University of South Bohemia in České Budějovice

Faculty of Science

The role of Q tRNA modification in the virulence of *Leishmania mexicana*

Master thesis

Bc. Michala Boudová

Supervisor: RNDr. Zdeněk Paris, Ph.D.

Co-Supervisor: Msc. Sneha Kulkarni

České Budějovice, 2018

Boudová, M., 2018: The role of Q tRNA modification in the virulence of *Leishmania mexicana*. Mgr. Thesis, in English. – 66 p, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

The aim of the thesis was to indentify the orthologues of TGT1 and TGT2 genes in *Leishmania mexicana* and to verify the involvement of the respective gene products in the formation of Q-tRNAs in this species. Additionally, infection experiments *in vitro* as well as *in vivo* were carried out in order to test the plausible effect of Q-tRNA modification presence and/or absence on the virulence of the parasite.

Affirmation

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice, 11th December 2018

……

Michala Boudová

Acknowlegements

First of all, I would like to thank my supervisor Zdeněk P. for the opportunity to work in his laboratory and to take part in this project. My thanks also go to Julius L. for the invaluable support he provided in terms of the laboratory equipment and facilities.

Second, I would like to express my gratitude to everyone who directly or indirectly contributed to the establishment as well as completing this project; V. Yurchenko and his research team, especially Aygul I. and Natalie K., who kindly provided the cell line and vectors used in this study as well as many useful advices. I am particularly grateful for the assistance and training given by Zuzka V. regarding animal experiments. I also wish to acknowlage B. Sak and his colleagues for their patient guidance on histopathology, as well as J. Chmelař who provided some essential chemicals and last but not least Radek Š. who introduced me to the RT-PCR cycler.

Next, many thanks and my very great appreciation go to my co-supervisor Sneha K., who provided many priceless discussions and vital insights that greatly assisted the research. Moreover, she also contributed many practical advices and spent time supervising me, in an attempt to identify the reason why things were not working for me, on some of the hectic days (of macrophage isolation) she would also help me pipetting, just to prevent me from developing so-called pipetting thumb. Additionaly, at times she also performed many other important functions such as my decidion-maker, time-manager, mood- and motivation-booster and a buffering agent. To make a long story short, I am eternally greatful to her for the kind guidance which has led me through to whole process and helped me to overcome many tough situations.

I would like to extand my thanks to all the members of our lab, who provided vital mental support and friendly atmosphere throughout the time of my study, as well as members of surrounding labs such as Priscila P. D. and Paula A. C. who were always opened to a debate and ready to share their expert knowlage (days and nights), Ambar who provided emotional support as well as food supplies particularly over the time of writing the thesis, this process was also greatly assisted by our coffee manager Eva K.

Finally, I must express my gratitude to my family for providing me with continuous encouragement throughout all the years of my study.

This work was supported by the project "Centre for research of pathogenicity and virulence of parasites" (No. CZ.02.1.01/0.0/0.0/16_019/0000759) funded by European Regional Development Fund (ERDF) and Ministry of Education, Youth and Sport (MEYS).

Table of contents

List of abbreviations

AA	amino acid
ACK lysis buffer	ammonium-chloride-potassium lysis buffer
Ag	antigen
APB	3-(acrylamido)phenylboronic acid
APC	antigen-presenting cells
APS	ammonium persulfate
BSA	bovine serum albumin
bTGT	bacterial TGT
CD	cervical dislocation
cDNA	complementary DNA
CL	cutaneous leishmaniasis
DAPI	4‘,6-diamidino-2-phenylindole
DC	dendritic cell
dH_2O	distilled water
DNA	deoxyribonucleic acid
E. coli	E*scherichia coli*
e.g.	*exempli gratia*
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
etc.	*et cetera*
eTGT	eukaryotic TGT
EtOH	ethyl alcohol
FBS	fetal bovine serum
gDNA	genomic DNA
HF	high fildelity
hpi	hours post infection
Hyg	hygromycin
i.p.	intraperitoneal
IAA	isoamyl alcohol
IFNγ	interferon gamma
IFN-γ	interferon gamma
IL-4/5/12	interleukin 4/5/12
IR	immune response

IS	immune system
JAK/STAT	Janus kinase/signal transducers and activators of transcription
KO	knock-out
LPS	lipopolysaccharides
MAPK	mitogen-activated protein kinases
MCL	mucocutaneous leishmaniasis
mRNA	messenger RNA
NA	nucleic acid
NEB	New England BioLabs inc.
Ntc	nourseothricin
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PECs	peritoneal exudate cells
PKC	protein kinase C
pre-tRNA	precursor tRNA
Puro	puromycin
q	queuine
Q	Queuosine
QTRT1	queuine tRNA ribotransferase 1
QTRTD1	queuine tRNA ribotransferase domain containing 1
RBC	red blood cell
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse-transcription PCR
qRT-PCR	quantitative reverse transcription PCR
sgRNA	single guide RNA
TAE buffer	tris base/acetic acid/EDTA buffer
TG	thioglycollate
TGFβ	transforming growth factor beta
TGT	tRNA-guanine transglycosylase
TGT	tRNA-guanine transglycosylase
Th cell	T helper cell
TNFα	tumor necrosis factor alpha

tRNA	transfer RNA
WT	wild-type

1. Introduction

1.1. *Leishmania* & leishmaniasis

Leishmania is a genus of obligate protozoan parasites belonging to the order Trypanosomatida, class Kinetoplastida. The genus comprises about 50 species described to date, out of which more than 20 are capable of infecting humans and/or domestic animals, causing them to suffer from a disease called leishmaniasis (Akhoundi et al 2016). The parasites are typically transmitted to mammals by infected insect vectors leading to the eruption of skin sores at the site of the bite, which may eventually disseminate throughout the skin and thereupon to other tissue types. The clinical manifestation of leishmaniasis ranges from often asymptomatic or self-healing cutaneous (CL) to potentially life-threatening mucocutaneous (MCL) or systemic visceral forms, depending on the species of *Leishmania* as well as of the host genetic background. The observed wide clinical spectrum reflects the complexity of leishmaniasis epizootiology; not only is it caused by several *Leishmania* species, but also many different invertebrate and vertebrate species have been implicated in the transmission of the disease, whether as vectors or reservoir hosts (Reithinger et al 2007). Consequently, the abundance of insect vectors and mammalian reservoirs is tied to the geographical distribution of leishmaniasis, which thus affects the populations of almost a hundred countries mainly in tropical and subtropical regions. Globally, there are an estimated 12 million cases of leishmaniasis, with 1,5-2 million new cases occurring annually and about 350 million people at risk of the infection; however, incidence numbers are likely underestimated, as many infections may be asymptomatic or misdiagnosed (Alvar et al 2012). Additionally, surveillance data indicate that the global number of cases has increased over the past decade, hence leishmaniasis constitutes a major public health problem (Bern et al 2008). Rationally, the ailment is most prevalent in regions where people live in poverty, without adequate sanitation and, in close contact with the infectious vectors, domestic animals and livestock. Therefore, it is classified by the WHO as a severely neglected disease, with little interest by financial donors, public-health authorities, and professionals to implement activities to research, prevent or control the disease (WHO 2018).

With regard to the varied spectrum of symptoms and signs, leishmaniasis is often referred to as a group of diseases, out of which the cutaneous form is the most prevalent. CL is endemic to all sub/tropical areas of the world, with about one-third of cases occurring in each of the three epidemiological regions, the Americas, the Mediterranean basin, and western to central Asia (Fig.1) (Alvar et al 2012). Currently, more than 15 *Leishmania* species are recognized as the disease causative agents (Reithinger et al 2007). These can be grouped

between the Old World species of the parasite (e.g. *L. major, L. infantum*, and *L. tropica*) and the ones from the New World (e.g. *L. amazonensis, L. chagasi, L. mexicana, L. braziliensis* and other), consecuently transmitted by the sandflies of genera *Phlebotomus* or *Lutzomyia* respectively (de Vries et al 2015).

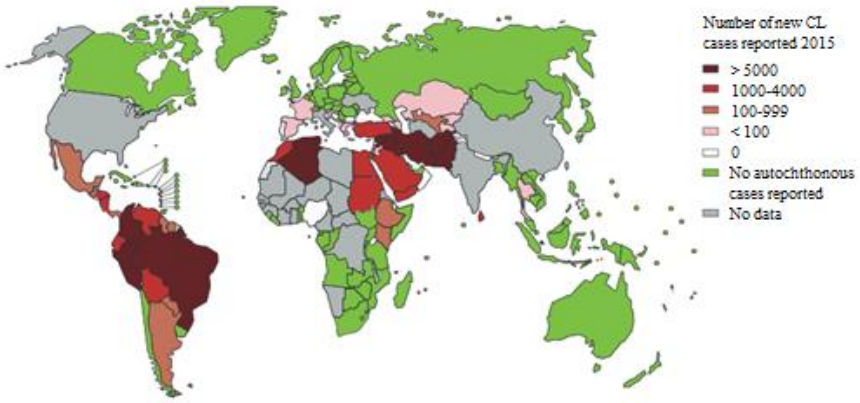

Fig.1: Status of endemicity of cutaneous leishmaniasis worldwide 2015 (WHO 2017).

Whereas the *Leishmania* species from the Old World cause, in most cases, self-limiting ulcers, CL induced by *Leishmania* New World species often develops into the disseminated form of the disease, resulting in mucocutaneous ulcerations. Unfortunately, almost all the current options for CL treatment are toxic and exhibit significant side effects, therefore, no definitive treatment guidelines are presently available (González U 2009).

In the Americas, CL is mainly caused by *L. braziliensis* and *L. mexicana* (Martins et al 2014), the latter representing our model organism of choice. In order to better understand the epidemiology of CL and to develop new treatment and preventive strategies, investigation of genes influencing *Leishmania* growth and/or differentiation, followed by experimental validation of such putative virulence factors, is required.

1.2. *Leishmania* life cycle

Leishmania is characterized by a dixenous life cycle consisting of the extracellular, flagellated promastigotes, which multiply and develop within the sand fly vector and the intracellular, sessile amastigotes, which reside within the phagocytes of their mammalian hosts (Fig. 2).

The journey of *Leishmania* begins in the midgut of sand flies, where *Leishmania* procyclic promastigotes proliferate and mature into the infectious metacyclic forms, which then migrate through the digestive tract into the oesophagus. When the sand fly is taking a blood meal, the parasites are transmitted into the mammalian skin tissue along with the vector saliva. Once in the mammalian host, phagocytes such as macrophages or dendritic cells, are

chemoattracted to the site of infection and eventually engulf the promastigotes, which reside within the newly formed phagosomes, where many are destroyed. However, some manage to evade the microbicidal power thereby modifying this organelle into a parasitophorous vacuole, which favors parasite growth and their transformation into the amastigote stage. Finally, once the host cell is brimful of the parasites, it may either rupture, leading to the release of amastigotes or become apoptotic and pass its amastigote cargo to other surrounding phagocytes. Either way amastigotes ultimately continue to proliferate and may in turn be ingested by another sand fly while taking a blood meal on the infected individual. At this stage the parasites transform again into procyclic promastigotes, which multiply within the hindgut of the insect vector and, subsequently migrate and accumulate in the anterior midgut, where they undergo the differentiation into metacyclic promastigotes (Sacks et al 2001).

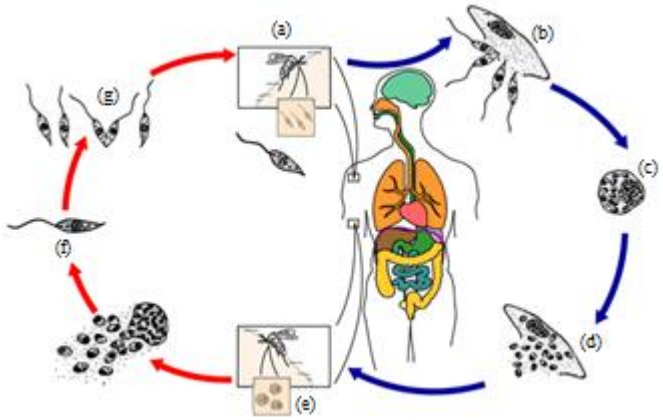

Fig.2: *Leishmania* life cycle; (a) when an infected sandfly takes a bloodmeal, *Leishmania* promastigotes are transmitted into the mammalian skin tissue along with vector saliva, (b) promastigotes are phagocytosed by macrophages, where they (c) transform into the amastigote stage, which multiplies intracellularly until (d) the cell eventually bursts, whence amastigotes are released and infect other phagocytes. (e) Once a sandfly takes a bloodmeal on the infected individual, parasitized macrophages are ingested, hereupon amastigotes transform into promastigotes (f, g) within the insect gut. (Modified from CDC 2018).

The establishment of *Leishmania* infection and the development of pathogenesis in the mammalian host is largely dependent upon multiple factors of the parasite, the sand fly and the host, such as the amount of vector saliva or the size of infectious inoculum egested (Abdeladhim et al 2014, Bates 2007, Belkaid et al 1998, Belkaid et al 2000, Norsworthy et al 2004). Yet, the most crucial determinant is the complex network of interactions among the innate and acquired immune responses of the host (Muller et al 1989, Rosas et al 2005, Soo et al 1998), as the resulting inflammatory reaction mediates the disease expression, ultimately manifested as either symptomless/self-healing local CL or chronic disseminated (M)CL.

Typically, two types of CD4+ T helper cell (Th) responses may be induced by antigen presenting cells (APC; i.e. dendritic cells (DC), macrophages and others), designated Th1 and Th2, triggering either APC activation (and the consequent secretion of pro-inflammatory

cytokines) or the maintenance of an inert inactive state, which eventually develops as a disease resistant or susceptible phenotypes, respectively (Scott 2005).

Current knowledge of the immune response to leishmaniasis stems mainly from studies employing *L. major* murine model of infection, which could be summarized as follows: (i) pathogen clearance is mediated via cell-mediated rather than humoral immunity; (ii) the primary activation of specific T cell subsets is crucial for the development of either Th1 or Th2 responses and the consequent course of infection, as (iii) there is a strong correlation between activation of different T-cell subsets and the final outcome of the disease (Reithinger 2007).

1.3. Evolutionary arms race: host immune response & *Leishmania* immune evasion

Once the parasites are injected into the host skin tissue, its immune system reacts by secreting a large numbers of cytokines, i.e. small proteins involved in the balance between humoral and cell-based immune responses (IR), acting via the chemoattraction of particular cell populations, and the regulation of their maturation, growth, and responsiveness to extracellular stimuli (Zhang et al 2009). The parasites are therefore rapidly taken up by various phagocyte populations recruited to the site of inoculation, including neutrophils, macrophages, monocytes and DCs, ultimately triggering their transformation into the amastigote stage, which then continuously proliferate within the host macrophages (Sacks 2001).

Macrophages act as the sentinels of the immune system (IS); they are present in all mammalian tissues (Ovchinnikov 2008), where they patrol for the occurrence of pathogens which are, upon their recognition, engulfed in the process of phagocytosis. Subsequently, the newly emerged pathogen-containing phagosome undergoes membrane fusion with the endogenous lysosomes leading to the exposure of the pathogen to the activity of hydrolytic enzymes and consequently, partial degradation. Next, immunogenic peptides of pathogen origin are transported to the cell surface in order to facilitate antigen (Ag) presentation to T cells, eventually leading to the activation and differentiation of CD8+ and various CD4+ T cell subsets, the latter resulting in the development of either Th1 or Th2 IRs with their typical cytokine profiles. Consequently, the cytokines released by Th1 cells (IL-12, TNFα, and most importantly, IFNγ) may induce macrophage activation resulting in the production of oxygen and/or nitrogen reactive compounds linked to intracellular pathogen killing. In contrast, cytokines produced by Th2 cells (IL-4, IL-5, TGFβ) render macrophages inactive and shift the balance of the IR towards humoral immunity (Fig. 3) (Chakraborty 1997). Thus, macrophages play a critical role in linking the innate to adaptive IR and therefore, perform a pivotal role in the regulation of microbial invasion and the progression of their infection within the host.

Hence *Leishmania* persistence within the host is determined by the subversion mechanisms that allow it to survive and multiply within the phagocytic cells. Although living in an extremely hostile environment specialized for pathogen destruction, this mechanism represents an ideal way to pass unnoticed by the IS. Therefore, *Leishmania* evolved ingenious ways to adapt to life inside the macrophage and, sophisticated mechanisms to dodge the IR by disabling key microbicidal activities though the manipulation of macrophage signaling pathways and the downstream transcription factors, eventually leading to the modulation of cytokine production in response to extracellular stimuli or more precisely, unresponsiveness to the stimuli (Olivier et al 2012).

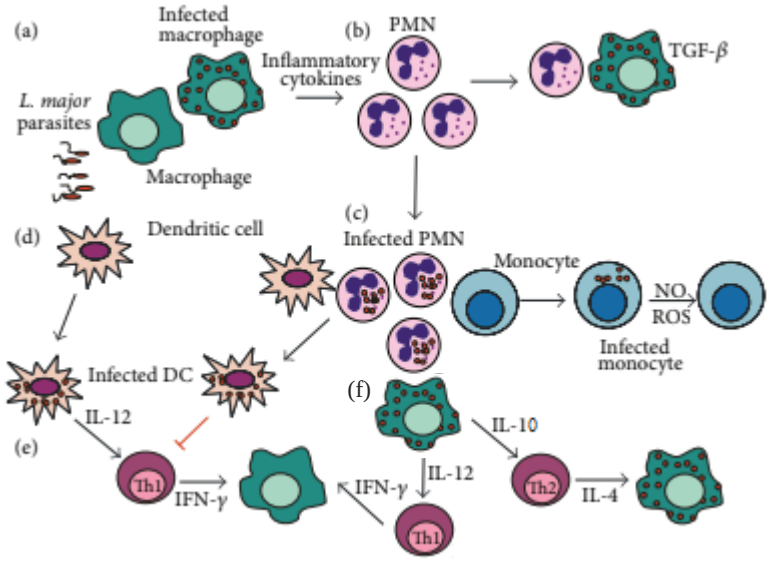

Fig. 3: Interactions among phagocytes in *Leishmania* infection; (a) upon infection tissue resident macrophages produce inflammatory cytokines that attract (b) polymorphonuclear leukocytes (PMN), some of which may secrete products that attract monocytes and macrophages. Neutrophils (of BALB/c mice) are incapable of parasite clearance, hence the infection is propagated (c) to macrophages, monocytes and DCs. Phagocytes infected via efferocytosis of neutrophils fail to activate T lymphocytes, otherwise (d) infected DCs migrate to lymph nodes to induce Th1 response, (e) cytokines produced by Th1 cells promote macrophage activation. (f) Infected macrophages, depending on their cytokine profiles, may induce Th1 as well as Th2 response, the latter rendering macrophages inactive, thus parasite-permissive (modified from Lopes et al 2014).

In order to survive, the parasites interact with host IS at multiple levels, for instance counteracting the innate IR by complement inactivation (Brittingham et al 1995) or inhibition of natural killer cell proliferation (Lieke et al 2008). Furthermore, while many virulence factors interfere with basic macrophage functions such as the phagolysosome biogenesis (Desjardins & Descoteaux 1997), consequently affecting Ag processing and presentation (Reiner et al 1988), others manipulate the cellular gene expression pattern. This is achieved either through direct degradation of transcription factors (Contreras et al 2010) or by the modulation of macrophage signaling pathways, primarily through the activation of protein tyrosine phosphatases, which leads to the disruption of e.g. PKC activity, JAK/STAT or

MAPK signaling (Gomez 2009). Finally, the above-mentioned alterations altogether result in the inhibition of macrophage activation, thus unresponsiveness to external stimuli reflected by repression of cytokine production, thereby preventing the induction of protective adaptive IR. The main virulence factors and the respective molecular mechanisms involved in the subversion of macrophage physiology have been thoroughly reviewed (Arango Duque & Descoteaux 2015, Basu & Ray 2005, Gregory & Olivier 2005, Kima 2014, Olivier et al 2012, Séguin & Descoteaux 2016).

1.4. Murine models of infection

Leishmania infection in different mouse strains is a widely used model for the study of Th1 or Th2 responses that control resistance or susceptibility to the parasite, respectively (Müller et al 1989, Scott & Farrell 1998). The conventional model typically utilizes high doses ($1x10^5$-$1x10^7$) of parasites inoculated subcutaneously into the footpad, where the development of the lesion and the onset of immunity is relatively rapid as compared to natural infection (Belkaid et al 2000). The model has, however, been successfully employed for the detailed identification of cells, cytokines and effector molecules involved in the development of acquired resistance, which has been shown to be dependent upon the IL-12-driven activation of Th1 cells for the production of IFNγ, which in turn activates the microbicidal mechanisms of infected macrophages (Sypek et al 1993). The extent to which the high dose model accurately recapitulates the pathology following the natural transmission of *Leishmania* by insect vectors has not been addressed until recently. Importantly, the Th1/Th2 dichotomy observed in murine models does not seem to be fully applicable to humans (Valencia-Pacheco et al 2014).

Perhaps, a model of cutaneous leishmaniasis resulting from a low-dose infection challenge can mimic the evolution of self-limiting infections that occur in mammalian reservoirs and human hosts more precisely. Thus, another model of *Leishmania* infection has later been established, which combines two main features of the natural transmission – low dose ($1x10^2$-$1x10^3$ parasites) and the inoculation into a dermal site (ear dermis) (Belkaid et al 2000, Kimbin et al 2008). This natural infection model revealed two discrete stages in the evolution of pathogenesis, which were previously not recognized – an initial silent phase, lasting for 4-5 weeks favoring parasite amplification in the absence of any overt pathology, followed by the second phase corresponding to the lesion development coincident with parasite clearance (Belkaid et al 2000). Furthermore, it has been demonstrated that the chronic phase of the disease, maintaining the reservoir potential of mammalian hosts, was established more

efficiently and lasted longer in the low-dose as compared to the high-dose infection (Lira et al 2000).

The high-dose inoculum results in the rapid onset of lesion formation as well as the IR, where the development of lesion is associated with an increasing number of parasites at the site, whereas healing is correlated with the activation of infected macrophages and the consequent destruction of the parasites (Lira et al 2000). The relatively fast disease progression is likely caused by the excessive amounts of parasites and antigens that may be taken up primarily by DCs. These cells are, in contrast to macrophages, capable of IL-12 production in response to *Leishmania* infection (Gorak et al 1998, van Stebut et al 1998), hence providing a potent source of APCs for T cell activation (Konecny et al 1999, Will et al 1992). Thus, this model undermines the ability of the parasite to initiate the infection in a quiescent manner.

