

Video Article

# Myeloid Cell Isolation from Mouse Skin and Draining Lymph Node Following Intradermal Immunization with Live Attenuated *Plasmodium* Sporozoites

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## Abstract

Malaria infection begins when the sporozoite stage of *Plasmodium* is inoculated into the skin of a mammalian host through a mosquito bite. The highly motile parasite not only reaches the liver to invade hepatocytes and transform into erythrocyte-infective form. It also migrates into the skin and to the proximal lymph node draining the injection site, where it can be recognized and degraded by resident and/or recruited myeloid cells. Intravital imaging reported the early recruitment of brightly fluorescent Lys-GFP positive leukocytes in the skin and the interactions between sporozoites and CD11c<sup>+</sup> cells in the draining lymph node. We present here an efficient procedure to recover, identify and enumerate the myeloid cell subsets that are recruited to the mouse skin and draining lymph node following intradermal injection of immunizing doses of sporozoites in a murine model. Phenotypic characterization using multi-parametric flow cytometry provides a reliable assay to assess early dynamic cellular changes during inflammatory response to *Plasmodium* infection.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/53796/>

## Introduction

Malaria is one of the deadliest infectious diseases in the world, killing more than half a million people per year. Infection by *Plasmodium*, the causal agent of the disease, begins by a pre-erythrocytic (PE) phase. During this phase, sporozoites injected into the host skin by a female Anopheline mosquito reach the liver via the bloodstream and differentiate inside hepatocytes into the parasite forms that infect red blood cells and cause the symptoms of the disease.

The PE stages of *Plasmodium* represent a privileged target for anti-malaria vaccination. Indeed, live attenuated vaccines against these stages, such as radiation attenuated sporozoites (RAS), genetically arrested parasites (GAP) or chemoprophylaxis and sporozoites (CPS) have demonstrated their capacity to protect both rodent and human hosts<sup>1-9</sup>. In the rodent model, most vaccination studies are conducted utilizing intravenous immunization, which is the gold standard in terms of protective efficacy. However, the description of a skin stage and the importance of the skin-associated draining lymph node (dLN) in eliciting protection has changed our perception of the PE phase and emphasized the importance of the intradermal route of injection. Intravital imaging of *P. berghei* sporozoites injected into the skin of rodents has shown that only ~25% of the inoculum reaches the liver via the bloodstream. The remaining ~75% distributes between the proximal dLN (~15%) and the skin (~50%)<sup>10,11</sup>, where a small proportion can transform and remain alive for weeks inside skin cells<sup>12,13</sup>. Moreover, subsequent studies described that the establishment of effective protective immunity after intradermal immunization mainly takes place in the skin-dLN, where parasite specific CD8<sup>+</sup> T cells are activated, and only marginally in the spleen or liver-dLNs<sup>14,15</sup>.

While most studies have concentrated on the characterization of the effector cells implicated in the establishment of protective immune response, much less is known about the fate of live attenuated parasites injected into the skin, especially their interactions with the innate immune system. In particular, characterization of antigen-presenting cells involved in parasite antigen uptake, processing and presentation to CD8<sup>+</sup> T cells is of critical importance, knowing that PE antigen acquisition can occur both in the skin and dLN compartments. Previous intravital imaging studies described an early influx of brightly fluorescent Lys-GFP positive cells in the skin following an infectious mosquito bite<sup>16</sup> while early interactions between sporozoites and dendritic cells were observed in the dLN<sup>10,17</sup>. More recently, it has been reported that sporozoites inoculated in the skin by mosquitoes increases the motility of both dendritic and regulatory T cells in the skin of mice, while a decrease number of antigen presenting cells was observed in the dLN<sup>18</sup>.

We aimed to identify and quantify more precisely the leukocyte subsets recruited in the skin and corresponding dLN as well as those interacting with the parasite following intradermal injection of immunizing doses of RAS<sup>19</sup>. In this context, we isolated myeloid cells (CD45<sup>+</sup>CD11b<sup>+</sup>) from both tissues and characterized subpopulations of interest by multi-parametric flow cytometry. Consistent with the immune response described in the early stage of *Leishmania major* skin infection<sup>20</sup>, the primary host response to sporozoite injection consists of a successive recruitment

of polymorphonuclear neutrophils (CD45<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>int</sup>) followed by inflammatory monocytes (CD45<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>-</sup>Ly6C<sup>+</sup>) which are identified on the basis of differential expression of the Ly6G and Ly6C surface markers.

