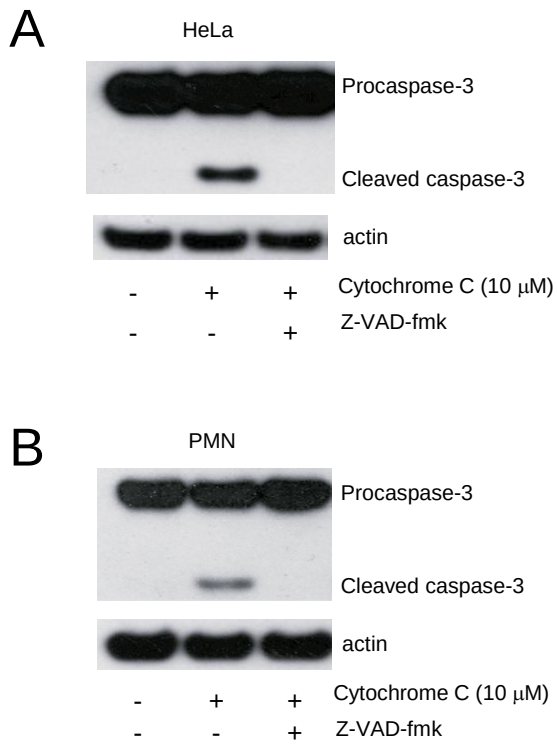

Figure S1. (A) Pan-caspase inhibitor z-VAD-fmk inhibits cytochrome c-mediated procaspase-3 cleavage. The recombinant procaspase-3 was incubated at 37'C for 30 min with the cytosolic fraction of Hela cells in the presence or absence of cytochrome c and/or z-VAD-fmk as indicated. The procaspase-3 cleavage was analyzed as described in Figure 1. **(B) Neutrophils are capable of conducting cytochrome c-mediated procaspase-3 cleavage.** The recombinant procaspase-3 was incubated at 37'C for 30 min with the cytosolic fraction of freshly prepared human neutrophils in the presence or absence of cytochrome c and/or z-VAD-fmk as indicated. The procaspase-3 cleavage was analyzed as described in Figure 1.

