RT 01 - RECENT ADVANCES TOWARDS VACCINE DEVELOPMENT FOR MALARIA, LEISHMANIOSE AND CHAGAS DISEASE

RT.01 – 001 - **Advancing towards a vaccine for Chagas disease using distinct strategies based on Trans-sialidases sequences**

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Trans-sialidases (TS), present on the surface of *Trypanosoma cruzi* and expressed in different stages of the parasite life cycle, are virulence factors encoded by the largest gene family identified in the parasite genome. Only a subgroup of this protein family has catalytic activity, which is responsible for transferring sialic acid residues from host glycoconjugates to mucins on the parasite surface, a mechanism related to parasite evasion from the host immune system. Active TS also contains a C-terminal immunogenic domain with amino acids repeats known as SAPA (shed acute parasite antigen). Shed in the blood of the infected host, TS mediates several biological effects and because of its essential role during infection, it has been tested recurrently as a vaccine candidate against Chagas disease (CD). Using TS sequences, we tested different strategies to develop a CD vaccine including mice immunization with recombinant TS proteins with and without SAPA repeats, as well as with RNA formulations encoding the same TS sequences. We have also tested a chimeric protein named TRASP, composed of two distinct members of the TS family. Besides confirming the immunodominance of the SAPA domain, we showed that immunization with TS, TRASP or RNA encoding TS results in a potent cellular immune response that confers robust protection against challenge with *T. cruzi*. We also showed that the presence of the repeats did not significantly impact protection but, immunization with TS protein or RNA encoding TS with the repeat domain resulted in increased production of IL-10 compared to mice immunized with TS without SAPA, and this increased IL-10 production correlates with a significant reduction in the inflammatory infiltrate in heart tissues of infected animals. Finally, we also tested mice immunization with a mutant *T. cruzi* cell line in which all genes encoding active TS were disrupted using CRISPR/Cas9. When inoculated into mice, TS mutants were unable to establish infection even in the highly susceptible gamma interferon (IFN-g) knockout mice. Importantly, mice immunized with TS mutants were fully protected against a challenge infection with a virulent strain. Altogether, our results confirmed the role of TS as a *T. cruzi* virulence factor essential for parasite infection as well as its relevance as a target antigen for vaccine studies. Furthermore, we showed that RNA vaccines are powerful alternatives to vaccines based on recombinant proteins. Finally, our results also indicated that an avirulent mutant lacking TS activity, tested as a live attenuated vaccine against CD, is the strategy that resulted in the highest protection efficacy since it activates a wide range of immune responses.

Supported by: CAPES, FAPEMIG, CNPq and INCTV
Keywords: Trypanosoma cruzi;Trans-sialidase;RNA vaccine.

RT.01 – 002 - **Immunotherapy of *Trypanosoma cruzi* chronic infection with a therapeutic vaccine**

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Chronic infections with *Trypanosoma cruzi* can lead to Chagas disease, which clinical manifestations include cardiac and/or digestive disease associated with severe morbidity and mortality. Efficacy of drug treatments decreases as disease progresses, so that new alternatives are needed for infected patients. Immunotherapeutic approaches, aimed at modulating the ongoing immune response against the parasite using various vaccine formulations to increase the efficacy of host responses and prevent disease progression, have shown promise in mouse models. These encouraging results have prompted the extensive pre-clinical evaluation of therapeutic vaccines in several animal models including dogs and non-human primates. In particular, a vaccine candidate based on parasite antigens Tc24 and TSA1 formulated with a TRL4 agonist is in advanced stages of development. Tc24 is a flagellar calcium binding protein and TSA1 belongs to the trans-sialidase multigene family of the parasite. This vaccine was found to be safe, immunogenic and able to reduce cardiac disease in multiple animal models, alone or in combination with benznidazole treatment. Nonetheless, several challenges remain for the further development of an immunotherapeutic vaccine against Chagas disease, that will need to be addressed to ensure a successful clinical development. We expect that ongoing studies will help pave the way to a key new tool for Chagas disease treatment and patient care.
Keywords: Chagas disease;vaccine;treatment.

RT.01 – 003 - **Preclinical evaluation of a multi-allelic CSP-based vaccine candidate against *Plasmodium vivax.***

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Plasmodium vivax remains a major obstacle to malaria elimination efforts worldwide due to its unique biological features, including the formation of dormant liver-stage hypnozoites. Targeting the pre-erythrocytic stage through vaccines based on the Circumsporozoite Protein (CSP) offers a promising strategy to prevent infection and relapse. We previously reported that a chimeric recombinant protein (PvCSP-All epitopes), containing the three variant repeats of PvCSP (VK210, VK247, and *P. vivax*-like), combined with Polyinosinic:polycytidylic acid, or Poly(I:C), and its derivative Poly-ICLC (Hiltonol®), elicited strong antibody-mediated immune responses in C57BL/6 mice and New Zealand White Rabbits against each of the three allelic forms of PvCSP when administered subcutaneously. Here, we evaluated the immunogenicity, efficacy, and safety of PvCSP-All epitopes adjuvanted with CTVad (a MF59-based adjuvant). Intramuscular immunization of C57BL/6 mice induced high antibody titers against the homologous protein as well as against all three PvCSP allelic forms. Toxicity and safety tests performed in mice and rhesus macaques demonstrated that PvCSP-All epitopes was safe and well tolerated in both species. In addition, mice that received the vaccine formulation were protected after challenge with Plasmodium berghei sporozoites expressing P. vivax CSP repeats (Pb/Pv-VK210), achieving the highest level of protection observed to date (>80%), and exceeding that previously obtained with the Poly-ICLC adjuvant. Based on these results, this formulation represents a promising candidate for future clinical testing.
Supported by: FAPESP, CNPq-INCTV, FINEP
Keywords: Plasmodium vivax;circumsporozoite protein;vaccine candidate.

