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The *Fasciola hepatica* growth factor; FhTLM, and actions in immune evasion and parasite survival

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Abstract

Helminth parasites promote the development of antigen-specific anergy and regulation which often limits pathology but allows parasite survival. Parasite effectors mediating this are the subject of intense study as they may be useful as future vaccine targets. *Fasciola hepatica* possesses a TGF-like molecule (FhTLM), part of a larger TGF family, which play an important role in regulating parasite development and modulating host immune responses. We hypothesized that *F. hepatica* may utilise FhTLM to down modulate host immune responses *via* induction of regulatory. Consequently, in this study, the immuno-modulatory properties of FhTLM with regards to its effect on T-cell mediated immunosuppression and modulation of host immune cells and signals to benefit parasite survival were studied.

The *in vitro* effect of FhTLM on induction of FoxP3 T-regs cells and anergic markers, PD-1, PD-L1 and CTLA-4, was studied in murine splenic CD4 T-cells. FhTLM failed to upregulate FoxP3 T-reg cells and all investigated anergic T-cell markers. However, production of IL-10, a key immunomodulatory cytokine was significantly enhanced in response to FhTLM stimulation. The interaction of FhTLM with naïve CD4 T-cells also promoted the development of inflammatory responses evidenced by enhanced production of pro-inflammatory cytokines and chemokines (TNF-α, IL-12 $_{P}$70, IL-1β, IP-10, TARC, MIP-1β, IL-1ra, RANTES and CXCL12). The chemokines induced by FhTLM were biologically functional as supernatants from FhTLM stimulated cells promoted *in vitro* migration of both activated CD4 T-cells and eosinophils. Lastly, immune signals generated from CD4 T-cells, eosinophils and monocytes failed to support enhanced survival of newly excysted juvenile (NEJs) flukes and this response

was not affected by the presence FhTLM. Whilst FhTLM did not directly modulate immune cells to promote parasite survival, induction of pro-inflammatory responses as evidenced by production of cytokines; IL-6, TNF-α, RANTES, MCP-1, Lipocalin -2, Endocan, Pentraxin-3 and ICAM-1 by LPS or IL-4-stimulated monocytes was suppressed or inhibited in the presence of FhTLM.

Taken together, our data highlight an indirect mechanism used by the parasite to benefit parasite persistence and also implicate a role for IL-10 in suppression of protective host immune response to *F. hepatica* infection. In addition, our data also indicates that FhTLM has an anti-inflammatory potential and may have implications in the treatment of allergic or pro-inflammatory diseases.

Declaration

I confirm that the work in this dissertation is my own work and contain no material that has been submitted for any other degree at the University of Nottingham or other institution.

All of the procedures in this thesis have been done under the regulation of the University of Nottingham.

Mayowa Musah-Eroje

November 2019

Publication, Conferences, Posters and Oral Presentations

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List of abbreviations.

α	Alpha
β	Beta
γ	Gamma
-/-	knockout
µl	Microlitre
ω	Omega
%	Percentage
°C	Degree Celsius
AAMϕ	Alternatively activated macrophage
ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen-presenting cells
ATP	Adenosine triphosphate
BMDE	Bone marrow-derived eosinophils
BMDM	Bone marrow-derived macrophages
CAMϕ	Classically activated macrophages
CD	Cluster of differentiation
CFSE	Carboxyflourescein diacetate succinimidyl ester
CL	Cathepsin
CTLA	Cytotoxic T-lymphocyte-associated protein
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay

ES	Excretory secretory
FABP	Fatty acid binding protein
FhCL	*Fasciola hepatica* cathepsin
FhHb	*Fasciola hepatica* haemoglobin
FhESP	*Fasciola hepatica* excretory secretory products
FhTeg	*Fasciola hepatica* tegumental coat antigen
FhTLM	*Fasciola hepatica* transforming growth beta-like molecule
FoxP3	Forkhead box P3
g	Gram
GLUT	Glucose transporter
GM-CSF	Granulocyte macrophage colony-stimulating factor
GRAIL	Gene related to anergy in lymphocytes
GSTs	Glutathione S-Transferases
Hb	Haemoglobin
hrs	Hours
ICOS	Inducible T-cell costimulatory
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ITIM	Immuno-receptor tyrosine-based inhibitory motif
LAP	Leucine aminopeptidase
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant proteins
mg	Milligram

MHC Major histocompatibility complex

mins Minutes

MIP Macrophage inflammatory proteins

ml Millilitre

MΦ Macrophages

NEJs Newly excysted juveniles

NF-κB Nuclear factor kappa-light chain enhancer of activated B cells

ng Nanogram

NK Natural killer cells

NES *Nippostrongylus brasiliensis* excretory secretory

NO Nitric oxide

OVA Ovalbumin

PAMP Pathogen-associated molecular patterns

PBMC Peripheral blood mononuclear cells

PBS Phosphate buffered saline

PD Programmed death

PDL Programmed death ligand

pg Picogram

PRR Pattern recognition receptors

Prx Peroxiredoxin

RANTES Regulated on activation, normal T-cell expressed and secreted

RBC Red blood cells

RFU Relative fluorescent unit

RNA Ribonucleic acid

ROS Reactive oxygen species

RT	Room temperature
SEA	Schistosoma mansoni soluble egg antigen
SODs	Superoxide dismutases
Spp	Species
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation regulated chemokine
TCBZ	Triclabendazole
TCR	T-cell receptor
TGF	Transforming growth factor
Th	T helper cells
TLR	Toll like receptor
TNF-α	Tumour necrosis factor-alpha
T-reg	Regulatory T-cells
US$	United State dollar
WHO	World Health Organisation

1 Introduction and Literature Review

1.1 Helminth parasites

Helminths are multicellular eukaryotic parasites that infect both humans and ruminants worldwide causing morbidity and mortality (Muller and Wakelin, 2002). Parasitic helminths invade their definitive hosts in various ways, including penetration by the infective larvae into the skin, ingestion of the infective larvae or eggs containing infective larvae in contaminated food. Infective stages can also be transmitted by blood feeding arthropods such as mosquitoes (Anderson, 2000). Parasitic helminths are grouped into two major phyla and these include, the Nematodes (round worms) and Platyhelminthes (flat worms). Parasitic flatworms constitute one of the most diverse groups of acoelomate metazoan parasites that are highly ubiquitous, occurring in diverse aquatic and terrestrial environments. They are grouped into three major classes which includes; (1) Monogenea - predominantly ectoparasites of cold-blooded animals although some may be found attached to internal organs; (2) Cestoda - endoparasites of vertebrate hosts, they are commonly found attached by means of the scolex to the lumen of the gastrointestinal mucosa of their hosts; and (3) Trematoda- this is further grouped into two sub-classes. The first sub-class constitute the Aspidogastrea which are endoparasite of aquatic vertebrates while the second sub-class represents the Digenean which are endoparasites of human and animals. They are commonly found in the intestinal tract, blood stream, bile duct and lungs of their hosts. Generally, Digenean parasites possess a complex life cycle involving one or more intermediate hosts where asexual reproduction takes place and a vertebrate host where maturation and sexual reproduction occurs. All members are hermaphroditic in nature except *Schistosoma* species

which have separate sexes. *Fasciola hepatica*, the common liver fluke was the first trematode parasite to be recognised (Jean de Brie., 1379).

1.2 *Fasciola hepatica*.

F. hepatica is an important parasite of livestock mainly sheep and cattle, although it can also infect other vertebrates such as goats, pigs, buffalos, horses and mules (Mas-Coma and Bargues, 1997). *F. hepatica* infection, is also an emerging zoonotic disease of human, infecting millions of people worldwide (Mas-Coma, 2004). The major species (*spp*) of *Fasciola* are *F. hepatica* and *F. gigantica*. *F. hepatica* is widely distributed worldwide with high prevalence in temperate regions of America, Australasia and Europe (Torgerson and Claxton, 1999). In contrast, *F. gigantica* is found mostly in tropical regions of Asia and Africa (Torgerson and Claxton, 1999). *Fasciola spp* have a complex life cycle involving a molluscan intermediate host where asexual reproduction occurs and a vertebrate definitive host where sexual reproduction takes place. While *F. hepatica* utilizes *Lymnaea (Galba) truncatula* as the intermediate snail host for development and transmission, *L. auricularia sensu lato* is the principal intermediate host for *F. gigantica*.

1.2.1 Life cycle of *F. hepatica.*

The life cycle (Figure 1.1) begins with the release of unembryonated eggs in host faeces into the environment. 2-3 weeks after release of the eggs, the eggs undergo embryonation and then hatch into miracidia (Kaplan, 2001). This occurs in the presence of favourable environmental conditions such as

temperature (23-30^{0}C), moisture, oxygen tension (aerobic conditions), pH (7.0) and exposure to light (Rowcliffe and Ollerenshaw, 1960, Thomas, 1883). The active miracidium then swims to locate a suitable intermediate snail host. Death of miracidia occurs within 24hrs of hatching if they are unable to locate and infect an appropriate snail host (Hope Cawdery et al., 1978). Following successful invasion of a snail host, the miracidium undergoes clonal expansion and asexual reproduction producing sporocysts, which then develop into rediae and are released into the digestive gland where their migratory movement results in extensive damage to the host tissues. The rediae in turn, develops multiple germinal balls from which the next generation of larvae (called cercariae) develop and are then liberated through a birth pore. The cercaria migrate through snail tissue and are released 4-7 weeks post infection. The cercaria swim freely in water and attach to vegetation such as grasses by means of ventral suckers. Two hours after emergence, the cercaria encyst by shedding their epithelium and tail to produce the infective metacercariae. Once the infective metacercaria are ingested by the vertebrate host, excystation occurs within the small intestine and newly excysted juvenile flukes (NEJs) are then released (Kaplan, 2001). The NEJs penetrate the intestinal mucosa entering into the peritoneal cavity and migrating towards the liver. The fluke reaches the liver capsule 4-6 days after infection and first becomes established in the liver parenchyma. During this process the fluke causes hepatic necrosis and haemorrhage as a result of migration through the liver parenchyma as well as feeding on host blood and hepatic cells. This migration usually takes about 5-6 weeks (Molina-Hernández *et al.*, 2015). After 7-8 weeks of infection, flukes emerge in the bile duct where they develop into adult fluke, undergo sexual maturation and establish a patent

infection. The adult fluke produces 20,000–24,000 eggs per day (Boray *et al.*, 1969). The lifecyle of *F. hepatica* from egg to adult fluke takes about 10-12 weeks, dependent on the definitive host; being longer in cattle than sheep.

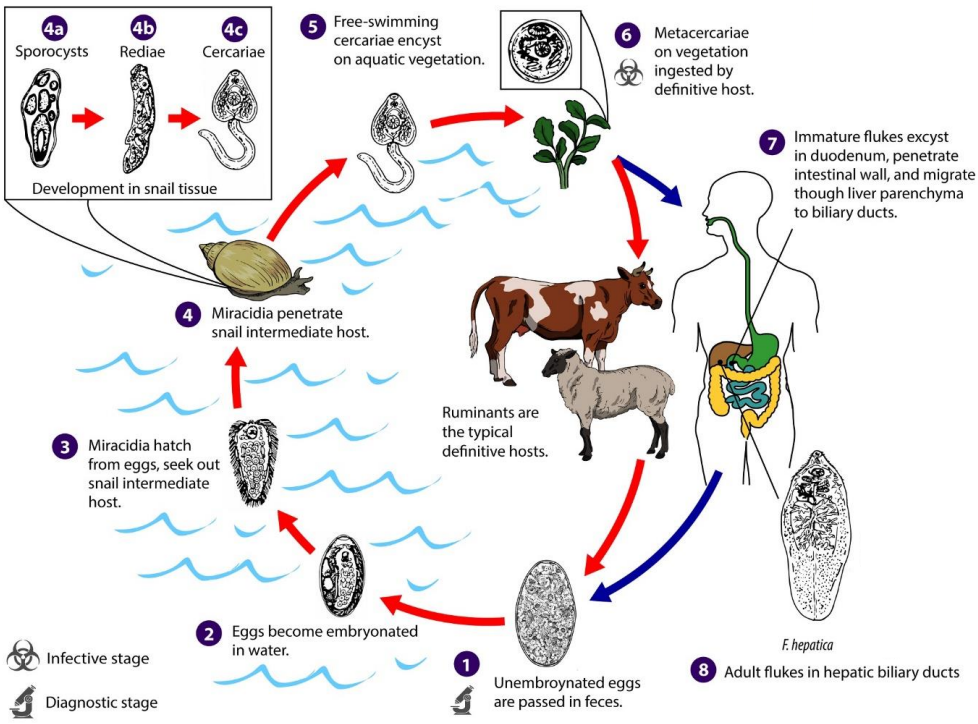


Figure 1:1: The lifecycle of *F. hepatica*. Figure adapted from https://www.cdc.gov/dpdx/fascioliasis/modules/Fasciola_LifeCycle_lg.jpg. Undeveloped eggs released in host faeces (1) undergo embryonation in water (2) to become the miracidium which swims to locate a snail host (3). Miracidia undergo asexual reproduction in the snail to produce cercariae (4). The cercariae are released from the snail host (5) and becomes encysted on aquatic vegetation as the infective metacercariae (6). The metacercariae excyst within the duodenum of ruminant or human host as NEJs prior to migrating to the bile ducts to establish a patent infection (7).

1.2.2 Pathology and economic importance of *Fasciola spp* infection.

The migratory activities within the liver parenchyma and the biliary duct coupled with the blood feeding and digestion of the host tissue by flukes make this an infection of significant economic importance. Global loss as a result of *Fasciola spp.* infections has been estimated to be over US$3 billion annually (Spithill, 1999). These losses could be attributed to sudden death of livestock in the case of heavy parasite burdens, reduced milk and meat yield, reduced wool production, loss of weight and reduced animal production or infertility as well as costs associated with treatments (Hillyer and Apt, 1997, Dargie, 1987).

Infection with *F. hepatica* may be acute or chronic depending on the host. While acute infection may result in sudden death, chronic infection manifests in diverse ways including weight loss, reduced milk and wool production. Morbidity and mortality attributed to fasciolosis is mainly due to loss of blood and plasma proteins, e.g. albumin, during the crossing of the parenchyma and the biliary migratory phase of fluke infections. Previous studies have investigated the pathological changes in the hepatic tissues of infected ruminants (Rahko, 1969, Marcos *et al*., 2007, Doy and Hughes, 1984). Migration of immature flukes within the hepatic parenchyma of cattle harbouring natural *F. hepatica* infection resulted in thrombosis and haemorrhage (Rahko, 1969). During chronic infection extensive damage to the mucosa of the bile duct was also observed (Rahko, 1969). In addition, naturally infected cattle showed pronounced fibrotic lesions (Marcos *et al*., 2007) and metaplasia of the hepatic tissue (Doy and Hughes, 1984). Conversely, sheep often succumb to acute and sub-acute fasciolosis. A recent review of an outbreak of natural ovine fasciolosis in Brazil suggested a morbidity and mortality rate of 3 - 66.7% and 5 - 50% respectively

(Fiss *et al*., 2013). In addition, the migratory ability of the immature flukes together with the anaerobic condition in the liver may result in the activation and proliferation of *Clostridium novyi*; a ubiquitous anaerobic bacteria which causes black disease (infectious hepatic hepatitis). This co-infection often result in exacerbation of fascioliasis and animals (sheep and cattle) may die as a result of black disease (Scott et al., 2005). Similar to ruminant fascioliasis, human infection may be acute to chronic. Acute phase is established when the immature flukes penetrate the intestinal mucosa into the peritoneal cavity and migrate to the bile duct. This stage is often characterised by extensive damage to the liver, internal bleeding, fever, nausea, body rash and abdominal pain. Conversely, chronic infection becomes established when the worm reaches adulthood and becomes patent in the bile duct. Infected human manifest diverse symptoms including epigastric pain, loss of appetite and weight, fever, malaise and arthralgia (Price et al., 1993).

1.2.3 Epidemiology and prevalence of fasciolosis.

F. hepatica has been reported to infect approximately 300 million cattle and 250 million sheep worldwide (Mas-Coma *et al*., 2005). Animal fasciolosis has been reported across Africa, Americas, Asia and Europe (Torgerson and Claxton, 1999) with the highest prevalence being reported in Bolivia, Peru, Iran, Portugal, France and Egypt (Mas-Coma *et al*., 1999)

In the United Kingdom, prevalence has also increased over recent years. For instance, a study on the prevalence of fasciolosis in dairy herds in 2003 for both England and Wales showed a prevalence of 48% and 86% respectively (Salimi-Bejestani *et al*., 2005). In addition, seroprevalence of fasciolosis estimated by

detection of *F. hepatica* antibodies in bulk tank milk samples of dairy herds between 2006/7 indicated a prevalence of 72% and 84% for England and Wales respectively (McCann *et al*., 2010). Moreover, a recent study by Howell *et al*., (2015) reported 77%, 88% and 73.45 prevalence in England, Wales and Scotland respectively (Howell *et al*., 2015). It is evident from these studies that prevalence of bovine fasciolosis is on the increase in England.

Human infection by *F. hepatica* has been recognised as an emerging important zoonotic disease. Between the years 1970 and 1990, there were 2594 clinical cases of human infection across 42 countries worldwide (Chen and Mott, 1990). The World Health Organisation (WHO) claimed that over 180 million people are at risk of infection (WHO, 1995). In addition, further research suggests that between 2.4 and 17 million people were infected worldwide (Rim *et al*., 1994). Prevalence of human fasciolosis has been documented in various countries across Europe, Asia, Africa, America and Oceania (Mas-Coma *et al*., 1999). For instance, in South American countries including Bolivia, Peru and Ecuador, *F. hepatica* is a significant threat to public health. In Bolivia, a high prevalence of 72% and 100% was recorded in coprological and serological samples respectively while in Peru, infection is widely distributed throughout the country (Mas-Coma *et al*., 1995).

1.2.4 Control and treatment of fasciolosis

Different approaches have been utilized to control the spread of fasciolosis. For instance, in *Fasciola* endemic areas, individual human prevention is achieved through avoiding the consumption of aquatic plants such as water cress which may be contaminated with the infective metacercaria. In addition, good farm

practices including grazing managements aimed at keeping ruminant hosts off wet areas especially in late summer/early autumn (the period that favours the release of cercariae from the snail host) are ideal, but this would mean significant loss of grazing pastures. Control of the snail host using molluscicides as well as drainage or fencing off wet areas may also prevent spread of pasture infection. However, these are often cost ineffective and impracticable in developing countries. In addition, the use of molluscicides are not reliable as efficacy is not balanced with concerns regarding environmental pollution and dangers to other aquatic species such as fish, crabs and shrimps. Moreover, rapid repopulation of lymnaeid snails due to their high biotic ability also renders snail control impractical (Roberts, 1996).

Treatment of fasciolosis relies on the use of anthelminthics. Within the past decades, various drugs have been used for the control of fasciolosis and these include derivatives of halogenated phenols, salicylanilides, benzimidazoles, sulphonamides, and phenoxyalkanes (Fairweather and Boray, 1999). Triclabendazole (TCBZ), a derivative of the benzimidazole group, has been used for the treatment of fasciolosis for the past 20 years (Fairweather, 2005). It has an advantage over other drugs because of its efficacy against both ruminant and human fasciolosis (Apt *et al*., 1995) and is effective against both juvenile and adult flukes (Boray *et al*., 1983, Keiser *et al*., 2005). The efficacy of TCBZ as a front-line drug for fascioliasis relies upon its ability to bind the fluke's tubulin molecules and causing disruption to the microtubule-based processes (a process critical for regulating important activities of the worm) of the parasite (Fairweather and Boray, 1999). For instance, loss of microtubule process due to the actions of TCBZ have been shown to cause damage to the vitelline cells of

the fluke and prevent the translocation of secretory bodies from the cell bodies to the parasites tegumental surface and this results in significant damage to the parasites tegument (Stitt and Fairweather, 1996, Stitt and Fairweather, 1994). Indeed, incubation of parasites with TCBZ-sulphoxide (an active component of TBCZ) revealed major destruction to the tegument of *F. hepatica* susceptible strains (Robinson *et al*., 2002). Previous studies have shown that TCBZ used at a dosage of 10mg/kg and 2.5-15mg/kg in goats and sheep experimentally-infected with *F. hepatica* showed 94-100% and 92-100% efficacy against immature and adult flukes respectively (Martinez-Moreno *et al*., 1997, Boray *et al*., 1983). Efficacy was found to be dependent on the time when TBCZ was administered as early treatment protected the host from development of lesions in the parenchyma and hepatic tissue while late treatment was not effective in control of lesion development (Martinez-Moreno *et al*., 1997). A recent report suggested that mass drug administration (MDA) of TCBZ was highly efficacious in reducing human fasciolosis in endemic area of Batallas and Huacullani in the Bolivian Altiplano from 12.6% and 26.9% reported in 1999 to 0.7% and 1% respectively (Mollinedo *et al*., 2019). However, the sustainability of this preventive chemotherapy approach remains uncertain with the emergence of resistance to TCBZ (Overend and Bowen, 1995).

1.2.5 Drug resistance

Resistance to TCBZ treatment in ruminants was first reported in Australia (Overend and Bowen, 1995) and subsequently in parts of Europe including the Netherlands (Moll *et al*., 2000), Ireland, Scotland, Wales, Northern Ireland and Spain (Alvarez-Sanchez *et al*., 2006). The effect of resistance is clear as, for

example on a farm in the Netherlands, mortality was reported in sheep due to sub-acute to chronic fascioliasis despite repeated treatment with TBCZ (Moll *et al*., 2000). Furthermore, experimental infection of sheep with isolates of either *F. hepatica*-TBCZ resistant or susceptible strains, showed an efficacy of 10.8% and 99.8% respectively following TBCZ treatment (Gaasenbeek *et al*., 2001). Whilst the genetic mechanism of drug resistance is not yet known, altered uptake and enhanced efflux of TCBZ in resistance strains have been shown to be mediated by ABC transporter; p-glycoprotein (Reed et al., 1998, Alvarez et al., 2005). Wilkinson et al., (2012) showed that a single nucleotide substitution in the nucleotide binding domain of p-gycoprotein differs between resistance and susceptible strains of *F. hepatica* (Wilkinson et al., 2012).

In order to control the increasing resistance to anthelmintic treatments whilst also maintaining drug efficacy, 'refugia 'which involves the maintenance of a proportion of population of parasite that have not been exposed to anthelmintic treatment, have been considered logic and best practice (Van Wyk, 2001). Refugia-based control strategies have been shown to be efficacious in slowing resistance development while preserving susceptibility gene within a parasite population (Melville et al., 2016, Van Wyk and Bath, 2002, Soulsby, 2007).

Refugia-based control approach has gained much attention in the control of gastrointestinal nematode infections (Kenyon et al., 2009). For instance, the use of refugia approach *via* targeted selective treatment (TST, treating a proportion of animals) have been shown to be efficacious in lowering number of anthelmintic treatment and preserving drug efficacy in *Teladorsagia circumcincta* dominant regions (Kenyon et al., 2013). Noteably, the applicability of this approach to control anthelmintic resistance in *F. hepatica* infection has

been previously discussed (Hodgkinson et al., 2019). The presence of *F. hepatica* stages within the snail intermediate host, viable eggs in pasture as well as the presence of infective metacercariae on vegetation suggest that refugia for *F. hepatica* exists. Despite the pre-existence of refugia in *F. hepatica*, resistance to drug treatment is still on the increase (Moll et al., 2000, Gaasenbeek et al., 2001). Till date, refugia-based control strategies such as maintenance of a proportion of untreated animals for reducing development of anthelmintic resistance in *F. hepatica* still remains to be evaluated (Hodgkinson et al., 2019). Although refugia based approached may represent best practice to control drug resistance in *F. hepatica*, factors such as the high pathogenicity of *F. hepatica*, and the ability of the parasite to undergo clonal expansion within the snail host (a condition that allows for the development of resistance gene (Beesley et al., 2017)), may impair the use of refugia to control liver fluke infection. Taken together, due to the problems of drug resistance, the presence of drug residues in the food chain, and the cost of TCBZ, there is an urgent need for the development of an alternative effective vaccine.

1.2.6 Vaccine candidates.

Studies have investigated the potential of several vaccine candidates for the control of fasciolosis. A number of parasite immunomodulatory molecules including Cathepsin L proteases (CL), glutathione S-transferases (GSTs), Leucine aminopeptidase (LAP), Fatty acid binding protein (FABP), peroxiredoxin (Prx), and haemoglobin (Hb) have been shown as attractive vaccine targets (McManus and Dalton, 2006, Toet *et al*., 2014). For instance, sheep immunized with *F. hepatica* GST prior to infection have a reduced worm

burden at post mortem, lower egg counts and reduced liver damage when compared to infected unimmunized controls (Sexton *et al*., 1990). Similarly vaccination of rabbits with rFhLAP, in Freund's adjuvant, induced a high IgG responses and conferred 78% protection as determined by a reduced worm burden at post mortem following *F. hepatica* challenge (Acosta *et al*., 2008). Moreover, vaccination of cattle with rFhCL1 formulated in mineral based adjuvant inhibited alternative activation of macrophages and also yielded a 48.2% reduction in fluke burden when compared to control groups (Golden *et al*., 2010). Vaccine trials which employed a combination of two or more immunomodulatory enzymes have been shown to be more efficacious in achieving protection against *F. hepatica* infections. For instance, vaccination of sheep with either FhCL1 or FhCL2 only resulted in 33 and 34% protection respectively measured by burden of flukes at post mortem. However, the combination of both molecules increased protection to 60% while a combination of three molecules FhCL1, FhCL2 and FhLAP further boosted protection to 78% (Piacenza *et al*., 1999). Moreover, vaccination trials in cattle using a combination of FhCL2 and FhHb yielded a 72.4% protection determined by evaluating worm burden at post mortem (Dalton *et al*., 1996). Despite the advances in vaccine development, efficacy required for commercialization has not been attained due to the powerful immunomodulatory capacity of the parasite together with the need to develop commercially acceptable adjuvants (Dalton *et al*., 2013, Spithill and Dalton, 1998). Hence, for a successful development of vaccines against *F. hepatica* infection, there is a need to improve our understanding of the parasite immune evasion strategies used to penetrate the host and hijack the accompanying immune response.

1.3 Immunity to helminths

The host immune response elicited to eliminate helminth parasites involves the co-operation of both innate and adaptive immune cells. Paramount to the initiation of the immune response is the ability of the host to distinguish self from non-self-antigens. This is particularly important for the protection against invading pathogens as well as preventing autoimmune disorders and cellular proliferation of mutant or malignant cells (Wakelin, 1996). In other to elicit an appropriate immune response, there is the need for processing and antigen presentation by antigen presenting cells (APCs) to naïve T-cells. Major histocompatibility complexes (MHC) are important for proper functioning of APCs. Antigen recognition processes begins when foreign antigens are taken up by APCs and are broken-down into peptide through enzymatic processes within the cellular endosomes (Straube, 2016). The peptides then bind to MHC molecules forming MHC-Peptide complex which are expressed on the cell surface of APCs for recognition by T-cell receptors (TCR). Whilst CD4 T-cells responses are associated with class II MHC-peptide complexes, CD8 T-cells recognises that of class I (Figure 1.2).

CD4 T-cells are a sub population of T lymphocytes that mediate cellular immune responses. They play a crucial role in immunity to helminth infection via regulation of the activities of various immune cells including helping B cells, mediating cytotoxic T lymphocyte function, regulating macrophage function; ultimately controlling autoimmunity and inflammatory responses (Luckheeram *et al*., 2012, Mosmann and Coffman, 1987, Zhu *et al*., 2009).

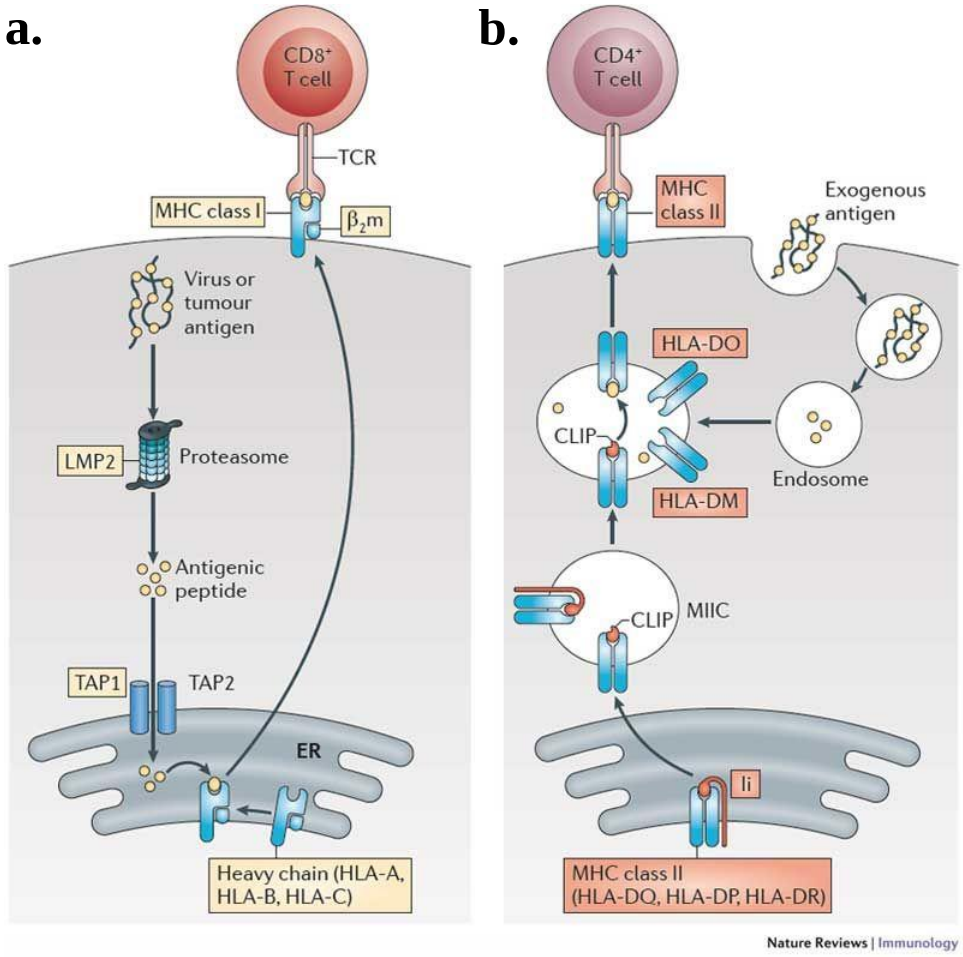


Figure 1:2: The antigen processing pathways of MHC class I and II. For the MHC class I pathway (a), intracellular antigens are first broken down into peptides by the proteasome prior to being transported to be taken up by MHC-I molecules. The MHC-I/peptide complex is then relocated to the cell surface where antigen-peptide is presented to $CD8^+$ T-cells. Conversely, extracellular antigens (b) are processed into peptide within the endosomes before being bound to the MHC-II molecule which then present antigen peptide to $CD4^+$ T-cells on cell surface. Figure was adapted from (Kobayashi and Van Den Elsen, 2012).

1.3.1 T-helper (Th) cells in helminth infections

Once antigen recognition has occurred naïve CD4 T-cells can differentiate into specific effector populations including Th1, Th2, Th17, follicular helper T cells (Tfh), induced T regulatory cells (iTregs), regulatory type 1 cells (Tr1) and Th9 cells (Luckheeram *et al*., 2012) (Figure 1.3).

The major T-cell responses implicated in the defence against parasitic infection are Th1, Th2 or T regulatory responses. Th1 cells promote type1 immune responses which are targeted at eliminating intracellular pathogens including protozoan, bacteria and viruses. Th1 cells are characterised by the production of tumour necrosis factor (TNF)-α, interferon gamma (IFN)-γ, interleukin (IL)-2 and IL-12. Th1 cytokines help to mediate the production of antibodies, classically activate macrophage to produce nitric oxide (NO) and also promote delayed type hypersensitivity (DTH) and inflammatory responses (Zhu *et al*., 2009). T-bet has been shown to be a Th1-specific transcription factor which upregulates production of IFN- γ and hence is crucial to the differentiation of naïve CD4 T cells to the Th1 phenotype. Conversely, the Th2 subset promotes a type 2 immune response which is involved in the host defence against extracellular pathogens. Th2 cells secret IL-4, IL-5, IL-9, IL-10 and IL-13 (Luckheeram *et al*., 2012). Th2 cells are associated with several cellular activities including antibody production and class switching, recruitment of eosinophil and mast cells as well as enhancing immediate type hypersensitivity (ITH). The major master regulator of the Th2 lineage differentiation is the GATA-binding protein (GATA3) which is upregulated by an IL-4 induced STAT6 dependent pathway (Zhu *et al*., 2001).

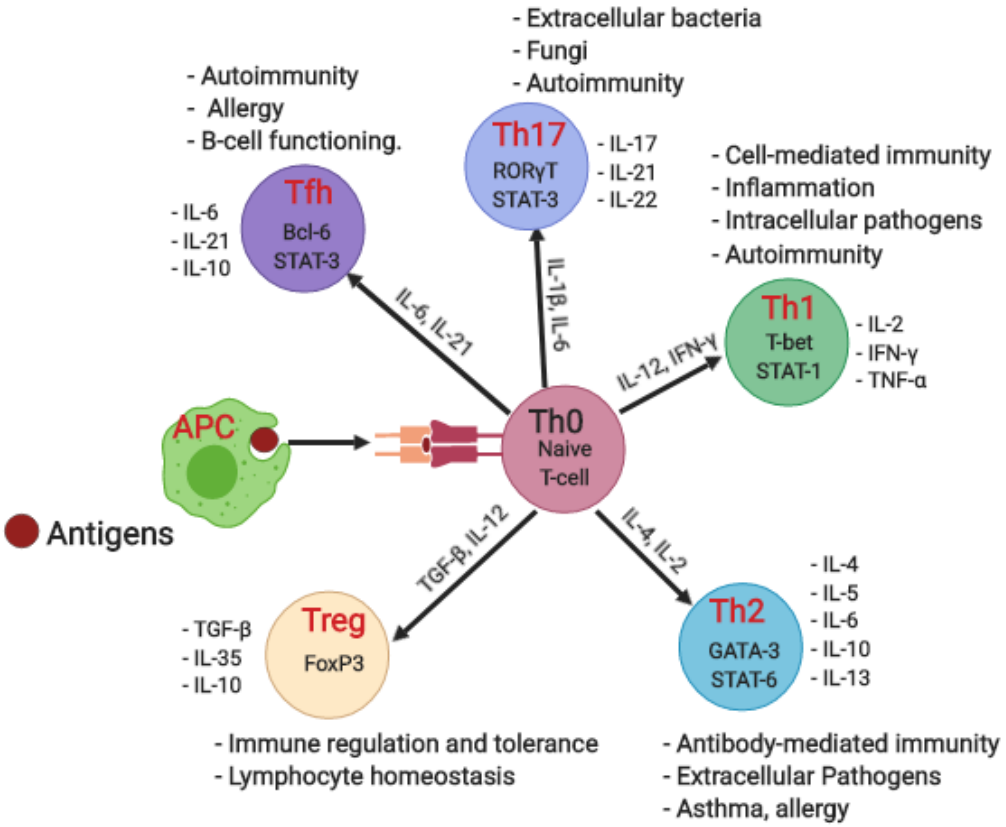


Figure 1:3: The diffentiation of naive T-cells into specific effector subsets. Upon antigen presentation by APCs, naïve $CD4^+$ T-cells (Th0) differentiate into specific effector lineages (Th1, Th2, T-reg, Tfh, and Th17) depending on the cytokine environment. Specific T-cell subsets produce an array of cytokines and participate in various immunological functions. Figure was adapted from (Yu *et al*., 2012) using BioRender (https://biorender.com/).

1.3.2 Th2-induced effector cells

Multiple innate effector cells have been shown to participate in the response to parasitic helminths. Eosinophilia, mastocytosis and recruitment of basophils together with activation of DCs and macrophages are critical mediators of the host response to helminth infection including *F. hepatica*.

DCs participate in the initiation and maintenance of adaptive immunity. DCs become activated upon stimulation by pathogens and recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRR) that are expressed on DCs. Depending on the stimulating ligand and the accompanying cytokines released upon activation, DCs promote polarization of naïve T cells to either Th1, Th2 or T-reg phenotype. Activated DCs classically expresses activation markers OX40L, CD80, CD86, and MHCII as well as secreting pro-inflammatory cytokines TNF-α, IFN-γ, IL-6 and IL-12. However, these responses have been shown to be altered in response to various helminth products.

Helminth parasite are capable of modulating DC function in both Toll-like receptor (TLR) dependent and independent signalling pathways. Helminth antigens provide either a stimulatory or inhibitory effect on DC activation, maturation and cytokine production as well as determining the fate of the ensuing immune response. For instance, lacto-*N*-fucopentaose III (LNFPIII), a carbohydrate molecule of *S. mansoni*, drives maturation of murine bone marrow DC *in vitro* via a TLR-4 signalling pathway and promotes the generation of Th2 responses in naïve T-cells in response to OVA-peptide stimulation (Thomas *et al*., 2003). Consistent with Thomas *et al*., TLR-2 dependent activation of human monocyte-derived DCs by lysophosphatidylserine (Lyso-PS; a lipid component

of *S. mansoni*) drives the development of IL-4 expressing Th2 cells and functionally active IL-10 producing T-reg cells (van der Kleij *et al*., 2002). In addition, murine bone marrow derived DC (BMDCs) exposed to *N. brasiliensis* excretory secretory (NES) products prior to adoptive transfer to the murine host, induced Th2 immune responses and also displayed enhanced upregulation of activation markers; CD86, OX40L, IL-6 and IL-12$_P$40. However, this response was found to be dependent on the heat labile component of NES, as heat treatment of NES abrogated DC-dependent Th2 responses *in vivo* and *in vitro* (Balic *et al*., 2004).

Contrary to promoting DC activation and maturation, some helminth products have an inhibitory effect on DC responses. For instance, stimulation of murine DCs with *S. mansoni* egg antigen (SEA) down regulated the expression of activation markers and secretion of cytokines by LPS-stimulated cells (Kane *et al*., 2004). ES-62 a secreted molecule of *Acanthocheilonema viteae* suppressed the inflammatory and Th1 responses in DC both *in vitro* and *in vivo* evidenced by inhibiting the production of IL-12p35, IFN-β and TNF-α (Goodridge *et al*., 2004). Moreover, classical production IL-2, IL-15 and TNF-α by DCs activated with various TLR ligands were inhibited in response to treatment with *Taenia crassiceps* ES (TcES). (Terrazas *et al*., 2010).

Consistent with other helminths, previous studies have reported a suppression of DC responses and dominance of Th2 immune responses in DCs stimulated with various *F. hepatica* secreted molecules (Falcón *et al*., 2010, Falcón *et al*., 2014, Hamilton *et al*., 2009). FhESP failed to induce activation of DCs but also suppresses LPS-mediated maturation of DCs. DCs pulsed with FhESP in an ova-peptide sensitization assay drive differentiation of $CD4^+$ T-cells into Th2 and T-

reg phenotype (Falcón *et al*., 2010). Furthermore, a low molecular weight serine protease inhibitor termed *kunitz* inhibitor present in *F. hepatica* total extracts, ES and tegumental coat antigen has been shown to be responsible for suppressing pro-inflammatory responses on DC stimulated with LPS (Falcón *et al*., 2014) .

