UNIVERSIDADE FEDERAL DE OURO PRETO
NÚCLEO DE PESQUISA EM CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

ADENOSINE PRODUCTION VIA CD39/CD73 PATHWAY PROMOTES *Leishmania amazonensis* SURVIVAL IN MACROPHAGES

BIJAY BAJRACHARYA

OURO PRETO

2014

UNIVERSIDADE FEDERAL DE OURO PRETO
NÚCLEO DE PESQUISA EM CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

ADENOSINE PRODUCTION VIA CD39/CD73 PATHWAY PROMOTES *Leishmania amazonensis* SURVIVAL IN MACROPHAGES

BIJAY BAJRACHARYA

SUPERVISOR: Prof. Dr. Luis Carlos Crocco Afonso

Tese apresentada ao programa de Pos-Graduação em Ciências Biológicas da Universidade Federal de Ouro Preto, como parte integrante dos requisitos para a obtenção do titulo de Doutor em Ciências Biológicas, área de concentração: Imunobiologia de Protozoários

OURO PRETO

2014

B594a Bijay, Bajracharya
Adenosine production via CD39/CD73 pathway promotes *Leishmania amazonensis* survival in macrophages [manuscrito] / Bajracharya Bijay - 2014.
95f.: il. color.; graf.; tab.

Orientador: Prof. Dr. Luís Carlos Crocco Afonso.

Tese (Doutorado) - Universidade Federal de Ouro Preto. Instituto de Ciências Exatas e Biológicas. Núcleo de Pesquisas em Ciências Biológicas. Programa de Pós-Graduação em Ciências Biológicas.
Área de concentração: Imunobiologia de Protozoários.

1. Células Killer - Teses. 2. Adenosina trifosfato - Teses. 3. Células imunocompetentes - Teses. 4. Leishmaniose - Teses. I. Afonso, Luis Carlos Crocco. II. Universidade Federal de Ouro Preto. III. Título.

CDU: 616.993.161

Catalogação: sisbin@sisbin.ufop.br

Meeting summary of the Evaluation Committee for the PhD defense of the thesis entitled:

"Adenosine production via CD39/CD73 pathway promotes *Leishmania amazonensis* survival in macrophages"

On March 21st, 2014 at 09:30 a.m. in the Auditorium of the Centre of Research in Biological Sciences (NUPEB) at the Universidade Federal de Ouro Preto (UFOP), the members of the evaluation committee met to evaluate the PhD thesis of the student **Bijay Bajracharya**. The meeting was initiated with the oral presentation of the thesis by the student and then the committee members interviewed the candidate. At the end, the members of the examining committee met in private, discussed the presentation and the work and decided to APROVE the candidate. The title will be granted after the compliance with all other requirements of the Post-Graduated Program in Biological Sciences, as stated in the Program rules.

Members of the examining committee:

Dr. Alexandre Barbosa Reis
Examiner (UFOP)

Dr.ª Fátima Ribeiro Dias
Examiner (UFG)

Dr.ª Lis Ribeiro do Valle Antonelli
Examiner (FIOCRUZ)

Dr. Wagner Luiz Tafuri
Examiner (UFMG)

Dr. Luís Carlos Crocco Afonso
President (UFOP)

DATE OF DEFENSE: **03/21/2014**

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
NUPEB – ICEB II – sala 49 | Universidade Federal de Ouro Preto
Campus Universitário Morro do Cruzeiro | CEP 35.400-000 – Ouro Preto – MG – Brasil
Tel: 55 (31) 3559 1680 | E-mail: cbiol@nupeb.ufop.br | www.nupeb.ufop.br/cbiol

This work was developed in the Laboratory of Immunoparasitology/Center for Research in Biological Sciences at Federal university of Ouro Preto under the supervision of Prof. Dr. Luis Carlos Crocco Afonso with the financial support from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), National Council for Scientific and Technological Development (*CNPq)* and The World Academy of Sciences (TWAS).

Oh tell me Lord how could it be, That though our cells make ATP, It's not all used for energy, But sometimes is secreted free. It puzzles you, it puzzles me, While Geoffrey Burnstock smiles with glee At the many roles of ATP. (Poem by Samuel C. Silverstein; extracted from Purinergic signaling by Geoffrey Burnstock)

ACKNOWLEDGMENT

First and the foremost, I would like to express my heartfelt gratitude to my supervisor **Prof. Dr. Luís Carlos Crocco Afonso** for continuous support of my PhD study and research, for his patience, motivation, enthusiasm and immense knowledge. His guidance throughout this period of time helped me to grow both professionally and scientifically. It was his belief and his optimistic vision that I and my wife are here today accomplishing our dreams. He, not only as a professor, but also as a friend, assisted me in solving problems both in the lab and off the lab. I would not have imagined living a good life or doing science in Brazil without his help. I owe him a debt of gratitude for all he had done for me.

I am equally grateful to **Prof. Dr. Ricardo Gonçalves** for supervising me in my work. It was his contribution that helped me to solve some of the experimental problems that I was struggling initially with. Without his efforts, this present work would not have had this shape. I am also indebt to **Prof. Dr. Andre Talvani**, who not only accepted my wife as her doctorate student but also gave us all support, friendship, love, and care. He and his family were so generous all the time that we felt like we are in our own home. I would also like to offer my special gratitude to **Prof. Leda** for being so welcoming and nice all the time and for being such an amazing person. Her energy is exceptional. She owes my respect.

I am so obliged to the funding agencies; **CNPq, TWAS, CAPES, FAPEMIG and UFOP** for bearing all the expenses of my living and studying in Brazil. Without their financial support, it would not have been possible to complete my doctorate.

I would also like to express thanks to all professors from NUPEB for allowing me to use their equipments and reagents whenever necessary. Particularly, I am grateful to the assistance given by **Prof. William, Prof. Riva and Prof. Ieso** in some of my experiments. Many thanks to Secretary sector of NUPEB, people from animal facility and from RU for their outstanding dedication, efforts and performance which somehow assisted me in living and doing things properly at the university.

I am also grateful to **Mr**. **Leandro** and his family who gave all the support in the beginning when I was accustoming to the language and to new culture. He not only helped me in

the lab but also did a lot to settle down in Ouro Preto. I also thank **Mr**. **Marcorelio** for technical assistance, his friendship, generosity and positive thoughts about life.

I am thankful to my fellow lab mates Hellem, Tiago, Rodrigo, Pauline, Amanda, Renata, Miriam, Jiame, Pedro, Marina, Leandro, Rodringinho, Luana, Glauber, Marcia, Hebert, Priscila (**too many to list here but you know who you are**!) for the healthy discussions, assistance in the experiments and giving me company in the lab and out of the lab. I thank you all for the patience, friendship and cooperation throughout my stay. I would also like to appreciate Prof. Sandra, Prof. Cristiane, Prof. Denise and Prof. Simone for their generosity and suggestions. I also equally thank to people from LDC and friends from other laboratories for offering their help whenever possible.

I am grateful to my dearest friends; **Hellem, Joao and Fabiano** for friendship, love, attention and brotherhood. Without you all, life would not have been so fun filled in Brazil. I also thank to their families for treating me and my wife as their own children. My appreciation also goes to all my beloved friends from Nepal who constantly motivated me although millions of miles away.

My deepest appreciation goes to my wife, **Mrs. Deena Shrestha**, for her support, love, positive energy and cheering me up all the times. She never complained me of coming home late and even when I stayed whole night in the lab. I appreciate for her patience and trusting me all the time.

Last but not the least, I would like to show profound gratitude to **my parents, my sister, Little and Anju, sweet Ridyan, rest of my family as well as Deena's family** who stood by me in both hard and good times. I would not imagine having my mind focused in my work without their continuous unconditional love and support. Thank you for being with me all the time. **This work is dedicated to all of them who inspired me to be a researcher.**

RESUMO

A leishmaniose cutânea (CL), causada por *L. amazonensis*, é caracterizada por uma intensa imuno- supressão e multiplicação descontrolada do parasito em modelos experimentais e é geralmente grave em humanos, variando desde a forma cutânea até a cutâneo-difusa. Não existem mecanismos precisos conhecidos sobre como *L. amazonensis* modula a resposta imunológica para que os macrófagos (MΦ) infectados com *L. amazonensis* se tornem refratários à ativação por células T efetoras. Aqui, nós investigamos o possível mecanismo regulador que *Leishmania* provavelmente pode induzir em MΦ residentes durante a interação precoce, de modo a impedir ativação das células. Neste estudo, analisou-se a expressão de CD39 e CD73, por citometria de fluxo, em MΦ peritoneais murinos infectados com promastigotas metacíclicas de *L. amazonensis* e também a porcentagem dessas células que expressam a CD39 e CD73 foi avaliada. Nossos resultados mostraram que em 72hrs inativos os MΦ tiveram baixa expressão de CD73. Curiosamente, no entanto, ao contrário de MΦ tratados com LPS os infectados com *L. amazonensis* expressaram altos níveis de CD73. Esta informação foi posteriormente validada pelos resultados de estudos no contexto *ex-vivo* que mostrou igualmente que MΦ infectados são predominantemente $CD73^{+}$. Quando as atividades enzimáticas de CD39 e CD73 foram bloqueadas, tal como pelo uso de DIDS e MAD αβ, tanto a infecção quanto o número de amastigotas diminuiu significativamente após 48 horas de incubação. Da mesma forma, a inibição dos receptores de adenosina A2a e A2b de ZM241385 e MRS1754 também apresentou os mesmos efeitos sobre a sobrevivência do parasito e infectividade. Em estudo posterior, em busca de um possível papel da HIF- 1α na infecção por *Leishmania*, investigamos os efeitos da FM19G11, inibidor do HIF- 1α, na expressão de CD39 e CD73, bem como na infecção parasitária . Observou-se que, apesar de HIF - 1α poder influenciar na sobrevivência do parasito, os seus efeitos sobre a expressão de CD39 e CD73 não eram visíveis. Também foi avaliada, por PCR em tempo real, a expressão de receptores de adenosina em populações infectadas, nas quais não se observou nenhuma mudança significativa na expressão após 24 horas de infecção. Além disso, também foi avaliada a produção de citocinas, tais como TNF- α e IL-10 a partir da produção de NO nos grupos tratados. Surpreendentemente, não houve variação nos níveis destes mediadores, sugerindo a existência de outros mecanismos independentes da mediação por citocina para produção de Óxido Nítrico, tais como a produção de ROS ou efeitos leishmanacidas independentes do triptofano. Concluindo, nossos dados mostram que a infecção

por *L. amazonensis* regula a expressão CD73 durante 24 horas de infecção e sua sobrevivência depende de atividades enzimáticas, bem como de receptores A2a e A2b.

ABSTRACT

Cutaneous leishmaniasis (CL) caused by *L. amazonensis* is characterized by intense immune-suppression and uncontrolled parasite multiplication in experimental models and is usually severe in humans ranging from cutaneous to diffuse cutaneous leishmaniasis. There are no precise mechanisms known how *L. amazonensis* modulates immune response so that macrophages (MΦ) infected with *L. amazonensis* are refractory to activation by effector T cells. Here, we investigated the possible regulatory mechanism that *Leishmania* can likely induce in host MΦ during early interaction so as to prevent their host cells from activation. In this study, we analyzed the expression of CD39 and CD73, by flow cytometry, in murine peritoneal MΦ infected with metacyclic promastigotes of *L. amazonensis* and percentage of those cells expressing CD39 and CD73 was evaluated. Our results showed that 72hrs rested MΦ down regulated CD73 expression. Interestingly, however, unlike LPS treated MΦ, *L. amazonensis* infected MΦ up regulated CD73 expression. This data was further validated by the findings from *in ex-vivo* studies which equally support that infected MΦ are predominantly CD73 positive. When CD39 and CD73 enzymatic activities were blocked such as by the use of DIDS and αβ MAD, both infection and amastigote number decreased significantly within 48hrs of incubation. Similarly, inhibition of adenosine receptors A2a and A2b by ZM241385 and MRS1754 also had the same effects on the parasite survival and infection. In another study, in search of a possible role of HIF-1α in *Leishmania* infection, we investigated the effects of FM19G11, inhibitor of HIF-1α, on expression of CD39 and CD73 as well as parasitic infection. We observed that although HIF-1α can influence in the parasite survival, their effects on CD39 and CD73 expression were not visible. We also evaluated the expression of adenosine receptors in infected population by real time PCR in which we observed no significant change in the expression after 24hrs of infection. Moreover, we also evaluated cytokine production such as TNF-alpha, IL-10 and NO production from the treated groups. Surprisingly, there was no alternation in the levels of these mediators suggesting other mechanisms, independent of cytokine mediated nitric oxide production such as ROS production or tryptophan independent oxygen anti-leishmanacidal effects, involved in it. In conclusion, our data show that *L. amazonensis* infected up regulates CD73 expression during 24hrs of infection and its survival is dependent on enzyme activities as well as A2a and A2b receptors.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

RESULTS

LIST OF ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CFSE	carboxyfluorescein diacetate sccinimidyl ester
CL	cutaneous leishmaniasis
CREB	cyclic responsive element binding protein
Ct	threshold cycle
DC	dendritic cell
DIDS	4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
dNTPs	deoxynucleotide triphosphate
Ecto-NTPDase	ectonucleoside tri-phosophodihydrolases
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
Fig	figure
FM19G11	2-oxo-2-(p-tolyl) ethyl]3-[(2,4-dinitrobenzoyl)amino]benzoate,3-[(2,4-initrobenzoyl)amino]- benzoic acid 2-(4-methylphenyl)-2-oxoethyl ester
G	gauge
Gp63	glycoprotein63
GPCRs	G-protein coupled receptors
HIF-1	hypoxia-inducible factor 1
IFN-γ	interferon-gamma
IL-1	Interleukin-1
IL-10	interleukin-10
IL-12	interleukin-12
IL-13	Interleukin-13
IL-1β	interleukin-1β
IL-4	Interleukin-4
IL-6	interleukin-6
InsP3	inositol triphosphate
LPG	lipophosphoglycan
LPS	lipopolysaccharide
MFI	mean fluorescence intensity
MHC-II	Major histocompatibility complex class II
mRNA	messenger ribonucleic acid
MRS 1724	4-[((4-Cyanophenyl) carbamoylmethyl) oxy]phenyl]-1,3-di(n-propyl) xanthine hydrate

MRS	MRS 1724
MΦ	macrophages
NO	nitric oxide
P	parasite
PAMPs	pathogen associated membrane patterns
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PHDs	prolyl hydroxylases
RNA	ribonucleic acid
ROS	reactive oxygen species
SD	standard deviation
SP1	specific protein 1 transcription factor
TGF-β	transforming growth factor-Beta
Th1	type 1 helper T-cell
Th2	type 2 helper T-cell
TNF-α	tumor necrosis factor- alpha
Treg	regulatory T cells
UFOP	Universidade Federal de Ouro Preto (Federal University of Ouro Preto)
VL	visceral leishmaniasis
ZM	ZM241385
ZM241385	4(-2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5} triazin-5-yl-amino]ethyl
αβ MAD	α,β-Methyleneadenosine 5′-diphosphate sodium salt

INTRODUCTION

1. INTRODUCTION

1.1 Leishmaniasis

Leishmaniasis is a vector born protozoan infection and is a potentially fatal disease threatening about 350 million people in more than 80 countries around the world. In addition, 12 million people are currently at the risk of this infection, with the estimated 1-2 million of new cases occurring every year (Bern et.al., 2008). Today, this disease presents a huge global challenge to clinicians due to the development of drug resistant forms and occurrence of co-infections with various other diseases such as AIDS making it hard to treat. Leishmaniasis in humans, which presents remarkably different pathological responses, is caused by more than 20 different species of *Leishmania*. The disease can have diverse clinical manifestations which may be cutaneous**,** mucocutaneous or visceral. Cutaneous leishmaniasis (CL) is the most common form which accounts for more than 50% of new cases, and is usually characterized by the formation of self-resolving skin ulcers at the bite site mainly on exposed parts of the body whereas visceral leishmaniasis (VL) is the most severe form in which vital organs of the body are affected. It has been reported that approximately 20,000-30,000 deaths occur annually and yet this disease is categorized as neglected tropical disease (http://www.who.int/leishmaniasis/burden/en/) (http://www.cdc.gov/parasites/leishmaniasis/).

1.2 Life cycle of Leishmania

Leishmania is a dimorphic protozoan parasite which shuttles between promastigote and amastigote forms in two different hosts (Liew & O'Donnell, 1993). Typical life cycle of leishmania is shown in **figure I**. When an infected female sandfly from the genera *Phlebotomus* (Old World) *or Lutzomyia* (New World) probes into the skin of mammalian host, it inoculates elongated flagellated motile promastigotes in the skin which are quickly engulfed by mononuclear phagocytic cells such as macrophages (MΦ), the primary host of the parasites. Once inside the phagocytic cells, the parasitophorous vacuole fuses with the secondary lysosomes leading to the formation of phagolysosome, where *Leishmania* can differentiate and proliferate into amastigotes. Amastigotes are mainly intracellular and they come outside occasionally when their host cells burst. They can infect new cells or they can be taken up by healthy sandflies during their blood feeding, completing their cycle of replication or transmission respectively (Sacks & Noben-Trauth, 2002; Zandbergen G et.al., 2004)

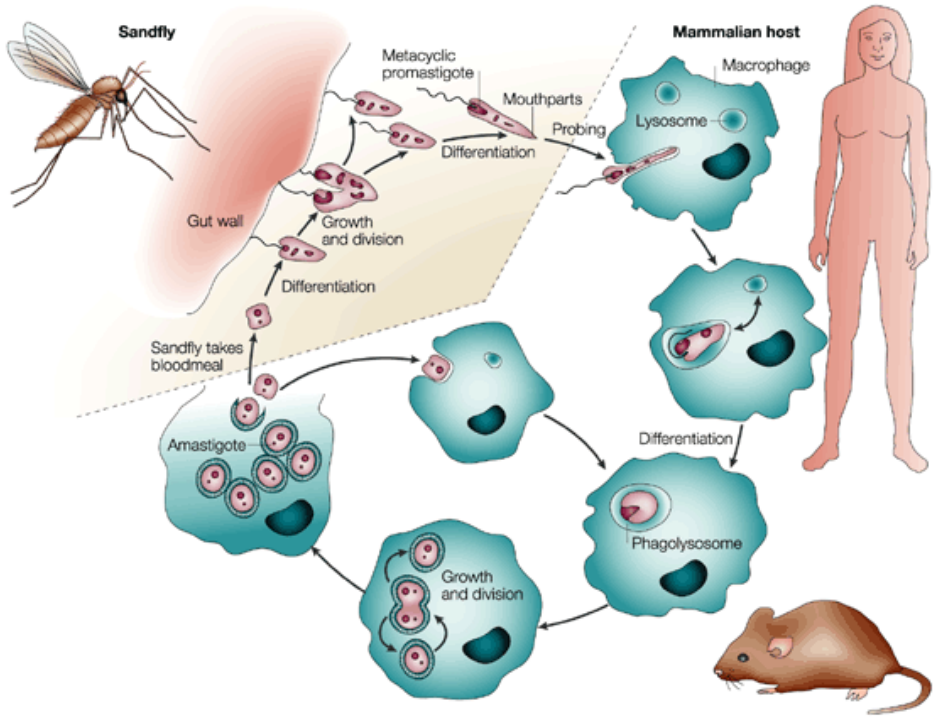


Fig I. Life cycle of *Leishmania*: Infective forms of *Leishmania* inoculated by female sandflies are taken up by the phagocytic cells mainly MΦ via complemented mediated phagocytosis. Inside MΦ, promastigotes multiply within phagolysomes and then transform into amastigotes. Once they multiply sufficiently, these parasites are released from MΦ which are again taken up by the female sandflies completing a cycle. Adapted from (Sacks & Noben-Trauth, 2002)

1.3 Host parasite interaction and immunomodulation during leishmaniasis

Several immune cells participate in immunopathogenesis of leishmaniasis. The outcome of disease is determined by the interaction and activation of these immune cells and how the parasites modulate the immune response (Nylen & Gautam, 2010).

Early events of leishmaniasis demonstrate that as soon as the parasites are inoculated, they are engulfed by a mixed population of neutrophil and monocytic infiltrate within an hour of intra dermal inoculation of *L. donovani* in hamsters (Wilson et.al., 1987) or neutrophils arriving within 3 hours post inoculation of *L. amazonensis* or *L. majo*r in footpads of BALB/c (Pompeu et.al., 1991) or C57Bl/6 mice respectively (Beil et.al., 1992). In a recent paper, it has been demonstrated that neutrophils migrate to the site quickly within 30 mins after intraperitoneal

inoculation of *L. major* in mice (Goncalves et.al., 2011). By the use of two-photon intra-vital microscopy, it is confirmed that neutrophils are the primary cells that infiltrate at the site of inoculation of *L. major* in the dermis of C57Bl/6 (Peters et.al., 2008). Neutrophils act as intermediate hosts where these parasites live shortly. Following ingestion, parasites induce apoptosis in neutrophils by the virtue of which MΦ that are recruited at the site of infection engulf neutrophils together with the parasites. They are popularly regarded as Trojan horses allowing the parasites to silently get inside MΦ (Laufs et.al., 2002; Zandbergen G.et.al., 2004)

Dendritic cells (DC) are professional antigen presenting cells and are distributed throughout the body. The interaction between *Leishmania* and dendritic cells depends on several factors including parasite species and type of DC, their maturation and activation. Function of DC in leishmaniasis is mainly known for its ability to induce naïve T cell polarization as *Leishmania* infected MΦ cannot secret IL-12 and thus cannot produce antigen specific Th1 response (Kima et.al., 1996)). It has been described that dermal migratory dendritic populations are the principal antigen presenting cells that migrate to draining lymph nodes for antigen presentation to T lymphocytes (Moll et.al., 1993; Ritter et.al., 2004). Other studies also indicate that the origin of DC in lymph nodes, as early as 24hrs post infection by *L. major*, can stimulate T cell proliferation (Iezzi et.al., 2006). In *L. major* infection, DC from BALB/c mice shows upregulation of IL-4 receptor expression but downregulates IL-12p40 production indicating that this parasites can inhibit DC Th1 polarization functions (Moll et.al., 2002). *L amazonensis* can modulate several functions of DC by modulating the expression of MHC-II, CD80, CD86 and CD40 as well as IL-10 and IL-12 production (Favali et.al., 2007; Xin et.al., 2008). Together with MΦ, infected DC can determine the outcome of the disease in resistant and susceptible mice (Liu & Uzonna, 2012).

In human and experimental leishmaniasis, the fate of parasite-MΦ interaction is chiefly determined by T cell differentiation and proliferation. The susceptibility and resistance to *Leishmania* are associated with unique subsets of T cells. The response to *Leishmania* -specific lymphocytes is essential for the control of infection with this parasite. CD4 + T cells polarization into Th1 cells secrete IFN -γ and / or TNF-alpha that contribute to the elimination of the parasite, mainly by activation of MΦ. Th2 cells differentiated in the absence of IL-12, secrete large amounts of IL- 4, IL -5 and IL -13, which facilitate parasitic growth and multiplication. CD8 + T cells also help in combating parasites and are a major source of IFN-γ (Mougneau et.al., 2011).

Th1 and Th2 paradigm has been explained in relation to resistance and susceptibility of towards *L. major* infection where the disease is determined based on dominance of either of Th1or Th2 cytokines (Sacks & Noben-Trauth, 2002). However, this observation is not always true such as in *L. amazonensis* infection where a mixed Th1/Th2 response can be observed (Afonso & Scott, 1993; Ji et.al., 2002). In the case of leishmaniasis, Treg cells suppress effector T cell functions through production of IL-10 (Rai et.al., 2012). IL-10 is a potent inhibitor of IFN-γ production and has been shown to be a key cytokine that favors the persistence of *Leishmania* in the skin lesions (Belkaid et.al., 2001)

1.4 Macrophage and *Leishmania* interaction:

MΦ are innate immune cells that have an indispensable role in early pathogen immune responses, and at the same time, they play important role in the healing of excessive inflammation (Stempin et.al., 2010). Although several immune cells including neutrophils, dendritic cells, and monocytes can migrate to the site of infection and interact with *Leishmania*, MΦ are the key immune cells in leishmaniasis serving as the primary host cells as well as the major effector cells (Mauel, 1990). Parasites first attach to MΦ via complement receptors CR1, CR3 (Mac-1), fibronectin receptor, and the mannose-fucose receptor (MR) present on the surface of MΦ (Kane & Mosser, 2000). Once attached, surface molecules such as lipophosphoglycan (LPG), GP3, and Proteophosphoglycans can enhance phagocytosis of the parasites (Naderer et.al., 2008; Yao et.al., 2003).

MΦ can be stimulated by number of stimuli producing different subsets of MΦ that can determine the outcome of the disease (Liu & Uzonna, 2012; Mosser, 2003). Based on the stimulus, MΦ can be broadly divided into classical activation or alternative activation of MΦ. Classically activated MΦ induced by Th1 cytokines secret nitric oxide (NO) (Liew et.al., 1990) and number of inflammatory cytokines such as TNF-alpha and IL-1 (Mosser, 2003). NO is detrimental to leishmania and its production in undetectable in absence of IFN-γ implicating IFN-γ as being the major cytokine for MΦ activation and parasite elimination (Santos et.al., 2006). Alternatively, MΦ induced by Th2 cytokines such as IL-4 and IL-13 are characterized by low IL-12, low TNF and low ROS production and increase TGF-β, IL-10 and arginase activities 13 (Gordon & Martinez, 2010).

