



## Research paper

## A comprehensive three-dimensional assay to assess neutrophil defense against bacteria

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## ABSTRACT

Neutrophil antibacterial capacity is measured in animal models and *in vitro* as an important indicator of neutrophil function. To be able to extrapolate their conclusions, *in vitro* experiments should mimic the *in vivo* situation. *In vivo*, antibacterial capacity depends on multiple steps of bacterial sensing, priming, chemotaxis, phagocytosis and intracellular killing. Therefore, we developed a simply executed assay that involves multiple steps in one assay. The neutrophils were incorporated into a three-dimensional matrix of fibrin fibers, in which they could freely migrate. The fibrin matrix provided a more physiological representation of tissue structure than a shaken suspension and extended *ex vivo* survival of neutrophils. Staphylococci endogenously producing GFP (Green Fluorescent Protein) provided a real-time quantification of the bacterial load without the need for lysing the fibrin matrix or counting of colony forming units on agar plates. The delay in bacterial outgrowth serves as a measure for the relative antibacterial capacity of the neutrophils. Additionally, neutrophil capacity could easily be measured high-throughput in a 96-wells format.

In this new assay we study neutrophil behavior in a physiologically relevant setting and explore many functions of the neutrophil in a single test. The functional capacity of neutrophils from different *in vitro* treatments or different donors can directly be compared.

## 1. Introduction

The main function of neutrophils is to defend the host against bacterial threats. This defense process requires many steps to be successful, including priming, rolling along endothelial cells, extravasation, chemotaxis and finally recognition, phagocytosis and killing of the bacteria (Kolaczowska and Kubes, 2013). After phagocytosis, neutrophils can kill bacteria intracellularly by causing a marked bactericidal milieu inside the phagolysosome, with lysosomal enzymes released from the granules and reactive oxygen species (ROS) formed by the NADPH-oxidase (Flannagan et al., 2009). Neutrophils can also kill extracellularly by releasing toxic compounds via degranulation, by releasing ROS or by releasing Neutrophil Extracellular Traps (NETs) (Brinkmann et al., 2004; Lacy, 2006). If the antibacterial capacity of

neutrophils is impaired, severe clinical symptoms might develop, such as seen in Chronic Granulomatous Disease (CGD) or Leukocyte Adherence Deficiency (LAD) (Berendes et al., 1957; Kishimoto et al., 1987). Hence, the capacity of neutrophils to control and kill bacteria is an important research subject.

When studying the antibacterial capacity of neutrophils, clear advantages of *in vitro* assays are (I) the possibility to manipulate cellular functions and (II) the possibility to discriminate and sort different subsets that can be assessed in parallel. Additionally, *in vitro* neutrophil function can be tested in high-throughput assays (Smirnov et al., 2017; Simons, 2010; Yona et al., 2010; Kuhns et al., 2001; Chow et al., 2001).

However, these types of assays have several shortcomings as they typically test only one of the steps in the antibacterial cascade, most often the step of phagocytosis or intracellular killing. To this end,

**Abbreviations:** CFU, colony forming unit; fMLF, *N*-formylmethionine-leucyl-phenylalanine; GFP, Green Fluorescent Protein; MOI, multiplicity of infection; PI, propidium iodide; PMN, polymorphonuclear leukocyte; RFU, relative fluorescence units; SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*

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neutrophils and bacteria are brought together in a shaken suspension to enforce interaction. This method bypasses steps such as bacterial sensing and chemotaxis. *Vice versa*, assays focused on migration do not involve subsequent phagocytic capacity of neutrophils. But in case of neutrophil pathology, it is not always possible to predict which step of the antibacterial cascade is affected. When neutrophils are tested *in vitro*, the existing assays may be focusing on the wrong step. In summary, many *in vitro* approaches may overlook valuable information, which can lead to biased conclusions on neutrophil *in vivo* behavior.

Performing experiments with human neutrophils *in vitro* is challenging because they are extremely sensitive for *in vitro* manipulations and conditions. Neutrophil isolation and/or culturing conditions can introduce a selection bias or artefacts. Many types of *in vitro* stimuli, not in the least the plastic or glass laboratory environment itself, can activate the neutrophils (Ginis and Tauber, 1990; Shalekoff et al., 1998; Ottonello et al., 1998), affecting the experimental results. Certainly, incubation of neutrophils in tissue culture plates and flasks does not resemble the complex environment found in the human body.

Mouse models have been used to complement *in vitro* human data with *in vivo* data. Even though murine studies have provided valuable data, they have revealed that murine neutrophils share but also lack characteristics of human neutrophils (O'Connell et al., 2015; Eruslanov et al., 2017; Condliffe et al., 2005; Hajjar et al., 2010; Kalupov et al., 2009). Certain surface markers and immunoglobulin receptors defining neutrophils in humans are not expressed on murine neutrophils (Rehli, 2002; Risso, 2000; Bruhns, 2012), and the kinetics of the neutrophil compartment is not the same (Copeland et al., 2005; Hulsdunker and Zeiser, 2015; Doring et al., 2015). *In vitro* experiments with human neutrophils, therefore, remain indispensable, but should approach the *in vivo* situation.

