

Characterisation of novel *F. hepatica* activated immune cells

A thesis submitted for the degree of PhD

by

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Declaration

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Abstract

The helminth parasite *Fasciola hepatica* causes fasciolosis in mammals infecting cattle and sheep, causing significant economic loss to the agricultural community annually. It is also a zoonotic disease, affecting approximately 2.4 million people worldwide. Infection is associated with Th2/Treg immune responses with a direct suppression of Th1/Th17 responses. With a view to understanding how *F. hepatica* interacts with its host's immune system we have isolated the tegumental coat antigens (FhTeg) which is a rich source of glycoproteins. Previous studies have examined its interaction with macrophages, mast cells and dendritic cells demonstrating the induction of novel cell populations that fail to drive Th1 immune responses. This thesis advances our understanding of FhTeg by examining its interaction with dendritic cells and CD4⁺ T-cells and a number of novel findings were identified. The interaction of FhTeg with C-type lectin receptors on dendritic cells is important for its immunomodulatory effect, while mannose glycans are involved, studies in MR knockout mice did not reverse the immune-suppression of FhTeg. We have confirmed that the novel dendritic cells population (CD11c^{low}, MR^{high}, CD40^{low} and SOCS3^{high}) induced by FhTeg drives anergic CD4⁺ T-cells. We also demonstrated for the first time that anergy is associated with *F. hepatica* infection. More importantly, FhTeg drives these responses and MR is critical for cell to cell communication. We have also demonstrated that FhTeg can directly interact with CD4⁺ T-cells, enhancing markers of anergy directly and it can suppress cytokine secretion from CD4⁺ T-cells. Finally, studies on human PBMCs also show a role for FhTegs inhibition, TNF- α was inhibited when PBMCs and CD4⁺ cells were co-stimulated with both LPS and PMA/Ionomycin but not CD14⁺ cells. This thesis presents a number of key findings that have never been reported previously in the literature and advance our understanding of helminth immune modulation.

Acronyms and abbreviations

APC: Antigen presenting cell

BCA: Bicinchoninic acid

BMDC: Bone-marrow derived dendritic cells

Casitas B-lineage Lymphoma Proto-oncogene b: CblB

CD: Cluster of differentiation

CLR: C-type lectin receptors

DC: Dendritic cell

ELISA: Enzyme-linked immunosorbent assay

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

GalNAc-4-Sulphate: N-Acetylgalactosamine-4-Sulphate

GM-CSF: granulocyte-macrophage cell stem factor

GRAIL: Gene related to anergy in lymphocytes

IFN γ : Interferon gamma

Ig: Immunoglobulin (e.g. IgG)

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

i.p.: Intraperitoneal

LPS: Lipopolysaccharide

M2: Alternatively activated macrophages

mAb: Monoclonal antibody

MFI : Mean fluorescence intensity

MGL: Macrophage galactose-type lectin

MHC: Major histocompatibility complex

MR: Mannose receptor

NF- κ B: Nuclear factor kappa B

NP-40: nonyl phenoxypolyethoxylethanol-40

PBS: Phosphate-buffered saline

PD-: Programmed death

RT-PCR: Reverse-transcription polymerase chain reaction

PMA: Acetate Phorbolmyristate

PRR: Pattern recognition receptor

RPMI: Roswell Park Memorial Institute

RNA: Ribonucleic acid

RNF128: Ring Finger Protein 128

SD: Standard deviation

TGF β : Transforming growth factor beta

Th: T helper cell

TLR: Toll-like receptor

TNF α : Tumor necrosis factor alpha

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

T-reg: T-regulatory

TSLP: Thymic stromal lymphopoietin

Chapter 1.

Introduction

1. Introduction

Parasitic worms (helminths) can infect both humans and animals causing a wide range of diseases (Maizels, Hewitson and Smith 2012). In general, infection leads to a polarised T-helper cell 2 (Th2) immune response followed by the induction of regulatory networks that induce tolerance to control immune mediated pathology. This is beneficial to the survival of the parasite within the host as reducing host pathology can prolong the survival of both the host and the helminth (Anthony et al. 2007). Helminth infections are characterised by an increase in immunoglobulin E (IgE) and the recruitment of cells such as alternatively activated macrophages (M2) macrophages, mast cells, eosinophils and basophils (Maizels et al. 2004). Helminths also induce T-regulatory (T-reg) cells which are important for maintaining tolerance (Belkaid et al. 2002) and can also induce another regulatory network of T-cells called T-cell anergy, this is a cellular hypo-responsive state which helps in the maintenance of tolerance, but few studies have examined this cell type in the context of helminth infection (Taylor, van der Werf and Maizels 2012b, Smith et al. 2004).

We are interested in the helminth parasite *Fasciola hepatica* which causes fasciolosis in mammals infecting cattle, sheep and is a zoonotic disease in humans, affecting approximately 2.4 million people worldwide (Mas-Coma, Bargues and Valero 2005). Infection with *F. hepatica* generally leads to a Th2/T-reg immune response with a decrease in Th1 responses observed (O'Neill et al. 2000). Our studies are focused upon the modulatory properties of *F. hepatica* tegumental antigens as these are a rich source of glycol-conjugates that induce novel dendritic cell, mast cell and M2-like populations, which suppress Th1 immune responses. While we have made great strides in understanding the immune-modulatory properties of this antigen source, no studies to date have fully characterised FhTeg driven T-cell responses

during infection (Hamilton et al. 2009b, Adams et al. 2014, Vukman et al. 2013b, Vukman et al. 2013a, Vukman, Adams and O'Neill 2013). Further studies are also required to fully characterise the interaction of FhTeg with dendritic cells and their subsequent effect on the adaptive immune response. These unanswered questions form the central premise of this doctoral project where we shed new light on the immune modulatory properties of helminth parasites.

1.1. Characteristics of helminth infections

Helminths, derived from the Greek word, *helminθ*, meaning worm, cause a plethora of neglected tropical diseases, infecting up to one third of the world's population with a high prevalence in the developing world. The term helminth encompasses a number of different family members including roundworms such as *Ascaris lumbricoides*, filarial worms which cause filariasis, cestodes and trematodes which contain the parasites amongst others, *Fasciola hepatica* and *Schistosoma mansoni* (Hotez et al. 2008). Helminths can infect both domestic and wild animals, causing a significant economic burden to the agricultural community. Infection in cattle can lead to weight loss, a poor growth rate and a lower milk yield (Bamaiyi 2012).

In humans, helminths infection is rarely fatal but can lead to morbidity and chronic inflammation; other characteristics are anaemia, weight loss and similar to cattle and sheep an increased susceptibility to secondary bystander infections is observed. It is difficult to estimate the incidence and burden of infection in human populations but there are a number of methods used such as disability-adjusted life year (DALY; to measure the extent of the

burden,) or more recently QALY (quality-adjusted life year). DALY is a time based unit which compares two disease related parameters, persons-years lost to disability (YLD) and persons-years life lost (YLL) to determine the disability burden for the health condition or infection. QALY is based not on what infection or health condition the person may have but it is based on quality of life based on the patient status (King, 2010.). Both of these methods give a clear picture of the burden of infection on the economy and on the impact on the quality of life. In 2010 a global burden of disease study found a total of nearly 15 million DALYs due to helminth infection (WHO, 2002). An increase in susceptibility to bystander infections is also common during helminth infections. Studies have shown co-infection with helminths can inhibit the clearance of TB in patients and it can also have an effect on the treatment of the disease itself (Resende Co et al. 2007). Some studies are also on-going on the effect of helminth infection with concurrent HIV infection and have showed that infection with both helminths and HIV impairs the patients Th1 immune response and this alters the clearance of the virus (Bentwich et al. 1999, Mulu, Maier and Liebert 2013).

Infection with helminths can have some evolutionary advantages as proposed by the hygiene hypothesis that demonstrates a link between the increase in immune mediated disease such as autoimmunity and allergy and the decrease in the numbers of helminth infections in the developed world (Yazdanbakhsh, Kremsner and van Ree 2002). Studies in the developed world demonstrate a correlation between helminth infections and the low occurrence of autoimmune diseases (Jackson et al. 2009). This points to the potential therapeutic benefit of helminth infection and clinical trials using “worm therapy” are underway in a variety of inflammatory disorders. Studies in mouse models of multiple sclerosis, experimental autoimmune encephalitis (EAE), have shown that early intervention with helminth therapy can reduce Th1 immune responses and bias towards a Th2 immune response, limiting tissue

damage and migration of lymphocytes to the CNS (Gruden-movsesijan et al. 2010, Reyes et al. 2011) but these therapies were only successful upon early treatment, as starting treatment once symptoms had progressed had no impact on disease progression.

Investigations into colitis have also shown promising results. Treatment with helminths showed a decrease in Th1 cytokines and in most cases, symptoms were attenuated or diminished completely (Heylen et al. 2014). Studies have also looked at the treatment of celiac disease with hookworms, a study using *Necator americanus* larvae and challenges of increasing gluten consumption showed an increase in T-reg cells and a decrease in disease severity (Croese et al. 2015, Croese, Gaze and Loukas 2013). Some studies have also looked at graft versus host disease (GVHD) and infection with *Heligmosomoides polygyrus* reduces the effect of GVHD by reducing inflammatory cytokines and priming the donor T-cells to become T-regs (Kuijk et al. 2012b, Li et al. 2015).

Human clinical trials have also been conducted, on the efficiency of helminths as therapeutics. The majority of these trials have focused on the use of *T. suis*, a study using five patients with relapsing MS showed an improvement and decrease in neurological and CNS lesions while therapy was on-going using *T. suis* but following the end of the study and the expulsion of the parasite, the lesion number increased again but no adverse events were noted during the trial (Fleming et al. 2011). Another number of studies also investigated the use of *T. suis* for the treatment of inflammatory bowel disease with an improvement in clinical symptoms and remission in some cases. As was seen with MS, continuing treatment with follow up doses of parasite is needed or relapses are possible (Summers et al. 2003, Summers et al. 2004, Summers et al. 2005, Fleming 2013). While there have been promising results,

whole worm therapy cannot always be used as there can be unpredictable side effects such as invasion into other tissues and pathology, also certain people would not be able to receive worm therapy such as children, pregnant women and individuals who may be immunocompromised.

Research into helminth products as an alternative to whole worm therapy is crucial for new therapeutic approaches to these diseases and helminth products have shown potential for the treatment of MS, with a shift in cytokine responses towards a Th2 immune response (Zheng et al. 2008, Kuijk et al. 2012a). Studies using *S. mansoni* SEA in the treatment of colitis have shown that the administration of SEA significantly improved inflammatory factors studied but this was time dependent as after 6 weeks of administering the SEA, no effects of these were seen whereas at an earlier time point of four weeks, Th2 responses were increased in the colon of mice (Heylen et al. 2015). Using ES from the hookworm, *Ancylostoma caninum*, and investigators found intestinal pathology could be suppressed in a mouse model of colitis; this was due to a potent Th2 immune response and the recruitment of alternatively activated macrophages to the site of ES injection (Ferreira et al. 2013). Investigations into inflammatory bowel disease (IBD) have also shown promising results, with studies using *T. suis* ova showing an improvement in ulcerative colitis compared to placebo controls, with 43% of patients recording an improvement in disease score compared to 16% of controls (Heylen et al. 2014, Heylen et al. 2015). Studies on psoriasis and rheumatoid arthritis have also shown positive results. Investigations using flaky skin mice which develop lesions similar to psoriasis and a glycan isolated from *S. mansoni*, LNFPIII, reduced lesion development with a decrease in IFN- γ production (Atochina and Harn, 2006). ES-62 isolated from *Acanthocheilonema viteae*, has been shown to reduce the initiation of collagen induced inflammatory arthritis and also inhibited the progression and severity of the disease (McInnes

et al. 2003). To develop these therapies it is important that we understand the immune properties of helminths and the products that release.

1.2. Immunology of helminths

The immune response to helminths is well characterised with a Th2/T-regulatory immune response observed. This response leads to the production of IL-4, IL-5, IL-10 and IL-13, an increase in immunoglobulin E (IgE) and the recruitment and expansion of cells such as mast cells, eosinophils, basophils and M2 macrophages (Maizels et al. 2004). This environment in the host is beneficial to the parasite as these responses suppress Th1 immune responses that can either lead to worm expulsion or immune pathology. The role of eosinophils is not entirely clear, however very early *in vitro* studies have shown them to be involved in parasite killing in combination with antibodies and complement and their granular products can also mimic this (David, Butterworth and Vadas 1980). *In vivo* their role is less understood and are linked to the production of IL-4 and IL-13 in conjunction with basophils (Anthony et al. 2007). Infection with helminths has also been shown to increase the numbers of M2 macrophages (Kreider et al. 2007) which differ from M1 macrophages in that they produce Arginase1 (Arg1) and show an up-regulation of Ym1 and Relm- α genes but not iNOS (Gordon 2003). During helminth infection M2 macrophages are important for controlling inflammation, tissue repair and for the killing and expulsion of parasites (Maizels et al. 2009). While these cells are important in driving the immune response associated with helminth infection the communication between dendritic cells with CD4⁺ T-cells is important to the development of these responses and is therefore a focus in this thesis.

1.3. Helminth infections and Dendritic Cells

Dendritic cells are hematopoietic stem cells in origin and are potent antigen presenting cells which are an important bridge between adaptive and innate immunity (Ardavín et al. 2001). While in an immature state, dendritic cells are constantly patrolling the body in search of foreign antigens. Following internalisation of antigen, dendritic cells generally become mature and enhance their expression of co-stimulatory markers like CD80, CD86 and MHC Class II which they then use to bind to receptors on other cell types like T-cells or mast cells and initiate an effective immune response (Banchereau, Briere and Caux 2000, Bonasio and von Andrian 2006). Dendritic cells decide whether an immune outcome will be pro-inflammatory or anti-inflammatory depending on the antigen it has encountered and can produce cytokines like IL-12p70, TNF- α or IL-10 depending on the immune response that is to be initiated (Macatonia et al. 1995, Cella, Sallusto and Lanzavecchia 1997, Guermonprez, Valladeau and Zitvogel 2002).

