell death was assessed by uptake of 1 μM ethidium bromide and flow cytometric analysis (FACSCalibur; BD Biosciences) (3, 16). To determine whether cell death was apoptosis, redistribution of phosphatidylserine (PS) in the presence of propidium iodide (PI) and DNA fragmentation was measured by flow cytometry (3, 16). For morphologic analysis, an Axiovert 35 microscope equipped with a 630/1.4 oil objective lens was used (Carl Zeiss, Jena, Germany). Images were processed with Adobe Photoshop 5.0 software (Adobe Systems, San Jose, CA).

Enzymatic caspase-3 assay

Neutrophils were cultured at the indicated conditions, washed with cold PBS, and subsequently lysed in cell lysis buffer (50 mM HEPES [pH 7.4]/0.1% CHAPS/5 mM DTT/0.1 mM EDTA) using a Teflon glass homogenizer (WVR International, Ismaning, Germany) on ice for 10 min. Caspase-3–like activity was measured as enzymatic conversion of the colorimetric substrate Ac-DEVD-pNA at 405 nm according to the manufacturer’s instructions (QuantZyme caspase-3 cellular activity kit; Biomol, Plymouth Meeting, PA).

Macrophage phagocytosis assay

Uptake of dead neutrophils by monocyte-derived macrophages was investigated as previously described (21).

Oxidative burst measurements

Neutrophils were cultured as indicated and subsequently incubated with 1 μM DHR at 37°C for 30 min, placed on ice, and analyzed by flow cytometry (22). In additional experiments, we measured superoxide production with a ferricytochrome c reduction assay (23). A total of 65 nM PMA for 15 min was used as a positive control in these experiments.

Gel electrophoresis and immunoblotting

Neutrophils (1 × 10⁶/ml) were washed with PBS supplemented with protease inhibitor mixture (Sigma-Aldrich) and lysed with RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, and 1 mM EGTA supplemented with protease inhibitor mixture). In case of Akt and phospho-Akt detection, neutrophils were lysed with Triton X-100 buffer (50 mM Tris-HCl, 150 mM NaCl, 1% w/v Triton X-100, and 1 mM EDTA supplemented with protease inhibitor mixture). After a 10 min centrifugation to remove insoluble particles, equal amounts of the cell lysates were loaded on NuPage-Gels (Invitrogen, Groningen, Netherlands). Separated proteins were electrophoresed onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The filters were incubated overnight at 4°C in TBS/0.1% Tween-20/5% nonfat dry milk (all 1/1000). For loading controls, stripped filters were incubated with anti-GAPDH (1/3000) mAb. Filters were washed in TBS/0.1% Tween-20/5% nonfat dry milk for 30 min at room temperature and incubated with the appropriate HRP-conjugated secondary Ab (Amersham Pharmacia Biotech, Duesseldorf, Switzerland) in TBS/0.1% Tween-20/5% nonfat dry milk for 1 h. Filters were developed by an ECL technique (BCL-Kit; Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Histologic examination

Tissue sections from cystic fibrosis, leukocytoclastic vasculitis, folliculitis, and psoriasis patients were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 5 μm were stained with H&E and examined by light microscopy (Axiovert 35; Carl Zeiss).

Confocal laser scanning microscopy

Cytospins (2 × 10⁶/ml) were made from freshly purified neutrophils or neutrophils cultured in the presence or absence of GM-CSF and anti-CD44 mAb for 1 h on noncoated slides. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature and washed three times in PBS (pH 7.4). Permeabilization of cells was performed with 0.05% saponin in buffer A (3% BSA in PBS) for 5 min at room temperature and with acetone for 15 min at −20°C. To prevent nonspecific binding, slides were incubated in blocking buffer (33% human Igs, 5% normal goat serum, 33% BSA) for 1 h at room temperature. Indirect immunostainings of CD44, LC3, Atg7, beclin1, LAMP-1, lactoferrin, MPO, and cathepsin G were performed by using the respective primary antibodies diluted in blocking buffer (dilutions 1/50–1/100). Mouse and rabbit control Abs, respectively, were used at the same concentrations in each experiment. Following incubation with primary Abs, neutrophils were incubated with appropriate TRITC- and FITC-
conjugated secondary Ab (1/500) for 1 h in the dark at room temperature. The anti-fading agent Mowiol (Calbiochem) was added. Slides were covered by coverslips and analyzed by confocal laser scanning microscopy (LSM 510; Carl Zeiss) equipped with Ar and HeNe lasers.