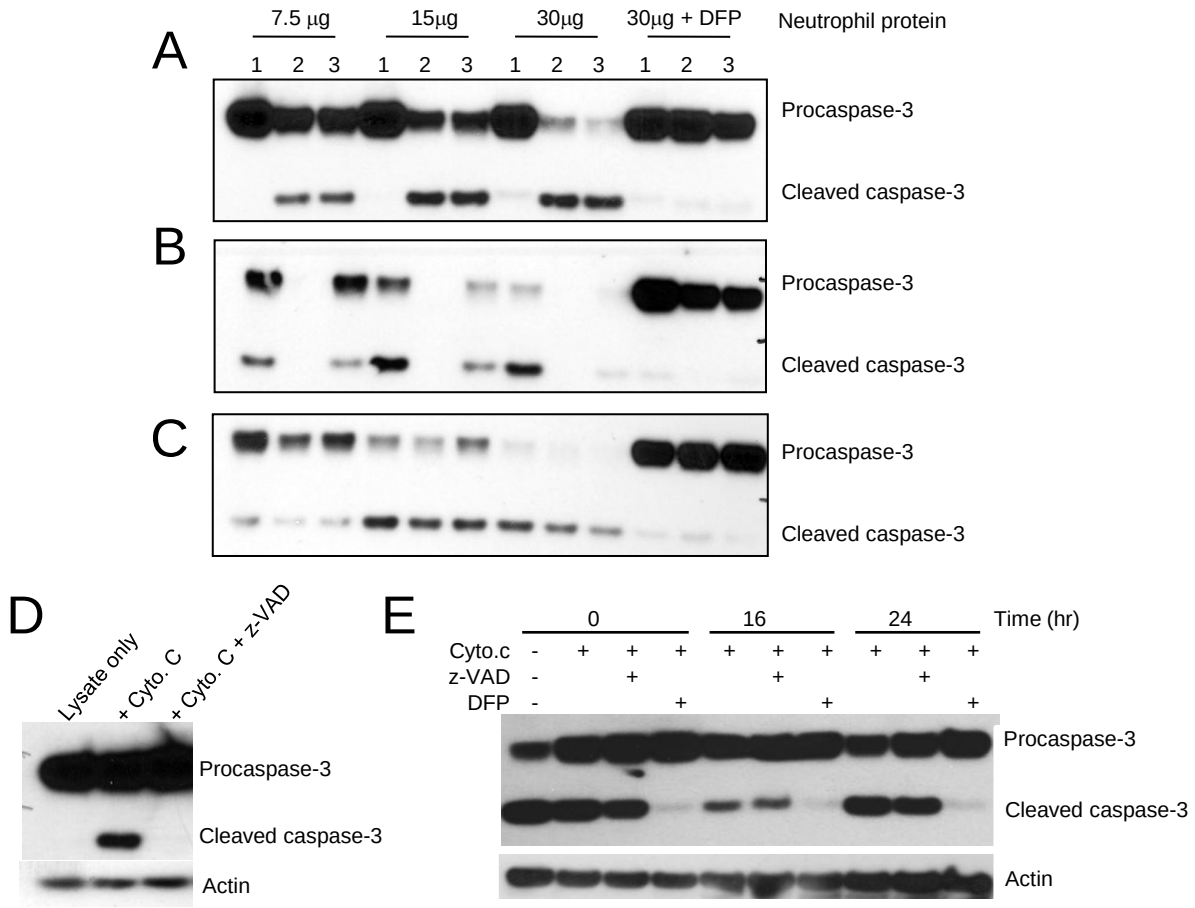

Figure S2. (A-C) The serine protease activity responsible for procaspase3 cleavage is also expressed in non-apoptotic neutrophils. The procaspase3 cleavage assay was performed as previously described. rhCasp3 was incubated with 7.5, 15 or 30 ug of neutrophil protein extracts before performing anti-his-tag western blotting. Neutrophil proteins were extracted from freshly isolated neutrophils as described in the Materiel and Methods, with the following modifications. **(A)** Neutrophils were resuspended in hypotonic Buffer A followed by sonication (lane 3) or not (lane 1) and lyzed with a douncer. Alternatively, cells were resuspended in Buffer A containing 0.1%-triton (lane 2). After centrifugation at 100,000g for 1 hour, the supernatant was kept at -80 'C. **(B)** The cell pellets resulting from cell lysis in (A) were frozen, thawed and soluble proteins were isolated once more using buffer A, douncing and ultracentrifugation. **(C)** Neutrophil s were frozen as dry cell pellet immediately after isolation, and soluble protein extraction was processed as described for panel (A). **(D-E) The serine protease activity responsible for procaspase-3 cleavage can be released to the cytosol of neutrophils by freeze/thaw lysis. (D)** Caspase-dependent procaspase-3 cleavage activity, but not Pr3 activity exists in the cytosol of freshly isolated neutrophils. Fresh neutrophils were resuspended in hypotonic Buffer A following isolation. Soluble protein extraction was prepared as described in the Materiel and Methods. Procaspase-3 was then incubated with the neutrophil protein extracts in the presence of cytochrome c (activator of the apoptosome) and/or z-VAD (pan-caspase inhibitor). Procaspase-3 cleavage was detected using an anti-his-tag antibody. Actin was used as a loading control. **(E)** Isolated neutrophils were frozen as dry cell pellets after cultured for indicated time periods (0, 16 and 24 hrs), and then were resuspended in hypotonic Buffer A. The cleavage of procaspase-3 by the cell extract in the presence of cytochrome c , z-VAD and/or DFP was analyzed as described above.

