Video Article

Intravital Imaging of Neutrophil Priming Using IL-1β Promoter-driven DsRed Reporter Mice

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Abstract

Neutrophils are the most abundant leukocytes in human blood circulation and are quickly recruited to inflammatory sites. Priming is a critical event that enhances the phagocytic functionality of neutrophils. Although extensive studies have unveiled the existence and importance of neutrophil priming during infection and injury, means of visualizing this process *in vivo* have been unavailable. The protocol provided enables monitoring of the dynamic process of neutrophil priming in living animals by combining three methodologies: 1) DsRed reporter signal — used as a measure of priming 2) *in vivo* neutrophil labeling — achieved by injection of fluorescence-conjugated anti-lymphocyte antigen 6G (Ly6G) monoclonal antibody (mAb) and 3) intravital confocal imaging. Several critical steps are involved in this protocol: oxazolone-induced mouse ear skin inflammation, appropriate sedation of animals, repeated injections of anti-Ly6G mAb, and prevention of focus drift during imaging. Although a few limitations have been observed, such as the limit of continuous imaging time (~ 8 hr) in one mouse and the leakage of fluorescein isothiocyanate-dextran from blood vessels in the inflammatory state, this protocol provides a fundamental framework for intravital imaging of primed neutrophil behavior and function, which can easily be expanded to examination of other immune cells in mouse inflammation models.

Video Link

The video component of this article can be found at http://www.jove.com/video/54070/

Introduction

Neutrophils are the most abundant and short-lived leukocytes in circulation. They are rapidly recruited to the sites of infection or injury, where they serve as professional phagocytes through release of reactive oxygen and nitrogen intermediates along with granules containing antimicrobial peptides and proteases[1]. During their recruitment, neutrophils are "primed" by various agents including microbial products, chemoattractants, and inflammatory cytokines, resulting in markedly enhanced phagocyte functionality upon arrival at a site of inflammation[2]. The mechanisms of neutrophil priming have been extensively studied *in vitro*[3,4]; however, dynamic monitoring of the process *in vivo* has not been possible to date.

Recently, intravital imaging has become an important technique for visualizing and quantifying the cellular dynamics of biological processes in living organisms. Intravital imaging can be performed via conventional one-photon excitation microscopy (*e.g.,* confocal) or multiphoton microscopy approaches[5]. Over time, substantial improvements have been achieved in this technique enabling increased image resolution, improved imaging depth, decreased tissue photodamage, and enhanced image stabilization[6,7]. Given its unique ability to enable dynamic visualization of cellular migration and interaction over time, intravital microscopy has been extensively applied to diverse areas of study in immunology[8]. Intravital imaging enables immunologists to better understand and contextualize immune responses at both the cellular and molecular level in living animal models.

Recent advances in transgenic as well as knock-in reporter mice have provided useful tools for monitoring the dynamic behaviors of neutrophils in living animals. Lysozyme M promoter-driven enhanced green fluorescent protein knock-in mice have been broadly used to characterize motility of neutrophils, monocytes, and macrophages during various inflammatory processes including extravasation, bacterial infection, and sterile inflammation[9-15]. Further, transgenic mice expressing a cytoplasmic fluorescence resonance energy transfer biosensor have been employed in studying the activities of neutrophil extracellular-regulated mitogen kinase and protein kinase A within inflamed intestine[16]. A murine model with high specificity for fluorescence expression in neutrophils is the Catchup knock-in mouse, which produces Cre recombinase as well as the fluorescent protein tdTomato, which itself is coupled to expression of lymphocyte antigen 6G (Ly6G)[17]. Visualization of Ly6G-deficient neutrophils via this model has demonstrated that these cells exert normal functionality in a variety of sterile or infectious *in vivo* inflammatory contexts. Transgenic mice expressing the DsRed fluorescent protein gene under the control of the mouse interleukin-1β (IL-1β) promoter (pIL1-DsRed)

have been utilized to visualize the motile behaviors of IL-1β producing cells — believed to include neutrophils, inflammatory monocytes, and activated macrophages — emerging in inflamed skin[18].

