RT.01 - EXPLORING GENOMES TO UNDERSTAND PARASITES AND VECTORS BIOLOGY

RT.01.001 - **EXPLORING MECHANISMS OF GENE EXPRESSION REGULATION IN LEISHMANIA**

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It is a challenge to understand how the protozoan parasite *Leishmania* survives in the extremely diverse and hostile environments of the insect gut and vertebrate macrophages. *Leishmania* genetic organization is crucial for parasite success, and this organization has several peculiarities. *Leishmania* genes are arranged as long polycistronic units, and mature mRNAs are produced from the primary transcripts by trans-splicing and polyadenylation. There are no canonical promoters for mRNA transcription, and modulation of gene expression happens at the post-transcriptional level through a combination of distinct mechanisms. Regulatory non-coding RNAs are a growing class of molecules known to be relevant in the post-transcriptional control of gene expression in eukaryotes. Therefore, we decided to search for novel putative non-coding RNA (ncRNA) involved in gene expression regulation in *Leishmania major*. We will present the analysis of ODD3, which was originally rescued from a *Leishmania major* cDNA library and is a regulatory non-coding RNA candidate. We observed noticeable phenotypic changes in parasites that ectopically express this putative ncRNA. We identified one negatively regulated mRNA target of ODD3. In addition, we observed that changes in the secondary structure or in the primary sequence of ODD3 prevented the original ODD3 phenotype. Our results indicate that ODD3 is a regulatory ncRNA that modulates the level of a target mRNA by base pairing with it. In the absence of the classical RNAi pathway in *Leishmania major*, our results point to the existence of an alternate route allowing regulatory RNAs to modulate gene expression in this parasite. In a complementary route of investigation to improve understanding of gene expression regulation in *Leishmania* we conducted a computational comparative genome analysis to disclose cis-elements present in untranslated regions (UTR) of *Leishmania* transcripts. The premise for the study was that the identification of conserved sequence motifs in a divergent genomic landscape might lead to the discovery of new functional cis-elements and the comparative analysis of *Leishmania* genomes of distinct species has revealed that conservation of sequences at coding regions (CDS) is not accompanied by conservation at untranslated sequences (intercoding regions). Therefore, we conducted an in silico investigation to find conserved intercoding sequences (CICS) in the genomes of *L. major*, *L. infantum*, and *L. braziliensis*. We have developed a computational pipeline to identify CICS (Vasconcelos et al, 2012) and we selected two of them to investigate their putative functional role using reverse genetics. We confirmed that one of them is necessary for the control of transcript levels during *in vitro* differentiation. **Supported by:**FAPESP e CNPq

RT.01.002 **- THE GENOME OF ANOPHELES DARLINGI, THE MAIN NEOTROPICAL MALARIA VECTOR.**

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Anopheles darlingi is the principal neotropical malaria vector, responsible for more than a million cases of malaria per year on the American continent. Anopheles darlingi diverged from the African and Asian malaria vectors ~100 million years ago (mya) and successfully adapted to the New World environment. Here we present an annotated reference A. darlingi genome, sequenced from a wild population of males and females collected in the Brazilian Amazon. A total of 10 481 predicted protein-coding genes were annotated, 72% of which have their closest counterpart in Anopheles gambiae and 21% have highest similarity with other mosquito species. In spite of a long period of divergent evolution, conserved gene synteny was observed between A. darlingi and A. gambiae. More than 10 million single nucleotide polymorphisms and short indels with potential use as genetic markers were identified. Transposable elements correspond to 2.3% of the A. darlingi genome. Genes associated with hematophagy, immunity and insecticide resistance, directly involved in vector-human and vector-parasite interactions, were identified and discussed. This study represents the first effort to sequence the genome of a

neotropical malaria vector, and opens a new window through which we can contemplate the evolutionary history of anopheline mosquitoes. It also provides valuable information that may lead to novel strategies to reduce malaria transmission on the South American continent. The A. darlingi genome is accessible at www.labinfo.lncc.br/index.php/anopheles-darlingi. **Supported by:**CNPq