We describe here a protocol for isolating myeloid cells from the mouse skin and dLN following intradermal injection of immunizing doses of RAS extracted from infected mosquito salivary glands. Reproducible intradermal injections and tissue processing are critical steps to quantify phenotypic changes of infiltrating cell population within infected tissues. The approach detailed below provides a reliable assay to assess the skin and dLN inflammatory response to *Plasmodium* parasite and can be extended to various experimental systems.

## Protocol

All procedures were approved by the committee of the Pasteur Institute and by the local Ethics Committee on Animal Experimentation (Ethical committee IDF-Paris 1, Paris, France; agreement number: 2012-0015) and performed in accordance with the applicable guidelines and regulations.

### 1. Materials and Reagents

1. Use female *Anopheles stephensi* mosquitoes (Sda500 strain) that feed on infected mice 3-5 days after emergence and rear as described previously<sup>21</sup>.
2. Use parasites *Plasmodium berghei* ANKA clone expressing the Green Fluorescent Protein (GFP) gene under the control of the constitutive Heat Shock Protein 70 (HSP70) promoter. This yields bright fluorescence throughout the parasite life cycle<sup>22</sup>.
3. Use female C57BL/6JRj mice (7 week-old).

NOTE: All the reagents used in the described protocol are listed in the **Table of Materials/Equipment**.

### 2. Radiation-attenuated Sporozoite Isolation from Mosquito Salivary Glands

1. Between 18-25 days after the infectious blood meal, collect and cold-anesthetize mosquitoes into a 15 ml tube on ice as described previously<sup>21</sup>. Carefully transfer them onto a Petri dish by inverting the tube. Maintain the insects on ice and expose the mosquitoes to a 12 krad dose of  $\gamma$ - or x-irradiation.
2. Isolate attenuated sporozoites from irradiated mosquito salivary glands as previously described<sup>21</sup>. Collect infected salivary glands in a small volume (around 15  $\mu$ l) of 1x Dulbecco's phosphate-buffered saline (DPBS) on ice and gently crush them to release sporozoites.  
NOTE: The injection of a highly concentrated parasite suspension in a small volume being critical, it is therefore essential to harvest a sufficient number of salivary glands from a high number of preselected well-infected mosquitoes, as evidenced by their robust expression of green-fluorescence.
3. Filter the sporozoite suspension through a 35  $\mu$ m cell strainer cap adapted to a low adhesion 1.5 ml microcentrifuge tube in order to eliminate clumps and mosquito debris.
4. Count the total number of isolated sporozoites as previously described<sup>21</sup> and adjust the concentration of parasite suspension with cold 1x DPBS to obtain 83,000 sporozoites/ $\mu$ l. Store the parasites on ice while continuing the next steps.
5. Collect the equivalent number of salivary glands from uninfected mosquitoes that will be used as negative control and process the sample as described in steps 2.2 and 2.3. Dilute the sample as indicated above to obtain similar concentration of salivary gland extracts in the suspension.

NOTE: Sporozoites can be stored on ice for a maximum of 2 hr. However, ideally they are injected within an hour after crushing the salivary glands.