In vivo labeling can serve as an alternative approach for tracing the cellular and molecular behaviors of neutrophils in inflamed tissues. After intravenous injection of low doses of fluorescently labeled anti-Gr-1 monoclonal antibody (mAb), the recruitment cascade of Gr-1$^+$ neutrophils has been visualized in mouse skin lesions infected with *Staphylococcus aureus*[19]. *In vivo* administration of conjugates containing streptavidin-conjugated 705 nm quantum dots and biotinylated anti-Ly6G mAb specifically label circulating neutrophils[20]. Moreover, endocytosis of such conjugates into neutrophil vesicles allows tracking of high-speed vesicle transport in neutrophils migrating into the interstitium. *In vivo* labeling with fluorescence-conjugated antibodies against P-selectin glycoprotein ligand-1 (PSGL-1), L-selectin (CD62L), integrin αM (CD11b) and chemokine (C-X-C motif) receptor 2 (CXCR2) in a TNFα-induced inflammatory model has elucidated the regulatory mechanisms at play during early inflammation[21]. Polarized neutrophils protrude PSGL-1-enriched uropods to interact with CD62L present on activated platelets, resulting in the redistribution of CD11b and CXCR2, receptors that drive neutrophil migration and initiate the inflammation.

IL-1β is one of the signature genes that is elevated in primed neutrophils[22]. In pIL1-DsRed reporter mice, DsRed fluorescence signals (*i.e.*, activation of IL-1β promoter) positively correlate with IL-1β mRNA expression and IL-1β protein production.[18] To monitor the process of neutrophil priming, an intravital microscopy method was developed involving induction of skin inflammation with oxazolone (OX) in the pIL1-DsRed mouse model following *in vivo* labeling of neutrophils with fluorescence-conjugated anti-Ly6G mAb. Via this model, it is possible to study the behavior and function of primed neutrophils in animal models of various diseases and disorders.

Protocol

All animal experiments are performed in accordance with the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of the University of Toledo.

1. Phenotyping of pIL1-DsRed Mice

NOTE: Offspring are generated by breeding heterozygous pIL1-DsRed mice with wild-type (WT) C57BL6 mice. Three to four week old pups are considered ready for phenotyping. Submandibular bleeding of mice follows an established protocol with minor modifications[23].

1. **Isolation of White Blood Cells from the Whole Blood of Mouse Pups**
    1. Add 20 µl of heparin to each 1.5 ml microcentrifuge tube.
    2. Acquire one pup from the cage. Record the identification tag of the pup. It is not required to anesthetize pups before blood collection from the submandibular vein.
    3. Hold pup by neck scruff and pierce the submandibular vein in the cheek pouch using a 5 mm lancet. Apply adequate force to create a small puncture, so that drops of blood exude from the point of penetration. Use a new needle for each pup.
    4. Collect 3 - 5 drops of blood per pup in a heparin-containing microcentrifuge tube. Clean the puncture site and apply pressure to facilitate hemostasis.
    5. Put pup in new cage and observe for 30 min before returning the cage to the animal facility.
    6. Collect blood from adult mice of a known phenotype as positive and negative controls.
    7. Add 500 µl of red blood cell lysing buffer to each tube, vortex tubes, and incubate for 5 - 10 min on ice.
    8. Slowly add 400 - 500 µl of fetal bovine serum (FBS) underneath the cell suspensions in each tube. A clear interface can be observed between the upper-layer cell suspension and the lower-layer of FBS.
    9. Cap tubes and centrifuge samples at 1,500 × g for 5 min.
    10. Repeat steps 1.1.7 - 1.1.9 to further remove red blood cells. Do not repeat if lysis is sufficient the first time. The pellet should be difficult to discern after centrifugation.

2. **Lipopolysaccharide (LPS) Stimulation and Culture**
    1. Resuspend cells in 200 µl of complete Roswell Park Memorial Institute (RPMI) 1640 medium.
    2. Transfer the cell suspension to a flow cytometry tube.
    3. Make LPS working solution by mixing 10 µl of LPS stock solution (1 mg/ml) with 990 µl of complete RPMI 1640 medium.
    4. Add 20 µl of 10 µg/ml LPS working solution to 200 µl of cell suspension.
    5. Cap tubes loosely and incubate samples for 4 hr at 37 °C with 5% CO_2.

