Material and methods

Subjects and study design

The institutional ethics committees from each hospital involved approved the studies, and written informed consent was obtained from all patients or their legal representatives in accordance with the protocols. Anonymous, healthy volunteers working at the University Medical Center Utrecht between the age of 18–65 years, male and female, provided blood samples for experiments with normal neutrophils. Peripheral blood was obtained from CGD patients in the Academic Medical Center Amsterdam. These subjects had mutations identified in the genes encoding p47phox (AR-CGD, homozygous Δdeletion in NCF1) or gp91phox (X-linked CGD, mutations in CYBB). Healthy male volunteers were enrolled after screening in Radboud University Medical Center Nijmegen for the endotoxemia model. Subjects participated in experiments, which were part of larger endotoxin trials (NCT01766414, NCT02602977, NCT01978158, NCT01835457 www.clinicaltrials.gov). Subjects were prehydrated with 1500 ml glucose/saline infusion. Reference E.Coli endotoxin (11:H 10:K negative) was used in these studies, which was reconstituted in 5 ml saline 0.9% and injected as a single i.v. bolus (2 ng/kg) during 1 min at t=0. Blood samples were taken from the arterial catheter at t=180 after administration of LPS since this time point was characterized by the presence of sufficient amounts of different neutrophil subsets as was known from previous studies[1].

Isolation of neutrophils and sorting of neutrophil subsets

Blood was collected in sterile collection tubes containing sodium heparin as anticoagulant. Neutrophils were isolated by a two-step procedure based on a method described previously[2]. Briefly, 9 ml of whole blood was diluted with an equal volume of PBS2+ (phosphate buffered saline supplemented with 0.38% trisodium citrate and 10% pasteurized plasma solution) and

centrifuged (2000 rpm for 20 min) after a layer of 18 ml Ficoll (GE Healthcare Life Sciences) was carefully underlain. After removing the interphase with mononuclear cells, the remaining erythrocytes were lysed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4°C.

For isolation of subsets of neutrophils whole blood sample was put on ice, and erythrocytes were lysed by adding three parts of isotonic ice-cold NH_4Cl. Total leukocytes obtained after lysis of erythrocytes were resuspended in Hepes3+ buffer (NaCl (132 mM), KCl (6 mM), potassium phosphate (1,2 mM), MgSO4 (1 mM), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes) supplemented with CaCl2 (1 mM), glucose (5.5 mM), and HSA (0.5% wt/vol)) and incubated with mouse anti-human CD16 (FITC, clone 3G8, BD Pharmingen) and CD62L (PE-CY5, clone DREG-56, BD Pharmingen) antibodies for 30 min at 4°C. After staining of the cells samples were washed before sorting on the MoFlo Astrios Eq (Beckman Coulter Life Sciences). Neutrophils were initially gated based on their characteristic forward scatter and side scatters. To divide three neutrophil subsets, cells were gated by taking the lower threshold of $CD62L^{bright}$ lymphocytes as the cut-off value for determination of CD62L dimness/brightness. After gating for CD62L the lower threshold for CD16 of $CD62L^{dim}$ neutrophils served as a cut-off value for division of CD16 dimness/brightness. After sorting, neutrophil populations were checked for their purity, and cellular concentration was determined by a Cell-Dyn 1800 cell counter (Abbott Diagnostics).

Bacterial strains and growth conditions

As described by Pang et al. community acquired methicillin-resistant *Staphylococcus aureus* MW2 strain (clone USA 400; Centers for Disease Control and Prevention, 1999) was transformed with pCM29 that expresses a robust superfolder fluorescent proteint green (GFP) from the sarAP1 promotor[3]. Bacteria were grown overnight on an orbital shaker at 37°C in

Tod Hewitt Broth (THB), always in the presence of chloramphenicol (10 ug/ml) to maintain expression of the plasmid. Subsequently, bacteria were cultured in fresh THB for 3 hours at 37°C to obtain bacteria in mid-log phase growth. Bacteria were washed, suspended in Hepes3+ and stored in -80°C freezer until use in a concentration of 1 x 10^{10} CFU's/ml. Evaluation of fluorescence was performed using a Fluostar Optima plate reader (BMG Labtech, Ortenberg, Germany). To ascertain a direct correlation between fluorescence and amount of CFU the amount of viable bacteria was determined using a pour-plate method. In short, samples of 100 µl of THB inoculated with SA-GFP were placed in 96-wells plate and incubated at 37 degrees whilst fluorescence was determined. Simultaneously, samples were serially diluted in PBS at the start and after each 30 minutes during 3 hours. Dilutions were pipetted on Todd Hewitt agar and were incubated overnight at 37°C and colonies were counted manually.

For experiments in which bacteria were grown in medium with variable pH THB was supplemented with HCl until the desired pH was reached. 1 µl of SA-GFP was added to a well containing 200 µl pH titrated THB. 96-wells plates covered with gas permeable adhesive plate seals were incubated in the platereader and growth curves were followed for 4 hours. After 4 hours the amount of CFU was determined as described above. Labeling of methanol inactivated S.aureus, or 'bioparticles', was performed as published previously[4]. In order to detect uptake as well as pH changes we made use of commercially available *Staphylococcus aureus* bioparticles pre-labeled with the pH sensitive dye pH-rodo red (Life Technologies) and co-labeled them with the pH-insensitive dye Alexa Fluor647[4]. In experiments in which bioparticles were quenched with phagosomal content we added 2 µl of stock solution bioparticles to 100 µl of constituents of human phagosomes in Hepes3+. Sequential titration was performed with variable concentrations of phagosomal content whereas the non-quenching factors pH, H_2O_2 and proteins (albumin) were tested in high/supra-physiological

concentrations whilst sub-physiological concentrations were used for the quenching agent HOCL[5].

