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Exploring the Macrophage Response to *Leishmania* infection: Immunometabolism and Pathway Biocuration

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This work is dedicated to my mother and grandmother
Martha silva (QEPD) and Gladys peña for your love and support.

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Exploring the Macrophage Response to *Leishmania* infection: Immunometabolism and Pathway Biocuration

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ABSTRACT

Leishmaniasis is a pro-inflammatory immunopathology developed as a consequence of infection with *Leishmania* parasites. These reside and replicate in macrophages, phagocytic cells in charge of the elimination of intracellular microbes. As in all microbial infections of a similar nature, the resolution of Leishmaniasis involves two key events. First, a transient activation of the pro-inflammatory response that enhances the microbicidal function of the macrophage, followed by a longer-term anti-inflammatory response, which allows the expression of tissue repair mechanisms. In symptomatic infections with *Leishmania*, microbicidal processes in the macrophage are not activated efficiently even under highly pro-inflammatory conditions. What explains the inability of the macrophage to respond to these signals? In analogous immunopathologies it is known that macrophages have the ability to dynamically reprogram immune and metabolic functions after exposure to pro-inflammatory stimuli. This phenomenon has not been proposed or studied in Leishmaniasis. The main hypothesis of my doctoral research work is that in macrophages infected with *Leishmania*, immunometabolic pathways are dynamically reprogrammed after pro-inflammatory stimuli. Depending on the immunometabolic program, the macrophage favours or not the survival of the parasite. My work derived two main scientific contributions on this topic. (1) Using RNA-seq data, enrichment analysis, and extensive literature review, I reconstructed the pathways involved in macrophage immunometabolism 24 hours after infection with *Leishmania* parasites. (2) In addition, transcriptome data and mathematical modelling allowed me to build a model of the reprogramming of macrophage immunometabolism pathways during the first 24 hours of infection. In summary, we found that: during the first hours of the *Leishmania*-macrophage interaction, the host cell activates a strong inflammatory as well as oxidative response, evidenced by the expression of transcription factors, cytokines, ROS-producing enzymes (reactive oxygen species), among others. Additionally, the host cell bioenergetics is sustained mainly by glycolysis, as opposed to oxidative phosphorylation

in mitochondria during basal conditions (without infection). However, damping mechanisms of these responses are mostly activated in parallel. By 24 hours after infection, the macrophage has a predominantly anti-inflammatory, anti-oxidative profile and with basal-type bioenergetics. These are precisely the conditions that favour the survival of the parasite. In Chapter 1 we provide a detailed description of the molecular interactions that support this profile. We hope that this molecular network will serve as a starting point to identify key intervention nodes in the reversion of macrophage permissiveness to infection. Additionally, we hope that the parameterization and structuring of the mathematical model, discussed in chapter 2, will motivate both *in silico* and *ex vivo* /*in vitro* experiments to study the system in the presence of pro-inflammatory stimuli, molecular factors secreted by the parasite, among other scenarios. Our work also has a third scientific contribution. We also set out to address the problem of extracting meaningful biological information from RNA-seq data in the context of Leishmaniasis. We consider that many of the transcriptomes available from macrophages infected with *Leishmania* are underexploited to explore mechanisms that transcend the classical mechanisms studied in Leishmaniasis. For this, we built the first repository of signalling pathways of importance in Leishmaniasis, in the public database Reactome. In Chapter 3 we discussed the importance of structuring the representation of biological processes considering the context of the immunopathology of this disease. Thus, we group signalling pathways according to their participation in: internalization of the parasite, induction of pro-inflammatory responses and induction of anti-inflammatory responses that favour parasite survival. The signalling pathways with which we initialized "Leishmania infection pathways" are of high importance in the control of the infection, based on the recent literature. After re-analyzing previously published transcriptomics data, we show how our database enhances the discernment of molecular mechanisms underlying any of the aforementioned categories, which were not reported in the original studies. For example, the ADORA2B signalling pathway contributes to the maintenance of an anti-inflammatory profile in macrophages infected with *Leishmania*. This is possible through the signalling cascade that leads to the production of interleukin 10. In general, we hope that the scientific community in Leishmaniasis will make use and contribute to the expansion of this repository, and thus accelerate our understanding of the biology of infection. Together, this doctoral research work integrates the use of computational tools and omics data to perform a comprehensive analysis of the macrophage response to infection with *Leishmania* parasites.

RESUMEN

La Leishmaniasis es una inmunopatología de carácter pro-inflamatorio desarrollada como consecuencia de la infección con parásitos de *Leishmania*. Éstos residen y se replican en los macrófagos, células fagocíticas a cargo de la eliminación de microbios intracelulares. Como en toda infección microbiana de similar naturaleza, la resolución de la Leishmaniasis implica dos eventos claves. Primero, una activación transitoria de la respuesta pro-inflamatoria que potencie la función microbicida del macrófago, seguido de una respuesta amortiguadora que permita la expresión de los mecanismos reparadores de tejido. A esto le sigue una respuesta anti-inflamatoria a más largo plazo, que permite la expresión de los mecanismos de reparación tisular. En la infección sintomática con *Leishmania*, los procesos microbicidas en el macrófago no se activan eficientemente incluso en condiciones altamente proinflamatorias. ¿Qué explica la incapacidad del macrófago para responder a estas señales? En inmunopatologías análogas se conoce que los macrófagos tienen la capacidad de reprogramar manera de dinámica funciones inmunológicas y metabólicas después de su exposición a estímulos pro-inflamatorios. Éste fenómeno ha sido propuesto o estudiado en la Leishmaniasis. La principal hipótesis de mi trabajo de investigación doctoral es que en macrófagos infectados con *Leishmania*, rutas inmunometabólicas son reprogramadas de manera dinámica después estímulos pro-inflamatorios. Dependiendo del programa inmunometabólico, el macrófago favorece o no, la supervivencia del parásito. Mi trabajo derivó dos principales contribuciones científicas en este tema. (1) A partir de datos de RNA-seq, análisis de enriquecimiento y una extensiva revisión de la literatura, caractericé las rutas involucradas en el inmunometabolismo del macrófago a las 24 horas de la infección con *Leishmania*. (2) Complementariamente, datos de transcriptómica y modelamiento matemático, me permitieron construir un modelo de la reprogramación que sufren rutas del inmunometabolismo del macrófago durante las primeras 24 horas de infección. En resumen, encontramos que: durante las primeras horas de la interacción *Leishmania*-macrófago, la célula hospedera activa una fuerte respuesta inflamatoria, así como oxidativa, evidenciada por la expresión de factores de transcripción, citoquinas, enzimas productoras de ROS (especies reactivas de oxígeno), entre otros. Adicionalmente, la bioenergética de la célula hospedera se sostiene principalmente en glicólisis, a diferencia de fosforilación oxidativa en mitocondria durante condiciones basales (sin infección). No obstante, mecanismos amortiguadores de éstas respuesta son en su mayoría activados paralelamente. Hacia las 24 horas de la infección, el macrófago presenta un perfil predominantemente antinflamatorio, anti-oxidativo y con una bioenergética de tipo basal. Éstas son precisamente las condiciones que favorecen la

supervivencia del parásito. En el capítulo 1 ofrecemos una descripción detallada de las interacciones moleculares que sostienen este perfil. Esperamos que esta red molecular sirva como punto de partida para identificar nodos de intervención clave en la reversión de permisividad del macrófago a la infección. Adicionalmente, esperamos que la parametrización y estructuración del modelo matemático, discutido en el capítulo 2, motive tanto experimentos *in silico* como *ex vivo/in vitro*, para estudiar el sistema en presencia de estímulos pro-inflamatorios, factores moleculares secretados por el parásito, entre otros escenarios. Nuestro trabajo además tiene una tercera contribución científica. También nos propusimos abordar el problema de extraer información biológica significativa de los datos de RNA-seq en el contexto de la Leishmaniasis. Consideramos que muchos de los transcriptomas disponibles de macrófagos infectados con *Leishmania* están subexplotados para explorar mecanismos que trasciendan los mecanismos clásicos de estudio en la Leishmaniasis. Para esto, construimos el primer repositorio de rutas de señalización de importancia en la Leishmaniasis, en la base de datos publica Reactome. En el capítulo 3 discutimos la importancia de estructurar la representación de procesos biológicos teniendo en la cuenta el contexto de la inmunopatología de esta enfermedad. Así, nosotros agrupamos rutas de señalización según su participación en: internalización del parásito, inducción de respuesta pro-inflamatorias e inducción de respuestas anti-inflamatorias que favorecen la supervivencia del parásito. Las rutas de señalización con las que inicializamos "Leishmania infection pathways" son de alta importancia en el control de la infección, con base en la literatura reciente. Tras reanalizar datos de transcriptómica previamente publicados, mostramos cómo nuestra base de datos potencia el discernimiento de mecanismos moleculares subyacentes a alguna de las categorías mencionadas, que no fueron reportados en los estudios originales. Por ejemplo, la ruta de señalización ADORA2B, contribuye al mantenimiento de un perfil antinflamatorio en macrófagos infectados con *Leishmania*. Esto es posible a través de la cascada de señalización que conduce a la producción de la interleucina 10. En general, esperamos que la comunidad científica en Leishmaniasis, haga uso y contribuya a la expansión de este repositorio, y así acelerar nuestro entendimiento de la biología de la infección. En conjunto, en este trabajo de investigación doctoral se integra el uso de herramientas computacionales y datos ómicos para realizar un análisis comprensivo de la respuesta del macrófago a la infección con parásitos de *Leishmania*.

Key words: Leishmaniasis, macrophage, immunometabolism, RNA-seq, biocuration, Reactome, dynamic model

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I want to finalize this session with this quote by Peter Medawar, Nobel Prize in Medicine:

"To be creative, scientists need libraries and laboratories and the company of other scientists; certainly a quiet and untroubled life is a help. A scientist's work is in no way deepened or made more cogent by privation, anxiety, distress, or emotional harassment. To be sure, the private lives of scientists may be strangely and comically mixed up, but not in ways that have any special bearing on the nature and quality of their work."

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DISSEMINATION

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Title of the poster: Curating and Modelling of Early Macrophages Responses in Chronic-Human Cutaneous Leishmaniasis. **Julieth Murillo**, Maria Adelaida Gómez.

Basel Computational Biology Conference. Workshop: BioNetVisa. Basel-Switzerland. September 2021.
Title of the talk: Enhancing the usefulness of transcriptome data in the context of *Leishmania*-infected macrophages with pathways biocuration and mathematical modelling. **Julieth Murillo Silva**, Henning Hermjakob, Bijay Jassal, Carolina Clavijo, Mauricio Quimbaya.

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GENERAL INTRODUCTION

Leishmaniasis is a parasitic disease transmitted by a vector fly from the subfamily Phlebotominae. The etiological agent of Leishmaniasis is constituted by the unicellular protozoan of the genus *Leishmania*. These are intracellular parasites of a wide variety of mammalian phagocytic cells, such as macrophages, neutrophils and dendritic cells. The genus *Leishmania* is divided into two subgenera: *Leishmania* and *Viannia*. *Leishmania* (*Leishmania*) holds the species: *amazonensis, tropica*, *major*, *donovani*, *mexicana*, among others. *Leishmania* (*Viannia*) includes the species: *panamensis*, *braziliensis*, *guyanensis, naiffi* among others (1). The clinical manifestations depend to a great extent on the infecting species and the immune status of the host; cutaneous (CL), mucocutaneous (MCL) and visceral (VL) leishmaniasis constitute the main presentations of the disease.

An old paradigm governed the understanding of the immunopathology of Leishmaniasis. This consisted in that a host capable of eliciting a predominant T helper 1 (Th1) response would be more resistant to the infection, than the ones with a marked T helper 2 (Th2) response. Mechanistically, this premise is based upon the effect of molecular factors produced by the Th1/Th2 on *Leishmania*-infected macrophages. The feature IFN-γ produce by Th1 contributes to the induction or maintenance of pro-inflammatory macrophages, known as M1. M1 are characterized for having enhanced microbicidal functions and therefore, are better suited to control the infection. On the other hand, Th2 cells characteristically produce the interleukins IL-4 and IL-10, which contribute to the maintenance of anti-inflammatory macrophages, known as M2. M2 naturally are more permissive to the infection. However, this conceptual frame only worked for one mouse infection model (2, 3). Nowadays, we know that a pro-inflammatory environment mediated by Th1 is not enough to induce killing mechanisms in infected macrophages (4–7).

How is it possible that some macrophages still harbour *Leishmania* parasites, in such pro-inflammatory conditions? What accounts for the inability of this signalling event to activate a killing response? Macrophage ontogeny and metabolic reprogramming might be the concepts that could help us to understand this phenomenon. Ontogeny accounts for the origin of either embryonic or hematopoietic of the macrophage. It has been shown that upon environmental cues (e.g. cytokines, infection), macrophages have different responses based on their ontogeny (8–11). Thus, a virulent strain of *L. major* is preferentially phagocytosed by tissue-resident macrophages

(which come from embryonic origin) than from monocyte-derived macrophages (12). The former keeps an anti-inflammatory profile even with pro-inflammatory environmental cues. On the other hand, monocyte-derived macrophages have also been shown to be effectively manipulated to provide a long-term niche for *Leishmania* parasites (11, 13, 14). Therefore, ontogeny alone does not explain what makes a macrophage to be permissive to harbour *Leishmania* parasites even in pro-inflammatory conditions. Here is when immunometabolic reprogramming comes along.

We can define macrophage immunometabolism as an interconnected set of inflammatory signalling pathways, together with metabolic pathways involved with bioenergetics, uses of carbon, catabolic and anabolic processes. Immunometabolic reprogramming deals with the changes that these pathways experience in macrophages over time. This is in nature a dynamic process in which environmental and phagocyted agents (e.g., debris or microbial pathogens) shape such reprogramming (15). Little is known about how *Leishmania* parasites shape the immunometabolic rewiring in their host macrophage. First, few studies in Leishmaniasis address the immunology and metabolism of the macrophages altogether (16, 17). Second, the existing studies employ reductionist approaches. This limits the finding of mechanistic patterns of macrophage responses to the *Leishmania* infection. Meanwhile, holistic approaches to characterize the macrophage's pathways in response to the *Leishmania* infection, often are focused on detailing the immune-related processes. If metabolic processes come across in many of such studies, they are often unconnected with the immunological side (18–20).

With the above context, my doctoral research work is based on the hypothesis that macrophages infected with *Leishmania* parasites undergo a dynamic reprogramming of their immunometabolism. This allows them to maintain an immunological and metabolic profile permissive to infection, even when they are embedded in a pro-inflammatory environment that, otherwise, stimulates them to eliminate the parasite. Given the complexity of characterizing the pathways involved in immunometabolism, large-scale measurement methodologies are very useful. However, there is a great barrier in the interpretability of this type of data. Going from a list of genes to an assembly of pathways with biological and contextual meaning is far from being trivial. Scientists often turn to query databases of pathways, where it is difficult to find contextualized information. In this sense, my second working hypothesis was that the unified biocuration of a set of signalling pathways of importance in Leishmaniasis, would enhance the interpretability of high throughput data.

My work derived three main scientific contributions. **1.** A detailed description of immunometabolic pathways of macrophages (derived from monocytes) infected for 24 hours with *L. panamensis*.

For this, I performed a secondary analysis of RNA-seq data from *Leishmania*-infected macrophages. The conceptual and methodological reasoning underlaying the description of the immunometabolic pathways are shown in chapter 1. **2.** Construction of a dynamic model of the interplay of immunometabolic pathways of macrophages infected with *Leishmania* spp. I assembled this mathematical model from three sets of gene expression data from macrophages infected with *L. panamensis* or *L. major*. I use the model to give a dynamic and mechanistic representation of the immunometabolic switch in the macrophage along the first 24 hours of infection. The methodology and findings are described in chapter 2. **3.** In chapter 3 I address the second hypothesis of my doctoral research. In this section I share the biocuration work of the repository: “Leishmania infection” pathways, in Reactome database. In a collaborative work with the Reactome team, I designed the structure of the repository, as well as selecting and bio-curating the first subpathways. In Chapter 3 I show the main utility of having pathways structured according to the biological context of Leishmaniasis. I final, in chapter 4 I discussing future directions motivated by the main findings in this thesis.

CHAPTER 1: Building a description of the *ex vivo* immunometabolism of *Leishmania panamensis*-infected macrophages from RNA-seq data.

ABSTRACT I
PART I.

In Colombia *Leishmania panamensis* is one of the most prevalent *Leishmania* species causing cutaneous leishmaniasis (CL). It leads to either self-healing (Sh) or chronic (Chr) localized cutaneous lesions in infected patients. In CL patients treated with glucantime, up to 30% may fail the treatment. It is unknown if there are distinguishing mechanisms driving non-healing or healing outcomes in CL. We performed a secondary analysis on RNA-seq data from non-healing models (NHM) and healing models (HM) of *ex vivo*, *L. panamensis*-infected macrophages, 24 hours post-infection. NHMs represent chronic (Chr) CL and therapeutic failure. Meanwhile, HMs represent self-healing (Sh) and therapeutic cure. We set out to identify distinguishing biological processes in the dataset. To do so, we first identified commonly modulated genes exclusive to the NHM and HM datasets. We then attempted to extract statistically likely distinguishing mechanisms involving these genes, using the Reactome database. Nine genes were found exclusive to NHM (n = 8) and HM (n = 1) infections, respectively. For each of the four datasets, we obtained a list of biological processes from Reactome. We then apportion this list of candidate processes into functional clusters, defined by participation in some common, macro-process (e.g. mitochondrial respiration and tricarboxylic acid (TCA) cycle grouped in the same cluster). Next, we attempted to identify distinguishing clusters in the NHM or HM datasets. Instead, we found common clusters in the NHM and Cure datasets, but not any distinguishing clusters of NHM or HM. The goals we established in my Ph.D. research proposal depended upon the recognition of such clusters. Therefore, we exploited the secondary findings from the above analysis to address a different research gap. We now explain the biological relevance of our new research question, our results, and our perspectives.

PART II.

Macrophages are the main host cell of *Leishmania* parasites. The stimulus of the infection switches the basal immunometabolism of the macrophage to contain the infection. A holistic understanding of the immunometabolic network in response to intracellular parasites, has helped to pin down therapy and drug targets in different pathogen infections. Despite the prevalence of *L. panamensis* causing localized CL or more aggressive clinical manifestations, we do not know how the immunometabolism of the human host is reprogrammed during the infection. In chapter 1, we use RNA-seq data to reconstruct the immunometabolic network displayed at 24 hours post-

infection, known as the adaptation or late stage, of *L. panamensis*-infected macrophages. We found evidence of an activated Hypoxia Transcription Factor potentially directing an early glycolytic flux. Alongside, negative regulators of glycolysis were upregulated at 24 hours post-infection. The redox metabolism was also regulated at the level of transcription by FOS, NOTCH3 and NFE2L2. The first two modulate the production of reactive oxygen species (ROS), while the last one contributes to the activation of a ROS scavenging system. Regarding the bioenergetic of the macrophage we found positively regulated components of oxidative phosphorylation, coupled with enzymes of the TCA cycle and fatty acid synthesis. On the immunological side, purinergic receptors might be directing inflammatory responses and adenosine receptors might be counteracting it. Despite the overexpression of receptors driving pro-inflammatory responses, we found some of the required downstream molecules either lacking, downregulated or negatively modulated by inhibitors. Overall, the immunometabolism in the adaptation stage of *L. panamensis*-infected macrophages recalls a suitable anti-inflammatory / modulatory profile for the survival of the parasite. Our immunometabolic network serves as a useful null hypothesis on the biological processes driving the adaptation phase of the *L. panamensis*-macrophage interaction. It provides explicit hypotheses testable by further, reductionist experiments, and serves as the groundwork for a dynamic mathematical model of the interaction, which we build in Chapter 2.

INTRODUCTION I

A primer on the immunometabolism of macrophages

Macrophages, despite comprising a key part of the immune system, are hosts of an array of different bacterial and parasitic pathogens. Their bioenergetic, biosynthetic and immune status over the course of such infections, is dynamic and changes according to the intrinsic genetic characteristics of both the host and the pathogen. Resting macrophages present low levels of catabolic and anabolic activity and produce their energy in an efficient manner through a metabolic pathway known as oxidative phosphorylation (OXPHOS), engaging a complete tricarboxylic acid (TCA) cycle (21). Upon infection, they shift their metabolism to arrange the inner machinery towards the production of anti-microbial molecules like reactive oxygen species-ROS- and inflammatory cytokines, so the infection is contained (21, 22). Many macrophage-infection models have shown that the macrophage display negative feedback to buffer the damage as a way of self-preservation as the infection progresses. Together with the evolved microbe strategies to subvert the host cell machinery, an adaptation stage may be reached where the

immunometabolism shifts again towards OXPHOS-TCA, anti-inflammation, and biosynthetic and anabolic pathways that may or may not favor the microbes. This will depend upon the nutritional requirements of the microbes. For instance, repressing glucose availability affects the growth of the pathogens like *Shigella flexneri* and *Trypanosoma cruzi*, while reducing the amino acid arginine, limits the growth of *Leishmania* spp. (23, 24).

Scientific literature in the *Mycobacterium tuberculosis* infection field, finds a clear biphasic dynamic in the macrophage-*Mycobacterium* interaction. During the early stage (4 to 8 hours post-infection approximately), the transcription factor (TF) HIF1-α orchestrates aerobic glycolysis, coupled with a potent oxidative and inflammatory response. As discussed above, later on, the immunometabolism switches to OXPHOS and an anti-inflammatory status, called the late or adaptation stage (around 24 hours post-infection) (25). Does a similar biphasic mechanism occur in *Leishmania* infection? Most studies do not monitor macrophage status longitudinally, a necessary prerequisite to constructing such an observation (16, 26, 27). Ramirez and colleagues (20) analysed microarrays expression data from *L. panamensis*-infected macrophages at 0.5, 4 and, 24 hours post-infection. They described the genes involved in the immunometabolic dynamics of the macrophages throughout the infection; inflammatory mediators are early produced, while mechanisms towards the dampening of inflammation are activated early and consistently up to 24 hours. Their description is limited by the technique used, but is consistent with our results, which we will discuss later.

A primer on the analysis of rna-seq data

Which aspects of the immunometabolic status in the macrophage are the key determinants of disease progression? RNA-seq (Ribonucleic acid sequencing) offers a systems biology perspective to answer this question, through quantitatively monitoring the simultaneous expression of genes in pathogen-infected macrophages. There is an increasing body of RNA-seq-derived data on the dynamics of macrophage-pathogen interactions. Although this corpus of data has been derived from a diversity of scientific studies, there are ongoing efforts to delineate a reproducible pipeline that goes from the transformation of raw sequencing data to meaningful gene counts, up to a list of differentially expressed (DE) genes. The pipeline is modular in nature, with multiple software/algorithm options. However, the output of the pipeline, for any given algorithm, is heavily dependent upon specific hyper-parameters chosen by the user. This leads to reproducibility issues (28), as pipeline results stored in different repositories do not always include information on hyper-parameters. Therefore, as good practice in a secondary analysis of RNA-seq data, one should derive standardized quality-assurance measures. Tables of raw gene

counts, with some exceptions, are found in the supplementary material of primary research papers. The rows of these tables provide the expressed genes, and the columns provide the sequenced samples. The intersection between the column and the row, therefore, corresponds to the number of sequence fragments that have been assigned to each gene in that sample. A sample is the unit of the experimental condition, which in turn it has different levels (commonly two, treated and untreated). For example, when studying pathogen-macrophage interactions at the *in vitro* or *ex vivo* level, the experimental condition could have two levels, uninfected macrophages, and infected macrophages; each condition must have replicates from which we extract the total RNA that becomes the samples to be sequenced. We now describe relevant considerations when processing tables of raw gene counts:

- quality assessment and visualization to define the batch removal method: this step is done to visually inspect the distribution of the count data based on the reduction of its dimensions by an unsupervised method such as the Principal Components Analysis (PCA). The counts are normalized to account for differences in the sequencing depth, before performing PCA. Often, the two first components are plotted to visually inspect if the clustering of the samples corresponds to the biological conditions or if there are technical factors influencing it. Since there are multiple technical steps, from the RNA extraction to the deduction of the gene counts, extreme deviation of a sample from samples of the same condition group may occur. This variance is known to be due to "batch effects", which brings negative impacts to the power of the differential expression analysis and downstream feature selection (29), therefore, one wants to account for them and remove them (29). Several methods have been proposed to this end, ranging from linear regression models such as ComBat (30) and limma (31) and, surrogate variables calculation such as in SVA (32).
- Before performing differential expression analysis, filtering low count genes may optimize computational speed (33). Also, depending on the algorithm used for the differential expression, eliminating lowly expressed genes, may be fundamental to reduce statistical burden (33). One common way of filtering is by calculating the scaling factor, "counts per million" (cpm). This considers the difference in sequencing depths for each sample, and normalized the counts, accordingly (34). Once the counts are normalized, come the filtration steps that consider two things: 1st, the number of samples in the smaller biological condition (let us call this number *X*) and, 2, by a rule of thumb, a threshold of 0.5 cpm.

Thus, genes are kept if they have 0.5 or more transformed counts, in minimum *X* samples. Otherwise, the genes are filtered out (35)

- There are different methods to performed differential expression analysis, such as EdgR, limma and, DESEq2 (36). DESEq2 uses a negative binomial generalized linear model; the estimates of dispersion and logarithmic fold changes (FC) incorporate data-driven prior distributions (33). The method is implemented in R, where it can be also used in conjunction with batch removal methods (33).
- After differential expression analysis: the outcome of this is a long list of genes that must be interpreted in terms of functional gene networks to produce testable hypotheses. However, going from a list of genes to a gene network fulfilling a biological function is not a trivial step. The usual approach found in scientific literature is to explore biological databases such as Gene Ontology (GO) and Reactome, through exploiting the statistical method: over representation analysis (ORA). ORA finds the overlap between an input gene list and the functional categorized genes in the database and uses the hypergeometric distribution to test for the accuracy of the overlap. If ORA operates on GO and Reactome, it gives a list of ontologies and a list of pathways, respectively.

Based on the RNA-seq data from *Leishmania*-infected macrophages data at 24 hours post-infection we described the bioenergetic and immunological status of the host cell. We found residues of glycolytic flux and oxidative stress potentially regulated at the level of transcription by TFs such as HIF1A, NOTCH3 and FOS. Furthermore, we found positively regulated glutathione synthesis that plays as an oxidative counteracting agent, together with components of OXPHOS, coupled with enzymes of the tricarboxylic acid cycle (TCA) and fatty acid synthesis. On the immunological side, we found that purinergic receptors might be directing inflammatory responses and adrenergic receptors might be counteracting it. Complementary, we found that NFE2L2 transcription factor must be a key regulator in the immunometabolism shift from the early response towards the adaptation stage. In general, the immunometabolism in the adaptation stage of *L. panamensis*-infected macrophages recalls a suitable anti-inflammatory / modulatory profile for the survival of the parasite.

MATERIAL AND METHODS I

Data set 1:

From the primary research of Gomez *et al.* (37) we requested the table of raw count genes regarding the RNA-seq of primary macrophages infected with two kinds of strains of *L. panamensis*. One is associated with self-healing (Sh) CL and the other with chronic (Chr) CL. These strains were characterized during a study by Weigle *et al*. (38), about the natural history of the leishmaniasis infection in endemic areas in Colombia. The isolated parasites were typed at the species and zymodeme level, and posteriorly stored in the biobank of CIDEIM (Centro de Internacional de Entrenamiento e Investigaciones y Médicas). The follow up of their study allowed them to identify people that after approximately a year had self-healed (Sh) the CL while others kept the lesions active, considered as Chr-CL. The stored strains were then classified according to the disease development of the donor in Sh or Chr. In figure 1.1 we summarized the experiment design that derived the RNA-seq data.