For a productive infection to occur, *Leishmania* must establish themselves in MΦ since MΦ contain very potent antimicrobial functions, *Leishmania* need to avoid MΦ activation and antigen presentation to T cells. For this, the parasites use several strategies of immune evasion; for instance alteration of host complement system (Brittingham & Mosser, 1996), protection against leishmanicidal products such as impairment of oxidative burst (Buchmuller-Rouiller & Mauel, 1987; Chan et.al., 1989; Passwell et.al., 1994; Zandbergen G et.al., 2004), cytokine modulation (Cummings et.al., 2010), inhibition of antigen presentation (Kaye et.al., 1994; Pinheiro et.al., 2004), and alteration of T cell differentiation and function (Alexander & Bryson, 2005). Understanding the possible mechanisms how these parasites avoid activation of macrophages is a key question in leishmaniasis.

1.5 Leishmania amazonensis

L. amazonensis, a member of the *Leishmania mexicana* complex, constitutes important group of parasites which causes severe infections with diverse clinical forms in humans (Almeida et.al., 1996). The disease caused by this parasite is characterized by uncontrolled parasite replication and profound immunosuppression (Afonso & Scott, 1993; Ji et.al., 2002; Ji et.al., 2003; Jones et.al., 2002). This parasite has been shown to alter the host cell defense mechanisms in several ways such as inhibition of antigen presentation and inhibition of reactive oxygen species (ROS) and NO production (Gomes et.al., 2003; Horta et.al., 2012; Meier et.al., 2003). Studies conducted in experimental cutaneous leishmaniasis mouse models demonstrate that most inbred mouse strains, which are resistant to *L. major* infection, are susceptible to *L. amazonensis* representing different modes of pathogen induced regulatory mechanisms for immune down modulation (Afonso & Scott, 1993; Ji et.al., 2002).

1.6 Virulence factors of *Leishmania* and role of ectonucleotidases during infection.

Survival of *Leishmania* in the host cells is governed by various factors primarily by their efficient capacity to remain inside the host cells and secondly triggering a powerful immune modulation. Because *Leishmania* amastigotes can rarely access to the extracellular environment, any nutritional demand must be fulfilled from the reservoir of the host cells or brought inside. *Leishmania* possesses several virulence factors such as LPG, amastigote specific protein (A2), cysteine proteinases, and gp63 (Matlashewski, 2001). A group of enzymes (ecto-NTPDase and

5'-nucleotidase) that have ability to cleave extracellular adenosine triphosphate (ATP) into adenosine has been reported in the parasites as a potential regulator of immune response in leishmaniasis (Sansom et.al., 2008). These enzymes are specifically used to obtain purine nucleosides from the extracellular environment in order to compensate for the lack of *de novo* synthesis (Marr et.al., 1978). Adenosine, the end product from ATP hydrolysis by ecto-enzymes, is a very strong immunomodulatory molecule which can suppress immune responses (Ohta & Sitkovsky, 2001). Our laboratory has discovered that the level of ectonucleotidase activity can determine the severity of the infections. *L. amazonensis* shows higher ectonucleotidases activity comparing with other species of *Leishmania* and usually presents severe infections in experimental models (de Almeida Marques-da-Silva et.al., 2008; de Souzaet.al., 2010; Maioli et.al., 2004). In addition, it has also been proposed that the level of ectonucleotidase activity may also determine the clinical manifestations of the disease in humans. Clinical isolates having high ectonucleotidase activities have been found to inhibit DC activation as well as NO production by MΦ *in vitro* (Leite et.al., 2012).

1.7 Purinergic signaling and immune response

The purinergic signaling system comprises of ectonucleotidases, extracellular purines (mainly ATP and adenosine) and their receptors. Extracellular nucleotides produced from living or dying cells act as indicators for various physiological or pathological conditions (Abbracchio et.al., 2009; North & Verkhratsky, 2006; Pankratov et.al., 2006). The role of ATP and adenosine released extracellularly during the complex process of inflammation or infection is chiefly known for its potential to alert defense system for the presence of pathogen associated membrane patterns (PAMPs). It is regarded as a "danger signal" (Bours et.al., 2006) as it promotes P2X7 mediated activation of inflammasome with the release of several proinflammatory cytokines such as IL-1β, IL-6, TNF-alpha and IFN-γ from activated MΦ, DC, and other immune cells (Atarashi et.al., 2008; Bours et.al., 2006; Langston et.al., 2003). ATP participates in maturation, differentiation and proliferation of immune cells. It attracts leukocytes to the sites of infection and inflammation (Chen et.al., 2006; Salmi & Jalkanen, 2005). It assists in neutrophil adhesion to vascular endothelium and subsequent migration to the sites of inflammation followed by increase phagocytosis as well as microbicidal activities (Bours et.al., 2006). It also affects

number of other immune cells such as the effector T cell functions. Lymphocytes proliferation and activation are also driven by the presence of extracellular ATP (Bours et.al., 2006)

Adenosine, the final product of ATP hydrolysis, is a potent immunosuppressive molecule. The extracellular concentration of adenosine is usually low, however, its concentration increases during ATP hydrolysis by ectonucleotidases or by suppression of adenosine kinase (Deussen, 2000; Pastor-Anglada et.al., 2001). During excessive inflammation and infection, both activated MΦ and neutrophils produce adenosine (Deussen, 2000; Hasko & Cronstein, 2013; Pastor-Anglada et.al., 2001). Adenosine can inhibit recruitment of neutrophils to vascular endothelium (Cronstein et.al., 1983) and also reduces phagocytosis as well as oxidative toxic metabolites such as ROS in activated neutrophils and MΦ (Cronstein et.al., 1983; Hasko & Pacher, 2012; Taylor et.al., 2005). It is observed that in presence of adenosine, maturation and differentiation of monocytes are considerably decreased when MΦ are treated with extracellular adenosine thereby leading to reduce pro-inflammatory cytokine production. Adenosine has been found to inhibit IL-12 and TNF alpha production from MΦ. Recently, studies have indicated that adenosine can transform into alternatively activated MΦ (Hasko & Pacher, 2012). Furthermore, it has also been shown that accumulation of extracellular adenosine, driven by ATP hydrolysis, is important to induce LPS stimulated MΦ into regulatory cells (Cohen et.al., 2013). Regulatory MΦ are characterized by low production of IL-12 and TNF and increase IL-10 and TGF-β that can favor for parasitic survival and growth (Mosser, 2003; Noel et.al., 2004).

Once outside, both ATP and adenosine can trigger different classes of receptors (**Figure II**); ATP acts on P2 receptors which are further subdivided into P2Y and P2X sub-classes (Abbracchio et.al., 2006; Abbracchio et.al., 2009; Burnstock, 2007; Ralevic & Burnstock, 1998). Among P2X receptors, P2X7 is also known for its function as a permeable channel through which ATP is released extracellularly. *L. amazonensis* infected MΦ have been shown to pump ATP outside through P2X7 channel and induce apoptosis (Marques-da-Silva et.al., 2011). There are infectious pathogens that can modulate P2X7 receptors (Miller et.al., 2011). P2Y receptors are nucleotide-sensitive G protein-coupled receptors (GPCRs) which act through cyclic adenosine monophosphate (cAMP) or inositol triphosphate (InsP3) (Abbracchio et.al., 2006). Extracellular ATP and P2Y receptor activation have shown to play important role in several infections (Save & Persson, 2010; Seror et.al., 2011).

Adenosine specifically binds to P1 receptors (**Figure II**). The P1 class has four types of G protein-coupled adenosine receptors A1, A2a, A2b and A3. A1, A2a, and A3 receptors can be activated by low concentration of adenosine; whereas A2b has low affinity towards adenosine (Hasko.,et.al., 2007). MΦ can express all four adenosine receptors depending on the activation state (Kobie et.al., 2006). A1 and A3 have proinflammatory and A2a and A2b have anti-inflammatory activities (Abbracchio et.al., 2009; Fredholm et.al., 2001). A2 receptors are associated with delayed maturation, differentiation of phagocytic cells as well as decreased activation and reduced phagocytic activities (Xaus et.al., 1999). A2 receptors also inhibit production of IL-12 production by DC and interfere with the differentiation of naïve T cells into the Th1 phenotypes (Haag et.al., 2007). Adenosine receptors have also been studied in association with tumors. The progression of the disease is delayed when A2a receptor is blocked (Ryzhov et.al., 2008). In addition, adenosine receptor activation can allow for the expansion of T cells lacking effector functions in presence of excessive adenosine (Ernst et.al., 2010)

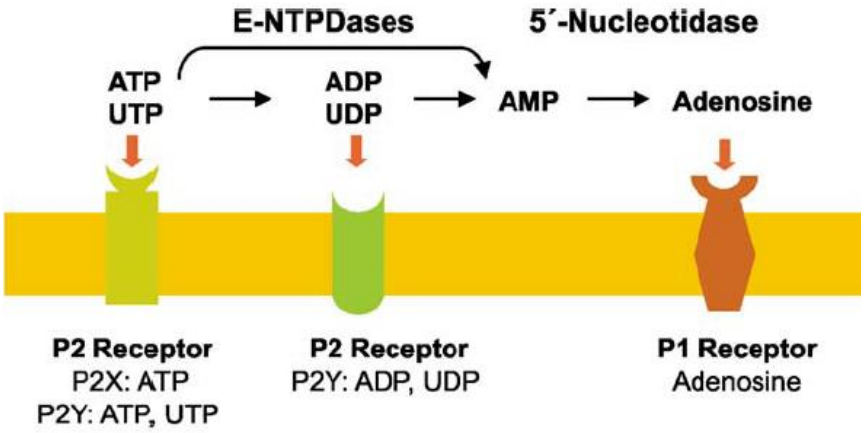


Figure II: Cell surface-located catabolism of extracellular nucleotides and potential activation of receptors for nucleotides (P2 receptors) and adenosine (P1 receptors). Taken from (Robson et.al., 2006)

CD39, ectonucleoside triphosphate diphosphohydrolase, is the major determinant for the regulation of extracellular ATP levels in blood. It is virtually present in all different types of immune cells such as B cells, natural killer cells, dendritic cells, monocytes, MΦ. CD39 plays critical role on recruitment, activation of neutrophils, DC and MΦ. It also helps in polarization of naive T cells (Mizumoto et.al., 2002). CD73, also known as 5´ectonucleotidases, is expressed by

a number of cells but its expression varies differently according to maturation and activation state (Zimmermann, 1992). It has been demonstrated that CD73 is the main source of adenosine production and depletion of this enzyme reduces adenosine production while it increases leukocyte adhesion in endothelial barriers (Grunewald & Ridley, 2010). A group of studies have demonstrated that in $CD73^{-/-}$ mice, leukocyte infiltration is reduced in inflammatory situations (Mills et.al., 2008; Reutershan et.al., 2009; Takedachi et.al., 2008). CD73 mediated adenosine production participates in specific immunosuppressive functions through the inhibition of TNF-alpha, IL-1, IL-6, and IL-12 synthesis (Bours et.al., 2006; Desrosiers et.al., 2007; Hasko et.al., 2008).

CD39 and CD73 work together to produce an adenosine rich environment. Expression of these enzymes on the surfaces of immune cells largely determines the pathophysiological conditions. It has been noted that immune suppression such as inhibition of proinflammatory cytokines release (Imai et.al., 2000) or DC inactivation (Borsellino et.al., 2007) occurs via combined actions of CD39 and CD73 expressed on the activated Treg cells (Borsellino et.al., 2007; Deaglio et.al., 2007). Adenosine is able to hamper lymphocyte migration in to draining lymph nodes (Takedachi et.al., 2008) as well as hinders TCR-mediated T cell proliferation through stimulation of adenosine receptors (Huang et.al., 1997; Koshiba et.al., 1997). Furthermore, Th17 cells expressing CD39 and CD73 also participate in adenosine derived immunomodulations (Longhi et.al., 2014).

Classically activated MΦ have less ectonucleotidase activity whereas alternatively activated MΦ show increase capacity to ATP hydrolysis (Hasko & Pacher, 2012; Zanin et.al., 2012). It has been found that MΦ expressing CD39 are able to self-regulate LPS induced activation by producing adenosine via CD39 activities. Therefore, the immunomodulation by adenosine production via the actions of CD39/CD73 can convert classically activated MΦ into regulatory type macrophages (Cohen et.al., 2013). The effects of ectonucleotidases on the immune response can be one of the processes of immune suppression that are likely to be exploited by *Leishmania* to ensure their survival inside the host MΦ.

The role of CD39 and CD73 has been extensively studied in infections and inflammation. Several pathogens have been found to regulate host ectonucleotidase enzymes suppressing immune response and thereby increasing their viability in the host cells. *Staphylococcus aureus*

produces adenosine as a virulence factor to escape from the immune response (Thammavongsa et.al., 2009). Similarly, *Streptococcuss anguinis* survives from immune patrol through the immunosuppressive actions of adenosine (Fan et.al., 2012). Fungus such as *Candida parapsilosis* inhibits phagocytosis by the production of adenosine (Russo-Abrahao et.al., 2011). AIDS progression has been found to be associated with CD39/CD73 activities. CD39 expression on Treg cells exhibits high immunosuppressive activities via A2a receptors in HIV positive patients (Nikolova et.al., 2011). In parasites, such as in *Leishmania*, *Toxoplasma gondii*, *T. cruzi*, the disease outcome is believed to be associated with ectonucleotidase activities of the parasites as well as of the host enzymes. In addition, the role of these enzymes has been also described in autoimmune diseases for instance, rheumatic arthritis or inflammatory bowel diseases and in the progression of tumor. Adenosine release by Treg has also been shown to increase progression of cancer (Antonioli et.al., 2013)

1.8 Hypoxia inducing factor and its role in immune response

During excessive inflammation, extracellular ATP release and its degradation can be governed by several factors. Hypoxia is a common phenomenon at the foci of severe inflammation and is characterized by low local tissue oxygen levels due to congestion of leukocyte trafficking termed as inflammatory hypoxia (Karhausen et.al., 2005). Hypoxia-inducible factor 1 (HIF-1), a transcription factor, is induced in hypoxic tissues (Pugh & Ratcliff e, 2003; Semenza, 2003). It is a heterodimeric protein that consists of HIF-1α and HIF-1β subunits (Wang et.al., 1995). The overall activity of this molecule is determined by intracellular HIF-1α levels. HIF-1β is constitutively expressed while HIF-1α is tightly regulated by level of oxygen tensions in the tissues. Under normal physiological conditions, prolyl hydroxylases (PHDs) together with ubiquitin cascades degrade HIF-1 α (Ivan et.al., 2001). In contrary, deficiency of oxygen in hypoxic condition halts the functions of PHDs so that HIF-1 α is no longer further degraded and is accumulated inside the hypoxic cells. However, there is also evidence saying that HIF-1α can be induced under non-hypoxic conditions (Richard et.al., 2000). It has been reported that HIF-1α can be stablized in normoxic tissues and is important regulator of tissue homeostasis (Stroka et.al., 2001).

Myeloid cells can function in HIF-dependent manner in hypoxic tissues. HIF-1α has been shown to affect phagocytic properties, antigen presentation, processing and effector functions

(Cramer et.al., 2003). HIF-1α–null phagocytes, for example, cannot effectively kill bacteria. HIF-1α also regulates generation of ATP molecules at the inflamed tissues thereby stimulating aggregation, motility, invasiveness, and bactericidal activity of myeloid cells (Cramer et.al., 2003; Peyssonnaux et.al., 2005). It may be advantageous for some parasites such as *Leishmania* to regulate HIF-1α in order for to survive within their host cells.

Interestingly, a number of adaptive immune cells are also influenced by the presence of HIF-1α (Sitkovsky & Lukashev, 2005). Existing evidence supports that increased production of HIF-1α in T cells can shift type 1 helper T-cell (Th1) phenotype, which is essential for MΦ activation and cytotoxic T cells proliferation, to a type 2 helper T-cell (Th2) phenotype (Ben-Shoshan et.al., 2009) which, via release of IL-10 and TGF-β, can suppress IFN-γ, TNF-alpha and IL-12 production. HIF-1α also upregulates differentiation and proliferation capacity of Treg (Ben-Shoshan et.al., 2008) as well as enhances extracellular adenosine production (Deaglio et.al., 2007) which prevents tissues from aggressive effector T activities to maintain homeostasis (Sitkovsky, 2009).

As illustrated in figure III, ATP degradation is enhanced by HIF-1α driven increased enzymatic conversion into adenosine (Colgan et.al., 2006; Eltzschig et.al., 2003; Eltzschig et.al., 2009). HIF-1α can stimulate release of extracellular adenosine (Synnestvedt et.al., 2002) and at the same time can inhibit the process of its uptake into the intracellular compartment as well as its intracellular metabolism (Morote-Garcia et.al., 2009) preventing cells from generating effective innate immune responses (Ohta & Sitkovsky, 2001; Sitkovsky et.al., 2004). HIF-1α can also influence adenosine receptors thereby leading to enhanced adenosine receptor signaling (Eckle et.al., 2008; Kong et.al., 2006) and elevating intracellular cAMP and CREB activation (Stiles, 1992). HIF-1α induced adenosine receptors, such as A2b, have shown to produce increased intracellular cAMP levels by human DCs inducing Th2 cell polarization (Yang et.al., 2010).

One study indicates that hypoxia directly activates CD73 transcription *in vitro* (Kobayashi et.al., 2000). HIF-1α binds to the promoter region of CD73 (Synnestvedt et.al., 2002) while HIF-1α can regulate CD39 via specific protein (SP1) (Eltzschig et.al., 2009; Synnestvedt et.al., 2002) and that they can together regulate ATP degradation in inflammatory

conditions. Therefore, the effects of these enzymes can be altered by HIF-1α levels during inflammation.

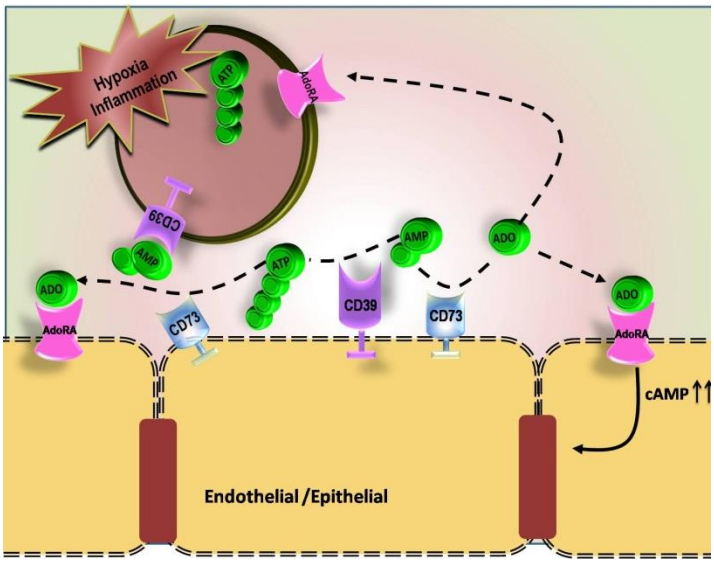


Figure III. Model of coordinated nucleotide metabolism and nucleotide signaling in hypoxia and inflammation (Colgan et.al., 2006)

Studies done in cutaneous lesions in experimental models infected with *Leishmania amazonensis* have shown that *Leishmania* can induce HIF-1α and HIF-1β (Arrais-Silva et.al., 2005) during chronic phase of infection. However, recently it becomes clear that the cells do not need to be hypoxic for HIF-1α expression in *Leishmania* infected HIF-1α (Degrossoli et.al., 2007). It is suggested that induction of HIF-1α (relative to hypoxia) could affect the microbial activities and protein expression of MΦ yielding different phenotypes from that of the normoxic counterparts so that this phenomenon may take part in modulating immune responses in pathological conditions (Degrossoli & Giorgio, 2007). It may be possible that *Leishmania* can alter immune functions of immune cells such as mononuclear phagocytes in *Leishmania* infection with the regulation of HIF-1α regardless whether cells are hypoxic or not.

The study of role of CD39 and CD73 in pathogen derived immunomodulation is a growing area and has not previously been explored in cells directly from the inflamed sites during leishmaniasis. Because expression of these enzymes can significantly influence on immune-modulation, *Leishmania* in *Leishmania*-infected MΦ may utilize these molecules during

their early host MΦ interaction as a part of their nutritional acquisition and then as a part of down modulation of immune response leading to uncontrolled parasite multiplication and MΦ inactivation. In brief, this study will try to address if CD39/CD73 pathway can be one of the possible regulatory mechanisms induced by *L. amazonensis* in MΦ. Since at the inflamed site, there is likely an inflammatory derived hypoxic environment, *L. amazonensis* induced HIF-1α may further regulate adenosine production via CD39 and CD73 pathway. Our findings from this study will help immunologists to understand early phase of host parasite interaction and subsequently for the effective treatments.

2. JUSTIFICATION

2. JUSTIFICATION

CL associated with *L. amazonensis* infection is usually severe in experimental mice as well as in humans. It often causes diffuse cutaneous leishmaniasis or in rare cases, VL in South American countries (Almeida et.al., 1996). The severity of the disease is further complicated due to failure in several treatment regimens and emerging co-infections (Chkravarty & Sundar, 2010). The underlying mechanisms for the pathogenesis of non-healing lesions are, however, still obscure. *L. amazonensis* can dampen both innate as well as adaptive immune responses at the site of infection where conditions can further be worsened by drastic changes in tissue metabolism, for example, increased nucleotide metabolism (Cramer & Johnson, 2003) and with the up-regulation of HIF-1α (Degrossoli et.al., 2007). The possibility that *L. amazonensis* may exploit host properties (such as host ectonucleotidases in this context) has not yet been explored in the early stages of their interaction with MΦ. Production of adenosine may also regulate MΦ activation state by triggering A2a and A2b receptors. These receptors can further be influenced by HIF-1α.

From the standpoint of the parasites, CD39/CD73 pathway can be crucial as it may lead to interference in T cell priming, differentiation and proliferation favoring generation of inefficient effector T cells and inhibition of MΦ activation through repertoires of cytokines. Anti-inflammatory response induced by adenosine, the final product of enzymatic conversion of extracellular ATP, is associated with an increase in intracellular cAMP that inhibits cytokine responses of many immune/inflammatory cells including T cells and MΦ activation (Ernst et.al., 2010). In summary, in case of non-healing infections, such as in *L. amazonensis* infected subjects, we propose that *L. amazonensis* during its interaction with MΦ may regulate extracellular nucleotides by the use of host ectonucleotidases (CD39 and CD73) and release potent anti-inflammatory molecule (adenosine), which via A2a and A2b, induces development of regulatory MΦ favoring more parasite growth. Study of these series of intertwined processes will help to improve understanding of mechanisms related to host-*Leishmania* interactions.

3. OBJECTIVES

3. OBJECTIVES

3.1 GENERAL OBJECTIVE:

Determine the effects of *L. amazonensis* infection on the expression of CD39 (ecto-NTPDase), CD73 (ecto-5'ectonucleotidases) and the involvement of adenosine receptors in the parasitic survival in resident MΦ

3.2 SPECIFIC OBJECTIVES:

1. Determine the expression of CD39 and CD73 in resting resident MΦ
2. Determine the role of CD39 and CD73 in survival of *L. amazonensis*
3. Determine the role of A2a and A2b receptors in survival of *L. amazonensis*
4. Determine the effects of HIF-1α on the expression of CD39 and CD73 as well as in parasite survival and infection in MΦ *in vitro*
5. Investigate mechanisms involved in parasite survival in resident MΦ
6. Determine mRNA expression of A2a and A2b receptors in *L. amazonensis* infected MΦ and evaluate role of HIF-1α on the adenosine receptors in infected MΦ.

4. MATERIALS AND METHODS

4.1 Animal:

C57BL/6 mice (8-12 weeks, both male and female) were used for the purpose of our study. Mice were housed and maintained at the central animal facility in the Universidade Federal de Ouro Preto (UFOP). All animal experiments and procedures were approved by the institution's committee on ethical handling of laboratory animals. (Protocol 2012/56)

4.2 Preparation of parasites:

Leishmania amazonensis (IFLA/BR/1967/PH8) were cultivated at the initial concentration of $1x10^5$ at 25^0C in grace's base (Sigma Aldrich Inc, St.Louis, MO, USA) supplemented with 10% inactivated fetal bovine serum (FBS) (SFB-LGC Biotecnologia, Cotia, SP, Brasil), 2mM L-glutamine (GIBCO BRL-Life Technologies, Grand Island, NY, MO, EUA), 100U/ml penicillin G (USB Corporation, Cleveland, OH, USA), pH 6.5. Five days old stationary phase promastigotes were used for metacyclic isolation and purification as described in protocol by Spath e Beverely (Spath & Beverely, 2001) and adapted from Marques-da-Silva (de almeida Marques- da- Silva et.al. 2008). Parasites were washed twice with phosphate buffered saline (PBS), and centrifuged at a speed of 1540 x *g*, 4^0C for 10 min. Parasites were resuspended in dulbecco's modified eagle medium (DMEM) (Sigma Aldrich Inc) pH 7.2, and 2ml of parasitic suspension was distributed in tubes. This was followed by Ficoll® (Amersham Biosciences do Brasil, Sao Paulo, Sp, Brasil) gradient by adding 2ml of 10% Ficoll. This preparation was centrifuged at a speed of 1070 x *g*, 25^0C, for 15 min. After centrifugation, supernatant was collected in another tube without disturbing the pellet. Supernatant, rich in metacyclics, was further washed twice with PBS to remove any remaining contamination from Ficoll and other ingredients. Parasites were always kept in ice in an interval between proceeding steps.