Some three-dimensional biological models have been proposed as a more physiological environment to study neutrophils *ex vivo*. For example, neutrophils can be added on top of a monolayer of endothelial cells to study the process of extravasation. The endothelial cells can grow on a membrane in a transwell system (Marinkovic et al., 2014), or on a matrix of collagen (Luo et al., 2015). Collagen is the most abundant protein in the extracellular matrix and is also a main constituent of Matrigel. Neutrophils can directly be embedded in a matrix of collagen or Matrigel (Steadman et al., 1997; Loike et al., 2001). Matrices can also be made from a mixture of collagen and fibrin (Guggenberger et al., 2012), or fibrin fibers alone (Koenderman et al., 2010). Conveniently, the biological matrices can be formed in different scaffolds such as 96-well plates, chemotaxis slides or angiogenesis slides (Gegenfurtner et al., 2018; Kramer et al., 2013).

Fibrin matrices form when the plasma protein fibrinogen is converted to fibrin by the enzyme thrombin, which *in vivo* occurs in blood clots and wound exudates. Since bacteria as well as neutrophils invade wounds *in vivo*, a fibrin matrix represents a natural environment for the migration of bacteria and neutrophils (Mosesson, 2005). Li et al. adapted a fibrin matrix system and showed that neutrophils could efficiently kill *Staphylococcus epidermidis* (SE) in this three-dimensional model (Li et al., 2002). After 90 min of co-incubation, the reduction in viable bacteria was measured. The matrices as well as the neutrophils were lysed to isolate the remaining bacteria. The bacterial numbers were quantified by plating dilution series on agar plates and counting colony forming units (CFU). Although counting CFUs might be considered the gold standard, this method is very laborious and prone to intra-assay and inter-assay variability. Furthermore, these agar plates represent single time points in a very dynamic process.

Surewaard et al. circumvented the need for CFU plating by using a genetically engineered *Staphylococcus aureus* (SA) strain that endogenously expresses green fluorescent protein (GFP) (Surewaard et al., 2013). A suspension of living bacteria produced a fluorescence signal, which was decreased after phagocytosis by neutrophils (Surewaard et al., 2013).

In our laboratory, these two ideas have been combined to create an

assay that examines diverse steps important for effective bacterial clearance *in vivo*, all in one quickly executed *in vitro* assay. Relative bacterial numbers are provided by direct measurements of fluorescence intensity, without lysis of the fibrin matrix. Importantly, the three-dimensional fibrin matrix allows for chemotactic migration of neutrophils and is more similar to a site of infection in the tissue than a shaken suspension. Since many steps of the antibacterial cascade are implicated in the readout, this is a sensitive method to detect neutrophil defects.

## 2. Materials & methods

### 2.1. Reagents

The incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.0 mM CaCl<sub>2</sub>, 5 mM glucose and 5 mg/ml human serum albumin (Albuman 200 g/l, Sanquin, Amsterdam, The Netherlands). The pH was adjusted to 7.4. Cells, bacteria and all other reagents were suspended and diluted in the incubation buffer unless stated otherwise.

### 2.2. Isolation of neutrophils from blood

Human blood samples were collected from anonymous, healthy volunteers between the age of 18–65 years, male and female, using sodium heparin as an anticoagulant. All donors gave informed consent under protocols approved by the Medical Ethical Committee of the University Medical Center Utrecht. Granulocytes were isolated by density gradient centrifugation over a single layer of Ficoll-Paque Plus. The erythrocytes in the granulocyte layer were lysed using a lysis buffer that consisted of 150 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub> and 0.1 mM Na<sub>2</sub> ethylenediaminetetraacetic acid (EDTA) dissolved in ddH<sub>2</sub>O and adjusted to a pH of 7.4. Hereafter, the cells were washed twice and resuspended in incubation buffer. Even though the protocol was standardized, the time between blood collection and the start of the isolation procedure could differ by *ca.* 1 h between experiments.

After FACS-sorting neutrophils based on CD16 expression, similar lag times as for Ficoll-isolated neutrophils were observed (data not shown).

### 2.3. Bacterial strains

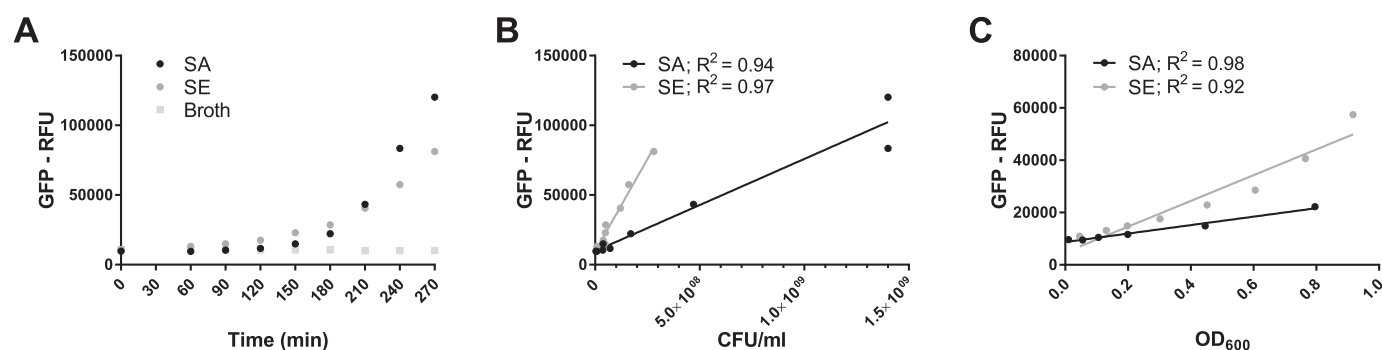
The methicillin-resistant *S. aureus* strain MW2 had been transformed with the pCM29 plasmid (Pang et al., 2010) containing the GFP gene and chloramphenicol resistance as described before (Surewaard et al., 2013). The *S. epidermidis* strain 2600 was transformed with the pCM29 plasmid using a protocol described before (Monk and Foster, 2012).

