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Supplementary Materials for

Targeting multiple cell death pathways extends the shelf life and preserves the function of human and mouse neutrophils for transfusion

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Materials and Methods

Analysis of cell proliferation by EdU incorporation

Cell proliferation in vitro was determined using the Click-iT Plus EdU Flow Cytometry Assay Kit (Invitrogen). EdU was added to the cell culture medium (1 μg per well in 1 mL medium). At the indicated time points, cells were collected and stained with allophycocyanin (APC)-CD16 (BioLegend, 51.1, 1 μg/mL) (for human cells) or APC-Ly6G (BioLegend, 1A8, 1 μg/mL) (for mouse cells) at 4 °C for 15 minutes and then fixed, permeabilized, and stained with azide dye (Invitrogen) following a protocol provided by the manufacturer. Finally, the samples were washed and analyzed using a BD FACSCanto II flow cytometer (BD Biosciences).

In vitro neutrophil death

Isolated neutrophils were cultured at a density of 1×10^6 cells/mL. The total initial cell number was counted using a hemocytometer before culture or treatment. At each indicated time point, the total cell number was counted again. Since the neutrophils undergoing lytic cell death (puffed cells) were destroyed by pipetting, the total number of cells in the culture gradually decreased. The morphology of cultured neutrophils was recorded by light microscopy (600×) at the indicated time points. Apoptotic cells were detected by Annexin V- fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining using an Annexin V Detection Kit (BD Biosciences) following the manufacturer's protocol. Flow cytometry was performed using a FACSCanto II flow cytometer and analyzed using FACSDiva software (BD Biosciences). Annexin V and PI double-negative cells were defined as healthy cells.

Chemotaxis assay

The EZ-TAXIScan device (Effector Cell Institute) was used to visualize the details of neutrophil chemotaxis. The EZ-TAXIScan chamber was assembled with a 260 μm-wide × 4 μm-thick silicon chip on a 2 mm untreated glass base as described by the manufacturer and filled with filtered RPMI-1640 medium. 1 μL freshly isolated or treated neutrophils (1×10^7/mL) were added to the lower reservoir and allowed to line up by pulling medium from the upper reservoir. 1 μl fMLP (1 μM for mouse neutrophils and 100 nM for human neutrophils) was then added to the upper reservoir. Neutrophil migration (at 37°C) in each channel was captured sequentially every 30 seconds for 20 minutes using a 10× lens. Migrating cells were tracked using Tracking Tool software. Chemotaxis velocity and directionality were analyzed as previously described (*21*).

In vitro phagocytosis assay

Fluorescein-conjugated pHrodo Red *E. coli* BioParticles or Zymosan A *S. cerevisiae* BioParticles (Invitrogen) were reconstituted in phosphate-buffered saline (PBS) and opsonized with 10% serum at 37°C for 30 minutes. Neutrophils were incubated with serum-opsonized bioparticles at a ratio of 1:10 (neutrophils:bioparticles) at 37 °C for 0.5 hours. APC-Ly6G (BioLegend, 1A8, 0.2 μg/mL) or APC-CD16 (BioLegend, 51.1, 0.2 μg/mL) antibody was added to the sample to label mouse or human neutrophil membranes. The labeling was achieved by incubating the samples at room temperature for 3 minutes. The number of internalized particles was counted under a spinning disk confocal microscope (UltraVIEW VOX). Phagocytosis efficiency was expressed as the percentage of neutrophils that engulfed at least one bioparticle. Phagocytosis index was expressed as the average number of internalized particles per cell. At least 200 cells were counted from random fields per coverslip for each group.

Chemoattractant-elicited NADPH oxidase activation

ROS, particularly superoxide anions, produced during NADPH oxidase activation were detected using luminol chemiluminescence (*40, 41*). To determine fMLP-induced NADPH oxidase activation, 0.5×10^6 mouse or human neutrophils were resuspended in 100 μL saline containing 1% BSA and then loaded into a 96-well MaxiSorp plate (Nunc). Chemiluminescence was measured using a TriStar LB941 microplate luminometer (Berthold Technologies USA). Saline (100 μL) containing 4 U/ml HRP, 50 μM luminol, and 500 nM fMLP was injected into the mixture via the injection port of the luminometer. Luminescence (arbitrary light units) was recorded every 12 seconds. Data are represented as mean ± SD of n=3 wells assayed simultaneously.

In vitro bacterial killing assay

Freshly isolated, G-CSF-treated, or CLON-G-treated aged mouse or human neutrophils were washed with saline and resuspended in RPMI-1640 medium without antibiotics at 1×10^6 cells/ml. Cells were incubated with opsonized *E. coli* (strain 19138, American Type Culture Collection (ATCC); Multiplicity of infection (MOI)=5) at 37°C for one hour. After incubation, neutrophils were lysed by adding distilled H_2O. The samples were then serially diluted and spread onto Luria-Bertani (LB) agar plates. The colony-forming units (cfus) were counted after incubating the plates overnight at 37°C. Bacterial suspension without incubating with neutrophils was used as input control. In vitro bacterial killing capabilities were reflected by the decrease in cfu (*17, 18*).

Establishment and confirmation of CPM-induced neutropenia

Cyclophosphamide powder (Cytoxan/CPM; Bristol-Myers Squibb) was dissolved in saline for injection at a final concentration of 20 mg/mL. Cyclophosphamide was injected intraperitoneally (i.p.) at a total dose of 250 mg/kg (150 mg/kg on day 0 and 100 mg/kg on day 3). Blood samples (20 μL) were taken

from the retroorbital sinuses of anesthetized mice using heparinized capillary tubes (Modulohm). Total and differential white blood cell counts (neutrophils, lymphocytes, and monocytes) were performed using a Hemavet 850 hematology system (Drew Scientific), a multiparameter, automated hematology analyzer designed for in vitro diagnostic use.

Relative death of transfused neutrophils in inflamed peritoneal cavity

Freshly isolated $CD45.1^+$ or CLON-G-treated (3 days) green fluorescent protein $(GFP)^+$ bone marrow neutrophils were mixed (1:1, a total of 5×10^6/mouse) and injected i.p. into neutropenic recipient mice challenged with thioglycollate (TG) (3% in 1mL PBS, i.p. injected) for 1 hour. Peritoneal lavage fluid was harvested at the indicated timepoints after cell injection. Cells in peritoneal lavage fluid were collected and resuspended in 100 μL ice-cold PBS and stained with phycoerythrin (PE)-CD45.1 (BioLegend, A20, 2 μg/mL) at 4 °C for 15 minutes. The relative amount of transfused freshly isolated $CD45.1^+$ and CLON-G-treated (3 days) GFP^+ neutrophils were analyzed by flow cytometry using a BD FACSCanto II flow cytometer.

In vivo recruitment of transfused neutrophils in a mouse peritonitis model

Freshly isolated $CD45.1^+$ or CLON-G-treated (1 day) GFP^+ bone marrow neutrophils were mixed (1:1, a total of 5×10^6/mouse) and injected intravenously into neutropenic recipient mice challenged with TG for 1 hour. At the indicated time points after cell injection, peritoneal cells were harvested by peritoneal cavity lavage with 5 mL of ice-cold PBS/15 mM EDTA, flushed back and forth three times. The relative amount of transfused freshly isolated $CD45.1^+$ and CLON-G-treated (1 day) GFP^+ neutrophils were analyzed by flow cytometry using a BD FACSCanto II flow cytometer as described above.

Neutropenia-related *E. coli*-induced pneumonia model

Mice were anesthetized by ketamine hydrochloride (100 mg/kg intraperitoneally) and xylazine (10 mg/kg intraperitoneally) injection, mice trachea were surgically exposed, and a dose of 5×10^3 cfu (for neutropenic mice) or 1×10^6 (for normal mice) *E. coli* (strain 19138; ATCC) per mouse was instilled intratracheally to the left bronchus (total volume 50 μL). The mice were placed on warm heating pads during post-surgery recovery. As soon as they were ambulatory (approximately 5 to 15 minutes), they were returned to the home cage with immediate access to food and water. At the end of the experiments, mice were euthanized with CO_2.

Bacterial burden

Freshly isolated or CLON-G-treated bone marrow neutrophils (5×10^6/mouse) were injected into neutropenic mice challenged with 5×10^3 cfu *E. coli* for 4 hours. For the bacterial burden assay (the number of live bacteria in the lungs), mice were euthanized at 24 hours after bacterial challenge, bronchoalveolar lavage fluids (BALF) were collected using 1 ml of cold PBS/15 mM EDTA flushed back and forth for three times, cells in BALF were lysed by adding distilled H_2O, then BALF was diluted and spread onto Luria broth (LB) agar plates. The cfu were counted manually by an independent blinded examiner after incubating the plates overnight at 37°C.

BALF cytokine and total protein concentrations

BALF samples were obtained from mice 24 hours after *E. coli* challenge using 1 ml of cold PBS/15 mM EDTA flushed back and forth three times. TNF-α and IL-6 concentrations in BALF were measured with ELISA kits following a protocol provided by the manufacturer (R&D Systems). Protein concentration

was measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories). The standard curve was constructed using BSA (*42*).

Histopathology

In pneumonia model, lungs were fixed by intratracheal instillation of Bouin's solution at 23 cmH_2O pressure. Tissues were embedded in paraffin, and 6 μm sections were stained with hematoxylin and eosin (H&E) and then examined by light microscopy. Non-quantitative histological analysis was performed by a pathologist blinded to the groups. ImageJ software (National Institutes of Health) was used to manually trace edema and neutrophil-containing regions of the tissue section. The pixel area of each edema and neutrophil-containing region was calculated using ImageJ software. Edema formation was calculated as the percentage of pixel area of all the edema-containing regions relative to the pixel area of the whole image.

Examination of GTX-induced organ damage

After anesthesia with ketamine hydrochloride (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.), mouse tracheas were surgically exposed and a total volume of 50 μl of saline or LPS (5 mg/kg body weight) was instilled intratracheally via an angiocatheter inserted through the trachea and into the left bronchus. After surgery and wound closure, mice were suspended by their front legs to help deliver the instillate deep into the left lobe before being placed back into the cage with soft and warm bedding for recovery. GTX was performed via intravenous injection 4 hours after the LPS instillation. Mice were euthanized by CO_2 24 hours after neutrophil transfusion. Kidneys, livers, spleens, hearts, and lungs were dissected, fixed in 10% neutral buffered formalin (Sigma-Aldrich), and then embedded in paraffin. Paraffin-embedded sections (~6 μm thick) were stained with H&E and examined by light microscopy.

In vivo recruitment of transfused neutrophils in bacterial pneumonia

Freshly isolated or CLON-G-treated bone marrow neutrophils were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 5 μM) or seminaphthorhodafluor-1 acetate (SNARF-1, 5 μM) at 37 °C for 10 minutes; labeled cells were mixed (1:1, a total of 5×10^6/mouse) and transfused via the tail vein into the same neutropenic recipient mice challenged with 5×10^3 cfu *E. coli* for 4 hours. Whole lungs were homogenized 3 hours after neutrophil transfusion. The number of adoptively transferred neutrophils recruited to the lungs was analyzed using a FACSCanto II flow cytometer and FACSDiva software. Relative recruitments of CLON-G-treated and freshly isolated neutrophils were calculated as the ratio of the indicated populations.

Neutrophil accumulation in inflamed lungs

Neutropenic mice were anesthetized and instilled with bacteria as described above. GTX was performed 4 hours after *E. coli* instillation. At the indicated time points, mice were euthanized by CO_2. The chest cavity was opened and a catheter was tied to the trachea. Bronchoalveolar lavage (BAL) was performed (1 mL of PBS/15 mM EDTA) in each group. The BALF was centrifuged at 450 x g for 10 minutes, and the total and differential cell counts were determined from the pelleted cell fraction by flow cytometry analysis. For flow cytometry, the cells were suspended in 100 μL ice-cold PBS and stained with FITC-F4/80 (BioLegend, QA17A29, 1 μg/mL) and APC-Ly6G (BioLegend, 1A8, 1 μg/mL) at 4 °C for 15 minutes. Neutrophil numbers were determined with the FACSCanto II flow cytometer and FACSDiva software (BD Biosciences).