3. **Flow Cytometry Analysis**
    1. Open the data acquisition program for flow cytometry. Set up acquisition template prior to sample acquisition.
    2. Click the Dot-Plot tool in the tool palette. Draw a forward scatter (FSC) vs side scatter (SSC) plot. Set the FSC and SSC voltage parameters to linear scale.
    3. Select the lowest thresholds of FSC and SSC allowed by the cytometer. Apply thresholds 200 and 50 for FSC and SSC, respectively.
    4. Within the FSC vs SSC plot, draw a gate G1 which includes monocytes, granulocytes, and dendritic cells, and excludes debris, red blood cells and lymphocytes.
    5. Click the Histogram tool in the tool palette. Draw a FL2 (red fluorescence channel) histogram. Use a negative control sample to set the baseline of FL2 signals and a positive control sample to draw a gate to include all DsRed positive events.
    6. On the Fluidics Control panel of the cytometer, set the fluid mode to "RUN" and the flow rate to "HI" (approximately 60 µl/min). Acquire 10,000 events for each sample.
    7. Determine pup phenotype by measuring the percentage of DsRed positive cells from the G1 gate.

2. Induction of Skin Inflammation in pIL1-DsRed Mice

1. Anesthetize mouse by intraperitoneal (i.p.) injection of an anesthetic cocktail containing 100 mg/kg ketamine, 10 mg/kg xylazine, and 1 mg/kg acepromazine. Adequately anesthetized mice do not show hind paw withdrawal to toe pinch.
2. Apply hair-removal cream to the dorsal surface of both ears of the mouse. Wait 30 sec to 1 min. Wipe ear surface with water-wetted cotton and allow to air-dry.
3. Measure the ear thickness using micrometer and replace mouse in a separate cage. Observe until recovery from anesthesia (~ 15 - 30 min). To resolve skin inflammation induced by the hair removal product, allow mouse to rest for 3 days before further experimentation is carried out.
4. Prepare 1.25% (w/v) OX by dissolving 16.7 mg of OX in 1 ml acetone and mixing 750 µl of OX/acetone solution with 250 µl of olive oil. Prepare vehicle solution by mixing 750 µl of acetone with 250 µl of olive oil.
5. Re-anesthetize mouse by i.p. injection of anesthetic cocktail (2.1). Measure the ear thickness as before.
6. Apply 12.5 µl of 1.25% OX onto each side of right ear. Apply 12.5 µl of acetone/olive oil vehicle solution onto each side of left ear.
7. Keep mouse separate from cage-mates until fully recovered to prevent insult to its ears by other mice, then replace the mouse in its original cage and return to animal housing facility.
   NOTE: Hair removal may result in minor skin inflammation following by change of ear thickness. This minor inflammation will be resolved 3 days later confirmed by normal ear thickness. After topical application for 24 hr, OX-treated ears show significant ($p < 0.01$) swelling compared vehicle solution alone-treated ears.

3. Labeling of Neutrophils in pIL1-DsRed Mice

NOTE: *In vivo* labeling of neutrophils by low dose fluorescence-conjugated neutrophil-specific mAb follows a recently developed protocol[19]. Retro-orbital injections are performed according to an established protocol with a few modifications[24].

1. Prepare anti-Ly6G mAb working solution by diluting 10 µl of 0.5 mg/ml Alexa Fluor 647-conjugated anti-Ly6G mAb (clone 1A8) in 90 µl of phosphate buffered saline (PBS).
2. Transfer 100 µl of anti-Ly6G mAb working solution (50 µg/ml) into a U-100 insulin syringe with 28 gauge needle.
3. Anesthetize an adult pIL1-DsRed mouse by i.p. injection of the previously described anesthetic cocktail (2.1). Place the anesthetized mouse abdomen down on a clean working board.
4. Apply gentle downward pressure to the skin dorsal and ventral to one mouse eye to partially protrude the eyeball from the socket.
5. Carefully place the needle, bevel down, at an angle of approximately 30° at the medial canthus into the retro-orbital sinus.
   NOTE: The operator can feel pressure, penetration and relieved resistance once the needle inserts into the sinus.
6. Slowly and smoothly inject 100 µl of anti-Ly6G mAb working solution (5 µg mAb/mouse/injection) into the mouse. Remove the needle quickly. A small amount of bleeding suggests a successful injection.
7. Immediately apply 1.25% OX and vehicle solution onto the right and left mouse ears.
8. After 8 hr, administer 100 µl of freshly prepared anti-Ly6G mAb working solution (5 µg mAb/mouse/injection) via retro-orbital injection into the same mouse.
9. To visualize the blood vessels, immediately before imaging, administer 100 µl of 30 mg/ml fluorescein isothiocyanate (FITC)-conjugated dextran (150 kDa) through retro-orbital injection.