SA was grown in Todd Hewitt broth and SE in Brain Heart Infusion broth, both supplemented with 10 µg/ml chloramphenicol, until the optical density at 600 nm was 0.5. The bacteria were centrifuged and resuspended in incubation buffer until OD<sub>600nm</sub> = 0.5. The bacterial suspension was aliquoted and stored at –70 °C until it was used in the (co)culture assays.

For counting of colony forming units (CFU), bacterial suspensions were diluted in PBS and plated on Todd Hewitt agar or Brain Heart agar plates, for overnight incubation.

### 2.4. Fibrin matrices

Wells of 96-well imaging plates (black, clear bottom; Corning Life Sciences, Tewksbury, MA, USA) were first filled with neutrophil suspensions containing human fibrinogen (25 mg/ml; FIB3 free of Plg, vWF & Fibronectin; Stago, Asnières sur seine, France). Then, neutrophil suspensions were mixed with bacterial suspensions containing human AB serum (H4522; Sigma-Aldrich, MO, USA) and human thrombin (10 U/ml; Sigma-Aldrich) by gently pipetting up and down 10 times. The total volume of the resulting gel was 100 µl and contained 40%



**Fig. 1.** GFP signal as a direct readout of bacterial proliferation.

SA was grown in Todd Hewitt Broth and SE was grown in Brain Heart Infusion, shaking at 37 °C. RFU = relative fluorescence units. A: At indicated time points samples were taken out and measured in the fluorescence plate reader. Background signal of BHI is shown, signal of THB was similar. B: Samples were plated on agar plates and bacterial colonies were counted the next day. C: Optical density (OD) of samples was measured in a spectrophotometer with a 600 nm filter. Data are a representative example of three experiments.

(vol/vol) human serum, 2 mg/ml fibrinogen and 1 U/ml thrombin. When the assay was performed in suspension instead of gel, the volume of fibrinogen and thrombin was replaced by the same volume of incubation buffer. Matrices were allowed to set for 10 min before plates were closed with adhesive film to prevent dehydration during incubation.

For detecting neutrophil survival propidium iodide (PI) was added to the matrix to a final concentration of 25 µg/ml.

In some assays, a solution of Cytochalasin D (final concentration 10 µM, Santa Cruz Biotechnology, Dallas, TX, USA), Triton X-100 (final concentration 0.5% vol/vol) or DMSO in incubation buffer was put on top of the matrices.

## 2.5. Settings of fluorescence plate reader

Fluorescence intensity measurements of GFP or PI were obtained with the FLUOstar Optima and FLUOstar Omega (BMG Labtech, Ortenberg, Germany) with bottom optics. For GFP the excitation filter was 485/10 nm and the emission filter was 520/10 nm. For PI the excitation filter was 530/10 nm and the emission filter was 610/10 nm. The incubation temperature was 37 °C. Eight positions per well were measured every 20 min and averaged. When the assay was performed in suspension, the plate was shaken at 150 rpm before every measurement cycle.

## 2.6. Live imaging

Fibrin matrices were transferred to 6-channel µ-slides (VI 0.4 iBiTreat; ibidi GmbH, Martinsried, Germany). Matrices with a final volume of 50 µl were formed after incubating them for 10 min at 37 °C, after which they were overlayed with the incubation buffer to prevent dehydration during imaging. Images were obtained with the Deltavision RT fluorescence microscope (GE Healthcare, Little Chalfont, UK).

## 2.7. Calculation of lag time

From the raw data on the fluorescence intensity over time, the first derivative of all time points was calculated with Graphpad Prism, in arbitrary fluorescence units/20 min. The growth curve without neutrophils was used to determine what derivative correlated with unrestricted outgrowth. When the derivative was above this threshold for three time points in a row, the first of the three time points was defined as the end of the lag time.

## 2.8. Fibrinolysis

A solution of human plasminogen (Biopur AG, Reinach, Switzerland) and streptokinase from beta-hemolytic *Streptococcus* (Sigma-Aldrich) was added on top of the matrices to a final concentration of 25 µg/ml plasminogen and 10 U/ml streptokinase. After a short vortex, the plate was incubated on a shaker at 37 °C for 30 min. Solutions were resuspended and incubated until all clots had dissolved. To maximize cell yield, wells were washed once with PBS.

## 2.9. Statistical analysis

Statistical tests as indicated in figure legends were performed in Graphpad Prism. Tests were performed on pooled data rather than on individual experiments. Results were regarded as significant when  $p < 0.05$ .

