Fasciola hepatica's tegumental antigens modulate macrophage phenotype and function

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Msc

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Acronyms and abbreviations

M2: Alternatively activated macrophages

APC: Antigen presenting cell

APC: Allophycocyanin

BCA: Bicinchoninic acid

BMDC: Bone-marrow derived dendritic cells

CD: Cluster of differentiation

CLR: C-type lectin receptors

DC: Dendritic cell

ELISA: Enzyme-linked immunosorbent assay

ES-62: *Acanthocheilonemavitae* Excretory-secretory 62

E/S: Excretory/secretory products

FACS: Fluorescence-activated cell sorter

FCS: Fetal calf serum

FhES: *Fasciola hepatica* excretory/secretory products

FhTeg: *Fasciola hepatica* tegumental coat

FITC: Fluorescein isothiocyanate

GalNAc: N-Acetylgalactosamine

GM-CSF: granulocyte-macrophage cell stem factor

IFN: Interferon (e.g., IFN-γ)

Ig: Immunoglobulin (e.g., IgG)

IL: Interleukin (e.g., IL-12)

iNOS: Inducible nitric oxide synthase

i.p.:Intraperitoneal

LPS: Lipopolysaccharide

mAb: Monoclonal antibody

MGL: Macrophage galactose-type lectin

MHC: Major histocompatibility complex

MR: Mannose receptor

NF-κB: Nuclear factor kappa B

NO: Nitric oxide

NP-40: nonyl phenoxypolyethoxylethanol-40

PBS: Phosphate-buffered saline

PC: Phosphorylcholine

PD-L: Programmed death-ligand

PEC: Peritoneal exudate cells

RT-PCR: Reverse-transcription polymerase chain reaction

PMA: Acetate Phorbolmyristate

PRR: Pattern recognition receptor

RELM α: Resistin-like molecule

RPMI: Roswell Park Memorial Institute

RNA: Ribonucleic acid

SCF: Stem cell factor

SCID: Severe Combined Immunodeficiency

SEA: Schistosome soluble egg antigens

SEM: Standard error of the mean

TGF: Transforming growth factor

Th: T helper cell

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TMB: 3,3',5,5'-Tetramethylbenzidine

TSLP: Thymic stromal lymphopoietin

Abstract: *Fasciola hepatica's* tegumental antigens modulate macrophage phenotype and function – Paul Adams

Alternatively activated macrophages (M2) are antigen presenting cells that have a critical role in host tissue repair, regulation of host metabolism and modulation of adaptive immune responses. During helminth infection, they also act as powerful immune suppressors by suppressing Th1 immune responses. *Fasciola hepatica* infection drives a Th2 immune response, which is associated with the induction of M2 macrophages, in its mammalian host. The induction of Th2 immune responses and M2 macrophages can be mimicked by *Fasciola* excretory-secretory products (FhES). Here a second Fasciola antigen preparation consisting of the tegumental coat of *F. hepatica* was examined for its immune-modulatory properties on macrophages. In contrast to FhES, FhTeg does not induce antigen specific Th1 (IFN-γ) or Th2 (IL-4/IL-5/IL-13) cytokine responses during *F. hepatica* infection or following treatment intra-peritoneally with FhTeg.Despite the lack of Th2 cytokines, similar to FhES, FhTeg can modulate macrophages *in vivo* by inducing a M2-like phenotype that exhibited T-cell suppressive functional ability. This M2-like phenotype was largely STAT6 dependent and while FhTeg cannot induce Th2 specific adaptive immune responses it can induce IL-13 producing macrophages *in vivo*. FhTeg could not induce the M2-likemacrophages directly *in vitro* but rather indirectly through the stimulation of dendritic cells. M2 macrophages express c-type lectin receptors (CLR), a family of receptors that recognize specific pathogen-associated glycoconjugate structures. CLRs are involved in helminth antigen recognition, influencing immune responses associated with helminth infection. The CLRs, Mannose receptor and Macrophage Galactose Lectin were up-regulated during *F. hepatica* infection and this was mimicked by FhTeg *in vivo* and *in vitro*. This study was important because it helps us further understand the role FhTeg plays in *F. hepatica* host/parasite interactions.

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Chapter 1

Introduction

1.1. Introduction:

Helminths are a diverse group of parasitic worm-like organisms that spend a portion of their lifecycle in a vertebrate host. They are prominent infectious agents in developing countries with approximately 3 billion humans infected globally and in both the developing and developed world helminth infection is highly prevalent in domestic and wild animals (Hotez et al., 2008).

Once helminths infect their host, they seek to modulate the host immune system for their own benefit. They are master regulators of host immunity, evolving strategies of evasion and suppression to survive and propagate within their host (Daniłowicz-Luebert et al., 2011). Helminths induce strong Th2/T_{reg} immune responses and are potent suppressors of Th1/Th17 immunity (Maizels et al., 2009; Dowling et al., 2010). This skewing of Th1/Th17 immune responses increases the risk of the host to bystander bacterial, viral and protozoan infections that require Th1 immunity for protection (Brady et al., 1999; Wolday et al., 2002; Hartgers & Yazdanbakhsh, 2006).The molecules that helminths release cause this skewing of the immune response which help the parasite to avoid immune detection and subsequent damage (Hewitson et al., 2009).

Helminths are categorised into two phylum Platyhelminthes (flatworms) and Nematoda (roundworms). The focus of this research is on the helminth *Fasciola hepatica* that belongs to the phylum Platyhelminthes (flatworms), which have bilaterian unsegmented soft-bodies and range in length from a few millimetres to thirty centimetres (Dalton, 1999). *F. hepatica*, as a member of the trematode family, has both an oral and ventral

sucker which can be used to adhere to host tissues (Dalton, 1999). The outer protein coating of flukes, called tegument (FhTeg), is morphologically and physiologically complex; it is shed every 2-3 hours during infection and is in constant contact with host immune cells (Threadgold, 1976). A second source of immune modulatory molecules is the released excretory-secretory products (FhES) from their branched intestine that contain numerous enzyme filled excretory vesicles (Threadgold, 1976). Both of these antigen sources were shown to display potent immune modulatory responses (Donnelly et al., 2005; Hamilton et al., 2009).

Using *F. hepatica* as a model, we seek to understand how these antigen sources modulate the host immune system. Previous studies have shown that FhES induce T helper type 2 (Th2)/regulatory T-cells ($T_{reg)}$responses (Donnelly et al., 2008; Donnelly et al., 2005) *in vivo* which in turn suppress immune responses to bystander infections (Brady, 1999) and autoimmune diseases (Walsh et al., 2009). FhES can drive alternative activated macrophages and modulate dendritic cell function altering its ability to drive Th1/Th17 immune response (Donnelly et al., 2005; Dowling et al., 2010).

FhTeg has been studied in the context of dendritic cells (DC) and mast cells. In DC, FhTeg was shown to inhibit pro-inflammatory cytokines in a model of septic shock and in response to Toll-like receptor (TLR) and non-TLR ligands. FhTeg was also shown to impair dendritic cell phagocytic capacity and their ability to prime T-cells (Hamilton et al., 2009). In mast cells, FhTeg fails to produce Th2 associated cytokine production but instead impairs their ability to drive Th1 immune responses (Vukman et al., 2013;

Vukman et al., 2013) by inducing suppressor of cytokine secretion-3 (SOCS3) a negative regulator of the TLR4 pathway. The focus of this work is to understand how FhTeg interacts with macrophages.

1.2. Helminth immunology

Helminth infections drive Th2/T_{reg} immune responses from their host (Maizels et al., 2009) as characterised by the differentiation of Th2 cells and the production of high levels of interleukin (IL)-4, IL-5, IL-9 and IL-13 and low levels of interferon (IFN)-γ. These cytokines affect antibody class switching, resulting in IgE and IgG1 in mice (IgG4 in humans) production. IgE antibodies bind to the surface of helminths allowing eosinophils and mast cells to attach through Fcε receptors (Hogan et al., 2008).

Regulatory cytokines such as TGF-β and IL-10, which suppress pro-inflammatory Th1 immune response, are released by host immune cells upon contact with helminth molecules and it has been shown that helminth molecules can directly impair immune cells to drive a Th1/Th17 immune response (Mulcahy et al., 2004; Song et al., 2011; Whelan et al., 2012; Hogan et al., 2008; Allen & Maizels, 2011). Helminths therefore leave their host more susceptible to bystander infections such as malaria and tuberculosis while simultaneously protecting them against Th1-mediated autoimmune disorders such as multiple sclerosis and Crohn's disease (Mulcahy et al., 2004; De Winter et al., 2012; Nacher, 2011; Song et al., 2011).

1.3. Helminth elicited Th2 induction

There are a number of endogenous candidate molecules that have been proposed to be responsible for Th2 responses such as the cytokines IL-4, IL-13, IL-33, IL-25 cytokines and thymic stromal lymphopoietin (TSLP). IL-4 can trigger antigen-specific Th2 responses but it has been shown that Th2 cell differentiation can also occur in the absence of IL-4 signalling (Jankovic et al., 2000; Finkelman et al., 2000). IL-25 and IL-33 have the ability to induce rapid production of Th2 cytokines independently of T or B cells (Hurst et al., 2002; Humphreys et al., 2008). IL-25 is a member of the IL-17 family but is functionally different in that it induces eosinophilia and IgE production (Fort et al., 2001), while IL-33 is a member of the IL-1 family. Active IL-33 is released during necrotic cell death and functions as an alarmin which stimulates various cell types such as mast cells to produce IL-5 and IL-13 (Cayrol & Girard, 2009; Ho et al., 2007). TSLP is thought to support growth and differentiation of T and B cells. TSLP stimulated DC can induce the differentiation of $CD4^+$ cells to Th2 in an OX40L dependent manner (Soumelis et al., 2002; Ying et al., 2005; Ito et al., 2005).

Some heterologous mixtures of molecules that can induce Th2 cytokines and associated physiological changes have been identified, but to date no single molecule has been shown to be critical for the induction of a Th2 response, which suggests a level of redundancy that makes no one molecule indispensable. What makes the task of understanding Th2 immunity difficult is that the cells responsible for producing Th2 cytokines remain obscure (Koyasu & Moro, 2011). An early theory for inducing Th2 responses was put forward, called the "default" hypothesis. This hypothesis stated that Th2 responses occurred in the absence of Th1 signals. The idea for this hypothesis came

forward due to the muted phenotype observed when DC were exposed to helminth products. Also, addition of IL-12 (Th1 associated) was seen to be able to reverse the Th2 response after injection with *S. mansoni* eggs (Th2 provoking) (Oswald et al., 1994). Although this theory seemed plausible, contrary evidence showed that helminth products could alter Toll-like receptor (TLR)-mediated Th1 responses to Th2 (Van Riet et al., 2009; Kane et al., 2004). When exposed to microbial pathogens that normally produce a Th1 response, IL-12 deficient mice did not develop a Th2 response (Jankovic et al., 2002). Other Th2 theories have been put forward such as reduced TLR triggering caused by helminth products or tissue factors that are not seen *in vitro* (Everts et al., 2010).

1.4. T-regulatory immune response

Successful parasitic infections are related to the down-regulation on Th2 immune responses. A T_{reg} response is commonly seen during chronic infection and is responsible for suppressing the host immune response once the infection is established. Host-helminth interactions can also lead to the induction and expansion of T_{reg} cells that are characterised by TGF-β and IL-10 production. Although the molecular mechanisms of how T_{reg} responses are primed have not yet been elucidated (Everts et al., 2010). This knowledge is lacking despite studies showing that helminth-derived components can drive a T_{reg} response (Van der Kleij et al., 2002; Segura et al., 2007). The presence of apoptotic cells during infection have also been linked with producing a T_{reg} response (Steinman et al., 2000).

The beneficial T_{reg} response that the host uses to dampen the immune response can also be beneficial for the parasite. For a parasite, a T_{reg} response can dampen the Th2 response, which is associated with parasite expulsion. For the host, an overzealous Th2 response during persistent parasite infection can lead to aggravation of pathology in the form of tissue fibrosis. Evidence for this was found in IL-10 deficient helminth mice models where increased mortality and pathology were observed (Bliss et al., 2003; Schopf et al., 2002; Sadler et al., 2003).

1.5. Dendritic cells in helminth infections

Critical to propagating an immune response are a group of antigen presenting cells (APC). The most well defined APC are dendritic cells (DC) that link innate and adaptive immunity by controlling the development of adaptive immune responses. DC sense their environment and when they encounter pathogens they become activated, present antigens on MHC complexes and up-regulate co-stimulatory molecules on their surface (Sher et al., 2003; Banchereau & Steinman, 1998; Wu & Liu, 2007). The activated DC then stimulate naïve T-cells to differentiate and proliferate (Banchereau & Steinman, 1998; Banchereau et al., 2000). Cytokine mixtures are released by activated DC which produces an appropriate T-cell mediated immune response dependent on the initial stimulant.

When the initial stimulant is an intracellular pathogen, virus, tumour or bacteria DC can drive a Th1 response. This is mediated in many cases by bioactive IL-12p70, released by DC, and by the up-regulation of the co-stimulatory molecules CD80, CD86 and

CD40. These Th1 inducing DC are commonly known as "classically activated" DC. When DC are exposed to helminth products they only display partial maturation (Balic et al., 2004; Kane et al., 2004; Hamilton et al., 2009). This partial maturation refers to the low expression of the co-stimulatory receptors, MHC molecules and muted secretion of both cytokines and chemokines. While these helminth-activated DC are not as phenotypically mature they do still possess the ability to drive a Th2/T_{reg} response (Balic et al., 2004).

Helminth studies have largely focused on using heterogeneous mixtures of soluble preparations from whole or partial parasites. The most studied helminth product on DC is schistosome soluble egg antigens (SEA). DC pulsed with SEA antigens do not conventionally mature; this is seen by a lack of co-stimulatory marker expression and low cytokine secretion (MacDonald et al., 2001). However, SEA has been shown to drive Th2 responses (Vella & Pearce, 1992). This Th2 response can be mimicked by isolated glycans from SEA (Faveeuw et al., 2003). SEA suppresses IL-12 production and co-stimulatory marker up-regulation in response to TLR activation. It also has the ability to suppress gene expression linked to LPS activation in DC (Kane et al., 2004). Lipids from *S. mansoni* have also been shown to promote Th2 responses by suppressing IL-12 and inducing T_{reg} cells through TLR2 (Van der Kleij et al., 2002). Conversely, SEA has been shown to produce Th2 responses independently of TLR2 or TLR4 suggesting TLR are not involved (Kane et al., 2008).

The nematode *Nippostrongylus brasiliensis* excretory/secretory antigens cause partial maturation of DC with up-regulation of CD86 and OX40L. These DC can prime a Th2 response (Balic et al., 2004). Excretory/secretory antigens from another nematode *Heligmosomoides polygrus* failed to induce DC maturation, suppressing both Th1 and Th2 responses and promoted a T_{reg} phenotype (Segura et al., 2007). Also two *T. spiralis* preparations were shown to partially up-regulate DC co-stimulatory molecules and induce a Th2 response (Ilic et al., 2008).