RT.01.003 **- T. CRUZI GENOME SEQUENCING AS A TOOL TO ADDRESS SPECIFIC BIOLOGICAL QUESTIONS**

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The protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas disease, a devastating neglected disease that affects more than 10 million people, mainly in Latin America but it is spreading to other parts of the world. We have been part of a large project to investigate *T. cruzi* strain diversity and epidemiology, and we have therefore performed comparative whole genome sequencing of several *T. cruzi* strains. These include the first sequence of a strain, Sylvio X10/1, from the TcI clade, which is the dominating clade north of the Amazon, and a clone of the bat-specific subspecies *T. cruzi marinkellei*, as well as a possibly divergent TcIV strain from Venezuela, with additional TcIV genomes being sequenced. In addition, we have sequenced two additional TcI strains, from Venezuela and El Salvador, in order to explore the degree of geographic genome-wide variation within TcI. I will present a comparative analysis of all of these genomes, as well as other sequencing and analysis projects that are in progress. Previously published result include the fact that the Sylvio X10/1 and the TcVI reference strain CL Brener core genomes were found to be highly similar and only 6 open reading frames from CL Brener were missing in Sylvio X10/1. The genetic diversity between the two strains is large and many genes show rapid evolution due to selective pressure. Large multicopy gene families were found to contain a significantly smaller number of genes in Sylvio X10/1. The *T. c. marinkellei* genome was found to be ~11% smaller than that of the human infective parasite Sylvio X10/1 and also to contain fewer repeats for most gene families. The level of polymorphism was low, ~0.19%, in both *T. c. marinkellei* and Sylvio X10/1. In coding regions, *T. c. marinkellei* and Sylvio X10/1 differed by ~7.5% at the nucleotide level, which is higher than between the *T. c. cruzi* strains. A unique acetyltransferase gene, not present in any other known parasite genomes, was found in *T. c. marinkellei*. Thanks to improved assemblies, it was possible to identify chromosome rearrangements, structural variation as well as segmental duplications in both *T. c. marinkellei* and Sylvio X10. The Venzuelan TcIV genome and the additional TcI sequences have been analyzed for unique features by comparison with all available reference genomes, in order to identify possible virulence candidates and other variants. Possible functional variants have been identified.

RT.02 - HUMAN AND AVIAN MALARIA

RT.02.001 - MALARIA NEW DIAGNOSTIC ASSAY AND ITS POTENTIAL FOR RAPID MONITORING IN HUMANS AND VECTORS IN ASSOCIATION WITH CLIMATE CHANGES

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Diagnosis of malaria in humans and vectors with the use of LAMP-based technologies that combine simplicity, rapidity and high efficiency represents an effective approach. Loop-mediated isothermal amplification (LAMP) is a novel molecular method that accelerates and facilitates DNA amplification and detection under isothermal conditions. It represents a revolution in molecular biology by reducing the high cost, turnaround time and technicality of polymerase chain reaction and other amplification methods. LAMP simplifies the diagnosis of malaria for epidemiological purposes and can contribute greatly to the control of malaria and its epidemiology due to its high sensitivity and specificity to detect malaria infections in the host and in the vector in an an early stage.
The assay has has been developed and applied for the diagnosis of a variety of viral, bacterial, parasitic and other diseases in the biomedical field. LAMP has been involved in studies concerning the diagnosis of malaria which is still a major cause of morbidity and mortality in different parts of the world.
The present review deals with a short summary of LAMP aüpplications in the biomedical field and the use of LAMP in the diagnosis of malaria in samples from Thailand and related investigations to make a view on what has been investigated and highlights the future perspectives regarding the possible applications of LAMP in diagnosis of the disease.
Because 99% of the results of the genus-specific LAMP technique were original investigations consistent with those of nPCR, we can propose that this assay is as reliable as nPCR for the clinical diagnosis of malarial infections of the genus *Plasmodium*. The results for the species-specific tests were considerably different in sensitivity and specificity of the different *Plasmodium* species. This might be caused by multiple genotypes of parasites in natural infections, implicating to design new primer sets that amplify regions that universally conserve the species. Therefore, indicating that further development is needed
In conclusion, LAMP is a promising technique, enabling Malaria cases diagnosis by a molecular tool in a resource-limited setting. However, in this study the species-specific tests still need further development before being used as routine diagnosis in field settings and epidemiological studies using material from clinical sources and from vectors.

RT.02.002 - HABITAT ELEMENTS IMPACT THE PREVALENCE AND HOST SPECIFICITY OF AVIAN HAEMOSPORIDIAN PARASITES IN TROPICAL ECOSYSTEMS.

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Human domination of the planet has rapidly led to increases in the relative fractions of deforested and agricultural land on the Earth's surface. Here we give a cross-continental perspective on how land use changes, especially deforestation and agricultural use can affect the prevalence and lineage diversity of avian blood parasites. Work from Sub-Saharan Africa demonstrates that anthropogenic habitat change can affect host-parasite systems and result in opposing trends in prevalence of haemosporidian parasites in wild bird populations. We conducted a study of the effects of deforestation and habitat type on the prevalence and diversity of blood parasites in African rainforest birds. Over the past 18 years, in collaboration with the Center for Tropical Research at UCLA, we have collected more than 10,000 individual blood samples from over 200 rainforest bird species in a variety of habitats across Africa. Significantly, the samples were collected from sites pristine and degraded sites, permitting a unique examination of the direct effects of human-induced habitat alterations. Using complementary techniques of blood smear analysis and molecular biology, samples are assayed for *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Trypanosoma*. We have obtained results regarding the host-specificity, prevalence and lineage diversity of these parasites in rainforest birds. As part of the larger project, we collected blood samples from two