**Electron microscopy**

The classical chemical fixation high-pressure freezing method was applied. High-pressure freezing and freeze substitution were performed as described (24). The cells were mounted onto sapphire discs and frozen via high pressure directly from the living state. The freeze substitution medium consisted of acetone with 0.2% osmium tetroxide, 0.1% uranyl acetate, and 5% water. Both chemically fixed and high-pressure frozen samples were embedded in EPON. Ultra-thin sections (80 nm) were cut on a Leica microtome E, stained with 0.2% lead citrate, and imaged using an EM 10 (Carl Zeiss) at an accelerating voltage of 80 kV.

**Statistical analysis**

Statistical analysis was performed with the Mann–Whitney U test. The figures show mean levels ± SD. For multiple comparisons, differences of the mean levels were analyzed using ANOVA followed by Tukey’s HSD test. A p value <0.05 was considered statistically significant.

**Results**

**CD44 ligation induces cell death in GM-CSF–primed neutrophils: possible role in inflammatory diseases**

CD44 is expressed on the surface leukocytes, including both neutrophils (11) and eosinophils (25). Ligation of CD44 has been shown to either inhibit (26) or to accelerate apoptosis in T cells (27) depending on the death trigger. Interestingly, a nonapoptotic death following anti-CD44 Ab treatment was observed in leukemic cells (28, 29). To investigate the effect of CD44 ligation on primary granulocytes, we used an anti-CD44 mAb in conjunction with F(ab’2) fragments of a polyclonal anti-mouse Ab (GaM) to enhance possible cross-linking. Activation of GM-CSF–primed neutrophils through CD44 resulted in the induction of significant neutrophil death in a time- (Fig. 1A) and concentration-dependent manner (Fig. 1B). In the absence of GM-CSF, we observed significantly less death induction following CD44 ligation. GM-CSF priming was done using a preincubation time of 30 min that did not result in changes of CD44 expression (data not shown).

To exclude the possibility of Fc receptor-mediated effects, several control experiments were performed (Supplemental Fig. 1A). First, isotype-matched (IgG1) control mAb was used with GaM, resulting in no effect on neutrophil death both in the presence and absence of GM-CSF. Second, we used, instead of a nonspecific control mAb, an isotype-matched (IgG1) anti-CD84 mAb that was able to positively stain neutrophils (data not shown). Ligation of CD84 using this mAb in conjunction with GaM again had no effect both in the presence and absence of GM-CSF (Supplemental Fig. 1A). Third, we used F(ab’2) fragments of the IgG1 control mAb in combination with GaM and/or anti-CD44 mAb (Supplemental Fig. 1A). Fourth, HA induced neutrophil death, particularly in the presence of GM-CSF (Supplemental Fig. 1B). Fifth, eosinophils, which carry both CD44 (25) and Fc receptors (30), did not demonstrate accelerated death following CD44 ligation both in the presence and absence of GM-CSF (Fig. 1C).

Besides GM-CSF, we also tested additional cytokines and inflammatory mediators, respectively, for their capacity to enhance the CD44-mediated cytotoxic activity in neutrophils. Similar to GM-CSF, priming with IMLF, IL-1, or IL-6 in conjunction with CD44 ligation resulted in significant cell death (Fig. 1D). In contrast, IL-8, G-CSF, as well as IFN-α and IFN-γ, were not or less efficacious. Although both GM-CSF and G-CSF significantly delayed spontaneous neutrophil death, only GM-CSF but not G-CSF pretreatment resulted in significant CD44-mediated cell death, suggesting that the cytokine priming of this death pathway was not linked to the capacity of increasing neutrophil survival. Because neutrophils are exposed to proinflammatory cytokines during inflammatory responses, we hypothesized that inflammatory neutrophils derived from patients may respond with an enhanced death rate following CD44 ligation compared with normal neutrophils. For instance, in sepsis, neutrophils are exposed to high levels of GM-CSF (31), giving rise to the possibility that these cells gain increased susceptibility toward CD44-mediated cytotoxic effects in vivo. Indeed, compared with normal blood neutrophils, neutrophils from patients suffering from acute septic shock responded with an enhanced in vitro death rate following CD44 ligation, and this effect could not be increased by GM-CSF preincubation (Fig. 1E). Similar results were observed using joint fluid neutrophils from patients with RA that had likely been exposed to GM-CSF in vivo, too (Fig. 1E) (32). Clearly, besides GM-CSF, other cytokines could act as priming agents for neutrophils in both sepsis and RA patients.

