

Targeting *Leishmania*: Exploring Novel Antileishmanial Compounds and Drug Targets in the Context of Parasite Cell Death

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Abstract

Leishmaniasis is a neglected tropical disease with limited therapeutic options. This thesis employed two complementary approaches and human primary cell models to identify novel biological concepts which may contribute to therapeutic advancements. In a series of phenotypic screenings, pateamines and arylmethyaminosteroids were identified as chemical entities eliciting *in vitro* antileishmanial effects. While pateamines exhibited excessive cytotoxicity towards host macrophages, both arylmethyaminosteroids could significantly reduce intracellular parasite burden. Based on their activity, authorized compounds fulfilling pharmacophore criteria were tested for antileishmanial activity with an extraordinary hit rate. Repurposing of established drugs is a pragmatic strategy to accelerate the development of new antiparasitic therapies.

As a second approach, putative drug targets in *Leishmania major* linked to cell death were investigated. Many cellular processes in these parasites remain poorly characterized. Programmed cell death is a potential drug target, as not only regulates parasite survival and death but also facilitates immunosuppressive mechanisms. Genome editing was employed to delete candidate genes hypothesized to be associated with this process. However, no links to cell death pathways were discovered.

Instead, a quantitative proteomics study identified p1/s1, a 3'-nucleotidase/nuclease, as enriched in sub-lethal stress and potentially involved in DNA degradation. Extensive characterization of this versatile ecto-enzyme revealed no link to cell death, but its dual activity was confirmed in an inducible knockout system. Degradation of free 3'-nucleotides and nucleic acids by p1/s1 provides *L. major* with essential purines while simultaneously facilitating immune evasion. Specifically, during *in vitro* infection, 3'-AMP hydrolysis led to decreased pro-inflammatory $\text{TNF}\alpha$ secretion and reduced T cell proliferation via the generation of immunomodulatory adenosine. Furthermore, p1/s1-mediated endonuclease activity enabled *L. major* to degrade and escape neutrophil extracellular traps.

In summary, this thesis provides new insights into the molecular biology of *Leishmania* and outlines several novel antileishmanial strategies, highlighting potential directions for the development of future therapeutics.

Leishmaniose ist eine vernachlässigte Tropenkrankheit mit begrenzten therapeutischen Möglichkeiten. Diese Dissertation nutzt zwei komplementäre Herangehensweisen und humane Primärzellmodelle, um neue biologische Konzepte zu identifizieren, die zu therapeutischen Fortschritten beitragen können.

In phänotypischen Testungen wurden Pateamine und Arylmethylaminosterioide als Substanzklassen identifiziert, die *in vitro* antileishmaniale Effekte hervorrufen. Wohingegen Pateamine übermäßige Zytotoxizität auf Makrophagen zeigten, konnten beide Arylmethylaminosterioide die intrazelluläre Parasitenlast signifikant reduzieren. Basierend auf deren Aktivität wurden bereits zugelassene Wirkstoffe, die Pharmakophor-Kriterien erfüllen, mit außergewöhnlich hoher Trefferquote auf antileishmaniale Aktivität getestet. Die Umwidmung von etablierten Medikamenten stellt einen pragmatischen Ansatz dar, um die Entwicklung neuer antiparasitärer Therapien zu beschleunigen.

Als zweiter Ansatz wurden mutmaßliche Wirkstoff-Zielproteine in *Leishmania major* untersucht, die in Verbindung mit Zelltod stehen könnten. Viele zelluläre Prozesse dieser Parasiten sind nur unzureichend charakterisiert. Programmierter Zelltod in Leishmanien ist ein potenzielles Ziel für Wirkstoffe, da er Überleben und Tod der Parasiten steuert, aber auch immunsuppressive Mechanismen ermöglicht. Mittels Genom-Editierung wurden Kandidatengene deletiert, die in Verbindung mit diesem Prozess stehen könnten. Allerdings wurden keine direkten Verbindungen zu Zelltod gezeigt.

Stattdessen identifizierte eine quantitative Proteomstudie die 3'-Nukleotidase/Nuklease p1/s1, die unter subletalem Stress an DNA Abbau beteiligt sein könnte. Eine umfangreiche Charakterisierung dieses vielseitigen Ektoenzym zeigte keine Verbindung zu Zelltod auf. Dagegen wurde dessen duale Aktivität durch eine induzierte Gendeletion bestätigt. Der Abbau von freien 3'-Nukleotiden und Nukleinsäuren durch p1/s1 versorgt *L. major* mit essentiellen Purinen, ermöglicht aber auch Mechanismen der Immunevasion. Insbesondere führte die Hydrolyse von 3'-AMP während *in vitro* Infektionen durch Generierung von immunmodulatorischem Adenosin zu verminderter Sekretion von proinflammatorischem $\text{TNF}\alpha$ und T-Zell Proliferation. Weiterhin erlaubte die p1/s1-induzierte Endonuklease-Aktivität den Abbau von Neutrophilen Extrazellulären Fallen und damit den Austritt aus solchen.

Zusammengefasst bietet diese Thesis neue Erkenntnisse in der Molekularbiologie von Leishmanien und beschreibt mehrere neuartige antileishmaniale Strategien, die potenzielle Ansätze für die Entwicklung zukünftiger Therapien aufzeigen.

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List of Abbreviations

Further used abbreviations including amino acid symbols, chemical elements and units correspond to IUPAC nomenclature.

Ado	Adenosine
AMP	Adenosine monophosphate
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
Cas	CRISPR-associated gene
CC	Cytotoxic concentration
CD	Cluster of differentiation
cDNA	Complementary DNA
CL	Cutaneous leishmaniasis
CRISPR	Clustered regularly interspaced palindromic repeats
DMDAPatA	Desmethyl-desamino-Pateamine A
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DNDi	Drugs for Neglected Diseases initiative
DRUID	Novel Drug Targets against Poverty-related and Neglected Tropical Infectious Diseases

dsM13	Double-stranded M13 phage genome
DTT	Dithiothreitol
EC	Effective concentration
EDTA	Ethylenediaminetetraacetic acid
eIF4A	Eukaryotic translation initiation factor 4A
ELISA	Enzyme-linked Immunosorbent Assay
FCS	fetal calf serum
FSC	Forward scatter
gDNA	Genomic DNA
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HIV	Human immunodeficiency viruses
hMDM	Human monocyte derived macrophage
HR	Homology recombination
IFN	Interferone
iKO	Induced knockout
IL	Interleukine
kb	Kilobases
KO	Knockout
LB	Lysogeny broth
Ld	<i>Leishmania donovani</i>
Lm	<i>Leishmania major</i>
LOEWE	Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz
logPh	Logarithmic growth phase
loxP	Locus of X-over P1

LIST OF ABBREVIATIONS

LPS	Lipopolysaccharide	rpm	Rounds per minute
M-CSF	Macrophage colony-stimulating factor	rt-qPCR	Quantitative real-time polymerase chain reaction
MACS	Magnetic cell separation	rt	Room temperature
MFI	Mean fluorescence intensity	SDS-PAGE	Sodiumdodecylsulfate polyacrylamide gel electrophoresis
MOI	Multiplicity of infection	SDS	Sodium dodecyl sulfate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	sgRNA	Single guide RNA
NET	Neutrophil extracellular trap	SI	Selectivity index
NMT	N-myristoyltransferase	SOC medium	Super optimal broth with catabolite repression medium
NNN	Novy-Nicolle-McNeal medium	SSC	Site scatter
PatA	Pateamine A	ssM13	Single-stranded M13 phage genome
PBL	Peripheral blood leukocyte	SSU	Small subunit
PBMC	Peripheral blood mononuclear cell	statPh	Stationary growth phase
PBS	Phosphate buffered saline	TAE	TRIS-acetat-EDTA
PCD	Programmed cell death	TBST	Tris-buffered saline with Tween20
PEI	Paul-Ehrlich-Institut	TEMED	Tetramethylethylenediamine
P_i	Free phosphate	Th	T helper cell
PI	Propidiumiodide	TMD	Transmembrane domain
pi	Post infection	TNF	Tumour necrosis factor
PMA	Phorbol-12-Myristat-13-acetat	TRIS	Tris(hydroxymethyl)aminomethane
PMN	Polymorphonuclear neutrophil	TTM	Ammouniumtetrathiomolybdate
PtdSer	Phosphatidylserine	VDAC	Voltage-dependent anion channel
QSAR	Quantitative structure-activity relationship	VL	Visceral leishmaniasis
RNA	Ribonucleic acid	WHO	World Health Organization
ROS	Reactive oxygen species		
RPMI	Roswell Park Memorial Institute		

Introduction

1.1 Biology of *Leishmania* spp.

Leishmania spp. are unicellular, parasitic protozoans that are the infective agents of leishmaniasis. They share the family of trypanosomatidae, which is characterized by a single flagellum and the presence of a kinetoplastid, with other pathogens like *Trypanosoma*. The genus *Leishmania* (L.) is divided in the four subgenera *Leishmania*, *Mundinia*, *Viannia*, together harboring up to 20 human pathogenic species and subspecies, and *Sauroleishmania*, with non-pathogenic species found in reptiles (88). Taxonomic classification within the genus is based on several molecular markers such as internal transcribed spacer of ribosomal RNA loci or heat-shock proteins (96).

In 1903, first descriptions of the causative agent of a fever-characterized disease in India were independently published by William Boog Leishman and Charles Donovan and later identified as a protozoan organism. Named after both of them, the pathogen *Leishmania donovani* was named after both of them (76, 162, 223). All pathogenic *Leishmania* species share a similar dixerous life cycle, alternating between an insect and a mammalian host, see Figure 1.1. As motile promastigotes of $\approx 15\text{-}20\text{ }\mu\text{m}$ body length, they colonize the digestive tract of phlebotomine sand flies found in tropical and subtropical areas. In the Old World, the genus *Phlebotomus* serves as main vector, in the New World transmission is dominated by *Lutzomyia*. The earliest evidence of leishmanial ancestors, in the alimentary tract of sand flies, termed *Paleoleishmania*, dates back to a 100-million year old fossil (210).

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As procyclic promastigotes, *Leishmania* are highly proliferative in the abdominal midgut, have a slender form and possess a flagellum up to twice their body length. After maturation into non-dividing nectomonad promastigotes, they migrate to the anterior midgut, transform into leptomonad promastigotes, initiate another proliferative stage and later differentiate into metacyclic promastigotes. After metacyclogenesis, the now quiescent and infective promastigotes localize to the proboscis, where they can attach to the inner lining of the stomodeal valve. This part of the life cycle can be completed within 10 days (77, 112, 225).

Upon the next bloodmeal of a female sand fly, infective metacyclic promastigotes are delivered into the skin of the mammalian host together with saliva and apoptotic-like parasites (254, 268). Parasite transmission can be enhanced by the parasites themselves through destruction of the stomodeal valve and plugging of the midgut by proteophosphoglycan filaments (77). Several mammals can serve as reservoir for pathogenic *Leishmania*, including humans, dogs, cats and rodents. Following inoculation, the sand fly saliva and parasites trigger leukocyte migration to the site of the bite, especially of neutrophils (257). Promastigotes are then phagocytosed into neutrophils, where they cannot multiply but use them as a "Trojan horse": They delay host cell apoptosis and attract macrophages by inducing secretion of chemotactic factors. After the final apoptosis of neutrophils, macrophages can internalize them together with the parasites during silent phagocytosis without eliciting an inflammatory immune response (3, 255). In macrophages, their final host cell, *Leishmania* modify the initial phagosome into a parasitophorous vacuole, which is guided by lipophosphoglycans on the parasite surface by reducing fusion with endosomes and lysosomes (72). Parasitophorous vacuoles can be communal or individual, holding one or several parasites, depending on the species (8, 58). About 24 to 48 hours post internalization, differentiation into proliferating amastigotes is completed. Amastigogenesis is tightly regulated by an interplay of environmental changes, like temperature and pH, and a cellular response (18). Intracellular amastigotes are non-motile, about 3-5 μm in size with an ovoid phenotype and a very short flagellum (77).

The mechanism by which *Leishmania* amastigotes spread within a mammalian host is still under debate. Whereas rupture of the macrophage membrane and thus release of amastigotes was assumed previously (192), recent results describe a direct cell-to-cell transfer via parasite proliferation induced macrophage apoptosis (14). The life cycle is

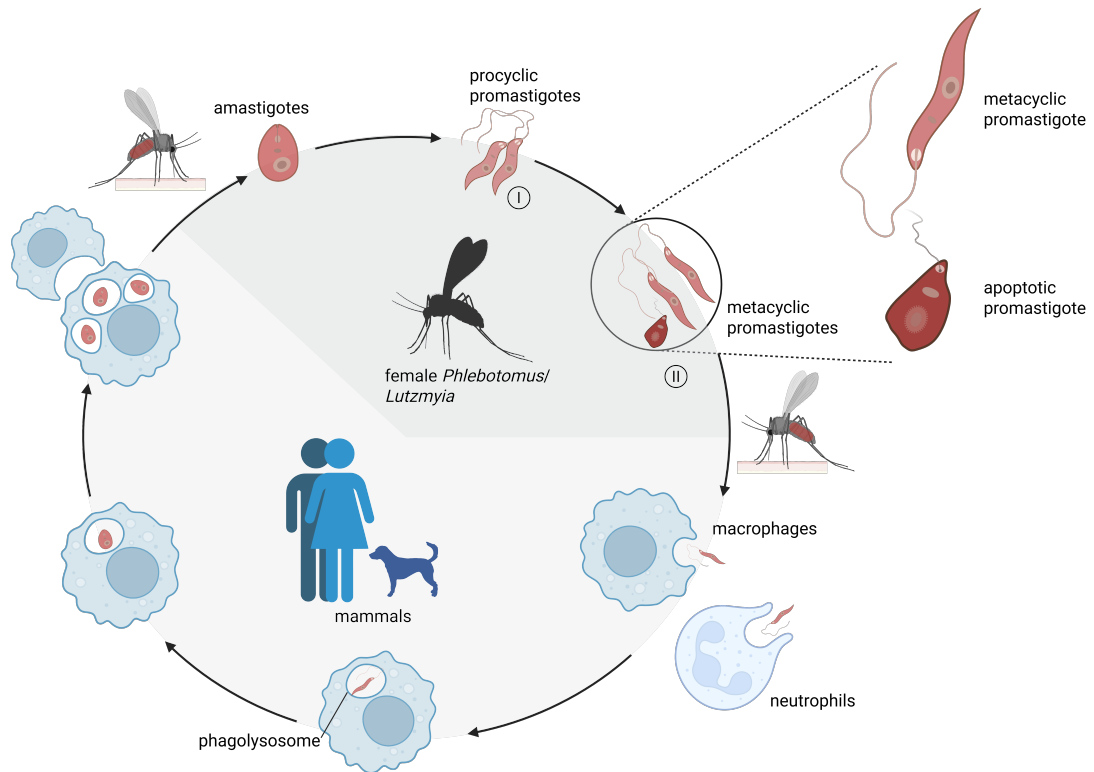


Figure 1.1: Schematic diphasic lifecycle of *Leishmania* - In female sand flies, procyclic promastigotes (I) proliferate in the sand fly midgut, differentiate into infective metacyclic promastigotes (II, upper right) and migrate to the stomodeal valve. During blood-meal on a mammalian host, metacyclic promastigotes are transferred together with apoptotic parasites and saliva in the inoculum. In the skin, they are internalized by attracted phagocytes (lower right). Neutrophils can be used "Trojan horse" (255) for silent transfer into macrophages, the final host cell. Inside of phagolysosomes, the parasites differentiate into round, non-motile amastigotes (lower left), proliferate and spread by cell-to-cell transfer. The lifecycle is completed when a sand fly takes up infected cells and amastigotes transform back into promastigotes in the sand fly midgut (upper left). Figure created with BioRender.

1. INTRODUCTION

completed when a female sand fly feeds on an infected host and amastigotes retransform into procyclic promastigotes in the sand fly midgut.

1.1.1 *Leishmania* genetics

Although trypanosomatids are eukaryotic organisms, their genomic architecture combines both prokaryotic and eukaryotic characteristics. Typically, open reading frames are organized in polycistronic clusters of functionally unrelated genes, and general transcription factors. Consensus promoter sequences are absent (132). The genome of Old World *Leishmania* consists of 36 chromosomes, whereas, due to chromosome fusions, New World species only possess 34 or 35 chromosome. Additionally, aneuploidy is very common in *Leishmania* (41, 222). The *L. major* genome is predicted to contain 8272 protein-coding genes, 3083 of which are arranged in clusters. Potentially, single locus clusters formed through tandem duplication, multi-locus clusters often encode *Leishmania*-specific proteins (132). Only a small number of unique genes differentiate *Leishmania* species (222).

Gene regulation in *Leishmania* is primarily post-transcriptional, since most of the genome is constitutively expressed (161). Due to very high genome instability, the parasites can rapidly adapt gene expression through haplotype changes, single nucleotide polymorphisms, and copy number variations (32, 44, 130, 212). However, gene dosage and mRNA levels do not always correlate with protein abundance (64, 65, 161). This plasticity allows rapid gain of fitness under selective pressure such as drug exposure (189), while a phenotypic robustness is ensured by high genetic redundancy, which balances the functionality of the parasitic organism against genomic instability. Additionally, translational or post-translational regulation of protein expression is commonly found in *Leishmania* during nutrient deprivation (50, 233) and may involve various mechanisms such as the action of non-coding RNAs and RNA binding proteins, including initiation factors (215, 235).

Due to the absence of DNA repair by non-homologous end joining and RNA interference mechanisms in most *Leishmania* species (170, 190), the toolbox for targeted genetic manipulation in these parasites was limited for many years. Reverse genetic studies relied on the parasites' predisposition to undergo homologous recombination for transgene insertion. Templates for genomic integration were supplied as linear DNA,

containing flanking regions specific for the gene of interest, and a selection marker (61). However, due to the laborious workflow, by 2018 only approximately 200 genes from various *Leishmania* species were assessed by targeted deletion (137).

The introduction of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated gene 9 (Cas9)-guided genome editing rapidly advanced reverse genetic studies in trypanosomatids. Cas9 can precisely introduce breaks on double-stranded DNA when bound to a single guide RNA (sgRNA) that can be engineered to target any locus. After introducing *Leishmania* strains with stably expressed Cas9 and a T7 RNA polymerase, knockouts can easily be generated by electroporation with sgRNA templates and a repair cassette. DNA cleavage at the targeted site facilitates the homologous recombination of a repair cassette encoding a resistance marker and homology flanks of ≈ 30 nucleotide length. Alternatively, the gene of interest can be tagged for various purposes, like fluorescence or proximity labeling. Through easily accessible resources for primer design and PCR templates, this scalable method has accelerated genetic studies of *Leishmania* (27). Combined with barcoding strategies, first pooled screenings enabled large-scale reverse genetic studies (26).

By now, DNA editing approaches that precisely introduce premature stop codons have been established in *Leishmania* (84). This allows the generation of a knockout library containing parasites with mutations in the vast majority of protein-coding genes, including multi-copy genes that are otherwise difficult to target. Such pooled screenings allow identification of phenotypes on the whole-genome level, allowing the discovery of genes linked to fitness, drug susceptibility or infectivity, for example (70).

Claiming essentiality of a *Leishmania* gene is crucial for drug target research. Whether the failure to delete a gene is due to technical or true biological reasons can be tested using methods besides CRISPR. In case of essentiality, null mutants can be rescued through complementation, either genetically by providing an additional copy of the gene or nutritionally by supplementing a metabolite. If a gene knockout is complemented by an episomally expressed gene copy, the retention of this copy without drug selection is a strong indicator of essentiality. Otherwise, deletion of a locus can be achieved through by inducing dimerizable Cre (DiCre). This recombinase can mediate recombination of loxP sites integrated up- and downstream of the locus of interest (81), as depicted in Figure 1.2. If excision of the gene post DiCre induction is lethal, essentiality is also very likely. Application and combination of reverse genetic methods can

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enhance the validation of drug targets and thus inform specific drug design, although genomic instability in response to gene deletion and high functional redundancy can complicate definitive conclusions (137).

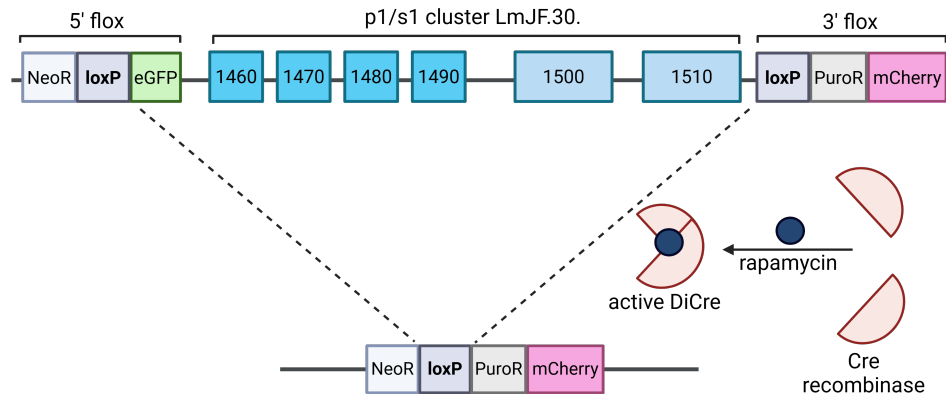


Figure 1.2: Schematic principle of dimerizable Cre (DiCre) recombination for inducible knockout generation - Flox cassettes containing loxP sites, a resistance and a fluorescence marker flank the locus of interest, here *p1/s1*. Rapamycin induces dimerization of the Cre recombinase to excise the floxed locus. NeoR: Neomycin resistance, PuroR: Puromycin resistance, flox: LoxP site flanked, loxP: Locus of X-over P1. Figure created with BioRender

1.1.2 Purine metabolism of *Leishmania*

As common for all trypanosomatids, *Leishmania* are purine auxotrophs, as they lack any *de novo* biosynthesis pathways for purine rings (117, 174). To maintain supply of their nucleotide pools, they depend on nutrient salvage from the host environment. However, macromolecules such as nucleotides and nucleic acids need to be degraded extracellularly prior to uptake, as they cannot be translocated across the plasma membrane.

To accomplish extracellular purine salvage, *Leishmania* employ ecto-enzymes to sequentially degrade nucleotides and nucleic acids. Such enzymes can either be secreted or membrane-bound with their active site facing the extracellular space. In addition to 5'-nucleotidases, which hydrolyze 5'-nucleotides, *Leishmania* also utilize class I nucleases with dual 3'-nucleotidase and nuclease activity (Figure 1.3) (113). Nucleoside hydrolases further participate in the generation of free nucleobases (35, 63).

1.1 Biology of *Leishmania* spp.

Nucleoside transporters, which are specific either for adenosine and pyrimidines or inosine, xanthine and guanosine, then mediate transport across the parasite membrane into the cytoplasm (167, 258). Amastigotes express a specific purine nucleoside transporter optimized for the acidic environment of the phagolysosome (109). Nucleobase transport is executed by distinct permeases (197, 226).

Intracellularly, *Leishmania* convert acquired purines in an interwoven metabolic network that contains several enzymatic redundancies and involves a multiplicity of enzymes, such as phosphoribosyltransferases, kinases and hydrolases. In contrast to human purine metabolism, *Leishmania* partly compartmentalize this pathway within the glycosome (35).

Due to the essential nature of purine salvage in trypanosomatids, this process can be exploited for drug development. Purine nucleoside derivatives can be toxic to the parasites following intracellular enzymatic conversion, but non-toxic to mammals due to differences in purine derivative transport efficiency (13, 19, 46, 124, 252). This principle also applies for allopurinol, a nucleobase analogue, that is in therapeutic use to treat canine leishmaniasis (107). Alternatively, enzymes or transporters along the purine salvage cascade can be inhibited. However, such approaches can be hampered by increased drug efflux and functional redundancy in the pathways (124).

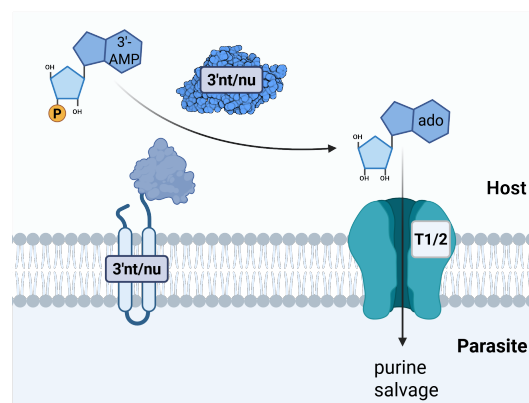


Figure 1.3: Schematic depiction of extra-cellular 3'-AMP salvage in *Leishmania* - 3'-nucleotidases/nucleases, such as p1/s1, are as ecto-enzymes either membrane-bound or secreted, thus facing the host environment. By degrading free 3'-nucleotides to nucleosides, for example 3'-AMP to adenosine (ado), they facilitate the uptake of purine precursors into the parasite. Transporters then mediate the absorption of nucleosides through specific transporters. Figure created with BioRender.

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1.1.3 3'-nucleotidases/nucleases in *Leishmania*

3'-nucleotidases/nucleases of *Leishmania* belong to the class I nucleases and are part of the upstream purine salvage cascade (Figure 1.3). This enzyme class is exclusively present in protozoans, plants and fungi and has no homologues in vertebrates (191). To hydrolyze their substrates, these enzymes mediate nucleophilic hydrolysis through a coordinated Zn^{2+} ion (264, 282), with a preference for single-stranded nucleic acids and 3'-AMP as substrates, but they can also cleave double-stranded substrates and diverse 3'-nucleotides with lower efficiency (47, 191). Five conserved domains are characteristic of class I nucleases in *Leishmania* that share high similarity to the nucleases P1 and S1 of *Penicillium citrinum* and *Aspergillus oryzae* (133, 264). A highly similar catalytic site is also shared with phospholipases C (126). Typically, eukaryotic enzymes of this class are N-glycosylated to enhance stability and solubility of the protein. Although *Leishmania* 3'-nucleotidases/nucleases possess putative glycosylation sites, neither phospholipase activity nor effects of linked oligosaccharides have been tested (138).

In *Leishmania*, genes encoding 3'-nucleotidases/nucleases are present in all species and the respective proteins have been characterized in a broad range of them. These proteins are often stage-specifically expressed and can be induced by nutrient depletion (69, 138, 140, 200, 224, 261). Consistent with the observed ecto-3'-nucleotidase/nuclease activity in promastigotes, these enzymes were described as membrane-bound or secreted (138, 209). Concerning *L. major*, a promastigote-specific enzyme with plasma membrane localization and an amastigote-specific class I nuclease were described (90, 158). Further genes annotated as putative class I nucleases are predominantly expressed in amastigotes (161). However, the functions of 3'-nucleotidases/nucleases in amastigotes remain hypothetical. On promastigotes, they are described as part of extracellular purine salvage, although reducing their activity by an unspecific inhibitor does not decrease parasite viability (114, 200).

Besides their relevance in metabolism, 3'-nucleotidases/nucleases are well characterized players in host-pathogen interactions to enhance parasite infectivity. Increased association to macrophages is suspectedly linked to the generation of adenosine from 3'-AMP through their enzymatic activity (200, 261), likely through the activation of purinergic signalling on the host cells, see Section 1.2.1.1. Additionally, their ecto-nuclease activ-

ity allows *Leishmania* to escape from neutrophil extracellular traps by degrading their DNA scaffold, that otherwise can prevent infection establishment (99, 114).

Class I nucleases of *Leishmania* have also been studied as potential vaccine antigens. The amastigote-specific antigens P4 of *L. pifanoi* and LmaC1N of *L. major* have been shown to elicit a T helper 1-mediated protection in mice, exploiting their specificity for the parasites (91, 140, 241).

1.2 Leishmaniasis

Leishmaniasis are a group of neglected tropical diseases caused by human pathogenic *Leishmania spp.* Depending on the infective agent and its tissue tropism, the disease can manifest in distinct clinical forms with varying incidence and fatality rates, but not every individual infected with *Leishmania* develops symptoms. Only a fraction of cases is reported to the World Health Organization, a high number of unrecorded cases are assumed (273, 274). Mainly, leishmaniasis can be distinguished into three main conditions:

Cutaneous leishmaniasis (CL) is the most common form globally, with over one million new cases estimated each year, marked by prominent lesions and ulcers on skin regions exposed to the sand fly vector (Figure 1.4a). Such lesions can be high in number but also heal spontaneously without treatment. Nevertheless, remaining scars or late complications can lead to social stigma and disability (273, 274). The disease is also known as "Oriental sore", "Aleppo-" or "Baghdad boil" and "forest yaws", referring to the visual and geographical appearance of CL lesions (243). Often, visible symptoms develop locally at the site of the insect bite but in case of insufficient immune responses, they can also disseminate as diffuse nodules. Typical species responsible for CL are *L. amazonensis* in South America, *L. mexicana* in Central America and Mexico, *L. aethiopica* in Ethiopia and Kenya as well as *L. tropica* and *L. major* in widespread regions of Central Asia, the Middle East and north and East Africa (218).

With about 50-90,000 new cases annually and a near 100% fatality rate within two years if left untreated, visceral leishmaniasis (VL), also known as kala-azar ("black fever"), is considered the most severe form with significant outbreak and mortality potential. It is characterized by irregular fever waves, cachexia, enlargement and inflammation of

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spleen and liver and anaemia, as visible in Figure 1.4b (227, 273, 274). Epidemiologically, the etiological agents of VL belong to the *L. donovani* complex, with *L. donovani* being mostly spread by anthroponotic and zoonotic infections in the Old World, especially South Asia and sub-Sahara Africa, and *L. infantum* transmitted zoonotically in South America and the Mediterranean region (216). Symptoms usually develop 2-8 months post infection (22). Up to 10% of VL patients face post-kala-azar dermal leishmaniasis as a sequel to VL, appearing usually 6-12 months after initial cure. It appears as a skin rash on the upper body, accompanied by hypopigmentation (273, 274).

Mucocutaneous leishmaniasis occurs mainly in Bolivia, Brazil, Peru and Ethiopia.



Figure 1.4: Spectrum of clinical manifestations of different forms of leishmaniasis.

- **a)** Cutaneous leishmaniasis lesions of Afghan patients infected with *L. tropica*, visible as acute (above) or relapsing (below) ulcer (218); **b)** Sudanese patient with visible splenomegaly and signs of malnutrition before treatment for visceral leishmaniasis (290); **c)** Lesion of the nasal mucosa as seen during acute mucocutaneous leishmaniasis (1).

It is considered as the most disabling disease form, leading to severe inflammatory destruction and lesions of the oral and nasal mucosal tissue (Figure 1.4c), often causing disfigurement and thus social isolation (273, 274). *L. braziliensis* is responsible for most cases which only rarely resolve spontaneously (1).

Comorbidities and superinfections, such as secondary bacterial infections, often accompany and potentiate leishmaniasis (6). Immunosuppression, as caused by coinfection with human immunodeficiency viruses (HIV) can increase the chances for relapses and mortality. Specialized treatment options are recommended for patients with a HIV-*Leishmania* coinfection (274).

Risk factors for leishmaniasis are heavily linked to poverty and socioeconomic factors. Exposure to the sand fly vector is directly connected to crowded housing and poor sanitary facilities. Additionally, malnutrition, migration and environmental changes increase the risk of transmission and disease outbreak, reflected by the geographical

distribution of leishmaniasis in tropical or sub-tropical countries (Figure 1.5) (274). Foci of infection in such regions often align with low living standards. Usually, when living standards are improved, the incidence of new cases declines (216). There, preventive measures to reduce the risk of infection include vector control and surveillance besides pre-exposure precautions like the use of bed nets. Due to progressing climate change and high human mobility, autochthonous infections in previously non-endemic regions are expected to emerge, also in the European Union (30, 220).

Besides human leishmaniasis, veterinary leishmaniasis is a widespread issue, as many mammals can serve as *Leishmania* reservoirs (1). Most actions focus on companion animals as leishmaniasis in this cohort is expanding beyond typical endemic areas. Several treatments or vaccines are available for canine and feline leishmaniasis (186, 207).

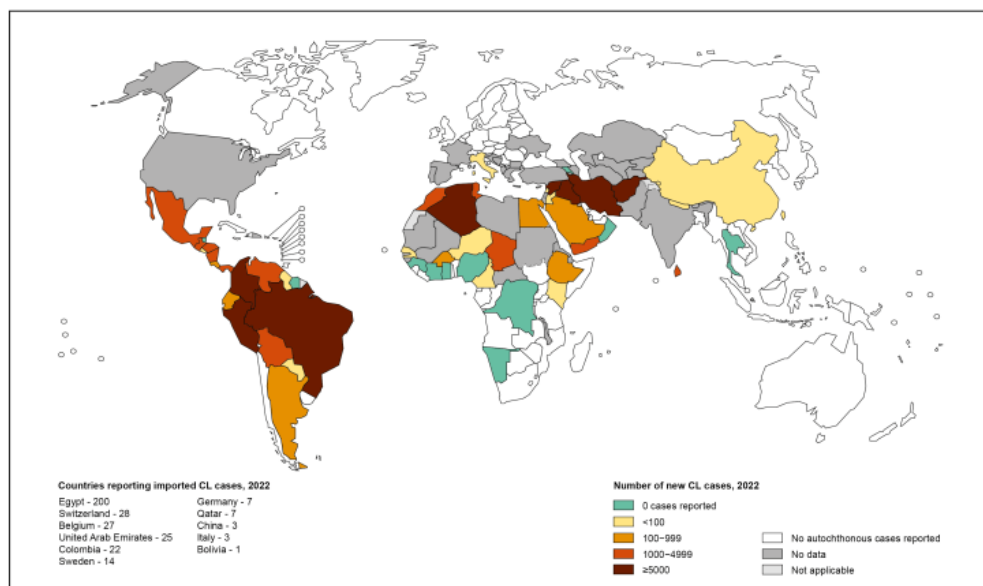
1.2.1 Immune response during infection

The clinical outcomes of leishmaniasis are largely dependent on the immune response developed by the host. As intracellular pathogens, *Leishmania* cannot be neutralized by antibodies. The humoral response is less critical for parasite control than a strong T cell response that stimulates intracellular parasite restriction. However, exuberant T cell activation can also lead to destructive immunopathology (15, 51). Thus, a balanced immune response is crucial to successfully control the disease.

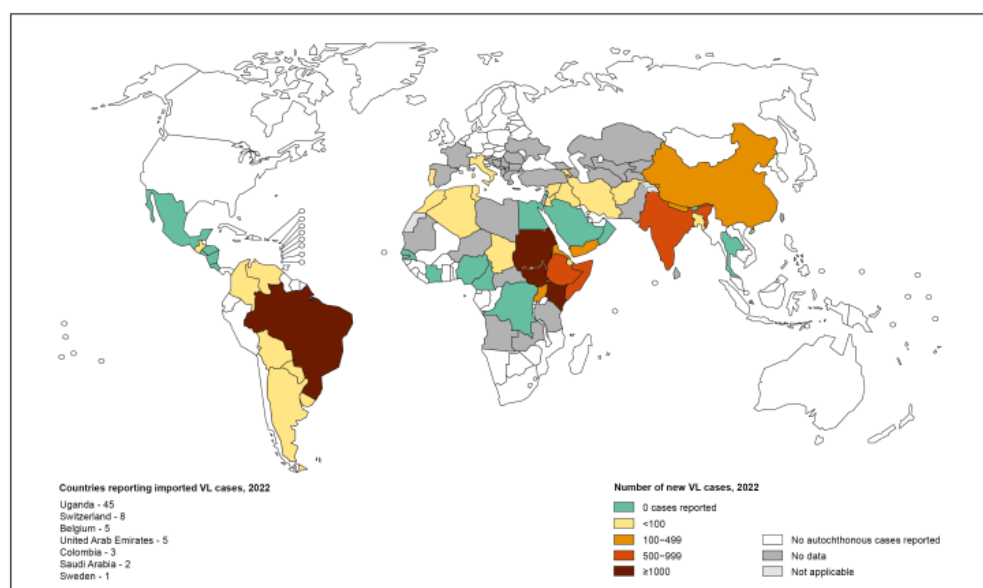
Early innate immune responses after *Leishmania* inoculation are not only steered by the parasites, but also by components of the sand fly saliva (49, 143, 257). Additionally, *Leishmania* can release a chemotactic factor to attract neutrophils (253). They are among the first immune cells recruited to the infection site, alongside dermis-resident immune cells, but their role in disease development is double-edged concerning disease promotion or suppression (208). Neutrophils are able to induce the formation of neutrophil extracellular nets (NETs), extracellular structures composed of chromatin and granule proteins with antipathogenic capacities (40, 115). Typically, NET extrusion is preceded by NETosis, a specialized form of cell death, induced through the reactive oxygen species (ROS) pathway, followed by nuclear disassembly and membrane rupture. Alternatively, non-lytic NETosis is a very rapid process leaving behind anucleated but still phagocytic cytoplasts (reviewed in (202)). Presumably, both pathways can be induced by *Leishmania* promastigotes (102, 221).

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a Status of endemicity of cutaneous leishmaniasis (CL) worldwide, 2022 (as reported by November 2023)



b Status of endemicity of visceral leishmaniasis (VL) worldwide, 2022 (as reported by November 2023)



The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement. © WHO 2023. All rights reserved

Data Source: World Health Organization
Map Production: Control of Neglected
Tropical Diseases (CNTD)
World Health Organization



Figure 1.5: Endemic distribution of leishmaniasis. - Global numbers of reported **a)** cutaneous (CL) and **b)** visceral leishmaniasis (VL) cases as reported to the World Health Organization (WHO) for 2022, also listed are imported cases. The number of non-reported cases is expected to exceed reported cases. Regions predominantly affected by CL are Middle and South America, Central Asia, the Arabic peninsular and Northern and East Africa. Regions and countries predominantly affected by VL are Brazil, East Africa, Southern Asia and China. Picture sources: WHO Status of endemicity of cutaneous leishmaniasis worldwide 2022 (275); WHO Status of endemicity of visceral leishmaniasis worldwide 2022 (276)

NETs exert general antimicrobial effects through several components, but likely histones are mediating toxicity towards *Leishmania* promastigotes (115, 269). However, nucleases, including 3'-nucleotidases/nucleases, deriving from the parasites or sand fly saliva are able to degrade the DNA scaffold of NETs to allow escape and thus survival (54, 114). Such activities can explain why antileishmanial activity of NETs is limited for promastigotes of several *Leishmania* species (102, 114). Similar escape mechanisms have been observed for several other pathogens, for example *Streptococcus* or *Vibrio* (52, 67, 232).

After phagocytic uptake into attracted neutrophils, *Leishmania* are able to temporarily delay host cell apoptosis. Infected neutrophils are stimulated to release the chemotactic factor MIP-1 β to attract macrophages, that then eventually can clear apoptotic neutrophils together with intracellular *Leishmania*. This silent phagocytosis elicits no inflammatory immune response but is accompanied by the release of anti-inflammatory transforming growth factor β , enabling efficient infection establishment (3, 255). Thus, using neutrophils as "Trojan horses" for entry into their final host cells is beneficial for the parasites, but they can also be directly taken up by macrophages via receptor-mediated phagocytosis (166). In this case, several receptor types, including complement receptors and Fc receptors, can mediate parasite uptake and determine the subsequent immune response (reviewed in (204)).

Intracellular restriction of parasites relies on ROS formation in a respiratory burst following phagocytosis. However,

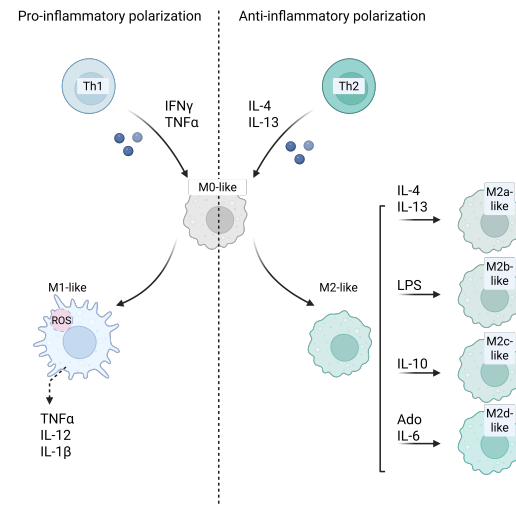


Figure 1.6: Simplified principle of macrophage polarization. - Resting M0-like macrophages can either be activated into a pro-inflammatory M1-like phenotype through Th-1 response cytokines (left) or alternatively activated into an anti-inflammatory M2-like phenotype during by Th-2 cytokines (right). Several M2-like subtypes can be differentiated, depending on present stimuli. Macrophage polarization can only roughly be categorized into strict phenotypes, their activation rather represents a broad spectrum. Figure modified after (4) and created with BioRender.

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non-activated macrophages cannot sufficiently kill intracellular *Leishmania* as the latter are able to suppress ROS synthesis in the phagolysosome (177). In mice, nitric oxide mediated parasite killing is the superior pathway for *Leishmania* elimination. NO is formed by the inducible nitric oxide synthase, either in the host cell or in a bystander cell, but in humans, its role in parasite control is conflicting (reviewed in (230)).

The equilibrium between a T-helper (Th)-1 and Th-2 dominated response contributes in shaping the clinical outcome of leishmaniasis. In mice, an IL-12 induced Th-1 based response confers resistance towards an infection, whereas an IL-4 promoted Th-2 based response mediates susceptibility, but the overall outcome is dictated by a more complex adaptive immune response. Recruited CD4⁺ T cells mainly steer the activation of infected macrophages by secretion of interferon (IFN) γ , tumour necrosis factor (TNF) α and several more cytokines (204, 230). A variety of immune cells impact *Leishmania* restriction through cytokine secretion, including natural killer cells, CD8⁺ T cells and dendritic cells (111). The latter can also phagocytose *Leishmania* but do not serve as a long-term niche for proliferation. Rather, their ability to present antigens and produce IL-12 is pivotal for developing immunity. After clearance of the primary infection, T cells can mediate long-lasting immunity (164).

The Th-1/Th-2 balance determines activation and differentiation of macrophages originating from their resting M0-like state. Macrophages can exhibit diverse phenotypes along an axis of non-inflammatory to pro-inflammatory states. Classically activated macrophages of an M1-like phenotype are exposed to cytokines secreted by Th-1 cells, including IFN γ and TNF α , but also granulocyte-macrophage colony-stimulating factor (GM-CSF). They are characterized by an increased mitochondrial metabolism and ROS generation, thereby limiting *Leishmania* survival (251). Alternatively activated macrophages of an M2-like phenotype are stimulated by Th-2 cytokine patterns to adopt a regulatory, tissue-protective phenotype. They have an enhanced polyamine metabolism, favoring *Leishmania* persistence (172). Further M2-like phenotypes can be distinguished: M2a-like macrophages are polarized by macrophage colony-stimulating factor (M-CSF), IL-4 and IL-13, M2b-like by lipopolysaccharides, M2c-like by IL-10 and M2d by IL-6 and adenosine. They all possess different functions in terms of immune cell recruitment, regulation, and tissue regeneration. Thereby, macrophage polarization is central in determining the fate of a *Leishmania* infection (11, 251).

Due to significant differences in the immune system of mice and humans and thus the response towards *Leishmania* infection, experimental results derived from animal experiments must be carefully interpreted for transferability to human patients. Additionally, heterogeneity of laboratory mice strains and in most cases the absence of the sand fly vectors can influence experimental outcomes. Especially the absence or diminished role of nitric oxide in human macrophages reduces parasite elimination in comparison to mice models (106). Thus, for *in vitro* experiments, the human primary immune cells are the model of choice.

1.2.1.1 Purinergic signalling

Various stress stimuli, including *Leishmania* infection, can lead to the release of adenosine 5'-triphosphate (ATP) as danger-associated molecular pattern to the extracellular space (25, 55). ATP can bind to P2 receptors, a family of membrane-bound receptors divided into P2X and P2Y subtypes, both of which generally initiate danger signals to promote a pro-inflammatory immune response (228). Ectonucleotidases, mainly CD39, sequentially dephosphorylate ATP, thereby reversing this effect by limiting extracellular ATP accumulation. Ecto-5'-nucleotidases like CD73 can then convert 5'-adenosine monophosphate (5'-AMP) to adenosine, a "retaliatory metabolite" (120, 284). Extracellular adenosine can bind to P1 receptors, eliciting tissue-protective signaling to counteract previous ATP signaling effects.

