Investigation into the dysregulation of pattern recognition receptor signalling in *Leishmania mexicana* infected THP-1 cells

A thesis submitted for the degree of Doctor of Philosophy

Rebecca Jane Barker

Department of Biomedical and Life Sciences

Lancaster University

2023

Declaration

I declare that this thesis is my own work and has not been submitted in part, or as a whole, for the award of a higher degree elsewhere.

Table of Contents

List of Tables

List of Figures

Abstract

Leishmaniasis is a neglected tropical disease caused by parasites of the *Leishmania* genus and is spread to humans by sandfly bite. Leishmaniasis is the second largest parasitic killer after Malaria and manifests a wide variety of symptoms from disfiguring skin lesions to fatal liver damage. Despite approximately 1 billion people living in endemic areas, with 1 million new cases occurring each year, treatment options for the disease remain limited, and a successful vaccine is yet to be developed.

The lack of successful control of Leishmaniasis can be partially attributed to an incomplete knowledge of how *Leishmania* establishes infection. The parasites are unusual as they infect and multiply inside macrophages; specialised innate immune cells that usually detect and destroy invading pathogens. Therefore to replicate, *Leishmania* successfully evades the hosts innate immune response in order to infect macrophages and persist within them. *Leishmania* express numerous virulence factors to aid infectivity by inhibiting host signalling pathways, some of which are downstream of pathogen recognition receptors (PRRs). After recognition of pathogens by the various PRRs, downstream signalling can activate host microbicidal and pro-inflammatory responses to eliminate the infection.

By combining parasite cell culture and molecular biology techniques, we examined how *Leishmania mexicana* may disrupt the pathogen sensing and communication pathways of the human macrophage. We focused on parasite interactions with PRRs, especially the cytoplasmic nucleic acid sensors as there has been little investigation concerning them in the context of *Leishmania* infection.

After establishing a successful *in vitro L. mexicana* infection within human THP-1 macrophages, we discovered that these cells had an enhanced response to transfected dsDNA following *L. mexicana* infection. This was observed by the increased activation of a key adaptor protein stimulator of interferon genes (STING), in the cGAS-STING DNA sensing pathway and its downstream targets, in addition to an increased cytokine response to dsDNA. Through protein analysis we also observed that STING and other key components of the DNA sensing pathway are modified during infection.

Through further experiments we identified the modification of STING as a cleavage event resulting in partial loss of its C-terminal tail. While cleavage to this form is highly

upregulated during infection, we have suggested that this alternative form of STING is a naturally occurring variant and may have a regulatory role under normal conditions that *Leishmania* parasites could exploit for their own benefit.

As with THP-1 cells, cleavage of the C-terminal tail of STING also occurs after infection of mouse bone marrow derived macrophages (BMDMs) and human keratinocytes (HaCaT line). This confirms that this parasite virulence activity is not isolated to one host species, or to the primary host cell type of *Leishmania.* However, the cleavage of STING in the mouse BMDMs and human keratinocytes was not accompanied by the enhancement of the dsDNA seen in infected THP-1 cells.

Intriguingly DNA sensors cGAS and IF16 also appear to be modified during *Leishmania mexicana* infection of both THP-1 macrophages and HaCaT keratinocytes. Unlike STING we have not yet been able to ascertain the exact nature of these modifications, but we have observed that they do not seem to prevent their function or downstream signalling of the cGAS-STING DNA sensing pathway.

Investigation of extracellular vesicles (EVs) released from infected THP-1 cells revealed that their cargo was altered during infection to likely include *Leishmania* derived proteins and DNA. Uninfected macrophages treated with these modified EVs also had an upregulation of cleaved STING, demonstrating whole parasite infection is not required for its modification. This also suggests that the immunomodulatory actions of *Leishmania* can be mediated by exploiting host vesicle secretion.

As the initial immune response to *Leishmania* infection has a significant impact on the course of the disease, identifying new host targets of parasite virulence factors will be crucial to increasing understanding of Leishmaniasis and may provide future therapeutic targets. Therefore, investigation of STING and other components of the cGAS-STING DNA sensing pathway is necessary to fully understand their role during *Leishmania* infection and thus identify therapeutic targets.

Acknowledgements

I would firstly like to thank my supervisor Dr Leonie Unterholzner for her support, guidance, and wisdom throughout this project. In addition, I must also thank Professor Paul Bates, who was crucial to the project development and provided all of the *Leishmania mexicana* stocks used in this thesis. I would also like to thank the Faculty of Health and Medicine for providing funding throughout my PhD and making it possible for me to complete this research. Thank you to the many past and present members of the Unterholzner Lab, especially fellow PB's Jessica and Gillian, and more recently Tom and Otto. Your company and support in the lab as well as feedback during lab meetings has been invaluable to the progress of my PhD.

A special thank you to the newly qualified Dr Jessica Hammond for her encouragement, cake, and advice over the years. and as well as the rest of office C17 for your friendship when most needed. Thank you to the technicians and department staff that have supported my research and helped me to learn essential skills over the last few years especially members of the Jackson-Jones lab and Professor Nigel Fullwood.

Finally thank you to all my supporters outside of Lancaster including my family and Mattea for encouraging me in so many ways throughout several challenging years.

Abbreviations

Acyl-CoA – Acyl co-enzyme A

APC – Antigen presenting cells

AP-1 – Activator protein 1

APS – Ammonium persulfate

ATP - Adenosine Triphosphate

BMDMs- Bone marrow derived macrophages

BME – Basal Medium Eagle

CARD - Caspase Activation and Recruitment Domains

CCL5 - Chemokine (C-C motif) ligand 5

CCL20 – C-C Motif Chemokine Ligand 20

CCR7 - C-C Chemokine Receptor Type 7

cDNA – Complementary DNA

cGAMP – Cyclic GMP-AMP

cGAS - Cyclic GMP-AMP Synthase

CL-Cutaneous Leishmaniasis

CPs – Cysteine protease

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

CSK – C-terminal Src kinase

CTD –Carboxy terminal domain

CTT - C-Terminal Tail

CXCL10 –C-X-C Motif Chemokine Ligand 10

DAI – DNA-dependent activator of IRFs

DALY – Disability-adjusted life year

DAMP – Damage Associated molecular patterns

DAPI – 4',6-Diamidino-2-phenylindole

DCL – diffuse cutaneous Leishmaniasis

DDX41–DEAD box helicase 41

DENV – dengue fever virus

DMEM - Dulbecco's Modified Eagle's Medium

DNA – Deoxyribonucleic acid

DNA-PK – DNA-dependent Protein Kinase

dNTPs - Deoxynucleotide Triphosphates

dsDNA – Double-stranded DNA

EDTA – Ethylenediamine tetraacetic acid

EF-1α – elongation factor-1α

EGTA – egtazic acid

ER – endoplasmic reticulum

ERK – Extracellular Signal Regulated Kinases

EV – Extracellular Vesicle

FCS – Fetal Calf Serum

GP63 – glycoprotein63

GTP – Guanosine-5'-triphosphate

HA – hemagglutinin

HASPB – hydrophilic acylated surface protein B

HD– Huntington's disease

HEK293T – Human Embryonic Kidney 293 (SV40 large T antigen)

HIV – Human Immunodeficiency Virus

HRP – Horse Radish Peroxidase

HSP –Heat shock protein

HSV-1 - Herpes Simplex Virus 1

HT-DNA – Herring Testis DNA

IFI16 – Interferon-γ Inducible Protein 16

IFN – Interferon

IgG – Immunoglobulin G

IKK – IκB Kinase

IL – Interleukin

IRAK - Interleukin 1 Receptor Associated Kinase

IRF – Interferon Regulatory Factor

ISG – Interferon Stimulated Genes

JAK – Janus Kinase

JNK – Jun N-terminal kinase

kDa – Kilodalton

kDNA-Kinetoplast Deoxyribonucleic acid

KMP-11– kinetoplastid membrane protein-11

KO – Knock Out

LACK –*Leishmania* homologue for receptors of activated C kinase

LB – Luria broth

LBD – Ligand binding domain

LGP2– Laboratory of Genetics and Physiology 2

LPG –lipophosphoglycan

LPS – Lipopolysaccharide

LRV1 –*Leishmania* RNA virus 1

M199 – Medium 199

MAPK – mitogen-activated protein kinases

MAVS – Mitochondrial Antiviral Signaling Protein

M-CSF – macrophage colony-stimulating factor

MDA5 –melanoma differentiation-associated protein 5

MHC – major histocompatibility complex

mHTT – mutation of the N-terminal polyglutamine of huntingtin protein

ML –mucocutaneous leishmaniasis

MRE11 – Meiotic Recombination 11

MR –Mannose receptor

mRNA – messenger Ribonucleic acid

MS – Mass spectrometry

MyD88 - Myeloid Differentiation primary response gene 88

NEMO– NF-kappa-B essential modulator

NETs–neutrophil extracellular traps

NFκB - Nuclear Factor Kappa-light-chain enhancer of activated B cells

NLR – NOD-Like Receptor

NLRP3– NLR family pyrin domain containing 3

NO – Nitric oxide

NOD - Nucleotide-binding domain,

NTD – Neglected Tropical Disease

PAMP – Pathogen-Associated Molecular Pattern

PBS - Phosphate-buffered saline

PCR – Polymerase Chain Reaction

PKC – protein kinase C

PMA - Phorbol 12-myristate 13-acetate

PMSF – phenylmethylsulfonyl fluoride

Poly(I:C) – polyinosinic–polycytidylic acid

PRR – Pattern Recognition Receptor

PTMs – Post-Translational Modifications

PTP– protein tyrosine phosphatase

qRT-PCR – quantitative Reverse Transcription Polymerase Chain Reaction

RIG-I - Retinoic acid-Inducible Gene I

RLRs – RIG-I-like receptors

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RPA – Replication Protein A

RPMI – Roswell Park Memorial Institute Medium

RUP – regulated ubiquitin/proteasome-dependent processing (RUP).

SAVI– STING-associated vasculopathy with onset in infancy

SDS – Sodium Dodecyl Sulphate

SDS-PAGE – Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SFM –serum free media

SHP-1 –Src homology region 2

SIDSP– STING independent DNA sensing pathway

SOD– superoxide dismutase

ssDNA – Single-stranded DNA

ssRNA – Single-stranded RNA

STAT1 (Signal Transducer and Activator of Transcription-1) 1

STING – Stimulator of Interferon Genes

SUMO – Small Ubiquitin-like Modifier

TAE –Tris-acetate-EDTA

TAK 1–TGF-β- activated kinase 1

TBK1– TANK-Binding Kinase 1

TBS – Tris Buffered Saline

TEM –Transmission electron microscopy

TGF-β– Transforming growth factor-β

Th – T helper

TIR – Toll/Il-1 Receptor

TLR – Toll-like Receptor

TMD– transmembrane domain

TMEM173 - Transmembrane Protein 173

TNF – Tumour Necrosis Factor

TRAF– TNF receptor associated factors

TRAIL – TNF-related apoptosis-inducing ligand

TRIF - TIR domain containing adaptor inducing IFN

ULK1– Unc-51 like autophagy activating kinase

VL– Visceral leishmaniasis

VSG– Variant Surface Glycoprotein

WT – Wild Typ

Chapter 1: Introduction

1.1. Introduction to Leishmaniasis

Leishmania parasites are the causative agents of a group of diseases collectively named Leishmaniasis. Leishmaniasis is thought to affect around 12 million people worldwide (Alvar et al., 2012), although it is thought that over 1 billion people live in endemic areas and are susceptible to infection (WHO, 2023). Leishmaniasis affects both humans and animals and is spread by the bite of infected sand flies of the phlebotomine species and manifests a wide spectrum of symptoms that is dependent on the species of infecting parasite and immune status of the host.

Leishmaniasis has been named a neglected tropical disease (NTD) by the World Health Organisation due to its prevalence in low-income populations and limited treatments, many of which have unwanted side effects. Poverty is key risk factor for developing the disease (Alvar et al., 2006) and the associated disability that can result from Leishmaniasis is thought to perpetuate the poverty cycle (Okwor and Uzonna, 2016). Among the NTDs Leishmaniasis has both a high mortality and morbidity rate. In the Global Burden of Disease study 2019, (Vos, 2020) it was estimated that up to 862,000 new cases of all forms of the disease may occur each year. This is predicted to result in almost 19,000 deaths and 1.6 million Disability-adjusted life years (DALYs) lost (Vos, 2020).

The disease has been brought to the public eye in the past decade as several countries in the Middle East have seen a re-emergence of Leishmaniasis due to ongoing conflict and political upheaval (Bizri et al., 2021; Karami et al., 2022; Salam et al., 2014). Other contemporary issues concerning Leishmaniasis include the exponential increase in canine Leishmaniasis cases in Spain and other southern European countries (Le Rutte et al., 2018; Mas et al., 2020; Morales-Yuste et al., 2022), as well as the emergence of human Leishmaniasis cases in Europe (Vaselek, 2021; Arce et al., 2013; Ready, 2010). Similarly

there has recently been an increase in both autochthonous and imported human infections being reported in southern USA (Bradley et al., 2013; Curtin and Aronson, 2021; McIlwee et al., 2018).

In the future the number of those at risk of *Leishmania* infection could increase dramatically- in both canine and human cases. While the recognition of Leishmaniasis as a public health problem is increasing, there is still a clear lack of safe and effective drugs and vector control methods. More than ever a deeper understanding of this parasite and how it can infect and propagate within human cells is necessary if more successful therapeutic treatments and vaccines are to be developed to allow more successful control of the disease.

1.1.1 Disease Presentation

More than 20 species of *Leishmania* are known to cause disease in humans (Pearson and Sousa, 1996) and the clinical presentation and outcome of the disease can vary widely depending on the parasite species. The main disease-causing species are summarised in Table 1. The three major forms of the disease are cutaneous, mucocutaneous and visceral Leishmaniasis and vary greatly in symptoms, pathologies and clinical outlook.

Cutaneous Leishmaniasis

Cutaneous Leishmaniasis (CL) is the most common and widespread form of the disease and generally considered to be the mildest, especially as the associated cutaneous lesions tend to self-heal without intervention and are rarely fatal. In 2021 there were 221, 953 new CL cases reported to the WHO, although it is expected that many new cases remain unreported (Ruiz-Postigo et al., 2022). *Leishmania* species such as *L.major, L.mexicana, L.braziliensis* and *L.guyanensis* cause this form among many others (WHO, 2010).

The disease is characterised by the presence of painful ulcerous skin lesions localised at the site of the sand fly bite. These lesions can vary between patients in severity, size and clinical appearance. Some lesions will self-heal without intervention within 3- 9 months, with the time generally depending on which *Leishmania* species is present. However resolved lesions usually result in scarring which can be socially debilitating for some patients and can cause isolation and social exclusion (Bennis et al., 2018; Bilgic-Temel et al., 2019).

Cutaneous Leishmaniasis can progress into more severe forms of the diseases such as diffuse cutaneous Leishmaniasis (DCL) or Mucocutaneous Leishmaniasis (ML). DCL is a rare variant of CL characterized by multiple widespread, slowly progressive, non-ulcerating nodules (Couppié et al., 2004).

Mucocutaneous Leishmaniasis

This form of the disease is characterised by the ulceration and destruction of mucocutaneous tissue. *Leishmania* species causing ML promote metastasis to mucosal tissues of the mouth and respiratory tract by lymphatic or haematogenous dissemination. Commonly the nasal mucosa is affected and perforation of the septum can occur (Reithinger et al., 2007). The lips, cheeks and larynx can also be involved leading to severe facial disfigurement and psychological trauma as ML does not heal spontaneously and is very difficult to treat successfully (Reithinger et al., 2007). Complications arising from this form of the disease include breathing difficulties and frequent secondary bacterial infections. The most common cause of death from this form of *Leishmania*sis is Pneumonia (WHO, 2010). ML is most commonly associated with *L.braziliensis* but other species have also been found to cause this form (Marsden, 1986).

Table 1: The main species of *Leishmania* that cause disease in humans. Adapted from (WHO, 2010).

Disease manifestation and geographical location	***Leishmania* Species**	
Old World (Africa, Asia, Middle East and Europe)	**Subgenus *Leishmania***	**Subgenus *Vianna***
Visceral Leishmaniasis	*Leishmania donovani and Leishmania infantum*	-
Cutaneous Leishmaniasis	*Leishmania major, Leishmania tropica and Leishmania aethiopica*	-
Diffuse Cutaneous Leishmaniasis	*Leishmania aethiopica*	-
New World (The Americas)	**Subgenus *Leishmania***	**Subgenus *Vianna***
Visceral Leishmaniasis	*Leishmania infantum*	-
Cutaneous Leishmaniasis	*Leishmania infantum, Leishmania mexicana, Leishmania pifanol and Leishmania amazonensis*	*Leishmania braziliensis, Leishmania guyanensis, Leishmania panamensis and Leishmania peruviana*
Diffuse Cutaneous Leishmaniasis	*Leishmania mexicana and Leishmania amazonensis*	-
Mucocutaneous Leishmaniasis	-	*Leishmania braziliensis and Leishmania panamenis*

Visceral Leishmaniasis

Visceral *Leishmania*sis, more commonly known as Kala-azar in India is the most severe form of *Leishmania*sis and without treatment has a high fatality rate. *Leishmania donovani* is the primary cause of visceral *Leishmania*sis in the Indian subcontinent and East Africa, *L.infantum* in the Mediterranean region, and *L.chagasi* in the New World (Guerin et al., 2002).

In 2021 11, 743 new VL cases were reported (Ruiz-Postigo et al., 2022) with many more predicted to be unreported. Malnutrition and immune suppression, notably due to Human Immunodeficiency Virus (HIV) infection, are key risk factors (WHO, 2010). This form of the disease occurs when the parasites migrate to internal organs such as the liver, spleen and the bone marrow. Patients can present with a wide variety of symptoms from fever, malaise, weight loss, abdominal pain, splenomegaly, hepatomegaly and pancytopenia. Death from visceral *Leishmania*sis can be indirect as opportunistic infections (i.e. pneumonia and tuberculosis) occur as a result of a highly depleted immune system (WHO, 2010).

A complication of VL that is becoming more and more prominent is co-infection with HIV. The number of cases of co-infection continue to grow as *Leishmania* is endemic in similar regions as HIV. These cases present further challenges to medical staff as the diagnosis of co-infected patients is difficult, and often challenging to treat (Guerin et al., 2002). Infection of immune cells with *Leishmania* has been shown to enhance the replication of HIV, for example by causing overexpression of CCR5, a co-receptor that allows HIV entry to CD4+ and CD8+ lymphocytes (Bernier et al., 1995; Garg et al., 2009; Vallejo et al., 2015). This can lead to the acceleration of both HIV and VL progression as both pathogens exploit and suppress host cell immune responses in a mutually beneficial manner.

1.1.2 Epidemiology

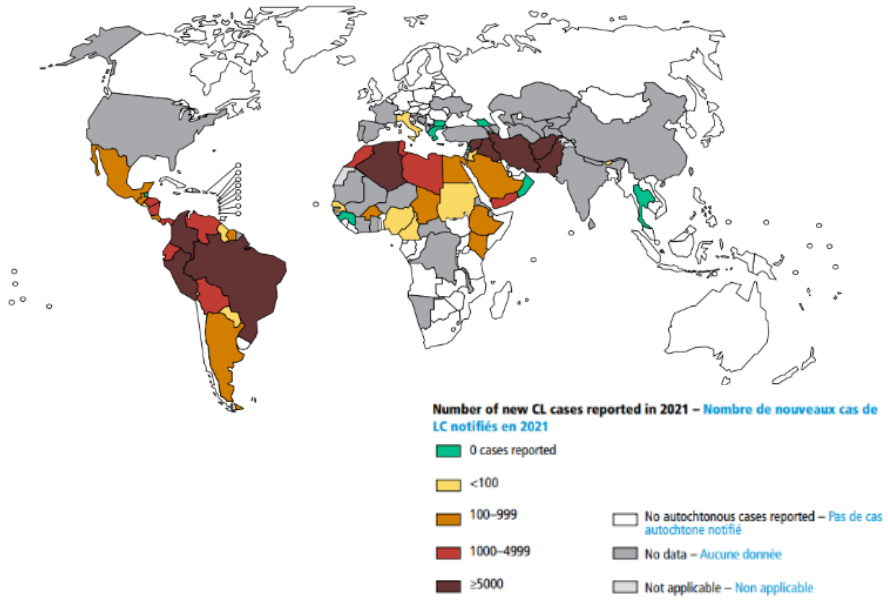
Leishmaniasis is a disease found worldwide, and in 2022 99 countries and territories were considered endemic for the disease; with 89 considered endemic for CL and 80 for VL (Ruiz-Postigo et al., 2022). According to data from WHO, more than 80% of new CL cases in 2020 were reported from just seven countries; Brazil, Columbia, Afghanistan, Pakistan, Syria, Iraq and Algeria (Ruiz-Postigo et al., 2021). Leishmaniasis is found throughout much of the Americas, from Argentina to southern Texas and often referred to as New World Leishmaniasis. It is also found in Asia and the Middle East as well as parts of southern Europe and Africa- collectively known as Old World Leishmaniasis. The global spread of different forms of the disease is highlighted in Diagram 1.

Factors such as conflict, population movement and poor vector control have seen new outbreaks of the disease and dramatic changes in the epidemiology and incidence of cases in the last decade. In the 2010s following the ongoing conflict in Syria, major outbreaks of Leishmaniasis occurred in refugee camps and cities affected by conflict (Al-Salem et al., 2016; Hayani et al., 2015). Piles of rubbish and standing water caused by the destruction provided ideal breeding grounds for sand flies, and a lack of health infrastructure prevented adequate control and medical intervention for those affected. A striking example of the impact of these outbreaks can be seen from the incidence of cases of CL in Lebanon. During the 2000-2012 period only 6 cases of CL were reported in Lebanon, however in 2013 there were 1,033 cases - the majority among the Syrian refugee populations (Al-Salem et al., 2016). The presence of sand fly populations in the same areas as refugee camps where patients were living in poor living, and sanitary conditions led to an ideal environment for the re-emergence of Leishmaniasis in this country.

As *Leishmania*sis is spread by the activity of a vector, the expansion of the sand fly population can be linked with cases of the disease. With climate change, it has been

predicted that sand fly populations will continue to move northwards and by the 2060s sand fly populations will be present in southern UK, Germany and France (Trájer, 2013). While in North America, even the most conservative modelling scenario in one paper predicts that the number of people currently at risk from *Leishmania*sis will double by 2080 and that the disease will be found as far north as Canada (González et al., 2010).

A



B

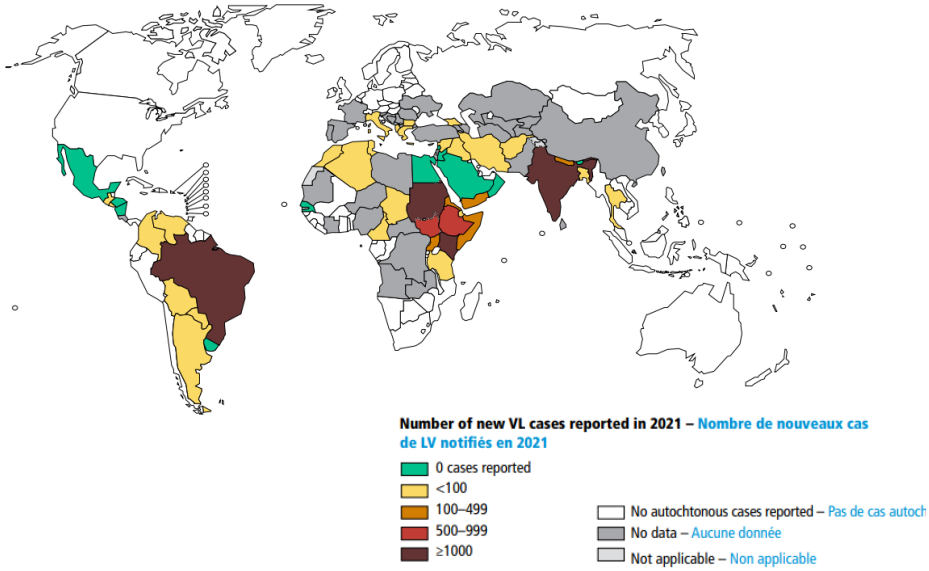


Diagram 1: Status of endemicity of **A)** Cutaneous Leishmaniasis and **B)** Visceral Leishmaniasis worldwide in 2021 (Figure adapted from Ruiz-Postigo et al, 2022)

1.1.3 Lifecycle of the *Leishmania* parasite

Leishmania spp, the causative agents of Leishmaniasis are flagellated parasitic protozoa belonging to the kinetoplastida class, characterised by the presence of an organelle with a large mass of circular DNA called a kinetoplast. Other notable parasitic members of this class include *Trypanosoma brucei* and *Typanosoma cruzi* which are the causative agents of Human African Trypanosomiasis and Chagas disease respectively (McGhee and Cosgrove, 1980).

Leishmania has a complex lifecycle requiring two hosts, female *Phlebotomine* sandflies of the genera Phlebotomus and Lutzomyia, and a mammal such as a human or canine. The parasite also has two distinct structural variants depending on their lifecycle stage, amastigotes and promastigotes. Promastigotes, the human infective form of *Leishmania* are elongated in shape with flagella located at the anterior end to allow motility. However, when promastigotes infect mammalian macrophages to multiply and transform into amastigotes, the shape of the parasites cell body becomes more ovoid, and their motility is mostly lost. The transformation of promastigotes to amastigotes inside the macrophage phagolysosome is triggered by what is termed a differentiation signal- in this case concomitant exposure of the parasite to 37ºC and pH 5.5 (Barak et al., 2005). The replication of amastigotes is most commonly associated with parasitism of mononuclear phagocytes where it occurs within the phagolysosome compartment. However, *Leishmania* parasites may also infect a variety of host cells including neutrophils, monocytes, tissue resident dermal macrophages and stromal cells.

Amastigotes can leave their initial host macrophage in order to infect more for continued replication. The exact mechanism by which amastigotes disseminate to more cells is still relatively unknown and thought to vary between *Leishmania* species. Cell rupture due to increasing and unrestricted amastigote replication had been suggested as one way amastigotes are released from the initial host cell and can go on to infect further cells

(Handman, 1999; Handman and Spira, 1977). Another method of cell-cell spreading seen in both *L.amazonensis* and *L.major* infections involves the proliferating parasite driving host cell death by apoptosis. The resultant apoptotic bodies are phagocytosed by nearby macrophages thus allowing the parasites be taken up by a new host cell (Baars et al., 2023; DaMata et al., 2015). Other studies have suggested that *L. aethiopica* and *L. mexicana* parasites have developed a mechanism of spreading in which they do not need to destroy their host cells- as the parasites appear to be slowly released from host cells without damaging them (Rai et al., 2017). This non-damaging method may be similar to how *L. amazonensis* amastigotes have been shown to transfer from cell to cell in *in vitro* studies. Amastigotes were transferred to new host cells within membrane blebs rich in phagolysosome membrane components. The extrusions containing amastigotes were selectively internalized by macrophages (Real et al., 2014).

Sandflies become infected by ingesting amastigote containing macrophages during a blood meal and provide an environment for the amastigotes to transform to promastigotes. Outside the mammalian host the lifecycle of *Leishmania* is confined to the digestive tract of female sandflies. Once again the change in conditions from the mammalian host macrophage- a lower temperature and higher pH triggers differentiation of the parasite within the sand fly midgut (Bates and Rogers, 2004; Kamhawi, 2006). This culminates in highly infective metacyclic promastigotes that are transmissible to a new mammalian host when the sand fly takes another blood meal (Rogers et al, 2002). This lifecycle is summarised in diagram 2.

The stationary phase of the parasite is a term applied to the metacyclic stage. Proteins with functions in protein synthesis, protein folding and mRNA processing are down-regulated by post-translational modification during this stage, leading to arrested growth, while infectivity of the parasite is increased due to the upregulation of proteins concerned with motility (Mojtahedi et al., 2008).

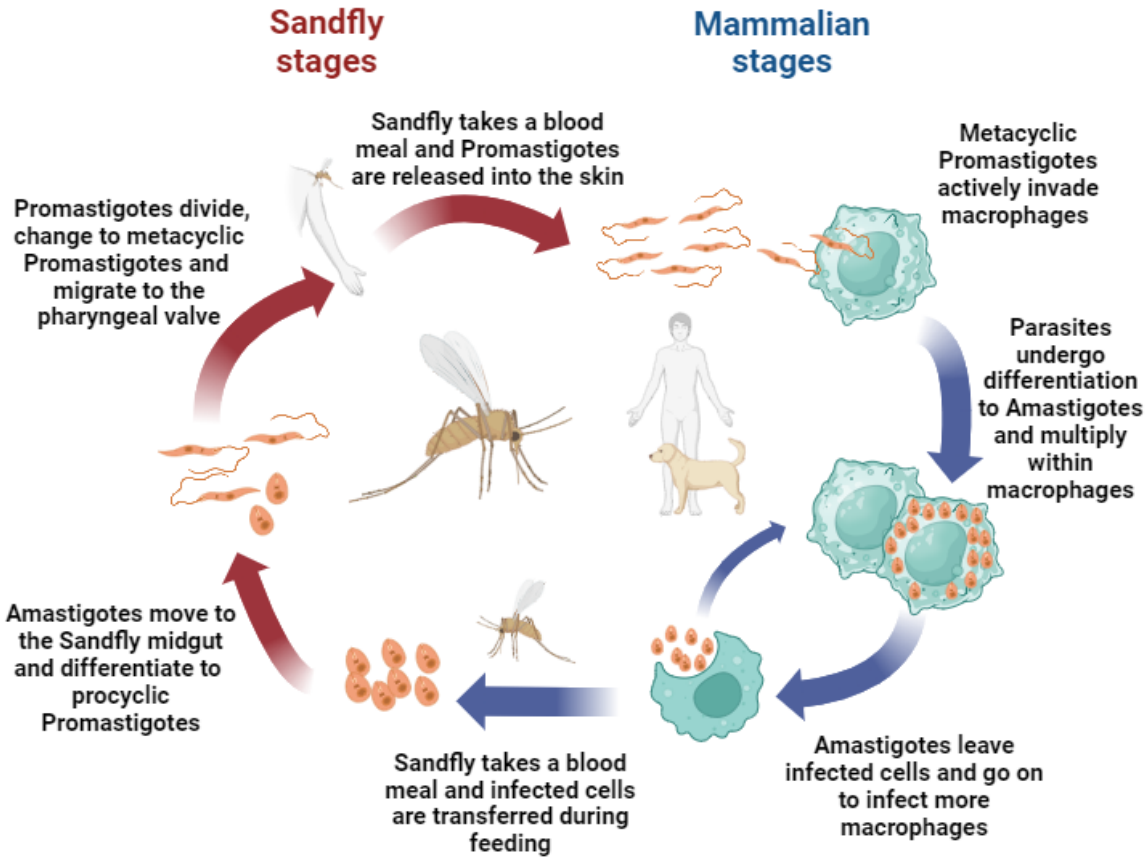


Diagram 2: Life cycle of *Leishmania* parasite
When a sandfly takes a blood meal, infective promastigotes are inoculated into a susceptible host mammal. These promastigotes infect host macrophages and transform into amastigotes, multiplying by simple division within these cells. Amastigotes continue to infect phagocytic cells either at the site of the bite (CL) or can disseminate to secondary lymphoid organs (VL). When sandflies feed on an infected host, they become infected with amastigotes. These amastigotes transform into promastigotes in the midgut of the sandfly and migrate from the midgut to the pharyngeal valve. Lifecycle described in (Bates and Rogers, 2004; Harhay et al., 2011). Diagram created in Biorender.

1.1.4 Treatment Options

Treatments for Leishmaniasis are limited, with chemotherapeutic agents being the primary options. While these can be reasonably effective, toxic side effects and a high cost can limit the use of these treatments. Distribution of these therapeutics is made more complicated as many of those affected live in rural areas far from health care access and may also be unable to afford appropriate treatment (Croft and Olliaro, 2011; Eid et al., 2019; O. P. Singh et al., 2016). Many of the treatments listed in Table 2 can also only be administered in complex regimens by injection or infusion which is likely to affect patient compliance (Croft and Olliaro, 2011). Drug resistance is also a growing problem especially for pentavalent antimonials- the first line treatment options for many cases. (Chakravarty and Sundar, 2010; Croft et al., 2006; Ponte-Sucre et al., 2017) Table 2 summarises the main therapeutic options currently available for Leishmaniasis and their limitations.

To overcome limitations of current therapies, research has focused on identifying novel drug targets from identified *Leishmania* virulence factors that are crucial for its pathogenicity. Similarly, a fuller understanding of *Leishmania* metabolism could also reveal targets crucial for maintaining its viability within cells. These could lead to a more targeted approach to treating the disease, as most of the current options are drugs that have been repurposed from use against other diseases (Ejazi and Ali, 2013) such as the anti-fungal medication Amphotericin B (Coukell and Brogden, 1998). Several compounds targeting key parasite factors and enzymes are now in development. These include *Leishmania* Heat shock protein 78 (Das et al., 2020) and *Leishmania* aurora kinase- a mediator of cell division (Chhajer et al., 2016).

Table 2: Available treatments for Leishmaniasis and their key limitations of use. Table adapted from *(Kumari et al., 2021)*

Treatment	Mechanism of action	Route of administration	Limitations	Advantages	References
Pentavalent antimonials	Inhibition of glycolysis, fatty acid oxidation, ATP and GTP synthesis	Intramuscular injection or intravenously either by infusion or slow injection. Topical administration can be performed for cutaneous *Leishmania*sis treatment	-Toxic side effects -Drug resistance reported -Long and invasive treatment course	-Usually the first line treatment for Leishmaniasis -Easily available -Intralesional route for CL showed reduced after-effects	(Mansuri et al., 2020; Arboleda et al., 2019; Ponte-Sucre et al., 2017; K. Singh et al., 2016; Sundar et al., 2000)
Amphotericin B	Pore formation in membrane and membrane synthesis disruption	Intravenous Infusion	- Difficult to administer -More toxic than pentavalent antimonials -High Cost	-Highly effective with decreased toxicity -Short course of treatment	(Roatt et al., 2020; No, 2016; Nagle et al., 2014)
Mitelfosine	Modulates cell surface receptors and alters sterol and phospholipid composition	Oral	-Unsuitable for pregnant women -Gastrointestinal side-effects -Long half-life -Nephrotoxicity and hepatotoxicity	-Only oral drug available and effective in antimonial resistant cases- higher patient compliance is likely	(Nagle et al., 2014; No, 2016; Sinha et al., 2011)

Paromomycin	Obstruct the machinery of protein synthesis as it binds to 30S-smaller subunit of the ribosomal complex	Intramuscularly, or topical application for CL	-Common side effects such as nausea and diarrhoea -Pain at the site of injection -Other side effects include hepatotoxicity, nephrotoxicity, and ototoxicity	-Seen as a first-line alternative drug in the setting of resistance to classical anti-Leishmanial drugs -Cost-effective and used in combination therapy	(Sundar and Singh, 2018; K. Singh et al., 2016; Hussain et al., 2014; Wiwanitkit, 2012)

1.1.5 Vaccines

Many of the diseases collectively known as Leishmaniasis are thought to be vaccine-preventable diseases, as they are characterised by a state of partial immunity after disease resolution. Additionally, there have been many promising results in pre-clinical trials for potential vaccines, and three commercial approved vaccines against canine Leishmaniasis are currently licensed (Velez and Gállego, 2020). However, at this time no licenced vaccines for human use have attained registration.

The reasons behind the lack of clinical trials for vaccine candidates is not due to a lack of vaccine candidates. However, many other factors have contributed to significant roadblocks in the development of a vaccine. These include a lack of funding; especially as Leishmaniasis is relatively unknown compared to other NTDs such as Malaria. Linked with this is the potential lack of commercial incentive due to the lack of well-evidenced estimates of vaccine demand (Malvolti et al., 2021). The disease also disproportionately affects middle- or low-income countries. As discussed previously poverty is a clear risk factor for the disease, and contracting the disease is likely to contribute to the poverty cycle in those affected due to expensive treatments and loss of income due to poor health (Okwor and Uzonna, 2016) .

The numerous differing species of disease-causing *Leishmania* may also add complexity to vaccine development, although many key virulence and structural proteins are highly conserved between species. Other issues that include the transition from results in animal models to human studies which can be challenging (Coutinho De Oliveira et al., 2020). Despite these challenges many different types of *Leishmania* vaccines have been proposed, some which have achieved promising first results and passed safety requirements.

The progression in the development of *Leishmania* vaccines can clearly be seen through the different generations of vaccines. The first-generation vaccines are comprised of

whole parasites, killed by heat or chemical processes. Second generation vaccines utilise purified parasite antigens or recombinant molecules, or even synthetic peptides representing epitopes of antigen. Examples include virulence factor glycoprotein 63 (GP63) and *Leishmania* homologue for receptors of activated C kinase (LACK) (Duthie et al., 2012). Finally, third generation vaccines are those that utilise *Leishmania* specific DNA or RNA, delivered either naked or within a viral vector. Currently one DNA based vaccine has passed initial safety trials in rodent and simian models and has shown to be safe and effective in initial human trials of healthy volunteers and patients with complicated visceral leishmaniasis. This adenovirus-based vaccine, ChAd63-KH, encodes *Leishmania* antigens, highly conserved kinetoplastid membrane protein-11 (K; KMP-11) and hydrophilic acylated surface protein B (H; HASPB). Both proteins are expressed by both amastigote and promastigote forms of *Leishmania* (Younis et al., 2021). This vaccine is undergoing further human trials to assess the therapeutic benefit of vaccination with results expected this year.

Another current and promising vaccine candidate is the live attenuated vaccine *L. major* centrin$^{-/-}$. A new generation of live but genetically attenuated whole parasite vaccines are now of current interest, especially as they closely mimic the normal *Leishmania* infection process. They normally involve attenuation of parasite genes essential for its survival within the host or virulence activities. *Leishmania* centrin is necessary for parasite growth and the differentiation process- it is a calcium-binding protein located in the basal body of the parasite that regulates centrosome duplication and segregation (Volpedo et al., 2022). Pre-clinal trials have demonstrated the safety and efficacy of this vaccine against new world cutaneous leishmaniasis in mouse models. Advancement of the candidate to human trials will be the next step for this vaccine (Karmakar et al., 2022).

The rise of interest in mRNA vaccines due to the COVID-19 pandemic and vaccine development could open some new directions for mRNA *Leishmania* vaccines in the near future. mRNA vaccine candidates for malaria (Mallory et al., 2021) and other NTDs are under investigation and it is likely that the use of this technology for many other NTDs, including Leishmaniasis could be rapid.

1.2 The Innate Immune response

The immune system in mammals consists of the innate immune response and the adaptive immune response. The adaptive immune response is primarily composed of T and B lymphocytes that are activated by exposure to specific pathogens and use immunological memory. The adaptive immune response is usually much slower at responding to threats than the innate immune response, although much more targeted.

The innate immune response is the first line of defence of the immune system against pathogenic threats to the host. As soon as a pathogen breach one of the host's anatomical barriers, such as the skin, a variety of soluble molecules present in the blood and extracellular fluid start to act. These innate immune mechanisms include antimicrobial peptides and enzymes in addition to the complement system- a group of plasma proteins that can prime pathogens for lysis and phagocytosis by innate immune cells such as macrophages. In addition to macrophages, a variety of cell types contribute to the innate immune response, including dendritic cells, neutrophils, natural killer cells and innate lymphoid cells.

An important aspect of the innate immune response is the presence of germline encoded pattern recognition receptors (PRRs) (Akira et al., 2006) on the surface or within the cytosol of innate immune cells. PRRs detect conserved motifs and patterns present on micro-organisms but not host cells. These are known as pathogen associated molecular patterns (PAMPs) and include a wide variety of molecules from bacterial cell wall components to pathogen derived nucleic acids. This allows the recognition of invading

pathogens as non-self, thus allowing an appropriate response to take place. Activation and downstream signalling of PRRs is essential for the production of intracellular signals that activate the innate immune response by the production of inflammatory cytokines, as well as supporting the adaptive immune response via the recruitment of other immune effector cells.

In addition to PAMPs, PRRs can recognise Damage Associated Molecular Patterns (DAMPs) (Janeway and Medzhitov, 2002). DAMPs are endogenous danger molecules normally not visible to the immune system, but often released during cellular stress from damaged or dying cells. Examples of DAMPs include Uric acid (Shi et al., 2003) double stranded DNA (dsDNA) (Jounai et al., 2013) or mitochondrial DNA (Zhang et al., 2010).

1.2.1 Toll-like receptors (TLRs)

There are many classes of PRRs, but perhaps the most well studied and known are the membrane bound Toll-like receptors (TLRs). In humans 10 TLRs have been identified, each able to detect a variety of PAMPs from bacteria, viruses, fungi and protozoa (Takeda, 2004). The ligands of each of the TLRs are summarised in Diagram 3. The majority of the TLRs are expressed on the extracellular cell surface, however TLRs 3, 7, 8 and 9 are found inside cell endosomes. Therefore further detection can occur after pathogen entry into cells via receptor mediated endocytosis or within phagosomes containing pathogens engulfed by macrophages (Takeda, 2004). This includes detection of obligate intracellular pathogens such as herpes simplex virus and *Leishmania*.

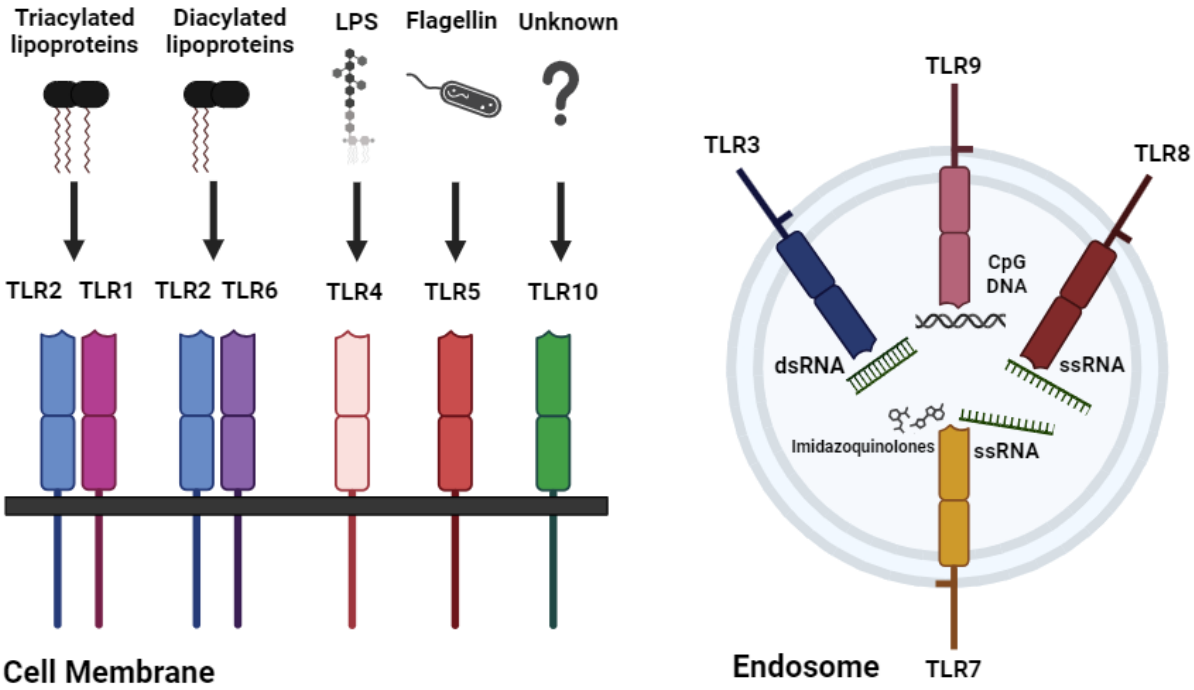


Diagram 3: Location and ligands of the human TLRs

TLRs recognize a variety of microbial products: TLR2 (together with TLR 1 and TLR 6) is specific for lipoproteins; TLR3 is specific for double-stranded RNA; TLR4 is specific for lipopolysaccharide and lipoteichoic acids; TLR5 is specific for bacterial flagellin; TLR 7 and TLR 8 are structurally highly conserved and can bind the same ligand in some cases TLR 7 can bind synthetic imidazoquinolines and both bind SSDNA.TLR9 is specific for CpG DNA. Currently the ligand for TLR 10 is unknown. Dimerization of TLRs is required to activate downstream signalling but has not been shown in this diagram. TLR ligands have been summarised in McInturff et al 2005 and Kawaii and Akira 2006. Diagram created in Biorender.

A principal response after the activation of a TLR with its cognate ligand is the production of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-8 and IL-12, to help control the pathogenic infiltration (Kawai and Akira; 2006). Pro-inflammatory cytokines, together with chemokines act to recruit and stimulate other immune cells to the site of infection. Different intracellular responses to TLR activation include the production of antimicrobial peptides and antiviral cytokines.

There are two major signalling cascades downstream of TLRs (Diagram 4), one results in the activation of NF-κB transcription factors and the other in members of the Interferon regulatory factor (IRF) transcription factors. NF-κB primarily regulates transcription of pro-inflammatory cytokines and chemokines, whereas IRF factors regulate the type I interferons.

The type I interferons include several structurally similar IFN-α proteins, and one IFN-β protein. These interferons can activate transcription of the interferon stimulated genes, which induce many antiviral strategies within infected cells to inhibit viral replication. This includes the upregulation of major histocompatibility complex (MHC) class II expression and activation of the adaptive immune response (Kumar et al., 2011).

NF-κB activation

To activate the NF-κB transcription factor, TLR signalling downstream of ligand binding involves the interaction of the TLR cytoplasmic Toll–IL-1-resistance (TIR) domains with adaptor molecules. There are four different adaptor molecules including myeloid differentiation factor 88 (Myd88) and all TLRs, except TLR 3, recruit Myd88.

Once activated, Myd88 recruits the serine-threonine kinases Interleukin 1 Receptor Associated Kinase (IRAK) 4 and IRAK 1/2 which phosphorylate the E3 ubiquitin ligase TNF receptor associated factors 6 (TRAF 6). Once active, TRAF 6 generates ubiquitin chains, both on itself and on the protein NF-kappa-B essential modulator (NEMO), a component of the IkB kinase (IKK) complex. These polyubiquitin chains create a scaffold for the activation of the serine-threonine TGF-β- activated kinase 1 (TAK1) to occur. Once activated TAK1 can activate both MAP kinases and the IKK complex.

MAP kinases, including p38 and Jun N-terminal kinase (JNK) phosphorylate and activate Activator protein 1 (AP-1) transcription factors, whereas the activated IKK complex allows the release of NF-κB and its subsequent entry into the nucleus to activate the transcription of many genes involved in the innate immune response (Hayden et al., 2006; Kumar et al., 2011; Takeda, 2004).

IRF transcription factors

Activation of IRF transcription factors occurs in a similar process after TLR binding of nucleic acids. However, TLR-3, which senses double-stranded RNA, uses a different adaptor protein in place of Myd88- TRIF. The interaction of TIR domain containing adaptor inducing IFN (TRIF) and TRAF6 activates TAK 1 kinases similarly to that in the Myd88 dependent pathway (Kumar et al., 2011).

TRIF also activates kinases TANK-Binding Kinase 1 (TBK1) and IkKe through the involvement of TRAF3, which are able to phosphorylate transcription factor IRF3. Once activated IRF3 enters the nucleus and can induce transcription (Kumar et al., 2011).

TLRs and anti-inflammatory cytokines

In addition to their role in regulating inflammation, TLRs can also be significant in the prevention of excessive inflammation. This is achieved via the production of anti-inflammatory cytokines (Lang and Mansell; 2007), including interleukin-10 (IL-10). The production of anti-inflammatory signals and negative feedback mechanisms are essential to prevent unchecked inflammation and damage to self.

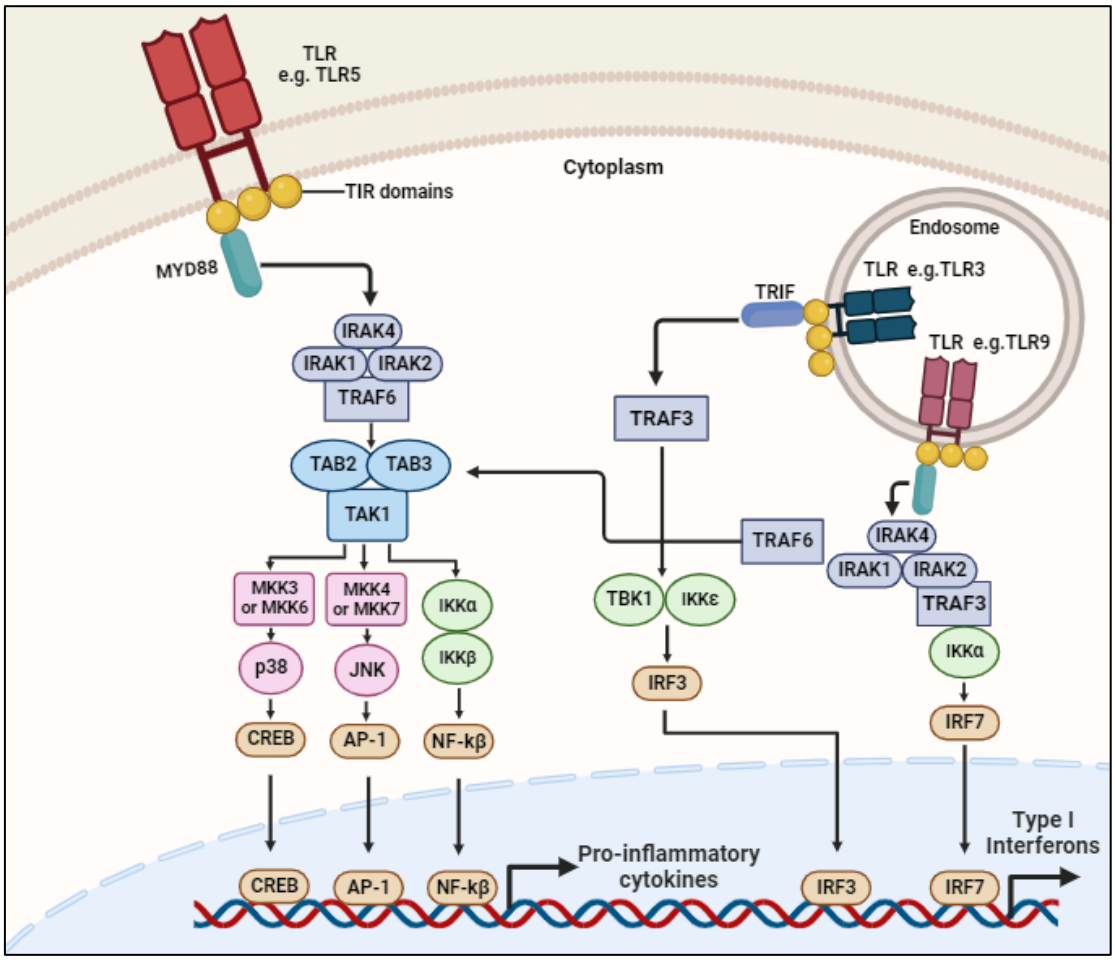


Diagram 4: TLR signalling pathways

Ligand activated TLRs on the cell surface, or at the endosomal membrane dimerize on activation, and engage with adaptor molecules such as Myd88 or TRIF, via TIR domains**.** Association with signaling adaptor molecules stimulates downstream signaling pathways through IRAKs and the E3 ubiquitin ligase TRAF 6. This leads to the activation of various protein kinases and transcription factors including AP-1 and NFκB. TRIF signaling also activates IRFs to contribute to Type-I interferon (IFN) gene expression. These pathways are described in (O'Neill et al., 2013) and (Kumar et al., 2011) Diagram created in Biorender.

1.2.2 RIG-I like receptors (RLRs)

The RLRs are a group of RNA sensors located in the cytosol that sense RNA. The family of receptors includes Retinoic acid-Inducible Gene I (RIG-I), Melanoma Differentiation Associated protein 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2). These sensors detect RNA present within the cytosol of virally infected cells and induce an inflammatory response in addition to the production of type I interferons.

All RLRs have a central helicase domain and a so-called carboxy-terminal domain (CTD). RIG-I and MDA5 contain a Caspase Activation and Recruitment Domain (CARD) that is essential for downstream signalling (Kumar et al., 2011). LPG2 lacks this CARD, and it has been suggested it acts as a positive regulator of RIG-I and MDA5 signalling (Satoh et al., 2010). While the RLRs are free within the cytosol, upon RNA detection a conformational change in the receptor exposes the CARDs, which interact with the CARD domains of adaptor protein Mitochondrial Antiviral-Signalling Protein (MAVS). MAVS localises to the mitochondria, where downstream signalling involving kinases such as TBK1 lead to IRF3 transcription factor activation (Kawai et al., 2005). MDA5 and RIG-I are also able to recognise Poly(I:C), a synthetic double-stranded RNA often used to simulate RNA virus infection (Kato et al., 2006).

1.2.3 NOD-like receptors (NLRs)

NLRs act as intracellular sensors of microbial PAMPs and activate NF-κB to initiate inflammatory cytokine production similar to that of the TLRs. Examples of PAMPs recognised by the NLRs include the bacterial cell wall components peptidoglycan (McDonald et al., 2005) and muramyl dipeptide (Mo et al., 2012). NLRs have also been reported to also detect viral, fungal and protozoal ligands (Kawai et al., 2005).

NLRs are also able to activate assembly of the inflammasome, a multiprotein complex that can initiate apoptosis or inflammatory cytokine production upon activation. The NLRP3 inflammasome is the most well studied and PAMPs from all classes of pathogen, in addition to danger associated molecular patterns (DAMPs) can lead to its activation (Kumar et al., 2011).

1.2.4 Cytoplasmic dsDNA sensors

In addition to endosomal DNA sensors such as TLR9, DNA sensors located within the cytosol have also been identified and their activation is able to trigger the innate immune response and a strong type I interferon response. Significant research over the past decade has identified many of these sensors, including cyclic GMP-AMP synthase (cGAS) (Sun et al., 2013), DNA-dependent activator of IFN-regulatory factors (DAI) (Takaoka et al., 2007), IFN inducible gene 16 (IFI16) (Unterholzner et al., 2010), DEAD/H-box helicase (DDX41) (Zhang et al., 2011), Meiotic Recombination 11 (MRE11) (Kondo et al., 2013), and DNA-dependent protein kinase (DNA-PK) (Ferguson et al., 2012). All these receptors have been shown to strongly activate and signal through the stimulator of IFN genes (STING). STING is located at the Endoplasmic Reticulum (ER) and activates the downstream kinase TBK1 and subsequently the transcription factor IRF3 (Ishikawa et al., 2009; Tanaka and Chen, 2012). A robust Type-I IFN response to cytosolic DNA requires both IRF-3 and TBK1 activation.

More recently a STING-independent DNA sensing pathway (SIDSP) has been discovered to be active in the human U937 monocyte cell line in addition to primary human hepatocytes (Burleigh et al., 2020). It has been proposed that DNA-PK is the DNA sensor of this SIDSP. While this SIDSP is strongly activated in human and primate cells, is appears to be absent from mouse cells (Burleigh et al., 2020).

1.2.5 cGAS

After its discovery in 2013 (Sun et al., 2013), cGAS came to be thought of as one of the most important cytosolic DNA sensors. cGAS and the associated cGAS-STING pathway has emerged as critical for the pairing of DNA sensing to the activation of the innate immune system and induction of a strong type I interferon response. cGAS has a crucial role in anti-viral cell immunity, but also has a role in the response to cellular stress and antitumour immunity. cGAS recognizes nucleic acids from DNA viruses as well as damaged mitochondrial and genomic DNA from self. DNA produced by retroviruses such as HIV may also be sensed by cGAS (D. Gao et al., 2013) and cytosolic RNA:DNA hybrids can also activate cGAS and its downstream adaptor STING (Mankan et al., 2014) Intracellular bacteria and parasites such as *M. tuberculosis* (Wassermann et al., 2015) and *P.falciparum* (Gallego-Marin et al., 2018) have also been shown to be detected by cGAS, leading to Type-I IFN induction.

Structurally cGAS consists of a non-conserved N-terminal domain and a highly conserved catalytic C-terminal domain (Civril et al., 2013). This C-terminal domain contains a nucleotidyltransferase (NTase) core domain, that is key to its enzymatic activity (Sun et al., 2013). Additionally, within the C-terminal domain are multiple positively charged DNA-binding sites (Civril et al., 2013; P. Gao et al., 2013; Li et al., 2013), which bind the sugar–phosphate backbone of DNA. This includes a strong DNA-binding site , weaker DNA-binding site , and a further DNA-binding site that aids cGAS activation (Xie et al., 2019). DNA binding induces a conformational change in cGAS, that specifically rearranges the catalytic pocket of the enzyme and allowing optimal interaction of the catalytic domain with ATP and GTP substates (Zhang et al., 2014). Cyclic GMP-AMP (cGAMP), a diffusible cyclic dinucleotide is then produced as a second messenger after DNA binding by cGAS.

The importance of this DNA sensor can be demonstrated in cGAS knockdown or knockouts, in which an ablation of the immune response to DNA viruses such as HSV-1 is observed. cGAS−/− mice have not only have a reduced immune response after HSV-1 infection but a higher virus titre, and much reduced survival when compared to the WT (Li, X.D., 2013). cGAS−/− mouse and human cell lines have also been shown to be more susceptible to HIV and unable to mount an effective cytokine response when compared to the WT (D. Gao et al., 2013).

Cytosolic DNA generated from various sources of DNA damage can also be sensed by cGAS and lead to its activation. This includes DNA damage from chromosome instability, radiation, and chemotherapy. cGAS has a role in tumour suppression as activation of the cGAS-STING pathway and type I IFN expression can have a suppressive effect on tumours. cGAS-STING signalling also facilitates crosstalk between tumour cells and immune cells. Dendritic Cells can be activated by tumour derived DNA or cGAMP which can trigger tumour clearance by the immune system.

The cGAS/STING pathway plays a crucial role in the pathogenesis of many inflammation-related diseases, including such as cardiovascular disease, neurodegenerative disease, inflammatory bowel disease, arthritis fibrosis, lupus, and psoriasis (Chen et al., 2016; Yu and Liu, 2021) For example, in Huntington's disease (HD) cGAS is up-regulated and shown to be linked to the promotion of an increased inflammatory response that may contribute to HD pathology. It is suggested that the cause of HD- the mutation of the N-terminal polyglutamine of huntingtin protein (mHTT), is associated with DNA damage and could cause upregulation of cGAS and activation. As cGAS depletion decreases inflammatory response in HD striatal cells it could be future therapeutic target (Sharma et al., 2020).

1.2.6 STING

STING (also known as ERIS, MPYS, MITA, and TMEM173) functions as adaptor protein for many cytosolic DNA sensors including cGAS, linking DNA sensing with an innate immune response and type I interferon signalling. STING was first reported by the Glen Barber lab as an endoplasmic reticulum adaptor protein that facilitates innate immune signalling downstream of intracellular DNA detection (Ishikawa and Barber, 2008). STING can also act as a PRR and was additionally identified to be a sensor of bacterial cyclic dinucleotides (Burdette et al. 2011).

After cGAS binds to dsDNA and catalyses the formation of second messenger cGAMP, cGAMP binds to STING which is resident at the ER. Activated STING then traffics to the Golgi apparatus and perinuclear compartments (Dobbs et al., 2015; Ishikawa and Barber, 2011), where it recruits and activates TANK Binding Kinase 1 (TBK1) and transcription factor IRF3. This behaviour is referred to as STING clustering. Activated IRF3 dimerizes and enters the nucleus leading to the transcription of type I interferons. Activation of the cGAS–STING pathway also results in the induction of NF-κB and transcription of proinflammatory cytokines such as IL-6 and tumour necrosis factor (TNF) (Ishikawa and Barber, 2008)

Aside from its role in innate immunity and production of type I interferon and cytokines, STING is also associated with other biological processes. This includes the adaptive immune response where STING has been shown to have a role in inhibiting T cell proliferation (Cerboni et al., 2017). Recent research has also shown that STING can directly impact cellular metabolism, including the modulation of insulin secretion and glucose metabolism (Qiao et al., 2022; Rong et al., 2022). STING also has a role in cellular death pathways such as apoptosis and autophagy. The translocation of STING after activation has been linked to the activation of autophagy (Saitoh et al., 2009).

STING has been reported to mediate autophagy through direct interaction with autophagy related protein LC3 (Liu et al., 2019).

In terms of structure, STING is split into three main domains, an N-terminal transmembrane domain (TMD) containing four transmembrane helices, a cytoplasmic ligand binding domain (LBD) and a C-terminal tail (CTT) (Shang et al., 2019). The CTT contains a TBK1 binding motif and phosphorylation motif.

Crystal structure analysis has revealed that the cytoplasmic ligand binding domain of STING forms a dimer (Shang et al., 2019). It is at the cleft at the middle of this dimer that cGAMP binds to STING in order to activate it. Upon cGAMP binding, conformational changes in the ligand binding domain are induced which leads to a high-order oligomerization of STING (Ishikawa et al., 2009; Tanaka and Chen, 2012) that is essential for TBK1 recruitment and subsequent phosphorylation events triggering the downstream signalling pathways (Shang et al., 2019; Zhang et al., 2019). Without STING oligomerization, STING phosphorylation by TBK1 is impaired as the active site of bound TBK1 will be unable to reach the phosphorylation site Ser366 in the STING tail (Zhang et al., 2019; Zhao et al., 2019).

TBK1 has been shown to be essential for the innate immune response to transfected DNA. The phosphorylation of STING at Serine 366 by TBK1 facilitates the recruitment and phosphorylation of IRF-3 by TBK1 (Tanaka, 2012). Both TBK1 and IRF3 phosphorylation are required for the induction of type I interferons.

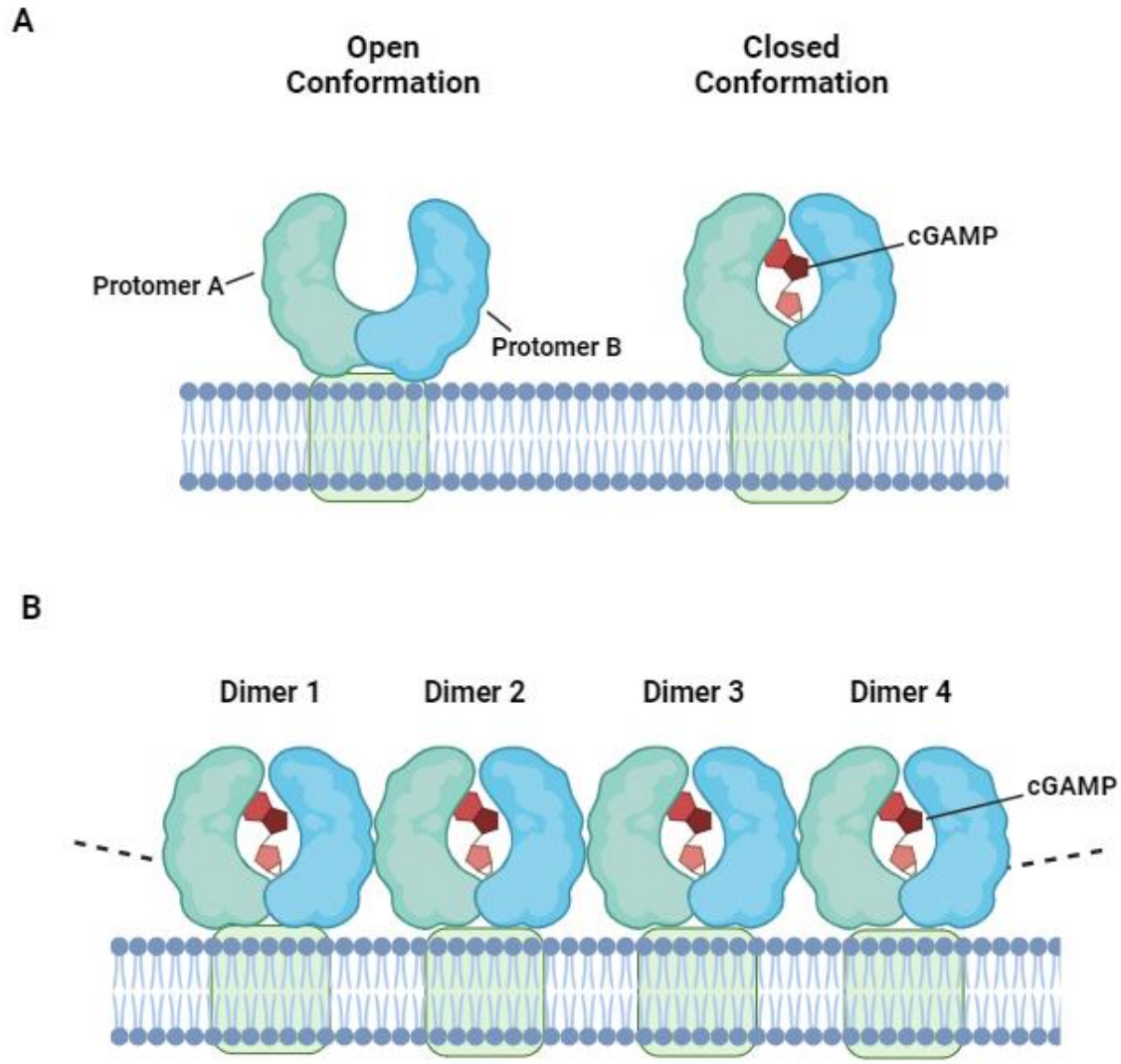


Diagram 5: Changes in STING structure during ligand binding

(A) Binding of ligand cGAMP to STING induces huge conformational changes; the two protomers in the complex undergo inward rotations relative to the twofold axis, creating a deeper crevice between the two protomers. Second, LBDβ2–β3 loops from two protomers move close to each other, forming a lid of a four-stranded antiparallel β sheet, which is disordered in the apo state. Therefore, the cGAMP-bound STING is in the closed state because of the lid formation of two protomers, while ligand free STING in the absence of this lid is in an open conformation. **(B)** After ligand binding oligomerisation of STING dimers can occur through interactions of the LBD. Structural changed and oligomerisation described in (Hussain et al., 2022; Shang et al., 2019). Diagram created in Biorender.

It has also been shown in human macrophages and keratinocytes that optimal STING activation is also dependent on another cytosolic DNA sensor: IFI16 (Almine et al., 2017; Jønsson et al., 2017). IFI16 appears to be essential for the full activation of STING and co-operates with cGAMP to achieve this (Almine et al., 2017). IFI16 may also have a role in promoting the production of cGAMP, but currently this has only been observed in macrophages (Jønsson et al., 2017). The role of IFI16 in the cGAS-STING pathway could provide additional regulation to prevent over activation of STING and subsequent downstream signalling.

Under normal conditions, levels of STING are tightly regulated as constant activity of STING and downstream gene induction after dsDNA stimulation would lead to excessive inflammation and damage to self. After activation, STING is normally degraded after approximately 3-6 hours (Abe, 2014). This degradation has been shown to occur through the autophagy pathway in the lysosomal compartment. Cells deficient in autophagy proteins or treated with drugs that inhibit lysosomal acidification display enhanced type I interferon production (Gonugunta et al., 2017).

A plethora of post-translational modifications (PTMs) are also important for both the activation and regulation of STING. These not only aid successful activation and downstream signalling but can also mark STING for degradation. An example of a PTM that aids activation, SUMOylation of STING at K338 by TRIM38 inhibit STING degradation and promotes oligomerization of STING and IRF3 recruitment (Hu et al., 2016). Conversely, after activation STING can be deSUMOylated by SUMO-specific protease (SENP)2 leading to STING degradation and inhibiting innate immune responses (Hu et al., 2016).

As mentioned earlier, phosphorylation at Ser366 by TBK1 is important for the activation of STING (Liu et al., 2015). Additionally other phosphorylation events at Y240 and Y245 by tyrosine kinase C-terminal src kinase (CSK) activates the immune responses after

HSV-1 infection (Gao et al., 2020). Equally, the STING pathway can be downregulated by both phosphorylation and dephosphorylation events. After activation STING can be successively phosphorylated by UNC-51-like kinase (ULK1) at S366 so that sustained innate immune responses are prevented (Konno et al., 2013). Dephosphorylation at S358 by Mg2+/Mn2+-dependent protein phosphatase 1A (PPM1A) can also inhibit STING aggregation and signalling (Li et al., 2015). Additional PTMs that STING undergoes for regulation include glycosylation, palmitoylation, Ubiquitylation, nitro alkylation and Carbonylation (Kang et al., 2022).