Unlike when stimulated with microbial antigens, dendritic cells stimulated with helminth products undergo semi-maturation with low levels of major histocompatibility complex classes I and II (MHC I/II), CD80 and CD86 being expressed and little or no cytokines like IL-12p70, TNF- α or IL-10 being produced. Studies using *S. mansoni* derived SEA have shown that dendritic cells become semi-mature and fail to up-regulate co-stimulatory markers, these cells when injected inter-peritoneal into naïve mice induced strong Th2 immune responses from splenocytes (MacDonald et al. 2001). Similarly, dendritic cells stimulated with products from *Nippostrongylus brasiliensis* and injected into naive mice

primed a Th2 immune response in recipients but in this case there was an up-regulation of co-stimulatory markers CD86, CD40 and OX40L (Balic et al. 2004). We have also shown that *F. hepatica* tegumental antigen can inhibit the maturation of bacterial ligand stimulated dendritic cells also, inhibiting the expression of maturation markers and the production of cytokines (Hamilton et al. June 2009). FhTeg also enhances the expression of suppressor of cytokine signalling 3 (SOCS3), a negative regulator of cytokine signalling and inhibits mitogen-activated protein kinase (MAPK) signalling (Vukman, Adams and O'Neill 2013).

1.4. Signalling Pathways

During helminth infections a number of different signalling molecules and pathways have been identified as playing important roles in the induction of the suppressive phenotypes seen. These include toll like receptors (TLRs) and C-type lectin receptors (CLRs) which are both pathogen recognition receptors (PRRs). Both TLRs and CLRs recognise pathogen associated molecular patterns (PAMPs) which are highly conserved across species. Signalling through both TLRs and CLRs contribute to activation of the immune response but can also switch off the response. TLRs have been implicated in helminth infections with both TLR2 and 3 being used for dendritic cell activation following exposure to *S. mansoni* eggs and leads to an inflammatory response being seen but is not required to control infection and pathology as studies using TLR2 and TLR3 knockout mice showed no difference in worm burden or the numbers of eggs in tissues compared to wild type mice (Vanhoutte et al. 2007). In filariasis TLR expression is down-regulated following infection leading to immune tolerance and diminished immune responses (Babu et al. 2005).

CLRs also play an important role in the immune response to helminths. Several helminth products have been shown to signal through CLRs (Tundup, Srivastava and Harn 2012b) and it is thought they signal through CLRs as an escape mechanism from the hosts' immune response (van Die and Cummings 2010b). *S. mansoni* soluble egg antigens (SEA) have been shown to signal through several CLRs; DC-SIGN, MGL and MR and inhibit DC maturation and induce a Th2 immune response (van Liempt et al. 2007). The nematode *Trichuris suis* has also been shown to be involved in CLR signalling. Glycans isolated from the parasite, signal through MR and DC-SIGN and induce, as was seen with *S. mansoni*, a DC phenotype which inhibits bacterial TLR activation and the activation of an inflammatory immune response. It has been shown that *F. hepatica* is rich in glycans on its structure and in the ES products. This may lead to signalling through CLRs rather than traditional routes like TLRs. In studies using mannan to block the interaction between the mannose receptor and FhES, the alternative activation of macrophages can be prevented and also using blocking antibodies for MR and Dectin 1 this can also be achieved (Guasconi et al. 2011). However it has also been shown that TLR2 plays a role in the FhES inhibition of macrophages during *Mycobacterium bovis* activation (Flynn et al. 2009). As some of the major components of FhTeg are carbohydrates, CLRs may play a role in the immunomodulatory effect seen on cells in the immune system.

1.5. The Mannose Receptor

The macrophage mannose receptor (MR) is a carbohydrate binding receptor which plays a role in a number of actions in the cell. It is involved in the clearance of endogenous molecules, the activation of cells and also the enhancement of antigen presentation from APCs. The receptor is found on a number of cell types including dendritic cells,

macrophages, mast cells and non-vascular endothelium (Martinez-Pomares 2012). MR is a type-I membrane protein with a cytoplasmic domain involved in antigen processing and receptor internalisation and three different types of binding domains at its extracellular region. It features multiple C-type lectin-like carbohydrate-recognition domains (CRDs) responsible for calcium dependent binding to terminal mannose, fucose or *N*-acetyl glucosamine (Gazi and Martinez-Pomares 2009) a fibronectin type II (FNII) domain involved in collagen binding (Martinez-Pomares et al. 2006) and an *N*-terminal cysteine-rich (Cys-MR) domain that mediates calcium-independent binding to sulfated sugars (Figure 1.1).

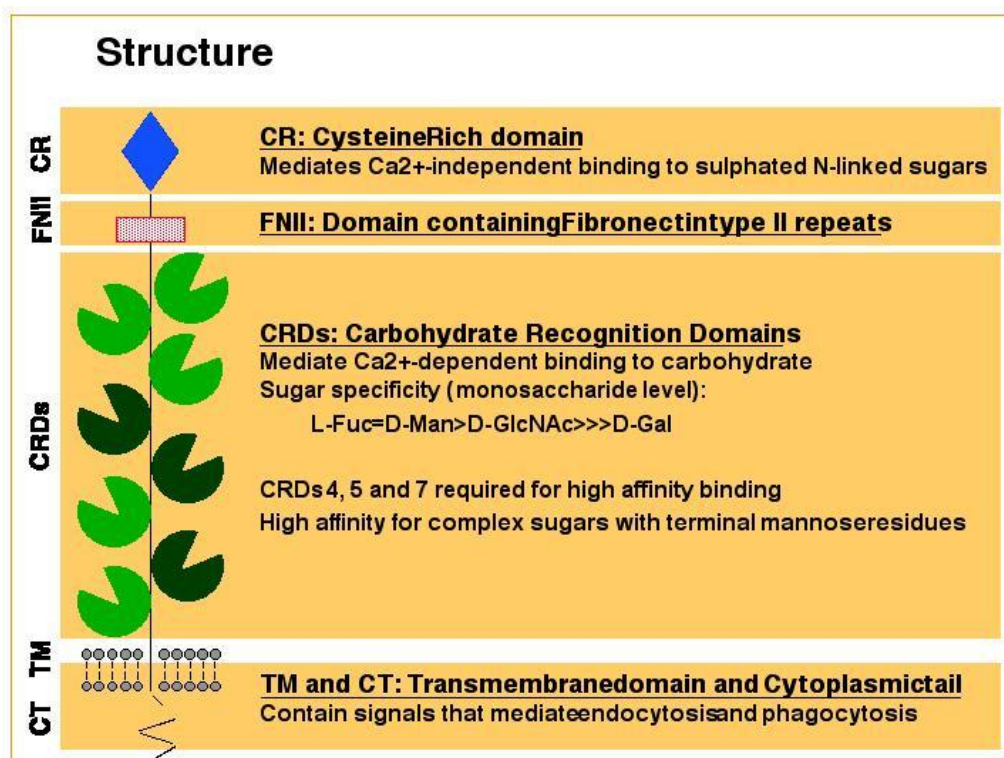


Figure 1.1 The structure of the mannose receptor. The mannose receptor has three binding domains. The cysteine rich domain controls calcium independent binding to sulphated N-linked sugars. The fibronectin type II binding domain is involved in binding to fibronectin or collagen. The carbohydrate recognition domains or CRDs are involved in calcium dependent binding of sugars.

The mannose receptor has been implicated as a receptor involved both *in vivo* and *in vitro* after stimulation with helminth products (Vázquez-Mendoza, César Carrero and Rodríguez-Sosa 2013). During *S. mansoni* infection, IFN- γ production was seen to be modulated by MR (Paveley et al. 2011) and antigens isolated from the soluble egg antigens also from *S. mansoni* which are highly glycosylated signal through MR and induce a Th2 priming DC population (Everts et al. 2012). During *T. muris* infection the MR receptor is also involved and the excretory/secretory products bind through MR but it is not essential for the generation of the immune response seen and knockout of the gene does not lead to the expulsion of the parasite in a quicker manner than wild-type mice (DeSchoolmeester et al. 2009a).

1.6. Helminth infections and CD4⁺ cells

CD4⁺ T-cells are a crucial link, mediating adaptive and innate immune responses. They are involved in antibody production from B-cells (MacLennan et al. 1997), regulate macrophage function (Chan et al. 2011), mediate CD8⁺ T-cell responses including memory effector cells following re-infection (Sun and Bevan 2003) and are important in allergic responses and auto-immunity (Wambre, James and Kwok 2012, Burkett et al. 2014). Following recognition of antigens presented by APCs like dendritic cells or macrophages they can differentiate into various subsets including Th1, Th2, Th17, T-reg or anergic T-cells (Yamane and Paul 2013). Following differentiation CD4⁺ cells produce cytokines according to their subset, Th1 cells tend to produce pro-inflammatory cytokines like TNF- α and IFN- γ whereas Th2 subsets produce IL-5, IL-4 and IL-13. T-reg cells produce TGF- β and IL-10 but anergic T-cells are hypo responsive and do not mount an effective immune response or produce cytokines following antigen re-challenge (Schwartz 2005, Schwartz 2003, Knoechel et al. 2006, Zhu, Yamane and Paul 2010).

Helminth parasites typically establish themselves for extended periods of time within their host by regulating the immune response using a number of different mechanisms, these include the pushing of the immune response towards a Th2 response and a decrease in a Th1 responses, the production of regulatory T-cells (T-reg) and also anergic T-cells (Maizels et al. 2004). Helminth infections are characterised by an increase in mast cell numbers, eosinophilia and increased production of immunoglobulin E (IgE) (MacDonald, Araujo and Pearce 2002). Th2 responses are predominant during helminth infection and are associated with the production of IL-4, IL-5, IL-9 and IL-13 which are produced by CD4⁺ T cells (Maizels, Hewitson and Smith 2012). The priming of a Th2 immune response can lead to gross pathology and to prevent this the helminth can induce FoxP3⁺ T-regulatory (T-reg) cells that secrete IL-10 and TGFβ that can dampen Th2 immunity (Maizels et al. 2004). Treg cells are important cells used to control inflammatory responses and help maintain tolerance to self-antigens. During infection FoxP3⁺ CD4⁺ cells can be observed as early as 3-7 days post infection pushing the response towards a regulatory phenotype. *Brugia malayi* infection induces a regulatory response with enhanced FoxP3 expression and increased cell surface expression of CTLA4, a negative regulator of T-cell function (McSorley et al. 2008). *Schistosoma haematobium* infection in humans has also been associated with FoxP3⁺ T-reg cells with the highest percentage of this cell population observed in younger patients (Nausch et al. 2011).

Helminth infections are also associated with anergic T-cells, a second type of inhibitory T-cell phenotype that maintains tolerance to self-antigen. In this case there is no FoxP3 expression or production of IL-10 and TGF-β. T-cell anergy is defined as a hypo responsive state with the failure of T-cells to proliferate or produce cytokines when re-stimulated with parasite antigens *in vitro* (Schwartz 2003). Anergic cells do not secrete IL-2, a factor

important for T-cell proliferation and effector responses. The addition of IL-2 can overcome this anergic response restoring the proliferative and cytokine producing ability of the T-cell (Carter et al. 2002). Gene analysis studies have shown a number of genes including RNF128 (GRAIL), ITCH, early growth response genes 2 and 3, (Egr2 and 3) and casitas B-lineage Lymphoma Proto-oncogene b (CblB) are involved in the induction and maintenance of T-cell anergy (Lechner et al., Mueller 2004b) . The expression of PD1 and CTLA4 by T-cells are also a defining mark of anergy (Greenwald et al. 2001, Okazaki and Honjo 2006). T-cell anergy rather than FoxP3 T-reg cells are associated with chronic infection which may be due to the persistence of parasite antigen and the constant contact of the cells with the antigens. T-cell anergy has been shown to one of the main causes of T-cell unresponsiveness in *S. mansoni* infection mediated by the up-regulation of PD-L1 on the surface of macrophages (Smith et al. 2004) and also with the up-regulation of RNF128 (GRAIL) in T-cells isolated from chronic infection (Taylor et al. 2009).

1.7. Dendritic cell/T-cell Crosstalk

To mount an effective immune response to antigens, effective crosstalk must occur between different cell types. As dendritic cells are the most potent antigen presenting cells in the immune system we are interested in how they drive T-cell responses to different helminth antigens. Following processing by dendritic cells, they present antigens to T-cells on markers like MHC class I and II. They then bind to their respective receptors on T-cells like the T-cell Receptor complex (TCR) (Signal 1) and also bind through co-stimulatory markers like CD80 and 86 to CD28 (Signal 2) to initiate immune responses. If insufficient signals are received by T-cells from dendritic cells like Signal 1 alone this may induce anergy (Sadegh-Nasseri et al. 2010).

There are a number of co-stimulatory ligands and receptors involved in dendritic cell/T-cell communication which can drive or inhibit T-cell activation (Guermonprez et al. 2002, Jung and Choi 2013). Presentation through CD80 (B7.1) and CD86 (B7.2) to CD28 and the TCR on T-cells results in stimulation of T-cells by lowering the threshold needed for activation and the subsequent production of IL-2 (Lenschow, Walunas and Bluestone 1996) but signalling through CTLA4 on T-cells terminate T-cell responses and maintains tolerance to self-antigens by inhibiting IL-2 production and subsequent proliferation (Slavik, Hutchcroft and Bierer 1999). Other ligands such as PDL1 and PDL2 are also inhibitory; they bind through the receptor PD1 on T-cells and terminate T-cell responses (Carter et al. 2002, Okazaki and Honjo 2006, Okazaki and Honjo 2007). C-type lectin receptors also have ligands on T-cells for communication and crosstalk. CD45 has been implicated as a co-receptor for the mannose receptor (Martínez-Pomares et al. 1999). CD45 regulates Src family kinases and can be both a positive and a negative regulator of T-cell function affecting cytokine secretion, migration and TCR signalling (Saunders and Johnson 2010).

1.8. *Fasciola hepatica*

The helminth parasite *Fasciola hepatica* causes fasciolosis in mammals infecting cattle, sheep and is a zoonotic disease in humans, affecting approximately 2.4 million people worldwide. Although generally not fatal, infection causes weight loss and anaemia (Mas-Coma, Bargues and Valero 2005). In sheep and cattle, infection causes a huge economic burden, estimated at more than 100 million dollars a year in the US and more than 80 million

pounds in the UK and Ireland. Infection can cause a lower rate of milk production and a susceptibility to tuberculosis in cattle (Brady et al. 1999b).

