Expression of Green fluorescent protein (GFP) in *Leishmania spp*. to identify survival strategies the parasite imposes on the human host

BY

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Declaration

"I certify that the work contained in this thesis, or any part of it, has not been accepted in substance for any previous degree awarded to me, and is not concurrently being submitted for any degree other than that of Doctor of Philosophy being studied at the University of Greenwich. I also declare that this work is the result of my own investigations, except where otherwise identified by references and that the contents are not the outcome of any form of research misconduct."

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Abstract

Leishmania protozoa are the etiological agent of human disease known as leishmaniasis. There are three main forms of this disease, whose manifestation depends on a number of factors including the infecting *Leishmania* species and the immunological status of the individual. Disease development requires extracellular form of the parasite, the promastigotes to be internalised, transform and replicate as intracellular amastigotes within mammalian cells. Once inside host cells parasites they regulate and control immune responses from inside the host cells through the modulation of protein expression including cytokines. The changes involving *Leishmania* promastigote and host cells is well documented during the early stage of infection. However, little is known on the modulative effect of *L. aethiopica* and *L. mexicana* during infection of host cells. It is key to have an altruistic approach to when studying the different *Leishmania spp* during host infection, as it is a key factor in the outcome of type of leishmaniasis.

The study of infection is complicated by the inherent difficulty of detecting intracellular parasites, as many methods are laborious and time consuming (microscopy with Giemsa staining). Implementation of fluorescent expressing parasites has been successfully used in a number of species to observe and quantify cellular infection. Although successful stable genetically modified *Leishmania* parasites are not available for *L. mexicana* and *L. aethiopica*, this research aims to investigate survival mechanisms such as cytokine and protein expression during the early stages of infection. This requires monitoring infection as the first step, which involved developing for the first time constitutively expressing GFP in *L. aethiopica*, *L. major, L. mexicana* and *L. tropica*. The effect of infection on host macrophages was directly quantified via comparative proteomics which identified significant differences in protein expression not only between uninfected and infected cells at late stages of infection but also between different species. The effect of infection on the ability of controlling host and neighbouring cells was further characterised by looking at cytokine & antimicrobial peptide expression, including TNFα, IL-10, TGFβ, IL-1RA and cathelicidin at various time points during *L. aethiopica* infection. As cytokine expression is closely linked to manipulation of T-cells, which are also know to be ultimately responsible for disease exacerbation/resolution, the effect of the parasites on such cells was further investigate. A novel role for T cells on infection was explored, specifically their ability to host parasites.

The successful stable integration of GFP expression in four *Leishmania spp*. was confirmed by PCR, western blot, fluorescent microscopy and flow cytometry (N=3). These parasites allowed the monitoring of infection in macrophages whilst investigating the expression of cytokines, proteins, and novel human cell types. Cathelicidin expression was shown to increase during the early stages of infection at 8hrs following by a steady decline towards 48hrs post infection. The anti-inflammatory cytokine IL-10 expression was shown to increase from 2-48hrs peaking at the latest point with >3-fold difference post infection. Interestingly, the inflammatory cytokine TNFα showed expression increased from 2-48hrs, peaking with >5-fold at 48hrs post infection. Both these cytokines have been shown to peak at 8 hours followed by a decline, which may indicate subtle differences at a species (Lapara and Kelly 2010). Proteinomic study revealed *L. mexicana* and *L. aethiopica* both up-regulated a total of eight proteins with only two that were influenced by both species (N=3). In addition, down regulation was only identified during infection with *L. aethiopica* and not *L. mexicana*. However, during upregulation, the proteins identified were found to be involved in endocytosis (actin), immunological responses (MHC I), free radical scavenging and apoptosis (metallothionein). When comparing with similar proteomic studies by Singh *et al*, all modulation effects identified by *L. aethiopica* and *L. mexicana* were not previously shown during *L. donovani* infection. Infection was tested against human T lymphocytes as they have been implicated in the production of cytokines for the activation of macrophages allowing the elimination of intracellular parasites within macrophages. This novel work has shown *L. aethiopica* and *L. mexicana* are capable of infecting Jurkat cells (N=3) and enriched human T lymphocytes extracted from five different individuals. This finding has uncovered that infection is not only includes T cells (including $CD4^+$ subsets) but percentage of infection is dependant on the species of parasites and the donor lymphocytes.

In conclusion, this research showed for the first time that *L. aethiopica* and *L. mexicana* modulate a repertoire of protein expression at 24hrs post infection. The changes of cytokines expression highlights differences between different species and the discovery that *Leishmania* has the ability to infect human T cells may commence the start of the complete understanding of the survival mechanisms imposed by the parasite on the wider cellular network. This will change the approach to studying immunology of leishmaniasis infection and potentially clarifying the role of these cells for which contrasting evidences in human and mice models have caused much debate.

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Abbreviations

18S ribosomal RNA	18SrRNA
4-(2-hydroxyethyl)-1-piperzineethanesulphonic acid	HEPES
Acquired Immune Disease Syndrome	AIDS
Adenosine diphosphate	ADP
Adenosine monophosphate	AMP
Adenosine triphosphate	ATP
Bacillus stearothermophilus restriction endonuclease XI	BstXI
Base pairs	Bps
Bicinchoninic acid	BCA
Caspase-asociated recruitment domain	CARD
Chaperone-assisted selective autophagy	CASA
Chemokine (C-X-C motif) ligand	CXCL
Chemokine ligand	CCL
Cluster of differentiation	CD
Complement protein 3	C3
Complement protein 5	C5
Complement receptor	CR
C-reactive protein	CRP
Cutaneous leishmaniasis	CL
Cycle threshold	cT
Cyclin-dependant kinases regulatory subunit	CKS1B
Dendritic cell	DC
Deoxyribonucleic acid	DNA

Diffuse cutaneous leishmaniasis	DCL
Disability-adjusted life years	DALYs
Dimethyl sulphoxide	DMSO
Eosinophil derived neurotoxin	EDN
Escherchia coli	*E.coli*
Ethylenediaminetetraacetic acid	EDTA
Fatty acid synthase	FAS
Fluorescein diacetate	FDA
Fluorescent active cell sorting	FACS
Forward Scatter	FSC
Geneticin sulphate	G418
Genomic DNA	gDNA
Granulocyte macrophage colony-stimulating factor	GM-CSF
Green fluorescent protein	GFP
Heat-inactivated foetal bovine serum	HI-FBS
High performance liquid chromatography	HPLC
Human acute monocytic leukaemic cell line	THP-1
Human immunodeficient virus	HIV
Human serum	HS
Human T cells	hT cells
Interferon	IFN
Interleukin	IL
Isobaric tags for relative and absolute quantification	iTRAQ
Interleukin-1 receptor antagonist	IL-1RA
Invariant natural killer cell	iNKT

Kinetoplast DNA	kDNA
Kilobase pairs	Kbps
Leishmania aethiopica	*L. aethiopica*
Leishmania amastigote medium	JH-30
Leishmania amazonensis	*L. amazonensis*
Leishmania braziliensis	*L. braziliensis*
Leishmania chagasi	*L. chagasi*
Leishmania infantum	*L. infantum*
Leishmania leishmania	*L. leishmania*
Leishmania major	*L. major*
Leishmania mexicana	*L. mexicana*
Leishmania tropica	*L. tropica*
Leishmania Viannia	*L. Viannia*
Langerhan cell	LC
Liquid chromatography tandem mass spectrometry	LC-MS/MS
Lipopolysaccharide	LPS
Lysogeny broth agar	LB agar
Macrophage colony-stimulating factor	M-CSF
Major histocompatability class	MHC
Messenger ribonucleic acid	mRNA
Metallothionein	MT
Molecular weight marker	MW
Monocyte attracting chemokine	MIP-1
Mucocutaneous leishmaniasis	ML
Multi-locus enzyme electrophoresis	MLEE

Natural T killer cell	NKT cell
Neomycin phosphotransferase gene	NEO
Neutrophils/polymorphonuclear leukocyte	PMN
Nitric oxide	NO
Optical density	OD
Peanut lectin agglutinin	PNA
Pencillin-Streptomycin-Glutamine	Penstrep
Phosphate buffer solution	PBS
Phosphatidylmyoinositol mannoside	PIM
Phospholipase D4	PLD4
Phycoerthrin	PE
Plasmid DNA	pDNA
Polymerase chain reaction	PCR
Post kala-azar dermal leishmaniasis	PKDL
Propidium iodide	PI
Quantitative PCR	qPCR
Reactive oxygen species	ROS
Retinoic acid	RA
Retinoic acid inducible gene	RIG
Red blood cell	RBC
Ribonuclease inhibitor	RI
Ribosomal ribonucleic acid	rRNA
Room temperature	RT
Side Scatter	SSC
Strong cation exchange	SCX

Super optimal broth with catabolite repression	SOC
Synaptopodin 2	SYNPO2
Tandem mass tag	TMT
TNF-related apoptosis inducing ligand	TRAIL
Tumour growth factor	TGF
Tumour necrosis factor	TNF
Tumour necrosis factor alpha-induced protein 8-like protein 2	TP8L2
T cell receptor	TCR
T helper cell	Th cell
T helper 1 response	Th1
T helper 2 response	Th2
Toll-like receptor	TLR
Toxoplasma gondii	*T. gondii*
Tumour necrosis factor	TNF
T regulatory cell	Treg cell
To be completed	TBC
Tris/Borate/EDTA	TBE
Western Blotting	WB
Wild-type	WT
World Health Organisation	WHO
Visceral leishmaniasis	VL
α-tubulin intergenic region	α-tubIR

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Chapter 1

Introduction

1.1 *Leishmania*

Leishmania are parasitic protozoa which infect mammalian hosts following a blood meal from their sandfly vector. They have a digenetic life cycle and exist as a motile, flagellated, promastigote (10-12μm) in the midgut of sandflies and as non-motile, obligate, intracellular amastigotes (3-7μm) in mammalian host cells (macrophages, neutrophils, basophils and eosinophils) (Gangneux, *et al.,* 2007; Sukmee *et al.,* 2008; Kaye and Scott 2011). There are more than 21 species of *Leishmania* infecting humans and causing leishmaniasis (Stuart *et al.,* 2008). These can manifest as localised cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (ML), visceral leishmaniasis (VL) or post-visceral leishmaniasis (PKDL) (Croft and Coombs 2003; Chappuis *et al.,* 2007; Stuart *et al.,* 2008). Leishmaniasis causes approximately 0.2-0.4 and 0.7-1.2 million VL and CL new cases, annually (http://www.who.int/mediacentre/factsheets/fs375/en/).

1.2 Taxonomy

Leishmania parasites belong to the order Kinetoplastida and Trypanosomatida family (Figure 1) (WHO, 1990) which means they are characterized by the presence of a kinetoplast. The initial classification of *Leishmania spp*. was based on geographic location, tropism, vector, clinical manifestation and antigenic properties (Bray 1974; Lumsden 1974; Pratt and David 1981; Lainson and Shaw 1987; Bañuls *et al.,* 2007). This lead to the application of biochemical and molecular analysis of proteins, antigens and kDNA (kinetoplast DNA) (Bañuls *et al.,* 2007)

have also been included to produce a more precise classification of *Leishmania* (Figure 1) (Chance *et al*., 1974; Kreutzer and Christensen, 1980; Simpson *et al*., 1980; Arnot and Barker, 1981; Miles *et al*., 1981; Pratt and David, 1981; De Ibarra *et al*., 1982; Handman and Curtis, 1982; Wirth and Pratt, 1982; Anthony *et al*., 1985; Saravia *et al*., 1985; Barker *et al*., 1986; Le Blancq *et al*., 1986a-e). All members of the *Leishmania* genus are parasitic to mammals (Bañuls *et al.,* 2007).

Leishmania taxonomy is split into two main subgenera (Figure 1), *Leishmania Leishmania* and *Leishmania Viannia* depending on their location in the intestine of the sandfly (Lainson and Shaw, 1987; Real, *et al*., 2013). There is a third subgenus, *Sauroleishmania* (Figure 1) which represents parasites with the ability of infecting lizards (Real *et al*., 2013) Isoenzyme analysis allowed further definition from subgenera into their respective complexes (Rioux, *et al*., 1990). The expansion of this technique with multi-locus enzyme electrophoresis (MLEE) allowed *Leishmania* species/intra-species distinguishing ability (Schönian *et al.,* 2008).

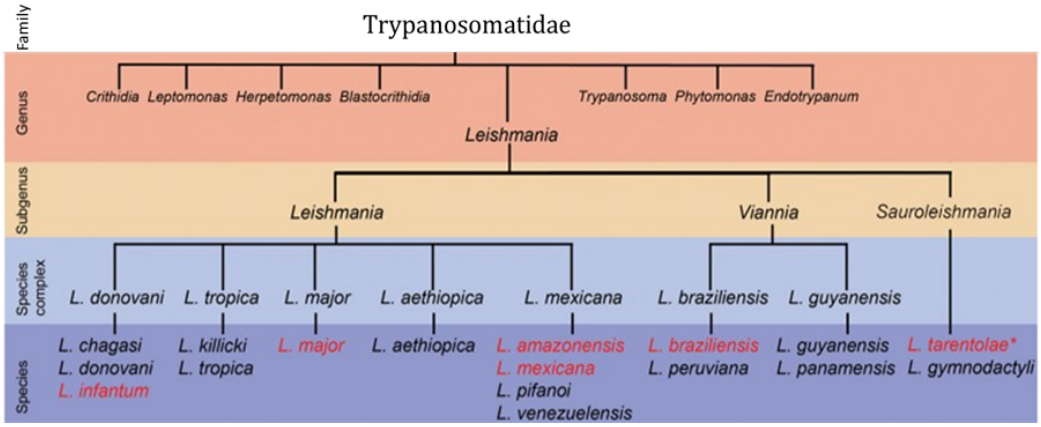


Figure 1: Overview of *Leishmania spp*. based on the MLEE taxonomic system. Classification of pathogenic and non-pathogenic *Leishmania* into genera, complexes and species. The colourimetric changes represents the phylogenetic catergorisation of *Leishmania* parasites which includes the *Trypanosomatidae* Family & *Leishmania* Genus (Red), *Leishmania Leishmania* & *Leishmania Viannia* Subgenus (Amber), Species Complex (Blue) and finally to the species level (Purple). (Adapted from Real *et al*., 2013)

1.3 Leishmaniasis

All species that have the ability to infect humans are able to cause cutaneous leishmaniasis (Stuart *et al*., 2008). Cutaneous lesions develop after the bite of an infected female sandfly (*Phlebotomus* or *Lutzomyia spp.*) (Bates, 2006). The initial swelling due to the bite is subsequently replaced with an erythema or a rash followed by the formation of a papule (Reithinger *et al*., 2007). This progresses into an ulcer weeks or months post-sandfly bite. In some cases it has been documented that infection can be found within the lymph node post-sandfly bite such as *L. braziliensis* (Barral *et al*., 1995). Clinical manifestation is dependent upon a variety of factors which are only partially understood, including the *Leishmania spp*., the immunological status and genetic background of the individual (*e.g.* compromised immune system, e.g. Acquired Immune Disease Syndrome, AIDS) and the zone of the bite etc (Reithinger *et al*., 2007). There is a strong association with members of the *L. donovani* complex causing VL and members of *L. Viannia* subgenus causing ML (Reithinger *et al*., 2007).

Some *Leishmania spp*. (*L. mexicana*, *L. aethiopica* and *L. amazonensis*) have the ability to spread away from the site of infection through the lymphatic system and develop nodules that do not ulcerate known as diffuse cutaneous leishmaniasis (DCL) (Masmoudi *et al*., 2013; Calvopina *et al*., 2006b; Van Griensven *et al*., 2016; Azeredo-Coutinho *et al*., 2007). The lesions developed during DCL do not self-heal or recover slowly and are resistant to treatment (Masmoudi *et al*., 2013; Berger, 2016) and are often confused with leprosies (Dassoni *et al*., 2013). Delayed diagnosis of localised or diffuse cutaneous leishmaniasis can result in enlarged lesions leading to scaring and increase the possibility of secondary bacterial infections (David and Craft, 2009).

Some species of *Leishmania* such as *Leishmania braziliensis* (*L. braziliensis*) have the ability to migrate to the mucosa causing disfiguration, this known as ML (Guerra *et al*., 2011). *L.*

braziliensis is found in the Americas (Central and South America) (Reithinger *et al.*, 2007) and is characterised by severe inflammation of the muscosa resulting in the progressive destruction of the mucosal region (nose/mouth to internal oropharyngeal organs including the possibility of the perforation of the septum) (Reithinger *et al.*, 2007; Chappuis *et al*., 2007). MCL lesions are not self-healing and often noticed months or years after the initial CL infection when infection of macrophages within the naso-oropharyngeal mucosa is established (Chappuis *et al*., 2007).

The most severe disease manifestation is VL, which is systemic and fatal if untreated (Chappuis *et al*., 2007). The main causative species are *L. donovani* (in East Africa and Indian subcontinent) and *L. infantum* (North Africa, Europe and Latin America) (Chappuis *et al*., 2007). VL develops 2-6 months post infection and involves the parasites migrating via blood vessels to blood filtering organs resulting in the infection of the liver, spleen and lymph nodes causing organ enlargement (Chappuis *et al*., 2007; McCall *et al*., 2013). Symptoms of individuals suffering from VL include fever, fatigue, splenomegly (swelling of the liver and spleen) and loss of appetite (Chappuis *et al*., 2007; Varma and Naseem, 2010). In some cases, including immunocompromised patients, following VL resolution a complication known as post kala-azar dermal leishmaniasis (PKDL) can develop (WHO, 2012). This dermal complication occurs during or after treatment which is difficult to report as it mimics clinical presentation of leprosy sufferers such as hypo-pigmented macular lesions (Trinidade, *et al*., 2015).

1.4 Epidemiology and Geographical distribution

*Leishmania*sis is endemic in 88 countries over four continents, only not present in Antarctica (Figures 2 and 3). It affects 12 million people with 350 million at risk, over 2 millions new cases are reported annually (http://www.who.int/csr/resources/publications/CSR_ISR_2000_1leish-/en/). Cutaneous leishmaniasis is more wide spread than VL and occurs in three epidemiological

regions (in the Americas, Mediterranea and from Middle East to Central Asia) (WHO, 2010). It is estimated that >70% of all CL cases occur in ten countries, including Afghanistan, Algeria, Columbia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru. Ninety percent of all visceral *leishmania*sis (VL) occurs in Bangladesh, Nepal, India, Brazil, Ethiopia and Sudan (Chappuis *et al.,* 2007). There are between 20,000-40,000 annual deaths due to leishmaniasis (Alvar *et al*., 2012).

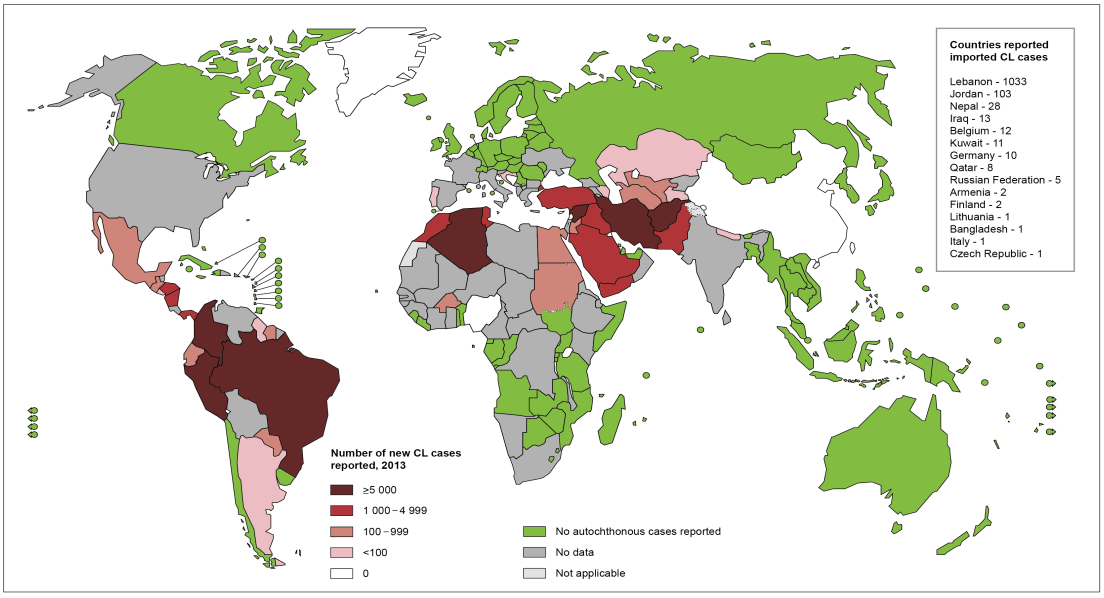


Figure 2: Geographical location of cutaneous leishmaniasis. The World Health Organisation's status of endemicity of cutaneous leishmaniasis (http://gamapserver.who.int/-mapLibrary/Files/Maps/Leishmaniasis_2013_CL.pn)

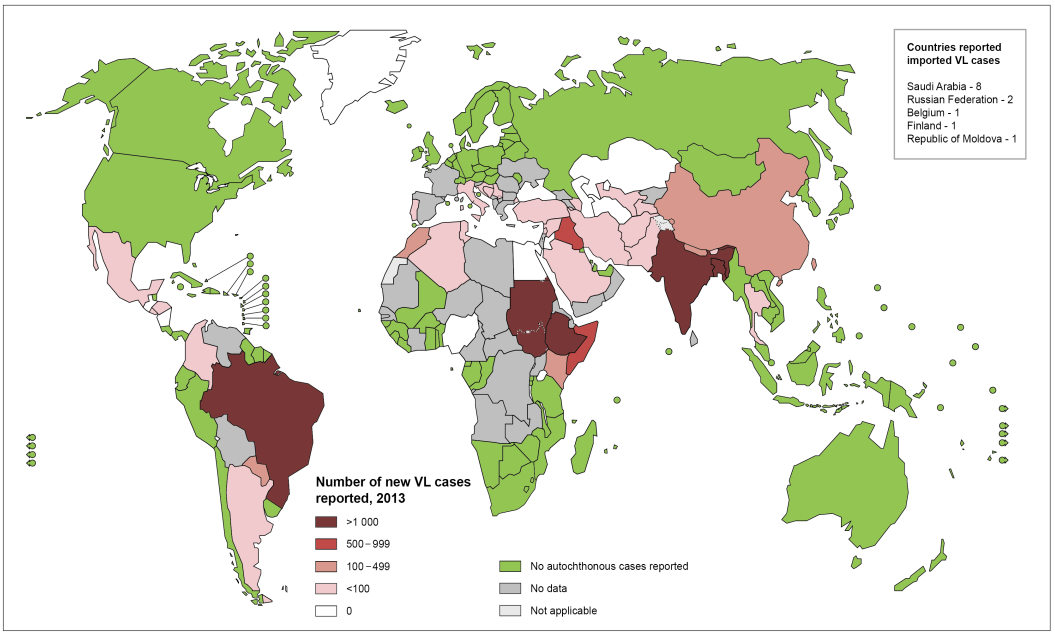


Figure 3: Geographical location of visceral leishmaniasis. The World Health Organisation's reported glocbal incidences of visceral leishmaniasis. (http://gamapserver.who.int/-mapLibrary/Files/Maps/Leishmaniasis_2013_VL.png)

The emerging of leishmaniasis into developed countries is due to a number of reasons including increased travelling, conflict (including U.S Gulf War veterans involved in Operation Desert Storm), urbanisation and co-infection of leishmaniasis with human immunodeficient virus (HIV) infection in rural locations (Magill *et al*., 1993; Lopez-Velez *et al*., 1998; Herwaldt, 1999). *Leishmania spp*. that cause VL are opportunistic with HIV infected individuals as it can lead to the rapid development of active VL in asymptomatic cases (http://www.who.int/leishmaniasis/burden/ hiv_coinfection/burden_hiv_coinfection/-en/). Levels of *Leishmania* infections in certain regions can be elevated when including high incidences of individuals suffering the immunocompromising HIV. The number of VL (*L. infantum*) and HIV infections has increased in the Mediterranean since the 1980's (Santos-Gomes *et al*., 2000). Ninety percent of >2000 co-infections identified in 2001 within southern Europe were in France, Italy, Portugal and Spain (WHO, 2007). This occurred due to geographical overlapping of HIV

with leishmaniasis and newly acquired infections among intravenous drug users (Alvar *et al*., 2008). In the Americas, the most co-infections are reported in Brazil, which has 620,000 individuals suffering HIV. The cases of VL increased from 700 cases in 1980 to 3,500 in 2005. Additionally, within the same time frame the tegumentary leishmaniasis increased from 4000 to 32,000 cases (WHO, 2007). In regions of Africa co-infection has increased due to migration and conflict *e.g*. In Humera, Ethiopia, 30% of all individuals suffering VL are co-infected with HIV. In Syria, prevalence of CL was only found in its endemic areas (Aleppo and Damascus) and pre-conflict (year 2010) incidence was 23,000 cases per annum (Hayani *et al*., 2015). In 2013 this elevated to 41,000 CL cases with high incidences within areas of Islamic State control (6,500 cases in Ar-Raqqah, Diyar Al-Zour and Hasakah) (Hayani *et al*., 2015; Haddad *et al*., 2015; Al-Salem *et al*., 2016).

In North America, it was thought VL was imported from infected dogs in southern Europe (*L. infantum*) and South America (*L. chagasi*) (Jeronimo *et al*., 2006; Herwalt *et al*., 2006). However, canine leishmaniasis was identified in foxhounds that had no history of travelling to enzootic *leishmania*-infected regions suggesting the transmission had been established within this continent (Anderson *et al*., 1980; Schantz *et al*., 2005). In 1999, *Leishmania* infections were identified at a New York foxhunting club which experienced >44% of dogs were infected and >11% died (Gaskin *et al*., 2002). A 3-year study (2000-2003) into canine VL in the U.S and Canada involved testing >12,000 hounds showed that it is enzootic in 18 U.S states and 2 Canadian provinces (Figure 4) (Duprey *et al*., 2006). Transmission was identified to be dog-to-dog as no dog-associated individual tested positive for human VL (Duprey *et al*., 2006). There are four species of *Lutzomyia* with *L. shannoni* overlapping the locations of many of the clubs where infected dogs were identified (Duprey *et al*., 2006). This species of sandfly has been proven capable of being infected after feeding on *L. infantum*-infected dogs and has been

suggested as an initiation for enzootic leishmania transmission from a canine reservoir (Travi *et al*., 2002; Duprey *et al*., 2006).

Figure 4: Distribution of confirmed canine VL cases in the U.S and Canadian provinces**.** States with confirmed cases of canine VL shade. Positive foxhounds were identified in Nova Scotia and Ontario, Canada (Duprey *et al*., 2006).

1.5 Life cycle

The *Leishmania* life cycle involves the initial inoculation of infectious metacyclic promastigotes into the human host during the blood meal of the sandfly which leads to infection of macrophages either directly or via phagocytosis of infected neutrophils (Figure 5). It is inside macrophages that intracellular amastigote parasites are either killed or replicate then spread and disease symptoms develop. The cycle is completed after another sandfly bites the individual resulting in the uptake of *Leishmania*-infected macrophages. Human cells are digested within the sand fly and amastigotes transform into procyclic promastigotes (Figure 5) (Dostalova and Volf

2012). Within sandflies, procyclic promastigotes develop into metacyclics to become infective to humans (Dostalova and Volf 2012). Transmission in mammals occurs during the bite from the female sandfly *Phlebotomus spp*., in the Old World (Africa, Europe, Asia) and *Lutzomyia spp*. in the New World (Americas) (Stuart *et al.,* 2008; Rogers *et al.,* 2008). Once inside the human host, infectious promastigotes avoid the innate human immune responses in order to be taken up and replicate as amastigotes in macrophages (Teixeira *et al*., 2013). Intracellular amastigotes manipulate the immune system in order to survive and spread within the human host (Gupta *et al*., 2013).

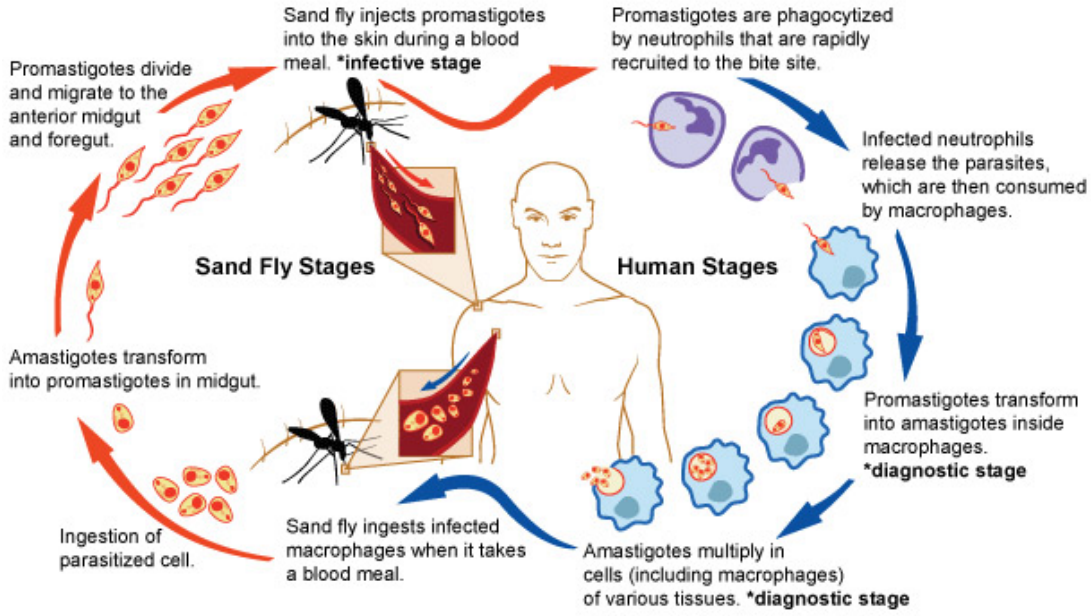


Figure 5 Life cycle of the *Leishmania* parasite.

The life cycle involves the inoculation of the promastigotes during the sandfly bite and the protozoa are phagocytosed by neutrophils. Afterwards, the macrophages phagocytose the infected neutrophils where the parasites are either eliminated or survive and replicate as amastigotes. The life cycle is completed upon the following a subsequent feed on the individual taking up the amastigotes which then transform back into their promastigotes form in the sandfly vector. (http://www.niaid.nih.gov/topics/- leishmaniasis/pages/lifecycle.aspx)

1.6 *Leishmania* and the immune system

In order for the parasite to successfully establish itself inside the human host and cause disease, *Leishmania* interacts closely with the human immune system to do so. In order to understand this interaction a brief overview of the immune system involved during infection is provided below.

The human immune system is comprised of innate and adaptive immunity (Delves and Roitt 2000a; Delves and Roitt 2000b) (Tables 1 and 2). The innate immune system includes biochemical and immune cells present prior to infection:

1) physical or chemical barriers *e.g.* epithelia and epithelial antimicrobial substances. 2) phagocytic cells *e.g.* neutrophils, macrophages and DC's.

3) natural killer cells

4) complement & mediators of inflammation in the blood

5) cytokines which are involved in the regulation and coordination of cellular responses during adaptive regulation (Table 2) (Delves and Roitt 2000).

The adaptive immune system on the other hand is characterised by cells (B, T lymphocytes), which mount a highly specific response to active infections as well as a protective response to following exposures (Table 2) (Delves and Roitt 2000a; Delves and Roitt 2000b).

Table 1: Innate human immune system that aid in the elimination of infections.

Human Innate defenses		Role	References
Physical Barriers	*Epithelial layer*	Physical barrier against invading microorganisms; the skin has the ability to produce a number of antimicrobial peptides and proteins (e.g. β-defensins, cathelicidin LL-37 and lysozymes)	Niyonsaba and Ogawa 2002
	Defensins and cathelicidins	Produced by different immune cells to help eliminating invading pathogens	Niyonsaba and Ogawa 2002; Lehrer and Ganz 2002
Circulating effector cells	*Neutrophils*	Phagocytosis; possess receptors for antibodies and complement	Delves and Roitt 2000a
	Macrophages	Possess limited repertoire of antibodies that bind to carbohydrate residues of pathogens; phagocytosis; possess receptors for antibodies and complement	Delves and Roitt 2000a
	Dendritic cells	Antigen-presenting cells when pattern-recognition receptors (PRRs) on their surface recognise PAMPs on the surface of pathogens; Activation leads to up-regulation of B7costimulatory molecules (CD80 and CD86) on their surface; Costimulatory molecules with the antigen receptor activates T cells at lymph nodes; Antigen is processed intracellularly prior to presentation by MHC molecules on the surface of DC's	Delves and Roitt 2000a; Medzhitov and Janeway 1997
	Natural killer cells	Destroys and eliminates infected and malignant cells	Biron *et al*., 1999
Circulating effector proteins	*Complement (all pathways)*	Aids in phagocytosis and lysis of pathogens via the MAC complex	Niyonsaba and Ogawa 2002; Black *et al*., 2004; Wallis and Drinkamer 1999
	C-reactive protein (CRP)	Binding to phagocytes via IgG, FcγRI and FcγRII leading to phagocytosis, respiratory burst and secretion of cytokines	Black *et al*., 2004
	Cytokines & chemokines	Pleiotrophic effects including inflammatory and anti-inflammatory responses	Dinarello 2007; Shaikh 2011; Zhang and An 2007
Cells receptors	*Pattern recognition receptors*	1) Toll-like receptors (targets pathogens and host components)	Akira *et al*., 2006
		2) Nod-like receptors (target bacteria and viruses)	Kanneganti *et al*., 2007
		3) Complement (tags pathogens for phagocytosis)	Gorgani *et al*., 2008
		4) CARD-containing proteins (apoptosis and cytokine maturation)	Bouchier-Hayes and Martin 2002
		5) C-type lectins (diverse and multifunctional)	Dambuza and Brown 2015
		6) Scavenger receptors (bind to host and pathogen components)	Mukhopadhyay and Gordon 2004
		7) Formyl peptide receptors	Kim *et al*., 2009
		8) RIG-I-like receptors (targets RNA viruses)	Loo and Gale 2011

Table 2: Cellular members of the adaptive human immune system.

Lympho cytes	Induction	Role	References
Th1 (CD4)	IL-12	Characterised by transcription factor T-bet and STAT4; produce IFN-γ, IL-2, TNFα, lymphotoxin/TNβ involved in cellular immunity	Zhu *et al*., 2010; Saito *et al*., 2010
Th2 (CD4)	IL-2, IL-4	Mediators of humoral immunity; produce IL-4, IL-5, IL-13 by GATA-3 and STAT6; TNFa, IL-9, IL-2	Zhu *et al*., 2010; Mosman *et al*., 1986; Saito *et al*., 2010
Th17 (CD4)	TGF-β1, IL-6 and IL-1 cytokines	Induction of inflammation signalling via RORγt; produce IL-17A, IL-17F and IL-22; host defense against bacteria, fungi and viruses	Zhu *et al*., 2010; Saito *et al*., 2010; Lee *et al*., 2012
Tregs (CD4+ CD25+)	in vitro via IL-10; ex vivo via IL-10 and TGF-β	Maintenance of peripheral tolerance; differentiation into Treg cells is transcription factor Foxp3	Saito *et al*., 2010; Sakaguchi 2005; Akbar *et al*., 2007; Tiemessen *et al*., 2007
NKT cells (CD4)	Th1 and Th2 cytokines	Combine features of adaptive and innate immunity; innate immune effectors by producing IFN-γ and CM-CSF as early host defense against pathogens such as bacteria, viruses and parasites; two subsets: CD56(bright) CD16 (dim) (90%) and CD56(dim) and CD16 (bright) (10%); express IL-2 receptor and cells expand after IL-2 exposure and c-kit receptor tyrosine kinase for IL-2 proliferation; Activated by IL-15, IL-2; CD4- primarily produce Th1 cytokines (IFN-y and TNF); CD4+ produce Th1 and Th2 cytokines (IL-4, IL-10, IL-13); TCR interaction occurs with CD1d glycoprotein and not MHC I or II molecules for peptide antigen presentation	Tupin *et al*., 2007; Cooper *et al*., 2001; Robertson and Ritz 1990; Biron *et al*., 1999; Bancroft 1993; Scharton-Kersten and Sher 1997; Lanier *et al*., 1986; Caligiuri *et al*., 1990; Baume *et al*., 1992; Caligiuri *et al*., 1993; Matos *et al*., 1993; Carson *et al*., 1997; Nagler *et al*., 1989; Carson *et al*., 1994; Coquet *et al*., 2008; Godfrey and Kronenberg 2004
B cells	IL-7	cytokines, Th1/Th2 responses; production of antibodies	LeBien and Tedder 2008

1.6.1 Innate Immunity

Leishmania promastigotes survive complement lysis before entering host cells via mechanisms previously described through the presence of proteases and variety of surface antigens (Franke *et al.,* 1985; Dominguez *et al.,* 2002). Upon entry into the human host the *Leishmania* parasites meet a hostile environment within the blood of individuals (Cunningham, 2002). *Leishmania* first encounters the complement which will lead to either lysis or opsonisation prior to phagocytosis. The initial encounter of the pathogen with the complement system involves the deposition of C3 proteins via the Alternative pathway (Mosser and Edelson, 1984; Mosser and Edelson, 1987). As a result, leukocyte migration and up-regulation of complement receptors on mononuclear phagocytes takes place (Yancey *et al.,* 1985). Complement proteins are an important proteinaceous cascade system involved in the elimination of promastigotes after innoculation, and its effect has been shown to be dose dependant (Rezai *et al*., 1969; Moreno *et al*., 2007). The classical pathway is activated by *Leishmania* but it is known that the alternative pathway is the eventual cascade that results in the killing of *Leishmania* parasites (Hoover *et al*., 1985). *Leishmania spp*. ability to resist complement lysis fully develops after procyclic promastigtoes mature into infective metacyclic parasites (Nunes *et al*., 1997; Moreno *et al*., 2007). Past studies have revealed differences in levels of amastigotes serum resistance between *Leishmania* species (Hoover *et al*., 1985; Puentes *et al*., 1988).

The most studied model used to mimic CL in humans is through infecting mice with *Leishmania spp*. The application of *L. major* for infection studies using mice is a good CL model by representing clinical symptoms and immunological factors features which are observed in humans, *i.e*. lesion development and the Th1/Th2 paradiagm (Loria-Cervera and Andrade-Narvaez 2014). Past infection studies using *L. major* show the formation of a lesion at the site caused after the bite from an infected-sandfly spontaneously healing which is also representative

in human infections (Belkaid *et al*., 2000). Murine studies (with C57BL/6 and BALB/c) with DCL and CL amastigotes showed self-healing for both mice breeds and only severe consequences with BALB/c mice through the loss of limb for both *Leishmania* disease specific types (Perez *et al*., 1978). Studies using *L. amazonensis* to infect susceptible mice, unlike *L. major* do not show an enhanced Th2 interleukin response and hence, been classed as a good murine model for mucocutaneous leishmaniasis (Liew, 1989; Heinzel *et al*., 1991; Afonso and Scott, 1993; Soong *et al*., 1997; Cupolilo *et al*., 2003). To have a good model for leishmaniasis that is comparable between different *Leishmania spp*. can only aid in the greater understanding of the survival strategies used by the parasite.

Parasites are phagocytosed by dendritic cells and neutrophils within thirty minutes post-sandfly bite. After several days, macrophages and monocytes become the most *Leishmania*-infected cell type (Ribeiro-Gomes, *et al*., 2012; Ng, *et al*., 2008). During the early stages, infected neutrophils are manipulated by the parasite to produce elevated levels of monocyte attracting chemokine (MIP-1) and IL-8. The infected-neutrophils are then phagocytosed by macrophages. This leads to the production of anti-inflammatory cytokines including TFG-β and lowered secretion of inflammatory ones such as tumour necrosis factor alpha (TNF-α). This promotes the survival of *Leishmania* through driving an anti-inflammatory immunological response (van Zandbergen *et al*., 2004). The DC's within the dermis phagocytose infected apoptopic neutrophils attenuating antigen presentation, expression of cell surface markers and priming of $CD4^+$ T lymphocytes (Ribeiro-Gomes *et al*., 2012). Hence, neutrophils have been described as the internal carriers known as 'Trojan horses', which facilitate the infection of macrophages and DCs during the initial establishment of infection. One week post-infection, there is an additional neutrophil recruitment which also includes macrophages (Ribeiro-Gomes *et al*., 2012; Ribeiro-Gomes and Sacks 2012). The recruitment is thought to be dependant on IL-17 as there is poor cell

accumulation when studying IL-17 $^{-/-}$ mice (Lopez *et al*., 2009). Four to five weeks post initial infection is characterised by the rapid replication of the parasite without development of a cutaneous lesion (Belkaid *et al*., 2000). Interestingly, non-immune cells within the dermatological site such as keratinocytes modulate the secretion of chemokines (CCL2 & CCL5) which induces the chemoaxis of macrophages to the site of infection. They are involved in mediating a Th2 immune response through the production of IL-4 and IL-6 during eight hours post infection (Enrchen *et al*., 2010).

The second infection phase, in mice, occurs five weeks post infection (Belkaid *et al*., 2000). The inoculation at dermal sites of murine models with low *Leishmania* doses has been suggested to be representative of natural infection leading to the formation of lesions. At this stage, there is a mass migration of phagocytic cells (neutrophils, DCs and macrophages) to the site of infection with macrophages being the main site of replication. The successful clearance of infection is dependent on macrophages activation of iNOS and production of nitric oxide (Green *et al*., 1990a-c). This type of macrophage activation is known as M1, which is induced by exposure to secreted inflammatory cytokines such as IFN-γ and TNF (Galván-Peña and O'Neill 2014).

Leishmania inhibits the Th1 response via stimulation of host cells expressing IL-3, IL-10, IL-13 and TGF-β cytokines, which inhibits the production of NO in macrophages preventing the elimination of infection (Reybier *et al*., 2010; Liu and Uzonna, 2012). These M2 cells involve the induction of arginase-1 producing urea, polyamines and ornithine involved in wound healing of macrophages preventing the clearance of infected macrophages (Galván-Peña and O'Neill 2014). This metabolism of arginine revealing the polarisation of macrophage activation is the main factor in human samples (Geelhaar-Karsch *et al*., 2013).

The principal cellular components of the innate immune system are neutrophils, mononuclear phagocytes and NK cells. These cells derived from the myeloid lineage circulate in the blood and migrate into tissues to lay siege onto pathogens. The innate immune cells secrete cytokines, which can activate phagocytes stimulating inflammatory responses (Cassatella 1995). This process involves the recruitment of leukocytes, movement of plasma proteins into areas of infection and both becoming activated eliminating the infectious agent. Inflammatory molecules such as cytokines and leukotriene B4 are involved during *Leishmania* infection. These molecules promote the activation of phagocytosis and the production of nitric oxide enabling the elimination of the parasite (Rodrigues *et al*., 2015).

1.6.1.1 Neutrophils

Neutrophils or polymorphonuclear (PMN) leukocytes are the most common abundant circulating leukocytes (Kobayashi and De Leo 2009). Their roles include the cellular recruitment via chemotaxis, transmigration, phagocytosis and peripheral cell activation for eliminating infection (Muller 2013; Kobayashi and De Leo 2009). PMN's are involved in acute inflammatory response and chemotactic recruitment via the release of IL-8, leukotrienes, prostaglandins and complement C5a (Kobayashi and De Leo 2009; Waltz *et al*., 1987; Yoshimura *et al*., 1987; Ehrengruber *et al*., 1994). PMN's are the first immune cells to reach the *Leishmania* infection site (Müller *et al*., 2001; Pompeu *et al*., 1991). Neutrophils have a protective role through phagocytosing promastigotes and producing anti-pathogenic factors (nitric oxide, neutrophil elastase and platelet activating factor, neutrophil extracellular traps) (Mollinedo *et al*., 2010; Pearson & Steigbigel, 1981; Charmoy *et al*., 2007; Ribeiro-Gomes et al., 2007; Camussi *et al*., 1987; de Souza Carmo *et al*., 2010; Novais *et al*., 2009). However, the outcome of infection is dependant on the *Leishmania spp.*, host genetics and current state (e.g. apoptopic, necrotic) of the neutrophil cells (Afonso *et al*., 2008; de Souza Carmo *et al*., 2010; Filardy *et al*., 2010;

Novais *et al*., 2009; Ribeiro-Gomes *et al*., 2004). Neutrophil depletion murine studies have shown an increase in parasitemia in resistant mice (C57BL/6 and C3H/HeJ) but susceptible (BALB/c) ones have shown a reduction in the parasitic load (Ribeiro-Gomes *et al*., 2004; Tacchini-Cottier *et al*., 2000). This may indicate the importance of neutrophils during the early stages of *Leishmania* infection as the depletion of this cell population may have resulted in the substitution of their proportion of cellular infection onto macrophage populations.