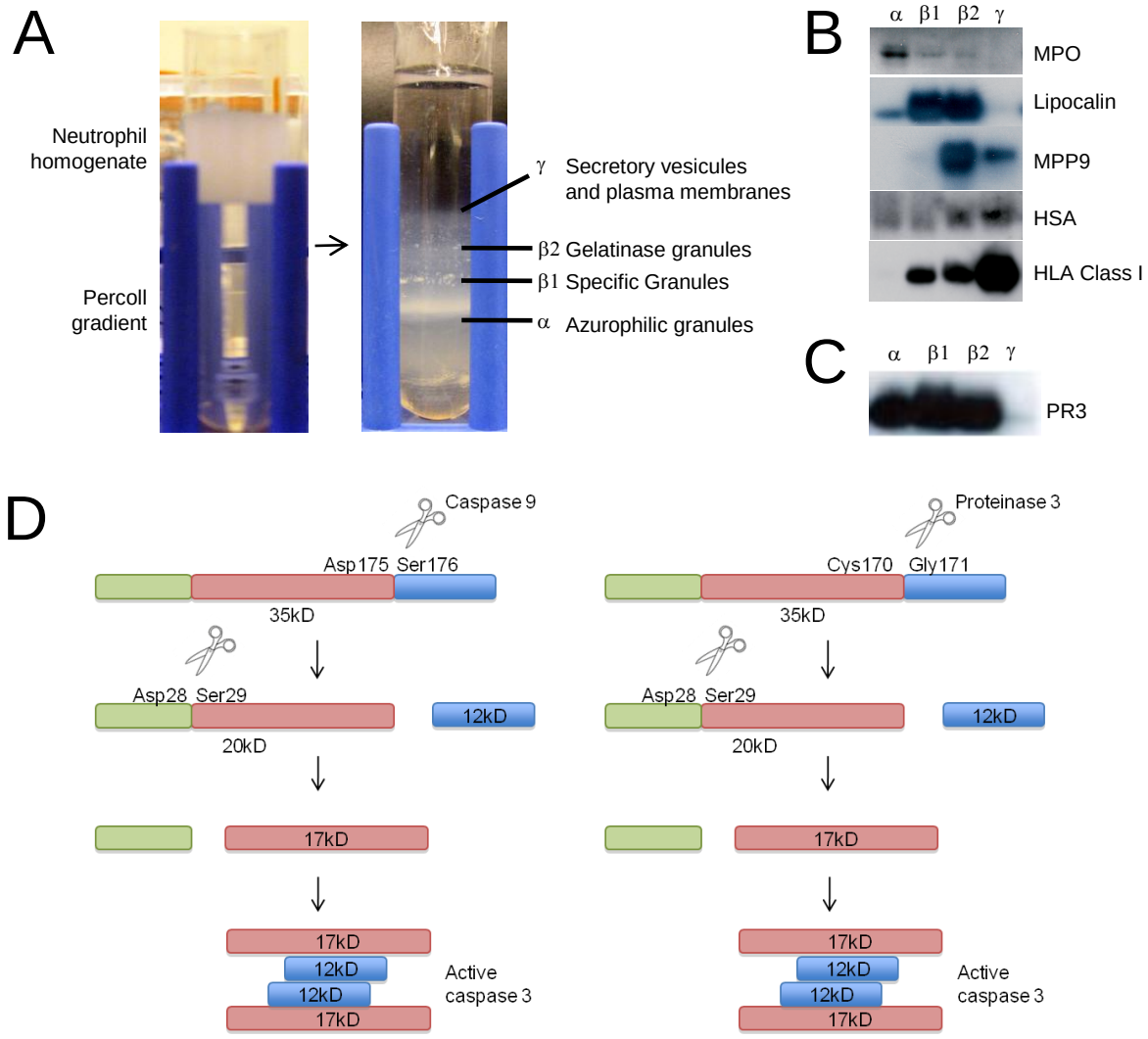

Figure S3. (A-C) Protease 3 is present in isolated neutrophil granules. (A) Isolation of neutrophil granules. Human neutrophil granules were isolated according to the method previously described (material and methods). Briefly, freshly isolated human neutrophil homogenate was obtained by nitrogen cavitation. After removal of the nuclei and unbroken cells, the post-nuclear supernatant was layered on top of discontinuous Percoll gradient of densities. The granules were separated by centrifugation at 37,000xg (35 min, 4°C) and four bands were visible and collected using a Pasteur pipette. **(B)** Subcellular fractionation of neutrophils was performed to isolate azurophil granules (a), specific (b1), gelatinase (b2), and secretory vesicles (g). Myeloperxidase (MPO) is a marker of azurophilic granules; Lipocalin is a marker of specific and gelatinase granules; Matrix Metalloproteinase 9 (MPP9) is a marker of gelatinase granules. Human Serum Albumin (HSA) is a marker of secretory vesicles and Human Leukocyte Antigen (HLA) is present on the plasma membrane. **(C)** PR3 is present in the isolated azurophil granules (a), specific (b1), gelatinase (b2), and secretory vesicles (g). **(D) Caspase 8/9 and protease 3 induced cleavage and activation of procaspase-3.**