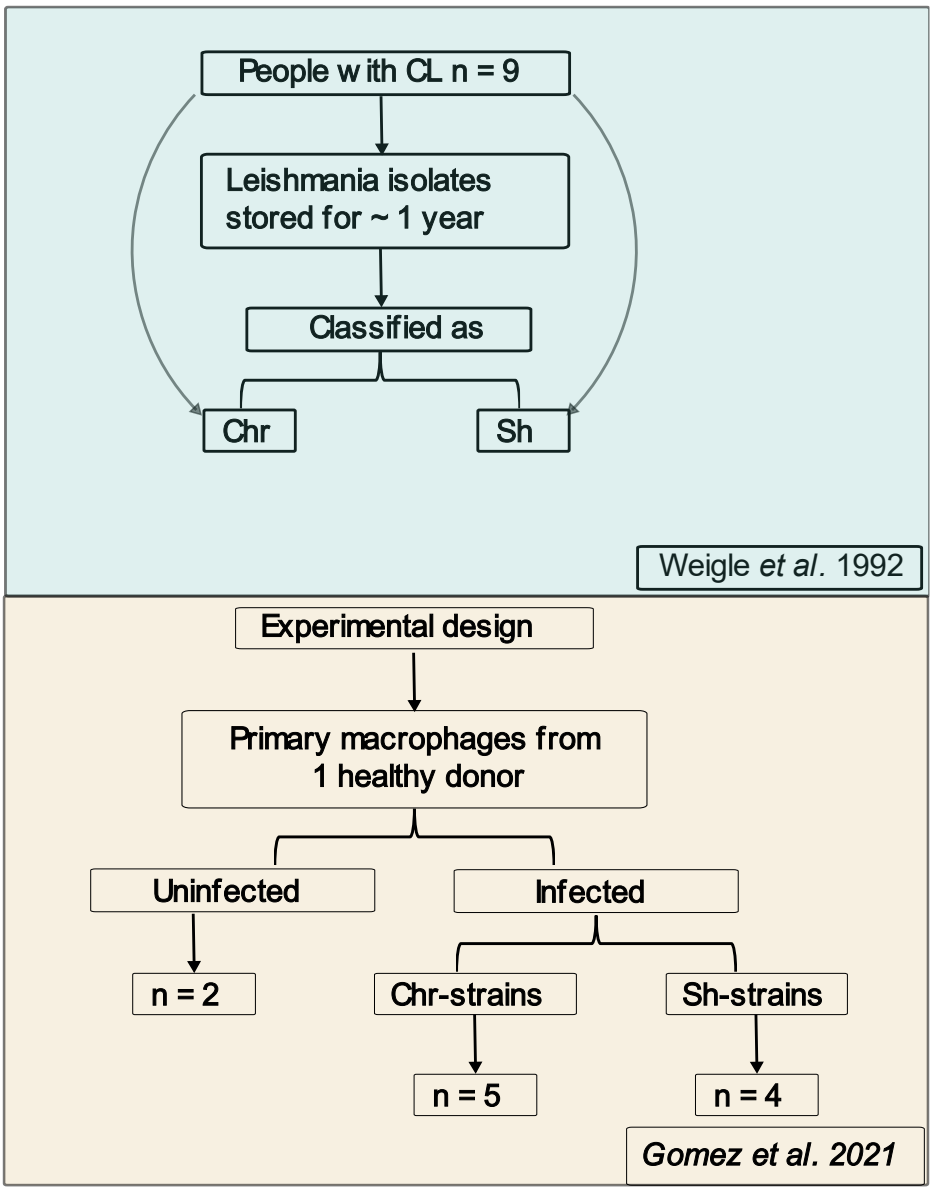


Figure 1.1: Origin of the data for the secondary analysis. *L. panamensis* strains were classified as Chr and Sh during a study by Weigle *et al* (39). Gomez and colleagues, in 2016, sequenced macrophages infected for 24 hours with one of two types of strains.

Data set 2:

From the research of Vargas *et al*.(40), we took the table of raw gene counts regarding the conditions of primary macrophages from patients with CL that prospectively cured or failed the treatment with Glucantime. Macrophages from each condition (cure or failure) were infected with a laboratory strain of *L. panamensis* and exposed to pentavalent antimony-Sb^{V} (simulating the

exposure to treatment) for 24 hours. In figure 1.2 we summarized the experimental design that derived the RNA-seq.

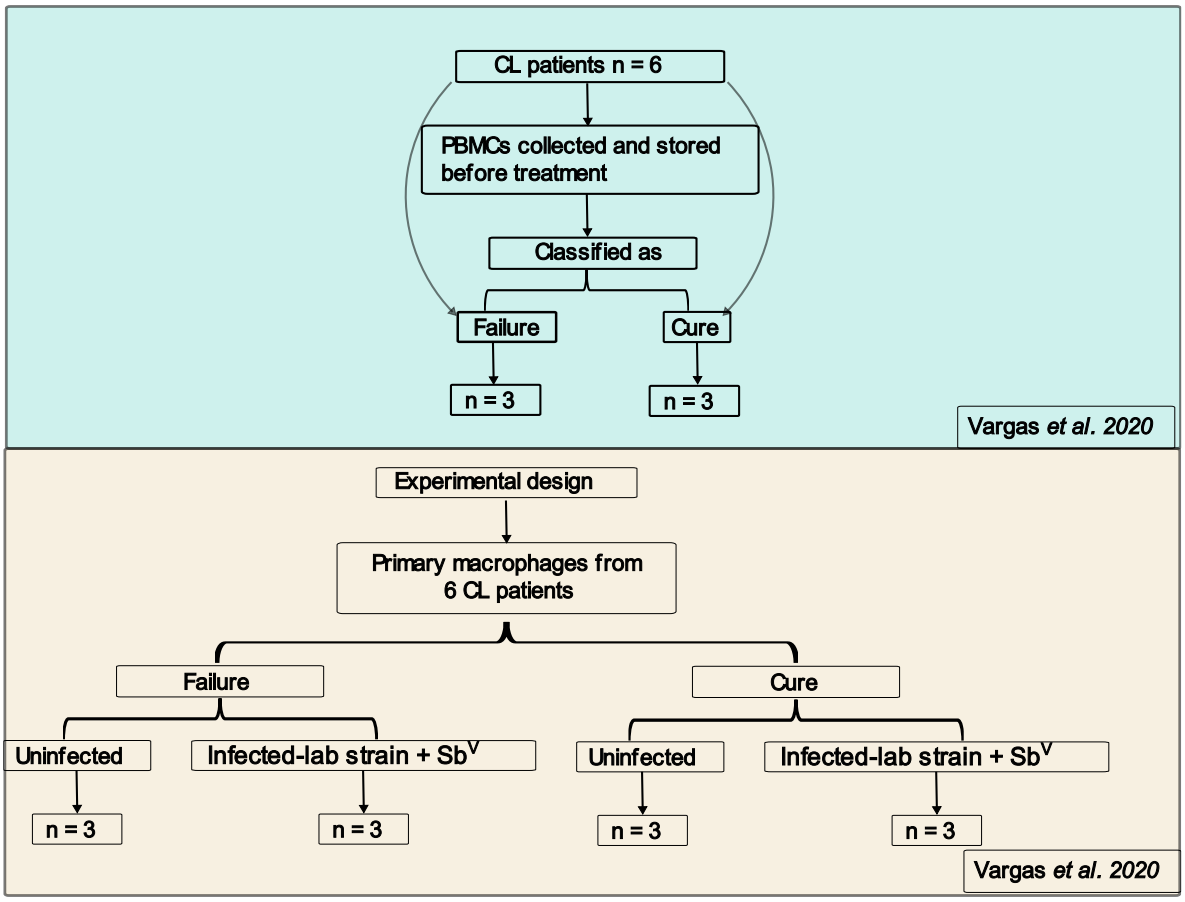


Figure 1.2: Origin of the data for the secondary analysis. Vargas and colleagues, infected with a laboratory-adapted line of *L. panamensis*, macrophages from patients that prospectively cured or failed the treatment with Glucantime. Alongside, they infected the macrophages for 24 hours followed by 24 hours drug exposure (Sb^V -pentavalent antimony).

Experimental design for the secondary data analysis

In figure 1.3 we summarized the overall flow of the methodology. It can also be accessed in more detailed in the attached R-scripts (annex 1.1). We used programmes implemented in R (version 3.6), mostly from Bioconductor repository. Also, we used some functions implemented in the HPGLTOOLS package from Najib El-Sayed laboratory (https://github.com/elsayed-lab/hpgltools/tree/master/R).

- **QA and Visualization:** We used PCA as the unsupervised method to visualize the clustering of the samples based on the expression data. To account for sequencing depth, the function normalize_expt allows to normalize through cpm, quantile, among others. A filter cut-off for the low gene counts is applied depending on the normalization method. Subsequently, the data is transformed to the base-2 logarithm (log2). To visualize the effects of potential batch effects in the expression data, we plotted two graphs. One in which we removed the technical variation that was acknowledged in the respective studies (with limma function in the R-scripts). The second one, plotted the PCA in which unknown nuisance factors were found and removed by the SVA algorithm.
- **Differential expression analysis:** We used DESeq2 algorithm to get the DE genes. As the developers of the methods stated, not much pre-processing is required since they have covered most of it in the algorithm that runs in R. We carried on the filtering step with the function they used, cbcb_filter_counts. This uses cpm as a normalization method and a cut-off of 1 in a minimum number of samples, that corresponds to the number of samples in the smaller group condition. Afterwards, we applied the batch effects removal that allowed for a better clustering in the previous step. Therefore, the raw gene counts input for DE analysis was filtered out by the low gene counts and the genes contributing to the variance caused by nuisance factors. We filtered the output by the adjusted p-value lesser than 0.05. From all the metrics the analysis produces, we left the log2 of the fold change (log2FC). Up to this point the genes were identified by the ENSEMBL identification (ID). Thus, we completed the annotation of the dataset with different IDs, like symbol, ncbi number and Kegg ID (see in the R-script, annex 1.1).
- **Searching potential explanatory mechanisms of non-healing and healing outcomes in CL:** We used ORA to identify common cellular mechanisms between the NHM (non-healing model) datasets (Chr and Failure data), as so for the HM (healing model) datasets (Sh and cure data). The consulted database was Reactome. In order to make more sense from the list of pathways we got from ORA applied to Reactome, we built a k-means clustering of them based on their genes. This way we went from a list of pathways arranged by statistic metrics to clusters of pathways, functional related. To do so, we created a function in R, from which we obtained a matrix of pathways x genes, filled up with 1 and 0 for presence and absence, respectively. With this binary matrix we calculated the distance matrix with the binary method and used K-means for clustering (see R-script, annex 1.4).

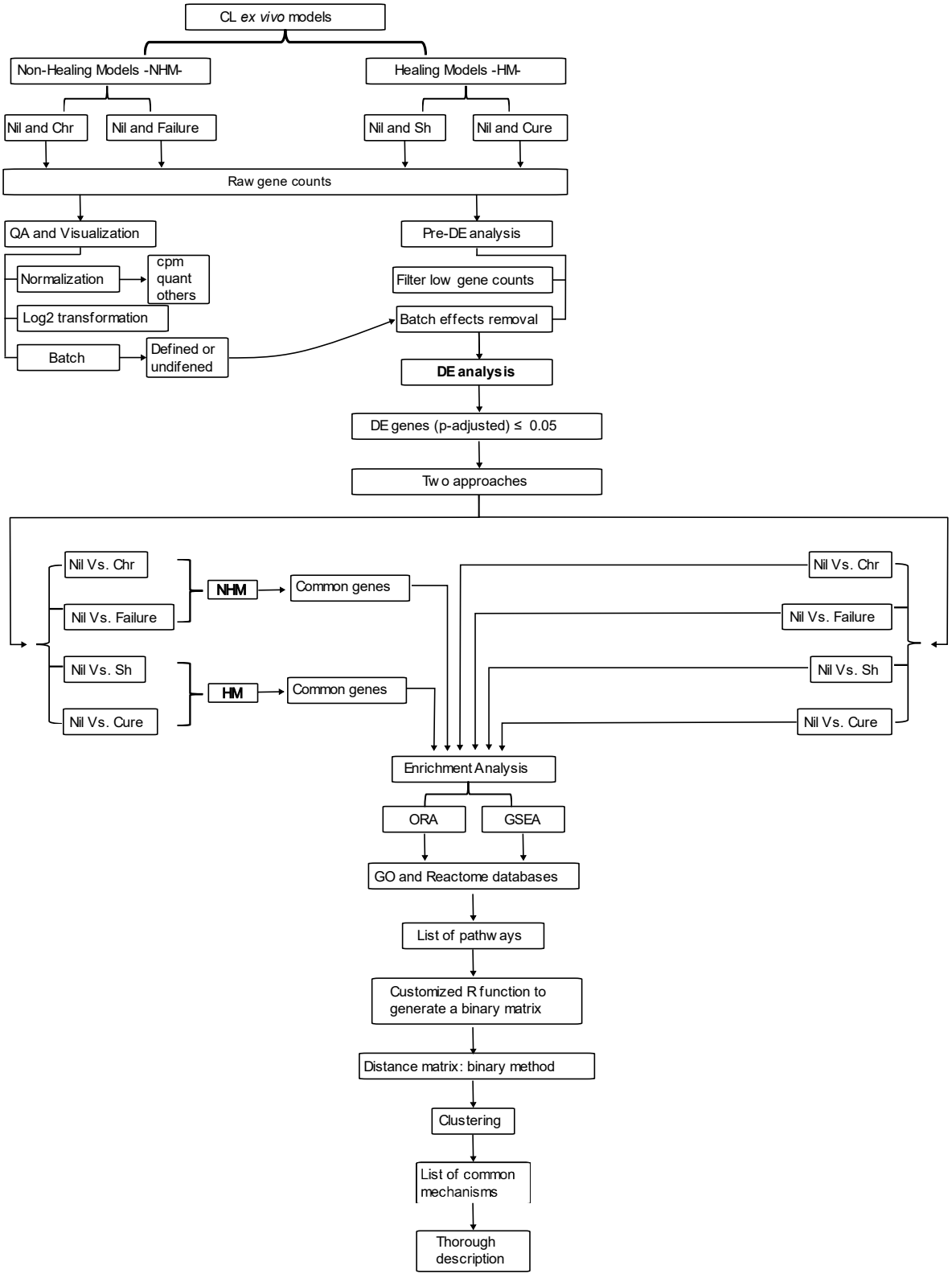

CL ex vivo models
Non-Healing Models -NHM-
Healing Models -HM-
Nil and Chr
Nil and Failure
Nil and Sh
Nil and Cure
Raw gene counts
QA and Visualization
Pre-DE analysis
Normalization
cpm
quant
others
Log2 transformation
Batch
Defined or
undifened
Filter low gene counts
Batch effects removal
DE analysis
DE genes (p-adjusted) ≤ 0.05
Two approaches
Nil Vs. Chr
Nil Vs. Failure
NHM
Common genes
Nil Vs. Sh
Nil Vs. Cure
HM
Common genes
Nil Vs. Chr
Nil Vs. Failure
Nil Vs. Sh
Nil Vs. Cure
Enrichment Analysis
ORA
GSEA
GO and Reactome databases
List of pathways
Customized R function to
generate a binary matrix
Distance matrix: binary method
Clustering
List of common
mechanisms
Thorough
description

Figure 1.3: Methodology pipeline. We used packages implemented in R (version 3.6), such as HPGLTOOLs, and packages from the Bioconductor repository. First, we used the function *normalize_expt*, that normalizes and calculates the log2 of raw data. Next, it accounts for batch effects. We applied two different methods of batch removal and visualized the distribution of the data according to PCA. The method that best allowed us to distinguish between conditions (e.g. Chr from Non-infection, annotated as Nil), was later used for the DE analysis. Afterwards, we filtered out low count genes, and use the batch correction method selected in the previous step. Next, we performed the DE analysis with DESeq2. We then kept the list of statistically differentially expressed genes (p adjusted value ≤ 0.05) in each dataset. Then, we performed pathway analysis (with ORA and GSEA) to: 1. common genes in the datasets of NHMs and HMs, respectively. 2. Each individual dataset. Next, we the list of pathways found represented by the respective gene sets, we built, a binary matrix, followed by a distance matrix, on which we applied the K-means, a clustering algorithm. Later, we looked for common clusters in the NHM and HM datasets, respectively. Finally, we made a through description of some selected pathways.

RESULTS I

Unsupervised clustering of RNA-seq data from NHM and HM datasets

Unsupervised methods of data analysis find underlying patterns in the data. When we apply such methods to RNA-seq data, and plot the output, we expect that such pattern reflects the commonalities in the samples from the same experimental/biological condition and set distance with the samples from another condition. This step is being traditionally applied in RNA-seq data analysis. However, such methods are only accurate when there are sufficient datapoints for the pattern to be exhibited. Having small number of replicates in biomedical research is the trend, not the exception. Situations such as the research budget or intrinsic characteristics to the phenomena under study (e.g. low frequency of patients who failed the CL treatment), limit the possible replicates per condition to be sequenced. Therefore, we should be aware of the limitations and the usefulness of the method and not just apply it to follow convections. Here, we visualized the output (in two dimensions) of applying PCA on the gene count tables, to decide what batch correction method to use before the differential expression analysis. The chosen one, allowed the biggest decrease of the variance intra condition, while maximized the variance inter conditions.

Figure 1.4 shows the PCA plots for the Chr dataset. Among the four options of normalization in the cbc_bc function (from hpgltools, https://github.com/elsayed-lab/hpgltools/tree/master/R), the

cpm method allowed for a better segregation of the samples according to the conditions “Chr and Nil”. We plotted three PCAs: not batch removal method was applied, technical batch variable declared in the study (37) was removed by *limma* function, and unknown batch variables were identified and removed by *svaseq* function. As it can be seen in figure 1.4, when removing unknown batch factors, the pattern outlined by the PCA, fits better the data regarding the experimental conditions. Therefore, *svaseq* batch removal may allow for a more accurate discovery of commonalities (during the differential expression analysis) between the replicates of the Chr condition that are not found in the Nil’s replicates, while at the same time, improve the usefulness of Nil’s replicates as comparative factor.

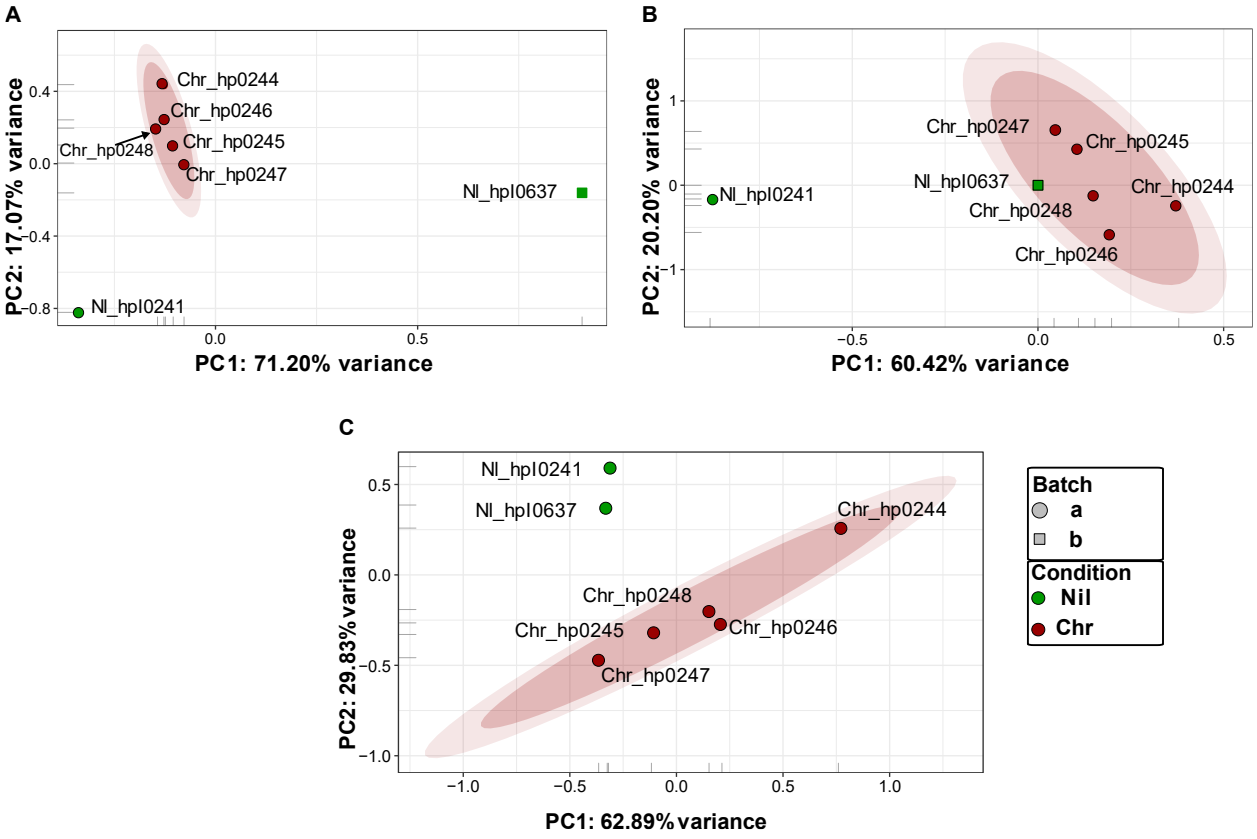


Figure 1.4: PCA plot of RNAseq data from the study of Gomez *et al*.(37). Each experimental sample is represented as a single point with color indicating the condition it belongs to, and shape indicating experimental batch (different technicians processing the RNA extraction). The normalization method applied was cpm. **A.** no batch correction, **B.** *limma* removal of technical batches, **C.** svaseq removal of unknow batches. Ovals represents confidence Interval-CI: 90% (inner) and 95% (outer). Symbols represent samples from each *L. panamensis* strain. Nil is for no-infection and Chr for the macrophages infected with the Chr strains of *L. panamensis* associated with chronic CL.

We followed the same logic for the other 3 datasets. The result for the Sh-Nil dataset was similar to the Chr dataset, we used cpm for normalization and, based on the three plots (figure 1.5), we selected *svaseq* for removal of nuisance factors.

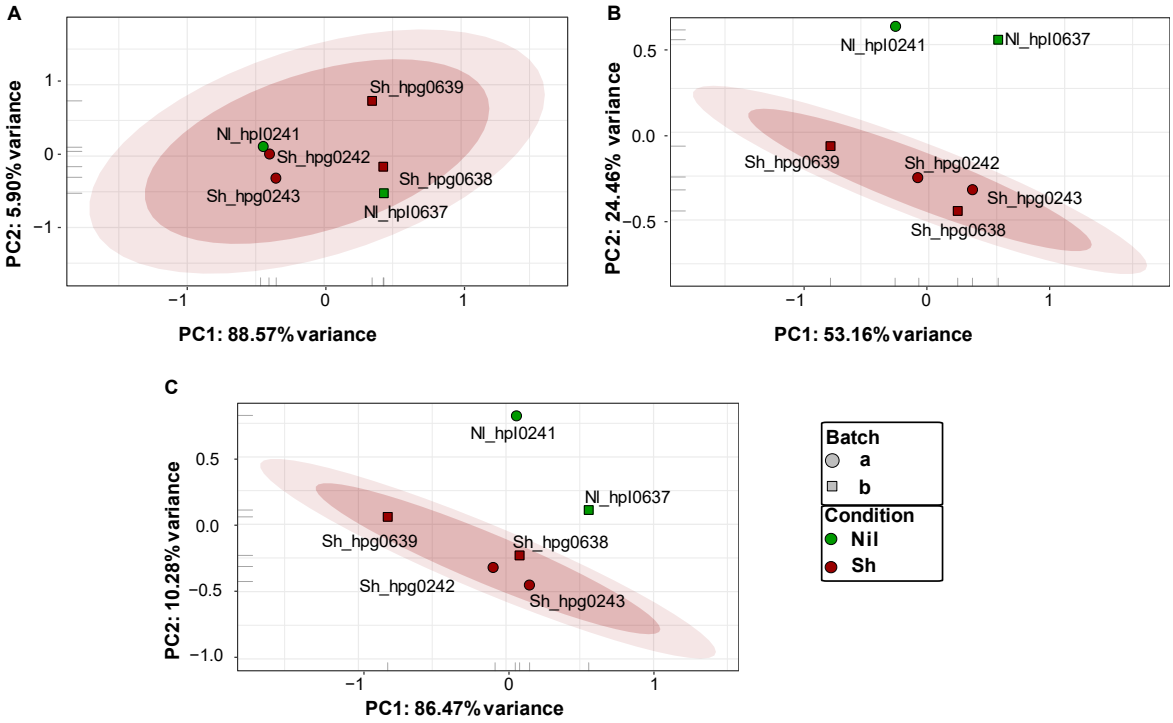


Figure 1.5: PCA plot of RNAseq data from the study of Gomez et al. Each experimental sample is represented as a single point with color indicating the condition it belongs to, and shape indicating experimental batch (different technicians processing the RNA extraction). The normalization method applied was cpm. **A.** no batch correction, **B.** *limma* removal of technical batches, **C.** svaseq removal of unknow batches. Ovals represents confidence Interval-CI: 90% (inner) and 95% (outer). Symbols represent samples from each *L. panamensis* strain. Nil is for no-infection and Sh for macrophages infected with a strain of *L. panamensis* associated with chronic CL.

Failure-Nil (figure 1.6) and Cure-Nil (figure 1.7) both exhibited a similar behavior, which was different from Chr-Nil and Sh-Nil. The quantile normalization method was used to best segregate the clusters given by the conditions “Failure and Nil” or “Cure and Nil”. Like in the other two datasets, *svaseq* was selected as the batch removal method.

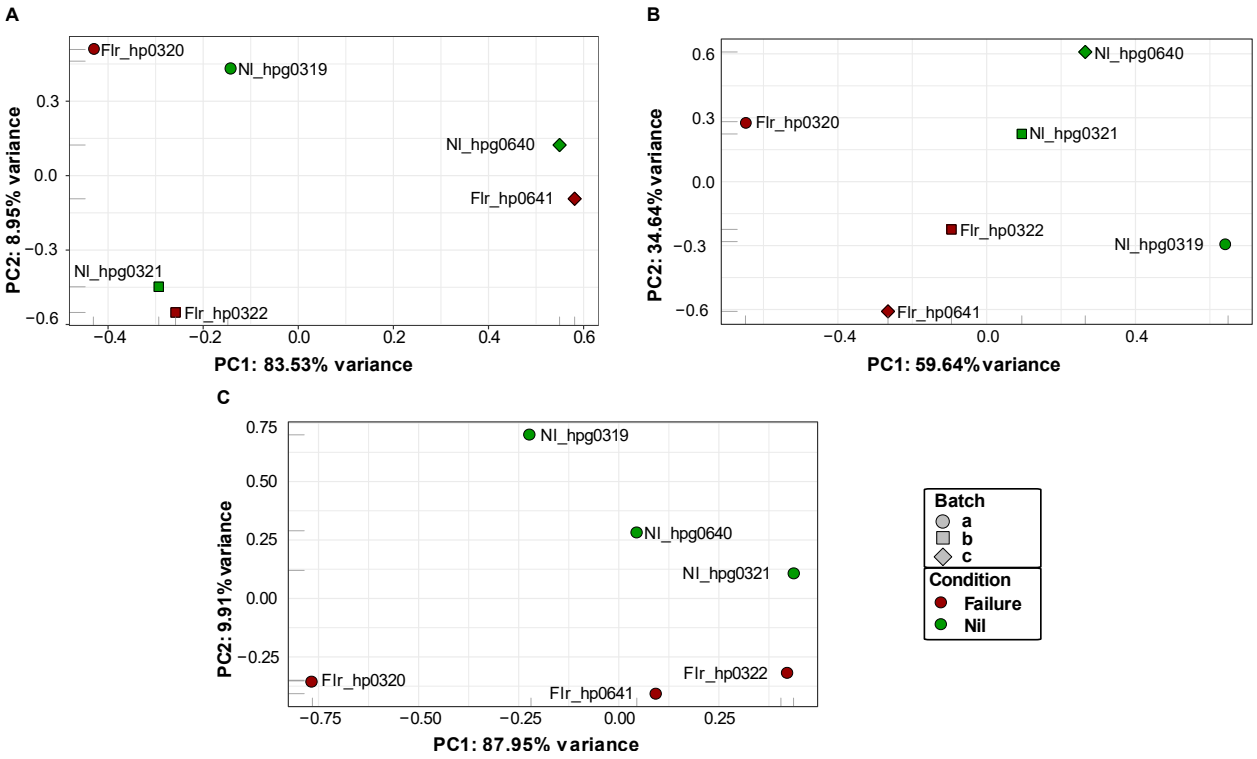


Figure 1.6: PCA plot of RNAseq data from the study of Vargas *et al* (41). Each experimental sample is represented as a single point with color indicating the condition it belongs to, and shape indicating experimental batch (different patients). Nil is for no-infection and Flr for *L.* panamensis-infected macrophages from patients that prospectively failed the CL treatment. The normalization method applied was quantile. **A.** no batch correction, **B.** *limma* removal of technical batches, **C.** svaseq removal of unknow batches. Symbols represent samples from each patient.