During helminth infection, macrophages act as both APC and effector cells. Macrophages involved in protection against intracellular pathogens are known to be classically activated (CAMϕ) (Figure 1.4). The CAMϕ pathway is activated in response to pro-inflammatory cytokines such as IFN-γ and stimulus from microbial pathogens including lipopolysaccharides (LPS) (Raes *et al*., 2002). This pathway is characterised by the expression of inducible nitric oxide synthase (iNOS) which metabolises L-arginine (Raes *et al*., 2002, Gordon, 2003). However, macrophages can deviate from this canonical pattern and become activated by an alternative pathway. These macrophages are termed alternatively activated macrophages (AAMϕ) (Kreider *et al*., 2007, Gordon and Martinez, 2010). In contrast to CAMϕ, this pathway is induced during helminth infection in response to Th2 cytokines IL-4 or IL-13 (Flynn *et al*., 2007a), although some studies suggest that macrophages could also be alternatively activated independent of IL-4 or IL-13 (Donnelly *et al*., 2005, Noel *et al*., 2002). AAMϕ preferentially expresses the molecular markers arginase-1 (Arg1), found in inflammatory zone (FIZZI) and macrophage protein Ym1 (Loke *et al*., 2002, Donnelly *et al*., 2005). AAMϕ play a key role in maintenance of Th2 responses and suppression of protective immunity during chronic helminth infections (Rodriguez-Sosa *et al*., 2002, Donnelly *et al*., 2005).

It is becoming increasingly apparent that *F. hepatica* infection converts macrophages from the classical to alternative phenotype suggesting that this ability could be used by the parasite to create an immune regulatory environment skewed towards Th2 responses and favourable to parasite survival. The role of macrophages in Th2 induction has been studied in various models of *F. hepatica* infection (Donnelly *et al*., 2005, Flynn *et al*., 2007a). Infection of mice with *F. hepatica* or injection with the molecule thioredoxin peroxidase (TPx) recruits peritoneal macrophages that exhibit an AAMϕ phenotype both *in vivo* and *in vitro* (Donnelly *et al*., 2005). Moreover, these macrophages induced expression of FIZZ1 and Ym1 as early as day 1 post infection, although low levels of iNOS were detectable at later stages of infection, suggesting an immune evasion strategy at the onset of infection. Moreover, *in vitro* stimulation of an ovine cell lines (MOC7) with *F. hepatica* derived molecules drives AAMϕ (Flynn *et al*., 2007a). Recent studies have demonstrated the plasticity of macrophages and have noted that aside from CAMϕ or AAMϕ pathways, macrophages could also exhibit a regulatory phenotype which is distinct from the other pathways by characteristic expression of IL-10 and suppression of IL-12 production. They have also been shown to play a role in limiting immunopathology and tissue damage during helminth infection (Fleming and Mosser, 2011). For instance, a mixed macrophage population constituting both AAMϕ and regulatory macrophages was induced by various *F. hepatica* molecules (Donnelly et al., 2005, Sulaiman et al., 2016).

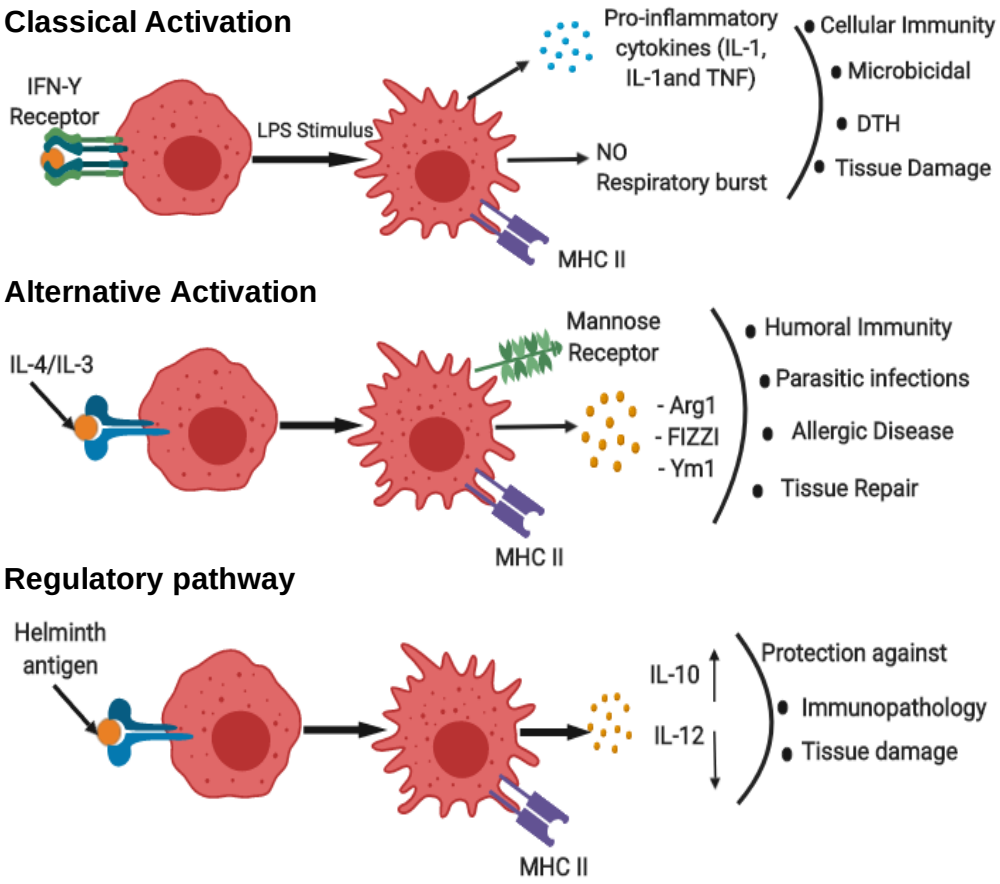


Figure 1:4: Macrophage activation pathways. Macrophages are classically activated in response to IFN-γ or LPS stimulation and they produce the indicated pro-inflammatory cytokines that mediate cellular immunity against microbial pathogens. Alternative macrophage activation on the other hand mediates defence against helminths and allergic diseases while regulatory macrophages regulates immunopathology. Image was adapted from (Gordon, 2003) using biorender.com.

Eosinophils are innate effector cells that participate in the immune response in parasitic and allergic diseases. Eosinophils are recruited from the circulation to inflammatory sites where they induce tissue damage and dysfunction by secretion of a wide range of cytotoxic granule proteins. The granule proteins secreted by eosinophils may include major basic protein (MBP), eosinophil peroxidase (EPO),eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) (Rothenberg and Hogan, 2006, Gleich, 2000). Degranulation of eosinophils and the release of cytotoxic granules onto the surface of the parasites results in significant structural damage and this has been shown to mediate the killing of some helminth parasites in an antibody dependent cellular cytotoxicity mechanism (ADCC) (Glauert *et al*., 1978). For instance, eosinophils were shown to adhere to and kill schistosomula of *S. mansoni in vitro* in an ADCC and compliment dependent manner (Mackenzie *et al*., 1977, Glauert *et al*., 1978). Moreover, Buys *et al*., (1981) showed that killing of new born *Trichinella spirallis* was mediated by eosinophil peroxidase activity (Buys *et al*., 1981).

Previous studies have suggested a role for eosinophils in the killing of *F. hepatica* in experimental rat infections. Davies & Goose (1981) demonstrated that injection of NEJ flukes into the peritoneal cavity of immunized rats resulted in recruitment of eosinophils which adhered to the parasite and then degranulated, releasing peroxidase that killed the fluke (Davies and Goose, 1981). Similarly, destruction of flukes in a challenged rat model was due to eosinophil adherence and coating of the parasite with IgG1 and IgG2a antibodies (Van Milligen *et al*., 1999, Van Milligen *et al*., 1998). Sensitization of rats with a single infection with *F. hepatica* enhances resistance to re-infection. The

finding that rats are resistant and mice susceptible to infection with *F. hepatica* is correlated with the eosinophil count. When rats and mice were infected with *F. hepatica* or injected with excretory secretory (ES) products, a dramatic increase in peripheral blood eosinophils was observed; however, this dropped one-week post treatment in mice but remained high in rats (Milbourne and Howell, 1990). Conversely, although Bossaert *et al*., (2000) noted an increase in the number of blood, skin and liver eosinophils in response to *F. hepatica* antigen, this did not correlate positively with resistance in infected cattle. Similarly, in experimental infection of sheep with *F. hepatica* a biphasic eosinophilia corresponding to 4 and 9 to 10 weeks post infection was noted and this did not result in reduced parasite burden (Raadsma *et al*., 2007). Although eosinophils are constantly recruited during liver fluke infection in the ruminant host, a definitive role for them has yet to be proven.

Mast cells are inflammatory cells that are developed from hematopoietic progenitor cells and are distributed in the blood vessels and all vascularized tissues where they develop into maturity. There are two types of mast cells and these include; (1) connective tissue mast cells which are commonly found in loose connective tissues and skin, (2) Mucosal mast cells which are mainly found in gastrointestinal mucosa and peripheral airways (Wasserman, 1990). They play an important role both in allergic disorders and helminth infections where they are associated with Th2 and IgE responses (Galli *et al*., 2005). For instance, the production of IL-4 has been shown to be crucial for inducing antibody class switching to the IgE subtype and the interaction of IgE via the high affinity receptor, FcεRI, expressed on mast cells. This leads to the activation of mast cells and subsequent degranulation with the release of several

mediators that regulates inflammatory responses (Galli and Tsai, 2008, Capron *et al*., 1981, Hagan *et al*., 1991, Paul and Zhu, 2010).

Persistence of parasites in *Strongyloides ratti* infected nu/nu mice (mice lacking thymus) was associated with a reduced number of mucosal mast cells. However, repeated stimulation with IL-13, (a mast cell growth factor), resulted in an increased numbers of mucosal mast cells and complete worm expulsion (Abe and Nawa, 1988). In addition, increased serum levels of mMCP-1 (a protease secreted by activated mast cells) was consistent with a reduced *Heligmosomoides polygyrus* fecundity rate in a murine wildtype or mast cell deficient host (W/W^v) mice reconstituted with bone marrow cells from mast cells sufficient (WBB6-$F1^{+/+}$) mice (Hashimoto *et al*., 2010). These studies suggest an indispensable role for mast cells in mediating protective immunity against some nematode parasites. Mast cells are also associated with *F. hepatica* infection. Following *F. hepatica* infection or injection of ES antigen, the number of peritoneal and hepatic mast cells were markedly increased (Vukman *et al*., 2013a), but *F. hepatica* antigen has been shown to suppress the mast cell's ability to initiate Th1 responses against microbial antigen (Vukman *et al*., 2013b).

In addition to eosinophils and mast cells, basophils also play a crucial role in Th2 immunity to helminth infections. Basophils have been previously shown to be capable of antigen presentation to CD4 T-cells and initiate the differentiation of $CD4^+$ $Th2^+$ cells both in *vivo* and *in vitro* in response to an allergen protease stimulation in mice (Sokol *et al*., 2009, Sokol *et al*., 2008). In addition, antibody-mediated depletion of basophils following infection of mice with *T. muris*

resulted in an impaired Th2 response and the inability of host to clear their parasite burden (Perrigoue *et al*., 2009). Moreover, infection with *Nippostrongylus brasiliensis*, drives the recruitment of basophils that secrets IL-4 in secondary lymphoid tissues of infected IL-4^{EGFP} mice (Min et al., 2004). However, basophil number was not increased in infected $Rag2^{-/-}$ mice (B/T-cell deficient) when compared to wildtype. Basophil recruitment and IL-4 secretion was enhanced when $Rag2^{-/-}$ mice were reconstituted with CD4 T-cells, implicating a role for CD4 T-cells in regulating basophil function during murine *N. braziliensis* infection (Min et al., 2004). Evidence was shown that the haematopoiesis of basophils from bone marrow progenitor cells and the subsequent polarization of Th2 responses was regulated by thymic stromal lymphopoietin (TSLP) in an IL-3 independent pathway. TSLP promoted basophil recruitment whereas the disruption of TSLP signalling pathway impaired basophil function (Siracusa *et al*., 2011).

Furthermore, basophils regulate the function of other innate effector cells including macrophages, eosinophils and DCs. Mice infected with *N. brasiliensis* undergo activation of basophils which in turn stimulates systemic accumulation of eosinophils and development of AAMϕ (Ohnmacht and Voehringer, 2009). Although basophils appear to participate in the development of Th2 immunity, recent evidence suggests that Th2 responses can still develop in the absence of basophils. Indeed, basophil-depleted mice mount an efficient Th2 response against *N. braziliensis* infection or allergens (Ohnmacht *et al*., 2010). Moreover, Mcpt8Cre mice (basophil lineage deletion) back-crossed with IL-4^{eGFP} mice demonstrated comparable secretion of IL-4, IL-5, IL-13 and IgE production as their wild type counterparts in murine schistosomiasis (Schwartz *et al*., 2014).

Taken together, the studies reviewed above suggest that parasitic helminths including *F. hepatica*, are capable of co-opting innate cells and modulating their functions early in the initial stages of antigen presentation /immune responses to generate a conducive niche favouring their long-time survival and the generation of chronic infection.

1.3.3 Th2 Response in Helminth infections

Helminth infection often generates a Th2 immune response typically characterised by the production of cytokines IL-4, IL-5, IL-10 and IL-13 together with recruitment of eosinophils, mast cells and basophils as well as secretion of immunoglobulin (Ig) E and IgG1 (Abbas *et al*., 1996, Maizels *et al*., 2004).

Selective induction of Th2 cells and the suppression of potentially-protective Th1 responses has been considered a common manifestation of most helminth infections (Maizels *et al*., 2004). Pearce *et al*., (1991) demonstrated that injection of mice with *S. mansoni* eggs induced the upregulation of the Th2 cytokines, IL-4 and IL-5. This response was shown to be highly dependent on egg antigen as a Th1 responses characterised by high production of IFN-γ and IL-2 dominated at the pre-patent stage of infection or when mice were vaccinated (Pearce *et al*., 1991). Similarly, in the filarial parasite *Brugia malayi* infection, adults induced a polarized Th2 response with production of IgE, IgG1 and IL-4, however, this response was altered in mice injected with microfilariae alone (first L1 stage of *B. malayi)*, suggesting that *B. malayi* induces a contrasting immune response depending on the parasite stage of its life cycle. Microfilaria infection induced a Th1 response as evidenced by enhanced

secretion of IFN-γ (Lawrence *et al*., 1994). Induction of the Th2 response in this model was shown to be dependent on the presence of IL-4 signalling, and this was supported by the finding that IL-$4^{-/-}$ mice infected with adult *B. malayi* showed decreased Th2 responses while possessing a boosted Th1 response (Lawrence *et al*., 1995).

In addition, Marechal *et al*., (1997) compared the cytokine responses of *Litomosoides sigmodontis* infected susceptible and resistant mouse strains and they showed that both strains exhibited very weak immune response at the early stage of infection as splenocytes from infected hosts had poor cytokine responses and cellular proliferation capacity. However, as infection progressed towards a chronic state, *L. sigmodontis* susceptible mice induced a stronger Th2 response when compared to the resistant strains (Marechal *et al*., 1997). This suggests that protective immunity to *L. sigmodontis* is dependent on Th1 responses and that the Th2 response elicited was not associated with protection.

Furthermore, T-cell responses during *F. hepatica* infection in various hosts including bovine (Clery et al., 1996, Flynn and Mulcahy, 2008), murine (O'Neill et al., 2000) and rat (Cervi *et al*., 1998) have been studied. Evidence from previous studies suggests protection against *F. hepatica* infection may be dependent on Th1 or a balance of Th1/Th2 response (Flynn and Mulcahy, 2008, Golden et al., 2010). However, several studies have shown that this protective immune response is down modulated during *F. hepatica* infection. For instance, Clery *et al*., (1996) showed that adult cattle responding to adult fluke antigen displayed no production of IFN-γ. Moreover, similar studies carried out in young cattle showed maximal expression of IFN-γ during acute phase of infection

(Clery and Mulcahy, 1998). This suggests the presence of Th1 immune response at the onset of infection which then remained suppressed as infection becomes chronic and allows for Th2 cells to dominate. Similarly, *F. hepatica* infection in mice showed a marked polarization of Th2 responses as evidenced by enhanced secretion of IL-4 and IL-5 with little or no production of IL-2 and IFN-γ (O'Neill et al., 2000). Brown *et al*., (1994) described the cytokine responses of *F. hepatica*-infected cattle peripheral blood mononuclear cells (PBMCs) to concavalin A (Con A) stimulation; high expression of IL-4 was noted with low or undetectable IL-2 or IFN-γ mRNA. Although non-specific Th clones designated (Th0) expressing combinations of Th1 and Th2 cytokine mRNA were detected, there was no specific Th1 cell response detected. Moreover, in the murine hosts, splenic CD4 T-cells taken from infected animals showed a high production of IL-4 and a down regulation of IFN-γ and IL-2. Induction of the Th2 response in this model was shown to be IL-4 dependent as IL-$4^{-/-}$ mice failed to generate a Th2 response but produced high levels of IFN-γ (O'Neill et al., 2000).

Notably, from these studies it is evident that type 1 and 2 responses counter regulate one another, suggesting a molecular mechanism driven by the parasite that allows for biased immune regulation. Evidently, the polarization of non-protective (Th2) immune response allows for parasite persistence and development of chronic infection.

1.3.4 Antibody responses to helminth infection

B-cells play an important role during Th2 response to helminth infections. Protective immunity against some helminth parasites haS been shown to be mediated by B-cells. Following infection of mice with an enteric nematode parasite *Heligmosomoides polygyrus*, an early production of non-specific IgG1 and IgE together with a late production of parasite-specific IgA and IgG resulted in inhibition of parasite fecundity and protected the host from re-infection, respectively (McCoy *et al*., 2008). In addition, prevention of worm establishment in a mouse mode of *T. muris* infection was associated with B-cell and antibody responses. µMT (B-cell deficient) mice demonstrated enhanced susceptibility to infection. However, resistance to infection was restored through administration of parasite-specific IgG1 or transfer of B-cells from naïve mice to µMT mice (Blackwell and Else, 2001).

It is well established that antibody of the IgG1 isotype predominates during *F. hepatica* infection in ruminants (O'Neill et al., 2000, Bossaert et al., 2000a, Chauvin and Boulard, 1996, Ortiz et al., 2000, Murphy et al., 1998). For instance, in a murine model of *F. hepatica* infection, O'Neill et al., (2000) reported that all mice with a dominant Th2 response had anti-fluke IgG1 antibodies in their serum, but IgG1 antibody was not detectable in IL-$4^{-/-}$ mice (O'Neill et al., 2000). This suggests that IL-4 plays an important role in the development of parasite-specific IgG1 responses in *F. hepatica* infection. Induction of IgG1 antibody isotype does not correlate with a reduced fluke burden, indicating that this response might not be protective (Raadsma *et al*., 2007, Mulcahy, 1998). Whilst non-immunized cattle displayed high specific serum IgG1 and high fluke burden, their vaccinated counterparts produced high

levels of IgG2 or mixed IgG1/IgG2 conferring high protection against infection (Mulcahy, 1998). Protection was associated with the level and avidity of IgG2 induced. These findings suggest that induction of IgG2 or a combination of both antibody isotypes may be needed for protection against liver fluke infection. Thus, preferential induction of non-protective antibody responses by *F. hepatica* has implications for the maintenance of parasites and their propagation.

1.3.5 T-Regulatory cells (T-reg) in helminth infections

T regulatory (T-reg) cells are a sub-population of CD4 T-cells that play a critical role in the maintenance of immune homeostasis. Induction of a T-reg dominant environment represents an endogenous immune mechanism to protect the host from immunopathology that may arise from excessive immune responses to foreign antigens. Notably, parasitic helminths have also exploited this arm of the immune system to prevent expulsion and maximize survival within the host (Scott-Browne *et al*., 2007, Taylor *et al*., 2005).

T-reg cells are classified into natural (nT-reg) and inducible or adaptive T-reg (i-Treg). Natural T-reg are $CD4^{+}CD25^{+}$ T-cells which originate from the thymus and they regulate the functioning of self-reactive T-cells in the periphery (Sakaguchi *et al*., 2008). They constitutively express the *forkhead* box P3 (Foxp3) gene. Conversely, iT-reg are generated from naïve $CD4^{+}$ T-cells in the peripheral circulation as a result of stimulation by APC, or regulatory cytokines such as TGF-β or IL-10 (Apostolou *et al*., 2002) . They may also express FoxP3. FoxP3 plays a crucial role in limiting excessive T-cell responses. For instance, *FoxP3* gene mutation gives rise to scurfy, an X-linked recessive mouse mutant with excessive CD4 T-cell proliferation and marked production of pro-

inflammatory molecules (Brunkow *et al*., 2001). Similarly, deficiency of the *FoxP3* gene in human patients results in multiple auto-immune disorders including polyendocrinopathy, enteropathy and X-linked syndrome (IPEX) (Ochs *et al*., 2005). Several studies have shown that helminth infection or their antigens drive the induction or expansion of host FoxP3 cells to suppress helminth directed responses (Belkaid *et al*., 2002, Taylor *et al*., 2005, Wilson *et al*., 2005, Grainger *et al*., 2010, Rausch *et al*., 2009, Layland *et al*., 2013). For instance, persistence of parasites during chronic *L. major* infection in mice that are known to be resistant to *L. major* infection was associated with the presence of $CD4^+CD25^+$ T-reg cells (Belkaid *et al*., 2002). In addition, infection with live *B. malayi* parasites promoted expansion of $CD4^+CD25^+Foxp3^+$ T-reg cells which prevented T-cell proliferation *in vitro* (McSorley *et al*., 2008). Moreover, induction and expansion of the natural $CD4^+CD25^+Foxp3^+$ T-reg cells has been shown to be mediated by the TGF-β signalling pathway (Peng *et al*., 2004, Marie *et al*., 2005, Liu *et al*., 2008). Peng *et al*., (2004) demonstrated that $CD4^+CD25^+Foxp3^+$ T-reg cells expanded dramatically within the pancreatic islets in response to a TGF-β pulse and prevented onset of diabetes in NOD mice (Peng *et al*., 2004). Reversal of T-reg cell activities has been shown to restore host resistance and promote parasite clearance (Taylor *et al*., 2005). For instance, *S. mansoni*-mediated suppression of development of allergic air way disease in an ovalbumin sensitized mouse was abrogated by depleting T-reg cells (DEREG mice with a diphtheria response deletion) (Layland *et al*., 2013). Moreover, these mice showed a reduced parasite egg burden when compared to uninfected controls at post-mortem (Layland *et al*., 2013). Sawant *et al*., (2014) demonstrated that FoxP3 T-reg depletion in DEREG mice following acute but

not chronic, infection with *T. muris* resulted in enhanced worm clearance associated with pronounced Th2 induction (Sawant *et al*., 2014). Conversely, depletion of T-reg activities at a later stage of infection abrogated protective immunity and resulted in increased worm burden. This finding implicates a role for T-reg cells in inhibiting protective immune response during *T. muris* infection in a time dependent manner.

T-reg induction promotes the production of IL-10 and TGF-β. These cytokines are critical for the maintenance of immune suppression in chronic infections (Beiting et al., 2007, Flynn and Mulcahy, 2008, Osborne and Devaney, 1999). Previous studies suggest that IL-10 and TGF-β co-operate in limiting inflammatory responses and promoting parasite survival in a murine model of *Trichinella spiralis* infection (Beiting *et al*., 2007). In the absence of IL-10 *T. spiralis* infected muscle cells produce high levels of IFN-γ and iNOS. However, neutralisation of TGF-β in IL-$10^{-/-}$ mice resulted in severe tissue inflammation and parasite death (Beiting et al., 2007). Moreover, IL-10 prevents the killing of the intracellular protozoan parasites *Toxoplasma gondii* and *S. mansoni* by inhibition of IFN-γ induced production of NO (Gazzinelli *et al*., 1992). In addition, IL-10 produced by innate effector cells and T-reg cells promotes parasite survival in murine schistosomiasis (Hesse *et al*., 2004). Furthermore, Flynn and Mulcahy (2008) demonstrated a role for IL-10 and TGF-β in contributing to immunosuppression during chronic fasciolosis. Expression of IL-10 and TGF-β was detected early in bovine PBMCs in response to parasite specific stimulation (Flynn and Mulcahy, 2008). Neutralization of TGF-β at the acute stage of infection resulted in enhanced secretion of IL-4. However, IL-10 neutralization at the chronic stage, dramatically enhanced secretion of IFN-γ

(Flynn and Mulcahy, 2008). These findings suggest a role for these cytokines in regulating the generation of type-2 immunity, immune-mediated pathology, and survival of helminth parasites during infection.

1.3.6 Anergic T-cells in helminth infections.

Another type of regulatory cell implicated in helminth infection is the anergic (suppressive) T-cell. These cells modulate immune responses to helminth infection via their characteristic suppressed proliferation and cytokine secretion capacities. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death 1 (PD-1) and PD ligand (PD-L) represents some of the molecules that are expressed on anergic T-cells during helminth infection.

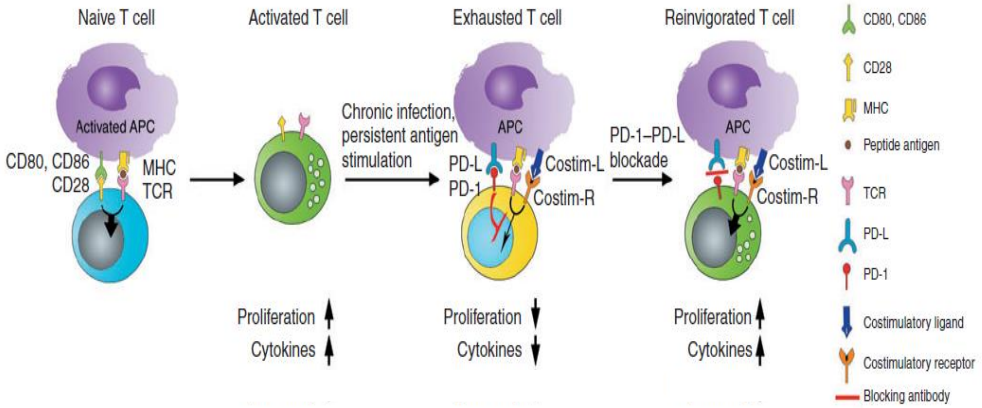


Figure 1:5: PD-1/PD-L pathways in chronic infections or persistence antigen stimulation. Naïve T cell becomes activated, proliferated and produced cytokines as a result of engagement of TCR with antigen-MHC complex and co-stimulation from binding of CD28 receptor expressed on naïve T cell to its ligands (CD80 and CD86). T cells become exhausted and lack proliferative and

cytokine production ability as a result of PD-1/PD-L1 signalling during chronic infection or persistent antigenic stimulation. Inhibition of this pathway and engagement of co-stimulatory receptor with its ligands, reinvigorates T-cells while restoring T-cell proliferation and cytokine production. Image was adapted from (Sharpe *et al*., 2007).

PD-1 (CD279) is a 56kDa type I transmembrane glycoprotein receptor that belongs to the immunoglobulin (Ig) superfamily. PD-1 is expressed on activated B cells, T-cells, monocytes and DCs (Khoury and Sayegh, 2004, Greenwald *et al*., 2005). There are two known ligands for PD-1 belonging to the B7 family of protein and these include, PD-L1 (B7-H1/CD274) and PD-L2 (B7-DC/CD273). It has been shown that engagement of PD-1 and its ligand PD-L1 delivers a down modulatory signals (Figure 1.5) on T-cell responses, inhibiting T-cell proliferation and production of cytokines in multiple helminth infections, including *F. hepatica* (Aldridge and O'Neill, 2016), *S. mansoni* (Smith *et al*., 2004) and *T. crassiceps* (Terrazas *et al*., 2005). Expression of PD-L1 on macrophages following *S. mansoni* infection, promoted T-cell anergy which was abrogated by application of a monoclonal antibody to PD-L1 (Smith *et al*., 2004). Similarly, in a murine model of cysticercosis infection, a down-modulation of the cellular response was observed in splenocytes following infection as evidenced by a poor proliferative response to parasite antigen (Terrazas *et al*., 2005). Peritoneal or splenic macrophages isolated from this infection model showed expression of anergic markers, PD-L1 and PD-L2.

Moreover, T-cells co-cultured with these macrophages failed to proliferate. T-cell proliferation was however restored by application of blocking antibody to PD-1 or its ligands (Terrazas *et al*., 2005).

Expression of markers of anergy is also known to occur during *F. hepatica* infection. T-cells recovered following *F. hepatica* infection or injection of *F. hepatica* tegumental coat antigens in a murine model of infection, lack the ability to proliferate and produce cytokines. Analysis of these T-cells showed enhanced expression of markers of anergy including genes related to anergy in lymphocytes (GRAIL), ICOS (inducible T-cell costimulatory), EGR2 (early growth response 2) and ITCH (itchy-E3 ubiquitin protein ligase) (Aldridge and O'Neill, 2016). Similarly, CD4 T-cells from a bovine infection demonstrated potent anergy within the hepatic lymph nodes with high expression of PD-1 (Sachdev *et al*., 2017). These CD4 T-cells lacked proliferative and cytokine production capability. However, anergy was fully reversed by a combined approach of the addition of exogenous IL-2 along with neutralization of TGF-β and IL-10 (Sachdev *et al*., 2017). Collectively, these studies demonstrate that PD-1/PD-L pathways plays a critical role in regulating immuno-pathology and maintenance of peripheral tolerance during helminth infection.

In addition to PD-1/PD-L pathways, CTLA-4 (CD152) is also expressed on anergic T-cells. CTLA-4 is a transmembrane receptor belonging to the same Ig superfamily of proteins as CD28 (a co-stimulatory receptor expressed on T-cells) (Sansom, 2000, Jago *et al*., 2004). The complimentary DNA sequence of CTLA is 76% identical to CD28. In addition, CTLA-4 and CD28 share a similar gene organisation, tissue expression, and chromosomal location (Harper *et al*., 1991, Waterhouse *et al*., 1995). They also bind to common ligands, which are

B7-1 (CD80) and B7-2 (CD86) that are expressed on APCs. While CD28 is constitutively expressed by T-cells, conversely CTLA-4 is expressed by T-reg cells and upregulated following T-cell activation (Takahashi et al., 2000, Read et al., 2000). Upon ligation, CD28 enhances T-cell activation and plays a major role in augmenting and maintaining T-cell responses (Thompson and Allison, 1997). Upregulation of CTLA-4 down modulates T-cell activation (Bluestone, 1997). A role for CTLA-4 in regulating lymphocyte homeostasis has been demonstrated (Khattri *et al*., 1999). CTLA-$4^{-/-}$ mice displayed severe lymphoproliferative disorders and premature death (Waterhouse et al., 1995, Tivol et al., 1995) . CTLA-$4^{-/-}$ mice exhibit excessive T-cell proliferation and cellular infiltration into lymphoid tissues including heart, lungs and liver (Waterhouse *et al*., 1995).

CTLA-4 plays an important role in initiation of T-cell anergy and maintenance of peripheral tolerance in various helminth infections (Jago *et al*., 2004, Takahashi *et al*., 2000, Taylor *et al*., 2007, Anderson *et al*., 2006). Studies of filarial infection suggests that susceptibility to *L. sigmodontis* infection was associated with enhanced expression of $CD4^{+}CTLA\text{-}4^{+}$ cells in the pleural cavity and the development of T-reg cells. Interestingly, neutralization of CTLA-4 and depletion of T-reg cells after the onset of infection resulted in parasite killing (Taylor *et al*., 2007). Similarly, infection of mice with *S. mansoni* resulted in enhanced expression of CTLA-4 in $CD4^{+}CD25^{-}$ splenic cells. However, CTLA-4 blockade via treatment with monoclonal antibody during acute but not chronic infection resulted in enhanced production of IL-4 and IL-5 as well as liver eosinophilia (Walsh *et al*., 2007). This suggests that CTLA-4 may be a

mechanism employed by the parasite during immune evasion to limit the extent of the Th2 immune response (Khattri *et al*., 1999).

1.4 *F. hepatica* immunomodulators

F. hepatica prolongs its existence by secretion of a number of molecules that target and suppress host protective immune responses. These molecules include cathepsin L proteinases (CL), Superoxide dismutases (SODs), Glutathione S-Transferases (GSTs), and Peroxidoxins (Prx).

CL is an abundant component of *F. hepatica* ES products representing about 60-80% of the total protein concentration. Synthesised in the epithelial cells lining the *F. hepatica* gut, they have been shown to participate in regulating the parasite's tissue penetration and ingestion of host tissue, as well as immune evasion during *F. hepatica* infection (Dalton *et al*., 2013). O'Neill *et al*., (2001) demonstrated that *F. hepatica* CL1 (FheCL1) mediates suppression of the Th1 responses *in vivo* in mice immunized with a *Bordetella pertussis* vaccine (O'Neill *et al*., 2001). This effect was abrogated in IL-$4^{-/-}$ mice, suggesting a role for IL-4 in suppression of protective immunity by *F. hepatica* proteases. FheCL1 also suppresses lymphocyte proliferation and reduces surface expression of CD4 in ovine and human T-cells (Prowse *et al*., 2002). T-cell proliferation was restored by incubating the cells with a cysteine protease inhibitor, E64. These findings suggest that FheCL is a potent molecule released by *F. hepatica* in defence against host effector responses.

Another enzyme used by the parasite to modulate the host responses is Prx. Prx is from a family of antioxidant enzymes which decompose alkyl-hydroperoxides and hydrogen peroxide (H_2O_2), thereby preventing the formation of harmful

hydroxyl radicals (McGonigle *et al*., 1998). Members of this family are highly conserved from lower to higher organisms. Through genome sequencing and expressed sequence tag (EST) analyses, Prx has been identified in parasitic helminths including *F. hepatica* (McGonigle *et al*., 1997) and they mediate protection from reactive oxygen species (ROS) generated as a result of metabolic activities and those released by host innate immune effector cells such as eosinophil, neutrophil and macrophages (Callahan *et al*., 1988). In *F. hepatica*, Prx has been shown to be actively secreted, and may have potential in acting as a major antioxidant that eliminates H_2O_2 and protects the parasite from damaging ROS (McGonigle *et al*., 1996, 1998). Prx has a role in modulation of the host immune response following infection. In murine models of schistosomiasis and fasciolosis, Prx has been shown to promote Th2 responses by inducing AAMϕ through expression of Ym1, FIZZI and Arg1 (Donnelly *et al*., 2005, Donnelly *et al*., 2008). However, treatment of mice with neutralising antibody to Prx or immunization of mice with recombinant Prx, abrogated both the AAMϕ and Th2 response (Donnelly *et al*., 2008).

SODs are also secreted by *F. hepatica* as an antioxidant thereby preventing parasite death due to ROS generated by the host. Free oxygen radicals released by the host immune effector cells contribute to the killing of parasites. Consequently, parasites have evolved a means to overcome this oxidative mediated damage. Three main SODs have been described based on the type of metal present at the active site and these includes; Manganese, Iron, Copper/Zinc SODs (Piacenza *et al*., 1998). SODs are present in several parasites including *F. hepatica*, *T. solium*, *Clonorchis sinensis*, *S. mansoni*, *B pahangi* as well as *O.*

volvulus (Hong *et al*., 1992, Henkle *et al*., 1991, Castellanos-González *et al*., 2002, Li *et al*., 2005, Kim *et al*., 2000, Tang *et al*., 1994, Mei *et al*., 1995).

F. hepatica utilises the Cu/Zn-SODs to prevent oxidative-mediated death by host cells (Kim *et al*., 2000). High levels of SODs detected in *F. hepatica,* and the differential migratory patterns observed in immature and adult fluke, suggests a role for SODs in the detoxification of superoxide radicals produced during parasite growth from the juvenile stage to adult forms (Piacenza *et al*., 1998).

A final class of enzymes that *F. hepatica* utilises to suppress host protective responses is the GST family. GSTs are multi-functional soluble proteins that have roles in detoxification and excretion of unwanted chemical and physiological substances (Cervi *et al*., 1999, Howell *et al*., 1988). They also function as major detoxifying enzymes in helminth parasites that do not possess a cytochrome P450 dependent detoxification mechanism such as *Schistosoma spp* (Smith *et al*., 1986, Smith *et al*., 1988, O'Leary and Tracy, 1988, Trottein *et al*., 1992). GSTs were found to be widely distributed in *F. hepatica* tissues (Howell *et al*., 1988, Chemale *et al*., 2006). GSTs are mainly produced in *F. hepatica* by parenchymal cells and peripheral tissues (Creaney *et al*., 1995). GSTs were shown to suppress proliferation of spleen mononuclear cells and also inhibit nitric oxide production (Cervi *et al*., 1999), suggesting a role for this molecule in immune evasion during *F. hepatica* infections.

1.5 Cytokine homologs in helminths

Aside from the secretion of various immunomodulatory molecules and induction of regulatory or anergic cells, as reviewed above, helminth parasites may also

modulate host immune responses by their ability to produce homologs of mammalian cytokines. These homologs may include TGF-β (Gomez-Escobar et al., 2000, Japa et al., 2015, McSorley et al., 2010, Freitas and Arasu, 2005, Gomez-Escobar et al., 1998) and macrophage inhibitory factor (MIF) (Pastrana *et al*., 1998, Pennock *et al*., 1998, Zang *et al*., 2002). The most highly studied cytokine homolog in helminths is TGF-β.

1.5.1 TGF-β homologs in helminths

TGFs are a large family of structurally-related proteins that control diverse cellular activities including proliferation, differentiation, adhesion, motility, recognition and apoptosis (Massagué, 1998). TGF-β plays a crucial role in animal development and immune regulation (Li *et al*., 2006a, Freitas *et al*., 2007) and its signalling pathways are highly conserved from lower to higher organisms. The TGF-β family comprises of several members which are grouped into two sub-families based on structural and functional differences and these include, the TGF- β and the bone morphogenic proteins (BMPs) sub-families.

TGF- β signal transduction involves two transmembrane receptors termed type I and II receptors. During activation of the TGF-β pathways, the type II receptor becomes phosphorylated and activates the type I receptor. The intracellular Smad protein becomes phosphorylated by the activated type I receptor. This Smad complex then transduces into the nucleus where it controls target genes (Shi and Massagué, 2003, Pang *et al*., 2011) (Figure 1.6).