**CD44-mediated cell death in GM-CSF–primed neutrophils is characterized by cytoplasmic vacuolization and caspase independency**

We next investigated whether the neutrophil death induced following GM-CSF priming and CD44 ligation was due to apoptosis. Apoptosis is mediated by caspases and can be blocked by pharmacological caspase inhibition. The pan-caspase inhibitor zVAD blocked spontaneous neutrophil death as expected (Fig. 2A). In contrast, anti-CD44–mediated death in the presence of GM-CSF was largely unaffected by pharmacological caspase inhibition (Fig. 2A). To more precisely investigate the potential involvement of caspases, we specifically analyzed the enzymatic activity of caspase-3, which represents a key effector caspase in neutrophil apoptosis (2). Whereas FAS ligation by anti-FAS mAb resulted in strong caspase-3 activation, CD44 ligation appeared to decrease caspase-3 activity both in the presence and absence of GM-CSF (Fig. 2B). Taken together, these data suggest that the initial cell death mediated by CD44 ligation in the presence of GM-CSF is caspase-independent. In contrast, it is possible that at least a subpopulation of those neutrophils, which are not rapidly killed, subsequently dies with the help of caspases.

Because we obtained evidence for a caspase-independent form of cell death in GM-CSF–primed and CD44-activated neutrophils, we thought to investigate its morphologic features. Cells were investigated 1 and 6 h after stimulation. Interestingly, priming with GM-CSF and subsequent CD44 ligation resulted in neutrophils characterized by cytoplasmic vacuolization (Fig. 3A). Approximately 20% of the cells had this aberrant morphology. Pharmacological inhibition of the NADPH oxidase (DPI) but not caspases (zVAD) prevented cytoplasmic vacuolization (Fig. 3A). Vacuolization was not seen in neutrophils, which were treated with anti-CD44 mAb or GM-CSF alone (Fig. 3A). At later time points, the vacuolated neutrophils demonstrated evidence for cell swelling and lysis; both events could not be prevented by pharmacological inhibition of caspases (zVAD), but were blocked by DPI (Fig. 3B). Moreover, in contrast to anti-FAS–treated neutrophils, enlarged cell sizes of GM-CSF–primed neutrophils upon CD44 ligation were noticed by forward light scatter versus side light scatter analysis using a flow cytometer (data not shown). Together, these morphologic characteristics are associated with necrosis (33). We also investigated whether neutrophils generated NETs. Up to 4 h after CD44 ligation, we did not obtain evidence for NET formation by GM-CSF–primed neutrophils (data not shown). Therefore, it is unlikely that the type of death seen in this study represented
NETosis, which has been described as another form of neutrophil death required for NET formation (6).

Redistribution of PS is a characteristic feature of apoptotic neutrophils and can be detected by Annexin V binding (2). PS redistribution was observed during spontaneous and FAS-mediated apoptosis as expected (Fig. 3C). However, in the presence of GM-CSF, ligation of CD44 resulted in a different picture. Besides normal PS redistribution in a small subpopulation of cells indicative for apoptosis, we observed a larger neutrophil subpopulation in which Annexin V binding was somehow low (Fig. 3C). Annexin V low-binding cells were seen within 2 h and further increased 4 h after CD44 ligation of GM-CSF–primed neutrophils, and the appearance of these cells was largely blocked by DPI but only little by zVAD (Fig. 3C). Up to 4 h, the Annexin V low-binding cells increased with time and were mainly observed in the presence of CD44 ligation of GM-CSF–primed neutrophils.

