WALMIR DA SILVA

Leishmania infantum NTPDASE 1 AND 2: OBTENTION OF OVEREXPRESSING AND NULL MUTANTS AND THEIR FUNCTIONAL CHARACTERIZATION

Thesis submitted to the Applied Biochemistry Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Juliana Lopes Rangel Fietto

Co-advisers: Gustavo Costa Bressan
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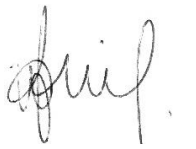

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I dedicate this work to my
beloved Waldir and Sandra.

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To the chaos that got organized on this little blue planet and allowed me to live!

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“I've never made it with moderation
No, I've never understood
All the feeling was all or nothing
And I took everything I could”

(Florence Welch)

ABSTRACT

DA SILVA, Walmir, D.Sc., Universidade Federal de Viçosa, December, 2020. ***Leishmania infantum*** **NTPDase1 and 2: obtention of overexpressing and null mutants and their functional characterization.** Adviser: Juliana Lopes Rangel Fietto. Co-Advisers: Gustavo Costa Bressan, Raphael de Souza Vasconcellos, Thiago Antonio de Oliveira Mendes and Márcia Rogéria Lamego.

Leishmaniasis is a serious public health problem mainly in tropical countries. They are considered neglected tropical diseases with approximately 900 thousand new cases per year according to WHO, 2019. The most severe form of the disease is visceral Leishmaniasis, which in the American continent is caused by the species *Leishmania infantum.* Although it has been described many virulence factors of this parasite, NTPDases have been showed to have a particular role not only in the infection of the parasite to host cells but also in the initial mechanisms of adhesion. NTPDases are described in several pathogenic organisms belonging to the Trypanosomatidae family. They hydrolyze pro-inflammatory ATP and ADP to their monophosphate form AMP, which in turn can be hydrolyzed by the 5'nucletidase to the anti-inflammatory Adenosine (ado), thus having the potential to subvert and prevent host defense mechanisms. In this thesis, we review Leishmania NTPDases biological roles and proposed their potential use as a target molecule for more efficient therapies against leishmaniasis. In addition, for the first time, we used genetic approaches to establish *Leishmania infantum* NTPDases null mutants using CRISPR / Cas9 and overexpressing using Conventional Homologous Recombination. We have successfully obtained clones overexpressing NTPDases. eGFP fused LiNTPDase2 at the C-terminal portion showed nuclear and kinetoplast localization. Furthermore, there were no metabolic changes in the clones overexpressing these enzymes. On the other hand, to obtain null mutants we initially obtained a clone of *Leishmania infantum* JPCM5 expressing SpCas9. Using these clones, we were able to obtain null mutants for NTPDases 1 and 2. However, the double knockout was lethal for Leishmania, evidencing the importance of at least one of these enzymes for the parasite's survival. The null mutants obtained showed a modified metabolism and growth, however, when supplemented with adenine and guanine, the phenotype was reversed. Analysis of

gene expression revealed that in both mutants there was a compensation in the expression of the other isoform that was not deleted. In the adhesion assays, only the null mutant clones showed decreased adhesion while the mutant overexpressing NTPDase2 showed increased adhesion. LiNTPDase1 ko clones showed no differences indicating that it has no role in adhesion. On the other hand, there was a decrease in macrophage infection by null mutants for LiNTPdase1 and 2, indicating their involvement in this mechanism. This report corroborates data from Nitric Oxide assay that showed to be elevated in null mutants indicating their inhibition in the wild type parasite. Enzyme activity using ATP, UDP, and AMP was also performed, but only clones overexpressing LiNTPDase2 showed an increase. Thus, in this thesis, we showed the involvement of LiNTPdases in adhesion, infection, and modulation of the initial response of the macrophage during the initial stages of infection.

Keywords: NTPDases. *Leishmania infantum*. Trypanosomatids. CRISPR/Cas9. Homologous Recombination.

RESUMO

DA SILVA, Walmir, D.Sc., Universidade Federal de Viçosa, dezembro de 2020. **NTPDase 1 e 2 de *Leishmania infantum*: obtenção de mutantes nulos e superexpressores e sua caracterização funcional.** Orientador: Juliana Lopes Rangel Fietto. Coorientadores: Gustavo Costa Bressan, Raphael de Souza Vasconcellos, Thiago Antonio de Oliveira Mendes e Márcia Rogéria Lamego.

Leishmanioses são consideradas um grave problema de saúde pública principalmente em países tropicais. São doenças negligenciadas com aproximadamente 900 mil novos casos anuais segundo a WHO, 2019. A Leishmaniose visceral é a forma mais grave da doença e no continente americano é causada pela espécie *Leishmania infantum*. Dos diversos fatores de virulência deste parasito as NTPDases tem sido descritas com um importante papel não somente na infecção do parasita a células hospedeira, mas também nos mecanismos iniciais de adesão. NTPDases são descritas em diversos organismos patogênicos. Elas hidrolisam de ATP e ADP pró-inflamatórios a sua forma monofosfatada AMP, que por sua vez pode ser hidrolisado pela 5'-nucletidase a Adenosina (ado) anti-inflamatória, tendo assim, o potencial de subverter e evitar mecanismos de defesa do hospedeiro. Neste trabalho, nós revisamos os papeis biológicos das NTPDases de Leishmania e propomos seu potencial uso como molécula alvo de terapêuticas mais eficientes contra as leishmanioses. Além disso, pela primeira vez nós utilizamos abordagens genéticas para estabelecermos mutantes nulos usando CRISPR/Cas9 e mutantes superexpressores usando recombinação homologa convencional para as NTPDases 1 e 2 de *Leishmania infantum*. Obtivemos com sucesso clones superexpressores das NTPDases. LiNTPDase2 fundida a eGFP na porção C-terminal mostrou localização nuclear e cinetoplastica. Além disso não houve modificação na taxa de crescimento nos clones superexpressores destas enzimas. Por outro lado, para obtenção de mutantes nulos inicialmente obtivemos um clone de *Leishmania infantum* JPCM5 expressando a SpCas9. Utilizando estes clones fomos capazes de obter mutantes nulos para as NTPDases 1 e 2. Contudo, dupla deleção foi letal para a Leishmania, evidenciando a importância de pelo menos uma destas enzimas para a sobrevivência do parasita. Os mutantes nulos obtidos mostraram um metabolismo e

crescimento modificado, porém quando suplementados com adenina e guanina, o fenótipo foi revertido. Analise de expressão genica revelou que em ambos os mutantes houve uma compensação na expressão da outra isoforma que não foi deletada. Nos ensaios de adesão somente os clones mutantes nulos mostraram diminuição da adesão enquanto o mutante superexpressor da NTPDase2 mostrou aumento da adesão. Mutante nulo da LiNTPDase1 não mostrou diferença indicando que ela não possui papel na adesão. Por outro lado, houve diminuição da infecção de macrófagos por mutantes nulos paras as LiNTPdase1 e 2 indicando seus envolvimentos neste mecanismo. Estes dados corroboram os dados de mensuração de Oxido Nítrico que se mostraram elevados nos mutantes nulos indicando a sua Inibição no parasita selvagem. A atividade das enzimas usando ATP, UDP e AMP também foram realizadas, porém somente os clones superexpressores da LiNTPDase2 mostraram aumento. Desta forma, neste trabalho nos mostramos o envolvimento da LiNTPdases na adesão, infecção e modulação da resposta inicial do macrófago durante as etapas iniciais da infecção.

Palavras-chave**:** NTPDases. *Leishmania infantum*. Trypanosomatideos. CRISPR/Cas9. Recombinação Homologa.

SUMMARY

1. CHAPTER 1: GENERAL INTRODUCTION

Leishmaniasis is a disease caused by flagellated protozoa belonging to the Trypanosomatidae family and Leishmania genus. Its clinical manifestations differ according to the infecting species, ranging from cutaneous, mucocutaneous to visceral lesions (MOSSER; EDELSON, 1987; WHO, 2019) . Leishmaniasis is considered a neglected disease affecting about 12 million people, mainly in tropical countries. It is estimated that over 900 thousand new cases occur annually, placing it as one of the most important tropical diseases associated with high mortality and morbidity worldwide (WHO, 2019) . Visceral leishmaniasis is considered the most severe form and in American continent, it is caused by *Leishmania infantum* (WHO, 2019).

Leishmania species have a digenetic life cycle, they occur both in a Phlebotomus or Lutzomyia genus invertebrate vector and in mammalian vertebrate hosts (MOSSER; EDELSON, 1987). In the urban environment, the dog is the main intermediate host of the parasite participating in the maintenance and transmission of the disease to human (FERRO et al., 1995; VASCONCELLOS et al., 2014). The parasite is transmitted to humans by inoculating the metacyclic promastigote forms during female blood-meal (FERRO et al., 1995). This initial stage is characterized by a high infiltration of neutrophils that initially phagocyte metacyclic promastigotes and produce cytokines for large recruitment of macrophages (MOSSER; EDELSON, 1987). Leishmania's invasion of macrophages occurs via phagocytosis and, in these cells, Leishmania lodges and multiplies by modulating macrophage signaling pathways involved in the elimination of the parasite (LIESE; SCHLEICHER; BOGDAN, 2008; SCHARTON-KERSTEN et al., 1995; SCOTT, 1991).

In macrophage phagolysosomes, promastigotes turn into amastigotes. The multiplication of amastigotes inside the cells culminates in cell lysis that allows the infection of new cells or infection of the vector insect, leading to the maintenance of the parasite's biological cycle (LAINSON et al., 1985; SHARMA; SINGH, 2008). The diagnosis of visceral leishmaniasis is made by a combination of clinical signs, parasitological, molecular, or serological tests. However, there is no standard, easy, inexpensive, and non-invasive method and there is a limited number of drugs for the treatment whose effectiveness varies widely (CHAPPUIS et al., 2007; ROMERO; BOELAERT, 2010; WHO, 2019). In addition, the attempt to control Leishmaniasis in dogs and humans results in an increase in parasitic resistance to drugs such as pentavalent antimonials, pentamidine, amphotericin B and miltefosine (DANTAS-TORRES, 2007; HENDRICKX et al., 2020; KAPIL; SINGH; SILAKARI, 2018; RAMESH et al., 2020; SANTOS et al., 2020).

The ability of Leishmania to reverse the infection control mechanism and anti-parasitic functions of macrophages rely on its ability to modulate various signaling pathways that regulate the host's defense systems. Leishmaniasis, therefore, is dependent on the early stages of infection and involves molecular and signaling interactions between the parasite and the host (ROGERS et al., 2009). Several mechanisms contribute to the evasion of the immune system by Leishmania. These mechanisms include: escape from complement system activation, inhibition of macrophage activation by apoptotic neutrophils and nitric oxide (NO) production, modulation not only of inhibitory cytokines by the macrophage and dendritic cells such as IL-10, TGF-β, but also of T cell activity. However, these are not the only mechanisms (MOSSER; EDELSON, 1987). In fact, several molecules present on the surface of Leishmania parasites have been linked in the parasite-host cell interaction and have been

described as potential virulence factors. For example, lipophosphoglycan, glycosyl inositol, phospholipids, proteophosphoglycans, secreted acid phosphatases, and cysteine protease B (CASTELLUCCI et al., 2014; ILG; DEMAR; HARBECKE, 2001; MOTTRAM; COOMBS; ALEXANDER, 2004; REMALEY et al., 1985). Additionally, several studies have pointed to the involvement of elements of Leishmania purinergic signaling as one of the mechanisms involved in controlling of the host immune system by the parasite (DA SILVA et al., 2020; DE FIGUEIREDO; SOUZA-TESTASICCA; AFONSO, 2016; PAES-VIEIRA; GOMES-VIEIRA; MEYER-FERNANDES, 2018; PIMENTEL et al., 2016; SANSOM, 2012).

Our research group and another groups have shown strong evidence of the potential of Nucleotides Triphosphate Diphosphohydrolases (NTPDases) as key molecules in modulating the parasite's virulence (DA SILVA et al., 2020). NTPDases are apyrases belonging to the family of CD39/GDA1. They are characterized by presenting five conserved regions called ACRs (apyrase conserved regions) involved in the catalytic domain of nucleotidase activity (HANDA; GUIDOTTI, 1996; KEGE et al., 1997; ZIMMERMANN; BRAUN, 1999). These enzymes hydrolyze extracellular nucleotides, generating nucleotides and nucleosides thus controlling the levels of tri and di-phosphate nucleotides and the pro-inflammatory response caused by them (DI VIRGILIO et al., 2009).

Enzymes homologous to mammalian NTPDases (CD39) have also been found in various pathogenic organisms (DE ALMEIDA MARQUES-DA-SILVA et al., 2008; DE SOUZA et al., 2013b; FIETTO et al., 2004; GOMES et al., 2015; LEITE et al., 2012; MAIOLI et al., 2004; MARIOTINI-MOURA et al., 2014; SANSOM; ROBSON; HARTLAND, 2008a; SILVERMAN et al., 1998; VIVIAN et al., 2010). In *Leishmania infantum* two

isoforms have been characterized, LiNTPDase 1 and LiNTPDase 2 (DE SOUZA et al., 2013a; VASCONCELLOS et al., 2014). The mechanisms by which pathogen-associated NTPDases can affect virulence are not fully understood (SANSOM; ROBSON; HARTLAND, 2008a). All experimental evidences suggest that the hydrolysis of ATP and ADP by these enzymes could have the potential to subvert and prevent host defense mechanisms. In addition, the hydrolysis of AMP to adenosine by the host or parasitic 5'-nucleotidase (5'-NT) enzymes could have several effects since adenosine (ADO) plays an important role in limiting the inflammatory response as well as parasites can use it to grow (DA SILVA et al., 2020; SANSOM; ROBSON; HARTLAND, 2008b).

Data from our research group have shown that *L. infantum* has two NTPDases enzymes (NTPDase1 and 2) homologous to mammalian NTPDases 5 and 6 and that they could be important for adhesion and infection of host cells (VASCONCELLOS et al., 2014). Nevertheless, those evidences were done using indirect assays (competition and/or blocking assays). In fact, a study of genetic manipulation of LiNTPDases 1 and 2 genes for the definitive characterization of their function has not yet been carried out and it is the focus of the present work.

In the post-genomic era, genome editing has become the main tool used in several experimental biological systems (DOUDNA; CHARPENTIER, 2014). Studies of gain or loss of function such as deletions, insertions, mutations of genes were particularly used to alter target genes through Homologous Recombination (RH). This molecular mechanism that naturally occurs to repair the damaged DNA molecule has been used for genomic editing of a target locus. This tool has been applied in gene therapy, studies of gene function, and in the production of transgenic organisms (KUWAYAMA, 2012). Genetic editing via RH has been an important tool for the characterization of metabolic

pathways and virulence mechanisms in Leishmania (CAPECCHI, 1989; CRUZ; COBURN; BEVERLEY, 1991; SANTRICH et al., 1997), as well as for many other pathogenic protozoa (CRABB et al., 1997; GUEDES AGUIAR et al., 2018).

A system capable of promoting the production of heterologous proteins in Leishmania in high yield, both via intracellular and extracellular production (secretion) was developed in 2002 by (BREITLING et al., 2002): the expression system in Leishmania (LEXSY -Leishmania Expression System). If the goal is to add extra copies of a gene or fuse it to a reporter gene, the use of LEXSY allows the approach to gain function of the genes of interest. This system allows the integration of the genes of interest to the locus of the 18S subunit of the ribosomal RNA (SSU) and the chosen proteins would be produced in extra copies via constitutive expression, under the control of RNA polymerase I (BREITLING et al., 2002; MISSLITZ et al., 2000). In this way, it would be possible to obtain clones that overexpress proteins of interest and, that express a fused protein, for example, eGFP, important for functional studies (DE OLIVEIRA et al., 2019). Several studies have generated mutant forms with eGFP in different Leishmania species and have pointed out advantages such as low toxicity, easy imaging, no need for substrate, permeabilization, or cell fixation in immunolocalization assays (BOLHASSANI et al., 2011; MEHTA et al., 2008; PULIDO et al., 2012).

Loss of function is another method employed in the study of the functional characterization of genes and involves the construction of cloning cassettes for genetic editing. This technique has been used in Leishmania and other pathogenic protozoa and uses the homology to the 5’ and 3' UTR regions of the gene of interest to flank a gene for drug resistance that will replace the target gene in the genome of the parasite. Insertion in the target locus allows the substitution and loss of the expression of the target gene

(FULWILER et al., 2011; GHASEMI NEJAD ALMANI et al., 2016; KUWAYAMA, 2012; SANSOM et al., 2014). However, these techniques still have some limitations since the success rates are extremely low and many rounds of transfections are necessary, laborious, and time-consuming (FULWILER et al., 2011).

Despite significant advances obtained in the study of some biological mechanisms in trypanosomatids, inefficiency in obtaining mutants due to “off targeting” effects on recombination makes it difficult to obtain null mutants, especially for Leishmania species, mainly due to the high rate of chromosomal aneuploidy. (CLAYTON, 2016). In addition, the use of interfering RNA (RNAi) is inefficient due to the absence of RNAi-related genes like Dicer and Argonauta in most species of Leishmania including *L. infantum* (LYE et al., 2010). Currently, the gold tool for genetic editing has been the Homologous Recombination mediated by the system of *Clustered Regularly Interspaced Short Palindromic Repeats associated with nuclease Cas9* (CRISPR / Cas9). Despite the high efficiency and specificity, to date, there is no record in the literature of the use of this technique to delete the genes of *Leishmania infantum* NTPDases (DA SILVA et al., 2020).

The CRISPR-Cas9 system was developed as a powerful technique for genome editing and has been used to study the function of genes in various organisms, including protozoa such as *Plasmodium*, *Toxoplasma*, *Trypanosoma cruzi and Leishmania* (BENEKE et al., 2019; GHORBAL et al., 2014; PENG et al., 2014; SHEN et al., 2014; SOARES MEDEIROS et al., 2017; WAGNER et al., 2014; ZHANG; LYPACZEWSKI; MATLASHEWSKI, 2017). The system is particularly interesting in Leishmania because the classic methods of manipulating the genome have serious limitations in this organism, one of which is the difficulty in performing gene knockouts due to its high genomic plasticity, in particular, to aneuploidy (LACHAUD et al., 2014; STERKERS et al., 2011). In

fact, in these classic techniques, two or more rounds of transfections may be necessary and, in the case of essential genes, additional copies of the target gene, chromosome fragment, or entire chromosome may appear in each transfection cycle (DUBESSAY et al., 2002). Thus, the use of a system based on delivery of the nuclease by a guide RNA, makes this system much more efficient than the systems used until now.

In view of the importance of NTPDases for the infection by Leishmania and in face of the importance of genetic approaches for the functional characterization of a gene, in this work, we review the known biological roles of Leishmania NTPDases bringing light their potential for biotechnological applications (chapter 2), and we used genomic editing tools to overexpress and knockout LiNTPDases 1 and 2 as well as we did their functional characterization (chapter 3).

1.1 HYPOTHESIS

Several evidences suggest that NTPDases 1 and 2 of *L. infantum* play a central role in the parasite infection, then the loss and gain of function of the genes encoding these enzymes will differentially modulate infection, adhesion, and the immune response of the host cells.

1.2 OBJECTIVES

Here, we have the general objectives to establish cellular models of Leishmania infantum genetically modified for the enzymes NTPDases1 and 2 for their biological characterization.

Specific objectives:

- Review on literature the biological roles of *Trypanosomatids* NTPDases and their biotechnogical applications;
- Obtention of *L. infantum* overexpressing NTPDases 1 and 2 fused or not to eGFP to their functional characterization;
- Establish *L. infantum* NTPDase1 and 2 null mutants by CRISPR/Cas9 to their functional characterization;

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2. CHAPTER 2: ENTPDASES FROM PATHOGENIC TRYPANOSOMATIDS AND PURINERGIC SIGNALING: SHEDDING LIGHT TOWARDS BIOTECHNOLOGICAL APPLICATIONS

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Abstract: *ENTPDases are enzymes known for hydrolyzing extracellular nucleotides and playing an essential role in controlling the nucleotide signaling via nucleotide/purinergic receptors P2. Moreover, ENTPDases, together with Ecto-5´-nucleotidase activity, affect the adenosine signaling via P1 receptors. These signals control many biological processes, including the immune system. In this context, ATP is considered as a trigger to inflammatory signaling, while adenosine (Ado) induces anti-inflammatory response. The trypanosomatids Leishmania and Trypanosoma cruzi, pathogenic agents of Leishmaniasis and Chagas Disease, respectively, have their own ENTPDases named "TpENTPDases," which can affect the nucleotide signaling, adhesion and infection, in order to favor the parasite. Besides, TpENTPDases are essential for the parasite nutrition, since the Purine De Novo synthesis pathway is absent in them, which makes these pathogens dependent on the intake of purines and nucleopurines for the Salvage Pathway, in which TpENTPDases also take place. Here, we review information regarding TpNTPDases, including their known biological roles and their effect on the purinergic signaling. We also highlight the roles of these enzymes in parasite infection and their biotechnological applications, while pointing to future developments.*

Keywords: ENTPDases, Purinergic signaling, Leishmaniasis, Chagas Disease, Parasites, Biotechnology

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2.1 INTRODUCTION

Nucleoside triphosphate diphosphohydrolases (NTPDases) belong to the CD39 gene family. They have been characterized by the presence of five apyrase conserved regions (ACR1 to ACR5). They also catalyze the hydrolysis of tri- and diphosphate nucleosides to their monophosphate forms. Ecto-5'-nucleotidases (5´-NTs), which belong to the CD73 gene family, take these monophosphate nucleosides to hydrolyze them to nucleoside. In a highly controlled manner, ENTPDases and 5´-NTs are able to modulate the nucleotide purinergic signaling [1,2].

These enzymes are ubiquitously expressed from mammals to unicellular pathogens, such as, the protozoan *Leishmania* genus [3], *Trypanosoma cruzi* [4], and the bacteria *Legionella penumophila* [5]. In the CD39 gene family, some enzymes are localized at the cell surface (named Ecto-NTPDases or ENTPDases), while others are localized in internal compartments of the cells[3]. In mammalian, these ENTPDases are involved in the control of nucleotide/purinergic signaling, which controls diverse biological systems, such as blood clotting and the immune system [6]. These enzymes are also present in pathogens and have been described as participants in the purine uptake by Purine Salvage Pathway and in infection and virulence mechanisms [5–10].

The roles of ENTPDases in adhesion, infection and virulence of pathogens have been studied by many research groups. The current understanding is that higher ENTPDase hydrolysis of ATP and ADP is associated with the production of Ado by 5´-NT, which drives to higher virulence and play a pivotal role on the subversion or blockage of host-defense mechanisms [1,3–6,11–17]. At least part of these effects is a consequence of the control of nucleotide/purinergic signaling on the host cells and tissues (Figure 1).

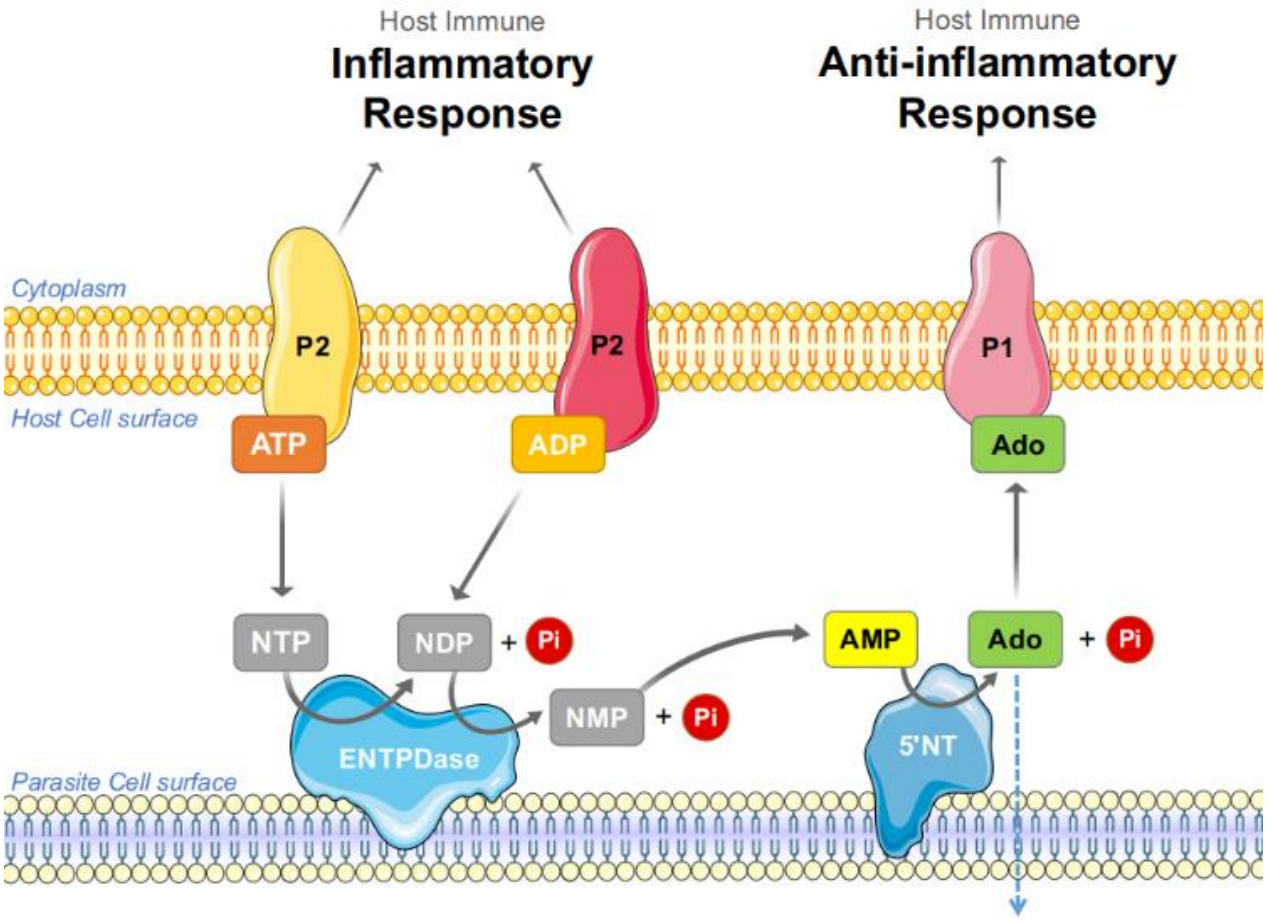
Host Immune
Inflammatory Response
Host Immune
Anti-inflammatory Response
Cytoplasm
Host Cell surface
P2
P2
P1
ATP
ADP
Ado
NTP
NDP
+ Pi
AMP
Ado
+ Pi
NMP
+ Pi
ENTPDase
5'NT
Parasite Cell surface

Figure 1 - Illustration of the effect of ENTPDases from the external surface of the parasite on the nucleotide/purinergic signaling in the host cells during the infection. The host cell surface in the context of immune cells with the presence of nucleotide/purinergic receptors P2 and the adenosine P1 receptor are presented at the upper part**.** In the infection, extracellular ATP is produced by the host as a danger signaling to induce the inflammatory response and control of the infection, which can be significantly decreased by the action of the ENTPDase in the surface of the parasite (lower part of the figure) converting ATP, ADP, UTP, and UDP into monophosphate nucleotides. If the parasite also hydrolyzes AMP into adenosine (Ado) via 5´-NT, it will lead to an anti-inflammatory response via P1 receptors. Considering this model, the parasites with the highest ENTPDase and 5´-NT activities could better modulate the host immune system in order to favor the establishment of the infection. Besides, Ado could be transported into the parasite and used in the purine salvage pathway.

It has been demonstrated that nucleotides are able to stimulate the purinergic P1 and P2 receptors on the host cell membrane [14]. These events play an important role in the modulation of the immune system in which P1 is involved in the anti-inflammatory response, while P2 is involved in pro-inflammatory stimuli (Figure 1). Each receptor has its own set of substrates and is stimulated in specific ranges of ligand concentrations[1]. The same mechanism of control has been demonstrated in different pathogenic agents[18], including the pathogenic trypanosomatids *Leishmania (Leishmania) tropica*[19], *Leishmania (Leishmania) amazonensis*[12,20,21], *Leishmania (Viannia) braziliensis*, *Leishmania (Leishmania) major*[11,15,22], *Leishmania (Leishmania) infantum* [3,23], *Trypanosoma cruzi* [4,11,20,24–27], *Trypanosoma rangeli*[28], and *Trypanosoma brucei* [29]. In this review, we focus on parasites that cause Leishmaniasis and Chagas Disease (different species of *Leishmania* genus and *Trypanosoma cruzi,* repectively). Moreover, we highlight the roles of ENTPDases in the adhesion, infection, and control of the immune system (Figure 1). We also discuss the potential biotechnological use of these enzymes on prophylactic vaccination, drug development, as well as diagnostic kits, bring new

insights into light, once none of the current drugs have been developed specifically against ENTPDases of these parasites.

2.2 PARASITES AND THEIR ASSOCIATED DISEASES

Trypanosomatids are unicellular protozoa classified as Kinetoplastidae, due to the presence of a specific organelle named as kinetoplastid. The kinetoplast is a specialized large unique mitochondria with a condensed mitochondrial DNA [30,31]. *Leishmania* genus and *T. cruzi* are pathogenic kinetoplastids that cause Leishmaniasis and Chagas Disease, respectively. In Table 1, we show a summary of the diseases, the part of the World affected, and their main pathogenic agents.

