Proteinase 3–dependent caspase-3 cleavage modulates neutrophil death and inflammation

Fabien Loison,[1,2] Haiyan Zhu,[3] Kutay Karatepe,[1] Anongnard Kasorn,[1,4] Peng Liu,[3] Keqiang Ye,[5] Jiaxi Zhou,[3] Shannan Cao,[3] Haiyan Gong,[3] Dieter E. Jenne,[6] Eileen Remold-O'Donnell,[7] Yuanfu Xu,[3] and Hongbo R. Luo[1]

[1]Department of Pathology, Harvard Medical School, Department of Lab Medicine, Children's Hospital Boston, and Dana-Farber/Harvard Cancer Center, Boston, Massachusetts, USA.

[2]Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand. [3]State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China. [4]Department of Community Medicine, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand. [5]Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia, USA. [6]Comprehensive Pneumology Center, Helmholtz Zentrum München, Munich, Germany. [7]Program in Cellular and Molecular Medicine and Division of Hematology/Oncology, Children's Hospital Boston, and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA.

Caspase-3–mediated spontaneous death in neutrophils is a prototype of programmed cell death and is critical for modulating physiopathological inflammatory responses; however, the underlying regulatory pathways remain ill defined. Here we determined that in aging neutrophils, the cleavage and activation of caspase-3 is independent of the canonical caspase-8– or caspase-9–mediated pathway. Instead, caspase-3 activation was mediated by serine protease proteinase 3 (PR3), which is present in the cytosol of aging neutrophils. Specifically, PR3 cleaved procaspase-3 at a site upstream of the canonical caspase-9 cleavage site. In mature neutrophils, PR3 was sequestered in granules and released during aging via lysosomal membrane permeabilization (LMP), leading to procaspase-3 cleavage and apoptosis. Pharmacological inhibition or knockdown of PR3 delayed neutrophil death in vitro and consistently delayed neutrophil death and augmented neutrophil accumulation at sites of inflammation in a murine model of peritonitis. Adoptive transfer of both WT and PR3-deficient neutrophils revealed that the delayed death of neutrophils lacking PR3 is due to an altered intrinsic apoptosis/survival pathway, rather than the inflammatory microenvironment. The presence of the suicide protease inhibitor SERPINB1 counterbalanced the protease activity of PR3 in aging neutrophils, and deletion of *Serpinb1* accelerated neutrophil death. Taken together, our results reveal that PR3-mediated caspase-3 activation controls neutrophil spontaneous death.

Introduction

Programmed cell death (PCD) is a broad term encompassing apoptosis, autophagy, lysosomal-mediated PCD (LM-PCD), and necroptosis. Constitutive neutrophil death is a form of PCD that plays a critical role in modulating neutrophil homeostasis, control of which is normally highly regulated to safely balance immune function and safe clearance (1–4). Dysregulated neutrophil death is implicated in a host of inflammatory, immunological, and infectious pathologies. Neutrophils undergo constitutive death even in the absence of extracellular stimuli, which indicates that an intrinsic spontaneous death program exists. While spontaneous neutrophil death shares many hallmark features with classical apoptosis, it also possesses several unique features, such as quicker death and independence from serum factors, which suggests that death in neutrophils is regulated by a unique signaling network (5).

Pathways that lead to apoptosis have been extensively studied, and most apoptosis is dependent on a set of cysteine proteases known as caspases (6, 7). The main effector caspase is caspase-3, which has a short prodomain and executes apoptosis after proteolysis by initiator caspases, caspase-8 and -9. Caspase-3 exists as an inactive 32-kDa proenzyme (procaspase-3; also known as zymogen) that undergoes proteolytic processing at conserved aspartate resi-

dues to produce a large 17-kDa subunit and a smaller 12-kDa subunit that dimerize to form the active enzyme. Activated caspase-3 then rapidly cleaves proteins in the cytoskeleton, membranes, and nucleus to effect the characteristic hallmarks of apoptosis. Caspase cascade can be activated by 2 independent mechanisms. The first, known as the intrinsic apoptosis pathway, is a mitochondria-mediated cellular process characterized by the loss of mitochondrial membrane potential and the release of proapoptotic factors such as cytochrome c to the cytosol. Cytochrome c activates caspase-9, which has been suggested to be the predominant initiator caspase in this pathway (6, 7). The second mechanism, the extrinsic death receptor pathway, is mediated mainly by TNF receptors (TNFRs) and Fas that can trigger downstream caspase cascade via activation of caspase-8. Caspase-8 ultimately drives the activation of caspase-3 either directly or indirectly, via activating the mitochondria-mediated intrinsic pathway (8). Both caspase-9 and caspase-8 cleave procaspase-3 at a conserved IXXD-S sequence to produce the large and small subunits of the active protease (6, 7, 9, 10). Caspase-3 activation during neutrophil death is well documented (1), but the full set of caspase activators remains unclear.

Here we showed that active caspase-3 was required for neutrophil spontaneous death. However, the cleavage and activation of caspase-3 in aging neutrophils was unexpectedly independent of caspase-8 or caspase-9, and instead was mediated by the serine protease proteinase 3 (PR3; encoded by *Prtn3*). Disruption of PR3 delayed neutrophil death both in vitro and in vivo. The protease
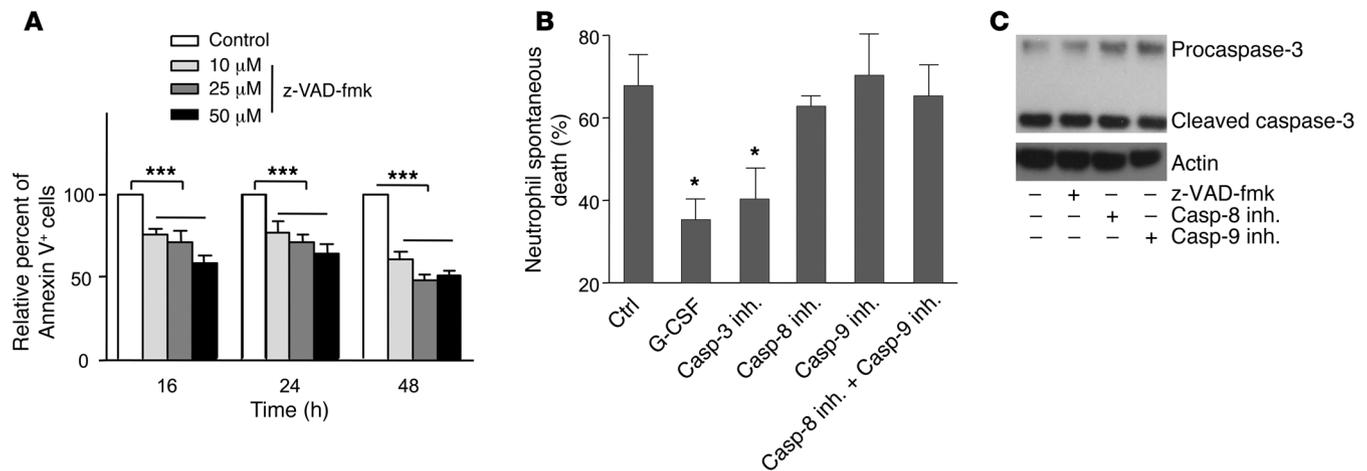
Figure 1. Caspase-3 activation during neutrophil spontaneous death is independent of caspase-8 or caspase-9. (A) Neutrophil spontaneous death was caspase dependent. Human primary neutrophils were cultured in the presence of the pan-caspase inhibitor z-VAD-fmk, and the percentage of apoptotic cells was measured, normalized to a no-inhibitor control. Results are mean ± SEM of 3 independent experiments. ***$P < 0.001$, 2-way ANOVA. **(B)** Neutrophil spontaneous death was mediated by caspase-3, but was independent of caspase-8 or caspase-9. Human neutrophils were cultured with the indicated specific caspase inhibitors for 36 hours; G-CSF (50 ng/ml) was used as a positive control. Results are mean ± SD of 3 independent experiments. *$P < 0.001$, Student's t test. **(C)** The cytosolic fraction of aging neutrophils (24 hours in culture) cleaved procaspase-3, independent of caspase-8 or caspase-9. Procaspase-3 cleavage was assayed using recombinant N-terminal His-tagged procaspase-3, and unprocessed procaspase-3 (32 kDa) and cleaved caspase-3 (N-terminal fragment; 20 kDa) were detected by Western blotting using anti-His tag antibodies, with actin as a loading control. Results are representative of 3 independent experiments.

activity of PR3 was inhibited by the suicide protease inhibitor SER-PINB1, and disruption of SERPINB1 accelerated neutrophil death. Thus, we discovered a novel mechanism by which caspase-3 is cleaved and activated during neutrophil spontaneous death, advancing our knowledge of the general molecular mechanisms that regulate cell death.

Results

Caspase-3 activation in aging neutrophils is caspase-8 and caspase-9 independent. Neutrophils isolated from peripheral blood undergo rapid and constitutive cell death following an apoptosis-type process (1, 11). We first confirmed the role of caspases in this process. Primary neutrophils isolated from the peripheral blood of healthy volunteers were cultured in the presence of increasing concentration of Z-Val-Ala-Asp(OMe)-fluoromethylketone (z-VAD-fmk), a cell-permeable pan-caspase inhibitor (1). The percentage of apoptotic neutrophils was measured using FACS and annexin V–propidium iodide (PI) staining, and neutrophil spontaneous apoptosis was found to be significantly delayed by up to 50% in the presence of increasing concentrations of z-VAD-fmk compared with controls (Figure 1A).

Caspase-8 and caspase-9 are initiators of the extrinsic and intrinsic apoptotic pathways, respectively, but their role in the initiation of neutrophil spontaneous death remains unclear (1); we therefore investigated their role in neutrophil spontaneous death using specific inhibitors. Surprisingly, there was no significant inhibition of apoptosis in response to caspase-8 and -9 inhibitors, either alone or in combination (Figure 1B). However, neutrophil spontaneous apoptosis was significantly delayed by 40% in the presence of the caspase-3–specific inhibitor z-DEVD-fmk, which suggests that caspase-3 activation is required and is mediated by a caspase-independent mechanism.