The parasite has a complex life cycle with an intermediate snail host and definitive mammalian host. Eggs are released from the definitive host which hatch and produce miracidium, these infect the intermediate snail host. The snail host sheds cercariae which then encyst as metacercariae (infective larvae) on water plants. The metacercariae are ingested by the definitive host and excyst in the duodenum to form newly excysted juvenile flukes that penetrate the intestinal wall and migrate through the peritoneal cavity and liver. Finally the adult worm resides in the bile ducts, releasing eggs for many years (Robinson and Dalton 2009) (Figure 1.2). Generally the process of migration and penetration through the small intestine does not outwardly show signs of clinical disease but movement to the liver can cause haemorrhaging and fibrosis.

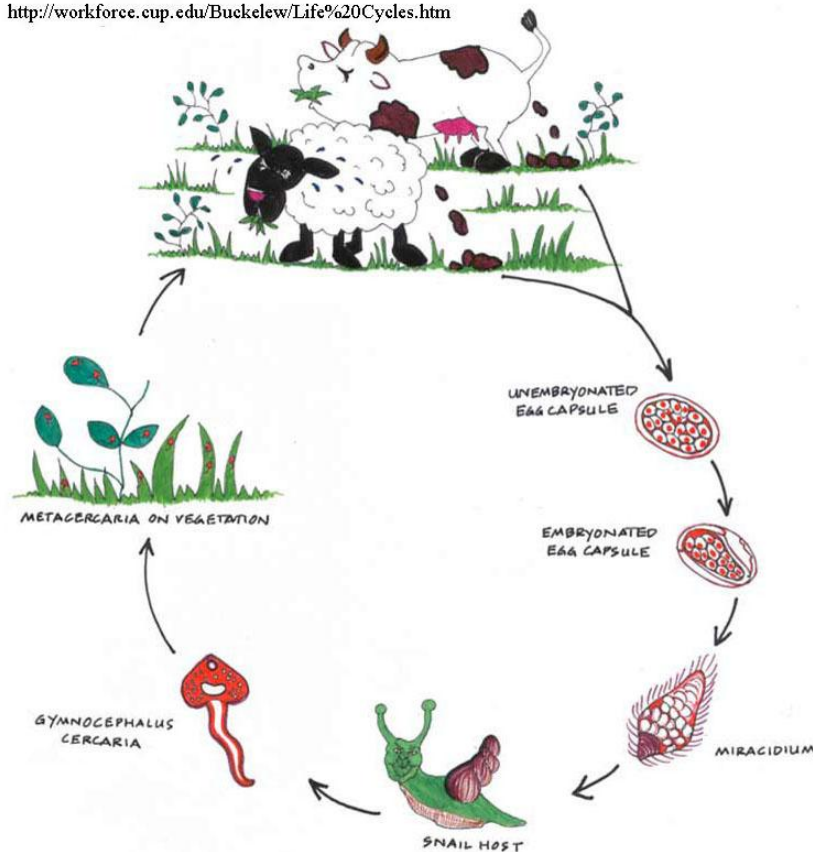


Figure 1.2 Life cycle of *F. hepatica*. The parasite has a complex life cycle with an intermediate snail host and definitive mammalian host. The intermediate snail hosts sheds cercariae which then encyst as metacercariae and the mammalian host ingests them. These metacercariae excyst in the duodenum to form newly excysted juvenile flukes that penetrate the intestinal wall and migrate through the peritoneal cavity towards the liver. Finally the adult worm resides in the bile ducts, releasing eggs for many years.

Infection typically exhibits a strong Th2/ T-reg immune response in the host with the production of IL-5, IL-4 and IL-10 with the inhibition of protective Th1 cytokines (O'Neill et al. 2000). Figure 1.3 outlines the hosts' immune response to *F. hepatica* infection. The juvenile fluke migrate through the gut and into the peritoneal cavity and reside in the liver, where they release antigens and eggs. The parasite secretes excretory/secretory molecules and sheds its tegumental coat, this causes the alternative activation of macrophages with the expression of Arg1, YM1, Fizz1 (Relm α) and IL-10. These molecules also suppress the

activation of dendritic cells with the down-regulation of MHC I and II, CD80 and 86 but the expression of CD40 is enhanced. The secretion of MIP1 α and MIP2 attracts mast cells to the site and the FhES and FhTeg cause the expression of SOCS3 to be enhanced and ICAM1 to be down-regulated. This affects downstream molecules, including MAPK and the mast cells fail to produce cytokines. The combined effect on macrophages, dendritic cells and mast cells when co-stimulated with bacterial ligands is that they do not respond, produce cytokines or mount an immune response to them. This fails to drive Th1 or Th17 immune responses and the immune response is pushed towards a Th2 or T-reg immune response.

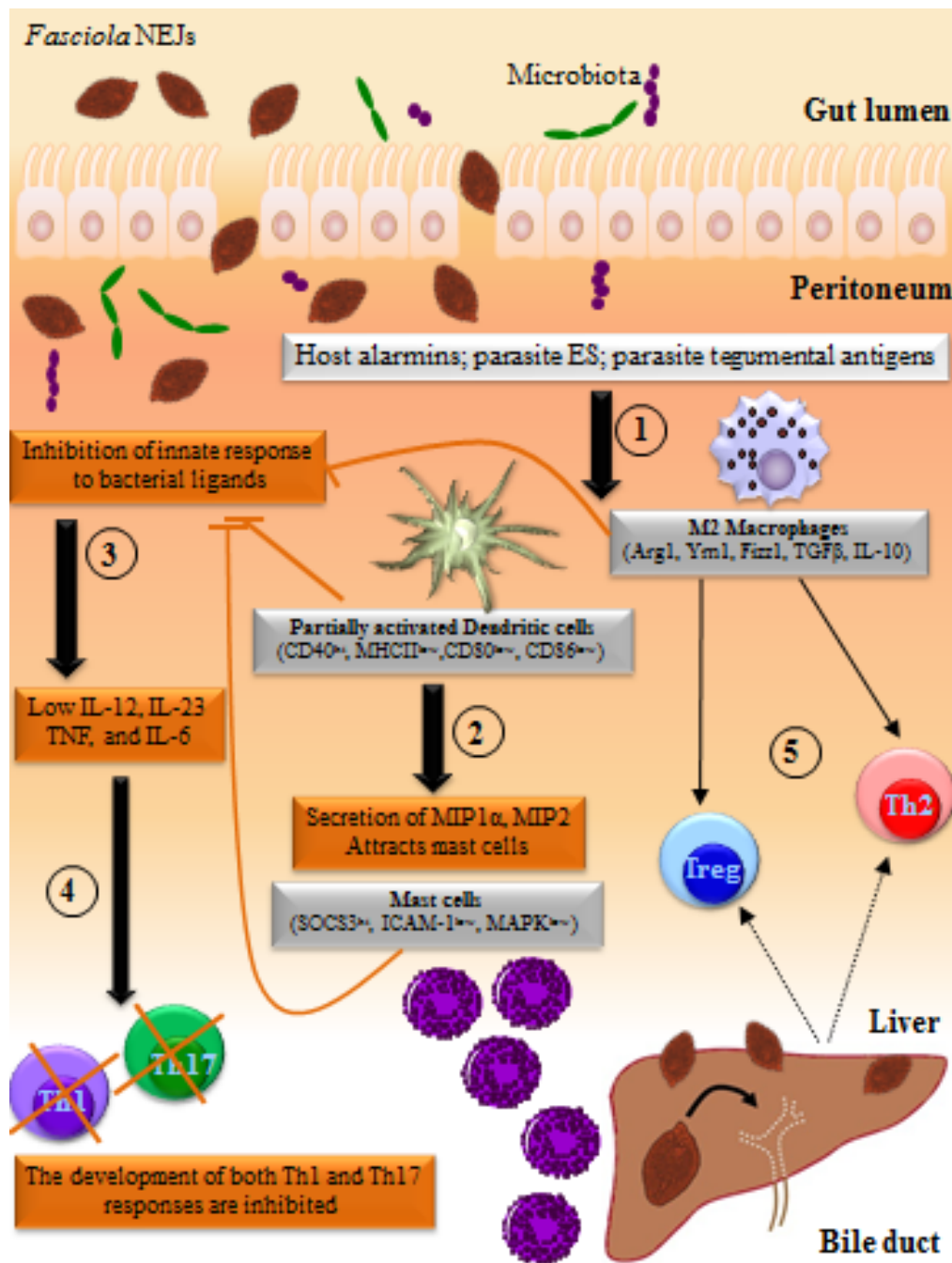


Figure 1.2 The hosts immune response to *F. hepatica*. 1. Parasite secretes excretory/secretory molecules and sheds its tegumental coat. This causes the alternative activation of macrophages with the expression of Arg1, YM1, Fizz1 (Relmα) and IL-10. These molecules also suppress the activation of dendritic cells with the down-regulation of MHC I and II, CD80 and 86 but the expression of CD40 is enhanced. 2. The secretion of MIP1α and MIP2 attracts mast cells the site and the FhES and FhTeg cause the expression of SOCS3 to be enhanced and ICAM1 to be down-regulated. This affects downstream molecules MAPK and the mast cell fails to produce

cytokines. 3. The combined effect on macrophages, dendritic cells and mast cells when stimulated with bacterial ligands is that they do not respond, produce cytokines or mount an immune response to them. 4. This fails to drive Th1 or Th17 immune responses. 5. The immune response is pushed towards a Th2 or T-reg immune response (O'Neill, 2014).

1.9. *Fasciola hepatica* Antigens

F. hepatica has two main sources of antigen which includes its excretory/secretory products which are constantly produced in the host and the tegumental coat which is shed every two to three hours. The excretory/secretory products (FhES) have been most widely studied and table 1 summarises the different antigen sources and our current understanding of their immune modulatory properties. FhES is composed mainly of cathepsin L, peroxiredoxin, helminth defence molecules and sigma class glutathione transferase. FhES modulates the immune response by producing a Th2 response. Studies have shown FhES molecules to have both *in-vitro* and *in vivo* effects. Thioredoxin peroxidase from FhES is involved in reducing oxidative stress in flukes (Selzer and Caffrey 2012), it also induces alternative activation of macrophages with the expression of YM1, Fizz (Relm α) and Arg1 (Donnelly et al. 2005).

Another major component of FhES, Cathepsin L cysteine peptidases, play a role in degrading host proteins into peptides (Robinson et al. 2013). Cathepsin L induces a suppressive dendritic cell phenotype which did not induce a Th2 immune response but attenuated a Th17 response (Dowling et al. February 2010) and in another study it was found that FhES induced a tolerogenic dendritic cell phenotype with the down-regulation of maturation markers and the priming of a Th2/T-reg response from CD4⁺ cells (Falcón et al.

2010). Cathepsin L also inhibited IFN- γ production when injected into a model of whole cell vaccination of *B. pertussis*, mimicking what was seen during *F. hepatica* co-infection (O'Neill, Mills and Dalton 2001, Brady et al. 1999a).

Another product from *F. hepatica* that is used to regulate the immune response is the tegumental antigen (FhTeg). FhTeg consists of a surface syncytial cytoplasmic layer bounded externally by a plasma membrane and covered by a glycocalyx which is rich in glycoproteins (Wilson et al. 2011). The surface layer contains many mitochondria and also two types of secretory bodies, T1 and T2. T1 secretory bodies are round and appear in a gradient within the syncytial cytoplasmic layer and are also more numerous in numbers. The T2 bodies are a biconcave shape and appear in a gradient opposite to that of the T1 bodies. The syncytial cytoplasmic layer is connected by cytoplasmic strands to cell bodies in the parenchyma below the muscle layers. The glycocalyx comprises of two layers, a continuous inner layer and an outer fibrillary layer. It consists largely of glycoproteins and has a net negative charge (Threadgold 1963). The tegument is shed from the fluke every 2-3 hours, so is in constant contact with the host and contributes to the evasion of the parasite by the hosts' immune response which is summarised in Table 1.

The tegument is involved in a number of different functions, like absorption of exogenous nutrients, synthesis of various substances and protection against bile and hosts enzymes (Dalton 1999). The removal of FhTeg from the fluke involves the use of detergents like Nonidet P40 and the subsequent removal of this detergent using detergent removal beads. This has some difficulties as the glycocalyx is insoluble and FhTeg contains a number of different proteins. The components of FhTeg and the glycocalyx have been mostly identified

and include a mixture of excretory-secretory proteins, membrane associated proteins, cytoskeleton and energy metabolism components (Morphew et al. 2013). Proteomic studies have identified some of the components of FhTeg, proteins such as acetylcholinesterase and alkaline phosphatase, annexins a,b and c and cathepsins B and L. FhTeg differs from FhES in that it is mostly composed of carbohydrates while FhES has high enzymatic activity and consists mostly of proteins but the biologically active component(s) of FhTeg are yet to be identified and further proteomic analysis are ongoing into the components of FhTeg with particular focus on the glyco-proteins (Wilson et al. 2011, Wuhrer and Geyer 2006).

Table 1. The effect of FhES and FhTeg on DCs, macrophages and mast cells.

	Excretory-secretory products	Tegumental antigens
Dendritic Cells	Immature phenotype ↑ (increase) IL-10 Induces Th2/Treg immune responses Inhibits TLR activation of dendritic cells	Immature phenotype Inhibits Th1 immune responses Inhibits phagocytosis Inhibits TLR and non-TLR activation of dendritic cells Inhibits NFkB and MAPKinases Induces SOCS3
Macrophages	Induces regulatory /M2 macrophages ↑ Fizz, Arginase and Relm1 ↑ IL-10, PGE2, TGFβ Promotes Th2 immune responses Inhibits Th1 immune responses	Induces Relmα
Mast Cells	Unknown	Immature phenotype Inhibits Th1 immune responses Induces Mast cell migration Inhibits TLR activation of mast cells Inhibits NFkB and MAP Kinases Induces SOCS3

The effect of FhTeg on a number of different immune cells has been studied. Dendritic cells stimulated with FhTeg fail to produce cytokines like IL-10 and they fail to induce Th2 or T-reg immune responses in CD4⁺ T-cells which differs from the effect of FhES. Stimulation with FhTeg and LPS, a TLR ligand, fail to enhance the expression of co-stimulatory molecules like CD80, CD86 and MHC class II and it also inhibits the production of inflammatory cytokines like IL-12p70 and TNF- α (Hamilton et al. June 2009). This may inhibit DC's ability to prime T-cells as enhanced co-stimulatory markers on DC's help in communication with T-cells. Further studies have shown that FhTeg induces suppressor of cytokines signalling-3 (SOCS3), a negative regulator of the TLR pathway, in dendritic cells and this may have an effect on the DC's ability to produce cytokines. FhTeg also inhibits NF- κ B and the MAPKs p38, JNK and ERK which are very important in inflammatory processes (Vukman, Adams and O'Neill 2013).

