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Microfluidic arenas for war games between neutrophils and microbes

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Abstract

Measurements of neutrophil activities such as cell migration and phagocytosis are generally performed using low-content bulk assays, that provide little detail activity at the single cell level, or flow cytometry methods, which have the single cell resolution but lack perspective on the kinetics of the process. Here, we present a microfluidic assay for measuring the essential functions that contribute to the antimicrobial activity of neutrophils: migration towards the target, and killing of microbes. The assay interrogates the interactions between isolated human neutrophils and populations of live, proliferating microbes. The outcome is measured in a binary mode that is reflective of *in vivo* infections, which are either cleared or endure the host response. The outcome of the interactions is also characterized at single cell resolution for both the neutrophils and the microbes. We applied the assay to test the response of neutrophils from intensive care patients to live *Staphylococcus aureus*, and observed alterations of antimicrobial neutrophil activity in patients, including those with sepsis. By directly measuring neutrophil activity against live targets at high spatial and temporal resolution, this assay provides unique insights into the life-or-death contest shaping the outcome of interactions between populations of neutrophils and microbes.

Graphical Abstract

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Nanoliter-scale microfluidic arenas for imaging war games between human neutrophils and microbes at cellular resolution

Introduction

Neutrophils, the most abundant circulating white blood cells in the human body, represent the first line of cellular defence against infection. Upon injury, they are rapidly recruited to sites of damage by both pathogen- and host-derived signals, where their primary function is to sterilize the wound using various antimicrobial strategies. Small pathogenic targets such as single bacteria are engulfed and destroyed by an antimicrobial cocktail of proteases and reactive oxygen species (ROS) in the phagolysosome¹. Larger pathogenic targets such as filamentous fungi or microbial aggregates induce additional, neutrophil-specific responses. These include induction of neutrophil swarming^{2, 3} and extrusion of neutrophil extracellular traps (NETs), extensive chromatin networks that act to immobilise pathogens and concentrate antimicrobial peptides⁴. A large body of work using genetically malleable macrophage and Dictyostelium models have dissected many of the molecular pathways underlying cellular motility, phagocytosis, phagosome maturation, and antigen processing and presentation^{5, 6}. Comparatively less is known about neutrophils because of difficulties in culturing the fragile, terminally-differentiated neutrophil lineage. Most conventional phagocytosis studies rely on end-point fluorescence activated cell sorting (FACS) readouts following bulk co-incubation in solution^{7, 8}, an approach that provides limited detail at the cellular level and is non-physiological with regards to neutrophil activities in tissues.

Microfluidic devices have proven useful for studying migration of neutrophils in response to chemoattractant gradients, which can be generated via diffusion down microscale channels from a chemoattractant reservoir⁹. A number of studies using microfluidic devices have measured neutrophil activity in response to microbial particles, microbial products^{10–13}, or potent stimulants¹⁴ that may not represent physiological conditions¹⁵. However, use of live bacterial targets has so far been limited to preliminary demonstration of stimulation of neutrophil ROS and NETosis by *Pseudomonas aeruginosa*¹⁶. So far, direct comparison of both neutrophil response and resulting microbe growth dynamics has been limited to interactions between neutrophil and fungi^{2, 17}.

Here, we present a new microfluidic device specifically designed to measure the ability of isolated human neutrophils to sense, chemotax toward, phagocytose and effectively neutralize actively growing microbes. The device is designed for use with standard pipettes and tips rather than requiring tubing and syringes, with the goal of making the assay more easily accessible a broad range of scientists compared to our previous designs¹⁸. We demonstrate use of the assay to provide binary outcomes of host-pathogen interactions, imaging of interactions at subcellular resolution, and detailed kinetics relating to neutrophil recruitment and bacterial proliferation. Finally, we compare the activity of neutrophils from a small number of intensive care patients recovering from major surgery, and report altered antimicrobial activity for neutrophils from patients with infections compared to those without.