RT.01 – 004 - **A new chimeric recombinant vaccine combining repetitive T cell epitopes for Visceral Leishmaniasis**

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As a neglected disease, visceral leishmaniasis (VL) remains a major cause of morbidity and mortality worldwide. In the absence of prophylactic vaccines, control measures rely on control of vector and reservoirs populations, efficient diagnosis and treatment. Data to be presented report on an end-to-end translational study from antigen discovery to definition of a vaccine formulation, named VaxLeish, to be used in a phase I clinical trial as a therapeutic vaccine. Antigen definition for VaxLeish was based on the use of epitopes derived from a previously well characterized vaccine candidate antigen, A2 and sequences from of a new antigen, Kinetoplast-Associated Protein-like from Leishmania infantum (LinKAP), discovered through a reverse vaccinology approach. Similarly to A2, LinKAP contains multiple tandemly repeated amino acid units, that have highly conserved predicted epitopes across various Leishmania species. But unlike A2, LinKAP is encoded by a single gene copy, that varies in numbers of repetitive units among Leishmania species. LinKAP is enriched in protein fractions like those found in mitochondria, as further supported by confocal microscopy. CRISPR-Cas9 knockout of the LinKAP gene did not impair parasite growth, differentiation, or infectivity in macrophages, suggesting that LinKAP is not essential for these functions in vitro. A recombinant truncated version of LinKAP was used to immunize mice and hamsters, which displayed a robust Th1-type immune response and partial protection, after challenge. Based on these attributes, we fused the mapped epitopes from A2 and LinKAP into a chimeric antigen, rKAPA2, which was produced under good laboratory practices. Poly ICLC (Hiltonol®) was selected as adjuvant for the final vaccine formulation. So far, VaxLeish (rKAPA2 + Poly ICLC) was extensively tested in animal models, as a prophylactic vaccine providing evidence that it is highly immunogenic, induced partial protection and it is safe. Several quality attributes of VaxLeish were defined, according to regulatory issues. With the aim of supporting future clinical translation studies as a therapeutic vaccine, the rKAPA2 (VaxLeish) formulation was evaluated to treat L. infantum-infected mice and hamsters. Animals displayed a shift in T cell responses toward a Th1 pattern, evidenced by increased frequencies of IFN-γ^+ and reduced IL-10 T cells, suggesting partial reversal of the immunosuppressed T cell responses induced by infection. This profile correlated with a marked reduction in parasite burden in treated animals, supporting the use VaxLeihs, not only as prophylactic, but also therapeutic vaccination strategy for visceral leishmaniasis. Based on these findings, we propose that VaxLeish testing advance towards clinical trials.

RT 02 - DIVERSITY, ECOLOGY AND EVOLUTION OF FREE-LIVING PROTISTS AND THEIR VIRUSES

RT.02 – 001 - **Mosaics of convergent evolution: understanding the natural history of Hypotricha (Ciliophora: Spirotrichea)**

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The Hypotricha Stein, 1859 (= Stichotrichia Small & Lynn, 1985) consist of a morphologically diverse clade of ciliates, comprising ca. 700–1000 known species, normally assigned to subclass rank within the class Spirotrichea Bütschli, 1889. Hypotrichs likely originated in the Neoproterozoic, evolving as complex unicellular eukaryotes exhibiting relatively sophisticated behaviour, highly differentiated organellar structures, and massively scrambled genomes, for which assembling during development is one of the most intricate genomic processes known in eukaryotes. Hypotrichs have a dorsoventrally flat body; at least one left and one right marginal row of compound cilia (= cirri); and a highly diversified fronto-ventral-transverse (FVT) cirral field, originated by numerous ontogenetic patterns. They are normally substrate-oriented organisms adapted to life in microporous sediments, and inhabit terrestrial, freshwater, brackish and saltwater ecosystems worldwide, where they feed on bacteria, other protists and even microscopic metazoans. Because of conflicting molecular phylogenies accumulated since around the early two-thousands (mostly based on the 18S-rDNA), and the perceived inconsistencies of such hypotheses with morphology-based classifications, Hypotrich systematics became one of the most confused subjects in ciliate biology. Thorough investigation of the 18S-rDNA sensitivity to multiple alignment parameters variation has shown insufficient signal at various hierarchical levels as one relevant cause for the aforementioned issue. Unfortunately, currently available hypotrich genomic/transcriptomic data are still insufficient for broad taxon sample phylogenetic inference. However, morphological data sets not only enable large taxonomic coverage, but also capture information from various unlinked genes, thus mitigating single gene biases when coupled with one or a few markers. Hence, analyses of morphological and 18S-rDNA data provided, for the first time, a robust phylogenetic tree of the Hypotricha, both in terms of clade support and resolution, which is on a par with recent big data trees (despite the narrower taxon sample). A systematic redefinition of the Hypotricha was then possible after the discovery of actual synapomorphies. Remarkably, most morphological and molecular characters exhibited low consistency but high synapomorphy retention, indicating an outstanding amount of convergent evolution associated with ontogenetic processes of the ventral and dorsal ciliature pattern building blocks, such as the FVC cirri groups, marginal rows and dorsal ciliary structures. Over geological time, supposed exhaustion of character states space resulted in various mosaic combinations of possible patterns for each of the main ciliature elements, rendering the Hypotricha an example of truly homoplasy-rich taxon.
Keywords: ciliates;systematics;taxonomy.

RT.02 – 002 - **Bromeliad Phytotelmata as Natural Microcosms: Exploring the Diversity and Roles of Ciliates**

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The study of the morphological and molecular diversity of ciliates in bromeliad tanks is both recent and scarce. Over the past twenty years, more than 200 ciliate species have been recorded in this microcosm, with at least 10% representing new taxa that are exclusive to this ecosystem. Our proposal for the presentation is to share results obtained at the Laboratory of Protozoology of the Federal University of Juiz de Fora, focusing on: (i) a checklist of ciliate species found in this ecosystem and their relationships with different bromeliad species; (ii) new ciliate taxa discovered in these environments; (iii) ecological studies addressing the specificity of ciliates to particular bromeliad species; (iv) a genomic study of a ciliate species involved in a unique ecological interaction among bromeliads, anurans, ciliates, and ectosymbiotic bacteria; (v) a macroevolutionary analysis including divergence dating and the possible origin of bromeliad-exclusive ciliate species based on molecular dating; and (vi) future perspectives for studies on ciliates and other protists in this still poorly explored ecosystem.
Keywords: Bromeliads; Endemic ciliates;Ciliophora.