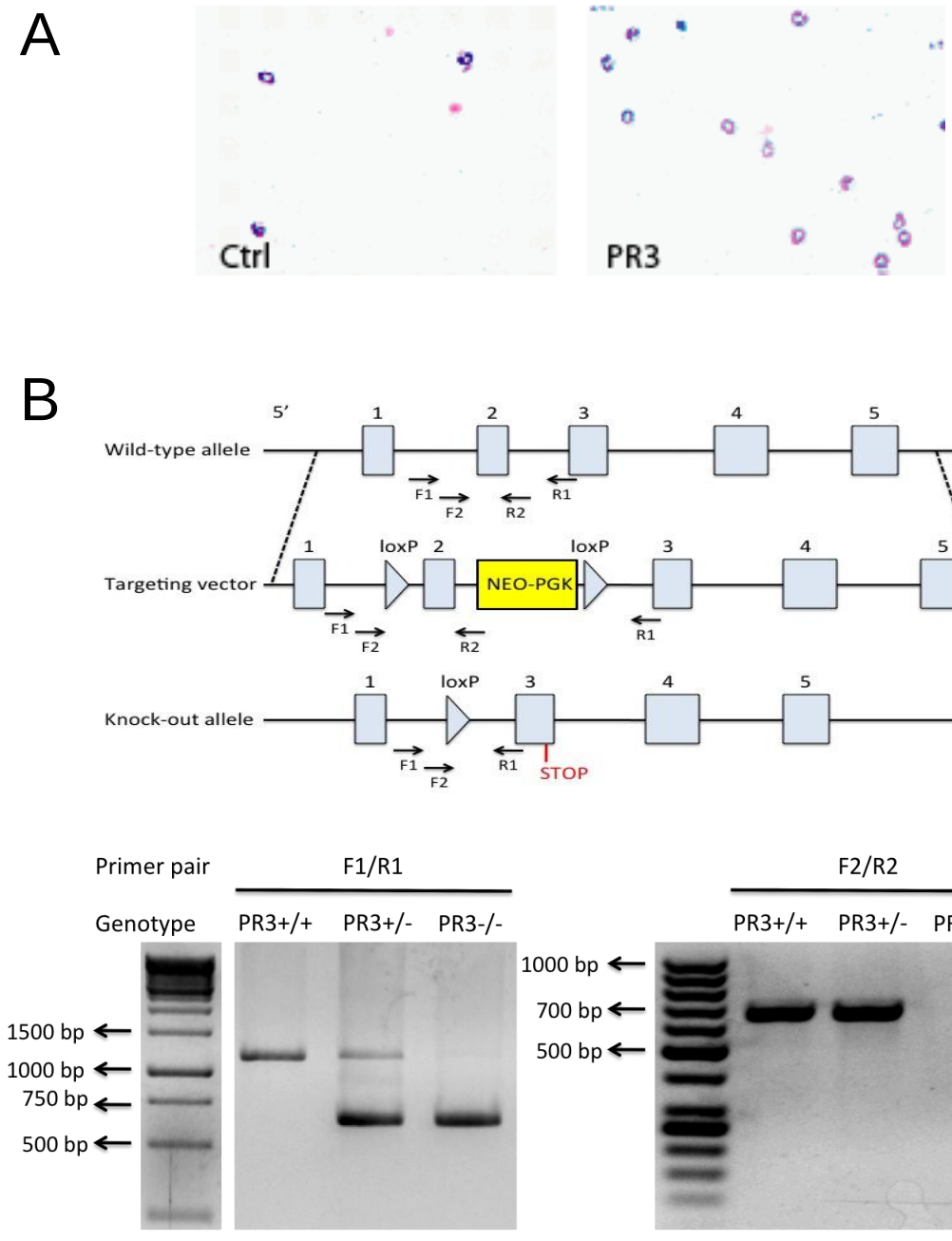

Figure S4. (A) Neutrophils after nucleofection. Wright Giemsa staining of cultured cells 24h after nucleofection showing typical PMN morphology, confirming that the surviving cells are neutrophils and not other myeloid cells. **(B) Generation of Pr3 deficient mice.** The PR3 knockout mouse was created on a pure C57BL/6 background using standard homologous recombination techniques. The primers used for genotyping and the PCR fragments from ET and KO allele are indicated.