Rather than soluble mixes of antigens, individual antigens from parasite preparations have also been examined, with ES-62 from the nematode *Acanthocheilonema vitae* being the most widely studied. ES-62 contains phosphorylcholine (PC) which is a structural component implicated in many immunological processes (Harnett &Harnett, 1999). ES-62 exposed DC increased CD40, CD80 and CD86 co-stimulatory molecules and induced a low level increase in the expression of IL-12p40 and TNF-α production. While ES-62 also increased TLR-4 expression, it inhibited IL-12 and TNF-α production induced by TLR ligation (Goodridge et al., 2005). ES-62 does induce a strong Th2 response and is not sufficient to skew the Th1 associated *T. gondii* infection to Th2 (Couper et al., 2005). Other examples of helminth antigens examined include both Neoglycoconjugate lacto-N-fucopentaose III derived from *S. mansoni* and purified antigens from *Echinococcus granulosus* that partially activate DCs and can produce a Th2 response (Thomas et al., 2005; Riganò et al., 2007). While most studies seem to confirm the partial activation of DC, there are slight variations as would be expected due to the vast variety of helminth species and molecules involved.

1.6. Macrophages in helminth infections

Macrophages are immune cells that have a great capacity to recognise and phagocytose invading pathogens along with acting as antigen presenting cells. Macrophages can also act as effector cells by directly eliminating pathogens. The secretion/presence of the Th2 associated IL-4 and IL-13 cytokines evokes macrophages to become alternatively activated (M2) and these cells are now recognised as a feature of Th2 responses associated with helminth infections (Zhang et al., 1997; Paul, 1991). However, helminth infections do not always result in M2, which are defined by their activation by IL-4/IL-13 (Gordon & Martinez, 2010). Macrophages can share phenotype characteristics of M2 without being induced by IL-4/IL-13 but they can be difficult to define as macrophages may retain a plasticity of function and phenotype depending on the environment and local stimulants at a given time (Cassetta et al., 2011).

Arginase out-competes inducible nitric oxide synthase (iNOS) for their common catalyst L-arginine, resulting in the production of urea and L-ornithine. This ultimately leads to the production of proline and polyamines which contribute to tissue repair and fibrosis (Witte & Barbul, 2003). Other makers distinguish M2 populations, such as IL-4Rα and mannose receptor cell surface up-regulation. Proteins from chitinase and FIZZ family members such as RELM α and Ym 1/2 are also nearly universally expressed by M2 (Nair et al., 2005). RELM α is a family member of cysteine-rich molecules related to a resistin which is involved in glucose metabolism (Steppan et al., 2001). Ym 1/2 while having no chitinolytic activity is a member of the chitinase family (Sutherland et al., 2009).

The presence of macrophages that display alternative activation markers can be seen throughout all classes of helminth infection. For example, peritoneal macrophages isolated by adherence from a mouse model of filariasis, where *B. malayi* adults were intraperitoneally introduced, displayed Arg 1 gene expression. When they were co-cultured with antigen-specific or naïve T-cells, an inhibited proliferation response was observed (MacDonald et al., 1998). The alternative activation and suppressive function was shown to be IL-4 dependent and IL-10 independent (Loke et al., 2000). Further studies using this model revealed RELM α and Ym 1/2 gene expression up-regulation (Loke et al., 2002). Experiments using another filarial model of *L. sigmodontis* resulted in very similar findings as in that of *B. malayi*. Macrophage suppressive activity was seen along with the expression of the Arg 1, RELM α and Ym 1/2 genes in macrophages found at the sites of parasite migration and in the pleural cavity (Nair et al., 2003; Nair et al., 2005).

Similarly, *N. brasiliensis* which causes lung fibrosis, produces M2 in the lungs as suggested by the presence of Arg 1, RELM α and Ym 1/2 (Reece et al., 2006). These M2 were seen in both wild-type (WT) and also severe combined immunodeficiency (SCID) mice. They were induced as early as day 2 post-infection in both WT and SCID mice and M2 were maintained in the WT mice whereas they decreased over time in the SCID mice, demonstrating that only mice with functioning T-cells could maintain the M2 phenotype which suggested that innate immunity was not sufficient for sustained presence of a Th2 phenotype. Another observation was that WT mice resolved damage and inflammation due to *N. brasiliensis* infection whereas in SCID mice the damage and inflammation persisted (Reece et al., 2006). This study clearly indicated that the

innate immune responses or direct pathogen-cell interactions can induce M2 but that a Th2 response maintains these M2. $STAT6^{-/-}$ mice, in which IL-4 or IL-13 have no effect, fail to expel *N. brasiliensis* and also fail to induce M2 (Sakamoto et al., 2004; Reece et al., 2008). Arginase was shown to have a protective role in *H. polygyrus* infection. During the infection arginase was chemically depleted and as a result a higher larval load was recovered when compared to untreated mice (Anthony et al., 2006).

The cestode *Taenia crassiceps* produces a Th2 response after a period of a mixed Th1/Th2 response (Toenjes et al., 1999). During acute infections, macrophages produce high levels of IL-12 and NO with low levels of IL-6 and PGE2. They also possess the ability to induce strong antigen specific $CD4^{+}$ T-cells. During chronic infections, the opposite is seen; macrophages produce higher levels of IL-6 and PGE2, suppress production of IL-12 and NO while failing to induce T-cell proliferation (Rodriguez-Sosa et al., 2002). These alternative chronic stage macrophages were however able to induce IL-4 producing T-cells. $STAT6^{-/-}$ proved essential for the expansion of these M2 macrophages by use of KO mice. It has also been indicated that PD-L1 and PD-L2 have a role in the suppressive activity of M2 during *T. crassiceps* infection (Terrazas et al., 2005). Interestingly, glycoproteins isolated from *T. crassiceps* also display some M2 induction properties (Gómez-García et al., 2006). In the trematode infection *S. mansoni* M2 are found in the liver granulomas and have been shown to have a suppressive function on T-cells. This suppression involves PD-L1 up-regulation on macrophages which interact with PD-1 on T-cells (Smith et al., 2004). Mice lacking IL-4Rα on their macrophages within a Th2 environment fail to recruit M2 and suffer acute schistosomiasis (Herbert et al., 2004). It is clear from the above studies that M2

macrophages have important roles in controlling helminth infection and are a worthy topic for study.

1.7. *Fasciola hepatica*

Fasciolosis is a disease caused by the trematodes *Fasciola hepatica* and *gigantica*. The disease has been designated as one of the neglected tropical diseases by the World Health Organisation (WHO, 2008). Human fasciolosis is a major food-borne zoonosis found through-out the world with no continent being free from infection. The risk of human cases is increased in regions where people and animals live in close proximity such as Bolivia, Peru, Iran, Cuba and Egypt (Moghaddam et al., 2004; Mas-Coma et al., 1999). The lack of sanitary conditions in developing countries has seen fasciolosis become particularly prevalent (Sierra et al., 2011). There is an estimated 2.4 million people infected world-wide each year with 180 million people thought to be at risk (Rim et al., 1994; Mas-Coma et al., 2005).

Fasciola hepatica, commonly known as liver fluke, is the more prevalent parasite when compared to *Fasciola gigantica*. *F. hepatica* inhabits temperate climates as opposed to the tropically located *F. gigantica*. Adult flukes, which typically measure 20-30mm by 13mm wide, reside in the bile ducts of its mammalian host (Dalton, 1999). Within the bile ducts individual flukes can release up to 50,000 eggs per day (Moxon et al., 2010). Hosts can regularly be infected with 50 or more flukes so the number of eggs released from an individual infected host can reach millions per day. Pathogenesis differs depending on the stage of disease. The disease occurs in two stages; the first when the

flukes are migrating through the liver tissue and secondly when they enter and reside in the bile ducts. Pathogenesis also depends on the host and fluke burden, where the smaller the host and larger fluke population the more detrimental effect on the hosts health (Mas-Coma et al., 2005). Symptoms are characterised by weight loss, anaemia and hypoproteinemia, reduction in body weight, milk and wool production which contributes to the loss in productivity (Dalton, 1999). Coupled with this is the cost of drugs to treat fasciolosis, as well as increased susceptibility to secondary bacterial infection. These examples deem *Fasciola* infection a great burden in the agricultural sector. In humans the severity of infection can vary from being asymptomatic to a severe and debilitating disease, if left untreated. Fever and abdominal pain are usually experienced.

The life cycle of *F. hepatica* is complex and requires a mollusc intermediate host (Figure 1.1.). Eggs are released from adult flukes which are then carried out along with its host's faeces through the intestine into the outside environment. When they contact water, free-swimming miracidia emerge from the eggs. The miracidia then seek out *Fasciola's* intermediate host which are predominantly freshwater snail species of the family Lymnaeidae (Gastropoda: Basommatophora) (Dalton, 1999). Upon finding a snail, the miracidia will penetrate the tissue and reach the digestive gland where it undergoes a series of developments from sporocysts to rediae to the eventual cercariae. This results in the production of thousands of free-swimming cercariae that escape the snail. The cercariae search for and encyst on water vegetation and are now called metacercariae. The metacercariae wait to be ingested by passing grazing mammals. Once in the mammalian host, they pass through the digestive tract until they reach the

intestine. Here they excyst and the juvenile flukes that emerge penetrate the host intestinal wall. Once they have burrowed through the intestinal wall the juvenile flukes enter the peritoneal cavity. The flukes then migrate along the peritoneal wall until they reach liver tissue. They enter into the liver tissue and migrate for several weeks until they reach the bile ducts. Once in the bile ducts they mature into full adult flukes where they feed on blood and begin to release eggs.

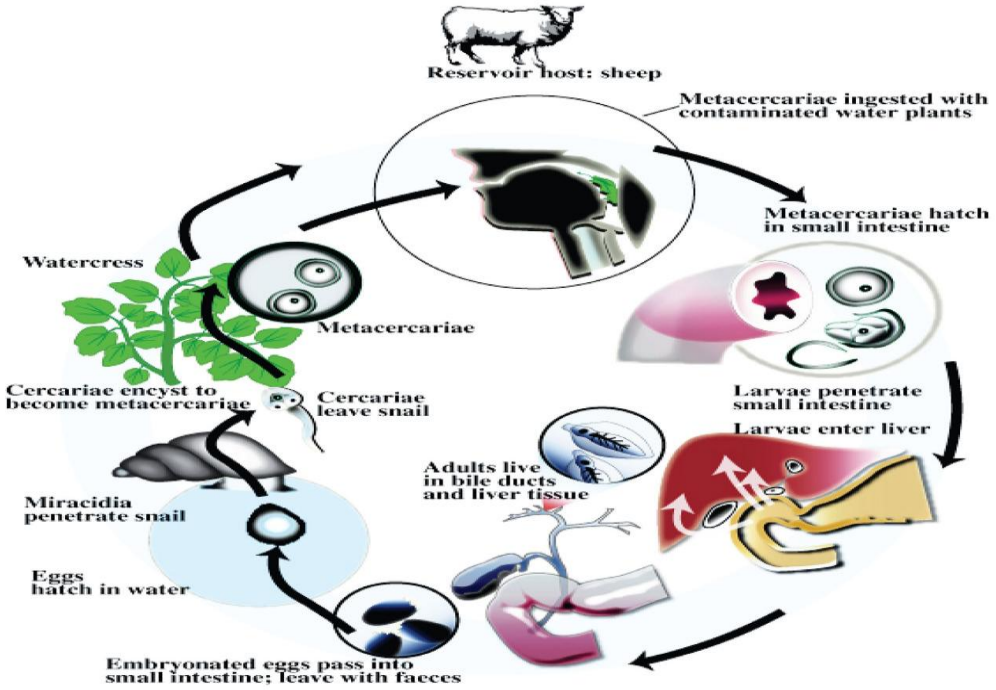

Figure 1.1. Life cycle of *Fasciola hepatica*. Image obtained from the Institute of Tropical Medicine (ITM), Antwerp, Belgium.

1.8. The immune response to *F. hepatica*

F. hepatica can live in its definitive hosts, such as cattle and sheep, for many years (Dalton, 1999). This feat is due to the fluke's ability to modulate the host's immune response for its own benefit. It does this by creating an immunological environment that

allows its migration from the intestine to the bile ducts. It has been shown in cattle, sheep and mouse models that infection with *F. hepatica* suppressed Th1/Th17 responses while promoting Th2/T_{reg} responses (Clery et al., 1998; Donnelly et al., 2005; Donnelly et al., 2008; Mulcahy et al., 1999; Walsh et al., 2009). This is characterised by production and presence of interleukin (IL)-4, IL-5, IL-10 and TGF-β and absence of IFN-γ and IL-2 (Donnelly et al., 2005; Donnelly et al., 2008). Th2 immune responses are non-protective for the host as it suppresses protective Th1 responses (Moreau & Chauvin, 2010). T_{reg} cells, that are also induced, limit the magnitude of the Th2 response, reducing its potential to produce excessive fibrotic damage. *F. hepatica* does this by manipulating the host's immune system through the molecules it secretes.

Hosts that are infected with *F. hepatica* are susceptible to secondary infections as their immune system has been compromised. A Th1 response has been shown to be protective against bystander infections such as tuberculosis and *Bordetella pertussis* infection in the context of *F. hepatica* infection (Flynn et al., 2009; Brady et al., 1999; Claridge et al., 2012). Mice which are co-infected with *F. hepatica* and *Bordetella pertussis* have a reduced ability to clear the bacterial infection. Mice infected with *F. hepatica* also reduced vaccine efficacy when they were immunized against whooping cough (caused by *Bordetella pertussis*) by suppressing IFN-γ production (Brady et al., 1999; Dalton, 1999; O'Neill et al., 2001). It has also been demonstrated that suppression of Th1/Th17 immune responses by FhES can attenuate experimental autoimmune encephalomyelitis (EAE) (Walsh et al., 2009).

During infection, M2 are produced in the peritoneum where Arg 1, RELM α and Ym 1/2 gene up-regulation is observed (Donnelly et al., 2005). This activation was also mimicked *in vivo* and *in vitro* by injecting protein fractions containing peroxiredoxin from *F. hepatica* excretory/secretory products (Donnelly et al., 2005; Donnelly et al., 2008). The stimulated macrophages also secrete PGE2, IL-10, and TGF-β which suggests a possible suppressive effect although this has not been shown. Peroxiredoxin can also activate macrophages independently from IL-4 or IL-13 as seen from the use of knockout mice. However in IL-4 deficient mice, IL-13 may have been causing activation and vice versa in IL-13 deficient mice (Donnelly et al., 2008). This possible redundancy needs to be addressed with either IL-Rα deficient ($^{-/-}$) or STAT6$^{-/-}$ mice.