Co-culture of neutrophils and bacteria in 3D-fibrin gels

We have adapted the assay described by Li et al. for culturing neutrophils and bacteria in tissue-like scaffolds[6]. 100 µl of fibrin gel was formed by mixing the indicated concentration of neutrophils in Hepes3+ and 2 mg/ml fibrinogen (Sigma-Aldrich , St. Louis, USA) with 40% human pooled serum (Sigma-Aldrich), 5 x 10^6 CFU/ml GFP-expressing MRSA and 1 U/ml thrombin (Sigma-Aldrich). Gels solidified after resuspending ten times in the well of a clear bottom black polysterene 96 wells plate (Corning Costar, New York, USA). Fluorescence of individual wells was determined every 20 minutes in the Fluostar Optima plate reader (BMG technologies, Ortenberg, Germany). Where indicated, lysis of neutrophils was achieved by pipetting 10 µl of triton X-100 solution suspended in Hepes3+(Sigma-Aldrich, 0,5% end concentration) on top of the matrices. The raw data from the fluorescence intensity over time was exported to excel, the derivative of all time points was calculated with Microsoft Excel, in arbitrary fluorescence units/20min. When the first derivative was above 100 points for three time points in a row, the first time point was defined as the time point of unrestrained outgrowth (TUO) (see also Figure S2).

Microscopy

For a visual confirmation of active migration through the fibrin gels and to validate the hypothesis that neutrophils phagocytose and contain SA-GFP in the phagolysosome, neutrophils and fluorescent MRSA were transferred after mixing with serum, thrombin and fibrinogen to µ-slides VI 0.4 (Ibidi GmbH, Planegg, Germany) and kept at 37°C using a climate chamber. Slides were imaged using a Deltavision RT fluorescence microscope (GE-

Healthcare, Little Chalfont, UK) attached to an EMCCD camera and videos were composed using ImageJ software (National Institutes of Health, Bethesda, USA). For determination of phagocytosis and intraphagosomal acidification bioparticles were used instead of living MRSA. 2 µl of stock solution bioparticles were used in these experiment and neutrophils were imaged in suspension instead of fibrin gel for adequate determination of in-focus bioparticles. Fluorescent z-stacks were made of three randomly selected spots per time point on the Deltavision microscope and image files were processed using Imaris software (Bitplane, Zurich, Switzerland). Neutrophils were identified and sorted based on FITC positive fluorescence after staining with CD16 (clone 3G8, BD Pharmingen, San Diego, USA) as described above. A mask was created using Imaris software based on FITC-positivity to determine neutrophil intra-and extracellular borders. Next, bioparticles were identified based on AF647 positivity and a mask was made to obtain mean fluorescence values for both fluorescent probes.

Viability

Apoptosis and necrosis of (sorted) neutrophil subsets was determined by annexin-V binding and 7-aminoactinomycin D (7-AAD) respectively (BD Biosciences, Oxford, UK). 1 x 10^5 neutrophils were stained with annexin-V (PE) and 7-AAD for 15 minutes in the dark at room temperature in annexin-binding buffer, 10 mM Hepes (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$. Flowcytometry of the samples was performed immediately on a Fortessa flowcytometer (Beckton Dickinson, Franklin Lakes, USA). A total of 10.000 events were collected for each sample. For determination of cell death in fibrin gels propidium iodide (Affymetrix, Santa Clara, USA) 1.6 ng/ml end concentration) was used and fluorescence was determined every 20 minutes during co-culture using the Fluostar Optima plate reader.

Chemotaxis

The chemotaxis of neutrophils towards the bacterial stimulus fMLP in µ-Slides was done according to the manufacturers' instructions (Ibidi GmbH). Neutrophils ($1x10^6$/ml) were mixed with human pooled serum (Sigma Aldrich, 40% vol/vol), thrombin (Sigma Aldrich, 1U/ml) and fibrinogen (Sigma-Aldrich, 2 mg/ml) equal to the co-culture as described above yet lacking bacteria. After resuspending, the cell sample was loaded in the chamber. Next, the upper reservoir was filled with fMLP (Sigma-Aldrich, 1 µM) in Hepes3+ and the lower compartment with Hepes3+ only in order to create a proper gradient. Slides were placed in an incubator, which provides constant temperature at 37 °C, on the stage of an inverted microscope connected to an image analysis system (Quantimet 570C; Leica, Wetzlar, Germany). Serial images were made every 30 seconds during 90 minutes to capture serial images of cell movement. Tracking individual neutrophils and measuring the absolute distance between end and starting point determined the net migration.

Phagosomal acidification

1.25 x 10^6 sorted neutrophils in 120 µl Hepes3+ were mixed with human pooled serum (Sigma Aldrich, 40% vol/vol). Maximal stimulation of neutrophils was achieved with 2 µl of stock suspension of dual labeled bioparticles corresponding to 10-50 organisms per neutrophil as verified by fluorescence microscopy and this concentration was used as saturating for the experiments reported here. The mixture was then placed in the incubator at 37°C and was shaken with 140 RPM during incubation. 20 µl of sample was serially transferred to a flowcytometer test tube containing 380 µl Hepes3+ and trypan blue (Sigma-Aldrich, 1.2 mg/ml) to quench extracellular particles. Fluorescence of internalized bioparticles was determined by using a Fortessa Flowcytometer (Beckton Dickinson) at indicated timepoints.

and phagocytosis of dual labelled SA bioparticles was affirmed by AF647 and pHrodo positivity.