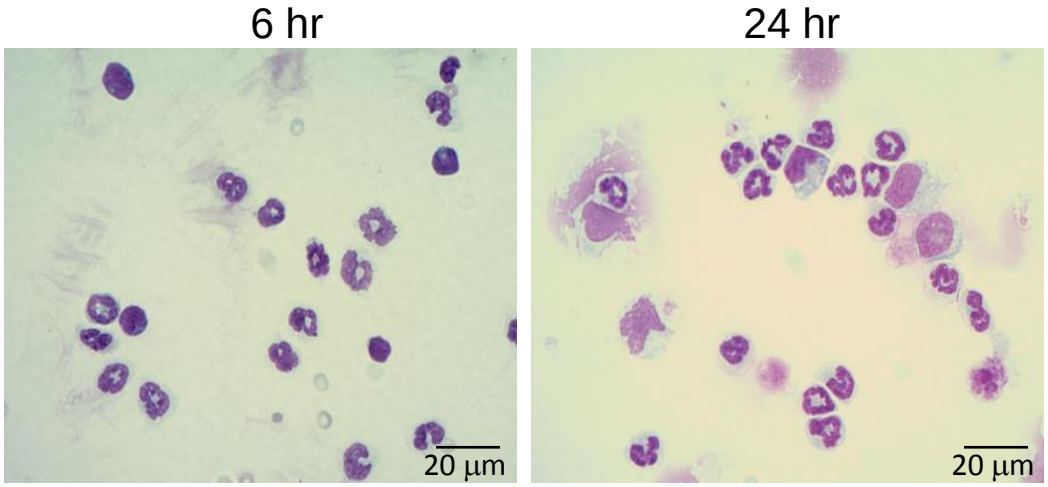

Figure S5. Wright Giemsa staining of murine peritoneal neutrophils. Peritoneal neutrophils were collected from WT mice at the indicated time points after the injection of 1 ml 3% thioglycollate.