To directly test whether procaspase-3 cleavage in aging neutrophils is mediated by caspase-8 and -9, we performed an in vitro cleavage assay using N-terminal His-tagged recombinant procaspase-3 as the substrate. In most cells, procaspase-3 processing and activation by caspase-8 and -9 first requires cleavage at its IETDS site, resulting in 12-kDa (p12) and 20-kDa (p20) peptides. Upon treatment of procaspase-3 with the cytosol of aging neutrophils, a 20-kDa peptide was detected by Western blotting using anti-His tag antibodies (Figure 1C). Recombinant procaspase-3 was not cleaved when incubated with the cytosolic fractions (50 mg) of either cultured HeLa cells or freshly isolated neutrophils (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI76246DS1). As expected, addition of cytochrome c led to cleavage of procaspase-3, and this cleavage was inhibited by the pan-caspase inhibitor z-VAD-fmk (Supplemental Figure 1). When the purified cytosolic fraction of aging neutrophils was incubated with procaspase-3, the 20-kDa peptide was detected in the untreated control and, surprisingly, also in the presence of z-VAD-fmk or specific caspase-8 and -9 inhibitors (Figure 1C). These results suggest that the cleavage of procaspase-3 in aging neutrophils is not mediated by caspase-8 or -9, and thus is independent of the mitochondrial or death receptor pathways.

A serine protease present in the cytosol of aging neutrophil cleaves procaspase-3. We next investigated which proteases might be responsible for procaspase-3 cleavage. His-tagged human procaspase-3 was incubated with aging neutrophil cytosolic fraction in the presence of different protease inhibitors. The cysteine protease inhibitor E-64D and the cathepsin inhibitor N-acetyl-L-leucyl-L-leucyl-L-methional prevented nonspecific cleavage of procaspase-3, but did not inhibit the processing of the inactive procaspase-3. Only diisopropylfluorophosphate (DFP), a potent and irreversible serine protease inhibitor, was able to completely
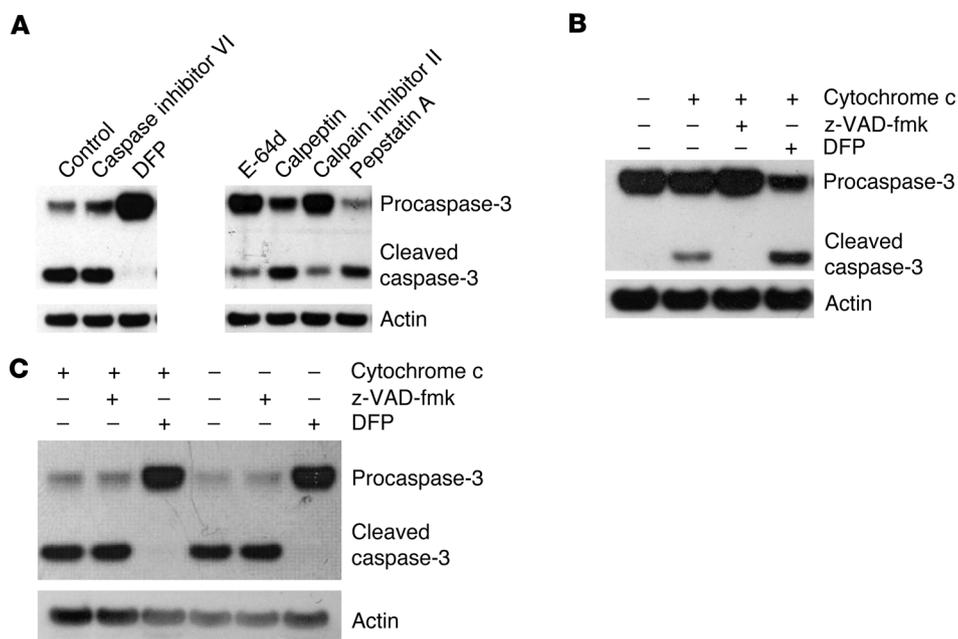
Figure 2. Serine protease activity is responsible for procaspase-3 cleavage during neutrophil spontaneous death. (**A**) Procaspase-3 cleavage in the cytosol of aging neutrophils was suppressed by a serine protease inhibitor. His-tagged procaspase-3 was incubated with the cytosolic fraction of aging human neutrophils (24 hours) at 37°C for 45 minutes in the presence of caspase inhibitor VI (20 mM), DFP (serine protease inhibitor, 20 mM), E-64d (calpain and cathepsins B, H, and L inhibitor, 20 mM), calpeptin (calpain-2 inhibitor, 20 mM), calpain inhibitor II (N-acetyl-L-leucyl-L-leucyl-L-methional; calpain-1 and cathepsin inhibitor, 20 mM), and pepstatin A (cathepsin D inhibitor, 1 mM). Procaspase-3 cleavage was assayed as described in Figure 1. (**B**) DFP did not inhibit procaspase-3 cleavage mediated by apoptosome (cytochrome c). Recombinant procaspase-3 was incubated with the cytosolic fraction of fresh human neutrophils in the presence or absence of cytochrome c (10 mM), z-VAD-fmk (20 mM), and/or DFP (20 mM). (**C**) Cleavage of procaspase-3 by the cytosolic fraction of aging neutrophils (24 hours) was independent of cytochrome c. Results are representative of at least 3 independent experiments.

prevent procaspase-3 processing (Figure 2A). The effect of DFP was not due to nonspecific inhibition of caspases, since the cleavage of procaspase-3 by the apoptosome of freshly isolated neutrophils, which is activated by the addition of cytochrome c, was not inhibited in the presence of DFP (Figure 2B). Finally, the cleavage of procaspase-3 by the cytosolic fraction of aged neutrophils did not require additional cytochrome c, since the 20-kDa caspase-3 peptide was detected even in the absence of an extra 50 mM of cytochrome c (Figure 2C).

Neutrophil spontaneous apoptosis does not need de novo protein synthesis, since concentrations of cycloheximide that completely block protein synthesis do not delay neutrophil death (12). Therefore, the serine protease is likely to be in an inactive state in freshly isolated neutrophils and become activated or unmasked. In fact, protease activity was detected in the soluble fraction of freshly isolated neutrophils after freeze-thaw lysis of cells (Supplemental Figure 2). Since DFP is a serine protease inhibitor, we focused on 3 neutrophil serine proteases (NSPs) known to be present in neutrophils: neutrophil elastase (NE), cathepsin G (CG), and PR3. Using nitrogen cavitation of freshly isolated neutrophils, we purified azurophil (α), specific (β1), and gelatinase (β2) granules as well as the secretory vesicles (γ) (Supplemental Figure 3, A–C). Procaspase-3 cleavage activity was mainly detected in the specific and gelatinase granules, with relatively lower levels

in secretory vesicles, all of which was inhibited by DFP (Figure 3, A and B). PR3 was present in all granules, strongly supporting its role as the serine protease responsible for the cleavage of procaspase-3 in apoptotic neutrophils. As previously reported (13), we detected PR3 in all neutrophil vesicles. However, the amount of PR3 was significantly lower in secretory vesicles (Supplemental Figure 3, A–C), consistent with the lower procaspase-3 cleavage activity in these vesicles. Interestingly, although the PR3 level in azurophil granules was high, the procaspase-3 cleavage elicited by azurophil granule extract was minimal, which suggests that the protease activity of PR3 may be inhibited in azurophil granules. A previous report showed that a PR3 inhibitor, SERPINA1, is highly localized in the azurophilic granules (14), which may contribute to the reduced activity.

We next incubated procaspase-3 with purified human NE, CG, and PR3; the 20-kDa peptide was only detectable in the presence of PR3. Incubation of procaspase-3 with either NE or CG led to detectable nonspecific proteolysis of the protein, but did not produce the specific 20-kDa caspase-3 peptide generated by the cytosol of aging neutrophils (Figure 3C). This in vitro result demonstrates that PR3 is the serine protease that cleaves caspase-3 in aging neutrophils.

PR3 cleaves procaspase-3 at a site upstream of the canonical caspase-9 cutting site. In order to determine the PR3 procaspase-3 cleavage site, the His-tagged recombinant protein was incubated with the cytosolic fraction of aging neutrophils, and the cleaved peptides were purified using Ni-NTA agarose beads and assessed using mass spectrometry (MS; Figure 4A). The PR3 cut site was located 5 amino acids upstream from the caspase-9/8 cut site (Figure 4B and Supplemental Figure 3D), consistent with the known preference of PR3 for small hydrophobic amino acids like Val, Cys, Ala, Met, Ser, and Leu at the P1 position (15).

Since PR3 cut caspase-3 at a site different than that of caspase-9, we next examined whether the processing of procaspase-3 by PR3 could also result in enzyme activation using a caspase-3 colorimetric assay (Figure 4C). Procaspase-3 was activated in the presence of PR3 or active caspase-9 positive control, but not on its own (16). Thus, similar to caspase-9, PR3 can also efficiently and effectively cleave and activate caspase-3.

Inhibition or disruption of PR3 delays constitutive neutrophil death. As constitutive neutrophil apoptosis was not inhibited by caspase-8 and -9 inhibitors, we next hypothesized that PR3 is a major activator of caspase-3 during constitutive neutrophil apop-
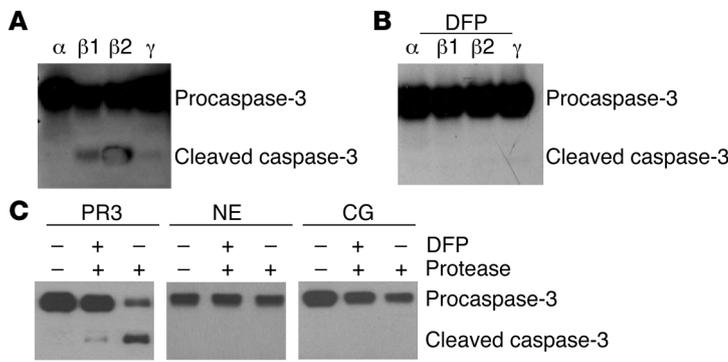
Figure 3. Caspase-independent cleavage of procaspase-3 is mediated by protease 3. (**A**) Cleavage of procaspase-3 by the lysates of purified neutrophil granules. Procaspase-3 cleavage activity was detected in specific (β1), gelatinase (β2), and secretory (γ) vesicles, but not in azurophilic (α) granules. (**B**) Cleavage of procaspase-3 by purified neutrophil granule lysates in the presence of DFP (20 mM). (**C**) Cleavage of procaspase-3 by purified serine proteases. Procaspase-3 (1 ng) was incubated with purified PR3 (12 nM), NE (12 nM), and CG (12 nM), alone or in the presence of DFP (20 mM) at 37°C for 30 minutes. Results are representative of 3 independent experiments.

tosis. Human neutrophils were cultured with the cell-permeable serine protease inhibitor DFP, which significantly delayed spontaneous apoptosis in a dose-dependent manner (Figure 5, A and B). Since DFP is a potent and irreversible anti-cholinesterase as well as a pan-serine protease inhibitor, we specifically targeted PR3 using siRNA. Serine proteases are predominantly expressed in myeloblasts and promyelocytes, and mature neutrophils therefore express low *PRTN3* mRNA levels (17) and cannot be used for efficient knockdown; we therefore used nucleofected murine BM-derived neutrophils (Figure 5, C and D), a heterogeneous population of precursor and mature neutrophils. Cytospins confirmed that the majority of the cultured cells after 24 hours were still neutrophils (Supplemental Figure 4A). Although the effect of siRNA-mediated PR3 knockdown was modest, the percentage of annexin V⁻ nonapoptotic neutrophils was significantly higher at each time point in PR3-knockdown cells than in controls (Figure 5, E and F).

siRNA knockdown could not completely abolish PR3 expression in neutrophils (Figure 5, C and D). In addition, siRNA is more likely to affect the less mature myeloid cells; thus, the results obtained from the siRNA knockdown experiments may just reflect events in immature BM myeloid cells rather than mature neutrophils. To circumvent these problems, we next sought to examine the survival of murine BM neutrophils completely lacking PR3 by generating *Prtn3*⁻/⁻ mice (Supplemental Figure 4B and Figure 5, G and H). PR3 protein expression was completely abolished in *Prtn3*⁻/⁻ mice, while expression of CG and ELA2 was essentially unaltered. Consistent with siRNA knockdown, BM neutrophils from *Prtn3*⁻/⁻ mice showed a significant increase in annexin V⁻ neutrophils compared with those of WT mice (Figure 5I). Thus, PR3 is an essential regulator of constitutive neutrophil death, and its disruption leads to delayed neutrophil death.

