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Short Communication

Video-rate two-photon imaging of mouse footpad – a promising model for studying leukocyte recruitment dynamics during inflammation

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Abstract. Leukocyte recruitment is a key host defense mechanism to infection and a salient feature of autoimmune diseases such as arthritis. The cell dynamics of these processes are difficult to study due to the challenge of tracking cells flowing in the circulation and migrating through light scattering tissues. Here, we describe a noninvasive two-photon (2P) microscopy approach to study leukocyte homing in the mouse footpad. In the absence of inflammation, cells moved >several hundred μm/s in vessels and only rarely adhered to endothelium or entered the tissue parenchyma. In response to bacterial infection, neutrophils moved in small capillaries at reduced speeds of (14–45 μm/min) and rolled in larger vessels at 5–60 μm/min. Within minutes of adoptive transfer, neutrophils entered the connective tissue and crawled with a median velocity of 7.3 μm/min. 2P imaging has excellent spatiotemporal resolution and is a promising *in vivo* approach to study the cellular basis of inflammation.

Key words: Multiphoton – Two-photon – Leukocytes – Neutrophil – Intravital imaging – Inflammation

Introduction

The phenomenon of leukocyte rolling and tethering within inflamed blood vessels was reported over 130 years ago by Julius Cohnheim [1]. In the last few decades, the molecular basis of this process has been an area of active interest. A host of molecules have been identified that act at different stages of leukocyte recruitment including selectins [2], integrins [3], junctional adhesion molecules [4] and chemokines [5] as well as factors that antagonize recruitment including cellular phosphatases [6] and nitric oxide [7]. However, our knowledge of the cell dynamics during the process are limited due in large part to the technical challenges associated with observing cells moving rapidly in the circulation and entering tissues under physiological conditions [8]. Considering the importance of leukocyte recruitment and migration in human health and disease [9], we developed a noninvasive two-photon (2P) microscopy approach that allows leukocytes to be observed with high spatiotemporal resolution within blood vessels and in the tissue parenchyma.

A major advantage of 2P imaging is that it does not involve a surgical preparation, as commonly required for epifluorescence intravital microscopy [10]. Because vessels and tissues can be imaged directly through skin, 2P imaging minimizes disruption to the physiological tissue state and permits basal trafficking behaviors to be studied. Moreover, since the mice are unharmed during the imaging process, it might be possible to perform longitudinal studies on individual research subjects. The first report of intravital 2P being used to study lymphoid tissues appeared in 2003 [11] and since then several variations of this approach have been widely adopted to study lymphocytes and antigen presenting cells *in vivo* [12]. This technique has changed our static view of the immune system and provided a tantalizing glimpse of the complex cellular dynamics that underlie the immune response [13].

The footpad is a classic site for assessing delayed type hypersensitivity (DTH) [14]. Here, we show that the mouse footpad is an ideal location to use 2P microscopy to study innate immune cell trafficking during inflammation. We envision 2P microscopy as an important new tool to study the single-cell dynamics of leukocyte trafficking *in vivo* during infection, vaccination and autoimmune disease.

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Methods

Bone marrow neutrophils were prepared as described by Graham et al. [15]. Briefly, bone marrow was harvested from the femurs and tibia of B6 mice. Neutrophils were isolated by Percol (Sigma Aldrich) gradient centrifugation and stained with 10 µm CFSE (Invitrogen) for 30 min at ~37 °C.

C57BL6 Mice were injected in the footpad with 20×10^6 *Listeria monocytogenes* in ~5 µl of PBS, and 2–4 h later, 5×10^6 CFSE labeled bone marrow neutrophils were adoptively transferred (i. v.). In other experiments we used CD11c-YFP mice (gift of the Nussenzweig lab) in which dendritic cells (DCs) express the enhanced yellow fluorescent protein. During the imaging experiment, mice were anesthetized with isofluorane for restraint and to avoid psychological stress on the animal. VetBond (3M) was used to secure the paw to the glass coverslip at the bottom of the imaging chamber and PBS was added to cover the tissue. The mouse's core body temperature was maintained with a warming pad (Braintree Scientific) set to 37 °C and supplemental fluids (Saline) administered i. p. or by retro orbital injection. We injected (i. v. or retro orbital) 1 mg dextran tetramethylrhodamine, 2,000,000 MW (Invitrogen), to label blood vessels during imaging. The rear footpad and toes were imaged for periods of 1–4 h before the mouse was euthanized while deeply anesthetized.

