FROM THE MARROW TO THE BLOOD: A MULTI-OMIC SIGNATURE ASSOCIATED WITH RESILIENCE TO MALARIA.

by

DIEGO MAURICIO MONCADA GIRALDO

(Under the Direction of Juan B Gutierrez)

ABSTRACT

Despite many years of research to control and eradicate the disease, malaria, caused by parasites of the genus *Plasmodium,* continues to be a worldwide health burden. While much focus has been placed on the properties of the infecting parasites, recently more attention has been given to interactions between the host and the parasite, which drive clinical outcomes. To investigate the underlying pathways in host responses, whole blood transcriptome, plasma metabolome, and proteome analysis were performed during peak of parasitemia from two longitudinal experimental malaria studies encompassing two different nonhuman primate host species (*Macaca mulatta* and *Macaca fascicularis*) infected with *P. knowelsi.*

INDEX WORDS: Multi-omic, Malaria infection, Resilience, Transcriptome, Proteome, Metabolome, *Macaca mulatta*, *Macaca fascicularis, Plasmodium knowelsi.*

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Chapter 1

Introduction and Literature Review

Malaria continues to be a worldwide health burden in spite of research efforts to develop novel disease treatments and intervention strategies [1]. New treatments are needed to continue to improve health outcomes and reduce the economic and social impacts of this disease. Historically, medical countermeasures have been pathogen-centric, which have placed evolutionary pressure on the parasite leading to the emergence of drug-resistance [2], [3]. Rather than exerting selective pressure with anti-parasitic drugs, favorable clinical outcomes may be promoted by increasing the hosts tolerance and resilience to infection [4]–[7].

Clinical manifestations associated with *Plasmodium* infections may be classified into asymptomatic, uncomplicated, and complicated cases with common symptoms that include fever, chills, and muscle aches [3], [8]. Asymptomatic cases most often occur in adults from regions of high malarial transmission and are characterized by the presence of circulating parasites, with parasitemias that may be up to 50,000 parasites per microliter, but no symptoms [3], [9], [10]. Uncomplicated malaria is characterized by parasitemias typically in the range of 1,000 to 50,000 parasites per microliter along with fever, sweating, chills and muscle aches but symptoms may include headache, nausea, vomiting, diarrhea, and anemia [3], [11]. Complicated or severe malaria happens more often in *P. falciparum* infections than with other infecting species and is deadly in 20% of adults and 10% of children [3], [12]. Since malaria symptoms are not always related to the level of parasitemia, other causes and treatments are needed to reduce the burden of the disease.

Even though pathogen load is not clearly correlated to severity of symptoms, previous intervention strategies have focused on mechanisms whereby parasite replication is prevented and the parasite is cleared [13]. This anti-parasitic immunity is in contrast with anti-disease or clinical immunity in which symptoms of the disease are prevented [13]. Anti-disease immunity, which can be quantified by tolerance curves, is associated with asymptomatic malaria cases [4], [13]–[15]. In malarial infections, the host's own immune system, with excessive inflammatory activation, can be responsible for much of the damage done by the disease [8], [15], [16]. Also for host-pathogen systems, exposure to the pathogen and the subsequent ability of the host to maintain health and productivity, termed resilience, is often used when quantification of the parasite load throughout the infection is not of interest or is not feasible, as with herd animals and livestock [17]–[23]. Tolerance, resilience and anti-disease immunity, with their focus on host processes and prevention of damaging immune responses, have been studied more in recent years [4]–[6], [14], [24]. This has resulted in the identification of tolerance pathways and mechanisms, including production of anti-inflammatory molecules, induction of anti-oxidant mechanisms and metabolic adaptation by the immune system, that reduce the impact of the disease without attacking the parasite [9], [25]–[27].

Non-human primates, which are also susceptible to many human diseases, as the commonly studied *Macaca mulatta*, and *Macaca fascicularis*, are natural hosts to several *Plasmodium* species [28]–[31]. For example, *M. fascicularis* is the natural host for *P. cynomolgi*, *P. inui* and *P. knowlesi* and experimentally is also susceptible to *P. coatneyi*, *P. fragile*, *P. gonderi*, and *P. fieldi* [28]. Within this host species*, M. fascicularis* show differing degrees of severity depending of geographical origin and genetic background [28], [32]–[34]. In addition, the degree of *M. mulatta* ancestry for an individual *M. fascicularis* is related to severity of *P. cynomolgi* infection [35]. Generally, *M. mulatta* exhibit higher parasitemias and more clinical signs for the same infecting species (*P. cynomolgi*, *P. inui*, *P. knowlesi, P. coatneyi*, *P. fragile*, *P. gonderi*, and *P. fieldi*) than *M. fascicularis* [28], [30], [31], [34]. Specifically, *P. knowlesi* infections in these two hosts show very different clinical outcomes, with *M. mulatta* developing fulminant parasitemias, which lead to death in the absence of treatment, and *M. fascicularis* developing a mild, chronic infection [28]–[31].

To take advantage of both systems biology approaches and high-throughput technologies, the Malaria Host-Pathogen Interaction Center (MaHPIC) and the Technologies of Host Resilience Host Acute Models of Malaria to Study Emerging Resilience (THoR's HAMMER) projects were designed to provide insight into malarial infections [36]–[38]. Systems biology approaches can provide powerful insight into the multi-level interactions of a biological system, while high-throughput technologies enable many variables to be simultaneously measured [39], [40]. Because the interactions between a host and its pathogens are complex, varied and hard to study in isolation, such systems approaches will enable a more integrated understanding than studying the systems in isolation [41], [42] . These projects provide a rich data set from controlled infections that provide a framework to investigate molecular, cellular, and clinical mechanisms of disease which will lead to the design of future strategies for interventions in malaria.

To investigate the underlying pathways in host responses, whole blood transcriptomic measures were assessed during acute parasitemia from two longitudinal experimental infection studies of malaria in NHPs. These experiments encompassed one infecting species of *Plasmodium* (*P. knowlesi*) and two different species of NHP host (*M. mulatta* and *M. fascicularis*). In this study, differential expression analyses of transcripts, plasma metabolome, and plasma proteome intersection were conducted, and unique molecular signatures for each host and infection were identified. Finally, a multi-omic approach was used to describe the common immune molecular signatures associated with clinical outcomes between *M. mulatta* and *M. fascicularis*. As a result, this work provided new host directed targets that can be used for future investigations in the development of new medical counter measures.

Chapter 2

FROM THE MARROW TO THE BLOOD: A MULTI-OMIC SIGNATURE ASSOCIATED WITH RESILIENCE TO MALARIA.

1. Diego Moncada, Elizabeth Trippe, and Juan Gutierrez To be submitted to BMC Bioinformatics.

Abstract:

Despite many years of research to control and eradicate the disease, malaria, continues to be a worldwide health burden. Historically, medical countermeasures as antimicrobials have been pathogen-centric, which have placed evolutionary pressure on the parasite, leading to the emergence of drug-resistance. Rather than exerting selective pressure with anti-parasitic countermeasures, host-centric strategies promote favorable clinical outcomes without directly attacking the parasite. To identify new host-directed drug targets against *Plasmodium* infections, a differential expression analysis from whole blood cells and bone marrow transcriptome, plasma proteome, and the plasma metabolome were performed.

Abbreviations: DEGs: Differentially Expressed, NHP: Nonhuman Primate

Background:

During an infection, a host's immune system can promote a defense strategy consisting of inflammation, that, when excessive, can lead to tissue damage [4], [24], [43]. However, medical countermeasures, such as antimicrobials, have been pathogen-centric, which have placed evolutionary pressure on parasites and have resulted in the emergence of drug resistance [2], [3], [43]. Rather than exerting selective pressure with anti-parasitic countermeasures, host-centric strategies promote favorable clinical outcomes without directly attacking the parasite [4]–[7], [44]. In infectious diseases, host-targeted therapies have been successfully used with viral and bacterial infections and may be an important addition to standard treatments in other infectious diseases [44]–[46].

The ability of an infected host to self-recover to health is termed resilience [47]. The contrast between resilient and non-resilient host responses can help drive host-directed therapies. In infectious diseases, the emergence of resilience relies on the strategy that a host exerts to control a pathogen. A resistance strategy is mainly focused on decreasing the parasite burden at the cost of tissue damage. In contrast, a tolerant response targets the restoration of homeostasis and tissue recovery without exerting a direct effect on the parasite load [4], [6], [43], [46]. In recent years, tolerance and resilience have been studied with focus on host prevention of damaging immune responses. This has resulted in the identification of tolerance pathways and mechanisms, including production of anti-inflammatory molecules, induction of anti-oxidant mechanisms, and metabolic adaptation by the immune system [4]–[6], [14], [24], [46], [48].

In malarial infections, resilience mechanisms reduce the impact of the disease without attacking the parasite [9], [25]–[27], [47]. In these situations, the stress imposed on the circulatory system leads to the disruption of the host homeostatic conditions, and vascular damage [4], [43], [49], thus, influencing the appearance of signs and clinical manifestations [4], [43]. In consequence, the emergence of resilience relies on the maintenance of the functional outputs produced between circulatory cells and the vascular endothelium [4], [27], [49]. Mainly, the establishment of resilience to malaria depends on host-centric mechanisms focused on the regulation of oxidative stress, limitation of the bioavailability of trace molecules, the restoration of metabolic disruptions, and the delivery of tissue repair signals into the circulatory environment [4], [26], [27], [47], [50], [51]. Especially, cross talking between mechanisms that supply the host's demand of energy from glycolysis, inflammation, and the capture of trace elements like glucose and iron, are critical to the emergence of resilience [27], [45], [50], [52], [53].

To date, evidence of tolerance and resilience to malaria come from studies in mice giving a panoramic view of the mechanisms present in small mammals [26], [27], [47], [50], [51]. However, translation of these mechanisms to human biology can be a challenge due to the intrinsic characteristics of the Murine model, such as animal size and the parasite species that can be used for infection. Alternative models, especially non-human primates (NHP), are susceptible to malaria and provide valuable settings in the study of resilience to *Plasmodium* infection [54], [55]. Particularly, *Macaca mulatta*, and *Macaca fascicularis,* which are natural hosts to several *Plasmodium* species, show different resilient responses to infection [28]–[31]. Specifically, *P. knowlesi* infections in these two hosts show very different clinical outcomes, with *M. mulatta* developing fulminant parasitemias, which lead to death in the absence of treatment, and *M. fascicularis* developing a mild, chronic infection [29]–[31], [56]. Accordingly, the unique molecular dysregulations developed between *M. fascicularis (resilient host)* and *M. mulatta (non-resilient host)* in response to the same infecting parasite should contain the resilience message to malaria.

To investigate the underlying resilience mechanisms in host responses to malaria, a longitudinal multi-omic profile of *P. knowlesi* infecting both *M. mulatta* and *M. fascicularis* was conducted. In this study, functional genomics, targeted proteomics, and targeted metabolomics analysis reveal specific molecular profiles that distinguish resilient and non-resilient host responses. Particularly, strong enrichments in the metabolism of soluble vitamins and cofactors, and type I interferon response, were identified as a common fingerprint of non-resilient hosts. In contrast, transport of bile salts and organic acids, metal ions and amine compounds were shown to be the molecular signature used by the resilient host. This research provides molecular targets for further investigation in host-directed therapies to promote resilience to infection.

Methods

Experimental Design:

Four malaria-naive male rhesus macaques (*M. mulatta*) and seven malaria-naive male long-tailed macaques (*M. fascicularis*), approximately five years of age, were inoculated intravenously with cryopreserved *P. knowlesi* Malayan strain salivary gland sporozoites, obtained from *Anopheles dirus* infected with parasites from the Pk1(A+) clone [57]. After inoculation, the macaques were profiled longitudinally according to the expected clinical progression. Venous blood and bone marrow samples were collected at five time points for *M. mulatta* and six time points for *M. fascicularis* for functional genomics, targeted proteomics, and targeted metabolomics. To access publicly available results from ‘E06 and E07' MaHPIC Experiments, including clinical results (specifics on time point definitions, drugs administered, diet, and veterinary interventions), visit http://plasmodb.org/plasmo/mahpic.jsp.