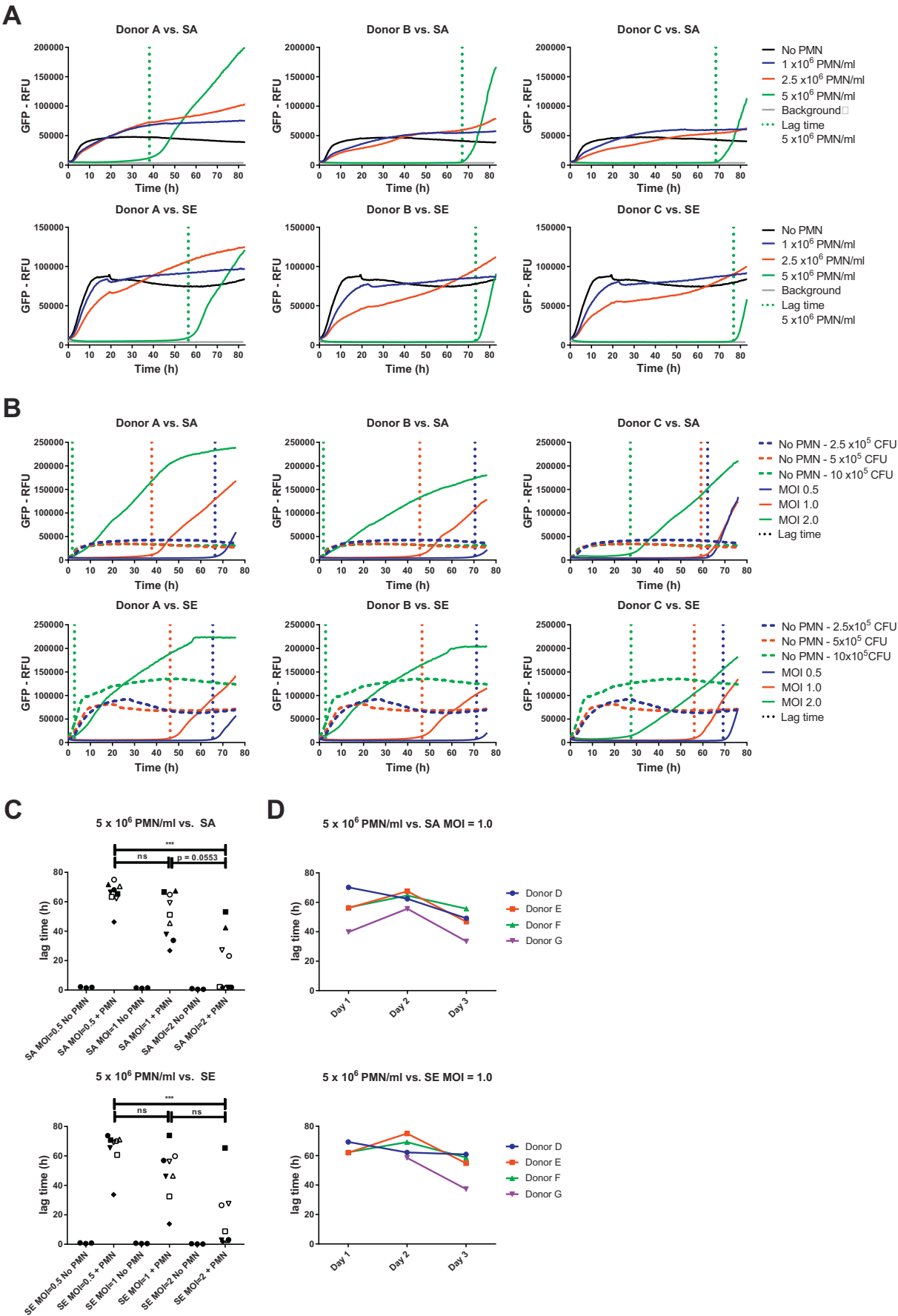
## 3. Results & discussion

We used derivatives of the methicillin-resistant SA strain MW2 and the SE strain 2600 that endogenously and constitutively produce GFP from the pCM29 plasmid (Surewaard et al., 2013). To confirm that the fluorescent signal could be used as a readout for bacterial numbers for both strains, outgrowth of the same bacterial culture was quantified by determining the fluorescence intensity and the optical density, as well as by plating and counting CFU (Fig. 1A-C). Fig. 1B demonstrates the linear correlation between GFP signal and CFU numbers, Fig. 1C the linear correlation between GFP signal and optical density. Thus, the GFP fluorescence signal represented the bacterial growth curve in real-time.

Fibrin matrices or ‘gels’ were formed upon mixing human fibrinogen with human thrombin, to obtain a tissue-like environment (see Methods). The mixing also dispersed the neutrophils and the staphylococci throughout the matrices. The occurrence of neutrophil migration and phagocytosis was confirmed by live imaging (Supplementary Video 1).

In the fluorescence plate reader the staphylococci proliferated well in the absence of neutrophils, as shown by a rapidly increasing GFP signal (Fig. 2A; “No PMN”). When the bacteria were co-cultured with neutrophils in the matrices, bacterial growth was inhibited. The GFP signal remained at background levels for long periods of time. The ‘lag time’ served as a quantitative measure of relative antibacterial capacity of the neutrophils. This value defines the time period until the fluorescent signal starts to show an exponential increase, i.e. the time until bacterial outgrowth.

The lag time depended on the concentration of neutrophils in the matrix (Fig. 2A). Since a fixed starting number of bacteria was used,



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**Fig. 2.** Antibacterial capacity depends on neutrophil concentration and MOI.

