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In vivo imaging of pathogen homing to the host tissues

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ABSTRACT

Hematogenous dissemination followed by tissue tropism is a characteristic of the infectious process of many pathogens including those transmitted by blood-feeding vectors. After entering into the blood circulation, these pathogens must arrest in the target organ before they infect a specific tissue. Here, we describe a non-invasive method to visualize and quantify the homing of pathogens to the host tissues. By using *in vivo* bioluminescence imaging we quantify the accumulation of luciferase-expressing parasites in the host organs during the first minutes following their intravascular inoculation in mice. Using this technique we show that in the malarial infection, once in the blood circulation, most of bioluminescent *Plasmodium berghei* sporozoites, the parasite stage transmitted to the host skin by a mosquito bite, rapidly home to the liver where they invade and develop inside hepatocytes. This homing is specific to this developmental stage since blood stage parasites do not accumulate in the liver, as well as extracellular *Trypanosoma brucei* bloodstream forms and liver-infecting *Leishmania infantum* amastigotes. Finally, this method can be used to study the dynamics of tissue tropism of parasites, dissect the molecular and cellular basis of their increased arrest in organs and to evaluate immune interventions designed to block this targeted interaction.

1. Introduction

The use of the host circulatory system to disseminate and infect specific tissues is a recurrent characteristic observed in the lifecycle of innumerous viruses, bacteria and parasites. This tissue tropism is frequently associated with the mode of transmission of these microorganisms, as observed for pathogens that invade salivary glands of arthropod vectors and are transmitted into the host skin with the vector saliva during an infectious bite [1,2]. Tissue tropism is also associated with the pathology caused by these microorganisms as known, for example, for the hepatitis or encephalitis-causing viruses [3]. In some cases this targeted infection can also be asymptomatic as observed for *Plasmodium* sporozoites, the infective malaria parasite stage inoculated in the skin during a mosquito bite. Following deposition in the skin tissue, sporozoites invade blood vessels [4,5] and enter into the blood circulation to specifically and transiently infect the liver [6–8].

Since mammals have a closed circulatory system, the use of the blood circulation to reach specific tissues requires the arrest of the pathogen in the target organ and the ensuing crossing of the endothelial barrier, which physically separates the blood compartment from the organ parenchyma. Some organs, such as the liver, spleen and adrenal glands, are directly exposed to blood-borne pathogens due to the presence of fenestrated vessels, allowing a direct extravasation of small pathogens (e.g., fenestra diameter of hepatic sinusoids:~100 nm [9]), or a local open circulation. Consequently, the arrest of pathogens can be expected to occur through their binding to molecules on the surface of either tissue-specific endothelial cells or resident immune cells, or to molecules of extravascular cells exposed to the blood circulation in the case of fenestrated sinusoids and in the open splenic circulation.

The dissection of the mechanisms used by pathogens to specifically infect a tissue is hampered by the lack of an easy and quantitative method to assess this first step of arrest in the host organs following blood dissemination. The localization and quantification of pathogens in tissues usually requires the dissection of organs

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and the measurement of pathogen load in the excised samples by PCR, radioactive labeling, subinoculation or *in vitro* culture. On the other hand, bioluminescence imaging can reveal the distribution of living luciferase-expressing cells in a non-invasive and quantitative manner in the whole body of small hosts, such as mice. This technique, which is based on an enzymatic reaction that generates photons following the oxidation of a substrate, has been frequently applied to study *Plasmodium* behaviour [10] in the blood [11,12], liver [13–15] and skin of rodents [8,16]. The firefly luciferase has been preferentially used for *in vivo* studies when compared to the NanoLuc and Gaussia luciferase, which despite emitting more light *in vitro*, seem to be less efficient *in vivo* [17]. This is in part due to the higher absorption and scatter by the host tissues of the blue light emitted by their substrates [18]. Accordingly, increased *in vivo* sensitivity has been successfully achieved by the red-shifted variant of firefly luciferase [19,20]. Here, using bioluminescence imaging we demonstrate the specific homing of firefly luciferase-expressing *Plasmodium berghei* sporozoites to the liver following the first minutes of their hematogenous dissemination.

2. Material and methods

2.1. Mice

All experiments were carried out in accordance with the IBMC. INEB Animal Ethics Committees and the Portuguese National Authorities for Animal Health guidelines or in accordance with the Animal Care and Use Committee of Institut Pasteur both according to the statements on the directive 2010/63/EU of the European Parliament and Council. Four to six weeks old C57BL/6, Swiss, NMRI or BALB/c mice were purchased from Elevage Janvier or Charles River.

2.2. Parasites

Throughout the experiments parasites used include *Plasmodium berghei* ANKA strain clone 676cl1 expressing a GFP-Luciferase fusion gene via the pbef1 α promoter [21], *Trypanosoma brucei brucei* Lister 427 expressing the redshifted luciferase gene (PpyRE9h) flanked by 5′VSG/3′tubulin [20,22], a cloned line of *Leishmania infantum* (MHOM/MA/67/ITMAP-263) expressing luciferase under the control of the intergenic region of α-tubulin [23].

