RT.01-A – Immunoregulation in cutaneous leishmaniasis: A role for Notch signaling

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Leishmania braziliensis infection results in a variety of immunological and clinical spectrum of disease, including the cutaneous (CL) and mucosal (ML) inflammatory forms of leishmaniasis. Upon stimulation with soluble *Leishmania* antigen, CD4+ T cells from CL and ML patients secrete high levels of IFN-gamma and TNF-alpha, cytokines known to participate in the pathogenesis of CL and ML, and low levels of IL-5 and IL-10. The use of recombinant IL-10 and antibodies against IL-12, failed to downmodulate *Leishmania* antigen-induced inflammatory responses by mononuclear cells from CL and ML individuals, suggesting other mechanism as responsible for the sustained IFN-gamma and TNF-alpha production in these patients. Recently, attention has been given to a role for Notch signaling in Th1 differentiation, T cell proliferation and mononuclear phagocyte activation. Notch signaling takes place upon the cleavage of Notch receptor after binding of the ligands Delta or Jagged. Here, we found that *Leishmania* antigen-induced IFN-gamma and TNF-alpha production is blunted by a gamma secretase inhibitor that prevents Notch signaling by interfering with the cleavage of Notch at the cell membrane. Moreover, we found that *Leishmania*-infected human monocytes become activated in a gamma secretase activity-dependent manner. These discoveries show a role for Notch signaling in controlling inflammatory responses in human leishmaniasis, opening perspectives for investigations of new forms of treatment for inflammatory diseases. Supported by NIH, USA.

RT.01-B - ROLE OF NEUTROPHILS IN RESISTANCE AGAINST EXPERIMENTAL *L. BRAZILIENSIS* INFECTION

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Experimenal cutaneous leishmaniasis caused by *L. braziliensis* is characterized by a necrotic lesion in the skin that heals spontaneously after ten weeks. In parallel to lesion development and cicatrization, an intense influx of polimorfonuclears neutrophils (PMNs) is observed at the infection site. It has been shown that neutrophil depletion leads to enhanced *Leishmania* infection and that that neutrophils cooperate with macrophages in eliminating *Leishmania*. Moreover, recent studies have also demonstrated the involvement of PMNs in the induction of the adaptive immune response. Data will be shown on the role of neutrophils in promoting resistance against *L. braziliensis* infection, considering the different scenarios described above.

RT.01-C- THE IMPORTANCE OF microRNAs IN MACROPHAGE POLARIZATION AND INTRACELLULAR SURVIVAL OF LEISHMANIA

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The *Leishmania* spp. protozoa reside intracellularly in their mammalian hosts, and are most often observed in host macrophages. Intracellular Leishmania cause dramatic changes in macrophage gene expression, suppressing microbicidal activities and IFN-gamma responses. Indeed, microarrays from several labs including our own show that infection with *Leishmania* promastigotes induces the expression of a set of genes characteristic of non-classical macrophage activation, similar but not identical to alternative (M2a) activation. This pattern is distinct from the set of classical activation (M1) genes induced by bacterial infection or LPS. MicroRNAs are 18-30 nt noncoding RNAs that globally regulate gene expression. We hypothesized that microRNAs could provide molecular triggers that underlie or modify the global changes in macrophage gene expression induced by leishmania infection or other polarizing stimuli. Therefore, we studied microRNA expression in human monocyte-derived macrophages (MDMs) treated with polarizing agents. Treatments were designed to induce MDMs to differentiate toward each of four distinct phenotypes: M1 (20 ng IFN-gamma/mL;10 ng LPS/mL), M2a (20 ng IL-4/mL), M2b (IgG complexes; 100 ng LPS/mL), and M2c (0.5 ng TGF-b1/mL). Polarization was verified by increased expression of the predicted chemokines or cytokines. Using multiplex TaqMan qPCR arrays (ABI), we identified microRNAs whose expression in MDMs was altered after incubation in polarizing conditions. Seven microRNAs were significantly altered in the different polarized states compared to control untreated MDMs. One of these microRNAs (miR-155) has already been reported to respond to LPS in the literature. The functions of significantly altered microRNAs were tested using either HEK-293 or the THP-1 monocytic cell line transfected with pre-miRs. First, studies of HEK-293 cells showed that several minor microRNA strands complementary to the strand loaded in the RISC complex were transiently altered by polarization. However, only the corresponding major microRNA strand,i.e. the strand loaded into RISC, was functional in suppressing expression of reporter luciferase activity. Second, studies of transfected THP-1 cells showed that IL-6, TNF-alpha, CXCL9, and IL-10 expression was modified both in unstimulated cells and in response to cytokines and/or LPS by several of the introduced pre-microRNAs. We conclude that microRNAs influence the type of macrophage polarization that occurs in response to external stimuli. Delineation of these microRNAs, and their effects on the intracellular survival of *Leishmania,* may provide novel therapeutic targets for *Leishmania* and other intracellular pathogens.