bird species from eight paired disturbed and undisturbed sites in Southern Cameroon. We describe the parasite lineages in 2 common bird species. Linking these DNA sequence lineages with identified parasite morphospecies, we describe significant differences in prevalence between habitat types in the haemosporidian parasites. We also present recent data on the evolution of specialist vs. generalist strategies in avian malaria. Our work incorporates satellite imagery data to quantify differences among the sites, and predict how changes in forest composition may affect the spread of diseases. Additionally, habitat impacts the degree of host specificity of parasites and their hosts. With further work in Costa Rica, we quantify how avian malaria in an abundant sedentary bird species responds to landscape features at fine scales. Debate over balancing agricultural production and biodiversity conservation has generated two opposing strategies: a “land sparing” approach involving large-scale nature reserves, versus a “land sharing” approach where agricultural areas support wildlife through fine-scale conservation. This debate however has largely ignored the effects of the different strategies on parasite dynamics. Data revealed no significant differences in habitat usage or home range size associated with infection status. However, we simulated “land sparing” and “land sharing” landscapes and modeled malaria prevalence to find that land sharing mitigates malaria prevalence more effectively at all agricultural scales. We emphasize that influences of land use changes on parasite prevalence are complex, and will require the detailed study of the vector ecology, and the further quantification of fine-scale habitat effects.

RT.02.003 - **EXPLORING THE DISTRIBUTION AND DIVERSITY OF AVIAN MALARIA PARASITES IN DIFFERENT BIOMES IN BRAZIL**

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It is increasingly evident that parasites and pathogens exert important influences on the distribution and abundance of host species. Brazil is a tropical country with continental extent which harbors one of the most diverse avifaunas in world. Moreover, Brazil has a remarkable diversity of ecosystems (six different biomes with some subdivisions) with peculiar aspects that influence the species composition of local communities as well as their population dynamics. About 10% of Brazilian bird species are considered threatened and some evidences have shown infectious diseases, particularly caused by haemosporidian parasites, can lead to changes in the local composition of bird species due to extinctions or changes in their distributions. Owing to the availability of DNA amplification and sequencing, information is accumulating on the geographic and host distribution of avian haemosporidian (“malaria”) parasites, which are becoming a model system for studying host-parasite relationships in natural systems. Our results from different Brazilian biomes have been evidencing that sites with a greater diversity of bird species showed a greater diversity of parasite lineages, providing evidence that areas with high bird richness also have high parasite richness. Our findings point to the importance of the neotropical region as a major reservoir of new haemosporidian lineages. However, a question remains: How these neotropical host and parasite communities are linked and how these host-parasite interactions influence the ecological and evolutionary dynamics of communities? In order to address these important questions we have started the use of two distinct ecological approaches, specificity index tools and network analysis which are providing useful information to the field.

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RT.03 - BIOLOGY AND PATHOGENICITY OF AMOEBAS

RT.03.001 - **PATHOGENIC FREE-LIVING AMOEBAE INTERACTIONS WITH HOST'S EXTRACELLULAR MATRIX: 2D, 3D, AND BEYOND.**

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Free-living amoebae are prevalent in the environment and can be isolated from fresh water bodies to soil samples. Amoebae also have been isolated from air conditioning vents, domestic water supplies, improperly chlorinated swimming pools, cooling towers from nuclear power plants, gardens, contact lens cases, and other medical paraphernalia. Although amoebae such as *Naegleria fowleri, Balamuthia mandrillaris,* and several species of *Acanthamoeba* are free-living, they can be pathogenic and cause fatal infections in humans and animals. *N. fowleri* causes Primary Amoebic Meningoencephalitis (PAM), a rapidly fatal disease of the central nervous system in humans in which the amoebae enter the nasal passages and migrate to the brain via the olfactory nerves in days. *Balamuthia mandrillaris*, a soil amoeba, is the causative agent of severe skin infections that can disseminate to the brain possibly by hematogenous route, leading to a fatal encephalitis called *Balamuthia amoebic encephalitis (BAE).* Amoebae of the genus *Acanthamoeba* can cause Granulomatous Amoebic Encephalitis (GAE), a severe disseminated infection of the brain, as well as infections of the skin, lungs and kidneys in immuno-compromised patients. In immuno-competent individuals, *Acanthamoeba* can cause amebic keratitis (AK), a sight-threatening corneal infection observed mostly in contact lens wearers. During AK, *Acanthamoeba* destroy the epithelial layers of the cornea, invading the stroma, causing severe inflammation and tissue destruction. A common trait in all three amoebic infections is their invasiveness behavior. From the host epithelium, amoebae can migrate to deeper tissues leading to the contact with components of the host extracellular matrix (ECM), an interaction that can facilitate and trigger disseminated infections. Amoeba-ECM interaction can be studied in a variety of ways: a) in two-dimensions (2D) in which ECM components are used to coat surfaces as an attachment substrate, and in (b) three-dimensions (3D) in which ECM scaffolds that are used to analyze attachment and invasion that resembles the ECM structure in vivo. Recent studies in our laboratory have shown that amoebae are able to specifically recognize ECM components, firmly attaching to ECM-coated surfaces containing laminin, fibronectin or collagen. Moreover, amoebae express ECM binding proteins similar to the integrin family of mammalian ECM receptors and are able to invade ECM scaffolds mimicking the basement membrane and fibrous connective tissue. Invasion appears to be a trait specific for pathogenic amoebae when compared to non-pathogenic amoebae. During invasion, two events are critical: migration and matrix remodeling. Host tissue is composed of a 3D scaffold of ECM components plus the resident cells in that specific tissue. Thus, for a better understanding of pathogen-host interactions, a new in vitro model of pathogen/host interactions that includes host cells and their respective ECM components is under development. In vitro models that contain fibroblasts and collagen I mimicking connective tissue, or models containing nasal cells and ECM components, can be an important platform to study virulence factors in amoebae including their ability of amoebae to bind to and degrade ECM components and invade tissues. Defining the molecular mechanisms by which *Acanthamoeba spp., Balamuthia mandrillaris*, and *Naegleria fowleri* invasiveness may yield novel insights into cellular targets that may be amenable to therapeutic manipulations. **Supported by:**National Institutes of Health (NIH)