Leishmaniasis is a zoonotic disease transmitted by female sandflies during blood meals, which can affect humans, and other mammals, such as dogs. It has been estimated that approximately 200,000 people are affected by this parasite, and 20,000 new cases emerge every year [32]. Clinical manifestations range from single ulcerative cutaneous lesions (Cutaneous Leishmaniasis); to ulcerative lesions at the mucous tissues (Mucocutaneous Leishmaniasis), non-ulcerative but scattered lesions through the entire body (Disseminated Leishmaniasis) and severe infection of internal visceral organs (Visceral Leishmaniasis), which could be fatal[32]. The disease manifestation depends on the species/strain of the pathogen and the host immunocompetence. The main cells infected during the development of the disease are the macrophages. It is well known that *Leishmania* genus can subvert the immune response of the host.

Table 1 - General data from Leishmaniasis and Chagas Disease

Disease/main manifestation	Main Pathogenic Trypanosomatids	Typical disease manifestation	Main infected cell	Part of the world endemically affected	Estimated # cases	References
Cutaneous leishmaniasis	*L. Leishmania aethiopica, L. (L.) amazonensis, L. (L.) major, L. (Viannia) peruviana, L. (Viania) panamemsis, L. (L.) tropica*	Single ulcerated cutaneous lesions	Macrophages	Central and South Americas, South of North America, Africa, Middle East, South Asia	200,000/year (1million/5years) >1 billion people living in risk areas	32
Diffuse cutaneous leishmaniasis	*L. (L.) aethiopica, L, (L.) amazonensis, L. (L.) mexicana*	Multi non ulcerated cutaneous lesions				32
Mucocutaneous leishmaniasis	*L. (V.) braziliensis*, *L. (V.) panamensis*	Disfiguration of mucosa (mouth, nose, pharynx)				32
Visceral leishmaniasis	*L. (L.) donovani, L. (L.) infantum*	Intense infection of visceral organs (liver, spleen, bone marrow)	Macrophages	Central and South Americas, Africa, Middle East, South of Asia	300,000/year (>20,000 death/year)	32
Chagas Disease	*T. cruzi*	Loss of muscle strength, increase of organs (mega heart, megacolon, mega esophagus	Smooth muscle cells	Latin America	8 million (total) 10,000 deaths/year	32

On the other hand, Chagas disease is caused by the protozoa *Trypanosoma cruzi*. Approximately 8 million people are infected with the parasite, and over 10,000 people die every year as a result of the disease, most of which in tropical areas (Table 1) [32]. This parasite is transmitted by bloodsucking invertebrate vectors and, in rare cases, by non-vector infection mechanisms, including blood transfusion, ingestion of contaminated food and congenital transmission[33]. The clinical presentation of Chagas disease comprises the acute and chronic phases. Due to the difficulty of diagnosing the acute phase untreated patients usually progress to the chronic phase, presenting cardiac dysfunction/failure or gastrointestinal dysregulation[34,35].

At the beginning of the infection, the main infected cells are macrophages, and in the long-term chronic phase, the main infected cells are smooth muscle cells. In consequence of the long-term infection of smooth muscle cells and the inflammatory reaction in the tissue, which leads to fibrosis, the respective organs usually increase in length and lose part of their motor functions [34].

The development of both Leishmaniasis and Chagas Disease includes the infection of immune system cells, and the infection maintenance is dependent on the host immune response. Taking this information into account and the known roles of ENTPDases in the control of the immune system, selective blocking the ENTPDases of the parasites could be a good approach for the development of new drugs against the diseases[4,12,15,22,25–27]. Leishmaniasis and Chagas disease are considered Neglected tropical diseases (NTDs), which account for high morbidity and mortality worldwide, and affect not only poor and low-income countries but also developed G20 countries[32,35]. It means that the pharmaceutical industry has made no efforts to develop new drugs, treatments, or even prophylactic tools. As a result, there are no vaccines to prevent the human disease, and the non-effective arsenal to treat the patients are very restricted once they show high toxicity and some strains have even developed resistance to treatment[36].

Currently, there are some drugs to treat these diseases based on different targets, as well as different administration approach, but none of them were specifically developed to treat either Leishmaniasis or Chagas disease. As a result, the existent drugs present high side

effects[37–42], this is the situation for example for the only two drugs used to treat Chagas disease Benznidazole and Nifurtimox.

The Leishmaniaisis first-line treatment has used pentavalent antimonial, which is known to inhibit glycolysis and fatty acid oxidation on parasite[42]. Besides presenting a range of side effects, these drugs are not specific for parasite metabolism[37,43]. Amphotericin B is another drug commonly used in four different formulations, including the liposomal one, which is more effective and has lower side effects, but still presents side effects and is more expensive than Amphotericin B Deoxycholate[37,41,44–46]. This antibiotic, as well as the antifungal Imidazole and Triazole bind to ergosterol molecules on parasite cytoplasmatic membrane and increases ion influx, but is not parasite specific either[43,47,48]. In addition, Paromomycin is an aminoglycoside antibiotic whose activity mechanisms are not completely known, but its usage is quite effective, with low toxicity, mainly against cutaneous leishmaniasis [38,49]. Pentamidine is an aromatic diamine presented as Isethionate and Mesylate, which decreases mitochondrial membrane potential[47]. The long term usage drives to chronic metabolic disease. The most recent drug used is Miltefosine, an anticancer drug that disrupts the transporters of the cell by accumulating the drug and could lead to several side effects[44,45,50,51]. In addition, it has been reported several resistant strains to Miltefosine which could be a problem to the control of the disease[45,50,52].

Taken together, all of the current drugs used on Trypanosomatid parasite diseases present several problems, such as severe side effects, low patient adhesion, high cost, parasite resistance, and are not specific to target the parasite molecules. Here, we highlight the advantages of using Trypanosomatids ENTPDases as target molecules, once their identity to mammalian ENTPDase is clearly divergent on aminoacid sequence[3].

The development of new drugs and the identification of possible new specific targets is urgent and extremely important to enhance leishmaniasis and Chagas Disease treatment or more effective and safer therapies against NTDs[39,40,44]. The right approach seems to be the one involving virulence molecules. Interestingly, ENTPDases play vital roles not only in trypanosomatids virulence mechanism but also in its survival during the infection. [3,9,26].

2.3 LEISHMANIA ENTPDases

Different species of *Leishmania* genus have two isoforms of ENTPDases: one with ~70 kDa and another with ~42 kDa. These enzymes do not have a broadly accepted standard terminology and, therefore, in this review, we will follow the nomenclature proposed by Vasconcellos [3]. Thus, the isoform with higher molecular mass will be named ENTPDase1, and the lower molecular mass isoform will be designated as ENTPDase2. When the enzyme name is not defined along with the review, both isoforms could be considered, since the study in matter evaluated the ENTPDase activity and did not discriminate the isoforms. The following topics will focus on the roles of the ENTPDases in different species of pathogenic *Leishmania* genus.

2.3.1 Leishmania *(L.) infantum* ENTPDases

Leishmania (L.) infantum ENTPDases (LiNTPDases) are capable of hydrolyzing extracellular nucleotides in a Ca^{+2}/Mg^{+2}-dependent manner [3,29,53]. In addition, they have been biochemically characterized and classified as genuine apyrases by cloning and expression not only in the classical bacteria system but also in mammalian COS-7 cell system [53]. More than its nutritional role, several studies have pointed out NTPDases as necessary on infectivity and virulence of different species of Leishmania [15,20,22,24,26,27], as well as to modulation of the host immune system, which favors the establishment of infection [12,54,55].

L. (L.) infantum has two putative NTPDases in its genome encoding, approximately 70 kDa LiNTPDase1 and 45 kDa LiNTPDase2 [3]. An N-transmembrane region or signal peptide was predicted, suggesting membrane anchorage of LiNTPDase2 and putative secretion for LiNTPDase1[3]. Using polyclonal antibodies produced against the recombinant LiNTPDase2 the LiNTPDases were found on the surface of the parasite and also in the intracellular space, such as the nucleus, flagellar pocket, mitochondria, endoplasmic reticulum and Golgi apparatus[3]. Since the polyclonal antibody recognizes both isoforms, it was not possible to discriminate between the isoforms in the localization

data. The same pattern of localization was observed for *L. (L.) amazonensis* and *L. (V.) braziliensis,* suggesting conserved localization between different Leishmania species [16,56].

Many aspects of the function of LiNTPDases have been described in the literature. It has been demonstrated a correlation between the ectonucleotidase activity and the parasite ability to infect macrophages, thus relating its activity to the virulence capacity of *L.(L.) infantum* [3]. Using an anti-LiNTPDases serum to block the enzymes during host-pathogen interaction, the authors showed a significant decrease in the adhesion and infection of the parasites on the macrophage. However, they did not find effects on the intracellular proliferation of amastigotes[3]. This result indicates that LiNTPDases could be crucial in the beginning of the infection, but could not be crucial after the onset of the infection, during the intracellular cycle of the parasite, at least for proliferation.

The action of *L. (L.) infantum* in subverting the host immune system was explored in the context of adenosine signaling. A promising publication described that *L.(L.) infantum* subvert the host inflammatory response through the adenosine A2a receptor to promote the establishment of infection [57]. It is essential to notice that the presence of LiNTPDases on the surface of *L.(L.) infantum* [3] and the ability of these enzymes to hydrolyze ATP and ADP [53] corroborates the possible action of these enzymes in the enzymatic cascade to produce adenosine during the establishment of infection. Furthermore, it has been suggested that NTPDases in other Leishmania species may interfere with the establishment of the immune response and favor the parasite [15].

Interestingly, we know the importance of tri and diphospho-nucleotide modulation and the production of adenosine ($P1_{A2a}$) to allow *L. infantum* interaction and infection on macrophages. No studies addressing the P2 receptor and nucleotide signaling on the host have been carried out, which could be a good topic for further research. Moreover, another important topic that could improve knowledge in the field is the study on the consequences of the null mutant or even the overexpression of the *L. (L.) infantum* LiNTPDase1 and LiNTPDase2 during experimental infection.

2.3.2 Leishmania *(L.) amazonensis* ENTPDases

The ATPase activity in *Leishmania (L.) amazonensis* has been characterized on the surface of living parasites, and virulent parasites have demonstrated the ability to hydrolyze more ATP than the non-virulent ones [20,21]. Interestingly, the higher AMP hydrolysis rate has shown a correlation to the emergence of more severe lesions in *L. (L.) amazonesis* infection, compared to *L. (V.) braziliensis*. This datum suggests that higher adenosine production in *L. (L.) amazonensis* infection may be related to increased anti-inflammatory response [58]. Indeed, Marques-da-Silva [22] showed that *L. (L.) amazonensis* presents higher extracellular nucleotide hydrolysis correlated with higher plasma membrane LaNTPDase2 expression, compared to *L.(V.) braziliensis* and *L. (L.) major*. They also evaluated the role of adenosine in the establishment of infection in the murine model. They showed that adenosine generation is related to higher parasite load, larger lesions and delayed resolution of lesions. These data suggest that not only the higher ATP hydrolysis but also the higher production of adenosine lead to the immunomodulatory effect, favoring the establishment of infection.

Additionally, the biochemical characterization of the parasite surface ENTPDases was demonstrated [21]. Two polypeptides of 43 and 54 KDa with promastigote ENTPDase expression and activity have decreased, over 96 h of in vitro culture compared to amastigotes. The higher and transitory activity was observed in the logarithmic phase, when the cell prepares for cell division, and nutrients are required. Moreover, this work showed that adenosine reduced Ecto-ATPase promastigote recognition in a dose-dependent manner [21]. The treatment of the parasite with adenosine and anti-ENTPDases antibodies reduced the interaction with macrophages. These results suggest the participation of ENTPDases in cellular differentiation and the adhesion of the *L. (L.) amazonensis* to the host cell [21].

More recently, Gomes [12] compared one virulent strain of *L. (L.) amazonensis* (named PH8) with one derivative less virulent clone (named 1IIId). They have demonstrated that the original strain PH8 has higher ectonucleotidase activity for ATP, ADP, and AMP and higher membrane expression of 42 kDa protein (LaNTPDase2) than the clone 1IIId. This fact was associated with a higher parasite survival in the host cell and decreasing NO

production in the original strain. They also showed that the adenine treatment reduced the expression and activity of ENTPDase in *L. (L.) amazonensis*. The adenine treatment also impaired the resistance of the parasite to macrophage activation, leading to higher NO production [12]. These findings suggest a main role of ectonucleotidases and, at least of LaNTPDase2 in *L. amazonensis* intra-species variations.

2.3.3 *Leishmania (V.) braziliensis* ENTPDases

Enzymatic characterization studies have shown ATP diphosphohydrolase activity in the promastigote form of *L. (V.) braziliensis*. ATP has proven to be the primary substrate, but other nucleotides have been hydrolyzed, such as ADP, UDP, GDP, UTP, GTP [59]. It has been demonstrated that Orthovanadate reduced ATPasic activity by 53%, which means that the observed activity could be a nonspecific hydrolysis from ENTPDases. In order to clarify the activity pattern of LbNTPDases, sodium azide, an ENTPDase inhibitor, depleted 58% of the ATPase activity. In addition, the combination of azide inhibition, calcium ion dependence and the hydrolysis of different di- and triphosphate substrates highlight the existence of ENTPDase in *L. (V.) braziliensis* promastigotes [59].

Since there is no specific information on the catalytic properties of the ENTPDases from *L. (V.) braziliensis*, many methodologies focused on the study of the native enzyme in the parasite and the use of antibodies against peptides derived from conserved regions in order to elucidate the cellular and antigenic roles [16,59,60]. It is crucial to notice that these researchers named the isoform of ~42 kDa as NTPDase1, but in the present work, we named it LbNTPDase2, as previously described [3].

It has been shown a conserved region named “B Domain,” which is shared between potato apyrase and the ENTPDases from pathogenic organisms [16,59,61,62]. Polyclonal antibodies against potato apyrase polypeptide (r-Domain B) identified a 48 and a 43 kDa bands in *L. (V.) braziliensis* promastigote preparations. Such antibodies were also able to partially inhibit ATPasic and ADPasic activities (22% and 72%, respectively) [16]. Considering the molecular masses these data should be related to the LbNTPDase2.

Moreover, mouse polyclonal antibodies raised against synthetic peptides from Domain B from LbNTPDase2 (LbB1LJ and LbB2LJ) also recognize bands of approximately 48 and

43 kDa. In that work, the antibodies against those peptides were able to reduce parasite NTPDase activity. Anti-LbB1LJ depleted approximately 30% of ATPase and 40% of ADPase activity, while anti-LbB2LJ depleted 53% and 44%, respectively. In addition, these antibodies reduced in vitro proliferation, pointing out the importance of ENTPDases for parasite growth, which is probably related to the purine salvage pathway [16]. The 43 and 48 KDa bands have been previously reported [59]; the smaller band was a result of natural partial proteolysis in the parasite. Nevertheless, once ENTPDases have putative N-glycosylation sites, we believe that the lower molecular mass could be the non-glycosylated intracellular form of the enzyme, and the higher molecular mass protein could be the fully glycosylated protein. It was possible to localize LbNTPDase2 on the membrane surface, flagellum, flagellar pocket, mitochondria and kinetoplast, using ultrastructural cytochemistry to access the subcellular localization of this enzyme[59]. A sequential study confirmed these results and identified the enzyme in the nucleus and cytoplasmic vesicles[16]. Considering the ubiquitous localization of LbNTPDase2, this protein may have other biological roles that should be further investigated.

Intra-species variation of ectonucleotidase activity and its effect on the outcome of Tegumentar Leishmaniasis were investigated on *L. (V.) braziliensis* strains related to cutaneous and mucocutaneous Leishmaniasis [15]. The authors compared the ectonucleotidase activity between 23 different isolates and observed that *L. (V.) braziliensis* isolates with different virulence presented polar activity of extracellular ATP, ADP and AMP hydrolysis. In addition, the increased hydrolysis of these extracellular nucleotides was associated with delayed lesion development and higher parasitic load. These findings suggest that ectonucleotidases could control the immune response by ATP decrease concomitant with increased Ado production, which allowed the multiplication of the parasite in the early stages of infection. Interestingly, they have shown that isolates of mucosal lesions present higher adenine nucleotide hydrolysis and delayed peak of lesion development, compared to isolates from cutaneous lesions. This datum suggests that the delayed lesion development of ML/MCL isolates could be due to higher ectonucleotidase activity [15]. In order to understand the infectivity and immune response, the authors have worked with two isolates (PPS6m and SSF). They observed that high ectonucleotidase activity parasites isolated from mucosal lesions (PPS6m) presented the highest delay in

lesion development and the most substantial parasite load, which corroborates the results previously shown for other isolates. This isolate was also able to modulate the immune response by reducing the dendritic cell activation and NO production, which indicates a possible association between enzymatic activity and clinical outcome [15].

Interestingly, when the authors evaluated the high activity of a cutaneous isolate (HPV6) and low activity of a mucosal isolate (JBC8m), they observed that HPV6 present similar behavior to PPS6m, despite being isolated from cutaneous lesions. It suggests that the ectonucleotidase activity could be more critical in macrophage resistance to activation than the type of clinical lesion. The authors also point out that mucosal lesions begin primarily in cutaneous form. Thus, they hypothesize that some high activity isolates from cutaneous lesions, such as HPV6, could progress to mucosal lesions, depending on the host environment. However, this hypothesis has yet to be deeply investigated [15].

In order to explain the different Ecto-ATPase and ADPase activities, it is reasonable to compare the sequences of ENTPDase2 of different isolates, in order to find single base polymorphisms (SNPs). The identification of some SNPs could, at least in part, justify the differences observed in the hydrolysis of ATP and ADP, and maybe explain the virulence profile[15]. However, no specific association of these polymorphisms with the direct enzymatic activity or with the clinical form was evaluated, and these proposals need investigations. Indeed, if the direct correlation between the mutations is proved in the future, this will probably be a good start point to explain the variation in the ENTPDase activity between the different species/strains of Leishmania and could be helpful to predict the prognosis of the disease.

2.3.4 *Leishmania (L.) major* ENTPDases

Similar to the other Leishmania species, *L. (L.) major* genome contains two genes encoding putative NTPDases. These putative enzymes are named here LmNTPDase 1 and LmNTPDase 2. Different papers described the presence of these genes and their predicted catalytic domains [3,4,10,63], but only one group deeply investigated them [11].

Sansom[11] generated not only overexpression of the C-terminal GFP-fused LmNTPDases but also the null mutants, using the classical homologous recombination approach, which made it possible to identify the subcellular location and function of each LmNTPDases. The LmNTPDase2 (~40KDa) has been located in the Golgi apparatus, and they evidenced that its function is related to the LPG elongation. Interestingly, the generation of a null mutant of LmNTPDase2 impaired the elongation of LPG and decreased the level of *in vitro* infection and a mouse model. The authors suggest that the enzyme acts indirectly on the Golgi glycosylation pathway, thus leading, for example, to the synthesis of smaller LPG. The authors also identified that the larger isoform (LmNTPDase1) is secreted, but its null mutant did not significantly impact the course of in vitro or mouse infection. They suggest that its function is unnecessary for infection or virulence in *L. major*.

Interestingly, that work described, for the first time, the roles of Leishmania ENTPDases in *L. (L.) major*, using a genetic approach [11]. Unfortunately, however, they used one species in which the sparse evidence about the roles of ENTPDases showed that *L. (L.) major* has very low ectonucleotidase (ENTPDase) activity, and no expression of ENTPDases on the surface of this parasite was detected [22]. In this context, it seems reasonable to consider that ENTPDases are unimportant molecules for *L. (L.) major* infection mechanisms. It means that the indirect effect of LmNTPDase2 and no effect of LmNTPDase1 on the infectivity and virulence on *L. (L.) major* could not be used as a model to say that these enzymes are not crucial to these events in other Leishmania species. Moreover, this point turns evident, considering the robust data previously described for other Leishmania species.

Furthermore, we could point out the need for better investigations of ENTPDases and the relationships with the variety of *L. (L.) major* strains/isolates and manifestations of the disease. Finding strains/isolates with higher levels of Ecto-ATPase, and Ecto-ADPase activity could be a good starting point to verify if the higher activities could be related to higher virulence, as found in other Leishmania species. Finally, using these cells could be better to do the null mutants to see the effects of those genetic deletions.

2.3.5 PURINERGIC RECEPTORS IN THE CONTEXT OF IMMUNE SYSTEM AND THEIR ROLES IN *LEISHMANIASIS*

NTPDases are components of a cellular communication system in which nucleotides and nucleosides, such as ATP and adenosine, act as signaling molecules, stimulating specific receptors (P2X, P2Y, and P1) and triggering a series of reactions that can alter cell physiology to control the damage/infection [64]. In this context, the extracellular nucleotides released during parasite infection can bind to specific receptors on the cell host surface, and these signaling could affect the establishment of infection (Figure 1).

P2 receptors are divided into two groups: P2X and P2Y. P2X (P2X1-7) is a family of NTP and NDP activated ion channels, while P2Y receptors are G protein-coupled receptors activated by ATP, ADP, UTP, UDP, ITP and sugar-bound nucleotides [65]. P2X7 has been the most studied receptor in the context of Leishmania infection. Its activation by ATP release leads to pro-inflammatory effects, such as the activation of host microbicidal mechanisms, including the production of reactive oxygen and nitrogen species, phagolysosomal fusion, acidification of parasitophorous vacuoles and release of cytokines and chemokines [66,67]. P2X7 has been involved not only in several protective effects but also in several deleterious effects in many pathogens species [64,66]. Savio [68] demonstrated an increased parasite load, lesion and deleterious effect using P2X7 KO mice, compared to the WT mice. It suggests that the modulation of this receptor is also significant to establish long term infection, once P2X7 could control both innate and adaptive responses against Leishmania [69]. Moreover, P2X7 KO and WT mice or treatment with an antagonist of P2X7 receptor in infection with *L. (L.) amazonensis*, *Toxoplasma gondii, T. cruzi,* and *P. falciparum* showed a vital role of this receptor in the control of the infection [68–72]. These data suggest the importance of this receptor for protozoan parasite infection, and studies concerning the roles of this receptor in other species of Leishmania, including those involved in the visceral form, would be interesting to clarify if P2X7 could be relevant to all types of Leishmaniasis.

The studies about other P2 receptors in the context of Leishmaniasis are rare [64]. Only one paper was found mentioning that P2Y2 and P2Y4 were upregulated in macrophages infected with *L. (L.) amazonensis,* which suggests a role of these receptors in Leishmaniasis [73]. In fact, Marques-da-Silva[73] showed a coupled mechanism in which

P2X7 and P2Y2-4 are activated to promote a boosting on immune response in *L. (L.) amazonensis* infection.

P1 receptors belong to another purinergic receptor family sensitive to the nucleoside Ado stimulation. There are four adenosine receptor subtypes, A1, A2A, A2B, and A3. These receptors vary in tissue distribution, affinity and function. The anti-inflammatory response is triggered by the activation of A2 receptors; On the other hand, A1 and A3 receptors are responsible for regulating the action of A2 receptors and preventing the excessive inhibition of immune cells [74].

A1 and A3 receptors present the highest affinity for adenosine, followed by A2A, while A2B has the lowest affinity. A1 receptors are distributed to virtually all tissues, and they are also well-characterized and present in the central nervous system (CNS). Therefore, A2B requires a higher concentration of adenosine to be activated; thereby, they are not activated at physiological adenosine concentrations. But in the case of pathophysiological conditions, the extracellular adenosine levels increase, leading to the activation of A2B. In fact, it has been demonstrated that during Leishmaniasis, circulating serum adenosine increases, probably leading to A2B activation [75,76]. In addition, it was observed that, in *L. (L.) amazonensis* infection, the adenosine production activates A2B receptors in dendritic cells (DC), inhibiting CD40, MHCII and CD86 expression, consequently inhibiting the ability of DCs to stimulate lymphocyte proliferation [15].

It was observed that A2B receptors in monocytes of visceral leishmaniasis (VL) patients have been increased, and, when treated with amphotericin B, these receptor expression levels decrease [77]. Moreover, they have demonstrated that the activation of A2B receptors increases IL-10 release by monocytes and decreases macrophage nitric oxide production in vitro. Interestingly, they observed that A2B levels are correlated with the disease severity, which demonstrates that VL patients have higher splenic parasite burden and higher expression of A2B receptors than those patients with lower infection rates [77].

Another role of the P1 receptor family is under macrophage chemotaxis. This cell movement process is critical for the recruitment of immune cells to the sites of inflammation. It was demonstrated the involvement of A2A, A2B, and A3 receptors under macrophage chemotaxis in a C5a gradient [78,79]. Moreover, A2A and A2B receptors are

also involved in the switch between macrophage subtypes. In this context, the activation of A2A adenosine dependent inhibits macrophage classical activation to subtype M1, which is necessary for parasite elimination. That activation of A2B can alternatively upregulate macrophage activation to M2, an anti-inflammatory phenotype that contributes to parasite survival within the macrophage [80].

Ado receptors are involved in Leishmania infection, and it is hypothesized that the production of adenosine from Leishmania ectonucleotidase activity modulates the host immune response in order to favor the infection. We believe that the development of selective inhibitors against the Leishmania enzymes can be potential new anti-leishmanial drugs, and, together with broader understanding of the effect of the parasite on purinergic signaling, it could lead to the use of drug modulators of hosts P2 and P1 to help in the treatment.

2.4 Trypanosoma *cruzi* ENTPDase1

Different from Leishmania, only one ENTPDase gene was found in *T. cruzi* genome named TcNTPDase1 [4]; our group has exhaustively searched another isoform in the gene data banks, without any success (data not shown). It means that *T. cruzi,* no matter the strain or clone, has just one ENTPDase. The TcNTPDase1 was cloned, expressed on the bacteria system, and biochemically characterized as an Apyrase/CD39/ENTPDase family member. Moreover, *T. cruzi* has already demonstrated to have TcNTPDase1 localization and activity on its surface. [26,27]. Its catalytic activity is Mg^{2+}-dependent, and it can hydrolyze ATP, ADP, UTP, UDP, GTP and GDP. The catalytic activity of the recombinant protein or at the surface of lived parasites was not inhibited by common ATPase inhibitors [4,25,26]. It has been demonstrated that *T. cruzi* treated with Ecto-NTPDase partial inhibitors (ARL67156, Gadolinium, and Suramine) significantly reduced *in vitro* infectivity and *in vivo* virulence. This datum has revealed a relationship between the activity of TcNTPDase1 and *T. cruzi* infectivity and virulence [26]. Suramin has been shown as a potent inhibitor of TcNTPDase1. However, when suramin was used in the experimental treatment of Chagas disease in a mouse model, the treatment led to enhanced parasitism, inflammation,

reactive tissue damage, aggravated myocarditis and increased mortality [81,82]. This unexpected result could be explained because Santos [26] used Suramin only in the parasites, previously to infection, but not in the host, contrarily to Novaes [82]. Furthermore, it is well known that Suramin is a P2 antagonist in mammalian cells [81]. Taking this into account; it is possible to hypothesize that the absence of specificity of Suramin (action on P2 receptors) could explain the discrepancy between these studies and highlight the importance of using specific inhibitors to inhibit only the pathogen ENTPDases. However, unfortunately, there is no such specific inhibitor. Thus, the development of specific inhibitors against the pathogen ENTPDases is needed.

Considering the actions of TcNTPDase1 as an Ecto-localized enzyme, as expected, TcNTPDase1 was shown to be located on the external surface of the parasite. However, it was also found in the flagellum, flagellum insertion region, the kinetoplast, nucleus, and intracellular vesicles, which may indicate different biological functions in these intracellular locations [27]. In addition, the authors evidenced that the TcNTPdase1 is vital to the parasite adhesion at the host cell [27] and also seems to be essential for the adaptation of *T. cruzi* to stress conditions, such as heat shock [70].

Several studies have shown that *T. cruzi* presents heterogeneity between strains or even clones. Interestingly, heterogeneity was also observed in Ecto-NTPDase activity. Santos [26] compared three strains and one clone (Y, Be-62, and CL strains/isolates and the clone CL-Brener). They showed the highest Ecto-ATPDase activity in the highest virulent strain Y. Moreover, Silva-Gomes [70], using isolates (Y, 3663 and 4167 strains, and Dm28c, LL014, and CL-14 clones), have shown higher mRNA expression of TcNTPDase1 in the higher virulent strains than in the CL-14 avirulent clone. These pieces of evidence reinforce the idea that the expression/activity of TcNTPDase1 is higher in more virulent forms of the parasite. Thus, similar to other trypanosomatids, it has been suggested a relationship between infectivity and ectonucleotidase activity of TcNTPDase1, but this hypothesis was tested only using indirect approaches. Thus, null mutant generation must be carried out in the future to prove this statement.

Finally, considering the knowledge about the Leishmania ENTPDases and their effect on the host immune system, we believe that it is crucial to take into account the role of

TcNTPDase in the modulation of the host immune response, but further studies are necessary.

2.4.1 *Trypanosoma cruzi*: PURINERGIC SIGNALING PATHWAY, HOST RECEPTORS AND THEIR ROLE IN CHAGAS DISEASE

It is a consensus hypothesis that ATP and ADP hydrolysis by TcNTPDase1 can change the extracellular balance of extracellular nucleotides modulating the purinergic signaling in the host and interfering in the immune response against the parasite. In this scenario, the involvement of P2 purinergic receptors has been reported by Coutinho [83], as illustrated by figure 1. It is well known that there is dramatic atrophy of the thymus during the acute phase of Chagas disease, a phenomenon investigated by Mantuano-Barradas [13] in an experimental model of Chagas disease (C57Bl/6 mice). They have demonstrated increased plasma membrane permeabilization. In addition, the death of thymocytes CD^{4+}/CD^{8+} was promoted by extracellular ATP during the acute phase. The results suggest that the P2X7 receptor could be a key factor in promoting cellular permeabilization.