RT.02-A – CHARACTERIZATION OF ARTEMISININ RESISTANT *Plasmodium falciparum*

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The emergence of artemisinin resistant falciparum malaria represents a major threat to malaria elimination and is among the most important issues that could undermine global malaria control efforts. Recent studies suggest that prolonged parasite clearance times in patients treated with an artemisinin drug is a harbinger of reduced cure rates in the near future. The problems associated with understanding artemisinin resistance range from a lack of understanding of the mechanism of resistance, the absence of validated molecular markers, and poor correlation of the resistance phenotype in current in vitro antimalarial drug susceptibility tests. In response to these pressing needs we have characterized the response of *P. falciparum* to various artemisinin derivatives in vitro and have identified artemisinin-induced dormancy in ring stages as a probable mechanism of recrudescence. Furthermore, we have developed several independent lines of *P. falciparum* that tolerate > 3 μM artemisinin. Characterization of these resistant lines has identified amplification of pfmdr1 as an accessory to developing resistance. Putative molecular markers of resistance have emerged from analysis of the unique transcriptional profile of resistant parasites as well as full length sequencing the genomes of parent and resistant lines. These data provide insight into the possible mechanisms of resistance and can be rapidly translated into studies with clinically resistant isolates in the field.

RT.02-B – STRUCTURE- AND LIGAND-BASED DRUG DESIGN APPROACHES FOR NEGLECTED TROPICAL DISEASES

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The modern drug discovery process is increasingly becoming more information driven. Recent years have seen a tremendous increase in new technologies and methods for the design of new chemical entities (NCEs). A broad variety of medicinal chemistry approaches can be used for the identification of promising hits, generation of leads, as well as to accelerate the development of high quality drug candidates. Structure- (SBDD) and ligand-based drug design (LBDD) methods are becoming increasingly powerful, versatile and more widely employed. The use of these drug design strategies has increased enormously because of the availability of databases with millions of commercially available compounds, as well as 3D structures of several target proteins. This presentation will provide a perspective of the utility of SBDD and LBDD approaches, and the integration of experimental and computational methods in medicinal chemistry, highlighting recent examples of successful applications in the area of neglected tropical diseases (NTDs).

RT.02-C – PHENOTYPIC HIGH-THROUGHPUT SCREENING FOR NEGLECTED DISEASES

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The Center for Neglected Diseases Drug Discovery (CND3) in Institut Pasteur Korea (IPK) develops high-content/high-throughput screening assays (HCS/HTS) for finding new drugs for the treatment of Leishmaniasis, Chagas disease and malaria. Taking advantage of automated confocal microscopy, we developed a whole cell-based approach for screening 200,000 compounds for their ability to inhibit *Leishmania donovani* amastigotes growth in the human macrophage cell line THP-1. Among the 200,000 compounds, the 179 most active compounds, able to inhibit parasite growth without harming macrophages host cells were selected for activity confirmation by dose response curves. We have also developed a whole-cell model assay for discovery of compounds active against *Trypanosoma cruzi*, the causative agent of Chagas disease. Two different *T. cruzi* strains, Y and Dm28c, representing two of *T. cruzi* six groups, were used in the screening of 4,000 compounds of know drug properties. Our data shows, for the first time to our knowledge, that Y and Dm28c strains differ both on their infectivity profile and also differ partly but significantly on their sensitivity to , 4,000 compounds. Currently both leishmaniasis and Chagas assays are being used for screening an additional library of 150,000 compounds in collaboration with Drugs for Neglected Diseases *initiative* (DNDi) and Pfizer, thus further increasing the number of potential drug candidates in the pipeline of Chagas and leishmaniasis drug discovery. In parallel to amastigote-host cell screening system, we developed a secondary high-content assay for kinetoplast-directed drug discovery for *Leishmania* and *T. cruzi* parasites. The kinetoplast is a single mitochondrion, exclusive to order Kinetoplastida (*Leishmania* and *Trypanosoma cruzi*, among other parasites) and contains a number of excellent chemotherapeutic targets that are very unlikely to be found in the human host. Among the kinetoplast targets, the kinetoplast DNA (kDNA) replication proteins are one of the best-known parasite specific machinery. The hits found in the amastigoste-host cell assays for *T. cruzi* and *Leishmania* will be further tested in the kinetoplast assay for assessment of their potential kinetoplast-targeting mechanism of action. This screening consists on incubating *Leishmania* promastigote forms with selected anti-leishmanial compounds in the presence of EdU, a thimidine analog that is incorporated into the replicating kDNA. Parasites are then fixed and stained for EdU-containing DNA. Desired hits will be able to inhibit kDNA, but not nuclear DNA, replication. Our approach to malaria consists on developing HCS/HTS that target the invasion of human red blood cells (RBCs) by *Plasmodium falciparum*, the deadliest of all malaria parasites. We have validated, by means of 4,000-compound screening, a quantitative enzymatic approach to assay for *P. falciparum* viability. This assay was used to screen 80,000 compounds, and the hits emerging from this assay will be tested on a secondary image based high-content assay for their ability to interfere with the red blood cell invasion pathway.