RT.03.002 - **CHEW YOUR FOOD: TROGOCYTOSIS-LIKE INGESTION CONTRIBUTES TO HUMAN CELL KILLING AND TISSUE INVASION BY *ENTAMOEBA HISTOLYTICA***

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Entamoeba histolytica is the causative agent of amoebiasis, a diarrheal disease that is a major source of morbidity and mortality in the developing world. Pathogenesis is associated with profound tissue destruction, manifesting as intestinal ulceration or extraintestinal abscesses.

Parasite cytotoxic activity is central to tissue destruction, but the mechanism for killing of host cells was unknown. Recently, by employing live confocal fluorescence microscopy, we discovered that amoebae kill by biting off and ingesting distinct pieces of living human cells. The process is reminiscent of trogocytosis (Greek trogo–, nibble) between immune cells. Amoebic trogocytosis initiates within one minute of host cell contact and precedes cell death, as assessed by permeabilization and DNA fragmentation. By using imaging flow cytometry to simultaneously quantify ingestion and killing, we find that pharmacological inhibitors of trogocytosis reduce host cell death in a dose-dependent manner. Trogocytosis is relevant to disease pathogenesis, since we demonstrated using live two-photon microscopy that trogocytosis occurs during invasion of colon explants from fluorescent-membrane mice. We are currently employing dominant negative mutants and recently developed gene knockdown approaches in *E. histolytica*, in order to define the pathways regulating trogocytosis. Interestingly, a C2 domain-containing protein kinase, EhC2PK, is required for both trogocytosis and conventional phagocytosis in *E. histolytica*, suggesting that some aspects of conventional phagocytic machinery may be common to *trogo*cytosis. We are using these and other trogocytosis mutants as valuable tools to further dissect tissue invasion and destruction in animal models of infection. These studies change the existing paradigms for cell killing and tissue destruction in amoebiasis and also suggest an ancient origin of trogocytosis as a form of intercellular exchange.

RT.03.003 - ***ACANTHAMOEBA CASTELLANII*** **AS AN UNIVERSAL ENVIRONMENTAL HOST FOR PATHOGENIC MICROORGANISMS: KEY FOR VIRULENCE FACTOR ORIGIN AND INTRACELLULAR PATHOGENESIS**

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According to WHO, about 3.4 millions of people die yearly due to polluted water consumption, lack of basic sanitation and hygiene. Free-living amoebaes (FLA) have enormously contributed to the microbiological contamination of water. FLA have displayed resistance to environmental adversities and germicides, therefore working as excellent reservoirs for microorganisms such as bacteria (Legionella pneumophila, Mycobacterium sp., Escherichia coli, etc) and virus (Adenovirus, Mimivirus, etc). Specifically, the free-living amoeba Acanthamoeba castellanii has been described as an environmental host for a wide variety of fungal pathogens capable of causing endemic mycosis such as Cryptococcus spp., Histoplasma capsulatum, Sporothrix schenkii e Blastomyces dermatitides. Studies have demonstrated that the interaction of pathogens with A. castellanii results in environmental selective pressure responsible for conservation of virulence factors and increase in pathogenicity to vertebrate hosts. A remarkable aspect of the interaction of Cryptococcus neoformans with mammalian hosts is a consistent increase in capsule volume. Given that many aspects of the interaction of C. neoformans with macrophages are also observed with amoebae, we hypothesized that the capsule enlargement phenomenon also had a protozoan parallel. Incubation of C. neoformans with Acanthamoeba castellanii resulted in C. neoformans capsular enlargement. Analysis of amoebae extracts showed that the likely stimuli for capsule enlargement were protozoan polar lipids. Purified phospholipids, in particular, phosphatidylcholine, and derived molecules triggered capsular enlargement with the subsequent formation of giant cells. These results implicate phospholipids as a trigger for both C. neoformans capsule enlargement in vivo and exopolysaccharide production. Protozoan- or mammalian-derived polar lipids could represent a danger signal for C. neoformans that triggers capsular enlargement as a non-specific defense mechanism against potential predatory cells. As this phenomenon usually requires contact of the protozoan with the fungus, and such fact occurs with a wide variety of pathogens, FLA therefore need to express a repertoire of related receptors or a class of universal receptors. Our aim is to characterize the molecular bases of interaction of FLA and human pathogens, which can emerge as causative agents of epidemics.