Additionally, using knockout $P2X^{-/-7}$ mice, they showed that the P2X7 receptor is not the central receptor involved in this phenomenon. Then, they used wild type mice and evidenced the possible involvement of P2X4 and P2Y in association with P2X7. The preincubation of thymocytes from infected mice with BBG, which is a blocker of P2X7, or UTP, showed no P2X4/P2X7 activity or permeabilization dependent of P2Y/P2X7, after incubation with extracellular ATP. This result reinforces the idea of cross-talk between receptors through the formation of heteromeric structures, interfering in its final function. The involvement of P2X7 is still unclear, and new studies are necessary to elucidate the participation of purinergic signaling on thymus atrophy in acute *T. cruzi* infection [84].

The chronic indeterminate form of Chagas Disease is known to have modulation between pro- and anti-inflammatory responses that establish the host-parasite adaptation[35]. The indeterminate form is associated with an anti-inflammatory cytokine profile, represented by a high expression of IL-10. In contrast, the cardiac form is associated with high production of IFN-gamma and TNF-alpha, compared to IL-10, which leads to an

inflammatory profile [85]. In a recent study with patients with an indeterminate form of Chagas disease to investigate the non-progression of the disease, Souza [86] evaluated the involvement of P2X7 receptors in lymphocytes and the cytokine profile of the patients. They evidenced no changes in P2X7 expression or the profile of IL-2, IL-10, IL-17, and IFN-γ. On the other hand, they showed significantly increased IL-4 and IL-6 and decreased TNF-α, compatible with an anti-inflammatory profile. Considering that P2X7 was not altered in the patients, this receptor may not be involved in the indeterminate form of the disease. However, since the author did not investigate the activity of P2X7, its role cannot be excluded based only on the expression levels. Moreover, considering that other P2 and the P1 receptors can modulate the production of cytokines, it is crucial to conduct an in-depth investigation in this area.

During the development of Chagas disease, disorders involving the modulation of platelet aggregation are considered essential. Besides, it is well known that the purinergic signaling is involved in the tromboregulation [34,87]. Souza [6] evaluated the activity of purinergic system Ecto-enzymes concerning the thromboregulation of patients with the indeterminate form of the disease. They found increased levels of Ecto-Nucleotide pirophosphase (E-NPP) and of Ecto-5´-NT and decreased Ecto-Adenosine deaminase (E-ADA) activity in platelets of the patients. Furthermore, they showed significantly decreased platelet aggregation in the patients, which suggests that the purinergic system is involved in the thromboregulation process.

More recently, it has been evidenced that purinergic Ecto-enzymes participate in thromboregulation in experimental Acute Chagas Disease [86]. This work demonstrated that the activities of E-NTPDase, E-5′NT and E-ADA in platelets significantly increased in infected mice. Additionally, they showed a negative correlation between the number of platelets and the E-NTPDase activity hydrolysis.

As observed, studies about the involvement of ENPDases and purinergic receptors in the clinical presentations of Chagas Disease are scarce. However, those that have already been carried out highlighted the importance of this research area. Genuine investigations could be relevant for future applications, such as the use of modulators of purinergic signaling and the respective enzymes to treat Chagas disease.

2.5 BIOTECHNOLOGICAL APPLICATIONS OF *Leishmania AND Trypanosoma cruzi* ENTPDases

Recombinant proteins from parasites can be used in numerous applications, including studies on structure-function correlations, the screening of drug candidates, engineering of immunogens for vaccination and development of diagnostic tools [88]. Due to the importance of the NTPDases from *Leishmania* genus and *T. cruzi* described above, it is possible to consider that these proteins can be exploited in the same ways. As an example, Figure 2 shows multiple biotechnological applications that could be explored with recombinant ENTPDases from *L. infantum*. One of those includes the immunodiagnosis for Canine Visceral Leishmaniasis, which has already been developed [89,90].

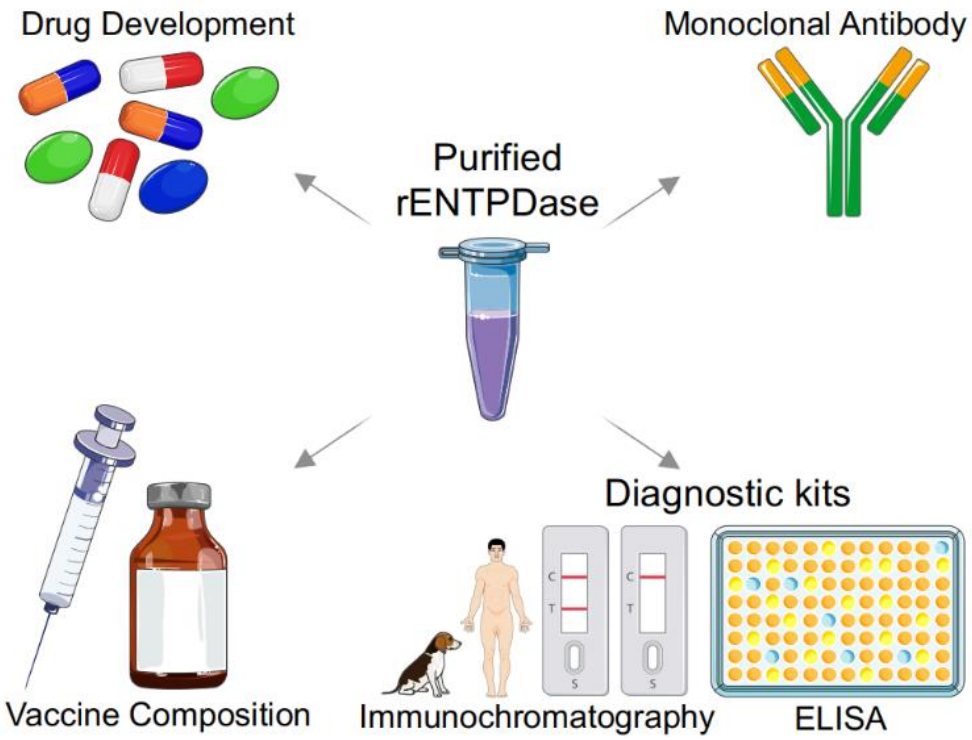

Figure 2 - Potential biotechnological applications of recombinant Leishmania genus ENTPDases. The arrows indicate possible applications for this protein: Upper left) Rational design of specific inhibitors. Lower left) Production of antigens for vaccine development. Upper right) Antibody production for diagnostic kits. Lower right) Antigens in diagnostic kits (e.g., Immunochromatographic and ELISA techniques).

Regarding the application to the development of new drugs, it is possible to exploit the direct interaction between ENTPDases and their substrates to design specific inhibitors. Another possibility is to explore the interaction between ENTPDases from parasites and the surface of the host cells, exemplified by the LiNTPDase2 and TcNTPDase1, which were evidenced as involved in the macrophage adhesion. It suggests the existence of an unknown receptor in the host cell which could recognize these proteins and facilitate adhesion and infection [3,27]. Thus, when the receptors are identified and characterized, this information could be used to design specific inhibitory drugs for treatments.

The development of monoclonal antibodies (mAbs) against the parasites ENTPDases was highlighted for *Toxoplasma gondii* NTPDases (TgNTPases). In fact, Feng Tan[91] developed two monoclonal antibodies (MNT1 and MNT2) against the recombinant TgNTPase2. The mAbs inhibited the ATPase activity of the enzymes. In addition, the treatment of cultured cells with these antibodies showed that cell invasion by the parasite is not halted, but *T. gondii* tachyzoite replication is reduced. Therefore, the authors suggested that reduced tachyzoite replication could be related to the inhibition of NTPase2.

Until now, there are no mAbs developed against the Leishmania genus or *T. cruzi* ENTPDases, but there are studies with polyclonal antibodies against the recombinant ENTPDases and one specific epitope. To *T. cruzi* [26]produced polyclonal antibodies against the recombinant TcNTPDase1 and achieved 50% inhibition of *in vitro* infection. Mariotini-Moura (2014) [27] complemented this work and demonstrated that the same polyclonal antibodies were capable of inhibiting the adhesion of the parasite to the host cell. Similarly, Vasconcellos [3] showed that antisera with polyclonal antibodies against the recombinant LiNTPDase2 inhibit at least 50% of adhesion and infection. Another approach was used by Maia[24]; they identified a catalytic and antigenic domain in LbNTPDase2, “the B domain” [23]. Using this epitope, they produced polyclonal antisera that inhibited the LbNTPDases.

The potential use of TpNTPDase inhibitors to block the infection and take part in the treatment was pointed out by Santos[27]. In this work, they showed that partial inhibitors of TcNTPDase1 decrease the in vitro infection and in vivo virulence of *T. cruzi*. They highlight Suramin as a partial inhibitor (70%) that may be tested in the experimental Chagas disease treatment. Novaes[70] evaluated the effects of suramin-therapy.

However, they showed that this drug increased the parasite load on the cardiac tissue, leading to the aggravation of cardiac inflammation and upregulation of oxidative stress. These undesired effects of Suramin are probably related to the unspecific inhibition of P2 receptors and other targets. This study highlights the need for the development of specific TpNTPDase-inhibitors.

Furthermore, ectonucleotidases have also demonstrated potential to be used in vaccine development. Indeed, Tlili[93] has shown that apyrase salivary proteins of *Phlebotomus papatasi*, a vector for *L. donovani,* are candidates to be used as a vaccine, or its component , against human cutaneous leishmaniasis. Moreover, Vijaymahantesh[76] evaluated the application of 3-ectonucleotidase on a polytope vaccine based on immunoinformatic tools.

The use of chimeric protein is another alternative for the development of vaccines. In this approach, it would be useful to build chimeric proteins containing not only TpNTPDase-1 epitopes but also TpNTPDase2 epitopes, for vaccine development. As an example, a chimeric protein has been based on *Schistosoma mansoni* glutathione S-transferase, which has been used to produce vaccine antigens that are immunogenic to more than one parasite [92]. The chimera can include epitopes for Leishmania, *T. cruzi* and other pathogens of interest.

The application of nucleotidases in diagnostics has also been explored. The recombinant rLiNTPDase2 from *L. (L.) infantum* was evaluated as antigen in the diagnosis of Canine Visceral Leishmaniasis (CVL), providing high sensitivity (100%) and specificity (99%) in the enzyme-linked immunosorbent assay (ELISA). In addition, the diagnosis showed low cross-reactivity with dogs infected by *T. cruzi* [89,90]. In a more recent study using immunohistochemistry, Vasconcellos[3] identified the presence of this protein in 100% (n=48) of tissue samples from dogs previously diagnosed as positive to CVL. These results indicate that NTPDases are promising antigens for new leishmaniasis diagnostic tests.

2.6 FINAL CONSIDERATIONS

In this paper, we review the knowledge about *Leishmania* genus and *T. cruzi* ENTPDases and the purinergic signaling. We concluded that there is enough evidence

suggesting that the parasites ENTPDases are important to the physiology of the parasites and involved in the infection by playing an essential role in the modulation of purinergic signaling, in adhesion and infection. Moreover, we verified that the importance of each enzyme is species, isolate, and clone dependent, which requires caution regarding generalizations. Concerning the purinergic signaling, it is possible to highlight that purinergic signaling affects the development and outcomes of Leishmaniasis and Chagas Disease. However, more details should be further investigated, such as the role of P2 receptors on infection and virulence, as well as the effects of SNPs in enzyme activity.

Additionally, there is enough evidence suggesting that the parasites ENTPDases can be exploited for biotechnological applications, such as diagnosis, vaccines, and drug design once they present low identity with the mammalian ENTPDases on aminoacids sequence. However, there is still a lack of structural data regarding ENTPDases from both *Leishmania* genus and *T. cruzi*, which can yield pivotal information for vaccine development efforts by shedding light upon possible new accessible epitopes. Such structural information would also enable further efforts on rational drug design by providing information regarding protein folding, active site structure and molecular recognition patterns of ligand-receptor complexes. Although some investigation venues are still open, the available information regarding the relationship between ENTPDases and purinergic signaling can already be exploited for the development of high-end products, such as diagnostic kits, vaccines and new drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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3 CHAPTER 3: *Leishmania infantum* LiNTPDase1 and LiNTPDase2 play central role on infection and macrophage early response

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Abstract

Leishmania infantum the causative agent of American visceral leishmaniasis is known for its ability to modulate the host immune response to its own favor. They have two putative NTPDases one with 70KDa and another with 45KDa which hydrolyze a broad range of nucleoside tri- and diphosphates and they have been poorly related to adhesion and to parasite virulence. Here, we use a genetic tool like Homologous recombination to get overexpressing clones and CRISPR/Cas9 to obtain null mutants to better understanding the role of these enzymes on macrophage infection. N-terminal eGFP tagged NTPDases2 showed nuclear and kinetoplast localization. Both NTPDases overexpressing clones showed no morphological or growth rate change with extra copies of the genes. On the other hand, JPCM5 L. infantum expressing Cas9 was obtained in order to get null mutants. NTPDase1 and NTPdase2 null mutants showed lag in growth profile which was reverted adding extra adenine and guanine to the culture media. In addition, their morphology even with supplemented media has changed to an amastigote-like form. Double knockout of the genes showed to be lethal do JPCM5 L. infantum and a mechanism of compensation of deletion of one isoform was detected. NTPDase 2 overexpressing showed to be important on macrophage adhesion while NTPDase2 overexpressing and null mutant showed to be important to infection. Interestingly NTPDase1 null mutant could also decrease infectivity in macrophages. Moreover, NO production was decreased during null mutant infection pointing out both NTPDase as important to inhibit macrophage response against the parasite highlight them as a potential drug target to control the disease.

Keywords: *Leishmania infantum*; NTPDases; CRISPR/Cas9; Homologous Recombination; macrophage infection

3.1 INTRODUCTION

Nucleosides Triphosphate Diphosphohydrolases, (NTPDases) are divalent cátion dependent enzymes that hydrolyze tri and diphosphated nucleosides to their monophosphate form (BISAGGIO et al., 2003; HANDA; GUIDOTTI, 1996; MEYER-FERNANDES et al., 1997, 2004; PLESNER, 1995). These monophosphate nucleotides could be used by the 5' ecto-nucleotidase (5´-NT) for nucleoside production (KNOWLES, 2011; MEYER-FERNANDES et al., 1997; ROBSON; SÉVIGNY; ZIMMERMANN, 2006).

NTPDases belong to CD39/apyrase family (HANDA; GUIDOTTI, 1996) and have five ubiquitous regions between species named *Apyrase Conserved Regions* (ACRs) which compose the NTPDases catalytic core (KIRLEY; CRAWFORD; SMITH, 2006; ZEBISCH; STRÄTER, 2008). Variations in amino acid residues between species is a possible factor that determines the difference in specificity by the substrate between the various enzymes that belong to the family (HANDA; GUIDOTTI, 1996; KEGE et al., 1997; PLESNER, 1995; ZIMMERMANN; BRAUN, 1999).

Enzymes homologous to mammalian NTPDases (CD39) have been described in pathogenic organisms as well as their nucleotidase activity (BERRÊDO-PINHO et al., 2001; BISAGGIO et al., 2003; MEYER-FERNANDES et al., 1997; SILVERMAN et al., 1998). Moreover, they have been described to play roles in virulence mechanisms of many pathogens such as *Trypanosoma cruzi* (FIETTO et al., 2004; MARIOTINI-MOURA et al., 2014), many Leishmania species (DE ALMEIDA MARQUES-DA-SILVA et al., 2008; DE SOUZA et al., 2013; GOMES et al., 2015a; LEITE et al., 2012; MAIOLI et al., 2004a; SANSOM et al., 2014; VASCONCELLOS et al., 2014), *L. pneumophila* (SANSOM; ROBSON; HARTLAND, 2008) and Toxoplasma gondii (SILVERMAN et al., 1998).

Leishmania infantum is the causative agent of visceral leishmaniasis which is considered a tropical neglected disease and the most severe clinic manifestation of Leishmaniasis (WHO, 2019). *L. infantum* has two putative NTPDases isoforms (LiNTPDase1 and LiNTPDase2) that have been partially characterized (BASTOS et al., 2017; DE SOUZA et al., 2013; VASCONCELLOS et al., 2014). NTPDase 1 has approximately 70kDa, is encoded by LINF 10.0006600 gene (TritrypDB) and it is annotated as Guanosine diphosphatase. This isoform is similar to NTPDase 1 of *T. cruzi* (FIETTO et al., 2004). The LiNTPDase 2 has approximately 45kDa, is encoded

by LINF 150005200 gene (TritrypDB) and it is annotated as ATP-diphosphohydrolase or Nucleoside diphosphatase.

In Leishmania, these enzymes have been described on the cell surface, organelles, intracytoplasmic membranes, or even been secreted (SANSOM et al., 2014; VASCONCELLOS et al., 2014). Moreover, many NTPDases present the active site facing the extracellular space or the lumen of an organelle, where they are called ecto-enzymes or ENTPDases (DA SILVA et al., 2020; KEGE et al., 1997; PINHEIRO et al., 2006; SANSOM; ROBSON; HARTLAND, 2008). It has been speculated that Leishmania species have the ability to subvert the host immune response in their favor by modulating extracellular levels of nucleotides and adenosine through the modulation of purinergic signaling (BERRÊDO-PINHO et al., 2001; DE ALMEIDA MARQUES-DA-SILVA et al., 2008; LEITE et al., 2012; ROBERTO MEYER-FERNANDES, 2002), for review see (DA SILVA et al., 2020). This hypothesis is based on the ENTPDases activity of these parasites that hydrolyze pro-inflammatory molecules like ATP and ADP to AMP, which in turn can be hydrolyzed to anti-inflammatory adenosine (ADO) by the action of 5'-NTs. The reduction of these pro-inflammatory purinergic signals would prevent the establishment of the inflammatory response against the parasite, while favor the anti-inflammatory action of ADO and the establishment of *Leishmania* infection. (LIMA et al., 2017; MAIOLI et al., 2004b; VIJAYAMAHANTESH et al., 2016). In addition to the role in the modulation of purinergic signaling, the Leishmania NTPDases seems to be important to the purine salvage pathway because of the absence of *de novo* nucleotide synthesis in those parasites (ULLMAN et al., 2008; COHN; GOTTLIEB, 1997; HAMMOND; GUTTERIDGE, 1984; MARR et al., 1978). These evidences point out Leishmania NTPDases as good targets for the development of new strategies to control the parasite infection.

Although there are evidences that suggest the main roles of LiNTPDases in virulence, infection, adhesion, control of host immune system, nutrition and development of the parasite, all the data were obtained using indirect techniques such as the use of anti-NTPDases antibodies, competition with recombinant NTPDases and/or partial chemical inhibition (DA SILVA et al., 2020; PAES-VIEIRA; GOMES-VIEIRA; MEYER-FERNANDES, 2018; SANSOM et al., 2014). Interestingly, NTPDases null mutants of *L. Major* was generated to direct point a new LmNTPDase2 role on lipophosphoglycan elongation and its involvement on lesion development (SANSOM et al., 2014) but

studies with genetic approaches to understand the biological roles of these enzymes in *L. infantum* have not yet been done. In this article, for the first time, we established null mutants of *L. infantum* NTPDases1 and 2 by CRISPR/Cas9 and overexpressing ones using the LESXY system as genetic approaches for an accurate understanding of the biological roles of these enzymes in *L. infantum*.

3.2 MATERIAL AND METHODS

3.2.1 Bioinformatic analysis

NTPDases sequences were identified on NCBI data base (Sequences ID available on supplementary table ST1. Available trypanosomatids and human genomes were compared to ACRs identification. Protein sequence alignments were done using Multiple Sequence Alignment by CLUSTALW. Phylogenetic tree was also generated on CLUSTALW using default mode and the clustering method Neighbour-joining (SAITOU; NEI, 1987) and edited with BioEdit tool.

3.2.2 Cloning to Overexpress LiNTPDases 1 and 2

Leishmania infantum JPCM5 NTPDases1 (LINF_100006600) and NTPDase2 (LINF_150005200) gene sequences were obtained from the TritrypDB (http://tritrypdb.org). Oligonucleotides for PCR were designed in Primer Quest IDT tool (IDT Technologies) and are described in table 1. Before being performed, the cloning of each gene was simulated *in silico* using Geneious 6.0.6 (Biomatters Ltda). In all cloning, genomic DNA from *L. infantum* JPCM5 MCAN/ES/98/LLM-724 (GONZÁLEZ-DE LA FUENTE et al., 2017) was used as a template.

For each gene, two different cloning approaches were performed for overexpression: (i) complete genes sequences were fused to a 6xHis-tag in the C-terminal end of the genes. Full length coding sequences of LiNTPDases were amplified with sense and antisense oligonucleotides containing sites for *Bgl*II in N-terminal end, and *Msp*CI in C-terminal end and cloned into the pGEM T-easy cloning vector (Promega) according to the manufacturer's instructions. Digestion of pGEM-T containing the LiNTPDase 1 or 2 allowed the insertion of the inserts into expression vector pLEXSY_neo2

previously digested with the same enzymes. PCR, digestion and sequencing confirmation were done.

Another approach was (ii) fuse eGFP sequence on C-terminal end of LiNTPDases gene, but only NTPDase2-eGFP was successfully done. In this strategy, initially the NTPDase sequences were amplified with oligonucleotides sense and antisense containing the sites for *Bgl*II in the C-terminal end, and *Xba*I in the C-terminal end. The eGFP gene from peGFP vector was amplified with oligonucleotides sense and antisense containing *Xba*I in the N-terminal end and *MspC*I in the N-terminal end. These amplicons were cloned into the vector pGEM T-easy (Promega) according to the manufacturer's recommendations. LiNTPDase2 cassette and the vector pEXSY_neo2 empty were initially digested with *Bgl*II and *Xba*I to link the gene of the each LiNTPDase separately. Subsequent digestion was performed with *Xba*I and *MspC*I to bind the eGFP gene to the C-terminal end of the NTPDase 2 gene. PCR, digestion and sequencing confirmed the cloning. *L. infantum* JPCM5 ($2x10^7$ cells) were electroporated (Bio-Rad system) according to the pLEXSY neo_2 manufacturer's recommendations with 5 µg of linearized plasmids with *Swa*I. After 24h, 10 µg/mL of neomycin was added and the cultures were maintained for 5 days for propagation and clonal selection by limiting dilution.

3.2.3 Knockout of LiNTPDase1 and LiNTPDase2 using CRISPR/Cas9

In order to get LiNTPDase1 e LiNTPDase2 *L. infantum* knockout (KO), we first engineered *L. infantum* JPCM5 cell line to express *Streptococcus pyogenes* Cas9 nuclease gene by electroporation (Bio-Rad system) with the vector pSpαhygroαSpCas9HA kindly given by Prof. Barbara Papadopoulou. Hygro represents hygromycin resistance gene and the symbol α represents the intergenic region of the *Leishmania enriettii* α-tubulin gene necessary for trans-splicing and polyadenylation. SpCas9 presents nuclear localization signal and tag from hemagglutinin. To address the DNA double strand break, gRNA for both genes were designed using Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool available on ‘http://grna.ctegd.uga.edu/". We use the entire ORF sequence of LiNTPDase1 and 2 as input to design gRNA to spCas9 with 20nt length and NGG PAM site on 3’end.

We search for gRNA with no T on NGG PAM sequence nor “T” on last 8 nucleotides to avoid undesirable secondary structure and off target effects. We use a target sequence as closer as possible to middle gene sequence, once we did just one DNA break. Alt-R System containing crRNA and tracrRNA was purched from IDT Technologies and the assemble was done according to manufacturer’s recomendation. Donor DNA to replacement of each gene allele by micro homologous recombination was built using sp72αneo and sp72αpuro as template to PCR amplification of the resistance genes to neomycin and puromycin. We design forward oligonucleotides containing 30 nucleotides of the 5’UTR flank of target genes and the 10 first nucleotides of resistance genes and reverse oligonucleotides containing the 10 last nucleotides of the resistance genes and 30 nucleotides of the 3’UTR flank of target genes (BENEKE et al., 2017). Cassettes were 1% agarose gel verified and purified using GenElute gel purification system (Sigma). Three strategies were chosen to get null mutants of *L. infantum:* Double knockout LiNTPdase1 and 2; LiNTPDase1 KO; and LiNTPDase2 KO. Cold mix of 10^7 cells of *L. infantum* expressing SpCas9, 3-5µg of Donor DNA of each drug resistance and 10µL of gRNA and TracrRNA (1:1) was transfected in Alt-R transfection buffer (Alt-R System, IDT Technologies) following the manufacture’s recommendation using one pulse with program X-001 in the Amaxa Nucleofector IIb (Lonza). Cells were transferred to 10mL of culture media without drugs to recovery for 24h. After that, 800µg/mL, 60µg/mL and 120µg/mL of Paramomycin, Puromycin and Hygromycin respectively were added to cells to pre-selection step. After 24 hours a 10 times dilution was done where 1mL of the cell culture was added in 9 mL of culture media supplemented with appropriated drug concentration. Here, no more hygromycin was necessary once spCas9 expression is no longer necessary. These Cell cultures were maintained for more 7 days to cell selection and limiting dilution was done to clonal selection. Clones were stored at -80°C.

3.2.4 Cloning to add back LiNTPDases 1 and LiNTPdse2 to null mutants

In order to add back the expression of NTPDase 1 and 2 of the null mutants obtained, two other strategies were outlined. In the first, the *Xba*I restriction site was added to the N-terminal end and the *Hindl*II site was added to the C-terminal end of the NTPDases genes by PCR. This sequence was ligated and digested from the cloning vector pGEMT-easy and then ligated into the vector αZeoα previously digested with these same enzymes. The vector was kindly given by Prof. Barbara Papadopoulou. Confirmation was performed by PCR and sequencing. In another approach where each LiNTPDases were fused to Hemagglutinin gene (HA), the sequences of LiNTPDases were amplified using primers with the *Xba*I restriction site in the N-terminus. In the C-terminal end the HA sequence was added to the oligonucleotide followed by the *Hind*III restriction site. The amplified sequences were separately ligated and digested from the cloning vector pGEM T-easy and again ligated into the expression vector αZeoα previously digested with these enzymes. Confirmation was performed by digestion, PCR and sequencing. Oligonucleotides used are described on supplementary table 1.

3.2.5 *L. infantum* e Macrophages culture conditions

JPCM5 *L. infatum* metacyclic promastigotes were grown at 26ºC, 5% CO_2 in 175cm^3 bottles containing DMEM medium (Gibco), 10% M-199 (Gibco), 25mM HEPES (Sigma-Aldrich) pH 7.2 and supplemented with 20% FBS, 200mM L-glutamine, Penicillin-Streptomycin 1µg / mL, 100/400 mM Adenine, 100/400 mM Guanine and 200 mM Hemin, 1% Biopterin, 1x MEM essential amino acids solution (Sigma-Aldrich), 1x MEM non-essential amino acids solution (Sigma-Aldrich). Parasite growth rate was assessed by cultivation with starting amount of $1x10^6$ parasites in 10mL of media. OD_{600} was measured every day during 8 days. RAW 264.7 macrophages were grown at 37ºC, 5% CO_2 atmosphere and in RPMI 1640 (Sigma) pH 7.2 medium, supplemented with 10% fetal bovine serum (FBS) (Sigma), 200mM L-glutamine, Penicillin-Streptomycin 1µg / mL.

3.2.6 DNA and RNA Isolation and cDNA synthesis

Genomic DNA was extracted from *L. infantum* (1 x 10^8 cells) using Phenol-Chlorophorm–Isoamylic Alcohol (Sigma Aldrich) following manufacturer's instructions. Total RNA from *L. infantum* (1 x 10^8 cells) was extracted using TRIzol Reagent (Invitrogen) and treated with DNAse I (Sigma Aldrich), following manufacturer's instructions. DNA and RNA quantity and purity were estimated by spectrophotometry at 260/280/230 nm. RNA integrity was verified through electrophoresis on an 1.0% (w/v) agarose gel (SAMBROOK; RUSSEL; 2000).

3.2.7 cDNA synthesis and Real Time qPCR

Reverse transcriptase reactions were performed from 400 ng of DNAse I treated total RNA using a Superscript III First-strand System (Invitrogen), according to the manufacturer's instructions. Real-time quantitative PCR assays were performed in ABI Prism 7300 sequence detection system using Power SYBR Green PCR Mastermix (Applied Biosystems) following manufacturer's instructions to cycling conditions. 300 nmol/L of primers were used and they are listed on supplementary table ST2. To monitor the primers specificity, melting curves were performed after each experiment, resulting in a single peak. Reactions were performed in triplicates using 1 µl of cDNA template, in a total volume of 10 µL reaction. The relative quantitative measurement of target gene levels were performed using the Relative Standard Curve (LIVAK; SCHMITTGEN, 2001; SCHMITTGEN; LIVAK, 2008). As endogenous control genes, G6PD and GAPDH genes were used. PCR assays were done in triplicate and data were pooled to plot the graph.