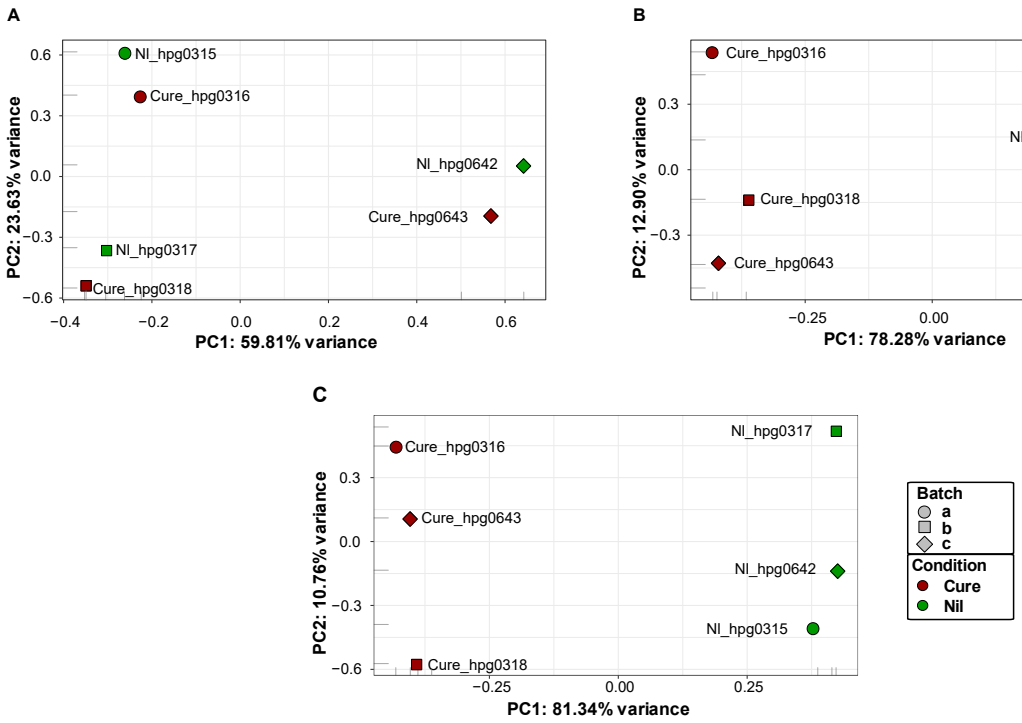


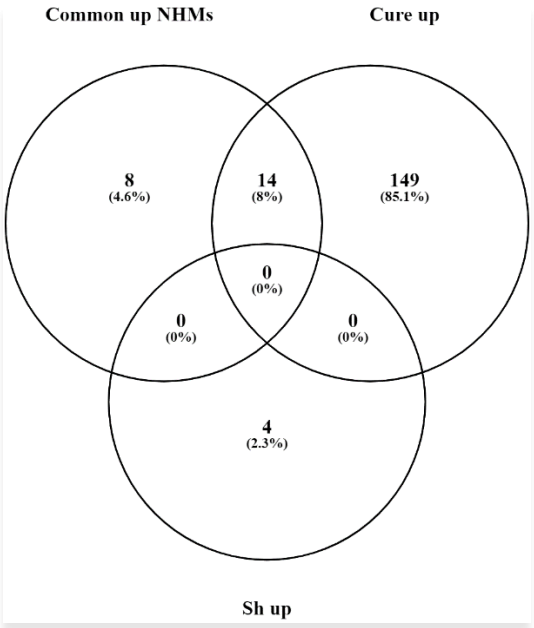
Figure 1.7: PCA plot of RNAseq data from the study of Vargas *et al* (41). Each experimental sample is represented as a single point with color indicating the condition it belongs to, and shape indicating experimental batch (different patients). Nil is for no-infection and Cure for *L.* panamensis-infected macrophages from patients that prospectively Cured the CL treatment. The normalization method applied was quantile. **A.** no batch correction, **B.** *limma* removal of technical batches, **C.** svaseq removal of unknow batches. Symbols represent samples from each patient.

OXPHOS and metabolism of lipids are common pathways across NHM and Cure datasets, albeit driven by different genes.

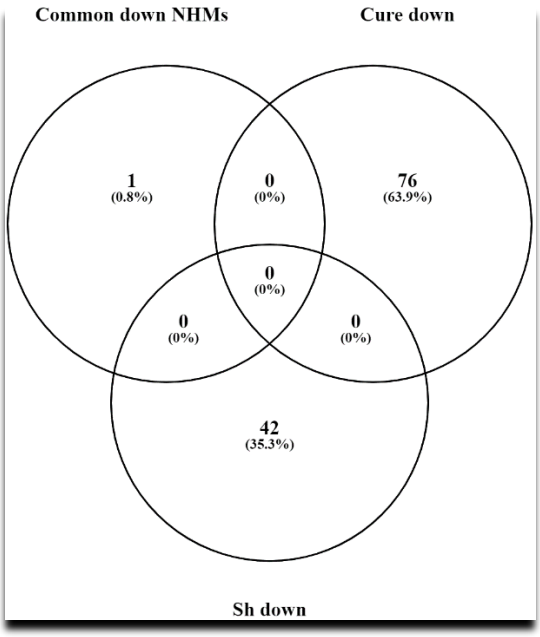
The NHMs had 69 genes in common, from which 21 were upregulated (table 1.1); 8 genes were only shared by the NHMs and not with any of the HMs datasets (figure 1.8-A): *DPYSL3*, *CYP4F11*, *KITLG*, *ARRDC4*, *IL3RA*, *ANKRD22*, *TSKU*, *FBXO30*. Meanwhile, only one was commonly downregulated in the NHMs datasets (table 1.1) and this gene, *CD1*, was not found in the HMs (figure 1.8-B). On the other hand, the HMs had only 2 genes in common, although they did not pass the cut-off of modulation (table 1.1).

Table 1.1: DE genes in NHM and HM datasets. The cut-off for the magnitude of the modulation (up or down) was defined by the absolute value of the log2FC ≥ 1.

	Nil vs. Chr	Nil vs. Failure	NHM	Nil vs. Sh	Nil vs. Cure	HM
Total DE genes	3253	266	69	67	247	2
Log2(FC) ≥ 1	431	162	22	4	163	0
Log2(FC) ≤ -1	236	88	1	42	76	0



A.



B.

Figure 1.8: Venn diagrams showing overlapping genes among the common NHM, Cure and, Sh datasets. A. Upregulated genes and B. downregulated genes.

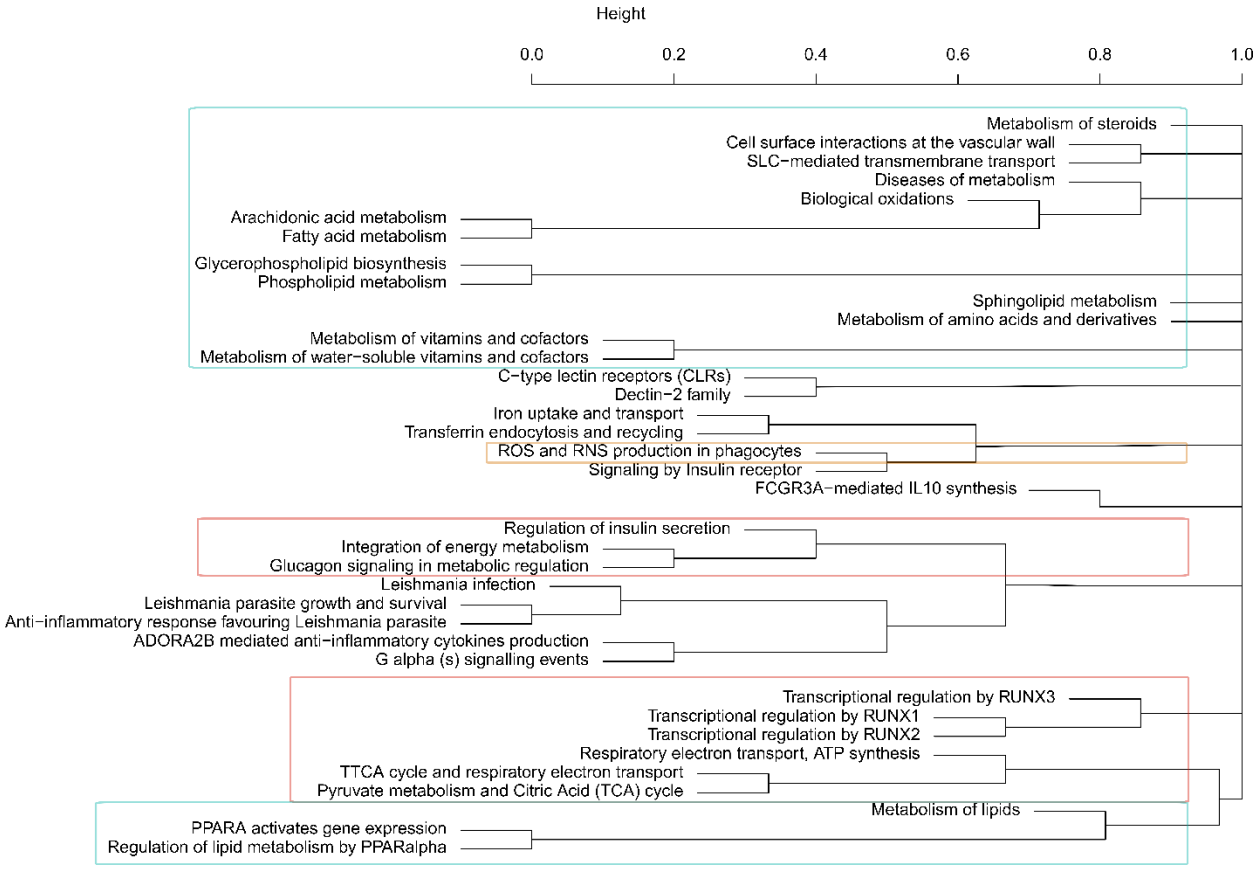
From the lists of upregulated and downregulated genes, we took the ones with a known symbol ID (annex 1) and performed ORA with clusterprofiler (version 3.14.3) in R, for each dataset at the time (annex 2 contains the genes with symbol ID for each of the four datasets). In figure 1.9 we plotted in one graph the over-represented pathways with the adjusted p-values ≤ 0.04 from each dataset. No common pathways, neither up nor downregulated were found in the HM datasets, nor in the upregulated genes in NHM datasets. Only two pathways with the labels: “Regulation of dendritic cell antigen processing and presentation” and “positive regulation of cytokine production”, were commonly found downregulated in NHMs. As many as the ontologies or

pathways found nowadays in biological databases, those two hold very vague names that may be misleading if they are interpreted only based on the label. The former label corresponds to a QuickGO: 0002606 term that states: “Any process that activates or increases the frequency, rate, or extent of dendritic cell antigen processing and presentation”. A wrong, although understandable interpretation, could be the following one: from the input gene list, the genes that matched that category, may be macrophages factors that could activate dendritic cells. However, these pathways/ontologies are curated based on heterogenous data, regarding the biological sources (organs, tissues, cell lines, etc.), methodological approaches, among other factors, that derived them. In other words, so far, the platforms where ORA can be performed to explore these pathways-databases, do not discern the biological source of the data, nor can match the processes that were built upon similar biological conditions as the ones that originated the input gene list (which is an expected goal to achieve by some of these databases through incorporating cell-sequencing data of specific tissues). Therefore, we cannot make inferences only by reading the label of the pathway.

Figure 1.9: List of over-represented pathways from the NHM and HM datasets, as predicted by ORA, using the gene ontology and Reactome databases. The gene ratio associated with each pathway gives the proportion of genes in the input list that are involved in said pathway. This is depicted by the size of the ellipses. The color of the ellipses gives the statistical confidence of the pathway being represented in the input list, using the Bonferroni-adjusted p-value as a metric. A pathway might have a high gene ratio (i.e. the overlap between genes represented in the input list and the pathway is high) but a low p-value. This would occur when the pathway itself involves many genes and only a low proportion (but high absolute number) of which were represented in the input data**.**

Since we did not get much information from the past approach, we attempted to get better mechanistical insights from the up/downregulated genes in the NHMs and HMs datasets, by using a different one. This time, we performed ORA in Reactome database web (annex 1.3), to make sure we were working with the most updated release. Additionally, we filtered by a minimum of 4 genes (from the input list) matched to a pathway. Next, we built a binary matrix of *pathways* times *genes,* (R-script in annex 1.4). This is, the pathway's labels were on the rows, the genes on the columns and, the intersection was fill up with 1 for presence and 0 for absence. From these matrices, we obtained the distance matrices required to perform the k-means clustering (R-script in annex 1.5). In figure 1.10 we showed a subset of the cladograms for the NHMs datasets (complete cladograms in annex 1.6).

A.



B.

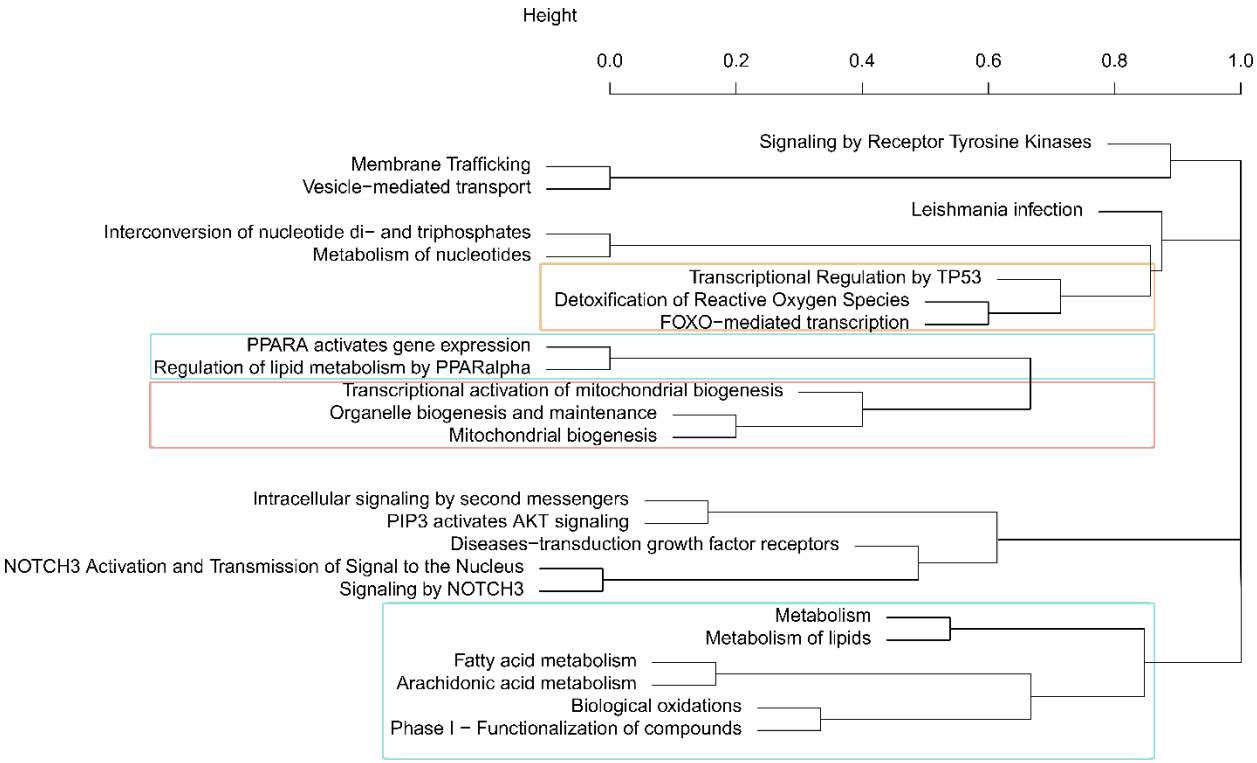


Figure 1.10: K-means clustering of over-represented Reactome pathways in upregulated genes from NHM datasets, A. Chr dataset, B. Failure dataset. Boxes sharing a color correspond to pathways involved in the same high-level biological process. Green: metabolism of lipids, red: mitochondrial production energy couple to the TCA, brown: ROS production.

From the pathways clustering, we were able to underpin a consistent modulation of immune and metabolic pathwyas across the Chr (figure 1.10-A), Failure (figure 1.10-B) and, Cure (annex 1.2) datasets.

- The red cluster in figure 1.10, holds pathways related with mitochondrial-ATP production, OXPHOS, which might be fed with electrons from the TCA cycle.
- The green cluster tells us that the macrophage catabolism might be dominated by lipid biosynthesis (42, 43). Whether the lipids are involved in anti or pro-inflammatory (or both) responses is unclear just from looking at the cluster.
- The brown cluster gives genes associated with the production of reactive oxygen species (ROS). However, it does not shed light on the specific mechanisms activating ROS production.

Addressing a different research gap

We could not identify distinct cellular mechanisms in NHM or HM datasets. By nature of the different infection outcomes, such cellular mechanisms are nevertheless likely to exist. Our failure to find them is potentially attributable to several issues. Firstly, our infection model considered the response of a single macrophage to infection. However, infection outcome depends not only on the response of individual macrophages in isolation, but also on patterns of communication and infection between macrophages, which our model cannot account for. Secondly, our data was collected at a single timepoint (24 hours post infection). It may be that the dynamics of cellular processes before or after this timepoint, distinguish the two infection outcomes within a single macrophage. Thirdly, our data only monitored the rates of transcription of different RNA within the macrophage. Differences in healing outcome could potentially be accounted for by post-translational or epigenetic factors that are independent of transcription rates.

Description of the energetic and inflammatory status during the adaptation state of *Leishmania*-infected macrophages: Anti-oxidative stress factors, Oxidative phosphorylation, TCA and anti-inflammatory mediators.

From the cluster analysis, we got some of the DE genes clustered base on processes related with immunometabolism. Next, we queried the scientific literature to reconstruct the molecular interactions intra (e.g how the function of enzymes and transporters overlapped with each other in the TCA cluster) and inter (e.g how the function of the enzymes in the glycolysis cluster overlapped or influence the enzymes in the TCA cluster) clusters. We complemented our immunometabolic reconstruction with pathways known to be involved in Leishmaniasis, other parasitic infections, or other immunopathologies. We forged a list of seemingly plausible processes and used this to make the reconstruction of the immunometabolic interactions taking place during the adaptation stage of *L. panamensis*-infected macrophages. This is depicted in figures 1.11 to 1.14. We only used the Chr dataset to build this network, because on one hand, the inter-subject variability in the Failure and Cure datasets was too high to infer consistent metabolic pathways at work. On the other hand, in the Sh dataset there were fewer modulated genes.

The magnitude of the expression in the DE gene list comes with the statistical confidence of such differential expression (maximum likelihood estimate of the change in expression). Usually, we describe a gene as up(/down)-regulated if the estimated change in expression exceeds a threshold that by a rule of thumb in the above exploratory analysis, we setup at the absolute value of log2FC = 1. For the following description, we incorporated all the DE genes (annex 1.2, tab: Nil_vs_Chr) to provide a richer list of genes from which to build hypothesized pathways. In particular, having more genes available allowed us to better discriminate between potential mechanisms from the literature involving highly overlapping subsets of genes that were differentially expressed in our data. The extra genes helped to implicate one candidate mechanism over another, based on which mechanism they were better associated with.

Remnants of oxidative stress and an activated glycolytic flux: Cytosolic and mitochondrial ROS production

The onset of the response of infected macrophages is characterized by glycolytic flux driving inflammation and ROS/RN production. The transcription factor H1F1A in part orchestrates these responses, by activating the transcription of many of the key enzymes in the glycolysis to lactate (44). Pyruvate oxidation in the mitochondria is decreased, which decreases the TCA cycle, therefore, carbon availability. NADPH oxidase complex transforms molecular oxygen to superoxide ($O2^-$) which is internalized to the cytosol. ROS production in the cell is optimized by inducing its production in the mitochondria through a process called, Reverse Electron Transport (RET). Based on the study on *Mycobacterium*-infected macrophages by Shi and colleagues (45), this early phase occurs during the first 8 hours.

In our data at 24 hours post-infection (annex 2, tab: Nil_vs_Chr), we found upregulated three subunits of the NADPH complex, *CYBB*, *DOX2* and *NCF2*. Alongside, positive (with *FOS* and *CIR1*), and negative (with *NOTCH3* and *RBPJ*) (46) feedback over the transcription of these genes were captured. Meanwhile, some remnants of the aerobic glycolysis and the RET, but mostly the evidence of their downregulation (figure 1.11) were represented among the modulated genes. On one side, key enzymes of the glycolytic flux were not even found modulated (such as hexokinases, the pyruvate kinase, or the lactate dehydrogenase). On the other hand, we found down-regulated the enzyme phosphofructokinase *PFKM*, that catalyzes the phosphorylation of fructose 6-phosphate (F-6-P) to fructose 1, 6-diphosphate (F-1-6-BP) (consider the “committed” step of the glycolysis). Complementary, *PFKFB3*, an indirect positive regulator of *PFKM* was also found downregulated. The enzyme *PFKFB3* catalyzes the production of F-2-6-BP (fructose 2,6-

bisphosphate), that is an allosteric activator of *PFKM* (47). More interesting, TIGAR, a causative divergent from glycolysis towards the Pentoses Phosphate Pathway is upregulated, together with its activator, *TP53INP1*. Furthermore, the aldolase *ALDOC* (Fructose-bisphosphate aldolase C) that cleaves F-1,6-BP into DHAP (dihydroxyacetone phosphate) and G-3-P (glyceraldehyde-3-phosphate) it is also downregulated (48). The G-3-P that might have been produce few hours after the infection, could be taken up into mitochondria and enhanced ROS production through the RET. In mitochondria, the upregulated G3P dehydrogenase (GPD2), oxidizes G-3-P to DHAP with simultaneous reduction of flavin adenine dinucleotide (FAD) to FADH2 that transfers electrons to coenzyme Q (general abbreviation for the members in the family: CoQ). We found upregulated: *NQO1)* (49). Under high membrane potential, CoQ transferred the electrons to complex II (with *SDHB*, *SDHC* and *SDHD* as the subunits that are positive modulated) or complex I, resulting in the production of superoxide (25, 49). Although mitochondria is considered to be the main source of ROS production in macrophages with an inflammatory profile (25), it is also documented that can be produced during normal respiration (48). Additionally, the enzyme *SDHD* that catalyses the conversion from succinate to fumarate during the TCA cycle, in the presence of NAD+ stop functioning (50). This leads to the accumulation of succinate, than afterwards through an unknow mechanisms, goes to the extracellular space an activate the *SUCNR1* receptor, which signalling is associated with stabilization of *HIF1A* (50).

If uninterrupted, the glycolysis flows to the production of pyruvate, this can be converted to lactate or contribute to the TCA cycle. In highly activated glycolytic conditions, lactate is exported to the extracellular space, to avoid its negative feedback (51). Here, we found downregulated one of its potential transporters, *SLC16A13*. Meanwhile, when pyruvate goes to mitochondria its oxidative decarboxylation by the pyruvate dehydrogenase complex (PDC) links glycolysis to the TCA cycle (25). In congruence with the data supporting a later state after aerobic glycolysis, we found upregulated two members of the PDC complex, DLAT and DLD (with NAD+ as cofactor), that in conjunction with the upregulation of other genes implicated in the TCA (discussed in a subsequent session), supports an activated OXPHOS and a decreased or suppressed glycolytic flux.

We also found *MyCL* and *MYBP* moderately upregulated. However, one of its known cofactors, *TRRAP*, was downregulated. Known genes to be modulated by Myc TF family are downregulated (like *POLG*, *SLC38A5*) except for GSL, or are not modulated at all. Myc is strongly associated with aerobic glycolysis through promoting the transcription of many of the involved enzymes. In fact, its functioning is correlating with reduction of the TCA cycle in the mitochondria (52).

Therefore, consistent with the data, Myc could have played an important role earlier during the infection.

In summary, at 24 hours post-infection, *L.panamensis*-infected macrophages, show traces of an activated oxidative response potentially product of an aerobic glycolysis directed by the *HIFA* TF (moderately upregulated) and perhaps Myc TF. Nonetheless, it seems that at this point they are almost silenced by the activation of negative feedback genes. Moreover, accumulated products such as G-3-P might been contributing to the production of ROS in the mitochondria.

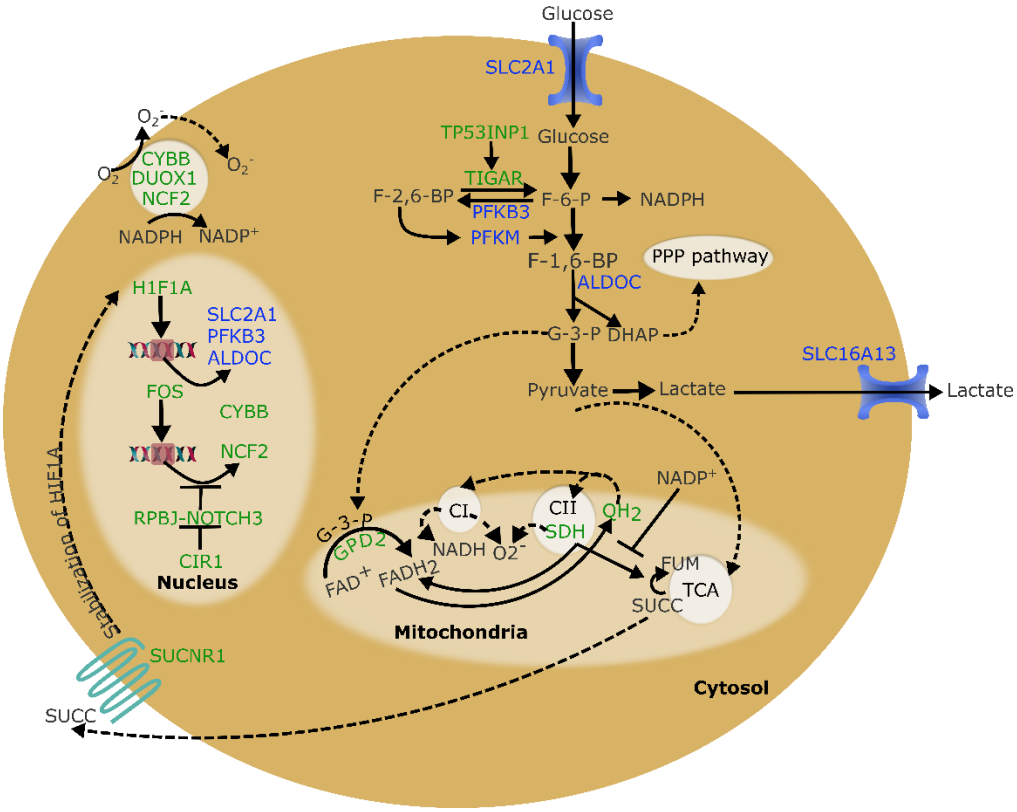


Figure 1.11: *L. panamensis*-infected macrophages at 24 hours post-infection. Controlled modulation of ROS production; positively by the expression of the TF *FOS* and *C1R1*, and with negative feedback of the complex *RPBJ-NOTCH3*. This is occurring during a downregulated glycolysis (due to the negative feedback from *TP53INP1* and *TIGAR*) that probably was activate earlier, as a result of the activation of the TF *HIF1A*. One potential mechanism of activation for the later, must be the accumulation of succinate, that upon interaction with is receptor *SUCNR1* induce a downstream cascade that stabilizes *HIF1A*. The accumulation of succinate occurs due to RET in the mitochondria. Green and blue, indicate up and downregulation, respectively, in the Chr dataset.