4. Intravital Imaging of Neutrophil Priming in pIL-1-DsRed Mice

1. Anesthetize mouse with i.p. injection of anesthetic cocktail (2.1). Apply veterinary ointment to mouse eyes to prevent dryness while under anesthesia for imaging.
   1. Prepare 1 ml half-dose anesthetic cocktail by diluting 500 µl of original anesthetic cocktail with an equal volume of PBS.
   2. Transfer half-dose anesthetic cocktail to 1 ml syringe with a butterfly 27 gauge needle.
   3. Insert needle of butterfly into mouse abdomen intraperitoneally and fix the butterfly wing to the abdomen by taping.
2. Place a glass coverslip in the center of the imaging stage. Tape the edges of the coverslip to hold it in place.
3. Place a drop of PBS on the ventral surface of the mouse ear as well as on the coverslip.
4. Position anesthetized mouse with its ear over the coverslip. Mount the tip of the ear, dorsal side down, by sandwiching the ear between the coverslip and a glass slide, also held in place via tape.
5. Turn on a multi-laser fluorescence confocal microscope and all the related equipment. Turn off room lights to minimize ambient light exposure.
6. Open the image acquisition software. Select the laser channels for detection of fluorescent dyes in the Dye List menu.
7. Adjust the focus on the inflamed ear skin by observation of green signals from the blood vessels and red signals from the $DsRed^+$ cells through eyepiece.
8. Perform a fast scan with scan speed of 2 µs/pixel and resolution of 512 × 512 pixels. Select a pseudocolor for each channel in the Live View menu. Adjust the focus knob to set the end and start location of scanning.
9. Perform slow scans with scan speed of 4 - 8 µsec/pixel and resolution of 800 × 800 or 1,024 × 1,024 pixels. Adjust the high voltage, gain, and offset on each individual channel to maximize the intensity of signals while avoiding saturation (red pixels). There should be only a few red pixels in each channel.
10. Create three-dimensional image sets by scanning the ear skin beginning superficially in the stratum corneum and progressing downward with x, y, z volumes of 317 µm × 317 µm × 30 µm (40X objective), 635 µm × 635 µm × 30 µm (20X objective), or 1,270 µm × 1,270 µm × 50 µm (10X objective) at 2 µm z-steps.
    NOTE: The stratum corneum is the outermost layer of epidermis and can be readily localized based on its strong autofluorescence signal.
11. In time-lapse imaging experiments, record three-dimensional images every 2 or 4 min for up to 8 hr.
12. Intermittently, as mouse begins to recover from anesthesia, noted based on observation of twitching whiskers, administer 5 - 10 µl of half-dose anesthesia cocktail through butterfly needle.

NOTE: Be cautious of the anesthesia dose - overdose may cause mouse death. Alternatively, inhalation anesthesia in a jar could be applied to the mouse for short-term anesthesia and a nose cone could be used for long-term imaging.

13. Remove the anesthetized mouse from the stage when imaging is complete. Detach tape and remove butterfly needle from the mouse's abdomen. Return the mouse to a separate cage in the animal housing facility.
NOTE: For short-term imaging (<1 hr), mice fully recover from anesthesia quickly in 1 - 3 hr. For long-term imaging (~ 8 hr), mice recover slowly, with a typical recovery period of 12 - 16 hr. Thus, oral water administration is recommended at least several times during the recovery period to prevent severe dehydration.
14. Process imaging datasets, track individual cells, generate migratory paths, and calculate the directionality and velocity of cell migration using image analysis software[25].