FIGURE 1. CD44 ligation induces cell death in vitro and in vivo cytokine-primed neutrophils. A, Viability assay. Neutrophils were cultured as indicated and analyzed in a time-dependent manner (n = 5). B, Viability assay. Neutrophils were analyzed in a concentration-dependent manner after 24-h cultures (n = 3). Note that the numbers here show agonist-specific cell death, meaning that the simultaneously occurring spontaneous cell death was subtracted. C, Viability assay. Eosinophils were cultured as indicated and analyzed after 24-h cultures (n = 4). D, Viability assay. Neutrophils were primed with multiple proinflammatory stimuli and analyzed after 24-h cultures (n = 4). E, Viability assay. Neutrophils from patients with sepsis and RA were cultured as indicated and analyzed after 24-h cultures (both n = 4). Please note specificity control experiments in Supplemental Fig. 1. All values are means ± SD. *p < 0.05, **p < 0.01.

FIGURE 2. CD44 ligation does not activate caspase-3. A, Viability assay. Neutrophils were cultured as indicated in the presence and absence of the pan-caspase inhibitor zVAD and analyzed after 24-h cultures (n = 8). Values are means ± SD. B, Caspase activity assay. Neutrophils were analyzed after a 9-h culture period. Values are means ± SD. (n = 3). *p < 0.05, **p < 0.01.
binding neutrophils did not show evidence for PI uptake, excluding the possibility that Annexin V would somehow stain these cells due to membrane permeabilization. Therefore, these data suggested that PS redistribution occurred in a caspase-independent manner. At later time points, the Annexin V low-binding cells were still detectable but showed PI uptake (data not shown). Taken together, these data demonstrate that the CD44-mediated neutrophil death in the presence of GM-CSF is nonapoptotic and caspase independent. Moreover, the death occurs rapidly, depends on NADPH oxidase activity, and is associated with vacuolization and detectable PS redistribution. It should be noted that both forms of neutrophil death described in this study resulted in DNA fragmentation as assessed by flow cytometry (Supplemental Fig. 2A) and in equal uptake by macrophages (Supplemental Fig. 2B).

**CD44-mediated cell death in GM-CSF–primed neutrophils depends on inducible NADPH oxidase and PI3K activities**

As mentioned above, pharmacological inhibition of NADPH oxidase using DPI blocked vacuolization (Fig. 3A) and cell death (Fig. 3B, 3C) of neutrophils following GM-CSF priming and CD44 ligation. Therefore, we analyzed the generation of ROS in neutrophils in more detail. CD44 activation of GM-CSF–primed neutrophils resulted in rapid increases of intracellular ROS concentrations as assessed by DHR mean fluorescence intensity measurements. Maximal ROS levels were reached within 15 min that declined thereafter within the next 2 h (Fig. 4A). Priming with fMLF was less sufficient but occurred with the same kinetics (Fig. 4A). CD44 ligation alone also resulted in ROS generation, but the response was delayed and with lower efficacy compared with the conditions in which priming was performed (Fig. 4A). In contrast, in inflammatory neutrophils obtained from patients suffering from sepsis or RA, CD44-mediated ROS generation was largely increased and independent of the presence of GM-CSF (Fig. 4B), further demonstrating the corelational relationship between ROS generation and cell death, as well as the possible relevance of the newly identified form of neutrophil death in inflammatory responses. In additional experiments, we quantified extracellular superoxide production with a ferricytochrome c reduction assay. Although stimulation of GM-CSF–primed neutrophils with FMLF resulted in detectable extracellular superoxide production, anti-CD44 mAb stimulation did not (data not shown).

In neutrophils, ROS are mostly generated by the inducible NADPH oxidase system, which can be blocked by DPI. Therefore, to better understand the potential role of ROS generation in CD44-mediated caspase-independent death, we used again a pharmacological approach. DPI completely abrogated CD44-mediated death in both the presence and absence of GM-CSF (Fig. 4C). Interestingly, DPI also partially blocked spontaneous neutrophil death (Fig. 4C). To confirm the data using DPI, we investigated neutrophils from three patients with chronic granulomatous disease (CGD) that are unable to generate ROS based on a genetic defect in the NADPH oxidase. The neutrophils of these three CGD patients exhibited no death responses toward CD44 stimulation both in the presence and absence of GM-CSF (Fig. 4D). In addition, compared with normal neutrophils, CGD neutrophils exhibited delayed spontaneous death rates (Fig. 4D), confirming the pharmacological data observed with DPI that also pointed to the possibility that ROS play a role in neutrophil apoptosis as well.