The P1 receptor family consists of A₁, A_{2a}, A_{2b} and A₃ transmembrane receptors, all coupled to G-proteins to initiate downstream signaling. Both A₂ receptors stimulate adenylyl cyclases via G_{αs} signaling, initiating anti-inflammatory pathways that promote cytokine secretion and reduce the respiratory burst (120, 122). The main stimulated receptor in pathologically high concentrations of extracellular adenosine is A_{2b} (98). Stimulation of adenosine receptors also mediates macrophage differentiation into the M2d-like phenotype, see Figure 1.6 (62, 86).

The relevance of adenosine and purinergic signaling in *Leishmania* infection has been investigated in various contexts, suggesting that parasites exploit anti-inflammatory adenosine signaling via A₂ receptors to establish infection. Adenosine, a suppressor of classical macrophage activation, has been detected in sand fly saliva (49, 143), and

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elevated levels were observed during active VL in human patients (262). Indeed, *Leishmania* are equipped with ecto-nucleotidases to generate adenosine from 5'-AMP or 3'-AMP (see Section 1.1.2). The latter is an intermediate product of the extracellular 2'-3'-cyclic AMP pathway (135). Substantial amounts of 3'-AMP are detectable in several rat organs, especially in liver and spleen, which are main organs targeted during visceral leishmaniasis (43). Numerous reports describe nucleotidase activity as a marker of *Leishmania* virulence and a promoter of infection, directly linked to A₂ receptor activation. This underscores its relevance as an immune escape mechanism used by the parasites (16, 25, 29, 66, 68, 165, 263).

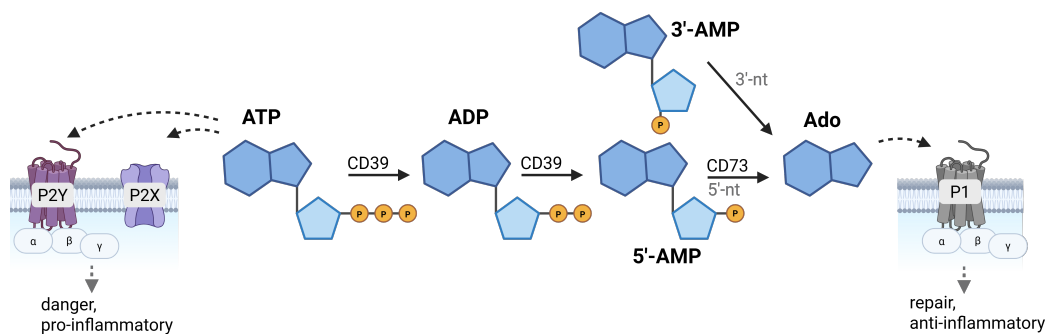


Figure 1.7: Purinergic signaling in an *Leishmania* infection setting. - ATP can be released from cells into the extracellular space during *Leishmania* infection as danger signal through activation of P2 receptors (left). Both G-protein coupled P2Y or ionotropic P2X transmembrane receptors promote pro-inflammatory signaling. ATP can be dephosphorylated by ecto-nucleotidases like CD39 in two steps to 5'-AMP, which can be degraded to adenosine through ecto-5'-nucleotidases like CD73 (middle). Adenosine then can counteract ATP danger signaling as retaliatory metabolite and induces anti-inflammatory signaling through binding on P1 receptors, including A₁, A_{2a}, A_{2b} and A₃ (right). *Leishmania* can accumulate adenosine to promote infection by providing additional parasite ecto-nucleotidases, denoted in gray, able to hydrolyze both 5'-AMP and 3'-AMP, an intermediate product of the 2'-3'-cyclic AMP pathway, to adenosine. Figure modified after (199) and created with BioRender.

1.2.2 Therapeutic options

1.2.2.1 Currently used drugs

To date, only a few conventional chemotherapeutic options for treatment of leishmaniasis are in use, but all face severe limitations (250). Pentavalent antimonials have been in clinical use for decades and remain among the first-line treatments. Their biologically active trivalent form targets several pathways, including a trypanothione reductase, thereby disrupting the parasite's redox homeostasis (278). However, their application is often accompanied with severe side effects like nephro- or cardiotoxicity (60). Amphotericin B was originally isolated from *Streptomyces nodusu* as anti-fungal agent, but also targets the ergosterol-rich *Leishmania* membrane through pore formation. A liposomal formulation improves bioavailability and safety, but side effects such as nausea and diffuse pain still occur (182). Other alternatives are pentamidine, which is especially effective against *L. guyanensis* (157), or paromomycin, a broad-spectrum antibiotic drug, but they also cause severe side effects (246). All these options share common drawbacks: As they follow injectable treatment regimens, therapy is very laborious, especially in remote areas. Additionally, high costs and side effects often cause patients to prematurely discontinue their therapy, resulting in treatment failure (217). Emerging resistances are described for all treatments, reducing efficacy as well (227). Thus, the development of miltefosine as first orally available drug was very promising and drastically decreased expenses. The alkylphospholipid was originally developed as neoplastic drug and resulted in very high cure rate of >90% upon approval in the early 2000s (244, 245). However, it also bears teratogenic potential and faces emerging resistances in many *Leishmania* species, linked to decreased drug influx and increased efflux by ABC transporter overexpression or alterations in the membrane composition (56, 87, 185). Its exact mode of action is still not completely resolved, but likely involves perturbation of the sterol metabolism (70, 169).

To improve therapeutic efficacy and safety, several combination therapies and chemotherapy for cutaneous lesions are currently under investigation (217, 285). However, due to compiling negative aspects of current treatment, there is an urgent need for the development of novel antileishmanial agents.

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1.2.2.2 Drugs under development

Due to very high costs and low commercial profit expectations, only very few antileishmanial drugs are currently in clinical trials. Organized by the Drugs for Neglected Disease initiative (DNDi) in cooperation with different pharmaceutical companies, several candidates with known mode of actions are currently tested in clinical programs (78): DNDI-6174, a cytochrome bc₁ inhibitor identified through phenotypical screenings of pyrrolopyrimidines, exhibits a very good pharmacological profile and is currently under evaluation in a clinical Phase I study for visceral and cutaneous leishmaniasis (39). Additional Phase I trials have been initiated for DNDI-6899, a cyclin-dependent kinase 12 inhibitor, and the proteasome inhibitor GSK245 (279). The latter selectively binds between $\beta 4$ and $\beta 5$ subunits to suppress chymotrypsin-like activity of the *Leishmania* proteasome (277). LXE408, another kinetoplastid-selective inhibitor of the proteasome with very similar mode of action already progressed into Phase II (187). Activities regarding other candidates, like the nitroimidazole DNDI-0690 or the oxaborole DNDI-6148, have been placed on hold due to abnormalities or toxicity signals in Phase I study participants. Further evaluation of the immune modulator CpG-D35 for use in combination therapy was stopped due to the lack of positive results (79).

1.2.2.3 Vaccines

Although vaccines for canine leishmaniasis are widely approved, no preventive therapy for human use has been successfully rolled out, despite substantial scientific efforts. The veterinary vaccine Letifend is based on different recombinant fragments of *L. infantum* proteins and is effective to reduce, but not to completely prevent, canine leishmaniasis upon exposure (89). Given the limitations of current chemotherapies, there is an urgent need for the development of an efficacious and safe vaccine for human use.

Historically, leishmanization by intradermal inoculation of *L. major* was used to elicit protection against re-infection, but today this strategy is neglected due to inadequate quality control, safety and ethical issues (146). First-generation vaccines, consisting of heat-inactivated parasites and tested both alone and with adjuvants, have failed to provide sustained protection (184, 266). Technological advances led to the unsuccessful

assessment of a multitude of second-generation vaccines composed of various recombinant antigens, epitopes or vector systems adapted to express leishmanial proteins (266). Similarly, nucleic acid-containing third-generation vaccines coding for leishmanial antigens were tested with negative results. Recent advances in targeted genetic manipulation of *Leishmania* (see Section 1.1.1) have facilitated the development of live attenuated strains suitable for vaccine development. For example, deletion of centrin-1 or a biopterin transporter is sufficient to generate non-infective strains (141, 201). Although some candidates of either technology progressed into clinical trials, none of them has yet received a final marketing authorization (168, 198).

1.3 Programmed cell death

1.3.1 Apoptosis in mammals

Cell death is a central process in every multicellular organism to abolish cells for various reasons like senescence, homeostasis, infections or damage. Multiple pathways exist to initiate and execute programmed cell death, all involving complex and tightly regulated cellular machineries. Mechanisms of regulated cell death can be divided into lytic and non-lytic outcomes, depending on definitive membrane integrity. Typically, lytic cell death is linked to activation of immunological processes through the release of intracellular components. Pyroptosis is triggered by inflammasome activation in response to danger- or pathogen-associated molecular patterns and results in gasdermin D-mediated pore formation in the cell membrane (57, 145). During necroptosis, membrane rupture is induced by Na^+ influx, following activation of receptor-interacting serine/threonine kinases (271).

In contrast, apoptotic cell death is immunologically silent as the cell disintegrates into apoptotic bodies to be removed by phagocytes without eliciting an inflammatory response. Apoptosis was the first describe type of programmed cell death (144) and is characterized by several hallmarks: Cell shrinkage, membrane blebbing, nuclear and DNA fragmentation, loss of mitochondrial potential, and exposure of phosphatidylserine (PtdSer) on the cell surface (103).

Principally, apoptosis can be initiated in two distinct manners: intrinsically or extrinsically (Figure 1.8). Intrinsic induction emerges from sensing of intracellular stressors

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like nutrient shortage or DNA damage, accumulating in an equilibrium of pro- and anti-apoptotic protein-protein interactions. When negative stressors predominate, pro-apoptotic BH-3 domain containing proteins can neutralize anti-apoptotic BCL-2 like proteins, thereby activating assembly of the BAK/BAX effector complex (139). This complex can disrupt the mitochondrial outer membrane, allowing permeabilization and release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm (247). There, cytochrome c triggers oligomerization of APAF-1, whose caspase recruitment domain (CARD) can interact with pro-caspase-9. The formed apoptosome, consisting of cytochrome c, APAF-1 and pro-caspase-9, can then cleave pro-caspase-9 into its active form (45). In turn, this effector caspase then activates the executioner caspases-3 and -7 (240). These cysteine-aspartic acid proteases, short caspases, then orchestrate the implementation of cell death by cleaving a multitude of substrates, including structural proteins, ATPases, polymerases and kinases (145), thus inducing the hallmarks of apoptosis and marking the point of no return in this process (211).

Amongst these substrates, a scramblase is cleaved and activated to translocate PtdSer in the outer membrane leaflet. PtdSer acts as "eat me" signal, facilitating cell clearance by silent phagocytosis (231). It is normally maintained on the inner membrane leaflet by flippase activity, which in turn is inactivated by caspases. Upon exposure, PtdSer can bind to receptors such as TIM-4 and, supported by further interactors, mediates engulfment into phagocytes without activation of an immune response (183).

Extrinsic apoptosis is mediated by death ligands secreted by neighboring cells, immune cells or other environmental factors, and their cognate receptors on the cell membrane (103). For example, TNF can act as ligand on TNF receptors 1 and 2 (206). Upon activation, the intracellular domains of death receptors recruit adaptor proteins through their death domains. Through homotypic interactions, the death-induced signaling complex recruits and cleaves pro-caspase-8 into its active form. Active caspase-8 then activates executioner caspase-3, culminating in the events characteristic of intrinsic apoptosis (149).

1.3.2 Apoptosis-like cell death in protozoans

While programmed cell death (PCD) is well characterized in metazoans, its pathways and functions in protozoa remain unclear. Several hallmarks of apoptosis have been

1.3 Programmed cell death

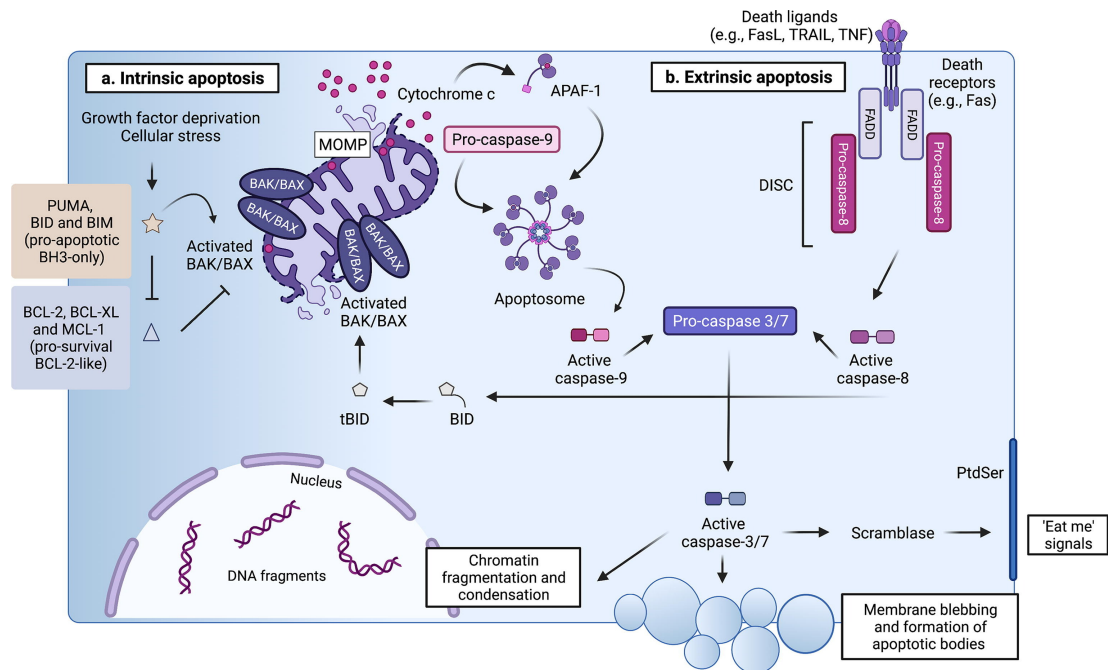


Figure 1.8: Cellular pathways of apoptosis. - Apoptosis can be either induced through complex cellular cascades intrinsically (left) or extrinsically (upper right), culminating in common execution (middle and bottom). Excess of internal stressors leads to inhibition of pro-survival BCL-2-like proteins and formation of pores in the outer mitochondrial membrane, formed by BAK/BAX. Released cytochrome c associates with APAF-1 and procaspase-9 in the apoptosome, cleaving caspase-9 into its active form. Extrinsic death signals can activate their respective receptors that mediate signaling through death domains to activate caspase-8. Effector caspase-8 or -9 activate executioner caspases-3 and -7 to implement final apoptosis. Figure taken from (145).



experimentally described in *Leishmania*, *Trypanosoma*, *Plasmodium*, and *Toxoplasma*, leading to the designation as apoptotic-like cell death (103, 136, 203, 256).

Homologs of metazoan apoptotic regulators, such as death domains, BH3- or Bcl2-like proteins and apoptosis executioners like caspases, are largely absent in *Leishmania*. Only one BH3-domain-containing aquaporin and a putative apoptosis-inducing factor have been identified, but experimental data on their involvement remain sparse (108, 259). Recently, *in silico* data suggested the formation of an apoptosis-regulating complex in *L. donovani* (154). However, since respective regulators and executioners of the molecular cell death machinery remain largely unknown, it is questioned whether this process indeed is regulated or rather incidental (213).

In *Leishmania*, hallmarks of PCD have been observed upon drug treatment, nutrient deprivation, or in metacyclic stationary phase promastigotes, both in *in vitro* cultures and in sand flies (136, 254) (see Figure 1.9): As typical for apoptosis, the parasites shrink and round, which is visible microscopically and by flow cytometry. Additionally, as early sign of apoptosis, PtdSer is exposed on the outer cell membrane leaflet, which can be quantified using AnnexinV or monoclonal antibodies binding to PtdSer (254). The presence of PtdSer in *Leishmania* was debated, as AnnexinV can also bind other anionic phospholipids (270). However, mass spectrometry of late stationary phase *L. major* confirmed the abundance of several distinct PtdSer species (28). Another prominent marker of intrinsic apoptosis, the loss of mitochondrial integrity accompanied by the release of cytochrome c, was observed on *Leishmania* under various pressures (239, 260). Similarly, nuclear condensation and DNA fragmentation have been reported to occur under manifold stressors: These are likely mediated by translocation of endonuclease G and involvement of Flap endonuclease-1 and TatD-like nucleases (28, 37, 105, 136, 219, 239). The accumulation of ROS often accompanies, and likely induces, PCD. For example, overexpression of a mitochondrial peroxidase confers tolerance to oxidative stress-induced cell death in *L. major* (75).

Unlike other apoptosis hallmarks, caspase homologs are absent from the *Leishmania* genome (82, 104). Although caspase-like protease activities have been repeatedly reported (203, 239), only one metacaspase has been described as potentially linked to protozoan PCD (160, 203, 239, 286). As a caspase orthologue, metacaspase shares structural similarity with caspases but exhibits distinct substrate specificity. Its function may be independent of cell death and instead connected to cell cycle progres-

1. INTRODUCTION

sion (5, 28, 53, 160). Instead, the role of metacaspase rather depends on the specific environmental stimulus. Otherwise, observed protease activity could also arise from cathepsin-like proteins or calpain-like activity (24, 83).

Whereas PCD in multicellular organism is well-defined, its evolutionary benefits for unicellular organisms remain disputed (213). However, accumulating data and hypotheses suggest that PCD serves as a protozoan strategy to balance population density, ensure clonality, and facilitate immune evasion (24, 104, 256). In host organisms, hyperparasitism can impair successful transmission of pathogens, which parasites must prevent to ensure their survival. Mechanisms similar to quorum sensing have already been described for *Plasmodium* and *Trypanosoma* (104). Thus, PCD can be beneficial for *Leishmania* as a form of "selfish altruism", eliminating less fit parasites and allowing only the fittest to proliferate in the nutrient-limited host environment (24).

Simultaneously, the presence of apoptotic-like parasites modulates the mammalian immune response to favor viable parasites and enhance their infectivity. Indeed, dead parasites enhance intraneutrophilic survival and suppressed secretion of pro-inflammatory cytokines. Thus, the absence of apoptotic-like parasites in the sand fly inoculum results in decreased virulence of the viable parasites (254, 268). The exposure of PtdSer might be crucial for such effects, as it can mediate a silent phagocytosis into macrophages without eliciting a pro-inflammatory response (254, 267). In trypanosomatids, PtdSer biosynthesis occurs exclusively via PtdSer synthase 2, which catalyzes the head-group exchange of phosphatidylethanolamine. PtdSer is likely externalized by an ATP-binding cassette half-transporter, whose deletion reduces parasite infectivity (48, 92). Additionally, apoptotic-like *L. major* enter an autophagy-like compartment in human macrophages, representing LC3-associated phagocytosis, leading to decreased T cell proliferation in response to infection (59). In conclusion, protozoan PCD represents an outstanding target for the development of novel antileishmanial therapeutics. However, the lack of detailed knowledge about apoptosis pathways currently limits its exploitation (24, 256).

2

Aims and Objectives

The emergence of leishmaniasis as a neglected tropical disease is a global health burden that demands attention to establish novel therapeutic options. To date, preventive vaccines for human use are absent and the few available chemotherapeutics face major drawbacks in terms of cost, side effects, and resistance development. Thus, identification of compounds with antileishmanial activity can strengthen the development of new agents against *Leishmania* infections. Proteins involved in programmed cell death of these parasites are very promising targets for drug development. They can be exploited either to trigger and direct the parasites' own suicide mechanisms against them or to suppress concomitant immunosuppressive strategies. Additionally, such proteins are parasite-specific and lack human homologs. However, knowledge about the underlying cell death pathways and mediators in *Leishmania* remains very limited.

Therefore, the overall explorative aim of this thesis is to advance knowledge relevant for the development of future therapeutic options by two distinct approaches, as depicted in Figure 2.1.

1: Compounds will be tested for their potential as leishmanicidal drugs. These compounds are either repurposed based on their known activity against other pathogens or they are drugs already in use for different indications with predicted antiparasitic activity. A workflow will be established, assessing cytotoxicity on *Leishmania* and human primary macrophages as a control, followed by activity testing in *in vitro* infected host cells. Based on the comprehensive testing conducted through this workflow, it can be hypothesized that a small subset of the analyzed compounds will emerge as promising candidates. The data generated from these experiments can provide a foundation for

2. AIMS AND OBJECTIVES

identifying lead compounds with significant potential for advancing into subsequent stages of drug development.

2: Proteins will be studied for their potential involvement in the regulation or execution of programmed cell death in *L. major* and their applicability as drug targets. Assuming regulated cell death pathway in *L. major*, it can be hypothesized that these processes represent highly promising drug targets. The focus will be on candidates selected by homology to cell death-linked domains of higher eukaryotic proteins, literature surveys and a quantitative proteomics screen of parasites in sub-lethal stress conditions. The latter was conducted and described in the PhD thesis of Michaela Bergmann (28) and now will be reanalyzed. Functions of these candidates will be assessed by targeted gene deletions and subsequent phenotypic screen for cell death hallmarks, based on the hypothesis that regulated pathways orchestrate programmed cell death in *L. major*. Virtual screens for p1/s1 inhibitors, conducted by cooperation partners, may enable the identification of potential antileishmanial agents.

In conclusion, this project aims to combine two strategies - phenotype-based compound screens and drug target identification - to establish novel options for antileishmanial therapies. To accomplish this, interdisciplinary approaches will be applied, encompassing biochemical, genetic, and immunological methods as well as proteome analyses.

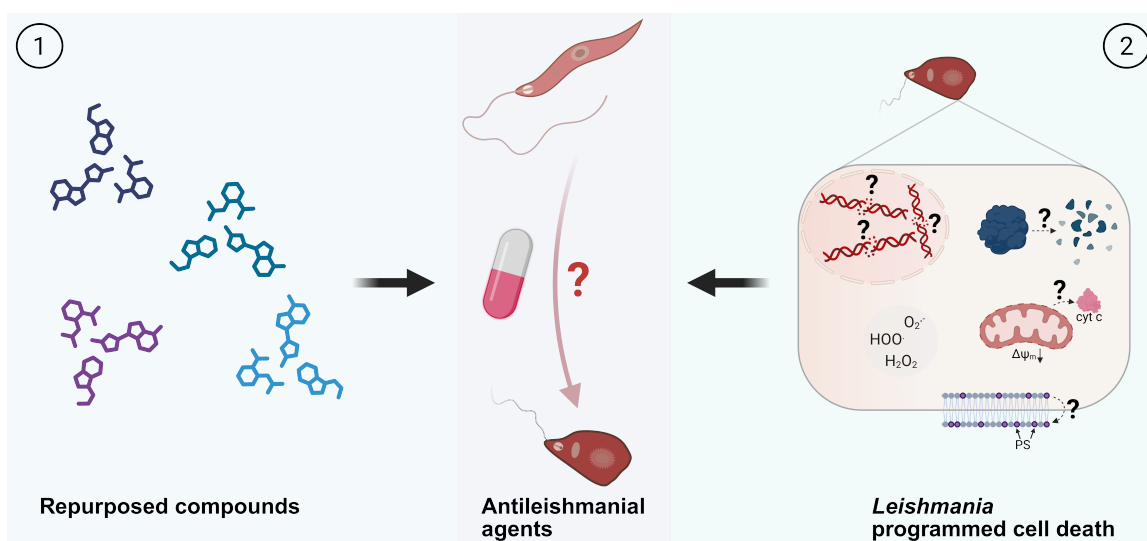


Figure 2.1: Two objectives of this thesis - In this project, two complementary approaches will be applied in to identify novel therapeutic approaches against leishmaniasis:

1: Repurposed compounds, either with known antipathogenic activity or approved drug with predicted antiparasitic activity, shall be phenotypically screened for antileishmanial activity (left).

2: Potential drug targets involved in programmed cell death mechanisms of *Leishmania* shall be identified (right).

2. AIMS AND OBJECTIVES

3

Material & Methods

3.1 Material

3.1.1 Cell culture media

Table 3.1: Cell culture media list - Overview of all used cell culture media and their compositions.

Description	Composition	Supplier
Alex' amastigote medium (AAM)	RPMI + 12.5% FCS + 4 mM L-Glutamine + 100 U/ml Penicillin + 0.1 mg/ml Streptomycin, adjusted to pH 5.5 with HCl	
Complete medium (CM)	RPMI + 10% FCS + 10 mM HEPES + 2 mM L-Glutamine + 100 U/ml Penicillin + 0.1 mg/ml Streptomycin + 50 μ M β -Mercaptoethanol	
Histopaque-1077	as per supplier	Sigma-Aldrich
Histopaque-1119	as per supplier	Sigma-Aldrich
LB Agar + Ampicillin	LB medium + 3.7% agar + 100 μ g/ml Ampicillin	PEI central lab

3. MATERIAL & METHODS

<i>Lm</i> freezing medium	<i>Lm</i> medium + 40% FCS	
<i>Lm</i> liquid medium	M199 + 2mM L-Glutamine + 10% FCS + 100 U/ml Penicillin + 0.1 mg/ml Streptomycin + 10 mM HEPES + 0.1 mM Adenin + 0.05% (w/v) Hemin + 0.041% (w/v) Biotin	
<i>Lm</i> medium	RPMI medium + 5% fetal calf serum (FCS) + 2 mM L-Glutamine + 100 U/ml Penicillin + 0.1 mg/ml Streptomycin + 10 mM HEPES	
Lysogeny broth (LB) medium	2.5% LB bouillon (Miller) in water	PEI central lab
M199 agar	16.06g/l M199 powder + 0.35 g/l NaHCO ₃ + 5 mg/ Hemin + 0.6 mg/l Biopterin + 20% FCS + 100 U/ml Penicillin + 100µg/ml Streptomycin + 1.5% LB Agar in water	M199 powder: Gibco
M199 medium with Hank's salt	as per supplier	Sigma-Aldrich/ Biowest
Novy-Nicolle-McNeal (NNN) bloodagar	16% defibrinated rabbit blood + 3.4% brain heart infusion agar + 66.2 U/ml penicillin + 66.2 µg/ml streptomycin in PBS	
PBL freezing medium	CM + 30% FCS + 10% DMSO	
Percoll	as per supplier	GE Healthcare
RPMI 1640 medium	as per supplier	Sigma-Aldrich (R0883)/ Biowest (L0501)

3.1 Material

RPMI 1640 medium without phenolred	as per supplier	Sigma-Aldrich
Super optimal broth with catabolite repression (SOC) medium	20 µg/ml Tryptone + 5 µg/ml Yeast Extract + 0.5 µg/ml NaCl + 2.5 µl/ml 1 M KCl + 10 µl/ml 1 M MgCl ₂ + 20 mM D-glucose	PEI central lab

3.1.2 Antibiotics

Table 3.2: Antibiotics list - Overview of antibiotics used for medium complementation and their respective final concentrations.

Antibiotic	Supplier	Final concentration
Ampicillin	VWR	100 µg/ml
Blasticidin	InvivoGen	10 µg/ml
Bleomycin	Jena Bioscience	100 µg/ml
G418	InvivoGen	40 µg/ml
Hygromycin B Gold	InvivoGen	30 µg/ml
Nourseothricin	Jena Biosciences	30 µg/ml
Penicillin-Streptomycin	Sigma-Aldrich	100 U/ml - 0.1 mg/ml
Puromycin	InvivoGen	40 µg/ml

3.1.3 Buffer

Table 3.3: Buffer list - Overview of all used buffers and their composition.

Description	Composition	Supplier
Antibody staining blocking buffer	PBS pH 7.4 + 10% FCS + 10% human serum	-
Blotting buffer	48 mM Tris + 38mM Glycin + 10% SDS + 20% Methanol	PEI central lab
CASYton	as per supplier	Omni Life Science

3. MATERIAL & METHODS

ELISA reagent diluent	PBS pH 7.4 + 1% BSA (w/v), filtered	-
ELISA washing buffer	PBS + 0.5% Tween20	-
Endonuclease reaction buffer	30 mM sodium acetate pH 5.3 + 100 mM NaCl + 2 mM ZnCl ₂	recipe from (140)
Laemmli buffer	500 mM Tris/HCl pH 6,8 + 38% glycerin + 10% SDS + 0.6M DTT + 0,01% bromophenolblue	-
Magnetic-activated cell sorting (MACS) buffer	PBS + 0.5% bovine serum albumine fraction V (BSA)	PEI central lab
MACS1 buffer	Ringer solution + 0.5% BSA	-
Nucleotidase reaction buffer	116mM NaCl + 5.4mM KCl + 5.5mM D-Glucose + 50mM HEPES pH 7-7.6	recipe from (200)
Phosphate buffered saline (PBS) pH 7.1	137 mM NaCl + 2.7 mM KCl + 8.1 mM Na ₂ HPO ₄ + 1.5 mM KH ₂ PO ₄ pH 7.1	PEI central lab
PBS pH 7.4	137 mM NaCl + 2.7 mM KCl + 8.1 mM Na ₂ HPO ₄ + 1.5 mM KH ₂ PO ₄ pH 7.4	PEI central lab
Permeabilisation & blocking buffer	10% FCS + 10% human serum + 0.5% saponin in PBS	-
Permeabilisation & blocking wash buffer 1	1% FCS + 1% human serum in PBS	-

3.1 Material

Permeabilisation & blocking wash buffer 2	1% FCS + 1% human serum + 0.5% saponin in PBS	-
Ringer solution	as per supplier	B. Braun
SDS-PAGE running buffer	125 mM Tris + 1,25M Glycin + 0,5% SDS	PEI central lab
50x TAE (TRIS-acetat-EDTA) buffer	-	PEI central lab
Tris-HCl/SDS pH 6.8 buffer	0,5 M Tris-HCl pH 6,8 + 0,4% SDS	-
Tris-HCl/SDS pH 8.8 buffer	1,5 M Tris-HCl pH 8,8 + 0,4% SDS	-
Tris-buffered saline with Tween20 (TBST)	50mM Tris + 150mM NaCl + 25% HCl + 0,05% Tween 20	PEI central lab
Trypsin-EDTA	0.05% Trypsin + 0.1% EDTA	PEI central lab
Washing buffer	PBS + 5% complete medium	-

3. MATERIAL & METHODS

3.1.4 Antibodies

Table 3.4: Antibody list - Overview of all used antibodies including their working concentration and applications; WB: Western blot, FC: Flow cytometry.

Epitope	Status	Dilution	Organism	Supplier	Order No	Use
Human A_{2a}	Monoclonal IgG, Alexa Fluor 488 conjugated	1 μ g/ 1×10^6 cells	Mouse	R&D Systems	FAB9497RG	FC
Human A_{2b}	Monoclonal IgG, Alexa Fluor 647 conjugated	1 μ g/ 1×10^6 cells	Rabbit	R&D Systems	FAB104442R	FC
Human CD62L	Monoclonal IgG, PE conjugated	0.5 μ l/ 1×10^6 cells	Mouse	BD Pharmingen	555544	FC
Human CD66b	Monoclonal IgG, FITC conjugated	0.5 μ l/ 1×10^6 cells	Mouse	Beckman Coulter	IM0531U	FC
Human α Tubulin	Monoclonal IgG	1:1000	Mouse	GeneTex	GT114	WB
His ₆	Monoclonal IgG	1:1000	Mouse	Cell Signaling Technology	2366	WB
Mouse IgG $_{\kappa}$ binding protein	HRP-conjugated	1:1000	-	Santa Cruz Biotechnology	sc-516102	WB
Rabbit	Polyclonal IgG, Alexa Fluor 568 conjugated	1:10,000	Goat	Invitrogen	A-11036	FC

3.1.5 Reagents

Table 3.5: Reagents list - Overview of all used chemicals and reagents.

Description	Supplier
1o & 1c	kindly provided by Prof. Dr. Katja Becker, Justus-Liebig-University Gießen
Acrylamide/Bis Solution	Serva
Adapalene	Sigma-Aldrich
Adenin	Sigma-Aldrich
Agarose LE	Biozym
Ammonium persulfate (APS)	Serva
Ammoniumtetrathiomolybdat (TTM)	AppliChem PanReact
3'-AMP	SantaCruz Biotechnology
3'-AMP sodium salt	Sigma-Aldrich
5'-AMP monohydrate	Sigma-Aldrich
AnnexinV Alexa Fluor 647	Invitrogen
AnnexinV FITC	Miltenyi Biotec
AnnexinV microbead kit	Miltenyi Biotec
Biotin	Sigma-Aldrich
Brain-heart infusion agar	Becton Dickinson
BSA fraction V	PanReact AppliChem
CD14 human microbeads	Miltenyi Biotec
CellTrace Far Red	Invitrogen
Cetorelixacetate	Sigma-Aldrich
cOmplete Protease Inhibitor Cocktail-EDTA	Roche
Cytochalasin D from <i>Zygosporium mansonii</i>	Calbiochem
Dithiothreitol (DTT)	Invitrogen
DMSO pharma grade	AppliChem PanReact
Dry milk powder	Sucofin
Enzyme-linked Immunosorbent Assay (ELISA) substrate solution	BioLegend
Ethanol absolute	Carl Roth
Ethidium bromide	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	PEI central lab
FACS Clean	PEI central lab
FACS Flow	PEI central lab
FACS Rinse	PEI central lab
FCS	Sigma-Aldrich

3. MATERIAL & METHODS

Desmethyl-desamino-Pateamine A (DMDAPatA)	kindly provided by Prof. Dr. Arnold Grünweller, Philipps-University Marburg
GC Enhancer	Invitrogen
GelRed	Biotium
GeneRuler 1kb DNA ladder	Thermo Fisher Scientific
GeneRuler 1kb Plus DNA ladder	Thermo Fisher Scientific
200mM L-Glutamine	Sigma-Aldrich
Leukine GM-CSF (Sagramostine)	Sanofi
Hemin	BioChemika
1M (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)	Sigma-Aldrich
HIS-Select nickel magnetic agarose beads	Sigma-Aldrich
Honokiol	Sigma-Aldrich
Human interleukine (IL)10 DuoSet ELISA	R&D Systems
Human serum	Sigma-Aldrich
Hydrochloric acid (HCl)	Roth
Human tumour necrosis factor (TNF) α DuoSet ELISA	R&D Systems
Interferon (IFN) γ , human recombinant	Sigma-Aldrich
Immobilon Forte Western HRP substrate	Millipore
Lipopolysaccharide (LPS) from <i>Escherichia coli</i> O111:B4	Sigma-Aldrich
Lornoxicam	Sigma-Aldrich
M13mp18 RF I DNA	New England Biolabs
M13mp18 Single-stranded DNA	New England Biolabs
β -Mercaptoethanol	Sigma-Aldrich
MLN9708	Cayman Chemicals
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	abcam
PageRuler Plus prestained protein ladder	Thermo Fisher Scientific
Paraformaldehyde (PFA)	Sigma-Aldrich

3.1 Material

Pateamine A (PatA)	kindly provided by Prof. Dr. Arnold Grünweller, Philipps-University Marburg
Phorbol-12-Myristat-13-acetat (PMA)	Thermo Fisher Scientific
Phosphate assay	Sigma-Aldrich
Polyclonal anti- <i>Lm</i> serum	kindly provided by Uwe Ritter, Würzburg
Poly-D-lysine	Gibco
Polyvinylidene fluoride membrane	GE Healthcare
Potential p1/s1 inhibitors no. 36, 42	Mcule
Potential p1/s1 inhibitor no. 45	TargetMol
Potential p1/s1 inhibitor no. 46	Cayman Chemicals
Potential p1/s1 inhibitor no. 52	Sigma-Aldrich
Propidiumiodide (PI)	Sigma-Aldrich
Rabbit blood	Animal facility PEI
Recombinant M-CSF	R&D Systems
RNA loading dye	Thermo Fisher Scientific
Rupatadinefumarat	Sigma-Aldrich
Saponin	AppliChem PanReact
Silmitasertibsodiumsalt	TargetMol
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
2N sulphuric acid H ₂ SO ₄	Carl Roth
SYBR Blue	Invitrogen
SYBR Green	Invitrogen
Tetramethylethylenediamine (TEMED)	Roth
TriTrack DNA loading dye	Thermo Fisher Scientific
Tween20	Sigma-Aldrich
Ultrapure water	PEI central lab
Ultrapure water molecular biology grade	Thermo Fisher Scientific
Ultracomp eBeads Plus	Invitrogen
ZombieAqua	BioLegend

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3.1.6 Enzymes and enzyme buffers

Table 3.6: Enzyme list - Overview of all used enzymes, enzyme mixes and respective buffers.

Enzyme	Supplier
DNaseI	Invitrogen
AluI	New England Biolabs
SwaI	New England Biolabs
NcoI	New England Biolabs
NotI	New England Biolabs
KpnI	New England Biolabs
rCutSmart buffer	New England Biolabs
r3.1 buffer	New England Biolabs
MESA Blue qPCR MasterMix Plus	Eurogentec
NEBuilder HiFi DNA assembly Master Mix	New England Biolabs
RNase	Thermo Fisher Scientific
PWO-Master	Roche
Platinum SuperFi DNA Polymerase	Invitrogen

3.1.7 Kits

Table 3.7: Kit list - Overview of all used ready-to-use kits.

Kit	Supplier
DNeasy Blood and Tissue gDNA kit	Qiagen
Human T Cell Nucleofector Kit	Lonza
ImProm-II reverse transcription system	Qiagen
NucleoBond PC500 Maxiprep kit	Macherey Nagel
QIAprep Spin Miniprep kit	Qiagen
RNeasy kit	Qiagen
Wizard SV Gel and PCR Clean-Up System	Promega

3.1.8 Oligonucleotides

Table 3.8: Oligonucleotide list - Overview of used oligonucleotides and their corresponding sequences; iKO: Inducible knockout generation, rt-qPCR: real time quantitative PCR, Seq: Sequencing. Oligonucleotides were synthesized at Eurofins in salt-free purity. For knockout generation, high purity salt purification was chosen. Sequences for KO primers continue in Table 5.2.

Use	Target & Direction	5'-3' Sequence
iKO	<i>p1/s1</i> cluster 5' flox fwd	CATTAACGTTTGGGAGACCCCTGTGC AAAGGTATAATGCAGACCTGCTGC
iKO	<i>p1/s1</i> cluster 5' flox rev	AGGCGCGGGCGCATGGGCACGTGGA AAAAGCCAATTTGAGAGACCTGTGC
iKO	<i>p1/s1</i> cluster 5' sgRNA template	GAAATTAATACGACTCACTATAGGAA CCGTCGGCGATAAAAAATGTTTTA GAGCTAGAAATAGC
iKO	<i>p1/s1</i> cluster 3' flox fwd	GCCGCTCGAGAGCCTTTATGTTTGTG GCTTGCCCGATTACGCATGATCTA
iKO	<i>p1/s1</i> cluster 3' flox rev	GCCGAAAAGAACACAAGAAAAAAAT CTTGCGAGCAAAGAAAGCTGGGTTC CATGG
iKO	<i>p1/s1</i> cluster 3' sgRNA template	GAAATTAATACGACTCACTATAGGATT GTCGTGATGGGTTCTGGGTTTTAGAG CTAGAAATAGC
iKO, KO	sgRNA scaffold (27)	AAAAGCACCGACTCGGTGCCACTTTT TCAAGTTGATAACGGACTAGCCTTATT TTAACTTGCTATTTCTAGCTCTAAAAC
iKO confirmation	<i>LmjF.30.1500/10</i> fwd	TCCGTCTGCCTCTCACAGTGCT
iKO confirmation	<i>LmjF.30.1500/10</i> rev	CTCCTCGTGAATCGCCACCA
flox confirmation	<i>eGFP</i> fwd	CGTGACCACCCTGACCTACG
flox confirmation	<i>eGFP</i> rev	GGCGGATCTTGAAGTTCACCTTG
flox confirmation	<i>mCherry</i> fwd	CATGAGATTCAAAGTTCACATGGAAG GTT
flox confirmation	<i>mCherry</i> rev	TTGTATAGTTCATCCATGCCACCAGT AC
rt-qPCR	<i>LmjF.12.0400</i> fwd	TCCCCAAGAGCACCAACA

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rt-qPCR	<i>LmjF.12.0400</i> rev	TGGTGACCTTGACATCCT
rt-qPCR	<i>LmjF.31.2310</i> fwd	GGAGTACAAGACGAACGCCA
rt-qPCR	<i>LmjF.31.2310</i> rev	CTTCGGCAGGTAGCACTTGA
rt-qPCR	<i>LmjF.30.1500/10</i> fwd	GCTGTGCGTGACTGAGGTTC
rt-qPCR	<i>LmjF.30.1500/10</i> rev	GCATACTGACCCCAACGCT
rt-qPCR	<i>LmjF.30.1460-90</i> fwd	GACGCGGTGAAAGATGCCTA
rt-qPCR	<i>LmjF.30.1460-90</i> rev	AAACAGACCACCACAGCGAA
rt-qPCR	<i>NMT</i> fwd	CCGTCGACTGTGATTGGGAA
rt-qPCR	<i>NMT</i> rev	GTGAATGCGCCACGATCAAA
Gibson Assembly	<i>LmjF.30.1500/10</i> - His pLexLm fwd	TGCTGTGCCTTGCCACCAGATCTGC CATGGATGCCCCGCGTTTGTCGG
Gibson Assembly	<i>LmjF.30.1500/10</i> - His pLexLm rev	ACGTGAAAGGAACAAGAAAGGAGGA GGAGGGCGGCCGCTAGTGGTGG
Gibson Assembly	<i>LmjF.30.1500/10</i> pLexLm rev	GTGAAAGGAACAAGAAAGGAGGAGG AGGGCGGCCGCTTACGCACTCCGCT TCCAAC
pLexLm Seq	5'-UTR fwd	GAAGTTGGCCTTCTCGCTC
pLexLm Seq	IR cam rev	GTCGTCTGCCAGCTACAAC
<i>L. major</i> Seq	<i>LmjF.01.0770/80</i> fwd	GCGAGACCTTTGTGCGCG
<i>L. major</i> Seq	<i>LmjF.01.0770/80</i> rev	GCTGCGGAAGGTGTTTCATGAC

3.1.9 Plasmids

Table 3.9: Plasmid list - Overview of all used plasmids and their sources.

Plasmid	Use	Source
pGL2314	3' flox cassette template for inducible knockout generation	kindly provided by Jeziel Damasceno; (81)
pLexLm	<i>Leishmania</i> homologous recombination cassette template	based on pLexsy-ble2.1 from Jena Bioscience with the <i>L. donovani</i> promotor region replaced by a <i>L. major</i> promotor region
pTBLast	<i>Leishmania</i> CRSIPR repair cassette template	kindly provided by Tom Beneke; (27)
pTNeo	<i>Leishmania</i> CRSIPR repair cassette template	kindly provided by Tom Beneke; (27)
pTNeo-eGFP-loxP	5' flox cassette template for inducible knockout generation	based on pTNeo with an eGFP and loxP sequence inserted

3.1.10 Consumables

Table 3.10: Consumables list - Overview of used consumables.

Consumable	Supplier
96-well microtiter plates	Greiner BioOne
CASY cups	Omni Life Science
Cell culture plates	Sarstedt
Cell scraper (S, M)	Sarstedt
Conical tubes (15 ml, 50 ml)	Greiner BioOne
Cryo Freezing tube 2ml, round bottom	Greiner BioOne
FACS tubes, 5 ml	BD Bioscience
Half-area 96-well microplate	Greiner BioOne
LD columns	Miltenyi Biotec
LS columns	Miltenyi Biotec
Light Cyler 96-well plates white	Roche
Microcentrifuge tubes (1.5 ml, 2 ml)	Eppendorf
Nunc cell culture plates with UpCell surface	Thermo Fisher Scientific
PCR single cap soft strips 0.2 ml	Biozym
Pipette filter tips Green-Tip	NerbePlus

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Pipette tips	Biozym
Serological pipettes	Greiner BioOne
SteriCup quick release bottle top filters	Merck Millipore
Syringe Omnifix Luer Solo 20 ml	B. Braun
Tubes 1.4 ml, U-bottom	Micronic
T ₇₅ , T ₂₅ cell culture flask with filter	Greiner BioOne/ Sarstedt
Whatman Syringe Filter (0.2 µm, 0.45 µm)	Cytiva

3.1.11 Devices

Table 3.11: Device list - Overview of used devices for experiments.