Contrarily, in the natural infection model, the parasites most probably first interact with neutrophils; these were suggested to act as „Trojan horses“ as *Leishmania* is able to avoid their intracellular killing (Laskay et al 2003) therefore, the infected neutrophils are quickly undergoing apoptosis and subsequently being cleared by macrophages (Savill et al 2002). In this manner the parasites are selectively confined to macrophages early on, therefore delaying the development of acquired resistance (Peters & Sacks 2009, Ribeiro-Gomes et al 2012). Such a sequence of events is supported by a large number of *in vitro* studies indicating that *Leishmania* can avoid or actively inhibit the immune response of their host macrophages, and particularly production of IL-12 (Belkaid et al 1998, Carrera et al 1996, Piedrafita et al 1999, Reiner et al 1994, Sartori et al 1997). Additionally, this scenario is also consistent with the histopathological descriptions of human lesions (ElHassan et al 1995, Gaafar et al 1995, Gaafar et al 1999).

1.5. The complex life of tRNAs

Transfer RNAs (tRNAs) are the essential players in translation, acting as the adapter molecules that decode the genetic information into protein sequences, in accordance with the rules defined by the genetic code. Functionally, most important regions of tRNA structure include i) the 3’ acceptor stem which is the site for the amino acid attachment; and ii) the anticodon stem, critical for the recognition of the complementary bases of the messenger RNA (mRNA). Hence, in the process of translation, each tRNA is charged with a specific AA that matches its anticodon triplet; this triplet in turn matches with a codon in the mRNA, therefore delivering the relevant AAs to be added to the growing polypeptide chain (Hoper et al 2011).

The biogenesis of tRNAs begins with the transcription of the respective genes as precursor molecules (pre-tRNAs), which undergo an elaborate set of alterations to generate mature

tRNAs capable of passing the nuclear export quality control, a process that precedes their subsequent inclusion in the translation machinery. Although the sequence of these maturation steps may differ among individual tRNAs, ultimately, they are all subjects to the cleavage of their 5' and 3' end overhangs respectively. Further on, the 3' end is (usually) reshaped by the addition of CCA tail, which is essential for the later attachment of the cognate amino acid (AA). Some pre-tRNAs also encounter intron removal; yet most importantly many are heavily modified in order to become fully active (Hoper et al 2011).

In fact, tRNAs exhibit the highest density of post-transcriptional modifications among all RNAs (Lorenz et al 2017). While pre-tRNAs are composed of the four standard bases exclusively, many are later converted, resulting in mature tRNAs with 7-15% nucleosides substituted with their variants (Phizicky & Alfonzo 2010). Currently, about 100 different modified bases are recognized, and the variety of their functions is at least similarly diverse (The tRNA Modification Database). The presence of chemically modified residues at various positions of the tRNAs has been shown to influence a number of their functional properties, such as the structure, stability, aminoacylation rate and accuracy (Madore et al 1999), as well as tRNA cleavage (Klassen et al 2008). Although the modifications may be present throughout the whole structure of the molecule, there are some "modification hotspots" located chiefly at certain positions of the anticodon loop, hence performing a critical function in accurate codon selection and frame shift prevention (Manickam et al 2016).

Since the genetic code comprises 61 possible codons (4^3 minus 3 stop codons generally not recognized by tRNAs) and most organisms encode fewer than 45 tRNA species, some must pair with more than one codon and *vice versa*; since there are only 23 proteinogenic AAs, different tRNA species are necessarily decoded as one and the same AA. This phenomenon is termed codon degeneracy or genetic code redundancy and it has been explained (at least to some extent) by the wobble hypothesis. According to the theory, the 5' base on the anticodon, which binds to the 3' base on the mRNA, is not as spatially confined as the other two bases, and thus could exhibit non-standard base pairing (called wobble), for instance an anticodon sequence 5'-GAA-3' is able to recognize either 5'-UUC-3' or 5'-UUU-3' codon sequences (Crick 1966). Wobble base pairing had later been postulated to be genuinely facilitated by post-transcriptional modifications of tRNAs, particularly those located at position 34 of the anticodon loop, thus expanding the ability of tRNAs to read three or even four degenerate codons (Agris 1991, Agris et al 2006).

Importantly, modified nucleotides within the anticodon loop modulate the binding to certain codons, granting specific tRNAs with the ability to regulate gene expression (Agris et

al 2006). Although multiple codons may lead to the incorporation of the same AA, tRNAs read codons with differing speed and accuracy. Also, synonymous codons are not used in equal frequency throughout the genome, a phenomenon referred to as "codon bias". Consequently, codon bias is linked to tRNA isoacceptor abundance within the cell; this means that if multiple codons code for the same AA, one of them may be significantly more abundant than the other "rare" codons among the mRNAs (Gustafsson et al 2004, Plotkin & Kudla 2011). Consequently, rare codon clustering or positioning near initiation or termination codons cause ribosome stalling and frameshifting due to the low abundance of the tRNAs that read them. Interestingly, rare codons are contained in regulatory genes and those involved in DNA replication (Cruz-Vera 2004, Gurvich 2005, Sakamoto et al 2004).

In conclusion, modifications found at the wobble position of the anticodon (34 and 37) contribute to the decoding of rare codons, and restrict or expand codon recognition depending on their chemistries. Thus, the modifications present in the anticodon affect translation rate and control gene expression (Gustilo et al 2008).

1.6. Queuosine tRNA modification

As already mentioned, modified nucleosides are most often located at the wobble position of the anticodon, and play a direct role in translational efficiency and fidelity, as well as codon recognition, consequently affecting gene expression. One of such modified bases is queuosine (Q), a hypermodified analogue of guanosine (G), found at the wobble position 34 in tRNAs containing "$G_{34}U_{35}N_{36}$" anticodon sequence (where N is standing for any of the canonical nucleotides). These correspond to four tRNA species (Asn_{GUU}, Asp_{GUC}, His_{GUG}, Tyr_{GUA}), with their respective pre-tRNAs originally carrying G at the first position of the anticodon, which is post-transcriptionally modified to Q during the process of their maturation (Vinayak & Pathak 2009).

Notably, in *Xenopus laevis* it has been demonstrated that Q-modified tRNAs (Q-tRNAs) exhibit equal affinity to both NAU and NAC codons, unlike their unmodified counterparts (G-tRNA) (Meier et al 1985). Furthermore, *in silico* analysis of codon usage in the housekeeping versus onco-developmental gene transcripts suggests a significant difference in the codon bias, between C-ending and U-ending codons (Morris et al 1999). Additionally, in one case, Q-$tRNA^{Tyr}$ was shown to mediate stop codon read-through resulting in a longer translational product (Bienz & Kubli 1981). A recent study in mammals showed that depletion of Q-tRNAs is directly correlated with the levels of tRNA methylation at position 38. Consequently, the reduced levels of these modifications affect translational speed at Q-decoded as well as near-cognate codons, ultimatelly resulting in translation deregulation and activation of unfolded

protein response (Turto et al 2018). Taken together, these data indicate that Q-tRNAs influence gene expression (and thus the arising cellular functions) by codon bias-based regulation of protein synthesis, resulting in translational stalling or programmed frame-shifting.

Q is ubiquitously present in all domains of life; while prokaryotes can synthesize it *de novo* in a multi-step enzymatic reaction, this biosyntetic pathway is absent in eukaryotes, hence they acquire the fully modified free base queuine (q) either from their diet or gut microbiome (Kersten 1988). The final reaction of Q insertion with the concomitant elimination of the genetically encoded G is catalysed by highly conserved tRNA-guanine transglycosylase (TGT), thus it is nearly identical in both prokaryotes and eukaryotes, yet the manner of Q uptake varies largely (Iwata-Reuyl 2003).

Prokaryotes utilize cytosolic GTP for its conversion into a partially modified base preQ1, which is the substrate for the bacterial TGT (bTGT) that incorporates it into the respective tRNA species (Okada et al 1978, Okada et al 1979). PreQ1 is then further remodeled *in situ* by the action of two enzymes resulting in Q-tRNA (Frey et al 1988). Henceforth the Q-tRNA participates in normal cellular functions until degraded by natural tRNA turnover, whereupon Q and/or q are released to the environment as metabolic waste products (Vinayak & Pathak 2009).

Since eukaryotes lack the enzymes required for Q *de novo* biosynthesis, q is salvaged from the environment via the recently characterized DUF2419 protein family, which was demonstrated to participate in q uptake from the extracellular environment and proposed to take part in the nutrient recycling following Q-tRNA degradation (Zallot et al 2014). Contrary to prokaryotes, q is then directly and irreversibly exchanged for guanin (thus converting q into Q) in a single enzymatic step catalyzed by the eukaryotic TGT (eTGT) (Fig.4).

While bTGT was shown to form homodimers (Romier at al 1996), eTGT functions predominantly as a heterodimer (Howes & Farkas 1978, Slany & Müller 1995), consisting of the queuine tRNA ribotransferase 1 (QTRT1) and its splice variant queuine tRNA ribotransferase domain containing 1 (QTRTD1). Even though both of the subunits were shown to be essential for Q-tRNA formation *in vivo*, QTRT1 subunit alone accommodates residues responsible for the catalytic activity of the complex, while QTRTD1 has been implicated as a regulatory subunit possibly involved in the complex stabilization, as well as in the nutrient recycling (Chen et al 2010).

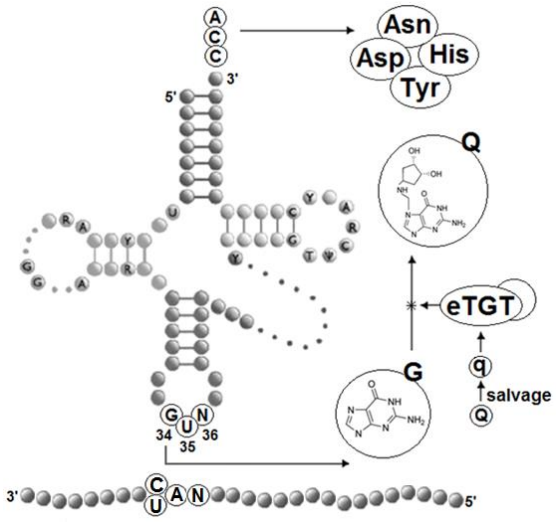

Fig. 4: Q tRNA modification in eukaryotes; Q, a base analogue of G, is found in position 34 of the anticodon loop in tRNAs containing "GUN" consensus sequence (Asn_{GUU}, Asp_{GUC}, His_{GUG}, Tyr_{GUA}), which is recognized by eTGT enzyme that facilitates post-transcriptional modification of such tRNAs (modified from Fergus et al 2015).

1.7. Physiological relevance of Q-tRNAs

The presence of Q-tRNAs in nearly all forms of life suggests a likely relevant role in physiology. Although several studies indicate the possible involvement of Q-tRNAs in diverse cellular processes, such as the survival of organisms under stress conditions, antioxidant defense systems, cell proliferation or signaling, up-to-date literature does not provide a satisfactory answer to any exact function and mechanism of action.

In an attempt to elucidate the physiological relevance, several studies have reported on the consequences of Q depletion in various experimental systems, no obvious defects were noted (Gaur et al 2007, Kirtland et al 1988, Ott et all 1982), except in one case (of *Drosophila melanogaster*) where the organisms exhibited decreased ability of response to certain stress stimuli (Siard et al 1991). Q deficiency did not cause an overt pathology any in vertebrate model (Farkas 1980), unless tyrosine had been withdrawn from the diet concurrently. This resulted in severe neurological symptoms ultimately leading to death of the studied individuals, indicating that in the absence of Q, tyrosine becomes an essential AA (Marks & Farkas 1997). This observation was later explained by the reduced capacity of conversion of phenylalanine to tyrosine, due to deficiency of phenylalanine hydroxylase (PAH), that stems from an increased oxidation of its essential co-factor BH4. Yet, this co-factor is not only critical for the activity of PAH, but also for the biosynthesis of various neurotransmitters, such as dopamine, epinephrine and others, thus the neuropathological symptoms observed may not necessarily be a direct consequence of tyrosine deficiency. The authors conclude that the increased oxidation of BH4 likely occurs due to changes in the intracellular homeostasis, since

queuine deficiency had previously been suggested to affect the activity of a number of antioxidant systems (Rakovich et al 2011). It is worthy of note that the experimental systems employed in the above-mentioned reports were not equivalent hence, the determination of the exact role of Q in this process will require further investigations.

The presence of Q was indeed shown to enhance the activity of anti-oxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase or glutathione reductase. Interestingly, the activity of these enzymes is decreased in cancerous cells, consequently increasing the accumulation of ROS, both of which can be counteracted by the exogenous administration of queuine (Pathank et al 2008). Inquisitively, Q-tRNAs of tumor cells were found to be hypomodified to various degrees that correlate with the histopathological grade of malignancy (Dirheimer et al 1995, Emmerich et al 1985). Additionally, q administration leads to the formation of Q-tRNAs, along with a decrease in the proliferation capacity of tissues. Although these data indicate a role for Q in cell proliferation (Pathak et al 2007), available literature provides an evidence for Q acting both as negative and positive regulator (Langgut et al 1993); this might be due to a number of factors, including the possible involvement of Q in cell signaling (Langgut 1995, Pathak et al 2008), the underlying molecular mechanisms of which remain to be elucidated.

Notably, the activity of TGT enzymes was shown to influence the virulence of *Shigella flexneri*, a human pathogen able to escape macrophage phagosomes *via* the expression of certain virulence factors, which are under the control of a transcriptional regulator termed VirF. Although TGT is well-known to function in tRNA modification, it has been demonstrated that VirF mRNA is recognized as a substrate of the enzyme leading to site-specific modification of a single base. Curiously, the translation of VirF is markedly reduced in TGT mutant strains, resulting in bacteria being incapable of host cell invasion. The authors suggest that Q may be acting on small molecule binding motifs of mRNAs ("riboswitches") consequently modulating their conformation and translation, thus TGT could promote the translation of VirF by mRNA modification (Hurt et al 2007). Additionally, the insertion of Q was also reported to occur in ribosomal RNA (Brooks et al 2012), leading to a hypothesis that there might be other potential TGT substrates, that resemble the secondary structure of the anticodon stem-loop containing the "UGUN" sequence.

In conclusion, the current knowledge on TGT as well as q and Q-tRNAs is rather incomplete. Therefore, the insufficiently defined signaling pathways adopted by q and/or Q-tRNA pose a challenge for the research in this field, as it is currently unclear, whether the biological functions are executed by q and/or Q-tRNA or due to a secondary effect to the

depletion of q and/or Q-tRNA therefore, the presented phenomena require further in-depth analysis.

1.8. Rational of the project

1.8.1. *Leishmania* reverse genetics (in brief)

Trypanosomatids, including *Leishmania* have, among eukaryotes, an unusual genome organization with protein-coding genes organized in tandem arrays (McDonagh et al 2000), which are transcribed as polycistronic units (Martinez-Cavillo et al 2004), encoding for multiple, usually functionally unrelated, mRNAs. Additionally, due to the lack of conventional RNA polymerase II promoters (Schimanski et al 2005), these protists largely rely on post-transcriptional mechanisms, regulating precursor mRNAs processing and abundance, or post-translational events (Shapira et al 2001).

Leishmania is a well-established model organism (Duncan et al 2017), with the whole genome sequence available (Ivens et al 2005, Rogers et al 2011), and readily searchable using the online source TriTrypDB (Aslett et al 2010). Although these protists are diploid, aneuploidy can occur (Sterkers et al 2014), therefore, in order to generate a null mutant by homologous recombination, two or more rounds of targeted gene replacement may be required, while the number of selectable markers is limited. Furthermore, the attempts to disrupt both alleles of target genes were reported to result in chromosomal amplification or aneuploidy (Cruz et al 1993). Additionally, this method does not allow for the study of essential genes, as it does not provide an option for conditional regulation of target gene expression.

Evidence for gene essentiality can be provided by complementation experiments where the ability to generate null mutants in the presence, but not absence, of episomes that express target gene is generally accepted. Yet, since episomal vectors lack active segregation mechanisms, equal distribution into daughter cells is not guaranteed, therefore the level of episomal expression varies among the cell population depending upon the episomal copy number. Consequently, the expression level of target gene may be insufficient for rescue (Roberts 2011).

Another tool for the regulation of gene expression is represented by RNAi, a technique well-established in *Trypanosoma brucei*, which operates at the transcriptional level, and is therefore not limited by the gene copy number, hence a single round of transfection usually allows for an efficient downregulation of the target mRNA. Moreover, the knock-down of the target gene can be designed as inducible, thus allowing for the study of essential genes (Kolev

et al 2011). However, in contrast to trypanosomes, most *Leishmania* species lack the RNAi machinery (Lye et al 2010).

One of widely used techniques for the regulation of gene expression at protein level relies on the expression of regulatory destabilizing domain (dd) fused to the protein of interest (Madeira da Silva et al 2009), where the protein stability is controlled by a chemical inducer. This system has been recently established in *L. mexicana* (Podešvová et al 2017), however it does not allow for generation of null mutants for essential genes.

Recently, a new tool based on a bacterial CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) /Cas9 (CRISPR-associated protein-9 nuclease) system has generated a considerable excitement among the scientific community, as it is extremely fast, cheap and so far, the most precise method for genetic manipulation. This technology allows for genome editing by removal, addition or alteration of target DNA sequences (Hsu et al 2014). The system was rapidly implemented in various model organisms, including *Leishmania* parasites (Sollelis 2015, Zhang 2017), and more recently its efficient use was demonstrated also in *L. mexicana* (Beneke et al 2017, Ishemgulova et al 2018). This method enables generation of null mutants in a single round of transfection, with the downside that it does not allow for study of genes essential for cell viability.

Promisingly, the recent development of a transgenic cell line of *L. mexicana* expressing tetracycline (Tet) repressor and T7 polymerase enabled conditional gene expression. This system has the potential to generate null mutants, which in parallel contain a Tet-inducible ectopic copy of a gene of interest, while the removal of Tet from the culture media allows for the null mutant phenotype to be assessed (Kareva et al 2014). Another study also revealed dramatically reduced expression of the constructs in both metacyclic promastigotes and amastigotes, making it unable to apply this system during *in vitro* macrophage and *in vivo* murine infections (Ishemgulova et al 2016).

1.8.2. Rationale of the project

Considering the large dependence of trypanosomatid species on post-transcriptional regulation of gene expression, the suggested roles for Q in the antioxidant defense system, cell proliferation and differentiation, as well as the preliminary results from our own laboratory suggesting a role for Q in the virulence of *T. brucei*, we hypothesized that these features might become particularly relevant in *Leishmania* residing in the hostile environment of host macrophages, which, if activated, utilize reactive oxygen and nitrogen compounds for the pathogen destruction.

Furthermore, in contrast to *T. brucei*, studies conducted in *Leishmania* allow the investigation of parasite virulence using an *in vitro* system. Additionally, the time period for phenotype assessment during *in vivo* infection is significantly prolonged, since contrary to trypanosomes, the pathology caused by these parasites is not fatal to the mammalian host.

Therefore, we decided to identify the homologues of TGT genes in *L. mexicana* and take an advantage of the recently established CRISPR/Cas9 system for the generation their respective knock-out (KO) cell lines, in order to test the possible influence on the virulence of the parasites *in vitro* as well as *in vivo*.

2. Aims of the thesis

1. Evaluation of the role of LmxTGT1 and LmxTGT2 in the formation of Q-tRNAs
2. Infection experiments *in vitro*
3. Infection experiments *in vivo*

3. Materials and methods

3.1. Cell culture

Leishmania mexicana isolate MNZC/BZ/62/M379 was utilized for the generation of the cell line stably expressing humanized *Streptococcus pyogens* Cas9 nuclease (hereafter referred to as WT cell line) as described elsewhere (Ishemgulova et al 2018). The WT lineage was kindly provided by doc. MSc. Vyacheslav Yurchenko, Ph.D. (Life Science Research Centre, Faculty of Science, University of Ostrava).

The WT culture was maintained in M199 medium (Sigma-Aldrich, Cat. No. M0393-10X1L) supplemented with 2 μg/ml biopterin (Sigma-Aldrich, Cat. No. B2517-25MG), 2μg/ml hemin (Sigma-Aldrich, Cat. No. 51280-5G), 25 mM HEPES (Applichem, Cat. No. A3268), 100 units/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich, Cat. No. P4333-100ML), 10% fetal bovine serum (FBS) (Sigma Aldrich, Cat. No. F7524-500ML) and 100 μg/ml hygromycin (Hyg) (Invivogen, Cat. No. ant-hg-1) at 25°C. The medium used for cultivation of the TGT KO cell line was additionally supplemented with 100 μg/ml nourseothricin (Ntc) (Jena Bioscience, Cat. No. AB-102L) and 50 μg/ml puromycin (Puro) (Invivogen, Cat. No. ant-pr-5) serving as selection markers.

In order to assess the cell density, samples were fixed with 10% formalin solution (3,6% formaldehyde, 150 mM NaCl, 15 mM $Na_3C_6H_5O_7$) and the cell count was determined using Neubauer haemocytometer (Sigma Aldrich, Cat. No. BR717805-1EA). The cells were kept in logarithmic phase of growth by diluting to $2x10^5$ cells/ml once per 72 hours, unless otherwise specified.

I*n vitro* differentiation was performed by varying pH and temperature as described previously (Bates 1994) with slight modifications as specified elsewhere (chapter 3.5. *In vitro* differentiation).

Murine (BALB/c) peritoneal macrophages were cultured in RPMI medium (Sigma Aldrich, Cat. No. R4130-10X1L), supplemented with 2 g/l sodium bicarbonate, 50 units/ml penicillin, 50 μg/ml streptomycin (Sigma Aldrich, Cat. No. P4333-100ML) and 10% FBS at 37°C (prior to infection) or 34°C (post infection) and 5% CO_2. Identical culture conditions were applied for murine splenocytes (RPMI, 37°C, 5% CO_2).

3.2. Generation of the TGT KO cell line(s)

3.2.1. Molecular cloning

L. mexicana TGT1 and TGT2 genes were identified by BLAST search based on the sequence of their respective counterparts known in *Trypanosoma brucei* (Kulkarni et al unpublished manuscript) and human (Boland et al 2009). Multiple sequence alignment (Larkin

et al 2007) has shown 48% and 32% sequence identity for LmTGT1 and LmTGT2 respectively as compared to the human homologues.

The cloning vector for the expression of sgRNA (under the control of U6 promoter) is a modified version (Ishemgulova et al 2018) of pLEXY-SAT2.1 (Jena Bioscience, Cat. No. EGE-274) (hereafter termed as pLEXY) carrying nourseothricin resistance marker. The vector was provided for the purpose of our study by Life Science Research Centre, Faculty of Science, University of Ostrava.

Gene-specific parts of the sgRNA sequence (hereafter referred to as seed) targeted for the ablation of TGT1 (GTCTCGCGGCCAACCAGAAT│CGG) and TGT2 (GAAGATGGTGCAGGTAAGCG│CGG) ORFs were designed using eukaryotic pathogen CRISPR guide RNA/DNA design tool at http://grna.ctegd.uga.edu (Peng & Tarleton 2015). The corresponding regions were amplified from the pLEXY vector using primers encoding the individual seed sequences, appropriate restriction sites (underlined) and partially annealing to the vector-encoded U6 promoter or terminator (highlighted) as shown in Tab. I (and Fig. 7); (i) P01/P02; (ii) P03/P04; (iii) P01/P04 for TGT1 and (i) P01/P05; (ii) P06/P04; (iii) P01/P04 for TGT2. The PCRs were performed using Q5® High-Fidelity DNA Polymerase (NEB, Cat. No. M0491S) in conformity with the manufacturer's instructions.