***Candida albicans* infection**

Neutropenic mice were injected intravenously with *C. albicans* blastospores in a 200 µL volume of sterile pyrogen-free PBS (1×10^3, strain SC5314; ATCC). Survival was assessed daily (for about 14 days). For histologic analysis, mice were euthanized by CO_2, and kidneys of subgroups were fixed in 10% neutral buffered formalin (Sigma-Aldrich). Paraffin-embedded sections were stained with H&E as described above.

Fungal burden

Freshly isolated or CLON-G-treated bone marrow neutrophils (3×10^6/mouse) were injected into neutropenic mice infected with *C. albicans* as described above for one hour. Mice were euthanized 3 days later. Whole kidneys were ground, filtered, and resuspended in 5 mL ice-cold PBS/15mM EDTA, cells were lysed by adding distilled H_2O, then the fluids were diluted and spread on Yeast Extract–Peptone–Dextros (YPD) agar plates. cfu were counted manually by an independent blinded examiner after incubating the plates overnight at 37°C.

Examination of the survival and function of transfused human neutrophils in vivo in NSG mice.
The experiment was conducted essentially as described by Trump et al. (*25*). Briefly, to deplete mice neutrophils, cyclophosphamide (CPM) was injected i.p. at a total dose of 250 mg/kg (150 mg/kg on day 0 and 100 mg/kg on day 3). Twenty-four hours after the last dose of CPM, untreated fresh or drug-treated human neutrophils isolated from apheresis-collected granulocyte concentrates were injected intravenously or intraperitoneally into neutropenic NSG mice in a final volume of 200 µl of PBS. To measure phagocytosis, pHrodo-*E. coli*, a nonviable fluorescently labeled bacteria (200 µg in 200 µl PBS, Invitrogen Molecular Probes), and the stimulant TG (3% in 800 µl distilled water) were co-injected intraperitoneally. At the conclusion of each experiment, the animals were euthanized by CO_2 asphyxiation and peritoneal exudates were harvested in two successive washes with 10 ml cold PBS

containing 15 mM EDTA and 0.2% BSA. Neutrophil recruitment, phagocytosis, and survival were assessed as described above. Transfused human neutrophils were identified by their unique surface markers by flow cytometry analysis. Cells in peritoneal lavage fluid were suspended in 100 μL ice-cold PBS and stained with PE-Cy7-anti-mouse-CD45 (BioLegend, 30-F11, 1 μg/mL), APC-Cy7-anti-human-CD45 (BioLegend, 2D1, 1 μg/mL), PE-anti-human-CD11b (BioLegend, LM2, 1 μg/mL), and APC-anti-human-CD16 (BioLegend, 51.1, 1 μg/mL) at 4 °C for 15 minutes. Samples were analyzed with a FACSCanto II flow cytometer (Becton Dickinson). Transfused human neutrophils were gated by their forward- and side-scatter characteristics and their CD45/CD11b/ CD16 expression patterns (**Fig. 8B and Fig. 8E**).

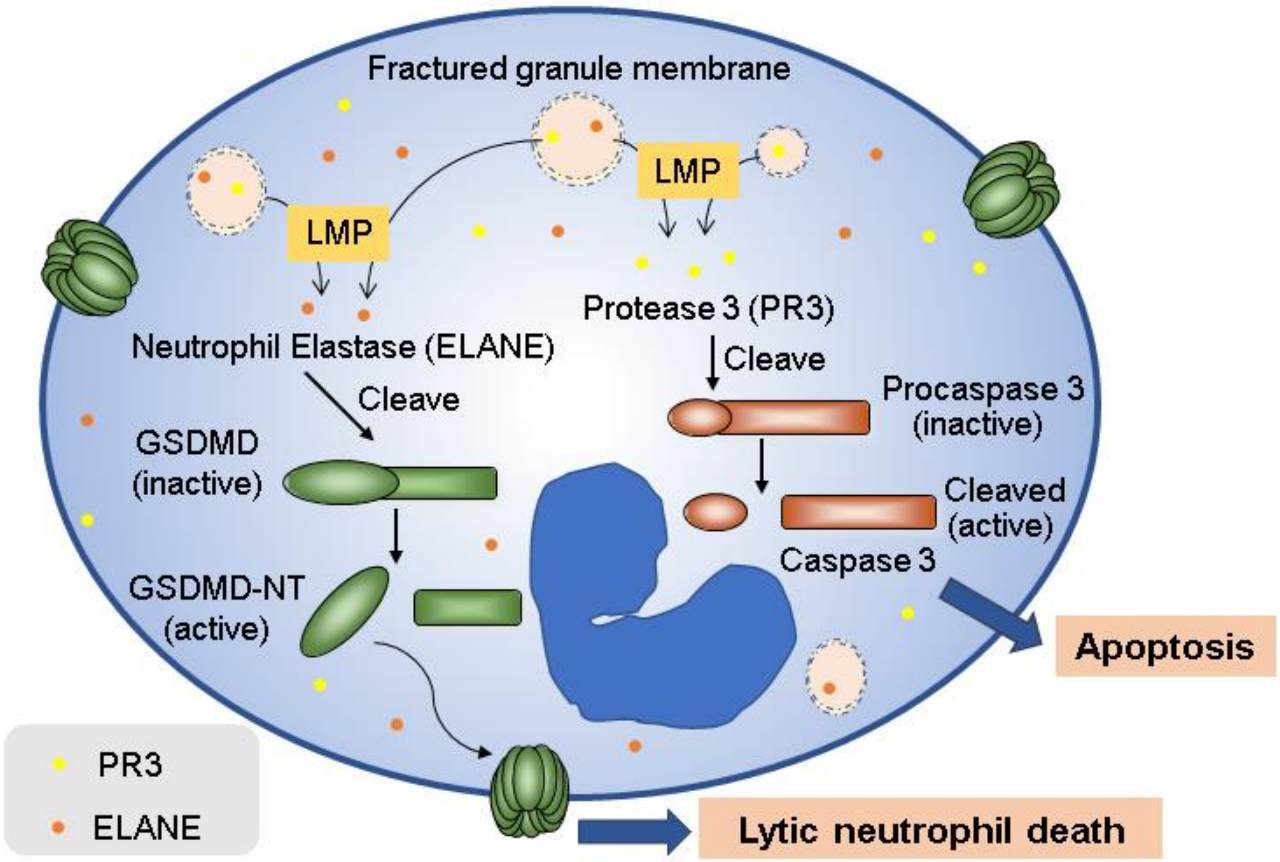

Fig. S1. Neutrophil death is mediated by multiple pathways.

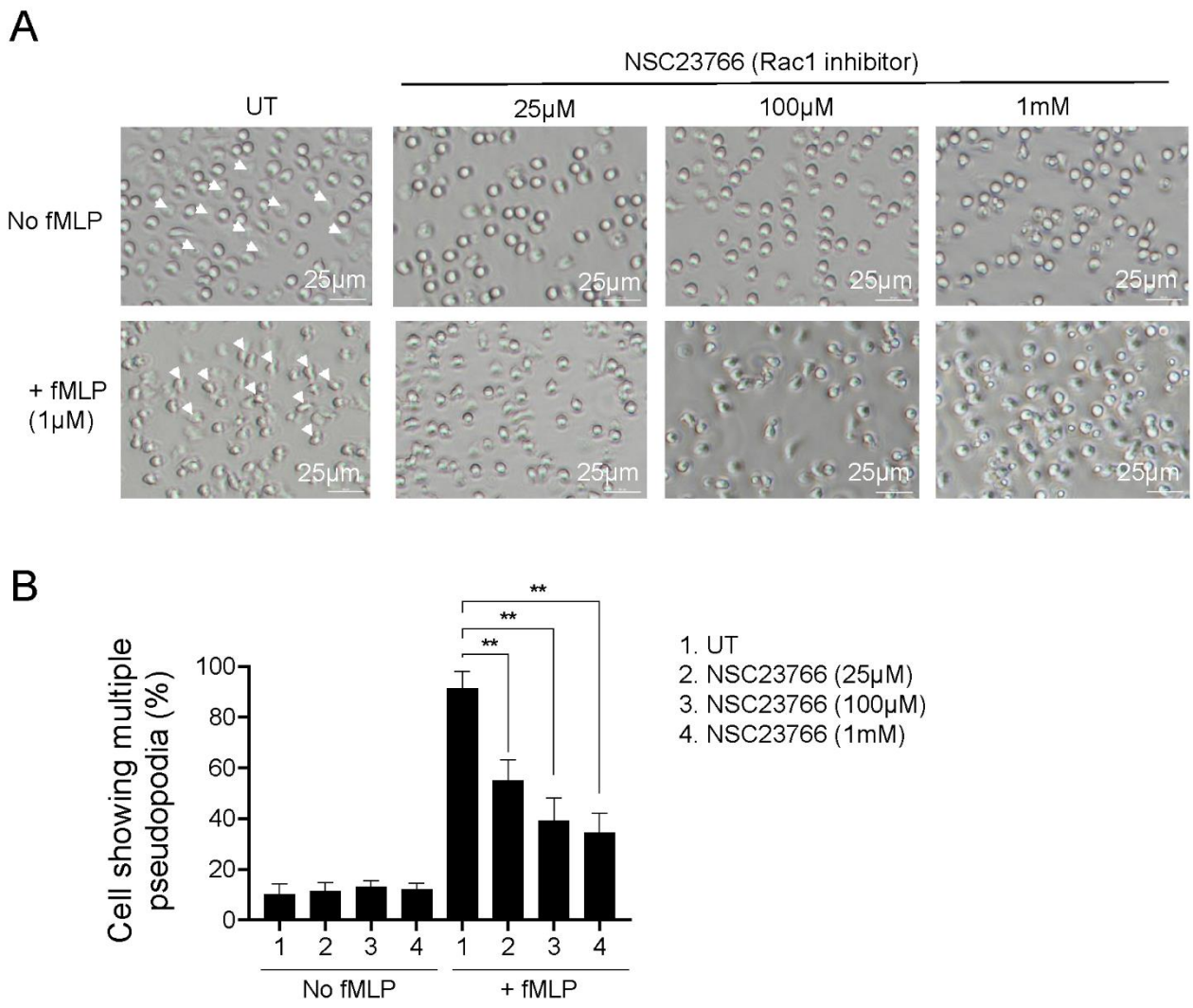

Fig. S2. Rac1 inhibition effectively suppresses chemoattractant-elicited neutrophil polarization.

(**A**) Chemoattractant-induced neutrophil ruffling. Neutrophils (2×10^5) purified from wild-type (WT) mice were plated on Labtek chamber slides and cultured in RPMI-1640 in the presence or absence of Rac1 inhibitor for 60 minutes. Cells were then uniformly stimulated with N-formyl-met-leu-phe (fMLP, 1μM). Images were taken 5 minutes after the fMLP stimulation (400×). (**B**) The percentage of cells that ruffled or extended pseudopods was calculated from images captured 5 minutes after stimulus was added. At least 200 cells were assessed for each sample in each experiment. Data are represented as mean ± SD of three experiments. ** $p<0.001$ versus untreated neutrophils.