Device	Supplier	Purpose
-20 °C Freezer	Liebherr	Storage of samples
-80 °C Freezer U725-G	Eppendorf	Storage of samples
Accu-jet Pro	Brand	Pipette controller
AxioVert25	Carl Zeiss	Wide field microscopy
Balance KB BA 100	Sartorius	Analytical weighing
CASY cell counter	Omni Life Science	Determination of viable cell number and diameter distribution
Centrifuge 5430/ 5430R	Eppendorf	Centrifugation
Centrifuge J2-MC	Beckman Coulter	Centrifugation
Chronos liquid nitrogen tank	Messer	Storage of cells
ClarioStar Plus 1.35	BMG LabTech	Absorption and fluorescence microplate reader
CO ₂ Incubator Forma Series II	Thermo Fisher Scientific	Cell culture incubator
CO ₂ Incubator Heracell VIOS 160i	Thermo Fisher Scientific	Cell culture incubator
ECL Imager Chemostar	Intas	Imaging of Western blots
Electronic Pipette Xplorer 1-channel	Eppendorf	Pipetting
Fortessa	BD Bioscience	Flow cytometry
Fridge FKS 3600	Liebherr	Storage of samples
Gel Doc XR+	Bio-Rad	Visualisation of agarose gels
Horizontal electrophoresis system 192 Cell	Bio-Rad	Agarose gels

3.1 Material

Laminar Flow Workbenches MSC Advantage	Thermo Fisher Scientific	Sterile cell culture work
LightCycler 480 System	Roche Diagnostics	Quantitative real-time PCR
MACS multistand	Miltenyi Biotec	MACS
Megafuge 40R, BIOLiner swinging rotor and buckets	Thermo Fisher Scientific	Centrifugation
MidiMACS separator	Miltenyi Biotec	MACS
Mini-centrifuge Sprout	Biozym	Centrifugation
Mini-PROTEAN Tetra Cell	BioRad	SDS-PAGE
Mr. Frosty	Nalgene	Freezing container
NanoDrop 2000c	PeQLab	Spectrophotometer
Neubauer counting chamber improved, 0.02 mm depth	VWR	Cell counting
Nucleofector 2b	Lonza	Electroporation of cells
pH-Meter PB-11	Sartorius	pH determination
Pipette Research Plus 1-channel	Eppendorf	Pipetting
Pipette Research Plus 8-channel	Eppendorf	Pipetting
Pipette Transferpette S 8-channel	Brand	Pipetting
PowerPac HC	Bio-Rad	Electrophoresis power supply
PrimoStar-1	Carl Zeiss	Wide field microscopy
Semi-dry transfer unit OWL HEP-1	Thermo Fisher Scientific	Semi-dry blotting
SORP	BD Bioscience	Flow cytometry
Symphony A3 Cell Analyzer	BD Bioscience	Flow cytometry
Thermal Cycler S-1000	Bio-Rad	PCR
Thermomixer Comfort with thermoblock	Eppendorf	Heating
Sonifier 450II Classic	Branson Ultrasonic	Sonification of samples
Vortex VV3	VWR	Mixing of samples
Waterbath	Köttermann	Heating of samples

3. MATERIAL & METHODS

3.1.12 Software

Table 3.12: Software list - Overview of software used for this thesis.

Description	Supplier	Usage
BioRender	BioRender	Illustrations
ChatGPT-4	OpenAI	Proofreading
Citavi 6.12.0.0	Swiss Academic Software	Literature management
FACSDiva 9.0.1	BD Bioscience	Control of flow cytometers
FlowJo 10.10.0	BD Bioscience	Flow cytometry data analysis
GraphPad Prism 9.5.0	GraphPad Software	Data visualization and statistical evaluation
ImageJ 1.53c	National Institute of Health, USA	Picture analysis
Image Lab 6.0.1	Bio-Rad	Agarose gel analysis
Inkscape 1.3	Inkscape	Arrangement of figure panels
LabImage1D	Intas	Capture of Western blot images
Le Chat	Mistral AI	Proofreading
LightCycler 480 SW 1.5.1	Roche	Analysis of quantitative real-time PCR data
Mars 4.00 R2	BMG Labtech	Evaluation of ClarioStar data
Office Package 2019	Microsoft	Documentation and evaluation of protocols and data
SnapGene 7.2	Dotmatics	Visualization of DNA sequences, primer & plasmid design

3.1.13 Organisms and human primary cells

Leishmania major MHOM/IL/81/FEBNI were kindly provided by Dr. Frank Ebert of the Bernhard Nocht Institute for Tropical Medicine Hamburg, Germany. This strain was isolated from a skin biopsy of an Israeli patient. *Leishmania donovani* MHOM/ET/67/HU3 Z18 Africa were provided by The *Leishmania* Collection in Montpellier, France by Patrick Bastien.

Parasites were modified by various genomic integrations to stably express transgenes. This includes a Cas9 endonuclease/ T7 RNA polymerase construct, a dimerizable Cre (DiCre) construct or fluorescence markers like eGFP or DsRed. Transgenic strains in the promastigote stage were always cultured under selection pressure, see Table 3.2.

Stocks of chemically competent *Escherichia coli* OneShot TOP10F' were obtained from Invitrogen.

Any human primary immune cells were isolated from human blood from either in-house donations or from buffy coats of anonymized, healthy donors provided by the German Red Cross blood donation service in Frankfurt am Main, Germany, see Section 3.2.2.

3. MATERIAL & METHODS

3.2 Methods

3.2.1 *Leishmania* cell culture

3.2.1.1 Culturing of *Leishmania major* promastigotes

Leishmania major MHOM/IL/81/FEBNI promastigotes were cultured on a biphasic rabbit-bloodagar-NNN/ *Lm* medium culture system for a maximum of eight passages. On days 2 to 4 post passage, parasites were in their logarithmic growth phase (logPh), 6 to 9 days after passage they reached stationary growth phase (statPh). When reaching statPh, promastigotes were counted in a Neubauer counting chamber, diluted to 0.5×10^6 viable promastigotes/ml in *Lm* medium, distributed on a new blood agar 96-well plate in 100 μ l/well and cultured at 27 °C, 5% CO₂ in a humidified atmosphere. Antibiotics were added to the medium accordingly (see Table 3.2).

To generate passage 0 promastigotes, axenic amastigotes were generated (see Section 3.2.1.5) and passaged on bloodagar to induce differentiation into promastigotes. After another passage, 100×10^6 viable passage 1 parasites were resuspended in *Lm* freezing medium and long-term stored in liquid nitrogen.

3.2.1.2 Preparation of rabbit-bloodagar plates

To prepare Novy-Nicolle-McNeal rabbit-bloodagar plates, 0.052 g/ml brain-heart-infusion agar was dissolved in ultrapure water and autoclaved. Sterile agar was heated to ≈ 45 °C and mixed 4:1 with defibrinated rabbit blood, 100 ml PBS and 4 ml Penicillin-Streptomycin. 50 μ l of blood agar was pipetted into each well of 96-well microtiter plates at an 45 ° angle in sterile conditions. Plates were stored at 4 °C and checked for contamination before first use.

3.2.1.3 Culturing of *Leishmania donovani* promastigotes

Leishmania donovani MHOM/ET/67/HU3 Z18 Africa promastigotes were cultured on a biphasic M199/ *Lm* medium culture system, similarly to *L. major* promastigotes. For passaging, they were seeded at 1.5×10^6 viable promastigotes/ml and cultivated at 26 °C, 5% CO₂ in a humidified atmosphere.

3.2.1.4 Preparation of M199 agarplates

To prepare M199 agarplates, 15 g LB agar were dissolved in 500 ml water and autoclaved. Meanwhile, 16.06 g M199 powder, 0.35 g NaHCO₃ and 2.5 ml of 2 g/l Hemin were dissolved in 400 ml water and adjusted to pH 7.4. After steril filtering, 200 ml FCS, 506.4 µl of 5 mM Biopterin and 10 ml penicillin/streptomycin stock solution were added. 50 µl blood agar per well were pipetted into 96-well microtiter plates at an 45° angle and stored at 4 °C. Plates were prepared under sterile conditions and checked for contamination before first use.

3.2.1.5 Culturing of *Leishmania major* axenic amastigotes

Generation of axenic amastigotes is based on (272). The liquid phase of 3-4 wells of *L. major* logPh promastigotes were transferred into 5 ml *Lm* liquid medium supplemented with 500 µl FCS plus antibiotics, if applicable, and expanded for 3 days at 27 °C, 5% CO₂ in a humidified atmosphere.

Alex' amastigote medium (AAM) was adjusted to pH 5.5 with HCl and sterile-filtered just before use. Sedimented parasites from the preculture were washed thrice in AAM, centrifuged and again resuspended in this medium. Viable parasites were counted in a Neubauer chamber, seeded at 20×10⁶ viable promastigotes per 24-well in AAM without antibiotics and incubated at 33 °C, 5% CO₂ in a humidified atmosphere. Transition into axenic amastigotes was checked phenotypically after about 11-18 days by microscopic assessment of the typical ovoid shape. Amastigotes were washed 1–2 times in the wells with pre-warmed PBS to remove cell debris and then resuspended. If they were further cultured axenically, they were kept in sterile-filtered, conditioned AAM.

3.2.2 Human primary cell culture

All centrifugation steps were performed at 143 g for 8 min, room temperature (rt) unless stated otherwise. Human cells were always cultured at 37 °C, 5% CO₂ in a humidified atmosphere.

3. MATERIAL & METHODS

3.2.2.1 Isolation of peripheral blood mononuclear cells (PBMCs) from human blood

Anonymized blood donations of healthy donors, obtained from the German Red Cross blood donation centre in Frankfurt am Main, were diluted with phosphate buffered saline (PBS) to ≈ 100 ml and separated by density gradient centrifugation using Histo-paque 1077 at 559 g at rt without acceleration and deceleration. PBMCs were collected from the interphase and washed once in washing buffer. Residual erythrocytes were lysed using an incubation of 10-15 min in 0.15 M NH_4Cl at rt, cells were then washed twice in washing buffer.

PBMC concentration and viability were determined in a 1:1000 dilution in CASYton using a CASY cell counter to distinguish viable from dead cells and to characterize cells according to their diameter.

3.2.2.2 Isolation of CD14^+ monocytes using magnetic-activated cell sorting (MACS)

PBMCs were incubated with CD14^+ magnetic beads at a ratio of 5 μl beads to 95 μl MACS buffer per 10×10^6 viable cells for 20 min at 4 °C. Cells were then positively selected on an LS column, yielding CD14^+ monocytes and CD14^- peripheral blood lymphocytes (PBLs), and counted again after three washing steps with MACS buffer. After elution in complete medium (CM), 20 to 25×10^6 viable CD14^+ monocytes were seeded in a T₇₅ cell culture flask or 7 to 9×10^6 viable monocytes in a T₂₅ cell culture flask in CM.

3.2.2.3 Isolation of human autologous peripheral blood lymphocytes

After MACS, 100×10^6 viable CD14^- PBLs per ml were frozen at -80 °C in PBL freezing medium and thawed when needed.

3.2.2.4 Isolation of monocytes using plastic adherence

Alternatively, 120 to 150×10⁶ viable PBMCs were seeded in CM supplemented with 1% human serum in a T₇₅ flask and incubated for 1-2 hours. Plastic adherent monocytes were washed twice to remove non-adherent lymphocytes before 15 ml differentiation medium was added.

3.2.2.5 Differentiation and stimulation of human monocyte derived macrophages (hMDMs)

For differentiation into macrophages, 30 ng/ml GM-CSF or 50 ng/ml M-CSF were added to the medium and cells were incubated for 5 to 7 days.

GM-CSF differentiated hMDMs could be further activated by subsequent incubation with 50 ng/ml IFN γ or activated with 100 ng/ml LPS for 24 hours.

3.2.2.6 Isolation of human primary granulocytes from human blood

Human neutrophils were either isolated from fresh blood or blood donations of healthy donors. Human fresh blood was collected in-house and polymorphonuclear neutrophils (PMNs) were isolated according to (153). For isolation from blood donations (253), blood was diluted 1:4 with PBS and loaded onto a double gradient composed of Histopaque-1077 and -1111, then centrifuged for 5 min at 218.4 g followed by 25 min at 1057 g without brake. Granulocytes were washed once in PBS by centrifugation for 10 min at 1057 g and then loaded onto a discontinuous gradient of Percoll in 1x PBS diluted to 85%, 80%, 75%, 70% and 65% to separate residual erythrocytes from granulocytes. Gradients were centrifuged for 25 min at 1057 g without brake.

The granulocyte fractions were pooled and cell number determined with a CASY cell counter as 1:1000 dilution in CASYton. Cells were further maintained in CM.

3. MATERIAL & METHODS

3.2.3 Genome editing methods

3.2.3.1 CRISPR-Cas9 in *Leishmania*

CRISPR-Cas9-mediated knockouts (KOs) in *Leishmania* were generated based on the LeishGEdit system (27). Primers for each gene locus were designed on LeishGEdit and checked for unique binding sites. If multiple binding sites were detected, primers were designed manually.

sgRNA templates were amplified in 35 cycles using 4 μ M primer, 2 μ M scaffold (see Table 3.8), and 1x PWO mastermix at 98 °C, 30 s for initial denaturation, 98 °C, 10 s for denaturation, 60 °C, 30 s for annealing and 72 °C, 15 s for extension. The repair cassette for homology recombination (HR) was amplified in 40 cycles using 1.5 ng/ μ l pT plasmid (see Table 3.9), 2 μ M forward and reverse primer, 3% DMSO, 1.375 mM MgCl₂ and 1x PWO mastermix at 94 °C, 5 min for initial denaturation, 94 °C, 30 s for denaturation, 65 °C, 2 min 15s for annealing and 72 °C, 15 s for extension. Amplicons were analyzed on an 1.4% TAE-agarose gel stained with 0.002% ethidium bromide, sterilized at 94 °C for 5 min and stored at -20 °C.

For electroporation, 60 $\times$ 10⁶ viable *L. major* from the logPh expressing a T7 RNA polymerase/ Cas9 construct were resuspended in T cell nucleofector buffer. 7.5 μ l of each sgRNA template and the repair cassette were added, and the suspension was pulsed once using program X-001 in a Nucleofector 2b. After electroporation, parasites were incubated overnight in 8.9 ml *Lm* medium and seeded on a 96-well bloodagar plate, supplemented with 10% extra FCS and the respective antibiotics for selection. A mock nucleofection without PCR products was used to control for recovery.

3.2.3.2 Dimerizable Cre (DiCre) recombination for inducible knockout generation in *Leishmania*

3' and 5' flox cassettes for Cre-mediated recombination were integrated sequentially upstream and downstream of the locus of interest in *L. major* expressing Cas9/T7 RNA polymerase and a dimerizable Cre construct. The method was adapted from (81). Cassettes were amplified using 0.5 μ M forward and reverse primer (see Table 3.8), 15 ng plasmid template (pGL2314 for 3' flox (80), pTNeo-eGFP-loxP for 5' flox, see Table 3.9) in 4 μ l GC Enhancer and 10 μ l Platinum Mastermix. A thermocycler was set

to 30 s at 98 °C, 35 cycles of 10 s at 98 °C and 3 (3' flox) or 4 min (5' flox) at 72 °C, with a final extension for 5 min at 72 °C. sgRNA templates were amplified as described in Section 3.2.3.1, followed accordingly by electroporation and single clone generation. In addition to subsequent procedure as in Section 3.2.3.3, integration of the flox cassettes was monitored by detection of the respective fluorescent label by flow cytometry. Cre-mediated recombination was induced in genetically confirmed full-floxed single cell clones by addition of 100 nM rapamycin. The fluorescence status was examined daily for up to 10 days after induction. Clones with eGFP positivity < 10% were subjected to serial dilution and analyzed for successful excision of the locus (see Section 3.2.3.3).

3.2.3.3 KO confirmation in *Leishmania*

Wells containing viable parasites were expanded, and genomic DNA (gDNA) was isolated from 3-4 logPh wells by using the Blood and Tissue gDNA kit, following the manufacturer's instructions. DNA concentration was measured at a NanoDrop 2000c, ≈ 20 ng/ μ l gDNA was used for a diagnostic PCR to confirm correct integration of the HR repair cassette into the target locus. Primer combinations were applied as in Figure 3.1, using gDNA of a parental strain as control.

Two confirmed clones were subjected to a 12-step 1:4 serial dilution to obtain a single-cell clone for use in further experiments. These single-cell clones were reconfirmed by the above described PCR. Follow-up experiments employing KO clones were performed with two independent clones, if possible.

3.2.3.4 Homology recombination in *Leishmania*

To integrate donor DNA into the SSU (18S rRNA) locus of the *Leishmania* genome, a pLexLm plasmid (see Table 3.9) was linearized to excise the recombination cassette containing the region of interest. To do so, 2.5 μ l SwaI were mixed with 5 μ g plasmid in 1x buffer r3.1 and 1-2 hours at 25 °C. Success of the digest was verified on a 1% agarose gel. The remaining DNA was heat-sterilized at 94 °C for 5 min and used for electroporation as in Section 3.2.3.1. Integration was confirmed and serial dilution performed according to Section 3.2.3.3. In case of tagged protein expression, the tag was also confirmed by Western blotting (Section 3.2.4.6).

3. MATERIAL & METHODS

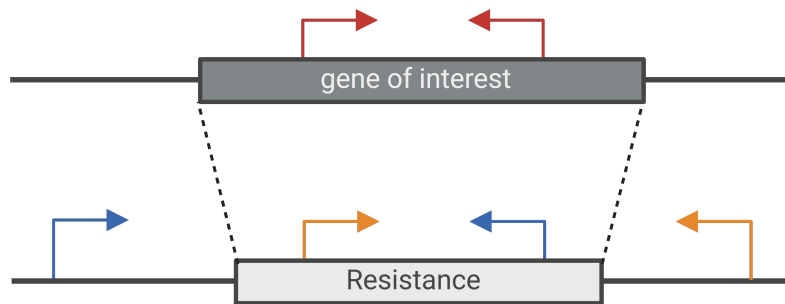


Figure 3.1: Schematic principle of diagnostic PCRs to confirm knockout status of *L. major* - Three PCRs were conducted to confirm knockout status of a strain:

Red: Primer pair binding within the open reading frame of the gene of interest. In case of at least one remaining allele, this combination will produce a positive band, whereas in case of a null mutant no band is visible.

Blue: Forward primer binding in the 5' untranslated region of the gene, reverse primer binding in the integrated resistance cassette. A band will confirm correct replacement of the gene by the resistance cassette.

Yellow: Same principle as for Blue, but using a forward primer binding in the integrated resistance cassette and a reverse primer in the 3' untranslated region of the gene.

Figure created with BioRender.

3.2.4 Molecular biology methods

3.2.4.1 Molecular cloning of plasmids

Regions of interest to be cloned into pLexLm plasmids were amplified from *L. major* wildtype gDNA using oligonucleotides with 30 nucleotide overhangs homologous to the plasmid insertion site. The size of PCR products was verified by agarose gel electrophoresis, and the products were purified using the Wizard SV Gel and PCR Clean-Up System. 5 µg of pLexLm plasmid were digested using the following restriction sites in the multiple cloning site: NcoI and NotI for untagged protein expression, and NcoI and KpnI for His₆-tagged protein expression. The digested plasmid was also purified. DNA concentration was determined using a NanoDrop 2000c. The insert and vector were combined in a 2:1 molar ratio, with a total DNA content of approximately 0.15 pmol, together with NEBuilder HiFi mastermix for Gibson assembly. After incubation for 20 min at 50 °C, constructs were used for transformation.

3.2.4.2 Chemical transformation of *E. coli*

2 µl of the assembly mix were added to 50 µl chemically competent *E. coli* TOP10 F'. After 20 min incubation on ice, the suspension was heat-shocked at 42 °C for 1 min, placed on ice for 2 min, and then recovered in 950 µl SOC medium for 1 hour at 37 °C. Approximately one fourth of the transformation mix was plated onto LB-Amp plates and incubated overnight at 37 °C.

Individual colonies were selected and expanded overnight in 3 ml LB medium supplemented with 100 µg/ml ampicillin at 37 °C, 6800 g. Plasmids were isolated using the QIAprep Spin Miniprep kit, verified by a control digest, and sent for Sanger sequencing to LGC Genomics using primers 5' UTR fwd and IR cam rev (see Table 3.8). Storage aliquots of plasmids were prepared from a 200 ml bacteria culture using the NucleoBond PC500 Maxiprep kit.

3.2.4.3 Reverse transcription

Approximately 1×10^8 viable *L. major* were washed in PBS and RNA was isolated according the RNeasy kit's manual. β -Mercaptoethanol was added to the lysis buffer accordingly. Purified RNA was eluted in 30 µl RNase-free water, its concentration was determined using a NanoDrop 2000c and integrity was verified by running 2 µl RNA on a 2% agarose gel after denaturation for 10 min at 70 °C in RNA loading dye. RNA samples were stored at -80 °C.

For reverse transcription, 50-100 ng RNA were heated up to 70 °C for 5 min and cooled at 4 °C for another 5 min, together with 1 µl random primers in a total of 5 µl. Immediately after, 6.5 µl water, 4 µl reaction buffer, 2 µl MgCl₂, 1 µl dNTP mix, 0.5 µl RNasin and 1 µl reverse transcriptase were added. All reagents were taken from the ImProm-II reverse transcription system. The transcription mix was then incubated for 5 min at 25 °C, 1 hour at 42 °C and 15 min at 70 °C. Synthesized cDNA was stored at -20 °C.

3.2.4.4 Quantitative real-time PCR

Reaction mix for quantitative real-time PCR (rt-qPCR) contained 2 µl cDNA, 1 µl of each 10 µM forward and reverse primer, 10 µl MESA Blue qPCR MasterMix Plus, and

3. MATERIAL & METHODS

6 µl water. All samples were prepared in duplicates, including an water control without cDNA, and measured using the SYBR Green program on an LC480 system (initial activation for 5 min at 95 °C, denaturation at 95 °C for 15 s, elongation at 60 °C for 1 min for 40 cycles). A final melting curve analysis (65 °C to 95 °C at 0.11 °C/s) was done to determine amplicon homogeneity. All primers used were tested before for a efficiency of 90-110%.

Threshold cycles were determined using the LightCycler 480 software and analyzed using the $\Delta\Delta C_t$ method with *NMT* as *Leishmania* housekeeping gene.

3.2.4.5 His₆-tag enrichment over magnetic nickel beads

12×10⁶ viable *L. major* logPh promastigotes and the respective volume of culture medium were separated into pellet and supernatant. Pelleted parasites were resuspended in 20 µl 1x Laemmli buffer and stored at -20 °C. 15 µl magnetic nickel beads were washed twice in PBS using a magnetic rack, resuspended in the supernatant fraction, and incubated for 30-60min at rt on an overhead shaker. Following two additional washing steps, His₆-tagged proteins were eluted in 20 µl 1x Laemmli buffer at 65 °C for 10 min. Samples in Laemmli buffer were denatured for 15 min at 95 °C and stored at -20 °C.

3.2.4.6 SDS-PAGE and Western blot

12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared with a stacking gel (1x Tris-HCl/SDS pH 6.8 buffer, 3,75% polyacrylamide, 0.25% TEMED, 0.1% APS) and a resolving gel (1x Tris-HCl/SDS pH 8.8 buffer, 12% polyacrylamide, 0.2% TEMED, 0.1% APS).

Samples containing lysate of 12×10⁶ *L. major* or equivalents in 20 µl Laemmli buffer were loaded onto the gel, along with a PageRuler Plus prestained protein ladder as a molecular weight marker. Electrophoresis was performed at 80 V for 20 min followed by 100 V for 90-120 min in 1x running buffer. Separated proteins were transferred on a polyvinylidene fluoride membrane in a semi-dry transfer chamber for 1 hour at 1.5 mA/cm². The membrane was blocked in 5% dry milk powder-TBST for 1 hour at rt and then incubated in primary antibody (see Table 3.4) diluted in the same buffer at

4 °C overnight with gentle shaking. After three 10 min washing steps at rt in TBST, the membrane was incubated for 1 hour at rt with secondary antibody diluted in 5% dry milk powder-TBST, washed again three times, and then imaged in Forte horseradish peroxidase substrate using a ECL Imager Chemostar.

3.2.4.7 AnnexinV staining of PtdSer on *L. major* cell surface

2×10^6 *L. major* were washed once in Ringer solution with centrifuging at 339 g for 4 min at 4 °C and incubated with 1:100 fluorophore-labeled AnnexinV in Ringer. A negative control was prepared by staining in PBS, since AnnexinV is Ca^{2+} -dependent. After another washing step, stained parasites were resuspended in 100 μl Ringer (or PBS) and analyzed using a flow cytometer (see Table 3.11). AnnexinV positive parasites were determined by gating on the control sample (Figure 3.2).

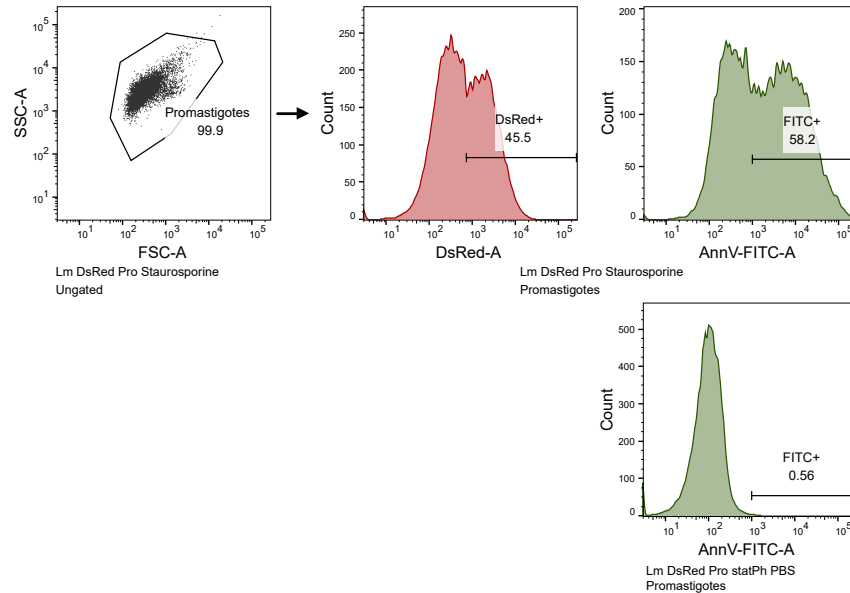


Figure 3.2: Gating strategy for AnnexinV staining - Analysis of PtdSer exposure by AnnexinV binding via flow cytometry. DsRed as viability marker of *L. major*^{DsRed} is quantified in parallel. A control stained in PBS is used to determine AnnexinV+ parasites.

3. MATERIAL & METHODS

3.2.4.8 AnnexinV MACS of PtdSer-positive *L. major*

100×10^6 *L. major* were washed in MACS1 buffer and centrifuged at 2400 g, 8 min, rt. Parasites were incubated in 160 μ l MACS1 buffer plus 40 μ l magnetic AnnexinV beads for 15 min at 4 °C and separated over an LS column, followed by three washing steps. AnnexinV negative parasites were collected in the flow-through, while positive parasites were eluted in MACS buffer. Both fractions were counted. The AnnexinV negative fraction was incubated again with 20 μ l AnnexinV beads and 40 μ l MACS1 buffer per 25×10^6 *L. major* for 15 min at 4 °C. The parasites were then purified over an LD column, washed, and eluted as described above. Purity of the fractions was determined by counting in a Neubauer chamber.

3.2.4.9 Cell cycle analysis of *L. major* using stoichiometric DNA staining

Cell cycle phases of *Leishmania* were determined using stoichiometric staining of DNA content. Cell undergoing cell death, accompanied by degradation of DNA, appear as a peak below the G₁ phase in a DNA staining histogram, see Figure 3.3. Thus, this cell cycle phase is termed SubG₁ phase.

$2-4 \times 10^6$ *L. major* were washed once in cold PBS by centrifugation at 2400 g, 8 min at 4°C. Cell pellets were resuspended in 100 μ l ice-cold 70% (v/v) ethanol and permeabilized for at least 30 min at -20 °C. After one wash in PBS pH 7.4 plus 50 mM EDTA, parasites were stained for 30 min in 100 μ l washing solution containing 1:1000 DNA dye plus 0.5 μ l RNase at 37 °C. For DNA staining, propidiumiodide (PI) or SYBR dyes were used. A control without dye was included. Samples were analyzed by flow cytometry. PI-stained parasites were determined by comparing them to the unstained sample. Cell cycle stages were defined based on the DNA content, see Figure 3.3.

3.2.4.10 MTT viability assay

Metabolic activity of viable cells was determined colourimetrically by measuring the conversion of MTT to formazan. Therefore, 5×10^6 *Leishmania* or 1×10^5 hMDM were kept in 1.1 mM MTT in RPMI without phenolred for 4 h at the respective incubation temperature (see Section 3.2.1, Section 3.2.2). If applicable, cells were pre-treated with

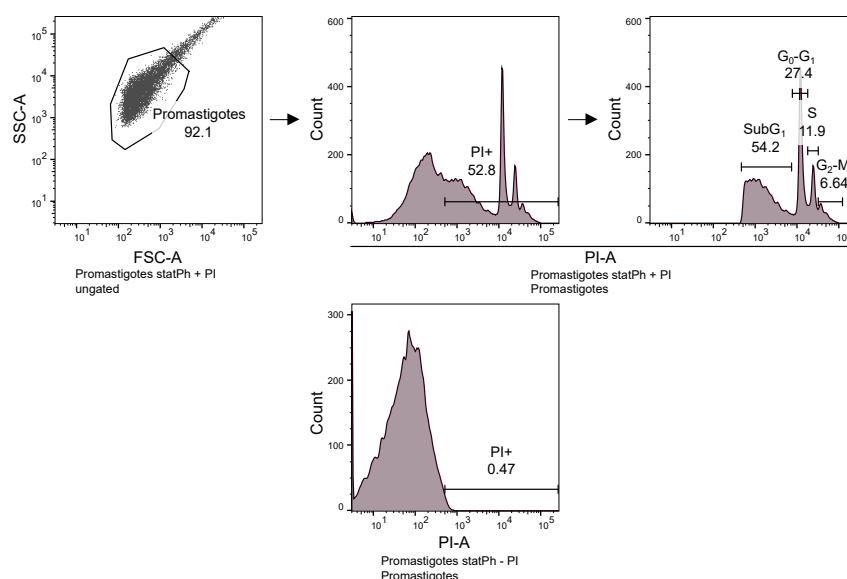


Figure 3.3: Gating strategy for cell cycle analysis - Stochastic DNA staining was evaluated by flow cytometry to quantify cells in the SubG₁ phase, marked by fragmented DNA. Here, DNA was stained by propidium iodide (PI). An unstained control was used to determine parasites positive for PI.

compounds for a specified time before the assay. Samples were set up in duplicate, if feasible. All compounds used for this assay were tested for false positive MTT reduction.

Reduced, insoluble formazan crystals were dissolved in 100 μ l DMSO, and their absorption was measured at 540 nm, along with a reference wavelength of 700 nm, using a ClarioStar plate reader. Each sample was corrected for its reference wavelength and for background absorbance using a blank sample containing cells without MTT incubation. Means of duplicates were used for normalisation to a control sample. The normalized viability values were visualized in GraphPad Prism, and an effective concentration (EC)₅₀ value determined by a four-parameter-regression with a bottom value constrained at ≥ 0 , and EC₅₀ ≥ 0 .

3.2.4.11 Nucleotidase assay

Nucleotidase activity was determined on 2×10^6 intact *L. major* by detecting the release of free phosphate (P_i) from adenosine nucleotides. 3'-AMP (Sigma) and 5'-AMP

3. MATERIAL & METHODS

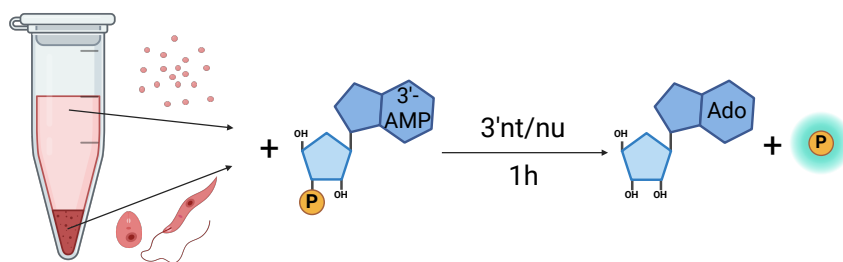


Figure 3.4: Principle of nucleotidase assay on intact *L. major* - Parasites were incubated for 3 hours in reaction buffer and then separated into pellet and supernatant. Intact, pelleted parasites expose ecto-enzymes on their surface, the supernatant contains secreted proteins. 3'-AMP was added as substrate for 1 hour, release of P_i by 3'-nucleotidase activity quantified using a malachite green based assay. 5'-AMP can be used as substrate for determination of 5'-nucleotidase activity. Figure created with BioRender.

were used as substrates for 3'-nucleotidase and 5'-nucleotidase activity, respectively. As a phosphate free environment is crucial for this assay, all reagents were pre-tested to ensure they were free of phosphate contamination. Each experiment included blanks without parasites and substrates to control for potential carry-over contaminations. Parasites were washed once in nucleotidase reaction buffer, and resuspended to a final concentration of 2×10^6 *L. major* in 100 μ l buffer and incubated at 27 °C (promastigotes) or 33 °C (axenic amastigotes) to allow for protein secretion. When inhibitors were tested, they were added to the buffer prior to incubation. After 3 hours, parasites were separated from the supernatant by centrifugation. Respective substrate was added for 1 hour to the supernatant or the parasite pellet at a final concentration of 100 μ M. Inhibitor concentrations were maintained during this incubation, if applicable. Parasites were removed by another centrifugation step, and 25 μ l reaction mix were diluted 1:1 in ultrapure water. To visualize the release of P_i , 100 μ l malachite green buffer was added per sample, and the phosphate assay was performed as per the manufacturer's protocol.

Turnover of P_i was measured colourimetrically as absorbance at 620 nm with a ClarioStar plate reader. Means of duplicates were taken and corrected using the blank without parasites. Concentration of P_i was determined by interpolating the measured values against a standard curve, following the technical bulletin. Nucleotidase activity was then expressed as μ M released P_i /2 $\times 10^6$ *L. major*/h.

3.2.4.12 Endonuclease assay

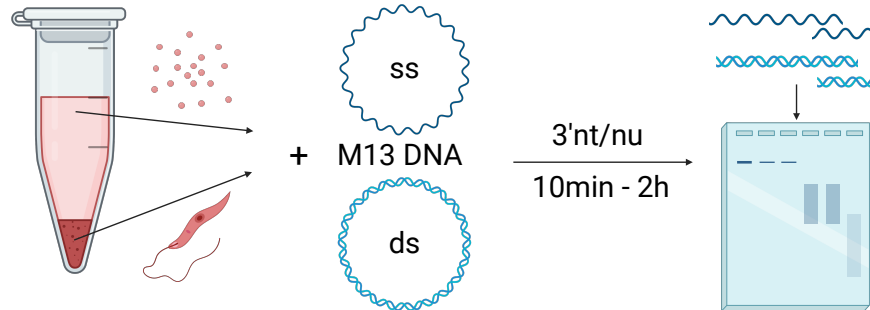


Figure 3.5: Principle of endonuclease assay on intact *L. major* - Parasites were incubated for 3 hours in reaction buffer and then separated into pellet and supernatant. Intact, pelleted parasites expose ecto-enzymes on their surface, the supernatant contains secreted proteins. Circular M13 phage genome, either single- or double-stranded (ss or ds), was added for the specified time as substrate for endonuclease activity. Reaction was stopped by addition of EDTA-containing DNA loading dye, the sample analyzed on an 0.8% agarose gel to visualize substrate integrity. Remaining intact substrate was semi-quantified using ImageJ. Figure created with BioRender.

Endonuclease activity was determined on 2×10^6 intact *L. major* promastigotes by monitoring the degradation of covalently closed, circular M13 bacteriophage genome. Double-stranded or single-stranded DNA was used as substrate (dsM13 or ssM13). Alternatively, NET-enriched supernatant (see Section 3.2.5.3) was used as substrate. Promastigotes were washed once in endonuclease reaction buffer with centrifugation at 2400 g, 8 min, rt, resuspended to a final concentration of 2×10^6 *L. major* in 25 μ l buffer, and incubated at 27 °C for 3 hours. Parasites were separated from the buffer by centrifugation. 0.5 μ g DNA substrate in buffer was added to either the pellet or the supernatant and incubated for the specified time. Controls included 130 U DNaseI and substrate or only parasites without substrate. After reaction time, cells were removed by centrifugation, and the reaction stopped by addition of EDTA-containing TriTrack loading dye.

Samples were analyzed on a 0.8% agarose-TAE gel stained with 1x GelRed and visualized using a Gel Doc XR+. The relative amount of intact substrate DNA was semi-quantified using densitometry tools in ImageJ 1.53c and the undigested control sample without parasites as reference value.

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3.2.4.13 Label-free mass spectrometry for quantitative proteomics

25×10^6 viable logPh *L. major* were pelleted and lysed in 40 μ l SDS-containing buffer (2% SDS, 5 μ M DTT, 1x cOmplete protease inhibitor, 50 μ M HEPES pH 8.0). Samples from seven consecutive passages were shipped to Ute Distler at the Core Facility for Mass Spectrometry Mainz. Any subsequent steps were conducted in their laboratory. Samples were further lysed by sonication in a Bioruptor and protein concentration was determined using a Pierce assay. 10 μ g total protein was digested as described in (127, 237).

Peptides were analyzed in triplicates by liquid chromatography and mass spectrometry on an Evosep One chromatography system coupled to a timsTOF SCP mass spectrometer. Samples were loaded onto EvoTips and separated on a reversed phase C18 column applying the Whisper Zoom 40 samples per day method provided by the manufacturer. Eluted fragments were analyzed as described in (178). A dual trapped ion mobility spectrometer was set to a fixed duty cycle.

Generated raw data were processed by DIA-NN version 1.9.2 (71) with default parameters for a library-free database search against the *L. major* proteome (UniProt release January 2025, Taxon ID 5664) and a list of common contaminants. QuantUMS high accuracy mode was applied for quantification using the build-in algorithm, peptide precursor false discovery rates were controlled below 1%. Data were deposited to the ProteomeXchange Consortium via jPOST partner repository (195) with identifiers PXD066928 (ProteomeXchange) and JPST003974 (jPOST).

3.2.5 Immunological methods

3.2.5.1 Infection of hMDMs with *L. major* promastigotes

Post differentiation and optional activation of macrophages (see Section 3.2.2.5), these were infected using either statPh *L. major* promastigotes at an multiplicity of infection (MOI)=10 or with axenic amastigotes using an MOI=2 in tissue culture plates. Plates were centrifuged at 339 g for 4 min to sediment parasites onto the macrophages. Samples were kept at 37 °C for 3 hours before remaining extracellular parasites were removed by washing once in PBS. Cells were subsequently maintained in CM.

If no surface markers were to be stained, cells were detached by trypsination. Infection rate of single, viable macrophages was analysed by flow cytometry at the indicated time post infection (pi). Parasite burden was defined as the mean fluorescence intensity (MFI) of infected macrophages (Figure 3.6). For fluorescent parasites, this marker was used to detect intracellular *Leishmania*. Alternatively, intracellular parasites were stained with anti-*L. major* serum. After live/dead staining, cells were fixed in 4% paraformaldehyde for 10 min on ice, followed by permeabilization and blocking in permeabilization & blocking buffer for 10 min at 4 °C. Parasites were stained in 1:10,000 anti-*L. major* serum for 30 min at 4 °C in buffer 2, then washed and stained with secondary antibody under the same conditions. After washing in buffer 1, cells were analyzed by flow cytometry.

If applicable, adenosine or 3'-AMP (SantaCruz) were added together with the parasites at the time of infection in indicated concentrations.

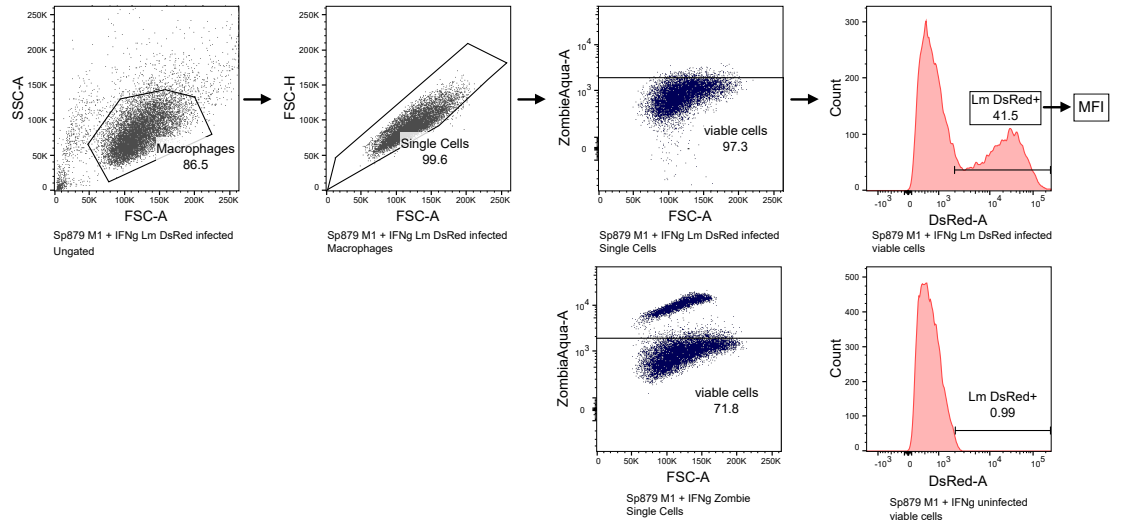


Figure 3.6: Gating strategy for analysis of *L. major* infection rate and parasite burden in hMDM - DsRed as fluorescent marker of *L. major*^{DsRed} was used to determine the percentage of infected, viable macrophages. A control containing viable and dead cells was used to set the viability gate. An uninfected control was used to determine DsRed positive macrophages. The DsRed geometric mean of infected macrophages was used to quantify the mean fluorescence intensity (MFI) as parasite burden. Intracellular parasites stained with *L. major* serum were analyzed accordingly.

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3.2.5.2 Infection of hMDMs with *L. major* promastigotes in co-culture with PBLs

Immediately before infection, macrophages and PBLs were stained in 1 μ M Cell Trace Far Red in PBS for 20 min at 37 °C and quenched by addition of CM for 5 min. 0.2×10^6 macrophages were infected with an MOI=20 *L. major* statPh promastigotes in 96-well U-shape plate for 3 hours, if applicable in the presence of adenosine or 3'-AMP (SantaCruz). Autologous PBLs were then added directly to the infection at a 5:1 PBL-to-macrophage ratio. Each well contained 2×10^4 macrophages, 10 technical replicates per condition were prepared and pooled just before analysis.

Four days pi, cells were detached by trypsination, and PBL proliferation was analyzed by flow cytometry as CellTrace_{low} fraction (see Figure 3.7). Infection rate and parasite burden of hMDM were determined as in Figure 3.6.

3.2.5.3 Isolation of neutrophil extracellular traps

Freshly isolated primary granulocytes (see Section 3.2.2.6) were seeded in a poly-D-lysine coated 12-well plate in a density of 1.5×10^6 PMNs per well in CM. To induce formation of NETs, Phorbol-12-Myristat-13-acetat (PMA) was added to a final concentration of 50 nM and cells were incubated for 3 h at 37 °C. To generate NET enriched supernatant, cells were gently washed once in PBS and incubated in PBS with 1x rCutSmart and 4 U/ml AluI for 20 min at 37 °C. Subsequently, NET enriched supernatants were collected and centrifuged for 5 min, 300 g, 4 °C to remove debris (23). Cleared supernatants were stored at -20 °C. DNA content was quantified using a Quant-it RiboGreen assay following instructions.