FhTeg also has an inhibitory role in mast cells. *In vivo*, the injection of FhTeg mimics infection with an increase in mast cell numbers in the peritoneal cavity due to the migration of cells to the area (Vukman et al. 2013a). *In vitro*, cells treated with FhTeg fail to mature and it inhibits Th1 responses. Like with dendritic cells, it inhibits TLR activation of cells with no production of IL-6, IFN- γ or TNF- α . FhTeg also enhances the expression of SOCS3 and affects the NF- κ B and MAPKs pathways. FhTeg treated mast cells also fail to drive Th2 immune responses from CD4⁺ cells and this may be due to the decrease in expression of ICAM1 on mast cells (Vukman et al. 2013b). As mast cells are critical in the clearance of microbial infections the suppression of Th1 responses by FhTeg may affect this. Mice injected with heat inactivated *Bordetella pertussis* antigen induce a strong Th1 immune response with the production of high levels of TNF- α from mast cells in the peritoneal cavity.

Co-injection with FhTeg markedly decreases the levels of TNF- α produced and the resulting Th1 immune responses (Vukman et al. 2013b).

Studies have also been recently done on the effect of FhTeg on macrophages. *In vivo*, the injection of FhTeg indirectly induces the alternative activation of macrophages in the peritoneal cavity and also increases the number of these cells to the area. These alternatively activated macrophages inhibit cytokine production from naïve CD4⁺ T-cells. Studies on Raw macrophage cell line have shown that FhTeg induces Relm- α from cells but not other markers of alternative activation like Arginase 1 (Adams et al. 2014).

1.11. Summary

Helminths and their antigens exert strong Th2/T-reg immune responses (Anthony et al. 2007) and can suppress Th1 immune responses but helminths can also have positive effects on autoimmune diseases and clinical trials are underway on a number of inflammatory diseases like multiple sclerosis and irritable bowel syndrome (Kuijk et al. 2012a, Heylen et al. 2015, Whary et al. 2001). We are interested in studying *F. hepatica* and its tegumental coat. Infection with *F. hepatica* down-regulated Th1 immune responses in mouse models (O'Neill et al. 2000) and also co-infection with *Bordetella pertussis* inhibits a Th1 immune response and the clearance of the bacteria (Brady et al. 1999a). The tegumental coat has also been shown to alter dendritic cell maturation, function and inhibits the production of Th1 and Th2 cytokines to TLR ligands (Hamilton et al. 2009a). The purpose of this study was to characterise the T-cell responses during *F. hepatica* infection and to investigate the role of FhTeg and CLRs on dendritic cells and T-cells from both murine and human origin.

1.12. Aims

The aims of the project are:

- To characterise further FhTeg treated dendritic cells and to show the role played by CLRs in FhTeg-dendritic cell interactions (Chapter 3)
- To characterise the T-cell responses during *F. hepatica* infection and to investigate if FhTeg stimulated DCs could mimic this response (Chapter 4)
- To investigate if FhTeg can interact directly with CD4⁺ cells and if CLRs play a role in this interaction (Chapter 5)
- To characterise the responses seen in human immune cells following treatment with FhTeg (Chapter 6)

Chapter 2.

Materials and Methods

2.1 Materials

2.1.1 Animals

PRODUCT	COMPANY
BALB/c mice (female)	Charles River (Kent, UK)
C57BL/6 mice (female)	Charles River (Kent, UK)
MR ^{-/-} mice	
Adult <i>Fasciola hepatica</i>	Abattoir (Navan, Ireland)
<i>F. hepatica</i> metacercariae	Baldwin Aquatics Ltd (USA)

2.1.2 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 (PBS)	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)

2.1.3 Antigens

PRODUCT	CATALOGUE #	COMPANY
anti-CD3	MAB4851	RnD (UK)
Ionomycin	I9657	Sigma-Aldrich (Wicklow, Ireland)
IL-2	MAB702	RnD (UK)
Lipopolysaccharide (LPS) (E. coli)	ALX-581-007	Alexis Biochemicals (Lausanne, Switzerland)
Phorbalmiristate acetate (PMA)	P8139	Sigma-Aldrich (Wicklow, Ireland)

2.1.4 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 Mouse	19852RF	Stemcell (France)
CD4⁺ Isolation kit Human	19662	Miltenyi Biotec, UK
Human IFN-γ ELISA kit	430103	Biolegend, (UK)
Human IL-2 ELISA kit	88-7025	eBiosciences, (UK)
Human IL-1β	88-7261	eBiosciences, (UK)
Human TNF-α	430203	Biolegend, (UK)
Mouse IFN-γ ELISA kit	555138	Biolegend (UK)
Mouse IL-10 ELISA kit	555252	Biolegend (UK)
Mouse IL-4 ELISA kit	555232	Biolegend (UK)
Mouse IL-5 ELISA kit	555236	Biolegend (UK)
Mouse IL-13 ELISA kit	88-7137-86	eBioscience (Hatfield, UK)
Mouse IL-2 ELISA kit	88-7024-77	eBioscience (Hatfield, UK)
Mouse TNF-α ELISA kit	88-7324-77	eBioscience (Hatfield, UK)
Mouse IL-12p70 ELISA kit	555256	BD Biosciences (UK)
PGD2 prostaglandin EIA kit	512031	CaymanChemical Company, (US)
PGE2 prostaglandin EIA kit	514531	CaymanChemical Company, (US)
Promofluor Protein Labelling kit	PKPFLK488P10	Promokine (Heidelberg, Germany)
Pyrogene Recombinant Factor C Endotoxin Detection System	50-658U	Lonza (Walkersville, USA)
MycoSensor PCR Assay Kit	302109	Agilent Technologies (Cork, Ireland)
RNA extraction kit	11828665001	Roche Diagnostics, (UK)
rt-PCR kit	04379012001	Roche Diagnostics, (UK)

2.1.5 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)
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)
Histopaque-1083	1083-1	Sigma-Aldrich (Wicklow, 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)
Lymphoprep		Stem Cell, France
Mannan	M7504	Sigma-Aldrich (Wicklow, Ireland)
Methanol	65543	Sigma-Aldrich (Wicklow, Ireland)
N-Acetyl-D-Galactosamine 4-sulphate (GalNAc-4-Sulphate)	A5926	Sigma-Aldrich (Wicklow, Ireland)
N,N,N',N'-Tetramethylethylenediamine	T9281	Sigma-Aldrich (Wicklow, Ireland)
Nonidet™ P 40 Substitute	74385	Sigma-Aldrich (Wicklow, Ireland)
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)
Triton X-100	BDH306324	VWR (East Grinstead, UK)
Tween 20	P1379	Sigma-Aldrich (Wicklow, Ireland)

2.1.6 Equipment

PRODUCT	CATALOGUE #	COMPANY
Analogue Stirred Water bath	NE4-22T	VWR (East Grinstead, UK)
Benchtop Microcentrifuge	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 (CO ₂) industrial	40-VK	BOC Gases Ireland (Dublin, Ireland)
FacsAria 1 flow cytometer		BD Biosciences (Oxford, UK)
Hemocytometer, Neubauer, Double cell		MSC Co. Ltd. (Dublin, Ireland)
Hotplate Stirrer	AGB1000	Jenway (Stone, UK)
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 (Merrington, 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 CO ₂ Water Jacketed Incubator	Model 3111	MSC Co. Ltd. (Dublin, Ireland)
Vortex mixer	SA8	Stuart (Stone, UK)
West balance	BL120S	Sartons (Goettingen, Germany)

2.1.7 Disposables

PRODUCT	CATALOGUE #	COMPANY
24 Well Plates	83.1836	Sarstedt. (Wexford,Ireland)
Coverslips	MLS17-20	Lennox Ltd (Dublin 12, Ireland)
15ml Tubes	62.554.502	Sarstedt. (Wexford,Ireland)
50ml Tubes	62.559.001	Sarstedt. (Wexford,Ireland)
25ml Pipettes	86.1685.001	Sarstedt. (Wexford,Ireland)
1.5ml Tubes	72.706.200	Sarstedt. (Wexford,Ireland)
96 Well Plates	82.1581	Sarstedt. (Wexford,Ireland)
Petri Dishes	82.1473.001	Sarstedt. (Wexford,Ireland)
6 Well Plates		Sarstedt. (Wexford,Ireland)

2.1.8 Software

PRODUCT	COMPANY
GraphPad Prism	GraphPad(USA)
FlowJo	Tree Star (Ashland, USA)
Origin	Origin Lab Corp. (Northampton, USA)

2.2 Methods

2.2.1 Animals

BALB/c and C57BL/6 mice 6-8 weeks old were purchased from Charles River (UK). Mannose receptor knockout mice on a C57BL/6 background were provided as a kind gift from Professor Padraic Fallon (Lee et al. 2002). An enhanced green fluorescent protein reporter gene was inserted at the start codon in Exon 1 of the MR gene. It contains a stop codon, which abolishes the expression of MR. The successful knocking out of the gene was detected by PCR genotyping and immunoblot. Mice were kept under specific pathogen free conditions at the Bio-resources unit, DCU. All mice were housed following guidelines from The Health Products Regulatory Authority and ethical approval was granted from the DCU ethics committee and The Health Products Regulatory Authority (HPRA.ie) before starting experiments, licence numbers B100/2833 and DCUREC/2010/033.

2.2.2 FhTeg Preparation

Fasciola hepatica tegumental antigen was prepared by adapting a previously published method (Hamilton et al. June 2009). Live worms were obtained from infected sheep or cattle from Irish Country Meats Abattoir, Navan. The presence of fluke in bile ducts was used to differentiate infected from non-infected livers. The flukes were transported back to the lab in warm RPMI media supplemented with 10% fetal calf serum (Biosciences, Dun Laoghaire) and 100u/ml penicillin/streptomycin (Sigma-Aldrich, Arklow). Back in the lab the flukes were washed three times in warm sterile PBS and 10 flukes were placed in 10ml of 1% Nonidet P40 for 30 minutes at 37 degrees. Nonidet P40 is a detergent which facilitates in the removal of FhTeg from the fluke, the supernatant was collected and the NP-40 was removed using detergent removal beads because detergent would interfere in cell assays (Bio-rad,US). Protein concentrations were measured using the bicinchoninic acid (BCA) protein assay kit

(see section 2.2.3) (Thermo Scientific, Leicestershire, UK), and endotoxin levels were assessed using the Pyrogene endotoxin detection system (see section 2.2.4). Negative values ensure that there is no contaminating endotoxin interfering in cell assays.

2.2.3 Bicinchoninic acid (BCA) assay

Determination of protein concentration on all samples used was performed using the bicinchoninic acid (BCA) assay in clear 96 well plates. The BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. In the first reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. In the second step, bicinchoninic acid reacts with the reduced cation that was formed in step one. 10 μl of each protein sample was placed in triplicate in a 96 well plate along with protein standards ranging from 2000 μg to 125 μg . The BCA reagents were mixed in a 1:50 dilution and 200 μl was added to each sample. The plate was incubated at 37 degrees for thirty minutes and the plate was then read in a micro-titre plate reader at 562nm. The averages of the triplicate values were used and standard curves were generated to determine the protein concentrations.

2.2.4 Endotoxin Testing

FhTeg was tested for endotoxin contamination using Pyrogene Recombinant Factor C Endotoxin Detection System (Lonza, USA). Endotoxin activates a serine protease catalytic coagulation cascade that results in the gelation of *Limulus* blood. Factor C which is isolated from *Limulus polyphemus* is 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. FhTeg gave similar or lower levels than background levels (0.01EU/ml).

2.2.5 *Fasciola hepatica* infection

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. The mice were infected by pipetting 20µl of the water containing the metacercariae orally. Following a two week infection, mice were sacrificed by cervical dislocation. To ensure an infection had taken place, pathology was investigated as monitored by tissue damage, migrating tracts in liver and enlarged spleens. Spleens were removed and processed (see section 2.2.13)

2.2.6 *Fasciola hepatica* injection

We have previously shown that by injecting FhTeg into the peritoneal cavity three times a week for three weeks, it will mimic infection by recruiting mast cells and alternatively activated macrophages (Adams et al. 2014, Vukman et al. 2013a). To investigate if FhTeg would mimic T-cell responses seen during infection, PBS (100µl) or FhTeg (10µg in 100µl) was injected intraperitoneally (i.p.) into mice and after 6, 24, 72 hours or 1 week, mice were sacrificed and mesenteric lymph nodes were removed and analysed for cell surface marker expression by flow cytometry. PBS or FhTeg (10µg in 100µl) was also injected over the sternum and 1 week later spleens were removed, analysed by flow cytometry for cell surface marker expression and re-stimulated with FhTeg (10µg/ml) with or without IL-2 (20ng/ml) or PMA (20ng/ml) and anti-CD3 (10µg/ml) to measure cytokine secretion. After 72 hours supernatants were removed and analysed for cytokine secretion (see section 2.2.10)

2.2.7 Generation of bone marrow derived dendritic cells

Bone marrow dendritic cells (BMDCs) were differentiated using a previously described method (Lutz, Manfred B 1999). Bone marrow was harvested from the tibia and fibia of a

female BALB/c, MR^{-/-} or C57BL/6 mouse by flushing with ice cold PBS. The cells were washed and seeded at 2×10^5 cells per ml in 10ml of RPMI supplemented with 10% fetal calf serum (Biosciences, Dun Laoghaire), 1% L-glutamine, 100u/ml penicillin/streptomycin (Sigma-Aldrich, Arklow) and 20ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF), GM-CSF obtained from a GM-CSF producing cell line X63 (a gift from Prof. Ton Rolink; University of Basel, Switzerland) is a growth factor which causes the differentiation of bone marrow cells into dendritic cells. Cells were fed on days 3, 6 and 8 and harvested on day 10. On day three, 10ml of fresh media was added with 20ng/ml of GMCSF, on day 6, 9ml of media was removed and discarded and 10ml of fresh media with 20ng/ml of GMCSF added. On day 8, 9ml of media was removed and spun down at 300g, the pellet was re-suspended in 10ml of fresh media containing 20ng/ml of GM-CSF and added back to the plate. On day 10, the loosely adherent cells were removed and dendritic cell purity was analysed by CD11c expression by flow cytometry, giving greater than 95% purity (93-99%) (Figure 2.2.7)

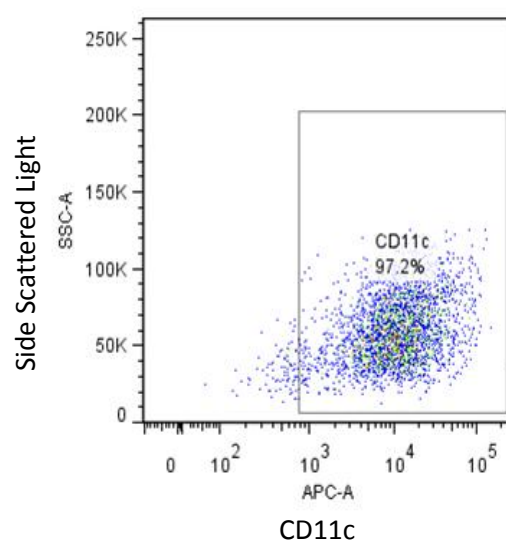


Figure 2.2.7. Purity of dendritic cells isolated from bone marrow. Dendritic cells were cultured for 10 days, on day 10, cells were analysed for CD11c expression as an indicator of purity using flow cytometry.

