Thermal injury can trigger a fulminant inflammatory cascade that can lead to sepsis and death. Although several prior studies have documented impairments in neutrophil chemotaxis concurrent with these post-burn inflammatory changes, cumbersome assays have prohibited further exploration of the significance of these findings. To address this need, we developed a microfluidic device that is simple to operate and allows for chemotaxis measurements at single-cell resolution. Our study on neutrophils from eight burn patients revealed impaired migration speed starting at 24 hours after injury, reaching a minimum at 72-120 hours, and potentially serving as an early indicator for concurrent infections.

KEYWORDS: Neutrophil, chemotaxis, trauma, clinical

INTRODUCTION

In the United States, 40,000 patients are hospitalized with severe burn injuries annually[1]. Of the possible complications, sepsis is the most serious one and contributes to 75% of deaths [2]. Among the various factors contributing to sepsis, an important role is played by neutrophil impairments in phagocytosis, bactericidal activity, adhesion, complement receptor expression after burn injury [3]. Moreover, neutrophils from burn patients have impaired ability to migrate. In 1975, Warden et al. evaluated the chemotaxis function of neutrophils from 46 burn patients with a Boyden chamber assay, and found defective chemotaxis indexes for neutrophils and, within 72 hours after burn injury, the degree of impairment correlated with burn size and was predictive of mortality from septic complications [4]. However, beyond these initial observations, technical and practical limitations of the existing chemotaxis assays significantly hindered the progress towards better understanding neutrophil chemotaxis in the context of burn injuries. For example, studies using the Boyden assay do not allow direct observation of the moving cells, and they could only measure an average chemotactic index that depends on several variables, including the number of cells in the test sample, the fraction of cells that moves, and their speed, directionality, and persistence. Consequently, these studies could not identify the source of the changes in neutrophil chemotaxis after burn injuries, or if one or more the chemotaxis parameters have changed at the same time. More detailed analysis using Zigmond assay that allows the examination of chemotaxis by direct observation of individual neutrophils, suggested that directionality of migration but not migration speed correlate with the overall magnitude of burn injuries, however, this assay is difficult to set up and impractical outside the research lab [5]. Overall, despite evidence suggesting a link between neutrophil chemotaxis and outcomes after burn injury, existing assays for neutrophil migration are difficult to implement in the clinical setting, and as such, the prognostic potential of chemotaxis measurements remains largely unexplored.

To address the need for a robust, yet practical assay to investigate the details of neutrophil chemotaxis in burn patients, we designed a no-flow microfluidic device to measure the directional migration speed in chemoattractant gradient.

Figure 1: Overview of the neutrophil chemotaxis device. A. Six chemotaxis devices are mounted on a glass slides and observed on a microscope stage. B. In the first step, the side channels are primed with the chemoattractant solution. In the second step, neutrophils in buffer are introduced in the main channel in suspension. The chemoattractant gradient is then established by diffusion, in the longitudinal direction of the side channels. C. Neutrophils inside the main channel follow this gradient and enter an array of side channels.
with high throughput, and at single cell resolution. We validated the device by measuring the migration speed of neutrophils in blood samples from healthy volunteers and established one reference value for healthy persons regardless of age and sex. We also employed this device to document the impairment of neutrophil migration speed after burn injury with a degree of precision previously unattainable.

EXPERIMENTAL

Microfluidic devices were manufactured using standard techniques, employing two layers of photopatterned resist on silicon wafers, PDMS casting and bonding to glass slides. The microfluidic devices were primed with the chemokine fMLP [100 nM] and the extracellular matrix protein fibronectin [100 µM] at least 15 minutes before the assay. Blood samples of 1 mL were obtained with written informed consent from healthy volunteers, and from burn patients admitted to the Massachusetts General Hospital and Shriners’ burn units. Patients were enrolled if they sustained burns covering at least 20% of their total body surface area. The first sample was obtained within 72 hours after burn injury, and two more samples were drawn at 48 hour intervals afterward. Neutrophils were isolated from whole blood by density gradient separation using Polymorphprep (Axis-Shield, Rodelloka, Oslo, Norway), and following the protocol from the manufacturer. Neutrophils were infused into the device and allowed to settle in the main channel by clamping the ports of the device. Neutrophil migration in the direction of chemoattractant started immediately, and was recorded using and time-lapse imaging (1 frame/min).