3.2.5.4 Neutrophil killing assay

2×10^6 PMNs were seeded in a 24-well plate and, if applicable, treated with 100 nM PMA for 30 min at 37 °C. To inhibit phagocytosis, cells were treated with 10 μ g/ml Cytochalasin D for 20 min. 130 U DNaseI were also added, if applicable. Then, *L. major* statPh promastigotes were added at an MOI=1 of viable parasites, spun down shortly and incubated for 2 hours at 37 °C. Control samples, containing additives and

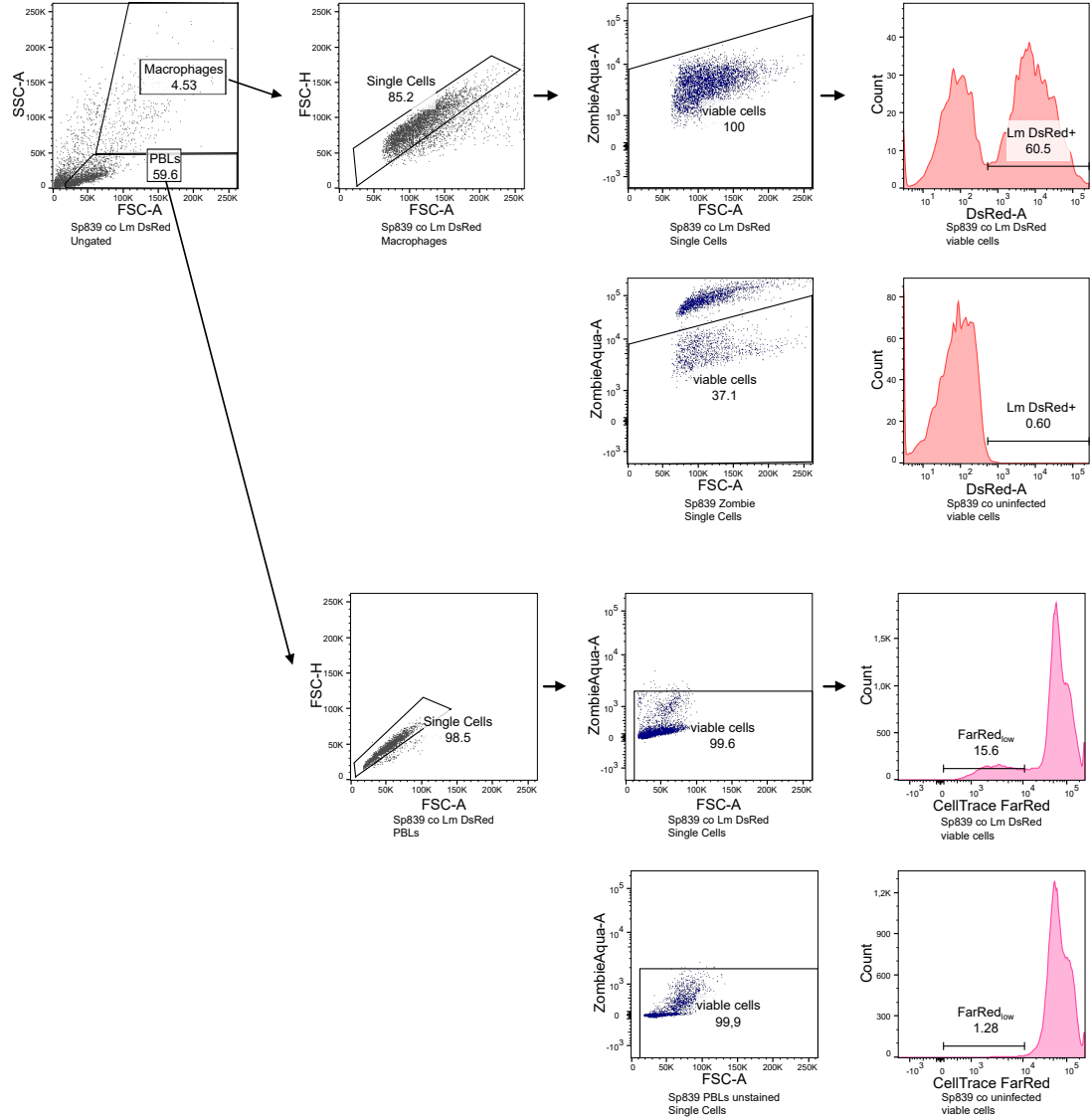


Figure 3.7: Gating strategy for analysis of *L. major* infection and PBL proliferation in a hMDM:PBL co-culture - hMDM and PBL populations are separated by forward and side scatter (FSC, SSC). Viable macrophages were analyzed for infection rate and parasite burden as in Figure 3.6. Viable lymphocytes were analyzed for CellTrace Far Red signal. This staining is diluted when cells divide. The FarRed_{low} share of PBLs was determined based on an uninfected control where no proliferation is expected. hMDMs: Human monocyte-derived macrophages, PBLs: Peripheral blood lymphocytes.

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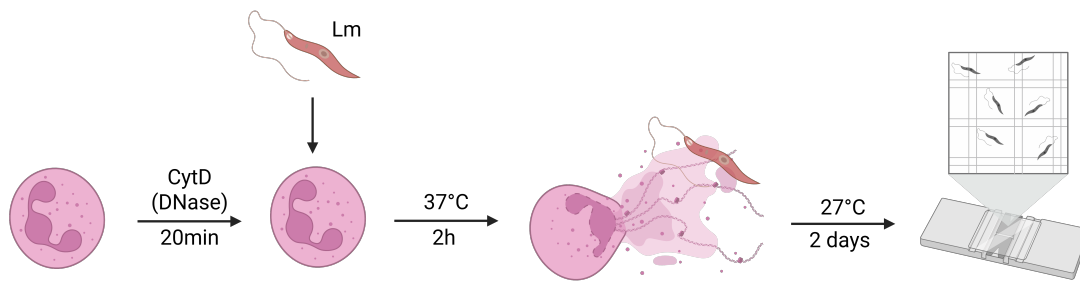


Figure 3.8: Schematic principle of the neutrophil killing assay - PMNs were treated with Cytochalasin D (CytD) and optionally with DNaseI for 20 min, then statPh promastigotes were added for 2 hours. Culture was transferred to 27 °C for two days, viable parasite numbers were counted and compared to control without PMNs. Figure created with BioRender.

parasites but no PMNs, were treated identically. After this incubation, samples were transferred to 27 °C and incubated for two days (Figure 3.8). Viable, motile parasites in each sample were counted in a Neubauer chamber. Cell counts were normalized to the respective control without PMNs, yielding the percentage of viable *L. major* relative to the control.

3.2.5.5 Extracellular antibody staining for flow cytometry

All centrifugation steps were carried out at 339 g, 4 °C for 4 min. Cells were detached by scraping to preserve surface protein integrity. 2×10^5 cells were washed in cold MACS buffer and incubated in blocking buffer for 15 min at 4 °C. Cells were stained with diluted antibodies in blocking buffer for 30 min at 4 °C in the dark. When ZombieAqua was used for live/dead discrimination, it was added 1:1000 to the staining mix. Cells were washed again, resuspended in 100 µl of buffer, and analyzed by flow cytometry. If SYTOX dyes were used for viability staining, they were added to a final concentration of 1 µM before analysis. Gating was set as depicted in Figure 3.9.

3.2.5.6 Enzyme-linked Immunosorbent Assay (ELISA)

If cell free culture supernatant from infection experiments were to be analyzed for cytokine content, it was stored at -80 °C and thawed upon usage, including a medium

3.2 Methods

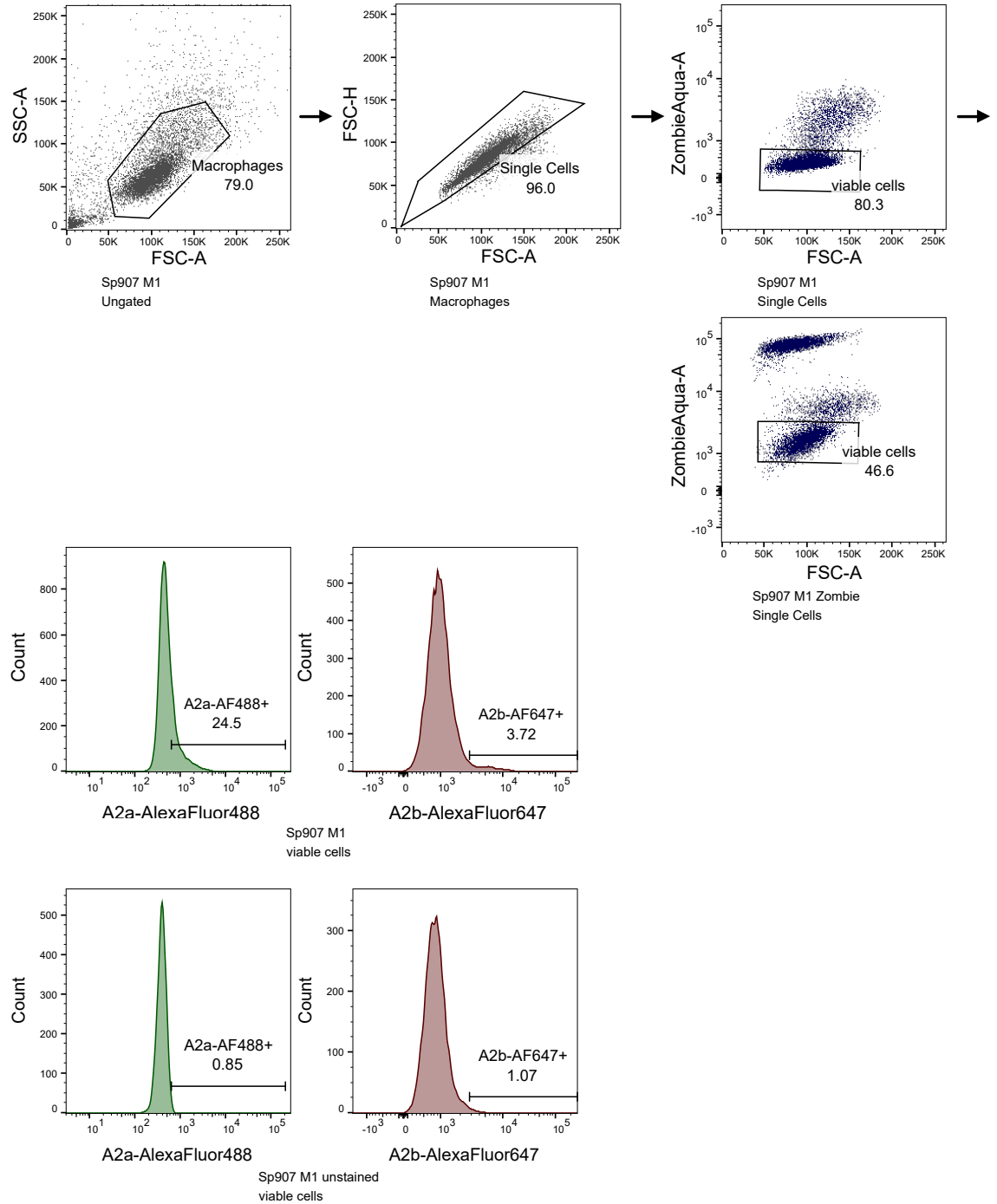


Figure 3.9: Gating strategy for extracellular antibody staining - Viable, single macrophages were analyzed for surface marker expression by gating on an unstained control. Respective gating controls are shown below the samples. Expression can be evaluated as positivity [%] or mean fluorescence intensity (MFI) of viable cells.

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control. Soluble cytokine content was determined using DuoSet ELISA kits according to the manual. All reagents were reconstituted and diluted as specified for the respective batch. The assay was conducted in half-area microplates with a working volume of 50 μ l per well. Samples, including standards, were measured in duplicate. Between each incubation step, wells were washed four times with ELISA washing buffer.

Wells were coated with capture antibody in PBS pH 7.4 overnight at rt and blocked in reagent diluent for 1 hour at rt. Samples and standards were added for 2 hours at rt, detection antibody in reagent diluent for another 2 hours at rt. Samples were diluted in reagent diluent to ensure they fell within the assay range. Detection antibodies were bound by Streptavidin-HRP and then developed using substrate solution. Each step was incubated for 20 min at rt in the dark. The reaction was stopped by adding 25 μ l H_2SO_4 , and absorption was measured at 450 nm using a ClarioStar plate reader. A reference wavelength at 570 nm was included to control for unspecific light scattering. Absorption values were corrected by the respective reference value and blank. A standard curve was calculated using a 4-parameter regression. Cytokine concentrations in samples were determined in pg/ml based on this regression, considering the respective dilution factor. All calculations were done in Mars software.

3.2.6 Statistics

For experimental setups involving biological or technical replicates, statistics were performed with GraphPad Prism 9.5.0. Data are presented as mean with standard deviation. Significance was determined using an unpaired, two-tailed t-Test, unless stated otherwise, with $p > 0.05$ gives no significance (ns), $p \leq 0.05$ *, $p \leq 0.01$ **, $p \leq 0.001$ ***.

3.2.7 Use of generative artificial intelligence

ChatGPT-4 and Le Chat were used to proofread texts for spelling, grammar and clarity. No scientific content was created using these chat bots, suggestions for corrections were carefully evaluated before implementation.

Results

4.1 Phenotypic screening for antileishmanial compounds

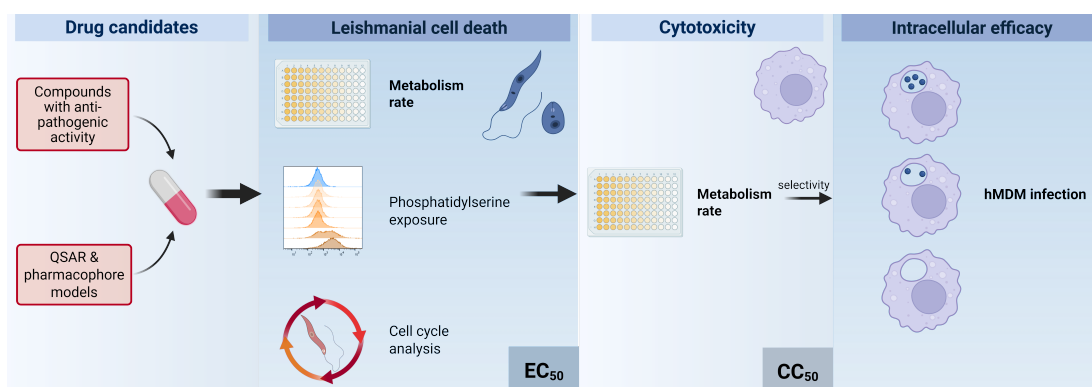


Figure 4.1: Workflow to identify novel antileishmanial compounds. - Several compounds were provided by cooperation partners, either due to their known antipathogenic activity or predictions as antiparasitics based on a QSAR model. These were tested for their leishmanicidal potential, mainly by reduction of metabolism, but also in regard to other cell death hallmarks. Active compounds were further tested for cytotoxicity on hMDM and, in case of suitable selectivity for *Leishmania*, progressed to *in vitro* infection experiments to assess intracellular efficacy. Figure created with BioRender.

In order to test compounds for their potential application as leishmanicidal drugs, they underwent a general selection scheme (Figure 4.1). Candidates were selected based on either their reported broad antipathogenic efficacy or by a model predicting their activity. All compounds, provided by cooperation partners from the LOEWE DRUID center, were tested for leishmanicidal activity based on their ability to reduce parasite

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metabolism. Additional hallmarks of PCD could also be tested. Compound activity was assessed by calculating the half-maximal effective concentration (EC_{50}) from dose-response experiments. Typically, for antileishmanial activity, an initial EC_{50} in the low micromolar range is sufficient to continue research for lead identification and optimization.

Active compounds were then subsequently for cytotoxicity, expressed as CC_{50} , on hMDM. Compounds that demonstrated selectivity for *Leishmania* over human host cells were further tested for their leishmanicidal potential in an *in vitro* infection model. There, intracellular efficacy was assessed by the compound’s ability to reduce the parasite burden or infection rate. *L. major* were used as standard parasite strain, an Old World species causing cutaneous leishmaniasis. Furthermore, activity against *L. donovani*, a clinically more relevant pathogen causing visceral leishmaniasis, could also be tested.

4.1.1 Antileishmanial activity of arylmethyaminosteroids

Arylmethyaminosteroids were previously identified as highly potent antimalarial and antischistosomal agents. This compound class has been continuously optimized by structure-activity relationship approaches based on phytochemical sarchines and possesses moderate antimicrobial activity against some bacteria and fungi species (152). The lead compound **1o** and its structurally highly similar derivative **1c** are characterized by a gonane steroidal core and a hydroxylarylmethylamino moiety (Figure 4.2a). They were tested against *Leishmania* to verify the potential of arylmethyaminosteroid as broad-spectrum antiparasitics.

Table 4.1: EC_{50} of arylmethyaminosteroids **1o** and **1c** on *Leishmania* - EC_{50} of **1o** and **1c** as determined by their ability to reduce metabolic activity after 48 hours, $\pm$ 95% asymptotic confidence interval.

	EC_{50} 1o [μ M]	EC_{50} 1c [μ M]
<i>L. major</i> promastigotes	4.76 ± 0.85	4.92 ± 0.53
<i>L. major</i> amastigotes	14.33 ± 4.74	30.84 ± 21.65
<i>L. donovani</i> promastigotes	6.32 ± 0.59	5.84 ± 0.71

4.1 Phenotypic screening for antileishmanial compounds

Both compounds were tested on axenic pro- and amastigote cultures and on hMDM to estimate their selectivity for parasites over host cells. Two concentrations of 1o and 1c were added to macrophages infected *in vitro* with *L. major* to test their ability to eliminate or reduce intracellular parasite numbers in an established infection setting.

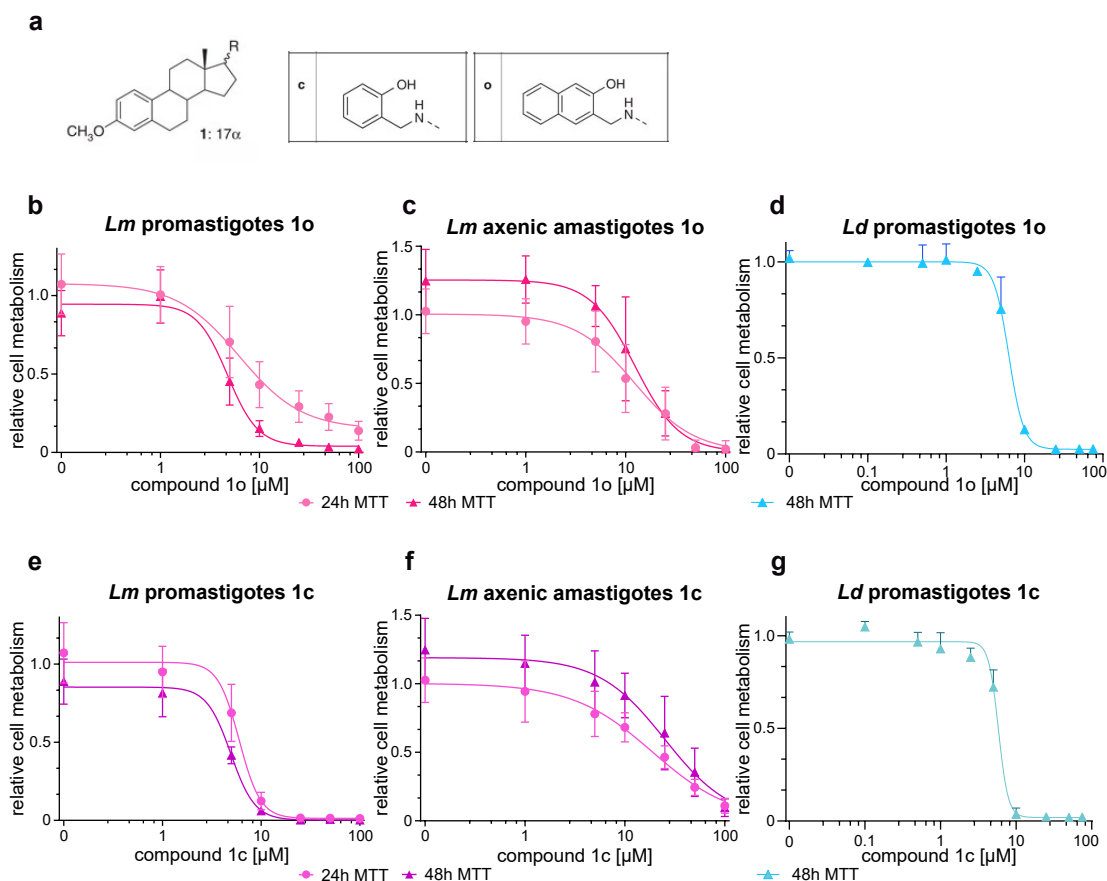


Figure 4.2: Dose-dependent effects of arylmethylaminosteroids 1o and 1c on the cellular metabolism rate of *Leishmania*. - a) Structural formula of 1o and 1c comprising a common steroid backbone and a specified residue; derived from (152); 1o was applied on b) *L. major* promastigotes, c) axenic amastigotes and d) *L. donovani* promastigotes, metabolism rate was assessed after 24 or 48 hours using an MTT assay; 1c was applied on e) *L. major* promastigotes, f) axenic amastigotes and g) *L. donovani* promastigotes, metabolism rate was assessed after 24 or 48 hours using an MTT assay; $n = 5$ (*Lm*), $n = 3$ (*Ld*), non-linear 4-parameter regression: solid line.

1o and 1c show apparent leishmanicidal activity on *L. major* promastigotes in an MTT assay, to a similar extent after 24 and 48 hours of treatment. The parasites' relative

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metabolic rate decreased to nearly zero with increasing compound concentration, visible as sigmoidal regression curve with an EC_{50} of 5 μ M (Figure 4.2b, e). Axenic amastigotes were less sensitive than promastigotes. 1o began to reduced the metabolism of this life cycle stage at $\approx 5 \mu$ M, no signs of viability were detected at 50 μ M, leading to an EC_{50} of about 15 μ M. Treatment with 1c did not achieve a stable bottom plateau phase close to zero for concentrations up to 100 μ M, making the EC_{50} determination imprecise. The half-maximal effect was estimated to be $\approx 30 \mu$ M (Figure 4.2c, f; Table 4.1). On *L. donovani* promastigotes, 1o and 1c efficacy is comparable to *L. major* promastigotes with EC_{50} values of around 6 μ M (Figure 4.2d, g).

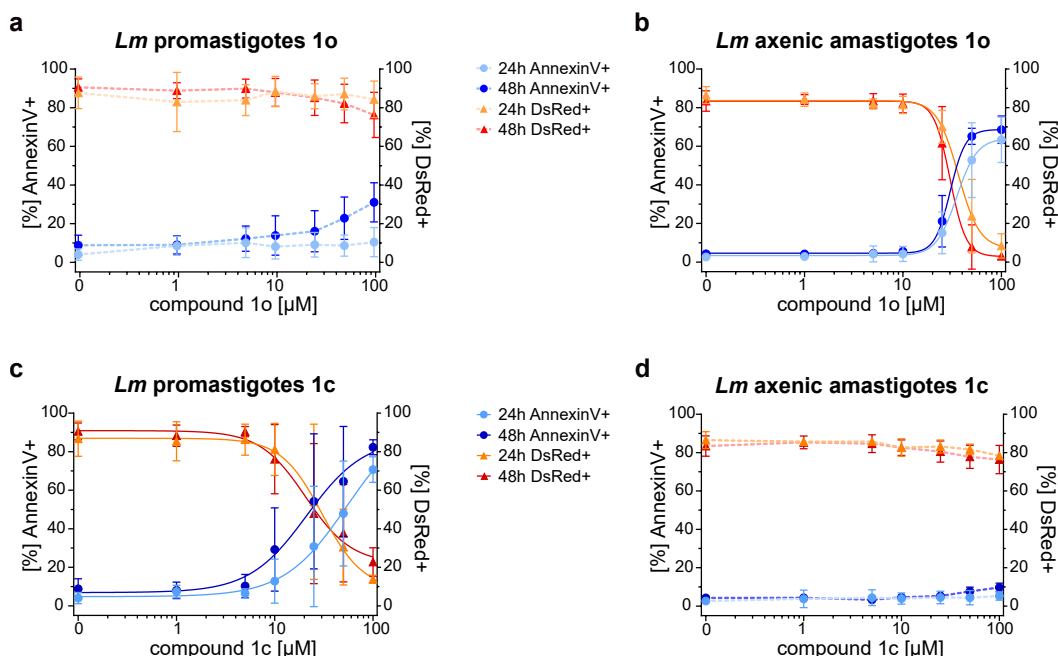


Figure 4.3: Dose-dependent effects of arylmethyaminosteroids 1o and 1c on phosphatidylserine exposure on *L. major*. - 1o was applied on a) *L. major* promastigotes and b) axenic amastigotes for 24 or 48 hours, PtdSer exposure was then assessed by AnnexinV staining and flow cytometry analysis; 1c was applied on c) *L. major* promastigotes and d) axenic amastigotes for 24 or 48 hours, PtdSer exposure was assessed. $n = 5$, non-linear 4-parameter regression: solid line

However, the two compounds differed in their concurrent induction of PtdSer exposure and loss of DsRed signal. Together with inclining AnnV positivity, declining fluo-

4.1 Phenotypic screening for antileishmanial compounds

cence of *L. major*^{DsRed} can be used to monitor parasite viability in strains expressing the fluorescence marker. Viable parasites, which do not expose PtdSer and are AnnV negative emit a DsRed signal, and vice versa for dead parasites. Within the tested concentration range, 1o only affected amastigotes and 1c only promastigotes, in a comparable manner (Figure 4.3). At 100 μ M compound concentration, an increase of AnnV positivity up to 60-80% was observed, along with a loss of DsRed signal down to 5-20%. Similarly, DNA fragmentation during cell death, as visible in the SubG₁ cell cycle phase, was potently induced by 1o on amastigotes in up to 90% of the population. The effect was less prominent in promastigotes, where only half of the parasites were in the SubG₁ phase at 100 μ M of 1o, and no stable plateau phase could be observed (Figure 4.4a-b). For 1c, this trend was similar. About 60% of promastigotes were quantified in the SubG₁ phase after treatment with 25-100 μ M 1c. On amastigotes, no sigmoidal curve was observed with increasing 1c concentration. Instead, the share of amastigotes in the SubG₁ phase continuously increased to $\approx$ 70% at 100 μ M 1c (Figure 4.4 c-d). Again, compound efficacy did not increase tremendously from 24 to 48 hours of treatment. Fragmentation of nuclear DNA is another typical hallmark of cell death. Thus, although both arylmethylaminosteroids similarly affect parasite metabolism, they differentially influence other hallmarks of cell death, depending on the *Leishmania* life stage. Potential reasons, such as different mechanisms of action or pH dependency of these compounds, were not investigated.

Before progressing to *in vitro* infection experiments, cytotoxicity of the compounds on human primary macrophages was assessed. Therefore, cellular metabolism of hMDM differentiated with M-CSF or GM-CSF, representing different polarization states, was measured post 24 or 48 hours of incubation with arylmethylaminosteroids. As reported previously, 1o is able to increase myeloid metabolism through enhanced glycolysis by up to threefold, reaching a plateau phase at $\approx$ 25 μ M (34). In contrast, for 1c at concentrations >10-25 μ M, the metabolic rate decreased after an initial increase, dropping below the untreated reference value for both macrophage subtypes (Figure 4.5). Hence, macrophage viability is likely impaired at these concentrations of 1c.

To proceed with *in vitro* infection experiments, 5 μ M and 50 μ M were selected as compound concentrations just below and above the EC₅₀ for axenic amastigotes and mostly non-toxic for the host cell (Table 4.1). Macrophages were infected with axenic amastigotes of *L. major*^{DsRed} for 3 hours. Extracellular parasites were then removed and

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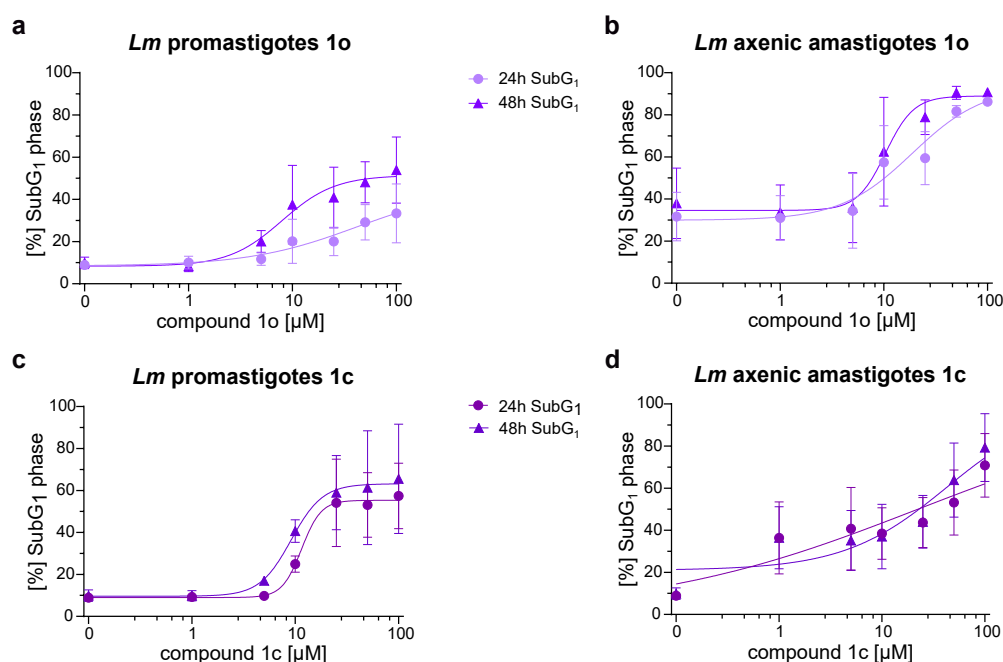


Figure 4.4: Dose-dependent effects of arylmethyaminosteroids 1o and 1c on DNA degradation of *L. major*. - 1o was applied on a) *L. major* promastigotes and b) axenic amastigotes for 24 or 48 hours, the share of cells with fragmented DNA in the SubG₁ phase was determined using stoichiometric DNA staining; 1c was applied on c) *L. major* promastigotes and d) axenic amastigotes for 24 or 48 hours, the share of cells with fragmented DNA in the SubG₁ phase was measured. $n = 5$, non-linear 4-parameter regression: solid line

4.1 Phenotypic screening for antileishmanial compounds

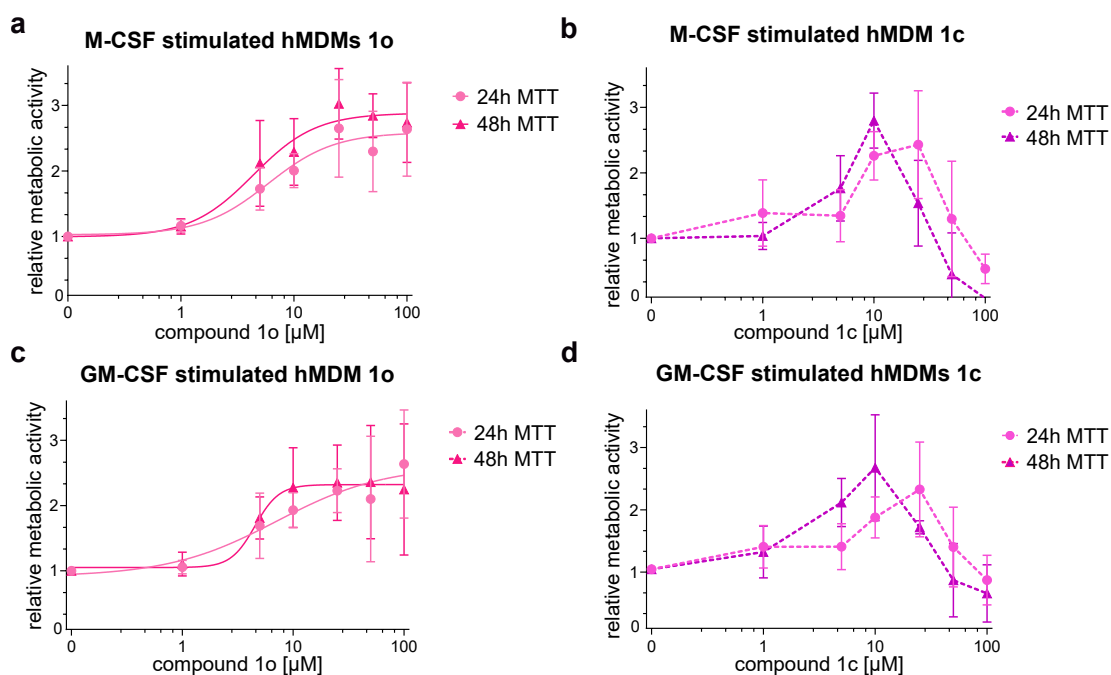


Figure 4.5: Dose-dependent effects of arylmethylaminosteroids 1o and 1c on cellular metabolism rate of hMDM. - 1o was applied on **a)** M-CSF and **c)** GM-CSF differentiated macrophages for 24-24 hours; 1c was applied on **b)** M-CSF and **d)** GM-CSF differentiated macrophages; Cellular metabolism rate was assessed and normalized to the carrier control. $n = 4$ donors in three independent experiments, non-linear 4-parameter regression: solid line.

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compounds in the respective concentrations were added. This procedure mimicked an established infection, as no promastigote-to-amastigote differentiation occurred during the experiment.

Infection rate and parasite burden were analyzed 24 and 48 hours pi and compared to an infected but untreated control (Figure 4.6). No significant decrease in the proportion of *L. major*^{DsRed} infected cells was observed, except for a marginal reduction at 48 hours pi. Parasite burden, determined as MFI of infected macrophages, increased from 24 to 48 hours pi for untreated samples, as amastigotes typically start to proliferate during this period. In treated cells, a dose-dependent reduction in parasite burden was apparent for both compounds 24 hours pi. This difference became more pronounced at 48 hours pi, particularly in hMDMs differentiated with GM-CSF. When normalizing the MFI to the respective donor's untreated but infected sample, this effect became even clearer. After 48 hours of infection, 5 μ M of 1o decreased the parasite burden by half, 50 μ M down to a third in hMDM(GM-CSF). 1c gave very similar results. For hMDM(M-CSF), 5 μ M of either compound decreased the parasite burden to approximately 60% of the untreated control, while 50 μ M reduced it to approximately 40%. Assessment of 1o and 1c effects on intracellular *L. donovani* is pending.

To summarize, both arylmethylaminosteroids showed robust antileishmanial activity in terms of metabolic rate, but demonstrated complementary, stage-dependent effects on other hallmarks of cell death. In macrophages, both compounds increased metabolic activity at lower concentrations. Whereas 1o reached a plateau, 1c induced a loss of metabolic activity at higher concentrations, likely indicating cytotoxicity. Nevertheless, at tolerable doses, both compounds effectively limited intracellular *L. major* proliferation. However, unfavorable oral bioavailability negatively impacts the potential of further development for these compounds (33, 152).

4.1.2 Antileishmanial activity of repurposed compounds derived from an arylmethylaminosteroid pharmacophore model

By leveraging knowledge of the antimalarial activity of the arylmethylaminosteroid compound set, a quantitative structure-activity relationship (QSAR) model was applied by Joachim Geyer and Michael Kirstgen (Justus-Liebig-University Gießen) to

4.1 Phenotypic screening for antileishmanial compounds

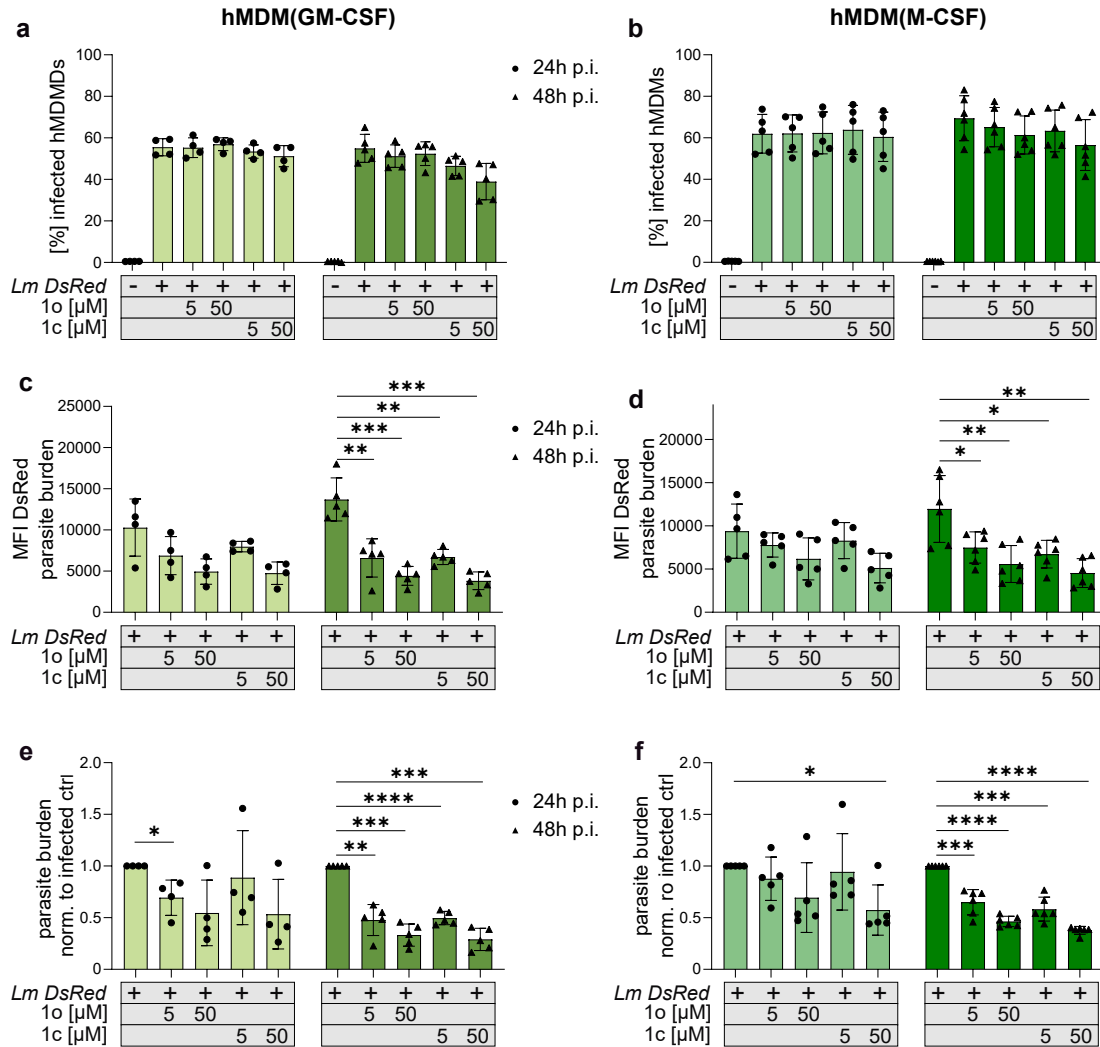


Figure 4.6: Effects of arylmethyaminosteroids 1o and 1c on intracellular *L. major* during hMDM *in vitro* infection. - Human macrophages were infected with *L. major* amastigotes, 1o and 1c were added 3 hours p.i. **a-b)** hMDM infection rate and **c-d)** parasite burden were assessed by flow cytometry as mean fluorescence intensity (MFI) 24 and 48 hours p.i. **e-f)** Relative parasite burden was normalized to the infected, untreated control.

$n = 4$ to 5 donors in three independent experiments, Welch's t-test

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map pharmacophores of this compound class (148). Pharmacophore models represent the three-dimensional chemical interactions of compounds that are required for biological activity (283). Based on this model, the APExBIO DiscoveryProbe FDA-approved drug library was screened for compounds that met these pharmacophore criteria. This library included 2320 drugs that are either approved or mentioned in a pharmacopoeia (10). Repurposing such compounds can be beneficial their advanced development in clinical trials and already defined pharmacological characteristics. Thus,

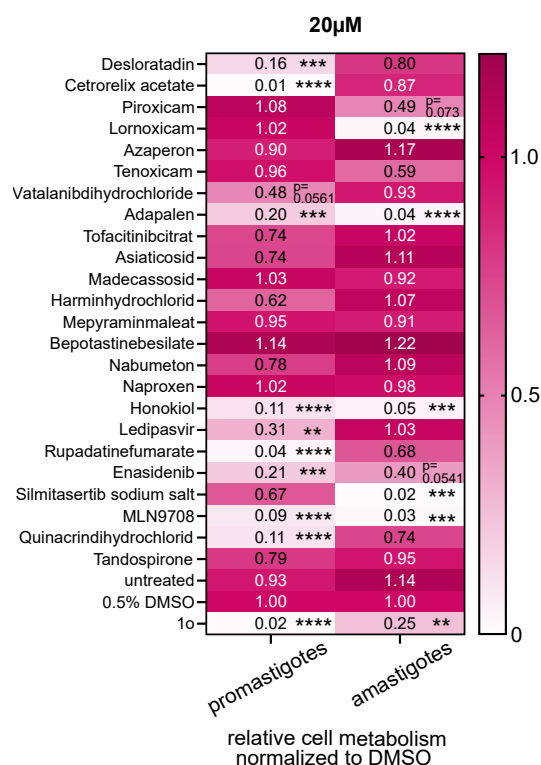


Figure 4.7: Hit identification on a single dose of repurposed compounds. - Effects of repurposed compounds identified in a pharmacophore model of arylmethylaminosteroids. Compounds were tested at a single dose of 20 μM for 72 hours, cellular metabolism rate was then assessed using an MTT assay and normalized to the 0.5% DMSO control. Data generated by Hicham Bouchrit. $n = 3$ (Pro); $n = 2$ to 3 (Ama), one-way ANOVA.

such an analysis might identify compounds that combine favorable antiparasitic activity while circumventing the disadvantages of the arylmethylaminosteroid class. Due to their ranking by a predicted EC_{50} on *Plasmodium*, 24 compounds were identified for testing in a phenotypic screening. Assuming a conserved arylmethylaminosteroid target, which is likely responsible for their broad antipathogenic activity, this set of compounds is also worth considering for leishmanicidal activity (152).

Thus, they were tested in a single-dose MTT-based screening on *L. major* promastigotes and axenic amastigotes to identify basal antileishmanial activity (Figure 4.7). Any compound that reduced relative metabolic activity of the parasites by at least 50%, compared to the carrier control, was designated as active. Still, activity below this threshold is also worth considering for pattern detection. The set consisted of highly diverse compounds, both in terms of chemical structure and mode of action

4.1 Phenotypic screening for antileishmanial compounds

in humans. However, some distinct accumulations of human targets and patterns of activity at 20 μ M were observed:

Among them were three anti-histamines of which two, Rupatadine and Desloratadine, exhibited a strong activity on promastigotes, whereas Bepostatine was inactive. The former two nearly eradicated metabolic rate of promastigotes, but amastigotes were only slightly affected.

The prostaglandin G/H synthase inhibitors Piroxicam, Tenoxicam and Lornoxicam share the oxicam scaffold and differ structurally by only a few atoms. Nevertheless, they showed a varying, predominantly amastigote-specific effects, ranging from about halved to no residual parasite metabolic activity after treatment with 20 μ M drug. In contrast, unrelated prostaglandin G/H synthase inhibitors exhibited a different pattern: Honokiol was active on both life stages, Nabumeton showed only minor activity against promastigotes. Looking further, quinacrine is well known for its antimalarial and antihelminthic activity. It also has antileishmanial effects, preferentially killing promastigotes (150).

Several tested compounds showed activity for either life stage. Applying a threshold for relative viability ≤ 0.5 considers compounds as active with an $EC_{50} \leq 20$ μ M. In consequence, 10 compounds exhibited leishmanicidal activity on promastigotes, 7 on axenic amastigotes, including 4 with activity on both, equaling to a 54.2% positive hit rate. According to their effects at 20 μ M on either life-stage, the 7 most potent compounds progressed to dose-response experiments on *L. major*, *L. donovani* and hMDM to assess toxicity against cutaneous and visceral *Leishmania* strains, as well as cytotoxicity on host cells (Figure 4.8, Figure 4.9). Based on non-linear regression of these dose-response experiments, EC_{50} on parasites and CC_{50} on macrophages are summarized in Table 4.2.