To obtain an idea about the proximal signaling events initiated by CD44 ligation in GM-CSF–primed neutrophils, we again used a pharmacological approach. Interestingly, inhibitors of PI3K prevented ROS generation in these cells (Fig. 4E). In contrast, pharmacological inhibition of cathepsins and calpains using CA, PepA, and E64d, respectively, had no effect on ROS generation in...
GM-CSF and CD44-stimulated neutrophils (Fig. 4E). Inhibition of RIP1 kinase activity using Nec-1 (34) slightly decreased ROS generation.

The possible involvement of class I PI3K in ROS generation, an event that was clearly required for vacuolization and caspase-independent cell death following CD44 ligation in GM-CSF–primed neutrophils, was somewhat surprising because PI3Ks are usually involved in cellular survival pathways (35, 36). To verify these findings, we indirectly measured class I PI3K activation by analyzing the phosphorylation of Akt by immunoblotting. Although both GM-CSF and anti-CD44 mAb treatment alone resulted in increased Akt phosphorylation, findings that are in line with previously published work (37, 38), combined stimulation appeared to result in an overadditive activation of PI3K (Fig. 5A).

Both LY294002 and wortmannin prevented Akt phosphorylation, demonstrating that both inhibitors indeed blocked PI3K activation under our stimulation conditions (Fig. 5A). In contrast, Nec-1, E64d, and DPI were not effective in inhibiting CD44-mediated Akt phosphorylation in GM-CSF–primed neutrophils (Fig. 5B). Moreover, when we investigated PI3K activation in inflammatory neutrophils from patients with sepsis or RA, we noticed strong PI3K activation following CD44 ligation compared with normal neutrophils in the absence of GM-CSF (Fig. 5C).

To more directly prove that the strong PI3K activation following combined GM-CSF and CD44 stimulation is part of a signaling pathway leading to caspase-independent death, we measured cell death in the presence of pharmacological inhibitors. LY294002 and wortmannin completely abrogated CD44-mediated death in the presence of GM-CSF (Fig. 5D). Interestingly, whereas wortmannin was unable to prevent GM-CSF–mediated survival in the absence of CD44 ligation, LY294002 was efficacious (Fig. 5D), perhaps due to blocking Pim-1 and not PI3K in this system (39, 40). Moreover, Nec-1 and E64d significantly prevented neutrophil death following GM-CSF priming and CD44 ligation (Fig. 5D), despite their reduced efficacy in blocking PI3K and NADPH oxidase activation (Figs. 4E, 5B), suggesting that RIP1 kinase and E64d-blockable protease(s) are also involved in the novel death pathway reported in this study. We subsequently tested the possible contribution of the cathepsins B and D, but obtained no evidence for their involvement in CD44-mediated neutrophil death (data not shown).

Vacuolization likely results from fusion events involving different organelles

The data reported so far suggested a death pathway that strikingly uses the survival cytokine GM-CSF and the survival signaling molecule PI3K, which is required for ROS production. Both PI3K activation and ROS production seem to be essential for cytoplasmic vacuolization and death induction. We next tried to obtain some understanding in the process of vacuolization. Recently, CD44 has been shown to be internalized upon activation in the cancer cell line JIMT-1 (41), and CD44 was identified as a cargo protein in HeLa cells (42). Using immunofluorescence analysis, we got strong evidence for rapid endocytosis of CD44 upon its ligation with anti-CD44 mAb both in the presence and absence of GM-CSF (Fig. 6A). Interestingly, anti-CD44 mAb also triggered a punc-
ongoing autophagic activity in untreated neutrophils (Fig. 6A). The presence of GM-CSF results in increased autophagic activity in these cells that, interestingly, correlated with low caspase-3 activity (Fig. 6B). These data confirm that CD44 stimulation of neutrophils both in the presence and absence of GM-CSF results in increased autophagic activity in these cells that, interestingly, correlated with low caspase-3 activity (Fig. 6B). To further explore the possibility that autophagosomes contribute to vacuolization, we analyzed two additional autophagy genes known to be involved in autophagosome formation, Atg7 and beclin1 (46, 47). Immunofluorescence analysis revealed that CD44 ligation resulted again in punctated staining patterns, suggesting autophagosome formation and that both proteins are present in the large vacuole structures (Fig. 6C, 6D).