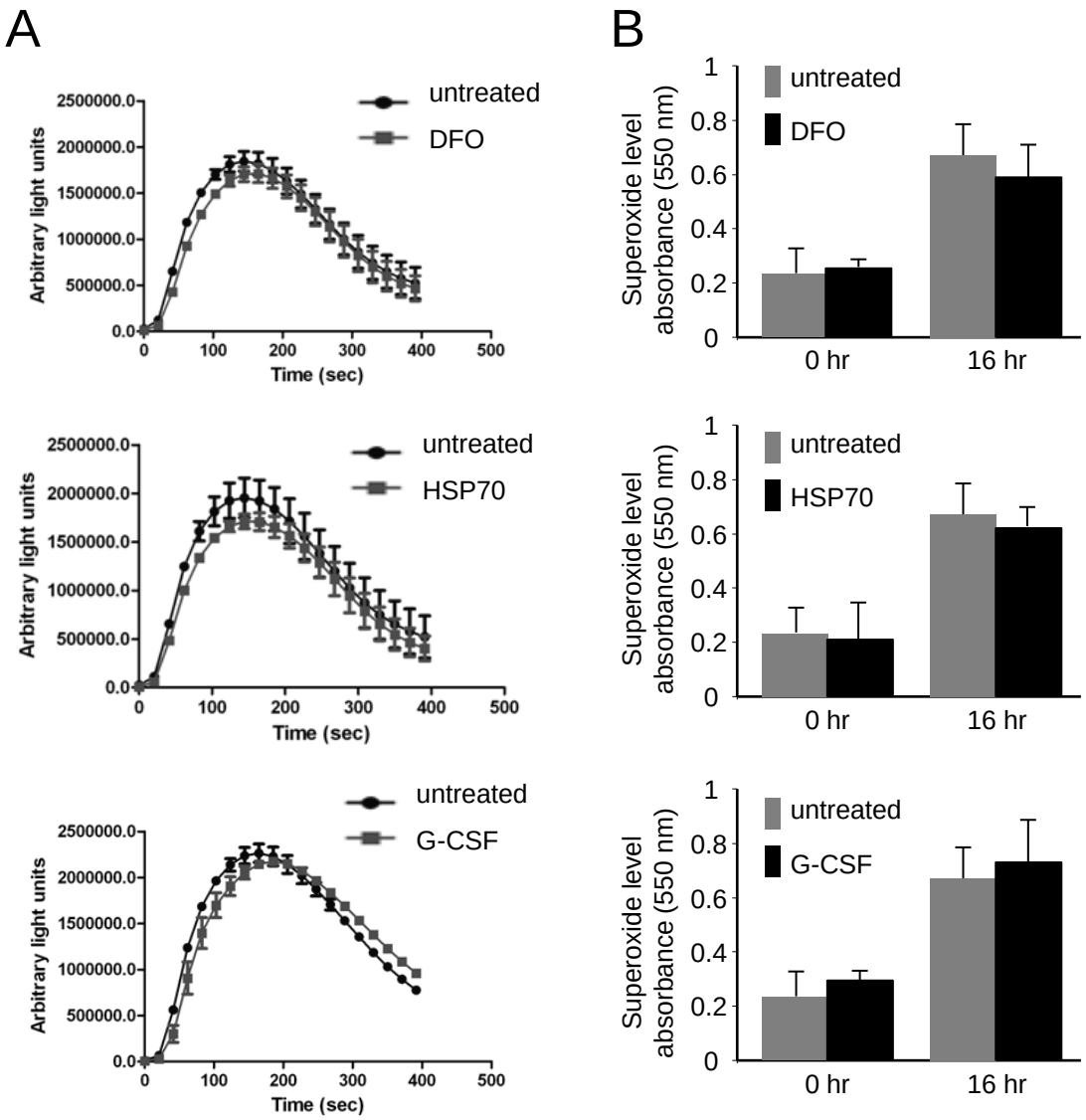

Figure S6. (A) Treatment with DFO, G-CSF, or HSP70 does not affect fMLP-elicited ROS production in neutrophils. Human primary neutrophils ($5x10^5$) were treated with DFO (200 uM), GCSF (100 ng/ml) or HSP70 (200 nM) for 5 hr, and them stimulated with 500 nM fMLP. ROS production was monitored in the presence of 50 μM isoluminol and 0.8U of HRP in a luminometer at 37°C. Chemiluminiscence (Arbitrary Light Units) was recorded (for 2 sec) at indicated time points. Untreated and drug treated neutrophils were assayed in parallel. Data are mean ± SD from one experiment representative of three. **(B) Treatment with DFO, G-CSF, or HSP70 does not alter total ROS level in freshly isolated or aging neutrophils.** The levels of reactive oxygen species in neutrophils were assessed using a cytochrome-C assay as previously described (Xu et al., 2010). Briefly, freshly prepared human neutrophils (10^7/data point) were culture for indicated periods of time, washed, and resuspended in 1 ml HBSS containing 1.5 mg/ml cytochrome-C. The cells were then filter-lysed through 2 layers of 5-μm filter membrane. After 5 min at RT, cytochrome-c reduction in each sample was detected by spinning down cells debris and reading absorbance (at 550 nm) of the supernatant in a spectrophotometer. The absorbance represents the level of superoxide ion in each sample. All values were normalized to the number of intact cells (PI^- cells) in the culture. Shown are means ± SD of three independent experiments. As reported previously (Xu et al., 2010), reactive oxygen species accumulate during the course of neutrophil spontaneous death. However, treatment with DFO, G-CSF, or HSP70 did not affect ROS level in freshly isolated or aging neutrophils.

