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Uncovering leishmaniasis resistance in mice: a transcriptomic and proteomic approach

Leishmaniasis is a zoonotic disease caused by the unicellular protozoan parasite *Leishmania* which comprises over 30 species. Leishmaniasis is associated with high morbidity and mortality, its incidence is 1.5 to 2 million cases per year which is a big challenge to public health, as other zoonotic diseases [1], [2], [3]. Leishmaniasis is endemic in tropical and subtropical regions like Asia, the Middle East, Northern Africa, the Mediterranean, and South and Central America [1]. Leishmania can be characterized in two distinct forms, cutaneous and visceral based on which subtype of phagocytic cells are infected [1]. The visceral form appears upon infection of phagocytic cells within secondary lymphatic organs, liver, and bone marrow. Hence the cutaneous form of leishmaniasis (CL), which is the most common form affecting about 10 million people, arises from an infection of epidermal tissue [4]. *Leishmania major* is a predominant cause of CL in the African and Asian continents and the reservoirs for this protozoan include many rodent species [3], [4].

The life cycle of *Leishmania spp.* involves a mammalian host and a vector stage, which is the reason why this disease is successfully transmitted among different hosts [5]. Figure 1 explains in detail the life cycle of this protozoan, which includes a sandfly vector and a mammalian host. Within the vector, *Leishmania spp.* presents a form of promastigotes, and when the sandflies are feeding, promastigotes are injected in the host which promotes an anti-inflammatory local immune response, benefiting the establishment of parasites, promoted by the presence of sandfly salivary proteins [2]. After this occurrence, promastigotes can be quickly phagocytized by macrophages where they reside in phagolysosomes and transform into replicating amastigotes and divide through simple division in a parasitophorous vacuole [4], leading to the burst of the macrophage and consequent infection of other cells. The change in form happens within 4 to 14 days. The cycle will continue because a sandfly can ingest blood from an infected mammalian giving rise to a new cycle [3]. This transmission is possible because female sandflies need blood meals, and the most common vectors that are infected with *L. major* are *Phlebotomus papatasi, Phlebotomus duboscqi, Phlebotomus salehi, Phlebotomus caucasicus* [2].

L. major can evade the immune system by interacting with various cell types, one of them being the macrophage. The major evasion strategy employed by Leishmania is to suppress nuclear factor-kappa B (NF-kB), which regulates the expression of several essential antimicrobial molecules suppressing them [5]. The production of IL-12 is suppressed which is promoted by the monarch-1 molecule since it negatively regulates NF-kB. Another way to

evade this system is by remodeling the phagosomal compartment and interfering with this signaling pathways that mediates parasitic clearance.

The response of the host upon infection is dependent on the signaling cascade expressed during the active stage of infection [5]. The signaling is controlled by genetic expression of the genes responsible in the response to infections. The loci involved in this response are called *L. major* response (*Lmr*), until now there are a few of these genes characterized and some of them contribute to a susceptible phenotype, this expression pattern is present in the susceptible mice strain SWR/J [6], [7]. It is also known that another possible phenotype upon infection with the protozoan is a resistant one, and the strain of mice where we can observe this is C57BL/6 [7].

This information leads us to hypothesize in what way the genomic response contributes to the susceptibility or resistance of different mice strains when encountering *L. major* infection. To address this issue transcriptomics and proteomics assays will be conducted in order to find a possible target for the future development of a new therapy.