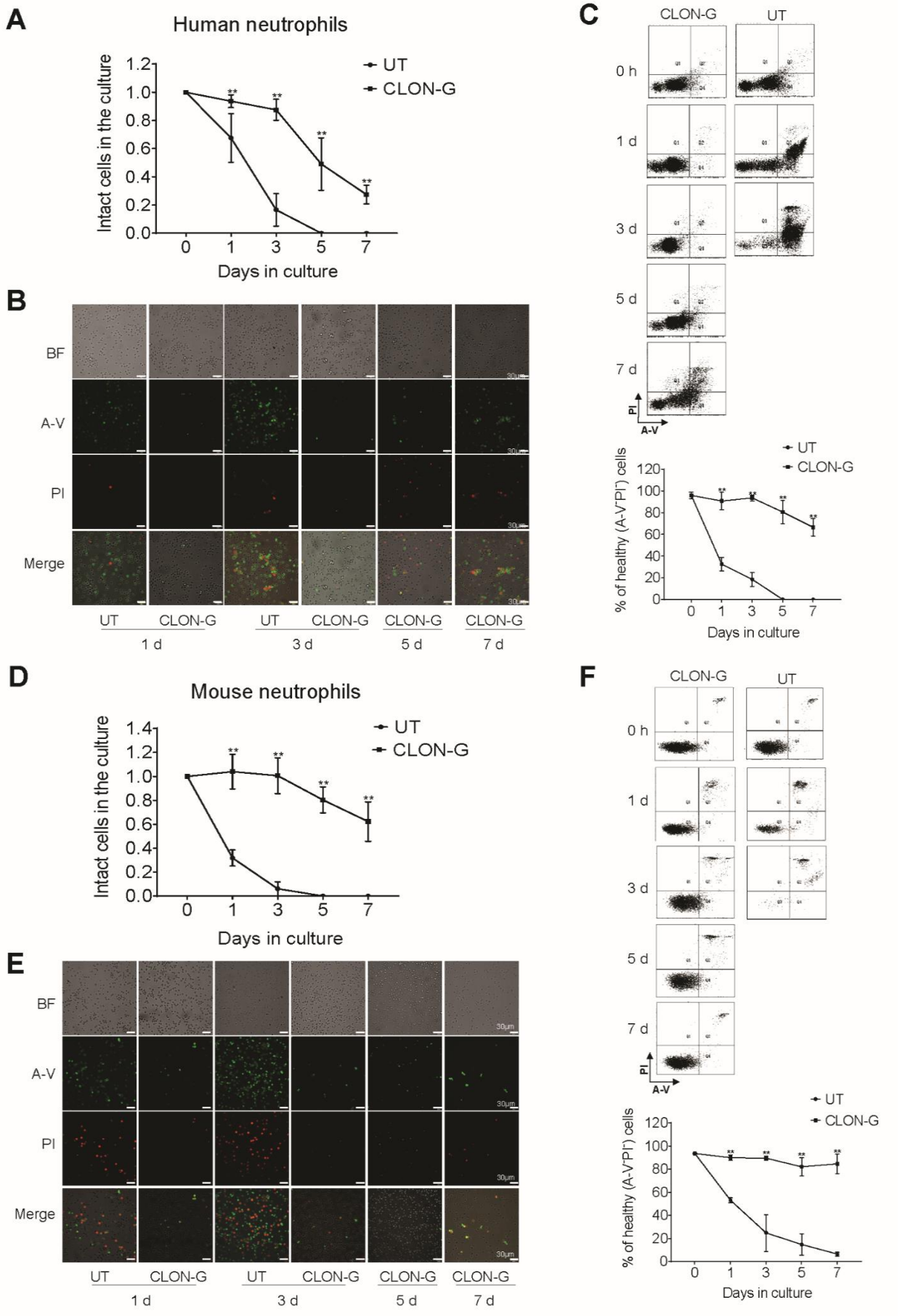
A
Human neutrophils
Intact cells in the culture
Days in culture
UT
CLON-G
B
BF
A-V
PI
Merge
UT
CLON-G
1 d
3 d
5 d
7 d
C
CLON-G
UT
0 h
1 d
3 d
5 d
7 d
PI
A-V
% of healthy (A-V-PI-) cells
D
Mouse neutrophils
E
F

Fig. S3. CLON-G treatment increases the half-life of both human and mouse neutrophils from less than 1 day to greater than 5 days.

(A) Freshly isolated human peripheral blood neutrophils were cultured in RPMI-1640 with or without CLON-G (caspases-LMP-oxidant-necroptosis inhibition plus granulocyte colony-stimulating factor (G-CSF)). The number of intact neutrophils in the culture was counted using a hemocytometer. **(B)** Human neutrophils were stained with FITC-Annexin-V (A-V, green) and PI (red) after culturing for the indicated time in vitro. Cell death was assessed by confocal fluorescence microscopy. Scale bars, 30μm. **(C)** The death of human neutrophils was assessed by flow cytometry. The percentage of healthy neutrophils was calculated in untreated (UT) and CLON-G-treated cell populations at the indicated time points. All data are presented as mean ± SD of three experiments. **, $P<0.001$ compared to the corresponding UT group. **(D)** The number of intact murine cells in the culture. **(E)** Murine neutrophil death was assessed by confocal fluorescence microscopy. Scale bars, 30μm. **(F)** The death of cultured mouse neutrophils was assessed by flow cytometry. All data are presented as mean ± SD of three experiments. ** $P<0.001$ compared to the corresponding UT group.

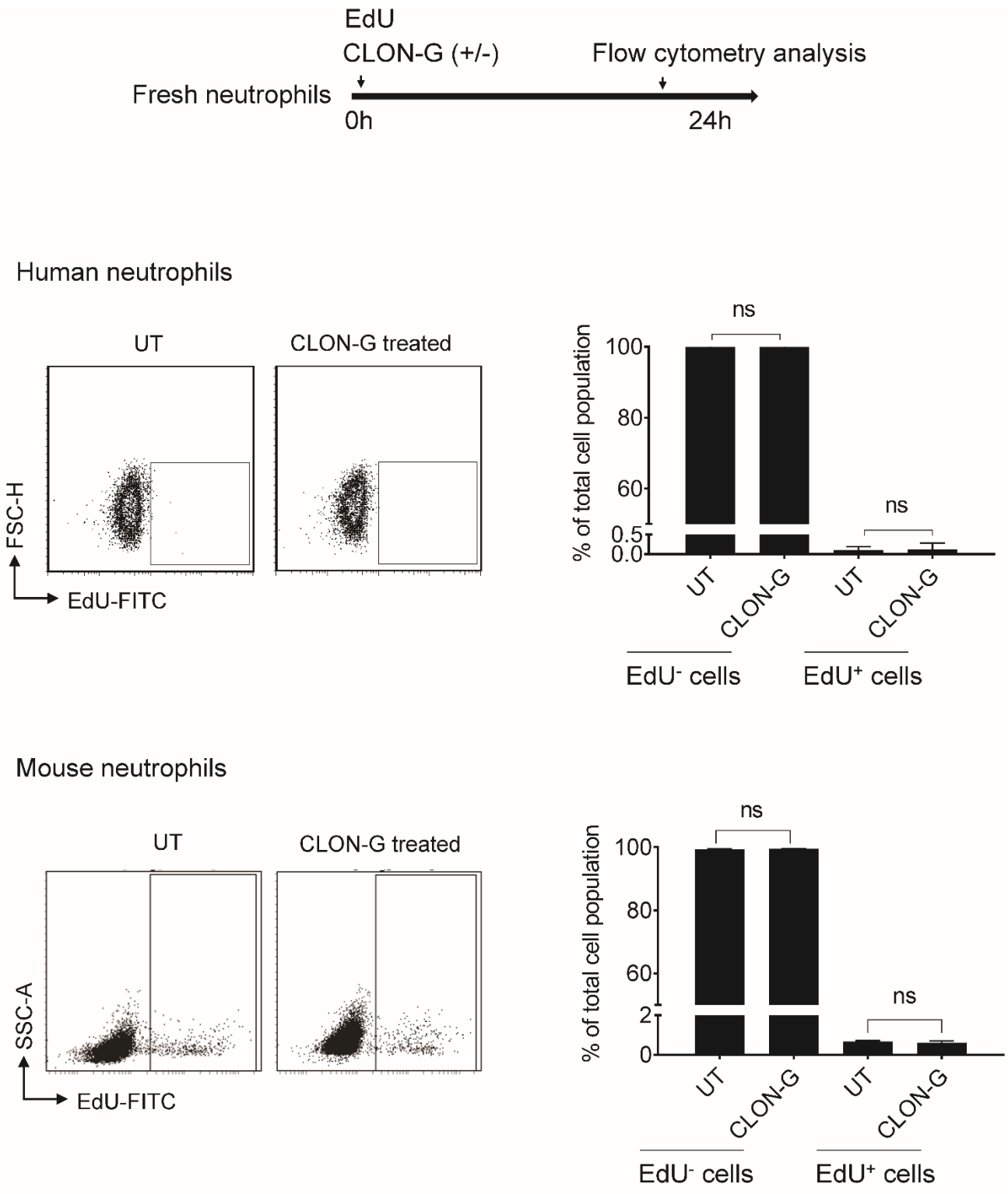

Fig. S4. CLON-G treatment does not influence the proliferative capacity of contaminated neutrophil progenitors in culture.

Human or mouse neutrophils were cultured in CLON-G-containing RPMI-1640 medium in the presence or absence of EdU (10 μM) for 24 hours. The frequency of EdU-positive cells was analyzed by flow cytometry. All data are represented as mean ± SD of three experiments. ns, no significant difference, FSC-H, forward scatter-height; SSA, side scatter.

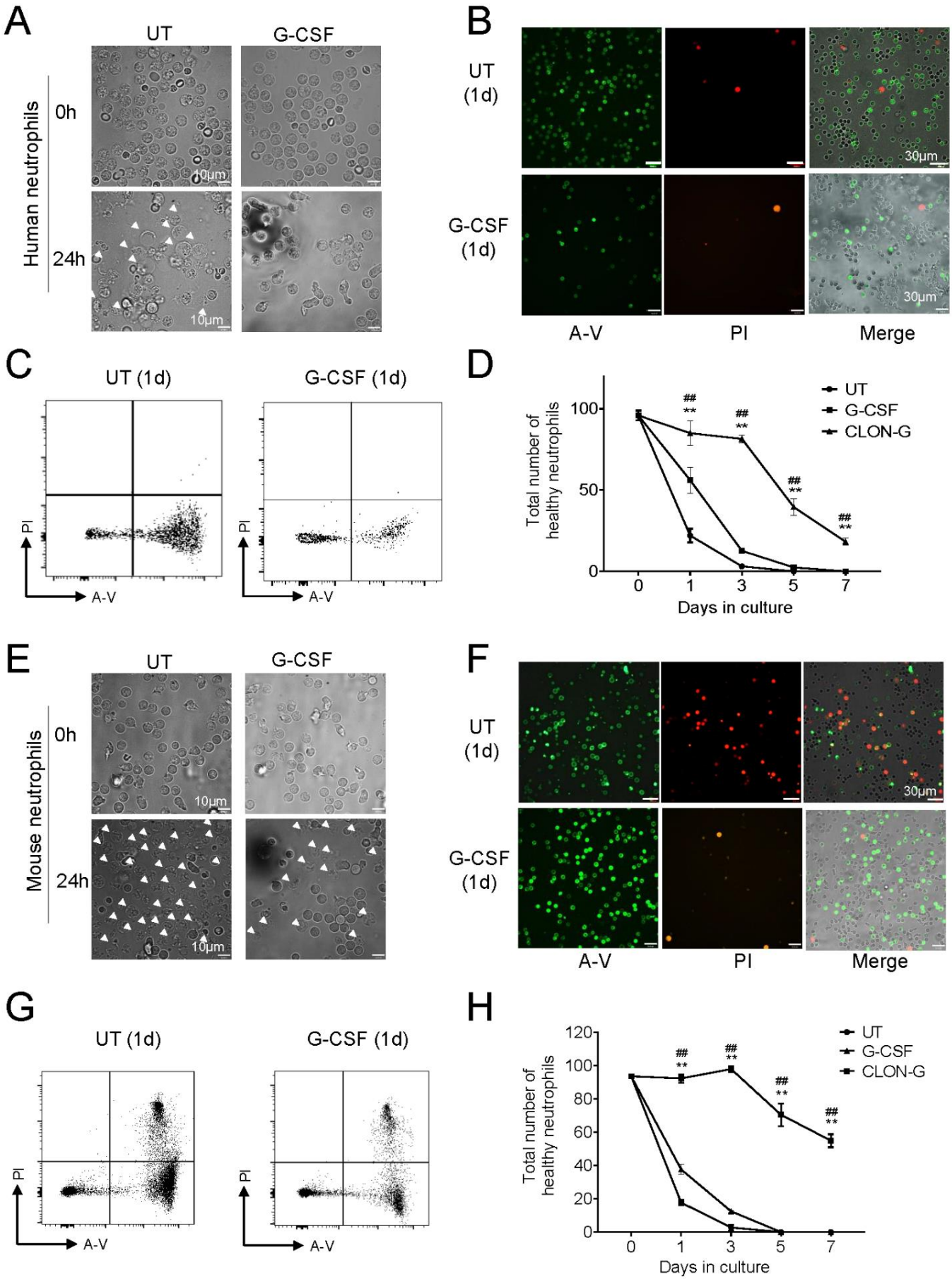
A
UT
G-CSF
Human neutrophils
0h
24h
10μm
B
UT (1d)
G-CSF (1d)
30μm
A-V
PI
Merge
C
UT (1d)
G-CSF (1d)
PI
A-V
D
Total number of healthy neutrophils
UT
G-CSF
CLON-G
Days in culture
E
Mouse neutrophils
F
G
H

Fig. S5. CLON-G is more effective in prolonging neutrophil survival than G-CSF alone.