4.3 CFSE labeling:

Purified metacyclics were suspended in PBS in a concentration of $6X10^7$ parasites /ml. carboxyfluorescein diacetate sccinimidyl ester (CFSE) dye was laid over the 50µl of PBS and then well mixed with parasites by inverting the tube. The final concentration of CFSE was maintained at 5µM. Whole suspension was incubated at 37^0C for 10 min (Goncalves et.al., 2005). Parasites were then washed with PBS/10%FBS once and it was followed by washing parasites twice with PBS at a speed of 1540 x *g*, 4^0C for 10 min. Parasites were finally suspended

in DMEM supplement medium/10% FBS and kept in ice until use. CFSE tagged parasites were always protected from direct light.

4.4 Harvest peritoneal macrophages:

Animals were euthanized and the abdomen was gently massaged and peritoneal lavage was collected by using ice cold PBS with 16G needle and peritoneal lavage was collected by injecting 10ml of ice cold PBS with 16G needle (Zhang, X et.al., 2008). Cells were centrifuged at a speed of 210 x *g*, 4^0C for 10 min and were suspended in DMEM supplemented with 10% FBS pH 7.2. Viability of the cells was confirmed by using Trypan blue (Sigma-Aldrich). Cells ($5x10^5$ cells) were distributed *in vitro* medium Dulbecco's modified eagle´s medium (DMEM-Sigma) supplemented with/10% FBS, 100 U/ml penicillin G, 2mM glutamine, 25mM HEPES (Sigma), 1.2mM sodium bicarbonate (Vetec Quimica Fina Ltd) and 50μM 2-mercaptoethanol (Pharmacia Biotech). Freshly harvested MΦ were pooled before treatment or any further incubation. Once harvested, MΦ were either analyzed *in ex-vivo* or cultivated *in vitro* culture medium. *In vitro* experiments, MΦ were rested for 24, 48 and 72hrs and subsequently incubated at 33^0C/5% CO_2 without any external stimulus. The shift in temperature was made considering the fact that *L. amazonensis* is sensitive to 37^0C (Ref). Therefore, in all experiments, resident cell population was rested for 72hrs at 37^0C/5% CO_2 prior to any further treatment and analysis. Further incubation was done at 33^0C/5%CO_2 for any longer period of time. For flow cytometry experiment, 6 mice were utilized whereas for *in vitro* infection in plates, 10 mice were used for each experiment.

4.5 *In vitro* infection of macrophages:

For analysis by flow cytometry, 72hrs rested resident cells were infected with metacyclics forms of *L. amazonensis* labeled with CFSE in a ratio of 1:3 in DMEM supplemented medium. In parallel, another group was treated with LPS obtained from *E. coli* (Sigma Aldrich Inc) at the concentration of 5μg /ml and mixed well. The cells were then incubated at 33^0C/5% CO_2 for 24hrs or 48hrs. The expression of CD39 and CD73 was then analyzed by flow cytometry.

In *in vitro* infection studies, harvested cell population was rested for 72hrs on 24-well plates provided in each well with grease free sterile cover slips of 13mm diameter. Any unbound

resident cells were removed by washing two times with PBS before infection or further treatment. New fresh medium was added to the rested resident MΦ and then infected with metacyclic forms of *L. amazonensis* in a ratio of 1:3. Cells were incubated at 33^0C/5% CO_2 for 3 hours and excess parasites were then removed by washing twice with PBS. Cells were further incubated at 33^0C/5% CO_2 for 24hours or 48hours.

4.6 CD39 and CD73 inhibition experiments

Inhibitors of CD39 and CD73, DIDS (4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate-Sigma) and αβ MAD (α,β-Methyleneadenosine 5′-diphosphate sodium salt-Sigma) were added at a concentration of 200μM after 3hrs of initial infection and were then kept throughout the infection study. These inhibitors were dissolved in PBS.

4.7 Study of role of A2a and A2b receptors *in vitro*

Resting MΦ were treated with MRS 1754 8-[4-[((4-Cyanophenyl) carbamoylmethyl) oxy] phenyl]-1,3-di(n-propyl) xanthine hydrate (Sigma-Aldrich), inhibitor for A2b receptor and ZM241385(4(-2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl) Phenol **(**Sigma-Aldrich), for A2a receptor, at the concentration of 5μM at the time of infection and left throughout the incubation time period. These antagonists were prepared in DMSO and therefore control groups were treated with DMSO not exceeding 1% in final volume.

In all conditions, coverslips were removed 3hrs, 24hrs and 48hrs post infection from macrophage culture plates. Coverslips were then fixed in methanol for 10 min (Vetec Fine Chemistry), dried and stained using the kit Panótico Rápido (Laborclin, Pinhais, PR, Brazil) following manufacturer's instructions. Coverslips were analyzed using an Olympus BX50 optical microscope (Olympus, Center Valley, PA, USA). A minimum of 200 macrophages per coverslip was examined and number of uninfected, infected and amastigotes in infected macrophages were recorded.

4.8 *In vivo* analysis of CD39 and CD73 expression in macrophages:

To determine the effects of *Leishmania* infection on the expression of CD39 and CD73 *in vivo*, CFSE labeled metacyclic forms of *L. amazonensis* were inoculated to C57BL/6 mice at the concentration of $50X10^6$parasites/ml intraperitoneally. PBS was inoculated in control groups.

Mice were euthanized after 24hrs inoculation. Peritoneal lavage by ice cold PBS was performed and cells were harvested and studied in *in ex-vivo* for the expression of CD39 and CD73 in resident MΦ.

4.9 Effects of HIF-1α on the expression of CD39 and CD73 in MΦ *in vitro* infection:

In *in vitro* infection, 72hrs rested cell population was treated with the FM19G11 (2-oxo-2-(p-tolyl)ethyl] 3-[(2,4-dinitrobenzoyl)amino]benzoate, 3-[(2,4- initrobenzoyl)amino]- benzoic acid 2-(4-methylphenyl)-2-oxoethyl ester) (Sigma-Aldrich), an inhibitor of HIF-1α, at a concentration of 100nM and 200nM. The inhibitor was added at the time of infection together with the parasites and was left there throughout the infection. This inhibitor was dissolved in DMSO. Control groups were always treated with DMSO. The expression of CD39 and CD73, in presence of FM19G11, was evaluated by flow cytometry after 24hrs of incubation with parasites.

Effects of the inhibitor were initially evaluated by cultivating *L. amazonensis* with the inhibitor and plotting a growth curve. *L. amazonensis* was cultivated in Grace´s medium/10% FBS. Inhibitor to HIF-1α in two different concentrations of 100nM or 200nM was added at the time of cultivation. Cultures were incubated at 25^0C for 6 days and growth curve was plotted. In a control group, DMSO was added.

4.10 Flow cytometry:

All samples were washed twice with PBS (210 x g, 40 C, 10 min). Cells were then resuspended in 0.2% bovine serum albumin (0.2% BSA/PBS), and Fc-blocked (purified rat anti-mouse CD16/CD32, clone 2.4G2, BD Pharmingen) for 15 min in ice. Cells were then washed and stained with anti-mouse F4/80 PECY7 antibody (Clone BM8, Biolegend), anti-mouse CD73PE antibody (Clone Ty/11.8, eBiosciences), and anti-mouse CD39 – alexa fluor 647 (clone 24DMS1, eBiosciences) antibodies in ice for 30 min. Cells were washed with PBS and then fixed in 250 µL fixation solution (1% paraformaldehyde, 47.7mM sodium cacodylate, 113mM NaCl, pH 7.2). Samples were analyzed using a BD FACSCaliburTM flow cytometer. All cytometric analyses were done by using Flow Jo version 7.6.5 (Tree Star, Ashland, OR, USA).

4. 11 Cytokine assays:

TNF-alpha, and IL-10 cell culture supernatants were determined by ELISA kits (Mouse TNF-alpha DuoSet catalogue DY410, Mouse IL-10 Duoset catalogue DY417E from R&D system). Assays were performed according to manufacturer´s instructions. Briefly, flat-bottom 96-well microtiter plates (Nunc) were coated with 100 µL/well of TNF-alpha and IL-10 specific monoclonal antibodies (0.2 µg/mL and 2.0 µg/mL, respectively) for 18hrs at 4^{o} C and then washed with PBS buffer (pH 7.4) containing 0.05% Tween 20 (wash buffer). Nonspecific binding sites were blocked with 300 µL/well of 1% BSA in PBS. Plates were rinsed with wash buffer, and 100 µL of samples and standards were added followed by incubation for 2hrs at room temperature. Seven fold serial dilution of standards of each these cytokines was prepared. Plates were then washed and 100 µL of the appropriate TNF-alpha (50 ng/mL) and IL-10 (100 ng/mL) biotinylated detection antibodies diluted in blocking buffer containing 0.05% Tween 20 were added for 1hr per well at room temperature. Plates were, then, washed and streptavidin-horseradish peroxidase (0.1 µg/mL) added for 30min of incubation at room temperature. Finally, plates were washed and 100 µL of the substrate solution (1:1-mixture H_2O_2 and Tetramethylbenzidine) was added per well and, after 30 min of a dark incubation at room temperature, the reaction was stopped by 50 µL/well of 1M H_2SO_4 solution. Plates were read at 450 nm with wavelength (Microplate Reader, model 680, BioRad). All samples were assayed in triplicate using DuoSet® ELISA Development System, R&D Systems®, Minneapolis, MN systems.

4.12 Nitric oxide measurement:

Nitric oxide (NO) in cell culture supernatant was measured by spectrophotometric assay based on the Griess reaction (Green et.al., 1982). Briefly, 100µl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediaminedihydrochloride in 2.5% H3PO4) was added to a 100µl sample and mixed immediately. After 10 min of incubation at room temperature in dark, absorbance was measured at 570 nm. The nitric oxide level in the given sample was determined by comparing with the standard curve obtained from sodium nitrate (Sigma) which was run together with the samples. Diluent (DMEM) was used as a blank for the assay. Levels of nitrite in the supernatant of cell culture were determined using the Griess reaction as an index of NO production.

4.13 Relative mRNA expression of adenosine receptors by real time PCR:

RNA was extracted from cultivated cells by treating with brazol (1ml for 5-10 x 10^6 cells). Cells were incubated with brazol for 5 min at 15-30^{0}C. Cells were then centrifuged at 12,000 x g/2-8^{0}C/15min. Supernatant was then collected in another tube. 0.2ml of chloroform was added to each 1ml of brazol used to lyse the cells and then vortexed vigorously for 15sec. It was incubated on ice for 2 min. It was then centrifuged at 12,000 x g/2-8^{0}C/15min. After centrifugation, top layer which contains RNA was collected. RNA was then treated with 0.5ml of isopropyl alcohol and incubated it for 10 min at 15-30^{0}C. It was then centrifuged at 12,000 x g/2-8^{o}C/10 min. Supernatant was removed and precipitated RNA was washed with 75% of alcohol followed by centrifugation at 7500 x g/2-8^{0}C/5 min. Precipitate was then dissolved in RNAse free water and was quantitated in spectrophotometer. Pure RNA will exhibit an A260/A280 ratio of 2.0.

cDNA was prepared using high capacity cDNA reverse transcription kit (Applied Biosystems). Mastermix was prepared containing 2µl of 10X RT buffer, 0.8µl of 25X deoxynucleotides mix (dNTPs), 1µl of RNase inhibitor, and 1µl of multiScribe reverse transcriptase, 2µl of 10 X random primers and 3.2 µl DEPC water and mixed with 10µl of sample (0.025µ/µl) to make the final volume 20µl. Then, the tubes were submitted in thermocycler with following temperatures: 25^{o}C 10 min, 37^{o}C 120 min, 85^{o}C 5 min and rest at 4^{o}C. After this process cDNA was ready for PCR and maintained at -20^{o}C.

Relative gene expression of adenosine receptors A2a and A2b in MΦ was performed by 7500 software V2.0.6 (Applied Biosystems), utilizing power SYBER® Green PCR Master mix. For each reaction, mix containing 7.5 µl of SYBER®Green Master Mix, 0.3µl of each sense and anti-sense primer (10µM), 2µl (10ng per reaction) of cDNA and 4.9µl of RNAse free water to make a volume of 15µl was used. The standard PCR conditions were as follows: 50ºC (2 min), 95°C (10 min); 40 cycles of 95°C (15sec) and 60°C (1 min). PCR amplification was performed in triplicates using standardized conditions. In the same run, the threshold cycle (Ct) values of the samples will be measured and relative expression levels were determined. Ct values obtained from triplicates were used to calculate the expression of target gene after normalizing by reference gene (β actin gene). Then, difference of Ct observed between test samples and Ct obtained from the amplification of uninfected and untreated groups was used for analysis.

Finally, it was then checked how many times the test samples expressed mRNA expression in relation to that uninfected group using formula $2^{-\Delta\Delta Ct}$.

Melting curve analysis was also performed during amplification to determine any non-specific amplification. Data were normalized to the expression levels of reference gene (β actin). Primer sequences for the target gene were illustrated below.

Primer Sequences

Gene	**Forward sequence**	**Reverse sequence**
β- actin	ACTGCTCTGGCTCCTAGCAC	ACATCTGCTGGAAGGTGGAC
A2a	GGCTCCTCGGTGTACATCAT	GTTCTGCAGGTTGCTGTTGA
A2b	TTCTTTGGGTGTGTCCTTCC	CCTGGAGTGGTCCATCAGTT

4.14 Statistical analysis:

Data were expressed as mean±SD. Several group data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni posttest or by Dunnett posttest against the control group. Two group comparisons were performed by paired Student t-test. p-value< 0.05 was considered statistically significant.

5. RESULTS

5. RESULTS:

Expression of CD39 and CD73 in resident MΦ has been illustrated here *in vitro*. Regulation of CD39 and CD73 expression by rested resident MΦ in *in vitro* culture medium has also been described. The effects of *L. amazonensis* on CD39 and CD73 expression have been reported for the first time here in resident MΦ and at the same time, the role of these molecules in parasite survival and infection has been discussed. Adenosine production via CD39 and CD73 enzyme activity binding to A2a and A2b receptors and its significance in parasite growth and survival has been shown. A possible link between HIF-1α and CD39 and CD73 has also been studied during *L. amazonensis* infection. The regulation of A2a and A2b receptors by HIF-1α during *L. amazonensis* infection at transcript levels was studied after 24hrs of incubation.

5.1 Resident macrophages are characterized by high CD39 and CD73 expression

In order to determine the effects of *L. amazonensis* on CD39 and CD73 expression in MΦ, we first analyzed expression of these enzymes in resident cell population based on F4/80 marker. When analyzed in flow cytometry, whole $F4/80^{+}$ population (MΦ) expressed CD39 **(Fig. 1A)** indicating CD39 as a predominant ectonucleotidase enzyme in MΦ while 39% of non-macrophage groups presented this enzyme. CD73 expression was equally higher in MΦ than non-MΦ **(Fig. 1B)**. In total, MΦ represented the major cell population expressing both ecto-nucleotidases CD39 and CD73 **(Fig 1C)**.

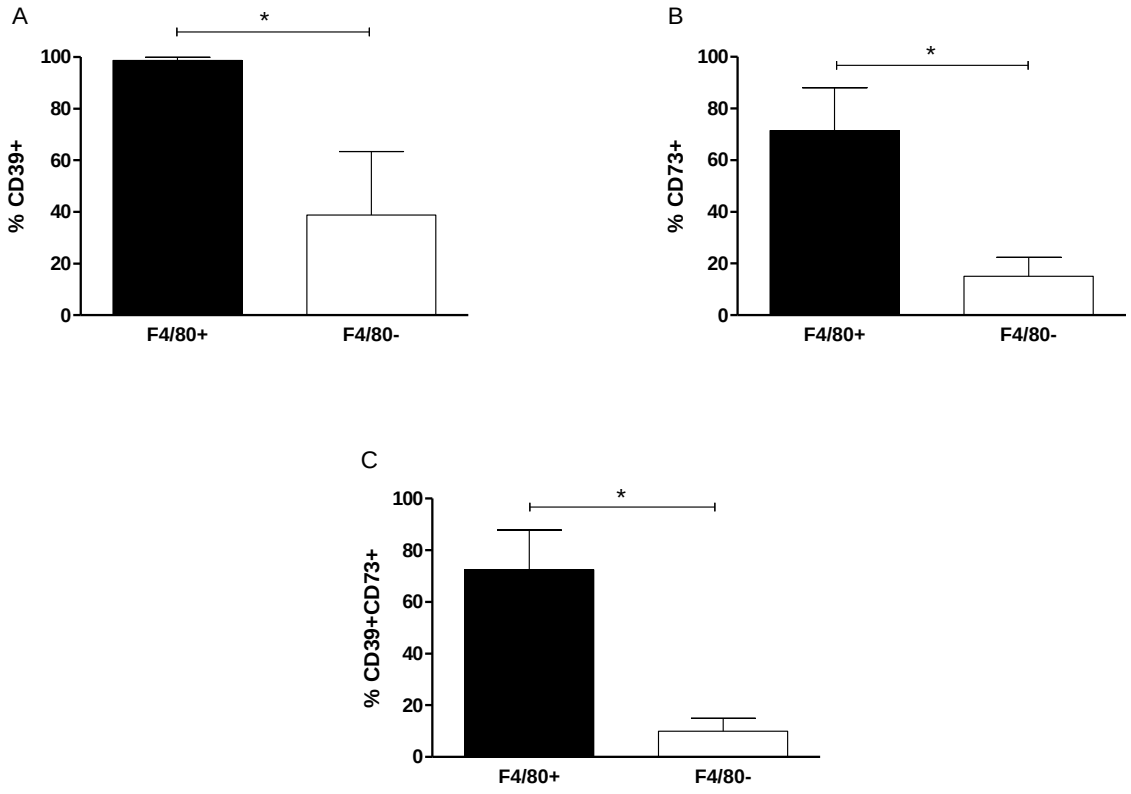


Figure 1: Peritoneal resident macrophages are major cell populations that express both CD39 and CD73 *in ex-vivo*. Resident MΦ were harvested and pooled from peritoneum of naïve C57BL/6 mice. Cells were labeled with anti-murine F4/80, anti-CD39 and anti-CD73 antibodies and then were analyzed by flow cytometry. In the figure, the percentage of cells expressing A. CD39 and B. CD73 and C. CD39CD73 is shown for $F4/80^{+}$ and $F4/80^{-}$ cells. This result is the mean±S.D of at least 3 independent experiments. *$p<0.05$ means the statistically different using Paired two tailed Student´s t- test.

5.2. Resident macrophages down regulate CD73 expression *in vitro*

Before studying the role of CD39 and CD73 in *Leishmania* infection, whole peritoneal cell population was cultivated *in vitro* medium in order to determine if these surface enzymes can self-regulate in *in vitro* conditions. As shown in **Fig. 2**, although the percentage of MΦ remained constant during incubation period **(Fig. 2B),** these cells surprisingly down regulated CD73 while their CD39 expression remained unaltered **(2C–2F)**. CD73 expression gradually decreased over the incubation period suggesting that the culture conditions may have an influence in the expression of CD73 *in vitro*. On the other hand, it may also be possible that the expression of CD73 could have been increased by the harvesting procedure and then slowly returned to the basal level.

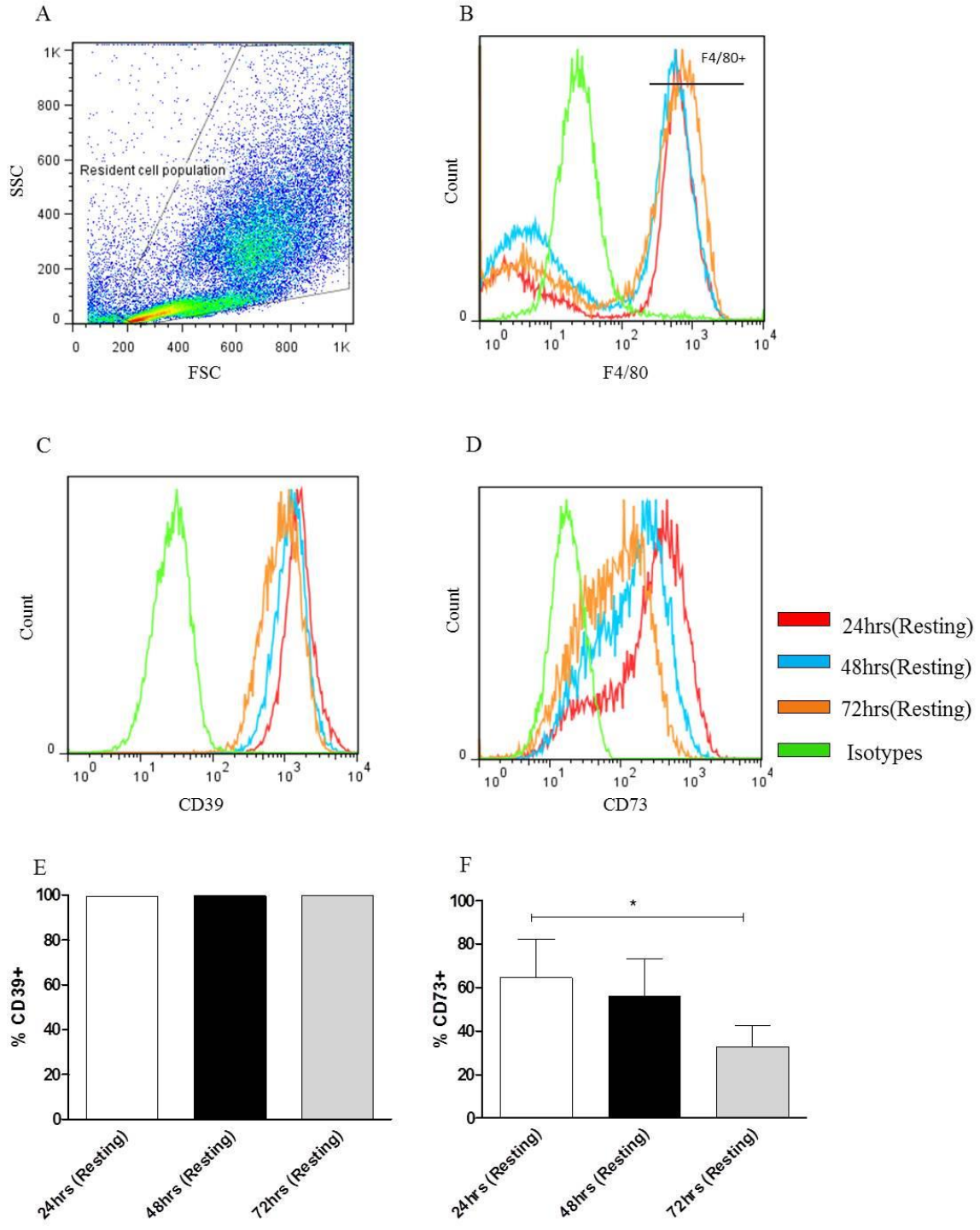

A
SSC
FSC
Resident cell population
B
F4/80+
Count
F4/80
C
Count
CD39
D
Count
CD73
24hrs(Resting)
48hrs(Resting)
72hrs(Resting)
Isotypes
E
% CD39+
24hrs (Resting)
48hrs (Resting)
72hrs (Resting)
F
% CD73+
*
24hrs (Resting)
48hrs (Resting)
72hrs (Resting)

Figure 2: Resident macrophages down regulate CD73 expression *in vitro*. Resident MΦ were harvested from naïve C57BL/6 mice. Total peritoneal cell population was counted and viability of the cells was determined by Trypan blue. Freshly harvested cells were rested for 24, 48 and 72hrs prior to further incubation at $33^{o}C/5\%CO_2$. Six mice were used for each experiment and cells were pooled for the analysis. Fig.2A shows size and granularity for total peritoneal population. In figure 2B, $F4/80^{+}$ cells were first gated and then cells expressing C. CD39 and D. CD73 in $F4/80^{+}$ population from 24, 48 and 72hrs rested MΦ were over laid in histograms. The percentage of cells expressing E. CD39 and F. CD73 is represented in bar diagrams for MΦ. This result is representative of 3 independent experiments. *$p<0.05$ indicates statistically different using Paired two tailed Student's test

5.3. *L amazonensis* increases CD73 expression in rested macrophages

Once it was determined that CD73 expression could be down regulated by the rested MΦ, our next approach was to observe if the infection by *L. amazonensis* can further influence in CD39 and CD73 expression in MΦ. Seventy two hours rested MΦ were incubated with CFSE labeled metacyclics of *L. amazonensis*. After 24hrs of infection, it was found that although the percentage of $CD39^{+}$ cells did not alter **(Fig. 3A)**, there was a significant increase in the percentage of cells expressing CD73 **(Fig. 3B)**. LPS treatment did not affect the expression of CD39 or CD73, suggesting that activation does not alter CD73 expression **(Fig 3A and 3B)**. **Figure 3C** demonstrated that the combined expression of CD39 and CD73 was higher amongst *L. amazonensis* infected MΦ when compared to unstimulated or LPS treated cells.