The consequences of a lack of regulation of STING activity can be demonstrated by the autoimmune disease STING-associated vasculopathy with onset in infancy (SAVI), caused by rare gain of function mutations in STING (Liu et al., 2014). This auto-inflammatory disease is associated with excessive systemic inflammation, cutaneous vasculopathy and interstitial lung disease (Liu et al., 2014). The mutations causing the disease give rise to the constitutive activation of STING (Melki et al., 2017). Within cells these mutations cause the spontaneous trafficking of STING to the golgi apparatus, enhanced IRF3 phosphorylation and activation of downstream signalling leading to abnormally increased production of type I interferons. Abnormal activation of STING has also been associated with SLE which is characterised by excessive inflammation and severe tissue damage (Kato et al., 2018). It remains controversial whether STING plays a necessary role in SLE (Motwani et al., 2021).

It has been shown that autoinhibition of STING can be mediated by the binding of the CTT tail to the ligand biding domain (Ergun et al., 2019; Qi et al., 2022; Yin et al., 2012). In this bound formation the oligomerisation of STING necessary for its activation cannot occur. The CTT tail is displaced from its inhibitory position by cGAMP binding to STING and triggering a conformational change in the LBD.

It was first hypothesised that the freed CTT would go on to initiate STING oligomerisation, however STING lacking the CTT was found to be highly susceptible to aggregation, even in the absence of an upstream signal and cGAMP binding. It was then proposed that autoinhibition is achieved by CTT binding to and blocking the polymerization interface located at the CBD (Ergun et al., 2019). This model was supported by the SAVI STING mutant R284S, which is unable to bind the CTT, meaning its polymerization interface is always free. The ability of STING to oligomerize in the absence of upstream signal may explain why this STING mutant is constitutively active. Due to the importance of STING polymerisation under normal conditions the prevention of this process could be a promising option to inactivate STING for therapeutic use (Hussain et al., 2022).

1.3 Immune Evasion Strategies

Many pathogenic organisms employ immune evasion strategies to enable them to establish infection and maximise the probability of being transmitted to a new host. For obligate intracellular infections, such as *Leishmania* species, the avoidance of immune detection long enough to gain entry to a host cell, replicate and disseminate to a new host cell is reliant on successful immune evasion strategies.

A vast spectrum of different evasion strategies from all types of microorganisms has been recorded, from passive avoidance of pattern recognition receptors to an active evasion and modulation of the immune system components to benefit the pathogen survival. Here a range of these immune evasion strategies used by bacterial, viral, fungal and protozoal threats are reviewed. *Leishmania* species evasion strategies will be described in the next section.

1.3.1 Avoidance dependent on structural changes

Immediately upon entry onto and into host tissues, pathogens must avoid detection from a wide variety of cell types. PAMPS are often the primary manner in which detection can occur. As these molecular structures are often essential to the structural integrity of the micro-organism- for example, the bacterial structural component Lipopolysaccharide (LPS)- eliminating expression of these molecular patterns is not an option. Therefore, approaches to hide these motifs to prevent detection is a key immune evasion strategy that many pathogens have evolved.

For some bacteria and fungi, a simple structural component, the outer capsule, is enough to prevent detection of foreign PAMPs. The capsule may surround antigenic structures present on the surface of a bacterium.

For example *Staphylococcus aureus* strains that express a capsule have been shown to exhibit enhanced virulence and have anti-phagocytic properties (Thakker et al., 1998).

Similarly, in certain fungi such as *C.neoformans, the* presence of a fungal capsule can provide protection from the host immune system (Doering, 2009). In addition to simply shielding antigens, the fungal capsule has been shown to induce the production of anti-inflammatory IL-10, downregulating inflammatory immune response (Syme et al., 1999). In other fungi, detection of PAMPS such as β-(1,3)-glucan can be reduced by the expression of other fungal structures, e.g. Mannans which effectively shield these PAMPS from recognition (Rappleye et al., 2007; Zhang et al., 2016)

Other pathogens may alter the structure of their PAMPS to prevent detection- *Salmonella typhimurium* can modify the structure of Lipid A, the host signalling portion of LPS in response to its environment. These LPS variants induce a weaker host immune response and enhance pathogenicity (Guo et al., 1997; Matsuura et al., 2012). Similarly, *Yersinia pestis* demonstrates a temperature dependent alteration of its Lipid A structures. When grown at 37ºC, expression of Lipid A genes that stimulate human macrophages much more weakly are upregulated and allow uninhibited growth of the bacteria (Matsuura et al., 2010).

Pathogens can degrade other PAMPs before detection can occur- *P. aeruginosa* is able to prevent detection of flagellin by TLR 5 by secretion of a protein alkaline protease which is able to degrade free flagellin (Bardoel et al., 2011). Some viruses also use this tactic to prevent detection by cytoplasmic DNA sensors. For example, coronaviruses rapidly degrade excess RNA that they produce before detection can occur (Kindler et al., 2017). Other RNA viruses are able to modify the RNA they produce, which may also prevent it from being recognised by RNA sensor RIG-I. *Caliciviridae* noroviruses covalently attach protein VPg to the 5' end of their RNA to allow ribosomal recognition and translation (Goodfellow et al., 2005). This activity can prevent recognition by the host as uncapped mRNA is able to be recognised by host RNA sensors (Rohayem et al., 2006).

Instead of altering their own host proteins to avoid detection, some strains of the bacterium *Borrelia burgdorferi* can coat themselves with host derived complement inhibitory factor H, promoting C3b degradation and avoiding complement mediated lysis (Alitalo et al., 2001). Similarly *S.mansoni* acquire host antigens such as immunoglobulins and erythrocyte antigens A,B,H and Lewis to mask target antigens on their surface (Goldring et al., 1976; Kemp et al., 1977)

Antigenic variation can also allow pathogens to evade surveillance from the immune system. A striking example of this is found in *Trypanosoma* parasites which are able to repeatedly change the structure of their major surface antigen during extracellular infection. These trypanosomes are coated with a variable surface glycoprotein (VSG) for which the trypanosome genome contains around 1000 genes, each with distinct antigenic properties. By using gene rearrangement to change the VSG expressed, Trypanosomes are able to persist and avoid elimination as antibodies to each new VSG will need to be continually produced by the host immune system (Mugnier et al., 2016).

Many viruses, such as influenza also are able to change the composition of their surface antigens via point mutation of genes encoding surface haemagglutinin or neuraminidase (Petrova and Russell, 2018). A more rare process of antigenic change termed antigenic shift, can also occur during reassortment of RNA viral genomes of different influenza viruses (Hale et al., 2010), resulting in a sudden change in the antigenicity of the virus.

1.3.2 Avoidance dependent on spatial separation of PAMPs and receptors

Instead of strategies to shield or alter PAMPs, recognition may be more simply avoided by some pathogens by the select invasion of specific anatomical sites where expression of PRRs is less common or are immunological privileged sites.

Some parasites, including *Opisthorchis viverrini* (Rana et al., 2007) reside in the hollow organs of the host such as the bile duct where serum IgM and IgG antibodies cannot reach. In pyelonephritis infection, bacteria such as E.coli are thought to infect human tubule cells from the renal cortex that do not express TLR4 receptors (Bäckhed et al., 2001).

Similarly, a technique used by some viruses to passively avoid recognition by cytoplasmic PRRs is to not replicate within the cytoplasm where they are abundant. Instead, some viruses form specific replication compartments within cellular membranes or organelles. The mosquito-borne flavivirus DENV replicates in convoluted membranes of the ER. This shields viral dsRNA from detection by cytoplasmic RLRs (Uchida et al., 2014). Similarly influenza virus limit PAMP availability to receptors such as RIG-I by encapsulating viral RNA in ribonucleoproteins (Hale et al., 2010). Replication of the HIV genome is carried out within the virus capsid where it is shielded from cGAS dsDNA sensor (Jacques et al., 2016) .

Bacteria and fungi may avoid detection and attack from the immune system by forming biofilms and residing within a matrix of polysaccharides, also known as the extracellular polymeric substance (Donlan, 2002). *Pseudomonas aeruginosa*, the cause of chronic respiratory infections, is one example of a pathogen able to persist within the host organism due to its capacity to form biofilms which seclude it from the immune response (Alhede et al., 2014).

1.3.3 Active evasion of the immune response

The active evasion of the immune response includes strategies such as pathogen mediated degradation of receptors and the prevention of activating immune responses by preventing or subverting downstream PRR signalling.

Resistance to humoral defence mechanisms

Bacteria such as *Streptococcus pneumoniae* can degrade immunoglobulin, preventing opsonisation and FC-receptor mediated phagocytosis (Wikström et al., 1984). The parasite *Trypanosoma cruzi* is also able to digest all human IgG subclasses via the cation of its cysteine protease Cruzipain (Berasain et al., 2003). Helminths can prevent antibody detection and opsonisation by trapping antibodies in excretory products present on the parasite surface. Parasite-derived proteases are then able to degrade the trapped antibody. This mechanism has been demonstrated by the trematode *Echinostoma caproni* but is expected also to be employed by other Helminths (Cortés et al., 2017).

Bacteria can express proteases that degrade complement molecules C1q, C3, C4 and C5-9 (Würzner, 1999) or acquire soluble complement inhibitors from the host to prevent the destructive action of the complement cascade. Viruses, parasites and yeasts have also shown the ability to evade complement in this way .(Abou-El-Hassan and Zaraket, 2017; Meri et al., 2004; Norris et al., 1991)

Recognition Receptor modification and degradation

In contrast to strategies that prevent detection by PRR receptors by changing or shielding PAMPs, a more active approach is taken by some viruses. These strategies involve degradation or interference of these receptors to prevent them from initiating downstream signalling.

Degradation of RLR sensors can be caused by the direct activity of viral proteins, or some viral proteins can interact with RLRs so that they are targeted for degradation by host cell machinery. West Nile virus NS1 protein targets both RIG-I and MDA5 for degradation by the host proteasome (Zhang et al., 2017), while viral proteases such as the 3C and 2A proteases employed by several picornaviruses can directly cleave RIG-I or adaptor protein MAVS (Barral et al., 2009; Feng et al., 2014; Mukherjee et al., 2011).

Even if RLRs are left intact, other evasion strategies include the prevention of RIG-I mitochondrial localization, as RIG-I and MDA5 need to localise to the mitochondria to initiate downstream signalling, (Chan and Gack, 2016). The active sequestration of MDA5 and RIG-I to viral inclusion bodies (Lifland et al., 2012) and cytoplasmic endosomes (Santiago et al., 2014) can also prevent optimal sensor activity.

Cytosolic DNA sensors are can also be targeted by viruses and other intracellular pathogens. HSV-1 can inhibit activity of both cGAS and STING with ICP27 protein targeting STING and VP22 protein inhibiting the enzymatic activity of cGAS. Viral NS2B proteases produced by multiple viruses can cause the cleavage of STING to prevent activity (Aguirre et al., 2012; Ding et al., 2018). Finally STING degradation can be induced by human cytomegalovirus IE286 protein. Interestingly, IE286 protein facilitates the proteasome-dependent degradation of STING (Kim et al., 2017)

Inhibition of host cell signalling and cytokine production

Inhibition of cell signalling downstream of PRRs can be subverted and inhibited by some pathogens to evade the action of inflammatory cytokines and proteins. *Yersina pestis* can suppress production of TNF-a, IL-1 and IL-8 pro-inflammatory cytokines by inhibiting activation of transcription factor NF-kB (Schesser et al., 1998).

Studies have also shown that NS1 influenza proteins prevents the translocation of IRF-3 (Talon et al., 2000) and NF-κB (Wang et al., 2000) into the nucleus after viral activation of upstream signalling pathways, and preventing the expression of genes vital to mounting an immune response against the virus such as the type I interferons. Similarly, the parasite *T.gondii* also interferes with NF-kB activation pathways in macrophages (Butcher et al., 2001), preventing inflammatory cytokine release and aiding survival within the host.

Surviving inside the cell

Pathogens intent on surviving within the intracellular environment must avoid anti-microbial mechanisms within the host cell. A prime example of this is acidification within the phagosome, as many intracellular pathogens enter their host cell during phagocytosis.

The parasite *Trypanosoma cruzi* survive within the phagolysosome by producing an array of anti-oxidant enzymes after phagocytosis. These include peroxidase, catalase and superoxide dismutase (SOD) and act to detoxify ROS before parasite damage and death can occur (Ding et al., 2004). In a slightly different approach to phagosome survival *Mycobacterium tuberculosis* can prevent the phagolysosome fusion event, by depleting H+ ATPase molecules from the vacuolar membrane (Sturgill-Koszycki et al., 1994). This prevents acidification and maturation of the phagosome protecting the bacterium from degradation. *Listeria monocytogenes* simply escape from the phagosome after entry to host cell due to the activity of a bacterial toxin able to disrupt the endosomal membrane (Dramsi and Cossart, 2002). These bacteria then multiply within the cytoplasm of macrophages.

Various fungal species are able to survive phagocytosis due to the presence of the capsule which protects the fungal cell from ROS and radicals and other anti-fungal molecules (Hernández-Chávez et al., 2017). *C.albicans* yeast can also escape phagocytosis by filamentation, as the growth of hyphae can perforate phagocytic cells (McKenzie et al., 2010). Alternatively, *C.albicans* has also demonstrated high levels of catalase which allows degradation of H_2O_2 (Komalapriya et al., 2015).

1.4 *Leishmania* and the innate immune response

Leishmania is a complex parasite that has evolved while in strict contact with its host cell's immune system. This has allowed the development of many different evasion mechanisms that will enable it to evade destruction and survive inside host cells. Many of its immune evasion mechanisms, virulence factors and immunomodulatory effects have been described (Atayde et al., 2016; Gomez et al., 2009; Gregory and Olivier, 2005; Hallé et al., 2009; Kumari et al., 2022; Shio et al., 2015) and are vital for progressing our understanding of host-parasite interaction. This is especially important if more effective therapeutics that target crucial *Leishmania* proteins or their evasion mechanisms are to be developed.

However, it is important to consider that every *Leishmania* spp. infection can manifest a different disease state in the host (Gurung and Kanneganti, 2015), as infection with different species can lead to several different host immune responses. In addition to species-specific infection strategies, the host's genetic background and immune state may also impact on the course of disease progression and how an immune response against this parasite is shaped. It is important to understand that there is currently no defined or standard series of events that occurs upon the interaction of *Leishmania* parasites and the innate immune response. Additionally, achieving consensus between studies, especially those that feature different *Leishmania* species is a challenge, especially between species that cause distinct forms of the disease.

1.4.1 Initial infection

Once inoculated into the host by the sand fly, it is imperative that *Leishmania* promastigotes can quickly locate and enter a host cell. Upon entry to the epidermis *Leishmania* will also encounter many host cells, including keratinocytes.

Keratinocytes have a role in host defence against invading pathogens and release cytokines and chemokines to attract immune cells to the site of infection. This also occurs during *Leishmania* infection where keratinocyte interaction with *Leishmania* can lead to the recruitment of neutrophils, activation of T cells and can have an impact on the course of the infection (Ehrchen et al., 2010; Passelli et al., 2021; Roebrock et al., 2014; Scorza et al., 2017; Teixeira et al., 2006).

The final host cells of Leishmania parasites are long-lived tissue-resident or monocyte-derived macrophages, where they will differentiate from promastigotes to amastigotes and proliferate (Rossi and Fasel, 2018). Other immune cells such as neutrophils (Chaves et al., 2020; Peters et al., 2008), monocytes (Romano et al., 2017) and dendritic cells (Feijó et al., 2016) will be recruited to the initial infection site and may also be infected during the course of infection.

Neutrophils are most rapidly recruited to the site of infection and the first immune cells to encounter *Leishmania* parasites (Müller et al., 2001; Peters et al., 2008). In addition to keratinocyte mediated recruitment (Passelli et al., 2021), it has been shown that promastigote secretory gel that is synthesized by *Leishmania* within the sand fly gut, and also delivered into the skin also acts as a neutrophil attractant (Rogers et al., 2009). Sand fly gut bacteria inoculated into the host at the same time as the parasite induce inflammasome derived IL-1β secretion, which also aids in neutrophil recruitment (Dey et al., 2018).

Generally, the role of neutrophils in infection is complex, as they can both assist in clearance and survival of the parasite depending on the *Leishmania* species present. However, a study has shown that neutropenic mice are resistant to non-healing lesions caused by *L.mexicana,* suggesting that neutrophils have a role in disease progression. It is suggested that they may promote infection by preventing the recruitment of other immune cells to clear the parasite (Hurrell et al., 2015). This is in contrast to other

Leishmania species such as *L.amazonesis* which are effectively killed by Neutrophil extracellular traps (Hurrell et al., 2016).

Neutrophils can be infected by *Leishmania* parasites and even allow parasite replication (Hurrell et al., 2017). They also have a key role in *Leishmania* transmission to their final host cell, as phagocytosis of infected or apoptotic neutrophils by macrophages allows transmission of parasites (Ritter et al., 2009). This means of delivery to macrophages prevents macrophage activation (van Zandbergen et al., 2004) aiding in parasite evasion.

1.4.2 Entry to the Macrophage

As *Leishmania* must enter a host cell in order to replicate, strategies that allow entry to the macrophage without detection are necessary for successful disease progression. *Leishmania* has developed multiple different approaches, including neutrophil aided phagocytosis as described above. *Leishmania* virulence factors can also mediate many of these methods of entry. Different species of *Leishmania* may rely on a variety of macrophage receptors, including complement receptors (CRs), mannose receptors (MR), fibronectin receptors and Fcγ receptors (FcγRs).

Virulence factor and surface protease GP63 can cleave the complement protein C3b to its inactive form, iC3b. This prevents detection and destruction of the parasite by complement-mediated lysis, and instead allows parasite entry via the CR3 (Brittingham et al., 1995). CR3 has been shown to be the common entry receptor for several species of *Leishmania* and allows entry to the parasite without triggering nitric oxide production. GP63 on the parasite surface can also bind to human fibronectin receptors and allow entry via this receptor (Brittingham et al., 1999).

In some species of *Leishmania,* the binding of host C-reactive protein (CRP) to the cell surface virulence factor LPG triggers phagocytosis via the CRP receptor, without activating macrophages and providing favourable conditions for intracellular replication (Culley et al., 1996).

After transformation to amastigotes, the parasite must leave and re-infect nearby macrophages. Due to a decrease in surface expression of LPG and GP63, amastigotes have been shown to instead enter via a Fc receptor dependent method following antibody opsonisation, rather than the above mechanisms (Guy and Belosevic, 1993)

1.4.3 TLRs and *Leishmania* infection

The TLRs are known to contribute to the innate immune response to *Leishmania* and have a clear role in recognition and subsequent parasite control. The TLRs are also the first receptors to recognize *Leishmania*-associated PAMPs after entry to the skin (Bamigbola and Ali, 2022). Mice deficient in TLR adaptor MyD88 have been shown to be highly susceptible to *L.major* infections (Muraille et al.2003), and mice deficient in TLR2 and TLR4 have larger lesions and higher parasite burdens than WT controls (Halliday et al., 2016) confirming an important role for TLRs in controlling parasite infection. However, some *Leishmania* spp subvert TLR signalling, exploiting TLR activation for their own benefit or inhibiting it to ensure their survival.

TLR2, TLR3, TLR 4 and TLR 9 have all been implicated in the detection of *Leishmania*. Glyco-sphingophospholipid (GSPL) (Karmakar et al., 2012) and proteoglycolipid complex (P8GLC) (Whitaker et al., 2008) from *Leishmania* induce TLR 4 activation. TLR 9 recognizes unmethylated CpG DNA sequences, which are commonly found in *Leishmania* (Gupta et al., 2015) and TLR 2 is able to recognise LPG (Becker et al., 2003; de Veer et al., 2003; Srivastava et al., 2013), the most abundantly expressed surface molecule of *Leishmania*. Finally, the endogenous TLR 3 recognises dsRNA and has also

shown to be activated during *Leishmania* infection, specifically by the *Leishmania* RNA virus 1 (LRV1) that is harboured by some *Leishmania* spp (Ives et al., 2011).

Activation of these TLRs by *Leishmania* can be essential for parasite control, with the recognition of *Leishmania* DNA by TLR9 able to induce IL-12 production and lysis of infected cells (Liese et al. 2007). Similarly LPG activation of TLR2 results in parasite control through IL-12, NO and ROS production (Kavoosi et al., 2010, 2009; Rojas-Bernabé et al., 2014).

However, as with many aspects of *Leishmania* infection, parasite-TLR interactions in both activation and regulation can vary between species. Some TLRs can play a species-specific role in activating a suitable immune response (Rossi and Fasel, 2017) for example, TLR 2 promotes the clearance of *L.mexicana, L. major* and *L. aethiopica* infection by promoting downstream NO and ROS production (Becker et al., 2003; de Veer et al., 2003). However in other *Leishmania* species such as *L.braziliensis* (Vargas-Inchaustegui et al., 2009) and *L. amazonensis* (Guerra et al., 2010) TLR 2 activation is thought to have a regulatory role that favours parasite survival, as infections with parasites deficient of TLR 2 had better parasite control compared to the WT. These differing roles may depend on variable expression of different PAMPS on the surface of different *Leishmania* species, but this is yet to be confirmed (Rossi and Fasel, 2018).

Another TLR interaction with *Leishmania* that varies between species is the activation of TLR 4. During *L.major* (CL causative) infection, the *Leishmania* protein known as inhibitor of serine peptidases (ISP) 2 inactivates host neutrophil elastase (NE) present at the macrophage surface, resulting in blockade of TLR4 activation leading to enhanced parasite survival (Faria et al., 2011). However in *L. donovani* (VL causative) ISP2 is absent or only expressed at low levels, and instead *L. donovani* uses the host NE-TLR activation for its own benefit, as the downstream production of IFN-β is beneficial for *L. donovani* parasite survival and growth during early infection (Dias et al., 2019). This

confirms how different *Leishmania* species can have completely different evasion mechanisms to facilitate early infection and the establishment of infection.

The suppression of TLR signalling pathways following some *Leishmania* infections can also be demonstrated by a lack of response to PAMPs derived from pathogens other than *Leishmania. Leishmania* infection can also inhibit LPS (a TLR 4 agonist) induced IL-12 production (Belkaid et al., 1998; Cameron et al., 2004; Chandra and Naik, 2008; Lapara and Kelly, 2010) and downregulate Pam3cys (a TLR 2 agonist) stimulated IL-12p40 production (Chandra and Naik, 2008). This suggests that *Leishmania* infection has a wider impact on the innate immune response that is not limited to its own evasion, but also that of other pathogens. This may contribute to why secondary bacterial and viral infections can be so serious in Leishmaniasis patients and are often the ultimate cause of death in severe cases.

1.4.4 NLRs and *Leishmania* infection

After the TLRs, the interaction of *Leishmania* and the NLR pathway is probably the next most well researched of the PRRs, although studies vary in demonstrating whether NLR activation positively or negatively affects the host during disease.

One of the first studies concerning *Leishmania* and the NLRP3 inflammasomes showed that many *Leishmania spp*, including *L. mexicana*, induce caspase-1 activation and IL-1β production upon macrophage infection (Lima-Junior et al., 2013) promoting *Leishmania* killing through NO production. Disruption to this pathway's activation by the parasite, just like that of the TLRs, has also been demonstrated; GP63 prevents NLRP3 inflammasome activation by directly cleaving NLRP3 (Shio et al., 2015). In contrast to the observed protective role of the NLRP3 inflammasome activation in *L.mexicana*, Gurung et al (2015) have shown that during *L.major* infection of susceptible mice, activation of the NLRP3 inflammasome is detrimental to the host, partially due to IL-18

induction (Gurung et al., 2015). Again, as with the TLRs there seems to be significant species variation in the interaction with host PRRs.

The NOD2 receptor has also been shown to important in the recognition of new world *Leishmania* species such as *L.amazonensis* or *L.braziliensis* in human PMBCs. NOD2 was shown to be crucial for the subsequent intracellular killing of *Leishmania* and induction of innate and adaptive immune responses after recognition (dos Santos et al., 2017). In contrast the NOD1 receptor was not deemed to be important for these responses. Another study in mice also showed that activation of the NOD2-RIP2 pathway by *L. infantum* was necessary for the development of a protective Th1 immune response (Nascimento et al., 2016).

More recently *Leishmania* infection has been shown to cleave and subvert the normal functions of Gasdermin-D (de Sá et al., 2023). Gasdermin-D is an important inflammasome component (He et al., 2015) required for pyroptosis and NLRP3 inflammasome activation. Despite subversion of this pathway, this study also showed that Gasdermin-D KO mice exhibited less NLRP3 inflammasome activation and were highly susceptible to infection by several *Leishmania* species, therefore confirming the role of Gasdermin-D and NLRP3 activation for inflammasome-mediated host resistance (de Sá et al., 2023).

Further research will be needed to allow a complete picture of whether different aspects of NLR signalling are host detrimental or host protective when activated by different species of *Leishmania.*

1.4.5 RLRs and *Leishmania* infection

To date there has been very little research into interactions between *Leishmania* and the RLRs. It could be expected that RLRs would have a role in the detection of the dsRNA virus LRV1 harboured by some *Leishmania* species in a similar way to how TLR3 has been shown to detect LRV1 (Ives et al., 2011). However, experiments by Hartley et al (2018) have shown that in mice cells lacking RLR adaptor molecule MAVS did not affect the response to parasites positive for LRV1. This suggests that LRV1 dsDNA is not detected by RLRs upstream of MAVS during infection (Hartley et al., 2018).

1.4.6 Cytoplasmic DNA sensors and *Leishmania* infection

There has been a lack of investigation into interactions between *Leishmania* and the various cytoplasmic DNA sensors. This is somewhat surprising as *Leishmania* completes its lifecycle within parasitophorous vacuoles located within the cytoplasm of macrophages. While the parasites confinement to this vacuole will provide protection from some detection, secreted virulence factors have been shown to consistently reach the cytoplasm and even nucleus of the host cell to interact with host signalling pathways. This suggests some transfer of parasite molecules into the cytoplasm does occur. It is possible that cytosolic DNA sensors will be able to detect *Leishmania* DNA if it leaks into the macrophage cytosol during parasite uptake and replication within the parasitophorous vacuoles. (Sun and Cheng, 2020)

While there has been limited research on *Leishmania* and the cytoplasmic DNA sensors, a very recent paper has described activation of the cGAS-STING DNA sensing pathway during *L.major* infection of human THP-1 macrophages (Yilmaz et al., 2022). Here it was shown that the delivery of isolated *L.major* kinetoplast DNA (kDNA) to the cytosol was able to activate cGAS and downstream signalling to promote a pro-parasitic state.

Collectively, these results suggest that *Leishmania* parasites may hijack the cGAS-STING-TBK1 signalling pathway to their own advantage.

STING has also been identified as a protein of interest following *Leishmania major* and *Leishmania amozenesis* infections in BALB/c mice and *in vitro* assays using the murine J774 macrophage cell line. Proteomic analysis of these Leishmaniasis models found that STING was the most upregulated protein after infection with these *Leishmania* species. Additionally, upstream analysis from cutaneous lesions in these infected mice predicted STING activation. This suggests that STING activation and upregulation is likely to occur during *Leishmania* infection and appear to agree with findings by *Yilmaz et al* (2022) described above that also suggest that STING activation induced by *Leishmania* occurs in human cells during infection.

IFI16 is another cytoplasmic DNA sensor and known to have a role in the full activation of STING where it co-operates with STING ligand cGAMP to achieve this. Blood transcriptomics of cutaneous leishmaniasis patients has identified IFI16 as a biomarker for healed CL. IFI16 was found to be overexpressed in the skin and blood of *Leishmania tropica* CL patients who had resolved the disease (Bahrami et al., 2022). This would appear to suggest that IFI16 upregulation is associated with CL resolution. More research will be required to understand if IFI16 has an active role in this resolution, and if this is due to its activity as a DNA sensor or involvement with STING activation.

1.4.7 Cytokine release

The initial host innate immune response to *Leishmania* is important during the early stages of infection, however parasite control and clearance also depends largely on T cell-mediated immunity and cytokine release from many different immune cells.

The cytokine response is important for determining disease outcome, whether it be to the benefit or detriment to the host. Host cytokine production can also be inhibited by *Leishmania* through the degradation of transcription factors that regulate their gene expression. For example *Leishmania* virulence factor GP63 mediated degradation of the AP-1 transcription factor may cause inhibition of TNF-α, , IL-1β and IL-12 production (Foletta et al., 1998; Gomez et al., 2009; Hallé et al., 2009; Newell et al., 1994; Zhu et al., 2001), adding further to the complex regulation of cytokines during *Leishmania* infection.

Following much investigation within animal models, the cytokine response to *Leishmania* can be generally simplified into either a strong Th1 or Th2 immune response. (Ajdary et al., 2000; Dayakar et al., 2019; Reiner and Locksley, 1995; Sacks and Noben-Trauth, 2002; Scott, 1991; Torres-Guerrero et al., 2017). A Th1 response is known to be host protective in models of both CL and VL (Castellano et al., 2009; Samant et al., 2021), whereas a Th2 response can be permissive for *Leishmania* growth and progression of the disease.

A Th1 response is associated with the production of cytokines IL-12, IFN-γ and TNF- α in addition to nitric oxide (NO), and reactive oxygen species (ROS) for parasite control (Kaye and Scott, 2011). IL-12 is mainly produced by activated macrophages and dendritic cells and can bridge the innate and adaptive immune responses (Abdi, 2002; Sartori et al., 1997), as it drives the TH1 response, and promotes T cell proliferation (Manetti et al., 1993; Yoo et al., 2002) *In vivo* studies showed that IL-12 produced in response to *Leishmania* in mice controls Th2 expansion in addition to increasing the Th1 type response (Dayakar et al., 2019; Heinzel et al., 1995; Sypek et al., 1993) Neutralization of IL-12 during early and late infection has shown to lead to a more serious disease in *L.donovani* infections, again highlighting its importance in parasite control (Engwerda et al., 1998)

The Th2 response is associated with the secretion of Interleukin IL-4, IL-10 and transforming growth factor-beta (TGF-β) (Dayakar et al., 2019).

IL-4 is associated with down-modulation of IFN-γ-mediated macrophage activation (1, 17) while IL-10 suppresses macrophage killing of *Leishmania* (Bhattacharyya et al., 2001; Vieth et al., 1994). IL-10 KO mice are also much more resistant to *Leishmania* infection (Belkaid et al., 2001; Murphy et al., 2001), confirming the role of IL-10 in aiding parasite survival.

However, while still useful for experimental models of Leishmaniasis, a clear functional dichotomy in CD4 T cells during human Leishmaniasis has not been documented and the Th1-Th2 balance is more complex. For example, while important for parasite clearance, excessive inflammatory cytokine production also directly contributes to the central pathology of CL by causing tissue damage (Maspi et al., 2016). The various roles of TNF-α during infection can demonstrate this complexity. Macrophage production of TNF-α assists in *Leishmania* clearance by increasing macrophage activity and promoting NO synthesis (Liew et al., 1997) and mice deficient in TNF-α , and infected with *L. major* parasites showed fatal visceral *Leishmania* infection (Wilhelm et al., 2001), despite the production of other inflammatory cytokines by infected macrophages. However, although TNF-α production seems to be essential for proper control of *Leishmania*, an increased level of TNF- α is associated with tissue destruction and development of cutaneous lesions (Antonelli et al., 2005; Bacellar et al., 2002; Oliveira et al., 2011).

Furthermore, host production of anti-inflammatory IL-10 to prevent excessive tissue damage can be induced but may be inadvertently beneficial to *Leishmania* by allowing them to persist within the cell and disease progression to occur (Castellano et al., 2015; Maspi et al., 2016).

1.4.8 *Leishmania* virulence factors

The virulence of *Leishmania* parasites relates to parasite infectivity, invasiveness, and the degree of disease establishment within the host. Virulence factors are molecules produced by a pathogen that loss of would significantly impact their virulence but not necessary their viability (Bifeld and Clos, 2015). *Leishmania* parasites have evolved a wide range of membrane-bound and secreted virulence factors that act in many different ways to aid pathogenesis and evade the host immune response. Here several of the most well researched and abundant virulence factors are described.

1.4.7.1 Lipophosphoglycan (LPG)

As with other parasites of the Trypanosomatid family, the surface of *Leishmania* is composed of a dense glycocalyx composed of glycosylated proteins and glycans (Ferguson, 1997). During the promastigote stage, Lipophosphoglycan (LPG) is the most abundant surface glycoconjugate and aids parasite virulence in many ways. This is in contrast to the amastigotes stage when LPG expression is highly downregulated (Franco et al., 2012; Turco and Sacks, 1991). This supports the idea that different immune evasion strategies are used by *Leishmania* at different life cycle stages and suggests that LPG is most crucial for aiding promastigotes in the initial infection of host macrophages.

LPG is known for its wide range of functions that aid in the virulence of *Leishmania* during the initial stages of infection, allowing the parasite to establish infection within its chosen host cell. After entry to the host, LPG can protect metacyclic promastigotes from complement-mediated lysis. The long length of LPG during this lifecycle stage prevents the attachment of complement membrane attack complex (MAC) and pore formation on parasite surface (Franco et al., 2012). LPG is also key for facilitating silent entry of promastigotes into the macrophage through the interaction of several host receptors.

LPG can bind to complement receptor CR3 (Puentes et al., 1988; Talamás-Rohana et al., 1990) and intergrin receptor p150/95 (Mosser and Brittingham, 1997; Talamás-Rohana et al., 1990).

Once phagocytosed LPG allows survival of the parasites by delaying the fusion of the phagosome and lysosome (Vinet et al., 2009). Additionally LPG can interfere with the assembly of a functional NADPH oxidase complex at the phagosome membrane, again interfering with host innate defence mechanisms (Lodge et al., 2006). LPG can also prevent damage to *Leishmania* from reactive oxygen intermediates by acting as a scavenger of oxygen radicals (Chan et al., 1989).

LPG is also able to disrupt more macrophage functions including inhibiting the activation of Protein kinase C (PKC) a key macrophage signalling protein, (Descoteaux et al., 1992), and inhibition of cytokines IL-12 and IL-1β at the transcriptional level (Hatzigeorgiou et al., 1996; Piedrafita et al., 1999)..

The many activities of LPG are crucial to virulence and survival of many *Leishmania* species and helps to provide a permissive environment within the host cell for differentiation and replication of amastigotes. Despite this, the activity of LPG is likely to have redundancy with other *Leishmania* virulence factors as it was shown that LPG deficient *L.mexicana* is just as virulent the wild-type (Ilg, 2000).

1.4.7.2 Proteases

Many different classes of proteases have been shown to have a role in the establishment of *Leishmania* infection. These include serine proteases, cysteine proteases, aspartic proteases, metalloproteases and threonine proteases among others. During infection proteases can degrade a multitude of target proteins and peptides to aid tissue invasion, parasite propagation and dysregulate the immune response.

GP63

Discovered in the 1980s, glycoprotein 63, also known as Leishmanolysin was considered the major surface antigen of *Leishmania* (Bouvier et al., 1985; Chang et al., 1986), GP63 is found in many different *Leishmania* species and is thought to be critical for immune evasion by the parasite. There are multiple genes encoding GP63, and these are highly conserved between different *Leishmania* species (Webb et al., 1991). GP63 also has structural homologs in other protozoa such as *Trypanosoma sp* (Cuevas et al., 2003) and *Trichomonasvaginalis* (Ma et al., 2011).

GP63 is a zinc-dependent metalloprotease found on the cell surface of both the promastigote and amastigote form of *Leishmania*. GP63 is highly abundant, especially on promastigotes where it comprises 1% of the total proteome, approximately 500,000 molecules per cell (Bouvier et al. 1995). Expression of the protein varies between the two primary lifecycle forms, with amastigotes expressing significantly less GP63 than the promastigote form (Schneider et al., 1992). This appears to suggest that GP63 activity will be much higher in the promastigote form; however, it has been noted that on amastigotes there is also significantly less expression of other surface proteins (e.g. LPG) that may normally restrict GP63 substrate access. In the promastigote form the majority of GP63 is anchored to the parasite cell surface by means of Glycosylphosphatidylinositol (GPI)- a short glycolipid that may be attached to the C-terminus of a protein during posttranslational modification. GP63 is also secreted directly from the parasite or within extracellular vesicles (Arango Duque et al., 2019). GPI anchored GP63 is released continuously from the parasite cell surface due to autoproteolysis mechanism (McGwire et al., 2002; Yao et al., 2007).

GP63 can cleave a wide range of proteins, a quality which allows it to disrupt multiple signalling pathways and mechanisms at once within the host macrophage cell to benefit

the parasite. It has been shown that GP63's target site is LIAY//LKKAT (Bouvier et al., 1990) suggesting that GP63 cleaves between polar and hydrophobic residues at position P1 and P'1 with basic residues at positions P'2 and P'3. However, GP63 has also been shown also to degrade extracellular matrix proteins such as fibrinogen aiding in parasite migration towards host cells and tissue infiltration (McGwire et al., 2003). GP63 also has a role in facilitating parasite entry to the macrophage via complement receptors.

Notably, GP63 can subvert the macrophages own complex signalling regulatory system for its own benefit. Through cleavage events, it is able to activate multiple intracellular protein tyrosine phosphatases (PTPs) such as SHP-1, TCPTP and PTP1B, which then negatively regulate various cell signalling pathways, many downstream of TLRs, by cleaving/degrading key molecules (Gregory and Olivier, 2005). GP63 is also able to act directly on other key signalling components leading to their degradation or cleavage to smaller protein fragments. *Leishmania* GP63 cleaves major transcription factors of macrophages such as STAT1, NF-κB and AP-1 (Atayde et al., 2016). GP63 partially degrades NF-κB subunit p65 to produce p35, which heterodimerizes with another NF-κB subunit p50. This heterodimer prevents transcription of NF-κB-regulated genes, such as *iNOS* and *IL-12* that important for parasite control, but do induce transcription of Leishmaniasis-promoting chemokines such as macrophage inflammatory protein (MIP) 1α and MIP1β (Gregory et al., 2008a; Guizani-Tabbane et al., 2004). Key innate immune signalling components affected by GP63 directly or indirectly within the macrophage are summarised in Table 3.

An abundance of research into the activities of GP63 have demonstrated that it has a key role in the virulence of *Leishmania* sp. However, a recent re-examination of the activities of the metalloprotease have suggested it may not degrade as many intracellular targets as previously suggested (Guay-Vincent et al., 2022). Further research will be needed to confirm this re-evaluation.

Table 3: GP63 interaction partners within the macrophage

Many of these parasite targets are involved in host signalling or pathogen control activities. Blue highlighting indicates when activity on targets is indirect.

Target	Mechanism (Indirect)	Species first reported to occur	Reference
AP1	GP63 enters the nucleus of the host macrophage to cleave c-Jun and c-Fos subunits	*L.major, L.donovani, L.mexicana*	(Contreras et al., 2010)
C3b	Cleaved to inactive C3bi	*L.major, L.mexicana, L.donovani, L.amazonensis*	(Brittingham et al., 1995)
CD4	Cleaved by GP63	*L.major, L.donovani*	(Hey et al. 1994)
c-JUN	Degraded by GP63	*L.mexicana*	(Contreras et al., 2010)
ELK	Inhibited by SHP-1 and PTB-1B	*L. amazonensis; L.donovani*	(Martiny et al., 1999; Nandan et al., 1999)
IRAK 1	Dephosphorylation by SHP-1	*L.donovani, L.mexicana, l.major*	(Abu-Dayyeh et al., 2010)
Janus Kinase (JAK) 2/3	Dephosphorylation by SHP-1 and PTB-1B	*L.donovani*	Blanchette et al 1999

Mitogen-activated protein kinases (MAPKs)	Dephosphorylation by PTPs and SHP-1	*L.amazomensis, L.donovani*	(Martiny et al., 1999; Nandan et al., 1999)
MARCKS MRP	Cleaved by GP63	*l.major*	(Corradin et al., 1999)
mTOR/mTORC1	Cleaved by GP63	*L.major*	(Jaramillo et al., 2011)
NF-kB p65	Degraded by GP63 and induces the translocation of a modified form of NF-kB to the nucleus, named P35 RelA, shown to pair with P50 instead of the canonical P65 RelA	*L. donovani, L. major, L. mexicana, and L. (Viannia) braziliensis,*	(Gregory et al. 2008)
NLRP3	Formation abrogated due to observed GP63-dependent cleavage of inflammasome and inflammasome-related proteins	*L.mexicana*	(Shio et al., 2015)
Nucleoporins	Degraded by GP63, and allows access to TFs.		(Isnard et al., 2015)
P130 cas	Cleaved by GP63	*L.major*	(Hallé et al., 2009)
Pro-IL-1B	Cleaved by GP63	*L.major/ L.mexicana*	(Shio et al 2015)

PTP-1B	Cleavage of C' terminal portion by GP63 to activate	*L.mexicana L.major*	(Gomez et al., 2009)
SHP-1	Cleavage of C' terminal portion by GP63 to activate	*L.major, L.mexicana*	(Gomez et al., 2009)
SNARE-VAMP8	Cleaved by GP63	*L.major, L.donovani*	(Matheoud et al., 2013)
STAT 1	Degraded by GP63 (and CPB)	*L.mexicana*	(Casgrain et al., 2016)
TC-PTP	Cleavage of C' terminal portion by GP63 to activate	*L.major*	(Gomez et al., 2009)

Cysteine Proteases

Another of the most-studied virulence components of *Leishmania sp* are the cysteine proteases (CPs), a broad group of papain-like enzymes that have an essential role in *Leishmania* biology as well as their virulence activities. CPB is the most studied CP and has many different host targets.

Similar to GP63, CPB has been shown to modulate macrophage protein tyrosine phosphatases and transcription factors NF-κB , STAT1 and AP-1 (Abu-Dayyeh et al., 2010; Cameron et al., 2004) during *L. Mexicana* infection. However, in contrast to GP63 CPB completely degrades NF-κB subunit p65, rather than cleaves it to a smaller form (Cameron et al., 2004).

Besides transcription factors, CPB can also cleave MHC-II protein within the macrophage, preventing antigen presentation and thus activation of the Th1 immune response (De Souza Leao et al., 1995)

CPs have also been shown to potentially be able to modulate parasite GP63 expression (Casgrain et al., 2016), as expression of GP63 is inhibited in CPB deficient *L.mexicana* parasites. CPB has also been implicated in parasite survival within macrophages by participating in the formation of parasitic vacuoles within macrophages that *L.mexicana* can replicate within (Casgrain et al., 2016).

Interestingly CPs have shown to be important for determining Leishmaniasis disease localisation. In *L.donovani* and *L.major* a single nucleotide polymorphism in genes encoding CPs has been identified as the determining factor in whether the resulting parasite infection will be dermatropic or viscerotropic (Hide and Bañuls, 2008).

1.4.7.3 Elongation Factor 1α

Elongation Factor 1α (EF-1α) is a known GTP-binding protein involved in protein synthesis across eukaryotes. In addition to this function within *Leishmania* parasites, EF1α also acts as a successful virulence factor. The most well-known virulence activity of EF1α is to bind and activate host SHP-1 (Nandan et al., 2002) leading to macrophage inactivation, similar to an activity of virulence factor GP63.

It has also been established that *Leishmania* EF-1α binds with SHP-1 in a distinct manner compared to host EF-1α, and also that host EF-1α binding to SHP-1 does not result in its activation (Nandan and Reiner, 2005). These differences between host and *Leishmania* EF-1α have identified it as a possible therapeutic target.

1.4.7.4 Heat shock proteins

The heat shock proteins (HSPs) are essential for allowing survival of *Leishmania* as they transition between vastly different conditions within the sandfly gut and mammalian host. Leishmanial HSPs allow the parasite to survive harsh conditions such as the acidic pH of macrophage vacuoles and any potential heat induced damage as they transition to a new host with a 10°C temperature increase (Prasanna and Upadhyay, 2021). HSPs ensure parasite survival by assisting in protein folding to prevent aggregation and degradation, but are also involved in parasite differentiation and virulence (Kumari et al., 2022).

HSP100 in particular has an immunomodulatory role during infection involving the regulation of extracellular vesicles (EVs) secreted by *Leishmania.* These EVs are able to suppress the host immune response and can promote disease progression. In *Leishmania donovani* parasites lacking HSP100, the EVs released had a different composition compared to the WT and lost some of their immunosuppressive impact on host immune cells (Silverman et al., 2010). HSP100 is also implicated in the successful differentiation

of promastigotes to amastigotes (Krobitsch and Clos, 1999) demonstrating that a loss of these proteins will have a significant impact on parasite virulence and disease development.

1.4.9 *Leishmania* derived extracellular vesicles

Another way that *Leishmania* parasites prepare for and can perpetuate infection is by the secretion of extracellular vesicles (EVs). EVs, also referred to as exosomes are endosomal vesicles between 30-150 nm of diameter and are produced by the majority of eukaryotic and prokaryotic cells (Johnstone et al., 1987; Rajput et al., 2022). These small vesicles can encapsulate a variety of proteins, lipids, DNA, RNA, miRNAs and lipids (Mathivanan et al., 2012) which are delivered from the host cell into the external environment where they may be delivered or taken up by recipient cells.

After uptake these EVs can release their cargo which may influence physiological and pathological processes (Rajput et al., 2022). These EVs have been described to have a role in cell to cell communication (Mathivanan et al., 2010; Yuan et al., 2017; Zhou et al., 2017), tumour development (Skog et al., 2008), autoimmune diseases (Turpin et al., 2016) and during infectious diseases (Hosseini et al., 2013). EVs are proposed to be used as biomarkers, especially for cancer (Araujo-Abad et al., 2022; Skog et al., 2008) and for therapeutic purposes in diseases as a drug delivery system (Chung et al., 2020).

EVs are a significant component of the *Leishmania* secretome. They are secreted both within the sandfly midgut (Atayde et al., 2015) and when inoculated into the skin of their mammalian host (Hassani et al., 2011; Silverman and Reiner, 2012).

EVs containing *Leishmania* derived proteins are able to enter macrophages before the parasite and prime them for parasite invasion by mediating immunosuppression and can contribute to disease progression. (Barbosa et al., 2018; da Silva Lira Filho et al., 2021;

Das et al., 2021; Silverman and Reiner, 2012). *Leishmania* virulence factors such as GP63 and elongation factor 1a have been identified as key components of these exosomes (Silverman and Reiner, 2012). GP63 was shown to be crucial to the suppression of the immune response by these EVs, as GP63-deficient EVs had much reduced immunomodulatory abilities and also a different EV protein content (Hassani et al., 2014)

It has also been discovered that *Leishmania* infection can alter the cargo of host macrophage EVs (Gioseffi et al., 2020; Hassani and Olivier, 2013). Other intracellular pathogens have also been shown to alter the EVs produced by their host cells (Rezaie et al., 2021; Schorey et al., 2015; Zhang et al., 2018) and this is seen as an additional strategy to mediate immune modulation and disease progression during infection from the safety of a host cell. This seems to also be the case with EVs released by *Leishmania donovani* infected cells, as these altered EVs may play a role in lesion development during disease by promoting vascular changes during infection (Gioseffi et al., 2020). Currently this area of EV research is understudied, and further work will be required to further determine the significance of altered EVs released from infected cells *Leishmania* infection.

1.5 Gaps in knowledge

There is still much to be discovered surrounding the innate immune response to *Leishmania* infection and the many different strategies *Leishmania* utilises to evade the immune response. For example, a more complete understanding of the role of different PRRs during infection with varying species of *Leishmania* could help to develop an adjuvant for a *Leishmania* vaccine or a more targeted drug without the limitations of chemotherapeutic agents.

While a complete understanding of how *Leishmania* interacts with the TLRs and PRRs may be complicated by the many species-specific interactions, it is clear that these PRRs and their downstream signalling pathways are targeted as part of their immune evasion strategy. Therefore, whether *Leishmania mexicana* can inhibit additional PRRs other than the TLRs and NLRs is a pertinent question. In comparison to the TLRs and NLRs, there is a clear lack of research into the other key PRRs- namely the cytoplasmic sensors including RLRs and cytoplasmic DNA sensors.

The immune response necessary to control *Leishmania* infection can be species specific (Rossi and Fasel, 2018) which can complicate and confound efforts to develop a more effective vaccine or standard treatment for the disease. While a species-specific formulation may be most effective for this, it is possible that with greater understanding and research a more suitable therapeutic target, effective against a range of *Leishmania* species, will be identified. While treatments based on inhibiting *Leishmania* virulence factors have been suggested (Chhajer et al., 2016; Das et al., 2020; Olivier and Hassani, 2010), it would first be desirable to more fully characterise the range of virulence factors.

Despite the discovery of several key *Leishmania* virulence factors, including the major surface protease GP63, as well as many of their host interaction partners, we are still far from a full understanding of how *Leishmania* exploit and evade the human immune system to perpetuate their infection. The haploid genome of *Leishmania spp* is organised into 30

chromosomes (Ivens et al., 2005). An analysis of the *L.mexicana* genome predicted a total of 9,169 putative protein-coding genes, of which 936 have not been previously described (Fiebig et al., 2015). It is highly possible that more proteins involved in the host-parasite interaction are still yet to be characterised and could play an essential role in immune evasion. This study also revealed that proteins of unknown function are among the most upregulated genes during amastigote stage of *Leishmania* suggesting many could have a key role during this stage (Fiebig et al., 2015).

Furthermore, due to the promiscuous enzymatic action of GP63 it is also likely that many targets of GP63, are yet to be identified. While the interaction of GP63 and other virulence factors with some components of signalling cascades downstream of the TLRs has been reported, there has been little to no investigation of *Leishmania* infection involvement with cytosolic PRRs reported e.g. RLRs and cytosolic DNA sensors.

1.6 Project Aims

In this thesis, changes to the innate immune response during the early hours after *in vitro* *Leishmania mexicana* infection within macrophages is investigated. In this project, we will aim to investigate how *Leishmania mexicana* parasites modulate and/or inhibit the host immune innate response to evade detection by the macrophages they live in.

Our aims for this project are:

- To determine if *Leishmania mexicana* can dysregulate pattern recognition receptors and downstream signalling during *Leishmania mexicana* infection of macrophages.
- To determine if intracellular DNA and RNA receptors play a role in detecting *Leishmania* within macrophages, and if *Leishmania* infection can inhibit cytosolic DNA sensing pathways or RLRs.
- To determine if any additional *Leishmania* virulence factors that target host proteins involved in innate immune signalling can be identified.

Chapter 2: Materials and Methods

2.1 Composition of buffers used in experiments

- **10x Running Buffer**

  0.25M Tris base, 1.92M Glycine, 1% (w/v) Sodium Dodecyl Sulphate (SDS)

- **10x Transfer Buffer**

  0.25M Tris base, 1.92M Glycine

- **10x Tris Buffered Saline (TBS)**

  0.2M Tris base, 1.5M NaCl

- **10x Phosphate buffered Saline (PBS)**

  0.1M Na_2HPO_4, 1.37M NaCl, 0.018M KH_2PO_4, 0.027M KCl

- **3x SDS Sample buffer**

  62.5mM Tris-HCl (pH 7.4), 2% (w/v) SDS, 10% (v/v) Glycerol, 0.1% (w/v) Bromophenol Blue, 50mM Dithiothreitol (added just before use)

- **50x TAE**

  2M Tris base, 5.7% (v/v) Glacial acetic acid, 50mM EDTA (pH 8)

- **Staining Phosphate Buffer**

  7.2 mM Na_2HPO_4, 2.8mM NaH_2PO_4 (pH 7.2)

- **Mammalian Cell Lysis Buffer**

  50mM Tris-HCl (pH 7.5), 1mM EDTA, 1mM EGTA, 1% (v/v) Triton X-100, 1mM Sodium Orthovanadate, 50mM Sodium Fluoride, 5mM Sodium pyrophosphate, 10mM Sodium β-glycerophosphate, 0.1mM PMSF, 10µl/ml Aprotinin

- **3x DNA Loading Buffer**

  30% (v/v) Glycerol, 0.025% (w/v) Bromophenol blue, 0.025% Xylene cyanol

2.2 Cell Culture

THP-1 cells (ECACC) were grown in complete RPMI (Roswell Park Memorial Institute) 1640 medium (Life Technologies) supplemented with 10% (v/v) Fetal Calf Serum (FCS) (Sigma), 50µg/ml Gentamycin (Life Technologies) and 1mM sodium pyruvate (Life Technologies). Cells were grown in suspension and maintained at cell densities in the range 2-9x10^5cells/ml at 37 °C, 5% CO_2. STING-/- and IFI16 -/- THP-1 cell lines (Jønsson et al., 2017) were also used and kindly gifted by Martin Roelsgaard Jakobsen (Aarhus University, Denmark).

For differentiation and use in experiments the THP-1 cell suspension was pelleted and resuspended in complete RPMI at a concentration of 0.8x10^6 cells/ml. The cells were treated with 100nM PMA (phorbol-12-myristate-13-acetate) (Sigma-Aldrich) in the culture media for 48 hours. After 48 hours, the cells became adherent and resembled spindle-shaped macrophages. PMA containing media was then washed away and cells were rested in fresh media for a minimum of 2 hours before further use.

Mouse bone marrow derived macrophages were obtained from C57BL/6 WT Mice and differentiated using macrophage colony-stimulating factor (M-CSF). Cells were seeded at 1 x 10^6/well in 6 well plates and cultured for 7 days in RPMI-1640 medium (Sigma Aldrich), 2mM L-Glutamine (GIBCO), 10% (v/v) FCS (Sigma) and 20 ng/ml M-CSF (Peprotech), fresh medium was added on days 4 and 6. Cells were harvested on day 7 with PBS containing 3mM EDTA and 10mM glucose. Harvested cells were seeded at 0.8 x 10^6/ml and left 2 hours to adhere before subsequent use in experiments.

Human Embryonic Kidney 293 (SV40 large T antigen) (HEK293 T) cells (Thermo, #HCL4517) were cultured in DMEM (Dulbecco's Modified Eagle's Medium) (Life Technologies) supplemented with 10% (v/v) FCS (Sigma) and 50µg/ml Gentamycin (Life Technologies). Cells were split every 2-3 days.

Immortalised human keratinocytes (HaCaTs) were grown in DMEM (Dulbecco's Modified Eagle's Medium) (Life Technologies) supplemented with 10% (v/v) FCS (Sigma) and 50µg/ml Gentamycin (Life Technologies).

2.3 *Leishmania mexicana* culture

Promastigotes

Strain MNYC/BZ/62/M379 *Leishmania mexicana* parasites were grown in Medium 199 (M199) with hanks salts, HEPES and L -glutamine (LONZA), which was supplemented by the addition of 10% heat-inactivated FCS (Sigma), 100x BME (Basal Medium Eagle) vitamins (Sigma-Aldrich) and 25µg/ml Gentamycin sulphate (Sigma-Aldrich).

This medium was further supplemented with 2% sterile human urine (Innovative Research Inc) and 20% heat inactivated FCS (Sigma) for use when promastigote growth was slow. The parasites were passaged to $5x10^5$ parasites/ml and incubated at 26°C. For infections, stationary phase (6-7days old) promastigote parasite forms were used.

Amastigotes

For transformation of *Leishmania* promastigotes to amastigotes, 10ml of stationary phase (6-7 days old*) L.mexicana* M379 promastigote culture was centrifuged for 10 minutes at 3000xg and washed with PBS. The pellet was re-suspended in 10ml of Grace's insect medium (Thermo Fisher Scientific) supplemented with 100x BME vitamins (Sigma-Aldrich), 2% sterile human urine (Innovative Research Inc), 25µg/ml Gentamycin sulphate (Sigma-Aldrich) and 20% heat-inactivated FCS. The medium was pH adjusted to pH 5.5. The parasites were incubated at 32°C and passaged every ~7 days.

2.4 Infections

Stationary phase *L. mexicana* promastigotes or amastigotes were pelleted and resuspended in complete RPMI or serum free RPMI (when subsequent PAMP stimulation was to take place). After initial optimisation experiments were carried out, in all subsequent infections cells were infected with a multiplicity of infection (MOI) of 10. Multiplicity of infection refers to the ratio of infectious agents to cells in a culture. Therefore cells were infected with a ratio of 10:1 parasites to cells. Infected cells were incubated at 37°C, 5% CO_2 for the required time. Uninfected control samples were incubated with fresh RPMI only.

After incubation, the media and any free parasites were washed away in PBS before cells were lysed.

2.5 Extracellular vesicle isolation and analysis

Isolation

THP-1 cells in T75 flasks were differentiated with 100nM PMA for 48 hours, washed twice, and refreshed with complete RPMI medium containing Exosome depleted FBS (Thermo) prior to a standard infection. After 8 hours the media was collected, filtered to remove parasites, and pooled for extracellular vesicle (EV) isolation with the exoEasy Maxi Kit (Qiagen) according to the manufacturers protocol. This process was repeated for an uninfected control.

60µl of the isolated EVs was added to plated THP-1 cells per ml cell media for 4 hours as a pre-treatment. Cells were then stimulated with 5µg/ml Herring Testis DNA. Cells were then lysed for qRT-PCR and western blot analysis.

Transmission electron microscopy

10µl of purified extracellular vesicles sample was added to a Formvar carbon copper grid (Agar Scientifc) and allowed to dry for 4 minutes. Excess liquid was removed with filter paper before staining with 10µl 1% phosphotungstic acid (Sigma) in Milli-Q water for 2 minutes. The grid was then washed with 10µl Milli-Q water and excess water again removed with filter paper. Imaging was carried out on the JEM-2100 Transmission electron microscope.

Analysis of EV DNA content

DNA from the EV isolate was extracted using QuickExtract DNA Extraction solution (Epicentre, Illumina) using the QuickExtract DNA Extraction Protocol. Amplification of *Leishmania* alpha tubulin gene and human IFN- β was carried out on the extracted EV DNA using the primers listed in Table 6.

The PCR products were run on a 1% agarose gel in combination with 3x DNA loading dye alongside appropriate positive controls. Further details on agarose gel composition and running conditions are detailed in section 2.17 Agarose Gel Electrophoresis.

2.6 Cell Stimulations

For Lipopolysaccharide stimulation (LPS) (O26:B6**,** Sigma-Aldrich) cells were incubated with 1µg/ml LPS for 1 hour.

For stimulation with herrings testes (HT)-DNA and polyinosinic–polycytidylic acid (Poly(I:C)) (both Sigma-Aldrich), serum free medium (SFM) was mixed with 1µl/ml Lipofectamine2000 (ThermoFisher Scientific) for 5 minutes. Mock-stimulated cells were treated with medium and lipofectamine only. HT-DNA and Poly(I:C) were incubated with

the SFM and Lipofectamine mix for 15 minutes prior to addition to the appropriate wells. HT-DNA was added at a concentration of 5µg/ml and Poly(I:C) at 1µg/ml, both for a 4-hour stimulation. After stimulation the PAMP containing media was washed away in PBS before cells were lysed.

For stimulation with recombinant human interferon alpha 2a (Sigma) either 2ng/ml or 20ng/ml was added to THP-1s for a 2h pre-treatment before subsequent infections.

2.7 Inhibitors

Proteasome inhibitor MG132 (Sigma) was dissolved in H_2O and used at 10µM, applied to cells 1 hour before infection. Autophagy inhibitor Bafilomycin (Sigma) was dissolved in H_2O and used at 50nM and applied to cells 1 hour before infection.

2.8 Lentivirus Transduction of STING KO THP-1 cells

Lentivirus preparation

Our gene of interest- heamagglutinin (HA) tagged STING was cloned into the pLVX-TetOne Vector (TaKaRa Bio) using the In-Fusion® HD kit (TaKaRa Bio) and according to the manufacturers protocol.

For virus production $2x10^6$ Hek293T cells were seeded on a 10cm plate and incubated overnight at 37°C, 5% CO^2 until cells reached ~80% confluence. The pLVX-TetOne Vector containing STING-HA (7µg) was then combined with Lenti-X packaging single shots (VSV-G) (TaKaRa Bio)- an optimized packaging pre-mix lyophilized with Xfect Transfection Reagent- in 600µl DMEM. The transfection mixture was vortexed for 20 seconds and incubated at room temperature for 20min. The entire mixture was added dropwise to cells and incubated overnight.

Lentiviral supernatants were harvested 48h post transfection and additionally at 60h and 72h post transfection. To remove cellular debris the viral media was passed through a 0.45uM low protein-binding filter. The viral supernatant was concentrated using Amicon® Filters (Merck) in a tabletop centrifuge at 3000 rpm for 20 min at 4^{O}C. Concentrated virus was aliquoted and stored at -80^{O}C before use in transduction.

Lentiviral transduction

2 million STING KO THP-1 cells were resuspended in 1ml complete RPMI media containing 16µg/mL Polybrene (Sigma). A 1ml lentiviral stock of WT HA tagged STING (generated using the Lenti-X Tet-One system, TaKaRa) was added to the cell suspension in a 6 well plate and tilted immediately to mix. The plate was spun for 120 min at 1,200 x g at room temperature for centrifugal enhancement, before incubation at 37°C, 5% CO_2 for 2h. After 2h the virus cell mix was diluted 2-fold with complete media before 20h further incubation. At 24h post transduction cells were harvested and resuspended in complete growth medium with 1µg/mL puromycin (selection medium) for a further 48h.

At 72h post transduction cells were resuspended at 2x10^{5}/ml in fresh selection medium. Transgene expression was induced by culturing cells with 1µg/mL doxycycline for 24h.

2.9 Microscopy

Light Microscopy

Differentiated THP-1 cells were grown on coverslips and then infected with *Leishmania* parasites as described in **2.4**.

After infection coverslips were fixed for 30 seconds in -20°C Methanol, and air dried before staining by flooding coverslips for 15 minutes with 10% Giemsa (VWR Avantor).

Giemsa stain was diluted in 10mM staining phosphate buffer. After staining, coverslips were washed in dH_2O, air dried and mounted in MOWIOL 4-88 (Calbiochem). After curing, coverslip edges were sealed with clear nail varnish and imaged using Zeiss axio-cam 1Cc1 camera.

Confocal Microscopy

Cells grown on glass coverslips were fixed for at least an hour in -20^{o}C methanol and washed prior to permeabilization at RT for 12 minutes in 0.5% Triton X- 84 100/PBS. Coverslips were blocked in 5% FCS/0.05% Tween-20/PBS for 1 hour and stained overnight with primary antibodies in blocking buffer at 4^{o}C. Coverslips were then washed in PBS and stained with fluorescent secondary antibodies in blocking buffer (1:1500) for 3 hours. Coverslips were mounted in MOWIOL 4-88 (Calbiochem) containing 1µg/ml DAPI. Images were taken on a Zeiss LSM880 confocal microscope.

The antibodies used were anti-IFI16 (1:600 Santa Cruz), anti-STING (1:600 Cell Signalling Technology), anti-Vimentin (1:600 Cell Signalling Technology) anti-acetylated tubulin (1:1000 Sigma-Aldrich) anti-rabbit-AlexaFluor488 (1:1500, Invitrogen), antimouse-AF488 (1:1500, Invitrogen),anti-rabbit-594 (1:1500 Cell Signalling) anti-rabbit-AF647 (1:1500, Cell Signalling Technology), anti-mouseAF647 (1:1500, Invitrogen). Image files were analysed using image J and Photoshop software.

2.10 Immunoblotting

Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate cell proteins by size.

For immunoblotting samples 150µl cold complete mammalian cell lysis buffer was added to each well and adherent cells scraped. Cells were incubated at 4°C for 30 mins with the lysis buffer before centrifuging at 12,000 rpm for 10 minutes at 4°C. Pre-cleared lysates were denatured in SDS sample buffer at 99ºC for 5 minutes. EV isolate was also denatured in SDS sample buffer at 99ºC for 5 minutes in preparation for protein analysis.

Samples were run on SDS-PAGE gels (composition described in Table 4) within a Mini-Protean Tetra Cell Tank (Bio-Rad) ran at 120-150V for 1.5 hours. Transfer of the gels to PVDF or nitrocellulose membranes (Millipore) was carried out using a semi-dry transfer apparatus (Biometra FastBlot). Transfer was carried out at 0.1A per gel for 1 hour.

Table 4: 10% polyacrylamide gel composition

	dH20	**40% N,N'-Methylenebisacrylamide**	**Tris (0.5/1.5M)**	**10% SDS**	**10% Ammonium Persulfate**	**TEMED**
Resolving Gel (10%)	4.9 ml	2.5 ml	2.5ml 1.5M Tris	2.5ml	50µl	5µl
Plug to seal plates	250µl of resolving gel mix without Ammonium Persulfate and TEMED				5µl	0.5µl
Stacking gel (4%)	1.9 ml	300µl	750µl 0.5M Tris	30µl	30µl	3µl

After transfer, blocking of membranes was carried out in either 5% Bovine serum albumin, 0.1% Tween in TBS or 5% non-fat milk (Marvel), 0.1% Tween in TBS, depending on compatibility with the antibody used. Primary antibody incubation was carried out overnight at 4^{o}C at a dilution of 1:1000 in blocking buffer (Antibodies used listed in Table 5). After primary incubation membranes were washed 3 x 5 minutes in TBS-Tween. Secondary antibodies, either anti-Mouse-IgG-HRP (Horse Radish Peroxidase) or anti-rabbit-IgG-HRP, were used at a dilution of 1:3000 and incubated at room temperature for 3 hours. HRP activity was detected with Clarity or Clarity Max Enhanced chemiluminescence (Biorad). Membranes were analysed using a Chemidoc (Bio-Rad) or iBright system (Thermo).

Table 5: Antibodies used in Immunoblotting

Target	**Species**	**Company**	**Catalogue Number**
Anti-mouse-HRP	Horse	Cell Signalling Technology	7076
Anti-rabbit-HRP	Goat	Cell Signalling Technology	7074
Beta-actin	Mouse	Sigma Aldrich	A2228
CD63	Mouse	AbCam	ab59479
cGAS	Rabbit	Cell signalling Technology	15102
Flag-tag	Mouse	Sigma Aldrich	F1804
HA-tag	Mouse	Cell Signalling Technology	2367
IFI16	Mouse	Santa Cruz	Sc-8023
IRF3	Rabbit	Cell Signalling Technology	11904/4302
NF-KB p65	Mouse	Cell Signalling Technology	6956
Phospho-IRF3 (Ser396)	Rabbit	Cell Signalling Technology	4947

Phospho-NF-KB p65 (Ser536)	Rabbit	Cell Signalling Technology	3033
Phospho- STAT1 (Tyr701)	Rabbit	Cell Signalling Technology	7649
Phospho-STING (Ser366)	Rabbit	Cell Signalling Technology	85735
Phospho-TBK1/NAK (Ser172)	Rabbit	Cell Signalling Technology	5483
STING	Rabbit	Cell Signalling Technology	13647
TBK1/NAK	Rabbit	Cell Signalling Technology	3504

2.11 Co-Immunoprecipitation

Cells were lysed in Mammalian Cell Lysis Buffer (50mM Tris-HCl (pH 7.5), 1mM EDTA, 1mM EGTA, 1% (v/v) Triton X-100, 1mM sodium orthovanadate, 50mM sodium fluoride, 5mM sodium pyrophosphate, 10mM sodium β-glycerophosphate, 0.27M sucrose, supplemented with cOmplete™ mini protease inhibitor cocktail (Roche) and incubated for 1 hour at $4^{O}C$.

Samples were centrifuged at 8,000xg for 10 min at $4^{O}C$ to remove cell debris. The supernatant was then pre-cleared with (100µl/sample) Protein G Sepharose 4 Fast Flow beads (GE Healthcare) rolling overnight at 4°C. The samples were then centrifuged at 3,000xg for 10 minutes and the supernatant incubated with Protein G Sepharose 4 Fast

Flow beads (GE Healthcare) covalently coupled to rabbit IgG (Sigma) or STING ab (Cell Signalling Technology) for 3h.

Beads were washed four times in the mammalian cell lysis buffer and bound proteins were eluted from the beads by boiling in SDS sample buffer for 10 minutes. The initial lysate before immunoprecipitation took place, in addition to the supernatant recovered post-immunoprecipitation was retained for use as controls in western blotting experiments.

2.12 Mass Spectrometry

Sample preparation

30 µl of each STING IP sample for analysis was heated to 70°C in Nu Page LDS Sample buffer (Invitrogen) for ten minutes prior to loading on a 4-12% Bis-Tris Nu-Page pre-cast gel (Invitrogen) and running for 20mins at 200V in MOPS running buffer (Invitrogen) according to manufacturer's instructions. The resulting gel was stained with the NOVEX Colloidal blue kit (Invitrogen) for 3 hours and then de-stained overnight in deionised water. Slices were cut and stored in water in preparation for sending to FingerPrints Proteomics Facility at The University of Dundee. On this occasion only the STING IP samples were sent for analysis and not the IgG control IP samples.

At FingerPrints Proteomics Facility

Instruments Used: Ultimate 3000 RSLCnano system (Thermo Scientific) coupled to a Q Exactive Plus Mass Spectrometer (Thermo Scientific).