RT.02 – 003 - **The viral factory of Nucleocytoviricota (Giant-Viruses) and their peculiarities.**

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Introduction:
The Mimivirus genus (phylum Nucleocytoviricota) of giant viruses that infect several eukaryotic cells, are particularly interesting due to their capsid size, large genome, and replication cycle, challenging established paradigms of the virosphere. These viruses form dense structures known as viral factory (VF) where virion replication occurs with the disruption of the host's intracellular structure and virions release.
Three viruses, isolated from Brazilian sources, are of particular interest; the Niemeyer virus (NYMV), Tupanvirus (TPV) and Naiavirus (NV), as they have diverse genome structures, gene composition, capsid structures and infection strategy.
This study aims to analyze the formation of the viral factory (VF) and the changes in host cell organization during virus replication in Acanthamoeba castellanii, using X-ray imaging techniques.
The viral particles were produced from A. castellanii infected cultures. Infected cells were fixed at different stages of infection and prepared for Cryo Soft X-ray Tomography (Cryo-SXT) and Nanotomography Ptychography (CXDI) experiments. The combination of Cryo-SXT and Ptychography revealed new images of the viral factory formation in the cytoplasm, highlighting significant structural changes during the infection and viral biosynthesis in its native three-dimensional form.
These techniques offer new structural analysis tools of the complex formed by the virus, contributing to a better understanding of the role of the viral factory and the changes in host cells architecture during infection, with minimal interference with the native structure of the cells and target complexes.
Keywords: Viral Factory;Nucleocytoviricota;Amoeba, Acanthamoeba castellanii.

RT 03 - Host defenses against protozoa

RT.03 – 001 - **CRISPR/Cas9 genomic edition in the context of the Neospora caninum-host relationship**

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Our research group aims to gather knowledge about cellular and molecular aspects of the interaction between hosts and two phylogenetically related protozoa, *Toxoplasma gondii* and *Neospora caninum*, with a view to developing prophylactic and therapeutic protocols aimed at diseases resulting from these infections. While *T. gondii* is a zoonotic agent that may cause severe disease in immunocompromised individuals and fetuses, *N. caninum* does not cause disease in humans, but is recognized worldwide as an important pathogen for dogs and cattle. In this context, strategies to control both infections are extremely valuable, while there are currently no commercially available vaccines for either parasite. For that purpose, we have described new secreted parasitic proteins from *N. caninum* that may act in the activation and subversion of host innate pathways during the interaction, especially those related to the composition of the parasitophorous vacuole and secretion of effector molecules into the cytoplasm and nucleus of the host cell. Using techniques such as CRISPR/Cas9-induced genome editing, RNASeq, and proximal biotinylation assays, associated with measurements of the elicited immune response, with a comparative approach between both parasites, we seek to clarify hidden aspects of the host-parasite interaction in order to enable the development of common approaches to control these infections.

Keywords: Neosporosis;Genomic edition;Innate immunity.

RT.03 – 002 - **Single-cell RNA sequencing reveals subset-specific immune signatures and unusual imune-cell populations differentiating asymptomatic and cardiac Chagas disease patients**

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Human infection with Trypanosoma cruzi leads to Chagas disease and induces profound changes in the immune response across different immune cell subsets, influencing parasite control and disease pathology. Dissecting the functional characteristics of distinct immune cells in patients with the asymptomatic (indeterminate – IND) or with the cardiac (CCC) clinical forms is crucial for unveiling mechanisms of disease progression and pathology and identifying disease markers. Here, single-cell RNA sequencing was applied to peripheral blood mononuclear cells (PBMCs) obtained from IND and CCC patients to unravel the immune landscape in this polar, well-characterized, clinical groups. Our findings revealed different myeloid and lymphoid cell clusters in both cohorts, each exhibiting unique gene expression patterns. CCC was characterized by an increased frequency of KLRB1+CD4+, TBX21+CD8+ T cells, and NK cells, which exhibited upregulation of genes associated with inflammatory, cytotoxic and apoptotic responses. Furthermore, we observed monocyte and B cell subsets, along with dendritic cells, expressing inflammatory and notably cytotoxic genes. Functional in vitro analyses confirmed the existence of these unusual cell populations in patients with CCC, which were activated by parasite-specific stimuli. These results reveal cell-specific changes in CCC compared to IND chronic Chagas disease patients, highlighted by distinct gene expression patterns. These nuanced changes indicate immune signature linked to the clinical forms of chronic Chagas disease, which provide information regarding disease pathology, indicating potential markers related to the disease progression.

Supported by: CNPq, INCT-DT, NIH
Keywords: Chagas disease;cardiomyopathy;single-cell mRNA analysis.

RT.03 – 003 - **Directing the Plasmodium vivax 19kDa merozoite surface protein (MSP1 19) to two distinct dendritic cell subpopulations through prime-boost vaccination strategies.**

