Parasitology International xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Intravital imaging of the immune responses during liver-stage malaria infection: An improved approach for fixing the liver

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ARTICLE INFO

Article history:
Received 2 November 2015
Received in revised form 10 February 2016
Accepted 23 February 2016
Available online xxxx

Keywords:
Imaging
Liver
Malaria
CD8$^+$ T cells

ABSTRACT

The host–parasite relationship is one of the main themes of modern parasitology. Recent revolutions in science, including the development of various fluorescent proteins/probes and two-photon microscopy, have made it possible to directly visualize and study the mechanisms underlying the interaction between the host and pathogen. Here, we describe our method of preparing and setting-up the liver for our experimental approach of using intravital imaging to examine the interaction between *Plasmodium berghei* ANKA and antigen-specific CD8$^+$ T cells during the liver-stage of the infection in four dimensions. Since the liver is positioned near the diaphragm, neutralization of respiratory movements is critical during the imaging process. In addition, blood circulation and temperature can be affected by the surgical exposure due to the anatomy and tissue structure of the liver. To control respiration, we recommend anesthesia with isoflurane inhalation at 1% during the surgery. In addition, our protocol introduces a cushion of gauze around the liver to avoid external pressure on the liver during intravital imaging using an inverted microscope, which makes it possible to image the liver tissue for long periods with minimal reduction in the blood circulation and with minimal displacement and tissue damage. The key point of this method is to reduce respiratory movements and external pressure on the liver tissue during intravital imaging.

1. Background

A new era in the visualization of immune systems began a decade ago following the development of various fluorescent proteins and two-photon microscopy (TPM) [6,10]. With TPM, researchers can observe deep tissues with minimal photo damage [16]; moreover, real-time intravital imaging of live tissue has allowed scientists to study the dynamic biological behaviors of microbes, cells, and organs as they occur within the body [4,7].

Intravital imaging of the liver using TPM is an attractive tool for various studies in biological science, since the liver plays a variety of vital roles in several biological systems. In particular, TPM and intravital imaging have recently made it possible to directly observe the interactions between host immune cells and infectious microbes such as *Plasmodium* species, *Leishmania* parasites, *Mycobacterium*, and *Listeria monocytogenes* in the liver [2–5,7]. However, the anatomical position of the liver makes it a challenging target for intravital imaging. The liver is positioned near the diaphragm, and neutralization of respiratory movements, especially during respiratory anesthesia, is critical. Another challenge is the surgical approach to the liver. The liver has a dense microvascular network, and any manipulation can lead to massive hemorrhaging and impairment of the microcirculation system. Additionally, any hemorrhaging or blood clotting can significantly reduce the quality of intravital imaging.

Technically, the most challenging part of performing live imaging of the liver is to set up the liver on the microscope with minimal damage and movement during the imaging procedure. Recently, different groups of scientists have invented several protocols for intravital imaging of the liver [8,9,12,14,15]. Here, by modifying a previously reported protocol [14], we developed an improved approach for fixing the liver during intravital imaging.

2. Objective

Our goal was to establish a protocol for stably preparing the mouse liver for intravital imaging with TPM, while minimizing respiratory movements, to yield the best possible intravital imaging results with fewer artifacts and detrimental effects (in the liver tissue and microcirculation) according to our experience. Specifically, we used our experimental setup to image the interaction of malaria parasites and antigen-specific CD8$^+$ T cells during the liver-stage of malaria infection.

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http://dx.doi.org/10.1016/j.parint.2016.02.011
1383-5769/

The pre-erythrocytic stage is a short asymptomatic step in the malaria lifecycle in which the amount of the parasite is at a minimal level; thus, this stage can be a good target for developing treatment or vaccine strategies [1,17]. Here, we studied the behavior of antigen-specific $CD8^+$ T cells during the liver-stage of malaria infection using inverted TPM. In this model system, we visualized parasites by using *Plasmodium berghei* ANKA, which expresses a fusion protein of the ovalbumin $(OVA)_{257–264}$ epitope attached to the C-terminus of green fluorescent protein (PbA-gfpOVA). We used OVA-specific T-cell receptor transgenic mice (OT-I), which were crossed with DsRed transgenic mice to visualize $CD8^+$ T cells [7]. Transfer of activated antigen-specific $CD8^+$ T cells prior to infection with sporozoites stops the development of *P. berghei* in the liver. Using intravital imaging, we showed that antigen-specific $CD8^+$ T cells form clusters around the infected hepatocytes and then destroy the parasite [7]. We imaged each individual cluster for around 60 sequential images with a 2-μm Z-stack (>30 slices) with 30-s intervals. The interval between each two sequential images can be increased to up to 5 min, which makes it possible to image each cluster for 2–3 h before photo bleaching. The area of the left lobe that was under the glass coverslip was available for intravital imaging for several hours. In our suggested preparation below, we present the protocol for this imaging set-up.

3. Methods

3.1. Materials

We used *P. berghei* ANKA expressing a fusion protein of green fluorescent protein and OVA (PbA-gfpOVA) [7]; the fusion protein is constitutively expressed throughout the life cycle of the parasite and is driven by the hsp70 promoter of *P. berghei*. The five N-terminal amino acids are derived from hsp70, and this fusion protein is expressed in the cytoplasm of the parasite.

The T-cell receptor transgenic mice specific for OVA (OT-I) and DsRed transgenic mice were from Jackson Laboratories (Bar Harbor, ME, USA), and the C57BL/6 mice were from SLC (Shizuoka, Japan).

3.2. Reagents

We used the following reagents: isoflurane and 3 M™ Vetbond™ tissue adhesive (3 M, Maplewood, MN, USA).