RESULTS AND DISCUSSION

We developed a microfluidic device that is simple to operate, and that allows for precise and robust chemotaxis measurements at single-cell resolution. A chemical gradient of chemoattractant (fMLP, 100nM) is established between a central, buffer-filled channel that acts as a sink, and an array of smaller dead-end channels that act as chemoattractant reservoirs (Fig.1). First, Priming the device with chemoattractant fills these side channels. Later, during infusion of neutrophils into the device, the buffer solution in which neutrophils are suspended replaces the chemoattractant from the central channel, and finally, after the flow ceases, progressive diffusion of the chemoattractant establishes a gradient in the longitudinal direction of the side channels (Fig.1B). Neutrophils from the central channel will follow this gradient and move along the side channels at uniform speed [6]. In control experiments, in the absence of fMLP, neutrophils did not migrate through the side channels.

![Image](image_url)

**Figure 2:** Neutrophil motility inside the channels. A. Sequential images illustrating the migration of one representative neutrophil inside the device. B. Distribution of average speed of migration for 800 neutrophils from one healthy donor.. C. Average neutrophil motility in 18 healthy donors (mean and standard error) The motility of at least 50 neutrophils was calculated for each sample. Validation of the repeatability of neutrophil motility using samples from the same healthy donors at two weeks time interval.

A total of 23 blood samples were collected from 18 healthy volunteers aged 19-68 years. Average neutrophil velocity for all volunteer samples was $18 \pm 5$ µm/min (range 14-24 µm/min) (Fig.2), in agreement with values for normal neutrophil velocity documented in the literature by standard techniques[4]. To verify the reproducibility of the neutrophil velocity measurements, we repeated experiments with neutrophils isolated from 4 healthy volunteers, with samples drawn between one week and one month apart. The experiment yielded equivalent neutrophil velocities, thus suggesting high reproducibility (Fig.2C, $p \geq 0.05$).
Neutrophil motility was measured in a total of 24 samples from 2 pediatric and 6 adult patients (range 1-48 years) admitted to the Massachusetts General Hospital after sustaining burns on 20-60% total body surface. Across the entire data set, the average neutrophil velocity in burn patients was 9 ± 6 µm/min, significantly lower than in controls ($p < 0.01$) (Fig.3A-C). One exception to this pattern was a 42 year-old male who sustained 60% TBSA burns, who had blood cultures positive for Acinetobacter. Neutrophils from this patient displayed higher motility speed compared to all other patients and higher than healthy controls. In patients who were not bacteremic at 72-120 hours post-injury, neutrophil velocity was negatively correlated with severity of burn injury (Fig.3D, $R^2 = 0.6$). Neutrophil motility was most depressed between 72 and 120 hours after injury (Fig.3E), with an average velocity of 8 ± 2 µm/min. After this time frame, neutrophils demonstrated a tendency toward motility recovery, with an average velocity of 11 ± 3 µm/min one week after the burn injury.

CONCLUSION

These results establish the assay as a valuable tool for measuring neutrophil motility, provide a unique set of values characteristic of neutrophils from healthy subjects, and validate the use of the assay in the context of disease. Further characterization of neutrophil chemotaxis with this assay may have important diagnostic implications not only for burn patients, but could also become a valuable indicator for the potential vulnerability to sepsis for patients afflicted by other diseases that compromise neutrophil functions when standard diagnostic parameters fail, and may assist in clinical decisions and patient outcomes.

REFERENCES


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