3.2.8 Southern Blotting

Genomic DNA from JPCM5 *L. infantum* were used to hybridizations following standard procedures (RIO, 2015). Double-stranded DNA was prompt digested with restriction enzymes. To NTPDase1 UTRs fragmentation was used *Xho*I; and to NTPDase2 were used *Cla*I restriction enzymes. Digested DNA was separated on agarose gel,

transferred to nitrocellulose membrane and incubated with the specific probes. LiNTPDase1_5'UTR and LiNTPDase2_3'UTR probes were radiolabeled with [α-^{32}P] dCTP using random oligonucleotides and Klenow fragment DNA polymerase I (New England Biolabs). Membranes were incubated with radiolabeled probes overnight and reveled according to (RIO, 2015).

3.2.9 Western Blotting

Total proteins were extracted from 1x 10^8 cells using PKB Lysis Buffer (200mM TRIS HCl pH 7.2, 200mM KCl, 100mM MgCl2, 1% Triton X100, 20% Glycerol, 200mM Protease Inhibitor Cocktail, 200µM PMSF, 200mM Sodium Orthovanadate, 200mM Okadaic Acid, 200mM DTT) and disrupted on cell homogenizer (5 pulses with 30 seconds intervals) on ice-cold bath (Polytron). Proteins were quantified by Bradford method (Bradford, 1964) and the 40µg was load on 10% SDS-PAGE. Proteins were semi-dry transferred to the Nitrocellulose membrane using a Transblotting device (Bio-Rad). The total of 2% of non-fat dry milk in PBS was used to block the membrane for 1h at room temperature followed by antibodies incubation. Anti-mouse alpha-tubulin antibody (1:3000 dilution; Sigma) and anti-mouse HA tag monoclonal antibody (1:3000; ABM) were used as primary antibodies and incubated for 1h at room temperature. HRP conjugated Goat anti-mouse IgG (1:10000, Cell Signaling) was used as secondary antibody and incubated during 1h at room temperature. IBright FL1500 Imaging System (Thermo Fisher Scientific) was used to detect the chemiluminescent signal provided by Clarity Max Western ECL Substrate Kit (Bio-Rad) according to the manufacture's recommendations.

3.2.10 Adhesion and Infection Assay

Macrophages J774 were grown in RPMI medium at 37°C, 5% CO_2. The cells were seeded in 24-well plates with 1x10^6 cells/mL per well, and allowed to adhere for 90–120 min. The medium was removed, and the wells were washed twice with sterile PBS to remove the non-adherent cells. The parasites were suspended in RPMI containing 10% FBS and added to each well. We used 30 parasites per macrophage to interact

for 2 h (adhesion) or for 48 h (infection). After adhesion or infection time the wells were washed twice with sterile PBS to remove free parasites. Slides were stained with Panoptic stain method (LaborClin) according to manufacturer's recommendations.

3.2.11 Image acquisition

eGFP fused LiNTPdase2 localization in promastigotes during exponential phase growth was performed by fluorescence microscopy (Evos Digital XL, Invitrogen). The live parasites ($1x10^8$ cells) were washed twice in 5 ml of PBS and a 30µl drop mixed with 4% paraformaldehyde (1:1) was spread on glass slides coated with 1% poly-lysine and dried at room temperature. Slides were mounted using DAPI mount slide (Invitrogen) and images were acquired on white, blue and green channel.

3.2.12 Activity Assay

L. infantum JPCM5 were grown in media previously described. $1x10^7$ parasites from the log phase were washed and suspended in reaction buffer. NTPDase activities were measured according to Fietto (2004). Briefly, the hydrolysis of 2,5µM of ATP, UDP and AMP was measured by incubation of live parasites for 30 min at 37°C in reaction buffer (50 mM Hepes-Tris, 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, 5.0 mM $MgCl_2$) in presence of 5 mM nucleotide. The reaction was stopped by adding 0.1 M ice-cold HCl and the suspensions were centrifuged. Inorganic phosphate (Pi) was measured in aliquots of the supernatant using the malachite green method (EKMAN; JAGER, 1993).

3.2.13 Measurement of macrophage Nitric Oxide production

After infection assay with overexpressing and null mutants of LiNTPDase1 and 2 the culture supernatants were collected by centrifugation at 500 g, 25° C for 2 min and the measurement of NO production was carried out using the Griess method (GREEN et al., 1982). LPS from Escherichia coli O111:B4 (Sigma Aldrich, MO, USA) (100 ng / mL) diluted in non-supplemented RMPI medium was used as positive control. The

absorbance at 570 nm was measured in a microplate reader. Results were expressed as μM of nitrite based on a standard curve established by known concentrations of sodium nitrite dissolved in non-supplemented RMPI medium. Measurements were made in biological duplicates for each cell line.

3.2.14 Statistical Analysis

All the assays were performed in biological triplicates and experimental duplicates. Data are shown as arithmetic mean +/- standard deviation. We used the Graphpad Prism 5.0 program to perform the Analysis of Variance-Tuckey test and to plot the data. Differences were considered statistically significant when confidence was superior to 95% ($p<0.05$).

3.3 RESULTS AND DISCUSSION

3.3.1 *L. infantum* NTPDases share high sequence identity on ACR between trypanosomatids and mammalian

A deep investigation of *L. infantum* JPCM5 genome reveals that it contains single-copy genes for *LiNTPDase1* and *LiNTPDase2* despite the high aneuploidy rate on its genome. The *LiNTPDase1* one is located on chromosome 10 (Linf_100006600) and encodes an approximately 70kDa protein known as Guanosine diphosphatase and the smaller one *LiNTPDase2* located on chromosome 15 (Linf_150005200) encodes an approximately 45kDa protein known as ATP diphosphohydrolase (description from the TritrypDB). Here, we refer to 70kDa NTPDase as LiNTPDase1 and 45kDa NTPDase as LiNTPPDase2 according to VASCONCELLOS (2014). *LiNTPDase1* gene encodes for a protein with 685 amino acids with a putative N-terminal peptide signal sequence from 1–20 and a cleavage site between 20-21 residues (Signal-P 5.0) (NIELSEN et al., 1997) (Fig. S1). On the other hand, *LiNTPDase2* gene encodes for a smaller protein with 432 amino acids with a putative N-terminal transmembrane domain (residues

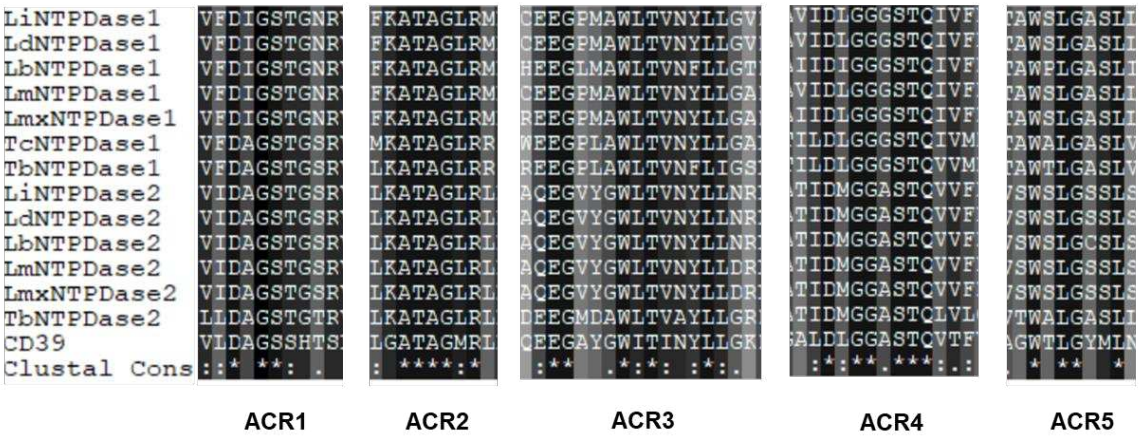
between 17–39) (Signal-P 5.0)(NIELSEN et al., 1997) (Fig.S1). These data suggest putative secretion for LiNTPDase1 and membrane anchorage for LiNTPDase2.

Sequences alignment and phylogenetic tree using Clustalw (SAITOU; NEI, 1987; SIEVERS et al., 2011) revealed that sequences are conserved between trypanosomatids species *(L. infantum, Leishmania donovani, Leishmania braziliensis, Leishmania major, Leishmania mexicana, Trypanosoma cruzi and Trypanosoma brucei)* and encode proteins with five putative ACR domains (Fig 1A). These domains have amino acid residues that characterize them as apyrases from CD39 family when compared to other trypanosomatid sequences and the reference mammalian CD39 (Fig. 1A and Fig. S2). These residues compose the catalytic core which make them important to enzyme function (ZEBISCH; STRÄTER, 2008). In fact, the soluble domains of LiNTPDase1 and LiNTPDase2 enzymes have been biochemically characterized after cloning and expression not only in bacteria but also in mammalian expression systems, setting them as authentic apyrases (BASTOS et al., 2017). Phylogenetic analysis showed that LiNTPDase1 is more related to other Leishmania NTPDase1, and *T. cruzi* NTPDase1 than with its ortholog isoform LiNTPDase2. Indeed, it has been demonstrated that LiNTPDase 1 shows 90% of identity compared to species belonging to the same Leishmania complex and ~69% of identity compared to other Leishmania complex (VASCONCELLOS et al., 2014). In addition, LiNTPDase2 is closer related to NTPDase2 from other species of Leishmania (Fig.1B) than to that from mammals. In fact, VASCONCELLOS et al., (2014) showed that LiNTPDase2 has 27% of identity with mammalian CD39. The low identity between LiNTPdases and Mammalian NTPDase can make them good drug targets. In addition, LiNTPDase 2 has been shown to have ~92% of identity to the same Leishmania complex and ~79% similar to a different Leishmania complex (VASCONCELLOS et al., 2014).

Even belonging to the same family, LiNTPDases are divided in two clades (Fig. 2B). This phylogenetic divergence evidences the differences in amino acids sequences including on the ACR domains and may indicate possible nucleotidase specificity for the *L. infantum* enzymes. To further understand how it could affect NTPDases function, a point mutation studies should be performed.

Finally, search for annotated function we assembled a functional related proteins network based on protein association and gene ontology using STRING (JENSEN et al., 2009), where we could design a network of linked functional prediction and partners

were taken. All partners of NTPDase1 are involved on purine and pyrimidine metabolism e.g. XP_001469194.1, XP_001470128.1, XP_001464138.1, XP_001469286.1, XP_001464314.1, XP_001467945.1, XP_001464543.1, XP_001467573.1, XP_001468477.1, XP_003392492.1, XP_003392832.1 and no other function has been proposed for the enzyme. Looking at LiNTPDase 2, a completely different network based on linked function prediction was assembled and some partners were identified e.g. XP_001466235.1, XP_001470024.1, XP_003392546.1 and LPG2. The same metabolism pathways were identified but here, lipophosphoglycan biosynthesis from *L. major* was included (SANSOM et al., 2014). Indeed, Sansom (2014) using genetic approach showed the involvement of this enzyme on lipophosphosglycan elongation on Golgi apparatus revealing a new function of NTPDase2 on *L. major*. All putative function related proteins are described on table ST3. Another possible function has been proposed on macrophage infection (ENNES-VIDAL et al., 2011; MOREIRA et al., 2009; PINHEIRO et al., 2006; VASCONCELLOS et al., 2014) but no genetic tools were used on those studies.


A
LiNTPDase1
LdNTPDase1
LbNTPDase1
LmNTPDase1
LmxNTPDase1
TcNTPDase1
TbNTPDase1
LiNTPDase2
LdNTPDase2
LbNTPDase2
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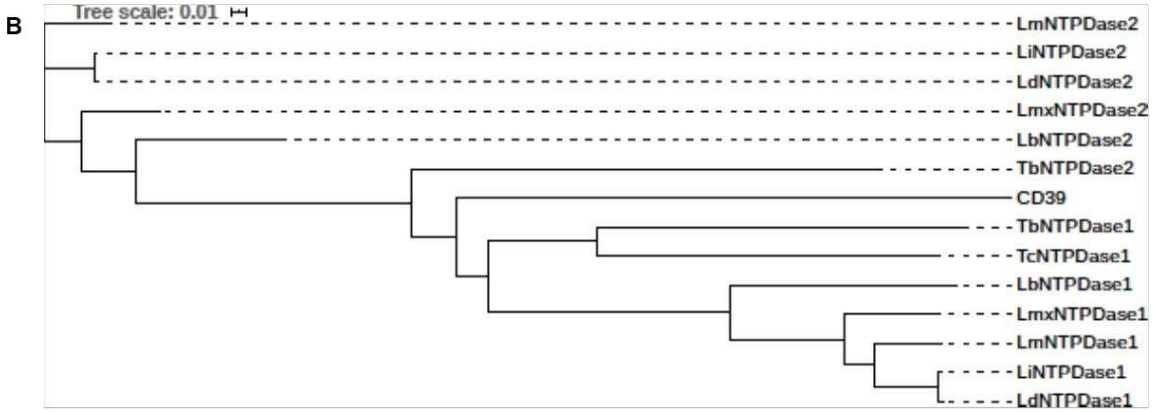
ACR3
ACR4
ACR5


B
Tree scale: 0.01
LmNTPDase2
LiNTPDase2
LdNTPDase2
LmxNTPDase2
LbNTPDase2
TbNTPDase2
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TcNTPDase1
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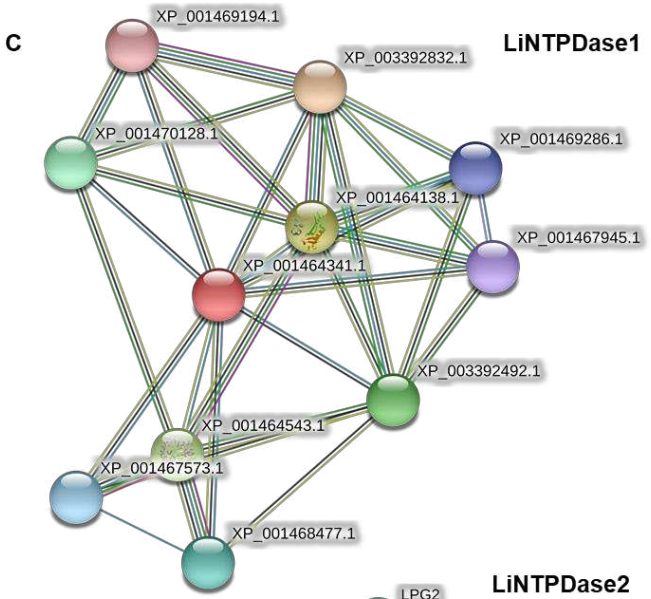
LmxNTPDase1
LmNTPDase1
LiNTPDase1
LdNTPDase1


C
LiNTPDase1
XP_001469194.1
XP_003392832.1
XP_001470128.1
XP_001469286.1
XP_001464138.1
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XP_001468477.1

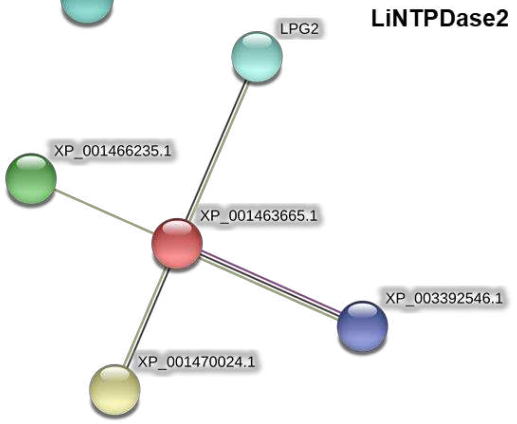

LiNTPDase2
LPG2
XP_001466235.1
XP_001463665.1
XP_003392546.1
XP_001470024.1

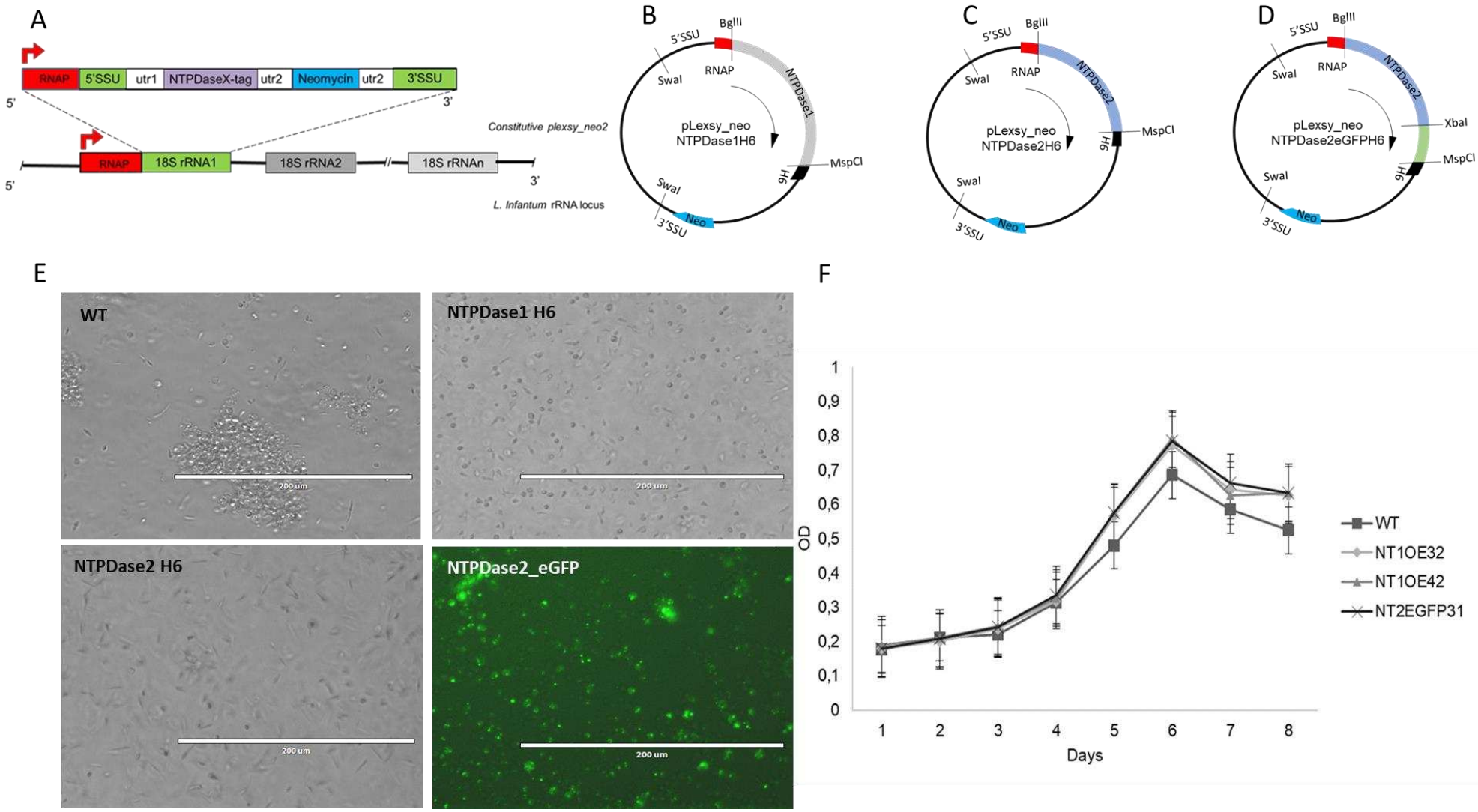
Figure. 1. Putative ACR regions of trypanosomatids NTPDases aligned with human CD39. A. ACR alignment shows the conserved residues dashed on dark color, grey color represent sequences with similar residues. Apyrase characteristics residues are starred on down below consensus sequence, revealing that they are present in the putative Leishmania NTPDases. Alignment was performed using ClustalW. B. Phylogenetic tree of trypanossomatids NTPDases and CD39. The tree was constructed from the ClustalW alignment showed in A. The tree was edited using Bioedit tool. C. Putative Network of functional related proteins based on protein association using STRING. Graph was designed using LiNTPDases 1 and 2 protein sequences. Sequences accession numbers used for Fig. 1A ,1B and 1C and Sequences used on the alignment are described on supplementary table ST1. Complete trypanosomatids alignment is on Fig. S3. All putative NTPdases function related proteins are described on table ST3.

3.3.2 *Obtention* of *L. infantum* overexpressing NTPDase1 and 2

Gain of function studies has been largely used to characterize biological roles of genes and would be equally useful on putative coding sequences from leishmania (BOLHASSANI et al., 2011; MISSLITZ et al., 2000). Once Leishmania expression system (pLEXSY, Jena Biosciences) has largely been used to produce recombinant proteins (BREITLING et al., 2002; DE OLIVEIRA et al., 2019) here, we chose the constitutive system pLEXSY neo_2 to clone and add extra copies to *L infantum* of LiNTPdase1 and LiNTPDase2 in order to get the overexpressing mutants. This system was used by Taheri et al (2014) and showed efficient obtention of tagged mutants using LEXSY constitutive system. Then, the LiNTPdase1 and LiNTPDase2 were independently cloned into pLEXSY_neo_2 (Fig S3 and S4). The full-length coding sequence of each gene were cloned using the restriction sites *Bgl*II and *Msp*CI. By using these restriction sites, we removed the vector secretion signal peptide SP at 5´end of the cloning site avoiding its interference in the localization of target LiNTPDases. In addition, this digestion allowed us to fuse our sequences to a 6x Histidine tag (here referred to as H6 tag) (Figures 2B and C). Moreover, to access the precise localization of LiNTPDase2 into the parasite cells we also cloned this enzyme fused to eGFP (unfortunately we did not have success using this approach to LiNTPDase1). To have LiNTPDase2-GFP, after the cloning of *LiNTPDase2* into pLESXY_neo_2 we subcloned this construction with eGFP using *XhoI* and *Msp*CI cloning site (Fig. 2D). Every construction was confirmed by digestion, PCR and sequencing. Cloning details are described in Fig. S3 and S4.

The linear cassettes used to homologous recombination were obtained from plasmid digestion using *Swa*I site (Fig. 2B, C, and D). The obtention of *L. infantum* mutants overexpressing LiNTPDase1 and LiNTPDase2 were achieved independently after electroporation with the cassettes and drug selection with neomycin (Fig. 2E). Isolated clones were obtained from limiting dilution and PCR genotyping confirmed the integration of the cassettes on the ribosomal locus under control of Leishmania RNA polymerase I (Fig S5). The first analyses we did using the overexpressing clones were the evaluation of morphology and growth curve. We did not observe any differences between the wild type strain and the overexpressing mutant clones (Fig. 2F) indicating

no chromosomal abnormalities that could take place during homologous recombination (LACHAUD et al., 2014).


A
RNAP
5'SSU
utr1
NTPDaseX-tag
utr2
Neomycin
utr2
3'SSU
5'
3'
Constitutive plexsy_neo2
RNAP
18S rRNA1
18S rRNA2
18S rRNAn
L. Infantum rRNA locus
B
BglII
5'SSU
SwaI
RNAP
NTPDase1
pLexsy_neo
NTPDase1H6
H6
MspCI
SwaI
Neo
3'SSU
C
BglII
5'SSU
SwaI
RNAP
NTPDase2
pLexsy_neo
NTPDase2H6
H6
MspCI
SwaI
Neo
3'SSU
D
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5'SSU
SwaI
RNAP
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NTPDase2eGFPH6
XbaI
H6
MspCI
SwaI
Neo
3'SSU
E
WT
NTPDase1 H6
NTPDase2 H6
NTPDase2_eGFP
200 um
F
OD
Days
WT
NT1OE32
NT1OE42
NT2EGFP31

Figure 2. Cloning and obtention of *Leishmania infantum* JPCM5 overexpressing LiNTPDases1 and 2. A. Schematic representation of the recombination site on *L. infantum* ribosomal locus. RNAP represents the promoter of Leishmania RNA polymerase I. 5' and 3' SSU represents the homologous recombination site. NTPDaseX is the site where target sequences were cloned on vector pLexsy_neo2.1. Neomycin is the drug resistance gene. B. Cloned vector to express C-terminal H6 tagged LiNTPDase1 with the restriction site used to digest the vector. C. Cloned vector to express C-terminal H6 tagged LiNTPDase2 with the restriction site used to digest the vector. D Cloned vector to express C-terminal H6 tagged LiNTPDase1 with the restriction site used to digest the vector. E. White light microscopy of the WT parasite, clones LiNTPdases1H6, LiNTPDase2H6 and green light fluorescence microscopy of the clone tagged LiNTPDase2_eGFP. All images were collected with 60x magnification. F. Growth curve of WT, LiNTPDase1H6,Li NTPDase2H6 and LiNTPDase2_eGFP. The SD represent the average of three independent experiments performed in triplicate. No significant differences were detected.

3.3.3 Nuclear and Kinetoplast localization of eGFP fused LiNTPDase2 on *L. infantum*

Trust on genetic tool to fluoresce tag protein has been valuable to precisely localize a protein on intracellular compartments (GUEDES AGUIAR et al., 2018; SANSOM et al., 2014). In several lines, the pLEXSY system has been used to generate leishmania mutants expressing fluorescent proteins (BREITLING et al., 2002; DE OLIVEIRA et al., 2019; BASTOS et al., 2017). Using this system, we were able to generate JPCM5 *L. infantum* expressing eGFP fused to C-terminal NTPDase2. Exponential phase promastigotes were taken to slides preparation. Images were taken in white, blue, and green channels (Fig. 3A). The white channel clearly showed leishmania promastigote needle shape. Parasite overexpressing eGFP fused NTDase2 (LiNTPDase2_eGFP) showed no morphological difference compared to wild type. In addition, on the blue channel, DAPI specifically stained nuclear and kinetoplast organelles (Fig. 3A and B). Moreover, using the green fluorescent channel, *L. infantum* expressing C-terminal eGFP tagged NTPDase2 revealed a non-diffuse signal (Fig. 3B). Overlapping the images showed us a colocalization between NTPDase2-eGFP, Nuclei, and Kinetoplast indicating possible roles of this protein on these organelles. In fact, in a previously work our research group, VASCONCELLOS (2014) using polyclonal antibodies produced against the recombinant LiNTPDase2 showed LiNTPDases on the parasite surface and also in the intracellular space, such as the nucleus, flagellar pocket, mitochondria, endoplasmic reticulum and Golgi apparatus. However, in the Vasconcellos 2014 work they used the polyclonal antibody that recognizes both isoforms and it was not possible to discriminate between the isoforms in the localization data as we did here with engineered eGFP tagged LiNTPDase2. The same pattern of localization was observed to *L. amazonensis*, *L. braziliensis and L. major* suggesting a conserved localization between different Leishmania species (DETONI et al., 2013; SANSOM et al., 2014), but again, no specific antibody against each isoforms was used. Even using a more accurated tool to localize NTPdase2 on *L. infantum* cell further analysis using a better resolution microscopy is still needed to achieve the precise localization.

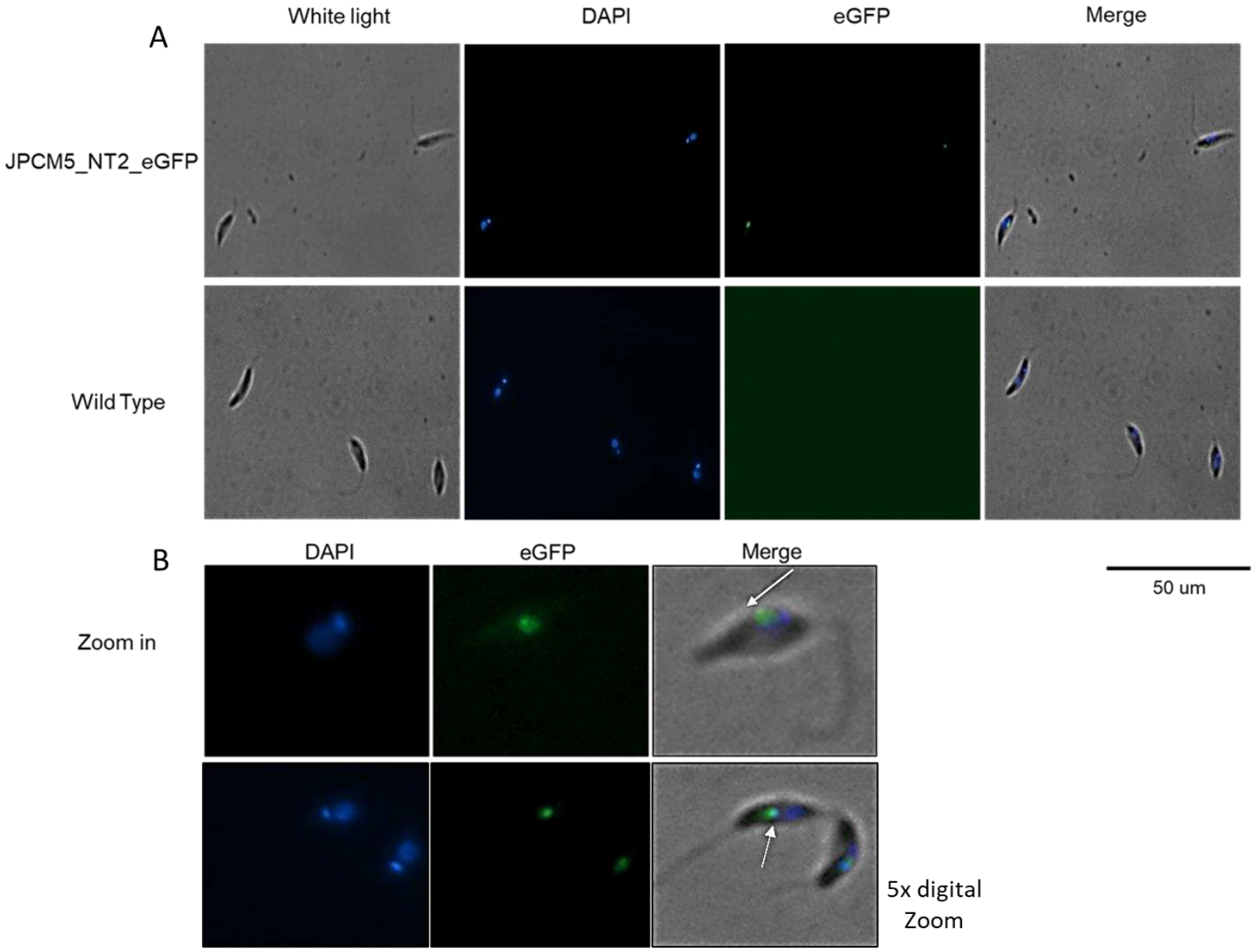
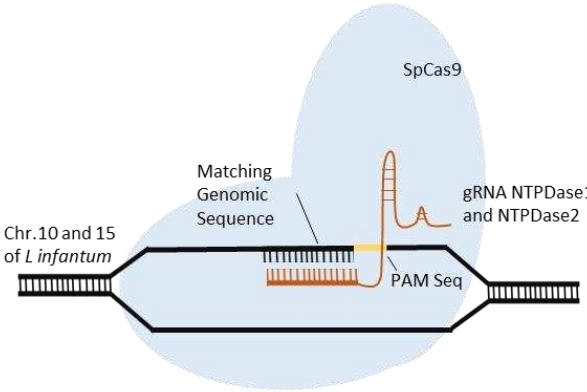

A
White light
DAPI
eGFP
Merge
JPCM5_NT2_eGFP
Wild Type
B
DAPI
eGFP
Merge
Zoom in
50 um
5x digital
Zoom

Figure 3. Cellular localization of eGFP fused NTPDase2 on *L. infantum.* A. Images showing Wild type *L. infantum* and eGFP fused LiNTPDase2 on white, blue (DAPI) and green channel (eGFP). B. Different slide images were 5x digital magnified to evidence the localization on overlayed pictures. White arrows point to eGFP tagged LiNTPdase2 localized on nuclei and kinetoplast. All images were taken on EVOS XL 60x magnification.