Functional Genomics:

Whole blood and bone marrow samples were processed using the PAXgene Blood RNA System, which allowed immediate preservation of the RNA profile following experimental conditions. All samples were processed according the modified Illumina TruSeq Stranded mRNA Sample Preparation Protocol (see supplementary material for details information about RNAseq).

Targeted Proteomics:

EDTA-Plasma extracted from whole blood was used to conduct targeted quantification of 1317 proteins for each time point using SOMAscan® Assay 1.3k kit. The collection and preparation of samples for each assay on SOMAscan at SomaLogic were conducted following the manufacturer recommendations (see supplementary material for detail information about targeted proteomics).

Targeted Metabolomics:

The Biocrates p180 kit was used for quantification of metabolites via liquid chromatography mass spectrometry and flow injection analysis using the SCIEX Exion LC and QTRAP 5500 mass spectrometer. The plasma sample preparation and data acquisition were performed using the Biocrates system following the manufacturer recommendations from Biocrates Absolute IDQ p180 Kit (see supplementary material for detail information about targeted metabolomics).

Molecular Signature Identification:

The differential expression analysis was conducted using the DEseq algorithm in the Matlab Bioinformatics Toolbox™. The Benjamini-Hochberg procedure was used to correct for multiple testing hypothesis with a false discovery rate of 10%. Genes were considered differentially expressed if the *q*-value < 0.1 and the fold change was more than two-fold. The differentially up and down regulated genes were then compared across host species to determine unique and common genes to each group.

To understand the functions of the differentially expressed genes (DEGs) the Cytoscape (v 3.5) plug-in ClueGO (v 2.5.2) and Enrichr pathway analysis were conducted using the overlap with the curated gene sets on BioCarta, Reactome, Humancyc and HMDB metabolites [58]–[67]. The code of the differential expression and the enrichment analyses can be found in the supplemental file 1. Finally, coupled with the enrichment analysis, we generate unsupervised clustergrams using the fold change of the enriched genes. We define as a unique molecular signature the pathways that were enriched for the distinctive dysregulations and fold change.

Metabolome Analysis:

The differential abundance of the target metabolites was calculated using a two-sample t-test between baseline (BL) and acute parasitemia (AP) measurements. The differential abundance of a protein or a metabolite between time points was considered significant if the associated q-value< 0.1 after the Benjamini-Hochberg procedure. Finally, a volcano plot was used to visualize the significance and analyze the abundance ratio (fold change) for each protein and metabolite (supplementary material 1).

Multi-omic Analysis and Integration:

To identify changes in the functional outputs from host whole blood cells and parenchymal during the peak of parasitemia, the related evidence from whole blood transcriptome dysregulations, and the plasma proteome abundance was intersected. Subsequently, the transcriptomic and proteomic signatures from whole blood were compared to the targeted metabolomic abundance using Enrichr. Finally, to identify gene coexpression patterns and metabolites abundance between the whole blood and the bone marrow the unique DEGs and the differentially expressed metabolites in both tissues were intersected.

Results

Identification of Distinctive Transcriptomic signatures Between Resilient and Non-resilient Hosts.

To understand the transcriptional changes in the host during acute parasitemia, differential expression analyses were conducted between baseline and acute parasitemia using whole blood samples. All hosts displayed substantial transcriptional changes during the peak of parasitemia. The *M. fascicularis* hosts, when infected with *P. knowlesi* (E07), displayed 836 and 528 significantly up regulated and down regulated genes, respectively. Likewise, the non-resilient host *M. mulatta*, when infected with *P. knowlesi* (E06), showed 908 up regulated and 515 down regulated genes (**Figure 1**a).

To select the unique gene sets that represent the distinctive molecular signatures between resilient and non-resilient hosts during acute parasitemia, an intersection of DEGs was conducted. The three subsets partition reveals 285 transcriptionally up regulated genes in both hosts. For the specific host signatures, a unique transcriptomic up regulation of 623 genes were detected for the non-resilient host. In comparison, 551 genes were contained outside the intersection exclusively up regulated for the resilient host. In contrast, fewer down regulated genes were observed in both hosts during *P. knowlesi* infections (**Figure 1**b). Finally, a share down regulation of 98 genes was observed, and 430 and 417 genes were uniquely down regulated for *M. fascicularis and M. mulatta,* respectively*.*

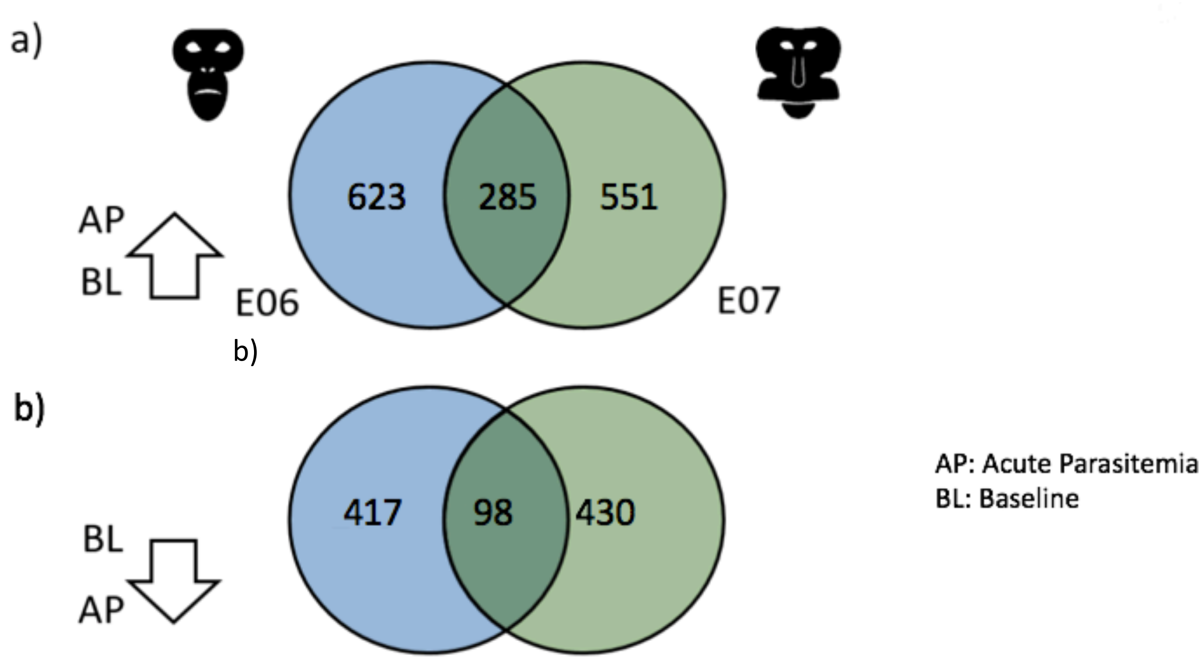


Figure 1: *Intersection between uniquely up and down regulated genes between resilient and non-resilient host. a) Up regulated genes. b) Down regulated genes.*

Enrichment Analysis of Unique Up and Down Regulated Genes During Acute Parasitemia in the Non-resilient host.

To associate the unique up regulated genes in the non- resilient host to a biological process during acute parasitemia, an enrichment analysis was conducted. Among the up regulated transcriptomic signature of *M. mulatta*, 70 percent of the genes were enriched for the terms cellular responses to external stimuli, immune system, cytokine signaling, and toll-like receptor cascades. Furthermore, 30 percent of the genes showed associations with 11 processes including HSP90 chaperone cycle of steroid hormone receptor (SHR), nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways, and collagen formation (**Figure 2**a,b). In addition, in the exclusively down regulated genes for the non-resilient host, 9 pathways were enriched. Within these pathways, 48.65 percent of genes were associated with platelet activation, syndecan interactions and immunoregulatory interactions between lymphoid and non-lymphoid cells (**Figure 3**a,b).

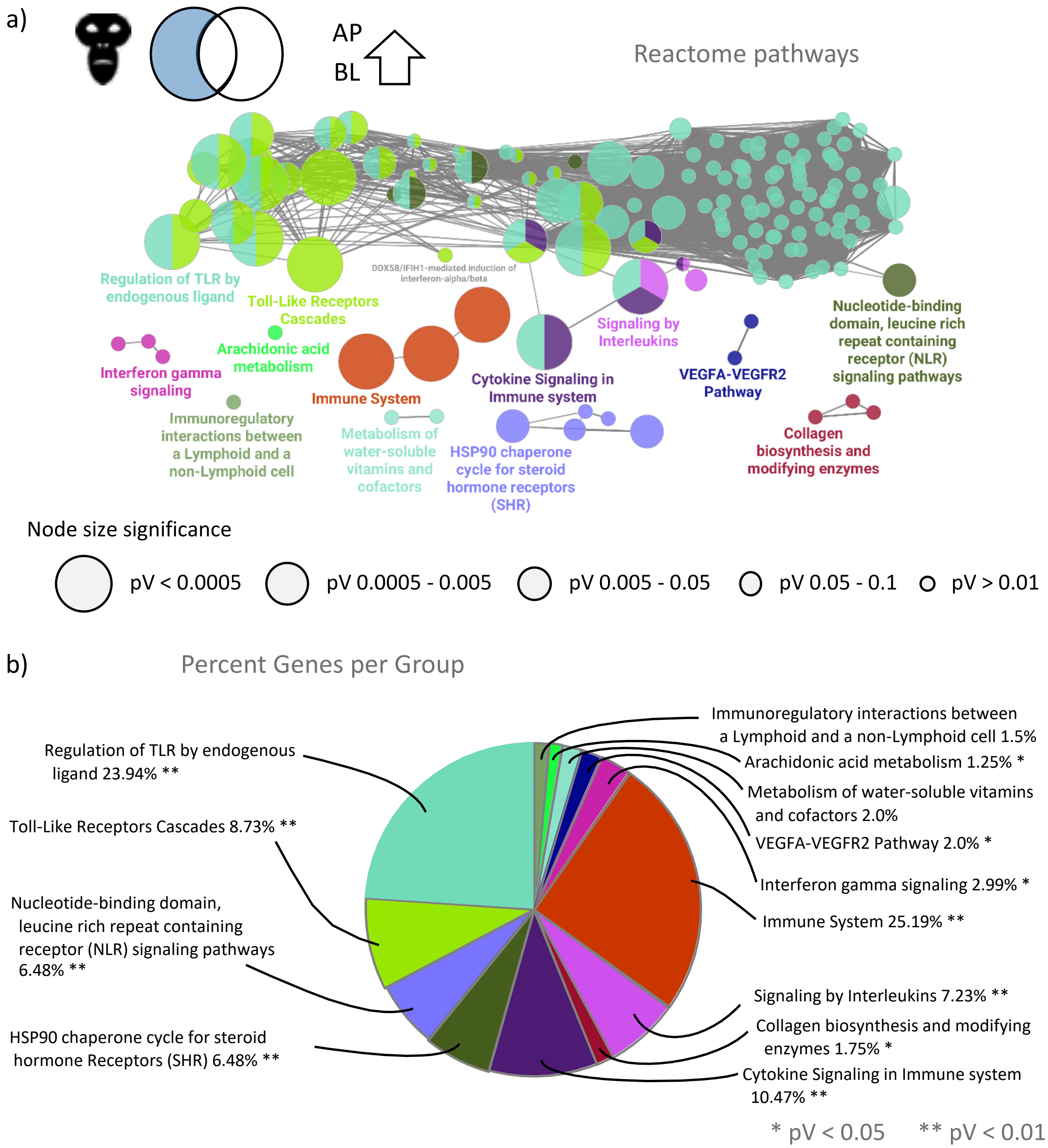


Figure 2: *Enrichment analysis of the unique up-regulated genes in the non-resilient host. a- shows the network for the associated terms for the up-regulated genes. The size of the circle represents the approximated p-value of the enrichment. b- shows the pie chart of the percentage of genes per group.*