Cetrorelixacetate, a Gonadotropin-releasing hormone antagonist (196), exhibited exclusive activity on *L. major* promastigotes. Similarly, rupatadinefumarate, a histamine H1 antagonist (180), was toxic only to promastigotes of both species. Neither compounds had cytotoxic effects on hMDMs. Lornoxicam, one of the prostaglandin G/H synthase inhibitors (17), only effected *L. major* amastigotes and had no effect on hMDM viability. Adapalene, an agonist of the retinoic acid receptor, and silmitasertib, a casein kinase II inhibitor (129, 236), had moderate antileishmanial activity on promastigotes of both species but strong effects on *L. major* amastigotes. The proteasome $\beta 5$ inhibitor

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MLN9708 (116) demonstrated exceptional activity on *L. major* axenic amastigotes, promastigotes were also mildly susceptible. Those three compounds also induced notable cytotoxicity on hMDMs. Conversely, *L. major* promastigotes were more susceptible to Honokiol, a lignan isolated from *Magnolia* inhibiting prostaglandin G/H synthases (100), but it also killed amastigotes and *L. donovani* promastigotes to a lesser extent. Its cytotoxicity to macrophages was apparent only at very high concentrations. Overall, those repurposed compounds were slightly less effective against *L. donovani* promastigotes than against *L. major* promastigotes. A direct comparison of susceptibility of axenic amastigotes was not possible, as a stable *L. donovani* amastigote culture could not be established.

A selectivity index (SI) was calculated as the ratio of $\frac{Ama\ EC_{50}}{CC_{50}}$. In the context of *in vitro* antileishmanial drug research, a $SI \geq 5$ or 10, depending on the source, is considered as sufficient for progression to infection experiments (147, 193). Due to time constraints, data on the efficacy of these compounds against intracellular amastigotes were not available by the end of this project. At this point, several compounds demonstrated promising selectivity for *L. major* amastigotes over the human host macrophage, especially MLN9708, Adapalen and Lornoxicam, all with an $SI > 10$.

4.1 Phenotypic screening for antileishmanial compounds

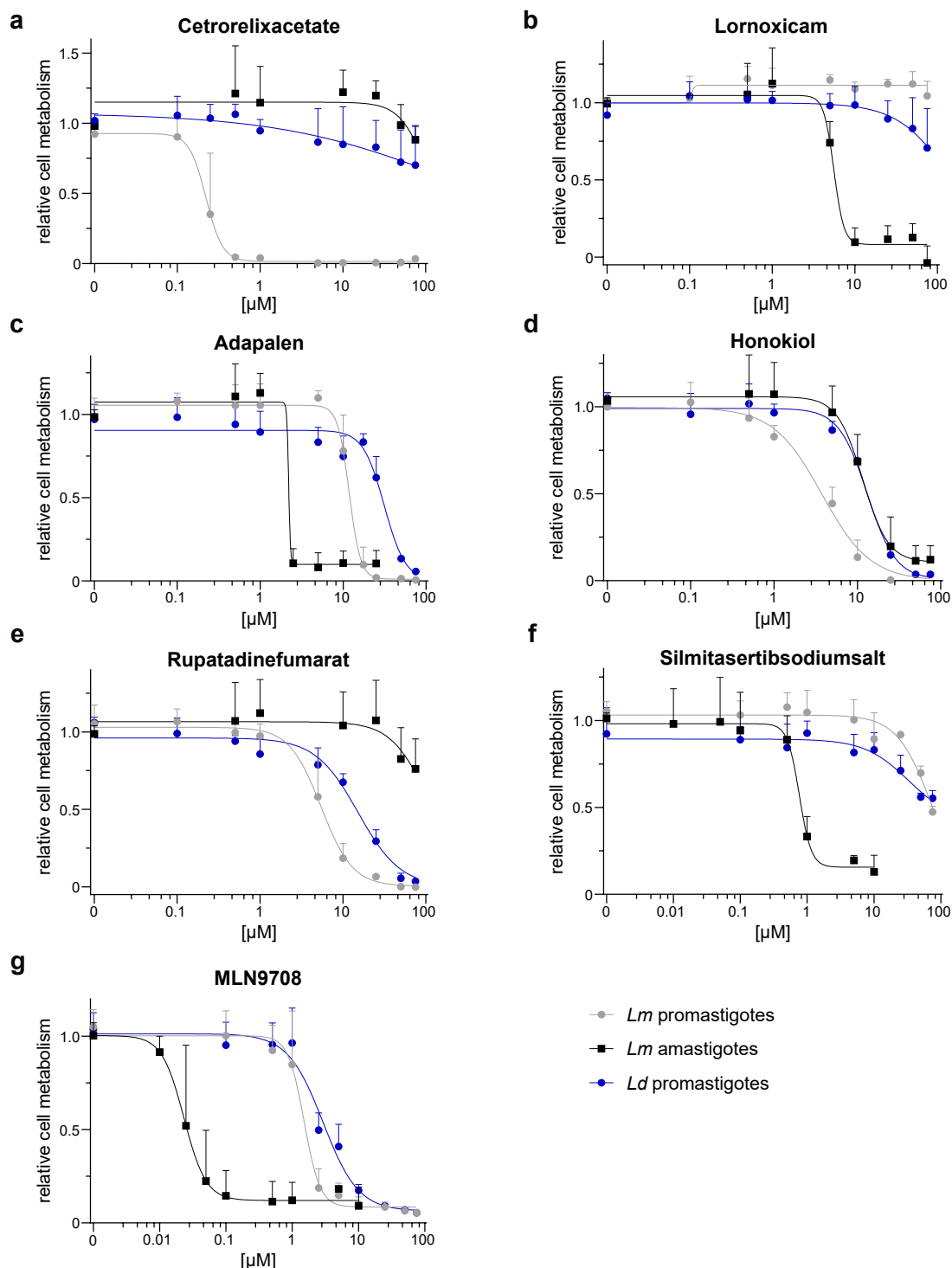


Figure 4.8: Dose-dependent antileishmanial activity of repurposed compounds.

- Dose-response curves of compounds derived from a pharmacophore model of arylmethylaminosteroids on the viability of *L. major* promastigotes, axenic amastigotes and *L. donovani* promastigotes after 72 hours of treatment: **a)** Cetorelixacetate, **b)** Lornoxicam, **c)** Adapalene, **d)** Honokiol, **e)** Rupatadinefumarate, **f)** Silmitasertib sodium salt, **g)** MLN9708; $n = 3$ (*L. major*), $n = 3$ to 5 (*L. donovani*), non-linear 4-parameter regression: solid line

4. RESULTS

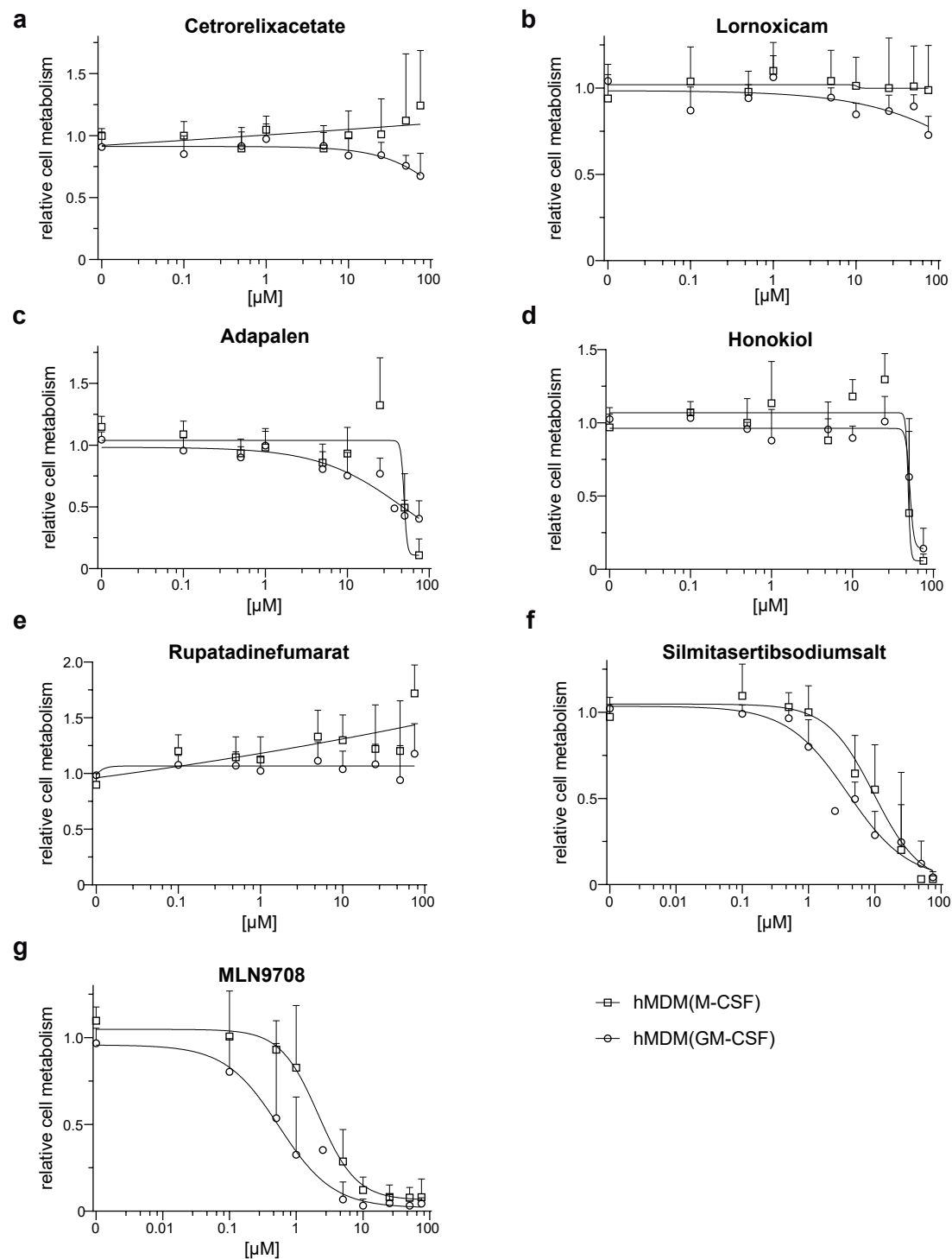


Figure 4.9: Cytotoxicity of repurposed compounds on human macrophages. - Dose-response curves of compounds derived from a pharmacophore model of arylmethylaminosteroids on the viability of hMDM differentiated either with GM-CSF or M-CSF after 72 hours of treatment: **a)** Cetorelixacetate, **b)** Lornoxicam, **c)** Adapalen, **d)** Honokiol, **e)** Rupatadinefumarat, **f)** Silmitasertibsodiumsalt, **g)** MLN9708; $n = 5$ to 7 donors in 3 to 4 independent experiments, non-linear 4-parameter regression: solid line

Table 4.2: Half-maximal effective or cytotoxic concentrations of pharmacophore-derived repurposed compounds on *Leishmania* and hMDM - Listed are EC_{50} on axenic *Leishmania* cultures and CC_{50} on human primary macrophages, differentiated with either GM-CSF or M-CSF, after 72 hours of treatment, calculated based on 4-parameter non-linear regression. $\pm$ 95% asymptotic confidence interval are stated if applicable. For graphs, see Figure 4.8 and Figure 4.9.

	EC_{50} <i>Lm</i> Pro [μ M]	EC_{50} <i>Lm</i> Ama [μ M]	EC_{50} <i>Ld</i> Pro [μ M]	CC_{50} hMDM (GM-CSF) [μ M]	CC_{50} hMDM (M-CSF) [μ M]	SI hMDM (GM-CSF)/ <i>Lm</i> Ama	SI hMDM (M-CSF)/ <i>Lm</i> Ama
Cetrorelix- acetate	0.22 ± 0.05	>75	>75	>75	>75	na	na
Lornoxicam	>75	5.57 ± 1.84	>75	>75	>75	>13.46	>13.46
Adapalene	11.8 ± 1.46	2.2	31.49 ± 9.65	49.24	49.28	22.41	22.35
Honokiol	3.75 ± 1.15	11.59 ± 3.34	13.12 ± 2.1	51.09	48.73	4.41	4.2
Rupatadine- fumarate	5.44 ± 1.07	>75	20.33 ± 14.17	>75	>75	na	na
Silmitasertib- sodiumsalt	>75	0.77 ± 0.21	>75	3.88 ± 2.2	9.74 ± 6.38	5.07	12.73
MLN9708	1.51 ± 0.4	0.023 ± 0.01	2.92 ± 0.85	0.55 ± 0.34	2.14 ± 1.11	23.73	92.22

4. RESULTS

4.1.3 Antileishmanial activity of pateamines

Pateamine A (PatA), a macrodiolide isolated from the marine sponge *Mycale hentscheli*, and its derivate desmethyl-desamino-PatA (DMDAPatA) are inhibitors of eukaryotic translation (36, 151, 156). Both compounds specifically inhibit the eukaryotic translation initiation factor 4A (eIF4A), an RNA helicase. By clamping RNA substrates onto the surface of eIF4A, they prevent correct helicase function. Opposing to other eIF4A inhibitors, PatA does not dependent on a polypurine stretch in the RNA or on a Phe163 residue (134, 171, 194). Pateamines are highly potent antivirals and their activity against *L. major* was proposed based on sequencing data of the interacting eIF4A amino acids, as *Leishmania* express all amino acids required for interaction. The presence of a serine, instead of a phenylalanine, at position 163 confers resistance to other eIF4A inhibitors but not to pateamines (Figure 4.10a)) (171, 194).

Table 4.3: EC₅₀ and CC₅₀ of pateamines PatA and DMDAPatA - Half-maximal efficacy of PatA and DMDAPatA on *L. major* and human primary macrophages differentiated with GM-CSF after 72 hours of treatment. Values were calculated based on a 4-parameter non-linear regression, $\pm$ 95% asymptotic confidence interval are stated if possible. For graphs, see Figure 4.10.

	EC ₅₀ PatA [μ M]	EC ₅₀ DMDAPatA [μ M]
<i>L. major</i> promastigotes	0.55 $\pm$ 0.25	12.57 $\pm$ 3.44
<i>L. major</i> amastigotes	42.05	32.3
	CC ₅₀ PatA [μ M]	CC ₅₀ DMDAPatA [μ M]
hMDM(GM-CSF)	0.0093	0.12 $\pm$ 0.06

Indeed, PatA exhibited pronounced activity against *L. major* promastigotes in an MTT assay, with an EC₅₀ of 0.55 μ M. However, PatA showed only modest activity against axenic amastigotes, as did DMDAPatA on both life cycle stages. In these cases, both pateamines started to reduce the parasites' metabolism at 25 to 50 μ M but induced no complete parasite killing at concentrations up to 75 μ M (Figure 4.10b-c, Table 4.3). This reduced efficacy against amastigotes was reproduced when promastigotes were cultivated and treated in medium at pH 5.5 (Figure 4.10d), suggesting that acidic pH influences inhibitor functionality. When treated with pateamines, human primary

4.1 Phenotypic screening for antileishmanial compounds

macrophages exhibited significant lethality with a higher predisposition for PatA and CC₅₀ values in the low nanomolar range (Figure 4.10e, Table 4.3).

Consequently, susceptibility of intracellular amastigotes could not be assessed, as host cell toxicity exceeded that against the parasite. In summary, PatA exhibited antileishmanial activity on *L. major* promastigotes, but effects on axenic amastigotes were limited to concentrations >50 µM. Similarly, DMDAPat only moderately reduced *L. major* metabolism at these high concentrations. Combined with the predominant cytotoxicity against hMDM, pateamines were not considered as promising antileishmanial candidates.

4. RESULTS

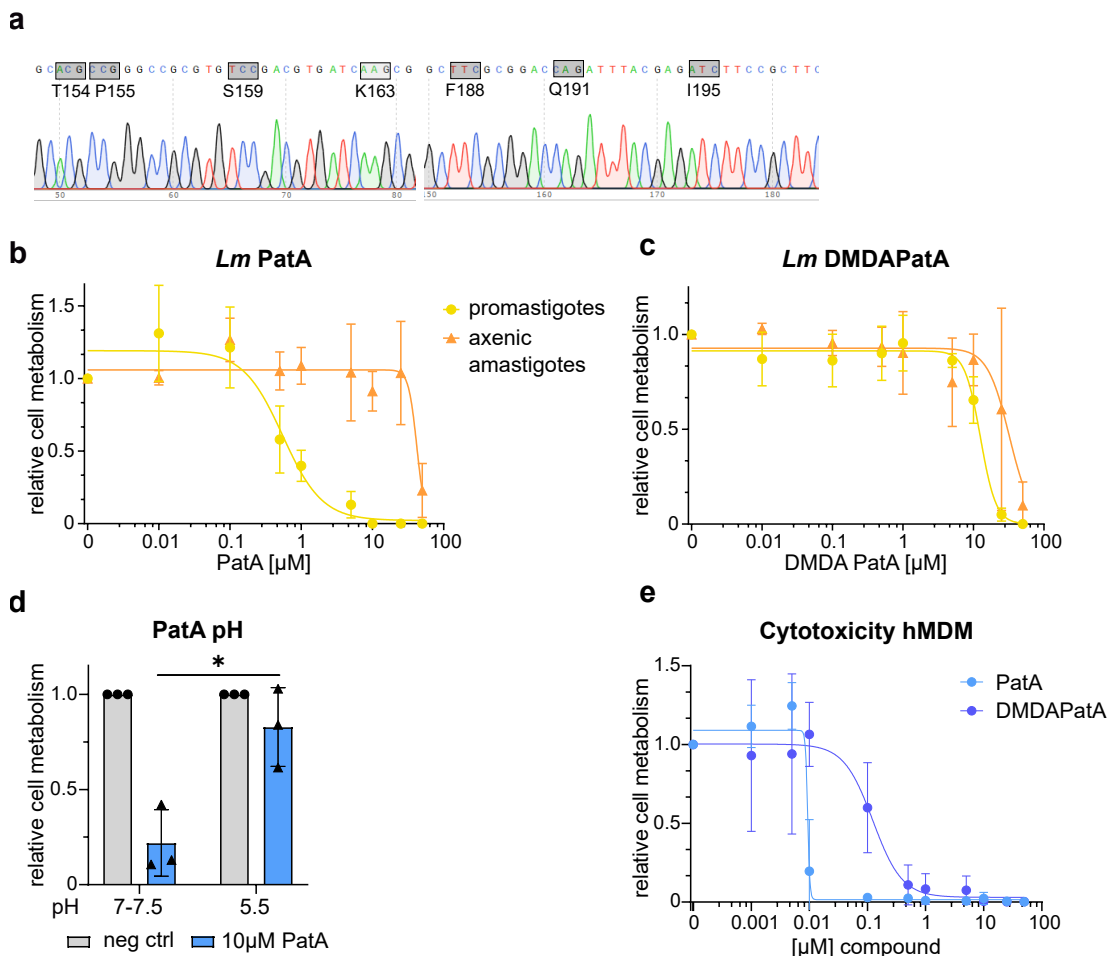


Figure 4.10: Leishmanicidal activities of pateamines PatA and DMDAPatA are pH sensitive - a) Sanger sequencing trace of the PatA binding pocket of *L. major* eIF4a, interacting amino acid codons are denoted in dark gray, position 163 in light gray (171, 194);

b) PatA and c) DMDAPatA were incubated with *L. major* promastigotes and axenic amastigotes for 72 hours at constant 0.75% DMSO, cellular metabolism rate was then assessed in an MTT assay. $n = 3$, non-linear 4-parameter regression: solid lines;

d) Activity of PatA on *L. major* promastigotes was tested at different pH, representing growth conditions of promastigotes and amastigotes. Cellular metabolism rate was assessed after 24 hours of treatment in an MTT assay, $n = 3$, Welch's t-test;

e) Cytotoxic effect of PatA and DMAPatA on hMDM(GM-CSF) at constant 0.75% DMSO. $n = 4$ donors from 2 independent experiments, non-linear 4-parameter regression: solid lines.

4.2 Identification of potential antileishmanial drug targets linked to parasite cell death or stress

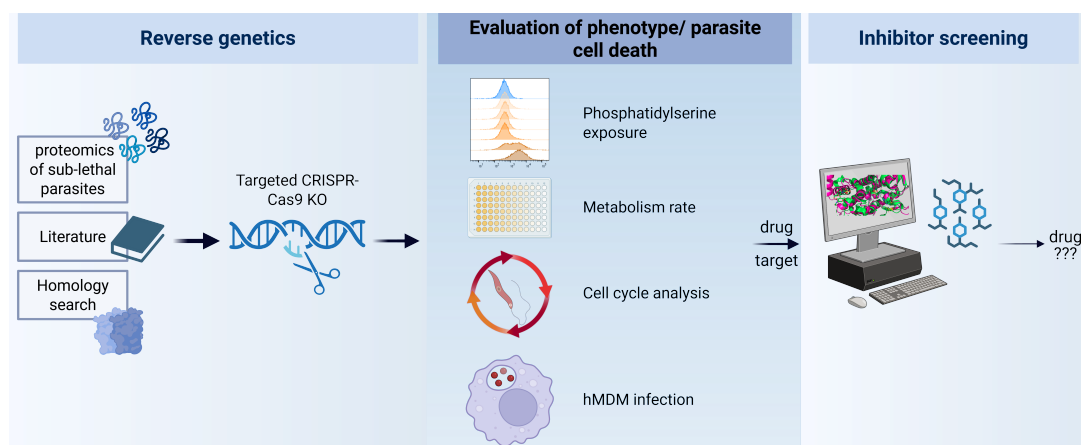


Figure 4.11: Workflow to study potential cell death-related proteins in *Leishmania* as druggable targets. - Proteins of interest were identified in a quantitative proteomics dataset of sub-lethal parasites, by literature research of homology searches. Respective genes were subjected to gene deletion. If mutants could be generated, they were assessed for a phenotype concerning cell death and infectivity on human macrophages. Proteins identified as potential drug targets undergo a virtual screening for specific inhibitors that can be tested as antileishmanial agents. Figure created with BioRender.

Since cell death and stress-related proteins are promising targets for drug development against *Leishmania*, a list of candidates was compiled to test their involvement in parasite cell death or stress responses and to evaluate their potential as drug targets. On the one hand, activation of central PCD regulators by small molecules can potentially induce antileishmanial effects. On the other hand, inhibition of the execution of certain PCD hallmarks, such as PtdSer exposure, might suppress immunosuppressive mechanisms during infection establishment.

For this project, proteins of interest were drawn from several sources: A quantitative proteomics study on sub-lethally stressed parasites, educated guesses from literature research, and predicted homology to human cell death domains. Their genes were targeted by CRISPR Cas9- or DiCre-mediated gene deletion and successfully established mutants were evaluated for cell death-related phenotypes in different assays. For a protein with established function, that represents a promising drug target, *in silico*

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inhibitor design was initiated to screen for potential antileishmanial compounds (Figure 4.11).

4.2.1 Phenotypic characterization of proteins putatively linked to *Leishmania* programmed cell death

To identify proteins potentially involved in programmed cell death of *Leishmania*, a subset of candidates was compiled in this project that might act as regulators or executioners. By knocking out these selected genes of interest in *L. major*, resulting deletion strains were evaluated for their phenotype relating different cell death hallmarks, depending on the putative function. These genes were identified either through homology comparisons to human apoptosis-related proteins or through literature findings. Table 4.4 summarizes the studied candidates, the success of knockout attempts, and experimentally observed phenotypes of the mutants.

The voltage-dependent anion channel (VDAC) is a conserved porin localized in the outer mitochondrial membrane and is associated with transport of ions and small uncharged molecules. In *Trypanosoma brucei*, it was described as only essential under growth conditions favoring oxidative phosphorylation as main energy source (214). Mammalian VDAC homologues regulate Ca^{2+} flux at the mitochondria-endoplasmic reticulum interface and for control of cytochrome c release, both are hallmarks of apoptosis (287). In *L. major*, the two genes *LmjF.02.0450* and *LmjF.02.0460* are annotated as putative VDACs, but no prior results on the functions of these channels were available. Expression of the latter gene is upregulated during metacyclogenesis in promastigotes Zitat (74). This life stage is associated with the occurrence of apoptotic-like parasites (254), highlighting these VDAC as interesting candidates for potential involvement in PCD.

After knocking out *LmjF.02.0460*, parasites did not show a growth deficiency under normal growth conditions, but further characterization concerning intracellular Ca^{2+} levels or cytochrome c localization was not conducted. A double knockout of both genes was not attempted within this project.

Homology of *LmjF.13.1460* (PCD-like 6) and *LmjF.28.1920* (PCD protein family 5) to parts of the mammalian apoptosis machinery was confirmed by HHpred with > 99.8%

4.2 Identification of potential antileishmanial drug targets

probability (101). Human PCD-like 6 is an EF-hand domain-containing protein promoting apoptosis through the caspase-3 dependent pathway (159). Programmed cell death 5 accelerates DNA damage-induced apoptosis in humans (281). The leishmanial homologue is also predicted by InterPro to bind DNA (205).

For these genes, knockout success in *L. major* varied. A heterozygous knockout, still retaining at least one copy of the *LmjF.28.1920* locus, was generated, but no complete deletion was attempted. A null mutant of *LmjF.13.1460* was established. However, neither of these knockouts were studied for a phenotype in this project.

As annotated by InterPro, *LmjF.34.0240* contains a CARD, a death fold motif characteristic for apoptotic signaling (125, 205). After complete deletion was successful, mutants were subjected to various cell death assays, including PtdSer exposure and DNA fragmentation after drug treatment or in the statPh. No phenotypes related to cell death hallmarks or growth deficiency were observed.

Exposure of PtdSer on the parasite membrane is a typical characteristic of apoptotic-like *Leishmania*, probably serving as apoptotic mimicry to be phagocytosed without eliciting an inflammatory response (97, 254, 267). Thus, parasites lacking the associated immune escape capacities could serve as attenuated vaccine strain. In *T. brucei*, PtdSer-synthase 2 (PSS) and PtdSer-decarboxylase (PSD) are the two enzymes directly connected to PtdSer biosynthesis through head exchange of phosphatidylethanolamine. In these trypanosomatids, PSD is not essential, localized in the mitochondria and its depletion disturbs ATP production and mitochondrial integrity (92). Attempts to delete close genetic homologues of both enzymes in *L. major* failed to generate null mutants. For both genes, inducible knockout attempts were also unsuccessful. In fact, any direct knockout approaches targeting PSS were lethal. Nevertheless, correct primer design was verified by generating a heterozygous knockout of the endogenous PSS locus when another gene copy was expressed from the SSU locus. Essentiality of PSS is supported by data from *T. brucei*, where PSS transferase activity is the only biosynthetic pathways for PtdSer synthesis (92).

Analyses of PSD functionality were only possible in heterozygous mutants, as any attempt to generate null mutants were lethal. For these heterozygous strains, no changes in PtdSer exposure were detected, although PSD mRNA levels were reduced to approximately half of the respective parental level. Measurements of ATP production or mitochondrial membrane potential were not conducted.

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Overall, the initial assessment of the involvement of these proteins in cell death was not encouraging enough to continue this approach. An intended genome-wide screening to identify genes related to PtdSer exposure using random sgRNA sequences for generation of a CRISPR-Cas9-mediated knockout library was abolished, as no feasible methodology could be established. Instead, such a screening was conducted in cooperation with Tom Beneke and Jorge Arias del Angel (University Würzburg) using DNA editing to introduce premature stop codons in 85.8% of the protein-coding genome of *L. major*, but these preliminary results are not discussed in this thesis (84).

4.2 Identification of potential antileishmanial drug targets

Table 4.4: Attempted knockout generation and phenotypes of proteins hypothesized as linked to programmed cell death in *L. major* - CARD: Caspase recruitment domain; PtdSer: phosphatidylserine; (i)KO: (induced) knockout; PCD: Programmed cell death; VDAC: Voltage-dependent anion channel.

Gene	Annotation	Rational	Knockout attempt	Phenotype
<i>LmjF.02.0450</i> ; <i>LmjF.02.0460</i>	VDAC in outer mitochondrial membrane	Literature	<i>0450</i> : not determined <i>0460</i> : null mutant	No growth deficiency
<i>LmjF.13.1460</i>	PCD-like 6	Homology	Null mutant	Not determined
<i>LmjF.14.1200</i>	Phosphatidylserine-synthase	Literature	Direct KO lethal, iKO failed, facilitated KO with genetic complementation: heterozygous	-
<i>LmjF.28.1920</i>	PCD protein family 5	Homology	Heterozygous	Not determined
<i>LmjF.34.0240</i>	CARD domain-like protein	Homology	Null mutant	No growth deficiency PtdSer exposure: negative DNA fragmentation: negative
<i>LmjF.35.4590</i>	Phosphatidylserine-decarboxylase	Literature	Heterozygous, iKO failed	Heterozygous: reduced mRNA levels, growth deficiency, PtdSer exposure: negative

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4.2.2 Relevance of p1/s1, an ecto-3'-nucleotidase/nuclease, in *L. major* cell death and host-pathogen interactions

4.2.2.1 3'-nucleotidase/nuclease activity is increased in *L. major* statPh promastigotes

Table 4.5: Proteins exclusively detected or enriched under sub-lethal cell death pressure in a quantitative proteomics analysis of *L. major* - Intersection of all comparisons from Figure 4.12. Fold changes and *p*-values are listed in Section 5.4.

UniProt code	UniProt annotation	Exclusive	Enriched
Q4Q7F3	p1/s1 nuclease	x	
Q4Q165	Putative polyubiquitin	x	
Q4QEI8	Elongation factor 1 α	x	
E9AEM1	Arrestin-like N-terminal domain-containing protein	x	
Q4QDQ9	Mitochondrial carrier protein		x
Q4QF48	EF-hand domain-containing protein		x
Q4Q6D6	p-glycoprotein e		x
P48157	Large ribosomal subunit protein uL5		x
Q4QHI2	Putative folate/biopterin transporter		x

In this part of the project, the aim was to identify proteins induced or enriched in stress conditions eventually leading to cell death in *L. major*. Such proteins may play a role in cell death induction, execution, or survival mechanisms and thus are of interest to better understand these cellular processes and identify novel drug targets. By focusing on sub-lethal conditions, early responses of the parasites to stimuli otherwise inducing an apoptotic-like cell death could be observed.

To quantify protein abundance in such sub-lethally stressed *L. major* promastigotes, a quantitative proteomics approach, conducted by Stefan Tenzer and Ute Distler of the Core Facility for Mass Spectrometry at the Johannes Gutenberg University Mainz, was utilized. This dataset was previously generated in (28). This stress was either extrinsically induced in promastigotes by 3 hours of treatment with miltefosine, an

4.2 Identification of potential antileishmanial drug targets

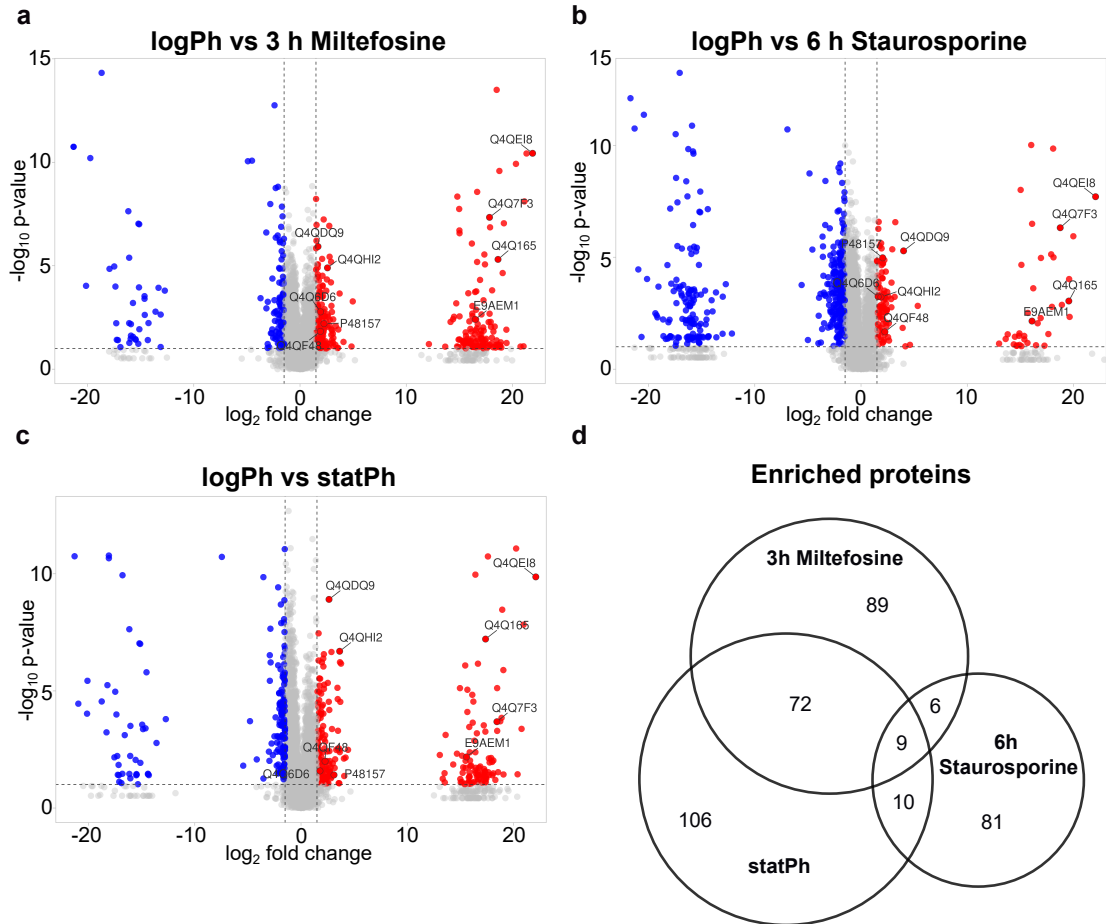


Figure 4.12: Quantitative proteomics to compare protein levels of pre-cell death stressed *L. major* - Vulcano plots comparing quantified protein levels of promastigotes treated with **a)** 25 μ M miltefosine for 3 hours, treated with **b)** 25 μ M staurosporine for 6 hours, or **c)** statPh promastigotes to untreated logPh promastigotes. Plots visualized with (110). Denoted are the nine commonly enriched hits, see Table 4.5. Cut-offs: $\log_2$ fold change > 1.5 , < -1.5 ; $p \leq 0.01$.

d) Venn diagram illustrating intersections of enriched proteins between the different comparisons a)-c), template created using (128).

Data from $n = 7$ of consecutive passages, measured in three technical repeats. Reanalysis of data by Tanja Ziesmann, originally described in (28), data generated by Stefan Tenzer and Ute Distler.

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antileishmanial drug, or 6 hours of treatment with staurosporine, a non-specific kinase inhibitor and known apoptosis inducer (31, 203). Intrinsic cell death induction was represented by statPh promastigotes, which consist of about 50% apoptotic-like parasites (28, 254). Each treatment was compared to an untreated logPh culture of viable promastigotes. In total, 4116 proteins were detected across those three comparisons, equivalent to 51.73% of the *L. major* reference proteome.

Reanalysis of these data by Tanja Ziesmann (Johannes-Gutenberg University Mainz) revealed that each comparison included distinct sets of proteins either exclusively detected in the control or sub-lethal sample and proteins with differentially regulated levels (Figure 4.12a-c). Focusing on proteins enriched in or exclusively present under stress conditions, nine hits were shared by all comparisons (Figure 4.12d). This intersection likely includes only proteins participating in drug-unspecific responses, since drug-specific hits are found only in their respective comparisons. Four of these proteins were undetectable in the control logPh sample, only in sub-lethally stressed parasites or in the statPh. Five proteins were more abundant upon stress induction (Table 4.5). Among these hits, several proteins are involved in protein biosynthesis, turnover, and cellular transport, whereas one enzyme with a distinct predicted function was detected only in treated or statPh promastigotes: p1/s1, annotated as 3'-nucleotidase/nuclease. Peptides annotated to this protein potentially derived from six genes arranged in an array on chromosome 30 of the *L. major* genome (Figure 4.13a). All these genes share a conserved C-terminus, but differ in their predicted localization signal: Copies *LmjF.30.1460-90* encode a putative transmembrane domain (TMD), whereas copies *LmjF.30.1500-10* an N-terminal signal sequence for secretion. Consistently, when a C-terminally His₆ tagged *LmjF.30.1500* was expressed from the SSU locus, this tag was detected only in the parasite culture supernatant and not in parasite lysate, suggesting secretion of p1/s1 (Figure 4.13b). Localization of proteins encoded by *LmjF.30.1460-90* could not be verified, as genomic integration of His₆ tagged constructs failed in multiple attempts.

p1/s1 is classified as a member of the class I nuclease family. Such proteins are typically expressed in a stage-specific manner (90, 138). Our proteomics dataset suggested expression in statPh promastigotes and amastigotes, but not in logPh promastigotes (28). rt-qPCR of *LmjF.30.1460-90* and *LmjF.30.1500-10* did not indicate highly significant changes in mRNA levels between the different life-stages. Only a minor trend of

4.2 Identification of potential antileishmanial drug targets

a 1.5-fold mRNA enrichment in statPh promastigotes and amastigotes was observed, suggesting a post-transcriptional or translational regulation of gene expression (Figure 4.13c).

As predicted ectoenzyme with 3'-nucleotidases/nuclease activity, p1/s1 is crucial for the parasite's purine salvage by degrading extracellular 3'-nucleotides to nucleosides and by hydrolyzing nucleic acids (see Figure 1.3). The former can be quantified by the release of P_i from 3'-AMP, as catalyzed by intact parasites, or their respective supernatant containing secreted proteins. In line with observed p1/s1 protein levels, this activity increased from logPh to statPh promastigotes in both fractions. On the parasite pellet, 3'-nucleotidase activity increased from about 30 to 80 $\mu\text{M } P_i/\text{h}/2 \times 10^6$ *L. major*, in the supernatant, it doubled from ≈ 10 to 20 $\mu\text{M } P_i/\text{h}/2 \times 10^6$ *L. major*. The latter likely derived from the secretion of p1/s1, as observed in Figure 4.13b. Surprisingly, no activity was detected on axenic amastigotes (Figure 4.14a), despite the assumed expression of p1/s1 based on proteomics data (28).

To investigate whether increased 3'-nucleotidase activity in the statPh originated from viable or apoptotic-like parasites, statPh promastigotes were separated based on PtdSer positivity. However, both fractions hydrolyzed 3'-AMP to similar extent (Figure 4.14b). Thus active 3'-nucleotidases/nucleases are present on PtdSer positive and negative parasites.

As trypanosomatids are purine auxotrophs, they rely on the uptake of adenosine from their environment. 3'-nucleotidases, such as p1/s1, as well as 5'-nucleotidases can serve as sources of extracellular adenosine. Nonetheless, *L. major* apparently relies primarily on 3'-nucleotidases, as all life stages exhibited very low 5'-nucleotidase activity (Figure 4.14c).

Alongside the distinct nucleotidase activity pattern observed on promastigotes, *L. major* shows a highly similar trend in endonuclease activity (Figure 4.14d-e). This enzymatic activity was semi-quantified as the degradation of dsM13 phage genome in an experimental setup analogous to 3'-nucleotidase activity measurements. Quantification was only possible relative to the control incubated without parasites. In the pellet fraction, endonuclease activity increased from ≈ 0.7 on logPh promastigotes to 1 in statPh promastigotes. In the latter samples, no distinct bands of the substrate DNA were detected post incubation with parasites. In the supernatant, activity from logPh to

4. RESULTS

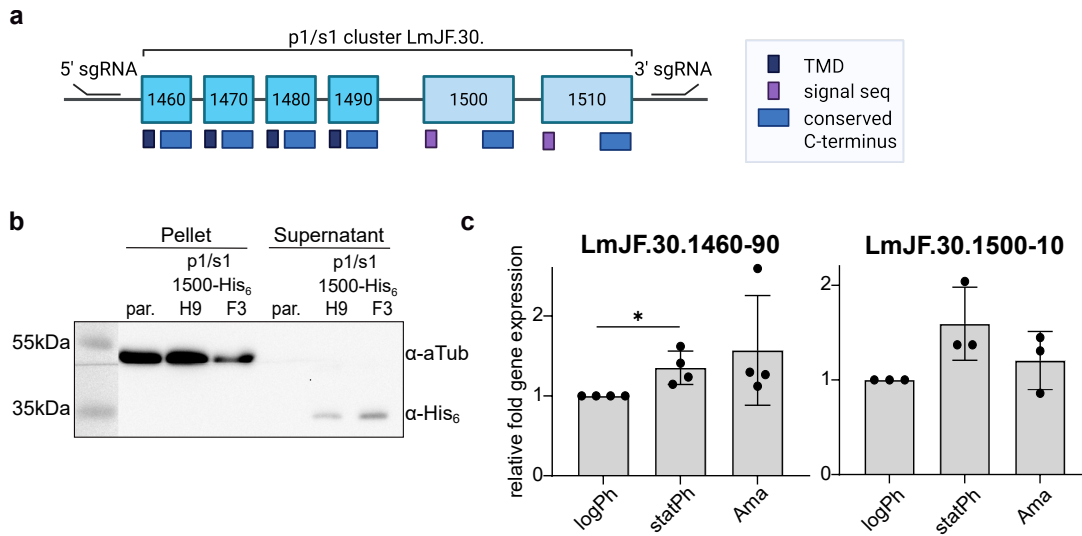


Figure 4.13: p1/s1 of *L. major* is encoded in a genomic cluster, secreted from promastigotes, but not differentially transcribed - **a)** Scheme of the genomic *p1/s1* cluster on chromosome 30, consisting of six genes 1460 - 1510. TMD: Transmembrane domain, sgRNA binding sites are marked. **b)** Western blot detection of His₆ tagged proteins in *L. major*^{LmJF.30.1500-His} lysate and culture supernatant enriched over Ni-NTA beads, compared to a parental strain. Representative blot of *n* = 3. **c)** rt-qPCR to determine relative fold gene expression of *LmjF.30.1460-1490* and *LmjF.30.1500-10* in logPh, statPh and axenic amastigotes. *n* = 3.