Time-lapse imaging was performed using a custom-built dual-laser video-rate 2P microscope. CFSE labeled BM neutrophils and Rhodamine dextran labeled blood vessels were excited by a Chameleon XR Ti:sapphire laser (Coherent) tuned to 820 nm. For experiments with CD11c-YFP mice we used simultaneous laser excitation at 915 nm and 820 nm. Fluorescence emission was passed through 490 nm and 560 dichroic mirrors placed in series and detected as red (560–650 nm), green (490–560 nm), and blue (<490 nm) channels by three head-on Bi-alkali PMTs. A customized version of ImageWarp (A&B software) was used to control the various hardware devices during real-time acquisition and to process and archive the image data. Each plane consists of an image of 200 µm by 225 µm (x and y = 2 pixel/µm). Z-stacks were acquired by taking between 21 and 61 sequential steps at 2.5 µm spacing. To increase signal contrast, we averaged between 10–20 video frames for each z-slice. Time points between stacks ranged from 18 to 48 s depending on the settings for each experiment. Multi-dimensional rendering was performed with Imaris (Bitplane) and cell tracking was performed with PicViewer Software (John Dempster University of Strathclyde) [16].

Results

To characterize the 3D tissue structure, we examined the footpads of CD11c-YFP mice with 2P microscopy (Fig. 1A). The native tissue appeared as discrete strata (Fig. 1B, supplemental movie 1); the surface of the skin, which gave off a diffuse blue-green autofluorescence, a sessile network of DCs positioned 10–50 µm below the skin surface (green cells, Fig. 1B), and a third region 50–150 µm deep that produced a distinctive second harmonic generation signal (blue fibers, Fig. 1B), presumably due to collagen in the connective tissue along the bone. Rhodamine dextran injection revealed a complex network of microvessels (red structures, Fig. 1B) within the connective tissue (blue). These vessels were between 3–15 µm in diameter and displayed a highly kinked and branched morphology.

Next we examined the homing dynamics of adoptively transferred neutrophils. In the absence of inflammation, neutrophils flowed in microvessels of the footpad at several hundred µm/sec (data not shown). Only rarely did we find evidence of neutrophil extravasation in the absence of inflammation (0–2 transferred neutrophils per footpad). In contrast, if mice were challenged with bacteria (Listeria monocytogenes) 2–4 h before adoptive transfer, large numbers of neutrophils were recruited from the circulation into inflamed tissues. Within 15 min after transfer, many neutrophils extravasated into the tissue and were crawling in the connective tissue (Fig. 1C, supplemental movie 2) with velocities between 5–10 µm/min (median velocity 7.3 µm/min, SD ± 2.5). At this time we also found neutrophils with a "torpedo like" morphology in small capillaries (3–15 µm in diameter) moving at speeds of 14–45 µm/min (Fig. 1C, supplemental movie 2). In larger vessels (20–50 µm in diameter), cells moved at speeds of 5–60 µm/min, similar to the rolling and tethering behaviors observed for monocytes with confocal imaging [17] but slower than what has been observed in the ear skin model [18]. Neutrophils often formed clumps in these larger vessels, which over 20–30 min, dissociated as cells entered the tissue parenchyma or dislodged and flowed away (data not shown).

Because each z-step in our image stack is an average of 10–20 video-rate frames, rapidly moving cells appeared as a single spot or a sequence of spots in our images. Each spot corresponds to a single video-rate frame and the distance between spots, equals the distance the cell traveled in $^1/_{30}$ of a second. An example of a cell moving ~300 µm/sec is shown in Figure 1D. Using this approach, we could measure neutrophil velocities up to 1.5 mm/s in the circulation.