Bacteria were cultured in fibrin matrices with or without human neutrophils, in a 96-well plate. Every 20 min the GFP signal of every well was recorded by the fluorescence plate reader. A:  $5 \times 10^6$  CFU/ml of SA or SE were cultured with or without different concentrations of neutrophils of three different healthy donors. A matrix without bacteria was measured as a background control. The vertical, dotted line represents the lag time calculated as explained in Materials & Methods. B: Different concentrations of SA or SE were cultured with or without  $5 \times 10^6$  neutrophils/ml of three different healthy donors. C: For matrices from B the lag time was calculated. Three experiments, each with three healthy donors, were pooled. Every symbol represents another donor, when applicable the mean of duplo's was used. \*\*\* =  $p < 0.001$ , Friedman test with Dunn's *post hoc* test. D: For four donors the assay was repeated on three different days (in a five-week period) and the lag time was calculated. With a Friedman test SA lag times were found to significantly differ between days ( $p < 0.05$ ), for SE the difference was not statistically significant ( $p = 0.1944$ ).

this dependency can be explained by a varying multiplicity of infection (MOI) for the varying neutrophil concentrations. The MOI is the ratio between the number of bacteria and the number of neutrophils in the matrix. Indeed, changing the MOI by varying the bacterial number and using a fixed neutrophil concentration, similarly influenced the neutrophil antibacterial capacity (Fig. 2B, C).

When an MOI of 0.5 was used, the lag time varied between 46 and 75 h for the different healthy donors (Fig. 2C). The donors delaying SA growth the most (highest lag time), often also delayed SE the most (Fig. 2C). However, the lag time was not a set value for each donor, since the same donors displayed different lag times on three different days (Fig. 2D). Seemingly, the antibacterial capacity of neutrophils differs slightly day by day, possibly depending on the donor's lifestyle or inflammatory status or depending on minor variations in the standardized neutrophil isolation procedure. Hence, to confirm the 46 h lag time as a benchmark lag time for healthy donors, a higher number of donors should be included.

Notably, we observed that not only the lag time but also the final plateau of GFP signal changed with the number of neutrophils in the matrix (Fig. 2A). The cells did not generate a background signal in the GFP channel (data not shown). Probably, dying neutrophils serve as a source of nutrients for the bacteria, allowing the bacteria to reach higher numbers in the presence of higher numbers of neutrophils. To test this hypothesis, neutrophil death was induced by culturing in suspension for 24 h before bacteria were added. Indeed, the bacteria immediately started to grow out, and the GFP signal reached different plateaus with different concentrations of dying neutrophils (Supplementary Fig. 1).

In our experiments lag times extending up to 75 h were observed (Fig. 2C), contradicting reports on short survival and low functionality of *ex vivo* neutrophils (e.g. Hannah et al., 1995; Costantini et al., 2010; Simon, 2003). To assess neutrophil survival in the conditions of our assay, we measured propidium iodide (PI) signal over time. PI becomes fluorescent after binding to DNA. Only necrotic or late apoptotic cells are permeable to PI and will thus have a PI+ nucleus. By adding PI to the wells from the start, an increase in the number of dead cells could be detected over time (Fig. 3A). As a control, neutrophils in a duplo well were lysed with Triton X-100 to determine the maximum PI signal. The maximum PI signal was stable over time (Fig. 3A), demonstrating that DNA dispersing or degrading after neutrophil lysis does not reduce the total fluorescence signal.

Fig. 3B and C show that in fibrin matrices the neutrophils survive better than in shaken suspensions in the same plate, presumably by reducing the contact with artificial surfaces such as cell culture plastic. Without shaking the survival effect of fibrin matrices is less pronounced and did not prove statistically significant. However, non-shaken suspensions do not permit bacterial containment because neutrophils quickly sink to the bottom of the well while bacteria remain dispersed in the suspension (unpublished observations). Therefore, when studying antibacterial capacity, the fibrin matrices are favorable not only because they are a more physiological model for the infection site but also because they allow improved neutrophil survival.

In fibrin matrices, neutrophil death remains below 30% for 40 h and markedly increases only after > 40 h (see Fig. 3B for a representative example). This time course roughly fits with the observed time lag in bacterial growth. In conclusion, the observed delay in bacterial

outgrowth is effectuated by live neutrophils.

Our results show that, with an MOI of 0.5 or 1, healthy neutrophils can contain bacterial growth for prolonged periods. Still 100% sterility was never reached. Nevertheless, we always observed a delay in SA outgrowth of > 25 h. In most *in vivo* infections such a time period would be enough to recruit more neutrophils or other immune cells that can finish clearance of the last bacteria (Kim et al., 2008). When at an infection site the first neutrophils to arrive have a decreased capacity to delay, it can be a turning point in the antibacterial defense. As the bacteria will start to divide exponentially, they will potentially overwhelm any new waves of immune cells entering the site of infection.

A reduced lag time until outgrowth of bacteria in our assay indicates reduced antibacterial capacity of the neutrophils. As a model, we compared the lag time between untreated control or fMLF-treated neutrophils, as fMLF is known to inhibit the antibacterial capacity of neutrophils in fibrin matrices (Li et al., 2002). Indeed, in the presence of fMLF the measured lag time was reduced (Fig. 4A,B).