(**A**) Freshly isolated human peripheral blood neutrophils were cultured in RPMI-1640 plus 20% fetal bovine serum (FBS) with or without G-CSF. The morphologies cells were observed after 1 day by light microscopy as described in Fig.1. White arrows indicate dying cells. (**B and C**) Human neutrophils were stained with FITC-Annexin-V (AV, green) and PI (red) after culturing for 1 day. Cell death was assessed by confocal fluorescence microscopy (B) or flow cytometry (C). (**D**) The total number of healthy neutrophils was calculated in UT, G-CSF-treated, and CLON-G-treated cell populations at the indicated time points as described in **Fig.1**. All data are represented as mean ± SD. (**E**) The morphologies of untreated (UT) or G-CSF-treated (Treated) mouse neutrophils were observed after 1 day by light microscopy as in (A). (**F and G**) Murine neutrophils were stained with FITC-Annexin-V (AV, green) and PI (red) after culturing for 1 day. Cell death was assessed by confocal fluorescence microscopy (F) or flow cytometry (G) as in (B) and (C). (**H**) The total number of healthy neutrophils was calculated in UT, G-CSF-treated, and CLON-G-treated cell populations at the indicated time points. All data are presented as mean ± SD. **, $P<0.001$ compared to the corresponding UT group. ##, $P<0.001$ compared to the corresponding G-CSF-treated group.

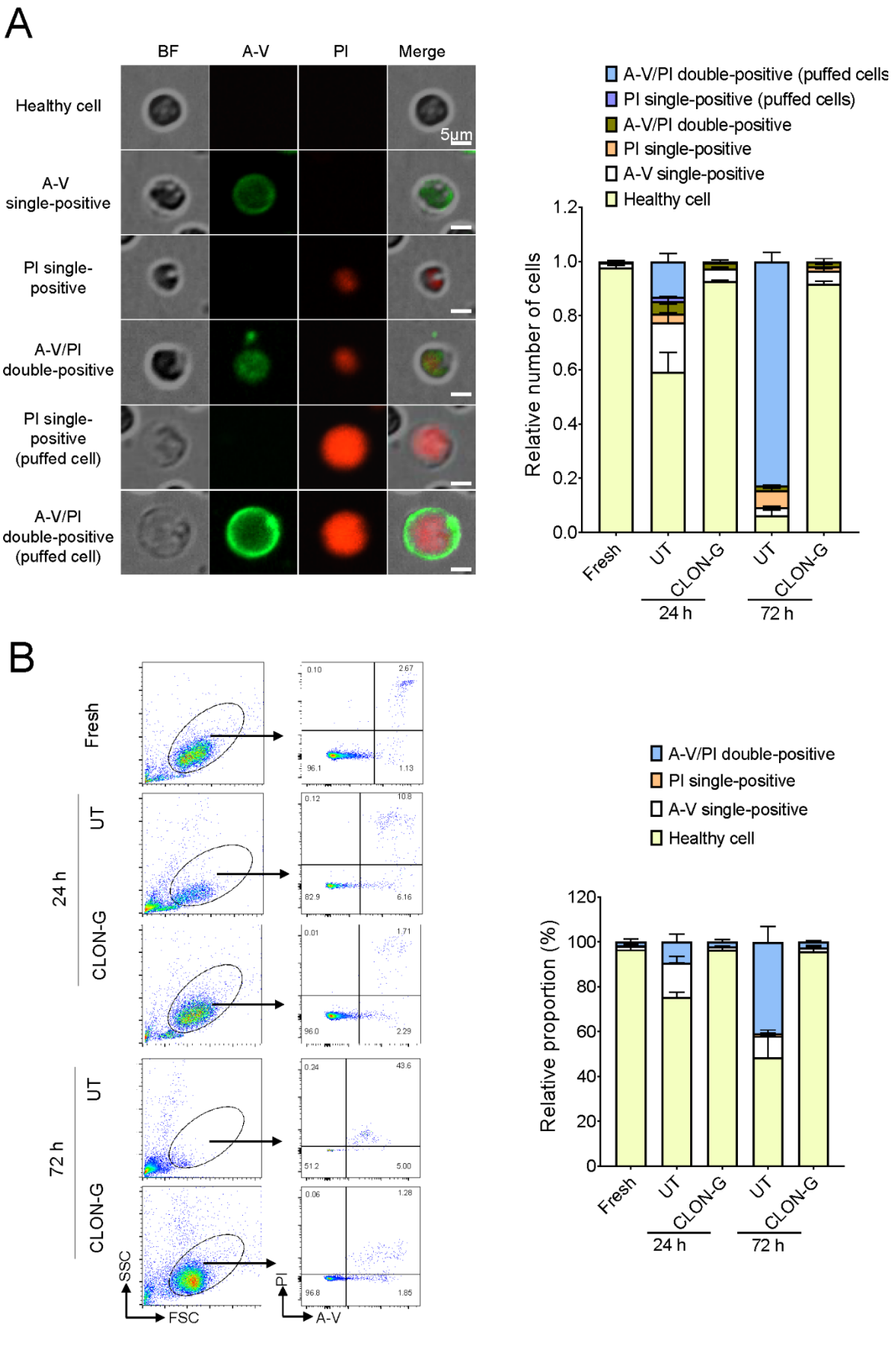
A
BF
A-V
PI
Merge
Healthy cell
A-V single-positive
PI single-positive
A-V/PI double-positive
PI single-positive (puffed cell)
A-V/PI double-positive (puffed cell)
5μm
A-V/PI double-positive (puffed cells
PI single-positive (puffed cells)
A-V/PI double-positive
PI single-positive
A-V single-positive
Healthy cell
Relative number of cells
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fresh
UT
CLON-G
UT
CLON-G
24 h
72 h
B
Fresh
24 h
UT
CLON-G
72 h
UT
CLON-G
0.10
2.67
96.1
1.13
0.12
10.8
82.9
6.16
0.01
1.71
96.0
2.29
0.24
43.6
51.2
5.00
0.06
1.28
96.8
1.85
SSC
FSC
PI
A-V
A-V/PI double-positive
PI single-positive
A-V single-positive
Healthy cell
Relative proportion (%)
120
100
80
60
40
20
0
Fresh
UT
CLON-G
UT
CLON-G
24 h
72 h

Fig. S6. CLON-G treatment inhibits both apoptotic and lytic cell death of mouse neutrophils.

(**A**) Freshly isolated mouse neutrophils were cultured in vitro for 24 hours and 72 hours as in Fig. 1. Untreated (UT) or CLON-G treated (Treated) cells were stained with FITC-Annexin V (A-V, green) and PI (red). Classification of cell types based on A-V/PI staining and morphology by confocal fluorescence microscopy. Bar graphs show the percentage of each cell type at 0 hour (fresh), 24 hours, and 72 hours. (**B**) Cells were collected at 0 hour, 24 hours, and 72 hours followed by staining for the different types of cell death by flow cytometry. Data are represented as mean ± SD of three experiments. In this experiment, to eliminate the noise cause by late-stage fragmented dead cells ($PI^+A\text{-}V^-$ or $PI^+A\text{-}V^+$), we gated and analyzed cells with normal forward scatter (FSC) and side scatter (SSC).

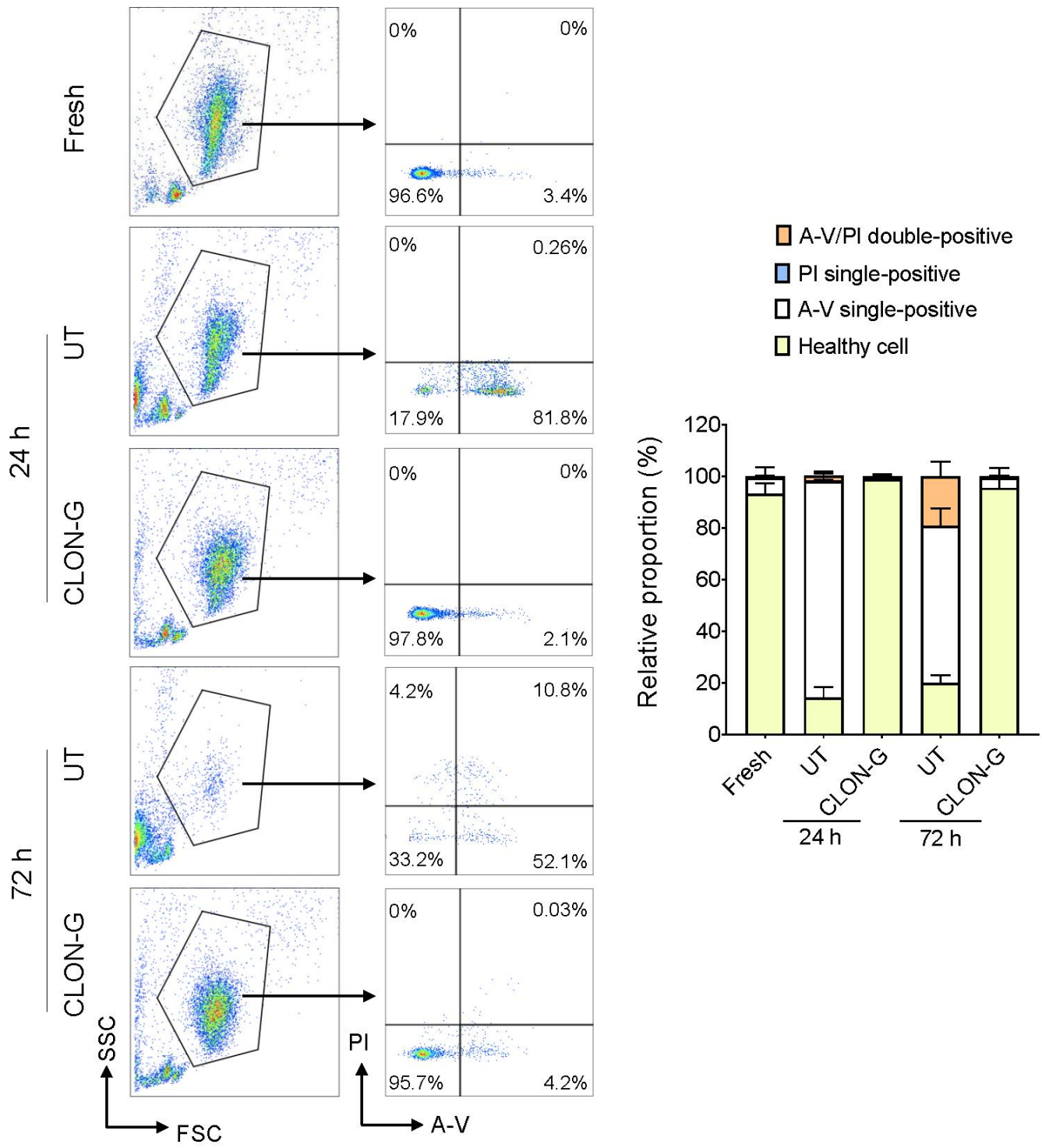

Fig. S7. CLON-G treatment inhibits both apoptotic and lytic cell death of human neutrophils.

Freshly isolated human neutrophils were cultured in vitro for 24 and 72 hours as described in Fig. 1. Untreated (UT) and CLON-G treated (Treated) cells were stained with FITC-Annexin V (A-V) and PI. Cells were collected at 0, 24, and 72 hours post treatment. The different types of cell death based on A-V and PI staining were calculated by flow cytometry. Data are represented as mean ± SD of three experiments. In this experiment, to eliminate the noise cause by late-stage fragmented dead cells ($PI^+A\text{-}V^-$ or $PI^+A\text{-}V^+$), we gated and analyzed cells with normal forward scatter (FSC) and side scatter (SSC).