Antioxidative defence responses or dampening of killing mechanisms?

Approximately at 24 hours post-infection, transcriptomic analysis of pathogen-infected macrophages indicates the arrival to an adaptation phase (25). One of the characteristics of this phase is the prevalence of anti-oxidative stress responses and ions detoxification. We now describe one mechanism that we ensemble based on the DE genes in the Chr dataset (represented in figure 1.12). The synthesis of the tripeptide glutathione (GHS) leads to the neutralization of ROS. GHS is composed by cysteine (Cys), glutamate (Glu) and glycine (Gly). The Cystine/Glutamate transporter xCT (gene *SLC7A11*) makes up an antiporter system, that takes up Cys in its oxidized dimeric form (cystine) and simultaneously secretes Glu. Cystine is then reduce to Cys as a result of the catalysis by a set of enzymes among which *TXNRD1* is found. Next, the glutamate cysteine ligase, *GCLM*, catalyzes the formation of GSH (25). Afterwards, peroxidases (like *PRDX1* or *GLRX*) catalyze the reaction of GSH with H_2O_2 that renders the reduction of the later to water and the oxidation of the former to GSSG. To ensure the availability of GSH, the glutathione reductase, GSR, reduces GSSG back to GSH. On the other hand, the availability of Cys seems to be optimized through the downregulation of *SLC1A4*, a Cys transporter from the cytosol to the extracellular space. Moreover, to compensate the loss of Glu during the internalization of Cys, we found positively modulate the Glutamine receptor *SLC38A2*. Glutamine is then deaminated in the mitochondria by the enzyme GSL to produce Glu, keeping on track the anti-oxidative response (53).

Glu does not just contribute to the redox balance through xCT, but also by enhancing the transcription of some of the afore mentioned enzymes. Glu is a source for the metabolism of other amino acids such as arginine (Arg). Arg, induces the activation of the transcription factor *NEF2L2*, whose DNA target is the motif ARE, found in the transcriptional units of the genes: *GLRX*, *TXNRD1*, *SLC7A11* and *GSR*.

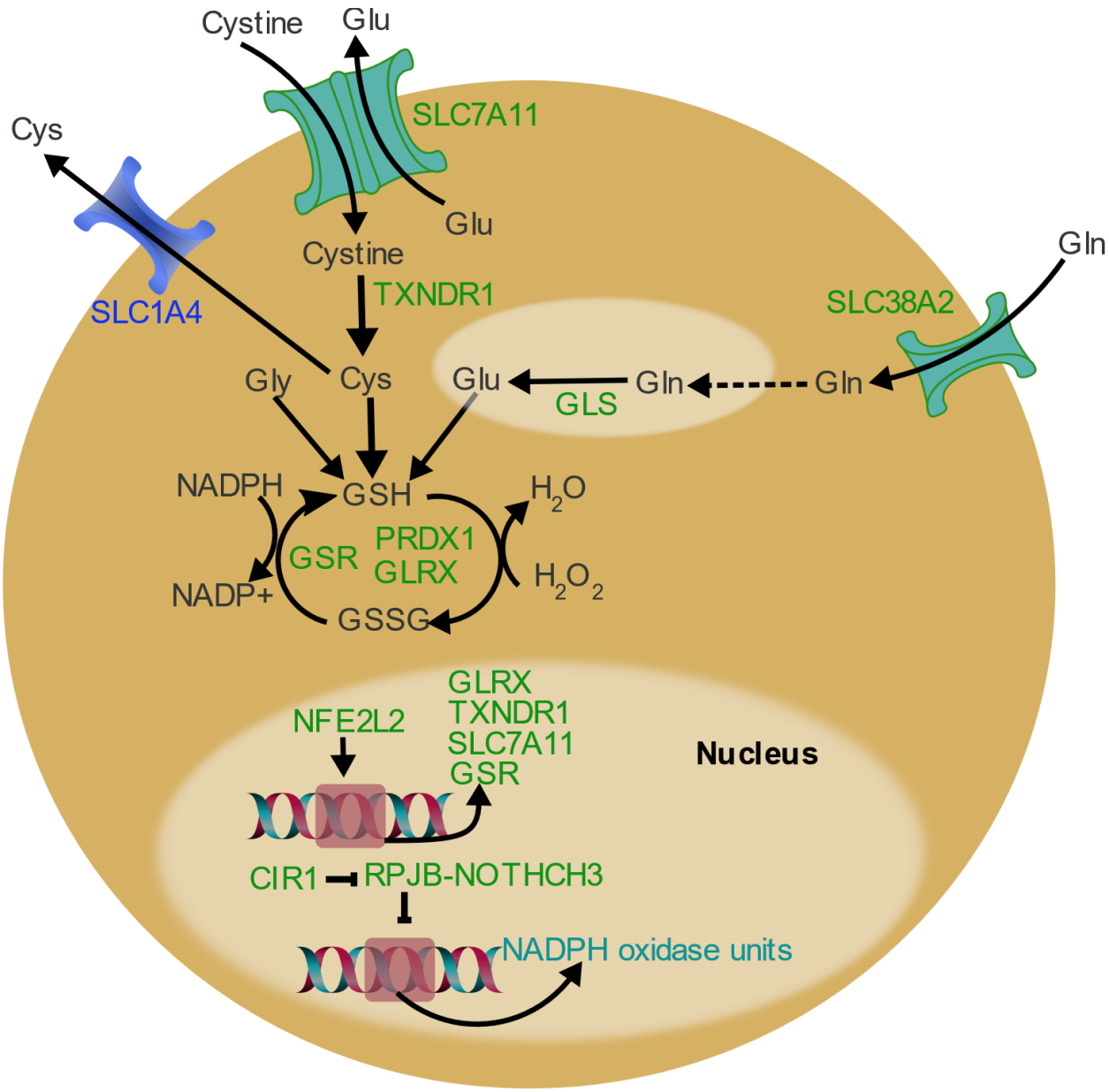


Figure 1.12: *Leishmania*-infected macrophages at 24 hours post-infection. Anti-oxidative stress responses through the production of glutathione (GSH). It is depicted the downregulation of *SLC144*, a transporter of Cys from the cytosol to the extracellular, together with the upregulation of the intake of Gln by *SLC38A2*. Both events, positively impact the production of GSH, ergo, the anti-oxidative stress. Transcriptionally, some of the above transporters and enzymes, are positively modulated by the transcription factor *NFE2L2*. Moreover, *RPJB-NOTHCH3* known to inactivate the transcription of NADPH oxidase units, are negatively modulated by CIR1. Green and blue, indicate up and downregulation, respectively, in the Chr dataset.

Oxidative Phosphorylation and the TCA cycle

As discussed earlier we found upregulated many of the genes involve in OXPHOS and the TCA cycle. On one hand, we found several subunits of the electron transport complexes in the mitochondria membrane (table 1.2), as well as some enzymes involve in the TCA cycle. In figure 1.13 we plotted the metabolic interactions. The TCA cycle begins with the acetyl-CoA, that can be synthesized in the cell or imported through transporter *SLC33A1*. Acetyl-CoA is combined with oxaloacetate to generate citrate. In the second step, citrate is converted into its isomer, isocitrate. The cycle continues with the decarboxylation in which isocitrate is converted into α-ketoglutarate (α-KG) with the generation of NADH (54). α-KG can be produce by two additional reactions: in mitochondria, *GLUD1* (glutamate dehydrogenase 1) catalyzes the conversion of Glu to α-KG (53). In the cytosol, *IDH2* (isocitrate dehydrogenase) catalyzes the oxidative decarboxylation of isocitrate to α-KG that can be transported to mitochondria and fuel the TCA cycle (55). α-KG can be found in the cytosol, due to the conversion of citrate to isocitrate by the enzyme *ACO1* (aconitase 1) (56). Back in the mitochondria, α-KG suffers oxidate decarboxylation that produces succinyl-coA. Next, the succinyl coenzyme-A synthetase (in figure 1.13 represented by the subunit *SUCLA2*) catalyzes the reversible reaction of succinyl-CoA to succinate (57). Subsequently, the enzymatic complex *SDH*, catalyzes the oxidation of succinate that generates the metabolite fumarate, alongside the transferring of two hydrogen atoms to FAD, producing $FADH_2$. Moreover, as mentioned earlier, *SDH* conforms the complex III in the electron transport chain (54). Afterward, the fumarate hydratase catalyzes the conversion of fumarate into malate than further is converter to oxalacetate (closing the TCA cycle) (54). During limited glucose conditions, the enzyme NAD-dependent malic enzyme, *ME2*, catalyzes the oxidative decarboxylation of malate to pyruvate, ergo, increasing TCA cycle flux. Finally, the electrons released from NADH and $FADH_2$ are eventually transferred to O_2, forming H_2O as shown in figure 1.13. The phosphorylation of ADP to form ATP is driven by the generation of the proton-motive force during the electron transport (58).

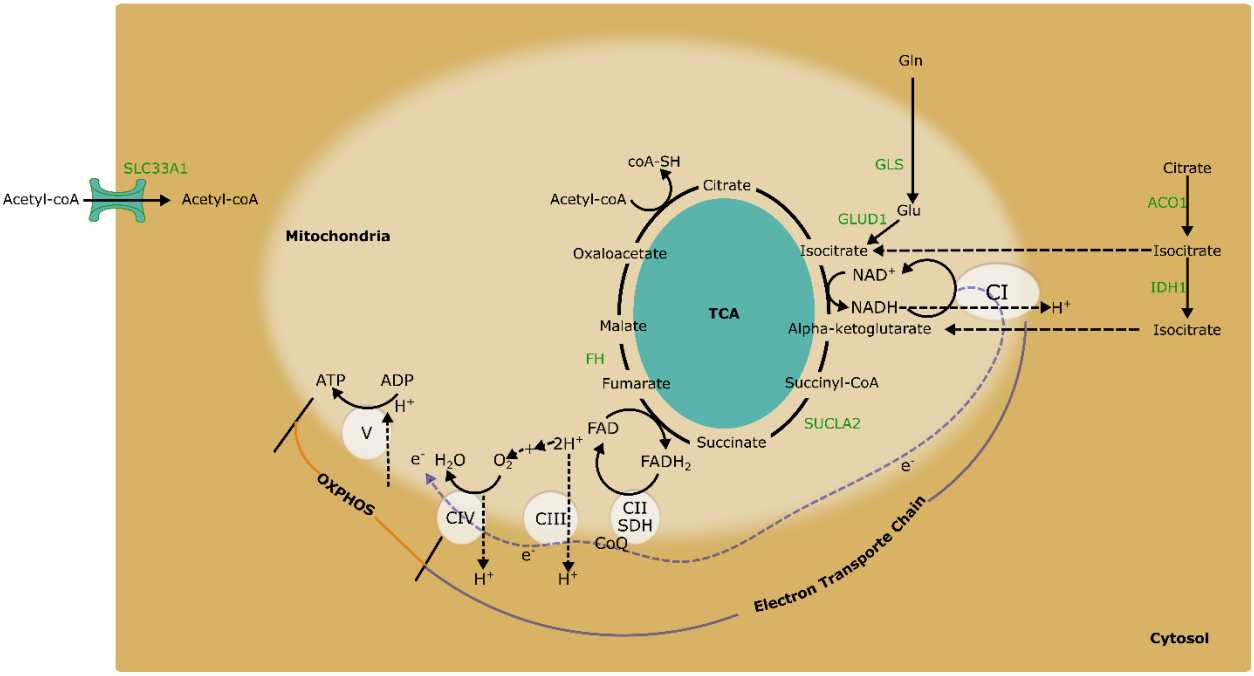


Figure 1.13: Enzymatic reactions in the TCA cycle, Electron Transport Chain, and the OXPHOS, in *Leishmania*-infected macrophages. The equivalent reducers NADH and $FADH_2$ are formed through the TCA cycle. Both are required to transfer electrons to the mitochondrial respiratory chain. The respiratory chain is composed of five complexes (CI, CII, CII, CIV and CV), the upregulated subunits from each one, found upregulated in our analysis are listed in the table 2. As the electrons are funnelled through the complexes in the inner mitochondrial membrane, a mitochondrial membrane potential is generated with the transfer of protons (H^+ ions) across the membrane. This membrane potential powered the synthesis of ATP. Green indicates upregulation in the Chr dataset.

Table 1.2: Expression magnitude of subunits of the electron transport chain, found modulated in the Chr dataset

Log_2FC	Gene symbol ID	Complex type
0.93	NDUFAF5	CI
0.90	NDUFAF6	
0.87	NDUFAF4	
0.61	NDUFB5	
0.48	NDUFB1	
0.40	NDUFA6	
0.40	NDUFA4	
0.39	NDUFA12	

1.16	SDHB	CII
0.36	SDHC	
0.55	SDHD	
0.71	CYCS	CIII
1.20	UQCRB	
0.53	UQCRC2	
0.68	COA	CIV
0.63	COX7B	
0.52	COX11	
0.33	COX5A	
0.63	ATP5MD	CV
0.58	ATP5PB	
0.50	ATP5F1C	
0.48	ATP5PF	
0.47	ATP5MPL	
0.46	ATP5PD	
0.43	ATP5MC3	

Another event (not depicted in figure 1.13) that also contributes to OXPHOS, is the mitochondrial fusion. Through this, the mitochondria keeps the morphology of the cristae, the folds in the inner membrane that extend into the matrix, increasing the functional surface area to produce O_2, required for OXPHOS (59). Although, one of the consequences of an activated HIF1A, is the occurrence of a process called mitochondrial fission, we found upregulated some of the factors that counteract the fission. Firstly, the mitochondrial fission is characterized by a disruption in the morphology of the organelle, where the cristae is broken. The above enhances some of process depicted in figure 1.13, like RET, and ultimately, ROS (60). Nonetheless, the presence of the HIF-induced protein, HIGD1A, BNIP3L (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like), OPA1 (Dynamin-like 120 kDa protein), indicates that rather than fission, the mitochondria is going through fusion. During fusion the tubular networks of the cristae are form/repair, favoring OXPHOS and ATP production (60).

Synthesis of bioactive lipid mediators.

Lipids and fatty acids are abundant elements in the plasma membrane where they fulfill a structural role. Non-structural lipids, whose turnover, has cellular functional impact, are called bioactive (61). Bioactive lipid metabolism is a common target among macrophages-intracellular parasites (62, 63). The liberation of lipids from the plasma membrane depends upon the catalysis of phospholipases whose activation are regulated to some extend by Ca^{2+} concentration (64). In the Chr dataset, we found upregulated the gene for a soluble phospholipase (sPLA) called, Group IID secretory phospholipase A2 (*PLA2G2D*). Depending on the substrate, sPLA may be involved

in the production of both pro and anti-inflammatory bioactive lipids (65). *PLA2G2D* in particular has been found expressed in macrophages with an anti-inflammatory profile, associated with the mobilization of omega-3 polyunsaturated fatty acids (PUFA) (66). There are three types of omega-3 PUFA the importance for humans: α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). As stated by Gutierrez and colleagues (67), their anti-inflammatory effects in infected macrophages has been broadly studied. It is not clear if *PLA2G2D* can also fulfill cytoplasmatic functions converting structural lipids to bioactive lipids (68). Complementary we found a negative feedback of phospholipases, the acyl CoA synthase long-chain family members, *ACSL1* and *ACSL4*. They lead the incorporation of long-chain fatty acids, like the arachidonic acid (AA), into membrane phospholipids (25).

When PUFA are released from complex membrane or storage lipids, they can be transformed to bioactive lipid mediators. Among them: arachidonic acid, eicosanoids, endocannabinoids, and sphingolipids. Importantly, the bioactivity of catabolized lipids depends to a great extent on the prevalence of the precursor PUFA and the bioavailability of the enzyme (64). For instance, we found upregulated the enzyme *CYP4F11*, which can have as substrate the precursors: EPA and DHA, which as mentioned before, are associated with anti-inflammatory responses. Nonetheless, CYP4F11 can also act on arachidonic acid PUFA, giving place to the bioactive lipids: HETE (hydroxyeicosatetraenoic acid) that has been associated with inflammatory conditions (69). But also, it can give rise to EET (epoxyeicosatetraenoic acid) that has anti-inflammatory effects on blood vessels and in the kidney, promote angiogenesis, and protect ischemic myocardium and brain (70). Moreover, the enzyme LTAH4 can catalyze the conversion from EET to DHET (dihydro-eicosatetraenoic acid), which is a more stable metabolite, but is less anti-inflammatory (64).

We also found evidence that other types of lipids, the sphingolipids, are being metabolized in macrophages infected with *Leishmania*. These lipids are defined by their amino-alcohol backbone. We found enzymes involved in the novo-synthesis of sphingolipids. Beginning by the action of the serine palmitoyltransferase (we found upregulated the Serine palmitoyltransferase small subunit A, SPTSSA and SPTL1), continuing with the conversions that renders the sphingomilin by a series of reactions catalyze by: KDSR 3-keto dihydrosphingosine reductase, *DEGS1* Sphingolipid delta(4)-desaturase *DES1*, *SGMS2* Phosphatidylcholine:ceramide cholinephosphotransferase 2 (64) .

The overall anti-inflammatory status in the L. panamensis-macrophage interaction is guarded by pro-inflammatory receptors.

TLR5 and *TLR4-LY96* downstream signaling cascade activates the TF *NF-kB*, that is a key positive regulator of pro-inflammatory mediators in pathogen-infected macrophages. Additionally, *TREM1* and *CLEC10A* receptors which shares many downstream signaling molecules with *TLR5*, were also found upregulated (71, 72). We found two of the downstream signaling molecules upregulated, *SYK* and *MAP4K3*. However, many of the reported downstream signaling molecules were downregulated, like *IRAK2*, *TRAF1*, *TRAF4*, *TRAF5*. Moreover, the inhibitors of TLR signaling, *IRAK3*, *TIFAB* and *NLRP12*, were upregulated (73–77). Consequently, and despite the upregulation of the mentioned receptors, two of the NF-kB subunits, are highly downregulated, *NFKB1* and *NFKB2*, while *RELA* is moderately downregulated. However, we can see some of the NF-kB targets still upregulated, like *ANKRD1* (78). *ANKRD1* is a nuclear DNA-binding protein commonly reported induced by inflammatory cytokines in human dermal vascular endothelial cells. Nevertheless, its activation has been associated with repression of MMP (matrix metalloproteinases) proteins. This means *ANKRD1* negatively regulates inflammation, trough the reduction of the anabolisms of the extracellular matrix (79, 80)

Contributing to the maintenance of an anti-inflammatory status, we found *CCL24* (C-C motif chemokine 24) upregulated. CCL24 is a chemokine that in leishmaniasis has been involved in allowing infected macrophages to keep an anti-inflammatory profile even in an inflammatory environment (5). CCL24 is induced in macrophages as a result of the stimulation of IL-10 and IL-4. CCL24, chemoattracts eosinophils which in the study by Lee and colleagues, were found to be an important source of IL-4, that keeps the loop of *CCL24* production by *Leishmania*-infected macrophages (5). Complementary, the chemoattraction of eosinophils seems to be enhanced in the Chr model, through the secretion of the C-C motif chemokine 7 (*CCL7*), which can also serve as a recruiter of that type of immune cell (81). One of *CCL7* receptors was found upregulated, CCR5, which transduces a signal by increasing the intracellular calcium ion level. We can track in the literature of *CCL7* and CCR5 links to inflammatory process (82), however, the signaling *CCL7-CCR5* might complement anti-inflammatory responses(81, 83) Complementarily, we found upregulated the long non-coding RNA, *CCR5AS* that positively regulates the expression of *CCR5* in $CD4^+$ T-cells in response to HIV infection (84).

On the other hand, we found upregulated the purinergic receptors *P2RY13*, *P2RY14* and *P2RX7* which are involved in signals induced by ATP. A broadly documented event that occurs during the early or acute response to infection or injury, is the release of ATP by immune cells, including

macrophages. Once in the extracellular milieu, it can activate purinergic receptors that will promote pro-inflammatory responses. In leishmaniasis ATP-P2X7 signaling allows for the elimination of *L. amazonensis* in infected macrophages during the early response. Nonetheless, nucleosidases in the membrane can act as negative regulators of this inflammatory process, through catalyzing the conversion of ATP all the way up (ADP to AMP) to ADO (adenosine). Furthermore, ADO can act as a ligand for adrenergic receptors such as *ADORA2B*, whose downstream effects contributes to build or sustained, an anti-inflammatory environment. In the Chr dataset, we found upregulated *ADORA2B* alongside with some of its downstream signaling molecules, *PRKACB* and *CREBL2*. *CREB* TFs are known for activating the transcription of anti-inflammatory molecules, like IL-10, that was also found upregulated.

The above indicates that at 24 hours post-infection, macrophages infected with strains of *L. panamensis* causing chronic CL, present an anti-inflammatory profile that is in congruent with an OXPHOS metabolism. However, the over-expression of receptors such TREM1, CLEC10A, TLRs and purinergic receptors, reflects the susceptibility of the macrophage to shift towards a pro-inflammatory state, based on the interaction with the recruited inflammatory cells.

DISCUSSION I

We could not find distinct pathways to non-healing or healing models in *L. panamensis*-infected macrophages. Instead, we pin down processes that were consistently modulated across three out the four datasets (the Sh dataset did not have enough genes to do the enrichment analysis) representing the above mentioned CL outcomes. These results showed that *L. panamensis* perturbs immunometabolic pathways in their macrophage host cells. Therefore, we moved our focus to characterize an immunometabolic network of the macrophage response at 24 hours post-infection with *L. panamensis*. To do so, we took the extensive list of differentially regulated genes in the Chr dataset and thorough scientific literature survey, we match them with genes involved in immune and metabolic pathways. As a result, we built a hypothetical network of immunometabolic interactions consistent with the transcriptome data.

Our hypothetical immunometabolic network serves a variety of uses. Firstly, specific proposed interactions can be tested by reductionist experiments, eventually allowing for complete verification of the network, or modification of those interactions not shown to be supported by experiments. The network also suggests candidate mechanisms of particular importance in

sustaining the adaptation stage of the host-parasite interaction. If these mechanisms are verified, then their drug-induced disruption offers a path to CL treatment.

One issue with our scientific predictions is that they are underconstrained by the analyzed data. RNA-seq data does not unambiguously determine the production of the actual proteins involved in the biological pathways of interest. Even if it did, our data only provides a static snapshot of the immunometabolomic interaction network. However, the interplay between host and pathogen is inherently dynamic, and a full understanding of the different possible paths of pathogenesis (as well as how they can be clinically disrupted) must take account of this. Encouragingly, we were able to use our data to make predictions on the state of the interaction network at earlier stages of the infection, and these predictions matched conclusions from the literature. How was this possible? We identified not only pathways directly involved in immunometabolism, but also the modulators of such pathways. This allowed us to predict whether such pathways were in the process of being up or downregulated. Illustrating this was the glycolysis pathway, which was downregulated in our data, as an example. The TF *HIF1A* was found moderately upregulated. This TF is a potent inductor of glycolysis by promoting the expression of glycolytic enzymes (85). This suggests that glycolysis must have been upregulated at an earlier point of the infection. Indeed, TIGAR and TP53INP1 inhibitors of glycolysis were strongly upregulated, further supporting the case for a recent downregulation of glycolysis. This is consistent with the literature: a unifying feature of studies of the early stages of host-pathogen interactions is that glycolysis is strongly upregulated (23).

Our interaction network serves as a tool to understand different aspects of the *Leishmania*-macrophage interaction. As mentioned previously, our network motivates a host of validation experiments. It can also serve as a base for asking relevant scientific questions. One notable feature of host-parasite interactions is that parasites commonly hijack parts of the host metabolism for their benefit. As an example, the activation of pathways that favor the survival of infected hosts allows the parasite a stable medium from which to spread. Reductionist experiments can identify the particular immunometabolic targets of parasite-derived factors. By investigating the role of such targets within our interaction network, one could ideally identify specific ´hijacked´ mechanisms operating for the parasite´s benefit and distinguish them from defence mechanisms intrinsic to the macrophage.

CONCLUSIONS I

Our analysis reveals that immunometabolic pathways are modulated in *Leishmania panamensis*-infected macrophages. Moreover, it gives clues that a glycolytic efflux was early activated and that a potential metabolic reprogramming conduced to an OXOPHOS metabolism. Lipid metabolism might be producing both, pro and anti-inflammatory bioactive lipids. A glutathione-based ROS-scavenger system is active, and this is probably allowing parasite survival. An extra benefit for the parasites comes with the production of anti-inflammatory cytokines and chemokines, such as IL-10 and CCL24, respectively. These have already been shown to benefit parasite survival in *in vivo* infection models with other *Leishmania* species (5).

STRENGHTS AND LIMITATIONS I

RNA-seq data constitutes an unbiased and holistic approach to study *L. panamesis*-infected macrophage systems. This allowed us to make a broad description of immunemetabolic processes taking place 24 hours post-infection. The generalizability of our findings is limited by several factors. First, our description and hypothesis are done on the basis that all the transcriptome is translated and fully expressed as proteins. Additionally, we described the molecular function of a given heterodimer or a molecular complex, based on the presence of a subunit(s) in the RNA-seq data. We addressed these limitations by an extensive literature survey supporting the tailored immunometabolic interactions and their reported occurrence in different infection models. Nonetheless, as we mentioned before, our description urges reductionist experiments to validate the existence of the specific interactions. Another limiting factor is that we made the description based on the Chr dataset that only accounts for the genetic background of one human donor. Although the macro-processes described have also been reported in other infectious models, the factors that we describe here supporting them must be confirmed by low-level molecular methodologies.

FUTURE WORK I

Network-validation: We built a description of the molecular interactions underlying the immunometabolism of *L. panamesis*-infected macrophages based on RNA-seq data. However, this type of data comes with intrinsic limitations, as every mRNA follows downstream regulatory steps that might allowed them or not to be expressed as functional proteins. Therefore, in future work we should use protein and metabolic assays to validate the occurrence of specific processes. For instance, we may validate glutathione as a critical ROS-scavenger system that

supports parasite persistence. First, we may validate the production of glutathione and evaluate the existence of a correlation between its production and both, ROS production and parasite load.

Earlier predictions: Our analysis makes predictions regarding earlier (before 24 hours) activated immunometabolic pathways, such as glycolysis, a higher concentration of TFs such HIF1A, accumulation of succinate, among others. One way to validate these predictions would require molecular assessment at earlier points in the infection.

Dynamic understanding: We believe that a dynamic understanding of the *Leishmania*-macrophage system is required to better understand how the immunometabolic programming is occurring.

Parasite perspective: Alongside characterising the immunometabolic network in the macrophage, we may also integrate the parasite's molecular factors. Are there *Leishmania* parasites secreted factors influencing the macrophage reprogramming?