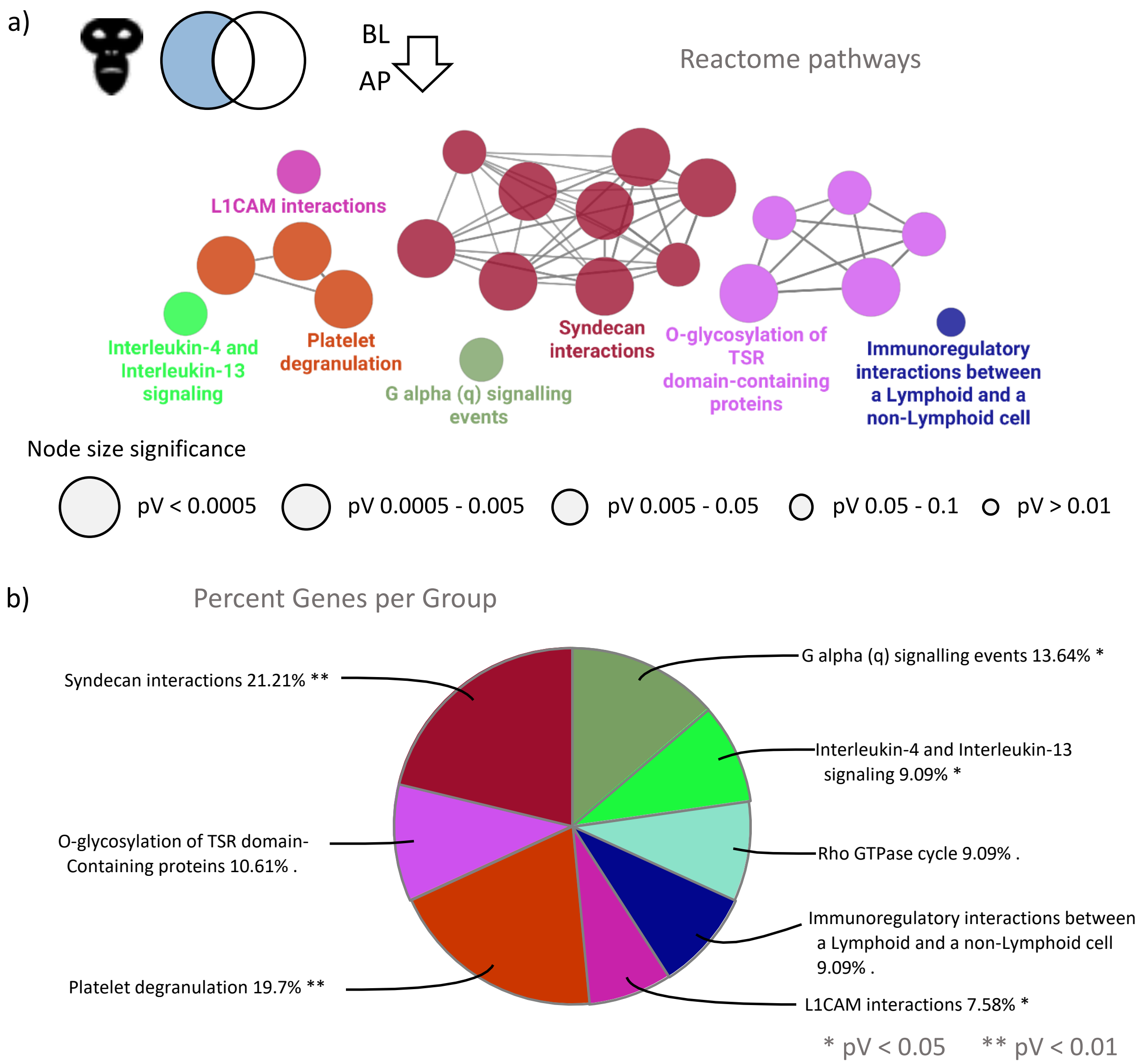


Figure 3: *Enrichment analysis of the unique down-regulated genes in the non-resilient host. a- shows the network for the associated terms for down-regulated genes. The size of the circle represents the approximate p-value of the enrichment. b- shows the pie chart of the percentage of genes per group.*

Enrichment Analysis of Unique Up and Down Regulated Genes During Acute Parasitemia in the Resilient Host.

To describe the functional enrichment of the uniquely up and down-regulated genes among the resilient host, the over-represented terms in reactome were identified. Within the up regulated genes, 56 percent were associated with cell cycle and mitotic phases. Additionally, 19 terms were enriched, being polo-like kinase mediate events, kinesins, complement cascade, and hemostasis

(

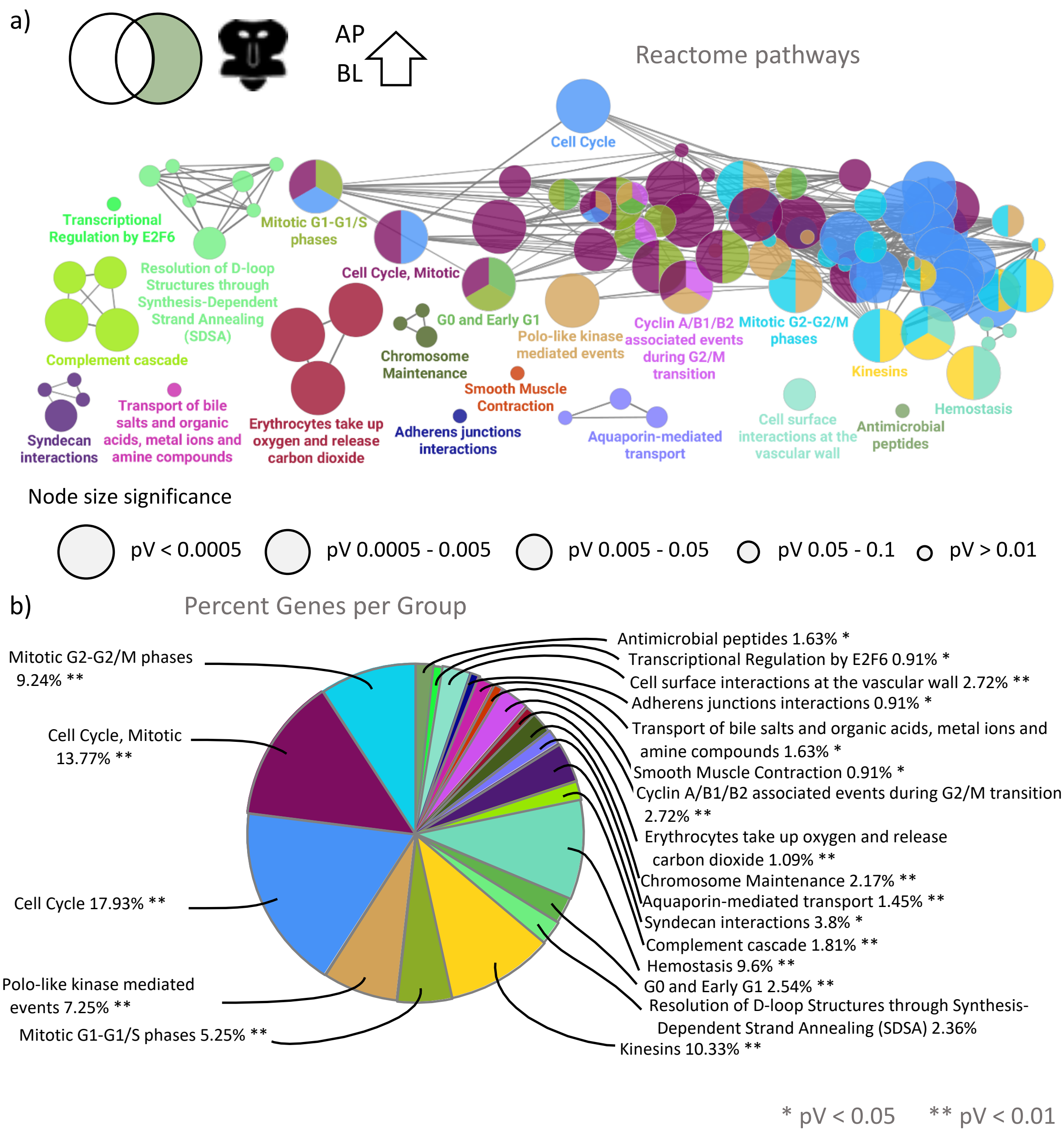

a)
AP
BL
Reactome pathways
Cell Cycle
Transcriptional Regulation by E2F6
Mitotic G1-G1/S phases
Resolution of D-loop Structures through Synthesis-Dependent Strand Annealing (SDSA)
Cell Cycle, Mitotic
Complement cascade
G0 and Early G1
Chromosome Maintenance
Polo-like kinase mediated events
Cyclin A/B1/B2 associated events during G2/M transition
Mitotic G2-G2/M phases
Kinesins
Smooth Muscle Contraction
Syndecan interactions
Transport of bile salts and organic acids, metal ions and amine compounds
Erythrocytes take up oxygen and release carbon dioxide
Adherens junctions interactions
Aquaporin-mediated transport
Cell surface interactions at the vascular wall
Antimicrobial peptides
Hemostasis
Node size significance
pV < 0.0005
pV 0.0005 - 0.005
pV 0.005 - 0.05
pV 0.05 - 0.1
pV > 0.01
b)
Percent Genes per Group
Mitotic G2-G2/M phases 9.24% **
Cell Cycle, Mitotic 13.77% **
Cell Cycle 17.93% **
Polo-like kinase mediated events 7.25% **
Mitotic G1-G1/S phases 5.25% **
Antimicrobial peptides 1.63% *
Transcriptional Regulation by E2F6 0.91% *
Cell surface interactions at the vascular wall 2.72% **
Adherens junctions interactions 0.91% *
Transport of bile salts and organic acids, metal ions and amine compounds 1.63% *
Smooth Muscle Contraction 0.91% *
Cyclin A/B1/B2 associated events during G2/M transition 2.72% **
Erythrocytes take up oxygen and release carbon dioxide 1.09% **
Chromosome Maintenance 2.17% **
Aquaporin-mediated transport 1.45% **
Syndecan interactions 3.8% *
Complement cascade 1.81% **
Hemostasis 9.6% **
G0 and Early G1 2.54% **
Resolution of D-loop Structures through Synthesis-Dependent Strand Annealing (SDSA) 2.36%
Kinesins 10.33% **
* pV < 0.05
** pV < 0.01

Figure 4a,b). The characteristic transcriptional down regulation among resilient subjects were over-represented in 4 terms. As a result, 51.52 percent of genes were grouped in chemokine receptors bind chemokines. In addition, interleukin-10 signaling, class B/2 secretin, and collagen chain trimerization were enriched, representing the 48.83 percent of genes in these three terms (**Figure 5**a,b)