1.9. *F. hepatica's* immunomodulatory properties

F. hepatica is clearly a very successful parasite with its ability to survive within its hosts for years without being eliminated (Dalton, 1999). Its success is due to its manipulation of the host immune system by secreting and shedding a variety of molecules into its environment which can render the host immune system ineffective against the fluke. The main antigen source that the host is exposed to during *F. hepatica* infection is its secretory/excretory products (FhES) that originate in the gut of the parasite and are expelled into its host. The major components of FhES have been studied, these include; cathepsin L1 and L2, peroxiredoxin, helminth defence molecule, leucine aminopeptidase, glutathione s-transferase and fatty acid-binding protein (Robinson et al. 2013).

Cathepsin L cysteine peptidases are secreted in large quantities into *F. hepatica* gut where they are then expelled from the parasite into the host environment. They are secreted by both immature and adult flukes. They are important as they degrade host proteins which help with fluke migration and also serve in degrading host blood and tissue that are used by the fluke as nutrients (Robinson et al., 2011). *F. hepatica* cysteine peptidases have been shown to alter the function of innate immune cells and lead to prevention of the formation of Th1/Th17 cells. This suppression is independent of Th2 cytokine production (Donnelly et al., 2005; Donnelly et al., 2008). Vaccines against Cathepsin L have shown a marked reduction of 55-72% in fluke burden (Golden et al., 2010; Dalton et al., 1996). Antibodies produced after vaccination prevented Cathepsin L from suppressing Th1 response resulting in less fluke surviving (Mulcahy et al., 1999).

Exposure to reactive oxygen species (ROS) can severely damage helminths during infection. Peroxiredoxins are a family of antioxidant enzymes that counteract the effects of ROS. *F. hepatica* expresses peroxiredoxin at different levels during each stage of infection (Robinson et al., 2009). This variation in peroxiredoxin production may be attributed to the differing levels of attack the fluke experiences due to ROS. Inactivation of ROS is not the only function of peroxiredoxin. As previously mentioned, peroxiredoxin has been shown to induce M2 in the peritoneum of mice. This occurred when both a protein fraction of FhES containing peroxiredoxin and a recombinant peroxiredoxin were injected intraperitoneally into mice (Donnelly et al., 2008). The same experiment was also performed in IL-4/IL-13 knockout mice suggesting that peroxiredoxin acts directly to induce M2. An inactive form of peroxiredoxin was also

examined, this induced M2 showing that this M2 phenotype was independent of its antioxidant activity (Donnelly et al., 2008).

A breach of the integrity of the intestinal wall barrier within the peritoneum is characteristic of helminth infections. Following this, immune cells are exposed to LPS from bacterial sources. *F. hepatica* compromises the intestinal wall during the juvenile fluke infection stage yet pro-inflammatory responses are absent. This may be due to the presence of *F. hepatica* host defence molecule (HDM)-1 which is secreted by the fluke. It has been shown that HDM-1 can reduce inflammatory mediator release from macrophages (Robinson et al., 2011). This process may explain the reduced effectiveness of the host immune response to bystander infections, during *F. hepatica* infection (O'Neill et al., 2001).

Glutathione transferases (GST) constitute 4% of the total soluble protein of *F. hepatica*. Proteomic and EST (expressed sequence tag) analysis has uncovered two new classes of GST (Chemale et al., 2006), one of which, Sigma, is closely related to the Schistosome vaccine candidate Sm28 (Capron et al., 2005). A recent paper has shown that a recombinant form of *F. hepatica* Sigma class GST possesses prostaglandin synthase activity and influences activity of host immune cells (LaCourse et al., 2012). This study also assessed the Sigma class GST vaccine potential but no reduction in worm burden was found, however there was a significant reduction in the pathology normally associated with liver fluke infection.

1.10. *F. hepatica's* tegumental coat

The tegumental coat of *F. hepatica* is shed and constantly replenished every 2 to 3hr during infection and so represents a constant source of antigen that directly contacts the host's immune cells. The surface contains a single membrane which is covered by a polyanionic glycocalyx composed of glycoproteins with side chains of oligosaccharides and gangliosides terminating in sialic acids (Threadgold, 1976). The membrane is 15 μm thick and contains mitochondria and secretory vesicles. The tegument has a number of functions such as osmoregulation, absorption of nutrients and secretions of substances (Dalton, 1999). It also acts in a sensory role and confers protection against the host immune response.

It has been shown that complement proteins attach to the surface of *F. hepatica* (Dalton, 1999). The complement cascade can directly kill cells or act as beacons for other immune cells to find the target. This strategy is countered by the fluke as it sheds its glycocalyx coat; thereby discarding the complement proteins that had become attached. These shed products may also act as a decoy for immune complexes (Duffus & Franks, 1980). The surface proteins of tegument and their antigenicity were found to change during *F. hepatica's* development (Bennett & Threadgold, 1973; Dalton & Joyce, 1987). A recent study has explored the tegumental proteome and it found the presence of an inhibitor to the complement pathway along with proteins that have lectin, cubulin and von Willebrand factor domains, among others (Wilson et al., 2011).

Few studies have looked specifically at the immune response of FhTeg. Our group was the first to study the effects of FhTeg on DC maturation and function. Here DC failed to produce a mature phenotype but they were found to be hypo-responsive to a range of TLR ligands, with both decreased production of cytokines and expression of co-stimulatory markers. The suppressive effect is not mediated through the common MAPKs found in the TLR pathway and is independent of MyD88 and TLR4. DC phagocytic function was also investigated; with FhTeg primed DC displaying reduced phagocytic ability. FhTegs effect on DC was not affected by the lack of TLR4 either in TLR4 mutant or knockout mice. FhTeg was found to suppress the transcription factor NF-κBp65, ERK, P38 and JNK (Vukman et al., 2013) along with suppressor of cytokine signalling (SOCS) 3, a negative regulator of the TLR pathway, which could explain the decrease in pro-inflammatory cytokine release. FhTeg maintains DC in an immature state, impairing their function and hence the development of the adaptive immune response (Hamilton et al., 2009; Vukman et al., 2013).

Recent studies by our group, which have focused on mast cells, have shown FhTeg to impair mast cells ability to drive Th1 immune responses by inhibiting the release of key mediators, such as TNF-α, IL-6, IFN-γ, and IL-10. FhTeg also induced the TLR negative regulator SOCS3 in mast cells (Vukman et al., 2013). SOCS3 is a member of a family of molecules that play an important role in auto regulation of pathogen induced inflammatory responses (Yoshimura et al., 2012). ICAM1, which is a molecule that has an important role in mast cell/T-cell crosstalk (Brill et al., 2004), was also shown to have its expression inhibited by FhTeg (Vukman et al., 2013). We also found that FhTeg suppressed LPS-induced NF-kβ and MAPK pathway activation in mast cells

(Vukman et al., 2013). NF-kβ and MAPKs are important signalling molecules that lead to the expression of ICAM1 and the secretion of pro-inflammatory cytokines (Tsang et al., 2005).

Another group completed a study using synthetically produced short peptides that had homologous sequences to that found in FhTeg proteins. They found degranulation of rat peritoneal mast cells by measuring histamine release was caused by these peptides (Trudgett et al., 2003). Another study alluded to the possible increase in arginase activity in rat peritoneal macrophages due to FhTeg exposure (Haçarız et al., 2011). Our group has found that FhTeg treated mast cells do not produce Th2 cytokines, drive Th2 immune responses or inhibit IgE degranulation of mast cells. FhTeg does however cause increase in mast cell number *in vivo* and also inhibits their ability to drive protective Th1 immune responses by suppressing cytokine secretion. The increase in mast cell numbers occurs in a STAT6 independent manner demonstrating that a Th2 environment is not required for mast cell activation during infection (Vukman et al., 2013).

1.11. Study Objectives

The overall objective of the study was to further understand the immune response to FhTeg. A main goal of the study was to determine the effect that FhTeg had on macrophages, their phenotype and function. This is crucial to understand as macrophages are vital immune cells and there has been little reported work on macrophages in relation to FhTeg exposure. Investigating innate and adaptive immune

responses both *in vitro* and *in vitro* was also a key objective along with studying C-type lectin receptor expression patterns in various immune cells post FhTeg exposure to try build a more complete picture of how FhTeg interacts with the immune system.

The project aimed to complete this objective through:

1. Determining if FhTeg can drive Th2/T_{reg} immune responses.
2. Examining macrophage phenotype and function following exposure to *F. hepatica* tegument.
3. Examining the role of $STAT6^{-/-}$ in *F. hepatica* infection and *F. hepatica* tegument treatment in mice.
4. Examining C-type lectins receptor expression on macrophages during *F. hepatica* infection and after exposure to *F. hepatica* tegumental antigens.

Chapter 2

Materials and Methods

2.1. Animals and Cell Lines

PRODUCT	COMPANY
BALB/c mice (female)	Harlan UK Ltd (Oxfordshire,UK)/Charles River (Kent, UK)
C57BL/6 mice (female)	Harlan UK Ltd (Oxfordshire,UK)/Charles River (Kent, UK)
$STAT6^{-/-}$ mice (female)	Charles River (Ireland)
Adult *Fasciola hepatica*	Abattoir (Ballyjamesduff, Ireland)
F. hepatica metacercariae	Baldwin Aquatics Ltd (USA)
RAW 264.7 macrophages	LGC Standards (Middlesex, UK)

2.2. Cell Culture

PRODUCT	CATALOGUE #	COMPANY
Fetal Calf Serum (FCS)	10270-106	Gibco, Invitrogen (Paisley, UK)
L-Glutamine	G7513	Sigma-Aldrich (Wicklow, Ireland)
Phosphate Buffer Saline	14190	Gibco, Invitrogen (Paisley, UK)
Penicillin/Streptomycin	1570-063	Gibco, Invitrogen (Paisley, UK)
RPMI 1640	31870-074	Invitrogen (Paisley, UK)
Trypan blue	T8154	Sigma-Aldrich (Wicklow, Ireland)
X VIVO-15	BE04-48Q	Lonza (Walkersville, USA)
DMEM	12491-015	Gibco, Invitrogen (Paisley, UK)

2.3. Stimulants

PRODUCT	CATALOGUE	COMPANY
Lipopolysaccharide (LPS) (E. coli)	ALX-581-007	Alexis Biochemicals (Lausanne, Switzerland)
Phorbalmyristate acetate (PMA)	P8139	Sigma-Aldrich (Wicklow, Ireland)
anti-CD3	16-0031-86	eBioscience (Hatfield, UK)

2.4. Commercial Kits

PRODUCT	CATALOGUE	COMPANY
Annexin V-FITC apoptosis detection kit	556547	BD Biosciences (Oxford, UK)
BCA protein kit	23288, 23224	Promega (Madison, USA)
$CD4^+$ Isolation kit	130-095-248	Milltenyi Biotech (UK)
DNAse kit	18068-015	Invitrogen (Paisley, UK)
Griess reagent system	G2930	Promega (Madison, USA)
Mouse IFN-γ ELISA Set	555138	BD Biosciences (Oxford, UK)
Mouse IL-10 ELISA Set	555252	BD Biosciences (Oxford, UK)
Mouse IL-4 ELISA Set	555232	BD Biosciences (Oxford, UK)
Mouse IL-5 ELISA Set	555236	BD Biosciences (Oxford, UK)
Mouse IL-13 ELISA Set	88-7137-86	eBioscience (Hatfield, UK)
PCR $Mastermix^+$ DNAse free water	M7505	Promega (Madison, USA)
Pyrogene Recombinant Factor C	50-658U	Lonza (Walkersville, USA)
MycoSensor PCR Assay Kit	302109	Agilent Technologies (Cork, Ireland)
rt-PCR kit	A5001	Promega (Madison, USA)

2.5. Reagents

PRODUCT	CATALOGUE	COMPANY
2-Mercaptoethanol	63689	Sigma-Aldrich (Wicklow, Ireland)
2-Propanol	24137	Sigma-Aldrich (Wicklow, Ireland)
3,3′,5,5′-Tetramethylbenzidine dihydrochloride hydrate	T8768	Sigma-Aldrich (Wicklow, Ireland)
Agarose	BIO-4125	Bioline (London, UK)
Buffer solution pH 10.0 (20 °C)	33668	Sigma-Aldrich (Wicklow, Ireland)
Buffer solution pH 4.0 (20 °C)	33665	Sigma-Aldrich (Wicklow, Ireland)
Buffer solution pH 7.0 (20 °C)	33666	Sigma-Aldrich (Wicklow, Ireland)
Calcium chloride	383147	Sigma-Aldrich (Wicklow, Ireland)
Chloroform	3505	Fisher Scientific (Dublin, Ireland)
Coverslips	MLS17-20	Lennox Ltd (Dublin 12, Ireland)
Dimethyl sulfoxide	D2650	Sigma-Aldrich (Wicklow, Ireland)
Ethanol	E7023	Sigma-Aldrich (Wicklow, Ireland)
Ethylenediamine-tetraacetic acid (EDTA)	E9884	Sigma-Aldrich (Wicklow, Ireland)
FACS tubes	352054	Unitech/BD (Dublin 24, Ireland)
FACS clean	340345	BD Biosciences (Oxford, UK)
FACS flow sheath	342003	BD Biosciences (Oxford, UK)
FACS rinse	340346	BD Biosciences (Oxford, UK)
Formaldehyde solution	F8775	Sigma-Aldrich (Wicklow, Ireland)
Glycine	G/0800	Fisher Scientific (Dublin, Ireland)
Hydrochloric acid	H1758	Sigma-Aldrich (Wicklow, Ireland)
HyperLadder IV	BIO33029	Bioline (London, UK)

HEPES	H3375	Sigma-Aldrich (Wicklow, Ireland)
Immobilon Western Chemiluminescent HRP Substrate	WBKLS0100	Millipore (MA, USA)
Magnesium chloride	M8266	Sigma-Aldrich (Wicklow, Ireland)
Methanol	65543	Sigma-Aldrich (Wicklow, Ireland)
N,N,N′,N′-Tetramethylethylenediamine	T9281	Sigma-Aldrich (Wicklow, Ireland)
Nonidet™ P 40 Substitute	74385	Sigma-Aldrich (Wicklow, Ireland)
Paraffin wax	107250	Merck (Darmstadt, Germany)
Phosphate-Citrate Buffer with Sodium Perborate	P4922	Sigma-Aldrich (Wicklow, Ireland)
Protease inhibitor Cocktail	P8340	Sigma-Aldrich (Wicklow, Ireland)
Sodium azide	13412	Sigma-Aldrich (Wicklow, Ireland)
Sodium carbonate	S7795	Sigma-Aldrich (Wicklow, Ireland)
Sodium Chloride	S/3160	Fisher Scientific (Dublin, Ireland)
Sodium hydroxide	S5881	Sigma-Aldrich (Wicklow, Ireland)
Sodium phosphate	S8282	Sigma-Aldrich (Wicklow, Ireland)
Sulfuric acid	435589	Sigma-Aldrich (Wicklow, Ireland)
SYBRSafe DNA gel stain	S33102	Invitrogen (Paisley, UK)
TEMED	T9281	Sigma-Aldrich (Wicklow, Ireland)
TMB Substrate Reagent Set	421101	Biolegend (San Diego, USA)
Trizma base	93352	Sigma-Aldrich (Wicklow, Ireland)
Tri-sure	BIO-38033	Bioline (London, UK)
Triton X-100	BDH306324	VWR (East Grinstead, UK)
Tween 20	P1379	Sigma-Aldrich (Wicklow, Ireland)