Ultimate 3000 Buffer conditions: Buffer A: 0.1% formic acid

Buffer B: 80% acetonitrile in 0.1% formic acid

Q Exactive Plus conditions: Top 15 Method:1 MS plus 15 MS/MS (150 min acquisition) Operating in data dependent acquisition mode, Lock Mass 455.120024.

On arrival samples were dried and suspended to 50 µl 1% Formic Acid. Samples were then injected (15µl) and washed on the C18 trap column with Buffer A. After 5 minutes a gradient was formed with buffers A and B.

Peptides were initially trapped on an Acclaim PepMap 100 (C18, 100 µM x 2 cm) and then separated on an Easy-Spray PepMap RSLC C18 column (75 µM x 50 cm) (Thermo Scientific). Samples were transferred to mass spectrometer via an Easy-Spray source with temperature set at 50˚C and a source voltage of 2.0 kV.

In data-dependent acquisition mode Full MS1 scans were performed at 70,000 resolution followed by 15 sequential dependent MS2 scans where the top 15 ions were selected for collision-induced dissociation (CID, normalized collision energy NCE = 35.0) and analysis in the Ion Trap with an MSn AGC target of 5000. An isolation window of 2.0 m/z units around the precursor was used and selected ions were then dynamically excluded from further analysis.

Data Analysis

Orbitrap XL.RAW files converted to MSF files (Proteome Discoverer Version 2.2). Extracted data then searched against *Leishmania Mexicana* and SwissProt-human database version Mascot Search Engine (Version 2.3.2). For each protein match, Mascot calculates an overall Protein Score. This number reflects the combined scores of all observed mass spectra that can be matched to amino acid sequences within that protein. A higher score indicates a more confident match.

Table 6: Search parameters used for Mass spectrometry Data analysis

Type of search	MS/MS Ion Search
Enzyme	Trypsin/P
Fixed modifications	Carbamidomethyl (C)
Variable modifications	Acetyl (N-term), Dioxidation (M), Gln->pyro-Glu (N-term Q) and Oxidation (M)
Mass values	Monoisotopic
Protein Mass	Unrestricted
Peptide Mass Tolerance	± 10 ppm (# ^{13}C = 2)
Fragment Mass Tolerance	± 0.06 Da
Max Missed Cleavages	2
Instrument type	ESI-TRAP

:

2.13 qRT-PCR

For RNA extraction 350µl TRK lysis buffer (E.Z.N.A total RNA kit, Omega Bio-Tek) was added directly to washed cells in each well (12 well plate) before storing at -80°C until RNA extraction could be performed.

RNA was extracted from cell lysates using the E.Z.N.A total RNA kit (Omega Bio-Tek). A DNase step was also carried out using 1 µl Dnase to 9µl Dnase buffer (Thermo Scientific) and incubated with each extracted RNA sample for 15 minutes at room temperature. The DNase was inactivated by the addition of 1 µl of 25 mM EDTA solution to the reaction mixture and heated for 10 min at 65°C. The concentration of RNA was measured by using a Nanodrop 2000c spectrophotometer (Thermo Scientific) and 200ng of RNA were taken from each sample.

Reverse transcription of RNA was completed using an iScript cDNA Synthesis Kit (Bio-Rad) on an Eppendorf Master Cycler. For each reaction 0.5µl iScript reverse transcriptase, 2µl 5x iScript reaction mix was combined and made up to 10µl with RNA and nuclease-free H2O. The cycle used was 5 minutes at 25^{o}C, 30 minutes at 46^{o}C and 1 minute at 95^{o}C before cooling.

qRT-PCR was performed using a LightCycler 96 realtime PCR instrument (Roche) or BioRad CFX96 (Bio-Rad). For each 10µl reaction 5µl 2x Fast SYBR green mastermix (Thermo Scientific) combined with 1 µl primer mix (500nM), (primers detailed in Table 7) (500nM), and 4µl of the cDNA to amplify the target genes.

The following cycling conditions were used for the qRT-PCR program:

Holding stage (1 cycle): 1 minute at 95^{o}C.

Cycling stage (40 cycles): 15 seconds at 95^{o}C; 1 minute at 60^{o}C.

Melt Curve stage (1 cycle): 15 seconds at 95^{o}C; 1 minute at 60^{o}C; 15 seconds at 95^{o}C; 15 seconds at 60^{o}C.

Table 7: Primers used in qRT-PCR

They were designed in NCBI Primer BLAST and synthesised by Eurofins

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
β-actin (human)	CGCGAGAGAAGATGACCCAGATC	GCCAGAGGCGTACAGGGATA
CCL5 (human)	CTGCTTTGCCTACATTGCCC	TCGGGTGACAAAGACGAC
CXCL10 (human)	AGCAGAGGAACCTCCAGTCT	AGGTACTCCTTGAATGCCACT
IL-6 (human)	CAGCCCTGAGAAAGGAGACAT	GGTTCAGGTTGTTTTCTGCCA
IL-10 (human)	AAGACCCAGACATCAAGGCG	AATCGATGACAGCGCCGTAG
IL-12 (human)	GCACAGTGGAGGCCTGTTTA	GCCAGGCAACTCCCATTAGTT
IFN-β (human)	ACACTGGTCGTGTTGTTGAC	GGAAAGAGCTGTCGTGGAGA
ISG56 (human)	CAAAGGGCAAAACGAGGCAG	CCCAGGCATAGTTTCCCCAG
TNFα (human)	GCCCATGTTGTAGCAAACCC	TATCTCTCAGCTCCACGCCA
β-actin (mouse)	TCCAGCCTTCCTTCTTGGGT	GCACTGTGTTGGCATAGAGGT
CCL5 (mouse)	CTCACCATATGGCTCGGACA	CGACTGCAAGATTGGAGCAC

CXCL10 (Mouse)	CGATGACGGGCCAGTGAGAATG	TCAACACGTGGGCAGGATAGGCT
IL-6 (mouse)	CTCTGCAAGAGACTTCCATCCA	AGTCTCCTCTCCGGACTTGT
IFN-β (mouse)	ATGGTGGTCCGAGCAGAGAT	CCACCACTCATTCTGAGGCA
18S (*Leishmania*)	GGGAAACCCCGGAATCACAT	GGTGAACTTTCGGGCGGATA
Alpha tubulin (*Leishmania*)	GAACTCGGTGTTTGAGCCTG	ATCCTTCGGCACGACATCAC

2.14 Cloning *L.mexicana* GP63 into a plasmid

L.mexicana DNA was extracted using QuickExtract DNA Extraction solution (Epicentre, Illumina) using the QuickExtract DNA Extraction Protocol.

Amplification of the GP63 gene by PCR was carried out using Herculase II Fusion DNA Polymerase (Agilent Technologies).

Primers for GP63, with added restriction sites EcoR1 and Kpn1 compatible for insertion into the pCMV-HA vector (Clontech) were designed in NCBI Primer Blast and are detailed below:

GP63 primers:

Forward Primer (5'-3')	**Reverse Primer (5'-3')**
AAGAATTCTAATGCCCGTCGACAGCAGCA	TAGGTACCTCAAGCTAGTGGGCCGTC

Optimised parameters for the amplification of GP63 from the genomic DNA is outlined in tables 8 and 9.

Table 8: Optimised PCR reaction mixture for the amplification of GP63

Component	
5 x Herculase II reaction buffer	10µl
dNTPs	250µM each dNTP
Parasite DNA template	70ng
Primers (each)	0.25µM
Herculase II polymerase	1µl
dimethyl sulfoxide (DMSO)	0.5µl
dH_2O	32.5µl

Table 9: Optimised cycling conditions for GP63 PCR amplification

Segment	Temperature	Duration	Number of cycles
1.Denaturing	98^{o}C	2 Minutes	1
2.Denaturing	98^{o}C	15 seconds	30
2.Anneaing	56^{o}C	20 seconds	
2.Extension	72^{o}C	30 seconds	
3.Extension	72^{o}C	3 minutes	1

Amplification was verified by agarose gel electrophoresis (see section 2.17) and the correct size PCR product was purified using the QiaQuick PCR gel purification kit (Qiagen) according to the manufacturer's instructions.

The purified GP63 insert was ligated into the pCMV-HA vector after restriction digest of both with Kpn I and EcoR I using Cut Smart Buffer (all NEB) and subsequent gel purification. Ligation was performed using the Rapid DNA Ligation Kit (Thermo Scientific) with components listed in Table 10 and incubated for 22 ^{o}C for 5 minutes.

Table 10: Reaction mixture for the ligation of GP63 insert into pCMC-HA vector

Component	**Volume for reaction**	**Concentration**
Vector DNA	1.5 µl	22ng
Insert DNA	6 µl	60ng
5x Rapid ligation buffer	4µl	-
T4 DNA ligase	1 µl	5u/ µl
dH20	7.5 µl	-

2.15 Bacterial transformation

Plasmid DNA was transformed to NovaBlue Competent *E. coli* cells. 0.5µl of the ligation mix was added to 10µl competent cells, stirred gently, and incubated on ice for 5 minutes before heat shock at 42^{o}C for 30 seconds. The mixture was then cooled on ice for a further 5 minutes. For plating to Ampicillin-resistant agar plates, 100µl of Super Optimal Broth with Catabolic repressor medium (Novagen) was first added to the mixture. The plates were then left overnight at 37^{o}C.

2.16 Maxiprep of plasmid containing GP63

Single colonies were selected from the plate and incubated in 3ml of Luria broth (LB) containing 100µg/ml Ampicillin in a shaking incubator for 6 hours. Following this step, the bacteria were transferred to 100ml Ampicillin-LB broth and left overnight in the shaking incubator at 37^{o}C. Bacteria were subsequently pelleted, lysed and plasmid purified using Maxiprep kit (Qiagen) according to manufacturer's instructions.

2.17 Agarose Gel Electrophoresis

1% agarose gels were made with 0.7g Ultrapure Agarose (Invitrogen) and 70ml TAE buffer heated together in the microwave. When the mixture had just boiled, it was removed and allowed to cool slightly before addition of 1X SYBR-Safe Nucleic Acid Gel stain (Biotium). The gel was then poured and allowed to set. 3x DNA loading buffer was added to the DNA samples before loading into the gel. A 2-log DNA ladder (Peqlab) was also added with the DNA loading buffer to provide size markers.

Gels were run in 1x TAE running buffer at 5-7V/cm for around 1 hour. A GelDoc EZ Imager machine (Bio-Rad) was used to image the gels using Image Lab software (Bio-Rad).

2.18 Transfection of HEK293T cells

HEK293T cells were seeded at a density of 0.35 x 10^6 cells in 6 well plates the day before transfection. DNA transfections were performed using GeneJuice (Millipore) to manufacturer's instructions, using 1 µg DNA: 3µl of GeneJuice per ml of medium.

Co-transfections of various other plasmids were carried out with a total of 1ug/DNA per ml between both plasmids and empty pcDNA3.1(+) vector as a negative control.

24 hours after the transfection cells were lysed directly into 3x SDS Sample buffer and boiled for 10 minutes before immunoblotting.

2.19 Plasmids used in this thesis

- pCMV-HA (Clontech)
- pCDN3.1(+): hSTING-HA (Unterholzner lab)
- pCDNA3.1(+) (Thermo Fisher)
- pCMV6Entry:IFI16-mycFlag (OriGene)
- pCDNA3.1 (+): mSTING-Flag (Lei Jin, University of Florida))
- pCMV6Entry:cGAS-mycflag (OriGene)

2.20 Statistics

Results from real-time PCR are shown as the average of experimental triplicate samples. Error bars represent the standard deviation of the mean. Statistical significance was determined using Student's unpaired t tests in GraphPad Prism 5. P-Values; *= $P \leq 0.05$, ** = $P \leq 0.01$, *** $P \leq 0.001$.

Chapter 3: Infection of THP-1 macrophages with *Leishmania mexicana* promastigotes and amastigotes

3.1 Chapter Introduction

The *in vitro* infection of human cell lines has long been an important way to study many aspects of Leishmaniasis, including the early infection process. We chose to use the THP-1 cell line in our experiments. THP-1 cells are immortalised pre-monocytes, committed to the monocytic cell lineage (Takashiba et al., 1999), that exist in suspension resembling monocytes that reside in the blood. After treatment with phorbol 12-myristate 13-acetate (PMA) they differentiate, become adherent and acquire phenotypic and functional characteristics similar to those of primary macrophages in the tissue (Lund et al., 2016). PMA drives this differentiation by strongly inducing protein kinase C (PKC). PKC activates downstream kinases and transcription factors regulating differentiation (Schwende et al., 1996). These differentiated THP-1 cells would therefore be a good model for the macrophages resident in the skin at the site of *Leishmania* entry, or those cells that are recruited to the skin from the circulation after a sandfly bite.

In 1990 successful *in vitro Leishmania* infection of the THP-1 human monocytic cell line was demonstrated for the first time (Ogunkolade et al., 1990). Since then, many others have used THP-1 cells to investigate leishmaniasis, including for the screening of anti-leishmanials, (Mehta et al., 2010), investigation into the host response (Millar et al., 2015) and research into the activity of parasite virulence factors (Shio et al., 2015) to name a few.

Many different *Leishmania* species have been used for *in vitro* experiments, making comparisons between different species possible. For this project we focused on *L.mexicana* which is the causal agent of the most common form of leishmaniasis - cutaneous leishmaniasis. Strain (MNYC/BZ/62/M379) was used in all of our experiments, and has been used by many other groups investigating the immune response to

Leishmania (Bhardwaj et al., 2005; Cameron et al., 2004; Rosas et al., 2005). There are also several other *L.mexicana* strains used for *in vitro* experiments, these include (MHOM/VE/80/NR*)* (Dagger et al., 2017), (MHOM/MX/92/UADY68) (Escalona-Montaño et al., 2016) and (MHOM/MX/2011/Lacandona) (Escalona-Montaño et al., 2016; Soto-Serna et al., 2020).

3.2 *In vitro* infection of THP-1 macrophages with *Leishmania mexicana*

Before any other experiments were considered it was essential that a robust and reproducible early infection could be achieved in our own cultured THP-1 cells. We chose to infect the THP-1 cells initially with both the promastigote and amastigote forms of the parasite. It is the promastigote form of the parasite that is deposited in the skin after a sandfly bite and will infect macrophages during a normal infection. However, after the differentiation of promastigotes within their macrophage host, it is the amastigote form of the parasite that goes on to infect further macrophages and propagate the infection. As well as having distinct morphologies, amastigotes and promastigotes have different surface molecule compositions, which includes distribution of key virulence factors. While it has been shown that both forms of the parasite are able to dysregulate the host immune system, it is possible that they may do so in different ways (Abu-Dayyeh et al., 2010).

Flagellated promastigotes, imaged in Figure 1A were cultured *in vitro* until they had reached the stationary phase.. This stage of growth contains a high proportion of the infective metacyclic promastigote form and was used for all infections. The stationary phase is reached within 5-7 days of cell culture after the parasites were passaged (Figure 1B). Stationary phase promastigotes were also used for *in vitro* differentiation of promastigotes to amastigotes, a process summarised in Figure 1C. A change in cell culture pH and temperature mimics environmental changes that prompts promastigotes to differentiate *in vivo* (Bates et al., 1992; Bates and Tetley, 1993; Pan et al., 1993).

Morphological change to a non-flagellated ovoid shape started to occur within the first 24h. Figure 1D shows the distinct change in parasite morphology after differentiation. Amastigotes used to infect THP-1 cells were cultured for at least 7 days post differentiation to ensure full maturation of the amastigote form. A growth curve of amastigotes (Fig.1E) demonstrates clear differences in the growth kinetics between different parasite forms. Unlike promastigotes, amastigotes do not yet reach a stationary phase within 5-6 days of cell culture.

Proof of this infection was demonstrated by microscopy and quantitative PCR (qPCR) (Figures 2 and 3) which became a standard for how we confirmed successful infection throughout the project. For quantification of intracellular parasite load, a standard curve was constructed using *L.mexicana* DNA extracted from a known quantity of cultured parasites ranging from $1x10^4$ to $1x10^8$ parasites per ml. Cycle threshold (Ct) values of the samples for the *Leishmania* gene 18s were plotted against log(parasite number) (Fig. 2A). This standard curve was then used to determine approximate intracellular parasite number by using the 18S Ct values from DNA extracted from cell samples after infection. The 18S rDNA region is commonly used for *Leishmania* detection and in diagnosis due to it being highly conserved among trypanosomatids and the large number of copies it has within the genome (Filgueira et al., 2020). As well as a simple tool to confirm intracellular entry, this standard curve can also be used to compare estimated intracellular parasite number between experimental repeats to confirm similar levels of infection are occurring in each experiment. Additionally, successful cellular entry of parasites can be compared between wild type (WT) THP-1 cells and different cell treatments or knock out (KO) cells lacking a gene of interest to determine if parasite entry or replication is affected.

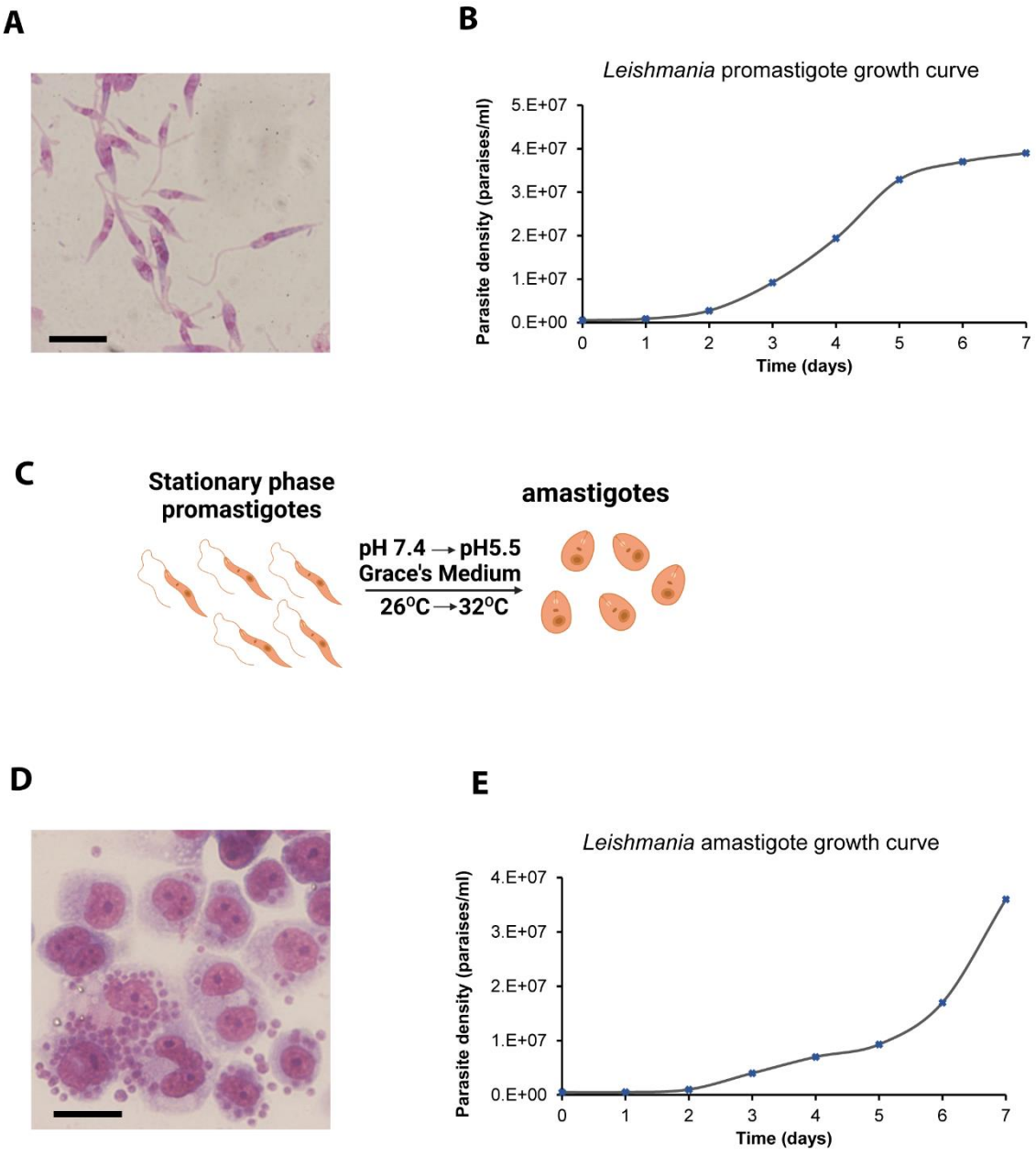


Figure 1: Cell culture of *L.mexicana* promastigotes and amastigotes

(A) Morphology of *Leishmania mexicana* promastigotes in cell culture. The parasites were fixed and stained using 10% Giemsa. Scale bar = 10µm. **(B)** Growth curve of promastigotes cultured at 26˚C in M199 medium. **(C)** Schematic showing *in vitro* change in conditions to induce promastigote to amastigote differentiation. **(D)** Morphology of *Leishmania mexicana* amastigotes observed after 2h infection of THP-1 cells. The cells were fixed and stained using 10% Giemsa. Scale bar = 20µm. **(E)** Growth curve of amastigotes cultured at 32˚C in Grace's medium.

In figure 2B the estimated intracellular parasite number is shown over a 48h period after initial infection with both promastigotes and amastigotes. Initial infection with amastigotes is much more successful than promastigotes over all three time points. In cells infected first with amastigotes, the estimated intracellular parasite number peaks at 24h and then decreases between the 24h to 48h infection. However, in promastigotes it peaks at 6h, and then decreases between 6h to 48h infection. The decrease at 48h in both sets of cells is perhaps unexpected but could be explained by an increase in the cell death of heavily infected cells. This cell death could be due to apoptosis following cellular stress or cell rupture due to increasing and unrestricted amastigote replication (DaMata et al., 2015). This is one of the suggested methods that *Leishmania* parasites use to disseminate further in a human host.

Light microscopy using Giemsa stain to visualise parasites was also used to observe how infection changes over a 48h time course starting at 2h post infection (Fig. 2C). Giemsa stain has been used for many years as to diagnose Leishmaniasis from biopsies (Gottstein et al., 1975) and peripheral blood smears (Sundar and Rai, 2002) as well as to determine morphological attributes of the parasites (Bates, 1994). In our experiments we utilised the stain to identify *Leishmania* parasites within the THP-1 macrophages after as little as 2h post infection.

In the cells initially infected with promastigotes we can observe the differentiation of promastigotes to amastigotes occurring over the time course. From 2h to 6h promastigotes distinguished by the presence of flagella can clearly be observed (marked with ‘P’ on enlarged image section). However, from 4h to 6h we can also observe parasites that resemble ovoid amastigotes (arrowed on enlarged image section). In cells infected with *in vitro*-differentiated amastigotes, amastigotes can be seen infecting cells successfully from 2h. At each subsequent time-point an increasing number of amastigotes can be observed accumulating within the macrophages.

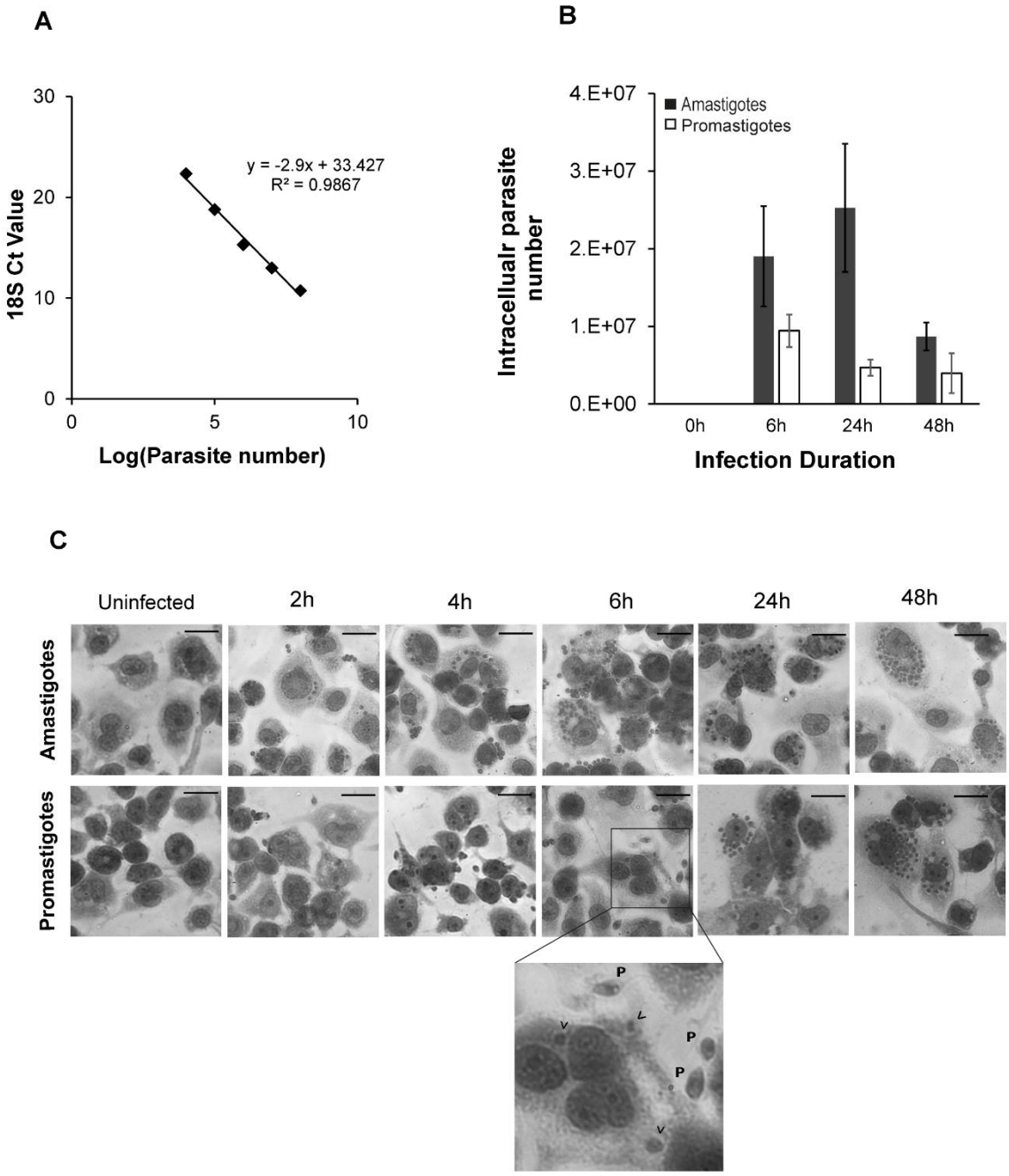


Figure 2: Successful Infection of THP-1 cells with *L.mexicana*

(A) Standard curve of parasite number created using a known number of parasites and using qRT-PCR with parasite reference gene 18S. **(B)** THP-1 cells were infected with amastigotes or promastigotes at a MOI of 5 for the specified time, then washed and lysed for qRT-PCR analysis of mRNA, where the estimated intracellular parasite number present in cells was determined using a standard curve of known parasite numbers. Data are presented as mean values of biological triplicates. Error bars indicate standard deviations. Data are representative of at least three experiments. **(C)** WT THP-1 cells were seeded onto coverslips, differentiated with PMA for 48h before infection with *L.mexicana* amastigotes or promastigotes over 48 hours. Cells were then fixed in methanol and stained with 10% Giemsa to visualize the parasites and imaged with light microscopy. **P**= promastigotes **arrows**= parasites more resembling ovoid amastigotes Scale bar = 20µm.

For further confirmation of infection, confocal microscopy on promastigote infected cells was performed utilizing a primary antibody against the endogenous parasite protein (acetylated tubulin) to visualise the *Leishmania* parasites, and a fluorescently labelled secondary antibody. Primary antibody against Vimentin was used as a cytoskeletal marker, and DAPI as nuclear DNA stain. Here the clear presence of parasites within infected cells can be observed after 6 hours (Fig. 3) and that the promastigotes had again undergone morphological change to an amastigote shape.

Both these different microscopy techniques clearly demonstrated visual evidence of intracellular infection in addition to the differentiation of flagellated promastigote to an amastigote form which only occurs upon a change to its environmental conditions associated with entry to the macrophage phagolysosome (Barak et al., 2005; Garlapati et al., 1999; Zilberstein and Shapira, 1994).

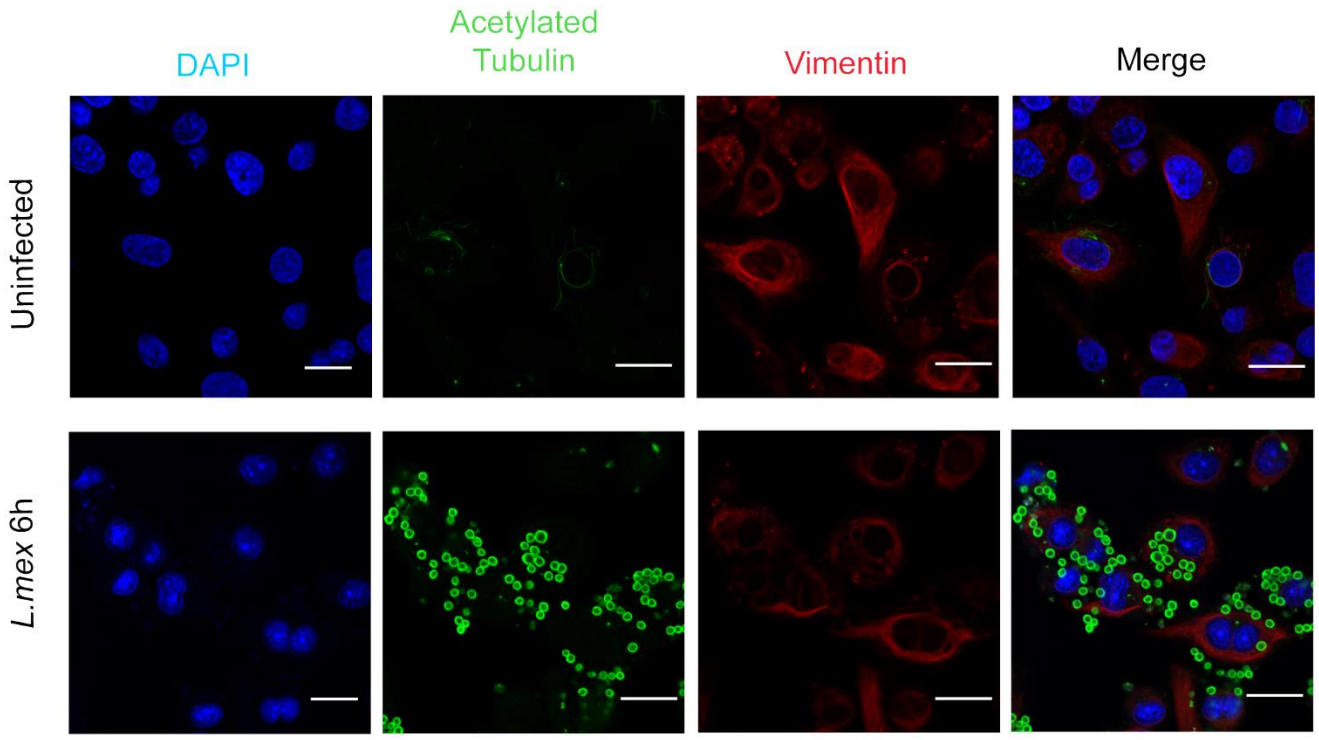


Figure 3: Monitoring of *L.mexicana* infection by confocal microscopy

THP-1 cells were seeded onto coverslips, differentiated with PMA for 48h before infection with *L.mexicana* promastigotes at a MOI of 5 for 6 hours. Cells were then fixed in methanol and stained for acetylated tubulin to visualize the parasites (green), vimentin (red) and DAPI nuclear stain (blue) and imaged by confocal microscopy. Scale bar = 20µm.

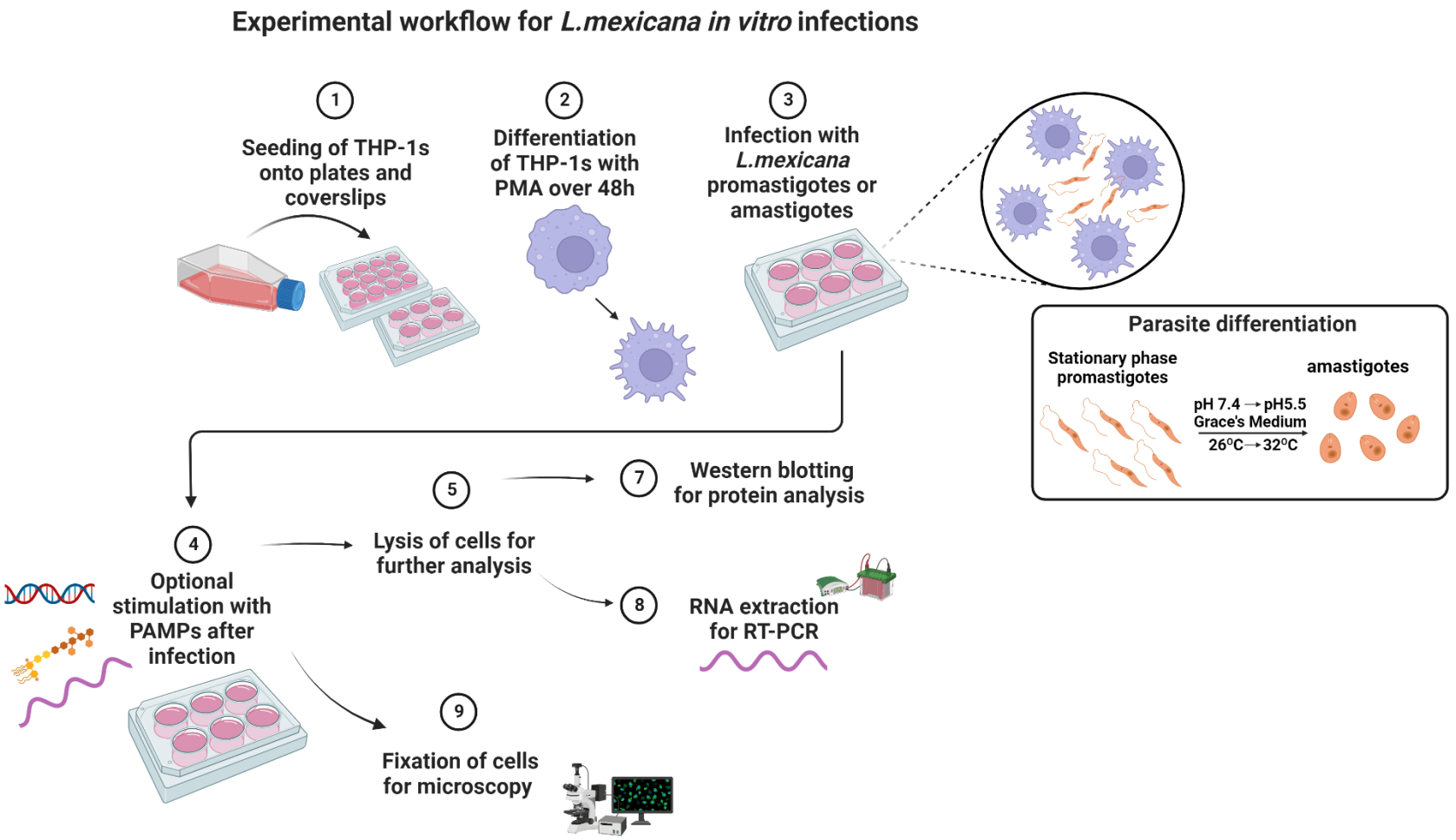


Figure 4: Experimental work-flow for *L.mexicana in vitro* infections

Diagram detailing the standard experimental procedures consistently used for *L.mexicana* in vitro infection of THP-1 macrophages. Before infection with *L.mexicana* promastigotes or amastigotes, THP-1 cells were differentiated with PMA. Infected cells were often stimulated with various PAMPs after infection, for example, LPS. Cells were then lysed for analysis of cell signalling proteins components by western blotting or gene expression by qRT-PCR of isolated RNA. Additionally, infected cells could also be fixed for visualisation by microscopy.

3.3 THP-1 cytokine response to *L.mexicana* over 48h

The interaction between macrophages and *Leishmania* has a crucial role in the pathogenesis of the infection. Macrophages are important cells for the survival and replication of the parasites upon entry, but also for their elimination. Analysis of cytokine production by macrophages after *Leishmania in vitro* infection is an important way to study the impact of *Leishmania* infection on the early macrophage response, as the cytokine and chemokine response of macrophages during infection can initiate and modulate local immune responses in the tissue.

Usually, the host response to infection is regulated by a carefully controlled production of cytokines that can aid resistance to the pathogen. However, the cytokine response to *Leishmania* infection in murine models can be variable: it can be simplified to a clear dichotomy between a Th1 cytokine response that favours protection and a Th 2 cytokine response that is associated with disease susceptibility (Dayakar et al., 2019; Sacks and Noben-Trauth, 2002; Reiner and Locksley, 1995). The success of the infection is usually dependent on the infecting species, host immune status and the modulation of host response by virulence factors (Scott, 1991).

To measure the innate immune response to *Leishmania mexicana* promastigotes in human THP-1 macrophages we used qRT-PCR (quantitative real-time polymerase chain reaction). Promastigotes were selected for this experiment as it would be promastigotes that would first be detected by the immune system during physiological infection following a sandfly bite. In figure 4 the different steps taken for our *in vitro* infection and further processing are elucidated, from the differentiation process to when optional further stimulation of infected cells was performed. Infected and uninfected cells are washed first then lysed and processed to enable RNA extraction from each sample. The resulting RNA undergoes a DNase treatment to remove any cellular DNA contamination before

complimentary DNA (cDNA) is produced by reverse transcription of the RNA. qRT-PCR uses oligonucleotide probes specific for genes of interest- in this case key cytokines and chemokines. The oligonucleotides hybridise with the target sequence and amplify it using Taq DNA Polymerase. This amplified DNA is then bound by the fluorescent DNA dye SYBR Green. This fluorescence in then quantified and for each experiment a housekeeping gene such as β-actin is amplified and quantified to normalise results to.

THP-1 cells seem to have a limited inflammatory cytokine response to *Leishmania* promastigotes, with IL-12, IL-6 and TNF-α not induced by infection at either 6h, 24h or 48h (Fig.5A-C).

In figure 5D we can see that IL-10 is significantly induced during *L.mexicana* infection of our THP-1 cells at all timepoints. IL-10 , an anti-inflammatory mediator, is also known to inhibit production of inflammatory cytokines such as IL-12, IL-6 and TNF- α (Dayakar et al., 2019). This could be a possible immune subversion strategy of *L.mexicana* as IL-10 is known to downregulate macrophage activation and ability to control *Leishmania*.

The next cytokine investigated was CXCL10 (Fig 5E) which is the one of the few in our panel to be significantly upregulated during infection, although not at the 6h timepoint. CXCL10 is a pro-inflammatory chemokine usually secreted by monocytes after stimuli such as viral infection, (IFN)-γ and PAMP detection. Here we can demonstrate that CXCL10 is induced after *L.mexicana* infection and it can be hypothesised that during an *in vivo L.mexicana* infection CXCL10 could have a role in recruiting macrophages and T cells to the infection site.

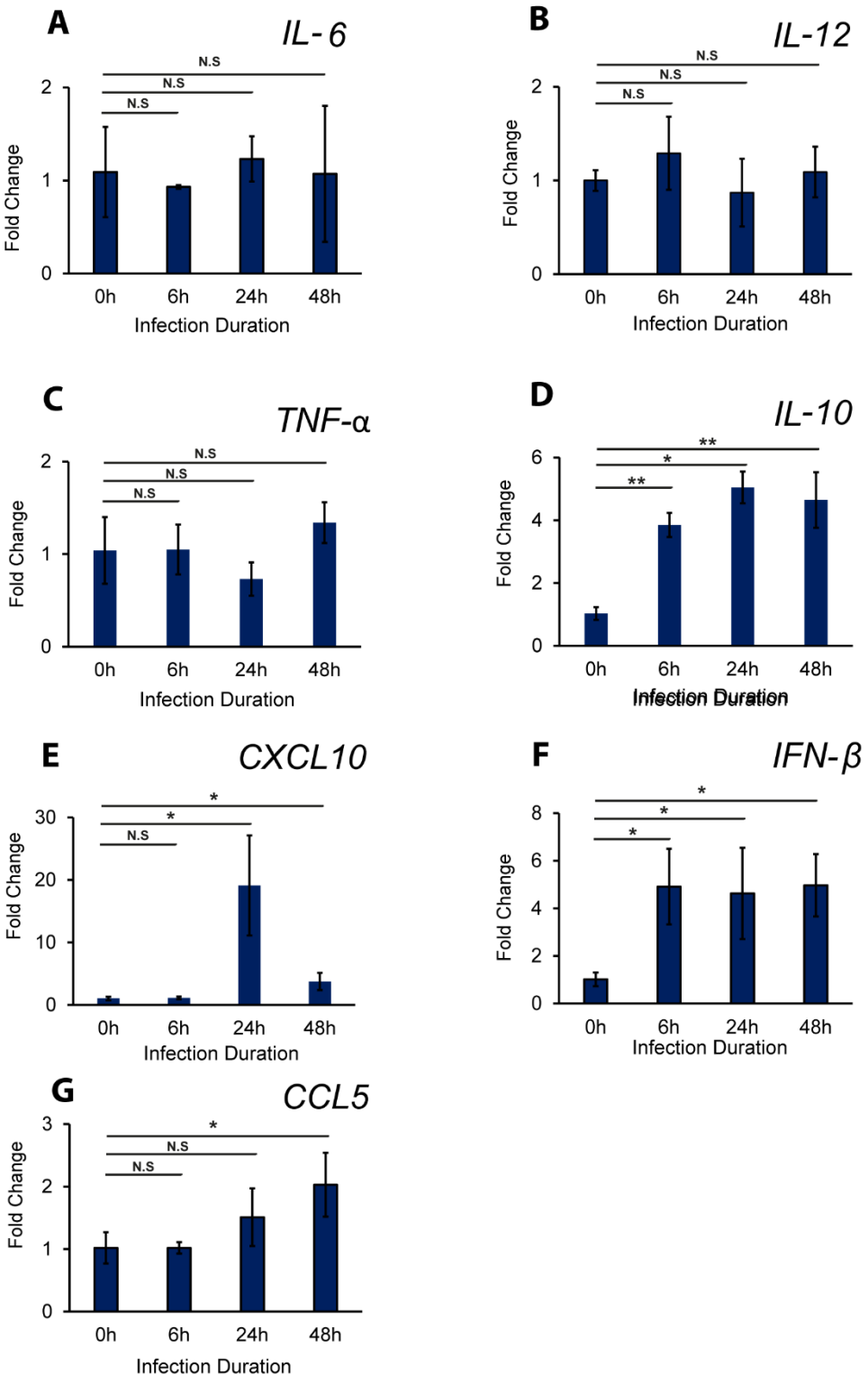


Figure 5: Response to *L.mexicana* infection over 48h

WT THP-1s were infected with *L.mexicana* promastigotes at a MOI of 5 for over 48h as indicated. At each time point cells were lysed for qRT-PCR analysis of **(A)** IL-6, **(B)** IL-12 **(C)** TNF-α , **(D)** IL-10 , **(E)** CXCL10 **(F)** IFN-β and **(G)** CCL5 mRNA. Data are presented as mean values of biological triplicates. Error bars indicate standard deviations. N.S = non-significant, * $p \leq 0.05$; ** $p \leq 0.01$ as determined by Student's t-test. Data are representative of at least three experiments.

The type-I interferon, IFN-β is also induced by *L.mexicana* infection (Fig 5F), with all three time points showing a significant increase in IFN- β mRNA levels over mock infections. Like CXCL10, type -I interferon is more prominently associated with the response to viral infections. However, a growing body of literature suggests an important protective role for type I interferons during protozoan infections as well, although the timing and duration of type I interferon induction may cause a shift from a host protective to host detrimental outcome (Beiting, 2014; Silva-Barrios and Stäger, 2017).

Finally, chemokine CCL5 was investigated (Fig 5G). There is a very slight upregulation of CCL5 with infection, but only at 48h. Ultimately our data indicates that *L.mexicana* in THP-1s does not lead to the expression of pro-inflammatory cytokines, but some induction of IL-10, IFN- β and CXCL10 is observed.

After we demonstrated only a very minor cytokine response following *L.mexicana* infection in these THP-1 cells (Fig.5), it was essential that we confirmed that the cells were fully responsive when stimulated with different agonists. Cells were stimulated with either TLR 4 agonist LPS or transfected dsDNA and the cytokine response was again measured by qRT-PCR (Fig.6). The expected cytokine response to these agonists could be observed with a strong inflammatory cytokine response upregulated following both LPS and dsDNA stimulation. A strong interferon response was also observed after dsDNA stimulation. This was able to confirm that these THP-1s were indeed fully responsive, just not to *L.mexicana* infection suggesting the parasite is successful at subverting the standard host response in these cells.

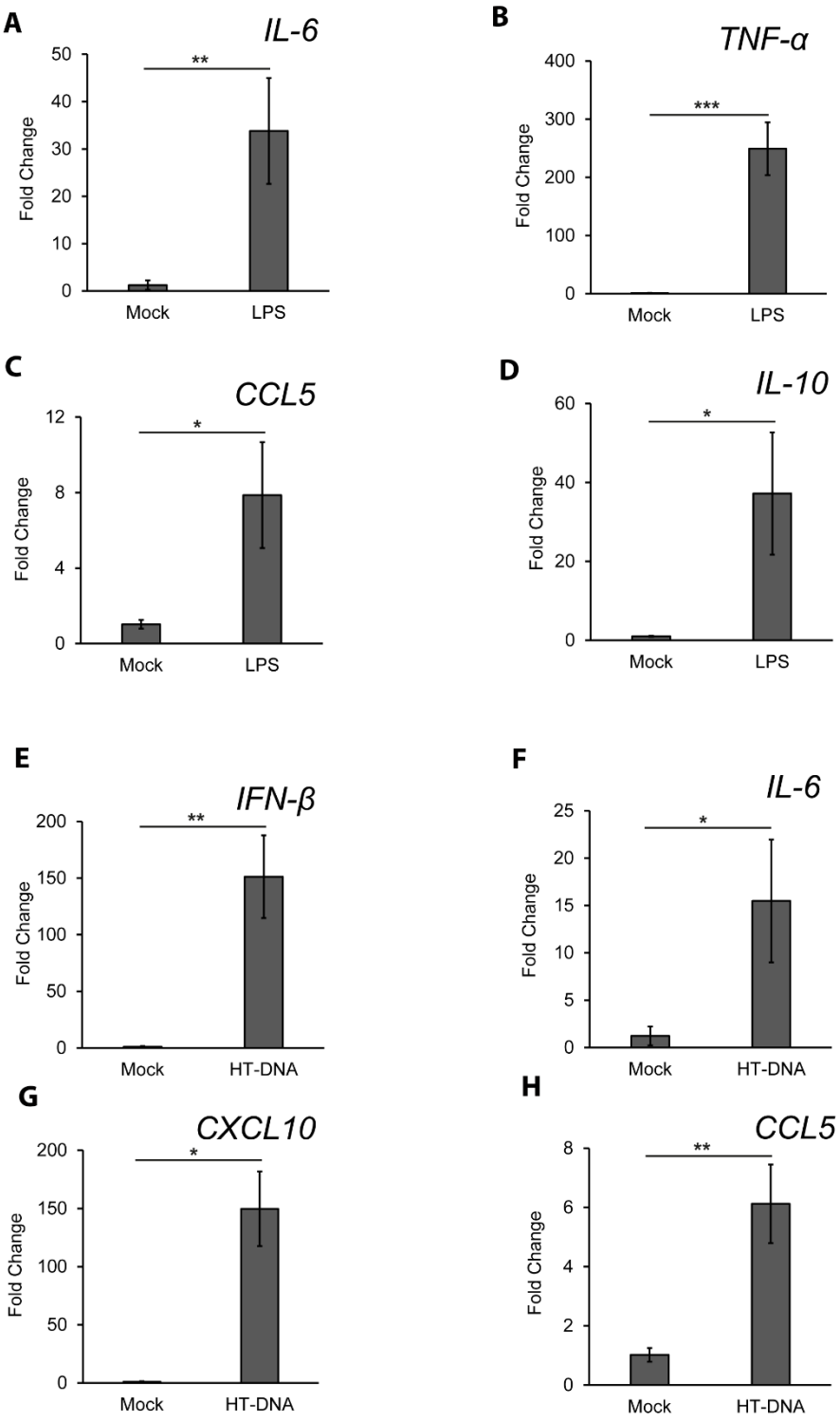


Figure 6: THP-1 cells are responsive to LPS and dsDNA

WT THP-1s were infected with *Leishmania mexicana* promastigotes at a MOI of 5 for 6h before subsequent stimulation with either LPS for 1 hour **(A)**, **(B)**, **(C)** and **(D)** or with HT-DNA for 4h **(E)**, **(F)**, **(G)** or **(H)**. Cells were then lysed for qRT-PCR analysis of appropriate cytokine genes as indicated. Data are presented as mean values of biological triplicates. Error bars indicate standard deviations. N.S = non-significant, * p≤ 0.05; ** p≤0.01 and *** p ≤ 0.001 as determined by Student's t-test.

3.4 Chapter Discussion

In this chapter we have demonstrated successful *in vitro* infection of THP-1 macrophages with *L.mexicana* amastigotes and promastigotes. Both microscopy of infected cells and qPCR, using the standard curve of parasite gene 18s created from known parasite numbers, confirmed parasite entry in infections with both lifecycle forms of *Leishmania*. It should be noted that in Figure 2B, time point 0h reflects an uninfected control, rather than the number of parasites used as the infection inoculum. This must be considered during any future growth analysis, so it is not erroneously concluded that there is an increase from 0 to ~ 1x 10^7 parasites within these THP-1 cells in just 6h.

Surprisingly, we also were able to observe visual changes in *Leishmania* that is suggestive of differentiation from promastigotes to amastigotes from the early 4 and 6h time point during our infection (Fig.2C). In general, the peak of promastigote-to-amastigote morphogenesis has been shown to occur between 10h and 24h. Full amastigote maturation is thought to be achieved between 24h and 120h after the differentiation signal is received (Tsigankov et al., 2014). However, previous *in vitro* studies have shown that following infection of macrophages by *L.major* promastigotes, changes in morphology to a more characteristicly amastigote shape occurred very rapidly- even from 2h post infection (Mandell et al., 2022). While complete differation will not occur until later, visually promastigotes seem to be able to undergo morphological change *in vitro* rapidly. This is also likely to be occuring in our infection based on the fairly rapid appearance of more ovoid parasites.

Interestingly, our qPCR data suggested that infection with amastigotes was more successful (Fig.2B) due to a higher number of intracellular parasites at all timepoints of the infection time course compared to promastigotes. Despite this, we continued to use both forms of the parasite throughout the project and even used the promastigote form alone in many experiments as promastigote infection more closely resembles

physiological infection by *Leishmania* after deposition in the skin. This is particularly relevant as we were aiming to look at the early immune response to *Leishmania*

It would be beneficial in future to carry out amastigote infections using amastigotes obtained from differnet methods for comparison. It has been shown that the amastigotes used here, obtained by *in vitro* differentiation and known as axenic amastigotes, may not be fully comaprable to amastigotes derived from *in vitro* infected macrophages or mouse lesion derived amastigotes (Fiebig et al., 2015; Holzer et al., 2006; Pacakova et al., 2022). Proteomic analysis of the three types revealed differences in the abundance of metabolic enzymes, virulence factors and proteins involved in DNA translation (Pacakova et al., 2022). This could suggest that while axenic amastigotes may resemble amasitgotes, they may not be equal to an amastigote that differentiates during a normal infection, as the differentiation process does not exaclty replicate what would occur within the macrophage.

The differences we observed between amastigotes and promastigotes could be explained by differing mechanisms of entry to THP-1 cells. Additionally, promastigotes need to undergo differentiation before they can proliferate which could suggest why at 24h there is a much higher number of intracellular parasites in the amastigote infected cells. At 48h post infection both undergo a decrease. The light microscopy images (Fig 2C) of cells at 48h infection timepoint include infected cells containing large numbers of parasites. We therefore know that at least some cells are heavily infected at this time point. It is possible that eventually these cells would burst by the density of parasites or undergo apoptosis and these free parasites would not be included in the quantification process due to cell washing steps. In future experiments it would be interesting to additionally count free parasites in the cell culture media.

This highlights one of the disadvantages of the qPCR parasite quantification method we used. While it is a quick and easy tool for the confirmation of parasite entry, the values for approximate intracellular parasite number produced by this method are unlikely to be

completely accurate. Therefore, we have been careful not to over-interpret comparisons between these values in different conditions. Improvements to the creation of the standard curve such as a larger range of known parasite numbers could improve this method for future use. Within this thesis this method will primarily be used to confirm infection and parasite entry rather than a predictor of exact numbers of intracellular parasites.

We also investigated the initial cytokine response to *Leishmania* over the first 48h of infection within THP-1 cells. We first looked at IL-12, IL-6 and TNF- α, of which there was no significant induction over the course of infection. These three inflammatory cytokines are important for host resistance against infection, with IL-12 especially shown to be detrimental to parasite survival (Dayakar et al., 2019; Liese et al., 2007; Tripathi et al., 2007).

IL-6 is a cytokine with pleiotropic activities in inflammation, the immune response, and haematopoiesis (Tanaka et al., 2014) as well as important for the inflammatory control of parasites after PAMP recognition (Kumar et al., 2011). Together with IL-12, macrophage IL-6 production can also be partially inhibited during *L.major* infection (Belkaid et al., 1998).

TNF-α is another inflammatory cytokine produced by macrophages and is crucial for mediating cell resistance to infection and also cancers (Idriss and Naismith, 2000). TNF-α can mediate host protection against cutaneous Leishmaniasis by increasing macrophage activity and promoting NO synthesis (Liew et al., 1990). Mice deficient in TNF-α, and infected with *L. major* parasites have also developed fatal visceral *Leishmania* infection (Wilhelm et al., 2001), despite the production of other inflammatory cytokines by infected macrophages. This suggests it can be an important cytokine for host resistance. Overall, macrophage-driven inflammation helps clear the parasites during infection.

In order to evade anti-parasite inflammatory responses, *Leishmania* is known to target the transcription factor NF-κB which promotes the expression of pro-inflammatory cytokines (Gregory et al., 2008a; Liu et al., 2017). The lack of induction of three important pro-

inflammatory cytokines IL-6, IL-12 and TNF-α in our infection (Fig. 5) is likely to be in part due to the known inhibitory role of *Leishmania* infection on NF-κB subunit p65. *Leishmania* virulence factors GP63 and CP3 are both known to degrade p65 during infection (Abu-Dayyeh et al., 2010; Gregory et al., 2008). Normally,activation and nuclear translocation of NF-κB lead to increased transcription of several genes, including those encoding IL-12 due to the IL-12 p40 promoter having two NF-κB binding sites (Yoshimoto et al., 1997). The promoter region of the interleukin-6 (IL-6) gene also has a putative NF-κB binding site (Libermann and Baltimore, 1990). TNF-α production can also be regulated by NF-κB, or by AP-1 (Liu et al., 2000; Newell et al., 1994) another transcription factor targeted by *Leishmania* (Contreras et al., 2010). Past studies have shown that IL-12 induction was impaired in *L.major* promastigote (Belkaid et al., 1998; Carrera et al., 1996) and *L.mexicana* amastigote (Weinheber et al., 1998) infected macrophages. This inhibition is thought be important for prolonging parasite intracellular survival.

In contrast to the pro-inflammatory cytokines, anti-inflammatory mediator IL-10 was induced by *L.mexicana* infection here. It is well known that IL-10 is a crucial cytokine for parasite survival with IL-10 KO mice resistant to infection (Belkaid et al., 2001; Murphy et al., 2001), suggesting that induction of IL-10 production by the parasite may be a immune subversion strategy. IL-10 can also downregulate macrophage activation and their ability to control Leishmaniasis through the prevention of nitric oxide production (Kane and Mosser, 2001). Pre-treatment of *in vitro* macrophages with IL-10 resulted in a dramatic enhancement in parasite intracellular survival (Kane and Mosser, 2001). Our data seems to confirm that IL-10 induction is occurring with successful infection. Thus, while *Leishmania* directly blocks the induction of pro-inflammatory cytokines by targeting NF-kB, it may also induce negative feedback signalling and anti-inflammatory functions of macrophages by enhancing IL-10 production.

One immunomodulator which was induced in our infection experiments was the chemokine CXCL10 which was expressed particularly at the later time points of

Leishmania infection (from 24h, Fig. 5). The biological function of CXCL10 involves the recruitment of macrophages and T cells to sites of infection, particularly in the anti-viral response. Past studies have indicated that cells infected with *Leishmania* can produce CXCL10 and that production has been linked to phagocyte activation and parasite control (de Souza et al., 2010; Vargas-Inchaustegui et al., 2010; Vasquez and Soong, 2006). There does however seem to be a lack of knowledge surrounding *L.mexicana* and this chemokine.

There is also currently limited research into the potential role of IFN-β in *Leishmania* infection (reviewed by Silva-Barrios and Stäger, 2017), and none involving *L.mexicana*. Our data indicates that IFN- β is induced by the THP-1 cells during infection. Whether this induction would benefit the parasite or host more is unclear, as previous studies have shown differing results on if IFN- β would play a protective or detrimental role during the parasite infection. Interferons are essential to viral control and act as the cells first line of antiviral defence, with their release resulting in the expression of hundreds of IFN-stimulated genes, many with antiviral functions (Samuel, 2001; Stetson and Medzhitov, 2006). Within the context of protozoan (Beiting, 2014; Silva-Barrios and Stäger, 2017) and intracellular bacterial infections (Boxx and Cheng, 2016; Snyder et al., 2017) the role of type I IFNs is less clear. For example, they can protect mice during *Plasmodium falciparum* infection (Miller et al., 2014), but exacerbate infections with *Trypanosoma cruzi* (Chessler et al., 2011). Clearly, further exploration into type I interferons and *Leishmania mexicana* infection will be needed to elucidate the role of type I IFNs during infection.

We also investigated induction of CCL5 during infection (Fig 5G) with a slight upregulation observed only after a 48h infection. Currently little is known about its role in *L.mexicana* infection, although its upregulation has shown to be induced in *L.major* infected mice (Antoniazi et al., 2004). A correlation between CCL5 expression and resistance to infection has also been demonstrated during *L. major* infection in resistant C57BL/6 mice and susceptible BALB/C mice.(da Costa Santiago et al., 2004).

Figure 5 gives us an overview over some of the major cytokines and chemokines induced during infection of *L. mexicana* in macrophages. Our data presented here is in agreement with what has been reported previously and demonstrates there are parallels between our experiments with *L. mexicana* and those using other *Leishmania* species. In the future, qPCR arrays or RNA sequencing could be used to obtain a fuller picture of how infection affects gene expression levels over time.

Chapter 4: *Leishmania mexicana* infection subverts pattern recognition receptor signalling pathways in THP-1 macrophages

4.1 Chapter introduction

While the data in Chapter 3 confirmed that parasite entry into the THP-1 macrophages was occurring, it was also important to establish that previously documented activities of *Leishmania* virulence factors, such as GP63, were also occurring in our *in vitro* infections. GP63 is found on the parasite surface and is one of the key virulence factors of the parasite, as well as having a key role in the receptor-mediated uptake of *Leishmania* (Yao et al., 2003).. Studies have demonstrated that GP63 is directly involved in subverting host signalling pathways and can interact with transcription factors NF-κB, STAT1, and AP-1 (Abu-Dayyeh et al., 2010; Contreras et al., 2010; Gregory et al., 2008b; Forget et al., 2005).

The NF-κB subunit p65, or RelA as it is also known, is a key part of the cell's inflammatory response. The NF-κB pathway can be induced by a variety of stress stimuli including PAMPs through the TLR receptors and pro-inflammatory cytokines including TNF-α and IL-1. NF-κB is a key transcription factor required for the activation of many pro-inflammatory genes including those encoding cytokines such as IL-6 and IL-12p40, making it an important regulator of inflammation.

4.2 Infection of THP-1 cells causes degradation of the NF-κB subunit p65

We performed western blot protein analysis of p65 on amastigote and promastigote infected cells to investigate if cleavage of p65 would occur (Fig.7A). Cells were lysed at various time points over 24h to determine how quickly this cleavage would occur after the initial infection. In figure 7A we can clearly see a decrease in the level of total p65 and phosphorylated p65 from as little as 2h after promastigote infection. In contrast, at the 2h and 4h infection time point with the amastigotes there does not seem to be much degradation of total p65 at all. However, at later timepoints, both promastigotes and amastigotes cause reductions in p65 protein levels.

Further investigation into p65 degradation during promastigote infection was carried out using a longer time course (Fig 7B) and varying parasite numbers (Fig.7C). In figure 7B, at 6h post infection, we can again observe almost a complete loss of total p65 and the appearance of a 35kD band. This represents the known cleavage product of p65 after GP63 activity and is known as p35 (Gregory et al., 2008b). However, the cells are able to recover p65 over time as at 24h and 48h post infection both total p65 and phosphorylated p65 is present again within the cell. This suggests that p65 is not continually cleaved throughout infection, perhaps just initially as a part of the early infection process. Although in Fig.7A we can observe some cleavage of p65 still occurring at 24h.

These changes to p65 occurred when cells are infected at a ratio of 5 parasites to each cell. While this ratio is shown to be sufficient to establish infection and show observable virulence factor activity, similar *in vitro* infection experiments in the literature can use up to a ratio of 20:1 parasite to cells. We therefore explored the affect that increasing the infection ratio would have on p65 cleavage. A ratio of 10:1 and 20:1 parasites to cells causes complete loss of total p65 at 6h infection, compared to 5:1 where a faint band of p65 is still visible (Figure 7C). Going forward we adjusted our protocol to use an infection ratio of 10:1 parasite to cells, or MOI of 10. This ratio can be seen used in the literature

(Ogunkolade et al., 1990; Bhardwaj et al., 2005; Chandra and Naik, 2008; Mehta et al., 2010; DaMata et al., 2015) which together with our observations in Figure 7C helped shape our standard infection protocol used in all future experimental procedures (Figure 4).

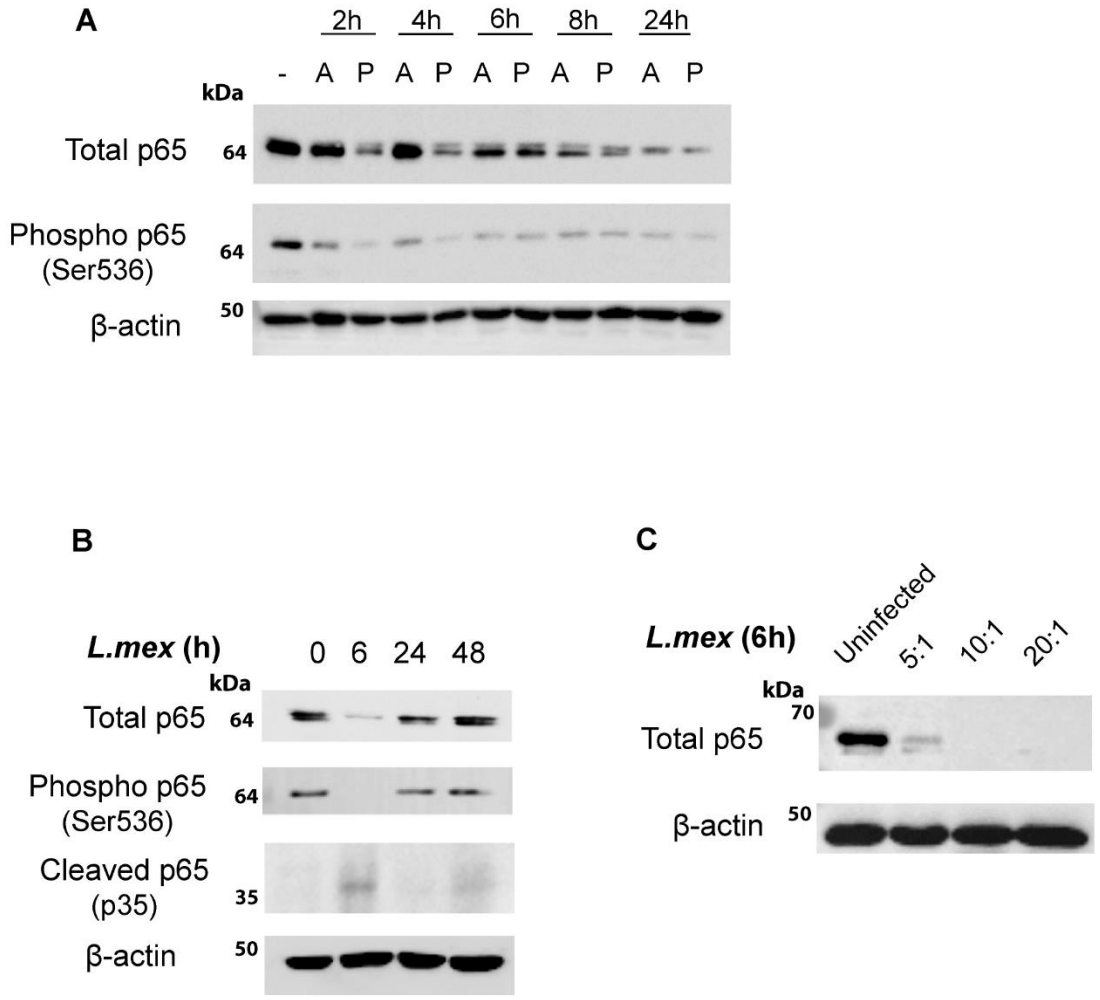


Figure 7: Infection of THP-1s with *L.mexicana* infection causes degradation of the NF-κB subunit p65

(A) WT THP-1 cells were infected with either the amastigote or promastigote form *mexicana* at a ratio of parasites to cells of 5:1 (MOI of 5) over a 24h time course. Cells were then washed and lysed for western blot analysis of p65 protein expression. **(B)** WT THP-1 cells were infected with *L.mexicana* promastigotes at a ratio of parasites to cells of 5:1,10:1 and 20:1 (MOI of 5, 10 or 20) over 48 hours as indicated before lysis for western blot analysis of protein expression of p65. Data are representative of at least three experiments. **(C)** WT THP-1s were infected with increasing numbers of *L.mexicana* promastigotes as indicated for 6 hours before lysis for western blot analysis of protein expression of p65. Data are representative of at least three experiments.

4.3 Changes to pattern recognition receptor responses during *L.mexicana* infection

Cytokine production after *L.mexicana* infection, such as that observed in Figure 5, can only occur after detection of the parasite or parasite derived molecules takes place. This is the case with all invading pathogens, making recognition a crucial process to start protective responses within the immune system. It has been well established that cells within the innate immune system can recognise specific pathogen associated molecular patterns (PAMPs) by germline encoded pattern recognition receptors (PRRs) (Akira et al., 2001; Broz and Monack, 2013; Janeway and Medzhitov, 2002; Li and Wu, 2021). Inhibiting macrophage cell signalling downstream of PRRs enables *Leishmania* parasites to prevent normal immune responses that would otherwise control *Leishmania* infection, allowing them to persist within the macrophage long enough to complete its life cycle and replicate.

To further investigate which other PRR signalling cascades are inhibited or modulated by *Leishmania*, various PAMPs were used to stimulate infected macrophages. The cytokine response to these PAMPs between infected and uninfected cells were then compared using qRT-PCR. A significant decrease in the gene expression of these normally induced cytokines could indicate that *Leishmania* is inhibiting the pathways induced by PRR activation. In the following figures 8, 9 and 10, changes in gene expression are shown as a fold change from the expression of each gene in the uninfected and unstimulated control and have been normalised using β-actin.

We stimulated THP-1 macrophages with three different agonists; lipopolysaccharide (LPS), herrings testis (HT)- DNA and polyinosinic–polycytidylic acid (poly(I:C)). LPS is a cell wall component of gram-negative bacteria that is detected by TLR4 and has been the subject of investigation during *Leishmania* infection before (Belkaid et al., 1998; Cameron et al., 2004; Lapara and Kelly, 2010). HT-DNA is double-stranded DNA which can be

detected by cytoplasmic DNA sensors such as cGAS within cells, similar to how foreign pathogen-derived DNA would if it entered the cytosol (Sun et al., 2013). Poly(I:C) is a synthetic analogue of double-stranded RNA which is structurally similar to dsRNA associated with viral infection. Poly(I:C) can bind to the membrane-bound TLR3 (Alexopoulou et al., 2001) in endosomes, in addition to intracellular RNA sensors such as MDA5 and RIG-I in the cytosol (Kato et al., 2006). In this experiment, both HT-DNA and poly(I:C) were transfected into the macrophages to activate cytosolic nucleic acid sensors.