Inhibition of lysosomal membrane permeabilization (LMP) delays constitutive neutrophil death. We next examined how PR3, a granule-specific proteinase, could be present in the cytosol with

caspase-3. Total PR3 protein level in whole cell lysate only slightly decreased during the course of spontaneous neutrophil death (Figure 6A). PR3 was present in azurophilic, specific, and gelatinase granules and secretory vesicles of freshly isolated neutrophils, and the peroxidase myeloperoxidase (MPO) was present exclusively in the azurophilic compartment (Supplemental Figure 3, A–C). However, neutrophil fractionation at different time points after isolation showed the presence of high levels of PR3 in the cytosol after overnight culture. Cytosolic PR3 was essentially undetectable in freshly isolated neutrophils (Figure 6A). MPO, stored exclusively
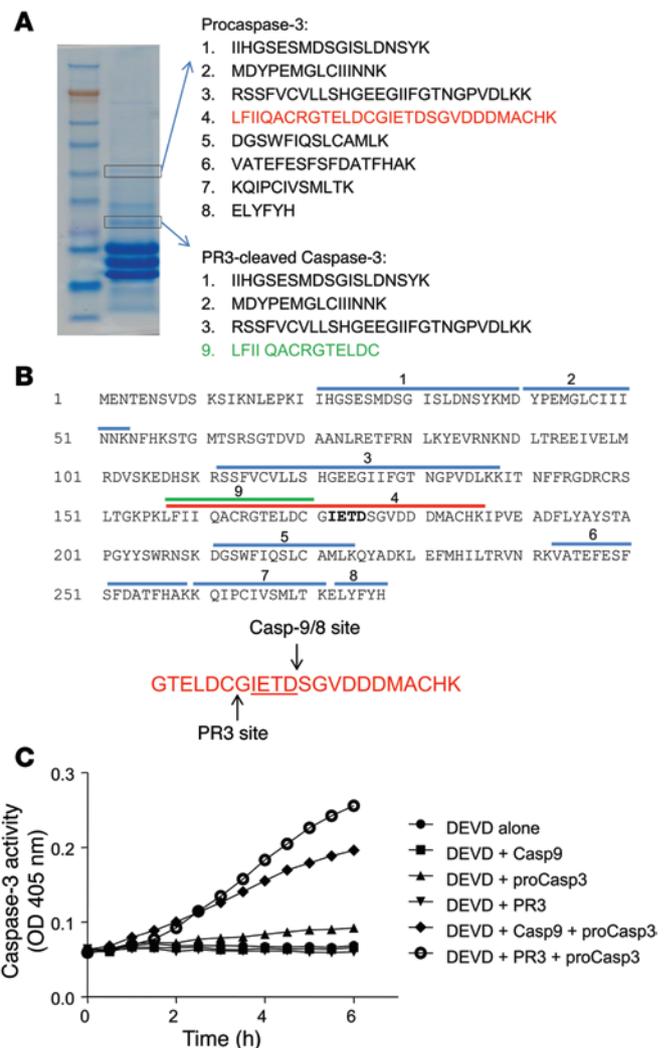

Figure 4. PR3 cleaves procaspase-3 at a site upstream of the canonical caspase-9 cutting site. (**A**) MS analysis of procaspase-3 and PR3-cleaved caspase-3. The expected position of procaspase-3 and the cleaved p20 fragment are indicated. The full-length procaspase-3 (top) and the 20-kDa fragment (bottom) were digested with endoproteinase Lys-C and analyzed by MS. (**B**) PR3 and caspase-9/8 cleaved procaspase-3 at different sites. Peptides in which one end followed the pattern of digestion by the endoproteinase Lys-C are underlined, and caspase-9/8 and PR3 cleavage sites are indicated. (**C**) Cleavage of procaspase-3 led to caspase-3 activation. PR3 was not able to cleave the DEVD substrate by itself. Results are representative of 3 independent experiments.
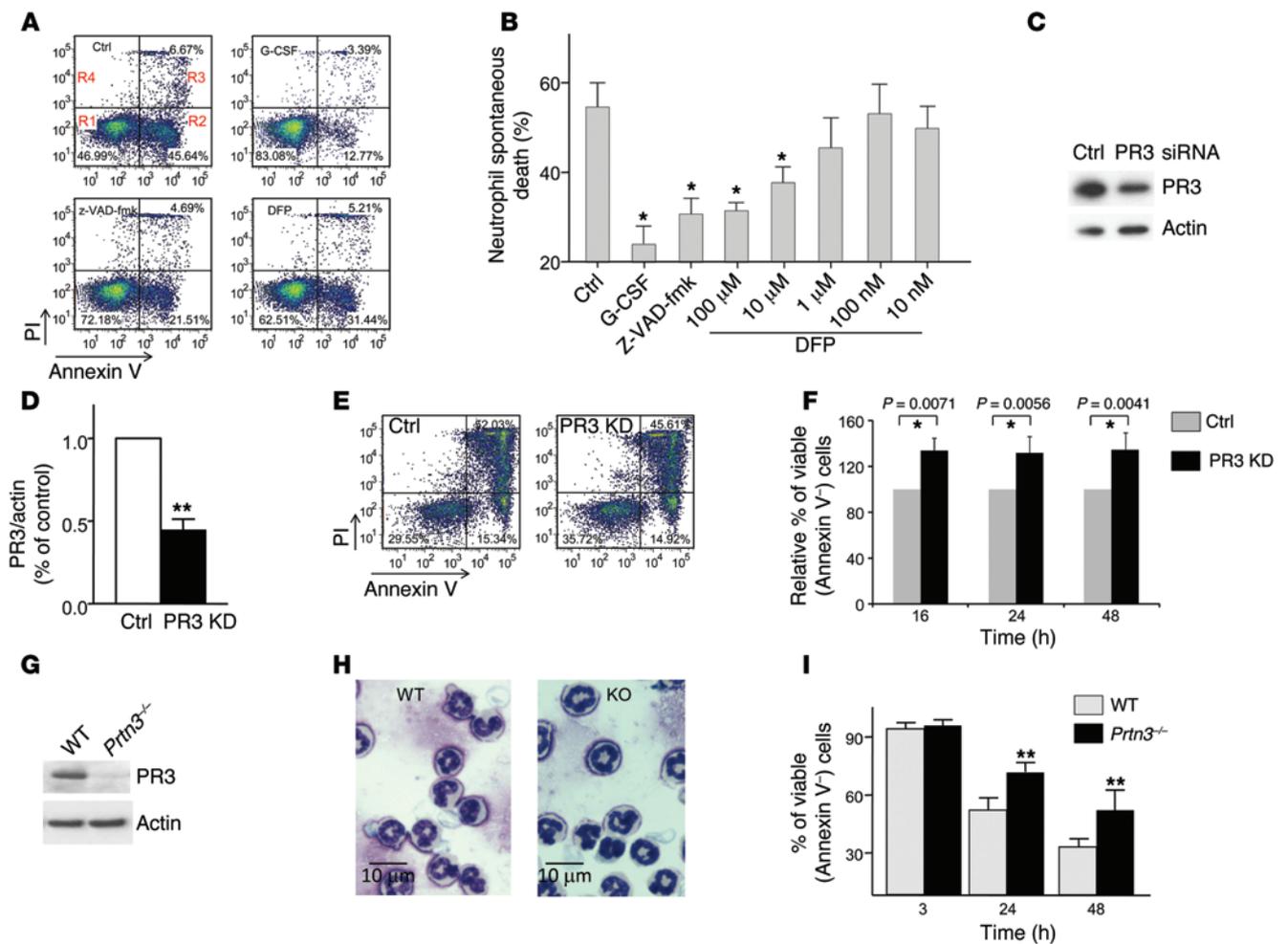
Figure 5. Inhibition of serine protease activity delays neutrophil spontaneous apoptosis in vitro. (**A**) Spontaneous death of human neutrophils, analyzed by annexin V and PI staining. Human neutrophils were cultured in the presence or absence of the indicated growth factor and protease inhibitor (G-CSF, 50 ng/ml; z-VAD-fmk, 50 mM; DFP, 100 mM) for 16 hours. The percentage of viable cells (region R1), early apoptotic cells (region R2); and late apoptotic and necrotic cells (regions R3 and R4) is indicated. (**B**) Quantification of neutrophil spontaneous death. Results are mean ± SEM of 3 independent experiments. *$P < 0.01$, Student's t test. (**C**) siRNA-mediated PR3 knockdown (KD) in murine neutrophils. Results are representative of 3 independent experiments. (**D**) Quantification of siRNA-mediated PR3 knockdown. Results are mean ± SEM of 3 independent experiments. **$P < 0.01$, Student's t test. (**E**) siRNA-mediated PR3 knockdown prolonged survival of murine neutrophils. Neutrophil death was analyzed 24 hours after siRNA nucleofection. Results are representative of 5 independent experiments. (**F**) Quantitative analysis of neutrophil spontaneous death. The percentage of viable cells at each time point was normalized to the sample transfected with control siRNA. Results are mean ± SD of 5 independent experiments. *$P < 0.01$, Student's t test. (**G**) PR3 expression was abolished in PR3-deficient murine neutrophils isolated from the femurs and tibias of 10-week-old mice. (**H**) Neutrophil morphology, examined by Wright-Giemsa staining. Scale bars: 10 μm. (**I**) Spontaneous death of WT and PR3-deficient murine neutrophils isolated from BM. Results are mean ± SD of 3 independent experiments. **$P < 0.005$.

in azurophilic granules, was also detected in the cytosolic fraction of aging cells, but to a much lesser extent (Figure 6A). Confirmatory immunofluorescence (IF) staining of young and aging neutrophils showed that PR3 signal was highly abundant and concentrated in vesicle-shaped structures in freshly isolated human neutrophils, but had a highly diffuse distribution in cells cultured for 24 hours (Figure 6, B and C), which suggests that PR3 protein concentrations in the granules decrease during neutrophil aging, with eventual PR3 release into the cytosol.