ANNEX I

1. **Folder: Differential expression analysis:** Contains 4 folders with R scripts, raw count data, and annotated DE gene list for each of the 4 data sets.
2. **DEgenes_in_Chr_Failure_Sh_&_Cure_datasets.xlsx**
3. **ORA-Reactome_web_results.xlsx**
4. **Binary_matrix**
5. **Clustering_Pathways_NHM_and_HM**
6. **Cladograms**

CHAPTER 2: Dynamics of the mechanism of a potential immunometabolic switching in macrophages infected with *Leishmania*

ABSTRACT II

The high functional and metabolic plasticity of the macrophage is re-emerging as a fundamental characteristic for studying intracellular microbial infections. Recently, studies on macrophages associated with tumours and bacterial infections have been consistently showing a dynamic reprogramming in their metabolism and immune profile. This phenomenon has not been explicitly studied for *Leishmania*-infected macrophages, although individual studies seem to be suggesting it. We consider that it is important to understand the basis of the immunometabolism rewiring in macrophages to predict how it can be manipulated by pathogens trying to establish their optimal growth conditions. To this end, our work aims to shed light on the dynamics of the immunometabolism in *Leishmania*-infected macrophages. We built a simplified structure of the TLR4 signalling pathway, in which we represented the link between its activation and the immune and metabolic functions in the macrophage. This included the regulation by the transcription factors: *HIF1A*, *STAT3*, *CREB*, *NFKB*, and *NFE2L2*, as well as the crosstalk with the purinergic signalling pathways. The quantitative data came from the fold-change of expression derived from transcriptomics applied on *Leishmania*-infected macrophages *ex vivo* models. Our model exposes, for the first time for *Leishmania*-infected macrophages, an immunometabolic switching in the host cell, during the first 24 hours of infection. In the period in which the cellular bioenergetics shifts from glycolysis to oxidative phosphorylation, the redox balance is achieved, possibly through increasing glutathione synthesis, and anti-inflammatory cytokines predominate over the inflammatory cytokines. Our model helps to explain discrepancies found in the literature regarding TLR4 and its association with resistance or permissiveness to the infection. Overall, our model exploited transcriptome data to build a dynamic mechanism of the immunometabolic switching in *Leishmania*-infected macrophages.

INTRODUCTION II

Metabolic reprogramming in macrophages is becoming the concept that helps to explain the old M1/M2 macrophage polarization paradigm (86, 87). As opposed to present an “M2” phenotype,

now there is more evidence that tumour associated macrophages present a dynamic rewiring immunometabolism that changes upon their localization in the tumour environment (88). Similarly, in bacteria-infected macrophages it has been shown that upon infection the metabolism and immunological profile, recalls more the M1 polarization type. However, as time goes by, the same infected host cells rewired their metabolism towards an M2-like phenotype (23, 89). For *Leishmania*-infected macrophages there are few studies addressing the mechanistic bases of this phenomenon. Moreira and colleagues proposed a bioenergetic switching mechanism based on a few molecules and they do not connect it with the immune profile of the macrophage (16). Although more studies designed to explore the basis of this rewiring are required, some previous literature give some clues about it might be happening (4, 90, 91). Notably, transcriptome data from *ex vivo* models (18–20), as well as our results described in the Chapter 1, seem to be supporting that an inflammatory and metabolic profile of *Leishmania*-infected macrophages undergoes a switch during the 24 hours post-infection. The problem is transcriptomics data provides an incomplete characterisation of the switch, as it does not point to the underlying metabolic interactions, and only provides data at a few, sparse timepoints. In this work, we amalgamated known immunometabolic pathways from the literature, and combined them with transcriptomics data from 4 *Leishmania*-infected macrophages studies (18–20) and Gomez *et al.* (37), to build a dynamic model of the aforementioned switching behaviour.

Classically, Toll-like receptors (TLRs) have been recognized as the hallmark of the activation of an early pro-inflammatory response in pathogen-infected macrophages (92). Their activation leads to the activation of inflammatory mediators such as NF-kB (nuclear factor kappa-light-chain enhancer of B-cell), IL1-β (interleukin 1 beta) and TNF-α (tumour necrosis factor-alpha) (92, 93). This activation might be boosted due to the crosstalk between TLRs and purinergic receptors, such as P2RX7 (94). These pro-inflammatory factors are meant to be involved in parasite contingency, by activating killing mechanisms in the phagocytic cell, and recruiting more inflammatory cells. However, there are several functional studies in *Leishmania* infection models, which consider that the activation of specific TLRs confers susceptibility (95–99) or resistance (100–102) to the infection. How is this possible? First, such associations are highly time-dependent (93, 95, 103). The discrepancies might resolve if they are carefully examined and contrasted based on the infection time point where the measurements are taking place.

Additionally, activated TLRs also contribute to the metabolic rewiring in macrophages through the downstream activated transcription factors (TFs) (104, 105). For instance, TLR/ NFkB supports the stabilization of the TF HIF1A (Hypoxia-induced factor 1 alpha), the master TF for the glycolytic

enzymes. This conduces to a shift towards a glycolytic-based bioenergetic, that otherwise depends upon the ATP generated through the oxidative phosphorylation (OXPHO) in the mitochondria. Besides, downstream activation of TLRs also impacts the metabolic redox balance, by contributing to the activation of both oxidative and anti-oxidative mediators (106, 107).

Overall, TLRs constitute a central core for the immune and metabolic functionality of the macrophage. With such importance, it is to expect that its signal is tightly and dynamically regulated with different negative feedback loops over time (108). We hypothesize that the apparently contradictory findings of TLR either supporting or not Leishmania growth in macrophages, can be explained if we study the *Leishmania*-host system from a holistic and dynamic perspective. Moreover, based on the literature mentioned above, we believe that by modelling the dynamics of TLR and its influence on the immunometabolic status of the macrophage, we might provide a mechanistic representation of the metabolic switching. Our model makes explicit predictions on the dynamics of (anti-) inflammatory and metabolic agents over 24 hours, post-infection. It provides a minimal set of interactions that are sufficient to explain the metabolic reprogramming of macrophages from a pro-inflammatory, to an anti-inflammatory state, oxidative to anti-oxidative status, and from glycolysis to OXPHO metabolism, in agreement with the transcriptomics data.

MATERIAL AND METHODS II

2.1 Basis of the structure

TLR4 is activated in macrophages upon *Leishmania* spp. infection (109). Among its downstream activator molecules there are: TRAF6, MAPK and a set of TFs such as NFKB, CREB, HIF1A, NFE2L2 and FOS (110). The TF STAT3 has been described as key regulatory element in TLR4-mediated metabolic switching (111). Thus, we integrated STAT3 through the well-known pathway IL10-STAT3 (112, 113). From each TF we included the activation of an effector molecule, either a cytokine (IL1B, TNFA, IL10 or CCL24), an enzyme (NADPH oxidase subunit, NT5E, QH2, HSK3 representing glycolysis or SUCLA as an indicator of succinate production and TCA dynamics, ATP5B representing mitochondrial ATP production or TXNRD1 representing glutathione production) or receptors/membrane channels (ADORA2B, P2RX7 or PANX). Metabolites such as adenosine, pyruvate, lactate, and ROS, are not constrained by data, but are produced by the model dynamics. Additionally, we incorporated the TLR4-dependent ATP efflux, through the pannexin channel PANX (114). Once ATP is in the extracellular (eATP)

milieu it acts as an activator of the pathway P2RX7-IL1B (115). In figure 2.1 we drew the 57 modelled interactions and in table 2.1 we collected the supportive literature of such interactions.

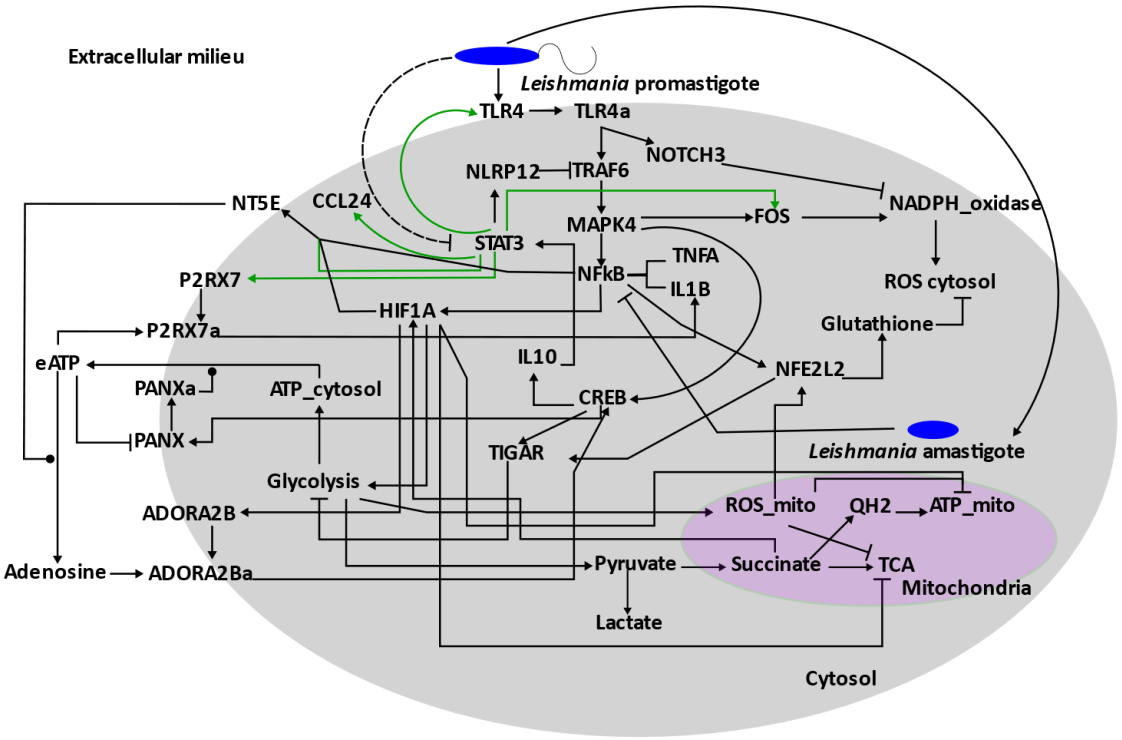


Figure 2.1: Model structure of the immunometabolic pathways in *Leishmania*-infected macrophages

Table 2.1: Literature references supporting the immunometabolic model of *Leishmania*-infected macrophages.

Differential equation	Interaction (+) or (-)	Literature reference
D(TLR4)	(+) STAT3	CHEA Transcription Factor Targets https://maayanlab.cloud/Harmonizome/gene/TLR4
D(TLR4a)	(+) L. promastigote*	(93, 109)
D(NOTCH3)	(+) TLR4a	(116)
D(TRAF6)	(+) TLR4a	(117)
D(TRAF6)	(+) NLRP12	(108)
D(MAPK)	(+) TRAF6	(118)
D(NFkB)	(+) MAPK	(118)
D(NFkB)	(-) Leishmania	(119)
D(IL1B)	(+) NFkB	(120)
D(IL1B)	(+) P2RX7a	(121)

D(TNFA)	(+) NFkB	(120)
D(CREB)	(+) MAPK	(122)
D(CREB)	(+) ADORA2B	(123)
D(IL10)	(+) CREB	(123, 124)
D(STAT3)	(+) IL10	(112)
D(STAT3)	(-) L. promastigote	(119)
D(NLRP12)	(+) STAT3	CHEA Transcription Factor Targets https://maayanlab.cloud/Harmonizome/gene/NLRP12
D(CCL24)	(+) STAT3	(125)
D(TIGAR)	(+) CREB	(126)
D(FOS)	(+) MAPK4	(127)
D(FOS)	(+) STAT3	CHEA Transcription Factor Targets https://maayanlab.cloud/Harmonizome/gene/FOS
D(NADPH_oxidase)	(+) FOS	(128)
D(ROS cytosol)	(+) L. promastigote	(129)
D(ROS cytosol)	(+) L. amastigote**	(130, 131)
D(ROS cytosol)	(-) Glutathione	(132)
D(Leish_amas)	(+) L.promastigote	(133)
D(P2RX7a)	(+) eATP	(134)
D(NFE2L2)	(+) NFKB	(135, 136)
D(NFE2L2)	(+) ROS mitochondria	(137)
D(Glutathione)	(+) NFE2L2	(138)
D(PANX)	(+) CREB	(139)
D(PANXa)	(+) ATP cytosol & L.promastigote	(140)
D(ATP_ext)	(+) ATP_cytos & L.promastigote	(140)
D(ATP_ext)	(-) Adenosine	(140, 141)
D(NT5E)	(+) NFkB	(142)*for the model to publish, would be better to have
D(NT5E)	(+) STAT3	(143, 144)
D(NT5E)	(+) HIF1A	(145, 146)
D(Adenosine)	(+) NT5E	(147)
D(ADORA2B)	(+) HIF1A	(123, 148)
D(ADORA2Ba)	(+) Adenosine	(147)
D(ATP_cytos)	(+) Glycolysis	(149)
D(ATP_cytos)	(-) L.promastigote *PANX	(140, 150)
D(Glycolysis)	(+) HIF1A	(151)
D(Glycolysis)	(-) TIGAR	(126, 152)
D(Pyruvate)	(+) Glycolysis	(149)
D(Succinate)	(+) Pyruvate	(54)

D(TCA)	(+) Succinate	(54)
D(TCA)	(-) ROS_Mitochondria	(153)
D(QH2)	(+) Succinate	(153)
D(ATP_Mito)	(+) QH2	(153)
D(ATP_Mito)	(-) ROS_Mito	(153)
D(ATP_Mito)	(-) HIF1A	(153–155)
D(ROS_Mito)	(+) L.promastigote & Glycolysis	(156)
D(HIF1A)	(+) NFkB	(157)
D(HIF1A)	(+) Succinate	(158)
D(Lactate)	(+) HIF1A	(159)
D(Lactate)	(+) Pyruvate	(159)

*L. promastigote = *Leishmania* promastigote. ** L. amastigote = *Leishmania*

2.2 Model formulation

Our model charts the dynamics of several different molecular species over time, in response to infection by *Leishmania* promastigote. Fundamentally, there are three types of species in the model:

1. Proteins (e.g. transcription factors, receptors)
2. Metabolites (e.g. Reactive oxygen species, glutathione, adenosine)
3. *Leishmania* parasite (both the promastigote and amastigote forms).

The contribution of receptors (e.g. TLR4, ADORA2B) to the overall dynamics of the system depends heavily upon whether they are activated. Their activation rate changes over time, and depends upon the relative concentrations of other species (e.g. *Leishmania* promastigote, adenosine). As such, for each modelled receptor, we incorporate the fold change (over time) of both the receptor itself, and the activated receptor. The fold change (FC) data comes from Ramirez (20), Gomez (37), Dillon (18) and Fernandes (19). It can be found in annex 2.1.

Our model attempts to distil the minimal ingredients necessary to explain the transcriptomics data and provide mechanistic insight into the dynamics of macrophage reprogramming post-infection, while still being easily interpretable. As such, we did not model every known, involved metabolite. In particular, we did not incorporate all the details of known signalling cascades and metabolic pathways. For instance, Glycolysis involves

a chain of metabolites and reactants, with complicated but well-characterised dynamics. We just included a single model species representing the fold-change rate of Glycolysis over time, with some important constituents metabolites (Pyruvate, Lactate and Succinate) incorporated as separate species whose dynamics are dependent upon the ongoing rate of glycolysis. Other model species that are up/downregulated by unmodelled metabolites produced during glycolysis, were then modelled as directly being up/downregulated by the 'Glycolysis' species.

Our model contains three spatial compartments: the extracellular space, the cytosol, and the mitochondria, each of which includes a different set of modelled interactions. We separately chart the dynamics of some species (e.g., ATP) within these separate compartments.

The different species represented in the model all have dynamics: they are produced and degraded over time. We do not chart the change in the absolute concentration of different species over time within the model. Not only do we not have data on absolute concentrations, but these can be highly variable between different cells. Instead, we chart the fold change of the different species over time. The FC of all species that are present at time zero, is 1 (figure 2.2 a). If the FC of a particular species is x, at time t, then our model predicts that the absolute concentration of the species has changed by a factor x, relative to its initial concentration at time zero. So, for instance, a FC of 3 after 4 hours, means that the concentration of a species has tripled in 4 hours.

The overall production and degradation rates of different species depend upon a set of constituent reaction/degradation processes. We chose the reaction / degradation processes incorporated in the model through a survey of the literature (see table 2.1). We can divide the reaction/degradation processes, which we shall hereby collectively refer to as interactions, into six types. We list them below. A description of how they are incorporated mathematically into the model follows later.

1. Constitutive production and degradation. All proteins are assumed to degrade at some constitutive rate, due to natural intracellular protein turnover (160). They also require some basal level of production to counteract this degradation, else they would disappear (160). The degradation and production rates for all proteins are set such that, in the absence of

Leishmania infection, these terms balance each other out and result in a constant fold change of 1, over time.

2. Upregulation: If species A is upregulated by species B, then the production rate of A increases as the relative concentration of B increases.

3. Downregulation: If species A is downregulated by species B, then the degradation rate of A increases as the relative concentration of B increases.

4. Enzymatic conversion: species A may be converted into species B through an enzymatic reaction depending upon the availability of an enzyme C, which may/may not be explicitly modelled.

5. Receptor activation: A receptor may be involved in metabolic interactions only when it is an activated state. Activation occurs by the binding of some ligand to form a complex, and the proportion of activated receptors depends upon the ligand concentration.

6. Transport / conversion: species A may be transported from one spatial compartment to another, with some rate. It may also be converted to species B. Both of these cases are treated similarly, as our model treats the fold changes of a single species in separate compartments as the fold changes of two separate species. Transport / conversion may be upregulated or downregulated by separate species.

The absolute fold change of species A at time t, relative to time zero, is represented symbolically as A(t). The rate of change of the fold change, meanwhile, can be represented by a mathematical derivative: dA(t)/dt. We use the law of mass action to determine how the six described interaction types quantitatively influence dA(t)/dt. The mathematics is described below.

1. Constitutive production and degradation: The degree of constitutive production of species A is constant over time. The rate of constitutive degradation is also constant, but the degree is not: if we have twice the concentration of species A, then the degradation per unit time is also doubled. Thus, for a species undergoing both constitutive production and degradation, we can write the dynamics as

$$\frac{dA(t)}{dt} = x + c_A - d_A * A(t)$$

where x represents the collective contribution of other processes producing / degrading A, following the law of mass action (161). c_A is a parameter representing the strength of constitutive production, and d_A is a parameter representing the strength of constitutive degradation.

2. Upregulation: Suppose species A is upregulated by species B. Then we write the dynamics as

$$\frac{dA(t)}{dt} = x + u_{AB} * B(t)$$

where x represents the collective contribution of other processes producing / degrading A, and u_{AB} is a parameter representing the strength of upregulation. So, if the relative fold change of B(t) is doubled, so too is the production of A attributable to the upregulation mechanism. This equation is a consequence of the law of mass action, assuming first-order kinetics. Some upregulation mechanisms within the model depend upon the concurrent effects of two different species. In such cases, the dynamics are given by

$$\frac{dA(t)}{dt} = x + u_{AB} * B(t) * C(t)$$

In other words, if either B or C doubles, so too does the upregulation, but if either is absent, then so too is the upregulation.

3. Downregulation: Suppose species A is downregulated by species B. The dynamics of this downregulation are given by:

$$\frac{dA(t)}{dt} = x + d_{AB} * A(t) * B(t)$$

where x represents the collective contribution of other processes producing / degrading A, and d_{AB} is a parameter representing the strength of downregulation. Note that, unlike upregulation, the degree of downregulation depends upon A(t) itself. For a constant

downregulation rate, if the concentration of A(t) halved, so too would the degree of downregulation.

4. Enzymatic conversion: Some interactions are upregulated by an enzyme. For instance, the conversion of ATP to adenosine depends, at the last stage, upon the availability of an enzyme NT5E, which catalyses this reaction. The reaction rates of such interactions are modelled by standard Michaelis-Menten kinetics (162). Specifically, for an enzyme E(t), and the substrate S(t), the rate of production of a product P(t) is taken as

$$\frac{dP(t)}{dt} = v\_max * E(t) * (\frac{S(t)}{k_m + S(t)})$$

where v_max is a parameter that sets the maximum possible reaction rate, and k_m is a parameter representing the Michaelis constant.

5. Receptor activation: Several interactions in our model consist of the activation of receptors by a ligand. To model this, we use standard first-order Hill kinetics (163). First-order kinetics are chosen as the degree of ligand co-operativity is unknown for the interactions we are modelling. Given time-dependent fold changes of a ligand L(t), a receptor R(t), and an activated receptor A(t), Hill kinetics give

$$\frac{dA(t)}{dt} = R(t) * (\frac{L(t)}{k_h + L(t)})$$

where k_h is a parameter representing the ligand concentration at which receptor activation is 50%.

6. Transport/conversion: Suppose species A exists in both the cytosol and the extracellular compartments. We represent the fold change of A in these two compartments, at time t, as A_cyt(t) and A_ext(t). If extracellular A were transported into the cytosol at some rate R(t), then we would have

$$\frac{dA\_cyt(t)}{dt} = R(t) * A\_ext(t)$$

$$or$$

$$\frac{dA\_ext(t)}{dt} = -k * R(t) * A\_ext(t)$$

The parameter k exists to convert between the units of A_cyt and A_ext. Recall that all species in the model are charted in relative units, specifically their FC relative to time zero. So while A_cyt(0) and A_ext(0) are both one (in units of fold change), they may have different absolute quantities. If (for example) there was initially a much higher amount of extracellular A, then the transport of half of the extracellular A to the cytosol would result in a much larger than 0.5 FC in A_cyt. The parameter k thus represents the ratio of the initial values of A_cyt and A_ext in units of absolute quantity, basically following the law of mass action.

2.3 Parameter estimation

The previous part of the Methods section detailed how we constructed our model. The model has many free parameters: numerical quantities without dynamics, that represent the constitutive strengths of various interactions. We now describe how we tuned the values of these parameters so that the dynamics of the model matched available transcriptomics data.

The transcriptomics data we have available measures mRNA FC at different time points. We used the approximation that these mRNA FC corresponded to the corresponding FC of the modelled proteins that they coded for. In actuality, there could be some delay between the realisation of a particular mRNA FC, and the corresponding protein FC, due to the time is taken to transcribe mRNAs into proteins, post-translational regulation, among other events. However, this delay does not change the qualitative dynamics of the model, it only delays the times at which particular protein FCs are produced.

Let us briefly describe the experiment from which our transcriptomics data was taken. Leishmania promastigote was introduced to an *ex vivo* preparation of macrophages. The preparation on average was left for two hours, over which time some of the *Leishmania* promastigote was internalised into the macrophages as *Leishmania* amastigote. Then, the remaining *Leishmania* promastigote was removed. We assumed that *Leishmania* promastigote concentration, therefore, experienced an exponential decay, with an

exponential rate constant fitted so that the FC would be 0.01 after 24 hours. At two hours, however, we applied a discrete removal of all *Leishmania* promastigote.

We modelled *Leishmania* amastigote internalization as growing exponentially over the first two hours, with an exponential rate constant dependent upon the amount of *Leishmania* promastigote. After removal, Ramírez (20), Fernandes (19) and Gomez (37) reported a fairly constant parasite load throughout the 24 hours of infection. We modelled *Leishmania* amastigote FC based on Ramírez data (20).

An important modelling constraint we imposed was that, in the absence of *Leishmania* promastigote, the FCs of all species should not change. After all, the initial concentrations of the different species is a `steady state' of the system, in the absence of promastigote. To enforce this, we manually calculated the degradation rate so that the degree of degradation was equal to the degree of production at time zero, in the absence of promastigote. To illustrate, suppose we have a species A, that has dynamics:

$$\frac{dA(t)}{dt} = f * [A, B, C, LP] - d_A * A(t)$$

where f is some function representing all of the production and degradation processes involving A, which also depends upon species B and C, and LP, which represents the concentration of *Leishmania* promastigote. We would then set

$$\frac{dA(t)}{dt} = \frac{1}{A(0)} * f[A(0), B(0), C(0), LP(0)]$$

Therefore, dA(0)/dt = 0, and the FC of A has no initial dynamics in the absence of *Leishmania* promastigote.

2.4 Loss function construction

We then constructed a 'loss function', that quantified how badly a given set of parameter values fitted the data. Let D_{ij} be the FC of the i^{th} species, at the j^{th} timepoint. Let $y_i(j, \theta)$ be the FC predicted by the model at the j^{th} timepoint, where θ is a vector representing the numerical values of the model parameters. Then we take

$$Loss(\theta) = \sum_i \left(y_i(\theta, j) - D_{ij}\right)^2$$

Finding a set of model parameters that best fit the data corresponds to finding a vector θ, for which Loss(θ) is minimal.

We initially took all parameters values as equal to one. We then used an optimisation algorithm to iteratively change the parameters to better fit the data. In particular we used the LBFGS algorithm (164). We asked the algorithm to constrain all parameters to be greater than zero, which is biophysically necessary since they quantify the rates of reactions. This provided us with a final set of parameters that best fitted the data (annex 2)

The final value of Loss(θ), after fitting, was 33. There were 89 datapoints on FC that the model was fitted to. Thus, the error in the model predictions of FC was, on average, 33/89, which is less than 0.37. So, we can say that, on average, model predictions on FC were different from the data by less than 0.2.

3.3 Coding

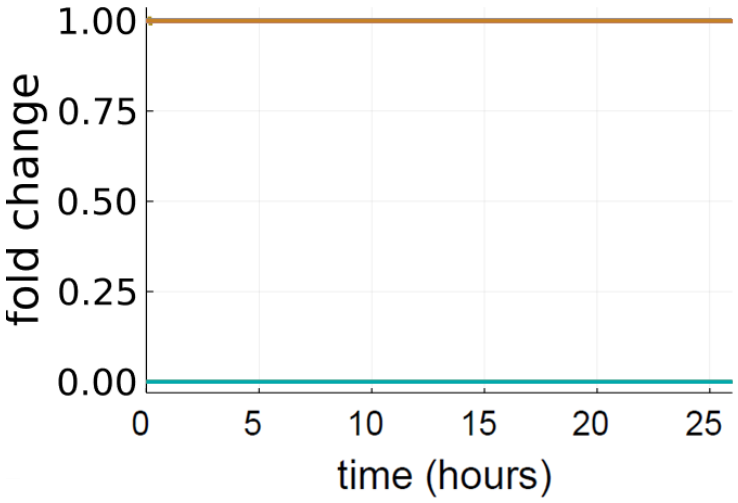
We simulated our model using the Julia programming language (165), and in particular the ModelingToolkit.jl package (166). Parameter estimation was carried out using the package Optim.jl (167). See the code in annex 2.2.

RESULTS II

We studied the dynamics of the immune-metabolic profile of macrophages infected with *Leishmania* parasites, through a simplified model of the crosstalk between the TLR4 signalling pathway, STAT3 activation, ATP extracellular efflux, activation of the purinergic receptors, glycolysis, ATP-mitochondrial production, and redox-regulated stress responses. Data on any given represented gene was only available at a limited set of time points. Therefore, the model predicts FC at intermediate time-points. The model contains 40 differential equations, 96 parameters (annex 2.3), and 89 FC expression data.