Experimental

Device design and fabrication

Devices were designed using AutoCAD. Chrome masks for photolithography were printed by Front Range Photo Mask (Palmer Lake, Colorado). Silicon wafers were fabricated using standard techniques. Briefly, clean silicon wafers were spin-coated with two layers of negative photoresist (SU-8, Microchem, Newton, MA), the first layer 5 μm thick and the second 50 μm . The wafer was then patterned by sequential UV exposure through two photolithography masks, and processed according to manufacturers instructions. The patterned wafer was then used as a mold for PDMS (Polydimethylsiloxane, Fisher Scientific, Fair Lawn, NJ) soft-lithography to produce the final PDMS devices. Inlets were punched using a 1.0 mm punch (Harris Uni-Core™) the whole device cut free using a scalpel. Following oxygen plasma treatment, devices were irreversibly bonded to glass-bottom well plates by heating to 75 °C for 10 mins.

COMSOL model

Fluid flow in the chips during washing steps (Fig. 2) was modeled using COMSOL Multiphysics® (COMSOL Inc.). In order to reduce the computation time, reduced models were used for both chip designs by exploiting the repeating patterns (in the x direction) and the symmetry (xy plane for the new design, xy and xz plane for the old design). Simulations were run with either pressure or flow-rate boundary conditions.

Bacterial culture and preparation

Staphylococcus aureus (SH1000-GFP) was cultured using standard approaches. Briefly, single colonies were picked from a BHI agar plate and used to inoculate 5 mL BHI starter cultures containing 5 $\mu\text{g/mL}$ tetracycline for selection of GFP-expression, which were incubated overnight at 37 °C with shaking. On the morning of the assay, 10 mL secondary BHI cultures were inoculated with 100 μL of the overnight culture and incubated for 2 hours with shaking. A 100x dilution was then scored using a hemocytometer to determine bacterial concentration in the culture and adjusted to the required concentration. Prior to loading, bacteria were pelleted at 25,000 g for 3 mins and re-suspended in cell culture medium (IMDM + 20 % FBS). Staining of *S. aureus* with pHrodo™ and CellROX™ was performed as described previously¹⁹.

Neutrophil isolation and treatments

Neutrophils were isolated from 9 mL of whole blood by density separation and negative selection (Neutrophil Enrichment Kit, STEMCELL Technologies, Vancouver, Canada) protocols as previously described^{20, 21}. Cells were then stained with 32 μM Hoechst 33342 dye for 10 mins, washed, and re-suspended in IMDM + 20% FBS at 5.0×10^7 cells/mL prior to loading. Drug conditions tested were based on data of mean plasma levels during treatment²²: Vancomycin [23 $\mu\text{g/mL}$]; Trimethoprim (TMP) [68 $\mu\text{g/mL}$]; Dicloxacillin [4.74 $\mu\text{g/mL}$]; Doxycycline [2.6 $\mu\text{g/mL}$]; Dapsone [2.3 $\mu\text{g/mL}$], and Cephalexin [32 $\mu\text{g/mL}$]. For pre-treatment with antibiotics, isolated neutrophils were co-incubated with drug for 30 mins,

washed, pelleted, and re-suspended prior to loading. Neutrophils were loaded by pipette using a gel-loading tip, until cells were observed to exit the device outlet.

Patient Diagnostic Criteria and Study design

Subjects for this observational study were consenting adults > 18 years and < 80 years. Patients were clinically defined as having sepsis when they were found to have a source of infection confirmed by blood, urine, or sputum culture, and evidence of organ dysfunction. Blood samples were drawn after obtaining informed written consent from patients with existing venous lines expected to remain in place for more than 48 hours. All experimental procedures were approved by the Massachusetts General Hospital Institutional Review Board (protocol 2017-P-000388). This study complied with the U.S. Department of Health and Human Services (HHS) Protection of Human Subjects regulations as stipulated by the Office for Human Research Protections (OHRP).

