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Intravital microscopy technique to study parasite dynamics in the labyrinth layer of the mouse placenta

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ABSTRACT

Intravital imaging techniques are the best approach to investigate in situ cellular behavior under physiological conditions. Many techniques have emerged during these last few years for this purpose. We recently described an intravital imaging technique that allows for the observation of placenta physiological responses at the labyrinth layer of this tissue. This technique will be very useful to study many placental opportunistic infections and in this article we reinforce its usefulness by analyzing placental physiological entrapment of beads and parasites. In particular, our results show that small beads (1.0 μm) or *Plasmodium chabaudi*-GFP-infected-Red Blood Cells (Pc-GFP-iRBCs) cannot get trapped inside small or large blood vessels of popliteal lymph nodes (PLNs). Inside the placenta, clusters of beads could only be found inside the maternal blood vessels. However, Pc-GFP-iRBCs were found inside and outside the maternal blood vessels. We observed that trophoblasts can ingest infected-Red Blood Cells (iRBCs) in vitro and immunofluorescence of placenta revealed Pc-GFP-iRBCs inside and outside the maternal blood vessels. Taken together, we conclude that fast deposition of particles inside blood vessels seems to be an intrinsic characteristic of placenta blood flow, but iRBCs could be internalized by trophoblast cells. Thus these results represent one of the many possible uses of our intravital imaging technique to address important questions inside the parasitological field.

1. Introduction

Intravital imaging microscopy has contributed to a deeper understanding of the dynamics of physiological processes that occur within organs. Together with two-photon microscopy technology, which allows deep tissue excitation of fluorescent molecules, intravital imaging has become a powerful tool in a broad set of scientific areas [1,2].

We recently have published an intravital imaging technique that provides the observation of blood flow dynamics in mouse placenta, specifically in the labyrinth zone [3]. This technique has been used to characterize the dynamics of *Plasmodium berghei*-infected Red Blood Cell (Pb-iRBCs) inside the placentas of infected BALB/c mice [4]. By adapting our broadly applicable technique, we show in this article how we have been able to address other relevant questions associated with parasites that infect this tissue.

Infections with *Salmonella enterica* serovar *Typhimurium* [5], *Listeria monocytogenes* [6], *Fusobacterium nucleatum* [7], *P. chabaudi* [8], *P. berghei* [9], and *Toxoplasma gondii* [10] can compromise placenta physiology. These placental infections share some common characteristics but are distinct in others. In *S. enterica*, a virulent strain reaches the labyrinth zone, invades trophoblasts, and causes abortion [5]. Ineffective immune responses against *L. monocytogenes* occur in the labyrinth zone because very few inflammatory cells reach this area until late in the course of infection [11]. Maternal *F. nucleatum* infections, unlike *Listeria* sp. infections, generate a transient systemic colonization, with bacteria entering the labyrinth zone only after decidual ischemic necrosis [7]. This order of events raises questions about the labyrinth bacterial invasion and decidual pathology. In experimental malaria infections, the parasite may enter and accumulate inside trophoblast cells, thereby causing abortion [8,9], however it neither crosses the placenta nor infects the embryos. In vitro *T. gondii* trophoblast cell infections lead to the apoptosis of uninfected trophoblasts [12], but nothing is known about the nature of such trophoblast infections or the resulting apoptosis. Therefore, observations of parasite–tissue cell interactions in the labyrinth zone can provide new clues about the pathology of these infections.

In this work, we further demonstrate how this technique can be used in experimental parasitological studies. We intravenously injected freshly isolated *P. chabaudi* AJ-GFP [13] infected-Red Blood

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Cells (Pc-GFP-iRBCs) or fluorescent beads into pregnant mice and compared parasite vs. bead dynamics inside the placenta. We also observed these parasite/bead dynamics inside popliteal lymph nodes (PLNs) to compare them with placenta data. Our results showed that placenta accumulation of clusters of beads/oversized beads occurred inside the maternal blood vessels, while no parasite or almost no clusters of bead/oversized bead accumulations occurred inside the PLNs. However, Pc-GFP-iRBCs could be found outside maternal blood vessels, indicating that cells of fetal origin could be internalizing these infected cells. In fact, trophoblast cell line SM9-1 can ingest Pc-GFP-iRBCs, indicating that trophoblasts could be the in vivo candidates to phagocytose infected cells. Finally, by using immunofluorescence, we identified cytokeratin-positive cells as the cells capturing parasites inside the placenta, indicating that trophoblast-like cells are involved in this process. These results, together with other recently published data [4], clearly demonstrate the potential of our placenta intravital imaging technique to address important questions about the blood dynamics inside this tissue after infections, which are difficult to obtain with previously studied techniques.

2. Materials and methods

2.1. Mice, P. chabaudi parasites, and bead injection

Eight week old C57Bl/6 (B6) female mice were bred with isogenic males, and 5×10^6 *P. chabaudi*-GPF-infected Red Blood Cells (Pc-GFP-iRBC) or *P. berghei*-GFP-infected Red Blood Cells (Pb-GFP-iRBCs) were intravenously injected between days 16 and 18 of pregnancy. *P. chabaudi*-GFP parasites were described elsewhere [13]. For comparison, 10^7 fluorescent beads (Cat. # F8821, Invitrogen Inc., USA) were injected in a second group of pregnant females at the same gestational days. Images of placentas were acquired immediately after injections.