The resulting fusion PCR products (iii) as well as the pLEXY vector were double-digested using NotI HF (NEB, Cat. No. R3189S) and NcoI HF (NEB, Cat. No. R3193S) following the manufacturer's instructions. The pLEXY restriction reaction was run on 1% agarose gel and DNA was isolated from the band of appropriate size using GenElute™ Gel Extraction Kit (Sigma-Aldrich, Cat. No. NA1111-1KT) in compliance with the manufacturer's instructions. Ligation was carried out using T4 DNA ligase (NEB, Cat. No. M0202S) as reported by the instruction manual.

The ligation products were transformed by standard heat shock (Froger & Hall 2007) protocol into *E. coli* strain XL-1 Blue. Plasmid DNA was isolated from five individual colonies using Hybrid-Q™ Plasmid (GeneAll, Cat. No. 100-102) following the standard protocol stated in the manufacturer's instructions. The purified plasmids were screened for the presence of the correct insert by PCR using seed-specific primers (P07/P04 and P08/P04 for TGT1 and TGT2 respectively, Tab I), and confirmed by sequencing (Eurofins Genomics) using primers P09 and P10 (Tab. I). Diagnostic PCR was carried out using PPP Master Mix (Top-Bio, Cat. No. P126) according to manufacturer's instructions. For linearization we utilized SwaI (NEB, Cat. No. R0604S) restriction enzyme, the restriction reaction was cleaned

using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, Cat. No. NA1020-1KT). 10 ug of linearized and purified construct was used for electroporation (see next section).

Tab. I: List of oligonucleotides used.

labeling	5'-3' sequence	features		purpose
P01	CCTTGCCACCAGATCTGCCATGGCTATGCTCAAGTGCGTGTCTGC	Nco I	U6 term	gRNA TGT1
P02	GTCTCGCGGCCAACCAGAATGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC	seed	sgRNA	
P03	ATTCTGGTTGGCCGCGAGACTCCATCACTGAGCATACGGTCAAGACAAGG	seed	U6 term	
P04	AGGAGGAGGAGGGCGGCCGCGCACTACCCACCAAGCG	Not I	U6 prom	
P05	GAAGATGGTGCAGGTAAGCGGTTTTAGAGCTAGAAATAGCAAGTTAAAA	seed	sgRNA	gRNA TGT2
P06	CGCTTACCTGCACCATCTTCTCCATCACTGAGCATACGGTCAAGACAAGG	seed	U6 prom	
P07	GTCTCGCGGCCAACCAGAAT	seed-specific		TGT1
P08	CGCTTACCTGCACCATCTTC	seed-specific		TGT2
P09	CCGACTGCAACAAGGTGTAG	pLEXY sequencing		
P10	CATCTATAGAGAAGTACACGTAAAAG			
P11	GCAAAGCGTTCCATCCGGTGGCTTGACCGCAGGCTTGCACCCAGGCTCGT	5' HR		TGT1 HRs
P12	CCTGCACGATGCCAAAGATGCACTGCTTTTCCTCCCTATCTCTCTCTCTC	3' HR		
P13	GTGCGACTGCTTCACCTGCAAGCGGCACTAGGCTTGCACCCAGGCTC	5' HR		TGT2 HRs
P14	GAATGTCGCTGTTCATTTCCTGAACGGTCCTCCCTATCTCTCTCTCTCCGCTC	3' HR		
P15	ACCACGGTAGCCGGTGATAA	WT TGT 2		
P16	ACCACCTGCGCAAGATTATG			
P17	ATGACCGAGTACAAGCCCAC	PuroR		
P18	GAGGCCTTCCATCTGTTGCT			
P19	CCTCCCGTTGGATTCGAAC	Asn		probes for APB
P20	TCCCGGCCGGGAATTGAA	Asp		
P21	GGGGAAACCGGGAATCGGAC	His		
P22	CCTTCCGGCCGGAATCGAAC	Tyr		
P23	CCAACAGGGGTCGAACCTGTGACC	Ile		
P23	GTTGTACACCGTCAGCTCCA	PfrD1		stage-specific primers for RT PCR
P24	CAAGGAGAACGAGGAGATGC			
P25	ACCAGGAGACAAGGGACCA	Sherp		
P26	CGCTTATCTTGTCCTTGATGC			
P27	TTCCTCGCGTTTCTCTTTGT	Ama		
P28	AAAATGGAAATGACCGCAAG			
P29	AACCAGTTCACGAAGGTGCT	60 S		
P30	CTTCGTCGACACCGTCTTCT			

In order to facilitate targeted mutagenesis and to enable selection of the transfectants, we discerned 30 bp regions flanking the expected (3‘ and 5‘) sites of the double strand break to produce a template for DNA repair by homologous recombination. These regions were employed for primer design to generate a cassette containing the puromycin resistance gene flanked by the 30 bp regions targeted for the homology recombination. The puromycin resistance was amplified (by Q5® HF Polymerase, using primers P11/P12 for TGT1 and P13/P14 for TGT2; Tab.I) from the cloning vector termed pVY013, a modified version of pLS6-PFR2 (Sollelis et al 2015), which was kindly provided by doc. MSc. Vyacheslav Yurchenko, Ph.D. (Life Science Research Centre, Faculty of Science, University of Ostrava). The amplicon was purified by GenElute™ PCR Clean-Up Kit. 10 ug of purified PCR product were used for electroporation.

All PCR reactions were performed using T100™ Thermal Cycler (Bio-Rad), run on 1% agarose gel containing ethidium bromide (at the final concentration of 0,2 µg/ml) and visualized using ChemiDoc™ MP Imaging System (Bio-Rad).

3.2.2. Electroporation

In total 20 µg purified DNA were resuspended in 10 ul Nucleofactor™ Solution (Amaxa™ Human T Cell Nucleofactor™ Kit, Lonza, Cat. No. VPA-1002). $5x10^7$ cells per transfection were harvested by centrifugation (at 1000 g, 10 min, 4°C), washed once with ice-cold PBS (137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4, 1,8 mM KH_2PO_4, pH 7,4) and resuspended in 90 µl of Nucleofactor™ Solution, DNA was added in 10 ul volume. The cell/DNA suspension was then transferred into certified cuvettes, which were placed into Nucleofactor™ Cuvette Holder and programme U-033 was applied. One electroporation was carried out without the addition of DNA serving as negative control.

Subsequently the cells were deposited into 5 ml of pre-warmed M199 medium (Hyg) and allowed to recover for 18 hours at 25°C prior to the addition of selection drugs (Ntc, Puro) in appropriate concentrations. At the same time the cultures were distributed into 24-well plates, clones were selected on liquid medium (supplemented as mentioned above).

3.3. Isolation of nucleic acids

Isolation of genomic DNA (gDNA) was performed as previously described (Medina-Acosta & Cross 1993) with slight modifications. In brief, $5x10^7$ cells were harvested by centrifugation (at 1000 g, 10 min, RT), washed with PBS and resuspended in lysis buffer (100 mM NaCl, 20 mM Tris pH 8, 20 mM EDTA pH 8, 1% SDS). Upon the addition of RNAse A (Quiagen, Cat. No. 19101) to the final concentration of 80 µg/ml the sample was incubated at 37°C for 30 min, proteinase K (MP Biomedicals, Cat. No. 193504) was then added to the final concentration of 80 µg/ml and the sample was further incubated at 37°C for 2 hours. The DNA was purified by phenol-chloroform extraction (1V phenol, pH 8; 0,3V chloroform/IAA, 24:1), following centrifugation (at 12000 rpm, 10 min), the aqueous layer was transferred to a new tube, EtOH-precipitated and dissolved in MiliQ® water.

Isolation of RNA was carried out as described previously (Chomczynski & Saschi 2006) with modifications. Concisely, $5x10^7$ cells were harvested by centrifugation, washed with PBS and resuspended in 0,5 ml solution D (4 M guanidine isothiocyanate, 25 mM sodium citrate pH 7, 0,5% sarcosyl, 0,1% β-mercaptoethanol). Sodium acetate (pH 4) was added at 200 mM concentration, the sample was then mixed with equal volume of (H_20 saturated) phenol and 0,3V chloroform/IAA (24:1), vortexed for 1 min, placed on ice for 10 min and spun (at 12000 rpm, 15 min, 4°C). The aqueous phase was then precipitated using equal amount of isopropanol, following centrifugation RNA pellet was dissolved in MiliQ® water and purified afresh using 0,5V phenol (pH 8), 0,5V chloroform/IAA. Subsequent to the second

phenol-chloroform extraction, the aqueous phase was EtOH-precipitated and dissolved in MiliQ® water.

Concentration and purity of nucleic acid samples was determined using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific).

3.4. Boronate affinity electrophoresis

Boronate affinity electrophoresis was performed as described elsewhere (Gabar & Kössel 1985) with modifications. Briefly, 5 µg of total RNA was deacetylated by incubation in 100 mM TRIS-acetate (pH 9) at 37°C for 30 min, EtOH-precipitated and resuspended in urea loading dye (8 M urea, 20 mM TRIS-HCl pH 8, 1 mM EDTA, 5% bromophenol blue/xylene cynaol). Periodate-oxidized RNA was employed as technical control, this sample was prepared by incubation of 5 µg of deacylated RNA in 50 mM Na-acetate (pH 4,5-5,2) and 2,5 mM $NaIO_4$ in the dark, at 37°C for 2 hours, followed by addition of glucose to the final concentration of 2 mM, further incubation at 37°C for 30 min and purification by Sephadex™ G-25 (GE Healthcare, Cat. No. 17-0572-02) column. RNA was then EtOH-precipitated and resuspended in urea loading dye. The samples were denatured at 70°C for 10 min prior to loading on the gel.

3-(Acrylamido)phenylboronic acid (APB) (2,5 mg/ml; Sigma-Aldrich, Cat.No. 771465-1G) was dissolved at RT for 30 min in the gel solution (7 M urea, 8% acrylamide (40%, 19:1) in 1x TAE (i.e. 40 mM TRIS-acetate, 1 mM EDTA)), once dissolved APS (final concentration 0,06%) and TEMED (1 µl/1 ml gel solution) were added to facilitate polymerization. Gel electrophoresis was performed at 4°C, 75V for 5,5 hours, the gel was then stained with EtBr (visualized using ChemiDoc™) serving as loading control.

3.4.1. Northern blotting and hybridization

Following boronate-affinity electrophoresis, RNA was transferred on ZETA probe membrane (Bio-Rad) at 150 mA for 90 min in 0,5x TAE. Subsequently, the membrane was dried, UV cross-linked (Stratagene, Stratalinker® UV Crosslinker) and pre-hybridized at 48°C for at least 30 min (hybridization solution: 5x SSC, 20 mM P_i, 7% SDS, 1x Denhardt's solution, and 1 mg/ml salmon sperm DNA; Denhardt's solution: 0,02% Ficoll 400, 0,02% polyvinylpyrrolidone, 0,02% BSA).

The sequences of oligonucleotides used for northern hybridization are listed in Tab I (P19 – P23). The primers were radioactively end-labelled with γ-^{32}P ATP (Hartmann Analytic, Cat. No. FP-401, 9,25MBq) using T4 Polynucleotide kinase (NEB, Cat. No. M0201S) according to manufacturer's instructions, purified by Sephadex™ G-25 column and denatured at 98°C for 5 min prior to transfer into the hybridization solution. The membrane was hybridized at

48°C overnight, washed at 48°C 2x 20 min (wash I: 3x SSC, 5% SDS, 25 mM NaH_2PO_4, 10x Denhadt's solution; wash II: 1x SSC, 1% SDS), exposed to Phosphoimager screen (GE Healthcare) overnight and developed using Typhoon scanner (Molecular Dynamics). The membrane was then stripped by heating at 80°C 2x 20 min (stripping solution: 0,1x SSC, 0,1% SDS) and re-hybridized with the control probe.

3.5. *In vitro* differentiation

In vitro differentiation was performed as described previously (Bates 1994) with slight modifications. In brief, isolated lesion amastigotes were cultivated in biphasic medium composed of blood agar (1,5% Neopepton, 1,5% Bacto agar, 0,5% NaCl, 20% human blood) overlaid with equal volume of M199 medium. Once the promastigotes reached logarithmic phase of growth (usually within 5 days) they were transferred into M199 medium pH 7, 10% FBS.

In order to differentiate these into the infective metacyclic form cells were seeded at the density of $2x10^5$/ml and cultured in M199 medium pH 5,5, 10% FBS, the parasites were regarded as metacyclic 4 days after reaching stationary phase (i.e. cell density dropped by 10% over the preceding 24 hours), at this point (day 10) the cultures were harvested for macrophage and/or murine infection studies. Amastigotes could be observed within one week following transfer of the metacyclic culture to 32°C (otherwise incubated under the same conditions).

In order to assess stage-specific gene expression by quantitative reverse transcription PCR (qRT-PCR) RNA was harvested on days 0, 8, 10, 13 and 17 of differentiation.

3.5.1. RT PCR

Reverse transcription of RNA into cDNA was performed using QuantiTect Reverse Transcription Kit (Qiagen, Cat. No. 205311) following the instruction manual. The cDNA was utilized for qRT-PCR using stage-specific primers (Tab.I, P24-P29), the reaction was carried out using *Power* SYBR™ Green PCR Master Mix (ThermoFisher Scientific, Cat. No. 4367659) according to manufacturer's instructions and executed on LightCycler® 480 Real-Time PCR System (Roche).

3.6. *In vitro* infection of macrophages

3.6.1. Isolation of peritoneal exudate cells (PECs)

Isolation of murine macrophages was performed as described previously (Zhang et al 2008). Thioglycollate (TG) - elicited PECs were harvested 4 days after i.p. injection of 2 ml 3% TG medium (Difco™, Cat. No. 225640).

Concisely, 8 - 10-week-old female BALB/c mice were euthanized by cervical dislocation (CD), their peritoneal cavity was injected with 5 ml ice-cold PBS supplemented

with 3% FBS and gently massaged for approximately 3 min, the cell suspension was then withdrawn using 24 G needle and deposited into falcon tubes, this step was repeated twice for each mouse, whilst the isolated PECs were kept on ice. The cellular exudates were washed with PBS (at 1000 g, 10 min, 4°C), resuspended in RPMI medium (supplemented as mentioned above) and checked for presence of any contamination potentially originating from the dissection procedure.

The number of viable cells was determined using Trypan blue (Sigma-Aldrich, Cat. No. T8154) exclusion assay performed according to manufacturer's instructions. The cells where then pooled, seeded at the concentration of $3x10^5$/well (in 1 ml volume) on glass cover slips (P-lab, Cat. No. H101112) situated in 24-well plates and incubated at 37°C, 5% CO_2 overnight. Non-adherent cells were removed by repeated washes with PBS, while the remaining (adherent) cell population was used for infection studies and will be referred to hereafter as macrophages. For some experiments macrophages were primed with IFN-γ (5ng/ml; Invitrogen, Cat. No. PMC4031) 24 hours prior to infection and activated with a combination of IFN-γ (5 ng/ml) and LPS (10 ng/ml; Sigma-Aldrich, Cat. No. L2630-10MG) 4 hours post infection (corresponding to time point 0 as mentioned in the following chapter). Macrophage-parasite interaction experiments were carried out at 34°C, 5% CO_2.

3.6.2. Macrophage infection *in vitro*

Infection of murine macrophages was performed as described previously (Sousa-Franco et al 2005) with modifications.

The parasites were differentiated into metacyclic form *in vitro* as described above (seeding log-phase promastigotes into M199 medium pH 5,5, 10% FBS) and harvested (at 1000 g, 10 min, RT) on day 10 of differentiation, washed once and resuspended in RPMI medium to the required density ($3x10^6$/ml).

Macrophage cultures were exposed to parasites (at 1:10 macrophage:parasite ratio; in 2 ml final volume) for 4 hours at 34°C, 5% CO_2. Non-internalized parasites were removed with four successive washes with PBS, subsequently replaced with fresh medium with or without stimulants. This point was considered as initial time of infection (i.e. 0 hpi). Cultures of infected macrophages were incubated up to 5 days under the same conditions.

Samples were collected during the course of the experiment (at 0, 24, 72 and 120 hpi) for determination of the number of intracellular parasites per infected macrophage and the percentage of macrophages infected. For this purpose, the slides were washed twice with 1x PBS, fixed in 4% paraformaldehyde for 20 min, washed twice afresh, permeabilized with ice-cold methanol for 20 minutes at -20°C, washed three times with PBS and subsequently stained

using a mountant with DAPI (ThermoFisher Scientific, Cat. No. P36935). The slides were stored in the dark, 4°C until analysed using fluorescence microscope (Zeiss, Axioplan 2 imaging Universal Microscope), at least 230 macrophages were inspected per sample.

3.7. *In vivo* infection of mice

Female BALB/c mice were purchased from VELAZ, s.r.o. (Prague, Czech Republic) and maintained under controlled conditions by the Animal facility, Institute of Parasitology, BC CAS in České Budějovice, in accordance with the institutional guidelines. The animals were used for infection at 8 - 10 weeks of age. The infection of animals was carried out as previously described (Belklaid et al 2000) with modifications.

L. mexicana WT and TGT2 KO strains were differentiated in M199 medium pH 5,5, 10% FBS as mentioned above. Infective-stage metacyclic promastigotes were harvested from stationary-phase cultures (day 10), washed and resuspended in PBS to the desired concentration. The cells were inoculated intra-dermally in 25 µl volume into left ear pinna using 29 G needle. Injection of 25 µl PBS served as negative control.

In the pilot infection experiment two groups of five and six animals were infected with $2{,}5x10^6$ cells per mouse of WT and TGT2 KO strains respectively, for the dosage experiment the size of inoculum varied from $1x10^2$ to $1x10^5$ parasites utilizing four groups of three mice. Disease progression was monitored at 1 - 2 week-intervals (up to 15 weeks post infection) by measuring the diameter of the ear induration with a digital calliper. The results are expressed as the increase in thickness of the left ear, compared to the uninfected right ear.

Peripheral blood samples (30 µl) were obtained during the course of the pilot infection experiment (at 1 week-intervals) by tail snapping, plasma samples were acquired following centrifugation at 200 g, 30 min, 4°C. The blood plasma (present in the form of supernatant) was collected for studies on cytokine production using ELISA (Osuchowski et al 2005), while the cell pellet could be utilized for the same purpose employing RT-PCR. Both samples respectively were therefore stored at -80°C for further examination.

Once the experiment was terminated, mice were euthanized by CD, ears were collected for histopathological analysis and the lesions were detached for estimation of parasite load. Total blood volume was collected for analysis of cytokine production by ELISA and/or RT-PCR. The weight of spleen was considered as an indicator of the immune response, and the splenocytes were isolated for further inspection of cytokine profiles.

3.7.1. Estimation of parasite load

Relative parasite burdens were determined by limiting dilution analysis as described previously (Titus et al 1985). In brief, the tissue was incubated in M199 medium at 37°C for approximately 2 hours, homogenized using a syringe plunger and filtered through 70 μm pore size nylon cell strainer (Sigma-Aldrich, Cat. No. CLS431751-50EA). The tissue homogenate was then serially diluted in a 96-well plate containing biphasic medium (100 μl volume per well in total). The number of parasites in each sample was determined from the highest dilution at which viable promastigotes could be observed after 7 days of incubation at 25°C, the results are expressed as log parasite titer.

3.7.2. Histopathology

For histopathological examination, the ears were first fixed in 40% formalin for 10 days, then dehydrated by series of ethanol solutions of increasing concentrations (70% EtOH 60 min, 80% EtOH 2x 45 min, 90% EtOH 2x 45 min, 100% EtOH 3x 30 min). Xylene was applied as a clearing agent (1:1 100% EtOH:xylene 2x 10 min, xylene I 10 min, xylene II 40 min), finally the samples were embedded in paraffin (2 days incubation) and cut into 5 μm sections. Subsequently, these sections were re-hydrated (xylene I 5 min, xylene II 5 min, 100% EtOH 5 min, 96% EtOH 5 min, dH_2O 5 min) and stained by routine hematoxylin-eosin staining (hematoxylin 5 min, acid alcohol 5 min, washed with dH_2O 20 min, 0,1% eosin 3 min, and dehydrated as mentioned above with 5 min incubation at each step).

3.7.3. Splenocyte culture

The spleen was weighed, placed in a 6-well plate containing PBS and homogenized using a syringe plunger. The tissue homogenates were filtered through 70 μm pore size nylon cell strainer and then spun down at 200 g, 10 min, 4°C. The pellet was resuspended in equal amounts of PBS and ACK lysis buffer (also referred to as RBC lysis buffer; 150 mM NH4Cl, 10 mM KHCO3, 0,1 mM Na2EDTA). The cell suspension was incubated at 37°C for 3 min, spun under the same conditions, washed three times with PBS and resuspended in RPMI medium (supplemented as described above).

The number of viable cells was determined using Trypan blue exclusion assay and the concentration was adjusted to $5x10^6$/ml corresponding to the cell seeding concentration. Splenocytes were then stimulated using concanavalin A at the final concentration of 2,5 μg/ml (Sigma-Aldrich, Cat. No. C2010-100MG), with some samples left untreated to serve as negative controls. The cultures were incubated at 37°C, 5% CO_2 up to 72 hours; culture supernatants were collected every 24 hours and stored at -80°C for further analysis by ELISA, the cells were harvested for examination by RT-PCR at the last time point of the experiment.

Ethical statement

All experimental procedures were conducted in accordance with the law of the Czech Republic on the use of experimental animals, safety and use of pathogenic agents. The study was approved by the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic and Institutional and National Committees (protocols no. 73/2017).

4. Results

4.1. Identification of LmxTGT1 and LmxTGT2 genes

The orthologues of TGT1 and TGT2 genes in *L. mexicana* were identified by BLAST search using the sequences of their respective human counterparts. The analysis has revealed that LmxTGT1 (XP_003877324.1) and LmxTGT2 (XP_003876577.1) share 48% and 32% sequence identity respectively, as compared to the human equivalents. When aligned, LmxTGT1 displays all the conserved aspartate residues critical for the catalytic activity as well as the cysteine and histidine residues involved in co-factor binding. On the contrary, LmxTGT2 only displays the residues important for co-factor binding (Fig. 5).