Acidification of hypoxic phagosomes was established by resuspending neutrophils and bioparticles in Hepes3+ and serum that had been pre-equilibrated using an InvivO2 1000 hypoxic work station (Baker Ruskin, Wales, UK). Oxygen concentration was set to 0.1% with a CO_2 concentration of 5%. The pO2, pCO2, and pH of the Hepes3+/serum were measured at the beginning of each experiment to confirm the delivery of a consistent hypoxic environment. Samples were incubated in gas impermeable test tubes and samples were transferred to hypoxic Hepes3+/trypan blue seconds before measuring fluorescence.

Protease activity

Neutrophils (5 x 10^5 per subset) were incubated with cytochalasin B (Sigma-Aldrich, 5 μg/ml) TNF-α (R&D systems, Minneapolis, USA, 10 ng/ml) for 10 minutes at room temperature. Next neutrophils were stimulated to degranulate by adding fMLP (Sigma-Aldrich, 1 μM). After incubating 15 more minutes cells were spun down (2 min 5000 RPM) and the substrate containing degranulation products were evaluated for serine protease activity by using highly sensitive Abz-peptidyl-EDDnp fluorescence resonance energy transfer (FRET) substrates that fully discriminate between the three human NSPs as previously described[7,8].

MPO content

A detailed description of the proteomics approach has been described elsewhere[9]. In short 3 x 10^6 cells from each neutrophil subset were digested with trypsin, labeled with a different label for each subset in a 1:1:1 ratio before analysis by Mass Spectrometry. Samples were fractionated using strong cation exchange chromatography. Fractions were analysed using a LTQ- Orbitrap LC-MS/MS. MPO was identified by comparing the resulting peak list with the

Unipro database (Homo Sapiens) with exclusion of common contaminants.

Data-analysis and statistics

Data analyses used GraphPad Prism version 6.0. Data are plotted as mean +/- SEM as indicated in the legends. Applied statistical analyses are indicated in corresponding legends. Differences with $P < 0.05$ were considered statistically significant.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on request.

References

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Figure and Video legends supplemental material

Figure S1: Linear relationship between amount of bacteria (CFU/ml) and magnitude of fluorescence as determined in the fluorescence plate reader. Fixed amounts SA-GFP (5 x 10^6/ml) were cultured in 100 µl in the well of a 96 wells plate containing 40% human pooled serum and 60 µl Hepes3+ and were placed in the fluorescence plate reader. Relative fluorescence intensity was determined by correcting for background. Fluorescence was determined every 30 minutes and simultaneously a sample was diluted and plated on Todd Hewitt agar plates. These plates were placed in an incubator overnight and CFU were determined by manual counting. Dots represent the mean of two consecutive experiments.

Figure S2: Schematic representation of determination of TUO after culturing increasing amounts of neutrophils with $5x10^6$ CFU/ml of SA-GFP. Fluorescence was determined every 20 minutes in all cases. From the raw data on the fluorescence intensity over time, the derivative of all time points was calculated with Microsoft Excel, in arbitrary fluorescence units/20min. When the derivative was above a set threshold (100 RFU) for three time points in a row, the first time point was defined as the 'time point of unrestrained outgrowth' or 'TUO'.

Figure S3: Identical growth curves for control and Triton X-100 incubated bacteria. Fibrin gels containing human pooled serum and $5x10^6$ CFU/ml SA-GFP were placed in the incubator after addition of either 10 µl Hepes3+ or Triton-X100 0,5% end-concentration. No differences were found indicating that bacteria are not influenced by the addition of triton and delay of bacterial outgrowth in gels with neutrophils are not biased by the lysing agent.

Figure S4: Similar kinetics of bacterial growth curves after neutrophil lysis. Co-cultures of 1 x 10^7 neutrophils/ml from healthy controls and bacteria in fibrin gel were started a t=0. Neutrophils were lysed after 0, 8, 20, 30 and 40 hours by addition of triton X-100, 0,5% end-concentration, on top of the gels. In all cases bacterial outgrowth was encountered and bacteria showed equal potential to start logarithmic growth as well as comparable fluorescent plateaus indicating that no putative culture artifacts were present. Bold lines represents mean of n=4; dashed line represent SEM.

Figure S5: AF647 is quenched by HOCL. (A), Fluorescence of dual labeled SA bioparticles incubated with various concentrations of different phagosomal constituents measured by a fluorescence plate reader (n=4). For non-quenching constituents supra-physiological concentrations are displayed to illustrate that even in these circumstances no quenching was apparent[56]. Hypochlorous acid, which is formed from hydrogenperoxide and myeloperoxidase, was the only oxidative constituent that could quench AF647 even in a concentration (5 mM) far lower than expected to be present in the phagosome[56]. **(B)**, Flowcytometric analysis after incubation of dual labeled particles with neutrophils treated with the MPO inhibitor azide and NADPH-oxidase inhibitor DPI (control n=6, azide n=4, DPI n=6). AF647 fluorescence was determined 120 minutes after starting incubation with dual labeled SA bioparticles. Significantly higher values of AF647 were found when either NADPH-oxidase or MPO were inhibited. These data confirm that quenching of AF647 was caused by ROS and only occurred when MPO is active in the phagosome.