2.2.8 BMDC activation

On day 10 of culture dendritic cells were removed and spun down at 300g to pellet the cells and re-suspend in fresh media containing growth factors. The pellet was re-suspended in 1ml of PBS and cell number and viability was determined using trypan blue staining. The cells were spun down again and re-suspended in RPMI to a final concentration of 1×10^6 cells/ml. They were seeded at 1×10^6 cells/ml and stimulated with FhTeg (10 μ g/ml) for 2.5 hours before LPS (100ng/ml) was added.

To determine if the mannose receptor was playing a role in signalling following FhTeg stimulation, blocking experiments were performed using antibodies and sugars to block the interaction of FhTeg and MR. In blocking experiments, anti-MR (10 μ g/ml) (Abcam, Uk), Mannan (50 μ g/ml) and GalNAc-4-Sulphate (1mM) (Sigma-Aldrich, Ire) were added 30 minutes before the addition of FhTeg (10 μ g/ml). After a further 2.5 hours LPS (100ng/ml) was added to appropriate wells. After 18 hours supernatants were removed and analysed for cytokine secretion using ELISAs (see section 2.2.11)

2.2.9 Generation and stimulation of Splenocytes and CD4⁺ cells

In order to measure antigen specific immune responses following infection with *F. hepatica* metacercariae, spleens were isolated from control and infected mice and splenocytes obtained by passing the spleens through a 40 μ m and 70 μ m filter before counting and plating at 5×10^6 cells per ml in X-Vivo media (Lonza,USA) supplemented with 10% fetal calf serum (Biosciences, Dun Laoghaire) and 100u/ml penicillin/streptomycin (Sigma-Aldrich, Arklow). Splenocytes were stimulated with FhTeg (10 μ g/ml) in the presence or absence of

recombinant IL-2 (20ng/ml), phorbol 12-myristate 13-acetate, (PMA), (20ng/ml) and anti-CD3 (10µg/ml). PMA and anti-CD3 were used as positive controls as they will cause proliferation and cytokine production from splenocytes and CD4⁺ T-cells.

CD4⁺ T-cells were isolated using a negative selection CD4⁺ isolation kit (Stemcell, France) and cells with greater than 95 per cent purity were used as analysed by flow cytometry (Figure 2.2.9). CD4⁺ T-cells were plated at 2x10⁶ in X-Vivo media (Lonza, USA) and stimulated with PMA (20ng/ml) (Sigma-Aldrich,IRE) and anti-CD3 (10µg/ml) (RnD) in the presence or absence of FhTeg (10µg/ml). After 72 hours supernatants were removed and analysed for the production of IL-2, IL-4, IL-5, IFN-γ and IL-10 using commercial ELISA (see section 2.2.11), following manufacturer's instructions (Biolegend,UK).

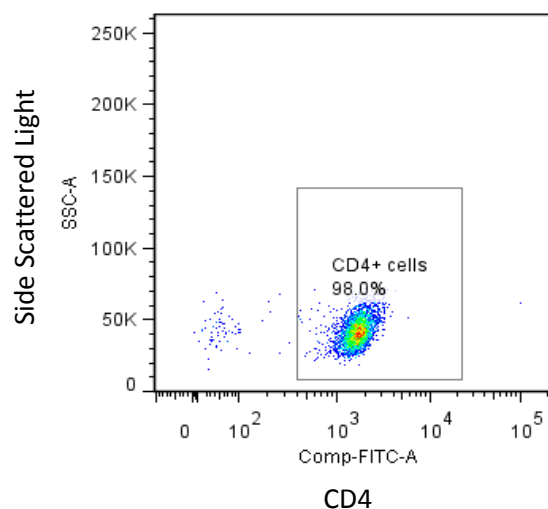


Figure 2.2.9 Analysis of purity of CD4⁺ T-cells used after isolation with negative selection kit. CD4⁺ cells isolated from spleens were labelled with a FITC CD4⁺ antibody and purity was found to be over 95% in each case, one representative experiment shown, purity at 98%.

2.2.10 Isolation and stimulation of human PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats which were sourced from The Irish Blood Transfusion Service, St James' Hospital, Dublin. PBMCs were isolated by density gradient centrifugation using Histopaque-1083 (Sigma Aldrich, Ire). Histopaque was layered at the bottom of a 50ml tube, isolated blood which was diluted 1:1 in sterile PBS was layered onto the histopaque. Tubes were centrifuged at 800g for 40 mins with no brake applied as sudden braking can disrupt the separating of blood into its different layers. During centrifugation, erythrocytes are aggregated and rapidly sediment as a pellet at the bottom of the tube. Lymphocytes and other mononuclear cells remain at the plasma/Histopaque interface. The layer of mononuclear cells are removed and washed three times with PBS before being counted.

PBMCs were plated at 1×10^6 cells/ml in complete RPMI and stimulated with FhTeg (10 μ g/ml) for 2.5 hours before the addition of PBS, LPS (100ng/ml) or PMA (20ng/ml) and Ionomycin (1 μ M). Supernatants were removed following a 24 hour stimulation and analysed by ELISA (see section 2.2.11). CD14⁺ cells were isolated by positive selection using CD14⁺ microbeads (Miltenyi Biotec, UK). Cells were stimulated with FhTeg (10 μ g/ml) for 2.5 hours before the addition of LPS (100ng/ml). Supernatants were removed and analysed for the production of TNF- α and IL-1 β by commercial ELISA. CD4⁺ cells were isolated by negative selection (Miltenyi Biotec, UK), isolated cells were stimulated with FhTeg (10 μ g/ml) for 2.5 hours before the addition of PMA (20ng/ml) and Ionomycin (1 μ M). PMA and Ionomycin were used to activate PBMCs to proliferate and produce cytokines. Following a 72 hour stimulation supernatants were removed and analysed by ELISA.

2.2.11 Cytokine Analysis

Cytokine secretion was measured using commercially available sandwich ELISA kits and following the manufacturer's guidelines (Biolegend UK, eBioscience UK, BD Biosciences UK). Briefly a monoclonal capture antibody for the cytokine of interest was coated onto the bottom of wells of a 96-well plate and left over night at four degrees. The plate was then washed three times with wash buffer (1X PBS with 0.05% Tween-20) to remove any unbound antibody. The wells were blocked to inhibit non-specific binding using 200µl of PBS containing 10% FCS for one hour at room temperature. The plate was then washed three times with wash buffer to remove unbound FCS and samples and standard dilutions (100µl to each well) were added in triplicate to the plate and left for two hours at room temperature. Following five washes to remove unbound sample, a detection antibody was then added to the plate and left for an hour at room temperature. The plate was then washed five times to remove unbound detection antibody, and a horse radish peroxidase- streptavidin labelled antibody was added to the plate for thirty minutes at room temperature. The plate was then washed seven times, to remove any unbound horse radish peroxidase and 100µl of TMB substrate reagent was added to each well, the plate was left in the dark for up to thirty minutes or until the colour change has become strong enough. 50µl of stop solution (0.16M Sulphuric Acid) was added to each well and the plate is read at 450nm on a plate reader.

2.2.12 Flow Cytometry

Cells were harvested after stimulation or isolation, washed twice in FACS buffer (100µl PBS with 2% FCS, 1mM EDTA) and then incubated with the following antibodies listed in Table 2.2.1, or the relative isotype control for thirty minutes at four degrees in the dark to stop any unwanted reactions taking place. The cells were then washed twice with FACS buffer to remove any unbound antibody and analysed on a BD FACS Aria.

For intracellular staining the cells were fixed with 4% formaldehyde for 10 minutes at 4 degrees after antibody incubation and then lysed with 0.5% Tween20 for fifteen minutes. The relevant antibody or isotype control was then added for thirty minutes at four degrees in the dark. The cells were then washed twice and analysed on a BD FACS Aria. The data was analysed using Flow Jo software (Treestar, USA). In all experiments unstained and single stained controls were used for gating and compensation.

Gating Strategy

For gating, splenocytes isolated from spleens from control and infected mice were gated on by excluding dead cells using forward and side scatter, (Figure 2.2.12 B). For gating on CD4⁺ cells, splenocytes were gated on by excluding dead cells and then by using CD4⁺ antibody to gate on CD4⁺ cells (Figure 2.2.12C). For BMDCs, cells were gated using CD11c antibody (Figure 2.2.7)

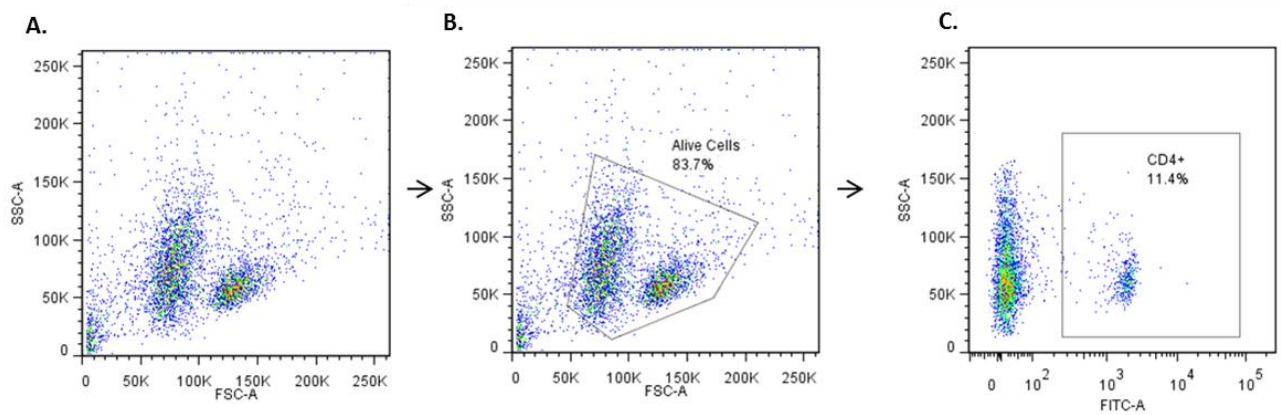


Figure 2.2.12. Gating strategy. **A.** Forward and side scatter of splenocytes before gating. **B.** Alive cells gated according to their cell size and granularity. **C.** CD4⁺ T-cells gated on.

Table 2.2.1. Antibodies used for flow cytometry

Antibody	Fluorochrome	Clone
CD4	FITC	GK1.5
CD4	APC	GK4.5
CTLA4	APC	UC10-4B9
CD206	FITC	C068C2
CD206	APC	C068C2
MGL	FITC	ERMP23
SIGNR1	Alexa Fluor647	eBio22D1
PD1	APC	J43
CD11c	APC	N418
F4/80	FITC	BM8
F4/80	APC	BM8
CD86	FITC	GL1
CD80	PE	16-10A1
CD40	PE	IC10
$\gamma\delta$-TCR	PE-Cy7	eBioGL3
CD49	PE-Cy7	DX5
CD62L	PerCP/CY5.5	MEL-14
CD44	PE-CY7	IM7

2.2.13 Proliferation Assay

Proliferation of cells was measured using the incorporation of the CFSE dye into cells and the halving of the fluorescence seen after each cell division (eBioscience, UK). Cells were labelled with 5 μ M CFSE dye for 10mins at room temperature in the dark before the remaining CFSE that had not been taken up by cells was quenched using ice cold PBS. The cells were washed three times with PBS before being re-suspended at 5x10⁶ cells per ml in X-vivo media (Lonza, USA) and stimulated with FhTeg (10 μ g/ml), with and without rIL-2 (20ng/ml) (RnD, UK), PMA (20ng/ml) (Sigma-Aldrich, Ire) and anti-CD3 (10 μ g/ml) (RnD, UK). After 24, 48 and 72 hours cells were removed and labelled with CD4⁺ APC, a second set of cells were also labelled with PI to check for viability. Cells were then analysed by flow cytometry (see section 2.2.12).

2.2.14 Cell Binding Assay

FhTeg and bovine serum albumin (BSA) were fluorescently labelled with a FITC-488 label using the Promofluor labelling kit according to the manufacturer's recommendations (Promokine, Germany). The labelling was performed using a reactive succinimidyl-ester of the dye of interest. The conjugates result from the formation of a stable covalent amide linkage of the dye to the protein. The protein-dye conjugates have fluorescence-excitation and fluorescence-emission at wavelengths of interest. Cells (50,000/well) were seeded in a 96 well plate. Where indicated cells were pre-incubated with EDTA (10mM), (Sigma-Aldrich), anti-MR (1 µg/ml), (Abcam, UK), mannan (100µg/ml), (Sigma-Aldrich) or GalNAc-4S (1mM), (Sigma-Aldrich) for forty five minutes at 37 °C. Subsequently, cells were incubated with 1 or 5 µg/ml of 488 labelled FhTeg at 37 °C for forty five minutes and washed in ice cold PBS before analysis using flow cytometry. As control for non-specific binding, cells were incubated with 5 µg/ml of FITC-488 labelled BSA.

2.2.15 Protein Extraction

Cells were isolated from control or infected spleens, or from cells stimulated overnight with antigens, washed twice and total protein was extracted using radio-immunoprecipitation assay (RIPA) buffer with protease and inhibitor cocktails added (Table 2.2.13). RIPA buffer is a detergent which aids in the lysis of cells and protein solubilisation but does not interfere with the activity of the protein and helps prevent protein degradation. A protease inhibitor was added to stop degradation of proteins by protease. Phosphatase inhibitor cocktails were also added to inhibit enzyme reactions which may degrade or modify proteins. After lysing in RIPA buffer the lysates were kept on ice for five minutes and centrifuged at 10000g for five minutes. The supernatants were transferred to new tubes and protein concentration was analysed by BCA (see section 2.2.3).