### 3. Injection of Sporozoites into the Dermal Layer of the Ear

1. Anesthetize animals by intraperitoneal injection of ketamine (50 mg/kg)/xylazine (5 mg/kg) mixture. Wait 5-8 min for the mouse to be in a state of unconsciousness and apply ophthalmic ointment to the eyes to prevent corneal drying. Evaluate the depth of anaesthesia by performing a gentle toe pinch on both rear feet.  
NOTE: The dose of anaesthesia lasts for less than 20 min, so the next steps must be done quickly.
2. Stabilize the ear pinna of the mouse by fixing the ventral side with clear tape previously placed under a stereomicroscope (**Figure 1A**). Gently press the ear to avoid damage of the vasculature.
3. Load a 10  $\mu$ l syringe/35 gauge bevelled needle with 0.6  $\mu$ l of resuspended parasites<sup>10</sup>.
4. Deliver the sporozoite suspension in 4 injection sites (0.15  $\mu$ l per site) by carefully inserting the needle on the dorsal side of the ear (**Figure 1A**), beneath the epidermis, with the bevel up, taking care to avoid any blood vessels. Allow the needle to remain in the dermis for few sec to prevent backflow of the injectant before removing it. Observe a characteristic papule at each injection site at the end of the procedure (**Figure 1B** and **1C**).
5. After injection, carefully remove the ear from the tape.
6. Inject the contralateral ear with the same dose of parasites by following the steps 3.2-3.4.
7. Inject 2 additional mice with either 0.6  $\mu$ l of 1x DPBS or 0.6  $\mu$ l of 1x DPBS + salivary gland extract from uninfected mosquitoes in order to evaluate the inflammatory response mediated by the injection alone and the deposition of mosquito material in the dermis.
8. Monitor mouse recovery from anaesthesia before placing it back into the cage.

NOTE: The proper sporozoite deposition in the skin can be monitored by fluorescence microscopy (**Figure 2A** and **2B**), the mouse being prepared as described previously<sup>23</sup>.

### 4. Skin and Draining Lymph Node Dissection

1. Prepare standard medium (SM) as follows: Dulbecco's Modified Eagle Medium (DMEM) + 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) supplemented with 400 U/ml collagenase and 50  $\mu$ g/ml deoxyribonuclease (DNase).

2. Prepare two 6 well flat-bottom tissue culture plates on ice with 4.5 ml SM + 70  $\mu$ m cell strainer per well for dLN samples (Plate A) and 4.5 ml SM per well for skin samples (Plate B).
3. At the desired time point following sporozoite inoculation, deeply anesthetize the mouse by intraperitoneal injection of ketamine (125 mg/kg)/ xylazine (12.5 mg/kg) mixture prior cervical dislocation. Make sure the mouse demonstrates signs of clinical death and start the dissection immediately.  
NOTE: The kinetics of myeloid cell recruitment can be examined at different times after injection (*i.e.* 2 hr, 4 hr and 24 hr as previously described<sup>14</sup>).
4. Disinfect the inoculated ear and the corresponding neck region with 70% ethanol to reduce the possibility of contamination.
5. Using sterile scissors and forceps, aseptically dissect the proximal auricular dLN without collecting the surrounding fat. Perform a first incision in the jugulo-carotidian sulcus and remove the hairs from the operating field. Stretch the incision with 2 forceps to expose the dLN that appears grayish compared to the surrounding tissue.
6. Pinch the connective tissues carefully on the top of the dLN with forceps to facilitate its removal. Extract the dLN by placing a forceps underneath the lymphoid tissue (**Figure 3A** and **3B**). Place the dLN in a well containing 4.5 ml SM + 70  $\mu$ m cell strainer (Plate A).
7. Harvest the entire inoculated ear using sterile sharp scissors and forceps (**Figure 4A**). Separate the dorsal and ventral aspects of the ear by pinching and spacing apart the cut ends with two forceps (**Figure 4B-4E**). Place them into a well containing 4.5 ml SM (Plate B) making sure both tissues are completely immersed in the medium.  
NOTE: In order to improve the reliability of the experiment and increase the number of cells of interest, pool 2 injected ears or 2 dLNs from the same mouse per sample.
8. Harvest and process in parallel the ears and dLNs of mice inoculated with either 1x DPBS or salivary gland extracts. Include 2 additional ear and lymph node samples harvested from a naive mouse for negative and single staining compensation controls.

## 5. Cell Isolation from Lymph Nodes

1. Incubate the lymph nodes (Plate A) at 37 °C + 5% CO<sub>2</sub> for 15 min under gentle agitation. Add 10 mM final Ethylenediaminetetraacetic acid (EDTA) per well to neutralize collagenase and DNase activities. Perform the next steps on ice.
2. Dissociate lymph nodes using the top end of a 5 ml syringe plunger. Press in circular motions against the strainer until all that remains is white connective tissues.
3. Rinse the cell strainer twice with 500  $\mu$ l 1x DPBS + 2% Fetal bovine serum (FBS) + 2mM EDTA. Transfer entire volume of the well in a 15 ml tube.
4. Rinse the well twice with 500  $\mu$ l 1x DPBS + 2% FBS + 2mM EDTA and transfer the volume into the 15 ml tube. Keep tubes on ice.