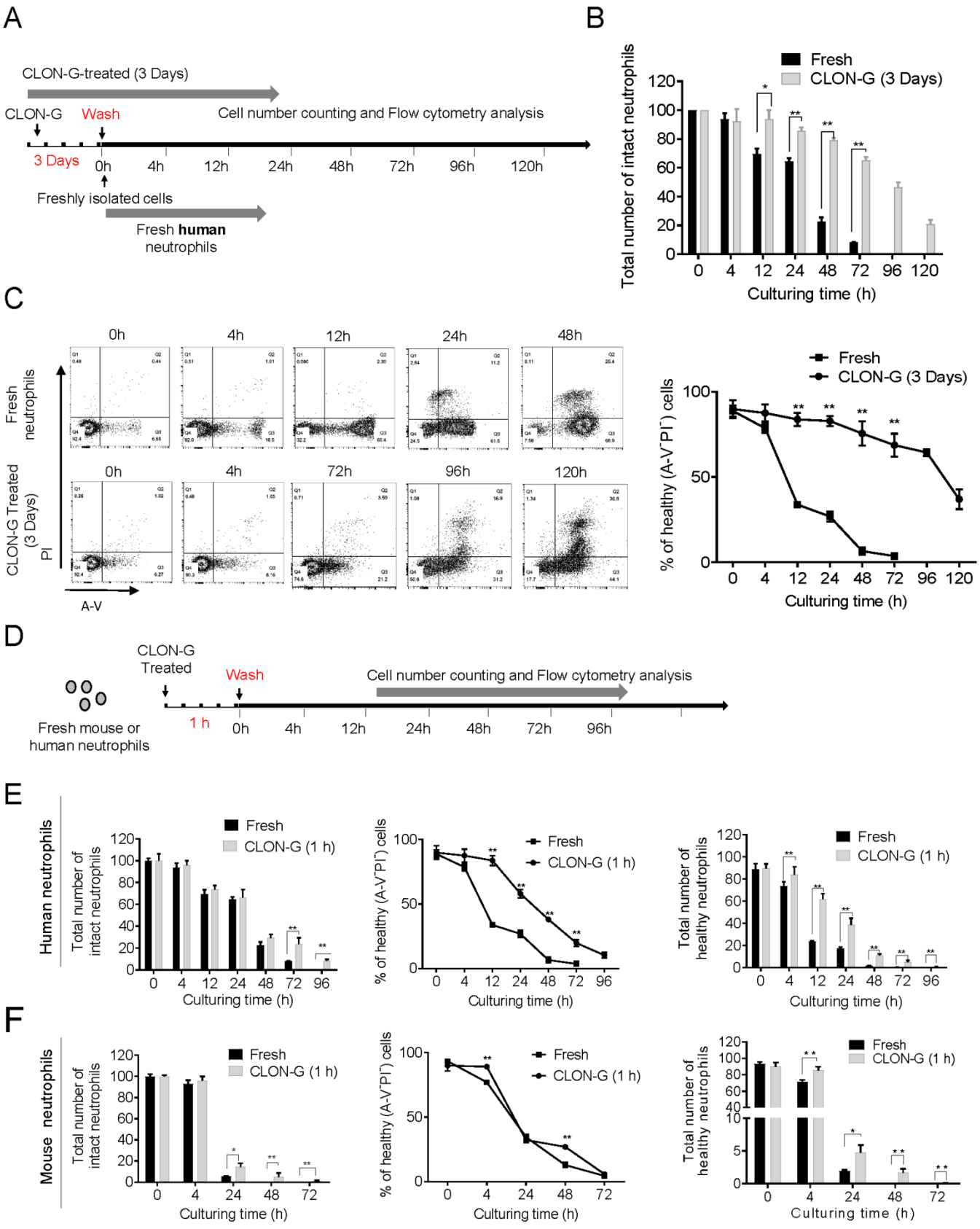

Fig. S8. Drug removal does not accelerate death of CLON-G-treated neutrophils.

(**A**) Human neutrophils were treated with CLON-G for 3 days in vitro followed by removing CLON-G by replacing the cell culture medium to normal medium (CLON-G-treated, 3 Days). At the same time, freshly isolated neutrophils were cultured in the same medium without CLON-G (Fresh neutrophils). (**B**) Total numbers of intact neutrophils were counted at the indicated time points. Data are presented as mean ± SD of three experiments. *, $P<0.05$ and **, $P<0.001$ compared to the corresponding UT (fresh neutrophil) group. (**C**) Cells were stained with FITC-Annexin V (A-V) and PI and then analyzed by flow cytometry. The percentage of healthy neutrophils was assessed. Data are presented as mean ± SD of three experiments. **, $P<0.001$ compared to the corresponding UT (fresh neutrophil) group. (**D**) Human and mouse neutrophils were treated with CLON-G for 1 hour in vitro followed by removing drugs by replacing the drug-containing medium to normal medium. (**E and F**) The number of intact cells, the percentage of healthy ($A\text{-}V^{-}PI^{-}$) cells, and the total number of healthy cells in UT and CLON-G-treated human (E) or mouse (F) neutrophil populations were measured and calculated. All data are represented as mean ± SD of three experiments. *, $P<0.05$ and **, $P<0.001$ compared to the corresponding UT group.

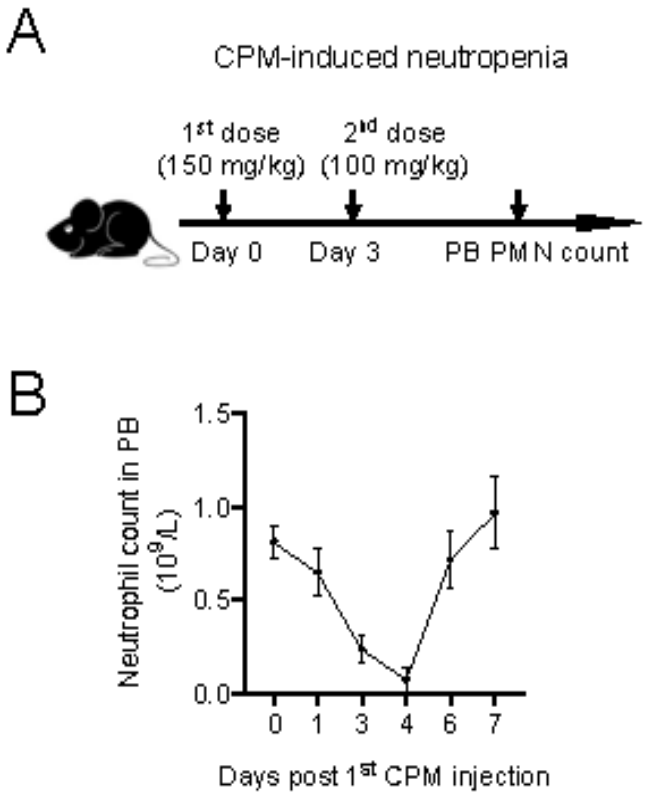

Fig. S9. Cyclophosphamide (CPM)-induced neutropenia in mice.

(**A**) The experimental scheme is shown. (**B**) Peripheral blood neutrophil counts in normal and CPM-treated mice are shown. Neutrophil number in the peripheral blood (PB) was assessed using a Hemavet 850 hematology system. Data are represented as mean ± SD of three experiments.

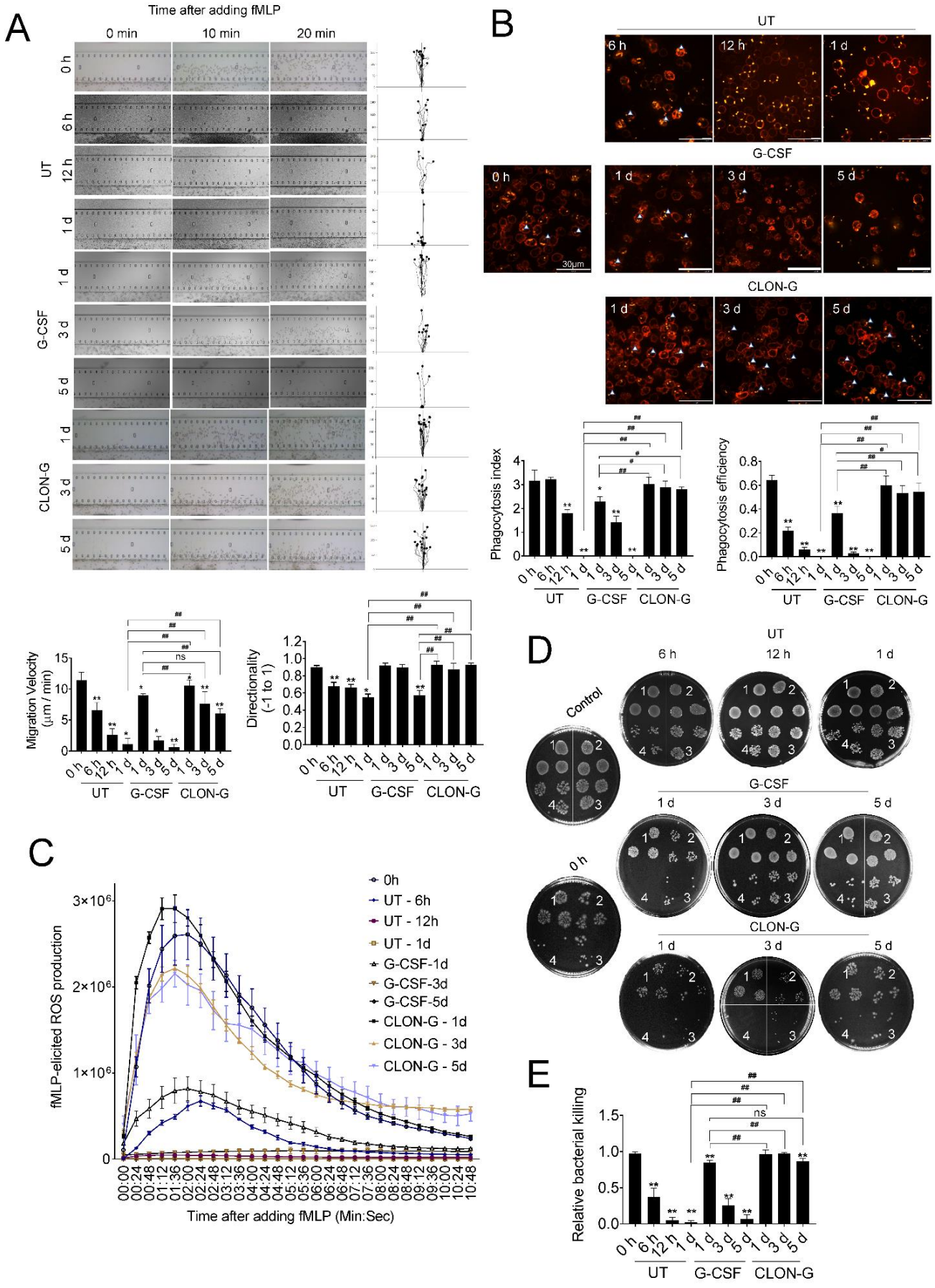
A
Time after adding fMLP
0 min
10 min
20 min
UT
G-CSF
CLON-G
0 h
6 h
12 h
1 d
3 d
5 d
Migration Velocity (μm / min)
Directionality (-1 to 1)
B
UT
G-CSF
CLON-G
30μm
Phagocytosis index
Phagocytosis efficiency
C
fMLP-elicited ROS production
Time after adding fMLP (Min:Sec)
0h
UT - 6h
UT - 12h
UT - 1d
G-CSF-1d
G-CSF-3d
G-CSF-5d
CLON-G - 1d
CLON-G - 3d
CLON-G - 5d
D
Control
E
Relative bacterial killing

Fig. S10. CLON-G treatment does not impair migration and bacterial killing capability of human neutrophils. Freshly isolated human neutrophils were cultured as described in Fig. 1 and fig. S5. The function of UT, G-CSF-treated, and CLON-G-treated neutrophils was assessed at indicated time points. (**A**) The chemotactic migration of human neutrophils to fMLP (100 nM) was assessed using an EZ-TAXIScan device. Migration velocity and directionality were calculated. (**B**) In vitro phagocytosis capacity of human neutrophils (APC-$CD16^+$, red) was measured using fluorescein-conjugated PhrodoTM *E. coli* bioparticles (yellow). At least 200 cells were assessed for each sample. The engulfed bioparticles are indicated with white arrowheads. (**C**) fMLP-induced ROS production in human neutrophils. (**D**) In vitro killing of *E. coli* by human neutrophils. The bacterial killing capabilities were reflected by the decrease in colony forming units (cfu). 1, 2, 3, and 4 indicate serial dilutions in the bacterial colony assay. Control, bacterial suspension without any cells; 0 h, fresh neutrophils. (**E**) The relative bacterial killing was measured as the proportion of bacteria killed in 60 minutes. All data are represented as mean ± SD of three experiments. * indicates $P<0.05$ and ** indicates $P<0.001$ compared to freshly isolated neutrophil group (0 h); $^{\#}$ indicates $P<0.05$ compared to UT (or G-CSF alone-treated) neutrophils cultured for 1 day; $^{\#\#}$ indicates $P<0.001$ compared to UT (or G-CSF alone-treated) neutrophils cultured for 1 day.