2.6. Flow cytometry antibodies

PRODUCT	CATALOGUE #	COMPANY
anti-CD16/CD32 (Fcγ III/II Receptor)	101301	Biolegend (San Diego, USA)
CD4 (FITC)	550280	BD Biosciences (Oxford, UK)
CD11c	117310	Biolegend (San Diego, USA)
F4/80 (APC)	17-4801-80	eBioscience Ltd (Hatfield, UK)
F4/80 (FITC)	123108	Biolegend (San Diego, USA)
IL-13	50-7133-80	eBioscience Ltd (Hatfield, UK)
MR (FITC)	C068C2	Biolegend (San Diego, USA)
MGL (Alexa Fluor-488)	MCA2392A488T	AbDSerotec (Oxford, UK)
Siglec-f (PE)	552126	BD Biosciences (Oxford, UK)
Hamster IgG	16-4888-81	eBioscience Ltd (Hatfield, UK)
Hamster IgG1	553971	BD Biosciences (Oxford, UK)
Hamster IgG	51-4888	eBioscience Ltd (Hatfield, UK)
Rat IgG1, κ	50-4301	eBioscience Ltd (Hatfield, UK)
Rat IgG2a	17-4321	eBioscience Ltd (Hatfield, UK)
Rat IgG2a, κ	400505	Biolegend (San Diego, USA)
Rat IgG2a	53-4321	eBioscience Ltd (Hatfield, UK)
Rat IgG2b, κ	553989	BD Biosciences (Oxford, UK)
Rat IgG2b	11-4031	eBioscience Ltd (Hatfield, UK)
Rat IgG2a, κ	554688	BD Biosciences (Oxford, UK)

2.7. Equipment

PRODUCT	CATALOGUE	COMPANY
Analogue Stirred Water bath	NE4-22T	VWR (East Grinstead, UK)
Benchtopmicrocentrifuge	4214	MSC Co. Ltd. (Dublin, Ireland)
BIOQUEL Microflow Class II ABS Cabinet	ABS1200F	VWR (East Grinstead, UK)
Block Heater	BBA series	MSC Co. Ltd. (Dublin, Ireland)
Consort nv electrophoresis power supply	AE-6450	Belgium
Compressed carbon dioxide (CO2) industrial	40-VK	BOC Gases Ireland (Dublin, Ireland)
Bio-Instrument		Bunkyo (Japan)
FacsAria 1 flow cytometer		BD Biosciences (Oxford, UK)
G-Box Gel Imaging system		Syngene (Cambridge, UK)
Hemocytometer, Neubauer, Double cell		MSC Co. Ltd. (Dublin, Ireland)
Hotplate Stirrer	AGB1000	Jenway (Stone, UK)
Homogenizer		Janke and Kunkel, Staufen (Germany)
Leica Inverted microscope	DMIL	Leica Microsystems (Wetzlar, Germany)
Mini horizontal electrophoresis unit 658	Z338796	Sigma-Aldrich (Wicklow, Ireland)
Olympus transmitted-reflected light microscope wit BF/DF/DIC/Polarised light	BX60	Olympus (Hamburg, Germany)
Sigma 4K15 Benchtop Refrigerated Centrifuge	10740	Sigma Centrifuges (Merringtn, UK)
Stuart Scientific combined incubator and orbital shaker	S150	MSC Co. Ltd. (Dublin, Ireland)
TECAN GeniosMicroplate Reader		Tecan (Mannedorf, Switzerland)
TECAN Safire2 UV/VIS/IR and fluorescence plate reader		MSC Co. Ltd. (Dublin, Ireland)
Thermo Scientific CO2 Water Jacketed Incubator	Model 3111	MSC Co. Ltd. (Dublin, Ireland)
Vortex mixer	SA8	Stuart (Stone, UK)

West balance	BL120S	Sartons (Goettingen, Germany)

2.8. Software

PRODUCT	COMPANY
Alpha View SA	Cell Bioscience Inc. (St Clara, USA)
BD CellQuest Pro	BD Biosciences (Oxford, UK)
FlowJo	Tree Star (Ashland, USA)
Origin	Origin Lab Corp. (Northampton, USA)

2.9. Animals

BALB/c and C57BL/6 mice, 6-8 weeks old were purchased from Harlan (Oxfordshire, UK) and Charles River (Kent, UK). STAT6$^{-/-}$ (B6.129S2(C)-Stat6tm1Gru/J) mice (breeding pair) were obtained from Jackson Laboratories and bred in house. STAT6$^{-/-}$ mice were developed using a targeting vector containing a neomycin resistance cassette. This was used to replace the region of the endogenous gene that encodes amino acids 505-584. The construct was electroporated into 129S2/SvPas-derived D3 embryonic stem cells. Embryonic stem cells were injected into BALB/c blastocysts. This resulted in chimeric animals which were then bred with BALB/c mice and maintained on that background for an unknown number of generations. The mutated mice were then mated to C57BL/6 for at least 10 generations before arrival at the Jackson Laboratory. Mice were kept under specific pathogen-free conditions at the Bioresource Unit, Faculty of Health and Science, Dublin City University, Ireland. All mice were maintained according to the guidelines of the Irish Department of Children and Health. Ethical

approval for mice experiments was obtained from DCU ethics committee and the Irish Department of Children and Health.

2.10. Antigens

2.10.1. Preparation of *Fasciola hepatica* excretory-secretory products (FhES)

To prepare *F. hepatica* excretory-secretory products, live adult *F. hepatica* worms were obtained from infected cattle freshly slaughter in a local abattoir. The flukes were transferred from the bile ducts into a vessel containing fresh bovine bile for transport to the laboratory. The flukes were washed in warm sterile Dulbecco's Phosphate-Buffered Saline (PBS; pH 7.0-7.3; KCl 2.67mM, KH2PO4 1.47mM, NaCl 137.93mM, Na2HPO4-7H2O 8.07mM). Washed flukes were placed in RPMI supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 2mM L-glutamine and 10% FCS at 37^{o}C and 5% CO_2 for 8hr. Culture medium containing excretory-secretory products (FhES) were stored at -20^{o}C.

2.10.2. Preparation of *F. hepatica* tegumental antigens (FhTeg)

FhTeg was prepared as described previously (Hamilton et al., 2009). 20-25 adult worms were twice washed in sterile PBS and then incubated in 1% NP40/PBS detergent for 30 min at 37^{o}C whilst shaking. NP40 detergent was removed from supernatant using extracti-gel D detergent removing gel (Pierce). The FhTeg extract was then dialysed against sterile PBS overnight in 12-30 ml Slide-A-Lyser dialysis cassette units (MWCO 10,000)

2.10.3. Preparation of *F. hepatica* liver fluke homogenate (FhHom)

Adult liver flukes were obtained from the infected livers of cattle at a local abattoir. The flukes were washed in sterile PBS and homogenized in a homogenizer. The homogenate was centrifuged at 13,000 g for 30 min and the supernatant (FhHom) was stored in PBS.

2.10.4. Determining antigen protein concentration

Protein concentrations of the prepared antigens were measured using a BCA assay kit (Pierce). This method is detergent compatible and quantifies total protein based on bicinchoninic acid (BCA). It relies on the reduction of Cu^{2+} to Cu^{1+} by protein; Cu^{1+} reacts with BCA producing a purple colour and exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 μg/mL).

2.10.5. Endotoxin testing of antigens

All prepared antigens were tested for endotoxin contamination using Pyrogene Recombinant Factor C Endotoxin Detection System (Lonza). It has been demonstrated that endotoxin activates a serine protease catalytic coagulation cascade that results in the gelation of *Limulus* blood. Factor C which is isolated *Limulus polyphemus* is the first component in the cascade. When activated by endotoxin binding, recombinant Factor C acts upon the fluorogenic substrate in the assay mixture to produce a fluorescent signal in proportion to the endotoxin concentration in the sample.

2.11. *F. hepatica* infection model

Metacercariae were obtained from Norman Baldwin (Baldwin Aquatics Ltd, USA). Using a 96 well plate, 20 *F. hepatica* metacercariae were aliquoted in 20 µl of the metacercariae storage water. 20 µl containing the 20 metacercariae were pipetted into the mouth of the mice. The mice were left for 2 weeks for the infection to take hold before sacrificing to examine adaptive immune responses. Livers were checked for signs of fascioliasis to confirm infection, such as tracts caused by the flukes migrating. Peritoneal exudate cells (PEC) and spleen cells were isolated and processed (section 2.13. and 2.14. respectively).

2.12. *F. hepatica* injection model

Mice were injected intraperitoneally (i.p.) with PBS, FhES (20 µg) or FhTeg (10 µg) and culled after 1, 6 and 24hr time points or injected three times per week for three weeks. PEC were then taken from the mice and either analysed by flow cytometry, RNA analysis or used to isolate PEC macrophages. Spleen cells were removed after 21days from initial injection for re-stimulation *in vitro* with PBS, FhTeg (10 µg/ml), FhES (20 µg/ml) or PMA (25 ng/ml)/anti-CD3 (1 µg/ml). After 72hr, supernatants were retained for analysis of cytokine levels (section 2.21.).

2.13. Isolation of Peritoneal Excudate Cells (PEC)

PEC were removed from mice by injecting 10ml of PBS into the peritoneal cavity and aspirating. The cells were centrifuged at 300g for 5mins and either used processed for

flow cytometric analysis or for macrophage isolation. To isolate macrophages, PEC were plated in 4ml of 10% FCS supplemented RPMI for 2hr, then the non-adherent cells were aspirated and the adherent cells washed. Adherent cells represented our macrophage population which were counted by trypan blue staining and were >90% $F4/80^+$ as checked by flow cytometry analysis (section 2.20.).

2.14. Isolation of spleen cells

Spleens were removed from mice using sterile forceps and scissors. They were then put into wash medium (DMEM with 30 mM HEPES and Pen/Strep) in sterile tubes on ice. The spleens were mashed in a 70 µM cell strainer using wash media. The cells were then counted and the required volume of cells was removed and spun at 300g for 5min and resuspended in X-Vivo-15 media supplemented with 1 ml per 100 ml of L-Glu, and 100 µl per 100 ml 2-Me.

2.15. Cell culturing

2.15.1. Culturing of *in vitro* macrophages

The macrophage cell line RAW 264.7 (ATCC) was originally established from a tumour induced by Abelson murine leukemia virus. The cells are from a BALB/c mouse origin and grown in a monolayer. They were cultured in RPMI media supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 2mM L-glutamine and 10% FCS in treated 75 cm^2 flasks. Media was replenished every 2-3 days. Sub-culturing was performed by scraping adherent cells and sub-cultivating at a 1:5 dilution in fresh

media. Cells were stimulated with media, FhES (20 µg/ml), FhTeg (10 µg/ml), and LPS (100 ng/ml) for 18hr.

2.15.2. Culturing of bone marrow-derived dendritic cells (BDMC)

BDMC were generated from bone marrow of BALB/c mice using a modified method as previously described (Lutz et al., 1999). In brief, bone-marrow was removed from the femurs and tibias of mice and cultured for 10 days in media containing 20 ng/ml of GM-CSF obtained from a GM-CSF producing cell line X63 (a gift from Prof. Ton Rolink; University of Basel, Switzerland). The cells were fed on days 3, 6 and 8 by adding 10 ml of fresh media containing 20 ng/ml GM-CSF; with removal of 9ml media on day 6 and 8. On day 10 loosely adherent cells were harvested, counted and re-suspended at $1X10^6$/ml ready for antigen stimulation. DC purity was >90% positive for the expression of CD11c as shown by flow cytometry analysis (section 2.20.).

2.15.3. Testing for Mycoplasma

Mycoplasma was tested for by using a commercial PCR kit (Agilent Technologies). In brief, 100 µl of supernatant from the cell culture is boiled for 5 min in a microcentrifuge tube. The tube is briefly spun in a microcentrifuge and resuspended in 10 µl of StrataClean resin to the supernatant. The tube is again spun and the supernatant taken and used as a template. A PCR master mix is made, containing PCR primers for mycoplasma. 5 µl of a positive and negative control along with our test template are added to their respective tubes along with 45 µl of the PCR mastermix. The samples were subjected to the following amplification conditions of 35 cycles at 94°C for 30

seconds, 55°C for 1 mi and 72°C for 1 min. PCR products were electrophorised on 1% agarose gels with SYBRSafe (Invitrogen) as our gel stain. A 315bp product signified the presence of mycoplasma in the cell culture.

2.16. Adoptive cell transfer of BMDC

BMDC were stimulated with Med, FhES (20 µg/ml) or FhTeg (10 µg/ml) for 18 hr and then washed followed by injection intraperitoneally into BALB/c mice ($5X10^5$ cells per mouse). After 7 days PEC macrophage isolation was performed. The adherent macrophages were examined for the markers of alternative (Arg 1, RELM α, Ym 1/2) and classical activation (iNOS) by PCR. β-actin was used as a reference (section 2.19).

2.17. Co-culturing experiments

2.17.1. Co-culturing of $CD4^+$T-cell with peritoneal macrophages

Following stimulation with antigens, peritoneal macrophages were incubated with naïve spleenic $CD4^+$ T-cells at a ratio of 1:4 on a plate pre-coated with 0.5 µg/well of anti-CD3 (e-Bioscience). $CD4^+$ T-cells were isolated using MACS $CD4^+$ T-Cell Isolation Kit (MiltenyiBiotec) and were used if the purity was >95% $CD4^+$ as determined by flow cytometry. Co-cultures were maintained at 37°C and 5% CO_2 for 72 hr when supernatants were taken and IFN-γ, IL-4 and IL-5 were then measured by commercial ELISA (BD Biosciences).

2.17.2. Co-culturing of BMDC with RAW 264.7 macrophages

DC were stimulated with Med, FhES (20 μg/ml) or FhTeg (10 μg/ml) and split into 2 groups, supernatant from the DC (SN) and washed DC alone (W). The washed DC group samples were co-cultured in a 1:1 ratio with RAW 264.7 macrophages and the SN was added 1:1 (v/v) to the media containing the macrophages. Both groups were co-cultured for 24hr. After 24hr the cells were counted and $1X10^6$ cells were used for an arginase activity assay. Total RNA was extracted from the remaining cells.