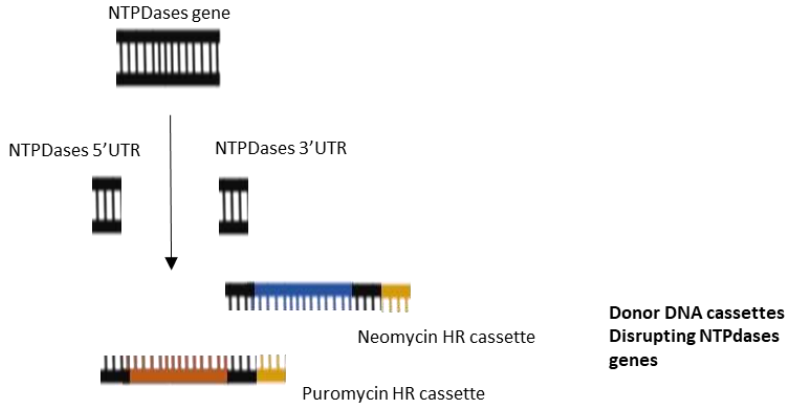
3.3.4 Generation *L. infantum* JPCM5 expressing SpCas9

In order to generate knockouts of LiNTPDase1 and 2 of *L. infantum* we first needed to establish a cell line expressing *S. pyogenes* Cas9 (SpCas9) since *L. infantum* are unable to express the nuclease Cas9. Cas9 is an endonuclease driven to a genomic target site by gRNA molecule to promote a DNA double-strand break (DOUDNA; CHARPENTIER, 2014) (Fig. 4A). Break on DNA needs to be repaired, which allows the homologous recombination mechanism (JINEK et al., 2012). Using a donor DNA that enable the disruption of the target gene at the same time that insert a resistance gene sequence we may generate specific target null mutants (BENEKE et al., 2017, 2019) (Fig. 4B). A plasmid construction cloned to express SpCas9 was kindly given by Prof. Barbara Papadopoulou. The leishmania expression plasmid Sp72αHygroαSpCas9HA was electroporated to generate *L infantum* episomally expressing Cas9. Different concentration of plasmid was used to transform the parasite (Fig. 4C) and all of them allowed the parasite to express SpCas9 protein. The α-tubulin protein was used as an internal control on Western Blotting analysis (Fig. 4C). Moreover, the growth curve showed no significant difference between wild type parasite and those expressing SpCas9 suggesting no toxic effect of the protein on parasite metabolism and reproduction. Indeed, using the episomal (BENEKE et al., 2017; LANDER et al., 2015) instead of the constitutive (HSU; LANDER; ZHANG, 2014; ZHANG; LYPACZEWSKI; MATLASHEWSKI, 2017; ZHANG; MATLASHEWSKI, 2015) systems to transformation of Leishmania showed to be the most efficient approach to achieve parasite expressing Cas9 with no toxic effects.

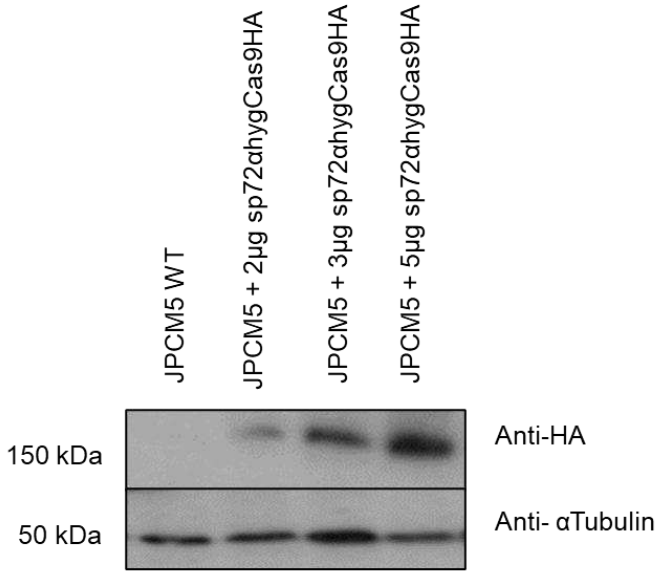
A


SpCas9
Matching
Genomic
Sequence
gRNA NTPDase1
and NTPDase2
Chr.10 and 15
of L infantum
PAM Seq

B


NTPDases gene
NTPDases 5'UTR
NTPDases 3'UTR
Neomycin HR cassette
Puromycin HR cassette
Donor DNA cassettes
Disrupting NTPdases
genes

C


JPCM5 WT
JPCM5 + 2µg sp72αhygCas9HA
JPCM5 + 3µg sp72αhygCas9HA
JPCM5 + 5µg sp72αhygCas9HA
150 kDa
50 kDa
Anti-HA
Anti- αTubulin

D

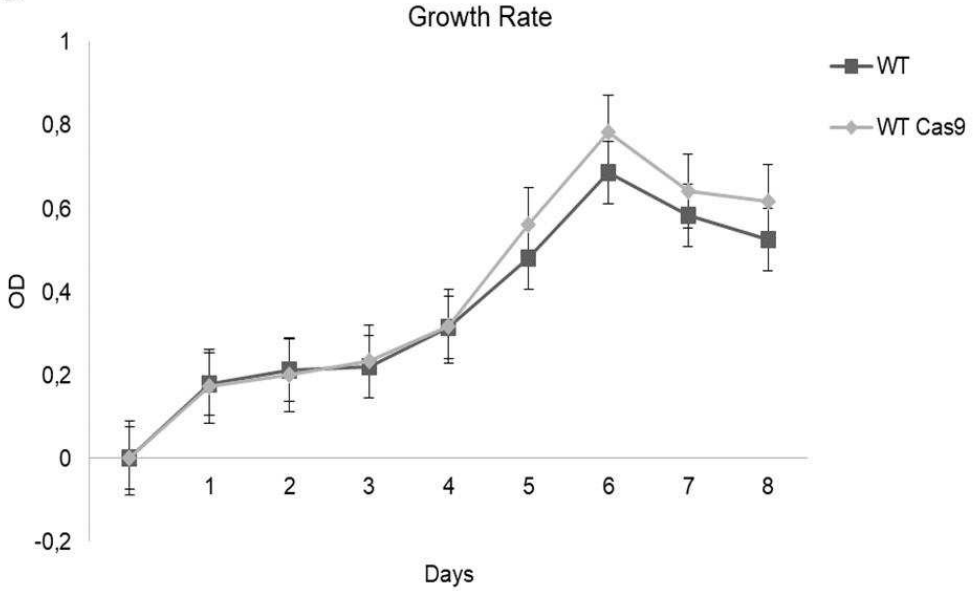

Growth Rate
OD
Days
WT
WT Cas9
1
0,8
0,6
0,4
0,2
0
-0,2
1
2
3
4
5
6
7
8

Figure 4. Generation of JPCM5 *L. infantum* expressing SpCas9. A. Schematic representation of CRISPR/Cas9 system to generate LiNTPDases knockouts. Cas9 nuclease has mainly two domains: The upper one that recognizes the secondary structure on sgRNA and the down one that recognizes PAM sequence on Genomic DNA and drives the recognition site of the gRNA to the target site increasing the specificity of the break. B. After DNA double strand break the donor DNA added to the system will uses the homologous recombination mechanism to incorporate the cassettes with drug resistance gene flanked by microhomology arms instead of the target gene. C. Western Blotting using mouse anti-HA and mouse anti-αtubulin as control. Successfully expressing SpCas9 parasite were labeled at 150KDa. D. Growth curves *of L infantum* expressing SpCas9. The SD represents the average of three independent experiments. No significant differences were detected.

3.3.5 Obtention of LiNTPDase1 and LiNTPDase2 *L. infantum* knockouts

SpCas9 *L. infantum* was used to generate NTPDase1 and 2 knockouts. Double knockout of NTPDase1 and NTPDase2 was not achieved after three attempts once the strategy seemed to be lethal to parasites. Homologous recombination cassettes were PCR generated using the sets of primers described in supplementary table ST2, and plasmids Sp72yneo and Sp72ypuro as template. Cassettes for both alleles of both genes were designed and amplified with neomycin and puromycin resistance gene with proximal 5' and 3' UTR from the target gene flanking their sequence (Fig. 4B and Fig. S6). Then, each drug resistance gene has a flank region of the proximal UTR of the target gene. The micro-homologous recombination mechanism occurs in trypanosomatids and were successfully used to replace gene targets locus and increase efficiency of deletion and selection in Leishmania (LANDER et al., 2015; ZHANG; LYPACZEWSKI; MATLASHEWSKI, 2017; ZHANG; MATLASHEWSKI, 2015). In fact, using two different drug resistance genes BENEKE (2017) achieved efficiently knockout of PF16 gene on *L. Mexicana* in just one round of transfection.

To evaluate the success of our deletion we used Southern blot analysis in the transfected populations. LiNT1 5'UTR and LiNT2 3'UTR probes to Southern Blotting were generated by PCR (Fig. S6). Probes were radiolabeled with P^{32} and used to hybridizes to its corresponding UTR sequence in order to detect wild type alleles or

recombinant drug resistance gene alleles. Genomic DNA from putative LiNTPDases1 and 2 null mutants and WT control were extracted and digested using *XhoI* and *Cla*I restriction enzymes chosen to digest leishmania DNA on flank region of LiNTPDase1 (1.1kb) and LiNTPDase2 (0.87kb) respectively (Fig. 5A e 5C). Wild type LiNTPDase1 allele was detected on Southern blotting developed with 3.2 kb which corresponds to 2.1kb of the LiNTPDase1 gene and 1.1kb of flank region digested (Fig 5B). In addition, neomycin gene insertion was detected by a fragment of 1.9kb relative to 0.8bp neomycin gene and 1.1kb of flank region to all analyzed subclones. The puromycin gene insertion was detected by a fragment of 1.7kb from 0.6kb of puromycin gene and 1.1kb of flank region (Fig. 5B). Although in all subpopulations we detected neomycin and puromycin replacing the target genes, we still note weak shadow representing a small number of WT alleles in the populations. We took the subpopulation LiNTPDase1 2040 to limiting dilution in order to isolate individual clones (Fig. S7) and expand the cell culture to further analysis (Fig. S7). In the same way, analyzing the subclones obtained from the LiNTPDase2 knockout approach, we detect wild types alleles with 2.1kb corresponding to 1.2kb LiNTPDase2 gene and 0.87kb of flank region (Fig. 5C). In addition, we also could detect neomycin allele in 2 subpopulations with 1.7kb corresponding to 0,8kb neomycin gene and 0.87bp of flank side (Fig. 5D). Puromycin was also detected on the same subclone with 1.5kb referred to 0.6kb puromycin resistance gene and 0.87bp of flank side (Fig. 5D). Both subclones were extremally pure but even that we decide to take one of them, the LiNTPDase2 2560 to limiting dilution to have true isolated clones.

The confirmation of the individual clones was done by genotyping (FigS7). Genomic DNA was extracted to diagnose as positive or negative to the presence of the analyzed gene. The other LiNTPDase gene was always used as a control. The clones were successfully isolated (Fig S7). Using homologous recombination approach Sansom, 2014 were also able to generate *L. major* null mutants for isoforms 1 and 2 from *L. major* (LmNTPDases). On the other hand, (SILVA-GOMES et al., 2020) were unable to generate null mutant of NTPDase1 in *T. cruzi*. Together with our failure on getting double knockout of LiNTPDases, seems plausible to consider that at least one NTPDase gene is necessary to trypanosomatids surviving.

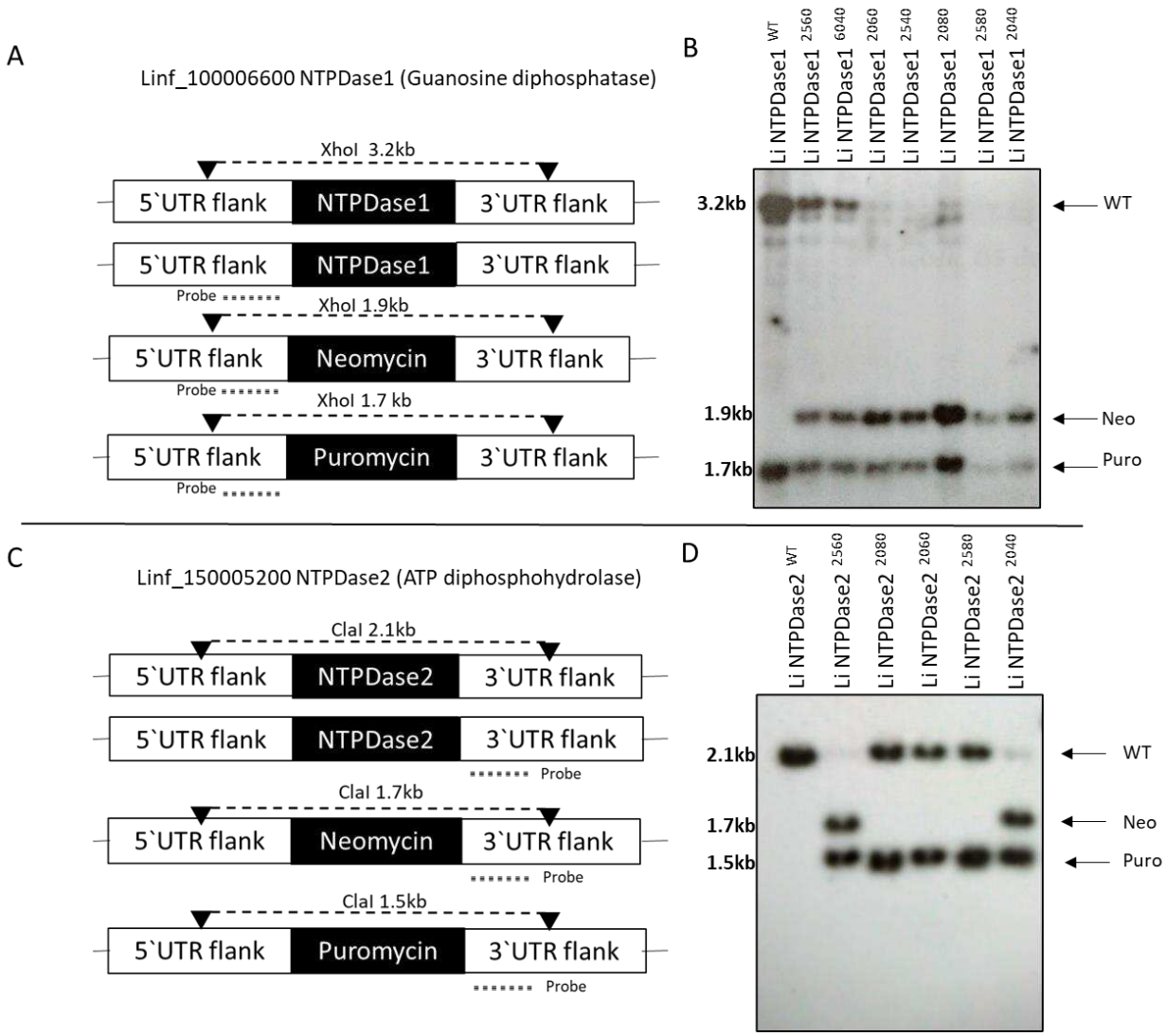


Figure 5. Southern blotting of Null mutants of *L. infantum* NTPDase1 and 2. A. Schematic representation of *L infantum* gDNA digestion using *Xho*I on NTPDase1 gene. WT, neomycin and puromycin alleles are represented, as well as, fragment size and probe localization. B. Southern blotting of the digested sample with *Xho*I. WT, neomycin and puromycin alleles can be observed. C. Schematic representation of *L infantum* gDNA digestion using *Cla*I on NTPDase2 gene. WT, neomycin and puromycin alleles are represented, as well as, fragment size and probe localization. D. Southern blotting of the digested sample with *Cla*I. WT, neomycin and puromycin alleles can be observed.

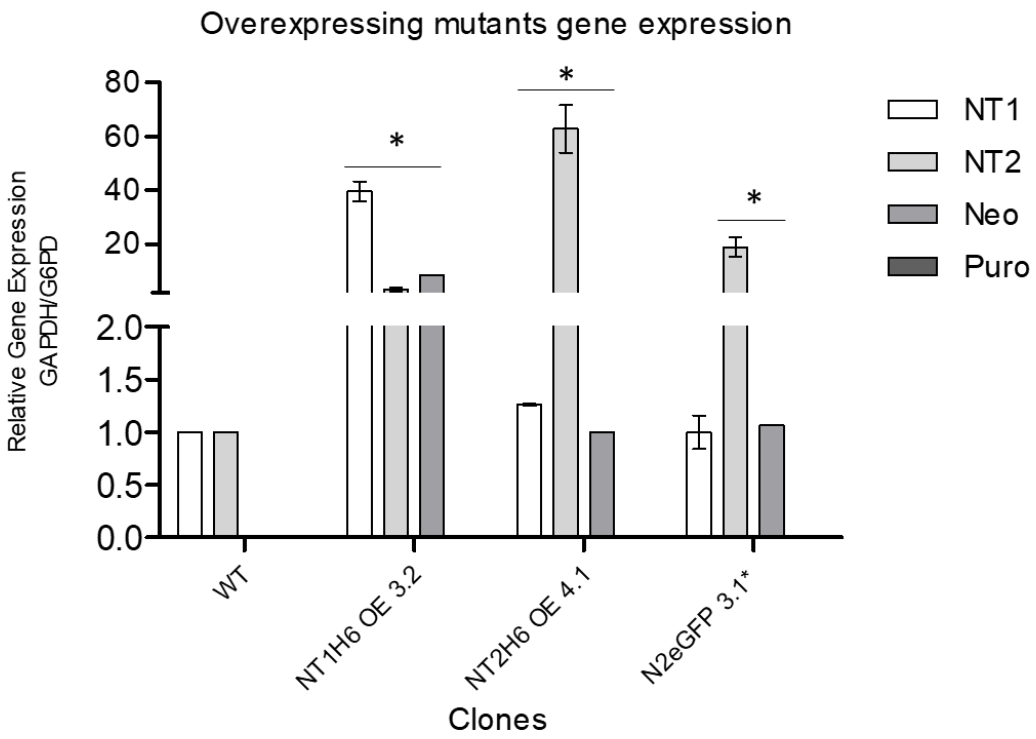
3.3.6 LiNTPDase1 and LiNTPDase2 gene expression are upregulated in LiNTPDase2$^{-/-}$ and LiNTPDase1$^{-/-}$ clones respectively

In order to access gene expression expression, we extracted total RNA from the KO clones in the promastigotes in the late exponential phase. RNA was reverse transcribed to cDNA and used to access expression level using specific oligonucleotides for each analyzed sequence (supplementary table ST2). Wild type samples showed a basal level of relative RNA expression of LiNTPDase1 and LiNTPDase2 genes and no expression of Neomycin and Puromycin resistance genes (Fig 6A). In addition, the RNA expression level was also accessed on overexpressing clones. LiNTPDase1H6 overexpressing clone showed an increase of 40x in LiNTPDase1 RNA and basal expression of LiNTPdase2 and basal expression of neomycin resistance gene indicating the successful of extra copies adding and drug selection (Fig.6 A). Likewise, overexpressing clones for LiNTPDase2H6 and NTPDase2_eGFP (Fig. 6A) showed an increase of LiNTPDase2 RNA expression (80x and 20x, respectively) and a basal expression of LiNTPDase1 and neomycin resistance gene confirming the achievement of LiNTPDase2 overexpression.

Moreover, RNA expression assessment of LiNTPDase1ko showed a complete abolishment of LiNTPDase1 RNA expression. Curiously, an increase in LiNTPDase2 RNA expression was detected. Neomycin and Puromycin resistance genes were also expressed in this sample (Fig. 6B) indicating gene replacement of the donor DNA during knockout achievement. Although some of our data suggest differential LiNTPDases RNA expression on different parasite life stage (RT-qPCR data not shown), the increase of isoform LiNTPdase1 on LiNTPDase2 null clone may indicate a compensation caused by the abolishment of the other isoform. The same pattern was also observed to LiNTPDase2ko where LiNTPDase2 RNA expression was undetected at the same time that LiNTPDase1 increased its level (Fig. 6B). Here again, neomycin and puromycin resistance genes were also expressed indicating successful obtention of LiNTPDase2 null mutant. Interestingly, this pattern of increasing RNA expression of the gene which was not deleted could indicate a simply mechanism of increase RNA to compensate the absent of the other isoform, but also some more drastic genetic events. Indeed, leishmania presents an absence of promoter mediated regulation at transcription level of nuclear genes (BEVERLEY et al., 2017). Changes in transcript levels within the parasite are associated to differences in the maturation

and stability of mRNAs, largely mediated by RNA-binding proteins. In addition, increase in gene copy number is a drastic usual genetic toll that Leishmania takes to increase gene expression as result of some environmental pressure (CLAYTON, 2016). Genome and transcriptome deep sequencing could be interesting in the future to better investigate the pleiotropic effects of the knockout of LiNTPDases.

A


Overexpressing mutants gene expression
Relative Gene Expression
GAPDH/G6PD
80
60
40
20
2.0
1.5
1.0
0.5
0.0
*
*
*
NT1
NT2
Neo
Puro
WT
NT1H6 OE 3.2
NT2H6 OE 4.1
N2eGFP 3.1*
Clones

B

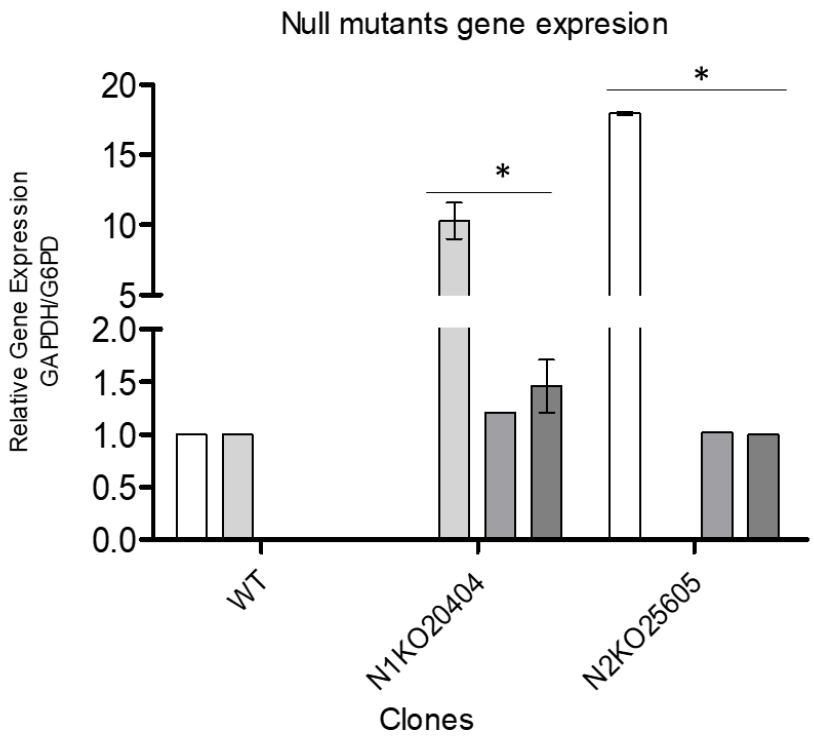
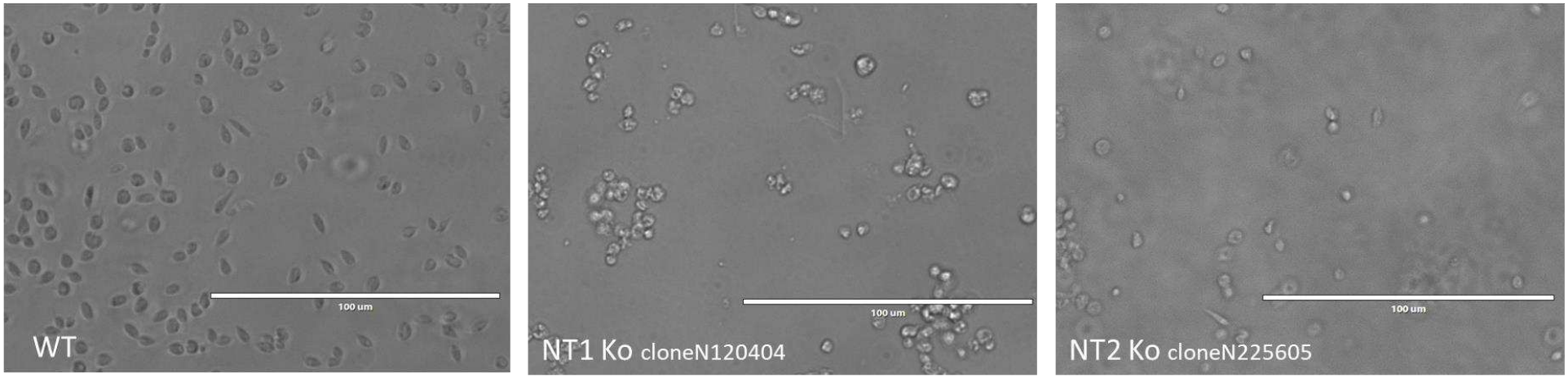

Null mutants gene expresion
Relative Gene Expression
GAPDH/G6PD
20
15
10
5
2.0
1.5
1.0
0.5
0.0
*
*
WT
N1KO20404
N2KO25605
Clones

Figure 6. Gene expression of null mutants and overexpressing isolated clones. Each gene NT1(LiNTPDase1), NT2(LiNTPDase2), Neo (Neomycin), Puro (Puromycin) for each sample WT (Wild type), NT1H6 OE3.2(NTPDase1 overexpressing), NT2H6OE 4.1(NTPDase2 overexpressing), N2eGFP 3.1*(eGFP tagged NTPDase2), NT1Ko20404 (NTPDase1 null mutant) and NT2Ko25605 (NTPDase2 null mutant) was compared to Wild type control. A. Relative gene expression accessed by Relative standard curve from RT-qPCR were plotted and showed successful achievement of Overexpressing clones with neomycin resistance gene expression. B. Mutant gene expression showed no RNA expression of the deleted gene while resistance genes neomycin and puromycin showed expression indicating success on getting null mutants clones isolated. Analysis of variance was performed and confidence of 95% was considered statistically significant. GaphPad prism was used to analyze and plot data. GAPDH and G6PD gene was used as internal control

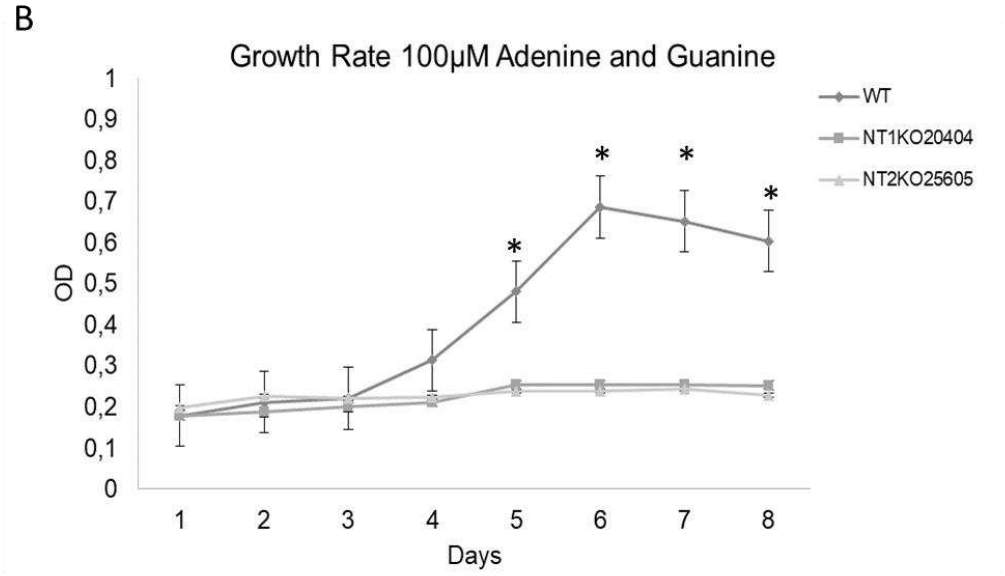
3.3.7 Knockout of LiNTPDase1 and 2 change *L. infantum* phenotype and purines uptake

After limiting dilution isolation and expansion of LiNTPDase 1 and 2 null mutants clones, they showed unusual morphology and difficulties to grow. Figure 7A shows the loss of classical needle shape of *L. infantum* at the late exponential phase, while wild type remains on its characteristic morphology indicating a change in parasite phenotype. As long as trypanosomatids cannot do *de novo* synthesis of purine nucleosides, they have to intake them from the environment in order to survive (COHN; GOTTLIEB, 1997). LiNTPDases could play a role in the purine *salvation pathway* once they are able to hydrolyze purine ecto-nucleotides and deliver ecto-nucleosides for 5´-NT that could provide purines for the parasite intake (VASCONCELLOS et al., 2014; MARR; et al., 1978; ULLMAN et al., 2008). Isolated clones NTPDase1ko_20405 and NTPDase2ko_25603 showed difficulties to grow on media usually used to growth the wild type *L. infantum* as showed on growth curve using 100μM of adenine and guanine (Fig. 7B). Not surprisingly, increasing the nucleotides composition of the media to 400μM (Fig. 7C) parasites get back to normal growth rate indicating that LiNTPDase 1 and LiNTPDase2 are important to parasite *purine salvage pathway* and normal development of the cells. Likewise, Sansom 2014, also used rich media to keep *L. major* LiNTPDase 1 and 2 null mutants viable. Moreover, LiNTPDases null mutant leishmania just thrive when the media is 4x supplemented with nucleosides. At the same time, the isoform which was not deleted increase its RNA expression level what would indicate a molecular compensation, unfortunately, no protein data was accessed. Interestingly, an approach of double knockout of these enzymes was designed (data not shown), but after three attempts of knocking out all of them failed and all cells died indicating that loss of both genes is lethal to the parasite, but at least one is enough to them thrive.