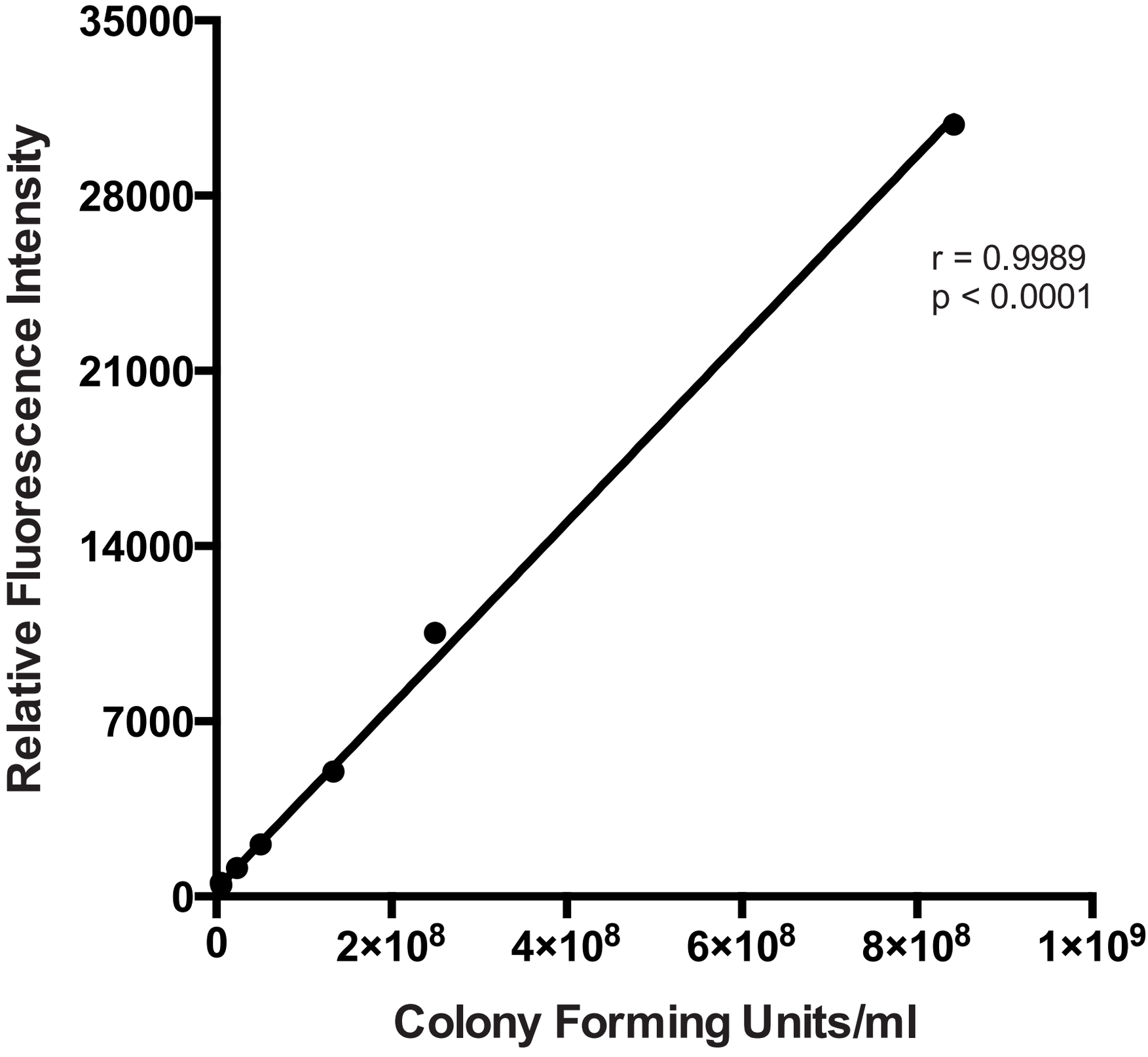
Figure S6: Viability of neutrophil subsets after sorting. Neutrophils were sorted based on expression of CD16 and CD62L as described in the methods section. After sorting, viability was assessed with Annexin-V and 7-AAD staining. Viability was found to be 97% (SD 3.8), no significance was found between neutrophil subsets (n=5).

Video 1: Fluorescence microscopy of co-culture of neutrophils and SA-GFP in fibrin gels. 5 x10^6 neutrophils/ml were cultured with 5 x10^6 CFU/ml SA-GFP in Ibidi μ-Slides and placed under Deltavision Fluorescence microscope. Images were made every 20 seconds during 11 hours. Transparent neutrophils move in and out of plane, SA is phagocytosed and transported intracellularly by neutrophils. Videos were produced using ImageJ software.

Video 2: Effect of phagocytosis on pHrodo signal of dual labeled SA bioparticles in fibrin gels; Calcein labeled neutrophils (5 x10^6 /ml) were cultured in fibrin gels with AF647 and pHRodo dual labeled SA bioparticles in Ibidi μ-Slides and placed under Deltavision Fluorescence microscope. Z-stack images were recorded every 15 seconds and imaged for 25 minutes. In the video time is displayed in the lower right corner and the movie is played back and forth 3 times. During the first 17 seconds of the video only neutrophils are shown, first by light microscopy only, then Calcein labeled neutrophils in red are displayed. After that AF647 signals on SA bioparticles are appended in blue. At 24 seconds pHRodo signals are blended in. This video illustrates how bioparticles (in blue) become pHRodo positive (green) once internalized by neutrophils. These images prove that neutrophils create a profound acidic environment around ingested bacteria. To illustrate this at 46 seconds the calcein label from neutrophils is removed: pHrodo positivity is only apparent after ingestion by a neutrophil.