Data acquisition and analysis

Cell migration was imaged at 10x magnification using a fully automated fluorescent Nikon TiE inverted wide field microscope with a biochamber heated to 37°C with 5% CO₂. Each field was imaged every 5 mins to allow accurate temporal tracking of cell recruitment.

Image analysis was performed with ImageJ (NIH) software, with automated tracking analysis for temporal tracking of cell migration using TrackMate and bacterial growth dynamics. Percent (%) neutrophil recruitment was calculated based on the number of cells recruited to each assay relative to the total number of neutrophils in each field of view divided by the number of assays visible in each field of view. Graphing and statistical analysis were performed using Graphpad Prism 7 software.

Results

A microfluidic device for studying the activity of isolated human neutrophils

We designed and optimized a microfluidic device for studying interactions between human neutrophils and bacteria. The devices include dozens of “war theatres” arrayed inside a larger chamber. Each “war theatre” consists of a central reservoir with a single entrance channel connecting to the outer chamber (Fig. 1B). Key design considerations included: delivery of appropriate number of bacteria and neutrophils to their respective compartments; providing enough volume in the inner chamber to support bacterial growth; large numbers of chambers in parallel channels to provide statistically meaningful output; minimal device footprint while allowing enough distance between arrayed assays to reduce the risk of interference; optimization of device design such that the flow through the outer chamber efficiently washes out bacteria; and, for practical purposes, we aimed for a robust design that performs well in the context of variable flow rate during operation with a pipette rather than the constant flow rate of a syringe pump and tubing.

To permit measurement of multiple assays in parallel, 33 devices were arrayed within a single outer channel into which neutrophils could be loaded (Fig. 1A). To ensure that assays progressed independently within the array, devices were spaced such that migration channels

were ~420 μm apart, 7 times larger than the previously reported distance necessary for establishment of independent neutrophil swarms²³. 3 independent channels were incorporated into each PDMS block (Fig. 1C). Bonding of 6 PDMS blocks to a glass-bottom well plate allowed testing of 18 different experimental conditions in parallel, with 33 technical replicates per condition (Fig. 1C).

Priming of the device is achieved by filling the outer chamber, then applying vacuum to fill the central reservoir. The outer chamber is then thoroughly washed by flowing 200 μL of media through the device over the course of 1 min, prior to loading of neutrophils (Fig. 1D).

To allow loading and washing steps to be performed using pipettes rather than tubing and syringes, we designed the outer channel to have ~100 fold lower hydraulic resistance ($3.27 \times 10^8 \text{ psi}/[\text{m}^3/\text{s}]$) compared to one of our previous designs ($3.59 \times 10^{10} \text{ psi}/[\text{m}^3/\text{s}]$)¹², and verified this by a finite element model using COMSOL (Fig. 2A–D). When used with a standard 200 μL pipette (which can provide approximately 1 psi of pressure), flow rates improved from 1.83 $\mu\text{L}/\text{min}$ to 200 $\mu\text{L}/\text{min}$, thus increasing average wall shear rates from 507.9 [1/s] to 13388 [1/s]. Enlarging of the outer channel also simplified neutrophil preparation by enabling loading of higher numbers of neutrophils using lower initial densities

We characterised neutrophil chemotaxis in our device by delivering the neutrophil chemoattractant fMLP (traced by a similarly sized fluorescein reporter) into the device, making sure that the inner microchambers were fully loaded. Washing the outer chamber established a gradient that was maintained for at least 8 hours, although the slope of the gradient dropped from ~7:1 to ~1.5:1 over the period measured (Fig. S1A,B). In response to this chemokine gradient, isolated human neutrophils were rapidly recruited over the first 6 hours of the assay (Fig. S1B), a significantly longer period than our previous designs with multiple migration channels and shorter-lived gradients¹⁸.

Bacteria induce antimicrobial responses from isolated human neutrophils

Because bacteria are known to produce multiple pathogen-associated molecular patterns (PAMPS) such as formylated peptides and lipopolysaccharides, which provide strong recruitment signals to neutrophils, we hypothesized that live bacteria would be sufficient to direct neutrophil migration in this device.