A


WT
100 um
NT1 Ko cloneN120404
100 um
NT2 Ko cloneN225605
100 um

B


Growth Rate 100µM Adenine and Guanine
OD
Days
WT
NT1KO20404
NT2KO25605
*
*
*
*

C

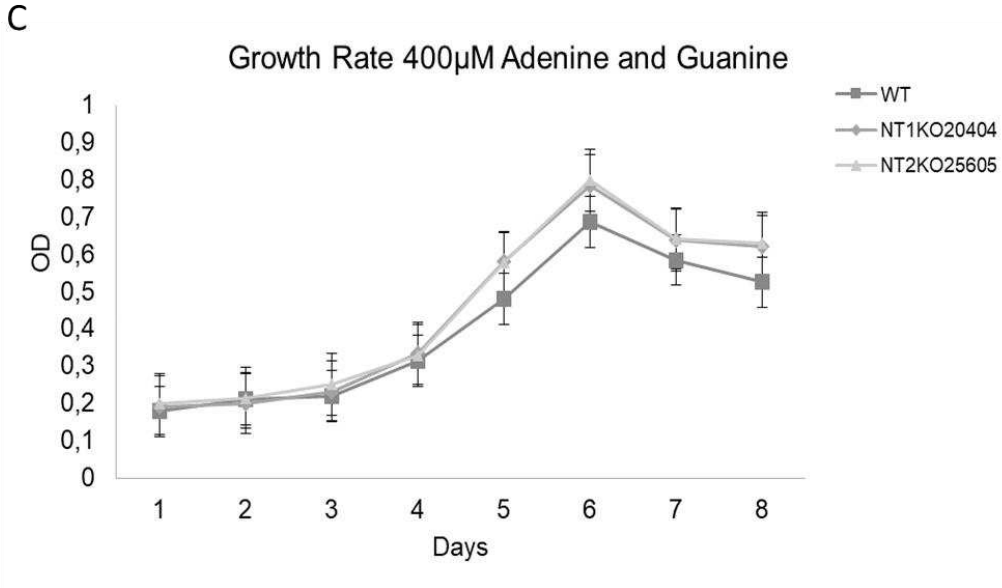

Growth Rate 400µM Adenine and Guanine
OD
Days
WT
NT1KO20404
NT2KO25605

Figure 7. NTPDase1KO and NTPdase2KO change *L. infantum* growth rate. A. White light microscopy showing considerable morphology difference between WT, NT1KO and NT2KO clones of *L infantum*. B. Growth curve using 100uM of adenine and guanine showed significative difference between WT and NT1KO and NT2KO clones. While, C. Growth curve using 400uM of adenine and guanine showed no difference between WT and NT1KO and NT2KO clones. Analysis of variance was performed and confidence of 95% was considered statistically significant. GraphPad prism was used to analyze and plot data.

3.3.8 LiNTPDase1$^{-/-}$ and LiNTPdase2$^{-/-}$ impair *L. infantum* to infect macrophages while increase NO production

The effects of Leishmania on cell hosts are firstly based on its ability to adhere and infect macrophages (DA SILVA et al., 2020; DE FIGUEIREDO; SOUZA-TESTASICCA; AFONSO, 2016; ROSSI; FASEL, 2018). In order to investigate the role of LiNTPDases on adhesion and infection of *L. infantum* on the host cell, we used null mutants and overexpressing clones to understand this mechanism. Overexpressing and null mutants of LiNTPDase1 showed no difference of adherent leishmania on the macrophage indicating that LiNTPDase1 is not important to adhesion on macrophage (Fig. 8A). On the other hand, LiNTPDase2 overexpressing clone showed an increase of ~50% in adhesion rate while LiNTPDase2Ko showed a decrease of 50% on adhesion rate when compared to wild type (Fig. 8A). In fact, previous work from our research group Vasconcellos (2014) using polyclonal serum produced against recombinant LiNTPDse2 leaded to significant reduction of *L. infantum* adhesion on macrophages. In addition, Sansom (2014) have also demonstrated that LmNTPDase2 of *L. major* is also required in the early stages of pathogen-host interaction. Put together these data, we highlight that LiNTPDase2 is really important to leishmania adhesion on macrophage may be due to some interaction to macrophage surface molecules. Interaction partners should be investigated. Moreover, LiNTPDase2 overexpressing clone were able to increase in ~30% the infection rate on macrophages while null mutant LiNTPDase2 was responsible for decrease the adhesion on ~92% corroborating our previous data that had shown important participation of LiNTPDases in the in vitro infection (VASCONCELLOS et al., 2014). Interestingly, LiNTPDase1 null mutant notably have its ability to infect macrophages decreased in ~90% of the infection rate (Fig. 8B). For the first time we showed the involvement of LiNTPDase1 on infection, note that infection was not abolished, indicating a partial role of this enzyme on infection ability. In fact, several works have shown that blocking or unspecific inhibition of *T. cruzi* NTPDase1 and Leishmania NTPDases decrease their ability to infect host cell indicating the key role of NTPDases on infectivity even without distinguish which isoform is involved (BISAGGIO et al., 2003; DE SOUZA et al., 2010; ENNES-VIDAL et al., 2011; MARIOTINI-MOURA et al., 2014; PINHEIRO et al., 2006). Here, using genetic approach we could clearly see the isolated effect of each gene on

adhesion and infectivity. On the other hand, overexpressing LiNTPdase1 was not enough to change adhesion and infection rate. In addition, looking the number of intracellular amastigotes after infection, both knockouts LiNTPDase1ko and LiNTPDase2ko showed a decrease in the average number of intracellular parasites indicating a change in the amastigote replication rate (Fig. 8C). Different of Sansom 2014 that proposed LmNTPDase1 has no importance on drug target on *L. major*, here we can see that absence on LiNTPDase1 decrease not only the infectivity but also the replication within macrophage, thus this isoform could also be good target to development of new therapy.

Leishmania parasites actively master the oxidative stress to their survival within host cell (ROSSI; FASEL, 2018). In fact, Leishmania have developed many strategies to interfere with NO production, such as the use of an LPG coat to avoid the iNOS assembly and induction of secretion of macrophage arginase, which competes with iNOS for arginine, decreasing the production of NO (ROSSI; FASEL, 2018). In order to access NO production during infection with the wild type and the mutants we collected the supernatant of infection assay and measured the NO level. Non infected macrophage was taken as negative control on NO production which was lower than in wild type parasites infected macrophages (Fig. 8D). LiNTPDase1 and 2 overexpressing clones have no difference when compared to wild type (Fig. 8D). Interestingly, LiNTPDases null mutants showed an increasing in NO production indicating that LiNTPDase1 and 2 expression play roles on the inhibition of NO production during infection (Fig. 8D). In fact, GOMES (2015) have suggested that the level of Leishmania NTPDase activity would influence parasite survival by inhibiting NO production as well modulating IL-12, IL-10 and TNF-α. Further analyzes concerning cytokines could be done to better understand this mechanism.

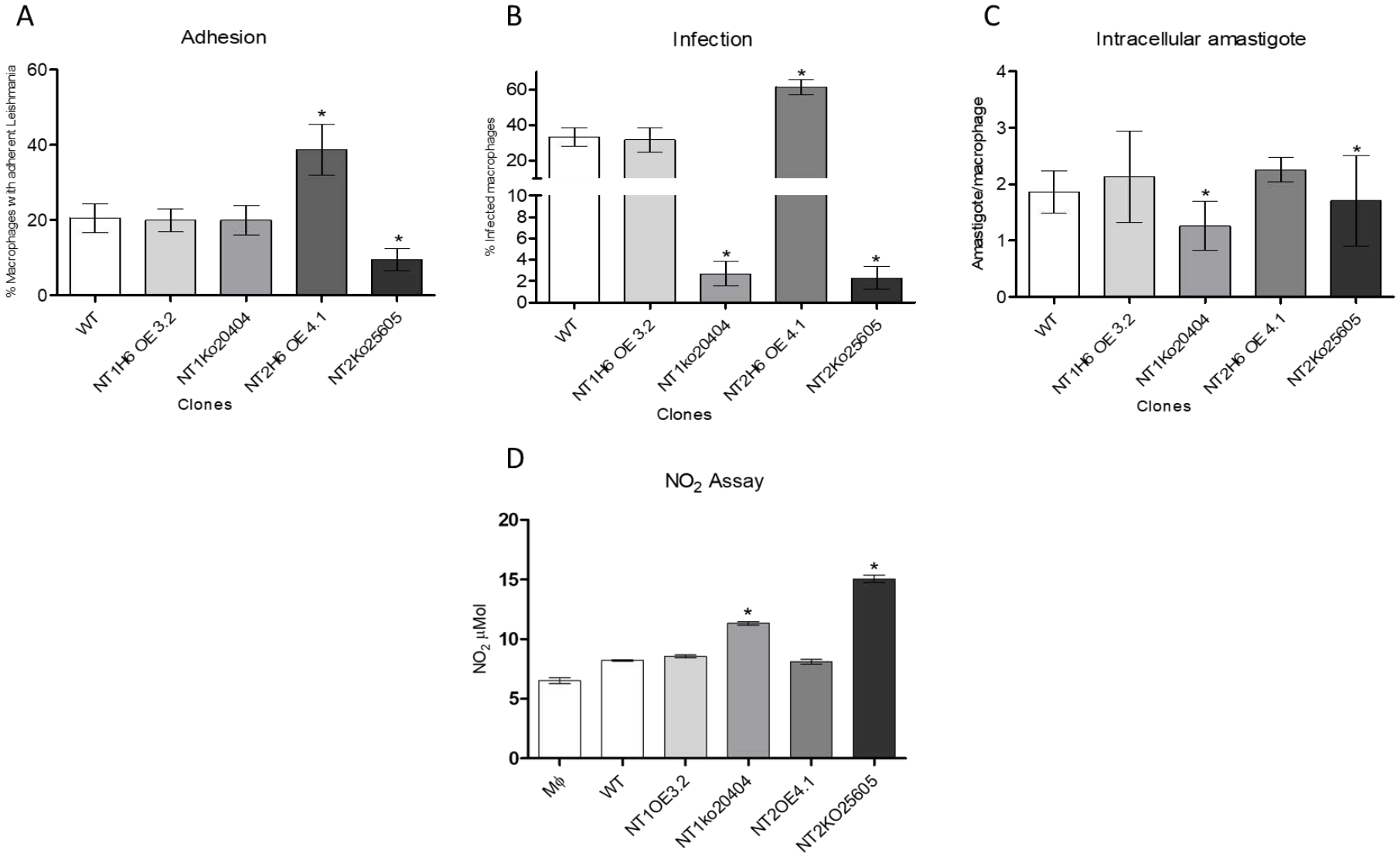

A
Adhesion
% Macrophages with adherent Leishmania
60
40
20
0
*
*
WT
NT1H6 OE 3.2
NT1Ko20404
NT2H6 OE 4.1
NT2Ko25605
Clones
B
Infection
% Infected macrophages
60
40
20
10
8
6
4
2
0
*
*
*
WT
NT1H6 OE 3.2
NT1ko20404
NT2H6 OE 4.1
NT2Ko25605
Clones
C
Intracellular amastigote
Amastigote/macrophage
4
3
2
1
0
*
*
WT
NT1H6 OE 3.2
NT1Ko20404
NT2H6 OE 4.1
NT2Ko25605
Clones
D
NO2 Assay
NO2 µMol
20
15
10
5
0
*
*
Mϕ
WT
NT1OE3.2
NT1ko20404
NT2OE4.1
NT2KO25605

Figure 8. Role of LiNTPDase 1 and 2 on adhesion, infection and influence in the NO production by the macrophage. A. Adhesion of overexpressing and null mutants to macrophages. B. Infection rate of overexpressing and null mutants to macrophages. C. Number of intracellular amastigotes of LiNTPDases overexpressing and null mutants within macrophages. D. Nitric Oxide levels produced by the macrophages after infection with null and overexpressing mutants. Data is representative of three independent experiments. Analysis of variance was performed and confidence bigger than 95% was considered statistically significant. GraphPad prism was used to analyze and plot data.

3.3.9 Ecto-NTPDase activity remains unchangeable in *L. infantum* null mutants

To access the ecto-NTPDases activity, we evaluate the ecto-nucleotidases activity on live *L. infantum* promastigotes clones using ATP, UDP and AMP (Fig. 9). In fact, activity on live parasite were previously done and all nucleotides were also used as substrate indicating a broad range of ectonucleotidase activity in *L. infantum* (MAIA et al., 2013; VASCONCELLOS et al., 2014). Our results show no differences were observed on LiNTPDass overexpressing and null mutants compared to wild type. As long as LiNTPDase1 have been described to be secreted (SANSOM et al., 2014) no change on its activity is expected on life parasite. On the other hand, LiNTPDase2 have been reported on parasite surface as well as in intracellular compartments (SANSOM et al., 2014; VASCONCELLOS et al., 2014) but surprisingly here we have no change on ectonucleotidase activity using our mutants. This data may indicate a compensation on the expression as previously showed by RNA upregulation on other isoform that was not deleted as well as a compensation on expression of others non-blocked nucleotidases. However, LiNTPDase1 overexpressing showed no change on its activity while LiNTPDase2 overexpressing clone showed an increasing on its activity using ATP and UDP as substrate (Fig. 9)

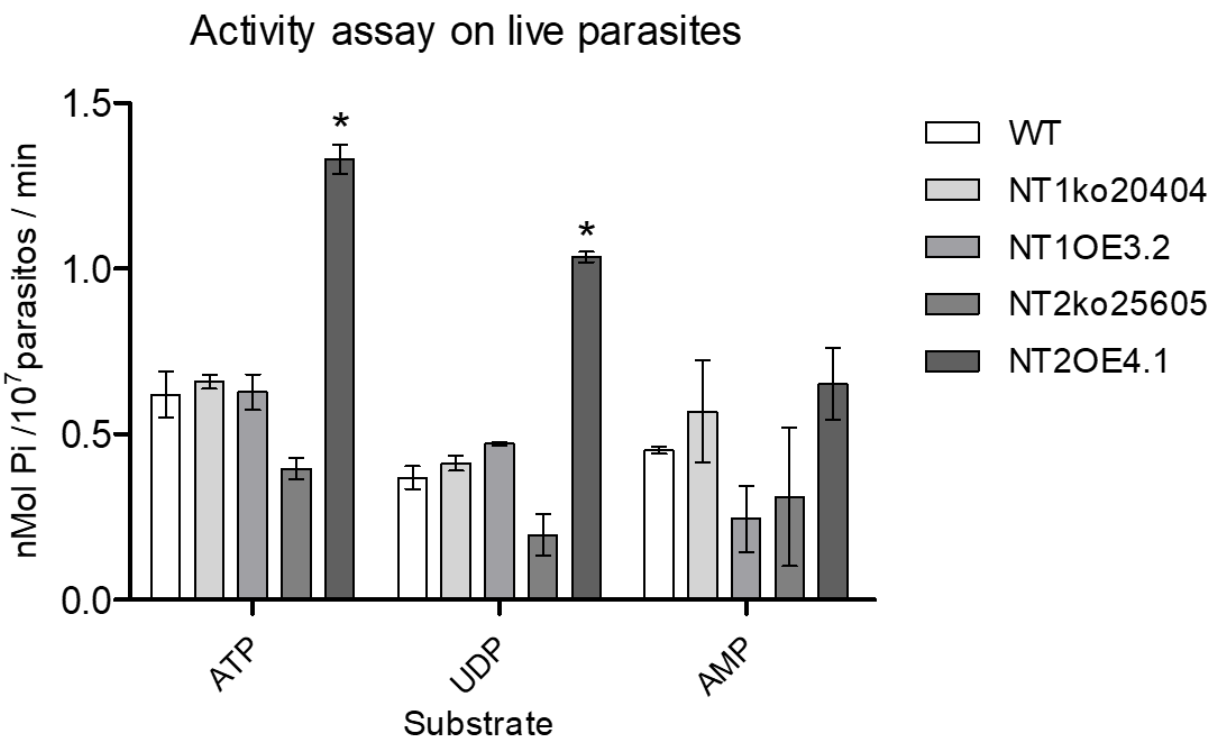


Figure 9. Nucleotidase acitivity assay on Live *L. infantum* wild type and LiNTPDases mutants. Activity assay was done measuring inorganic phosphate using Malachite Green method. A. Promastigotes live parasites was used to measure ecto-nucleotidase activity using ATP, ADP and AMP as substrate.

3.4 CONCLUSIONS

L. infantum genome encodes two single copy genes of NTPDases (LiNTPDase1 and 2) which they have been demonstrated to take place in parasite infectivity. They share high sequence similarity on ACR between trypanosomatids indicating a close related function. In fact, small differences on ACR amino acids sequences could indicate divergent function or even differential preference for substrate. Further investigation concerning punctual differences on ACR sequences like SNP or point mutation studies should be done.

Genetic approaches have been widely used to gain and loss of function studies on several organisms. Here, for the first time, we use this technology to engineer JPCM5 *L. infantum* in order get overexpressing and null LiNTPDase1 and LiNTPDase2 mutants by Homologous recombination and CRISPR/Cas9 respectively. We used the mutants to do their functional characterization. First, we have successful added extra copies on overexpressing clones with no negative effects on viability and growth of the parasite. We could also localize eGFP tagged LiNTPDase2 which revealed nuclear and kinetoplast localization. To see surface and internal specific localization deep investigations need to be done.

In addition, we could also efficiently knockout the gene LiNTPDase1 and LiNTPDase2 and the *L. infantum* KOs showed loss of viability and amastigote like form if not supplemented with extra adenine and guanine, reinforcing their role on parasite *purine salvage pathway* and survival. In fact, double knockout LiNTPDase1 and LiNTPDase2 on *L. infantum* showed to be lethal for the parasite indicating that at least one NTPDase isoform is necessary for them thrive.

Interestingly, mRNA of one isoform was differentially upregulated when the other NTPDase was absent, indicating a possible compensation mechanism. We also showed for the first time that LiNTPDase 1 is also important to infection of *L. infantum* once LiNTPDase1ko notably decreased the infection rate within macrophages as well as amastigote replication. Moreover, LiNTPDase2 overexpressing showed increased adhesion and infectivity rates, while LiNTPDase2ko decreased its adhesion and infection as well as intracellular amastigote reproduction. These data indicate that both LiNTPDase1 and LiNTPDase2 must be considered on drug target to rational design of therapy to control the disease.

Finally, NO production was changed when the infection was done with the LiNTPDases null mutants leading to increased levels when compared to wild type. This data indicates that LiNTPDases play role on inhibition of NO production in vitro infection. This data evidences main function of LiNTPDases in the subversion of host immune system during the infection. Concerning the ecto-nucleotidase activity our data showed higher levels of activity on the overexpressed mutant of LiNTPDase2, corroborating its expression on the cell surface of the parasite.

Taken together our data support the conclusion that both isoforms LiNTPDase1 and LiNTPDase2 are important to *L. infantum* development, growth, differentiation, adhesion, infection and subversion of host immune cell response.

Conflict of Interest

The authors declare no conflict of interest.

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3.6 SUPPLEMENTARY MATERIAL

Supplementary Table ST1. Sequences of protein used to align and design the phylogenetic tree on ClustalW.

Species	Protein	NCBI Identifier
H sapiens	Human CD39	NP_001767
L braziliensis	LbNTPDase1	XP_001562788
	LbNTPDase2	XP_001562178
L donovani	LdNTPDase1	CBZ32136.1
	LdNTPDase2	CBZ32820.1
L infantum	LiNTPDase1	XP_001463665
	LiNTPDase2	XP_001464341
L major	LmNTPDase1	XP_001681345.1
	LmNTPDase2	XP_001681917.1
L mexicana	LmxNTPDase1	CBZ24328
	LmxNTPDase2	CBZ25018.1
T brucei	TbNTPDase1	XP_845817.1
	TbNTPDase2	XP_847211.1
T cruzi	TcNTPDase	AAS75599

Supplementary Table ST2. Oligonucleotides sequences

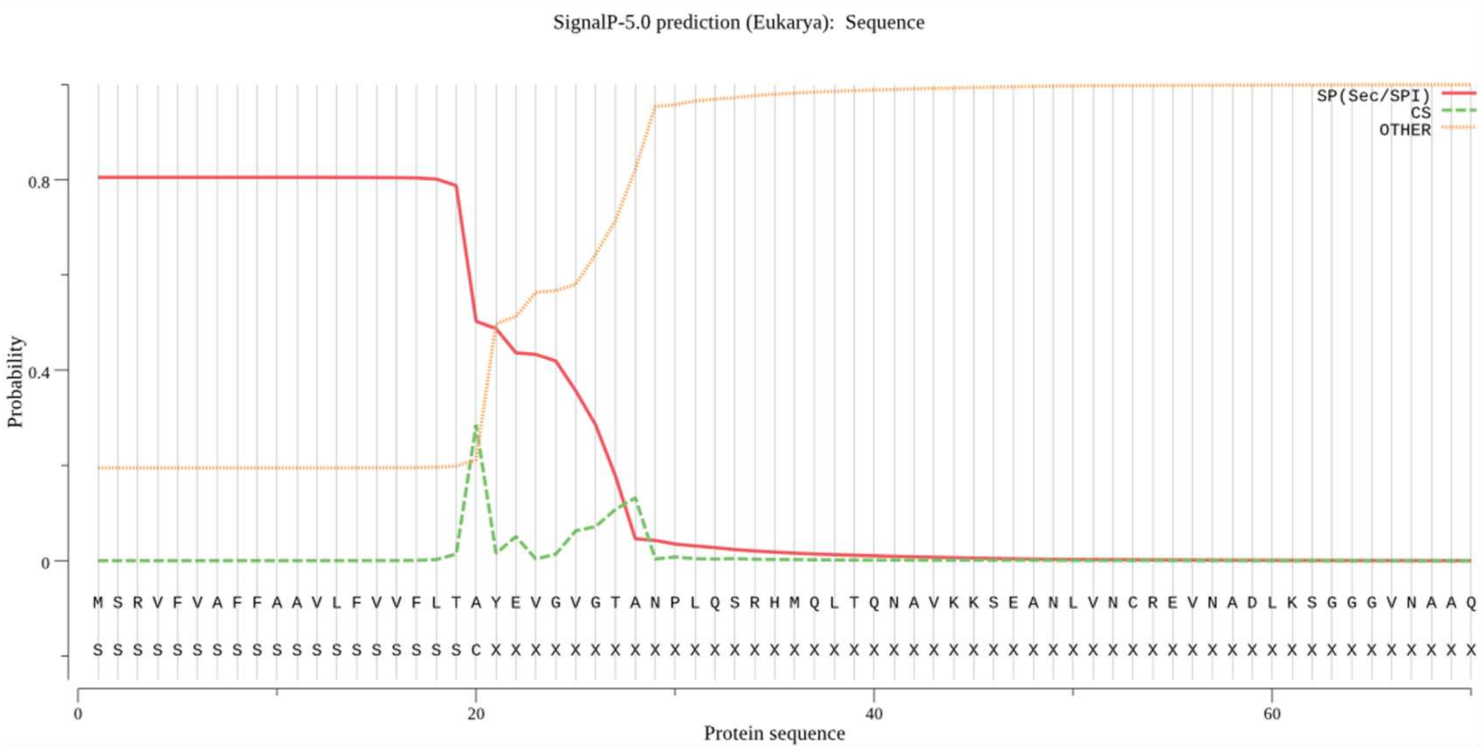
	Oligonucleotide	Sequence 5'>3'
Overexpressing clones	eGFP F XbaI	TCTAGAATGGTGAGCAAGGGCGAGGAG
	eGFP R MspcI	CTTAAGCTTGTACAGCTCGTCCATGC
	NT1 F BglII	AGATCTATGAGTCGCGTCTTTGTTGC
	NT1 R XbaI	TCTAGAGGTAAGAGAGAGGAGTGAGG
	NT1 R MspCI	CTTAAGGGTAAGAGAGAGGAGTGAGG
	NT2 F BgLII	AGATCTATGCGACCGTACTCCTCG
	NT2 R XbaI	TCTAGATTCCATCTTGAGCAGGGAGG
	NT2 R MspCI	CTTAAGTTCCATCTTGAGCAGGGAGG
NT1 null clones	NT1 F Cas9	CTTCGCCCCCGAAACAAAGCCTTTTGCATAcccgggtaccgagctctctctt
	NT1 Neo R Cas9	GCCCTCTCCTCTTCCCACGCCCAAATGCCGtcagaagaactcgtcaagaagg
	NT1 Puro R Cas9	GCCCTCTCCTCTTCCCACGCCCAAATGCCGtcaggcaccgggcttgcgggt
	gRNA NT1	GCTGCCGATGTCGAACACGACGG
	5UTR Probe F	ACGAGACAGACACACAGAGA
	5UTR Probe R	AGCAGACAGACACACAGAGA
NT2 null clones	NT2 F Cas9	GCCTCCTCATTCCTTTCTCCACGTTTGACGcccgggtaccgagctctctctt
	NT2 Neo R Cas9	GTGTAAAGGGCAAGATGTCCGTCAACACTTtcagaagaactcgtcaagaagg
	NT2 Puro R Cas9	GTGTAAAGGGCAAGATGTCCGTCAACACTTtcaggcaccgggcttgcgggt
	gRNA NT2	AGGTAGGAGCCGAAGGCCGGCGG
	3UTR Probe F	ACGAAACGGGCCGCCCTCT
	3UTR Probe R	AAGAAGGCCTGCATCTGAGA
Add back	NT1 F XbaI	GCTCTAGAATGAGTCGCGTCTTTGTTGC
	NT1 R HindIII	CCCAAGCTTGGTAAGAGAGAGGAGTGA
	NT1 HA R HindIII	CCCAAGCTTGGTAGCGTAGTCTGGCACGTCGTAAGGGTAAAGAGAGAGGAGTGA
	NT2 F XbaI	GCTCTAGAATGCGACCGTACTCCTCGGT
	NT2 R NdeI	GGAATTCCATATGTCATTCCATCTTGAGCAGCA
	NT2 HA R NdeI	GGAATTCCATATGTCAAGCGTAGTCTG
RT-qPCR	NT1 F	CGGAGTACGAGTGCATGTATTA
	NT1 R	AGGTAAGAGAGAGGAGTGAGG
	NT2 F	CTGCAAGTTTGATGCCTGTG
	NT2 R	AGTGGTAGAGTCGGTCGTAAA
	Neo F	GGTGCCCTGAATGAACTG
	Neo R	CACTTCGCCCAATAGCAG
	Puro F	GACATCGGCAAGGTGTG
	Puro R	CAGGAGGCCTTCCATCT
	GAPDH F	GGAGATAGACAAGGCCATCAAG
	GAPDH R	TGTCGTTGATGAAGTCAGAGC
	G6PD F	GTCCGAGGCTATGTATGTCAAG
	G6PD R	CATCGTACCGAGTGTGGTATG

Supplementary Table ST3. Predicted protein interaction partner to LiNTPDase1 and 2.