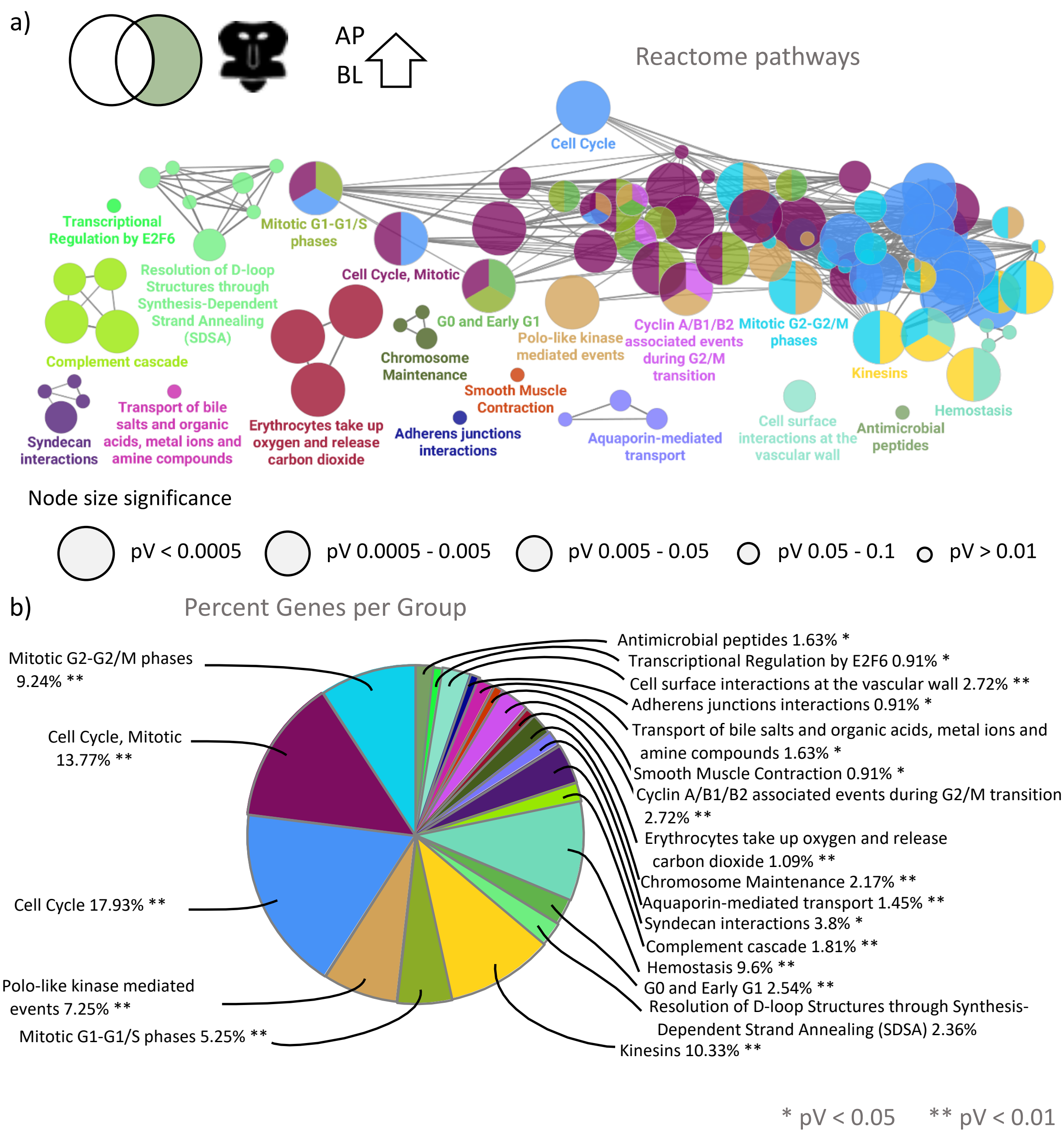


Figure 4: Enrichment analysis of the unique up regulated genes in the resilient host. a- shows the network for the associated terms for the up regulated genes, the size of the circle represents the approximate p-value of the enrichment. b- shows the pie chart of the percentage of genes per group.

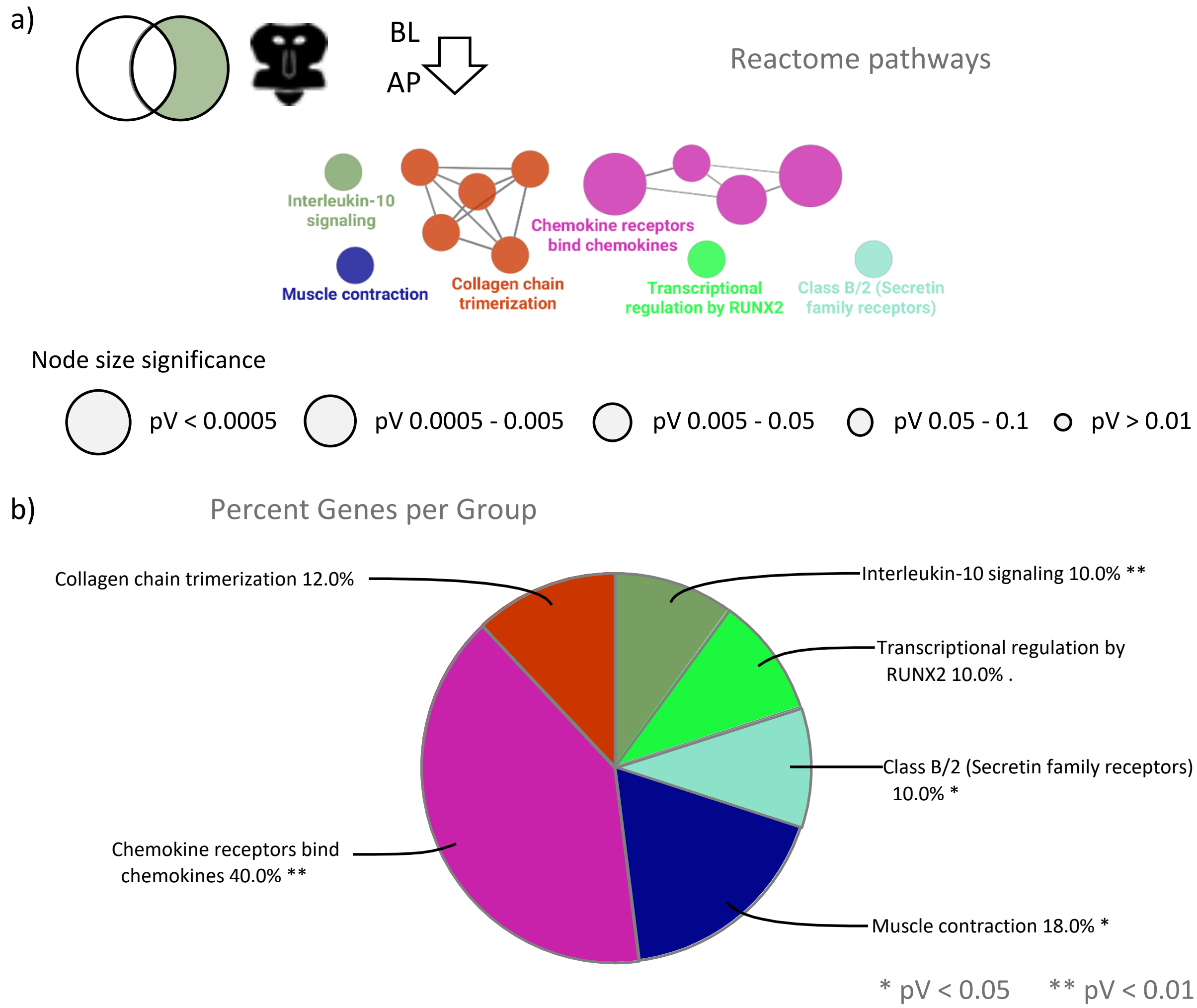


***Figure** 5: Enrichment analysis of the unique down regulated genes in the resilient host. a- shows the network for the associated terms for the down regulated genes, the size of the circle represents the approximate p-value of the enrichment. b- shows the pie chart of the percentage of genes per group.*

Enrichment Analysis of Shared Up and Down Regulated Genes During Acute Parasitemia Between Resilient and Non-resilient Host.

The up-regulated share response between hosts was enriched in 8 terms, with a 61.49 percent of the genes contained in the terms cytokine signaling in immune system and interferon Signaling. Furthermore, interleukin-4 and 13 signaling, ISG15 antiviral mechanism, regulation of IFNG signaling and insulin-like growth factor regulation were enriched with 27.32 percent of genes shared between hosts during the acute parasitemia. Finally, 6.84 percent of the genes were associated with cytosolic sensors of pathogen-associated DNA and TP53 regulates transcription of cell death (**Figure 6**a,b). Equally, genes that present a shared down regulation between resilient and non-resilient host showed 50.33 percent of the genes related to the term immunoregulatory interaction between a lymphoid and non-lymphoid cell. Likewise, 46.67 percent of elements were contained in the term degradation of the extracellular matrix (**Figure 7**a,b).

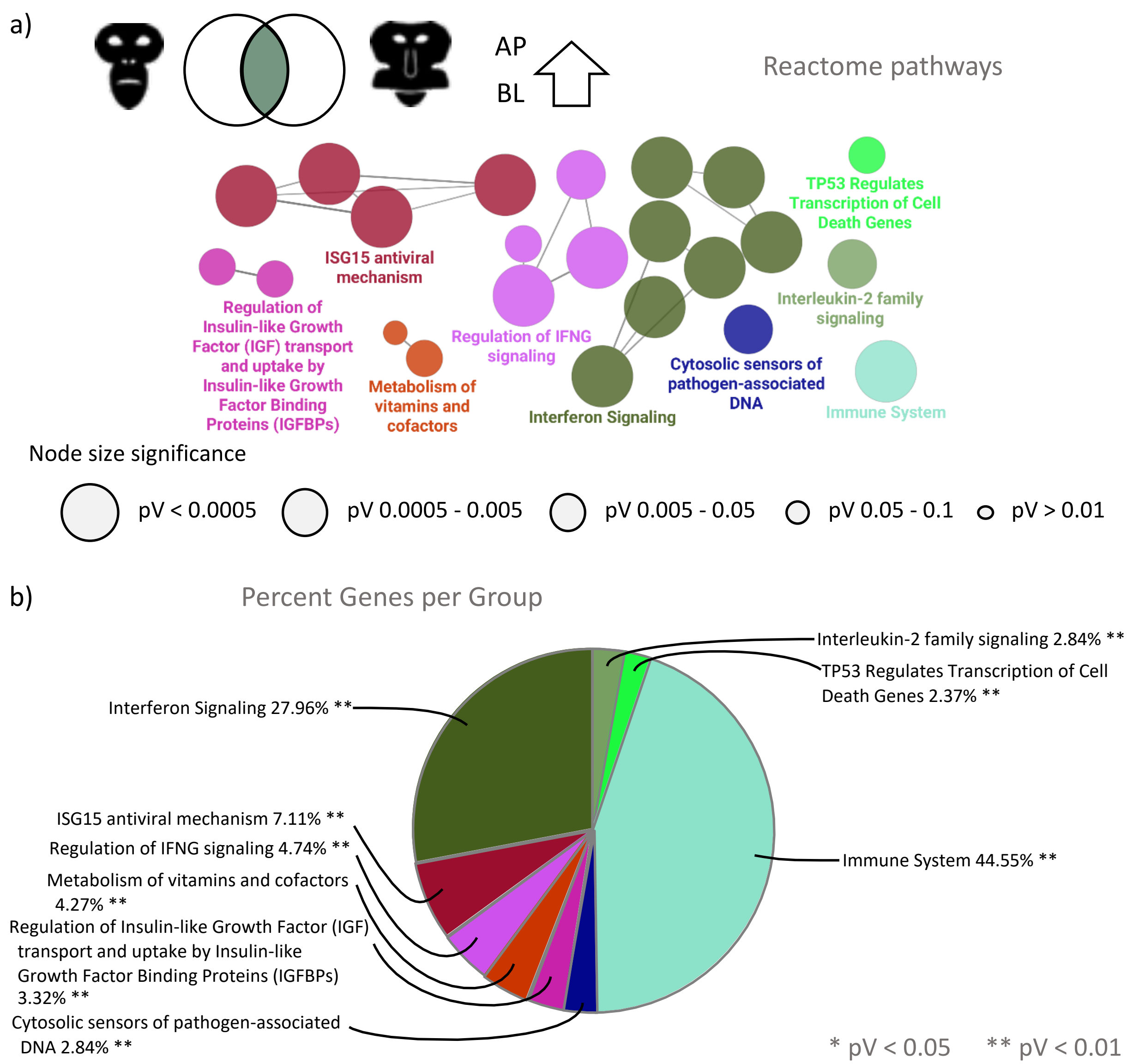


Figure 6: *Enrichment analysis of the shared up regulated genes between non-resilient host and resilient host. a- shows the network for the associated terms for the up regulated genes, the size of the circle represents the approximate p-value of the enrichment. b- shows the pie chart of the percentage of genes per group.*