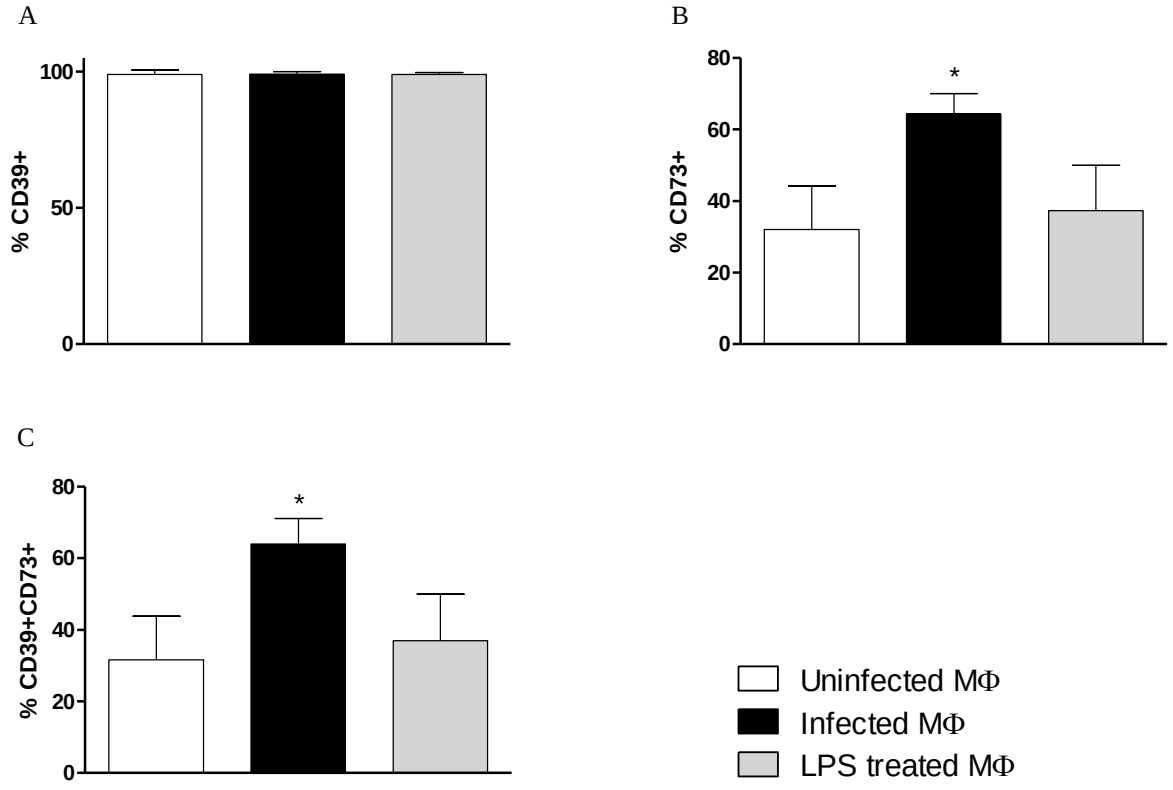


Figure 3: *L. amazonensis* upregulates CD73 expression in rested resident macrophages. Resident cells were collected and pooled from naïve C57BL/6 mice and rested for 72hrs prior to infection. They were infected with CFSE tagged metacyclics of *L. amazonensis* and additionally another group was treated with 5µg/ml of LPS. Cells were then further incubated for 24hrs at 33^0C/5% CO2 and flow cytometry was performed. The percentage of cells expressing A. CD39 B. CD73 and C. CD39CD73 in uninfected, infected or LPS treated MΦ was analyzed by flow cytometry. This result is the mean±SD of at least 3 independent experiments* $p<0.05$ indicates statistically significant between infected and control groups. One-way analysis of variance (ANOVA) followed by a posttest Bonferroni test.

Fig 5.4: ***L. amazonensis*** **increases CD73 expression in rested macrophages during 48hrs of infection**

Furthermore, seventy two hour rested MΦ were incubated with CFSE labeled metacyclics of *L. amazonensis* and infection was prolonged to 48 hours of incubation. We observed that the percentage of cells expressing CD73 was higher in infected groups than in control groups **(Fig 4A-C).**

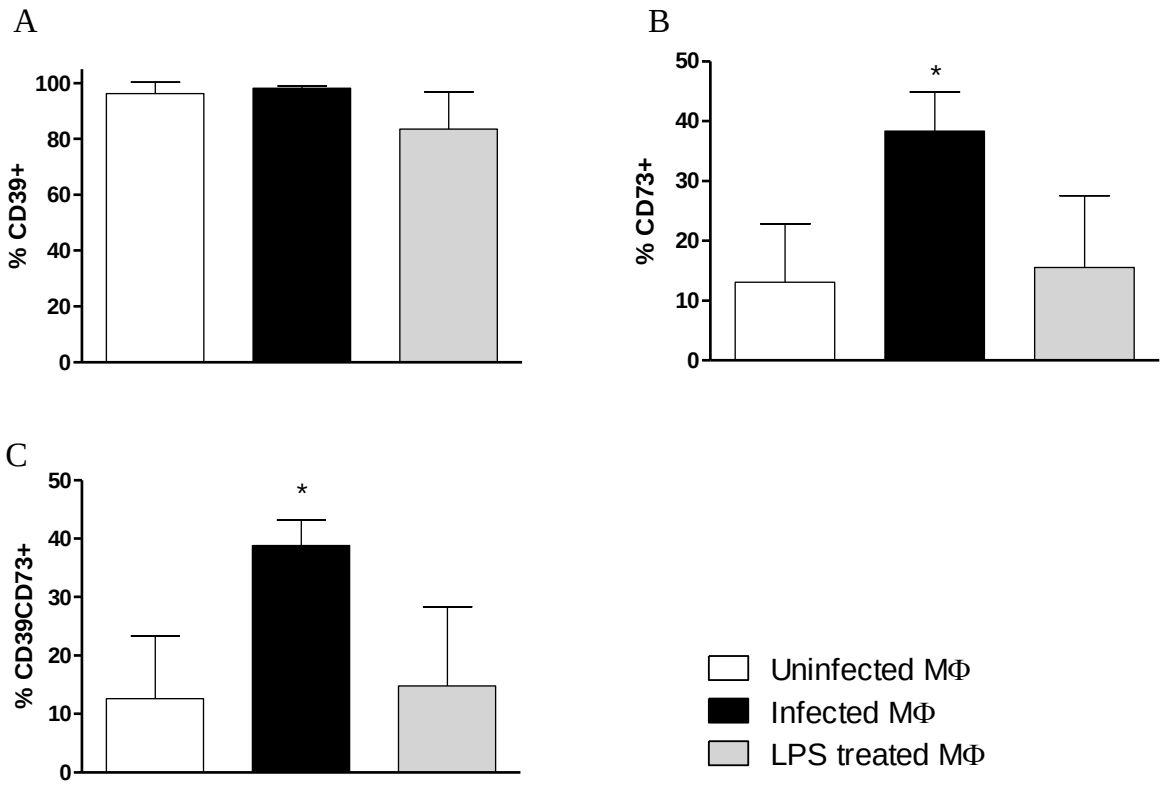


Figure 4: ***L. amazonensis*** **keeps CD73 expression high in infected macrophages during 48hrs of infection**. Seventy two hour rested MΦ were prepared and treated with parasite or LPS as described in materials and methods. Cells were incubated at 33^0C/5% CO2 for 48hrs infection. In given figure, the percentage of MΦ expressing A. CD39 and B. CD73 was analyzed for uninfected, infected and LPS treated MΦ by flow cytometry. Similarly, in figure C, the combined expression of CD39CD73 in all three populations is represented in bar diagram. This result is the mean±SD of at least 3 independent experiments. * $p<0.05$ indicates statistically significant between infected and control groups using One-way analysis of variance (ANOVA) followed by Bonferroni posttest.

.

5.5 L. amazonensis does not affect cytokine and NO production

Following the evaluation of expression of these enzymes, cytokine as well as NO production by treated MΦ were also measured. As shown in **Fig. 5A-5C**, *L. amazonensis* infected MΦ did not alter the production of TNF-alpha, IL-10, and NO. However, MΦ treated with LPS produced significantly higher levels of these inflammatory mediators compared to control and infected groups, indicating that these cells were fully capable to respond to inflammatory stimuli but not to the parasite. Up regulation in CD73 expression was, therefore, observed independently of cytokine as well as NO production. Therefore, these data together with increase CD73 expression in infected MΦ indicate that CD73 is regulated differently by *L. amazonensis*.

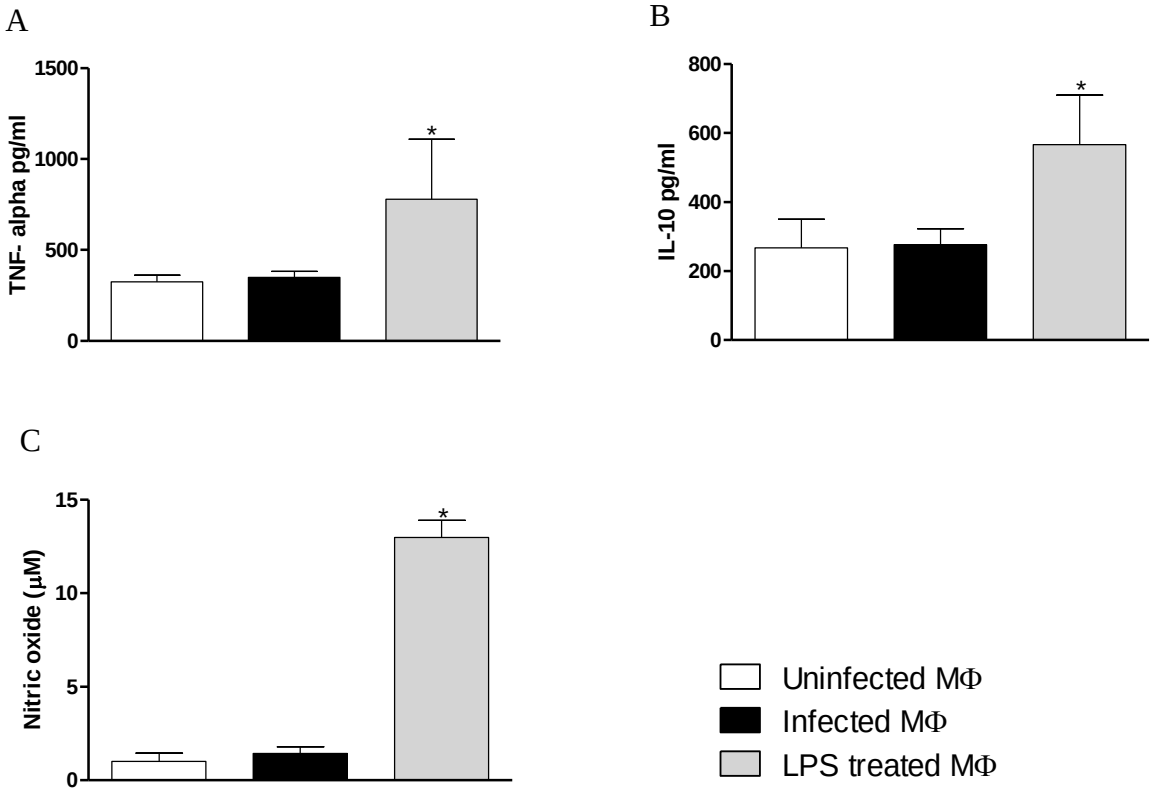
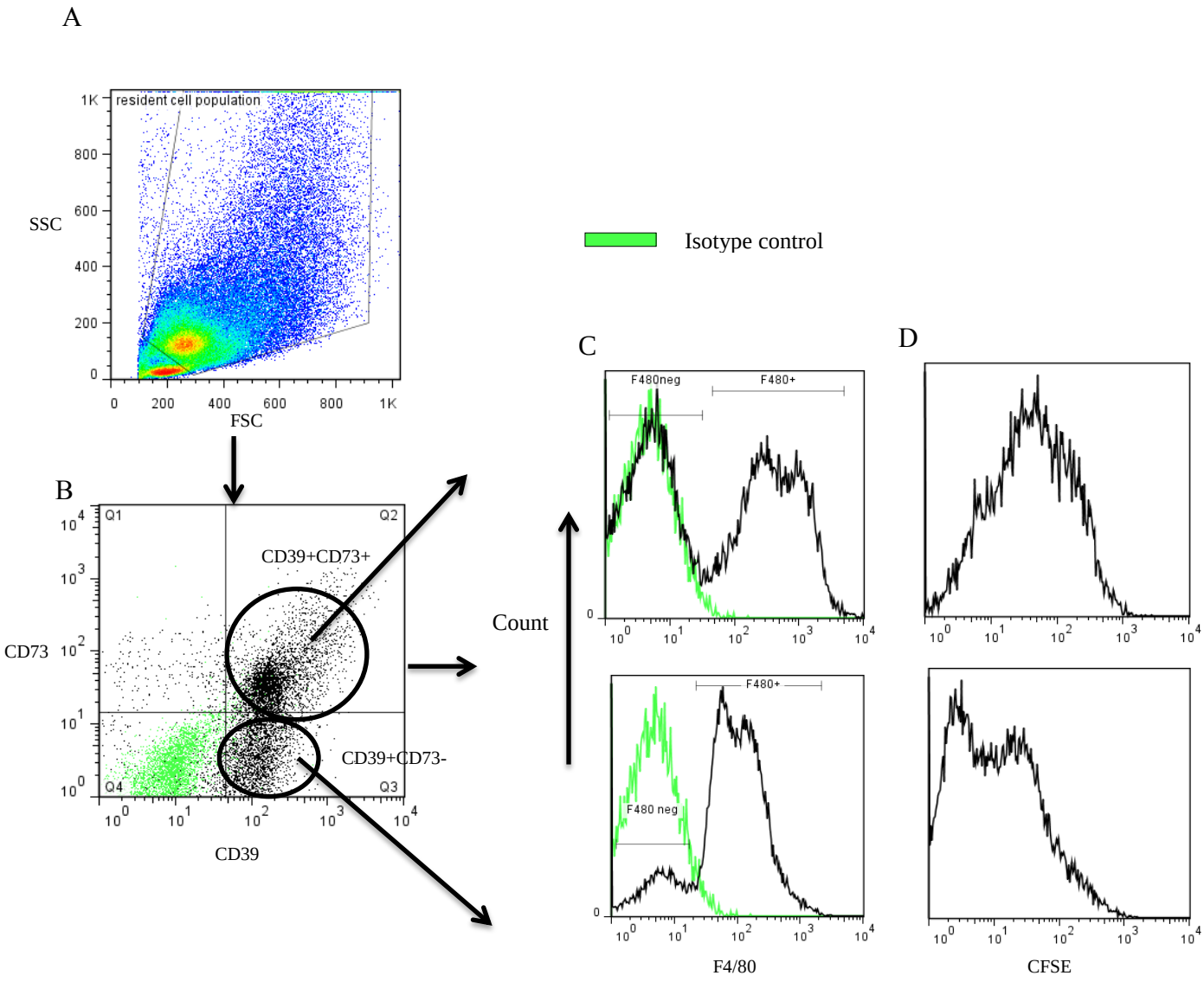


Figure 5: *L amazonensis* does not alter cytokine and NO production. Resident cells were collected from naïve C57BL/6 mice and rested for 72hrs prior to infection. They were either infected with *L. amazonensis* or treated with 5µg/ml of LPS. Cells were then further incubated for 24hrs at 33^0C/5% CO2. Supernatant from all groups was collected for the measurement of TNF-alpha, IL-10 and NO. In figures, A. TNF-alpha B IL-10 were measured by ELISA and C. NO production in treated MΦ was measured by Griess method. This result is the mean±SD of at least 3 independent experiments * $p<0.05$ indicates statistically significant between infected and control groups using One-way analysis of variance (ANOVA) followed by Bonferroni posttest.

.

5.6. Infected macrophages express high CD39 and CD73 in *in ex-vivo* studies

As an another approach, in order to demonstrate the effects of *L. ama*zonensis on the expression of CD39 and CD73 *in vivo*, CFSE tagged live metacyclics were injected in the peritoneum of mice and peritoneal cell population was harvested after 24hrs of inoculation. It was found that majority of the cells from whole peritoneal cell population could be divided into $CD39^{+}CD73^{+}$ or $CD39^{+}CD73^{-}$ **(Fig 6B)**. Inside these populations, total $F4/80^{+}$ cells **(Fig 6C)** were evaluated for CFSE positivity **(Fig 6D)**. Interestingly, it was noted that the intensity of CFSE positivity for $F4/80^{+}$ cells was high for CD73 positive population indicating that MΦ expressing CD73 are more infected **(Fig 6B-E)**.


A
resident cell population
SSC
FSC
B
Q1
Q2
Q3
Q4
CD39+CD73+
CD39+CD73-
CD73
CD39
Isotype control
C
D
F480neg
F480+
F480 neg
Count
F4/80
CFSE

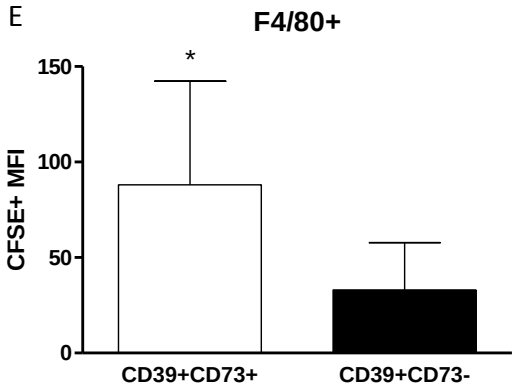

E
F4/80+
CFSE+ MFI
*
CD39+CD73+
CD39+CD73-

Figure 6: *L. amazonensis* infected macrophages show high CD73 expression in *in ex-vivo* studies. $50X10^6$ CFSE tagged live metacyclics were inoculated in the peritoneum of mice and then whole peritoneal cell population was harvested after 24hrs of inoculation. Cells were labeled with anti-mouse F4/80, anti-CD39 and anti-CD73 antibodies. Figure A. FSC X SSC shows size and granularity for whole cell population. B. Inside gated population; cells expressing CD39 and CD73 were shown. Cells expressing $CD39^+CD73^+$ or $CD39^+CD73^-$ were selected and then examined for C. $F4/80^+$ population and D. CFSE positivity was determined in $F4/80^+$ population. In the bar diagram E. MFI for CFSE in $F4/80^+$ population is shown. Data are the mean±SD from 2 independent experiments. *$p<0.05$ indicates statistically different between using Paired two tailed Student´s t- test. Two mice were used for each control group per experiment.

5.7. CD39 and CD73 activity determine parasite survival in infected macrophages

Having shown that *L. amazonensis* infection is characterized by increased CD73 expression, our next approach was to determine the importance of CD39 and CD73 surface enzymes in *L. amazonensis* survival and infection. Since the use of inhibitors at the time of infection could interfere with similar enzymes present on the surface of these parasites (de Souza et.al., 2010). DIDS and αβ-MAD, inhibitors for CD39 and CD73, respectively, were added after the parasites had been incubated with the MΦ for 3hrs and subsequently removed by washing. Our data showed that following inhibition of these enzymes, parasitism diminished within 24hrs of infection **(Fig 7A and 7B)** and this reduction in parasitic infection and amastigote number per 100 MΦ was further reduced after 48hrs of incubation in presence of these inhibitors **(Fig. 7C and 7D)**.

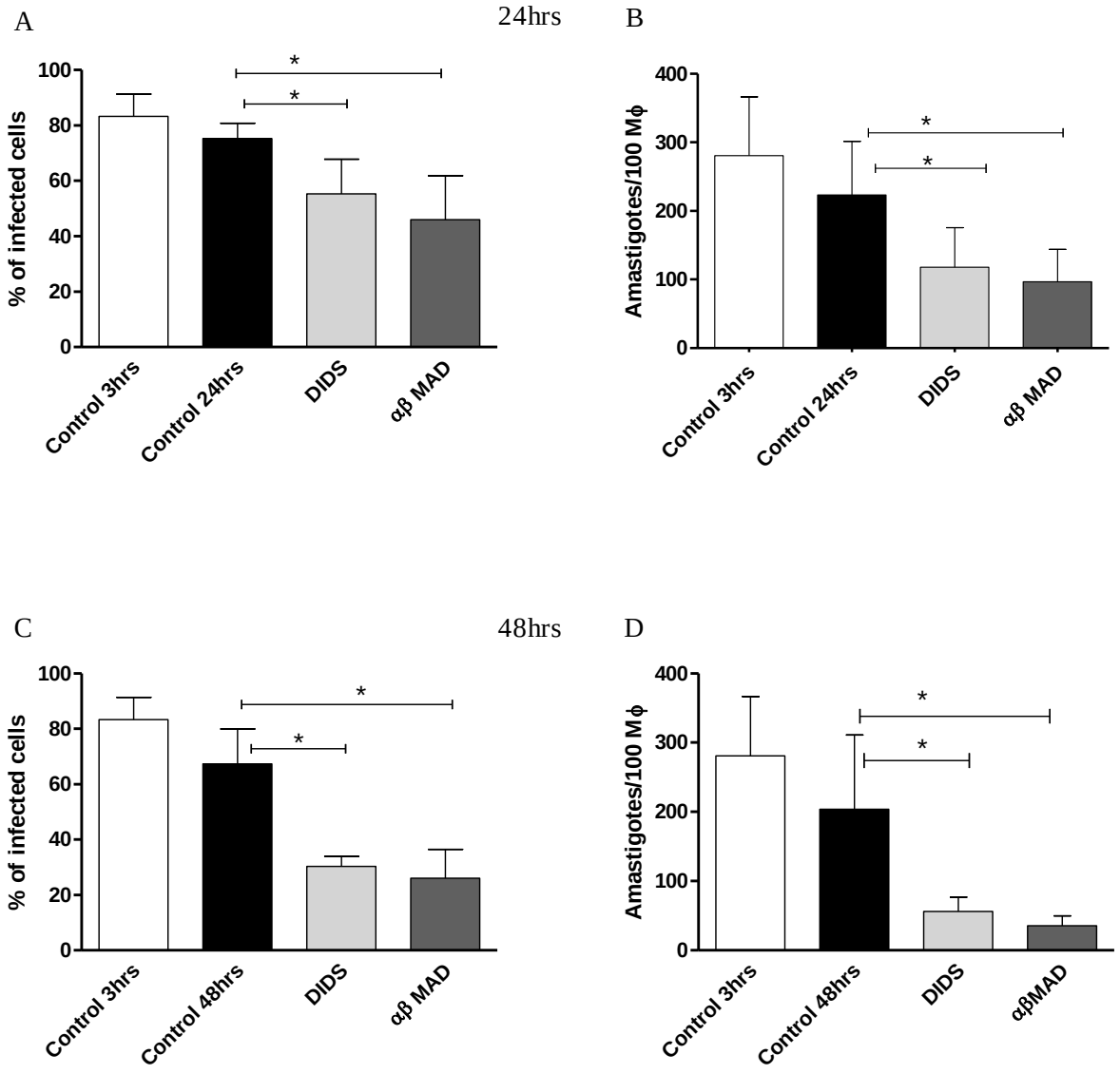


Figure 7: CD39 and CD73 activity determine survival of *L. amazonensis*. Resident MΦ were obtained from naïve mice by injecting 10ml of ice cold PBS in to the peritoneal cavity and rested for 72hrs at 37^0C/5% CO2 as described in materials and methods. Cells were then infected with metacyclic forms of the parasites in a ratio of 1:3 and allowed for the parasites to interact for 3hrs at 33^0C/5% CO2. Extracellular parasites were washed away and the inhibitors DIDS and αβ MAD were added against CD39 and CD73 at a concentration of 200µM respectively. In the figure, the percentage of infection for 24hrs (A) and for 48hrs (C) and the amastigote number per 100 MΦ for 24hrs (B) and for 48hrs (D) were calculated. Control represents infected MΦ treated with DMSO. Data are the mean±SD from 3 independent experiments. * $p<0.05$ is statistically different between control and treated groups using repeated measures of ANOVA followed by Dunnett posttest against the control group

5.8 Evaluation of cytokine and NO production from treated macrophages

In order to determine the mechanism of parasite killing in treated and untreated infected MΦ, cytokine and NO production were measured. Interestingly, reduction in parasite survival in MΦ was not related to the production of TNF-alpha, IL-10 or NO production as shown in **Fig. 8A-8C**. These results indicate that killing of the parasites under these circumstances is possibly mediated via pathways involving other than cytokine mediated NO production.