RT.03-A – CHARACTERIZATION OF THE *ANOPHELES AQUASALIS* IMMUNE RESPONSE TO *Plasmodium vivax*

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Malaria affects millions of people worldwide annually, and 450.000 only in Brazil. The interaction of malaria vectors and parasites has been extensively studied, but very little is known about the pair *Anopheles aquasalis-Plasmodium vivax*, of great importance in the malaria scenario in Brazil. One of the reasons for this lack of information relies on the virtual impossibility of *P. vivax* cultivation, and the recently challenged belief that disease caused by this parasite is not serious. We are characterizing the immune response of *A. aquasalis* to *P. vivax*. We constructed subtraction libraries, comparing infected and non-infected insects. Surprisingly, few immunity genes were identified 2 and 24 hours after infection (hAI). Among these were a serine proteinase with diminished expression, and a carboxipeptidase with increased expression. We also identified a GATA transcription factor, more expressed in males than females and induced (almost 15 times) 36 hAI. Infection increased 63% after GATA knock-down, confirming its importance in the immune response of *A. aquasalis* against *P. vivax.* In parallel, specific genes were amplified using degenerate primers and characterized. The immune response genes STAT, PIAS and NOS were induced by infection, demonstrating the importance of the JAK/STAT pathway in response against the parasite. Silencing of STAT caused an increase in oocysts count. In relation to the detoxification enzymes, we observed an increased expression of SOD and catalase 36 hAI and a decreased activity at 24 hAI. Fluorescence microscopy using a redox state probe showed a reduction of free radicals in both blood fed and infected insects when compared to sugar fed insects.

RT.03-B – PROTEOPHOSPHOGLYCAN CONFERS RESISTANCE OF LEISHMANIA MAJOR TO MIDGUT DIGESTIVE ENZYMES INDUCED BY BLOOD FEEDING IN VECTOR SAND FLIES

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Leishmania synthesize abundant phosphoglycancontaining molecules made up of [Gal-Man-PO4] repeating units, including the surface lipophosphoglycan (LPG), and the surface and secreted proteophosphoglycan (PPG). The vector competence of *Phlebotomus duboscqi* and *Lutzomyia longipalpis* sand flies was tested using *L. major* knockout mutants deficient in either total phosphoglycans (lpg2- or lpg5A-/5B-) or LPG alone (lpg1-) along with their respective gene add-back controls. Our results confirm that LPG, the major cell surface molecule of Leishmania promastigotes known to mediate attachment to the vector midgut, is necessary to prevent the loss of infection during excretion of the blood meal remnants from a natural vector, *P. duboscqi* , but not an unnatural vector, *L. longipalpis*. Midgut digestive enzymes induced by blood feeding pose another potential barrier to parasite survival. Our results show that 36–72 h after the infective feed, all parasites developed well except the lpg2- and lpg5A-/5B- mutants, which showed significantly reduced survival and growth. Protease inhibitors promoted the early survival and growth of lpg2- in the blood meal. PPG was shown to be the key molecule conferring resistance to midgut digestive enzymes, as it prevented killing of lpg2- promastigotes exposed to midgut lysates prepared from blood-fed flies. The protection was not associated with inhibition of enzyme activities, but with cell surface acquisition of the PPG, which appears to function similar to mammalian mucins to protect the surface of developing promastigotes against proteolytic damage.

RT.03-C – INFLUENCES OF THE MICROBIOME ON PATHOGEN TRANSMISSION: LESSONS LEARNED FROM THE TSETSE FLY

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The role of microbial organisms in eukaryotic functions is increasingly being recognized. These interactions range from transient environmental associations to coevolved symbiotic interactions. in particular insect are rich in such symbiotic interactions, which may have enabled them to exist in specialized niches. Similarly, tsetse flies, sole vectors of African trypanosomes, have coevolved with multiple symbionts: mutualistic endosymbiont *Wigglesworthia,* commensal *Sodalis* and parasitic *Wolbachia.* Recent results show that the functions of symbiotic bacteria in tsetse range from host nutrient provisioning to host immune competence and reproductive fitness outcomes. It has been possible to maintain tsetse lines through dietary supplementation that either lack *Wigglesworthia* or all three endosymbionts. Absence of *Wigglesworthia* alone compromises tsetse's fertility and also immune resistance to pathogenic trypanosomes. Comparison of select immune gene expression between *Wigglesworthia* cured flies and their age-matched normal counterparts indicate that the absence of *Wigglesworthia* during host immature development particularly affects the maturation of cellular immune responses in the adults.

RT.04-A – THE BIOLOGY OF NUCLEAR DNA REPLICATION IN TRIPANOSOME

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DNA replication is a complex multistep process that is comprised of initiation, elongation, and DNA damage repair. Chromosomal replication initiates with the assembly of the prereplication complex (pre-RC) at DNA sites along the chro- mosomes that are called origins of replication. In eukaryotes, the pre-RC is composed of an origin recognition complex (ORC) containing six proteins, Orc1 to Orc6, two proteins named Cdc6 and Cdt1, and the minichromosome maintenance (MCM) complex, which is composed of Mcm2 to Mcm7 proteins. Different from eukaryotes and similar to Archaea, tripanosomes contain a single protein, homologous to Orc1 and Cdc6, named Orc1/Cdc6 that presents a functional role at pre-replication machinery. This protein is expressed during the entire cell cycle in *Trypanosoma cruzi* and *Trypanosoma bruce* and it remains bound to DNA in any stage of the cell cycle. This result suggests that Orc1/Cdc6 is not involved in the control of DNA replication in these organisms. We analyzed the localization of Orc1/Cdc6 and PCNA, the replication machinery component, during the *T. cruzi* cell cycle. Therefore the organization of DNA replication in the nuclear space of *T. cruzi* will also be discussed.
Supported by FAPESP.