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In 2022, an estimated 249 million clinical cases and over 608,000 fatalities from malaria were recorded worldwide, imposing a significant burden on the economies of developing nations. There is a broad agreement that Plasmodium vivax (Pv) represents the most widespread human malaria parasite globally and poses the most formidable obstacle to malaria eradication. Consequently, the creation of an effective vaccine to fight Pv malaria remains a substantial challenge. For the past two decades, the precise delivery of antigens directly to conventional dendritic cells (cDC) utilizing chimeric monoclonal antibodies (mAbs) specifically designed to bind endocytic receptors on these cells has proven to be an effective method for eliciting robust immune responses. Therefore, this investigation aimed to selectively target two different cDC subsets in vivo by genetically coupling anti-DEC205 and anti-DCIR2 chimeric mAbs to the Pv 19 kDa merozoite surface protein (MSP1 19) and the Pan Allelic DR epitope (PADRE) antigenic sequences. C57BL/6 mice underwent two immunization rounds with combinations of these chimeric mAbs, with poly (I:C) included as an adjuvant. Subsequent assessments encompassed the cellular and humoral immune responses generated, in addition to the level of protection conferred against a challenge involving a transgenic P. berghei (Pb) strain engineered to express PvMSP1 19 . Our findings indicated that immunization with the anti-DCIR2-MSP1 19 _PADRE chimeric mAb (either administered twice or in conjunction with anti-DEC205-MSP1 19 _PADRE) stimulated potent humoral responses, primarily characterized by the presence of serum anti-PvMSP1 19 IgG antibodies. Conversely, when the anti-DEC205-MSP1 19 _PADRE mAb was given as the initial dose, it triggered a cellular immune reaction, orchestrated by CD4 + T lymphocytes that secreted pro-inflammatory cytokines, specifically IFN-γ + , IL-2 + , and TNF-α + . Ultimately, both the heterologous prime-boost regimen (anti-DEC-MSP1 19 _PADRE followed by anti-DCIR2-MSP1 19 _PADRE) and the homologous prime-boost approach (anti-DCIR2-MSP1 19 _PADRE administered twice) provided partial protection in 100% and 75% of the challenged subjects, respectively.

Keywords: malaria;monoclonal antibodies;dendritic cells.

RT.03 – 004 - **Gut microbes are associated with immune and metabolic responses to Plasmodium vivax malaria**

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Emerging evidence demonstrates that gut microbes are integral components influencing human health and disease. Recent studies suggest that gut bacteria promote susceptibility to severe malaria caused by *Plasmodium falciparum*, including the genus Bacteroides, whose abundance is also associated with infection with *P. vivax*. Although associations with infection or disease severity suggest an important role for the microbiome during human malaria, the mechanisms by which gut microbes influence host phenotypes remain unknown. Metabolism and host immune response are critical for protection but are also involved in malaria pathology. Therefore, balanced effector and regulatory mechanisms operate in concert to contain Plasmodium replication, while limiting potential tissue damage caused by inflammation. We formulated the hypothesis that gut microbes affect host metabolism and regulate the inflammatory response induced by *P. vivax* malaria. To test our hypothesis, we recruited patients with symptomatic *P. vivax* malaria during acute infection and 28 days after successful treatment, and endemic healthy controls. We collected feces from all individuals to evaluate the relative abundance of bacteria and fungi using 16S rRNA and ITS gene sequencing; and blood to investigate the relative abundance of plasma cytokines, chemokines, and growth factors using multiplex technology; and the relative levels of plasma metabolites using liquid chromatography tandem mass spectrometry (LC-MS/MS) platform. We collected samples from 42 patients and 20 controls, which presented

with similar demographic characteristics, including age and sex. Patients displayed variations in previous exposure to Plasmodium and registration of malaria, which varied from 1 to > 10. Parasitemia was not correlated with age, number of malaria episodes or clinical score, but the former was negatively correlated with both age ($p = 0.0006$) and number of malaria episodes ($p = 0.04$). Analysis of the gut bacteria composition revealed no significant differences in diversity between acute infection or the same samples after treatment and healthy controls, indicating that the infection does not promote dysbiosis. However, linear discriminant analysis (LDA) revealed acute malaria is associated with higher abundance of genus including, Eschirichia_Shigella, Roseburia, Megasphaera, and several members of the Lachnospiracea family, whereas abundance of the genus Weissella was higher after treatment and Monoglobus was higher in healthy controls. Multiplex protein analyses showed patients display higher abundance of IL-1alfa, IL-4, IL-6, IL-10, IL-18, IL-21, IL-22, IL-1Ra, CCL2, CCL3,CCL4, CCL11, CXCL1, CXCL8, CXCL10, IFN-gamma, SDF-1alfa, HGF, VEGF-alfa; and lower abundance of BDNF. Untargeted metabolomics analysis showed that differentially abundant metabolites in patient´s plasma were enriched for pathways including carnitine shuttle, tryptophan metabolism, vitamin A metabolism, Vitamin B5 – CoA metabolism and catabolism, and bile acid biosynthesis. The differential abundance of gut bacteria, proteins and metabolites in blood from patients with *P. vivax* malaria at the same time suggest there might be interactions between them affecting the patient´s phenotype, such as development of symptoms or parasitemia control, which will be further investigated in this study.
Keywords: malaria;gut microbiota;multiomics.

RT 04 – Vectors

RT.04 – 001 - **Lipid Flow from Vectors to Parasites: An Unexplored Cascade of Biochemical Mechanisms**

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Parasites have evolved sophisticated strategies to manipulate the lipid metabolism of their hosts, ensuring their survival and replication. Our laboratory investigates how parasites alter lipid uptake, storage, and biosynthesis athways, creating a favorable environment for their development. We have demonstrated that Plasmodium, *Trypanosoma cruzi*, and *Trypanosoma rangeli* incorporate and metabolize vector lipoproteins in a time- and dose-dependent manner, utilizing these lipids to organize membranes or store them in lipid droplets. In Rhodnius prolixus infected with *Trypanosoma cruzi*, we documented an 80.6% decrease in fat body TAG stores and 60% reduction in LSD-1 expression. The parasite activated AMPK phosphorylation, redirecting lipid flux to the midgut where we observed a 3-fold increase in lipid uptake. Despite 2-4 fold upregulation of PLA2 genes (RpPLA2 9995, 8617, 4037), overall PLA2 activity decreased by 40%, demonstrating complex tissue-specific regulation. Hemolymph analysis showed 50-70% reductions in cholesterol esters, diacylglycerols, and fatty acids. For Trypanosoma rangeli infections, we also observed a significant reduction in fat body lipid stores (TAG, MAG, PE, and PC), accompanied by a 2.5-fold increase in hemolymph free fatty acids. Microscopic analysis revealed a 30% decrease in lipid droplet size and high parasite adhesion (>50 parasites/μm²) to fat body surfaces. Molecular studies showed a 3.2-fold upregulation of acetyl-CoA carboxylase alongside 70% downregulation of lipophorin receptor, indicating parasite-induced metabolic reprogramming. In the Plasmodium-mosquito system, we demonstrated active lipid acquisition by oocysts, which incorporated FITC-labeled lipophorin at 12.5 ng/hr. Quantitative studies with ^{125}I-Lp revealed temperature-dependent uptake (Q10=2.3), with phospholipid transfer to sporozoites occurring at 8.2 pmol/hr. These findings represent the first direct evidence of Plasmodium lipid scavenging in the invertebrate host. Key discoveries include identification of miRNA-mediated communication (with rpr-mir-281-3p increasing 2.1-fold in infected hemolymph) and parasite induction of metabolic changes in non-colonized tissues. Our work reveals that these parasites employ convergent strategies to manipulate vector lipid metabolism, despite their distinct life cycles. These findings provide new targets for disrupting parasite development within vectors, offering novel approaches for disease control.
Keywords: lipids;Rhodnius prolixus;mosquito.