1.6.1.2 Mononuclear phagocytes

Mononuclear phagocytes include monocytes, macrophages, dendritic cells/DC's, Langerhans cells, and are part of the innate system however, they initiate and contribute to the adaptive immune responses. Monocytes and DC's circulate in the blood, monocytes represent 10% and 4% of nucleated cells in the blood stream in humans and mice, respectively (van Furth and Sluiter 1986; Swirski *et al*., 2009) and they terminally differentiate into highly phagocytic macrophages at the site of infection. All these cells are phagocytic, but the outcome of phagocytosis is dependant on the type of cell activation during infection (Liu and Uzonna 2012). Macrophages in tissues are replenished by arrival of recruited monocytes (van Furth and Cohn 1968), which classically occurs during inflammation. Macrophages function in cellular development, homeostasis and resolving inflammation.

Macrophages have the ability to recognise pathogens through the presence of specific receptors (C-type lectins, scavenger receptors, TLRs (Toll-like receptors), G protein-coupled and opsonins, *i.e.* antibodies, complement proteins, lectins). Pathogens are phagocytosed and destroyed through the formation of the phagolysosome where most of the antimicrobial products are concentrated (elastase, cathepsin G, reactive oxygen species - ROS, nitric oxide – NO).

It has been proposed that there are two main types of macrophage activation (Figure 6 and 7), M1 and M2 (M2a, 2b, 2c, 2d) (Mantovani, *et al*., 2004). The classical (M1) activation is characterised by increased capacity for antigen presentation, high expression of IL-12 and IL-23. This results in the production of toxic intermediates (nitric oxide, reactive oxygen intermediates) critical for killing *Leishmania* infected macrophages. IL-4 and IL-13 are known inhibitors of this and induce the alternative macrophage phenotype. During the infection of macrophages with *Leishmania* parasites, the M2 pathway has been linked with exposure to IL-10, glucocorticoids, or vitamin D3 hormones, and is associated with inhibiting the elimination of the intracellular parasite. Human macrophages simulated with GM-CSF (granulocyte macrophage colony-stimulating factor) or M-CSF (macrophage colony-stimulating factor) show M1 and M2 properties, respectively. The exposure of LPS and immune complexes to macrophages are characterised by elevated IL-10 and low levels of IL-12 (type II activated macrophages). Differentiated human monocytes by GM-CSF or M-CSF express M1 and M2 properties, respectively (Mantovani *et al.,* 2004; Kropf *et al.,* 2005).

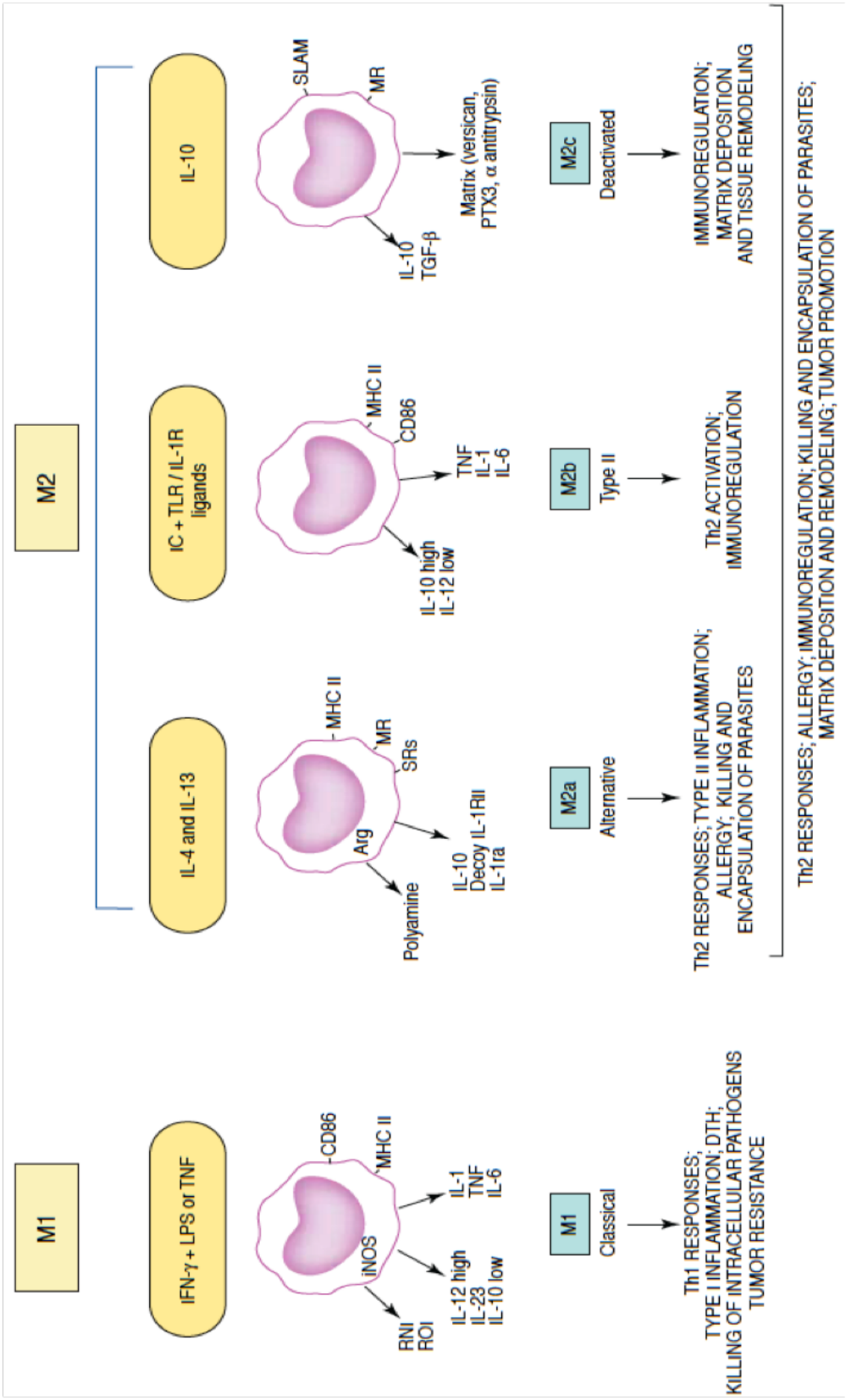


Figure 6: The various types of macrophage activation/inhibition.

(Adapted from Mantovani *et al.,* 2004)

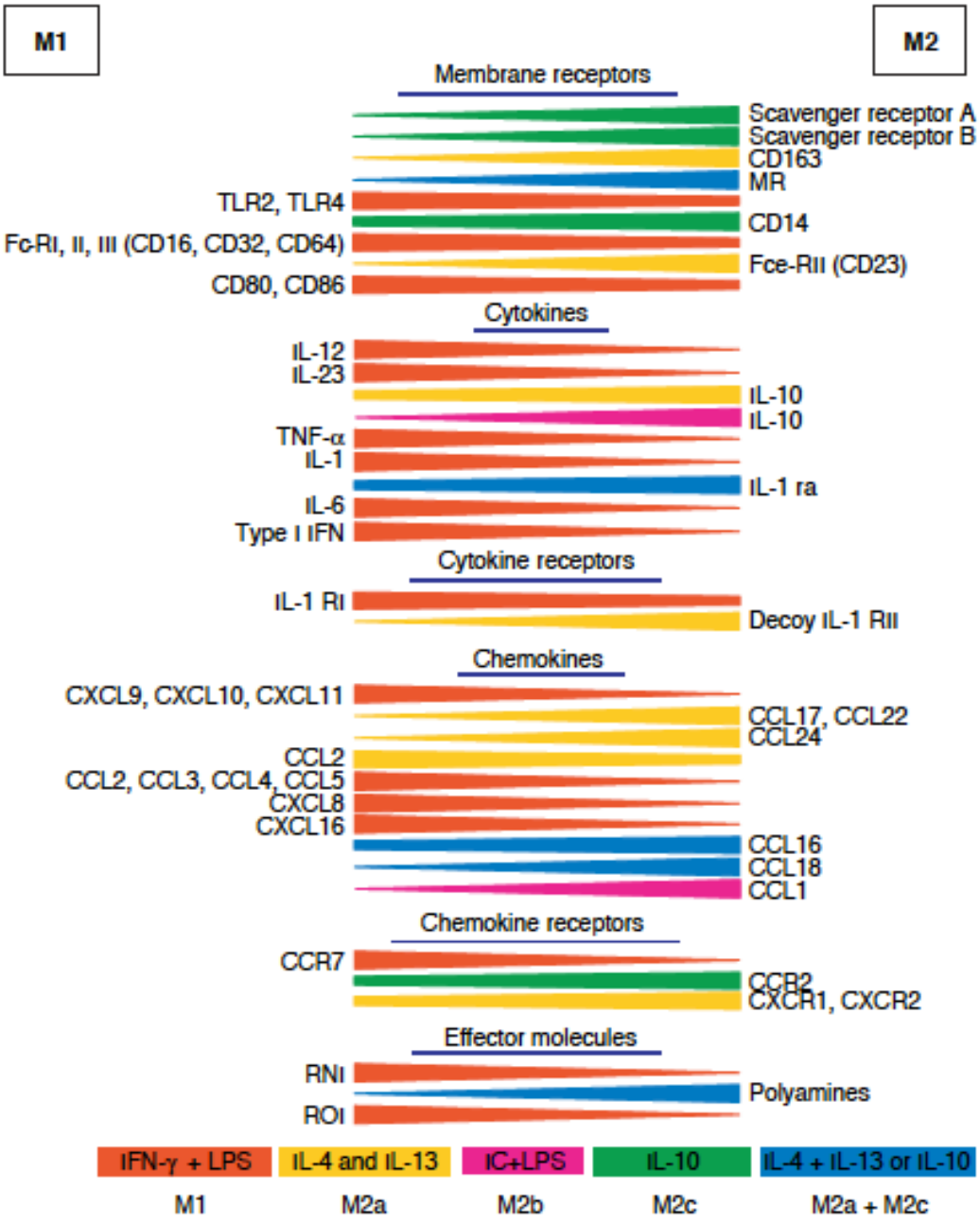


Figure 7: Macrophage phenotypes during the different activation pathways.

(Adapted from Mantovani *et al.,* 2004)

1.6.1.3 Dendritic cells (DCs)

DC progenitors (including epidermal Langerhan cells) become circulating precursors homing to tissues as immature phagocytes. They are an important link between the innate and the adaptive immune system. These cells have the ability to induce primary immune responses allowing the establishment of immunological memory. Additionally, DC's induce immunological tolerance and regulate the type of T lymphocyte cell mediated response (Banchereau 2000). They have long membrane projections, phagocytic abilities and are located around the body where lymphoid tissues are. They express pattern recognition receptors and respond to an infection with the secretion of cytokines. With respect to the adaptive immune responses they capture and display pathogen antigens to T lymphocytes (Bodey *et al*., 2004).

DCs have been implicated to have an important role in the immune responses during *L. major* infection (Gorak *et al*., 1998; von Stebut *et al*., 1998; Leon *et al*., 2007). In mice, *L. major* TCR-stimulated DC's indicate dermal DC's are important in secreting Th1 responses against the parasite (Leon *et al*., 2007). The epidermis contains three populations of DC's including the Langherhan cells and two migratory cells types. It is after antigen capture that immature DCs migrate to lymphoid organs where activated T cells contribute to their maturation allowing expansion and differentiation of the respective lymphocytes (Figure 8) (Banchereau 2000). *In vivo L. major* studies in mice have shown that the Langerhan cells (MHC II^{high}, CD11c+, $CD11b^{low}$, CD8αinter, $CD205^{high}$, and Langerin+ migrate to lymph nodes for the presentation to T cells (Moll *et al*., 1993). Further studies revealed that parasites within DCs in lymph nodes are $Langerin^{-}$ and express molecular markers, i.e. MHC II^{high}, $CD11c^{+}$, $CD11b^{+}$, $CD8\alpha^{-}$ and $CD205^{low}$ which is the population involved in the priming of $CD4^{+}$ T cells (Ritter *et al*., 2004). Mice genetically MHC class II deficient in Langheran cells (LCs) but not in dermal DC's manage to control *L. major* infection through Th1 differentiation into $CD4^{+}$ T cells producing

IFN-γ without the need of this subset of DC's (Lemos *et al*., 2004; Liu and Uzonna 2012). Additionally, LC's have been implicated as a possible pathogenic role in mice, as their absence (with the presence of Langerin^{+} dermal DCs) has shown to be responsible for reduced Treg cell migration and an appropriate Th1 response required for infection clearance (Kautz-Neu *et al*., 2011). Moreover, dermal DCs habouring *Leishmania* protozoa have been shown to most likely migrate and transport antigens to draining lymph nodes (Ritter *et al*., 2004; Ng *et al*., 2008). In addition, DCs derived from blood monocytes have been shown to differentiate in areas of dermal inflammation following the bite, phagocytosis of *Leishmania* transporting them to the draining lymph node for antigenic presentation to T cells (Leon *et al*., 2007).

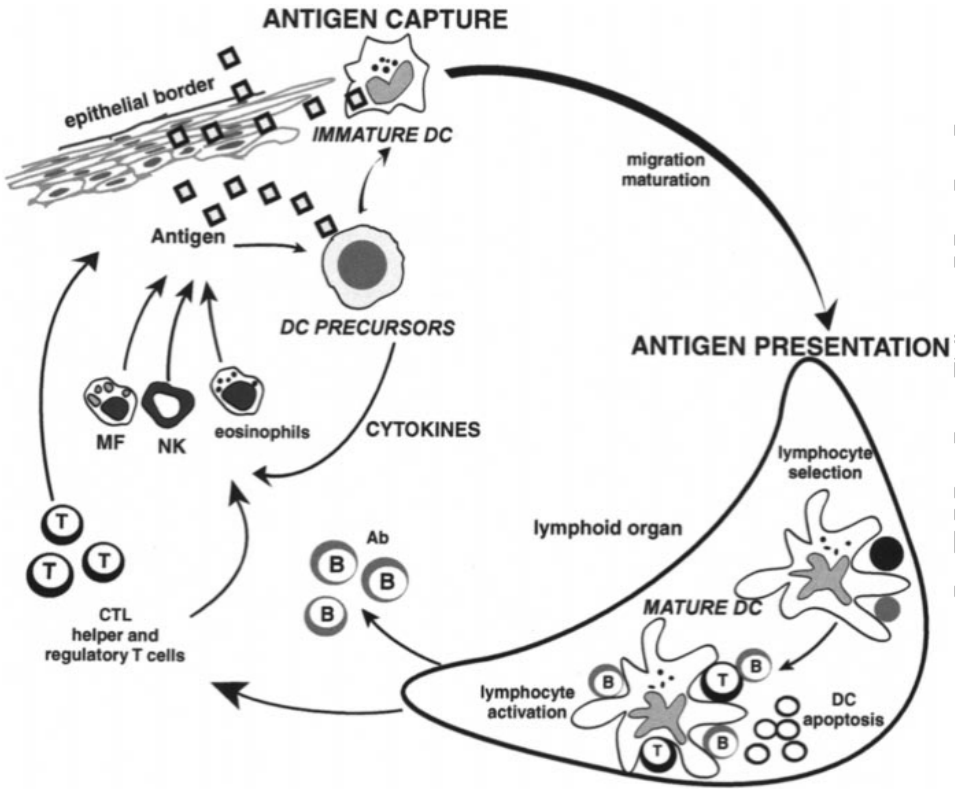


Figure 8: Interaction between lymphocytes and DC's (Banchereau 2000)

1.6.1.4 Natural Killer Cells (NK cells)

NK cells have the ability to kill a variety of different cells without the need for activation. Apart from their killing role they are a good source of IFN-γ cytokine with activate macrophages during infection.

1.6.2 Adaptive Immunity

The principal component of the adaptive immune system are lymphocytes, the only cells which have the ability to distinguish between antigenic epitopes and are responsible for the specificity and immune memory. Lymphocytes are distinguished within populations based on their membrane proteins and are divided into subsets based on their specific roles (Table 3). The main groups of lymphocytes are: T cells ($CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells, regulatory T cells [Tregs], γδ T lymphocytes), B cells and natural killer (NKT) cells (Table 3).

Table 3: Various classes of lymphocytes and lymphocyte-like cells

Lymphocyte name	Receptor chains	Surface markers	Secreted products	Affected cells	Role
Cytotoxic (CTLs)	αß	CD8	perforins, granenzymes,	all nucleated cells	killing infected cells
T Helper cells			cytokines (IFN-γ)		
Th1	αß	CD4	cytokines (IFN-γ)	macrophages	activation
Th2	αß	CD4	cytokines (IL-4, IL-5, Il-13)	B lymphocytes	proliferation,
					antibody production
Th17	αß	CD4	cytokines (IL-17)	stromal cells,	neutrophil production
				endothelium	and recruitment
Regulatory (Tregs)	αß	CD4	IL-10, TGF-ß	T, B lymphocytes,	inhibition
				macrophages	
γδ cells	γδ		IFN-γ	macrophages	activation, regulation
Natural killer T cells	αß	NK1.1	IFN-γ, IL-4	NK cells	activation, regulation
(NKT)					

(Adapted from Playfair and Bancroft, 2008)

Both B and T lymphocytes originate in the bone marrow; B cells produce antibodies while T cells guide the cellular immune responses through production of cytokines. The main subsets of B-lymphocytes include follicular, marginal zone and B-1 B cells. Studies on mice have shown that $CD4^{+}CD25^{+}$ T populations constitutively express IL-10 mRNA. Additionally, these cells also produce TGFß showing both their role in inflammatory responses and immunological homeostasis (Papiernik *et al.,* 1997; Mason and Powrie 1998; Pontoux *et al.,* 2002) (Table 3).

The are two types of NKT cells, type I and II with the latter associated with intestinal inflammation (Liao *et al*., 2013). However, during *Leishmania* infection in mice, type I NKT are

activated which is dependant on a TLR9 stimulation via DCs resulting in the production of IL-12 (Bogdan 2012). NK cells are not essential for clearance of *Leishmania* infection but still provide a benefical source of IFN-γ allowing antileishmanial activity in macrophages and protective Th cell responses (Bogdan 2012). The NK cell cytotoxity effect has been suggested to be largely inaffective as infected myeloid cells are résistant to natural killer cell lytic processes (Bogdan 2012). During human CL and VL, NK cells are suggested to be activated/inhibited by promastigotes and cells are suppressed during chronic non-healing infection (Bogdan 2012).

1.6.2.1 Antigen-presentation in T lymphocytes during *Leishmnaia* infection

Antigen presentation is the internalisation of antigens, proteolytic processing into peptides for their displayed by MHC I and II molecules. This presentation by DCs towards allows naïve T cells to become activated (Guermonprez *et al*., 2002). Successful antigen presentation is dependent on ligation of TCR with either CD4 or CD8, two soluble factors (*e.g.* IL-2) or ligation of co-stimulatory molecules complementing the TCR-CD4/CD8 engagement. The major T cell co-stimulatory molecule, CD28 interacts with the B7 family of ligands, which are essential for T-specific antigen specific T cell activation, expression of cytokines, and promotion in their expansion and differentiation (Chen and Flies 2013). CD28 is also involved/responsable for cytokine expression (IL-1, IL-2, IL-4, IL-5, TNF, IFN-γ) and affects the early development and differentiation of Th1/Th2 T cells (*in vitro* and *in vivo*) (Lenschow *et al.,* 1996).

The concept of Th cells being divided into lineages (Th1, Th2) based on their secreted cytokines and function was first proposed 30 years ago (Mosmann *et al.,* 1986; Mosmann *et al.,* 1996). The existence of additional T-helper subsets was confirmed following discovery of IL-23 dependant Th17 subsets, secreting IL-17 but not IFN-γ (Th1) or IL-4 (Th2) (Mosmann & Coffman 1989; Aggarwal *et al.,* 2003; Langrish *et al.,* 2005). Activation/inhibition of

macrophage is dependent upon which Th phenotypes is present. Th1 responses are dictated by their stimulation and concomitant expression of cytokines ligands/receptors *e.g.* CXCR3 & CCR5 on Th1 cells, CCR3, CCR4, CCR8 with Th2 phenotype. IL-12-driven cells express IL-12R-β2, IFN-γ, granenzymes, TNF-related apoptosis-inducing ligand, Fas ligand and CCL5 (chemokine ligand 5). However, in IL-23 driven cells, they express IL-23R, IL17A, IL-17F, IL-6, TNF, CCL6 and α3 integrin (McKenzie *et al.,* 2006) (Figure 9, below). The Th1 response is critical for the successful elimination of *Leishmania* infection. The expression of B7-1 (B7/CD80) and B7-2 (CD86) occurs on B cells, T cells and APCs (DCs, Langerhans cells, activated monocytes, activated T cells, and some tumour cells (Taylor *et al.,* 2004; Lenschow *et al.,* 1996).

During *Leishmania* infection, lymphocytes, as part of the adaptive immune system through expression of cytokines, activate or inhibit parasitic infection in macrophages (Gupta *et* al., 2013). IFN-γ producing by Th1 cells is critical for successful elimination of *L. major* through the formation of nitric oxide in macrophages. Susceptibility during infection arises by production of Th2 cytokines such as IL-4 and IL-13. The implementation of resistant and susceptible mice during *L. major* characterises the polarised cytokine profiles by C57BL/6 and BALB/c murine traits (Gupta *et al*., 2013). The importance of parasitic clearance via Th1 responses is documented during *L. donovani* infection in mice and humans (Kushawaha *et al*., 2011). In humans, $CD8^+$ T lymphocytes have been shown to contribute in the resolution of disease by IFN-γ production (Auriault *et al*., 1999; Rostami *et al*., 2010). However, in murine models the importance of $CD8^+$ cells appears to depend on the model in question (Belkaid *et al*., 2002; Erb *et al*., 1996; Gomes-Pereira *et al*., 2004; Huber *et al*., 1998; Tsagozis *et al*., 2005). In resistant mice, Treg cells have been shown to be important through dampening $CD4^+$ T responses by the production of IL-10 allowing the persistence of the parasite (Liu *et al*., 2009; Suffia *et al*., 2005;

Yurchenko *et al*., 2006). However, this contributes to the long-term immunity to *L. major* through the balancing between the effector and regulatory T lymphocytes (Belkaid, 2003; Belkaid *et al*., 2002). It should be stated that IL-17 was first discovered as a proinflammatory cytokine when produced by $CD4^+$ memory T cells (Rouvier *et al.,* 1993). This cytokine has been shown to act on stromal endothelial cells and monocytes inducing the secretion of proinflammatory mediators (IL-8, CXCL1, TNF, GM-CSF) which cause the rapid recruitment of neutrophils during acute infection (Figure 9). Previous immunological investigations on human-derived T cells during have involved the Jurkat cell line (ATCC TIB-152), which was originally obtained in 1977, from the peripheral blood of a 14-year old boy, by Schneider *et al*., 1977.

1.6.2.2 B lymphocytes

B cells have been shown to be important during the early stages of infection as deficient B-lymphocytes in mice infected with *L. donovani* cause lower levels of parasite (Smelt *et al*., 2000). Marginal zone B lymphocytes decrease the cytotoxic effect of CD8 T cells and production of IFNγ by $CD4^+$ T lymphocytes causing elevations in parasitemia (Bankoti *et al*., 2012).

Table 4: Various differences between MHC classes and CD1 group 1 molecules

MHC and CD1 molecules	Chains	Ligands (amino acids)	Cells expressing	Affected cells
MHC class I	α, ß2M	8-11	all nucleated cells	CD8+ T (TCR a/ß)
MHC class II	αß	10-30	DCs, macrophages, B lymphocytes	CD4+ T (TCR a/ß)
CD1 group 1	α, ß2M	glycolipids	CDs, B lymphocytes	CD4-/8- T, CD8+ T, CD4+ T (TCR a/ß, gd)
CD1 group 2	α, ß2M	glycolipids, PIM, a-galactosyl ceramide, protozoa	Monocytes Macrophages	NKT, iNKT

Key: PIM - phosphatidylmyoinositol mannoside, iNKT - invariant natural killer cells

(Adapted from Playfair and Bancroft, 2008)

Table 5: Similarities between the TCR and the antibody molecule

Similarities/differences	TCR	Antibody molecule
Recombination (V-J-C genes)	T-cells only	B-cells only
Junctional diversity	Yes	Yes
Sunsequent mutation	No	Yes
Chains	α/β or γ/δ	2 heavy (M,G,D,A or E) and 2 light (κ or λ)
Molecular Weight (kDa)	95	146-970
Recognition	1) Small linear peptides (9-15 amino acids) and MHC molecule 2) Glycolipids plus CD1 molecules	3D proteins and sugars
Secreted in blood	No	Yes

(Adapted from Playfair and Bancroft, 2008)

	Th1	Th17
IL-2	+	+
IL-4	–	–
IFNγ	+	–
IL-17A	–	+
IL-17F	–	+
IL-6	–	+
TNF	+	+
IL-12Rb1	+	+
IL-12Rb2	+	–
IL-23R	–	+
Granzyme	+	–
Fas ligand	+	–
TRAIL*	+	–
CCL5	+	–
CCL6	–	+
α3 Integrin	–	+

Figure 9: Differences in cytokine expression during Th1 and Th17 driven cells (McKenzie *et al.,* 2006). Key: Trail - TNF-related apoptosis-inducing ligand.

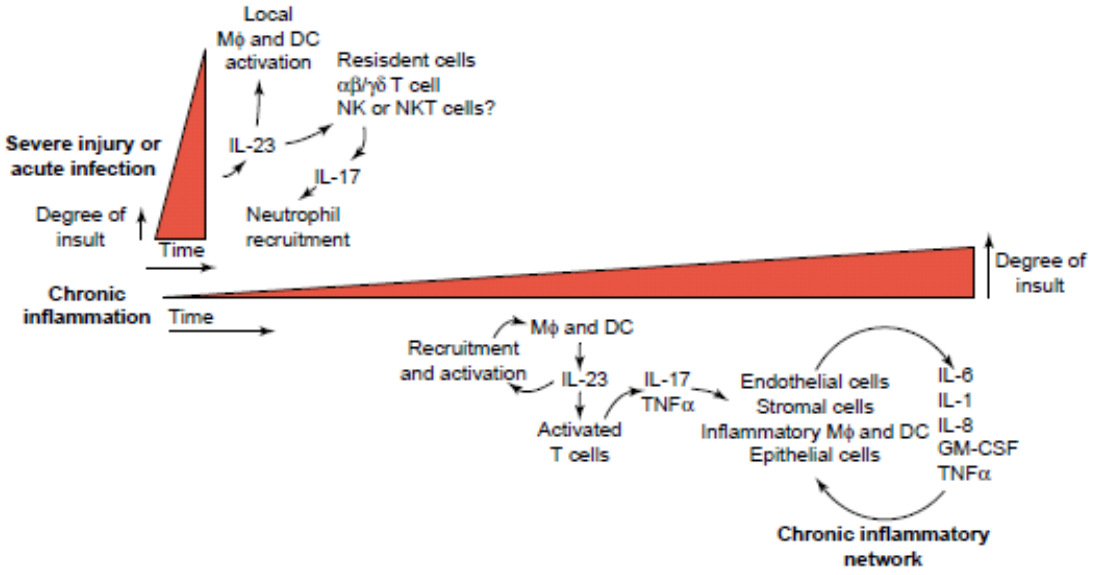


Figure 10: The effect of IL-17 and IL-23 during acute and chronic inflammation

(Adapted from McKenzie *et al.,* 2004)

Leishmania survival and disease development are the results of a complex and balanced interaction between the intracellular parasites and the immune system. The two most important components involved in this interaction are macrophages and T cells, where the former is the main host cell and T cells regulate to either allow the survival or death of *Leishmania*. In order to clarify *Leishmania* survival mechanisms within the human host the relationship between these two cell types and parasites needs to be further explored. To gain a greater insight into the survival strategies imposed by the parasite, development of green fluoresent protein (GFP) expression in *Leishmania spp*., T cell infections, proteomic and cytokine expression was investigated in this research. The involvement of T cells during infection remains controversial as they are only recognised as activators of macrophages, faciliatating the intracellular killing inside these phagocytes. Further investigation is required within this research area which involved the development GFP expressing parasites.

Aims

Leishmania species associated with causing CL (*L. major*), ML (*L. brazilensis*) and VL (*L. donovani*) manipulate a wide range of cellular pathways and responses to increase their likelihood of survival. This involves extracellular evasion and the hijacking of infected cells to prolong their existence, maximising their chances of continual spreading and continual transmission via their vector. In an attempt to increase our knowledge on the survival mechanisms by other *Leishmania spp.,* this work will investigate the influence species that are implicated with species that cause the diffuse form of the disease, *i.e*. *L. aethiopica* and *L. mexicana*. Hence, the following aims of this thesis are listed below.

1. To development novel GFP expression into *Leishmania* parasites (*L. mexicana*, *L. aethiopica*, *L. major*, *L. tropica*) allowing fluoresecent microscopy and flow cytometry analysis of infection in different human cell types.

2. To identify novel changes of cathelicidin and cytokine (TNFα, IL-10, IL-1RA, TGFβ) expression during *Leishmania aethiopica* infection between 2 and 48 hours.

3. To identify novel protein changes during the early stages of macrophage infection with *L. aethiopica* and *L. mexicana*.

4. To identify if DCL causing *L. aethiopica* and *L. mexicana* have the ability to infect human T lymphocytes.

Chapter 2

Methods & Materials

2.1. Cell culture

2.1.1 *Leishmania spp.* promastigote cultures

All *Leishmania spp.* (*L. major* MHOM/SU/73/5ASKH, *L. mexicana (*MNYC/B2/62/M379), *L. aethiopica* MHOM/ET/72/L-100, *L. tropica* (MHOM/SU/58/OD), and their green fluorescent protein (GFP) species equivalents were cultured using Drosophila Schneider medium (Invitrogen, UK) supplemented with 23% (v/v) heat-inactivated foetal bovine serum (HI-FBS) (Invitrogen, UK), 1% (v/v) penicillin, streptomycin, glutamine (Invitrogen, UK), 300μg/ml geneticin sulphate (G418) and incubated at 37^{o}C, 5% CO_2. All *Leishmania spp*. were initially subcultured weekly at a starting concentration of 5x10^{5} cells/ml at 24^{o}C. Cell counts were carried out following dilution in 0.04% (v/v) formaldehyde (Sigma, UK) on FASTREAD102 disposable counting slide (ImmuneSystems, UK).

2.1.2 THP-1 cell cultures

THP-1 cells (ECACC: 88081201, Human acute monocytic leukaemia cell line) were generously donated from Dr. Tim Paget, School of Pharmacy, University of Kent. THP-1 cell lines were maintained in RPMI-1640 (Fisher Scientific, UK) supplemented with 1% (v/v) penicillin, streptomycin, glutamine and 10% (v/v) HI-FCS (Complete RPMI-1640 media). These cells were subcultured every other day at a starting concentration of 2x10^{5} cells/ml and cell viability assessed microscopically by trypan exclusion method. This involved diluting the cells in 50%

trypan blue (0.04% v/v): 50% cell mixture (v/v) and performing a cell count using the FASTREAD102 disposable counting slides (ImmuneSystems, UK).

2.1.3 Jurkat cells

The Jurkat cell line (ATCC TIB-152) was originally obtained in 1977 from the peripheral blood of a 14-year old boy by Schneider *et al*., 1977. These cells were maintained in complete RPMI-1640 media (Method 2.1.2). Cells were counted and subcultured every 3 days at a starting concentration of $2x10^5$cells/ml. Viability was assessed microscopically via the trypan blue exclusion method previously mentioned (Methods 2.1.2).

2.1.4 Long-term storage of all *Leishmania spp*., Jurkat and THP-1 cells

The cryopreservation of all cells was carried out following centrifugation (1000 x g, 10 minutes for *Leishmania spp*. and 1000 x g, 10 minutes for THP-1 and Jurkat) of 10^6 cells/ml log-phase promastigotes or Jurkat and THP-1 cells. Cell viability was assessed using the trypan blue exclusion method (Methods 2.1.2) for the mammalian cell lines (Jurkat & THP-1 cells) and by fluorescent microscopy of the GFP-expressing parasites. The parasite viability involved placing 10μl of culture mixture onto a microscope slide by pipette, followed by a coverslip on top. The viability of the parasites was assessed through counting (>100 per count) the number of GFP expressing parasites from dead cells (non-GFP expressing parasites). Cells were resuspended in HI-FBS supplemented with 10% (v/v) DMSO (Invitrogen, UK). The cell suspension was mixed and aliquoted into 1.8ml freezing vials (Nalgene, UK). These were placed into a Cryo freezing container (Nalgene, UK) and stored at -80^{o}C for 48 hours to allow slow freezing of the cells. The vials were placed into a liquid nitrogen tank (Fisher Scientific, UK) for long-term storage. All cells were defrosted by removing the frozen vials from the liquid nitrogen tank, placing them in category level 2 hood and waiting 10 minutes for complete thawing. The cells were then

centrifuged as above and washed twice in their respective media. Finally, cells were placed into flasks cultured as previously mentioned (Method 2.1.1).

2.1.5 Growth curve of *Leishmania spp*.

The growth curve was carried out in triplicate over a period of 10 days. All *Leishmania spp*. (including their GFP-expressing equivalents) were cultured at $5x10^5$/ml in a 10ml culture flask at 24^oC. Daily counts were recorded microscopically following dilution in 0.04% (v/v) formaldehyde (Fisher Scientific, UK) in FASTREAD102 disposable counting slides (ImmuneSystems, UK). Growth curves were repeated three times and the mean results and corresponding standard deviations were plotted in Excel.

2.2 Development of green fluorescent protein (GFP) expression in *Leishmania spp*.

To develop GFP expression within *Leishmania spp*. two plasmids were used namely, pXG-GFP^+ (Figure 11) and pRib1.2aNEOaGFP (Figure 12). The pXG-GFP^+ and pRib1.2aNEOaGFP plasmids were generously donated from Professor Stephen Beverley (University of Harvard, U.S) and Professor Barbara Papadopoulos (Laval University, Canada), respectively. The pXG-GFP^+ plasmid was electroporated into the parasites' cytosol as previously done in *L. major* (Ha *et al*., 1991). The pRib1.2aNEOaGFP plasmid was cloned in *E. coli*, linearised by restriction enzyme to purify a DNA segment to be incorporated into the parasites' genome by homologous recombination. This successful genomic integration was previously carried out in *L. donovani* (Singh *et al*., 2009). The plasmids and the respective methodology will now be discussed.

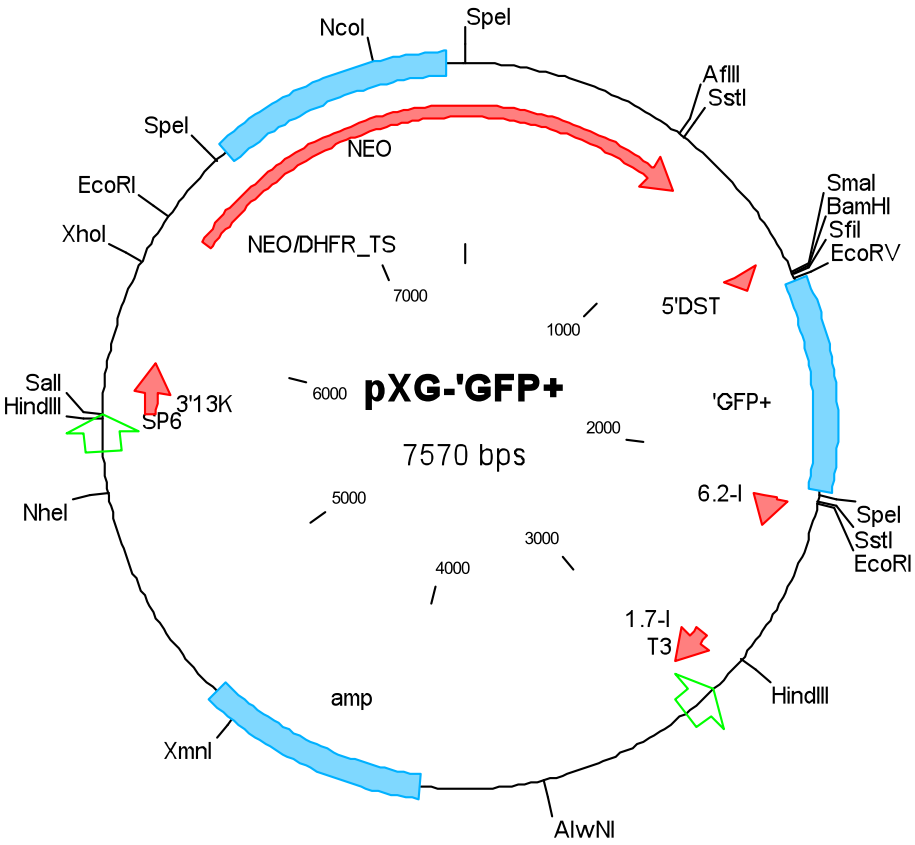


Figure 11: pXG-GFP+ plasmid for cyosolic GFP expression in *Leishmania spp*.

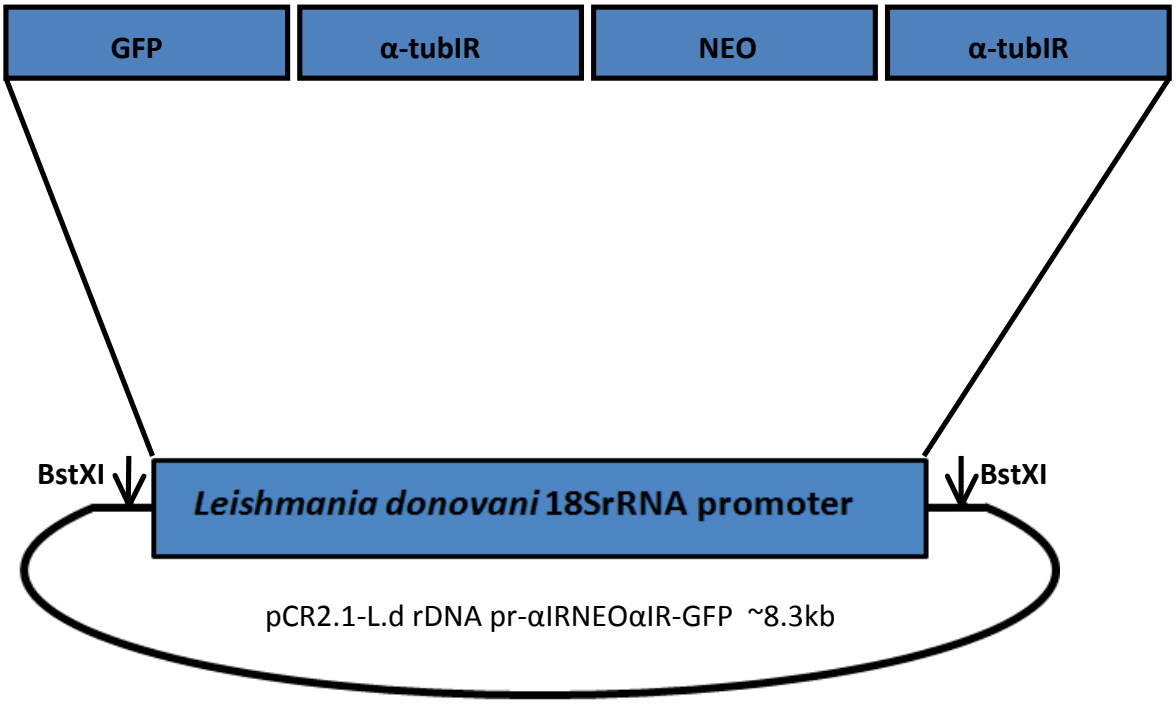


Figure 12: Genetic elements of pRib1.2aNEOaGFP

This was incorporated into the *Leishmania spp*. genome. Key: *L. donovani* 18sRNA promoter – ~1.2Kb, α-tubulin intergenic regions (α-tubIR) – 0.75-0.80kb, NEO - neomycin phosphotransferase gene (~0.8kb), GFP - Green fluorescent protein (~0.75kb from vector phGFP-S65T), pCR 2.1 vector – 3.9Kb. (Adapted from information provided by Dr Barbara Papadopoulos, Infectious Diseases Research Center, CHμL Research Center, Laval University, Quebec, Canada).

2.2.1 Cloning

pXG-GFP^{+} is an episomal plasmid that was amplified using the XL-Gold competent *Escherichia coli* (*E. coli*) cells (Stratagene, UK). Transformation was carried out according to the XL-Gold competent *E.coli* kit (Stratagene, UK) protocol, plus additional modifications. Briefly, 15ml falcon tubes were pre-chilled and the Super Optimal Broth with Catabolite repression (SOC) medium was pre-heated to 42^{o}C in a water bath (Fisher Scientific, UK). In the meantime, the competent cells were placed on ice. The 50μl XL-competent *E.coli* mix was put in one tube and 2μl β-mercaptoethanol (Sigma, UK) was added. The tube was incubated on ice for 10 minutes and swirled every 2 minutes, followed by the addition of 1μl DNA of 30μl pXG-

GFP+ DNA stock. The tube was incubated on ice for an additional 30 minutes, heat-pulsed at 45^{o}C in water bath for 30 seconds and incubated further on ice for 2 minutes. The NZY+ broth (500µl) was added and the tubes were incubated at 37^{o}C for 1 hr whilst shaking at 225-250 rpm (shaking incubator, New Brunswick Scientific, U.S). The transformation mixture (200µl) was put on LB agar supplemented with 100µg/ml (pXG-GFP+). Individual colonies were selected and incubated overnight in 10 ml culture flasks (37^{o}C, shaking at 225-250rpm) (shaking incubator, New Brunswick Scientific, U.S). Cultures were transferred into 50 ml falcon tubes for overnight incubation (37^{o}C, shaking 225-250rpm) (shaking incubator, New Brunswick Scientific, U.S). The two overnight incubations discussed were implemented to maximise bacterial replication.

2.2.2 Plasmid Purification

The overnight *E.coli* cultures (5ml) were divided into 1ml aliquots and stored at -20^{o}C. Plasmid purification was carried out with the QIAprep Spin Miniprep Kit (Qiagen, UK). Extracted pDNA was quantified using a spectrophotometer (260nm) and stored at -20^{o}C.

2.2.3 Transfection of pXG-GFP+ into *Leishmania spp.*

The log-phase cells (1 x 10^{8} total cells) were pelleted by centrifugation (1000 g for 10 minutes) and resuspended in half the original volume in ice cold cytomix. Cells were centrifuged again at 1000 g for 10 min and resuspended in ice-cold cytomix buffer to a final concentration of 2x10^{8} cells/ml. The pDNA (10-50µg/ml) was added into an ice chilled 4-mm gap cuvette containing 500µl of cells (BioRad, UK). Electroporation was carried out twice at 25 mF, 1500V (3.75kV/cm) pausing 10s between pulses. The cells were stored on ice for 10 min to allow to recover before being transferred into culture flask with 10ml complete Drosophila Schneider medium and incubated overnight at 24°C. Following 24-hour incubation, cells were treated with

geneticin sulphate (G418) (Sigma, UK). To select for successful integration of pXG-GFP+ into each *Leishmania spp*. 400µg/ml G418 was added and gradually increasing to the final concentration of 700µg/ml (Robinson & Beverley 2003). Clonal selection was carried on a 24 well plate format and transferred into 10ml culture flasks. Confirmation of GFP expression was achieved by fluorescent microscopy. This was completed by placing 10-20µl of neat *Leishmania* culture onto a microscope slide with a cover slip. The slides were then left for 10 minutes to allow the cells to settle to the bottom and the images were taken using the Nikon 90i fluorescent microscope combined with the Nikon Elements software.

2.2.4 Plasmid cloning, purification and genomic Integration

The plasmid cloning and purification was carried out as previously described for pXG-GFP^{+}. Exceptions include at the cloning step where the XL-Gold competent *E. coli* cells were streaked out on 50µg/ml ampillicin LB agar for the overnight incubation. After purification, the plasmid pRib1.2aNEOaGFP was linearised using FastDigest BstXI (Fermentas, UK) and separated by gel electrophoresis (in 1%, w/v agarose, 1x TBE, Fisher Scientific, UK). The extracted DNA fragment was purified directly from the agarose gel (QIAquick Gel extraction kit, Qiagen, UK). Digested product was resuspended in sterile diH_2O and quantified using a spectrophotometer (260nm). The electroporation of the DNA fragment was identical to the one used for the integration of pXG-GFP^{+}. The only difference included 20µg/ml of digested product being put into the 4mm electroporation cuvette with the 500µl cells (Singh *et al.,* 2009). The selection method was identical as the one used for pXG-GFP^{+} transfectants. After the overnight incubation, the GFP expressing cells were treated with gradually increasing concentration of G418 until 750µg/ml. This was optimised at 300µg/ml later with all *Leishmania spp*. Confirmation of genomic integration was carried using PCR, western blotting (WB), fluorescent microscopy and flow cytometric analysis, which will be now discussed.