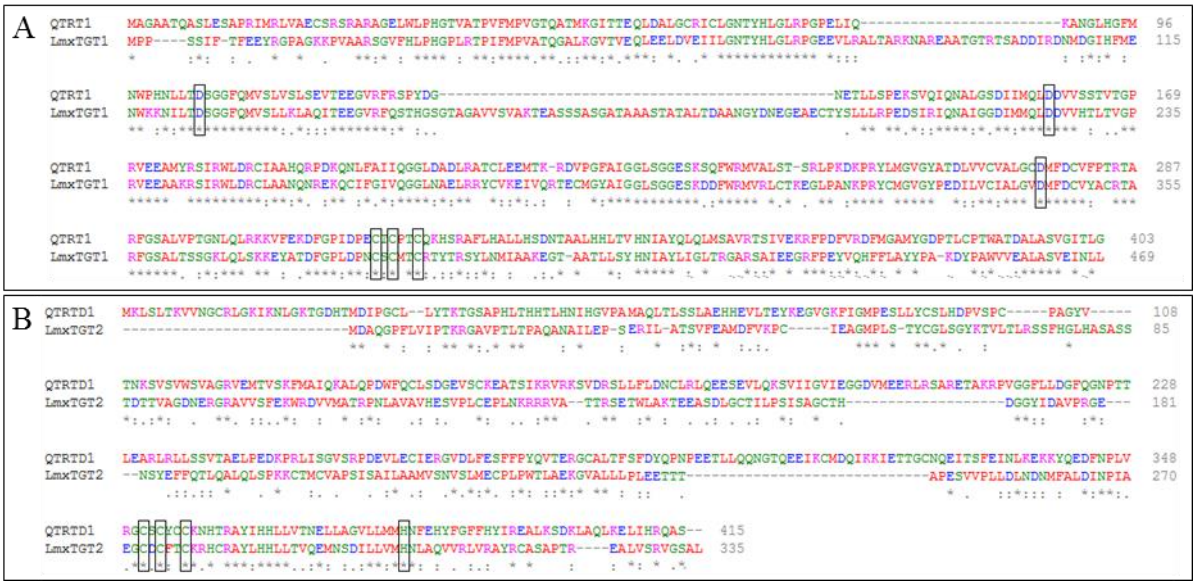

Fig. 5: Protein alignment of A) human QTRT1 with LmxTGT1 and B) human QTRTD1 with LmxTGT2. The catalytic (aspartate) and co-factor-binding (cysteine, histidine) residues are highlighted.

4.2. Determination of presence of Q in Lmx tRNA$^{Aspn, Asn, His, Tyr}$

In order to ascertain the presence of Q in the anticodon position of the relevant tRNA species, we performed boronate affinity electrophoresis of *L. mexicana* WT total RNA, followed by northern blotting and hybridization with the respective tRNA species-specific probes. Ethidium bromide gel staining showed good-quality RNA of equal loading (Fig. 6A), since the method is based on the affinity of cis-diol groups to APB resulting in the differential mobility of G- and Q-tRNAs (Gabar & Kössel 1985); oxidized RNA was employed as negative control of the technique, Q-non-containing tRNA (Ile) represents negative control. As a result, it was confirmed that at least three out of the four corresponding tRNAs are modified with Q to various levels (Fig. 6B).

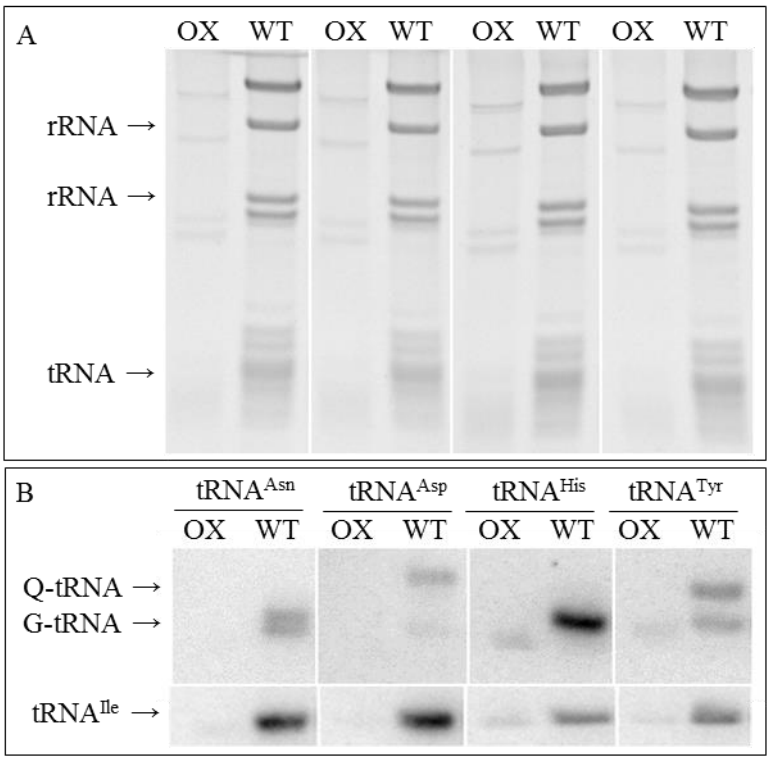

Fig. 6: Northern blot of *L. mexicana* WT total RNA; A) Ethidium bromide gel staining, B) hybridization of the northern blot with tRNA species-specific ^{32}P-labeled probes (as indicated above each image; see Tab. I). OX standing for periodate-oxidized RNA serving as negative control of the technique, $tRNA^{Ile}$ serving as negative control.

4.3. Generation of the LmxTGT knock-out cell lines

4.3.1. Generation of the constructs

To address the role of LmxTGT in Q-tRNA formation, CRISPR/Cas9 approach was utilized for generation of LmxTGT1 and LmxTGT2 KO cell lines. The construct for the expression of sgRNA was generated using the pLEXY vector (for details see Fig. 7) as a template for the amplification of the corresponding regions. The resulting fusion PCR products (Fig. 8A), as well as the pLEXY vector were subjected to double-digestion, by a matching pair of restriction enzymes (Fig. 8B), and subsequently ligated together in order to facilitate the final substitution of the original seed sequence with the genes-of-interest specific sequences. The homology template for DNA repair was produced using the pVY013 vector as a template for the amplification of puromycin resistance flanked by genes-of-interest regions of homology (Fig. 8C).

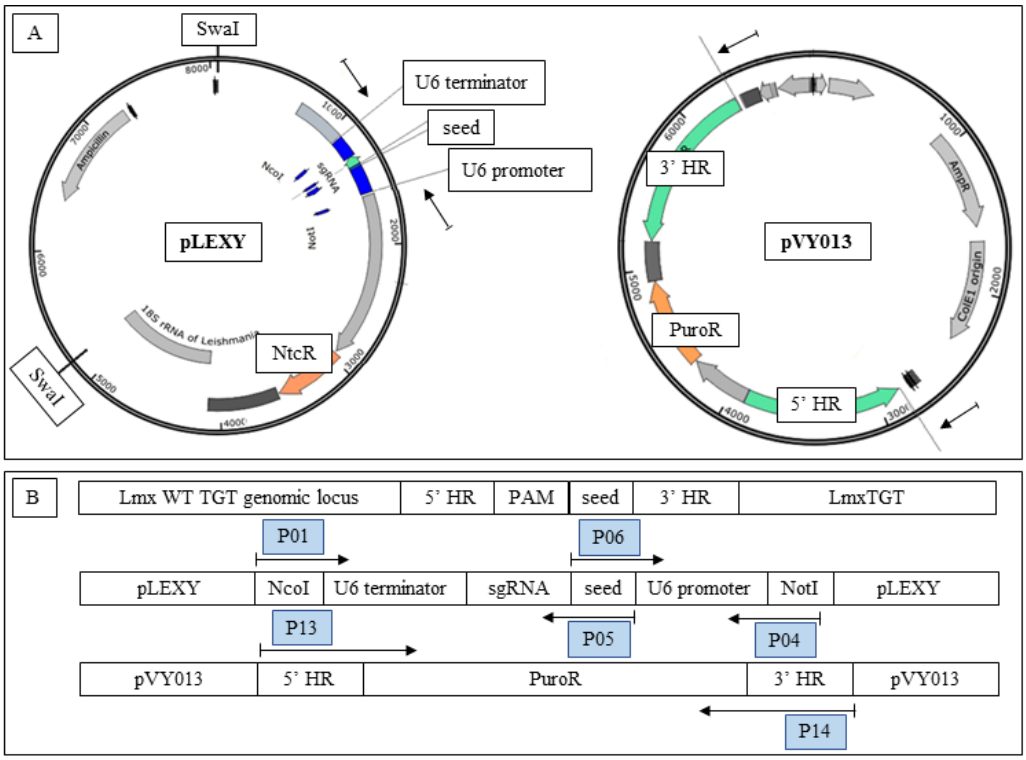

Fig. 7: Schematic depiction of the cloning strategy and primers used; A) vector maps; B) Replacement of gene-specific part of sgRNA (seed) in pLEXY and generation of template for DNA repair by homology recombination using pVY013 by PCR.

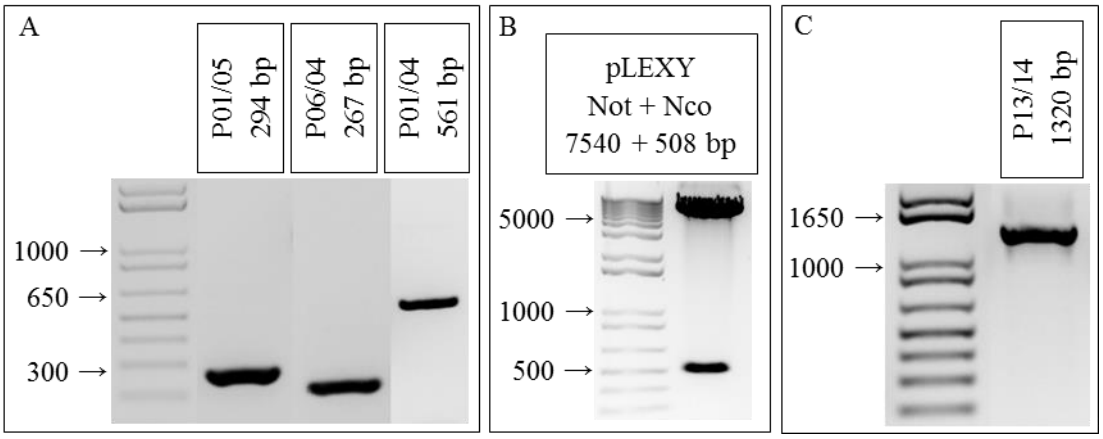

Fig. 8: Agarose gel electrophoresis; A) The sequential amplification of gene-of-interest-specific seed sequences of the sgRNA and the final fusion product subjected to double-digestion and followed by ligation into the restricted pLEXY vector; B) pLEXY double-digestion; C) The amplicon used as homology repair template. Primer combinations and the expected product sizes are indicated at the labels above each line. Negative controls not shown. Results for TGT1 not shown.

4.3.2. Confirmation of correct integration of the construct

Following the electroporation of the respective gene-specific constructs into the *L. mexicana* Cas9-expressing cell line (referred here to as WT), transfectants were selected in liquid medium containing appropriate antibiotics and individual clones were obtained by single-cell-dilution in tissue culture plates. Once recovered, gDNA was isolated for analysis by PCR (Fig.9).

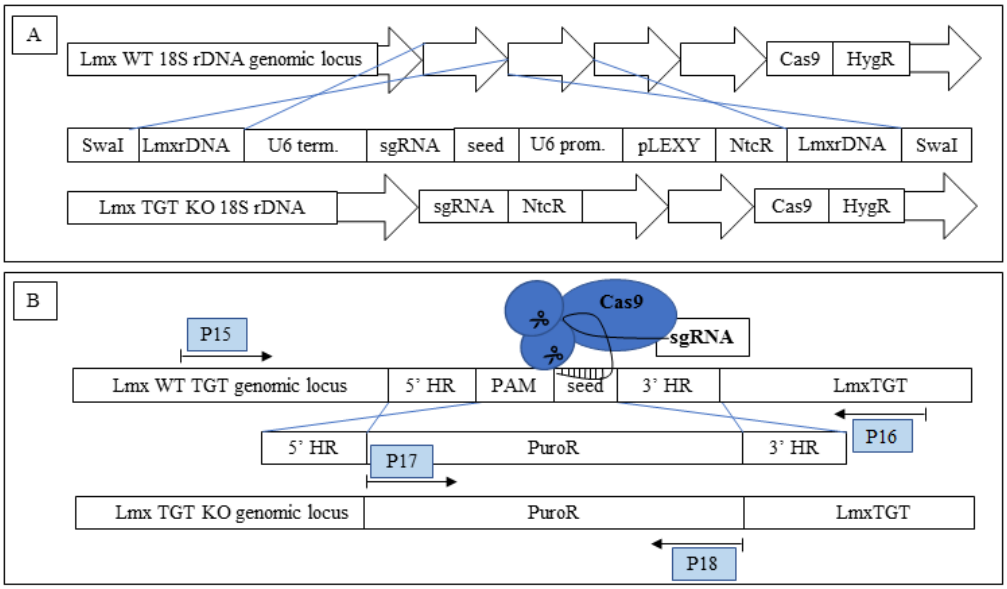

Fig. 9: Schematic depiction of recombination of the introduced constructs with endogenous genomic loci; A) recombination of the construct coding for sgRNA; B) double strand break introduction by Cas9 and repair by homology recombination with the template provided leading to the disruption of TGT2 ORF by PuroR.

Despite performing three independent repeats of the electroporation of constructs targeted for the ablation of LmxTGT1, we failed to produce the TGT1 KO cell line, most probably due to its lethal phenotype.

Correct integration of the repair template, following the induction of the double-strand break by sgRNA-guided Cas9, aimed for the disruption of LmxTGT2 ORF by puromycin resistance gene was verified by PCR, using primers annealing either to the WT TGT genomic locus or the resistance marker introduced in TGT2 KO cell line, additionally combinations of these primers were also utilized (for reference see Fig. 9). Here we provide an evidence for the successful generation of the TGT2 KO cell line (Fig. 10).

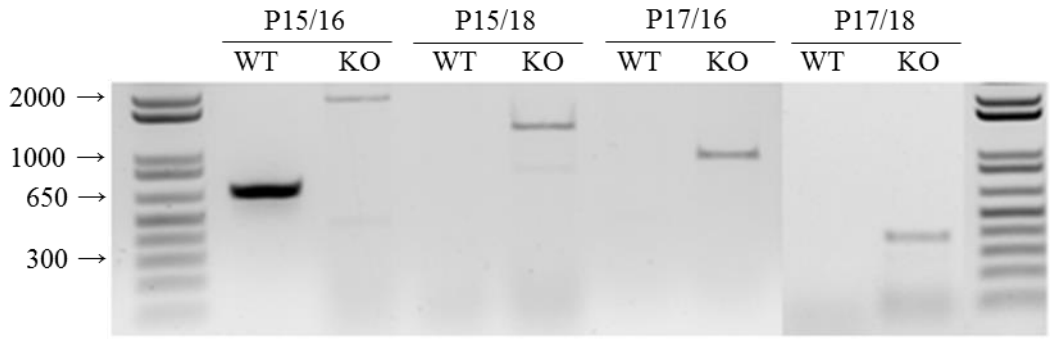

Fig. 10: Agarose gel electrophoresis of the PCR products resulting from the reactions indicated at the labels of respective lines. The expected sizes of the PCR products are listed in Tab. II. WT cell line was employed as negative control.

Tab.II: Expected product size [bp]

Primer combination		WT	KO
15	16	624	1922
17	16	0	925
15	18	0	1333
17	18	0	336

4.4. Evaluation of the role for LmxTGT2 in Q-tRNA(s) formation *in vivo*

For the assessment of Q-tRNA content in the TGT2-deficient cell line, total RNA was isolated and resolved by boronate affinity electrophoresis. Following ethidium bromide gel staining and subsequent transfer by northern blotting, the RNA was subjected to hybridization with Q-tRNA specific probes. The resulting radiographs showed complete depletion of Q in all corresponding tRNA species (Fig. 11), thus attesting the role for LmxTGT2 in Q-tRNA formation *in vivo*.

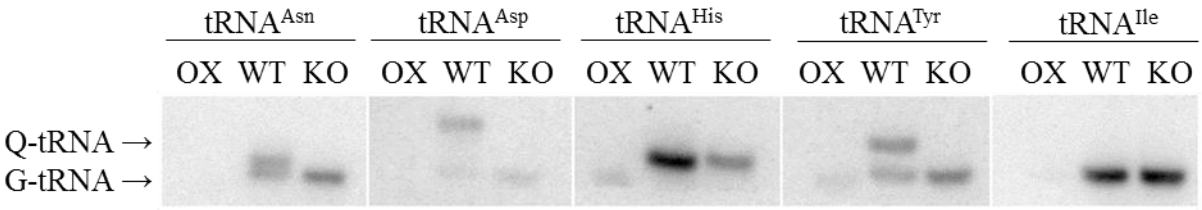

Fig. 11: Northen blot of total RNA isolated from WT and TGT2 KO cell line hybridized with Q-tRNA specific ^{32}P-labeled probes revealed complete depletion of Q-tRNAs. OX standing for periodate-oxidized RNA serving as negative control of the technique, tRNA^{Ile} serving as negative control.

4.5. The effect of LmxTGT2 ablation on cell growth *in vitro*

We first investigated the effect of LmxTGT2 ablation on *L. mexicana* growth by comparing the cell division kinetics of WT and TGT2 KO strains *in vitro*. The TGT2 KO strain did not exhibit any significant difference in growth rate, as compared to the parental cell line (Fig 12).

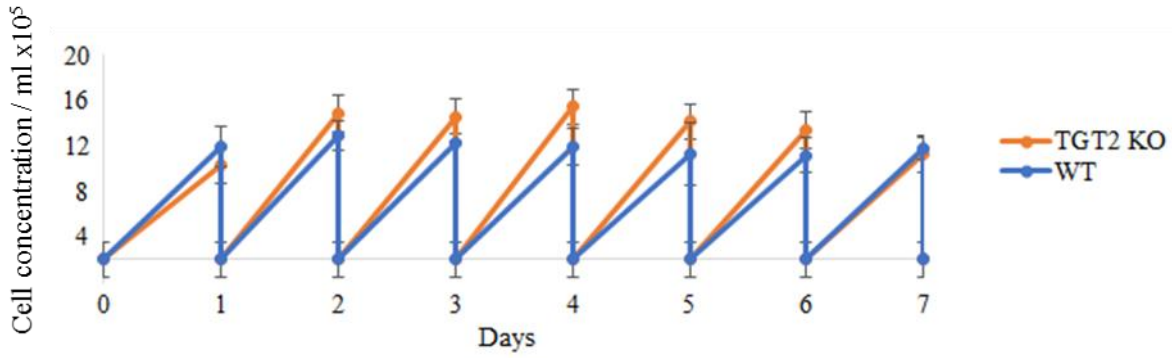

Fig. 12: Growth-curves of *L. mexicana* WT and TGT2 KO cell lines, the growth-curves represent an average value of two repeats, error bars indicate standard deviation.

4.6. *In vitro* infection of macrophages

Although the ablation of LmxTGT2 did not affect cell proliferation in the axenic culture, we presumed that the absence of Q could perhaps be demonstrated if the parasites were subjected to a more challenging environment within their host cells. To test the plausible impact of LmxTGT2 KO on the virulence of the parasites, we conducted *in vitro* infection of TG-elicited murine macrophages. First, we compared the ability of WT and TGT2 KO strains to infect and multiply within the unstimulated macrophages. The experiment revealed a slight,

yet significant, impairment of the ability of TGT2 KO strain to infect macrophages as well as to multiply within the phagolysosomal environment (Fig. 13, 14).

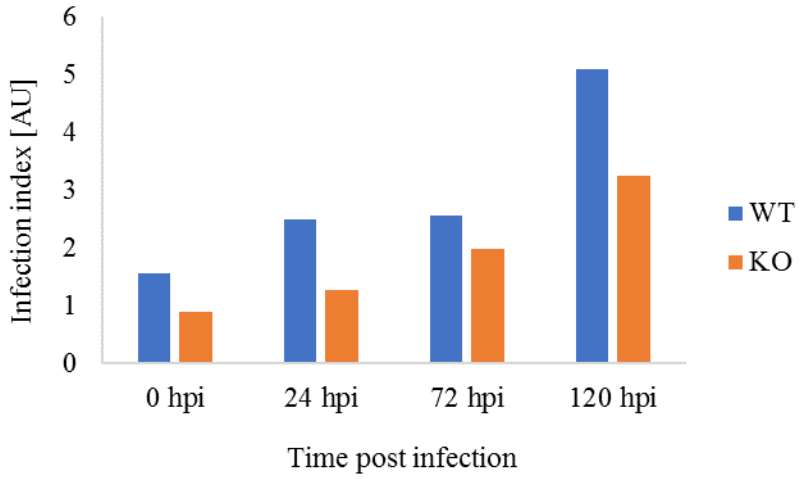

Fig. 13: Infection of unstimulated macrophages with WT and TGT2 KO cell lines; the data represent three experiments of two technical repeats, 230 cells were inspected per sample. Results are expressed in the form of infection index, which was calculated as the number of intracellular parasites per infected macrophage multiplied by the percentage of macrophages infected.

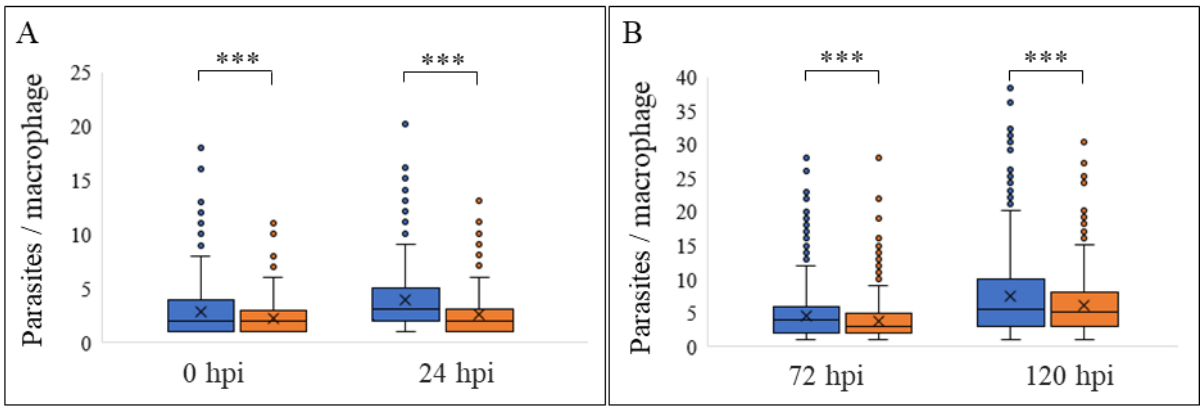

Fig. 14: Number of intracellular parasites per infected macrophage at different time points as indicated; the data represent three experiments of two technical repeats, 130 cells were inspected per sample. The experiment revealed a significant difference ($p < 0.0015$) between the strains (▪WT, ▪TGT2 KO) at all the time points examined. Statistical analysis of the results was performed using T-test paired two sample for means.