RT.04 – 002 - **Dissection of *Leishmania* parasite adhesion in the sand fly vector**

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Within its sand fly vector, *Leishmania* parasites have two major morphological forms, a motile promastigote and a haptomonad, which is adhered to the stomodeal valve through a shortened and modified flagellum. Dissecting haptomonad development and adhesion is critical to understanding parasite transmission. We have previously generated high-resolution 3D models of haptomonads adhered to the stomodeal valve using volume electron microscopy. This showed that the adhesion complex consisted of filaments that run through the flagellum to an electron-dense plaque, with connections across to the surface of the valve. Using comparative proteomic approaches, we identified three Kinetoplastid-Insect Adhesion Proteins (KIAPs) that locate to different regions of the adhesion complex. One of these proteins, KIAP2, is necessary for the formation of filament bundles in the flagellum of the promastigote, demonstrating this motile form is primed for adhesion to the insect. Furthermore, these proteins are present in other kinetoplastid parasites, suggesting a common mechanism of adhesion in the kinetoplastid parasites. Deletion analysis compromised *Leishmania* adhesion both in vitro and in the sand fly, confirming that we have identified the first critical components of the adhesion complex. Infection of sand flies with *Leishmania* parasites results in damage to the cuticle surface of the valve and distension of the midgut by secretion of the promastigote secretory gel, enhancing parasite transmission. Interestingly, we found that loss of parasite adhesion in the sand fly caused reduced distension of the midgut, with no observable damage to the cuticle surface of the valve. Overall, our study provides the first molecular insights into a kinetoplastid parasite vector adhesion interface and highlights the importance of *Leishmania* adhesion for the modification of the sand fly gut environment and onward transmission.
Keywords: adhesion;sand fly;pathogenicity.

RT.04 – 003 - ***Leishmania* transmission is disrupted in sandflies colonized by *Delftia tsuruhatensis* TC1 bacteria**

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Most human pathogenic *Leishmania* species are zoonotic agents; therefore, sandfly-based control strategies are essential to prevent parasite circulation. Here, we used a *Delftia tsuruhatensis* strain that inhibits the development of *Plasmodium* in mosquitoes, but in the context of *Leishmania*-infected sandflies. Using GFP-expressing *D. tsuruhatensis* TC1, we show that this bacterium colonizes the midgut of *Phlebotomus duboscqi* sandflies. Such colonization impacts the development of *L. major* parasites in the vector, as per the significantly lower number of both total and infectious metacyclic parasites detected in the midguts of bacteria-fed *versus* control sandflies (90% reduction). This phenotype was consistently observed, regardless of the timing of bacterial feeding (from 1 week prior to infection to 8 days after infection), and was even stronger in sandflies given a second, uninfected, bloodmeal. Curiously, our data suggests this phenotype is likely an indirect effect of TC1 colonization, related with the induction of sandfly gut dysbiosis. These results have biological significance, since we observed that *Leishmania*-infected, bacteria-fed sandflies are less able to transmit *Leishmania major* parasites and cause disease in a mouse model of cutaneous leishmaniasis (parasites detected in 27% of animals bitten by bacteria-fed flies *versus* 100% of animals in the control group). Relevantly, modelling studies based on our results support the disruption of disease endemicity in the field. Altogether, these results highlight TC1 as a promising vector-based approach for the control of leishmaniasis in the field. **Supported by:** This research was supported by the Intramural Research Program of the National Institutes of Health (NIH). **Keywords:** Leishmania-sand fly interactions;vector refractoriness;gut dysbiosis.

RT.04 – 004 - **Heme as a player in the control of hemoglobin digestion in *Rhodnius prolixus***

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The triatomine bug *R. prolixus* is a hematophagous hemipteran and an important vector of *Trypanosoma cruzi*, the etiological agent of Chagas disease. Due to the lack of an effective vaccine for Chagas disease, vector control remains the primary method for preventing or reducing disease transmission. Following each blood-feeding event, the digestion of hemoglobin leads to the release of substantial quantities of heme. This heme is rapidly converted into bioinert crystals called hemozoin (Hz). In this work we showed that at least two alpha-glucosidades associated with the perimicrovillar membranes play a role in the Hz formation. These enzymes likely cluster heme molecules during the initial nucleation step. Alpha-glucosidase knockdown caused a significant reduction both in the enzyme activity and Hz formation in the midgut. In addition, the blood meal was retained longer in the anterior midgut, and higher levels of intact hemoglobin were observed in the posterior midgut compared to controls, suggesting a disruption in hemoglobin digestion. This indicates that the digestive process, specifically the breakdown of hemoglobin, was likely affected by the treatment. The alpha-glucosidase knockdown also negatively affected *Trypanosoma cruzy* metacyclogenesis, what might be a consequence of higher levels of free heme. We then decide to investigate the possible effect of heme on the hemoglobin proteolysis. The incubation of the midgut extract with free heme revealed heme, but not Hz, can associate to a protein. Enzymatic assays, using midgut content, revealed free heme can inhibit the breakdown of hemoglobin and the use of specific protease inhibitors indicated the enzymatic activity corresponded to a cysteine protease. Our results suggest Hz may protect the protease from heme's inhibitory effect. The effect of free heme on the hemoglobin proteolysis might be a strategy to avoid free heme overload. The synchronized activities of membrane-associated alpha-glucosidase and a protease could ensure the success of blood digestion in the midgut of R. prolixus without hazard consequences to the vector. The interference in this process might represent a promising target for vector control and reducing parasite spread. **Keywords:** hemoglobin proteolysis;metacyclogenesis;heme.