The normal THP-1 response to TLR agonist LPS is partially abrogated during *Leishmania* infection (Fig.8). Pro-inflammatory cytokines IL-6, IL-12 and TNF- α (Figures 8A, 8B and 8C) are upregulated after LPS stimulation but this induction is significantly reduced in the infected cells. This is similar to what has been reported in the literature (Belkaid et al., 1998). Furthermore, confirming our observations in Figure 5, even basal induction of IL-6 in unstimulated cells was significantly decreased in infected cells. However, this trend was not observed in IL-12 and TNF- α where basal levels of these cytokines were not affected, but LPS-induced mRNA levels were inhibited following *L.mexicana* infection.

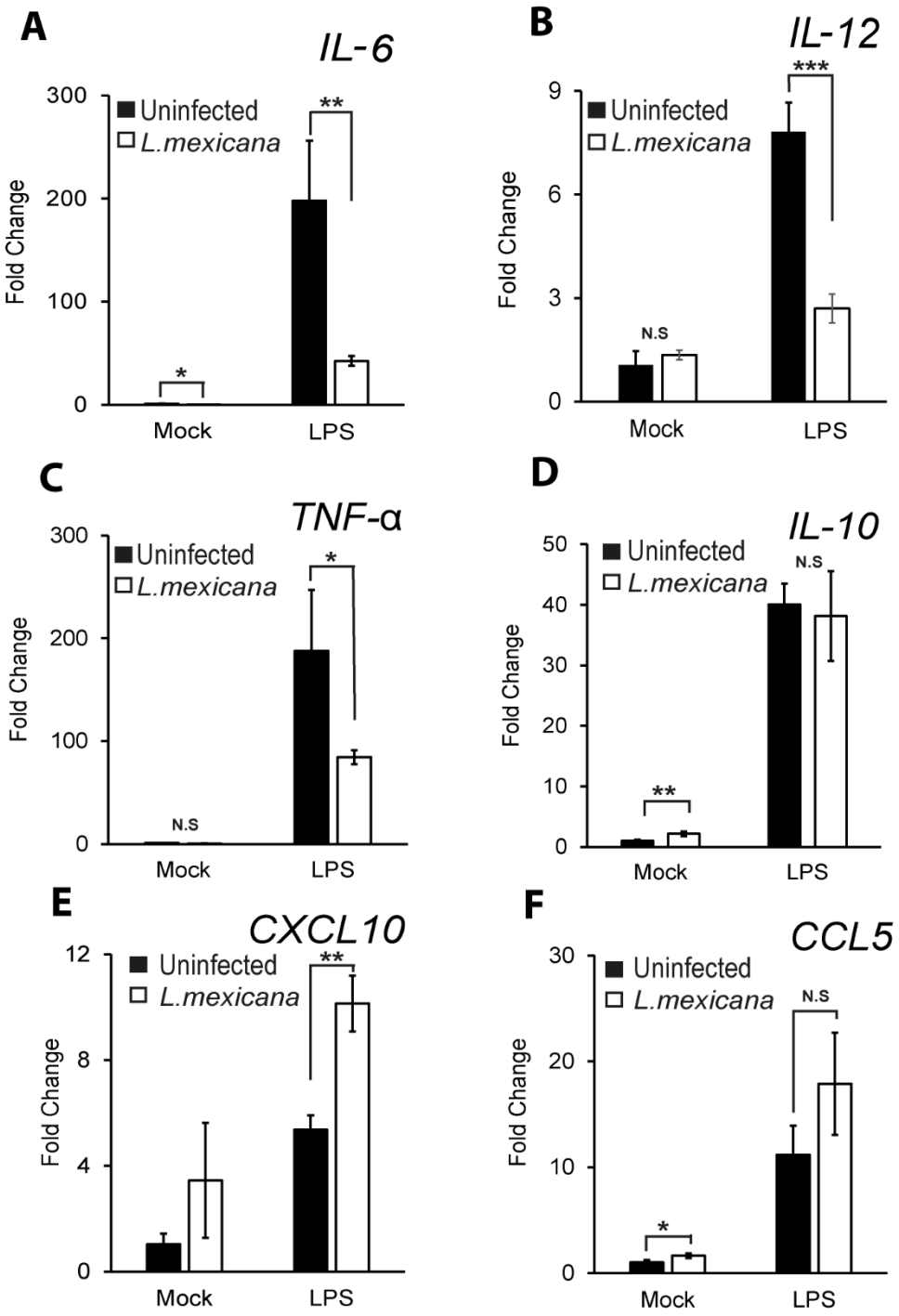


Figure 8: There is an altered response to LPS in infected THP-1 cells

WT THP-1 cells infected with *L.mexicana* at a MOI of 10 for 4 hours before stimulation with 1µg/ml LPS for 1 hour. Cells were then lysed for qRT-PCR analysis of **(A)** IL-6, **(B)** IL-12, **(C)** TNF-α, **(D)** IL-10, **(E)** CXCL10 and **(F)** CCL5 mRNA. Data are presented as mean values of biological triplicates. Error bars indicate standard deviations. N.s. = non-significant, * p≤ 0.05; ** p≤0.01 and *** p ≤ 0.001 as determined by Student's t-test. Data are representative of at least three experiments.

Interestingly, there is no difference in LPS induced IL-10 production between uninfected and infected cells (Fig. 8D). As IL-10 can be upregulated in response to infection (Dayakar et al., 2019; Kane and Mosser, 2001; Nandan et al., 2012), and we have shown that it is induced after *L.mexicana* infection in the absence of LPS (Figure 5D and Figure 8D). We find that LPS-induced IL-10 mRNA levels are not further modulated by *L. mexicana* infection.

CXCL10 induction (Fig.8E) was significantly increased in response to LPS in infected cells compared to uninfected THP1 macrophages. While we have seen that the parasites alone can induce CXCL10 production (Figure 5), this was not until the 24h infection timepoint. In these experiments (Figure 8) we chose to use a 4h infection before LPS stimulation to examine the immediate effects of infection on innate immune signalling. At this time point, we observe a trend towards enhanced CXCL10 expression in the absence of additional stimulation, but this is not significant. The CCL5 induced by LPS (Fig.8F) appeared to not be significantly increased or decreased by infection. Overall, our data shows that *L.mexicana* infection does not affect LPS detection per se, but rather affects the cytokine/chemokine profile in a gene-specific manner where the expression of proinflammatory cytokines which have anti-parasite functions is selectively inhibited, but other cytokines and chemokines are maintained or enhanced.

We next examined the response to cytosolic DNA. DNA sensors may play a role in the detection of *Leishmania* parasites in macrophages, in the case parasite DNA enters the cytosol. However, the DNA sensing pathway has not yet been extensively studied in the context of *Leishmania* infection. Here we observed a significant increase in the gene expression of cytokines normally associated with the response to cytoplasmic dsDNA in infected cells compared to the uninfected (Fig.9). Genes induced by the dsDNA sensing machinery include IFN-β, CXCL10, CCL5 and IL-6, and we find that all of these are further up-regulated when cells were infected with *L. mexicana* for 4h prior to stimulation with dsDNA (Fig.9A-D). The expression of TNF-α and IL-10 is also increased following infection

and dsDNA stimulation, but these cytokines are not strongly induced by DNA stimulation alone, and the increases with infection are more modest (Fig. 9 E-F). We originally hypothesised that the response to dsDNA might be decreased in this experiment if the DNA sensing pathway was also a target for *Leishmania* virulence factors. The enhancement of DNA-induced responses which we observe instead suggests that inhibition is not occurring, and indeed *L.mexicana* infection may promote activation of the DNA sensing pathway.

The THP-1 response to poly(I:C) shows no significant difference between the uninfected and *Leishmania* infected cells (Figure 10). This could suggest that *L.mexicana* neither inhibits this pathway or mediates an enhancement as is seen to be occurring with the THP-1 response to DNA. As the cytosolic DNA and RNA sensing pathways converge on the use of shared signalling factors and transcription factors, and ultimately result in the induction of a similar cytokine and chemokine profile, our data show that *L. mexicana* selectively enhances signalling through the DNA sensing pathway.

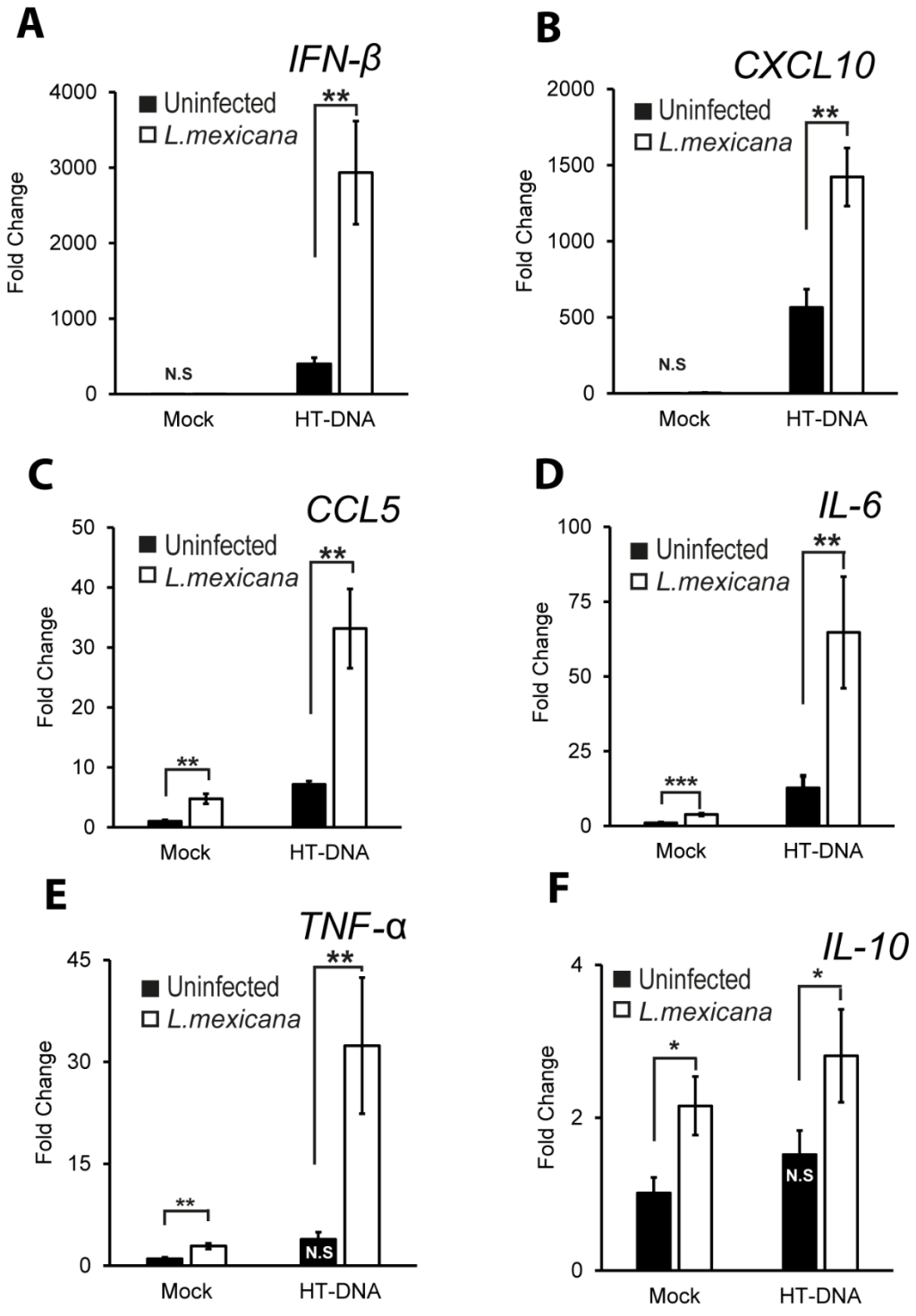


Figure 9: There is an enhanced response to dsDNA in infected THP-1 cells

WT THP-1 cells infected with *L.mexicana* promastigotes at a MOI of 10 for 4 hours before transfection with 5µg/ml HT-DNA for 4 hours. Cells were then lysed for qRT-PCR analysis of **(A)** IFN-β, **(B)** CXCL10, **(C)** CCL5, **(D)** IL-6, **(E)** TNF-α, and **(F)** IL-10 mRNA. Data are presented as mean values of biological triplicates. Error bars indicate standard deviations. * p≤ 0.05 and ** p≤0.01 as determined by Student's t-test. Data are representative of at least three experiments.

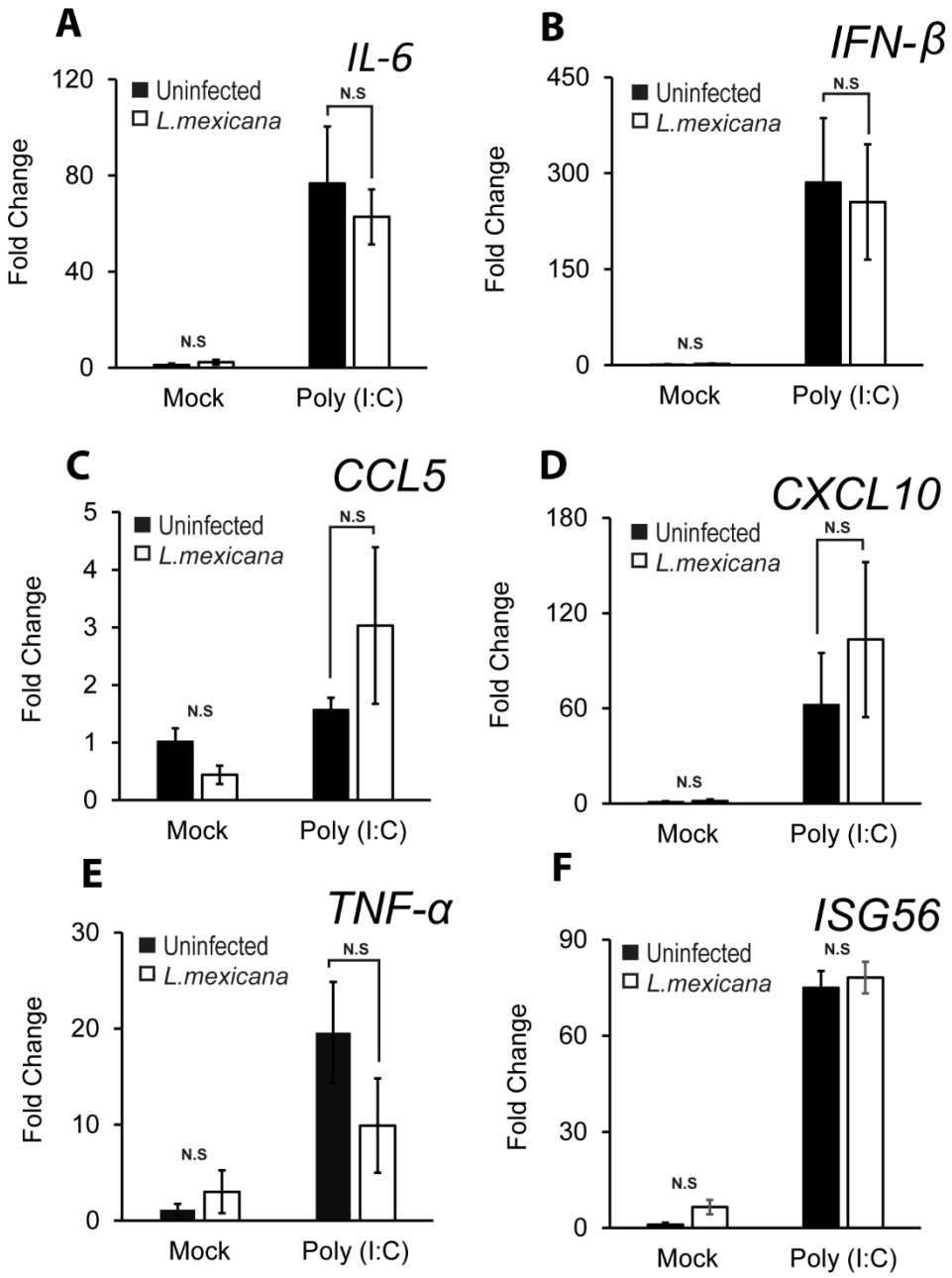


Figure 10: There is no significant difference in the THP-1 response to Poly(I:C) during *L.mexicana* infection.

WT THP-1 cells were infected with *L.mexicana* promastigotes at a MOI of 10 for 4 hours before stimulation with 5µg/ml Poly (I:C) for a further 4 hours. Cells were then lysed for qRT-PCR analysis of **(A)** IL-6, **(B)** IFN-β, **(C)** CCL5, **(D)** CXCL10, **(E)** TNF-α and **(F)** ISG56 mRNA. Data are presented as mean values of biological triplicates. Error bars indicate standard deviations. NS, not significant ($p>0.05$) as determined by Student's t-test. Data are representative of at least three experiments.

4.4 Investigation of the DNA sensing pathway and STING during *L.mexicana* infection

To date, there has been little investigation of *Leishmania* and its interaction with the cytoplasmic DNA sensing pathway, as the majority of *Leishmania* and PRR research has focused on the role of the TLRs during *Leishmania* infection (Rossi and Fasel, 2018). Therefore, the initial results seen in Figure 9 that suggest some interaction between *Leishmania mexicana* and the cytoplasmic DNA sensing pathway may be occurring, provide a new and promising avenue of investigation.

Key factors in the cytoplasmic DNA sensing pathway (simplified in Figure 11A) include the cytosolic DNA sensor cGAS and its adaptor proteins STING, which mediates the activation of the kinase TBK1 and the transcription factor IRF3. Protein expression of several key components of this pathway downstream of the DNA sensor cGAS were investigated during *L. mexicana* promastigote infection for 6, 24 or 48 hours (Fig.10B). The most striking difference between the infected and uninfected cells is the presence of a smaller, faster migrating STING band that appears with infection (Fig 11B). While a much fainter band of this size is present in the uninfected (0h) sample, its significantly stronger appearance in the 6 hour infected sample suggests its presence is influenced by the *Leishmania* infection. It could be suggested that this is a smaller variant of STING which is increasingly expressed during infection, or that STING has been cleaved or modified by the parasite.

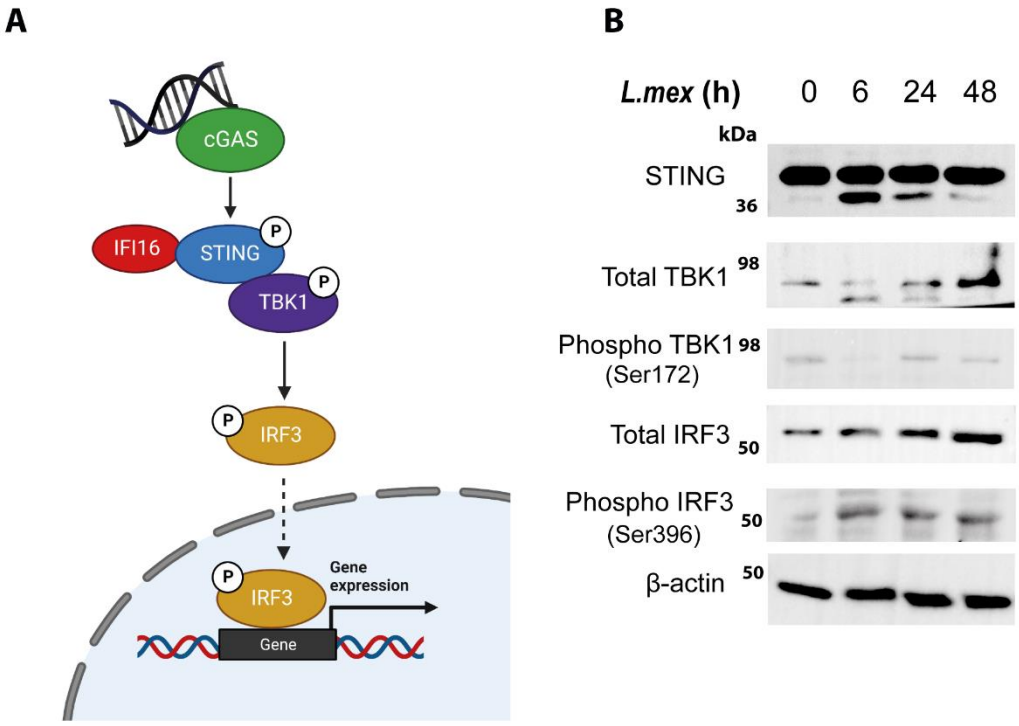


Figure 11: *L.mexicana* infection leads to changes in STING- a key component of the cytoplasmic DNA Sensing pathway

(A) Simplified diagram identifying the major components of the cytosolic DNA sensing pathway. cGAS binds to dsDNA and catalyses the formation of cGAMP, a second messenger, which binds to the essential adaptor protein STING which is resident at the ER. STING then activates downstream signalling factors TBK1 and IRF3 to activate the immune response. IFI16 is a DNA receptor found in the nucleus of the cell, that has been shown to promote STING signalling in response to dsDNA. **(B)** WT THP-1 cells were infected with *L.mexicana* promastigotes at a MOI of 10 over 48 hours as indicated before lysis for western blot analysis of protein expression. Data are representative of at least three experiments.

A smaller band can also be seen in the TBK1 blot at 6h and 24h after infection, however this was not seen as consistently as the STING lower band upon experimental repeats. Phosphorylated TBK1 does also not appear to be induced by infection and the band for the 6h timepoint is also very faint. In contrast to phosphorylated TBK1, phosphorylated IRF3 is slightly increased during infection compared to mock-treated cells, particularly at later time points.

As the lower STING band was consistently seen through repeats of this infection time course and western blot analysis, including at early time points, a series of further infection experiments were carried out to probe its induction more deeply (Figure 12).

By using an increasing number of parasites for the initial infection we demonstrated that the presence of this lower STING band can be induced with a parasite to cell infection ratio of 5:1 after a 6h infection period (Fig.12A). The intensity of the lower band does not increase as the ratio is increased to 10:1 and 20:1 (Fig.12A). Cells were also infected for 24h with these three different infection ratios. While the STING lower band is still present at this longer time point, what is unusual compared to the other times we have observed this band is that the normal size STING band was no longer detectable, leaving only the smaller STING. This could suggest that the longer the cells are infected, more of the STING within these cells is modified or cleaved to the smaller form. While we consistently observed the appearance of a smaller STING band upon *L. mexicana* infection, the relative ratio between the two forms of STING was variable between different experiments, possibly dependent on infection levels.

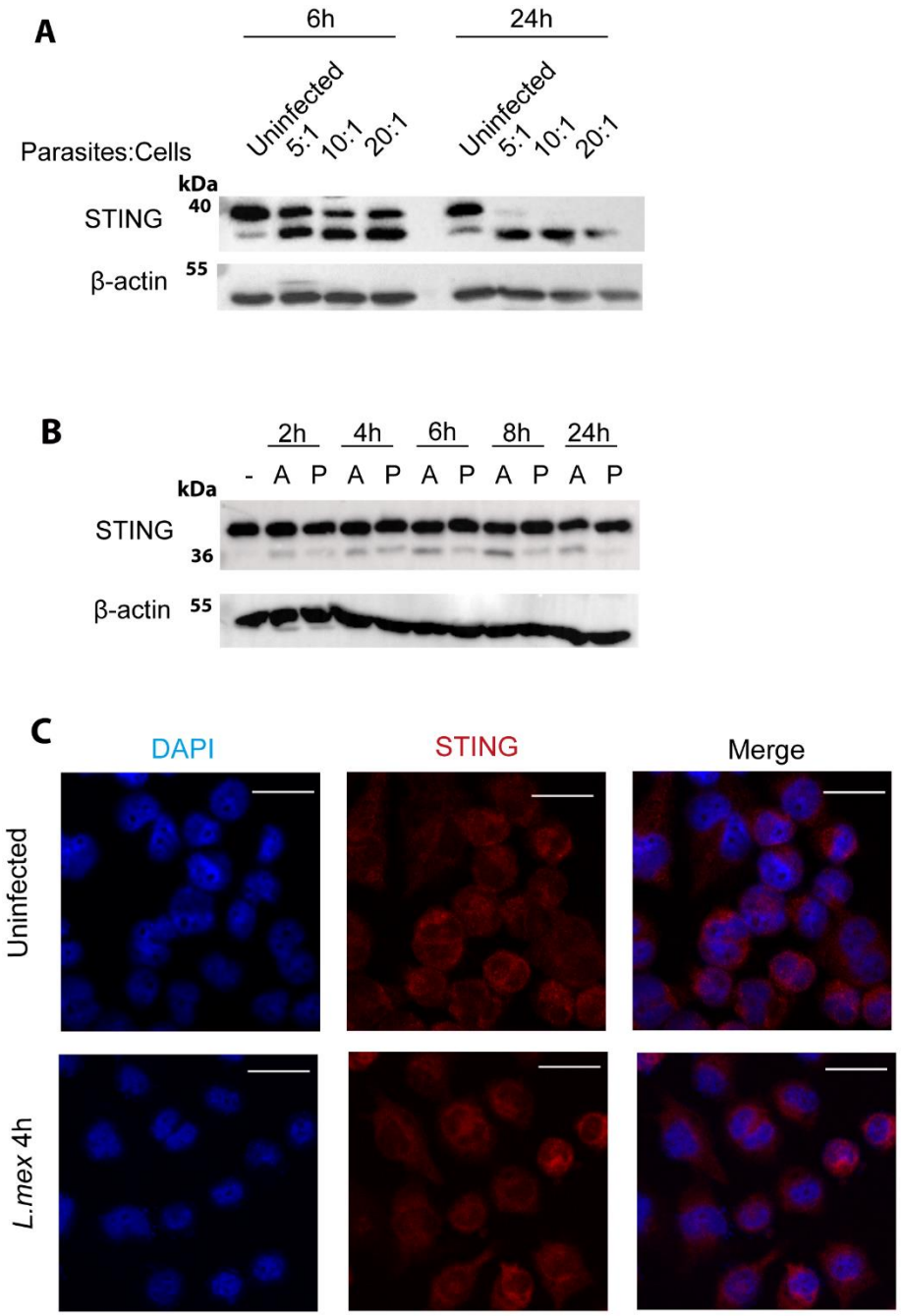


Figure 12: The presence of the STING lower band is not dependent on parasite load or life cycle form, and does not change STING intracellular localisation.

(A) WT THP-1 cells were infected with increasing numbers of *Leishmania* promastigote parasites for either 6h or 24h. Cells were then washed and lysed for western blot analysis of STING protein expression. **(B)** WT THP-1 cells were initially infected with either the Amastigote or Promastigote form at a MOI of 10 over a 24h time course, Cells were then washed and lysed for western blot analysis of STING and p65 protein expression. **(C)** WT THP-1 cells were seeded onto coverslips, differentiated with PMA for 48h before infection with *L.mexicana* promastigotes for 6 hours. Cells were then fixed in methanol and stained for STING (red) and DAPI nuclear stain (blue) and imaged by confocal microscopy. Scale bar = 20μm.

The STING band could be observed after both promastigote and amastigote infection (Fig. 12B), suggesting that its formation is not restricted to one lifecycle stage of the parasite.

The more extensive time course in Figure 12B also showed that induction of this band occurs as early as 2 hours into infection. This again occurs with both lifecycle forms.

Finally in Figure 12C we can observed that the presence of a smaller alternative form of STING does not appear to change its normal cellular localisation at 6h post infection. Confocal microscopy using a primary antibody against STING and nuclear stain DAPI, did not reveal a difference in STING localisation or overall abundance between the uninfected and infected cells.

4.5 The DNA response in *L.mexicana* infected THP-1 cells is STING dependent

We have discovered that a smaller form of STING is induced during *L.mexicana* infection. Despite this modification, STING localisation within the cell was unchanged (Fig.12C), DNA sensing could still proceed, and indeed was enhanced following infection (Fig.9). In some of our experiments a much fainter STING band at the same size of that induced by *Leishmania* can be seen in our uninfected control cells (for instance Figs.11B,12A,12B), suggesting it could be an alternative form of STING that is just more heavily induced with infection. We also needed to exclude the possibility that the lower migrating band is not derived from a *Leishmania* protein which cross-reacts with the polyclonal STING antibody. To definitively demonstrate that the lower band is derived from endogenous STING, we used THP-1 cells in which both STING alleles had been deleted using CRISPR (Jønsson et al., 2017).

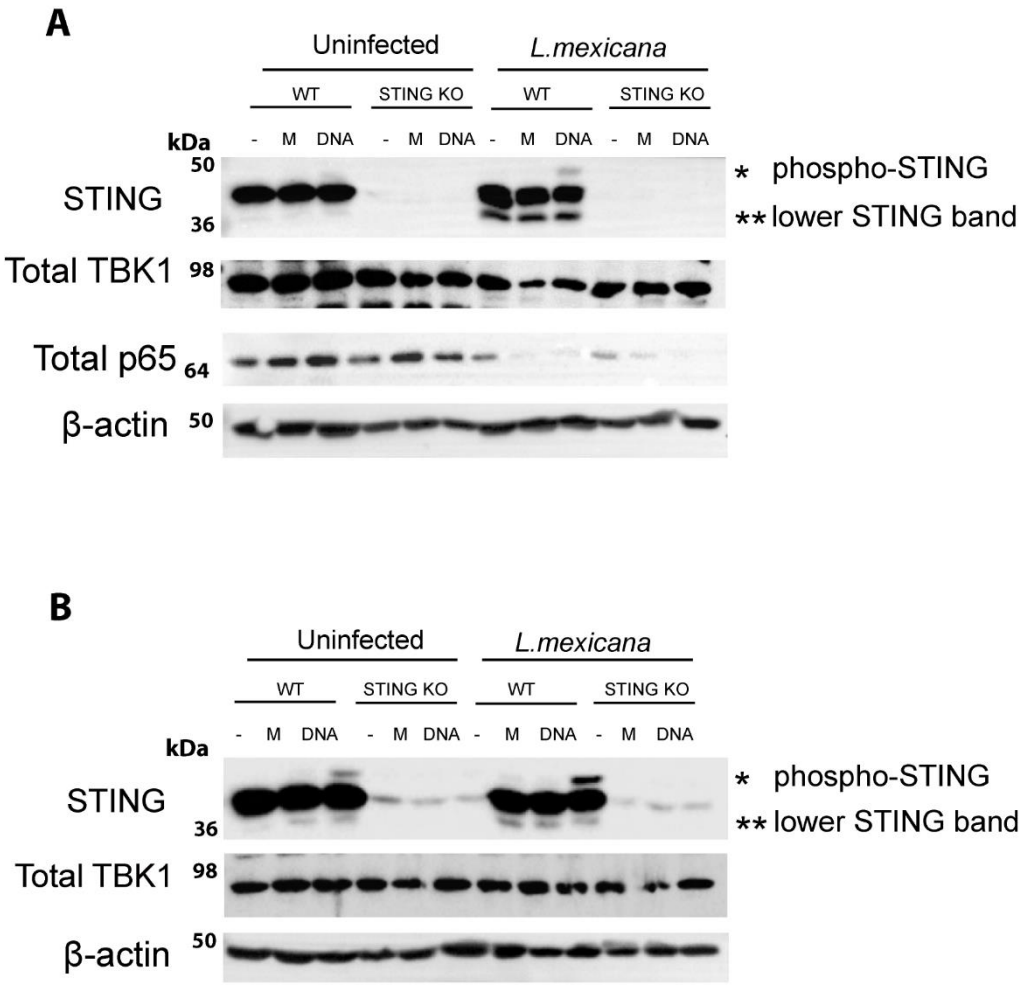


Figure 13: Confirmation that the lower STING band present in *L.mexicana* infection is derived from STING.

WT and STING KO THP1s were infected with *L.mexicana* **(A)** amastigotes and **(B)** promastigotes at a MOI of 10 for 6 hours before stimulation with 5µg/ml transfected HT-DNA for 4 hours or transfection reagent only (M). Cells were then lysed for western blot analysis of protein expression. * = Presence of activated phospho-STING. **= Lower STING band seen enhanced by infection.

We infected both WT and STING KO THP-1 cells and analysed protein expression by western blot. Both *Leishmania* amastigotes (Fig.13A) and promastigotes (Fig.13B) were used in this infection. These western blots clearly demonstrated that the lower STING band induced by infection was no longer present in the infected STING KO THP-1 cells, confirming that this band is indeed derived from STING (Fig.13). We also stimulated WT and STING KO cells by DNA transfection, 6h after they had been infected with *L. mexicana.* In analogy to our cytokine and chemokine expression data (Fig.9), we find that prior infection does not prevent STING activation. It even appears that DNA-induced STING phosphorylation is enhanced in infected cells, particularly promastigote infected cells, with stronger induction of the band representing its phosphorylated form seen on the western blot. This is in good correlation with the enhanced DNA-induced innate immune response following *L .mexicana* infection.

We then investigated if the enhanced cytokine response observed after dsDNA stimulation was also STING dependent. Both WT and STING KO THP-1 cells were infected for 4 hours with *Leishmania* amastigotes (Fig.14) or promastigotes (Fig.15) before stimulation with dsDNA for a further 4 hours. Cells were then lysed and processed for the analysis of gene expression by qRT-PCR.

IFN- β, IL-6 and TNF-α induction in response to DNA is again significantly enhanced by *L.mexicana* infection, and this induction is dependent on STING (Figs.14,15). CXCL10 induction is only enhanced with infection in the promastigote infected cells (Figs.14B, 15B). Cytokine upregulation in response to dsDNA is significantly reduced in the STING KO THP-1 cells, as expected. While the presence of STING is critical for the DNA response, and it's up-regulation during *L.mexicana* infection, we still observe a small, but significant increase in the production of some cytokines and chemokines when STING KO cells are infected with *L.mexicana*. This suggests that there might be additional, STING-independent pathways which can mediate a much more minor DNA sensing response in the absence of STING, and these also appear to be enhanced by infection.

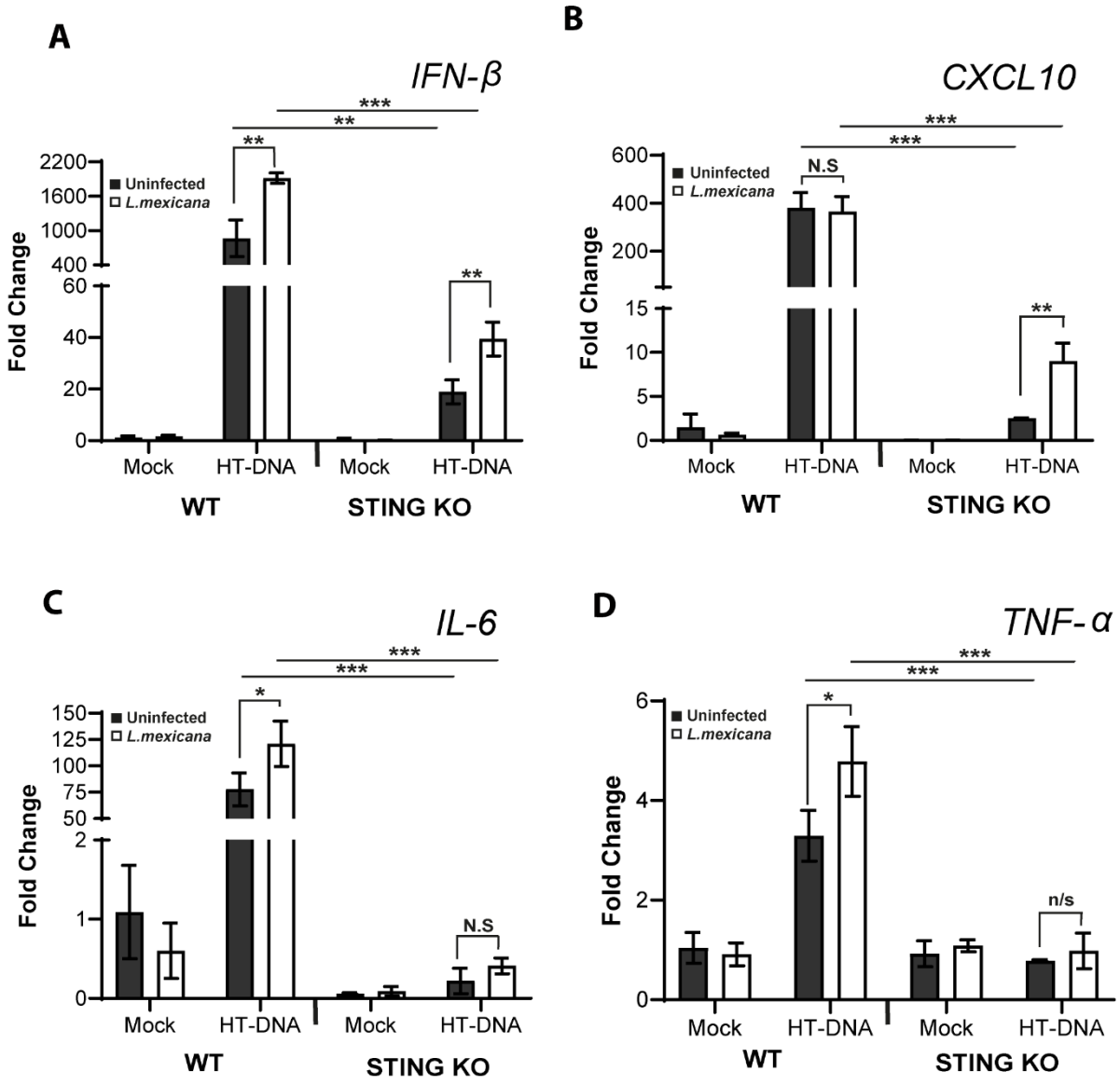


Figure 14: The enhanced response to DNA observed in *L.mexicana* amastigote infected cells is STING dependent.

WT or STING KO THP-1 cells infected with *L.mexicana* amastigotes at a MOI of 10 for 4 hours before stimulation with 5μg/ml HT-DNA or transfection reagent only for 4 hours. Cells were then lysed for qRT-PCR analysis of **(A)** IFN-β **(B)** CXCL10 **(C)** IL-6 and **(D)**TNF-α. Data are presented as mean values of biological triplicates. Error bars indicate standard deviations. n/s. = non-significant, * p≤ 0.05; ** p≤ 0.01 and *** p ≤ 0.001 as determined by Student's t-test. Data are representative of at least three experiments.

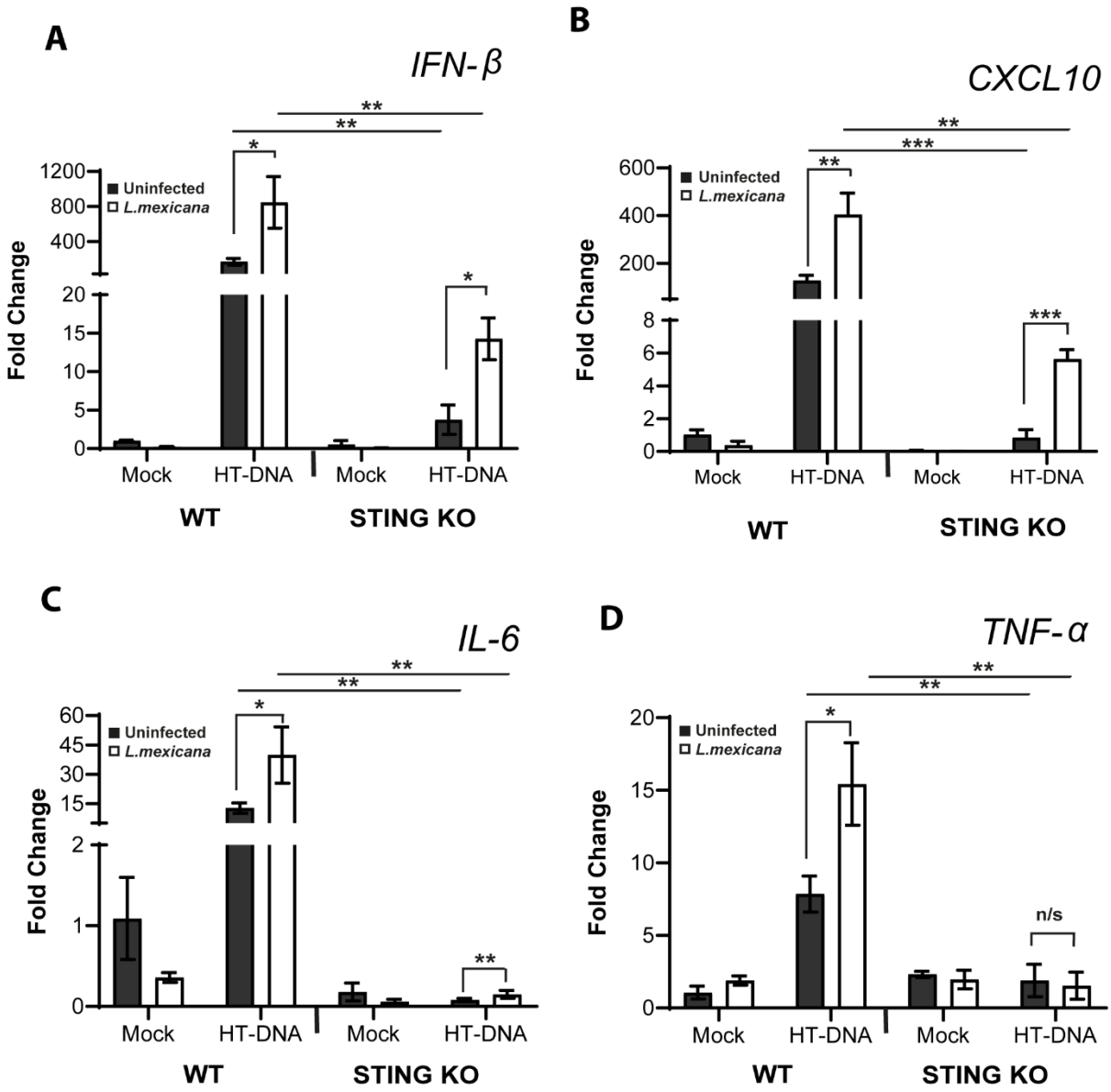


Figure 15: The enhanced response to DNA observed in *L.mexicana* promastigote infected cells is STING dependent.

WT or STING KO THP-1 cells infected with *L.mexicana* promastigotes at a MOI of 10 for 4 hours before stimulation with 5μg/ml HT-DNA or transfection reagent only for 4 hours. Cells were then lysed for qRT-PCR analysis of **(A)** IFN-β **(B)** CXCL10 **(C)** IL-6 and **(D)**TNF-α. Data are presented as mean values of biological triplicates. Error bars indicate standard deviations. n/s. = non-significant, * p≤ 0.05; ** p≤0.01 and *** p ≤ 0.001 as determined by Student's t-test. Data are representative of at least three experiments.

4.6 *Leishmania* enhances the phosphorylation of key signalling components after HT-DNA, but not poly(I:C) stimulation

In Figure 13 we demonstrated that the STING band was indeed derived from STING. Additionally, we were also able to observe by western blot that after stimulation with dsDNA, STING phosphorylation in infected cells seemed to be enhanced compared to the uninfected cells. To investigate STING signalling further we looked at the activation of different components of the DNA sensing pathway when combining a 4h dsDNA stimulation with prior *L.mexicana* infection in THP1 cells. This would be able to inform us if the enhancement of the cytokine response (Figs.9,14,15) could be linked to changes at the protein and phosphorylation levels of signalling components downstream of STING, and thus point towards a mechanism of action.

We specifically we looked at the phosphorylated forms of STING, TBK1 and IRF3 after infection and stimulation with DNA, as well as after LPS stimulation for comparison with a pathway we know to be downregulated during infection (Fig.8).

When stimulated with dsDNA, despite the presence of this alternative STING band, STING phosphorylation at serine 366 is still shown to occur (Fig.16 and Fig.17). Again, in fact STING activation by dsDNA appears to be enhanced with increasing infection time (2-6h) as shown by a more strongly induced phospho-STING band which is seen above STING by western blot. The phosphorylation of IRF3 after DNA stimulation is also noticeably more strongly induced with a longer infection time. Phosphorylated TBK1 is also much more strongly induced in both LPS and DNA stimulated cells following *L.mexicana* infection.

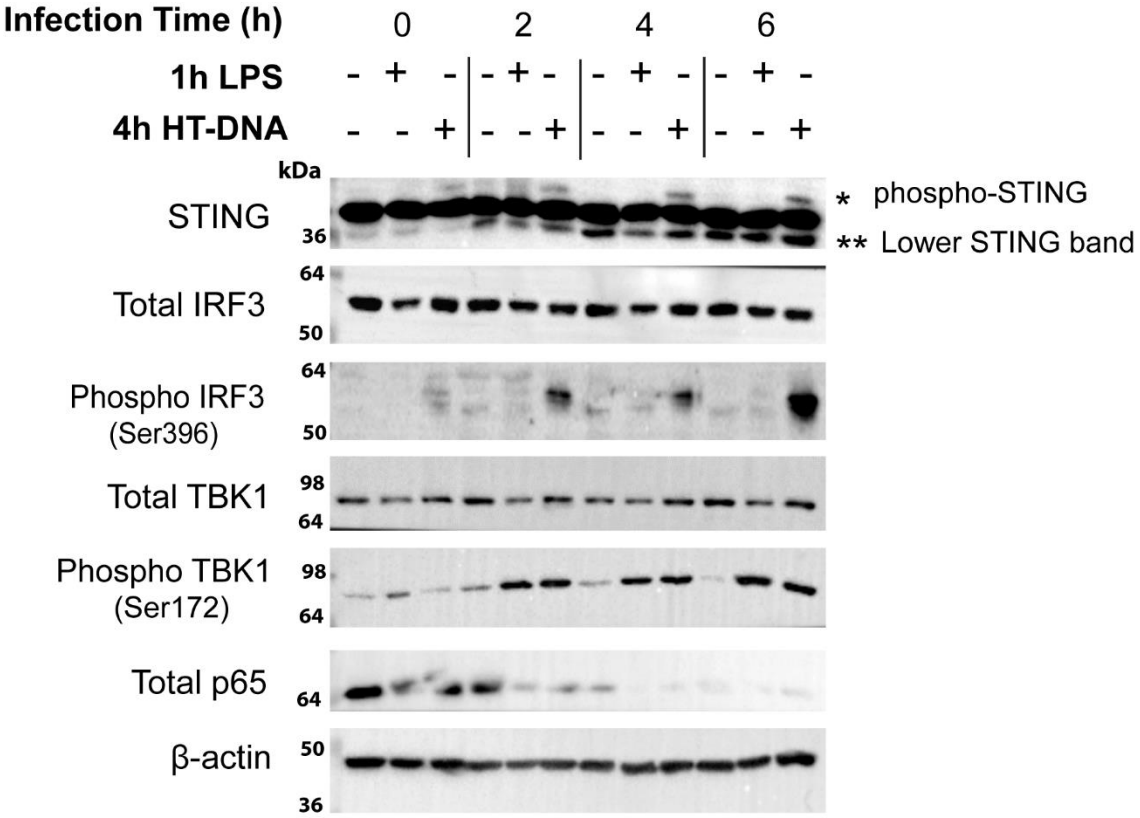


Figure 16: There is an altered response to LPS and HT-DNA in infected THP-1 cells

WT THP-1 cells were infected with media only (0h) or *L.mexicana* promastigotes at a MOI of 10 for 2, 4, or 6 hours as indicated before stimulation with 1µg/ml LPS or 5µg/ml transfected HT-DNA for 4 hours. Cells were then lysed for western blot analysis of protein expression. Phospho Data are representative of at least three experiments. * = Presence of activated phospho-STING. **= Lower STING band seen enhanced by infection.

After seeing an increasing enhancement in the phosphorylated forms of STING, TBK1 and IRF3 with infection time, a longer time course was carried out to confirm if this trend continued. We looked at similar protein expression after DNA stimulation of cells instead infected for 6h, 24h and 48h (Fig.17) and also stimulated cells with poly(I:C) as a comparison. We observed previously that the THP-1 cytokine response to poly(I:C) was not affected by infection as shown by qRT-PCR analysis (Fig.10) despite both DNA and RNA sensing pathways converging on components such as TBK1 and IRF3. Comparing their activation during infection can tell us more about how the enhancement may be mediated.

At the 6h infection time point, there is enhanced phosphorylation of IRF3 following DNA stimulation, while the IRF3 response to poly(I:C) is not affected. This enhancement at 6h is less obvious in phosphorylated STING and TBK1 in this experiment. The increased activation of IRF3 appears to be transient, and at the 24h time point no increase in the phosphorated forms of IRF3, STING and TBK1 compared to uninfected is seen. At 48h post infection instead there seems to be a decrease in the phosphorylation of IRF3, STING and TBK1 after both DNA and poly(I:C) stimulation. This could be due to the accumulated stress of a longer infection that has made cells less responsive to stimulation.

Overall, we observe that there is a specific enhancement of the DNA sensing response in THP-1 cells infected with *L.mexicana,* and that it seems to be more prominent at earlier timepoints such as 4-6h post infection when the initial innate immune response to infection occurs.

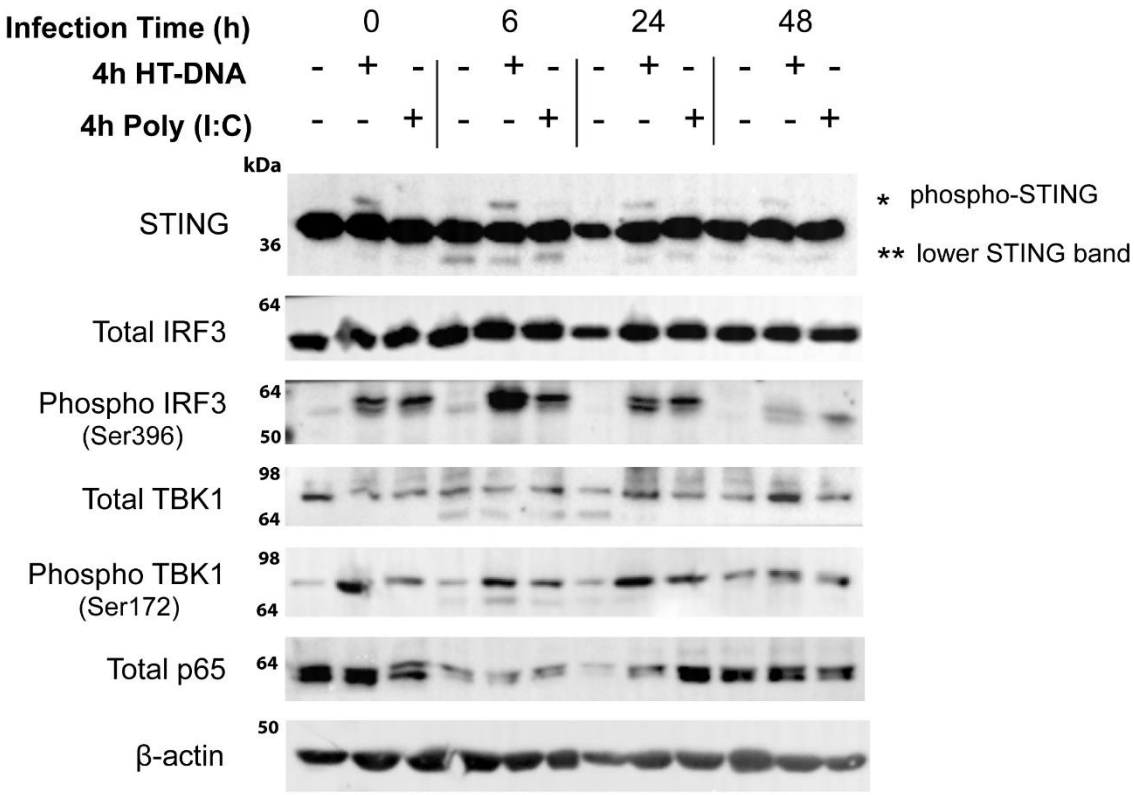


Figure 17: Stimulation of WT THP-1s cells with HT-DNA or Poly(I:C) during a 48h *L.mexicana* infection

WT THP-1 cells were infected with media only (0h) or *L.mexicana* promastigotes at a MOI of 10 over 48 hours as indicated before stimulation with transfected HT-DNA (5µg/ml) or Poly(I:C) (1µg/ml) for 4 hours. Cells were then lysed for western blot analysis of protein expression. Data are representative of at least three experiments. * = Presence of activated phospho-STING. **= Lower STING band seen enhanced by infection.

4.7 The presence of STING is not beneficial for the initial infection of *Leishmania mexicana in vitro*

Having shown that an alternative form of STING is induced by infection, and that STING is essential for the enhanced cytokine response to dsDNA, understanding why this enhancement may be taking place was our next question.

The enhancement is particularly interesting as *Leishmania* infection alone during early infection does not seem to strongly activate the DNA sensing pathway, especially when compared to HT-DNA transfection (Fig.9). Considering that *Leishmania* has evolved to survive within host macrophages and is known to cleave and modify many host proteins to help it do so, it is conceivable that any potential modification of STING by the parasite could be purposeful and beneficial for its own survival within host macrophages or for the evasion of an early local immune response.

To determine if STING, and by extension the DNA sensing pathway, is beneficial or not for *Leishmania* infection we examined how infection progressed in both WT and STING KO cells (Fig.18). To compare parasite entry between WT and KO cells, we determined the percentage of infected cells at each timepoint (Fig.18A). We seeded THP-1 cells onto coverslips prior to infection then after the indicated infection times washed, fixed, and stained the infected cells. After imaging we randomly selected a set number of the total cells and determined what percentage showed parasite infiltration.

From this data we can see that the percentage of infected cells is very similar at each timepoint between the WT and STING KO cells, and even slightly higher in the STING KO cells after 48 hours of infection. This suggests that a lack of STING does not prevent the initial infection of macrophages or limit cell entry. The microscopy images in figure 18B confirm that STING KO cells are infected to a similar extent at each time point.

We also counted parasite numbers per cell, which revealed that there were many more heavily infected cells in the STING KO cells compared to the WT (Fig.18C). This suggests that the lack of STING does not prevent successful parasite replication in addition to parasite entry, and in fact could perhaps lead to more successful infection of individual macrophages.

These preliminary results suggest that the presence of STING per se is not beneficial for infection and perhaps even somewhat detrimental for the infection of macrophages *in vitro*. As STING induces a type I interferon response, it could be possible that it is the increase in type I interferon production that affects *L.mexicana* growth in macrophages. We observe low levels of IFN-β induction between 6 and 48h after infection (Fig. 5), and infection further potentiates the interferon response to cytosolic DNA.

To investigate if type I interferons will limit or enhance *L.mexicana* infection in our cells, we first pre-treated THP-1 cells with two different concentrations of recombinant human IFN-α. THP-1 cells were pre-treated with either 2ng/ml or 20ng/ml IFN-α for 2 hours before a 6h infection with *L.mexicana* promastigotes. Protein analysis of phopho-STAT1 was carried out by western blot to ensure that a known cellular target of the type I IFNs was successfully activated at these concentrations. STAT1 is well established to be activated by interferons and treatment with IFN-α can induce phosphorylation of a specific tyrosine residue- Y701 (Darnell et al., 1994; Schindler et al., 1992).

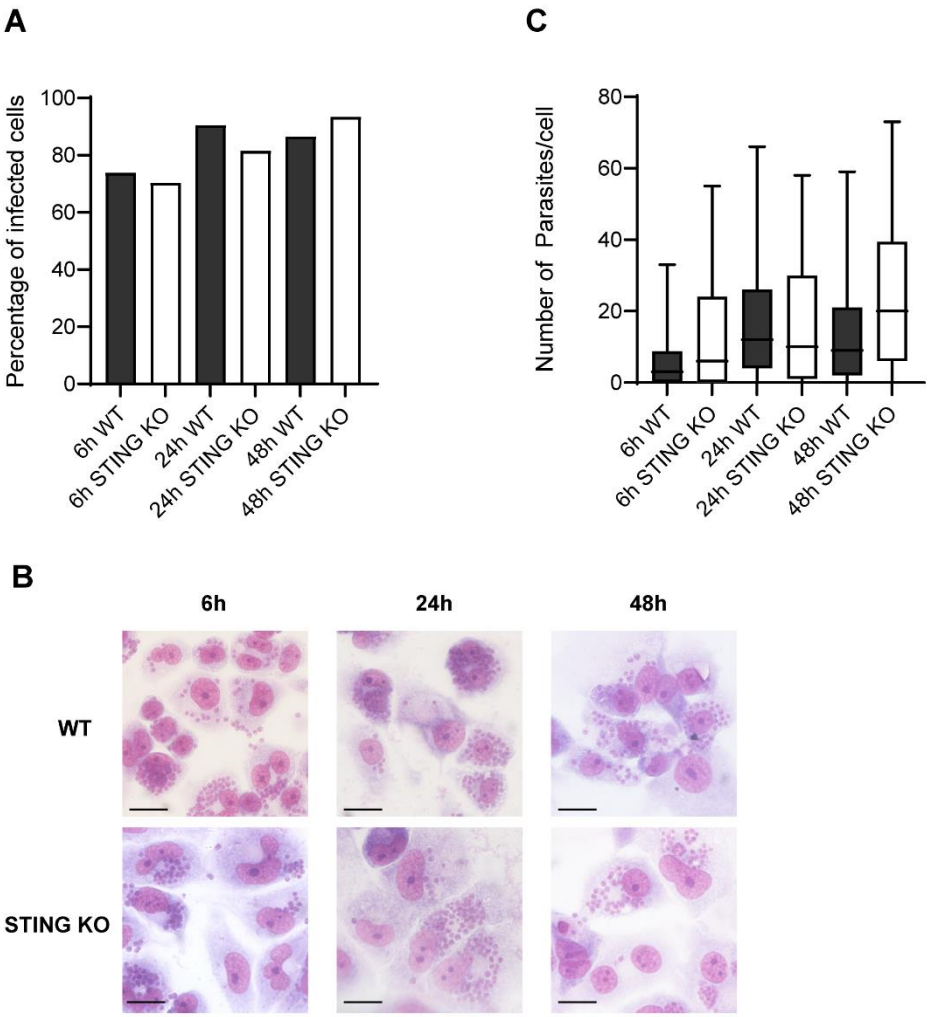


Figure 18: Changes in parasite replication and infectivity between WT and STING KO THP-1s

(A) WT and STING KO THP-1 cells were infected with *Leishmania* promastigotes at a MOI of 10 over a period of 48h.The percentage of infected cells in both WT and STING KO THP-1 cells was determined from images taken of infected cells. 200 cells were randomly chosen from each time-point and condition. **(B)** Before imaging WT and STING KO THP1 cells were seeded onto coverslips, differentiated with PMA for 48h and then infected with *L.mexicana* over 48 hours. Cells were then fixed in methanol and stained with 10% Giemsa to visualise the parasites and imaged with light microscopy. Scale bar = 20µm. **(C)** The number of parasites/cell was also determined from the images at each time point.

From figure 19A we can clearly see that phosphorylation of STAT1 was induced in both the 2ng/ml and 20ng/ml IFN- α pre-treated cells, although slightly more so at 2ng/ml. At both concentrations STAT1 phosphorylation is slightly reduced in the infected cells. This reduction is likely to be due to STAT1 also being a known target of *Leishmania* inhibition and degradation (Abu-Dayyeh et al., 2010; Forget et al., 2005). While total loss of STAT1 does not appear to occur in our infections, its activity is clearly impacted by infection.

We also looked at protein expression of STING in these treated cells (Fig.19A) where again the smaller form is induced with infection, although slightly less so in the IFN- α treated cells. We also can observe here the loss of the normal size STING as the lower band is induced, again less so in the treated cells.

To investigate if type I interferons will aid *L.mexicana* infection in our cells we pre-treated THP-1 cells with IFN- α prior to a 24h infection with both promastigote and amastigote forms of the parasite. After 24h we washed and lysed the cells and used qRT-PCR of parasite gene 18S to determine approximate intracellular parasite number between the treated and untreated cells (Fig.19B). For this longer infection time we selected the lower concentration of IFN- α (2ng/ml) to pre-treat the THP-1 cells with, based on the slightly stronger observed STAT1 phosphorylation in Figure 18A. Pre-treatment of cells with IFN-α did not significantly affect intracellular parasite numbers compared to untreated cells during infection with either amastigotes or promastigotes (Fig.19B). Overall, we conclude that the presence of STING or STING-induced type I interferons does not enhance initial infection or growth of *Leishmania* parasites within macrophages *in vitro*.

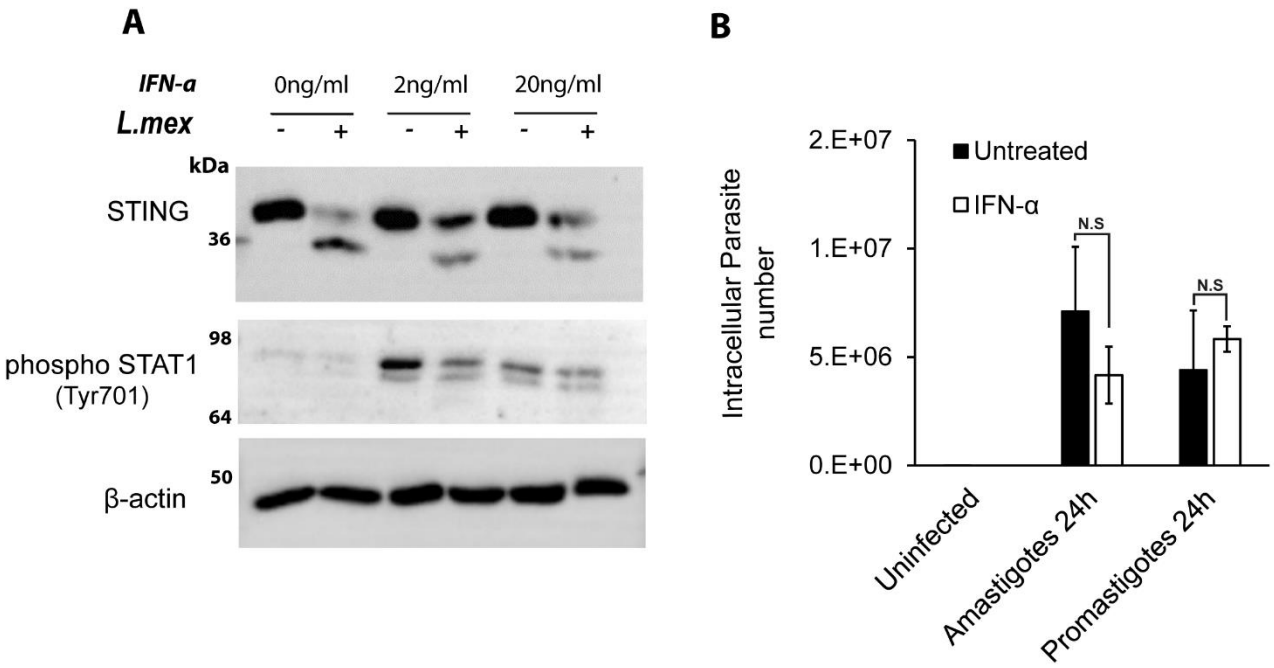


Figure 19: Interferon-α pre-treatment of THP-1 cells does not significantly affect *L.mexicana* growth

(A) WT THP-1 cells were pre-treated with either 2ng/ml or 20ng/ml IFN- α for 2h before infection with *L.mexicana* promastigotes at a MOI of 10 for 6h. Cells were then lysed for western blot analysis of protein expression. **(B)** WT THP-1 cells pre-treated with 2ng/ml IFN- α for 2h, and then infected with the amastigote or promastigote form of *Leishmania* parasites at a MOI of 10 for 24 hours. Cells were then washed and lysed for qRT-PCR analysis of mRNA, where the estimated intracellular parasite number present in cells was determined using a standard curve of known infection numbers. Error bars indicate standard deviations. N.S. = non-significant. Data based on two experiments.

4.8 Chapter Discussion

In this chapter we have explored the impact of the early *L.mexicana* infection on PRR signalling pathways within THP-1 cells.

We first demonstrated the active infection of *Leishmania mexicana* in these THP-1 cells by demonstrating cleavage of p65 - a known virulence activity of the parasite (Fig.7). The cleavage of p65 by GP63 was first described by Gregory *et al,* in a wide variety of *Leishmania* species infected macrophages, including *L.mexicana*. (Gregory et al., 2008). This cleavage resulted in the creation of a novel smaller subunit termed p35 (Gregory et al., 2008b), that we have also observed (Fig.7B). An additional *Leishmania* virulence factor CP3 is also known to degrade p65 and may also be contributing to p65 loss in these experiments. However, CP3 completely degrades p65, while GP63 cleaves it to the smaller p35 subunit (Cameron et al., 2004; Gregory et al., 2008b). Identification of p35 by western blot (Fig.7B) suggests that at least some of the degradation of p65 must be due to GP63.

In our experiments, we observed a total loss of total p65, but only when a ratio of 10:1 parasite to cells or more was used for the infection. It should be noted that our earliest infection time point was 2h, but cleavage of p65 has been observed within infected cells as early as 1h after infection (Gregory et al., 2008b).

p65 is one of five constituents that make up the NF-κB transcription factor family. p65 and another component, p50 form the most common heterodimer in the NF-κB signalling pathway present in most cell types (Bassères and Baldwin, 2006; Napetschnig and Wu, 2013). p65 (RelA) contains C-terminal transcriptional activation domains (TADs), allowing it to target gene expression (Schmitz and Baeuerle, 1991). The other half of the heterodimer p50, does not contain TADs and a p50 homodimer will instead repress transcription without p65 (Driessler et al., 2004; Zhong et al., 2002). This makes the downregulation of p65 by *Leishmania* an effective target to prevent a robust inflammatory

gene response being induced, and something that we have also demonstrated in Figure 8. Here we have shown the downregulation of the cytokine response to LPS within infected cells. LPS is a ligand of the PRR TLR4, and activation of signalling cascades downstream of the TLRs is well known to activate the NF-κB transcription factor.

As noted in Chapter 1, the TLRs are the most well studied group of PRRs and TLR2, TLR3, TLR 4 and TLR 9 have all been implicated in the detection of *Leishmania*. However, the lack of studies on the intracellular PRRs which were discovered more recently, including cytosolic DNA sensors and the RLRs led us to also investigate the impact of *Leishmania* to the normal response to dsDNA and dsRNA. Here we discovered that instead of inhibiting the response to dsDNA, like the LPS response, *L.mexicana* infection appears to enhance it (Fig.9).

This enhancement is particularly interesting as *Leishmania* infection alone does not appear to strongly induce the DNA sensing pathway at early time points post infection. IFN-β and CXCL10 are only induced in the infected cells after HT-DNA activation of the DNA sensing pathway. It could be that a longer infection period is necessary to induce the type I interferon response with just *Leishmania* alone, as we observe an increase in CXCL10 expression 24h post infection (Fig.5E).

Genomic DNA from other protozoan parasites is able to activate the cGAS-STING pathway, including *Plasmodium yoelii* (Du et al., 2022), *Plasmodium falciparum* (Gallego-Marin et al., 2018; Sharma et al., 2011) *Trypanosoma cruzi* (Vieira et al., 2021) and *Toxoplasma gondii* (Wang et al., 2019). As cytosolic DNA is sensed in a sequence-independent manner, it can be assumed that *Leishmania* DNA is equally immunostimulatory if it gains access to the cytosol of infected cells.

The increase in IFN-β response after dsDNA stimulation could also suggest that rather than *Leishmania* being detected directly by a DNA sensor and invoking a strong response, it is instead able to enhance or prime DNA sensor activation. In this way, *Leishmania*

infection may synergise with other danger signals, for instance the presence of DNA from dying or damaged cells, to activate the innate immune system during infection.

In contrast to this enhancement of the DNA sensing response, we observed no impact on the response to poly(I:C) during infection (Fig.10). Interestingly, both the cytoplasmic DNA sensing pathway and RLR pathway share some downstream signalling components, such as TBK1 and IRF3, and both pathways induce the same set of cytokines and chemokines. If modulation of one of these downstream components by *Leishmania* was occurring to cause the enhancement observed, we might also expect the response to poly(I:C) to also be enhanced. As no enhancement of the response to poly(I:C) was observed, we can conclude that *L.mexicana* specifically enhances the DNA sensing response at the level of the DNA sensors or the adaptor protein STING.

Initial investigation of STING and other components of the DNA sensing pathway during *L.mexicana* infection using western blot analysis revealed a potential modification of STING. This was in the form of a faster migrating band, observed when endogenous STING was probed for, suggesting a smaller form of STING was present within infected cells.