Representative Results

Screening of pIL1-DsRed mice is performed based on the phenotypic DsRed fluorescence signal produced by their peripheral blood leukocytes using flow cytometry. LPS stimulation is known to induce IL-1β production in myeloid cells including neutrophils, monocytes, and dendritic cells[26-28]. Thus, isolated leukocytes are incubated with LPS for 4 hr prior to flow cytometry analysis. Next, gating is set for circulating myeloid cells based on cell size (FSC) and internal complexity (SSC) (**Figure 1A**)[29], and DsRed signal is measured in this gated population. Circulating myeloid cells from WT mice minimally express DsRed, while the same cells from pIL1-DsRed mice express distinguishable DsRed signals (**Figure 1B**). The pIL1-DsRed mice identified by this phenotyping method are used for subsequent experiments. It should be noted that the complete phenotyping process takes only a few hours — much shorter than the traditional genotyping methods including tissue harvest, enzymatic digestion, DNA isolation, PCR and electrophoresis.

IL-1β promoter activation has recently been identified as a marker of neutrophil priming[22]. Thus, the dynamic process of neutrophil priming can be visualized and assessed in living animals using pIL1-DsRed mice. To induce skin inflammation, the skin sensitizer OX is topically applied on the right ear of pIL1-DsRed mice and vehicle alone is applied on the left ear of the same animal. To label neutrophils in living animals, a recently developed protocol is performed by administering a small amount of fluorescently labeled anti-Ly6G mAb immediately before and 8 hr after OX application. To locate blood vessels, FITC-dextran is injected immediately prior to imaging.

At 24 hr after OX application, a large number of $DsRed^+/Ly6G^+$ cells are found in the extravascular space (**Figure 2A**) and represent primed neutrophils. A small number of $DsRed/Ly6G^+$ cells are also found, which most likely represent resting neutrophils. Moreover, a small population of $DsRed^+/Ly6G^-$ cells are observed, which may comprise inflammatory monocytes and activated macrophages. In a 40 min time-lapse video, $Ly6G^+$ neutrophils emerging within an inflammatory skin lesion exhibit typical amoeba-like crawling movement (**Supplemental Movie 1**). Cell migratory paths are tracked to compare motile behaviors of the $DsRed^+/Ly6G^+$ and $DsRed^-/Ly6G^+$ neutrophil populations (**Figure 2B**). The track plot analyses reveal that both neutrophil populations display "random" migration once the extracellular space has been entered (**Figure 2C**). Interestingly, $DsRed^+/Ly6G^+$ neutrophils exhibit a significantly higher velocity compared with their $DsRed^-/Ly6G^+$ counterparts (**Figure 2D**). This suggests an association between neutrophil priming and accelerated motility in inflammatory lesions.

To monitor the timing of neutrophil priming, a series of time-lapse videos were recorded starting at different times following OX treatment (**Supplemental Movies 2 - 4**). $DsRed^-/Ly6G^+$ cells, the resting neutrophils, become detectable in the extravascular space as early as 8 - 12 hr after OX application and their numbers remain relatively low thereafter (**Figure 2E, Supplemental Movies 3, 4**). On the contrary, the number of $DsRed^+/Ly6G^+$ cells, the primed neutrophils, increase progressively at later time points, beginning at 14 - 16 hr after OX application. Some $DsRed^-/Ly6G^+$ cells gradually acquire DsRed signals after recruitment to the extravascular space during this period (**Figure 2F, Supplemental Movie 5**). At 16 - 20 hr, a majority of the extravascular $Ly6G^+$ neutrophils are considered as "primed" based on their DsRed expression. These observations reveal the magnitude, tempo, and location of neutrophil priming occurring at inflammatory lesions in living animals.