4.2 Identification of potential antileishmanial drug targets

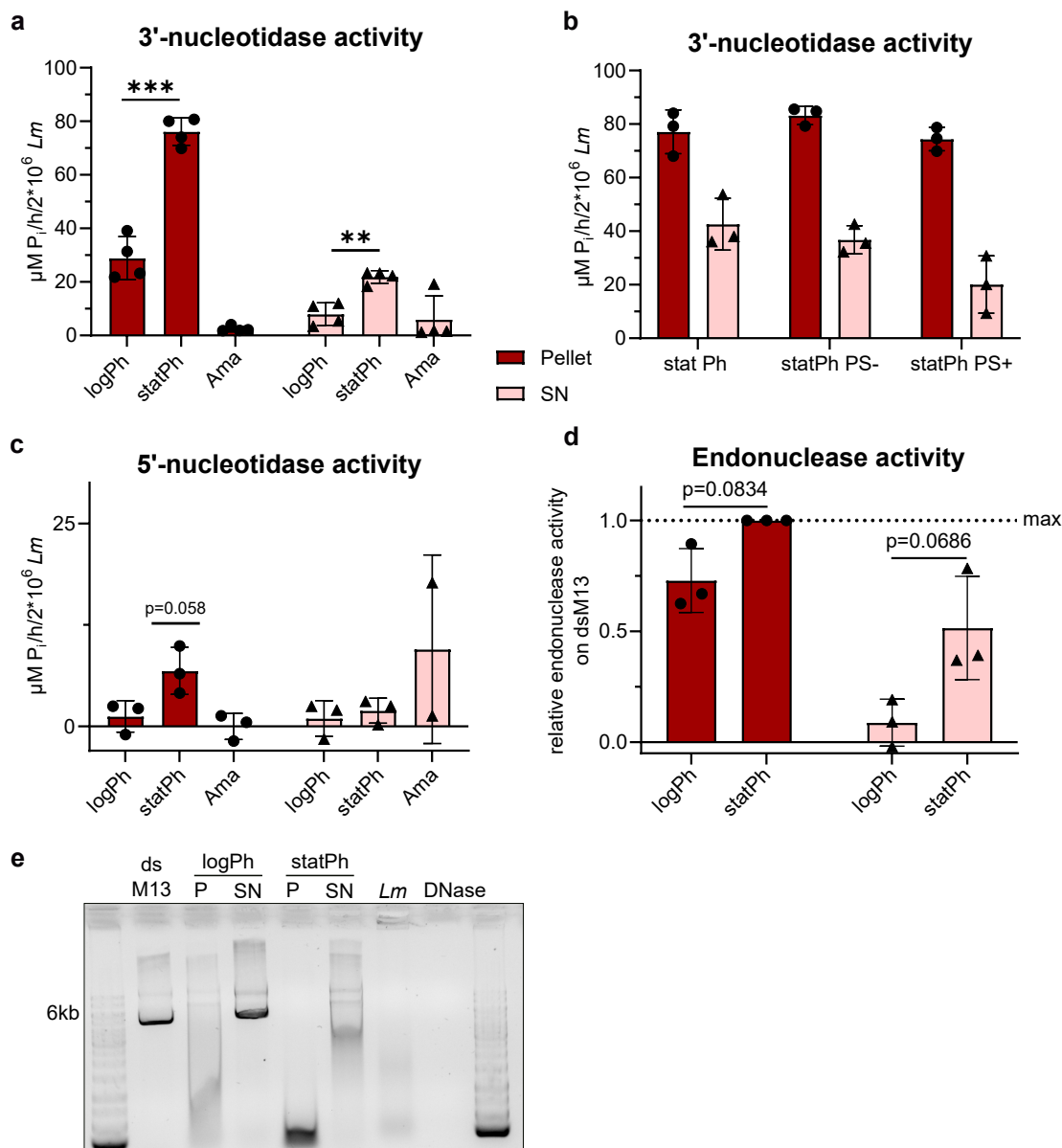


Figure 4.14: *L. major* exhibits increased ecto-3'-nucleotidase and -endonuclease activity in the statPh - a) Ecto-3'-nucleotidase activity was determined by release of P_i from 100 μ M 3'-AMP on intact *L. major*. Pellet and supernatant were separated, 3'-AMP was added for 1 hour and P_i detected using a malachite green assay, $n = 4$, Welch's t-test. b) 3'-nucleotidase activity after MACS of statPh promastigotes for PtdSer exposure, $n = 3$. c) 5'-nucleotidase activity was determined as in a) with 5'-AMP as substrate, $n = 3$. d) Ecto-endonuclease activity was evaluated by degradation of 500 ng circular, double-stranded M13 (dsM13) phage genome on intact *L. major*. Pellet and supernatant were separated, dsM13 was added for 1 hour, degradation visualized on an 0.8% agarose gel and semi-quantified using ImageJ, $n = 3$, Welch's t-test. e) Representative gel of d), controls: Without substrate (*Lm*), DNase + dsM13 (DNase).

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statPh promastigotes increased from ≈ 0.1 to 0.5. Activity on axenic amastigotes was not tested due to the absence of 3'-nucleotidase activity in this life stage.

When comparing the sequences of proteins derived from the *p1/s1* cluster to a close homologue in *L. donovani*, only the two copies *LmjF.30.1500-10*, which carry a signal sequence, possess the five domains participating in the 3'-nucleotidases/nuclease active site. In contrast, *LmjF.30.1460-90* lack these, suggesting these proteins cannot catalyze both functions, also see Figure 4.27 (138). As they still are annotated by similarity to class I nucleases, a residual nuclease activity may still be present (158). Beyond *p1/s1*, three additional proteins of *L. major* contain the characteristic 3'-nucleotidases/nuclease domains and thus could act redundantly to *p1/s1*. These are encoded by *LmjF.12.0400*, *LmjF.31.2300*, and *LmjF.31.2310*. The presence of several 3'-nucleotidases/nucleases on distinct chromosomes may indicate high functional importance to the parasite.

4.2.2.2 Anti-inflammatory effect of *L. major* ecto-3'-nucleotidase activity during hMDM infection

Generation of free adenosine by promastigotes of *L. major* not only facilitate purine salvage for the parasites. Additionally, extracellular adenosine can also act as anti-inflammatory immune modulator during early stages of infection, before parasites are phagocytosed. Adenosine mediates anti-inflammatory signaling through membrane receptors termed A_{2a} and A_{2b} (121). To assess expression levels of these receptors on human macrophages, both surface markers were stained on macrophages of different polarization and activation states. Thereby, different phenotypes along the pro- to anti-inflammatory macrophage spectrum were represented. First, both antibodies were titrated to optimize staining resolution. 1 μ g antibody per 1×10^6 cells was set as concentration with the best antibody quantity to staining index ratio not using excessive amounts of antibody (Figure 4.15a-c). Second, the percentage of A_{2a} and A_{2b} positive macrophages was quantified. hMDM(GM-CSF+IFN γ) were used for further experiments, as they showed the highest and most consistent expression of both adenosine receptors. Thus, these cells are expected to be sensitive towards immunosuppressive effects of extracellular adenosine. This phenotype represents pro-inflammatory M1-like macrophages. High inter-donor variability was observed for both receptors. Overall,

4.2 Identification of potential antileishmanial drug targets

the share of A_{2a} positive cells ranged from 75% among hMDM(GM-CSF+IFN γ) to 39% for hMDM(GM-CSF+LPS). A_{2b} positivity varied greatly, from 13% in hMDM(M-CSF) to 82% in hMDM(GM-CSF+IFN γ).

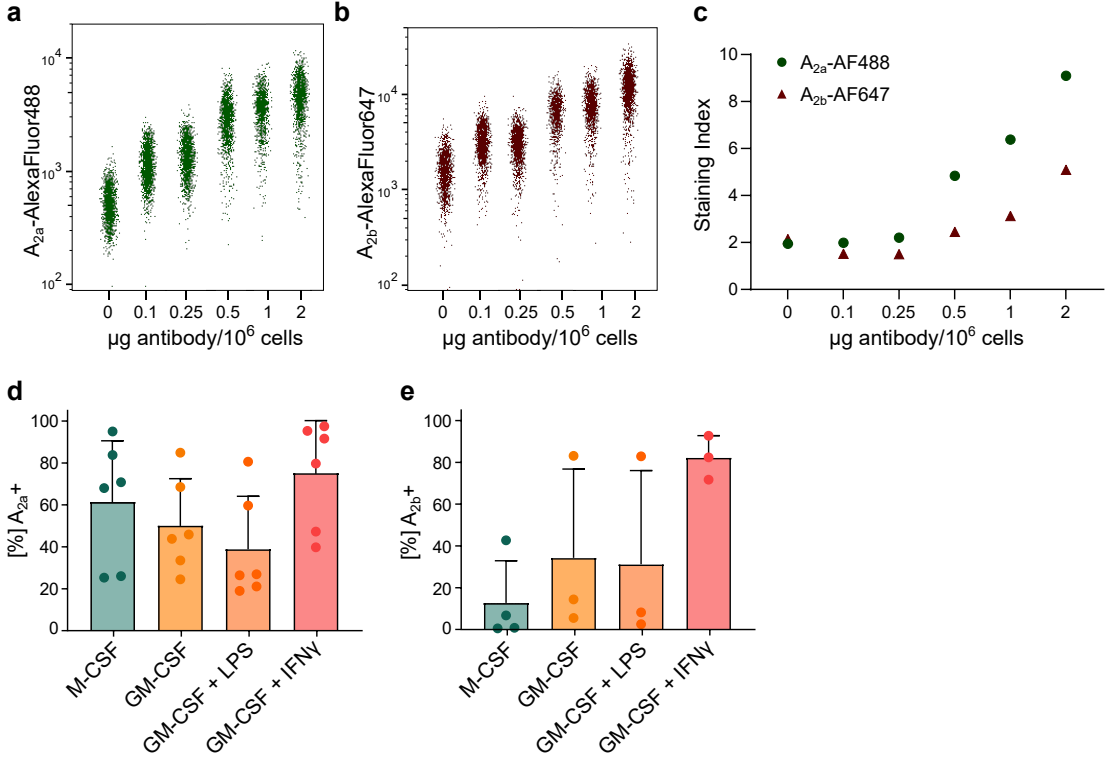


Figure 4.15: hMDM(GM-CSF+IFN γ) have the highest expression levels of adenosine receptors A_{2a} and A_{2b} - a) A_{2a} and b) A_{2b} antibody titration on hMDM(GM-CSF+IFN γ) to determine the final concentration for staining.

c) Staining index of A_{2a} and A_{2b} antibody titration on hMDM(GM-CSF+IFN γ), calculated as $\frac{\text{MFI}_{\text{pos}} - \text{MFI}_{\text{neg cells}}}{2 \text{ standard deviation}}$. A higher staining index suggests better resolution of stained vs unstained cells.

d) A_{2a} and **e)** A_{2b} expression on hMDM of different differentiation and activation states. $n = 3$ to 6 donors in 2 to 3 independent experiments, Welsh's t-test.

To test the effects of adenosine during *L. major*^{DsRed} infection, hMDM(GM-CSF+IFN γ) were infected with statPh promastigotes, with the addition of adenosine or 3'-AMP at increasing concentrations. Adenosine served as positive control to verify the outcomes of A_{2a} and A_{2b} activation, 3'-AMP was added as 3'-nucleotidase substrate, which is degraded to adenosine. An infected condition without additives served as basal control.

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No effects were observed 24 hours pi, neither on parasite burden nor on adenosine receptor expression levels (Figure 4.16a-d). However, secretion of $\text{TNF}\alpha$, a highly pro-inflammatory cytokine, decreased dose-dependently upon addition of adenosine, as expected from literature. With 100 μM adenosine, concentration of $\text{TNF}\alpha$ in the culture supernatant corresponded to that of the uninfected control. Suppression of $\text{TNF}\alpha$ release is a hallmark of A_{2a} and A_{2b} activation (119). Addition of 3'-AMP partly mirrored this observation. Release of this cytokine was greatly induced by infection with *L. major*, but adenosine or 3'-AMP had no effects on $\text{TNF}\alpha$ secretion in uninfected controls (Figure 4.16e-f). High donor variation made absolute values difficult to interpret, thus, normalization to the untreated, infected control was also performed.

Previously, we established a link between $\text{TNF}\alpha$ levels and PBL proliferation during *L. major* infection (12). As the majority of PBLs are T cells, their proliferation is critical for initiating an adaptive immune response. Thus, it was investigated whether the presence of adenosine or 3'-AMP during infection affects PBL proliferation, by employing a hMDM:PBL co-culture model.

Four days pi, a trend toward a higher parasite burden was now visible when adenosine or 3'-AMP were present (Figure 4.17a). This effect was not observed earlier when macrophages were infected without PBLs, suggesting that it likely depends on the presence of T cells. PBL proliferation was induced in response to *L. major* infection, from <5% in uninfected samples to 15% in infected samples, on average. However, proliferation decreased again with adenosine and 3'-AMP (Figure 4.17b-c). Consistent with the results of the preceding experiments, a decrease in $\text{TNF}\alpha$ secretion was observed for adenosine and 3'-AMP treated conditions four days pi, in both absolute and relative terms (Figure 4.17d-e). Still, these additives reduced $\text{TNF}\alpha$ levels only to $\approx 70\%$ of the infected, untreated control. The suppressive effects of A_2 receptor signaling likely diminish over time. All observed effects were infection-dependent and absent in uninfected controls.

When analyzing the correlation between relative PBL proliferation to $\text{TNF}\alpha$ secretion ($r = 0.33$) or parasite burden ($r = -0.4$), moderate correlations were found for both comparisons (Figure 4.17f-g). This suggests that additional factors induced by adenosine signaling may also contribute to the observed effects. In conclusion, ecto-3'-nucleotidase activity of *L. major* suppressed $\text{TNF}\alpha$ secretion and PBL proliferation during infection by generating extracellular adenosine from 3'-AMP.

4.2 Identification of potential antileishmanial drug targets

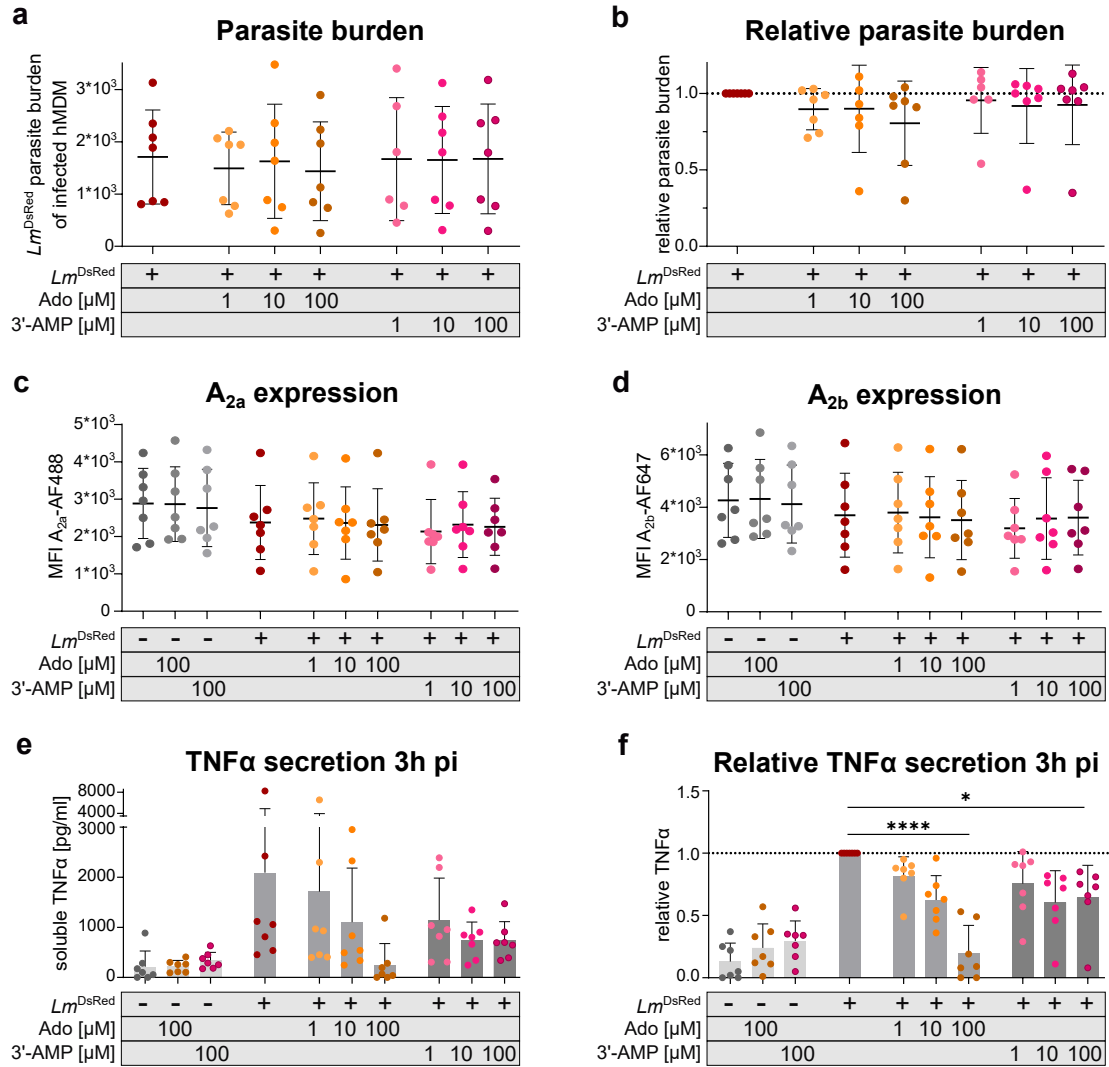


Figure 4.16: Extracellular adenosine or 3'-AMP dose-dependently decrease TNF α secretion during hMDM infection - a) Parasite burden expressed as DsRed MFI of hMDM(GM-CSF+IFN γ) infected with *L. major*^{DsRed} for 24 h in presence of adenosine (Ado) or 3'-AMP, b) normalized to the infected, untreated sample. c) A_{2a} and d) A_{2b} expression of hMDM(GM-CSF+IFN γ), infected in presence of adenosine (Ado) or 3'-AMP. e) TNF α levels in supernatants of hMDM(GM-CSF+IFN γ) 3 hours pi with *L. major*^{DsRed} in the presence of adenosine (Ado) or 3'-AMP, f) normalized to the infected, untreated sample.

$n = 7$ donors in 4 independent experiments, Welch's t-test

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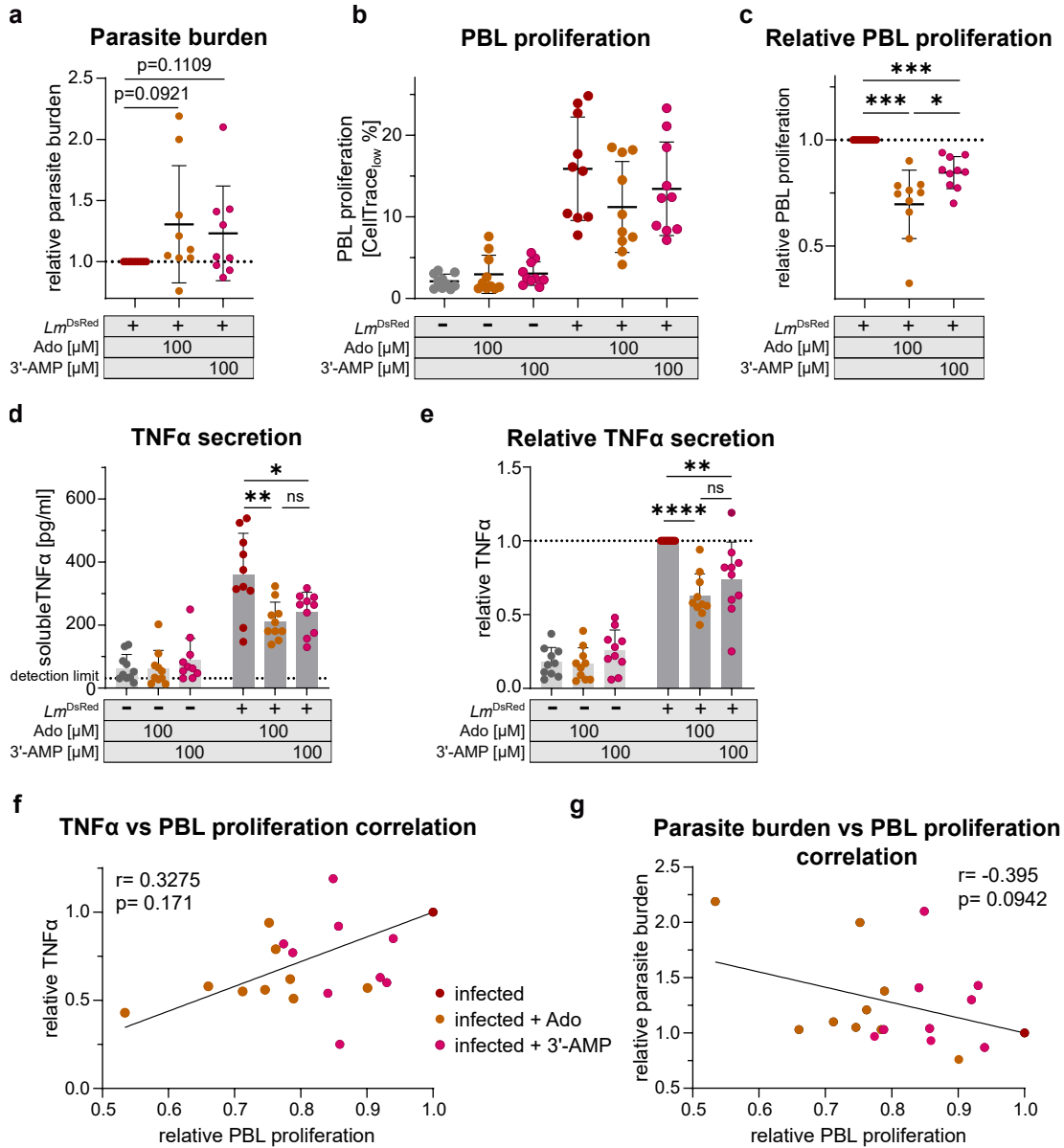


Figure 4.17: Extracellular adenosine and 3'-AMP decrease PBL proliferation during infection of a hMDM:PBL co-culture - A hMDM(GM-CSF+IFN γ):PBL co-culture was infected with *L. major*^{DsrEd} at an MOI = 20, in the presence of either 100 μ M adenosine or 3'-AMP, and analyzed four days pi:

a) Relative parasite burden of infected macrophages, normalized to the infected, untreated sample.

b) PBL proliferation expressed as CellTrace_{low} PBLs, c) normalized to the infected, untreated sample.

d) Soluble TNF α in the supernatant, e) normalized to the infected, untreated sample.

f) Pearsons correlation of PBL proliferation versus TNF α secretion or g) parasite burden versus PBL proliferation, linear regression: solid line with origin 1, 1.

$n = 9$ to 10 donors in 4 independent experiments, Welch's t-test.

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4.2.2.3 *L. major*^{Δ*p1/s1*} are viable upon inducible knockout induction and have reduced 3'-nucleotidase/nuclease activity

Acquisition of extracellular purines is essential for *Leishmania*, as they cannot synthesize these metabolites *de novo* (117, 174). In *L. major*, 3'-nucleotidase activity is the primary nucleotidase activity for generation of extracellular adenosine, which likely derives from *p1/s1* (Section 4.2.2.1). To determine whether *p1/s1* is essential for *L. major*, it was attempted to remove the full 11.2 kb of the *LmjF.30.1460-1510* cluster by applying CRISPR-Cas9 mediated gene deletion (27).

Table 4.6: Knockout attempts to delete the *p1/s1* locus - Several approaches were undertaken to delete the *LmjF.30.1460-1510* cluster. Heterozygous strains were used for a sequential round of deletion using an additional selection marker. Facilitated deletions were conducted either by regular addition of 100 μM adenosine to the medium or genetically by attempting *p1/s1* deletion during expression of a *LmjF.30.1500-His₆* construct integrated into the SSU locus. Only a DiCre-mediated inducible recombination successfully led to *p1/s1* null mutant strains.

Deletion approach	Details	Result
Sequential deletion of alleles	$n = 6$	heterozygous knockout or lethal
Facilitated, sequential deletion by nutritional complementation	$n = 3$, addition of 100 μM adenosine	heterozygous knockout or lethal
Facilitated, sequential deletion by genetic complementation	$n = 3$, in presence of genomically integrated <i>LmjF.50.1500-His</i> construct	heterozygous knockout or lethal
Inducible recombination	DiCre-mediated	two <i>p1/s1</i> null mutant strains

Unique sgRNA binding sites up- and downstream of the cluster are indicated in Figure 4.14. Targeting single copies within the cluster was infeasible due to the high homology among the genes and intergenic regions. However, direct *p1/s1* knockout attempts only yielded heterozygous strains or were lethal in sequential knockout trials. Successful replacement of at least one allele of this locus proved basic technical feasi-

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bility of the approach. Similarly, facilitated approaches, either by metabolic or genetic complementation, did not result in *p1/s1* null mutants (Table 4.6).

However, an inducible approach employing DiCre-mediated recombination successfully deleted the *p1/s1* locus, despite using the same sgRNA binding sites and homologous recombination sequences as in previous deletion attempts. Thus, initial knockout attempts did not fail due to erroneous oligonucleotide design.

For the inducible approach, a modified method as previously described in (81), was used. Two flox cassettes were sequentially integrated upstream and downstream of the *p1/s1* gene locus in an *L. major* strain expressing Cas9, T7 RNA polymerase, and DiCre. Each cassette contained a loxP site, a resistance marker and a fluorescence marker. Integration was verified by measuring the fluorescence signal. Dimerization of Cre was induced by addition of rapamycin and resulted in recombination of the genetic sequence between both loxP sites (see Figure 1.2).

As the *eGFP* gene shall also be lost during recombination, loss of this signal can be used to trace successful deletion. This sequential deprivation was observed in two *L. major*^{*p1/s1* full floxed} clones (E10 and F4) for up to 9 days post rapamycin addition (Figure 4.18a-b). To establish a clonal cell line, these induced knockouts (iKOs) were subjected to a serial dilution. In all resulting single-cell clones, eGFP signal was below the threshold set for an eGFP negative control strain. Likewise, a diagnostic PCR showed loss of the eGFP and *LmjF.30.1500-10* loci in most single cell clones. Clones marked in gray and were used for further experiments and denoted as *L. major* ^{$\Delta p1/s1$} (Figure 4.18c-f). Based on iKO clone E10, an untagged *LmjF.30.1500* construct was ectopically integrated into the parasite's SSU locus to yield an addback strain. When grown under the same conditions, density of both *L. major* ^{$\Delta p1/s1$} clones was reduced by half in the logPh and statPh, whereas the addback strain exhibited partially recovered growth (Figure 4.19).

Subjecting *p1/s1* null mutants to 3'-nucleotidase/nuclease assays revealed comparable effects of both knockout strains on all tested functions. Whether on 3'-nucleotidase or endonuclease activity on dsM13, *L. major* ^{$\Delta p1/s1$} showed about half the activity of the parental strain. By contrast, the addback strain behaved similarly to the parental strain (Figure 4.20a-c).

Endonuclease activity was also tested over time on a single-stranded substrate revealing delayed turnover in the absence of *p1/s1* (Figure 4.20d-f). A substrate preference for

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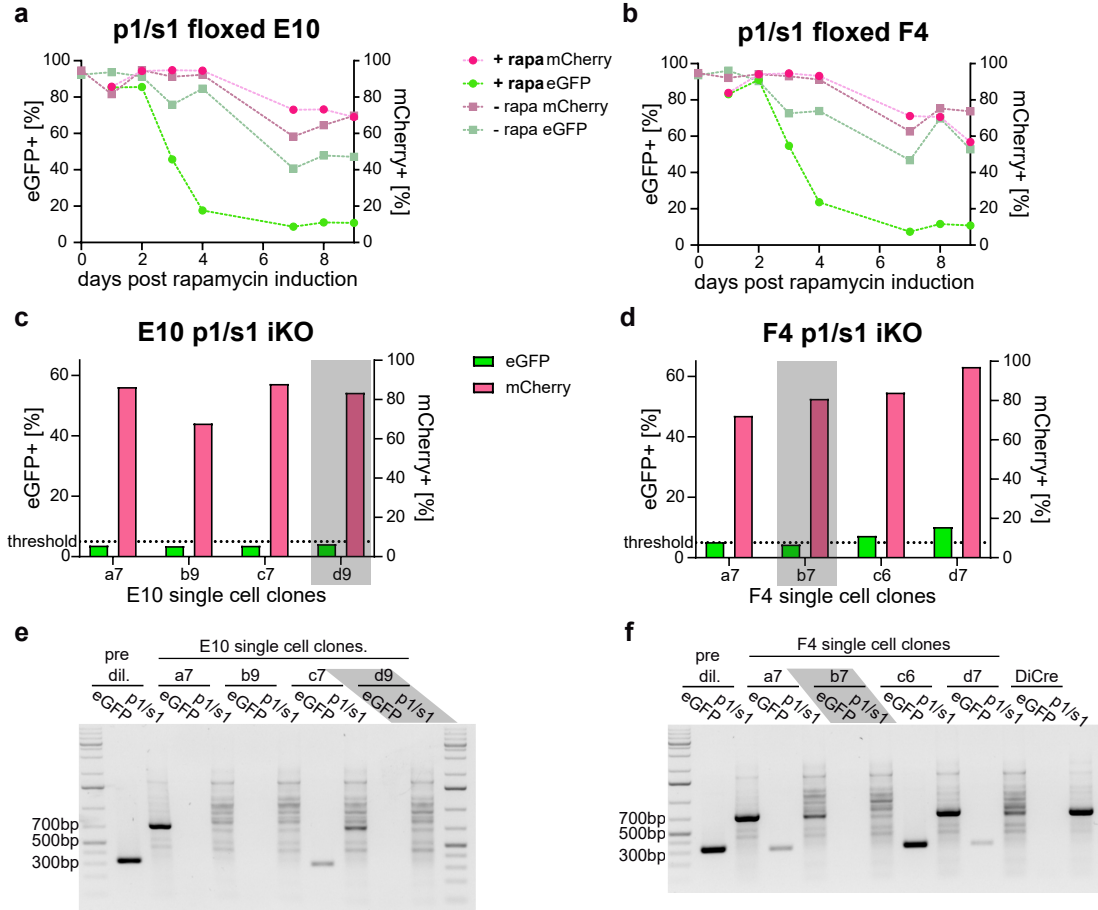


Figure 4.18: Upon inducible knockout induction, *L. major*^{Δp1/s1} are viable - a) eGFP and mCherry positivity was monitored for nine days post rapamycin induced Cre dimerization in *L. major*^{p1/s1} full floxed clone E10 and b) F4 to trace successful recombination. c) Post serial dilution of *p1/s1* iKO clones E10 and d) F4, single cell clones were checked for eGFP and mCherry fluorescence. A threshold was determined based on a wildtype strain. e) Diagnostic PCR of single cell *p1/s1* iKO clones E10 and f) F4 for the *LmjF.30.1500/10* and *eGFP* locus. A control pre serial dilution (pre. dil.) was used as control positive for both genes, *L. major*^{DiCre} as control negative for eGFP. Expected product sizes: *p1/s1* 713 bp, *eGFP* 326 bp. Gray boxes denote *L. major*^{Δp1/s1} used for further experiments.

4. RESULTS

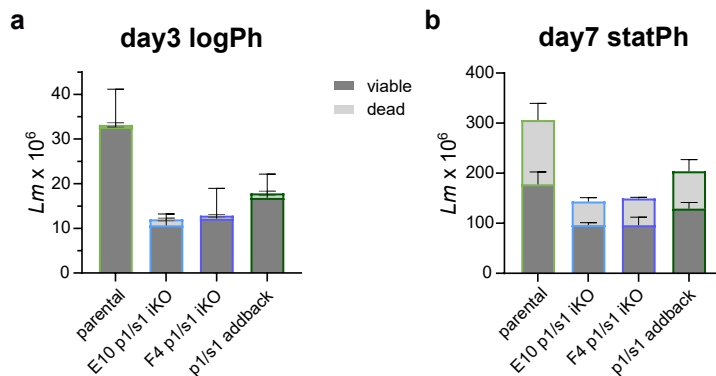


Figure 4.19: *L. major* $\Delta p1/s1$ show attenuated growth - Parasite density in *p1/s1* iKO strains in comparison to a parental strain and a *LmjF.30.1500/10* addback strain in **a)** logPh and **b)** statPh. All strains were grown under the same conditions. $n = 3$ from consecutive passages.

single-stranded DNA was evident, as this substrate was digested much a faster than the double-stranded one. This substrate specificity was previously also described for other class I nucleases (191). The partial depletion of nuclease activity in *L. major* $\Delta p1/s1$ strains strongly suggested that these functions are attributed to *p1/s1*. Reasons for the residual activity were investigated by comparative proteomics, as described in the next paragraph.

4.2.2.4 *L. major* $\Delta p1/s1$ compensate by enriching purine salvage proteins

To investigate why induced *L. major* $\Delta p1/s1$ clones are viable, when direct knockout approaches were lethal, experiments focused on how the null mutants vary from their parental strain beyond *p1/s1* deletion. As the induced recombination of the *p1/s1* locus progressed gradually within the parasite population (Figure 4.18a-b), may have adapted to compensate for the loss of *p1/s1* activity. *Leishmania* exhibits significant plasticity in genome architecture, protein expression, and metabolism (32, 44). Indeed, tendency toward increased nucleotidase activity was observed in the knockout strains (Figure 4.21a). Increased 5'-nucleotidase activity might allow alternative adenosine generation via 5'-AMP hydrolysis.

4.2 Identification of potential antileishmanial drug targets

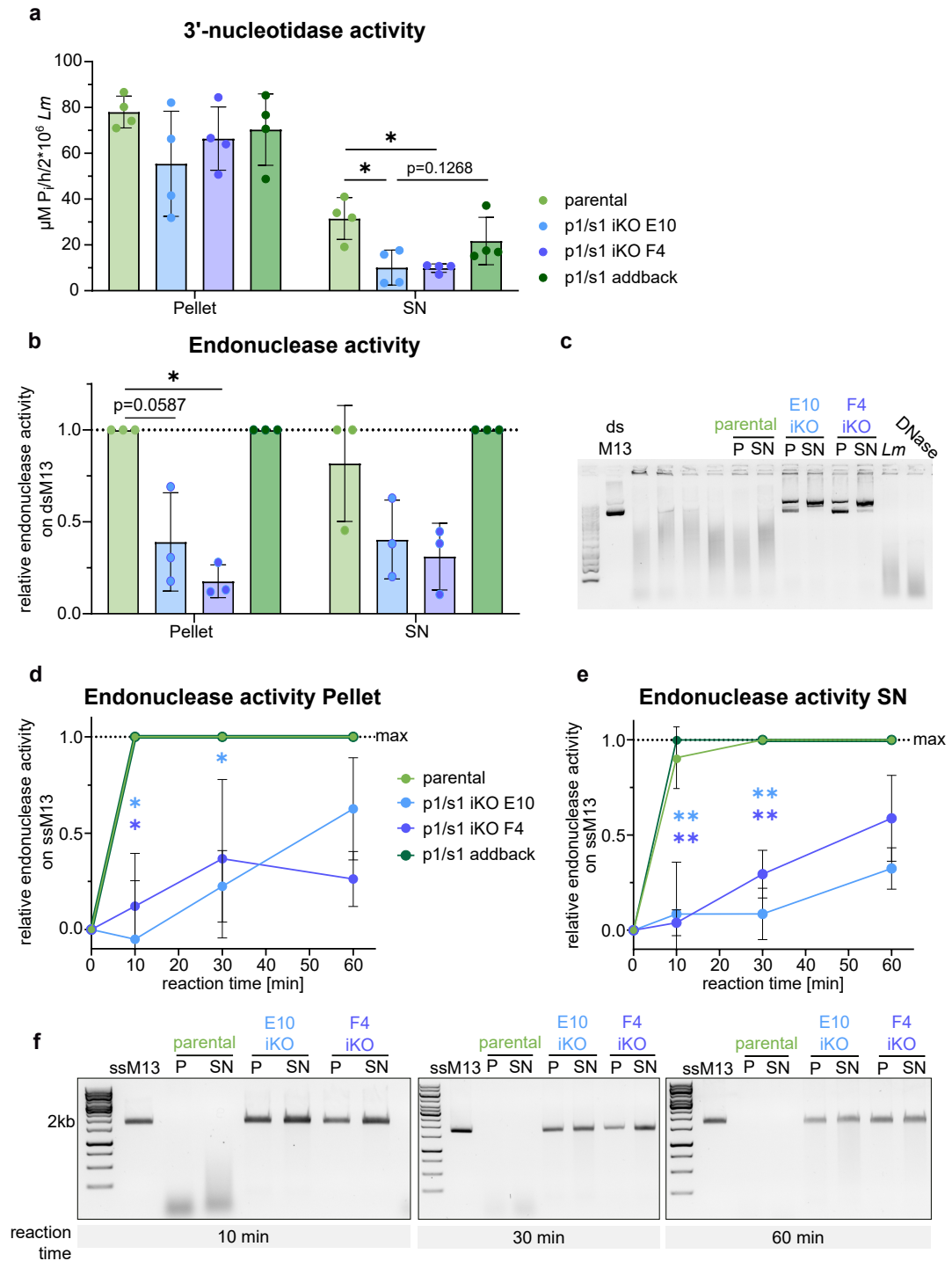


Figure 4.20: *L. major*^{Δp1/s1} have reduced 3'-nucleotidase/nuclease activity - a) 3'-nucleotidase activity was determined on *L. major* ^{p1/s1} 3'^{fl}ox, iKO clones E10 & F4 and *L. major*^{LmjF.30.1500} addback. *n* = 4.

b) Endonuclease activity on dsM13 was semi-quantified using *L. major* ^{p1/s1} 3'^{fl}ox, iKO clones E10 and F4 and *L. major*^{LmjF.30.1500} addback. **c)** Representative gel of *n* = 3.

d) Endonuclease activity was determined on ssM13 over time on parasite pellet or **e)** supernatant. **f)** Representative gels of *n* = 3. Welsh's t-test.

4. RESULTS

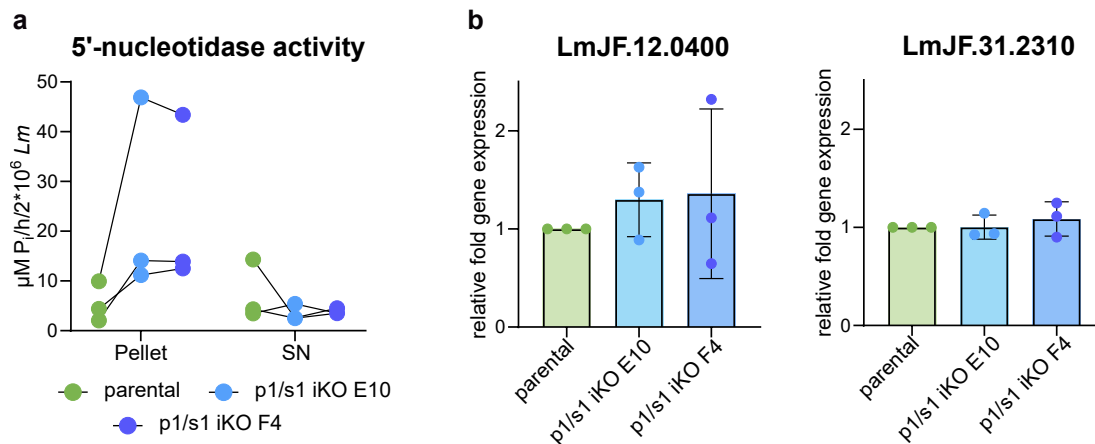


Figure 4.21: *L. major*^{Δp1/s1} show increased 5'-nucleotidase activity - a) 5'-nucleotidase activity was determined on *L. major* *p1/s1* 3'^{fl}ox and two *p1/s1* iKO clones E10, F4. *n* = 3.

b) Relative gene expression of *LmjF.12.0400* and *LmjF.31.2310*, two redundant 3'-nucleotidases/nucleases, was determined by rt-qPCR in *L. major* *p1/s1* 3'^{fl}ox and *p1/s1* iKO clones E10 and F4. *n* = 3, .

Alongside, upregulation of redundant 3'-nucleotidases/nuclease expression could produce similar effects, but no changes of their mRNA levels was observed (Figure 4.21b). However, under nutrient-restricted conditions, *Leishmania* can enhance adenosine uptake through translational remodeling of salvage proteins and transporter levels to sustain purine supply (50, 175, 234). Thus, analyzing protein expression beyond the transcriptome level may yield more conclusive insights.

Hence, a quantitative proteomics analysis was conducted at the Core Facility for Mass Spectrometry of the Johannes Gutenberg-University Mainz to enable a global comparison of protein levels between *L. major*^{Δp1/s1} and the parental strain. Transcriptomic analyses were not attempted, as protein expression in *Leishmania* is primarily regulated posttranscriptionally (161, 175). Principal component analysis revealed similarities between both *p1/s1* null mutants, while the parental control strains clustered separately, indicating knockout-specific changes in the proteome (Figure 4.22a).

Proteins enriched in *p1/s1* iKO strains may be upregulated in response to purine starvation to mitigate complete nutrient depletion. In total, 467 proteins were differentially expressed in both null mutant clones, 175 of which were enriched in both (Figure 4.22b-

4.2 Identification of potential antileishmanial drug targets

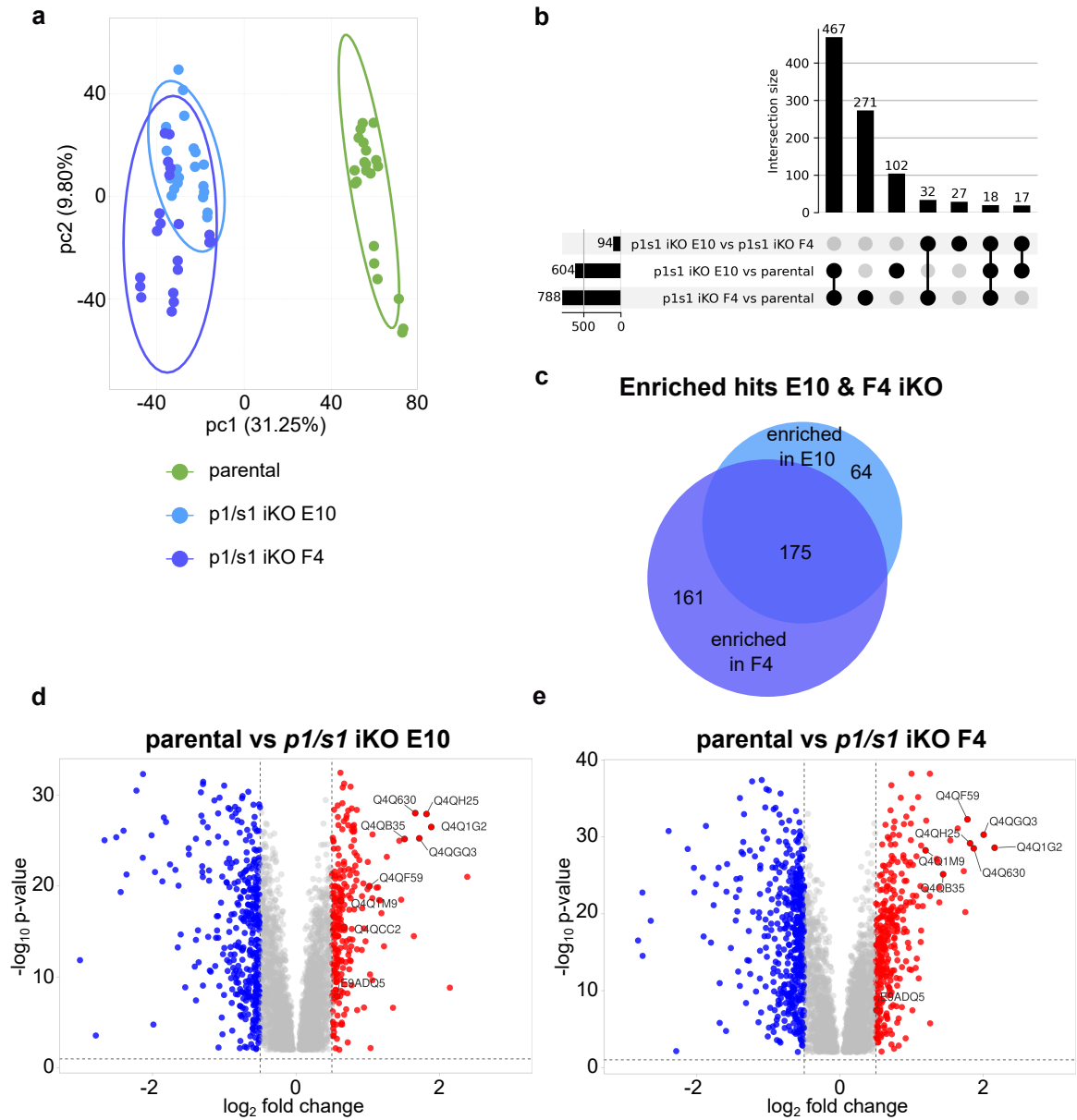


Figure 4.22: *L. major*^{Δp1/s1} enrich proteins of the purine salvage pathway - a) Principal component analysis of a quantitative proteome data of *L. major* *p1/s1*^{3'flo} and *p1/s1* iKO clones E10 and F4.

b) Intersections of differentially expressed proteins within the three samples.

c) Venn diagram showing the overlap in enriched protein numbers for both *p1/s1* knockout clones. Template created with (128).

d) Volcano plot comparing the quantified proteomes of *p1/s1* iKO clones E10 and e) F4 to *L. major* *p1/s1*^{3'flo} as parental strain. Cut-offs: $\log_2$ fold change > 0.5, < -0.5; $p \leq 0.01$. Denoted are enriched proteins of the purine salvage pathway, see Table 4.7. Plots visualized with (110).

Analysis conducted by Tanja Ziesmann. $n = 7$ from consecutive passages, measured in three technical repeats.