This smaller STING was induced during infection with either promastigote or amastigotes. Promastigotes and amastigotes can modulate and interact with the host immune system in different ways. This has already been demonstrated to some degree by various studies investigating stage-dependent host invasion strategies (Abu-Dayyeh et al., 2010; Wenzel et al., 2012). However, the results here seem to suggest that whichever unknown parasite virulence factor interacts with the host cell to modulate STING, it is present within both lifecycle stages. The smaller STING band did not appear to significantly increase in intensity when increasing number of parasites were used in the infection. This contrasts with the degradation of p65 mediated by *Leishmania* GP63 (Fig.6C), suggesting that these two modifications have different modes of action during an infection.

The presence of a much fainter band for the smaller form of STING in our uninfected controls suggested that this was a naturally occurring form of STING, that infection was able to enhance. We used STING KO THP-1 cells to confirm that the lower band is a form of STING and not a parasite derived molecule that could be cross reacting with the STING antibody (Fig.13). *Leishmania* virulence factors can interact directly with host proteins to modify them, or they can activate host regulators that will also modify host proteins (Atayde et al., 2016; Gupta et al., 2022; Lambertz et al., 2012). As it appears that a similar form of STING can be produced without infection (albeit at low levels), we could speculate that perhaps infection is able to enhance a host mechanism responsible for forming the smaller STING under normal conditions.

Investigation into the cytokine response to dsDNA during infection in both WT and STING KO THP-1 cells also revealed that the presence of STING is critical for the DNA response, and it's up-regulation during *L.mexicana* infection (Fig.14,15). However we still observe a small, but significant increase in the production of some cytokines and chemokines when STING KO cells are stimulated with DNA and infected with *L.mexicana*. This suggests that there might be additional, STING-independent pathways which can mediate a much more minor DNA sensing response in the absence of STING. One such STING independent DNA sensing pathway (SIDSP) has been described recently by (Burleigh et al., 2020) and involves sensor DNA-dependent protein kinase (DNA-PK). This pathway has been exclusively found to be active in human cells and not mice.

This could suggest that the up-regulation induced by infection is not restricted to STING-dependent DNA sensing pathways and other SIDSPs may also enhanced and affected by *Leishmania* infection, although these pathways contribute a much more minor response than the STING-dependent pathway in macrophages. If other pathways may also contribute to the enhanced response to dsDNA, it is possible that these are two distinct of activities of *Leishmania* infection. To identify the origin of this residual DNA sensing response, additional experiments utilising KO cells of different DNA signalling pathway

components or inhibitors of these components could be conducted. For example, if the residual enhancement is also still seen in cGAS KO THP-1 cells we could determine that this alternative pathway is downstream of a different dsDNA sensor. A panel of inhibitors could achieve similar results by inhibiting different DNA sensors and components until the residual signalling observed in STING KO cells is also lost.

To investigate STING and downstream signalling further we looked at the activation of different components of the DNA sensing pathway when combining a 4h dsDNA stimulation with *L.mexicana* infection in THP-1 cells (Figs 16,17). STING, IRF3 and TBK1 phosphorylation all appear to be enhanced after dsDNA stimulation during a 2h-6h *L.mexicana* infection compared to uninfected cells (Fig.16), further demonstrating that the enhancement of the DNA sensing pathway occurs at or above the level of STING, and not for instance at the level of transcription factor activation, as is the case for NF-kB. The enhancement of STING signalling does not seem to occur during later timepoints, and thus it might be linked to early immune modulatory activities deployed by

We next investigated if the changes to STING and downstream signalling during infection would be beneficial for parasite infectivity and replication. *Leishmania* mediated modification and degradation of host proteins are usually performed to the benefit the parasite. Thus, we speculate that enhanced activation of STING may benefit *Leishmania* infection or growth, or dissemination in the tissue.

The hypothesis that a STING-dependent type I interferon response could be beneficial to *Leishmania* proliferation may seem counter-intuitive, as a strong type I interferon response is usually associated with pathogen elimination. However, while the type I interferons are key effectors of the anti-viral response, their role in infections with other pathogens such as intracellular bacteria and parasites is more complicated. As mentioned in the previous chapter, type I Interferons have been shown to play both a host protective and detrimental

role during *Leishmania* infection that varies depending on the disease and causative species and timing of IFN induction (Dias et al., 2019; Diefenbach et al., 1998; Khouri et al., 2009; Rossi et al., 2017). Excessive type I interferon production may also to cause tissue damage and inflammation that may help dissemination of *Leishmania* parasites and cutaneous lesion development (Davidson et al., 2015)

One of the earliest studies linking *Leishmania* and type I interferons was in 1970 when Herman and Baron showed that poly(I:C) treatment prior to *L.donovani* infection triggered type I interferon production that helped in the control of parasite burden (Herman and Baron, 1970) *in vivo*. The neutralization of type I interferons during *L.major* infection in mice caused an increase in host susceptibility and increased parasite replication (Diefenbach et al., 1998). The role of type I interferons has also been studied *in vitro*, where again during *L.major* infection type I interferons activated macrophages for parasite elimination (Diefenbach et al., 1998; Mattner et al., 2000).

In contrast to these host-protective roles, another study demonstarted that IFN-β dose-dependently increases parasite burden in *L. amazonensis* and *L.braziliensis* infected human macrophages through a superoxide dependent mechanism (Khouri et al., 2009). Superoxide dependent killing of *Leishmania* has shown to be a key control mechanism early after parasite cell entry (Channon et al., 1984; Gantt et al., 2001) along with other ROS. IFN-β was able to induce superoxide dismutase (SOD) which regulates superoxide levels within the cell and promotes parasite survival. Similarly, the addition of IFN-α to *L. amazonensis* infected macrophages dramatically increased the parasite burden, again linked to an increase in SOD (de Carvalho Vivarini et al., 2011). *L.donovani* can also induce host IFN-β production that aids in parasite survival and intracellular growth (Dias et al., 2019). This relatively recent finding seems to be conflict with previous experiments that suggest type I IFNs contribute to the control of *L.donovani* parasite burden (Herman and Baron, 1970).

It is also known that type-I IFNs can negatively regulate IL-12 and IFNɣ production in macrophages (Cousens et al., 1997). As IL-12 and IFNɣ are known to be important for parasite control (Dayakar et al., 2019; Engwerda et al., 1998; Heinzel et al., 1991; Scott, 1991), this could be an alternative or additional way in which increased type I interferon production in macrophages may benefit the intracellular survival of the parasite as an immune evasion mechanism.

When comparing *L.mexicana* infection within STING KO and WT THP-1 cells we have observed similar numbers of infected cells at 6h, 24h and 48h (Fig.18A) suggesting that lack of STING does not prevent successful infection of macrophages, and may even lead to an increased number of parasites per cell at early time points. If STING has an effect on parasite entry, survival or growth, it may be independent of type I interferon induction, as pre-treatment with IFN- α did not affect infection levels (Fig.19).

In vitro the protective role of type I interferons may be dependent on when they are introduced to the cells. Interferon addition at the same time as *Leishmania* results in the most host protection (Mattner et al., 2000) which is not something we tested. It is clear that further investigation will be required to fully elucidate what type of modulatory affect type I interferons have in *L.mexicana* infection.

The association of STING and *Leishmania* are scarce in the literature, although one proteomics paper has recently reported an increase in levels of STING after infection (Negrão et al., 2019). In this study, they evaluated *Leishmania*-specific protein modulation in macrophages and found STING was the most upregulated protein after *Leishmania major* and *Leishmania amozenesis* infections in BALB/c mice and *in vitro* assays using the J774 macrophage cell line. Additionally, they utilised Ingenuity Pathways Analysis System (IPA) upstream analysis that predicted the activation of STING within infected macrophages and cutaneous tissue samples during infection. It should be kept in mind

that the upregulation of STING discovered by Negrão et al, was in cells infected by different *Leishmania* species to *L.mexicana*. It could be that these species have a different effect on STING. Nevertheless, this is confirmation that STING can be affected by other *Leishmania* infections, either in abundance or in activity, just perhaps in species-specific ways. An interesting future experiment would be to see if other species of *Leishmania* also cause induction of smaller STING, especially the two species used by Negrão et al, in which STING was upregulated.

A more recent study by Yilmaz et al (2022) has associated activation of the cGAS-STING pathway with increasing parasite burden within THP-1 cells. This study has several similarities to the work carried out in this thesis, although there are key differences. One central statement of Yilmaz et al (2022) is that the activation of cytosolic DNA sensors can aid parasite virulence. STING and TBK1 knockout THP-1 cell lines were found to be more resistant to *Leishmania* infection than the WT, suggesting that *Leishmania* parasites may exploit the cGAS-STING-TBK1 signalling pathway for their virulence.

The main similarities between this study and our work is the premise, methodology and that after stimulation of infected and differentiated THP-1 cells with DNA, there is a robust induction of the interferon response. This appears to confirm that *Leishmania* does not inhibit this pathway. There are also several differences between our work and this study, both in terms of methodology and results. Firstly it should be noted that different species of *Leishmania* were used (*L.mexicana* here and *L.major* by Yilmaz et al) and it is well known that that *Leishmania* can have species specific differences in virulence and infection kinetics.

Another interesting difference is that Yilmaz et al use *Leishmania* derived kDNA to stimulate already infected cells whereas we used double stranded HT-DNA. Our results have suggested that *Leishmania* infection alone does not activate the DNA sensing pathway strongly (Fig.9) which would mean kDNA derived from the infecting *Leishmania*

may not be able to activate cGAS. Yilmaz et al also stimulate already infected cells with additional *Leishmania* kDNA suggesting they also do not see an induction of this pathway with infection alone. It would be interesting to investigate if the results they see could also be achieved with stimulation using another DNA source such as the HT-DNA we used.

Another major difference is that this study also determines that activation of the cGAS-STING pathway is beneficial for the parasite as it increases parasite loads within THP-1 cells. While we did not explore differences in parasite loads between HT-DNA stimulated and unstimulated infected cells, our preliminary results show no difference in parasite load when pre-treating THP-1 cells with IFN-α (Fig.19). Yilmaz et al performed a similar experiment in that they pre-treated THP-1 cells with recombinant IFN- β 8 hours prior to infection and they observed an enhanced parasite infectivity and parasite load. Further repeats and more extensive experiments using the longer pre-treatment time used in this study (8h vs the 2h we used) could be carried out to further explore this finding.

Finally, Yilmaz et al observed that parasite proliferation was significantly hindered in cGAS, STING, and TBK-1 KO THP-1 macrophages when compared to wild type cells. When we investigated parasite infectivity and proliferation in STING KO cells, we saw no difference in infectivity but a slight increase in parasite proliferation (Fig.18). We did not however investigate cGAS and TBK1 KO cells. Overall, this study confirms the importance of the cGAS-STING pathway in *Leishmania* infection even if there are significant differences in how the pathway affects infection compared to our results.

Based on preliminary results we have shown that it appears that neither STING nor the production of type I IFNs are particularly beneficial for parasite infection in THP-1 cells *in vitro*. Further investigation will be necessary before we can say that STING and type I IFN production is host protective instead. In addition to inducing an interferon response, STING can also induce autophagy and cell death. Much like the impact of type I IFNs on infection, studies investigating host cell autophagy on parasite infection vary between favouring the

infection and promoting *Leishmania* clearance, often in a species specific way (Veras et al., 2019). Induction of autophagy in mouse macrophages can favour both *L. major* and *L. amazonensis* intracellular viability *in vitro*. *L.major* viability is increased much more than that of *L. amazonensis*, suggesting species differences (Dias et al., 2018). Further experiments should be planned to investigate the impact of both STING-dependent IFN production and autophagy induction during *Leishmania mexicana* infection. Determining which, if any, of these STING-dependent responses are host-protective and whether they are inhibited or subverted for the benefit of parasite infection will increase understanding of parasite virulence.

Chapter 5: Investigating the interaction between *L.mexicana* and STING during early infection

5.1 Chapter Introduction

In the previous chapter we have consistently demonstrated via protein analysis that STING is modified to a smaller form during *L.mexicana* infection. This change in size could be due to part of the protein being cleaved or could represent the expression of a smaller form of STING such as a splice variant. Cleavage of STING by an intracellular pathogen has been observed before by several viruses. For example, the Dengue virus NS2B3 protease complex cleaves STING to evade the type I interferon response and aid immune evasion (Aguirre et al., 2012).

Several splice variants of STING have also been described including MRP, EV-less Isoforms 1,2, 3 and STING-β (Chen et al., 2014; Liang et al., 2021; Rodríguez-García et al., 2018; Wang et al., 2018). All these STING variants are truncated forms of STING that have lost protein domains that can be key to the activity of STING. Due to this, many of these splice variants have a role in negatively regulating cGAS-STING signalling and the IFN response (Liang et al., 2021). In contrast, the smaller form of STING we have observed after *L.mexicana* infection is still able to induce downstream signalling and an enhanced IFN response to dsDNA stimulation. It is also possible that human STING splice variants can positively regulate cGAS-STING signalling, as STING splice variants tSTING-mini and tSTING-F found in Chinese tree shrews *(Tupaia*) and have a role positively regulating the IFN response have been described (Xu et al., 2020).

5.2 STING reconstitution reveals a potential cleavage site

For further analysis of how *Leishmania* interacts with STING during infection we reconstituted STING KO THP-1 cells with STING tagged with a HA tag at its C terminus via lentiviral transduction. This could allow us to look at potential protein modifications to STING rather than alternative splicing that may cause the size change. This is possible as there are no introns and exons in the plasmid, just the whole STING cDNA.

STING KO THP-1 cells were firstly treated with polybrene to increase the efficiency of viral uptake into the cells. After this treatment the lentivirus was added to the cell suspension and immediately centrifuged to increase transduction by centrifugal enhancement. The plasmid used to transfer STING also contained puromycin resistance genes to allow selection of successfully transfected cells. Transfected cells were treated with puromycin over several days to eliminate those which were not successfully transfected. This process is shown in Figure 20A.

The remaining cells were then allowed to recover before treatment with doxycycline to induce transgene expression of HA-tagged STING. These treated cells, along with untreated control cells were then lysed for western blot analysis. Successful re-constitution of STING was confirmed by immunoblotting using anti-STING and anti-HA tag antibodies (Fig.20B). The reconstitution was successful as only the doxycycline treated cells expressed both STING and HA (Fig.20B).

We next infected doxycycline treated cells with *L.mexicana* promastigotes for 6h. After the infection cells were lysed for further western blot analysis of STING protein (Fig 20C). This revealed again the presence of the smaller STING band. However, in a new observation immunoblotting for the HA tag revealed that when these cells were infected with *L.mexicana* the HA tag is lost.

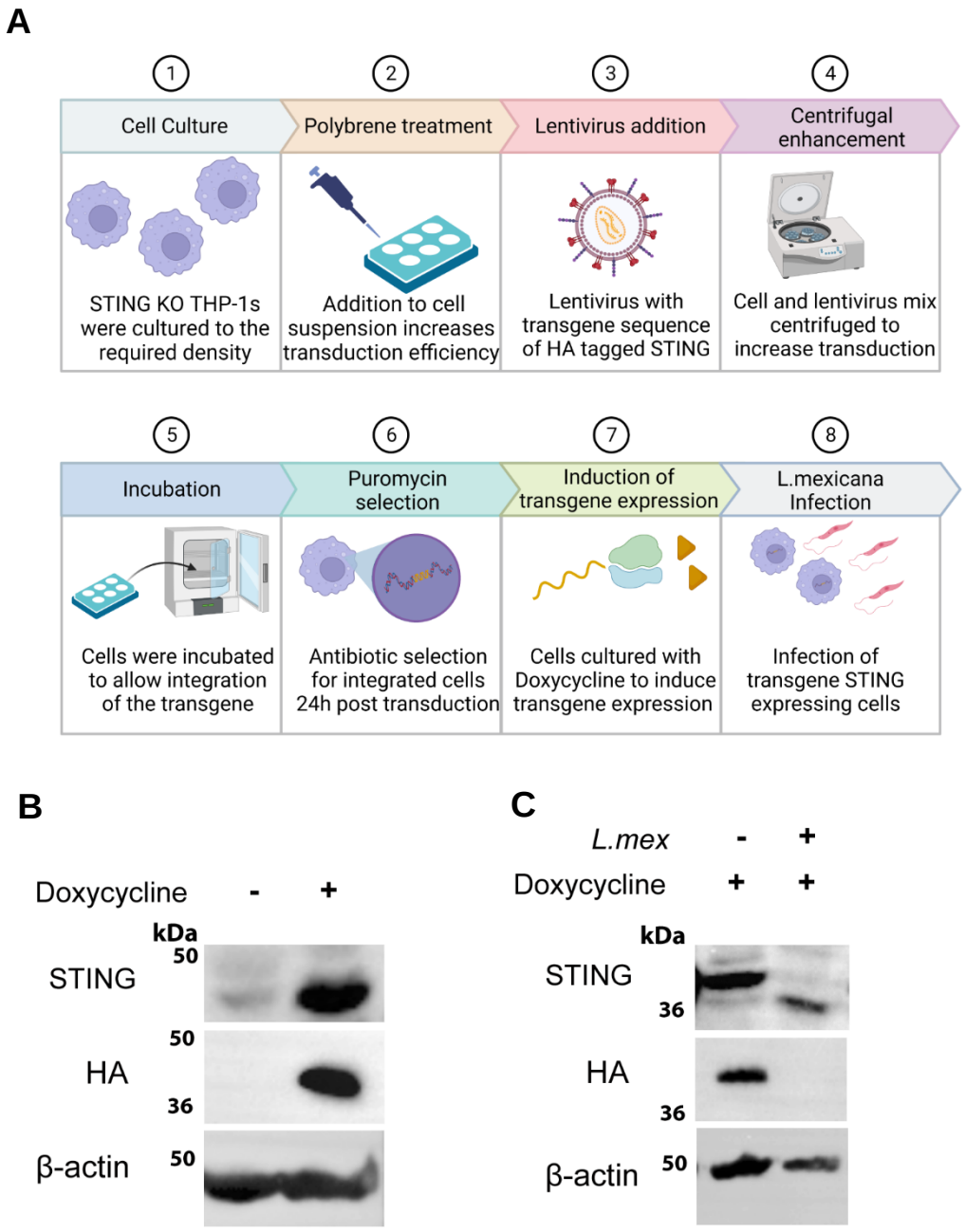


Figure 20: Reconstitution of STING reveals a potential site of modification by *L.mexicana* due to loss of C-terminal HA tag

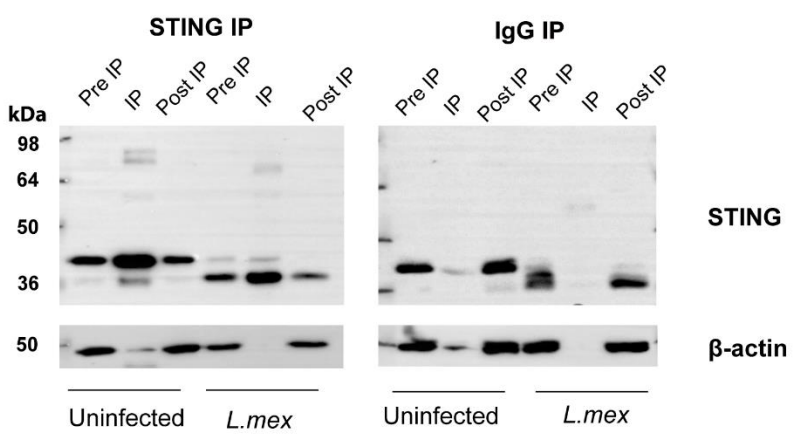
(A) Workflow of lentivirus transduction of STING KO THP-1 cells. Diagram created in Biorender **(B)** STING KO THP-1 cells were transduced with STING tagged at the C-terminus with HA via a lentiviral vector. Transgene expression was induced by culturing cells for 24h in the presence of 1µg/mL doxycycline. Cells were lysed for western blot analysis. **(C)** Reconstituted STING-HA THP-1 cells were infected with *L.mexicana* promastigotes at a MOI of 10 for 6 hours before lysis for western blot analysis of protein expression. Data is representative of at least 3 experiments.

This suggests that the smaller form of STING must be cleaved somewhere on its C terminal end so that the C-terminally attached HA tag would also be lost when this happened. Additionally, this suggested that the smaller form of STING was not being produced in response to infection like a splice variant could, but instead formed by an active cleavage event.

Further analysis of endogenous STING by mass spectrometry (MS) was performed to examine any peptide changes in STING during infection. We performed immunoprecipitations (IPs) on the lysates of uninfected and *Leishmania* infected cells. Beads coupled with anti-STING antibody, or rabbit IgG for a control, were incubated with the cell lysates overnight. After incubation the beads were washed, and bound proteins eluted into sample buffer. Confirmation of successful STING immunoprecipitation was carried out by western blot analysis of the IP sample in addition to samples of the pre- and post- IP. While there still is some STING found in the post-IP sample (Fig.21A), the IP has been successful and much of STING has been immunoprecipitated from the cell lysate during the process. We can also observe that much of STING immunoprecipitated from the infected sample is STING in its smaller cleaved form. The lack of STING in the IgG IP also confirms that non-specific binding of STING to the beads did not occur.

The remaining STING IP sample from both uninfected and infected cells was run out on a clean pre-cast polyacrylamide gel and stained with Coomassie dye. Gel slices of the sample were then excised and sent for MS analysis.

A



B

Peptide Sequences	a.a. localisation	Present in Uninfected	Present in Infected
GLAPAEISAVCEK	138-150	Yes	Yes
GNFNVAHGLAWSYYIGYLR	151-169	Yes	Yes
LILPELQAR	170-178	Yes	Yes
TYNQHYNNLLR	180-191	Yes	No
VYSNSIYELLENGQR	239-253	Yes	Yes
LIAYQEPADDSSFSLSQEVLR	311-331	Yes	Yes
HLRQEEKEEVTVGSLK	332-347	Yes	Yes
QEEKEEVTVGSLK	335-347	Yes	Yes
TSAVPSTSTMSQEPELLISGMEKPLPLR	348-375	Yes	No

C

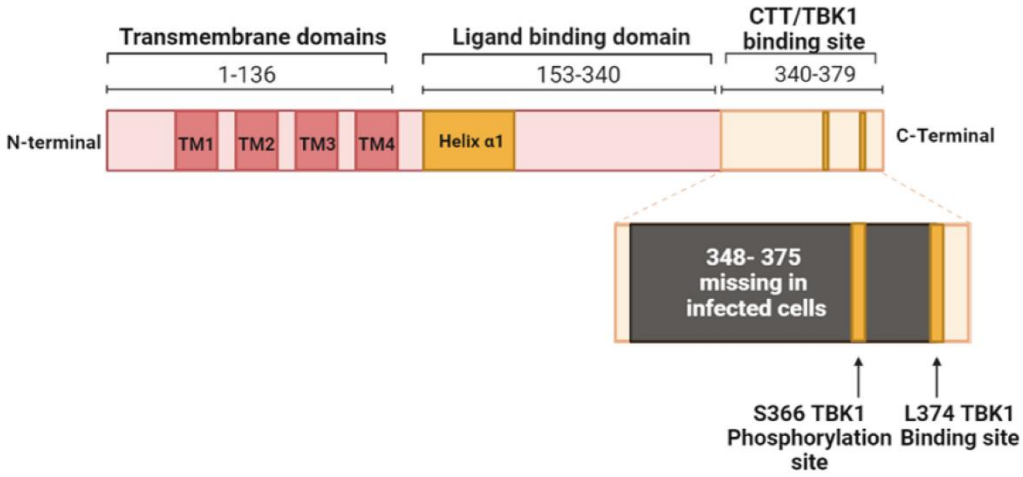


Figure 21: Mass Spectrometry analysis shows loss of C terminal STING peptide during *L.mexicana* infection

(A) WT THP-1 cells were infected with *L.mexicana* at a MOI of 10 for 6 hours before lysis for Immunoprecipitation prior to mass spectrometry analysis. Lysates were immunoprecipitated with anti-STING antibody or anti-IgG antibody for the control. Immunoprecipitation of STING was confirmed by western blot using samples taken pre- and post- immunoprecipitation. **(B)** Data from mass spectrometry analysis indicate that a 27 amino acid STING C-terminal peptide is lost during infection. A smaller peptide from the LBD binding domain is also no longer detectable in infected cells. **(C)** Schematic of STING protein showing key features and the location of the lost C-terminal peptide, with almost all the TBK-1 binding site missing.

Upon receiving the mass spectrometry data, we looked at any changes to the STING peptides that could be detected in the *Leishmania* infected sample compared to the uninfected. Figure 21B details a list of all the STING peptides that could be identified and if they were present in the uninfected and infected samples. Within the infected samples two STING peptides were not present. The most significant of these is 27 amino acid sequence localised at almost the very end of the STING c- terminus (Fig 21C). This would support what we observed in Figure 20C, when the c-terminal HA tag of reconstituted STING was lost during infection, indicating the cleavage site to be located around this area.

This schematic of STING in Figure 21C also shows in more detail the location and relevance of the longer missing c-terminal peptide. This peptide accounts for the majority of the TBK1 binding domain of STING, which is an important site for its activation. The cleavage and targeting of this part of STING would make sense for the activity of a *Leishmania* virulence factor if inactivation was its aim, as occurs with many of the other host proteins that *Leishmania* targets such as p65 and AP-1. The other missing peptide is 11 amino acids in length and located at the LBD binding domain of STING, towards the centre of the protein on the STING schematic.

5.3 Transfected *Leishmania* GP63 does not cleave STING

GP63 is a is a zinc-dependent metalloprotease found on the cell surface of both the promastigote and amastigote form of *Leishmania*. GP63 can cleave a wide range of proteins, a quality which allows it to disrupt multiple signalling pathways and mechanisms at once (Hallé et al., 2009; Olivier et al., 2012). To investigate if the degradation and cleavage of STING was due to the activity of the *Leishmania* virulence factor GP63, we cloned GP63 from genomic *L.mexicana* DNA into a plasmid containing an N-terminal HA tag (Fig.22). Amplification of the GP63 gene by PCR from extracted parasite DNA was

first carried out and confirmed by visualisation of a band on a DNA gel at the correct size for GP63 (Fig.22A). Ligation of the GP63 gene into the chosen vector in frame with the HA tag was carried out after digestion of both insert and vector by Kpn I and EcoRI restriction enzymes (Fig.22B). The success of this ligation and subsequent plasmid amplification was confirmed by restriction digest analysis of the plasmid, and visualization of the product by DNA gel, where bands corresponding to the size of empty vector and insert were observed (Fig.22C).

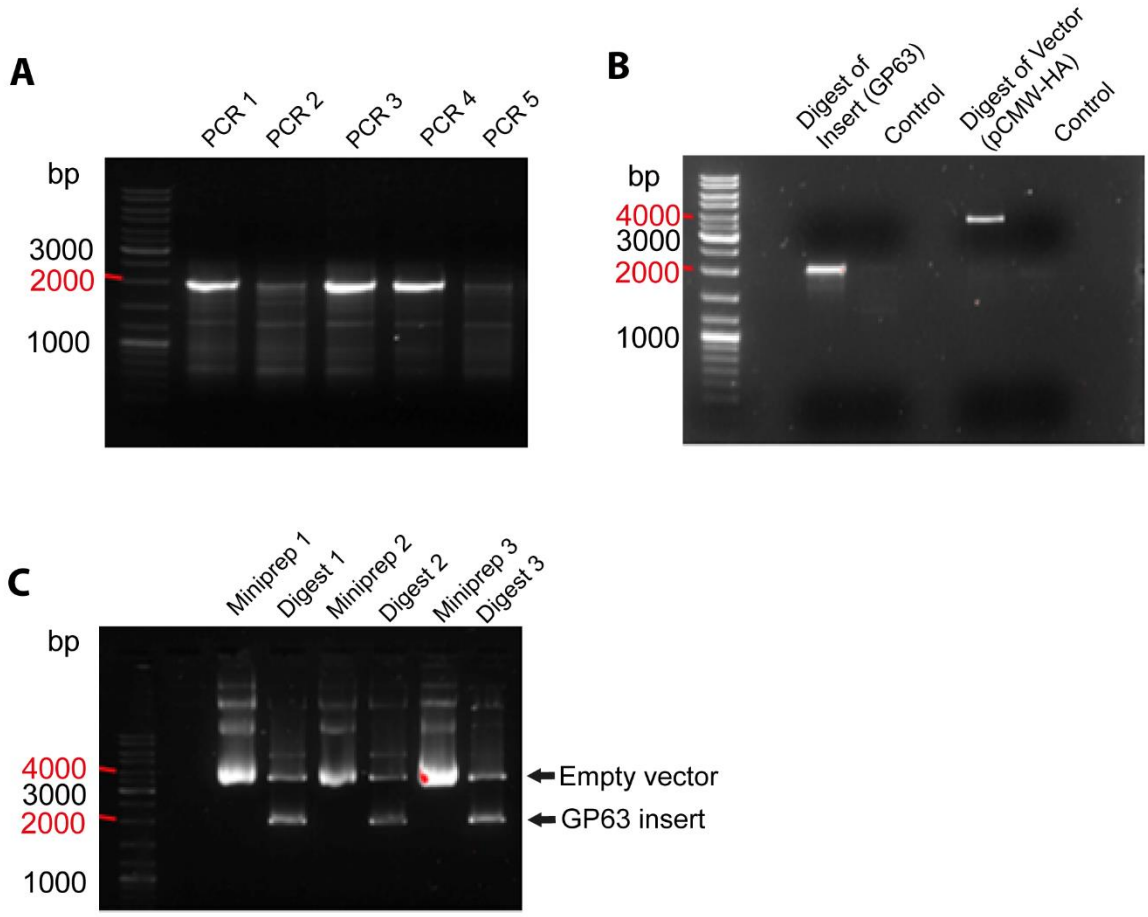


Figure 22: Cloning of *Leishmania* mexicana GP63 into an expression vector

(A) PCR products from the amplification of the GP63 gene from *L.mexicana* genomic DNA. The PCR product from 5 reactions is seen here as a PCR annealing temperature titration was used to optimise PCR cycling conditions. (Different annealing temperatures: 1) 52 ºC 2) 53 ºC, 3) 55.5 ºC, 4) 56.5 ºC, 5) 58 ºC) Amplification was confirmed by visualisation of a band corresponding to 1803bp- the size of GP63. **(B)** After purification of the PCR product, both the insert and chosen vector (pCMV-HA) were digested using restriction enzymes Kpn I and EcoR I prior to ligation and transformation. Control digests were performed without the DNA template. **(C)** After plasmid amplification, restriction digest analysis of minipreps indicated the presence of a product of around 1803bp, matching the size of the GP63 insert. The size of the empty vector is 3800bp.

The plasmid was then transfected into in HEK293T cells, a human cell line derived originally from embryonic kidney cells with a high propensity for transfection. This allowed expression of GP63 without the confounding effects of other *Leishmania* proteins and virulence factors and meant we could investigate its ability to cleave STING and other host proteins.

Successful GP63 expression was confirmed in the transfected cells by detection of the HA tag around the expected size of GP63 (Fig.23A). HA-tagged STING was also transfected as a positive control. Functional protease activity of transfected GP63 was also confirmed by protein analysis of endogenous p65, which has previously shown to be degraded by GP63 both in the literature and in our own previous experiments (Fig.) (Abu-Dayyeh et al., 2010; Gregory et al., 2008b). Levels of total p65 were almost completely ablated in cells with transfected GP63, but not in the mock transfected or empty vector-transfected control sample (Fig. 23B). This suggests that it is the presence of transfected GP63 alone that caused a decrease in p65 levels, and that the HA tag did not interfere with the protease activity of GP63.

We then co-transfected the HA-GP63 construct with a plasmid containing STING-FLAG (Fig.24), HEK293 T cells do not express detectable levels of endogenous STING, and hence do not have a functional DNA sensing pathway. Successful plasmid transfection was again confirmed with western blot analysis of both HA and FLAG tags (Fig.24). No smaller STING or loss of the FLAG tag was observed in cells transfected with both STING and GP63. This suggests that it is not GP63 alone that is responsible for this cleavage. As cleavage of p65 does occur with transfected GP63 alone, we can tentatively postulate that GP63 is not responsible for STING cleavage.

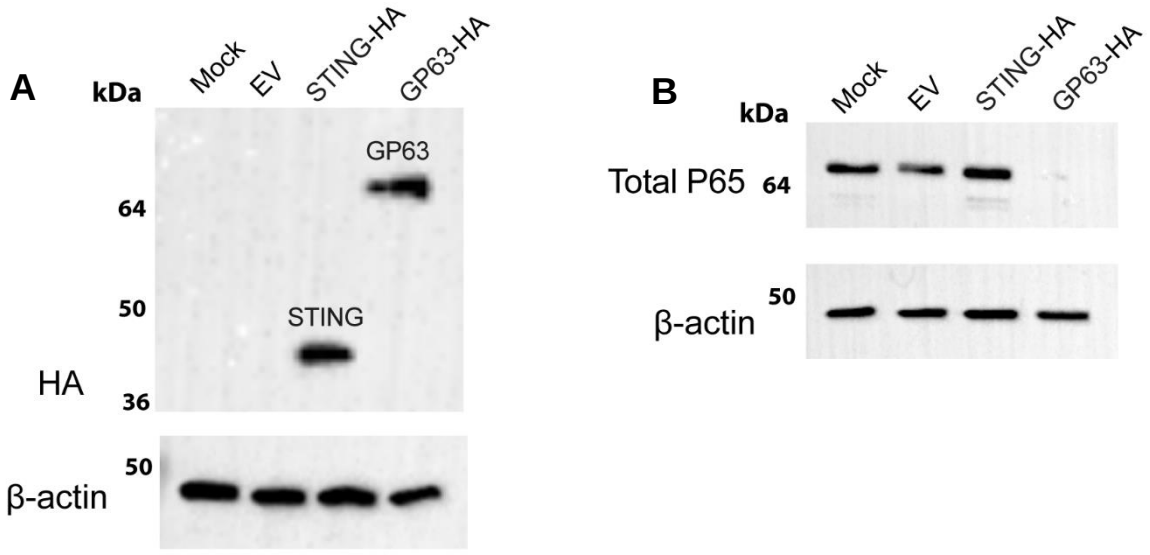


Figure 23*:* Transfection of *Leishmania* GP63 into HEK293 T Cells

HEK293 T cells were transfected for 24h with plasmid containing HA-tagged GP63. Transfection of empty vector was used as a negative control and transfection of plasmid containing HA tagged STING was used as a positive control. Cells were then lysed for western blot analysis of protein expression. **(A)** Detection of HA was used to confirm successful transfection of GP63-HA. **(B)** Confirmation of GP63 protease activity in HEK293 T was shown by cleavage of endogenous p65.

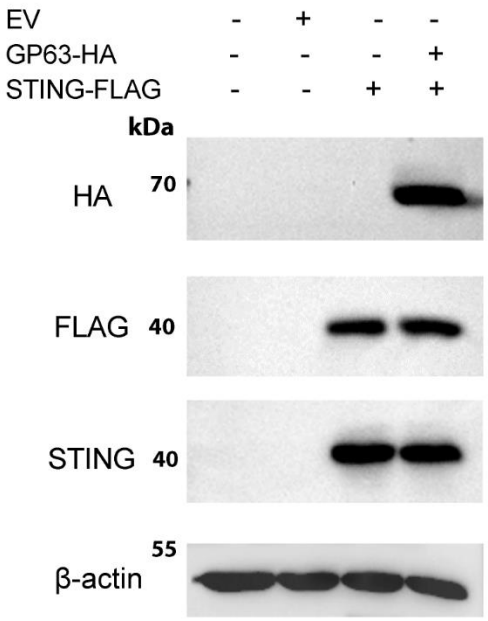


Figure 24: Co-transfection of *Leishmania* derived GP63 and STING

HEK293T cells were co-transfected with *Leishmania* derived GP63 tagged with HA and STING-FLAG to observe if changes to STING that are observed with whole *Leishmania* infection occur. Transfection of empty vector was used as a negative control. Plasmids were transfected using GeneJuice and cells lysed with SDS lysis buffer after 24h. Lysates were then analysed for protein expression by western blot.

5.4 The proteasome may be required for the formation of the slower migrating STING band

Leishmania virulence factors are well known to be able to initiate the degradation and modulation of many host proteins. They can do this in two main ways: direct cleavage by a *Leishmania* virulence factor or the manipulation of existing host regulatory pathways to initiate degradation by the host cell itself.

Many proteases are employed by *Leishmania* as virulence factors, the most well-known being the metalloprotease GP63 and the cysteine proteases that can cleave host JNK and Extracellular Signal Regulated Kinases (ERK) signalling proteins (Cameron et al., 2004). *Leishmania* is also able to hijack the cellular degradation machinery and increase the host proteasome mediated degradation of transcription factor STAT1α (Forget et al., 2005). Clearly with the activity of its own many proteases, in addition to accessing host mechanisms of degradation and inactivation, *Leishmania* could cause the modulation of STING in many ways.

We decided to investigate if host pathways of degradation were responsible for the changes to STING. This was because the smaller STING can often be seen faintly in the uninfected control cells on our western blots, suggesting that this may be a naturally occurring form of STING formed through normal cellular processes. Therefore, instead of a direct cleavage by a *Leishmania* protease such as GP63, perhaps *Leishmania* is able to drive activation of this alternative form of STING through interaction with or subversion of host pathways.

To determine if a host degradation pathway is involved in the changes we have observed to STING, we pre-treated THP-1 cells with known autophagy inhibitor bafilomycin or the proteasome inhibitor MG132. After 1h pre-treatment with each inhibitor, cells were infected with *L.mexicana* promastigotes over a time course starting at 2h and continuing to 6h. We started at a 2h infection based on where we have first observed STING lower band

induction previously (Figs.11B,15). At each time point cells were lysed for western blot analysis of STING. Pre-treatment of cells with bafilomycin did not prevent the formation of the lower STING band (Fig.25). We did also not observe a depletion of normal sized STING simultaneously with the appearance of the lower band in this experiment, even in the untreated cells. We were therefore unable to determine if inhibition of the autophagy pathway would prevent this loss. However, we can conclude that production of smaller STING is not dependent on the activity of the autophagy pathway.

Pre-treatment of cells with MG132 prior to infection did prevent formation of the lower STING band. This suggests that host proteasome activity is needed for the generation of this smaller STING. Although the proteasome usually catalyses the complete hydrolysis of cell proteins into small peptides, in some cases proteins are instead subject to proteasome-mediated processing (Rape and Jentsch, 2004). In this process, proteins undergo only a partial degradation that is termed regulated ubiquitin/proteasome-dependent processing (RUP). This process can yield a protein of different biological activity and is essential for the function and regulation of certain transcription factors including NF-κB (Palombella et al., 1994). It is possible that *Leishmania* infection could cause STING to undergo RUP.

As expected, infection at all time points was associated with a loss of p65 in these cells. However, a slight rescue of p65 cleavage can be observed with MG132 treatment at the 2h and 4h timepoints. This is unexpected as the current understanding is that p65 cleavage is directly mediated by GP63 (Gregory et al., 2008b) or other *Leishmania* derived proteases such as CP3 (Cameron et al., 2004). As pre-treatment of MG132 only partially rescues p65 loss, it could be possible that multiple routes exist for p65 degradation after *Leishmania* infection, and proteasome mediated degradation may be one of these.

Protein levels of cGAS and IFI16, additional components of the DNA sensing pathway were also investigated in the western blot. An immediate striking observation from these

blots is the dramatic changes that occur in the protein size of cGAS and IFI16 during infection. Both cGAS and IFI16 appear to undergo a change of size that is induced during *L.mexicana* infection and can be observed by western blot (Figure 25). This change was confirmed by subsequent repeats of this experiment.

cGAS is dsDNA sensor that can detect dsDNA, after this occurs it produces cGAMP which then binds to STING to activate it. Here we can observe that the band for cGAS undergoes a size change from 62kD in uninfected cells to just over double that in the infected cells. Due to approximate doubling in size, it is possible that this band may represent the formation of a cGAS dimer. This size change can be observed from 2 hours post infection similar to the kinetics of the appearance of the STING lower band. However, unlike with STING there is no remaining band observed at the usual size for cGAS.

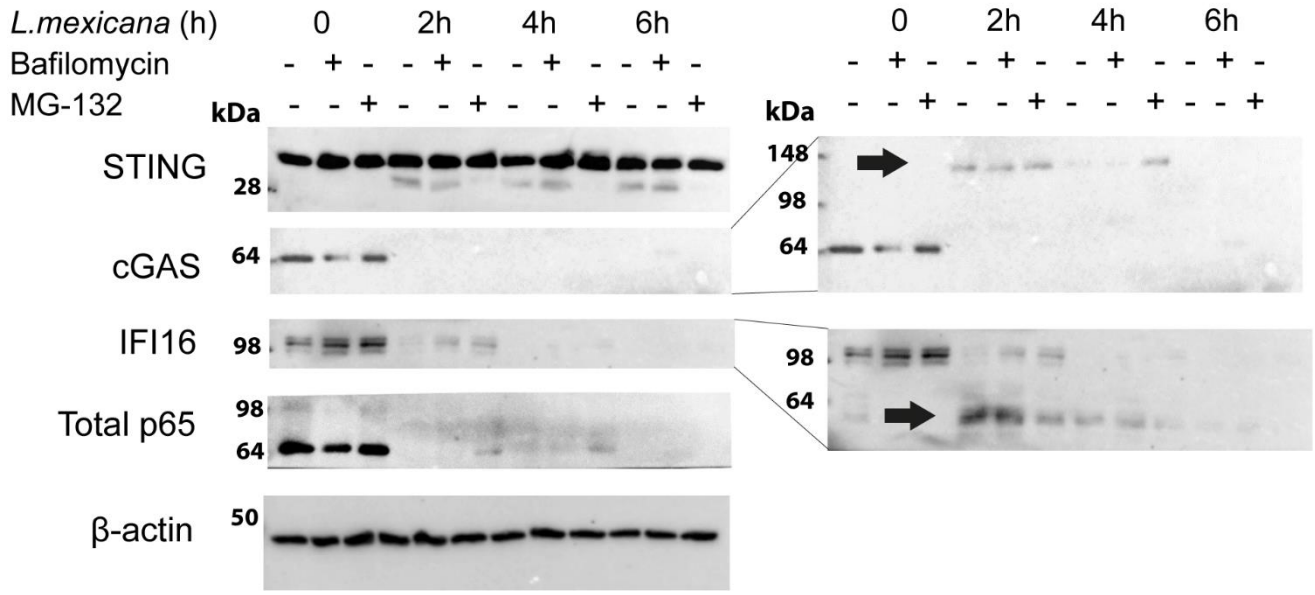


Figure 25: **The proteasome may be required for the appearance of a faster migrating STING band during *L.mexicana* infection**

WT THP1s were infected with *L.mexicana* at a MOI of 10 over 6 hours as indicated after a 1 hour pre-treatment of either 10μM MG132 or 50nM Bafilomycin. Cells were then lysed for western blot analysis of protein expression. Data are representative of at least three experiments.

The band for IFI16, another dsDNA sensor and promoter of STING signalling has a smaller size in the infected cells. The size changes from the normal 85-98kD range to just under 64kD. Unlike the change to cGAS this appears more gradual with some of the normal size IFI16 still being present at 2h. The change to IFI16 appears to be potentially prevented in MG132 treated cells. At the 4h timepoint, a faint band for IFI16 can still be seen at its normal size, which is not present in the untreated and bafilomycin treated cells. This perhaps could suggest that the cleavage of IFI16 is a mediated by the proteasome, similar to what may be happening to STING.

Despite the changes to cGAS and IFI16, we have already demonstrated that the DNA sensing pathway is not inhibited by *Leishmania* infection and even appears to be enhanced. This suggests that these changes to cGAS and IFI16 do not negatively affect their function, and it could be possible that these modifications are instead linked to the enhancement of the pathway. The possible cleavage event in IFI16 size does not change its localisation within infected cells (Fig.26). Confocal microscopy was performed using a primary antibody against IFI16 and a fluorescently labelled secondary antibody. A primary antibody against parasite tubulin was also used to visualise the *Leishmania* parasites in addition to nuclear stain DAPI. IFI16 is shown to be localised to the nucleus and predominantly nucleoli and this does not change upon infection.

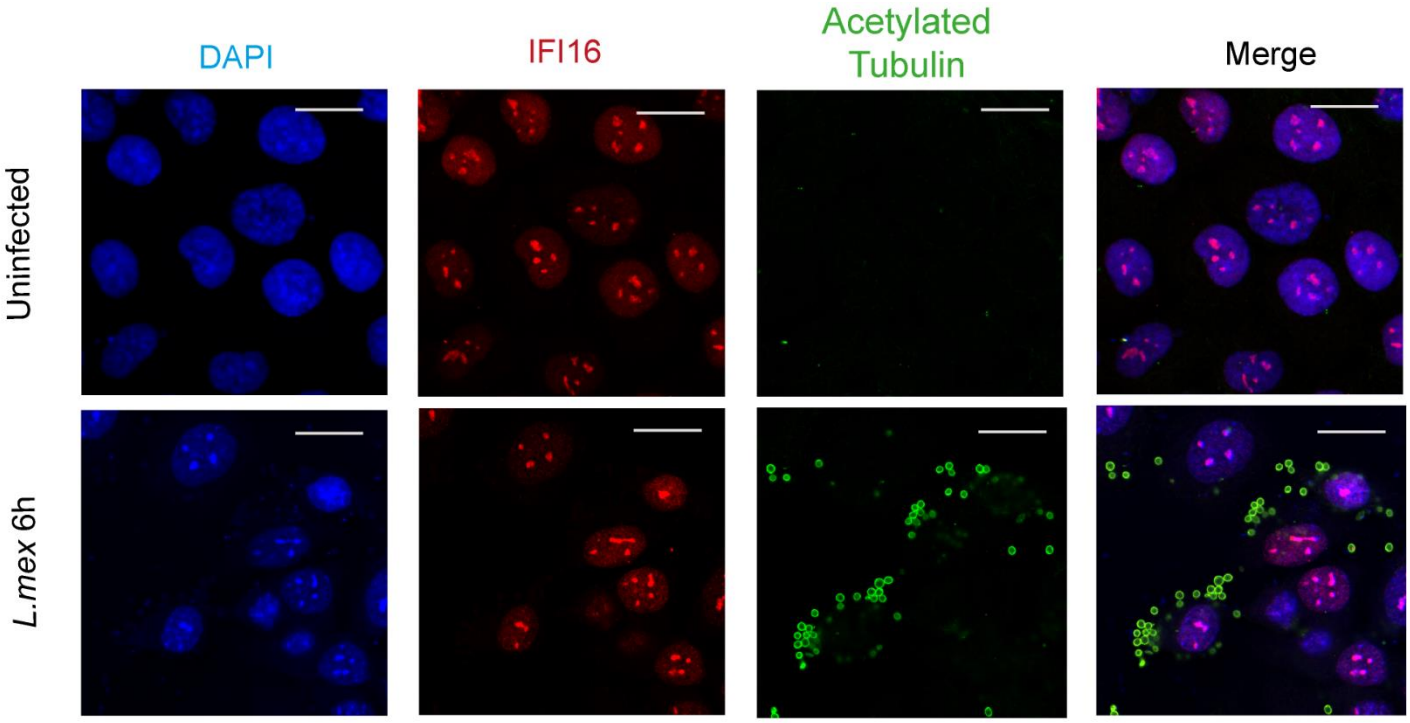


Figure 26: IFI16 localisation is unchanged in infected THP-1 cells

WT THP-1 cells were seeded onto coverslips, differentiated with PMA for 48h before infection with *L.mexicana* at a MOI of 10 for 6 hours. Cells were then fixed in methanol and stained for IFI16 (red), parasite tubulin (green) and DAPI nuclear stain (blue) and imaged by confocal microscopy. Scale bar = 20μm

5.5 Co-transfection of GP63 with cGAS or IFI16

We investigated whether GP63 was also able to have the same effect on the size of IFI16 and cGAS as infection by co-expressing HA-GP63 with FLAG-tagged IFI16 or cGAS (Fig.27). The levels of cGAS also appear unchanged when co-transfected with GP63, although there does seem to be slightly less FLAG tag. In the co-transfection of IFI16 and GP63 a slightly fainter band was seen for both FLAG and IFI16 compared to when IFI16 is transfected alone. This suggests that it could be starting to be cleaved by GP63 as is observed in *Leishmania* infection. Further experiments will be required to determine if IFI16 is cleaved by GP63.

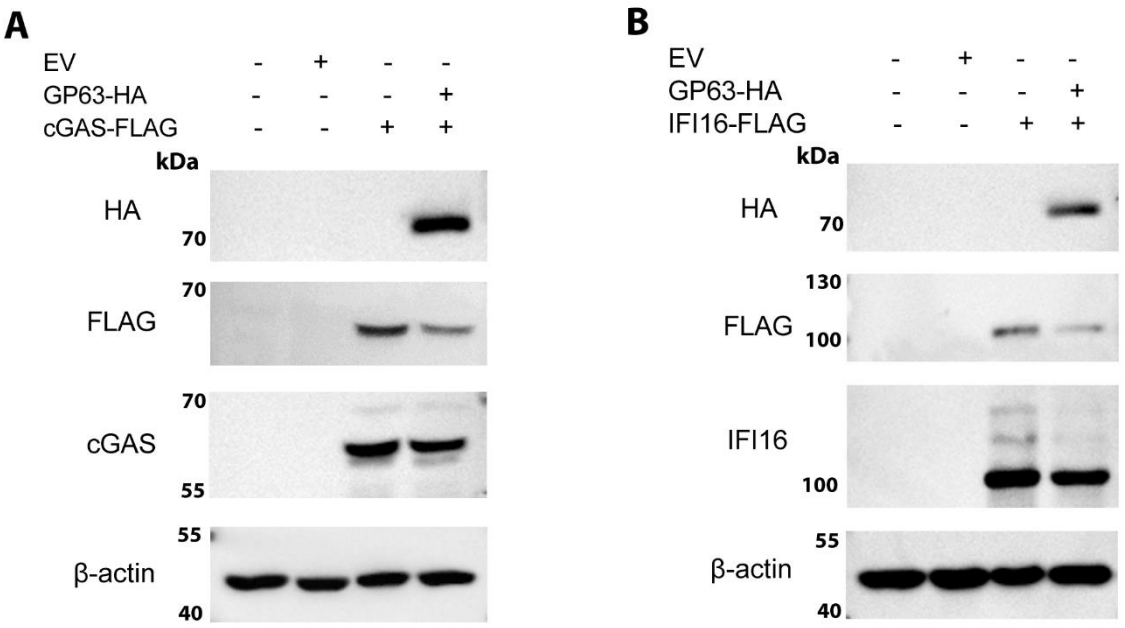


Figure 27: Co-transfection of *Leishmania* derived GP63 and cGAS or IFI16

HEK293T cells were co-transfected with *Leishmania* derived GP63 tagged with HA and either **(A)** cGAS-FLAG or **(B)** IFI16-FLAG to observe if changes to these that are observed with whole *Leishmania* infection occur. Transfection of empty vector was used as a negative control. Plasmids were transfected using GeneJuice and cells lysed with SDS lysis buffer after 24h. Lysates were then analysed for protein expression by western Blot.

5.6 Chapter Discussion

In this chapter, we planned experiments to help us understand the interaction between STING and *L.mexicana* and try to identify how the formation of a smaller STING during infection occurs.

We first reconstituted C-terminally HA-tagged STING into STING KO THP-1 cells to allow confirmation that the modification was occurring at the protein level rather than alternative splice variant of STING being produced (Fig.20). We confirmed that it was protein modification after infection of the reconstituted cells resulted in the appearance of the same lower band (Fig.20B). Additionally, we determined that cleavage was occurring at the C terminal end of STING due to the loss of the HA tag concurrent with STING cleavage and lower band formation (Fig.20C).

Mass spectrometry analysis of immunoprecipitated STING was utilised to probe peptide changes during infection. Samples were sent to a Proteomics facility and where acquisition was completed on a Q Exactive Plus Mass Spectrometer (Thermo Scientific). Extracted data was then searched against both *Leishmania Mexicana* and SwissProt human protein databases. One limitation of this process is that we did not send off our IgG IP control for analysis, meaning we were unable to control for protein abundance during infection. This would be especially crucial for carrying out protein interaction analysis where IgG acts as a negative control to help identify non-specific binding proteins that would give false positive interactions.

The mass spectrometry analysis led to the confirmation that a c-terminal peptide was absent during infection when STING peptides from uninfected and infected THP-1 cells were compared. This 27 amino acid C-terminal peptide was found to be located in the CTT binding site region of STING and included the TBK 1 binding site at Leu374 in addition to TBK1 phosphorylation site Ser366.

Research into the structure of STING has revealed the CTT (residues 340-379) to be essential for downstream activation of IRF3 leading to gene expression associated with activation of the cytoplasmic DNA sensors (Tanaka and Chen, 2012). This activation is mediated by the recruitment of TBK1 to the C-terminus of STING, which results in STING phosphorylation at Ser366. TBK1 recruitment is also necessary for IRF3 association with STING and its activation (Tanaka and Chen, 2012).

In addition to initiating downstream signalling, the CTT has also been implicated in a more regulatory role in which it participates in the autoinhibition of STING by binding to the LBD (Ergun et al., 2019; Qi et al., 2022; Yin et al., 2012) and masking the ST|NG polymerization interface. When DNA is sensed by cGAS, cGAMP will be produced as a second messenger. The CTT tail is displaced from its inhibitory position by cGAMP binding thus allowing STING polymerization to occur. This step is essential for STING downstream signalling to proceed (Ergun et al., 2019). The mechanism of autoinhibition proposed by Ergun et al is outlined in Figure 28A.

Loss of part or all of the STING CTT due to cleavage can therefore have multiple impacts on the activation and signalling of STING. On one hand, loss of this peptide should be associated with a loss of function, as TBK1 will no longer be able to interact with STING to activate downstream gene expression and type I interferon production. This is clearly not occurring in our infected THP-1 cells as we have observed that infection and therefore also the presence of the cleaved STING is associated with an enhanced activation of downstream DNA sensing pathway components. This is in addition to an increased DNA-dependent cytokine production.

However loss of the CTT is also expected to relieve autoinhibition and permit STING polymerisation even in the absence of an activation signal and cGAMP binding (Ergun et al., 2019). We therefore speculate that cleavage of the CTT mediated by *Leishmania* could result in spontaneous STING polymerisation in our infected cells. If a mixture of full-length

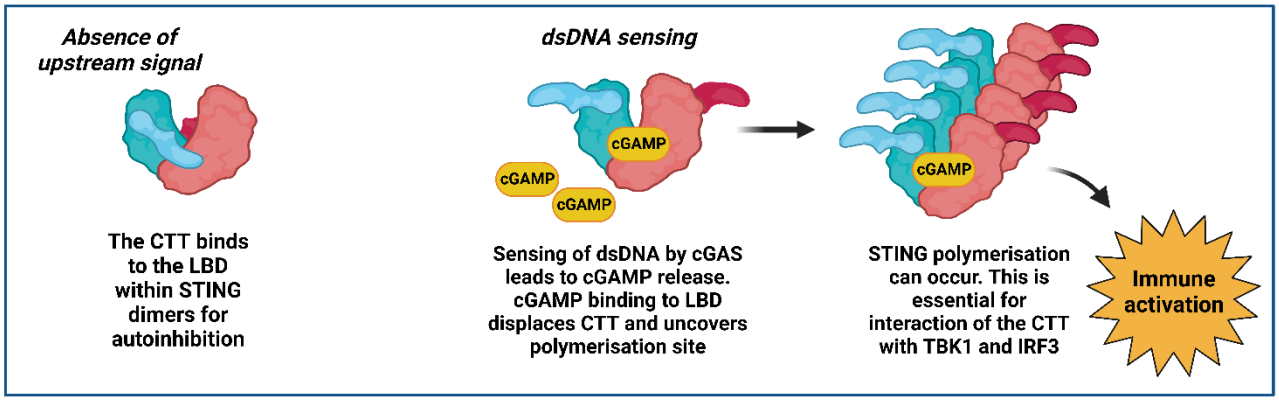
STING and STING lacking the CTT are present and polymerize together, downstream signalling may still be able to occur. Therefore, after additional stimulation, such as the transfected dsDNA in our experiments, it is conceivable that enhanced signalling may occur due to the primed and already polymerised STING. In many of our early infection time points we see both normal sized STING and the cleaved STING together in the western blot (Figs.11,12(6h),13,16,17,26). This therefore suggests that interaction of the two STING forms and polymerisation could occur. This potential mechanism that we have suggested is outlined in Figure 28B.

As we have also observed infections in which the whole of normal STING is absent in favour of the cleaved form (Figs.12(24h),20,21A), additional experiments should be carried out to fully determine if cleavage of all normal sized STING is still associated with enhanced DNA signalling. We could also test this hypothesis by co-transfecting WT STING and STING missing its CTT into cells and determining if this combination of STING forms display any enhancement in the response to dsDNA.

There was also a smaller missing peptide located at amino acid 180-191 within the ligand binding domain of STING. While an interesting discovery, we were unsure how detrimental this loss would be to the ligand binding function of STING as cGAMP was clearly able to still bind and activate STING polymerisation due to the enhancement of the pathway we have observed. We can hypothesise that this missing peptide could in some way allow more successful ligand binding, thus helping the enhancement of the STING signalling, or as the LBD is where the CTT binds during autoinhibition, perhaps this peptide could be located close to this area. To determine the importance of this region, STING mutants lacking this region could be created and its activity compared to that of full-length STING.

A

Model of CTT mediated STING autoinhibition proposed by Ergun *et al* (2019)



B

Proposed mechanism of how *Leishmania* mediated cleavage of STING may lead to an enhancement of STING siganlling and response to dsDNA

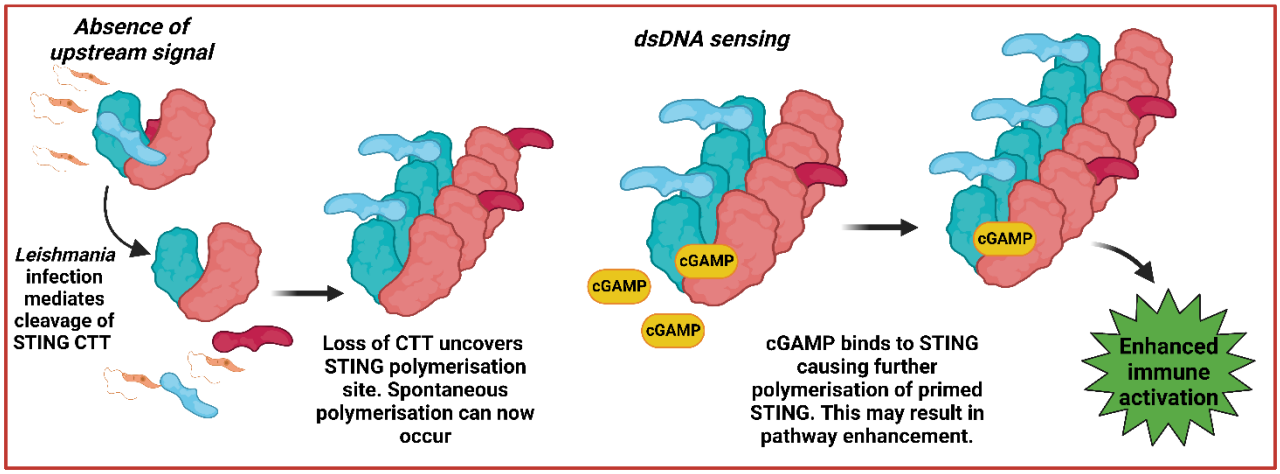


Figure 28: The autoinhibition of STING by its CTT may explain enhanced DNA sensing in *L.mexicana* infected cells

A) The model of CTT mediated STING autoinhibition proposed by Ergun et al (2019) (Blue and pink protein shape= STING monomers that form a dimer, light blue and red attachment = the CTT of each STING monomer.) **B)** A proposed mechanism for how partial CTT cleavage of STING by *L.mexicana* may lead to enhanced activation using the understanding of CTT autoinhibition.

Next, we cloned and overexpressed *Leishmania* virulence factor GP63 into HEK293T cells, confirming successful transfection by HA-tag protein analysis (Fig.23A). We then confirmed that GP63 was still active when transfected and that the HA tag did not interfere with any protease activity by determining if p65 cleavage would still occur in the transfected cells (Fig.23B).

As HEK293T cells are deficient in STING protein, we co-transfected a STING-FLAG plasmid with the GP63-HA plasmid into these cells. Upon co-transfection of STING and GP63 we did not see cleavage of normal size STING or the appearance of the STING lower band. While this seems to suggest GP63 alone is not the cause of the STING degradation, it does not completely rule out GP63 having a role in this cleavage. In this experiment we isolated GP63 from other *Leishmania* proteins that would be present during an infection. It is possible that GP63 activity may differ slightly when transfected alone as some virulence factors have been shown to work co-operatively to carry out *Leishmania* immune evasion strategies (Casgrain et al., 2016). GP63 is also known to cause inhibition by activating host proteins or regulators such as SHP-1 and PTP-1B (Gomez et al., 2009). If this is how it mediates STING degradation it is possible that host target of GP63 may not be present in these HEK 293T cells, preventing STING cleavage from occurring.

Next, we explored the possibility that *Leishmania* could be hijacking host pathways to modulate components of the DNA sensing pathway. For example, both GP63 and another *Leishmania* virulence factor, elongation factor-1α (EF-1α) can activate SHP-1 via cleavage or binding events (Gomez et al., 2009; Nandan et al., 2002). SHP-1 is a protein tyrosine phosphatase (PTP) involved in the regulation of cell signalling (Lorenz, 2009). Upon activation, it can inhibit cell signalling pathway components involved in the immune response including IRAK1 and JAK 2 among other kinases that would normally be activated in response to *Leishmania* infection.

Under normal conditions, levels of STING are tightly regulated as constant activity of STING and downstream gene induction after dsDNA stimulation would lead to excessive inflammation and damage to self. After activation, STING is normally degraded after approximately 3-6 hours (Abe, 2014). This degradation occurs through the autophagy pathway in the lysosomal compartment as this protein turnover can be inhibited by chloroquine and bafilomycin treatment (Gonugunta et al., 2017; Konno et al., 2013). Chloroquine inhibits autophagy by preventing autophagosome fusion with lysosomes (Mauthe et al., 2018), whereas Bafilomycin is able to inhibit lysosome acidification (Bowman et al., 1988; Yoshimoto et al., 1997). We therefore chose to pre-treat our THP-1 cells with bafilomycin and proteasome inhibitor MG132 prior to infection. MG132 reversibly blocks protein degradation by inhibiting the chymotrypsin-like activity of the proteasome complex (Rock et al., 1994). MG132 treatment partially rescues STING degradation, although not to the extent that inhibitors of autophagy can (Gonugunta et al., 2017).

While we saw no change to STING cleavage in cells pre-treated with bafilomycin, pre-treatment of cells with MG132 prior to infection did prevent formation of the lower STING band (Fig 25). This suggests that the proteasome plays a role in STING cleavage, either directly or indirectly. As STING is not completely degraded by infection, involvement of the proteasome could suggest that *Leishmania* infection could induce STING to undergo partial degradation or RUP (Rape and Jentsch, 2004). It is also possible that another protein needed for STING cleavage could also go through this process to be activated, or that the proteasome is responsible for the degradation of a protein which inhibits STING cleavage. We also saw some rescue of p65 degradation with MG132 pre-treatment of these cells which could suggest that the proteasome, in addition to GP63 activity, could contribute to p65 degradation. It is possible that *Leishmania* could also target p65 for proteasome degradation like STAT1, at least in part (Forget et al., 2005).

Another possibility we should consider is the impact of this inhibitor on the parasite's own proteasome. It could be that MG132 present in the cell media is also able to disrupt the *Leishmania* proteasome which may have a significant impact on its infectivity, virulence and even viability. Proteasome inhibitors can cause cell cycle arrest and cell death in trypanosomatids. It has been shown previously that MG132 treated *L.major* cannot replicate (Shaheen et al., 2021) although treatment did not affect the viability of *L.donovani* (Sharma et al., 2015). However as a new pre-clinical treatment for *Leishmania* specifically acts by inhibiting the proteasome (Wyllie et al., 2019), it is likely that parasite infectivity will be affected significantly.

It is therefore possible that the reason we could not observe formation of the STING band or as much p65 cleavage was because the parasite was less infective due to the MG132 treatment. To determine if this is the case we could instead pre-treat *L.mexicana* parasites with MG132 before using them in an infection, so we can then compare appearance of the STING band in cells infected with pre-treated or untreated parasites. We could also directly measure the infectivity and viability of parasites treated with this inhibitor.

These western blots also revealed that size modifications to cGAS and IFI16, additional components of the DNA sensing pathway, occur during *L.mexicana* infection. So far, no suggestion of modification or interaction between *Leishmania* parasites and cGAS or IFI16 has been reported in the literature, meaning this could be an important starting point for further investigation. To confirm these different sized bands were modified cGAS and IFI16, we could use cGAS KO and IFI16 KO cells to address potential antibody cross-reactivity with a *Leishmania* protein during infection.

Unlike the cleavage of STING, cGAS appears to increase in size during infection. As this increase is an approximate doubling in size, we have suggested it is possible that this band may represent the formation of a cGAS dimer. Other modifications that would cause an increase to the size of cGAS, such as acetylation or ubiquitylation would not cause

such a large size increase. Dimerization of cGAS does occur as part of its activation and is crucial for its activity (Kranzusch and Vance, 2013). However, it would be expected that these dimers would have separated to cGAS monomers during sample preparation steps prior to the running of SDS-PAGE gels. To confirm the modification is due to *Leishmania* infection future samples should be treated with varying concentration of denaturing agents to ensure the size change is not an artefact of sample preparation.

The size of IFI16 is shown to decrease during infection. This could be due to cleavage of the protein, similar to what we have shown is happening to STING during infection. Also similar to STING is that MG132 seems to partially prevent this size change at 4h infection (Fig.25). This would suggest that the size change may be mediated by the proteasome. IFI16 is known to be the target of immune evasion by other intracellular pathogens. For example a viral protein that inhibits its activation and can promote its degradation is the E3 ubiquitin ligase ICP0 from Herpes Simplex (Orzalli et al., 2016, 2012). This is in an attempt to inhibit its activation and prevent sensing of viral DNA.

Despite the changes to cGAS and IFI16, we have already demonstrated that the DNA sensing pathway is not inhibited by *Leishmania* infection and even appears to be enhanced. This suggests that these changes to cGAS and IFI16 do not negatively affect their function, and it could be possible that these modifications are instead linked to the enhancement of the pathway. We have also shown the change in IFI16 size does not affect is localisation to the nucleus and predominantly nucleoli, (Fig.26).

Co-transfections of GP63 with cGAS and IFI16 were performed (Fig 27) to determine if the size changes observed with infections could be mediated by GP63. We do not see much change in cGAS when transfected with GP63, although as the change to cGAS was an increase in size, it would be unlikely that the GP63 protease could be responsible, unless it was activating a host regulator that interacted with cGAS.

In the cells transfected with IFI16 and GP63 we do observe a small decrease in both FLAG and IFI16 total protein level. However, we do not see the appearance of the smaller IFI16 as was seen previously during whole parasite infection (Fig.25). We could speculate that some degradation of IFI16 could be starting to occur in these cells but perhaps without a whole parasite infection, there is either not enough GP63, or another condition missing to mediate complete cleavage to its smaller form. Additional experiments will be required to elucidate the precise molecular mechanism(s) by which DNA sensing factors are targeted by *Leishmania* virulence factors.

Chapter 6: Investigating EVs derived from *Leishmania* infected THP-1 cells

6.1 Chapter introduction

Leishmania parasites can release extracellular vesicles (EVs) that contain various parasite proteins including virulence factors that can contribute to disease establishment even before *Leishmania* enters a host cell. These EVs are taken up by naïve macrophages and prime them for parasite invasion by mediating immunosuppression (Silverman and Reiner, 2012). These EVs have been intensively studied and represent an important part of how *Leishmania* are able to establish infection.

However, a related area that has been studied much less is EVs that are released by *Leishmania* infected macrophages. Viruses, bacteria, and parasites are able to alter the EVs produced by their host cells (Schorey et al., 2015) and these infections can affect host cell EVs by altering the quantity of EVs generated in addition to changing the structure of the vesicle membrane as well as its cargo. Nucleic acids, proteins and lipids derived from intracellular pathogens have all been identified within the EVs of these infected cells (Rezaie et al., 2021; Schorey et al., 2015; Zhang et al., 2018). When taken up by nearby uninfected cells, the pathogen-derived components contained within these EVs can have significant immunomodulatory effects. Both immune cell activation and suppression have been observed with the outcome dependent on several factors, including the recipient cell type and type of components delivered.

Considering the capacity of *Leishmania* to modulate the innate immune response in macrophages and inactivate host proteins and signalling pathways, the study of EVs released from *Leishmania*-infected macrophages is likewise expected to be of considerable significance.