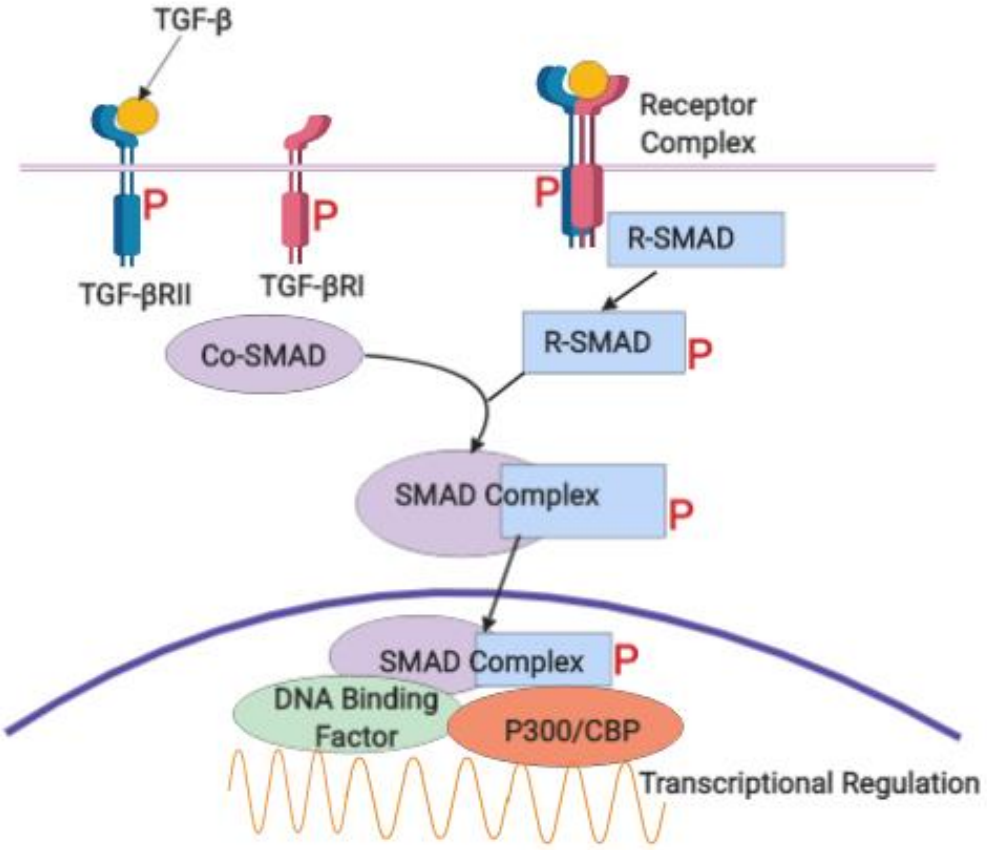


Figure 1:6: Diagram showing TGF-β pathway. Upon ligand binding, type II receptor becomes activated and phosphorylates type I receptor. Activated type I receptor in turn phosphorylates Smad protein. The Smad complex transduces into the nucleus to effect gene expression. Image was created at https://biorender.com/.

1.5.1.1 TGF-β homologs in free living helminths

Various ligands encoding the TGF-β family have been identified in free living helminths. In *Caenorhabditis elegans*, five genes that belong to the TGF-β family have been described with three well characterised (Patterson and Padgett, 2000). These genes include *DAF-7*, *DBL-1*, *UNC-129*, *TIG-2* and *CE27471*.

Signalling through these pathways has been shown to control growth and development in *C. elegans.*

DAF-7 has been shown to regulate the formation of dauer larva (a larva undergoing developmental arrest in response to harsh environmental conditions). Expression of *DAF-7* is tightly regulated by signals from the environment. Induction of *DAF-7* expression via enhanced food and pheromone signals has been shown to rescues dauer larva formation (Ren *et al*., 1996). A second TGF-β pathway described in *C. elegans* encodes the ligand *DBL-1* and it is a member of the bone morphogenic protein (BMP) family. Signalling through this pathway regulates *C. elegans* growth and development (Suzuki et al., 1999, Morita et al., 1999). DBL-1 mutant worms demonstrate decreased body length and a range of abnormalities in the male worm including fusion of tail rays and fragmented spicules, whereas overexpression results in increased body length (Morita *et al*., 1999).

1.5.1.2 TGF-β homologs in parasitic helminths.

In the parasitic filarial nematodes, *B. malayi* and *B. pahangi,* genes encoding the TGF-β family have been described (Gomez-Escobar *et al*., 1998, Gomez-Escobar *et al*., 2000). The genes designated *tgh-1* and *tgh-2,* were found to be differentially-expressed at various developmental stages including, microfilarial larvae, infective L3 stage, and the adult stages. *Tgh-1* represents a stage restricted gene with highest expression in the mosquito-derived infective larva that are undergoing growth and development within the mammalian hosts, implicating a role for this gene in regulating development in filarial parasites.

Moreover, adult worms showed limited expression while expression was found to be absent in the microfilaria (Gomez-Escobar *et al*., 1998). Conversely, *tgh-2* demonstrated highest expression in the microfilarial stage with no expression in larva undergoing moulting and expression was maintained in the adult stage (Gomez-Escobar *et al*., 2000). Although the precise function of these ligands is yet to be determined, their role may not be limited to regulation of development but also immune modulation. This is supported by their expression pattern together with the ability of the recombinant *tgh-2* to bind the mammalian TGF-β receptors and induce the transcription of plasminogen activator inhibitor-1 (a TGF-β target) on mink lung cells (MLEC) (Gomez-Escobar et al., 2000).

Tgh-2 homologues have been identified from four trichostrongyloid nematodes parasites including *H. polygyrus, N. braziliensis, Haemonchus contortus* and *Teladorsagia circumcincta* (McSorley *et al*., 2010). This ligand shares close similarity to *C. elegans, B. malayi* and mammalian TGF-β molecules. Expression analysis revealed that *tgh-2* transcripts were present in the infective stage of the parasites studied, suggesting a role in immune modulation and invasion.

In the parasitic nematode of dogs, *Ancyclostoma caninum*, two genes designated *Ac-daf-7* and *Ac-dbl-1* have been characterised (Freitas and Arasu, 2005). *Ac-dbl-1* and *Ac-daf-7* possess close similarity to the *C. elegans* TGF-β genes, *dbl-1* and *daf-7* respectively. Similar to the expression pattern of *C. elegans dbl-1*, *Ac-dbl-1* was found to be highly expressed in the adult male parasite while the *Ac-daf-7* transcript was maximal in the infective larva undergoing developmental arrest as well as in the infective larva reactivated in response to cues from the host (Freitas and Arasu, 2005, Brand *et al*., 2005). The detection

of *Ac-daf-7* during developmental arrest and in serum reactivated infective larvae suggests a role for this gene in the regulation of *A. caninum* growth.

The expression profile of *daf-7* in free-living nematodes is different to that in parasitic nematodes. For instance, in *C. elegans daf-7* is highly expressed in the L1 stage (when commitment to the dauer stage is governed) and undetectable in the dauer stage (Ren *et al*., 1996). Conversely, in parasitic nematodes, high levels of the *daf-7* homologs were associated with the developmentally arrested stages and maintained or reduced in the reactivated stages (Freitas and Arasu, 2005, Brand *et al*., 2005). Hence, it is thought that *daf-7* expression in the developmental arrest stages of parasitic nematodes play crucial roles in arrest and are conserved possibly to suppress reactivation until the parasite encounters a host specific signal that reactivates the arrested stage and thus resumption of the parasitic lifecycle. Indeed manipulation of expression levels of *daf-7* in the developmental arrest stage through molecular strategies, may have implications in the control of parasitic nematode infections (Viney *et al*., 2005).

In the parasitic trematodes TGF-β homologues have been well characterised in the genus *Schistosoma*. In *S. mansoni* TGF-β homologues designated *S. mansoni* Inhibin/Activin (SmInAct) has been described (Freitas *et al*., 2007). SmInAct was found to be maximally expressed in patent female worms and their eggs and *in situ* hybridization confirms presence of *SmInAct* transcripts in the reproductive tissue of the female worms. Moreover, the *SmInAct* transcript was found to be absent in single sex infection as well as in IL-7 Receptor$^{-/-}$ mice; conditions that do not allow for parasite reproduction and production of fertile eggs respectively. These findings suggest a role for the participation of host cytokine signalling in regulating transcription of *SmInAct* in the reproductive

biology of the parasite. Indeed, RNA interference targeted knockdown of this gene resulted in termination of egg development when compared controls, implicating a role in embryogenesis. In addition, *S. japonicum* BMP (SjBMP) demonstrated a similar pattern of expression and function with SmInAct protein (Liu *et al*., 2013), indicating the existence of co-evolutionary strategies within Schistosomes that allows for the regulation of host immune responses.

Furthermore, through genome screening for scaffolds that have homology to the conserved domain of TGF-β molecules within *F. hepatica,* three ligands were identified (Japa *et al*., 2015). Two of these ligands belong to the BMP subfamily while the third one assigned a functional role belongs to the TGF-β subfamily and this is designated *Fasciola hepatica* transforming growth factor-like molecule (FhTLM). FhTLM was found to be up to 35% identical in the active domain to TGF-β from *C. elegans,* bovine and human hosts. In addition, the amino acid sequence of FhTLM was 60% and 58% identical to inhibin-β B-chain of *C. sinensis* and SmInAct of *S. mansoni,* respectively. Expression analysis revealed that FhTLM is a stage restricted molecule with NEJs having the highest expression followed by the unembryonated eggs; however, low expression was observed in the adult worms. In addition, *in situ* hybridisation analysis revealed that mRNA probes bound throughout the NEJ parasite, lacking the tissue restriction seen in the related *Schistosome* trematodes (Freitas *et al*., 2007, Liu *et al*., 2013) reviewed above. However, this may be related to the hermaphrodite nature of *F. hepatica*. Notably, adults had low levels of mRNA and a correspondingly restricted *in situ* expression profile. This expression data corresponded to results seen when exogenous protein was added to *ex vivo*

parasite cultures. Parasite survival was improved in the NEJs but not the adults, suggesting that the components for TGF signal transduction might not be expressed within adults. Improved survival was also supported by the finding that NEJs were more active in their movement. A final developmental role was seen when eggs were incubated with exogenous protein and embryonation rate increased in the presence of FhTLM (Japa *et al*., 2015)

1.6 Research aims

F. hepatica induces a non-protective host response while promoting immune regulation and anergy. Increasing *F. hepatica* prevalence (McCann *et al*., 2010, Howell *et al*., 2015) together with the development of drug resistance (Overend and Bowen, 1995, Alvarez-Sanchez *et al*., 2006), makes the need for development of effective control strategies such as vaccines urgent. Parasite effectors mediating immunomodulating responses are an attractive target for vaccine development (McManus and Dalton, 2006, Toet *et al*., 2014). FhTLM plays an important role in parasite development and in inhibiting host protective immunity (Sulaiman *et al*., 2016, Japa *et al*., 2015) and represents a potential vaccine candidate for fasciolosis. Therefore, further characterisation of immune evasion properties of this molecule will be significant in future vaccine development since FhTLM is abundantly produced by the NEJs and the pathogenesis associated with *F. hepatica* infections often result from migratory and feeding activities of the NEJs. Therefore, the aims of this study were to determine:

1. The effect of FhTLM on modulation of host immune responses via induction of T-reg and anergic markers in CD4 T-cells
2. The range of cytokine and chemokine responses associated with FhTLM induction.
3. The effect of FhTLM in regulating parasite survival via modulation of host immune cell signals.

2 General Materials and Methods

2.1 Animal and Ethical Approval

The University of Nottingham School of Veterinary Medicine & Science Animal Welfare Ethical Review Board (AWERB) approved ethics and the use of animal materials locally. Animals were housed under standard conditions as described by the Home Office Code of Practice (ISBN 9781474112390). C57BL/6J mice were purchased from Charles River UK through the Bio Support Unit (BSU), University of Nottingham, Sutton Bonington campus. Transgenic mice which express green fluorescent protein (GFP) as a promoter for the FoxP3 gene previously described (Haribhai *et al*., 2007) were a kind gift from Professor Karen Robinson (University of Nottingham). All animals were housed in individually ventilated cages; a maximum of three females per cage or three males each in separate cages. Water and standard mouse chow were provided *ab lib.*

2.2 *F. hepatica* protein (FhTLM)

Recombinant FhTLM used in this study was produced previously (Japa *et al*., 2015) in *E. coli* Rosetta competent cells, purified over a Nickel resin column, free of endotoxin residues and was supplied by the laboratory of Robin Flynn. Protein concentration was routinely analysed by bicinchoninic acid (BCA) protein assay. In brief, an eight-point protein standard (bovine serum albumin-BSA) was prepared with concentration ranging from 2000-25µgmL. Following this, BCA working reagent (WR) was prepared by mixing reagent A and B in a 50:1 ratio. 25µL of either BSA or FhTLM protein was added in duplicate to wells of a 96 flat bottom plate. Equal volume of PBS was used as blank control.

Thereafter, 200µL of WR was added to each well and mixed on a plate shaker for 30 sec. The plate was then covered and incubated at 37^0C for 30 min. Following this incubation, the plate was allowed to cool to RT and absorbance was measured at 562nm using a multimode varioskan plate reader.

2.3 Animal preparation and tissue isolation.

Mice were humanely killed using an approved Schedule 1 method; exposure to an increasing concentration of carbon dioxide (CO_2) gas in an enclosed chamber. Mice were administered CO_2 to a final concentration of two litre/min for 5 minutes (min). Death was confirmed by dislocation of the cerebral region using a plastic blunt end knife. Following confirmation of death, mice were dissected using scissors and forceps. Tissues including spleens, tibia and femur were aseptically removed and transferred into a 50mL sterile tube containing Dulbecco's phosphate buffered saline (D-PBS; Sigma-Aldrich, UK). Tissues were then transported to the laboratory for preparation. Tissues were kept on ice to preserve integrity until used in cell separation.

2.4 Spleen cell preparation.

For the isolation of splenocytes, spleens were placed on a pre-wet 70µm cell strainer (BD Pharmingen) fitted into a 50mL falcon tube containing sterile D-PBS. Thereafter, a single cell suspension was prepared by crushing spleens with the end of a 5mL syringe (BD Pharmingen). The cell strainer was then washed with 2mL D-PBS to ensure complete cell retrieval. Cell suspensions were

pelleted by centrifugation at 300 x g for 8 min at 4^0C. Following this, red blood cells (RBCs) were lysed by incubating cells in red blood cell (RBC) lysis buffer (see appendix 1) at room temperature (RT) for 10 min. Thereafter, cells were washed twice in RPMI (Roswell Park Memorial Institute) 1640 medium (Sigma Aldrich) and pelleted by centrifugation. Supernatant was removed, and cells were re-suspended in 1mL RPMI media. The number of isolated splenocytes were determined by trypan blue exclusion methods (see below).

2.4.1 Cell viability determination by trypan blue exclusion method

In order to determine the number of cells isolated, pelleted cells were resuspended in 1mL RPMI medium and 10µL of cell suspension was placed in a 1.5mL Eppendorf tube and mixed with an equal volume of trypan blue. Following this, 10µL of the cell mixture was placed on a cleaned haemocytometer. The haemocytometer was then examined under a light microscope and viable cells were counted at 10X magnification. The number of viable cells was determined using the equation below;

$$\text{Total cells/ml} = \text{Total cells counted} \times \frac{\text{dilution factor}}{\text{\# of Squares}} \times 10{,}000 \text{ cells/ml}$$

2.4.2 CD4 T-cell separation from splenocytes

CD4 T-cells were separated from the spleen cell suspension prepared using a CD4 T-cell isolation kit (Miltenyi Biotec) which works on the principle of

depleting non-CD4 T-cells including CD8a, CD11b, CD11c, CD19, CD25, CD45R (B220), CD49b (DX5), CD105, Anti-MHC class II, Ter-119, TCRγ/δ, and CD44. Briefly, spleen cell suspensions were pelleted by centrifugation at 300 x g for 10 min and then re-suspended in 400µL of MACS running buffer (see Appendix 1). 100µL of biotin-conjugated antibody cocktail was used to label up to 1 $x10^8$ cells for 5 min at 4^0C in the dark. Thereafter, 200µL of MACS running buffer was added, followed by incubation with of 200µL anti-biotin microbeads and 100µL of CD44 microbeads at 4^0C for 10 min. Cells were then washed with 2mL MACS running buffer, pelleted by centrifugation and then re-suspended in 500µL MACS running buffer. Following this, a MACS LS column (Miltenyi Biotec) and a midi separator (Miltenyi Biotec) were assembled. The column was rinsed with 3mL MACS rinsing buffer (see Appendix 1), then cell suspension was loaded onto the column. CD4 T-cells were collected as the flow through. The eluted cells were pelleted by centrifugation, and cell number was determined as described (2.4.1)

2.5 RAW 264.7 Macrophage cell line maintenance

RAW 264.7 macrophage cell line was a generous gift from Prof. Paul Barrow (School of Veterinary Medicine and Science, Nottingham). Cryopreserved RAW264.7 cells were allowed to thaw on ice and then cells were transferred into a 5mL falcon tube. Thereafter, cells were washed 3 times by addition of 1mL RPMI medium to the tube and then mixed by pipetting, this was followed by centrifugation at 300 x g for 8 min at 4^0C. Supernatant was removed without disturbing the cell pellet and then cells were suspended in 1mL RPMI. The number of viable cells was determined as described (2.4.1). For the propagation

of RAW 264.7 cells, they were routinely cultured in a T75 flask (ThermoFisher Scientific) in complete RPMI medium and incubated at 37°C, 5% CO2 in air. Medium was changed regularly until cells were 70-80% confluent. Cells were harvested by gentle scraping with a cell scraper (Falcon) and pelleted by centrifugation. Cells were resuspended in 500uL RPMI and an equal volume of freeze mix (see Appendix 1) in a 1.2mL for cryopreservation. Cells passaged between 5-7 times were used in the described experiments.

2.6 Bone marrow cell separation

Bone marrow progenitor cells were isolated following published methods by (Zhang *et al*., 2008). Following isolation of the tibia and femur as described (2.3), excess muscle was removed by holding with forceps and gently scraping with a scalpel. The bones were held with forceps and cut at the two ends with sterile scissors. Thereafter, a 25-G needle (BD Pharmingen) was attached to a 10 mL syringe (BD Pharmingen) filled with sterile complete RPMI medium and inserted into one end of the bone. The bone marrow was then flushed into a 50mL Falcon tube and filtered through a 70µm cell strainer to remove any contaminating debris. Following this, the cells were centrifuged at 300 x g for 10 min at 4^0C and then resuspended in 1mL D-PBS. RBCs were lysed by incubating the cells in RBC lysis buffer for 10 min at RT. Following RBC lysis, cells were washed with 30mL of D-PBS and pelleted by centrifugation. Supernatant was discarded and cells resuspended in complete medium and the numbers of bone marrow cells was determined as described (2.4.1).

2.6.1 Macrophage differentiation from murine bone marrow.

In order to differentiate bone marrow cells to macrophages, bone marrow cells isolated as described above were cultured in complete medium containing 10ng/mL murine macrophage colony stimulating (M-CSF) factor (Peprotech). 1 x 10^6 cells/mL were placed in a sterile Petri-dish and incubated at 37^0 C, 5% CO_2. After 3 days, medium was removed by pipetting and replaced with medium containing 10ng/mL (M-CSF). At day 7, cells were re-fed with fresh medium and harvested on day 10 when the cells were fully matured. Bone marrow derived macrophages (BMDM) were harvested by discarding the culture medium and gently washing the cells with 5mL pre-warmed D-PBS. Macrophages were gently scraped off the plate with a cell scraper and centrifuged at 300 x g for 8 min. Cells were suspended in RPMI medium and cell number was determined as described (2.4.1). Differentiated macrophage phenotype and morphology was accessed by immunofluorescence and Diff-Quick staining, respectively as described below (2.6.1.1).

2.6.1.1 BMDM morphology

Morphology of BMDM was assessed by diff quick staining. Briefly, 5 x 10^4 cells were suspended in 100uL PBS in a 1.5 mL Eppendorf tube. In order to concentrate cells on a slide, cytospin apparatus was assembled by placing a filter card (Shandon) onto a superfrost plus adhesion microscope slide (ThermoFisher Scientific) and securing this with a plastic funnel in a cytospin slide clip. The assembled unit was placed into the cytocentrifuge (ThermoFisher Scientific) and

100uL of cell suspension was added onto the slide through the plastic funnel. Cells were cytospun at 400 rpm for 5 min. Following this, the slide was removed and allowed to dry for 5 min at RT. The cells on the slide were fixed by dipping 5 times in fast green in methanol solution, excess fixative was dabbed on a clean paper towel. Following this, cells were stained by dipping 10 times in Eosin G, excess stain was removed by washing gently in distilled water. Cells were counter stained by dipping 10 times in Thiazine dye and washed gently by dipping in water until excess stains were removed. The slide was allowed to dry at RT. The cells were then examined at 10x magnification using an upright Lieca microscope.

2.7 Eosinophil differentiation from murine bone marrow cells

Eosinophils were differentiated from low density bone marrow derived cells as described previously (Lu and Rothenberg, 2014). Briefly, bone marrow progenitor cells were isolated as described in section 2.8. Cells were centrifuged at 300 x g for 8 min at 4^0C, supernatant was removed, and cells were resuspended in 16mL D-PBS. Following this, 4mL of Histopaque 1083 (Sigma-Aldrich) was placed in a 15mL tube and subsequently, 4mL of cell suspension was layered on top. Thereafter, the cells were centrifuged at 400 x g for 30 min at RT with the centrifuge brake in the off position. After centrifugation, the low-density cell suspension at the interphase between the upper and the lower layers was collected carefully without disturbing the layers. The low-density cells were washed with 30mL D-PBS and then pelleted by centrifugation. The low-density cells were resuspended in 1mL Iscove's Modified Dulbecco's Medium (IMDM)

with Glutamax-1 (Gibco). Cells were cultured in a 6 well plate (ThermoFisher Scientific) at a density of 1 x 10^6/mL in complete IMDM medium (see Appendix 1) supplemented with 100ng/mL murine recombinant stem cell factor (SCF; Peprotech) and 100ng/mL FLT-3 ligand (Peprotech) at 37^0C, 5% CO_2 incubator. On day 2 of culture, medium was changed by aspirating the old medium without disturbing the cells and then an equal volume of new medium was added. On day 4 of culture, cells were collected and spun down and washed with 30mL D-PBS. Following this, cells were again cultured in IMDM complete medium supplemented with recombinant murine IL-5 (Peprotech). The cell culture medium was changed every other day. At day 14 of culture, eosinophils were harvested.

2.8 Monocyte separation from bone marrow cells.

For isolation of monocytes, a mouse monocyte isolation kit was used (BD, Pharmingen). Bone marrow cells were collected as previously described. Monocytes were separated from bone marrow following manufacturer's instructions. Briefly, cells were washed with MACs running buffer and then centrifuged at 300 x g for 10 min at 4^0C. Thereafter, 5 x 10^7 cells were resuspended in 175µL MACs running buffer and incubated with 25µL FcR reagent supplied to block non-specific antibody binding. Following this, cells were incubated with 50µL of monocyte biotin conjugated antibody cocktail supplied by the manufacturer against non-monocytes cells including T cells, B cells, NK cells, dendritic cells, erythroid cells, and granulocytes. Cells were subsequently washed by addition of 10mL MACs running buffer and pelleted

by centrifugation. Supernatant was aspirated and the cell pellet was resuspended in 400µL MACs running buffer. Thereafter, cells were incubated in 100µL anti-biotin microbeads for 10 min at 4^0C. Cell separation columns were assembled and primed by washing with 3mL MACs rinsing buffer. Then cell suspension was loaded onto the column. Monocytes were then collected as the unlabelled fraction that passed through the column. To ensure high monocyte recovery, 3mL of MACs rinsing buffer was placed into the column and the flow through was collected and added to the initial flow through. Isolated monocytes were centrifuged, and cell number determined as described (2.4.1). Cells were resuspended in RPMI medium only and placed on ice prior to cell culture.

2.9 Cell culture

2.9.1 CD4 T-cell culture

To differentiate CD4 T-cells into FoxP3 expressing T-reg cells, a 12 well tissue culture plate was coated overnight with 1µg/mL purified mouse anti-CD3 antibody (Appendix 2) at 4^0C to activate the cells. Following this, 1 x 10^6 $CD4^+$ T-cells isolated from $FoxP3^{EGFP}$ mice were placed per well and were either unstimulated (media alone) or stimulated with IL-2 (20ng/ml) alone or in combination with either 2ng/mL recombinant human TGF-β (rhTGF-β-Peprotech; positive control) or *F. hepatica* FhTLM. To determine expression of anergic markers (PD-1, PD-L1 and CTLA-4) on T-cells, naïve CD4 T-cells isolated from C57BL/6J mice were either cultured following the T-reg inducing protocol described above or cells were activated with plate bound anti-CD3 at

the indicated concentration and soluble anti-CD28 at 2µg/mL, prior to stimulation with either rhTGF-β or FhTLM at various doses (2-200ng/mL). Cells were stimulated in duplicate and incubated for 3 days in a 37^0C and 5% CO_2 incubator. After stimulation cells were harvested by pipetting into 1.5mL eppendorf tubes and pelleted by centrifugation at 1200 rpm for 5 min. Supernatant was then collected and stored at -20^0C prior to chemokine and cytokine analysis.

2.9.2 RAW 264.7 cell and BMDM cultures.

RAW 264.7 cells and BMDM were cultured at a density of 2 x 10^5 in 1mL complete medium in a 12 well plate and were either unstimulated (media alone; negative control) or stimulated with 100ng/mL LPS (Sigma), 20ng/mL IL-4 (Peprotech) and various doses of FhTLM as indicated. Cells were incubated at 37^0C and 5% CO_2 for 48hrs. Thereafter, cells were harvested by scraping and centrifuged at 300 x g for 8 min. Supernatant was collected and stored at -20^0C prior to cytokine ELISA.

2.9.3 Generation of conditioned medium

To determine the effect of immune signals on NEJ growth and viability, monocytes and CD4 T-cells were stimulated as described (2.9.3.1 and 2.9.3.2). The medium isolated from these cultures was collected and hereafter referred to as “conditioned medium”.

2.9.3.1 Monocyte stimulation

In order to generate monocyte conditioned medium, 1 x 10^6 cells were cultured in duplicate in a 12 well plate and were either unstimulated or stimulated with either LPS (100ng/mL), IL-4 (20ng/mL), or FhTLM (200ng/mL). For some wells, monocytes were stimulated with either LPS or IL-4 in combination with FhTLM (Table 2.1). Cells were incubated at 37°C, 5% CO_2 for 48hrs. Following this, supernatant was collected as conditioned medium and stored at minus 20^0C prior to NEJ viability assays.

2.9.3.2 CD4 T-cell stimulation

For the generation of T-cell conditioned medium, CD4 T-cells were activated as described (2.9.1). Cells were unstimulated or stimulated with combination of IL-2 (5ng/ml) and IL-4 (10ng/mL) to induce Th2 polarization, or FhTLM alone (200ng/mL) (Table 2.1). Cells were incubated at 37°C, 5% CO_2 for 72hrs. Following incubation, conditioned medium was harvested and stored at -20^0C prior to NEJ viability assays. CD4 T-cells were harvested for GLUT4 qPCR and Immuno-staining.

Table 2.1: Culture conditions used for generation of conditioned medium.

In order to assess NEJ viability and growth in the presence of immune cell signals (conditioned medium), monocytes and CD4 T-cells were stimulated as described in Table 2.1 below.

Culture conditions	
Monocytes	**CD4 T-cells** (Activated with anti-CD3 and anti-CD28)
Unstimulated cells	Unstimulated Cells
LPS + Cells	IL-4 + IL-2 + Cells (Th2 differentiation)
IL-4 + Cells	FhTLM + Cells
FhTLM + Cells	
LPS + FhTLM + Cells	
IL-4 + FhTLm + Cells	

2.10 Flow cytometry

All anti-mouse antibodies (Appendix 2) and isotype controls used for flow cytometry were purchased from BD pharmingen, UK. Anti-PD-1 was purchased from eBioscience, UK.

2.10.1 Determination of purity of isolated CD4 T-cells.

Prior to cell culture, the purity of the isolated CD4 T-cells was determined. 100µL of CD4 T-cell suspension containing 1 x 10^6 cells was placed in a 1.5mL tube and Fc receptors were blocked by incubation with an equal volumes of rat anti-mouse CD16/CD32 (BD Pharmingen) for 20 min at 4^0C, after which cells were pelleted by centrifugation at 300 x g for 5 min and supernatant was removed. Cells were placed on ice until ready to stain. For antibody staining, cells were incubated with various dilutions 1/50 (4µg/mL) of anti-mouse CD4: APC (BD Pharmingen or corresponding isotype controls diluted in FACs buffer for 1 hr at 4^0C (see Appendix 2 for details of all antibodies and isotype controls). Following staining, cells were washed 3 times in FACs buffer and then resuspended in 1mL FACS buffer and purity was analysed by flow cytometry using a BD FACS Canto II flow cytometer machine.

2.10.2 Determination of expression of Foxp3 and anergic markers on CD4 T-cells.

For the detection of Foxp3 T-reg cells and anergic markers, PD-1, PD-L1 and CTLA-4 on CD4 T-cells, cells were stimulated as described (2.9.1). Following cell culture, cells were collected by pipetting into a 1.5mL eppendorf tube and pelleted by centrifugation at 300 x g at 4^0C for 8 min. 100µL of CD4 T-cell suspension containing 1 x 10^6 cells was placed in a 1.5mL tube and Fc receptor was blocked as described (2.10.1). Cells were placed on ice until ready to stain. For antibody labelling, antibodies and isotype controls were diluted in FACS

buffer. Cells were then incubated with 1/50 anti-CD4: APC, 1/10 (20µg/mL) anti-PD-1: FITC, 1/10 (20µg/mL) anti-PD-L1: PE and 1/10 (20µg/mL) anti-CTLA-4. Corresponding isotype controls labelling was also performed at the indicated dilutions. Cell were allowed to incubate for 1 hr at 4^0C, after which reaction was stopped by addition of 1mL FACS buffer. Thereafter, cells were washed 3 times in FACs buffer by centrifugation and resuspended in 1mL FACS buffer and placed on ice prior to flow cytometry analysis. Expression of Foxp3 or anergic markers was analysed by flow cytometry using a BD FACS Canto II flow cytometer machine. $FoxP3^{EGFP}$ expression was detected on FITC channel.

2.11 Chemokine and cytokine profiling of FhTLM stimulated CD4 T-cells.

For the detection of various chemokines or cytokines from culture supernatants of stimulated CD4 T-cells (2.9.1), a mouse cytokine panel A (ARY006, details in chapter 4) was used (R&D sytems, UK). Assay was performed following manufacturer's instructions. The supplied lyophilized biotinylated antibody cocktail was prepared in 100µL deionised water and chemiluminescent reagents 1 and 2 supplied were mixed in equal volumes before use. Four nitrocellulose membranes spotted in duplicate with capture antibodies specific for each protein were placed in a four well multi-dish and blocked with 2mL of array buffer 6, as supplied, for 1 hr on a rocking platform shaker at RT. CD4 T-cell culture supernatant was prepared to a 1/3 dilution by adding 500uL to 1mL solution of buffer 4 as supplied. Thereafter, 15µL of the reconstituted antibody cocktail was added to each prepared culture supernatant (samples/antibody mixture) and then

incubated for 1 hr at RT. The blocking buffer was aspirated from the multi-dish, thereafter, 1.5mL of sample/antibody mixture was added to each nitrocellulose membrane and then incubated overnight on a shaking platform at 4^0C. The membrane was washed 3 times in 20mL 1x wash buffer supplied by the manufacturer for 10 min on a rocking platform. The membrane was then incubated in 2mL streptavidin conjugated to horseradish peroxidase (SA-HRP) for 30min at RT on a rocking platform, after which membranes were washed as described above. With identification number on the membrane facing upwards, 1mL of chemiluminescent reagent mixture was pipetted onto the membrane and following incubation with chemiluminescent reagent and exposure to imager, light was produced in a manner that was proportionate to the amount of protein detected. Images was acquired using an Odyssey FC image system (Li-cor) and pixel density was then analysed using Image studio lite software version 5.2 (Li-cor).

2.12 Detection of growth-promoting molecules on FhTLM-stimulated Monocytes

For the detection of various proteins that may regulate parasite development in the supernatant isolated from monocytes stimulated as previously described (2.9.3.1), a mouse adipokine array kit (ARY013) was used (R&D system, UK). Assay was carried out as described previously (2.11). Images were acquired using a ChemiDoc^{TM} MP Imaging System (Bio-Rad) and pixel density was analysed as described (2.11).

2.13 Cytokine ELISA

For the detection of various cytokines in the CD4 T-cell culture supernatants (2.9.1), mouse ELISA kits were purchased from R&D system except for an IL-10 detection kit that was purchased from BD Bioscience. All reagents were either diluted in reagent diluent as supplied or prepared assay diluent (Appendix 1).

For the detection of IL-10 protein (Mouse IL-10 ELISA Set, 555252), an anti-mouse IL-10 monoclonal capture antibody was diluted at a ratio of 1/250 in carbonate coating buffers (Appendix 1) and 100µL was used to coat 96 well ELISA plates overnight at 4^0C. Capture antibody was aspirated and the plate was washed 3 times using 300µL wash buffer (Appendix 1). The residual wash buffer was removed by blotting the plate on paper towel. After which, non-specific antibody binding was blocked by incubating with 200µL assay diluent at RT for 1 hr. Following this incubation, the blocking buffer was discarded, and the plate was washed 3 times. The lyophilized standard supplied (recombinant mouse IL-10) was reconstituted in 1mL deionized water, then a working stock concentration of 2000pg/mL was prepared in assay diluent. A 2-fold serial dilution of the standard was prepared from 2000pg/mL to 31.3pg/mL. 100µl of culture supernatant, standards and assay diluent (blank set of wells) were added to respective wells and incubated for 2 hrs at RT. Thereafter, samples were aspirated and the plate was washed 5 times with wash buffer. IL-10 detector reagent was prepared by addition of detection antibody (biotinylated anti-mouse

IL-10 monoclonal antibody) to assay diluent at 1/250 dilution and then after 15 min, SA-HRP was added to the mixture at 1/250 dilution. Following this, 100µL of working detector was then added to each well and incubated for 1 hr at RT. Following this, plate was washed 7 times and then incubated for 30 minutes with 100µl tetramethylbenzidine (TMB) substrate solution in the dark at RT. The reaction was stopped by addition of 50µL sulphuric acid. Absorbance values were acquired on an ELISA plate reader LT-4000 (Labtech) at 450nm. Sample ODs were blank corrected by subtracting average OD of individual samples from that of blank controls. A standard curve was plotted and concentration of IL-10 from each sample was interpolated.

For the detection of PD-1 (Mouse PD-1 DuoSet ELISA; DY1021), rat anti-mouse PD-1 capture antibody was reconstituted in PBS and used at a final concentration of 6µg/mL. 100 µL was then added to wells of 96 well plates, the plate was then sealed and incubated overnight at RT. Following overnight incubation, capture antibody was aspirated and washed as described above. The plate was blocked with 100µL of reagent diluent for 1 hr at RT and the wash process was repeated. The recombinant mouse PD-1 standard was reconstituted in reagent diluent and a seven-point serial dilution of standard from 8000 to 125pg/mL was prepared in reagent diluent. Thereafter, 100µL of either standard or samples was added to appropriate wells and incubated for 2 hrs at RT, reagent diluent was used as blank. After this, the wash process was repeated, and the plate was incubated for 2hrs at RT in 100µL detection antibody (biotinylated goat anti-mouse PD-1) diluted to a final concentration of 400ng/mL in reagent diluent. The wash process was repeated, and the plate was incubated in SA-HRP

(1/200) for 20 min at RT in the dark. Following this incubation, wash process was repeated and then plate was incubated in substrate solution for 20 min at RT in the dark. Thereafter, the reaction was quenched by addition of stop solution to each well. Optical density (OD) was then determined at 450nm using a multimode varioskan plate reader.

For the detection of various cytokines including PD-L1, CTLA-4, RANTES (Regulated on activation, normal T-cell expressed and secreted) and TARC (thymus and activation regulated chemokine), assay was carried out as described for PD-1 above with slight variation.

For PD-L1 detection (Mouse PD-L1/B7-H1; DY1019-05), rabbit anti-mouse capture antibody was used at a final concentration of 2µg/mL. Recombinant mouse PD-L1 standard dilution used was from 1200 to 18.75pg/mL. Biotinylated rabbit anti-mouse PD-L1 detection antibody was used at 250ng/mL and SA-HRP was diluted 1/40 before use.

For the detection of CTLA-4 protein (Mouse CTLA-4; DY476), goat anti-mouse CTLA-4 capture antibody was used at a final concentration of 400ng/mL. Recombinant mouse CTLA-4 standard dilution used was from 8000 to 125pg/mL. Biotinylated goat anti-mouse detection antibody was used at 100ng/mL and SA-HRP was diluted 1/200 before use.

To determine RANTES secretion (Mouse CCL5/RANTES kit; DY478-05), rat anti-mouse RANTES capture antibody was used at 2µg/mL. Recombinant mouse RANTES standard dilution used was from 2000 to 31.3pg/mL.

Biotinylated goat anti-mouse detection antibody was used at 12.5ng/mL and SA-HRP was diluted 1/40 before use.

For the detection of TARC (Mouse TARC kit; DY529-05), rat anti-mouse TARC capture antibody used was at 2µg/mL. Recombinant mouse TARC standard dilution used was from 2000 to 31.3pg/mL. Biotinylated goat anti-mouse detection antibody was used at 200ng/mL and SA-HRP was diluted 1/40 before use.

2.14 Glucose transporter 4 (GLUT4) detection

For the detection of GLUT4 (a glucose transporter molecule) protein and mRNA levels in CD4 T-cells stimulated as previously described (2.9.3.2) were analysed by immunofluorescence staining and real time qualitative polymerase chain reactions (rt-qPCR).

2.14.1 Immuno-fluorescence staining

For the detection of GLUT4 in CD4 T-cells, a GLUT4 primary antibody was used following the manufacturers instruction. Briefly, CD4 T-cells were stimulated as described (2.9.3.2). Cells were harvested and up to 1 x 10^6 cells were cytospun as described (2.6.1.1). In order to fix cells, cells were incubated in 4% paraformaldehyde in PBS for 15 min at RT and washed 3 times for 10 min per wash with 0.1% Triton-x 100 prepared in PBS. Cells were permeabilised with 0.5% Triton-x 100 for 10 min at RT, and the wash process was repeated.

Fc receptors were blocked using 10% normal goat serum (Thermofisher Scientific, UK) overnight at 4^0C. Thereafter, blocking buffer was discarded and the slide was washed 3 times. The cells were incubated in GLUT4 primary antibody (NBP1-49533) at a final concentration of 10µg/mL for 3hrs at RT. Cells were again washed three times and then incubated with Goat anti-Rabbit IgG (H+L) secondary antibody:DyLight 488 (Thermofisher scientific, UK) at a final concentration of 2µg/mL. Secondary antibody was aspirated then cells were washed again. Thereafter, excess wash buffer was removed from the slide by dabbing on a clean paper towel and the cell nuclei were counter stained with a DAPI mounting medium (Vector Laboratories). The slides were covered with clean cover slips and the images acquired using a Leica upright microscope at X40 magnification. Analysis of GLUT4 positive cells was performed using image J software version 1.50i (https://imagej.nih.gov/ij/)

2.14.2 Gene expression

Expression of Glut4 mRNA in CD4 T-cells was carried out as described below.

2.14.2.1 Ribonucleic acid (RNA) extraction

Total RNA was purified from CD4 T-cells stimulated as described (2.9.3.2) using RNeasy mini kit (Qiagen) following the manufacturer's instruction. Briefly, 4 volumes of absolute ethanol was added to buffer RPE supplied before use. The cell suspension was pelleted by centrifugation at 300 x g for 5 min at

4^0C and supernatant was completely removed without disturbing the cells. Following this, cells were lysed by addition of 350µL RLT buffer. Cells were subsequently homogenised by vortexing for 1 min. Thereafter, 350µL of 70% ethanol was added to the cell lysate and then mixed by pipetting. 700µL of the cell lysate was then transferred into a spin column and placed in a 2mL tube and centrifuged at 8000 x g for 15 sec. Following this, the spin column membrane was washed using 700µL RW1 buffer by centrifugation. The spin column membrane was washed with 500µL of RPE buffer by centrifugation and the flow through was discarded. 500µL of RPE buffer was placed into the spin column and centrifuged at same speed for 2 min and flow through was discarded. In order to minimise ethanol carry over contamination, the spin column was placed into a clean tube and centrifuged for 1 min. To elute the RNA, the spin column was placed into a 1.5mL collection tube and 50µL RNase-free water was added directly to the spin column membrane and centrifuged at 8000 x g for 1 min. The RNA elution process was repeated using the previous eluate to ensure high RNA yield. For RNA clean-up, a RNeasy mini spin column (Qiagen) protocol was used following the manufacturers instruction. Briefly, the volume of the purified RNA was adjusted to 100µL using RNase free-water. Thereafter, 350µL RLT buffer was added to the RNA and mixed thoroughly. Following this, 250µL of absolute ethanol was added to the RNA mixture and mixed by pipetting. 700µL of the RNA mixture was then transferred to spin column placed in a 2mL tube and centrifuged at 8000 x g for 15 sec. Subsequently, 500µL of RPE buffer was added to the spin column and centrifugation was repeated. Flow through was again discarded. 500µL of RPE buffer was again added to the spin column and centrifugation was repeated at same speed for 2 mins. To prevent ethanol

contamination, spin column was placed into a new collection tube and re-centrifuged for 1 min. Following this, RNA was eluted as described. RNA concentration was determined using Nanodrop 8000 (ThermoFisher Scientific, UK).