2.18. Arginase activity assay

$1X10^6$ cells per sample were washed once with 200 μL of PBS. Cells were centrifuged at 300g, resuspended with 100 μL of protein lysis buffer containing 0.1% Triton-X and incubated for 15min at room temperature. 100 μL of 50mM Tris-HCl buffer (pH 7.5) and 10 μL of 100mM MnCl2 was then added to each sample and mixed. 100 μL from each sample was transferred to 1.5ml tube and incubated for 7min at 56°C to activate the arginase enzyme. After incubation, the samples were mixed with 100 μL of 0.5M L-arginine (Sigma, St. Louis, MO) and incubated at 37°C for 60min. Meanwhile urea standards (100 μl) were prepared at concentrations of: 0 μg/ml, 1 μg/ml, 5 μg/ml, 10 μg/ml, 15 μg/ml, 30 μg/ml, 45 μg/ml, and 60 μg/ml. After incubation, the reactions were stopped by adding 800 μl acid mix (7:3:1 of H2O: H3PO4: H2SO4) to the samples and 900 μl acid mix to the standards. 40 μl of α-isonitrosopropiophenone (Sigma) was added to each sample and standard. Samples were vortexed and incubated for 5-30min at 95°C until samples were in range of the standards which turn a purple colour. Samples were then cooled and 200 μl of standards and samples were added in triplicate on a 96-well

micro-plate to determine the optical density at 540 nm. The concentration of urea was determined using the following equation: U of arginase activity per 10^6 cells/min = [x μg urea/(60 μg/μmol)] X [40/incubation time with L-arginine (min)]

2.19. RNA extraction and reverse-transcription PCR

Total RNA was extracted from cultured cells using TRIsure (MyBio) as recommended by the manufacturer. Briefly, cell grown in a monolayer were washed with ice cold PBS followed by the addition of 1 ml of TRIsure reagent per 10 cm^2 dish and scraped with a cell scraper. Cells were homogenized by pippetting and vortexing. The homogenate was incubated for 5 min at RT. 200 μl of chloroform was added per 1 ml of Tri Reagent used and samples vortexed for 15 sec and incubated for 2-3 min at RT followed by centrifugation at 12,000g for 15 min at 4^{o}C. Following centrifugation, the upper aqueous phase (RNA layer) was transferred into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with 500 μl isopropanol per 1 ml Tri reagent used. The sample was again vortexed for 5-15 sec and incubated at RT for 5-10 min followed by centrifugation at 12,000 g for 8 min @ 4-25^{0}C. The supernatant was discarded and the pellet resuspended in 1 ml 75% ethanol per 1 ml Tri regent used. The sample was then centrifuged at 7,500g for 5 min at 2-8^{o}C. Ethanol was removed by pipetting and the pellet was allowed to air dry until excess ethanol had evaporated. The pellet was then solubilized using pre-heated (70^{o}C) nucleus free water (20-50 μl). The tubes were then incubated at 70^{o}C for 10 min and transferred onto ice. RNA levels were measured using a Nanodrop machine.

First strand cDNA was synthesised with random primers from 1 µg total RNA using GoScript Reverse Transcription System (Promega) and then used as a template for PCR using primers specific for Arg 1, Ym 1/2, iNOS, RELM α, IL-4, IL-13 and β-actin (Table 2.1.). Each amplification step was preceded by a denaturation phase at 95^{o}C for 5min and preceded by a final extension phase of 72^{o}C for 5 min. PCR products were electrophorised on 1% agarose gels with SYBRSafe (Invitrogen) as our gel stain.

Table 2.1.

Gene	**Arginase 1**	**Ym 1/2**	**iNOS**	**IL-4**
Sense	ATGGAAGAG ACCTTCAGC TAC	TCACAGGTC TGGCAATTC TTCTG	CCCTTCCGA AGTTTCTGG CAGCAGC	ACGGAGATG GATGTGCCA AACGTC
Anti- sense	GCTGTCTTCC CAAGAGTTG GG	TTTGTCCTTA GGAGGGCTT CCTC	GGCTGTCAG AGAGCCTCG TGGCTTTGG	CGAGTAATC CATTTGCAT GATGC
Denaturation	60 sec at 94^{o}C	60 sec at 95^{o}C	40 sec at 95^{o}C	45 sec at 94^{o}C
Annealing	45 sec at 55^{o}C	5 sec at 63^{o}C	60 sec at 65^{o}C	30 sec at 60^{o}C
Elongation	60 sec at 72^{o}C	12s ec at 72^{o}C	60 sec at 72^{o}C	90 sec at 72^{o}C
Cycle #	30	40	30	35
Gene	**RELM α**	**β-actin**	**IL-13**	
Sense	TCCCAGTGA ATACTGATG AGA	TGGAATCCT GTGGCATCC ATGAAAC	GCCAGCCCA CAGTCTACA GC	
Anti- sense	CCACTCTGG ATCTCCCAA GA	TAAAACGCA GCTCAGTAA CAGTCCG	GTGATGTTG CTCAGCTCCT CA	
Denaturation	60 sec at 94^{o}C	60 sec at 95^{o}C	60 sec at 94^{o}C	
Annealing	45 sec at 55^{o}C	5 sec at 63^{o}C	30 sec at 60^{o}C	
Elongation	60 sec at 72^{o}C	12 sec at 72^{o}C	60 sec at 72^{o}C	
Cycle #	40	40	35	

2.20. Flow cytometry

Monoclonal antibodies with fluorescent tags were used which targeted cell surface markers (Table 2.2.). In brief, cells were counted by trypan blue staining with 100,000 cells added to each sample tube and washed twice with wash buffer (PBS buffer containing 2% FCS, 5mM EDTA and 0.05% NaN_3).Cells were blocked for 15min with anti-CD16/CD32 (Fcγ III/II receptor) prior to incubation with fluorescent antibodies, except in the cases when macrophages were being stained. The cells were again washed twice with wash buffer. Antibody incubations were performed in the wash buffer with the manufacturers recommended dilutions. Isotype controls corresponding to the above antibodies were used to rule out non-specific binding. Unstained cells were used to set up the acquisition settings and gates. Single stained cells were used for compensation of overlapping fluorochromes. Acquisition was performed using a FACSAria I cell sorter (BD biosciences, Oxford), and data was analysed using FlowJo software (Treestar).

Table 2.2.

Antibody	Purpose	Colour	Isotype Control
F4/80	Cell surface pan marker for macrophages	APC	Rat IgG2a
F4/80	Cell surface pan marker for macrophages	FITC	Rat IgG2a, κ
MR	Cell surface marker found on leukocytes	FITC	Rat IgG2b
MGL	Cell surface marker found on leukocytes	Alexa Fluor-488	Rat IgG2a
CD4	Cell surface pan marker for T-helper cells	FITC	IgG2a, κ

Siglec-f	Cell surface pan marker for eosinophils	PE	Rat IgG_2, κ
Cd11c	Cell surface pan marker for dendritic cells	APC	Hamster IgG
IL-13 ebio 50-7133-80	Intracellular stain	efluor 660	Rat IgG1, κ

2.21. Cytokines

Cytokines in cells supernatants were measured using enzyme-linked immunosorbent assays (ELISA) (BD biosciences, Oxford). Assays were carried out by following the manufacturer's instructions. In brief, a monoclonal capture antibody (mAb) specific for the cytokine of interest is added to the wells of a 96 well plate at the recommended concentration and incubated overnight at 4^oC. The following steps were carried out at room temperature. Excess or unbound antibodies are removed by washing with wash buffer (1X PBS with 0.05% Tween-20) 3 times. The plate was blocked for 1hr with 200 µl of assay diluent containing 10% FCS. The plates were then washed 3 times with wash buffer. Samples and standards are made in assay diluent and 100 µl of each added to the wells in triplicate and incubated for 2hr. The plate was washed 5 times in wash buffer and 100 µl working detector (detection Ab + SAv-HRP) added and incubated for 1hr. The plates were then washed and 100 µl of tetramethylbenzidine (TMB) substrate solution added to each well and incubated for 30 min in the dark. 50 µl of stop solution was added to each well and the plates were then read in a Tecan plate reader at 450 nm with λ correction 570 nm. Kits for IFN-γ, IL-4, IL-5, IL-13 and IL-10 were used.

2.22. Statistics

All data were analysed for normality prior to statistical testing by Origin® 6.1 software (OriginLab Corporation). Where multiple group comparisons were made, data were analysed using one-way ANOVA. For comparisons between two groups, the Student's t test was used. In all tests, $p < 0.05$ was deemed significant.

Chapter 3

Results

3.1. Introduction

Central to the Th2 response in helminth infections is the secretion/presence of two structurally and functionally similar cytokines, IL-4/IL-13 (Zhang et al., 1997). They exert various biological effects by regulating proliferation and differentiation of a wide variety of lymphoid and myeloid cells (Paul, 1991). It has been long known that IL-4/IL-13 evoke macrophages to become alternatively activated. However, helminth infections do not always result in M2, which are defined by their activation by IL-4/IL-13 (Gordan, 2010). Macrophages can share phenotype characteristics of M2 without being induced by IL-4/IL-13 but they can be difficult to define as macrophages may retain a plasticity of function and phenotype depending on the environment and local stimulants at a given time (Poli, 2011). They can be induced by stimulation with immune complexes in the presence of Toll-like receptor (TLR) ligands or by anti-inflammatory stimuli such as IL-10, TGF-β and glucocorticoids, rendering them "deactivated" macrophages (Mantovani et al., 2004).

The presence of macrophages that display alternative activation markers can be seen throughout all classes of helminth infection. For example the nematodes *B. malayi* and *L. sigmodontis* induce macrophages expressing Arg 1, RELM α and Ym 1/2 (MacDonald et al., 1998; Loke et al., 2000; Loke et al., 2002; Nair et al., 2003; Nair et al., 2005). Similarly, these macrophages are seen in the lungs during *N. brasiliensis* infection (Reece et al., 2006). The cestode, *T. crassiceps* can elicit a Th1 or Th2 response but only when the Th2 response is prevalent, during times of chronic infection, are the markers induced (Toenjes et al., 1998; Rodríguez-Sosa et al., 2002). Macrophages that have a suppressive function on T-cells are also found in the liver

granulomas during *S. mansoni* infection that anergize T-cells through PD-L1. (Smith et al., 2004).

Macrophages like other immune cells have the ability to recognise specific motifs on pathogens which distinguish them from self-antigens. These motifs are called pathogen-associated molecular patterns (PAMPs). PAMPs are recognised by pattern recognition receptors (PRRs) on immune cells. C-type lectin receptors (CLR) are a family of receptors that recognize specific pathogen-associated carbohydrate structures on glycoproteins present on pathogens, along with self-danger signals released from necrotic cells (Weck et al., 2008; Sancho et al., 2009; Shrimpton et al., 2009). In addition to other forms of pathogens, CLR have been associated with many different helminth infections (Meyer et al., 2005; Guasconi et al., 2011; van Vliet et al., 2005) and these are associated with M2 macrophages. Some of the most important CLR linked with helminth infections are mannose receptor (MR) and macrophage galactose-type lectin (MGL).

The mannose receptor (MR) has long been used as a cell surface marker for M2 (Stein et al., 1992). MR recognises high-mannose-type-structures (Kuijk & Van Die, 2010) but lacks any classical signalling motifs with its signalling mechanism remaining undefined (Vautier et al., 2012). It has been shown to be up-regulated by IL-4 and down regulated by IFN-γ (Harris et al., 1992; Doyle et al., 1994). Evidence of MR binding to helminths is seen in studies involving larvae of *T. spiralis*¸ excretory/secretory products of *T. muris* and the soluble schistosomal egg antigen (SEA) from *S. mansoni* (Gruden-

Movsesijan & Milosavljevic, 2006; deSchoolmeester et al., 2009; Linehan et al., 2003). Excretory/secretory products (E/S) from the larval stage of *S. mansoni* were shown to be internalised by MR. Using $MR^{-/-}$ mice they showed that a Th1 response was mediated after E/S exposure (Paveley et al., 2011). These studies implicate an important role for MR in helminth immunity.

The CLR MGL was also shown to internalise products from *S. Mansoni* (Van Liempt et al., 2007). MGL recognises terminal α- and β-linked GalNAc and also high-mannose-type-structures (Kuijk & Van Die, 2010). These structures are present on many different helminths such as *S. mansoni, F. hepatica, M. corti, T. sprilis*and *T. canis* (Van Die & Cummings, 2010; Casaravilla et al., 2003), but data showing binding of MGL to natural ligands is lacking. Two different forms of β-GalNAc present in SEA from *S. mansoni* were shown to bind to MGL, displaying its potential role in helminth infections (Van Vliet et al., 2005).

During *F. hepatica* infection, macrophages are also seen in liver tracts caused by fluke migration but they are not seen in the immediate area around the fluke (Dalton, 1999). This may suggest these macrophages are more responsible for host tissue repair than parasite killing/expulsion. When examined, macrophages isolated from the peritoneal cavity during *F. hepatica* infection, or following immunisation with FhES, have expressed Arg 1, RELM α and Ym 1/2 (Donnelly et al., 2005) and FhES stimulated macrophages express CLR (Guasconi et al., 2011). We know peroxiredoxin which is a constituent of the flukes FhES products can cause M2 formation both *in vivo* and *in*

vitro in an IL-4/IL-13 independent manner (Donnelly et al., 2008). Both a protein fraction of FhES containing peroxiredoxin and a recombinant *F. hepatica* peroxiredoxin, lacking antioxidant activity, induced M2.

The tegumental coat from *F. hepatica* is in continuous contact with the host, but it has not been previously shown if FhTeg is capable of inducing strong Th2 responses either during infection or following injection of FhTeg into mice. While FhES can produce Th2 responses and induce M2, it is unknown if FhTeg can drive M2 *in vivo* or *in vitro* making it a worthy topic for investigation. As of yet, no study has characterised macrophage phenotype due to FhTeg exposure. This study addresses this lack of knowledge and helps in the understanding of the role FhTeg plays during infection. We also sought in this study to determine if the expression of MR and MGL are altered by FhTeg in macrophages.

3.2. Results

3.2.1. FhTeg does not induce Th2 antigen specific immune responses during *F. hepatica* infection

We have previously shown that *F. hepatica* infected mice induce strong FhES specific Th2 immune response as characterised by the presence of IL-4, IL-5 and IL-13 with no antigen specific IFN-γ detected (Donnelly et al., 2008). Here mice were infected with 20 *F. hepatica* metacercariae and after 2 weeks spleens were then removed and re-stimulated with PBS, FhTeg (10 µg/ml), FhHom (10 µg) and PMA (25 ng/ml)/anti-CD3 (1 µg/ml). FhTeg stimulated spleen cells from infected mice failed to secrete IFN-

γ, IL-4, IL-5 and IL-13 cytokines in response to FhTeg (Figure 3.1.). PBS stimulated spleen cells did not produce cytokines while cells stimulated with PMA/anti-CD3 secreted all cytokines tested. Spleen cells from uninfected mice secreted no cytokines in response to FhTeg (Figure 3.1).