To test this hypothesis, we delivered bacteria expressing green fluorescent protein (GFP) into the inner microchambers of the device in place of a chemoattractant. Washing of the device efficiently removed bacteria in the outer chamber, while those in the inner chambers remained (Fig. 2E,F). *Staphylococcus aureus* was used as an archetypal Gram-positive pathogen, while *Escherichia coli* was chosen as a representative Gram-negative pathogen.

Neutrophils delivered into the outer chamber directionally migrated into the central microchamber in response to bacteria, initiating direct host-pathogen interactions (Fig. 3A). The outcome of these interactions was classified in a binary manner for each microchamber in the array as either “cleared”, where neutrophils successfully suppressed bacterial growth (Fig. 3A, top time series), or “overgrown”, where neutrophil failed to suppress bacterial

proliferation (Fig. 3A, bottom time series). Scoring of binary outcomes was performed at the time point at which bacterial growth in the control condition lacking neutrophils became confluent, usually around 6–8 hours. This binary approach provided a simple readout for the final result of host-pathogen interaction for each sample, as an integrative measure of both host and pathogen factors contributing to the interaction outcome.

To observe host-pathogen interactions in more detail, GFP-expressing bacteria were either stained with pHrodo, a pH-sensitive reporter that increases in red fluorescence at low pH, or labelled with CellROX, a dye that exhibits increased fluorescence in the far-red spectrum when exposed to reactive oxygen species (ROS). Upon entering the bacteria reservoir, neutrophils rapidly phagocytosed bacteria, with phagosomal pH acidification indicated by increased fluorescence of the pHrodo reporter within 12 mins following internalization (Fig. 3B). Production of reactive oxygen species, as visualized by CellROX fluorescence, was observed later both within the phagosome and throughout the cell (Fig. 3C). To visualize loss of neutrophil viability and NETosis²⁴ during interactions with *S. aureus*, we co-loaded the bacteria with 1 µg/mL propidium iodide (PI) (Fig. 3D). Loss of neutrophil viability could be identified by bright PI staining following permeabilization of the nuclear membrane (Fig. 3D, solid white arrowheads), while NETosis was associated with diffuse PI staining (Fig. 3D, open white arrowheads; Fig. S2A, PMA positive control, open yellow arrowheads). Interactions in which neutrophils were unable to control bacterial growth were associated with increased loss of neutrophil viability and NETosis compared to examples where neutrophils efficiently cleared the bacteria (Fig. S2C,D).

Factors influencing the outcome of neutrophil-microbe interactions

To establish the effect of bacterial “dosage” on interaction outcome and to identify what bacterial dose would provide a good baseline for testing experimental perturbations, we titrated bacterial dose against isolated human neutrophils delivered at 60×10^6 cells/mL. As expected, the ability of neutrophils to effectively suppress bacterial growth depended on the number of bacteria initially loaded into the device (Fig. 4A,B).

We hypothesised that the number of bacteria initially loaded into each microchamber of a single array might follow a Gaussian distribution, resulting in the range of binary outcomes observed for some of the bacterial concentrations tested (Fig. 4A,B). Indeed, close examination of bacterial loading demonstrated considerable variation across an array (Fig. 4C). To test whether initial bacterial delivery influenced neutrophil recruitment kinetics, chambers were binned into “High” (>15 bacteria per chamber average), “Medium” (~5–10 bacteria per chamber average) and “Low” (<5 bacteria per chamber average) categories, and representative plots of neutrophil recruitment kinetics were graphed for each category (Fig. 4D). Neutrophil recruitment scaled according to the initial number of bacteria loaded into each microchamber, with higher initial numbers resulting in greater recruitment of neutrophils.