2.14.2.2 DNase treatment

To remove any DNA contamination from the purified RNA, a TURBO DNA-free kit (Invitrogen, UK) was used. Briefly, 5µL of 10x turbo DNase buffer and 1µL of DNase enzyme supplied was added to 50µL RNA sample in a 0.5mL tube. This was mixed gently and incubated at 37^0C for 30 mins. Thereafter, 5µL of DNase inactivation reagent supplied was added to the mixture and mixed by pipetting and incubated for 5 mins at RT. Following this, DNase inactivation reagent was pelleted by centrifugation at 10,000 x g for 1.5 min and the supernatant containing the RNA was collected and stored at -80^0C prior to cDNA synthesis.

2.14.2.3 Complimentary deoxyribonucleic acid (cDNA) synthesis

To synthesize the first strand cDNA template, a high capacity cDNA reverse transcription kit (ThermoFisher Scientific, UK) was used. For the preparation of 2x reverse transcription (RT) master mix, the following kit components was combined in a 1.5mL PCR tube; 2µL of 10x RT buffer, 0.8µL of 25x dNTP mix (100nM) 2µL of 10x RT random primers, 1µL of multiScribe reverse

transcriptase and 4.2µL nuclease free water. The mixture was placed on ice until ready to use. For the preparation of the reverse transcription reaction, 10µL of the prepared 2x RT master mix was placed in a PCR tube and 10µL of the RNA sample containing 1µg of total RNA was added. The content of the tube was mixed gently on ice by pipetting. The tube was then sealed and centrifuged at 2000 x g for 10 sec to bring the reaction to the bottom of the tube and to eliminate air bubbles. The reaction was placed on ice until ready to load on the thermal cycler. Reaction tubes was placed in thermal cycler (Bio-Techne). The thermal cycling conditions used are displayed in Table 2.2. The cDNA samples were diluted 1/10 in nuclease free water and stored at -80^{0}C before use.

Table 2.2: Thermal cycling conditions used for the reverse transcription of RNA to cDNA.

Settings	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Temp. (^{0}C)	25	37	85	4
Time (mins)	10	120	5	∞

2.14.2.4 Real time polymerase chain reaction (RT-PCR)

For the quantification of GLUT4 transcript levels a Taqman GLUT4 PCR assay (Mm00436615_m1) and Taqman master mix (4444556) were used. Beta-actin (Mm00607939_s1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mm99999915_g1) were used as the housekeeping genes. All assays and

reagents were purchased from Applied Biosystems, UK. Taqman master mix was allowed to thaw on ice and mixed thoroughly before use in the reaction. Taqman GLUT4 assays and RNA samples were also thawed on ice, then vortexed and centrifuged at 2000 x g for 10 sec before use. Following this, PCR reaction mix was prepared by addition of 10µL of 2x master mix to 7µL nuclease free water into a PCR tube. The PCR reaction mix was then mixed by vortexing and then centrifuged to collect the mixture and eliminate air. 10µL of the PCR reaction mix was then transferred into each well of 96 well PCR reaction plate (Bio-Rad) and placed on ice. Following this, 1µL of either GLUT4 assay, Beta-actin or GAPDH were added to appropriate wells. Thereafter, 2µL (10ng) of cDNA samples was added to appropriate wells. Nuclease-free water was used as no template controls (NTC). Reactions were performed in triplicate wells per sample. The reaction plate was then sealed with an optical adhesive film (Bio-Rad) and then centrifuged to collect the mixture. Subsequently, the plate was placed in a CXF96 thermal cycler (Bio-Rad) for GLUT4 gene amplification. The amplification conditions used is shown in Table 2.3. Following gene amplification, expression levels of GLUT4 mRNA in treated cells relative to actin was quantified using the delta-delta-cycle threshold methods.

Table 2.3: Thermal cycling conditions used for detection of GLUT4 mRNA.

Uracil-N-glycosylase (UNG) incubation	**Polymerase activation**	**PCR (40 cycles)**	
Hold 50^0C	Hold 95^0C	Denature 95^0C	Anneal/extend 60^0C
2 min.	2 sec.	1 sec.	20 sec.

2.15 Metacercariae

Metacercariae were generously supplied by Prof Jane Hodgkinson of University of Liverpool, Institute of Infection and Global Health. The supplied metacercariae were shed by F. *hepatica* infected snails maintained on algae in a controlled laboratory environment. They were supplied on Visking tubing placed in a Bijou containing double distilled water. Metacercariae were kept at 4^0C prior to NEJs excystation assay.

2.15.1 NEJ excystation

To prepare NEJs for excystation, the following protocol was adopted. Exystation buffers; 0.9% sodium chloride (NaCl) and 1.2% sodium bicarbonate ($NaHCO_3$) was prepared in a 50mL falcon tube containing distilled water. Thereafter, 40mg of sodium tauroglychocolate (Sigma) was added to this solution. This solution was designated excystation solution 1. Following this, 40mg L-cysteine (Sigma) was added in a fume hood to a solution containing 2.34M hydrochloric acid (HCl) diluted in water. This solution was designated excystation solution 2. Excystation buffers were placed in a water bath at 37^0C until ready to use, but not for more than 1hr. All plastic ware, tips, petri-dishes and watch glasses used were serum lined to prevent sticking of metacercariae. Thereafter, metacercariae on visking tubing were placed in a petri-dish. Metacercariae were popped out of their outer cyst using a scalpel blade under a dissecting microscope. Metacercariae were carefully removed from the petri-dish placed into a clean watch glass. Metacercariae were then incubated for 4.5 mins at RT in solution containing 1:10 thin bleach (Tesco) in ddH_20. During this incubation, the watch glass containing the metacercariae was gently agitated to facilitate metacercariae excystation. After this incubation, the bleach was removed and metacercariae were washed 3 times in double distilled water. Thereafter, metacercariae were transferred into a clean watch glass and the wash process was repeated twice. Following this, equal volumes of excystation solution 1 and 2 was mixed together in a 5mL falcon tube and then poured onto the metacercariae. The watch glass containing the metacercariae was placed in a lunch box lined with damp paper towel and then the lunch box was securely covered. The metercariae were

incubated at 37^0C for 1hr. Thereafter, metacercariae were exposed to air for 5min and then covered and returned to 37^0C incubator. After 15mins incubation, excystation was observed and subsequently NEJs were collected using a serum lined tip into a tissue culture plate containing pre-warmed RPMI medium. NEJ were placed in 37^0C incubator prior to NEJs viability and growth assays.

2.15.2 NEJ culture in monocytes and CD4 T-cells conditioned medium

To determine NEJ viability in conditioned medium, monocytes and CD4 T-cells were stimulated as previously described (2.9.3.1 and 2.9.3.2) and supernatant was harvested. 20 NEJs were placed per well in 96 well plates containing 250µL of conditioned medium and parasites were cultured in duplicate for 12-18 hours at 37°C, 5% CO_2 incubator. Control worms were cultured in RPMI medium alone. Following incubation, NEJs were photographed at 10X magnification to assess growth and subsequently collected using a serum lined 20µL tip and transferred to 96 well flat bottom plates (Thermofisher Scientific) to determine viability using MTT reagent (Sigma).

2.15.2.1 Assessment of NEJ growth and viability following culture in conditioned medium

Following parasite culture in either monocytes or CD4 T-cell conditioned medium, parasites were collected and photographed as described above. Parasite growth was assessed by measuring worm area using ImageJ software version

1.50i (https://imagej.nih.gov/ij/). Thereafter, parasite viability was determined using MTT reagent as described by (Comley *et al*., 1989). Briefly, NEJs were transferred into a 96 well plate and incubated for 1hr at 37^0C for 30 min in 100uL solution of MTT reagent diluted to a final concentration of 5mg/mL in PBS. Thereafter, the MTT solution was replaced by an equal volume of DMSO and incubated at RT for 1hr. Following incubation, the solution was transferred to an ELISA plate without disturbing the parasites and OD was read at 540nm using a varioskan multimode plate reader.

2.15.3 NEJ culture in FhTLM primed Eosinophils

To determine the viability of NEJs in the presence of eosinophils, BMDEs were generated as described (2.7). Prior to eosinophil and NEJ co-culture, eosinophils were primed with either 200ng/mL FhTLM or BSA (control protein) prepared RPMI medium and incubated for 24hrs in a 37^0C and 5% CO_2. Following this, eosinophils were harvested by centrifugation at 300 x g for 8 mins. NEJs were prepared as previously described (2.15.1) and were co-cultured with eosinophils using 10 NEJs per well at a ratio of 5:1 (eosinophils: NEJs) in RPMI medium. Plates were incubated at 37^0C and 5% CO_2 incubator for 24hrs. Thereafter, plates were centrifuged, and supernatant was carefully recovered and transferred into 1mL Eppendorf tubes. The supernatant was frozen at minus 20^0C prior to NEJ viability analysis.

2.15.3.1 Assessment of NEJ viability following culture in FhTLM-primed eosinophils

Following co-culture of NEJs in eosinophils primed with either in FhTLM or BSA as described (2.15.3), the supernatant was collected for the detection of NEJ viability. Parasite viability was assessed using a CellTiter-Glo luminescent assay kit (Promega) that measures ATP levels as a readout for viable cells. Briefly, 100µL/well of the isolated supernatant was placed in triplicate in an opaque-walled 96 well plates (Greiner Bio-one) and incubated with an equal volume of CellTiter-Glo reagent as supplied. RPMI was used as blank control. The content of the plate was mixed using a plate shaker and incubated for 10 min at RT. Following incubation, luminescence was measured using a varioskan multimode plate reader.

3 The effect of FhTLM on regulatory and anergic T-cell induction in CD4 T-cells

3.1 Introduction.

In helminth infection, the two main regulatory cell phenotypes have been extensively characterised; $Foxp3^+$ T-reg cells and anergic (suppressive) T-cells (reviewed in chapter 1). Dominance of T-reg cells has been implicated in persistence of infection and inability of host to clear their helminth burden in various settings including *Leishmania major* (Belkaid *et al*., 2002), *Heligmosomoides polygyrus,* (Grainger *et al*., 2010), *Brugia malayi* (McSorley *et al*., 2008), and *S. mansoni* (Layland *et al*., 2013) infections.

Aside from induction of T-reg cells, helminths may also control host responses by generating a population of conditioned T-cells termed anergic T-cells. These cells are typically non-responsive to antigenic stimulation, hence they do not proliferate or produce effector cytokines that are critical for protection during infection (Taylor *et al*., 2005). Expression of the anergic markers PD-1, PD-L1, GRAIL and CTLA-4 are often associated with T-cell suppression during helminth infection including *F. hepatica* (Terrazas *et al*., 2005, Chen and Wahl, 2003, Smith *et al*., 2004, Aldridge and O'Neill, 2016). Reversal of T-reg or anergic T-cell activities have been shown to restore host resistance and promote parasite clearance (Taylor *et al*., 2005, Layland *et al*., 2013, Sawant *et al*., 2014, Taylor *et al*., 2009, Walsh *et al*., 2007, Taylor *et al*., 2007).

Previous studies by Johnston *et al*., (2017) have shown that Hp-TGM, a component of *H. polygyrus* ES binds mammalian TGF-β receptor and induced expression of $FoxP3^+$ T-reg cells (Johnston *et al*., 2017). EmACT, a TGFβ/Activin homologue present in the ES of *Echinococcus multilocularis* induced $CD4^+FoxP3^+$ *in vitro* in a TGF-β dependent manner (Nono et al., 2019). Moreover, previous work in our group, has shown that FhTLM (reviewed in

chapter 1) could also bind and initiate TGF-β signalling (Sulaiman *et al*., 2016). Given the important role played by regulatory cells during chronic helminth infections and the contribution of TGF-β in their development (Chen *et al*., 2003, Fantini *et al*., 2004), in the present chapter the aim was to determine the effect of FhTLM on induction of regulatory T-cells (PD-1, PD-L1, CTLA-4 and FoxP3). This will yield insight into understanding the parasite-mediated suppression of the T-cell response that is often associated with *F. hepatica.*

3.2 Methods.

3.2.1 CD4 T-cell separation and culture.

CD4 T-cells were either isolated (2.4.2) from Foxp3^{EGFP} mouse (for the detection of FoxP3) or C57BL/6J mice (for the detection of anergic markers) and cultured as described previously (2.9.1).

3.2.2 Determination of CD4 T-cell purity and expression of regulatory and anergic markers on CD4 T-cells by flow cytometry

Following CD4 T-cell separation, purity of isolated CD4 T-cells was assessed as described (2.10.1). For the detection of Foxp3 T-reg cells and anergic markers, PD-1, PD-L1 and CTLA-4, CD4 T-cells were processed as previously described (2.10.2).

3.2.3 Cytokine detection

IL-10, PD-1, PD-L1 and CTLA-4 protein production in stimulated CD4 T-cell supernatant was assessed by ELISA as previously described (2.13).

3.2.4 Statistical analysis

Expression of regulatory markers were analysed using KALUZA software version 2.1.1. Results were graphed and data analysed using GraphPad prism version 8.1.2 software. One-way ANOVA was used for analysis of significant difference between treatments and control groups; significant difference was based on Tukey's multiple comparisons test.

3.3 Results

3.3.1 CD4 T-cell purity

Following CD4 T-cell isolation from murine splenocytes, the antibody purity of these cells was determined by labelling with anti-CD4 antibody and analysing using flow cytometry (Figure 3.1).

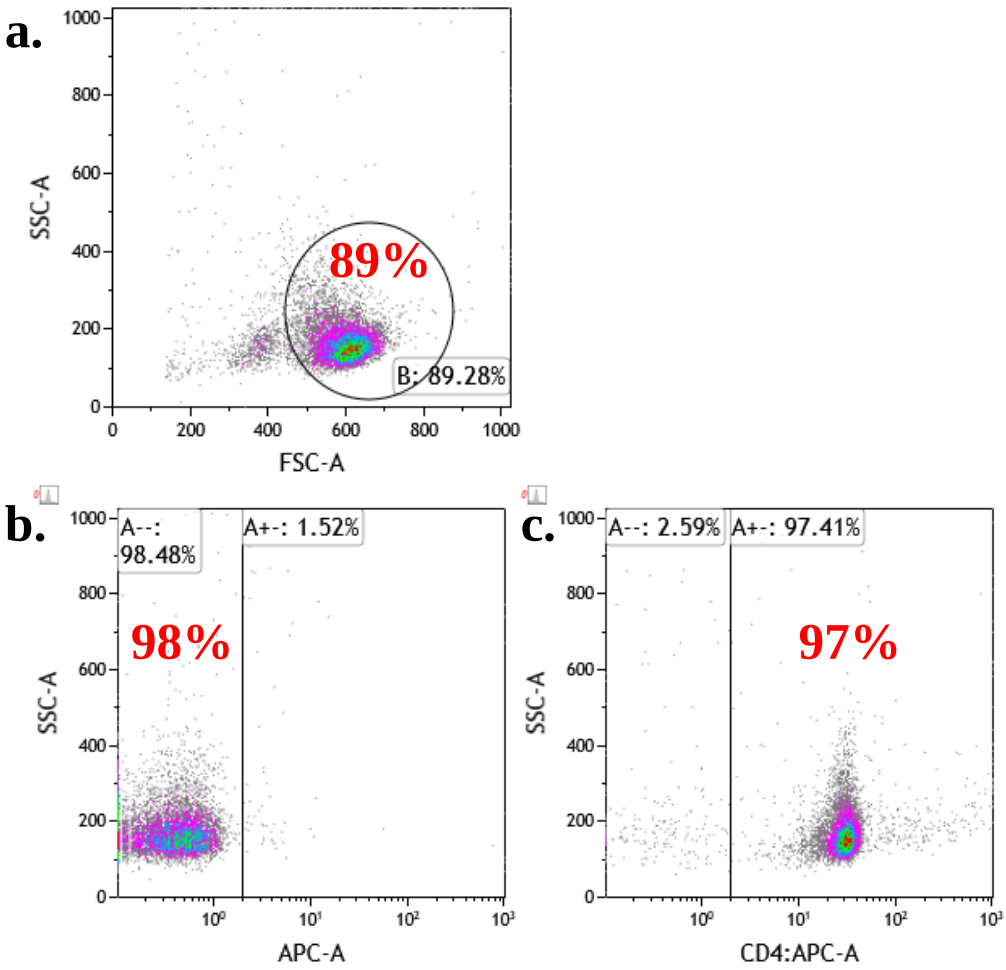


Figure 3:1: Percentage purity of CD4 T-cells. CD4 T-cells were isolated from murine splenic cells as previously describes (2.4.2) and the purity was analysed by flow cytometry. Dot plots indicate (a) Forward and side scatter analysis showing that the purified cell population contained greater than 89% lymphocytes, (b) Isotype control with no CD4 expression, (c) shift of dot plot to the right showing 97% $CD4^+$ cells incubated with Anti-CD4: APC antibody.

3.3.2 FhTLM fails to induce FoxP3 in $CD4^+$ T-cells

To determine if FhTLM could induce Foxp3 expression on CD4 T-cells, the T-cells were isolated from $Foxp3^{EGFP}$ mice and cultured following T-reg inducing conditions described in (2.9.1). The results indicated that background Foxp3 expression was only found in 0.91% of cells in the negative control treatments (Figure 3.2a). As expected, TGF-β induced expression of Foxp3 in greater than 41% of CD4 T-cells (Figure 3.2 b). However, FoxP3 expression was not significantly raised above control levels in FhTLM stimulated CD4 T-cells (Figure 3.2 c-e; $P>0.05$), as Foxp3 expression was only seen in less than 2% of FhTLM stimulated cells. In T-reg induction, representing the positive control, more than 30% of CD4 T-cells routinely expressed Foxp3 after T-reg culture (Figure 3.2 f; ***$p\leq0.0009$).

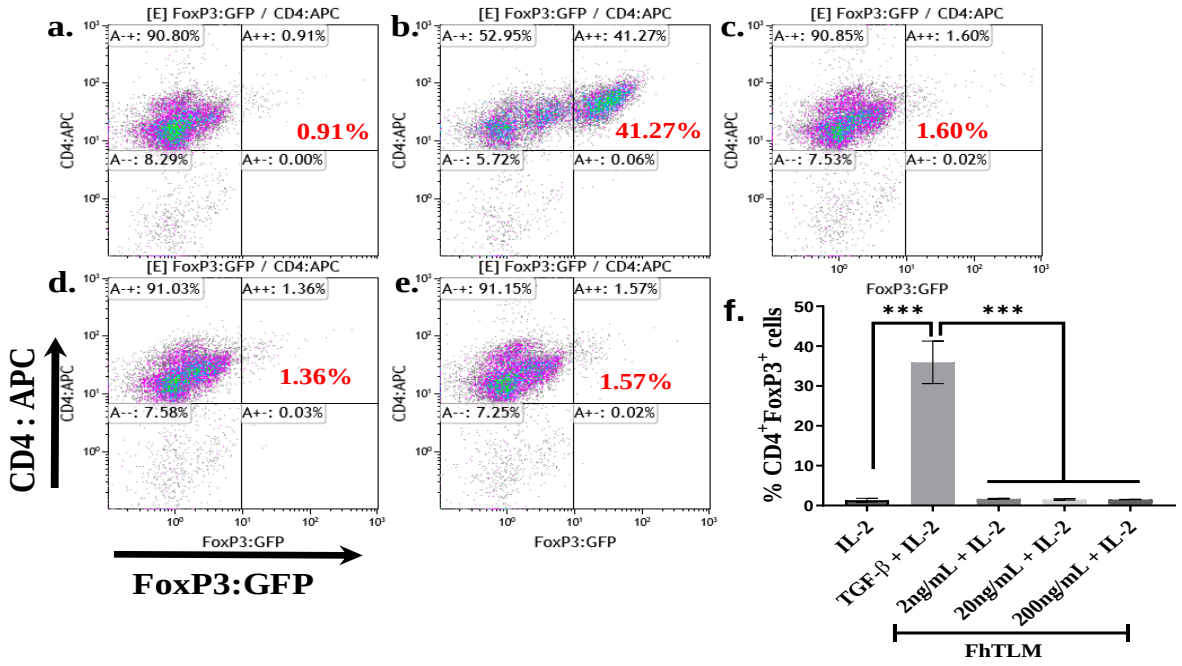


Figure 3:2: Induction of FoxP3 in CD4⁺ T-cells. $CD4^+$ T-cells isolated from $FoxP3^{EGFP}$ mice were either stimulated with IL-2 (20ng/ml) alone or in combination with either 2ng/mL recombinant human TGF-β or FhTLM. Cells were harvested following 3 days culture and percentage $CD4^+Foxp3^+$ cells were detected by flow cytometry on a FITC/GFP channel. Percentage $CD4^+Foxp3^+$ cells are shown on dot plots for (a) IL-2, (b) TGF-β + IL-2, (c) 2ng/mL FhTLM + IL-2, (d) 20ng/mL FhTLM + IL-2, (e) 200ng/mL FhTLM + IL-2, and (f) Pooled data from two independent experiments displaying mean ± SEM. Significant difference between treatments and control was determined using one-way ANOVA and ***indicates $p<0.0009$. CD4 T-cells were isolated from n=6 animals per experiments.

3.3.3 Expression of markers of anergy within FhTLM-stimulated CD4 T-cells.

In order to assess whether FhTLM could induce expression of markers of anergy we examined expression of PD-1 or PD-L1 and CTLA-4 in T-cells. CD4 T-cells isolated from C57BL/6J mice were cultured as described (2.9.1). FACs analysis of cultured CD4 T-cells indicated that FhTLM stimulation (Figure 3.3 d-f), at varying doses, does not induce a significant increase in the number of $CD4^+PD\text{-}L1^+$ cells ($P \geq 0.8008$) when compared to the IL-2 control (Figure 3.3b). Similarly, T-reg induction does not result in a significant increase in the percentage of $CD4^+PD\text{-}L1^+$ cells (Figure 3.3c; $P = 0.9998$) when compared to IL-2 only controls. Expression of PD-1 was not detected under any treatment conditions (Figure 3.4 and Figure 3.7). CTLA-4 expression was in less than 1% of FhTLM stimulated cells (Figure 3.5d-f) and this was comparable to the levels detected in IL-2 controls. Although the levels of $CD4^+CTLA\text{-}4^+$ cells were raised for T-reg induction (Figure 3.5c), this was not statistically significant when compared to IL-2 or FhTLM groups ($P \geq 0.6011$).

To determine if co-stimulation with anti-CD28 could enhance expression of anergic markers on $CD4^+$ T-cells, CD4 T-cells were activated with plate bound anti-CD3 and soluble anti-CD28 as described (2.9.1) prior to stimulation with FhTLM or TGF-β. Our result indicates that levels of PD-L1 induced following FhTLM stimulation (Figure 3.6d-f) was comparable to that seen in media alone controls (Figure 3.6b). T-reg induction, conversely, appeared to down modulate PD-L1 expression (Figure 3.6c), although this was not statistically significant when compared to media alone or FhTLM controls ($p \geq 0.9291$).

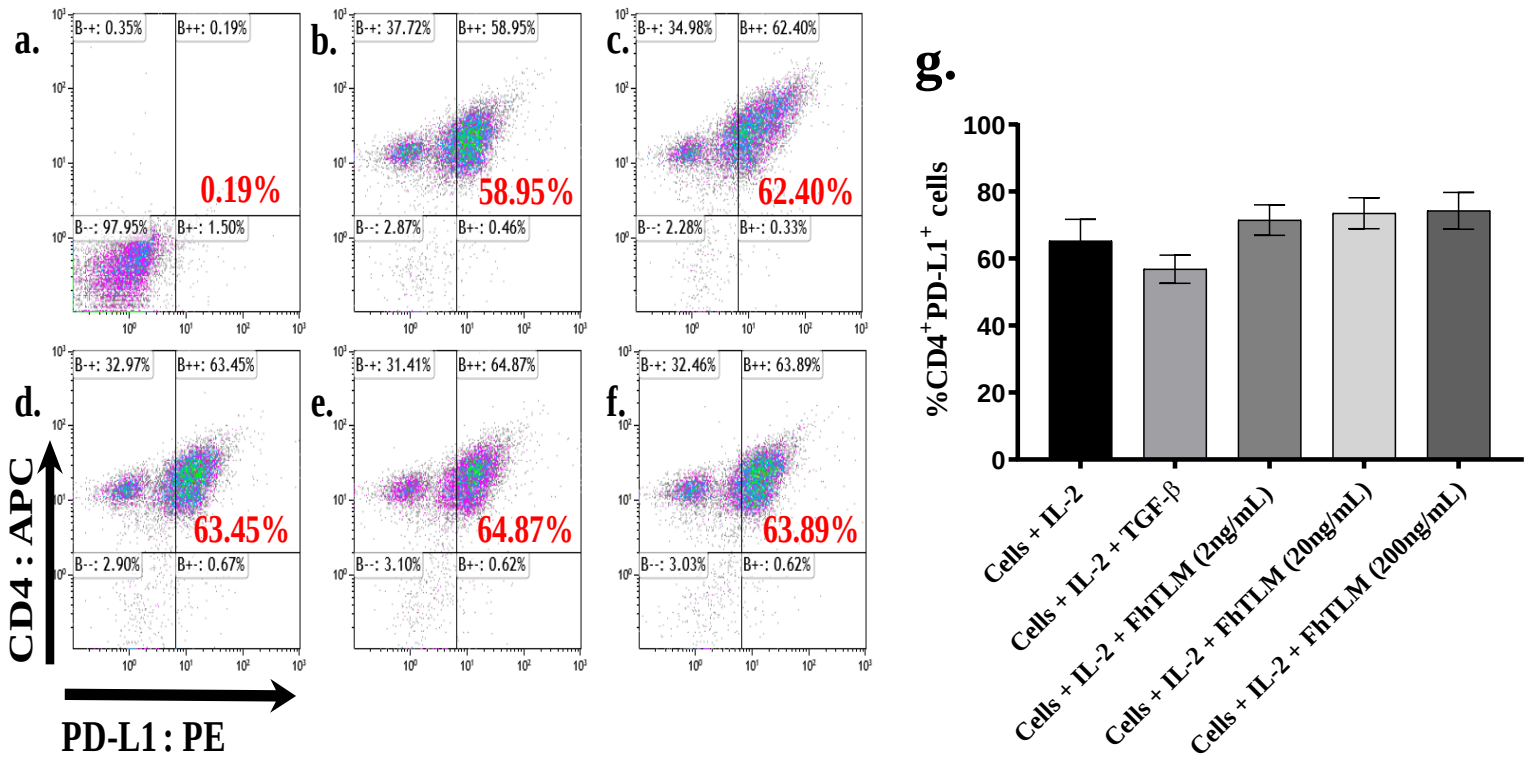


Figure 3:3: PD-L1 expression in FhTLM-stimulated CD4$^+$ T-cells. CD4$^+$ T-cells isolated from C57BL/6J mice were stimulated as described in Figure (3.2). Cells were harvested after 3 days culture and expression of PD-L1 was analysed by flow cytometry. Percentage CD4$^+$PD-L1$^+$ cells are shown on dot plots for (a) isotype control, (b) cells + IL-2, (c) cells + IL-2 + TGF-β, (d) cells + IL-2 + 2ng/mL FhTLM, (e) cells + IL-2 + 20ng/mL FhTLM, (f) cells + IL-2 + 200ng/mL FhTLM, (g) Mean ± SEM, of two independent experiments. Data were analysed using one-way ANOVA. CD4 T-cells were isolated from n=6 animals per experiment.

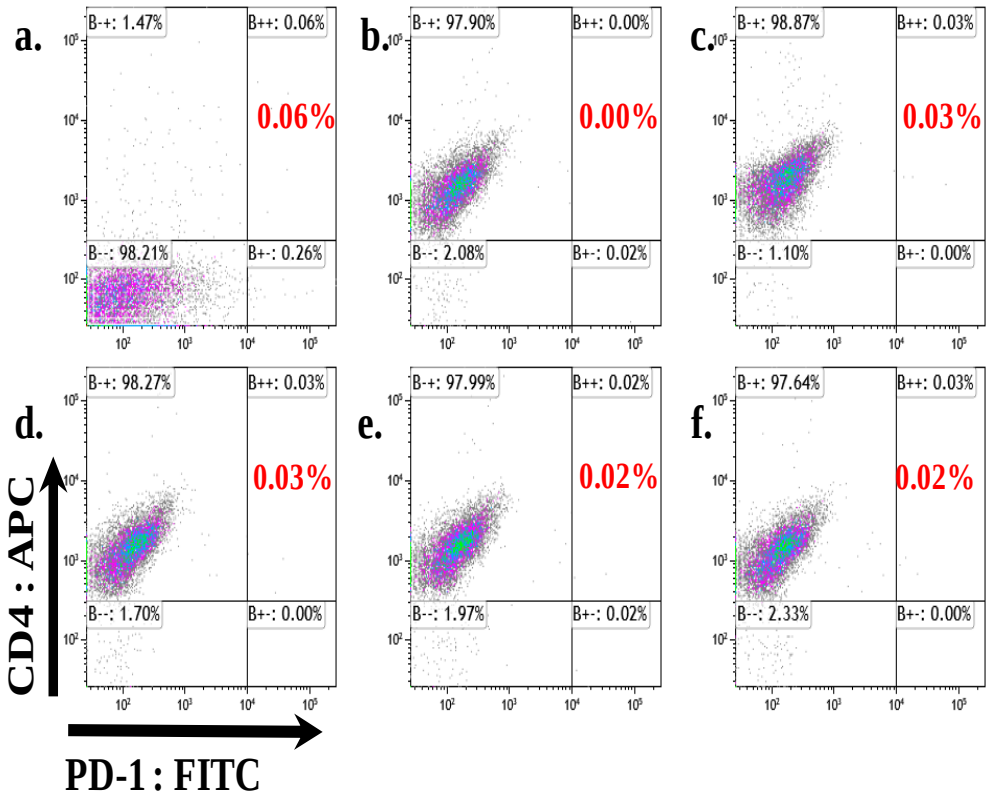


Figure 3:4: PD-1 expression in FhTLM-stimulated CD4$^+$ T-cells. CD4$^+$ T-cells isolated from C57BL/6J mice were stimulated as described in Figure (3.2). Cells were harvested after 3 days culture and expression of CD4$^+$PD-1$^+$ cells were analysed by flow cytometry. Percentage CD4$^+$PD-1$^+$ cells are shown on dot plots (red colour) for (a) isotype control, (b) cells + IL-2, (c) cells + IL-2 + TGF-β, (d) cells + IL-2 + 2ng/mL FhTLM, (e) cells + IL-2 + 20ng/mL FhTLM, (f) cells + IL-2 + 200ng/mL FhTLM. Dot plots are representative of two independent experiments with similar results.

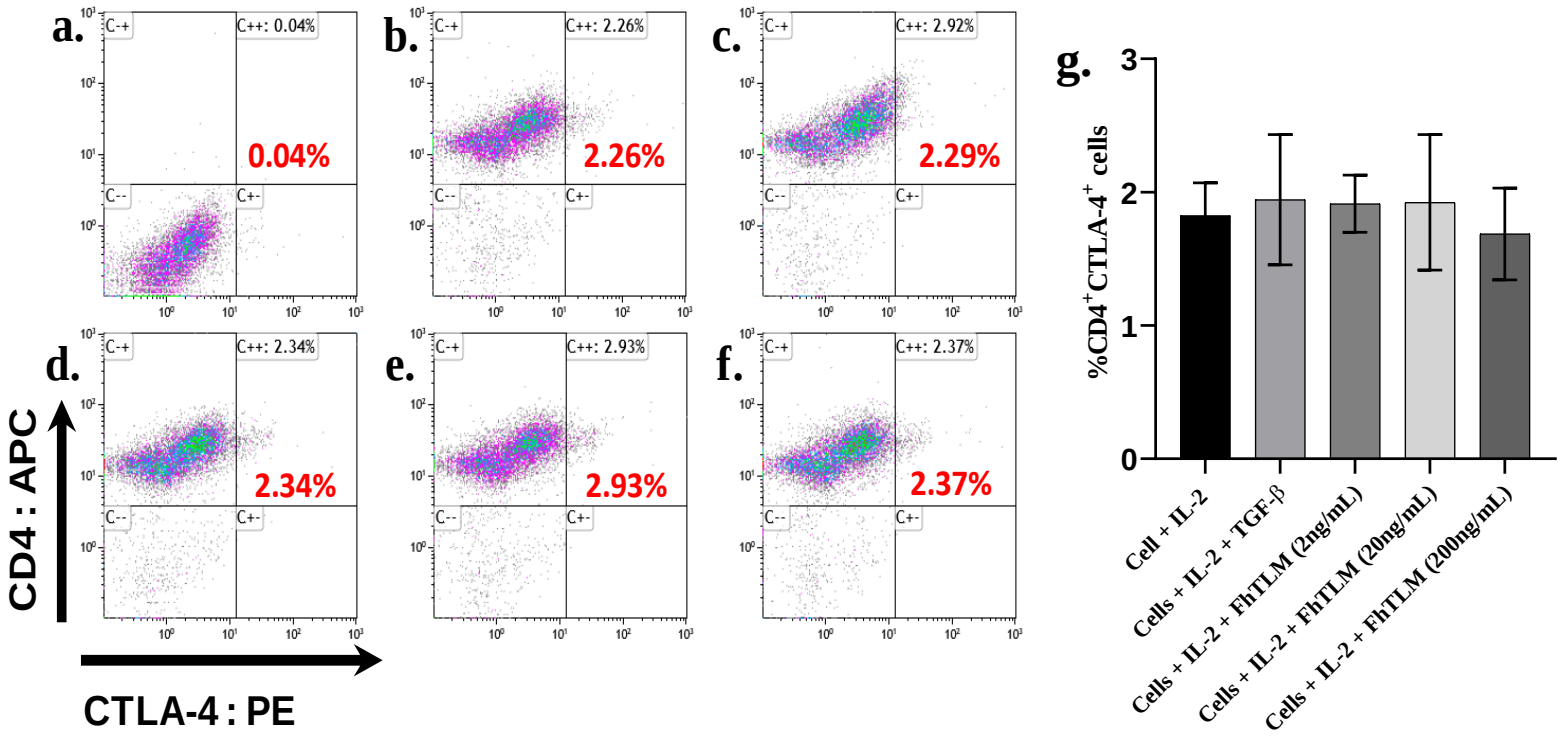


Figure 3:5: CTLA-4 expression in FhTLM-stimulated CD4$^+$ T-cells. CD4$^+$ T-cells isolated from C57BL/6J mice were stimulated as described in Figure (3.2). Expression of CD4$^+$CTLA-4$^+$ cells were analysed by flow cytometry. Percentage CD4$^+$CTLA-4$^+$ cells is shown on dot plots for (a) isotype control, (b) cells + IL-2, (c) cells + IL-2 + TGF-β, (d) cells + IL-2 + 2ng/mL FhTLM, (e) cells + IL-2 + 20ng/mL FhTLM, (f) cells + IL-2 + 200ng/mL FhTLM. (g) Mean ± SEM, of two independent experiments; data were analysed using one-way ANOVA. CD4 T-cells were isolated from n=6 animals per experiment.

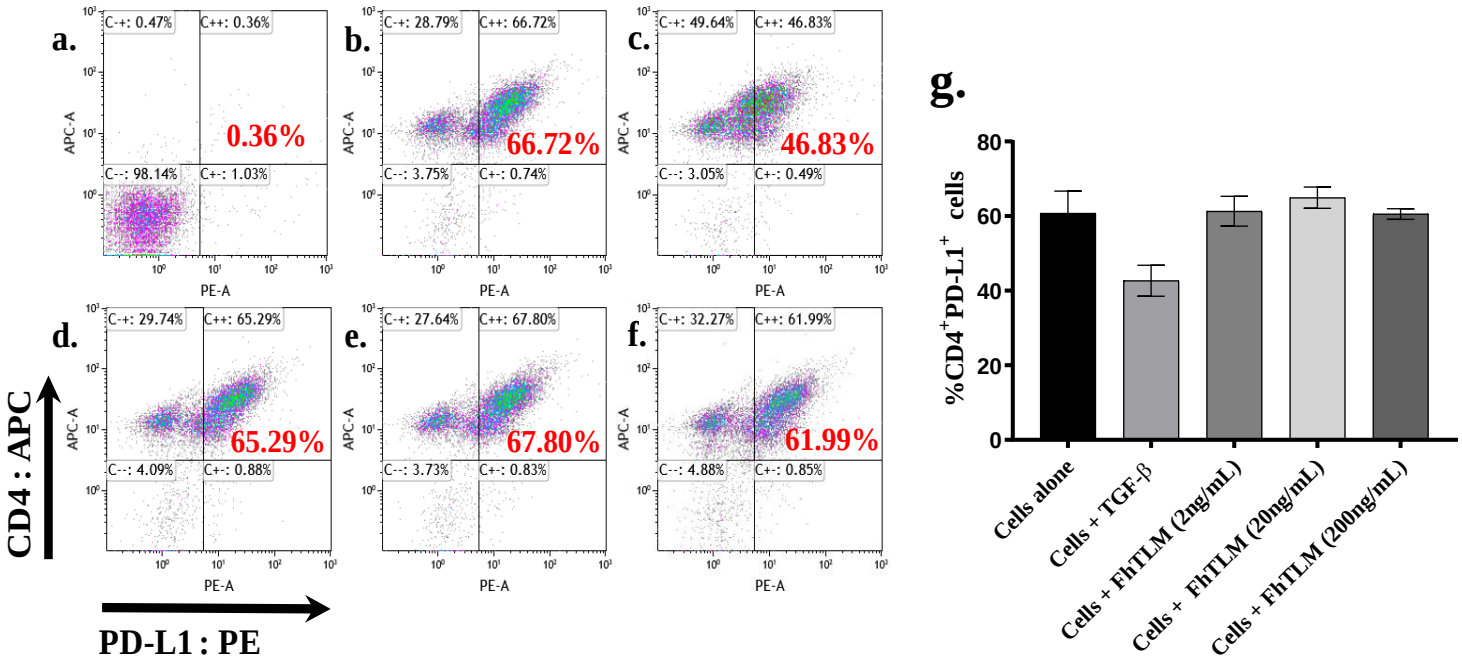


Figure 3:6: PD-L1 expression in FhTLM-stimulated CD4⁺ T-cells in the presence of co-stimulation of CD28 using anti-CD28.