Video 3: Representative recording of co-culture of hypersegmented neutrophils and SA in fibrin gels. Neutrophils were sorted and 5 x 10^6 neutrophils/ml were cultured with SA-GFP in Ibidi μ-Slides and placed under the Deltavision Fluorescence microscope. Fluorescence microscopy and light microscopy were combined to study the fate of bacteria. Bacteria are internalized and start to proliferate intracellularly and eventually escape the neutrophils.

Figure S1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28000
21000
14000
7000
0
Relative Fluorescence Intensity
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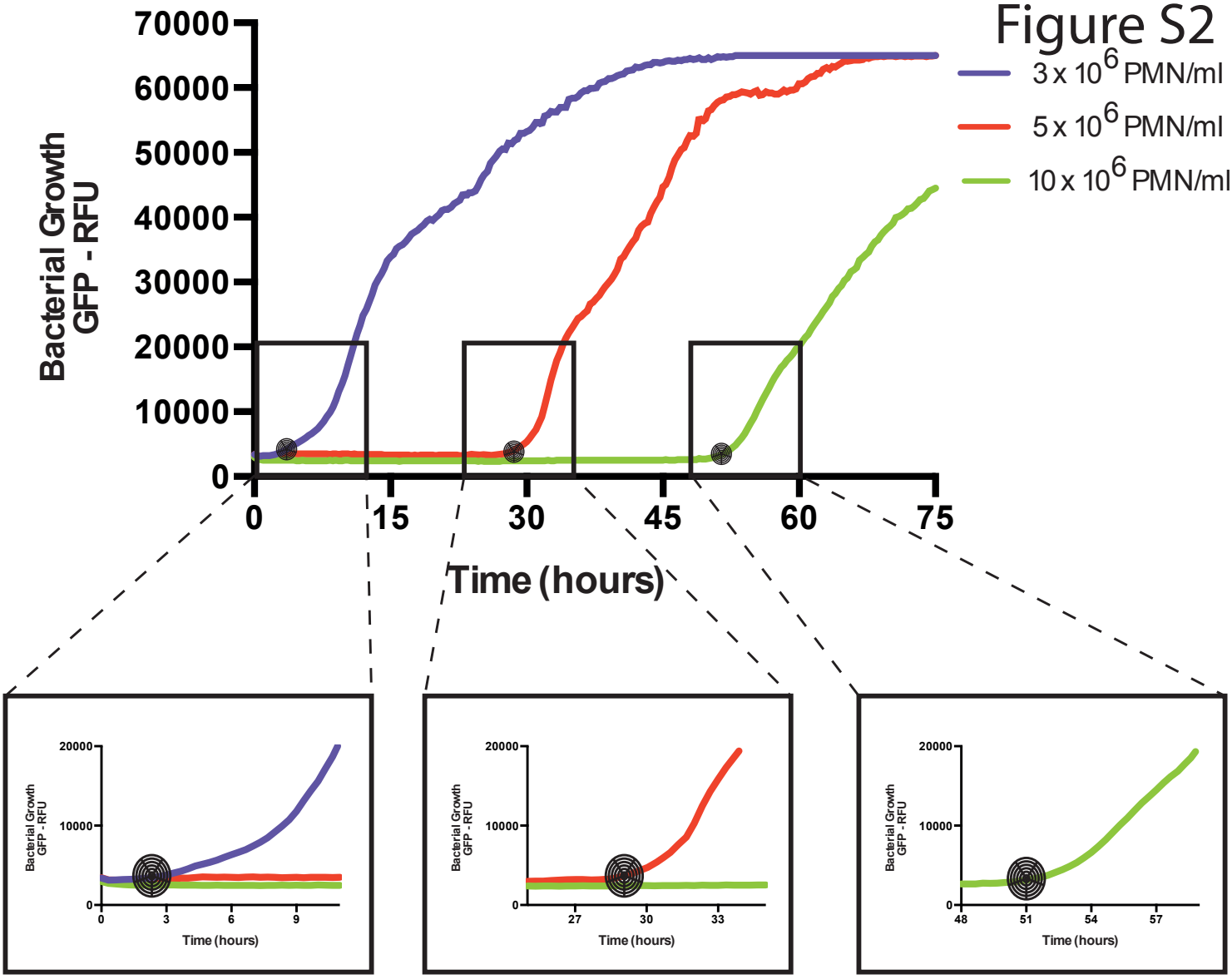
4×10^8
6×10^8
8×10^8
1×10^9
Colony Forming Units/ml
r = 0.9989
p < 0.0001