We investigated the influence of bacterial proliferation rate and neutrophil recruitment dynamics on assay outcome in more detail (Fig. 5). To test the effect of enhanced neutrophil recruitment, we co-loaded bacteria with the host-derived chemoattractant LTB4, which has been shown to improve neutrophil activity against microbes¹⁷ (Fig. 5C). This resulted in

immediate recruitment of large numbers of neutrophils, which efficiently suppressed bacterial proliferation compared to controls (Fig. 5A,B).

To test the effects of bacterial suppression on neutrophil recruitment, we inhibited bacterial proliferation using a low dose of ampicillin (5 µg/mL) (Fig. 5D). Lack of bacterial proliferation in this condition greatly attenuated neutrophil recruitment compared to controls (Fig. 5B), suggesting that viable, proliferative bacteria are required to recruit neutrophils in this assay.

Antibiotic pre-treatment does not affect neutrophil recruitment

Patients with suspected infection are routinely treated with antibiotics as a first course of action. Some antibiotics are thought to directly influence neutrophil activity, including adhesion and chemotaxis^{25–28}, so it was important to assess any potential influence that patient treatments might have on neutrophil activity *ex vivo*. To test whether antibiotic treatment affected neutrophil function in the context our microfluidic assay, we pre-treated neutrophils with a range of common antibiotics at relevant prescribed doses²² prior to challenging them with *S. aureus* in our microfluidic devices.

No consistent or significant changes were observed in neutrophil migration towards either live *S. aureus* or fMLP (Fig. 6A), suggesting that neutrophil chemotaxis was not affected by antibiotic pre-treatment.

Additionally, for 5 of the 6 antibiotics tested, no significant effect was observed on the ability of neutrophils to suppress *S. aureus* growth (Fig. 6B). However, pre-treatment of neutrophils with trimethoprim (TMP) resulted in temporal suppression of *S. aureus* growth (Fig. 6B). Detailed analysis of neutrophil recruitment and *S. aureus* growth (Fig. 6C) in the presence of TMP compared to controls demonstrated suppression of *S. aureus* growth even in the absence of significant neutrophil recruitment. Importantly, TMP-treated neutrophils migrated normally in response to fMLP (Fig. 6A), which suggests that antibiotic emanating from TMP pre-treated neutrophils is sufficient to suppress *S. aureus* growth, which in turn had the effect of reducing neutrophil recruitment, as previously demonstrated for ampicillin (Fig. 5D).

Detailed statistical analysis of neutrophil recruitment to live microbes versus chemoattractant revealed significant donor-donor variability in response to both *S. aureus* and fMLP (Fig. S3A,B). Responses to *S. aureus* were further complicated by more variable recruitment between microchambers by neutrophils from the same donor compared to fMLP (Fig. S3C). This is likely due to the Gaussian distribution of *S. aureus* loading into microchambers inducing different neutrophil recruitment between chambers, as was previously noted (Fig. 4C,D).

The effects of sepsis on neutrophil antimicrobial activity

Key neutrophil anti-microbial activities, including chemotaxis and phagocytosis, are deficient during sepsis, particularly among patients with poor clinical outcomes^{8, 13}. The altered activity of neutrophil populations observed in sepsis is thought to arise in response to systemic inflammatory signalling, also known as “cytokine storm”. This situation can induce

phenotypic changes in circulating neutrophils directly, as well as stimulate the influx of immature, functionally distinct cells from the bone marrow into the circulation²⁹. Our recent studies have revealed that both the plasma changes and sustained phenotypic changes of the neutrophils take place during sepsis and may potentially reinforce each other³⁰.