We had, however, questioned the feasibility of this observation to become more pronounced if macrophages were classically activated by the combination of IFN-γ and LPS, as macrophages activated in this manner typically produce nitric oxide, which is toxic to the parasites. With the literature suggestion for a role of Q-tRNAs under stress conditions and in the antioxidant defence mechanisms, we wondered whether TGT2 KO parasites could resist the microbicidal power with the same efficiency as WT cell line. Although the phagocytosis in IFN-γ-primed macrophages was generally enhanced, TGT2 KO strain was either taken up in lower numbers or its ability of intracellular survival was considerably decreased already in the first time point examined. Furthermore, while WT parasites were still capable of multiplication within the macrophages up to 24 hours post infection, this ability was

completely abolished with regard to TGT2 KO strain (Fig 15). In conclusion, the results suggested that the ability of TGT2 KO parasites to survive intracellularly were significantly decreased, pointing to a possibility that the cell line may be possessing certain developmental defects.

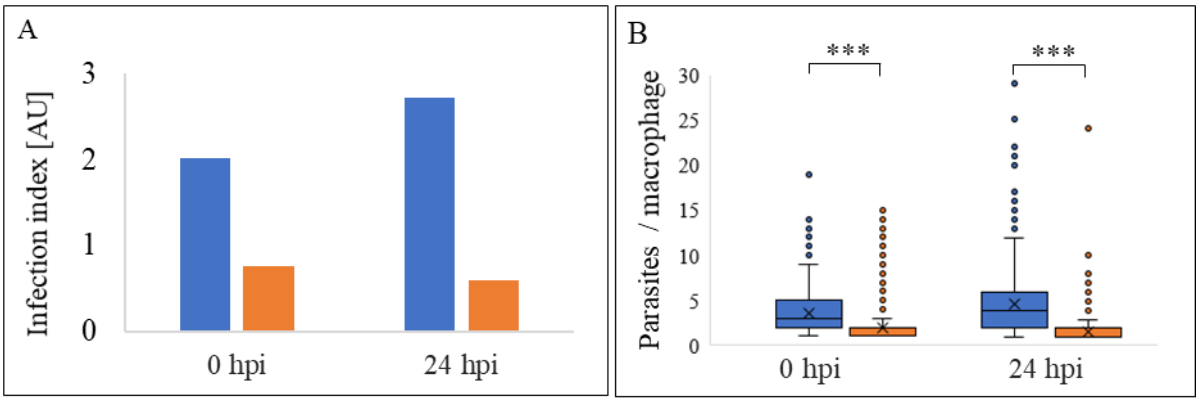

Fig. 14.: Infection of stimulated macrophages; the data represent three experiments of two technical repeats and were analysed as for *in vitro* infection of unstimulated macrophages; A) infection index, B) number of intracellular parasites per infected macrophage, for ▪WT and ▪TGT2 KO cell lines.

4.7. The effect of LmxTGT2 ablation on *in vitro* differentiation

In order to determine whether the disruption of LmxTGT2 and/or the consequent depletion of Q-tRNAs affected the development of the parasite, we induced complete life cycle differentiation in axenic culture. Total RNA was harvested at specific time points throughout the period of the *in vitro* differentiation for the expression analysis of previously identified stage-specific markers (Pfr1d for procyclic and metacyclic promastigotes, Amastin for amastigotes) by qRT-PCR. For normalization, the expression value of LmxM.07.0510 (gene encoding a 60S ribosomal protein L7a) was used (Ishemgulovat et al 2017). Whereas the expression of promastigote-specific marker was unaffected, the expression levels of the amastigote marker showed considerable differences in between the strains. Thus, the data obtained suggest that the absence of TGT2 presented difficulties for metacyclic-to-amastigote life stage transition (Fig. 15).

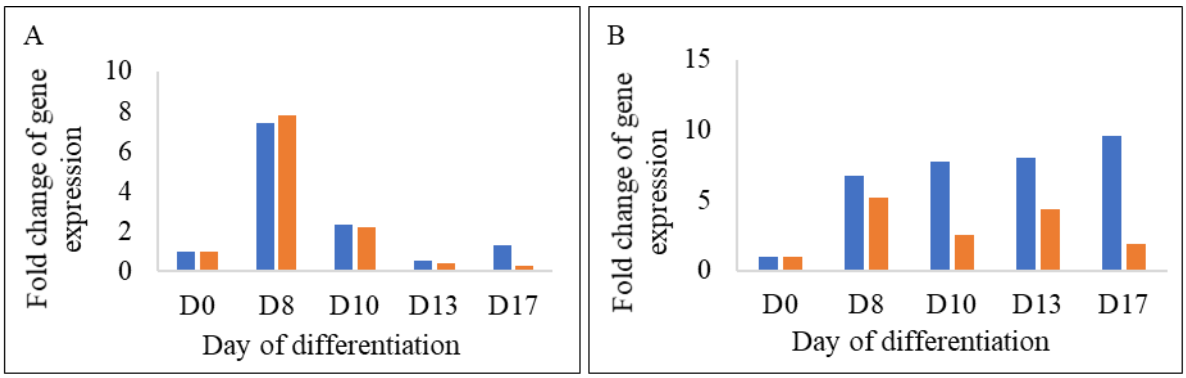

Fig. 15: qRT-PCR analysis of stage-specific markers for ▪WT and ▪TGT2 KO cell lines, the data represent mean value of three technical repeats. A) Pfr1d, marker of procyclic and metacyclic promastigotes, B) Amastin, marker for the amastigote stage.

4.8. Q levels throughout *L. mexicana* life cycle

The results obtained from the previous experiments led us to hypothesize that if TGT2 absence was to affect the development of parasites in the amastigote stage exclusively, it would imply that the requirements for Q modification levels differ among the individual life stages. In order to test this possibility, we performed boronate affinity electrophoresis of WT total RNA comparing the Q content of $tRNA^{Tyr}$. This was performed using logarithmic phase procyclic promastigotes as well as cells at specific time points of the differentiation, particularly day 0 when parasites are at the very beginning of their transition to the next life stage, day 10 representing metacyclic promastigotes and day 17, when the majority of cells are present in the amastigote form. The experiment revealed that the levels of Q modification were increasing throughout the life cycle, starting immediately from day 0 of differentiation until reaching metacyclic stage, while the amastigote culture, on the other hand, showed almost equal amount of Q- and G-tRNAs (Fig. 16).

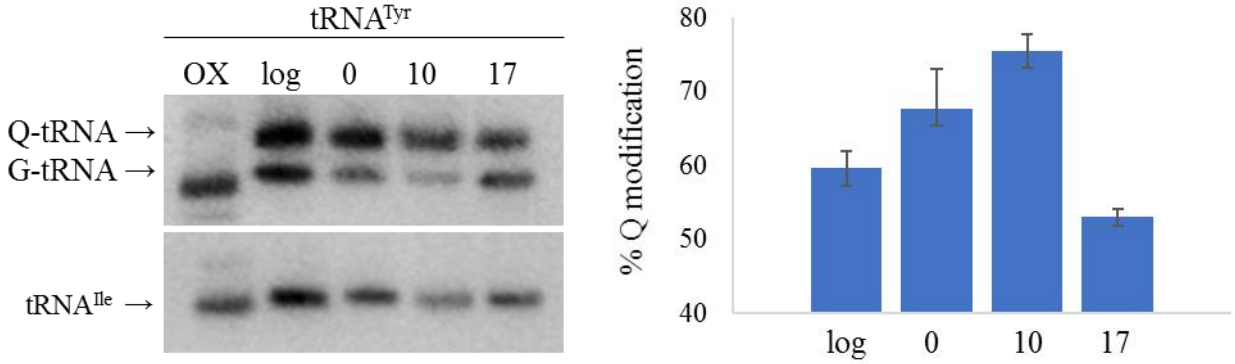

Fig.16: Q levels throughout the life cycle; northern blot of *L. mexicana* WT total RNA from log phase procyclic promastigote culture (log) and days 0, 10 and 17 of differentiation. Q percentage content was assessed using ImageQuant software and represents a mean value of three technical repeats, error bars represent standard deviation.

4.9. *In vivo* infection of mice

To test whether the outcome of the macrophage experiment could possibly be reflected in an altered susceptibility of mice to TGT2 KO as compared to WT parasites, murine infections were carried out.

First, we performed a pilot infection experiment using a high dose of parasites inoculated intradermally, which triggered off clinical symptoms in all mice. Although only two (2) out of six mice infected with TGT2 KO strain developed an apparent pathology, the size of these lesions was actually greater than those of mice infected with the WT cell line, while the ear thickness of the remaining four (4) mice was comparable to that of the uninfected control group. TGT2 KO-infected animals are therefore depicted as two separate groups (labelled as KO (2) and (4); Fig. 17). The mean size of lesions by the end of the experiment were likewise similar in between the two (2) mice infected with TGT2 KO and WT strains, respectively, both of which, however, differed significantly from the other four (4) mice infected with TGT2 KO parasites (Fig. 18).

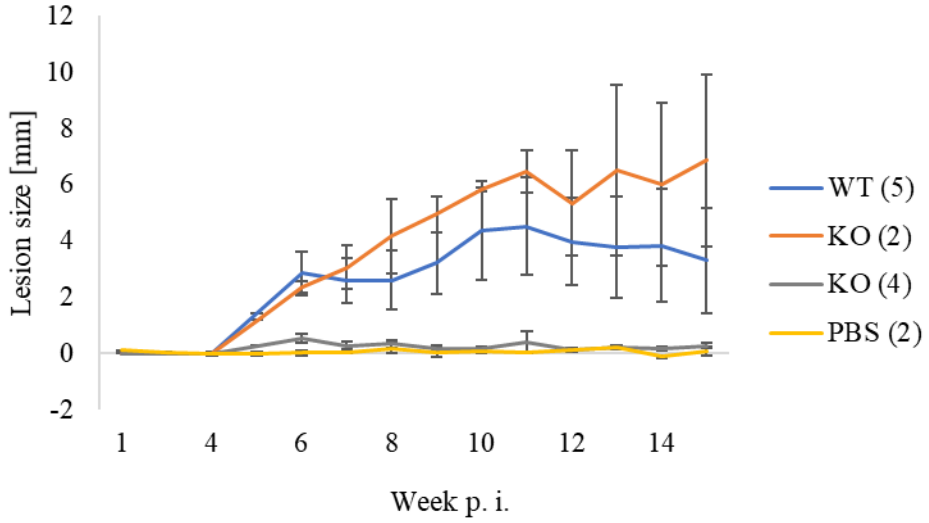

Fig. 17: Experimental infection of BALB/c mice with WT and TGT2 KO parasites.Each mouse was inoculated with $2{,}5x10^6$ parasites in 25 µl volume. Additionally, two mice were injected with equal volume of PBS, serving as negative control. Hence, in the legend of the chart numbers in brackets represent the number of animals per each group. Error bars represent standard deviations.

Additionally, the weight of the spleen was employed as an indicator of the immune response. Mice infected with TGT2 KO parasites showed overall lower values, however, one of the two (2) mice within this group exhibited an evident splenomegaly (Fig. 19).

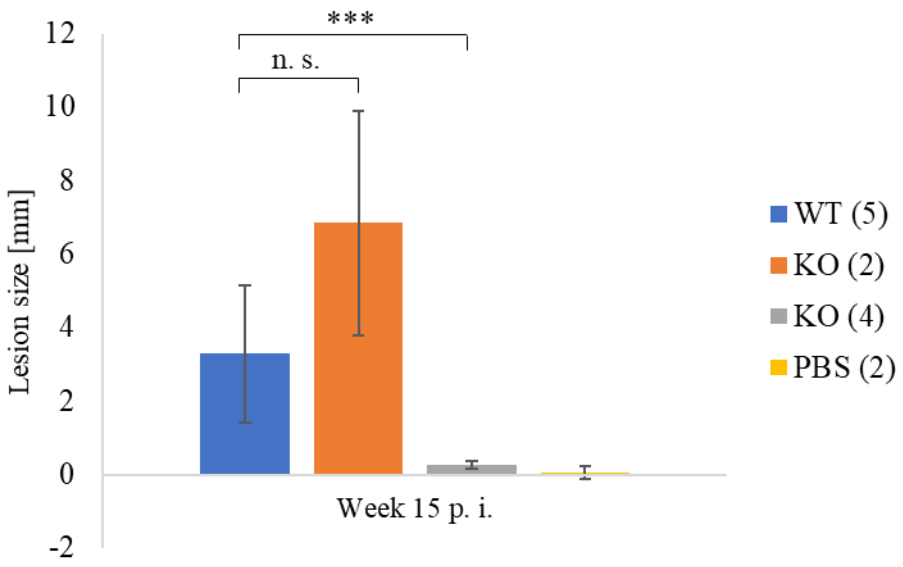

Fig. 18: Mean lesion size at week 15 post infection showing a non-significant difference between WT and TGT2 KO (2) as contrasted to the significant (p= 0.0015) difference between WT and TGT2 KO(4)-infected animals. Numbers in brackets are standing for the number of animals per each group, error bars represent standard deviations. Statistical analysis of the results was performed using T-test paired two sample for means.

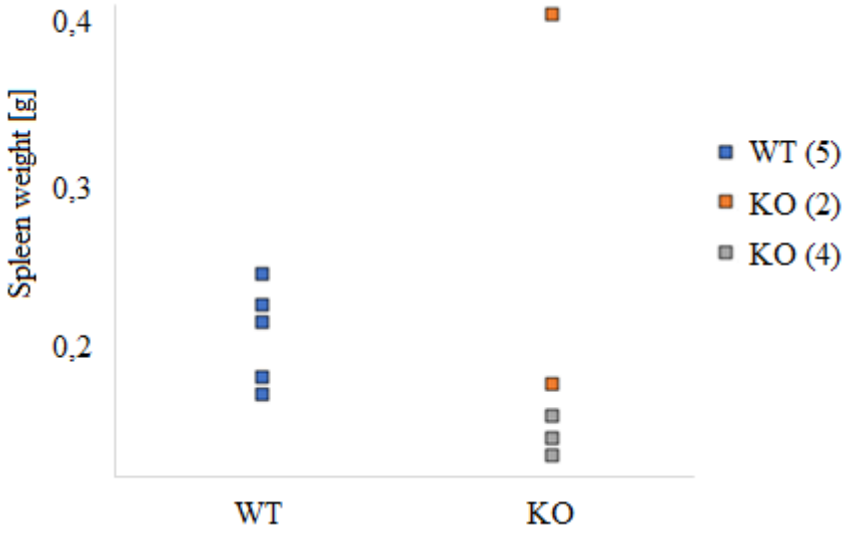

Fig. 19: Comparison of spleen weight between WT- and TGT2 KO- infected animals at week 15 post infection.

Furthermore, the assessment of the parasite load by serial dilution assay, demonstrated lower numbers of viable parasites in one of the two (2) mice infected with TGT2 KO as compared to WT strain, despite of the larger lesions observed in the former than the latter group. Interestingly, this experiment also revealed persistence of viable parasites at the site of infection even in TGT2 KO-infected animals (4) which did not develop discernible lesions (Fig. 20), albeit this fact could not be readily distinguished by histopathological examination, where these samples appeared as relatively preserved skin tissue. On the contrary, the inspection of ear skin originating from the animals infected with WT as well as TGT2 KO (2)

strain showed large influx of immune cells with a considerable number of infected macrophages harbouring the amastigotes (Fig. 21).

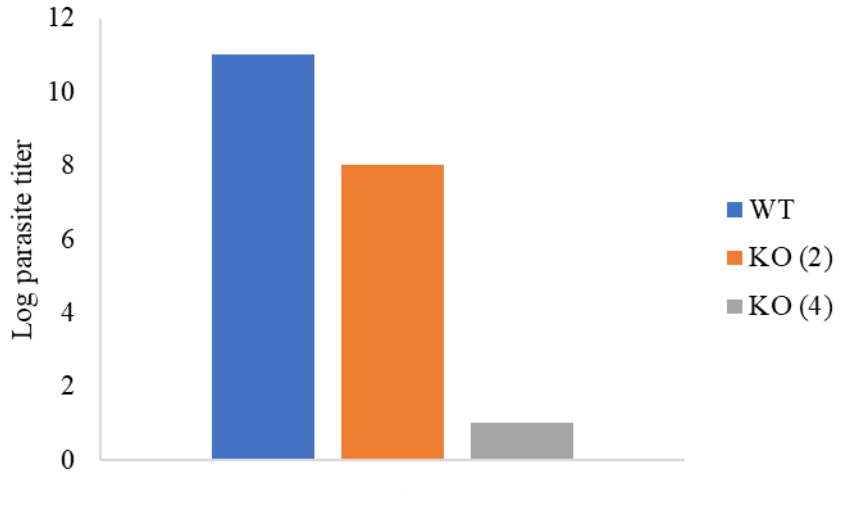

Fig. 20: Determination of parasite burden by serial dilution assay, average value of two samples is presented for WT- and a representative of each TGT2 KO-infected group is shown.

We conclude, that the discrepancy among the TGT2 KO-infected group was likely caused by different dose of parasites inoculated per each mouse due to an experimental and/or human error. Thus, the decreased susceptibility of the animals to TGT2 KO parasites seen in majority of the animals might be manifested in a dose-dependent manner.

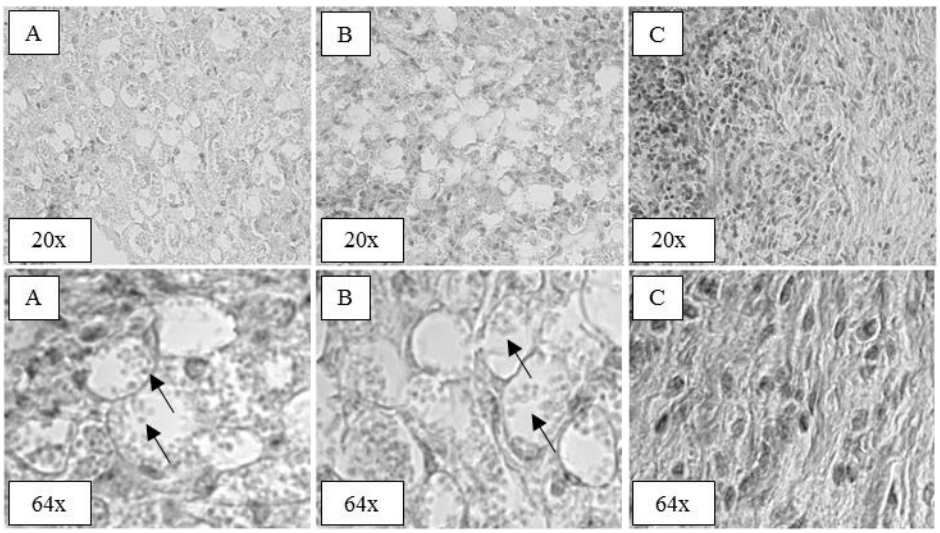

Fig. 21: Histopathological analysis of infected ears; A) WT and B) KO (2) showing ulceration and extensive tissue destruction with inflammatory infiltrate comprised of parasitized macrophages (arrows indicate intracellular parasites) and neutrophils. In contrast, section C) KO (4) displayed more preserved skin, in the absence of any infected macrophages.

Consequently, in an attempt to test the lowest possible number of parasites which would, in our hands, be capable of establishing the infection, mice were infected with four

various doses of parasites. We have, however, not noted any significant lesion development within 14 weeks post infection, hence the experiment was terminated (Fig. 22).

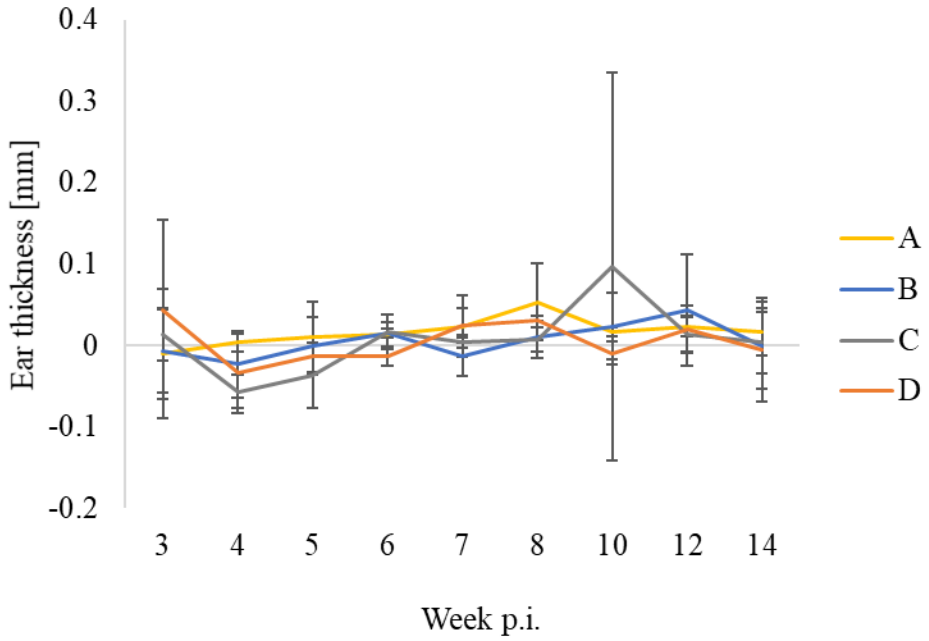

Fig. 22: The dosage experiment; each group represents three mice infected with different dosage of parasites; A) 1E5, B) 1E4, C) 1E3, D) 1E2, the graph depicts mean lesion size of all mice within the individual groups, error bars represent standard deviation. No significant lesion growth was observed within 14 weeks post infection.

5. Discussion

5.1. The presence of Q modification in Lmx tRNA$^{Asp, Asn, His, Tyr}$

Although the ubiquitous presence of Q modification at the wobble position 34 in $tRNA_{GUN}$ is well documented in both Bacteria and Eukarya (Vinayak & Pathak 2009), a few exceptions exist, such as *S. cerevisiae* (Globisch et al 2011, Walden T et al 1982). Therefore, we tested the presence of the modification in the corresponding tRNAs in *L. mexicana*. The analysis indeed proved the presence of Q in this species, yet the individual tRNAs seem to differ in the exact degree of the modification, with nearly equal quantities of G- and Q-containing tRNAs concerning the cellular pool of $tRNA^{Asn}$ as contrasted to >70% Q-modified $tRNA^{Asp}$. A similar trend was noted in *T. brucei* by other members of our laboratory (Kessler et al 2018). Additionally, in case of $tRNA^{His}$ we conclude that G- and Q-tRNAs were apparently not distinguished by this method, as the northern blot of WT RNA showed presence of a single band, which intensity was however later found to be decreased to about 30% in TGT2 KO cell line.

As the enzymatic reaction catalysed by TGT does not require any energy input (Okada & Nishimura 1979), it seems highly probable that the base-exchange reaction may be mediated by the energy released from the enzyme-tRNA binding interaction (Curnow & Garcia 1995). Despite of the 7 bp 'UGUN'-containing loop being the minimal recognition motif for bTGT, eTGT appears to require an intact tRNA molecule for efficient transglycosylation (Grosjean et al 1996). Therefore, not only the exact size and conformation of the anticodon loop, but presumably also the overall tRNA tertiary structure may influence the amount of energy released, and consequently the strength and duration of this interaction. Thus, the minimal differences in the structure of the individual tRNA species could eventually account for their varying degree of modification.