Figure S2


3 x 10^6 PMN/ml
5 x 10^6 PMN/ml
10 x 10^6 PMN/ml
Bacterial Growth GFP - RFU
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60000
50000
40000
30000
20000
10000
0
0
15
30
45
60
75
Time (hours)
Bacterial Growth GFP - RFU
20000
10000
0
0
3
6
9
Time (hours)
Bacterial Growth GFP - RFU
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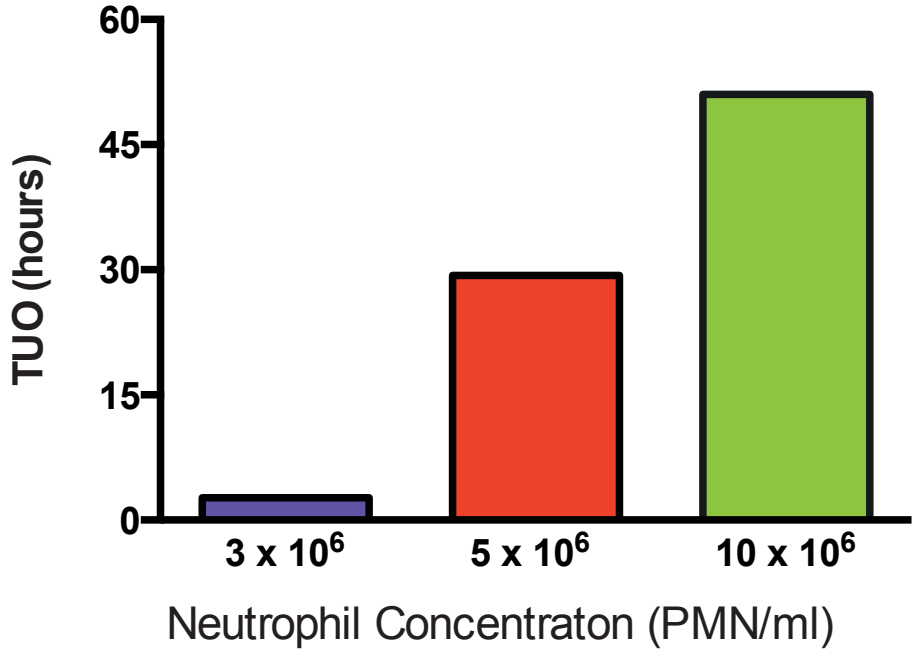
48
51
54
57
Time (hours)


TUO (hours)
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30
15
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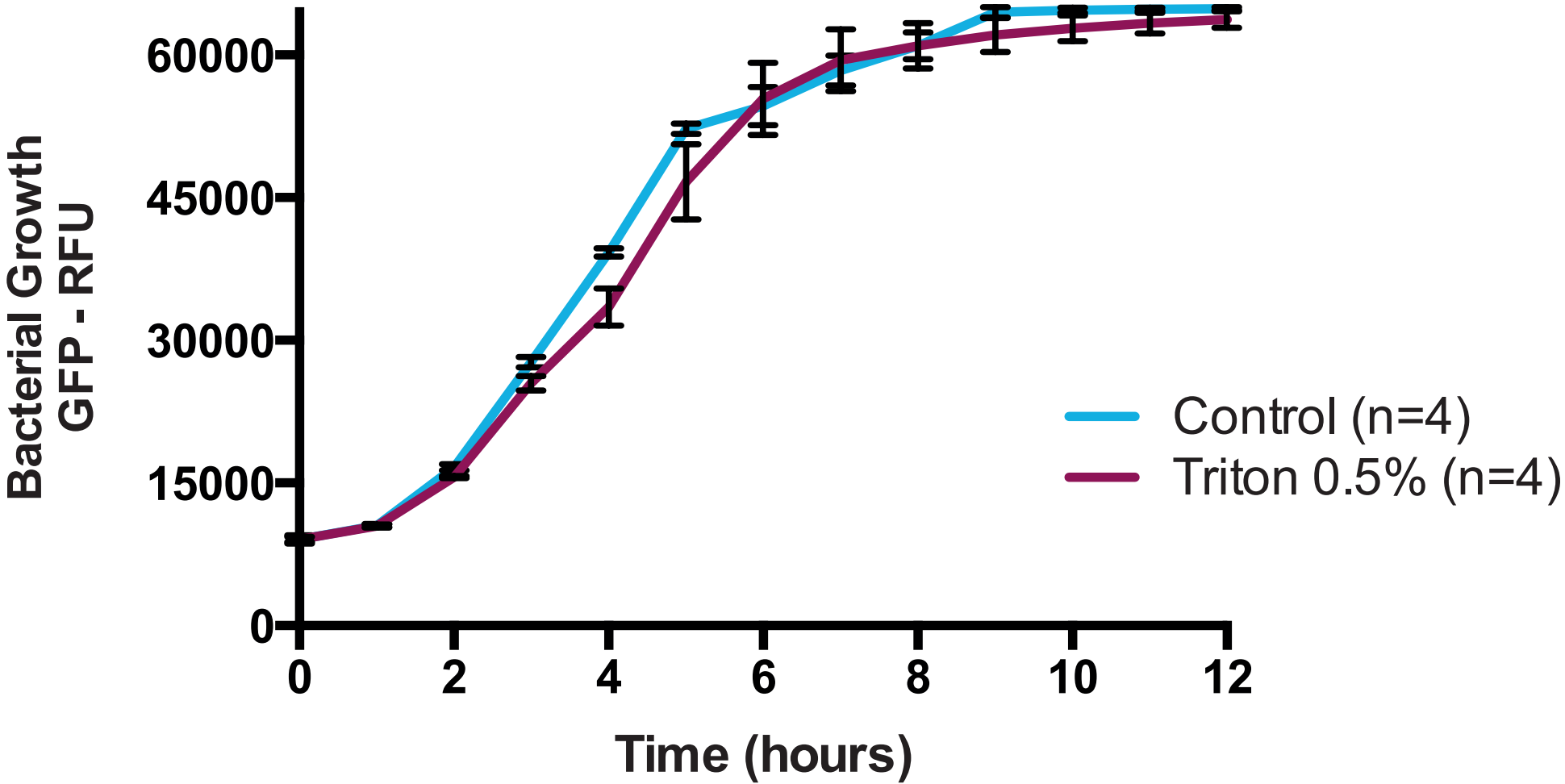
5 x 10^6
10 x 10^6
Neutrophil Concentraton (PMN/ml)