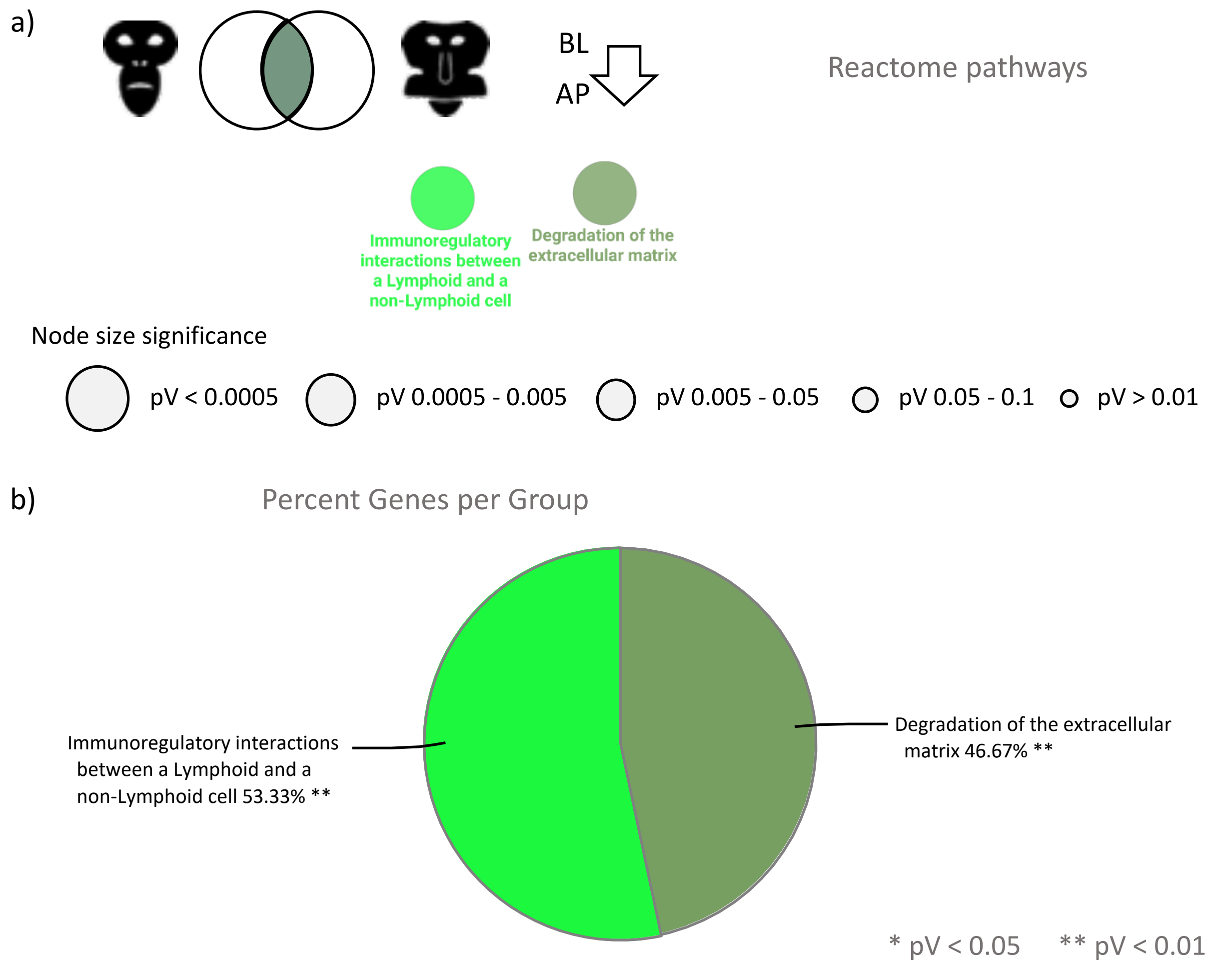


Figure 7: *Enrichment analysis of the shared up regulated genes between non-resilient host and resilient host. a- shows the network for the associated terms for the up regulated genes, the size of the circle represents the approximate p-value of the enrichment. b- show the pie chart of the percentage of genes per group.*

Table 1: *Contained genes per compartment and the associated term in Reactome.*

Host		Non-resilient Unique associated Terms 	Resilient Unique associated Terms 
Differential expression of associated genes	AP BL **Up regulated**	• *Immune System* • *Toll-Like Receptors Cascades* • *Cytokine Signaling in Immune system* • *Regulation of TLR by endogenous ligand* • *Signaling by Interleukins* • *HSP90 chaperone cycle for steroid hormone receptors (SHR)* • *Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways* • *Interferon gamma signaling* • *VEGFA-VEGFR2 Pathway* • *Collagen biosynthesis and modifying enzymes* • *Arachidonic acid metabolism* • *Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell* • *Metabolism of water-soluble vitamins and cofactors*	• *Hemostasis* • *Cell Cycle* • *Cell Cycle, Mitotic* • *Mitotic G1-G1/S phases* • *Polo-like kinase mediated events* • *Kinesins* • *Cyclin A/B1/B2 associated events during G2/M transition* • *Erythrocytes take up oxygen and release carbon dioxide* • *G0 and Early G1* • *Mitotic G2-G2/M phases* • *Complement cascade* • *Cell surface interactions at the vascular wall* • *Resolution of D-loop Structures through Synthesis-Dependent Strand Annealing (SDSA)* • *Syndecan interactions* • *Aquaporin-mediated transport* • *Chromosome Maintenance* • *Transport of bile salts and organic acids, metal ions and amine compounds* • *Adherens junctions interactions* • *Transcriptional Regulation by E2F6* • *Antimicrobial peptides* • *Smooth Muscle Contraction*
	BL AP **Down-regulated**	• *Platelet degranulation* • *Syndecan interactions* • *O-glycosylation of TSR domain-containing proteins* • *G alpha (q) signalling events* • *Interleukin-4 and Interleukin-13 signaling* • *Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell* • *Rho GTPase cycle* • *L1CAM interactions*	• *Class B/2 (Secretin family receptors)* • *Chemokine receptors bind chemokines* • *Collagen chain trimerization* • *Interleukin-10 signaling* • *Muscle contraction* • *Transcriptional regulation by RUNX2*

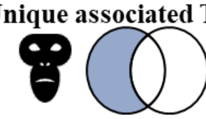
Comparison of Pathways with Differential Modulation Between Resilient and Non-resilient Hosts.

To identify if the resilient and non-resilient hosts can modulate differently the same pathway, uniquely up and down regulated genes that were enriched to the same term in different compartments were detected. Initially, it was identified that genes associated with syndecan interactions were up regulated for the resilient host and significantly down regulated in non-resilient subjects. Likewise, collagen chain trimerization was down regulated in resilient subjects in contrast to non-resilient host that showed significant up-regulation of collagen formation. Additionally, platelet activation, signaling and aggregation related genes showed to be enriched for the uniquely down-regulated genes in non-resilient subjects different from resilient individuals were platelet aggregation enrichment were over-represented for the uniquely up-regulated genes. Furthermore, the terms immunoregulatory interaction between a lymphoid and non-lymphoid cell and TP53 regulates transcription of cell death, and TP53 regulates metabolic genes were enriched **Table 1**.

Molecular Signature Selection.

The selection strategy allowed us to identify distinctive dysregulations associated to resilience. Initially, thirteen up regulated and eight down regulated pathways were identified through enrichment analysis in the non-resilient host. Then, three representative pathways were identified through unsupervised clustergrams, one up regulated and two down regulated. In comparison, the unique enrich pathways in the resilient host were associated with 21 up and 6 down regulated pathways, and through unsupervised clustergrams, we identified five dysregulations, three up, and two down regulated pathways that have different regulation and allow the separation between resilient and non-resilient hosts (**Table 2**, see supplemental material for clustergrams).

Table 2: *Distinctive transcriptomic dysregulations associated with resilience in both hosts.*

Host		**Non-resilient Unique associated Terms** 	**Resilient Unique associated Terms** 
Differential expression of associated genes	AP BL **Up regulated**	• *Metabolism of water-soluble vitamins and cofactors*	• *Erythrocytes take up oxygen and release carbon dioxide* • *Transport of bile salts and organic acids, metal ions and amine compounds* • *Smooth Muscle Contraction*
	BL AP **Down-regulated**	• *L1CAM interactions* • *G alpha (q) signaling events*	• *Transcriptional regulation by RUNX2* • *Chemokine receptors bind chemokines*

Cell Deconvolution from Transcriptome.

To estimate which cell populations are the main contributors to the transcriptomic signals, we identified changes between the baseline and acute parasitemia for each host. The non-resilient host showed an increment in transcripts contribution from neutrophils (most likely early myeloid cells) (**Figure 8**). In contrast, a delay in the neutrophils contribution was observed in the resilient host. Furthermore, an increment in the contribution of transcripts by monocytes was detected in the resilient host during the acute parasitemia, suggesting a monocyte-driven regulation during the acute parasitemia in the resilient host (**Figure 9**).

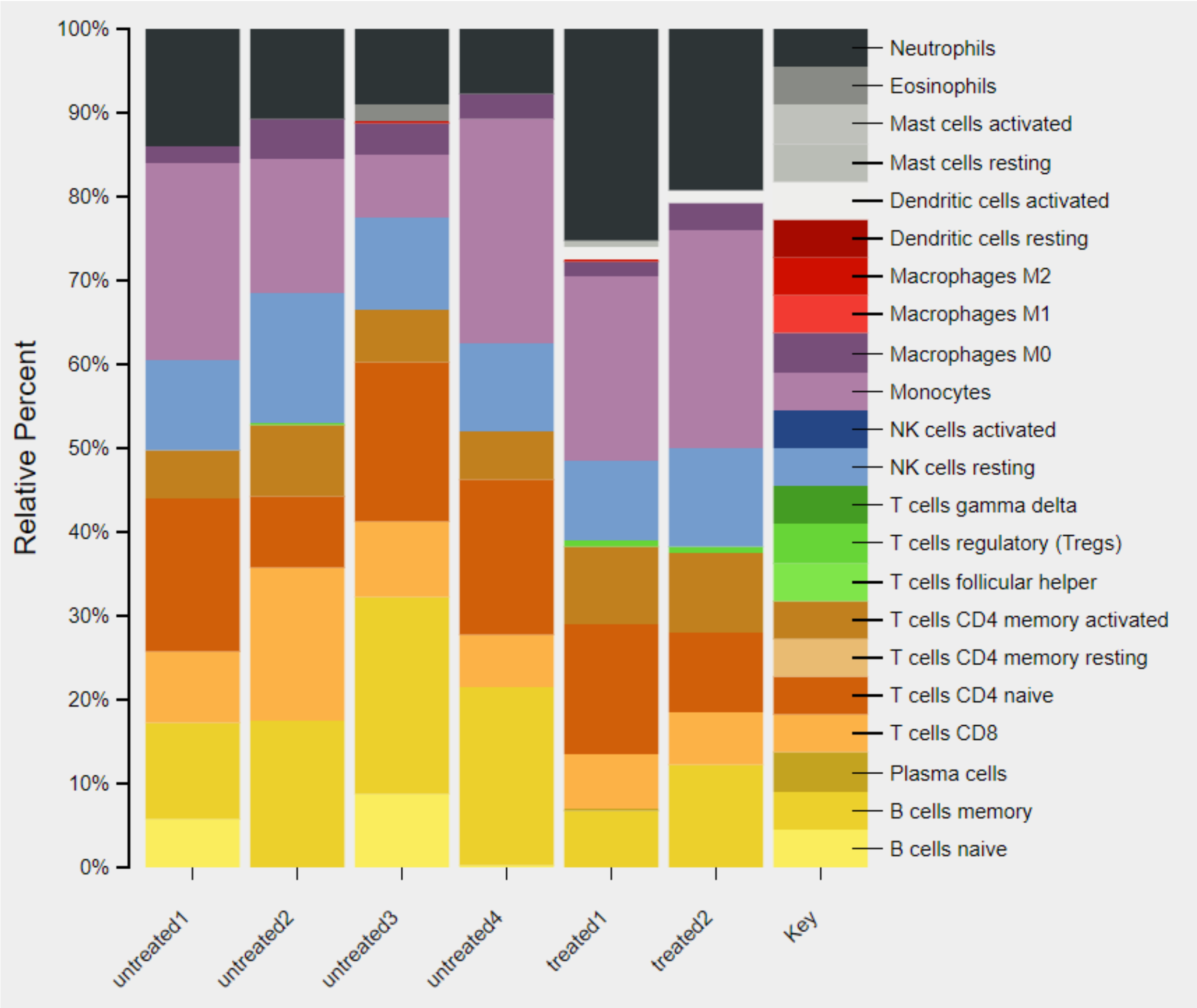


***Figure** 8:Cell deconvolution from the whole blood transcriptome in the non-resilient host. The figure shows the relative percentage of cells contribution to the transcriptome in the non-resilient host.*