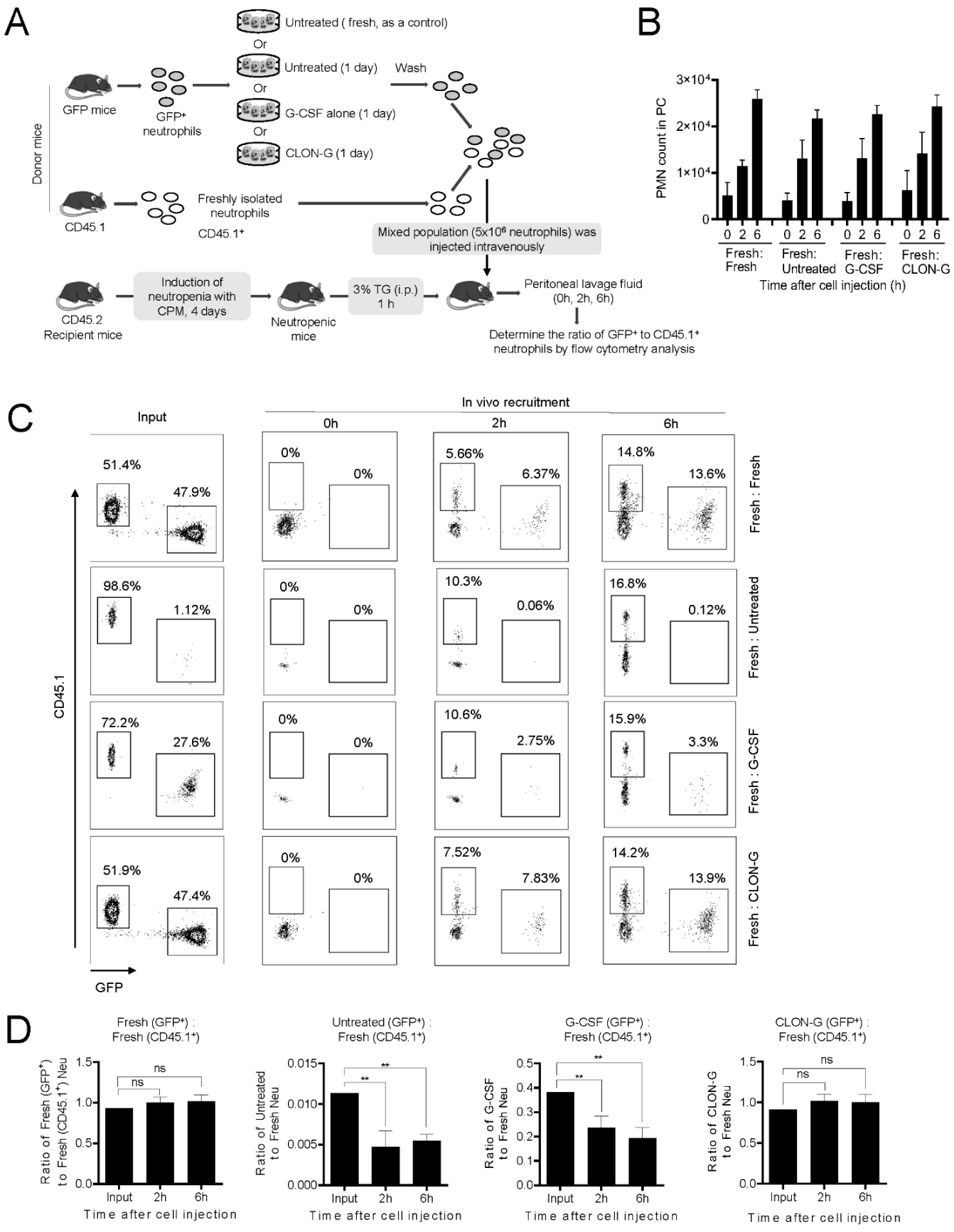
A
Untreated (fresh, as a control)
Or
Untreated (1 day)
Wash
Or
G-CSF alone (1 day)
Or
CLON-G (1 day)
Donor mice
GFP mice
GFP+ neutrophils
CD45.1
Freshly isolated neutrophils
CD45.1+
Mixed population (5x10^6 neutrophils) was injected intravenously
CD45.2 Recipient mice
Induction of neutropenia with CPM, 4 days
Neutropenic mice
3% TG (i.p.) 1 h
Peritoneal lavage fluid (0h, 2h, 6h)
Determine the ratio of GFP+ to CD45.1+ neutrophils by flow cytometry analysis
B
PMN count in PC
3×10^4
2×10^4
1×10^4
0
0 2 6 0 2 6 0 2 6 0 2 6
Fresh: Fresh
Fresh: Untreated
Fresh: G-CSF
Fresh: CLON-G
Time after cell injection (h)
C
Input
In vivo recruitment
0h
2h
6h
51.4%
47.9%
0%
0%
5.66%
6.37%
14.8%
13.6%
Fresh : Fresh
98.6%
1.12%
0%
0%
10.3%
0.06%
16.8%
0.12%
Fresh : Untreated
72.2%
27.6%
0%
0%
10.6%
2.75%
15.9%
3.3%
Fresh : G-CSF
51.9%
47.4%
0%
0%
7.52%
7.83%
14.2%
13.9%
Fresh : CLON-G
CD45.1
GFP
D
Fresh (GFP+) : Fresh (CD45.1+)
Ratio of Fresh (GFP+) to Fresh (CD45.1+) Neu
ns
ns
Input 2h 6h
Time after cell injection
Untreated (GFP+) : Fresh (CD45.1+)
Ratio of Untreated to Fresh Neu
**
**
Input 2h 6h
Time after cell injection
G-CSF (GFP+) : Fresh (CD45.1+)
Ratio of G-CSF to Fresh Neu
**
**
Input 2h 6h
Time after cell injection
CLON-G (GFP+) : Fresh (CD45.1+)
Ratio of CLON-G to Fresh Neu
ns
ns
Input 2h 6h
Time after cell injection

Fig. S11. The effect of CLON-G-treatment on the recruitment of transfused neutrophils in a mouse peritonitis model.

(**A**) Experimental scheme for assessing the relative in vivo recruitment of transfused neutrophils in a mouse peritonitis model. The peritonitis was induced by 3% TG (i.p.). The trafficking of transfused cultured green fluorescent protein $(GFP)^+$ and fresh $CD45.1^+$ neutrophils was assessed in the same neutropenic CD45.2 recipient mouse. The fresh, untreated, G-CSF alone-treated, and CLON-G-treated neutrophils were prepared as described in Fig. 2D. (**B**) Total cell number in peritoneal cavity (PC). The cells were stained with APC-Ly6g antibody and the percentage of neutrophils was analyzed by flow cytometry. Neutrophil number in peritoneal lavage was calculated as the product of percentage of neutrophils and total cell number. (**C**) Flow cytometry analysis of recruited transfused neutrophils in peritoneal lavage. Cells in peritoneal lavage fluid were stained with PE-CD45.1. Representative flow cytometry plots from one of three independent experiments are shown. (**D**) The ratio of indicated transplanted neutrophil populations in peritoneal lavage fluid. All data are represented as mean ± SD of three experiments. * * indicates $P<0.001$, compared to input. ns. not statistically significant ($P >0.05$).

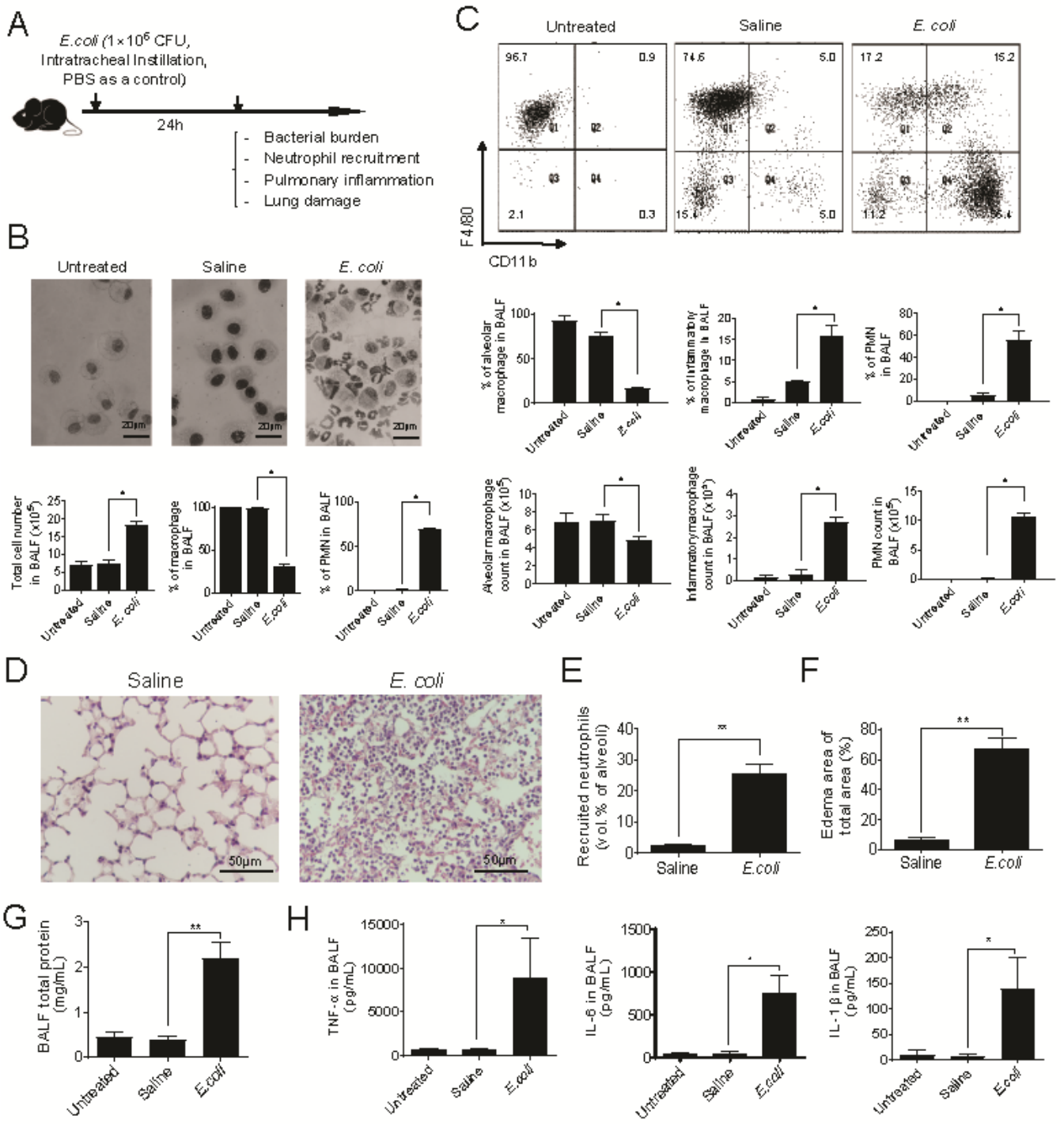

Fig. S12. A clinically relevant mouse *E. coli* pneumonia model.