	NCBI code	Gene name
LiNTPDase1	XP_003392832.1	*Inosine triphosphate pyrophosphatase;*
	XP_001464138.1	*Adenylosuccinate synthetase*
	XP_001464543.1	*Orotidine-5-phosphate decarboxylase/orotate phosphoribosyltransferase*
	XP_001470128.1	*Ribonucleoside-diphosphate reductase*
	XP_003392492.1	*Putative GMP synthase*
	XP_001467573.1	*Uridine kinase-like protein*
	XP_001468477.1	*Putative uracil phosphoribosyltransferase*
	XP_001466408.1	*Putative ribonucleoside-diphosphate reductase small chain*
	XP_001467945.1	*Putative AMP deaminase*
LiNTPDase2	XP_001470024.1	*Putative UDP-N-acetylglucosamine-dolichyl-phosphate N-acetylglucosaminephosphotransferase*
	XP_001466235.1	Putative acylphosphatase; Belongs to the acylphosphatase family
	LPG2	*Lipophosphoglycan biosynthetic protein (lpg2)*
	XP_003392546.1	*Putative ethanolamine phosphotransferase*

Supplementary Figure S1. Signal P prediction to LiNTPDase1 and LiNTPDase2 protein sequences (ST1) using Signal P prediction browser

Leishmania infantum NTPDase1

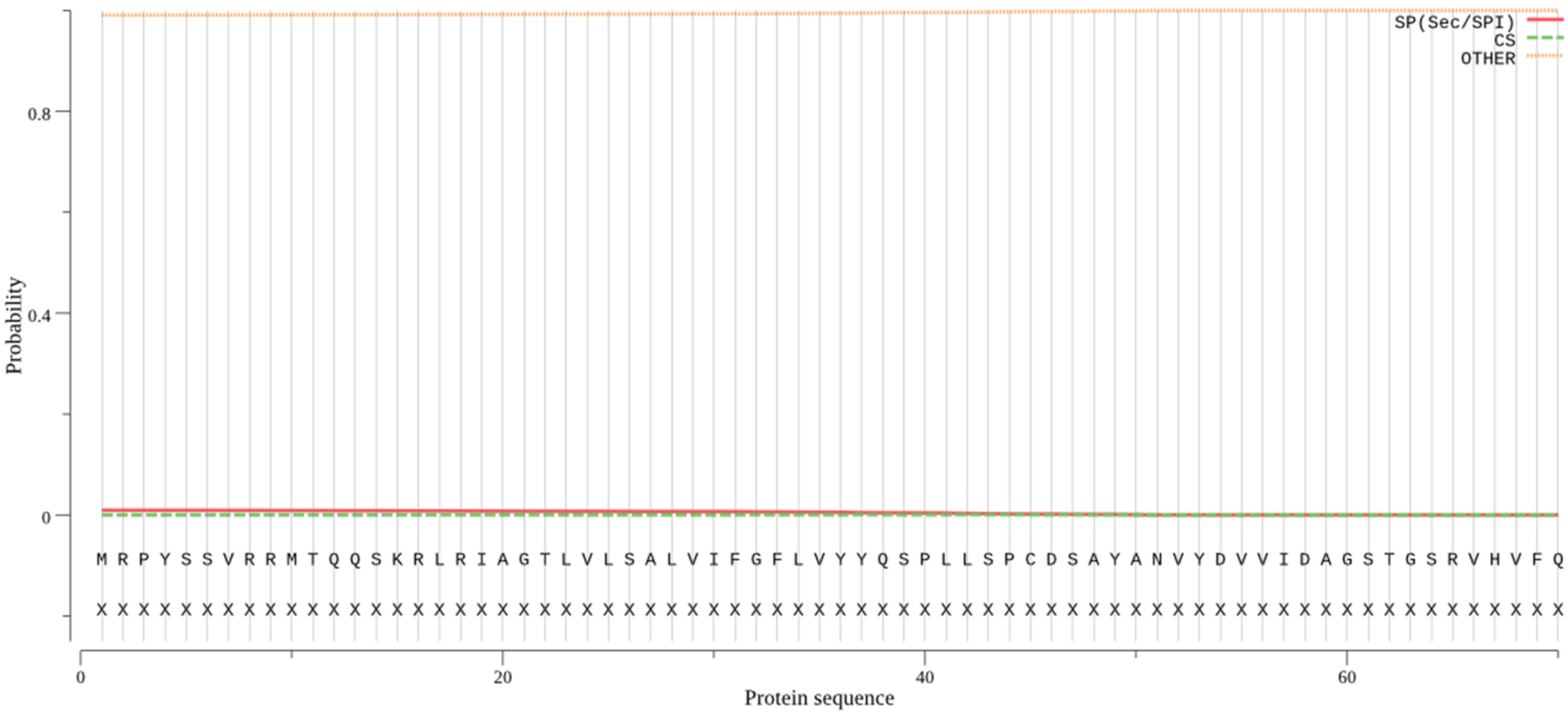


Protein type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.8053	0.1947

Prediction: Signal peptide (Sec/SPI)
Cleavage site between pos. 20 and 21: LTA-YE. Probability: 0.2852

Leishmania infantum NTPDase2

SignalP-5.0 prediction (Eukarya): Sequence



Protein type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.0092	0.9908

Supplementary Figure S2. Trypanosomatids protein sequences alignment using CLUSTALW. Sequences are described on ST1.

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50         60         70         80         90        100
CD39             ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -ME-------
LiNTPDase1       ---------- -----M-SRV FVAFFAAVLF VVFLTAYEVG VGTANPLQSR HMQLTQNAVK KSEANLVNCR EVNADLKSGG GVNAAQAIAE MRRQREELMN
LdNTPDase1       ---------- -----M-SRV FVAFFAAVLF VVFLTAYDVG VGTANPLQSR HMQLTQNAVK KSEANLVNCR EVNADLKSGG GVNAAQAIAE MRRQREELMN
LbNTPDase1       MAVVGGFTAG KPLRGM-GRI VLGLFAVMLV AFVITAYQVG VSTANPRQSR HIQLAQNAVA KSEAMLTGCR EANANLKNSG SVKGAQAIAE MARQKAELTS
LmxNTPDase1      ---------- -----M-GRV VVAFFAAVLV VVFLTSYEVG VGTANPLQSR HMQLTENAVM KSESNLASCR EVNGDLKSGG DVNAAQAVAE MKRQREELVS
LmNTPDase1       ---------- -----M-SRV VLAFFAAVLF VVFLTAYEVG VGTANPLQSR HMQLTQNAVK KSEENLVNCR EVNTDLRSGG DVNVARAMAE MRRQREELMN
TbNTPDase1       MLHSDHPKQS RFLTPF--VI FPLFTLLTLV LLYAAGYFIG LSRYHSLTRD EHA------L DADGRDSRCV SQQEMLRNTH KVA-AGTLKR LKEDRDELKK
TcNTPDase1       -MKQSMARSR RLKRPCISLT FALLCLFLVT VVYITAYTVG KFSMDARGRE KLDLTDRIIH SMTKRFQECQ AQKQLLKNGH TTA-RMALEI AQETKKKLEM
LiNTPDase2       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -MRPYSSVRR MTQ-------
LdNTPDase2       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -MRPYSSVRR MTQ-------
LbNTPDase2       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -MRPYSSVRR MTQ-------
LmxNTPDase2      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -MRPYSSVRR MTQ-------
LmNTPDase2       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -MRPYSSVRR MTQ-------
TbNTPDase2       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----MVTLR MAA-------
Clustal Cons

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         110        120        130        140        150        160        170        180        190        200
CD39             ---------- ---------- ---------- ---------- DTKESNVKTF CSKNILAIL- -------G-- -FSSIIAVIA LLAVGLTQ-- -NKAL-----
LiNTPDase1       IVALERERVV SARSLLQVCE DELASDLSVL FGVADHNFTA RIRSLEEKRK HLEGLHSMLN TTPFGAVELR RSSEIRALQA ALFHEMRASK KKAENGVANG
LdNTPDase1       IVALERERVV SARSLLQVCE DELASDLSVL FGVADHNFTA RIRSLEEKRK HLEGLHSMLN TTPFGAVELR RSSEIRALQA ALLHEMRASK KKAENGVANG
LbNTPDase1       TVALERERVV SARSLLQACE DGLASEHRTL FGTAHRNTTA HLLWLKQRRA HLKSEHEKLT EGPLGVVEPR RSSGIRALQA ALLQEIHPST GLAGNGVENR
LmxNTPDase1      IVALERERVA SARSLLQVCE DALARDLSVL FGVADHNITA RLRSLEEKRT HLESVISMLN TGPFGAVELR RSSDIRALQA ALFHEMRASK KKAEN----V
LmNTPDase1       IVALERERVV SARSLLQVCE DELASDLSVL FGVADHNFTA RLRSLEKKRK HLEGLHSMLN TDPFGAVQLR SSSEIRALQA ALFHEMRASK KKAENGVASG
TbNTPDase1       KLKLLEEGKG RTGEPAASCG GKD-ATSN-- NGEG------ ---------P SVNDGGGNM- -TGSAAVSLQ RAA---NLID SLTHQTESWL KKAADTTPSL
TcNTPDase1       ELGLMRNRNT LMLQSVEECK KELELLKE-- NGRGAVTLAL QLDRLTKERQ FYMDAIAVV- -NGSSDVGRR QTA---DVFA AIAKEFDEWE DAAQNKTELL
LiNTPDase2       ---------- ---------- ---------- ---------- QSKRLRIAGT LVLSALVIF- -------G-- -F-------- ----LVYY-- ---QSP--LL
LdNTPDase2       ---------- ---------- ---------- ---------- QSKRLRIAGT LVLSALVIF- -------G-- -F-------- ----LVYY-- ---QSP--LL
LbNTPDase2       ---------- ---------- ---------- ---------- QSNRLRVIGV LVLSAFVLF- -------G-- -F-------- ----VIYS-- ---ESP--WF
LmxNTPDase2      ---------- ---------- ---------- ---------- QSKRLRVAGT LVLSVLVLF- -------G-- -F-------- ----IVYH-- ---KSP--LF
LmNTPDase2       ---------- ---------- ---------- ---------- QSKRLRVAST LVLSALVIF- -------G-- -F-------- ----LVYH-- ---QSP--LF
TbNTPDase2       ---------- ---------- ---------- ---------- RRRGLHAKRN WHVSVFLFL- -------G-- -LTFLVALFA LLSLITGKGS DNSDLPKSTT
Clustal Cons                                                                 .    .

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         210        220        230        240        250        260        270        280        290        300
CD39             -----PENVK YGIVLDAGSS HTSLYIYKWP AEKENDTG-- ---------- ---VVHQVEE CRVKGPGISK FVQKVNE-IG IYLTDCMERA REVIPRSQHQ
LiNTPDase1       EACTK-TSDK YSVVFDIGST GNRVHVYKYR VAPATHTA-- ----AAAGSE LSDIDLVEEL FELNHKALSE LDNPVQD-AP EALWELFMKA KYFVPAELHA
LdNTPDase1       EACTK-TSDK YSVVFDIGST GNRVHVYKYR VAPATHTA-- ----AAAGSE LSDIDLVEEL FELNHKALSE LDNPVQD-AP EALWELFMKA KYFVPAELHA
LbNTPDase1       KACVD-AVAK YSVVFDIGST GNRVHVYKYR VNPLTQIS-- ----ASAPDE LSKIDLVGEL FELNYKALSE LNNPVQD-AP EALWELFAKA KNFVPAELHA
LmxNTPDase1      EPCTK-SSDK YSAVFDIGST GNRVHVYKYR VAPAAHTA-- ----AAAGSE LSDMDLAEEF FELNHIALSE LDNPVRD-AP EALWELFVKA KDFVPADLHA
LmNTPDase1       AACAK-SSVK YSVVFDIGST GNRVHVYKYR VAPATRTA-- ----AAAGSE LSDIDLVEEL FELNHKALSE LENSVQD-AP EALWELFVKA KDFVPAELHA
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TbNTPDase1      AACNK-SPRR YSVVFDAGST GSRVHVYRYN LTSNPYDNID SAPNGAHASL LPYLKLEDEL FIENHEPLSG FSDPR-G-AA ASLVPLLDAA KARVPTDMHA
TcNTPDase1      TNCNN-AMRR YSIVFDAGST GSRVHVFRYN LTSTPHAGN- FSWNGTRPSL TSFLRLDDEL FVENYEPLSG LANPN-D-AV ASLSPLIEAA KAYIPESLHA
LiNTPDase2      SPCDSAYANV YDVVIDAGST GSRVHVFQYE RG---RTG-- ---------- ---LVLLRER FKRVEPGLSS FATDPDG-AK ESLAGLLRFA DKVVPQSYQK
LdNTPDase2      SPCDSAYANV YDVVIDAGST GSRVHVFQYE RG---RTG-- ---------- ---LVLLRER FKRVEPGLSS FATDPDG-AK ESLAGLLRFA DKVVPQSYQK
LbNTPDase2      SPCNSPYSNV YDIVIDAGST GSRVHVFQYE RS---STG-- ---------- ---VILLRER FKRIEPGLSS FATDQEG-AK QSLAGLLRFA EKAVPRSYQR
LmxNTPDase2     SPCDSAYANV YDIVIDAGST GSRVHVFQYE RS---RTG-- ---------- ---VVLLKER FKRIEPGLSS FTTDPDG-AK ESLTGLLRFA DEVVPESYQR
LmNTPDase2      SPCDSAYANV YDVVIDAGST GSRVHVFQYE RG---RTG-- ---------- ---FVLLRER FKRAEPDLSS FATDLDG-AK ASLEGLLRFA DTVVPQSYQK
TbNTPDase2      GLCDT--PLM YDILLDAGST GTRVHIFEYN KW---R---- ---------- -----LVGEV YNSTHPGLSQ LASDDSANIS ANLQPLMATA VRAIPEVYHP
Clustal Cons               *. ::* **:  . :::::.:                              *         :*  :            *   :  *    :*   :

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                      310        320        330        340        350        360        370        380        390        400
CD39            ETPVYLGATA GMRLLRMESE ELADRVLDVV ERSLSNYPFD FQGA----RI ITGQEEGAYG WITINYLLGK FSQKTRWFSI VPYETNNQET FGALDLGGAS
LiNTPDase1      CTAVEFKATA GLRMLGMEKA ---TEILDGI RARYRNETFW LRGN-APVRI LDACEEGPMA WLTVNYLLGV FSRGTKA--- ------TAST VAVIDLGGGS
LdNTPDase1      CTAVEFKATA GLRMLGMEKA ---TEILDGI RARYRNETFW LRGN-APVRI LDACEEGPMA WLTVNYLLGV FSRGTKA--- ------TAST VAVIDLGGGS
LbNTPDase1      CTPIEFKATA GLRMLGLEKA ---TEILAEI RARYRKETFW LRGS-APVRI LDSHEEGLMA WLTVNFLLGT FARNTEA--- ------TAST AAIIDIGGGS
LmxNTPDase1     CTAVEFKATA GLRMLGMEKA ---TEILAGI RERYRNEAFW LRGN-APVRI LDPREEGPMA WLTVNYLLGA FSRGAMA--- ------AAST VAIIDLGGGS
LmNTPDase1      CTAAEFKATA GLRMLGMEKA ---NEILAGI RARYRNETFW LRGN-ASVRI LDACEEGPMA WLTVNYLLGA FSRGATA--- ------TDST VAVIDLGGGS
TbNTPDase1      CVGIELKATA GLRRIGAEQA ---GRVLDAV RREFQKGPFW VHSELGSVRI LEGREEGPLA WLTVNFLIGS LSGT------ -------KET ATILDLGGGS
TcNTPDase1      CVPIEMKATA GLRRIGREHA ---EAVLDVV RRLFARGPFW MQSELDSVRI LEGWEEGPLA WLTVNYLLGA LNGD------ -------KNT ATILDLGGGS
LiNTPDase2      CTFVTLKATA GLRLLPESVQ ---QVLLDAA QHTLNASPFQ SRGA----SI ISGAQEGVYG WLTVNYLLNR LDTDV----- --------AT VATIDMGGAS
LdNTPDase2      CTFVTLKATA GLRLLPESVQ ---QVLLDAA QHTLNASPFQ SRGA----SI ISGAQEGVYG WLTVNYLLNR LDTDV----- --------AT VATIDMGGAS
LbNTPDase2      CTSVTLKATA GLRLLPEADQ ---QVLLDAA QQTLKAFPFQ SRGA----SI VSGAQEGVYG WLTVNYLLNR LDKEG----- --------AT VATIDMGGAS
LmxNTPDase2     CTSVTLKATA GLRLLPESDQ ---QVLLDAA QQALNASPFQ SRGA----SI ISGAQEGVYG WLTVNYLLDR LDTDV----- --------AT IATIDMGGAS
LmNTPDase2      CTSVTLKATA GLRLLPESAQ ---QALLDVA QHTLNASPFQ SRGA----SI ISGAQEGVYG WLTVNYLLDR LDTDV----- --------AT VATIDMGGAS
TbNTPDase2      CTKVTLKATA GLRLLPPEKV ---EFILRET KKVLRRYPFV VGSV----SV MDGDEEGMDA WLTVAYLLGR FDRSAEGT-- ---PNSTAKR IATIDMGGAS
Clustal Cons     .   : *** *:* :           :*    .      *    .      : :   :**  . *:*: :*:.  :                      :*:**.*

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                      410        420        430        440        450        460        470        480        490        500
CD39            TQVTFVPQNQ TIESP--DNA LQFRLYGKDY NVYTHSFLCY GKDQALWQKL AKD------- ---------- ---------- ---------- ----------
LiNTPDase1      TQIVFEPGES AFHGMRTDLR YSATLGSRSV SAYQHSYEGY GLHAATKELL FHIQGKSQEK PGGGTTTSTA TTTTTTTPAN SGDKALSVWN VLGNLGADGS
LdNTPDase1      TQIVFEPGES AFHGMRTDLR YSATLGSRSV SAYQHSYEGY GLHAATKELL FHIQGKSQEK PGGGTTTSTA TTTTTTTPAN SGDKALPVWN VLGNLGADGS
LbNTPDase1      TQIVFEPGED TFYKMRADVR GSATLGGRSV KVYQHSYEGY GLHAATKALL FHIQGKRQER PGGGSATRAT TT----ARTS GDDSTPSVGN GVRNVSANDS
LmxNTPDase1     TQIVFEPDER AFDRMHTDFR YSATLGSRSV RAYQHSYEGY GLHAATKALL FHVQGKSQEK PGGGTTTSTA TTTTTTAPAN GDDKATSVWD GQGNLDADGS
LmNTPDase1      TQIVFEPGES TFHGMRTDFR YAATLGSRSV RAYQHSYEGY GLHAATKKLL YHIQGKSQEK PGGGTATNTA MTTTTTAPAN GGDEVLPVWK ALRNLGADGR
TbNTPDase1      TQVVMRPNDP EVLEGHEEFT DTFAINGKSI KLYQHSYEGN GLNAAREQLL ASVATSVAT- ---------- ---------- ---------- ----------
TcNTPDase1      TQIVMHPSDP KAIEAYAEFS YALKVNGRSF VVYQHSYEGN GLHAAKEQLL QAVAANNTGA NS-------- ---------- ---------- ----------
LiNTPDase2      TQVVFETTPT SGEWLPFNYA HQLRTPKRTI TMYQHSYLGL GMNEAKKKLM M--------- ---------- ---------- ---------- ----------
LdNTPDase2      TQVVFETTPT SGEWLPFNYA HQLRTPKRTI TMYQHSYLGL GMNEAKKKLM M--------- ---------- ---------- ---------- ----------
LbNTPDase2      TQVVFETKFT SGEWLPFNYA HQLRTPKRTI AMYQHSYLGL GLNEAKKTLM T--------- ---------- ---------- ---------- ----------
LmxNTPDase2     TQVVFETTPT SGEWLPFNYA HQLRIPKRTI SVYQHSYLGL GMNEAKKKLM T--------- ---------- ---------- ---------- ----------
LmNTPDase2      TQVVFETTPT SGEWLPFNYA YQLRTPKRTI AMYQHSYLGL GMNEAKKRLM T--------- ---------- ---------- ---------- ----------
TbNTPDase2      TQLVLQMSPG YTGETQ--FI TSLHFGVTEI PLYTYSFLGL GLRQATLEVL R--------- ---------- ---------- ---------- ----------
Clustal Cons    **:.:                            * :*:    *   *    :
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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         510        520        530        540        550        560        570        580        590        600
CD39             ---------- ---------- --IQVASNEI LRDPCFHPGY KKVVNVSDLY KTPCTKRFEM TLPFQQFEIQ GIGNYQQCHQ SILEL-FNT- -SYCPYSQCA
LiNTPDase1       SERD--DIVT KRAPPMPP-- -PPLPDAEAV EAFPCFAVGY EDPL-GVKNI KKNN------ -----TGEPA MPPNFQACAN LFRDRLLKPV GLTCEAANC-
LdNTPDase1       SERD--GIVT KRAPPMPP-- -PPLPDAEAV EAFPCFAVGY EDPL-GVKNI KKNN------ -----AGEPA MPPNFQACAN LFRDRLLKPV GLTCEAANC-
LbNTPDase1       KDEEDENMIT DTAPPK--TL PWLQLDTEAV DAFPCFAVGY EDQL-GVRNT KRND------ -----AGEPA RHPDFQACAN LFRDRLLKPV GLTCEEVNC-
LmxNTPDase1      GERD--DIVT KRAPPMLPPL PPPPPDAEAV EAFPCFAVGY EDPL-GVKNV KRNN------ -----VGKPV MPTNFQACAN LFRDRLLKTV GLTCEAANC-
LmNTPDase1       SERG--DIVT KRAPPMPP-- -P---DAEAM EAFPCFAVGY EDPL-GVKNV KRNN------ -----AEEPV MPPNFQACAN LFRDRLLKPV GLTCEAVNC-
TbNTPDase1       ---------- G--------G TTTAAKSGPL RAFPCFPQGY AHEKSGVS-- --NG------ -----VGGGG VTPSMEECVA LFRKHVVRS- GGECSSKSC-
TcNTPDase1       KVQEANKIAT GT------EA TPGASADVAV DAFPCFPKGY VHTETGIS-- --NT------ -----RD-GG KVPSMEECSA LFRRHVVRK- HQPCVSNSC-
LiNTPDase2       ---------- ---------- --SFAEANGT SSFPCFPRGY AKRLNDV--- ---------- -----ELRNS DATDFDACAG LFREHVITK- -TTCKFDAC-
LdNTPDase2       ---------- ---------- --SFAEANGT SSFPCFPRGY AKRLNDV--- ---------- -----ELRNS DATDFDACAG LFHEHVITK- -TTCKFDAC-
LbNTPDase2       ---------- ---------- --LFAKVNGT SPFSCFPRRH TEHLNGV--- ---------- -----ELRNG DSTDFDVCVN LFREHVITK- -PICRFDAC-
LmxNTPDase2      ---------- ---------- --LFAEANGT SSFPCFPRGY TKHLNGV--- ---------- -----ELRNS DATDFDACVG LFREHVITT- -ATCRFDAC-
LmNTPDase2       ---------- ---------- --LFAEANGT LSFPCFPRGY TKRVNGV--- ---------- -----ELRNS DATDFNACAG LFREHVITT- -STCKFDAC-
TbNTPDase2       ---------- ---------- --ELTA-VER TSFPCFPSRY RHRIDGEGGV ---------- --SFVENIDG NAQGFGACLR FFHRFINTR- -SSEHLAALR
Clustal Cons                                  **   :  .                                .   *    :

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                         610        620        630        640        650        660        670        680        690        700
CD39             -FNGIFLPPL ----QGDFGA FSAFYFVMKF LNLT------ -----SEKVS QEKVTEMMKK FCAQPWEEI- -----KTSYA GVKEKYLSEY CFSGTYILSL
LiNTPDase1       GIAGVMQPPL TN-FTGEIYV FSFIFDLLAL ANSSLVPAGA AVSREKFEVK LPDLATIAEG HCAAFSLTRI AE---ATAKE GLGSLKPEYE CMYYSYVYAL
LdNTPDase1       GIAGVMQPPL TN-FTGEIYV FSFIFDLLAL ANSSLVPAGA AVSREKFEVK LPDLATIAEG HCAAFSLTRI AE---ATAKE GLGSLKPEYE CMYYSYVYAL
LbNTPDase1       GVAGVFQPPL AN-FTGDIYA FSFLFDLLAM ANNSLAPVGA AVSNDKFEVK LPDLAKIGER HCAAFSLTRI AE---ATAKG GLGSLKPEYE CMYYSYTYAL
LmxNTPDase1      GIAGVMQPPL TN-FTGEIYA FSFIFDLLVL ANKSLVPAGA AVSKEKFEVK LPDLAKIAEG HCSAFSLTRI AE---ATAKE GLGSLKPEYE CMYYSYVYAL
LmNTPDase1       GIAGVMQPPL TN-FTGEIYA FSFIFDLLVL ANSSLVPAGA AVSKEKFEVK LPDLAKIAEG HCAAFSLTRI AE---ATAKG GLGSLKPEYE CMYYSYVYAL
TbNTPDase1       GFNGVFQPDV RTAVSGPVYA FSFYHDLLRP HMT----AG- -----TTVIR LQDIIGIASQ VCKSMKSVEQ LRRENKGKVS VHPSLRPELE CLHLSYVVAL
TcNTPDase1       GFNGVFQPDI SAVPMVPVYA FSFYYDRLKP YIK------- -----DEVIR VQDVLDIASQ VCRSMKSVRE LLEQNDTK-K NKGSLKPEME CFELSYLFTL
LiNTPDase2       GARGVPQPLF PS-RRHLIYA FSYFYDRLYH FSKE------ -----GSPVY VSSYKEVGQE VCHRESARR- -----TT--- ----APEETA CMELAYMYSF
LdNTPDase2       GARGVPQPLF PS-RRHLIYA FSYFYDRLYH FSKE------ -----GSPVY VSSYKEVGQE VCHRESARR- -----TT--- ----APEETA CMELAYMYSF
LbNTPDase2       GARGVPQPPL PS-KQHPIYA FSYFYDRLYH FRSE------ -----GFPVY VSSYKELGRE VCQRESADH- -----TT--- ----TPKETT CMELAYLYSF
LmxNTPDase2      GARGVPQPLF PS-RRHPVYA FSYFYDRLYH FHKE------ -----GRPVY VSLYKEVGKE VCHRESASR- -----AT--- ----TPEETA CMELAYLYSF
LmNTPDase2       GARGVPQPLF PS-RRHPIYA FSYFYNRLYD FLKE------ -----GSQVY VSSYKEVGQE VCHRESARR- -----TT--- ----APEETA CMELAYMYSF
TbNTPDase2       GGRHVLKFAE GA-QSVEIFA FSFF-HRLWR LR-P------ -----ADNVT VQYYKELGEQ VCSAGGKH-- ---------- -----NSGTN CMDVAYLYGF
Clustal Cons         :             . . **     :                    :        :     *                          *:  :*   :

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         710        720        730        740        750        760        770        780        790        800
CD39             LLQGYHFTAD SWEHIHFIGK IQGSDAGWTL GYMLNLTNMI PAEQPLSTPL SHSTYVFLMV LFSLVLFTVA IIGLLIFHKP SYFWKDMV.. ..........
LiNTPDase1       LRYGYEVPED R--VLHVVKK IRGYETAWSL GASLLSLT-- ---------- ---------- ---------- ---------- --------.. ..........
LdNTPDase1       LRYGYEVPED R--VLHVVKK IRGYETAWSL GASLLSLT-- ---------- ---------- ---------- ---------- --------.. ..........
LbNTPDase1       LRYGYEVPEG R--VLHVAKK ISGYETAWPL GASLISVT-- ---------- ---------- ---------- ---------- --------.. ..........
LmxNTPDase1      LRYGYEVPED R--VLHVAKK IRGYETAWSL GASLLSLI-- ---------- ---------- ---------- ---------- --------.. ..........
LmNTPDase1       LRYGYEVPED R--VLHVAKK IRGYETAWSL GASLLSLT-- ---------- ---------- ---------- ---------- --------.. ..........
TbNTPDase1       LRDGFHYPLD Q--ELHIAKK IDGYETAWTL GASLVSLEEH CV-------- ---------- ---------- ---------- --------.. ..........
TcNTPDase1       LRYGFGFPPE Q--KLHIAKK INGFETAWAL GASLVSLEGQ SA-------- ---------- ---------- ---------- --------.. ..........
LiNTPDase2       LTYGLGLSDA T--ALTVPNR IEGMAVSWSL GSSLSFLLKM E--------- ---------- ---------- ---------- --------.. ..........
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LdNTPDase2      LTYGLGLSDA T--ALTVPNR IEGMAVSWSL GSSLSFLLKM E--------- ---------- ---------- ---------- --------.. ..........
LbNTPDase2      LTHGLGLSDD R--TLEVPNR IEGIAVSWSL GCSLSFVLKM E--------- ---------- ---------- ---------- --------.. ..........
LmxNTPDase2     LTNGLGLGDD T--ALTVPNR IAGMSVSWSL GSSLSFLLKM E--------- ---------- ---------- ---------- --------.. ..........
LmNTPDase2      LTYGLGLSDA T--VLTVPNR IEGMAVSWSL GSSLSFLLKM E--------- ---------- ---------- ---------- --------.. ..........
TbNTPDase2      LTSGLGLSDK T--KLNVPYV LSDVKVTWAL GASLLSVEGK VRR------- ---------- ---------- ---------- --------.. ..........
Clustal Cons    *  *           : .    : .  . * * *  *                                                  .......... ..........