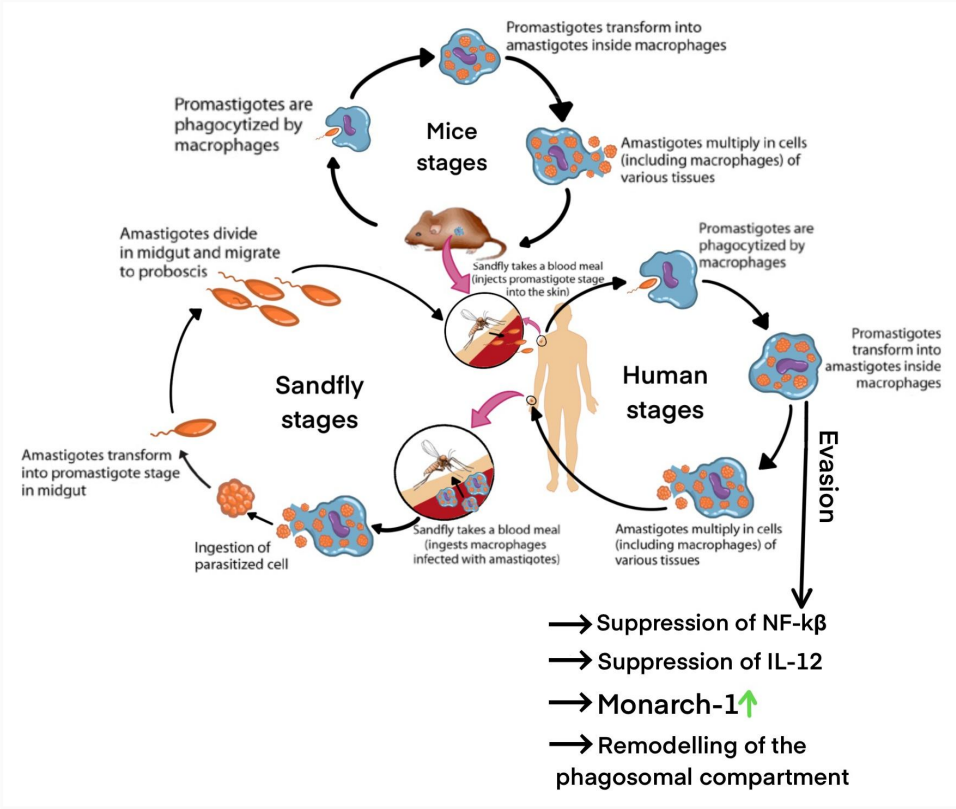

Figure 1: Leishmania spp. life cycle. Phlebotomus sandflies infect mammalian hosts with promastigotes being phagocytized by their resident macrophages. Promastigotes will turn into amastigotes, and these will multiply within macrophages. The infection can be contained at the site of cutaneous infection or progress into visceral infection. When sandflies are feeding on the host's blood which has cutaneous or visceral Leishmaniasis, they get infected with amastigotes that will be converted to promastigotes in the sandfly midgut. Afterwards, the promastigotes will migrate from the midgut to proboscis, being ready to infect when the sandfly stings again (Adapted from [3]).

Animal models, inoculation and sampling

Two strains of mice C57BL/6 (resistant) and SWR/J (susceptible) (Figure 2A) will be divided into two groups: Experimental group and Control group [8][7]. Each group is composed of 5 resistant and 5 susceptible mice. Each mouse will experience, on day zero (Figure 2B), stings of 50 *Phlebotomus perniciosus* [9] for 30 minutes, to increase pathogen burden. The experimental group will be stung by *Phlebotomus* sandflies infected with *Leishmania major* (*L. major LV 561 (MHOM/IL/67/LRC-L137 JERICHO II)* [6] and the control group will also be submitted to stings but with sandflies not carrying *L. major* parasite.

After 2 weeks, mice will undergo local anesthesia in the peritoneum to collect peritoneal fluid where the macrophages are in an adequate quantity to be analyzed (Figure 2B). For this extraction, the protocol [10] will be followed, excluding step one, with some adaptations. For macrophage isolation, the peritoneal fluid collected from each mouse will be submitted to Flow Cytometry Assisted Cell Sorting (FACS) so that the populations of cells present can be separated and categorized. After FACS, we divide the population of macrophages collected into two pools, one will be used for mRNA extraction with mRNA isolation kit [11], in order to do transcriptomics, and the other will be used for proteomic assay after protein extraction [12].

This is a single experiment, and the samples are randomized without reposition. The statistical power is 90% due to ethical reasons, since at the end of this experiment the infected mice will be euthanized, we cannot raise the number of controls. The susceptibility or resistance, which is the dependent variable in this study, will be categorized as binary (yes/no) and it will be compared between subjects.

Transcriptomics and Proteomics

After mRNA extraction we will perform a microarray assay to analyze mRNA transcription and build a heatmap with the use of computation tools. To complement our data, we will do a proteomic assay, Figure 2A, in order to detect the amount and what proteins are present in these cells of resistant and susceptible mice, so that we can see if there are any differences between them. The following step includes analysis of the data obtained from the proteomic and transcriptomic assays to see the differences between the two mice strains.
This procedure should be repeated (after step number 2) 8 weeks (56 days) after infection, because that's when the pathogen burden starts to decline, so we can search for differences in the response pattern related to the resistant and susceptible phenotype (Figure 2B) [13].