Figure S3


Bacterial Growth
GFP - RFU
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30000
15000
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2
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8
10
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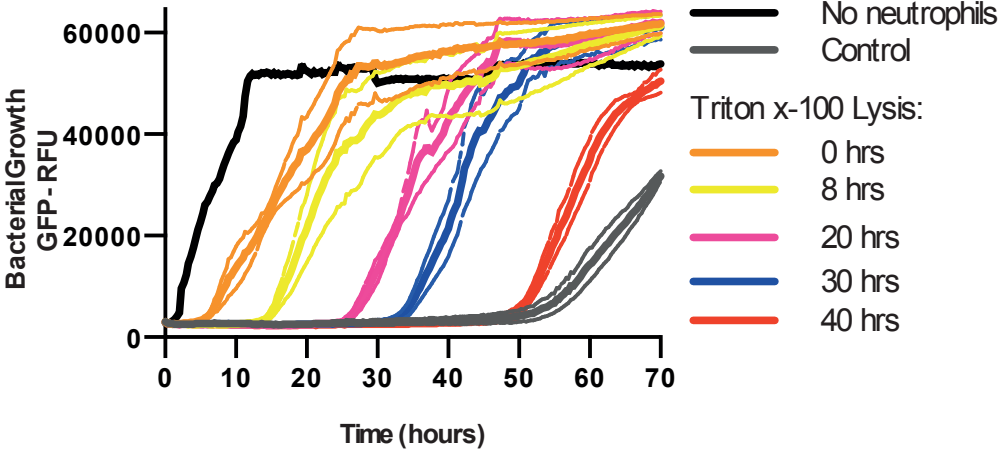
Time (hours)
Control (n=4)
Triton 0.5% (n=4)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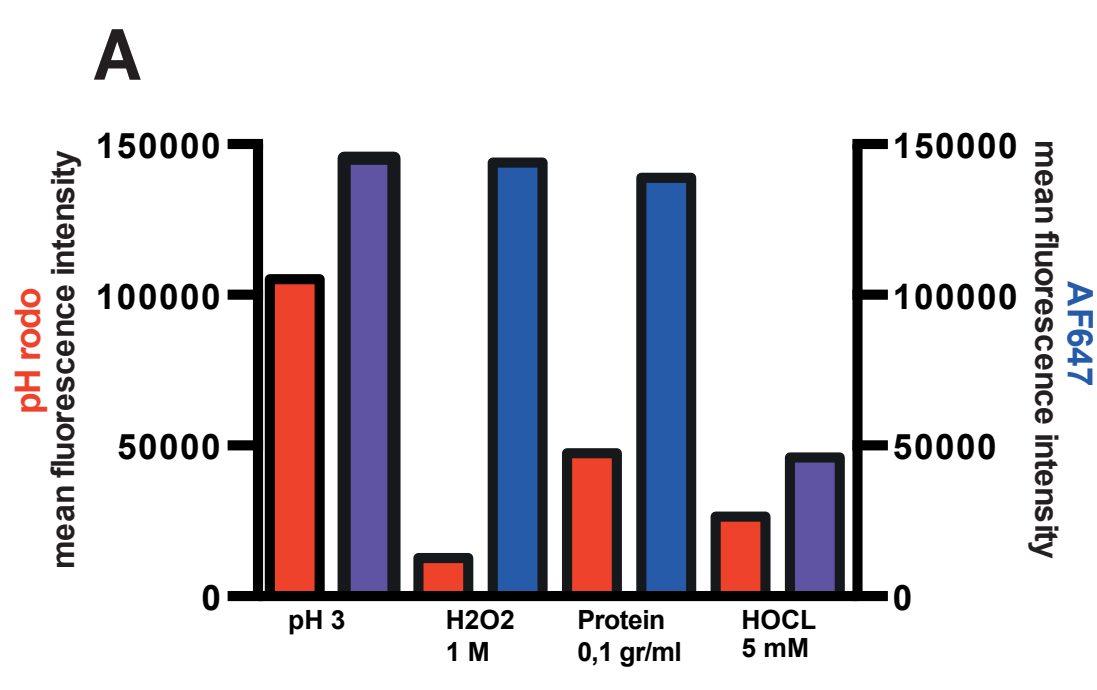
40000
20000
0
Bacterial Growth
GFP - RFU
0
10
20
30
40
50
60
70
Time (hours)
No neutrophils
Control
Triton x-100 Lysis:
0 hrs
8 hrs
20 hrs
30 hrs
40 hrs