## 6. Cell Isolation from Skin of the Ear

1. Incubate the ear leaflets (Plate B) at 37 °C + 5% CO<sub>2</sub> for 1 hr under gentle agitation.  
NOTE: After 20 min of incubation, cut the ear leaflets with scissors into small pieces to facilitate tissue digestion and put the sample back in the incubator for the remaining 40 min. Single cells can be observed in the medium at the end of the incubation time.
2. Add 10 mM final EDTA per well to neutralize collagenase and DNase activities. Perform the next steps on ice.
3. Transfer the ear tissue fragments and the whole volume of medium in a new well containing a 70  $\mu$ m cell strainer using a 1ml pipette. Cut 2 to 3 mm from the end of the tip to collect the ear tissue fragments more easily.
4. Dissociate ear tissue fragments using the top end of a 5 ml syringe plunger. Press in circular motions against the strainer until all that remains is white connective tissues.
5. Rinse the cell strainers twice with 500  $\mu$ l 1x DPBS + 2% FBS + 2 mM EDTA.
6. Transfer the entire volume of the well into a 50 ml-tube through a 70  $\mu$ m cell strainer.
7. Rinse the well with 500  $\mu$ l 1x DPBS + 2% FBS + 2 mM EDTA and transfer the volume into the 50 ml tube. Keep the tube on ice.  
NOTE: The different steps to isolate total cells from the skin and dLN tissues are summarized in **Figure 5**.

## 7. Count and Viability of Collected Cells

1. Centrifuge the skin and dLN samples for 5 min at 450 x g at 4 °C and discard the supernatant. Gently resuspend the pellet with 300  $\mu$ l 1x DPBS + 2% FBS + 2 mM EDTA.
2. Collect a small volume of each sample to count the total number of cells isolated from the skin and dLN using a hemocytometer. Add 0.04% trypan blue to determine the cell viability.

## 8. Blocking of Non Antigen-specific Binding

1. Add 10  $\mu$ g/ml of unlabelled CD16/CD32 Fc-block antibody. Incubate 20 min at 4 °C (can go longer).
2. Add 2 ml of cold 1x DPBS + 2% FBS + 2 mM EDTA. Centrifuge the sample for 5 min at 450 x g at 4 °C and discard the supernatant. Gently resuspend the pellet with 300  $\mu$ l 1x DPBS + 2% FBS + 2 mM EDTA and maintain the samples on ice.

## 9. Staining Skin and Lymph Node Myeloid Cells

1. Incubate previously blocked skin and dLN isolated cells with Live/Dead tracker (*i.e.* 4',6-diamidino-2-phenylindole or DAPI), anti-CD45 and anti-CD11b specific antibodies.  
NOTES: In order to enrich the myeloid cell subpopulations of interest, especially rare ones, CD45<sup>+</sup> cells and lymphocytes can be respectively purified from the skin or depleted from the dLN by magnetic bead facilitating cell sorting. Since most of the cells isolated from the dLN are CD45<sup>+</sup>, the use of anti-CD45 is not mandatory. Other markers can be included to identify myeloid cell subpopulations of interest by positive