3.3. Equipment

The set-up and surgery required the following pieces of equipment: electric hair clipper (BRAUN, model EP-25, P & G, Cincinnati, OH, USA), microsurgery scissors, coverslip (glass micro coverslip 50 × 40 mm, 0.12-mm thick, Matsunami, Osaka, Japan), gauze sponge (Unfolded, 25 × 25 cm), gauze folded two times in package (12.5 × 12.5 cm) (Hize Gauze NT-4, Tokyo, Japan), electric cautery (Bovie® Medical, USA), inhalant small-animal anesthesia system (MK-A110 Muromachi kikai Co., Ltd., Japan), and a two-photon microscope TCS SP5 TPM OPO (inverted type) (Leica Microsystems, Wetzlar, Germany) with Imaris software (Bitplane, Zurich, Switzerland).

3.4. Ethical approval

The experiments on animals were approved by the Institutional Animal Care and Use Committee of Nagasaki University and were conducted according to the guidelines for Animal Experimentation at Nagasaki University.

3.5. Protocol

3.5.1. Transfer of $CD8^+$ T cells and infection

First, we crossed OT-I mice with DsRed transgenic mice to prepare $DsRed^+$ OT-I cells. However, purified $CD8^+$ T cells with fluorescent dye can be labeled as described previously [5] if the cells do not proliferate too much *in vivo*. Next, we activated OT-I cells *in vitro*, as described previously [7], and intravenously transferred them to C57BL/6 mice *via* the tail vein. Two days after transferring the OT-I cells, mice were infected intravenously with sporozoites isolated from the salivary glands of *Anopheles stephensi*, which were infected with PbA-gfpOVA for approximately 20 days. The protocol for preparing sporozoites has been published previously [14]. We then performed intravital imaging of the liver approximately 44–48 h after sporozoite infection (Fig. 1). However, imaging can be performed at an earlier time point depending on the purpose of the imaging. Below, we describe our improved surgical procedures for preparing the liver for imaging studies using TPM.

3.5.2. Mouse preparation prior to imaging: exposing the liver

First, we induced the anesthesia. Some studies use an injectable cocktail of Ketamine® and Xylazine® for anesthesia [2,13,14]. However, we used isoflurane inhalant anesthesia because it allows better control of the depth of anesthesia. To use this method, place the mouse in an induction chamber that has been pre-filled with the anesthetic vapor (2% isoflurane by volume) *via* the vaporizer anesthesia system fresh gas delivery supply, and induce anesthesia. After 5 min, when a sufficient level of anesthesia has been reached, transfer the mouse from the induction chamber to the surgery stage and keep the mouse under anesthesia by approximating the muzzle to a small facemask that delivers the isoflurane/oxygen anesthetic to the animal. In this stage, avoid the agonal respiration that is typically caused by an overdose of isoflurane, as agonal breathing will cause severe chest excursion, which will induce movement in the target organ, the liver, thus distorting the images. After transferring the mouse to the surgery stage, we recommend using the same 2% concentration of isoflurane with approximately 500 cm^3/min flow of oxygen through the vaporizer. After 5 min, decrease the concentration of isoflurane to 1%. Our study demonstrates that keeping the concentration of isoflurane at 1% during the surgery and experiment will significantly reduce the risk of agonal breathing. If agonal breathing occurs, it will usually disappear a few minutes after reducing the isoflurane dosage. Next, shave the abdomen of the mouse and some parts of the thorax carefully with electric clippers to avoid injuries (skin irritation and sores). After shaving, remove the hair with gauze soaked in 70% ethanol. It is important to remove all of the hair to preclude evidence of the hair in the final image.

Once the animal is shaved, pull the skin up and then cut a 1-cm flap of skin with a pair of microsurgery iris scissors from the xiphoid to the left side of the mouse below the last rib (Fig. 1B). This results in an oval-shaped area below the last rib, wherein the left lobe of the liver is partially exposed (Fig. 1C). In this step, the liver can be observed under the muscle layer. Very carefully, pull up the muscle layer under the xiphoid and make an incision with a pair of microsurgery scissors (Fig. 1D). Extend the muscle to the left side of the mouse (Fig. 1E). It is crucial to avoid contacting the liver's surface with the scissor tips, as this could lead to hemorrhaging. Afterward, immediately cauterize the entire upper and lower edge of the incision with an electric cautery device (Fig. 1F). Any delay in cauterizing may lead to an accumulation of blood and blood clotting that could produce an unwanted layer between the glass coverslip and liver surface. Very carefully, using a blunt instrument, expose the tip of the left lobe of the liver. Then, using a small 3 × 3 cm piece of single-folded gauze sponge, touch the gauze to the tip of the liver lobe (Fig. 1G). Apply a small drop of Vetbond™ adhesive to the contact point between the tip of the liver lobe and the piece of gauze. The tip of the liver lobe will now be attached to the gauze (Fig. 1H). Check under the contact point to be sure no other tissue is connected to the gauze. Very carefully retract the gauze and the attached liver lobe until the entire left lobe is safely exposed. While the liver lobe is retracted, apply a drop of Vetbond™ adhesive to the skin under the retracted liver lobe (Fig. 1I). Then, carefully place the left lobe of the liver on the skin (Fig. 1J). After a few seconds, the left lobe of the liver will be fixed to the skin. Additional drops of Vetbond™

Please cite this article as: M. Akbari, et al., Intravital imaging of the immune responses during liver-stage malaria infection: An improved approach for fixing the liver, Parasitology International (2016), http://dx.doi.org/10.1016/j.parint.2016.02.011