Figure S5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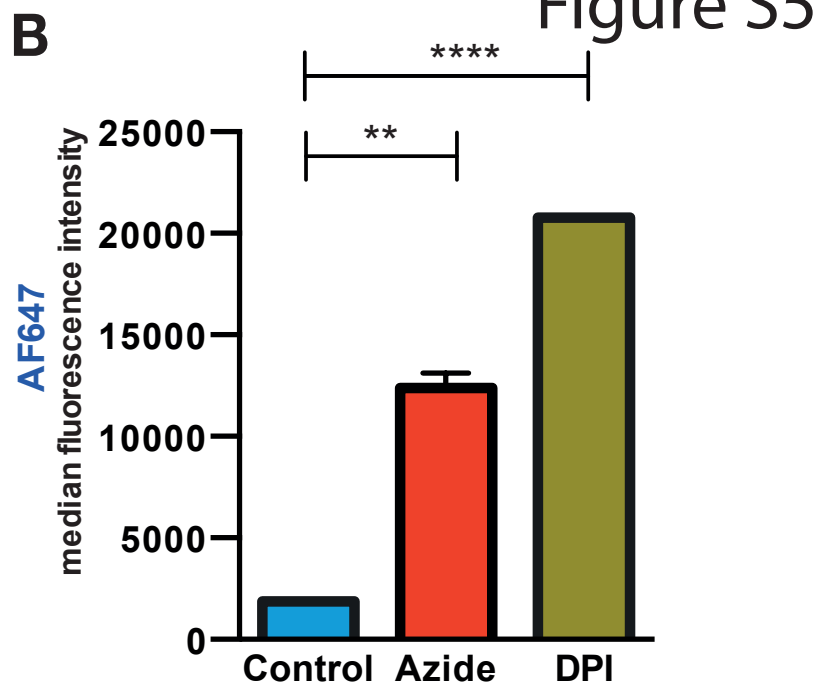
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15000
10000
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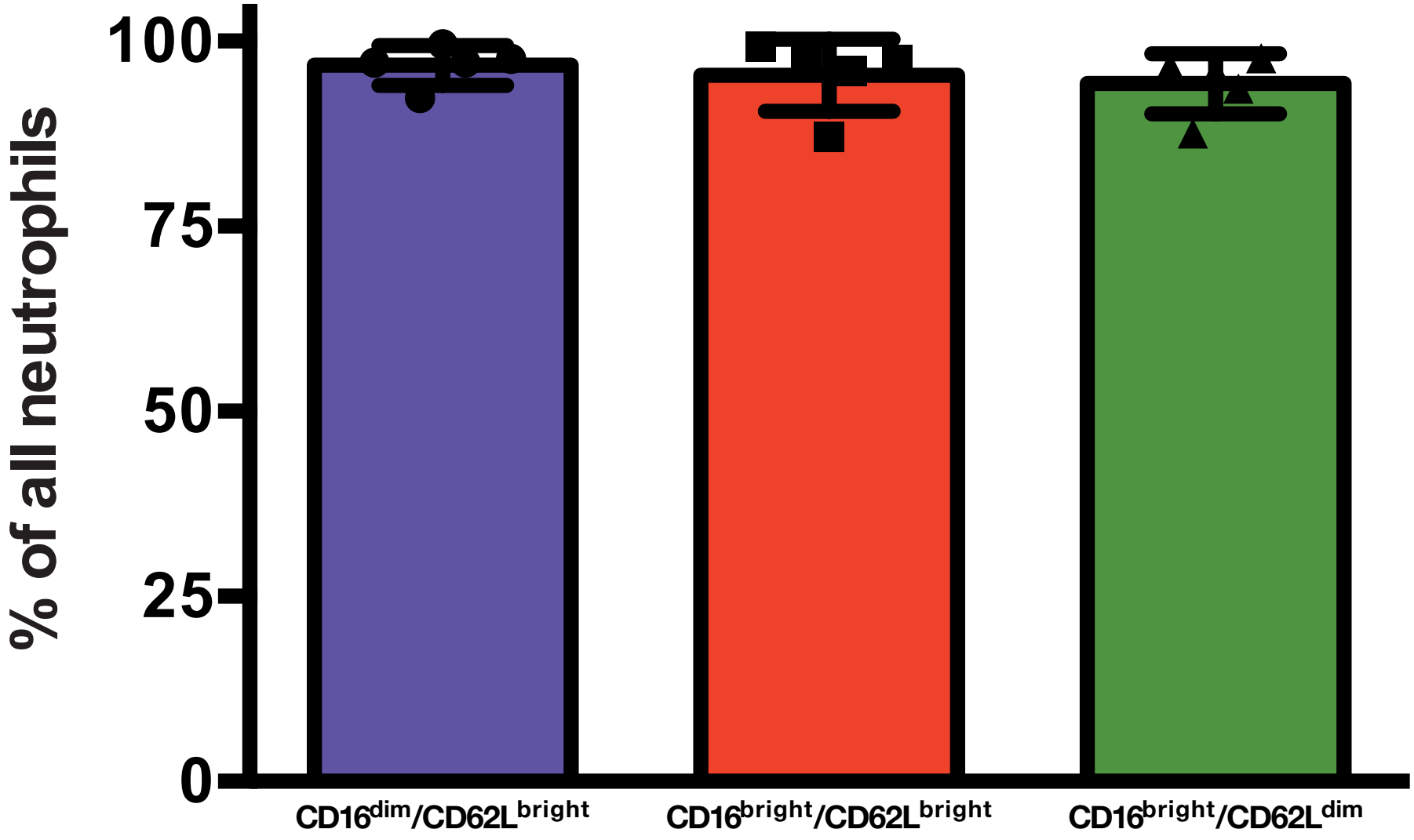
Control
Azide
DPI

Figure S6


100
75
50
25
0
% of all neutrophils
CD16dim/CD62Lbright
CD16bright/CD62Lbright
CD16bright/CD62Ldim