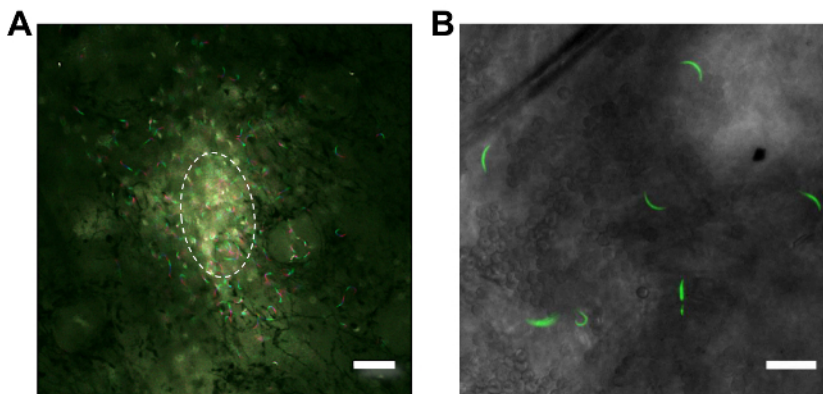
- or negative-gating strategy. In this protocol, the myeloid cells recruited in the dLN were enriched by excluding CD8 $\alpha$ <sup>+</sup> cells (the whole T cell compartment can be targeted by using anti-CD3 specific antibody).
2. Incubate 30 min at 4 °C in the dark. Add 500  $\mu$ l 1x DPBS + 2% FBS + 2 mM EDTA, centrifuge the sample 5 min at 450 x g at 4 °C and discard the supernatant. Repeat the wash step twice.
  3. Resuspend the samples with 300  $\mu$ l 1x DPBS + 2% FBS + 2 mM EDTA. Transfer the entire volume in a FACS tube through a 35  $\mu$ m cell strainer. Rinse the filter with 100  $\mu$ l 1x DPBS + 2% FBS + 2 mM EDTA.
  4. Run the stained cells in a flow cytometer. Gate live single cells (DAPI<sup>-</sup>,FSC-W) to exclude dead cells and doublets. Plot CD45<sup>+</sup>CD11b<sup>+</sup> events for the skin (**Figure 6A**) and (CD45<sup>+</sup>)CD8 $\alpha$ <sup>-</sup>CD11b<sup>+</sup> events for the dLN (**Figure 6B**).

## Representative Results

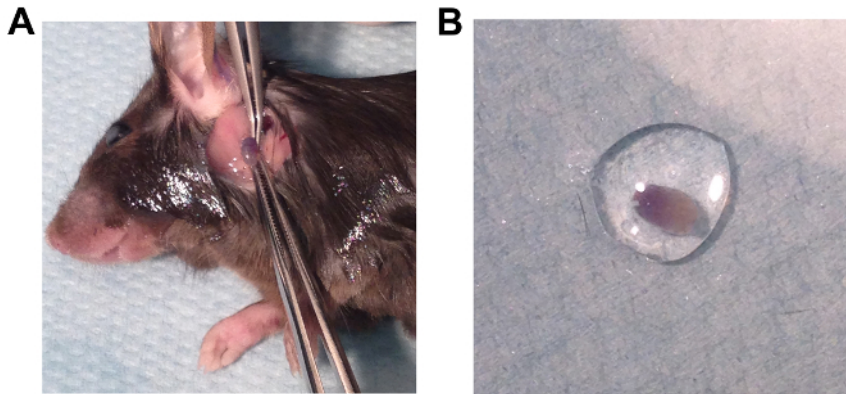
We recently demonstrated that needle-syringe injection of immunizing doses of *P. berghei* sporozoites in the mouse skin induces a successive recruitment of polymorphonuclear neutrophils followed by inflammatory monocytes in the skin and dLN<sup>19</sup>. The Protocol Section described above details the procedure used to successfully isolate live myeloid cells from both tissues following multiple injections of large number of sporozoites in the ear dermis (**Figures 1 and 2**). Within the first 24 hr, the dLN (**Figure 3**) and the skin injection site (**Figure 4**) were isolated and processed as summarized in **Figure 5** to analyse the cellular infiltration by multi-parametric flow cytometry. Overall, this technique allowed us to isolate an average 10<sup>4</sup> CD45<sup>+</sup>CD11b<sup>+</sup> and 2.10<sup>4</sup> CD11b<sup>+</sup> live single cells for the skin and dLN respectively. The acceptable viability of non-debris single cells ranged from 40-70% for the skin and 70-90% for the dLN. As shown in **Figure 6**, the frequency of myeloid cells in the skin (CD45<sup>+</sup>CD11b<sup>+</sup>) and the dLN (CD8 $\alpha$ <sup>-</sup>CD11b<sup>+</sup>) strongly increases following intradermal injection of RAS compared with non-infected mice.