To test whether additional organelles are involved in the process of vacuolization, we continued investigating marker proteins using immunofluorescence analysis in neutrophils upon CD44 ligation in the presence and absence of GM-CSF. LAMP-1 and LAMP-2 are lysosomal membrane proteins. In neutrophils, however, they have been identified in secretory vesicles and secondary granules (48, 49). Moreover, lactoferrin is a marker for secondary granules (50). Using specific Abs against LAMP-1 and lactoferrin, we obtained evidence that secondary granules and possibly secretory vesicles contribute to the fusion process of organelles in GM-CSF–primed and CD44-activated neutrophils (Supplemental Fig. 3). In contrast, primary (azurophilic) granules did not appear to participate in the process of vacuolization. We used two marker proteins of azurophilic proteins, MPO and cathepsin G, and obtained no evidence that these proteins are present in the vacuoles seen in activated neutrophils as assessed by immunofluorescence analysis (Supplemental Fig. 4).

To investigate whether neutrophils from patients with inflammatory diseases do not only exhibit an increased susceptibility to undergo caspase-independent death associated with vacuolization due to organelle fusion, but also actually undergo such events under in vivo conditions, we analyzed fresh neutrophils in blood and tissue fluids as well as neutrophils in tissue sections of patients suffering from inflammatory diseases. We detected vacuolized neutrophils in blood smears of sepsis patients and in fresh joint fluids of RA patients (Fig. 7A, upper left panel and right panel, which show quantitative analysis) as well as in purified sepsis blood neutrophils (Supplemental Fig. 5). Such data suggest that autophagy is a major contributor to cell death in these inflammatory diseases.

FIGURE 5. Role for PI3K, a papain-like protease, and RIP1 for CD44-mediated cell death in GM-CSF–primed neutrophils. A and B, Immunoblotting. Neutrophils were preincubated with pharmacological inhibitors and stimulated as indicated for 60 min and phosphorylation of Akt (Ser\(^{473}\)) detected. Results are representative of three independent experiments. C, Immunoblotting. Neutrophils from patients suffering from RA (left panel) and sepsis (right panel), respectively, were stimulated as indicated for 60 min and phosphorylation of Akt (Ser\(^{473}\)) detected. Results are representative of three independent experiments. D, Viability assays. Neutrophils were cultured as indicated in the presence and absence of different pharmacological inhibitors and analyzed after 24-h cultures (at least n = 6 for each condition). All values are means ± SD. *p < 0.05.
inflammatory neutrophils demonstrated colocalization of CD44 and LC3 upon CD44 activation in vitro without the need of GM-CSF priming (Fig. 7A, lower left panel). Moreover, in the lungs of cystic fibrosis patients and in the skin of patients suffering from leukocytoclastic vasculitis, folliculitis, or psoriasis, we observed multiple vacuolized neutrophils by regular histology (Fig. 7B).

Discussion

We investigated functional consequences of CD44 ligation in neutrophils under normal and inflammatory conditions and report the following new findings. First, CD44 transduces death signals into neutrophils, resulting in a rapid, nonapoptotic, programmed necrotic cell death. Second, although caspases are not involved, PS redistribution occurs under these conditions. Third, the newly described death pathway involves internalization of CD44, activation of PI3K, the generation of ROS, induction of autophagy, vacuolization, and RIP1 kinase activity. Fourth, vacuolization most likely results from a fusion process between several organelles, including endosomes, autophagosomes, and secondary granules, but not primary (azurophilic) granules. Fifth, noncaspase proteases are required for cell death execution, but their identity remains to be determined. Last, inflammatory neutrophils from patients with sepsis and RA, respectively, exhibit an increased susceptibility toward programmed necrotic death in vitro.