RT.04 - HOST-PARASITE INTERACTIONS

RT.04.001 - **DETERMINANTS OF PROTOZOAN VIRULENCE: FROM PARASITES AND GENOMES TO HOST CELLS AND SPECIES PREFERENCES**

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Whether seeking novel therapies, prophylaxis, specific diagnostics for pathogenic lineages or simply an understanding of disease pathology; the key steps remain the definition and characterization of the group of molecules which dictate the ability of a parasite to infect and cause disease. In our studies of kinetoplastid and amitochondrial protozoan parasites we exploit common characteristics for the identification and utilization of their virulence factors: ie that (i) they are normally externally exposed, either on the surface of the parasite or as secreted proteins; (ii) as a result of "Red Queen Evolution" they are often hypervariable between isolates and are encoded by genes with high dN:dS ratios (iii) they are often encoded telomerically or subtelomerically; (iv) the encoding genes may be multicopy or belong to gene families; and (v) as proteins they are frequently glycosylated and/or lipoylated. The variability of the genes encoding such proteins means that they are often lineage specific, this can be a useful property but as a result such genes tend to be listed, somewhat disappointingly, in genome annotations as encoding “hypothetical non-conserved” proteins. In this round table on parasite host interactions I aim to draw vignettes from genomics led discovery studies in trypanosomes, giardia, trichomonas and cryptosporidium to elicit discussion of routes towards real world outcomes, emphasizing the utility of these factors in potential disease interventions.
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RT.04.002 - **HEME UPTAKE MEDIATED BY LHR1 IS ESSENTIAL FOR *LEISHMANIA AMAZONENSIS* INFECTIVITY**

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Leishmania parasites and other trypanosomatids are heme auxotrophs, so must acquire this essential cofactor from the environment. Heme is not only a crucial component of cytochromes in the respiratory chain, but also functions as an essential co-factor for hemoproteins involved in the biosynthesis of polyunsaturated fatty acids and sterols [1]. Recently, our group has demonstrated that *Leishmania amazonensis* incorporates heme through the transmembrane protein LHR1 (*Leishmania* Heme Response-1). Heme deprivation in *L. amazonensis* increased *LHR1* transcript levels, promoted uptake of the fluorescent heme analog ZnMP, and increased the total intracellular heme content of promastigotes. *LHR1* double knockout mutants in *L. amazonensis* were not viable, suggesting that this is an essential gene [2]; however, it was possible to obtain a *LHR1*-single knockout strain, which was characterized regarding its biological properties, such as replication, differentiation, and in vitro and in vivo infectivity. Our results show that parasites lacking one copy of *LHR1* replicated poorly in heme-deficient media and had lower intracellular heme content when compared to wild type parasites. These promastigotes were also less effective in reducing ferric to ferrous iron, a reaction mediated by the heme-containing parasite enzyme LFR1 (*Leishmania* Ferric Reductase 1 [3]). Parasite virulence was also evaluated for this mutant strain and we observed that *LHR1*-single knockout parasites are incapable of replicating as amastigotes in macrophages, an effect that is fully rescued when the endosomal pathway of macrophages is loaded with red blood cells, a rich source of heme. As expected, this intracellular growth defect led to a strong inhibition in cutaneous lesion development in mice. Our data demonstrate for the first time that heme acquisition plays a critical role in the establishment of *L. amazonensis* infections in the mammalian host. These findings have important implications both for future drug development, and also for a better understanding of the role of erythrophagocytosis in the pathogenesis of leishmaniasis.