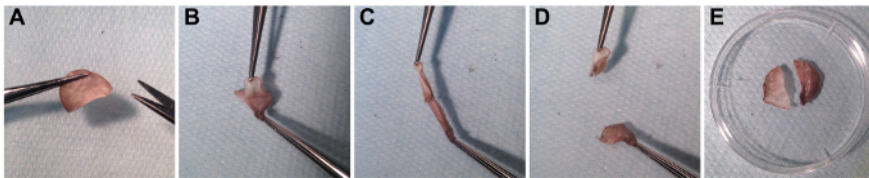
**Figure 1: Intradermal Injection of Sporozoites into the Ear of Mouse.** (A) Image showing intradermal injection of sporozoites in the ear pinna of an anesthetized mouse. The ventral side of the ear is stabilized with clear tape previously placed under a dissecting microscope. The needle is carefully inserted on the dorsal side of the ear, beneath the epidermis, with the bevel up. (B-C) Pictures showing the dorsal side of the ear pinna prior (B) and after (C) intradermal injection of 0.1-0.2  $\mu$ l of parasite suspension. A characteristic papule (black arrow) is observable at the injection site at the end of the operation. [Please click here to view a larger version of this figure.](#)



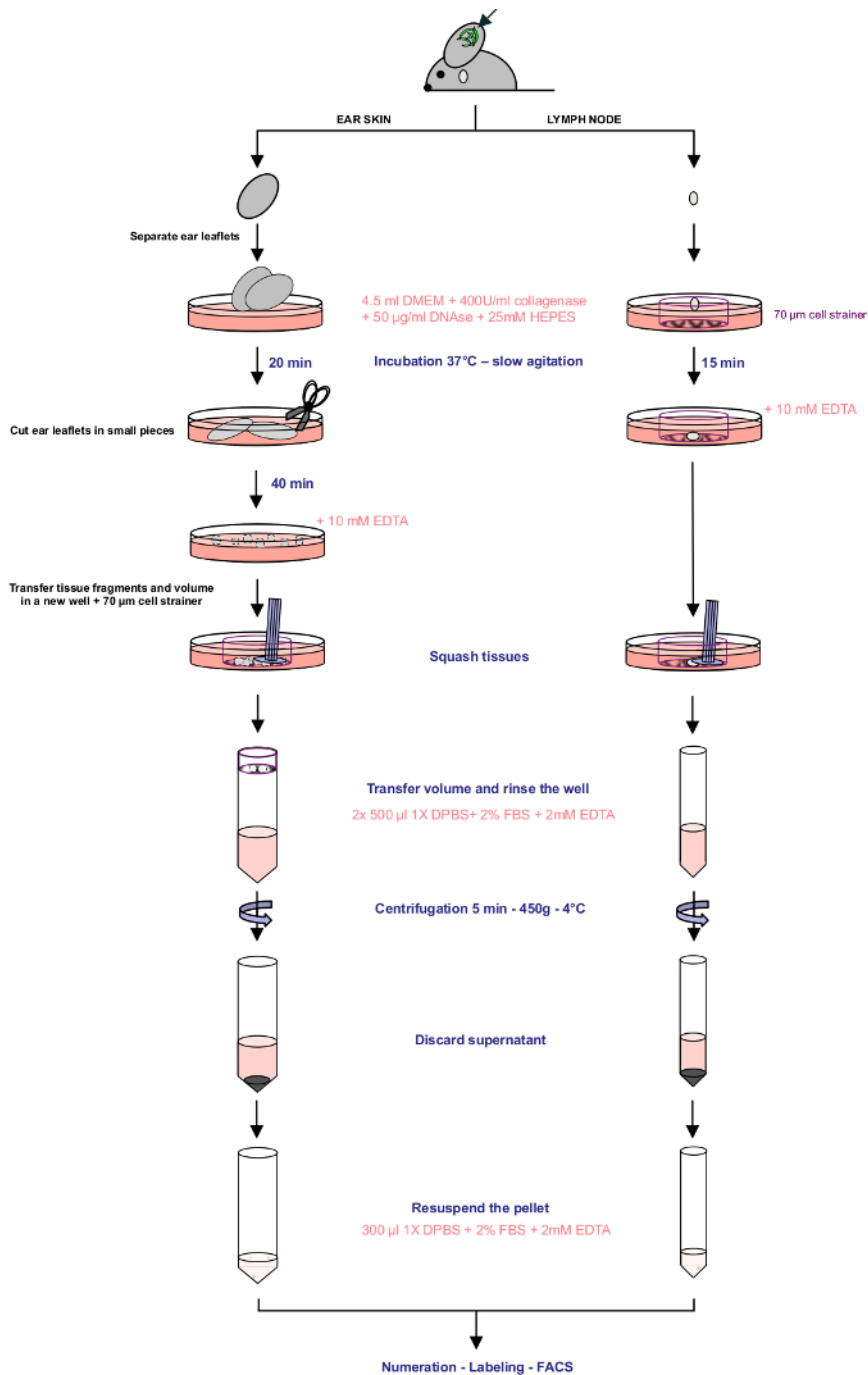
**Figure 2: Imaging of Sporozoites Deposited in the Ear Dermis of Mouse.** (A) Fluorescence microscopy showing RAS migrating from the injection site (indicated by the dashed lines) in the mouse skin 15 min post-injection (Scale bar = 60  $\mu$ m; 10X magnification). The path of sporozoites is represented by the maximum intensity projection of the fluorescent signal. Green and red fluorescence respectively show parasite position in the skin at the beginning and after 10 sec of acquisition. (B) Higher magnification of RAS (green) injected in the skin (Scale bar = 20  $\mu$ m; 25X magnification). [Please click here to view a larger version of this figure.](#)