Isolated CD4⁺ T-cells were activated using plate bound anti-CD3 and soluble anti-CD28. Cells were either unstimulated or stimulated with either rhTGF-β or FhTLM at various doses (2-200ng/mL). Following 3 days culture, cells were harvested and expression of $CD4^+PD\text{-}L1^+$ cells was analysed by flow cytometry. Percentage $CD4^+PD\text{-}L1^+$ cells is shown on dot plots for (a) isotype control, (b) cells + media alone, (c) cells + TGF-β, (d) cells + 2ng/mL FhTLM, (e) cells + 20ng/mL FhTLM, (f) cells + 200ng/mL FhTLM, (g) Mean ± SEM, of two independent experiments; data were analysed using one-way ANOVA. CD4 T-cells were isolated from n=6 animals per experiment.

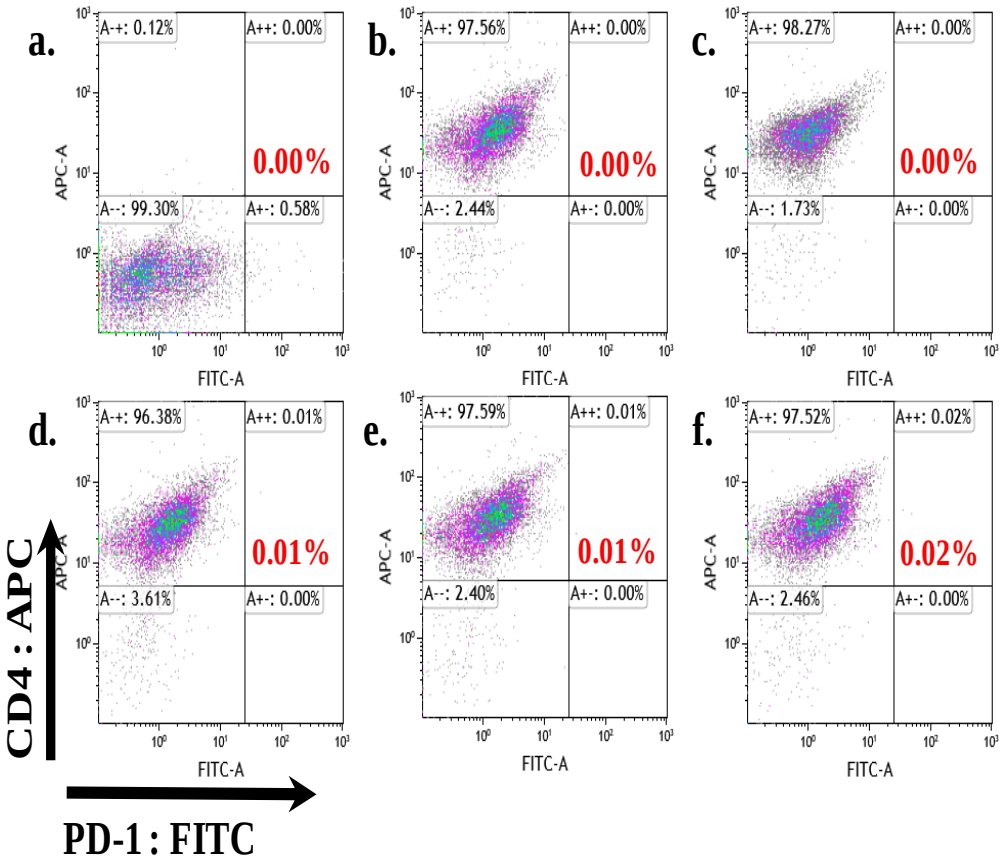
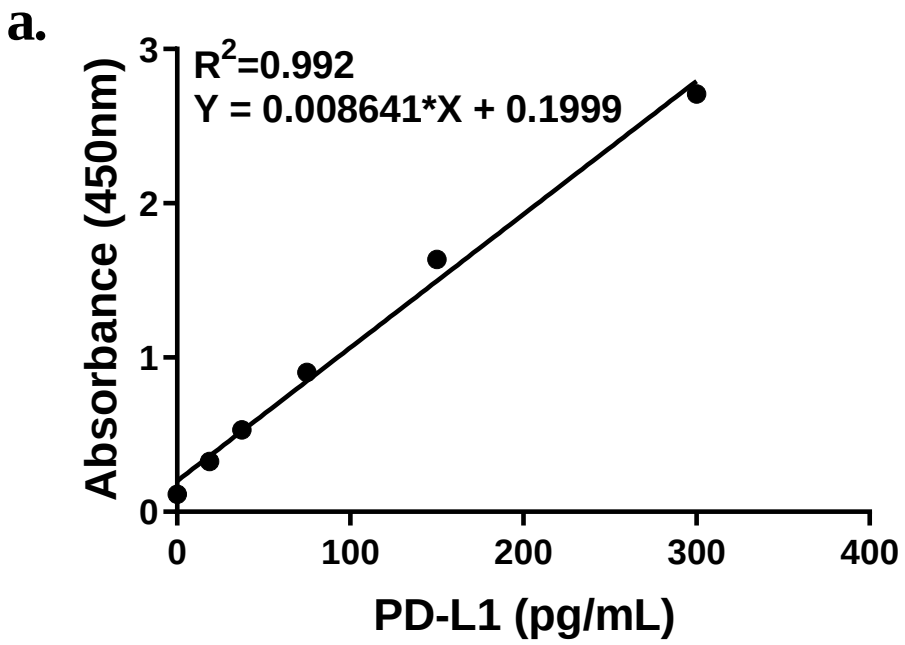


Figure 3:7: PD-1 expression in FhTLM-stimulated CD4 T-cells in the presence of co-stimulation of CD28 using anti-CD28. Cells were stimulated as described in Figure (3.6). Percentage $CD4^{+}PD\text{-}1^{+}$ cell is shown on dot plots for (a) isotype control, (b) cells + media alone, (c) cells + TGF-β, (d) cells + 2ng/mL FhTLM, (e) cells + 20ng/mL FhTLM, (f) cells + 200ng/mL FhTLM. Data shown is from one experiment. CD4 T-cells were isolated from n=6 animals.

3.3.4 Anergic molecules; secretion analysis

Under some circumstances, cellular markers of anergy are secreted (Jago *et al*., 2004, Davies *et al*., 2017). To determine if this was occurring in our system, we measured PD-1, PD-L1 and CTLA-4 in the cell culture supernatants by ELISA. Following stimulation of CD4 T cells as described previously, supernatants were harvested and analysed for detection of PD-1, PD-L1 and CTLA-4. No detectable level of any of these were observed in our control treatments (IL-2 alone). Similarly, neither FhTLM stimulation nor T-reg inducing conditions induced the secretion of these molecules. This finding was not due to the failure of the ELISA as standard curves for each yielded a concentration-dependent increase in signal spanning 300-18.75pg/mL, 4000-125pg/mL, and 2000-125pg/mL for PD-1, PD-L1 and CTLA-4 respectively (Figure 3.8).



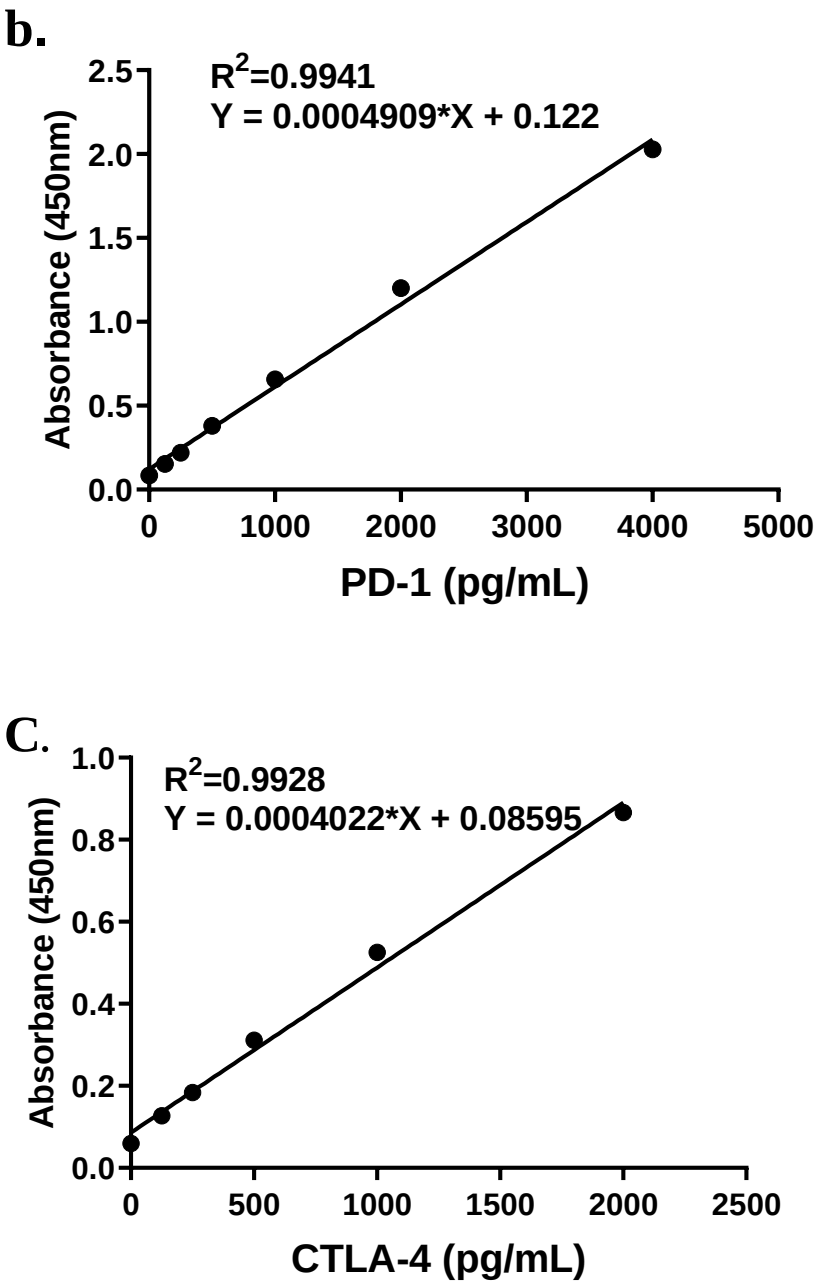


Figure 3:8: Standard curves for anergic molecule ELISA. 1 x 10^6 $CD4^+$ T-cells isolated from spleen cells of C57BL/6J mice were placed per well and were either unstimulated (media alone) or stimulated with IL-2 (20ng/ml) alone or in combination with either 2ng/mL TGF-β or FhTLM at various doses (2, 20 and 200ng/mL). Following stimulation of cells, supernatants were harvested and used for indicated cytokine analysis by ELISA. Figures represents standard plots

used for the detection of (A) PD-L1, (B) PD-1 and (C) CTLA-4 showing mean OD value determined at various concentrations.

3.3.5 IL-10 production in FhTLM stimulated CD4 T-cells

CD4 T-cells were stimulated as described (2.9.1) and IL-10 production in the culture supernatant was measured by ELISA (2.12) and compared between different treatments. (Fig. 3.9). Maximum expression of IL-10 was detected in FhTLM stimulated cells when used at 2ng/mL concentration ($p \leq 0.0002$). In addition, significant expression of IL-10 was also detected when cells were stimulated with 200ng/mL FhTLM ($p= 0.029$). In contrast, Treg induction did not induce significant IL-10 secretion into the supernatant ($p= 0.5774$).

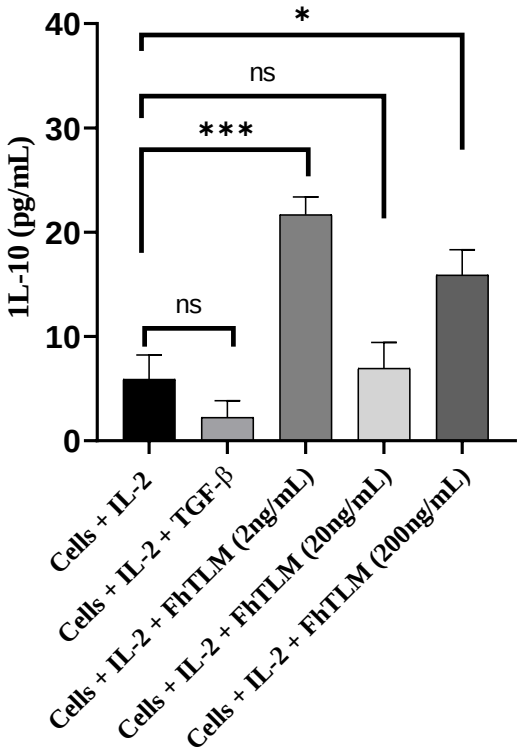


Figure 3:9: Secretion of IL-10 in FhTLM-stimulated CD4 T-cells. CD4 T-cells were isolated and stimulated as described in Figure 3.8 and the levels of IL-10 secretion were measured by ELISA. Data shown is mean ± SEM of three independent ELISAs from three independent cultures; data were analysed using one-way ANOVA with *** indicating p=0.0002 and * indicates p=0.0229. CD4 T-cells were isolated from n=6 animals per experiment.

3.4 Discussion

Helminth parasites secrete biologically active molecules that are critical for the generation and maintenance of conditions that enhance parasite persistence while suppressing protective immunity (Maizels *et al*., 2004, Hewitson *et al*., 2009). These molecules can direct the host immune response towards a non-protective phenotype via induction of regulatory or suppressive cell populations. Notably, *F. hepatica* infection is characterized by suppression of protective (Th1) immunity while generating a highly polarized non-protective Th2/T-reg immune responses and resulting in parasite persistence. Recent studies in our group suggests that *F. hepatica* possesses a TGF-β homolog, FhTLM (Japa *et al*., 2015), that was capable of altering host immune responses to benefit parasite survival (Sulaiman *et al*., 2016). In this study, we explored the effect of FhTLM on induction of immunomodulatory molecules using CD4 T-cells and flow cytometry.

To determine if FhTLM can induce expression of Foxp3 T-reg cells on CD4 T-cells, we stimulated naïve CD4 T-cells isolated from $Foxp3^{EGFP}$ mice with different doses of FhTLM. Our results showed that FhTLM stimulation failed to induce Foxp3 expression beyond that observed in baseline controls. This finding is in agreement with a previous report suggesting that Foxp3 expression was not raised beyond control levels in *F. hepatica* ES (FhES) treated splenocytes (Lund *et al*., 2014). However, Foxp3 could only be induced in this setting, when macrophages that were conditioned to a regulatory phenotype following intraperitoneal injection of FhES were co-cultured with naïve splenocytes (Lund *et al*., 2014). In addition, $Foxp3^{+}$ cells were only upregulated when FhES

primed DC were co-cultured with CD4 T-cells (Falcón *et al*., 2010). These findings indicate that the studied *F. hepatica* secreted molecules lack a direct capability to upregulate Foxp3 expression on CD4 T-cells. This argues for the presence of a molecular pathway that the parasite exploits via manipulation of APCs (including macrophages and DCs) for immune evasion to effectively dampen T-cell effector responses. However, our finding is contrary to other studies showing that various helminth products including *H. polygyrus* TGM (Johnston *et al*., 2017) and *S. mansoni* ω ‑ 1 (Zaccone *et al*., 2011), induces FoxP3 T-reg cells. Activation of Smad proteins are crucial for TGF-β-mediated induction of Foxp3 (Sundrud and Nolan, 2010). In addition, it has been shown that activated Smad3 interacts with the conserved non-coding promoter region of the Foxp3 gene by binding to other nuclear factors in the enhancer region to initiate Foxp3 expression (Xu *et al*., 2010). Although, FhTLM binds to mammalian TGF-β receptor complexes and initiates Smad2/3 phosphorylation (Sulaiman *et al*., 2016). Failure to induce Foxp3 expression may be attributed to inability of FhTLM-activated Smad complexes to interact with other nuclear factors such as nuclear factor of activated T-cells (NFAT) and cAMP response element-binding protein (CREB) that are critical for Foxp3 induction.

Previous studies have demonstrated that a number of other regulatory markers such as CTLA-4, PD-1 and PDL-1 operate in generating T-cell anergy and immune-suppression during chronic helminth infections (Taylor *et al*., 2005, Sharpe *et al*., 2007, Latchman *et al*., 2001). To determine the effect of FhTLM on upregulation of the indicated anergic markers, CD4 T-cells were stimulated with FhTLM and protein expression was determined by flow cytometry and validated by ELISA. Following stimulation of CD4 T-cells, flow cytometry

analysis showed that FhTLM failed to upregulate PD-L1 levels significantly and PD-1 was not detected. In line with our result, Guasconi et.al, (2015), showed that levels of PD-L1 were not significantly changed in peritoneal macrophages stimulated with FhES (Guasconi *et al*., 2015). However, our result is contrary to the previous finding that suggests that PD-L1 expression was enhanced on FhTLM stimulated bovine macrophages (Sulaiman *et al*., 2016). The discrepancies in the findings may be attributed to the capability of FhTLM to evoke different immunological responses in varying cell types. It may also reflect the presence of an immunomodulatory ability within the parasite that allows it to condition macrophages to a regulatory phenotype (Allen and Loke, 2001, Atochina *et al*., 2001, Donnelly *et al*., 2005), given the critical role of macrophages in mediating killing of juvenile flukes (Duffus and Franks, 1981, Sibille *et al*., 2004). In addition, T-cell anergy was mediated by induction of PD-L1 on splenic macrophages isolated following *S. mansoni* infection in mice (Smith *et al*., 2004). Our result showed that PD-1 was not expressed following stimulation of CD4 T-cells with FhTLM, contrary to a previous finding demonstrating an increase in the levels of PD-1 in CD4 T-cells upon injection of FhTeg in mice (Aldridge and O'Neill, 2016). However, it is not surprising that FhTeg evokes an immune response differing from that of FhTLM. Proteomic studies revealed that FhTeg is rich in multiple glycoproteins (Wilson *et al*., 2011) while FhTLM represent a single recombinant protein (Japa *et al*., 2015). In addition, the difference in findings may also be attributed to different experimental settings. While Aldridge and O'Neill employed an *in vivo* approach (which is capable of replicating the precise cellular conditions), ours utilized a simplified *in vitro* approach.

PD-1 is constitutively expressed on T-cells (Khoury and Sayegh, 2004, Greenwald *et al*., 2005) and upregulated upon T-cell activation (Keir *et al*., 2007). We attempted to use co-stimulation of CD4 T-cells with anti-CD28 antibody to promote PD-1/PD-L1 pathways. Following activation of CD4 T-cells and upon stimulation with FhTLM, we did not observe any increase in the expression levels of either PD-1 or PD-L1 compared to controls. This suggests that co-stimulation does not promote PD-1 or PD-L1 expression on FhTLM stimulated CD4 T-cells.

CTLA-4 on the other hand, negatively regulates T-cell activation and it delivers a down-regulatory effect on T-cell mediated immune responses (Bluestone, 1997), failing to respond to antigenic stimulation (Wells *et al*., 2001). Upon CD4 T-cell stimulation with FhTLM, we did not detect CTLA-4 expression. Our finding is contrary to Aldridge *et al*., (2017) who demonstrated that FhTeg induced markers of anergy including CTLA-4 in splenocytes isolated following *F. hepatica* infection in mice. CTLA-4 was found to be upregulated in response to various *in vivo* infections and are implicated in suppression of immune responses in multiple infections including *S. mansoni*, *N. brasiliensis*, *Trypanosoma cruzi*, and *L. donovani* (Walsh *et al*., 2007, Martins *et al*., 2004, McCoy *et al*., 1997, Murphy *et al*., 1998).

Under certain circumstances, for instance in cancer, cellular markers of anergy are secreted and this contributes to disease progression and pathogenesis (Jago *et al*., 2004, Davies *et al*., 2017, Jalali *et al*., 2018, Simone *et al*., 2014, AlFadhli, 2013, Gaber *et al*., 2018). To ensure this was not occurring in our system, we

assessed production of indicated markers in our culture supernatants. Consistent with our flow cytometry data, we did not detect any secretion of these markers. This suggests that recombinant FhTLM molecule may not be important for the *in vitro* generation of CD4 T-cell anergy via induction of PD-1/PD-L1 and CTLA-4. Perhaps, a condition of antigenic presentation or antigen-specific stimulation may be required to induce the studied pathways.

Immunoregulation during *F. hepatica* infections has been previously been shown to be associated with IL-10 production (Flynn and Mulcahy, 2008). Cellular production of IL-10 in FhTLM stimulated CD4 T-cells was assessed by ELISA. Our results showed that stimulation of CD4 T-cells with FhTLM resulted in production of IL-10 in the cell culture supernatant. This finding is consistent with production of IL-10 that has been reported in multiple parasite infections including *S. masoni* (Sher *et al*., 1991), *L. major* (Belkaid *et al*., 2001) and *F. hepatica* (Flynn and Mulcahy, 2008, Sulaiman et al., 2016) and suggests a role for this cytokine in the down-modulation of the host protective immune response.

In conclusion, in this chapter we have demonstrated that recombinant FhTLM failed to promote *in vitro* expression of FoxP3 or anergic T-cell markers. However, as indicated from previous studies that *F. hepatica* secretory molecules modulate T-cell anergy indirectly via induction of anergic markers on innate cells including macrophages and DCs (Lund *et al*., 2014, Aldridge and O'Neill, 2016), we therefore suggest that FhTLM may also have the capacity to potentiate this response. This remains to be investigated in a co-culture assay of FhTLM-primed macrophages or DC with naïve CD4 T-cells. Furthermore, we have also shown that FhTLM induced IL-10 production in CD4 T-cells and this

may have implications for the suppression of protective immunity and persistence of parasites during chronic *F. hepatica* infections.

4 The Cytokine and Chemokine responses associated with FhTLM stimulation of cells.

4.1 Introduction

Cytokines that specifically play roles in the trafficking of leukocytes to sites of infection are called chemokines (Luster, 1998). Inflammatory chemokines are produced at sites of inflammation in response to cues from invading pathogens (Laing and Secombes, 2004) where they mediate the recruitment of effectors to the site of pathogen invasion. Dixon *et al*., (2006) showed that infection with the intestinal nematodes *T. muris* and *T. spirallis* in $CCL11^{-/-}$ (eotaxin-1; an eosinophil chemoattractant) mice dramatically reduced the number of eosinophils recruited to gastrointestinal tract. However, detection of CCL24 (eotaxin-2) transcripts in the intestine of infected animals suggests that although CCL11 plays the major role in eosinophil recruitment, it may not represent the only chemotactic factor for eosinophils (Dixon *et al*., 2006).

Chemokines play a crucial role in the development of chronic helminth infections. A low parasite burden, reduced liver granuloma and poor eosinophil enzymatic activities was detected in $CCL3^{-/-}$ (macrophage inflammatory protein 1-alpha; MIP-1α) upon infection with *S. mansoni* (Souza *et al*., 2005). In addition, mesenteric lymph node cells isolated from these mice produced low levels of IL-4 and IL-10 in response to stimulation with SEA or adult worm antigens. This suggests a role for CCL3 in amplifying Th2 responses during *S. mansoni* infection. Moreover, $CCL2^{-/-}$ (monocyte chemotactic protein-1; MCP-1) mice have impaired Th2 responses characterised by poor IL-4 and IL-5 production and this is consistent with a reduced secondary granuloma size in mice intravenously challenged with *S. mansoni* eggs (Lu *et al*., 1998). Indeed Th2 granuloma formation in mice induced by SEA resulted in increased production of CCL2 when compared to granuloma formation induced by a Th1

promoting pathogen, *Mycobacterium tuberculosis* (Chensue *et al*., 1996). However, *in vivo* treatment with anti-CCL2 resulted in reduced Th2 granuloma size but had no effect on Th1 granuloma (Chensue *et al*., 1996), implicating a role for CCL2 in generation of Th2 responsive granulomas in murine schistosomiasis.

Few studies have investigated the role of chemokines during *F. hepatica* infection (Dowling *et al*., 2010, De Paula *et al*., 2010). It has been shown that $CCL3^{-/-}$ mice have reduced hepatic lesions with a low numbers of flukes at necropsy and a low mortality rate when compared to the wild type control (De Paula *et al*., 2010). Indicating that CCL3 during *F. hepatica* infection promotes chronic fasciolosis, parasite persistence, and host survival. In addition, various *F. hepatica* secreted antigens also mediate production of Th2 specific chemokines. For instance, DCs stimulated with rFhCL1 or rFhGST-si, produced high levels of CXCL2 (macrophage inflammatory protein 2-alpha; MIP-2α) a chemokine associated with selective migration of Th2 cells (Dowling *et al*., 2010, Müller *et al*., 2003). Enhanced production of CXCL2 by DCs in response to *F. hepatica* antigen may represent one of the mechanisms used by the parasite to modulate innate effector cells early during immune evasion to establish a Th2 response. *In vivo* injection of FhTeg or infection with *F. hepatica* metacercariae results in dramatic migration and accumulation of mast cells in the peritoneal cavity and liver of mice (Vukman *et al*., 2013a), suggestive of participation of chemokines during this infection. This was validated by *in vitro* experiments demonstrating that supernatants isolated from DCs stimulated with FhTeg, have high levels of CCL3 and CXCL2 and promote migration of mast cells in a chemotaxis assay (Vukman et al., 2013a).

Given the critical role played by chemokines in regulating the function of effector cells and development of non-protective immune responses during chronic helminth infections, it is thought that *F. hepatica* may target this aspect of the immune system. Indeed, since various *F. hepatica* secreted molecules including FhTeg, FhCL1 and FhGST (reviewed above) participate in induction of Th2 promoting chemokines, it was hypothesized that FhTLM may induce secretion of chemokines. Moreover, given the minimal impact of FhTLM on regulatory cell surface markers (Chapter 3), we performed a global profile of the cytokines and chemokines that may be induced by FhTLM stimulation. These initial findings show a skew towards upregulated chemokines, and these findings were confirmed by individual ELISA and bioassays.

4.2 Methods

4.2.1 CD4 T-cell chemokine and cytokine global profiling.

Various chemokines and cytokines (Table 4.1) were measured as described (2.11) in the culture supernatant of CD4 T-cell stimulated as described (2.9.1).

4.2.2 Chemokine ELISA.

The production of RANTES and TARC in CD4 T-cell culture supernatant described above were measured as described (2.13). In addition, production of RANTES in BMDM and RAW 264.7 cells stimulated as described (2.9.2) was measured as previously described (2.13).

4.2.3 BMDM morphology and expression of macrophage marker F4/80$^+$

BMDM cells were differentiated as described (2.6.1) and the morphology was assessed by Diff-Quick staining as described (2.6.1.1). Expression of the macrophage antigen F4/80$^+$ was determined on BMDM using rat anti-mouse F4/80: RPE antibody (Bio-Rad). The lyophilized F4/80: RPE antibody supplied was reconstituted in 0.25mL distilled water. Following this, 1 x 10^6 BMDM were labelled with 10µL of the reconstituted antibody (neat dilution as suggested by the manufacturer) for 1hr at 4^0C in the dark. After which labelled cells were washed 3 times in FACS buffer and resuspended in 1mL FACS buffer. Expression of F4/80$^+$ was analysed as described (2.10.1).

Table 4.1: Cytokines and chemokines that are targeted using the mouse array panel A (R&D).

CXCL13/ B lymphocyte chemoattractant **(**BLC)	IL-5	M-CSF
Complement component 5a (C5a)	IL-6	CCL2/ Monocyte chemoattractant protein 1 (MCP-1)
Granulocyte colony-stimulating factor (G-CSF)	IL-7	CCL12/MCP-5
Granulocyte macrophage colony-stimulating factor (GM-CSF)	IL-10	CXCL9/ Monokine induced by gamma interferon (MIG)
CCL1/I-309	IL-12 p70	CCL3/ Macrophage Inflammatory Proteins (MIP) 1 alpha
CCL11/Eotaxin	IL-13	CCL4/MIP-1 beta
Soluble intercellular Adhesion Molecule 1 (sICAM-1)	IL-16	CXCL2/MIP-2
IFN-γ	IL-17	CCL5/ (Regulated on activation, normal T cell expressed and secreted (RANTES)
IL-1 alpha	IL-23	CXCL12/ Stromal cell-derived factor (SDF) 1
IL-1 beta	IL-27	CCL17/ Thymus and activation regulated chemokine (TARC)

Table 4.1 continuation;

IL-1 receptor antagonist	CXCL10/ interferon γ-induced protein 10 (IP-10)	Tissue inhibitor of metalloproteinase (TIMP) 1
IL-2	CXCL11/ Interferon–inducible T Cell Alpha Chemoattractant (I-TAC)	TNF-alpha
IL-3	CXCL1/ Keratinocyte chemoattractant (KC)	Triggering receptor expressed on myeloid cells (TREM) 1
IL-1 receptor antagonist	CXCL10/ interferon γ	Tissue inhibitor of metalloproteinase (TIMP) 1
IL-4		

4.2.4 Chemotaxis (cell migration) assay.

In vitro chemotactic responses of various cell types in response to positive controls and supernatant isolated from FhTLM stimulated cells is described below;

4.2.4.1 RAW 264.7 Cells.

Chemotaxis was assessed in 6.5 mm transwell plates with 5μm pore size inserts (Corning). RAW 264.7 cells were cultured to 70-80% confluence as previously described and then suspended in serum free medium. 200uL of cell suspension containing 2 x 10^5 cells were added to the upper chamber of the transwell insert (Figure 4.2). Thereafter, 600uL of serum free medium containing various doses of MCP-1 (positive control, (Zhang et al., 2011)) was placed in the bottom collection chamber. Serum free medium without chemotactic factor was used as a negative control. Cells were allowed to migrate in a 37^0C and 5% CO_2 incubator 6 hrs. Following incubation, non-migrated cells were collected from the top of the insert by means of a cotton wool swab and discarded. Migrated cells at the bottom of the insert and bottom collection chamber were then collected for quantification.

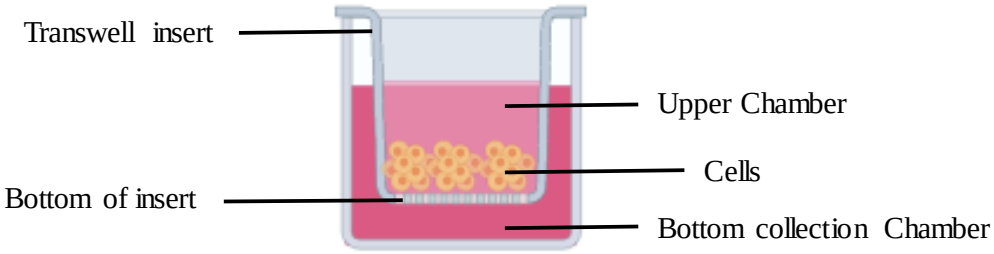


Figure 4:1: Schematic representation of the transwell chamber used for chemotaxis assay.

4.2.4.1.1 Optimisation for quantification of number of migrated RAW 264.7 cells.

To determine the number of cells that migrated from the upper insert to the lower chamber of the transwell plates, a number of methods were used.

4.2.4.1.1.1 Carboxyflourescein diacetate succinimidyl ester (CFSE) quantification of cell migration.

Prior to performing chemotaxis assay, cells were stained with CFSE (BD Pharmingen. Briefly, 10mM of CFSE was prepared by adding 90uL DMSO to the stock provided by the manufacturer. RAW 264.7 cells were washed thoroughly in PBS to remove any residual serum proteins and a single cell suspension was prepared in PBS by pipetting. Following this, 1mL of CFSE (10µM) was added to 1 x 10^6 cells in a 5mL centrifuge tube. Subsequently, cells were mixed thoroughly with CFSE solution by vortexing, followed by

incubation at 37^0C for 15 min. The reaction was quenched by addition of 9mL PBS to the cell solution. Cells were pelleted by centrifugation at 300 x g for 8 min and washed twice in RPMI medium containing 10% FBS. Following this, cells were resuspended in FACs buffer and CFSE uptake was analysed on a FACs CANTO II (BD). Chemotaxis of RAW 264.7 cells were subsequently performed as described above. Following chemotaxis, adherent cells were incubated in trypsin-EDTA solution at 37^0C, 5% CO_2 incubator for 15-20 min to detach adherent cells. Detached cells were pelleted by centrifugation at 300 x g for 8 min. Cells were subsequently transferred into a 96 well fluorescent plate (Greiner bio-one) and relative fluorescent unit (RFU) signals acquired using a multimode VarioSkan plate reader. For the quantification of the number of migrated cells, a standard curve was generated following a protocol described in cell migration assay (BioVision). Briefly, 50uL of cell suspension containing 5 x 10^4 CFSE labelled cells was added in duplicate to wells of a 96 well fluorescent plate. Thereafter, a 2-fold serial dilution in RPMI medium was performed to generate a standard curve of cells (50,000 to 781 cells) in 100uL total volume. 100uL of RPMI medium was used as blank. Following this, fluorescence was read at Em/Ex of 492/517nm using a multimode Varioskan plate reader. Subsequently, the number of cells was plotted against the relative fluorescent unit (RFU) and the equation for the standard curve (Figure 2.2) was used to determine the number of migrated cells

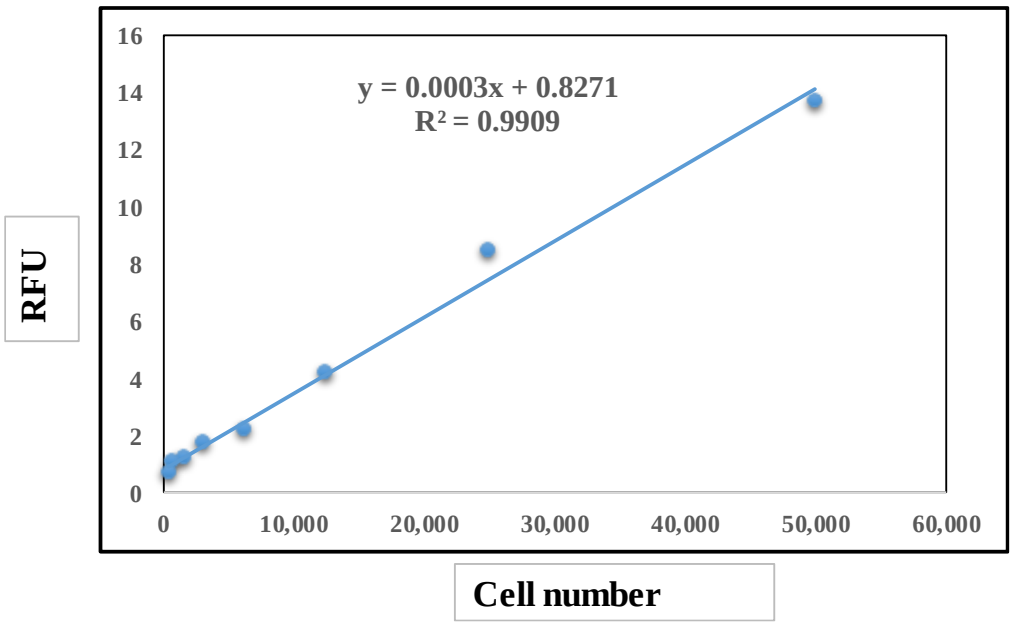


Figure 4:2: Standard curve for CFSE-labelled cells. Mean RFU determined at various cell concentration; ranging 5 x 10^4 – 3.9 x 10^2.

4.2.4.1.1.2 Crystal violet staining

In order to determine the number of cells that had migrated following migration assay, cells in the bottom insert were fixed with 4% paraformaldehyde in PBS at RT for 15 min. Thereafter, fixative was removed by gentle pipetting and then cells were stained with 500µL of 1% crystal violet (Sigma-Aldrich, UK) for 25 min at RT. The insert was transferred by means of forceps into a beaker container PBS for washing. Wash process was repeated until the washing medium becomes clear. Following this, the insert was allowed to air dry and then visualized under upright microscope (Leica) at 10X magnification. For the quantification of migrated cells, cells were detached as described (4.2.4.1.1.1) and then combined with the cells that fall to the lower chamber and their

numbers determined as described (2.4.1). Percentage of migrated cells was determined by the equation below;

$$\% \text{ Cell migration} = 100 \; X \; \frac{\textbf{No of migrated cells}}{\textbf{Total no of cells seeded}}$$

4.2.4.2 CD4 T-cells

The adherent nature of macrophages made quantification of cell migration difficult. Therefore, used other cells types including CD4 T-cells in our *in vitro* migration assay.

4.2.4.2.1 Unactivated CD4 T-cells

CD4 T-cells were isolated as previously described (2.4.2). Chemotaxis assay was carried out as described (4.2.4.1) with slight modification. Briefly, isolated CD4 T-cells were rested for 1hr at 37^0C and 5% CO_2 incubator in serum free medium prior to chemotactic assay. Thereafter, 200uL of serum free medium containing 1 x 10^5 CD4 T-cells was added to the upper insert. 600µL of medium containing 100ng/mL CCL19 (positive control) or supernatant from FhTLM stimulated BMDM (2.9.2) was placed at the bottom chamber and cells were allowed to migrate for 3hrs. Following migration, migrated cells at the bottom chamber were collected by centrifugation at 300 X g for 5 min. Number of migrated cells was quantified as described (2.4.1).

4.2.4.2.2 Activated CD4 T-cells.

CD4 T-cells were activated for 72hrs as previously described (2.9.1). Following activation, cells were harvested by centrifugation at 300 x g for 8 min and washed twice in serum free medium. Thereafter, cells were suspended in serum free medium and rested for 1 hr prior to use in the chemotaxis assay as described (4.2.4.2.1).

4.2.4.3 Eosinophils

Eosinophils were differentiated as previously described (2.7) and a chemotaxis assay was carried out as described (4.2.4.1) with a slight modification. 200uL of serum free medium containing 8 x 10^5 cells were placed in the upper insert. 600uL of either 100ng/mL recombinant murine eotaxin (Peprotech) used as a positive control or supernatant from FhTLM stimulated cells (BMDM or CD4 T-cell) was placed at the bottom chamber.