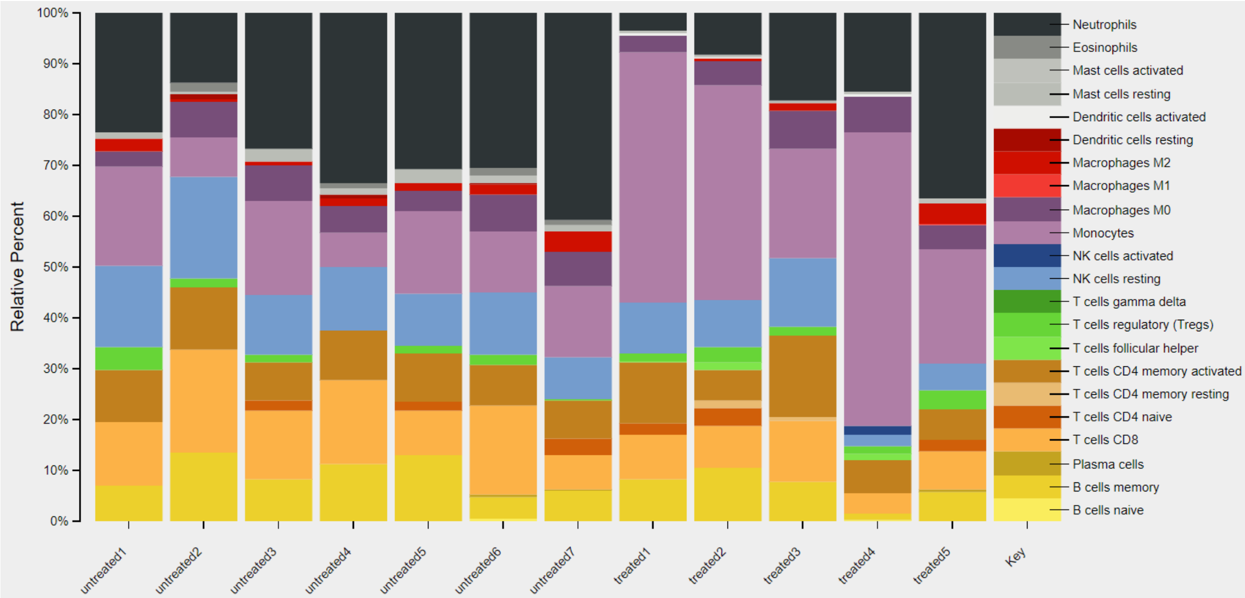


***Figure** 9: Cell deconvolution from the whole blood transcriptome in the resilient host. The figure shows the relative percentage of cells contribution to the transcriptome in the resilient host.*

Co-occurrence of Up Regulation Between Whole Blood Transcriptome and Plasma Proteome.

To identify the co-occurrence of signals between the PBMCs distinctive molecular signature and the plasma proteome, an intersection between the elements from both omic sets was conducted. In the case of the resilient host, the presence of kynureninase (KYNU) and the epidermal growth factor (EGF), suggest a response involving active tryptophan degradation and growth factors promotion. Also, the co-occurrence of lactoferrin and the unique up-regulation of iron bioavailability indicates an active transport and retention of in the whole blood. Particularly, the co-occurrence of GDF15, a protein that is known to be an activator of iron intracellular retention, suggest that a controlled depletion of the circulating iron is a crucial component of the resilient response to malaria

(**Figure 10**). This evidence indicates that these responses need to be localized between tissues especially in circulating cells and not in the bone marrow.

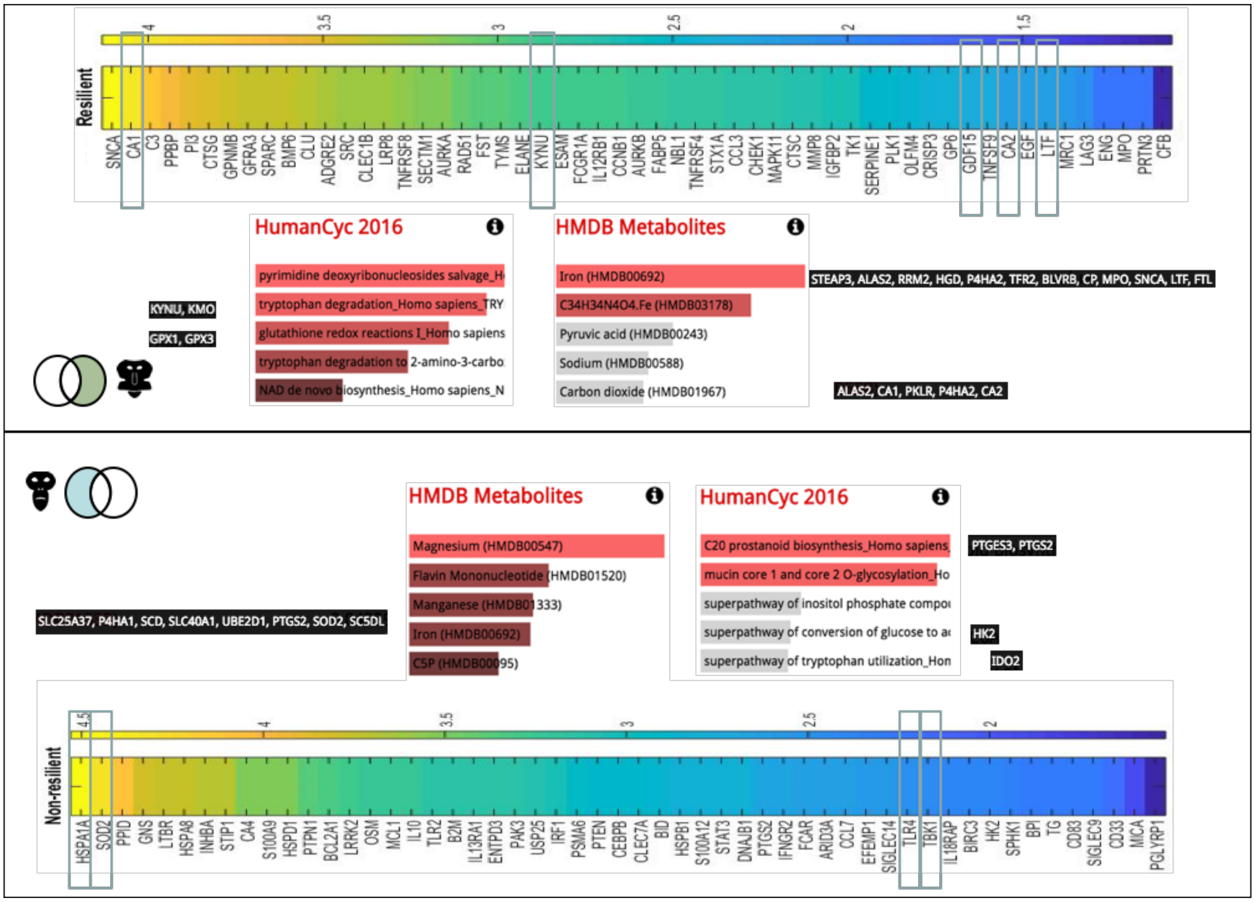


Figure 10: *Heatmap for the intersection between the upregulated genes in whole blood and the plasma proteome during acute parasitemia. The figure shows the heatmap for the Log10(RFU) from SOMAscan for the resilient and non-resilient host.*

Targeted Metabolomics Disruption in Whole Blood and Bone-marrow.

To identify associations between the up-regulated genes between tissues and the metabolic products in the plasma, a differential abundance of metabolites was performed. In the case of the whole blood metabolome, kynurenine showed a share up-regulation between host, probably as a signal of homeostasis disruption (Figure 3). Notably, despite the share up-regulation of kynurenine in both hosts, a unique co-occurrence of kynureninase (KYNU) was observed in the resilient host between the whole blood and the plasma proteome indicating an active degradation of kynurenine in plasma in contrast to the non-resilient host (Figures 2,3). Furthermore, a unique down-regulation of amino the amino acids Gln, Asn, and 14-OH-Proline was observed for the resilient host and can be related to the up-regulation of and the transport of amine compounds that was unique for the resilient host in the whole blood. Similarly, the bone marrow metabolome showed shared elements between the plasma and bone marrow metabolomes, especially the up-regulation of kynurenine in both hosts. Furthermore, we were able to identify that there was a significant down-regulation of different amino acids in both hosts, suggesting that a differential consumption of amino acids in the marrow (**Figure 11**).

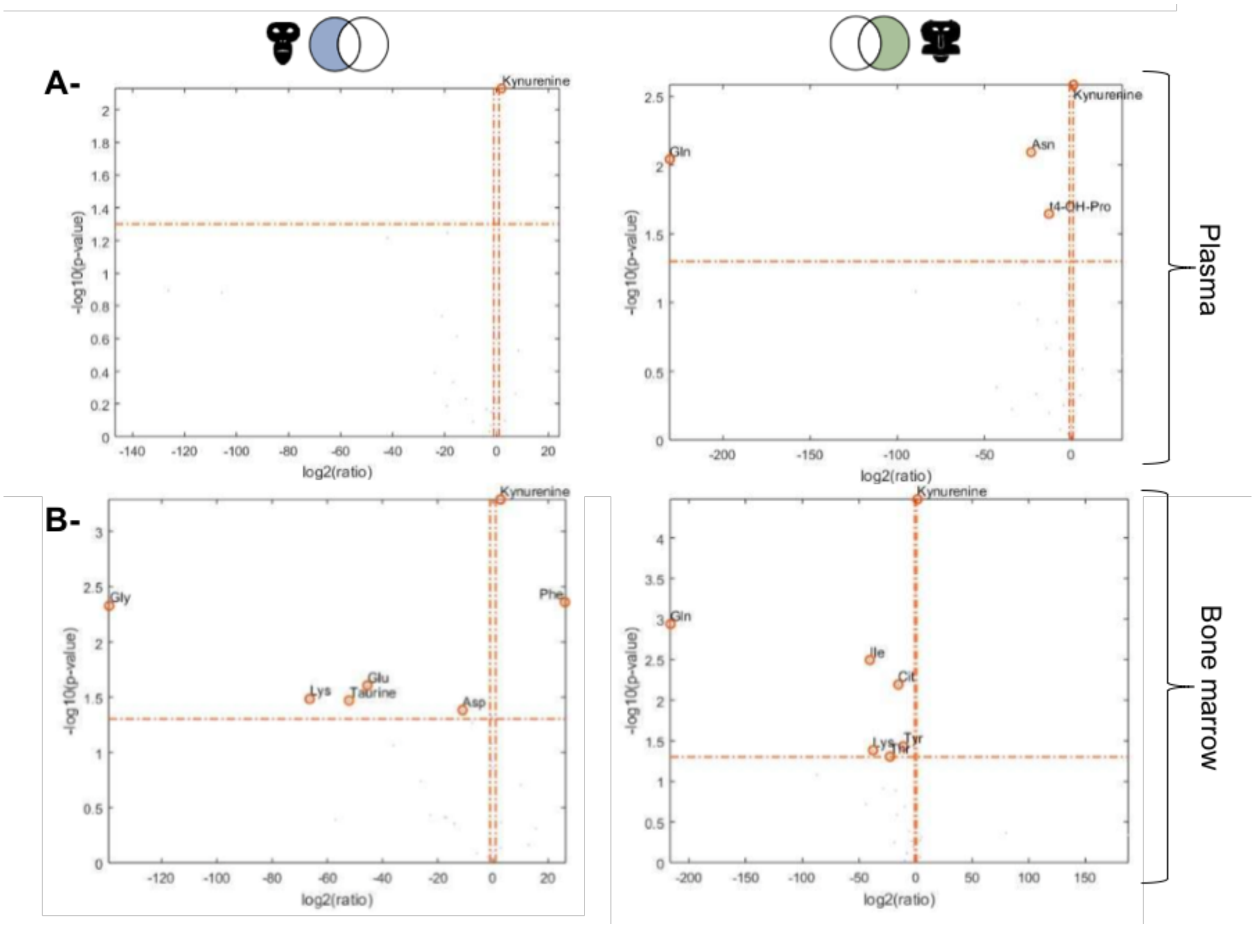


Figure 11: *Shows the up-regulated and down-regulated metabolites between the baseline and acute parasitemia in both hosts. At the left non-resilient host, and the right resilient host. A- Plasma metabolome; B- Bone-marrow metabolome.*

Parasite Transcriptomic Dysregulation is Related with Host Molecular Disruptions.