RT.04-B – *Plasmodium falciparum* ORIGIN RECOGNITION COMPLEX (ORC): ROLE IN THE REGULATION OF PARASITE DNA REPLICATION AND VIRULENCE GENE EXPRESSION

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DNA replication, a fundamental process central to the parasite proliferation and pathogenesis is poorly understood in human malaria parasite *Plasmodium falciparum*. A six-protein initiator complex known as origin recognition complex, is essential for DNA replication initiation in eukaryotes. *Plasmodium* ORC contains the largest subunit of ORC, ORC1 and putative ORC5 and ORC2 subunits only. The C-terminal PfORC1 shows the replication-specific function whereas N-terminus may have different function (1). We find that ORC5 forms distinct nuclear foci co-localized with replication foci marker proliferating cell nuclear antigen (PCNA) with the onset of DNA replication in the parasites. However, PCNA and ORC5 foci separate from each other at the end of DNA replication suggesting the presence of replication factory model in the parasites. Surprisingly, PfORC1 co-localizes with both PCNA and PfORC5 at the beginning of DNA replication but gets degraded at the end of the erythrocytic cycle possibly mediated through phosphorylation in a CDK-dependent manner ensuring the regulation of DNA replication in the parasites (2). *Plasmodium falciparum* sirtuin, PfSir2, contains histone deacetylase (HDAC) activity that may regulate virulence gene expression in the parasites (3). PfORC may co-operate with PfSir2 to regulate the telomeric gene silencing. The possible role of ORC in gene silencing apart from DNA replication will also be discussed.

References:

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RT.04-C – ANALYSIS OF THE ARCHITECTURE AND FUNCTION OF THE NUCLEAR DNA REPLICATION APPARATUS IN *TRYPANOSOMA BRUCEI*.

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DNA replication is central to the propagation of life and initiates by the designation of genome sequences as origins, where synthesis of a copy of the genetic material begins once per cell division. Despite considerable progress in understanding mitochondrial (kinetoplast) DNA replication in kinetoplastid parasites, little is known about nuclear DNA replication. The mechanism and machinery of DNA replication initiation is well-conserved amongst characterised eukaryotes. The six-protein origin recognition complex (ORC; Orc1-Orc6), Cdc6 and Cdt1 are recruited sequentially to DNA and, once bound, load the replicative helicase complex (Mcm2-7) to form a pre-replicative complex at origins. Orc1 and Cdc6 are related in sequence, indicative of derivation from a common ancestor. Such an ancestral molecule is found in present-day archaea, prokaryotes in which an Orc1/Cdc6 protein appears to provide all ORC functions, since orthologues of Orcs2-6 are absent. Comparative genome analysis of *Trypanosoma brucei* and related trypanosomatids revealed, remarkably, only a single ORC protein, equally related to eukaryotic Orc1 and Cdc6 (named TbORC1/CDC6 in *T. brucei*). In addition, no clear homologue of Cdt1 was found. These observations have been interpreted as suggesting that origin designation in trypanosomatids, although eukaryotic, may be archaeal-like, raising numerous mechanistic and evolutionary questions. To test this hypothesis, and to dissect the process of nuclear DNA replication in *T. brucei*, we have taken a number of approaches. First, we have used RNAi to examine TbORC1/CDC6 function, and our findings are in agreement with work from other labs in suggesting an essential nuclear replication function in the parasite. Second, we have used chromatin immunoprecipitation of TbORC1/CDC6, coupled with microarray hybridisation, to map genome-wide binding sites of the protein. These data suggest sparse binding in the conserved core of the *T. brucei* chromosomes as well as high-density binding in the more variant subtelomeres, perhaps consistent with origin and non-origin functions. Consistent with this, analysis of gene expression following RNAi suggests that TbORC1/CDC6 plays an important role in putative epigenetic silencing of some *T. brucei* genes. Finally, we have searched for factors that interact with TbORC1/CDC6 and the replicative helicase. This has revealed a number of diverged proteins with roles in DNA replication, which shed new light on the architecture and potential functioning of the origin designation machinery in these parasites.

RT.05-A – INNATE IMMUNE RESPONSES TO *Neospora caninum*

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Neospora caninum, the causative agent of neosporosis, is an obligate intracellular parasite considered to be a major cause of abortion in cattle throughout the world. Most studies concerning *N. caninum* have focused on life cycle, seroepidemiology, pathology and vaccination, while data on host–parasite interaction, such as host cell innate recognition, cell migration, mechanisms of evasion and dissemination of this parasite during the early phase of infection are still poorly understood. Recently, we have focused our research in order to unravel the initial interactions between *N. caninum* and innate immune cells and, along with comparative experiments with closely related *T. gondii*, the results yielded have clarified some key features of the parasite's biology and the generated knowledge may soon be applied in therapeutical or prophylactic protocols towards the disease.