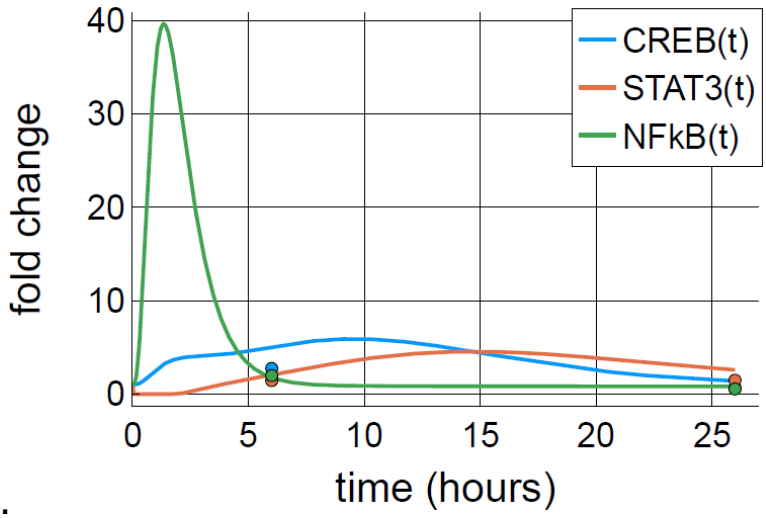
Immunological module: Cytokines and Transcription Factors

Downstream TLR4 we modelled the activation of TFs with the known dichotomic connotation of inducing pro or anti-inflammatory responses. These are NFkB and CREB, respectively. Besides, the activation of the other TF STAT3 was also included. As by-products of their activation, we modelled the set of pro-inflammatory cytokines IL1B and TNFA, together with the anti-inflammatory cytokines IL10 and CCL24. The model simulation after parameter estimation, predicts a sharp expression of pro-inflammatory mediators (TF and cytokines), that reach their highest activation level before the first 2 and half hours post-infection (figure 2.2-b and c). In contrast, the anti-inflammatory mediators are slowly activated, but they become predominant after roughly 8 hours post-infection (figure 2.2-b and c). Moreover, STAT3 contributes to the activation of a negative feedback loop on the TLR4 signalling at the level of TRAF6. This occurs by positively regulated the expression of the TRAF6 inhibitor, NLRP12. However, as shown in figure 2.2-c, NLRP12 must be a later negative regulator that guarantees the inactivation of the pathway.

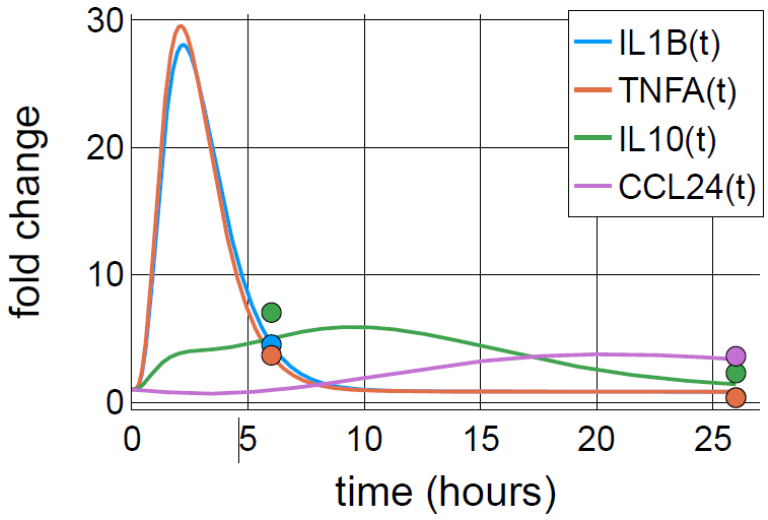


A.

B.


40
30
20
10
0
fold change
0
5
10
15
20
25
time (hours)
CREB(t)
STAT3(t)
NFkB(t)

C.


30
20
10
0
fold change
0
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15
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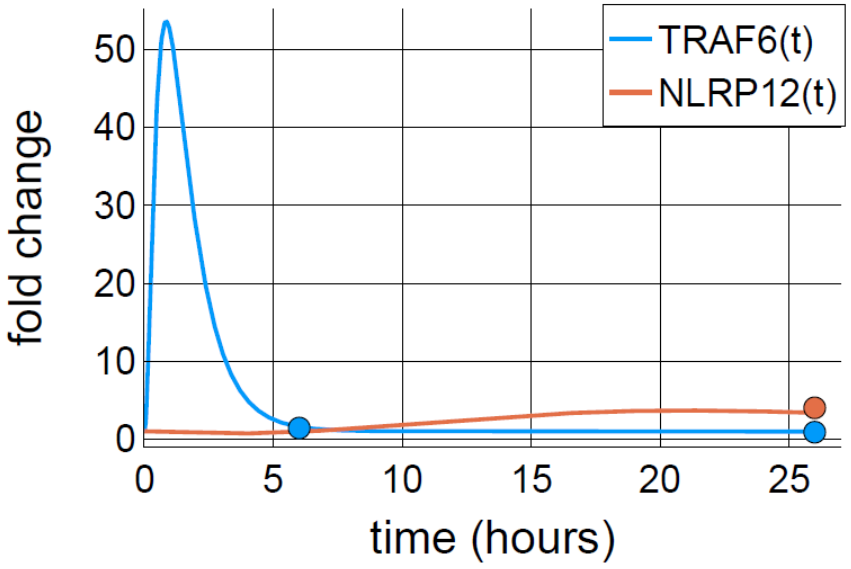
25
time (hours)
IL1B(t)
TNFA(t)
IL10(t)
CCL24(t)



C.

Figure 2.2: Solution of the ODE model of pathways representing the immunemetabolism of *Leishmania*-infected macrophages. Control simulation in the absence of the infection (A). Dynamics of TFs (A) and cytokines (B) are classically used to classify the macrophage inflammatory profile. Early, the pro-inflammatory mediators are dominant over the anti-inflammatory mediators. Later, the model shows a shift in these phenotypes. In C, is plotted a negative feedback loop contributing to such shift. NLRP12 attenuates TLR4 signalling at the TRAF6 level. The FC data are plotted as dots.

This part of our model represents the dynamics of TLR4 contribution to an early pro-inflammatory and a late anti-inflammatory profile, by inducing both TFs and cytokines with divergent roles in this regard. Besides, it contributes to its own attenuation through CREB-IL10-STAT3-NLRP12 pathway.

Immunological module: extracellular ATP and Purinergic signalling

On the other hand, our model integrates the crosstalk between TLR4, and the inflammation exerted by ATP-efflux/purinergic pathways. Some of the activated TFs described above, activate the transcription of enzymes and receptors involved in the metabolism of eATP. First, the ATP efflux channel, PANX is early upregulated by CREB. The first negative feedback loop in this mechanism, comes from the eATP itself, by inducing PANX internalization (139). However, the infection with *Leishmania* enhances up to around 2.6 FC the ATP efflux, as shown by (140), during the first hours post-infection. Thereafter, a second but stronger feedback loop takes place, through the enzymes ENTPD and NT5E (168). These enzymes are responsible for the hydrolysis

of eATP to the nucleotide Adenosine. In our model we integrated the dynamics of the latter. The model suggests that in *Leishmania*-infected macrophages, NT5E is key to ensure the buffering of pro-inflammation mediated by eATP. This is done by increasing the availability of adenosine, that in turns activates the ADORA2B signalling pathway receptor. Additionally, it downregulates the activation of P2RX7, that otherwise activates the production of IL1B. The availability of ADORA2B receptors (figure 2.3) is partially boosted by STAT3. Thus, generating a positive feedback loop regulation on STAT3 itself, through the pathway ADORA2B-CREB-IL10-STAT3. Accordingly, this is a dynamic exemplification of how through ADORA2B a positive feedback loop on the anti-inflammatory profile is sustained once TLR4 signalling has been attenuated.

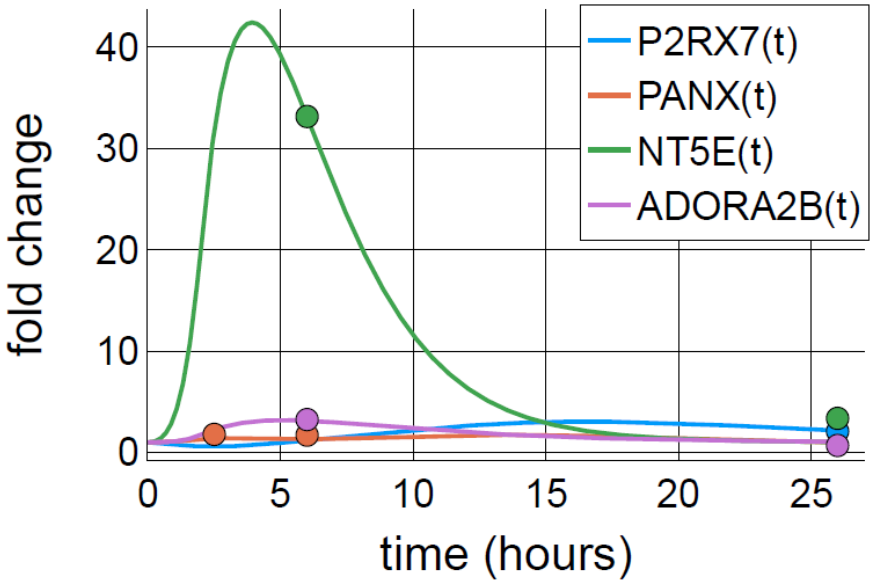
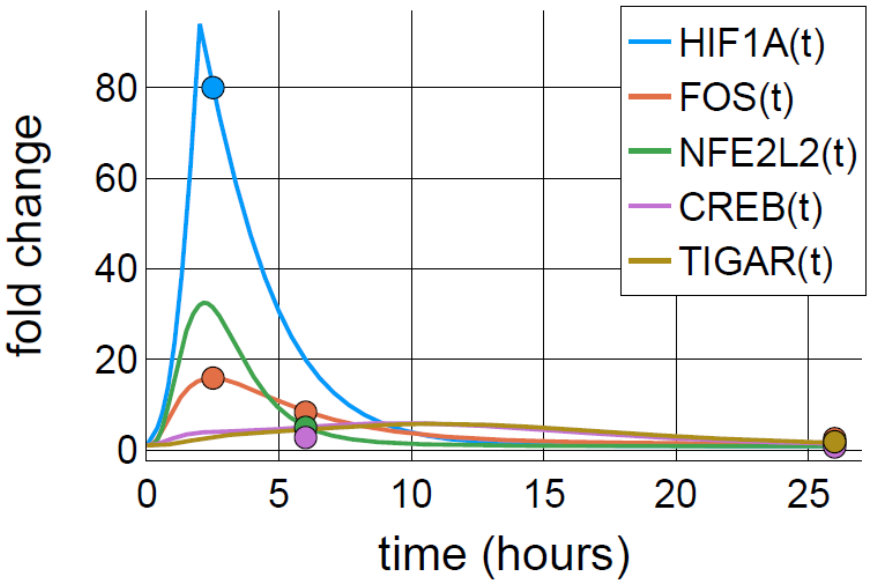


Figure 2.3: Solution of the ODE model of pathways representing the immunemetabolism of *Leishmania*-infected macrophages. Dynamics of the eATP channel and receptor PANX and P2RX7, respectively. Besides, dynamics of NT5E involved in the second step of the eATP hydrolysis to Adenosine, together with the adenosine receptor ADORA2B. The FC data are plotted as dots.

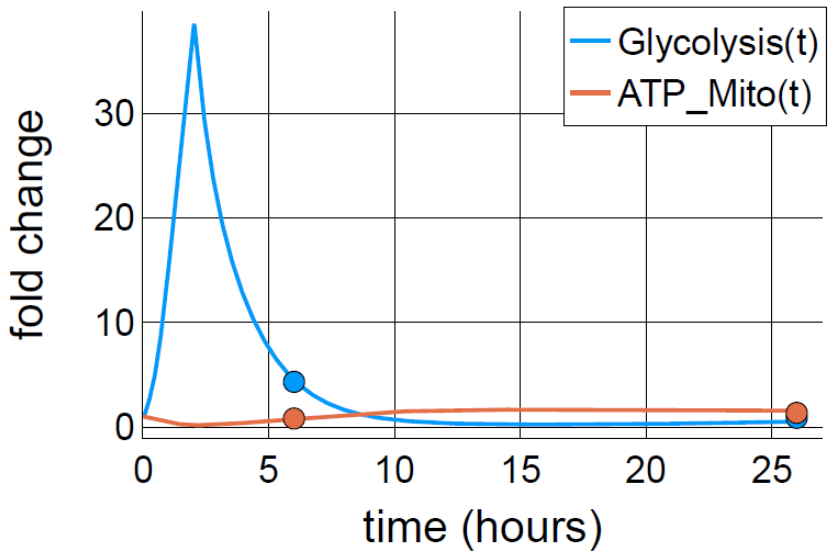
Metabolic module: Bioenergetics and Oxidative Stress

Simultaneously, we modelled the crosstalk between the immune module and the metabolic module. From the activation of TLR4, the activation of TFs involved in ROS (FOS and NFE2L2) and intracellular ATP (HIF1A and CREB) metabolism follows (figure 2.4). We plotted in figure 2.4

the dynamics of such TFs. FOS as part of the heteromeric TF factor AP-1 binds to the promoter region of the NADPH oxidase subunits. This enzyme catalyses the reaction that yields ROS, which is part of the killing mechanisms of the macrophage. Conversely, the TF NFE2L2 activates the transcription of the enzymes involved in ROS scavenging through glutathione synthesis. On the side of the energetic module, we followed the dynamics of the TFs HIF1A and CREB, and TIGAR. HIF1A is responsible for the upregulation of many of the glycolytic enzymes. It is early on activated via NFkB and is enhanced by the accumulation of succinate. Our model predicts a peak of FC higher than 80 around 4h post-infection (figure 2.4-a). Consequently, glycolysis is upregulated upon infection (figure 2.4-b), which generates intermediate metabolites that drop the production of ATP mitochondrial. Nevertheless, the TF CREB fulfils an antagonist role of HIF1A. CREB activates the transcription of TIGAR (figure 2.4-a), a negative regulator of glycolysis.



A.



B.

Figure 2.4: Solution of the ODE model of pathways representing the immunometabolism of *Leishmania*-infected macrophages. A. Dynamics of TFs involved in oxidative stress and the bioenergetics of the macrophage. B. Dynamics of glycolysis (approached through the enzyme HK3) and mitochondrial ATP production (also known as OXPHO).

Our model represents a mechanistic model of an immunometabolic switching in *Leishmania*-infected macrophages. It describes the regulatory feedback mechanisms around the activation of the TLR4 signaling pathway, which leads to the "ON" of an early pro-inflammatory response together with glycolysis-based bioenergetics. At the same time, our model depicts how TLR4 downstream activated effector molecules regulate the "OFF" of such responses. In particular, our model curated the key role of STAT3 that induces the expression of factors involved in anti-inflammation and OXPHO-based bioenergetics

DISCUSSION II

In this work, we presented a dynamic mechanism by which *Leishmania*-infected macrophages undergo a switch in immune-metabolic pathways during the first 24 hours post-infection. Our model dynamically exemplifies how TLR4 signalling early contributes to the mounting of a pro-inflammatory response but later some of its downstream molecular effectors are key anti-inflammatory mediators. Thus, contributing to a time-dependent shifting. These results shed light on found discrepancies in the literature regarding the association of TLR4 with both restriction

and permissiveness of *Leishmania* parasites. Additionally, we also showed a useful methodology to implement a dynamic mathematical model based on transcriptome data.

First, we curated into the model a widely known link between TLR4 and pro/anti-inflammatory responses. The former is represented by the module TLR4-NFKB-pro-inflammatory cytokines (IL1B and TNFA), and the latter by the module TLR4-CREB-IL10 and TLR4-CREB-IL10-STAT3-CCL24 (92, 124, 169). The pro-inflammatory module is strongly induced shortly after the infection, but it decreases during the first 5 hours post-infection. The attenuation in the signal is partially explained by negative regulatory feedback loops triggered after the infection (170), such as the one played by NLRP12. This Nod-like receptor is recurrently exploited by bacteria and viruses to favour their infection and growth (171–173) and it might be contributing to the shifting from pro to an anti-inflammatory profile. However, NLRP12 might be fulfilling a pleiotropic role that our model is not considering. Nonetheless, modelling the above set of interactions helped to mechanistically inform unsorted findings in the literature. Let us take as an example the findings reported by Gallego *et al* (93). When they compared the parasite survival between wild-type macrophages and tlr4$^{-/-}$, the former presented an increase after 24 hours post-infection, while the latter continued decreasing. In isolated macrophage infection models, through an autocrine manner, our model exposes how TLR4-CREB-IL10 contributes to the acquisition of an anti-inflammatory profile, that favours parasite growth, as in Gallego's study. Meanwhile, our model predicts that TLR4-CREB-IL10-STAT3-CCL24, when *in vivo*, supports the anti-inflammatory profile through the recruiting of eosinophils mediated by CCL24 (5).

Secondly, in the model, we also exposed an apparently unseen link in the literature between the TLR4 and a divergent regulatory role on eATP-mediated inflammation. Firstly, TLR4 activation is positively correlated with ATP efflux to the extracellular milieu, where eATP acts as an inflammatory signal (174, 175). We represented it with the interactions TLR4-CREB-PANX-eATP. In parallel, eATP is also a ligand of the purinergic receptor P2RX7, whose activation signal contributes to an early inflammatory response in *Leishmania*-infected macrophages (175). Interestingly, our model illustrates how through TLR4-NFKB-NT5E and TLR4-NFKB-HIF1A-ADORA2B, TLR4 is also contributing to buffering the above pro-inflammatory mechanisms. Consistently, our transcriptome data, as well as shown by others (140, 176), NT5E and ADORA2B are upregulated in *Leishmania*-infected macrophages. Hence, our model mechanistically illustrates how TLR4 is early contributing to eATP-mediated inflammation and later supporting anti-inflammatory responses that benefit *Leishmania* survival.

On the other hand, our model integrated the crosstalk between the immune response and the metabolic status of the macrophage. This was done by putting together the regulatory role of STAT3 and the TLR4-dependent activation of the TFs HIF1A and NFE2L2, key regulators of glycolysis and oxidative stress, respectively. Congruently, the immunological shifting is also displayed in energetic and oxidative metabolic pathways. First, our model represented the sharp increase in HIF1A production as reported by others (25, 106) (refs). This leads to an increased in the glycolytic enzymes (like HK3), hence, the glycolytic flux (151). However, the activation of glycolysis inhibitors, such as TP53INP1, might be contributing to a metabolic reprogramming towards OXOPH. Our model predicts this shifting is occurring during the first 20 hours post-infection. This prediction is congruent to the results by Moreira and colleagues (26) who found that 18 hours post-infection, *Leishmania*-infected macrophages upregulates OXOPH over glycolysis. Lastly, we modelled the dynamics of DUOX1, the NADPH oxidase subunit, and TXRD1, the enzyme involved in ROS neutralization by the production of glutathione. The model represents a potential tight control of ROS-mediated *Leishmania* killing, explained by an early upregulation of both ROS production and glutathione, but with a faster decay for the former. This might explain why Ramírez *et al.*(20) and Gomez *et al.*(37), reported a fairly steady parasite load during the 24 hours of infection.

CONCLUSIONS II

We managed to distil into a mathematical model a representation of a switching mechanism of the immunometabolism of *Leishmania*-infected macrophages. TLR4, purinergic receptors signalling and metabolic processes crosstalk, were modelled based on transcriptome data and parameter estimation. To highlight is the emphasis on the feedback mechanisms involved in the rewiring of an initial pro-inflammatory and glycolytic status of the macrophages. TFs play a central role in coordinating the dynamic control of such rewiring.

STRENGTHS AND LIMITATIONS II

Our model makes use of available transcriptome data to give an integrative and dynamic perspective of the macrophage response to *Leishmania* infection, which otherwise is static and disconnected. We assumed that the mRNAs are fully expressed as proteins. This, of course, should be validated at the experimental level. We cope with this limitation by supporting our model structure with a vast literature survey. Besides, we used TLR4 as our “case study” where we use the model to explain the apparent disparities in the role of this receptor and Leishmaniasis,

previously reported. The fact that this model agrees with different public studies is a good indicator of the model calibration.

FUTURE WORK II

Protein activation validation: Post-translational modifications and events of activation/deactivation might be interfering with the productive expression of the mRNAs. Therefore, a first step to validate our model should be the verification of the activation some of some the modelled molecules.

Model expansion from isolated macrophages to a multicellular system: The model represents a set of feedback mechanisms that hold the immunometabolic rewiring upon the 24 hours post-infection. However, it does not account for the regulatory mechanisms the macrophage might undergo when embedded into a pro-inflammatory environment. Therefore, PBMCs could be a suitable *ex vivo* model to test such mechanisms. My hypothesis is that the already upregulated inhibitory mechanisms during the adaptation stage, play an important role in keeping the macrophage as a permissive niche for *Leishmania* parasites.

Incorporate the parasite molecular factors: We know that the macrophage displays anti-inflammatory and oxidative mechanisms as a self-protection mechanism to buffer the initial robust pro and oxidative response. However, *Leishmania* is known for its elegant hijacking strategies of the host cell machinery. A mathematical model integrating both, the host and parasite factors regulating the rewiring of the immunometabolism could be a greatly useful tool for identifying drug targets.

ANNEXES II

1. Folder with the FC data
2. Folder with the files to generate the simulations in Julia

CHAPTER 3: Exploring *Leishmania*-Host Interaction with Reactome, a Database of Biological Pathways and Processes

ABSTRACT III

The use of Omics technologies to study *Leishmania*-host interaction is an understandably common approach. It allows for identification of multiple molecules (e.g. genes, proteins or metabolites), from both actors, at the same time. However, the list of molecules is a means not an end. Researchers need to figure out how those molecules are interacting with each other. Then, they need to abstract those interactions into biological processes and understand how these relate to the infection. Commonly, researches rely on ORA applied to databases such as Reactome, to transform the list of molecules, into a list of biological processes. However, these lists often contain vague labels (e.g. Immune system), whose biological meanings are disconnected from each other. This makes difficult to exploit to the fullest Omics data, that otherwise, offers a holistic perspective of the biological system under study, as we showed in chapter 1. In this chapter 3, we show our work towards representing detailed contextual knowledge on *Leishmania*-host interactions in the Reactome pathway database, to facilitate the extraction of novel mechanistic insights from Omics datasets. We conducted a systematic curation of published studies documenting different aspects of the *Leishmania*-host interaction. The "*Leishmania* infection pathway" was constructed and included four sub-pathways: phagocytosis, killing mechanisms, cell recruitment, and *Leishmania* parasite growth and survival. These subpathways contain the signaling cascades that involve the membrane proteins FCGR3A, FZD7, P2RX4, P2RX7, ADORA2B, and CD163. These proteins were chosen at an early point of our research based upon two factors. First, they were commonly modulated genes in Chr and Failure datasets. Second, they were relevant to Leishmaniasis according to the literature. However, as we mentioned in chapter 1, these datasets changed when we had to repeat the differential expression analysis, initially done by the authors that generated the data. Therefore, as proof-of-principle of the usefulness of the *Leishmania* infection pathway, we did not use our own data (this work was carried on before knowing the second mistake in the source data). Instead, we analyzed two previously released transcriptomic datasets, of human and murine macrophages infected with *Leishmania*. Our results provide insights on the participation of ADORA2B signaling pathway in the modulation of IL10 and IL6 in infected macrophages. This work opens the way for other researchers to contribute to, and make use of, the Reactome

database. Our work was released in March-2020 and can be accessed through this link: https://reactome.org/PathwayBrowser/#/R-HSA-9658195.

A primer on ORA and the Reactome database

Omics data sheds light on the multitude of activated mechanisms within the host-parasite interactome, by providing long lists of differentially expressed (DE) molecules. However, such data is a means, not an end. The ultimate goal is to interpret this data to build a mechanistic understanding of the interactions at hand. This exercise is made tractable by the existence of pathway databases that curate and organize current knowledge (177–179). Typically, the databases are used to find known biological processes that could underlie the data. A common methodology is Over-Representation Analysis (ORA). This takes the set of DE genes from the data, and iteratively compares them to the set of genes involved in each separate pathway in the database. It uses the overlap between these two sets to predict the statistical likelihood of the biological pathway being represented in the data (180). Mechanistic hypotheses on the processes underlying the data are then proposed by the researcher, based on the ORA results.

Biological process labels within a database often lack context (e.g. ´immune system´). Does the process occur within one particular cell type, or more? Across species? In a diseased organism? In the context of a pathogen-host interaction? It is difficult to build detailed hypotheses from such labels, using ORA or indeed other analytical approaches.

The Reactome database builds a hierarchy of abstractions into which the observed features of any biological process can be incorporated. At the top level of the hierarchy, high level characteristics are represented: Is it a disease? Is it infectious or metabolic? At lower levels, features such as specific, temporally-ordered sequences of cellular processes are represented (179). Choices such as how many levels of abstraction to include, and what each should represent, depend to some extent upon the expertise of the curator. Therefore, it is critical that the curator has expert domain knowledge or that they collaborate with an appropriate expert in the field.

The purpose of this work was to add representative features and variability of the *Leishmania* spp.-host interaction into the Reactome database. In the terminology of the database, this representation is known as a ‘pathway’. Our pathway is sufficiently flexible as to allow for expansion and revision as new datasets are published. It incorporates detailed information on

biological processes known to be activated during the *Leishmania*-host interaction. In particular, we focused on those processes correlated with the outcome of infection.

We explicitly demonstrate the utility of our database curation. We took two existing datasets to which ORA or manual revision of the literature was previously applied. With our expanded database, we uncover new mechanistic insights. In the long term, we hope that the research community will be able to use our pathways as a source of primary consultation, and as a curated database for functional and mechanistic interpretation of new data derived from omics technologies, functional tests, or *in-silico* experiments. We believe that this pathway will enable fast and curated access to the integrative mechanisms of importance in leishmaniasis.

MATERIALS AND METHODS III

The abstraction of *Leishmania* infection into a structure of pathways and reactions fulfilling the Reactome paradigm was accomplished by the Reactome working group. This consisted of a consortium of biocurators, software developers and leishmaniasis researchers. The latter selected the mechanisms of interest. The selection was based on biological pathways known to be associated with the infectious outcome, directly or indirectly. From here, domain experts and Reactome curators worked side by side to translate the selected pathways into the Reactome data structure using the curator tool version 3.3.

Reactome represents, categorizes, and annotates all known entities in each reaction. Different components can interact with each other only in ways prescribed by the Reactome data-model. For instance, the representation of an individual protein in the database requires several steps. In an example, for the protein ADAM17, the Uniprot ID (P78536) is retrieved, as well as the GO cellular compartment it functions in, and which species this protein belongs to (e.g., *Homo* sapiens). Note that if the represented process implies the transition of the same protein from one compartment to another, different instances of this protein must be created (e.g ADAM17 [endoplasmic reticulum], ADAM17 [golgi apparatus] and ADAM17 [plasma membrane]). However, all these instances still point to the same UniProt ID. On the other hand, if several proteins individually fulfil the same role in a reaction (e.g., phosphorylation), these are grouped together into a single entity called "*defined set*" (PKL2 [cytosol] and MAPK14 [cytosol] in the *defined set* PKL2, MAP14 [cytosol] that phosphorylates ADAM17 [plasma membrane]). The *defined set* entity is also specific to each cellular compartment. Therefore, each individual molecule within the *defined set* must have a compartment-specific representation. Otherwise, in the quality assurance procedure, this will come out as an error. Molecules of the same or different

types can form complexes, for example ADAM17:Zn2+ which represents the functional form of ADAM17. Once these entities are created, the reactions in which they take part, are created. Binding is a common type of reaction in Reactome. Here, the output is a complex type entity (e.g., the interaction between sCD163 and MYH9 is represented as a binding that ends in the formation of the sCD163:MYH9 complex). If we are not at the final point of the pathway or dealing with a secondary product, the complex can participate in subsequent reactions. In that case, the reaction from which this complex came from is indicated as the "preceding event" of the subsequent reaction.