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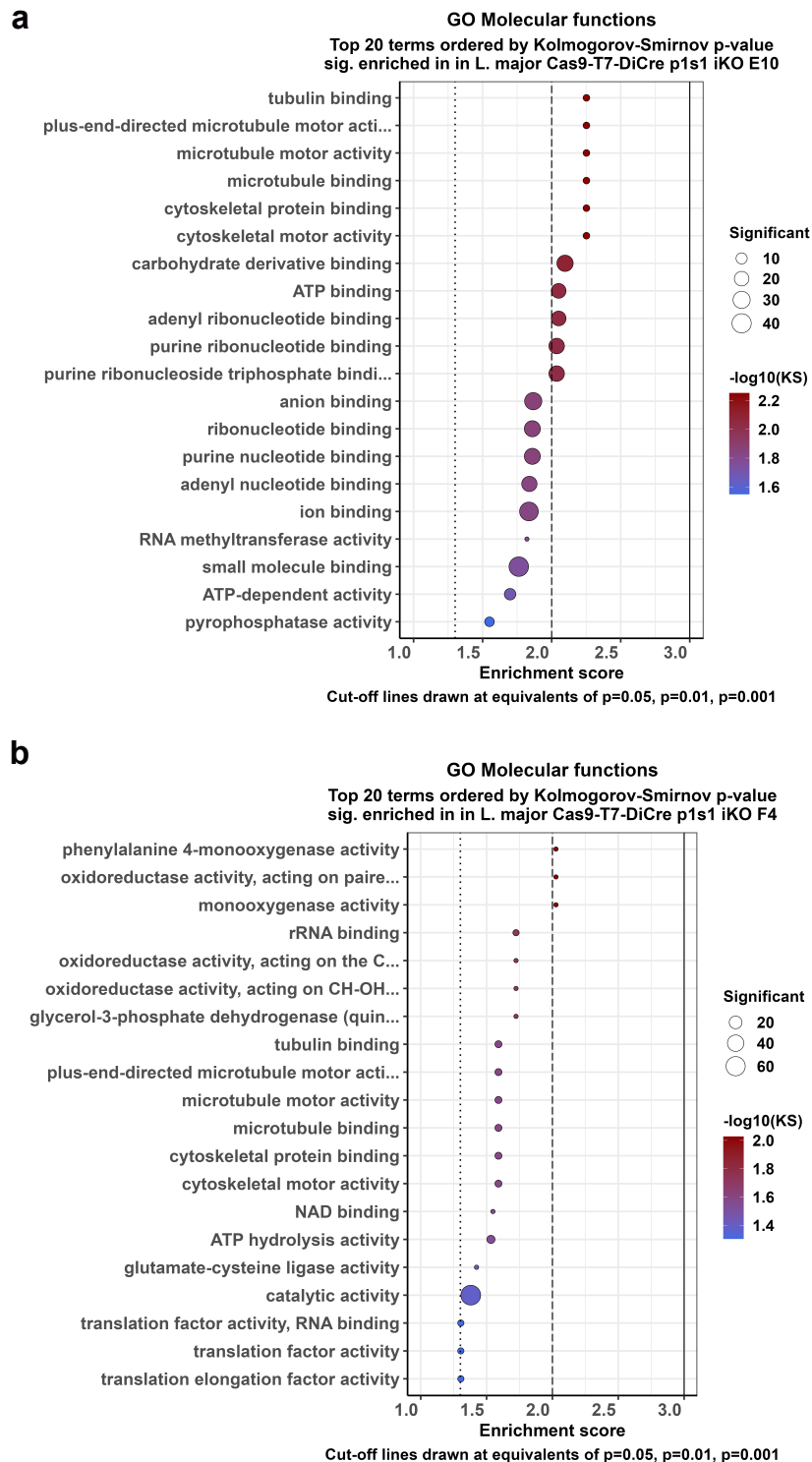


Figure 4.23: Gene ontology enrichment analyses of proteins enriched in *L. major* ^{$\Delta p1/s1$} - Gene ontology enrichment analysis for molecular functions enriched in a) iKO clones E10 and b) clone F4.

Analyses conducted by Tanja Ziesmann.

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Table 4.7: Proteins commonly enriched in *L. major* ^{$\Delta p1/s1$} - Sorted by their fold change in *p1/s1* iKO E10, proteins are denoted in Figure 4.22d-e.

UniProt code	UniProt annotation	log ₂ ratio E10 iKO	log ₂ ratio F4 iKO
Q4Q1G2	Putative membrane-bound acid phosphatase 2	1.89	2.16
Q4QH25	Nucleobase transporter	1.82	1.82
Q4QGQ3	Putative 3'-nucleotidase/nuclease	1.72	2
Q4Q630	Putative 3'-nucleotidase/nuclease	1.67	1.87
Q4QB35	Membrane bound acid phosphatase 2	1.51	1.44
Q4QF59; Q4QF58	Putative nucleoside transporter 1	1.02	1.78
Q4Q1M9	Inosine-guanosine transporter	0.63	1.2
E9ADQ5	Exonuclease domain-containing protein	0.58	0.51

c). Indeed, among these hits, redundant 3'-nucleotidases/nucleases likely contribute to the residual enzyme activity observed in Figure 4.20. Furthermore, proteins associated with extracellular purine generation and uptake were commonly enriched, see Table 4.7 and Figure 4.22d-e. Among the enriched proteins in iKO clone E10, gene ontology terms for purine and adenylyl nucleotide binding significantly overrepresented, whereas this was not observed in iKO clone F4 (Figure 4.23). In this clone, additional terms linked to other metabolic pathways or transport were enriched, likely attributing to the greater diversity of regulated proteins in this clone (Figure 4.22).

Notably, abundance of the purine salvage enzyme xanthine phosphoribosyltransferase (Q4QCC2) was also elevated in *L. major* ^{$\Delta p1/s1$} E10. All together, these enriched proteins may assist *p1/s1* null mutants to compensate for reduced adenosine supply by optimizing alternative or downstream purine salvage pathways.

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4.2.2.5 *L. major*^{Δp1/s1} do not exhibit altered cell death hallmarks

As observed previously, protein levels of p1/s1 were elevated upon stress induction prior to cell death (Figure 4.12). Potentially, this enzyme may be involved in executing programmed cell death hallmarks in *L. major*, particularly DNA fragmentation via its endonuclease activity. Additionally, another 3'-nucleotidase/nuclease was previously described as miltefosine resistance marker in *L. infantum*. To test whether *L. major*^{Δp1/s1} strains exhibit a distinct phenotype associated with miltefosine-induced cell death, the strains were treated with increasing drug concentrations and subjected to various cell death assays: an MTT assay measuring metabolic activity, AnnexinV staining to detect PtdSer exposure and a cell cycle assay to determine the proportion of cells harboring fragmented DNA. However, in none of these assays, consistent changes for both *p1/s1* iKO clones in response to miltefosine were detected (Figure 4.24, Table 4.8). All conducted assays were impaired by high standard deviations, so resulting sigmoidal dose-response curves required careful interpretation. Miltefosine decreased the metabolic rate of all tested strains, with EC₅₀ values of 7-8 μM and no significant differences were observed. PtdSer exposure in response to miltefosine treatment increased up to 100% with EC₅₀ values of 8-10 μM for the parental strain and both iKO strains. Only the addback strain exhibited slightly reduced sensitivity to miltefosine, with an EC₅₀ of 13.4 μM. Similarly, the fraction of parasites with fragmented DNA increased to up to 90% following treatment with 60 μM miltefosine. Here, EC₅₀ values ranged widely from 11 to 18.5 μM.

However, in all assays, the minor trends observed did not apply to both *p1/s1* iKO clones and are not complementary when comparing iKOs and the addback. In case of an unambiguous trend, the same deviations were expected to occur for both null mutants, whereas the addback strains were expected to reflect the behavior of the parental strain. Thus, no straightforward link between p1/s1 and the studied cell death hallmarks could be established.

4.2 Identification of potential antileishmanial drug targets

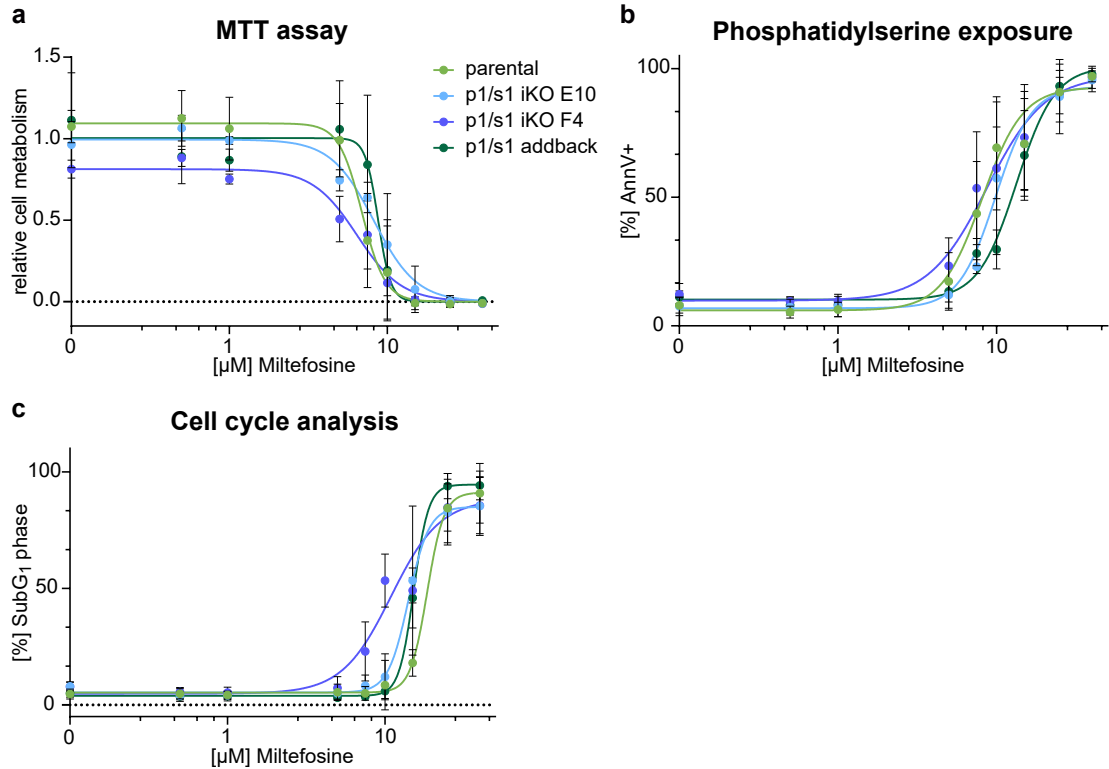


Figure 4.24: *L. major*^{Δp1/s1} do not show altered cell death hallmarks in response to miltefosine - a) *L. major* *p1/s1*^{3'floxed}, *p1/s1* iKO clones E10, F4 and *L. major*^{*LmjF.30.1500* addback} were treated with increasing concentrations of miltefosine for 48 hours. Cell death hallmarks were evaluated using an MTT assay for metabolic activity, b) detecting PtdSer exposure by AnnexinV (AnnV) binding and c) quantifying DNA degradation by the share of cells in the SubG₁ phase. $n = 3$ to 5, non-linear 4-parameter regression: solid line.

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Table 4.8: EC₅₀ of different cell death hallmarks of *L. major*^{Δp1/s1} - Post treatment of the parasites with miltefosine for 48 hours, graphs see Figure 4.24, ± 95% asymptotic confidence interval.

EC ₅₀ [μM]	parental	<i>p1/s1</i> iKO E10	<i>p1/s1</i> iKO F4	addback
MTT	6.9 ± 0.9	8.2 ± 1.4	6.5 ± 1.2	8.8 ± 1.3
AnnV	8.1 ± 1.2	9.8 ± 1.4	8.7 ± 2	13.4 ± 1.3
SubG ₁	18.5 ± 1.9	14.1 ± 1.4	10.9 ± 2	15.2 ± 0.4

4.2.2.6 *L. major*^{Δp1/s1} cannot decrease PBL proliferation upon hMDM infection in presence of 3'-AMP

As previously shown in Figure 4.16 and Figure 4.17, adenosine, a product of *L. major* 3'-nucleotidase activity, can decrease TNFα secretion and PBL proliferation during *in vitro* infection. Likewise, *p1/s1* null mutants have a diminished ability to degrade 3'-AMP to adenosine (Figure 4.20). Whether 3'-nucleotidases participate in nutrient acquisition within the phagolysosome remains unknown. Thus, it was investigated whether *L. major*^{Δp1/s1} are still able to suppress PBL proliferation upon addition of 3'-AMP or show an infection deficiency.

However, *p1/s1* strains exhibited neither a deviating basal infection rate nor differences in parasite burden or PBL proliferation at 4 days pi. Nevertheless, these strains lost the ability to attenuate PBL proliferation in the presence of 3'-AMP. While the addition of adenosine during infection produced a comparable reduction in proliferation across all strains, as seen in Figure 4.17, only the parental strain maintained this suppression by utilizing 3'-AMP. For samples infected with *p1/s1* null mutants, PBL proliferation was comparable between non-treated and 3'-AMP treated conditions (Figure 4.25a-d). In contrast, *L. major*^{Δp1/s1} did not differentially affect TNFα secretion. Upon infection with any strain, TNFα levels were reduced by addition of adenosine or 3'-AMP by about 25% (Figure 4.25e-f). Absolute TNFα concentration was lower in null mutants infections under untreated conditions. Likely, the residual 3'-nucleotidase activity in iKO strains remained sufficient to suppress the release of this cytokine. This again suggested that TNFα is not the sole mediator between adenosine signaling and PBL proliferation. Although these results did not establish *p1/s1* as a virulence factor, they

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still highlight its importance as a mechanism for evading the initial immune response.

4.2.2.7 *L. major* ^{$\Delta p1/s1$} lose their full ability to degrade and escape neutrophil extracellular traps

Not only can the 3'-nucleotidase activity of p1/s1 benefit *L. major* in terms of immune evasion. Earlier during infection, promastigotes encounter neutrophils and utilize them as "Trojan horse" for final uptake into macrophages (Figure 1.1) (255). As part of the innate immune response, these immune cells can eliminate pathogens by trapping them in NETs. Several pathogens have been shown to evade the antimicrobial effects of NETs by employing ectonucleases to degrade the DNA scaffold (40, 42, 67, 232). Therefore, endonuclease activity of p1/s1 might allow *L. major* to degrade and evade NETs.

Previously, *L. major* was shown to induce NET formation upon encountering neutrophils, but no antileishmanial effects of NETs were noted (73, 102). When NET-enriched supernatants from human neutrophils were used as substrates for an endonuclease assay, this activity was reduced for *L. major* ^{$\Delta p1/s1$} in the pellet and supernatant fraction by up to 50% (Figure 4.26a-b). These results closely resembled the activity observed with dsM13 (Figure 4.20) indicating p1/s1-mediated degradation of the DNA scaffold of NETs.

To the leishmanicidal activity of NETs, *L. major* promastigotes were incubated with PMNs whose endocytosis was blocked by cytochalasin D (40). Prior to each experiment, neutrophils of each donor were stained for CD62L and CD66b to confirm their naive phenotype. Figure 4.26d depicts representative staining, where CD62L was shed following control activation with PMA, as expected for naive neutrophils.

After two days of co-culture, the number of viable parasites was determined and compared to a reference sample lacking PMNs. Indeed, the parental strain exhibited no loss of viability. However, *p1/s1* iKO strains partially lost the ability to withstand NET-induced killing by about 30%. This effect was reversed by the addition of DNaseI, which underscored the nuclease dependency of this effect. In these samples, the survival of null mutants mirrored that of the parental strain (Figure 4.26c). Thus, the endonuclease activity of p1/s1 was demonstrated to be essential for *L. major* to

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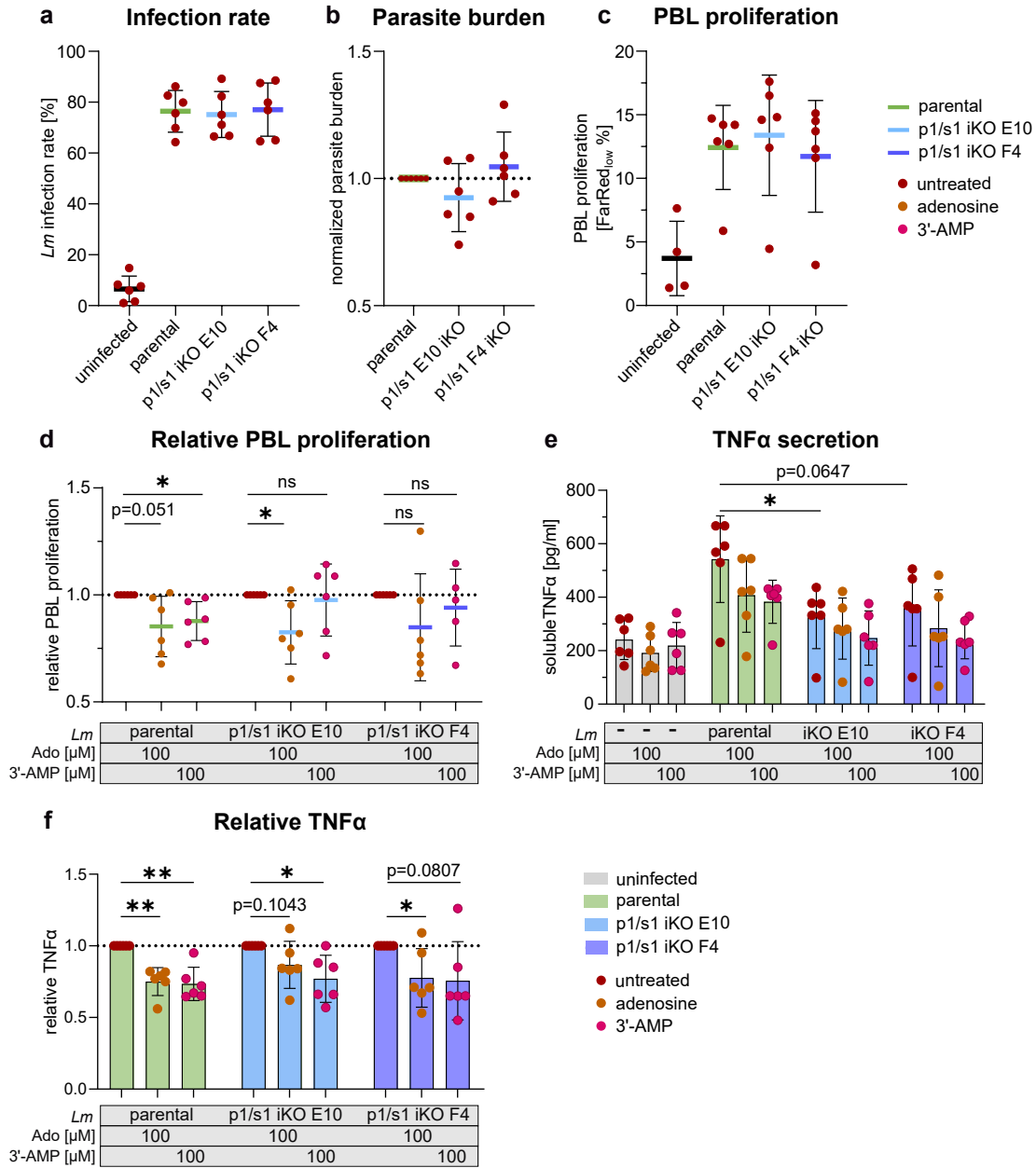


Figure 4.25: *L. major*^{Δp1/s1} cannot decrease PBL proliferation upon hMDM infection in presence of 3'-AMP - A hMDM:PBL co-culture was infected for 4 days with *L. major* *p1/s1* 3'^{fl}_{ox} or *p1/s1* iKO clones in presence of 100 μM adenosine or 3'-AMP; a) Infection rate of hMDMs; b) Relative parasite burden of hMDMs, normalized to the parental strain; c) Share of proliferating PBLs, d) normalized to the untreated sample infected with the parental strain; e) TNFα secretion, f) normalized to the untreated sample infected with the parental strain. *n* = 6 donors in three independent experiments, Welsh's t-test.

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degrade NETs and evade NET-mediated killing.

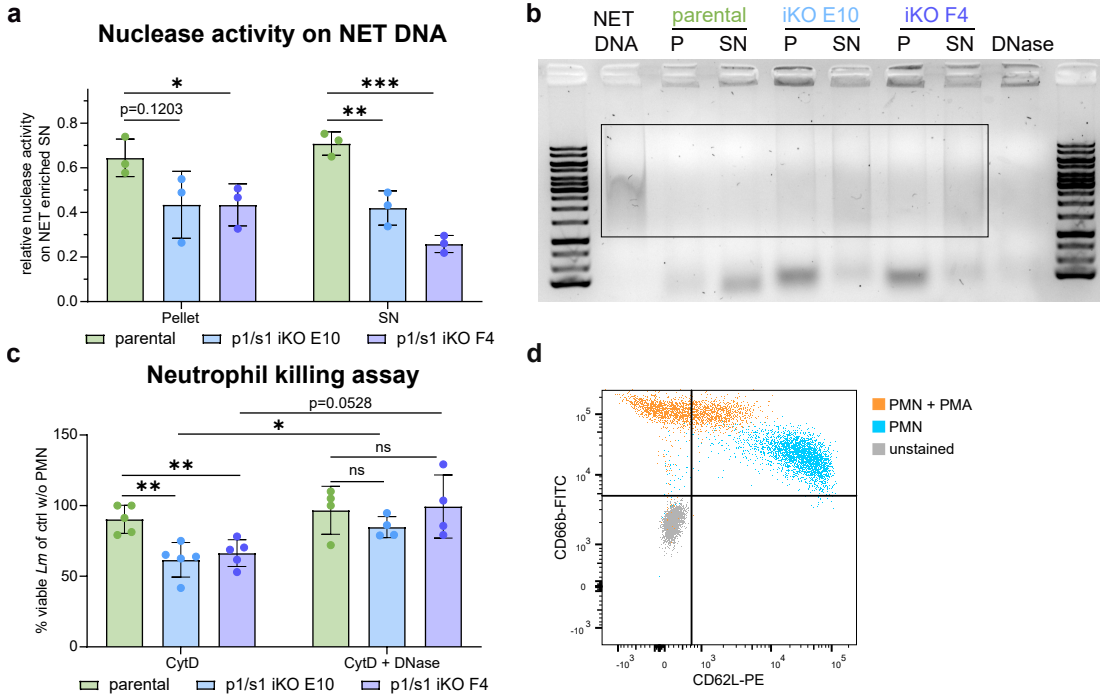


Figure 4.26: *L. major*^{Δp1/s1} partially lose the ability to withstand NET-induced killing - a) Nuclease activity of *L. major* *p1/s1*^{3'floxed} and *p1/s1* iKO clones E10 and F4 on NET enriched supernatants. *n* = 3 donors in three independent experiments, Welch's t-test.

b) Representative gel of **a)**, box denotes area used for semi-quantification.

c) PMNs were treated with cytochalasin D (CytD) and DNaseI, if applicable, before infection with *L. major* *p1/s1*^{3'floxed} or *p1/s1* iKO clones E10, F4. 2 days post infection, viable parasites were counted and normalized to a control without PMNs. *n* = 4 to 5 donors in 2 to 3 independent experiments, Welch's t-test.

d) Exemplary CD62L and CD66b staining of PMNs. If applicable, PMNs were activated with 100 nM PMA for 1 hour to control for an otherwise naive phenotype post isolation from blood.

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4.2.2.8 Virtual screening for specific p1/s1 active site binders did not yield a specific inhibitor

After establishing the central role of p1/s1 in host-parasite interactions and its relevance in purine salvage, it was aimed to identify compounds that bind into the active site of p1/s1 and have inhibitory capacity. Such inhibitors could be beneficial for further elucidating the suitability of p1/s1 as a drug target. Therefore, Jamal Shamsara and Peter Kolb (Core Facility Chemoinformatics & Molecular AI, Philipps-University Marburg) assigned residues participating in the enzyme's active site. Therefore, predicted structure of proteins encoded by *LmjF.30.1500* and *LmjF.30.1510* (Q4Q7F3-4) were superimposed onto close homologues with experimentally determined three-dimensional structures. Based on the crystal structure of endonuclease 2 from *Arabidopsis thaliana* (Q9C9G4), they identified five regions contributing to the active site, see Figure 4.27. According to a previous study (138), these regions were also characteristic for *LdNuc^S*, a close p1/s1 homologue in *L. donovani*. However, proteins of *LmjF.30.1460-1490* (Q4Q7F5-8) do not possess these domains. A zinc atom coordinated in the active site also contributes to enzymatic catalysis (264).

Using their homology model, our collaborators performed a virtual screen for drug-like compounds in the ZINC20 library that dock into the active site of *LmjF.30.1500* or *LmjF.30.1510* (131). A key residue at position 99 varies between the two p1/s1 variants and potentially influences binding of substrates or inhibitors, so two separate docking analyses were carried out.

Out of their ranking of 56 potential binding partners, 18 compounds available from vendors were ordered to test for their inhibitory potential on *L. major*. Initially, two concentrations (2.5 and 25 μ M) were tested to assess a potential dose-dependency inhibition of 3'-nucleotidase activity. TTM, DTT, and EDTA were included as inhibitor controls. Their inhibitory effects have been previously published and are rather non-specific, as they rely on either chelation of the zinc atom or reduction of disulfide bonds (113, 191, 200). These controls demonstrated stable inhibition in both the parasite pellet and supernatant fractions, reducing relative 3'-nucleotidase activity to 0.63 (TTM), 0.42 (DTT) and 0.24 (EDTA), confirming general suitability of the assay setup. All samples were evaluated against the carrier control containing the equivalent percentage of DMSO, which did not influence 3'-AMP hydrolysis compared to the untreated control.

4.2 Identification of potential antileishmanial drug targets

However, no obvious dose-dependent inhibition was observed for any of the inhibitor candidates (Figure 4.28a-c). No compound showed a clear decreasing effect on 3'-nucleotidase activity with increasing compound concentration. In contrast, some compounds rather exhibited an activating effect, for example compound 1, 3, or 36, which had a relative activity in the supernatant of ≈ 1.3 at 25 μM compound. Conversely, some molecules, such as compounds 46 and 47, showed minor inhibition, reducing activity to approximately 0.75, but this effect was not dose-dependent between 2.5 and 25 μM .



Figure 4.27: Alignment of proteins derived from the *p1/s1* cluster - Amino acid sequence alignment of proteins Q4Q7F3, encoded by *LmjF.30.1510*, Q4Q7F4 (*LmjF.30.1500*), Q4Q7F5 (*LmjF.30.1490*), Q4Q7F6 (*LmjF.30.1480*), Q4Q7F7 (*LmjF.30.1470*) and Q4Q7F8 (*LmjF.30.1460*). The signal sequence for secretion is marked in red, yellow boxes denote regions of the predicted p1/s1 active site. Homology of the C-terminal region is recognizable in dark blue. Light blue and gray backgrounds illustrate partial homology. Figure by Jamal Shamsara, alignment conducted using the Clustal Omega interface provided by UniProt (238, 248).

To confirm that no inhibition could be observed across a wider concentration range up to 100 μM of compound, a dose-response experiment was conducted using compounds

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36 and 47 as examples. These were chosen as representatives for the above mentioned observations. Still, no clear dose-dependency on 3'-nucleotidase activity, neither inhibiting nor activating, was found (Figure 4.28d-e). Notably, treatment with 0.5 μ M increased the relative nucleotidase activity of both compounds by 50%. However, this potentially activating effect was not stably observed with increasing compound concentrations. An additional dose-response experiment focusing on nanomolar compound concentrations might clarify the existence of a dose-dependent relationship.

Additional compounds were not subjected to dose-response experiments due the low likelihood of success. Inhibition on endonuclease activity was not checked, as this assay was not feasible for scale-up. In conclusion, no p1/s1 specific 3'-nucleotidase/nuclease was identified within the available compound subset. A potential inhibitor may still be identified among the remaining untested compounds.

4.2 Identification of potential antileishmanial drug targets

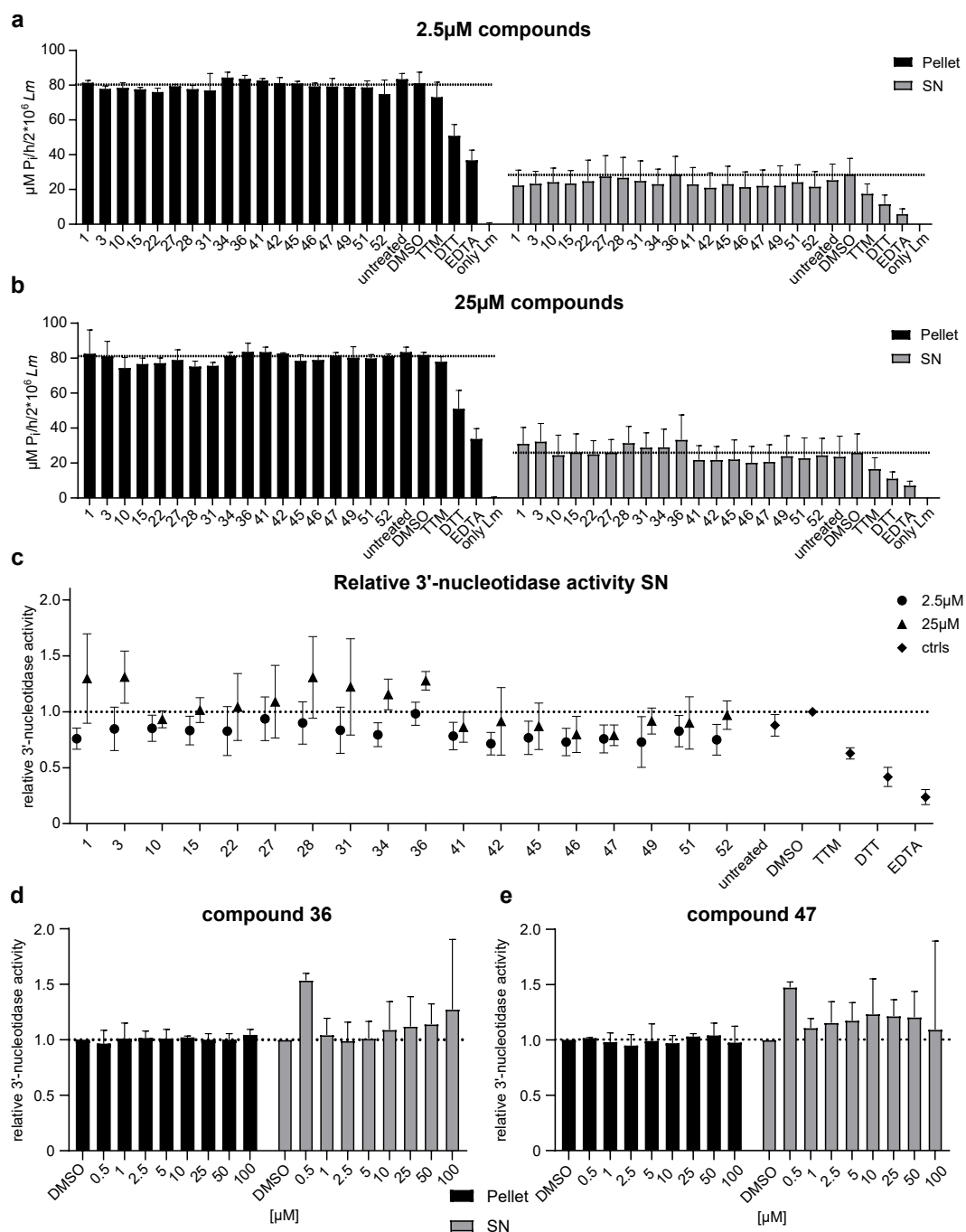


Figure 4.28: 3'-nucleotidase activity of *L. major* does not decrease dose-dependently by treatment with potential inhibitory compounds - a) 3'-nucleotidase activity of *L. major* treated with 2.5 µM compounds or b) 25 µM compounds. Compound concentration was maintained throughout the assay. 1% DMSO was used as carrier control, 25 µM TTM, 5 mM DTT and 2 mM EDTA were used as unspecific inhibition controls. $n = 4$.

c) Relative 3'-nucleotidase activity in the supernatant as depicted in a) and b)

d) Relative 3'-nucleotidase activity on a dose-response curve for compound 36 and e) 47, $n = 2$; dotted lines represent activity of the respective DMSO control.

4. RESULTS

Discussion

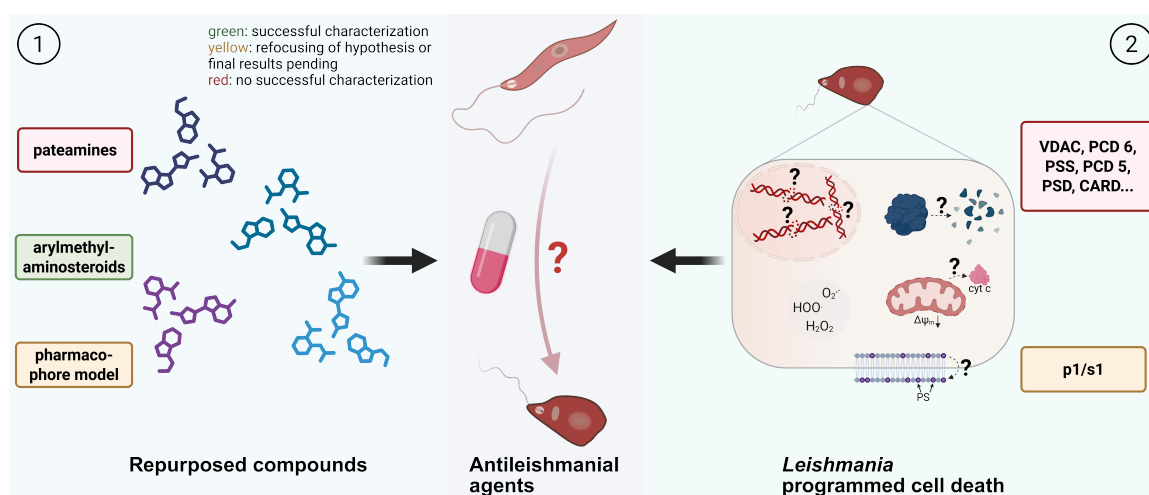


Figure 5.1: Evaluation of the two main objectives of this thesis - 1: Three compound classes were evaluated for their potential as antileishmanial drug: pateamines, arylmethylaminosteroids and repurposed compounds derived from a pharmacophore model of the latter. While pateamines exhibited limited effects on *L. major* and exceeding cytotoxicity on hMDM, arylmethylaminosteroids 1o and 1c reduced parasite burden in an *in vitro* infection model. Compounds selected by pharmacophore criteria of the latter show promising selectivity for *Leishmania* but tests on intracellular efficacy are pending.

2: Several candidates were subjected to gene deletion, but no auspicious phenotypes regarding PCD were observed. Similarly, p1/s1 was also tested negative for cell death involvement, but it was successfully characterized regarding the immunomodulatory impact of its enzymatic functions. Figure created with BioRender.

5. DISCUSSION

In summary, this project aimed to proceed knowledge on potential novel antileishmanial therapy strategies. Within the scope of the thesis, advancements were achieved in both *in vitro* approaches: phenotypic screenings of several compound classes and drug target exploration for inhibitor design (Figure 5.1). Although no direct links to parasite programmed cell death were discovered, valuable findings were made to expand the repertoire of approaches for promising therapeutic developments. Regarding objective **1**, several novel compounds from diverse backgrounds and classes were characterized for their *in vitro* antileishmanial activity and selectivity for *Leishmania* over host macrophages. Within objective **2**, no pathways, single effectors, or executioners of PCD could be identified or confirmed in *L. major*. Therefore, the hypothesized presence of designated and regulated cell death pathways in *Leishmania* remains unresolved.

5.1 Compounds eliciting axenic antileishmanial effects and their applicability in *in vitro* infected macrophages

5.1.1 Arylmethylaminosteroids

Although their exact mode of action remains elusive, arylmethylaminosteroids have been described as promising antiparasitic agents with potent activity against adult *Schistosoma mansoni* and *Plasmodium* *ssp.*, comparable to the respective gold-standard drugs (152). As 1o can chelate di- and trivalent metal ions, its ability to induce radical formation and disrupt intracellular redox homeostasis has been proposed as a mechanism of action for these compounds. Depletion of proteins linked to oxidative stress in *L. major* in response to 1o treatment, such as trypanothione peroxidase (28), may support this hypothesis. Additional experiments investigating the formation of reactive oxygen species in *L. major* after treatment with c1o and 1c could further substantiate this hypothesis.

Currently, a genome-wide screen in *L. major* genes associated with the mechanisms of resistance or action of 1o by employing DNA C-to-T base editing (84). In a respective setup for 1c in *L. mexicana*, resistance was conferred by alterations in the flagellar transport system, putatively by blocking 1c uptake. Further uncharacterized markers were identified as determinants of sensitivity and resistance (70). Given that aryl-

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methylaminosteroids are effective against multiple microbial and parasitic organisms, a commonly conserved target pathway is plausible.

Accordingly, 1o and 1c moderately reduce *Leishmania* cellular metabolism of promastigotes and axenic amastigotes, with a more pronounced effect on the former. Although both compounds share a steroid and hydroxyarylmethylamino moiety and differ only in an additional phenyl residue, they exert differential effects on both life cycle stages with respect to PtdSer exposure and intracellular DNA degradation. They may induce distinct cell death executioners in these parasite life stages, despite the disruption of mitochondrial metabolism in both. Elucidating the exact mode of action of 1o and 1c may also enhance our understanding of cell death mechanisms in *Leishmania*. Following the process of programmed cell death, it is apparent that the loss of mitochondrial integrity is upon the early sign of apoptosis and can be measured at lower concentrations than late apoptotic markers. Accordingly, as an MTT assay detects mitochondrial oxidoreductase activity, respective EC₅₀ values are lower in this assay than in those assessing later markers, such as PtdSer exposure and DNA fragmentation.

Concerning susceptibility of *L. donovani*, both molecules reduce the cellular metabolism rate in a manner comparable to the effects observed in *L. major*, suggesting a conserved mode of action between both species. As causative agent of visceral leishmaniasis, susceptibility of *L. donovani* is clinically more relevant than that of *L. major*, which causes the cutaneous form. However, assessing the effects on axenic amastigotes of *L. donovani* was not feasible due to instability of the culture system.

In addition to their toxic effects on pathogens, 1o exhibits known immunomodulatory effects on myeloid cells. Consistent with our observations on hMDM metabolic activity, the compound increases cellular metabolism by augmenting the glycolytic rate (34). As a decrease in metabolism at high concentrations of 1c was detected, this compound may also exert toxic effects that outweigh an initial activating effect. Notably, the used MTT assay used in this study may not be entirely suitable for assessing cytotoxic effects. The assay detects mixed effects of viable cell number and cellular metabolism. Thus, the increased signal may also result from multiple factors, including proliferation, activation, and toxicity. Comprehensive approaches based on membrane integrity or ATP quantification could add to the understanding.

Additionally, 1o can induce a shift towards a more pro-inflammatory phenotype of hMDMs (34). Macrophage activation and repolarisation by 1o can also contribute to

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intracellular parasite restriction by enhancing pro-inflammatory responses. No significant difference were observed in terms of infection numbers in untreated samples of both macrophage polarization states. However, such an immunomodulatory effect of 1o might contribute to the similar trends observed on both macrophage differentiation states.

Using a protocol employing infection with axenic amastigotes, we can resemble an established *Leishmania* infection without the influence of a promastigote-to-amastigote differentiation. In such a setting, compounds have to pass the macrophage cell membrane and the phagolysosomal membrane to reach the acidic compartment surrounding the amastigotes. As evidenced by an increase in parasite burden from 24 to 48 hours pi, intracellular amastigotes proliferate in the phagolysosome, but 1o or 1c treated samples do not show this trend. It is conceivable that the compounds are not sufficiently effective in the intracellular environment to eliminate amastigotes, but they can inhibit proliferation. Whether the inability to reduce the percentage of infected macrophages is caused by insufficient penetration of any of the membranes, unfavorable intracellular compound distribution, or high persistence of the parasites, remains elusive.

In first pharmacokinetic studies, the steroid moiety of 1o resulted in detrimental properties with respect to oral bioavailability and aqueous solubility (33). Additionally, developing a new compound class for neglected tropical diseases requires extensive and costly preclinical data collection before progressing into clinical trials, a process that is often not economically attractive to the pharmaceutical industry. Thus, identifying already characterized, pharmaceutically active molecules with similar antiparasitic effects as arylmethyaminosteroids is of high interest.

5.1.2 Repurposed compounds derived from an arylmethyaminosteroid pharmacophore model

Drug repurposing is highly beneficial for the development of new treatments against neglected tropical diseases (7). Resources already employed for development of a known drug can be reused to accelerate and facilitate preclinical and clinical development for novel indications. For approved drugs, extensive data on their mechanisms of action, pharmacology, tolerability, and safety can also be utilized in meta-analyses during lead identification. Thus, the goal of the pharmacophore model estimated by Joachim Geyer

5.1 Compounds eliciting antileishmanial effects and their efficacy in infected macrophages

and Michael Kirstgen (Justus-Liebig-University Gießen) was to identify compounds already approved or tested in clinical trials, that have similar antipathogenic effects as arylmethyaminosteroids. The use of a QSAR model enabled to virtually screen for compounds with interaction surfaces similar to those of arylmethyaminosteroids (148). Although they consulted activity data generated on *Plasmodium*, these results can also be applied to other species if the mode of action of arylmethyaminosteroids is conserved. Given that their activity has been observed across a broad range of pathogens, this hypothesis is highly conceivable. However, identifying the molecular targets of 1o or 1c can refine these predictions.

With a hit rate exceeding 50% in the phenotypic screening at 20 μ M on *L. major*, this rational prediction achieved an exceptional success rate far surpassing the hit rates of large-scale library approaches used in conventional drug development. Thus, applying the principle of pharmacophore modelling to previously identified compound hits can be a very promising strategy for future lead selection against neglected tropical diseases.

Observed differential effects on *Leishmania* life stages can be attributed to several amastigote-specific factors, like acidic environment, differential expression of molecular targets, metabolic requirements or membrane composition (38, 161). Although the use of axenic parasites is of limited clinical relevance, these data can be used to further refine prediction models. For example, comparison of the screened oxicams, that structurally only differ in a few atoms but had varying effects on amastigotes, can yield valuable information for future QSAR analyses. The diversity of compounds advancing into dose-response experiments reflects the heterogeneity of possible antileishmanial targets. Most of these compounds target cytosolic human proteins (see Table 5.1), so membrane permeability into the macrophage cytoplasm can be assumed. However, to reach an intracellular amastigote, a compound must also penetrate the phagolysosome and amastigote membranes. Additionally, a share of the compound can also be sequestered by its human target protein. Thus, data obtained from axenic cultures are not directly transferable to intracellular efficacy. The efficacy assessment of these compounds in *in vitro* infection is currently ongoing.

Exact antileishmanial mode of actions for these repurposed drug remains elusive, despite well-characterized human targets. Similarity searches for homologous *Leishmania* proteins or reverse docking could reveal target candidates. In the case of MLN9708,

Table 5.1: Descriptions and mode of actions of repurposed compounds with antileishmanial activity- Details for all compounds identified through a pharmacophore model of arylmethylaminosteroids. Plasma levels were taken from pharmacological studies in human patients treated with the standard therapeutic dosage. If not cited otherwise, information were derived from DrugBank 6.0 (150). Details on MLN9708 apply for its active form ixazomib.