To test whether our assay could identify changes in patients with sepsis, we isolated neutrophils from patients in the surgical intensive care unit (ICU) at Massachusetts General Hospital (Table S1) and compared those who had developed sepsis to those that did not (Fig. 7). Neutrophils from healthy donors exhibited robust recruitment and effective killing of bacteria in these experiments. Neutrophils isolated from non-septic (NS) ICU patients failed at suppressing *S. aureus*. Samples from two out of the three patients with infections showed heterogeneous antimicrobial functions, killing bacteria or suppressing bacteria growth in some but not all chambers of the assay (Fig. 7A). Suppression of bacterial proliferation was not the result of enhanced neutrophil migration, as relatively low recruitment was observed for neutrophils from patients with sepsis compared to non-septic patients (Fig. 7B). Interestingly, while all patients with sepsis were treated with antibiotics, patient 6, who was unable to suppress bacterial growth, was also receiving immunosuppressant therapy for rheumatoid arthritis (Table S1).

Discussion

The assay presented here allowed the measurement of neutrophil antimicrobial activity against living pathogens. The device was specifically designed to enable single cell resolution study of interactions between human neutrophils and bacteria. Several “war theatres” could be monitored in parallel, providing greater parallelization than devices previously designed to study neutrophil interactions with pathogenic fungi^{2, 17} or production of ROS and NETs¹⁶. Compared to previous dead-end reservoir arrays used to study neutrophil chemotaxis¹⁸, the device provides: temporally extended chemotactic gradients and optimized delivery of neutrophils to achieve appropriate multiplicity of infection (MOI). The design of the device enables enhanced flow through the outer chamber, allowing rapid and efficient loading and washing using pipettes and tips rather than syringes and tubing. This feature makes the device user-friendly and may help the translation of the technology to biology labs by improving collaborative accessibility.

Measurements were made either as a simple binary outcome of the interaction, where either the neutrophils or the bacteria populations prevailed. We also tracked the detailed dynamics of bacterial proliferation and neutrophil recruitment. Time-lapse fluorescent imaging of host-pathogen interactions was used to visualize specific antimicrobial activities of neutrophils in this device, including phagocytosis, generation of ROS, and NETosis. We found that NETosis could not be reliably measured in our assay by monitoring of Hoechst staining alone (an approach used by others¹⁶), due to cells moving out-of-focus and signal bleed from GFP fluorescence, and ambiguities of cell borders in the presence of neutrophil clusters. However, NET formation could be visualized using alternative fluorescent DNA dyes that cannot pass through intact nuclear membranes, such as SYTOX or PI. Using PI, NETosis could be distinguished from other forms of cell death by the intensity of the signal²⁴. This assay is also suitable for studying neutrophil interactions with pathogenic

fungi, particularly yeasts. In the context of filamentous fungi, such as *Aspergillus fumigatus* or dimorphic *Candida albicans* species, measurement of NETosis would be especially important.

Key design features of our new device include; a single neutrophil migration channel, which significantly increased the temporal stability of chemoattractant gradients compared to our previous designs; a more streamlined chamber design for more efficient washing of bacteria out of the outer channel following loading; and use of three parallel channels per well, providing eighteen experimental conditions within each six-well plate.

In this assay, neutrophil recruitment was dependent on initial bacterial number and proliferation rates. Faster recruitment of larger numbers of neutrophils favored an outcome in which neutrophils prevailed. Suppression of bacterial proliferation by antibiotics also favored the neutrophils. Pre-treatment of neutrophils with antibiotics did not affect their ability to migrate, despite previously published observations on the effect of dapsone treatment on neutrophil adhesion and chemotaxis signalling²⁸. This is likely due to the ten-fold lower, physiological dose used in our experiments.

Strikingly, potent antibiotics emanating from loaded neutrophils were sufficient to suppress bacterial proliferation, thereby resulting in reduced neutrophil recruitment. Loading of neutrophils with antibiotics and its subsequent diffusion out of the cells likely depends on the influx and efflux rates of the drug. For example, for beta-lactams such as dicloxacillin, entry into granulocytes is thought to be quite fast³¹ but the drug is retained poorly³², while glycopeptides such as vancomycin exhibit slow influx³¹ and tetracyclines such as doxycycline are efficiently loaded but irreversibly bound within the cell³². TMP is known to be efficiently taken up by neutrophils³³, but its effect on the microbiocidal activity of neutrophils is unclear^{34, 35}. In neutrophils from patients with chronic granulomatous disease (CGD), TMP in combination with sulfamethoxazole (SMX) is thought to induce higher levels of nitric oxide (NO), providing a functional rescue of the reduced neutrophil reactive oxygen species (ROS) production exhibited in this disorder^{35, 36}, however little effect was observed with healthy neutrophils.