We next investigated whether PR3 is also present in the cytosolic fraction of murine peritoneal neutrophils undergoing spontaneous death; peritoneal neutrophils were chosen since they represented a more homogenous cell population than BM-derived neutrophils (Supplemental Figure 5). Cells were collected 6 hours

after thioglycollate (TG) injection and lysed either immediately or after 24 hours of culture (Figure 6D). Similar to what was observed in human neutrophils, total PR3 did not change markedly in the first 24 hours during spontaneous neutrophil death. While no PR3 was present in the cytosolic fraction of freshly isolated murine neutrophils, PR3 was present at a substantially higher level in the cytosolic fraction of cells cultured for 24 hours (Figure 6D). Decreased PR3 IF signal was also observed in the granules of murine neutrophils cultured for 24 hours (Figure 6, E and F).

We hypothesized that increases in cytosolic PR3 in aging neutrophils were the consequence of the permeabilization of granule membranes. In support of this, electron microscopy of human neutrophils showed that the membrane integrity of granules was lost during spontaneous neutrophil death (Figure 6G). We next cul-
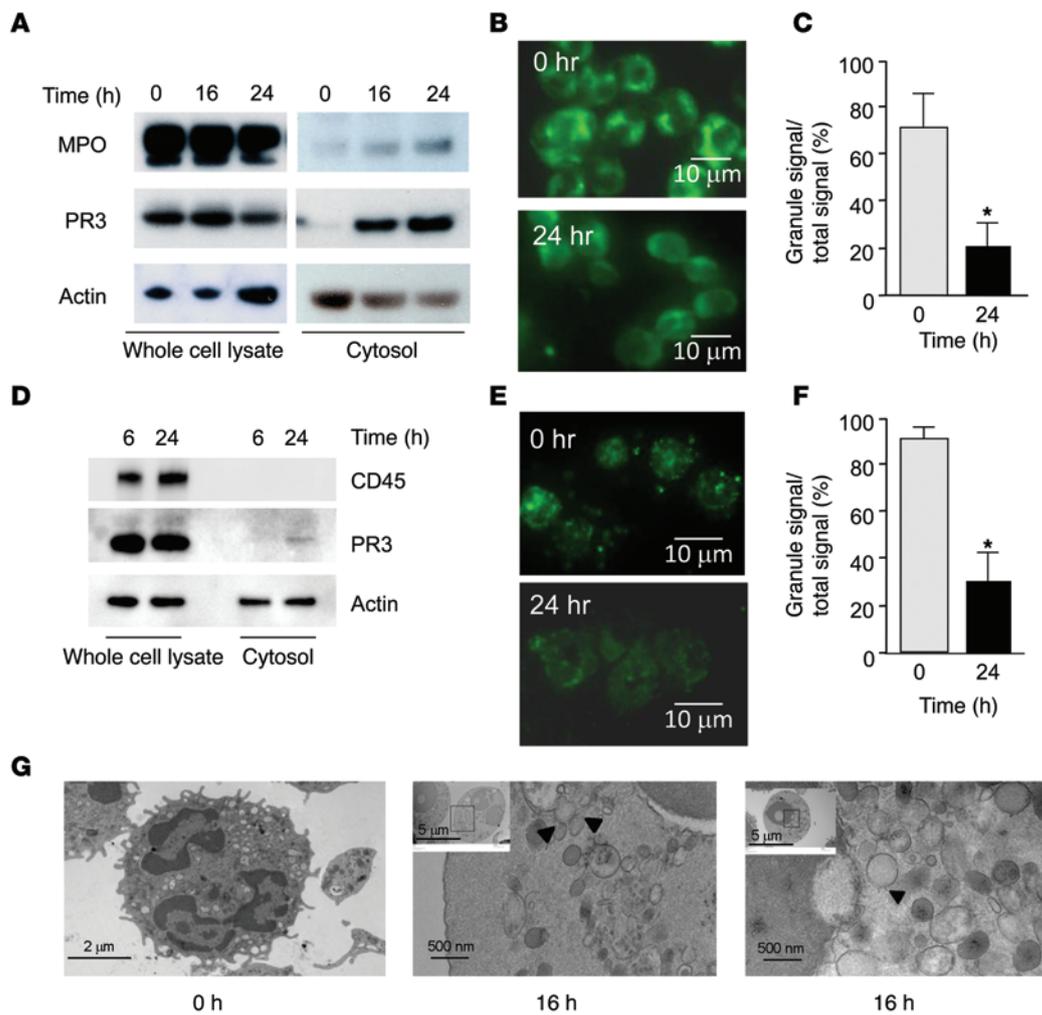
Figure 6. PR3 is released into the cytosol during the spontaneous death of both human and murine neutrophils. (**A**) PR3 and MPO protein expression in whole cell lysates and the cytosolic fraction of freshly isolated and aging human neutrophils. (**B**) Immunostaining of PR3 in freshly isolated and aging human neutrophils (24 hours). (**C**) Percentage PR3 in granules, calculated as the fluorescent intensity of granular structures in an individual cell relative to the total fluorescent intensity in the same cell. At least 15 cells were quantified. Results are mean ± SD. *$P < 0.001$, Student's t test. (**D**) PR3 expression in whole cell lysates and the cytosolic fraction of mature murine neutrophils. CD45 served as a plasma membrane marker. (**E**) Immunostaining of PR3 in freshly isolated and aging murine neutrophils. (**F**) Percentage PR3 in granules, calculated as in **C**. *$P < 0.001$, Student's t test. (**G**) Granule morphology, examined by electron microscopy. The granules in aging neutrophils were ruptured. Dark arrowheads indicate permeabilized granules. Scale bars: 10 μm (**B** and **E**); 2 μm (**G**, 0 h); 500 nm (**G**, 16 h); 5 μm (**G**, 16 h, insets).

tured human neutrophils in the presence of 2 well-characterized LMP inhibitors, Hsp70 and deferoxamine (DFO) (18, 19), as well as in the presence of G-CSF, which is known to potently delay spontaneous neutrophil death. After 24 hours of culture with or without the drugs, lysosomal membrane integrity was assessed using the acridine orange (AO) uptake method (20, 21), which enables detection of intact and permeabilized lysosomes. G-CSF, Hsp70, and DFO all decreased the percentage of cells with a reduced number of intact granules; inhibition of LMP was 30% for DFO, nearly 40% for Hsp70, and 50% for G-CSF (Figure 7, A and B). Treatment of neutrophils with DFO or Hsp70 consequently decreased the quantity of PR3 in the cytosolic fraction of aging neutrophils (Figure 7C). Culturing neutrophils in the presence of either Hsp70 or DFO led to nearly 50% inhibition of cell death, comparable to the inhibitory effect of G-CSF (Figure 7D); thus, the inhibition of LMP was highly correlated with the degree of inhibition of apoptosis.

It is noteworthy that treatment with DFO or Hsp70 did not affect chemoattractant-induced generation of ROS or the total ROS level in freshly isolated and aging neutrophils (Supplemental Figure 6, A and B), which suggests that the delayed neutrophil death by LMP inhibitors was not a result of general anti-oxidative effects.

SERPINB1 is a major regulator of PR3 activity during spontaneous neutrophil apoptosis. If PR3 can activate caspase-3 in the cytosol after release from neutrophil granules, PR3 might be released but be suppressed in freshly isolated neutrophils. The NSP responsible for procaspase-3 processing could be inhibited by a factor present in the cytosol of nonapoptotic neutrophils (Figure 8A). It is known that NSPs are the targets of serpins (22, 23), which covalently trap their targets and render them ineffective. SERPINA1 (also known as A1-AT) and SERPINB1 are major inhibitors of neutrophil serine proteinases. SERPINA1 is predominantly secreted and found in the serum and at sites of inflammation (22). SERPINA1 levels did not
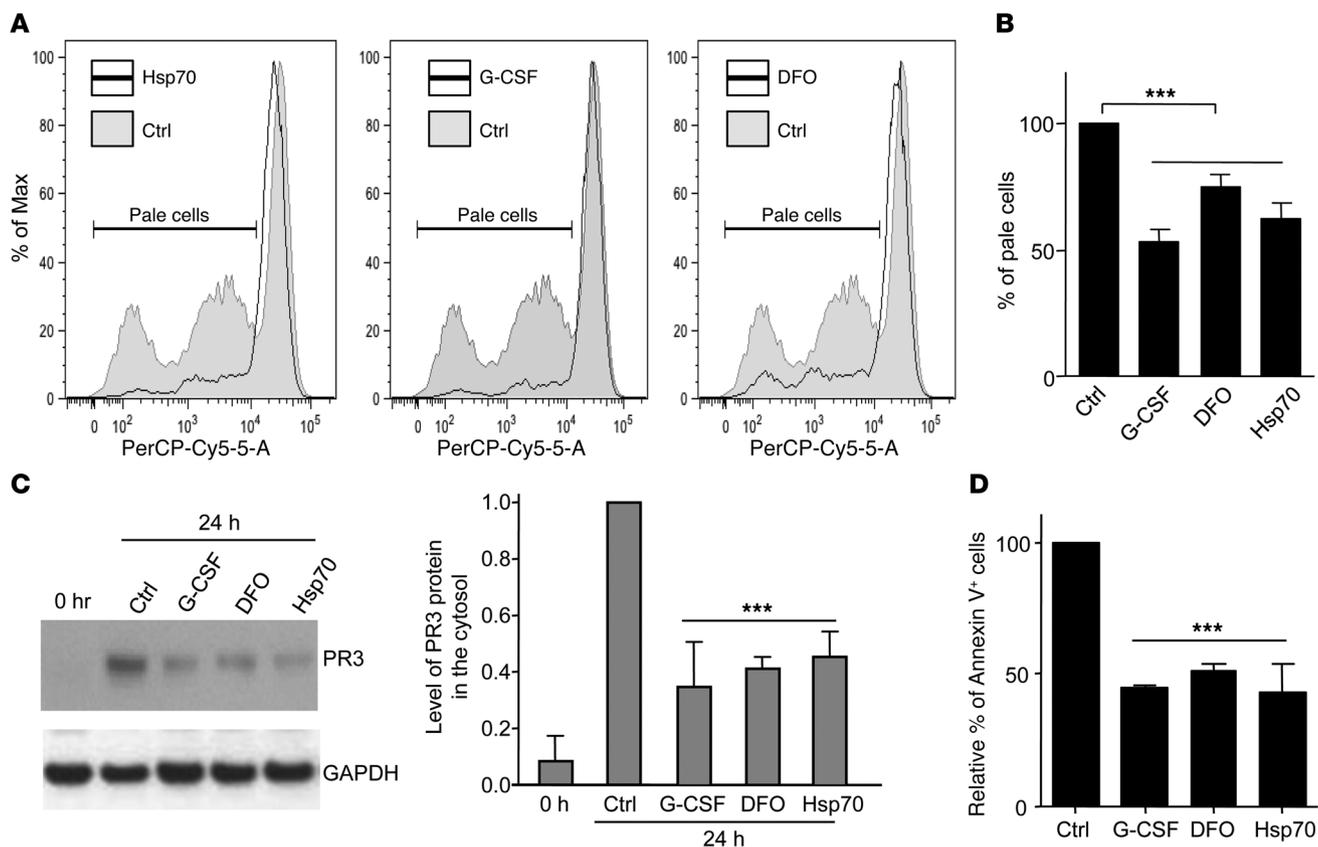
Figure 7. Inhibition of LMP delays PR3 release and neutrophil spontaneous death. (**A**) Neutrophils were assessed for lysosomal stability using the AO uptake assay of human neutrophils after 24 hours of culture in the presence of G-CSF (50 ng/ml), DFO (2 mM), or purified Hsp70 (150 nM). (**B**) Percentage of pale (AO negative) cells, normalized to that of untreated control cells. Data are mean ± SD of 3 independent experiments. ***$P < 0.001$. (**C**) LMP inhibition suppressed PR3 release to the cytosol during neutrophil spontaneous death. Relative amounts of PR3 were quantified using NIH Image and normalized to GAPDH. The level of PR3 in untreated aging neutrophils (Ctrl, 24 hours) was used as a basal signal. Data are mean ± SD of 3 independent experiments. ***$P < 0.001$. (**D**) LMP inhibition correlated with inhibition of neutrophil death, as measured by annexin V and PI staining. All samples were normalized to control (untreated) cell death. Data are mean ± SD of 3 independent experiments. ***$P < 0.001$.