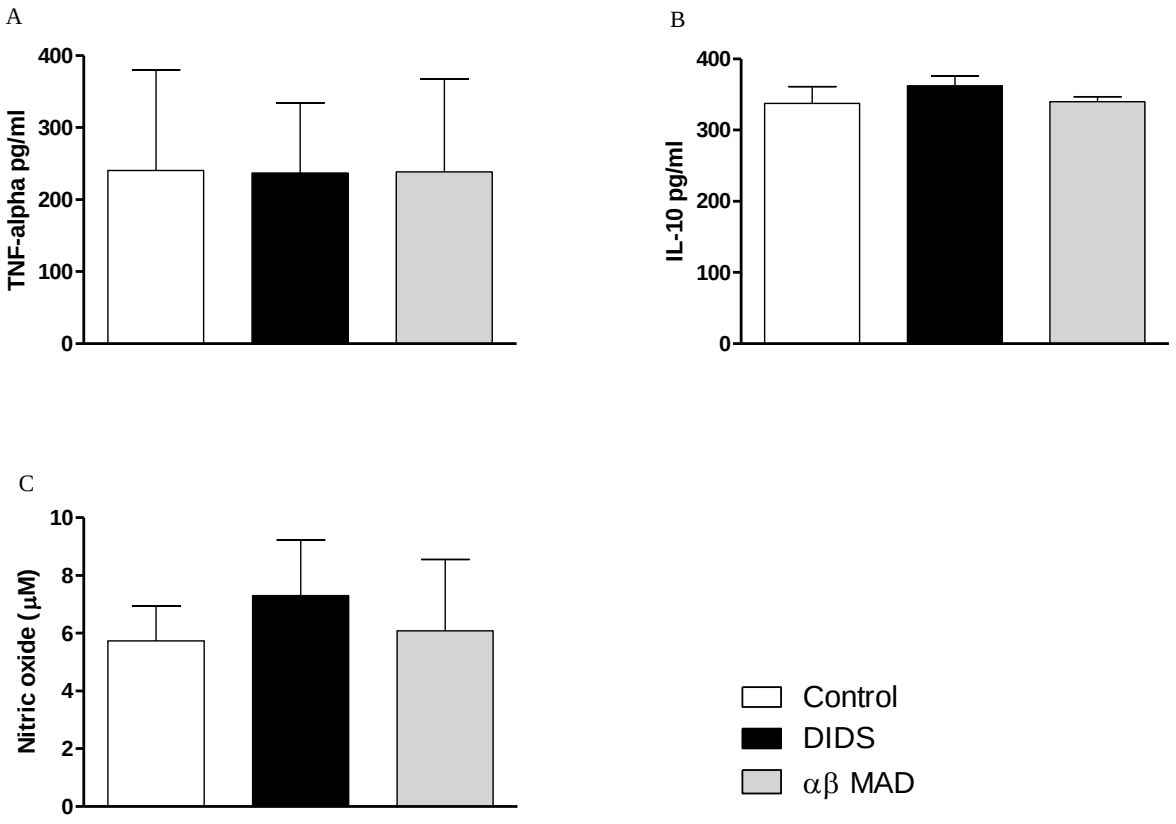


Figure 8: Inhibition of CD39 and CD73 activity does not alter cytokine and NO production by infected macrophages. Resident MΦ were obtained from naïve mice as described in materials and methods. Cells were then infected with *L. amazonensis* and inhibitors DIDS and αβ MAD were added after 3hrs of infection and then left them for 48hrs. Supernatant was collected from these groups after 48hrs incubation. Control represents infected MΦ treated with DMSO. Production of A.TNF-alpha B. IL-10 C. NO in treated groups is illustrated in the figure. Data are the mean±SD from 3 independent experiments.

.

5.9 Inhibition of A2a and A2b adenosine receptors reduces *L. amazonensis* survival in rested macrophages

Given the fact that inhibition of CD39/CD73 enzyme activity confines parasitic survival in MΦ, it was important to determine if adenosine receptors equally contribute in *L. amazonensis* infection and survival because the end of product of CD39/CD73 pathway is adenosine. To have an effect on the cells, adenosine produced by the ectonucleotidases should trigger one of the four adenosine receptors (A1, A2a, A2b, A3) and therefore, MΦ were treated with ZM241385 and MRS1754, antagonists of the adenosine receptors A2a and A2b (which are mostly involved in deactivation of MΦ) respectively, at the time of infection and kept throughout the infection. It was found that in 3hrs of infection, there was no alteration in the percentage of infected MΦ as well as the number of amastigotes per MΦ **(Fig. 9A and 9B)**. Within 24hrs of infection, however, percentage of infection and amastigote number was decreased significantly **(Fig 9C and 9D)**. After 48hrs of infection, in the presence of A2a and A2b antagonists, parasitism was further reduced **(Fig. 9E and 9F)**. In parallel, a group of MΦ treated with both antagonists together showed similar pattern of reduction **(Fig. 9C-9F)**.

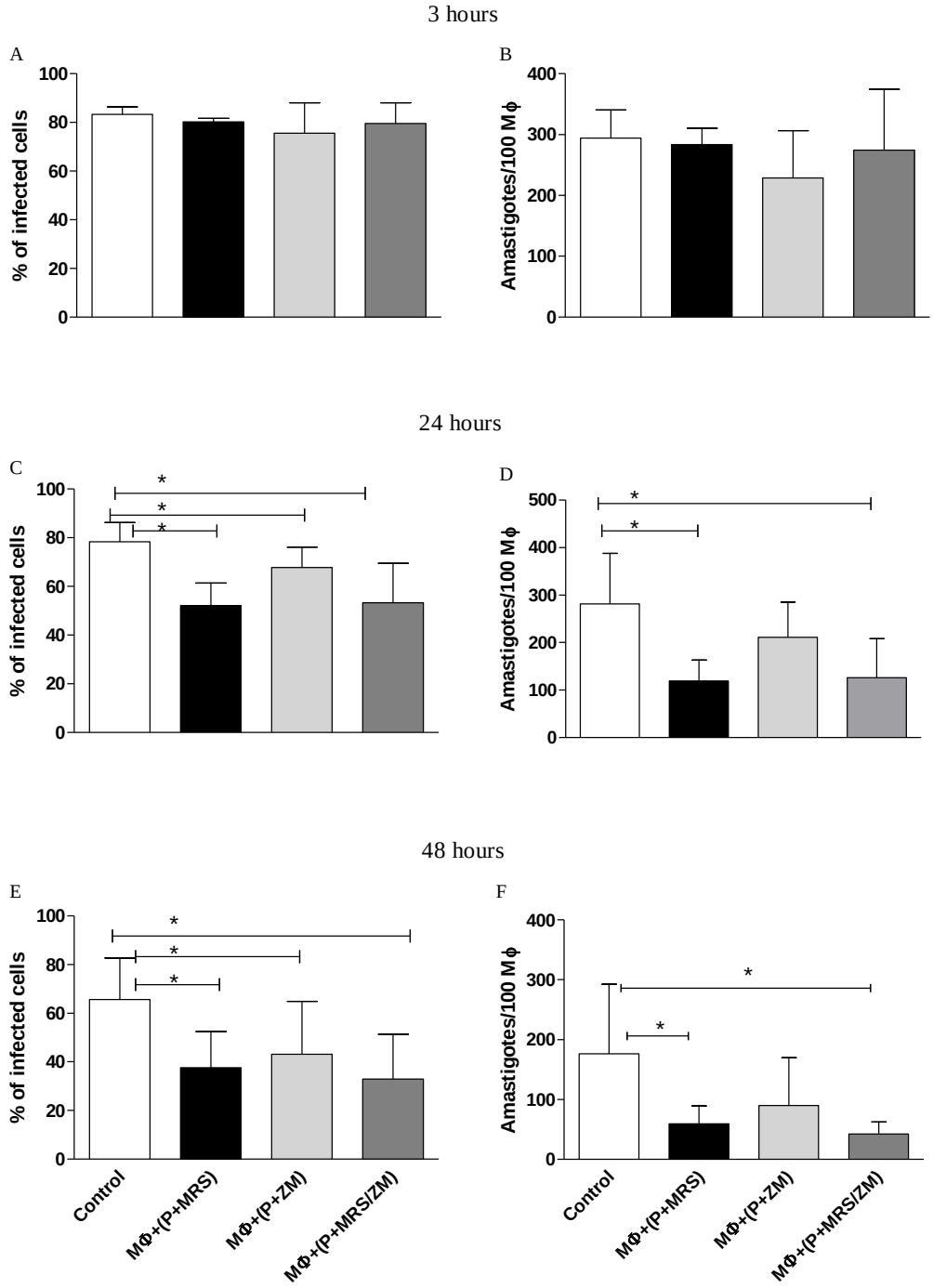


Figure 9: Survival of *L. amazonensis* depends on A2a and A2b receptors. MΦ were harvested and rested before infection. A2a and A2b antagonists ZM241385 and MRS1754 were added at a concentration of 5µM at the time of infection and kept throughout the infection. Percentage of infection and amastigote number were determined during 3hrs (A) and (B), 24hrs (C) and (D) and 48hrs (E) and (F) infection. Control represents infected MΦ treated with DMSO. These data indicate the mean±SD of at least 3 independent experiments. *$p<0.05$ represents statistical different between treated and control groups using repeated measures of ANOVA followed by Dunnett posttest against the control group

5.10 Evaluation of cytokine and NO production from treated macrophages

Similarly, as in CD39 and CD73 enzyme activity inhibition experiments, cytokine and NO production were evaluated from adenosine receptor inhibited MΦ culture supernatant. As observed in **Fig 8**, blockade of A2a and A2b adenosine receptors did not induce cytokine and NO production as shown in **Fig. 10A-C**, once again suggesting a possible common downstream pathway other than NO production, involving in CD39/CD73 enzyme actions as well as adenosine receptors.

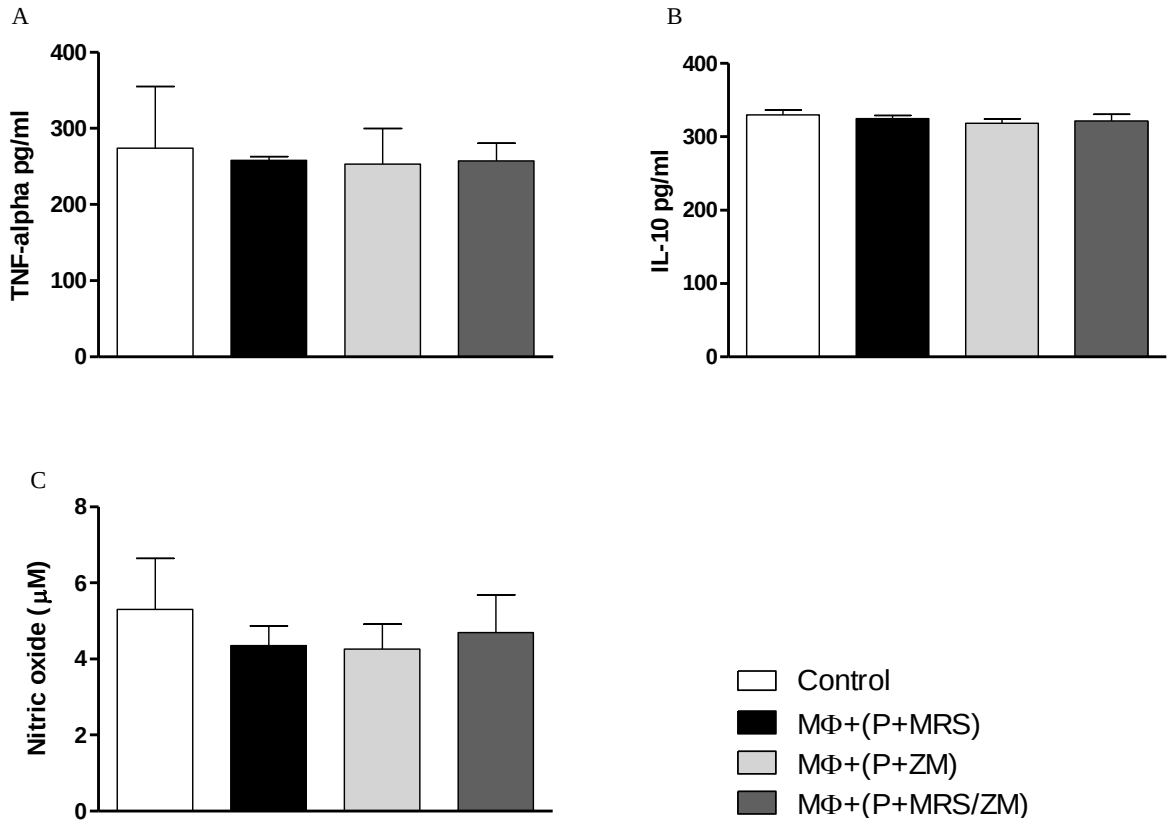


Figure 10: Inhibition of A2a and A2b receptors does not alter cytokine and NO production by infected macrophages. MΦ were harvested and rested as previously described in materials and method. A2a and A2b antagonists ZM241385 and MRS1754 were added at the time of infection and kept throughout the infection. Supernatant collected from these groups after 48hrs of infection and was evaluated for A. TNF-alpha B. IL-10 by ELISA and C. NO production by Griess method. Control represents infected MΦ treated with DMSO. These data indicate the mean±SD of 3 independent experiments.

5.11 Effects of HIF-1α inhibitor on growth of *L. amazonensis*

FM19G11 is an inhibitor to HIF-1α. Before studying its role in parasitic survival, parasite growth was determined in presence of FM19G11. It was observed that this inhibitor did not alter the growth of the parasites **(Fig 11)**.

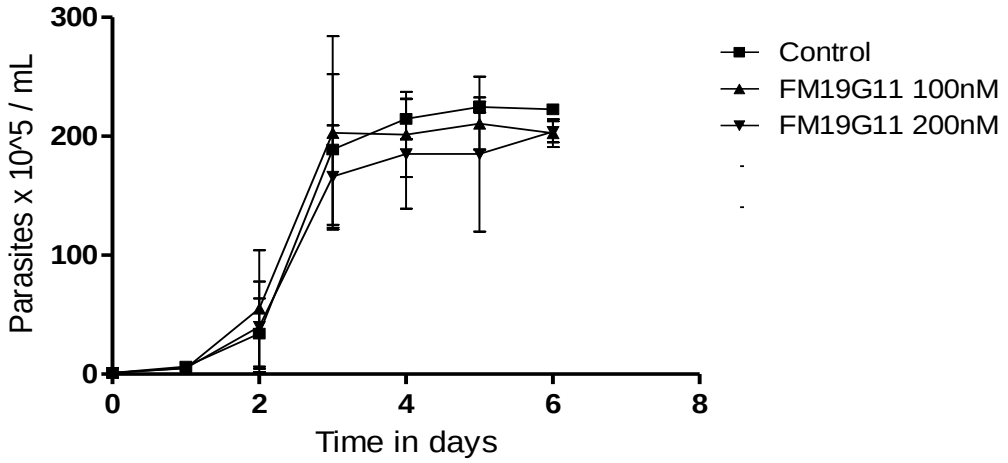


Figure 11: Growth Curve of *L. amazonensis* in presence of FM19G11 *L. amazonensis* was cultivated in Grace´s medium/10% FBS. Inhibitor to HIF-1α in two different concentrations of 100nM or 200nM was added at the time of cultivation. Cultures were incubated at 25^0C for 6 days and growth curve was plotted. In a control group, DMSO was added. Results are mean±SD of 3 independent experiments.

5.12 Inhibition of HIF-1α in resident macrophages and its role in *L. amazonensis* infection

Infection and inflammation induced by the parasite may lead to localized hypoxic conditions in host cells. However, *in vitro* studies, it has been found that *L. amazonensis* can induce HIF-1α even when the cells are not hypoxic (Degrossoli et.al., 2007). Therefore, we evaluated possible role of HIF-1α in parasitic survival in MΦ in the presence of HIF-1α inhibitor.

In order to study the possible role of HIF-1α in the survival of *L. amazonensis* in MΦ, 72hrs rested MΦ were treated with FM19G11 at a concentration of 100nM and 200nM. It was found that 3hrs of incubation with parasites in presence of the inhibitor did not affect the parasite infection and amastigote number (**Fig 12A and Fig 12B**). During 24hrs of infection, in presence of HIF-1α inhibitor, although no appreciable decrease in percentage of infection was observed, there was significant reduction in amastigote number per 100 MΦ (**Fig 12C and Fig 12D**).

However, after 48hrs of incubation in presence of FM19G11, parasitism decreased significantly (**Fig 12E-12F**). All these findings suggest that presence of HIF-1α is important for the parasitic survival in resident MΦ.

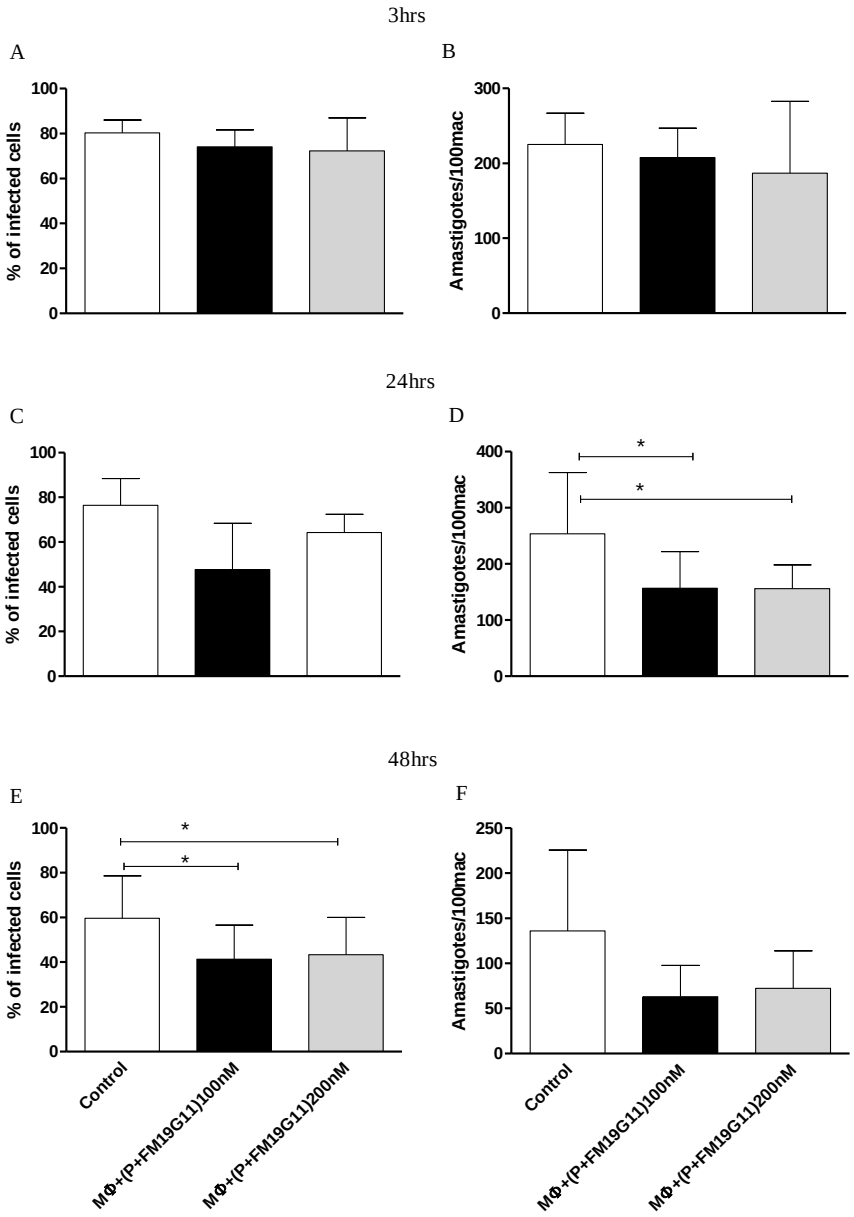


Figure 12: HIF-1α inhibition in resident macrophages interferes parasite survival Seventy two hour rested MΦ were infected with metacyclics of *L. amazonensis*. Inhibitor FM19G11 was added at a concentration of 100nM and 200nM and then incubated for 48hrs. A. the percentage of MΦ infected and B. amastigote number/100MΦ for 3hrs of infection was calculated. Similarly, C. the percentage of cells infected as well as D. amastigote number for 24hrs and E. the percentage of infection and F. amastigote number/100 MΦ for 48hrs were determined. Control represents infected MΦ treated with DMSO. These data indicate mean±SD of at least 3 independent experiments. *$p<0.05$ represents statistical difference between treated and control groups using repeated measures of ANOVA followed by Dunnett posttest against the control group

5.13. Role of HIF-1α on CD39 and CD73 expression in resident macrophages during *L. amazonensis* infection

Several studies support that CD73 is linked to HIF-1α and that it can regulate CD39/CD73 functions of adenosine production (Synnestvedt et.al., 2002). Since, *L. amazonensis* infection induces HIF-1α in MΦ in *in vitro*, the effects of HIF-1α on the expression of CD39 and CD73 were evaluated by treating infected MΦ with HIF-1α inhibitor and then the percentage of cells expressing CD39 and CD73 were analyzed by flow cytometry. In present study, it was found that infected MΦ, regardless of the treatment with FM19G11, always expressed high CD73 **(Fig 13A and 13B)**. The combined expression of CD39 and CD73 was also high in infected groups **(Fig 13C**). We did not find any change in the expression of CD39 and CD73 in between FM19G11 treated and untreated infected groups, suggesting that effects of HIF-1α is not visible after 24hrs of infection. The possible link in between these enzymes and HIF-1α expression may be at a different level of regulation.

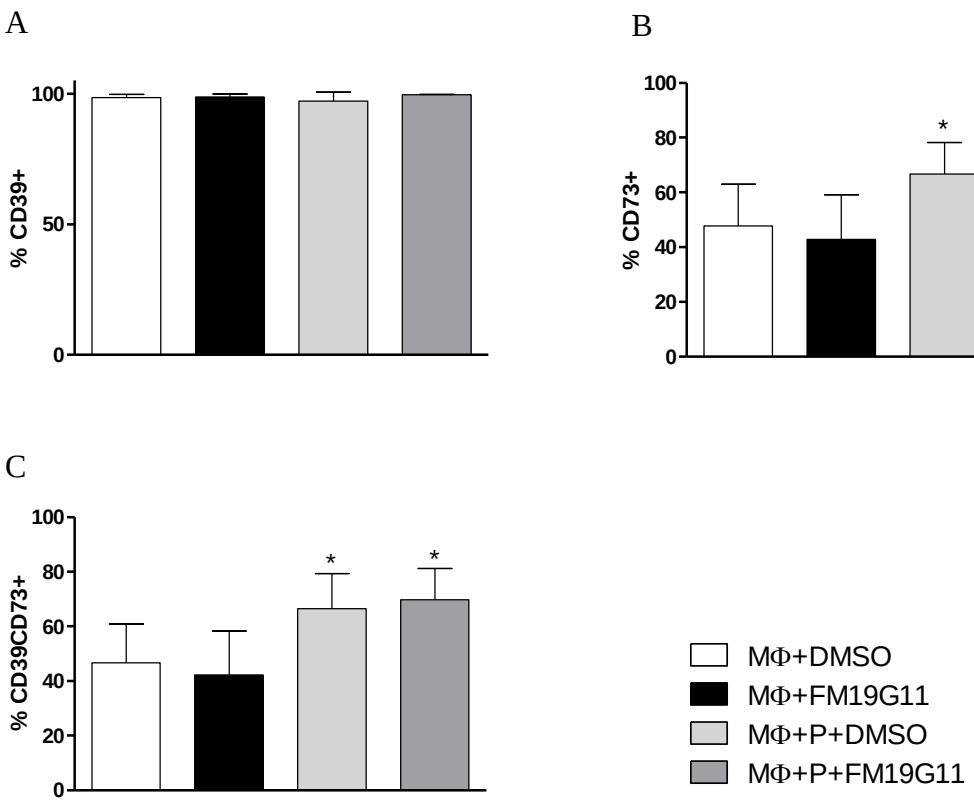


Figure 13: Inhibition of HIF-1α does not alter expression of CD39 and CD73 expression in macrophages during infection with *L amazonensis*. Seventy two hour rested MΦ were infected with *L. amazonensis* in presence of 100nM FM19G11 for 24hrs. Cells were analyzed for CD39 and CD73 expression in presence of FM19G11. Percentage of cells for A. CD39 and B. CD73 was evaluated in MΦ. In figure C. percentage of expressing both CD39 and CD73 was determined. These data indicate the mean±SD of 3 independent experiments. *p<0.05 represents statistical different between treated and control groups using repeated measures of ANOVA followed by Dunnett posttest against the control group

5.14. Expression of purine receptors in *L. amazonensis* infected macrophages

In order to determine whether *L. amazonensis* regulates adenosine receptors during infection, real time PCR was performed for each of the gene targets *A2a* and *A2b* using specific primers. It was observed that infection with *L. amazonensis* did not alter mRNA receptors for both A2a and A2b **(Fig 14A and 14B)**. Surprisingly, LPS treatment also did not induce any substantial change in the expression of these receptors indicating that the fold change in mRNA expression is possibly not evident after 24hrs of incubation **(Fig 14A and 14B)**. Similarly, mRNA expression for adenosine receptors A2a and A2b was also evaluated in presence of HIF-1α inhibitor. It was found that A2a and A2b mRNA expression in presence of FM19G11 did not

change during 24hrs of infection **(Fig 14 C and 14D)** indicating that the effects are not evident after 24hrs of infection.