Measurement of antimicrobial activity of neutrophils from ICU patients revealed consistently reduced recruitment of neutrophils from patients with sepsis compared to those without. None of the non-septic samples were able to suppress bacteria, despite relatively strong neutrophil recruitment for two of the three samples tested. This suggests that neutrophil activity in this patient cohort was generally suppressed, and highlights the ability of this assay to distinguish between different neutrophil functions, e.g. chemotaxis and antimicrobial activity. Interestingly however, partial bacterial suppression was observed for two of the three sepsis samples tested. It is possible that suppression of bacterial proliferation in these experiments stemmed from prolonged antibiotic treatment of these patients during their extended hospital courses. This may have reduced bacterial proliferation and consequently neutrophil recruitment, with the overall result that the small numbers of neutrophils recruited were able to prevail. The lack of neutrophil response from donor P6 may relate to their successful antibiotic treatment and resulting short and uncomplicated hospital stay. It may also relate to continuing immunosuppressive therapies

(adalimumab and methotrexate) prescribed for their rheumatoid arthritis, which highlights the difficulties introduced by the complex co-morbidities and previous medical histories inherent to patients at risk of developing sepsis.

These studies demonstrate the utility of microfluidic approaches for the study of host-pathogen interactions between *ex vivo* human neutrophils and live *S. aureus*. Experimental manipulation of leukocyte and microbial activity in the assay provided insights into the importance of both host and pathogen dynamics in the outcome of interactions, while preliminary clinical studies identified changes in activity in samples from patients at risk of infection.

For clinical use, the assay could be customized for use with off-the-shelf reagents such as fluorescently labelled bacterial particles to simplify assay preparation and avoid complications introduced by variable bacterial loading. Co-loading of pHrodo-labelled particles and a standard chemoattractant such as fMLP would provide easily automatable measurement of neutrophil chemotaxis and phagocytic activity.

Finally, the approach used in this study could easily be customized for studying interactions between a wide range of cell types, including host-host and microbe-microbe interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Microchamber array for studying host-pathogen interaction.

A) Cartoon of host-pathogen interaction assay. Microbes (yellow spots) loaded into the microchamber array (200 μm diameter $\times$ 50 μm height) stimulate purified human neutrophils (purple) loaded into the main channel (50 μm height) to migrate through the connecting channel (green, 125 μm long $\times$ 10 μm^2 cross section). Some microbes may be phagocytosed and destroyed by neutrophils, while unphagocytosed microbes may divide.

B) Diagram of microchamber design, showing dimensions of central chamber and connecting channel (green).

C) Final working layout. Each PDMS device contains 3 arrays, allowing 18 conditions to be tested in parallel within a 6-well plate.

D) Schematic of experimental workflow prior to imaging. Upper panels show loading and washing steps, lower panels show magnified view of workflow at the level of individual devices.

Figure 2. Optimization of device design for washing using pipettes.

A,B) Streamlines (A) and fluid velocity (B) for 50 μm high devices during washing using a pipette ($\sim 200 \mu\text{m}/\text{min}$).

C,D) Comparison of predicted shear rates during washing using a pipette ($\sim 1 \text{ psi}$) for a previously reported design (C) and new device (D). Model generated using COMSOL.

E) Representative images showing loading of the chambers and channel (left panel) with live GFP-expressing *Staphylococcus aureus* (green), followed by washing of the main channel to remove bacteria (right panel).

F) Scoring of bacterial number in the chambers and channel pre- and post-wash demonstrate the efficient washing of the channel while leaving bacteria in the chambers unaffected. N = 5 FOV (15 chambers) scored per condition.