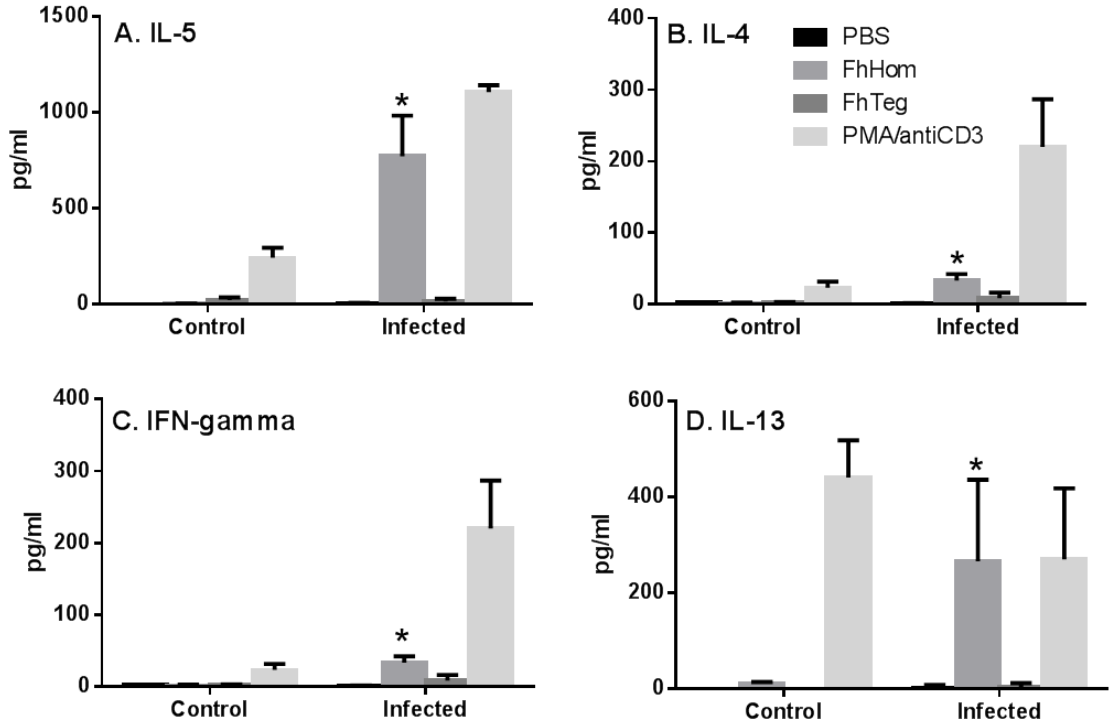

Figure 3.1 FhTeg does not induce Th2 antigen specific immune responses during *F. hepatica* infection. Mice were infected for two weeks with 20 *F. hepatica* metacercariae orally. Spleen cells were removed after a two week infection for re-stimulation *in vitro* with FhTeg (10 µg), FhHom (10 µg) or PMA (25 ng/ml)/anti-CD3 (1 µg/ml). After 72hr, spleen cell supernatants were analysed by ELISA for IFN-γ, IL-4, IL-5 and IL-13. Data, analysed by anova, is the mean (± SEM) of three individual wells

for four individual mice, and are representative of three experiments, *, $p \leq 0.05$; compared with controls.

3.2.2. FhTeg does not induce Th2 antigen specific immune responses in BALB/c treated mice

We have previously shown mice immunised with FhES induce strong FhES specific Th2 immune response as characterised by the presence of IL-4, IL-5 and IL-13 with no antigen specific IFN-γ detected. Using FhES as a control, we injected BALB/c mice with PBS, FhES (20 µg) or FhTeg (10 µg) three times per week for three weeks. Spleens were then removed and re-stimulated with PBS, FhTeg (10 µg/ml), FhES (20 µg/ml) and PMA (25 ng/ml)/anti-CD3 (1 µg/ml). FhES injected mice produced significant levels of IL-4 ($p \leq 0.001$), IL-5 ($p \leq 0.01$) and IL-13 ($p \leq 0.001$) cytokines following stimulation with FhES (Fig. 3.2.), while spleen cells from FhTeg injected mice failed to produce IFN-γ, IL-4, IL-5 and IL-13 cytokines in response to FhTeg. PBS stimulated spleen cells did not produce cytokines while cells stimulated with PMA/anti-CD3 secreted all cytokines tested.

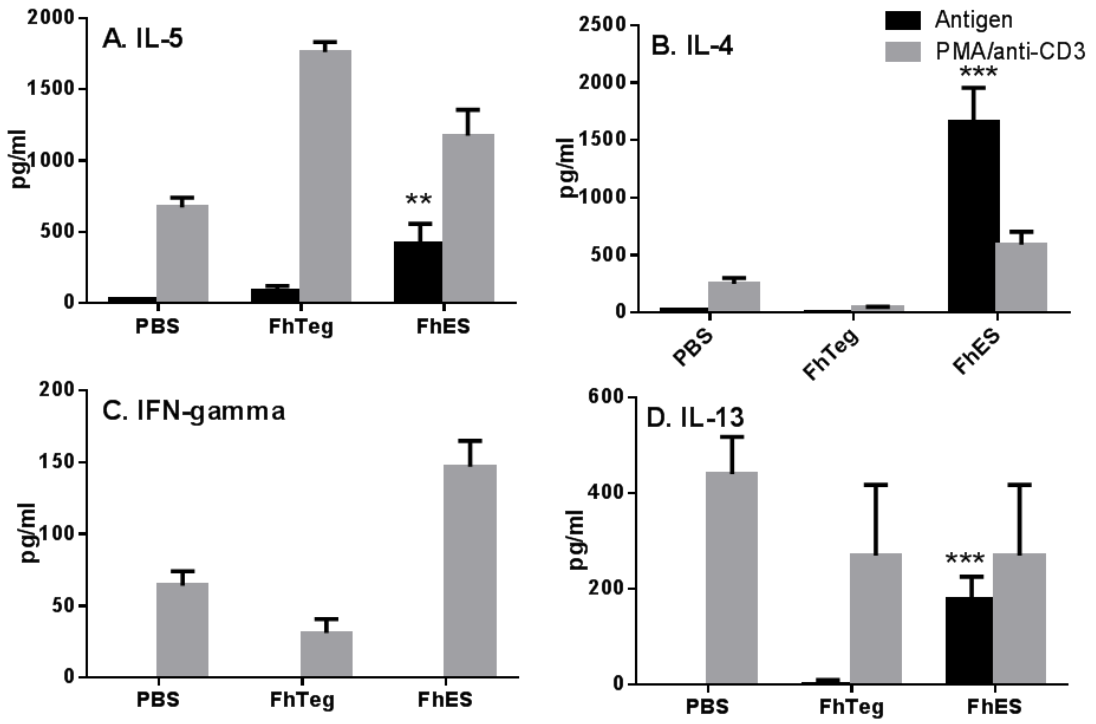

Figure 3.2.FhTeg does not induce Th2 antigen specific immune responses in BALB/c treated mice: Mice were injected three times per week for three weeks with PBS, FhES (20 µg) or FhTeg (10 µg). Spleens cells were removed and plated at 2 X 10^6/ml for re-stimulation *in vitro* with PBS, FhES (20 µg), FhTeg (10 µg) or PMA (25 ng/ml)/anti-CD3 (1 µg/ml). After 72 hr, spleen cell supernatants were analysed by ELISA for IFN-γ, IL-4, IL-5 and IL-13. Data, analysed by anova, is the mean (± SEM) of three individual wells for four individual mice, and are representative of three experiments, **, $p \leq 0.01$; ***, $p \leq 0.001$ compared with controls.

3.2.3. FhTeg induces a phenotype of macrophages that express M2 markers

The lack of FhTeg antigen specific Th2 response *in vivo* suggested that FhTeg would not induce M2 since this phenotype is dependent upon the presence of the IL-4 and IL-

13 cytokines. Mice were injected three times per week for three weeks with PBS, FhES (20 µg) or FhTeg (10 µg). PEC macrophages were isolated, RNA extracted and RT-PCR performed for the most common M2 markers (Arg 1, RELM α and Ym1/2). All markers were expressed by macrophages *ex vivo* from FhES and FhTeg injected mice while neither antigen induced iNOS expression, a marker of classical activation (Fig. 3.3.).

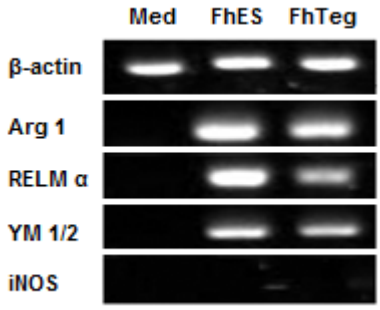

Figure 3.3.FhTeg induces a phenotype of macrophages that express alternative M2 markers: Mice were injected three times per week for three weeks with PBS, FhES (20 µg) or FhTeg (10 µg). PEC macrophages were isolated for measurement of Arg 1, Ym 1/2, RELM α, iNOS and β-actin gene expression by RT-PCR.

3.2.4 FhTeg induced macrophages functionally suppress cytokine secretion from $CD4^+$ T-cells

The ability of FhTeg stimulated macrophages to modulate T-cells has not been previously shown. PEC macrophages were isolated from PBS, FhES and FhTeg injected mice and co-cultured with naïve $CD4^+$ T-cells in the presence of anti-CD3. $CD4^+$ T-cells co-cultured with macrophages in the presence of anti-CD3 produced significant levels of IL-4 ($p \leq 0.001$), IFN-γ ($p \leq 0.001$), IL-13 ($p \leq 0.001$) but not IL-5 when

compared to un-stimulated T-cells. FhES and FhTeg caused significant decrease in the cytokines produced when compared to PBS IFN-γ (Fig 3.4A FhES $p \leq 0.01$, FhTeg $p \leq 0.0001$) and (Fig. 3.4B: IL-4 (FhES $p \leq 0.001$, FhTeg $p \leq 0.0001$) while no significant change in IL-13 was observed. Interestingly FhES ($p \leq 0.01$) but not FhTeg induced a significant increase in IL-5 although there is a trend not supported by statistics that IL-5 is induced by FhTeg. Macrophages stimulated with FhTeg can now be said to display an M2-like phenotype and possess a suppressive function *in vitro*.

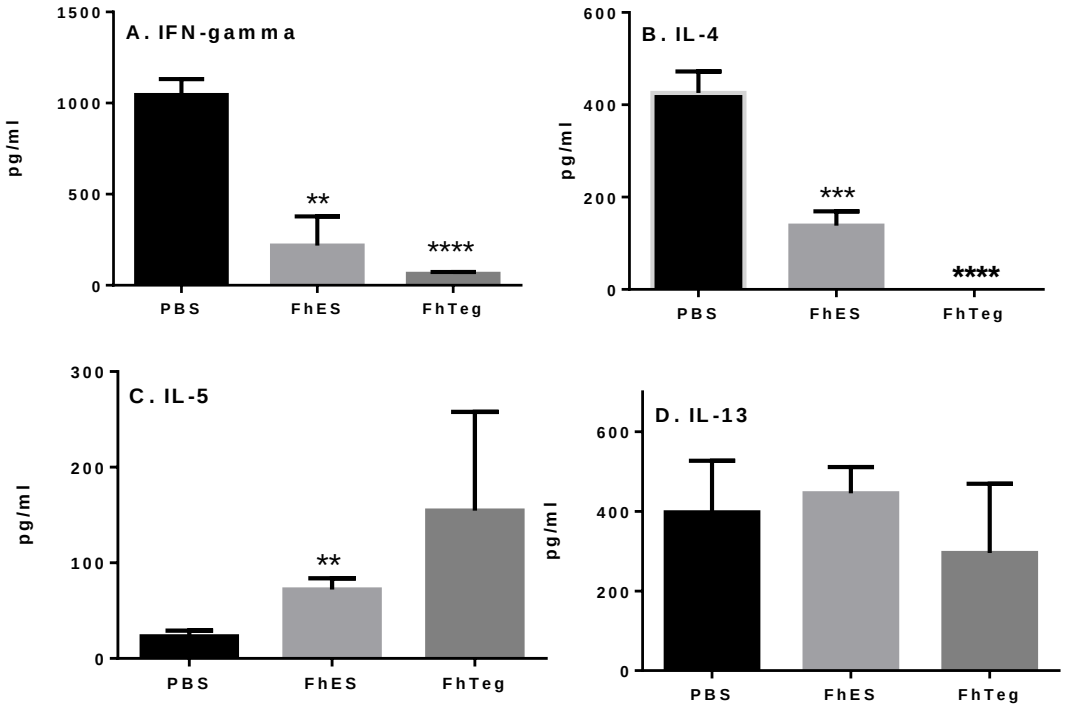

Figure 3.4.FhTeg induced M2-like macrophages functionally suppress cytokine secretion from $CD4^+$ T-cells: Mice were injected three times per week for three weeks with PBS, FhES (20 µg) or FhTeg (10 µg). PEC macrophages were co-cultured with naïve $CD4^+$ T-cells at a ratio of 1:4 on plates pre-coated with anti-CD3 (0.5 µg/well). After 72hr, supernatants were taken and analysed by ELISA for IFN-γ, IL-4, IL-5 and IL-13. Data, analysed by anova, is representative of four mice per group and the

experiment was repeated two times; **, $p \leq 0.01$; ***, $p \leq 0.001$, $p \leq 0.0001$compared with controls.

3.2.5. STAT6$^{-/-}$ mice infected with *F. hepatica* or injected with *Fasciola* antigens produce less arginase and fail to display a strong M2 phenotype

Since FhTeg does not produce antigen specific immune responses we sought to determine if FhTeg can induce M2 in the absence of IL-4/IL-13. Firstly we infected C57BL/6 and STAT6$^{-/-}$ mice with 20 *F. hepatica* metacecariae or administered PBS as a control. A peritoneal lavage was performed after 2 weeks and macrophages were isolated for RNA extraction to measure Arg 1, Ym 1/2, RELM α, iNOS and β-actin gene expression. Levels of arginase activity were also measured. Here we demonstrated that STAT6$^{-/-}$ mice infected with *F. hepatica* fail to display a strong M2 phenotype (Fig 3.5.A) with weak Arg 1, Ym 1/2, RELM α gene expression. This observation was supported by the arginase activity levels from infected STAT6$^{-/-}$ ($p \leq 0.05$) mice producing significantly lower levels of arginase activity than infected C57BL/6 mice (Fig. 3.5.B.).

We then repeated the experiment by injecting mice with PBS, FhES (20 µg) or FhTeg (10 µg) three times per week for three weeks, after which PEC macrophages were isolated for measurement of Arg 1, Ym 1/2, RELM α and β-actin gene expression by RT-PCR. Levels of arginase activity were also measured. STAT6$^{-/-}$ mice failed to induce Arg 1and Ym 1/2 for both FhES and FhTeg injected mice (Fig 3.5.C) indicating that IL-4/IL-13 is essential for FhTeg induced alternative activation. RELM α was

present however in FhTeg injected mice. There was a reduced level of arginase activity in the STAT6$^{-/-}$-FhES ($p \leq 0.05$) and FhTeg ($p \leq 0.001$) samples when compared to control mice (Fig. 3.5.D). Taken together, these show that FhTeg causes an M2 like phenotype in a STAT6 dependent manner.