change during neutrophil apoptosis, and alterations in SERPINA1 were not detectable in the cytosolic fraction of human neutrophils during the first 24 hours of culture (Supplemental Figure 7). SERPINB1 is an intracellular protease inhibitor that has been reported to protect neutrophil survival in inflamed lungs and in BM (24, 25). While total SERPINB1 did not change during spontaneous death, there was a dramatic decrease in free SERPINB1 levels in the cytosolic fraction in aging neutrophils (Figure 8, B and C). Furthermore, a band corresponding to a PR3/SERPINB1 complex was detected in the cytosolic fraction of aging neutrophils using anti-PR3 or anti-SERPINB1 antibodies (Figure 8D). These results suggest that SERPINB1 is important in preventing spontaneous death of neutrophils on release of PR3 to the cytosol. Thus, SERPINB1 possibly plays a counterbalancing role in protecting mature neutrophils from the proapoptotic activity of PR3. As previously reported (25), the rate of spontaneous death was accelerated for BM neutrophils isolated from *Serpinb1*−/− mice. The effect was significant but modest 4 hours after isolation, and was more pronounced at 24 and 48 hours (Figure 8, E and F).

Disruption of PR3 delays neutrophil death and augments neutrophil accumulation at sites of inflammation in vivo. Our data suggest that PR3 is causally related to spontaneous neutrophil death, at

least ex vivo. Neutrophil death under physiological or pathological conditions in vivo is complex and involves multiple pathways and mechanisms. To determine the relative contribution of PR3 to neutrophil death in vivo and to evaluate its physiological role in inflammation, we next investigated inflammation-associated neutrophil accumulation and death in WT and *Prtn3*−/− mice using a mouse peritonitis model. Very few peritoneal neutrophils were found in unchallenged WT and *Prtn3*−/− mice (Figure 9A). After induction of inflammation by intraperitoneal injection of live *E. coli*, the number of neutrophils in the WT peritoneal exudate reached nearly 6×10^6 after 12 hours, before decreasing at 24 hours (reflecting the rapid death of neutrophils and the replacement of neutrophils with macrophages). This decrease was attenuated in *Prtn3*−/− mice (Figure 9A), indicative of delayed neutrophil death. The number of apoptotic neutrophils was directly measured at multiple points, which confirmed that the percentage of apoptotic neutrophils decreased significantly in *Prtn3*−/− mice at the late stage of acute inflammation (Figure 9B), with more than 11% annexin V+ peritoneal neutrophils in WT mice and less than 7% in *Prtn3*−/− mice at 24 hours. Notably, gradually reduced neutrophil accumulation was still detected in *Prtn3*−/− mice (Figure 9A), although it was significantly delayed compared with WT mice. This suggests
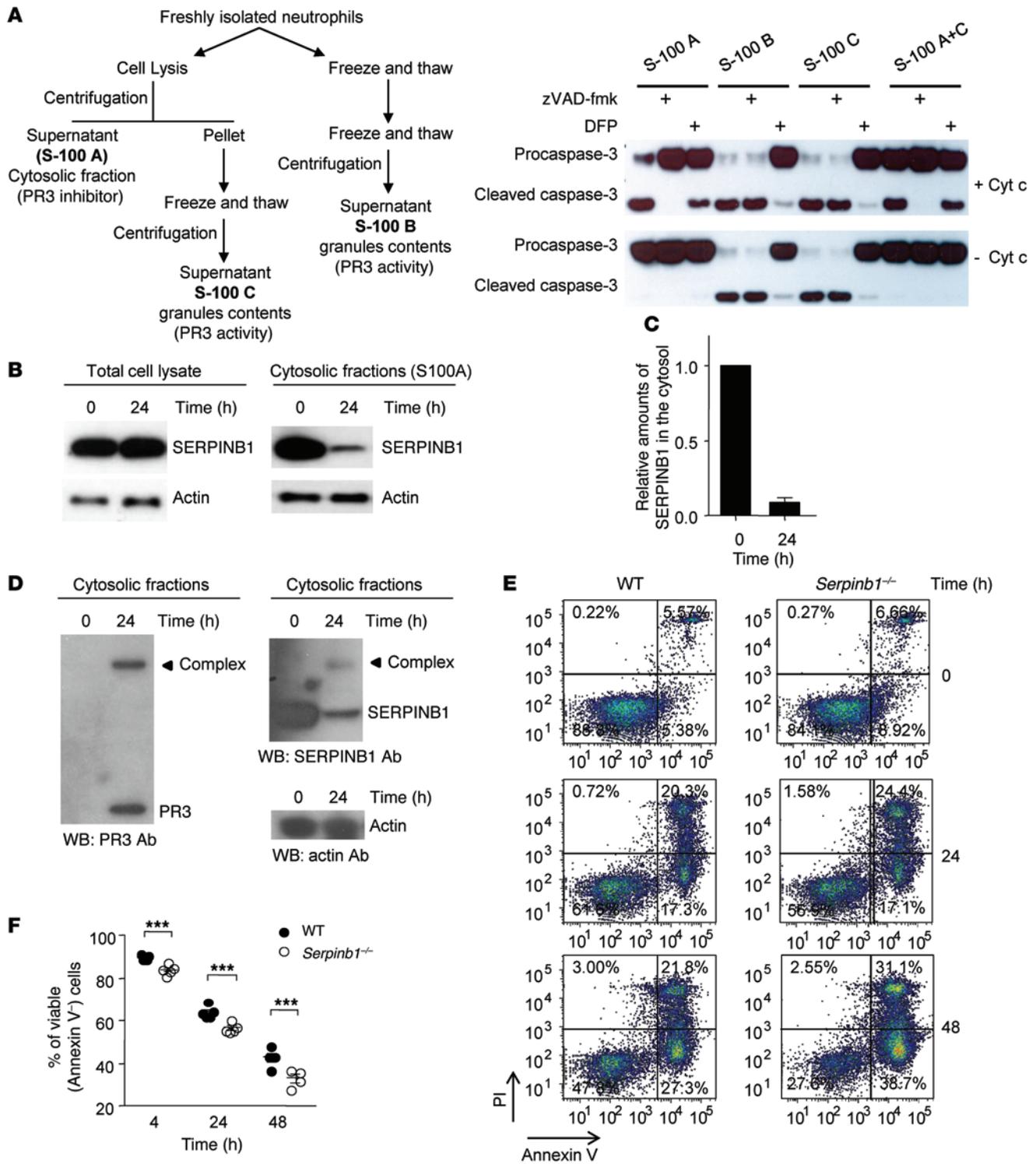
Figure 8. SERPINB1 negatively regulates neutrophil spontaneous death. (**A**) The serine protease responsible for procaspase-3 processing was inhibited by a factor present in the cytosol of nonapoptotic neutrophils. Proteins were extracted from freshly isolated neutrophils (processed as shown at left) and cultured in the presence or absence of cytochrome c, z-VAD-fmk, or DFP. (**B**) SERPINB1 expression in total cell lysates and in the cytosolic fraction was measured in freshly isolated and aging (24 hours) neutrophils by Western blotting. (**C**) SERPINB1 in the cytosolic fraction, quantified using NIH Image and expressed relative to actin. Data are mean ± SD of 3 independent experiments. (**D**) SERPINB1 protein levels decreased during neutrophil spontaneous death by complexing with proteases. The cytosolic fractions were prepared from freshly isolated and aging (24 hours) neutrophils, and levels of PR3 and SERPINB1 were assessed by Western blotting. PR3 and SERPINB1 were detected both as uncomplexed proteins (~27 and ~42 kDa, respectively) and in a complex (~65 kDa; dark arrowheads). (**E**) Spontaneous death of WT and SERPINB1-deficient neutrophils was analyzed by FACS. Mouse neutrophils were isolated from BM and cultured for the indicated times. Neutrophil spontaneous death was detected by annexin V and PI staining. Results are representative of 3 independent experiments. (**F**) Quantitative analysis of spontaneous death of WT and SERPINB1-deficient neutrophils. All results were normalized to the amount of viable WT neutrophils at the 4-hour time point. Results are mean ± SEM of 3 independent experiments. ***$P < 0.001$, Student's t test.
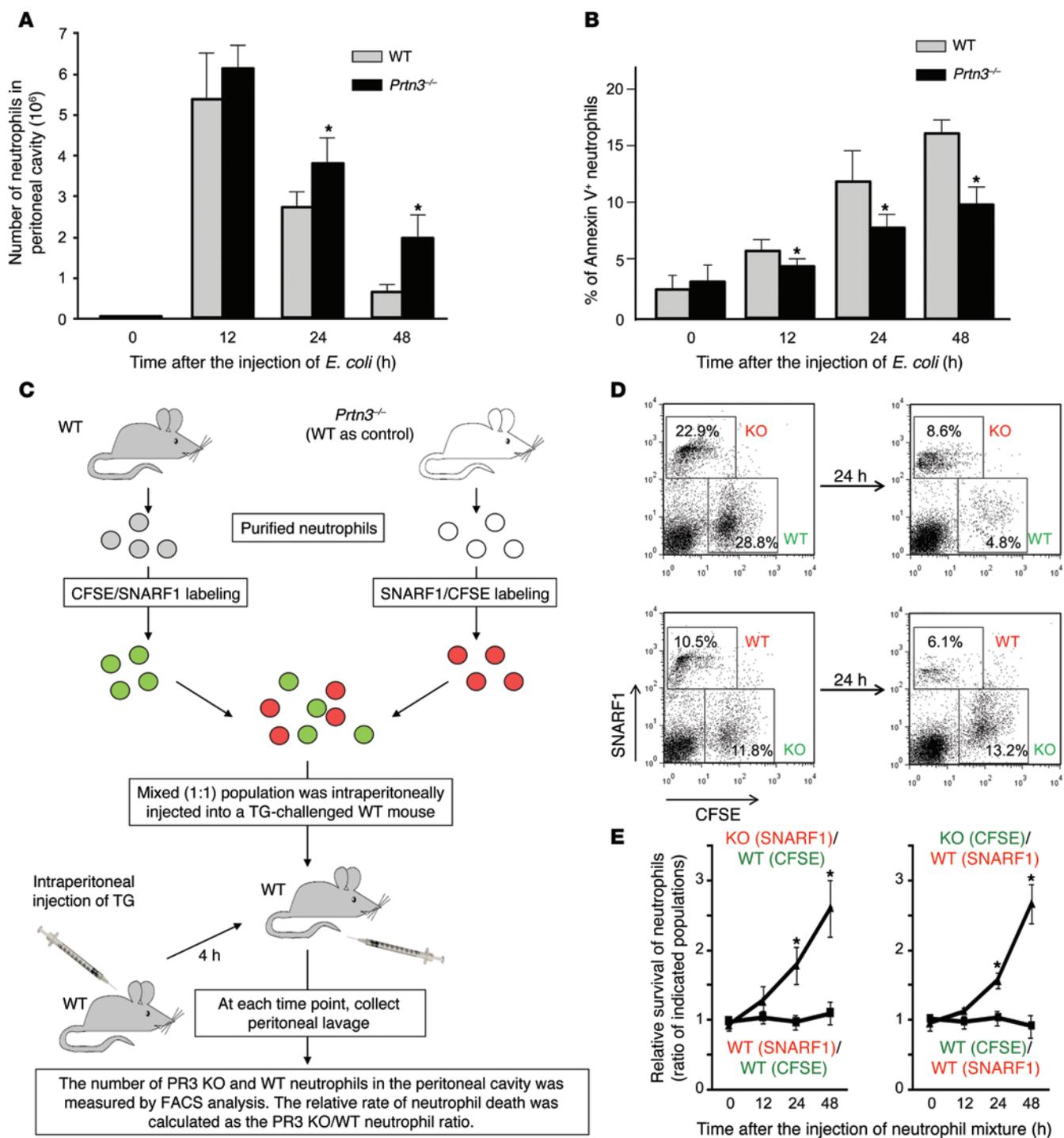
Figure 9. Disruption of PR3 delays neutrophil spontaneous death during the course of inflammation in vivo. (**A**) Enhanced neutrophil accumulation at sites of inflammation in *Prtn3*^−/−^ mice. WT and *Prtn3*^−/−^ mice were injected intraperitoneally with *E. coli*. Total neutrophil content in the lavage fluid was calculated by microscopic examination of Wright-Giemsa–stained cytospins. All values are mean ± SD of 3 separate experiments. *$P < 0.01$ vs. WT. (**B**) Reduced neutrophil spontaneous death at sites of inflammation in *Prtn3*^−/−^ mice. Apoptotic cells in peritoneal lavage fluid were detected as described in Figure 5. Neutrophils were recognized by Mac-1 and Ly6G staining. The annexin V^+^ cells included both PI^−^annexin V^+^ (early apoptotic) and PI^+^annexin V^+^ (late apoptotic and necrotic) cells. Since PI^+^annexin V^−^ cells were rarely detected, we used percentage of annexin V^+^ cells as the primary outcome measure. Data are mean ± SD (*n* = 3 mice). *$P < 0.01$ vs. WT. (**C**) Strategy for analyzing apoptosis in adoptively transferred neutrophils in the TG-induced peritonitis model. Since the purity of the neutrophils used for adoptive transfer was >90%, the majority of SNARF-1– or CFSE-labeled cells were neutrophils. (**D**) Apoptosis of adoptively transferred neutrophils. Results are representative of 3 independent experiments. (**E**) Relative neutrophil survival, calculated as the ratio of the indicated populations in the peritoneal cavity. The total number of viable cells (PI^−^annexin V^−^) was used for calculation. All values represent mean ± SD of 3 separate experiments. *$P < 0.01$ versus WT (SNARF1)/WT (CFSE) control.
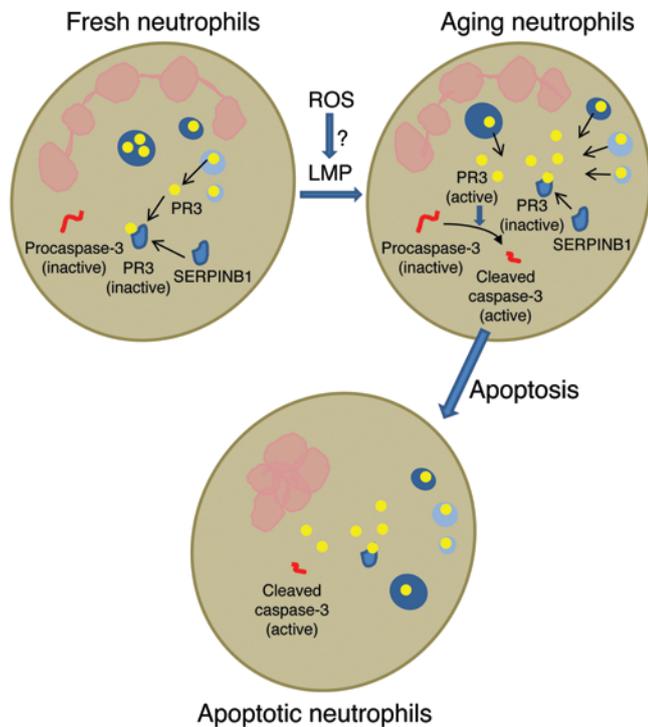
Figure 10. PR3-elicited caspase-3 activation during neutrophil spontaneous death. PR3 is sequestered in neutrophil granules in mature cells (left). If a small amount of proteases are released into the cytosol due to LMP, the protease activity can be inhibited by the suicide protease inhibitor SERPINB1. During neutrophil spontaneous death, more PR3 molecules are released from various granules, leading to cleavage and activation of procaspase-3 (right). This ultimately leads to apoptosis of aging neutrophils (bottom).