We also injected beads or Pc-GFP-iRBCs (at the same concentrations described above) in non-pregnant females and acquired blood flow images of PLN to compare with placenta bead or parasite results. Pc-GFP-iRBCs were harvested from infected mice during a period of the early evening/night, when the parasites were in the trophozoite/schizont phase of the erythrocytic cycle. Contrary to *P. chabaudi*, *P. berghei* parasites are asynchronous, where all forms of the erythrocytic cycle can be found, independently of the period of the day.

Rhodamine B isothiocyanate-Dextran of 70,000 KDa or FITC-Dextran of 2,000,000 KDa (both from Sigma-Aldrich, Inc., stock at 20 mg/ml) were used to allow blood vessel identifications.

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and they were in agreement with the Federation of European Laboratory Animal Science Associations (FELASA) directives, approval ID number AO10/2010.

2.2. Intravital imaging of placentas

This technique has been previously described [3]. Briefly, after anesthesia, one of the uterine horns was carefully exposed, the uterine wall and amniotic sac were cut, and the placentas were exposed, keeping the fetal umbilical cords intact. PBS-soaked cotton was placed over the fetuses to avoid dehydration. The whole animal was placed in our organ stage holder, and 2% low-melting agarose (NuSieve GTG agarose, Lonza Inc.) was placed on top of the preparation. The whole system was closed by a top heater with a hole in the middle where images were acquired. Finally, we transferred the final assembly to the microscope.

2.3. Two-photon microscopy of placentas

The details of our two-photon microscope setup used to image placentas were previously described [3]. In brief, we used 2 of our 4 upper PMTs, one containing a 495 dichroic mirror with a 525/50 nm filter (for Pc-GFP-iRBC or FITC-Dextran signals), and the other containing a 640 dichroic mirror with a 595/50 nm filter (for Dextran-Rhodamine B or bead signals). The combination of the detected wavelengths depends upon the different experimental setups and is specified in the figure legends. Sequential images of a 50 μm-depth tissue volume, divided into 4.0 μm z-steps constituting the 5D (x, y, z, t, and color), were acquired to allow observation of Pc-GFP-iRBCs, Pb-GFP-iRBCs, or beads inside placental blood vessels. Each acquisition volume required approximately 30 s to be scanned by the laser microscope. We used Fiji imaging analysis software (General public license, NIH, USA) to reconstruct our videos. Further details of popliteal lymph node intravital imaging acquisition was described elsewhere [14].

2.4. Image sequence stabilization of endogenous motion

Due to endogenous motion, such as that caused by intestinal peristaltic movement, these intravital image sequences require software based post-processing stabilization in order to identify the flow of beads or parasites inside the blood vessels. Since this is a critical technical step required for obtaining viable videos of the biological processes of interest, we developed a custom software application that stabilizes the full 3D image sequence over time. This software has been made available under a GNU open source license, and can be downloaded from a public repository at http://sourceforge.net/projects/stabitissue/.

As described, two-photon microscopy produces full 3D image stacks of the tissue. For our studies, the distance between images was 4 μm, the number of stacks in the z-direction was approximately 15, and the time between each image acquisition was approximately 3 s. Thus, between subsequent time-points of the image sequence, between 30 to 45 s transpires. Moreover, during such long time scales, significant endogenous movement in this tissue may arise (contributions coming from intestinal peristaltic motion amongst other organ motion) that greatly influence the quality of the acquired image sequences. Since adequate levels of stabilization, in all three directions (x-y and z-planes), are difficult to achieve with the experimental procedure alone, post-processing can greatly improve the qualitative and quantitative results obtained from the image data.

Thus, our post-processing software tool simultaneously stabilizes the image sequence in the x-, y-, and z-planes over in consecutive image planes over time. In particular, our algorithm uses a multi-parameter objective function for selecting the optimal linear displacement (Δx, Δy, Δz) between consecutive pairs of 3D image stacks in time. The optimal configuration between each pair is based upon a maximal pixel overlap (or correlation) between 3D stack pairs, at consecutive times t_k and t_{k+1}. Simply stated, image drift over time in the x, y, and z planes, with respect to some fixed anatomical feature, is minimized in order to achieve a better movie quality.

2.5. Immunofluorescence identification of cytokeratin-positive cells

To evaluate whether trophoblasts could internalize Pc-GFP-iRBCs, we incubated SM9-1 cells [15] with these infected cells, or harvested/frozen placentas used in our intravital image acquisitions.

To address the in vitro capacity of Pc-GFP-iRBC ingestion by SM9-1 cells, 10^5 of these trophoblast cells were cultured on chamber slides for 24 h and incubated for 6 h with beads or infected red blood cells (iRBCs). After this period of incubation, these slides were fixed in acetone, dried and incubated with a blocking solution (3% BSA in PBS) for 30 min at room temperature. The primary anti-human pan-cytokeratin antibody (SC-15367, Santa Cruz Biotechnology, USA) was used at a dilution of 1:200 and incubated with the specimens for 18 h at 4 °C in a humidified chamber. Each slide was washed, and then incubated with Cy3-conjugated secondary antibodies (A-10520, Invitrogen, USA) for 40 min at room temperature within a humidified chamber. All