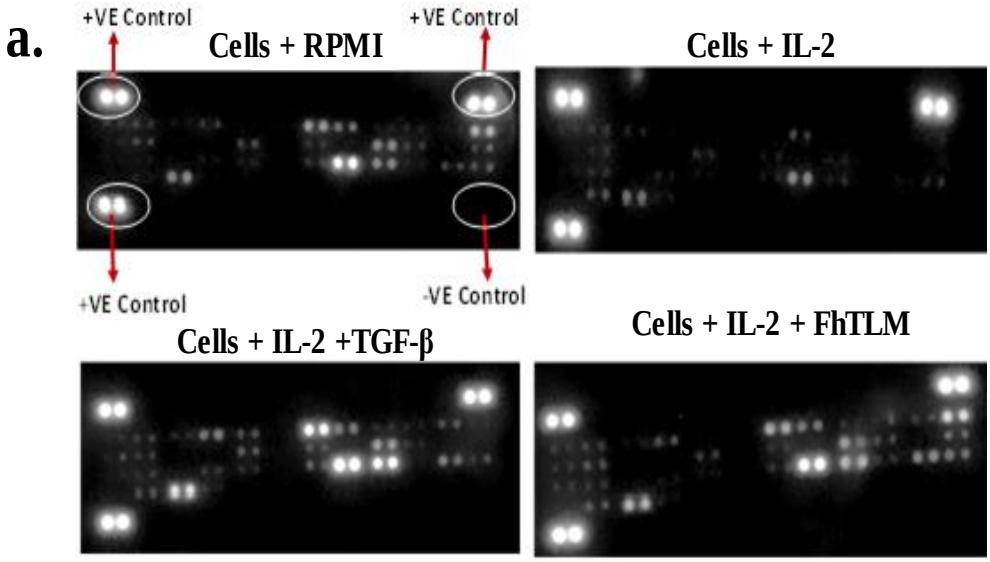
4.2.5 Statistical analysis

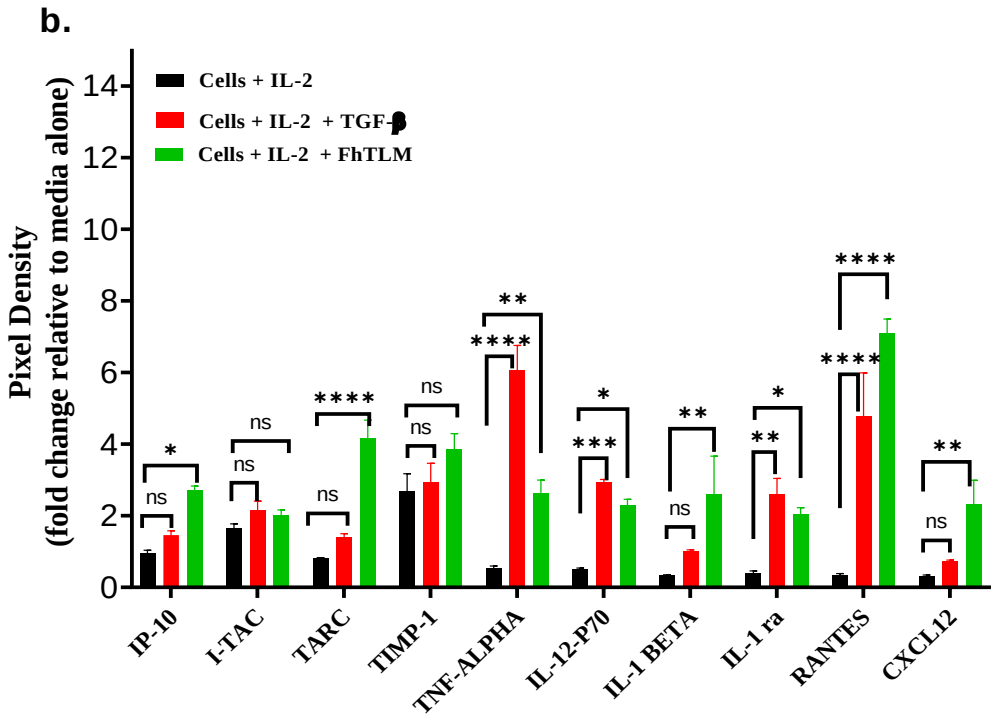
Results were graphed and data analysed using GraphPad prism version 8.1.2 software. Proteome profiler array results were expressed as mean fold change relative to unstimulated control and a Two-way ANOVA was used for analysis using Tukey's multiple comparisons test to determine significant difference between control and treatment groups. For the other graphs, One-way ANOVA was used for analysis and the significant difference was based on Dunnett's multiple comparisons test.

4.3 Results

4.3.1 Analysis of chemokines and cytokines secreted in response to FhTLM stimulation.

Following our finding in Chapter 3 that FhTLM fails to induce a strong T-reg profile we sought to assess the range of chemokines and cytokines that may be induced following treatment of CD4 T-cells with FhTLM as previously described (2.9.1). Supernatants were harvested and assayed for chemokines and cytokines using a mouse proteome profiler array kit. Following incubation of the nitrocellulose membranes with the isolated supernatant, representative blots were probed and analysed (Figure 4.3a). Differential chemokine or cytokine expression was obtained by subtracting the background values (designated negative control on the blot image) from each spot and calculating fold change relative to media alone control. Our results indicated that cells that were treated with IL-2 only have the least amount of differential protein expression with TIMP-1 the most upregulated protein (Figure 4.3b). TGF-β stimulation on the other hand, induced significant expression of multiple chemokines and cytokines including IL-1ra, IL-13, IL-12p70, M-CSF, CXCL9, TREM-1, MIP-1α, MIP-1β, TIMP-1, RANTES, CCL1, GM-CSF, TNF-α and sICAM-1. Similar to TGF-β, FhTLM also induced expression of multiple chemokines and cytokines (Figure 4.3b). Expression of TNF-α, IL-12 p70, IL-1β, IP-10, TARC, MIP-1β, IL-1ra, RANTES and CXCL12 were upregulated in supernatants from FhTLM stimulated cells. However, chemokines RANTES and TARC were found to be the most enhanced in response to FhTLM stimulation when compared to IL-2 and TGF-β treatments (Figure 4.3c), hence these chemokines were selected for individual validation by ELISA.


a.
+VE Control
+VE Control
Cells + RPMI
Cells + IL-2
+VE Control
-VE Control
Cells + IL-2 +TGF-β
Cells + IL-2 + FhTLM


b.
Pixel Density
(fold change relative to media alone)
Cells + IL-2
Cells + IL-2 + TGF-β
Cells + IL-2 + FhTLM
0
2
4
6
8
10
12
14
IP-10
I-TAC
TARC
TIMP-1
TNF-ALPHA
IL-12-P70
IL-1 BETA
IL-1 ra
RANTES
CXCL12

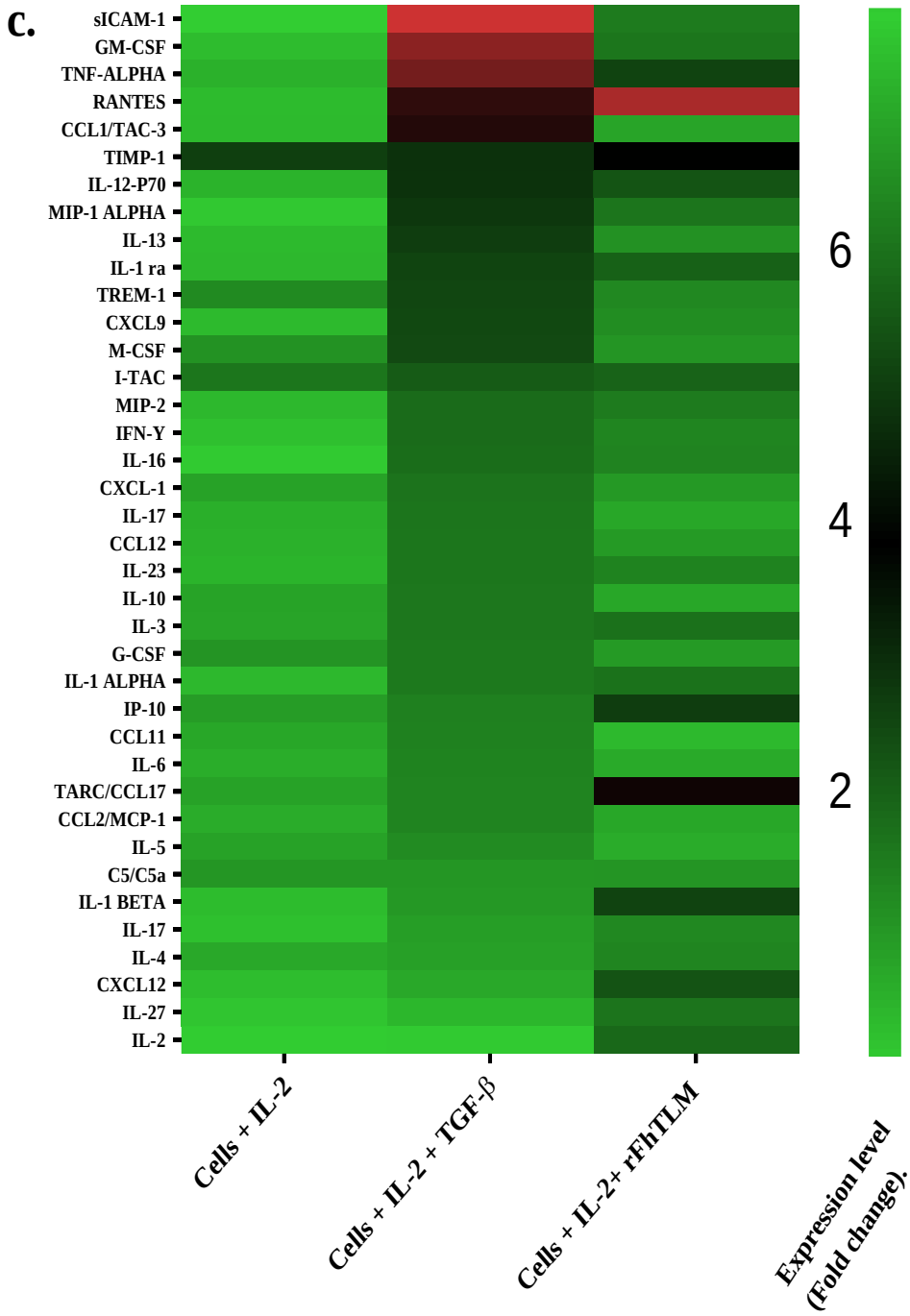


Figure 4:3: Chemokine and cytokine profile of supernatant from FhTLM-stimulated CD4 T-cells. CD4 T-cells were isolated from C57BL/6J mice as previously described (2.4.2). Cells were either unstimulated (media alone) or stimulated with IL-2 (20ng/ml) alone or in combination with either 2ng/mL TGF-β or FhTLM. Following 3 days culture, supernatant was harvested and

assayed for chemokine and cytokine secretion. Dot blots (a), representing the target proteins were acquired using an Odyssey FC imaging system (Li-cor) and pixel density of the blots was analysed using image studio lite software version 5.2. Average pixel density of each blot was subtracted from background signal. Mean fold change of the pixel density relative to media control of all proteins where FhTLM treatment induced a value of 2 and above is shown in (b) and statistical difference between treatment and control group (Cells + IL-2) was analysed using a two-way ANOVA. Heat map (c) was generated in Graph Pad Prism version 8.1.2. The average pixel density for the global cytokines and chemokines assayed is shown on the heat map with red and green colours corresponding to highly and under-expressed proteins respectively while black colour corresponds to intermediate expression.

4.3.2 Validation of chemokine secretion by ELISA.

ELISAs were carried out to confirm the results obtained in the array. Cell culture supernatants generated from CD4 T-cell cultures were assayed for secretion of RANTES and TARC. The level of RANTES secretion in IL-2 and TGF-β treated CD4 T-cells were comparable; however, this was not significantly raised beyond baseline control (Figure 4.4a). Conversely, FhTLM treatment induced a significant increase in the level of RANTES ($P < 0.0001$) when compared to TGF-β and IL-2 treatments (Figure 4.4a). Dose titration of FhTLM indicated that RANTES expression was highest when FhTLM was used at 20ng/mL.

Expression of TARC was slightly upregulated in response to IL-2 stimulation but not statistically significant when compared to medium alone controls (Figure

4.4b). Significant induction of TARC was detected in TGF-β stimulation in comparison to IL-2 and FhTLM (Figure 4.4b). Although FhTLM stimulation raised the levels of TARC above the medium and IL-2 controls, this was not statistically significant (Figure 4.4b).

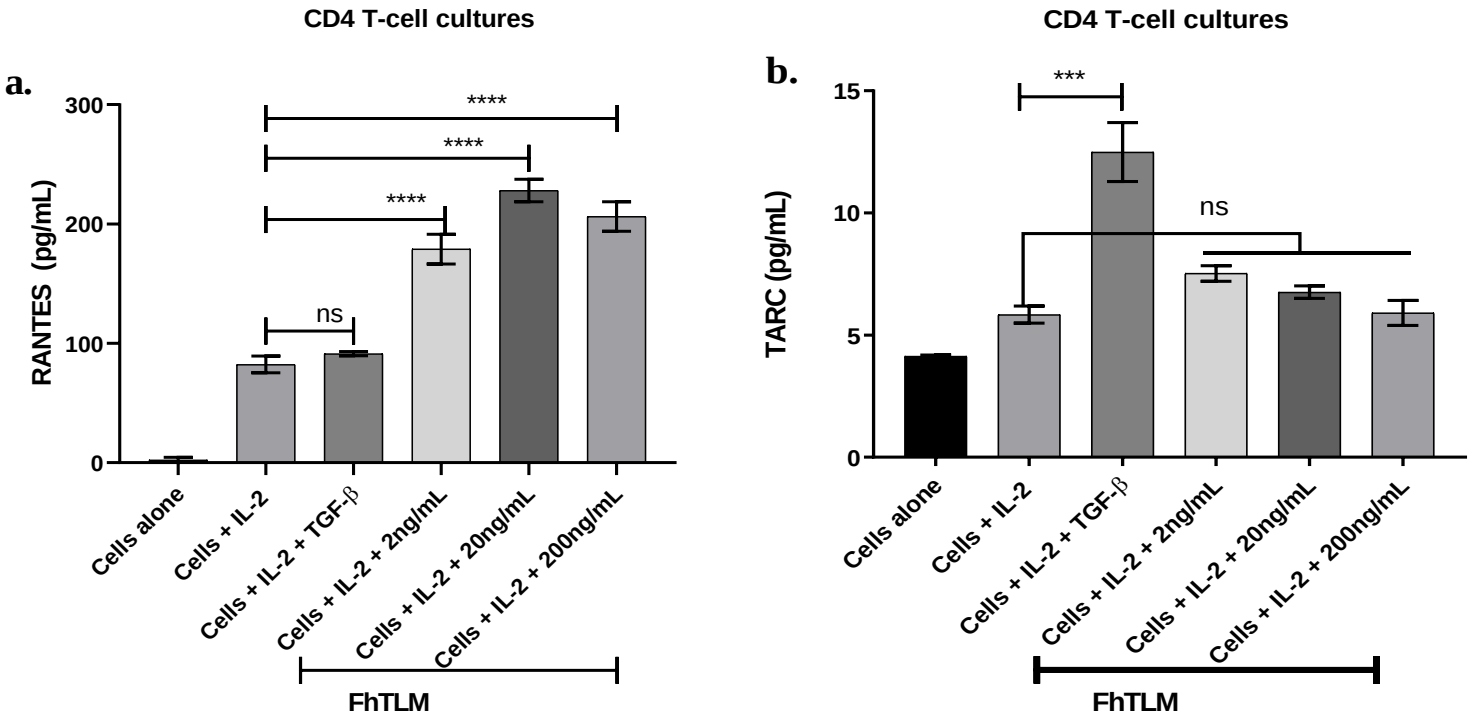


Figure 4:4: Validation of chemokine secretion by ELISA. Cells were stimulated as described in Figure 4.3 and supernatants was harvested for chemokine validation by ELISA. (a) RANTES, and (b) TARC. Data displayed represent mean ± SEM of triplicate wells of one experiment with n= 6 mice. Experiments were repeated three times and similar result was obtained. Results were analysed using one-way ANOVA and significant difference are indicated on the graph (****P <0.0001 and **P≤0.0068).

4.3.2.1 Detection of RANTES expression in BMDM and RAW 264.7 cells

We have previously shown that FhTLM can interact with host macrophages (Sulaiman *et al*., 2016). In order to determine if FhTLM could also modulate induction of RANTES in macrophages as seen in CD4 T-cells, BMDM and the RAW 264.7 macrophage cell lines were stimulated with either FhTLM, LPS or IL-4. Prior to macrophage culture, the morphology of BMDM was assessed by diff-quick stain. As shown in Figure 4.5a, the BMDM cells appeared to be mononuclear and possessing a large vacuolated cytoplasm. In addition, purity of BMDM was analysed by determining the percentage expression of $F4/80^+$ by flow cytometry on the PE channel. Our result indicated that 96.33% of the cells expressed $F4/80^+$ (Figure 4.5b).

Following macrophage stimulation as described previously (2.9.2), our results indicated that production of RANTES in BMDM was not detected in unstimulated cells (Figure. 4.6a). Stimulation with IL-4 did not raise the level of RANTES significantly when compared to that seen in the medium alone control. Conversely, induction of RANTES was significantly upregulated in response to LPS ($P < 0.0001$) or FhTLM (used at 1-10µg/mL range; $P < 0.0001$) when compared to IL-4 or medium alone controls. Notably, FhTLM induced RANTES secretion in a dose dependent manner. The levels of RANTES induced by FhTLM at lower concentrations (200 and 500 ng/mL) was comparable to that induced in response to IL-4 while that induced at higher concentrations (5 and 10µg/mL) was comparable to LPS induction. A similar pattern of expression was noted in stimulated RAW 264.7 cells (Figure. 4.6b). However, the levels of RANTES induced in medium alone control was comparable to that of IL-4 or FhTLM at lower concentrations (2 and 20 ng/mL). LPS stimulation induced the

highest level of RANTES (P <0.0001), followed by the levels seen in FhTLM at higher concentrations (5 and 10 µg/mL; P <0.0001).

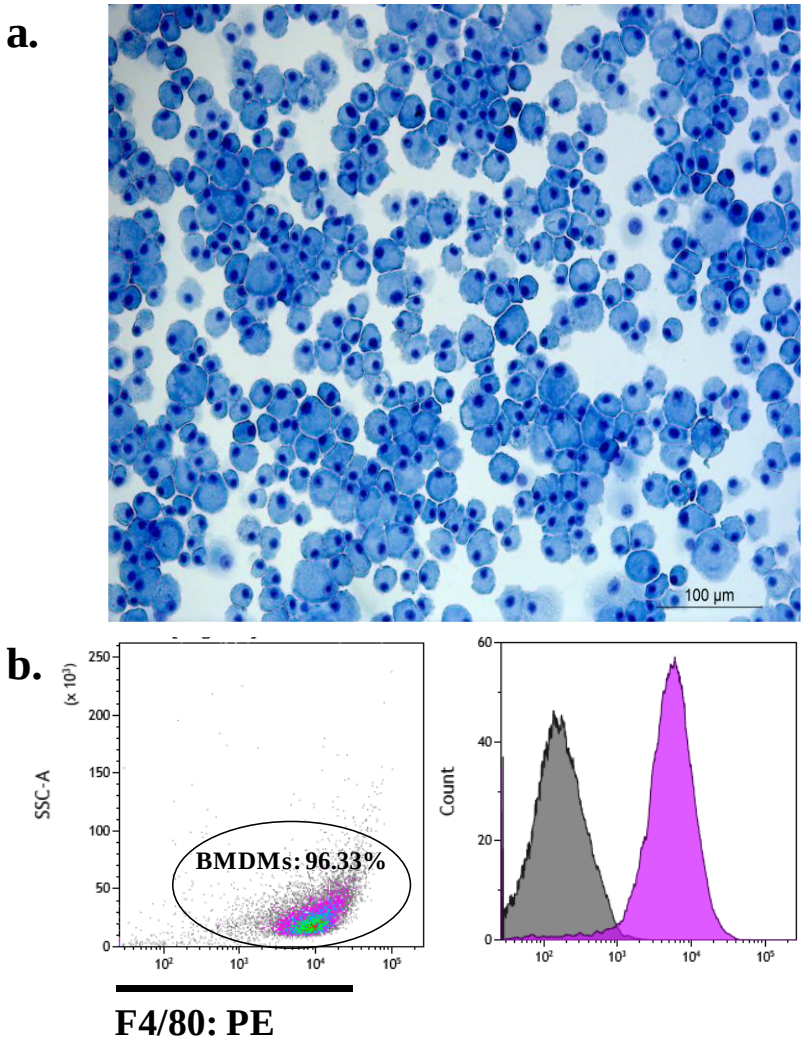


Figure 4:5: Morphology of and F4/80^{+} expression in BMDM. (a) Diff-quick stained cells showing one nucleus and vacuolated cytoplasm, image was acquired at 20x using LEICA upright microscope. (b) Dot plots showing percentage purity of BMDM (Left panel) and histogram (right panel) showing overlay of isotype control (grey colour) and PE conjugated anti-F4/80 (purple).

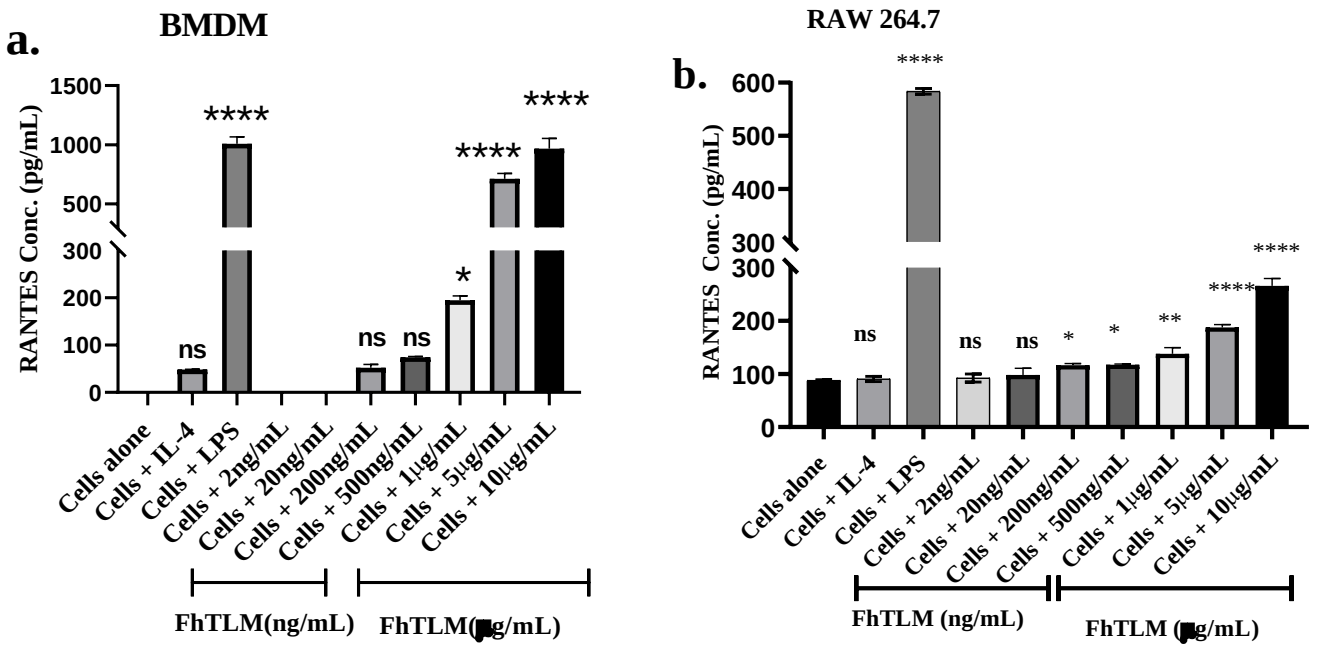


Figure 4:6: Secretion of RANTES in FhTLM-stimulated BMDM (a) and RAW 264.7 macrophage cell line (b). BMDM or RAW 264.7 cells were stimulated as previously described (2.9.2) and supernatant was harvested for RANTES detection by ELISA. Data is representative of three independent experiments (n=6 per experiment) showing mean ± SEM of duplicate wells. Results were analysed using one-way ANOVA and significant difference between treatment and controls were compared using Dunnett's multiple comparisons test. **** indicates P <0.0001, ** indicates P= 0.0012 and * indicates P= 0.0439. RANTES expression in RAW 264.7 (a) and BMDM (b) is shown.

4.3.3 Optimised methods for quantification of RAW 264.7 cell migration

Having confirmed FhTLM induction of chemokine secretion, so to confirm the functional effect of FhTLM supernatant on cell migration, an *in vitro* chemotaxis assay was first used. Chemotaxis of RAW 264.7 cells in response to MCP-1, used as the positive control was assessed as described (4.2.4). To quantify the number of migrated cells, RAW 264.7 cells were first labelled with CFSE at 10µM (Figure 4.7a) and relative fluorescent signals of migrated CFSE stained cells was measured successfully. However, following chemotaxis, we could not detect any cell migration, by CFSE proxy, in response to different doses of MCP-1 assayed (result not shown)

In separate experiments, migrated cells which had adhered to the bottom of transwell insert were stained with crystal violet and visualized by microscopy (Figure 4.7b), prior to detaching adherent cells by trypsin-EDTA treatments. The highest density of migrated cells was seen in MCP-1 used at 25ng/mL (Figure 4.7b). Following Trypsin-EDTA treatments, migrated cells were quantified. Our results indicated that in response to decreasing doses of MCP-1 there was a significant increase in Raw264.7 migrated cells (Figure 4.7c).

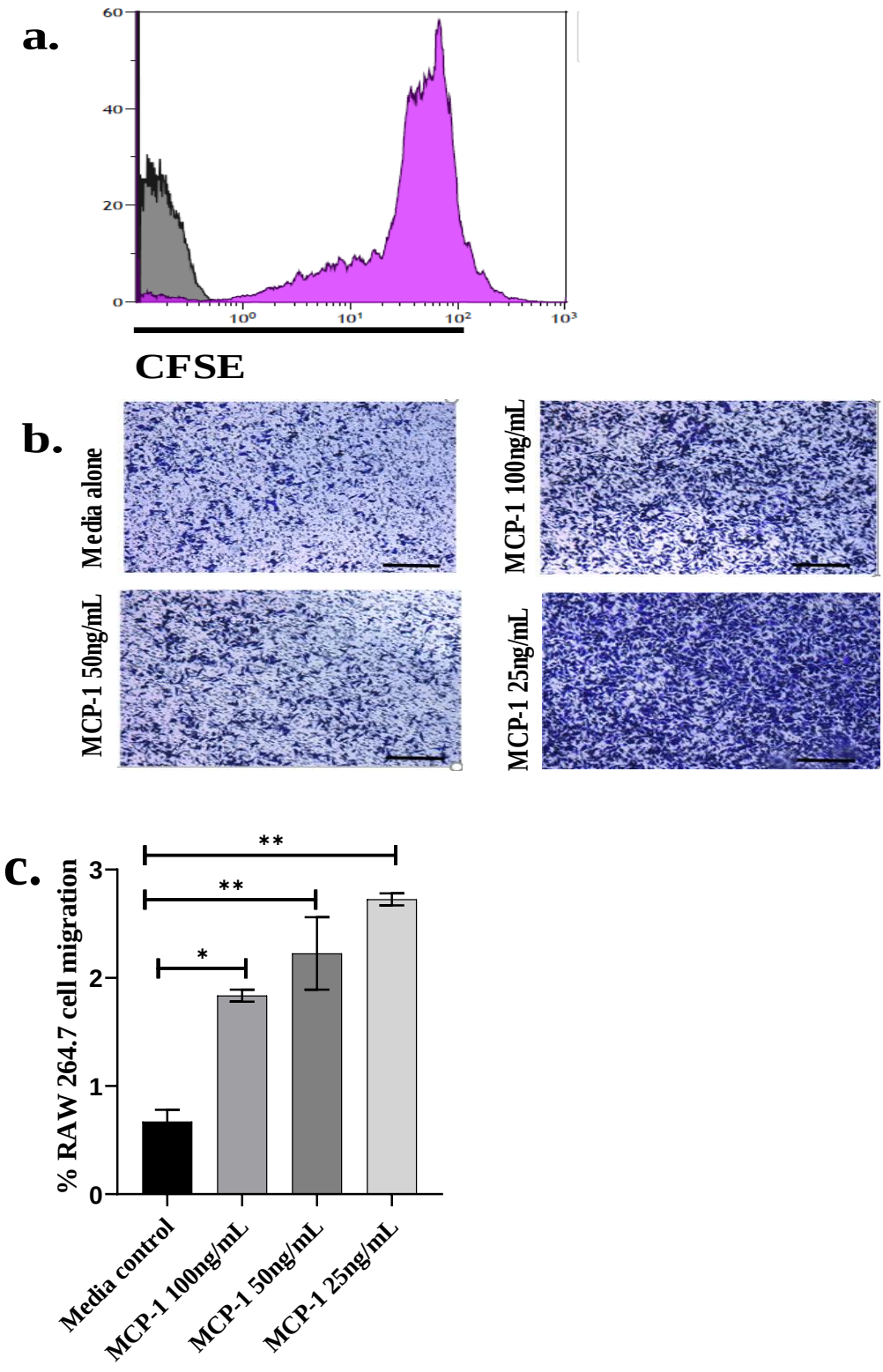


Figure 4:7: Cell number quantification methods for RAW 264.7 cell migration. RAW 264.7 cells were labelled with CFSE and allowed to migrate in response to MCP-1 for as described (4.2.4). (a) Histogram showing overlay

of CFSE stained RAW 264.7 cells (purple) and unstained cells (grey colour), (b) representative images of migrated cells in response to MCP-1 doses, images were taken at x10 and scale bar indicates 200µm, (c) percentage cell migration showing mean ± SEM of duplicate wells. Statistical difference between random cell migration and migration in response to chemotactic medium was analysed using one-way ANOVA. * indicates P= 0.0234 and ** indicates p=0.003.

4.3.4 FhTLM-stimulated cell supernatant induced *in vitro* migration of CD4 T-cells and eosinophils

Following optimisation, we tested supernatant from FhTLM stimulated cells for its capacity to induce migration of both CD4 T-cells and eosinophils. For the migration of CD4 T-cells, supernatant isolated from FhTLM stimulated macrophages was used. CD4 T-cells either naive or activated, were allowed to migrate for 3 hrs. After this, the number of migrated cells were determined by trypan blue exclusion methods (2.4.1). Random cell migration was seen in about 1-2.4% of activated and unactivated CD4 T-cells only (Figure 4.8a and b). CCL19 as a positive control, induced significant migration of unactivated (p= 0.0013) and activated CD4 T-cells (p= 0.0009) when compared to random cell migration in serum free medium (Figure 4.8a and b). However, the percentage cell migration of unactivated cells was greater than that of activated cells. Supernatant from FhTLM stimulated macrophages also promoted significant migration of activated CD4 T-cells (Figure 4.8a; p=0.0010). Although the levels of migration in unactivated CD4 T-cells in response to FhTLM supernatant was comparable to that induced in activated CD4 T-cells (Figure 4.8a and b), the percentage migration of unactivated CD4 T-cells was not significantly enhanced

when compared to random cell migration ($P>0.05$; Figure 4.8b). For the migration of eosinophils, supernatant from either FhTLM stimulated macrophages or CD4 T-cells was used. Chemotaxis of eosinophils was carried out as previously described (4.2.4.3) and the percentage of migrated cells were analysed by trypan blue exclusion (2.4.1). Our results indicated that migration was seen in 4.3% of cells in response to eotaxin as a positive control and this was not significant when compared to random cell migration (Figure 4.8c; $P>0.05$). Conversely, 15% and 12.5% eosinophils migrated in response to supernatant from FhTLM stimulated CD4 T-cells and macrophages, respectively (Figure 4.8c). These levels of cell migration were found to be statistically significant in comparison to random cell migration controls ($P\leq0.0037$)

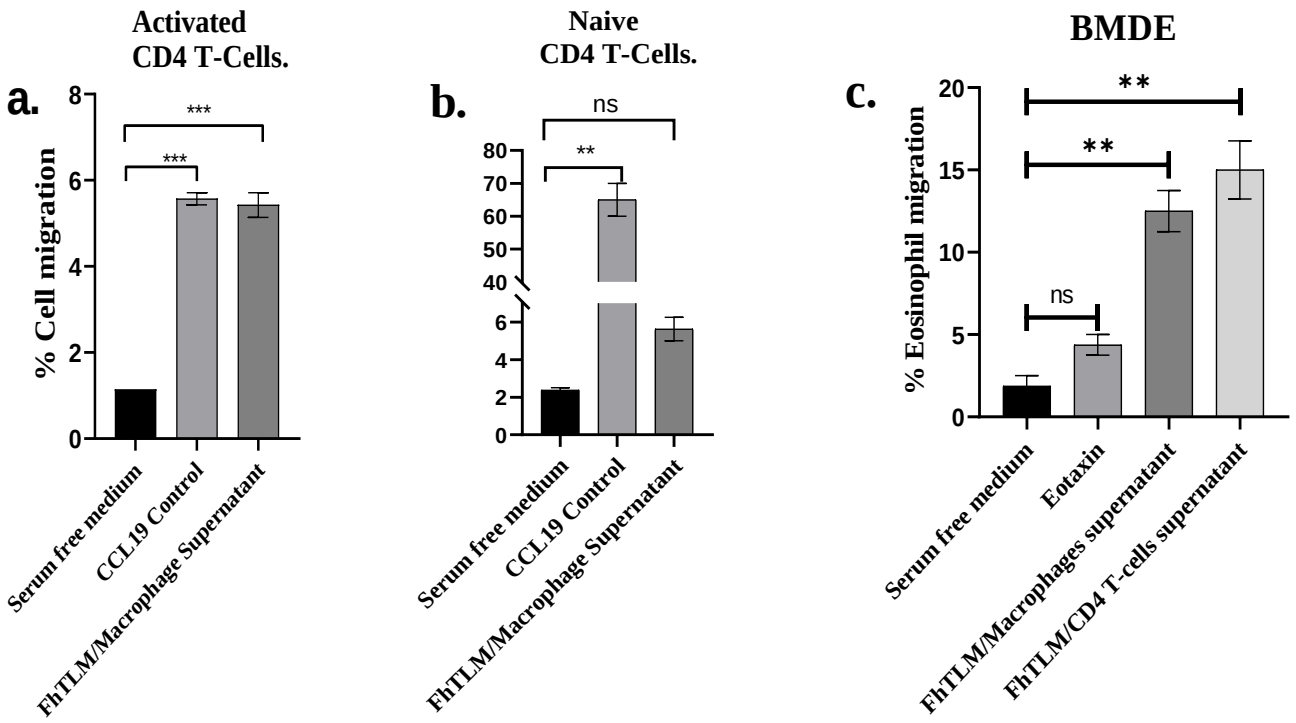


Figure 4:8: FhTLM supernatants induced *in vitro* migration of CD4 T-cells and eosinophils. CD4 T-cells or BMDM were stimulated and cultured with FhTLM as previously described (2.91 and 2.9.2). Following culture, supernatants were harvested and analysed for its capacity to induce the migration of BMDE and CD4 T-cells. Percentage migration of (a) activated CD4 T-cells, (b) unactivated CD4-T cells, and (c) BMDE, in response to FhTLM stimulated cell supernatants and controls are shown. Data displayed are one of two independent experiments with similar results showing mean ± SEM of duplicate wells. Significant difference between controls and treatments was analysed using one-way ANOVA. ** indicates $p \leq 0.0037$, and *** indicates $p = 0.0008$.

4.4 Discussion

The regulation of migration and activation of leukocytes during inflammatory responses is mediated by chemokines. Infiltration of leukocytes including eosinophils, lymphocytes, mast cells and macrophages are associated with ruminant *F. hepatica* infection (Bossaert et al., 2000b, Meeusen et al., 1995), implicating the participation of chemokine during *F. hepatica* infection. From our findings as reported in Chapter 3, FhTLM had no effect on regulatory cell surface markers expression. As a result, we aimed to investigate the effect of FhTLM on global cytokine and chemokine secretion.

Following stimulation of CD4 T-cells with FhTLM, cell culture supernatant was collected and analysed for the production of cytokines and chemokines using a proteome profiler array system. Our result indicated that FhTLM induced expression of multiple pro-inflammatory cytokines (TNF-α, IL-12 $_{p}$70, IL-1ra, and IL-1β) and chemokines (TARC MIP-1β, RANTES, CXCL10 and CXCL12). This suggests the activation of pro-inflammatory pathways in CD4 T-cells by FhTLM and supports a role for FhTLM in the development of early inflammatory responses associated with *F. hepatica* infection (Clery et al., 1996). Induction of inflammatory response in CD4 T-cells by FhTLM was not due to endotoxin contamination as recombinant FhTLM used in immuno-assays has previously been shown to be free of endotoxin residues (Japa et al., 2015). FhTLM does not induce production of pro-inflammatory molecules on monocytes (Chapter 5), indicating that this response was CD4 T-cell specific. Indeed Sulaiman et al., (2016) previously showed that FhTLM failed to

upregulate inflammatory responses in bovine macrophages (Sulaiman et al., 2016).

Upregulation of pro-inflammatory mediators by FhTLM reported in this study agrees with previous studies that showed that DCs stimulated with various *F. hepatica* secreted molelules, FhTeg, FhCL1 and FhGST-si, induced production of pro-inflammatory chemokines and cytokines including MIP-1α, MIP-2, IL-12_p40, and IL-6 (Dowling et al., 2010, Vukman et al., 2013b).

Amongst the chemokines that were expressed in response to FhTLM stimulation, TARC and RANTES were found to be the most highly upregulated, suggesting a possible role for these chemokines during *F. hepatica* infection. However, our ELISA validation assay revealed that levels of TARC protein expression were not significantly raised above control levels in FhTLM stimulated CD4 T-cells, suggesting that *in vitro* expression of TARC by FhTLM, may be transient. TARC expression was found to be transient in phytohemagglutinin (PHA) stimulated human PBMC (Imai et al., 1996). Conversely, enhanced RANTES expression in FhTLM stimulated CD4 T-cells was validated in our ELISA assay. This finding is consistent with increased level of RANTES reported in various helminth infections including *F. hepatica* (Rodríguez et al., 2017), *B. malayi* (Semnani et al., 2003), *S. mansoni,* (Park et al., 2001), *T. spirallis* (Conti and DiGioacchino, 2001) and *E. multiocularis* (Kocherscheidt et al., 2008).

Previous work in our group has shown that FhTLM interacts with host macrophages (Sulaiman *et al*., 2016). As a result, we assessed the ability of FhTLM to induce RANTES expression in macrophages, since RANTES was the top candidate molecule induced by FhTLM. Our result indicated that FhTLM

stimulation resulted in enhanced expression of RANTES in both BMDM and RAW 264.7 macrophage cell lines in a dose dependent manner. Production of RANTES by macrophages in response to FhTLM was found to be similar to that observed following LPS but not IL-4 stimulation, suggesting that AAMϕ does not promote RANTES expression. Induction of RANTES has been previously shown to be associated with CAMϕ (Jiang and Zhu, 2016).

Although *F. hepatica* infections are associated with increased RANTES expression (Rodríguez et al., 2017), the precise role of RANTES in regulating immune responses during *F. hepatica* infection has yet to be studied. However, previous studies have suggested that RANTES is differentially expressed by Th1 cells (Schrum et al., 1996, Chensue et al., 1999) and has a role in regulating inflammatory responses in various helminth infections including *T. spirallis* (Conti and DiGioacchino, 2001) and *T. muris* (Dénes et al., 2010). Our finding that FhTLM induced enhanced RANTES expression suggests a role for this chemokine in induction of the transient Th1/inflammatory responses during *F. hepatica* infections.

Furthermore, to confirm the functional effect of chemokine secretion in supernatants isolated from either FhTLM stimulated CD4 T-cells or macrophages, we performed an *in vitro* chemotaxis assay. We initially optimised an *in vitro* chemotaxis assay using RAW264.7 cells and found that MCP-1 used as a positive control induced greatest level of cell migration at a concentration of 25ng/mL. However, due to the adherent nature of macrophages, definitive quantification of cell migration was difficult. We therefore used other cell types including CD4 T-cells and eosinophils in our subsequent cell migration studies. We first assessed the migration of eosinophils in response to supernatants

isolated from FhTLM stimulated CD4 T-cells and macrophages. Our results showed that both supernatants promoted the migration of eosinophils, suggesting the presence of a potent eosinophil chemotactic factor within FhTLM stimulated cultures. In addition, supernatant isolated from FhTLM stimulated macrophages also drives the migration of activated but not naïve CD4 T-cells. It has previously been shown that polyclonally activated CD4 T-cells upregulate chemokine receptors that allows for their migration to sites of infection, where they differentiate and carry out effector functions (Sallusto et al., 1999, Krummel et al., 2016).