RT.05-B – TOXOPLASMA CYCLING INTO THE HOST CELL

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Cell-to-cell transmission, also termed intercellular transmission, is a critical step in the life cycle of any intracellular pathogen. To successfully make this treacherous transition, the pathogen must escape from one host cell, avoid a battery of immune insults, and hastily infect a new host cell. During acute infection, *Toxoplasma* replicates particularly well in lymphoid organs, which are laden with immune effector cells. Thus the parasite has presumably evolved maximally efficiency mechanisms for intercellular transmission. Here we show that *Toxoplasma* preferentially egresses in the early part of G1 when it has maximal invasion proficiency. Using cell cycle synchronized and non-synchronized populations, we demonstrate that G1 parasites are substantially more efficient at egress and invasion than parasites in S phase or mitosis/cytokinesis (M/C) phase. Although extracellular parasites display the cellular features of G1, they don't show a normal G1 transcription profile. Instead, they express a subset of genes from the "invasion transcriptome" normally seen in late S and M/C when invasion organelles are being made. This altered expression profile may be due to "spill over" of S/M/G transcripts into G1 parasites or it could represent a unique expression profile optimized for intercellular transmission. Upon invasion, the parasite quickly initiates progression through G1 with a "basal transcriptome" expression profile indicative of metabolic escalation and DNA replication. Interestingly, when the time of invasion is restricted, the parasite population is initially cell cycle synchronized by the invasion event through at least the first cell division. By obviating the need to chemically synchronize populations, this observation should greatly aid analysis of organelle biogenesis during daughter cell formation.

RT.05-C – RECOGNITION OF INTRACELLULAR PARASITE INFECTION BY NOD-LIKE RECEPTORS

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Immune responses against infectious agents are usually initiated upon recognition of pathogen-associated molecular patterns (PAMPs) by the pattern recognition receptors (PRRs) present in the host cells. Among PRR are the Toll-like receptors (TLRs), which have being extensively characterized and are well known to recognize intracellular and extracellular parasites. However, the recognition of parasites by non-TLR PRR remains largely obscure. A family of cytosolic proteins containing a nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) has being recently described as important sensors of microbial infection. Among the well characterized NLRs are proteins (such as Nod1, Nod2, Naip5, Nlrc4, Nlrp1 and Nalp3), which contributes for detection of different PAMPs. In contrast to TLRs, which detect extracellular and vacuole-containing PAMPs, the NLRs detect PAMPs contained in the host cell cytoplasm. This information has led to the speculation that NLRs are able to discriminate avirulent from the pathogenic microbes (because only the latter can survive intracellular and release PAMPs in the host cell cytoplasm). In fact, it has being recently demonstrated that in contrast to TLRs, NLRs are able to recognize virulent bacteria that either lyses the vacuolar membranes or that secrete proteins into the host cell cytoplasm by using specialized secretion system. Although important pathogenic parasites (such as *Leishmania, Trypanosoma, Toxoplasma, Plasmodium*) inhabits the host cell cytoplasm, there is virtually no information regarding the recognition of these pathogens by NLRs. Data to be presented will highlight the important role of NLRs for recognition of pathogenic parasites and the mechanisms by which NLRs triggers host resistance against infection.
Supported by FAPESP, CNPq, PEW and WHO/TDR.

RT.06-A – DARING TO BE DIFFERENT: UNUSUAL ASPECTS OF GENE TRANSCRIPTION IN *Trypanosoma brucei*

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I will present some contemporary observations on the regulation of gene transcription in *Trypanosoma brucei* — the agent of African Sleeping Sickness. Trypanosomes have three RNA polymerases, but that seems to be where the similarity to more intensively studied organisms ends. Evolving differently, trypanosomes have largely eschewed introns and —with a few exceptions — do not appear to regulate the initiation of RNA transcription. Differential mRNA trans-splicing and stability appear to be major determinants of mRNA abundance. Histone modifications are central to the regulation of gene transcription in all organisms, and we expected chromatin structure to play some role in the regulation of transcription in *T. brucei*, especially for the allelic exclusion involved in regulating variant surface glycoprotein transcription, where previous work has demonstrated differences in chromatin structure. Although histone sequences are generally highly conserved, the amino acid sequences of trypanosome histones are unusually divergent. This necessitated identifying histone modifications ab initio and raising specific antibodies to them, as no commercial antibodies were suitable. Using mass spectrometry and Edman degradation chemistry, we identified more than 30 modifications but have studied only a few of them in detail. Each of the four core histones are encoded by arrays of identical genes, preventing genetic approaches to identifying the role of specific amino-acid modifications until recently, when we have replaced both tandem alleles of H3 and H4 with single genes. For each core histone, there is a single-gene-encoded variant, allowing them to be studied by genetic manipulation. The variants of H3 and H4 are not essential, at least separately, but the variants of H2A and H2B are. To investigate the genome-wide distribution of various histone posttranslational modifications and all four histone variants in *T. brucei*, we used chromatin immunoprecipitation followed by high-throughput DNA sequencing (ChIP-seq). We have identified striking profiles at regions that are probably associated with the initiation and termination of polycistronic transcription by RNA Polymerase II. Identification of the precise points at which transcription starts is an ongoing challenge. The current status and proposed future directions of this work will be discussed.