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|..
                       810        820        830        840        850
CD39            .......... .......... .......... .......... .......... .......
LiNTPDase1      .......... .......... .......... .......... .......... .......
LdNTPDase1      .......... .......... .......... .......... .......... .......
LbNTPDase1      .......... .......... .......... .......... .......... .......
LmxNTPDase1     .......... .......... .......... .......... .......... .......
LmNTPDase1      .......... .......... .......... .......... .......... .......
TbNTPDase1      .......... .......... .......... .......... .......... .......
TcNTPDase1      .......... .......... .......... .......... .......... .......
LiNTPDase2      .......... .......... .......... .......... .......... .......
LdNTPDase2      .......... .......... .......... .......... .......... .......
LbNTPDase2      .......... .......... .......... .......... .......... .......
LmxNTPDase2     .......... .......... .......... .......... .......... .......
LmNTPDase2      .......... .......... .......... .......... .......... .......
TbNTPDase2      .......... .......... .......... .......... .......... .......
Clustal Cons    .......... .......... .......... .......... .......... .......
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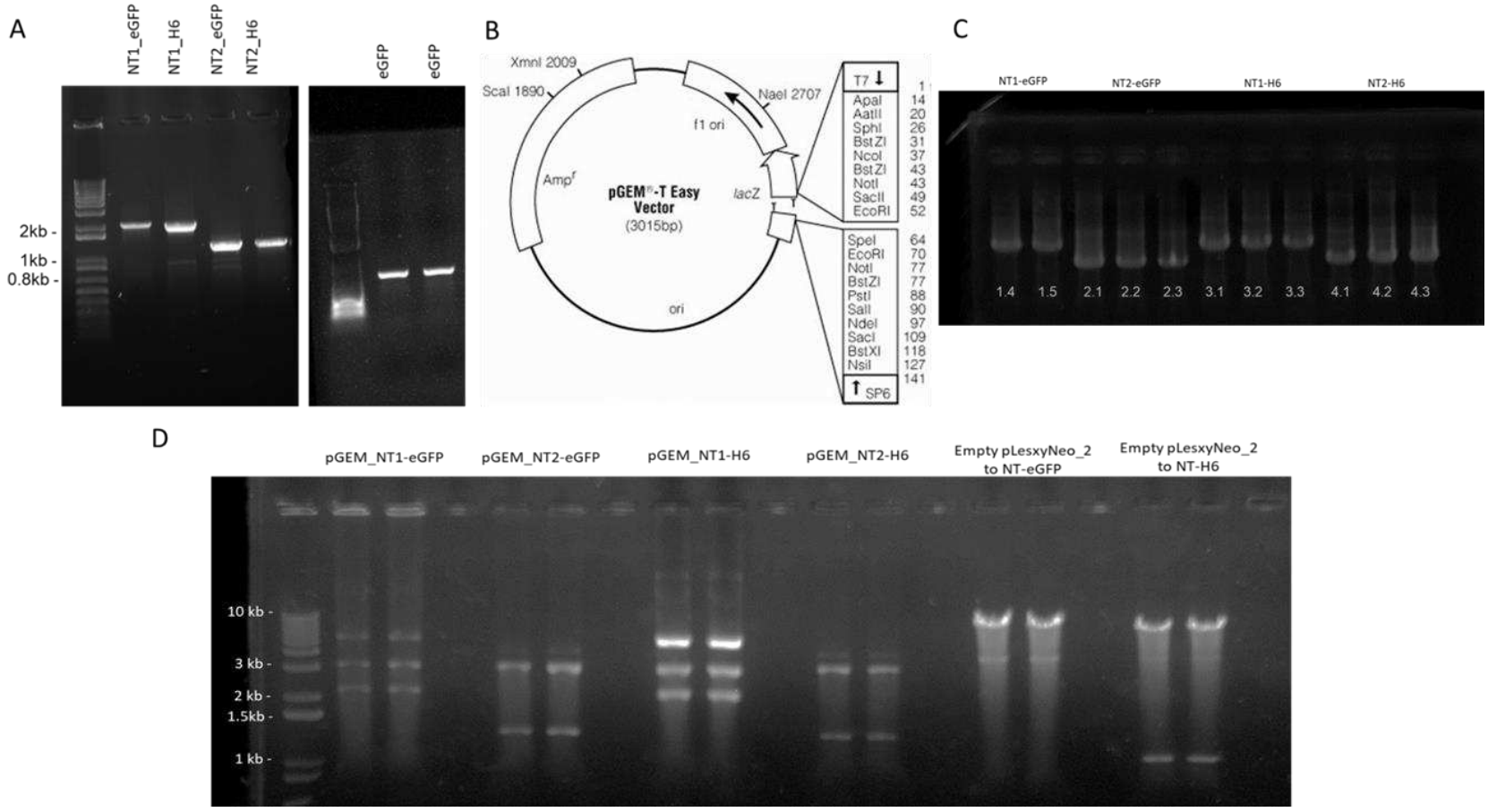
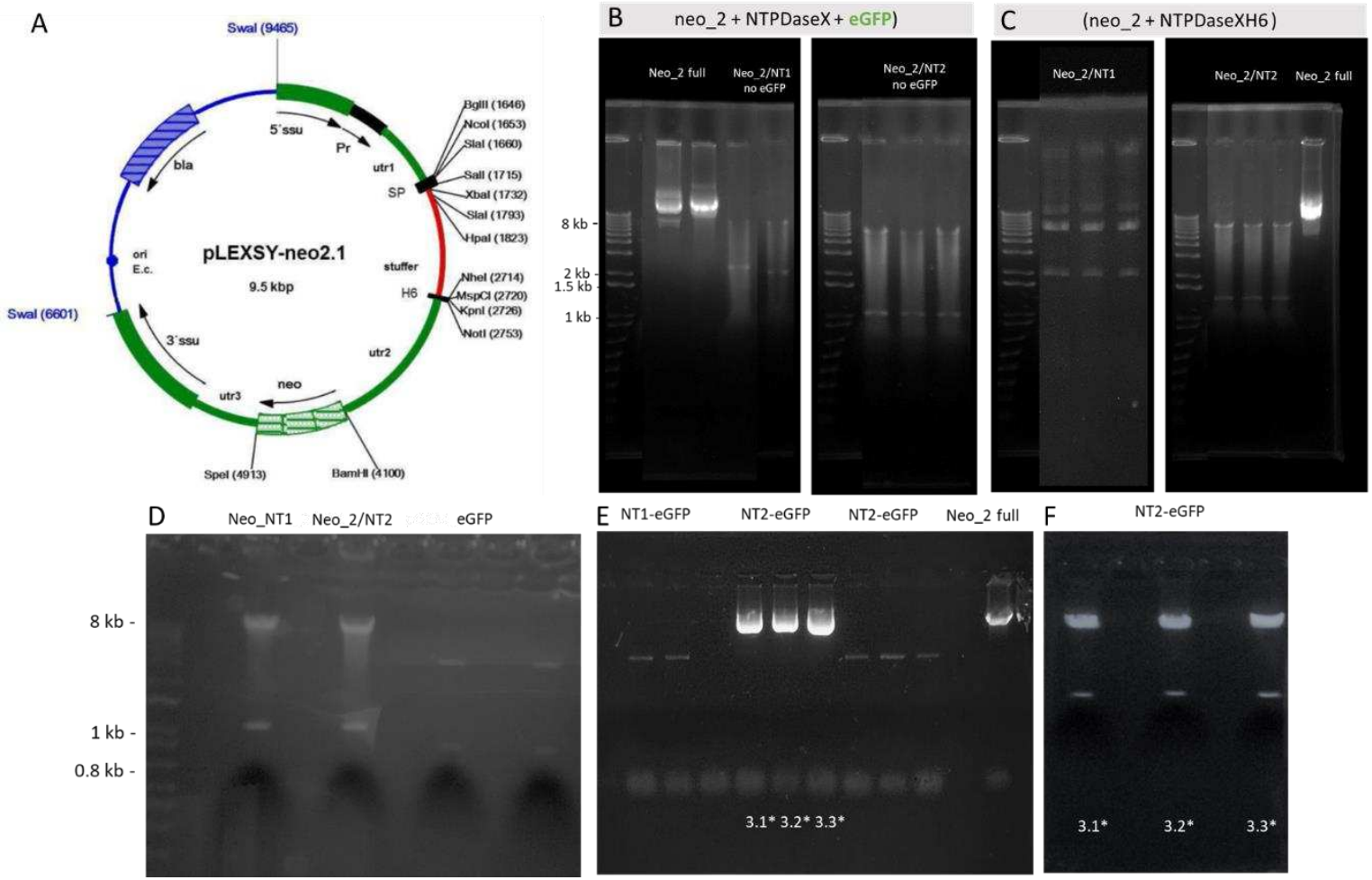
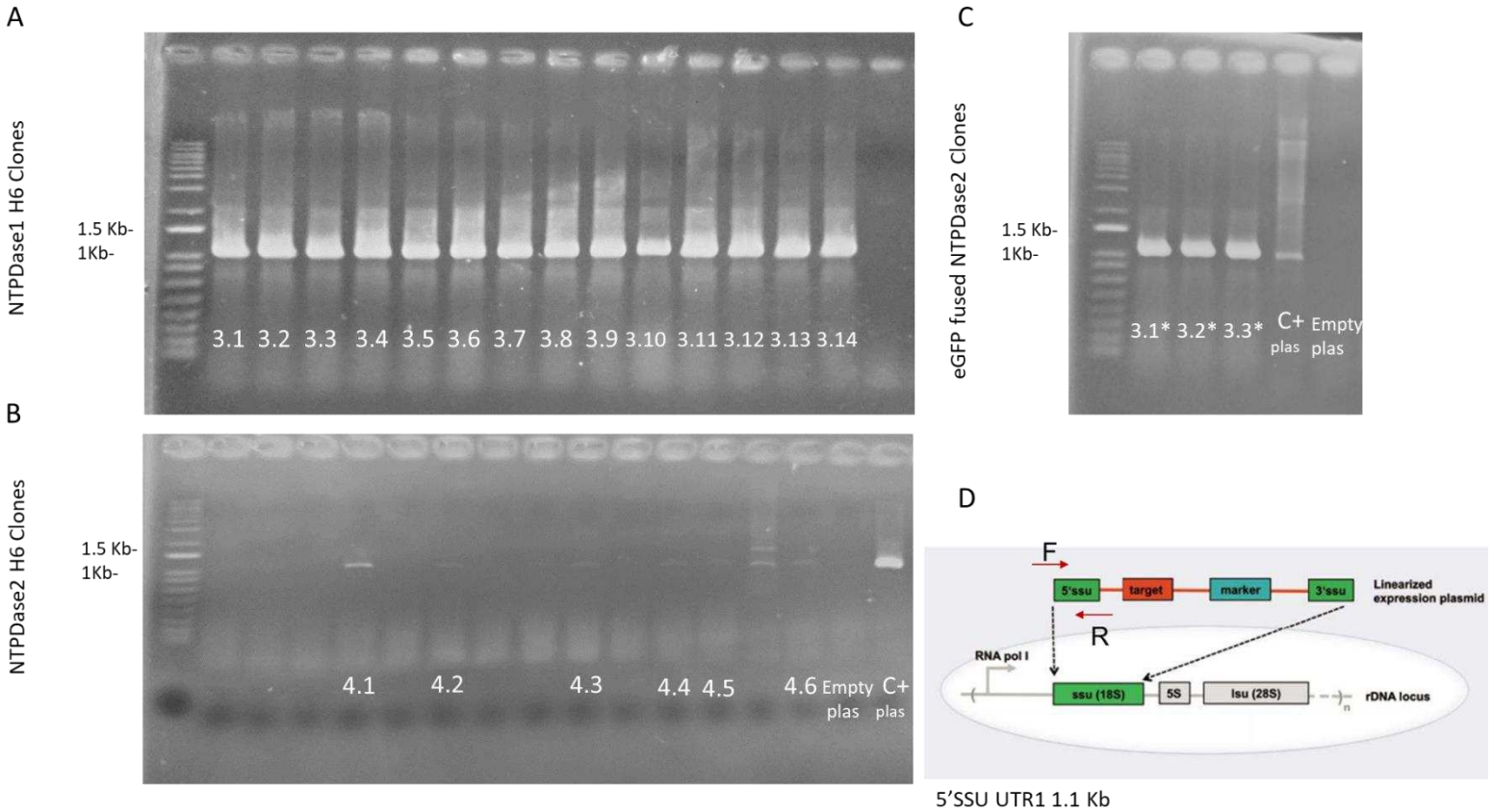
Supplementary Figure S3. Cloning of LiNTPDases in pGEM-T easy system to overexpress and tag. A. PCR amplification of genomic DNA from LiNTPDase1 and 2 for both strategies with eGFP or without eGFP. eGFP was amplified from peGFP vector system. B. Representation of PGEM T-easy vector system where the target sequeces was first cloned. B. PCR confirmation of the cloning into PGEM vector. D. Digestion using appropriate restriction enzimes to confirm the correct insertion of the target genes into PGEM vector and opening pLEXSY vector. See Material and Methods Cloning to Overexpress *L. infantum* NTPDases 1 and 2.



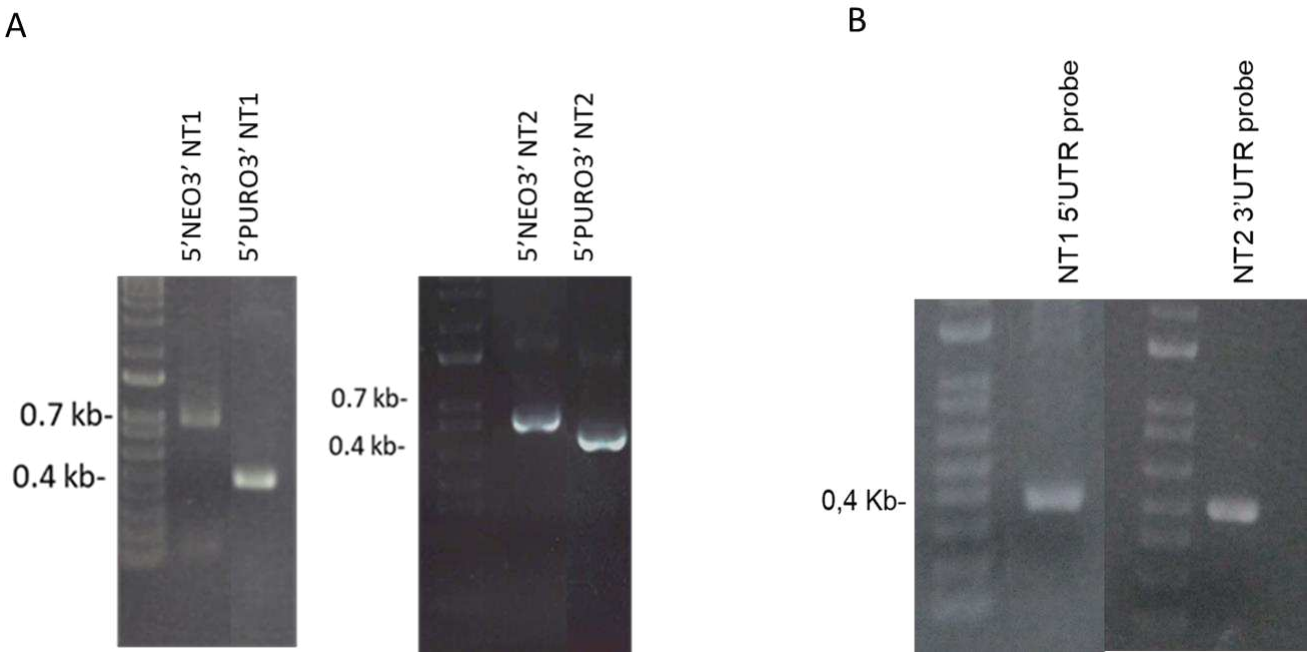
Supplementary Figure S4. Cloning LiNTPDases1 and 2 into expression vector pLexsy_neo2.1. A. Schematic representation of the pLEXSY neo_2.1 system. B and C. Digestion neo_2.1 to be used on eGFP tagged strategy and with 6X His tagged. D. Disgestion to confirmation the ligation of LiNTPDases_6xHis on pLEXSY and recover eGFP from PGEM. E. LiNTPDase2_eGFP miniprep and F. Digestion to confirmation of LiNTPDase2_eGFP cloning. See Material and Methods: Cloning to Overexpress *L. infantum* NTPDases 1 and 2.



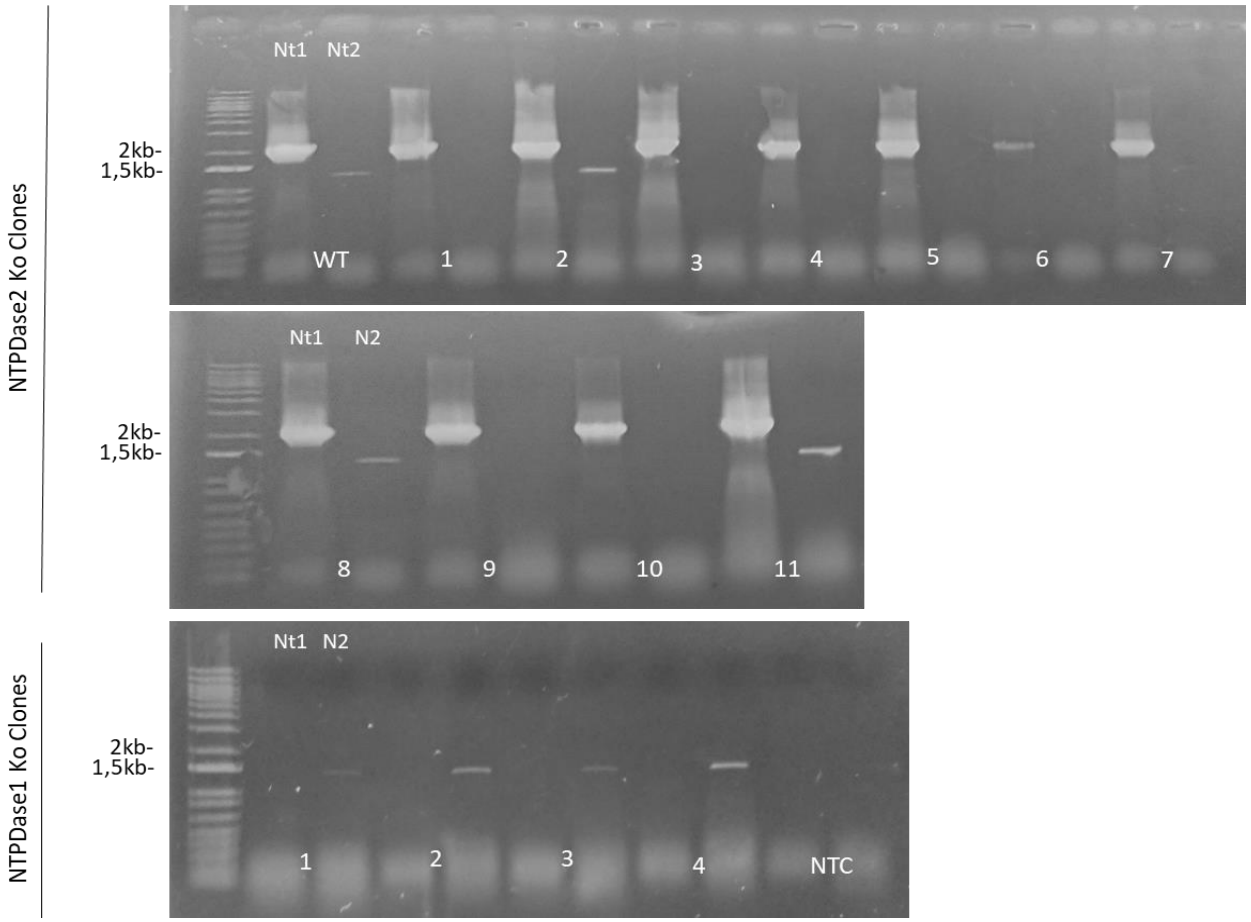
Supplementary Figure S5. Genotyping of overexpressing clones isolated from limiting dilution. A. 1.1Kb fragment amplified from 5'SSU locus of homologous recombination indicating the correct recombination to LiNTPDase1_6xHis into neomycin selected clones. B. Confirmation of clones LiNTPdase2_6xHis. C. Confirmation of clones LiNTPDase2-eGFP. Schematic representation of the site amplified to confirm the clones. All clones, independent of the gene overexpressing, must have 1.1Kb fragment amplification representing a fragment from 5'SSU.



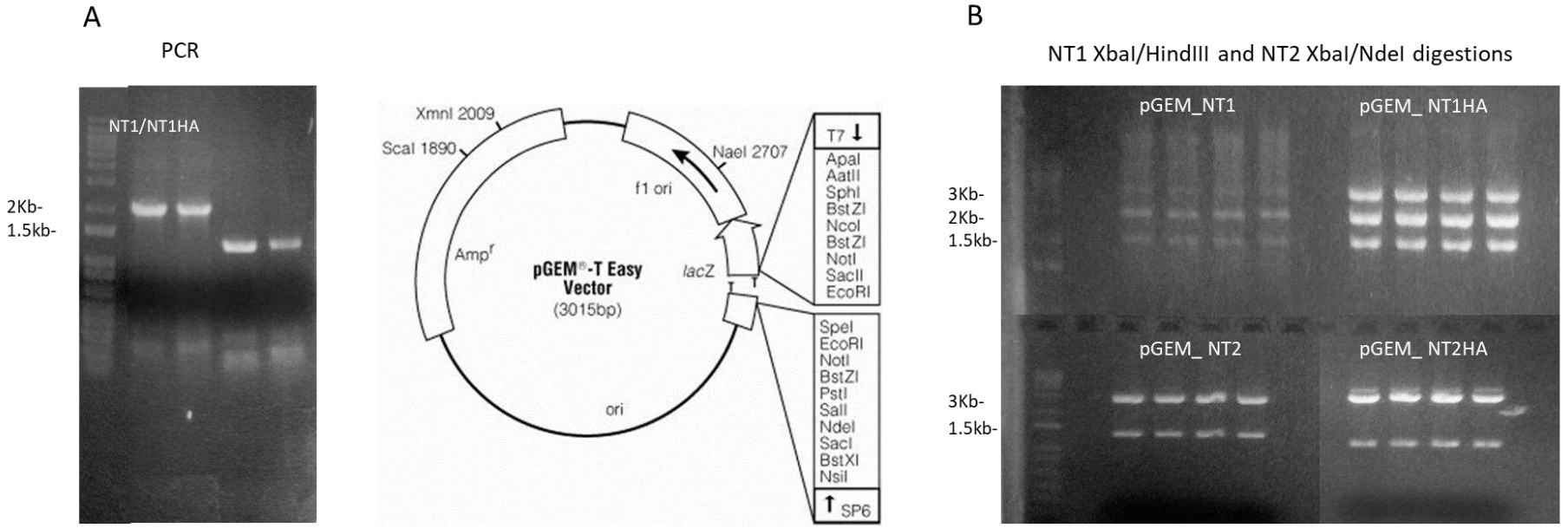
Supplementary Figure S6. Cassettes with resistance genes to homologous recombination on CRISPR/Cas9 and Probes to hybridization on UTR gene target to Southern Blotting development. A. Neomycin e Puromycin drug resistance genes flanked by UTR sequence of each gene. The cassettes were PCR generated to Homologous recombination after DNA double strand break B. Probes PCR generated to 5'UTR to LiNTPDase1 and 3'UTR to LiNTPDase2



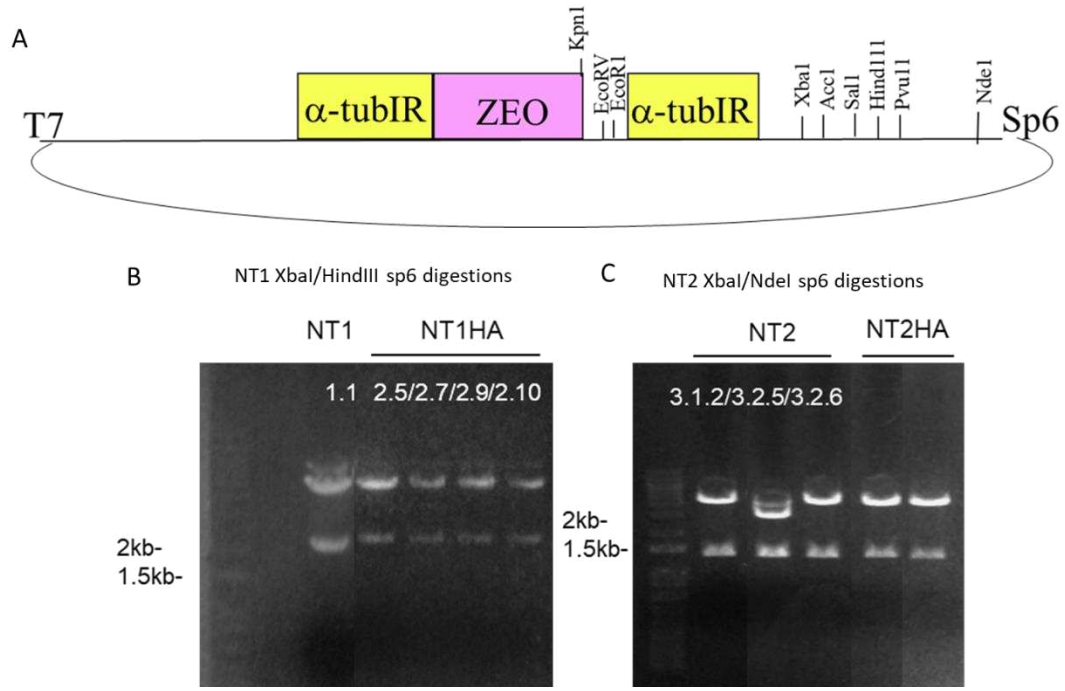
Supplementary Figure S7. Genotyping of knockout clones isolated from limiting dilution. LiNTPdase 1 and 2 were full length amplified to diagnose its presence or absence. The other isoform which was not deleted was always used as positive control.



Supplementary Figure S8. Cloning in pGEM to add back NTPDase1 and 2 on null mutants. A. PCR Generated of both LiNTPDase1 with or without Hemagglutinin tag and LiNTPdase2 with or without Hemagglutinin tag. B. These sequences were cloned into PGEM system and digested to confirmation their ligation. For more information see Material and Methods: Cloning to add back LiNTPDases 1 and LiNTPdse2 to null mutants.



Supplementary Figure S9. Cloning in SP6 expression vector to add back NTPDase1 and 2 on null mutants. A. Schematic representation of the sp6 vector system. B. LiNTPdase1 with or without Hemagglutinin tag was cloned and digested from sp6 to confirmation of the ligation. C. LiNTPdase2 with or without Hemagglutinin tag was cloned and digested from sp6 to confirmation of the ligation. For more information see Material and Methods: Cloning to add back LiNTPDases 1 and LiNTPdse2 to null mutants.



4 GENERAL CONCLUSIONS

Several evidences suggest that NTPDases 1 and 2 of *L. infantum* play a central role in the parasite infection, adhesion, nutrition and development. Here, we review on literature their biological roles proposed until now (Chapter 2). We found enough evidences that LiNTPDases are important to the physiology and infection by playing an essential role in the modulation of purinergic signaling, in adhesion and infection. Although, purinergic signaling affects the development and outcomes of Leishmaniasis, studies involving the role of P2 receptors on infection and virulence, as well as the effects of SNPs in enzyme activity are still few. Additionally, there is enough evidence suggesting that the parasites LiNTPDases can be exploited for biotechnological applications, such as diagnosis, vaccines, and drug design once they present low identity with the mammalian ENTPDases on amino acids sequence. However, no structural data regarding LiNTPDases were found. Structural information would also enable further efforts on rational drug design by providing information regarding protein folding, active site structure and molecular recognition patterns of ligand-receptor complexes as well as new epitopes.

We were also able to generate LiNTPDase 1 and 2 null and overexpressing mutants to their functional characterization (Chapter3). LiNTPDase2_eGPF was localized into nucleus and kinetoplast but further analyses need to be done. We generate LiNTPDase1 and LiNTPDase2 null mutant separately, but the double knockout for the both LiNTPDases genes was lethal, indicating that at least one is necessary to parasite survive. Additionally, we observe an RNA expression compensation on isoform that was not deleted. We also showed that LiNTPDase 1 is important to infection, but not to adhesion on macrophages. While LiNTPDase2 is important not only on adhesion but also on infection mechanism. Null mutants of LiNTPDases showed an increase on NO production indicating their role on NO production inhibition in vitro infection and their role on subversion of host immune system. Ecto-nucleotidase activity showed increased activity on LiNTPDase2 overexpressing mutant indicating its expression on cell surface. Our data showed that both isoforms LiNTPDase1 and LiNTPDase2 are important to *L. infantum* development, growth, differentiation, adhesion, infection and

subversion of host immune cell response what make these enzymes an attractive drug target.