Additionally, starvation experiments performed in murine model demonstrated that there is a distinct hierarchy for Q incorporation into $tRNA_{GUN}$ species, with $tRNA^{Asp}$ taking a precedence over $tRNA^{His}$ leading to a speculation about differential affinity of the enzyme for various substrates (Reyniers et al 1981).

Besides, the minor sequence and consequent structural dissimilarities as well as the varying Q content also result in diverse interactions, thus the rate of migration, when subjected to boronate affinity electrophoresis, leading to the disparity observed in the relative distance of Q- and G-bands among individual tRNAs.

5.2. LmxTGT KO cell lines & the role of LmxTGT2 in Q-tRNA formation

Based on the sequence homology, we identified the orthologues of TGT genes in *L. mexicana*, taking into consideration their constituent conserved residues, we hypothesize that likewise the human counterparts (Chen et al 2010), LmxTGT1 represents the catalytic subunit, while LmxTGT2 is responsible for the complex stabilization and/or nutrient recycling.

We failed to produce LmxTGT1 KO cell line, which could be due to its possible lethal phenotype. We have noted a similar observation in *T. brucei*, where the (RNAi-mediated) downregulation of TbTGT1, as opposed to TbTGT2, results in a growth phenotype. Of note, preliminary results of co-immunoprecipitation experiments indicate that TbTGTs form heterodimeric complex like their mammalian counterparts however, both subunits of the complex localize to nucleus (Kulkarni et al unpublished manuscript), suggesting a slightly different mode of action as compared to the one described for mammalian enzymes, which associate with mitochondrial outer membrane (Boland et al 2010).

On the other hand, LmxTGT2 KO cell line was successfully generated, and the gene product was demonstrated to participate in Q-tRNA formation *in vivo*. Whereas it is likely that a complex of LmxTGT1 and LmxTGT2 functions in Q-tRNA generation, if the phenotype for LmxTGT1 KO was to be lethal, the underlying reason for this must clearly result from the enzyme exhibiting an additional activity that would be TGT2- and Q-tRNA-independent.

Since TGT1 constitutes the catalytic subunit of the complex, with significant sequence identity to bTGT (Chen et al 2010), the primary reason for its essentiality could theoretically be originating from an action of the enzyme on alternative (*i.e*. other than tRNA) substrates, such as DNA (Thiaville et al 2016), ribosomal and messenger RNA (Brooks et al 2012, Hurt et al 2007) or plausibly additional, yet unidentified targets. Thus, such activity of TGT1 would be TGT2-independent and could eventually involve interaction with (an)other binding partner(s). Interestingly, the initial purification studies of eTGT reported on the recovery of a number of peptides, with no homology to either of the TGT subunits, including aspaginyl tRNA synthetase, coenzyme A reductase (Fergus et al 2015), elongation factor 2 and a deubiquitinating enzyme USP14 (Deshpande et al 1996). Moreover, the identification of USP14 has led to its assignment as a critical component of the TGT complex (Shinji et al 1996), eventually proposing eTGT to be a heterodimer of QTRT1:USP14, with USP14 representing the regulatory subunit (Morris et al 1995). Although the relevance of USP14 to the catalytic activity of TGT complex has been dismissed, by demonstration of the QTRT1:QTRTD1 heterodimer, the experiments conducted in this study did not address nor could rule out the possibility that USP14 could act as a regulator of TGT dimer (Chen et al

2010). Furthermore, recent literature indicates that both subunits of the complex can also exist, although in less quantity, as monomers and homodimers the function of which remains to be elucidated (Behrens et al 2018, Boland et al 2009, Chen et al 2010).

Even though the ultimate proof of essentiality of LmxTGT1 is missing, perhaps, it could be provided using the tetracycline-inducible gene expression system (Kareva et al 2014). Theoretically, in parallel with the constructs targeted for the ablation of LmxTGT1, the cells could be transfected with a copy of TGT1 gene, carrying mutation at the protospacer adjacent motif (a site critically required for Cas9-mediated cleavage (Hsu et al 2014)), under the control of T7 promoter.

Furthermore, co-immunoprecipitation experiments could be performed in order to verify LmxTGT1-TGT2 complex formation *in vivo* and the plausible ability of the individual subunits to form homodimers and/or to exist as monomers. Yet most importantly, the subsequent mass spectrometry analysis would clarify the possibility of LmxTGT1 interaction with alternative binding partners, which would provide us with valuable insights into the hypothesized other than Q-tRNA function for this subunit. Additonally, in order to determine wheter TGT1 was to act on other than tRNA substrates, RNA immunoprecipitation should be carried out.

5.3. *In vitro* infection of macrophages

Although no effect of TGT2 ablation was noted in the axenic culture, we questioned whether the presence of Q-tRNAs could potentially be vital for the survival of the parasite within the host macrophages. Hence, to verify the conceivable effect of Q absence on the parasite virulence, we first scrutinized the parasite-host cell interaction *in vitro*. Observing the infected cells at 24-48-hour intervals for a time period of up to 5 days, we quantified the proportion of infected macrophages as well as the numbers of intracellular parasites. Thus, the ability of parasites to complete the host cell entry, life stage transition and intracellular survival were examined.

The experiment revealed significantly lower numbers of intracellular parasites, as well as the overall percentage of infected cells for TGT2 KO- as compared to WT-infected macrophages. This difference was especially noticeable at earlier time points of the infection, suggestsing that the differentiation of TGT2 KO parasites into amastigote stage took place at lower rate, probably due to their increased sensitivity to the phagolysosomal environment. The decreased infectivity of TGT2 KO strain was, however, observed also in the later time points of the infection, leading to the assumption that, once transformed into the amastigotes, the ability of parasite to multiply within the host cells was also affected. A possible mechanism

for such a sequence of events could be an inefficient blockage of macrophage functions, for instance phagolysosome biogenesis, perhaps, as a consequence of (s)lower translation of amastigote-specific products.

This scenario is additionally supported by the observations made in classically activated macrophages where TGT2 KO parasites showed a complete lack of multiplication up to 24 hours post infection, in contrast to the WT strain. Furthermore, at 72 hours post infection, TGT2 KO parasites were completely eliminated, whereas WT-infected macrophages still harboured a negligible number of parasites (data not shown). Since this effect was IFNγ-dependent, it strongly suggests that the natural capability of *L. mexicana* to interfere with macrophage cytokine signalling pathways, particularly IFNγ-dependent JAK-STAT signalling that leads to the production of killing agents such as NO; was appreciably less efficient in the mutant, as compared to the WT cell line (Atayde et al 2016, Basu & Ray 2005). Hence, the determination of NO production by macrophages infected by the respective cell lines, measured as nitrate accumulation in the culture supernatants and/or iNOS expression levels, would be of interest for future experiments. Additionally, both strains could also be examined for the expression levels of gene products known to participate in the blockade of IFNγ signalling.

Alternatively, the increased susceptibility of TGT2 KO parasites to intracellular killing by the activated macrophages could be explained by the role for Q-tRNAs in the general enhancement of antioxidant defence mechanisms (Pathank et al 2008), which could also be determined as a measure of gene expression for the particular enzymes involved in this pathway. Althgouh, the phenotype observed may as well be resulting from a combination of both factors.

5.4. *In vitro* differentiation

Since several studies suggest a role for Q in differentiation and development (Beier et al 1987, Jacobson et al 1981, Singhal et al 1981) and the results of *in vitro* macrophage infection indicated that the ability of WT and mutant parasites to complete their life cycle was likely not equivalent, we examined this possibility by inducing the complete developmental cycle of *Leishmania* parasites of both strains in the axenic culture. RNA was isolated at particular time points throughout the period of *in vitro* differentiation for the expression analysis of stage-specific transcripts. Whereas the marker for procyclic and metacyclic promastigotes did not show any major differences in gene expression, among the strains tested, the situation was quite different in the case of amastigote-specific marker. While the expression levels of amastin in WT cell line were continually raising from day 8 until day 17 of differentiation,

TGT2 KO parasites did not achieve comparable levels of expression at any time point examined.

Although morphological examination did not reveal any major difference among the cell lines tested, the results suggest that the TGT2 KO cell line amastigotes were most probably unable to express the full spectrum of stage-specific transcriptome, consequently affecting the proteome to a degree which only became relevant when subjected to the challenging environment within host macrophages.

Therefore, it made us wonder whether the ratio of G- and Q-tRNAs within the cellular pool would undergo any major alterations as the parasites sequentially proceed through different life stages, facing dynamic environmental changes, which may require rapid translational adaptation. The comparison of Q content between different life stages indeed revealed a gradual increase in Q levels from procyclic to metacyclic promastigotes, with a subsequent fall in the value once transformed into the amastigote stage. Thus, the results confirmed that the translational requirement for Q-tRNAs may be particularly important during metacyclic-to-amastigote life stage transition.

Although more time points of differentiation and biological repeats should certainly be considered for any future investigation, perhaps, we may hypothesize that variability in codon usage bias among the individual life stages might be correlated to the varying degree of Q modification and may account for the hampered development seen in TGT2 KO cell line in amastigote stage. Transcriptome-wide stage-specific analysis of codon usage followed by proteomic studies will, however, be necessary to test this hypothesis.

5.5. *In vivo* infection of mice

5.5.1. Murine infection models revisited

It must be noted that, although animal infections are routinely performed by several laboratories, there is no validated animal model for CL which would appropriately reflect human condition (Mears et al 2015). Furthermore, most research aiming for the elucidation of the molecular factors that govern CL progression employs murine infection with *L. major*, while the mechanisms contributing to the chronic disease seen during infection with New World *Leishmania* species are poorly understood, yet some profound differences in the virulence factors and the consequent pathology are recognized (Alexander & Bryson 2005, McMahon-Pratt & Alexander 2004). A large variability exists in the experimental design utilized by different laboratories, particularly in terms of the strain of parasite used, the duration of *in vitro* cultivation since obtaining a field isolate, method of *in vitro* maintenance, differentiation and selection of infective stage parasites, route of inoculation, size of the

infective inoculum, the strain and sex of mice employed in the studies and so forth (Mears et al 2015).

Two distinct modes of experimental infection are described in the introduction of this thesis, with the past literature mostly reporting on murine infection employing high numbers of parasites inoculated subcutaneously to either footpad or back rump, leading to a rapid onset of the disease (Belklaid et al 2000). On the other hand, more recent literature introduces *in vivo* models for CL which pursue the objective to mimic the natural transmission of the parasites, hence utilizing low dose inoculum injected intradermally, typically into the ear (Kimblin et al 2008), revealing an initial silent phase favouring parasite amplification prior to the lesion formation. Thus, leading to the development of an animal model reminiscent of pathological features and immunological responses similar to humans (Belklaid et al 2000).

However, the combination of many experimental variables and natural variation generally seen in animal experimentation (Hilley et al 2000), results in no universal correlation regarding criteria such as the number of parasites inoculated and the time it takes for lesions to first appear. Hence, inoculation of lower numbers of parasites ($1x10^5$) occasionally lead to a more rapid onset of pathology (week 1-2) (Denise et al 2003) as compared to higher dose infection ($5x10^6$) with lesion development perceived significantly later (week 10) (Alexander et al 1998). Even though these particular studies were produced by a single research team, and the parameters utilized for cell culture, differentiation, route of inoculation or the choice of mice strain are seemingly identical.

Profound dissimilarities in pathogenesis may, first of all, be a result of parasite growth conditions, as the cells are frequently reported to be serially passaged in mice, either using isolated lesion amastigotes directly for subsequent infection (Bryson et al 2011, Hilley et al 2000, Pollock et al 2003), or performing *in vitro* differentiation into metacyclic promastigotes (Al-Mutairi et al 2010, Cummings et al 2012, García-Miss Mdel et al 2015, Rosas et al 2005). In other cases, the infective inoculum originates from apparently long-term *in vitro* cultures, with a wide variety of culture media employed (Aguilar Torrentera et al 2002, Buxbaum & Scott 2005, Denise et al 2003, Ramos-Martínez et al 2012).

Second, while *L. major* infective-stage metacyclic promastigotes (naturally transmitted by the sandfly bite) can be purified using negative selection with peanut agglutin (Sacks et al 1985), this method was proved to be ineffective for *L. mexicana* due to the differences in cell surface carbohydrates (Bates & Tetley 1993). Hence it appears that stationary-phase promastigotes are generally considered as metacyclic (Al-Mutairi et al 2010, Cummings et al 2012, Rosas et al 2005), although at times the inoculum is solely stated to consist of metacyclic

promastigotes, omitting detailed information of the culture conditions applied to induce such differentiation (García-Miss Mdel et al 2015, Ramos-Martínez et al 2012, Tuladhar et al 2015).

Since long, it has been known that *L. mexicana* cell culture at acidic pH (simulating the environment within the sandfly midgut (Bates 2007)) produces homogenous population of metacyclic forms (Bates & Tetley 1993). Despite the emphasis on the requirement for purified metacyclic promastigotes with regards to low dose infection challenge (Lira et al 2000), to the best of our knowledge, studies reporting *L. mexicana* low-dose infection do not employ any selection method (Cummings et al 2012).

5.5.2. Interpretation of the results

We tested differentiation from procyclic into metacyclic form, by means of allowing the cells to reach stationary phase of growth, in both neutral and acidic medium, since cultivation in low pH was likewise utilized in order to facilitate *L. mexicana* full life cycle development *in vitro*, as previously described (Bates 1994). Upon morphological evaluation of the samples, we could clearly distinguish a substantial enrichment of metacyclic forms within the cell population maintained at acidic pH, hence we found it reasonable to set up the cultures intended for infection experiments under such condition.

Since there was no previous experience with *Leishmania* murine infection studies in our laboratory, when designing the pilot infection experiment, we decided to utilize a supposed "medium" ($2{,}5x10^6$) among the high doses of parasites commonly used (Belklaid et al 2000), in order to determine, first of all, whether the pathogenesis could be established in our hands, under the conditions employed and furthermore, if the phenotype of TGT2 mutant cell line observed during *in vitro* macrophage infection experiment could, perhaps, become pronounced *in vivo*.

In spite of using high dose of parasites, with an inoculum consisting of metacyclic promastigotes, an apparent lesion formation was only noticed as soon as 4 weeks post infection. We cannot, however pinpoint the exact time of lesion appearance, as the ear thickness was unfortunatly not measured unless visible lesions occurred.

Subsequently, an inhibition of ear dermis swelling was observed in most of the mice that were injected with TGT2 KO parasites, leading to the significant decrease in lesion size by the end of the experiment. While the histological tissue sections originating from WT-infected mice showed persistent mononuclear cell infiltrates revealing heavily infected macrophages, the histological analysis of nearly all mice infected with TGT2 KO cell line failed to reveal any major changes in the dermal architecture, although small numbers of parasites were found to persist at the site. Since we never noted a significant lesion formation

in these animals, this result supports our already stated hypothesis on the hampered ability of TGT2 KO parasites to multiply within mammalian cells that most probably resulted in failure to establish peak parasite loads capable of eliciting a potent source of antigen presentation to $CD4^+$ T lymphocytes. The activation of CD4+ cells could in turn trigger an immune response that would lead to macrophage activation and complete parasite elimination. Thus, the TGT2 KO parasites appeared to induce rather chronic infection, hence not bringing about any major tissue damage.

Even though two of the TGT2 KO-infected mice developed a more severe form of the disease when compared to those infected with WT parasites, the difference in lesion size by the end of the experiment was insignificant, and we consider these to be outliers, explanations are as follows. When the experiment reached completion, one of the two individuals was found to suffer from splenomegaly, suggesting an occurrence of abnormalities in this particular animal, such as a disordered immunoregulation resulting from slight differences in genetic background, which could eventually lead to dissemination of CL into MCL. Yet, the exact cause of our observation could not be determined. Also, it is plausible that the dosage of parasites may have had variations from one animal to another, due to technical limitations. Considering the lack of experience, needle injection aimed at the borderline of the ear dermal and epidermal sheets represented a technical difficulty *per se*, furthermore, the dosage could have, perhaps, been more accurate if lower volume syringes, or ideally microliter syringes, would have been used.

The relationship between lesion progression and parasite numbers at the site of infection was examined by quantifying the numbers of viable amastigotes. There appeared to be a direct correlation between parasite amplification and the size of lesion in WT-infected animals. However, one of the two mice within the TGT2 KO-infected group (2), which developed larger lesions showed lower parasite load when compared to the former set. Moreover, the analysis also revealed persistence of parasites in the other four TGT2 KO-infected animals, which did not develop any significant lesions. Since this was a preliminary experiment, the sample size utilized for parasite load determination was insufficient for statistical evaluation. However, the results obtained suggest that the disease progression resembles that of the high dose model-like in case of WT-infected mice. Meanwhile, TGT2 KO infected animals, displayed a low dose-like infection thus providing additional support for the decreased ability of TGT2 KO parasites to multiply *in vivo* (Fig. 23). The infection experiment, however, clearly requires more repeats, hence drawing any final conclusions from

the results currently available would be premature. Therefore, the samples collected for the analysis of cytokine profiles are awaiting further validation of the results.

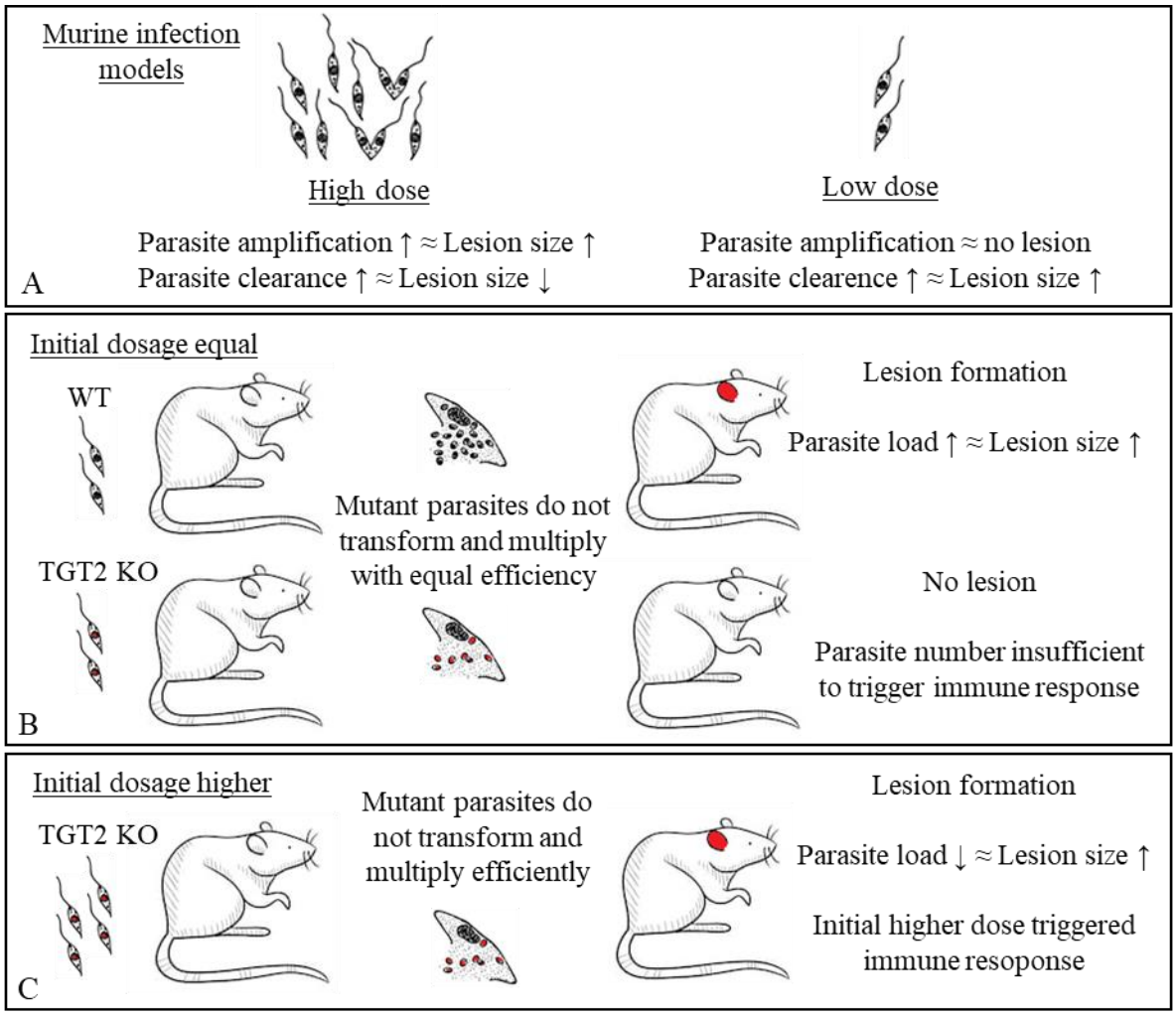

Fig. 23: Interpretation of the pilot *in vivo* infection experiment; A) *Leishmania* high-dose vs. low-dose murine models of infection, B) Disease progression in animals infected with equal dose of WT and TGT2 KO-parasites, representing KO (4) experimental group, C) representation of KO (2) experimental group.

Observing the effect of TGT2 absence on the infection propagation following large inoculum, we hypothesized that the impact would be more evident in an infection model employing a number of parasites comparable to the amount inoculated during the natural transmission of *L. mexicana* by sandflies. Also, the delayed parasitemia seen in mice infected with *T. brucei* TGT2 KO cell line appears to be manifested in a dose-dependent manner, with the routinely used high experimental dose of parasites causing a slight yet consistent effect and, meanwhile infection with a smaller inoculum results in a significantly prolonged survival of the animals (Kulkarni et al unpublished manuscript).

Therefore, we attempted to test the lowest possible infectious dosage of WT cells that would be capable of the disease manifestation. Regrettably, none of the doses utilized in this study led to a significant lesion formation, most probably due to long-term *in vitro* cultivation

of the parasites, which is known to result in decreased infectivity potential (da Silva et al 1987, Moreira et al 2012, Nolan et al 1985, Segovia et al 1992). Although we successfully isolated the parasites from the animals sacrificed for the pilot experiment, unluckily a contamination of these cultures later occurred, whence these could not be employed for the subsequent infection. Therefore, we have recently passaged both cell lines through mice and isolated the parasites afresh for use in an additional repeat of the dosage experiment.

6. Conclusion

In conclusion, we generated TGT2 KO cell line in *Leishmania mexicana* and demonstrated the involvement of this gene product in Q-tRNA formation. The results of *in vitro* infection experiments suggested that LmxTGT2 KO cell line exhibits difficulties with metacyclic-to-amastigote life stage transition, which was also confirmed *in vivo*. This hypothesis was supported by the gradual increase in Q levels from procyclic to metacyclic promastigotes noticed in WT parasites in combination with the expression analysis of stage-specific markers.

We hypothesize that Q-tRNAs may be crucial for the translation of the full-spectrum amastigote-specific proteome due to the differences in codon usage bias among the individual life stages (Fig. 24).