Table 2.2.13 Protein extraction

Reagent	Volume added	Concentration
RIPA Buffer	100µl	
Protease Inhibitor	1µl	1%
Phosphatase Inhibitor Cocktail 1	1µl	1%
Phosphatase Inhibitor Cocktail 2	1µl	1%

2.2.16 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to molecular weight using a previously described method (Laemmli 1970). SDS is an anionic detergent which denatures proteins and also imparts a negative charge evenly to the protein. This allows the proteins to be separated by size using acrylamide. 10% acrylamide resolving gels and 5% stacking gels were used (Table 2.3). Ammonium persulfate and temed were used to start a reaction in which methylenebisacrylamide forms cross links between two acrylamide molecules forming a gel. Stacking gels are also prepared which have a lower percentage to allow proteins to stack according to their size before being separated. An electric field is placed on the gel and the proteins migrate from the negative electrode through the gel towards the positive electrode. The larger proteins cannot move through small pores as they move through the gel and so the proteins are separated out based on size and charge.

Protein samples were prepared in SDS-PAGE loading buffer (Table 3) and boiled for 5 minutes to denature the proteins before being loaded onto the gels. A molecular weight marker was also loaded on each gel (LI-COR, UK), to distinguish protein sizes following separation. Gels were run at 150V for 1-2 hours using cold SDS-PAGE running buffer (Table 2.4).

Table 2.2.15. Reagents used for 10% acrylamide resolving gels and 5% stacking gels.

5% Stacking gels	Volume added	10% resolving gels	Volume added
30% Acylamide	670µl	30% Acylamide	4.62ml
1M Tris-HCL (pH6.8)	625µl	1M Tris-HCL pH (8.8)	3.8ml
dH ₂ O	2.7ml	dH ₂ O	5.6ml
10% SDS	50µl	10% SDS	150µl
10% APS	50µl	10% APS	75µl
Temed	25µl	Temed	25µl

Table 2.2.14. Components of SDS-Page loading buffer and SDS-Page running buffer.

SDS-PAGE Loading Buffer	Volume added	Concentration	Running Buffer	Volume added	Concentration
Tris	0.757g	0.625M	Tris	3g	25mM
Glycerol	5ml	50%	Glycine	14.4g	192mM
SDS	1g	10%	SDS	1g	0.2%
Bromophenol Blue	0.01g	0.1%	Total	1000ml	
2-Mercaptoethanol	0.5ml	5%			
Total	10ml				

2.2.17 Western Blot

Protein samples were run on an SDS-PAGE gel and then transferred to a PVDF membrane using an iBLOT (Lifesciences, Ire). Membranes were blocked with 1% skimmed milk to block non-specific interactions of primary antibodies or secondary antibodies with the membrane, before antibodies were added which are listed in Table 2.2.16. Blots were incubated with an anti-rabbit secondary for 1 hour at room temperature before being visualized with a chemiluminescent HRP substrate (Millipore, Ireland), exposed to film and processed using an FPM 100A processor (Fuji Film). Protein bands were quantified using

ImageJ analysis software (imagej.nih.gov). Levels of protein were normalised to a control gene, β -actin and the levels of protein are shown as fold increases over the untreated levels.

Table 2.2.16. Antibodies used for protein analysis

Primary Antibody	Dilution	Size of Product
RNF128	1:500	48kDa
β -actin	1:10000	42kDa

2.2.18 RNA extraction and cDNA synthesis

Cells from control or infected spleens or cells treated with antigens were harvested, washed twice with PBS and total RNA was extracted using a RNA isolation kit (Roche, UK), following manufacturer's guidelines. Cells were lysed in a lysis/binding buffer and RNases were inactivated. The mixture was added to a high pure filter tube which contains glass fiber fleece in a column and the RNA was trapped in this. DNase was added to the tube to eliminate genomic DNA and the RNA was then washed using two wash buffers which both contain ethanol to remove impurities and inhibitors which may affect downstream applications. The RNA was then eluted from the column using RNase and DNase free water. The quantity and purity of the extracted RNA was analysed using Nanodrop (Thermo-Fisher, Ire). cDNA was synthesised using a kit (Roche, UK) following manufacturers guidelines (Table 2.6). 1 μ g of RNA was used as starting material for cDNA synthesis.

Table 2.6.17. RTPCR reaction conditions

Temperature	Time
25 degrees	10 minutes
55 degrees	30 minutes
85 degrees	5 minutes

2.2.19 QPCR Gene Array Assays

RNA from control and infected spleenocytes was isolated using an RNA isolation kit (Roche, UK). The RNA from the control and infected samples were pooled and reverse transcribed to cDNA (Roche, UK). Samples were added to each well of a 96 well plate containing specific genes related to anergy, house-keeping genes and reverse transcription controls. The data was analysed using Qiagens online data analysis tool.

2.2.20 QPCR

RNA from control and infected spleenocytes was isolated using a high pure RNA isolation kit (Roche, UK). The quantity and quality of the RNA was checked using Nanodrop (thermo-science, Ire). RNA was reverse transcribed to cDNA using random primers and a reverse transcription kit (Roche, UK). Primer probes (Roche, U K) with primer efficiency of 2.0 were used to analyse gene expression, for the following genes listed in Table 2.2.19 A-B. Primer probe master mix with a 6-carboxyfluorescein (FAM) labelled enzyme was used (Table 2.2.19C). Three housekeeping genes were used as internal standards, β -actin(NM_007393.3), GAPDH (NM_008084.2) and GusB (β -glucuronidase) (NM_010368.1) Gene expression was analysed using a Light Cycler 96 (Roche, UK). Samples were maintained for 10 seconds at 95°C as initial step, then 15 seconds at 95°C and 60 seconds at 60°C through 40 cycles and Pfaffl's method was used to determine relative gene expression (Pfaffl 2001).

Table 2.2.19A. Forward and reverse primer sequences of primers used for qPCR analysis (Mouse).

Gene Name	Sense	Anti-sense
β-actin	GGATGCAGAAGGAGATTACTGC	CCACCGATCCACACAGAGTA
GAPDH	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG
GusB	GATGTGGTCTGTGGCCAAT	TGTGGGTGATCAGCGTCTT
CTLA4	TCACTGCTGTTTCTTTGAGCA	GGCTGAAATTGCTTTTCACAT

ICOS	AACCTTAGTGGAGGATATTTGCAT	CCTACGGGTAGCCAGAGCTT
RNF128	AAATGCAAGAGCTCAAAGCAG	GCAGCTGAAGCTTTCCAATAG
ITCH	TTGATGCGAAGGAATTAGAGG	GGTGTAGTGGCGGTAGATGG
Egr2	CCTCTACCCGGTGCAAGAC	GTCAATGTTGATCATGCCATCT
SOCS3	ATTCGCTTCGGGACTAGC	AACTTGCTGTGGGTGACCAT

Table 2.2.19B. Forward and reverse primer sequences of primers used for qPCR analysis (Human)

Gene Name	Sense	Anti-sense
β-actin	TTCCTCCCTGGAGAAGAGCTA	CGTGGATGCCACAGGACT
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
GusB	TCGCCATCAACAACACACTC	TCTGGACAACTAACCCTTGG
CTLA4	TCACAGCTGTTTCTTTGAGCA	AGGCTGAAATTGCTTTTCACA
RNF128	AACACGTGCAGTCAACAAATG	TGGGTTGTCAACATGAGGAA
Egr2	GGAGGGCAAAGGAGATACC	CAGAAAAGCCGTTTTGGAGA

Table 2.2.19C. Working solutions for qPCR

Component	Volume
Primer Probes master mix (FAM)	10μl
Primers	2μl
H₂O	3μl
cDNA	50ng in 5μl

2.2.21 Statistics

All data were analysed for normality prior to statistical testing by Prism[®] 6.0 (GraphPad Software Inc, La Jolla, CA, USA) software. Where multiple group comparisons were made, data were analysed using one-way or two 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.

High mannose and sulphated glycoproteins on *Fasciola hepatica* surface coat exhibit immune modulatory properties independent of the mannose receptor

3.1. Introduction

Infection with parasitic worms (helminths) modulates the host immune system by biasing T helper (Th) cells towards a Th-2/T-reg immune response (Allen and Maizels 2011, McSorley, Hewitson and Maizels 2013), while simultaneously impairing pro-inflammatory Th1/Th17 immunity. This polarisation is due to the interaction of helminth derived molecules with pattern recognition receptors on innate immune cells such as dendritic cells, macrophages and mast cells that drive the polarisation of T-cells (Taylor, van der Werf and Maizels 2012a). Many helminth proteins and lipids are glycosylated, and the initial interaction of these molecules with innate immune cells is *via* C-type lectin receptors (CLRs) (Vázquez-Mendoza, César Carrero and Rodriguez-Sosa 2013).

Several reports have pointed to a role for CLRs in mediating immune regulatory processes driven by helminth-derived glycoconjugates (Everts et al. 2012, Tundup, Srivastava and Harn 2012a, van Die and Cummings 2010a). *Schistosoma mansoni* soluble egg antigens signal through several CLRs, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), Macrophage galactose lectin (MGL) and Mannose receptor (MR), inhibiting dendritic cells maturation and influencing the development of Th2 immune response (van Liempt et al. 2007). Similarly, *Trichuris suis* antigens signal through the CLRs, MR and DC-SIGN which are involved in inhibiting a pro-inflammatory dendritic cell phenotype. Recent studies, demonstrated the direct interaction of MR with *Fasciola hepatica* and *S. mansoni* derived antigens (Everts et al. 2012).

Tegumental antigens (FhTeg) are released continuously by *F. hepatica* during infection and studies have demonstrated that it exhibits potent Th1 immune suppressive properties *in vivo*

by suppressing serum levels of the Th1 mediators IFN γ and IL-12p70 in the mouse model of septic shock. FhTeg-activated dendritic cells and mast cells are hypo-responsive to TLR activation thereby suppressing the production of inflammatory cytokines and co-stimulatory molecules important in driving adaptive immune responses (Hamilton *et al*, 2009). FhTeg mechanism of action is independent of TLRs and has been linked to the suppression of NF- κ B and MAPK pathway (Vukman *et al*, 2013) and enhanced expression levels of suppressor of cytokine signalling (SOCS) 3, a negative regulator of the TLR and STAT3 pathway. The tegument is a biological matrix rich in glycoconjugates that remains continually exposed to the host immune system and is unique to each *Fasciola* species.

The macrophage mannose receptor (MR) is a type-I membrane protein with a cytoplasmic domain involved in antigen processing and receptor internalisation and three different types of binding domains at its extracellular region, multiple C-type lectin-like carbohydrate-recognition domains (CRDs) responsible for Ca²⁺-dependent binding to terminal mannose, fucose or *N*-acetyl glucosamine, a fibronectin type II (FNII) domain involved in collagen binding and an *N*-terminal cysteine-rich (Cys-MR) domain that mediates Ca²⁺-independent binding to sulfated sugars such as SO₄-3-Gal or SO₄-3/4-GalNAc. Glycomic studies on FhTeg have revealed that it is rich in N-glycans with high mannose type terminal structures, which contain GlcNAc and/or Man and these structures would have high binding affinity for the CRDs on the MR. *F. hepatica* antigens have been shown to interact with MR (Guasconi L *et al* 2011) and to further expand our knowledge of the immuno-modulatory capacity of FhTeg on dendritic cells, this study will focus upon FhTeg-MR interactions.

3.2. Experimental Design

This chapter focuses on the interaction of FhTeg with dendritic cells (generated from Balb/c mice) and the role C-type lectin receptors play in this. Firstly the binding of FhTeg was examined and a possible role for MR and MGL was investigated by using specific antibodies and sugars for the receptors to block the interaction of FhTeg with cells. Cells were pre-incubated with EGTA (10mM), anti-MR (1 µg/ml), mannan (100µg/ml), or GalNAc-4S (1mM), for forty five minutes at 37 °C. Subsequently, cells were incubated with 1 or 5 µg/ml of 488 labelled FhTeg at 37 °C for forty five minutes and washed in ice cold PBS before analysis using flow cytometry. As control for non-specific binding, cells were incubated with 5 µg/ml of FITC-488 labelled BSA.

We have previously shown that FhTeg causes the induction of SOCS3 in dendritic cells (Vukman, Adams and O'Neill 2013). To investigate if MR plays a role in this and in the inhibition of cytokine secretion, cells were incubated with anti-MR (10µg/ml), Mannan (100µg/ml) or Gal-NAc-4-S for 30 minutes before the addition of FhTeg (10µg/ml). Following 2.5 hours stimulation cells were washed and lysed for RNA analysis. QPCR was used to measure the expression of SOCS3. Cells were also stimulated as above but after 2.5 hours LPS was added as a bacterial ligand which will enhance IL-12p70 production from cells, to investigate if using blocking antibodies could reverse cytokine secretion as previous studies have shown that FhTeg inhibits LPS mediated IL-12p70 production (Hamilton et al. June 2009). Following 18 hours stimulation, supernatants were removed and analysed for the production of IL-12p70.

To investigate the role MR plays in FhTeg/DC interactions, DC's were generated from background (C57bl/6) MR knockout mice and the ability of FhTeg to bind, to enhance SOCS3 and to inhibit cytokine secretion was measured as above.

3.3. Results

3.3.1. FhTeg binds to DCs in a calcium dependent manner

From previous studies we know that FhTeg has an inhibitory effect on the maturation of dendritic cells after co-incubation with bacterial antigens like LPS (Hamilton et al. June 2009). To investigate the binding of FhTeg and its possible receptors, FhTeg and BSA were labelled with a FITC-488 label. DCs were stimulated for 30 mins with or without EGTA (10mM) and then stimulated with FITC-488 labelled FhTeg (1, 5 and 10 μ g/ml) or BSA (10 μ g/ml) as a control for non-specific binding for 45 minutes. The cells were washed twice and analysed for binding using flow cytometry. FITC-488 labelled BSA showed no significant binding compared to unstained cells. FhTeg showed significant binding to cells at 1 μ g, 5 μ g and 10 μ g ($p < 0.0001$) (Figure 3.3.1). This binding was inhibited by the addition of EGTA to cells before incubation with FhTeg ($p < 0.0001$) but the binding was not completely blocked to unstained levels (Figure 3.3.1A).