(**A**) Pneumonia was induced by intrarectal instillation of 1×10^6 cfu *E. coli*. Lung inflammation and pathology were assessed 24 hours after *E. coli* challenge. (**B**) The macrophages and neutrophils in bronchoalveolar lavage fluid (BALF) of untreated, saline control, and *E. coli*-challenged mice were assessed by morphometric analysis. The morphology of BALF cells was analyzed by Wright-Giemsa staining. Macrophages and neutrophils were identified by morphometric analysis and their percentage was determined accordingly. At least 200 cells were examined for each sample. Scale bars, 20μm. (**C**) The macrophages and neutrophils in BALF of untreated, saline control, and *E. coli*-challenged mice were assessed by flow cytometry. The cells in BALF were stained with FITC-F4/80 and APC-CD11b

antibodies. The percentages of alveolar macrophage ($CD11b^{-}F4/80^{+}$), inflammatory macrophages ($CD11b^{+}F4/80^{+}$), and polymorphonuclear cells (PMN, $CD11b^{+}F4/80^{-}$) were analyzed by flow cytometry. (**D**) H&E staining of lung tissues from saline control or *E. coli*-challenged mice are shown. Scale bar, 50 μm. (**E**) Emigrated neutrophils in alveolar air spaces were quantified as volume fraction of the alveolar air space using standard point-counting morphometric techniques. The relative volumes of the parenchymal regions occupied by emigrated neutrophils were calculated by investigators blinded to the identities of the mice and were expressed as the percentage of the total parenchymal region volume (including both tissue and air space). (**F**) Pulmonary edema formation was quantified as the percentage of edema area in the total parenchymal region. (**G**) Protein accumulated in the BALF was measured using a protein assay kit. (**H**) BALF chemokine and cytokine concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kits. Data are represented as mean ± SD of three experiments. *, $P<0.05$; **, $P<0.001$.

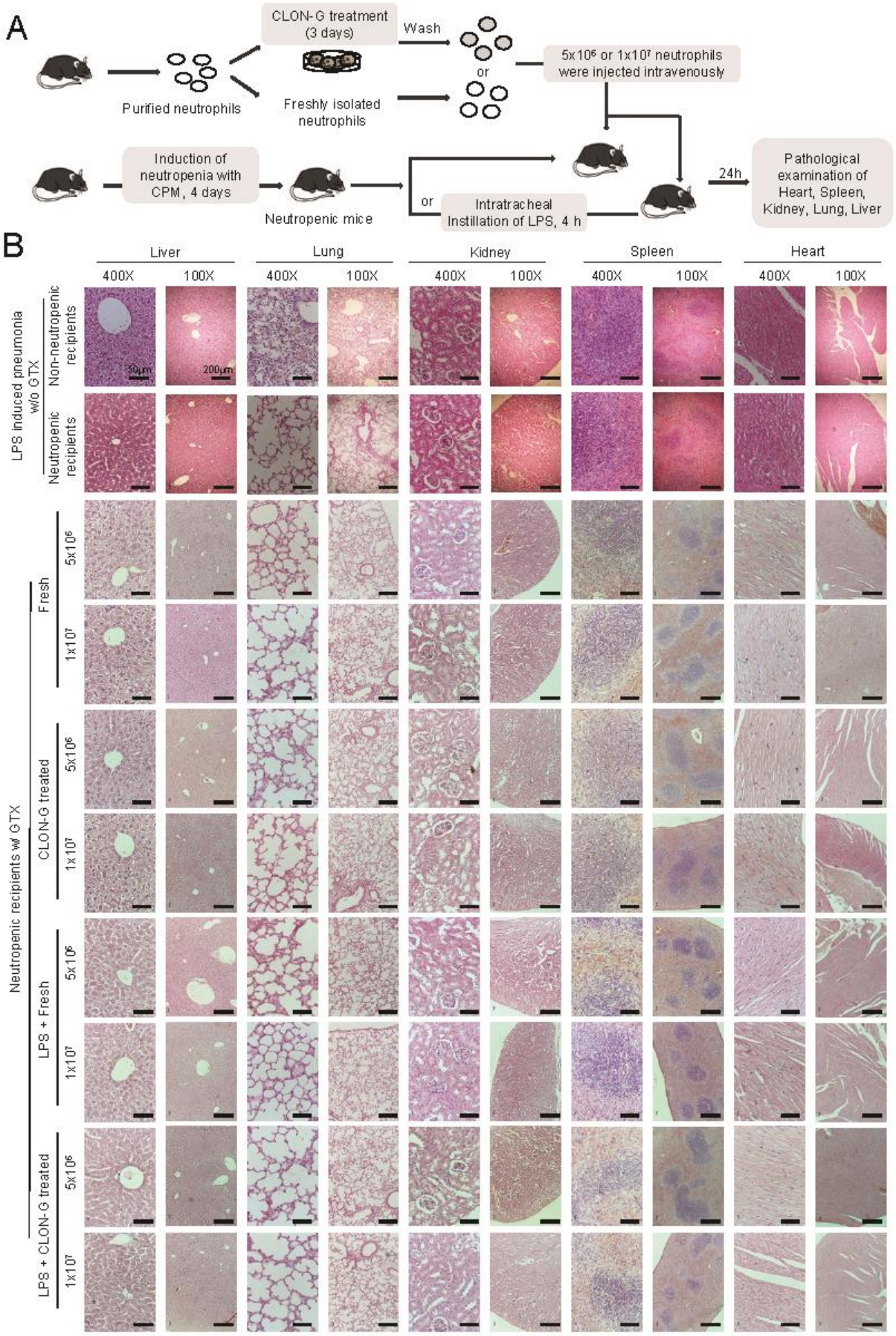
A
CLON-G treatment (3 days)
Wash
or
5x10⁶ or 1x10⁷ neutrophils were injected intravenously
Purified neutrophils
Freshly isolated neutrophils
Induction of neutropenia with CPM, 4 days
Neutropenic mice
or
Intratracheal Instillation of LPS, 4 h
24h
Pathological examination of Heart, Spleen, Kidney, Lung, Liver
B
Liver
Lung
Kidney
Spleen
Heart
400X
100X
LPS induced pneumonia w/o GTX
Non-neutropenic recipients
Neutropenic recipients
Neutropenic recipients w/ GTX
Fresh
CLON-G treated
LPS + Fresh
LPS + CLON-G treated
5x10⁶
1x10⁷
50μm
200μm

Fig. S13. Transfusion of fresh or CLON-G treated neutrophils does not induce tissue damage in unchallenged or lipopolysaccharides (LPS)-challenged neutropenic mice.

(**A**) Neutropenic mice were or were not challenged by intratracheal instillation of LPS (5 mg/kg body weight), and then transfused with fresh or CLON-G treated neutrophils. Pathological examination of heart, spleen, kidney, lung, and liver was performed 24 hours after neutrophil transfusion. (**B**) Histopathologic assessment of the heart, spleen, kidney, lung, and liver of mice transfused with indicted amounts of fresh or CLON-G treated neutrophils. Representative H&E-stained sections of indicated tissues are shown. Scale bars are indicated in each panel.

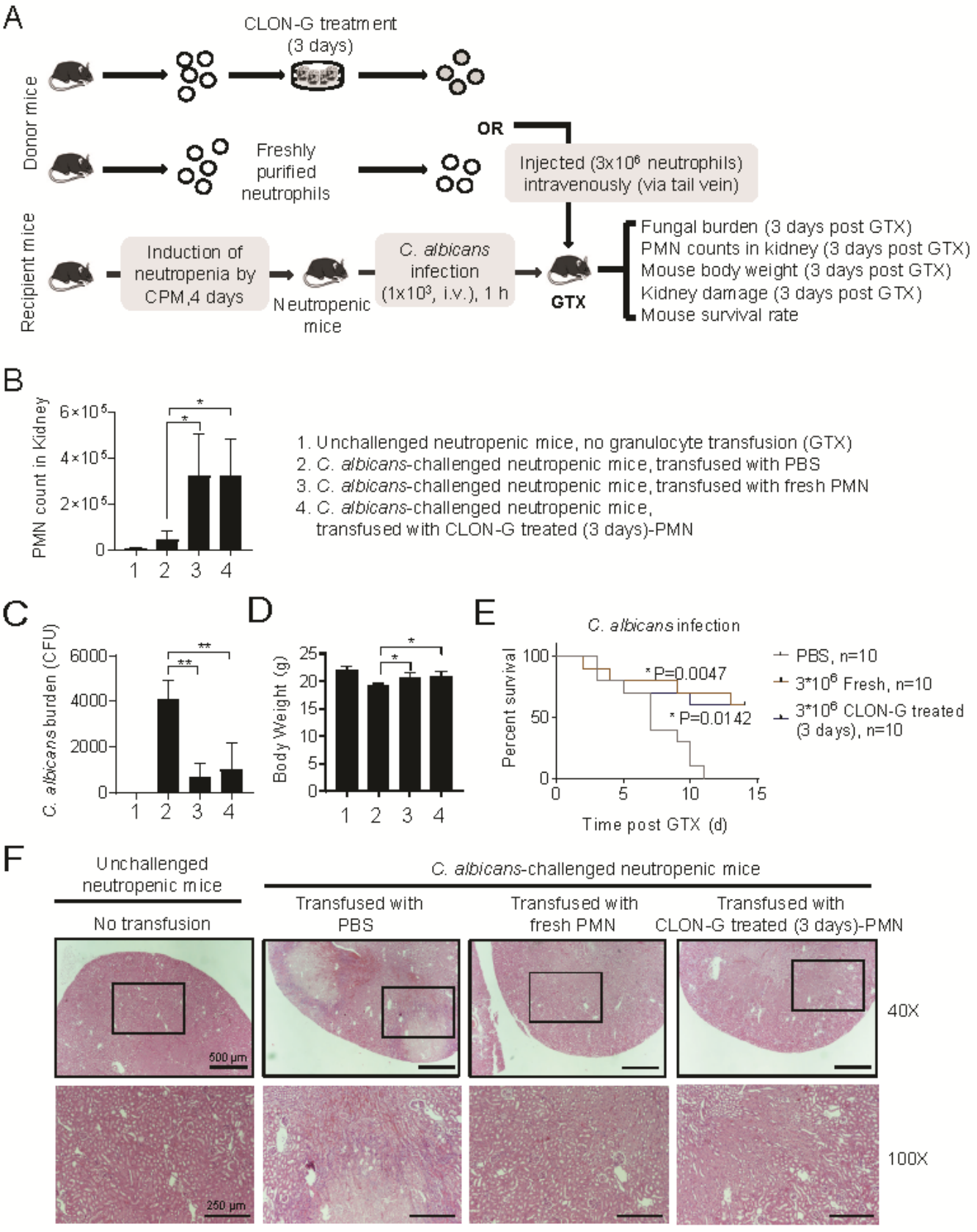

Fig. S14. Transfusion with stored CLON-G treated neutrophils enhances host defenses as effectively as transfusion with untreated fresh neutrophils in neutropenia-related fungal infection.

(**A**) Neutropenic mice were challenged with *C. albicans* (1×10^3 cfu). (**B**) Neutrophil cell counts in the kidney. On day 3, kidney tissues were harvested by grinding and filtration; the total cell number was counted with a hemocytometer. The harvested cells were stained with CD45-PE-Cy7 and Ly6G-APC; the percentage of PMNs ($CD45^+Ly6G^+$) was evaluated by flow cytometry. Neutrophil cell counts in the kidney were calculated accordingly. Data are represented as mean ± SD of three experiments. * indicates $P<0.01$. (**C**) The fungal burden in kidney tissue. Data are represented as mean ± SD of three experiments. *, $P<0.05$; **, $P<0.001$. (**D**) Body weights of *C. albicans*-challenged mice. Data are represented as mean ± SD of three experiments. * indicates $P<0.01$. (**E**) The survival curve for *C. albicans*-challenged mice transfused with fresh or CLON-G-treated neutrophils is shown. Survival was analyzed using a Log-rank test. P-values are indicated. (**F**) H&E staining of kidney tissues is shown. Representative images of three experiments are shown. Scale bars are indicated in each panel.