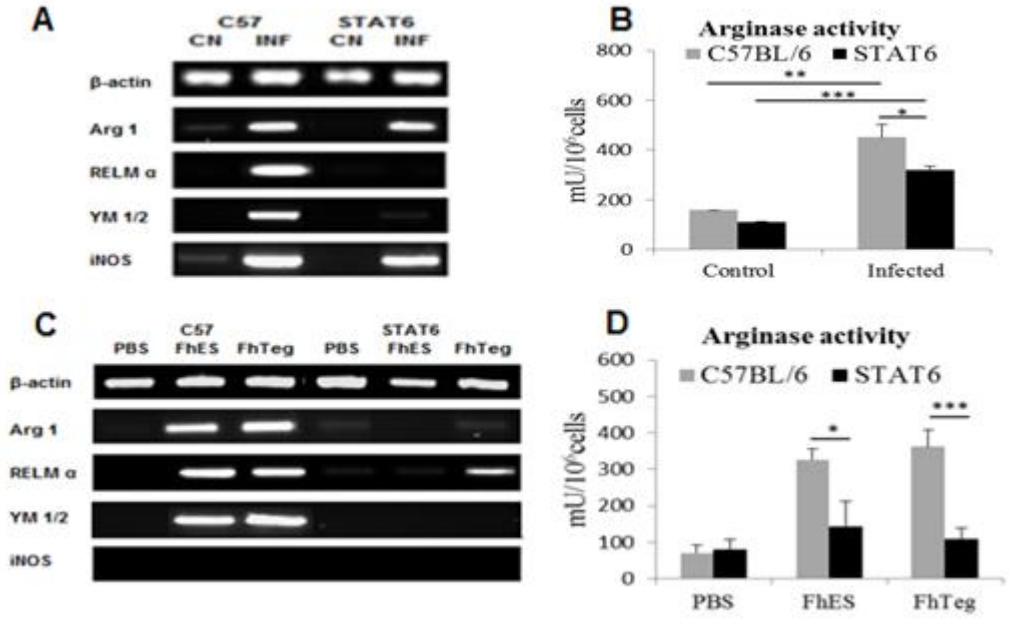

Figure 3.5. STAT6$^{-/-}$ mice infected with *F. hepatica* or injected with *Fasciola* antigens produce less arginase and fail to display M2 phenotype: C57BL/6 (background strain) and STAT6$^{-/-}$ mice were either infected with 20 *F. hepatica* metacecariae or given PBS as a control. After 2 weeks peritoneal macrophages were isolated for measurement of Arg 1, Ym 1/2, RELM α and β-actin gene expression by RT-PCR (A) or for measurement of arginase activity (B). Mice were injected three times per week for three weeks with PBS, FhES (20 µg) or FhTeg (10 µg) and PEC macrophages isolated for measurement of Arg 1, Ym 1/2, RELM α, iNOS and β-actin gene expression (C) or arginase activity (D). Data, analysed by anova, is representative of four mice per group and the experiment was repeated three times, *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ compared with controls.

3.2.6. FhTeg induces IL-13 but not IL-4 at 6 hours post injection

To investigate whether the presence of M2-like macrophages was due to innately expressed IL-4 or IL-13 we used an *in vivo* model where BALB/c mice were i.p. injected with either PBS, FhES (20 µg) or FhTeg (10 µg) and culled after 1, 6 and 24 hr time points. PEC were then analysed for M2 markers along with the presence of IL-4 and IL-13 (Figure 3.6. A). There was no detection of IL-4 from any of the samples by 24 hr. IL-13 was detected at the 6 hr and 24 hr time points which correlate to the increased expression of Arg 1 and RELM α at the same time points. This seems to suggest that IL-13 but not IL-4 is involved in the maintenance of M2-like macrophages. The initiation of M2-like macrophages is seen before IL-13 production. The source of this IL-13 was also investigated by analysing PEC from mice that had undergone three i.p. injections for three weeks. The PEC were then analysed by flow cytometry for intracellular IL-13 in both macrophages and eosinophils. FhTeg injected mice increased the numbers of macrophages and eosinophils, while only macrophages displayed a significantly higher fluorescent reading compared to the PBS injected mice. This indicates that FhTeg induces macrophages to express both IL-13 and M2-like macrophages markers. IL-13 may work in an autocrine manner to keep the macrophages in the same state.

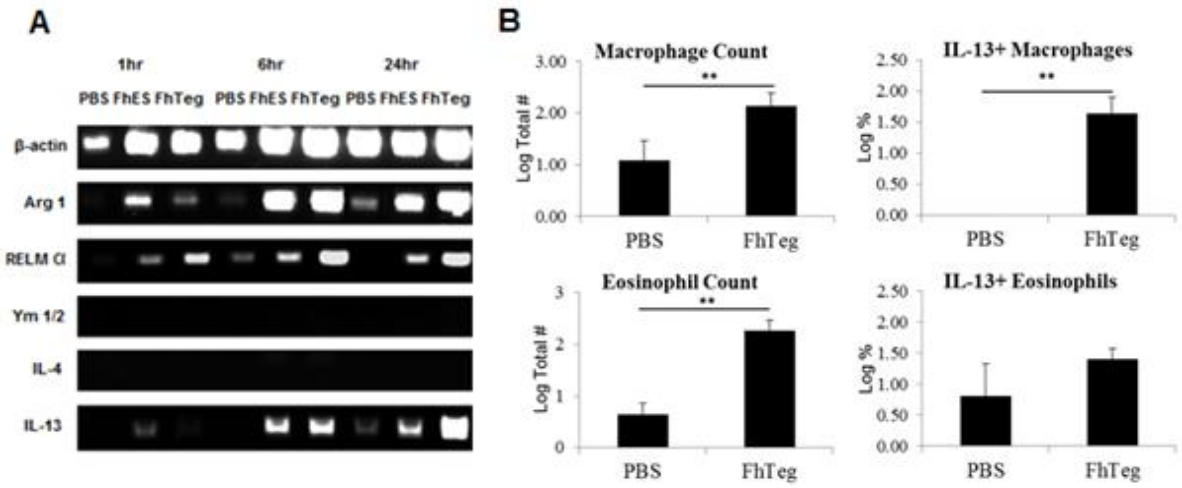

Figure 3.6. FhTeg–treated mice induce IL-13 producing peritoneal macrophages and eosinophils: BALB/c mice were i.p. injected with either PBS, FhES (20 µg) or FhTeg (10 µg) and culled after 1, 6 and 24hr time points. PEC were removed for RNA extraction and RT-PCR was performed for IL-4 and IL-13; along with Arg 1, Ym 1/2, and RELM α with β-actin as a reference gene (A). Data is representative of three mice per group. PEC isolated after three i.p. injections for three weeks were also analysed by flow cytometry for intracellular IL-13 in macrophages and eosinophils (B). The log of the total cell number of macrophages and eosinophils along with the log of the percentage of IL-13^{+} macrophages and eosinophils are shown. Data, analysed by anova, is representative of four mice per group and the experiment was repeated three times; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ compared with controls.

3.2.7. FhTeg does not directly induce M2-like macrophages *in vitro*

Since FhTeg induced alternative activation of macrophages *in vitro* we sought to determine if similar to FhES it could directly induce an M2 like phenotype in the RAW 264.7 macrophage cell line. RAW 264.7 macrophages were stimulated with Med, FhES

(20 µg/ml) or FhTeg (10 µg/ml) and after 18hr, RNA extraction and RT-PCR were performed for the M2 marker Arg 1. Cells were also lysed and arginase activity was measured. Unlike FhES, FhTeg did not induce Arg 1 expression directly in macrophages (Fig. 3.7.A), neither did it cause an up-regulation in arginase production (Fig. 3.7.B), As FhTeg *in vivo* had induced M2 it was expected that this could be repeated *in vitro*, however this was not observed.

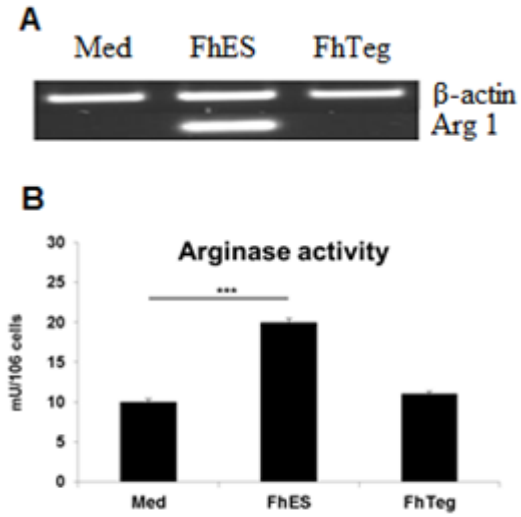

Figure 3.7. FhTeg does not directly induce alternative activation of macrophages *in vitro*: RAW 264.7 macrophages were stimulated with Med, FhES (20 µg/ml) or FhTeg (10 µg/ml) and after 18hr RNA was extracted and RT-PCR was performed for Arg 1 and β-actin gene expression (A). Arginase activity was also measured (B). Data, analysed by anova, is representative of three experiments, ***, $p \leq 0.001$ compared with controls.

3.2.8. FhTeg stimulated dendritic cells can induce M2 like phenotype *in vitro* and *in vivo*

Since we demonstrated that FhTeg does not induce M2 directly, we investigated whether FhTeg exposed DC could indirectly induce M2 features in PEC macrophages. Med, FhES (20 µg/ml) or FhTeg (10 µg/ml) stimulated, washed BMDC were injected intraperitoneally into BALB/c mice. After 7 days PEC macrophages were isolated for measurement of Arg 1, Ym 1/2, RELM α, iNOS and β-actin gene expression by RT-PCR. FhES and FhTeg stimulated PEC macrophages both induced M2 expressing Arg 1, Ym 1/2, RELM α but not iNOS, implying that DC can cause M2 to be produced by either direct or indirect means *in vivo* (Fig. 3.8.A).

This experiment was replicated *in vitro*; Med, FhES (20 µg/ml) or FhTeg (10 µg/ml) stimulated BMDC were either washed (W) or supernatant removed (SN) and cells or supernatant co-cultured with RAW 264.7 macrophages. After 24hr, cells were examined for Arg 1 gene expression by RT-PCR and for arginase activity. FhES-W ($p \leq 0.001$), FhTeg-W ($p \leq 0.001$) and FhES-SN ($p \leq 0.001$) samples produced significant amounts of arginase enzyme when compared to Med-W (Fig. 3.8.B). FhTeg-SN failed to produce a significant increase in arginase activity when compared to Med-SN indicating that cell contact after FhTeg stimulation was necessary to increase arginase activity in macrophages (Fig. 3.8.B). RT-PCR confirmed the finding that FhES-W and FhES-SN can induce M2 by inducing Arg 1 expression. FhTeg-W cells but not FhTeg-SN expressed Arg 1 (Fig. 3.8.C). These results suggest that FhTeg-SN alone is not sufficient for M2 induction but that the continual presence of dendritic cells that have been exposed to FhTeg can induce M2.

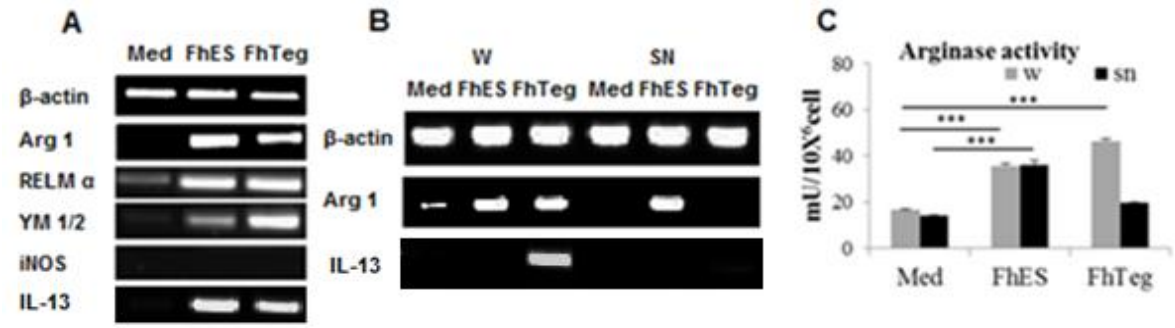

Figure 3.8.FhTeg stimulated dendritic cells can induce M2-like macrophages *in vitro* and *in vivo*: BMDC were stimulated with Med, FhES (20 µg/ml) or FhTeg (10 µg/ml) for 18hr and then washed followed by injection intraperitoneally into BALB/c mice ($5X10^5$ cells per mouse). After 7 days PEC macrophages were isolated for measurement of Arg 1, Ym 1/2, RELM α, iNOS and β-actin gene expression by RT-PCR (A). Data is representative of four mice per group and the experiment was repeated three times. BMDC were stimulated with Med, FhES (20 µg/ml) or FhTeg (10 µg/ml) for 18hr. Washed BMDC were co-cultured 1:1 with macrophages (W) or supernatant added 1:1 (v/v) to macrophages (SN) for culturing. After 24hr, cells were examined for Arg 1 gene expression by RT-PCR (B) and for arginase activity (C). Data, analysed by anova, is representative of four mice per group and the experiment was repeated three times, ***, $p \leq 0.001$ compared with controls.

3.2.9. *F. hepatica* infection induces the expression of C-type-lectin receptors on the cell surface of macrophages

We investigated whether CLR expression, which is normally associated with M2 was up-regulated on isolated peritoneal macrophages following *F. hepatica* infection. Peritoneal macrophages were isolated two weeks post-infection and MGL and MR

expression was measured by flow cytometry. *F. hepatica* infection enhanced MGL and MR expression on peritoneal macrophages (Fig. 3.9.A).

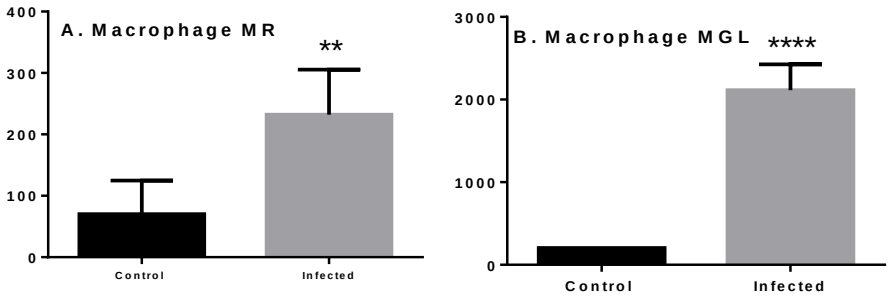

Figure 3.9. *F. hepatica* infection induces the expression of C-type-lectin receptors on the cell surface of peritoneal macrophages: Mice were infected with 20 *F. hepatica* metacecariae. After 2 weeks peritoneal exudate cells were removed and analysed using flow cytometry for markers previously associated with Th2 response MR (a) and MGL (b) on F4/80 positive macrophages. Graph displays the relative mean florescence (MFI) of unstained, control and infected cells. Data, analysed by anova, is representative of four mice per group and the experiment was repeated twice; **, $p \leq 0.01$; ****, $p \leq 0.0001$ compared with controls.