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RT.04.003 - **ABANDON THE SHIP! *LEISHMANIA AMAZONENSIS* AMASTIGOTES ARE RESCUED FROM APOPTOTIC MACROPHAGES IN ASSOCIATION WITH HOST LYSOSOMAL-ASSOCIATED MEMBRANE PROTEIN (LAMP)**

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The last step of *Leishmania* intracellular life cycle is the egress of amastigotes from the host cell and their uptake by adjacent cells. Using multidimensional live imaging of macrophage cultures infected for 15 days with *Leishmania amazonensis*, we observed that amastigotes were transferred from cell to cell when the donor host macrophage collapses; transfer between live cells was not detected. Amastigotes were extruded from the macrophage within zeiotic structures (blebs) rich in late endosome/lysosome components such as LAMP1 and Rab7. These structures were classified as parasitophorous extrusomes. The extrusomes were selectively internalized by vicinal macrophages and the rescued amastigotes carrying host lysosomal components attached to their surfaces (but dissociated from host cytoskeleton components such as actin) remain viable in recipient macrophages. Host cell apoptosis induced by microirradiation of infected macrophage nuclei promoted amastigote egress and rescue by non-irradiated vicinal macrophages, suggesting that amastigotes benefit from host cell apoptosis to spread among other cells. Transfer was also stimulated when macrophages were treated with streptolysin O, a pore-forming protein innocuous to amastigotes. Using amastigotes isolated from LAMP1/LAMP2 knockout fibroblasts, we observed that the presence of these lysosomal components on egressing amastigotes increases their uptake and modulates TGF-β cytokine production in macrophage cultures. Enclosed within host cell membranes, amastigotes can be transferred from cell to cell without full exposure to the extracellular milieu, what represents an important strategy developed by the parasite to evade host immune system.
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RT.05 - LEISHMANIASIS: ADVANCES IN CHEMOTHERAPY, VACCINES AND FLIPPASES

RT.05.001 - **PROSPECTION OF NATURAL PRODUCTS WITH LEISHMANICIDAL ACTIVITY**

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Pentavalent antimonials, amphotericin B (AMB) and miltefosina are the main drugs currently available for leishmaniasis treatment. All of them present problems that limit their use, such as, adverse side-effects, induction of parasite resistance and high costs. Natural products are an important source of bioactive substances with anti-trypanosomatids activity, in addition to its potential to provide sources of novel active molecules or structural prototypes for drug development. Natural products may also be associated with drugs currently used in the therapy of parasitic diseases in order to reduce adverse effects and improve effectiveness. Resveratrol is a polyphenol, naturally occurring in red wine, black grapes, berries, and peanuts, with biological activities such as, anti-inflammatory, anti-cancer and anti-oxidant. Thus we are interested in evaluate the leishmanicidal effect of Resveratrol, alone and in association with Amphotericin B. Our assays were done using *Leishmania amazonensis* promastigotes and intracellular amastigotes. We demonstrate that Resveratrol presents leishmanicidal activity for promastigotes, IC_{50} of 27 µM, and for intracellular amastigotes, IC_{50} of 42 µM. Evaluating the association of Resveratrol with Amphotericin B, we showed a synergic effect to promastigotes as well as, amastigotes. We also demonstrated that Resveratrol induce an increase in the percentage of promastigotes in the G0/subG1 phase of the cell cycle, beyond increasing the number of annexin V positive promastigotes. Parasite apoptosis induced by Resveratrol was confirmed by NMR spectroscopy analysis by elevated peaks in the region of choline and CH_2/CH_3 ratio. Alteration in the mitochondrial potential of Resveratrol treated-promastigotes was observed with the XTT and JC-1 assays. Resveratrol decreased arginase activity, either in infected or uninfected murine macrophages, an important mechanism that ensures parasite survival. Polyamines starvation induced by arginase inhibition activity could be the reason to apoptosis death Resveratrol-treated parasites. Investigating leishmanicidal activity of Resveratrol analogs, we analyzed Pterostilbene (PT), Piceatannol (PI), Polydatin (PO) and Oxyresveratrol (OX). Our data show that PT, PI, PO and OX shown an anti-*Leishmania amazonensis* activity with an IC_{50} of 18µM, 65µM, 95µM and 65µM respectively, for promastigotes, while for intracellular amastigotes the IC_{50} were 33.2µM, 45µM, 29µM and 30.5µM, respectively. Among all tested analogs only PI was able to induce apoptosis similarly to Resveratrol. Thus, PI alters the parasite cell cycle, increasing 5 times the G0/subG1 phase and decreasing 1.7 times the G2 phase. PI changed the mitochondrial membrane potential of promastigotes, and also increased the number of annexin V positive promastigotes, which suggests death by apoptosis. Our results demonstrate Resveratrol activity against *Leishmania amazonensis* and its synergistic association with Amphotericin B, pointing it as a possible drug for further studies of association therapy *in vivo*. **Supported by:** CAPES, FAPERJ, CNPq