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Figure 3. Neutrophil antimicrobial responses to live bacteria.

A) A binary readout (“Cleared”, top panels, or “Overgrown”, bottom panels) can be measured for interactions between isolated human neutrophils (blue nuclei, Hoechst) and microbe (*S. aureus* SH1000-GFP, green). Scale bar: 100 μm .

B) Labelling of *S. aureus* (GFP, green) with the pH-sensitive conjugate pHrodo (red at low pH), allows convenient tracking of bacterial phagocytosis, as the bacteria become red fluorescent upon acidification of the neutrophil phagosome. Upper panels show location of cell within chamber, magnified lower panels show cellular and subcellular detail. Scale bar: upper panels: 25 μm , lower panels: 10 μm .

C) Pre-staining of bacteria with CellROX, a dye that fluoresces in the far-red spectrum upon exposure to reactive oxygen species, allows tracking of ROS production by neutrophils following phagocytosis. Scale bar: 10 μ m.

D) Co-delivery of propidium iodide (1 μ g/mL) allows visualization of loss of neutrophil viability (bright red PI stain, solid white arrowheads) and NETosis (diffuse red PI staining, open white arrowheads). Scale bar: 100 μ m.

Figure 4. Influence of bacterial number on interaction outcome.

(A,B) Representative results from titration of *S. aureus* (A) and *E. coli* (B) loading concentrations against healthy neutrophils loaded at 60×10^6 cells per mL.

(C,D) Variability in bacterial loading into each chamber (C) induces variable neutrophil recruitment (D), with chambers containing more *S. aureus* attracting more neutrophils. N = 10 microchambers scored per condition, N = 2 representative neutrophil recruitment plots shown for each starting number.

Figure 5. Bacterial proliferation rate and neutrophil recruitment kinetics determine outcome of host-pathogen interactions.

A) Growth of *S. aureus* alone (black), and in the presence of [100 nM] LTB4 (blue) or low dose [5mg/mL] of Ampicillin (magenta). Bacteria were loaded at 1×10^6 cells/mL.

B) Neutrophils only partially suppress *S. aureus* growth when 1×10^6 bacteria/mL are loaded, delaying the onset of exponential growth by approximately 1 hour.

C) Addition of LTB4 results in a rapid influx of neutrophils and effective suppression of *S. aureus* growth.

D) Direct suppression of *S. aureus* growth with a low dose of Ampicillin results in few neutrophils being recruited to the chamber. Data is mean $\pm$ SEM.

Figure 6. Neutrophil-delivered antibiotics suppress extracellular bacterial growth.

A) Pre-treatment of neutrophils with a range of common antibiotics does not significantly alter recruitment in response to fMLP or *S. aureus*. N = 12 assays scored per condition per donor, from 3 healthy donors.

B) Temporal dynamics of chamber overgrowth by *S. aureus* alone and in the presence of untreated neutrophils or neutrophils pre-treated with antibiotics. Pre-treatment of neutrophils with TMP appeared to result in suppression of *S. aureus* overgrowth. Average percentage overgrowth calculated from pooled data for all three donors.

C) Detailed kinetics from assay with highly suppressed *S. aureus* growth in the presence of TMP-treated neutrophils. TMP-treated neutrophils show lower recruitment than DMSO-treated controls. Data is mean $\pm$ SEM, n = 12 microchambers. *DMSO diluent.

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Figure 7. Neutrophils from ICU patients with infections display more efficient suppression of *S. aureus* despite lower recruitment.

A) Binary readout of patient neutrophil activity at 12 hours shows that neutrophils from 2 out of 3 patients with sepsis (S) exhibit enhanced suppression of *S. aureus* growth compared to non-sepsis (NS) patients.

B) All patients with sepsis (S) and 1 of 3 non-sepsis (NS) patients exhibited low neutrophil recruitment in response to *S. aureus* compared to a healthy control.