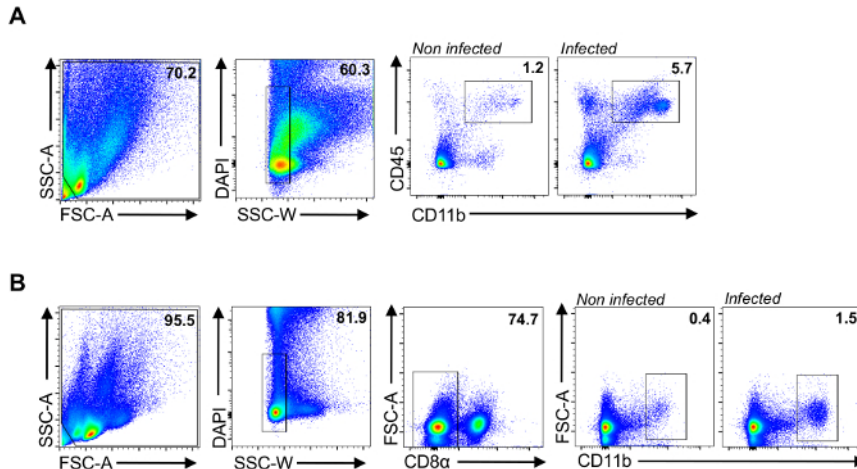
**Figure 3: Draining Lymph Node Processing.** (A) Picture showing the dissection of the proximal auricular dLN following intradermal injection of Evans blue for demonstration purposes. After cervical dislocation of the mouse, the neck is disinfected with 70% ethanol. A first incision is made in the jugular-carotid area with sharp scissors and the skin is removed from the operating field. The incision is stretched with 2 forceps to expose the dLN that appears grayish compared to the surrounding tissue. The connective tissues are then carefully pinched with forceps on the top of the dLN and progressively separated from it. Finally, the dLN is extracted by placing a forceps underneath the lymphoid tissue. (B) Picture showing the extracted dLN in a drop of PBS. [Please click here to view a larger version of this figure.](#)



**Figure 4: Ear Skin Processing.** Pictures showing the successive steps to process the inoculated ear. (A) After cervical dislocation of the mouse, the ear is disinfected with 70% ethanol and removed using sterile sharp scissors and forceps. (B-D) The dorsal and ventral aspects of the ear are lightly separated by pinching and spacing apart the cut ends with two forceps. (E) Picture showing the dorsal and ventral aspects of the ear prior enzymatic treatment. [Please click here to view a larger version of this figure.](#)



**Figure 5: Protocol Summary.** Schematic representation of the key steps to isolate total cells from the skin and dLN tissues. [Please click here to view a larger version of this figure.](#)



**Figure 6: Myeloid Cell Population Isolated From the Ear Skin and the Proximal dLN.** Representative FACS plots showing the gating strategy to analyse the myeloid compartment in the skin (A) and the dLN (B). CD45<sup>+</sup>CD11b<sup>+</sup> and CD8α<sup>+</sup>CD11b<sup>+</sup> cells were gated on total live singlet events for the skin and dLN respectively. For each condition, 5 x 10<sup>5</sup> total events were acquired (2 ears and 2 dLN pooled per sample). Please click here to view a larger version of this figure.