6.2 Extracellular vesicles derived from infected cells contain DNA

We extracted EVs from the media of infected THP-1 macrophages to determine whether they play a role in the modulation of innate immune responses that we observed during infection. For instance, it would be conceivable that EVs containing DNA would stimulate or prime DNA sensors, which could be an explanation for the enhanced DNA sensing response we observed. Interestingly EVs released from *P. Falciparum* infected cells contain parasite DNA that is able to activate the DNA sensing pathway within uninfected monocytes (Sisquella et al., 2017).

To ensure that any EVs isolated from our cells were derived from the macrophages, we cultured and differentiated our THP-1s in exosome depleted FCS. After differentiation of THP-1 cells they were washed twice with exosome-depleted media and the media was replaced. We then infected the THP-1s with *L.mexicana* promastigotes for 8 hours. After the infection a centrifugal isolation of EVs from the cell media was performed. Some of the extracted EVs were used in validation experiments while the rest were used in experiments with naïve THP-1s to determine what effect they may have on the DNA sensing pathway. These procedures are detailed in the workflow shown in Figure 29A.

Confirmation that we had correctly isolated EVs was carried out by transmission electron microscopy (TEM) of phosphotungstic acid-stained vesicle suspensions (Fig.29C). The resulting images indicated that our extraction contained EVs of a size between 30 and

150 nm in samples derived from both infected and uninfected THP-1 cells. We also lysed our EV sample for western blot analysis of EV marker CD63 (Fig.29B). Though faint, a band for CD63 can be detected in our EV sample and not in the elution buffer control.

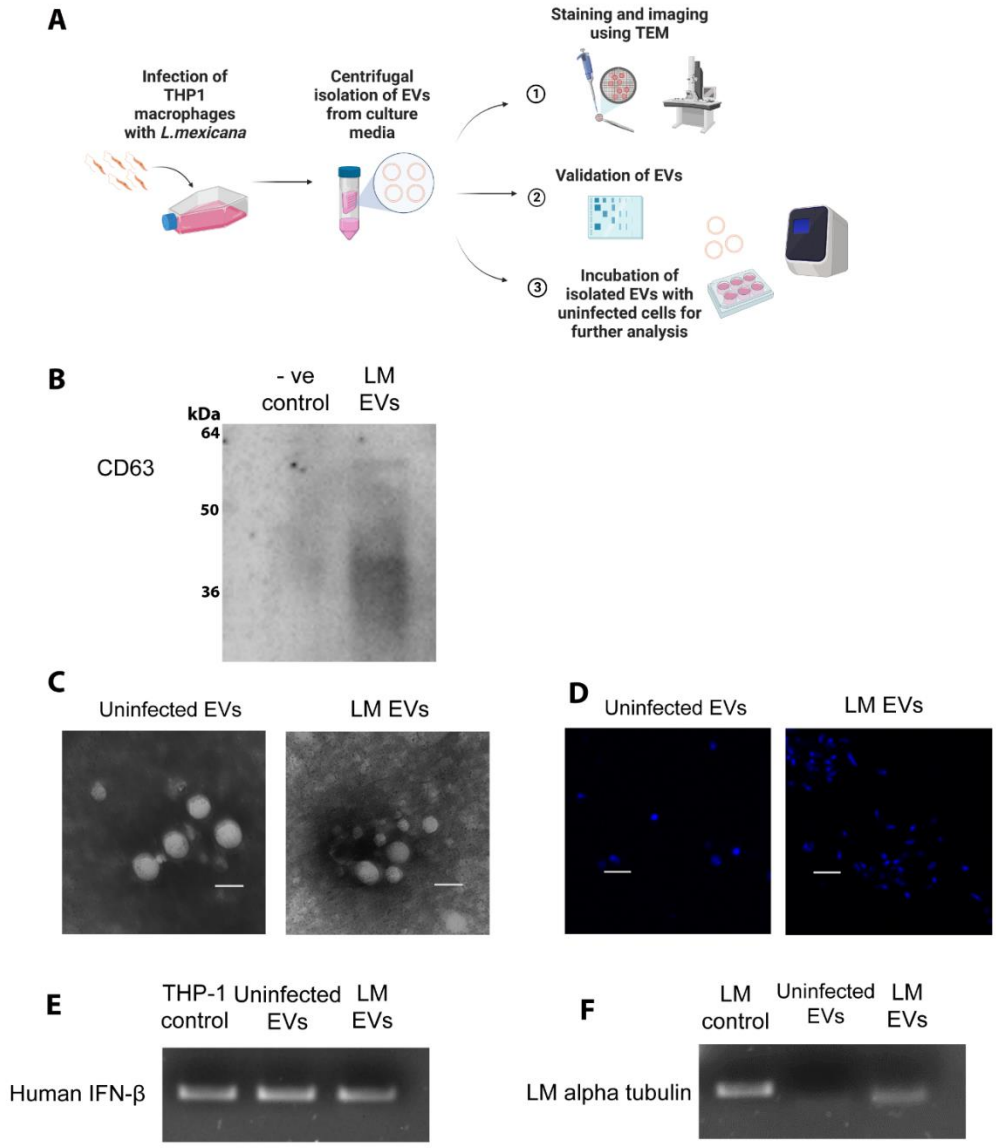


Figure 29: *L.mexicana* infection changes the composition of Extracellular Vesicles released from THP-1 cells

(A) Workflow of how the extracellular vesicles were isolated and further used in additional experiments. Diagram created in Biorender. **(B)** EVs were isolated from the cell media of $2x10^7$ THP-1 cells after infection at a MOI of 10 for 8h. EVs were lysed for western blot analysis of protein expression of vesicle marker CD63. Negative control is EV elution buffer only. **(C)** Confirmation of vesicle isolation from uninfected and *L.mexicana* infected cells by staining with 1% phosphotungstic acid and subsequent imaging using Transmission Electron Microscopy. Imaged with JEM-2100 TEM. Scale bar is 100nm. **(D)** Confocal images of extracellular vesicles stained with DAPI to reveal dsDNA**.** Scale bar is 2 µm **(E)** The presence of human DNA within EVs isolated from both uninfected and infected THP-1s was confirmed by amplification of human IFN- β gene from DNA extracted from the EV samples. A control of human IFN- β DNA amplified from uninfected THP-1s was used to compare band sizes when samples were run on an agarose gel. **(F)** The presence of *Leishmania* DNA within the vesicles was confirmed by amplification of *Leishmania* alpha-tubulin gene from DNA extracted from the EV samples. A control of DNA amplified from whole *Leishmania* parasites was also ran on the agarose gel.

Our EVs were also imaged by confocal microscopy to determine if DNA had been packaged into the EVs, with DAPI used to visualize any DNA present. DAPI is a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA. A small volume of each EV isolate was air dried onto coverslips and dipped into methanol for fixing. Coverslips were fixed to slides with a mounting medium containing DAPI. A positive DAPI signal was obtained for both samples of EVs (Fig. 29D).

As both human DNA and parasite DNA could be the source of the positive DAPI signal in our samples, we further investigated the DNA within our isolated EVs. A DNA extraction protocol was carried out on pooled EVs from either the uninfected or infected macrophages. We then used the extracted DNA as a template to amplify by PCR the intronless human gene IFN-β and the *Leishmania* gene alpha-tubulin. DNA extracted from uninfected THP-1 cells and whole *L.mexicana* parasites was used as a positive control for human and parasite gene amplification respectively. The resulting PCR products were run on an agarose gel and amplification was confirmed by the visualisation of a band corresponding to IFN- β or alpha tubulin (Figs. 29E,F).

A band corresponding to the amplified IFN-β gene was present in both EVs isolated from uninfected and infected THP-1 cells whereas a band corresponding to the correct size for the alpha-tubulin gene was only seen in the infected cell derived EVs. This confirmed that human DNA was present within EVs isolated from both uninfected and infected cells, but parasite DNA was only present in infected cells.

6.3 EVs derived from infected THP-1 cells do not enhance the DNA sensing of uninfected cells

After confirming successful isolation of EVs, we investigated the effect of infected macrophage derived EVs on uninfected cells. We were especially interested in changes to the DNA sensing pathway that had been observed in whole parasite infections.

Given that we see an activation and enhancement of the cytoplasmic DNA sensing pathway in cells infected with whole *Leishmania* parasites, whether this effect can also be observed in cells infected with only EVs derived from infected macrophages will tell us more about how this enhancement is occurring.

We treated uninfected THP-1 cells for 4h with either elution buffer from the EV isolation process, the EVs isolated from uninfected cells or those from *L.mexicana* infected cells. After 4h cells were then transfected with dsDNA for a further 4h. It is possible that *Leishmania* derived cargo within these EVs could prime the DNA sensing pathway, similar to what may be occurring in whole parasite infected cells. Cells were then analysed by western blot for protein changes in addition to the gene expression of cytokines associated with the response to dsDNA sensing including IFN-β, CXCL10 and IL-6 by qRT-PCR.

There was no induction of these cytokines from treatment with vesicles alone, either from uninfected or infected cells (Fig. 30A-F). While we have shown that these vesicles do seem to contain human and/or *Leishmania* DNA, it is possible that the amount of EV DNA was not enough to activate the cytoplasmic DNA sensing pathway. There was also no significant difference in the gene expression of cytokines induced by dsDNA transfection between the treatments (Fig.30A-F). This was different to what was observed in cells infected with whole parasites where an enhancement of gene expression was observed in the infected cells after HT-DNA transfection.

We also looked at the protein levels of STING and p65 after treatment of our isolated EVs (Fig. 30G). Previous investigations into EVs released by *Leishmania* infected macrophages have revealed that they can contain limited parasite derived proteins (Gioseffi et al., 2020; Hassani and Olivier, 2013). Western blot analysis of our cells treated with EVs derived from infected cells does show a very weak induction of the STING lower band (Fig.30G), suggesting cleavage is occurring, just much less than in whole parasite infected cells. This not only confirms uptake of these EVs by the uninfected macrophages,

but also suggests that the *Leishmania* virulence protein responsible for STING cleavage is contained within the EVs released from infected macrophages and that a whole parasite is not required for STING cleavage.

p65 degradation is however not observed in cells treated with infected cell derived EVs. This could suggest that GP63 is not contained within these EVs, although it is also possible that there was simply not enough present to see any cleavage activity in these cells.

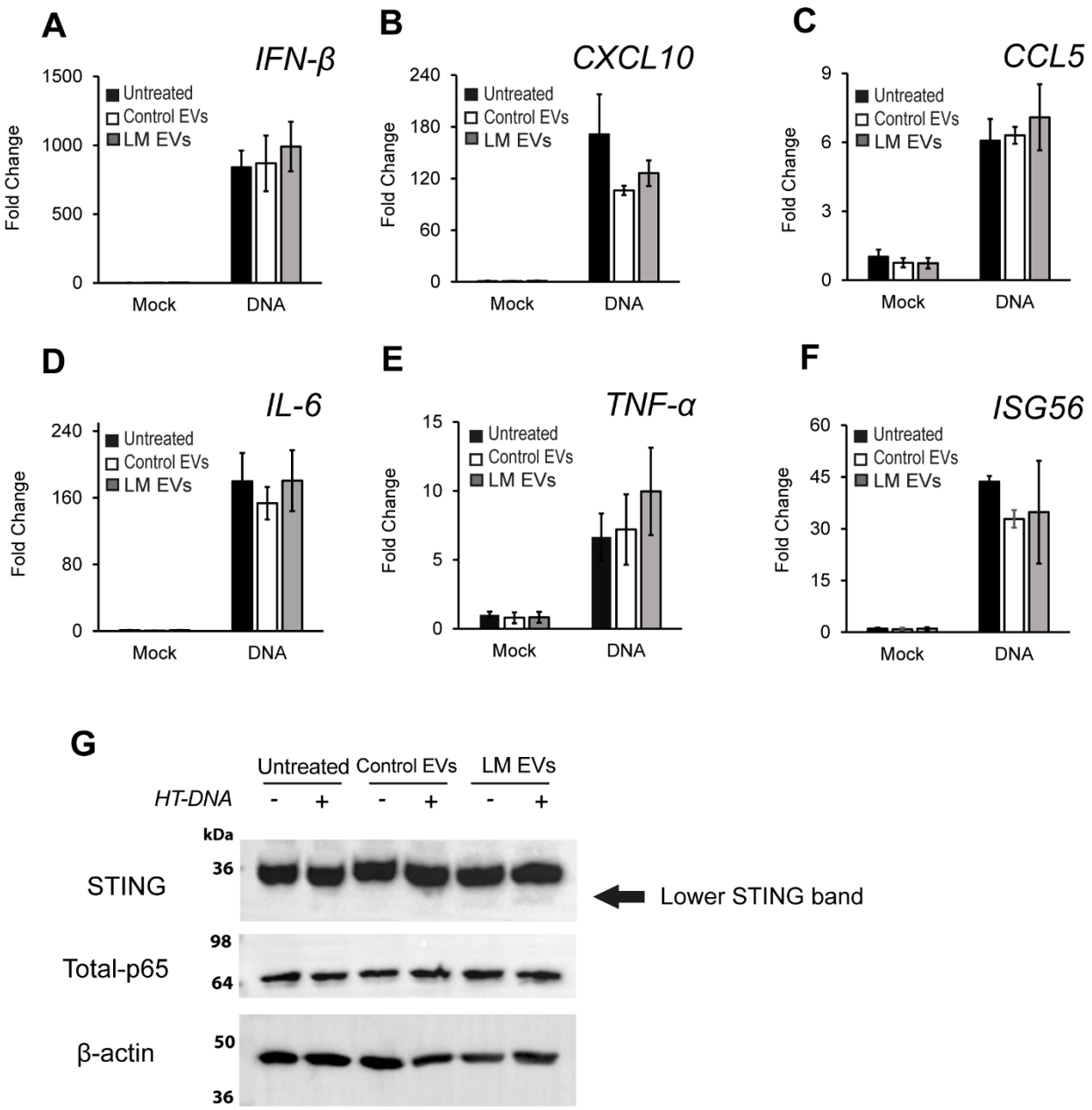


Figure 30: EVs derived from infected THP-1s do not enhance the DNA sensing of uninfected THP-1 cells

WT THP1s infected with *L.mexicana* derived EVs or control EVs for 4 hours before stimulation with 5µg/ml HT-DNA for 4 hours. Cells were then lysed for qRT-PCR analysis of **(A)** IFN-β **(B)** CXCL10 **(C)** CCL5 **(D)** IL-6 **(E)** TNF-α and **(F)** ISG56 mRNA. Data are presented as mean values of biological triplicates. Data are representative of at least three experiments. **(G)** WT THP1s were treated with control EVs from uninfected cells or treated with EVs derived from infected THP-1s for 4 hours before stimulation with 5µg/ml transfected HT-DNA or mock for 4 hours. Cells were then lysed for western blot analysis of protein expression. Data are representative of at least three experiments.

6.4 Chapter Discussion

In this short chapter we conducted preliminary investigations into EVs released from *Leishmania mexicana* infected THP-1 cells and if they could also mediate changes to STING as a whole parasite infection does. The release of host cell derived EVs that have undergone modification by intracellular pathogens can be another important way that pathogens, including *Leishmania* interact with the host immune system after infection. In some cases, these modified EVs can activate the host immune system and in others mediate suppression.

For an example of immune suppression, HIV infected T cells are able release EVs containing HIV virulence factor Nef (Lenassi et al., 2010) . These Nef-containing EVs have a possible role in T-cell depletion as they have can induce CD4+ T-cell apoptosis *in vitro* (Lenassi et al., 2010). In contrast, EVs released from THP-1 cells after intracellular infection with *Mycobacterium bovis* BCG and *M. tuberculosis* could activate non-infected cells to induce a pro-inflammatory response involving TNF-α production (Bhatnagar et al., 2007).

Previous studies of EVs derived from *Leishmania*-infected macrophage have suggested that *Leishmania* also modifies the contents of their host EVs, as parasite proteins have been detected in the EVs released from host cells (Gioseffi et al., 2020; Hassani and Olivier, 2013). *Leishmania* genomic DNA has not yet been reported to be included in the cargo of these modified host EVs, however given the associations we have discovered so far with infection and STING and the DNA sensing pathway, we decided to investigate if we could detect DNA in the EVs isolated from our infected THP-1 macrophages.

Using confocal microscopy, we also showed that DNA was present in EVs isolated from *Leishmania*-infected THP1 cells (Fig.29D). Further analysis showed that this DNA was of

human origin in the EVs from uninfected cells, but of both human and parasite origin in the EVs from cells infected with *Leishmania* (Figs. 29E,F).

We then treated uninfected THP-1s with the isolated EVs, but we did not observe an induction of cytokines associated with the DNA sensing response (Figs.30A-F). It is possible that not enough DNA was present within the EVs we collected to elicit a response from the cGAS-STING DNA sensing pathway. These cells were then additionally stimulated with dsDNA to see if any enhancement as observed with whole cell infection would also occur after incubation with the EVs isolated from infected cells. We did not see any enhancement, suggesting that either whole *Leishmania* infection was required or that the enhancement required a *Leishmania* virulence factor that was not packaged into the host EVs. Alternatively, it is possible that the amount of EVs used was not sufficient to elicit a response.

It was previously reported by Hassani and Olivier that within EVs isolated from *L.mexicana* promastigote infected macrophages, GP63 was the sole enriched *Leishmania* protein (Hassani and Olivier, 2013). Additional studies that used the amastigote form of *Leishmania* for initial macrophage infection have shown that macrophage derived EVs contained up to 59 parasite derived proteins, that does not include GP63 (Gioseffi et al., 2020). These differences perhaps suggest that the parasite proteins that are packaged into host EVs may vary by species, life-cycle form or from infection to infection. As we did not perform mass spectrometry analysis of the EVs we used to treat our uninfected THP-1 cells, we do not know exactly which *Leishmania* proteins were transferred to the uninfected cells. This would be a good area for further investigation and to would help determine if specific *Leishmania* proteins are always selected for EV enrichment or if it varies between infections.

While we do not know what exact *Leishmania* proteins are present within our isolated EVs, it is likely that the *Leishmania* virulence factor responsible for STING cleavage is within

these EVs. After treatment of our uninfected cells with infected cell-derived EVs the lower STING band can be faintly seen (Fig.30G). We do not see this faint band in untreated cells or cells treated with control EVs from uninfected THP-1 cells. This suggests that it is the presence of *L.mexicana* EVs that caused the lower STING band induction. Tp65 degradation does not occur in these treated cells so we can tentatively suggest that it must not be the same *Leishmania* derived protein that is responsible for the cleavage of both, ruling out GP63. However, while we can't observe p65 degradation here it is possible we would in experiments where a higher EV content is used.

A full characterisation and quantification of these EVs would be highly beneficial to expand and improve our experiments with these EVs. While the same number of THP-1 cells and *Leishmania* parasites were used for each repeat of the EV extraction process we were unable to quantify the EVs and compare extraction success between experimental repeats. Additionally, the international society for extracellular vesicles suggests that best practice in experiments involving EVs should include an ascertainment of EV population homogeneity through nanovesicle tracking assay, in addition to morphological assessment by TEM to understand more about the potential mixture of EVs isolated. This is in addition to protein content analysis of a single type of EV of interest (Théry et al., 2018). These experiments will be incredibly important to furthering understanding of how *L.mexicana* mediates changes to host macrophage

Chapter 7: Changes to the DNA sensing pathway occur in other cells types during *L.mexicana* infection

7.1 Chapter Introduction

In addition to carrying out infections in the human THP-1 macrophage cell line, we also infected mouse bone marrow derived macrophages (BMDMs). Mouse models have been extensively used to aid in the study of *Leishmania* and its impact on the immune system. Most commonly the two mouse strains Balb/c and C57BL/6 are used on the basis of their genetic susceptibility or resistance to infection by *Leishmania* (Howard et al., 1980). Many different species of *Leishmania* have been successfully used to infect these mouse models (reviewed by Loría-Cervera and Andrade-Narváez, 2014; Sacks and Melby, 2001) including models of cutaneous Leishmaniasis caused by *L.mexicana*. In addition to mouse models where the parasite is inoculated under the skin of the mouse, infections have also been carried out on mouse cells such as BMDMs *in vitro* (Aoki et al., 2019; Carrera et al., 1996; Loría-Cervera and Andrade-Narváez, 2014b; Naderer et al., 2006; Rabhi et al., 2013; Van Bockstal et al., 2020)

7.2 STING cleavage also occurs in mouse derived macrophages

For our *in vitro* infections, bone marrow was collected from C57BL/6 mice and macrophages were differentiated with macrophage colony-stimulating factor (M-CSF). On day 7 macrophages were harvested and seeded for *L.mexicana* infections. Proof of infection was confirmed by confocal microscopy after 6h infection (Fig.29A), again utilizing a primary antibody against the endogenous parasite protein acetylated tubulin to visualise the *Leishmania* parasites, and a fluorescently labelled secondary antibody.

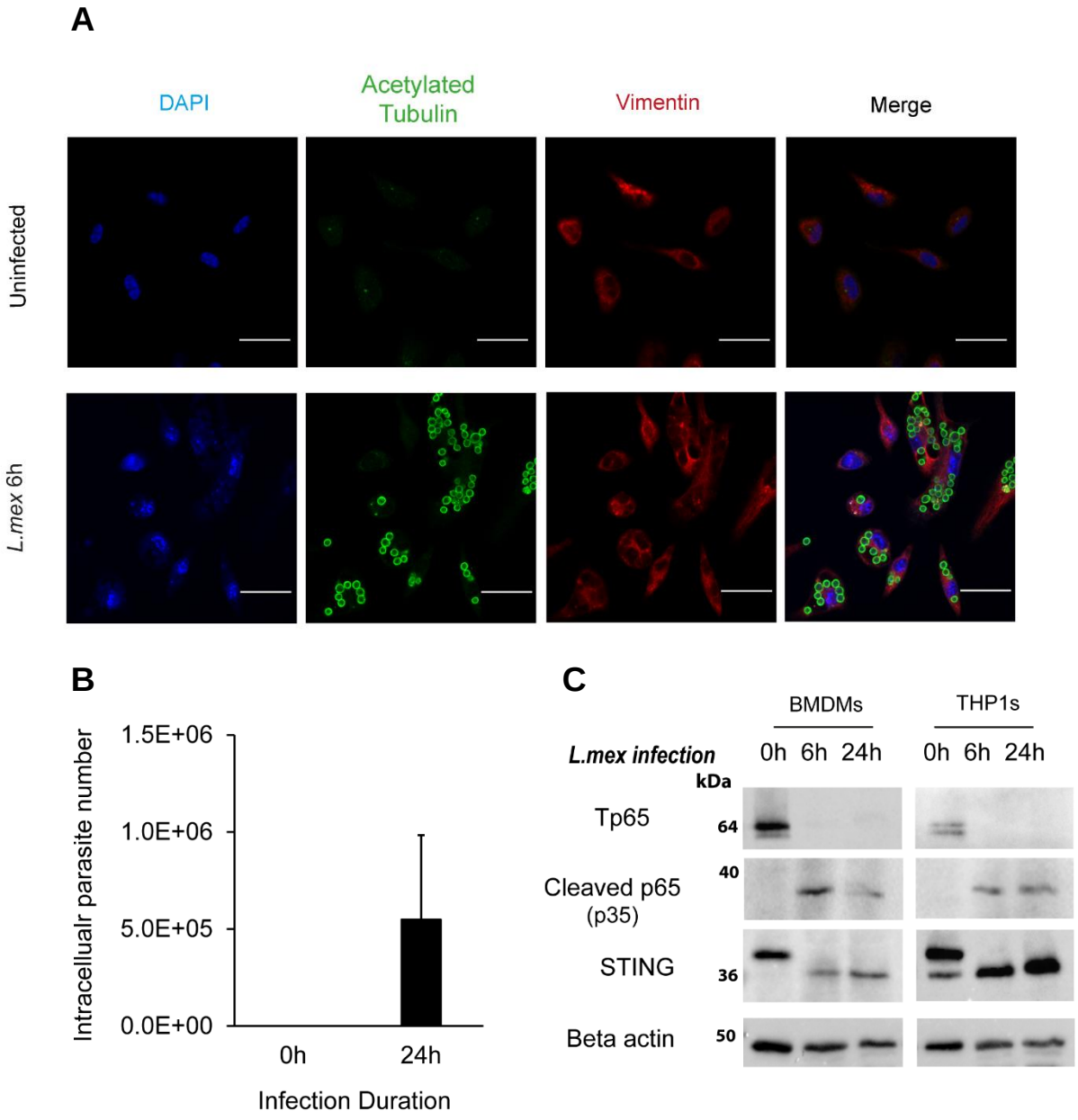


Figure 31: *L.mexicana* successfully infects Bone Marrow derived Mouse Macrophages and also causes cleavage of Tp65 and STING

(A) WT BMDMs were seeded onto coverslips and allowed to adhere for 1 hour before infection with *L.mexicana* at a MOI of 10 for 6 hours. Cells were then fixed in methanol and stained for Acetylated Tubulin to visualize the parasites (green), Vimentin (red) and DAPI nuclear stain (blue) and imaged by confocal microscopy. Scale bar = 20μm. **(B)** BMDMs were infected with parasites at a MOI of 10 for 24h, then washed and lysed for qRT-PCR analysis of mRNA, where the estimated intracellular parasite number present in cells was determined using a standard curve of known infection numbers. **(C)** WT BMDMs and THP-1s were infected with *L.mexicana* at a MOI of 10 over 24 hours as indicated before lysis for western blot analysis of protein expression. Data are representative of at least three experiments.

Vimentin was used as a cytoskeletal marker, and DAPI as a nuclear DNA stain. Parasites could be observed within cells at the 6h time-point and appeared to have undergone morphological change to the amastigote form due to their ovoid shape. To further confirm parasite entry, we determined the approximate intracellular parasite number in our samples by qPCR, using the standard curve of parasite gene 18s created from known parasite numbers (Fig.31B). This demonstrated that successful internalization of *Leishmania* was occurring in these cells.

To investigate the virulence activity of *Leishmania* within these cells we infected both THP-1 cells and BMDMs at the same time for 6h and 24h. After infection cells were washed and lysed for western blot analysis of key proteins known to be affected by infection, including STING (Fig.31C).

In both these cell types *Leishmania* infection can induce the cleavage of p65, as indicated by a loss of p65 at both 6h and 24h post infection (Fig.31C). The appearance of the smaller cleaved form p35 also appears at both time points indicating cleavage to this form has occurred. Induction of the faster migrating STING band was also seen in both THP-1 cells and the BMDMs after infection (Fig.31C). In both cell types, complete loss of the normal size of STING is also observed. The smaller STING band also gets stronger from 6h to 24h post infection suggesting that STING is continually being cleaved to this size until at least 24h post infection.

We next looked to see if the enhancement of the DNA sensing pathway we have observed after *Leishmania* infection in the THP-1 cells also occurred in the BMDMs. Using qRT-PCR we looked at the expression of key genes induced after HT-DNA stimulation in both uninfected and infected cells (Fig.32).

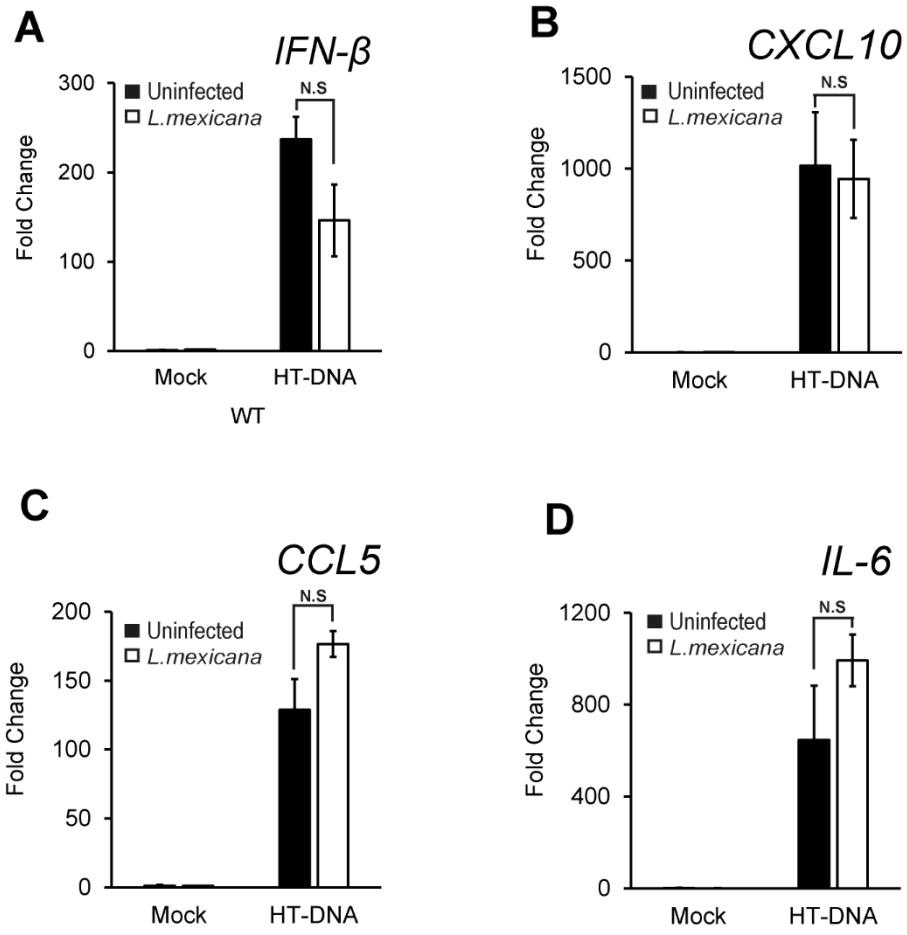


Figure 32: There is not an enhanced response to dsDNA in infected BMDMs

WT BMDMs infected with *L.mexicana* at a MOI of 10 for 4 hours before stimulation with 5µg/ml HT-DNA for 4 hours. Cells were then lysed for qRT-PCR analysis of **(A)** IFN-β **(B)** CXCL10 **(C)** CCL5 and **(D)** IL-6 mRNA. Data are presented as mean values of biological triplicates. Error bars indicate standard deviations. N.S= non-significant as determined by students T-test. Data are representative of at least three experiments

In contrast to what we have observed in the THP-1 cells (Figs.9,14,1) there was no significant difference in the induction of IFN-β, CXCL10, IL-6 or CCL5 after stimulation between the infected and uninfected cells (Fig.32). However, while we did not observe an enhancement of DNA sensing in *Leishmania*-infected mouse BMDMs, there was also no reduction in the response, despite clear STING cleavage in these cells (Fig. 31C).

7.3 *L.mexicana* can infect HaCaT keratinocytes

Infection with *Leishmania* begins when the parasites are deposited into the mammalian host skin during the blood meal of infected sand flies. The final host cells of *Leishmania* parasites are long-lived tissue-resident or monocyte-derived macrophages, where the parasites will differentiate from promastigotes to amastigotes and proliferate (Rossi and Fasel, 2018). Other immune cells such as neutrophils (Chaves et al., 2020; Peters et al., 2008), monocytes (Romano et al., 2017) and dendritic cells (Feijó et al., 2016) may also be infected during the course of infection. Upon entry to the epidermis, *Leishmania* will also encounter many other host cells, including keratinocytes. Keratinocytes are the primary constituent of the epidermis, accounting for around 90% of all skin cells and are an important source of cytokines and growth factors (Pivarcsi et al., 2004). Keratinocytes also have a role in host defence against invading pathogens and release cytokines and chemokines to attract immune cells to the site of infection. This also occurs during *Leishmania* infection where keratinocyte interaction with *Leishmania* can lead to the recruitment of neutrophils, activation of T cells and can have an impact on the course of the infection (Ehrchen et al., 2010; Roebrock et al., 2014; Scorza et al., 2017; Teixeira et al., 2006). Keratinocyte-derived cytokines released after activation by *Leishmania* parasites can also directly enhance the leishmanicidal activity of macrophages (Ehrchen et al., 2010; Jafarzadeh et al., 2021; Scorza et al., 2017).

Additionally, keratinocytes are key to the pathological changes to the skin observed during cutaneous leishmaniasis in which parasites induce inflammatory lesions and ulceration (Tasew et al., 2010). The number of apoptotic keratinocytes correlate with skin ulceration, a process which is mediated by Fas/TRAIL apoptotic pathway (Rethi and Eidsmo, 2012; Tasew et al., 2010; Eidsmo et al., 2007).

As there is limited research on *Leishmania* keratinocyte infection, it is not known if *Leishmania*-derived virulence factors would act upon them in the same way that has been shown in macrophages and other immune cell hosts. We therefore infected immortalised human keratinocyte cells, HaCaT cells, to determine if *L. Mexicana* parasites can infect these cells *in vitro*, and to test whether innate immune signalling cascades are also dysregulated.

HaCaT cells were incubated with *L.mexicana* promastigotes in the same manner that we have infected THP-1 cells. We probed if parasite entry was occurring by determining the intracellular parasite number after 24h infection using qRT-PCR analysis of parasite gene 18s in combination with our standard curve (Fig.33A). The result suggested successful infection of the HaCaTs.

An infection time course of 2h, 4h, 6h and 24h was also performed in the keratinocytes, with cells were washed and lysed at each point. Western blot analysis of host protein p65, known to be targeted by *Leishmania* virulence factors was then performed Fig.33B). Cleavage of p65 to p35, known to be mediated by *Leishmania* GP63, was seen as little as 2h into infection in these cells. However, the cleaved form p35 was only visible on our blots from the 4h timepoint. This, together with the qRT-PCR analysis of parasite 18S, confirmed successful infection of the HaCaT cells by *Leishmania*.

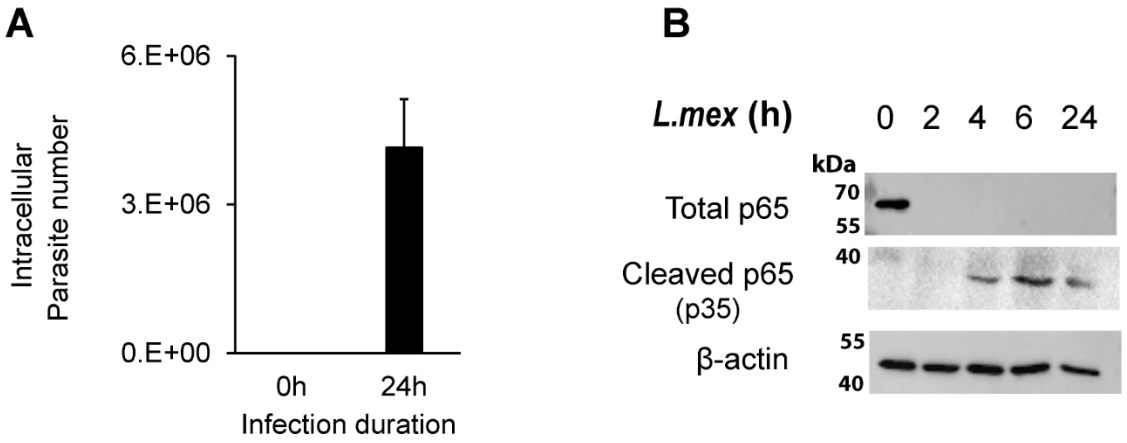


Figure 33: *L.mexicana* are able to infect HaCaTs and cause cleavage of p65 to p35

(A) WT HaCaTs were infected with parasites at a MOI of 10 for the specified time, then washed and lysed for qRT-PCR analysis of mRNA, where the estimated intracellular parasite number present in cells was determined using a standard curve of known infection numbers. **(B)** WT HaCaTs were infected with *L.mexicana* at a MOI of 10 over a time course of 24 hours as indicated before lysis for western blot analysis of protein expression. Data are representative of at least three experiments.

It has been reported that keratinocytes activated by *Leishmania in vivo* can secrete cytokines and chemokines to activate the immune response (Scorza et al., 2017). In our own infected cells, we have demonstrated that *Leishmania* virulence factors are active within the infected HaCaTs from 2h post infection by p65 cleavage (Fig.33B). This is likely to have some impact on the cells' ability to mount a response as we have seen in the THP-1 cells (Fig.8) as many inflammatory cytokines are under transcription control by NF-κB. We therefore used qRT-PCR to investigate the gene expression of key cytokines and chemokines at 6h and 24h post infection (Fig.34).

None of the genes we investigated showed any significant increase with *L.mexicana* infection for 6h or 24h. This is somewhat similar to the response of infected THP-1 cells (Fig.5) in which IL-6, IL-12 and TNF- α production were not induced by infection alone. We suggested this could be due to downregulation of signalling pathways as an immune evasion strategy. However in the THP-1 cells we did see some induction of IFN- β and CXCL10 after *L.mexicana* infection which is not observed in HaCaT keratinocytes.

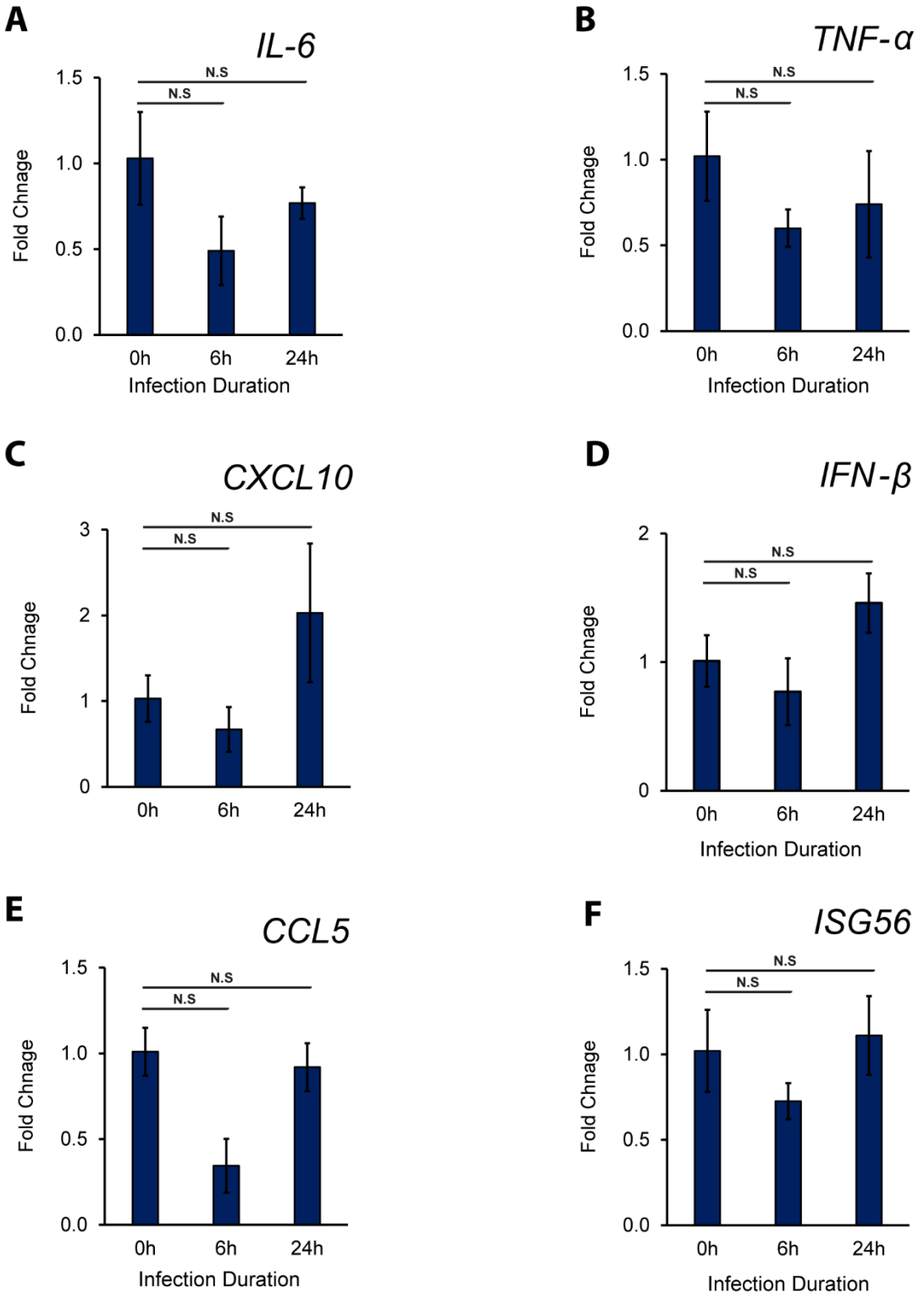


Figure 34: The HaCaT cytokine response to *L.mexicana* over 24h

WT HaCaTs were infected with *L.mexicana* at a MOI of 10 for over 24h as indicated. At each time point cells were lysed for qRT-PCR analysis of **(A)** IL-6 **(B)** TNF- α **(C)** CXCL10 **(D)** IFN-β **(E)** CCL5 and **(F)** ISG56 mRNA. Data are presented as mean values of biological triplicates. Error bars indicate standard deviations. Data are representative of at least three experiments.

7.4 Changes to the DNA sensing pathway occur in infected HaCaT cells

To determine if modifications to DNA sensing components STING, cGAS and IFI16 observed in infected THP-1 cells were also present in keratinocytes, we investigated protein changes by western blot analysis of infected cells during a 24h time course (Fig.35A). Modifications to STING, IFI16 and cGAS was also seen in these cells from as early as 2h post infection.

The smaller form of STING was strongly induced from 2h post infection and is simultaneously associated with loss of normal size STING, which after 24h post infection is completely absent from the HaCaTs.

We have suggested that the host proteasome could be involved in the parasite induced cleavage of STING in THP-1 cells (Fig.25), so to determine if the proteasome is also involved in STING cleavage in HaCaTs we pre-treated HaCaTs with proteasome inhibitor MG132 or the autophagy inhibitor Bafilomycin before infection over 6h (Fig.35B). As in the THP-1 cells, cleavage of STING is prevented in the MG132 treated cells at all infection timepoints from 2h to 6h.

In addition to STING we also see that the size modifications to cGAS and IFI16 seen in THP-1 cells are also present in the HaCaTs. This includes large increase in the size of cGAS that occurs by the 4h infection timepoint. We have tentatively suggested that this cGAS band could represent a cGAS dimer due to the size being approximately double of normal cGAS. Any normal post-translational modification to cGAS is also highly unlikely to cause such a large size increase.

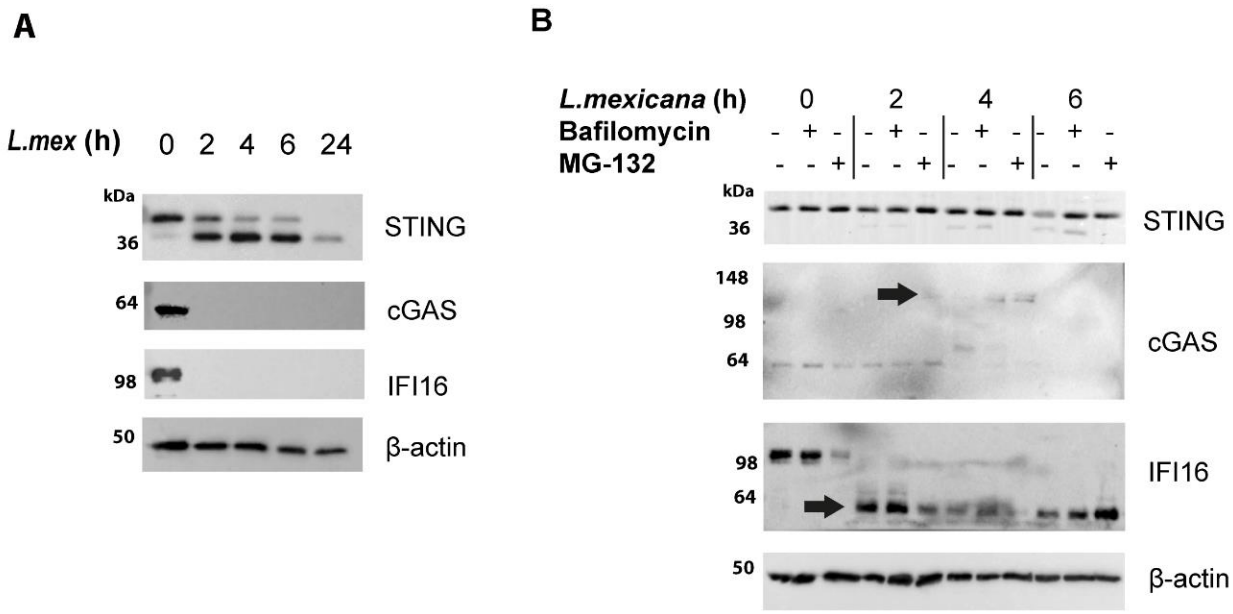


Figure 35: The proteasome may be required for the appearance of a faster migrating STING band during *L.mexicana* infection in HaCaTs

WT HaCaTs were infected with *L.mexicana* at a MOI of 10 over 24 hours as indicated before lysis for western blot analysis of protein expression. Data are representative of at least three experiments. **(B)** WT HaCaT cells were infected with *L.mexicana* over 6 hours as indicated after a 1 hour pre-treatment of either 10μM MG132 or 50nM Bafilomycin. Cells were then lysed for western blot analysis of protein expression. Arrows indicate the change in size of cGAS and IFI16 in infected cells. Data are representative of at least three experiments.

The faster migrating cGAS band observed on the western blot is no longer detected after 6h infection. This is similar to what was observed in the THP-1 cells where this alternative cGAS band also could no longer be detected by 6h infection (Fig.25).

We have suggested the IFI16 modification could be a cleavage due to the decrease in size during infection occurs as early as 2h after infection in the HaCaTs. This timing is similar to what was observed in the THP-1s. The difference in the timings of the modifications observed in cGAS (4h post infection) and IFI16 (2h post infection) suggest they are mediated by *Leishmania* infection in different ways.

So far we have demonstrated that entry into keratinocytes can occur quickly and that *Leishmania* virulence factors remain active, suggesting that methods of evasion are not limited to the parasites primary host cells such as macrophages.

7.5 STING localisation and translocation is unchanged during *L.mexicana* infection of HaCaT cells

We used confocal microscopy to not only visually confirm infection of these HaCaTs but also investigate the activity of cleaved STING in these cells. From the western blot analysis (Fig.35A) by 4h post infection the smaller cleaved STING is much more abundant within these cells than the full-length STING. A primary antibody against STING and a fluorescently labelled secondary antibody was used to investigate if STING localisation changed during infection.

Additionally, infected cells were also stimulated with dsDNA prior to fixation to examine if STING clustering can still occur within infected cells in which cleaved STING is the dominant form. Primary antibody against alpha tubulin was also used to visualise *Leishmania* and DAPI as a nuclear DNA stain.

The presence of internalised parasites within HaCaTs can be shown after a 6 hour infection (Fig.36). STING clustering after dsDNA stimulation is also occurs in both uninfected and infected cells (Fig. 36). In the canonical DNA sensing pathway cGAMP binding triggers a conformational change in STING, which induces STING clustering in the peri-nuclear region. It is here where the recruitment and activation of downstream signalling molecules TBK1 and IRF3 occur (Ishikawa, 2009; Zhang, 2013). Even though STING is cleaved, it still isable to translocate from the ER to signalling foci. From the images there also seems to be some co-localisation of STING and *Leishmania* (arrowed), including some parasites that look atypical. This could suggest that STING is able to sense damaged *Leishmania*.

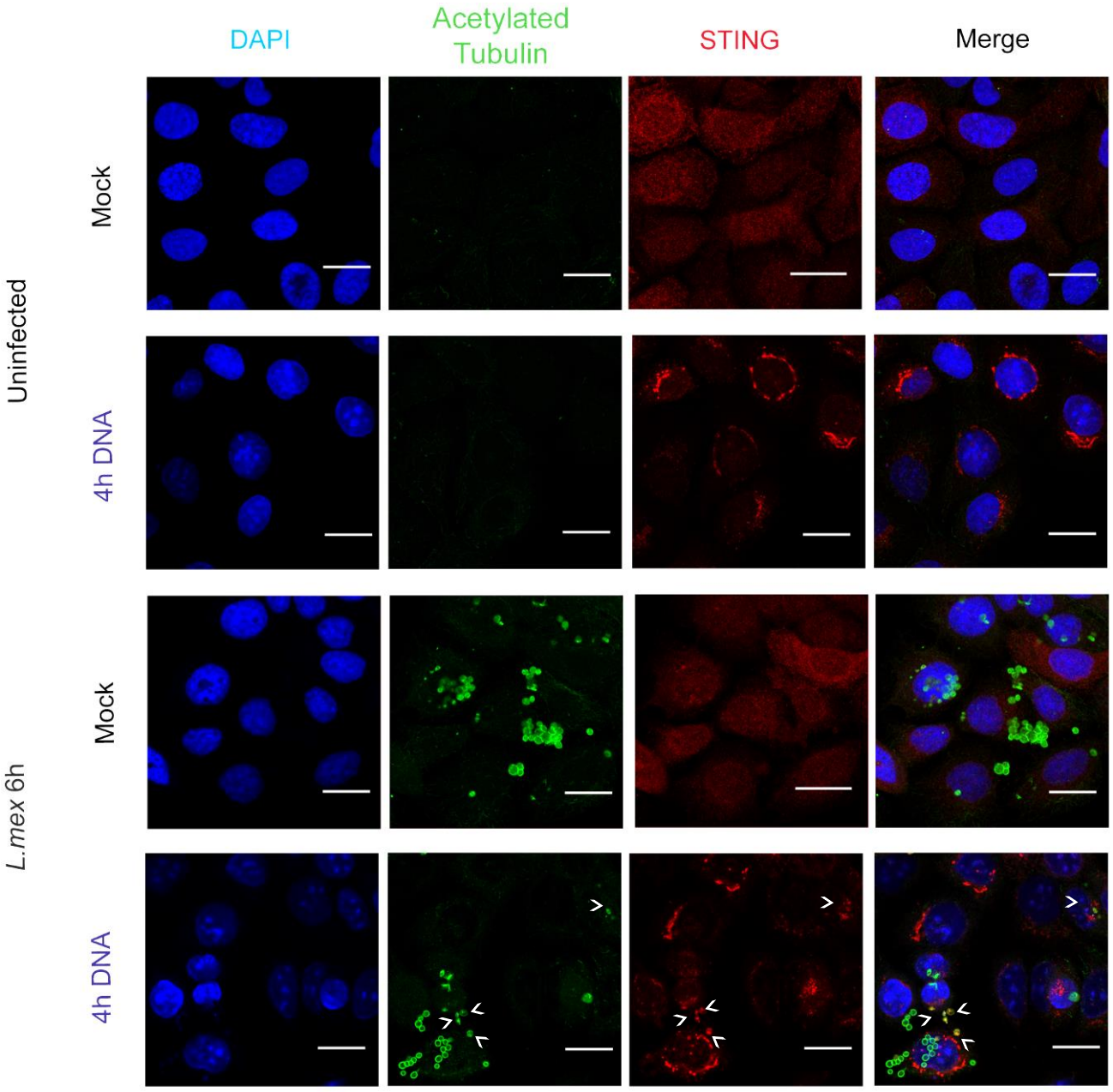


Figure 36: STING localisation is unchanged and translocation after DNA stimulation occurs in *L.mexicana* infected HaCaT cells

WT HaCaTs were seeded onto cover slips and differentiated with PMA for 48h before infection with *L.mexicana* at a MOI of 10 for 6 hours. Cells were then mock transfected (lipofectamine) or transfected with 5µg/ml HT-DNA for 4 hours. Cells were then fixed in methanol and stained for Acetylated Tubulin to visualize the parasites (green), STING (red) and DAPI nuclear stain (blue) and imaged by confocal microscopy. Arrows indicate STING and *L.mexicana* co-localisation Scale bar = 20µm

7.6 No enhancement of the DNA sensing pathway is observed in *L.mexicana* infected HaCaT cells

We examined activation of the DNA sensing pathway within infected HaCats by investigating gene expression after dsDNA stimulation. Using qRT-PCR we can show that gene expression of IFN- β and other key cytokines and chemokines are induced after dsDNA stimulation of infected cells (Fig.37) confirming the pathway is active. The dsDNA induced gene expression does not significantly differ between the infected and uninfected HaCaTs unlike the enhancement we have demonstrated in the THP-1 cells.

We also looked at enhancement at the protein level via western blot analysis. Keratinocytes were infected for 4h before 4h HT-DNA transfection (Fig.38). In the uninfected cells dsDNA transfection causes an increase in phosphorylated p65 and phosphorylated STING (indicated by the upper band). During infection no phosphorylated p65 is present in the mock or dsDNA treated cells, likely due to cleavage by GP63 or CP. Phosphorylated STING can be observed but the induction is very weak. This is in contrast with the enhancement of phosphorylated STING induced after dsDNA transfection of infected THP-1 cells (Figs.13,16). However, as we have seen no enhancement of cytokine production after dsDNA transfection in these cells (Fig.37) it makes sense that we also do not observe enhancement of STING phosphorylation. We do however again see a loss of normal size STING and the appearance of the lower STING band in both the mock and stimulated infected HaCaTs.

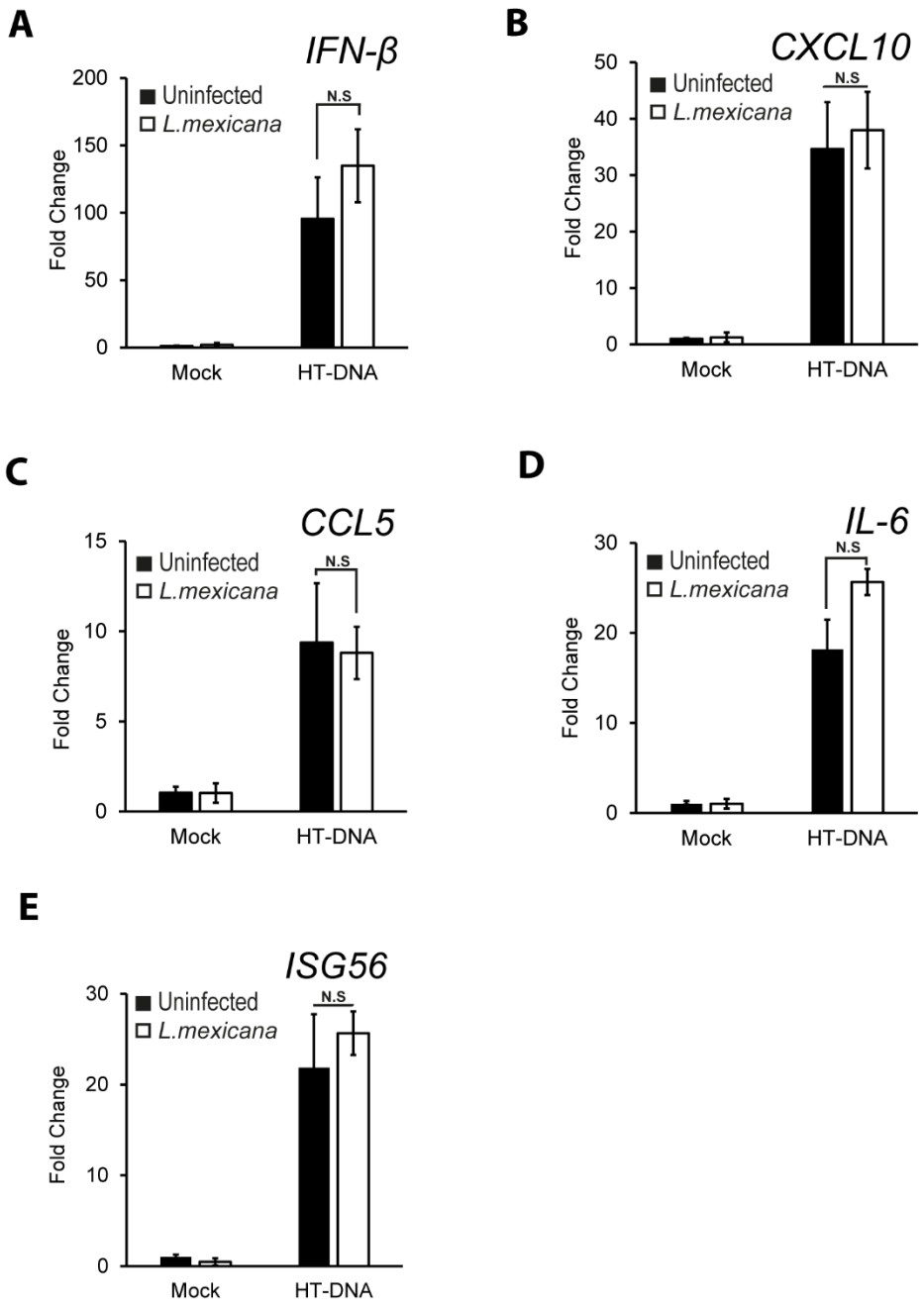


Figure 37: There is not an enhanced response to dsDNA in infected HaCaTs

WT HaCaTs infected with *L.mexicana* at a MOI of 10 for 4 hours before stimulation with 1µg/ml HT-DNA for 4 hours. Cells were then lysed for qRT-PCR analysis of **(A)** IFN-β **(B)** CXCL10 **(C)** CCL5 **(D)** IL-6 and **(E)** ISG56 mRNA. Data are presented as mean values of biological triplicates. Error bars indicate standard deviations. Data are representative of at least three experiments.

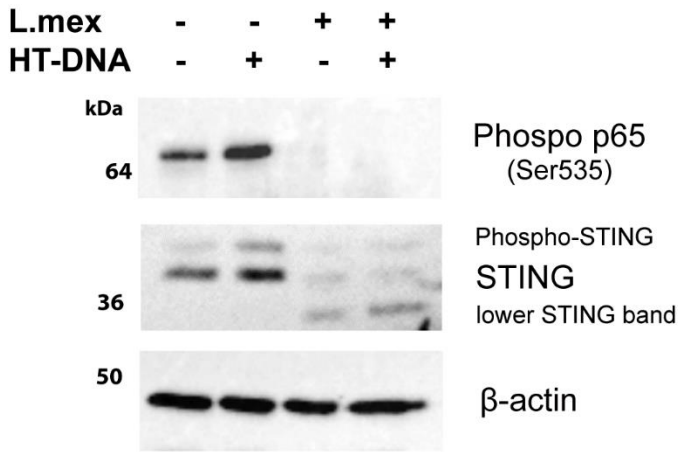


Figure 38: No enhancement of STING activation following dsDNA stimulation in *L.mexicana* infected keratinocytes

WT HaCaT cells were infected with media only or *L.mexicana* at a MOI of 10 for 4h before stimulation with 5µg/ml transfected HT-DNA for 4 hours. Cells were then lysed for western blot analysis of protein expression.

7.7 Chapter Discussion

In this chapter we demonstrated successful infection of mouse BMDMs and human keratinocytes by *L.mexicana* and that cleavage of both p65 and STING also occurs during these infections.

During infection of the primary mouse BMDMs, at both 6h and 24h post-infection we saw a complete loss of normal size STING in favour of its smaller cleaved form. This confirms that this interaction with STING is not restricted to human STING and suggests that the site of cleavage is conserved between human and mouse STING. It is also notable that we did not observe a faint band for cleaved STING in uninfected BMDMs, whereas we have in uninfected THP-1 cells in many of our previous experiments. Thus, it is possible that formation of this form of STING under non-infection conditions is restricted to human macrophages.

No enhancement of IFN-β, CXCL10, CCL5 or IL-6 production in response to HT-DNA transfection was observed in our infected BMDMs compared to the uninfected control. This suggests that there is no enhancement of the DNA sensing pathway in this cell type compared to what we have observed in the THP-1 cells. This variance could be explained by a difference in the interactions of parasite and cell components between human and mice cells. There is also known to be variation in how the cytoplasmic DNA sensing pathway is activated and regulated during normal activity between mice and humans (Brunette et al., 2012; Burleigh et al., 2020; Jønsson et al., 2017). However, based on our proposed possible mechanism for what may be occurring in THP-1 cells to mediate this enhancement (Fig.28), the lack of enhancement in these cells could be explained by the complete loss of normal STING in BMDMs infected with *L.mexicana* (Fig.31C). In these westerns blots there is no normal size STING left, and a key part of this proposed mechanism is the interaction of normal STING and the smaller variant to polymerise. Why there is a complete loss of normal STING in this infection compared to others we have

observed previously (Figs.11,12(6h),13,16,17,25) is currently unclear, but could be explained by a more effective infection and therefore more STING cleavage.

Investigating if changes to cGAS also occur in BMDMs could be a future experiment to help us to understand how *Leishmania* interacts with the DNA sensing pathway in mice other than cleaving STING.

While *Leishmania* parasites clearly interact with keratinocytes, their internalisation by these cells remains controversial. In the majority of previous studies it has been shown that *Leishmania* do not infect mouse-derived keratinocytes *in vivo* or *ex vivo* (Mbow et al., 2001; Ronet et al., 2019; Vasconcellos and Sotto, 1997). However, one study carried out by Scorza *et al.* has shown that internalization of parasites by human keratinocytes *in vitro* does occur, although intracellular replication and survival is not supported (Scorza et al., 2017). It is possible that mouse and human keratinocytes may exhibit different patterns of internalisation. Efficiency of internalisation may also depend on the parasite species, especially as different *Leishmania* species can have differing abilities in the activation of keratinocytes to produce immunomodulatory mediators (Scorza et al., 2017).

Keratinocytes are not a primary host for *Leishmania*, whether they are internalised or not, but they can have a significant impact on the course of infection, with keratinocyte activation by *Leishmania* generally associated with a host protective response that limits infection within monocytes and leads to parasite control (Jafarzadeh et al., 2021).

Our qRT-PCR and confocal microscopy data (Figs.33A and 36) strongly suggested successful infection of the human HaCaT keratinocytes by *Leishmania*, with clear internalisation of *Leishmania* parasite visible by confocal microscopy (Fig.36). It is still uncertain if parasite replication within these cells was also occurring. The approximate parasite number after 24h is comparable to that of the promastigote infection in THP-1s after 24h in Figure 2B, but much lower than what was observed in the amastigote infection over the same period (Fig.2B). More repeat experiments, including longer time points

could be carried out to determine the extent of *Leishmania* replication more accurately within these cells. For this experiment proof of parasite entry is still important as protein changes that we have already observed in the THP-1s and BMDMs such as the cleavage of p65 to p35 occurred within the first 6h of infection (Fig.31C). This suggests that initial infection of the parasites is sufficient for the activity of *Leishmania* virulence factors and ongoing parasite replication is not necessarily required.

The previous study that suggested that keratinocytes do not support intracellular growth also looked at parasite numbers after much longer infection period of 120 hours (Scorza et al., 2017). It is therefore possible that earlier in the infection some parasite replication may occur, but in the long-term keratinocytes would not support *Leishmania* growth. *In vivo* other factors may also influence the ability of keratinocytes to support long term parasite replication: the induction of apoptosis in many keratinocytes during cutaneous leishmaniasis is one example of why they are unlikely to be a suitable long-term host. However, as we are primarily investigating changes in the innate immune response that occurs early on in parasite infection (2h to 6h), achieving long term parasitisation of these keratinocytes is not crucial for these experiments.

We observed cleavage of p65 to p35 in the keratinocytes from 4h post infection (Fig.33) . This is the first time p65 cleavage has been shown to be mediated by *Leishmania* in this cell type. However p65 cleavage has also been observed in infected dendritic cells, suggesting it is not just limited to macrophage or keratinocyte derived p65 (Neves et al., 2010). No significant production of IL-6, TNF-a, CXCL10, IFN-β, CCL5 ISG56 cytokine after 6h and 24h post infection was observed with parasite infection alone in the HaCaTs (Fig.34). The absence of IL-6 and TNF-a are likely to be due to the p65 degradation. The lack of an induction of cytokines that are associated with the DNA response is similar to what we have seen in infected THP-1 cells, although there we did see some induction of IFN- β and CXCL10 in those cells at later time points (Fig.5). This could suggest that *in vitro* keratinocytes are less responsive to *Leishmania* parasites than macrophages.

Investigation of the DNA sensing pathway further in keratinocytes revealed that like infected THP-1 cells and mouse BMDMs, we also see cleavage of STING (Fig.33). Additionally, we have shown that proteasome inhibitor pre-treatment also prevents this cleavage in this cell type.

Confocal microscopy also allowed us to investigate if this cleavage prevents STING translocation and clustering during dsDNA treatment of keratinocytes. Based on previous experiments, we have suggested that it is a region of the C-terminal tail (CTT) is cleaved during infection to form the smaller STING we observe by western blot. Based on the confocal images, loss of most of the CTT does not prevent STING clustering. This could suggest that the STING C -terminal region is not required for cGAMP binding and STING translocation to the perinuclear signalling foci, which supports some of what we have suggested about the role of the STING CTT (Fig.28). However, we can also determine from these images that HaCaT cells were not all consistently infected. We cannot say that all of STING is cleaved within the individual infected cells visible, so the STING clustering observed could be the non-cleaved STING. These images also appear to show some co-localisation between STING and incoming *Leishmania* (Fig.36). This co-localisation primarily appears to be with *Leishmania* parasites that appear damaged or have an altered morphology. We could speculate that some DNA from damaged *Leishmania* may be sensed by STING. More repeats of the confocal experiments will be needed to verify this co-localisation.

Modifications to cGAS and IFI16 also were observed in these keratinocytes, again similar to in the THP-1 cells (Fig.27). As has been noted in the THP-1 cells, these modifications do not prevent subsequent downstream activation of the DNA sensing pathway or changes in the response, as seen by qRT-PCR of the cytokine response. The same is true for the keratinocytes as after dsDNA treatment we have seen that STING clustering still occurs by confocal microscopy and by qRT-PCR we still see a cytokine response (Fig.37). However, unlike infected THP-1 cells, we do not see any enhancement in the production

of any of these cytokines, including IFN- β, compared to uninfected cells. No enhancement in STING phosphorylation is observed by western blot after dsDNA transfection (Fig.38), which was seen in THP-1 cells (Figs.13,16).

In both the infected BMDMs and HaCaTs we have observed similar changes at the protein level that we first detected in THP-1 cells, such as the cleavage of STING. However, we only observe an enhancement in the activation of the DNA sensing pathway in THP-1 cells. However cell-type specific immune modulation is not completely unexpected as keratinocytes are not known to be widely parasitised by *Leishmania* and are not the primary host cell of the parasite. Modulations to the innate immune system that *Leishmania* may have evolved to favour growth in human macrophages are not necessarily also going to be observed in a non-host cell type like keratinocytes. It is possible that the enhancement to the DNA sensing pathway is one of these modulations although further investigation will be required to understand how it benefits *Leishmania* infection.

The observation that STING cleavage also occurs in primary mouse cells and keratinocytes strengthens this discovery and demonstrates that it is not isolated to cell line macrophages, nor is STING cleavage restricted to one species. STING cleavage does not seem to impair the function of the DNA sensing pathway but may also not necessarily enhance it, as STING size can be uncoupled from enhanced DNA sensing in these different cell types. Further investigation is required to understand how *Leishmania* infection primes or enhances STING signalling in human macrophages, and whether this is a detection mechanism that favours the host, or an immune evasion strategy of the parasite.

Chapter 8: Final Conclusions

Leishmaniasis continues to be a disease of significant global concern with high morbidity and mortality if left untreated. However, the development of therapeutic targets and vaccine candidates has seen slow progression. A full knowledge of *Leishmania* virulence factors and a fuller understanding of parasite survival mechanisms is key to identify potent therapeutic targets and expedite the drug discovery process.

In this project, we set out to further investigate the interplay between *Leishmania* and the innate immune system during initial infection by examining interactions of *Leishmania* and various PRRs, with a focus on the under researched cytosolic DNA sensors. One of our key aims was to explore if *L.mexicana* can inhibit the RLR pathway or cytosolic DNA sensing pathway during infection, similar to how they can inhibit signalling downstream of the TLRs.

Here we have shown that *Leishmania* infection alone does not significantly activate the cytosolic DNA sensing pathway by itself, but it does dysregulate the response to transfected dsDNA in human THP-1 macrophages. It does not appear to dysregulate the normal downstream response of the RNA sensing pathway. Through our initial experiments we discovered that *Leishmania* infection enhances the cytokine response to dsDNA. Further investigation demonstrated that phosphorylation of STING, a key adaptor protein for the cytosolic DNA sensors after dsDNA stimulation was enhanced in infected cells and also that STING was modified during infection. Through additional experiments we determined that this modification was a smaller form of STING formed through an active cleavage at the C-terminal tail (CTT) during infection.