RT 05 – Host-Protozoa Interaction

RT.05 – 001 - **Genome-scale profiling of gene function in African trypanosomes in culture, models and host.**

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High-throughput methods for gene function analysis are powerful tools to understand parasite biology. To date, genome-scale screening has only been possible in culture-adapted *Trypanosoma brucei*, meaning that significant aspects of parasite biology cannot be directly addressed – nor the biology of the most important agents of the disease in humans and cattle. To understand the biology of the parasite in contact with the host, we developed Direct RNAi-Fragment Sequencing (DRiF-Seq), a novel high-throughput genome-scale approach to create massively parallel libraries of 1000s of RNAi mutants in monomorphic and pleomorphic bloodstream-form T. brucei. We have used DRiF-Seq to quantitatively follow ~250,000 independent barcoded mutants over >20 generations, and robustly estimate clone- and gene-specific competitive growth rates in vitro. In pleomorphic cells, 60% of the core genome is associated with a significant loss-of-fitness in culture (similar to that seen by knock-outs in Plasmodium). As DRiF-Seq libraries are compatible to animal models, we can further look for genes necessary for survival in vivo. This shows that ~25% of mutants in invariant surface genes differ significantly in their importance in culture versus infection and uncovers novel modifiers of parasite virulence which do not act through canonical quorum-sensing. Parallel phenotyping over multiple timepoints reveals the characteristic timings and effect sizes associated with specific cellular processes, and allows the functional dissection of essentiality within protein complexes. Moreover, the method can be quickly modified for application to trypanosome species causing human and animal diseases, and intra- and inter-species DRiF-Seq comparisons reveal unexpected differences in specific biology between strains of *T. brucei*, and also substantial differences between *T. brucei* and the major agent of animal African trypanosomiasis, T. congolense. Additionally, we have transferred DRiF-Seq to two strains of the human-infective species *T. gambiens*e with differential virulence (chronic or acute) as a means to profile gene knockdown fitness associated with resistance to human serum. These data demonstrate the power of robust quantitation of mutant fitness at genome-scale in vitro and in the host, and point to aspects of parasite biology that are not well represented by common culture-adapted lines. They also point to a much more effective means to study gene function in multiple models of trypanosomiasis that would remove many confounding factors from analysis and massively reduces animal usage.

RT.05 – 002 - **DNA traps and extracellular vesicles: double-edged swords of neutrophil immunity during confrontation with Entamoeba histolytica.**

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Amebiasis is an intestinal and liver disease caused by the protozoan *Entamoeba histolytica*, characterized by severe tissue damage with extensive neutrophil infiltration. The role of these neutrophils is controversial, as our previous studies suggest that they may contribute to the damage by being manipulated by the amoeba. Furthermore, it is well known that parasites release extracellular vesicles (EVs) that modulate the host immune response, contributing to the establishment of infection. In this work, we confronted *E. histolytica* trophozoites with human neutrophils and evaluated NETosis and the release and content of EVs from amoebas and neutrophils themselves. We found that neutrophils die rapidly by NETosis rather than apoptosis, in a process independent of NOX2-derived ROS (the amoeba inhibits the respiratory burst) but dependent on exogenous ROS delivered by the amoeba itself, since trophozoites depleted of their ROS by pyrocatechol lose the ability to induce NETs. Interestingly, we found that the parasite"s ROS are transferred to neutrophils in their EVs, and that they are even produced within vesicles by a still unknown process. Using specific inhibitors, we identified hydrogen peroxide, hydroxyl radical and superoxide anion in trophozoites and amoebic EVs. Nanoparticle tracking analysis showed that amoebic EVs are variable in size, ranging from less than 50 nm to nearly 600 nm in diameter (average of 167 nm), whereas neutrophil EVs are more uniform with an average of 136 nm. In cocultures amoeba:neutrophil (1:100) most EVs are 98 nm in size, typical size of exosomes. The incorporation of EVs from amoebae carrying ROS into neutrophils was insufficient to induce NETosis, suggesting that other stimuli are required; however, they were able to downregulate phagocytosis and respiratory burst, while on their own they are able to induce some pro-inflammatory cytokines such as TNF alpha and IL-1beta. A comparative

proteomic analysis between the EVs of amoebae and neutrophils separately vs cocultures amoebae:neutrophils showed a similar distribution of protein categories in the GO analysis, but differences in the expression and abundance; thus, the cargo of EVs from cocultures showed higher levels of key proteins such as amoebic N-acetyl-D-galactosamine (GalNAc) inhibitable surface lectin and cysteine proteases and antimicrobial neutrophil molecules such as lactoferrin and myeloperoxidase. Finally, to assess the possible role of neutrophil DNA traps and amoebal EVs in pathogenesis, we purified amoeba-induced NETs and parasite EVs and demonstrated in vitro that both can cause extensive cytotoxic damage to colon and liver cell lines. In particular, the contribution of serine proteases such as neutrophils elastase and NET DNA was demonstrated by using specific inhibitors and DNAse. These results highlight the importance of EVs in the immunomodulatory effects exerted by the amoeba on human neutrophils, affecting their effector mechanisms at a distance, and possibly contributing, together with amoeba-induced NETs, to the extensive tissue destruction that accompanies tissue invasion by *E. histolytica*. **Keywords:** Entamoeba;NETosis;extracelular vesicles.