Overall, we curated *Leishmania* infection pathways following the general structure of a signaling pathway. Namely, a ligand binding to a receptor, then the stimulated receptor effecting a downstream signaling cascade, up to the activation of effector molecules (eg. cytokines, nitric oxide, etc.). However, other pathways were conceived with different starting points. For instance, the first step of the CD163 example pathway consisted of ADAM17 activation. This begins in the endoplasmic reticulum, and its maturation process follows through the Golgi apparatus until its translocation to the plasma membrane, where its phosphorylation by kinases (either PLK2 or MAPK14), activates the cleavage reaction of CD163. It is the soluble portion of CD163, sCD163, that has been found as a regulator of the inflammatory responses in leishmaniasis infection, through the inhibition of the proliferation of lymphocyte T cells.

Reactome's criteria for the acceptance of a particular molecular interaction and its supporting reference have been previously explained (179, 181). Once the reactions were successfully integrated into Reactome's central database, we reached out to an experienced researcher in leishmaniasis, Dr. David Gregory (ORCID: 0000-0001-6534-7150) (119, 182, 183) to review the curated material on the basis of his expertise in the field (6, 19 and 20). Only material reviewed by an independent domain expert is allowed to be published in a Reactome release. The contributions of authors and reviewers can be directly accessed through Reactome's search interface, for example https://reactome.org/content/query?q=David+Gregory shows a detailed description of credit attribution in Reactome (184).

Once an orderly list of reactions was created, these were transformed according to the Reactome paradigm by using the curator tool. Some reactions were created from scratch to populate a low-level pathway, while others that were present within other pathways were duplicated (or reused) in the context of *Leishmania* infection (table 3.1). Within a reaction, each component was re-used if it already existed as part of a different process in the database, otherwise it was created. Reuse of existing components and reactions ensures no redundancy in the database.

Reaction details, such as input/catalyst/output molecules, reaction type, preceding reactions and experimental species, were captured during the curation process. Reactions were linked together based on preceding-following relationships to generate pathways.

RESULTS III

Abstracting the top-level pathway: phagocytosis, killing mechanisms, cell recruitment and responses favoring *Leishmania* parasites.

The curation of a pathway in Reactome starts by structuring pathways into hierarchical sections. The parent *Leishmania* infection pathway was structured into four subpathways describing the major processes involved: phagocytosis, killing mechanisms, cell recruitment and *Leishmania* parasite growth and survival. For each subpathway, there is extensive literature on the specific host responses to *Leishmania* infection, and their implication in the outcome of infection (185–187).

Leishmania parasites are transmitted to the host through the bite of a sand-fly that injects the motile promastigote form into the dermis of humans and other warm-blooded animals. Therein, the parasite interacts with the host cell(s) to establish the intracellular niche, where it will adopt the amastigote form (188). Paradoxically, macrophages, professional phagocytes of the innate immune system, are the main host cells for *Leishmania*. The first interaction of the parasite with the host cell is crucial to the outcome of the infection (185). The type of phagocytic receptor or pattern recognition receptor stimulated might influence the signaling cascade(s) that will trigger or inhibit cellular mechanisms involved in parasite killing or permissiveness for infection. Deregulated immune responses contribute to pathology (189). Pro and anti-inflammatory mediators must be expressed at the “right” time and in the “appropriate” magnitude, in order to have a healing response (189). This discussion motivates our abstraction of existing knowledge into the categories of phagocytosis, killing mechanisms, cell recruitment (pro-inflammatory response), and *Leishmania* parasite growth and survival (figure 3.1).

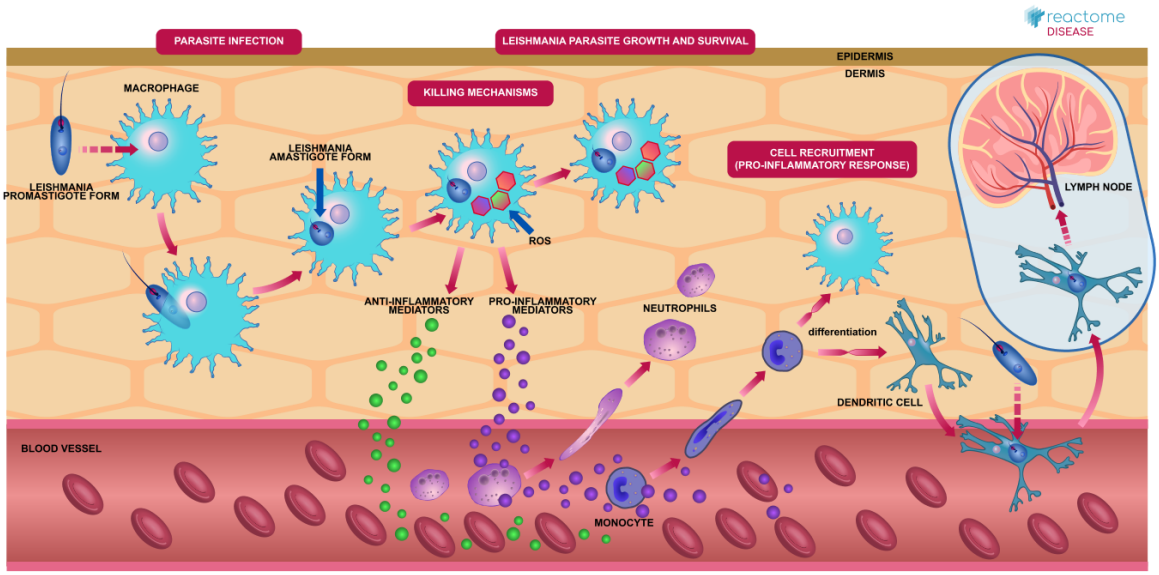


Figure 3.1: Textbook-style diagram representing the top-level pathway "*Leishmania* infection". The major steps occurring in the dermis were compartmentalized into four categories: phagocytosis, killing mechanisms, cell recruitment, and *Leishmania* parasite growth and survival. On the webpage (https://reactome.org/PathwayBrowser/#/R-HSA-9658195), the magenta rectangular labels are interactive and take the user to the content of each subpathway.

***Leishmania* infection pathways: From a sketch on paper to Reactome database.**

Reactome's curation tool is a graphical user interface (GUI), that connects to its central database with which new information can be added to existing or new pathways. We started with a sketch of the overall pathways we wanted to curate for the first version of the "*Leishmania* infection pathway". Then, we identified what molecules/entities and reactions already existed in the database and which ones needed to be added. Similarly, we accounted for the molecular interactions that were already described in existing Reactome pathways. If new reactions were required, they were created from scratch, supported by literature references. Table 3.1 summarizes these metrics.

Table 3.3: Curation and annotation metrics for the creation of *Leishmania* infection pathways in Reactome.

Pathway	Entities		Reactions/Catalysis/Regulation		New literature references
	New	Re-used	New	Re-used	
Phagocytosis	9	141	12	14	7

Killing mechanisms	5	18	9	0	8
Cell recruitment	3	28	3	24	9
Leishmania parasite growth and survival	15	51	19	12	17
Total	32	238	42	50	41

Structuring the lowest-level pathways: signaling cascades traceable from a membrane protein to the production of effector molecules.

As part of the host response against *Leishmania*, many signaling cascades are modulated (activated or inactivated). Once a cellular/membrane receptor is stimulated, the downstream signal transduction can result in the activation of many molecules with different effects in the system (e.g. interleukins inducing the polarization of several types of T-cells, or chemokines mediating the recruitment of immune cells to different tissues, among others). That is why general pathway labelling such as “TNF-signaling”, might not be informative enough, and can allow for erroneous biological interpretations if the gene lists contained within enriched pathways are not carefully analysed. There could be many regulatory processes that favor one direction rather than another in a specific signaling pathway (as it can be noticed in TNF-pathway in Reactome, R-HSA-75893 https://reactome.org/PathwayBrowser/#/R-HSA-75893 and KEGG, hsa04668- https://www.genome.jp/kegg-bin/show_pathway?hsa04668). Therefore, we structured the lowest-level pathways in each of four subpathways, starting off from an activated membrane protein (e.g., receptors, ion channels or enzymes). This was followed by inclusion of signalling and accessory molecules and finished with synthesis of effector molecules that are consistent with the overall biological processes underlying the subpathway (e.g., reactive oxygen species – ROS- for “killing mechanisms”).

For this first version of the *Leishmania* infection pathway, we chose the membrane proteins FCGR3A, FZD7, P2RX4, P2RX7, ADORA2B, and CD163 and their downstream signaling cascades. Although additional membrane molecules are known to also participate in the initial macrophage-*Leishmania* interaction, such as complement receptors, toll-like receptors -TLRs-, or chemokine receptors among others (190–192), the selected less “classical” membrane molecules allows for increasing the breath of mechanistic interpretation of host-*Leishmania* –omic datasets. Shown in figure 3.2 are the structures of the four subpathways, each of which contains a set of

reactions. Supporting references evidencing their relevance in the context of leishmaniasis will be discussed below.

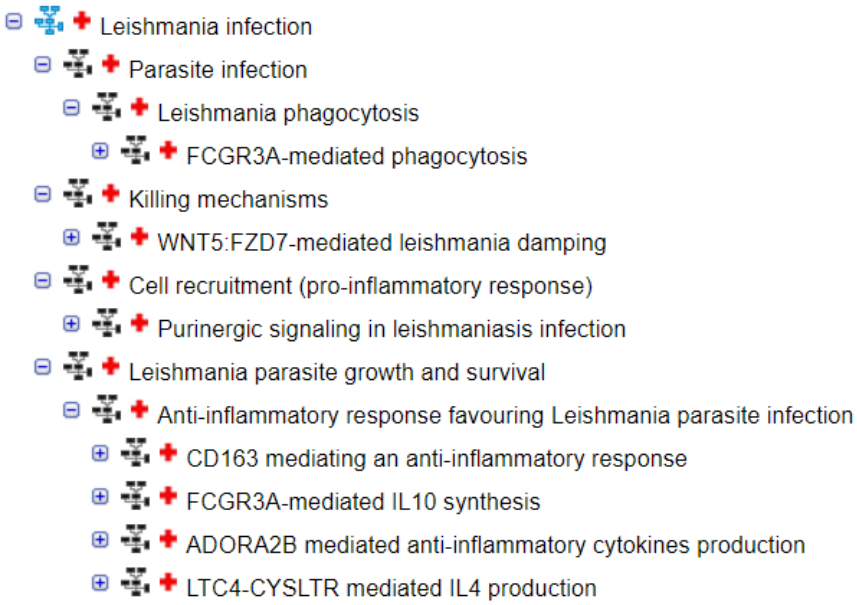


Figure 3.2: Hierarchical structure of the *Leishmania* infection pathway showing the four sub-pathways and their contents. Each indentation introduces a new subpathway; as an example, "parasite infection" is the parent pathway for the subpathway "*Leishmania* phagocytosis", which, to date only contains *FCGR3A*-mediated phagocytosis.

I. **Parasite Infection/*Leishmania* phagocytosis**: The phagocytosis subpathway was built to account for the different types of phagocytic receptors that *Leishmania* parasites can utilize for their entry point into host cells. We started by adding FCGR3A-mediated phagocytosis. To build it, we referenced the pre-existing Reactome pathway "FcG receptor (FCGR) dependent phagocytosis" (https://reactome.org/content/detail/R-HSA-2029480). Overall, 14 reactions were re-used from this pathway while 12 new reactions were created to represent phagocytosis in the context of *Leishmania* infection (table 1). The starting point was the binding of immunoglobulin G antibodies (IgG) to either an unknown *Leishmania* amastigote (abbreviate as: Lma) surface molecule or the glycoinositol phospholipid - GIPL (193, 194), as shown in figure 3.3 a. These interactions correspond to binding reaction types in Reactome, with the product of binding being a complex comprising the inputs. The complexes "IgG:Lma surface" and "IgG:GIPL" represent the opsonization of the *Leishmania* amastigote by the antibody IgG, during a "second round" of host-parasite

contact where the proliferative form and infective form in the host is the amastigote. We assumed the same course when opsonization occurs via these known, or other unknown molecules. Therefore, we collated the two complexes into one entity, which in Reactome is represented by the defined set “IgG:Lma antigens”. From here, downstream reactions continue towards the activation of actin filaments that then continue to the formation of the phagocytic cup. The overall diagram depicting each step of the pathway can be accessed through this link https://reactome.org/PathwayBrowser/#/R-HSA-9664422&PATH=R-HSA-1643685,R-

II. HSA-5663205,R-HSA-9658195,R-HSA-9664407,R-HSA-9664417. A close-up depicting a portion of the pathway is found in figure 3.3 a.

The route of entry into the macrophage can affect the fate of *Leishmania* parasites (185). We expect to incorporate the internalization processes that are mediated by other receptors into the *Leishmania* phagocytosis subpathway. This includes complement receptors (CR3 and CR1), mannose receptor-MR, and fibronectin receptors-FNRs (185). Similarly, the “Parasite infection” subpathway will be populated with the steps that describe the maturation of the phagocytic cup, and so on.

III. **Killing mechanisms:** this subpathway was designed to contain the signaling cascades that converge in the production of antimicrobial molecules in the context of leishmaniasis. We started by curating the activation of the receptor Frizzled-7 (FZD7) by the ligand WNT5 and its downstream cascade. To build this pathway we reviewed publications that contain the original experimental data used to determine the reactions details (195–199). WNT5 is known for being a highly specific regulated gene in response to microbial infection (200–202) including leishmaniasis (203), where it seems to be involved in mechanisms that dampen the parasite load within the macrophage. Complementary, FZD7 acts as a receptor of WNT5 which, upon binding, is implicated in the initiation of the non-canonical WNT pathway that leads to re-organization of the cytoskeleton to allow a process called planar cell polarity (PCP) (202). The activation of the WNT5:FZD7 non-canonical signaling cascade that drives PCP is being studied for its involvement in inflammatory responses (204). Treatment of RAW264.7 macrophages with recombinant WNT5 induced NADPH oxidase-mediated ROS production, which has been suggested to contribute to the macrophage control of *L. donovani*. Consequently, detailed understanding of how the WNT

signaling network defines host responses to infection could be important to identify new potential therapeutic targets (202).

We represented in 9 reactions, the activation of FZD7 by the WNT5 ligand, resulting in the production of ROS (table 1). Unlike the phagocytosis pathway, these reactions correspond to a host's response to the infection, even if no parasite components are depicted in the diagram (found at https://reactome.org/PathwayBrowser/#/R-HSA-9673324&PATH=R-HSA-1643685,R-HSA-5663205,R-HSA-9658195,R-HSA-9664420). In future versions we will incorporate the cross talk with signaling cascades, like TLR-signaling, that activate antimicrobial functions and synthesis of antimicrobial molecules.

IV. **Cell recruitment:** this subpathway was aimed at bringing together signaling pathways that converge in the induction of gene expression and synthesis of chemokines and pro-inflammatory cytokines. It is known that a proinflammatory response early in the infection enhances host cell microbicidal mechanisms (205). However, the recruitment of inflammatory cells to the site of infection, once the parasite load has been controlled, transforms the course of infection and can lead to immunopathology (189). Therefore, it is important to curate and represent specific pathways that have shown to be activated upon *Leishmania* infection, resulting in the production of pro-inflammatory mediators.

The first specific mechanism we curated was the activation of the purinergic receptors P2RX4 and P2RX7. The liberation of ATP normally occurs in tissues facing stressful stimuli such as infection (206). Binding of ATP to purinergic receptor activates the inflammasome leading to subsequent activation of interleukin 1 beta-IL1β, which promotes the recruitment and activation of macrophages (207). We represented this process in 27 reactions (table 1), that included a regulatory step mediated by NTPDase1 and NTPDase5, which reduces ATP to adenosine (141). The molecular diagram can be found at https://reactome.org/PathwayBrowser/#/R-HSA-9664424&SEL=R-HSA-9660826&PATH=R-HSA-1643685,R-HSA-5663205,R-HSA-9658195.

There are many other pathways promoting cell recruitment as a response to *Leishmania* infection with different consequences for the parasite and the host (208). In future expansions of this subpathway, it would be possible to highlight cross talk between different cascades that target the same effector molecules.

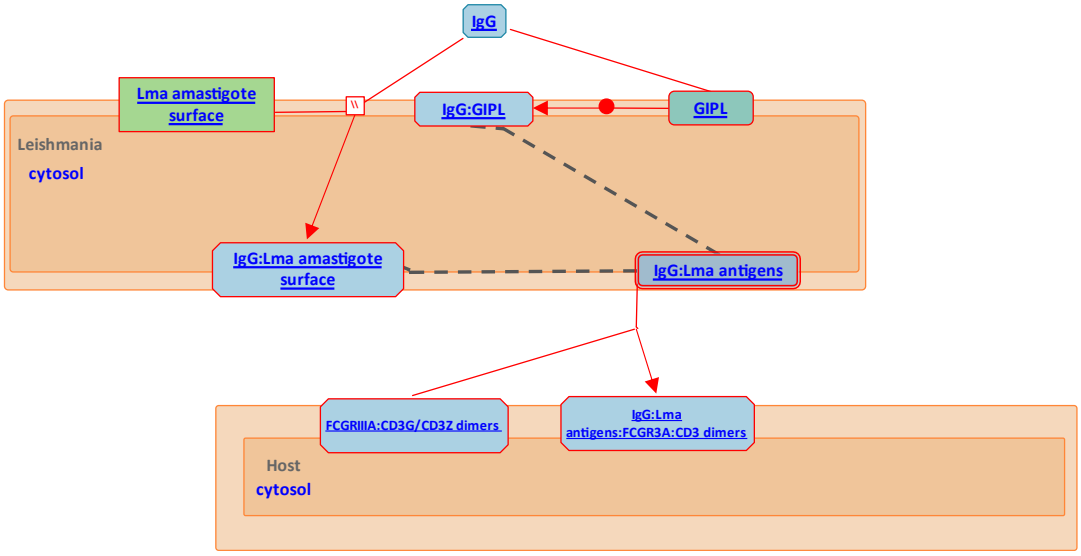
V. ***Leishmania* parasite growth and survival**: this subpathway covers the host responses that favor intracellular parasite survival, and the mechanisms used by the parasite to hijack host cell functions. To survive as an intracellular parasite, *Leishmania* evades the activation of host cell microbicidal machineries. Many mechanisms facilitate this purpose. On the host side, the production of anti-inflammatory mediators often occurs alongside the repression of expression of antimicrobial molecules, together with the recruitment of regulatory immune cells (e.g., regulatory T-cells). On the parasitic side, inactivation of host molecules through mechanisms such as cleavage or activation of phosphatases are part of its repertoire (189). Induction of anti-inflammatory molecules was the first mechanism that we curated, compiling the steps that describe the cleavage of the membrane protein CD163, the activation of the receptors FCGR3A and ADORA2B, and ending with the corresponding production of the known anti-inflammatory molecules sCD163, IL4, and IL10, as well as the dual functioning IL6.

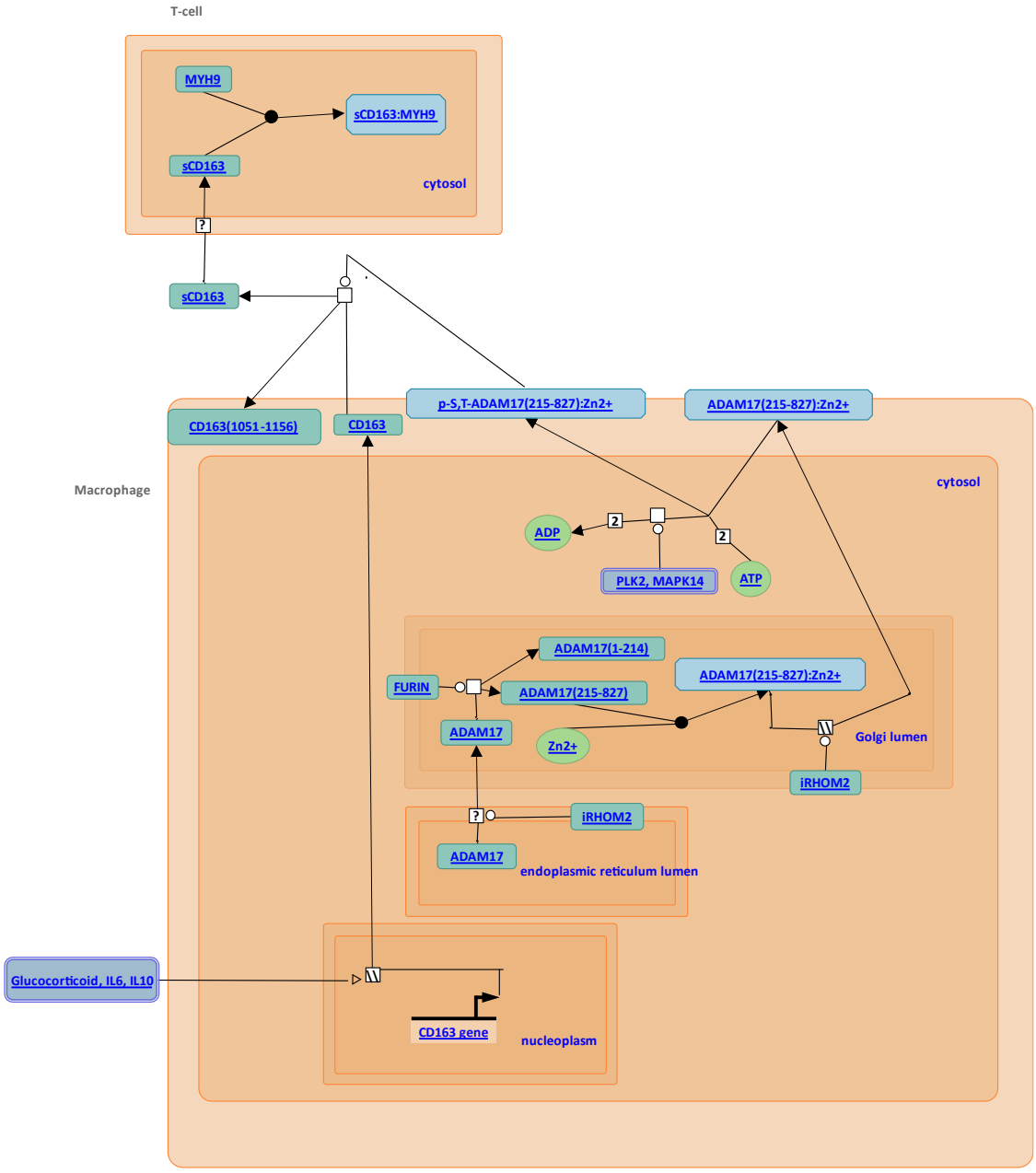
Macrophages infected with *L. amazonensis* or *L. donovani* strongly express the membrane protein CD163 (209–211), and soluble CD163 (sCD163) has been proposed as biomarker of visceral leishmaniasis. The hypothesis of the association between sCD63 and an anti-inflammation status is that it interferes with the proliferation of T-cells (212, 213). sCD163 is formed from the increased shedding of CD163 mediated by the metalloprotease ADAM17 (214, 215). Posteriorly, it might translocate to the cytoplasm of T-cells (through an unknown mechanism) where it binds with a protein involved in the proliferation process (212, 213). In “CD163 mediated anti-inflammatory responses” we represented, in 9 reactions, the production of sCD163 including the steps that precede the activation of ADAM17. Additionally, we included the positive regulation of glucocorticoids, IL6 and IL10 on CD163 gene expression (216–219). In figure 3.3b we show the molecular diagram depicting the pathway.

IL-10 is an important immunoregulatory cytokine produced by many cell populations; in macrophages it is induced after the stimulation of TLRs, FCG receptors or by TLR-FCGR crosstalk (220). Classically, its function is considered to be the limitation and termination of inflammatory responses and the regulation of differentiation of several immune cells (221). In the context of leishmaniasis, IgG-opsonized amastigotes have been shown to induce IL10 production through FCGRs, which in turn suppresses the killing mechanisms in phagocytic cells (194). We represented, in 21 steps, the activation of FCGR3A that leads to the activation of the transcription factor CREB1, ending with the production of IL10.

Finally, we curated ADORA2B-mediated anti-inflammatory responses. ADORA2B is a receptor for the ribonucleoside adenosine. Its activation leads to the production of anti-inflammatory cytokines which have been shown to favor *Leishmania* infection and survival (222–224). Apparently, this pathway exerts an opposing/regulatory response to the purinergic signaling pathway. The blockade in the production of pro-inflammatory cytokines may come with the inhibition of killing mechanisms (225). We represented this pathway with 6 reactions, starting with the binding of adenosine to ADORA2B, and ending with synthesis of IL6. Both FCGR3A and ADORA2B signaling pathways activate transcription factors, generating a positive feedback loop for transcription of more anti-inflammatory cytokines (226, 227). In future versions, these reactions might be incorporated, as well as other pathways leading to the synthesis of other anti-inflammatory mediators known to be induced during the *Leishmania*-host interactions. Moreover, other mechanisms that favor the persistence of *Leishmania* parasites must be added into new subpathways (e.g., Polyamine synthesis). For the representation of these pathways in Reactome, follow the link https://reactome.org/PathwayBrowser/#/R-HSA-9662851&PATH=R-HSA-1643685,R-HSA-5663205,R-HSA-9658195,R-HSA-9664433.

A.


IgG
Lma amastigote surface
IgG:GIPL
GIPL
Leishmania
cytosol
IgG:Lma amastigote surface
IgG:Lma antigens
FCGRIIIA:CD3G/CD3Z dimers
IgG:Lma antigens:FCGR3A:CD3 dimers
Host
cytosol



B.

Figure 3.3: Standard graphical representation of pathways in Reactome. **A.** Fragment of the diagram for FCGR3A-mediated phagocytosis. It shows the reactions corresponding to the parasite opsonization process by the IgG. Parasitic components are highlighted in red. **B.** CD163 mediating an anti-inflammatory response. Each diagram shows the participating entities in the granularity of chemical compounds (green ovals), proteins (green rectangles), complexes (blue rectangles) and sets (blue rectangles with a double border). The arrangement of the entities in the reactions can be easily followed on the web page, by clicking on the arrows that connect adjacent steps.

Leishmania infection pathways enhancing transcriptome data analysis

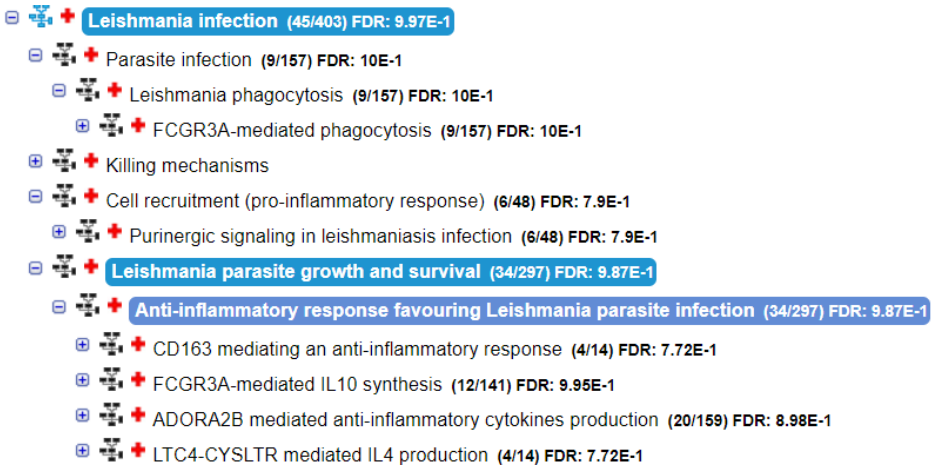
The first version of *Leishmania* infection pathways was released in March 2020. All released data can make use of Reactome pathway analysis tools. We used the ORA tool to test the impact of context-dependent labels on published datasets (prior march-2020) that explored the *Leishmania*-host interaction.