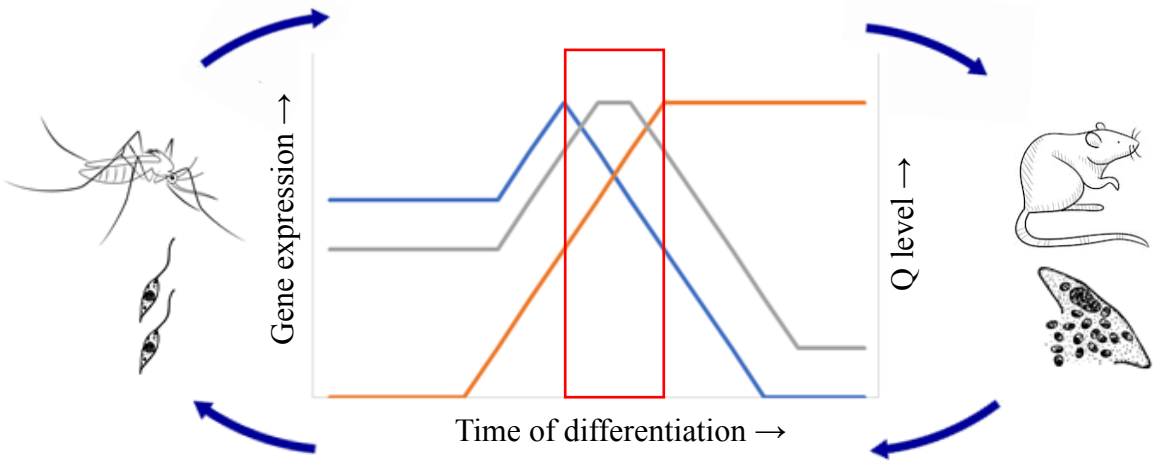

Fig. 24: Current model for the role of Q-tRNA modification in the virulence of *Leishmania mexicana*; ● procyclic/metacyclic promastigote-specific gene expresson, ● amastigote-specific gene expression, ● level of Q-tRNA modification, ○ time point when Q is critical.

7. List of references

▪ Abdeladhim M, Kamhawi S, Valenzuela JG (2014): What's behind a sand fly bite? The profound effect of sand fly saliva on host hemostasis, inflammation and immunity. Infect Genet Evol 28:691-703. PMID: 25117872.

▪ Agris PF (1991): Wobble position modified nucleosides evolved to select transfer RNA codon recognition: a modified-wobble hypothesis. Biochimie. 73(11):1345-9. PMID: 1799628.

▪ Agris PF, Vendeix FA, Graham WD (2006): tRNA's wobble decoding of the genome: 40 years of modification. J Mol Biol. 9;366(1):1-13. PMID: 17187822.

▪ Aguilar Torrentera F, Lambot MA, Laman JD, Van Meurs M, Kiss R, Noël JC, Carlier Y (2002): Parasitic load and histopathology of cutaneous lesions, lymph node, spleen, and liver from BALB/c and C57BL/6 mice infected with *Leishmania mexicana*. Am J Trop Med Hyg. 66(3):273-9. PMID: 12139220.

▪ Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, Sereno D (2016): A historical overview of the classification, evolution, and dispersion of *Leishmania* parasites and sandflies. PLoS neglected Tropical diseases 10(3): e0004349. PMID: 26937644.

▪ Alexander J, Bryson K (2005): T helper (h)1/Th2 and *Leishmania*: paradox rather than paradigm. Immunol Lett. 99(1):17-23. PMID: 15894106.

▪ Alexander J, Coombs GH, Mottram JC (1998): *Leishmania mexicana* cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. J Immunol.161(12):6794-801. PMID: 9862710.

▪ Alexander J, Coombs HG, Mottram CJ (1998): *Leishmania mexicana* cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. Journal of Immunology 161 (12) 6794-6801. PMID: 9862710.

▪ Al-Mutairi SM, Cadalbert CL, McGachy AH, Shweash M, Schroeder J, Kurnik M, Sloss MC, Bryant EC, Alexander J, Plevin R (2010): MAP Kinase phosphatase-2 plays a critical role in response to infection by *Leishmania mexicana*. PLoS Pathog. 6(11): e1001192. PMID: 21085614.

▪ Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990): Basic local alignment search tool. J Mol Biol 215(3):403-10. PMID: 2231712.

▪ Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M; WHO Leishmaniasis Control Team (2012): Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7(5): e35671. PMID: 22693548.

▪ Arango Duque G, Descoteaux A (2015): *Leishmania* survival in the macrophage: where the ends justify the means. Curr Opin Microbiol 26:32-40. PMID: 25988701.

▪ Aslett M et al (2010): TriTrypDB: a functional genomic resource for the Trypanosomatidae. Nucleic Acids Res. 38 (Database issue): D457-62. PMID: 19843604.

▪ Atayde VD, Hassani K, da Silva Lira Filho A, Borges AR, Adhikari A, Martel C, Olivier M (2016): *Leishmania* exosomes and other virulence factors: Impact on innate immune response and macrophage functions. Cell Immunol. 309:7-18. PMID: 27499212.

▪ Basu KM, Ray M (2005): Macrophage and *Leishmania*: an unacceptable coexistence. Crit Rev Microbiol;31(3):145-54. PMID: 16170905.

▪ Bates AP (1993): Complete developmental cycle of Leishmania mexicana in axenic culture. Parasitology 108: 1-9. PMID: 8152848.

▪ Bates AP (2007): Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. International Journal for Parasitology 37: 1097-1106. PMID: 17517415.

▪ Bates PA (1994): Complete developmental cycle of *Leishmania mexicana* in axenic culture. Parasitology. 108 (Pt 1):1-9. PMID: 8152848.

▪ Bates PA (2007): Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. Int J Parasitol. 37(10-3): 1097–1106. PMID: 17517415.

▪ Bates PA, Tetley L (1993): *Leishmania mexicana*: induction of metacyclogenesis by cultivation of promastigotes at acidic pH. Exp Parasitol. 76(4):412-23. PMID: 8513879.

▪ Behrens C, Biela I, Petiot-Bécard S, Botzanowski T, Cianférani S, Sager CP, Klebe G, Heine A, Reuter K (2018): Homodimer Architecture of QTRT2, the Noncatalytic Subunit of the Eukaryotic tRNA-Guanine Transglycosylase. Biochemistry. 57(26):3953-3965. PMID: 29862811.

▪ Beier H, Zech U, Zubrod E, Kersten H (1987): Queuine in plants and plant tRNAs: Differences between embryonic tissue and mature leaves. Plant Mol Biol. 8(4):345-53. PMID: 24301197.

▪ Belkaid Y, Butcher B, Sacks DL (1998): Analysis of cytokine production by inflammatory mouse macrophages at the single-cell level: selective impairment of IL-12 induction in *Leishmania*-infected cells. Eur J Immunol 28(4):1389-400. PMID: 9565379.

▪ Belkaid Y, Kamhawi S, Modi G, Valenzuela J, Noben-Trauth N, Rowton E, Ribeiro J, Sacks DL (1998): Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. J Exp Med. 188(10):1941-53. PMID: 9815271.

▪ Belkaid Y, Kamhawi S, Modi G, Valenzuela J, Noben-Trauth N, Rowton E, Ribeiro J, Sacks DL (1998): Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. J Exp Med 16;188(10):1941-53. PMID: 9815271.

▪ Belkaid Y, Mendez S, Lira R, *et al* (2000): A natural model of *Leishmania major* infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. J Immunol. 165(2):969-77. PMID:10878373.

▪ Beneke T, Madden R, Makin L, Valli J, Sunter J, Gluenz E (2017): A CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids. R Soc Open Sci. 3;4(5):170095. PMID: 28573017.

▪ Bern C, Maguire JH, Alvar J (2008): Complexities of assessing the disease burden attributable to leishmaniasis. PLoS Negl Trop Dis 2(10): e313. PMID: 18958165.

▪ Boland C, Hayes P, Santa-Maria I, Nishimura S, Kelly VP (2009): Queuosine formation in eukaryotic tRNA occurs via a mitochondria-localized heteromeric transglycosylase. J Biol Chem. 284(27):18218-27. PMID: 19414587.

▪ Brittingham A, Morrison CJ, McMaster WR, McGwire BS, Chang KP, Mosser DM (1995): Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. J Immunol 15;155(6):3102-11. PMID: 7673725.

▪ Brooks AF, Vélez-Martínez CS, Showalter HD, Garcia GA (2012): Investigating the prevalence of queuine in *Escherichia coli* RNA via incorporation of the tritium-labeled precursor, preQ(1). Biochem Biophys Res Commun. 17;425(1):83-8. PMID: 22819844.

▪ Bryson JK, Millington RO, Mokgethi T, McGachy AH, Brombacher F, Alexander J (2011): BALB/c Mice deficient in CD4+ T Cell IL-4Rα expression control *Leishmania mexicana* load although female but not male mice develop a healer phenotype. PLoS Negl Trop Dis. 5(1): e930. PMID: 21245915.

▪ Buffet PA, Sulahian A, Garin YJ, *et al*. (1995): Culture microtitration: a sensitive method for quantifying *Leishmania infantum* in tissues of infected mice. Antimicrob Agents Chemother. 39(9):2167-8. PMID: 8540741.

▪ Buxbaum LU, Scott P (2005): Interleukin 10- and Fcgamma receptor-deficient mice resolve *Leishmania mexicana* lesions. Infect Immun. 73(4):2101-8. PMID: 15784551.

▪ Carrera L, Gazzinelli RT, Badolato R, Hieny S, Muller W, Kuhn R, Sacks DL (1996): *Leishmania* promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. J Exp Med 183(2):515-26. PMID: 8627163.

▪ CDC (2018): Parasites – Leishmaniasis. Retrieved from https://www.cdc.gov/parasites/leishmaniasis/biology.html

▪ Chakraborty P, Basu MK (1997): *Leishmania* phagolysosome: Drug trafficking and protein sorting across the compartment. Critical Reviews in Microbiology 23(3):253-268. PMID: 9347223.

▪ Chen YC, Kelly VP, Stachura SV, Garcia GA (2010): Characterization of the human tRNA-guanine transglycosylase: confirmation of the heterodimeric subunit structure. RNA. 16(5):958-68. PMID: 20354154.

▪ Chomczynski P, Sacchi N (2006): The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nature Protocols 1(2):581-5. PMID: 17406285.

▪ Contreras I, Gómez MA, Nguyen O, Shio MT, McMaster RW, Olivier M (2010): *Leishmania*-induced inactivation of the macrophage transcription factor AP-1 is mediated by the parasite metalloprotease GP63. PLoS Pathog 14;6(10): e1001148. PMID: 20976196.

▪ Crick FH (1966): Codon-anticodon pairing: the wobble hypothesis. J Mol Biol. 19(2):548-55. PMID: 5969078.

▪ Cruz AK, Titus R, Beverley SM (1993): Plasticity in chromosome number and testing of essential genes in *Leishmania* by targeting. Proc Natl Acad Sci U S A. 90(4):1599-603. 8381972.

▪ Cruz-Vera LR, Magos-Castro MA, Zamora-Romo E, Guarneros G (2004): Ribosome stalling and peptidyltRNA drop-off during translational delay at AGA codons. Nucleic Acids Research 32:4462–4468. PMID: 15317870.

▪ Cummings HE et al (2012): Critical role for phosphoinositide 3-kinase gamma in parasite invasion and disease progression of cutaneous leishmaniasis. Proc Natl Acad Sci U S A. 109(4):1251-6. PMID: 22232690.

▪ Curnow AW, Garcia GA (1995): tRNA-guanine transglycosylase from Escherichia coli. Minimal tRNA structure and sequence requirements for recognition. J Biol Chem. 21;270(29):17264-7. PMID: 7615526.

▪ da Silva R, Sacks DL (1987): Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. Infect Immun. 55(11): 2802–2806. PMID: 3666964.

▪ DaMata PJ, Mendes PB, Maciel-Lima K *et al* (2015): Distinct macrophage fates after in vitro infection with different species of *Leishmania*: Induction of apoptosis *by Leishmania (Leishmania) amazonensis*, but not by *Leishmania (Viannia) guyanensis*. PLoS ONE (10). PMID: 26513474.

▪ de Vries CJH, Reedijk HS, Schallig HFDH (2015): Cutaneous leishmaniasis: Recent developments in diagnosis and management. Am J Clin Dermatol 16(2): 99–109. PMID: 25687688.

▪ Denise H, McNeil K, Brooks RD, Alexander J, Coombs HG, Mottram CJ (2003): Expression of multiple CPB genes encoding cysteine proteases is required for *Leishmania mexicana* virulence in vivo. Infect Immun. 71(6): 3190–3195. PMID: 12761098.

▪ Deshpande KL, Seubert PH, Tillman DM, Farkas WR, Katze JR (1996): Cloning and characterization of cDNA encoding the rabbit tRNA-guanine transglycosylase 60-kilodalton subunit. Arch Biochem Biophys. 326(1):1-7. PMID: 8579355.

▪ Desjardins M, Descoteaux A (1997): Inhibition of phagolysosomal biogenesis by the *Leishmania* lipophosphoglycan. J Exp Med 185(12): 2061–2068. PMID: 9182677.

▪ Dirheimer G, Baranowski W, Keith G (1995): Variations in tRNA modifications, particularly of their queuine content in higher eukaryotes. Its relation to malignancy grading. Biochimie. 77(1-2):99-103. PMID: 7599283.

▪ Duncan SM, Jones NG, Mottram JC (2017): Recent advances in *Leishmania* reverse genetics: Manipulating a manipulative parasite. Mol Biochem Parasitol. 216:30-38. PMID: 28629934.

▪ El Hassan AM, Gaafar A, Theander TG (1995): Antigen-presenting cells in human cutaneous leishmaniasis due to *Leishmania major*. Clin Exp Immunol. 99(3):445-53. PMID: 7882568.

▪ Emmerich B, Zubrod E, Weber H, Maubach PA, Kersten H, Kersten W (1985): Relationship of queuine-lacking transfer RNA to the grade of malignancy in human leukemias and lymphomas. Cancer Res. 45(9):4308-14. PMID: 4028017.

▪ Farkas RW (1980): Effect of diet on the queuosine family of tRNAs of germ-free mice. J Biol Chem. 255(14):6832-5. PMID: 6771278.

▪ Fergus C, Barnes D, Alqasem MA, Kelly VP (2015): The queuine micronutrient: charting a course from microbe to man. Nutrients. 7(4):2897-929. PMID: 25884661.

▪ Frey B, McCloskey J, Kersten W, Kersten H (1988): New function of vitamin B12: cobamide-dependent reduction of epoxyqueuosine to queuosine in tRNAs of *Escherichia coli* and *Salmonella typhimurium*. PMID: 3129401.

▪ Froger A, Hall EJ (2007): Transformation of plasmid DNA into E. coli Using the heat shock method. J Vis Exp. (6): 253. PMID: 18997900.

▪ Gaafar A, el Kadaro AY, Theander TG, Permin H, Ismail A, Kharazmi A, el Hassan AM (1995): The pathology of cutaneous leishmaniasis due to *Leishmania major* in Sudan. PMID: 7771611.

▪ Gaafar A, Veress B, Permin H, Kharazmi A, Theander GT, El Hassan MA (1999): Characterization of the local and systemic immune responses in patients with cutaneous leishmaniasis due to *Leishmania major*. Clin Immunol. 91(3):314-20. PMID: 10370377.

▪ Gabar IL, Kössel H (1985): Affinity electrophoresis for monitoring terminal phosphorylation and the presence of queuosine in RNA. Application of polyacrylamide containing a covalently bound boronic acid. Nucleic Acid Research 13: 6881-6898. PMID: 2414733.

▪ García-Miss Mdel R, Mut-Martín MC, Góngora-Alfaro JL (2015): β-Adrenergic blockade protects BALB/c mice against infection with a small inoculum of *Leishmania mexicana* (LV4). Int Immunopharmacol. 24(1):59-67. PMID: 25461918.

▪ Gaur R, Björk GR, Tuck S, Varshney U (2007): Diet-dependent depletion of queuosine in tRNAs in *Caenorhabditis elegans* does not lead to a developmental block. J Biosci. 32(4):747-54. PMID: 17762147.

▪ Globisch D et al (2011): Systems-based analysis of modified tRNA bases. Angew Chem Int Ed Engl. 50(41):9739-42. PMID: 21882308.

▪ Gomez MA, Contreras I, Hallé M, Tremblay ML, McMaster RW, Olivier M (2009): *Leishmania* GP63 alters host signaling through cleavage-activated protein tyrosine phosphatases. Sci Signal 29;2(90):ra58. PMID: 19797268.

▪ González U, Pinart M, Rengifo-Pardo M, Macaya A, Alvar J, Tweed JA (2009): Interventions for American cutaneous and mucocutaneous leishmaniasis. Cochrane Database Syst Rev 15(2):CD004834. PMID: 19370612.

▪ Gorak PM, Engwerda CR, Kaye PM (1998): Dendritic cells, but not macrophages, produce IL-12 immediately following *Leishmania donovani* infection. Eur J Immunol 28(2):687-95. PMID: 9521079.

▪ Gregory DJ, Olivier M (2005): Subversion of host cell signalling by the protozoan parasite *Leishmania*. Parasitology 130 Suppl: S27-35. PMID: 16281989.

▪ Grosjean H, Edqvist J, Stråby KB, Giegé R (1996): Enzymatic formation of modified nucleosides in tRNA: dependence on tRNA architecture. J Mol Biol. 255(1):67-85. PMID: 8568876.

▪ Gurvich OL, Baranov PV, Gesteland RF, Atkins JF (2005): Expression levels influence ribosomal frameshifting at the tandem rare arginine codons AGG_AGG and AGA_AGA in *Escherichia coli*. Journal of Bacteriology 187:4023–4032. PMID: 15937165.

▪ Gustafsson C, Govindarajan S, Minshull J (2004): Codon bias and heterologous protein expression. Trends Biotechnol. 22(7):346-53. PMID: 15245907.

▪ Gustilo ME, Vendeix PAF, Agris FP (2008): tRNA's modifications bring order to gene expression. Curr Opin Microbiol. 11(2): 134–140. PMID: 18378185.

▪ Hilley JD, Zawadzki JL, McConville MJ, Coombs GH, Mottram JC (2000): *Leishmania mexicana* mutants lacking glycosylphosphatidylinositol (GPI): protein transamidase provide

insights into the biosynthesis and functions of GPI-anchored proteins. Mol Biol Cell. 1183-95. PMID: 10749923.

▪ Hopper KA, Pai AD, Engelke RD (2011): Cellular dynamics of tRNAs and their genes. FEBS Lett. 21; 584(2): 310. PMID: 19931532.

▪ Howes NK, Farkas WR (1978): Studies with a homogeneous enzyme from rabbit erythrocytes catalyzing the insertion of guanine into tRNA. J Biol Chem. 25;253(24):9082-7. PMID: 721832.

▪ Hsu DP, Lander SE, Zhang F (2014): Development and Applications of CRISPR-Cas9 for Genome Engineering. Cell 157(6): 1262–1278. PMID: 24906146.

▪ Hurt KJ, Olgen S, Garcia AG (2007): Site-specific modification of *Shigella flexneri* virF mRNA by tRNA-guanine transglycosylase *in vitro*. Nucleic Acids Res. 35(14): 4905–4913. PMID: 17626052.

▪ Ishemgulova A et al (2017): A putative ATP/GTP binding protein affects *Leishmania mexicana* growth in insect vectors and vertebrate hosts. PLoS Negl Trop Dis. 24;11(7):e0005782. PMID: 28742133.

▪ Ishemgulova A, Hlaváčová J, Majerová K, Butenko A, Lukeš J, Votýpka J, Volf P, Yurchenko V (2018): CRISPR/Cas9 in *Leishmania mexicana*: A case study of LmxBTN1. PLoS One. 13(2):e0192723. PMID: 29438445

▪ Ishemgulova A, Kraeva N, Faktorova D, Podesvova L, Lukes J, Yurchenko (2016): T7 polymerase-driven transcription is downregulated in metacyclic promastigotes and amastigotes of *Leishmania mexicana*. Folia Parasitol (Praha) 18;63. PMID: 27311571.

▪ Ivens AC et al (2005): The genome of the kinetoplastid parasite, *Leishmania major*. Science. 309(5733):436-42. 16020728. PMID: 16020728.

▪ Jacobson BK, Farkas RW, Katze RJ (1981): Presence of queuine in Drosophila melanogaster: correlation of free pool with queuosine content of tRNA and effect of mutations in pteridine metabolism. Nucleic Acids Res. 9(10): 2351–2366. PMID: 6789305.

▪ Kelly S, Kramer S, Schwede A, Maini KP, Gull K, Carrington M (2012): Genome organization is a major component of gene expression control in response to stress and during the cell division cycle in trypanosomes. Open Biol. 2(4): 120033. PMID: 22724062.

▪ Kersten H (1988): The nutrient factor queuine: biosynthesis, occurrence in transfer RNA and function. Biofactors. 1(1):27-9. PMID: 3076430.

▪ Kessler AC, Kulkarni SS, Paulines MJ, Rubio MAT, Limbach PA, Paris Z, Alfonzo JD (2018): Retrograde nuclear transport from the cytoplasm is required for tRNATyr maturation in T. brucei. RNA Biol. 15(4-5):528-536. PMID: 28901827.

▪ Kima PE (2014): *Leishmania* molecules that mediate intracellular pathogenesis. Microbes Infect 16(9):721-6. PMID: 25107580.

▪ Kimblin N, Peters N, Debrabant A, Secundino N, Egen J, Lawyer P, Fay PM, Kamhawi S, Sacks D (2008): Quantification of the infectious dose of *Leishmania major* transmitted to the skin by single sand flies. Proc Natl Acad Sci U S A. 105(29): 10125–10130. PMID: 18626016.

▪ Kirtland GM, Morris TD, Moore PH, O'Brian JJ, Edmonds CG, McCloskey JA, Katze JR (1988): Novel salvage of queuine from queuosine and absence of queuine synthesis *in*

Chlorella pyrenoidosa and *Chlamydomonas reinhardtii*. J Bacteriol. 170(12):5633-41. PMID: 3142853.

▪ Klassen R, Paluszynski JP, Wemhoff S, Pfeiffer A, Fricke J, Meinhardt F (2008): The primary target of the killer toxin from *Pichia acaciae* is tRNA(Gln). Mol Microbiol. 69(3):681-97. PMID: 18532979.

▪ Kolev GN, Tschudi C, Ullu E (2011): RNA interference in protozoan parasites: Achievements and challenges. Eukaryot Cell. 10(9): 1156–1163. PMID: 21764910.

▪ Konecny P, Stagg AJ, Jebbari H, English N, Davidson RN, Knight SC (1999): Murine dendritic cells internalize *Leishmania major* promastigotes, produce IL-12 p40 and stimulate primary T cell proliferation *in vitro*. Eur J Immunol 29(6):1803-11. PMID: 10382742.

▪ Kraeva N, Ishemgulova A, Lukeš J, Yurchenko V (2014): Tetracycline-inducible gene expression system in *Leishmania mexicana*. Mol Biochem Parasitol. 198(1):11-3. PMID: 25461484.