that neutrophil death during inflammation is likely mediated by multiple pathways and mechanisms, and PR3-induced caspase-3 activation is probably only one of the involved mechanisms.

Programmed neutrophil death is an essential cellular event for maintaining neutrophil numbers in infection and inflammation. However, neutrophil accumulation at sites of inflammation can also be regulated by other cellular processes, such as neutrophil recruitment (trafficking) from the circulation, cytokine production, and clearance of apoptotic neutrophils. In *Prtn3⁻/⁻* mice, PR3 was also ablated in monocytes and macrophages, which may indirectly affect neutrophil accumulation by changing the inflammatory environment in the peritoneal cavity. Additionally, the proportion of apoptotic neutrophils may also be affected by engulfment and clearance of apoptotic neutrophils by phagocytes (i.e., efferocytosis) (26, 27). Thus, to investigate whether PR3 is directly involved in neutrophil death in vivo, we conducted an adoptive transfer experiment (Figure 9C). We labeled in vitro–purified WT neutrophils with intracellular fluorescent dye, 5-(and -6)-carboxyfluorescein diacetate succinimidyl esters (CFSE), and PR3-deficient neutrophils with another dye, 5-(and -6)-chloromethyl SNARF-1 acetate, or vice versa (Figure 9D). The mixed (1:1) population was intraperitoneally injected into a WT recipient mouse. In this study, the peritoneal inflammatory environment was induced by intraperitoneal injection of TG, a polysaccharide mixture that has been widely used for decades to induce a mild peritonitis,

yielding information about the early events of inflammation. Use of TG rather than *E. coli* eliminates the effect of potential variable phagocytosis, which also directly regulates neutrophil death (1). Using this adoptive transfer method, we were able to skip the step of neutrophil recruitment and evaluate neutrophil death directly. In addition, we compared the survival of WT and PR3-deficient neutrophils in exactly the same environment, and thus variability caused by differences of individual recipient animals was eliminated. Importantly, the WT and PR3-deficient neutrophils were treated identically before being mixed together and studied in parallel so that any effects of ex vivo manipulation were controlled for, and in all cases the comparison of interest was the effect of genetic deficiency, rather than effects of ex vivo manipulation. Consistent with the ex vivo data, significantly more viable transplanted PR3-deficient neutrophils were detected in the inflamed peritoneal cavity compared with transplanted WT neutrophils (Figure 9, D and E), directly demonstrating that PR3 disruption in neutrophils can lead to delayed neutrophil death in vivo due to alterations in their intrinsic apoptotic/survival pathway, rather than an altered peritoneal inflammatory microenvironment.

Discussion

Dysregulated neutrophil death is implicated in various inflammatory, immunological, and infectious diseases, and thus novel therapeutic strategies that target neutrophil lifespan in pathological conditions are warranted. However, the exact molecular mechanisms to target are incompletely defined. Here we showed that a caspase-8 and caspase-9–independent pathway mediates neutrophil spontaneous death. A serine protease, PR3, released from granules into the cytosol in aging neutrophils via LMP, leads to cleavage and activation of procaspase-3 and ultimately to apoptosis. These results demonstrate for the first time that PR3-mediated caspase-3 activation plays a critical role in a physiologically relevant programmed cell death process and advance our general understanding of the molecular mechanisms controlling cell death (Figure 10).