To obtain *P. berghei* (Pb) sporozoites, female *Anopheles stephensi* mosquitoes (SDA 500 strain) reared in the Center for the Production and Infection of Anopheles at the Pasteur Institute were infected 3–4 days after emergence and kept as previously described [24]. Sporozoites were collected from salivary glands 21–28 days after the infectious blood meal and kept on ice in PBS until inoculated into mice. Merozoites were obtained by culturing infected mouse blood in RPMI 1640 (Lonza) supplemented with 20% heat inactivated fetal bovine serum (Biowest) and 50 μg/ml neomycin (Sigma) and flushed 90 s at 1.5–2 bar pressure with a gas mixture of 5% CO_2, 5% O_2 and 90% N_2 for 16 h at 37 °C and under shaking. Mature schizonts were separated using a Nycodenz (Axis Shield) gradient and merozoites were isolated by filtration of schizonts through a 1.2 μm filter [25].

The bloodstream forms of *T. b. brucei* (Tb) Lister 427 were grown in HMI-9 medium (Sigma) supplemented with 10% heat inactivated fetal bovine serum (Biowest) and 100 IU/ml of penicillin/streptomycin (Lonza) at 37 °C, 5% CO_2 in a humidified atmosphere [26].

L. infantum (Li) axenic amastigotes were cultured at 37 °C, and 5% CO_2 in a cell-free medium called MAA/20 (medium for axenic amastigotes growth) as described previously [27]. Briefly, MAA/20 consisted of modified medium 199 (Invitrogen) with Hank's Balanced Salt Solution supplemented with 0.5% tryptocasein (Oxoid), 15 mM D-glucose (Sigma), 5 mM glutamine (Lonza), 4 mM $NaHCO_3$, 0.023 mM bovine hemin (Fluka) and 25 mM HEPES (Lonza) to a final pH of 6.5 and supplemented with 20% heat inactivated fetal bovine serum (Biowest).

2.3. Bioluminescence imaging

Parasite loads of mice infected with luciferase-expressing parasites were assessed by bioluminescence imaging using the IVIS Lumina LT system (Perkin Elmer). Prior to infection, animals had their ventral fur shaved with an appropriate clipper. In all the models, mice were infected following the intravenous injection into the tail vein of 100 μl of parasite suspension.

For the experiments with Pb, C57BL/6 mice were typically infected with either $\sim 1.5 \times 10^5$ salivary gland sporozoites or ~ 2 to 6×10^7 blood merozoites. Infections with Tb bloodstream forms or with Li axenic amastigotes were performed in BALB/c mice following the inoculation of respectively 1×10^6 or 1 to 2×10^8 parasites. Immediately after parasite inoculation, mice were anesthetized with 2.5% isoflurane and four minutes after the infection, injected subcutaneously with D-luciferin (2.4 mg, Perkin Elmer). Mice were then transferred to the stage of an intensified charge-coupled device photon-counting video camera box where anaesthesia was maintained with 2.5% isoflurane. After a three minute incubation allowing the distribution of the substrate in the body of the anesthetized animals, a five minute signal acquisition controlled by the Living Image software (Perkin Elmer) was initiated. At the end of this period animals returned to their cage and recovered from the anaesthesia. Measurement of infection by bioluminescence one day later was performed as described above.

The detection of the bioluminescence signals by the system resulted in the generation of signal maps automatically superimposed to the grey-scale photograph of the mice. The quantifications were performed using the Living Image software (Perkin Elmer). Bioluminescent signals on the regions of interest (ROI) encompassing most of the ventral view of the animal body, thorax, liver, spleen and lower abdomen were manually defined as described below and applied to all animals. The total flux (photons/s) and average radiance (photons/s/cm^2/steradian) within these ROIs was automatically calculated. ROI background signal was obtained from imaging non-infected mice that received D-luciferin as described above. The total flux or the average radiance background signal of the respective ROIs were subtracted to the respective total flux or average radiance measured on infected animals. The percentage of bioluminescence in the defined ROIs (thorax, liver, spleen and lower abdomen) was calculated by dividing the total flux of the respective ROI by the total flux of the ventral animal body ROI.

2.4. Statistical analysis

All the statistical analysis and graphical representation of the data were performed using *GraphPad Prism* 6 software. *Students t*-test or a two-way analysis of variance (ANOVA) with Sidak's multiple comparisons test was performed and statistical significance was asserted whenever the value of p was lower than 0.05 by $^{*}(p < 0.05)$, $^{**}(p < 0.01)$, $^{***}(p < 0.001)$ and $^{****}(p < 0.0001)$.

3. Results and discussion

In this study we applied 2D bioluminescence imaging to investigate the accumulation of blood-circulating Pb bioluminescent sporozoites in the liver of living mice during the first minutes fol-