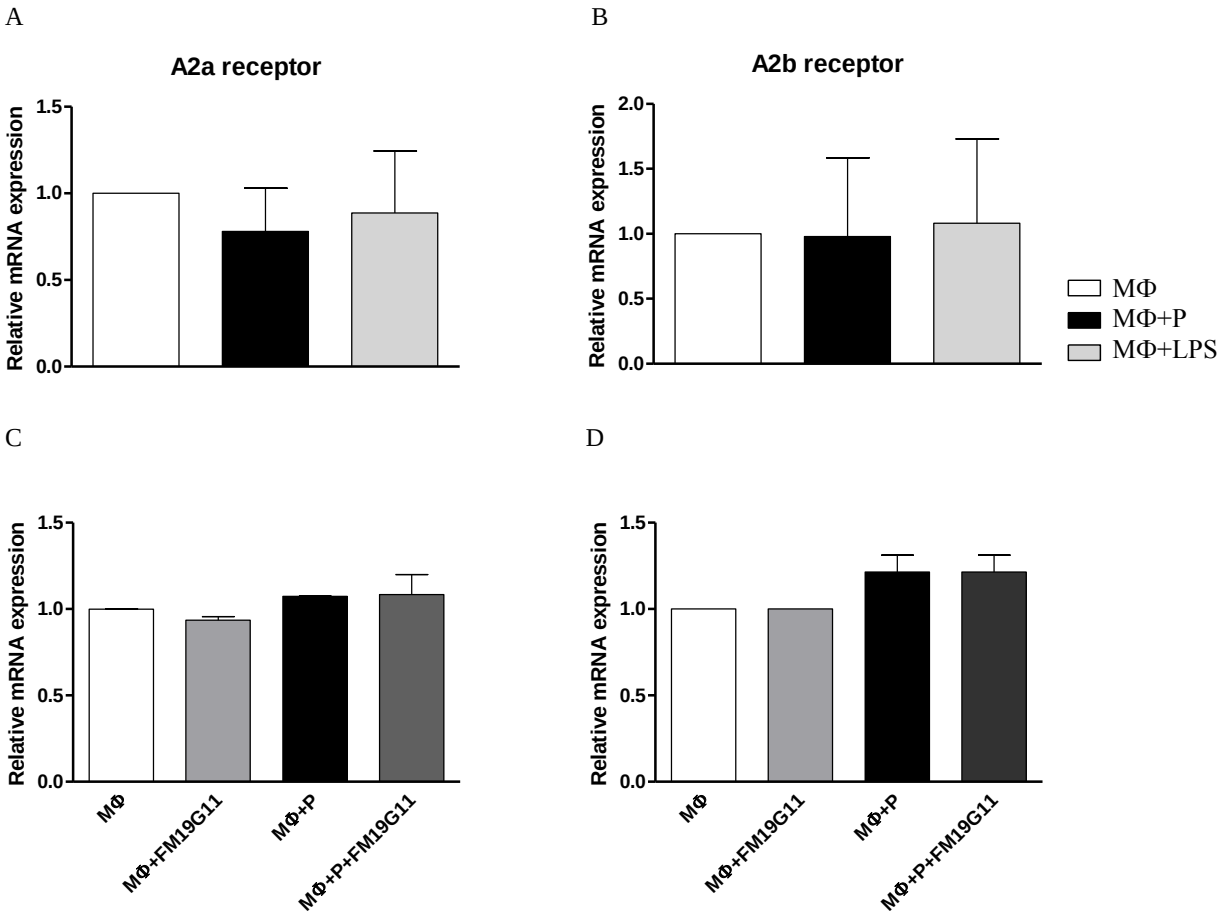


Figure 14: Relative expression of mRNA of A2a and A2b receptors in resident macrophages. Seventy two hour rested MΦ were either infected with *L. amazonensis* or treated with LPS and left for 24hrs at 33^0C/5% CO_2 in DMEM supplemented medium. Cells were then harvested using brazol. RNA was extracted followed by reverse transcription and real time PCR was performed. The relative mRNA expression of A. A2a B. A2b receptors were determined for uninfected, infected and LPS treated groups. Similarly, mRNA expression for C. A2a and D. A2b was also analyzed in presence of FM19G11. Relative expression of mRNA was normalized by reference gene.

In order to determine mRNA expression of A2a and A2b adenosine receptors during the course of infection, MΦ were infected with *L. amazo*nensis and the expression was evaluated by real time PCR at different time points. As shown in the **Fig 15**, both receptors A2a and A2b were found to be regulated by *L. amazonensis*. It was observed that the expression of mRNA for both A2a and A2b increased slowly and peaked at 8hrs and then it declined gradually. The point that we did not observe any change in mRNA expression after 24hrs may be due to the fact that these receptors stabilized after 24hrs of incubation. It is important to evaluate the effects at early time

points to observe the relationship between HIF-1α and adenosine receptors during *Leishmania* infection.

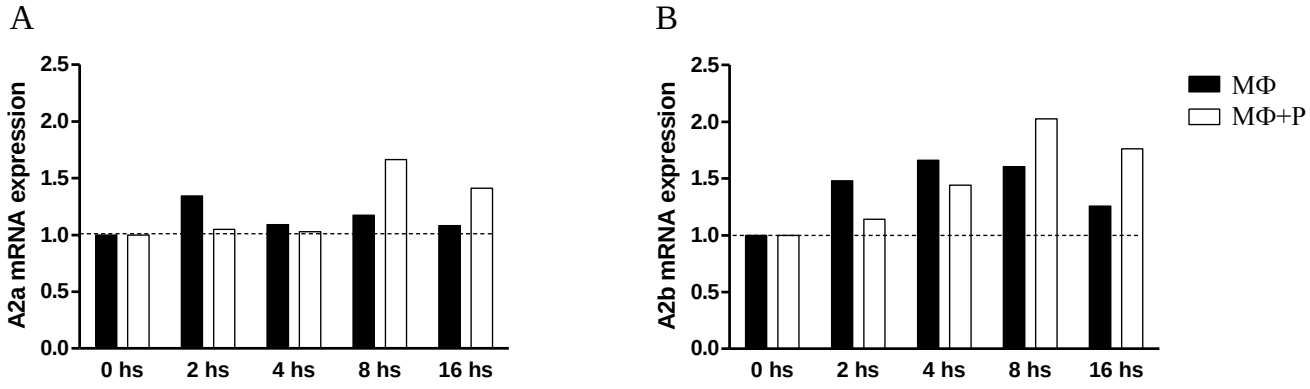


Figure 15: Kinetics of relative A2a and A2b mRNA expression in macrophages. MΦ were prepared as explained in materials and methods. Cells were infected with *L. amazonensis* and were harvested from uninfected and infected MΦ cultures cultivated for different time points. RNA from cells was extracted followed by reverse transcription and real time PCR. The relative mRNA expression of A. A2a B. A2b receptors was determined for uninfected and infected groups in different incubation time. Relative expression of mRNA was normalized by reference gene.

In summary, our results demonstrate that *L. amazonensis* increases CD73 in resident MΦ and its survival is dependent on both CD39 and CD73 activities. Furthermore, our findings also illustrate that A2a and A2b are equally important for parasitic growth and multiplication. No significant TNF-alpha, IL-10 and NO production suggest possible involvement of mechanisms other than cytokine derived activation of MΦ for killing of parasites. Although HIF-1α restricts *L. amazonensis* survival in MΦ, it has no significant effects on CD39, CD73 as well as A2a and A2b receptors studied during 24hrs of period incubation. In conclusion, our study indicates that *L. amazonensis* regulates CD39/CD73 in MΦ pathway during infection. Moreover, further analysis of mRNA expression of CD39, CD73 and adenosine receptors in relation to HIF-1α may aid in understanding mechanisms how these parasites are modulating the host MΦ. The involvement of these immunomodulators can determine the outcome of host and parasite interaction.

6. SUMMARY OF RESULTS

6. SUMMARY OF RESULTS:

1. Resident MΦ were the major cell populations which contain both CD39 and CD73 surface enzymes **(Fig 1)**
2. Unstimulated resident MΦ decreased CD73 expression without changing CD39 expression upon incubation **(Fig 2)**
3. *L. amazonensis* increased CD73 expression in MΦ during infection *in vitro* but did not alter cytokine as well as NO production **(Fig 3-5**)
4. Infection with *L. amazonensis in vivo* experiments was characterized by increase CD73 expression **(Fig 6)**
5. CD39 and CD73 were important enzymes for the survival of *L. amazonensis*. Interference in the parasite survival was not mediated via production of cytokines or NO **(Fig 7-8)**
6. A2a and A2b receptor inhibition restricted parasite survival *in vitro* but inhibition of these receptors was not associated with the release of cytokine and NO production**(Fig 9-10)**
7. Inhibition of HIF-1α protein reduced parasitism but did not alter the expression of CD39 and CD73 **(Fig 12-13)**
8. Change in fold of mRNA expression of A2a and A2b receptors was not evident during 24hrs of infection in presence of FM19G11 **(Fig 14)**

7. DISCUSSION

7. DISCUSSION:

Extracellular nucleotides such as ATP and its metabolite adenosine produced by dying or activated cells can modulate immune response during pathophysiological conditions. These molecules can trigger either inflammatory or anti-inflammatory responses, thus, playing significant role in determining the course or the outcome of the diseases (Almeida et.al., 1996; Bours et.al., 2006; Haag et.al., 2007; Vitiello et.al., 2012). In leishmaniasis, enzymes present in the parasites have demonstrated a strong correlation between enzymatic activities and disease severity (de Almeida Marques-da-Silva et.al., 2008; de Souza et.al., 2010; Maioli et.al., 2004). Two important ecto-enzymes CD39 and CD73 present on surfaces of the cells can directly regulate the ratio of ATP: adenosine under stress conditions. Adenosine release is associated with the impairment of immune response particularly in suppressing activation of antigen presenting cells such as MΦ (Hasko & Pacher, 2012). During leishmaniasis, MΦ infected with *Leishmania* constitute the core part in the host immune response which if properly activated in presence of Th1 responses, can eliminate the parasites such as *L. majo*r infection in resistant mice (Sacks & Noben-Trauth, 2002). The possible mechanism of *L. amazonensis* involving CD39 and CD73 expression in relation to survival of the parasites in MΦ has been described here.

Initially, in order to evaluate the expression of CD39 and CD73 in murine macrophages, peritoneal cells were harvested. Murine peritoneal cavity contains several immune cells that include B cells, macrophages, dendritic cells, eosinophils, mast cells, neutrophils, T cells, natural killer cells (Ghosn et.al., 2010). Therefore, in our present *in ex-vivo* study, based on F4/80 marker, it was broadly categorized into two major populations of $F4/80^{+}$ and $F4/80^{-}$ which displayed both CD39 and CD73 **(Fig 1)**. F4/80 antigens are extracellular membrane molecules which are highly restricted to mature MΦ subpopulation residing in tissues mainly (McKnight & Gordon, 1998). It was found that from the total peritoneal population, MΦ ($F4/80^{+}$) are the major cell populations that have higher CD39 and CD73 expression compared to non-macrophages ($F4/80^{-}$). It has already been demonstrated that peritoneal MΦ are capable of producing adenosine from ATP hydrolysis suggesting presence of enzymes on their surfaces (Beigi & Dubyak, 2000; Gordon et.al., 1971). In one of the recent studies, mRNA level of CD39 has been evaluated and in parallel, enzymatic activity was also studied. Their findings suggest that CD39

expressed on the surfaces of peritoneal macrophages is the dominant ectonucleotidase enzyme present in peritoneal macrophages (Levesque et.al., 2010). In agreement with this finding, our results also show that almost all F4/80^{+} cells expressed CD39 in peritoneal population. CD73, however, was positive to only 72% of the F4/80^{+} population. CD73 expression may vary with the type of cells, their maturation as well as activation (Zanin et.al., 2012).

It is important to note that the expression of ecto-5'-nucleotidase does not occur on all cells and its expression can be regulated by external factors (Edelson & Cohn, 1976). In our subsequent experiments, in order to determine if culture conditions can modify both CD39 and CD73 expression, cells were rested in DMEM/10% FBS supplemented medium as described in methods and methodology. In *in vitro* resting of MΦ demonstrated that CD73 expression decreased slowly upon incubation. Interestingly, the percentage of cells expressing CD73 decreased gradually from 24hrs to 72hrs of resting without altering CD39 expression **(Fig 2)**. It is possible that the culture conditions employed *in vitro* may have an influence in the expression of CD73. It may also be possible that these cells increased CD73 expression while harvesting them from their natural habitat so that the expression returned to their original level. Given the fact that only CD73 but not CD39 expression was changed may indicate that these MΦ are capable of ATP hydrolysis efficiently as per CD39 enzyme activity, but the production of adenosine, which is the end production of ATP hydrolysis, will be regulated differently by CD73 as it is the rate limiting enzyme for adenosine production (Friedman et.al., 1998; Lennon et.al., 1998). It has been shown that depending on the activation state of MΦ, 5´ecto-nucleotidase activities may vary. It has been reported that thioglycollate elicited MΦ show lower 5´ectonucleotidase activity *in vitro* than resident MΦ (Edelson & Cohn, 1976).

L. amazonensis is known for its ability to inhibit MΦ in several different ways making them refractory to the activation (Gomes et.al., 2003; Horta et.al., 2012; Meier et.al., 2003). Provided the activation of MΦ is regulated by 5´ectonucleotidase activity, it is likely possible that *L. amazonensis* infection would regulate CD39 and CD73 expression to inhibit MΦ activation. Remarkably, when these rested MΦ were infected with *L. amazonensis*, although there was no change in CD39 expression, CD73 was significantly increased in infected MΦ (**Fig 3 and Fig 4)**. This was in contrast with LPS stimulation which showed no effect on both CD39 and CD73 expression indicating that the change observed was specific to the parasite derived

immunomodulation. Similarly, the effects of *L. amazonensis* on CD39 and CD73 were also evaluated *in vivo* **(Fig 6)**. It was observed that the majority of infected MΦ were $CD73^+$ suggesting that *L. amazonensis* may regulate CD73 expression in infected MΦ and this change can influence MΦ activation in such a way that MΦ may favor parasite growth and survival during infection. It is, however, yet to be determined if infection increases CD73 or high $CD73^+$ cells are preferentially infected *in vivo.* Our present data showed that both *in vitro* and *in vivo* experiments, CD73 expression is high in infected macrophages. Although we did not use LPS control in *in viv*o studies, there is an evidence which shows that MΦ harvested from endotoxin inoculated mice have reduced 5´ecto-nucleotidase activity than naïve MΦ indicating reduced 5´ecto-nucleotidase activity in activated MΦ (Edelson & Cohn, 1976).

The role of CD73 expression and activity has been well described in several pathological conditions. In toxoplasmosis, for instance, increase in CD73 gene transcript levels after 2hrs of infection has already been recorded (Blader et.al.,2001). Similarly, cytomegalovirus infection is also associated with the increase expression and activity of CD39 as well as CD73 on endothelia cells (Kas-Deelen et.al., 2001). In addition, it is also reported in some diseases like AIDS or Helicobacterial infections where CD73 expression can significantly alter the disease outcomes (Alam et.al., 2009; Nikolova et.al., 2011). Moreover, in dendritic cells infected with *L. amazonensis*, it is shown that the expression of CD39 and CD73 increases significantly (Figueiredo et.al., 2012), suggesting that upregulation of CD73 expression is possibly a common mechanism for *L. amazonensis* to modulate host immune response.

The significance of CD39 and CD73 expression in MΦ is such that these molecules can hydrolyze extracellular nucleotides releasing adenosine thereby regulating MΦ activation (Hasko & Pacher, 2012). It is well established that *L. amazonensis* does not activate MΦ. In order to characterize these rested MΦ, cytokine and NO production were evaluated. It was observed that infected MΦ **(Fig 5)** did not induce production of TNF-alpha, IL-10 and NO resembling typical characteristics of *L. amazonensis* infection. However, LPS treatment induced sufficient production of cytokines as well as NO indicating that these MΦ were activated but this activation did not lead to the change in the expression of CD39 and CD73. Classically activated MΦ derived from LPS treatment have shown lower ATP hydrolysis ability than alternatively activated MΦ. The difference in the enzymatic activity is clearly explained by the difference in

the levels of enzymes expressed by these two groups of MΦ. Alternatively activated MΦ express high CD39 and CD73 (Zanin et.al., 2012). These groups of macrophages produce less IL-12 and NO as well as produces more TGF-β or IL-10, therefore favoring parasite growth (Stempin et.al., 2002). Increase CD73 expression can induce MΦ into regulatory MΦ. It should be noted that CD73 expressed by MΦ can suppress pro-inflammatory cytokine production (Grunewald & Ridley, 2010).

ATP produced during *L. amazonensis* infection via P2X7 receptor channel has been shown to induce apoptosis in infected MΦ (Lammas et.al., 1997; Chaves et.al., 2009; Coutinho-Silva et.al., 2009). One of the popular findings in relation to parasite survival is that Chlamydia-infected J774 murine MΦ resist to treatment to extracellular ATP, whereas uninfected cells undergo apoptosis via P2X7 receptor–dependent pathways (Coutinho-Silva et.al., 2001). Removal of extracellular accumulation of nucleotides via CD39/CD73 pathway may be one possible mechanism employed by the pathogens to survive against lethal effects of these molecules. In *Leishmania*, although role of parasite surface ectonucleotidase activities has been described in relation with the disease progression, function of CD39 and CD73 in *L. amazonensis* infected MΦ has not been determined. Once these parasites get inside, their contact to extracellular milieu becomes limited and their surface ectoenzymes should cease to influence extracellular nucleotide degradation. However, several pathogens can also exploit host ectonucleotidases present on the outer membranes of the cells or tissues generating adenosine rich environment (Antonioli et.al., 2013). Hence, role of these enzymes in *L. amazonensis* infection was evaluated in resident MΦ

Our findings illustrate that if CD39 and CD73 are inhibited by specific inhibitors such as by DIDS and αβ MAD respectively in infected resident MΦ, parasite infection and survival decreased significantly **(Fig 7)**. This data suggests that adenosine production via CD39/CD73 pathway is an important regulatory molecule that facilitates survival of *L. amazonensis* in MΦ. Since extracellular nucleotides are released during infection and inflammation, CD39/CD73 pathway directly participates in determining the outcome of several diseases. One such example is in toxoplasmosis in which an increase in CD73 expression is associated with *T. gondii* survival and if CD73 molecule is deleted, it can severely hinder the parasite growth and differentiation (Mahamed et.al., 2012). Also, extracellular adenosine synthesized by CD73 in *Salmonella*

infection inhibits anti-bacterial properties of MΦ favoring bacteria to grow in the host cells (Alam et.al., 2011). In dendritic cells infected with *L. amazonensis*, it has been shown that the enzyme activity is dependent on CD39 and CD73 expression and if these enzymes are blocked, their antigen presenting capacity increases (Figueiredo et.al., 2012). Together with these data, it is clear that *L. amazonensis* regulates CD39 and CD73 expression in host cells. Hence, blockade of these molecules can limit parasite survival and might be an alternative approach for the control of diseases including leishmaniasis

Having known that the effects of CD39 and CD73 can alter the parasite growth and survival, our next approach was to determine the effects of adenosine receptors on parasite viability. Adenosine produced via CD39/CD73 pathway binds specifically to adenosine receptors and under given circumstances, if adenosine receptors are not available, the effects of adenosine on MΦ will be absent. Adenosine is a potent immunosuppressive molecule which functions through four G-protein-coupled membrane receptors; of four receptors, A2a and A2b receptors are more importantly recognized for their actions on MΦ deactivation via cAMP production (Abbracchio et.al., 2009; Fredholm et.al., 2001). MΦ express all four adenosine receptors depending on the type of cells and activation state (Hasko et.al, 2007). LPS activated J774 MΦ for example; express all adenosine receptors whereas IFN-γ triggers all four receptors in RAW264 MΦ suggesting these receptors are differently regulated by activation signals (Watanabe K., 2005). Hence *L. amazonensis* induced MΦ adenosine release and adenosine receptor modulation can also regulate MΦ functions.

Our study in relation to A2a and A2b receptor and parasite survival confirms that if the receptors are inhibited, their survival is drastically reduced **(Fig 9)**. Given the fact that inhibition of adenosine receptors restricts *L. amazonensis* survival suggests that the release of adenosine via CD39/CD73 pathway specifically binds to A2a and A2b receptors and regulates MΦ promoting *L. amazonensis* survival. A2a and A2b are the principal receptors that have been shown to involve in down-regulation of classically activated MΦ and promoting development regulatory MΦ (Cohen et.al., 2013; Hasko & Pacher, 2012). In a recent study, Cohen and Mosser demonstrated that LPS induced activation of MΦ negatively regulates MΦ activation by up regulating A2breceptor, thus allowing adenosine biding to the receptor for effective control of inflammatory response (Cohen et.al., 2013).

The importance of adenosine receptors has been demonstrated in several immune cells during infection and inflammation. In dendritic cells, it has been shown that the effects of *L. amazonensis* in DC activation are mainly dependent on A2b receptor functions (Figueiredo et.al., 2012). In visceral lesihamanisis, high adenosine production has been found to participate in the disease indicating possible interference in in the actions of effector functions of immune cells such as T lymphocytes via adenosine receptors (Rai et.al., 2011). Similarly, in Clostridium infections, the severity of morbidity and mortality are related to A2b receptors activation (Warren et.al., 2012). Furthermore, adenosine receptors associated immunosuppression in septic mice such as activation of A2a reduces mortality from endotoxemia (Sullivan et.al., 2004). Since activation of A2a and A2b receptors is associated with the suppression of MΦ effector functions, from parasitic standpoint, it is plausible that *L. amazonensis* stimulates A2a and A2b receptors which impede activation of cells via downstream purinergic pathway leading to cAMP production. In future studies, it will be interesting to evaluate the effects of A2a and A2b receptors on other species of *Leishmania* including *L. infantum* in visceral leishmaniasis.

Extracellular release of nucleotides induces production of proinflammatory cytokines such as IL-1β, IL-6, TNF-alpha and IFN-γ from activated immune cells (Atarashi et.al., 2008; Bours et.al., 2006; Langston et.al., 2003). Ectonucleotidase enzymes have been found to shift from inflammatory to anti-inflammatory pathway via production of adenosine. Adenosine is known to suppress production of several of important proinflammatory cytokines such as TNF-alpha IL-1, IL-6, and IL-12 synthesis (Bours et.al., 2006; Desrosiers et.al., 2007; Hasko et.al., 2008) whereas enhance release of IL-10, IL-4, IL-13 (Koscso et.al., 2012; Koscso et.al., 2013). CD73 has been found to suppress NO production as well as proinflammatory cytokine production (Grunewald & Ridley, 2010). CD73 increased the survival of septic mice by diminishing bacterial growth, reducing inflammatory cytokine levels and ameliorating organ injuries (Hasko et.al., 2011). Furthermore, A2b receptor has also been implicated as the receptor responsible for down regulation of inducible NO synthase and MHC II expression in response to IFN-γ (Xaus et.al., 1999). NO and ROS are inhibited in presence of adenosine leading to suppression of MΦ (Xaus et.al., 1999; Hasko et.al., 1996).

In an attempt to determine the mechanism how these parasites are being killed, important cytokines such as TNF-alpha, IL-10 as well as NO production in the presence of ZM241385 or

with MRS1754 were evaluated but none of them was found to be involved in parasite killing **(Fig 10)**. Similarly, reduction of *L. amazonensis* growth in MΦ treated with DIDS and αβ MAD was also not associated with effector molecules such as NO and TNF alpha production in **(Fig 8)**, indicating that the mechanisms involved in killing *L. amazonensis* are other than cytokine derived NO production. It should be pointed out that, in the experiments described here, MΦ were not treated with IFN-γ/LPS as it is usually seen in several studies. Thus the ability of these cells to control parasitism is independent of the mechanisms induced by the "classical activation" pathway. Increased ROS accumulation or tryptophan depletion leading to oxygen independent anti-leishmanicidal effects are other possible mechanisms that need to be investigated. Moreover, some cytokines such as TGF-β has been reported to increase CD73 in immune cells such as CD4 cells (Regateiro et.al., 2013).