One of the key open questions in relation to this finding is if this cleaved STING contributes to the enhancement of the dsDNA response within infected cells, especially as this change in STING size can be uncoupled from enhanced DNA sensing in different cell types (mouse BMDMs and human keratinocytes). However, we have proposed a mechanism of

action in which cleavage of some STING could act to enhance STING signalling based on what is known about the autoinhibition of STING by the CTT (Fig.28). Ergun et al. (2019) have shown that CTT binding to the ligand binding domain of STING protects the polymer interface and that STING mutants lacking the CTT can undergo STING polymerisation without ligand activation first. Therefore, it is conceivable that cleavage of the CTT may also result in spontaneous STING polymerisation in our infected cells - a structural change thought to be essential for its activation. It is conceivable that if some full-length STING is also included in this polymerization event, downstream signalling may still be able to occur and could be enhanced, thus priming the DNA sensing response. In the future, additional experiments such as a co- transfection of WT STING and STING missing its CTT could be carried out to determine if it is this truncated STING that enhances the response to dsDNA.

Another open question that this work has not fully resolved, is whether this STING modification and enhanced DNA response is to the benefit or detriment of the parasite. A recent study conducted by Yilmaz et al (2022), and one of the only to investigate *Leishmania* infection and cytoplasmic DNA sensing, suggested that *Leishmania mexicana* may hijack the cGAS-STING-TBK1 signalling pathway to their own advantage to promote a pro-parasitic state. Our experiments suggested that neither the presence of STING nor type I interferons were beneficial to the initial infection of macrophages *in vitro*. However, there is room for further investigation in this area, as the secretion of interferons, cytokines and chemokines could have additional effects *in vivo*, for instance in the recruitment of further macrophages for the propagation of infection, or the dysregulation of adaptive immune responses in the local tissue.

While we have consistently demonstrated that modification to STING occurs across multiple cell types with infection, we have not definitively determined how this cleavage is mediated. One of our project aims was to try and identify additional *Leishmania* virulence factors that could have a role in subverting host innate immune signalling pathways. While

we have not been able to fully achieve this aim we have tentatively concluded that *Leishmania* virulence factor GP63 was not responsible for STING cleavage. GP63 was a protease of interest in our investigation due to its promiscuous enzymatic action and known ability to cleave a wide range of cellular targets. However, we have shown that without the influence of additional *Leishmania* factors, over-expressed GP63 cannot cleave STING, while it could still cause degradation of p65. There is still much work to be done in identifying additional *Leishmania* virulence factors, and it is likely that future work utilising global proteomics to study host-*Leishmania* interactions will make progress in this area where we have not yet been able to.

We have determined a potential role for the host proteasome pathway in STING cleavage, as known proteasome inhibitor MG132 prevented formation of smaller STING during infection. Additional experiments will first need to confirm that this is not due to MG132 affecting the parasite proteasome and thus parasite viability. Further investigation will then be necessary to determine if proteasome activity is directly or indirectly responsible for host cleavage, and how *Leishmania* may enhance a host process. We have noted that *Leishmania* has already been reported to be able to hijack host proteasome machinery to target STAT-1 for degradation (Forget et al., 2005).

Involvement of the host proteasome in the regulation of STING cleavage would support our early hypothesis that this STING variant can also occur naturally within the cell even without *Leishmania* infection. This is based on the consistent presence of a faint band representing the smaller STING within uninfected samples. Determining whether this smaller STING has a novel regulatory role within cells would be an interesting subject for future work.

Overall, this work contributes to the knowledge of how *Leishmania* interact with host proteins during early infection to establish infection. We propose that the cGAS-STING DNA sensing pathway is another PRR pathway that is dysregulated by *Leishmania.*

STING and potentially other components of this pathway such as IFI16 are shown to be modified by *Leishmania* infection for the first time. More research will be required to understand the extent of how the dysregulation of the pathway impacts on *Leishmania* parasite growth and disease progression. If further work does reveal it to be beneficial for the parasite this could make a potential therapeutic target. We also add to the knowledge of STING function and regulation and suggest that the discovery of a smaller STING form should be followed up to further understand a potential role within normal DNA sensing.

References

Abou-El-Hassan, H., Zaraket, H., 2017. Viral-derived complement inhibitors: current status and potential role in immunomodulation. Exp. Biol. Med. Maywood NJ 242, 397–410. https://doi.org/10.1177/1535370216675772

Abu-Dayyeh, I., Hassani, K., Westra, E.R., Mottram, J.C., Olivier, M., 2010. Comparative study of the ability of Leishmania mexicana promastigotes and amastigotes to alter macrophage signaling and functions. Infect. Immun. 78, 2438–2445. https://doi.org/10.1128/IAI.00812-09

Aguirre, S., Maestre, A.M., Pagni, S., Patel, J.R., Savage, T., Gutman, D., Maringer, K., Bernal-Rubio, D., Shabman, R.S., Simon, V., Rodriguez-Madoz, J.R., Mulder, L.C.F., Barber, G.N., Fernandez-Sesma, A., 2012. DENV Inhibits Type I IFN Production in Infected Cells by Cleaving Human STING. PLoS Pathog. 8, e1002934. https://doi.org/10.1371/journal.ppat.1002934

Akira, S., Takeda, K., Kaisho, T., 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. 2, 675–680. https://doi.org/10.1038/90609

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801. https://doi.org/10.1016/j.cell.2006.02.015

Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413, 732–738. https://doi.org/10.1038/35099560

Alhede, Maria, Bjarnsholt, T., Givskov, M., Alhede, Morten, 2014. Pseudomonas aeruginosa biofilms: mechanisms of immune evasion. Adv. Appl. Microbiol. 86, 1–40. https://doi.org/10.1016/B978-0-12-800262-9.00001-9

Alitalo, A., Meri, T., Rämö, L., Jokiranta, T.S., Heikkilä, T., Seppälä, I.J., Oksi, J., Viljanen, M., Meri, S., 2001. Complement evasion by Borrelia burgdorferi: serum-

resistant strains promote C3b inactivation. Infect. Immun. 69, 3685–3691. https://doi.org/10.1128/IAI.69.6.3685-3691.2001

Almine, J.F., O'Hare, C.A.J., Dunphy, G., Haga, I.R., Naik, R.J., Atrih, A., Connolly, D.J., Taylor, J., Kelsall, I.R., Bowie, A.G., Beard, P.M., Unterholzner, L., 2017. IFI16 and cGAS cooperate in the activation of STING during DNA sensing in human keratinocytes. Nat. Commun. 8, 14392. https://doi.org/10.1038/ncomms14392

Al-Salem, W.S., Pigott, D.M., Subramaniam, K., Haines, L.R., Kelly-Hope, L., Molyneux, D.H., Hay, S.I., Acosta-Serrano, A., 2016. Cutaneous Leishmaniasis and Conflict in Syria. Emerg. Infect. Dis. 22, 931–933. https://doi.org/10.3201/eid2205.160042

Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., Boer, M. den, the WHO Leishmaniasis Control Team, 2012. Leishmaniasis Worldwide and Global Estimates of Its Incidence. PLoS ONE 7, e35671. https://doi.org/10.1371/journal.pone.0035671

Alvar, J., Yactayo, S., Bern, C., 2006. Leishmaniasis and poverty. Trends Parasitol. 22, 552–557. https://doi.org/10.1016/j.pt.2006.09.004

Antoniazi, S., Price, H.P., Kropf, P., Freudenberg, M.A., Galanos, C., Smith, D.F., Müller, I., 2004. Chemokine gene expression in toll-like receptor-competent and -deficient mice infected with Leishmania major. Infect. Immun. 72, 5168–5174. https://doi.org/10.1128/IAI.72.9.5168-5174.2004

Aoki, J.I., Muxel, S.M., Zampieri, R.A., Müller, K.E., Nerland, A.H., Floeter-Winter, L.M., 2019. Differential immune response modulation in early Leishmania amazonensis infection of BALB/c and C57BL/6 macrophages based on transcriptome profiles. Sci. Rep. 9, 19841. https://doi.org/10.1038/s41598-019-56305-1

Arango Duque, G., Jardim, A., Gagnon, É., Fukuda, M., Descoteaux, A., 2019. The host cell secretory pathway mediates the export of Leishmania virulence factors out of the

parasitophorous vacuole. PLoS Pathog. 15, e1007982. https://doi.org/10.1371/journal.ppat.1007982

Araujo-Abad, S., Saceda, M., de Juan Romero, C., 2022. Biomedical application of small extracellular vesicles in cancer treatment. Adv. Drug Deliv. Rev. 182, 114117. https://doi.org/10.1016/j.addr.2022.114117

Arboleda, M., Barrantes, S., Úsuga, L.Y., Robledo, S.M., 2019. Successful treatment of cutaneous leishmaniasis with intralesional meglumine antimoniate: A case series. Rev. Soc. Bras. Med. Trop. 52, e20180211. https://doi.org/10.1590/0037-8682-0211-2018

Arce, A., Estirado, A., Ordobas, M., Sevilla, S., García, N., Moratilla, L., de la Fuente, S., Martínez, A.M., Pérez, A.M., Aránguez, E., Iriso, A., Sevillano, O., Bernal, J., Vilas, F., 2013. Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. Eurosurveillance 18. https://doi.org/10.2807/1560-7917.ES2013.18.30.20546

Atayde, V.D., Hassani, K., da Silva Lira Filho, A., Borges, A.R., Adhikari, A., Martel, C., Olivier, M., 2016. Leishmania exosomes and other virulence factors: Impact on innate immune response and macrophage functions. Cell. Immunol. 309, 7–18. https://doi.org/10.1016/j.cellimm.2016.07.013

Atayde, V.D., Suau, H.A., Townsend, S., Hassani, K., Kamhawi, S., Olivier, M., 2015. Exosome secretion by the parasitic protozoan Leishmania within the sand fly midgut. Cell Rep. 13, 957–967. https://doi.org/10.1016/j.celrep.2015.09.058

Baars, I., Jaedtka, M., Dewitz, L.-A., Fu, Y., Franz, T., Mohr, J., Gintschel, P., Berlin, H., Degen, A., Freier, S., Rygol, S., Schraven, B., Kahlfuß, S., van Zandbergen, G., Müller, A.J., 2023. Leishmania major drives host phagocyte death and cell-to-cell transfer depending on intracellular pathogen proliferation rate. JCI Insight 8, e169020. https://doi.org/10.1172/jci.insight.169020

Bäckhed, F., Söderhäll, M., Ekman, P., Normark, S., Richter-Dahlfors, A., 2001. Induction of innate immune responses by Escherichia coli and purified lipopolysaccharide correlate with organ- and cell-specific expression of Toll-like receptors within the human urinary tract. Cell. Microbiol. 3, 153–158.

Bahrami, F., Masoudzadeh, N., Van Veen, S., Persson, J., Lari, A., Sarvnaz, H., Taslimi, Y., Östensson, M., Andersson, B., Sharifi, I., Goyonlo, V.M., Ottenhoff, T.H., Haks, M.C., Harandi, A.M., Rafati, S., 2022. Blood transcriptional profiles distinguish different clinical stages of cutaneous leishmaniasis in humans. Mol. Immunol. 149, 165–173. https://doi.org/10.1016/j.molimm.2022.07.008

Bamigbola, I.E., Ali, S., 2022. Paradoxical immune response in leishmaniasis: The role of toll-like receptors in disease progression. Parasite Immunol. 44, e12910. https://doi.org/10.1111/pim.12910

Barak, E., Amin-Spector, S., Gerliak, E., Goyard, S., Holland, N., Zilberstein, D., 2005. Differentiation of Leishmania donovani in host-free system: analysis of signal perception and response. Mol. Biochem. Parasitol. 141, 99–108. https://doi.org/10.1016/j.molbiopara.2005.02.004

Barbosa, F.M.C., Dupin, T.V., Toledo, M.D.S., Reis, N.F.D.C., Ribeiro, K., Cronemberger-Andrade, A., Rugani, J.N., De Lorenzo, B.H.P., Novaes E Brito, R.R., Soares, R.P., Torrecilhas, A.C., Xander, P., 2018. Extracellular Vesicles Released by Leishmania (Leishmania) amazonensis Promote Disease Progression and Induce the Production of Different Cytokines in Macrophages and B-1 Cells. Front. Microbiol. 9, 3056. https://doi.org/10.3389/fmicb.2018.03056

Bardoel, B.W., van der Ent, S., Pel, M.J.C., Tommassen, J., Pieterse, C.M.J., van Kessel, K.P.M., van Strijp, J.A.G., 2011. Pseudomonas Evades Immune Recognition of Flagellin in Both Mammals and Plants. PLoS Pathog. 7, e1002206. https://doi.org/10.1371/journal.ppat.1002206

Barral, P.M., Sarkar, D., Fisher, P.B., Racaniello, V.R., 2009. RIG-I is cleaved during picornavirus infection. Virology 391, 171–176. https://doi.org/10.1016/j.virol.2009.06.045

Bassères, D.S., Baldwin, A.S., 2006. Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. Oncogene 25, 6817–6830. https://doi.org/10.1038/sj.onc.1209942

Bates, P.A., 1994. Complete developmental cycle of Leishmania mexicana in axenic culture. Parasitology 108 (Pt 1), 1–9. https://doi.org/10.1017/s0031182000078458

Bates, P.A., Robertson, C.D., Tetley, L., Coombs, G.H., 1992. Axenic cultivation and characterization of Leishmania mexicana amastigote-like forms. Parasitology 105, 193–202. https://doi.org/10.1017/S0031182000074102

Bates, P.A., Rogers, M.E., 2004. New insights into the developmental biology and transmission mechanisms of Leishmania. Curr. Mol. Med. 4, 601–609. https://doi.org/10.2174/1566524043360285

Bates, P.A., Tetley, L., 1993. Leishmania mexicana: induction of metacyclogenesis by cultivation of promastigotes at acidic pH. Exp. Parasitol. 76, 412–423. https://doi.org/10.1006/expr.1993.1050

Becker, I., Salaiza, N., Aguirre, M., Delgado, J., Carrillo-Carrasco, N., Kobeh, L.G., Ruiz, A., Cervantes, R., Torres, A.P., Cabrera, N., González, A., Maldonado, C., Isibasi, A., 2003. Leishmania lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. Mol. Biochem. Parasitol. 130, 65–74. https://doi.org/10.1016/s0166-6851(03)00160-9

Beiting, D.P., 2014. Protozoan parasites and type I interferons: a cold case reopened. Trends Parasitol. 30, 491–498. https://doi.org/10.1016/j.pt.2014.07.007

Belkaid, Y., Butcher, B., Sacks, D.L., 1998. Analysis of cytokine production by inflammatory mouse macrophages at the single-cell level: selective impairment of IL-12 induction in Leishmania-infected cells. Eur. J. Immunol. 28, 1389–1400.

https://doi.org/10.1002/(SICI)1521-4141(199804)28:04<1389::AID-IMMU1389>3.0.CO;2-1

Belkaid, Y., Hoffmann, K.F., Mendez, S., Kamhawi, S., Udey, M.C., Wynn, T.A., Sacks, D.L., 2001. The Role of Interleukin (IL)-10 in the Persistence of Leishmania major in the Skin after Healing and the Therapeutic Potential of Anti–IL-10 Receptor Antibody for Sterile Cure. J. Exp. Med. 194, 1497–1506. https://doi.org/10.1084/jem.194.10.1497

Bennis, I., De Brouwere, V., Belrhiti, Z., Sahibi, H., Boelaert, M., 2018. Psychosocial burden of localised cutaneous Leishmaniasis: a scoping review. BMC Public Health 18, 358. https://doi.org/10.1186/s12889-018-5260-9

Berasain, P., Carmona, C., Frangione, B., Cazzulo, J.J., Goñi, F., 2003. Specific cleavage sites on human IgG subclasses by cruzipain, the major cysteine proteinase from Trypanosoma cruzi. Mol. Biochem. Parasitol. 130, 23–29. https://doi.org/10.1016/s0166-6851(03)00139-7

Bernier, R., Turco, S.J., Olivier, M., Tremblay, M., 1995. Activation of human immunodeficiency virus type 1 in monocytoid cells by the protozoan parasite Leishmania donovani. J. Virol. 69, 7282–7285.

Bhardwaj, N., Rosas, L.E., Lafuse, W.P., Satoskar, A.R., 2005. Leishmania inhibits STAT1-mediated IFN-γ signaling in macrophages: increased tyrosine phosphorylation of dominant negative STAT1β by Leishmania mexicana. Int. J. Parasitol. 35, 75–82. https://doi.org/10.1016/j.ijpara.2004.10.018

Bhatnagar, S., Shinagawa, K., Castellino, F.J., Schorey, J.S., 2007. Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo. Blood 110, 3234–3244. https://doi.org/10.1182/blood-2007-03-079152

Bifeld, E., Clos, J., 2015. The genetics of Leishmania virulence. Med. Microbiol. Immunol. (Berl.) 204, 619–634. https://doi.org/10.1007/s00430-015-0422-1

Bilgic-Temel, A., Murrell, D.F., Uzun, S., 2019. Cutaneous leishmaniasis: A neglected disfiguring disease for women. Int. J. Womens Dermatol. 5, 158–165. https://doi.org/10.1016/j.ijwd.2019.01.002

Bizri, N.A., Alam, W., Khoury, M., Musharrafieh, U., Ghosn, N., Berri, A., Bizri, A.R., 2021. The Association Between the Syrian Crisis and Cutaneous Leishmaniasis in Lebanon. Acta Parasitol. 66, 1240–1245. https://doi.org/10.1007/s11686-021-00395-3

Bouvier, J., Etges, R.J., Bordier, C., 1985. Identification and purification of membrane and soluble forms of the major surface protein of Leishmania promastigotes. J. Biol. Chem. 260, 15504–15509.

Bouvier, J., Schneider, P., Etges, R., Bordier, C., 1990. Peptide substrate specificity of the membrane-bound metalloprotease of Leishmania. Biochemistry 29, 10113–10119.

Boxx, G.M., Cheng, G., 2016. The Roles of Type I Interferon in Bacterial Infection. Cell Host Microbe 19, 760. https://doi.org/10.1016/j.chom.2016.05.016

Bradley, K.K., Glowicz, J., Wright, J.H., Clarke, C.F., 2013. Emergence of Autochthonous Cutaneous Leishmaniasis in Northeastern Texas and Southeastern Oklahoma. Am. J. Trop. Med. Hyg. 88, 157–161. https://doi.org/10.4269/ajtmh.2012.11-0717

Brittingham, A., Chen, G., McGwire, B.S., Chang, K.P., Mosser, D.M., 1999. Interaction of Leishmania gp63 with cellular receptors for fibronectin. Infect. Immun. 67, 4477–4484.

Brittingham, A., Morrison, C.J., McMaster, W.R., McGwire, B.S., Chang, K.P., Mosser, D.M., 1995. Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. J. Immunol. Baltim. Md 1950 155, 3102–3111.

Broz, P., Monack, D.M., 2013. Newly described pattern recognition receptors team up against intracellular pathogens. Nat. Rev. Immunol. 13, 551–565. https://doi.org/10.1038/nri3479

Brunette, R.L., Young, J.M., Whitley, D.G., Brodsky, I.E., Malik, H.S., Stetson, D.B., 2012. Extensive evolutionary and functional diversity among mammalian AIM2-like receptors. J. Exp. Med. 209, 1969–1983. https://doi.org/10.1084/jem.20121960

Burleigh, K., Maltbaek, J.H., Cambier, S., Green, R., Gale, M., James, R.C., Stetson, D.B., 2020. Human DNA-PK activates a STING-independent DNA sensing pathway. Sci. Immunol. 5, eaba4219. https://doi.org/10.1126/sciimmunol.aba4219

Butcher, B.A., Kim, L., Johnson, P.F., Denkers, E.Y., 2001. Toxoplasma gondii Tachyzoites Inhibit Proinflammatory Cytokine Induction in Infected Macrophages by Preventing Nuclear Translocation of the Transcription Factor NF- B. J. Immunol. 167, 2193–2201. https://doi.org/10.4049/jimmunol.167.4.2193

Cameron, P., McGachy, A., Anderson, M., Paul, A., Coombs, G.H., Mottram, J.C., Alexander, J., Plevin, R., 2004. Inhibition of lipopolysaccharide-induced macrophage IL-12 production by Leishmania mexicana amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway. J. Immunol. Baltim. Md 1950 173, 3297–3304.

Carrera, L., Gazzinelli, R.T., Badolato, R., Hieny, S., Muller, W., Kuhn, R., Sacks, D.L., 1996. Leishmania promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. J. Exp. Med. 183, 515–526. https://doi.org/10.1084/jem.183.2.515

Casgrain, P.-A., Martel, C., McMaster, W.R., Mottram, J.C., Olivier, M., Descoteaux, A., 2016. Cysteine Peptidase B Regulates Leishmania mexicana Virulence through the Modulation of GP63 Expression. PLoS Pathog. 12, e1005658. https://doi.org/10.1371/journal.ppat.1005658

Chakravarty, J., Sundar, S., 2010. Drug Resistance in Leishmaniasis. J. Glob. Infect. Dis. 2, 167–176. https://doi.org/10.4103/0974-777X.62887

Chan, J., Fujiwara, T., Brennan, P., McNeil, M., Turco, S.J., Sibille, J.C., Snapper, M., Aisen, P., Bloom, B.R., 1989. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. Proc. Natl. Acad. Sci. U. S. A. 86, 2453–2457.

Chan, Y.K., Gack, M.U., 2016. A phosphomimetic-based mechanism of dengue virus to antagonize innate immunity. Nat. Immunol. 17, 523–530. https://doi.org/10.1038/ni.3393

Chandra, D., Naik, S., 2008. Leishmania donovani infection down-regulates TLR2-stimulated IL-12p40 and activates IL-10 in cells of macrophage/monocytic lineage by modulating MAPK pathways through a contact-dependent mechanism. Clin. Exp. Immunol. 154, 224–234. https://doi.org/10.1111/j.1365-2249.2008.03741.x

Chang, C.S., Inserra, T.J., Kink, J.A., Fong, D., Chang, K.P., 1986. Expression and size heterogeneity of a 63 kilodalton membrane glycoprotein during growth and transformation of Leishmania mexicana amazonensis. Mol. Biochem. Parasitol. 18, 197–210. https://doi.org/10.1016/0166-6851(86)90038-1

Channon, J.Y., Roberts, M.B., Blackwell, J.M., 1984. A study of the differential respiratory burst activity elicited by promastigotes and amastigotes of Leishmania donovani in murine resident peritoneal macrophages. Immunology 53, 345–355.

Chaves, M.M., Lee, S.H., Kamenyeva, O., Ghosh, K., Peters, N.C., Sacks, D., 2020. The role of dermis resident macrophages and their interaction with neutrophils in the early establishment of Leishmania major infection transmitted by sand fly bite. PLoS Pathog. 16, e1008674. https://doi.org/10.1371/journal.ppat.1008674

Chen, H., Pei, R., Zhu, W., Zeng, R., Wang, Yun, Wang, Yanyi, Lu, M., Chen, X., 2014. An alternative splicing isoform of MITA antagonizes MITA-mediated induction of type I

IFNs. J. Immunol. Baltim. Md 1950 192, 1162–1170. https://doi.org/10.4049/jimmunol.1300798

Chen, Q., Sun, L., Chen, Z.J., 2016. Regulation and function of the cGAS-STING pathway of cytosolic DNA sensing. Nat. Immunol. 17, 1142–1149. https://doi.org/10.1038/ni.3558

Chhajer, R., Bhattacharyya, A., Didwania, N., Shadab, M., Das, N., Palit, P., Vaidya, T., Ali, N., 2016. Leishmania donovani Aurora kinase: A promising therapeutic target against visceral leishmaniasis. Biochim. Biophys. Acta 1860, 1973–1988. https://doi.org/10.1016/j.bbagen.2016.06.005

Chung, I.-M., Rajakumar, G., Venkidasamy, B., Subramanian, U., Thiruvengadam, M., 2020. Exosomes: Current use and future applications. Clin. Chim. Acta Int. J. Clin. Chem. 500, 226–232. https://doi.org/10.1016/j.cca.2019.10.022

Civril, F., Deimling, T., de Oliveira Mann, C.C., Ablasser, A., Moldt, M., Witte, G., Hornung, V., Hopfner, K.-P., 2013. Structural mechanism of cytosolic DNA sensing by cGAS. Nature 498, 332–337. https://doi.org/10.1038/nature12305

Contreras, I., Gómez, M.A., Nguyen, O., Shio, M.T., McMaster, R.W., Olivier, M., 2010. Leishmania-Induced Inactivation of the Macrophage Transcription Factor AP-1 Is Mediated by the Parasite Metalloprotease GP63. PLoS Pathog. 6, e1001148. https://doi.org/10.1371/journal.ppat.1001148

Corradin, S., Mauël, J., Ransijn, A., Stürzinger, C., Vergères, G., 1999. Down-regulation of MARCKS-related Protein (MRP) in Macrophages Infected with *Leishmania*. J. Biol. Chem. 274, 16782–16787. https://doi.org/10.1074/jbc.274.24.16782

Cortés, A., Sotillo, J., Muñoz-Antolí, C., Molina-Durán, J., Esteban, J.G., Toledo, R., 2017. Antibody trapping: A novel mechanism of parasite immune evasion by the

trematode Echinostoma caproni. PLoS Negl. Trop. Dis. 11, e0005773. https://doi.org/10.1371/journal.pntd.0005773

Coukell, A.J., Brogden, R.N., 1998. Liposomal amphotericin B. Therapeutic use in the management of fungal infections and visceral leishmaniasis. Drugs 55, 585–612. https://doi.org/10.2165/00003495-199855040-00008

Couppié, P., Clyti, E., Sainte-Marie, D., Dedet, J.P., Carme, B., Pradinaud, R., 2004. Disseminated cutaneous leishmaniasis due to Leishmania guyanensis: case of a patient with 425 lesions. Am. J. Trop. Med. Hyg. 71, 558–560.

Cousens, L.P., Orange, J.S., Su, H.C., Biron, C.A., 1997. Interferon-α/β inhibition of interleukin 12 and interferon-γ production in vitro and endogenously during viral infection. Proc. Natl. Acad. Sci. U. S. A. 94, 634–639.

Coutinho De Oliveira, B., Duthie, M.S., Alves Pereira, V.R., 2020. Vaccines for leishmaniasis and the implications of their development for American tegumentary leishmaniasis. Hum. Vaccines Immunother. 16, 919–930. https://doi.org/10.1080/21645515.2019.1678998

Croft, S.L., Olliaro, P., 2011. Leishmaniasis chemotherapy—challenges and opportunities. Clin. Microbiol. Infect. 17, 1478–1483. https://doi.org/10.1111/j.1469-0691.2011.03630.x

Croft, S.L., Sundar, S., Fairlamb, A.H., 2006. Drug resistance in leishmaniasis. Clin. Microbiol. Rev. 19, 111–126. https://doi.org/10.1128/CMR.19.1.111-126.2006

Cuevas, I.C., Cazzulo, J.J., Sánchez, D.O., 2003. gp63 Homologues in Trypanosoma cruzi: Surface Antigens with Metalloprotease Activity and a Possible Role in Host Cell Infection. Infect. Immun. 71, 5739–5749. https://doi.org/10.1128/IAI.71.10.5739-5749.2003

Culley, F.J., Harris, R.A., Kaye, P.M., McAdam, K.P., Raynes, J.G., 1996. C-reactive protein binds to a novel ligand on Leishmania donovani and increases uptake into human macrophages. J. Immunol. Baltim. Md 1950 156, 4691–4696.

Curtin, J.M., Aronson, N.E., 2021. Leishmaniasis in the United States: Emerging Issues in a Region of Low Endemicity. Microorganisms 9, 578. https://doi.org/10.3390/microorganisms9030578

da Costa Santiago, H., Oliveira, C.F., Santiago, L., Ferraz, F.O., da Glória de Souza, D., Rodrigues De-Freitas, L.A., Crocco Afonso, L.C., Teixeira, M.M., Gazzinelli, R.T., Vieira, L.Q., 2004. Involvement of the Chemokine RANTES (CCL5) in Resistance to Experimental Infection with Leishmania major. Infect. Immun. 72, 4918–4923. https://doi.org/10.1128/IAI.72.8.4918-4923.2004

da Silva Lira Filho, A., Fajardo, E.F., Chang, K.P., Clément, P., Olivier, M., 2021. Leishmania Exosomes/Extracellular Vesicles Containing GP63 Are Essential for Enhance Cutaneous Leishmaniasis Development Upon Co-Inoculation of Leishmania amazonensis and Its Exosomes. Front. Cell. Infect. Microbiol. 11, 709258. https://doi.org/10.3389/fcimb.2021.709258

Dagger, F., Bengio, C., Martinez, A., Ayesta, C., 2017. Leishmania mexicana differentiation involves a selective plasma membrane autophagic-like process. Cell Stress Chaperones. https://doi.org/10.1007/s12192-017-0864-z

DaMata, J.P., Mendes, B.P., Maciel-Lima, K., Menezes, C.A.S., Dutra, W.O., Sousa, L.P., Horta, M.F., 2015. Distinct Macrophage Fates after in vitro Infection with Different Species of Leishmania: Induction of Apoptosis by Leishmania (Leishmania) amazonensis, but Not by Leishmania (Viannia) guyanensis. PloS One 10, e0141196. https://doi.org/10.1371/journal.pone.0141196

Darnell, J.E., Kerr, I.M., Stark, G.R., 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264, 1415–1421. https://doi.org/10.1126/science.8197455

Das, P., Mukherjee, A., Adak, S., 2021. Glyceraldehyde-3-phosphate dehydrogenase present in extracellular vesicles from Leishmania major suppresses host TNF-alpha expression. J. Biol. Chem. 297, 101198. https://doi.org/10.1016/j.jbc.2021.101198

Das, S., Banerjee, A., Kamran, M., Ejazi, S.A., Asad, M., Ali, N., Chakrabarti, S., 2020. A chemical inhibitor of heat shock protein 78 (HSP78) from Leishmania donovani represents a potential antileishmanial drug candidate. J. Biol. Chem. 295, 9934–9947. https://doi.org/10.1074/jbc.RA120.014587

Davidson, S., Maini, M.K., Wack, A., 2015. Disease-Promoting Effects of Type I Interferons in Viral, Bacterial, and Coinfections. J. Interferon Cytokine Res. 35, 252–264. https://doi.org/10.1089/jir.2014.0227

Dayakar, A., Chandrasekaran, S., Kuchipudi, S.V., Kalangi, S.K., 2019. Cytokines: Key Determinants of Resistance or Disease Progression in Visceral Leishmaniasis: Opportunities for Novel Diagnostics and Immunotherapy. Front. Immunol. 10.

de Carvalho Vivarini, Á., de Meirelles Santos Pereira, R., Teixeira, K.L.D., Calegari-Silva, T.C., Bellio, M., Laurenti, M.D., Corbett, C.E.P., de Castro Gomes, C.M., Soares, R.P., Silva, A.M., Silveira, F.T., Lopes, U.G., 2011. Human cutaneous leishmaniasis: interferon-dependent expression of double-stranded RNA-dependent protein kinase (PKR) via TLR2. FASEB J. 25, 4162–4173. https://doi.org/10.1096/fj.11-185165

de Sá, K.S.G., Amaral, L.A., Rodrigues, T.S., Ishimoto, A.Y., de Andrade, W.A.C., de Almeida, L., Freitas-Castro, F., Batah, S.S., Oliveira, S.C., Pastorello, M.T., Fabro, A.T., Zamboni, D.S., 2023. Gasdermin-D activation promotes NLRP3 activation and host

resistance to Leishmania infection. Nat. Commun. 14, 1049. https://doi.org/10.1038/s41467-023-36626-6

De Souza Leao, S., Lang, T., Prina, E., Hellio, R., Antoine, J.C., 1995. Intracellular Leishmania amazonensis amastigotes internalize and degrade MHC class II molecules of their host cells. J. Cell Sci. 108, 3219–3231. https://doi.org/10.1242/jcs.108.10.3219

de Souza, M.C., de Assis, E.A., Gomes, R.S., Marques da Silva, E. de A., Melo, M.N., Fietto, J.L.R., Afonso, L.C.C., 2010. The influence of ecto-nucleotidases on Leishmania amazonensis infection and immune response in C57B/6 mice. Acta Trop. 115, 262–269. https://doi.org/10.1016/j.actatropica.2010.04.007

de Veer, M.J., Curtis, J.M., Baldwin, T.M., DiDonato, J.A., Sexton, A., McConville, M.J., Handman, E., Schofield, L., 2003. MyD88 is essential for clearance of Leishmania major: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. Eur. J. Immunol. 33, 2822–2831. https://doi.org/10.1002/eji.200324128

Descoteaux, A., Matlashewski, G., Turco, S.J., 1992. Inhibition of macrophage protein kinase C-mediated protein phosphorylation by Leishmania donovani lipophosphoglycan. J. Immunol. Baltim. Md 1950 149, 3008–3015.

Dias, B.T., Dias-Teixeira, K.L., Godinho, J.P., Faria, M.S., Calegari-Silva, T., Mukhtar, M.M., Lopes, U., Mottram, J.C., Lima, A.P.C.A., 2019. Neutrophil elastase promotes Leishmania donovani infection via interferon-β. FASEB J. 33, 10794–10807. https://doi.org/10.1096/fj.201900524R

Diefenbach, A., Schindler, H., Donhauser, N., Lorenz, E., Laskay, T., MacMicking, J., Röllinghoff, M., Gresser, I., Bogdan, C., 1998. Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. Immunity 8, 77–87. https://doi.org/10.1016/s1074-7613(00)80460-4

Ding, M., Kwok, L.Y., Schlüter, D., Clayton, C., Soldati, D., 2004. The antioxidant systems in Toxoplasma gondii and the role of cytosolic catalase in defence against oxidative injury. Mol. Microbiol. 51, 47–61. https://doi.org/10.1046/j.1365-2958.2003.03823.x

Ding, Q., Gaska, J.M., Douam, F., Wei, L., Kim, D., Balev, M., Heller, B., Ploss, A., 2018. Species-specific disruption of STING-dependent antiviral cellular defenses by the Zika virus NS2B3 protease. Proc. Natl. Acad. Sci. U. S. A. 115, E6310–E6318. https://doi.org/10.1073/pnas.1803406115

Dobbs, N., Burnaevskiy, N., Chen, D., Gonugunta, V.K., Alto, N.M., Yan, N., 2015. STING activation by translocation from the ER is associated with infection and autoinflammatory disease. Cell Host Microbe 18, 157–168. https://doi.org/10.1016/j.chom.2015.07.001

Doering, T.L., 2009. How sweet it is! Cell wall biogenesis and polysaccharide capsule formation in Cryptococcus neoformans. Annu. Rev. Microbiol. 63, 223–247. https://doi.org/10.1146/annurev.micro.62.081307.162753

Donlan, R.M., 2002. Biofilms: microbial life on surfaces. Emerg. Infect. Dis. 8, 881–890. https://doi.org/10.3201/eid0809.020063

dos Santos, J.C., Damen, M.S.M.A., Oosting, M., de Jong, D.J., Heinhuis, B., Gomes, R.S., Araújo, C.S., Netea, M.G., Ribeiro-Dias, F., Joosten, L.A.B., 2017. The NOD2 receptor is crucial for immune responses towards New World Leishmania species. Sci. Rep. 7, 15219. https://doi.org/10.1038/s41598-017-15412-7

Dramsi, S., Cossart, P., 2002. Listeriolysin O: a genuine cytolysin optimized for an intracellular parasite. J. Cell Biol. 156, 943–946. https://doi.org/10.1083/jcb.200202121

Driessler, F., Venstrom, K., Sabat, R., Asadullah, K., Schottelius, A.J., 2004. Molecular mechanisms of interleukin-10-mediated inhibition of NF-kappaB activity: a role for p50. Clin. Exp. Immunol. 135, 64–73. https://doi.org/10.1111/j.1365-2249.2004.02342.x

Du, Y., Luo, Y., Hu, Z., Lu, J., Liu, X., Xing, C., Wu, J., Duan, T., Chu, J., Wang, H.Y., Su, X., Yu, X., Wang, R., 2022. Activation of cGAS-STING by Lethal Malaria N67C Dictates Immunity and Mortality through Induction of CD11b+Ly6Chi Proinflammatory Monocytes. Adv. Sci. 9, 2103701. https://doi.org/10.1002/advs.202103701

Duthie, M.S., Raman, V.S., Piazza, F.M., Reed, S.G., 2012. The development and clinical evaluation of second-generation leishmaniasis vaccines. Vaccine 30, 134–141. https://doi.org/10.1016/j.vaccine.2011.11.005

Ehrchen, J.M., Roebrock, K., Foell, D., Nippe, N., Stebut, E. von, Weiss, J.M., Münck, N.-A., Viemann, D., Varga, G., Müller-Tidow, C., Schuberth, H.-J., Roth, J., Sunderkötter, C., 2010. Keratinocytes Determine Th1 Immunity during Early Experimental Leishmaniasis. PLOS Pathog. 6, e1000871. https://doi.org/10.1371/journal.ppat.1000871

Eid, D., San Sebastian, M., Hurtig, A.-K., Goicolea, I., 2019. Leishmaniasis patients' pilgrimage to access health care in rural Bolivia: a qualitative study using human rights to health approach. BMC Int. Health Hum. Rights 19, 12. https://doi.org/10.1186/s12914-019-0196-4

Eidsmo, L., Fluur, C., Rethi, B., Eriksson Ygberg, S., Ruffin, N., De Milito, A., Akuffo, H., Chiodi, F., 2007. FasL and TRAIL Induce Epidermal Apoptosis and Skin Ulceration Upon Exposure to Leishmania major. Am. J. Pathol. 170, 227–239. https://doi.org/10.2353/ajpath.2007.060068

Ejazi, S.A., Ali, N., 2013. Developments in diagnosis and treatment of visceral leishmaniasis during the last decade and future prospects. Expert Rev. Anti Infect. Ther. 11, 79–98. https://doi.org/10.1586/eri.12.148

Engwerda, C.R., Murphy, M.L., Cotterell, S.E.J., Smelt, S.C., Kaye, P.M., 1998. Neutralization of IL-12 demonstrates the existence of discrete organ-specific phases in the control of Leishmania donovani. Eur. J. Immunol. 28, 669–680. https://doi.org/10.1002/(SICI)1521-4141(199802)28:02<669::AID-IMMU669>3.0.CO;2-N

Ergun, S.L., Fernandez, D., Weiss, T.M., Li, L., 2019. STING Polymer Structure Reveals Mechanisms for Activation, Hyperactivation, and Inhibition. Cell 178, 290-301.e10. https://doi.org/10.1016/j.cell.2019.05.036

Escalona-Montaño, A.R., Ortiz-Lozano, D.M., Rojas-Bernabé, A., Wilkins-Rodriguez, A.A., Torres-Guerrero, H., MondragóN-Flores, R., MondragóN-Gonzalez, R., Becker, I., GutiéRrez-Kobeh, L., Aguirre-Garcia, M.M., 2016. Leishmania mexicana: promastigotes and amastigotes secrete protein phosphatases and this correlates with the production of inflammatory cytokines in macrophages. Parasitology 143, 1409–1420. https://doi.org/10.1017/S0031182016000949

Faria, M.S., Reis, F.C.G., Azevedo-Pereira, R.L., Morrison, L.S., Mottram, J.C., Lima, A.P.C.A., 2011. Leishmania inhibitor of serine peptidase prevents TLR4 activation by neutrophil elastase promoting parasite survival in murine macrophages. J. Immunol. Baltim. Md 1950 186, 411–422. https://doi.org/10.4049/jimmunol.1002175

Feijó, D., Tibúrcio, R., Ampuero, M., Brodskyn, C., Tavares, N., 2016. Dendritic Cells and Leishmania Infection: Adding Layers of Complexity to a Complex Disease. J. Immunol. Res. 2016, 3967436. https://doi.org/10.1155/2016/3967436

Feng, Q., Langereis, M.A., Lork, M., Nguyen, M., Hato, S.V., Lanke, K., Emdad, L., Bhoopathi, P., Fisher, P.B., Lloyd, R.E., van Kuppeveld, F.J.M., 2014. Enterovirus 2Apro

targets MDA5 and MAVS in infected cells. J. Virol. 88, 3369–3378. https://doi.org/10.1128/JVI.02712-13

Ferguson, B.J., Mansur, D.S., Peters, N.E., Ren, H., Smith, G.L., 2012. DNA-PK is a DNA sensor for IRF-3-dependent innate immunity [WWW Document]. eLife. https://doi.org/10.7554/eLife.00047

Ferguson, M.A., 1997. The surface glycoconjugates of trypanosomatid parasites. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 352, 1295–1302. https://doi.org/10.1098/rstb.1997.0113

Fiebig, M., Kelly, S., Gluenz, E., 2015. Comparative Life Cycle Transcriptomics Revises Leishmania mexicana Genome Annotation and Links a Chromosome Duplication with Parasitism of Vertebrates. PLoS Pathog. 11, e1005186. https://doi.org/10.1371/journal.ppat.1005186

Filgueira, C.P.B., Moreira, O.C., Cantanhêde, L.M., Farias, H.M.T. de, Porrozzi, R., Britto, C., Boité, M.C., Cupolillo, E., 2020. Comparison and clinical validation of qPCR assays targeting Leishmania 18S rDNA and HSP70 genes in patients with American Tegumentary Leishmaniasis. PLoS Negl. Trop. Dis. 14, e0008750. https://doi.org/10.1371/journal.pntd.0008750

Foletta, V.C., Segal, D.H., Cohen, D.R., 1998. Transcriptional regulation in the immune system: all roads lead to AP-1. J. Leukoc. Biol. 63, 139–152. https://doi.org/10.1002/jlb.63.2.139

Forget, G., Gregory, D.J., Olivier, M., 2005. Proteasome-mediated Degradation of STAT1α following Infection of Macrophages with Leishmania donovani*. J. Biol. Chem. 280, 30542–30549. https://doi.org/10.1074/jbc.M414126200

Franco, L.H., Beverley, S.M., Zamboni, D.S., 2012. Innate immune activation and subversion of Mammalian functions by leishmania lipophosphoglycan. J. Parasitol. Res. 2012, 165126. https://doi.org/10.1155/2012/165126

Gallego-Marin, C., Schrum, J.E., Andrade, W.A., Shaffer, S.A., Giraldo, L.F., Lasso, A.M., Kurt-Jones, E.A., Fitzgerald, K.A., Golenbock, D.T., 2018. Cyclic GMP–AMP Synthase Is the Cytosolic Sensor of Plasmodium falciparum Genomic DNA and Activates Type I IFN in Malaria. J. Immunol. 200, 768–774. https://doi.org/10.4049/jimmunol.1701048

Gantt, K.R., Goldman, T.L., McCormick, M.L., Miller, M.A., Jeronimo, S.M.B., Nascimento, E.T., Britigan, B.E., Wilson, M.E., 2001. Oxidative Responses of Human and Murine Macrophages During Phagocytosis of Leishmania chagasi. J. Immunol. 167, 893–901. https://doi.org/10.4049/jimmunol.167.2.893

Gao, D., Wu, J., Wu, Y.-T., Du, F., Aroh, C., Yan, N., Sun, L., Chen, Z.J., 2013. Cyclic GMP-AMP Synthase is an Innate Immune Sensor of HIV and Other Retroviruses. Science 341, 10.1126/science.1240933. https://doi.org/10.1126/science.1240933

Gao, P., Ascano, M., Wu, Y., Barchet, W., Gaffney, B.L., Zillinger, T., Serganov, A.A., Liu, Y., Jones, R.A., Hartmann, G., Tuschl, T., Patel, D.J., 2013. Cyclic [G(2′,5′)pA(3′,5′)p] Is the Metazoan Second Messenger Produced by DNA-Activated Cyclic GMP-AMP Synthase. Cell 153, 1094–1107. https://doi.org/10.1016/j.cell.2013.04.046

Garg, R., Barat, C., Ouellet, M., Lodge, R., Tremblay, M.J., 2009. Leishmania infantum amastigotes enhance HIV-1 production in cocultures of human dendritic cells and CD4 T cells by inducing secretion of IL-6 and TNF-alpha. PLoS Negl. Trop. Dis. 3, e441. https://doi.org/10.1371/journal.pntd.0000441

Garlapati, S., Dahan, E., Shapira, M., 1999. Effect of acidic pH on heat shock gene expression in Leishmania. Mol. Biochem. Parasitol. 100, 95–101. https://doi.org/10.1016/s0166-6851(99)00037-7

Gioseffi, A., Hamerly, T., Van, K., Zhang, N., Dinglasan, R.R., Yates, P.A., Kima, P.E., 2020. Leishmania-infected macrophages release extracellular vesicles that can promote lesion development. Life Sci. Alliance 3. https://doi.org/10.26508/lsa.202000742

Goldring, O.L., Clegg, J.A., Smithers, S.R., Terry, R.J., 1976. Acquisition of human blood group antigens by Schistosoma mansoni. Clin. Exp. Immunol. 26, 181–187.

Gomez, M.A., Contreras, I., Halle, M., Tremblay, M.L., McMaster, R.W., Olivier, M., 2009. Leishmania GP63 Alters Host Signaling Through Cleavage-Activated Protein Tyrosine Phosphatases. Sci. Signal. 2, ra58–ra58. https://doi.org/10.1126/scisignal.2000213

Gonugunta, V.K., Sakai, T., Pokatayev, V., Yang, K., Wu, J., Dobbs, N., Yan, N., 2017. Trafficking-Mediated STING Degradation Requires Sorting to Acidified Endolysosomes and Can Be Targeted to Enhance Anti-tumor Response. Cell Rep. 21, 3234–3242. https://doi.org/10.1016/j.celrep.2017.11.061

González, C., Wang, O., Strutz, S.E., González-Salazar, C., Sánchez-Cordero, V., Sarkar, S., 2010. Climate change and risk of leishmaniasis in north america: predictions from ecological niche models of vector and reservoir species. PLoS Negl. Trop. Dis. 4, e585. https://doi.org/10.1371/journal.pntd.0000585

Goodfellow, I., Chaudhry, Y., Gioldasi, I., Gerondopoulos, A., Natoni, A., Labrie, L., Laliberté, J.-F., Roberts, L., 2005. Calicivirus translation initiation requires an interaction between VPg and eIF4E. EMBO Rep. 6, 968–972. https://doi.org/10.1038/sj.embor.7400510

Gottstein, U., Steiner, K., Hauk, H., Klimaschewski, G., Sedlmeyer, I., 1975. [Visceral leishmaniasis (kala-azar), an important disease not only in the tropics (author's transl)]. Dtsch. Med. Wochenschr. 1946 100, 2022, 2025–2027. https://doi.org/10.1055/s-0028-1106495

Gregory, D.J., Godbout, M., Contreras, I., Forget, G., Olivier, M., 2008a. A novel form of NF-kappaB is induced by Leishmania infection: involvement in macrophage gene expression. Eur. J. Immunol. 38, 1071–1081. https://doi.org/10.1002/eji.200737586

Gregory, D.J., Godbout, M., Contreras, I., Forget, G., Olivier, M., 2008b. A novel form of NF-kappaB is induced by Leishmania infection: involvement in macrophage gene expression. Eur. J. Immunol. 38, 1071–1081. https://doi.org/10.1002/eji.200737586

Gregory, D.J., Olivier, M., 2005. Subversion of host cell signalling by the protozoan parasite Leishmania. Parasitology 130, S27. https://doi.org/10.1017/S0031182005008139

Guay-Vincent, M.-M., Matte, C., Berthiaume, A.-M., Olivier, M., Jaramillo, M., Descoteaux, A., 2022. Revisiting Leishmania GP63 host cell targets reveals a limited spectrum of substrates. PLOS Pathog. 18, e1010640. https://doi.org/10.1371/journal.ppat.1010640

Guerin, P.J., Olliaro, P., Sundar, S., Boelaert, M., Croft, S.L., Desjeux, P., Wasunna, M.K., Bryceson, A.D.M., 2002. Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. Lancet Infect. Dis. 2, 494–501.

Guerra, C.S., Silva, R.M.M., Carvalho, L.O.P., da Silva Calabrese, K., Bozza, P.T., Côrte-Real, S., 2010. Histopathological analysis of initial cellular response in TLR-2 deficient mice experimentally infected by Leishmania (L.) amazonensis. Int. J. Exp. Pathol. 91, 451–459. https://doi.org/10.1111/j.1365-2613.2010.00717.x

Guizani-Tabbane, L., Ben-Aissa, K., Belghith, M., Sassi, A., Dellagi, K., 2004. Leishmania major Amastigotes Induce p50/c-Rel NF-κB Transcription Factor in Human Macrophages: Involvement in Cytokine Synthesis. Infect. Immun. 72, 2582–2589. https://doi.org/10.1128/IAI.72.5.2582-2589.2004

Guo, L., Lim, K.B., Gunn, J.S., Bainbridge, B., Darveau, R.P., Hackett, M., Miller, S.I., 1997. Regulation of lipid A modifications by Salmonella typhimurium virulence genes phoP-phoQ. Science 276, 250–253.

Gupta, A.K., Das, S., Kamran, M., Ejazi, S.A., Ali, N., 2022. The pathogenicity and virulence of Leishmania - interplay of virulence factors with host defenses. Virulence 13, 903–935. https://doi.org/10.1080/21505594.2022.2074130

Gupta, C.L., Akhtar, S., Waye, A., Pandey, N.R., Pathak, N., Bajpai, P., 2015. Cross talk between Leishmania donovani CpG DNA and Toll-like receptor 9: an immunoinformatics approach. Biochem. Biophys. Res. Commun. 459, 424–429. https://doi.org/10.1016/j.bbrc.2015.02.121

Gurung, P., Kanneganti, T.-D., 2015. Innate immunity against Leishmania infections. Cell. Microbiol. 17, 1286–1294. https://doi.org/10.1111/cmi.12484

Gurung, P., Karki, R., Vogel, P., Watanabe, M., Bix, M., Lamkanfi, M., Kanneganti, T.-D., 2015. An NLRP3 inflammasome-triggered Th2-biased adaptive immune response promotes leishmaniasis. J. Clin. Invest. 125, 1329–1338. https://doi.org/10.1172/JCI79526

Guy, R.A., Belosevic, M., 1993. Comparison of receptors required for entry of Leishmania major amastigotes into macrophages. Infect. Immun. 61, 1553–1558.

Hale, B.G., Albrecht, R.A., García-Sastre, A., 2010. Innate immune evasion strategies of influenza viruses. Future Microbiol. 5, 23–41. https://doi.org/10.2217/fmb.09.108

Hallé, M., Gomez, M.A., Stuible, M., Shimizu, H., McMaster, W.R., Olivier, M., Tremblay, M.L., 2009. The Leishmania surface protease GP63 cleaves multiple intracellular proteins and actively participates in p38 mitogen-activated protein kinase inactivation. J. Biol. Chem. 284, 6893–6908. https://doi.org/10.1074/jbc.M805861200

Halliday, A., Bates, P.A., Chance, M.L., Taylor, M.J., 2016. Toll-like receptor 2 (TLR2) plays a role in controlling cutaneous leishmaniasis in vivo, but does not require activation by parasite lipophosphoglycan. Parasit. Vectors 9, 532. https://doi.org/10.1186/s13071-016-1807-8

Handman, E., 1999. Cell Biology of Leishmania, in: Baker, J.R., Muller, R., Rollinson, D. (Eds.), Advances in Parasitology. Academic Press, pp. 1–39. https://doi.org/10.1016/S0065-308X(08)60229-8

Handman, E., Spira, D.T., 1977. Growth of Leishmania amastigotes in macrophages from normal and immune mice. Z. Parasitenkd. Berl. Ger. 53, 75–81. https://doi.org/10.1007/BF00383117

Harhay, M.O., Olliaro, P.L., Costa, D.L., Costa, C.H.N., 2011. Urban parasitology: visceral leishmaniasis in Brazil. Trends Parasitol. 27, 403–409. https://doi.org/10.1016/j.pt.2011.04.001

Hartley, M.-A., Eren, R.O., Rossi, M., Prevel, F., Castiglioni, P., Isorce, N., Desponds, C., Lye, L.-F., Beverley, S.M., Drexler, S.K., Fasel, N., 2018. Leishmania guyanensis parasites block the activation of the inflammasome by inhibiting maturation of IL-1β. Microb. Cell Graz Austria 5, 137–149. https://doi.org/10.15698/mic2018.03.619

Hassani, K., Antoniak, E., Jardim, A., Olivier, M., 2011. Temperature-induced protein secretion by Leishmania mexicana modulates macrophage signalling and function. PloS One 6, e18724. https://doi.org/10.1371/journal.pone.0018724

Hassani, K., Olivier, M., 2013. Immunomodulatory impact of leishmania-induced macrophage exosomes: a comparative proteomic and functional analysis. PLoS Negl. Trop. Dis. 7, e2185. https://doi.org/10.1371/journal.pntd.0002185

Hassani, K., Shio, M.T., Martel, C., Faubert, D., Olivier, M., 2014. Absence of Metalloprotease GP63 Alters the Protein Content of Leishmania Exosomes. PLoS ONE 9, e95007. https://doi.org/10.1371/journal.pone.0095007

Hatzigeorgiou, D.E., Geng, J., Zhu, B., Zhang, Y., Liu, K., Rom, W.N., Fenton, M.J., Turco, S.J., Ho, J.L., 1996. Lipophosphoglycan from Leishmania suppresses agonist-induced interleukin 1β gene expression in human monocytes via a unique promoter sequence. Proc. Natl. Acad. Sci. U. S. A. 93, 14708–14713.

Hayani, K., Dandashli, A., Weisshaar, E., 2015. Cutaneous Leishmaniasis in Syria: Clinical Features, Current Status and the Effects of War. Acta Derm. Venereol. 95, 62–66. https://doi.org/10.2340/00015555-1988

Hayden, M.S., West, A.P., Ghosh, S., 2006. NF-kappaB and the immune response. Oncogene 25, 6758–6780. https://doi.org/10.1038/sj.onc.1209943

He, W., Wan, H., Hu, L., Chen, P., Wang, X., Huang, Z., Yang, Z.-H., Zhong, C.-Q., Han, J., 2015. Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion. Cell Res. 25, 1285–1298. https://doi.org/10.1038/cr.2015.139

Heinzel, F.P., Sadick, M.D., Mutha, S.S., Locksley, R.M., 1991. Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by CD4+ lymphocytes in vivo during healing and progressive murine leishmaniasis. Proc. Natl. Acad. Sci. 88, 7011–7015. https://doi.org/10.1073/pnas.88.16.7011

Herman, R., Baron, S., 1970. Effects of interferon inducers on the intracellular growth of the protozoan parasite, Leishmania donovani. Nature 226, 168–170. https://doi.org/10.1038/226168a0

Hernández-Chávez, M., Pérez-García, L., Niño-Vega, G., Mora-Montes, H., 2017. Fungal Strategies to Evade the Host Immune Recognition. J. Fungi 3, 51. https://doi.org/10.3390/jof3040051

Hide, M., Bañuls, A.L., 2008. Polymorphisms of cpb multicopy genes in the Leishmania (Leishmania) donovani complex. Trans. R. Soc. Trop. Med. Hyg. 102, 105–106. https://doi.org/10.1016/j.trstmh.2007.09.013

Holzer, T.R., McMaster, W.R., Forney, J.D., 2006. Expression profiling by whole-genome interspecies microarray hybridization reveals differential gene expression in procyclic promastigotes, lesion-derived amastigotes, and axenic amastigotes in Leishmania mexicana. Mol. Biochem. Parasitol. 146, 198–218. https://doi.org/10.1016/j.molbiopara.2005.12.009

Hosseini, H.M., Fooladi, A.A.I., Nourani, M.R., Ghanezadeh, F., 2013. The role of exosomes in infectious diseases. Inflamm. Allergy Drug Targets 12, 29–37. https://doi.org/10.2174/1871528111312010005

Howard, J.G., Hale, C., Chan-Liew, W.L., 1980. Immunological regulation of experimental cutaneous leishmaniasis. 1. Immunogenetic aspects of susceptibility to Leishmania tropica in mice. Parasite Immunol. 2, 303–314. https://doi.org/10.1111/j.1365-3024.1980.tb00061.x

Hurrell, B.P., Beaumann, M., Heyde, S., Regli, I.B., Müller, A.J., Tacchini-Cottier, F., 2017. Frontline Science: Leishmania mexicana amastigotes can replicate within neutrophils. J. Leukoc. Biol. 102, 1187–1198. https://doi.org/10.1189/jlb.4HI0417-158R

Hurrell, B.P., Regli, I.B., Tacchini-Cottier, F., 2016. Different Leishmania Species Drive Distinct Neutrophil Functions. Trends Parasitol. 32, 392–401. https://doi.org/10.1016/j.pt.2016.02.003

Hurrell, B.P., Schuster, S., Grün, E., Coutaz, M., Williams, R.A., Held, W., Malissen, B., Malissen, M., Yousefi, S., Simon, H.-U., Müller, A.J., Tacchini-Cottier, F., 2015. Rapid Sequestration of Leishmania mexicana by Neutrophils Contributes to the Development of Chronic Lesion. PLoS Pathog. 11, e1004929. https://doi.org/10.1371/journal.ppat.1004929

Hussain, B., Xie, Y., Jabeen, U., Lu, D., Yang, B., Wu, C., Shang, G., 2022. Activation of STING Based on Its Structural Features. Front. Immunol. 13.

Hussain, H., Al-Harrasi, A., Al-Rawahi, A., Green, I.R., Gibbons, S., 2014. Fruitful decade for antileishmanial compounds from 2002 to late 2011. Chem. Rev. 114, 10369–10428. https://doi.org/10.1021/cr400552x

Ilg, T., 2000. Lipophosphoglycan is not required for infection of macrophages or mice by Leishmania mexicana. EMBO J. 19, 1953–1962. https://doi.org/10.1093/emboj/19.9.1953

Ishikawa, H., Barber, G.N., 2011. The STING pathway and regulation of innate immune signaling in response to DNA pathogens. Cell. Mol. Life Sci. 68, 1157–1165. https://doi.org/10.1007/s00018-010-0605-2

Ishikawa, H., Barber, G.N., 2008. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 455, 674–678. https://doi.org/10.1038/nature07317

Ishikawa, H., Ma, Z., Barber, G.N., 2009. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature 461, 788–792. https://doi.org/10.1038/nature08476

Isnard, A., Christian, J.G., Kodiha, M., Stochaj, U., McMaster, W.R., Olivier, M., 2015. Impact of Leishmania Infection on Host Macrophage Nuclear Physiology and Nucleopore

Complex Integrity. PLOS Pathog. 11, e1004776.
https://doi.org/10.1371/journal.ppat.1004776

Ivens, A.C., Peacock, C.S., Worthey, E.A., Murphy, L., Aggarwal, G., Berriman, M., Sisk, E., Rajandream, M.-A., Adlem, E., Aert, R., Anupama, A., Apostolou, Z., Attipoe, P., Bason, N., Bauser, C., Beck, A., Beverley, S.M., Bianchettin, G., Borzym, K., Bothe, G., Bruschi, C.V., Collins, M., Cadag, E., Ciarloni, L., Clayton, C., Coulson, R.M.R., Cronin, A., Cruz, A.K., Davies, R.M., De Gaudenzi, J., Dobson, D.E., Duesterhoeft, A., Fazelina, G., Fosker, N., Frasch, A.C., Fraser, A., Fuchs, M., Gabel, C., Goble, A., Goffeau, A., Harris, D., Hertz-Fowler, C., Hilbert, H., Horn, D., Huang, Y., Klages, S., Knights, A., Kube, M., Larke, N., Litvin, L., Lord, A., Louie, T., Marra, M., Masuy, D., Matthews, K., Michaeli, S., Mottram, J.C., Müller-Auer, S., Munden, H., Nelson, S., Norbertczak, H., Oliver, K., O'neil, S., Pentony, M., Pohl, T.M., Price, C., Purnelle, B., Quail, M.A., Rabbinowitsch, E., Reinhardt, R., Rieger, M., Rinta, J., Robben, J., Robertson, L., Ruiz, J.C., Rutter, S., Saunders, D., Schäfer, M., Schein, J., Schwartz, D.C., Seeger, K., Seyler, A., Sharp, S., Shin, H., Sivam, D., Squares, R., Squares, S., Tosato, V., Vogt, C., Volckaert, G., Wambutt, R., Warren, T., Wedler, H., Woodward, J., Zhou, S., Zimmermann, W., Smith, D.F., Blackwell, J.M., Stuart, K.D., Barrell, B., Myler, P.J., 2005. The genome of the kinetoplastid parasite, Leishmania major. Science 309, 436–442. https://doi.org/10.1126/science.1112680

Ives, A., Ronet, C., Prevel, F., Ruzzante, G., Fuertes-Marraco, S., Schutz, F., Zangger, H., Revaz-Breton, M., Lye, L.-F., Hickerson, S.M., Beverley, S.M., Acha-Orbea, H., Launois, P., Fasel, N., Masina, S., 2011. Leishmania RNA virus controls the severity of mucocutaneous leishmaniasis. Science 331, 775–778.
https://doi.org/10.1126/science.1199326

Jacques, D.A., McEwan, W.A., Hilditch, L., Price, A.J., Towers, G.J., James, L.C., 2016. HIV-1 uses dynamic capsid pores to import nucleotides and fuel encapsidated DNA synthesis. Nature 536, 349–353. https://doi.org/10.1038/nature19098

Jafarzadeh, A., Nair, A., Jafarzadeh, S., Nemati, M., Sharifi, I., Saha, B., 2021. Immunological role of keratinocytes in leishmaniasis. Parasite Immunol. 43, e12870. https://doi.org/10.1111/pim.12870

Janeway, C.A., Medzhitov, R., 2002. Innate Immune Recognition. Annu. Rev. Immunol. 20, 197–216. https://doi.org/10.1146/annurev.immunol.20.083001.084359

Jaramillo, M., Gomez, M.A., Larsson, O., Shio, M.T., Topisirovic, I., Contreras, I., Luxenburg, R., Rosenfeld, A., Colina, R., McMaster, R.W., Olivier, M., Costa-Mattioli, M., Sonenberg, N., 2011. Leishmania Repression of Host Translation through mTOR Cleavage Is Required for Parasite Survival and Infection. Cell Host Microbe 9, 331–341. https://doi.org/10.1016/j.chom.2011.03.008

Johnstone, R.M., Adam, M., Hammond, J.R., Orr, L., Turbide, C., 1987. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J. Biol. Chem. 262, 9412–9420.

Jønsson, K.L., Laustsen, A., Krapp, C., Skipper, K.A., Thavachelvam, K., Hotter, D., Egedal, J.H., Kjolby, M., Mohammadi, P., Prabakaran, T., Sørensen, L.K., Sun, C., Jensen, S.B., Holm, C.K., Lebbink, R.J., Johannsen, M., Nyegaard, M., Mikkelsen, J.G., Kirchhoff, F., Paludan, S.R., Jakobsen, M.R., 2017. IFI16 is required for DNA sensing in human macrophages by promoting production and function of cGAMP. Nat. Commun. 8, 14391. https://doi.org/10.1038/ncomms14391

Jounai, N., Kobiyama, K., Takeshita, F., Ishii, K.J., 2013. Recognition of damage-associated molecular patterns related to nucleic acids during inflammation and

vaccination. Front. Cell. Infect. Microbiol. 2, 168.
https://doi.org/10.3389/fcimb.2012.00168

Kamhawi, S., 2006. bbatb. Trends Parasitol. 22, 439–445.
https://doi.org/10.1016/j.pt.2006.06.012

Kane, M.M., Mosser, D.M., 2001. The Role of IL-10 in Promoting Disease Progression in Leishmaniasis. J. Immunol. 166, 1141–1147.
https://doi.org/10.4049/jimmunol.166.2.1141

Karami, M., Gorgani-Firouzjaee, T., Chehrazi, M., 2022. Prevalence of cutaneous Leishmaniasis in the Middle East: a systematic review and meta-analysis. Pathog. Glob. Health 0, 1–10. https://doi.org/10.1080/20477724.2022.2133452

Karmakar, S., Bhaumik, S.K., Paul, J., De, T., 2012. TLR4 and NKT Cell Synergy in Immunotherapy against Visceral Leishmaniasis. PLoS Pathog. 8, e1002646.
https://doi.org/10.1371/journal.ppat.1002646

Karmakar, S., Volpedo, G., Zhang, W.-W., Lypaczewski, P., Ismail, N., Oliveira, F., Oristian, J., Meneses, C., Gannavaram, S., Kamhawi, S., Hamano, S., Valenzuela, J.G., Matlashewski, G., Satoskar, A.R., Dey, R., Nakhasi, H.L., 2022. Centrin-deficient Leishmania mexicana confers protection against Old World visceral leishmaniasis. NPJ Vaccines 7, 157. https://doi.org/10.1038/s41541-022-00574-x

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C.-S., Reis e Sousa, C., Matsuura, Y., Fujita, T., Akira, S., 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441, 101–105.
https://doi.org/10.1038/nature04734

Kato, Y., Park, J., Takamatsu, H., Konaka, H., Aoki, W., Aburaya, S., Ueda, M., Nishide, M., Koyama, S., Hayama, Y., Kinehara, Y., Hirano, T., Shima, Y., Narazaki, M.,

Kumanogoh, A., 2018. Apoptosis-derived membrane vesicles drive the cGAS-STING pathway and enhance type I IFN production in systemic lupus erythematosus. Ann. Rheum. Dis. 77, 1507–1515. https://doi.org/10.1136/annrheumdis-2018-212988

Kavoosi, G., Ardestani, S.K., Kariminia, A., 2009. The involvement of TLR2 in cytokine and reactive oxygen species (ROS) production by PBMCs in response to Leishmania major phosphoglycans (PGs). Parasitology 136, 1193–1199. https://doi.org/10.1017/S0031182009990473

Kavoosi, G., Ardestani, S.K., Kariminia, A., Alimohammadian, M.H., 2010. Leishmania major lipophosphoglycan: discrepancy in Toll-like receptor signaling. Exp. Parasitol. 124, 214–218. https://doi.org/10.1016/j.exppara.2009.09.017

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K.J., Takeuchi, O., Akira, S., 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat. Immunol. 6, 981–988. https://doi.org/10.1038/ni1243

Kemp, W.M., Merritt, S.C., Bogucki, M.S., Rosier, J.G., Seed, J.R., 1977. Evidence for adsorption of heterospecific host immunoglobulin on the tegument of Schistosoma mansoni. J. Immunol. Baltim. Md 1950 119, 1849–1854.

Khouri, R., Bafica, A., Silva, M. da P.P., Noronha, A., Kolb, J.-P., Wietzerbin, J., Barral, A., Barral-Netto, M., Van Weyenbergh, J., 2009. IFN-beta impairs superoxide-dependent parasite killing in human macrophages: evidence for a deleterious role of SOD1 in cutaneous leishmaniasis. J. Immunol. Baltim. Md 1950 182, 2525–2531. https://doi.org/10.4049/jimmunol.0802860

Kindler, E., Gil-Cruz, C., Spanier, J., Li, Y., Wilhelm, J., Rabouw, H.H., Züst, R., Hwang, M., V'kovski, P., Stalder, H., Marti, S., Habjan, M., Cervantes-Barragan, L., Elliot, R., Karl, N., Gaughan, C., van Kuppeveld, F.J.M., Silverman, R.H., Keller, M., Ludewig, B., Bergmann, C.C., Ziebuhr, J., Weiss, S.R., Kalinke, U., Thiel, V., 2017. Early

endonuclease-mediated evasion of RNA sensing ensures efficient coronavirus replication. PLoS Pathog. 13, e1006195. https://doi.org/10.1371/journal.ppat.1006195

Komalapriya, C., Kaloriti, D., Tillmann, A.T., Yin, Z., Herrero-de-Dios, C., Jacobsen, M.D., Belmonte, R.C., Cameron, G., Haynes, K., Grebogi, C., de Moura, A.P.S., Gow, N.A.R., Thiel, M., Quinn, J., Brown, A.J.P., Romano, M.C., 2015. Integrative Model of Oxidative Stress Adaptation in the Fungal Pathogen Candida albicans. PLOS ONE 10, e0137750. https://doi.org/10.1371/journal.pone.0137750

Kondo, T., Kobayashi, J., Saitoh, T., Maruyama, K., Ishii, K.J., Barber, G.N., Komatsu, K., Akira, S., Kawai, T., 2013. DNA damage sensor MRE11 recognizes cytosolic double-stranded DNA and induces type I interferon by regulating STING trafficking. Proc. Natl. Acad. Sci. 110, 2969–2974. https://doi.org/10.1073/pnas.1222694110

Kranzusch, P.J., Vance, R.E., 2013. cGAS Dimerization Entangles DNA Recognition. Immunity 39, 992–994. https://doi.org/10.1016/j.immuni.2013.11.012

Krobitsch, S., Clos, J., 1999. A novel role for 100 kD heat shock proteins in the parasite Leishmania donovani. Cell Stress Chaperones 4, 191–198.