Apoptosis of neutrophils has intensively been studied by several groups (2). In contrast, nonapoptotic forms of neutrophil death were only recently reported. For instance, we previously reported that Siglec-9 ligation on neutrophils in the presence of GM-CSF priming results in cell death, which was dependent on ROS, caspase independent, and associated with cytoplasmic vacuolization (3). However, molecular signaling events as well as the process and role of vacuolization in death induction were not investigated in this previous report. Clearly, vacuolization of neutrophils occurs under in vivo conditions, and it is therefore not only of interest to identify the surface receptors that are able to trigger this phenomenon, but also its molecular mechanisms and functional consequences.

Besides the role of CD44 in cellular adhesion and migration, recent studies suggest that CD44 acts as a phagocytic/endocytic receptor for large particles and HA, respectively, that involve internalization of CD44 itself (51–53). Therefore, we were not surprised to see that CD44 ligation by anti-CD44 mAb was followed by translocation of CD44 from the surface to the cytoplasm. The endosomal uptake of CD44 was recently confirmed using a proteomic approach, in which CD44 was identified as one of the cargo proteins present in early endosomes of HeLa cells (42). CD44 internalization has been shown to be dependent on its association to lipid rafts (52) and triggered IL-1β release in macrophages (53). The CD44-containing signaling platform may involve Syk, Rac1, and PI3K (51), and its translocation might be required to somehow meet the inducible NADPH complex.

We established a functional role of ROS for CD44-mediated death in GM-CSF–primed neutrophils by both genetic and pharmacological means. ROS were required for autophagosome formation and vacuolization. It should be noted that inflammatory neutrophils from patients suffering from septic shock or RA exhibited significantly stronger ROS production and cell death induction upon CD44 ligation in the absence of GM-CSF priming compared with normal neutrophils, supporting the view that ROS is critical for cell death induction. It should be noted that we did not measure detectable levels of extracellular superoxide generation following CD44 ligation of GM-CSF–primed neutrophils, implying that ROS generation largely occurs intracellularly upon...
mediated survival pathways are stopped or continue to be active, under inflammatory conditions. Whether possible PI3K-
lecular switch in neutrophils that initiates the PI3K-mediated death appears that PI3K-mediated ROS production represents the mo-
eutrophils are also highly susceptible for PI3K activation. It role for PI3K in this process and demonstrated that inflammatory generation remain to be investigated, we established a functional caspase activation and subsequent neutrophil apoptosis (58).

And phagocytosis of bacteria enhance ROS production, leading to vacuolization and cell death. Interestingly, CD44 apparently colocalized with marker proteins of autophagosomes (LC3) and secondary granules (LAMP-1 and lactoferrin) in the large vesicles, suggesting that vacuolization was likely the consequence of fusion between different organelles. It should be noted that we obtained no evidence that primary granules participated in the formation of vacuolization. Recently published work demonstrated that autophagosomes and endosomes are able to fuse, at least in vitro, forming the so-called amphisomes (61), and that both GTPases and SNARE proteins are involved in this process (62, 63).

But how is CD44-mediated caspase-independent neutrophil death executed? We tested several protease inhibitors to determine whether they would be able to block CD44-mediated death in GM-CSF–primed neutrophils. In agreement with the view that primary granules were not involved in vacuolization, we did not observe any effect of blockers of cathepsin B and cathepsin D, respectively. We previously reported that cathepsin D, which is CD44 internalization. The exact intracellular ROS levels required for vacuolization and cell death in single neutrophils need to be determined in future studies. It is possible that lower ROS levels induce apoptosis and certain threshold levels of ROS must be reached to initiate the nonapoptotic death pathway described in this study.

ROS have previously been implicated in the regulation of neutrophil apoptosis. For instance, in agreement with this study, delayed spontaneous death of CGD neutrophils compared with neutrophils from normal donors has been observed (3, 16, 54–56). In contrast, other previous work did not report different death kinetics between normal and CGD patients (57). The reason(s) for this discrepancy remains unclear, although differences in cell isolation, culture conditions, and cell death measurements could play a role. Moreover, other earlier work suggested that TNF-α and phagocytosis of bacteria enhance ROS production, leading to caspase activation and subsequent neutrophil apoptosis (58).