3.2.10. Mice injected with FhTeg can mimic MR and MGL C-type-lectin receptor up-regulation seen in *F. hepatica* infection

With the aim of understanding what the specific effect FhTeg has on CLR *in vivo* we injected mice intraperitoneally three times per week for three weeks with FhTeg (10 µg)

and PBS as a control. PEC were isolated and analysed using flow cytometry for MGL and MR on F4/80^{+} macrophages (Fig. 3.10.A). Macrophages showed an increase in the expression of MGL ($p \leq 0.0001$) and MR ($p \leq 0.0001$) when compared to macrophages in PBS injected mice. This indicates that FhTeg has the ability to mimic CLR expression as seen during *F. hepatica* infection.

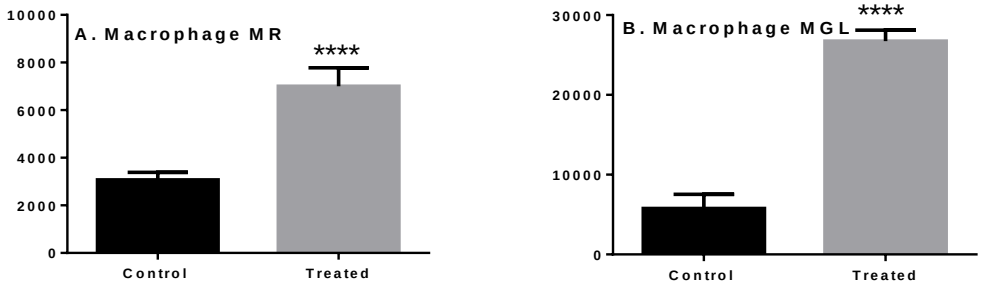

Figure 3.10. Mice injected with FhTeg can mimic C-type-lectin receptor up-regulation seen in infection: Mice were injected intraperitoneally three times per week for three weeks with 10µg of FhTeg. PEC were removed and analysed using flow cytometry for MR and MGL on F4/80 positive macrophages. Data, analysed by anova, is representative of four mice per group and the experiment was repeated twice; ****, $p \leq 0.0001$ compared with controls.

3.2.11. *In vitro* macrophages express MGL and MR in response to FhTeg

In vivo studies indicated that CLR expression is up-regulated by *F. hepatica* antigens during infection and that FhTeg antigen injection can partially mimic this CLR up-regulation. We sought to determine if FhTeg had a similar effect on CLR expression *in vitro*. RAW 264.7 macrophages were stimulated with Med and FhTeg (10 µg/ml).

Macrophages were then analysed by flow cytometry for the expression of MR and MGL. FhTeg stimulated macrophages showed an up-regulation of MR and MGL when compared with media stimulated cells (Fig. 3.11. A+B). This suggests that FhTeg up-regulates CLR through direct interaction with macrophages.

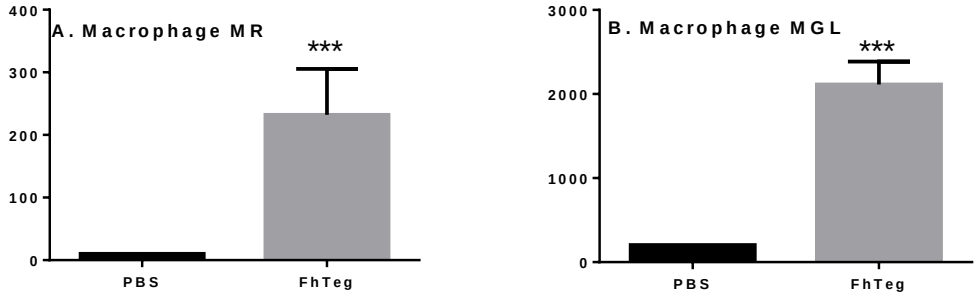

Figure 3.11: FhTeg induces expression of MGL and MR on macrophages *in vitro*: The macrophage cell line RAW 264.7 was cultured with Med and FhTeg (10ug/ml) at 37^{o}C and 5% CO2 for 18hr. The macrophages were then analysed by flow cytometry for the expression of the CLR MR (A) and MGL (B). Data, analysed by anova, is representative of four mice per group and the experiment was repeated twice; ***, $p \leq 0.001$ compared with controls.

3.3. Discussion

The tegumental coat of *F. hepatica* (FhTeg) is in constant contact with the host immune cells and it was shown to be a good source of fluke immune modulators. This study was the first to demonstrate that FhTeg can modulate macrophages indirectly, inducing a

M2-like phenotype and conferring them with a T-cell suppressive functional ability. This M2-like phenotype was demonstrated to be largely STAT6 dependent and while FhTeg cannot induce Th2 specific adaptive immune responses it can induce IL-13 producing macrophages *in vivo*.

This study's findings show that FhTeg does not produce antigen-specific cytokines for either Th1 (IFN-γ) or Th2 (IL-4/IL-5/IL-13) subsets using a *F. hepatica* infection model. Furthermore, FhTeg does not produce any antigen specific response when $CD4^+$ T-cells are re-stimulated after mice are treated with FhTeg intra-peritoneally. This data is in contrast to FhES which is able to produce a strong Th2 response characterized by the presence of IL-4, IL-13 and IL-5. The lack of a T-cell response due to FhTeg is significant as it may point to a mechanism of immune suppression that *F. hepatica* uses to avoid or suppress its host's immune system. It is possible however that FhTeg could induce Th9 or T_{reg} cell populations, as these cell types have been previously associated with helminth infection (Veldhoen et al., 2008; Wilson & Maizels, 2004) and further studies are required to address this.

Given that FhTeg lacks Th2 specific adaptive immune response it was surprising that the macrophage phenotype is STAT6 dependent. STATs (signal transducers and activators of transcription) are a family of transcription factors that respond to cytokines by activating gene transcription. They are cytoplasmic proteins that are activated via tyrosine phosphorylation by JAK kinases. IL-4 and IL-13 both specifically activate STAT6 through the IL-4R. Not only is STAT6 specific for IL-4R but also for a STAT

DNA binding site that no other STAT is able to bind (Mikita et al., 1996). STAT6$^{-/-}$T-cells are unable to differentiate into IL-4 and IL-13 producing Th2 cells *in vitro* or *in vivo* and their proliferating ability is also hampered (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996). With regard to helminths, STAT6$^{-/-}$mice were unable to mount an immune response to soluble egg antigen (SEA) from *S. mansoni* (Kaplan et al. 1998). STAT6$^{-/-}$ mice have also been shown to fail to develop airway hyper-responsiveness after allergen provocation (Kuperman et al., 1998). Thus, mice deficient in STAT6 present a great tool to study the effects of IL-4/IL-13 and therefore Th2 immunity. The use of STAT6$^{-/-}$ mice demonstrated that while FhTeg could readily induce an M2-like phenotype *in vivo*, FhTeg significantly reduced arginase production which is a marker for M2 and also failed to display M2 gene markers except for RELM α. RELM α is a family member of cysteine-rich molecules related to a resistin which is involved in glucose metabolism (Steppan et al., 2001). While the function of RELM α remains elusive, a study using IL-13 receptor 1 deficient mice concluded that IL-13 is dispensable for expression of RELM α in the liver during *S. mansoni* infection (Jenkins & Allen, 2010; Ramalingam et al., 2008). This apparent contradiction that FhTeg works through both STAT6 dependent and independent pathways should not come as a surprise as a previously described parasite immunomodulator that only demonstrated arginase activity to be STAT6 dependent and not other M2 associated genes (Marshall et al., 2011). It is plausible that RELM α expression is induced by different antigens within the FhTeg preparation, antigens that may drive IL-4/IL-13 production and therefore bypass the need for STAT6 involvement.

FhTeg could induce Arg1 and RELM α one hour following injection into the peritoneal cavity, while IL-13 was not detected at this time point. This demonstrates that IL-13 is not required for the initial activation of RELM α expression post FhTeg exposure and this was confirmed in the studies with STAT6$^{-/-}$ mice. While Arg-1 is observed at one hour in the absence of IL-13 it is clear that IL-13 is required to maintain this expression as it is not observed in STAT6$^{-/-}$ mice treated with FhTeg. IL-13 was observed in the PEC at 6 hr and 24 hr time points and this correlated with increased expression of the M2 markers Arg 1 and RELM α at the same time points. No IL-4 was detected at these time-points indicating that IL-13 is the early source of Th2 cytokines. While Th2 required for the activation of an M2 phenotype during helminth infection the required to maintain it (Jenkins et al., 2011).

The secretion/presence of two structurally and functionally similar molecules IL-4/IL-13 is central to the Th2 response in helminth infections (Zhang et al., 1997). They exert various biological effects by regulating proliferation and differentiation of a wide variety of lymphoid and myeloid cells (Paul, 1991). Some studies have identified non-redundant role for both IL4 and IL-13. These have shown IL-4 to mediate most events involved in the generation of high-affinity IgE antibodies while IL-13 mediates localized tissue effects such as chemokine secretion, goblet cell hyperplasia, mucus production, and smooth muscle alterations (Finkelman et al., 2010; Liang et al., 2012). Macrophages were identified as the innate source of IL-13 by intracellular staining in the antigen injection model. It is possible that the innate macrophage source of IL-13 may work in an autocrine manner keeping the macrophages in a M2-like state without leading to a Th2 immune response.

While M2-like IL-13 producing macrophages are seen at an early time-point after FhTeg exposure they failed to be induced by FhTeg directly *in vitro*. In fact, the induction of an M2 phenotype by FhTeg can only be induced indirectly *in vitro* by co-culturing macrophages with FhTeg exposed DC. This phenomenon of FhTeg exposed DC inducing M2-like macrophages can also be observed *in vivo* after adoptive transfer. Cell cross-talk can be mediated either through secreted products, binding or being internalized by other cells, or through direct binding between cells. The data here is the first to demonstrate that an M2-like macrophage phenotype can be induced by another cell type. FhTeg exposed DC have also been shown to induce mast cell migration via the secretion of MIP1α and MIP2 chemokines (Vukman et al., 2013). This serves as evidence that FhTeg exposed DC can influence other cells types. The question of direct or indirect induction of M2-like macrophage by FhTeg-DC was answered as FhTeg-SN from DC failed to induce M2-like macrophages. The continual presence of FhTeg-DC in culture with macrophages induced and sustained the M2-like macrophages. The mechanism of how FhTeg stimulated DC can induce M2-like macrophages needs to be investigated further. Preliminary studies could not observe an enhancement of cell surface markers important for DC-macrophage communication (CD14, OX40L, CD40 and ICAM-1) and it is yet to demonstrate that FhTeg can bind to macrophages. This could be determined through analysis with confocal microscopy following the biotinylation and fluorescent labeling of FhTeg. It may prove difficult as FhTeg is a complex mix of molecules which may require the isolation and characterization of individual molecules to identify the bioactive component.

FhTeg exposed cells not only have an effect on surrounding innate cells, macrophages exposed to FhTeg functionally suppress cytokine secretion from $CD4^+$ T-cells. This functional altering ability of FhTeg was also seen in dendritic cells, where their phagocytic capacity and their ability to prime T-cells was impaired (Hamilton et al. 2009). When DC are exposed to FhTeg their maturation is restricted and their normal function to TLR ligands is altered. It was shown to inhibit pro-inflammatory cytokines *in vivo* in a model of septic shock and *in vitro* in response to Toll-like receptor (TLR) and non-TLR ligands (Hamilton et al., 2009). It has also been established that FhTeg impairs masts cells ability to drive Th1 immune responses. This is accomplished by inducing suppressor of cytokine secretion-3 (SOCS3) a negative regulator of the TLR4 pathway which would explain the suppression of NF-κB and MAPKs in these cell populations (Vukman et al., 2013).

The suppressive cytokines IL-10 and TGF-β are produced by macrophages in response to FhES. A study blocking the CLR MR caused partial inhibition in the increase of arginase activity, Arg 1 expression along with IL-10 and TGF-β secretion in macrophages both *in vitro* and *in vivo* (Guasconi et al., 2011). This prompted an investigation to see if any CLR were up-regulated on FhTeg exposed macrophages as we saw FhTeg conferred them with a suppressive function. FhTeg was found to induce an increased expression of both MR and MGL CLR. Furthermore FhTeg possesses the ability to mimic CLR up-regulation in macrophages as observed during *F. hepatica* infection. CLR have been reported to be up-regulated in many different helminth infections and they are associated with a M2 phenotype (Meyer et al., 2005; Guasconi et al., 2011; van Liempt et al., 2007; Stein et al., 1992). CLR have also been recognized as

being important receptors on antigen presenting cells as they can bind and internalise glycosylated antigens which leads to their processing and presentation on MHC molecules which elicits a T-cell response (Zelensky & Gready, 2005). While our data demonstrated that FhTeg does not elicit a T-cell response, it has been demonstrated that antigen uptake by CLR may not always cause the induction of T-cell responses but can enhance antigen processing ability (Van Kooyk, 2008). It has been suggested that CLR antigen uptake without TLR ligation may lead to antigen-specific tolerance which may help to suppress inflammatory responses (Van Kooyk, 2008).

The suppressive nature of FhTeg along with its lack of T-cell response has been demonstrated by our data and correlates to what is currently known about CLR. These results represent a first step in confirming FhTegs involvement in the up-regulation of CLR on innate immune cells during *F. hepatica* infection. Further work should be completed to determine if FhTeg is binding to CLR. CHO cells could be used to express individual CLR and determine if FhTeg binds them. CLR expression in $STAT6^{-/-}$ mice was not explored; this would have been interesting as they may be linked to M2-like macrophages being induced. While this study has demonstrated some novel findings and expanded on the sparse knowledge of the role of FhTeg in *F. hepatica* infection, especially in a macrophage context, further work is needed to determine what FhTeg fully contributes to the overall effect of *F. hepatica* infection.

3.4. Conclusion

This study makes it evident that FhTeg places an important role in *F. hepatica* infection. The interaction it has with macrophages is now better known. As innate cells are critical in host defense and in the development of adaptive immune responses, the findings in this study give us an insight into how *F. hepatica* modulates host immunity. FhTeg was found to induce M2-like macrophages which have a functional suppressive ability. These M2-like features are largely STAT6 dependent, meaning they are evoked by IL-4/IL-13 means. While we found no evidence for a role of IL-4 we did see that an early source of IL-13 in a macrophage population *in vivo* may be critical in the maintenance of M2-like macrophages. Further studies are required to determine what role this innately produced IL-13 is having on the immune environment. The discovery that the CLR MR and MGL were significantly expressed by macrophages exposed to FhTeg and that this pattern mimics infection CLR expression is an exciting prospect for further work on the function of these receptors. While direct exposure with FhTeg antigen fails to induce the M2 phenotype; an interesting finding demonstrated that DC exposed to FhTeg were capable of inducing M2-like macrophages both *in vitro* and *in vivo*. This novel finding of cell cross-talk adds to the complexity of how FhTeg interacts with immune cells during *F. hepatica* infection. The use of DC knockout and DC/STAT6 knockout mice would further enhance our knowledge how DC and STAT6 fit into the induction of the M2 phenotype. Further work is needed to fully understand FhTeg role in *F. hepatica* infection.

Chapter 4

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