Kumar, H., Kawai, T., Akira, S., 2011. Pathogen Recognition by the Innate Immune System. Int. Rev. Immunol. 30, 16–34. https://doi.org/10.3109/08830185.2010.529976

Kumari, D., Mahajan, S., Kour, P., Singh, K., 2022. Virulence factors of Leishmania parasite: Their paramount importance in unraveling novel vaccine candidates and therapeutic targets. Life Sci. 306, 120829. https://doi.org/10.1016/j.lfs.2022.120829

Kumari, D., Perveen, S., Sharma, R., Singh, K., 2021. Advancement in leishmaniasis diagnosis and therapeutics: An update. Eur. J. Pharmacol. 910, 174436. https://doi.org/10.1016/j.ejphar.2021.174436

Lambertz, U., Silverman, J.M., Nandan, D., McMaster, W.R., Clos, J., Foster, L.J., Reiner, N.E., 2012. Secreted virulence factors and immune evasion in visceral leishmaniasis. J. Leukoc. Biol. 91, 887–899. https://doi.org/10.1189/jlb.0611326

Lapara, N.J., Kelly, B.L., 2010. Suppression of LPS-induced inflammatory responses in macrophages infected with Leishmania. J. Inflamm. 7, 8. https://doi.org/10.1186/1476-9255-7-8

Le Rutte, E.A., van Straten, R., Overgaauw, P.A.M., 2018. Awareness and control of canine leishmaniosis: A survey among Spanish and French veterinarians. Vet. Parasitol. 253, 87–93. https://doi.org/10.1016/j.vetpar.2018.01.013

Lenassi, M., Cagney, G., Liao, M., Vaupotič, T., Bartholomeeusen, K., Cheng, Y., Krogan, N.J., Plemenitaš, A., Peterlin, B.M., 2010. HIV Nef is secreted in exosomes and triggers apoptosis in bystander CD4+ T cells. Traffic Cph. Den. 11, 110–122. https://doi.org/10.1111/j.1600-0854.2009.01006.x

Li, D., Wu, M., 2021. Pattern recognition receptors in health and diseases. Signal Transduct. Target. Ther. 6, 1–24. https://doi.org/10.1038/s41392-021-00687-0

Li, X., Shu, C., Yi, G., Chaton, C.T., Shelton, C.L., Diao, J., Zuo, X., Kao, C.C., Herr, A.B., Li, P., 2013. Cyclic GMP-AMP synthase is activated by double-stranded DNA-induced oligomerization. Immunity 39, 1019–1031. https://doi.org/10.1016/j.immuni.2013.10.019

Liang, J., Hong, Z., Sun, B., Guo, Z., Wang, C., Zhu, J., 2021. The Alternatively Spliced Isoforms of Key Molecules in the cGAS-STING Signaling Pathway. Front. Immunol. 12.

Liese, J., Schleicher, U., Bogdan, C., 2007. TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. Eur. J. Immunol. 37, 3424–3434. https://doi.org/10.1002/eji.200737182

Lifland, A.W., Jung, J., Alonas, E., Zurla, C., Crowe, J.E., Santangelo, P.J., 2012. Human respiratory syncytial virus nucleoprotein and inclusion bodies antagonize the innate immune response mediated by MDA5 and MAVS. J. Virol. 86, 8245–8258. https://doi.org/10.1128/JVI.00215-12

Lima-Junior, D.S., Costa, D.L., Carregaro, V., Cunha, L.D., Silva, A.L.N., Mineo, T.W.P., Gutierrez, F.R.S., Bellio, M., Bortoluci, K.R., Flavell, R.A., Bozza, M.T., Silva, J.S., Zamboni, D.S., 2013. Inflammasome-derived IL-1β production induces nitric oxide-mediated resistance to Leishmania. Nat. Med. 19, 909–915. https://doi.org/10.1038/nm.3221

Liu, D., Wu, H., Wang, C., Li, Y., Tian, H., Siraj, S., Sehgal, S.A., Wang, X., Wang, J., Shang, Y., Jiang, Z., Liu, L., Chen, Q., 2019. STING directly activates autophagy to tune the innate immune response. Cell Death Differ. 26, 1735–1749. https://doi.org/10.1038/s41418-018-0251-z

Liu, T., Zhang, L., Joo, D., Sun, S.-C., 2017. NF-κB signaling in inflammation. Signal Transduct. Target. Ther. 2, 17023. https://doi.org/10.1038/sigtrans.2017.23

Liu, Y., Jesus, A.A., Marrero, B., Yang, D., Ramsey, S.E., Sanchez, G.A.M., Tenbrock, K., Wittkowski, H., Jones, O.Y., Kuehn, H.S., Lee, C.-C.R., DiMattia, M.A., Cowen, E.W., Gonzalez, B., Palmer, I., DiGiovanna, J.J., Biancotto, A., Kim, H., Tsai, W.L., Trier, A.M., Huang, Y., Stone, D.L., Hill, S., Kim, H.J., St Hilaire, C., Gurprasad, S., Plass, N., Chapelle, D., Horkayne-Szakaly, I., Foell, D., Barysenka, A., Candotti, F., Holland, S.M., Hughes, J.D., Mehmet, H., Issekutz, A.C., Raffeld, M., McElwee, J., Fontana, J.R., Minniti, C.P., Moir, S., Kastner, D.L., Gadina, M., Steven, A.C., Wingfield, P.T., Brooks, S.R., Rosenzweig, S.D., Fleisher, T.A., Deng, Z., Boehm, M., Paller, A.S., Goldbach-Mansky, R., 2014. Activated STING in a vascular and pulmonary syndrome. N. Engl. J. Med. 371, 507–518. https://doi.org/10.1056/NEJMoa1312625

Lodge, R., Diallo, T.O., Descoteaux, A., 2006. Leishmania donovani lipophosphoglycan blocks NADPH oxidase assembly at the phagosome membrane. Cell. Microbiol. 8, 1922–1931. https://doi.org/10.1111/j.1462-5822.2006.00758.x

Lorenz, U., 2009. SHP-1 and SHP-2 in T cells: two phosphatases functioning at many levels. Immunol. Rev. 228, 342–359. https://doi.org/10.1111/j.1600-065X.2008.00760.x

Loría-Cervera, E.N., Andrade-Narváez, F.J., 2014a. ANIMAL MODELS FOR THE STUDY OF LEISHMANIASIS IMMUNOLOGY. Rev. Inst. Med. Trop. São Paulo 56, 1–11. https://doi.org/10.1590/S0036-46652014000100001

Loría-Cervera, E.N., Andrade-Narváez, F.J., 2014b. ANIMAL MODELS FOR THE STUDY OF LEISHMANIASIS IMMUNOLOGY. Rev. Inst. Med. Trop. São Paulo 56, 1–11. https://doi.org/10.1590/S0036-46652014000100001

Lund, M.E., To, J., O'Brien, B.A., Donnelly, S., 2016. The choice of phorbol 12-myristate 13-acetate differentiation protocol influences the response of THP-1 macrophages to a pro-inflammatory stimulus. J. Immunol. Methods 430, 64–70. https://doi.org/10.1016/j.jim.2016.01.012

Ma, L., Meng, Q., Cheng, W., Sung, Y., Tang, P., Hu, S., Yu, J., 2011. Involvement of the GP63 protease in infection of Trichomonas vaginalis. Parasitol. Res. 109, 71–79. https://doi.org/10.1007/s00436-010-2222-2

Mallory, K.L., Taylor, J.A., Zou, X., Waghela, I.N., Schneider, C.G., Sibilo, M.Q., Punde, N.M., Perazzo, L.C., Savransky, T., Sedegah, M., Dutta, S., Janse, C.J., Pardi, N., Lin, P.J.C., Tam, Y.K., Weissman, D., Angov, E., 2021. Messenger RNA expressing PfCSP induces functional, protective immune responses against malaria in mice. NPJ Vaccines 6, 84. https://doi.org/10.1038/s41541-021-00345-0

Malvolti, S., Malhame, M., Mantel, C.F., Le Rutte, E.A., Kaye, P.M., 2021. Human leishmaniasis vaccines: Use cases, target population and potential global demand. PLoS Negl. Trop. Dis. 15, e0009742. https://doi.org/10.1371/journal.pntd.0009742

Mandell, M.A., Beatty, W.L., Beverley, S.M., 2022. Quantitative single-cell analysis of Leishmania major amastigote differentiation demonstrates variably extended expression of the lipophosphoglycan (LPG) virulence factor in different host cell types. PLoS Negl. Trop. Dis. 16, e0010893. https://doi.org/10.1371/journal.pntd.0010893

Mankan, A.K., Schmidt, T., Chauhan, D., Goldeck, M., Höning, K., Gaidt, M., Kubarenko, A.V., Andreeva, L., Hopfner, K.-P., Hornung, V., 2014. Cytosolic RNA:DNA hybrids activate the cGAS–STING axis. EMBO J. 33, 2937–2946. https://doi.org/10.15252/embj.201488726

Mansuri, R., Singh, J., Diwan, A., 2020. An Insight into the Current Perspective and Potential Drug Targets for Visceral Leishmaniasis (VL). Curr. Drug Targets 21, 1105–1129. https://doi.org/10.2174/1389450121666200422083735

Martiny, A., Meyer-Fernandes, J.R., de Souza, W., Vannier-Santos, M.A., 1999. Altered tyrosine phosphorylation of ERK1 MAP kinase and other macrophage molecules caused by Leishmania amastigotes. Mol. Biochem. Parasitol. 102, 1–12.

Mas, A., Martínez-Rodrigo, A., Orden, J.A., Viñals, L.M., Domínguez-Bernal, G., Carrión, J., 2020. A further investigation of the leishmaniosis outbreak in Madrid (Spain): low-infectivity phenotype of the Leishmania infantum BOS1FL1 isolate to establish infection in canine cells. Vet. Immunol. Immunopathol. 230, 110148. https://doi.org/10.1016/j.vetimm.2020.110148

Matheoud, D., Moradin, N., Bellemare-Pelletier, A., Shio, M.T., Hong, W.J., Olivier, M., Gagnon, É., Desjardins, M., Descoteaux, A., 2013. Leishmania Evades Host Immunity

by Inhibiting Antigen Cross-Presentation through Direct Cleavage of the SNARE VAMP8. Cell Host Microbe 14, 15–25. https://doi.org/10.1016/j.chom.2013.06.003

Mathivanan, S., Fahner, C.J., Reid, G.E., Simpson, R.J., 2012. ExoCarta 2012: database of exosomal proteins, RNA and lipids. Nucleic Acids Res. 40, D1241–D1244. https://doi.org/10.1093/nar/gkr828

Mathivanan, S., Ji, H., Simpson, R.J., 2010. Exosomes: extracellular organelles important in intercellular communication. J. Proteomics 73, 1907–1920. https://doi.org/10.1016/j.jprot.2010.06.006

Matsuura, M., Kawasaki, K., Kawahara, K., Mitsuyama, M., 2012. Evasion of human innate immunity without antagonizing TLR4 by mutant Salmonella enterica serovar Typhimurium having penta-acylated lipid A. Innate Immun. 18, 764–773. https://doi.org/10.1177/1753425912440599

Matsuura, M., Takahashi, H., Watanabe, H., Saito, S., Kawahara, K., 2010. Immunomodulatory effects of Yersinia pestis lipopolysaccharides on human macrophages. Clin. Vaccine Immunol. CVI 17, 49–55. https://doi.org/10.1128/CVI.00336-09

Mattner, J., Schindler, H., Diefenbach, A., Röllinghoff, M., Gresser, I., Bogdan, C., 2000. Regulation of type 2 nitric oxide synthase by type 1 interferons in macrophages infected with Leishmania major. Eur. J. Immunol. 30, 2257–2267. https://doi.org/10.1002/1521-4141(2000)30:8<2257::AID-IMMU2257>3.0.CO;2-U

Mbow, M.L., DeKrey, G.K., Titus, R.G., 2001. Leishmania major induces differential expression of costimulatory molecules on mouse epidermal cells. Eur. J. Immunol. 31, 1400–1409. https://doi.org/10.1002/1521-4141(200105)31:5<1400::aid-immu1400>3.0.co;2-j

McDonald, C., Inohara, N., Nuñez, G., 2005. Peptidoglycan signaling in innate immunity and inflammatory disease. J. Biol. Chem. 280, 20177–20180. https://doi.org/10.1074/jbc.R500001200

McGhee, R.B., Cosgrove, W.B., 1980. Biology and physiology of the lower Trypanosomatidae. Microbiol. Rev. 44, 140–173.

McGwire, B.S., Chang, K.-P., Engman, D.M., 2003. Migration through the Extracellular Matrix by the Parasitic Protozoan Leishmania Is Enhanced by Surface Metalloprotease gp63. Infect. Immun. 71, 1008–1010. https://doi.org/10.1128/IAI.71.2.1008-1010.2003

McGwire, B.S., O'Connell, W.A., Chang, K.-P., Engman, D.M., 2002. Extracellular release of the glycosylphosphatidylinositol (GPI)-linked Leishmania surface metalloprotease, gp63, is independent of GPI phospholipolysis: implications for parasite virulence. J. Biol. Chem. 277, 8802–8809. https://doi.org/10.1074/jbc.M109072200

McIlwee, B.E., Weis, S.E., Hosler, G.A., 2018. Incidence of Endemic Human Cutaneous Leishmaniasis in the United States. JAMA Dermatol. 154, 1032–1039. https://doi.org/10.1001/jamadermatol.2018.2133

McKenzie, C.G.J., Koser, U., Lewis, L.E., Bain, J.M., Mora-Montes, H.M., Barker, R.N., Gow, N. a. R., Erwig, L.P., 2010. Contribution of Candida albicans cell wall components to recognition by and escape from murine macrophages. Infect. Immun. 78, 1650–1658. https://doi.org/10.1128/IAI.00001-10

Mehta, S.R., Zhang, X.-Q., Badaro, R., Spina, C., Day, J., Chang, K.-P., Schooley, R.T., 2010. Flow cytometric screening for anti-leishmanials in a human macrophage cell line. Exp. Parasitol. 126, 617–620. https://doi.org/10.1016/j.exppara.2010.06.007

Melki, I., Rose, Y., Uggenti, C., Van Eyck, L., Frémond, M.-L., Kitabayashi, N., Rice, G.I., Jenkinson, E.M., Boulai, A., Jeremiah, N., Gattorno, M., Volpi, S., Sacco, O., Terheggen-Lagro, S.W.J., Tiddens, H.A.W.M., Meyts, I., Morren, M.-A., De Haes, P., Wouters, C.,

Legius, E., Corveleyn, A., Rieux-Laucat, F., Bodemer, C., Callebaut, I., Rodero, M.P., Crow, Y.J., 2017. Disease-associated mutations identify a novel region in human STING necessary for the control of type I interferon signaling. J. Allergy Clin. Immunol. 140, 543-552.e5. https://doi.org/10.1016/j.jaci.2016.10.031

Meri, T., Blom, A.M., Hartmann, A., Lenk, D., Meri, S., Zipfel, P.F., 2004. The hyphal and yeast forms of Candida albicans bind the complement regulator C4b-binding protein. Infect. Immun. 72, 6633–6641. https://doi.org/10.1128/IAI.72.11.6633-6641.2004

Millar, J.A., Valdés, R., Kacharia, F.R., Landfear, S.M., Cambronne, E.D., Raghavan, R., 2015. Coxiella burnetii and Leishmania mexicana residing within similar parasitophorous vacuoles elicit disparate host responses. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.00794

Mo, J., Boyle, J.P., Howard, C.B., Monie, T.P., Davis, B.K., Duncan, J.A., 2012. Pathogen sensing by nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is mediated by direct binding to muramyl dipeptide and ATP. J. Biol. Chem. 287, 23057–23067. https://doi.org/10.1074/jbc.M112.344283

Mojtahedi, Z., Clos, J., Kamali-Sarvestani, E., 2008. Leishmania major: identification of developmentally regulated proteins in procyclic and metacyclic promastigotes. Exp. Parasitol. 119, 422–429. https://doi.org/10.1016/j.exppara.2008.04.008

Morales-Yuste, M., Martín-Sánchez, J., Corpas-Lopez, V., 2022. Canine Leishmaniasis: Update on Epidemiology, Diagnosis, Treatment, and Prevention. Vet. Sci. 9, 387. https://doi.org/10.3390/vetsci9080387

Mosser, D.M., Brittingham, A., 1997. Leishmania, macrophages and complement: a tale of subversion and exploitation. Parasitology 115 Suppl, S9-23. https://doi.org/10.1017/s0031182097001789

Motwani, M., McGowan, J., Antonovitch, J., Gao, K.M., Jiang, Z., Sharma, S., Baltus, G.A., Nickerson, K.M., Marshak-Rothstein, A., Fitzgerald, K.A., 2021. cGAS-STING Pathway Does Not Promote Autoimmunity in Murine Models of SLE. Front. Immunol. 12, 605930. https://doi.org/10.3389/fimmu.2021.605930

Mugnier, M.R., Stebbins, C.E., Papavasiliou, F.N., 2016. Masters of Disguise: Antigenic Variation and the VSG Coat in Trypanosoma brucei. PLoS Pathog. 12, e1005784. https://doi.org/10.1371/journal.ppat.1005784

Mukherjee, A., Morosky, S.A., Delorme-Axford, E., Dybdahl-Sissoko, N., Oberste, M.S., Wang, T., Coyne, C.B., 2011. The coxsackievirus B 3C protease cleaves MAVS and TRIF to attenuate host type I interferon and apoptotic signaling. PLoS Pathog. 7, e1001311. https://doi.org/10.1371/journal.ppat.1001311

Müller, K., van Zandbergen, G., Hansen, B., Laufs, H., Jahnke, N., Solbach, W., Laskay, T., 2001. Chemokines, natural killer cells and granulocytes in the early course of Leishmania major infection in mice. Med. Microbiol. Immunol. (Berl.) 190, 73–76. https://doi.org/10.1007/s004300100084

Murphy, M.L., Wille, U., Villegas, E.N., Hunter, C.A., Farrell, J.P., 2001. IL-10 mediates susceptibility to Leishmania donovani infection. Eur. J. Immunol. 31, 2848–2856. https://doi.org/10.1002/1521-4141(2001010)31:10<2848::AID-IMMU2848>3.0.CO;2-T

Naderer, T., Ellis, M.A., Sernee, M.F., De Souza, D.P., Curtis, J., Handman, E., McConville, M.J., 2006. Virulence of Leishmania major in macrophages and mice requires the gluconeogenic enzyme fructose-1,6-bisphosphatase. Proc. Natl. Acad. Sci. 103, 5502–5507. https://doi.org/10.1073/pnas.0509196103

Nagle, A.S., Khare, S., Kumar, A.B., Supek, F., Buchynskyy, A., Mathison, C.J.N., Chennamaneni, N.K., Pendem, N., Buckner, F.S., Gelb, M.H., Molteni, V., 2014. Recent

Developments in Drug Discovery for Leishmaniasis and Human African Trypanosomiasis. Chem. Rev. 114, 11305–11347. https://doi.org/10.1021/cr500365f

Nandan, D., Lo, R., Reiner, N.E., 1999. Activation of phosphotyrosine phosphatase activity attenuates mitogen-activated protein kinase signaling and inhibits c-FOS and nitric oxide synthase expression in macrophages infected with Leishmania donovani. Infect. Immun. 67, 4055–4063.

Nandan, D., Oliveira, C.C. de, Moeenrezakhanlou, A., Lopez, M., Silverman, J.M., Subek, J., Reiner, N.E., 2012. Myeloid Cell IL-10 Production in Response to Leishmania Involves Inactivation of Glycogen Synthase Kinase-3β Downstream of Phosphatidylinositol-3 Kinase. J. Immunol. 188, 367–378. https://doi.org/10.4049/jimmunol.1100076

Nandan, D., Reiner, N.E., 2005. Leishmania donovani engages in regulatory interference by targeting macrophage protein tyrosine phosphatase SHP-1. Clin. Immunol., Renovations for an intracellular life style 114, 266–277. https://doi.org/10.1016/j.clim.2004.07.017

Nandan, D., Yi, T., Lopez, M., Lai, C., Reiner, N.E., 2002. Leishmania EF-1alpha activates the Src homology 2 domain containing tyrosine phosphatase SHP-1 leading to macrophage deactivation. J. Biol. Chem. 277, 50190–50197. https://doi.org/10.1074/jbc.M209210200

Napetschnig, J., Wu, H., 2013. Molecular basis of NF-κB signaling. Annu. Rev. Biophys. 42, 443–468. https://doi.org/10.1146/annurev-biophys-083012-130338

Nascimento, M.S.L., Ferreira, M.D., Quirino, G.F.S., Maruyama, S.R., Krishnaswamy, J.K., Liu, D., Berlink, J., Fonseca, D.M., Zamboni, D.S., Carregaro, V., Almeida, R.P., Cunha, T.M., Eisenbarth, S.S., Silva, J.S., 2016. NOD2-RIP2-Mediated Signaling Helps

Shape Adaptive Immunity in Visceral Leishmaniasis. J. Infect. Dis. 214, 1647–1657. https://doi.org/10.1093/infdis/jiw446

Negrão, F., Diedrich, J.K., Giorgio, S., Eberlin, M.N., Yates, J.R., 2019. Tandem Mass Tag Proteomic Analysis of *in Vitro* and *in Vivo* Models of Cutaneous Leishmaniasis Reveals Parasite-Specific and Nonspecific Modulation of Proteins in the Host. ACS Infect. Dis. 5, 2136–2147. https://doi.org/10.1021/acsinfecdis.9b00275

Neves, B.M., Silvestre, R., Resende, M., Ouaissi, A., Cunha, J., Tavares, J., Loureiro, I., Santarém, N., Silva, A.M., Lopes, M.C., Cruz, M.T., Cordeiro da Silva, A., 2010. Activation of Phosphatidylinositol 3-Kinase/Akt and Impairment of Nuclear Factor-κB: Molecular Mechanisms Behind the Arrested Maturation/Activation State of Leishmania infantum-Infected Dendritic Cells. Am. J. Pathol. 177, 2898–2911. https://doi.org/10.2353/ajpath.2010.100367

Newell, C.L., Deisseroth, A.B., Lopez-Berestein, G., 1994. Interaction of nuclear proteins with an AP-1/CRE-like promoter sequence in the human TNF-alpha gene. J. Leukoc. Biol. 56, 27–35. https://doi.org/10.1002/jlb.56.1.27

No, J.H., 2016. Visceral leishmaniasis: Revisiting current treatments and approaches for future discoveries. Acta Trop. 155, 113–123. https://doi.org/10.1016/j.actatropica.2015.12.016

Norris, K.A., Bradt, B., Cooper, N.R., So, M., 1991. Characterization of a Trypanosoma cruzi C3 binding protein with functional and genetic similarities to the human complement regulatory protein, decay-accelerating factor. J. Immunol. Baltim. Md 1950 147, 2240–2247.

Ogunkolade, B.W., Colomb-Valet, I., Monjour, L., Rhodes-Feuillette, A., Abita, J.P., Frommel, D., 1990. Interactions between the human monocytic leukaemia THP-1 cell

line and Old and New World species of Leishmania. Acta Trop. 47, 171–176. https://doi.org/10.1016/0001-706x(90)90023-s

Okwor, I., Uzonna, J., 2016. Social and Economic Burden of Human Leishmaniasis. Am. J. Trop. Med. Hyg. 94, 489–493. https://doi.org/10.4269/ajtmh.15-0408

Olivier, M., Atayde, V.D., Isnard, A., Hassani, K., Shio, M.T., 2012. Leishmania virulence factors: focus on the metalloprotease GP63. Microbes Infect. 14, 1377–1389. https://doi.org/10.1016/j.micinf.2012.05.014

Olivier, M., Hassani, K., 2010. Protease inhibitors as prophylaxis against leishmaniasis: new hope from the major surface protease gp63. Future Med. Chem. 2, 539–542. https://doi.org/10.4155/fmc.10.17

O'Neill, L.A.J., Golenbock, D., Bowie, A.G., 2013. The history of Toll-like receptors - redefining innate immunity. Nat. Rev. Immunol. 13, 453–460. https://doi.org/10.1038/nri3446

Pacakova, L., Harant, K., Volf, P., Lestinova, T., 2022. Three types of Leishmania mexicana amastigotes: Proteome comparison by quantitative proteomic analysis. Front. Cell. Infect. Microbiol. 12.

Palombella, V.J., Rando, O.J., Goldberg, A.L., Maniatis, T., 1994. The ubiquitinproteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell 78, 773–785. https://doi.org/10.1016/S0092-8674(94)90482-0

Pan, A.A., Duboise, S.M., Eperon, S., Rivas, L., Hodgkinson, V., Traub-Cseko, Y., McMahon-Pratt, D., 1993. Developmental life cycle of Leishmania--cultivation and characterization of cultured extracellular amastigotes. J. Eukaryot. Microbiol. 40, 213–223. https://doi.org/10.1111/j.1550-7408.1993.tb04906.x

Passelli, K., Billion, O., Tacchini-Cottier, F., 2021. The Impact of Neutrophil Recruitment to the Skin on the Pathology Induced by Leishmania Infection. Front. Immunol. 12.

Peters, N.C., Egen, J.G., Secundino, N., Debrabant, A., Kimblin, N., Kamhawi, S., Lawyer, P., Fay, M.P., Germain, R.N., Sacks, D., 2008. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science 321, 970–974. https://doi.org/10.1126/science.1159194

Piedrafita, D., Proudfoot, L., Nikolaev, A.V., Xu, D., Sands, W., Feng, G.-J., Thomas, E., Brewer, J., Ferguson, M.A.J., Alexander, J., Liew, F.Y., 1999. Regulation of macrophage IL-12 synthesis by Leishmania phosphoglycans. Eur. J. Immunol. 29, 235–244. https://doi.org/10.1002/(SICI)1521-4141(199901)29:01<235::AID-IMMU235>3.0.CO;2-S

Pivarcsi, A., Kemény, L., Dobozy, A., 2004. Innate immune functions of the keratinocytes. A review. Acta Microbiol. Immunol. Hung. 51, 303–310. https://doi.org/10.1556/AMicr.51.2004.3.8

Ponte-Sucre, A., Gamarro, F., Dujardin, J.-C., Barrett, M.P., López-Vélez, R., García-Hernández, R., Pountain, A.W., Mwenechanya, R., Papadopoulou, B., 2017. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. PLoS Negl. Trop. Dis. 11, e0006052. https://doi.org/10.1371/journal.pntd.0006052

Prasanna, P., Upadhyay, A., 2021. Heat Shock Proteins as the Druggable Targets in Leishmaniasis: Promises and Perils. Infect. Immun. 89, e00559-20. https://doi.org/10.1128/IAI.00559-20

Puentes, S.M., Sacks, D.L., da Silva, R.P., Joiner, K.A., 1988. Complement binding by two developmental stages of Leishmania major promastigotes varying in expression of a surface lipophosphoglycan. J. Exp. Med. 167, 887–902. https://doi.org/10.1084/jem.167.3.887

Qi, S., Wang, C., Zhang, R., Zhu, J., Hou, X., Jiang, Y., Li, J., Ren, M., Li, M., Hou, F., 2022. Human STING Is Regulated by an Autoinhibitory Mechanism for Type I Interferon Production. J. Innate Immun. 14, 518–531. https://doi.org/10.1159/000521734

Qiao, J., Zhang, Z., Ji, S., Liu, T., Zhang, X., Huang, Y., Feng, W., Wang, K., Wang, J., Wang, S., Meng, Z.-X., Liu, M., 2022. A distinct role of STING in regulating glucose homeostasis through insulin sensitivity and insulin secretion. Proc. Natl. Acad. Sci. U. S. A. 119, e2101848119. https://doi.org/10.1073/pnas.2101848119

Rabhi, I., Rabhi, S., Ben-Othman, R., Aniba, M.R., Trentin, B., Piquemal, D., Regnault, B., Guizani-Tabbane, L., Sysco Consortium, 2013. Comparative analysis of resistant and susceptible macrophage gene expression response to Leishmania majorparasite. BMC Genomics 14, 723. https://doi.org/10.1186/1471-2164-14-723

Rai, R., Dyer, P., Richardson, S., Harbige, L., Getti, G., 2017. Apoptotic induction induces Leishmania aethiopica and L. mexicana spreading in terminally differentiated THP-1 cells. Parasitology 144, 1912–1921. https://doi.org/10.1017/S0031182017001366

Rajput, A., Varshney, A., Bajaj, R., Pokharkar, V., 2022. Exosomes as New Generation Vehicles for Drug Delivery: Biomedical Applications and Future Perspectives. Molecules 27, 7289. https://doi.org/10.3390/molecules27217289

Rana, S.S., Bhasin, D.K., Nanda, M., Singh, K., 2007. Parasitic infestations of the biliary tract. Curr. Gastroenterol. Rep. 9, 156–164. https://doi.org/10.1007/s11894-007-0011-6

Rape, M., Jentsch, S., 2004. Productive RUPture: activation of transcription factors by proteasomal processing. Biochim. Biophys. Acta 1695, 209–213. https://doi.org/10.1016/j.bbamcr.2004.09.022

Rappleye, C.A., Eissenberg, L.G., Goldman, W.E., 2007. Histoplasma capsulatum alpha-(1,3)-glucan blocks innate immune recognition by the beta-glucan receptor. Proc. Natl. Acad. Sci. U. S. A. 104, 1366–1370. https://doi.org/10.1073/pnas.0609848104

Ready, P.D., 2010. Leishmaniasis emergence in Europe. Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 15, 19505.

Real, F., Florentino, P.T.V., Reis, L.C., Ramos-Sanchez, E.M., Veras, P.S.T., Goto, H., Mortara, R.A., 2014. Cell-to-cell transfer of Leishmania amazonensis amastigotes is mediated by immunomodulatory LAMP-rich parasitophorous extrusions. Cell. Microbiol. 16, 1549–1564. https://doi.org/10.1111/cmi.12311

Reiner, S.L., Locksley, R.M., 1995. The regulation of immunity to Leishmania major. Annu. Rev. Immunol. 13, 151–177. https://doi.org/10.1146/annurev.iy.13.040195.001055

Reithinger, R., Dujardin, J.-C., Louzir, H., Pirmez, C., Alexander, B., Brooker, S., 2007. Cutaneous leishmaniasis. Lancet Infect. Dis. 7, 581–596. https://doi.org/10.1016/S1473-3099(07)70209-8

Rethi, B., Eidsmo, L., 2012. FasL and TRAIL signaling in the skin during cutaneous leishmaniasis - implications for tissue immunopathology and infectious control. Front. Immunol. 3.

Rezaie, J., Aslan, C., Ahmadi, M., Zolbanin, N.M., Kashanchi, F., Jafari, R., 2021. The versatile role of exosomes in human retroviral infections: from immunopathogenesis to clinical application. Cell Biosci. 11, 19. https://doi.org/10.1186/s13578-021-00537-0

Ritter, U., Frischknecht, F., van Zandbergen, G., 2009. Are neutrophils important host cells for Leishmania parasites? Trends Parasitol. 25, 505–510. https://doi.org/10.1016/j.pt.2009.08.003

Roatt, B.M., de Oliveira Cardoso, J.M., De Brito, R.C.F., Coura-Vital, W., de Oliveira Aguiar-Soares, R.D., Reis, A.B., 2020. Recent advances and new strategies on leishmaniasis treatment. Appl. Microbiol. Biotechnol. 104, 8965–8977. https://doi.org/10.1007/s00253-020-10856-w

Rodríguez-García, E., Olagüe, C., Ríus-Rocabert, S., Ferrero, R., Llorens, C., Larrea, E., Fortes, P., Prieto, J., González-Aseguinolaza, G., Nistal-Villan, E., 2018. TMEM173 Alternative Spliced Isoforms Modulate Viral Replication through the STING Pathway. ImmunoHorizons 2, 363–376. https://doi.org/10.4049/immunohorizons.1800068

Roebrock, K., Sunderkötter, C., Münck, N.-A., Wolf, M., Nippe, N., Barczyk, K., Varga, G., Vogl, T., Roth, J., Ehrchen, J., 2014. Epidermal expression of I-TAC (Cxcl11) instructs adaptive Th2-type immunity. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 28, 1724–1734. https://doi.org/10.1096/fj.13-233593

Rohayem, J., Robel, I., Jäger, K., Scheffler, U., Rudolph, W., 2006. Protein-Primed and De Novo Initiation of RNA Synthesis by Norovirus 3Dpol. J. Virol. 80, 7060–7069. https://doi.org/10.1128/JVI.02195-05

Rojas-Bernabé, A., Garcia-HernáNdez, O., Maldonado-Bernal, C., Delegado-Dominguez, J., Ortega, E., GutiéRrez-Kobeh, L., Becker, I., Aguirre-Garcia, M., 2014. Leishmania mexicana lipophosphoglycan activates ERK and p38 MAP kinase and induces production of proinflammatory cytokines in human macrophages through TLR2 and TLR4. Parasitology 141, 788–800. https://doi.org/10.1017/S0031182013002187

Romano, A., Carneiro, M.B.H., Doria, N.A., Roma, E.H., Ribeiro-Gomes, F.L., Inbar, E., Lee, S.H., Mendez, J., Paun, A., Sacks, D.L., Peters, N.C., 2017. Divergent roles for Ly6C+CCR2+CX3CR1+ inflammatory monocytes during primary or secondary infection of the skin with the intra-phagosomal pathogen Leishmania major. PLoS Pathog. 13, e1006479. https://doi.org/10.1371/journal.ppat.1006479

Ronet, C., Passelli, K., Charmoy, M., Scarpellino, L., Myburgh, E., Hauyon La Torre, Y., Turco, S., Mottram, J.C., Fasel, N., Luther, S.A., Beverley, S.M., Launois, P., Tacchini-Cottier, F., 2019. TLR2 Signaling in Skin Nonhematopoietic Cells Induces Early Neutrophil Recruitment in Response to Leishmania major Infection. J. Invest. Dermatol. 139, 1318–1328. https://doi.org/10.1016/j.jid.2018.12.012

Rong, Y., Zhang, S., Nandi, N., Wu, Z., Li, L., Liu, Y., Wei, Y., Zhao, Y., Yuan, W., Zhou, C., Xiao, G., Levine, B., Yan, N., Mou, S., Deng, L., Tang, Z., Liu, X., Kramer, H., Zhong, Q., 2022. STING controls energy stress-induced autophagy and energy metabolism via STX17. J. Cell Biol. 221, e202202060. https://doi.org/10.1083/jcb.202202060

Rosas, L.E., Keiser, T., Barbi, J., Satoskar, A.A., Septer, A., Kaczmarek, J., Lezama-Davila, C.M., Satoskar, A.R., 2005. Genetic background influences immune responses and disease outcome of cutaneous L. mexicana infection in mice. Int. Immunol. 17, 1347–1357. https://doi.org/10.1093/intimm/dxh313

Rossi, M., Castiglioni, P., Hartley, M.-A., Eren, R.O., Prével, F., Desponds, C., Utzschneider, D.T., Zehn, D., Cusi, M.G., Kuhlmann, F.M., Beverley, S.M., Ronet, C., Fasel, N., 2017. Type I interferons induced by endogenous or exogenous viral infections promote metastasis and relapse of leishmaniasis. Proc. Natl. Acad. Sci. U. S. A. 114, 4987–4992. https://doi.org/10.1073/pnas.1621447114

Rossi, M., Fasel, N., 2018. How to master the host immune system? Leishmania parasites have the solutions! Int. Immunol. 30, 103–111. https://doi.org/10.1093/intimm/dxx075

Ruiz-Postigo, J.A., Jain, S., Madjou, S., Maia-Elkhoury, A.N., Valadas, S., Warusavithana, S., Osman, M., Yajima, A., Lin, Z., Beshah, A., Kim, S., 2022. Global leishmaniasis surveillance: 2021, assessing the impact of the COVID-19 pandemic. Wkly. Epidemiol. Rec. 575–590.

Ruiz-Postigo, J.A., Jain, S., Maia-Elkhoury, A.N., Warusavithana, S., Osman, M., Lin, Z., Beshah, A., Yajima, A., 2021. Global leishmaniasis surveillance: 2019–2020, a baseline for the 2030 roadmap. Wkly. Epidemiol. Rec.

Sacks, D., Noben-Trauth, N., 2002. The immunology of susceptibility and resistance to Leishmania major in mice. Nat. Rev. Immunol. 2, 845–858. https://doi.org/10.1038/nri933

Sacks, D.L., Melby, P.C., 2001. Animal Models for the Analysis of Immune Responses to Leishmaniasis. Curr. Protoc. Immunol. Ed. John E Coligan Al 0 19, Unit-19.2. https://doi.org/10.1002/0471142735.im1902s28

Saitoh, T., Fujita, N., Hayashi, T., Takahara, K., Satoh, T., Lee, H., Matsunaga, K., Kageyama, S., Omori, H., Noda, T., Yamamoto, N., Kawai, T., Ishii, K., Takeuchi, O., Yoshimori, T., Akira, S., 2009. Atg9a controls dsDNA-driven dynamic translocation of STING and the innate immune response. Proc. Natl. Acad. Sci. U. S. A. 106, 20842–20846. https://doi.org/10.1073/pnas.0911267106

Salam, N., Al-Shaqha, W.M., Azzi, A., 2014. Leishmaniasis in the Middle East: Incidence and Epidemiology. PLoS Negl. Trop. Dis. 8, e3208. https://doi.org/10.1371/journal.pntd.0003208

Samuel, C.E., 2001. Antiviral Actions of Interferons. Clin. Microbiol. Rev. 14, 778–809. https://doi.org/10.1128/CMR.14.4.778-809.2001

Santiago, F.W., Covaleda, L.M., Sanchez-Aparicio, M.T., Silvas, J.A., Diaz-Vizarreta, A.C., Patel, J.R., Popov, V., Yu, X., García-Sastre, A., Aguilar, P.V., 2014. Hijacking of RIG-I signaling proteins into virus-induced cytoplasmic structures correlates with the inhibition of type I interferon responses. J. Virol. 88, 4572–4585. https://doi.org/10.1128/JVI.03021-13

Satoh, T., Kato, H., Kumagai, Y., Yoneyama, M., Sato, S., Matsushita, K., Tsujimura, T., Fujita, T., Akira, S., Takeuchi, O., 2010. LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. Proc. Natl. Acad. Sci. U. S. A. 107, 1512–1517. https://doi.org/10.1073/pnas.0912986107

Schesser, K., Spiik, A.-K., Dukuzumuremyi, J.-M., Neurath, M.F., Pettersson, S., Wolf-Watz, H., 1998. The yopJ locus is required for Yersinia-mediated inhibition of NF-kappaB activation and cytokine expression: YopJ contains a eukaryotic SH2-like domain that is

essential for its repressive activity. Mol. Microbiol. 28, 1067–1079. https://doi.org/10.1046/j.1365-2958.1998.00851.x

Schindler, C., Shuai, K., Prezioso, V.R., Darnell, J.E., 1992. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. Science 257, 809–813. https://doi.org/10.1126/science.1496401

Schmitz, M.L., Baeuerle, P.A., 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. EMBO J. 10, 3805–3817.

Schneider, P., Rosat, J.P., Bouvier, J., Louis, J., Bordier, C., 1992. Leishmania major: differential regulation of the surface metalloprotease in amastigote and promastigote stages. Exp. Parasitol. 75, 196–206.

Schorey, J.S., Cheng, Y., Singh, P.P., Smith, V.L., 2015. Exosomes and other extracellular vesicles in host–pathogen interactions. EMBO Rep. 16, 24–43. https://doi.org/10.15252/embr.201439363

Schwende, H., Fitzke, E., Ambs, P., Dieter, P., 1996. Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. J. Leukoc. Biol. 59, 555–561.

Scorza, B.M., Wacker, M.A., Messingham, K., Kim, P., Klingelhutz, A., Fairley, J., Wilson, M.E., 2017. Differential Activation of Human Keratinocytes by Leishmania Species Causing Localized or Disseminated Disease. J. Invest. Dermatol. 137, 2149–2156. https://doi.org/10.1016/j.jid.2017.05.028

Scott, P., 1991. IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. J. Immunol. Baltim. Md 1950 147, 3149–3155.

Shaheen, F., Stephany-Brassesco, I., Kelly, B.L., 2021. Dynamic modulation of Leishmania cytochrome c oxidase subunit IV (LmCOX4) expression in response to

mammalian temperature. Mol. Biochem. Parasitol. 244, 111391. https://doi.org/10.1016/j.molbiopara.2021.111391

Shang, G., Zhang, C., Chen, Z.J., Bai, X.-C., Zhang, X., 2019. Cryo-EM structures of STING reveal its mechanism of activation by cyclic GMP-AMP. Nature 567, 389–393. https://doi.org/10.1038/s41586-019-0998-5

Sharma, M., Mandal, G., Mandal, S., Bhattacharjee, H., Mukhopadhyay, R., 2015. Functional role of lysine 12 in Leishmania major AQP1. Mol. Biochem. Parasitol. 201, 139–145. https://doi.org/10.1016/j.molbiopara.2015.07.005

Sharma, M., Rajendrarao, S., Shahani, N., Ramírez-Jarquín, U.N., Subramaniam, S., 2020. Cyclic GMP-AMP synthase promotes the inflammatory and autophagy responses in Huntington disease. Proc. Natl. Acad. Sci. U. S. A. 117, 15989–15999. https://doi.org/10.1073/pnas.2002144117

Sharma, S., DeOliveira, R.B., Kalantari, P., Parroche, P., Goutagny, N., Jiang, Z., Chan, J., Bartholomeu, D.C., Lauw, F., Hall, J.P., Barber, G.N., Gazzinelli, R.T., Fitzgerald, K.A., Golenbock, D.T., 2011. Innate Immune Recognition of an AT-Rich Stem-Loop DNA Motif in the Plasmodium falciparum Genome. Immunity 35, 194–207. https://doi.org/10.1016/j.immuni.2011.05.016

Shi, Y., Evans, J.E., Rock, K.L., 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. Nature 425, 516–521. https://doi.org/10.1038/nature01991

Shio, M.T., Christian, J.G., Jung, J.Y., Chang, K.-P., Olivier, M., 2015. PKC/ROS-Mediated NLRP3 Inflammasome Activation Is Attenuated by Leishmania Zinc-Metalloprotease during Infection. PLoS Negl. Trop. Dis. 9, e0003868. https://doi.org/10.1371/journal.pntd.0003868

Silva-Barrios, S., Stäger, S., 2017. Protozoan Parasites and Type I IFNs. Front. Immunol. 8, 14. https://doi.org/10.3389/fimmu.2017.00014

Silverman, J.M., Clos, J., Horakova, E., Wang, A.Y., Wiesgigl, M., Kelly, I., Lynn, M.A., McMaster, W.R., Foster, L.J., Levings, M.K., Reiner, N.E., 2010. Leishmania exosomes modulate innate and adaptive immune responses through effects on monocytes and dendritic cells. J. Immunol. Baltim. Md 1950 185, 5011–5022. https://doi.org/10.4049/jimmunol.1000541

Silverman, J.M., Reiner, N.E., 2012. Leishmania Exosomes Deliver Preemptive Strikes to Create an Environment Permissive for Early Infection. Front. Cell. Infect. Microbiol. 1. https://doi.org/10.3389/fcimb.2011.00026

Singh, K., Garg, G., Ali, V., 2016. Current Therapeutics, Their Problems and Thiol Metabolism as Potential Drug Targets in Leishmaniasis. Curr. Drug Metab. 17, 897–919. https://doi.org/10.2174/1389200217666160819161444

Singh, O.P., Singh, B., Chakravarty, J., Sundar, S., 2016. Current challenges in treatment options for visceral leishmaniasis in India: a public health perspective. Infect. Dis. Poverty 5, 19. https://doi.org/10.1186/s40249-016-0112-2

Sisquella, X., Ofir-Birin, Y., Pimentel, M.A., Cheng, L., Abou Karam, P., Sampaio, N.G., Penington, J.S., Connolly, D., Giladi, T., Scicluna, B.J., Sharples, R.A., Waltmann, A., Avni, D., Schwartz, E., Schofield, L., Porat, Z., Hansen, D.S., Papenfuss, A.T., Eriksson, E.M., Gerlic, M., Hill, A.F., Bowie, A.G., Regev-Rudzki, N., 2017. Malaria parasite DNA-harbouring vesicles activate cytosolic immune sensors. Nat. Commun. 8. https://doi.org/10.1038/s41467-017-02083-1

Skog, J., Würdinger, T., van Rijn, S., Meijer, D.H., Gainche, L., Sena-Esteves, M., Curry, W.T., Carter, B.S., Krichevsky, A.M., Breakefield, X.O., 2008. Glioblastoma

microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat. Cell Biol. 10, 1470–1476. https://doi.org/10.1038/ncb1800

Snyder, D.T., Hedges, J.F., Jutila, M.A., 2017. Getting “Inside” Type I IFNs: Type I IFNs in Intracellular Bacterial Infections. J. Immunol. Res. 2017, e9361802. https://doi.org/10.1155/2017/9361802

Soto-Serna, L.E., Diupotex, M., Zamora-Chimal, J., Ruiz-Remigio, A., Delgado-Domínguez, J., Cervantes-Sarabia, R.B., Méndez-Bernal, A., Escalona-Montaño, A.R., Aguirre-García, M.M., Becker, I., 2020. Leishmania mexicana: Novel Insights of Immune Modulation through Amastigote Exosomes. J. Immunol. Res. 2020, 8894549. https://doi.org/10.1155/2020/8894549

Srivastava, S., Pandey, S.P., Jha, M.K., Chandel, H.S., Saha, B., 2013. Leishmania expressed lipophosphoglycan interacts with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. Clin. Exp. Immunol. 172, 403–409. https://doi.org/10.1111/cei.12074

Stetson, D.B., Medzhitov, R., 2006. Type I Interferons in Host Defense. Immunity 25, 373–381. https://doi.org/10.1016/j.immuni.2006.08.007

Sturgill-Koszycki, S., Schlesinger, P., Chakraborty, P., Haddix, P., Collins, H., Fok, A., Allen, R., Gluck, S., Heuser, J., Russell, D., 1994. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science 263, 678–681. https://doi.org/10.1126/science.8303277

Sun, L., Wu, J., Du, F., Chen, X., Chen, Z.J., 2013. Cyclic GMP-AMP Synthase is a Cytosolic DNA Sensor that Activates the Type-I Interferon Pathway. Science 339, 10.1126/science.1232458. https://doi.org/10.1126/science.1232458

Sun, Y., Cheng, Y., 2020. STING or Sting: cGAS-STING-Mediated Immune Response to Protozoan Parasites. Trends Parasitol. 36, 773–784. https://doi.org/10.1016/j.pt.2020.07.001

Sundar, S., More, D.K., Singh, M.K., Singh, V.P., Sharma, S., Makharia, A., Kumar, P.C., Murray, H.W., 2000. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 31, 1104–1107. https://doi.org/10.1086/318121

Sundar, S., Rai, M., 2002. Laboratory diagnosis of visceral leishmaniasis. Clin. Diagn. Lab. Immunol. 9, 951–958. https://doi.org/10.1128/cdli.9.5.951-958.2002

Sundar, S., Singh, B., 2018. Emerging therapeutic targets for treatment of leishmaniasis. Expert Opin. Ther. Targets 22, 467–486. https://doi.org/10.1080/14728222.2018.1472241

Syme, R.M., Bruno, T.F., Kozel, T.R., Mody, C.H., 1999. The capsule of Cryptococcus neoformans reduces T-lymphocyte proliferation by reducing phagocytosis, which can be restored with anticapsular antibody. Infect. Immun. 67, 4620–4627.

Takaoka, A., Wang, Z., Choi, M.K., Yanai, H., Negishi, H., Ban, T., Lu, Y., Miyagishi, M., Kodama, T., Honda, K., Ohba, Y., Taniguchi, T., 2007. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature 448, 501–505. https://doi.org/10.1038/nature06013

Takashiba, S., Van Dyke, T.E., Amar, S., Murayama, Y., Soskolne, A.W., Shapira, L., 1999. Differentiation of monocytes to macrophages primes cells for lipopolysaccharide stimulation via accumulation of cytoplasmic nuclear factor kappaB. Infect. Immun. 67, 5573–5578.

Takeda, K., 2004. Toll-like receptors in innate immunity. Int. Immunol. 17, 1–14. https://doi.org/10.1093/intimm/dxh186

Talamás-Rohana, P., Wright, S.D., Lennartz, M.R., Russell, D.G., 1990. Lipophosphoglycan from Leishmania mexicana promastigotes binds to members of the CR3, p150,95 and LFA-1 family of leukocyte integrins. J. Immunol. Baltim. Md 1950 144, 4817–4824.

Talon, J., Horvath, C.M., Polley, R., Basler, C.F., Muster, T., Palese, P., García-Sastre, A., 2000. Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. J. Virol. 74, 7989–7996.

Tanaka, Y., Chen, Z.J., 2012a. STING Specifies IRF3 phosphorylation by TBK1 in the Cytosolic DNA Signaling Pathway. Sci. Signal. 5, ra20. https://doi.org/10.1126/scisignal.2002521

Tanaka, Y., Chen, Z.J., 2012b. STING Specifies IRF3 phosphorylation by TBK1 in the Cytosolic DNA Signaling Pathway. Sci. Signal. 5, ra20. https://doi.org/10.1126/scisignal.2002521

Tasew, G., Nylén, S., Lieke, T., Lemu, B., Meless, H., Ruffin, N., Wolday, D., Asseffa, A., Yagita, H., Britton, S., Akuffo, H., Chiodi, F., Eidsmo, L., 2010. Systemic FasL and TRAIL Neutralisation Reduce Leishmaniasis Induced Skin Ulceration. PLoS Negl. Trop. Dis. 4, e844. https://doi.org/10.1371/journal.pntd.0000844

Teixeira, M.J., Teixeira, C.R., Andrade, B.B., Barral-Netto, M., Barral, A., 2006. Chemokines in host–parasiteinteractions in leishmaniasis. Trends Parasitol. 22, 32–40. https://doi.org/10.1016/j.pt.2005.11.010

Thakker, M., Park, J.S., Carey, V., Lee, J.C., 1998. Staphylococcus aureus serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. Infect. Immun. 66, 5183–5189.

Trájer, A.J., 2013. THE EFFECT OF CLIMATE CHANGE ON THE POTENTIAL DISTRIBUTION OF THE EUROPEAN PHLEBOTOMUS SPECIES. Appl. Ecol. Environ. Res. 11, 189–208. https://doi.org/10.15666/aeer/1102_189208

Tripathi, P., Singh, V., Naik, S., 2007. Immune response to leishmania: paradox rather than paradigm. FEMS Immunol. Med. Microbiol. 51, 229–242. https://doi.org/10.1111/j.1574-695X.2007.00311.x

Tsigankov, P., Gherardini, P.F., Helmer-Citterich, M., Späth, G.F., Myler, P.J., Zilberstein, D., 2014. Regulation dynamics of Leishmania differentiation: deconvoluting signals and identifying phosphorylation trends. Mol. Cell. Proteomics MCP 13, 1787–1799. https://doi.org/10.1074/mcp.M114.037705

Turco, S.J., Sacks, D.L., 1991. Expression of a stage-specific lipophosphoglycan in Leishmania major amastigotes. Mol. Biochem. Parasitol. 45, 91–99. https://doi.org/10.1016/0166-6851(91)90030-A

Turpin, D., Truchetet, M.-E., Faustin, B., Augusto, J.-F., Contin-Bordes, C., Brisson, A., Blanco, P., Duffau, P., 2016. Role of extracellular vesicles in autoimmune diseases. Autoimmun. Rev. 15, 174–183. https://doi.org/10.1016/j.autrev.2015.11.004

Uchida, L., Espada-Murao, L.A., Takamatsu, Y., Okamoto, K., Hayasaka, D., Yu, F., Nabeshima, T., Buerano, C.C., Morita, K., 2014. The dengue virus conceals double-stranded RNA in the intracellular membrane to escape from an interferon response. Sci. Rep. 4, 7395. https://doi.org/10.1038/srep07395

Unterholzner, L., Keating, S.E., Baran, M., Horan, K.A., Jensen, S.B., Sharma, S., Sirois, C.M., Jin, T., Latz, E., Xiao, T.S., Fitzgerald, K.A., Paludan, S.R., Bowie, A.G., 2010. IFI16 is an innate immune sensor for intracellular DNA. Nat. Immunol. 11, 997–1004. https://doi.org/10.1038/ni.1932

Vallejo, A., Abad-Fernández, M., Moreno, S., Moreno, A., Pérez-Elías, M.J., Dronda, F., Casado, J.L., 2015. High levels of $CD4^+$ $CTLA\text{-}4^+$ Treg cells and CCR5 density in HIV-1-infected patients with visceral leishmaniasis. Eur. J. Clin. Microbiol. Infect. Dis. Off. Publ. Eur. Soc. Clin. Microbiol. 34, 267–275. https://doi.org/10.1007/s10096-014-2229-1

Van Bockstal, L., Bulté, D., Van den Kerkhof, M., Dirkx, L., Mabille, D., Hendrickx, S., Delputte, P., Maes, L., Caljon, G., 2020. Interferon Alpha Favors Macrophage Infection by Visceral Leishmania Species Through Upregulation of Sialoadhesin Expression. Front. Immunol. 11.

van Zandbergen, G., Klinger, M., Mueller, A., Dannenberg, S., Gebert, A., Solbach, W., Laskay, T., 2004. Cutting edge: neutrophil granulocyte serves as a vector for Leishmania entry into macrophages. J. Immunol. Baltim. Md 1950 173, 6521–6525.

Vargas-Inchaustegui, D.A., Hogg, A.E., Tulliano, G., Llanos-Cuentas, A., Arevalo, J., Endsley, J.J., Soong, L., 2010. CXCL10 production by human monocytes in response to Leishmania braziliensis infection. Infect. Immun. 78, 301–308. https://doi.org/10.1128/IAI.00959-09

Vargas-Inchaustegui, D.A., Tai, W., Xin, L., Hogg, A.E., Corry, D.B., Soong, L., 2009. Distinct Roles for MyD88 and Toll-Like Receptor 2 during Leishmania braziliensis Infection in Mice. Infect. Immun. 77, 2948–2956. https://doi.org/10.1128/IAI.00154-09

Vasconcellos, C., Sotto, M.N., 1997. Experimental cutaneous leishmaniasis: transmission electron microscopy of the inoculation site. Int. J. Exp. Pathol. 78, 81–89. https://doi.org/10.1046/j.1365-2613.1997.d01-243.x

Vaselek, S., 2021. Systematic Review: Re-emergence of human leishmaniasis in the Balkans. Trop. Med. Int. Health 26, 1189–1199. https://doi.org/10.1111/tmi.13653

Vasquez, R.E., Soong, L., 2006. CXCL10/gamma interferon-inducible protein 10-mediated protection against Leishmania amazonensis infection in mice. Infect. Immun. 74, 6769–6777. https://doi.org/10.1128/IAI.01073-06

Velez, R., Gállego, M., 2020. Commercially approved vaccines for canine leishmaniosis: a review of available data on their safety and efficacy. Trop. Med. Int. Health TM IH 25, 540–557. https://doi.org/10.1111/tmi.13382

Veras, P.S.T., de Menezes, J.P.B., Dias, B.R.S., 2019. Deciphering the Role Played by Autophagy in Leishmania Infection. Front. Immunol. 10, 2523. https://doi.org/10.3389/fimmu.2019.02523

Vieira, R. de S., Nascimento, M.S., Noronha, I.H., Vasconcelos, J.R.C., Benvenuti, L.A., Barber, G.N., Câmara, N.O.S., Kalil, J., Cunha-Neto, E., Almeida, R.R., 2021. STING Signaling Drives Production of Innate Cytokines, Generation of CD8+ T Cells and Enhanced Protection Against Trypanosoma cruzi Infection. Front. Immunol. 12, 775346. https://doi.org/10.3389/fimmu.2021.775346

Vinet, A.F., Fukuda, M., Turco, S.J., Descoteaux, A., 2009. The Leishmania donovani lipophosphoglycan excludes the vesicular proton-ATPase from phagosomes by impairing the recruitment of synaptotagmin V. PLoS Pathog. 5, e1000628. https://doi.org/10.1371/journal.ppat.1000628

Volpedo, G., Bhattacharya, P., Gannavaram, S., Pacheco-Fernandez, T., Oljuskin, T., Dey, R., Satoskar, A.R., Nakhasi, H.L., 2022. The History of Live Attenuated Centrin Gene-Deleted Leishmania Vaccine Candidates. Pathogens 11, 431. https://doi.org/10.3390/pathogens11040431

Wang, Peiyan, Li, S., Zhao, Y., Zhang, B., Li, Y., Liu, S., Du, H., Cao, L., Ou, M., Ye, X., Li, P., Gao, X., Wang, Penghua, Jing, C., Shao, F., Yang, G., You, F., 2019. The GRA15 protein from Toxoplasma gondii enhances host defense responses by activating the

interferon stimulator STING. J. Biol. Chem. 294, 16494–16508. https://doi.org/10.1074/jbc.RA119.009172

Wang, P.-H., Fung, S.-Y., Gao, W.-W., Deng, J.-J., Cheng, Y., Chaudhary, V., Yuen, K.-S., Ho, T.-H., Chan, Ching-Ping, Zhang, Y., Kok, K.-H., Yang, W., Chan, Chi-Ping, Jin, D.-Y., 2018. A novel transcript isoform of STING that sequesters cGAMP and dominantly inhibits innate nucleic acid sensing. Nucleic Acids Res. 46, 4054–4071. https://doi.org/10.1093/nar/gky186

Wang, X., Li, M., Zheng, H., Muster, T., Palese, P., Beg, A.A., García-Sastre, A., 2000. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. J. Virol. 74, 11566–11573.

Wassermann, R., Gulen, M.F., Sala, C., Perin, S.G., Lou, Y., Rybniker, J., Schmid-Burgk, J.L., Schmidt, T., Hornung, V., Cole, S.T., Ablasser, A., 2015. Mycobacterium tuberculosis Differentially Activates cGAS- and Inflammasome-Dependent Intracellular Immune Responses through ESX-1. Cell Host Microbe 17, 799–810. https://doi.org/10.1016/j.chom.2015.05.003

Webb, J.R., Button, L.L., McMaster, W.R., 1991. Heterogeneity of the genes encoding the major surface glycoprotein of Leishmania donovani. Mol. Biochem. Parasitol. 48, 173–184.

Weinheber, N., Wolfram, M., Harbecke, D., Aebischer, T., 1998. Phagocytosis of Leishmania mexicana amastigotes by macrophages leads to a sustained suppression of IL-12 production. Eur. J. Immunol. 28, 2467–2477. https://doi.org/10.1002/(SICI)1521-4141(199808)28:08<2467::AID-IMMU2467>3.0.CO;2-1

Wenzel, U.A., Bank, E., Florian, C., Förster, S., Zimara, N., Steinacker, J., Klinger, M., Reiling, N., Ritter, U., van Zandbergen, G., 2012. Leishmania major parasite stage-

dependent host cell invasion and immune evasion. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 26, 29–39. https://doi.org/10.1096/fj.11-184895

Whitaker, S.M., Colmenares, M., Pestana, K.G., McMahon-Pratt, D., 2008. Leishmania pifanoi Proteoglycolipid Complex P8 Induces Macrophage Cytokine Production through Toll-Like Receptor 4. Infect. Immun. 76, 2149–2156. https://doi.org/10.1128/IAI.01528-07

World Health Organisation. 2023. Leishmaniasis Fact sheet: Website https://www.who.int/newsroom/fact-sheets/detail/leishmaniasis.

World Health Organisation. 2023. Leishmaniasis Health Topics Overview: Website https://www.who.int/health-topics/leishmaniasis#tab=tab_1

WHO Expert Committee on the Control of the Leishmaniases, World Health Organization (Eds.), 2010. Control of the leishmaniases: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010, WHO technical report series. World Health Organization, Geneva.

Wikström, M.B., Dahlén, G., Kaijser, B., Nygren, H., 1984. Degradation of human immunoglobulins by proteases from Streptococcus pneumoniae obtained from various human sources. Infect. Immun. 44, 33–37.

Wiwanitkit, V., 2012. Interest in paromomycin for the treatment of visceral leishmaniasis (kala-azar). Ther. Clin. Risk Manag. 8, 323–328. https://doi.org/10.2147/TCRM.S30139

Würzner, R., 1999. Evasion of pathogens by avoiding recognition or eradication by complement, in part via molecular mimicry. Mol. Immunol. 36, 249–260.

Wyllie, S., Brand, S., Thomas, M., De Rycker, M., Chung, C., Pena, I., Bingham, R.P., Bueren-Calabuig, J.A., Cantizani, J., Cebrian, D., Craggs, P.D., Ferguson, L., Goswami, P., Hobrath, J., Howe, J., Jeacock, L., Ko, E.-J., Korczynska, J., MacLean, L., Manthri, S., Martinez, M.S., Mata-Cantero, L., Moniz, S., Nühs, A., Osuna-Cabello, M., Pinto, E., Riley, J., Robinson, S., Rowland, P., Simeons, F.R.C., Shishikura, Y., Spinks, D.,

Stojanovski, L., Thomas, J., Thompson, S., Viayna Gaza, E., Wall, R.J., Zuccotto, F., Horn, D., Ferguson, M.A.J., Fairlamb, A.H., Fiandor, J.M., Martin, J., Gray, D.W., Miles, T.J., Gilbert, I.H., Read, K.D., Marco, M., Wyatt, P.G., 2019. Preclinical candidate for the treatment of visceral leishmaniasis that acts through proteasome inhibition. Proc. Natl. Acad. Sci. 116, 9318–9323. https://doi.org/10.1073/pnas.1820175116

Xie, W., Lama, L., Adura, C., Tomita, D., Glickman, J.F., Tuschl, T., Patel, D.J., 2019. Human cGAS catalytic domain has an additional DNA-binding interface that enhances enzymatic activity and liquid-phase condensation. Proc. Natl. Acad. Sci. U. S. A. 116, 11946–11955. https://doi.org/10.1073/pnas.1905013116

Xu, L., Yu, D., Peng, L., Wu, Y., Fan, Y., Gu, T., Yao, Y.-L., Zhong, J., Chen, X., Yao, Y.-G., 2020. An Alternative Splicing of Tupaia STING Modulated Anti-RNA Virus Responses by Targeting MDA5-LGP2 and IRF3. J. Immunol. Baltim. Md 1950 204, 3191–3204. https://doi.org/10.4049/jimmunol.1901320

Yao, C., Donelson, J.E., Wilson, M.E., 2007. Internal and surface-localized major surface proteases of Leishmania spp. and their differential release from promastigotes. Eukaryot. Cell 6, 1905–1912. https://doi.org/10.1128/EC.00073-07

Yao, C., Donelson, J.E., Wilson, M.E., 2003. The major surface protease (MSP or GP63) of Leishmania sp. Biosynthesis, regulation of expression, and function. Mol. Biochem. Parasitol. 132, 1–16. https://doi.org/10.1016/s0166-6851(03)00211-1

Yilmaz, I.C., Dunuroglu, E., Ayanoglu, I.C., Ipekoglu, E.M., Yildirim, M., Girginkardesler, N., Ozbel, Y., Toz, S., Ozbilgin, A., Aykut, G., Gursel, I., Gursel, M., 2022. Leishmania kinetoplast DNA contributes to parasite burden in infected macrophages: Critical role of the cGAS-STING-TBK1 signaling pathway in macrophage parasitemia. Front. Immunol. 13, 1007070. https://doi.org/10.3389/fimmu.2022.1007070

Yin, Q., Tian, Y., Kabaleeswaran, V., Jiang, X., Tu, D., Eck, M.J., Chen, Z.J., Wu, H., 2012. Cyclic di-GMP Sensing via the Innate Immune Signaling Protein STING. Mol. Cell 46, 735–745. https://doi.org/10.1016/j.molcel.2012.05.029

Younis, B.M., Osman, M., Khalil, E.A.G., Santoro, F., Furini, S., Wiggins, R., Keding, A., Carraro, M., Musa, A.E.A., Abdarahaman, M.A.A., Mandefield, L., Bland, M., Aebischer, T., Gabe, R., Layton, A.M., Lacey, C.J.N., Kaye, P.M., Musa, A.M., 2021. Safety and immunogenicity of ChAd63-KH vaccine in post-kala-azar dermal leishmaniasis patients in Sudan. Mol. Ther. 29, 2366–2377. https://doi.org/10.1016/j.ymthe.2021.03.020

Yu, L., Liu, P., 2021. Cytosolic DNA sensing by cGAS: regulation, function, and human diseases. Signal Transduct. Target. Ther. 6, 1–15. https://doi.org/10.1038/s41392-021-00554-y

Yuan, D., Zhao, Y., Banks, W.A., Bullock, K.M., Haney, M., Batrakova, E., Kabanov, A.V., 2017. Macrophage Exosomes as Natural Nanocarriers for Protein Delivery to Inflamed Brain. Biomaterials 142, 1–12. https://doi.org/10.1016/j.biomaterials.2017.07.011

Zhang, C., Shang, G., Gui, X., Zhang, X., Bai, X.-C., Chen, Z.J., 2019. Structural basis of STING binding with and phosphorylation by TBK1. Nature 567, 394–398. https://doi.org/10.1038/s41586-019-1000-2

Zhang, H.-L., Ye, H.-Q., Liu, S.-Q., Deng, C.-L., Li, X.-D., Shi, P.-Y., Zhang, B., 2017. West Nile Virus NS1 Antagonizes Interferon Beta Production by Targeting RIG-I and MDA5. J. Virol. 91, e02396-16. https://doi.org/10.1128/JVI.02396-16

Zhang, Q., Raoof, M., Chen, Y., Sumi, Y., Sursal, T., Junger, W., Brohi, K., Itagaki, K., Hauser, C.J., 2010. Circulating Mitochondrial DAMPs Cause Inflammatory Responses to Injury. Nature 464, 104–107. https://doi.org/10.1038/nature08780

Zhang, S.Q., Zou, Z., Shen, H., Shen, S.S., Miao, Q., Huang, X., Liu, W., Li, L.P., Chen, S.M., Yan, L., Zhang, J.D., Zhao, J.J., Xu, G.T., An, M.M., Jiang, Y.Y., 2016. Mnn10 Maintains Pathogenicity in Candida albicans by Extending α-1,6-Mannose Backbone to Evade Host Dectin-1 Mediated Antifungal Immunity. PLoS Pathog. 12, e1005617. https://doi.org/10.1371/journal.ppat.1005617

Zhang, W., Jiang, X., Bao, J., Wang, Y., Liu, H., Tang, L., 2018. Exosomes in Pathogen Infections: A Bridge to Deliver Molecules and Link Functions. Front. Immunol. 9, 90. https://doi.org/10.3389/fimmu.2018.00090

Zhang, Xu, Wu, J., Du, F., Xu, H., Sun, L., Chen, Z., Brautigam, C.A., Zhang, Xuewu, Chen, Z.J., 2014. The Cytosolic DNA Sensor cGAS Forms An Oligomeric Complex with DNA and Undergoes Switch-like Conformational Changes in the Activation Loop. Cell Rep. 6, 421–430. https://doi.org/10.1016/j.celrep.2014.01.003

Zhang, Z., Yuan, B., Bao, M., Lu, N., Kim, T., Liu, Y.-J., 2011. The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. Nat. Immunol. 12, 959–965. https://doi.org/10.1038/ni.2091

Zhao, B., Du, F., Xu, P., Shu, C., Sankaran, B., Bell, S.L., Liu, M., Lei, Y., Gao, X., Fu, X., Zhu, F., Liu, Y., Laganowsky, A., Zheng, X., Ji, J.-Y., West, A.P., Watson, R.O., Li, P., 2019. A conserved PLPLRT/SD motif of STING mediates the recruitment and activation of TBK1. Nature 569, 718–722. https://doi.org/10.1038/s41586-019-1228-x

Zhong, H., May, M.J., Jimi, E., Ghosh, S., 2002. The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol. Cell 9, 625–636. https://doi.org/10.1016/s1097-2765(02)00477-x

Zhou, Y., Tian, T., Zhu, Y., Jaffar Ali, D., Hu, F., Qi, Y., Sun, B., Xiao, Z., 2017. Exosomes Transfer Among Different Species Cells and Mediating miRNAs Delivery. J. Cell. Biochem. 118, 4267–4274. https://doi.org/10.1002/jcb.26077

Zhu, C., Gagnidze, K., Gemberling, J.H.M., Plevy, S.E., 2001. Characterization of an Activation Protein-1-binding Site in the Murine Interleukin-12 p40 Promoter: DEMONSTRATION OF NOVEL FUNCTIONAL ELEMENTS BY A REDUCTIONIST APPROACH *. J. Biol. Chem. 276, 18519–18528. https://doi.org/10.1074/jbc.M100440200

Zilberstein, D., Shapira, M., 1994. The role of pH and temperature in the development of Leishmania parasites. Annu. Rev. Microbiol. 48, 449–470. https://doi.org/10.1146/annurev.mi.48.100194.002313