2.2.5 Confirmation of GFP integration into the *Leishmania*

2.2.5.1 GFP integration confirmation via PCR

To confirm the successful integration of the GFP gene within the parasites' genome by PCR it was important to extract and purify gDNA from each of the *Leishmania spp*. gDNA was extracted and purified from all *Leishmania* species expressing GFP using the DNAeasy Blood and Tissue kit (Qiagen, UK). Briefly, *Leishmania* cells were grown between 4-5 days and harvested (at 10^{7-8} cells/ml). The 10ml culture was centrifuged at 1000 x g for 10 minutes to pellet the cells. The supernatant was removed and the cell pellet was resuspended in 180µl Buffer ATL and 20µl proteinase K was added by pipette. The solutions were mixed thoroughly by vortexing, and incubated at 56°C for 1 hour. Afterwards, 200µl Buffer AL was added to the sample, and mix thoroughly by vortexing. This was followed by adding 200µl of 100% ethanol and mixed again by vortexing. The solution was placed into the DNeasy Mini spin column (Qiagen, UK) by pipette and centrifuged at 6000 x g for one minute. The flow-through was discarded. The Buffer AW1 (500µl) was added by pipette and centrifuged like before (6000 x g, 1 minute). The flow-through was discarded and the column was put into a fresh 2ml collection tube. The AW2 buffer (500µl) was added and the microcentrifuge tubes were centrifuged at 12,000 rpm for 3 minutes. This was carried out to dry the DNeasy membrane removing all ethanol residues. The DNeasy Mini spin column was put into a clean 1.5ml microcentrifuge tube and 100µl AE Buffer was added directly onto the membrane. The samples were incubated for 1 minute and centrifuged at 6000 x g for 1 minute to elute the gDNA. This was repeated three times to increase the DNA yield.

2.2.5.2 PCR

Primers were designed as described in (2.7.1) for full length GFP amplification (5'-GFP 829 - GTGAGCAAGGGCGAGGAGCT and 3'-GFP 1543 - ACTTGTACAGCTCGTCCATGC) and to assess site-specific homologous recombination of the reporter gene (5'-18SRibo. - GCATTGCTTCGCGTGTGAGT and 3'- sequence primer, as above) at the 18S ribosomal locus of all four species. Amplification was achieved using the REDtaq® ReadyMix™ PCR reaction mix (Sigma, UK) under the following conditions: 94 ºC 2 min, 30 cycles of 94 ºC 30 sec, 64 ºC 30 sec and 72 ºC 2 min followed by extended 72 ºC 10 min elongation cycle. Amplification conditions to assess site-specific genomic integration of GFP at 18S ribosomal 111 locus were 94 ºC 2 min, 30 cycles of 94 ºC 30 sec, 50 ºC 30 sec and 72 ºC 2 min followed by 112 an extended 72 ºC 10 min elongation cycle.

2.2.5.3 GFP integration confirmation via western blot

Log-phase promastigotes (~$4x10^6$/ml) were harvested by centrifugation (1000 x g for 10 minutes) and were washed twice in ice cold PBS and resuspended in cells lysis buffer (930 mg EDTA, 4350 mg NaCl, 3028.5 mg Tris 2.5 ml IGEPAL® CA-630 and 497.5 ml H_2O). The parasites were snap-frozen in liquid nitrogen and immediately thawed at 80ºC three times to maximise cell lysis. The lysed cell mixtures were centrifuged at 12,500 x g for 10 minutes to remove cell debris. Protein concentration was determined using the Bradford reagent according to manufacturers instructions (Sigma, UK).

Total proteins (40µg) were separated on precast SDS-Tris-Glycine (Pierce) gels and transferred to HYBOND (Amersham) membranes by electroblotting. The membranes were blocked overnight at 4 ºC in blocking solution (PBS, 0.01 % v/v Tween and 5 % v/v milk). Afterwards, the membranes were incubated with rabbit anti-GFP immunoglobulin (1: 1000 dilution) at 37 ºC

for 1 hour followed by three 5-min washes each in PBS with 0.01 % v/v Tween. A secondary ECL™ peroxidase labelled anti-rabbit immunoglobulin was applied to the membranes and incubated under the same conditions as the primary antibody and washed as above to remove unbound excess antibody. Detection of GFP was performed using The ECL™ Plus Western Blotting Detection Reagents (GE Healthcare).

2.2.5.4 Flow cytometry of *Leishmania* promastigotes

GFP-expressing promastigotes from all four transgenic *Leishmania* species were analysed on the Accuri C6 flow cytometer for GFP expression using a solid state blue laser with a 488 nm 171 excitation spectrum and a detector of FL1 path with filter 530/30 nm. A minimum of >2,000 events was acquired from the promastigote population and data analysed using the Accuri C6 software (Figure 13). The detection of GFP-expressing parasites involved running volumes of 10-50µl from culture of log-phase promastigotes (~10^6/ml).

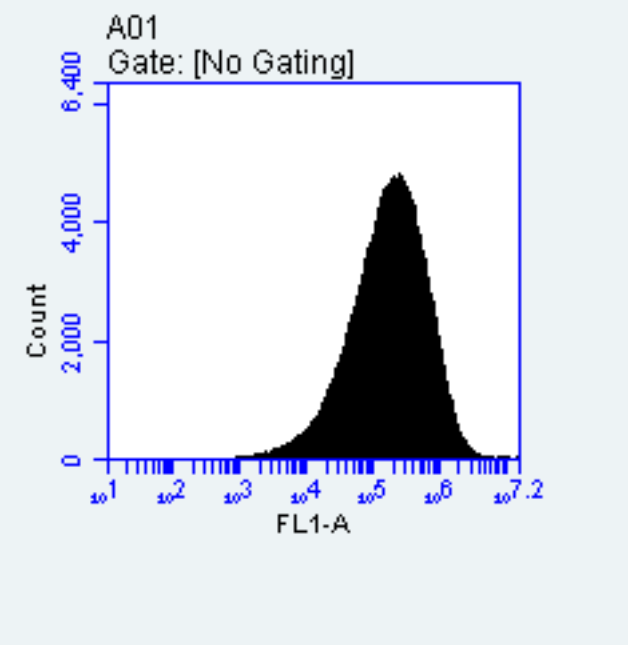


Figure 13: GFP detection of *L. aethiopica* promastigotes using the FL1 filter.

Note: This was carried out for all *Leishmania spp* detecting a minimum of 50,000 events/GFP clone.

2.2.5.5 Fluorescent microscopy of GFP expressing promastigotes.

A total of 5µl of fixed (0.04% formaldehyde, Fischer Scientific, UK) log phase transgenic promastigotes (10^6 cells/ml) or infected THP-1 cells ($2.5\text{-}5x10^5$/ml) were placed onto glass microscopy slides and a coverslip was placed on top. Slides were allowed to settle for 2 minutes before microscopy analysis. Images were acquired using the Nikon ECLIPSE 90*i* overhead epifluorescent microscope attached to a Nikon digital camera (DS-Qi1Nc) and a computer running Nikon NIS-Elements Advanced Research software (Figure 14). The principal objective used for fluorescent imaging was an oil immersion CFI Plan Apochromat VC 60X N2 (NA1.4, WD 0.13 mm).

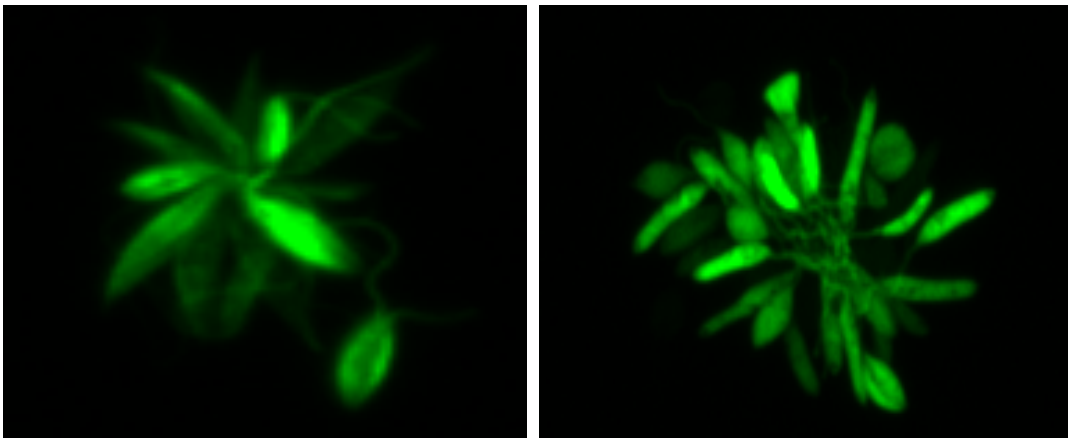


Figure 14: *L. aethiopica* (left) and *L. mexicana* (right) promastigotes expressing GFP when cultured in complete Schneider media. Images were obtained using the Nikon ECLIPSE 90*i* overhead epifluorescent microscope.

2.3 Infections of mammalian cells

Infection of the three types of mammalian cells (THP-1, Jurkat and human T lymphocytes) was carried out using metacyclic promastigotes isolated from parasites cultured infecting at a ratio of 10:1. Metacyclic purification was obtained following peanut agglutination (PNA).

2.3.1 Isolation of metacyclic promastigotes by PNA

Infective metacyclic promastigotes were obtained from stationary phase *Leishmania spp*. culture via peanut agglutination as previously described (Ready and Smith, 1988). Briefly, stationary phase parasites were harvested (1000 x g, 10 minutes) and resuspended in fresh Schneider's Drosophila media at a concentration of 10^8 cells/ml. The peanut lectin agglutinin (PNA, 1mg/ml stock, Vector Laboratories, UK) was added to 1ml cell suspension at a concentration of 50 µg/ml in a sterile 1.5ml Eppendorf tube. After 20 minutes incubation (at RT), the cells were centrifuged at 100 x g for 5 minutes agglutinated procyclics pelleted to the down of the eppendorf and the metacyclic containing supernatant was isolated.

2.3.2 Infection of THP-1 cells

THP-1 was differentiated through the incubation with retinoic acid (RA) (1µM) for three days. The working solution was made through diluting RA at 1mM in DMSO (Fisher Scientific, UK), respectively. After washing twice (PBS 1x, Fisher Scientific), THP-1 cells were resuspended at $2.5x10^5$ cells/ml in complete RPMI-1640 media and the total cell count and percentage of viability of THP-1 cells was completed using the trypan blue exclusion method (Sigma-Aldrich, UK) (1:10 dilution in 1x PBS, Fisher Scientific). A 50:50 dilution of THP-1 cell sample to 0.04% trypan blue solution was analysed onto the disposable FASTREAD102 histocytometer (Immune Systems, UK). Only >90% cell viability was used for experiments with THP-1 cell lines and infected with *Leishmania spp* (10:1, ratio of *Leishmania* parasite: THP-1).

2.3.2.1 Flow cytometry analysis of THP-1 cells

THP-1 cells were run on the Accuri C6 flow cytometer (BD, UK) involving gating the population of interest (FSC v SSC), removing cell doublets (SSC-H v SSC-A & FSC-H v FSC-H) and selecting the percentage of infection with GFP expressing parasites (Count v FL1)

(Figure 15). All cells were washed twice to remove unattached parasites and >2000 total events were analysed.

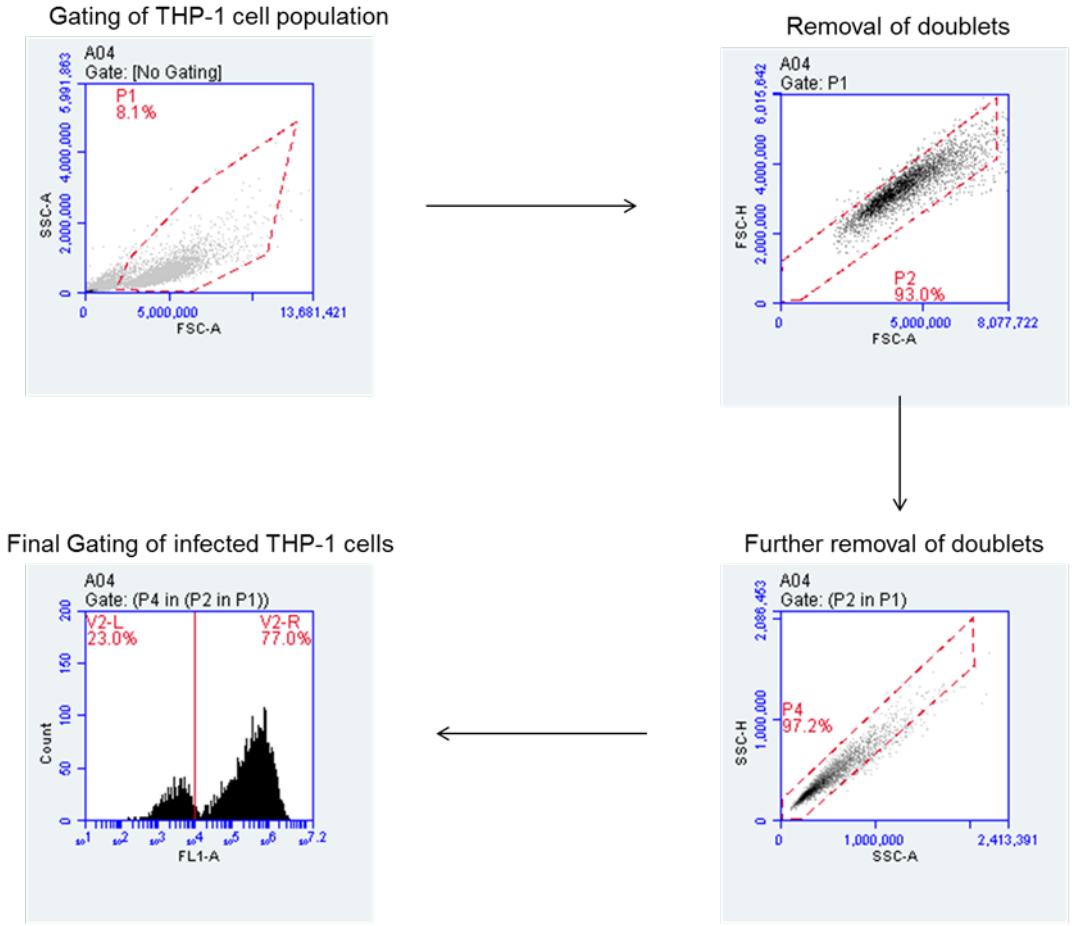


Figure 15: Flow cytometric example of how the gating of THP-1 cells infected with all GFP-expressing *Leishmania* species was carried out.

2.3.3 Infection of Jurkat T lymphocytes

Log-phase Jurkat cells ($5x10^5$/ml) were infected at a ratio of 10:1 (T cells: *Leishmania spp.*) with *L. mexicana* and *L. aethiopica* as previously described for THP-1 cells (Method 2.3.2). Infected cells were transferred in a 24 well plate and incubated at 37°C, 5% CO_2 over a period of 5 days. Infection was detected every 24 hours via flow cytometry. Before analysis, samples were washed twice in 1xPBS to eliminate unbound parasites and resuspended in fresh complete RPMI-1640 media.

2.3.3.1 Flow cytometry analysis of Jurkat cells

Jurkat cells were run on the Accuri C6 flow cytometer (BD, UK) involving gating the population of interest (FSC v SSC), removing cell doublets (SSC-H v SSC-A & FSC-H v FSC-H) and selecting the percentage of infection with GFP expressing parasites (Count v FL1) (Figure 16). All cells were washed twice to remove unattached parasites and >2000 total events were analysed.

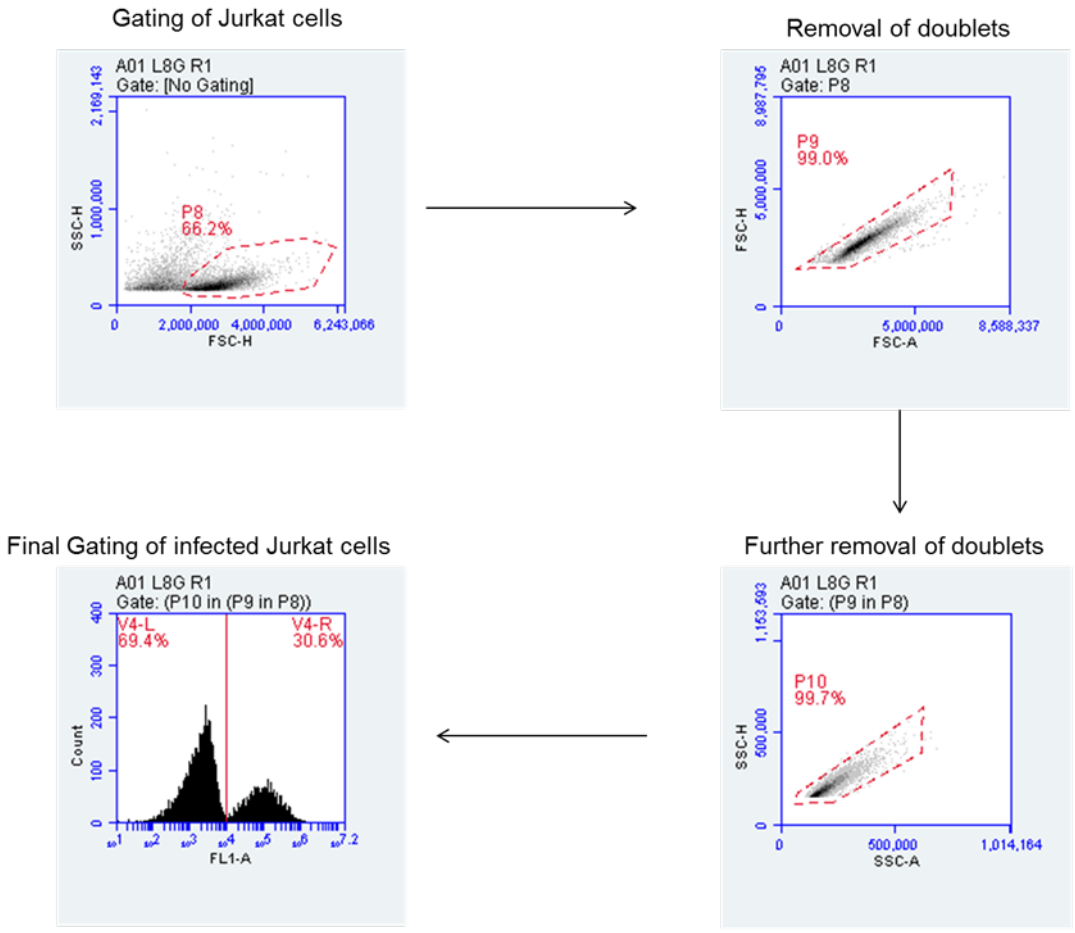


Figure 16: Flow cytometric example of how the gating of Jurkat cells infected with GFP-expressing *Leishmania spp* was carried out. The example provided is of *L. aethiopica* GFP infection post 24 hours.

2.3.4 Infection of human T lymphocytes

Before commencing the experiment ethical approval was granted with all appropriate standards met. This included providing background information to the blood volunteers and gaining their written consent prior to the blood extraction (Appendix I). Peripheral blood (~30ml) from healthy individuals was obtained from the forearm by venepuncture and transferred into 50ml centrifuge tubes containing 37.5mg (6000 USP units) of heparin, sodium salt (Santa Cruz Biotechnology, USA) diluted in 100µl 1x phosphate buffer solution (PBS, Sigma, UK, A). A minimum of 20-140 USP/ml of human blood was used to avoid coagulation. The heparinised blood was centrifuged at 3000 rpm for 10 minutes to pellet the cells and to remove plasma serum. The cells were resuspended in complete RMPI-1640 media at a ratio of 1:1 and well mixed by inverted the centrifuge tube. The cell mixture was layered onto lymphoprep (Stem Cell Technologies, UK) very slowly by using a sterile Pasteur pipette. The cells were centrifuged at 2400 rpm for 30 minutes and allowed to stand for an additional 30 minutes at room temperature to allow the polymorphomononuclear cells (PBMCs) to settle. The PBMC's were removed slowly from the interphase and centrifuged at 1000 x rpm for 10 minutes to remove platelets and red blood cells. The PBMCs were washed twice in complete RPMI-1640 media by removing half the volume (ratio of 1:1 of PBMCs: RPMI-1640 complete media) and resuspending the cell mixture with complete RPMI-1640 media. After washing, the cells were centrifuged at 500 x g for 10 minutes and the supernatant was poured off and the cells were resuspended in fresh complete RMPI-1640 media. Afterwards, the T cell populations within the PBMCs mixture were purified as described below.

2.3.4.1 Purification of human T lymphocytes

The total T cell population was purified by depletion of non-target cells according to Pan T Cell Isolation kit protocol (Milteny biotec, UK) in conjugation with a MACS MultiStand (Milteny

biotec, UK), a MidiMACS separator (Milteny biotec, UK) and a LS column (Milteny biotec, UK).

Briefly, PBMCs were resuspended in pre-chilled cell buffer and Pan T Cell Biotin-Antibody Cocktail was added to the cells at 10µl per 10^7 total cells. Following 5 minutes incubation at 4^oC, pre-chilled cell buffer (30µl per 10^7 total cells) and Pan T Cell Microbead Cocktail (20µl per 10^7 total cell count) were added. The solution was mixed and incubated at 4^oC for 10 minutes. To complete the T cell enrichment the LS column placed in the MidiMACS separator (held by MACS Stand) and prepared by passing 3ml of cell buffer through the column (held by the magnetic separator). The PBMCs cell mix was passed through the column and T cells flowed through and were collected by negative selection as the all other white blood cells remained in the column. The T cells were washed twice in complete RPMI-1640 media and treated with red blood cell (RBC) lysis buffer according to the manufacturer's instructions (RBC lysis buffer, Santa Cruz Biotechnology, U.S). Briefly, the RBC buffer was added to the cell PBMC's suspension (1 part cell mixture: 2 parts RBC lysis buffer). The tube was mixed by inverting and then incubated for 5 minutes at room temperature on a shaking platform. The cells were centrifuged at 1000 x g, for 5 minutes and fresh media was added to the cell pellet after removing the supernatant.

2.3.4.2 Infection of human T lymphocytes

Cells (set at 2.5 or 5 $x10^5$/ml) were infected at a 10:1 ratio (*Leishmania* parasites: human T lymphocytes) on a 24-well plate format. The 1ml cell mixtures were put into their respective well and an additional 1ml of fresh complete RPMI-1640 media was added. The cells were incubated at 37^oC, 5% CO_2 for up to 96hrs. The sampling was carried out at 24, 48 and 72hrs

post infection. The cell mixtures were 1) human T cells (hT cells) 2) hT cells+ *L.mexicana* and 3) hT cells+ *L. aethiopica*.

2.3.4.3 Antibody labeling of human T lymphocytes (hT cells)

This was carried using the antiCD4 and antiIgG1k antibodies (BD, UK). The antiCD4 antibody was tested against the control hT cells and infected cells. The antibody control antiIgG1k was tested against hT cells as additional confirmation of the purification of human lymphocytes. Briefly, this involved washing cells twice in ice-cold 1xPBS (2000 rpm, 5 minutes) and resuspending them at 10^6/100µl 1xPBS. The antibodies were added (20µl) by pipette and incubated on ice for 20 minutes. The cells were then washed twice as previously mentioned and were analysed on the C6 Accuri flow cytometer. This involved running 10-20µl gaining a minimum of 2000 events per sample.

2.3.4.4 Flow cytometric analysis of human T cell populations

Jurkat and extracted human T cell populations run on the flow cytometer involving gating the population of interest (FSC v SSC), removal of cell doublets (SSC-H v SSC-A & FSC-H v FSC-H), gating PE/CD4+ populations (SSC-A v FL2) and the final gating of GFP fluorescence (Count v FL1) with the total T cell population and within $CD4^+$ positive cells (Figure 16). All cells were washed twice to remove unattached parasites and >2000 total events were analysed.

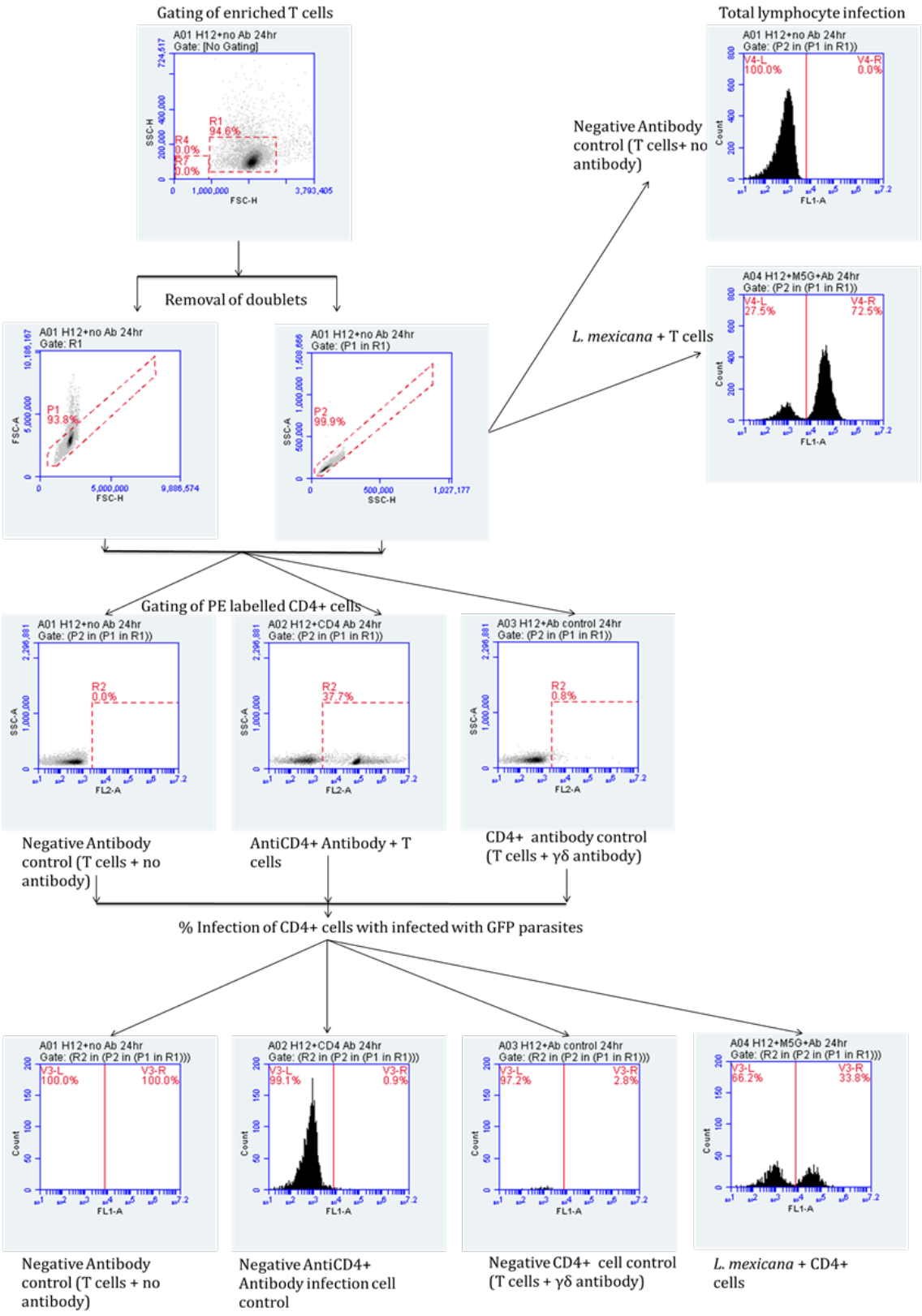


Figure 17: Flow cytometric analysis for identifying the percentage of infection within the total T cell population and within the $CD4^+$ subsets.

2.4 Cytokine expression during *Leishmania spp.* infection

This work is investigating the effects of *Leishmania aethiopica* infection on the mRNA expression of cytokines (IL-1RA, TNFα, IL-10, TGFβ) and cathelicidin at different times (2, 4, 24 ad 48hr), *i.e*. post-infection. To evaluate this effect comparative mRNA expression β-tubulin was used as the house-keeping gene control.

2.4.1 mRNA primer design

All primers were designed via BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), CLUSTALΩ (http://www.ebi.ac.uk/Tools/msa/clustalo/) and Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 6). The only exception was the cathelicidin primers, which were previously designed in 2007 (Liu *et al*., 2007). The aim was to a get universal annealing temperature of ~60ºC and target amplications <200 base pairs for all cytokine primers.

Table 6: Primers designed for targeting different cytokines and cathelicidin

Primers	Sequence	Primer Length (bp)	GC (%)	Primer3 Tm (^{0}C)	Optimal Tm (^{0}C)	PCR Product Size (bp)
IL-1ra Forward	GGAATCCATGGAGGGAAGAT	20	50	60.1	58	97
IL-1ra Reverse	TCTCGCTCAGGTCAGTGATG	20	55	60.14		
IL-10 Forward	TTACCTGGAGGAGGTGATGC	20	55	60.07	60	127
IL-10 Reverse	GGGAAGAAATCGATGACAGC	20	50	59.63		
TGF-beta1 Forward	GAGCCTGAGGCCGACTACTA	20	60	59.6	58	131
TGF-beta1 Reverse	CGGAGCTCTGATGTGTTGAA	20	50	59.98		
TNFalpha Forward	CAACCTCCTCTCTGCCATCAA	20	50	59.8	60	150
TNFalpha Reverse	ATAGTCGGGCCGATTGATCT	20	50	60.82		
Cathelicidin forward	GGACCCAGACACGCCAAA	18	61.1	58	60	52
Cathelicidin reverse	GCACACTGTCTCCTTCACTGTGA	23	52.1	70		
b-tubulin Forward	CTCTTCCAGCCTTCCTTCCT	20	55	59.95	60	116
b-tubulin Reverse	AGCACTGTGTTGGCGTACAG	20	55	59.97		

2.4.2 Sampling for the optimisation of cytokine mRNA primers

Three different samples were initially set up to test the designed primers against undifferentiated THP-1 (negative control), RA (retinoic acid) treated THP-1 cells (negative control) and RA treated THP-1 with LPS (positive control). This was carried out to validate the primers during active dividing monocyte-like cells, differentiated THP-1 into macrophage-like cells and macrophage-like cells undergoing inflammatory activation by bacterial LPS. THP-1 cells ($2.5x10^5$/ml) were differentiated with RA (See Method 2.3.2), washed and resuspended in complete RPMI-1640 media. After the trypan exclusion method (Method 2.3.2), the 1ml ($2.5x10^5$/ml) of cells were put into 24-well plate. The cells (undifferentiated THP-1, differentiated THP-1+RA and THP-1+RA+ 1µg/ml LPS (Sigma, UK) were incubated for 8 hours at 37^{o}C, 5% CO_2. Afterwards, cells were frozen and RNA extraction was carried out.

2.4.3 RNA Extraction

RNA was extracted via Trizol Reagent with PureLink RNA Mini Kit (Invitrogen, UK). Briefly, the THP-1 cells were centrifuged at 1000 x g for 5 minutes to form a pellet. The supernatant was removed and 1ml Trizol Reagent was added. The cells were mixed by pipette until the pellet was equally distributed to maximise cellular lysis, and incubated at room temperature for 5 minutes. This incubation allowed complete dissociation of nucleoprotein complexes. Chloroform (0.2ml) was added to the lysate and the tubes were shaken vigorously by hand for 15 seconds and incubated for 3 minutes at room temperature. The samples were centrifuged at 12,000 x g for 15 minutes at 4^{o}C allowing separations into the lower red-chloroform phase containing proteins, the middle interphase (cell debris) and the upper clear aqueous phase containing the RNA. Approximately 600µl of the clear aqueous phase was obtained and transferred to a fresh RNAse free eppendorf tube (Ambion, UK). An equal volume (600µl) of 70% ethanol was added forming a final concentration of 35% ethanol. The tubes were thoroughly mixed using a vortex and inverted several times.

2.4.4 Binding, washing and eluting

The RNA aqueous mixture (700µl) was put into spin cartridges within columns and centrifuged at 12,000 x g for 15 seconds. The spin cartridges allow the passage of regents and the capture of RNA within its mesh. The flow through was removed and this was repeated until all the solution had passed through the spin cartridge. Wash buffer I (350µl) was added to the spin cartridge and centrifuged at 12,000 x g for 15 seconds at room temperature. The flow through was discarded and cartridges were placed into fresh columns. The PureLink DNase mixture (80µl) was added directly to the spin cartridge membrane to digest any possible DNA contamination. The DNase mixture was prepared as per PureLink DNase protocol (8µl 10x DNase I Reaction Buffer, 10µl Resuspended DNase, 62µl RNase-free water; 80µl total volume) for every sample. After the incubation (15 minute, R.T), 350µl Wash buffer I was added to the spin column and centrifuged

at 15,000 x g, 15 seconds. The flow-through was removed and the spin column was placed into a fresh collection tube. The Wash Buffer II (500µl) was added to the spin column and centrifuged (15,000 x g, 15 seconds). The flow-through was removed from the collection tube and this step was carried out twice in total. The spin column was centrifuged (15,000 x g, 1 minute) again to dry the membrane bound with RNA. The collection tubes were discarded and the spin cartridge was placed into a sterile RNase-free 1.5 microcentrifuge tube. To elute the RNA, 30µl TE buffered RNase-free water was added directly on the top of the membrane and the tubes were incubated at R.T for 1 minute. The tubes were centrifuged for 1 minute at 12,000 x g at R.T. This was repeated through using the flow-through onto the spin cartridge maximising the final quantity of RNA. All RNA samples were stored at -70^{o}C for long term storage.

2.4.5 Quantification of RNA

The total RNA was quantified by adding 1µl of RNA solution onto the BioDrop (BioDrop, UK) and the optical density (OD) of every sample was measured against the 1x TE buffer (Sigma-Aldrich, UK) blank control. The total RNA (total RNA - 260nm and RNA purity at 260nm/280nm) was measured. The $OD_{260/280}$ values >1.8 were considered sufficiently clean of proteins and UV chromophores (Invitrogen PureLink MiniRNA kit, UK).

2.4.6 Reverse Transcriptase of RNA into cDNA

The first strand cDNA synthesis was carried out using the SuperScript^{TM} III Reverse Transcriptase protocol (Invitrogen). Briefly, 1µl of random hexamer primers (0.2µg/µl) (Fermentas), 5µg total RNA, 1µl 10mM dNTP mix (10mM each dATP, dGTP, dCTP and dTTP) were added to a total volume of 13µl with RNase-free DEPC-treated water. This mixture was heated to 65^{o}C for 5 minutes (PCR thermocycler) and incubated on ice for 1 minute. The tubes were briefly centrifuged to collect all contents within the tubes. The SuperScript II RT (1µl, 200

units/µl) (Invitrogen) was added to tubes alongside with 4µl 5x First-Strand Buffer (Invitrogen), 1µl 0.1M DTT (Invitrogen), 1µl RNaseOUT Recombinant RNase Inhibitor (Invitrogen). The tubes were well mixed by pipette and incubated at 25^{o}C for 5 minutes (PCR Thermocycler). The tubes were incubated at 50^{o}C for 60 minutes and to inactivate the reaction, the tubes were heated at 70^{o}C for 15 minutes (PCR Thermocycler). The removal of complementary RNA to cDNA, 1µl of *E. coli* RNase H was added and incubated at 37^{o}C for 20 minutes.

2.4.7 Standard PCR and gel electrophoresis of primers

To identify the optimal annealing temperature for all primers designed, they needed to be tested using a temperature gradient PCR and the products run on electrophoretic gel. PCR of samples generated (THP-1, THP-1+RA, THP-1+RA+LPS) (Methods 2.4.2) was carried out using the RedTaq ReadyMix PCR reaction mix (Sigma, UK). Briefly, 12.5µl RedTaq ReadyMix PCR reaction mix (20mM Tris-HCl, pH 8.3, with 100 mM KCl, 3 mM $MgCl_2$, 0.002 % gelatin, 0.4 mM dNTP mix - dATP, dCTP, dGTP, TTP, stabilizers, and 0.06 unit/mL of *Taq* DNA Polymerase) (Sigma-Aldrich, UK), 0.5µl forward primer (10µM) (Sigma, UK), 0.5µl reverse primer (10µM) (Sigma, UK), 9.5µl DNase-free water and 2µl total cDNA per reaction on a BioRad thermocycler, (BioRad, UK). All the PCRs were tested at 94^{o}C for 2 minutes, cycling at 94^{o}C for 30 seconds, annealing temperature (58, 60, 62 and 64^{o}C) for 30 seconds and 72^{o}C for 30 seconds for 40 cycles. The reaction was terminated at 72^{o}C for 10 minutes. PCR products were run on a 1% agarose (1g, Sigma-Aldrich, UK) in 1x TBE (Fisher Scientific, UK). The PCR products were run on the gel at for 1 hour and 30 minutes at 100mA, 70v. A 1kb plus ladder (Invitrogen, UK) was used as a molecular weight marker. Images were generated using the SYNGENE UV lamp with SYNGENE camera CAMFLXCM-1.

2.4.8 cDNA generation for experimental qPCR

Differentiated THP-1 cells were washed, counted and prepared as mentioned before (Method 2.3.2) except 2ml of $5x10^5$/ml solution were added per well on a 24-well plate. *L. aethiopica* cell cultures were previously treated with PNA as previously mentioned (Method 2.3.1). Afterwards, half of the purified metacyclics were killed by heating at 70°C for 10 minutes. Samples for this experiment included THP-1+RA (negative control), *L. aethiopica*-infected THP+RA cells, killed *L. aethiopica*-infected THP+RA cells (phagocytic control) and THP-1+RA treated with LPS (1µg/ml *E. coli* 111:B4 LPS) (inflammatory control). All infections were conducted as previously described at a ratio of 10 parasites per THP-1 cell (Method 2.3.2) and samples were taken at 2, 8, 24 and 48 hours post infection/treatment. The RNA extraction, purification and quantification were carried out as previously described (Methods 2.4.3, 2.4.4 & 2.4.5). The generation cDNA differed from samples used for the standard PCR (Method 2.4.7) as the remainder of reverse transcription was carried out using the RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, UK). Briefly, 1µg total RNA, 1µl Random hexamer primer and nuclease-free water to a total volume of 12µl. Afterwards the following were added in order: 4µl 5X reaction buffer, 1µl RiboLock RNase Inhibitor (20U/µl), 10mM dNTP mix and 1µl RevertAid M-MulV RT (200U/µl). The microcentrifuge was placed onto the BioRad thermocycler to complete the cDNA synthesis through heating the samples at 25°C for 5 minutes, followed by 42°C for 1 hour and terminated at 70°C for 5 minutes.

2.4.9 qPCR of experimental cDNAs

The qPCR standard curves were produced at their respective optimal annealing temperature. The 10µl qPCR mastermix comprised of 5µl SYBR GREEN, 1µl forward primer, 1µl reverse primer, 1µl cDNA (from 1µg RNA to cDNA synthesis) and TE buffer (Sigma-Aldrich, UK). The standard curves were completed after conducting four ten-fold serial dilutions in triplicate.

Confirmation of optimal PCR was identified by the melt curve and standard curve. All qPCR of the experimental samples were carried out as above maintaining the 1µg total RNA and 1/10 dilution of cDNA to total SYBR GREEN mastermix volume of 10µl.

2.5 Proteomics analysis of THP-1 infection

THP-1 cells were differentiated, washed and counted as previously mentioned (Method 2.3.2). Afterwards, $5x10^5$/ml of each cell mixture (THP-1+RA, THP-1+RA+*L. aethiopica* and THP-1+RA+*L. mexicana*) were put into 15 wells on a 24-well plate per biological replicate. This carried out to pool 12 wells ($1x10^7$ total cell number) to maximise protein concentration and use the remaining three wells to validate the percentage of infection on the Accuri C6 flow cytometer. The cells were incubated for 24hrs at 37^{o}C, 5% CO_2, and the procedure was repeated three times (N=3).

2.5.1 Protein extraction

The proteins were extracted using 1x RIPA buffer (Thermoscientific, Pierce, UK). Briefly, the cells were pelleted by centrifugation (1000 x g, 10mins), washed twice in 1xPBS and resuspended in 500µl of ice-cold 1x RIPA buffer. All samples (on ice) were well mixed by pipette and placed onto a shaker at 200 rpm for 15mins to maximise cell lysis. Cell debris was removed by centrifuging at 10,000 rpm for 10 minutes at 4^{o}C. The supernatants containing proteins were transferred to a new eppendorf and stored at -20^{o}C prior to protein quantification.

2.5.2 Protein Quantification

This was carried as advised by the supplier's instructions (Pierce BCA Protein Assay Kit, UK). Briefly, BSA standards (2000, 1500, 1000, 750, 500, 250, 125, 25, 0µg/ml) were made from 2mg/ml stock through diluting in diH_2O. Protein samples were diluted (1:10, 10µl: 90µl) in 1x

RIPA buffer. BCA reagent A and B were mixed at 50:1 ratio (WR mixture) and 200ul was added into each well on a 96 well plate. The protein samples and BSA standards were added (25µl) to the wells containing the WR mixture and mixed thoroughly on a plate shaker for 30 seconds at 200 rpm. The plate was incubated at 37^{o}C for 30 minutes, allowed to cool to RT (room temperature) and absorbance was measured at 540nm. Protein concentration was calculated using the standard curve.

2.5.3 Proteomic analysis

Afterwards, the samples from Method 2.5.1 were sent to Bristol University to be tested using tandem mass tagging peptide analysis using LC-MS/MS, which provides full proteomic profiles for all samples. Advise obtained by Dr. Karen Heesom, at the proteomic department at Bristol University, only values ≥1.5 (under expression) or ≤0.5 (under expression) were regarded as statistically significant (P≥0.05, 95% confidence). Once all significant values were pooled from each biological replicate (N=3), only proteins that were identified in all biological replicates were considered for further investigation.

2.6 Statistical analysis

All data obtained involved in this research were statistically tested by student t-test against their respective controls. The only exception was the proteomic data (≥1.5 or ≤0.5 fold change) since all data obtained from Bristol University were provided at 95% confidence. The non-parametric data analysis of flow cytometry was carried using Wilcoxon signed rank test.

Chapter 3

Can THP-1 infection be detected using GFP expressing parasites?

Novelty statement

This is the first time that the successful chromosomal integration of GFP in *L. mexicana*, *L. aethiopica*, *L. tropica* and *L. major*.

Impact

The development of *Leishmania* parasites expressing GFP allows the monitoring of the percentage of infection *in vitro* and *ex vivo* during all aspects of this work.

Publication

Asha Parbhu Patel, Andrew Deacon and Giulia Getti. (2014). Development and validation of four *Leishmania* species constitutively expressing GFP protein. A model for drug discovery and disease pathogenesis studies. *Parasitology*. 141. 501-510.

3.1 Introduction

Leishmania parasites interact with a number of mammalian cells in order to establish infection, interactions are based on binding and often entering mammalian cells and making them their host. Macrophages are arguably the most important hosts for *Leishmania* as the interaction between the two ultimately determine the parasites survival. Parasites can enter macrophages directly or after briefly residing within neutrophils, which act as 'Trojan horses' during early stages of infection (Peters *et al*., 2008; Peters and Sacks 2009). Once inside macrophages, the

parasite needs to establish itself inside the parasitophorous vacuole, down regulate reactive oxygen species (ROS) and nitric oxide species (NOS) as well as expression of inflammatory cytokines in order to replicate and spread (Bodgan and Rollinghof 1998; Real and Mortara 2012).

Investigations of the processes behind *Leishmania* infection as well as identification of novel treatments and/or vaccines (which are targeted to intracellular amastigotes) require reliable determination of infection. Such studies are hindered by the fact that monitoring infection mostly requires time consuming and laborious procedures based on Giemsa staining and microscopic counting (Nwaka & Hudson, 2006). Alternative methods are available for some *Leishmania* species and include development of parasites expressing reporter genes such as B-galactosidase and luciferase (Okuno *et al*, 2003; Buckner and Wilson, 2005; Ashutosh *et al*., 2005; Lang *et al*., 2005; Roy *et al*., 2000; Sereno *et al*., 2001) and GFP (Singh *et al*., 2009).