Discussion

To date, most intravital imaging approaches require surgery to access the blood vessels as described in depth by Mempel et al. [19]. Moreover, only cells in the circulation or attached to the endothelium can be clearly seen; once cells extravasate, tissue-induced light scattering makes it difficult to track cell movement. Because surgery in itself causes tissue damage and inflammation, this might adversely affect leukocyte behavior [10] and essentially precludes obtaining a physiological base line level of homing, which is an important control parameter. Recently, several groups have used non-invasive single-photon techniques such as confocal and near IR imaging to study leukocyte homing [17, 18, 20]. While these studies represent significant technological advances in their own right, 2P imaging has the advantages of decreased phototoxicity [21] and superior imaging depth that should make it possible to follow cells longer and deeper into tissues. Because the neutrophils stay motile for several hours in the imaged regions we don't think we have generated significant heat or photodamage, however it is not possible to rule this out formally. Furthermore, the footpad is a classic site for assessing inflammation via a DTH reaction [14] and is arguably more physiological than other sites, such as the cremaster muscle preparation described by Baez in 1973 [22].

Considering that leukocyte extravasation has been studied over the last century, it is surprising that only a few studies have yielded quantitative data regarding leukocyte velocities in the circulation [17, 18]. In fact the most common form of analysis is to measure changes in leukocyte abundance in the images over time in relatively large vessels (>100 µm in diameter) [2]. The velocities we measured for adoptively transferred neutrophils were similar to values obtained for monocytes (5–30 µm/min) [17]. A detailed analysis of veloc-