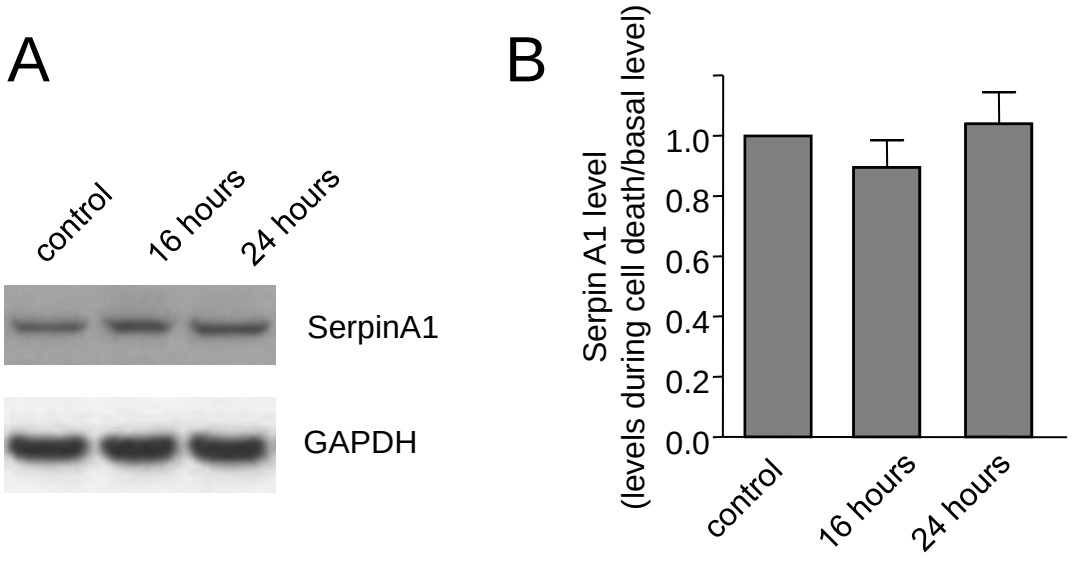

Figure S7. The level of SerpinA1 in the cytosol does not change during neutrophil spontaneous death. **(A)** Proteins in the cytosolic fraction of freshly isolated and aging neutrophils were resolved on SDS-PAGE. The levels of Serpin A1 and GAPDH were detected by Western blot using specific antibodies. Representative results of 3 experiments are shown. **(B)** Relative amounts of Serpin A1 were quantified using NIH Image software. All samples were normalized to the amount of GAPDH. "Basal signal" refers to the level of Serpin A1 in freshly isolated neutrophils. Data presented are the means (± SD) of three independent experiments.

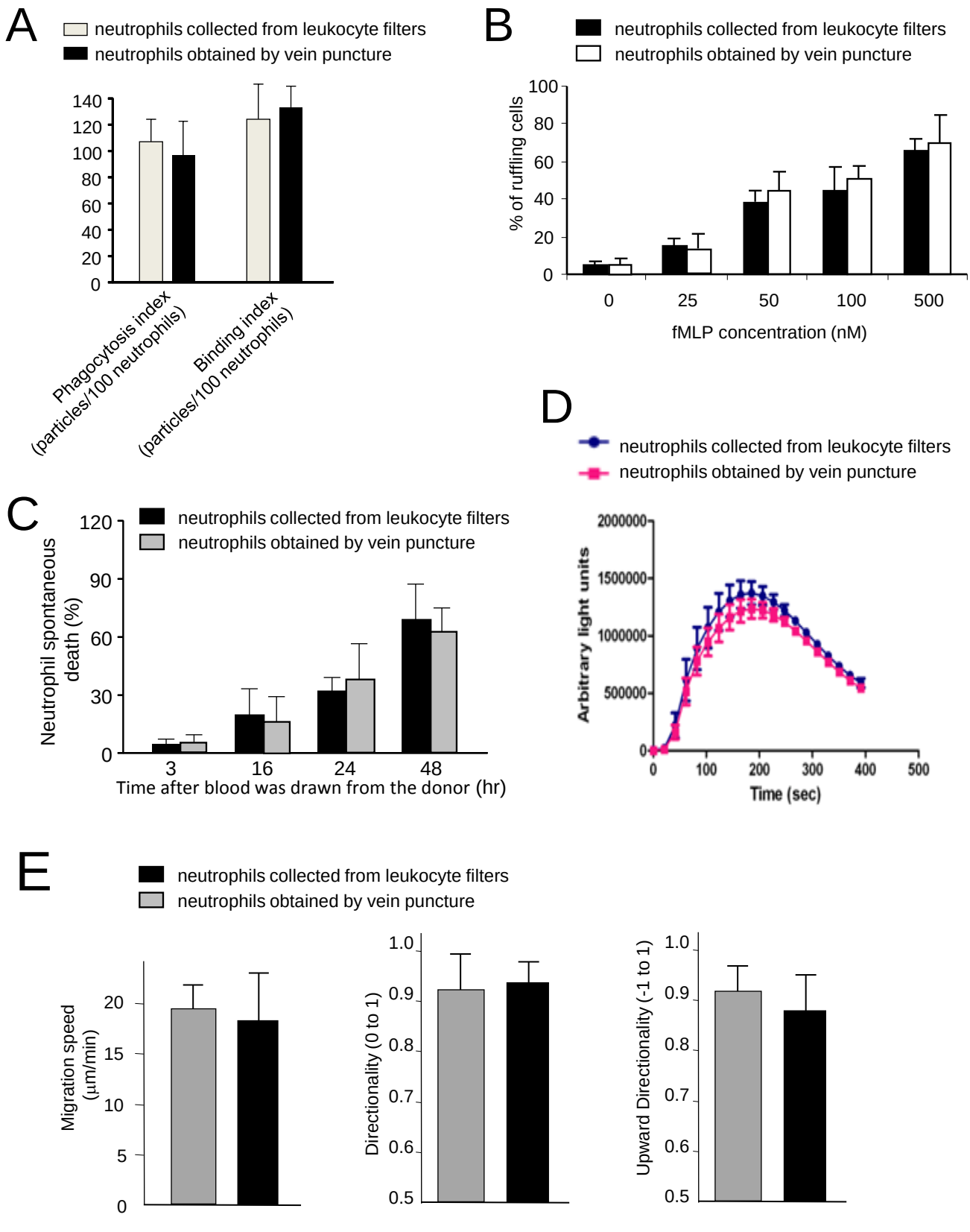
A
neutrophils collected from leukocyte filters
neutrophils obtained by vein puncture
140
120
100
80
60
40
20
0
Phagocytosis index (particles/100 neutrophils)
Binding index (particles/100 neutrophils)
B
neutrophils collected from leukocyte filters
neutrophils obtained by vein puncture
% of ruffling cells
100
80
60
40
20
0
0
25
50
100
500
fMLP concentration (nM)
C
neutrophils collected from leukocyte filters
neutrophils obtained by vein puncture
Neutrophil spontaneous death (%)
120
90
60
30
0
3
16
24
48
Time after blood was drawn from the donor (hr)
D
neutrophils collected from leukocyte filters
neutrophils obtained by vein puncture
Arbitrary light units
2000000
1500000
1000000
500000
0
0
100
200
300
400
500
Time (sec)
E
neutrophils collected from leukocyte filters
neutrophils obtained by vein puncture
Migration speed (μm/min)
20
15
10
5
0
Directionality (0 to 1)
1.0
0.9
0.8
0.7
0.6
0.5
Upward Directionality (-1 to 1)
1.0
0.9
0.8
0.7
0.6
0.5