The intrinsic caspase-9–mediated mitochondrial pathway and extrinsic caspase-8–mediated death receptor pathway are well recognized. Our results revealed that neither inhibition of caspase-8 activity nor inhibition of caspase-9 activity was sufficient to prevent neutrophil spontaneous apoptosis. Previous reports have indicated that caspase-8 is essential for ligand-induced (e.g., TNF-α or Fas ligand) apoptosis of neutrophils. However, neutrophils from mice lacking Fas still undergo normal spontaneous apoptosis, which suggests that the extrinsic pathway is not crucial for neutrophil spontaneous death (28). In traditional intrinsic apoptosis pathways, cytochrome c released from mitochondria is involved in the assembly of a multimolecular complex known as the apoptosome, consisting of cytochrome c, Apaf1, dATP, and caspase-9 (29), which plays an essential role in activating caspase-9 and the downstream caspase cascade (29). Intriguingly, the amount of cytochrome c in neutrophils is extremely low (1). This new caspase-9–independent mechanism could explain the lack of dependence on cytochrome c in neutrophils.

We discovered that in aging neutrophils, procaspase-3 cleavage was mediated by PR3, which is 1 of 4 active serine proteases identified in neutrophils (30). These serine proteinases are present at different concentration in different granules and have anti-

bacterial activity, but are also emerging as important mediators of the inflammatory process (15, 23, 31–34). PR3 can convert or activate many inflammatory molecules, such as IL-8, IL-32, IL-1β, TNF-α, or the antiinflammatory progranulin PGRN (32, 35). In addition, PR3 is one of the antigens recognized by anti-neutrophil cytoplasmic antibodies (ANCAs) present in Wegener's granulomatosis. PR3 has been shown to inactivate intracellular proteins such as p21, which indicates that this neutrophil proteinase could also play a role in the induction of epithelial cell apoptosis (13, 36). Cleavage of procaspase-3 by PR3 has been previously reported in a transfected mast cell line; however, this caspase-3 activation does not result in apoptosis (16).

Caspase-independent cleavage and activation of caspase-3 was also reported in other cases. For instance, the induction of apoptosis by etoposide in a myelodysplastic cell line was shown to be depend on the activation of caspase-3 and lysosomal compartment dysfunction, but independent of the mitochondrial pathway (37). A lysosomal apoptosis pathway has also been described in human leukemia HL60 cells after exposure to a synthetic retinoid (38), and in the same cell line, activation of a serine proteinase–mediated death pathway was reported in response to staurosporine (39).

Lysosomes and azurophilic granules are acidic (40). It is thought that PR3 is inactive in granules and that a neutral pH is required for full proteinase activity (41). Consistent with this, we showed that PR3 was sequestered in granules in mature neutrophils, but released from various granules to the cytosol via LMP during spontaneous neutrophil death, leading to cleavage and activation of procaspase-3 and ultimately apoptosis. Inhibition of LMP significantly inhibited release of PR3 from neutrophil granules and neutrophil apoptosis. Thus, our results identified PR3 as one of the key factors mediating LMP-elicited neutrophil death, although other components may also contribute to this type of cell death. The mechanisms leading to LMP formation are still incompletely understood. Numerous stressors, including ROS and cell death factors such as Bax, can induce LMPs. ROS are recognized as one of the causal mediators of neutrophil death (5), while glutathione (GSH), an H_2O_2 scavenger, and catalase inhibit neutrophil death (42–44). Presumably, accumulation of ROS in aging neutrophils provides a mechanism leading to LMPs.

Our data also showed that the proteinase activity of PR3 in the cytosol was neutralized by the serine proteinase "suicide" inhibitor SERPINB1, via formation of a covalent and irreversible complex. SERPINB1, a member of the clade B serpins, is mainly expressed in the cytosol (22, 23). If a small amount of PR3 is released into the cytosol due to LMP, protease activity is likely to be inhibited by SERPINB1. Thus, intracellular SERPINB1 may protect against misdirected granule proteases in mature neutrophils (45). During neutrophil spontaneous death, more PR3 molecules are released, leading to cleavage and activation of procaspase-3 (Figure 10). Consistent with this model, both our own and previously published (24, 25) data show that neutrophils lacking SERPINB1 demonstrate accelerated death. However, SERPINB1 can also inhibit other serine proteases, such as NE and CG (46). Baumann et al. recently reported that SERPINB1 is critical for neutrophil survival in the BM through cell-autonomous inhibition of CG (47). Interestingly, in contrast to the caspase-3–dependent PR3-mediated neutrophil spontaneous death described here, CG-mediated neutrophil cyto

toxicity is mediated by a caspase-3–independent mechanism via cleavage of a distinct set of targets during neutrophil apoptosis (47).

The homeostatic and pathological relevance of neutrophil death is increasingly recognized. A better understanding of the molecular mechanisms underlying neutrophil apoptosis is necessary in order to search for novel therapeutic targets and develop strategies to modify neutrophil apoptosis. Neutrophils die even in the absence of any extracellular stimuli, indicative of the existence of an intrinsic spontaneous death program. Under in vivo conditions, neutrophil death is controlled by both the intrinsic "death program" and numerous extrinsic pro- and antiapoptotic factors (reviewed in ref. 1). All extrinsic factors must exert their function by modulating the intrinsic death program. For example, G-CSF elicits activation of the prosurvival factor AKT. Fas ligand promotes neutrophil death via caspase activation. Thus, elucidating the cellular and molecular nature of the intrinsic death program is of great important for understanding the regulation of neutrophil lifespan under both homeostatic and inflammatory conditions. Indeed, in the present study, we showed that deficiency of PR3 could lead to persistence of neutrophils in an infectious peritonitis model, which suggests that this PR3-mediated spontaneous death pathway is also important in "elicited" cell death that occurs in vivo during infection or inflammation. Collectively, the results presented here provide insight into the mechanism of action of PR3 in neutrophil spontaneous death, which may represent a novel therapeutic target for the treatment of various acute and chronic inflammatory diseases.

Methods

Mice. The *Prtn3$^{-/-}$* mouse was created on a pure C57BL/6 background using standard homologous recombination techniques. Briefly, LoxP sites were inserted around exon 2 of the *Prtn3* gene in the targeting vector. Gene targeting was performed by electroporation of the C57BL/6 embryonic stem (ES) cell line Bruce 4. ES cells undergoing homologous recombinant were identified by Southern blot analysis and injected into blastocysts of C57BL/6 mice. The offspring were mated with C57BL/6-NCr mice to remove exon 2 flanked by LoxP sites in all tissues to generate whole-body knockout mice. Offspring were backcrossed to C57BL/6 mice. Germline transmission was confirmed by PCR of tail genomic DNA using the following primers: forward 1, 5′-TGCTTGGGAAGGAAAGAATG (forward in intron 1); reverse 1, 5′-GAGCTGCAGTGAAGGTTGTG (reverse in intron 2 and exon 3); forward 2, 5′-CCCTCATGCCTCATCTCAGT (forward in intron 1); reverse 2, 5′-CCTCGCTGTCTCCTGAACTC (reverse in intron 2) (Supplemental Figure 4). *Serpinb1$^{-/-}$* mice were generated as previously described (24). In all experiments performed with knockout mice, we always used corresponding littermates as WT controls.

Human primary neutrophil isolation and culture. Human primary neutrophils were isolated from discarded white blood cell filters (WBF2 filter; Pall Corp.) as described previously (11). The filters were provided by the Blood Bank Lab at Children's Hospital Boston. Erythrocytes were sedimented by adding an equal volume of dextran/saline solution (3% dextran T-500 in 0.9% NaCl) at room temperature for 25 minutes. The erythrocyte-depleted supernatants were then layered on Lymphocyte Separation Medium (1.077 g/ml Ficoll-Hypaque solution; Voigt Global Distribution LLC) and centrifuged at 400 *g* at room temperature for 20 minutes. Contaminated erythrocytes in the neutrophil pellets were lysed after a brief (<30 seconds) treatment with 0.2% NaCl. The purity of neu

trophils was >97%, as determined by both Wright–Giemsa staining and FACS analysis with CD15 antibody. We routinely obtained about $2–5 \times 10^8$ neutrophils from 1 filter (450 ml blood from a healthy donor). Neutrophil "spontaneous" death needs to be investigated using minimally manipulated neutrophils. Neutrophils isolated using this method were mostly inactive, as evidenced by minimal polarization and low F-actin level. We have compared the neutrophils collected from leukocyte filters with those obtained by vein puncture and stored in anticoagulant testing tubes, and found that the filtration method did not induce more neutrophil activation or impair any neutrophil functions (e.g., polarization, chemotaxis, phagocytosis, and time course of cell death; Supplemental Figure 8). We always coordinated with the blood bank technicians to ensure that the filters were prepared using the same standard procedure and delivered to us in time. Thus, the time points used in our experiments are all accurate. Isolated neutrophils were resuspended in RPMI 1640 supplemented with 10% heat-inactivated low endotoxin (≤5 EU/ml, usually ≤1 EU/ml), FCS (Gibco), and penicillin-streptomycin antibiotics and cultured at a density of 2×10^6 cells/ml at 37°C in a 5% CO_2 incubator. Cells were incubated in 6-well plates without swirling or agitation. In selected experiments, cells were cultured in the presence of z-VAD-fmk (Caspase Inhibitor I; Calbiochem), Caspase-9 Inhibitor II, Caspase-8 Inhibitor I, Caspase-3 Inhibitor II (Calbiochem), human recombinant Hsp70 (Stressgen), DFO (Sigma-Aldrich), or DFP (EMD).

Preparation of cell-free extracts. Purified neutrophils were washed once with ice-cold PBS, then incubated for 30 minutes on ice in Buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EGTA, 1 mM sodium EDTA, and 1 mM DTT) supplemented with protease inhibitors (0.1 mM PMSF, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 25 μg/ml ALLN). Cells were thereafter lysed by 20–30 strokes of Dounce homogenizer with pestle B. Extracts were then centrifuged at 16,000 g at 4°C for 30 minutes. The supernatants (S-100 A) were collected and stored at –80°C. The pellets resulting from the cell lysis were frozen and thawed, and the soluble proteins were isolated once more using buffer A, douncing, and ultracentrifugation (S-100 C). In some experiments, neutrophil proteins were extracted from freshly isolated neutrophils after freezing of the cell pellet (S-100 B).

Western blot analyses. Western blots were performed as previously described (11). The cleavage of N-terminal His-tagged recombinant human procaspase-3 (R&D Systems) was detected using a His-tag antibody (Cell Signaling). The antibodies against human PR3 (H-60), mouse PR3 (P20), and SERPINA1 (48D2) were purchased from Santa Cruz. MPO (2C7), human serum albumin (15C7), MMP9, lipocalin 2, HLA class I (W6/32), and SERPINB1 (ab114774) antibodies were obtained from Abcam.

Murine BM neutrophil isolation. Murine BM neutrophils were isolated by negative selection using an EasySep method following a protocol provided by the manufacturer (Stem Cell Technology). Briefly, tibias and fibulas were crushed in MACS buffer (0.5% BSA, 2 mM EDTA in PBS). The samples were passed through a 40-mm mesh and then centrifuged. The cells were resuspended and incubated with an antibody mix for the negative selection of neutrophils for 15 minutes on ice and washed. Cells were subsequently incubated for 15 minutes with a biotin selection cocktail, and (without any additional wash) for 10 minutes with streptavidin-coupled magnetic beads. The purity of neutrophils was >95%, as determined by Wright-Giemsa staining. The negatively selected neutrophils were collected and cultured in RPMI 1640 supplemented with 10% FCS and penicillin-streptomycin antibiotics.