In conclusion, we have shown that FhTLM elicits the production of pro-inflammatory cytokines and chemokines in CD4 T-cells, suggesting that FhTLM secretion may be associated with inflammatory responses that are associated with the early phase of *F. hepatica* infection. In addition, we showed that chemokines produced by FhTLM are biological active as it induced *in vitro* migration of both eosinophils and activated CD4 T-cells. Whilst our proteome profiler data results showed TARC and RANTES to be the most highly induced targets, our ELISA validation assay confirmed that RANTES, but not TARC, was significantly enhanced in FhTLM stimulated CD4 T-cells. This finding suggests a role for RANTES during *F. hepatica* infection. However, chemokine depletion and *in vivo* studies will allow for the precise role of these chemokines during *F. hepatica* infection to be determined.

5 The effect of FhTLM in regulating parasite survival *via* modulation of host immune signals.

5.1 Introduction

Through co-evolution with their hosts, parasitic helminths have developed immune regulatory strategies that allows them to divert the host immune attack targeted at eliminating them. This ultimately results in facilitating parasite survival (Maizels *et al*., 2004). Indeed, it has been hypothesized that organisms are able to adjust their developmental timing in response to environmental (host) cues that predicts their life cycle (Babayan *et al*., 2010). Infection with *F. hepatica* is chronic, and the parasite can survive for a long time within the host (Clery et al., 1996), suggesting that the host effector response may, on balance, benefit rather than impair parasite survival.

Evidence from several studies have shown that developmental processes of parasitic helminths can be initiated by host immune signals (Huang *et al*., 2015, Babayan *et al*., 2010, Lamb *et al*., 2007, Lamb *et al*., 2010). Signals from both innate and adaptive effector cells have been shown to be important in modulating blood fluke development during the pre-patent stages of infection (Davies *et al*., 2001, Lamb *et al*., 2007, Lamb *et al*., 2010). *S. mansoni* in RAG-$1^{-/-}$ mice have stunted developmental characteristics, including reduced adult worm pairing and egg production. However, parasite development was not impaired in immunoglobulin heavy chain, Igh-$6^{-/-}$, deficient mice, suggesting that T- cells but not B-cells were required for normal *S. mansoni* development. Normal parasite pairing and egg production was restored when infected RAG-$1^{-/-}$ mice were reconstituted with naïve $CD4^{+}$ T-cells. The developmental effect of CD4 T-cells on *S. mansoni* was shown to be indirect and mediated via interaction with innate immune cells. Lamb *et al* (2010) showed that in the absence of CD4 T-cells, direct stimulation of innate cells, mainly monocytes and macrophages,

with the TLR ligand LPS was sufficient to trigger parasite development in RAG-$1^{-/-}$ mice (Lamb *et al*., 2010). These results show that pro-inflammatory cytokines may provide a benefit to developing flukes.

Modulation of parasite development by immune signals has also been reported in other helminth infections. For instance, *T. spiralis* grew rapidly and exhibited increased survival as a result of eosinophil induced accumulation of $CD4^{+}IL\text{-}4^{+}$ cells at the site of infection, as well as prevention of NO synthesis by macrophages and neutrophils (Gebreselassie *et al*., 2012). Similarly, it has been shown STAT6/IL-4 signalling in eosinophils promotes the growth of *T. spiralis* in the skeletal muscle, and eosinophils mediate parasite survival by inhibiting NO production, while supporting enhanced metabolic activity of the parasite (Huang *et al*., 2015) Finally, the filarial parasite *L. sigmodontis* has been shown to reach adulthood faster and produce more microfilarial in response to host IL-5 induced eosinophilia (Babayan *et al*., 2010). *B. malayi* development was also facilitated by the presence of natural killer (NK) cells and CD4 T-cells (Shultz and Rajan, 1999, Babu *et al*., 1998). Together these studies support a role for immune signals generated by both innate and adaptive effector cells in promoting parasite development and maturation.

Aside from utilizing host immune signals for developmental cues, parasitic organisms also must scavenge resources, such as glucose, for energy metabolism and survival. Glucose is key to the survival of any living organism. In vertebrates, it is transported to the skeletal muscles where it is stored as glycogen for the production of energy, mediated by GLUT (glucose transporter) molecules. Upregulation of host GLUT complexes during parasitic infection has been shown to be consistent with enhanced parasite growth (Meireles *et al*.,

2017). Blockade of host GLUT pathways resulted in stunted parasite development and inhibited infectivity during malaria infection (Wei *et al*., 2018). Infection of mice with *T. spiralis* and *T. pseudospiralis* have been shown to trigger host insulin signalling pathways consistent with reduced blood glucose and enhanced parasite growth and development (Wu *et al*., 2009). In addition, GLUT4 expression in nurse cells was shown to be associated with eosinophil induced development of *T. spiralis* (Huang *et al*., 2015).

We have previously shown that *F. hepatica* can influence induction of host chemokines, and our chemotaxis studies revealed that supernatant from FhTLM culture is capable of chemo-attracting both innate and adaptive immune cells including CD4-T cells and eosinophils (Chapter 4). In order to understand if FhTLM can alter host immune effector responses to facilitate growth/viability, NEJs were cultured in the presence of conditioned media generated from innate and adaptive immune cells; thereafter parasite viability and growth was assessed. To explore a potential mechanism, we examined expression of GLUT4 in FhTLM stimulated CD4 T-cells. An understanding of the interaction between *F. hepatica* and host immune mediators that either facilitate or impede *F. hepatica* growth will be of great significance in the development of new control measures.

5.2 Materials and Methods

5.2.1 NEJs

NEJs were prepared for excystation from metacercariae as described previously (2.15.1).

5.2.2 Assessing NEJ viability in the presence of FhTLM primed eosinophil.

To assess NEJ viability in the presence of FhTLM or BSA (protein control) stimulated eosinophil, NEJs were cultured as previously described (2.15.3) and viability was determined by measuring ATP content as previously described (2.15.3.1).

5.2.3 NEJ culture in the presence of conditioned medium isolated from FhTLM stimulated CD4 T-cells and Monocytes

To determine the viability and growth of NEJs in the presence of conditioned medium (immune signals) generated from monocytes and CD4 T-cells, NEJs were cultured as previously described (2.15.2). NEJs viability and growth were measured as previously described (2.15.2.1).

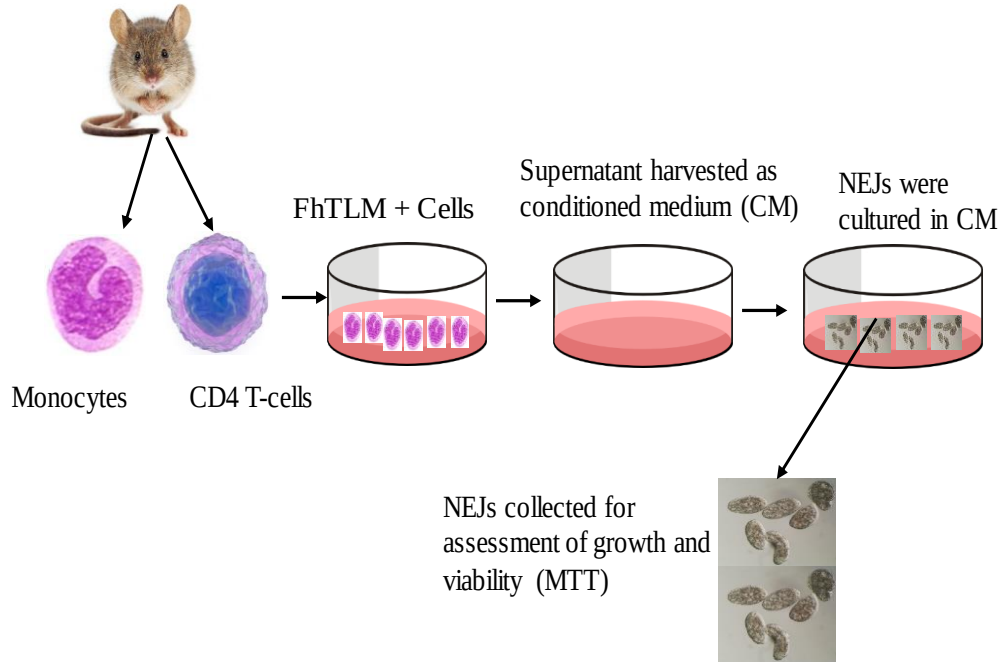


Figure 5:1: Schematic of methods used for assessing NEJ viability and growth in conditioned medium generated from either stimulated monocytes or CD4 T-cells. Cells were isolated from mice and cultured as described previously (2.9.3.1 and 2.9.3.2). Following cell culture, conditioned medium was collected and used for NEJ culture. After 12-18hrs, NEJs were collected for viability and growth assessment.

5.2.4 Detection of growth-promoting molecules in conditioned medium isolated from FhTLM stimulated Monocytes

Conditioned medium generated from the monocytes to be used for incubating with NEJs (5.3.2) was assayed for the detection of any proteins that may regulate growth and development using a mouse adipokine array kit (see table 5.1 for list of targeted proteins). The assay was performed as previously described (2.12).

Table 5.1: Growth-related proteins that are targets using the mouse adipokine array kit.

Adiponectin	Insulin-like growth factor (IGF) I	M-CSF
Agouti-related peptide (AgRP)	IGF-II	Oncostatin (OSM)
Angiopoietin-Like 3 (ANGPT-L3)	Insulin-like Growth Factor Binding Proteins 1 (IGFBP-1)	Plasminogen activator inhibitor-1 (PAI-1) /Serpin E1
CCL2/MCP-1	IGFBP-2	Pentraxins (PTX) 2
CCL5/RANTES	IGFBP-3	PTX3
C-reactive protein (CRP)	IGFBP-5	Preadipocyte factor 1(Pref-1)
Dipeptidyl peptidase IV (DPPIV)/CD26	IGFBP-6	Receptor for advanced glycation endproducts (RAGE)
Endocan 1	IL-6	Retinol Binding Protein (RBP) 4
Fetuin A	IL-10	Resistin
Fibroblast growth factors (FGF) acidic	IL-11	TIMP-1

Table 5.1 continuation;

FGF-21	Leptin	TNF-alpha
Hepatocyte growth factor (HGF)	Leukemia inhibitory factor (LIF)	Vascular endothelial growth factor (VEGF)
ICAM-1	Lipocalin-2	

5.2.5 Glucose transporter 4 (GLUT4) detection

For the detection of GLUT4 protein and mRNA in CD4 T-cells, CD4 T-cells were stimulated as previously described (2.9.3.2) and analysed by immunofluorescence staining and real time qualitative polymerase chain reaction (rt-qPCR) as described previously (2.14).

5.2.6 Statistical analysis

Results were graphed and data analysed using GraphPad prism version 8.1.2 software. Data were analysed using either two-way ANOVA where statistical significance was based on Sidak's multiple comparison test or one-way ANOVA where significant difference was based on Dunnett's multiple comparisons test.

5.3 Results

5.3.1 *In vitro* effect of eosinophils on viability of NEJs.

We sought to determine if FhTLM can alter eosinophil function to benefit parasite viability. NEJs were incubated for 24hrs in the presence of eosinophils treated with either FhTLM or BSA. Thereafter, parasite viability was determined by measuring ATP levels using a CellTitre-Glo luminescence assay. In order to determine the internal background effect of either BSA or FhTLM treated eosinophil on NEJ viability, signals acquired for BSA or FhTLM treated eosinophil controls were subtracted from that of NEJs incubated in eosinophils treated with either protein ((NEJ + Eos/BSA) - (Eos/BSA) or (NEJ + Eos/FhTLM) - (Eos/FhTLM)). Following this, fold change was calculated relative to medium only control. As shown in Figure 5.2, the viability of NEJs was significantly increased in BSA-treated eosinophils ($P<0.0001$) when compared to NEJs only controls. In addition, worm viability in FhTLM-treated eosinophils was also increased, however, this was not statistically significant level ($P>0.005$).

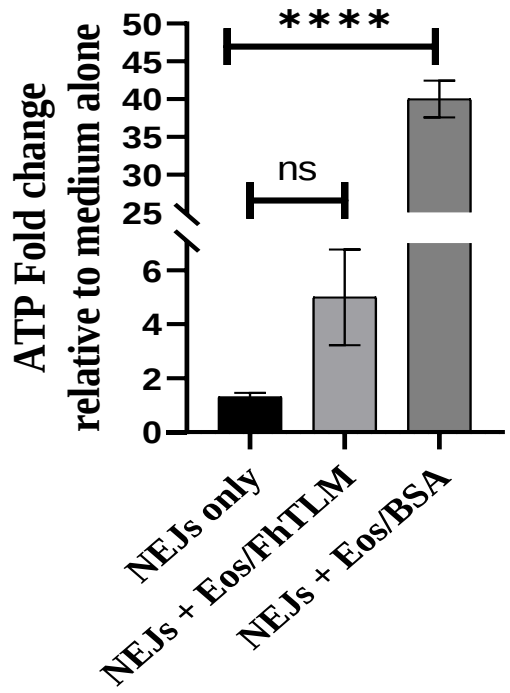


Figure 5:2: Viability of NEJs in the presence of eosinophils. BMDE was primed with BSA or FhTLM for 24hrs in a 37^0C and 5% CO_2 incubator. Following this, NEJs were cultured alone or in the presence of either BSA or FhTLM-primed eosinophils at a ratio of 5:1 (eosinophils:NEJs). Supernatant was harvested and the viability of NEJs were determined by measuring the ATP content. Fold change in NEJ viability in various treatment relative to medium alone control was determined. Data displayed is mean ± SEM of two independent experiment. Significant differences in parasite viability between treatment and control groups was analysed with one-way ANOVA using Dunnett's multiple comparison test. **** indicates $P<0.0001$ and ns indicates $P > 0.05$.

5.3.2 *In vitro* effect of conditioned medium isolated from stimulated CD4 T-cells on NEJ viability.

In order to determine if signals from activated CD4 T-cells is capale of benefiting parasite survival, NEJs were incubated for 12-18hrs in conditioned media generated by stimulating CD4 T-cells with FhTLM or Th2 inducing conditions. Following culture, NEJs were collected and viability and growth were assessed. Parasite viability was determined by MTT assay and parasite growth was assessed by measuring worm area using imageJ software. As shown in Figure 5.3a, the viability of NEJs was suppressed in the presence of Th2 or FhTLM media when compared to NEJs incubated in medium from unstimulated cells. While a trend for decreased NEJ viability was apparent, this was not statistically significant ($P>0.05$). In addition, analysis of worm growth in response to the various treatments showed that there was no diffence in parasite growth in all treatment conditions assayed (Figure 5.3b; $P>0.05$).

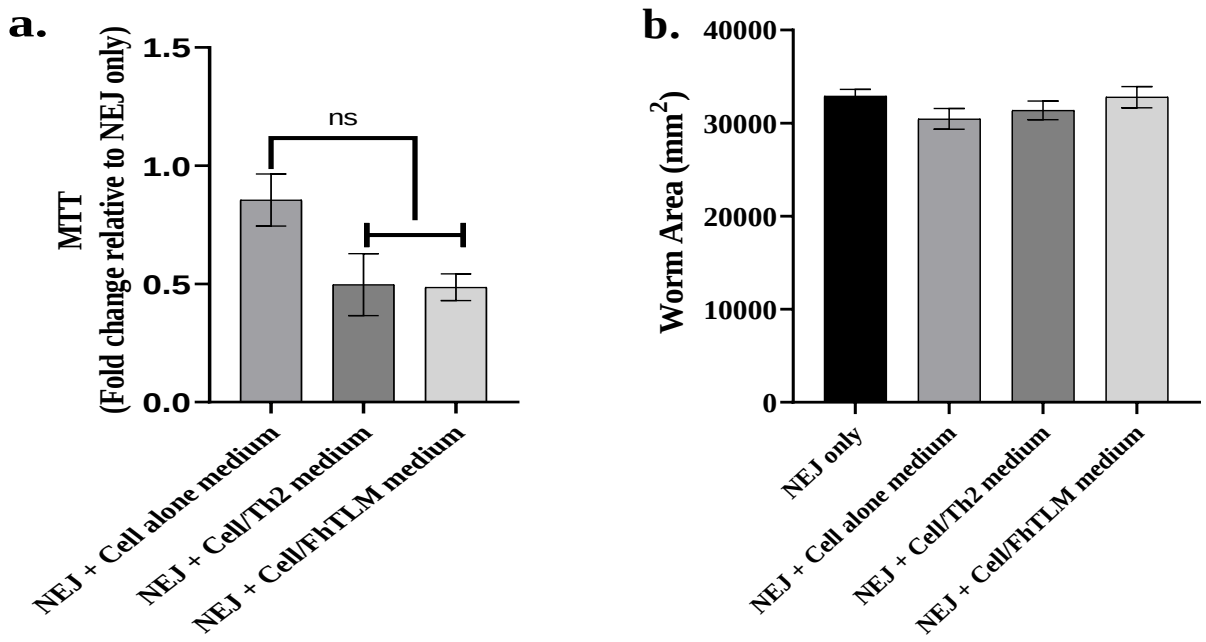
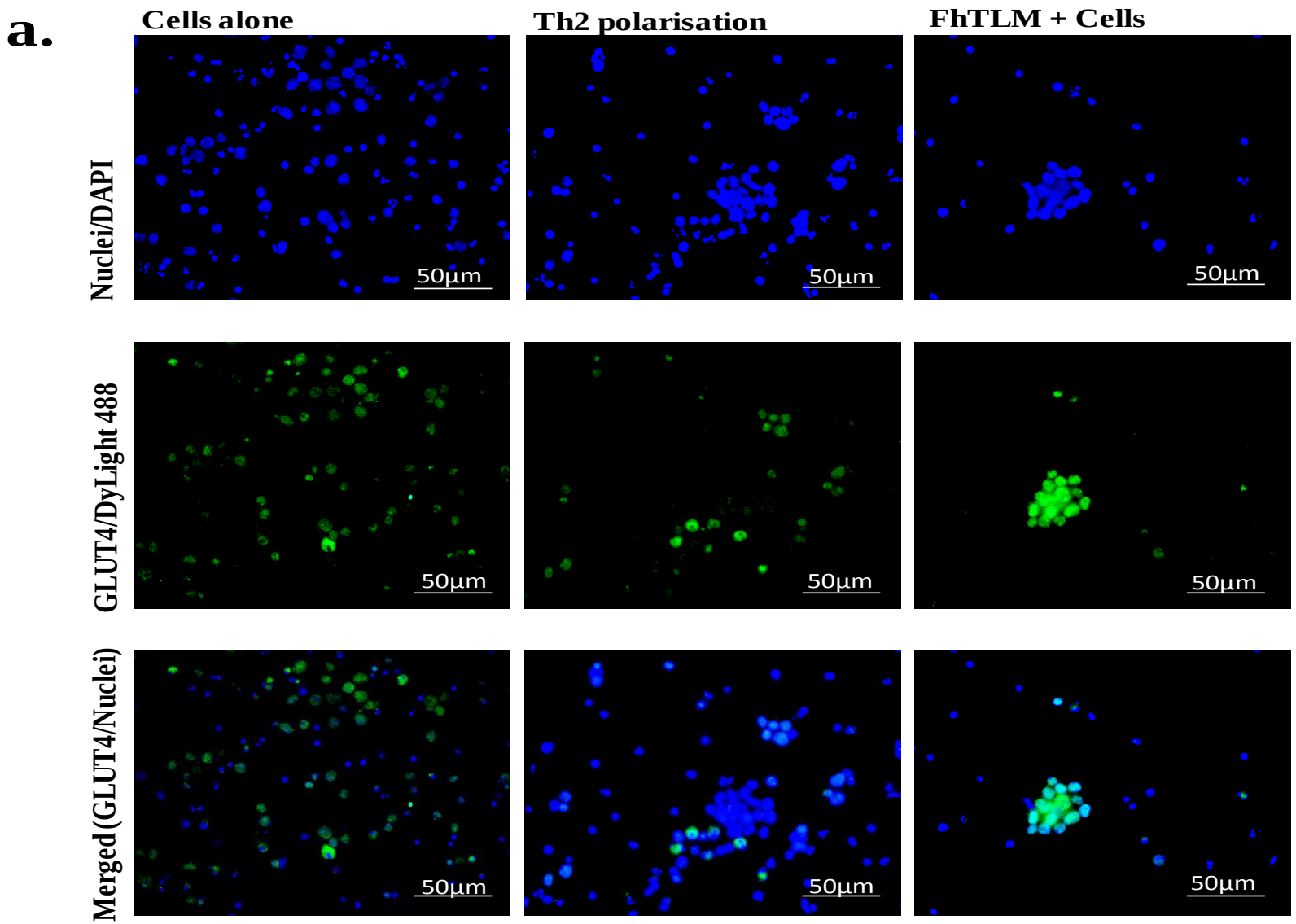


Figure 5:3: Viability of NEJs in CD4 T-cell conditioned medium. Conditioned medium was generated from CD4 T-cells as described (2.9.3.2). NEJs were cultured in duplicate wells (20 worms/well) in 37^0C and 5% CO_2 incubator for 12-18hrs in conditioned medium. Following incubation, NEJ viability was determined by MTT reduction and fold change in NEJ viability in various treatments relative to NEJs only controls was determined (a). Worm area (b) was measured in at least 10 individual worms per treatment. Data (a) is mean ± SEM of one experiment and (b) represents mean ± SEM of two independent experiments. Differences between treatment and control groups were analysed with one-way ANOVA using Dunnett's multiple comparison test. (ns indicated P>0.005).

5.3.3 Analysis of GLUT4 expression on FhTLM stimulated CD4 T-cells.

Upregulation of GLUT4 during parasitic infection has been shown to be associated with a period of enhanced parasite growth and development (Huang *et al*., 2015). Therefore, to investigate a possible mechanism that may promote parasite growth, GLUT4 expression on FhTLM stimulated CD4 T-cells was examined. Expression of GLUT4 mRNA and protein was determined by rt-PCR and immunofluorescence staining, respectively. Immunofluorescence images of GLUT4 positive cells are shown in Figure 5.4a. Quantification showed that percentage of GLUT4 expressing cells during FhTLM stimulation was comparable to control (cell alone) (Figure 5.4b; $P>0.05$). While Th2 polarisation caused a significant decrease in the levels of GLUT4 staining (Figure 5.4b) compared to the FhTLM ($P=0.0023$) or controls ($P=0.0039$). In addition, the levels of GLUT4 mRNA detected in FhTLM stimulated cells were comparable to that seen in controls (Figure 5.4c). Again, Th2 polarisation resulted in decrease in GLUT4 mRNA when compared to FhTLM or controls. However, was not statistically significant ($P>0.05$).


a.
Cells alone
Th2 polarisation
FhTLM + Cells
Nuclei/DAPI
GLUT4/DyLight 488
Merged(GLUT4/Nuclei)
50µm

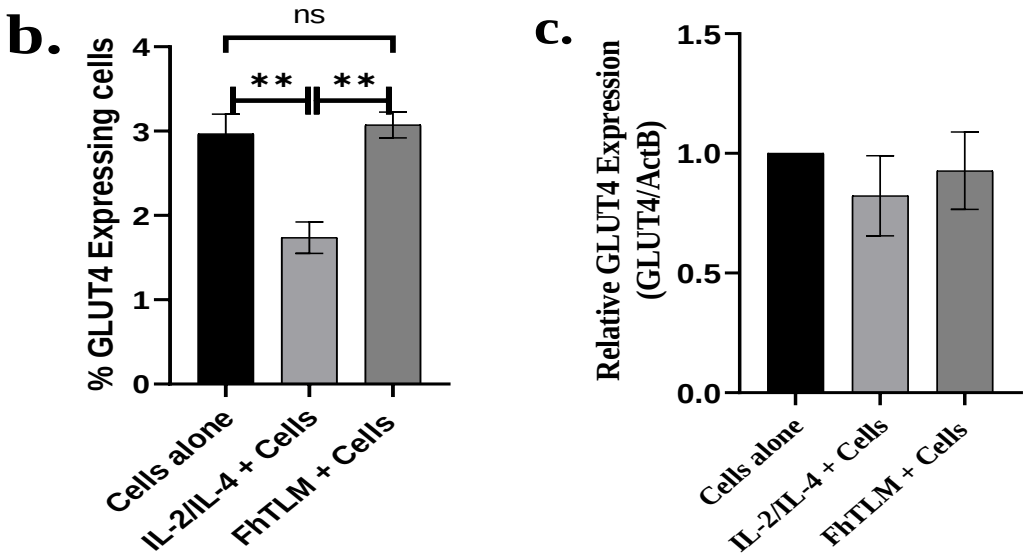


Figure 5:4: Expression of GLUT4 in FhTLM-stimulated CD4 T-cells. CD4 T-cells were activated in the presence of anti-CD3/CD28 and stimulated as described (2.9.3.2). Following culture, CD4 T-cells were analysed for the detection of (a) GLUT4 positive cells by immunofluorescence using GLUT4 primary antibody and goat anti-rabbit IgG:DyLight 488 secondary antibody as described (2.14.1). After staining, cell preparations were examined using a Leica upright microscope at x40 magnification. In image (a), the upper row are cells that are DAPI stained, the middle row is stained with anti-DyLight 488 (green) and the bottom row shows DAPI overlay on anti-DyLight 488. The columns represent (from left to right) unstimulated CD4 T-cells, Th2 polarized CD4 T-cells and

FhTLM stimulated CD4 T-cells. (b) Percentage GLUT4 positive cells analysed using imageJ software and showing mean ± SEM of percentage GLUT4 expressing cells of images taken in 5 different fields of view from one experiment. (c) Quantification of expression levels of GLUT4 mRNA normalised to actin using delta-delta-cycle threshold (methods and data are mean ± SEM of triplicate wells pooled from two independent experiments. Data were analysed with one-way ANOVA using Dunnett's multiple comparisons test and statistical difference are indicated on the graph. ** indicates P=0.0023 or 0.0039.

5.3.4 *In vitro* effect of conditioned medium isolated from stimulated monocytes on NEJ viability.

Stimulation of monocytes with LPS has been shown to provide immune signals that facilitate parasite development (Lamb *et al*., 2010). Following incubation of NEJs in monocyte conditioned medium, NEJ viability and growth were assessed. As shown in Figure 5.5a below, the results indicated that the viability of NEJs under any treatment condition did not significantly change ($p>0.05$) when compared to NEJs cultured in unstimulated monocyte conditioned medium control. However, a trend over two independent experiments showed that NEJs demonstrated greater viability in FhTLM medium, followed by IL-4 medium when compared to other treatments. In addition, NEJs showed a reduced viability in LPS medium when compared to other treatments and there was no increase in the viability of NEJs cultured in IL-4 or LPS medium in the presence of FhTLM. Furthermore, analysis of worm growth in repsonse to various treatments indicated there was no significant difference in worm size under all treatment conditions compared to NEJs only (Figure 5.5b, $P>0.05$).

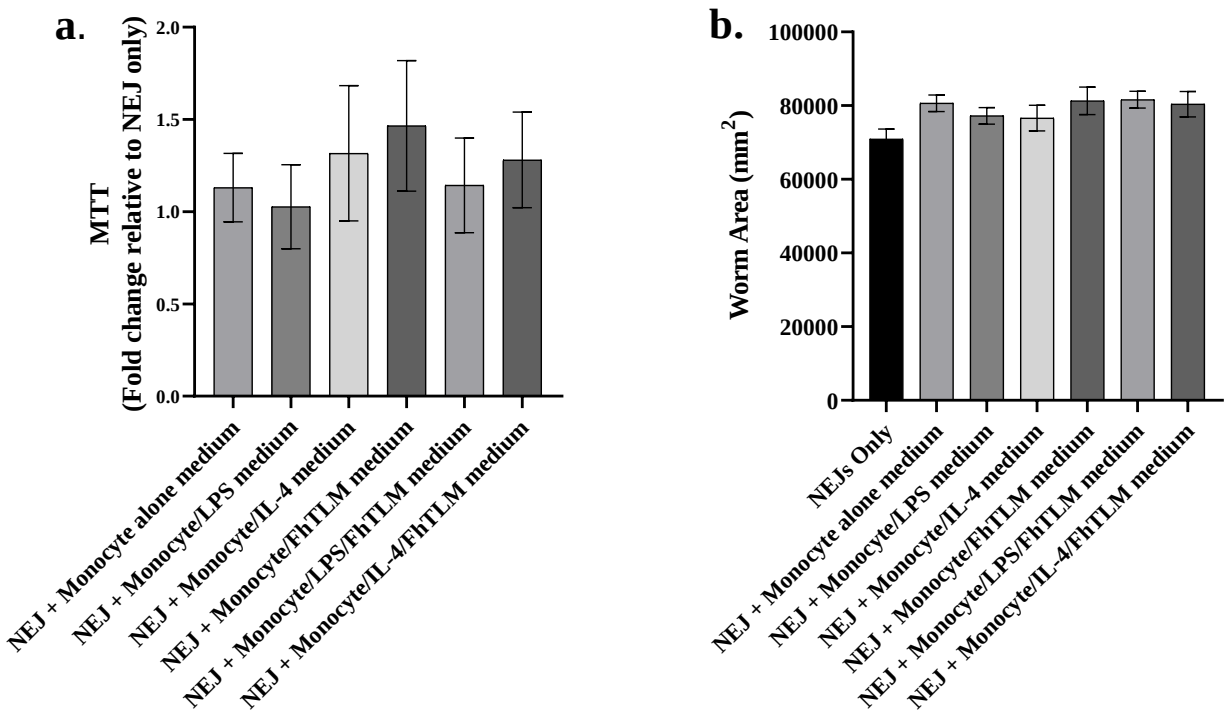


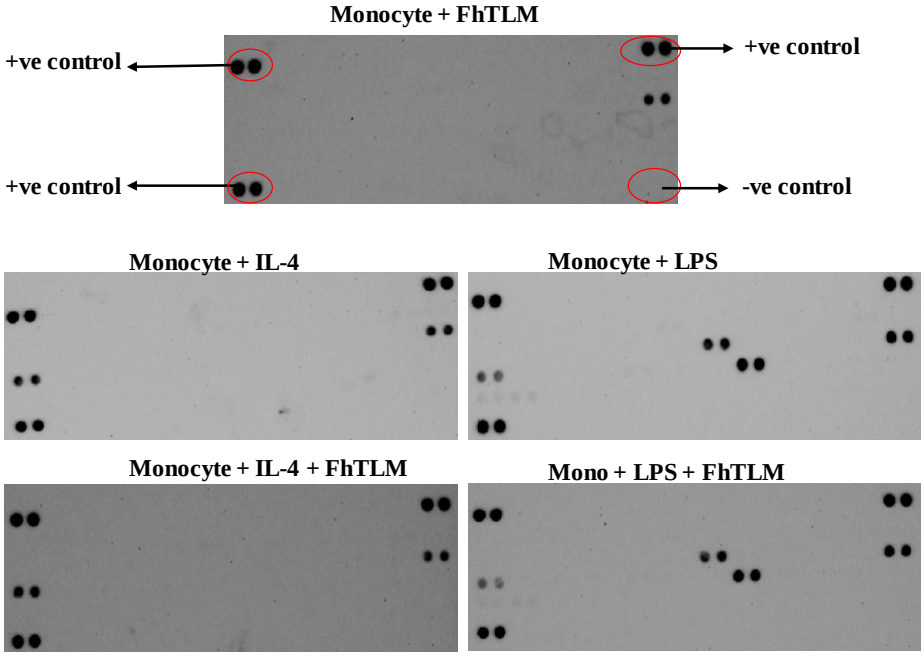
Figure 5:5: Viability of NEJs in monocyte conditioned medium. Conditioned medium was generated from monocytes as described (2.9.3.1). NEJs were cultured in duplicate wells (20 worms/well) in 37^0C and 5% CO_2 incubator for 12-18hrs in conditioned medium. Following incubation, NEJ viability was determined by MTT reduction and fold change in NEJ viability in various treatments relative to NEJs only controls was determined (a). Worm area (b) was measured in at least 10 individual worms per treatment. Data displayed in a, and b are mean ± SEM of two independent experiment. Significant differences between treatment and control groups was analysed using one-way ANOVA using Dunnett's multiple comparison test.

5.3.5 Analysis of growth-related proteins in conditioned medium from FhTLM stimulated monocytes.

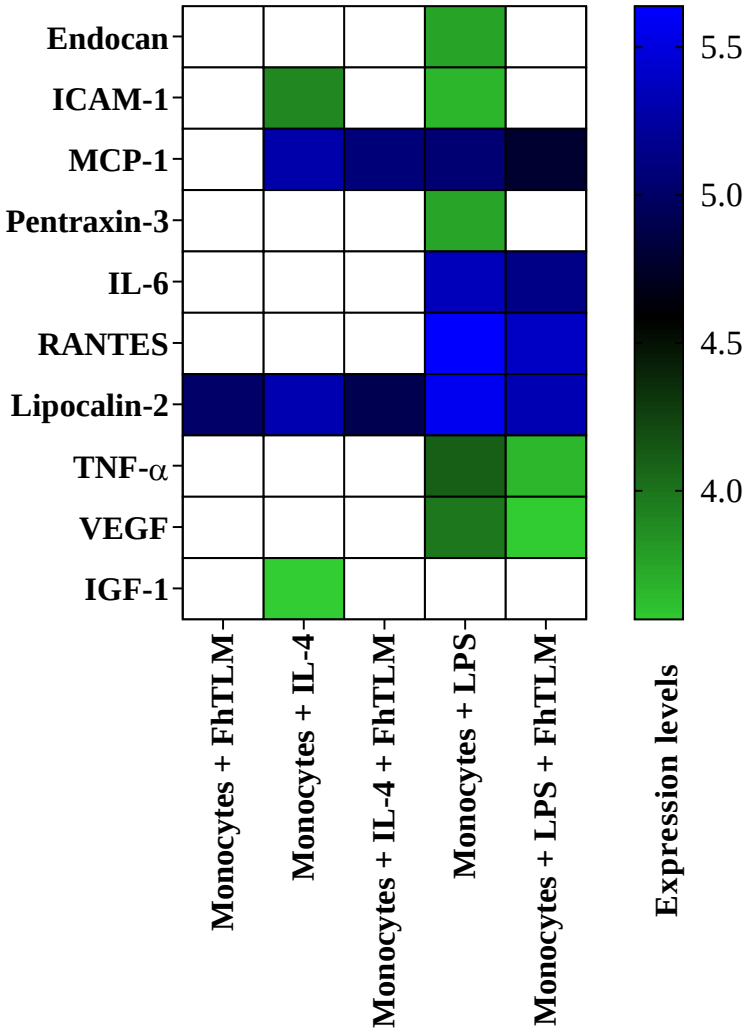
Following the observation that FhTLM conditioned medium may promote parasite survival (Figure 5.6) but in a GLUT4 independent fashion, we sought to determine what, if any, host growth promoters may be upregulated following treatment of monocytes with FhTLM. Conditioned medium generated from stimulated monocytes was assayed for presence of growth-related proteins using a mouse adipokine array kit. Upon incubation of the supplied membrane with conditioned medium, blots were developed, and representative signals were acquired (Figure 5.6a). Signals developed were analysed using an image studio-lite software (Licor) and differential protein expression was obtained by subtracting the negative control (background) values from each spot. Out of the 40 target proteins (Table 5.1), 9 proteins including IL-6, lipocalin-2 (LCN2; also called neutrophil gelatinase-associated lipocalin, MCP-1, RANTES, Endocan 1 (ESM-1), ICAM-1, pentraxin-3, TNF-α and VEGF were induced in LPS stimulated monocytes (Figure 5.6b and c), while IL-4 stimulation induced expression of four target proteins including MCP-1, lipocalin-2, IGF-1 and ICAM-1 (Figure 5.6b and c). Stimulation of monocytes with FhTLM on the other hand, only resulted in expression of LCN2. Interestingly, expression of pro-inflammatory molecules; IL-6 ($P=0.0008$), RANTES ($P= <0.0001$), MCP-1 (0.0267), and lipocalin-2 (<0.0001) by LPS stimulated cells were significantly down-modulated when co-stimulated with FhTLM (Figure 5.6d). TNF-α appeared suppressed in the presence of FhTLM but this was not statistically significant (Figure 5.6d, $P>0.05$). Moreover, expression of endocan, ICAM, and

pentraxin-3 by LPS treatment were completely inhibited in the presence of FhTLM (Figure 5.6b and c). Similarly, while expression of MCP-1 ($P<0.0001$) and lipocalin-2 ($P<0.0001$) in monocytes stimulated with IL-4 were significantly suppressed in the presence of FhTLM (Figure 5.6e), expression of ICAM and IGF were also inhibited (Figure 5.6b).

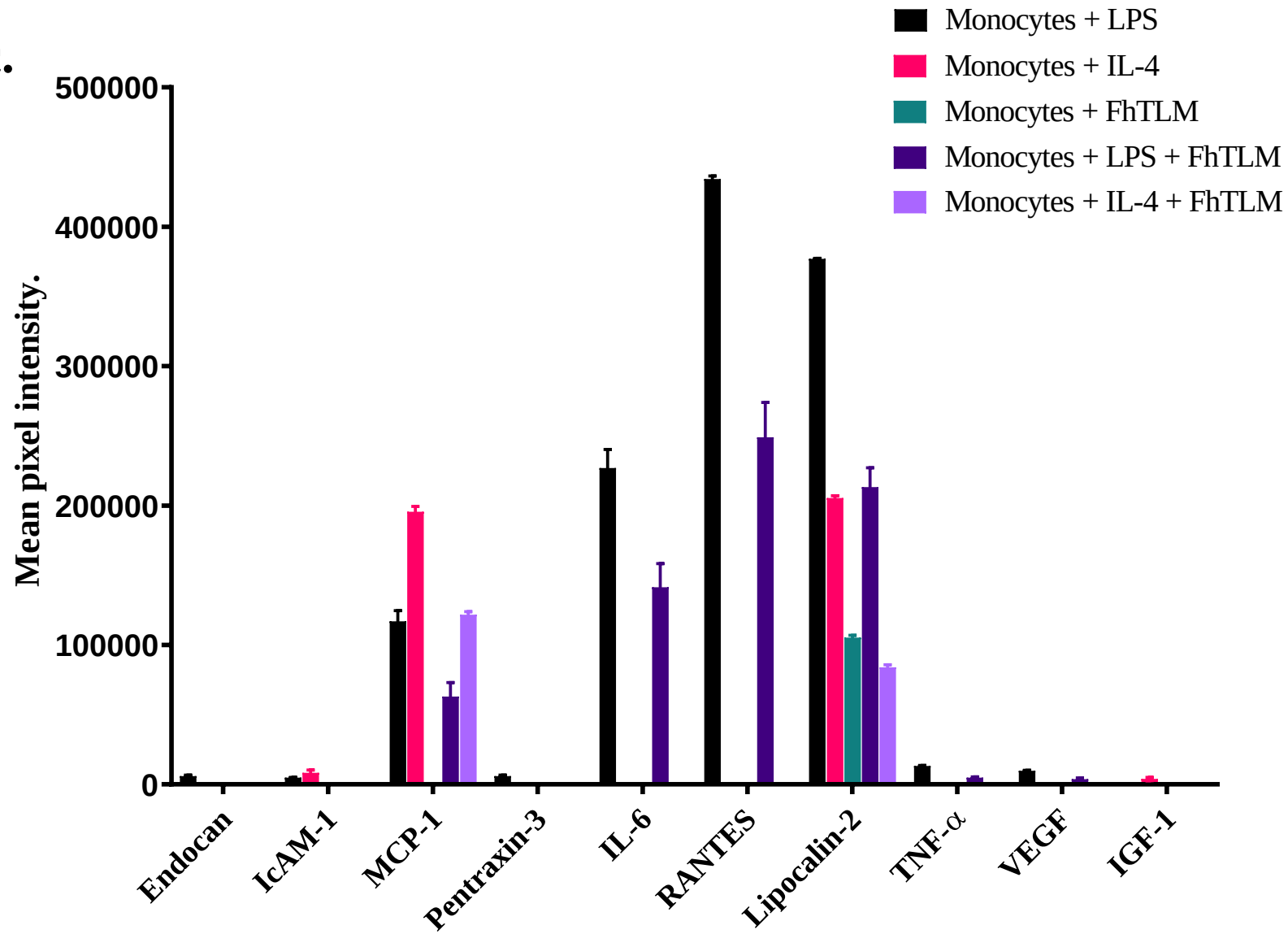
a.