HIF-1α has been found to be regulated by several pathogens during host pathogen interaction for the benefits of their survival (Werth et.al., 2010). While HIF-1 α activation in some bacterial infections can eliminate the pathogens by activating phagocytic activities, in some others such as *Bartonella henselae*, viruses and *Toxoplasma gondii* can induce HIF-1 α in host cells for their survival and growth (Kempf et.al., 2005; Wiley et.al., 2010; Smith et.al., 2013). In leishmaniasis, it has been revealed that HIF-1α increase parasite persistence in cutaneous lesions of mice infected with *L. amazonensis* (Arrais-Silva et.al., 2005). Furthermore in vitro studies demonstrated that *L. amazonensis* can induce HIF-1α even when the cells are not hypoxic (Degrossoli et.al., 2007). In our present study, it has been found that when the MΦ were infected with *L amazonensis* in presence of a potent inhibitor of HIF-1α, parasitism reduced significantly **(Fig 12).** Similar to our findings, it has been demonstrated *in vitro* studies that if HIF-1α is inhibited, *L. amazonensis* survives less in MΦ (Degrossoli et.al., 2007). Furthermore, the role of HIF-1α in *L. major* as well as in *L. donovani* infection has similarly demonstrated that if HIF-1α is blocked, their growth and survival are inhibited, suggesting that the presence of HIF-1α is essential and is possibly a general mechanism of survival of *Leishmania* conserved by these parasites. (Lemaire et.al., 2013; Singh et.al., 2012)

One of the consequences of the local tissue inflammation is an induction of hypoxic microenvironment where extracellular ATP release is predominant. HIF-1α can facilitate ATP degradation by increasing enzymatic conversion of ATP hydrolysis into adenosine (Synnestvedt

et.al., 2002). For example, HIF-1α dependent CD73 activity can influence adenosine production which via activation of A2b receptors suppresses excessive inflammation (Hart et.al., 2011). Interestingly, release of adenosine can also trigger induction of HIF-1α in MΦ and thereby altering the catalytic activities of surface ectonucleotidases (Takeda et.al., 2010). Inhibition of HIF-1α expression results in significant decrease in hypoxia inducing CD73 expression (Synnestvedt et.al., 2002). Our findings demonstrated that infection did not alter the expression of CD39 and CD73 in the presence of HIF-1α inhibitor. However, the percentage of cells expressing CD73 was always high in infected groups **(Fig 13)** independently of treatment. It is important to mention here that the analysis of expression of CD39 and CD73 was done after 24hrs of infection. It is therefore possible that effects of HIF-1α on CD39 and CD73 may have already disappeared. One of the recent studies demonstrate that activated MΦ show increase ATP release with an induction of HIF-1 within 1-2 hrs post stimulation (Cohen et.al., 2013). Furthermore, induction of HIF for *L. donovani* in MΦ of parasitic growth has been reported *in L. donovani* within 8hrs of incubation period (Singh et.al., 2012). Moreover, the studies related with CD39 and CD73 and HIF-1α have always been performed at the transcriptional level (Lemaire et.al., 2013; Singh et.al., 2012) Furthermore, HIF is one of the most degradable proteins known and is highly susceptible to oxygen. It will be interesting to analyze mRNA expression of CD39 and CD73 using HIF-1α antisense oligonucleotides and observe the kinetics of the effects of HIF on the expression of CD39 and CD73.

Adenosine receptors are regulated differently by several activation signals. LPS, for example, can induce upregulation of A2b receptors in MΦ (Cohen et.al., 2013). Adenosine released by activated macrophages can trigger adenosine receptors mainly A2a and A2b to transform into alternatively activated MΦ (Vega & Corbi., 2006). HIF-1α can also influence adenosine receptors thereby leading to enhanced adenosine receptor signaling (Poth et.al., 2013). HIF-1α induced adenosine receptors, such as A2b, have also shown to induce Th2polarization by increasing cAMP production (Yang et.al., 2010). In our study, although the parasitic survival was dependent on both adenosine receptors and HIF-1α, *L. amazonensis* did not demonstrate upregulation of mRNA expression of A2a and A2b receptors during 24hrs of infection **(Fig 14)**. Similarly LPS treated MΦ also did not show significant change in the mRNA expression of A2a and A2b receptors. Furthermore, we also observed no change in the mRNA expression of A2a and A2b receptors when HIF-1α was inhibited by FM19G11. This analysis was also made after

24hrs of infection. Recently, study shows that change in adenosine receptor expression after LPS induced activation of MΦ is time dependent. It is shown that after 8hrs of treatment, mRNA expression of A2b increases significantly which after 16hrs, returns to its normal level. In our present context, it is likely possible that the effects were already gone by the time the cells were analyzed for CD39 and CD73 or A2a and A2b receptor expression in relation to HIF-1α. Our preliminary data for the kinetics of mRNA expression of A2a and A2b receptors indicates that *L. amazonensis* may regulate A2a and A2b receptors and we found that the receptors were upregulated and were maximum at 8hrs of incubation **(Fig 15)**. mRNA expression of A2a and A2b receptors slowly decreased after 16hrs. It is therefore important to analyze these receptors at a different time point and evaluate the effects of HIF on adenosine receptors during *L. amazonensis* infection.

Our results demonstrate that *L. amazonensis* upregulates CD73 in resident MΦ and their survival is strictly dependent on CD39 and CD73 activities as well as A2a and A2b receptors. Although HIF-1α influences parasite growth in resident MΦ, in present laboratory conditions, HIF-1α was not found to be involved in expression of CD39 and CD73 as well as A2a and A2b receptor regulation. In summary, our findings indicate that *L. amazonensis* regulates purinergic pathways during early infection and suggest possible involvement of these immunomodulators in the outcome of host and *Leishmania* interaction.

8. CONCLUSION

8. CONCLUSION:

In the current study, we have confirmed the effects of *L. amazonensis* on the expression of ecto-nucleotidases in MΦ *in vitro*. It is demonstrated that *L. amazonensis* can upregulate CD73 expression in rested resident MΦ and that its survival within the host cell is dependent on CD39 and CD73 activities as well as on the activation of A2a and A2b adenosine receptors without producing TNF-alpha, IL-10 and NO. Furthermore, although inhibition of HIF-1α restricts parasite growth and survival, both CD39 and CD73 remain unaltered. *L. amazonensis* may regulate adenosine receptors at the transcription level in early period of time. Altogether, our data supports that adenosine production via CD39/CD73 pathway activates adenosine receptors and leads to the induction of more regulatory type of MΦ, making them more suitable for parasite development and proliferation.

9. PERSPECTIVES

9. PERSPECTIVES

1. Study of possible mechanisms involved (such as measurement of ROS accumulation) in killing of *L. amazonensis* in relation to CD39, CD73 as well as to adenosine receptors
2. Study of kinetics of A2a and A2b receptors expression during infection.
3. Evaluate proliferation and cytokine production of CD4+ T cells purified from the spleen of C57BL/6 mice infected with *L. major* and co-incubated with MΦ infected with *L. amazonensis* in relation to adenosine receptors as well as HIF-1α
4. Assess cytokine production such as TNF-alpha, IL-10, IL-12, IFN-γ and cAMP *in vitro* from cocultivation of macrophage and T lymphocyte assay.
5. Evaluate the role of HIF-1α on expression of CD39 and CD73 as well as adenosine receptors at transcript levels (mRNA) in MΦ infected with *L. amazonensis* at different time points
6. *In vivo* expression of CD39 and CD73 at the lesion site by means of immunohistochemistry assays

10. REFERENCES

10.REFERENCES

ABBRACCHIO M.P., BURNSTOCK G., BOEYNAEMS J.M., BARNARD E.A., BOYER J.L., KENNEDY C., KNIGHT G.E., FUMAGALLI M., GACHET C., JACOBSON K.A. & WEISMAN G.A. (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol.Rev.* **58**, 281-341.

ABBRACCHIO M.P., BURNSTOCK G., VERKHRATSKY A. & ZIMMERMANN H. (2009) Purinergic signalling in the nervous system: an overview. *Trends Neurosci.* **32**, 19-29.

AFONSO L.C. & SCOTT P. (1993) Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. *Infect.Immun.* **61**, 2952-2959.

ALAM M., GAINES D., KUO J., PEREIRA M., BILEY E., ERNST P., WILLIAMS K. (2011) Adenosine synthesized by CD73 and CD39 regulates inflammation during Salmonella infection. *J.immunol*. **186**,162.10

ALAM M.S., KURTZ C.C., ROWLETT R.M., REUTER B.K., WIZNEROWICZ E., DAS S., LINDEN J., CROWE S.E. & ERNST P.B. (2009) CD73 is expressed by human regulatory T helper cells and suppresses proinflammatory cytokine production and Helicobacter felis-induced gastritis in mice. *J.Infect.Dis.* **199**, 494-504.

ALEXANDER J. & BRYSON K. (2005) T helper (h)1/Th2 and Leishmania: paradox rather than paradigm. *Immunol.Lett.* **99**, 17-23.

ALMEIDA R.P., BARRAL-NETTO M., DE JESUS A.M., DE FREITAS L.A., CARVALHO E.M. & BARRAL A. (1996) Biological behavior of *Leishmania amazonensis* isolated from humans with cutaneous, mucosal, or visceral leishmaniasis in BALB/C mice. *Am.J.Trop.Med.Hyg.* **54**, 178-184.

ANTONIOLI L., PACHER P., VIZI E.S. & HASKO G. (2013) CD39 and CD73 in immunity and inflammation.*Trends Mol.Med.* **19**, 355-367.

ARRAIS-SILVA W.W., PAFFARO V.A., Jr., YAMADA A.T. & GIORGIO S. (2005) Expression of hypoxia-inducible factor-1alpha in the cutaneous lesions of BALB/c mice infected with *Leishmania amazonensis*. *Exp.Mol.Pathol.* **78**, 49-54.

ATARASHI K., NISHIMURA J., SHIMA T., UMESAKI Y., YAMAMOTO M., ONOUE M., YAGITA H., ISHII N., EVANS R., HONDA K. & TAKEDA K. (2008) ATP drives lamina propria T(H)17 cell differentiation. *Nature* **455**, 808-812.

BEIGI R.D. & DUBYAK G.R. (2000) Endotoxin activation of macrophages does not induce ATP release and autocrine stimulation of P2 nucleotide receptors 1. *J.Immunol.* **165**, 7189-7198.

BEIL W.J., MEINARDUS-HAGER G., NEUGEBAUER D.C. & SORG C. (1992) Differences in the onset of the inflammatory response to cutaneous leishmaniasis in resistant and susceptible mice. *J.Leukoc.Biol.* **52**, 135-142.

BELKAID Y., HOFFMANN K.F., MENDEZ S., KAMHAWI S., UDEY M.C., WYNN T.A. & SACKS D.L. (2001) The role of interleukin (IL)-10 in the persistence of *Leishmania major* in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. *J.Exp.Med.* **194**, 1497-1506.

BEN-SHOSHAN J., AFEK A., MAYSEL-AUSLENDER S., BARZELAY A., RUBINSTEIN A., KEREN G. & GEORGE J. (2009) HIF-1alpha overexpression and experimental murine atherosclerosis. *Arterioscler.Thromb.Vasc.Biol.* **29**, 665-670.

BEN-SHOSHAN J., MAYSEL-AUSLENDER S., MOR A., KEREN G. & GEORGE J. (2008) Hypoxia controls CD4+CD25+ regulatory T-cell homeostasis via hypoxia-inducible factor-1alpha. *Eur.J.Immunol.* **38**, 2412-2418.

BERN C., MAGUIRE J.H. & ALVAR J. (2008) Complexities of assessing the disease burden attributable to leishmaniasis. *PLoS.Negl.Trop.Dis.* **2**, e313.

BLADER I.J., MANGER I.D. & BOOTHROYD J.C. (2001) Microarray analysis reveals previously unknown changes in Toxoplasma gondii-infected human cells 2. *J.Biol.Chem.* **276**, 24223-24231.

BORSELLINO G., KLEINEWIETFELD M., DI M.D., STERNJAK A., DIAMANTINI A., GIOMETTO R., HOPNER S., CENTONZE D., BERNARDI G., DELL'ACQUA M.L., ROSSINI P.M., BATTISTINI L., ROTZSCHKE O. & FALK K. (2007) Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* **110**, 1225-1232.

BOURS M.J., SWENNEN E.L., DI V.F., CRONSTEIN B.N. & DAGNELIE P.C. (2006) Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol.Ther.* **112**, 358-404.

BRITTINGHAM A. & MOSSER D.M. (1996) Exploitation of the complement system by *Leishmania* promastigotes. *Parasitol.Today* **12**, 444-447.

BUCHMULLER-ROUILLER Y. & MAUEL J. (1987) Impairment of the oxidative metabolism of mouse peritoneal macrophages by intracellular *Leishmania spp*. *Infect.Immun.* **55**, 587-593.

BURNSTOCK G. (2007) Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev.* **87**, 659-797.

CHAN J., FUJIWARA T., BRENNAN P., MCNEIL M., TURCO S.J., SIBILLE J.C., SNAPPER M., AISEN P. & BLOOM B.R. (1989) Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. *Proc.Natl.Acad.Sci.U.S.A* **86**, 2453-2457.

CHAVES S.P., TORRES-SANTOS E.C., MARQUES C., FIGLIUOLO V.R., PERSECHINI P.M., COUTINHO-SILVA R. & ROSSI-BERGMANN B. (2009) Modulation of P2X(7) purinergic receptor in macrophages by Leishmania amazonensis and its role in parasite elimination. *Microbes.Infect.* **11**, 842-849.

CHEN Y., CORRIDEN R., INOUE Y., YIP L., HASHIGUCHI N., ZINKERNAGEL A., NIZET V., INSEL P.A. & JUNGER W.G. (2006) ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* **314**, 1792-1795.

COHEN H.B., BRIGGS K.T., MARINO J.P., RAVID K., ROBSON S.C. & MOSSER D.M. (2013) TLR stimulation initiates a CD39-based autoregulatory mechanism that limits macrophage inflammatory responses. *Blood* **122**, 1935-1945.

COLGAN S.P., ELTZSCHIG H.K., ECKLE T. & THOMPSON L.F. (2006) Physiological roles for ecto-5'-nucleotidase (CD73). *Purinergic.Signal.* **2**, 351-360.

COUTINHO-SILVA R., CORREA G., SATER A.A. & OJCIUS D.M. (2009) The P2X(7) receptor and intracellular pathogens: a continuing struggle. *Purinergic.Signal.* **5**, 197-204.

COUTINHO-SILVA R., PARSONS M., ROBSON T. & BURNSTOCK G. (2001) Changes in expression of P2 receptors in rat and mouse pancreas during development and ageing. *Cell Tissue Res.* **306**, 373-383.

CRAMER T. & JOHNSON R.S. (2003) A novel role for the hypoxia inducible transcription factor HIF-1alpha: critical regulation of inflammatory cell function. *Cell Cycle* **2**, 192-193.

CRAMER T., YAMANISHI Y., CLAUSEN B.E., FORSTER I., PAWLINSKI R., MACKMAN N., HAASE V.H., JAENISCH R., CORR M., NIZET V., FIRESTEIN G.S., GERBER H.P., FERRARA N. & JOHNSON R.S. (2003) HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* **112**, 645-657.

CRONSTEIN B.N., KRAMER S.B., WEISSMANN G. & HIRSCHHORN R. (1983) A new physiological function for adenosine: regulation of superoxide anion production *Trans.Assoc.Am.Physicians* **96**, 384-391.

CUMMINGS H.E., TULADHAR R. & SATOSKAR A.R. (2010) Cytokines and their STATs in cutaneous and visceral leishmaniasis. *J.Biomed.Biotechnol.* **2010**, 294389

DE ALMEIDA MARQUES-DA-SILVA, DE OLIVEIRA J.C., FIGUEIREDO A.B., DE SOUZA LIMA J.D., CARNEIRO C.M., RANGEL FIETTO J.L. & CROCCO AFONSO L.C. (2008) Extracellular nucleotide metabolism in *Leishmania*: influence of adenosine in the establishment of infection. *Microbes.Infect.* **10**, 850-857.

DE SOUZA M.C., DE ASSIS E.A., GOMES R.S., MARQUES DA SILVA E.A., MELO M.N., FIETTO J.L. & AFONSO L.C. (2010) The influence of ecto-nucleotidases on

Leishmania amazonensis infection and immune response in C57B/6 mice. *Acta Trop.* **115**, 262-269.

DEAGLIO S., DWYER K.M., GAO W., FRIEDMAN D., USHEVA A., ERAT A., CHEN J.F., ENJYOJI K., LINDEN J., OUKKA M., KUCHROO V.K., STROM T.B. & ROBSON S.C. (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J.Exp.Med.* **204**, 1257-1265.

DEGROSSOLI A., BOSETTO M.C., LIMA C.B. & GIORGIO S. (2007) Expression of hypoxia-inducible factor 1alpha in mononuclear phagocytes infected with *Leishmania amazonensis*. *Immunol.Lett.* **114**, 119-125.

DEGROSSOLI A. & GIORGIO S. (2007) Functional alterations in macrophages after hypoxia selection. *Exp.Biol.Med.(Maywood.)* **232**, 88-95.

DESROSIERS M.D., CEMBROLA K.M., FAKIR M.J., STEPHENS L.A., JAMA F.M., SHAMELI A., MEHAL W.Z., SANTAMARIA P. & SHI Y. (2007) Adenosine deamination sustains dendritic cell activation in inflammation. *J.Immunol.* **179**, 1884-1892.

DEUSSEN A. (2000) Metabolic flux rates of adenosine in the heart 2. *Naunyn Schmiedebergs Arch.Pharmacol.* **362**, 351-363.

ECKLE T., GRENZ A., LAUCHER S. & ELTZSCHIG H.K. (2008) A2B adenosine receptor signaling attenuates acute lung injury by enhancing alveolar fluid clearance in mice. *J.Clin.Invest* **118**, 3301-3315.

EDELSON P.J. & COHN Z.A. (1976) 5'-Nucleotidase activity of mouse peritoneal macrophages. I. Synthesis and degradation in resident and inflammatory populations. *J.Exp.Med.* **144**, 1581-1595.

ELTZSCHIG H.K., IBLA J.C., FURUTA G.T., LEONARD M.O., JACOBSON K.A., ENJYOJI K., ROBSON S.C. & COLGAN S.P. (2003) Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: role of ectonucleotidases and adenosine A2B receptors. *J.Exp.Med.* **198**, 783-796.

ELTZSCHIG H.K., KOHLER D., ECKLE T., KONG T., ROBSON S.C. & COLGAN S.P. (2009) Central role of Sp1-regulated CD39 in hypoxia/ischemia protection. *Blood* **113**, 224-232.

ERNST P.B., GARRISON J.C. & THOMPSON L.F. (2010) Much ado about adenosine: adenosine synthesis and function in regulatory T cell biology 2. *J.Immunol.* **185**, 1993-1998.

FAN J., ZHANG Y., CHUANG-SMITH O.N., FRANK K.L., GUENTHER B.D., KERN M., SCHLIEVERT P.M. & HERZBERG M.C. (2012) Ecto-5'-nucleotidase: a candidate virulence factor in Streptococcus sanguinis experimental endocarditis 2. *PLoS.One.* **7**, e38059

FAVALI C., TAVARES N., CLARENCIO J., BARRAL A., BARRAL-NETTO M. & BRODSKYN C. (2007) Leishmania amazonensis infection impairs differentiation and function of human dendritic cells.*J.Leukoc.Biol.* **82**, 1401-1406.

FIGUEIREDO A.B., SERAFIM T.D., MARQUES-DA-SILVA E.A., MEYER-FERNANDES J.R. & AFONSO L.C. (2012) Leishmania amazonensis impairs DC function by inhibiting CD40 expression via A2B adenosine receptor activation 5. *Eur.J.Immunol.* **42**, 1203-1215.

FREDHOLM B.B., IJZERMAN A.P., JACOBSON K.A., KLOTZ K.N. & LINDEN J. (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol.Rev.* **53**, 527-552.

FRIEDMAN G.B., TAYLOR C.T., PARKOS C.A. & COLGAN S.P. (1998) Epithelial permeability induced by neutrophil transmigration is potentiated by hypoxia: role of intracellular cAMP. *J.Cell Physiol* **176**, 76-84.

GHOSN E.E., CASSADO A.A., GOVONI G.R., FUKUHARA T., YANG Y., MONACK D.M., BORTOLUCI K.R., ALMEIDA S.R., HERZENBERG L.A. & HERZENBERG L.A. (2010) Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc.Natl.Acad.Sci.U.S.A* **107**, 2568-2573.

GOMES I.N., CALABRICH A.F., TAVARES R.S., WIETZERBIN J., DE FREITAS L.A. & VERAS P.S. (2003) Differential properties of CBA/J mononuclear phagocytes recovered from an inflammatory site and probed with two different species of Leishmania 1. *Microbes.Infect.* **5**, 251-260.

GONCALVES R., VIEIRA E.R., MELO M.N., GOLLOB K.J., MOSSER D.M & TAFURI W.L. (2005). A sensitive flow cytometric methodology for studying the binding of L. chagasi to canine peritoneal macrophages. *BMC Infec Dis.* **5,**39

GONCALVES R., ZHANG X., COHEN H., DEBRABANT A. & MOSSER D.M. (2011) Platelet activation attracts a subpopulation of effector monocytes to sites of Leishmania major infection. *J.Exp.Med.***208**, 1253-1265.

GORDON S. & MARTINEZ F.O. (2010) Alternative activation of macrophages: mechanism and functions. *Immunity.* **32**, 593-604.

GORDON S., RIPPS C.S. & COHN Z. (1971) The preparation and properties of macrophage-L cell hybrids.*J.Exp.Med.* **134**, 1187-1200.

GREEN L.C., WAGNER D.A., GLOGOWSKI J., SKIPPER P.L., WISHNOK J.S. & TANNENBAUM S.R. (1982) Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids.*Anal.Biochem.* **126**, 131-138.

GRUNEWALD J.K. & RIDLEY A.J. (2010) CD73 represses pro-inflammatory responses in human endothelial cells. *J.Inflamm.(Lond)* **7**, 10

HAAG F., ADRIOUCH S., BRASS A., JUNG C., MOLLER S., SCHEUPLEIN F., BANNAS P., SEMAN M. & KOCH-NOLTE F. (2007) Extracellular NAD and ATP: Partners in immune cell modulation. *Purinergic.Signal.* **3**, 71-81.

HART M.L., GRENZ A., GORZOLLA I.C., SCHITTENHELM J., DALTON J.H. & ELTZSCHIG H.K. (2011) Hypoxia-inducible factor-1alpha-dependent protection from intestinal ischemia/reperfusion injury involves ecto-5'-nucleotidase (CD73) and the A2B adenosine receptor. *J.Immunol.* **186**, 4367-4374.

HASKO G. & CRONSTEIN B. (2013) Regulation of inflammation by adenosine 7. *Front Immunol.* **4**, 85

HASKO G., CSOKA B., KOSCSO B., CHANDRA R., PACHER P., THOMPSON L.F., DEITCH E.A., SPOLARICS Z., VIRAG L., GERGELY P., ROLANDELLI R.H. & NEMETH Z.H. (2011) Ecto-5'-nucleotidase (CD73) decreases mortality and organ injury in sepsis. *J.Immunol.* **187**, 4256-4267.

HASKO G., LINDEN J., CRONSTEIN B. & PACHER P. (2008) Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat.Rev.Drug Discov.* **7**, 759-770.

HASKO G. & PACHER P. (2012) Regulation of macrophage function by adenosine 4. *Arterioscler.Thromb.Vasc.Biol.* **32**, 865-869.

HASKO G., PACHER., P., DEITCH EA., VIZI ES. (2007) Shaping of monocytes and macrophage function. *Pharmacol Ther*. 2(9),2295-301

HASKO G., SZABO C., NEMETH Z.H., KVETAN V., PASTORES S.M. & VIZI E.S. (1996) Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice 1. *J.Immunol.* **157**, 4634-4640.

HORTA M.F., MENDES B.P., ROMA E.H., NORONHA F.S., MACEDO J.P., OLIVEIRA L.S., DUARTE M.M. & VIEIRA L.Q. (2012) Reactive oxygen species and nitric oxide in cutaneous leishmaniasis.*J.Parasitol.Res.* **2012**, 203818

HUANG S., APASOV S., KOSHIBA M. & SITKOVSKY M. (1997) Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. *Blood* **90**, 1600-1610.

IEZZI G., FROHLICH A., ERNST B., AMPENBERGER F., SAELAND S., GLAICHENHAUS N. & KOPF M. (2006) Lymph node resident rather than skin-derived dendritic cells initiate specific T cell responses after Leishmania major infection. *J.Immunol.* **177**, 1250-1256.

IMAI M., GOEPFERT C., KACZMAREK E. & ROBSON S.C. (2000) CD39 modulates IL-1 release from activated endothelial cells. *Biochem.Biophys.Res.Commun.* **270**, 272-278.

IVAN M., KONDO K., YANG H., KIM W., VALIANDO J., OHH M., SALIC A., ASARA J.M., LANE W.S. & KAELIN W.G., Jr. (2001) HIF alpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* **292**, 464-468.

JI J., SUN J., QI H. & SOONG L. (2002) Analysis of T helper cell responses during infection with *Leishmania amazonensis*. *Am.J.Trop.Med.Hyg.* **66**, 338-345.

JI J., SUN J. & SOONG L. (2003) Impaired expression of inflammatory cytokines and chemokines at early stages of infection with Leishmania amazonensis. *Infect.Immun.* **71**, 4278-4288.

JONES D.E., ACKERMANN M.R., WILLE U., HUNTER C.A. & SCOTT P. (2002) Early enhanced Th1 response after Leishmania amazonensis infection of C57BL/6 interleukin-10-deficient mice does not lead to resolution of infection. *Infect.Immun.* **70**, 2151-2158.

KANE, M.M AND MOSSER,D.M. (2000) Leishmania parasites and their ploys to disrupt macrophage activation. *Curr.Opin. Hematol.* **7**, 26-31

KARHAUSEN J., HAASE V.H. & COLGAN S.P. (2005) Inflammatory hypoxia: role of hypoxia-inducible factor. *Cell Cycle* **4**, 256-258.

KAS-DEELEN A.M., BAKKER W.W., OLINGA P., VISSER J., DE MAAR E.F., VAN SON W.J., THE T.H. & HARMSEN M.C. (2001) Cytomegalovirus infection increases the expression and activity of ecto-ATPase (CD39) and ecto-5'nucleotidase (CD73) on endothelial cells. *FEBS Lett.* **491**, 21-25.