Figure S8. The filtration method does not induce more neutrophil activation or impair any neutrophil functions. (A) In vitro phagocytosis assay was conducted as previously described (49,50). Human neutrophils obtained by vein puncture or collected from leukocyte filters were prepared as described in Methods. Cells were incubated with FITC-labeled zymosans (opsonized with human serum) at 37°C for 1 hour. Extracellular fluorescence was quenched by trypan blue. Phagocytosis index was expressed as the number of bioparticles engulfed by 100 neutrophils. Binding index was expressed as the number of bioparticles bound to 100 neutrophils. Data shown are mean ± SD of three experiments. **(B)** Chemoattractant-induced neutrophil polarization. Human neutrophils were settled down for 5 min on Labtek chamber cover-glass coated with 30 μg/mL bovine fibronectin. Cell polarization was induced by uniform stimulation with indicated concentrations of fMLP. The percentage of neutrophils extending pseudopods or ruffling was calculated from fields captured 4-8 minutes after fMLP stimulation (49,50). Data shown are mean ± SD of three experiments. **(C)** Neutrophil spontaneous apoptosis was assessed as described in Figure 5. Data shown are mean ± SD of three experiments. **(D)** Chemoattractant-elicited superoxide production by human neutrophils. Production of reactive oxygen species was monitored after stimulation with indicated concentrations of fMLP in the presence of 50 μM isoluminol, 0.8U HRP, and 0.2%BSA in a luminometer at 37°C. Chemiluminescence (arbitrary light units) was recorded at indicated time points (49,50). Data are mean ± SD from one experiment representative of three. **(E)** Neutrophil chemotaxis analyzed using a EZ-taxiscan chamber (Effector Cell Institute, Tokyo, Japan). Directionality (0 to 1), upward directionality (-1 to 1), and chemotaxis speed were calculated as previously described (49,50). Data shown are mean ± SD of three experiments.