In the present chapter, a fast and sensitive method to quantify infection of four *Leishmania* species in terminally differentiated THP-1 cell was developed. This method is based on the development and validation of stable expression of GFP in Old and New world *Leishmania* species (*L. mexicana*, *L. aethiopica*, *L. tropica* and *L. major*) through homologous recombination in the 18s rRNA locus.

3.2 Methods

The cell culture practices for THP-1 and all *Leishmania* promastigotes were carried as previously mentioned (Methods 2.1.1 pp. 32, 2.1.2 pp. 32, 2.1.5 pp.34, respectively). The purification of metacyclic promastigotes, infection of THP-1 cells and the flow cytometric analysis was carried as seen in methods 2.3.1 pp. 43, 2.3.2 pp. 43 and 2.3.2.1 pp. 43.

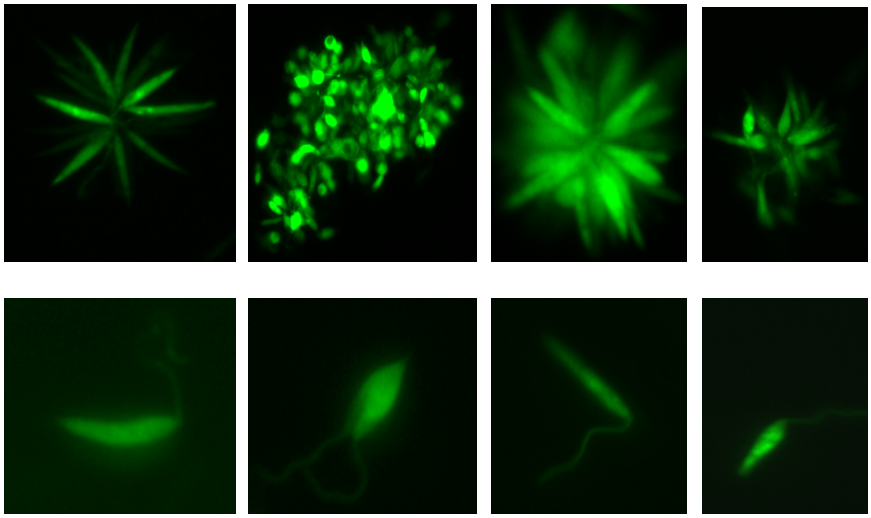
The methods involved in the development of GFP expression in *Leishmania* using pXG-GFP+ and pRib1.2αNEOαGFP plasmids can be found in the Methods chapter (Development of green fluorescent protein (GFP) expression in *Leishmania spp*. 2.2 pp.34, Cloning 2.2.1 pp.36, Plasmid purification 2.2.2 pp.37, Transfection of pXG-GFP+ into *Leishmania spp*. 2.2.3 pp. 37, Plasmid cloning, purification and genomic integration 2.2.4 pp. 38, GFP integration confirmation via PCR 2.2.5.1 pp. 39, PCR 2.2.5.2 pp. 39, GFP integration confirmation via western blot 2.2.5.3 pp. 40, Flow cytometry of *Leishmania* promastigotes 2.2.5.4 pp. 41 and fluorescent microscopy of GFP expressing promastigotes 2.2.5.5 pp. 42).

3.3 Results

3.3.1 Confirmation of GFP expression in *Leishmania spp.*

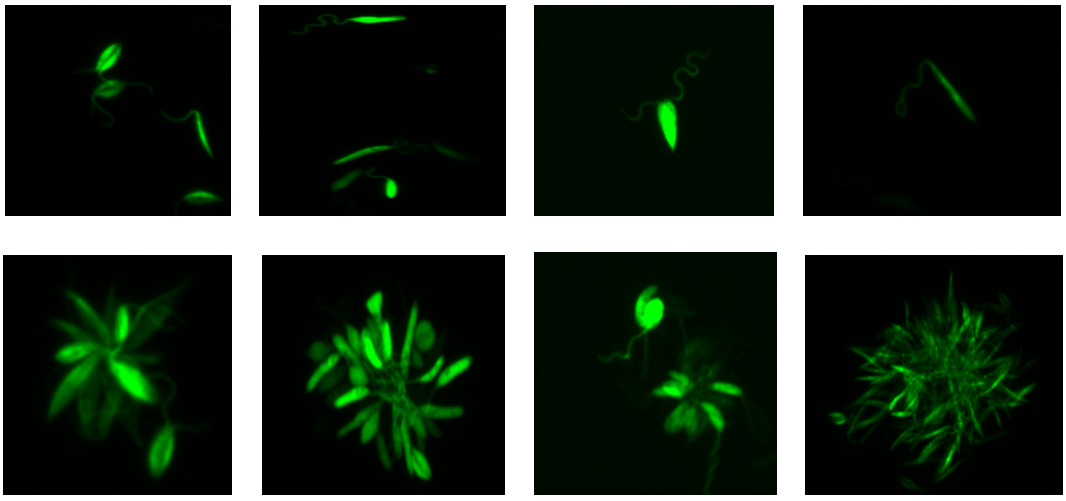
Successful transfection and expression of GFP in *L. mexicana, L. aethiopica*, *L. tropica* and *L. major* was confirmed via fluorescent microscopy, PCR, Western blot and flow cytometry.

3.3.1.1 Fluorescent microscopy of GFP expressing promastigotes



(A) (B) (C) (D)

Figure 18: GFP expression via the successful electroporation of episomal pXG-GFP+ into the cytosol of each *Leishmania spp.* (*L. mexicana* (A), *L. aethiopica* (B), *L. major* (C) & *L. tropica* (D).



(A) (B) (C) (D)

Figure 19: GFP expression via the successful integration of the GFP gene downstream of the 18S rRNA promoter in each *Leishmania spp.* (*L. mexicana* (A), *L. aethiopica* (B), *L. major* (C) & *L. tropica* (D).

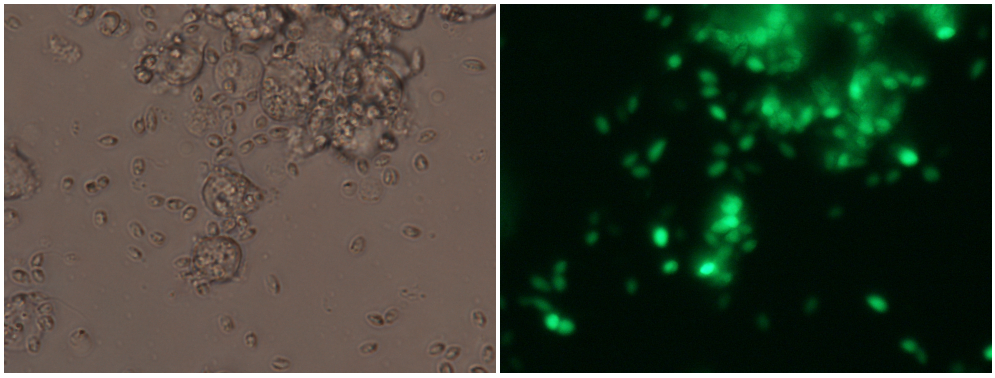


Figure 20: Differentiated THP-1 cells infected with *L. mexicana* GFP after 24hrs exposure. All biological replicate infections (N3) were counted, i.e. >100 cells counts three times/biological replicate (All infections were validated at a 20X and 60X magnification).

3.3.1.2 Flow cytometric analysis of GFP-expressing promastigotes

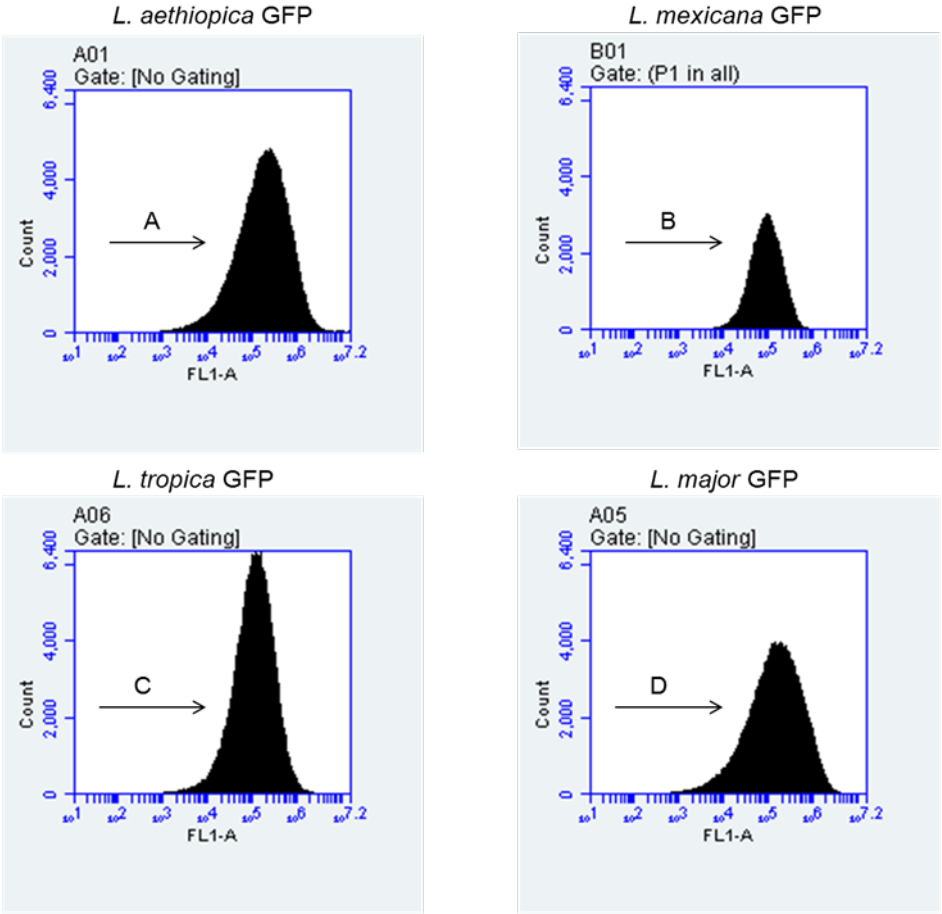


Figure 20: Pure GFP expressing clones for each *Leishmania* through maximum presence ≥10^4 whilst using the FL1 filter used to detect GFP signal on the Accuri C6 flow cytometer.

3.2.1.3 Confirmation of the integration of GFP gene by PCR

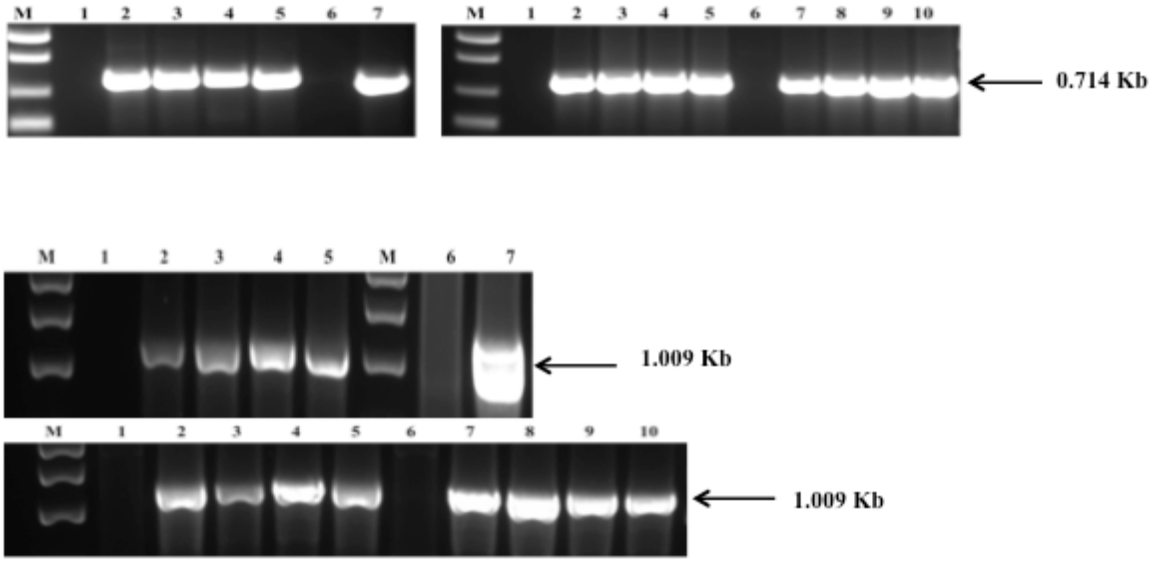


Figure 22: Integration of GFP into the *Leishmania spp*. (Top left): M = 1kb plus molecular weight maker. Lane 1: *L. mexicana* WT, lanes 2-5 *L. mexicana*-GFP clones; Lane 6: *L. maior* WT; Lane 7 *L. major*-GFP clone; In the top right: Lane 1: *L. aethiopica* WT, lanes 2-5 *L. aethiopica*-GFP clones; lane 6: *L. tropica* WT, lanes 7-10 *L. tropica*-GFP clones. In middle and bottom shows successful integration though amplication of 18S and GFP gene. M = 1Kb molecular weight marker; Lane 1: *L. mexicana* WT, Lane 2-5 *L. mexicana*-GFP clones; Lane 6: *L. major* WT, Lane 7 *L. major*-GFP clone; (Bottom image) Lane 1: *L. aethiopica* WT, Lanes 2-5 *L. aethiopica*-GFP clones; Lane 6: *L. tropica* WT; *L. tropica*-GFP clones.

3.2.1.4 Western blot analysis of GFP expression by *Leishmania spp.*

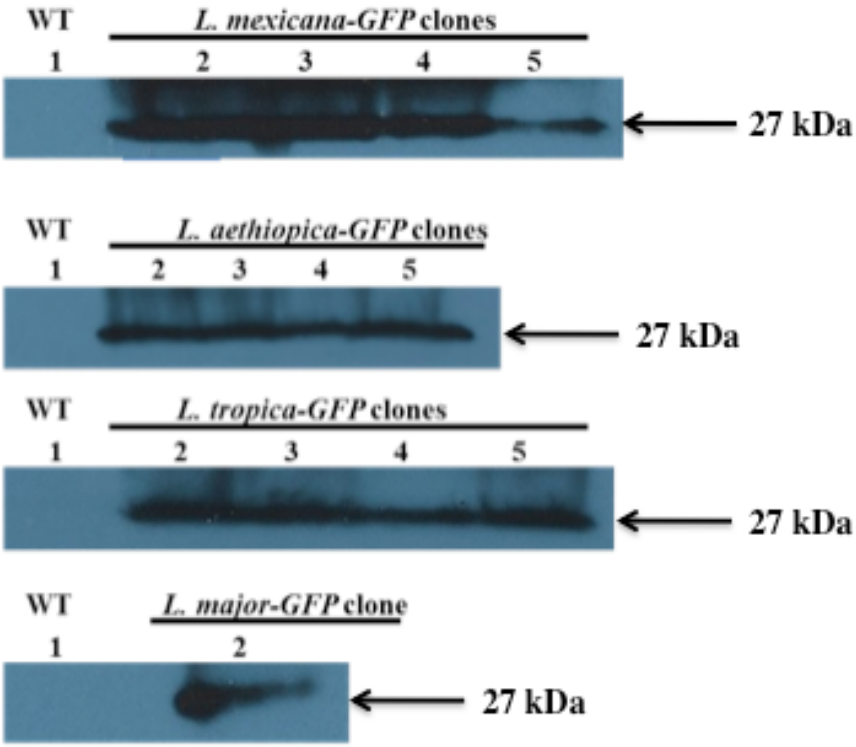


Figure 23: Successful GFP expression at a protein level for all four species. As seen in the PCR validation four GFP clones were successfully identified to express GFP protein except for *L. major* were only 1 clone was integrated. A colleague carried out the western blotting work, as I was absent due to illness during this stage of my research.

3.1.5 Growth curve analysis of WT and GFP-expressing *Leishmania spp.*

Promastigotes of transgenic GFP-expressing species were monitored for growth to determine infective metacyclic stationary phase and to detect whether introduction of GFP gene affected parasites' growth. The growth cycle of transgenic species are comparable to each other in that stationary phase is reached at day 5 for all species (Figures 23-26).

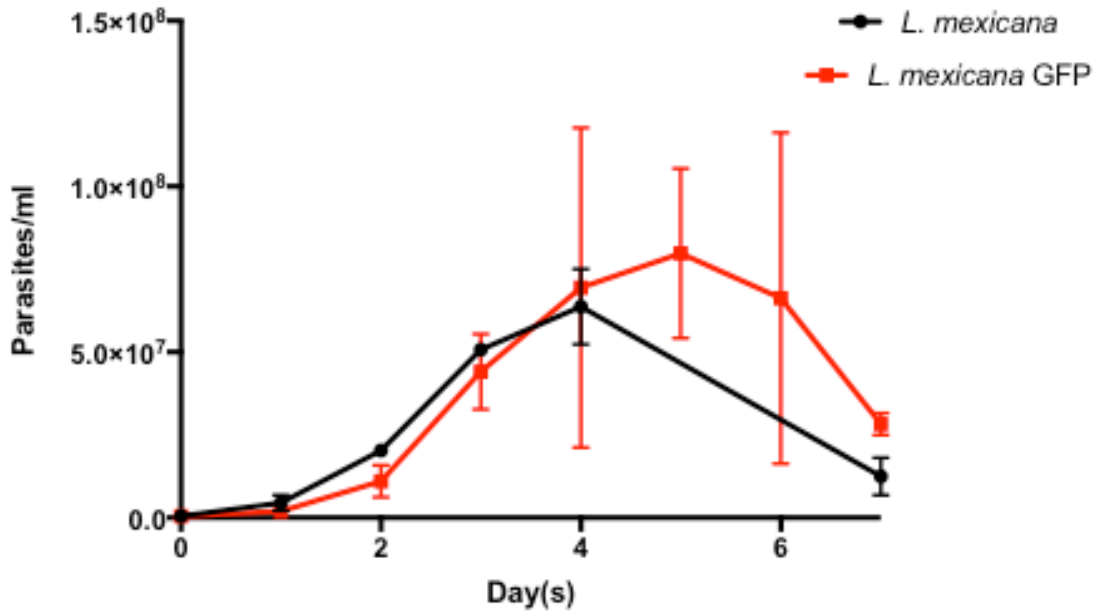


Figure 24: *L. mexicana* WT and GFP growth curve over 7 days.

All biological replicates (N3) were fixed in 0.04% formaldehyde and counted via FASTREAD102 disposable counting slides. The means, SD, SEM and t test values were calculated ($P<0.05$) using Excel which showed no significant difference between the WT and GFP expressing *L. mexicana* at the various times points (except for on Day 7).

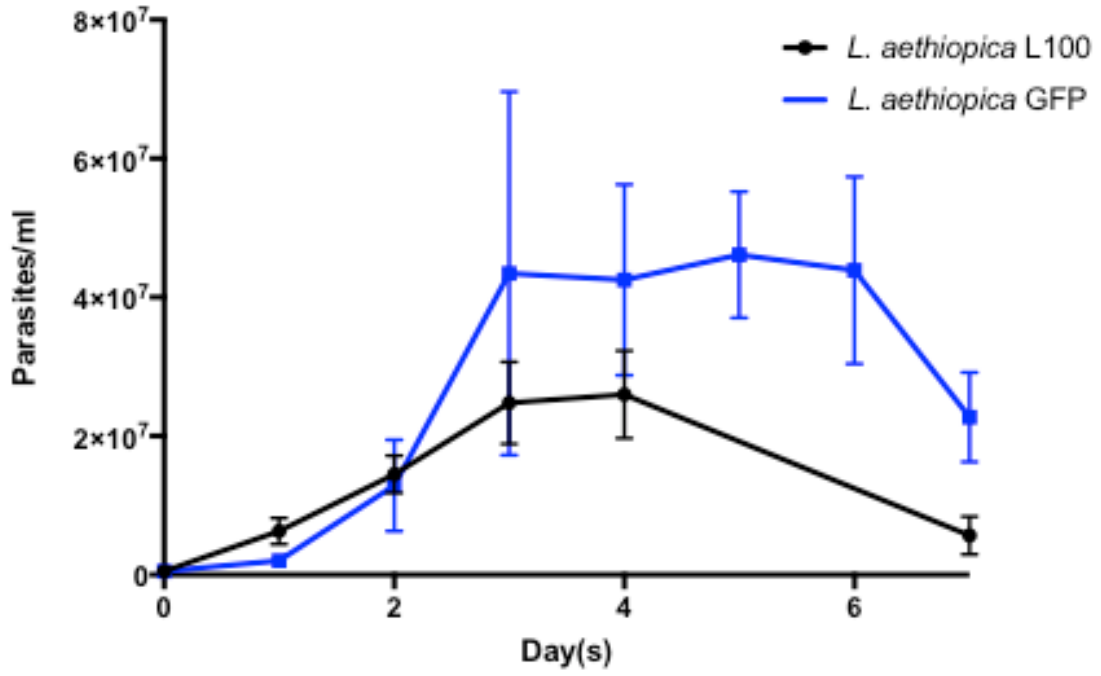


Figure 25: *L. aethiopica* WT and GFP growth curve over 7 days.

All biological replicates (N3) were fixed in 0.04% formaldehyde and counted via FASTREAD102 disposable counting slides. The means, SD, SEM and t test values were calculated using Excel, which showed no significant difference ($P<0.05$) between the day 1 and 2. However, after completing the two-t test, it was found that day 3, 4 and 7 were significantly different between the WT and GFP expressing *L. aethiopica.*

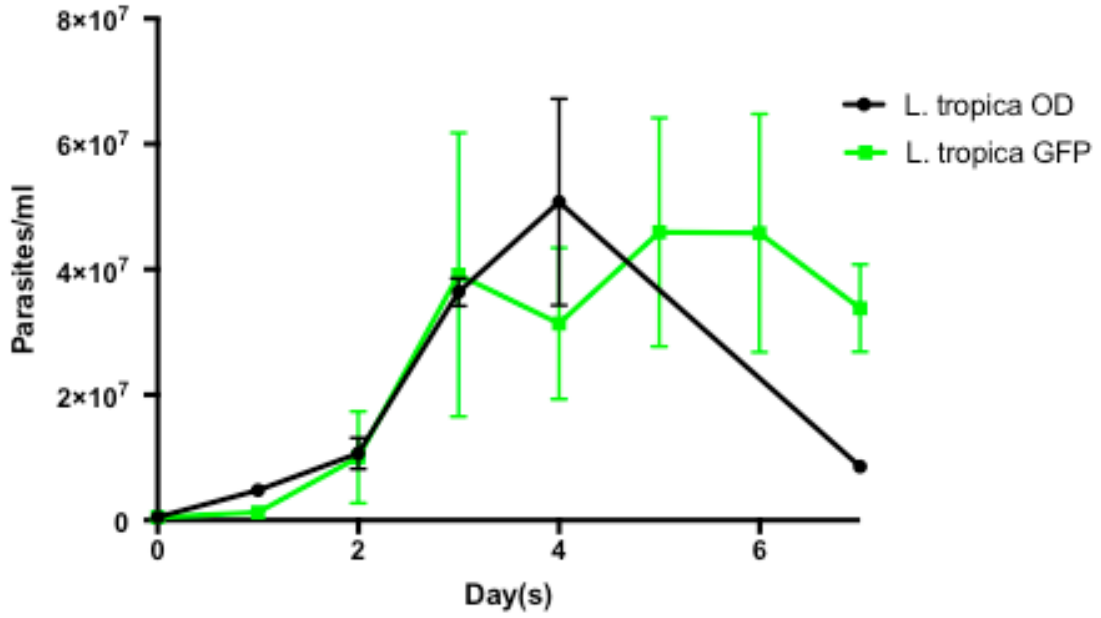


Figure 26: *L. tropica* WT and GFP growth curve over 7 days.

All biological replicates (N3) were fixed in 0.04% formaldehyde and counted via FASTREAD102 disposable counting slides. The means, SD, SEM and t test values were calculated ($P<0.05$) using Excel, which showed no significant difference between the day 1 and 4. However, on day 7 a significantly difference between the WT and GFP expressing *L. aethiopica* was found.

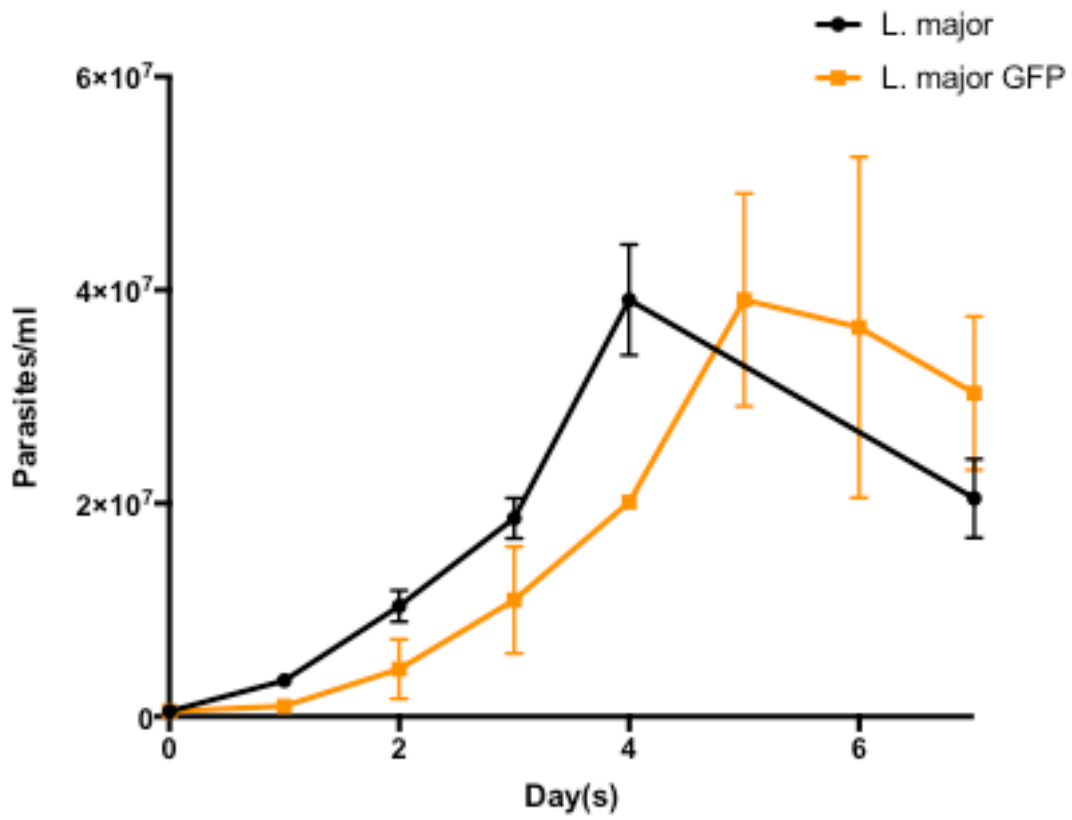


Figure 27: *L. major* WT and GFP growth curve over 7 days.

All biological replicates (N3) were fixed in 0.04% formaldehyde and counted via FASTREAD102 disposable counting slides. The means, SD, SEM and t test were calculated using Excel, which showed no significant difference ($P<0.05$) on any day apart on day 7.

3.2.1.6 Fluorescent microscopy analysis of infected THP-1 cells

The metacyclic form of all four stable GFP-expressing species, isolated from the late stationary phase promastigotes were successfully used to infect terminally differentiated THP-1 cells. Infection was monitored at 24, 48, 72 and 96 hr through both fluorescent microscopy and flow cytometry (Figure 28 and 29).

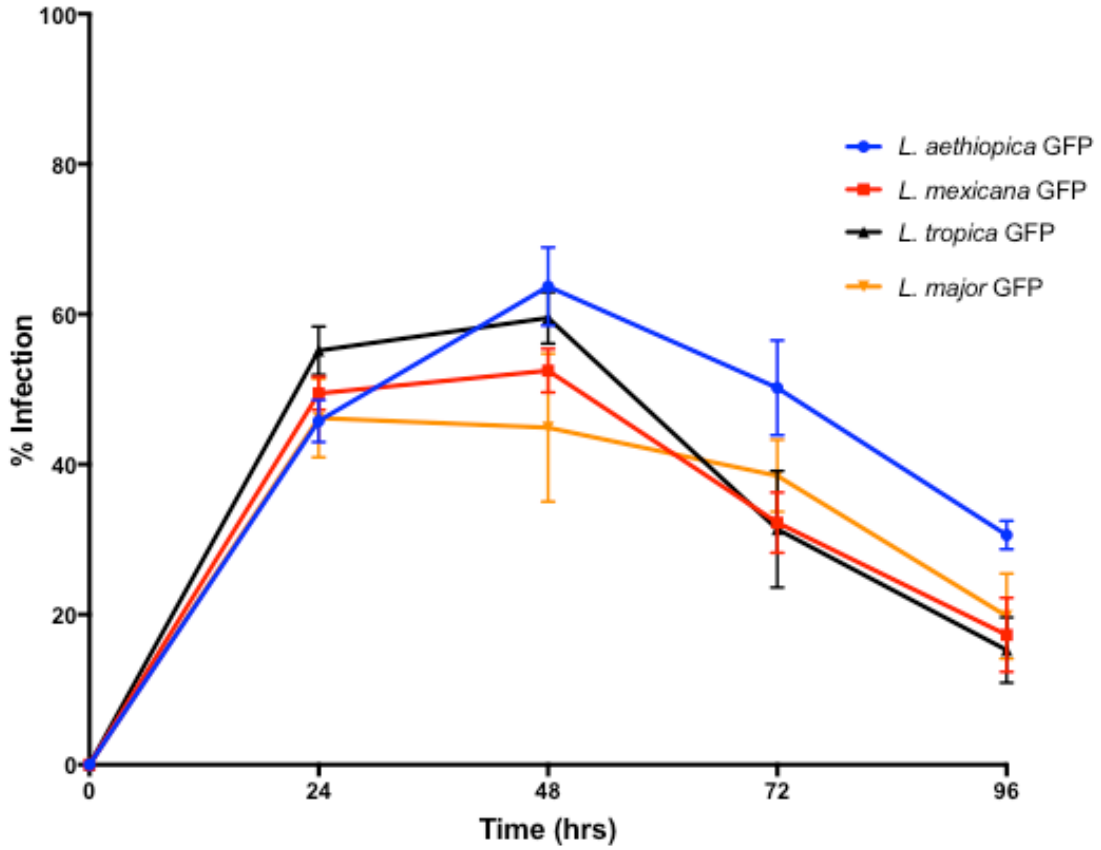


Figure 28: Percentage of infected THP-1 cells using fluorescent microscopy**.**

All biological replicates (N=3) of infected and non-infected cells were counted using the Nikon inverted fluorescent microscopy. The means, SD, SEM and t test values were calculated (95% confidence, P≤0.05) using Excel.

3.2.1.7 Flow cytometric analysis of infected THP-1 cells

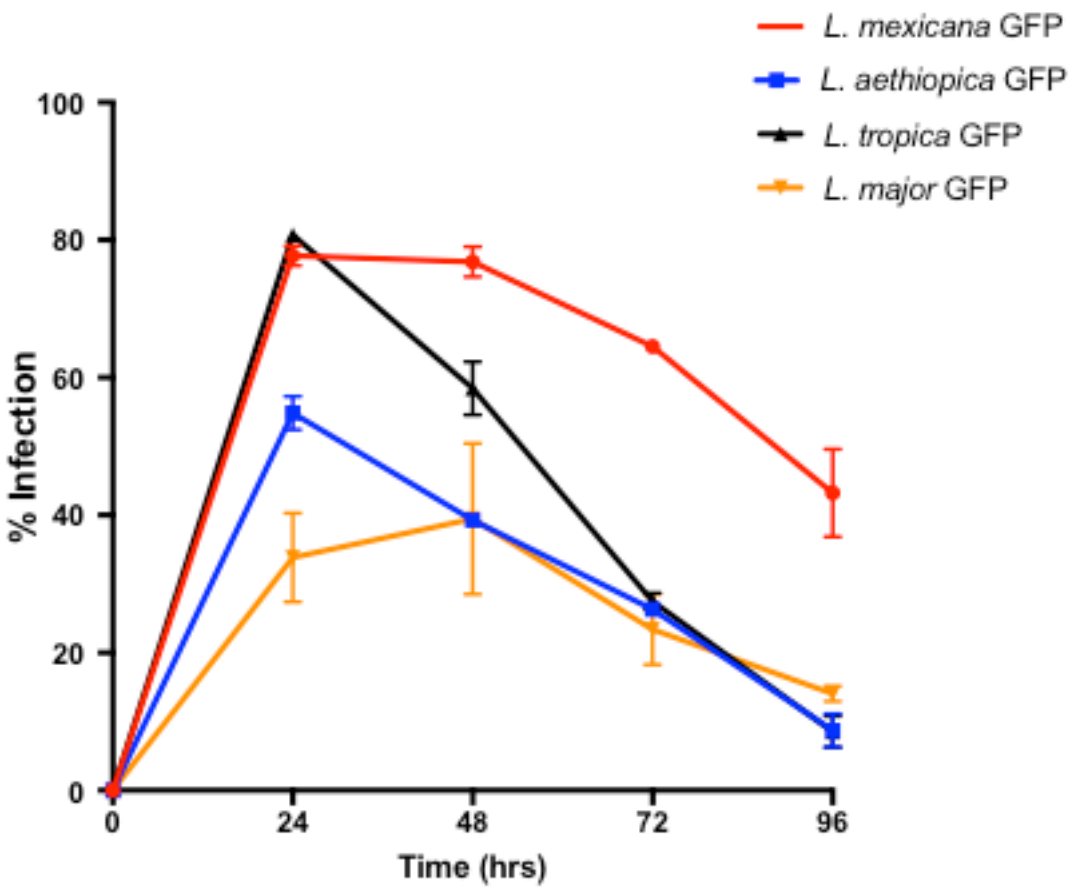


Figure 29: Percentage of infected THP-1 cells using flow cytometry.

All biological replicates (N=3) of infected and non-infected cells were counted using the Nikon inverted fluorescent microscopy. A colleague carried out the flow cytometry work, as I was absent due to illness during this stage of my research. The means, SD, SEM and Wilcoxon signed rank test were calculated using Excel ($P<0.05$).

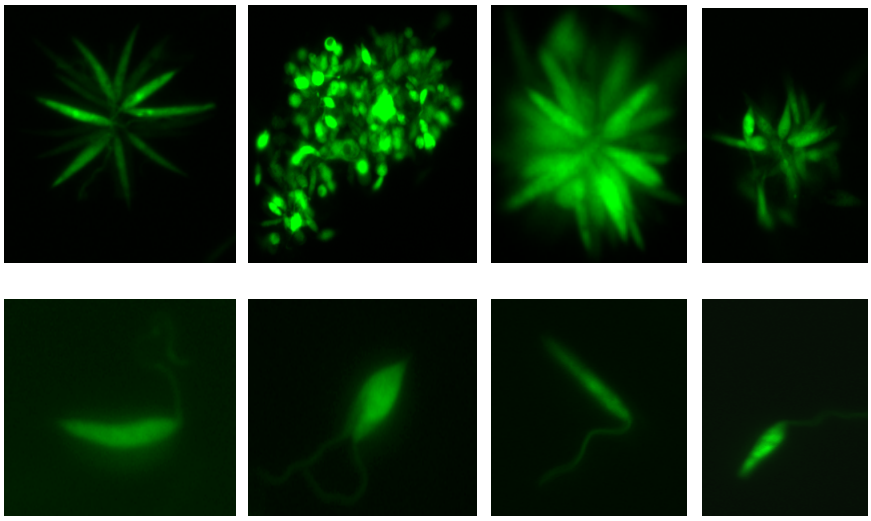
3.4 Discussion

3.4.1 Confirmation of GFP expression in *Leishmania spp.*

Successful transfection and expression of GFP in *L. mexicana, L. aethiopica*, *L. tropica* and *L. major* was confirmed via fluorescent microscopy, PCR, Western blot and flow cytometry.

3.4.1.1 Fluorescent microscopy of GFP expressing promastigotes

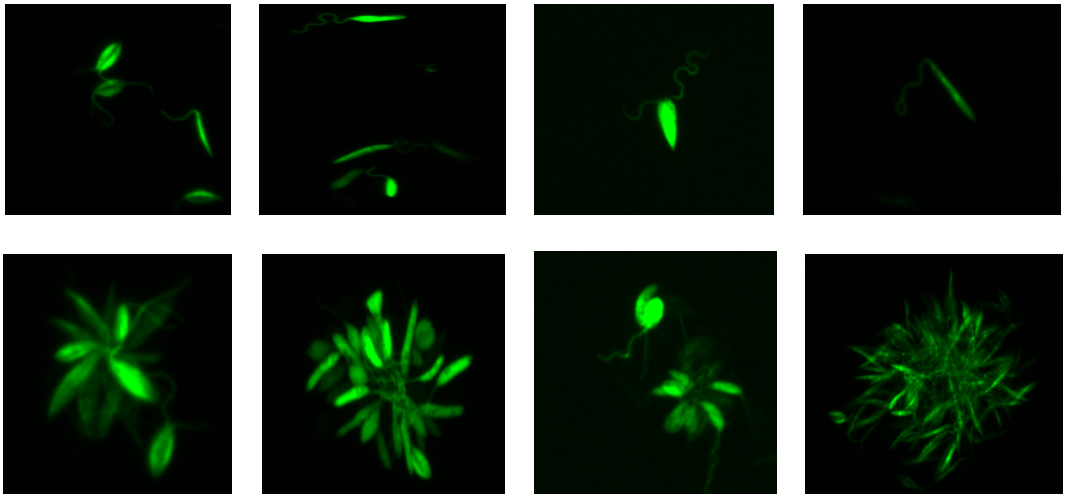
Fluorescence of GFP was observed throughout the cytoplasm and flagella in viable extracellular promastigotes (Figures 17 & 18) and throughout the cytosol in intracellular amastigotes (Figures 19). Clonal selection procedure enabled isolation of a homogenous population of several clones expressing GFP for each of the species. In this study, four representative clones are shown for each species except *L. major* for which a single clone was isolated. Fluorescent microscopy analysis demonstrated a distinct and high level of GFP fluorescence both for the promastigotes and the intracellular amastigotes.



(A) (B) (C) (D)

Figure 18: GFP expression via the successful electroporation of episomal pXG-GFP+ into the cytosol of each *Leishmania spp.* (*L. mexicana* (A), *L. aethiopica* (B), *L. major* (C) & *L. tropica* (D).

Fluorescent microscopy confirmed successful uptake of both the GFP plasmid (pXG-GFP+) into each of the *Leishmania spp*. (no further work was carried out on these parasites).



(A) (B) (C) (D)

Figure 19: GFP expression via the successful integration of the GFP gene downstream of the 18S rRNA promoter in each *Leishmania spp.* (*L. mexicana* (A), *L. aethiopica* (B), *L. major* (C) & *L. tropica* (D).

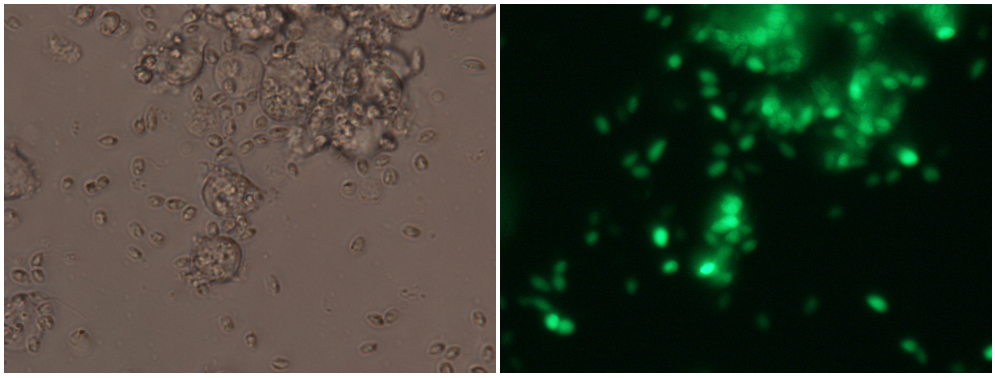


Figure 20: Differentiated THP-1 cells infected with *L. mexicana* GFP after 24hrs exposure. All biological replicate infections (N3) were counted, i.e. >100 cells counts three times/biological replicate. All infections were validated at a 20X and 60X magnification using the Nikon ECLIPSE 90*i* overhead epifluorescent microscope.

3.4.1.2 Flow cytometric analysis of GFP-expressing promastigotes

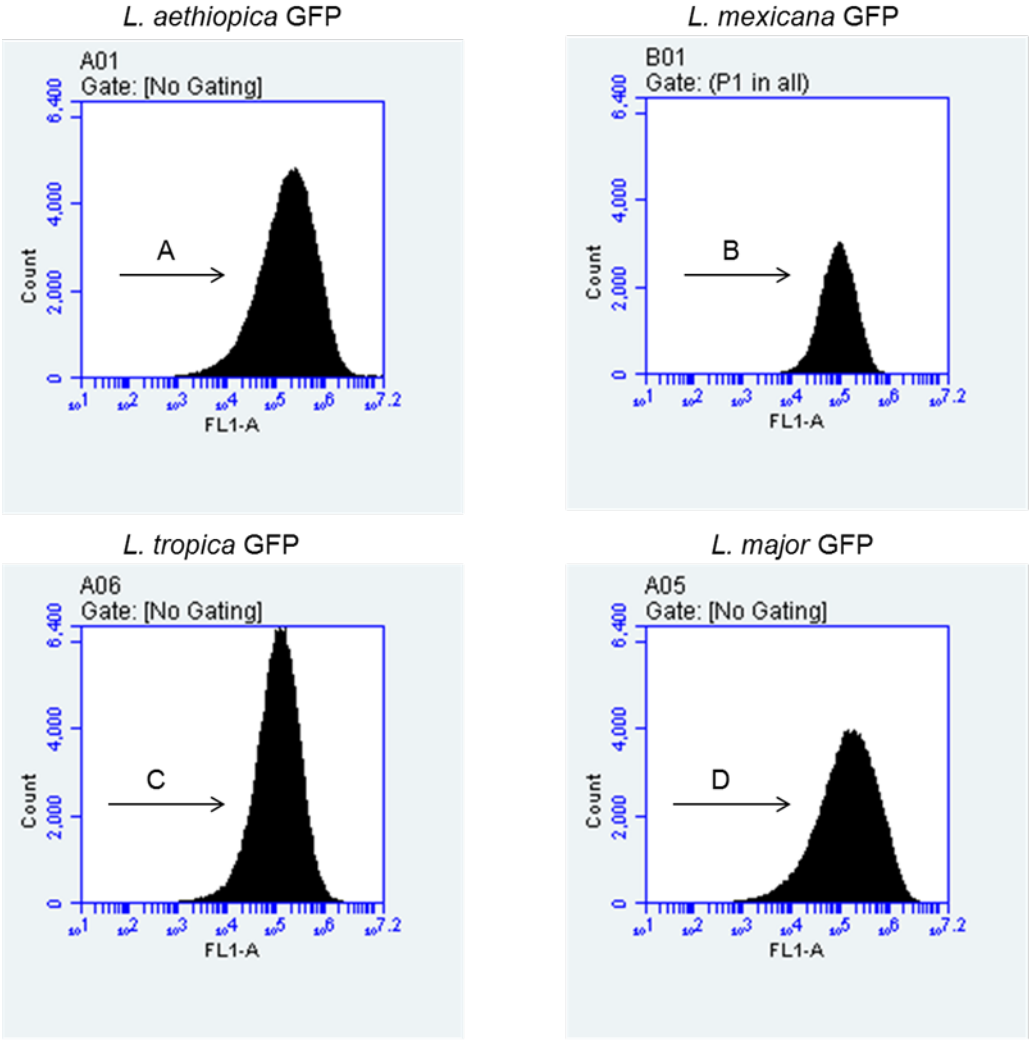


Figure 21: Pure GFP expressing clones for each Leishmania through maximum presence ≥10^4 whilst using the FL1 filter used to detect GFP signal on the Accuri C6 flow cytometer.

Similarly to microscopy, flow cytometry easily differentiated WT parasites from GFP expressing parasites, and demonstrated a distinct and high level of GFP fluorescence on the promastigotes (*L aethiopica* and *L. tropica* in Figure 21).