Neutrophil spontaneous death analysis by FACS. Murine or human neutrophils were cultured for the indicated times. Cells were then harvested, washed twice with ice-cold PBS, and stained with human annexin V (APC labeled; Invitrogen) and PI following a protocol provided by the manufacturer (see Figure 5). FACS was performed using a FACS Canto flow cytometer (BD). At least 10^4 events were recorded and analyzed using FlowJo software (Tree Star). In this FACS analysis, cell debris were eliminated by appropriate gating on forward and side scatter. Neutrophil spontaneous death was calculated as the percentage of annexin V$^+$ cells (both PI$^-$annexin V$^+$ and PI$^+$annexin V$^+$) at each time point. In other experiments, neutrophil spontaneous death was assessed by the reduction of the percentage of PI$^-$annexin V$^-$ viable cells (%PMN_{viable}). At each time point (t^n), the total number of neutrophils ($PMN_{total\text{-}t^n}$), including both healthy and apoptotic cells, were counted using a hemocytometer. This number decreased gradually during neutrophil spontaneous death, particularly at the later stage. The percentage of viable cells in this population (%$PMN_{viable\text{-}t^n}$) was obtained by FACS analysis. At each time point, %PMN_{viable} was calculated relative to the total cells at time point 0 ($PMN_{total\text{-}t^0}$), as follows:
$$\%PMN_{viable} = (PMN_{total\text{-}t^n} \times \%PMN_{viable\text{-}t^n})/PMN_{total\text{-}t^0}.$$

LMP assay. Human neutrophils were isolated as described above and cultured for 24 hours. LMP was then assayed as previously described (18). Briefly, cells were stained with AO (150 nM) in RPMI plus 10% FCS for 15 minutes at 37°C, then washed once in PBS. Red fluorescence was measured by FACS in the PercP/Cy5 channel. The percentage of pale (AO-negative) cells was quantified and normalized to the percentage in untreated cells.

Caspase-3 activity assay. Caspase-3 activity was measured using a colorimetric substrate for caspase-3 (CPP32) (Enzo Life Sciences). The sequence of this substrate is based on poly(ADP ribose) polymerase (PARP) cleavage site Asp216 for caspase-3. The full-length and the cleaved procaspase-3 were incubated with the substrate for the indicated times. The rate of cleavage was monitored colorimetrically at 405 nm. Caspase-9 was used as a positive control.

IF staining. For immunostaining of PR3 in mouse and human neutrophils, cells were plated into a 35-mm glass-bottom dish (MatTek Corp.) and fixed for 5 minutes in 3% paraformaldehyde at different times after their isolation. The fixed cells were washed 3 times in PBS, then permeabilized for 10 minutes in PBS plus 0.1% Triton X-100 (PBST). Blocking was done with 1% BSA plus 0.3 M glycine in PBST for 30 minutes at room temperature. The diluted primary antibodies (1:2,000) were added and incubated overnight at 4°C. After washing 3 times with PBST, the Alexa Fluor dye-conjugated secondary antibody (1:1,000) was added and incubated for 1 hour at room temperature. Staining was visualized under a fluorescent microscope (Olympus IX71), and images were taken using a ×60 oil objective lens.

Isolation of neutrophil granules. Human neutrophil granules were isolated according to a previously described method (48). Briefly, neutrophil homogenate was obtained by nitrogen cavitation (375 psi, 5 minutes; Parr). Unbroken cells and nuclei were removed by centrifugation at 400 g (15 minutes, 4°C), and the resulting postnuclear supernatant was layered on top of a discontinuous Percoll gradient of densities (1.05, 1.09, and 1.12 g/ml). After centrifugation at 37,000 g (35 minutes, 4°C), 3 bands were visible, and these were collected with a Pasteur pipette. The bands were characterized by specific protein markers and denoted α (azurophil granules, myeloperoxidase as marker), β1 (specific granules, lipocalin 2 as marker), β2 (gelatinase

granules, MMP9 as marker), and γ (secretory vesicles/plasma membranes, serum albumin as marker) in order of decreasing density. For procaspase-3 cleavage assay, procaspase-3 was incubated with 25 mg granule proteins for 30 minutes at 37°C.

Mouse peritonitis model. $Prtn3^{-/-}$ or WT mice were intraperitoneally injected with 3×10^6 *E. coli* (strain 19138; ATCC) in 0.9% saline. At the indicated times after injection, mice were sacrificed by euthanizing with CO_2. Peritoneal exudate cells were then harvested by 3 successive washes with 10 ml HBSS containing 0.2% BSA and 20 mM EDTA. The exudate cell count (total cell number) was determined using a hemacytometer, and the differential cell count was determined by microscopic analysis of Wright-Giemsa–stained cytospins. Neutrophils were identified based on their characteristic nuclear morphology. The neutrophil composition in the exudates was determined by analysis of 10 images captured using a ×100 objective. The total number of neutrophils in the peritoneal exudates was then calculated accordingly. Results were represented as mean ± SD of 5 WT or $Prtn3^{-/-}$ mice.

Apoptosis of adoptively transferred neutrophils. The in vitro purified WT and PR3-deficient neutrophils were labeled with intracellular fluorescent dyes CFSE (final concentration, 0.2 μM) and SNARF-1 (final concentration, 1.0 μM) at 37°C for 10 minutes. These fluorescent dyes are routinely used for tracing transplanted hematopoietic cells. Nearly 100% neutrophils can be labeled, and cell labeling with CFSE or SNARF-1 does not alter the lifespan or functions (e.g., chemotaxis, polarization, phagocytosis, and NADPH oxidase activity) of in vitro cultured neutrophils (49). Induction of peritonitis was conducted in WT mice. At 4 hours after TG injection (3% in 1 ml PBS), fluorescent-labeled WT and PR3-deficient neutrophil mixture (10^6 cell for each population) was injected into the inflamed peritoneal cavity of the same receipt mice. 24 hours after injection, mice were sacrificed, and peritoneal exudate cells were recovered by peritoneal lavage. The numbers of adoptively transferred WT and PR3-deficient neutrophils were measured at each indicated time point using a BD FACSCanto II flow cytometer and BD FACSDiva software. Relative death of WT and PR3-null neutrophils was calculated as the ratio of indicated populations.

Superoxide production. Measurement of ROS in stimulated neutrophils was conducted as previously described (49, 50). Briefly, murine neutrophils were resuspended at a density of 1×10^7/ml in HBSS and kept on ice until use. A reaction mixture containing 20 ml of 0.5 mM isoluminol, 10 ml of 80 U/ml horseradish peroxidase (Type XII; Sigma-Aldrich), 40 ml of cells, and 110 ml of HBSS was added into each well of a 96-well Maxisorp plate (Nunc) and allowed to equilibrate to 37°C for 4 minutes in a 1420 Wallac Victor² (PerkinElmer) multilabel counter. Chemoattractant (20 ml of 10× concentrated) was then added to the reaction mixture via the injection port of the luminometer, and luminiscence was recorded (for 2 seconds) at fixed time intervals.

Knockdown of PR3 in murine BM neutrophils. To knock down PR3 in murine BM neutrophils, we used a predesigned ON-TARGETplus SMARTpool siRNA reagent for mouse *Prtn3* (L-062718-01; Dharmacon). This siRNA reagent is a mixture of 4 siRNAs provided as a single reagent, providing advantages in both potency and specificity. The reagent is guaranteed to silence by 75% or better. The patented modifications on the siRNAs also significantly reduce the off-targets (by up to 90% compared with unmodified siRNA). The negative control used was an ON-TARGET plus nontargeting siRNA1 (Dharmacon). For gene silencing, BM neutrophils were purified as previously described, and 3×10^6 cells were resuspended in 100 μl Cell Line V nucleofector solution,

mixed with 5 μM siRNA (negative control or *Prtn3*), and nucleofected using program Y-001 per the manufacturer's recommendations. Cells were then cultured in 2 ml RPMI plus 20% FBS for 2 hours, washed, and harvested at different time points for Western blot and FACS analysis (to measure knockdown efficiency and cell survival, respectively). Efficient PR3 knockdown was confirmed by Western blotting.

Electron microscopy. After culture in RPMI supplemented with 10% FCS for different times, human neutrophils were pelleted and fixed with 2.5% glutaraldehyde, 1.25% paraformaldehyde, and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4). Pellets were then washed in cacodylate buffer, postfixed with 1% osmiumtetroxide (OsO_4) and 1.5% potassium ferrocyanide ($KFeCN_6$), washed, and dehydrated in grades of alcohol, before finally being embedded in TAAB Epon. Ultrathin sections (~60 nm) were cut using a Reichert Jung Ultracut S microtome, mounted on copper grids, stained with lead citrate, and analyzed under a JEOL 1200EX transmission electron microscope.

MS analysis of procaspase-3 cleavage. Cell free extracts (7 mg protein) of cultured neutrophils (S-100 fraction) were prepared as described above and incubated with N-terminal His-tagged recombinant procaspase-3 (20 mg) at 37°C for 1 hour. His-tagged proteins were then enriched by precipitation using Ni-NTA beads in the presence of DFP (100 mM). After 3 washes, the His-tagged proteins were eluted using imidazole and resolved on a SDS-PAGE. Bands at 32 and 20 kDa were cut from the gel and analyzed by MS at the Proteomics Center at Children's Hospital Boston. Samples were digested using endoproteinase Lys-C. The resulting MS/MS spectra were searched against a human protein sequence database with emphasis on Lys-C and semi–Lys-C peptides.

Statistics. Analysis of statistical significance for the indicated datasets was performed by 2-tailed Student's *t* test or ANOVA using Graph-Pad Prism 5. A *P* value less than 0.05 was considered significant.

Study approval. All procedures involving mice were approved and monitored by the Children's Hospital Boston IACUC. Human peripheral blood was drawn from healthy blood donors. Subjects provided informed consent prior to their participation in the study. All protocols related to human samples were approved by the Children's Hospital Boston IRB and subjected to annual review.

Acknowledgments

Address correspondence to: Hongbo R. Luo, Enders Research Building, Room 811, Boston, Massachusetts 02115, USA. Phone: 617.919.2303; E-mail: Hongbo.Luo@childrens.harvard.edu. Or to: Yuanfu Xu, Institute of Hematology and Blood Diseases Hospital, 288 Nanjing Rd., Tianjin 300020, China. Phone: 0086.22.23909415; E-mail: xuyf@ihcams.ac.cn.

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