A unique up-regulation of parasite genes related to vitamin B6 and pyridoxal 5 phosphate transcripts was observed in the parasite transcriptome in the non-resilient host. Particularly, the up-regulation pyridoxal five phosphate associated genes in the parasite suggest that the parasite is targeting vitamin B6, and the biosynthesis of pyridoxal phosphate, this is probably to the high rate nucleotide production needed for the parasite replication. In combination, the increase in the water-soluble vitamins up-regulation showed by the host and the parasite competition for the soluble vitamins in the environment can be a source for impairment of the tryptophan degradation and the kynurenine pathways due to the role of vitamin B6 as a cofactor in this set of reactions (Figure 12).

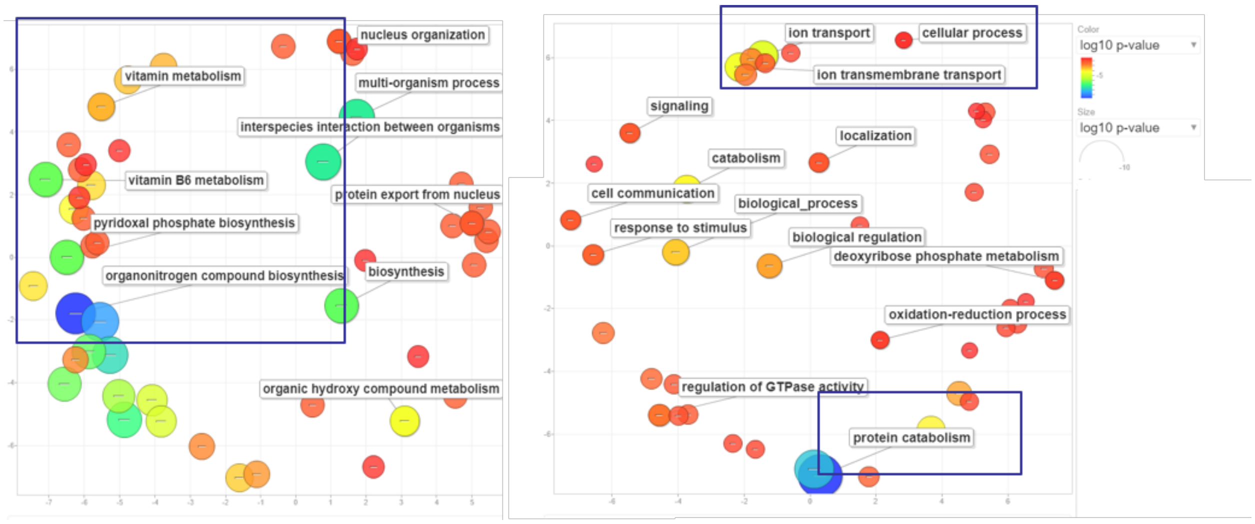


Figure 12: Shows the enrichment analysis of the unique up-regulated parasite genes in the both hosts (left: non_resilient host, righ: resilient host). The size of the circle represents the log10 p-value of the enrichment.

Discussion

In this study, a multi-omic approach was used to identify specific molecular profiles that distinguish resilient (*M. fascicularis*) and non-resilient (*M. mulatta*) host responses to the same infecting species (*P. knowlesi*) during acute parasitemia. First, the multi-omic signature associated with *M. fascicularis* suggests that the resilience mechanism against *P. knowlesi* is a nutritional immunity strategy that prioritizes the host sequestration of trace molecules in an effort to limit essential elements for the parasite survival. Furthermore, the intersection of these signatures between whole blood and bone marrow implied that coordination between tissues is needed to promote resilience. In contrast, the non-resilient host developed a resistance strategy composed by strong enrichment in the Immune system, Cytokine Signaling, and Toll-Like receptor cascades in combination with the distinctive signature of metabolism of soluble vitamins and cofactors. Finally, our data suggest that the host transcriptional signatures conditioned the parasite gene expression driving the parasite to mirror the host response.

As resilience strategy, *M fascicularis* exert a nutritional immune mechanism that relies upon control of the bioavailability of iron. In infectious diseases, a successful approach to limit iron depends on the lifestyle of the parasite, being sequestration of iron into intracellular compartments the best strategy against extracellular pathogens; in contrast, iron export showed to be successful against intracellular pathogens like *Mycobacterium tuberculosis, M. bovis,* and *Leishmania donovani* [4], [6], [27], [46], [68], [69]. In mammals, macrophage orchestrates the systemic iron homeostasis through the capture or the release of this element [4]–[6], [14], [48], [68]. Accordingly, the import of iron into macrophages showed to be a crucial factor in establishing resilience to extracellular pathogens, and the export of this element is essential to counter-attack the growth of intracellular parasites [46], [48], [70]. Although *Plasmodium* parasites are obligate intracellular pathogens, our data suggest that the successful strategy against malaria is treated Plasmodium parasites as extracellular agents.

Our data suggest that *M. mulatta* used an intracellular resistant strategy via the reduction of intracellular Fe content in combination with oxidative stress response due to an intracellular accumulation of ROS promoted by the activation of the electron transport chain. Thus, affecting the dynamics between the production and elimination of H_2O_2 in the mitochondria. Primarily, the mitochondria ROS scavenger system compose by the superoxide dismutase (SOD2) and GPX1. Particularly, our data suggest that the oxidative stress dysregulation in the non-resilient host may be promoted through the SOD2 up-regulation may be related to the bioavailability of intracellular iron and antioxidant promoters, thus driving impairment in the tolerance signals in circulation and between tissues [71]–[73]. Principally, the SOD2 unique upregulation in the non-resilient host is contrasted by a unique up-regulation GPX1 in the resilient host, suggesting that imbalance in the ratio of production of H_2O_2 by SOD2 and the detoxification of H_2O_2 by the Glutathione peroxidase-1 GPX1 is a crucial factor in the systemic response to the pathogen.

Even though SOD2 is an essential factor in the elimination of superoxides O_2, its antioxidant properties can turn to a deleterious pro-oxidant role due to the accumulation of H_2O_2 [72], [73]. Mainly, diffusion of H2O2 out of mitochondria can act as a potent redox agent affecting the plasticity of cell signaling pathways due to its activity on active amino acids on regulatory proteins, thus having systemic effects on signaling pathways [73].

While the distinctive molecular signatures between resilient and non-resilient hosts showed differences in the strategies to combat the parasite, a share response regarding the activation of interferon-stimulated genes was observed. Specially, the activation of Cytosolic sensors of pathogen-associated DNA, and Cytokine Signaling in Immune system and Interferon Signaling. Although, both hosts displayed activation of the innate immune system, particularly nucleic acid sensing pathways, the evidence presented in this study suggested that the share response to the parasite in combination with the distinctive molecular signatures from each host are relevant to the establishment of resilience.

Particularly, the activation of cytosolic nucleic acid sensors and the subsequent Type I interferon response has been reported to be activated by parasite nucleic acids from hemozoin, lysates from infected red blood cells, and microvesicles [16], [74]–[79]. In liver, microglia, and PBMCs, DNA sensors (as the cGAS-STING pathway), and RNA sensors (as MDA5 and TLR7), have been related to parasitemia dynamics and host mortality in malaria [75]–[77], [80]–[82]. While OAS family proteins, which are viral RNA sensors, have been systematically found to be upregulated in malaria studies, they have not been described as potential RNA sensors for this pathogen [81], [83]–[88]. Pathways related to interferon Type I and viral-like immune response have been shown to be very effective against viral infections but are detrimental to the host during bacterial infections, as *Mycobacterium tuberculosis* and *M. leprae*, and other intracellular parasites, as *Toxoplasma gondii* [89]–[92]. The above evidence suggests a relationship between these signatures, immune pathology, and host resilience.

Finally, the enrichment analysis of the parasite transcriptome exhibited similar enriched terms regarding the molecular signatures from their corresponding host. Accordingly, these transcriptomic profiles suggested that there is a direct influence in the parasite gene expression in reply to the host response. In combination, the multi-omic approach allow us to identify trends in the host-pathogen interaction that enable the establishment of a resilience outcome.

Conclusions

Our analysis provides novel insight into the molecular and cellular basis for the development of resilience in two hosts infected with *P. knowlesi* parasites. The resilience mechanism against *P. knowlesi* infections in *M. fascicularis* is a nutritional immunity strategy from the host to sequester trace molecules in an effort to limit pathogenicity during infection. The resilient host can promote resilience using an approach against extracellular pathogens (sequestering iron) in a selected fashion between tissues. In contrast, oxidative stress dysregulation in the non-resilient host through the SOD2 up-regulation may be related to the bioavailability of iron and antioxidant promoters, thus driving impairment in the tolerance signals between tissues.

Future directions

To estimate the contribution from the whole blood cells to the plasma proteome, the Pearson correlation coefficient between transcripts and their corresponding protein will be calculated. Then, the corresponding correlation will be considered significant if the associated q-value is < 0.1 after Benjamini-Hochberg adjustment. Next, the returned matrix of correlation coefficients will be visualized as a heatmap.

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Supplemental Material

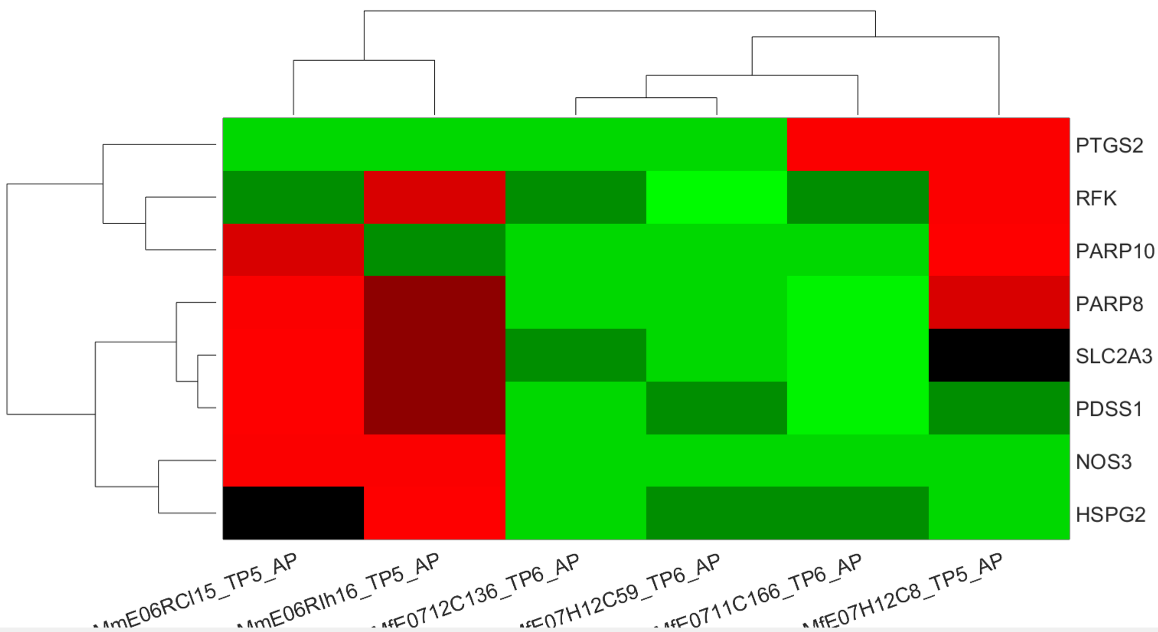
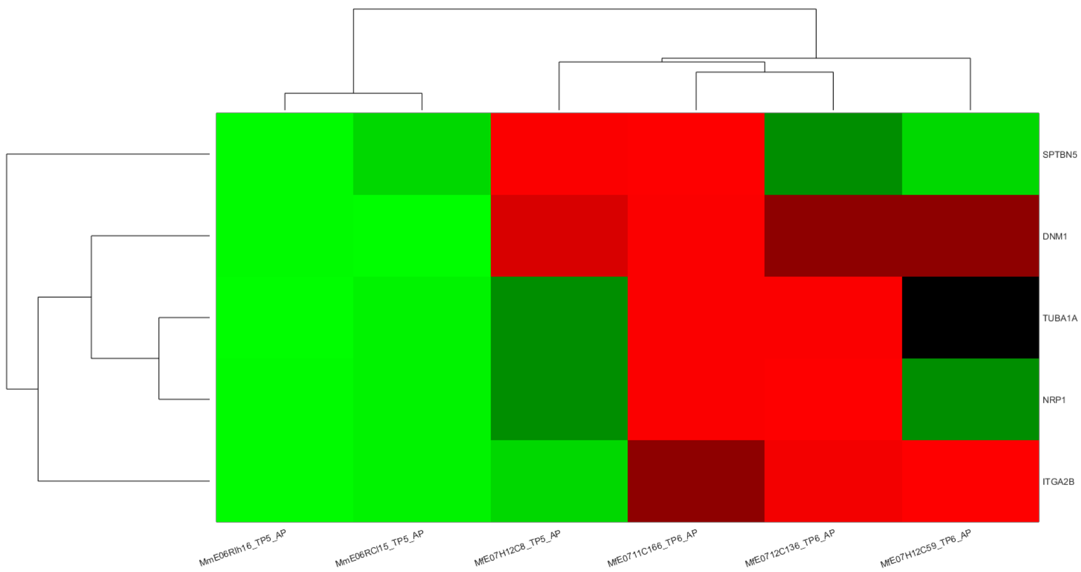


Figure S1:Unsupervised clustergram for uniquely upregulated genes in E07. This figure shows the clustergram for the genes enriched to the terms: A-Metabolism of water-soluble vitamins and cofactors (Mm=*M. mulatta*, Mf=*M.fascicularis*).