In 2015, Dillon *et al.* (18) explored the early response of macrophages to infection with *L. major*, using RNA-seq. With the differentially expressed (DE) gene list (>|2|-fold, uninfected macrophages versus infected macrophages at 4 hours post-infection), they performed ORA against the curated pathways in the KEGG database. For the up-regulated genes, the results included cytokine-cytokine receptor interactions, TNF-signaling pathway and NFkappa B-signaling pathway, among other pathways. An overall interpretation of these enriched pathways may suggest that the early infection of macrophages by *L. major* leads to induction of pro-inflammatory responses; as an example, TNF-signalling as represented in KEGG (PATHWAY: hsa04668), classically leads to the recruitment of inflammatory cells. However, transduction of the signal may induce the activation of factors that contribute to opposite responses, such as tissue regeneration (with VEGF and EDN1) (228, 229). Interestingly, among their gene-specific analyses, Dillon *et al*. identified a set of genes involved in anti-inflammatory responses (*Csf1, Csf3, Il10, Il11r, Il1rn, Socs3, Hmox1, Egfr* and*, Vegf*). However, the mechanisms that lead to production of these effector molecules, or how they contribute to achieve or maintain the underlying immune status couldn't be inferred from the KEGG pathway analysis. To overcome this gap, we implemented ORA from the same DE gene list (annex 3.1 table ST1) in Reactome. Among enriched pathways (ST2), were some of the *Leishmania* infection subpathways. As reported by Dillon and colleagues, FCGR3A subpathway (figure 3.4a) was found downregulated. Interestingly, the most over-represented pathway was the ADORA2B mediated anti-inflammatory cytokine production (Fold change-FC = 3.25). This pathway contributes to the expression of IL6 (FC = 7.68) and IL10 (FC = 17.05), through the activation of the transcription factor CREB (FC = 2.75) (figure 3.4b). This pathway, simultaneously, leads to the activation of killing mechanisms, as well as to the production of IL10. Neither authors nor we found pathways involved in the former. Therefore, this suggests an alternative mechanism mediated by ADORA2B, that leads to production of IL10, and does not induce parallel pro-inflammatory consequences or the activation of antimicrobial molecules

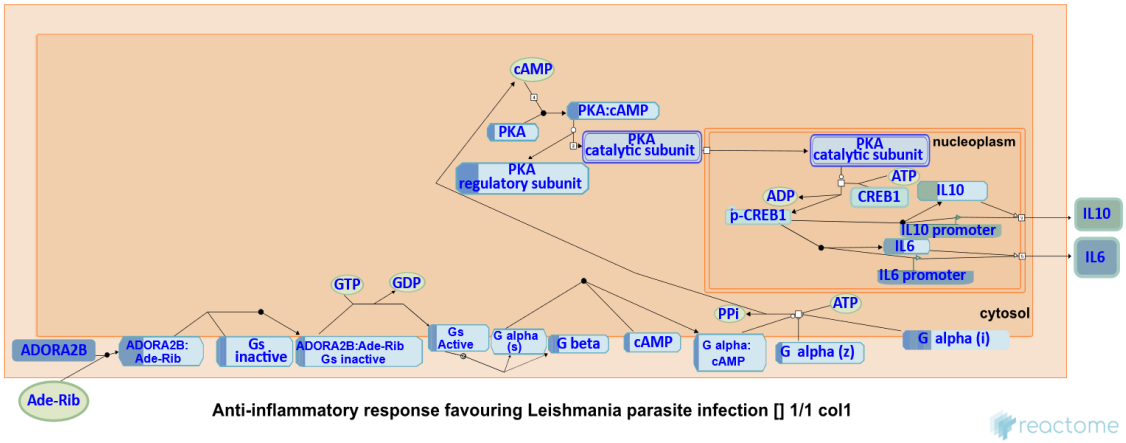
Complementary, we analyzed the microarray data from Gregory *et al.* (182) which represent the transcriptomic response of murine macrophages to infection with *L. major* and *L. donovani*. The

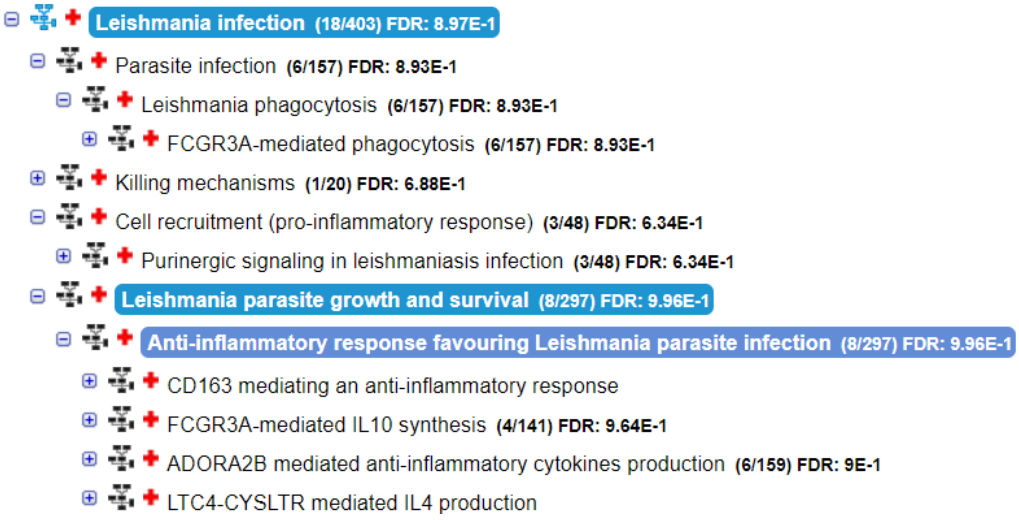
authors stated that there were few differences in the number of genes and the magnitude of the expression with both species. Like Dillon's dataset, the ADORA2B subpathway was enriched among upregulated genes (figure 1.4-c and annex 3 table ST1 and ST2). These results highlight the identification of ADORA2B-pathway driving anti-inflammatory cytokine production, consistently activated in macrophages infected with different *Leishmania* species. These results substantiate the usefulness of the *Leishmania* infection pathways for gaining mechanistic insights from new and previously published data. In expanded versions of this pathway, secondary analysis like the one we performed would shed light on response patterns against the infection with different *Leishmania* species, as well as species-dependent responses.

A.



B.





C.

Figure 3.4: Results of applying ORA in Reactome, on the datasets generated by Dillon *et al.* and Gregory *et al.* A. Number of genes found in each subpathway from Dillon's dataset, with the associated false discovery rate (FDR) B. ADORA2B pathway enriched in Dillon's dataset shows upregulated genes involved in ADORA2B signaling cascade leading to the production of IL6 and IL10. C. Number of genes found in each subpathway from Gregory's dataset, and associated FDR.

DISCUSSION III

Over-representation analysis (ORA) has become one of the standard methods for extracting mechanistic information from omics data. As part of the workflow, ORA matches the omics data with molecular data curated in pathway databases. As a result, it gives a list of pathway names in which the genes are known to be involved. Mechanistic insight must then be built from a list of labels, a problem made much harder when labels lack biological context. There are several specific cases in literature, relating to *Leishmania*-host interaction, where this has proven to be an issue (18, 19, 230). In this work, we addressed this by creating leishmaniasis-context labels pathway in Reactome database. We created four subpathways, labelled as: *Leishmania* phagocytosis, killing mechanisms, cell recruitment (pro-inflammatory response), and *Leishmania* parasite growth and survival. Inside each, we labeled the pathways according to the receptor or membrane protein directing the signaling cascade. This structure facilitates the generation of high-level mechanistic insights from low-level processes highlighted by ORA, which are sensitive to particular experimental contexts (e.g. the type of source of the biological sample that derived the data).

Manual literature search is often required for in depth interpretation of the outcome of ORA for extracting biological/functional insights. However, this strategy is time consuming, prone to omissions, and certainly incompatible with unbiased exploration of novel mechanisms/functions in the data. This is because the search is necessarily constrained to cover a small number of topics within the researcher's area of expertise, and it is unachievable to manually trawl the entirety of biological literature relating to a particular gene, microorganism or disease.

We have re-analyzed two previously published transcriptomic datasets (from *Leishmania*-infected macrophages), to provide proof-of-concept of the usefulness of context-dependent databases such as the one described in this study, and recently released Reactome *Leishmania* infection pathways. Results from this secondary analysis revealed the putative participation of a signaling pathway (ADORA2B mediated anti-inflammatory cytokines production), in the parasite-mediated induction of anti-inflammatory molecules.

Although *Leishmania* infection pathways in its first version is far from representing the full current knowledge about the interaction between the parasite and the host, we have shown that our database curation has led to new mechanistic insights from existing datasets. Moreover, these findings are generated from the use of a workflow that skips particular time-consuming, problematic manual curation steps, aligning the available data interpretation tools to the nature of unbiased hypothesis generation from –omics datasets.

CONCLUSIONS III

In *Leishmania* infection pathways we curated detailed information on biological processes known to be activated during the *Leishmania*-host interaction. We focused on those processes correlated with the outcome of infection. This allowed to uncover new mechanistic insights about macrophage permissiveness to *Leishmania* infection, from available transcriptome data of infected macrophages. This pathway is sufficiently flexible as to allow for expansion and revision as new datasets are published. With the participation of the scientific community in Leishmaniasis, this pathway has the potential to enable fast and curated access to the integrative mechanisms of importance in this disease.

CHAPTER 4: General discussion

Macrophages are the all-purpose soldiers of the immune system. Not only do they go into battle, eliminating foreign pathogens, but they are also critically involved in tissue repair after the immediate threat has been negotiated. Individual macrophages contain a huge suite of metabolic pathways that are programmed in response to a wide variety of external stimuli, whether from surrounding tissue or from invading pathogens. Understanding, predicting, and being able to manipulate the metabolic programs underlying macrophage behaviour is a pathway towards understanding and treating the overall immune response to pathogenic infection (90, 231–233)

Macrophages can be fooled. A successful strategy of *Leishmania*, the pathogen of interest in this thesis, highlights this. During the initial, pro-inflammatory phase of most infections, the microbicidal functions of macrophages are activated. During the subsequent anti-inflammatory phase, after the pathogen has been eliminated, macrophages become more benign neighbours, repairing tissue damaged by the pathogen and the inflammation (231). *Leishmania*, however, seems to manipulate the behaviour of macrophages so that their microbicidal processes are quickly deactivated, even in a highly pro-inflammatory environment. This helps the parasite to survive the initial pro-inflammatory cascade, and thrive in the damaged, inflamed tissue that results (186).

How can we get *Leishmania*-infected macrophages to 'wake up' and activate their microbicidal response to a pro-inflammatory environment? Answering this question is key to producing treatment strategies that eliminate chronic infection. Helping to answer this question was the key goal of this thesis.

Understanding the dynamics of the macrophage response to infection by *Leishmania* parasites.

We now give a summary of the basic motivations, methodology, and limitations of the first three chapters of the thesis. This forms a springboard from which we can jump into some of the specific findings, and the scientific questions they generate.

To understand how the *Leishmania* parasite manipulates the immunometabolic pathways within a macrophage, one first needs an understanding of how immunometabolism is altered during *Leishmania* infection. Omics techniques constitute a great approach to indirectly gain information on the whole suite of immunometabolic pathways, by observing `snapshots' of the overall concentrations of large libraries of RNA/protein types over time.

The first contribution of our thesis was to reconstruct the immunometabolic pathways modulated in *L. panamensis*-infected macrophages at 24 hours post-infection, by analysing RNA-seq data. This initially involved `differential expression analysis': a statistical comparison of the RNA expression data for infected vs uninfected macrophages. This provided us with a list of genes that were differentially expressed during *L. panamensis* infection, together with an estimate of the corresponding fold-change in expression.

We comprehensively queried databases of biological pathways, as well as existing literature on the functionality of genes in our list, to find candidate pathways whose modulation would be consistent with our list of differentially altered genes. We laid more emphasis on pathways that have been previously studied in the context of other *Leishmania* species. Our database search was algorithmic, using an approach known as 'pathway enrichment analysis'.

The described methodology can never unambiguously uncover the exact suite of altered metabolic pathways for several reasons. Firstly, the immunometabolic reconstruction is based on mRNA expression and therefore ignores downstream modifications, such as enzymatic phosphorylation, that may affect eventual gene expression. Secondly, the statistical methodology has several issues. The fidelity of the statistical comparison is unavoidably constrained by the noisiness of the data, and the limited numbers of experimental replicates available. Different choices of parameters in the differential expression analysis led to slightly altered lists of final genes, which biases the search for pathways. Inferring likely pathways from the candidate gene list also suffers from several issues. Multiple pathways can share similar genetic profiles, in which case pathway selection relies on intuition. Furthermore, there is a limited list of pathways available to select from, given that not all pathways have yet been explored in the literature and curated in databases.

Nevertheless, our proposed set of pathways is important for several reasons. Firstly, it provides strong hypotheses for the existence of particular pathways, which can be tested with more targeted experiments. Secondly, it allows us to weave together a holistic proposal for how the macrophage deals mechanistically with the infection. Indeed, we made several important observations/hypotheses based on our reconstructed set of immunometabolic pathways, some of which we outline subsequently in this discussion.

Scientific understanding is difficult to distil from a large array of altered immunometabolic pathways. The second main contribution of this thesis was the construction of a mathematical model of the dynamics of this immunometabolic alteration in the 24 hours post infection, which

was tied to transcriptomic data. The model showed that the transcriptomic data supports a paradigm whereby macrophages dynamically rewire their metabolism post infection, moving from a pro-inflammatory, microbicidal state to an anti-inflammatory state more conducive to tissue repair. Interestingly, the final anti-inflammatory state charted by our model, and data, possessed upgregulated, but potentially inactivated, pro-inflammatory pathways. The existence of these provides a means for macrophages to shift back to a pro-inflammatory state, a transition more extensively reported in the literature.

The construction of dynamic mathematical models of metabolism, built from mathematical objects known as `differential equations', has a long and distinguished history (234–236). Such models (and indeed nearly all mathematical models in biology) are coarse approximations of the underlying reality, used to generate hypotheses or help answer scientific questions, rather than to be a truly faithful representation of reality. In the past, such models have been constructed using time-course data of metabolite concentrations within a cellular preparation (237). By contrast, we had transcriptomic data, which has the advantage of simultaneously monitoring a large host of mRNAs molecules, but at the cost of only measuring fold changes, rather than absolute values, of RNA concentrations. To our knowledge, our construction and fitting of a differential-equation model from fold change data is methodologically novel. We believe that it could serve as an important example to modellers in other areas of biology, given the wide availability of transcriptomic data, as well as its advantages in highly parallel measurement-taking.

An important extra constraint introduced in our model was that fold changes should be one (i.e. the modelled molecules concentrations should be static) in the control experiment where *Leishmania* has not being introduced into the macrophage yet. We built a methodology whereby this constraint was satisfied automatically. As such, our model accounts for not only alterations to the immunometabolism post-infection, but also the lack of alterations in the absence of infection.

Fitting large differential equation models to data has traditionally been a highly involved and rate-limiting step in the scientific process. This exercise was often achieved by evolutionary / genetic algorithms, which are highly inefficient in models with tens of parameters and require huge amounts of both computational power and time (238, 239). There have been recent advancements in this field, however. Automatic or algorithmic differentiation, a subfield of computer science, has existed for several decades as a way to quickly and accurately acquire the sensitivity of a computer program's output to changes in its input (240–242). Recent advances in the Julia language, and in particular the DifferentialEquations.jl package, allow one to use this technique to quickly and accurately find the sensitivity (i.e. the 'mathematical derivative' of

calculus) of the output of a differential-equation model to changes in its parameters. This allows the use of more sophisticated optimisation algorithms, such as gradient descent, to efficiently fit differential-equation models to data (240–242).

The scientific meaning of fitting complicated mathematical models to data has always been a contentious topic. As Von Neumann joked, "with four parameters I can fit an elephant, and with five I can make him wiggle his trunk". The initial phase of Systems Biology took the parameter values gained from fitting metabolic models to data seriously. These represented hypothetical values for e.g. the reaction rate of particular metabolic processes. In the last decade, many computational papers have shown (through both example and theory) that such parameter values are meaningless: in a model with more than a few parameters, one can maintain a very good fit with the data even as individual parameter values vary by orders of magnitude (237, 243, 244). This phenomenon is often known as `sloppiness' or 'unidentifiability' of the model (243). The purpose of our mathematical model is not to assign importance to synthesised parameter values. Instead, it is to show that a parsimonious set of mechanisms, proposed via our pathway enrichment analysis, are capable of explaining the transcriptomic data of this thesis. Our model generates explicit scientific predictions, and is integral to the generation and validation of specific hypotheses that we will soon discuss.

Several of the research steps taken in this thesis were unnecessarily time-consuming and difficult. The third contribution of this thesis was aimed at avoiding the repetition of such steps for researchers in the wider Leishmaniasis community. We set up a project to insert and annotate known “*Leishmania* infection pathways” in the broadly used Reactome database (179), for the benefit of the wider research community. These database entries are designed for easy expansion and revision as new datasets are published.

The first difficult step was the extensive literature review required to extract scientific meaning from each biological pathway flagged by the transcriptomic data. We inserted selected pathways into the Reactome database, along with detailed contextual knowledge gained from each literature review. Unfortunately, Leishmaniasis is a neglected disease. Relatively few biological pathways associated with infection by Leishamaniasis strains have been documented in such databases. This is a first step towards rectifying this systemic issue.

The second difficult step was reasoning about the mechanistic insight provided by knowledge of the presence/alteration of a particular pathway in Leishmaniasis infection. We chose the Reactome database to insert our pathways as the very structure of the database helps with this.

The hierarchical nature of the database allows one to connect low-level biological processes (e.g. receptor upregulation) with higher-level processes (e.g. inflammation). One can easily query the list of intermediate and higher-level processes associated with very specific biological pathways, which aids in the process of hypothesis generation from transcriptomic data (e.g CD163 receptor mediating anti-inflammatory responses https://reactome.org/content/detail/R-HSA-9662834)

Summary of main findings

We now provide a condensed summary of some of the important, specific findings and questions that arise from this thesis, subject to the caveats noted in the previous section. This paves the way for a subsequent, more open-ended discussion of the significance and relevance of the thesis.

We gained several insights from our reconstruction of the immunometabolic pathways modulated in *Leishmania panamensis* at 24 hours post-infection. We emphasise that these insights are not definitive and have discussed their caveats more fully in earlier chapters. We now list some important insights.

- A glycolytic efflux was activated soon after infection.
- Lipid metabolism apparently produces both pro and anti-inflammatory bioactive lipids, simultaneously.
- A glutathione-based reactive oxygen species-scavenger system is active at 24 hours post-infection. Since ROS is a microbicidal agent, this could be aiding parasite survival.
- Production of anti-inflammatory cytokines and chemokines, such as IL10 and CCL24, are upregulated. These have previously been shown to benefit parasite survival in *in vivo* models with other *Leishmania* species, and are presumably fulfilling a similar role with *L. panamensis*.
- The over-expression of receptors that transduce pro-inflammatory signals in *L. panamensis*-infected macrophages. This shows the susceptibility of the macrophage to shift towards a pro-inflammatory profile when exposed to the “right” inflammatory cues or the “right” magnitude.

While each of these insights constitutes nuggets of information, integrating such insights into a holistic view of the overall workings of the immunometabolic system is an additional challenge. We distilled a holistic proposal of this type into our mathematical model, and validated its compatibility with the transcriptomics data. Our proposal is that several important aspects of the macrophage immunometabolism shift over the first 24 hours following infection. Our model

proposes a time course of the respective shifts, which are otherwise hidden in temporally sparse transcriptomics data.

- The metabolism of the macrophage, post-infection, shifted from one based on glycolysis to one based on oxidative phosphorylation.
- The metabolism similarly shifted from oxidative stress (low degradation of reactive oxygen species) towards anti-oxidative stress (high degradation).
- The immune profile of the macrophage, post-infection, shifted from a pro-inflammatory state to an anti-inflammatory state. The TLR4 receptor plays a dual role in this regard. Early on, it contributes to the pro-inflammatory state through the activation of NFkB. As a later consequence of TLR4 activation, STAT3 gets activated. This transcription factor mediates anti-inflammatory mechanisms that also positively regulate its own expression. Additionally, STAT3 induces a negative feedback on TLR4, and thus indirectly on NFkB, therefore, downregulating the pro-inflammatory response.

Direction for future research

We now discuss the potential of the insights we have summarised in informing new immunotherapies. A key theme of our research was that the pro-inflammatory, microbicidal functions of the macrophage, which are turned on upon the initial entrance of the *Leishmania* parasite, seem to be speedily turned off, even before *Leishmania* is eliminated. This allows the *Leishmania* parasite to thrive in a more benign environment. We hypothesise that this premature switch (which is necessary for tissue repair after parasite elimination) is hastened by manipulation of the immunometabolism by the parasite itself.

Assuming our hypothesis is correct, this survival strategy is by no means unique to the *Leishmania* parasite. We, therefore, propose a future research program of identifying pathological biological systems in which similar macrophage reprogramming seems to occur, and testing effective immunotherapies for these pathologies on Leishmaniasis. Indeed, this *"modus operandi"* can be extended to considering pathologies in which the macrophage immunometabolic response shares any notable features with our reconstruction. This research program makes sense given that Leishmania is a neglected disease, which is allocated very little research time and funding as compared to pathologies such as cancer, with which there are nevertheless commonalities in terms of macrophage response.

We now provide some background on pathologies sharing analogous aspects of macrophage immunometabolic response. We begin by considering tumoral-associated macrophages (TAMs)

and *M. tuberculosis*-infected macrophages. There has been an explosion of research articles and reviews describing plasticity in the immunometabolism of macrophages in the context of cancer (233, 245–248). TAMs (as well as *M. tuberculosis*-infected macrophages) share an "M2" like, anti-inflammatory profile, in common with *Leishmania*-infected macrophages (249). Their bioenergetic system is based on oxidative phosphorylation rather than glycolysis, and that their anti-oxidative stress response is mediated by glutathione production (250). Interestingly, STAT3 plays a critical role in TAM inducing tumour progression (251–253). Our simulations for *Leishmania*-infected macrophages showed that STAT3 is one of the dominant upregulated transcription factors after about 5 hours post-infection, and together with IL-10, positively regulates the production of CCL24. Both this chemokine and IL-10 are produced by TAMs (254, 255). As previously discussed, these inflammatory mediators are fundamental to maintaining the “M2”-like profile in *Leishmania*-infected macrophages. It appears that they also play an analogous role in TAMs.

Reprogramming of TAMs towards a pro-inflammatory profile that kills cancer cells has been applied as immunotherapy for solid tumours (248, 256). One approach consists in targeting CD40 receptors expressed in TAM with agonist agents (256–259). This stimulates the production of NFkB-dependent pro-inflammatory mediators (260, 261). Interestingly, functional activity of CD40 in Leishmaniasis has also been associated with protective effects during the infection with different species of *Leishmania* (262–265). For instance, the ligand for CD40 (CD40L) confers protection in Visceral Leishmaniasis (262) and the murine Cutaneous Leishmaniasis *by L. amazonensis* (264). Moreover, adding a CD40 agonist to ex-vivo models of *Leishmania*-infected macrophages reduces the parasite load while increasing the secretion of pro-inflammatory cytokines (266). The authors of the latter study, highlight that the mechanism by which the macrophage controls the infection is unknown.

We propose extending our mathematical model to explore the mechanism by which CD40 agonists reduce parasite load. First, we would incorporate CD40 into the model structure, specifying that it positively regulates the same species as the TLR-TRAF-NFkB signalling pathway, and that it is negatively regulated by adenosine receptors (see (267, 268) for justification, and Figure 2.1 for a graphical depiction of this description). Next, we would calibrate the extended model with the transcriptomics data provided in (18, 19)

We would then use a cycle of modelling and experiment, starting with our extended model, to research the relevance of targeting CD40 to reprogram macrophage immunometabolism to a more pro-inflammatory state. This research program would proceed in several stages:

- It is paramount that any Leishmania therapy be effective regardless of the specific *Leishmania* species. Therefore, to validate CD40 as an immunotherapeutic target, we propose to start by evaluating in parallel, the effect of activating CD40 on the immunometabolic profile of macrophages independently infected with the *L. panamensis, L. major* and *L. amazonensis* species.
- Second, with a customized microarray chip containing only the genes integrated into the extended mathematical model, we could chart the dynamics of the infection over time, with and without the addition of CD40L to the ex-vivo *Leishmania*-infected macrophage system.
- The fold-change data on both RNA and parasite load coming from this experimental setup can then be used to further inform the mathematical model. This way, we could test the validity of CD40L-CD40 in reprogramming permissive macrophages towards resistant macrophages. Moreover, the model could be used to predict the critical CD40L concentration required to induce such an immunometabolic shift, in case the CD40 strategy was indeed valid. This model-based hypothesis could be again tested on the bench, with data again further informing the model.

A key constraint of immunotherapies targeting neglected tropical diseases is affordability. Chemotherapy-based approaches often fare better on this count than the recombinant protein-based approaches discussed previously. Fortunately, we found that TAMs and macrophages harbouring *M.tuberculosis* are also reprogrammed by chemotherapy approaches. In particular, metformin (245, 269) and curcumin (270, 271) have shown promise.

Metformin acts as an inhibitor of the respiratory complex I (245). As we depicted in figures 1.11 and 1.13 in Chapter 1, this complex is of great importance for the oxidative phosphorylation metabolism. Its inhibition skews the immunometabolic profile of the macrophage towards an "M1"-like, pro-inflammatory state, as has been shown for TAMs (245, 272). Furthermore, metformin reduces the intracellular growth of the bacteria (273), for macrophages harbouring *M. tuberculosis*. This is associated with the interruption of the electron transport in mitochondria and the positive regulation of NFkB (273) (a readout of inflammation). In the tuberculosis murine model, metformin improved lung pathology, reduced chronic inflammation, and enhanced the efficacy of conventional tuberculosis drugs (30). In a case-control study, metformin was also found to be protective against M. tuberculosis in diabetic patients (274).

Curcumin, and its isolated bioactive ingredients (e.g. Puerarin), also induces reprogramming towards a resistance pro-inflammatory state in both TAMs and THP1(human monocytic cell line

derived from an acute monocytic leukemia patient)-derived macrophages. This is done by targeting several pathways, including the inactivation of STAT3 (275, 276) and the inhibition of TLR4 (277). The Leishmanicidal effects of curcumin were confirmed through testing on several species of *Leishmania* (270). This motivated the use of curcumin encapsulated nanoparticles to target macrophages in CL lesions, with promising results (271).

Both metformin and curcumin have huge potential to be used in a neglected disease such as Leishmaniasis. The pathways that are modulated by these agents seem to be consistently modulated in TAMs, M. Tuberculosis-infected macrophages, and Leishmania-infected macrophages. Our mathematical model already provides mechanistic insight into how modulation of these pathways promotes a pro-inflammatory macrophage state. We propose an analogous research program to that we proposed for CD40 based therapy, incorporating a cycle of targeted experiments (as opposed to -omics) that precisely measure dynamics of metabolites associated with these pathways, and mathematical model refinement.

There is growing interest in the dynamics of macrophage immunometabolism for different diseases. In particular, immunometabolic reprogramming seems to be an interesting approach to fight diseases that show a high macrophage involvement. Our detailed reconstruction of the immunometabolism of *Leishmania*-infected macrophages revealed the potential of “repurposing” immunotherapies applied in fields that are financially more supported. Additionally, our mathematical model would enable the outcomes of different therapeutic strategies to be assessed computationally before expensive experimental testing.

Nevertheless, there are some future challenges that need to be considered in the study of macrophage immunometabolism. A greater understanding of the interplay between the metabolism of the host and the parasite is necessary, as the adaptation of one to the other might hamper immunometabolic targeting. Likewise, our work has been informed by *ex vivo* data. However, during the infection macrophages must deal with a complex and dynamic microenvironment. Hence, *in vivo* data will be critical to bringing Leishmaniasis therapies based on immunometabolic manipulation one step closer to the clinic.

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