KAYE P.M., ROGERS N.J., CURRY A.J. & SCOTT J.C. (1994) Deficient expression of co-stimulatory molecules on *Leishmania*-infected macrophages. *Eur.J.Immunol.* **24**, 2850-2854.

KEMPF V.A., LEBIEDZIEJEWSKI M., ALITALO K., WALZLEIN J.H., EHEHALT U., FIEBIG J., HUBER S., SCHUTT B., SANDER C.A., MULLER S., GRASSL G., YAZDI A.S., BREHM B. & AUTENRIETH I.B. (2005) Activation of hypoxia-inducible factor-1 in bacillary angiomatosis: evidence for a role of hypoxia-inducible factor-1 in bacterial infection. *Circulation* **111**, 1054-1062.

KIMA P.E., SOONG L., CHICHARRO C., RUDDLE N.H. & MAHON-PRATT D. (1996) Leishmania-infected macrophages sequester endogenously synthesized parasite antigens from presentation to CD4+ T cells. *Eur.J.Immunol.* **26**, 3163-3169.

KOBAYASHI S., ZIMMERMANN H. & MILLHORN D.E. (2000) Chronic hypoxia enhances adenosine release in rat PC12 cells by altering adenosine metabolism and membrane transport. *J.Neurochem.* **74**, 621-632.

KOBIE J.J., SHAH P.R., YANG L., REBHAHN J.A., FOWELL D.J. & MOSMANN T.R. (2006) T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. *J.Immunol.* **177**, 6780-6786.

KONG T., WESTERMAN K.A., FAIGLE M., ELTZSCHIG H.K. & COLGAN S.P. (2006) HIF-dependent induction of adenosine A2B receptor in hypoxia. *FASEB J.* **20**, 2242-2250.

KOSCSO B., CSOKA B., KOKAI E., NEMETH Z.H., PACHER P., VIRAG L., LEIBOVICH S.J. & HASKO G. (2013) Adenosine augments IL-10-induced STAT3 signaling in M2c macrophages.*J.Leukoc.Biol.* **94**, 1309-1315.

KOSCSO B., CSOKA B., SELMECZY Z., HIMER L., PACHER P., VIRAG L. & HASKO G. (2012) Adenosine augments IL-10 production by microglial cells through an A2B adenosine receptor-mediated process. *J.Immunol.* **188**, 445-453.

KOSHIBA M., KOJIMA H., HUANG S., APASOV S. & SITKOVSKY M.V. (1997) Memory of extracellular adenosine A2A purinergic receptor-mediated signaling in murine T cells. *J.Biol.Chem.* **272**, 25881-25889.

LAMMAS D.A., STOBER C., HARVEY C.J., KENDRICK N., PANCHALINGAM S. & KUMARARATNE D.S. (1997) ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X7) receptors. *Immunity.* **7**, 433-444.

LANGSTON H.P., KE Y., GEWIRTZ A.T., DOMBROWSKI K.E. & KAPP J.A. (2003) Secretion of IL-2 and IFN-gamma, but not IL-4, by antigen-specific T cells requires extracellular ATP. *J.Immunol.* **170**, 2962-2970.

LAUFS H., MULLER K., FLEISCHER J., REILING N., JAHNKE N., JENSENIUS J.C., SOLBACH W. & LASKAY T. (2002) Intracellular survival of Leishmania major in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. *Infect.Immun.* **70**, 826-835.

LEITE P.M., GOMES R.S., FIGUEIREDO A.B., SERAFIM T.D., TAFURI W.L., DE SOUZA C.C., MOURA S.A., FIETTO J.L., MELO M.N., RIBEIRO-DIAS F., OLIVEIRA M.A., RABELLO A. & AFONSO L.C. (2012) Ecto-nucleotidase activities of promastigotes from Leishmania (Viannia) braziliensis relates to parasite infectivity and disease clinical outcome.*PLoS.Negl.Trop.Dis.* **6**, e1850

LEMAIRE J., MKANNEZ G., GUERFALI F.Z., GUSTIN C., ATTIA H., SGHAIER R.M., DELLAGI K., LAOUINI D. & RENARD P. (2013) MicroRNA expression profile in human macrophages in response to Leishmania major infection. *PLoS.Negl.Trop.Dis.***7**, e2478

LENNON P.F., TAYLOR C.T., STAHL G.L. & COLGAN S.P. (1998) Neutrophil-derived 5'-adenosine monophosphate promotes endothelial barrier function via CD73-mediated conversion to adenosine and endothelial A2B receptor activation. *J.Exp.Med.* **188**, 1433-1443.

LEVESQUE S.A., KUKULSKI F., ENJYOJI K., ROBSON S.C. & SEVIGNY J. (2010) NTPDase1 governs P2X7-dependent functions in murine macrophages. *Eur.J.Immunol.* **40**, 1473-1485.

LIEW F.Y. & O'DONNELL C.A. (1993) Immunology of leishmaniasis. *Adv.Parasitol.* **32**, 161-259.

LIEW F.Y., LI Y. & MILLOTS. (1990) Tumor necrosis factor (TNF-alpha) in leishmaniasis II. TNF-alpha induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. *Immunology*. **71**,556-559

LIU D. & UZONNA J.E. (2012) The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. *Front Cell Infect.Microbiol.* **2**, 83

LONGHI M.S., MOSS A., BAI A., WU Y., HUANG H., CHEIFETZ A., QUINTANA F.J. & ROBSON S.C. (2014) Characterization of Human CD39(+) Th17 Cells with Suppressor Activity and Modulation in Inflammatory Bowel Disease. *PLoS.One.* **9**, e87956

MAHAMED D.A., MILLS J.H., EGAN C.E., DENKERS E.Y. & BYNOE M.S. (2012) CD73-generated adenosine facilitates Toxoplasma gondii differentiation to long-lived tissue cysts in the central nervous system. *Proc.Natl.Acad.Sci.U.S.A* **109**, 16312-16317.

MAIOLI T.U., TAKANE E., ARANTES R.M., FIETTO J.L. & AFONSO L.C. (2004) Immune response induced by New World *Leishmania species* in C57BL/6 mice. *Parasitol.Res.* **94**, 207-212.

MARQUES-DA-SILVA C., CHAVES M.M., RODRIGUES J.C., CORTE-REAL S., COUTINHO-SILVA R. & PERSECHINI P.M. (2011b) Differential modulation of ATP-induced P2X7-associated permeabilities to cations and anions of macrophages by infection with Leishmania amazonensis. *PLoS.One.* **6**, e25356

MARR J.J., BERENS R.L. & NELSON D.J. (1978) Purine metabolism in *Leishmania donovani* and *Leishmania braziliensis*. *Biochim.Biophys.Acta* **544**, 360-371.

MATLASHEWSKI G. (2001) *Leishmania* infection and virulence. *Med.Microbiol.Immunol.* **190**, 37-42.

MAUEL J. (1990) Macrophage-parasite interactions in *Leishmania* infections. *J.Leukoc.Biol.* **47**, 187-193.

MCKNIGHT A.J. & GORDON S. (1998) Membrane molecules as differentiation antigens of murine macrophages. *Adv.Immunol.* **68**, 271-314.

MEIER C.L., SVENSSON M. & KAYE P.M. (2003) Leishmania-induced inhibition of macrophage antigen presentation analyzed at the single-cell level. *J.Immunol.* **171**, 6706-6713.

MILLER C.M., BOULTER N.R., FULLER S.J., ZAKRZEWSKI A.M., LEES M.P., SAUNDERS B.M., WILEY J.S. & SMITH N.C. (2011) The role of the P2X(7) receptor in infectious diseases. *PLoS.Pathog.***7**, e1002212

MILLS J.H., THOMPSON L.F., MUELLER C., WAICKMAN A.T., JALKANEN S., NIEMELA J., AIRAS L. & BYNOE M.S. (2008) CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. *Proc.Natl.Acad.Sci.U.S.A* **105**, 9325-9330.

MIZUMOTO N., KUMAMOTO T., ROBSON S.C., SEVIGNY J., MATSUE H., ENJYOJI K. & TAKASHIMA A. (2002) CD39 is the dominant Langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness. *Nat.Med.* **8**, 358-365.

MOLL H., FUCHS H., BLANK C. & ROLLINGHOFF M. (1993) Langerhans cells transport Leishmania major from the infected skin to the draining lymph node for presentation to antigen-specific T cells.*Eur.J.Immunol.* **23**, 1595-1601.

MOLL H., SCHARNER A. & KAMPGEN E. (2002) Increased interleukin 4 (IL-4) receptor expression and IL-4-induced decrease in IL-12 production by Langerhans cells infected with Leishmania major. *Infect.Immun.* **70**, 1627-1630.

MOROTE-GARCIA J.C., ROSENBERGER P., NIVILLAC N.M., COE I.R. & ELTZSCHIG H.K. (2009) Hypoxia-inducible factor-dependent repression of equilibrative nucleoside transporter 2 attenuates mucosal inflammation during intestinal hypoxia. *Gastroenterology* **136**, 607-618.

MOSSER D.M. (2003) The many faces of macrophage activation. *J.Leukoc.Biol.* **73**, 209-212.

MOUGNEAU E., BIHL F. & GLAICHENHAUS N. (2011) Cell biology and immunology of Leishmania. *Immunol.Rev.* **240**, 286-296.

NADERER T., WEE E. & MCCONVILLE M.J. (2008) Role of hexosamine biosynthesis in Leishmania growth and virulence. *Mol.Microbiol.* **69**, 858-869.

NIKOLOVA M., CARRIERE M., JENABIAN M.A., LIMOU S., YOUNAS M., KOK A., HUE S., SEDDIKI N., HULIN A., DELANEAU O., SCHUITEMAKER H., HERBECK J.T., MULLINS J.I., MUHTAROVA M., BENSUSSAN A., ZAGURY J.F., LELIEVRE J.D. & LEVY Y. (2011) CD39/adenosine pathway is involved in AIDS progression 2. *PLoS.Pathog.***7**, e1002110

NOEL W., RAES G., HASSANZADEH G.G., DE B.P. & BESCHIN A. (2004) Alternatively activated macrophages during parasite infections. *Trends Parasitol.* **20**, 126-133.

NORTH R.A. & VERKHRATSKY A. (2006) Purinergic transmission in the central nervous system. *Pflugers Arch.* **452**, 479-485.

NYLEN S. & GAUTAM S. (2010) Immunological perspectives of leishmaniasis. *J.Glob.Infect.Dis.* **2**, 135-146.

OHTA A. & SITKOVSKY M. (2001) Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* **414**, 916-920.

PANKRATOV Y., LALO U., VERKHRATSKY A. & NORTH R.A. (2006) Vesicular release of ATP at central synapses. *Pflugers Arch.* **452**, 589-597.

PASSWELL J.H., SHOR R., SMOLEN J. & JAFFE C.L. (1994) Infection of human monocytes by *Leishmania* results in a defective oxidative burst. *Int.J.Exp.Pathol.* **75**, 277-284.

PASTOR-ANGLADA M., CASADO F.J., VALDES R., MATA J., GARCIA-MANTEIGA J. & MOLINA M. (2001) Complex regulation of nucleoside transporter expression in epithelial and immune system cells. *Mol.Membr.Biol.* **18**, 81-85.

PETERS N.C., EGEN J.G., SECUNDINO N., DEBRABANT A., KIMBLIN N., KAMHAWI S., LAWYER P., FAY M.P., GERMAIN R.N. & SACKS D. (2008) In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science***321**, 970-974.

PEYSSONNAUX C., DATTA V., CRAMER T., DOEDENS A., THEODORAKIS E.A., GALLO R.L., HURTADO-ZIOLA N., NIZET V. & JOHNSON R.S. (2005) HIF-1alpha expression regulates the bactericidal capacity of phagocytes. *J.Clin.Invest* **115**, 1806-1815.

PINHEIRO R.O., PINTO E.F., BENEDITO A.B., LOPES U.G. & ROSSI-BERGMANN B. (2004) The T-cell anergy induced by *Leishmania amazonensis* antigens is related with defective antigen presentation and apoptosis. *An.Acad.Bras.Cienc.* **76**, 519-527.

POMPEU M.L., FREITAS L.A., SANTOS M.L., KHOURI M. & BARRAL-NETTO M. (1991) Granulocytes in the inflammatory process of BALB/c mice infected by Leishmania amazonensis. A quantitative approach. *Acta Trop.* **48**, 185-193.

POTH J M., BRODSKY K., EHRENTRAUT H., GRENZ A., ELTZSCHIG HK (2013) Transcriptional control of adenosine signaling by hypoxia-inducible transcription factors during ischemic or inflammatory disease. *J Mol Med (Berl)*. **91(2)**, 183-193

PUGH C.W. & RATCLIFFE P.J. (2003) Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat.Med.* **9**, 677-684.

RAI A.K., THAKUR C.P., SINGH A., SETH T., SRIVASTAVA S.K., SINGH P. & MITRA D.K. (2012) Regulatory T cells suppress T cell activation at the pathologic site of human visceral leishmaniasis. *PLoS.One.* **7**, e31551

RAI A.K., THAKUR C.P., VELPANDIAN T., SHARMA S.K., GHOSH B. & MITRA D.K. (2011) High concentration of adenosine in human visceral leishmaniasis despite increased ADA and decreased CD73. *Parasite Immunol.* **33**, 632-636.

RALEVIC V. & BURNSTOCK G. (1998) Receptors for purines and pyrimidines. *Pharmacol.Rev.* **50**, 413-492.

REGATEIRO F.S., COBBOLD S.P. & WALDMANN H. (2013) CD73 and adenosine generation in the creation of regulatory microenvironments. *Clin.Exp.Immunol.* **171**, 1-7.

REUTERSHAN J., VOLLMER I., STARK S., WAGNER R., NGAMSRI K.C. & ELTZSCHIG H.K. (2009) Adenosine and inflammation: CD39 and CD73 are critical mediators in LPS-induced PMN trafficking into the lungs. *FASEB J.* **23**, 473-482.

RICHARD D.E., BERRA E. & POUYSSEGUR J. (2000) Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. *J.Biol.Chem.* **275**, 26765-26771.

RITTER U., MEISSNER A., SCHEIDIG C. & KORNER H. (2004) CD8 alpha- and Langerin-negative dendritic cells, but not Langerhans cells, act as principal antigen-presenting cells in leishmaniasis. *Eur.J.Immunol.* **34**, 1542-1550.

ROBSON S.C., SEVIGNY J. & ZIMMERMANN H. (2006) The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic.Signal.* **2**, 409-430.

RUSSO-ABRAHAO T., COSENTINO-GOMES D., GOMES M.T., ALVIANO D.S., ALVIANO C.S., LOPES A.H. & MEYER-FERNANDES J.R. (2011) Biochemical properties of Candida parapsilosis ecto-5'-nucleotidase and the possible role of adenosine in macrophage interaction. *FEMS Microbiol.Lett.* **317**, 34-42.

RYZHOV S., NOVITSKIY S.V., ZAYNAGETDINOV R., GOLDSTEIN A.E., CARBONE D.P., BIAGGIONI I., DIKOV M.M. & FEOKTISTOV I. (2008) Host A(2B) adenosine receptors promote carcinoma growth. *Neoplasia.* **10**, 987-995.

SACKS D. & NOBEN-TRAUTH N. (2002) The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat.Rev.Immunol.* **2**, 845-858.

SALMI M. & JALKANEN S. (2005) Cell-surface enzymes in control of leukocyte trafficking. *Nat.Rev.Immunol.* **5**, 760-771.

SANSOM F.M., ROBSON S.C. & HARTLAND E.L. (2008) Possible effects of microbial ecto-nucleoside triphosphate diphosphohydrolases on host-pathogen interactions. *Microbiol.Mol.Biol.Rev.* **72**, 765-81, Table.

SANTOS J.L., ANDRADE A.A., DIAS A.A., BONJARDIM C.A., REIS L.F., TEIXEIRA S.M. & HORTA M.F. (2006) Differential sensitivity of C57BL/6 (M-1) and BALB/c (M-2) macrophages to the stimuli of IFN-gamma/LPS for the production of NO: correlation with iNOS mRNA and protein expression. *J.Interferon Cytokine Res.* **26**, 682-688.

SAVE S. & PERSSON K. (2010) Extracellular ATP and P2Y receptor activation induce a proinflammatory host response in the human urinary tract. *Infect.Immun.* **78**, 3609-3615.

SEMENZA G.L. (2003) Targeting HIF-1 for cancer therapy. *Nat.Rev.Cancer* **3**, 721-732.

SEROR C., MELKI M.T., SUBRA F., RAZA S.Q., BRAS M., SAIDI H., NARDACCI R., VOISIN L., PAOLETTI A., LAW F., MARTINS I., AMENDOLA A., BDUL-SATER A.A., CICCOSANTI F., DELELIS O., NIEDERGANG F., THIERRY S., SAID-SADIER N., LAMAZE C., METIVIER D., ESTAQUIER J., FIMIA G.M., FALASCA L., CASETTI R., MODJTAHEDI N., KANELLOPOULOS J., MOUSCADET J.F., OJCIUS D.M., PIACENTINI M., GOUGEON M.L., KROEMER G. & PERFETTINI J.L. (2011) Extracellular ATP acts on P2Y2 purinergic receptors to facilitate HIV-1 infection. *J.Exp.Med.* **208**, 1823-1834.

SINGH A.K., MUKHOPADHYAY C., BISWAS S., SINGH V.K. & MUKHOPADHYAY C.K. (2012) Intracellular pathogen Leishmania donovani activates hypoxia inducible factor-1 by dual mechanism for survival advantage within macrophage. *PLoS.One.* **7**, e38489

SITKOVSKY M. & LUKASHEV D. (2005) Regulation of immune cells by local-tissue oxygen tension: HIF1 alpha and adenosine receptors. *Nat.Rev.Immunol.* **5**, 712-721.

SITKOVSKY M.V. (2009) T regulatory cells: hypoxia-adenosinergic suppression and re-direction of the immune response. *Trends Immunol.* **30**, 102-108.

SITKOVSKY M.V., LUKASHEV D., APASOV S., KOJIMA H., KOSHIBA M., CALDWELL C., OHTA A. & THIEL M. (2004) Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. *Annu.Rev.Immunol.* **22**, 657-682.

SMITH G.L., BENFIELD C.T., MALUQUER DE M.C., MAZZON M., EMBER S.W., FERGUSON B.J. & SUMNER R.P. (2013) Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. *J.Gen.Virol.* **94**, 2367-2392.

SPATH G.F & BEVERELY S.M. (2001) A lipophosphogly can-independent method for isolation of infective Leishmania metacyclic promastigotes by density gradient centrifugation. *Exp.Parasitol*. **99**,97-103

STEMPIN C., GIORDANENGO L., GEA S. & CERBAN F. (2002) Alternative activation and increase of Trypanosoma cruzi survival in murine macrophages stimulated by cruzipain, a parasite antigen. *J.Leukoc.Biol.***72**, 727-734.

STEMPIN C.C., DULGERIAN L.R., GARRIDO V.V. & CERBAN F.M. (2010) Arginase in parasitic infections: macrophage activation, immunosuppression, and intracellular signals. *J.Biomed.Biotechnol.* **2010**, 683485

STILES G.L. (1992) Adenosine receptors. *J.Biol.Chem.* **267**, 6451-6454.

STROKA D.M., BURKHARDT T., DESBAILLETS I., WENGER R.H., NEIL D.A., BAUER C., GASSMANN M. & CANDINAS D. (2001) HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *FASEB J.* **15**, 2445-2453.

SULLIVAN G.W., FANG G., LINDEN J. & SCHELD W.M. (2004) A2A adenosine receptor activation improves survival in mouse models of endotoxemia and sepsis. *J.Infect.Dis.* **189**, 1897-1904.

SYNNESTVEDT K., FURUTA G.T., COMERFORD K.M., LOUIS N., KARHAUSEN J., ELTZSCHIG H.K., HANSEN K.R., THOMPSON L.F. & k S.P. (2002) Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. *J.Clin.Invest* **110**, 993-1002.

TAKEDA N., O'DEA E.L., DOEDENS A., KIM J.W., WEIDEMANN A., STOCKMANN C., ASAGIRI M., SIMON M.C., HOFFMANN A. & JOHNSON R.S. (2010) Differential activation and antagonistic function of HIF-{alpha} isoforms in macrophages are essential for NO homeostasis. *Genes Dev.* **24**, 491-501.

TAKEDACHI M., QU D., EBISUNO Y., OOHARA H., JOACHIMS M.L., MCGEE S.T., MAEDA E., MCEVER R.P., TANAKA T., MIYASAKA M., MURAKAMI S., KRAHN T., BLACKBURN M.R. & THOMPSON L.F. (2008) CD73-generated adenosine restricts lymphocyte migration into draining lymph nodes. *J.Immunol.* **180**, 6288-6296.

TAYLOR P.R., MARTINEZ-POMARES L., STACEY M., LIN H.H., BROWN G.D. &GORDON S. (2005) Macrophage receptors and immune recognition. *Annu.Rev.Immunol.* **23**, 901-944.

THAMMAVONGSA V., KERN J.W., MISSIAKAS D.M. & SCHNEEWIND O. (2009) Staphylococcus aureus synthesizes adenosine to escape host immune responses. *J.Exp.Med.* **206**, 2417-2427.

VEGA MA & CORBI AL (2006) Human macrophage activation: Too many functions and phenotypes for a single cell type. *Immunologia*. **25(4)**,248-272

VITIELLO L., GORINI S., ROSANO G. & LA S.A. (2012) Immunoregulation through extracellular nucleotides. *Blood* **120**, 511-518.

WANG G.L., JIANG B.H., RUE E.A. & SEMENZA G.L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc.Natl.Acad.Sci.U.S.A* **92**, 5510-5514.

WATANABE K (2005) Differentiation by LPS and IFN-γ of expression of adenosine receptors in macrophage cell lines RAW264 and J774.*Pediatr Dent.* **15(1)**, 43-51.

WARREN C.A., LI Y., CALABRESE G.M., FREIRE R.S., ZAJA-MILATOVIC S., VAN O.E., FIGLER R.A., LINDEN J. & GUERRANT R.L. (2012) Contribution of adenosine A(2B) receptors in Clostridium difficile intoxication and infection. *Infect.Immun.* **80**, 4463-4473.

WERTH N., BEERLAGE C., ROSENBERGER C., YAZDI A.S., EDELMANN M., AMR A., BERNHARDT W., VON E.C., BECKER K., SCHAFER A., PESCHEL A. & KEMPF

V.A. (2010) Activation of hypoxia inducible factor 1 is a general phenomenon in infections with human pathogens. *PLoS.One.* **5**, e11576

WILEY M., SWEENEY K.R., CHAN D.A., BROWN K.M., MCMURTREY C., HOWARD E.W., GIACCIA A.J. & BLADER I.J. (2010) Toxoplasma gondii activates hypoxia-inducible factor (HIF) by stabilizing the HIF-1alpha subunit via type I activin-like receptor kinase receptor signaling. *J.Biol.Chem.* **285**, 26852-26860.

WILSON M.E., INNES D.J., SOUSA A.D. & PEARSON R.D. (1987) Early histopathology of experimental infection with Leishmania donovani in hamsters. *J.Parasitol.* **73**, 55-63.

XAUS J., MIRABET M., LLOBERAS J., SOLER C., LLUIS C., FRANCO R. & CELADA A. (1999) IFN-gamma up-regulates the A2B adenosine receptor expression in macrophages: a mechanism of macrophage deactivation5. *J.Immunol.* **162**, 3607-3614.

XIN L., LI K. & SOONG L. (2008) Down-regulation of dendritic cell signaling pathways by Leishmania amazonensis amastigotes. *Mol.Immunol.* **45**, 3371-3382.

YANG M., MA C., LIU S., SHAO Q., GAO W., SONG B., SUN J., XIE Q., ZHANG Y., FENG A., LIU Y., HU W. & QU X. (2010) HIF-dependent induction of adenosine receptor A2b skews human dendritic cells to a Th2-stimulating phenotype under hypoxia. *Immunol.Cell Biol.* **88**, 165-171.

YAO C., DONELSON J.E. & WILSON M.E. (2003) The major surface protease (MSP or GP63) of Leishmania sp. Biosynthesis, regulation of expression, and function. *Mol.Biochem.Parasitol.* **132**, 1-16.

ZANDBERGEN G., KLINGER M., MUELLER A., DANNENBERG S., GEBERT A., SOLBACH W. & LASKAY T. (2004) Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. *J.Immunol.* **173**, 6521-6525.

ZANIN R.F., BRAGANHOL E., BERGAMIN L.S., CAMPESATO L.F., FILHO A.Z., MOREIRA J.C., MORRONE F.B., SEVIGNY J., SCHETINGER M.R., DE SOUZA WYSE A.T. & BATTASTINI A.M. (2012) Differential macrophage activation alters the expression profile of NTPDase and ecto-5'-nucleotidase. *PLoS.One.***7**, e31205

ZHANG X., GONCALVES R. & MOSSER D.M (2008) The isolation and characterization of murine macrophages. *Curr.Protoc.Immuno*l. **Chapter 14**, Unit

ZIMMERMANN H. (1992) 5'-Nucleotidase: molecular structure and functional aspects. *Biochem.J.* **285 (Pt 2)**, 345-365.