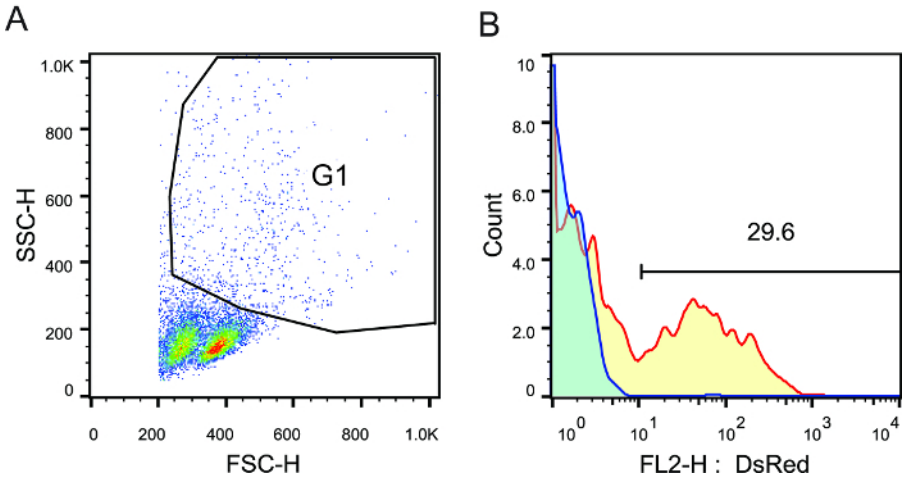

Figure 1: Phenotyping of pIL1-DsRed Mice. (**A**) Flow cytometry analyses demonstrate a population of circulating myeloid cells (G1) based on FSC and SSC parameters. (**B**) Histograms at the FL2 channel show the percentage of $DsRed^+$ cells within G1 gate of the cells from pIL-DsRed mice (red) or from WT mice (blue). Data are representative of at least three independent experiments. Please click here to view a larger version of this figure.