▪ Kramer S (2012): Developmental regulation of gene expression in the absence of transcriptional control: The case of kinetoplastids. Mol. Biochem. Parasitol. 181, 61–72. PMID: 22019385.

▪ Kulkarni S, Stanzl H, Kessler A, Hegedűsová E, Alfonzo DJ, Paris Z: Queuosine: The role of an essential tRNA modification in parasitic protist *Trypanosoma brucei*. Unpublished manuscript.

▪ Langgut W (1995): Regulation of signalling by receptor tyrosine kinases in HeLa cells involves the q-base. Biochem Biophys Res Commun. 207(1):306-11. PMID: 7857281.

▪ Langgut W, Reisser T, Nishimura S, Kersten H (1993): Modulation of mammalian cell proliferation by a modified tRNA base of bacterial origin. FEBS Lett. 20;336(1):137-42. PMID: 8262197.

▪ Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD *et al* (2007): Clustal W and Clustal X version 2.0. Bioinformatics 23(21): 2947-2948. PMID: 17846036.

▪ Laskay T, van Zandbergen G, Solbach W (2003): Neutrophil granulocytes -Trojan horses for *Leishmania major* and other intracellular microbes?. Trends Microbiol 11(5):210-4. PMID: 12781523.

▪ Lieke T, Nylén S, Eidsmo L, McMaster WR, Mohammadi AM, Khamesipour A, Berg L, Akuffo H (2008): *Leishmania* surface protein gp63 binds directly to human natural killer cells and inhibits proliferation. Clin Exp Immunol 153(2):221-30. PMID: 18713141.

▪ Lira R, Doherty M, Modi G, Sacks D (2000): Evolution of lesion formation, parasitic load, immune response, and reservoir potential in C57BL/6 mice following high- and low-dose challenge with *Leishmania major*. Infect Immun. 68(9):5176-82. PMID: 10948141.

▪ Loeuillet C, Bañuls LA, Hide M (2016): Study of *Leishmania* pathogenesis in mice: experimental considerations. Parasites & Vectors 9: 144. PMID: 26969511.

▪ Lopes MF, Costa-da-Silva AC, DosReis GA (2014): Innate immunity to *Leishmania* infection: within phagocytes. Mediators Inflamm.2014:754965. PMID: 25110400.

▪ Lorenz C, Lünse EC, Mör M (2017): tRNA modifications: Impact on structure and thermal adaptation. Biomolecules. 7(2): 35. PMID: 28375166.

▪ Lye LF, Owens K, Shi H, Murta SM, Vieira AC, Turco SJ, Tschudi C, Ullu E, Beverley SM (2010): Retention and loss of RNA interference pathways in trypanosomatid protozoans. PLoS Pathog. 6(10):e1001161. PMID: 21060810.

▪ Madeira da Silva L, Owens KL, Murta SM, Beverley SM (2009): Regulated expression of the L*eishmania major* surface virulence factor lipophosphoglycan using conditionally destabilized fusion proteins. Proc Natl Acad Sci U S A. 106(18):7583-8. PMID: 19383793.

▪ Madore E, Florentz C, Giegé R, Sekine S, Yokoyama S, Lapointe J (1999): Effect of modified nucleotides on *Escherichia coli* tRNAGlu structure and on its aminoacylation by glutamyl-tRNA synthetase. Predominant and distinct roles of the mnm5 and s2 modifications of U34. Eur J Biochem. 266(3):1128-35. PMID: 10583410.

▪ Mallinson JD, Coombs HG (1989): Interaction of *Leishmania* metacyclics with macrophages. International Journal for Parasitology, 19: 647-656. PMID: 2807719.

▪ Manickam N, Joshi K, Bhatt MJ, Farabaugh PJ (2016): Effects of tRNA modification on translational accuracy depend on intrinsic codon-anticodon strength. Nucleic Acids Res. 44(4):1871-81. PMID: 26704976.

▪ Marks T, Farkas WR (1997): Effects of a diet deficient in tyrosine and queuine on germfree mice. Biochem Biophys Res Commun. 230(2):233-7. PMID: 9016755.

▪ Martínez-Calvillo S, Nguyen D, Stuart K, Myler PJ (2004): Transcription initiation and termination on *Leishmania major* chromosome 3. Eukaryot Cell. 3(2):506-17. PMID: 15075279.

▪ Martins AL, Barreto JA, Lauris JR, Martins AC (2014): American tegumentary leishmaniasis: correlations among immunological, histopathological and clinical parameters. An Bras Dermatol 89(1):52–58. PMID: 24626648.

▪ McDonagh DP, Myler JP, Stuart K (2000): The unusual gene organization of *Leishmania major* chromosome 1 may reflect novel transcription processes. Nucleic Acids Res. 28(14): 2800–2803. PMID: 10908338.

▪ McMahon-Pratt D, Alexander J (2004): Does the *Leishmania major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease?. Immunol Rev. 201:206-24. PMID: 15361243.

▪ Mears ER, Modabber F, Don R, Johnson GE (2015): A Review: The current in vivo models for the discovery and utility of new anti-leishmanial drugs targeting cutaneous leishmaniasis. PLoS Negl Trop Dis. 9(9):e0003889. PMID: 26334763.

▪ Medina-Acosta E, Cross AMG (1993): Rapid isolation of DNA from trypanosomatid protozoa using a simple 'mini-prep' procedure. Molecular & Biochemical Parasitology (59): 327-329. PMID: 8341329.

▪ Meier F, Suter B, Grosjean H, Keith G, Kubli E (1985): Queuosine modification of the wobble base in tRNAHis influences '*in vivo*' decoding properties. EMBO J 4: 823–827. PMID: 2988936.

▪ Moreira D, Santarém N, Loureiro I, Tavares J, Silva AM, Amorim AM, Ouaissi A, Cordeiro-da-Silva A, Silvestre R (2012): Impact of continuous axenic cultivation in *Leishmania infantum* virulence. PLoS Negl Trop Dis. 6(1):e1469. PMID: 22292094.

▪ Morris RC, Brooks BJ, Eriotou P, Kelly DF, Sagar S, Hart KL, Elliott MS (1995): Activation of transfer RNA-guanine ribosyltransferase by protein kinase C. Nucleic Acids Res.; 23(13):2492-8. PMID: 7630727.

▪ Müller I., Pedrazzini T., Farrell JP, Louis J (1989): T-cell responses and immunity to experimental infection with *Leishmania major*. Annual Review of Immunology, 7(1), 561–578. PMID: 2653376.

▪ Nolan TJ, Herman R (1985): Effects of long-term in vitro cultivation on *Leishmania donovani* promastigotes. J Protozool. 32(1):70-5. PMID: 3989752.

▪ Norsworthy BN, Sun J, Elnaiem D, Lanzaro G, Soong L (2004): Sand fly saliva enhances *Leishmania amazonensis* infection by modulating interleukin-10 production. Infect Immun 72(3): 1240–1247. PMID: 14977924.

▪ Okada N, Nishimura S (1979): Isolation and characterization of a guanine insertion enzyme, a specific tRNA transglycosylase, from Escherichia coli. J Biol Chem. 25;254(8):3061-6. PMID: 107167.

▪ Okada N, Noguchi S, Kasai H, Shindo-Okada N, Ohgi T, Goto T, Nishimura S (1979): Novel mechanism of post-transcriptional modification of tRNA. Insertion of bases of Q precursors into tRNA by a specific tRNA transglycosylase reaction. J Biol Chem. 254(8):3067-73. PMID: 372186.

▪ Okada N, Noguchi S, Nishimura S, Ohgi T, Goto T, Crain PF, McCloskey JA (1978): Structure determination of a nucleoside Q precursor isolated from E. coli tRNA: 7-(aminomethyl)-7-deazaguanosine. Nucleic Acids Res. 5(7):2289-96. PMID: 353740.

▪ Olivier M, Atayde VD, Isnard A, Hassani K, Shio MT (2012): *Leishmania* virulence factors: focus on the metalloprotease GP63. Microbes Infect 14(15):1377-89. PMID: 22683718.

▪ Osuchowski MF, Siddiqui J, Copeland S, Remick DG (2005): Sequential ELISA to profile multiple cytokines from small volumes. J Immunol Methods 302(1-2):172-81. PMID: 16023134.

▪ Ott G, Kersten H, Nishimura S (1982): *Dictyostelium discoideum*: a useful model system to evaluate the function of queuine and of the Q-family of tRNAs. FEBS Lett. 146(2):311-4. PMID: 7140979.

▪ Ovchinnikov DA (2008): Macrophages in the embryo and beyond: much more than just giant phagocytes. Genesis 46(9):447-62. PMID: 18781633.

▪ Padigel UM, Alexander J, Farrell JP (2003): The role of interleukin-10 in susceptibility of BALB/c mice to infection with *Leishmania mexicana* and *Leishmania amazonensis*. J Immunol. 171(7):3705-10. PMID: 14500669.

▪ Padilla-Mejía NE, Florencio-Martínez LE, Figueroa-Angulo EE, Manning-Cela RG, Hernández-Rivas R, Myler PJ, Martínez-Calvillo S (2009): Gene organization and sequence analyses of transfer RNA genes in Trypanosomatid parasites. BMC Genomics. 10:232. PMID: 19450263.

▪ Pathak C, Jaiswal YK, Vinayak M (2008): Queuine mediated inhibition in phosphorylation of tyrosine phosphoproteins in cancer. Mol Biol Rep. 35(3):369-74. PMID: 17514433.

▪ Pathak C, Jaiswal YK, Vinayak M (2008): Queuine promotes antioxidant defence system by activating cellular antioxidant enzyme activities in cancer. Biosci Rep. 28(2):73-81. PMID: 18290765.

▪ Peng D, Tarleton R (2015) EuPGDT: a web tool tailored to design CRISPR guide RNAs for eukaryotic pathogens. Microb Genom 1: e000033. PMID: 28348817.

▪ Peters NC, Sacks DL (2009): The impact of vector-mediated neutrophil recruitment on cutaneous leishmaniasis. Cell Microbiol 11(9):1290-6. PMID: 19545276.

▪ Phizicky EM, Alfonzo JD (2010): Do all modifications benefit all tRNAs?. FEBS Lett. 21;584(2):265-71. PMID: 19931536.

▪ Piedrafita D, Proudfoot L, Nikolaev AV, Xu D, Sands W, Feng GJ, Thomas E, Brewer J, Ferguson MA, Alexander J, Liew FY (1999): Regulation of macrophage IL-12 synthesis by *Leishmania* phosphoglycans. Eur J Immunol 29(1):235-44. PMID: 9933105.

▪ Plotkin BJ, Kudla G (2011): Synonymous but not the same: the causes and consequences of codon bias. Nat Rev Genet. 12(1): 32–42. PMID: 21102527.

▪ Podešvová L, Huang H, Yurchenko V (2017): Inducible protein stabilization system in *Leishmania mexicana*. Mol Biochem Parasitol. 214:62-64. PMID: 28373094.

▪ Pollock KG, McNeil KS, Mottram JC, Lyons RE, Brewer JM, Scott P, Coombs GH, Alexander J (2003): The *Leishmania mexicana* cysteine protease, CPB2.8, induces potent Th2 responses. J Immunol. 170(4):1746-53. PMID: 12574338.

▪ Rakovich T, Boland C, Bernstein I, Chikwana VM, Iwata-Reuyl D, Kelly VP (2011): Queuosine deficiency in eukaryotes compromises tyrosine production through increased tetrahydrobiopterin oxidation. J Biol Chem. 286(22):19354-63. PMID: 21487017.

▪ Ramos-Martínez E, Villaseñor-Cardoso MI, López-Vancell MR, García-Vázquez FJ, Pérez-Torres A, Salaiza-Suazo N, Pérez-Tamayo R (2013): Effect of 1,25(OH)2D3 on BALB/c mice infected with *Leishmania mexicana*. Exp Parasitol. 134(4):413-21. PMID: 23707346.

▪ Ray A, Dittel NB (2010): Isolation of mouse peritoneal cavity cells. JoVE Immunology 35. PMID: 20110936.

▪ Reiner NE, Ng W, Ma T, McMaster WR (1988): Kinetics of gamma interferon binding and induction of major histocompatibility complex class II mRNA in *Leishmania*-infected macrophages. Proc Natl Acad Sci U S A 85(12):4330-4. PMID: 2967971.

▪ Reiner SL, Zheng S, Wang Z-E, Stowring L, Locksley MR (1994): *Leishmania* promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4+ T cells during initiation of infection. J Exp Med 179(2): 447–456. PMID: 7905017.

▪ Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S (2007): Cutaneous leishmaniasis. Lancet Infectious Diseases 7(9):581-96. PMID: 17714672.

▪ Reyniers JP, Pleasants JR, Wostmann BS, Katze JR, Farkas WR (1981): Administration of exogenous queuine is essential for the biosynthesis of the queuosine-containing transfer RNAs in the mouse. J Biol Chem. 256(22):11591-4. PMID: 6795188.

▪ Ribeiro-Gomes FL, Peters NC, Debrabant A, Sacks DL (2012): Efficient capture of infected neutrophils by dendritic cells in the skin inhibits the early anti-*Leishmania* response. PLoS Pathog 8(2): e1002536. PMID: 22359507.

▪ Rogers MB et al (2011): Chromosome and gene copy number variation allow major struct.ural change between species and strains of *Leishmania*. Genome Res. 21(12):2129-42. PMID: 22038252

▪ Romier C, Reuter K, Suck D, Ficner R (1996): Crystal structure of tRNA-guanine transglycosylase: RNA modification by base exchange. EMBO J. 15(11):2850-7. PMID: 8654383.

▪ Rosas LE, Keiser T, Barbi J, Satoskar AA, Septer A, Kaczmarek J, Lezama-Davila CM, Satoskar AR (2005): Genetic background influences immune responses and disease outcome of cutaneous L. mexicana infection in mice. PMID: 16141242.

▪ Sacks D, Kamhawi S (2001): Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. Annu Rev Microbiol 55:453-83. PMID: 11544364.

▪ Sacks DL, Hieny S, Sher A (1985): Identification of cell surface carbohydrate and antigenic changes between noninfective and infective developmental stages of *Leishmania major* promastigotes. J Immunol. 135(1):564-9. PMID: 2582050.

▪ Sakamoto K, Ishimaru S, Kobayashi T, Walker JR, Yokoyama S (2004): The *Escherichia coli* argU10(Ts) phenotype is caused by a reduction in the cellular level of the argU tRNA for the rare codons AGA and AGG. J Bacteriol. 186(17):5899-905. PMID: 15317795.

▪ Sartori A, Oliveira MA, Scott P, Trinchieri G (1997): Metacyclogenesis modulates the ability of *Leishmania* promastigotes to induce IL-12 production in human mononuclear cells. J Immunol 159(6):2849-57. PMID: 9300708.

▪ Satoskar A, Bluethmann H, Akexander J (1995): Disruption of the murine interleukin-4 gene inhibits disease progression during *Leishmania mexicana* infection but does not increase control of *Leishmania donovani* infection. Infection and Immunity 63: 4894-4899. PMID: 7591152.

▪ Savill J, Dransfield I, Gregory C, Haslett C (2002): A blast from the past: clearance of apoptotic cells regulates immune responses. Nat Rev Immunol 2(12):965-75. PMID: 12461569.

▪ Schimanski B, Nguyen TN, Günzl A (2005): Characterization of a multisubunit transcription factor complex essential for spliced-leader RNA gene transcription in *Trypanosoma brucei*. Mol Cell Biol. 25(16):7303-13. PMID: 16055738.

▪ Scott P (2005): Immunologic memory in cutaneous leishmaniasis. Cellular Microbiology 7(12): 1707–1713. PMID: 16309457.

▪ Scott P, Farrell JP (1998): Experimental cutaneous leishmaniasis: induction and regulation of T cells following infection of mice with *Leishmania major*. Chem Immunol 70:60-80. PMID: 9509670.

▪ Segovia M, Artero JM, Mellado E, Chance ML (1992): Effects of long-term in vitro cultivation on the virulence of cloned lines of *Leishmania major* promastigotes. Ann Trop Med Parasitol. 86(4):347-54. PMID: 1463354.

▪ Séguin O, Descoteaux A (2016): *Leishmania*, the phagosome, and host responses: The journey of a parasite. Cell Immunol 309:1-6. PMID: 27531526.

▪ Shapira M, Zilka A, Garlapati S, Dahan E, Dahan I, Yavesky V (2001): Post transcriptional control of gene expression in *Leishmania*. Med Microbiol Immunol. 190(1-2):23-6. PMID: 11770103.

▪ Siard TJ, Jacobson KB, Farkas WR (1991): Queuine metabolism and cadmium toxicity in *Drosophila melanogaster*. Biofactors. 3(1):41-7. PMID: 1905548.

▪ Singhal RP, Kopper RA, Nishimura S, Shindo-Okada N (1981): Modification of guanine to queuine in transfer RNAs during development and aging. Biochem Biophys Res Commun. 99(1):120-6. PMID: 6165359.

▪ Slany RK, Müller SO (1995): tRNA-guanine transglycosylase from bovine liver. Purification of the enzyme to homogeneity and biochemical characterization. Eur J Biochem. 230(1):221-8. PMID: 7601103.

▪ Sollelis L, Ghorbal M, MacPherson CR, Martins RM, Kuk N, Crobu L, Bastien P, Scherf A, Lopez-Rubio JJ, Sterkers Y (2015): First efficient CRISPR-Cas9-mediated genome editing in *Leishmania* parasites. Cell Microbiol. 17(10):1405-12. PMID: 25939677.

▪ Soo SS, Villarreal-Ramos B, Anjam Khan CM, Hormaeche CE, Blackwell JM (1998): Genetic control of immune response to recombinant antigens carried by an attenuated *Salmonella typhimurium* vaccine strain: Nramp1 influences T-helper subset responses and protection against leishmanial challenge. Infect Immun 66(5):1910-7. PMID: 9573069.

▪ Sousa-Franco J, Arajujo-Mendes E, Silva-Jardim I *et al* (2006): Infection-induced respiratory burst in BALB/c macrophages kills *Leishmania guyanensis* amastigotes through apoptosis: possible involvement in resistance to cutaneous leishmaniasis. Microbes and Infection 8: 390-340. PMID: 16242371.

▪ Sterkers Y, Crobu L, Lachaud L, Pagès M, Bastien P (2014): Parasexuality and mosaic aneuploidy in *Leishmania*: alternative genetics. Trends Parasitol. 30(9):429-35. PMID: 25073852.

▪ Sypek JP, Chung CL, Mayor SE, Subramanyam JM, Goldman SJ, Sieburth DS, Wolf SF, Schaub RG (1993): Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. J Exp Med 177(6):1797-802. PMID: 8098733.

▪ Teixeira ED, Benchimol M, Rodrigues FCJ, Crepaldi HP, Pimenta PFP, de Souza W (2013): The cell biology of *Leishmania*: How to teach using animations. PLoS Pathog 9(10): e1003594. PMID: 24130476.

▪ The tRNA Modification Database: http://mods.rna.albany.edu/home

▪ Thiaville JJ et al (2016): Novel genomic island modifies DNA with 7-deazaguanine derivatives. Proc Natl Acad Sci U S A. 113(11):E1452-9. PMID: 26929322.

▪ Tuorto F, Legrand C, Cirzi C, Federico G, Liebers R, Müller M, Ehrenhofer-Murray AE, Dittmar G, Gröne HJ, Lyko F (2018): Queuosine-modified tRNAs confer nutritional control of protein translation. EMBO J. 14;37(18). PMID: 30093495.

▪ Titus GR, Marchand M, Boon T, Louis AJ (1985): A limiting dillution assay for quantifying *Leishmania major* in tissues of infected mice. Parasite Immunology 7:545-555. PMID: 3877902.

▪ Tuladhar R, Oghumu S, Dong R, Peterson A, Sharpe AH, Satoskar AR (2015): Ox40L-Ox40 pathway plays distinct roles in regulating Th2 responses but does not determine outcome of cutaneous leishmaniasis caused by *Leishmania mexicana* and *Leishmania major*. PMID: 25447125.

▪ Roberts CS (2011): The genetic toolbox for *Leishmania* parasites. Bioeng Bugs. 2(6): 320–326. PMID: 22067831.

▪ Valencia-Pacheco G, Loría-Cervera EN, Sosa-Bibiano EI, Canché-Pool EB, Vargas-Gonzalez A, Melby PC, Andrade-Narvaez FJ (2014): In situ cytokines (IL-4, IL-10, IL-12, IFN-γ) and chemokines (MCP-1, MIP-1α) gene expression in human *Leishmania (Leishmania) mexicana* infection. Cytokine 69(1):56-61. PMID: 25022962.

▪ Vinayak M, Pathak C (2009): Queuosine modification of tRNA: its divergent role in cellular machinery. Biosci Rep. 2009 Nov 23;30(2):135-48. PMID: 19925456.

▪ von Stebut E, Belkaid Y, Jakob T, Sacks LD, Udey CM (1998): Uptake of *Leishmania major* amastigotes results in activation and interleukin 12 release from murine skin–derived dendritic cells: Implications for the initiation of anti-*Leishmania* immunity. J Exp Med 188(8): 1547–1552. PMID: 9782133.

▪ Walden T, Reyniers JP, Hiatt V, Farkas WR (1982): Yeast cells cannot incorporate queuine into their tRNA. Proc Soc Exp Biol Med. 170(3):328-32. PMID: 7045884.

▪ WHO (2017): Status of endemicity of cutaneous leishmaniasis worldwide, 2015. Retrieved from http://www.who.int/leishmaniasis/burden/Status_of_endemicity_of_CL_worldwide_2015_with_imported_cases.pdf?ua=1

▪ WHO (2018): WHO Report on global surveillance of epidemic-prone infectious diseases – Leishmaniasis. Retrieved from http://www.who.int/csr/resources/publications/CSR_ISR_2000_1leish/en/

▪ Will A, Blank C, Röllinghoff M, Moll H (1992): Murine epidermal Langerhans cells are potent stimulators of an antigen-specific T cell response to *Leishmania major*, the cause of cutaneous leishmaniasis. Eur J Immunol 22(6):1341-7. PMID: 1601029.

▪ Zallot R, Brochier-Armanet C, Gaston KW, Forouhar F, Limbach PA, Hunt JF, de Crécy-Lagard V (2014): Plant, animal, and fungal micronutrient queuosine is salvaged by members of the DUF2419 protein family. ACS Chem Biol. 9(8):1812-25. PMID: 24911101.

▪ Zhang JM, An J (2007): Cytokines, Inflammation and Pain. Int Anesthesiol Clin. 45(2): 27–37. PMID: 17426506.

▪ Zhang W, Lypaczewski P, Matlashewski G (2017): Optimized CRISPR-Cas9 genome editing for *Leishmania* and its use to target a multigene family, induce chromosomal translocation, and study DNA break repair mechanisms. mSphere. 2(1): e00340-16. PMID: 28124028.

▪ Zhang X, Goncalves R, Mosser MD (2008): The isolation and characterization of murine macrophages. Curr Protoc Immunol 14: 1-18. PMID: 19016445.