RT.05.002 - **THE USE OF ADJUVANTS AND VITAMINS TO VACCINE DEVELOPMENT AGAINST *LEISHMANIA AMAZONENSIS***

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We have studied the association of LaAg vaccine (the whole *Leishmania amazonensis* antigens) with adjuvants (innate immune potentiators) by intramuscular route and with vitamin D by intranasal and oral routes against *Leishmania amazonesis* infection. Mice were immunized twice and seven days after second dose mice were infected with promastigotes of *Leishmania amazonensis* ($5x10_5$ to $2x10^6$). Clinical parameters were evaluted (lesion development, parasite load, citokines and celularity). INTRAMUSCULAR ROUTE: It was demonstrated that intramuscular immunization with LaAg enhanced susceptibility to infection in BALB/c and there is no effect in C57BL6 (Pinheiro, 2005). We evaluted the association of LaAg (100µg) with Saponin (100µg /SIGMA), MPLA(5µg/InvivoGen), LTA (20µg/InvivoGen), AddaVax™ (50%/InvivoGen) and combinations. The association of LaAg plus saponin induced protection on Balb/c mice, but not in C57BL6. The protective effect is dependent of Eosinophils and a

mixed Th1, Th2 and Th17 responses. We evaluated the association with other adjuvants in C57BL6. Only the association of LaAg with MPLA/AddaVax™ induced a small improvement of vaccine efficacy. MUCOSAL ROUTES: LaAg is a protective vaccine by oral and nasal routes (Pinto et al 2003;2004). Vitamin D is a potent inducer of cell migration to skin. Our hypothesis was to increase cell migration to the skin of the cells generated from vaccination with LaAg in the mucosa through the association with vitamin D. Association of LaAg (10µ-100µg) plus vitamin D2 (40µ-200µg/SIGMA) and vitamin D3 (40µ-200µg/SIGMA) improved both oral and nasal vaccinations in C57BL6, with better results observed for Vitamin D3. Protection was observed by increase of Th1 response in infected footpad of vaccinated mice. In conclusion, the studies with LaAg, adjuvants and vitamin D indicate the advantage in mucosal routes with possibility to improve protective efficacy by association with Vitamin D3.
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RT.05.003 - **TRACKING DOWN LIPID FLIPPASES IN THE PROTOZOAN PARASITE LEISHMANIA**

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The protozoan parasite *Leishmania* causes serious infections in humans all over the world. After being inoculated into the skin through the bite of an infected sandfly, *Leishmania* promastigotes must gain entry into macrophages to initiate a successful infection. Specific, surface exposed phospholipids have been implicated in *Leishmania*–macrophage interaction but little is known about the mechanisms controlling and regulating the plasma membrane lipid distribution.
Growing evidence indicates that the transbilayer distribution of lipids is largely determined by a diverse group of lipid translocators that use the energy of ATP hydrolysis to move specific lipids across the bilayer. Among these ATP-driven translocators, P4-ATPases comprise lipid flippases that catalyze the translocation of phospholipids from the exoplasmic to the cytosolic leaflet of cell membranes. A second class of ATP-dependent translocators includes members of the ATP binding cassette (ABC) transporters family which catalyze the outward movement of lipids from the cytoplasmic to the extracellular/luminal leaflet. Members of both transporter families have also been implicated in the development of drug resistance in *Leishmania*, which includes resistance to alkyllysophospholipids. This suggests that the mechanism by which the drugs are extruded from cells is closely related to the flippase mechanism by which lipids are translocated across membranes, and that these processes involve structurally similar, if not identical transporters. We have found that the activity and substrate specificity of the inward translocation machinery varied between *Leishmania* species. The differences in activity of inward phospholipid transport correlated with the different sensitivities of the various species towards the alkyl phospholipid analogue miltefosine. In addition to the ATP-dependent translocators, we have provided evidence that *Leishmania* parasites contain a Ca2+- activated phospholipid scramblase, a putative membrane protein that upon activation facilitates a rapid bidirectional movement of phospholipids across the two plasma membrane leaflets, disrupting the lipid asymmetry created by the ATP-dependent translocators. The implications of these findings will be discussed.

RT.06 - ROUNDTABLE - BIOLOGY OF APICOMPLEXAN PARASITES AND IMMUNE RESPONSES OF INFECTED HOSTS

RT.06.001 - **CD14+CD16+ MONOCYTES PLAY DISTINCT ROLE IN *PLASMODIUM VIVAX* MALARIA**

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The regulation and function of the immune response triggered during malaria is complex, poorly understood and undermined by a paucity of studies conducted in humans infected with *Plasmodium vivax*. During the syndrome, the *Plasmodium* triggers high levels of cytokines, which both help the immune response controlling the parasite and contribute to the symptoms observed during the diseases. Our data show that monocytes are the main source of cytokines upon *P. vivax* infection, suggesting their important role during disease. While it is now recognized that circulating monocytes are heterogeneous, the physiological relevance of this is not yet completely understood. The different monocyte subsets seem to reflect developmental stages with distinct physiological roles, such as recruitment to inflammatory lesions or entry to normal tissues. Our overall goal is to define the role of the circulating monocyte during malaria caused by *P. vivax*. Our data show that patients infected with *P. vivax* display higher levels of cytokines that are accompanied by increased frequencies of circulating monocytes. The infection triggers the expression of activation markers, adhesion molecules and chemokine receptors on circulating monocytes. Moreover, these cells can be distinguished into three monocyte subsets based on the expression of CD14 and CD16. Each of these subsets display an distinct profile, suggesting that they distinctly act during *P. vivax* infection to induce inflammation, control the infection and modulate immune response. Importantly, CD14+CD16+ monocytes displayed higher phagocytic activity and killing potential than their other counterparts. Identification of mechanisms that regulate monocyte responses during malaria will provide important information on the development of therapeutic strategies that are targeted to modify their particular cell subsets.
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RT.06.002 - **IMPORTANCE OF NEOSPORA CANINUM INFECTIONS AND THEIR PATHOLOGY.**