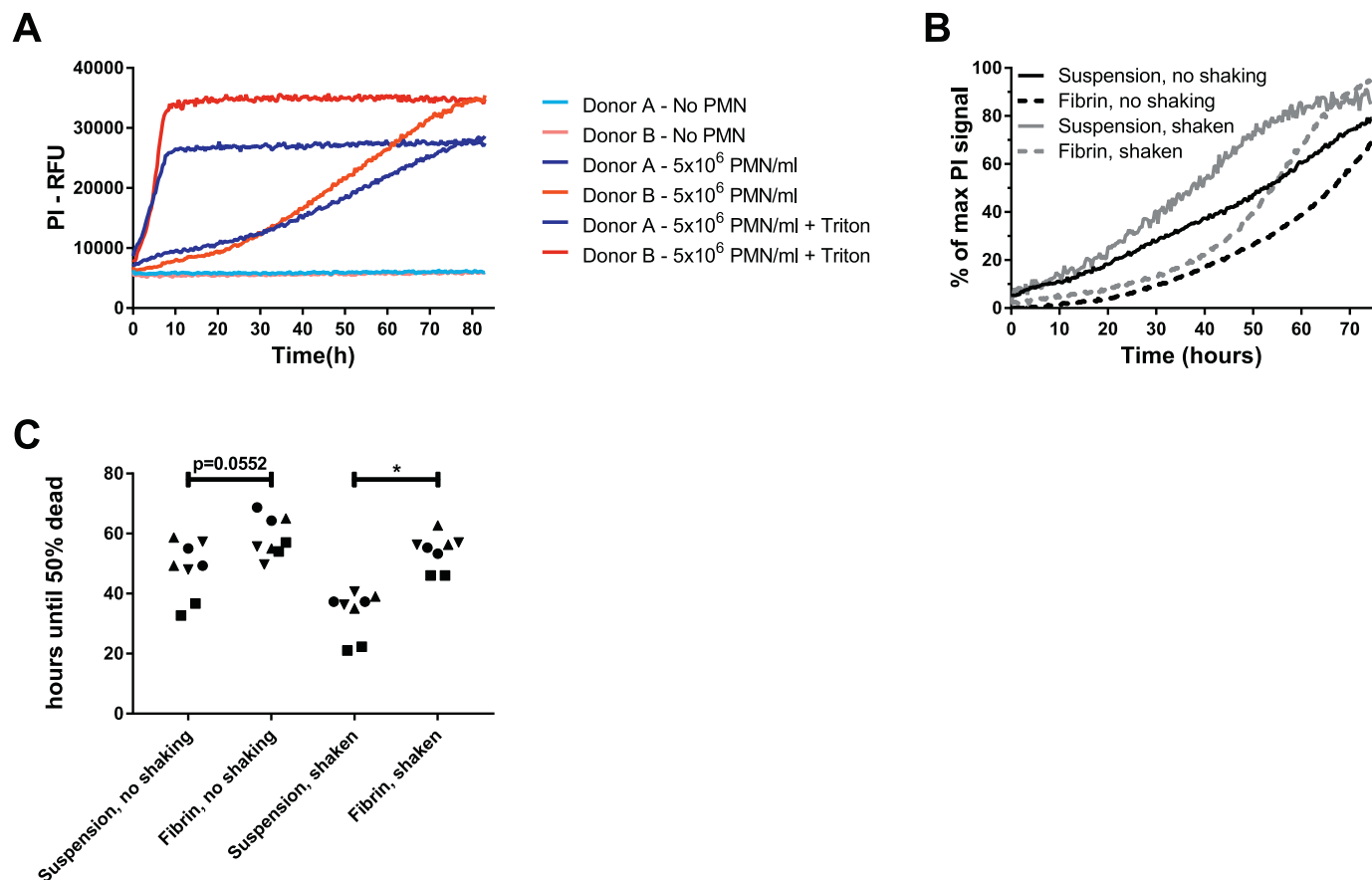
The neutrophil survival assay with PI can be run in the same plate as the bacterial inhibition assay, and this confirmed that the adverse effect of fMLF did not result from reduced neutrophil survival (Fig. 4C). When testing the antibacterial capacity of e.g. *in vitro* treated neutrophils or patient neutrophils, complementary data on neutrophil *ex vivo* survival can support data interpretation.

If the measured lag time is comparable to the untreated control, any effect on either migration, phagocytosis, intracellular containment or neutrophil survival is simultaneously deemed unlikely. A decreased lag time calls for further investigation of neutrophil function. Even though the strength of our assay lies in testing many functions of the neutrophil in a single assay, the assay also allowed us to zoom in on specific steps of the antibacterial cascade. For example, bacterial sensing is an important component of neutrophil antibacterial functionality. When we used complement-deficient serum in our fibrin matrices, the resulting lag times were significantly decreased compared to matrices with complement-sufficient serum (Supplementary Fig. 2). This result implicated that the role of the complement system in bacterial sensing could be studied in our assay.

To zoom in on the role of migration and phagocytosis in postponing bacterial outgrowth, we added Cytochalasin D, an inhibitor of actin remodeling and thus of migration and active phagocytosis. When Cytochalasin D was added after 10 min of coculturing the SA with the neutrophils, the SA almost immediately started growing out, indicating that the antibacterial capacity of the neutrophils was severely compromised by Cytochalasin D (Fig. 5A,B; compared to DMSO control). But when Cytochalasin D was added after 90 min of coculture instead of 10 min, the lag time was extensive (> 60 h; Fig. 5A,B). Thus, adding Cytochalasin D after 90 min did not significantly decrease antibacterial capacity (Fig. 5B; compared to DMSO control).