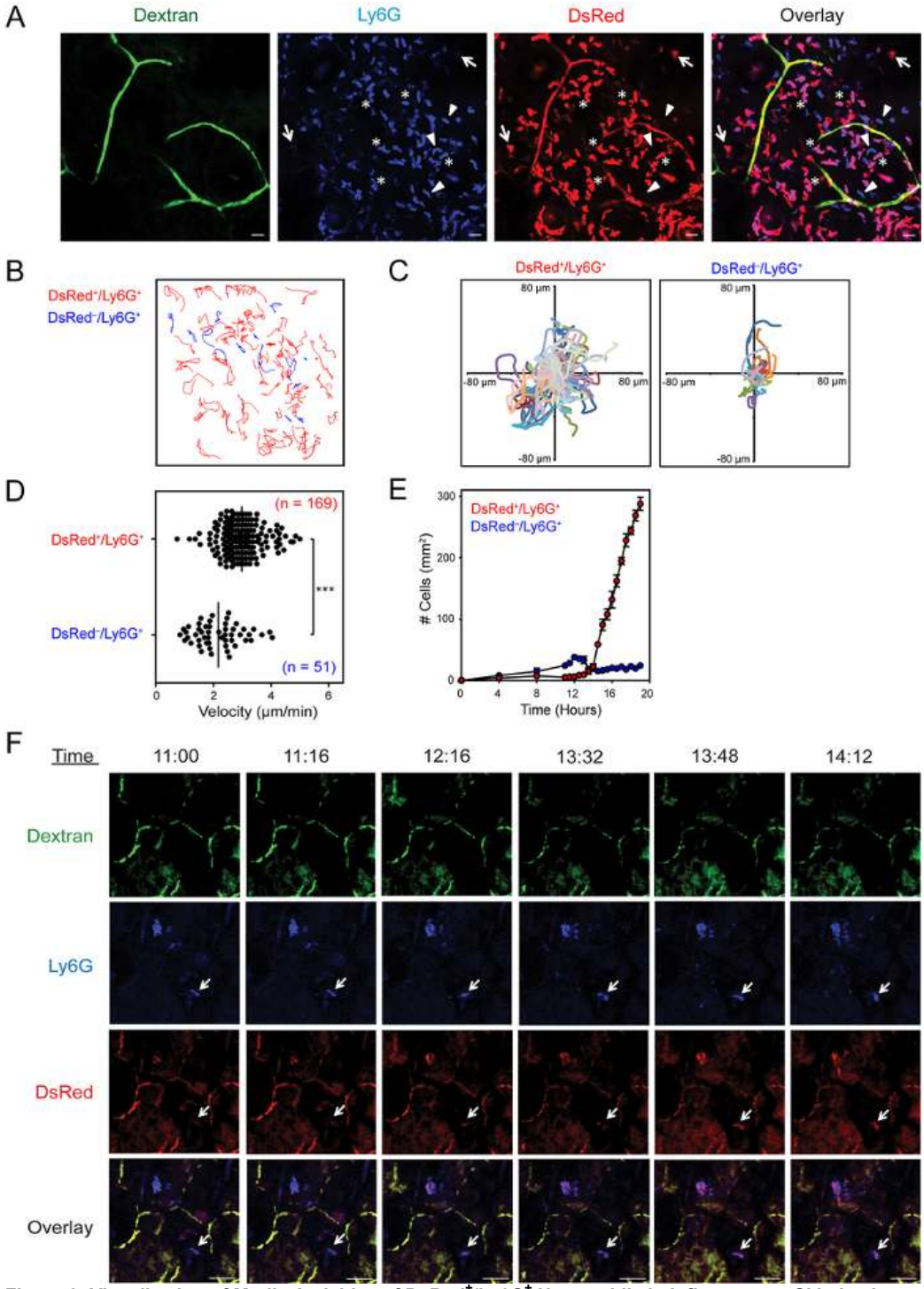

Figure 2: Visualization of Motile Activities of DsRed$^+$/Ly6G$^+$ Neutrophils in Inflammatory Skin Lesions. (**A**) Static confocal microscopy images recorded 24 hr after OX treatment show three leukocyte populations in the extravascular space, *i.e.*, DsRed$^+$/Ly6G$^+$ cells (asterisks), DsRed$^-$/Ly6G$^+$ cells (triangles), and DsRed$^+$/Ly6G$^-$ cells (arrows). Bar = 20 µm. DsRed$^+$/Ly6G$^+$ cells (red) and DsRed$^-$/Ly6G$^+$ cells (blue) are compared for migratory paths (**B**), track plots (**C**), and velocity (**D**) from a 40 min time-lapse movie recorded by confocal microscopy starting 24 hr after OX application (**Supplemental Movie 1**). (**D**) Bars indicate mean velocity values. *** P <0.001. (**E**) The numbers of DsRed$^+$/Ly6G$^+$ cells and DsRed$^-$/Ly6G$^+$ cells are counted from a series of time-lapse movies recorded at different time-points after OX application (**Supplemental Movies 2 through 4**). The data shown are the cell numbers/mm^2 (means ± SD) calculated from three consecutive images. (**F**) Static images are created from a time-lapse movie recorded from 11 to 14.5 hr after OX painting (**Supplemental Movie 5**). The arrow indicates a Ly6G$^+$ cell that acquires DsRed expression in the extravascular space during the observation period. Bar = 50 µm. Images are reproduced from reference 22 with permission (Copyright 2015. The American Association of Immunologists, Inc.). Please click here to view a larger version of this figure.

Supplemental Movie 1: Motile Behavior of DsRed$^+$ Neutrophils in Inflammatory Skin Lesions (at 24 hr after OX painting). Confocal microscopy images show the motility of DsRed$^+$/Ly6G$^+$ cells (pink) and DsRed$^-$/Ly6G$^+$ cells (blue) in the extravascular space. Blood vessels are seen in green. Bar = 20 µm. The video is reproduced from reference 22 with permission (Copyright 2015. The American Association of Immunologists, Inc.). Please click here to view this video.

Supplemental Movie 2: Motile Behavior of DsRed$^+$ Neutrophils in Inflammatory Skin Lesions (at 4 hr after OX painting). Confocal microscopy images show DsRed$^+$/Ly6G$^+$ cells (pink) and DsRed$^-$/Ly6G$^+$ cells (blue) in the extravascular space. Blood vessels are seen in green. Autofluorescence is present and associated with hair follicles. Bar = 100 µm. The video is reproduced from reference 22 with permission (Copyright 2015. The American Association of Immunologists, Inc.). Please click here to view this video.

Supplemental Movie 3: Motile Behavior of DsRed$^+$ Neutrophils in Inflammatory Skin Lesions (at 8 hr after OX painting). Confocal microscopy images show DsRed$^+$/Ly6G$^+$ cells (pink) and DsRed$^-$/Ly6G$^+$ cells (blue) in the extravascular space. Blood vessels are seen in green. Autofluorescence is present and associated with hair follicles. Bar = 100 µm. The video is reproduced from reference 22 with permission (Copyright 2015. The American Association of Immunologists, Inc.). Please click here to view this video.

Supplemental Movie 4: Motile Behavior of DsRed$^+$ Neutrophils in Inflammatory Skin Lesions (at 11 - 19 hr after OX painting). Confocal microscopy images show DsRed$^+$/Ly6G$^+$ cells (pink) and DsRed$^-$/Ly6G$^+$ cells (blue) in the extravascular space. Blood vessels are seen in green. Autofluorescence is present and associated with hair follicles. Bar = 100 µm. The video is reproduced from reference 22 with permission (Copyright 2015. The American Association of Immunologists, Inc.). Please click here to view this video.