3.4.1.3 Confirmation of the integration of GFP gene by PCR

Integration of the complete *gfp* gene was confirmed by PCR amplification in the genome of each of the transfected species mediated via site-specific integration (Figure 22). The amplified PCR products showed successful integration of the GFP gene downstream of the 18S rRNA promoter. The integration was checked by the PCR of 1) the sole GFP gene and 2) 18S rRNA promoter upstream within genetically manipulated *Leishmania spp*. against their respective non-GFP controls. During the genetic manipulation process four GFP expressing clones were identified per *Leishmania spp*. except for *L. major* were only 1 GFP clone was successful.

Transfection of *L. mexicana*, *L. major*, *L. aethiopica* and *L. tropica* with linearised pRib1.2αNEOαGFP enabled chromosomal integration of the complete GFP gene at the 18S rRNA locus downstream of the DNA Pol I- driven promoter.

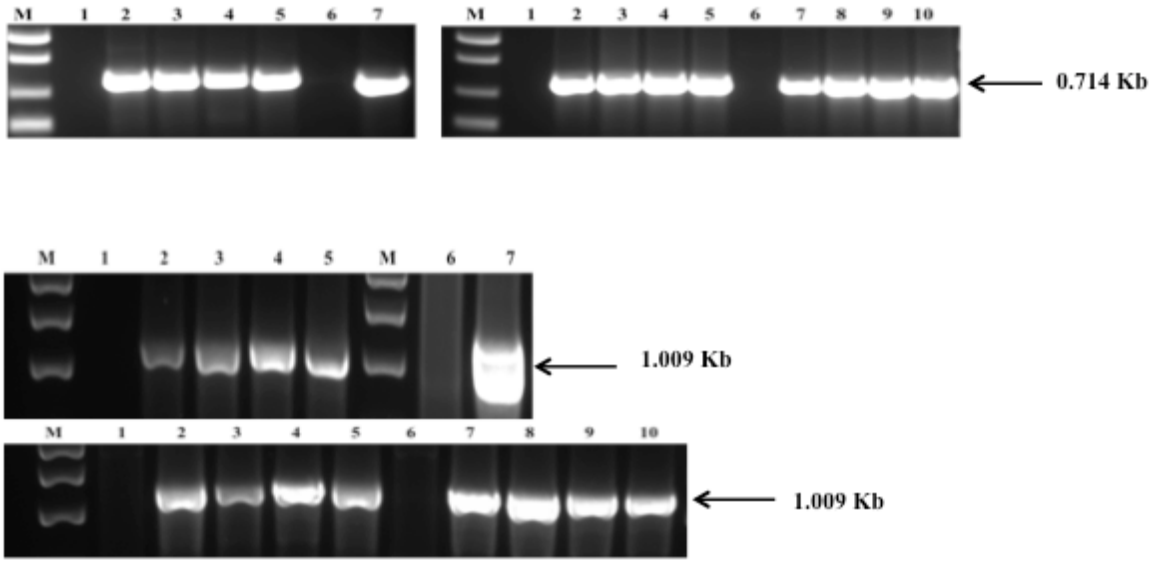


Figure 22: Integration of GFP into the *Leishmania spp*. (Top left) M = 1kb plus molecular weight maker. Lane 1: *L. mexicana* WT, lanes 2-5 *L. mexicana*-GFP clones; Lane 6: *L. maior* WT; Lane 7 *L. major*-GFP clone; In the top right: Lane 1: *L. aethiopica* WT, lanes 2-5 *L. aethiopica*-GFP clones; lane 6: *L. tropica* WT, lanes 7-10 *L. tropica*-GFP clones. In middle and bottom shows successful integration though amplication of 18S and GFP gene. M = 1Kb molecular weight marker; Lane 1: *L. mexicana* WT, Lane 2-5 *L. mexicana*-GFP clones; Lane 6: *L. major* WT, Lane 7 *L. major*-GFP clone; (Bottom image) Lane 1: *L. aethiopica* WT, Lanes 2-5 *L. aethiopica*-GFP clones; Lane 6: *L. tropica* WT; *L. tropica*-GFP clones.

The successful genetic integration of GFP downstream of the 18S rRNA promoter revealed a good level expression and fluorescence within the cytosol and the flagellum of all *Leishmania spp* (Patel *et al*., 2012). The amplification of the GFP gene within each of the GFP-expressing *Leishmania* clones showed the desired product of 714 base pairs. This amplification controls against their respective WT showed the no PCR product as expected. The amplication of the 18S rRNA gene and GFP gene in each of *Leishmania spp*. further provided evidence for successful integration downstream of the 18S rRNA promoter through homologous recombination. The desired PCR product of 1.009Kb in all GFP-expressing clones revealed this. The identical

amplification protocol against their respective WT counterparts showed no PCR product as expected.

3.4.1.4 Western blot analysis of GFP expression by *Leishmania spp.*

The presence of GFP in all four species (*L. mexicana*, *L. major*, *L. aethiopica* and *L. tropica*) was further validated by Western blot analysis. Green fluorescent protein was detectable in whole cell lysates of transgenic parasites using polyclonal rabbit antiserum. An expected dominant band of 27 kDa protein was visible in all transgenic clones while no protein of this molecular weight was detected in the negative controls, *i.e*. whole cell lysates prepared from wild-type promastigotes of all four species (Figure 22)**.**

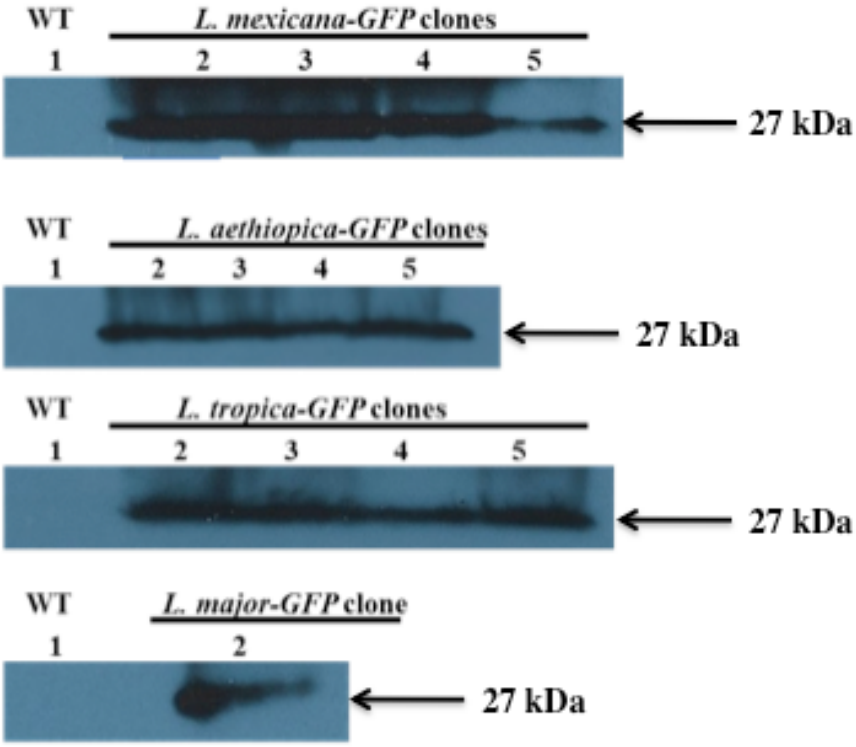


Figure 23: Successful GFP expression at a protein level for all four species.

As seen in the PCR validation four GFP clones were successfully identified to express GFP protein except for *L. major* were only 1 clone was integrated. A colleague carried out the western blotting work, as I was absent due to illness during this stage of my research.

The results show successful protein expression of GFP by all *Leishmania spp*. As with the PCR validation four GFP expressing clones were identified except for *L. major* were only 1 GFP clone was validated (Figure 23). This provides additional evidence that successful integration of GFP occurred into the *Leishmania* genome. The western blotting test against GFP protein expressed in GFP-expressing *Leishmania spp*. indicated a good level of protein expression with a product of 27KDa. All WT *Leishmania spp*. showed no protein expression when conducting the WB testing.

3.4.1.5 Growth curve analysis of WT and GFP-expressing *Leishmania spp.*

Promastigotes of transgenic GFP-expressing species were monitored for growth to determine infective metacyclic stationary phase and to detect whether introduction of GFP gene affected parasites' growth. The growth cycle of transgenic species are comparable to each other in that stationary phase is reached at day 5 for all species.

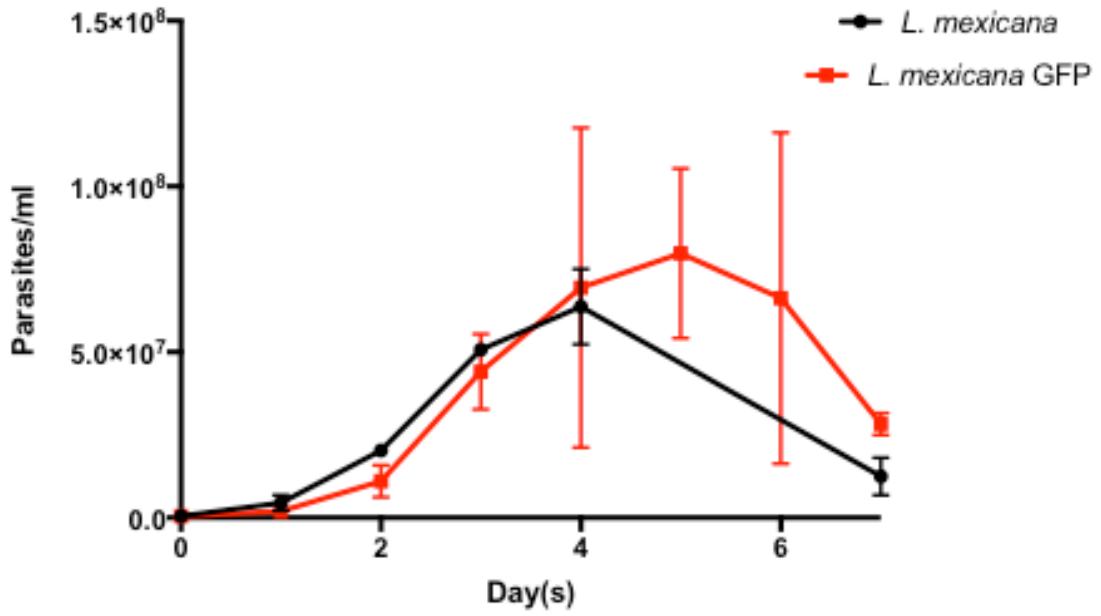


Figure 24: *L. mexicana* WT and GFP growth curve over 7 days.

All biological replicates (N3) were fixed in 0.04% formaldehyde and counted via FASTREAD102 disposable counting slides. The means, SD, SEM and 2-t test values were calculated ($P<0.05$) using Excel which showed no significant difference between the WT and GFP expressing *L. mexicana* at the various times points (except for on Day 7).

The *L. mexicana* growth curve of promastigotes shows increasing cell number after the initial starting concentration of $5x10^5$/ml parasites in complete Schneider media (Methods). The *L. mexicana* WT and GFP expressing promastigotes reached their highest concentrations on day 4 and 5, respectively. There was no significant difference ($P\geq0.05$), difference in the promastigote growth cycle between the GFP expressing and WT *Leishmania* species except for on Day 7. This

highlights that only during the death stage there were differences. All growth curve graphs displayed here were used to decide how to maximise the metacyclic yield through peanut agglutination for all *Leishmania spp*.

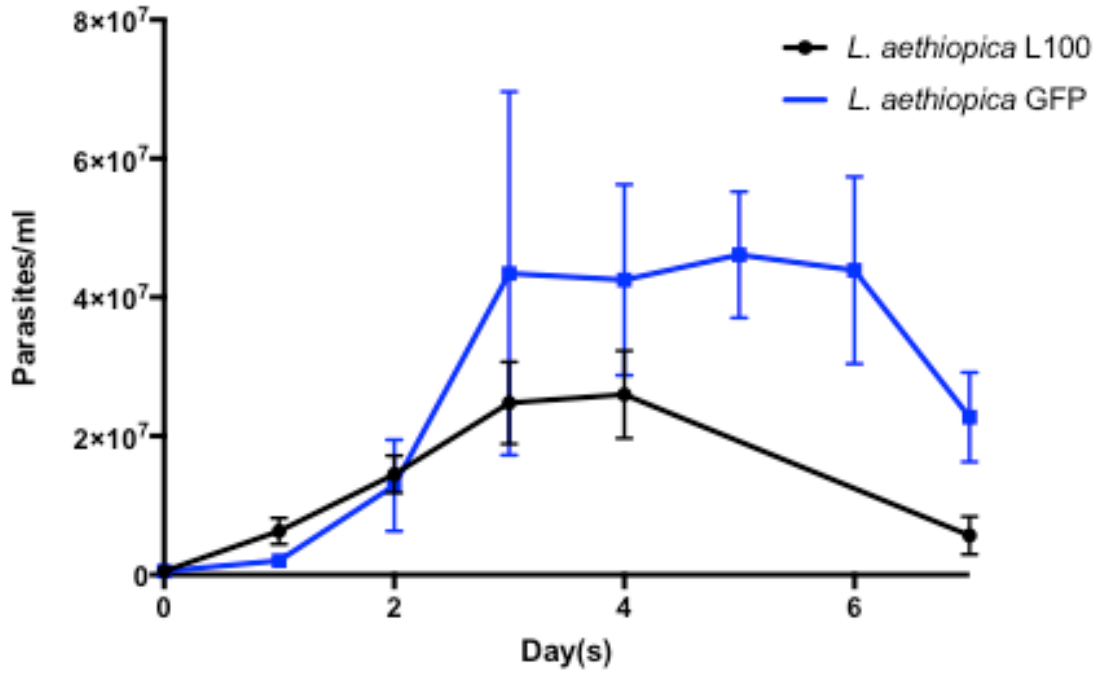


Figure 25: *L. aethiopica* WT and GFP growth curve over 7 days.

All biological replicates (N3) were fixed in 0.04% formaldehyde and counted via FASTREAD102 disposable counting slides. The means, SD, SEM and t test values were calculated using Excel, which showed no significant difference ($P<0.05$) between the day 1 and 2. However, after completing the two-t test, it was found that day 3, 4 and 7 were significantly different between the WT and GFP expressing *L. aethiopica.*

The *L. aethiopica* growth revealed the parasites divided successfully reaching their maximum concentration between day 3 & 4 for the WT and between days 3-6 for GFP expressing parasites. There was no significance between the GFP and WT growth between day 1 and 2. However, on day 3, 4 and 7, significant difference was observed ($P\leq0.01$). The reasoning is

unclear for the significant difference found between the GFP expressing and WT *Leishmania aethiopica* on these days.

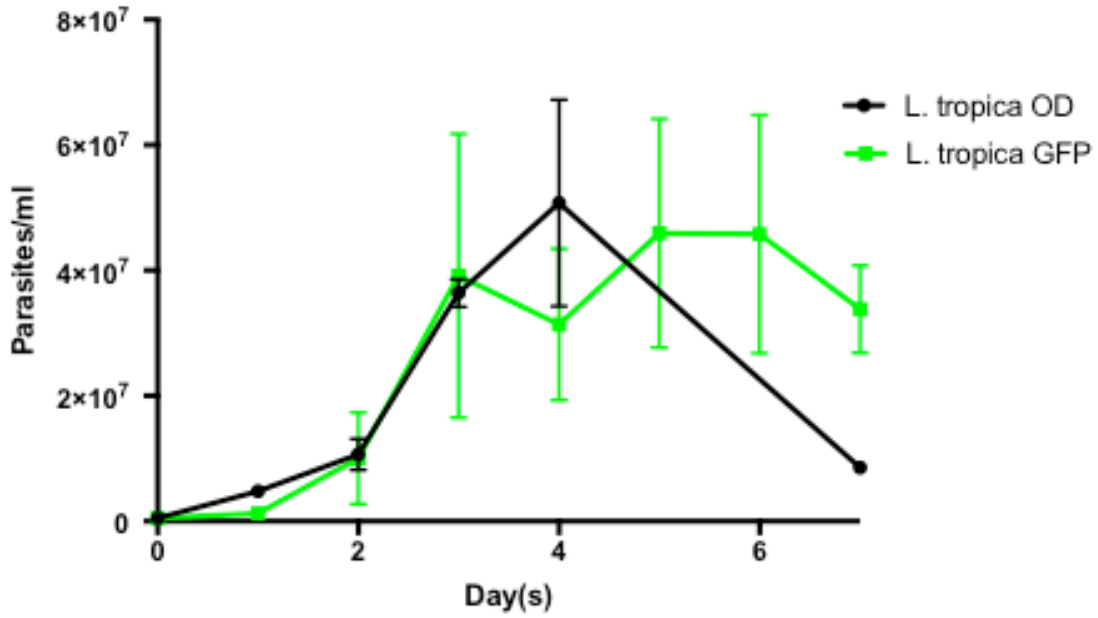


Figure 26: *L. tropica* WT and GFP growth curve over 7 days.

All biological replicates (N3) were fixed in 0.04% formaldehyde and counted via FASTREAD102 disposable counting slides. The means, SD, SEM and t test values were calculated ($P<0.05$) using Excel, which showed no significant difference between the day 1 and 4. However, on day 7 a significantly difference between the WT and GFP expressing *L. aethiopica* was found.

The *L. tropica* growth curve revealed that the parasites reached their maximum concentration between days 3-4 for the WT and between days 3-6 for the GFP expressing equivalent. No significant difference was shown between Day 1 and 3. However, on Day 7 it should be stated that this death phase there was a significant difference ($P\leq0.01$). It is unclear why the integration of GFP gene into the *Leishmania spp*. would have this affect.

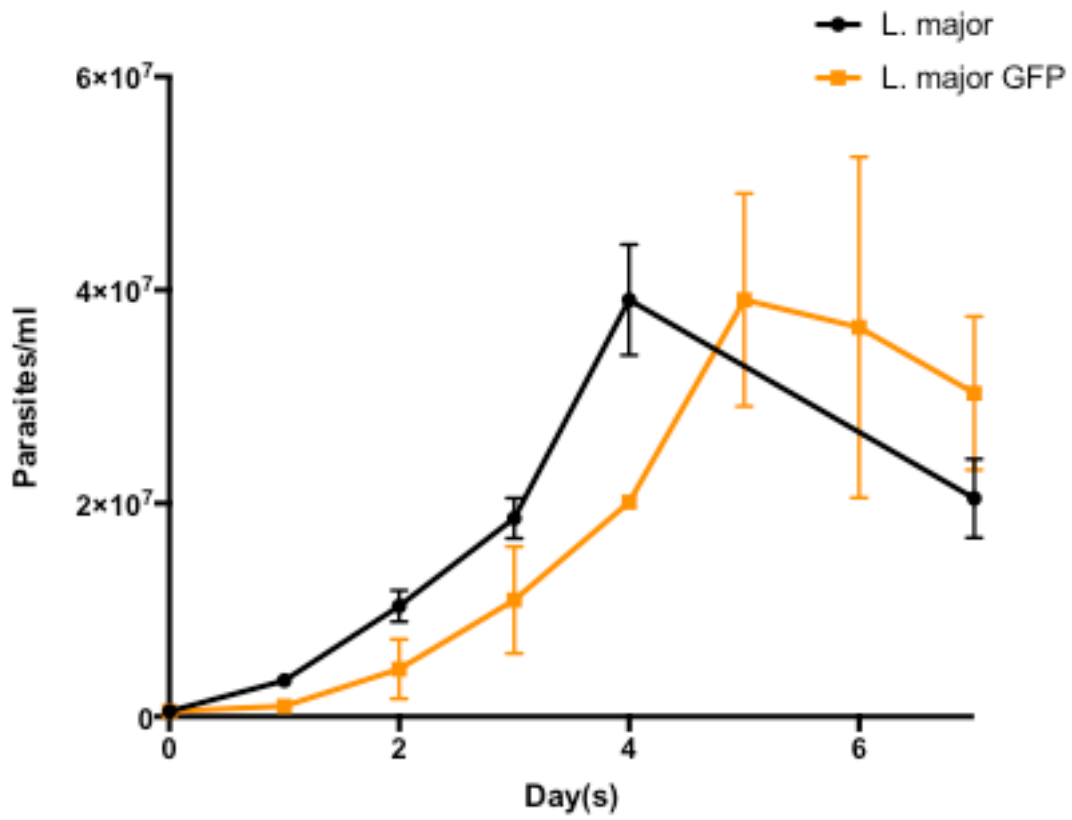


Figure 27: *L. major* WT and GFP growth curve over 7 days.

All biological replicates (N3) were fixed in 0.04% formaldehyde and counted via FASTREAD102 disposable counting slides. The means, SD, SEM and t test were calculated using Excel, which showed no significant difference ($P<0.05$) on any day apart on day 7.

The *L. major* growth curve revealed that the parasites reached their maximum concentration on days 4 for the WT, and between days 5-6 for the GFP expressing equivalent. There was no significant difference between the WT and the GFP expressing *L. major* on any day except for on day 7 ($P\leq0.01$) The two growth curves displayed here were used to decide how to maximise the metacyclic yield through peanut agglutination for *L. major* parasites. Hence, it was decided that for all experiments involving infection studies with *L. major* GFP promastigotes, the parasites would be harvested on day 5.

All WT *Leishmania spp*. showed their cell growth peaked on day 4 (Figures 23-26). However, no cell counts were recorded on day 5 or 6 preventing a comparative count analysis with their GFP equivalents. The GFP expressing *Leishmania spp*. showed a varied growth curve when compared to their WT counterparts. These promastigotes, *i.e*. *L. mexicana* GFP, *L. aethiopica* GFP and *L. tropica* GFP reached the stationary phase between days 4-6, 3-6 and 5-6 respectively (Figures 24-27). There were obvious differences between the *Leishmania* WT and their respective GFP counterparts indicating no negative effect has been caused through the integration of the GFP gene downstream of the 18S promoter of the *Leishmania spp*. The growth curves show that all *Leishmania spp*. WT or GFP expressing reached their maximum carrying capacity at 10^{7-8} cells/ml. This is consistent with literature; as it represents the beginning of the stationary phase and the population containing the highest number of metacyclic promastigotes.

3.4.1.6 Fluorescent microscopy analysis of infected THP-1 cells

The metacyclic form of all four stable GFP-expressing species, isolated from the late stationary phase promastigotes were successfully used to infect terminally differentiated THP-1 cells. Infection was monitored at 24, 48, 72 and 96 hr through both fluorescent microscopy and flow cytometry **(**Figure 28 and 29). As previously reported (Singh *et al*., 2009), flow cytometry analysis enabled distinct separation of uninfected macrophage from an infected macrophage population based on differences in fluorescence levels between the two populations**.** The percentage of infection in THP-1 cells calculated by either method followed a similar trend; percentage of infected cells peaked between 24 and 48 hours and a significant decrease in infection ratio was detected after 48 hours during infection with all four species (Figures 27 and 28).

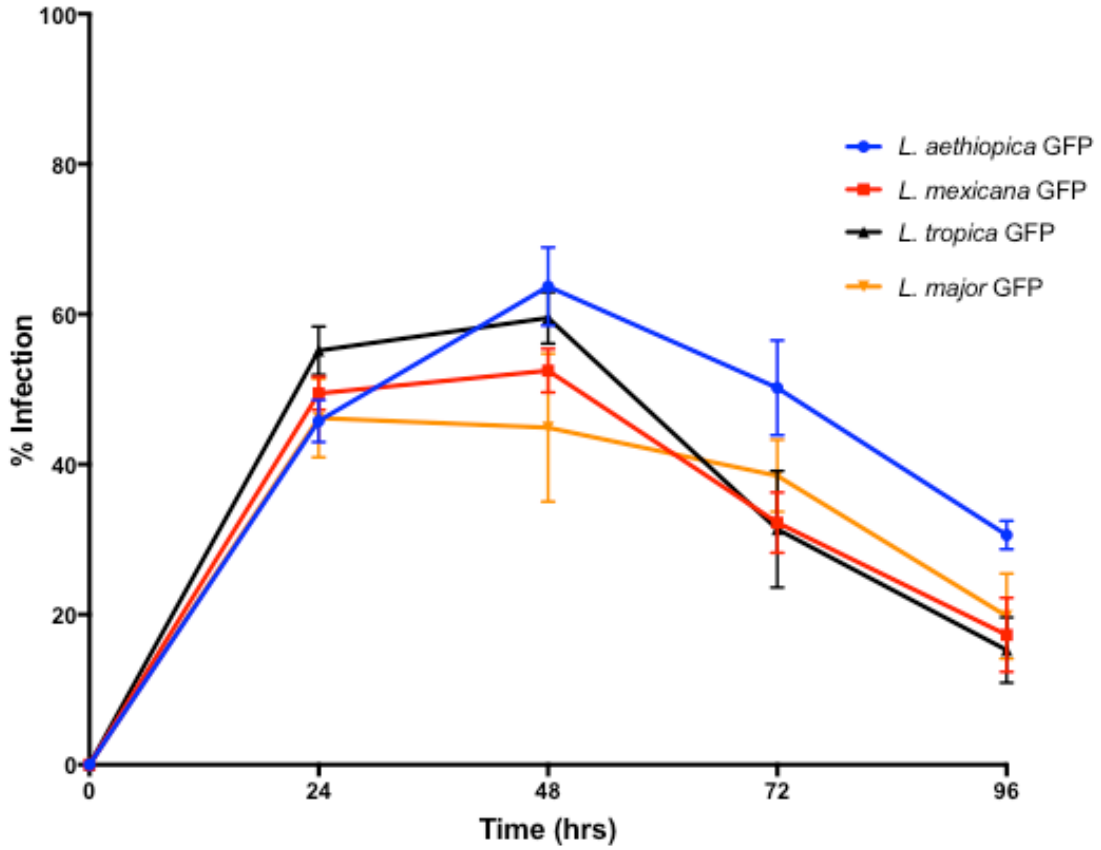


Figure 28: Percentage of infected THP-1 cells using fluorescent microscopy.

All biological replicates (N=3) of infected and non-infected cells were counted using the Nikon inverted fluorescent microscopy. The means, SD, SEM and t test values were calculated (95% confidence, $P<0.05$) using Excel.

The fluorescent microscopy data reveals that the highest level of infection occurs between 24-48hrs; after which all infections decrease as time passes. All *Leishmania spp*. show slightly increasing levels of infection from 24 to 48hrs except for *L. major*, which shows a slight decline. *L. aethiopica* GFP was found to be significantly different from *L. mexicana* GFP, *L. major* GFP and *L. tropica* GFP between 48-96hrs, 48-96hrs and 72-96hrs, respectively ($P \leq 0.05$). In addition, *L. tropica* GFP and *L. major* GFP was only found to be significantly different at 48 hours post infection. The differences between *L. mexicana* with *L. tropica* or *L. major* GFP were not found to be significant ($P \geq 0.05$) at any of the time points.

3.4.1.7 Flow cytometric analysis of infected THP-1 cells

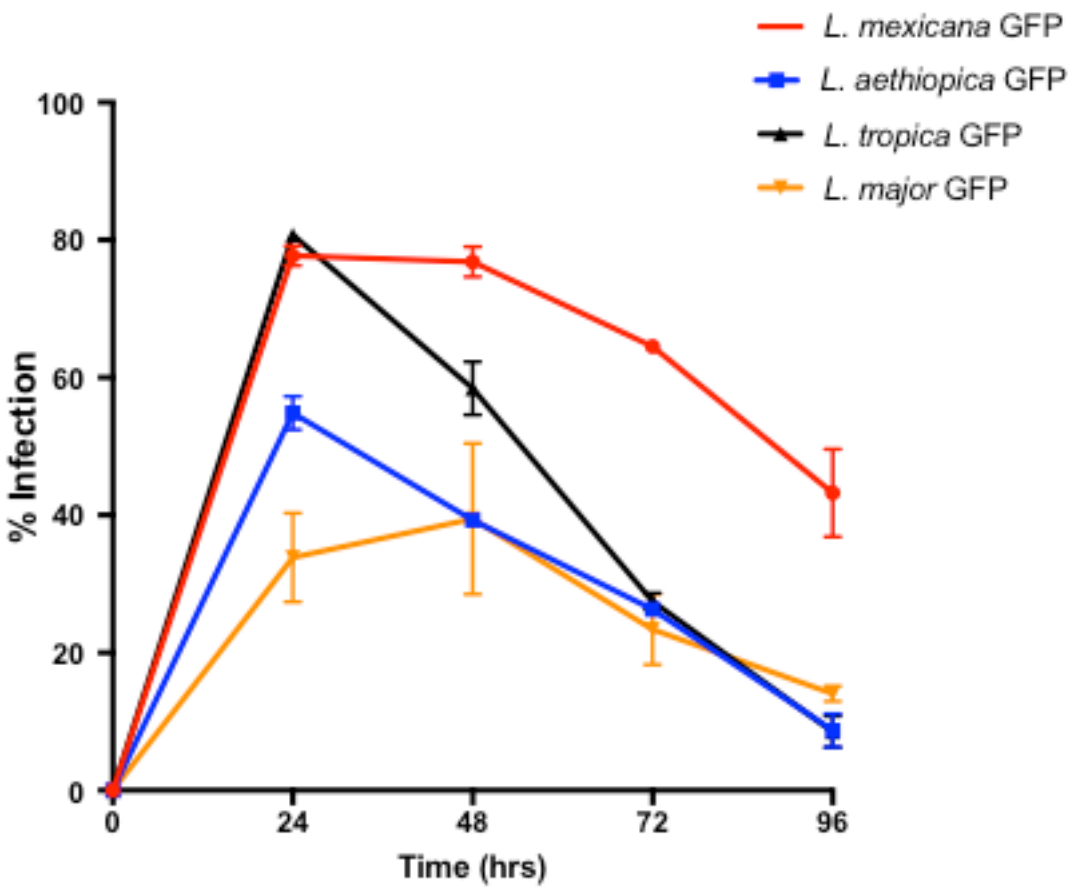


Figure 29: Percentage of infected THP-1 cells using flow cytometry.

All biological replicates (N=3) of infected and non-infected cells were counted using the Nikon inverted fluorescent microscopy. The means, SD, SEM and Wilcoxon signed rank test were calculated using Excel ($P<0.05$). A colleague completed this work, as I was absent due to illness during this stage of research.

The flow cytometric data on the *Leishmania*-infected THP-1 cells show the highest levels at 24hrs, which is followed by a gradual decline towards 96hrs. This occurs for all species except for *L. major,* which peaked at 48hrs. The gradual decline was mostly recognised in *L. mexicana* infected cells occurred post 48hrs infection. This was due to the fact that the percentage of infection only decreased from 77.7% to 76.8% at 24 and 48hrs post infection, respectively. When comparing infection between different *Leishmania spp*., *L. aethiopica* GFP was found to significantly differ from *L. mexicana* GFP (24-96hrs), *L. tropica* GFP (at 24hrs only) and *L.*

major GFP (at 24hrs). Additionally, *L. mexicana* GFP was shown to be significantly different from *L. tropica* GFP (48-72hrs), with the latter also differing from *L. major* GFP (24-48hrs) ($P \leq 0.05$).

Fluorescent microscopy showed mean THP-1 infections did not surpass 65% and the highest percentages of infections were at 24 and 48hrs. The mean percentage infections ranged between 24 & 48hrs at 45.77 to 63.71 with *L. aethiopica* GFP, 49.46 to 52.50 with *L. mexicana* GFP, 55.18 to 59.51 with *L. tropica* GFP and 46.18 to 44.92 with *L. major* GFP. 48hrs post infection, the percentages decline until 96hr is reached for all *Leishmania* expressing GFP. The Flow cytometric analysis showed higher mean percentages of infection than observed when using fluorescent microscopy. The mean percentage infections ranged between 24 & 48hrs at 59.76 to 37.67% with *L. aethiopica* GFP, 77.71 to 76.82 with *L. mexicana* GFP, 80.74 to 66.15% with *L. tropica* GFP and 33.85 to 39.47% with *L. major* GFP. It is clear there are differences in the mean percentages of infection when comparing flow cytometry with counting infected cells using fluorescent microscopy. In flow cytometry, the general trend was that the percentage of infections decreased from 24 to 96hrs. The exceptions to this were found with *L. aethiopica* GFP and *L. major* GFP between 24 and 48hrs where infection either was similar or increased, respectively. In addition, flow cytometry showed the highest levels of infection at 24hrs when THP-1 cells were infected with *L. mexicana* and *L. tropica* where as the fluorescent microscopy highest mean was *L. aethiopica* at 48hrs. The level of discrepancies between the two techniques in evaluating *Leishmania* infection in THP-1 highlights the superiority of flow cytometry in resolving power. This stems from the fact that during microscopy only a total of >900 THP-1 cells per *Leishmania* species test were counted whereas the flow cytometry was able to analyse and identify the percentage of infected cells in 50,000 events which greatly enhances the accuracy.

3.5 Conclusions

This research has highlighted the successful expression of GFP in *Leishmania spp*. by using the two different plasmids. The electroporation of pXG-GFP+ into the cytosol of *Leishmania spp*. showed good GFP expression using fluorescent microscopy. The successful integration of GFP downstream of the 18S rRNA promoter via homologous recombination was confirmed by fluorescent microscopy, flow cytometry, PCR (targeting sole GFP gene & 18S rRNA and GFP gene) and WB. The successful integration of GFP into *Leishmania spp*. provides a huge advantage for research and development when using this fluorescent marker constituently expressed by the parasites. This study succeeded in generating, for the first time, transgenic *L. aethiopica* and *L. tropica* parasites constitutively expressing GFP. Such strains as well as transgenic *L. major* and *L. mexicana* were successfully validated as representing the same characteristic as their wild type counterparts. Specifically, because such parasites maintain infectivity and drug susceptibility comparable to their wild type counterparts, they are an ideal model, both for infection studies and drug assays. The transgenic parasites were used throughout this PhD work to further study infection.

Chapter 4

Does *L. aethiopica* affect cytokine expression in macrophages?

Novelty statement

This is the first time that the expression of IL-1RA, IL-10, TGFβ, TNFα and cathelicidin mRNA have been investigated simultaneously using GFP expressing *L. aethiopica* parasites.

Impact

This preliminary study indicates that further investigation is required as no significant relationship was identified when comparing mRNA expression of cathelicidin and cytokines during *L. aethiopica* infection.

4.1 Introduction

The ability of *Leishmania* to manipulate cellular cytokine expression infection has been shown to be critical for the parasites' survival (Rodgers and Titus 2004; Oliveira *et al*., 2014). Cytokine expression is in involved in controlling cellular mediated responses which in turn control parasites survival through specific CD4 T helper lymphocytes (Th cells). The balance between the pro- and anti-inflammatory expression of cytokines dictates the outcome of infection. This novel research has involved studying this cytokine balance in differentiated THP-1 cells infected with *L. aethiopica* at various time points as little is known regarding the cytokine profile during infection with this species. Similar studies using *L. major* and *L. amazonensis* showed elevation anti-inflammatory cytokines (IL-4 and IL-10) increase from 2-8hrs followed by a decline at 19hrs (Lepara and Kelly 2010). Inflammatory cytokines (TNFα, IL-12p40 & IL-1α showed increased expression from 2-19hrs except for TNFα which peaked at 8hrs. There is very little

understanding into the effect of *L. aethiopica* on the expression of cathelicidin and cytokines during infection. This novel research chapter focuses on the expression of TNFα, IL-1RA, IL-10, TGFβ and cathelicidin at various time points (2, 8, 24 & 48hrs). The results of which will now be discussed.

4.2 Methods

The cell culture practices for THP-1 and *L. aethiopica* promastigotes were carried as previously mentioned (Methods 2.1.1 pp. 32 and 2.1.2 pp. 32). The infection of THP-1 cells, purification of metacyclic promastigotes, and the flow cytometric analysis was carried as seen in Methods 2.3.1 pp. 43, 2.3.2 pp. 43 and 2.3.2.1 pp. 43. All the cytokine expression methods including mRNA primer design, sampling, RNA extraction, RNA quantification, reverse transcription of RNA into cDNA, standard PCR/gel electrophoresis and qPCR were carried as previously mentioned (Methods 2.4.1 pp 50, 2.4.2 pp 51, 2.4.3 pp 52, 2.4.4 pp 52, 2.4.5 pp 53, 2.4.6 pp 53, 2.4.7, pp 54, 2.4.8 pp 55 and 2.4.9 pp 56).

4.3 Results

4.3.1 Flow cytometric analysis of *L. aethiopica* infection

At all time points (2, 8, 24 and 48hrs) the levels of infection were tested using the Accuri C6 flow cytometer (Method 2.3.2.1). The percentages of infections (N=3) were similar to the previous infection data at 24 and 48 hours post infection (Chapter 3, Section 3.4.1.7 pp 90).

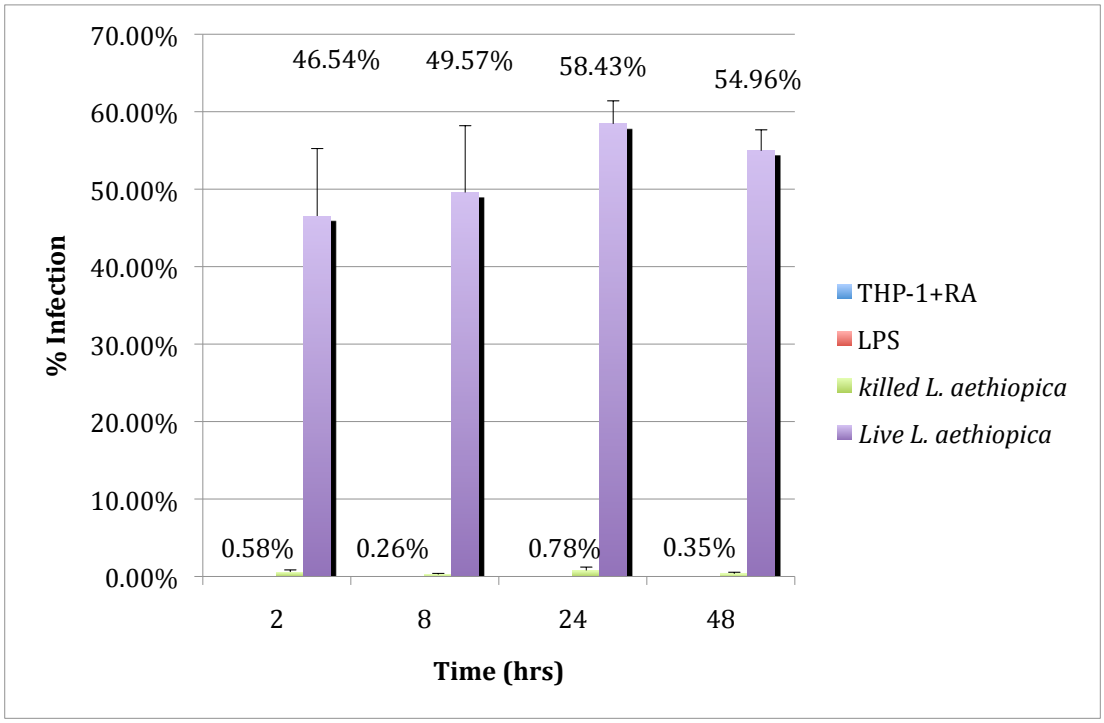


Figure 29: Percentage of *L. aethiopica* infection at the different time points.
All biological replicates (N3) were analysed using the Accuri C6 flow cytometer with a minimum of >2000 events recorded. GFP was only detected in the differentiated THP-1 cells infected with live and dead *L. aethiopica*. The mean, SEM and Wilcoxon signed rank test were calculated using Excel ($P<0.05$).

4.3.2 Optimisation of PCR

The identification of the optimal annealing temperatures for all PCR primers was required to before commencing the cytokine qPCR experiment. To identify the optimal annealing temperature for each pair of primers, an annealing temperature gradient (58-64^{o}C) was applied.

Table 7: Annealing temperatures of the cytokine and cathelicidin primers designed.

Primers	Sequence	Primer Length (bp)	GC (%)	Optimal Tm (^{O}C)	Product Size (bp)
IL-1ra Forward	GGAATCCATGGAGGGAAGAT	20	50	58	97
IL-1ra Reverse	TCTCGCTCAGGTCAGTGATG	20	55		
IL-10 Forward	TTACCTGGAGGAGGTGATGC	20	55	60	127
IL-10 Reverse	GGGAAGAAATCGATGACAGC	20	50		
TGFβ Forward	GAGCCTGAGGCCGACTACTA	20	60	58	131
TGFβ Reverse	CGGAGCTCTGATGTGTTGAA	20	50		
TNFα Forward	CAACCTCCTCTCTGCCATCAA	20	50	60	150
TNFα Reverse	ATAGTCGGGCCGATTGATCT	20	50		
Cathelicidin forward	GGACCCAGACACGCCAAA	18	61.1	60	52
Cathelicidin reverse	GCACACTGTCTCCTTCACTGTGA	23	52.1		
b-tubulin Forward	CTCTTCCAGCCTTCCTTCCT	20	55	60	116
b-tubulin Reverse	AGCACTGTGTTGGCGTACAG	20	55		

4.3.3 qPCR of cytokine expression during infection

To identify the changes in cytokine and cathelicidin mRNA expression, the qPCR was run on all the samples types (THP-1+RA – negative control, LPS – positive control, killed *L aethiopica* GFP and *L. aethiopica* GFP). Comparative cT analysis was conducted to obtain the fold difference of expression of the gene of interest (cytokine type or cathelicidin) from the house-keeping gene (β-tubulin) providing the relative quantification. All cTs results for all mRNA expression targets were obtained from setting the threshold of 1.

4.3.3.1 Cathelicidin mRNA expression during *L. aethiopica* infection

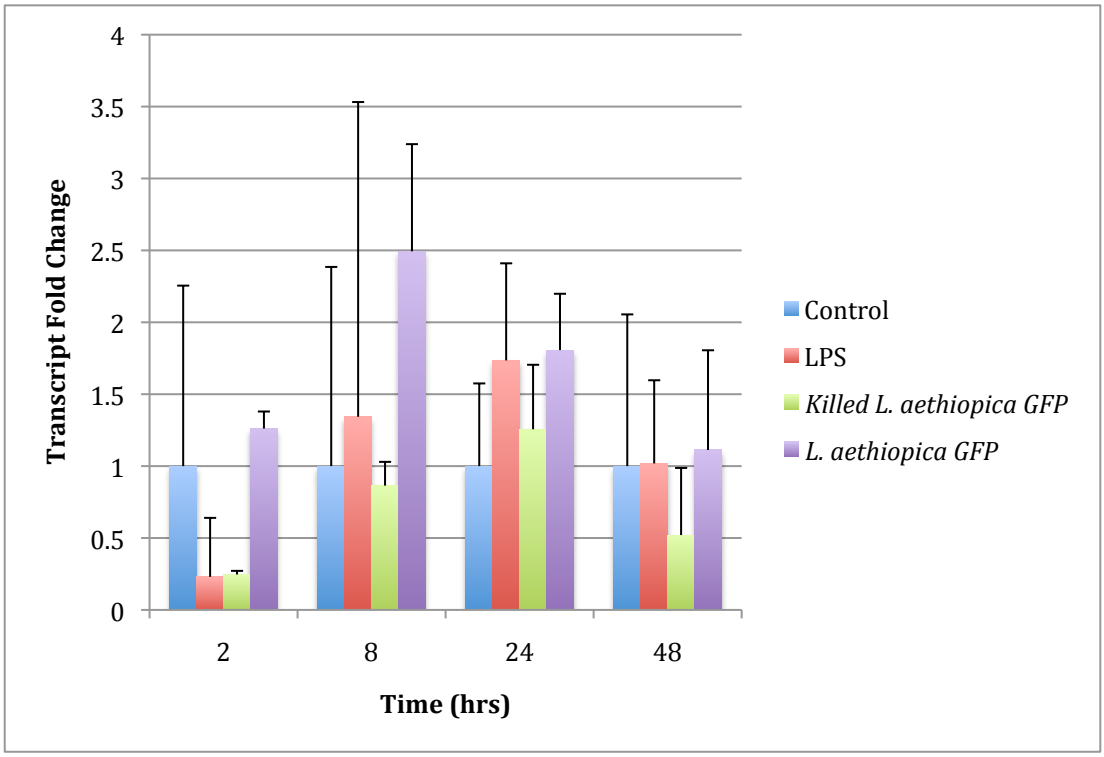


Figure 30: mRNA Expression levels of cathelicidin during *L. aethiopica* infection. All biological replicates (N2) cT values were compared by calculating the fold change (2^-ΔΔCt) against their respectively controls i.e. β-tubulin as the housekeeping gene control, cathelicidin as the gene of interest, control and experimental samples as the comparative cT value of either THP-1+RA (negative control), LPS (positive control), killed *L. aethiopica* GFP or *L. aethiopica* GFP. The SEM was generated through using the mean cT values against the negative control at that time point.