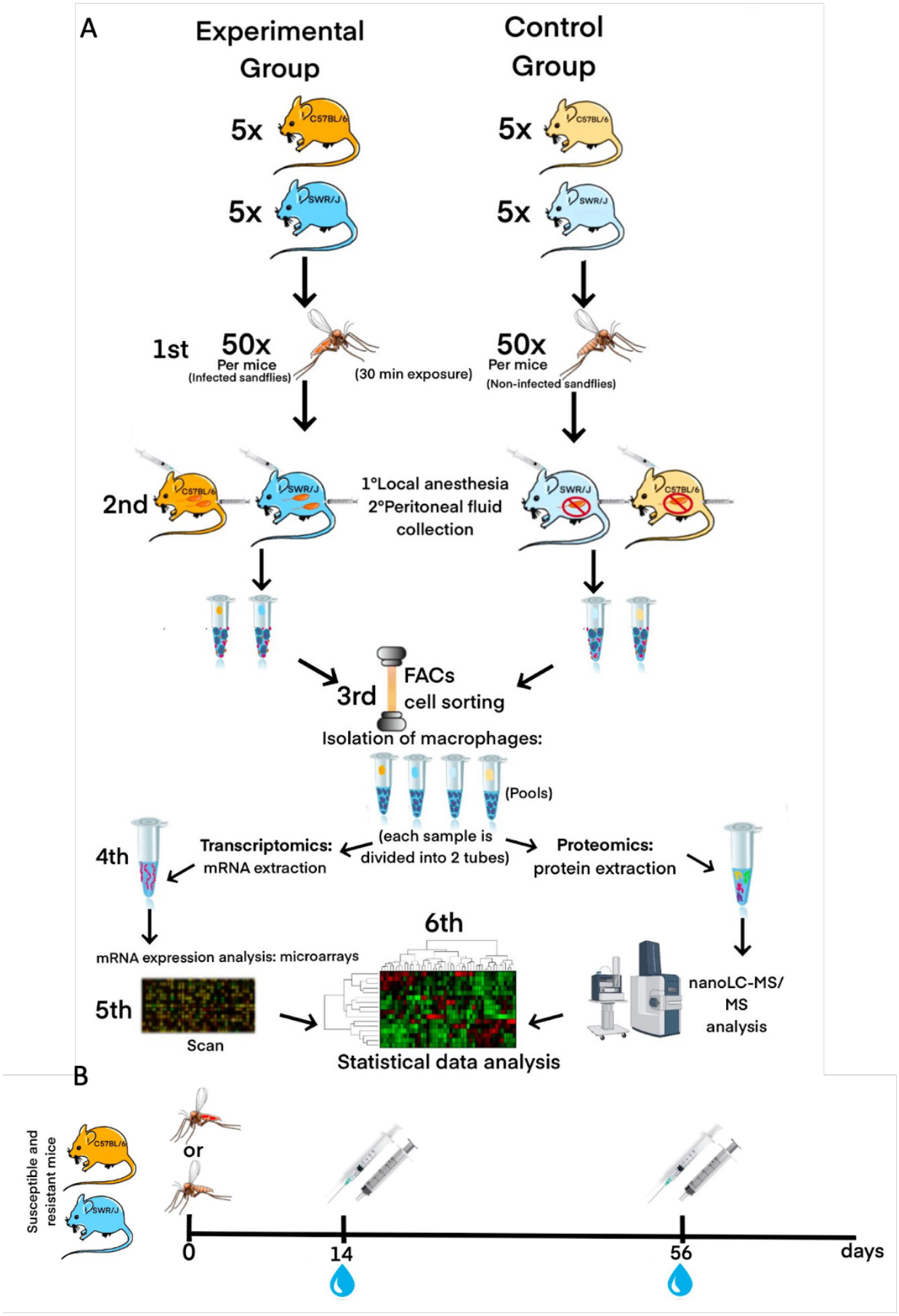
A
Experimental Group
Control Group
5x
C57BL/6
5x
SWR/J
1st
50x
Per mice
(Infected sandflies)
(30 min exposure)
50x
Per mice
(Non-infected sandflies)
2nd
1°Local anesthesia
2°Peritoneal fluid collection
3rd
FACs
cell sorting
Isolation of macrophages:
(Pools)
4th
Transcriptomics:
mRNA extraction
(each sample is divided into 2 tubes)
Proteomics:
protein extraction
mRNA expression analysis: microarrays
6th
5th
Scan
nanoLC-MS/MS analysis
Statistical data analysis
B
Susceptible and resistant mice
or
0
14
56
days

Figure 2: Experimental design for comparison of resistant and susceptible mice. A - General workflow for this experiment. The mice will be divided into two groups: the experimental group and the control group. Each group will be constituted by 5 C57BL/6 (resistant) and 5 SRW/J (susceptible) mice. First, the experimental group of mice will be stung by *Phlebotomus* sandflies and infected with *L. major* meanwhile the control group will also be stung but will not be infected. After the exposure to the sandflies, samples of the peritoneal fluid will be collected after local anesthesia administration. The experimental group will give rise to two sample pools, one from C57BL/6 and the other from SWR/J mice and the same will happen in the control group. The isolation of macrophages will be processed using FACS and each pool will be divided into two tubes, one will be used to do mRNA extraction (transcriptomics) and the other to protein extraction (proteomics). Transcriptomics screening will be performed by microarrays to mRNA expression analysis, while proteomics will be realized by MS. Lastly, the data obtained needs to be analyzed and statistics must be done. **B - Extraction of peritoneal fluid.** On day 0, both SRW/J and C57BL/6 mice strains will be stung by sandflies carrying *L. major* (experimental group) or without *L. major* (control group). After 14 days, the first extraction of peritoneal fluid will be performed and only 56 days post-infection the second extraction will be done.

Expected results and future perspectives

The differences found between transcriptomic and proteomic results in the two strains should be accounted as a target for further studies. To confirm if this could be a good target, sequencing could be done, as well as knockdown, knockout, and up-regulation assay.

If all results are equal in transcriptomic and proteomics, then we should also check for metabolomics and post-translational modifications, in order to understand furthermore the action and effect that this parasite has on the host.

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