Although the exact molecular mechanisms responsible for ROS generation remain to be investigated, we established a functional role for PI3K in this process and demonstrated that inflammatory neutrophils are also highly susceptible for PI3K activation. It appears that PI3K-mediated ROS production represents the molec-
ular switch in neutrophils that initiates the PI3K-mediated death pathway under inflammatory conditions. Whether possible PI3K-mediated survival pathways are stopped or continue to be active,

**FIGURE 7.** Vacuolized neutrophils occur under in vivo inflammatory conditions. A, Upper panels. Nonpurified blood and joint fluid neutrophils from sepsis and RA patients, respectively. Cells were stained with Giemsa–May–Grünewald (Diff-Quik). Original magnification ×1000. Arrows indicate neutrophils with cytoplasmic vacuolization. Right panel, Statistical analysis of nonpurified blood and joint neutrophil data (control, n = 9; RA, n = 4; sepsis, n = 9). Values are means ± SD. Lower panels, Sepsis blood and RA joint fluid neutrophils were activated with anti-CD44 Ab in the absence of GM-CSF priming for 1 h, stained (green: CD44; red: LC3), and analyzed by confocal microscopy. Colocalization between CD44 and LC3 was observed (white arrows). Scale bars, 10 μm. B. Tissue sections from patients suffering from inflammatory diseases were stained with H&E. Arrows indicate neutrophils with cytoplasmic vacuolization. Data are representative of at least three independent experiments for each indicated disease. **p < 0.01, ***p < 0.001.

**FIGURE 8.** Schematic diagram showing the molecular pathway of programmed neutrophil necrosis in inflammation.
released by primary granules, triggers caspase-8 activation in neutrophil apoptosis (16). We also tested E64d, an inhibitor of cathepsins and cathepsins, and observed a significant death-preventing effect. It is, therefore, possible, but clearly not proven, that cathepsins are activated in the death pathway described in this paper. This hypothesis is supported by the observation that cathepsins target necrotic death in neutrophils (64). E64d did not block Akt phosphorylation, ROS generation, and vacuolization, suggesting that the protease(s) blocked by this inhibitor is a distal event (Fig. 8). It is not clear whether permeabilization of the vacuoles is required for protease activation and subsequent cell death. The term necroptosis has been used to designate one particular type of programmed necrosis that depends on the serine/threonine kinase activity of RIP1 (17). Nec-1 is a specific inhibitor of RIP1, which has been shown to block death receptor-mediated necrotic signaling (34). Necrostatins are tools for evaluating the contribution of necrotic cell death in experimental in vitro and in vivo systems. The observation that Nec-1 was able to block CD44-mediated death in GM-CSF–primed neutrophils indicates that the type of death could be considered as programmed necrosis. However, in the absence of a death receptor, it is unclear how RIP1 is activated under these conditions. Previously published work suggested that TNFR1 induces necrosis by RIP1-dependent recruitment of the Nox1 NADPH oxidase complex in murine fibroblasts (65). However, in neutrophils, RIP1 kinase activity was not required for CD44 internalization, P3K activation, generation of ROS, induction of autophagy, and vacuolization, indicating that RIP1 acts rather distal in the pathway. Future work may answer the question of whether classical autophagic cell death also depends on RIP1 and how the kinase would be activated under such conditions. Taken together, we provide evidence for a novel form of necrotic cell death in neutrophils that depends on P3K activation, ROS generation, increased autophagic activity, vacuolization, RIP1 kinase activity, and noncaspase proteases. Vacuolization of neutrophils occurs under in vivo pathological conditions, and inflammatory neutrophils are more prone to undergo this type of cell death. Interestingly, PS redistribution occurs in the absence of caspase activation, supporting earlier published work reporting PS redistribution mediated by an alternate oxidant-dependent pathway (66). The neutrophils, which undergo the newly described programmed necrotic death, were recognized and taken up by phagocytes. Therefore, it is likely that these neutrophils do not necessarily induce additional inflammation as one might expect from classical necrotic cells. The distinction between autophagic cell death and programmed necrosis needs further evaluation, because it appears that there are common molecular features between these two forms of nonapoptotic cell death. It should be noted that common morphologic features of autophagic and necrotic cell death have been described (67), suggesting the existence of a continuum of cell death phenotypes (33). Characterizing the precise mechanisms of different forms of regulated neutrophil death will likely provide new candidate molecules, which could be targeted by future anti-inflammatory drug treatments.

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Disclosures
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References