RT.06-B – UNVEILING PROTEIN:PROTEIN INTERACTIONS AT *LEISHMANIA* SPP. TELOMERES

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In most eukaryotes, telomere binding proteins such as POT1 and TRF2 play crucial roles in telomere biology by interacting with several other telomere regulators to ensure proper telomere maintenance and to form high order complexes known as telosome or shelterin. *Leishmania* spp. telomeres are composed by the conserved TTAGGG repeats which are maintained by telomerase. The basic *Leishmania* telomeric protein complex is formed by the proteins LaRPA-1 and LaRbp38, which bind *in vitro* and *in vivo*, with high affinity, to the G-rich single-stranded DNA, and by proteins that interact with the double-stranded region of telomeres such as the recently described TRF homologue. The *Leishmania* spp. genome, like other trypanosomatid, lacks many of the conserved single-stranded telomeric proteins found in other eukaryotes, such as the CDC13 and POT1 protein homologues. Thus, we speculate that the *Leishmania* RPA-1 homologue may play the same roles as POT1/CDC13 at parasite telomeres, although it can also bind to other single-stranded DNA with high affinity and in a sequence-independent manner. LaRPA-1 together with the multifunctional LaRbp38 protein, which also interacts with a wide range of GT-rich sequences, including telomeres, seems to form part of a parasite telomeric complex that resembles the recently described CST complex. The CST complex is being considered a second telomere capping mode occurring in a broad variety of species, except budding yeast, and is mainly formed by RPA-like proteins. In this report we used different approaches to show that LaRPA-1 interacts with both LaRbp38 and with telomerase. We are currently investigating if these protein:protein interactions occur in a cell-cycle dependent manner. In addition, LaRPA-1 partially co-localizes with both proteins, probably reflecting its functions in DNA metabolism. We speculate whether these protein interactions reflect the entire telomeric complex or the presence of functionally distinct subcomplexes at parasite telomeres.

RT.06-C – DNA REPAIR AND TELOMERE EXPRESSION IN *LEISHMANIA MAJOR*.

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The *Leishmania* genome presents hallmarks of genome instability such as gene amplification and chromosome polymorphisms that are compatible with the presence of a robust mechanism to deal with such high plasticity. Genome stability is constantly challenged by DNA damage that is caused by the metabolism of the cell or inflicted by exogenous factors. Several types of DNA damage result in the formation of single stranded DNA which is recognized by the single strand binding protein RPA. The RPA-coated DNA serves as the substrate for the recruitment of checkpoint protein complexes. One of the first players recruited to the DNA lesion sites in eukaryote genomes is a PCNA-related trimeric complex formed by the proteins Rad9, Rad1 and Hus1. Loading of the 911 complex onto the chromatin initiates the signaling events necessary to halt cell cycle progression and to allow DNA repair to take place. In mammals, the 911 complex also associates with telomeres and plays a role in chromosome stability. Despite its importance in genome maintenance, the 911 complex has not been studied in trypanosomatids. We have investigated the existence and functioning of the 911 complex in the protozoan *Leishmania major*. We identified the parasite gene that is a homolog of Hus1 from other organisms. The predicted secondary structure of the parasite Hus1 revealed the presence of putative beta-sheets and alpha-helices in conserved positions indicating the presence of the PCNA-like domains that may enable this protein to participate in DNA metabolism. Using specific antibodies we found that the parasite Hus1 protein localizes to the nucleus. Upon exposure to DNA damaging drugs Hus1 not only relocates to the nuclear periphery, but also exhibit an increased co-localization with RPA1, which has been shown to be a telomeric protein in *L. amazonensis*. In fact, Hus1 also associates to telomeres and seems to affect the expression of a selectable marker integrated at a subtelomere. Transfectant cell lines with an increased expression of Hus1 are clearly resistant to hydroxyurea and methyl methanesulfonate. However, resistance to phleomycin is not observed upon Hus1 overexpression. Interestingly, exposure to phleomycin leads to a significant increase in telomeric repeat-containing transcripts. Transcription of telomere repeats in protozoa have been investigated and regarded as result from read-through downstream of telomeric genes. However, it has been recently demonstrated that the transcription of mammalian and yeast telomeres is finely regulated resulting in telomeric repeat-containing RNA (TERRA) that associates with chromosomal ends. *Leishmania* TERRA transcription proceeds exclusively toward the telomeres irrespective of the direction of the polycistron suggesting that it is not a mere transcription by-product. The data gathered so far support our hypothesis that LmTERRA is a functional RNA class that might be explored by the cell to regulate its genome expression and maintenance.
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RT.07-A – MULTIDIMENSIONAL IMAGING OF MACROPHAGES DOUBLY INFECTED BY *LEISHMANIA AMAZONENSIS* AND *LEISHMANIA MAJOR*

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Protozoan parasites of the genus *Leishmania* alternate between flagellated, elongated extracellular promastigotes found in insect vectors, and round-shaped amastigotes enclosed in phagolysosome-like Parasitophorous Vacuoles (PVs) of infected mammalian host cells. *Leishmania amazonensis* amastigotes occupy large PVs which may contain many parasites; in contrast, single amastigotes of *Leishmania major* lodge in small, tight PVs, which undergo fission as parasites divide. To determine if PVs of these *Leishmania* species can fuse with each other, mouse macrophages in culture were infected with non-fluorescent *L. amazonensis* amastigotes and, 48h later, superinfected with fluorescent *L. major* amastigotes or promastigotes. Fusion was directly investigated by time lapse image acquisition of living cells and inferred as well from the presence of parasites of the two species in the same PVs. Survival, multiplication and differentiation of parasites that did or not share the same vacuoles were also investigated. Fusion of PVs containing *L. amazonensis* and *L. major* amastigotes was not found. However, PVs containing *L. major* promastigotes did fuse with pre-established *L. amazonensis* PVs. In these chimeric vacuoles, *L. major* promastigotes remained motile and multiplied, but did not differentiate into amastigotes. In contrast, within their own, unfused PVs in doubly infected cells, metacyclic-enriched *L. major* promastigotes, but not log phase promastigotes - which were destroyed - differentiated in proliferating amastigotes. The results indicate that PVs presumably customized by *L. major* amastigotes or promastigotes, differ in their ability to fuse with *L. amazonensis* PVs. Additionally, a species-specific PV was required for *L. major* destruction or differentiation – a requirement which mechanisms remain unknown. This descriptive experimental work should be useful in further studies of the permissiveness of PVs to different species of *Leishmania* parasites, and of the mechanisms involved in the recognition and fusion of PVs.