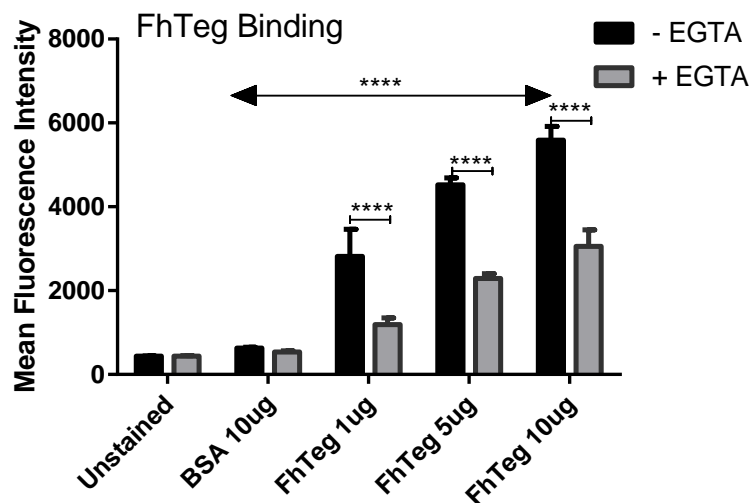


Figure 3.3.1. FhTeg binds to dendritic cells and this is calcium dependent. FhTeg significantly binds to dendritic cells at 1, 5 and 10 μ g/ml ($p < 0.0001$). This binding can be inhibited using EGTA for all concentrations ($p < 0.0001$). Data is presented as the mean \pm SD of one experiment with the mean of triplicate values shown. The

experiment was repeated three times and one representative experiment shown. Data analysed using one-way anova.

3.3.2. Binding of FhTeg to dendritic cells can be inhibited using sugars and antibodies specific to the mannose receptor.

FhTeg is composed of high mannose glycans and both MR and MGL have binding specificities for this, we investigated if those receptors are involved with binding of FhTeg to dendritic cells and if the binding could be suppressed with antibodies and sugars. Dendritic cells were stimulated with 488-FhTeg in the presence or absence of anti-MR (1µg/ml), mannan (50µg/ml) or GalNAc-4-sulphate (1mM). FhTeg bound significantly to DC's ($p<0.0001$), anti-MR, Mannan and GalNAc-4-Sulphate significantly inhibited the binding of 488-FhTeg ($p<0.01$) to dendritic cells by both mean fluorescence intensity (MFI) and percentage of cells (Figure 3.3.2A-B).

To examine the role of MGL in binding of FhTeg to DC's, cells were also incubated with antibodies and sugars specific to the MGL receptor, anti-MGL (1µg/ml) and Gal-NAc (10µg/ml). FhTeg significantly bound to DC's ($p<0.0001$) and both anti-MGL and Gal-NAc did not significantly inhibit binding of FhTeg to dendritic. The percentage of cells that FhTeg bound to was significantly inhibited using both anti-MGL and Gal-NAc (Figure 3.3.2 C-D).

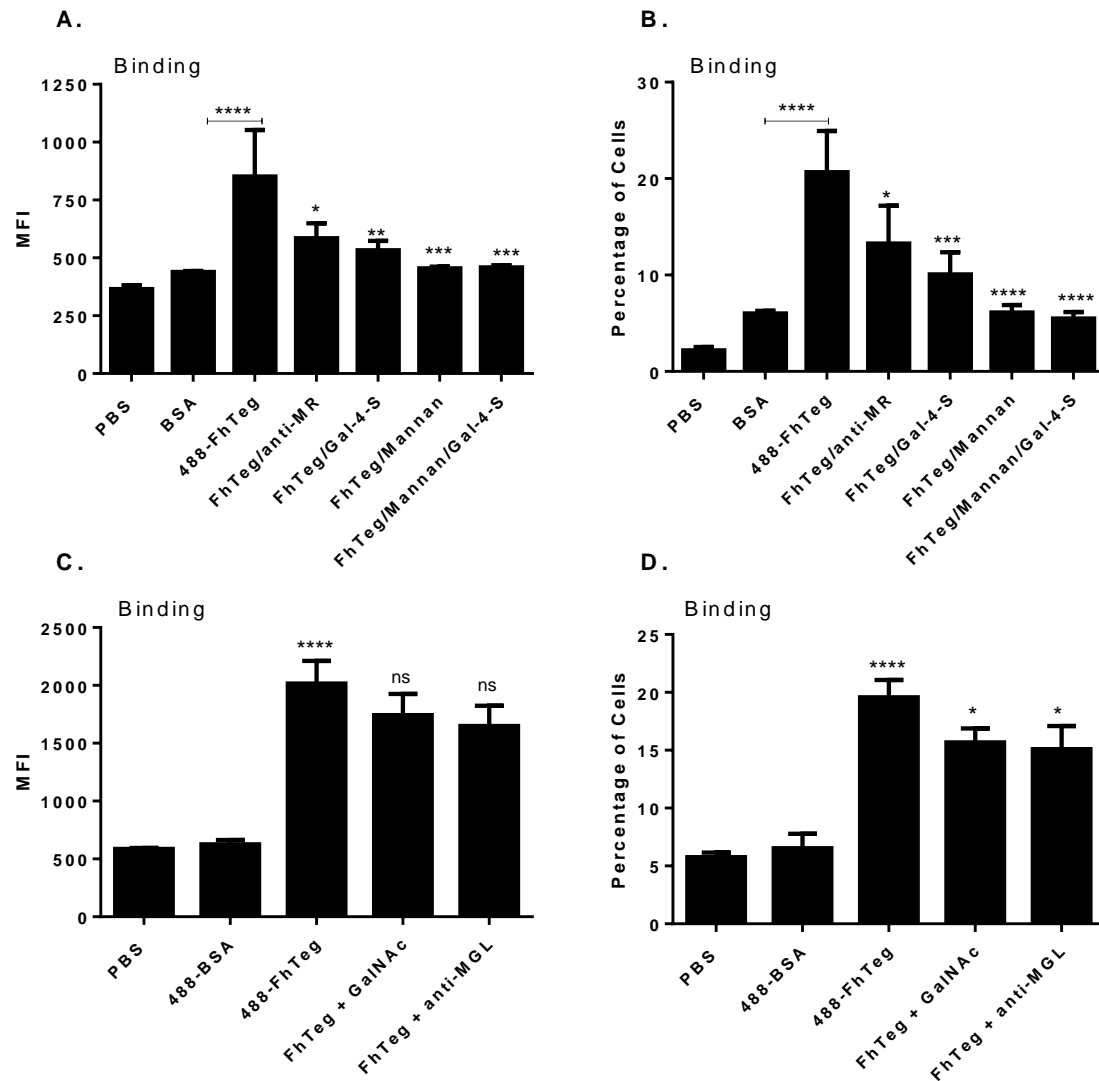


Figure 3.3.2 The mannose receptor has a role to play in the binding of FhTeg to dendritic cells but the MGL receptor does not. A-B. FhTeg significantly binds to DC's compared to BSA controls ($p < 0.0001$). Mannan ($p < 0.001$, $P < 0.0001$), anti-MR ($p < 0.05$) and Gal-4-S ($p < 0.001$, $p < 0.0001$) inhibit the binding of FhTeg to DCs. **C-D.** Anti-MGL and Gal-NAc did not have a significant effect on the binding of FhTeg to DC's by MFI but significantly inhibited the percentage of cells which FhTeg bound to ($p < 0.05$). Data shown is the mean \pm SD of one experiment with the mean of triplicate values shown. The experiment was repeated three times and one representative experiment shown, data analysed using one-way anova.

3.3.3. The macrophage galactose lectin does not play a role in the suppressive ability of FhTeg

The macrophage galactose lectin (MGL) does not seem to play a role in the binding of FhTeg to dendritic cells but there are binding epitopes specific for MGL on the surface of *F. hepatica* (manuscript in preparation). To examine if sugars or antibodies against MGL would inhibit the suppressive activity of FhTeg, DC's were stimulated with Gal-NAc (10 μ g/ml) or anti-MGL (10 μ g/ml) for 30 mins before the addition of FhTeg for a further two hours, the expression of SOCS 3 was measured by qPCR. FhTeg significantly enhances SOCS3 expression ($p<0.05$) but neither Gal-NAc nor anti-MGL inhibit this (Figure 3.3.3 A).

To investigate cytokine responses, DC's were stimulated with anti-MGL (10 μ g/ml) for 30 minutes before the addition of FhTeg (10 μ g/ml), following a further 2.5 hours stimulation LPS (100ng/ml) was added. Cells were incubated over-night and cytokines were measured for levels of IL-12p70. FhTeg significantly suppresses IL-12p70 production ($p<0.05$), the addition of anti-MGL does not reverse this (Figure 3.3.3 B).

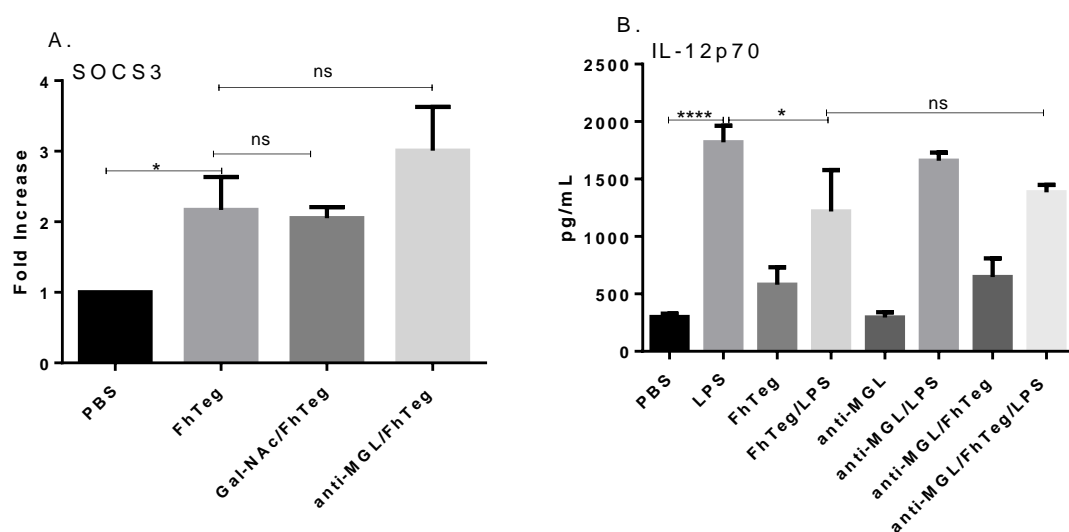


Figure 3.3.3. The macrophage galactose lectin does not play a role in the suppressive ability of FhTeg. A. FhTeg significantly enhances SOCS3 expression compared to PBS ($p<0.05$) but neither Gal-NAc nor anti-MGL

inhibit this. **B.** LPS significantly enhances IL-12p70 production compared to PBS ($p < 0.0001$) and FhTeg significantly suppresses this ($p < 0.05$). The addition of anti-MGL does not reverse FhTeg suppression of IL-12p70. The data shown is the mean \pm SD of two independent experiments. Data analysed using one-way anova.

3.3.4. Inhibition of FhTeg with the carbohydrate inhibitor GalNAc-4S suppresses SOCS3 expression while mannan reverses FhTeg's ability to modulate cytokine secretion in BMDCs.

In previous studies we demonstrated that FhTeg up-regulates SOCS3 *in vitro* (Vukman *et al*, 2013), a negative regulator of the TLR4 signalling pathway (Morgensen *et al*, 2009) and here we determine if SOCS3 RNA levels can be inhibited by the addition of GalNAc-4S and mannan to DCs in culture prior to stimulation with FhTeg (10 μ g/ml). Incubation with 1mM GalNAc-4S for 30 min prior to FhTeg stimulation reversed SOCS3 transcription to basal levels, whereas mannan did not alter SOCS3 expression (Figure 3.3.4 A). Another property of FhTeg is its ability to suppress the LPS-induced production of pro-inflammatory cytokines in BMDCs (Hamilton *et al*, 2009). Pre-incubation of BMDCs with mannan and not GalNAc-4S prior to LPS and FhTeg stimulation reversed IL12p70 secretion (Figure 3.4.4 B-C).

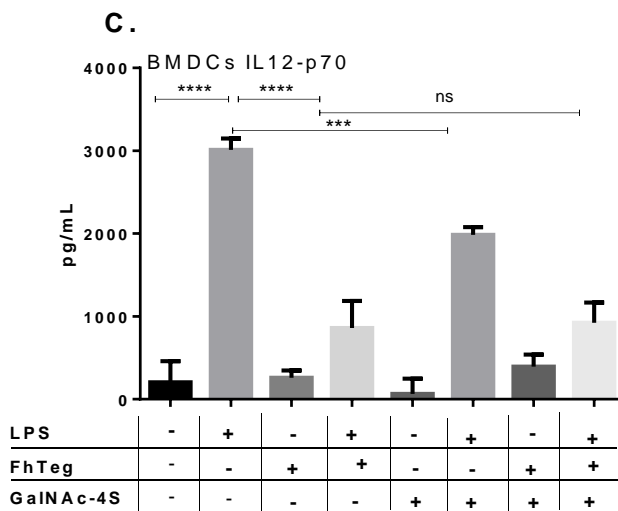
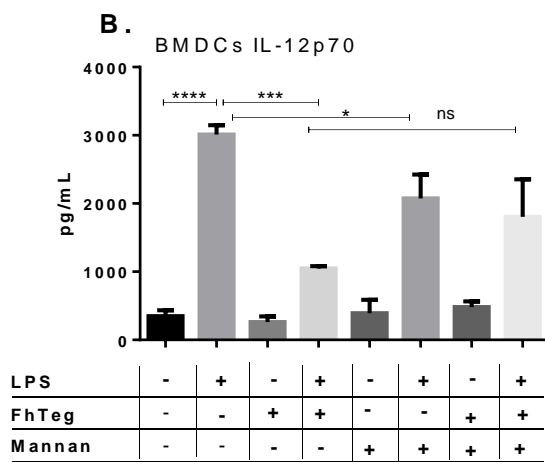
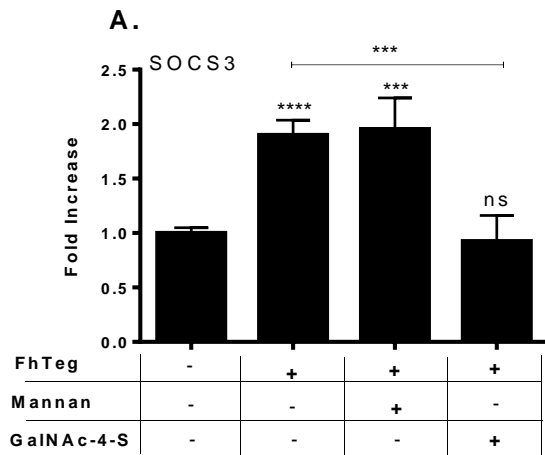


Figure 3.3.4 Inhibition of FhTeg with the carbohydrate inhibitor GalNAc-4S suppresses SOCS3 expression while mannan reverses FhTeg's ability to modulate cytokine secretion in BMDCs. **A.** FhTeg significantly enhances SOCS3 expression compared to PBS ($p<0.0001$). Mannan does not inhibit SOCS3 expression but GalNAc-4-S does ($p<0.001$). Data are presented as the mean \pm SD of three independent experiments. **B-C.** LPS significantly enhances IL-12p70 production compared to PBS ($p<0.0001$) and FhTeg significantly inhibits this ($p<0.0001$). Mannan restores IL-12p70 levels but GalNAc-4-S does not. Data are presented as the mean \pm SD of two independent experiments. Data analysed using one-way anova.