RT.05 – 003 - **Two Keys to the Engine: Methylation Control of Gliding Initiation in Apicomplexa**

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Apicomplexan parasites such as *Toxoplasma gondii* rely on finely tuned cytoskeletal regulation to transition between intracellular replication and active motility during invasion and egress. At the heart of this transition lies the apical complex, and more specifically the conoid—a dynamic microtubule-based structure that functions as a regulatory hub for motility initiation. While the lysine methyltransferase AKMT (Apical complex lysine methyltransferase) has been shown to localize to the conoid and regulate gliding by promoting the recruitment of the glideosome-associated connector (GAC), the mechanisms that coordinate conoid activation and motility onset remain poorly understood. In this study, we identify and characterize PCKMT, a second SET-domain methyltransferase that localizes to the preconoidal rings. PCKMT is essential for parasite motility, but dispensable for replication. Loss of PCKMT leads to severe defects in gliding, invasion, and egress. Although the overall architecture of the apical complex remains intact in PCKMT knockouts, conoid protrusion is completely blocked, implicating PCKMT in a key early step in motility activation. We show that PCKMT is required for the stable apical recruitment of the formin-like protein FRM1, which likely catalyses F-actin polymerization at the apical tip. In the absence of PCKMT, FRM1 is not recruited, and actin assembly is disrupted. Interestingly, the SET domain of PCKMT may not be catalytically active, as we observe no detectable changes in global methylation patterns or FRM1 methylation in PCKMT mutants. In contrast, AKMT retains full catalytic activity and remains associated with the conoid until motility is triggered, at which point it disengages and relocalizes toward the basal pole—this translocation occurs even in PCKMT knockouts, suggesting that AKMT dynamics are independent of PCKMT-mediated structural changes. Proteomic analyses reveal distinct interactomes for AKMT and PCKMT: while AKMT is enriched for cytoskeletal and conoid-associated proteins, PCKMT specifically associates with FRM1 and DAP1. Importantly, reintroduction of wild-type PCKMT rescues the FRM1 localization defect and restores motility, while a point-mutant version of PCKMT only partially rescues both, leading to reduced FRM1 recruitment and diminished gliding. Together, our findings support a model in which two methyltransferases—AKMT and PCKMT—act as “keys” that unlock the parasite's motility machinery in a coordinated but distinct manner. PCKMT functions as a scaffold-like anchor at the preconoidal ring, enabling FRM1 recruitment and conoid protrusion, while AKMT modifies downstream cytoskeletal elements and disengages to license actin flow and motor engagement. This dual-methyltransferase mechanism defines a two-step logic for initiating parasite motility, highlighting the conoid as a platform for both structural assembly and dynamic signalling. These insights advance our understanding of cytoskeletal regulation in *Toxoplasma* and provide a framework for dissecting conserved strategies of motility control across the Apicomplexa.
Keywords: Toxoplasma gondii;gliding motility;cytoskeletal regulation.

RT.05 – 004 - **Detection of Leishmania and sand fly borne viruses coinfection in dogs and studies of the role of the phlebovirus Non-structural protein N (NSs) in Leishmania infection.**

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Phlebovirus cocirculation in *Leishmania* transmission areas occurs in various regions of the World. However, few papers have described natural coinfection in sand flies, and just one report has demonstrated the acquisition of *L. infantum* and Toscana virus in the field. The experimental coinfection of *Phlebovirus Icoaraci* (ICOV) and *Leishmania amazonensis* revealed the potentiation of the parasite infection. We addressed the following questions regarding the possible importance of sand fly-borne viruses and Leishmaniasis:

- Does the main virulence factor of Phlebovirurs (NSs) play a role in the modulation of Leishmania infection?
- Are we able to detect natural sand fly-borne viruses in Leishmaniasis transmission focus?

To address the first question, we cloned the ICOV/NSs in the retroviral vector pHIV-zeo and transduced them into RAW 264-7 macrophage cell lines. We could confirm the Nss expression in some selected cell clones by DNA sequencing and West blot analysis (RW-NSs). The sole expression of ICOV/NSs in RW-NSs increased the infection index of *L. amazonensis* and the expression of IL-10 and IFNbeta. Moreover, we observed the upregulation of nuclear factor erythroid 2-related factor 2 (Nrf2), superoxide dismutase 1 (SOD1), and heme oxygenase 1 (HO-1). These results prompted us to test the hypothesis that RW-NS macrophages resisted oxidative stress. As we have predicted, RW-NSs were resistant to H2O2 treatment compared to RW-Zeo (empty vector). Oximetric assays revealed an elevated consumption of O2 by RW-NSs cells, while DNA PCR analysis showed a higher ratio of mtDNA/nDNA. The RT-qPCR assays revealed the higher expression of mt genes such as Nrf1 and the mitochondrial co-transcriptional factor PGC-1α. To address the second question,we developed an RT-PCR assay to detect sand fly-borne viruses in Leishmania reservoirs and vectors. Our assays successfully detected coínfection in two out of twenty dogs with visceral Leishmaniasis in the western region of the Municipality of Rio de Janeiro. Moreover, we isolated the virus from the serum in BHK cells, and the virome analysis identified it as a member of the Pacui virus group, formerly described as Phlebovirus, which was isolated from rodents and sand flies from the North of Brazil. In conclusion, our data support the notion that a Phlebovirus virulent factor contributes to Leishmania infection in macrophages by developing an antioxidative cell milieu and sand fly-borne viruses related to Phlebovirus coinfect dogs with *L. infantum*.

Keywords: L; amazonensis;Phlebovirus;Co-infection.