RT.07-B – HOST CELL MEMBRANE-LYSOSOMES INTERPLAY DURING *TRYPANOSOMA CRUZI* INVASION

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Trypanosoma cruzi, the etiological agent of Chagas´Disease, can invade a wide variety of cell types, varying from phagocytic to non-pahgocytic cells. The parasitic invasion of the latter occurs through two convergent paths: host membrane invagination with subsequent lysosomal fusion or direct lysosomal fusion at the parasite attachment site. The interaction between parasite and host cell membrane is then one of the critical steps to the establishment of a well-succeeded invasion. Two relevant steps during this interaction are: 1. the interaction of *T. cruzi* with host cell plasma membrane, which will direct signaling events that will culminate with host cell lysosome recruitment for the formation of the parasitophorous vacuole; 2. *T. cruzi* interaction with its parasitophorous vacuole membrane which will help its invasion in host cell and will influence parasite vacuole escape during parasite intracellular development. Concerning parasite interaction with host plasma membrane, recently, it has been shown that cholesterol and cell microdomains might influence parasite-host cell invasion in fibroblasts. Our work demonstrated that these host cell microdomains, also known as membrane rafts, are also important during parasite invasion in cardiomyocytes, important cells during *T. cruzi* infection in humans. These host cell membrane domains seem to be hot spots of parasite interaction and entrance. We also investigated the participation of the membrane rafts in lysosome fusion during *T. cruzi* entry. Cardiomyocytes treatment with MβCD, a drug which can deplete cellular cholesterol content, led to decreased *T. cruzi* invasion in a dose dependent manner without interfering with cell viability, showing that cholesterol is important for efficient *T. cruzi* entry. However upon MβCD treatment lysosomal fusion during parasite entry became altered, suggesting that these regions might be involved with directing lysosome fusion to the site of parasite attachment. After lysosome recruitment and fusion, *T. cruzi* interaction with lysosomal membrane is imperative for *T. cruzi* retention in host cells. Studies of parasite infection in cells lacking LAMP 1 and 2 (LAMP-KO cells), two integral lysosome membrane proteins, show that this two proteins interfere with parasite entry and later with its intracellular development in the host. Absence of these two proteins lead to decreased cell infection, however it facilitates parasite intracellular development, suggesting that parasite interaction with its vacuolar membrane might determine the success of host infection.

RT.07-C – *Toxoplasma gondii* INTERACTION VARIES ACCORDING TO HOST CELL MICRODOMAINS

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Lipid rafts are dynamic nanoscale assemblies enriched in sterol-sphingolipid. The exploitation of rafts by intracellular pathogens may facilitate invasion. *Toxoplasma gondii* is a pathogen that actively invades host cells. Most host cell proteins do not pass beyond the moving junction and are excluded from the parasitophorous vacuole (PVM). However, many components of PVM derive from host cell membrane, including microdomains. We evaluated the participation of cholesterol enriched microdomains in invasion of *T. gondii* into LLC-MK2 and murine macrophages through transient depletion of host cells cholesterol with either methyl-beta-cyclodextrin (MβCD) [final concentrations of 5, 10 and 20 mM for 30 min before interaction]; or Filipin [final concentrations of 1, 3 and 6 nM for 30 min before interaction]. Reversibility for MβCD was also tested. The interaction of the parasite with the host cells after treatment with cholera toxin B subunit (CTB) that binds to GM1 ganglioside was also tested. After interaction (10 min, 50 parasites per cell), adhesion and internalization indexes were determined by light microscopy. These were significantly diminished in cells treated with MβCD compared to controls. With 20 mM of MβCD, inhibition of internalization in LLC-MK2 reached 80% and almost 100% in macrophages. Pretreatment with MβCD followed by cholesterol reposition before interaction completely reverted inhibition in macrophages, but not in LLC-MK2. Filipin did not interfere in interactions with LLC-MK2 cells, but in macrophages, inhibition reached 76%. CTB did not interfere in adhesion to LLC-MK2 cells, but internalization was inhibited in 70%. Furthermore, macrophages treated with CTB inhibited the adhesion and internalization of the parasite in almost 80%. These results indicate that host cell membrane cholesterol enriched domains participate in the process of adhesion and active invasion of Toxoplasma and the differences observed between macrophages and LLC-MK2 cells may be due to different levels of this molecule in its membranes. Support: CNPq, CAPES, FAPERJ.

RT.08-A – *Trypanosoma rangeli*: A BANE OR A BLESSING FOR CHAGAS DISEASE?