4.3.3.2 IL-1RA expression during *L. aethiopica* infection

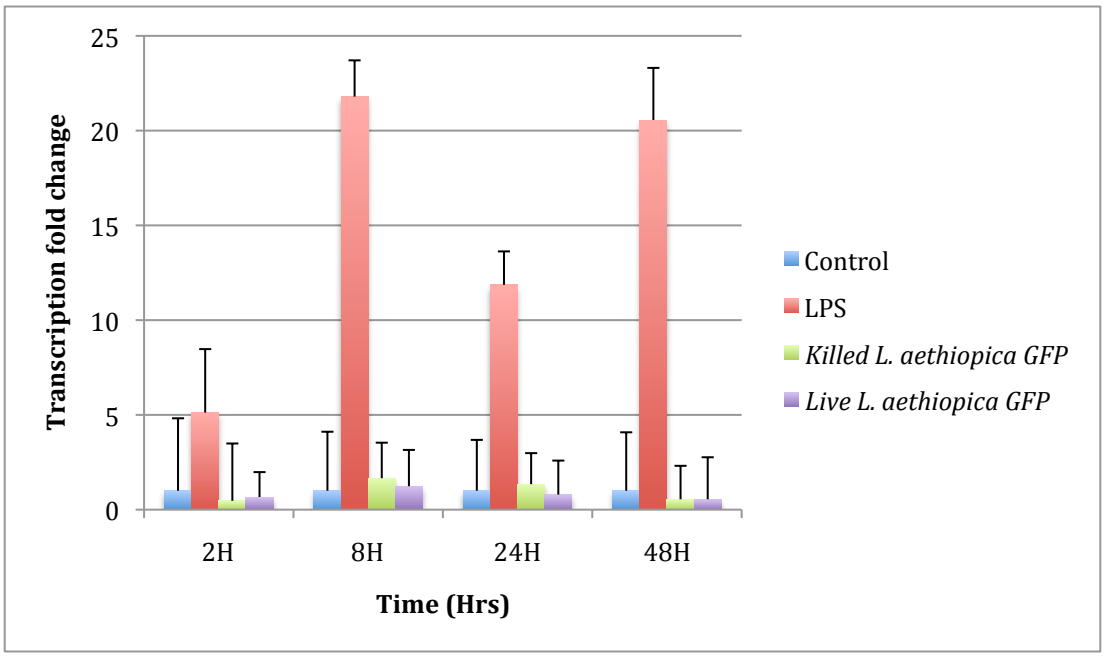


Figure 31: mRNA expression levels of IL-1RA during *L. aethiopica* infection**.**
All biological replicates (N2) cT values were compared by calculating the fold change (2^-ΔΔCt) against their respectively controls i.e. β-tubulin as the housekeeping gene control, IL-1RA as the gene of interest, control and experimental samples as the comparative cT value of either THP-1+RA (negative control), LPS (positive control), killed *L. aethiopica* GFP or *L. aethiopica* GFP. The SEM was generated through using the mean cT values against the negative control at that time point.

4.3.3.3 IL-10 expression during *L. aethiopica* infection

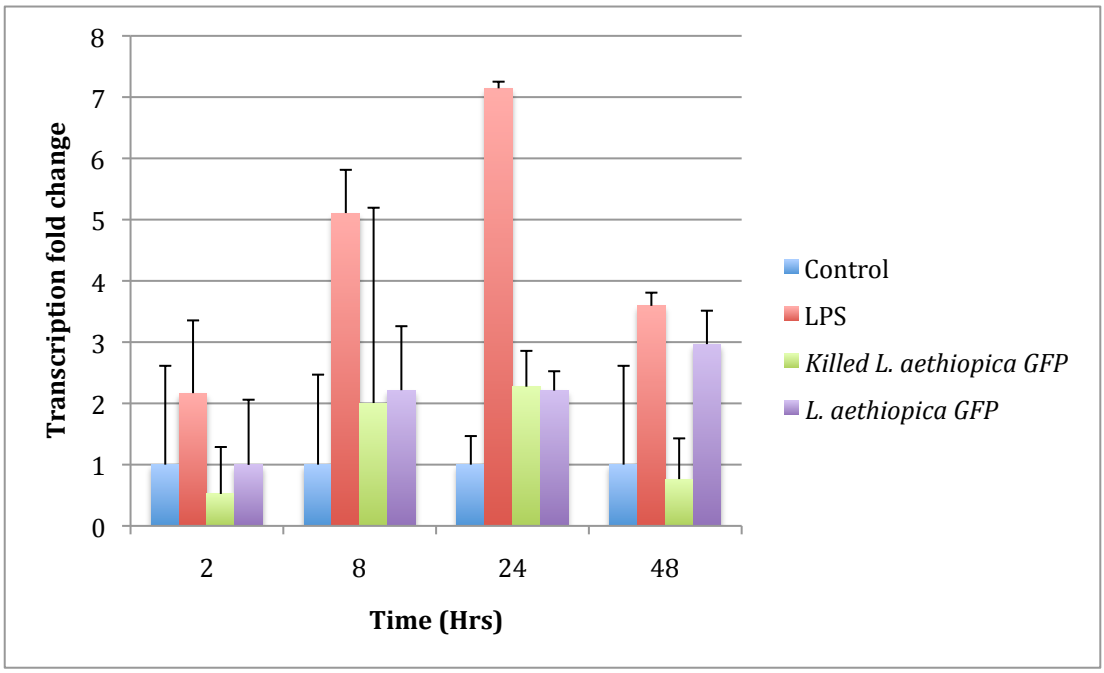


Figure 32: mRNA expression levels of IL-10 during *L. aethiopica* infection.
All biological replicates (N2) cT values were compared by calculating the fold change (2^-ΔΔCt) against their respectively controls i.e. β-tubulin as the housekeeping gene control, IL-10 as the gene of interest, control and experimental samples as the comparative cT value of either THP-1+RA (negative control), LPS (positive control), killed *L. aethiopica* GFP or *L. aethiopica* GFP. The SEM was generated through using the mean cT values against the negative control at that time point.

4.3.3.4 TGFβ expression during *L. aethiopica* infection

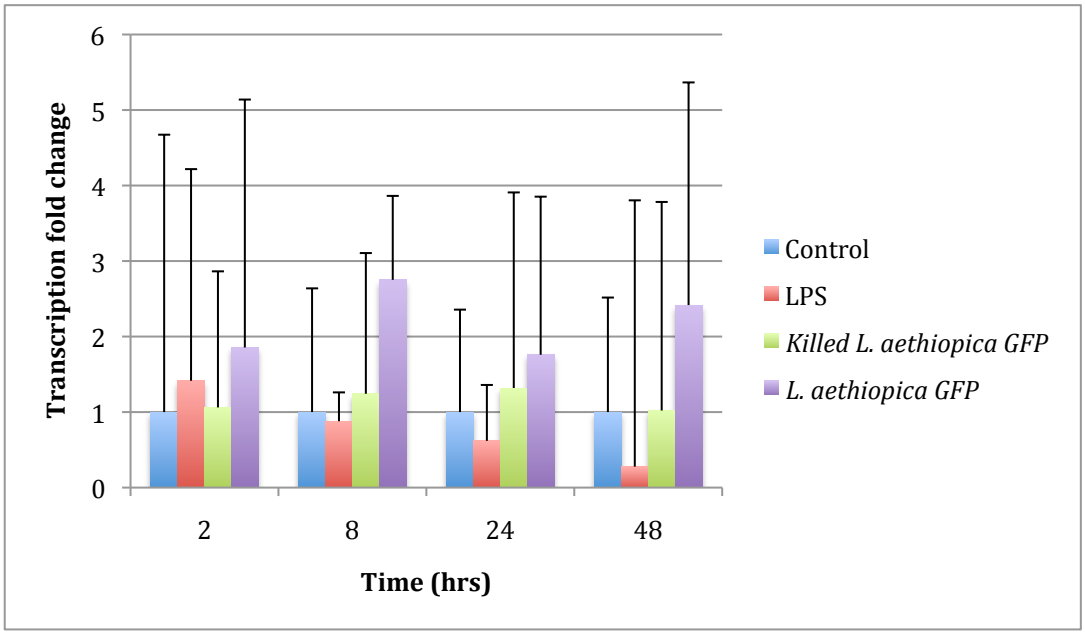


Figure 33: mRNA expression levels of TGF-β during *L. aethiopica* infection.
All biological replicates (N2) cT values were compared by calculating the fold change (2^-ΔΔCt) against their respectively controls i.e. β-tubulin as the housekeeping gene control, TGF-β as the gene of interest, control and experimental samples as the comparative cT value of either THP-1+RA (negative control), LPS (positive control), killed *L. aethiopica* GFP or *L. aethiopica* GFP. The SEM was generated through using the mean cT values against the negative control at that time point.

4.3.3.5 TNFα expression during *L. aethiopica* infection

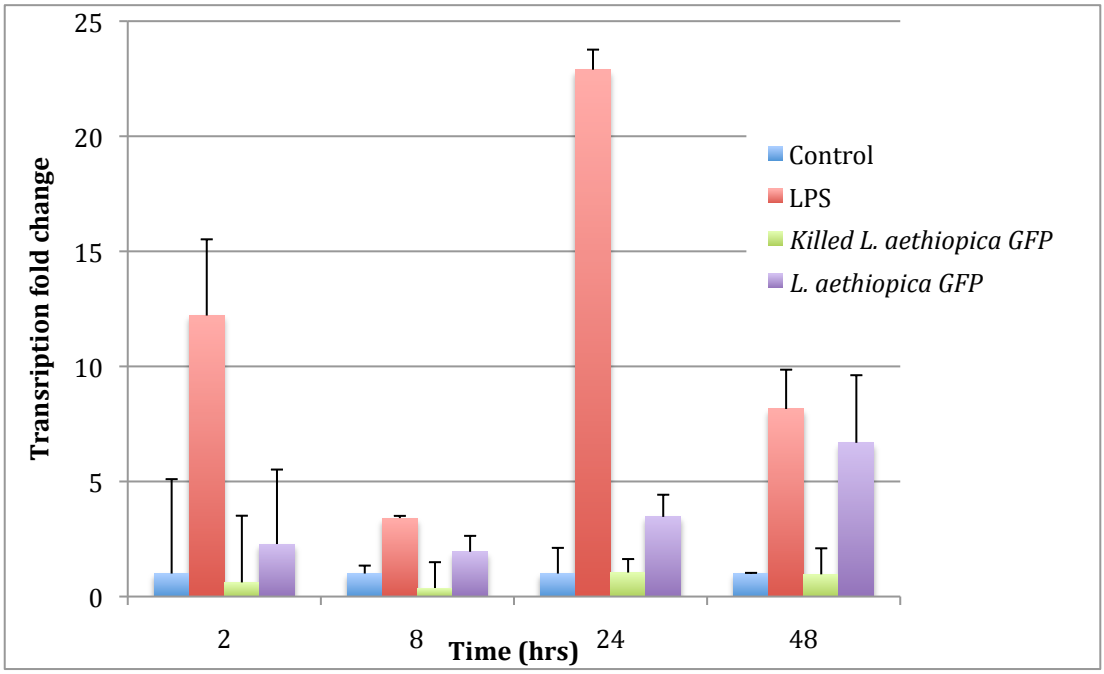


Figure 34: mRNA expression levels of TNFα during *L. aethiopica* infection.
All biological replicates (N2) cT values were compared by calculating the fold change (2^-ΔΔCt) against their respectively controls i.e. β-tubulin as the housekeeping gene control, TNFα as the gene of interest, control and experimental samples as the comparative cT value of either THP-1+RA (negative control), LPS (positive control), killed *L. aethiopica* GFP or *L. aethiopica* GFP. The SEM was generated through using the mean cT values against the negative control at that time point.

4.4 Discussion

4.4.1 Flow cytometric analysis of *L. aethiopica* infection

At all time points (2, 8, 24 and 48hrs) the levels of infection were tested using the Accuri C6 flow cytometer (Method 2.3.2.1). The percentages of infections (N=3) were similar to the previous infection data at 24 and 48 hours post infection (Chapter 3, Section 3.4.1.7 pp 90).

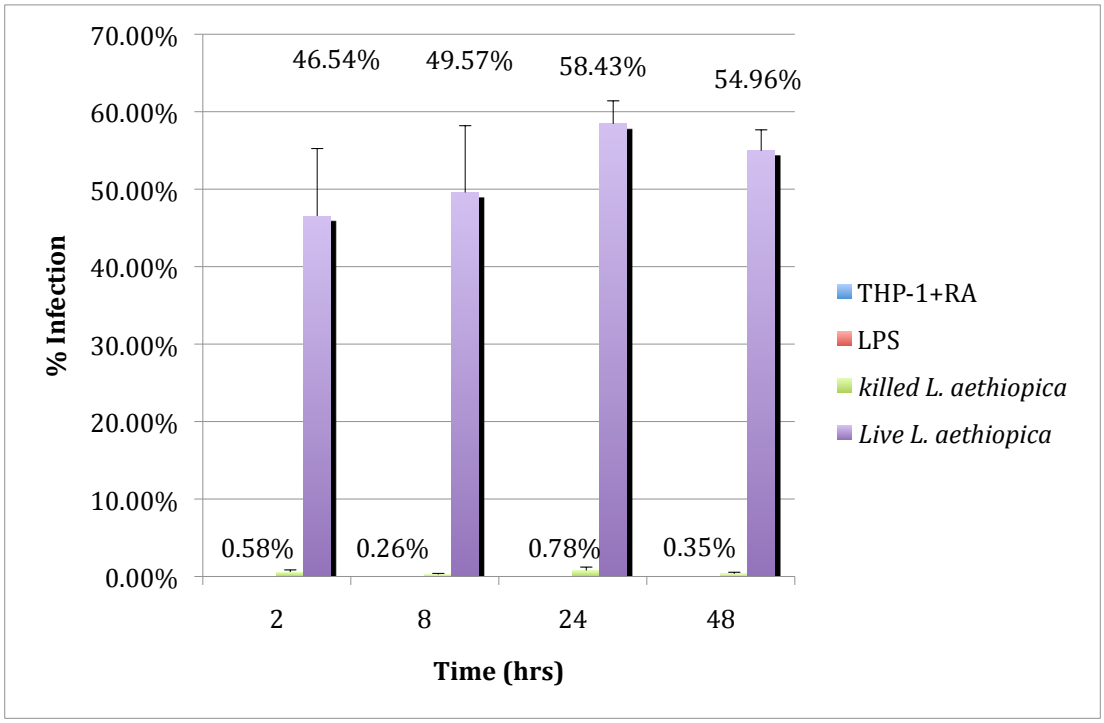


Figure 29: Percentage of *L. aethiopica* infection at the different time points.
All biological replicates (N3) were analysed using the Accuri C6 flow cytometer with a minimum of >2000 events recorded. GFP was only detected in the differentiated THP-1 cells infected with live and dead *L. aethiopica*. The mean, SEM and Wilcoxon signed rank test were calculated using Excel ($P<0.05$).

The flow cytometry data shows that only infection was detected with differentiated THP-1 infected with live *L. aethiopica*. The live parasite infection increases from 46.54 to its peak at 58.43% at 24hrs, which is then followed by a slight decline in its mean to 54.96%. It should be stated that only the internalisation of the parasite was observed at 24hrs post infection. The earlier stages of infection indicate permanent physical attachment of the parasite to the cell membrane. Additionally, residual GFP signal observed in dead *L. aethiopica* which ranged from 0.26-0.78%. This likely reflects the residual GFP protein detected by the FL1 filter within attached or internalised dead parasite in the differentiated THP-1 cells.

4.4.2 Optimisation of PCR

The identification of the optimal annealing temperatures for all PCR primers was required to before commencing the cytokine qPCR experiment. To identify the optimal annealing temperature for each pair of primers, an annealing temperature gradient (58-64^{o}C) was applied. After PCR, the products were run on a 1% TBE/agarose gel for 1hr 30 minutes (Appendix II). The products with the strongest signal were selected as optimal. All primers were designed to be 20 base pairs in length, have an annealing temperature of ~60^{o}C and a product size ~100-200 bp for successful amplification (Table 7). The only exception was for the cathelicidin primers, which were previously designed by Liu *et al* 2007.

Table 7: Annealing temperatures of the cytokine and cathelicidin primers designed

Primers	Sequence	Primer Length (bp)	GC (%)	Optimal Tm (^{O}C)	Product Size (bp)
IL-1ra Forward	GGAATCCATGGAGGGAAGAT	20	50	58	97
IL-1ra Reverse	TCTCGCTCAGGTCAGTGATG	20	55		
IL-10 Forward	TTACCTGGAGGAGGTGATGC	20	55	60	127
IL-10 Reverse	GGGAAGAAATCGATGACAGC	20	50		
TGFβ Forward	GAGCCTGAGGCCGACTACTA	20	60	58	131
TGFβ Reverse	CGGAGCTCTGATGTGTTGAA	20	50		
TNFα Forward	CAACCTCCTCTCTGCCATCAA	20	50	60	150
TNFα Reverse	ATAGTCGGGCCGATTGATCT	20	50		
Cathelicidin forward	GGACCCAGACACGCCAAA	18	61.1	60	52
Cathelicidin reverse	GCACACTGTCTCCTTCACTGTGA	23	52.1		
β-tubulin Forward	CTCTTCCAGCCTTCCTTCCT	20	55	60	116
β-tubulin Reverse	AGCACTGTGTTGGCGTACAG	20	55		

4.4.3 qPCR of cytokine expression during infection

To identify the changes in cytokine and cathelicidin mRNA expression, the qPCR was run on all the samples types (THP-1+RA – negative control, LPS – positive control, killed *L aethiopica*

GFP and *L. aethiopica* GFP). Comparative cT analysis was conducted to obtain the fold difference of expression of the gene of interest (cytokine type or cathelicidin) from the house-keeping gene (β-tubulin) providing the relative quantification. All cTs results for all mRNA expression targets were obtained from setting the threshold of 1.

4.4.3.1 Cathelicidin mRNA expression during *L. aethiopica* infection

This antimicrobial peptide was shown to increase from 2hr (>1.2 fold) to 8hrs (2.5 fold) and then gradually decreased towards 48 hours (>1 fold) post infection with *L. aethiopica* GFP (Figure 30). The positive control showed increased transcription from 2hrs to 24 hours and then decreased to 1 fold in line with the control expression level. There were low levels of transcription observed at 2hrs for LPS and killed *L. aethiopica* GFP treatment (<0.25 fold). In addition, the killed parasite treated cells showed increased mRNA expression after 24 hours followed by a decrease at 48 hours. Although the t test did not indicate any significance differences when comparing the mean cT values nevertheless the data suggests cathelicidin is up-regulated 8 hours post infection. The increase of cathelicidin transcription at 8hr post infection may indicate up-regulation of these innate antimicrobial peptide cathelicidin, which suggests the activation of the host innate immune response. The slow increase of mRNA expression of killed parasite and LPS treated THP-1 cells may indicate cathelicidin requires 8-24hrs to be successful induced by these factors. Interestingly, live parasite induces faster antimicrobial responses than LPS or dead parasite. However, the additional biological replicate would provide the sufficient support needed for statistical relevance. This preliminary study suggests for the first time *L. aethiopica* may cause an increase in cathelicidin expression at 8hrs, suggesting an involvement in the control of *Leishmania* infection.

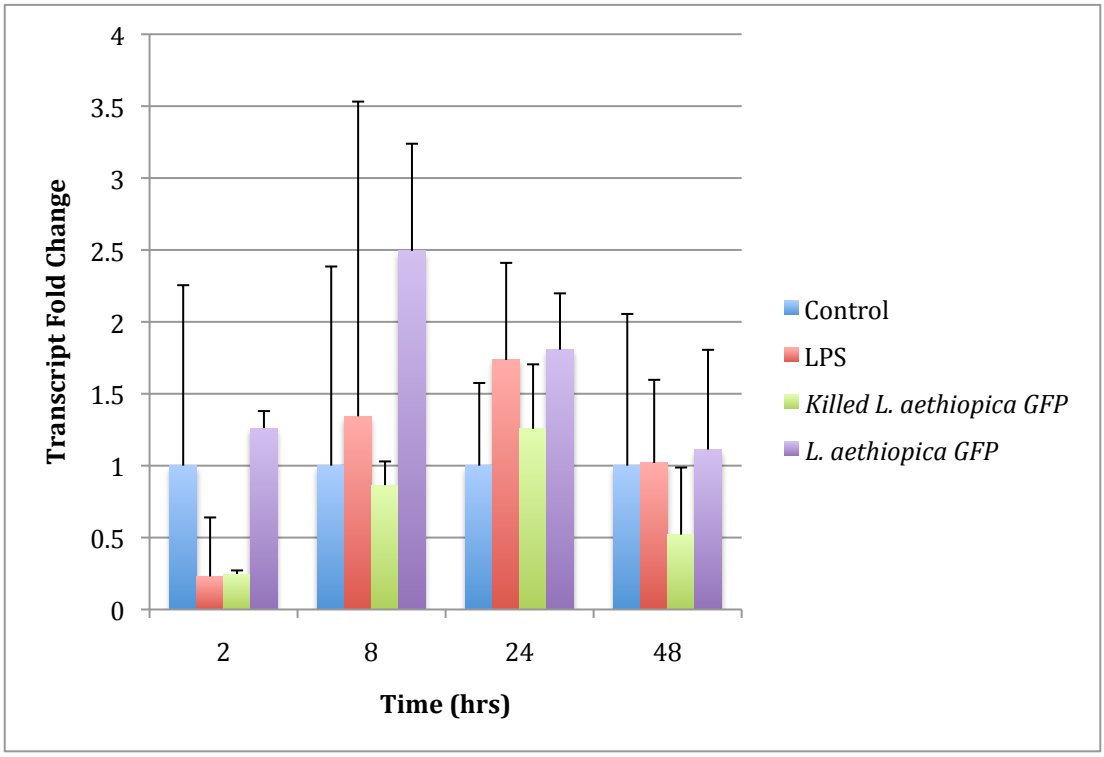


Figure 30: mRNA Expression levels of cathelicidin during *L. aethiopica* infection. All biological replicates (N2) cT values were compared by calculating the fold change (2^-ΔΔCt) against their respectively controls i.e. β-tubulin as the housekeeping gene control, cathelicidin as the gene of interest, control and experimental samples as the comparative cT value of either THP-1+RA (negative control), LPS (positive control), killed *L. aethiopica* GFP or *L. aethiopica* GFP. The SEM was generated through using the mean cT values against the negative control at that time point.

4.4.3.2 IL-1RA expression during *L. aethiopica* infection

The anti-inflammatory cytokine was weakly expressed in THP-1 cells treated with live and dead parasite. It is unclear why the expression was so low but I feel confident due to the positive signal obtained from the LPS treated cells. This anti-inflammatory cytokine was weakly expressed by the killed and live *L. aethiopica* GFP parasites at all time points. The lack of IL-1RA up regulation at any time point was surprising but may be explained by the fact that inflammatory cytokines like TNFα were not induced until 48hrs post infection (Figure 34). However, the levels of IL-1RA during the LPS treatment dropping at 24 hours when it was >20 at 8 and 48 hours post treatment is unclear. These results reveal the transcription levels of IL-1RA did not vary from the negative control when treated with live or dead parasite and only strong signal was observed when LPS was applied.

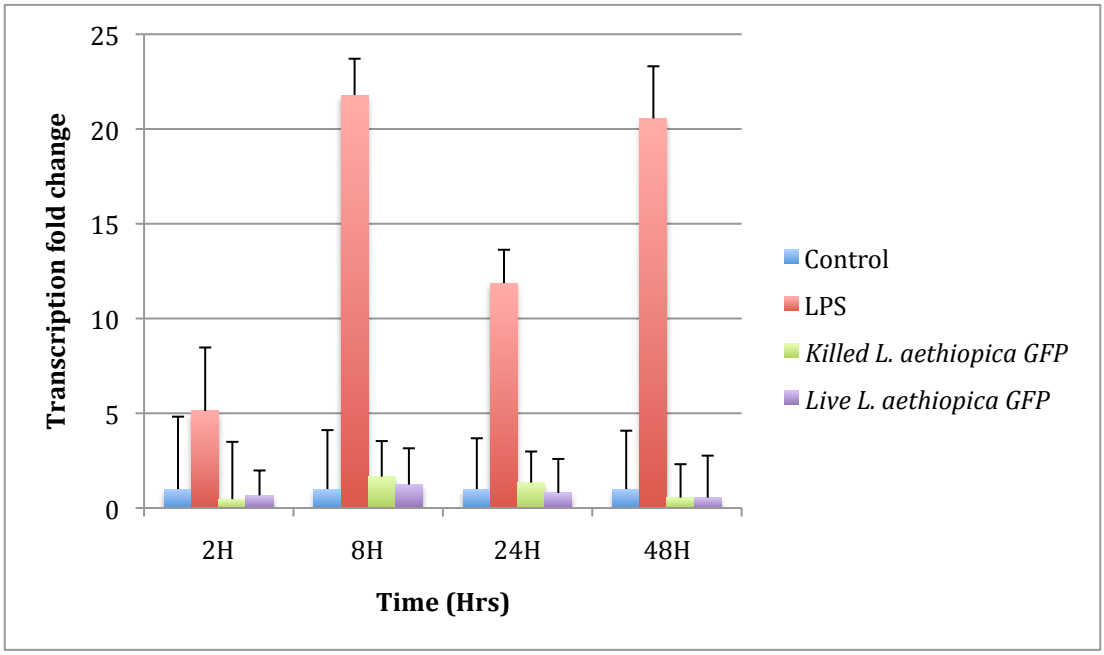


Figure 31: mRNA expression levels of IL-1RA during *L. aethiopica* infection**.**
All biological replicates (N2) cT values were compared by calculating the fold change (2^-ΔΔCt) against their respectively controls i.e. β-tubulin as the housekeeping gene control, IL-1RA as the gene of interest, control and experimental samples as the comparative cT value of either THP-1+RA (negative control), LPS (positive control), killed *L. aethiopica* GFP or *L. aethiopica* GFP. The SEM was generated through using the mean cT values against the negative control at that time point.

4.4.3.3 IL-10 expression during *L. aethiopica* infection

This anti-inflammatory cytokine transcription was shown to increase from 2hr (1-fold) to 48 hours (3-fold) when cells were infected with *L. aethiopica* GFP. This was as expected as over time a greater anti-inflammatory response would be beneficial to the parasites' survival within macrophages. The dead parasite showed a similar effect up to 24hrs but afterwards (at 48 hours) the levels decreased below the control (<1 fold). No differences were observed between the live and dead parasite at 8 and 24 hours post treatment. The treatment of LPS on the THP-1 cells showed strong transcript signal by increasing from 2-fold at 2 hours, peaking at 24 hours at >7-fold and later decreasing to >3.5-fold. IL-10 transcription peaked between 8 and 24hrs after LPS treatment, which is consistent with previous publications (Lepara and Kelly 2010). This confirmed LPS as a good inducer of IL-10 when in THP-1 cells. The results reveal that the dead parasites may provide an increase in the transcription on IL-10 up to 24 hours similar to live

parasites. The ability of the live parasites to increase IL-10 transcription from 2-48 hours is beneficial for the protozoan survival. IL-10 is known to be involved in inhibiting the elimination of infected macrophages via a M1 response.

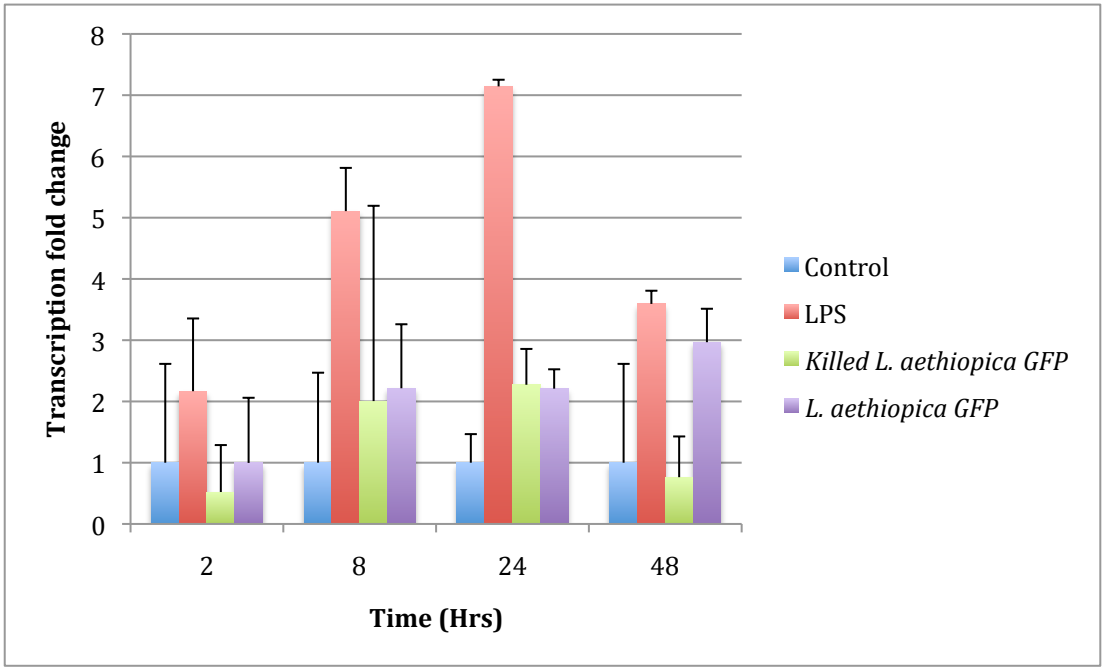


Figure 32: mRNA expression levels of IL-10 during *L. aethiopica* infection.
All biological replicates (N2) cT values were compared by calculating the fold change (2^-ΔΔCt) against their respectively controls i.e. β-tubulin as the housekeeping gene control, IL-10 as the gene of interest, control and experimental samples as the comparative cT value of either THP-1+RA (negative control), LPS (positive control), killed *L. aethiopica* GFP or *L. aethiopica* GFP. The SEM was generated through using the mean cT values against the negative control at that time point.

4.4.3.4 TGFβ expression during *L. aethiopica* infection

TGFβ is potent anti-inflammatory cytokine, which causes the down regulation of Th1 cytokines including IL-1, IL-12 and TNF (Duque and Descoteaux 2014). This cytokine expression is increased in infected cells at all stages of infection with live parasites while infection with dead parasites has no impact on TGFβ expression as its mean fold change is similar to the negative control. The mean fold change during infection from 1.75 to >2.75, but it is unclear why the levels dropped at 24 hours post treatment to >1.75 fold. TGFβ mRNA expression during LPS treatment was highest at 2 hours (>1.4) and afterwards decreased to 48 hours (0.27 fold). The

THP-1 cells treated with killed parasite showed minimal increase from 2-24 hours (>1 to >1.3) and then decreased at 48hours similar to the control at >1. This cytokine is implicated in the activation of macrophages towards M2 responses, which is important for survival of the parasite (Gong *et al*., 2012).

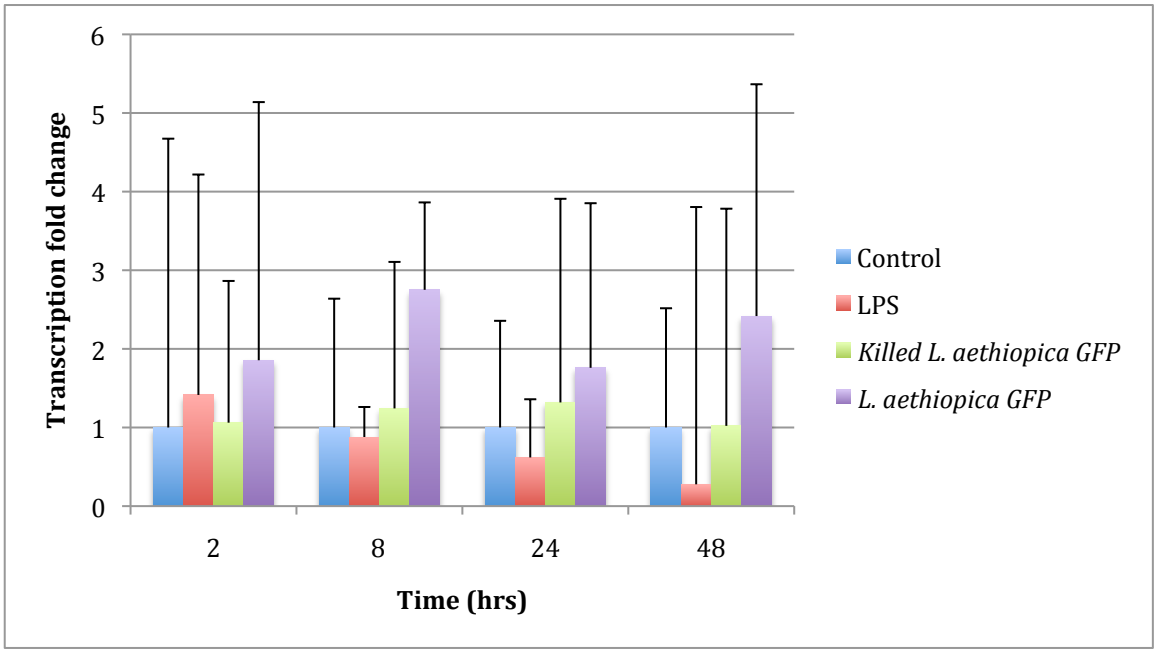


Figure 33: mRNA expression levels of TGF-β during *L. aethiopica* infection.
All biological replicates (N2) cT values were compared by calculating the fold change (2^-ΔΔCt) against their respectively controls i.e. β-tubulin as the housekeeping gene control, TGF-β as the gene of interest, control and experimental samples as the comparative cT value of either THP-1+RA (negative control), LPS (positive control), killed *L. aethiopica* GFP or *L. aethiopica* GFP. The SEM was generated through using the mean cT values against the negative control at that time point.

4.4.3.5 TNFα expression during *L. aethiopica* infection

The transcription of this cytokine increased when the THP-1 cells were infected with *L. aethiopica* GFP from >2.2 at 2hours to >6.6 fold at 48 hours. Comparatively, the cells treated with dead parasites indicated low levels of mRNA expression with the highest fold at >1 at 24 hours. Lapara & Kelly, who worked on infection with different *Leishmania spp.* infection (*L.*

major and *L. amazonensis*) showed that TNFα expression peaked at 8hrs. This may highlight differences on the modulative effect that different species of *Leishmania spp*. may have on the host cell. LPS treated cells showed a good level of expression at all time points (>12.2 at 2hrs, >3.4 at 8 hours, >22 at 24 hours and >8 at 48 hours). It is unclear why the levels decreased at 8 and 48 hours post treatment and that the previous studies show TNFα expression peaking at 8hrs whereas this study has shown good levels of expression at all time points (Lapara and Kelly 2010). This may highlight subtle differences of TNFα expression between mice and human macrophage cell lines. The slow increase of TNFα mRNA expression from 2 to 48 hours post treatment is suggesting an inflammatory response resulting from the parasite infection. It is unclear why this cytokine is increasing up to 48 hrs as this pattern is similar to the anti-inflammatory cytokine IL-10. This may represent an example where not all the mRNA TNFα transcripts are being translated into protein.

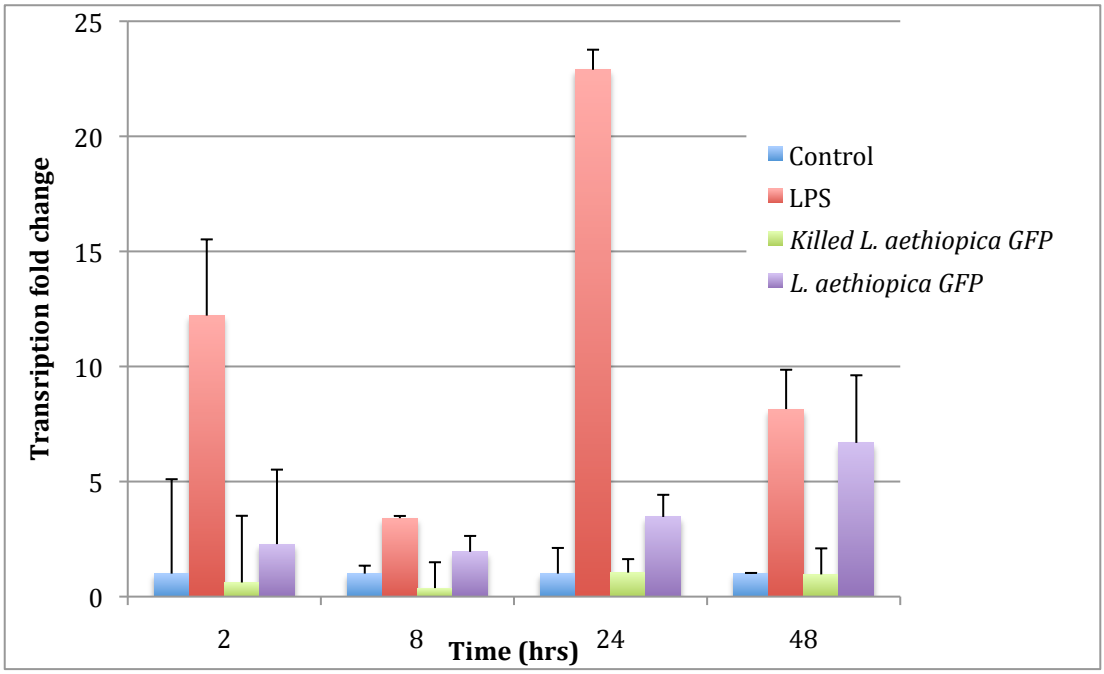


Figure 34: mRNA expression levels of TNFα during *L. aethiopica* infection.
All biological replicates (N2) cT values were compared by calculating the fold change (2^-ΔΔCt) against their respectively controls i.e. β-tubulin as the housekeeping gene control, TNFα as the gene of interest, control and experimental samples as the comparative cT value of either THP-1+RA (negative control), LPS (positive control), killed *L. aethiopica* GFP or *L. aethiopica* GFP. The SEM was generated through using the mean cT values against the negative control at that time point.

4.5 Conclusions

This investigation has shown that *L. aethiopica* infection influence mRNA expression of inflammatory and anti-inflammatory cytokines. Past literature reveal a clear link between the expression of cytokines and antimicrobial peptides for successful clearance of infection. This is the first study looking at a range of time points from 2-48 hours post infection allowing an overview of expression changes over time. However, the time points indicated how the transcription varied for the mRNAs of interest. The cathelicidin transcription peaked at 8 hours during infection may indicate its importance as part of the early innate immune response against *Leishmania* parasites. However, the poor mRNA expression of IL-1RA may indicate this as not an important anti-inflammatory cytokine during the infection process. The gradual increased expression of IL-10 of infected cells could highlight the importance of time and the parasites' influence from attachment (2-8 hours) to becoming internalised (post 24 hours infection). The similar IL-10 transcription of the dead and live parasites between 8 and 24 hours post infection may indicate innate immunological factors *Leishmania* possess. If infection and an inflammatory effector such as LPS were used at the same time the anti-inflammatory effect by IL-10 may have become more profound. The mRNA expression of TGFβ during infection is likely to result in the M2 activation leading the survival of the parasites. The gradual increased transcription of TNFα may indicate the lack of the parasites' ability to down regulate such an inflammatory pathway or that the transcripts are not being translated into proteins. The main consensus that inflammatory cytokines are expressed early on and decrease after *Leishmania* parasites have established may not always follow. Unfortunately, as this study required a further biological replicate is difficult to comprehensively conclude the findings. This chapter on mRNA expression leads onto the next one investigating changes in protein expression during infection with *L. aethiopica* and *L. mexicana*.

Chapter 5

Does *L. aethiopica* and *L. mexicana* affect protein expression during infection?

Novelty statement

This study is the first example where the proteomic levels in macrophages have been altered post 24hrs infection with *L. aethiopica* and *L. mexicana*. The up-/down-regulation of proteins showed similarities between the two *Leishmania* species but also differences (no downregulation observed during *L. aethiopica* infection). This suggests subtle differences of protein expression during infection. The proteins affected by both *Leishmania* species were identified as knowingly to be involved in attachment & endocytosis (Actin), immunological receptors (MHC I), inhibition of apoptosis (ribosome S2), reduction of intracellular ROS (Metallothenein), antiparasitic, inflammation, Th2 polarisation (ribonuclease inhibitors).

Impact

This investigation is first time where the proteomic levels in macrophages have been altered post 24hrs infection with *L. aethiopica* and *L. mexicana*. The up-/down-regulation of proteins showed similarities between the two *Leishmania* species but also differences (no downregulation observed during *L. aethiopica* infection). This suggests subtle differences of protein expression during infection.

5.1 Introduction

The entrance of *Leishmania* parasites into host macrophages is only the beginning of the infection process. Promastigotes need to establish themselves inside the parasitiophorous vacuole and survive lysis following phagolysomal fusion in order to survive inside the human host. Studies involving the infection of murine macrophages with *L. amazonensis* have shown

increases in infected cells and high levels of parasitic replication within the parasitophorous vacuole (Soong 2012). It has been proposed that this dilutes the effect of NO and ROS when infected with *L. amazonensis* or *L. mexicana* (Wilson *et al*., 2008). This would facilitate the continual transmission through subsequent parasitic inoculation in a sandfly during the next blood meal. *Leishmania* have adapted several mechanisms to manipulate the human immune system through immunosuppression or promotion of advantageous parasitic factors inside host macrophages (Oliver *et al*., 2005). Molecular tactics implemented by *Leishmania* include suppression of antimicrobial NO, manipulation of critical pathways including selective induction/suppression of apoptosis, cytokines manipulation and activation of T lymphocytes (Proudfoot *et al*., 1995; Forget *et al*., 2001; Moore *et al*., 1994; Carrera *et al*., 1996; Reiner 1987). Previous work using quantitative proteomics using THP-1 infected with the visceral causative agent *L. donovani* has shown up-/down-regulation of proteins associated with 1) signal transduction, 2) apoptosis, cell death, and differentiation, 3) immune and inflammatory responses and 4) chromatin remodelling and RNA splicing (Singh *et al*., 2015). This investigation used a similar analytical technique (TMT with LC-MS/MS) to identify protein changes (i.e. up-regulation with ≥1.5 and down regulation with ≤0.5) at 24 hours post infection. Singh *et al* revealed up-regulation was observed with camp-responsive element binding protein 3-like protein 4, coronin 1C, high-motility-group protein (HMG-I/HMG-Y), mitochondrial antiviral signalling protein and histone H4. Less abundant expression was seen with the 78-kDa glucose-regulated protein and heterogeneous nuclear ribonucleoprotein K (Singh *et al*., 2015). Selection of such proteins was made possible by an isobaric tagging (iTRAQ and LC-MS/MS) approach where protein samples had undergone treatment (precipation, protein dissolving, denaturation, reduction and tryptic digestion) and iTRAQ labelling (-114, -115, -116, -117) of peptides. The differentially labelled samples were pooled together and fractions separated by strong-cation-exchange (SCX) chromatography with a high-performance liquid chromatography

(HLPC) system (1200 Agilent series) with a UV detector. After the peptide elutions were collected and further separated by reverse-phase chromatography (Nano-LC column, Agilent Technologies, Germany) the samples were analysed using a hybrid quadrupole linear ion trap (LIT) mass spectrometer (4000 Q Trap LC-MS/MS system, AB Sciex). In this study a similar approach was applied where after producing the protein solutions at 24 hours post infection, the samples were sent to Bristol University to process and analysis using TMT and LC-MS/MS.

The aim of this chapter is identify proteins which are changed/regulated following parasitic infection with *L. aethiopica* and *L. mexicana*. The human monocytic cell line, THP-1 was used and comparative proteomics was conducted between cells infected with each of the two species and within the past literature. The use of the THP-1 monocytic cell line as a model of *Leishmania* infection is well established (Getti *et al*., 2008). Infected samples were analysed 24 hours from infection both via flow cytometry to detect the percentage of infected cells and by TMT LC-MS/MS.

5.2 Methods

The cell culture practices for THP-1 and all *Leishmania* promastigotes were carried as previously mentioned (Methods 2.1.1 pp. 32, 2.1.2 pp. 32, 2.1.5 pp. 34, respectively). The purification of metacyclic promastigotes, infection of THP-1 cells and the flow cytometric analysis was carried as seen in methods 2.3.1 pp. 43, 2.3.2 pp. 43 and 2.3.2.1 pp. 43. All proteomic extraction, quantification and analysis were carried out as previously mentioned (Methods 2.5 pp 56, 2.5.1 pp 56, 2.5.2 pp 57 and 2.5.3 pp 57).