RT 06 – Drug Development and Resistance

RT.06 – 001 - **The metabolic basis of amphotericin B resistance in Leishmania**

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Amphotericin B has become increasingly important in the treatment of Leishmaniasis. The drug targets ergosterol in the membrane of the parasites, which contain this as their primary sterol, as do fungi which are also sensitive to amphotericin B. We have selected dozens of Leishmania lines resistant to amphotericin B. In each case we find that ergosterol (and closely related ergostane-type sterols) are absent from the parasites using mass spectrometry based approaches. Genome analysis reveals that several different points in the sterol biosynthesis pathway can be targeted including sterol C14 demethylase (Cyp51: ERG11), Sterol C5 desaturase (ERG 3), sterol C4 methyltransferase (ERG6) and Sterol C22-desaturase (Cyp710C). Other work has revealed additional enzymes involved in the sterol biosynthesis pathway to also associate to resistance when mutated. Analysis of the sterol content of parasites mutated in these different genes reveal that they replace ergosterol as the major sterol with different cholestane type sterols explaining why the drug no longer binds. Other genetic changes that can associate with resistance are also observed such as loss of the miltefosine transporter which appears to be involved in distribution of different lipids in the membrane. The resistant parasites develop hyper-sensitivity to oxidative stress inducing agents and also to pentamidine offering opportunities for treatment of amphotericin B resistance should it emerge.

Keywords: Leishmania;macrophage;immunometabolism.

RT.06 – 002 - **From Mechanism to Resistance: Lessons from a Metal-Based Compound in Leishmaniasis Drug Discovery**

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Drug discovery for leishmaniasis is characterize not only by biological complexity and therapeutic resistance but also by a persistent gap between basic research and translational application. In this presentation, we examine the case of CP2, a cyclopalladated compound with multi-target activity against *Leishmania amazonensis*, to illustrate both the scientific advances and the systemic barriers that hinder progress from mechanistic insight to therapeutic innovation. Our studies We demonstrated that CP2 disrupts mitochondrial respiration through inhibition of ubiquinol-cytochrome creductase (Complex III), alters calcium homeostasis, and induces oxidative stress leading to parasite death. We also developed and characterized a CP2-resistant strain, revealing adaptive responses such as overexpression of Complex III and diminished mitochondrial Ca^{2+} uptake. These mechanistic findings not only strengthen our understanding of drug resistance in Leishmania but also illustrate the challenges of advancing early-stage discoveries into effective therapeutic interventions. While CP2 has not yet advanced beyond the preclinical stage, its characterization exemplifies the level of mechanistic and functional insight that is often achieved in academic settings but rarely supported through the next translational steps. This gap reflects persistent regulatory, economic, and infrastructural barriers that continue to limit innovation in neglected disease contexts. Addressing these challenges requires the early integration of mechanistic validation, resistance profiling, and rational combination strategies to build a more feasible and impactful drug development pipeline.

Keywords: Leishmaniasis;Drug Resistance;Drug Development.

RT.06 – 003 - **Preclinical research of novel therapeutic options for toxoplasmosis: from in silico screening to animal testing**

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Toxoplasmosis, caused by the protozoan *Toxoplasma gondii*, remains a major public health concern worldwide, particularly affecting immunocompromised individuals and congenitally infected infants. Despite its high global prevalence, current treatment options are limited, often associated with toxicity, poor tolerability, and lack of efficacy against latent forms of the parasite. These limitations underscore the urgent need for safer and more effective therapies, driving the search for new drug candidates through innovative preclinical strategies. This presentation will provide an overview of our research pipeline dedicated to the discovery and development of novel therapeutic options for toxoplasmosis. Our integrative approach spans multiple stages, beginning with the rational selection of candidate compounds through in silico methodologies, including virtual screening, molecular docking, and prediction of pharmacokinetic and toxicological properties. These computational tools allow the prioritization of molecules with favorable drug-like profiles and predicted activity against validated or putative *T. gondii* targets. Selected compounds then undergo in vitro evaluation in phenotypic assays using tachyzoites infecting mammalian host cells. This step includes the assessment of antiparasitic efficacy, host cell cytotoxicity, selectivity index, and mechanism-of-action studies involving mitochondrial dysfunction, reactive oxygen species generation, plasma membrane rupture, and other cell death-related parameters. Promising compounds are further evaluated through the generation of *T. gondii* parasites resistant to drug pressure, followed by whole-genome sequencing and bioinformatic analysis to identify potential genes and mutations associated with the resistance phenotype. This strategy provides valuable insights into drug targets, mechanisms of action, and potential pathways of resistance. Promising hits are further validated in vivo, including murine models of acute and chronic toxoplasmosis, to evaluate therapeutic efficacy. In addition to synthetic compound libraries and natural products, our research also explores drug repurposing, aiming to identify new indications for approved drugs. This strategy provides a time- and cost-effective avenue for accelerating translational research and clinical repositioning. Finally, we will discuss current challenges and future directions in preclinical research for toxoplasmosis, including assay development, target validation, and the translation of laboratory findings into clinical candidates. Our findings highlight the need of combining computational, experimental, and translational tools to advance the pipeline of new therapeutic options for toxoplasmosis.

Keywords: Toxoplasmosis;Therapy;Preclinical Assays.

RT.06 – 004 - **A cell surface transporter mediates phenanthridine resistance in African trypanosomes**

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The unicellular protozoan parasite Trypanosoma congolense is the primary cause of African animal trypanosomosis (AAT), a debilitating livestock disease that is also caused by T. vivax and to a lesser extent, T. brucei. This disease poses a significant threat to livestock health and agricultural productivity across sub-Saharan Africa, with an estimated 3 million cattle deaths annually. Isometamidium remains the only drug available with both prophylactic and curative properties. Despite reports of resistance since the 1970s, a definitive molecular mechanism of resistance remains unresolved, particularly in the clinically relevant species, T. congolense. In this study, the role of a previously identified protein, TcoDMT was validated via the analysis of in vitro-derived mutants. We show that expression levels of this protein correlate strongly with isometamidium sensitivity - overexpression and knock-out result in hypersensitivity and resistance, respectively. Functional analyses reveal that the protein is a phenanthridine transporter localised to the trypanosome plasma membrane, and is closely related to nucleotide sugar transporters. Interestingly, ablation of expression of the DMT orthologue in T. brucei has no effect on isometamidium sensitivity, suggesting that the role of this protein may be species-specific. This study validates, for the first time, a transporter with a defined role in isometamidium action and resistance, advancing our understanding of drug resistance mechanisms in parasitic protists, and informing strategies to combat trypanosomosis in endemic regions.