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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 (Kolaczkowska 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 $MgSO_4$, 1.2 mM $KH_2PO_4$, 1.0 mM $CaCl_2$, 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 $NH_4Cl$, 10 mM $KHCO_3$ and 0.1 mM $Na_2$ ethylenediaminetetraacetic acid (EDTA) dissolved in $ddH_2O$ 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 $OD_{600nm} = 0.5$. 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, Tewskbury, 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%