5.3 Results

5.3.1 Percentage of *Leishmania* infection post 24 hours

Table 8: Infection of differentiated THP-1 cells post 24hrs.

Leishmania spp.	Replicates	% Infection	Mean % infection	SD	Significance
L. mexicana (M5G clone)	Replicate 1	39.20%	39.13%	4.90	P>0.05
	Replicate 2	34.20%			
	Replicate 3	44.00%			
L. aethiopica (L8G clone)	Replicate 1	43.30%	41.57%	4.74	P>0.05
	Replicate 2	36.20%			
	Replicate 3	45.20%			
Controls (THP-1+RA)	Replicate 1	0.00%	0.00%	0.000	n/a
	Replicate 2	0.00%			
	Replicate 3	0.00%			

All biological replicates (N=3) were analysed by flow cytometry with a minimum of >2000 events tested to identify the percentage of infection at 24hrs. The means, standard deviations and Wilcoxon signed rank test was performed using Excel (P>0.05).

5.3.2 Protein quantification

To quantify the concentrations of all protein samples a standard BCA assay with known concentrations of BSA was set up to create an excel graph (Figure 35). This was important in order to quantify the protein lysates from all the samples (negative control - THP-1+RA, and infected THP-1 with *L. mexicana* and *L. aethiopica*. The linear line equation (Figure 35) was used to calculate the absorbance values into protein concentrations for all protein samples generated. All protein samples were run with the BCA assay and the concentrations of proteins were identified to range from 3.63 to >9.00μg/ml (Table 9).

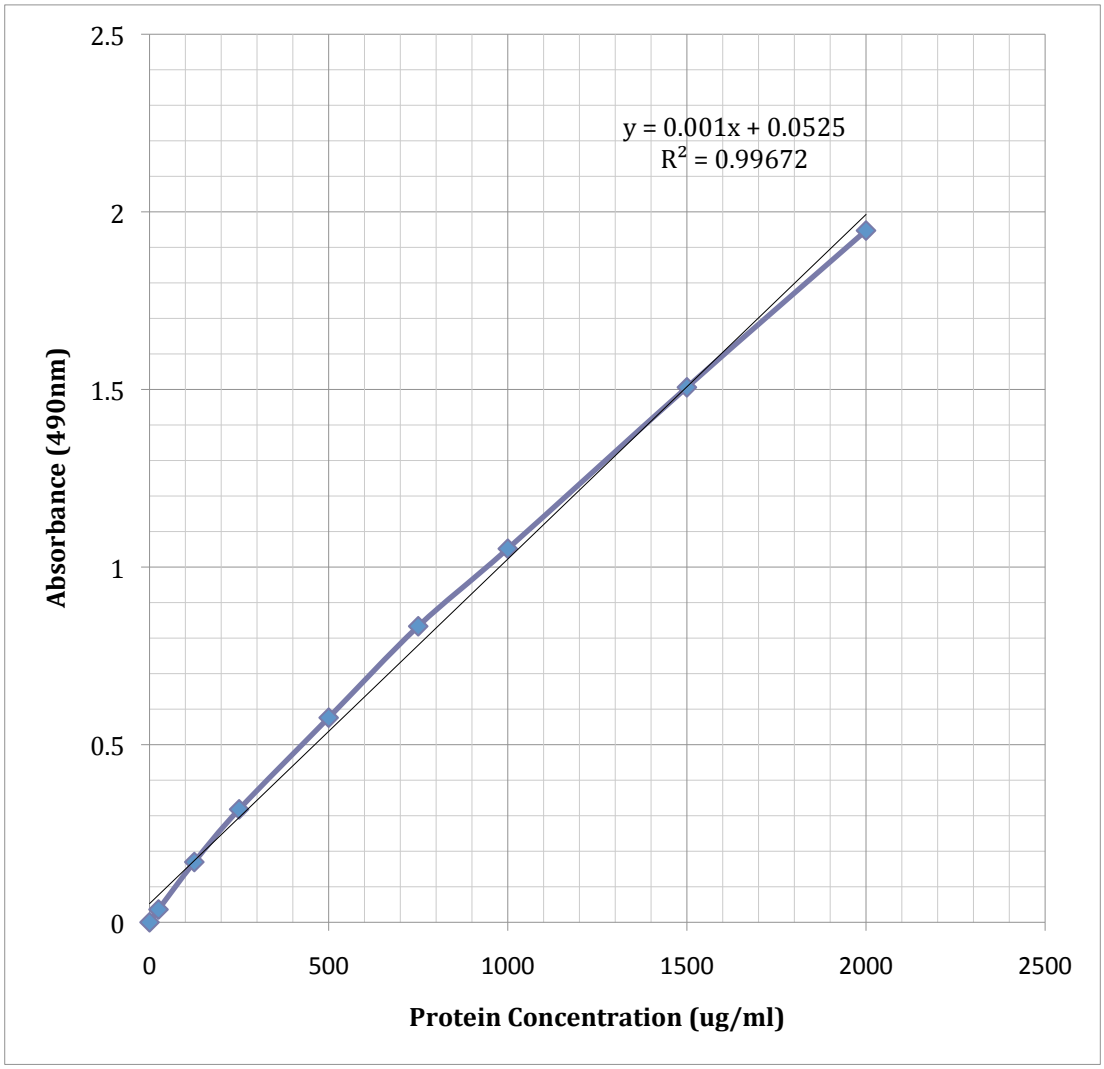


Figure 35: Linear curve obtained from protein standard quantified by BCA assay.

Table 9: Protein concentration of infected and non-infected RA-treated THP-1 cells.

Leishmania spp.		Absorbance (540nm)			Mean	Protein Concentration (µg/ml)
M5G	Replicate 1	0.377	0.427	0.454	0.419	3668.33
	Replicate 2	0.549	0.541	0.529	0.540	4871.67
	Replicate 3	0.528	0.694	0.726	0.649	5968.33
L8G	Replicate 1	0.995	0.94	0.933	0.956	9035.00
	Replicate 2	0.824	0.796	0.793	0.804	7518.30
	Replicate 3	0.688	0.537	0.563	0.596	5435.00
Control	Replicate 1	0.563	0.671	0.589	0.608	5551.67
	Replicate 2	0.99	0.826	0.957	0.924	8718.33
	Replicate 3	0.436	0.562	0.607	0.535	4825.00

5.3.3 Up-regulation of proteins during *Leishmania* infection

The comparative proteomic study was carried out against the non-infected differentiated THP-1 cells. All protein scores ≥ 1.5 showed statistically significant differences when compared against non-infected cells. The results highlight that 8 proteins are up regulated during infection with both *Leishmania spp* (Table 10). The biological importance of this will be mentioned in the Discussion.

Table 10: Proteins over expressed 24hrs post infection with *Leishmania spp.*

Protein Name	Post 24hr infection				
	Accession	*L. mexicana*		*L. aethiopica*	
		Score (Mean)	SD	Score (Mean)	SD
Actin	E7EVS6	20.61	10.71	-	-
Heat shock cognate 71 kDa protein	E9PN89	10.68	9.28	-	-
MHC class I antigen	F6KRQ8	5.51	5.23	22.85	27.36
Epididymis luminal protein 176	V9HVZ7	22.20	34.75	16.62	3.99
Coiled-coil domain-containing protein 68	K7EN04	-	-	15.25	10.29
Ribosomal protein S2	Q8NI62	-	-	3.30	0.85
Metallothionein-1H	P80294	-	-	5.25	1.64
Ribonuclease inhibitor	H0YCR7	-	-	5.67	4.33

All biological replicates (N3) were aligned with their identical protein accession name. All over expressed proteins tabulated are statistical significant at 95% confidences. This was maintained by only analysing protein scores ≥1.5.

5.3.4 Under expression of proteins during infection with *L. aethiopica*

A reduction of protein expression was only identified in differentiated THP-1 cells infected with *L. aethiopica*. From this data, 10 proteins were found whose expression was influenced by the parasite when compared from the non-infected control (Table 12). The under expression statistical confidence is based on the stringent analysis carried by the proteomic department at the University of Bristol who informed us that all samples ≤0.5 has a P value ≤0.05 with 95% confidence. The biological importance of this will now be discussed.

Table 11: Proteins under expressed 24hrs post-infection with *Leishmania spp*.

Protein Name	Post 24hr infection		
	Accession	*L. aethiopica*	
		Score (Mean)	SD
HCV p7-transregulated protein 3	Q2V8N0	0.43	0.07
40S ribosomal protein S30	P62861	0.21	0.23
Tumor necrosis factor alpha-induced protein 8-like protein 2 (TP8L2)	Q6P589	0.34	0.21
Ribonuclease T2	D6REQ6	0.41	0.06
Synaptopodin 2	B9EG60	0.4	0.1
Catechol-O-methyltransferase	F2WW55	0.2	0.22
Cyclin-dependent kinases regulatory subunit	Q5T179	0.27	0.16
Phospholipase D4	G3V3J8	0.25	0.15
Epididymis secretory protein Li 55	V9HW35	0.31	0.12
Cytochrome c oxidase subunit 6C	P09669	0.27	0.11

All biological replicates (N3) were aligned with their identical protein accession name. All under expressed proteins tabulated are statistical significant at 95% confidences. This was maintained by only analysing protein scores ≤0.5.

5.4 Discussion

5.4.1 Percentage of *Leishmania* infection post 24 hours

The percentage of infection after 24hours was consistent with data previously repeated and ranged from 34.2 to 44% for *L. mexicana* and 36 to 45.2% for *L. aethiopica* (Table 8). A Wilcoxon signed rank test was used against the *L. mexicana* and *L. aethiopica* infected cells identifying its statistical significance ($P>0.05$ for $N=3$).

Table 8: Infection of differentiated THP-1 cells post 24hrs

Leishmania spp.	Replicates	% Infection	Mean % infection	SD	Significance
L. mexicana (M5G clone)	Replicate 1	39.20%	39.13%	4.90	P>0.05
	Replicate 2	34.20%			
	Replicate 3	44.00%			
L. aethiopica (L8G clone)	Replicate 1	43.30%	41.57%	4.74	P>0.05
	Replicate 2	36.20%			
	Replicate 3	45.20%			
Controls (THP-1+RA)	Replicate 1	0.00%	0.00%	0.000	n/a
	Replicate 2	0.00%			
	Replicate 3	0.00%			

All biological replicates (N=3) were analysed by flow cytometry with a minimum of >2000 events tested to identify the percentage of infection at 24hrs. The means, standard deviations and Wilcoxon signed rank test was performed using Excel.

5.4.2 Protein quantification

To quantify the concentrations of all protein samples a standard BSA assay with known concentrations was set up to create an excel graph (Figure 35). The linear line equation (Figure 35) was used to calculate the absorbance values into protein concentrations for all protein samples generated. All protein samples were run with the BSA assay and the concentrations of proteins were identified to range from 3.63 to >9.00μg/ml (Table 9).

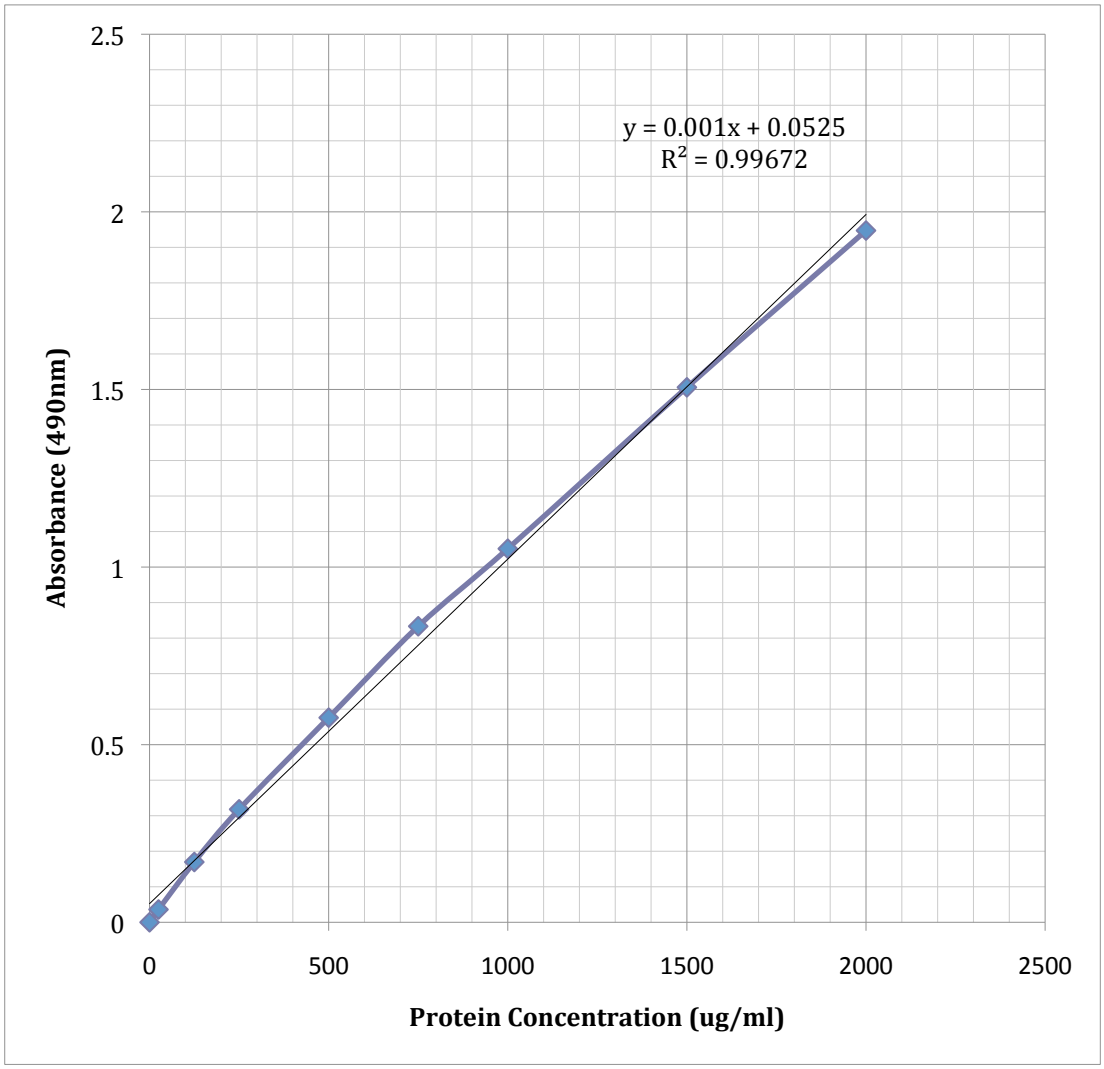


Figure 35: Linear curve obtained from protein standard quantified by BCA assay.

Table 9: Protein concentration of infected and non-infected RA-treated THP-1 cells

Leishmania spp.		Absorbance (540nm)			Mean	Protein Concentration (µg/ml)
M5G	Replicate 1	0.377	0.427	0.454	0.419	3668.33
	Replicate 2	0.549	0.541	0.529	0.540	4871.67
	Replicate 3	0.528	0.694	0.726	0.649	5968.33
L8G	Replicate 1	0.995	0.94	0.933	0.956	9035.00
	Replicate 2	0.824	0.796	0.793	0.804	7518.30
	Replicate 3	0.688	0.537	0.563	0.596	5435.00
Control	Replicate 1	0.563	0.671	0.589	0.608	5551.67
	Replicate 2	0.99	0.826	0.957	0.924	8718.33
	Replicate 3	0.436	0.562	0.607	0.535	4825.00

5.4.3 Up-/down-regulation of proteins during *Leishmania* infection

The TMT LC-MS/MS based protein investigation revealed that 5927-7069 proteins were identified from the biological replicates (R1 – 6969, R2 – 7069, R3 – 5927). From the identified proteins only 18 revealed up or down regulation expression that was of significance, i.e. ≥1.5 or ≤0.5 fold difference when compared to the un-infected differentiated THP-1 cell control ($P \leq 0.05$, N=3). From these 18 proteins, 8 were identified as over expressed by the two species of *Leishmania* (*L. mexicana* and *L. aethiopica*) and 10 were down regulated only when infected with *L. aethiopica* (Tables 10 & 11, pp.123 & 125).

5.4.3.1 Over expression of proteins

The protein levels between the two *Leishmania spp*. show varied elevation of many proteins (Table 12, pp 123). The MHC I antigen (>5.5 & >22.8 ratios) and Epididymis luminal protein 176 (>22.2 & >16.6) increased when infected with both *L. mexicana* and *L. aethiopica*, respectively. In addition to the proteins previously mentioned increased expression was observed with actin (>20 ratio) and heat shock cognate 71 kDa protein (>10.6 ratio) during *L. mexicana* infection. Infected cells with *L. aethiopica* showed elevated expression levels with Coiled-coil domain-containing protein 68 (>15.2), ribosomal protein S2 (>3.3), Metallothionein-1H (>5.2) and Ribonuclease inhibitor (>5.6).

Table 10: Summarised role of all proteins up regulated during infection.

Protein Name	Roles	Reference
Actin gamma	cell motility, cytokinesis and endocytosis	Hansen *et al*., 2013
Heat shock cognate 71 kDa protein	associated with heat shock factor cochaperones; binds to polypeptides contained abnormal amino acids; dissociate clathrin form clathrin coats; promote lysosomal degradation of cytosol proteins; contains signal peptides, which allow its nucleolar or its cytoplasmic localisation; expressed in non-malignant mammary cells as well as breast cancer cells; overexpression of Hsp/hsc70 in chemoresistant cancer cells	Morimoto 1998; Rothman and Schmid 1986; Chiang *et al*., 1989; Tsukahara and Maru 2004; Soulier *et al*., 1996; Vargas-Roig *et al*., 1998; Kao *et al*., 2003 ; Nirdé *et al*., 2010
MHC class I antigen	Critical for antigen presentation to CD8 T cells; present on most nucleated cells	Grommé and Neefjes 2002; Townsend *et al*., 1988; Bjorkman *et al*., 1987a; Bjorkman *et al*., 1987b
Epididymis luminal protein 176	aka Actin gamma (See above)	Hansen *et al*., 2013
Coiled-coil domain-containing protein 68 (CCDC68)/cutaneous T-cell lymphoma-associated antigen Se57-1 (CTCL-associated antigen se57-1)	T cell associated lymphoma antigen identified as a tumour-specific antigen in colorectal cancer	Eichmuller *et al*., 2001 Gerhardt *et al*., 2004
Ribosomal protein S2	Part of the translation machinery for protein synthesis	Aseev *et al*., 2008
Metallothionein-1H	involved in cell proliferation and apoptosis; selectively bind to heavy metals (zinc, copper, cadmium); heavy metal detoxification; free radical scavenging; implicated in resistance to anti-cancer drugs induced by stress including toxic heavy metals, glucocorticoid, LPS, IL-1, α IFN	Miles *et al*., 2000 Kägi and Schäffer 1988 Jayasurya *et al*., 2000 Roesijadi 2000 Ebadi *et al*., 1996
Ribonuclease inhibitor	bind and inhibit ribonucleases; determinant for ribonuclease cytotoxicity; implications as it is know that bovine ribonuclease; have immunosuppressive, embryotoxic, aspermatogenic and anti-tumour activities	Leland and Raines 2001 Matousek 2001 D'Alessio *et al*., 1997 Youle *et al*., 1997

5.4.3.2 Under expression of proteins

There were 10 proteins identified as under expressed when infected post 24 hours with *L. aethiopica*. The decreased protein expressed was shown in HCV p7-transregulated protein 3 (0.433), 40S ribosomal protein S30 (0.212), Tumour necrosis factor alpha-induced protein 8-like protein 2 (0.339), ribonuclease T2 (0.407), synaptopodin 2 (0.403), catechol-O-methyltransferase (0.202), cyclin-dependent kinases regulatory subunit (0.270), Phospholipase D4 (0.248), epididymis secretory protein Li 55 (0.308), and cytochrome c oxidase subunit 6C (0.273) (Table 12). No significant under expression was observed with differentiated THP-1 infected with *L. mexicana*. The proteins of interest will now be discussed.

Table 11: Summarises proteins down regulated when infected with *L. aethiopica.*

Protein	Role	References
HCV p7-transregulated protein 3 (transmembrane 50B)	protein marker in Down's Syndrome	Moldrich *et al*., 2008
40S ribosomal protein S30	initiation of protein synthesis	Rabl *et al*., 2011
Tumour necrosis factor alpha-induced protein 8-like protein 2 (TP8L2)/Tumour necrosis factor-alpha-induced protein 8-like 2 (TIP2)	negative regulator of innate and adaptive immune response; powerful negative regulator of TGF beta-activated kinase 1 (TAK1) in mice; negatively regulating signalling via T cell receptors and Toll-like receptors (TLRs) binds to caspase-8, inhibits the activating protein-1 and nuclear factor-κB activation; promotes Fas-induced apoptosis	Oho *et al*., 2016; Luan *et al*., 2013; Lou and Liu 2011
Ribonuclease T2	catalyse the cleavage of RNA; localisation in lysosomes and vacuoles	Luhtala and Parker 2010
Synaptopodin 2/Myopodin	actin binding protein and biomarker for invasive cancer; its suppression leads to in reduction of human prostate cancer (PC3) cell migration	Kai *et al*., 2015; Kai *et al*., 2012
Catechol-O-methyltransferase (COMT)	enzyme involved in the metabolising cateholamines neurotramitters associated with cranial disorders e.g. schizophrenia, obsessive-compulsive disorder, bipolar disorder, anxiety, panic disorder and Parkinson's disease	Qayyum *et al*., 2015; dos Santos Passos *et al*., 2015; Brisch *et al*., 2009; Hamaue *et al*., 2010;
Cyclin-dependent kinases regulatory subunit	subunit of serine/threonine kinases involved in cell-cycle progression, transcription and neuronal function	Malumbres and Barbacid 2005
Phospholipase D4 (PLD4)	important in brain development; expressed in activated microglia; involved in phagocytosis and proliferation	Chiba *et al*., 2016
Epididymis secretory protein Li 55	involved in sperm maturation in epididymis; possible oxidoreductase activity	Lareyre *et al*., 1998; http://www.ebi.ac.uk/
Cytochrome c oxidase subunit 6C	subunit of enzyme that catalyses the final step in mitochondrial electron transport chain; its release results in cell apoptosis	*Li et al*., 2006; Shaha *et al*., 2006

5.4.4 Proteins of parasitological interest

5.4.4.1 Actin (cytoplasmic)

This is an essential protein in all eukaryotic cells as it is involved in structural, assembling actin into filaments critical for cell motility, cytokinesis and endocytosis (Hansen *et al*., 2013). The over expression of actin during infection may be due to the fact that the ingestion of particles into macrophages by phagocytosis involves the interaction and rearrangement of the actin skeleton and membrane (Diakonova *et al*., 2002). Actin is known to be involved in the attachment of promastigotes in macrophages. Destabilising of the actin cytoskeleton by cytochalasin showed a dose-dependant reducing in attachment of *L. donovani* promastigotes and intracellular amastigote load (Roy *et al*., 2014). There were similar observations in *L. brazilensis*-infected macrophages when the association index (% of infected macrophages x mean number of parasites/cell) decreased without F-actin or microtubules independent of the activation of the cell line (Azevedo *et al*., 2012). The same study identified the loss of the cytosketal components and/or presence of actin decreased the levels of IL-10. The past studies imply that increased levels of actin are critical for initial phagocytosis and establishment of infection by effecting not only structurally but also through molecular process such as decreasing IL-10 expression.

5.4.4.2 MHC I antigen and the CCDC68/CTCL-associated antigen Se57-1

The major histocompatability complex class I molecules present on most nucleated human cells are critical for peptide presentation to cytotoxic T lymphocytes (Grommé and Neefjes 2002; Townsend *et al*., 1988; Bjorkman *et al*., 1987). In abbearant or viral infected cells, peptides are presented with native proteins for recognition (Bailey *et al*., 2015). The up-regulation of MHC I molecules during *Leishmania* infection in this study may occur to intracellular endogenous detection possibly involved during recognition of *Leishmania* parasites within infected cells.

MHC molecules e.g. MHC II are known to be critical for antigen presentation to T helper cell and subsequent recognition of infected cells. *Leishmania* is known to attenuate the expression of MHC II for this reason (Kwan *et al*., 1992). The upregulation MHC I molecules normally occur through IFN-γ induction which was not present in past literature investigations (Zhou 2009; Grommé and Neefjes 2002). The ability of eliminating *Leishmania spp*. during infection is dependant on the activation of macrophages via interaction with T helper cells. The decrease of MHC II has been observed during *Leishmania* (*L. major* and *L. donovani*) infection of murine BALB/c macrophages (Gregory *et al*., 2008). In addition, the colorectal cancer marker CCDC68 has shown to be increased during *L. aethiopica* infection. Patients with colorectal cancer have shown natural and induced T lymphocyte responses against MHC I epitopes (Nagorsen *et al*., 2000; Engleman *et al*., 2003; Gerhardt *et al*., 2004). The importance of this up-regulation of this protein during infection remains unclear.

5.4.4.3 Ribosomal protein S2 (RPS2)

Ribosome S2 is an essential and conserved protein required for translation protein synthesis in prokaryotes, eukaryotes, mitochondria and chloroplasts (Aseev *et al*., 2008; Ardini *et al*., 1998; Wilson and Nierhaus 2005). In higher eukaryotes, the S2 protein evolved to as a 67kDa laminin-binding receptor (Ardini *et al*., 1998). The expression of this surface laminin-binding protein has been shown to increase in human adenocarcinomas (Sobel 1993). *In vitro* and *in vivo*, inhibition of RPS2 has been linked to increase apoptosis and cell death of murine prostrate cells (Wang *et al*., 2009). A wide range of ribosomal proteins has been implicated in diverse cellular process such as cell growth, apoptosis and tumour suppression (Naora and Naora 1999). This suggests a link between infection and regulation of apoptosis by elevated ribosomal S2 protein concentration during *Leishmania* infection. However, this was not observed by Singh *et al* which may highlight differences between *Leishmania* species and the time point at which sampling

took place (Singh *et al*., 2015). One possible reason maybe that an abundance of S2 ribosomal protein is needed for the translation of proteins required during the replication of *Leishmania* amastigotes within macrophages continuing their survival.

5.4.4.4 Ribonuclease inhibitor (RI)

This protein is involved in the binding and inhibition of ribonucleases (Leland *et al*., 2001). Human RI's are cytosolic proteins and potent inhibitors of mammalian pancreatic-type ribonucleases such as angiogenin and human pancreatic ribonuclease (Lee *et al*., 1993; Boix *et al*., 1996; Papageorgiou *et al*., 1997). Bovine ribonucleases (*e.g*. RNase A) have shown immunosuppressive, embrygotoxic, aspermatogenic and anti-tumour activity (Matousek 2001; D'Alessio *et al*., 1997). Additionally, it has been proposed that human cytotoxic ribonucleases have antiparasitic and anticancer activity in immune cells such as eosinophils (Youle *et al*., 1993). There are 8 different RNases (RNase 1-8), which show a multitude of effects on the immune responses, which involve infection (antibacterial, anti-viral, anti-fungal, antihelmintic), pro-inflammatory stimulation, cytokine & chemokine activation/maturation of DCs, Th2 polarisation and cytotoxicity (Koczera *et al*., 2016). RNase 2/eosinophil derived neurotoxin (EDN) is secreted from eosinophils and macrophages. Treatment of 1μg/ml RNase 2 protein to human DC's showed elevated levels of cytokines (IL-12 p40 - 6-fold, MIP-1α - 36.5 fold and MIP-1β - 28.8 fold) after 16 hours exposure (Yang *et al*., 2004). As RNase 2 is capable of eliciting a skewed inflammatory Th1 and stimulating MIP-1 & IL-12 in DCs, it would seem plausible that the *Leishmania* parasite, induce over expression of RI within the host cell to elicit a Th2 response (Maurer and Stebut 2004). Comparatively, the expression of MIP-1α/CCL3 and MIP-1β/CCL4 (and CCL5/RANTES) increase IL-12 production derived from DCs enhancing Th1 immune responses enabling successful clearance of *Toxoplasma gondii* (*T. gondii*) infection (Maurer and Stebut 2004). The up-regulation of RI protein may aid the survival of *Leishmania*

through directing the infected macrophage towards a Th2 response through direct inhibition of RNase 2.

5.4.4.5 Metallothionein (MT)

MTs have many proposed functions including metal detoxification, scavenging of free radicals, apoptosis and modulating redox balance intracellularly (Miles *et al*., 2000). However, the actual biological role is complicated by the presence of different isoforms and complex induction mechanisms (Miles *et al*., 2000). Interestingly, host macrophage MTs involvement in providing protection in patients infected with *L. panamensis* were treated with antimonial compounds was previously suggested (Gómez *et al*., 2013). The cytosolic expression of MTs decreases the levels of ROS and has been shown to be cytoprotective when murine fibroblasts were exposed to oxidising agents (Woo and Lazo 1997). This suggests a mechanism to decrease the presence of reactive oxidative species would likely result in survival of the parasite within the host cell. Previous work has shown that the levels of superoxide (O_2^-) decreased when MT was induced *in vitro* rat peritoneal macrophages (Irato *et al*., 2001). MTs have been implicated in the regulation of M-CSF mRNA expression via zinc in mice lung fibroblasts (Kanekiyo *et al*., 2002). MTs show a 5-fold increase in protein expression during *L. aethiopica* infection. This is consistent with previous studies on MT mRNA expression during *Leishmania* infection (*L. donovani* and *L. major*) have shown increased levels when infecting murine macrophages (Gregory, *et al*., 2008). The known influence of *Leishmania* on the elevated expression of MTs is likely to aid the parasites' survival through the quenching of free radicals critical in an infected macrophage to eliminate infection.

5.4.4.6 Tumour necrosis factor alpha-induced protein 8-like protein 2 (TIPE2)

Tumour necrosis factor alpha-induced protein 8-like protein 2 (TIPE2) is a death-effector domain (DED) protein, which is a negative regulator of immune responses (Oho *et al*., 2016). It is a powerful inhibitor of TGF-β-activated kinase (TAK1), which is important in the regulation of inflammatory and immune signals in mouse primary and macrophage-like cells (Oho *et al*., 2016). TAK1 is a member of MAPK kinase family and acts upstream of NIK in the activation of the IL-1 signalling through its associated with TNF Receptor Associated Factor 6 (TRAF6) and stimulation of TAK1 results in the activation of NF-κβ (Ninomiya-Tsuji *et al*., 1999). Numerous inflammatory signals (e.g. TNFα, IL-1, LPS) activate JNK and NFκβ via TAK1 in mammals (Delaney and Mlodzik 2006). JNK can be pro-apoptotic in combination with TNFα and in the absence of NFκβ (Liu and Lin 2005). However, the requirement of caspases during apoptosis is inhibited by NFκβ, which are members of inhibitors of apoptosis family (IAPs) (Liu and Lin 2005). The down regulation of TIPE2 in *L. aethiopica* infected THP-1 cells post 24 hours could lower its inhibitory effect against TAK1 induction of apoptosis as a result of the down regulation of an inflammatory response (*e.g*. IL-1). The ability to prolong the cell viability in macrophages would allow the parasite to persist and replicate. Apoptosis during *Leishmania* infection is thought to be a hijacked molecular process maximising the replication and spreading of the parasite within the human host (Getti *et al*., 2008).

5.4.4.7 Ribonuclease T2

Ribonucleases are ubiquitous in cells that catalyse the cleavage of RNA (ssRNA, dsRNA orDNA-RNA hybrids) (Luhtala and Parker 2010). They are secreted from the cell and compartmentised within lysosomes and vacuoles (Luhtala and Parker 2010; Deshpande *et al*., 2002; Irie 1999). Ribonuclease T2 (RNASET2) proteins are found in animals, plants, protozoa, bacterial and viruses (Deshpande *et al*., 2002). T2 ribsonucleases with RNA degradation have

additional functions including scavenging of nucleic acids, influencing host immune responses and acting as extra/intra-cellular cytotoxins (Luhtala and Parker 2010). Omega-1 a known RNase T2 family member is known to prime DCs for $CD4^+$ T lymphocytes to be polarized towards a Th2 immune response (Everts *et al*., 2009; Steinfelder *et al*., 2009). Macrophages infected with *L. aethiopica* have been shown to decrease levels of RNASET2, which is beneficial to the parasites' survival as it inhibits the Th1 response, required for a successful clearance of infection.

5.4.4.8 Synaptopodin 2 (SYNPO2)

SYNPO2 is an actin binding protein and biomarker for invasive cancer (Kai, *et al*., 2012). In response to serum stimulation, this protein promotes PC3 prostrate cancer cell migration (Kai *et al*., 2015). SYNPO2 is important in the development of autophagosomes during chaperone-assisted selective autophagy (CASA) through its interaction with BAG3 (BAG family molecular chaperone regulator 3) (Ulbricht *et al*., 2013). Autophagosomes are responsible for the degradation of cytoplasmic organelles and macromolecules to lysosomes (Xie and Klionsky 2007). Autophagy has been implicated as part of the innate immune response though pathogens within vesicles fusing with lysosomes (Pinheiro *et al*., 2009). Autophagy and apoptotic processes are both cellular response to stress, interestingly they are both are linked as the former requires the inhibition of caspases (Amer and Swanson 2005; Martin and Baehrecke 2004; Yu *et al*., 2004). Studies on autophagy reveal its importance in preventing efficient replication of *Legionella pneumophila* in infected murine macrophages (Amer and Swanson 2005). In *Leishmania*-infected macrophages, it has been shown that the parasites are able to block the transportation of the vacuole containing the parasite to the lysosome is thought to be caused by alteration of the phagosomes' membrane or regulatory associated factors (Swanson and Fernandez-Moreia 2002). It has been shown that *Leishmania spp*. are able to block the

maturation of phagosome via their surface glycoconjugate LPG (Turco and Descoteaux 1992; Desjardins and Descoteaux 1997; Turco *et al*., 2001). *L. donovani* LPG^- mutants showed the parasites were readily killed via the lysosome within macrophages (Desjardins and Descoteaux 1997). This is important during the early stage of infection as promastigotes because the amastigote form readily resides within the phagolysosome (Turco *et al*., 2001). The down-regulation of SYNPO2 by *L. aethiopica* could represent defensive strategy by intracellular parasites to prevent the fusion of the autophagosome with the lysosome whilst increasing the time of survival allowing the transformation of the promastigote into the amastigote form.

5.4.4.9 Cyclin-dependent kinases regulatory subunit (CKS1B)

CKS1B is a subunit of serine/threonine kinases, which are involved in cell-cycle progression, transcription and neuronal function (Malumbres and Barbacid 2005). Studies involving the removal of CKS1B in multiple melanoma (MM) cell lines caused the stabilization of Cyclin-dependant kinase inhibitor 1B ($p27^{kip1}$), cell cycle arrest and apoptosis (Zhan *et al*., 2007). CKS1B has been implicated in the inhibition of viral replication during Hepatitis C virus (HCV) infection (Lee *et al*., 2014). The reasoning for *L. aethiopica* to down regulate this protein is not clear. One possible reason could be that it is a known wild-type *L. aethiopica* can habour RNA viruses, and thus could be a protective mechanism, as it is known to be beneficial for the parasite during infection. In addition, the down regulation of CKS1B could aid the parasites' survival through delaying the cycle progression of monocytes into macrophages.

5.4.4.10 Phospholipase D4 (PLD4)

PLD4 is important in brain development of humans, expressed in activated microglia and involved in phagocytosis and proliferation (Chiba *et al*., 2016). Following LPS stimulation, PLD4 is up regulated in nuclei and cyosolic vesicles and accumulates in phagosomes during

phagocytosis (Chiba *et al*., 2016). Further more, PLD4 is an ER transmembrane glycoprotein but the exact enzymatic role is not known (Nelson and Frohman 2015; Yoshikawa *et al*., 2010). Studies on gliol-derived cell lines (U-87 MG, glioblatoma astrocytoma) show that inhibition of PLD4 at a protein level causes a reduction of intracellular production of dNTPs (Matthews *et al*., 2015). The increased expression of PLD4 results in activation of microglial macrophages and elevations in phagocytosis (Nelson and Frohman 2015; Yoshikawa *et al*., 2010; Otani *et al*., 2011). Previous work using siRNA down regulation of PLD4 inhibited phagocytosis, and a role during infection and injury to the central nervous system (CNS) has been suggested (Yoshikawa *et al*., 2010; Otani *et al*., 2011). There is no evidence in the literature linking the down regulation PLD4 to *Leishmania* infection. However, this proteomic study revealed this effect during infection, which might be one of many mechanisms permitting the parasites' survival through inactivation of macrophages.

5.4.4.11 Cytochrome c oxidase subunit 6C

Cytochrome c oxidase catalyses the final step in mitochondrial electron transport chain involving the transfer of electrons from ferrocytochrome c to molecular oxygen reduced to water (Li *et al*., 2006; Hüttemann *et al*., 2012). It has been suggested to be involved in apoptosis through allosteric ATP-inhibition of cytochrome c oxidase at high matrix ATP/ADP ratios maintaining low levels of mitochondrial membrane potential ($\Delta\Psi_m$) (Kadenbach *et al*., 2004). Cell apoptosis occurs either by type 1 or 2 apoptotic pathway. Initiation occurs in 1) Type 1 activation of death receptors (Fas, TNF-receptors) present on the plasma membrane resulting in the activation of caspase 8 and 2) Type 2 through changes in mitochondrial integrity induced by effectors (Ca^{2+}, ROS, Bax or ceramide) leading to the release of cytchrome c and activation of caspase 9 (Kadenbach *et al*., 2004). The decrease of $\Delta\Psi_m$ occurs following the release of cytochrome c. The ability of *L. aethiopica* to down regulate a constituent of cytochrome c oxidase may inhibit

the host's cell apoptosis providing further time to replicate, spread infection and maximize the likelihood of continual transmission during the next sandfly's blood meal.

5.4.5 Conclusions

It is clear that both *Leishmania* species manipulate the host cells' molecular pathways. Concerning the over expressed proteins; it appears to differ with some manipulation at a species level with only 2 proteins (Epididymis luminal protein 176 & MHC I) overlapping between *L. aethiopica* and *L. mexicana*. It poses no surprises that the proteins in question are involved in attachment & endocytosis (Actin), immunological receptors (MHC I), inhibition of apoptosis (ribosome S2), reduction of intracellular ROS (Metallothenein) and antiparasitic, inflammation, Th2 polarisation (ribonuclease inhibitors). However, it remains unclear why only 4 proteins (Actin aka Epididymis luminal protein 176, Heat shock protein cognate 71kDa and MHC I) were *L. mexicana* species specific. The role of this heat shock protein remains unclear but the results are suggesting that actin is an important factor during infection of *L. mexicana* in macrophages. Additionally, the definitive molecular reason for the up regulation of MHC I requires further study. It may be as a result of the intracellular presence of the parasite peptides. *L. aethiopica* on the other hand appears to up regulate additional proteins involved in apoptosis (ribosome S2), lowering intracellular ROS (metallothenein) and anti-parasitic, inflammatory and Th2 polarisation (ribonuclease inhibitor). *L. aethiopica* was observed to down regulate 9 proteins involved in apoptosis (TP8L2 & cytochrome c oxidase), autophagy (SYNPO2) and phagocytosis (PLD4).

The role that transmembrane protein 50B and the ribosomal 40S subunit S30 in aiding the parasite during infection is unclear. The ribosomal subunit S30 is part of the protein synthesis machinery that binds to initiation factors facilitating the scanning of mRNA (Rabl *et al*., 2011).

It has been documented that a wide range of ribosome proteins and their subsequent subunits can influence extraribosomal processes including tumour suppression, oncogenic, immune signalling and the development of diseases (Zhou *et al*., 2015). However, neither the 40S ribosome nor the S30 subunit has been implicated in affecting such processes. It is therefore, not possible to propose a theory on the benefits the parasite would gain from such a protein down-regulation. Transmembrane proteins are involved in cell adhesion, energy & material transportation and signal transduction (Kozma and Tusnady 2015). TMEM50 has been identified as a protein marker for Down's syndrome and is thought to be involved in the developmental regulation of ER and Golgi apparatus needed for correct brain function via glial and precursor cells (Moldrich *et al*., 2008). There is very little known about the function of this protein and as such, is difficult to provide sufficient reasoning for its down regulation during *L. aethiopica* infection.

There is a lack of understanding with two proteins that influence cellular apoptosis. TP8L2 has been implicated with inducing apoptosis and inhibiting cytokine inflammatory response such as IL-1. Whereas the down regulation of cytochrome c oxidase appears to be a mechanism to inhibit apoptosis and as both proteins are relatively down regulated to the same degree suggesting a complex interaction. The role of ribonuclease T2 in inhibiting Th1 responses is based on the fact a similar ribonuclease omega-1 causes a polarisation towards a Th2 response and needs further investigation. However, if this was to be confirmed it would identify a novel survival mechanism implemented by *L. aethiopica*. The down regulation of SYNPO2, which is involved in actin binding and autophagy, is of particular interest. The fact that LPG has been shown to cause poor fusing with lysosomes is clearly a useful strategy allowing the parasites' survival. The decrease in expression of PLD4 may be as important as it is involved in phagocytosis and activation of macrophages both of which are needed for successful clearance of infection.

This research has revealed *L. aethiopica* and *L. mexicana* within macrophages have some proteins elevated during infection are either shared by both species or are species specific. In addition, it has been shown that down regulation occurred only during *L. aethiopica* infection further empathising species-specificity. At 24 hours post infection, the parasites are still promastigotes requiring time to transform into amastigotes in order to successfully replicate. The ability of *Leishmania spp*. to establish through the modulation of proteomic pathways is a critical step for intracellular establishment. This study has shown clear distinction of protein expression between the two species in which they have evolved to persist within their human host.

Chapter 6

Can Leishmania parasites infect human T lymphocytes?

Novelty statement

This preliminary study is the first example where *Leishmania* species (*L. aethiopica* and *L. mexicana*) have been demonstrated to be capable of infecting human T lymphocytes *ex vivo*. This work involved using flow cytometric analysis of extracted human T lymphocytes and JURKAT T cells to identify percentage of infection.

Impact

This preliminary study suggests that the first plausible case of human T cells as a site of infection when infected with *L. aethiopica* and *L. mexicana*.

6.1 Introduction

As previously described, on infection, *Leishmania* parasites are able to enter a number of phagocytic cells (including macrophages, dendritic cells and neutrophils) but they are only known to replicate within macrophages. Macrophages together with T cells are the two major players in parasite survival. The two interact in a complex manner, which has not yet been completely elucidated. It is known that infected macrophages secrete cytokines to activate specific subsets of T-cells, which are then responsible for guiding the immune response towards successful resolution of the infection and/or spreading, and ultimately disease outcomes. How T cells guide the organism towards either outcome in humans remains unclear.

The immunology of the disease is complex and studies in mouse models have only partially clarified its bases. In the murine model, the development of T cell Th1 response is associated

with control of infection, and Th2 response is associated with disease progression. However, such dichotomy in the human system is not as distinct suggesting a Th1/Th2 paradox. The functional role of T cells is largely considered to be via cytokine regulation, which activates macrophages to actively eliminate intracellular *Leishmania*. Current literature does not suggest the possibility of these parasites infecting T cells. However, recent findings suggest that T cells might have an even more direct role on infection. Phagocytic T cells subpopulations have been described (Novais *et al*., 2009) and at the same time novel associations between *Leishmania* and immune cells have been reported showing that B cells are capable to host intracellular parasites (Geraldo *et al*., 2016). This highlights the fact that infection of host cells with *Leishmania* parasites might not be limited to DC, PMN and macrophages.

This chapter investigates whether *Leishmania spp.* have the ability to infect human T lymphocytes (Jurkat and T cells) *in vitro* and *ex vivo*. T cells are extremely important for successful clearance of *Leishmania* infection, as they are able to activate macrophages by producing cytokines such as IFNγ. This allows the activation of the M1 pathway involving the production of ROS in order to kill the internalized protozoan. This preliminary work investigates a hypothesis never tested before, and furthers current knowledge into the interactions between *Leishmania* parasites (*L. aethiopica* & *L. mexicana*) and human T cells.

6.2 Methods

The cell culture practices for Jurkat cells and all *Leishmania* promastigotes were carried as previously mentioned (Methods 2.1.1 pp. 32, 2.1.3 pp. 33). The purification of metacyclic promastigotes, infection of Jurkat cells, extraction human T lymphocytes, infection human T cells and the flow cytometric analysis was carried as previously mentioned (Methods 2.3.1 pp. 43, 2.3.3-2.3.4.4 pp. 44-48).