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Soon after it was named *Neospora caninum* in 1988 by Dr. J. P. Dubey working at the USDA in Beltsville, Maryland, USA this parasite exploded on to the Veterinary Medical scene worldwide. It was first recognized as the cause of a devastating neuromuscular disease in young dogs. Researcher soon learned that an infected female dog could repeatedly infect her puppies. The first evidence that N. caninum caused abortion in cattle was demonstrated the next year in 1989. *Neospora caninum* is now recognized as a major cause of abortion in dairy cattle worldwide. The parasite is easily maintained in cattle by maternal transmission. Repeat abortion is common. *Neospora caninum* has many similarities to the related parasite *Toxoplasma gondii*. The dog was identified as a true definitive host in 1998 by a group of researchers led by Dr. Milton M. McAllister working at the University of Wyoming, USA. Researchers are presently looking in to the sylvatic cycle of N. caninum focusing on transmission between wild ruminants and canines. The importance of the N. caninum oocyst in the urban and sylvatic cycles is also an active area of investigation.

RT.06.003 - **GENE EXPRESSION STUDIES PROVIDE EVIDENCE FOR JUST-IN-TIME TRANSCRIPT PRODUCTION IN *EIMERIA* PARASITES**

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Seven species of the genus *Eimeria* can infect chickens and produce coccidiosis, an enteric disease that leads to important economic losses in poultry production. Draft sequences of *E. tenella* and *E. maxima* are already available, and genome sequencing of the remaining species are currently underway. Our group has carried out a comparative EST sequencing study of the three most economically relevant *Eimeria* species: *E. tenella*, *E. maxima* and *E. acervulina* (Rangel *et al*., *Int. J. Parasitol.* 42: 39-48, 2012). Combined sets of ORESTES/EST reads were used, covering samples from several developmental stages. Annotation data and all supporting evidences are publicly available at http://www.coccidia.icb.usp.br/eimeria_tdb (Rangel *et al*., Database, 2013:bat006). Digital expression profiles obtained in this work revealed highly conserved patterns across different species and correlated to distinct developmental stages. To better assess these expression patterns, we decided to extend our study by performing differential gene expression analyses including digital profiles using ORESTES/EST read counting (digital Northern), LongSAGE, and qRT-PCR of selected genes. Statistical tests were used to accurately identify differentially expressed genes. Read abundance analysis in digital Northerns revealed that most contigs of the reconstructed cDNA are composed of a very small number of reads, whereas only a few contigs are populated by large amounts of reads. This kind of distribution was observed in all individual stages of the three analyzed *Eimeria* species. Also, hierarchical clustering analysis clearly showed a specific high-level expression of small sets of genes in each developmental stage. As a second approach, we performed LongSAGE analysis on sporozoite and second-generation merozoite stages of *E. tenella*, obtaining a total of 9,516 unique tags. This number is relatively close to the estimated complexity of circa 8,000 genes found in coccidian transcriptomes. An analysis of the tag profiles revealed that circa 66.5% of the SAGE tags present single counts, whereas more than 88% of the unique tags show counts below to five. We believe that this highly asymmetric distribution is most probably not related to experimental artifacts, since we used a very conservative sequence quality filtering. This result also suggests that the eimerian transcriptome is very narrowly expressed in any stage, with some few genes presenting a very high expression, while most of the genes are expressed in low amounts. A comparison of the digital Northern and LongSAGE results in *E. tenella* showed good agreement, and many differentially expressed genes have been mutually identified in both techniques. Finally, we also carried out a preliminary experimental validation by quantitative RT-PCR using twelve genes selected from sets of differentially and non-differentially expressed genes. In all cases, the expression status observed on LongSAGE and/or digital Northern has been confirmed by real-time PCR. In four tested genes, the expression ratio observed between the tested developmental stages has been experimentally confirmed with very high correlation. Altogether, our body of evidence indicates that a small and specific set of genes is highly expressed in any developmental stage, with expression levels starting to increase in the immediately preceding stage and then gradually decreasing in the subsequent stage. We now intend to extend the validation of differential expression to identify genes involved in some particular steps of the parasite life cycle. For instance, the mechanisms and specific genes involved in the sporulation process are still poorly understood. Thus, by using our expression data and selecting the most promising candidates for experimental validation, we expect to better define proteins associated with this essential step of the life cycle. This knowledge may help us to devise new strategies to control this important disease.

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