A-



B-

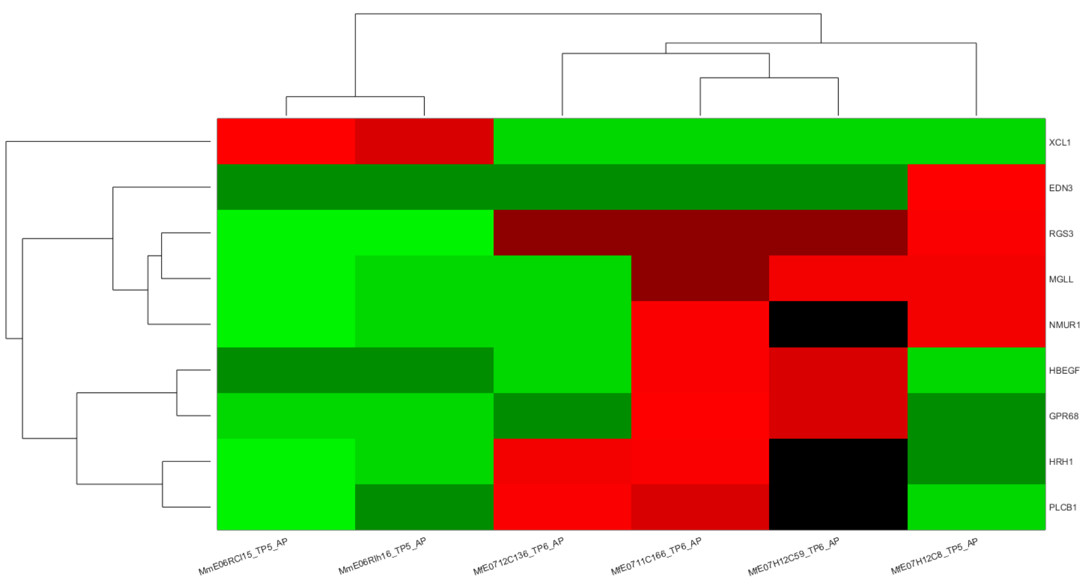


Figure S2: Unsupervised clustergram for uniquely down-regulated genes in E06. This figure shows the clustergram for the genes enriched to the terms: A-L1CAM interactions and B- G alpha (q) signaling events(Mm=*M. mulatta*, Mf=*M.fascicularis*).

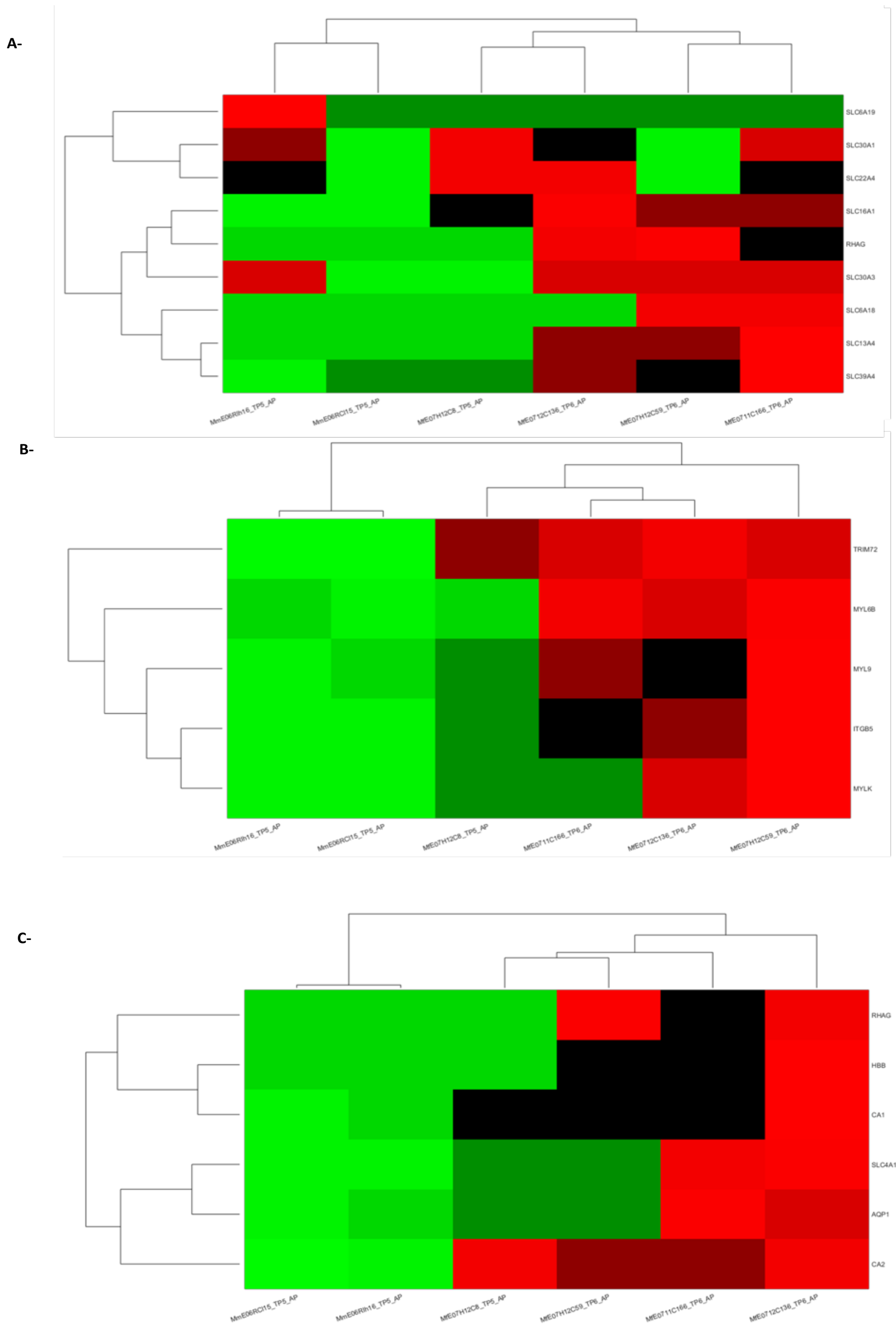


Figure S3: Unsupervised clustergram for uniquely up-regulated genes in E07. This figure shows the clustergram for the genes enriched to the terms: A- Transport of bile salts and organic acids, metal ions and amine compounds . B- Smooth Muscle Contraction. C-

Erythrocytes take up oxygen and release carbon dioxide (Mm=*M. mulatta*, Mf=*M.fascicularis*).

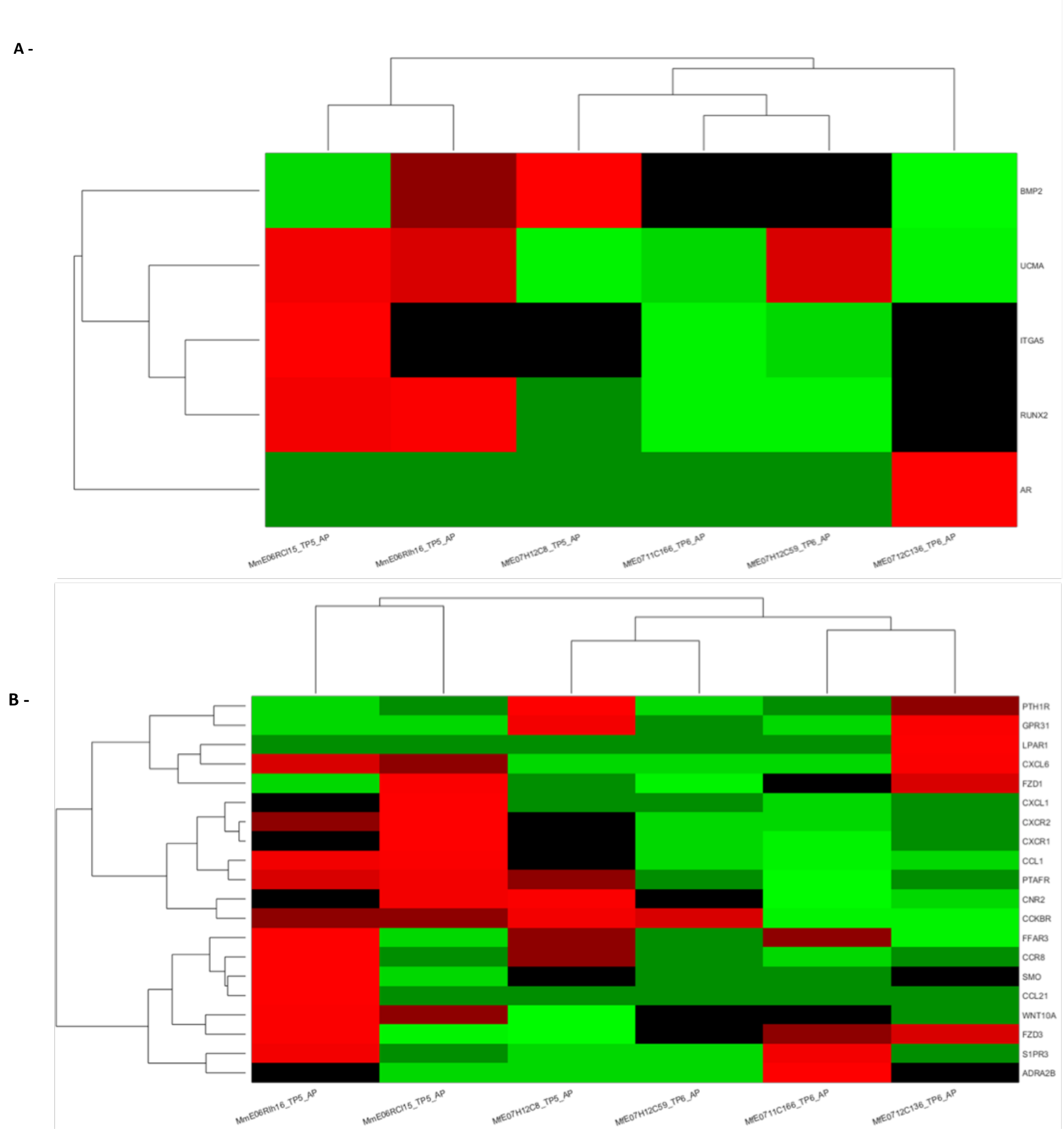


Figure S4: **Unsupervised clustergram for uniquely Upregulated genes in E07.** This figure shows the clustergram for the genes enriched to the terms: **A-** Transport of bile salts and organic acids, metal ions and amine compounds. **B-** Smooth Muscle Contraction. C- Erythrocytes take up oxygen and release carbon dioxide (Mm=*M. mulatta*, Mf=*M.fascicularis*).