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Trypanosoma rangeli is non-pathogenic, but is frequently mistaken for the related Chagas disease agent, *T. cruzi*, with which it shares vectors, hosts, significant antigenicity and a sympatric distribution over a wide geographical area. Trypanosomes infective to humans have been extensively studied at a cell and molecular level, but study of *T. rangeli*, remains in relative infancy. Since human infection by *T. rangeli* can induce cross-reactivity with *T. cruzi* confounding serological detection of Chagas disease, reliable discrimination of *T. rangeli* from *T. cruzi* remains of utmost importance.

Aiming to increment the knowledge on the *T. rangeli* genome and to perform comparative analyzes, assessment of the parasite transcriptome through the generation of EST and ORESTES tags from both epimastigote and trypomastigote forms increased 26-fold the number of parasite sequences on the GenBank. The results allowed an estimate of ~8,500 genes for *T. rangeli* with an average G+C content of 55%. Biological functions were assigned to around 70% of the generated sequences, allowing the establishment of an annotated *T. rangeli* gene expression database.

Comparative analyses of *T. rangeli* sequences were carried out with *T. cruzi* and a wide range of pathogenic and non-pathogenic protozoan sequences now available, resulting in a relatively low number of unmatched sequences. Most *T. rangeli* sequences showed similarity to their homologs on the TriTryps genomes (3,128), revealing 625 exclusive hits with *T. cruzi*, which is consistent with former reports on the close phylogenetic relationship of these two taxa. Almost 23% of the sequences failed to yield blast hits with the TriTryps genomes. The presence of these *novel T. rangeli* specific sequences may provide new biological insights and/or diagnostic targets. Excluding the 289 *T. rangeli* protein coding sequences described in this study which were similar to those already in the GenBank database, a total of 2,228 sequences are described for this taxon for the first time.

Genes associated with virulence in pathogenic kinetoplastids, including GP63, TS, Cruzipain, Oligopeptidase B and Cysteine proteases, were found to be expressed in *T. rangeli*, but no transcripts related to mucin-associated proteins (MASPs) were among the generated ESTs. Groups of cDNA related to transcription and translation (RNA editing, RNA genes, RNA binding proteins and ribosome proteins), division (cyclins and protein kinases) and metabolism pathways like sterol synthesis were observed. Also, several retrotransposon hot spot proteins, like RHS1, RHS2c and RHS4 (a, e, f and g), are now described for this taxon. Based on their similarity to proteins of unknown function in related species a total of 1,076 hypothetical and hypothetical conserved proteins were observed for *T. rangeli*, representing 36.6% of all annotated sequences described.

These results represent the first large-scale analysis of the parasite genome, describing a draft of the gene expression profile of epimastigote and trypomastigote forms from polar strains. Furthermore, comparative analysis of these sequences with kinetoplastid genomes suggests that the gene repertoire of *T. rangeli* is closely related with pathogenic *T. cruzi*, reinforcing the importance of *T. rangeli* as a model to address studies involving mechanisms of virulence and pathogenicity. Currently, the Brazilian Genome Consortia is sequencing the *T. rangeli* genome, which will dramatically increase the knowledge of this neglected parasite, allowing comparative and functional genomics studies.

RT.08-B – ANALYSIS OF PARASITE METABOLISM IN VIVO USING METABOLOMIC APPROACHES

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The genomes of many parasitic protozoa have now been sequenced providing a detailed overview of the metabolic capacity of these pathogens. However, it is likely that many metabolic and regulatory processes remain to be discovered as a large number of genes within the sequenced parasite genomes (typically >50%) have no assigned function. Moreover, almost nothing is known about the relationship between gene/protein expression and metabolic fluxes in these organisms. This is particularly striking in some trypanosomatid parasites that constitutively express the majority of genes in all life-cycle stages. Metabolomics is complementary to other profiling approaches (transcriptomics, proteomics) and is increasingly being used to identify stage-specific changes in parasite metabolism and physiology and parasite responses to various environmental and chemical stimuli (drugs etc). Here we provide an overview of analytical methods that are being used in metabolomic studies. We also highlight the unique insights that can be obtained using ^{13}C stable isotope labeling methods to measure metabolic dynamics and develop quantitative models of metabolism. We have applied these approaches to assess the metabolic state of the major developmental stages of *Leishmania*. Our results demonstrate that each stage enters a unique physiological state that are not predicted from other profiling approaches.

RT.08-C – UNDERSTANDING TRYPANOSOMATIDS ANTIOXIDANT MECHANISMS: HOW FAR ARE WE?

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Trypanosoma brucei and *Trypanosoma cruzi* are responsible for two major tropical diseases: human African trypanosomiasis and Chagas disease, respectively. With no immediate prospect of vaccines, and no satisfactory treatment, the search for targets to develop a more specific therapy is a priority. One potential Achilles? heel is the systems the parasite employs to detoxify reactive oxygen species (ROS). Since the pioneering studies which identified trypanothione (N^1,N^8-bis-glutathionylspermidine), a protein unique to the members of the Kinetoplastida order and ascribed nowadays to an important role in ROS detoxification, numerous work have been undertaken trying to elucidate the molecular mechanisms that underlie the parasite's complex network of antioxidant devices. A review of the advances made throughout the years and the biological relevance of the proteins involved in this system will be presented.