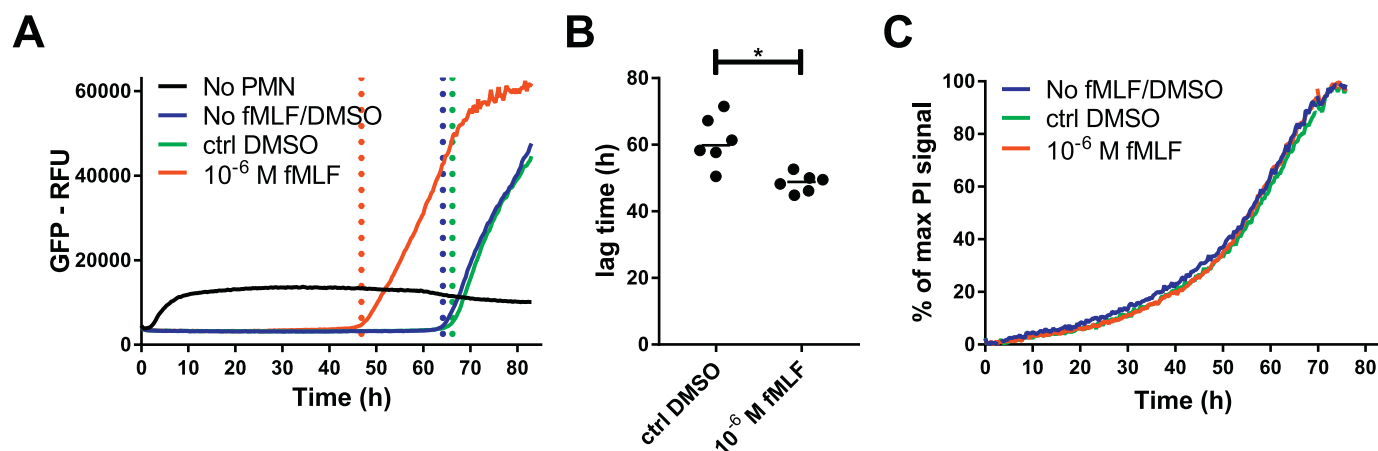
Proficient antibacterial capacity while actin remodeling was inhibited, implied that virtually all bacteria had been internalized within 90 min. Only when bacteria had still been extracellular, immobilizing neutrophils with Cytochalasin D would have allowed bacterial growth and thus have shortened the lag time. Whether the Cytochalasin D was added after 90, 120, 180, 240 or 360 min, the lag time was similar, supporting the idea that phagocytosis had already been completed at 90 min (Fig. 5A). Thus, in fibrin matrices SA are phagocytosed on a similar time scale as in shaken suspensions (Hellebrekers et al., 2017).





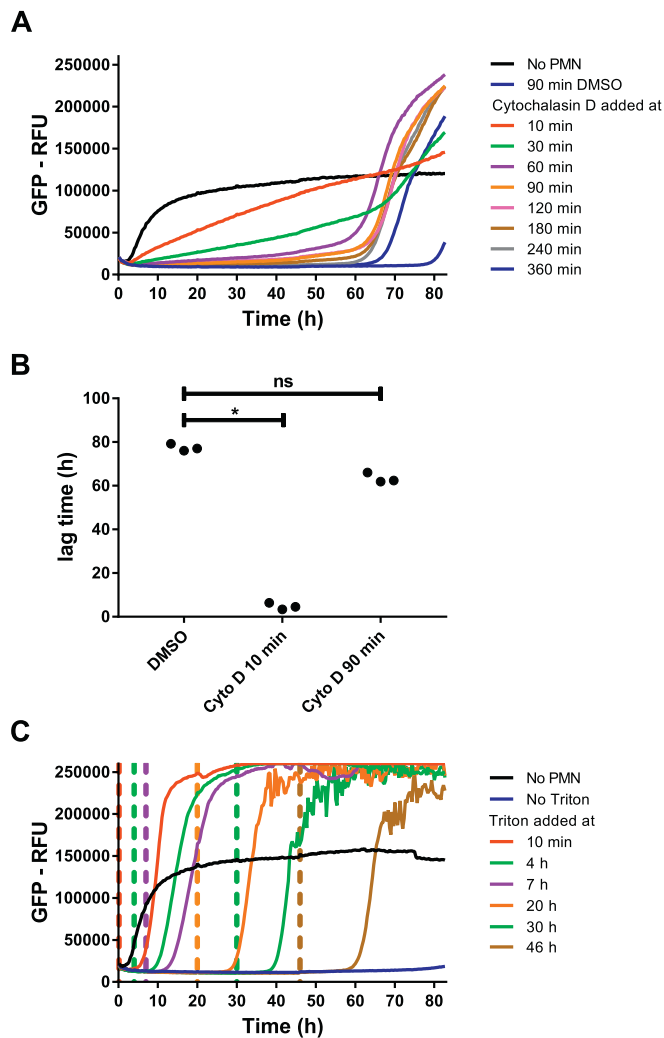
**Fig. 3.** Increased neutrophil survival in fibrin matrices compared to shaken suspensions.

Human neutrophils were cultured at  $5 \times 10^6$ /ml in the presence of PI in a 96-well plate. Every 20 min the PI signal was recorded by the fluorescence plate reader. **A:** Raw PI signal, with or without neutrophil lysis by Triton X-100 at time point 0 h. **B:** Neutrophils of the same donor were cultured either in suspensions or in fibrin matrices. Duplo plates were measured simultaneously in two plate readers, one shaking and one not shaking. At the end of the assay, the maximum PI signal was determined by lysing all neutrophils with Triton X-100. A representative experiment is shown. **C:** For curves as in B the time point at which the PI signal reached 50% of maximum was determined. Every symbol represents another donor (in duplo). \* =  $p < 0.05$ , Friedman test with uncorrected Dunn's *post hoc* test.