Monocyte + FhTLM
+ve control
+ve control
+ve control
-ve control
Monocyte + IL-4
Monocyte + LPS
Monocyte + IL-4 + FhTLM
Mono + LPS + FhTLM

b.


Endocan
ICAM-1
MCP-1
Pentraxin-3
IL-6
RANTES
Lipocalin-2
TNF-α
VEGF
IGF-1
Monocytes + FhTLM
Monocytes + IL-4
Monocytes + IL-4 + FhTLM
Monocytes + LPS
Monocytes + LPS + FhTLM
5.5
5.0
4.5
4.0
Expression levels

C.


Monocytes + LPS
Monocytes + IL-4
Monocytes + FhTLM
Monocytes + LPS + FhTLM
Monocytes + IL-4 + FhTLM
Mean pixel intensity.
500000
400000
300000
200000
100000
0
Endocan
IcAM-1
MCP-1
Pentraxin-3
IL-6
RANTES
Lipocalin-2
TNF-α
VEGF
IGF-1

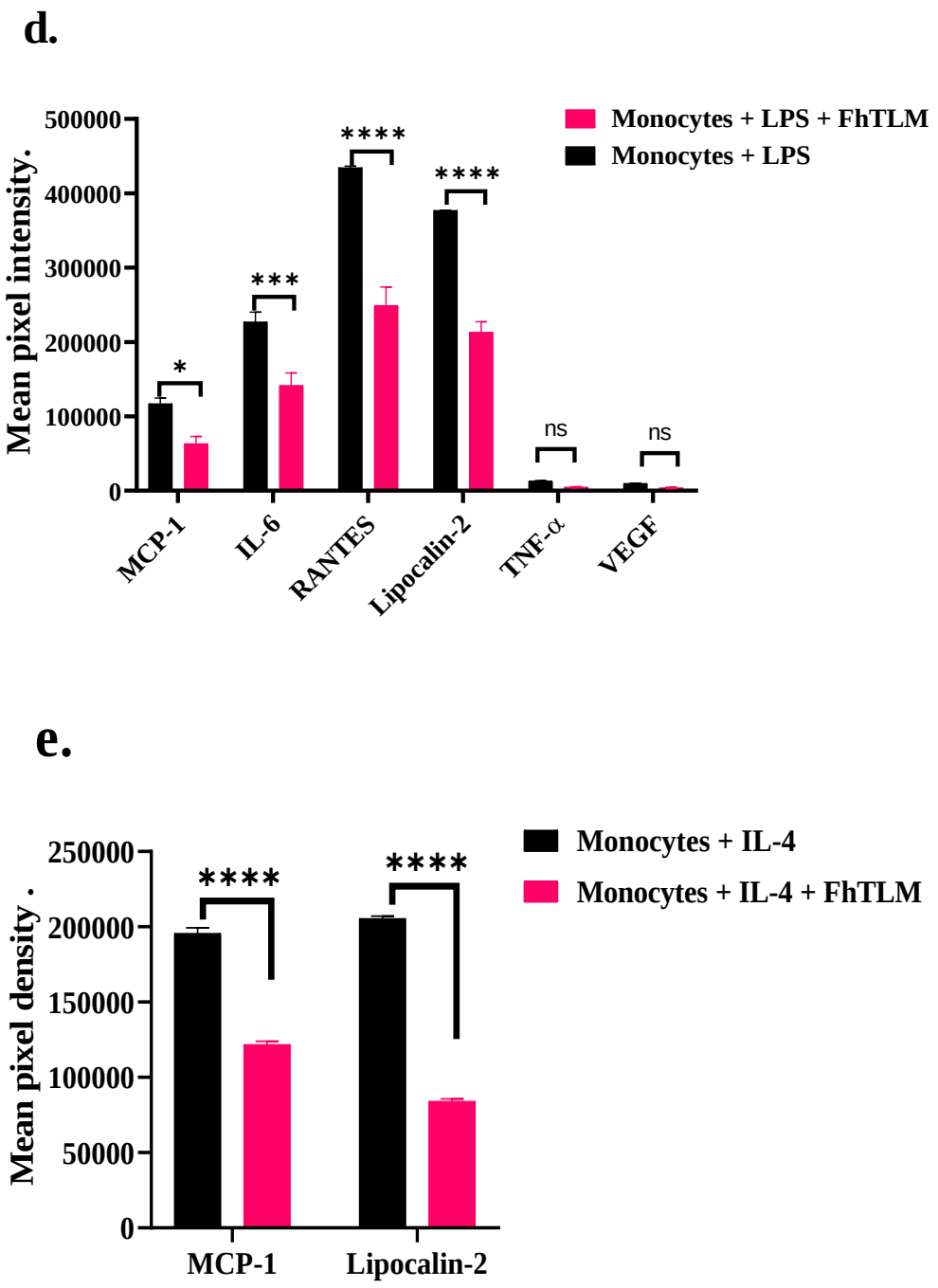


Figure 5:6: FhTLM down modulates expression of pro-inflammatory molecules in LPS-stimulated monocytes. Monocytes were stimulated as described (2.9.3.1) and supernatant was harvested and analysed for detection of growth promoting molecules using an adipokine array kit. Following incubation of the supplied membrane with supernatant and antibody binding, dot blots (a)

were acquired using ChemiDoc^{TM} MP Imaging System and pixel density of the blots were analysed using image studio lite software (Licor). Average pixel density of each blot was subtracted from the background signal. (b) Heat map was plotted for the induced protein targets using Graph Pad Prism version 8.1.2 with red and green colours corresponding to highly and under-expressed proteins while black colour corresponds to intermediate expression. (c) Graph showing mean ± SEM of intensities of proteins that were expressed in indicated treatments. Proteins that were down-modulated in the presence of LPS (d) and IL-4 (e) are shown. Statistical difference was analysed using a two-way ANOVA using a Sidak's multiple comparisons test. **** indicates P <0.0001, *** indicates P=0.0008 and * indicates P= 0.0267.

5.4 Discussion

In some models it is becoming increasingly apparent that triggering of developmental processes in parasitic helminths occurs in response to host immune signals, thereby representing an important adaptative mechanism used by the parasite to promote survival and maximize transmission between hosts. This indicates that both host and parasite factors may synergize, ultimately benefitting long term parasite persistence. In this chapter we sought to determine if FhTLM secretion by the parasite could alter host effector cell secretions in order to benefit parasite survival.

The viability of NEJs in the presence of eosinophils primed with either FhTLM or BSA (control protein) was assessed. Eosinophilia is one of host innate effector responses that mediates protective immunity against some helminth infections including *F. hepatica* (Van Milligen *et al*., 1999, Van Milligen *et al*., 1998, Ramalho-Pinto *et al*., 1978, Folkard *et al*., 1996). However, recent findings suggest that eosinophils can promote rather than impair the growth of some helminth parasites (Huang *et al*., 2015, Babayan *et al*., 2010). Following NEJ incubation, our results indicated that NEJ viability was not significantly enhanced in the presence of FhTLM-treated eosinophils. However, NEJs demonstrated greatest viability in the presence of BSA-treated eosinophils as indicated by higher levels of ATP. Enhanced worm viability seen in NEJs cultured in BSA treatments is consistent with previous reports which showed that uptake of serum albumin and its proteolysis was required for the optimum growth and progression of the malaria parasite, *P. falciparum* (El Tahir *et al*., 2003). Therefore, we can speculate that the possibility of BSA uptake and

subsequent degradation by NEJs may result in increased parasite metabolic activities reported in this study. Moreover, BSA was previously shown to promote the *in vitro* growth and development of NEJs (McCammick et al., 2016). In addition, our finding that NEJ viability was not enhanced in the presence of FhTLM-treated eosinophils differs from a previous study that showed that FhTLM could promote parasite survival by preventing the killing of NEJs by macrophages pulsed with immune sera from infected cattle in an ADCC dependent mechanism (Sulaiman *et al*., 2016). It would appear that *F. hepatica* may have evolved ways of sensing the host killing mechanism (ADCC) and consequently secreting molecules that alters the host's effector functions to benefit its survival. Indeed, Carmona *et al*., (1993) showed that a *F. hepatica* CL-like molecule prevented eosinophil attachment to NEJs in an ADCC mechanism, suggesting a mechanism to enhance parasite survival (Carmona *et al*., 1993)

Furthermore, evidence from various studies has shown that signals from the host's innate and adaptive immune system modulate parasite development (Lamb *et al*., 2007, Lamb *et al*., 2010, Davies *et al*., 2001, Blank *et al*., 2006). As a result, we investigated the effect of immune signals generated from innate (monocytes) and adaptive cells (CD4 T-cells) on the survival of NEJs by assessing growth and viability. Conditioned medium was generated by stimulation of monocytes with various ligands including FhTLM, LPS or IL-4. Analysis of worms following 12-18hrs incubation in LPS or IL-4 conditioned medium showed that parasite growth and viability was not enhanced in these treatments (Figure 5.5). This may imply that signals from classical or

alternatively activated monocytes were not required for worm survival in this setting. This finding is contrary to other studies that showed that repeated stimulation of monocytes/macrophages with LPS provides the necessary signals that facilitated *S. mansoni* development *in vivo* (Lamb *et al*., 2010, Riner *et al*., 2013). While our study induced inflammatory responses by a one-time *in vitro* activation of monocytes with either IL-4 or LPS, the previous studies utilized a-repeated injection of either ligands into mice. The enhanced worm development reported in the latter studies may be favoured by induction of endotoxin tolerance (ET) associated with repeated exposure to endogenous danger signals such as LPS (Biswas and Lopez-Collazo, 2009). ET has previously been shown to promote a poorly controlled inflammatory responses characterised by the down modulation of inflammatory cytokines upon re-stimulation with LPS in human and murine monocytes/macrophages (Draisma *et al*., 2009, Foster *et al*., 2007). Indeed, down regulated inflammatory responses have been shown to allow for *S. mansoni* growth and development (Riner *et al*., 2013). Conversely, our own study induced inflammatory responses by a one-time *in vitro* activation of monocytes.

Furthermore, while FhTLM treatment does not induce enhanced worm growth and viability, a trend over two independent experiments (Figure 5.5a) suggests that FhTLM may have the capacity to promote worm viability. Perhaps, a longer incubation time is required to produce a clearer picture of changes in parasite viability, this remains to be investigated. It has previously been shown that *F. hepatica* calmodulin proteins (*Fh*CaMs) facilitate *in vitro* NEJ growth over 21 days in a bovine serum dependent manner (McCammick et al., 2016).

In line with our observation that FhTLM medium showed a potential to promote worm viability, we sought to determine the possibility of upregulation of any growth promoters in this medium. As expected, analysis of LPS stimulated monocyte conditioned medium showed induced expression of multiple pro-inflammatory molecules including IL-6, TNF-α, RANTES, MCP-1, lipocalin-2, ICAM-1 and pentraxin-3. In addition, expression of lipocalin-2, MCP-1 and ICAM was also induced by IL-4 treatments. Perhaps the inability of these exogenous cytokines to stimulate parasite survival may reflect the negative effect of host pro-inflammatory mediators on NEJs. Indeed, it has been shown that the cytotoxic effect and the killing of NEJs *in vitro* by LPS-stimulated monocytes/macrophages was mediated by the release of the pro-inflammatory molecule NO. Notably, Lipocalin-2 was the only upregulated protein in response to FhTLM stimulation. Lipocalin-2 is an anti-microbial host molecule that sequester iron by binding to bacterial siderophores (iron-binding sites) (Flo et al., 2004). *F. hepatica* lives in the host bile duct where it feeds on host blood and hepatic tissues rich in iron for development (Pantelouris and Hale, 1962). Although iron is essential during *F. hepatica* development, excess/poor regulation of iron content can be detrimental as it can participate in free radical reactions, which are known to be harmful to the parasite (Piedrafita et al., 2000). Hence, there is need for regulation of iron content during development within the vertebrate host. We reasoned that the secretion of lipocalin-2 by the parasite might be essential for regulation of iron uptake.

Surprisingly, while FhTLM had minimal effect on cytokine expression, it significantly suppressed or inhibited the expression levels of multiple pro-

inflammatory molecules (IL-6, RANTES, MCP-1, lipocalin-2, ICAM-1, endocan and pentraxin-3) in LPS stimulated monocytes. These pro-inflammatory molecules have been shown to play important role in the defence against some bacterial infections (Cavalcanti *et al*., 2016, He *et al*., 2007, Huttunen *et al*., 2011, Flo *et al*., 2004, Humlicek *et al*., 2004). In addition, expression of lipocalin, MCP-1 and ICAM by IL-4 was also suppressed by FhTLM. Our data concur with a previous study which showed that FhTeg failed to induce cytokine production but promoted marked suppression of pro-inflammatory cytokines including IL-12, IL-6, TNF-α and NO on DCs stimulated with LPS (Hamilton *et al*., 2009). Down modulation of inflammatory responses by FhTLM would appear to be important in promoting parasite survival. This is consistent with Riner *et al*., (2013) who showed that normal *S. mansoni* development in $Rag^{-/-}$ mice correlated with down modulation of pro-inflammatory molecules. Moreover, blockade of host inflammatory responses by neutralizing antibody to TNF-α also restores parasite development (Riner *et al*., 2013).

Several studies have shown that immune signals from host CD4 T-cells facilitate the growth and development of various helminth parasites including *B. malayi* and *S. mansoni* (Shultz and Rajan, 1999, Davies *et al*., 2001, Lamb *et al*., 2007) This prompted us to investigate the effect of signals from CD4 T-cells and the contribution of FhTLM to the growth and viability of NEJs. Conditioned medium was generated following activation and stimulation of CD4 T-cells with Th2 polarizing conditions (Sekiya and Yoshimura, 2016) or FhTLM. Following NEJ incubation, analyses of NEJs showed no increase in worm size in all treatment conditions. This finding is contrary to previous findings indicating that

presence of CD4 T-cells in immuno-deficient mice provides the necessary signals that facilitated growth and development of various helminth parasites including *S. mansoni* and *B. malayi* (Shultz and Rajan, 1999, Blank *et al*., 2006, Davies *et al*., 2001). The discrepancies in findings may reflect the contribution of other components of the immune system to parasite development, as these studies employed an *in vivo* approach while ours utilized purified CD4 T-cells in our *in vitro* studies. For instance, it has been shown that CD4 T-cells do not directly modulate *S. mansoni* development rather, they provides essential assistance to innate cells which in turn promote parasite development (Lamb *et al*., 2010) and NK cells have been shown to participate in filarial parasite development (Babu *et al*., 1998).

Glucose metabolism has been shown to play an important role in regulating T-cell function (Palmer *et al*., 2015) and upregulation of GLUT4 during T-cell activation was consistent with glucose uptake and increased cell metabolic activities such as proliferation, differentiation and effector function (Fischer *et al*., 2017). Moreover, it has been previously shown that some parasites including *T. spiralis* and *Plasmodium berghei* have exploited host metabolic glucose demand to facilitate their growth and development (Wu *et al*., 2009, Meireles *et al*., 2017). Increased worm growth was associated with upregulation of GLUT4 and activation of PI3K-Akt pathways (signal transduction pathway that regulates a number of cellular activities including glucose metabolism) in nurse cells during *T. spiralis* infection in mice (Huang *et al*., 2015). In order to investigate a possible mechanism that may promote parasite growth, expression of GLUT4 on activated CD4 T-cells stimulated with either FhTLM or Th2 was

examined. As expected, expression of GLUT4 was upregulated in response to T-cell activation and this level was maintained by FhTLM stimulation (Figure 5.3b and c). Th2 induction on the other hand, resulted in significant down modulation of GLUT4 expression. The finding that FhTLM stimulation had no effect on GLUT4 expression may imply that other GLUT molecules such as GLUT1 (Meireles *et al*., 2017) may be important in regulating glucose transport during *F. hepatica* infection or it may reflect the inability of FhTLM to induce GLUT4 expression. This remains to be investigated.

In conclusion, we have shown from this study that FhTLM failed to promote parasite survival in the presence of host innate (eosinophils and monocytes) and adaptive (CD4 T-cells) immune signals/cells. Furthermore, we also demonstrated that FhTLM possess an anti-inflammatory capacity as evidenced by down modulation of host inflammatory responses on activated monocytes. This finding will have implications in supporting parasite persistence in the face of host immune responses. The mechanisms mediating FhTLM action on monocytes remains to be investigated. However, targeted interventions against parasite molecules that promote immune down modulation will result in parasite clearance.

6 General Discussion and Conclusion

6.1 General Discussion

In this study, the immune-modulatory potential of FhTLM (a *F. hepatica* secreted growth factor belonging to the TGF-β superfamily) (Japa *et al*., 2015) has been researched. T-cell responses to FhTLM have been studied in terms of expression of anergic T-cell markers and induction of Foxp3 - a key transcriptional factor of T-reg cells - using flow cytometry and ELISA. FhTLM failed to induce $Foxp3^+$ expression when added to an *in vitro* naïve CD4 T-cell culture. This finding differed from other studies that showed that various helminth secreted molecules promote $Foxp3^+$ induction on CD4 T-cells (Grainger et al., 2010, Johnston et al., 2017, Smyth et al., Nono et al., 2019), indicating that the parasite uses various means to down-regulate T-cell responses but this apparently does not extend to FhTLM. One of the most potent immunological functions of TGF-β is its ability to initiate T-cell suppression via Foxp3 expression (Li *et al*., 2006b). Despite FhTLM possessing several similar biological activities with its mammalian TGF-β counterpart (Japa *et al*., 2015, Sulaiman *et al*., 2016), our results indicate that it differs from FhTLM in the transcriptional regulation of Foxp3 (as evidenced by enhanced secretion of $CD4^+$ $Foxp3^+$ cell by TGF-β and not FhTLM). Sulaiman *et al* (2016) have previously observed that in comparison to mammalian TGF-β, FhTLM demonstrated delayed kinetics in signal transduction and less ability to stimulate TGF-β responsive cells as measured in Smad phosphorylation and luciferase reporter assays (Sulaiman *et al*., 2016).

Apart from induction of Foxp3, upregulation of anergic markers on T-cells during chronic infections has also been implicated in suppression of protective immunity and persistence of helminth infections (Borkow *et al*., 2000). Chronic

F. hepatica infections are characterised by anergic CD4 T-cells that neither proliferate nor produce effector cytokines (Sachdev *et al*., 2017). In this study, we assessed the *in vitro* effect of FhTLM on upregulation of various anergic markers including PD-1, PD-L1 and CTLA-4 in CD4 T-cells. FhTLM did not promote the upregulation of expression of these markers beyond those seen in controls. The ELISA validation assay confirmed that these molecules were not upregulated and secreted in response to FhTLM. Therefore, in conclusion, we have shown that FhTLM is not required for the *in vitro* generation of anergic CD4 T-cells via PD-1/PD-L1 and CTLA-4 pathways. Perhaps, *in vivo* approach via injection of FhTLM would be necessary to stimulate these responses. For instance, immunization of the ovine host with recombinant FhCL-1 has previously been shown to promote Foxp3 expression during acute *F. hepatica* infection (Pacheco et al., 2018). In addition, injection of FhTeg antigen in mice drives expression of anergic markers in splenic $CD4^+$ T-cells (Aldridge and O'Neill, 2016). Whilst our finding indicates that the interaction of FhTLM with CD4 T-cells does not promote expression of immune regulatory markers, we cannot completely rule out the possible participation of FhTLM in the regulation of T-cell anergy via induction of other genes such as *Grail, Egr2/3, Itch* and *CblB* that are associated with T-cell hyporesponsiveness during chronic *F. hepatica* infections (Aldridge and O'Neill, 2016). For instance, enhanced expression of Grail, was shown to be associated with the development and maintenance of suppressive T-cells during chronic *S. mansoni* infection in mice both *in vivo* and *in vitro* (Taylor *et al*., 2009). Small hairpin RNA (shRNA) mediated KO of GRAIL expression reversed T-cell hyporesponsiveness (Taylor *et al*., 2009). Therefore, there remains other regulatory pathways to be explored.

We did measure the production of IL-10 in FhTLM stimulated CD4 T-cells. FhTLM induced significant production of IL-10 in CD4 T-cells. This is in keeping with several studies that have reported secretion of IL-10 during chronic *F. hepatica* infection (Cervi et al., 2001, Moreau et al., 1998, Mendes et al., 2013, Flynn and Mulcahy, 2008, Hacariz et al., 2009). In addition, experimental infection of young cattle with *F. gigantica* or injection of a cysteine proteinase (CP) molecule in sheep resulted in secretion of IL-10 (Eman et al., 2012, Ingale et al., 2010). Production of IL-10 has been shown to suppress cellular proliferation of various cells while also inhibiting protective (Th1) immune responses against *F. hepatica* infection in multiple hosts (Cervi *et al*., 2001, Mendes *et al*., 2013, Moreau *et al*., 1998). For instance, IL-10 has been shown to inhibit IFN-γ and NO production during natural bovine and experimental ovine *F. hepatica* infections respectively (Mendes *et al*., 2013, Moreau *et al*., 1998). FhTLM is secreted by the NEJs, and it has been shown that peritoneal cells in close contact with the NEJs produced increased levels of IL-10 and suppression of NO production (Cervi *et al*., 1998). It would seem reasonable to assume that in order to facilitate parasite migration and survival, the cytotoxic effector responses that mediate parasite death must first be dampened. The production of IL-10 by FhTLM reported in this study will be a contributing factor to the suppression of the host protective response while ensuring parasite persistence. Increased IL-10 secretion following experimental infection of cross-bred sheep with *F. hepatica* was associated with high fluke burden (Hacariz *et al*., 2009). In addition, in other parasitic infections including *S. mansoni*, *T. gondii* and *T. spiralis*, IL-10 has been shown to prevent parasite death by suppressing the host's inflammatory responses (Gazzinelli et al., 1992,

Gazzinelli et al., 1996, Beiting et al., 2004, Beiting et al., 2007). Moreover, persistence of *L. major* in resistant mice following healing of skin lesions was associated with the presence of IL-10 (Belkaid *et al*., 2001).

Following our finding that FhTLM had no effect on T-reg cell surface markers, a global profile of the cytokines and chemokines that were induced in CD4 T-cells cultured with FhTLM was performed using a cytokine profiler array that allows for the detection of up to 40 proteins. Stimulation of CD4 T-cells along with FhTLM promoted a Th1/pro-inflammatory response evidenced by production of pro-inflammatory cytokines including TNF-α, IL-12p70, IL-1β, and IL-1ra, MIP-1β, CXCL12 and RANTES. Our results that FhTLM induces secretion of inflammatory mediators is in keeping with other studies demonstrating increased expression of IL-6, IL-12p40, and MIP-2 on DC in response to both *F. hepatica* secreted FhGST-si and FhCL (Dowling *et al*., 2010). This indicates that FhTLM likely contributes to the early inflammatory response that is associated with *F. hepatica* infections (Tliba et al., 2002, Clery et al., 1996, Clery and Mulcahy, 1998).

From the chemokine and cytokine analyses, RANTES and TARC were the most highly induced targets in response to FhTLM stimulation. While significant expression of RANTES in response to FhTLM was validated by ELISA, expression of TARC protein was not raised above control levels. In order to validate the biological relevance of chemokine secretion by FhTLM, an *in vitro* chemotaxis assay was performed using cell culture supernatant isolated from FhTLM stimulated cells or medium with/without chemoattractant as controls. FhTLM supernatants significantly induced the migration of both eosinophils and activated CD4 T-cells above those seen in medium alone controls, indicating

that the chemokine(s) secreted by FhTLM are biologically functional. To date, studies on chemokine responses during *F. hepatica* infections are limited (Rodríguez *et al*., 2017). However, because of the increased expression levels of RANTES, a chemoattractant for various cells including T-cells and eosinophils in response to FhTLM supernatants, the results might suggest a possible participation of these chemokines in leukocyte recruitment during *F. hepatica* infection. For instance, a previous study has shown that increased mast cell number was associated with secretion of chemokines, MIP-1α and MIP-2 by DC stimulated with *F. hepatica* antigen (Vukman *et al*., 2013a).

Following on from the findings that FhTLM induced functional host chemokines together with recent studies that suggest that the effector function of host immune cells extends beyond host protection to providing signals that serve as cues for helminth parasites to speed up their developmental processes (Huang et al., 2015, Lamb et al., 2010, Davies et al., 2001), we investigated the *in vitro* effect of immune signals generated from adaptive immune cells (CD4 T-cells) and innate cells (eosinophils and monocytes) on the survival of NEJs. NEJ survival as measured by viability and growth was not significantly enhanced following incubation in conditioned supernatants generated from either LPS/IL4 activated monocytes or Th2 polarized CD4 T-cells, when compared to NEJs incubated in medium alone control. This indicates that signals from monocytes and CD4 T-cells stimulated in this way does not affect *in vitro* survival of NEJs in culture. This finding is contrary to previous *in vivo* studies that showed that host macrophages/monocytes and CD4 T-cells were important in modulating *S. mansoni* growth and development in immunodeficient mice (Lamb *et al*., 2010, Riner *et al*., 2013, Davies *et al*., 2001, Lamb *et al*., 2007). The discrepancies in

findings, may reflect the contribution of other components of the immune system affecting parasite development, as the latter studies employed an *in vivo* approach while ours utilized purified CD4 T-cells and monocytes in our *in vitro* studies. It has been shown that CD4 T-cells do not directly modulate *S. mansoni* development, rather they provide essential signals that initiates the development of macrophages/monocytes, which in turn promote parasite development (Lamb *et al*., 2010). Moreover, regulation of *S. mansoni* development by CD4 T-cells was shown to be dependent on IL-7 signalling pathways (Blank *et al*., 2006). In this study, we showed that incubation of NEJs in conditioned supernatants generated from either FhTLM stimulated CD4 T-cell or monocytes does not improve NEJ survival. In addition, NEJ survival was not enhanced following culture in conditioned supernatants isolated when monocytes were stimulated with FhTLM in combination with either IL-4 or LPS.

Whereas FhTLM failed to promote worm survival in the experiments described above, analysis of monocyte supernatants indicated that FhTLM significantly down modulated or inhibited expression of inflammatory chemokines and cytokines stimulated by both LPS and IL-4. LPS mediated-expression of IL-6, MCP-1, RANTES and lipocalin-2 were suppressed by FhTLM and expression of other inflammatory mediators including pentraxin-3, ICAM-1 and endocan were also completely inhibited in the presence of FhTLM. In addition, we also noted that expression of lipocalin-2 and MCP-1 induced by IL-4 treatment was significantly suppressed and ICAM-1 was inhibited in the presence of FhTLM. This finding suggests an indirect mechanism used by *F. hepatica* to promote its survival via interaction with innate immune cells that results in suppression of inflammatory responses. The finding that FhTLM suppressed expression of

inflammatory responses is in keeping with previous studies that showed that FhTeg impaired the ability of mast cell to drive a Th1 response evidenced by suppression the production of TNF-α, IL-6, IFN-γ and ICAM (Vukman *et al*., 2013b). Consistent with our finding, a previous study in our group showed that macrophages pre-pulsed with FhTLM have suppressed levels of NO and IL-12 and lost their ability to kill NEJs in the presence of parasite-specific antibody (Sulaiman *et al*., 2016). Our observation that FhTLM promotes pro-inflammatory responses in CD4 T-cells and down modulates this response in monocytes supports a role for FhTLM in possessing the capability to evoke different immunological responses in varying cell types. It may also reflect immunomodulatory capability of FhTLM that allows it to suppress monocyte function, given the critical role of monocytes in mediating killing of juvenile flukes (Duffus and Franks, 1981, Sibille et al., 2004, Piedrafita et al., 2001).

Furthermore, down modulation of inflammatory responses together with the production of IL-10 that we have reported in this study would also indicate that FhTLM possesses an anti-inflammatory potential. Notably, *F. hepatica* infection has been shown to generate anti-inflammatory responses (via production of IL-10 and TGF-β) and also promote a bystander suppressive effect on autoantigen-specific secretion of IFN-γ and IL-17 (Walsh *et al*., 2009) . In addition, down modulation of inflammatory responses by FhTLM would have implications in understanding the relationship between *F. hepatica* and co-infection with other pathogens that initiate innate immune responses such as bacterial infection. Several studies have shown that co-infection with *F. hepatica* could down modulate these responses (Flynn *et al*., 2007b, Claridge *et al*., 2012, Brady *et al*., 1999, Flynn *et al*., 2009). For instance, co-infection of *F.*

hepatica with the bacterial pathogens *Bordetella pertussis* and *M. bovis* resulted in exacerbation of bacterial infection and reduced the efficacy of tests used to diagnose bovine tuberculosis (Brady *et al*., 1999, Claridge *et al*., 2012).

We further assessed if FhTLM could alter eosinophil function to benefit parasite survival and we showed that incubation of NEJs in the presence of FhTLM-primed eosinophils does not result in enhanced worm survival when compared to those induced by irrelevant protein control. Although a previous study in our group indicated that NEJs survival was improved when FhTLM was added to an *ex vivo* parasite culture (Japa *et al*., 2015), in this study, our findings indicate that in the presence of signals from both innate and adaptive immune cells, FhTLM failed to promote NEJ survival. Given that NEJs express the highest levels of FhTLM mRNA (Japa *et al*., 2015), it is possible that an additive or synergistic effect between endogenous and exogenous protein was delivering a down modulatory effect on NEJ viability in the presence of immune cell signals. Experimental designs that allow for the detection of endogenous protein concentration or target knock down of FhTLM in NEJs via RNAi approaches will be of great benefit in understanding the role of FhTLM in *F. hepatica* development.

While immune signals from CD4 T-cells failed to promote NEJ survival, we investigated a mechanism that is associated with parasite survival as seen in other infections (Huang *et al*., 2015, Wu *et al*., 2009). Glucose uptake via GLUT4 pathways has been shown to coincide with enhanced parasite growth in multiple infections (Huang *et al*., 2015, Wu *et al*., 2009). Therefore, we examined expression of GLUT4 in FhTLM stimulated CD4 T-cells. We showed that FhTLM does not upregulate GLUT4 expression (mRNA and protein levels)

above those seen in unstimulated cells, suggesting that FhTLM does not utilize GLUT4 expression to promote parasite survival, at least under our experimental conditions. During development within the vertebrate host, NEJs breakdown glucose aerobically to release energy while the adult parasite depends on anaerobic conditions in the bile duct for glucose degradation (Tielens *et al*., 1984). This indicates that glucose metabolism is critical for *F. hepatica* development in the host. Therefore, study of the GLUT pathway that mediates glucose transport during this infection will be important in control interventions as blockade of GLUT activities may result in reduced glucose availability to the developing parasite and consequently impair disease transmission as seen in other infections such as cancer (Liu *et al*., 2012).

6.2 Conclusion and future work.

In conclusion, our data demonstrates that FhTLM and TGF-β have distinct mechanisms in terms of driving Foxp3 in murine splenic CD4 T-cells *in vitro*. Notably, $CD4^{+}Foxp3^{+}$ expression was induced in response to TGF-β but not FhTLM. We have also shown that stimulation of CD4 T-cells with FhTLM does not promote *in vitro* expression of anergic markers in CD4 T-cells, indicating no role for FhTLM in promoting T-cell anergy *via* the studied pathways. It will be interesting to investigate the capacity of FhTLM to induce other important markers of anergy such as *Grail* that regulates T-cell hypo-responsiveness as seen in a related trematode infection (Taylor *et al*., 2009). We also demonstrated that induction of inflammatory cytokines and chemokines by CD4 T-cells in response to FhTLM may suggest the participation of these molecules in the development of early/transient inflammatory responses that are often associated

with *F. hepatica* infections and may also reflect the display of a conserved mechanism within the parasite that allows for its secreted molecules to induce temporary inflammatory responses (Dowling *et al*., 2010). Enhanced production of RANTES in cells stimulated with FhTLM suggests a role for the participation of this chemokine during *F. hepatica* infection. Studies have shown that RANTES is a potent chemoattractant for eosinophils (Teran et al., 1996). Therefore, we reason that RANTES may contribute to eosinophilia that is associated with *F. hepatica* infections. Indeed, we have shown that FhTLM induced chemokines are functional as they modulate *in vitro* migration of both eosinophils and activated CD4 T-cells. However, chemokine depletion and *in vivo* studies will allow for the precise role of these chemokines during *F. hepatica* infection to be determined. During this study, we attempted to investigate the *in vivo* effect of FhTLM on host immune response with regards to chemokine secretion and leukocyte recruitment (Chapter 4). However, obtaining a license that would allow for *in vivo* studies was not possible at the time of this work.

Furthermore, we have also demonstrated that FhTLM does not modulate NEJ survival in the presence of both adaptive (CD4 T-cells) and innate immune signals/cells (monocytes/eosinophils) *in vitro*. Recent studies that have demonstrated a positive role for both adaptive and innate immune cells in facilitating worm growth and survival suggests that various components of the immune system participate in worm survival. Therefore *in vivo* studies through the use of transgenic mice such PHIL (mice devoid of eosinophils) or IL-1R$^{-/-}$ (mice with altered innate inflammatory responses as a result of deficiency in IL-

1 receptor) will allow for the precise role of immune cell in *F. hepatica* development to be investigated.

Finally, we demonstrate that FhTLM possesses an anti-inflammatory potential evidenced by suppression of inflammatory responses induced by both LPS and IL-4 stimulated monocytes. This finding suggest that FhTLM indirectly modulates innate cells to promote parasite persistence and potentially to ensure host survival. Down modulation of inflammatory responses would protect the both the parasite and the host from excessive immune response that may modulate parasite killing and also expose the host to various immune-pathology. For instance, anti-inflammatory effect mediated by AAMϕ during acute murine *S. mansoni* infection was critical for host survival by preventing destructive immunopathology associated with egg-induced inflammation (De'Broski et al., 2004). Moreover, IL-10 also protected the host from hepatic pathology during murine *T. spiralis* infection (Bliss et al., 2003). Collectively, production of an immunomodulatory molecule, IL-10 together with suppression of inflammatory responses by FhTLM that we have reported in this study, would suggest that FhTLM represents an immune evasion molecule that can alter host responses for the benefit of parasite survival. Future studies on secretion/expression of FhTLM during host infection, could yield insight into understanding the precise function of FhTLM in host-parasite interactions. The ability to test this *in vivo* is dependent on the capacity to develop a robust RNAi approach for *F. hepatica* that would need to be stage-specific given the expression pattern of FhTLM. Furthermore, the anti-inflammatory properties of FhTLM suggests that this molecule may be used for the treatment of allergic and/or inflammatory disorders (Harnett, 2014).

7 Appendices

Appendix 1; Buffer and media formulation

Complete cell culture medium

RPMI 1640 (Sigma Aldrich, UK) .....……450 ml

Heat inactivated foetal calf serum (Sigma Aldrich, UK) ……..……50 ml

Penicillin-streptomycin (10 mg/ml)…..……5 ml

L-glutamine (5 M) ……..……1 ml

Eosinophil cell culture medium

IMDM with Glutamax (ThermoFisher, UK) ……..……..450 ml

Heat inactivated foetal calf serum (Sigma Aldrich, UK)……..……50 ml

Penicillin-streptomycin (10 mg/ml)..……..……5 ml

Freeze mix medium

Heat inactivated foetal calf serum (Sigma Aldrich, UK)…..…..……450 ml

DMSO……..50 ml

MACS running buffer

Bovine serum albumin …….……5g

0.5M EDTA (pH 8.0) ……..……..……4 ml

Bring the volume to 1 litre with phosphate buffer saline and store at room temperature

MACS rinsing buffer

0.5 M EDTA (pH 8.0) ……...……4 ml

Bring the volume to 1 litre with phosphate buffer saline and autoclave then store at room temperature

FACS buffer

Heat inactivated foetal calf serum (Sigma Aldrich, UK)…….……50 ml

Bring the volume to 1 litre with phosphate buffer saline and store at 2-8^{0}C.

RBC lysis buffer

Ammonium chloride (NH4Cl)…….8.3 g

Potassium bicarbonate (KHCO3)…….…….……1.0 g

0.5M EDTA…… ……..…….. 200 µl

Bring the volume to 1litre with distilled water and adjust the PH to 7.2 - 7.4.

IL-10 ELISA Coating Buffer

Sodium Bicarbonate ($NaHCO_3$)…….……7.13g

Sodium Carbonate (Na_2CO_3) …….……..……..……...1.59g

Bring the volume to 1 litre with distilled water, adjust PH to 9.5 and store at 2-8^{0}C.

IL-10 ELISA Assay Diluent

Heat inactivated foetal calf serum (Sigma Aldrich, UK)……..……100 ml

Bring the volume to 1 litre with phosphate buffer saline and adjust the PH to 7.0

ELISA Wash Buffer

Tween 20……..500µl

Bring the volume to 1 litre with phosphate buffer saline and store at 2-8^{0}C.

Appendix 2; Table 7.1: List of Antibodies

Name	Target	Clone	Isotype	Use	Conjugate	Supplier.
Rat anti- CD4	Mouse	RM4-5	Rat (DA) IgG2a, k	Flowcytometry (FC)	APC	BD Pharmingen
Rat IgG2a		R35-95	Rat (LOU) IgG2a, k	FC	APC	BD Pharmingen
CF594 Hamster anti-CD152	Mouse	UC10-4F10-11	Armenian Hamster IgG1, k	FC	PE	BD Pharmingen
CF594 Hamster IgG1, k		A19-3	Armenian Hamster IgG1, k	FC	PE	BD Pharmingen
Rat anti-CD274	Mouse	MIH5	Rat (SD) IgG2a, λ	FC	PE	BD Pharmingen
Rat IgG2a, λ		B39-4	Rat (LOU) IgG2a, λ	FC	PE	BD Pharmingen
Purified NA/LE Hamster anti-CD3	Mouse	145-2C11	Armenian Hamster IgG1, k	Co-stimulation	Unconjugated	BD Pharmingen
Purified NA/LE Hamster anti-CD28	Mouse	37.51	Syrian Hamster IgG2, λ1	Co-stimulation	Unconjugated	BD Pharmingen

Table 7.1 Continuation;

Rat IgG2b, k		A95-1	Rat (LOU) IgG2b, k	FC	PE	BD Pharmingen
Rat anti-IL10	Mouse	JES5-16E3	Rat IgG2b	FC	PE	BD Pharmingen
Rat anti-CD25	Mouse	3C7	Rat (LEW) IgG2b, k	FC	FITC	BD Pharmingen
Rat IgG2b, k		A95-1	Rat (LOU) IgG2b, k	FC	FITC	Rat (LOU) IgG2b, k
Recombinant Human IL-2				Cell culture	Unconjugated	Peprotech
GLUT4 primary antibody		Polyclonal		Immunocytochemistry (ICC)	Unconjugated	Novous Biological
Rabbit IgG		Polyclonal		ICC	DyLight® 488	ThermoFisher Scientific, UK
Purified Rat anti-CD16/32	Mouse	2.4G2	Rat IgG2b k	Fc Receptor Block	Unconjugated	BD Pharmingen
Anti-CD279	Mouse	RMP1-30	Rat IgG2b k	FC	FITC	eBioscience

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