Targets	Inhibitor/drug	Source	Concentration (Final)	Neutrophil survival after 3 days - normalized to fresh neutrophils (100)
Untreated (Control)				< 25
Caspase-1/4	Z-YVAD-FMK	Adooq Bioscience	12.5 µM	< 25
			25 µM	< 25
			50µM	< 25
			100µM	< 25
			200µM	< 25
	Belnacasan (VX-765)	Selleck chem	12.5 µM	< 25
			25 µM	< 25
			50µM	< 25
			100µM	< 25
			200µM	< 25
Caspase-2	Z-VDVAD-FMK	Adooq Bioscience	25 µM	< 25
Caspase-3	Z-DQMD-FMK	Adooq Bioscience	25 µM	< 25
	Boc-D-FMK	Adooq Bioscience	25 µM	< 25
	Ivachtin	Adooq Bioscience	25 µM	< 25
	Z-DEVD-FMK	Selleck hem	12.5 µM	< 25
			25 µM	< 25
			50µM	< 25
			100µM	< 25
			200µM	< 25
Caspase-4	Belnacasan (VX-765)	Selleck chem	12.5 µM	< 25
			25 µM	< 25
			50µM	< 25
			100µM	< 25
			200µM	< 25
Caspase-5	Z-WEHD-FMK	Adooq Bioscience	25 µM	< 25
Caspase-6	Z-VEID-FMK	Adooq Bioscience	25 µM	< 25
Caspase-8	Z-IETD-FMK	Selleck chem	12.5 µM	< 25
			25 µM	< 25
			50µM	< 25
			100µM	< 25
			200µM	< 25
Caspase-9	Ac-LEHD-CHO	Sigma-Aldrich	12.5 µM	< 25
			25 µM	< 25
			50µM	< 25
			100µM	< 25
			200µM	< 25
Caspase-11	Wedelolactone	Adooq Bioscience	25 µM	< 25
Pan-caspase	Q-VD-Oph	Selleck chem	12.5 µM	65 ± 9.6
			25 µM	60 ± 0.8
			50 µM	65 ± 3.9
			100 µM	62 ± 4.9
			200 µM	47 ± 8.3
	Z-VAD-FMK	Selleck chem	12.5 µM	< 25
			25 µM	< 25
			50 µM	< 25
			100 µM	< 25
			200 µM	< 25
	Emricasan	Selleck chem	100 nM	< 25
			1 µM	< 25
			10 µM	< 25

			100 µM	< 25
			200 µM	< 25
	Ac-DEVD-CHO	Selleck chem	100 nM	< 25
			1 µM	< 25
			10 µM	< 25
			25 µM	< 25
			100 µM	< 25
			200 µM	< 25
LMP	DFO	Sigma-Aldrich	1 µM	< 25
			10 µM	< 25
			100 µM	< 25
			1 mM	< 25
			10 mM	< 25
	Hsp70	Enzo Life Sciences	10 pM	< 25
			100 pM	< 25
			1 nM	< 25
			10 nM	< 25
			100 nM	< 25
			200 nM	< 25
Serine protease	DFP	Sigma-Aldrich	1 nM	< 25
			10 nM	< 25
			100 nM	< 25
			1 µM	< 25
			10 µM	< 25
NADPH oxidase	DPI	Sigma-Aldrich	1 nM	< 25
			10 nM	< 25
			100 nM	< 25
			1 µM	< 25
			10 µM	< 25
			50 µM	< 25
Antioxidant	N-acetylcysteine (NAC)	Sigma-Aldrich	3.125 mM	< 25
			6.25 mM	< 25
			12.5 mM	< 25
			25 mM	< 25
			50 mM	< 25
Necroptosis	Nec-1s	EMD Millipore (852391-15-2)	100 nM	< 25
			1 µM	< 25
			10 µM	< 25
			100 µM	< 25
			200 µM	< 25
	Necrox-2	Enzo Life Sciences (ALX-430-166)	10 nM	< 25
			100 nM	< 25
			1 µM	< 25
			10 µM	< 25
			100 µM	< 25
	Necrox-5	Enzo Life Sciences (ALX-430-167)	10 nM	< 25
			100 nM	< 25
			1 µM	< 25
			10 µM	< 25
			100 µM	< 25
Growth factor	G-CSF	Amgen	1 ng/ml	31 ± 2.1
			10 ng/ml	35 ± 4.5
			100 ng/ml	30 ± 5.5
			200 ng/ml	31 ± 2.1
			1 µg/ ml	34 ± 3.2
			10 µg/ ml	32 ± 5.4

Rho-GTPase	NSC23766 (Rac inhibitor)	Zheng Lab	100 nM	< 25
			1 μM	< 25
			10 μM	< 25
			100 μM	< 25
Combined treatment	Z-YVAD-FMK+ Z-DEVD-FMK			< 25
	Belnacasan (VX-765)+ Z-DEVD-FMK			< 25
	Q-VD-Oph +NAC			74 ± 5.2
	Q-VD-Oph +DFO			74 ± 5.0
	Q-VD-Oph +Hsp70			76 ± 2.2
	Q-VD-Oph + Nec-1s			75 ± 4.9
	Q-VD-Oph + G-CSF			70 ± 2.4
	Z-VAD-FMK + DFO + DPI + G-CSF			35 ± 3.8
	Emricasan + DFO + DPI + G-CSF			36 ± 9.1
	Ac-DEVD-CHO + DFO + DPI + G-CSF			28 ± 10.3
	Z-DEVD-FMK + Hsp70+ NAC + G-CSF			26 ± 9.6
	Z-VAD-FMK + Hsp70+ NAC + G-CSF			74 ± 13.1
	Ac-DEVD-CHO + Hsp70+ NAC + G-CSF			79 ± 6.8
	Q-VD-Oph + DFO + DPI + G-CSF			39 ± 7.7
	Q-VD-Oph + human Hsp70 + DPI + G-CSF			43 ± 9.4
	Q-VD-Oph + DFO + NAC + G-CSF			56 ± 7.5
	Q-VD-Oph + mouse Hsp70+ DPI + G-CSF			51 ± 5.4
	Q-VD-Oph + Hsp70+ NAC + G-CSF			85 ± 10.5
	Q-VD-Oph + DFO + NAC + DFP + G-CSF			51 ± 12.5
	Q-VD-Oph + Hsp70+ DPI + DFP + G-CSF			55 ± 7.0
	Q-VD-Oph + Hsp70+ NAC + DFP + G-CSF			63 ± 16.2
	Q-VD-Oph + Hsp70+ NAC + DFO + G-CSF+ Nec-1s			91 ± 6.6
	Q-VD-Oph + Hsp70+ NAC + DFO + G-CSF+ Nec-1s + NSC23766			83 ± 8.5

Table S1. Screening for treatments that delay human neutrophil death. Neutrophil survival after 3 days assessed as described in Fig. 1 was normalized to untreated fresh neutrophils (counted as 100). LMP, lysosomal membrane permeabilization. All data are represented as mean ± SD of at least three experiments.

Targets	Inhibitor/drug	Source	Concentration (Final)	Neutrophil survival after 3 days - normalized to fresh neutrophils (100)
Untreated (Control)				< 10
Caspase-1/4	Z-YVAD-FMK	Adooq Bioscience	12.5 µM	< 10
			25 µM	< 10
			50µM	< 10
			100µM	< 10
			200µM	< 10
	Belnacasan (VX-765)	Selleck chem	12.5 µM	< 10
			25 µM	< 10
			50µM	< 10
			100µM	< 10
			200µM	< 10
Caspase-3	Z-DEVD-FMK	Selleck hem	12.5 µM	< 10
			25 µM	< 10
			50µM	< 10
			100µM	< 10
			200µM	< 10
Caspase-8	Z-IETD-FMK	Selleck chem	12.5 µM	< 10
			25 µM	< 10
			50µM	< 10
			100µM	< 10
			200µM	< 10
Caspase-9	Ac-LEHD-CHO	Sigma-Aldrich	12.5 µM	< 10
			25 µM	< 10
			50µM	< 10
			100µM	< 10
			200µM	< 10
Pan-caspase	Q-VD-Oph	Selleck chem	12.5 µM	49 ± 4.4
			25 µM	51 ± 2.5
			50 µM	52 ± 0.2
			100 µM	46 ± 2.4
			200 µM	50 ± 3.6
	Z-VAD-FMK	Selleck chem	12.5 µM	< 10
			25 µM	< 10
			50 µM	< 10
			100 µM	< 10
			200 µM	14 ± 0.3
	Emricasan	Selleck chem	100 nM	< 10
			1 µM	< 10
			10 µM	< 10
			100 µM	< 10
			200 µM	< 10
	Ac-DEVD-CHO	Selleck chem	100 nM	< 10
			1 µM	< 10
			10 µM	< 10
			100 µM	< 10
			200 µM	< 10
LMP	DFO	Sigma-Aldrich	1 µM	< 10
			10 µM	< 10
			100 µM	< 10
			1 mM	< 10
			10 mM	< 10

	Hsp70	Abcam	10 pM	< 10
			100 pM	< 10
			1 nM	< 10
			10 nM	< 10
			100 nM	< 10
			200 nM	< 10
Serine protease	DFP	Sigma-Aldrich	1 nM	< 10
			10 nM	< 10
			100 nM	< 10
			1 μM	< 10
			10 μM	< 10
NADPH oxidase	DPI	Sigma-Aldrich	1 nM	< 10
			10 nM	< 10
			100 nM	< 10
			1 μM	< 10
			10 μM	< 10
			50 μM	< 10
Antioxidant	N-acetylcysteine (NAC)	Sigma-Aldrich	3.125 mM	< 10
			6.25 mM	< 10
			12.5 mM	< 10
			25 mM	< 10
			50 mM	< 10
Necroptosis	Nec-1s	EMD Millipore (852391-15-2)	100 nM	< 10
			1 μM	< 10
			10 μM	< 10
			100 μM	< 10
			200 μM	< 10
	Necrox-2	Enzo Life Sciences (ALX-430-166)	10 nM	< 10
			100 nM	< 10
			1 μM	< 10
			10 μM	< 10
			100 μM	< 10
	Necrox-5	Enzo Life Sciences (ALX-430-167)	10 nM	< 10
			100 nM	< 10
			1 μM	< 10
			10 μM	< 10
			100 μM	< 10
Growth factor	G-CSF	Amgen	1 ng/ml	19 ± 2.3
			10 ng/ml	21 ± 2.3
			100 ng/ml	21 ± 1.5
			1 μg/ ml	21 ± 1.8
			10 μg/ ml	21 ± 1.2
Rho-GTPase	NSC23766 (Rac inhibitor)	Zheng Lab	100 nM	< 10
			1 μM	< 10
			10 μM	< 10
			100 μM	< 10
Combined treatment	Belnacasan (VX-765) + Z-DEVD-FMK			< 10
	Z-DEVD-FMK + Z-IETD-FMK			< 10
	Z-DEVD-FMK + Ac-LEHD-CHO			< 10
	VX-765+ Z-DEVD-FMK+ Z-IETD-FMK+Ac-LEHD-CHO			< 10
	Q-VD-Oph +NAC			61 ± 7.0
	Q-VD-Oph +DFO			64 ± 9.0
	Q-VD-Oph +Hsp70			64 ± 11.3
	Q-VD-Oph + Nec-1s			67 ± 11.7
	Q-VD-Oph + G-CSF			66 ± 0.9
	Q-VD-Oph + G-CSF+NAC			63 ± 4.3

	Q-VD-Oph + G-CSF+DFO	77 ± 6.0
	Q-VD-Oph + G-CSF+ Hsp70	73 ± 4.8
	Q-VD-Oph + G-CSF+ Nec-1s	87 ± 4.1
	Q-VD-Oph + G-CSF+ Necrox-2	70 ± 8.6
	Q-VD-Oph + G-CSF+ Necrox-5	72 ± 3.6
	Q-VD-Oph + Hsp70+ NAC + DFO + G-CSF+ Nec-1s	90 ± 0.4
	Q-VD-Oph + Hsp70+ NAC + DFO + G-CSF+ Nec-1s + NSC23766	78± 6.2

Table S2. Screening for treatments that delay murine neutrophil death. Neutrophil survival after 3 days assessed as described in Fig. 1 was normalized to untreated fresh neutrophils (counted as 100). All data are represented as mean ± SD of at least three experiments.