## Discussion

In the perspective of large-scale vaccination of humans using a whole sporozoite malaria vaccine, one of the main challenges to overcome is to develop optimized routes and methods of parasite administration to ensure successful immunization and protection<sup>24,25</sup>. In humans, the evaluation of protective efficacy mediated by live attenuated parasites (LAP) has been performed following natural mosquito bites<sup>2</sup>, as well as intradermal, subcutaneous<sup>25,26</sup> and IV immunizations<sup>27</sup>. As reported in rodents<sup>28</sup> and non-human primates<sup>25</sup>, IV administration of LAP induces stronger protective immune responses compared to the above-mentioned routes. Nevertheless, non-IV routes of administration using needle and syringe still remains more suitable for human mass vaccination and efforts are currently being made to optimize their protective efficacy.

Recent studies conducted in mice demonstrated, especially in the case of the ID route, that the deposition of a small volume of sporozoite suspension (range of 1 µl) in multiple injection sites (4 points) significantly increased parasite infectivity compared to a single inoculation of parasites diluted in larger volumes (50 µl)<sup>24</sup>. In our experimental system (C57BL/6 mouse - *P. berghei*), complete protection was achieved after multiple injections in the ear dermis of highly concentrated suspension of parasites (50,000 RAS in 0.6 µl, 4 injection sites)<sup>19</sup>. In this context, we established the protocol described above to analyze more precisely the local host immune response to immunizing doses of RAS that allowed us to identify 2 major myeloid cell subsets recruited in the skin and dLN in response to the parasite<sup>19</sup>.

Multi-parametric flow cytometry allows accurate identification and quantification of immune cell changes in the context of host response to infection<sup>19,20</sup>. Isolation of large number of viable cells is therefore critical to perform reproducible phenotypic analysis. For this purpose, the enzyme concentration and the duration of tissue digestion must be optimized. Indeed, low concentration of enzymes or insufficient incubation time may lead to incomplete digestion with low yield of cells while prolonged tissue digestion may result in decrease cell viability and alteration of cell surface antigen expression<sup>29</sup>. Nevertheless, well-established protocols using collagenase treatment have been shown to have marginal impact on the detectability of relevant surface molecules frequently used for phenotypic analyses<sup>30</sup>.

Among the several parameters that can optimize the quality of cell isolation, the separation of the dorsal and ventral sections of the ear skin is essential, since it allows the collagenase/DNAse to access the dermis. In addition, mincing the skin into small pieces increases the surface area accessible to the enzymes, therefore allowing shorter duration of digestion. Finally, the use of mechanical dissociation helps to increase the yield in cell number for both the skin and dLN. However, excessive dissociation can cause additional stress that could negatively impact cell viability.

We favoured acquisition of live cells since fixation leads to suboptimal staining, complicating distinction of rare positive events of interest (data not shown). Moreover, the discrimination of dead cells was more problematic. In cases where cell fixation is required, we would suggest the use of LIVE/DEAD fixable dead cell stains.

Using the defined protocol, we successfully isolated myeloid cells infiltrating into the skin and dLN in response to parasite injection (Figure 6A and B). We usually obtain higher levels of viable single cells from the dLN (70-90%) compared to the skin (40-70%). This difference can be explained by the longer incubation time needed for efficient enzymatic digestion together with additional steps of mechanical dissociation to isolate skin cells (skin layer separation, mincing and stronger mashing).

Finally, an important parameter to take into account when analysing the host response to ID injection of sporozoites is the level of inflammation induced by both needle insertion and mosquito salivary gland extract deposition in the skin<sup>19,20</sup>. We therefore suggest to inject additional mice with either 1x PBS alone or salivary gland extracts from the same number of non-infected mosquitoes to evaluate the immune response specifically induced by the parasite. Altogether, we advise to dissect infected mosquitoes with high parasite load in the salivary glands and to filter the parasite suspension to limit the amount of mosquito material that could be deposited in the skin.

In summary, the isolation of myeloid cells from the skin and dLN through the described protocol provides a reliable assay to assess dynamic cellular changes during inflammatory response to *Plasmodium* infection and can be extended to other pathogens transmitted into the skin of the host.

## Disclosures

The authors declare that they have no competing financial interests.

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