**Fig. 4.** Decreased antibacterial capacity after fMLF treatment.

$2.5 \times 10^6$  CFU/ml of SA were cultured in fibrin matrices with or without  $5 \times 10^6$  human neutrophils/ml, in a 96-well plate. Every 20 min the GFP signal of every well was recorded by the fluorescence plate reader. **A:** fMLF was added to the matrix in a final concentration of  $10^{-6}$  M, or the same volume of solvent (DMSO) was added as a control. The vertical, dotted line represents the lag time. **B:** For experiments such as in A the lag time was calculated and pooled for six donors. \* =  $p < 0.05$ , Wilcoxon matched-pairs signed rank test. **C:**  $5 \times 10^6$  human neutrophils/ml were cultured in fibrin matrices in the presence of PI, in a 96-well plate. fMLF was added to the matrix in a final concentration of  $10^{-6}$  M, or the same volume of solvent (DMSO) was added as a control. Every 20 min the PI signal of every well was recorded by the fluorescence plate reader. At the end of the assay, the maximum PI signal was determined by lysing all neutrophils with Triton X-100. Representative graph of six donors.



**Fig. 5.** Cytochalasin D and Triton X-100 treatment.  $2.5 \times 10^6$  CFU/ml of SA were cultured in fibrin matrices with or without  $5 \times 10^6$  human neutrophils/ml, in a 96-well plate. Every 20 min the GFP signal of every well was recorded by the fluorescence plate reader. **A:** At indicated time points incubation buffer, with Cytochalasin D (final concentration 10  $\mu$ M) or DMSO, was added on top of matrices. Representative graph of 3 healthy donors. **B:** Lag times for 3 healthy donors tested in conditions as shown in **A**. \* =  $p < 0.05$ , Friedman test with uncorrected Dunn's *post hoc* test. **C:** At time points indicated by dashed lines incubation buffer, with or without Triton X-100, was added on top of matrices. Representative graph of 3 healthy donors.

Cytochalasin D could also have inhibited processes other than migration and phagocytosis. For example, granule trafficking also relies on the activity of the actin skeleton (Lerm et al., 2007). The lag time for neutrophils treated with Cytochalasin D after > 90 min did seem somewhat decreased compared to DMSO-treated neutrophils, but this difference did not prove statistically significant (Fig. 5B).

In conclusion, Cytochalasin D can be exploited to estimate the duration of migration and phagocytosis, which can be used to distinguish migration-deficient neutrophils from healthy neutrophils.

Similarly, we could also zoom in on the efficiency of intracellular killing of bacteria by using the membrane lysing agent Triton X-100. In our assay some bacteria survived the phagolysosome, since viable SA could be recovered from inside neutrophils by adding Triton X-100 to the matrix. At all time points neutrophil lysis released SA that started to grow, even after the bacteria had remained intracellular for over 40 h (Fig. 5C). The increasing delay between neutrophil lysis (dashed line) and bacterial outgrowth (solid line) showed that the longer the

intracellular stay, the slower the bacteria grew upon neutrophil lysis (Fig. 5C). This suggested that fitness and/or survival of intracellular bacteria did decrease, but as stated before 100% sterility was never reached. This could be due to intracellular SA activating immune escape mechanisms (Guerra et al., 2017).

Apart from SA and SE we have also successfully measured the human neutrophil response to *Streptococcus pneumoniae* (Supplementary Fig. 3), and the assay could be extended to yet other pathogens. Our assay, however, cannot be used to compare the capacity of bacterial strains to escape neutrophil killing. Because not all bacterial species have the same growth characteristics, the basal lag time without neutrophils added already differs (Supplementary Fig. 4). Therefore, when we compare different bacterial species in the presence of neutrophils, we cannot reliably conclude that a difference in lag time between bacterial species directly corresponds to a difference in immune evasion.

As an optional step, viable neutrophils can be extracted again from the fibrin matrices (Supplementary Fig. 5). To dissolve the fibrin matrices, human plasminogen and streptokinase were added on top of the matrices (Tillett and Garner, 1933). Streptokinase binds and activates plasminogen to produce plasmin, an endogenous fibrinolytic enzyme. After fibrinolysis the resulting single cell suspensions can be further analyzed, for example with flow cytometry (Supplementary Fig. 5).

#### 4. Conclusions

In conclusion, this assay allows a direct comparison of the functional capacity of neutrophils under different *in vitro* treatments or from different sources. The assay explores not one but many functions of the neutrophil, resulting in high sensitivity for detecting neutrophil defects without biasing processes of neutrophils lysis or bacterial plating. It is quickly executed in a high-throughput format and can replace multiple smaller experiments. The assay offers a starting point as well as a tool for further investigation of a defect in antibacterial function. This assay can be extended to work with other pathogens as long as a strain can be engineered to endogenously express a fluorescent protein.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jim.2018.09.001>.

#### Authorship

E.v.G. designed and performed experiments, created the SE strain, analyzed results and wrote the paper. P.H.C.L. designed the method, designed and performed experiments and analyzed results. J.P. designed the method, supervised experiments and edited the paper. C.W.v.A. performed experiments. N.V. and L.K. provided daily supervision, interpreted results and edited the paper.

#### Conflict of interest disclosure

Conflicts of interest: none.

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