6.3 Results

6.3.1 Infection of Jurkat cells

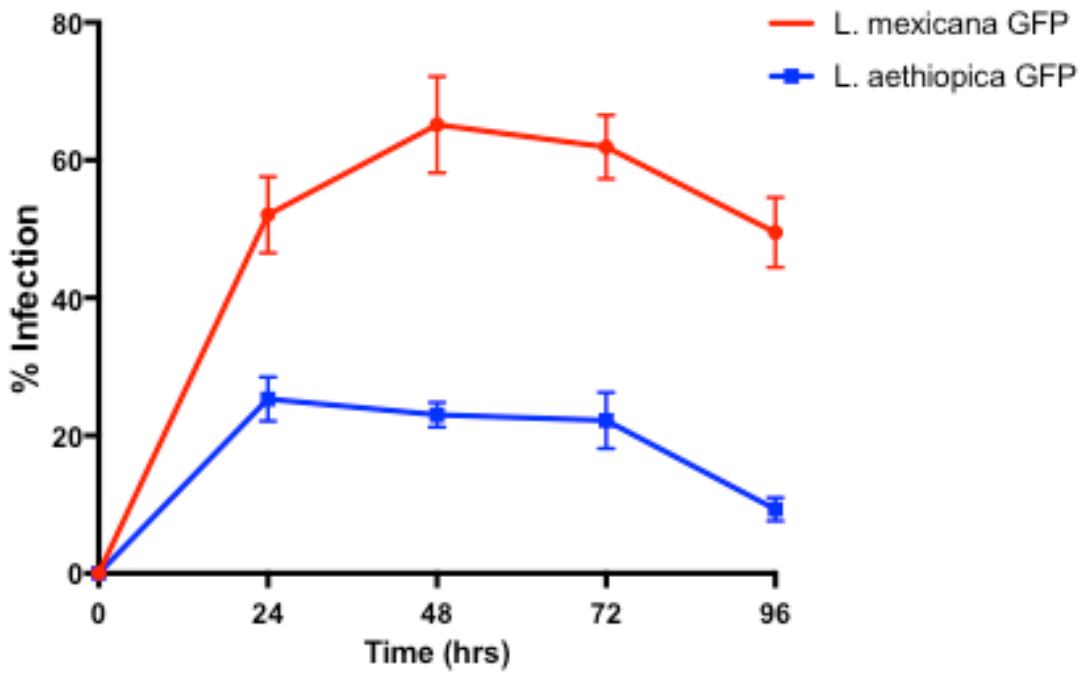


Figure 36: Percentage of infected Jurkat cells with *L. aethiopica* and *L. mexicana* over 96hrs. The biological replicates (N3) were obtained using flow cytometry analysis of gated Jurkat cells infected with GFP parasites. The means, standard deviations and Wilcoxon signed rank test was performed using Excel ($P<0.05$).

6.3.2 Infection of human T cells *ex vivo*

To support the theory that *Leishmania spp*. infects T lymphocytes four volunteers (H1, H2, H4 and H5) generously donated their blood for this research. T cells were infected for a maximum of 72hrs, analysed by flow cytometry and the data obtained was presented by their respective donor (Figure 37-40).

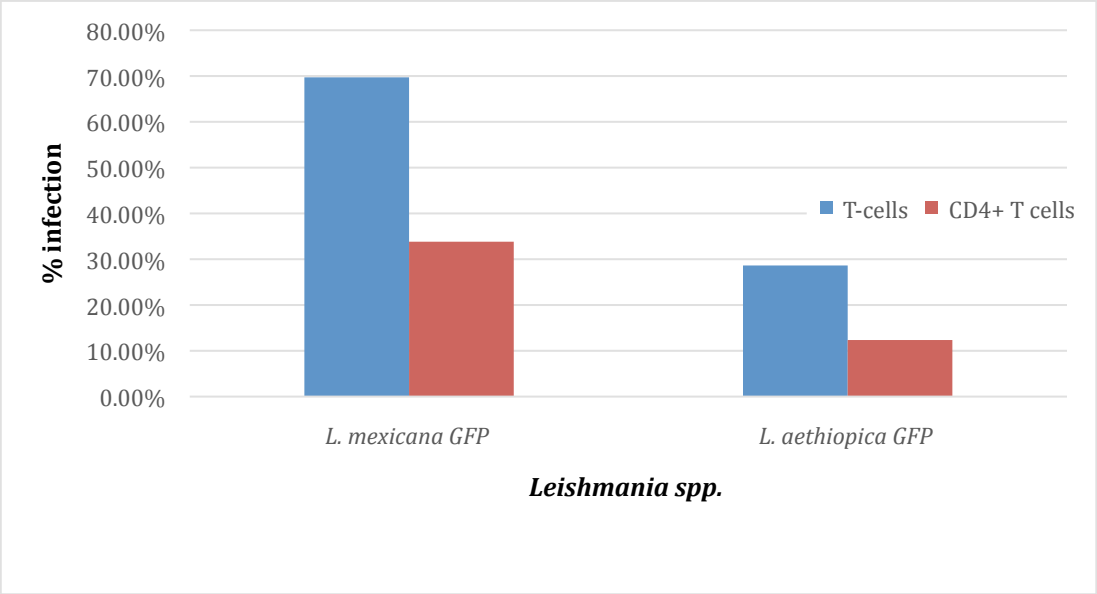


Figure 37: Percentage of *L. mexicana* and *L. aethiopica* infection in human T (H4) cells. This represents flow cytometric infection data on T cells obtained from individual H4 after 24hrs.

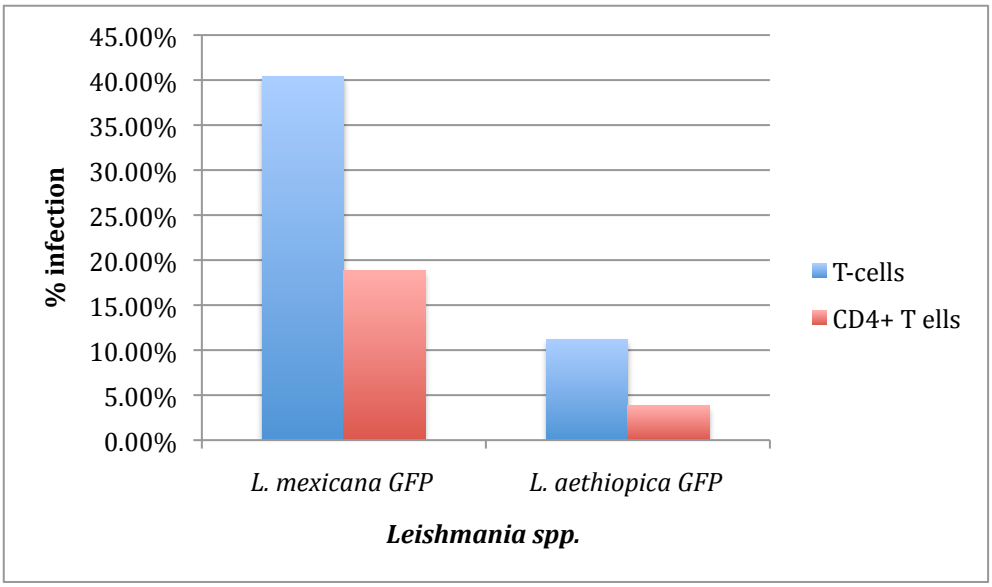


Figure 38: Percentage of *L. mexicana* and *L. aethiopica* infection in human T (H5) cells. This represents flow cytometric infection data on T cells obtained from individual H5 after 24hrs.

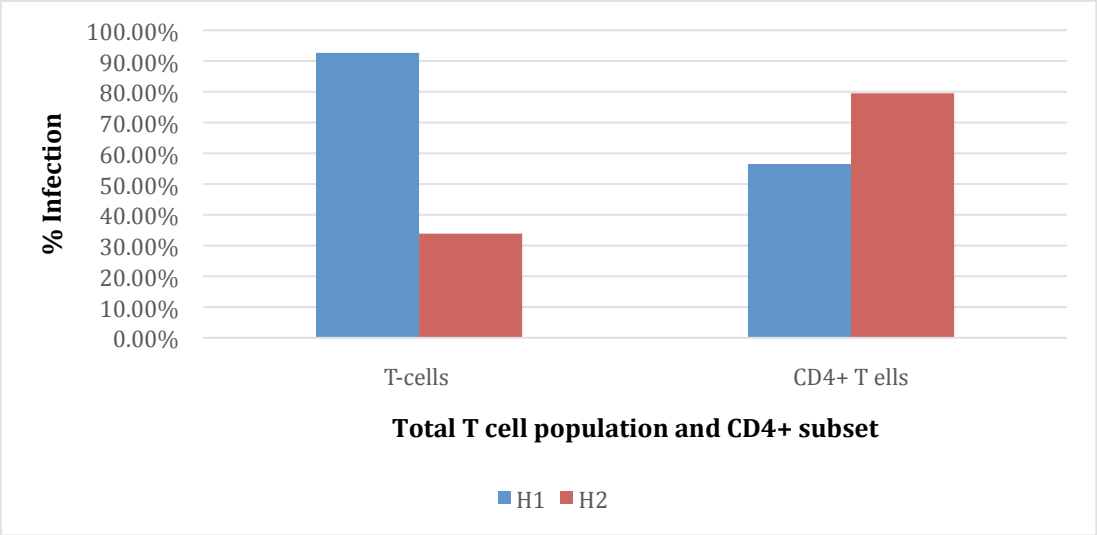


Figure 39: Percentage of *L. mexicana* infection in T cells (H1 & H2) at 48hrs. This represents flow cytometric infection data on T cells obtained from individual H1 and H2.

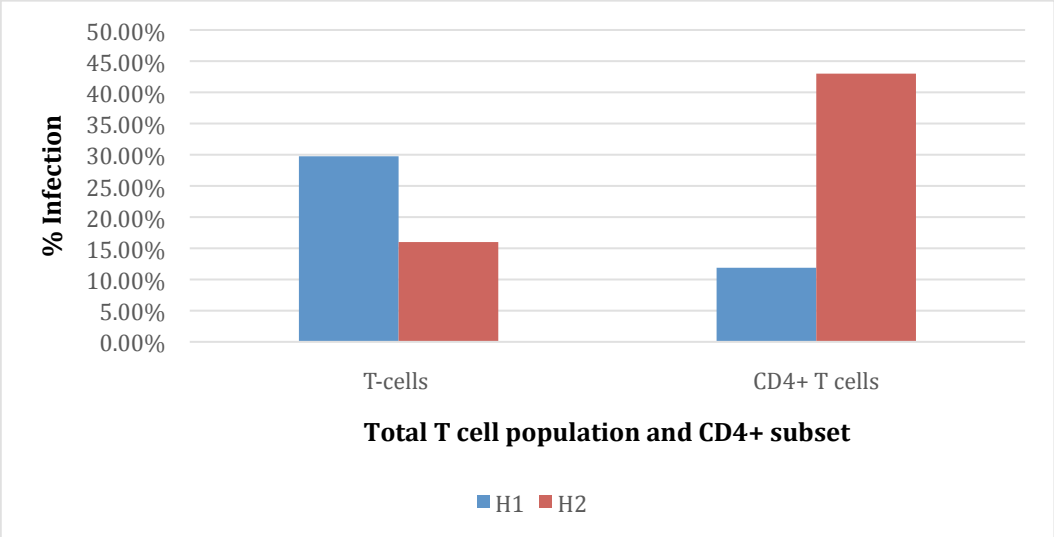


Figure 40: Percentage of *L. aethiopica* infecting human T cells at 72hrs**.** This represents flow cytometric infection data on T cells obtained from individual H1 and H2.

6.4 Discussion

6.4.1 Infection of Jurkat cells

The first step to test the hypothesis that *Leishmania spp*. have the ability to infect T cells, involved the use of the human Jurkat T cell line. It was thought to be unlikely that *Leishmania* would be able to infect human T cells *ex vivo* if they weren't able to infect the human T

lymphocyte cells first. The two species that were used, both capable to cause DCL: *L. aethiopica* and *L. mexicana*. The experimental rationale behind selecting *Leishmania aethiopica* and *L. mexicana* was the plausible mechanism for spreading as they migrate between the lymphatic system. The Jurkat cells were infected during log-phase cell growth and no treatment was applied during infection at a 10 parasite to 1 cell ratio. The percentage of infected cells was detected through flow cytometry over a period of 96 hours (Figure 36).

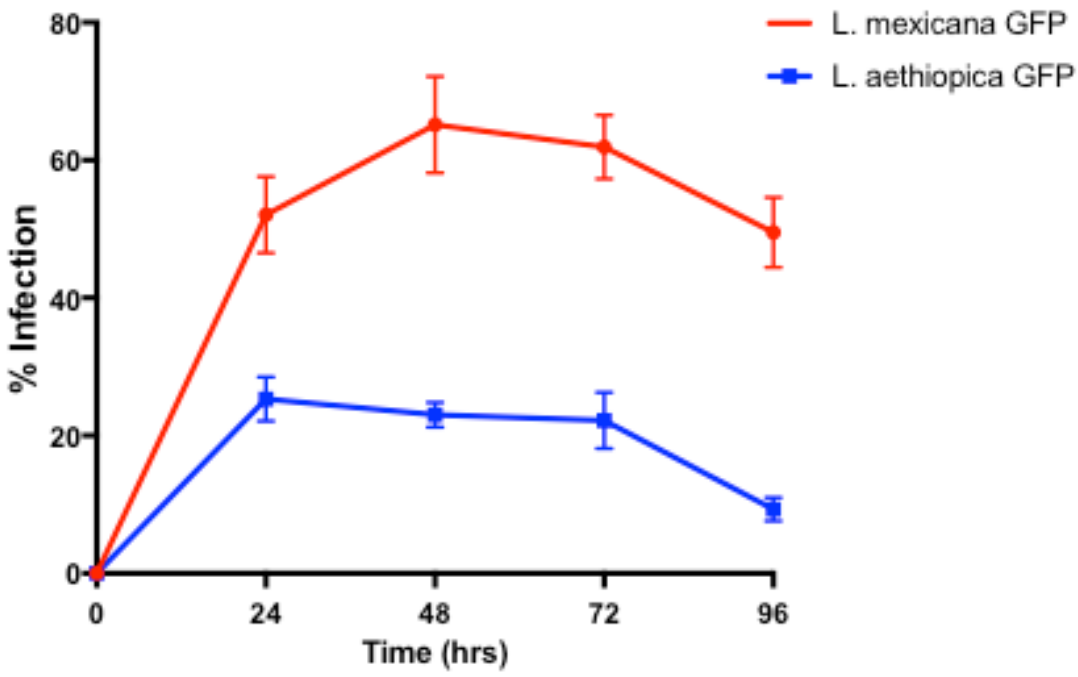


Figure 36: Percentage of infected Jurkat cells with *L. aethiopica* and *L. mexicana* over 96hrs. The biological replicates (N3) were obtained using flow cytometry analysis of gated Jurkat cells infected with GFP parasites. The means, standard deviations and Wilcoxon signed rank test was performed using Excel ($P<0.05$).

The percentage of infected Jurkat cells was higher during *L. mexicana* than *L. aethiopica* infection. *L. mexicana* infection increased to its peak of >65% at 48 hours and then slowly declined to 49.5% at 96 hours. *L. aethiopica* infection appeared to plateau from 24-72 hours where mean infection varied from 26.4% (at 24 hours) to 23% (at 48 and 72 hours). At 96 hours post infection, there was a decrease in *L. aethiopica* incidence with a mean of no more than 9%

infection. The levels of infection were found to less than with THP-1 cells. In THP-1 cells, *L. mexicana* infection peaked at 24hrs at 77.7% and declined to 43% at 96hrs whereas in Jurkat cells, it remains ~50% from 24-96hrs peaking with 65.16% at 48hrs post infection. In *L. aethiopica*, THP-1 infections peaked at 24hrs at 54.87% followed by a decline to 8.69% at 96hrs. In Jurkat cells, *L. aethiopica* infections remained ~20% between 24-72hrs (25-22%) followed by a decline to 9.30% at 96hrs. The differences between percentage infections of THP-1 and Jurkat cells may explain why the highest levels were detected in macrophages, as this is the only site where the parasite can replicate. In addition, the fact the Jurkat cells were not terminally differentiated may explain the sustained levels of infection for both *Leishmania* species through the continual replicating cell population for the parasites to infect. The differences observed between the two species during Jurkat infection may be possible due to species-specificity. This led to the hypothesis that *Leishmania aethiopica* and *L. mexicana* could possibly use human T lymphocytes as a reservoir of infection. To further investigate this hypothesis and in consideration of the fact that Jurkat cells are not T cells, infection of peripheral blood derived T cells was carried out.

6.4.2 Infection of human T cells *ex vivo*

To support the theory that *Leishmania spp*. infects T lymphocytes four volunteers (H1, H2, H4 and H5) generously donated their blood for this research. T cells were infected for a maximum of 72hrs, analysed by flow cytometry and data were presented by their respective donor (Figure 37-40). The results will now be discussed.

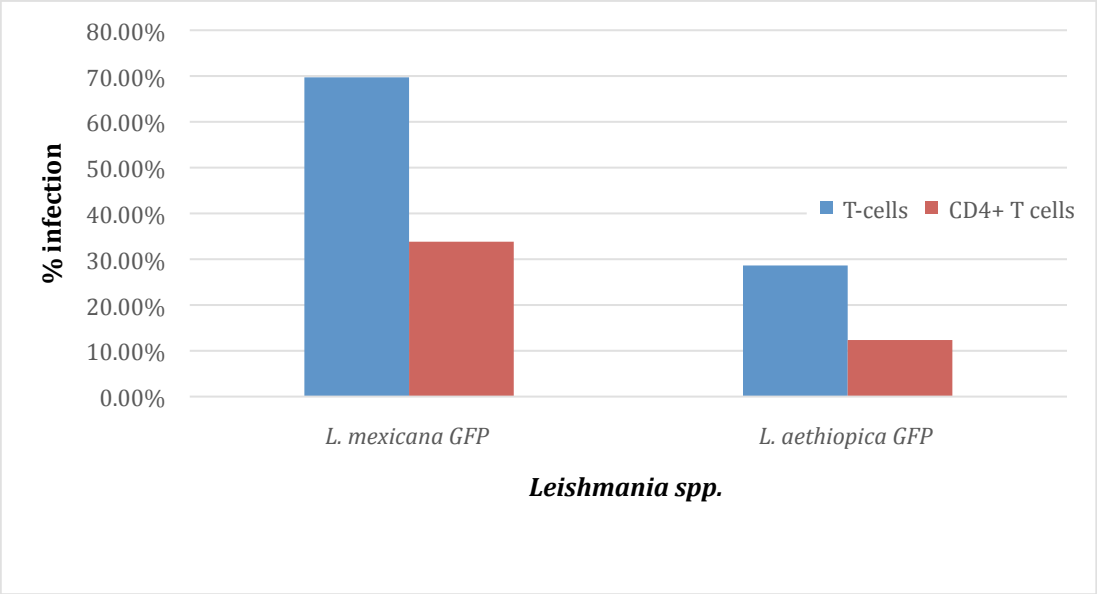


Figure 37: Percentage of *L. mexicana* and *L. aethiopica* infection in human T (H4) cells over 24hrs**.** This represents flow cytometric infection data on T cells obtained from individual H4.

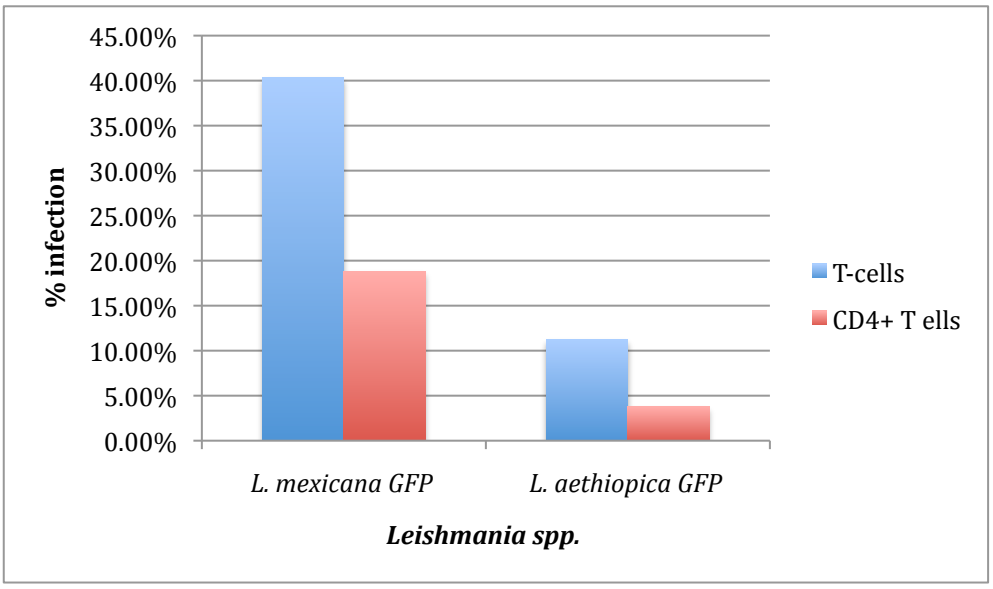


Figure 38: Percentage of *L. mexicana* and *L. aethiopica* infection in human T (H5) cells at 24hrs. This represents flow cytometric infection data on T cells obtained from individual H5.

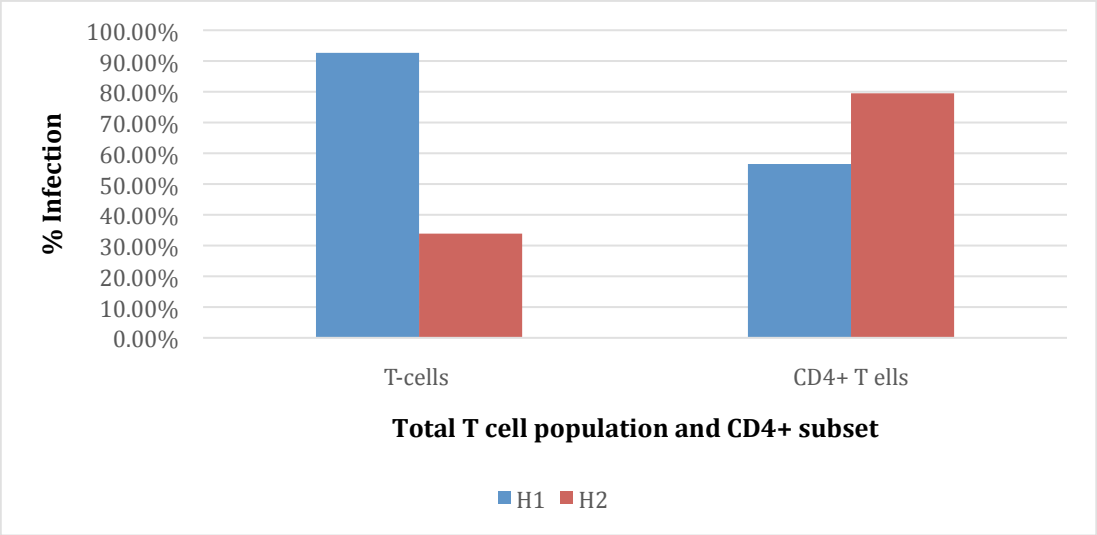


Figure 39: Percentage of *L. mexicana* infection in T cells (H1 & H2) at 48hrs. This represents flow cytometric infection data on T cells obtained from individual H1 and H2.

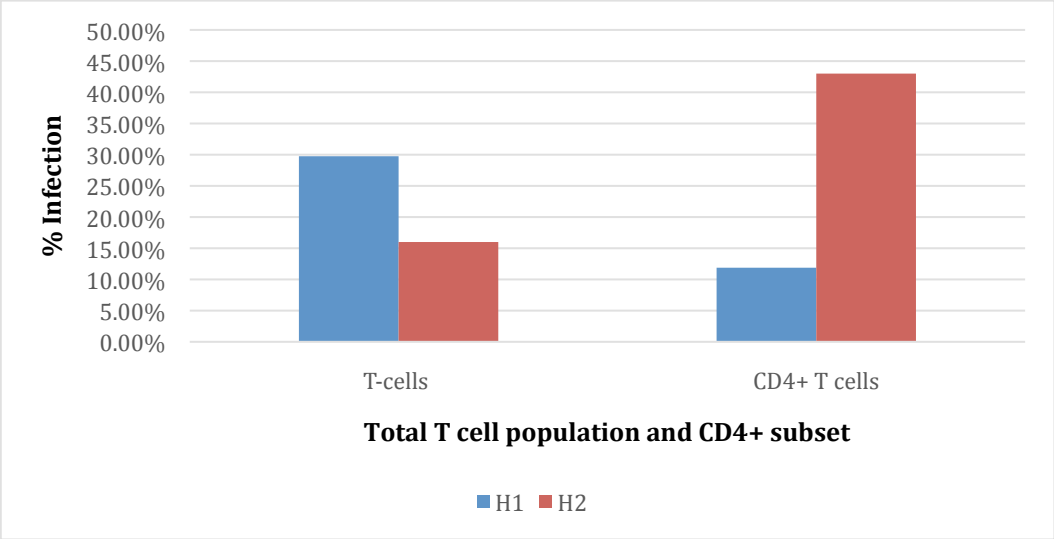


Figure 40: Percentage of *L. aethiopica* infecting human T cells at 72hrs**.** This represents flow cytometric infection data on T cells obtained from individual H1 and H2.

The preliminary data clearly shows that infection took place with both species over a 72 hours period. The percentage of infected cells varied greatly between individuals and was therefore not averaged at this stage. Further analysis was carried out to confirm whether the subpopulation of infected T cells expresses CD4. After 24 hours, *L. mexicana* infection with the total T cell population (H4 and H5) shows the highest levels with >69 and >40%, respectively. In addition, through the gating of this population with the $CD4^+$ antibody the cells were found to have >33 and 18% infection, respectively. *L. aethiopica* on the other hand, showed >28 and 11% total T cell infection with 12 & 3.8% $CD4^+$ infection, respectively. This shows that of the total T cell populations, between >45-47% infection was represented during *L. mexicana* infection in both human $CD4^+$ cells tested (H4 and H5). *L. aethiopica* on the same human T cell populations (H4 and H5) showed that this proportion ranged between 34.5-42.8%.

During 48 and 72hrs post infection, the trend was similar with the total T cells (H1) infection at 92.65% and 29.75%, respectively. Additionally, from the T total cells, infection of $CD4^+$ T cells was 56.51 & 11.8%, respectively. The gated $CD4^+$ population at 48 & 72hrs post infection represented 60% and 39.6%, respectively. It should be stated this proportional trend was not observed throughout the experiment as infection of H2 T cells showed highest levels of infection in the gated $CD4^+$ populations with 79.5% compared with the total T cell infection at 33.8%. This result has been regarded as an anomaly requiring further experimental testing. The preliminary findings show that *Leishmania spp*. are capable of infecting T cells including the $CD4^+$ subset.

6.5 Conclusions

The preliminary studies on *Leishmania* infection on human T lymphocytes indicate infection by both species. The ability of *Leishmania spp.* to infect Jurkat cells has been previously documented (Novais *et al*., 2009). Moreover, this is the first time that *L. aethiopica* and *L. mexicana* have indicated the ability to infect T lymphocyte cells. *L. mexicana* has been shown to consistently have the highest level of T cell infection when compared with *L. aethiopica*. This research has confirmed successful infection of T helper $CD4^+$ cells, which are regarded as a main source of IFNγ for activation of macrophages. This work is suggesting that the remaining infected lymphocyte populations may include the $CD8^+$ T cells. The investigation into the ability of *Leishmania spp*. to infect human T cells *in vitro* and *ex vivo* has proved true. Further work is required to fully contemplate the parasites intracellular influence within T lymphocytes. *Leishmania spp*. able to persist within T cells may aid the parasites' survival through the modulation of the host cell responses.

Chapter 7

Conclusions

The hypothesis that stable expression of GFP in these four *Leishmania* species was fulfilled for the first time, enabling fast and accurate monitoring of infection. This was validation by PCR, WB, fluorescent microscopy and flow cytometric analysis. Moreover validation of transgenic species was carried out and proved that GFP insertion did not significantly affect the parasites' life cycle and most importantly its ability to infect host cells. This has represented an invaluable tool for work carried out in this thesis as it has allowed confirmation of infection and quantification of percentage of infected cells during all experiments. Infected samples containing similar percentages of infected cells were analysed to establish intracellular parasites' effect on mRNA (cytokines and cathelicidin) and protein expression. In addition, GFP expression coupled with fluorescent microscopy and flow cytometry represented a powerful tool in studying not only infection in terminally differentiated THP-1, but also other host cell types.

Leishmania spp. survival relies on a tightly regulated control of the immune system of the host. Once promastigotes are released inside the mammalian host, parasites need to establish a long-term relationship with macrophages in order to survive. It is through the manipulation of such host cells that intracellular parasites replicate and spread effectively initiating infection. In order for infection to develop into disease a more complex interaction between innate and immune system needs to be established. The interaction culminates in activation of T cells, which are finally responsible for disease development or resolution. Previous infection studies in mice have revealed the importance of this mechanism. It is clear though that *Leishmania*-induced

cytokine expression is a major contributor to the cellular communication between innate and adaptive immunity and is likely to be a decisive contributor to disease.

A particularly small amount of information is available for *L. aethiopica*, an Old World species of particular interest as it is capable of causing all forms of CL. Unfortunately, there are no mice models available for this species. This research concentrated on *L. aethiopica* and on *L. mexicana*, a New World species also able to cause all forms of diseases.

Both species were able to control expression of both inflammatory and anti-inflammatory cytokines in host macrophages, including the antimicrobial peptide cathelicidin. The expression of cathelicidin peaked at 8 hours during infection possibility indicating its importance as part of the early innate immune response against *Leishmania* parasites. During infection, the levels of IL-10 increased over time in line with the expression of TNFα, which may highlight the parasite up regulating IL-10 to dampening inflammatory responses. However, the hypothesis that early stages of infection results in mRNA inflammatory cytokines are expression followed by anti-inflammatory cytokines was not met requiring further investigation. The effect of infection on host macrophages is not limited to controlling cytokine expression, it extends to a number of intracellular pathways. A cell wide analysis of cytosolic protein expression changes during infection has only been carried out in VL species (*L. donovani*) during infection of THP-1 cells. This work is the first analysis of differential proteomics, not only between infected and non-infected cells, but also between cells infected with different species. A relatively small number of proteins were identified to be differentially expressed (N=3) which are thought to be involved in phagocytosis/phagosomes (Actin, TIPE2, Synaptopodin 2), apoptosis (Ribosome S2, Cytochrome c oxidase subunit 6C), possible immunomodulation towards a Th2 response (Ribonuclease inhibitor, Ribonuclease T2), quenching of free radicals (Metallothionein) and cell

cycle (CKS1B). All of these cellular processes would likely need to be modulated for continual survival of the parasite.

Finally, the ability of *Leishmania spp*. to interact with T cells in a more direct manner than previously thought was investigated. The hypothesis that *Leishmania spp*. have the ability to infect human T cells follows two recent discoveries (infection of B cells and phagocytic T cells) as well as the knowledge that it is not clear how parasites control T cell differentiation and various disease manifestations. Initial data on a T-cell immortal cell line showed that infection was indeed possible and was maintained over a 96 hours period, even in actively replicating Jurkat cells. But as Jurkat cells greatly vary from T cells, data was confirmed in human T cells *ex vivo*. This has never been observed before showing the even closer relationship the parasite has with lymphocytes. The work shows that the T cell subsets $CD4^{+}$ are infected, further strengthening the interaction between parasite and host lymphocyte.

In conclusion, *L. aethiopica* and *L. mexicana* can control macrophages both in terms of cytokine expression and intracellular pathways and do so in a tightly regulated and balanced manner that insures survival while maintaining infection hidden by the immune system. In addition, this work highlighted that *L. aethiopica* and *L. mexicana* can use T cells as hosts and indicates that they are able to do so for as long as 96 hours from infection. The parasites ability to infect T lymphocytes is likely to be an incredibly an important find considering the parasitological clearance is mainly dependant on Th cells activating macrophages to eliminate infection. This discovery will undoubtedly further aid our understanding in the strategies that *Leishmania spp*. imposes on the human host.

Chapter 8

Future Work

Transgenic parasites constitutively expressing GFP can further be used to confirm infection on T cells via immunostaining and confocal microscopy. The molecular/cellular effect on the T cells remains unclear at this point and requires further research.

It is clear that not all types of cytokines are up or down regulated at the same time. Hence, there is scope for expansion of the cytokine expression work. Specifically, the repertoire of cytokines in macrophages can be expanded tools at IL-12, IL-1, IL-6 etc, as well as during infection with additional *Leishmania spp*. This would provide further understanding of the inflammatory and anti-inflammatory pathways that are important during *L. aethiopica* infection comparatively to other species. In addition, the ability to test the protein expression (e.g. ELISA) would confirm the link between the mRNA and actual protein expressed during infection. The same technique can be expanded to look at cytokine expression during infection of T cells.

The proteomic study revealed 18 proteins that were modulated by the *Leishmania spp*. However, further validation of expression could be carried out involving mRNA and additional protein tests such as WB or ELISA. Also, the role of each of protein modulated by the parasites could be researched through creating lacking mutants, protein labeling systems, which would allow further understanding in role and identify if any close association occurs during phagocytosis.

Proteomic (WB, ELISA, TMT, LC-MS/MS), and mRNA expression assays could give a further understanding of the effect the parasites have on human T cells. As this is novel territory, further

flow cytometric analysis could identify specific sub-populations of T lymphocytes (including CD8 and Tregs) that could possibly support infection. Only through the confirmation of infection can proteomic or mRNA expression assays be applied to additional T subsets. Research into the spreading of infection with possible T cells within the host would require further work as this is still unknown. This chapter has revealed a novel finding and only through progressive further work will we gain an altruistic understanding of the T cell function during infection.

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Note: All Web site references were obtained September 2016.

Appendices

Appendix I - Blood Collection Procedure and Consent forms

1. Process of Blood Collection

1.1 Blood cannot be taken without first obtaining informed consent (written). Research workers are not permitted to take blood from themselves. Volunteers should not donate if they may be infected with blood-borne virus and should not become regular donors if they may be at risk of infection.

1.2 Blood should be taken only in a quiet area set aside for this purpose away from people not critical to the procedure.

1.3 The donor should always sit, or preferably lie down before taking blood.

1.4 If taking blood with the donor seated, ensure there is sufficient space immediately adjacent to lie the donor down should s/he faint.

1.5 For samples of >50ml the sample must be collected in a designated "clinical" room.

1.6 On the very rare occasion that it is necessary for samples of >200ml to be taken in one donation, a haemoglobin estimation should be carried out prior to collection.
Samples should not be taken from men if haemoglobin is lower than 13.0 g/dl.
Samples should not be taken from women if haemoglobin is lower than 12.0 g/dl.

1.7 A procedures tray must be prepared with all the necessary equipment needed for blood collection.

1.8 Gloves and protective garment (gown or laboratory coat) should be worn during venepuncture and the handling of blood.

1.9 Blood should be drawn in accordance to the guidelines recommended by the professional training body responsible for training University staff and students to take blood (see the training requirements (section 1.2) of the University of Greenwich Blood Collection Policy). For the purposes of this document, finger prick blood collection may be defined as collecting a volume of less than 1mL via a route that doesn't involve venepuncture.

1.10 The blood collection site must be cleansed.

1.11 Finger prick samples are collected using appropriate capillary tubes. Undergraduates should be closely supervised by a trained member of staff (see the training requirements (section 1.2) of the University of Greenwich Blood Collection Policy).

1.12 Where mL quantities of blood are required, best practice should be followed *i.e.* using BDs Vaccutainer® systems to avoid sharp stick injury.

1.13 Needles and lancets must be dealt with carefully, to avoid a sharps injury. Needles must never be recapped. Needles must be discarded directly into a sharps box. Unsheathed needles should not be handed from one person to another.

1.14 Discard syringes and needles as a unit and only dispose of via a proprietary sharps container; never carry used sharps. Discard sharps containers following biohazard disposal procedures when three-quarters full.

1.15 All phlebotomy equipment is single use only. Never re-use disposable equipment.

1.16 All samples collected must be appropriately labelled.

1.17 After blood is drawn steps will be taken to ensure that the bleeding is stopped after the procedure. Bleeding of the venepuncture site should be controlled by exerting pressure with a sterile gauze pad, in line with best practice.

1.18 Blood and body fluids should not be forced from syringes into tubes or bottles in order to avoid popping of stoppers and spraying of contents or generating aerosols.

1.19 Before glove removal, inspect the blood collection tubes for contamination on the outside. Any blood should be removed with an alcohol wipe before further processing.

1.20 Disposable paper covers around the work area help to absorb possible spills and should be discarded as contaminated material in the appropriate infectious waste containers.

1.21 As always, report all accidents to the Local Safety Officer and the Safety Unit on an accident/incident form and seek advice as appropriate. In emergencies phone (internally) 43333 or 01634 883138 (from an external phone).

2. Handling Blood Specimens

2.1 Again best practice should be followed. These should be in line with any guidelines stipulated during training regarding the taking of blood (see above). These include the following local rules:

2.1 All blood specimens must be contained in a tightly closed, non-leaking container and placed in a secondary container for transport (see 2.2.4 and 2.2.5).

2.2 Blood collected in capillary tubes should be secured properly on a protective flat surface prior to further processing.

2.3 The person collecting the specimen is responsible for removing any contamination on the outside of a collection container with a suitable substance when the specimen is transported.

2.4 Specimens stored in collection areas or in the laboratory must be placed in a safe area *e.g.* cupboard or refrigerator that is clearly marked with the BIOHAZARD sign and identified as inaccessible to the public and not used for food storage. This container should be fitted with a lock that should be engaged when the container is not in use.

2.5 Specimens to be transported should be handled with gloved hands and on easily disinfected surfaces.

2.6 Individuals transporting specimens must be instructed in universal precautions having been trained locally (See University of Greenwich Blood Collection Policy section 1.2 (specifically 1.2.4)).

3. Disposing of used needles, sharps and other human tissue wastes
3.1 All needles, used or unused, must be discarded in a yellow sharps disposal bin and disposed of as clinical waste via high temperature incineration.

Under no circumstances must any sharps be discarded in the general waste bin or other bin. It is the responsibility of the investigator to ensure that needles are not placed inappropriately so as to pose a danger to others.

3.2 All items contaminated with blood and other body fluids must be discarded using the appropriately labelled hazardous waste disposable bins. Such waste material must never be disposed of in the general waste bins. Biological waste material must be appropriately labelled and the necessary arrangements made to transport them for incineration after alerting facilities management.

Failure to comply with these directives will be deemed a serious breach of procedure and appropriate action taken. This may result in disciplinary action and or legal action being taken against the offender.

Best practice can be found in:
"WHO guidelines on drawing blood: best practices in phlebotomy" Printed by the WHO Document Production Services, Geneva, Switzerland ISBN 978 92 4 159922 1

Available as PDF at: www.who.int/injection_safety/phleb_final_screen_ready.pdf .

V. 01/11/13

Blood Sample Consent Form

First name:_____

Surname: _____

d.o.b.:_____

I agree to provide a blood sample for use by_____

I have been informed of:

- the quantity of blood to be taken
- the use that will be made of the sample
- any tests for disease, or markers of disease, that will be undertaken whilst the sample can be identified as originating from me.

I can confirm that to the best of my knowledge:

- I do not have any communicable blood borne diseases
- I am comfortable with the procedures outlined
- I am not pregnant
- I am not taking anticoagulant medication
- I am not suffering from any form of haemophilia or bleeding disorder

I agree to the sample being stored for possible further uses in future.

Signed_____

Date_____

Consent form for recurrent donation

First name:_____

Surname: _____

d.o.b.:_____

I agree to provide a blood sample for use by_____

I have been informed of:

- the amount and frequency of donations
- the use that will be made of the sample
- any tests for disease, or markers of disease, that will be undertaken whilst the sample can be identified as originating from me.

I can confirm that to the best of my knowledge:

- I do not have any communicable blood borne diseases
- I am comfortable with the procedures outlined
- I am not pregnant
- I am not taking anticoagulant medication
- I am not suffering from any form of haemophilia or bleeding disorder

I agree to the sample being stored for possible further uses in future. I agree to inform the researcher if I:

- donate blood elsewhere
- become a formal National Blood Service donor

Signed_____

Date_____

PARTICIPIANT INFORMATION SHEET

As the participant of this research you are aware of the following:

1. You will not be personally identified and kept anomalous, *i.e.* though naming samples as human sample 1, 2 and 3 *etc* (HS1, 2, 3).

2. Your donated blood will be used for research purposes only.

3. Your blood sample(s) will only be used for infection of human cells by *Leishmania* parasites for a maximum duration of 1 week. This will involve the extraction of peripheral blood mononuclear cells from the blood sample by centrifugation. The infection study may involve further analysis of infection using flow cytometry and microscopy (confocal, fluorescent, TEM, SEM).

4. No human DNA or cells will be stored after the 1 week usage.

PARTICIPANT CONSENT FORM

To be completed by the participant..

- I have read the information sheet about this study
- I have had an opportunity to ask questions and discuss this study
- I have received satisfactory answers to all my questions
- I have received enough information about this study
- I understand that I am / the participant is free to withdraw from this study:
    - At any time (until such date as this will no longer be possible, which I have been told)
    - Without giving a reason for withdrawing
    - (If I am or intends to become, a student at the University of Greenwich) without affecting my future with the University
- I understand that my research data may be used for a further project in anonymous form, but I am able to opt out of this if I so wish, by ticking here. ☐
- I agree to take part in this study

Signed (participant)	Date
Name in block letters	
Signature of researcher	Date
This project is supervised by: Dr. Giulia Getti	
Researcher's contact details (including telephone number and e-mail address): Andrew Deacon [**Redacted**] Da65@gre.ac.uk	

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Our Ref UREC/15.1.5.1
Date: 12 August 2015

Dear Andrew,

University Research Ethics Committee – Minute 15.1.5.1

TITLE OF RESEARCH: Investigation of Leishmania infection

I am writing to confirm that the above application has been **approved** by Chair's Action on behalf of the Committee and that you have permission to proceed.

I am advised by the Committee to remind you of the following points:

- You must notify the Committee immediately of any information received by you, or of which you become aware, which would cast doubt upon, or alter, any information contained in the original application, or a later amendment, submitted to the Committee and/or which would raise questions about the safety and/or continued conduct of the research;
- You must comply with the Data Protection Act 1998;
- You must refer proposed amendments to the protocol to the Committee for further review and obtain the Committee's approval thereto prior to implementation (except only in cases of emergency when the welfare of the subject is paramount).
- You are authorised to present this University of Greenwich Research Ethics Committee letter of approval to outside bodies in support of any application for further research clearance.

On behalf of the Committee may I wish you success in your project.

Yours sincerely

[redacted]

John Wallace
Secretary, University Research Ethics Committee

Cc: Dr Giulia Getti

QUEEN'S
SARY PRIZES
2008

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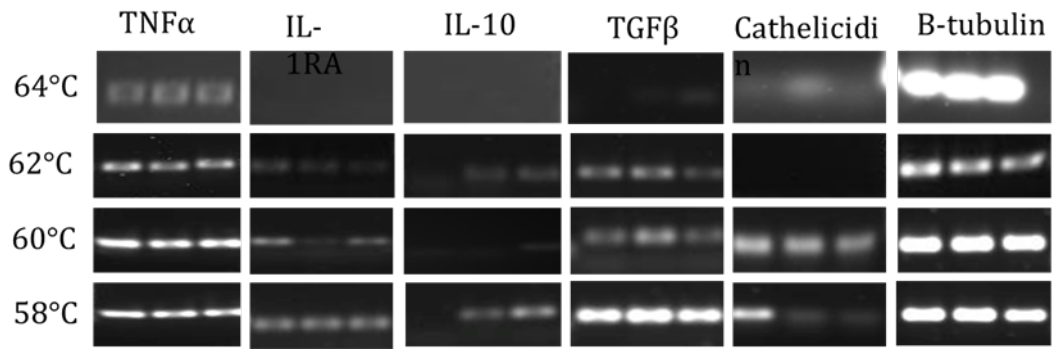

Appendix 2: Electrophoresis images of primers optimisation prior to qPCR. Each image shows the PCR products (Lane 1: THP-1, Lane 2: THP-1+RA and Lane: THP-1+RA+LPS post 8 hours) from the different primer sets at the various annealing temperatures. These gel electrophoresis images were used to decide the optimal annealing temperatures for the qPCR expression system.