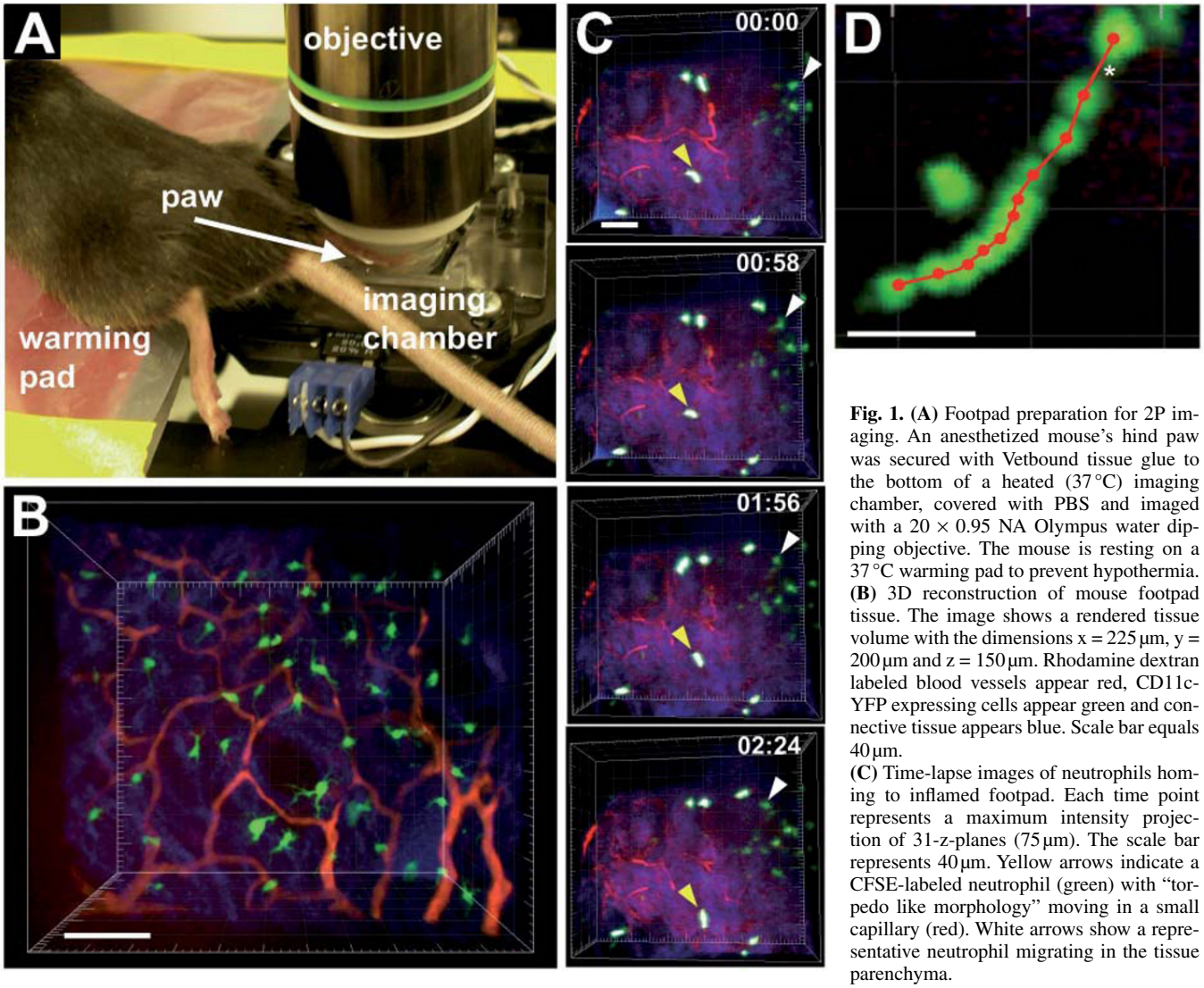

Fig. 1. (A) Footpad preparation for 2P imaging. An anesthetized mouse's hind paw was secured with Vetbound tissue glue to the bottom of a heated (37 °C) imaging chamber, covered with PBS and imaged with a 20 × 0.95 NA Olympus water dipping objective. The mouse is resting on a 37 °C warming pad to prevent hypothermia. **(B)** 3D reconstruction of mouse footpad tissue. The image shows a rendered tissue volume with the dimensions x = 225 µm, y = 200 µm and z = 150 µm. Rhodamine dextran labeled blood vessels appear red, CD11c-YFP expressing cells appear green and connective tissue appears blue. Scale bar equals 40 µm. **(C)** Time-lapse images of neutrophils homing to inflamed footpad. Each time point represents a maximum intensity projection of 31-z-planes (75 µm). The scale bar represents 40 µm. Yellow arrows indicate a CFSE-labeled neutrophil (green) with "torpedo like morphology" moving in a small capillary (red). White arrows show a representative neutrophil migrating in the tissue parenchyma. **(D)** Measuring high-speed cell movement in microvessels. A continuous 1-second video-rate record (single plane) showing a CFSE labeled neutrophil flowing through a vessel at 300 µm/s. Each dot is the cell captured in successive frames. By measuring the distance traveled between frames (see asterisk, each $^{1}/_{30}$ of a second apart) cell velocity can be calculated. Scale bar equals 20 µm.

ity and migratory behavior in physiological and pathological settings will provide fresh insight regarding the molecular machinery involved in this process. In particular, transgenic and knockout mouse models with genetically defined defects in chemokine [5] or integrin signaling [23] will be extremely informative. In these models *in vivo* imaging will be essential to distinguishing between closely related defects, such as, decreased rolling interactions or failed leukocyte firm adhesion, which would be difficult to discern in fix tissue sections. In terms of pharmaceutical development, we anticipate that 2P imaging will be useful for determining the precise mechanism of a drug's action, e. g., effects on cell homing, motility or chemotaxis, and hence will facilitate the rational design of more potent and specific therapeutics.

In summary our imaging approach is simple, robust and has high spatiotemporal resolution. The 2P method described here has higher resolution than bioluminescence and PET approaches, allows single-cell dynamics to be studied in 3D unlike conventional pathology or near IR imaging, and can image cells functioning in their native tissue environment at greater depths and with less photodamage than confocal microscopy. 2P *in vivo* imaging will deliver new insights for understanding how the host responds to infection or vaccination and how the regulation of leukocyte trafficking contributes to autoimmune diseases such as arthritis and lupus.

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