Supplemental Movie 5: Acquisition of DsRed Signals by Ly6G$^+$ Neutrophils. From the time-lapse movie shown in supplemental Video 4, this higher magnification video shows the process in which a Ly6G$^+$ cell (indicated with an arrow) acquires DsRed expression in the extravascular space during the observation period (11-14.5 hr after OX painting). Bar = 50 µm. The video is reproduced from reference 22 with permission (Copyright 2015. The American Association of Immunologists, Inc.). Please click here to view this video.

Discussion

The aim of this study is to develop a technology for monitoring the process of neutrophil priming in living animals, which has not yet been fulfilled by the currently available techniques. To achieve this goal, three established methodologies are performed: 1) induction of skin inflammation in IL-1β promoter-driven DsRed reporter mice as a measure of priming, 2) *in vivo* labeling of neutrophils with low doses of fluorescence-conjugated anti-Ly6G mAb, and 3) intravital confocal microscopy imaging. The combination of these three methods enables visualization of the motile activities of primed neutrophils (DsRed$^+$/Ly6G$^+$) in inflammatory skin lesions. The migration direction and velocity of the primed neutrophils is further quantified via migratory path tracking. By recording a series of time-lapse videos, the real-time kinetics of primed neutrophil emergence is monitored at inflammatory site and acquisition of DsRed expression by individual neutrophils is also observed in the extravascular space. Thus, the dynamic process of neutrophil priming has been successfully visualized in living animals for the first time using the technology presented here.

This technology is employed in several critical steps. First, skin tissues are the most convenient body parts for intravital imaging. Topical application of OX on the mouse ear allows direct visualization of immune cell behaviors in inflamed skin under confocal microscopy. Second, the mouse must be sedated constantly during imaging which necessitates repeat injections of anesthetic. However, over-sedation may cause mouse die during imaging. To avoid overdose, low doses of anesthetic (a half or a third of the full dose) are recommended for long-term imaging. Third, fluorescence-conjugated neutrophil-specific mAb must be injected intermittently due to the short lifespan of murine neutrophils (half-life of 8-10 hr[30]). Last, focus drift is a major issue during time-lapse imaging, which could be caused by multiple factors including temperature variations, mouse body movement, ear swelling over time, and vibrations arising from operation of the instrument[31]. Several methods have been developed to prevent drifting, such as administration of a constant dose of sedation during imaging, fixing the mouse ear in place by taping a glass slide over it on the stage, installing an environmental chamber to provide oxygen and warmth to the sedated organism as well as by manually re-focusing the scope when necessary[32,33].

It is equally important to state the limitations of this protocol. First, a slow mouse recovery (12 - 16 hr) after an imaging period 8 hr in length is observed due to continuous sedation, indicating that this protocol may not be suitable for such lengthy experiments. An alternative approach is to use several mice in sequence over the imaging period rather than using only one. Second, a gradual leak of circulating FITC-dextran is observed during imaging over time, which is likely caused by increased permeability of skin blood vessels in the inflammatory state[34]. Thus, visualization of blood vessels is markedly compromised given reduced fluorescence in circulation at later stages of imaging. Alternatively, one may use fluorescently conjugated lectins such as isolectin B4 to mark blood vessels as these adhere to vascular endothelial cells[35].

In addition to visualization of the process of neutrophil priming in skin inflammatory lesions, the technology described here can also be applied in many other experimental settings. In combination with recently developed imaging windows, intravital microscopy delineating the behaviors of primed neutrophils could be assessed in deep tissues and organs including brain, mammary glands, lungs, and abdominal organs[36-39]. Moreover, visualization of priming/activation of other cell types such as monocytes and macrophages could be achieved using pIL1-DsRed mice along with *in vivo* labeling via fluorescence-conjugated mAb specific to the cell type of interest. Taken together, not only does this technology provide a fundamental framework for intravital imaging of the priming process of neutrophils in living animals, but it also unveils new approaches for studying the behavior and function of other immune cells in various inflammatory states unfolding in different tissues.

Disclosures

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