Drug	Human target	Mechanism	Approved indication	Administration route	Plasma levels C_{max}	Antipathogenic effect	Literature
Cetrorelix-acetate	Gonadotropin-releasing hormone receptor	Antagonist	Prevention of premature ovulation in reproduction medicine	Subcutaneous	≈ 36.1 nM (85)		(196)
Lornoxicam	Prostaglandin G/H synthases	Inhibitor	Mild to moderate pain, rheumatoid arthritis	Oral	≈ 1.83 μ M (20)		(17)
Adapalene	Retinoic acid receptors	Agonist	Acne vulgaris	Topical	≈ 1.34 nM (93)	<i>L. panamensis</i> (181)	(236)
Honokiol	Prostaglandin G/H synthases, lipoxygenase	Inhibitor	In clinical trials for: anti-tumourigenic, anti-inflammatory, neuro-protective effects			Several (123, 289)	(180)
Rupatadine-fumarate	Histamine H1 receptor, platelet activating factor	Antagonist	Rhinitis, urticaria	Oral	≈ 4.13 nM (142)	<i>Mycobacteria, Schistosoma</i> (249, 280)	(180)
Silmitasertib-sodiumsalt	Casein kinase II	Inhibitor	In clinical trials for several tumours	Oral		Anti-fungal (176)	(129)
MLN9708	Proteasome subunit $\beta 5$ (ixazomib)	Inhibitor	Multiple myeloma	Oral	≈ 142.33 nM (179)	<i>Babesia</i> (2)	(179)

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5.1 Compounds eliciting antileishmanial effects and their efficacy in infected macrophages

a proteasome $\beta 5$ inhibitor, the respective leishmanial proteasome is a plausible target. Proteasome inhibitors are very well-characterized antileishmanial drugs, since several amino acid residue differences from the human homologue can confer parasite-specific inhibition. Two drugs are currently progressing into clinical phases I and IIa, both targeting a similar proteasome site as MLN9708 (78, 179, 187, 277). Among the compounds identified through pharmacophore modeling, only adapalene had been previously described for its antileishmanial activity (181), but several more are known for diverse antipathogenic effect and may thus enable the development of broad-spectrum antimicrobial treatments.

Of course, as all of these compounds have characterized human targets and are not parasite specific, they require a sufficient therapeutic window to circumvent adverse side effects. Selectivity indices vary for these compounds. In antileishmanial drug development, an SI of ≥ 5 to 10, depending on the source, can be sufficient for lead identification (147, 193). Nevertheless, before conducting *in vivo* treatments, host toxicity should be minimized, particularly since the EC_{50} values for axenic amastigotes are often higher than the pharmacologically determined plasma concentrations in human patients (see Table 5.1). From a practical perspective, the evaluation of administration routes should prioritize topical or oral routes as they are the most feasible for field use in areas with poor medical infrastructure. Ideally, formulations should also be stable in tropical environments without constant requiring constant refrigeration, to minimize costs of transport.

Although ultimate efficacy of these compounds in infection settings remains to be tested, this workflow demonstrates the significant advantages of using a QSAR approach to identify molecules that are already in clinical tests or use and are feasible for drug repurposing against leishmaniasis.

5.1.3 Pateamines

Due to key differences in leishmanial and human eIF4A sequences and substrate affinities, this initiation factor was previously described as druggable protein in *Leishmania* (21, 118). Whereas rocaglates, another natural eIF4A inhibitor class, depend on a Phe163 interaction, PatA does not require this residue for efficient clamping. Thus, *Leishmania* are rocaglate-insensitive but sensitive towards PatA, since they possess a

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serine residue at position 163 of eIF4A. These *in vitro* data strongly support data obtained from recombinant proteins to elucidate the PatA mode of action (171, 194). However, DMDAPatA still requires interaction with Phe163 (171, 188), reflected by the negligible sensitivity of *L. major* to this compound. Additionally, PatA activity may be impaired when promastigotes are cultured under acidic pH conditions. Thus, the reduced PatA activity against axenic amastigotes is likely due to changes in the protonation state of the compound rather than amastigote-specific factors. As PatA efficiently binds to the human eIF4A binding pocket (171), a distinct cytotoxicity on human cells was anticipated and indeed observed in primary macrophages.

Given the low efficacy of PatA against axenic amastigotes, combined with notable host cell cytotoxicity that surpasses its leishmanicidal activity combined with its poor safety and bioavailability profile (229) renders this compound unsuitable for *in vitro* infection experiments. Due to its very high antiviral potency, this agent is of further interest for development of such therapies, unlike its potential antileishmanial applications. Still, characterisation of PatA activity on *L. major* corroborated its proposed mode of action by providing a proof of principle on axenic parasite cultures (171).

5.2 Programmed cell death mechanisms of *Leishmania* remain elusive

To date, pathways of programmed cell death in *Leishmania*, whether extrinsic or intrinsic, remain mostly uncharacterized. Orthologs of mammalian apoptosis-linked proteins, such as caspases, are absent in the *Leishmania* genome and the role of a metacaspase and endonuclease G remain debated (28, 213). Only a limited number of proteins that directly influence parasite cell death rates have been described, but none of these proteins have been placed within the context of a signaling cascade (53, 83, 108, 155, 219, 259). Only recently, first putative protein-protein interactions of potentially pro- and anti-apoptotic domains involved in leishmanial apoptosis were proposed (154).

Advancing our understanding of parasite cell death can also promote drug development, as regulators and executioners of apoptosis might be exploited to reduce parasite viability. Additionally, interfering with PtdSer exposure can reduce immunosuppressive apoptotic mimicry used by *Leishmania* for silent phagocytosis into mammalian hosts, potentially enabling the development of a live-attenuated vaccine strain (254, 268). Therefore, further investigations of putative cell death-related proteins focused on candidates identified either by homology to human cell death-related proteins and domains or through a rational literature search. These genes were subjected to targeted gene deletion via CRISPR-Cas9 or DiCre mediated recombination to study the occurrence of cell death-related phenotypes.

None of the candidate proteins tested yielded promising results. Either, no viable null mutants could be generated or no prominent phenotype was observed following cell death induction. Noteworthy, some mutants were not tested in any phenotype assays due to time restrictions. Essentiality of phospholipid biosynthesis-related proteins is conceivable, as PSS and PSD are part of the sole synthesis pathway of PtdSer encoded by the *L. major* genome (288). The absence of altered cell death behavior in other mutants can be attributed to several potential reasons. In cases of incomplete or single knockouts of multicopy genes, residual gene expression may be sufficient to maintain functionality. Alternatively, redundant proteins might compensate the deletion. For null mutants, these proteins are either not participating in cell death regulation or execution, have redundant pathways, or the assays conducted were not appropriate.

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In conclusions, these attempts to identify pathways of cell death regulation in *Leishmania* were not promising enough to justify further experiments in this direction. To advance research in this field, combining sophisticated approaches to screen the entire *Leishmania* genome might be more effective. For example, molecular docking or phylogenetic analyses could identify orthologous cell death pathways or protein complexes in protozoa (154). Previously, we conducted a quantitative proteomics screen to assess changes in protein expression in response to different PCD-inducing stimuli (28). Reanalysis of these data might be used to examine previously unidentified proteins enriched upon death induction. However, this approach is not sufficient to reproduce protein localisation, enzymatic activity, protein-protein interactions or post-translational modifications that can be part of a PCD pathway. Thus, it might rather reflect protein levels that are regulated as part of a stress response (see Section 4.2.2.1). To address these knowledge gaps regarding PCD-related pathways, a phenotypic whole-genome screen for *L. major* mutants with deficiencies in PtdSer exposure is currently under evaluation.

5.3 p1/s1 as potential antileishmanial drug target

Manifold implications and biochemical characterizations of ecto-nucleotidase and nuclease activities of trypanosomatids have been described. In particular, 3'-nucleotidase/nuclease activity, arising from class I nucleases, has been in the focus of research. For *L. major*, several proteins have been linked to this activity (90, 158). Due to the inability of *Leishmania* to synthesize purines *de novo*, these enzymes are essential for the extracellular degradation of nucleotides and nucleic acids, as only nucleosides can be transported across the parasite membrane (167). But also involvements in host-parasite interactions mediated by 3'-nucleotidases/nucleases have been reported, including replication advantages in sand flies, degradation of neutrophil extracellular traps (NETs), and promotion of macrophage infection (95, 114, 200).

In order to study p1/s1, a 3'-nucleotidase/nuclease of *L. major*, in detail, genome editing, quantitative proteomics, and *in vitro* human primary immune cell models were combined to assess its involvement in *L. major* cell death and suitability as potential drug target. Class Romannum1 nucleases are expressed in a stage-specific manner. In accordance with previous reports on homologous proteins, p1/s1 was detected in axenic amastigotes, but also in statPh promastigotes (90, 138). Expression in statPh promastigotes may suggest a role for p1/s1 in infection establishment within mammalian hosts, expression amastigotes implies involvement in intracellular survival. Promastigotes in the statPh mostly consist of non-proliferating metacyclic promastigotes and apoptotic-like parasites (254). Promastigotes in the logPh are in their procyclic and non-infective form, as found as proliferating stage in the sandfly midgut. The lack of p1/s1 in this stage reduces purine availability and thereby potentially contributes to enhanced metacyclogenesis and colonization within the insect vector (95, 233).

In accordance with the expression pattern of p1/s1 in promastigotes, higher 3'-AMP hydrolysis and DNA degradation were observed for statPh compared to logPh promastigotes. As the dominant ecto-nucleotidase activity of *L. major*, 3'-nucleotidase activity is essential for the parasite to sustain its purine salvage pathways. Conversely, no 3'-nucleotidase activity was detected on amastigotes, despite p1/s1 being expressed in this stage. Several potential reasons may explain this observation: Peptides detected by mass spectrometry of axenic amastigotes could also derive from Q4Q7F8-F5, which

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lack the p1/s1 active site. Otherwise, p1/s1 might be retained intracellularly in amastigotes or assay conditions interfered with 3'-nucleotidase activity.

Expression or activity of proteins involved in the purine salvage cascade can be induced under nutrient-depleted conditions (50, 234, 242). p1/s1 is also expressed following sublethal, drug-induced stress. Possibly, through its role in nucleotide and nucleic acid catabolism, p1/s1 activity aids the parasites in acquiring essential purines required for survival and thus to combat stress. However, no connection between p1/s1 and the execution of cell death in *L. major* was established, as deletion of p1/s1 did not affect any of the tested hallmarks of apoptosis. Neither did the knockout of *p1/s1* influence the parasites' sensitivity towards miltefosine, as it currently has been described for a 3'-nucleotidase/nuclease of *L. infantum* (95). Mechanisms of miltefosine resistance may be species-specific, a phenomenon also observed for other antileishmanial drugs (173). Whether p1/s1 is essential for the survival of *L. major* still remains elusive. The lethality observed in direct or facilitated knockout approaches suggested essentiality, but a DiCre based induced recombination yielded viable $\Delta p1/s1$ strains, although identical sgRNA and homologous recombination sequences were used in all experiments. Facilitated knockouts may have failed due to insufficient adenosine supplementation in the medium or the absence of other purines. Genetical complementation could have been hampered because *LmjF.30.1500* in these experiments was expressed together with a C-terminal His₆ tag. Genomical integration of such a construct did not restore 3'-nucleotidase activity during addback generation, only integration of untagged *LmjF.30.1500* did. It is plausible that the His₆ tag interfered with enzymatic activity of p1/s1, either through steric hindrance or accumulation of negative charge of the histidine residues. Repeating facilitated knockout approaches in strains overexpressing untagged *LmjF.30.1500* could clarify this.

Observed growth deficiencies of the induced null mutants still implied a central role of p1/s1 for the parasites. Insufficient purine uptake due to reduced nucleoside acquisition may impair proliferation in the culture medium. During induction of *p1/s1* excision, *L. major* can adapt to the altered metabolite supply by upregulation of alternative or downstream purine salvage processes. *Leishmania* are known for their dynamic responses to environmental changes at the genomic and proteomic levels (32, 44). Functionally, a trend of enhanced 5'-nucleotidase activity was indeed observed.

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No changes concerning copy number variations were detected in the genome of $\Delta p1/s1$ strains (NCBI SRA submission number: SUB15491365). Such metabolic adaptations have previously been described as primarily regulated post-transcriptionally (50). Accordingly, comparative proteomics revealed similar changes in both *L. major* $\Delta p1/s1$, compared to a parental strains, underlining the common need for adaption. Enhanced expression of proteins associated with purine transport and salvage, including redundant 3'-nucleotidases/nucleases, could explain how *Leishmania* bypass essentiality of p1/s1. By enhancing alternative extracellular nucleoside generation and downstream purine salvage, they can still meet their nutrient requirements.

Final confirmation that 3'-nucleotidases/nuclease activity arises from p1/s1 was demonstrated by reduced or delayed enzymatic activities of *L. major* supernatant lacking the *p1/s1* cluster. In line with these results, complementation of *L. major* $\Delta p1/s1$ clone E10 with a genomically integrated *LmjF.30.1500* restored both activities. Additionally, secretion of p1/s1 was confirmed by detection in the culture supernatant, which is coherent with previous data on the *L. major* secretome and the *L. donovani* p1/s1 homologue *LdNuc^S* (138, 209). Localization of p1/s1 in intracellular parasites by fluorescence microscopy is pending. Both accumulation in the phagolysosomal lumen and intracellular retention within the parasite are conceivable.

iKO effects on the parasite pellet differ between 3'-nucleotidase and nuclease activity. While nucleotidase activity was not significantly reduced, nuclease activity was. It is plausible that this effect may be attributed to the enzymatic activity of *LmjF.30.1460-1490* products. These are putatively membrane-bound and are assigned as class I nucleases, but do not possess the p1/s1 active site. Potentially, they may only exhibit endonuclease, but not nucleotidase, activity.

The generated *L. major* $\Delta p1/s1$ parasites are only partial functional knockouts, as a remaining enzymatic activity was observed. Likely, redundant proteins that possess the same active site as p1/s1 and that are enriched in both knockout clones, are responsible for the remaining 3'-nucleotidases/nuclease activity. Presence of homologous 3'-nucleotidase/nuclease genes in the genome underline the importance of these enzymes for the parasites.

The endonuclease activity of p1/s1 is capable of degrading NETs and thereby enabling *L. major* to evade their leishmanicidal capacities, supporting previous reports on a

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3'-nucleotidase/nuclease from *L. infantum* (99, 114). Typically, *L. major* promastigotes are not killed when co-incubated with neutrophils, although they induce NET formation, which was consistent with the survival rate of the control strain observed in our experimental setup (102, 115). In contrast, *p1/s1* null mutants partially lost their ability to withstand NET-induced killing, which could be reversed by addition DNaseI as exogenous nuclease. Thus, a reduction in ecto-nuclease activity is sufficient to significantly affect parasite survival in the presence of neutrophils. Efficient escape from NETs through 3'-nucleotidase/nuclease function can enhance subsequent infection establishment. *Leishmania* thereby circumvent leishmanicidal effects of NETs and instead can efficiently utilize neutrophils as "Trojan horses" for later phagocytosis by macrophages (255).

Furthermore, ecto-nucleotidase activities of *Leishmania* have been described as virulence factors in mice infections (29, 68, 163, 200). Beyond the described effects on neutrophils, the generation of extracellular adenosine from nucleotides influences disease progression. Adenosine receptor-mediated signaling suppresses the immune response, linked to reduced pro-inflammatory cytokine secretion and nitric oxide synthesis (16, 25, 66). Elevated adenosine levels and adenosine receptor expression have also been associated with pathogenicity in patients with visceral leishmaniasis and immunosuppressive effects of *Phlebotomus* saliva can also be attributed to adenosine occurrence (143, 262, 263).

In the applied *in vitro* infection setting using human primary immune cells, addition of adenosine or 3'-AMP can slightly enhance parasite burden in macrophages, but only when hMDMs are co-cultured with PBLs. This hints to a T cell-mediated mechanism of intracellular parasite control. A stronger effect might be limited by the lack of active inducible nitric oxide synthase in hMDM upon *L. major* infection (265), a factor often associated with parasite restriction through adenosine signaling (163).

However, the expected dose-dependent reduction of $\text{TNF}\alpha$ following exposure to adenosine during infection was observed, which can be partially replicated by exposure to 3'-AMP. This implies the generation of adenosine from 3'-AMP by 3'-nucleotidases, providing a direct link to *p1/s1*. $\text{TNF}\alpha$ is strongly induced by *L. major* infection, but the presence of adenosine or 3'-AMP can prevent this induction, potentially suppressing an effective inflammatory immune response. Adenosine-dependent induction of IL-10 secretion from macrophages was tested, but could not be observed (data not shown).

5.3 p1/s1 as potential antileishmanial drug target

Alterations of further cytokines in response to adenosine were not tested, but adenosine is known to affect a broad range of cytokines, overall shifting the response from pro- to anti-inflammatory, also by repolarizing macrophages toward a M2d-like phenotype (9, 62, 94). Preliminary data on macrophage surface markers did not associate the presence of adenosine or AMP during infection with the loss of a pro-inflammatory M1-like phenotype (data not shown). However, low $\text{TNF}\alpha$ secretion already suggest a polarization toward this phenotype.

Additionally, no changes in A_{2a} or A_{2b} expression on hMDM upon infection were detected, in contrast to enhanced A_{2b} expression described for monocytes of human patients with active visceral leishmaniasis (263). This effect may be strain specific and not applicable for the cutaneous species *L. major*.

T cell proliferation and the balance between Th-1/-2 response are major determinants of *Leishmania* restriction and thus disease resolution. Previously, a link between $\text{TNF}\alpha$ and T cell proliferation during *L. major* infection was described (12). Indeed, application of adenosine or 3'-AMP reduced PBL proliferation four days post infection. Thus, degradation of 3'-AMP to adenosine can affect not only host cells themselves, but also modulates the cytokine milieu to orchestrate effector immune cells. How T cell differentiation and activation are affected during proliferation was not investigated, but could provide further insights into the immunomodulatory effects of extracellular adenosine. Conceivably, the observed inhibitory effect was mediated by effects originating from both macrophages and lymphocytes, as A_{2a} receptor activation can also directly suppress T cell function (9). The only mild correlation between $\text{TNF}\alpha$ levels and PBL proliferation underscores the influence of additional factors beyond this cytokine.

In summary, p1/s1 is a protein with manifold functions and is involved at multiple levels in the life cycle of *L. major*, see Figure 5.2. Justified by its dual activities as 3'-nucleotidase and endonuclease, as well as its extracellular localization, it is crucial for both nutrient acquisition and immune evasion. Thereby, it is not only providing the parasite with essential purines, but is also involved in the vast network of host-parasite interactions. By manipulating and shaping immune responses during the early stages of infection, 3'-nucleotidase/nuclease activity favors disease progression. While these properties apply to the promastigote stage prior to phagocytosis, the role of p1/s1 for intracellular amastigotes remains unsolved, but protein expression in this stage indicates relevant tasks. Recent results suggest that deletion of a 3'-nucleotidase/nuclease

5. DISCUSSION

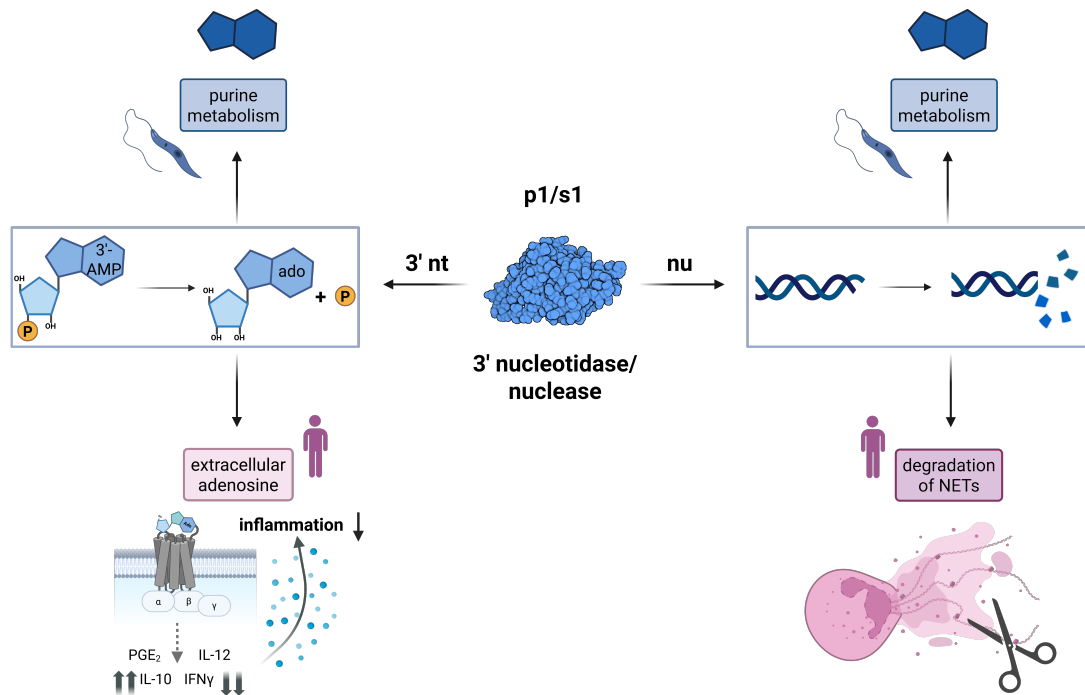


Figure 5.2: p1/s1, a 3'-nucleotidase/nuclease of *L. major*, has dual activity with implications in purine metabolism and immune evasion - The ecto-enzyme p1/s1 has dual 3'-nucleotidase and endonuclease activity, thus can hydrolyze 3'-nucleotides such as 3'-AMP (left) and degrade nucleic acids (right). Both functions are key in extracellular purine acquisition, an essential process for *Leishmania* that are otherwise purine auxotroph (upper). By generating extracellular adenosine from 3'-AMP, the parasites can also suppress inflammatory signaling on immune cells to evade an effective host immune response. The endonuclease activity allows promastigotes to degrade neutrophil extracellular traps (NETs). Escaping their antileishmanial capacities promotes infection establishment (lower). 3' nt: 3'-nucleotidase, ado: adenosine, NETs: neutrophil extracellular traps, nu: nuclease. Figure created with BioRender.

5.3 p1/s1 as potential antileishmanial drug target

of *L. infantum* promastigotes confers advantages in metacyclogenesis and transmissibility (95). Potentially, implications and consequences of 3'-nucleotidase/nuclease activity during the *Leishmania* life cycle vary, as reflected by stage-specific expression and activity. This may be a consequence of parasite adaptation to the challenges posed by different host microenvironments.

Whether p1/s1 and 3'-nucleotidases/nucleases in general are suitable drug targets for future therapeutic developments remains questionable. The effects observed in null mutant strains are limited due to the compensatory capacities of the parasite, which involve redundant enzymes. A pan-3'-nucleotidase/nuclease inhibitor could be used to study the role of this enzyme class for parasite survival and infectivity in sand flies and mammals. But due to sequence variations in close proximity to the active site of all *L. major* 3'-nucleotidases/nucleases, the rational design of such an inhibitor is highly challenging. A virtual screening for compounds binding into the active site of p1/s1 was complicated by the presence of a zinc atom in its active site. Therefore, our cooperation partners Peter Kolb and Jamal Shamsara estimated an expected hit rate of approximately 10%. As only a fraction of their predicted hits could be purchased from vendors, the lack of positive results does not permit a definitive conclusion regarding the success of their method. Among the predicted hits was tenofovir (no. 10), an adenine analog with antiviral activity, and L-ascorbic acid-2-phosphate derivatives (no. 45 & 46). Affinity of these compounds to the p1/s1 active site is conceivable due to their structural similarity with natural substrates.

In theory, active site binders can also evoke activation of the enzyme, so dose-response experiments on additional compounds should be conducted. Establishing a robust, upscalable 3'-nucleotidase/nuclease assay on recombinant p1/s1 could also advance inhibitor identification. So far, attempts to produce p1/s1 in *E. coli* have failed due to low solubility of the protein. Switching the expression system to non-pathogenic *Leishmania tarantolae* might resolve this issue, a suitable expression vector based on the pLexsy system is already available. In case of a potential inhibitor, assessing its effect on the endonuclease activity of p1 would also be necessary, although the semi-quantitative method used so far is not suitable for high- or medium-throughput screening. In contrast, the colorimetric determination of 3'-nucleotidase activity as plate-based method can easily be applied for extended screens.

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Despite these limitations, targeting proteins in the purine salvage pathway is of significant interest for antileishmanial drug development. Purine salvage is essential for trypanosomatids, but essentiality of 3'-nucleotidases/nuclease remains elusive. Enzymes further downstream of protozoan purine salvage pathways have been in the focus of drug research, given their essential role and the distinct nature of their processes compared to mammalian pathways. Consequently, numerous base derivatives have been characterized for their antileishmanial activity (13, 19, 252).

Several characteristics of 3'-nucleotidases/nucleases make them promising potential drug targets, see Figure 5.3: Such class I nucleases are expressed in the infective stages of a wide range of protozoan parasites, but they do not have mammalian homologues, thereby reducing the likelihood of host toxicity (138, 161, 191).

Eliciting effects on intracellular pathogens is challenging, especially in the case of *Leishmania*. A drug needs to cross three differently composed membranes: The macrophage membrane, the phagolysosomal membrane, and the amastigote membrane, while encountering pH differences along the way. Since 3'-nucleotidases/nucleases are ectoenzymes, they are relatively accessible to an inhibitor, as they face the parasites' extracellular environment. Whether a prompt inhibition of p1/s1 is sufficient to eliminate intracellular amastigotes or is too prone to eliciting resistance remains to be studied. How p1/s1 influences host–amastigote interactions during the later stages of infection is also unknown. Although its immunosuppressive activities are beneficial for *Leishmania* during early infection, these activities may be negligible following parasite phagocytosis. Nevertheless, developing a specific p1/s1 inhibitor could be crucial for elucidating the role of nucleases in established infections and transmissibility in sand flies.

This comprehensive study on p1/s1, a secreted bifunctional 3'-nucleotidase/nuclease of *L. major*, elucidated and emphasized its central role through its multifaceted involvement in infection establishment. These findings underscore the importance of this enzyme class in understanding the molecular dynamics of *Leishmania* infection. Overall, protozoan 3'-nucleotidases/nucleases are highly relevant across multiple aspects of the life cycle and play central roles for parasite nutrition and host-pathogen interactions. Additionally, they represent promising drug targets for future therapeutic development.

5.3 p1/s1 as potential antileishmanial drug target

Properties of p1/s1 as a drug target

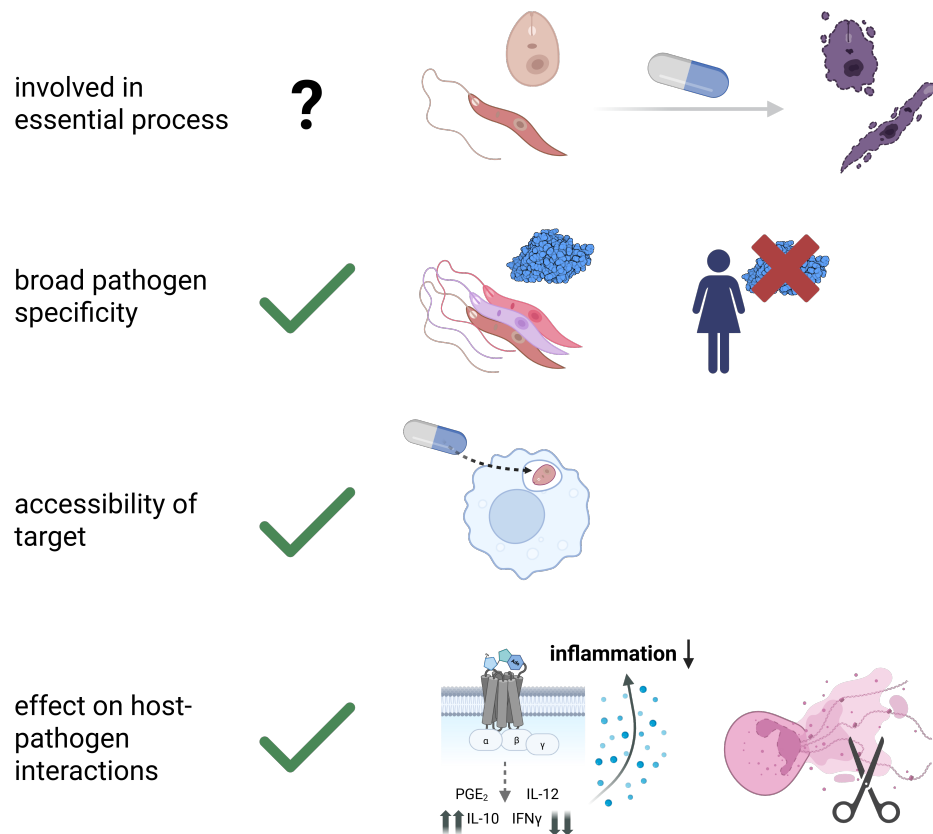


Figure 5.3: p1/s1 as 3'-nucleotidases/nuclease fulfills several properties of a beneficial drug target - Foremost, inhibition of a drug target should lead to death of the parasite. Essentiality of p1/s1 remains unclear but a central role in purine metabolism can be assumed (top). Whether complete inhibition of 3'-nucleotidase/nuclease activity is lethal needs to be determined in the future.

Otherwise, several characteristics of a promising drug target are fulfilled: 3'-nucleotidases/nucleases such p1/s1 are expressed in the infective stages of a broad range of pathogens. As ecto-enzymes, an inhibitor would not need to cross the parasite membrane, enhancing accessibility. Additionally, mechanisms of immune evasion mediated by 3'-nucleotidases/nucleases can be prevented through inhibition of these activities.

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5.4 Conclusion and Outlook

In the first part of this project, several compounds were provided by cooperation partners for testing of previously unidentified *in vitro* antileishmanial potential. Although pateamines elicited stronger toxic effects on macrophages than on *L. major* amastigotes, their basic activity against *L. major* promastigotes served as a proof-of-principle for the molecular mode of action of this compound class. Arylmethylaminosteroids successfully completed the testing workflow and reduced intracellular parasite burden, but their pharmacological profile discourages further development. In contrast, well-established drugs were selected based on pharmacophore criteria of the arylmethylaminosteroid class and identified as leishmanicidal with an exceptionally high hit rate. These compounds are scheduled for intracellular efficacy assessment and may benefit from established pharmacological characterization in future development. In summary, the initial hypothesis was supported by these findings: Among these repurposed compounds, several are promising candidates for upcoming lead identification and subsequent development as therapeutic agents.

Selectivity of compounds for parasite targets remains a major challenge. Host toxicity is often a severe drawback of antileishmanial compounds, as relatively high concentrations are required for axenic parasite killing. Intracellular elimination in an infection setting is even more challenging due to the presence of multiple membranes that act as physical barriers. Beyond these biological obstacles, the substantial effort required for a treatment to reach market authorization is economically unattractive to the pharmaceutical industry due to the status of leishmaniasis as a "disease of the poor". Whether any of the characterized compounds are suitable for preclinical testing must be determined in further experiments, particularly using suitable *in vivo* infection models.

In the second part of this thesis, no cell death-linked proteins were discovered in *L. major* to confirm the existence of a programmed cell death machinery in these parasites that may be exploited for drug development. Thus, whether cell death events in *Leishmania* are incidental or regulated remains still unanswered. Until the identification and characterization of dedicated signaling cascades, their presence or absence cannot be declared for certain.

Although p1/s1 was initially hypothesized to be a potential cell death executioner participating in DNA fragmentation due to enrichment in sub-lethal stress, it could not be

linked to parasite cell death mechanisms. Instead, its dual enzymatic activities appear to be part of a general stress response. Nevertheless, p1/s1 was established as a crucial component of the parasite's salvage and immunomodulatory machinery, with multiple functions related to host-parasite interactions and parasite metabolism. p1/s1 fulfills several criteria for a promising drug target, but at the same time, it is very challenging target due to its structure and redundancy to other enzymes.

Leishmaniasis remains a global health burden requiring continued attention to develop new therapeutic strategies. This project contributes valuable insights into the novel antileishmanial activities of several compounds and advances our understanding of the molecular strategies employed by *L. major* to survive in its diverse host environments.

5. DISCUSSION

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Acknowledgments

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Appendix

Appendix: Oligonucleotide List - Oligonucleotides used for knockout generation of putative PCD-linked genes and their corresponding sequence; iKO: Inducible knockout generation, KO: Knockout generation, CDS: Coding sequence, UTR: Untranslated region

Use	Target & Direction	5'-3' Sequence
KO	<i>LmjF.34.0240</i> CARD-like protein 5' sgRNA template	GAAATTAATACGACTCACTATA GGCTCGCACGGTCCTCGAGA ATGTTTTAGAGCTAGAAATAGC
KO	<i>LmjF.34.0240</i> CARD-like protein 3' sgRNA template	GAAATTAATACGACTCACTATA GGGTGTGCGTACGCGCCTGT CGGTTTTAGAGCTAGAAATAGC
KO	<i>LmjF.34.0240</i> CARD-like protein 5' HR	TGCAGCGGTCTGCCATCGTCGACC GCCCCGTATAATGCAGACCTGCTGC
KO	<i>LmjF.34.0240</i> CARD-like protein 3' HR	GGGATGGGGCAGCGAAGAGTGAGAG TACCGCCAATTTGAGAGACCTGTGC
KO confirmation	<i>LmjF.34.0240</i> CARD-like protein 5' UTR fwd	CGCGCTTGATCCAATGGC
KO confirmation	<i>LmjF.34.0240</i> CARD-like protein 3' UTR rev	CAGATGAGCGACACGTATGTG
KO confirmation	<i>LmjF.34.0240</i> CARD-like protein CDS fwd	GGACAGAGTGGGTGTCCAAG
KO confirmation	<i>LmjF.34.0240</i> CARD-like protein CDS rev	CGTCCGCTTTTTTCACAGCTC

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KO	<i>LmjF.02.0450</i> VDAC 5' sgRNA template	GAAATTAATACGACTCACTATAGGTG GGGTGTGCCGATTAACAGGT TTTAGAGCTAGAAATAGC
KO	<i>LmjF.02.0450</i> VDAC 3' sgRNA template	GAAATTAATACGACTCACTATAGGGT GGAGTCCACTACGCTCGCGT TTTAGAGCTAGAAATAGC
KO	<i>LmjF.02.0450</i> VDAC 5' HR	CGTGTCTCTGTCTCTCTCTCTCTCT CTCCTGTATAATGCAGACCTGCTGC
KO	<i>LmjF.02.0450</i> VDAC 3' HR	AGGGCGCGCGTCTCGGCAAGAGCAAGA TGCCGCCAATTTGAGAGACCTGTGC
KO confirmation	<i>LmjF.02.0450</i> VDAC 5' UTR fwd	CCACCATCGCCATCACCATC
KO confirmation	<i>LmjF.02.0450</i> VDAC 3' UTR rev	CATCCACACATCCACACCTCC
KO confirmation	<i>LmjF.02.0450</i> VDAC CDS fwd	GTGGAAGGTCGAGAGCAAGG
KO confirmation	<i>LmjF.02.0450</i> VDAC CDS rev	CATCGTCTGGTGGTTGACCT
KO	<i>LmjF.02.0460</i> VDAC 5' sgRNA template	GAAATTAATACGACTCACTATAGGCG CTGAAAAGGGTGATGGAAGT TTTAGAGCTAGAAATAGC
KO	<i>LmjF.02.0460</i> VDAC 3' sgRNA template	GAAATTAATACGACTCACTATAGGCG CTGAAGGACATGCAAGCAGT TTTAGAGCTAGAAATAGC
KO	<i>LmjF.02.0460</i> VDAC 5' HR	TTCCTTGCTTGCCCCCCTCCCCCCT CTCCCGTATAATGCAGACCTGCTGC
KO	<i>LmjF.02.0460</i> VDAC 3' HR	CGTAGATACTGCCCCACTTTCGCTAT TGCCACCAATTTGAGAGACCTGTGC
KO confirmation	<i>LmjF.02.0460</i> VDAC 5' UTR fwd	CGGCAGTATCATTTGCTGTCTGC
KO confirmation	<i>LmjF.02.0460</i> VDAC 3' UTR rev	CGCGAGTGAGGTGGAGACAG

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KO confirmation	<i>LmjF.02.0460</i> VDAC CDS fwd	GACTTCCCCACCGCGAATAA
KO confirmation	<i>LmjF.02.0460</i> VDAC CDS rev	CGTGACTGACCTCGTAGTGG
KO	<i>LmjF.28.1920</i> PCD5 5' sgRNA template	GAAATTAATACGACTCACTATAGGTG ACAGGCACAGGAACTTGCGT TTTAGAGCTAGAAATAGC
KO	<i>LmjF.28.1920</i> PCD5 3' sgRNA template	GAAATTAATACGACTCACTATAGGGA AGTAACAAGACCGGTGCAGT TTTAGAGCTAGAAATAGC
KO	<i>LmjF.28.1920</i> PCD5 5' HR	GCACGCACACCAACCGAGCCACGTA CTCCGGTATAATGCAGACCTGCTGC
KO	<i>LmjF.28.1920</i> PCD5 3' HR	ACTCGCCAACTTTGCGGCTCGCCAC CGCCCCCAATTTGAGAGACCTGTGC
KO confirmation	<i>LmjF.28.1920</i> PCD5 5' UTR fwd	CTTGACAAGGCACGCCTTC
KO confirmation	<i>LmjF.28.1920</i> PCD5 3' UTR rev	CGATTCTCATCTGCTGCAGTG
KO confirmation	<i>LmjF.28.1920</i> PCD5 CDS fwd	GCTCCCAGGCTGTAGAGATG
KO confirmation	<i>LmjF.28.1920</i> PCD5 CDS rev	CAGTCATCATCCGTGCGCTT
KO	<i>LmjF.13.1460</i> PCD6 5' sgRNA template	GAAATTAATACGACTCACTATAGGTA GACGAGACGCAAGCGACAGT TTTAGAGCTAGAAATAGC
KO	<i>LmjF.13.1460</i> PCD6 3' sgRNA template	GAAATTAATACGACTCACTATAGGGA GTGCGAGTAGGTGAGTCTGT TTTAGAGCTAGAAATAGC
KO	<i>LmjF.13.1460</i> PCD6 5' HR	TCTTTCTCTTTCGCCCCACGAACAGC TGCCTGTATAATGCAGACCTGCTGC
KO	<i>LmjF.13.1460</i> PCD6 3' HR	CCTTCGTCCCTCCCCTCCCCCTCTC CGCCGCCAATTTGAGAGACCTGTGC

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KO confirmation	<i>LmjF.13.1460</i> PCD6 5' UTR fwd	CTCTTGTTCTCCCTCACCGC
KO confirmation	<i>LmjF.13.1460</i> PCD6 3' UTR rev	CGGAGAGCCAGAAGAATGACC
KO confirmation	<i>LmjF.13.1460</i> PCD6 CDS fwd	CGCATACCTCGGCTACACTC
KO confirmation	<i>LmjF.13.1460</i> PCD6 CDS rev	CGGAACCACTCCATCAGCTC
KO, iKO	<i>LmjF.35.4590</i> PSD 5' sgRNA template	GAAATTAATACGACTCACTATAGGGC TGTGCAGCGCTGCGACAGGT TTTAGAGCTAGAAATAGC
KO, iKO	<i>LmjF.35.4590</i> PSD 3' sgRNA template	GAAATTAATACGACTCACTATAGGGT GACGAAAGACGAACCTTAGT TTTAGAGCTAGAAATAGC
KO, iKO	<i>LmjF.35.4590</i> PSD 5' HR fwd	CTCTTGAGTGTTGCACGACGCACCAT CCCAGTATAATGCAGACCTGCTGC
iKO	<i>LmjF.35.4590</i> PSD 5' HR rev	CAACGGAGGCGTGATGCAAAATGGC GCCATCCAATTTGAGAGACCTGTGC
KO	<i>LmjF.35.4590</i> PSD 3' HR	GAGCCCTTTACACACACACACACAC AAGCTCCAATTTGAGAGACCTGTGC
iKO	<i>LmjF.35.4590</i> PSD 3' HR fwd	CTCTTGAGTGTTGCACGACGCACCA TCCCAGCCCGATTACGCATGATCTA
iKO	<i>LmjF.35.4590</i> PSD 3' HR rev	GAGCCCTTTACACACACACACACAC AAGCTAAGAAAGCTGGGTTCATGG
KO confirmation	<i>LmjF.35.4590</i> PSD 5' UTR fwd	GCTGCTTAGGCACCGACAC
KO confirmation	<i>LmjF.35.4590</i> PSD 3' UTR rev	CAACGTCCGTTTTCGAACCACC
KO confirmation	<i>LmjF.35.4590</i> PSD CDS fwd	AACGCGAATGTGGAAAGCAC

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KO confirmation	<i>LmjF.35.4590</i> PSD CDS rev	TATGAGGCGTTTCGTTGAGGG
KO, iKO	<i>LmjF.14.1200</i> PSS 5' sgRNA template	GAAATTAATACGACTCACTATAGGTG CGTGATGCGTGCTATGTAGT TTTAGAGCTAGAAATAGC
KO, iKO	<i>LmjF.14.1200</i> PSS 3' sgRNA template	GAAATTAATACGACTCACTATAGGAG AGAAGGCGACGGACAAACGT TTTAGAGCTAGAAATAGC
KO, iKO	<i>LmjF.14.1200</i> PSS 5' HR fwd	CCCATCTTCTCCCTCTTTTCTGCC TATCCTGTATAATGCAGACCTGCTGC
iKO	<i>LmjF.14.1200</i> PSS 5' HR rev	CGACGCATGCGTGTGCGTCAAGGA CGAACCCCAATTTGAGAGACCTGTGC
KO	<i>LmjF.14.1200</i> PSS 3' HR	TGAGCTGCCCCACCCACCCACCCCCC CCCCTCCAATTTGAGAGACCTGTGC
iKO	<i>LmjF.14.1200</i> PSS 3' HR fwd	TTTGGCGCTAGCGCTGACAGAGCGA GAGGTGCCCCGATTACGCATGATCTA
iKO	<i>LmjF.14.1200</i> PSS 3' HR rev	TGAGCTGCCCCACCCACCCACCCCCC CCCCTAAGAAAGCTGGGTTCATGG
KO confirmation	<i>LmjF.14.1200</i> PSS 5' UTR fwd	GACGCTCCATGGTTGCTGC
KO confirmation	<i>LmjF.14.1200</i> PSS 3' UTR rev	ATGAGAGAAGGAAGGTGGAGCAGAC
KO confirmation	<i>LmjF.14.1200</i> PSS CDS fwd	ATGCGTCGAGGTCCGGATGA
KO confirmation	<i>LmjF.14.1200</i> PSS CDS rev	CGTACAGCTCTCGCAAGCACGA
KO confirmation	Blasticidin resistance fwd	GCCGCATCTTCACTGGTGTCAA
KO confirmation	Blasticidin resistance rev	TCGGCTGTCCATCACTGTCCT
KO confirmation	Neomycin resistance fwd	CCATTGAACAAGATGGATTGCACGC

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KO confirmation	Neomycin resistance rev	CGGCCATTTTCCACCATGATATT
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Appendix: Proteins exclusively detected or enriched under cell death pressure in a proteomics analysis of *L. major*
- Log₂ ratio and *p*-value derived from a Benjamini-Hochberg corrected multiple t-test for each comparison against the untreated logPh sample, details to Table 4.5.

UniProt code	UniProt annotation	log ₂ ratio statPh	<i>p</i> -value statPh	log ₂ ratio 3 h Miltefosine	<i>p</i> -value 3 h Miltefosine	log ₂ ratio 6 h Staurosporine	<i>p</i> -value 6 h Staurosporine
Q4Q7F3	p1/s1 nuclease	18.43	4.59×10^{-8}	17.83	2.06×10^{-4}	18.75	4.82×10^{-7}
Q4Q165	Putative polyubiquitin	18.63	4.95×10^{-6}	17.37	3.46×10^{-6}	19.56	9.05×10^{-4}
Q4QE18	Elongation factor 1 α	22.09	1.36×10^{-10}	21.89	3.67×10^{-11}	22.07	1.98×10^{-8}
E9AEM1	Arrestin-like N- terminal domain- containing protein	15.58	6.59×10^{-3}	16.49	3.95×10^{-3}	16.097	7.12×10^{-3}
Q4QDQ9	Mitochondrial carrier protein	2.63	1.23×10^{-9}	1.69	1.18×10^{-6}	4	5.2×10^{-6}
Q4QF48	EF-hand domain- containing protein	2.26	0.01	1.92	1.49×10^{-2}	2.24	0.02
Q4Q6D6	p-glycoprotein e	1.78	2.6×10^{-2}	1.93	1.7×10^{-3}	1.61	5.82×10^{-4}
P48157	Large ribosomal subunit protein uL5	3.11	3.87×10^{-2}	2.23	6.32×10^{-3}	2.04	1.06×10^{-5}
Q4QH12	Putative folate/ biopterin trans- porter	3.63	2.03×10^{-7}	2.58	1.27×10^{-5}	1.88	5.99×10^{-4}

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Curriculum Vitae

CURRICULUM VITAE

CURRICULUM VITAE