3.3.5. The immune properties of FhTeg are independent of the MR receptor.

Since high mannose and sulphated glycans interact with the MR receptor we obtained BMDCs from MR knockout mice to determine the role of FhTeg-MR interactions. In the absence of MR there was a statistically significant decrease in the expression of MR on the surface of BMDCs (Figure 3.3.5 A-B). However in the absence of MR FhTeg induced SOCS3 expression and suppressed LPS induced cytokine expression of BMDCS (Figure 3.3.5 C-D).

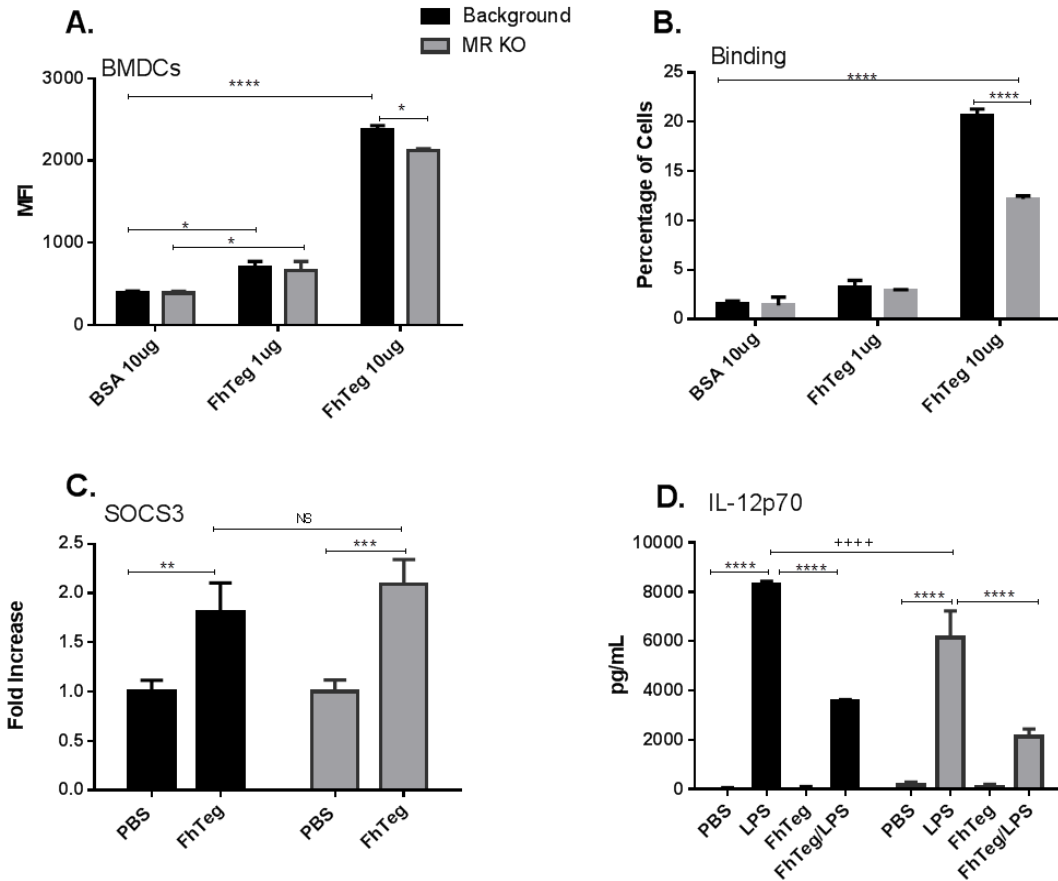


Figure 3.3.5 The immune properties of FhTeg are independent of the MR receptor. **A.** FhTeg binds significantly at both 1 and 10 μ g/ml ($p<0.05$, $p<0.0001$) to both background and knockout mice compared to BSA. There was a significant difference in binding between background and MR knockout mice at 10 μ g/ml ($p<0.05$). **B.** Binding of FhTeg to DC's from both background and knockout mice was analysed using percentage of cells that bound to FhTeg. FhTeg binds significantly to DC's from both background and knockout mice ($p<0.0001$) and this was significantly inhibited in MR knockout mice ($p<0.0001$). **C.** FhTeg significantly enhances SOCS3 expression in both background and MR knockout DC's compared to PBS, with no significant differences between the two groups ($p<0.01$, $p<0.001$). **D.** LPS enhanced IL-12p70 production in both background and MR knockout DC's ($p<0.0001$) and FhTeg significantly inhibited this ($p<0.0001$). Data are presented as the mean \pm SD of two independent experiments with 3 mice in each experiment. Data analysed using one-way anova for differences within groups, for differences between groups two-way anova was used.

3.4. DISCUSSION

We have previously shown that FhTeg has potent anti-inflammatory properties; it can inhibit the production of IL-12p70 and TNF- α from dendritic cells when co-stimulated with a wide range of TLR ligands, it can also modulate cell surface expression with no enhancement of CD80, CD86 or MHCII receptors (Hamilton et al. 2009a). FhTeg also induces SOCS3 expression in dendritic cells which is a negative regulator of cytokine signalling pathways (Vukman, Adams and O'Neill 2013). In this study we have characterised dendritic cells following FhTeg stimulation and demonstrated that the mannose receptor is not involved in priming the dendritic cell phenotype seen.

We have recently shown that FhTeg-derived *N*-glycans are composed abundantly of high mannose type structures. These glycans are widely distributed on the tegument of adult flukes with abundance especially on the spines and suckers (manuscript in preparation). These high mannose glycans may have binding specificities to CLRs like MR, MGL, SIGN-R1 or Dectin 1. The macrophage mannose receptor (MR) is a type-I membrane protein with a cytoplasmic domain involved in antigen processing and receptor internalisation and three different types of binding domains at its extracellular region, multiple C-type lectin-like carbohydrate-recognition domains (CRDs), a fibronectin type II (FNII) domain and an *N*-terminal cysteine-rich (Cys-MR) domain (Martinez-Pomares 2012). The macrophage galactose type lectin has two orthologues in mouse, MGL1 and MGL2. MGL1 has binding specificities for Lewis X, galactose and terminal Gal-NAc structures and MGL2 binds terminal Gal-NAc (Tsuiji et al. 2002). Here we have shown that FhTeg binds to dendritic cells partially in a calcium dependent way, as EDTA did not completely block binding of FhTeg to dendritic cells. MR

has both calcium dependent and independent binding and as FhTeg is a complex mix of over 70 proteins some differences in how they may bind was expected. Interestingly, only antibodies and sugars specific to the mannose receptor significantly inhibited binding of FhTeg to DC's, antibodies and sugars for MGL had little or no effect on binding. The role of CLRs has been studied for a number of helminth infections; MR has been implicated in *S. mansoni* infection and *in vitro* after stimulation of dendritic cells with antigens. In early stages of infection the ES of *S. mansoni* is rich in glycoproteins which bind to MR, this regulates IFN- γ from CD4⁺ cells and inhibits a Th1 immune response (Paveley et al. 2011), similarly *S. mansoni* derived omega 1 also signals through MR and drives Th2 immune responses (Everts et al. 2012). Studies using *T. crassiceps* antigens showed less binding to macrophages generated from MGL1 knockout mice than wild type controls and during infection also favoured a Th2 immune response with a higher parasite load than in controls (Montero-Barrera et al. 2014).

We have previously shown that FhTeg enhances the expression of SOCS3 in dendritic cells (Vukman, Adams and O'Neill 2013). SOCS3 is an intracellular protein regulating the duration or intensity of cytokine-induced signal *via* a negative feedback inhibition mechanism. SOCS 3-transduced DCs exhibit low expression levels of stimulatory signals (i.e. MHC, CD86, IL-12p70 and IFN- γ) and a tolerogenic/DC2 phenotype with decreased ability to induce T cell proliferation (Li et al. 2006), similarly to FhTeg-stimulated DCs. SOCS proteins are induced *via* JAK/STAT signaling and, among other signals, stimulation of TLRs (Murray 2007). Here we have shown the reversal of SOCS3 expression using GalNAc-4-Sulphate but not Mannan which seems to enhance the expression. This points to sulphated and non-sulphated glycans having an immuno-modulatory role in FhTeg/dendritic cell interactions. Using antibodies and sugars specific for MGL had no reversal of SOCS3 expression. In previous studies FhTeg has

inhibited the TLR activation of dendritic cells by inhibiting the secretion of IL-12p70 and TNF- α from LPS stimulated cells (Hamilton et al. June 2009). As we can reverse the expression of SOCS3 which may be playing a role in the cytokine suppression we investigated if the cytokine suppression could also be reversed. Pre-incubation with mannan reversed IL-12p70 suppression but GalNAc-4-Sulphate did not. As FhTeg is a complex mix of over 70 proteins, the mechanism of action of FhTeg may be more complex than affecting just one pathway.

Other studies have found the MR receptor to be involved *in vivo* following *F. hepatica* infection and *in vitro* following stimulation of macrophages with excretory/secretory products (Guasconi et al. 2011). They found that the interaction between macrophages and FhTeg was significantly decreased by pre-incubation with mannan or anti-MR blocking antibody in binding assays by flow cytometry and they also observed that the high levels of Arginase I activity, IL-10 and TGF- β expression induced on peritoneal macrophages by *F. hepatica* infection *in vivo* or FhES stimulation *in vitro* were partially inhibited by the use of MR blocking antibodies or sugar ligands both *in vivo* and *in vitro*. To investigate the importance of MR in FhTeg and dendritic cell interactions, DC's were generated from MR knockout mice and a number of experiments were repeated. FhTeg binding to BMDCs was significantly reduced but its suppressive effect was not abrogated. An inhibition of IL-12p70 was still observed when DCs were co-stimulated with FhTeg and LPS and an enhancement of SOCS3 was also observed. These results point to a role for MR in binding of FhTeg but not to its suppressive ability. This could be due to another role for MR other than signal transduction such as cell to cell communication. FhTeg is a complex mix of many proteins so it may not work through one receptor but may require a number of receptors to exert its influence. A number of studies into helminth infections have used knockout mice to try to

identify the receptors responsible for modulating the immune system. Studies using MR knockout mice and *T. suis* infection have shown that although IL-6 production from bone marrow derived macrophages generated from MR knockout mice partially depended on MR, the clearance of the parasite did not differ in knockout mice compared to wild-type mice (DeSchoolmeester et al. 2009b).

As some of the major components of FhTeg are carbohydrates, and we have shown that MR plays a role in the binding of FhTeg but not in the signalling pathways, other CLRs may play a role in the immunomodulatory effect seen on cells. Several helminth products have been shown to signal through CLRs (Tundup, Srivastava and Harn 2012b) and it is thought they signal through CLRs as an escape mechanism from the hosts' immune response (van Die and Cummings 2010b). *S. mansoni* soluble egg antigens have been shown to signal through several CLRs; DC-SIGN, MGL and MR and inhibit DC maturation and induce a Th2 immune response (van Liempt et al. 2007). The nematode *T. suis* has also been shown to be involved in CLR signalling. Glycans isolated from the parasite signal through MR and DC-SIGN and induce, as was seen with *S. mansoni*, a DC phenotype which inhibits bacterial TLR activation and the activation of an inflammatory immune response (Klaver et al. 2013). Dectin 2 has also been implicated in the induction of IL-1 β production from DCs following *S. mansoni* SEA stimulation (Ritter et al. 2010). Further studies need to be completed to identify the receptor or receptors responsible for the induction of the signalling pathways in BMDCs following FhTeg stimulation.

In this study we have shown that FhTeg binds to dendritic cells in a calcium dependent way and the binding can be inhibited by using specific antibodies and sugars for the mannose

receptor but MR is not involved in the immunomodulatory effects seen in DC's following FhTeg incubation. We have previously shown that FhTeg induces SOCS3 and inhibits cytokine secretion following bacterial ligand stimulation. Here we show that this is independent of FhTeg binding through MR, as studies on MR knockout DC's showed no difference in SOCS3 expression and on cytokine secretion. Further studies would need to be carried out to identify the receptor or receptors involved in initiating the immune response in DC's following FhTeg stimulation.

Chapter 4.

***Fasciola hepatica* tegumental antigens induce anergic T-cells via dendritic cells in a mannose receptor dependent manner.**

4.1 Introduction

Helminth parasites can survive within their hosts for many years by suppressing T-cell driven protective immune responses and Th1/Th2 mediated pathology through the induction of regulatory networks that suppress inflammatory responses (Taylor, van der Werf and Maizels 2012a, Belkaid and Rouse 2005, McKee and Pearce 2004, Grainger et al. 2010). These regulatory networks include regulatory T cells (T-reg) that in the context of helminth infection is widely understood. However, less is currently known about regulatory response termed *in-vivo* anergy or adaptive tolerance. T-reg cells suppress the immune response to helminth driven Th1/Th2 immune pathology through the induction of IL-10 and TGF- β . *Brugia malayi*, infection induces a regulatory response with enhanced FoxP3 expression and increased cell surface expression of CTLA4, a negative regulator of T-cell function (McSorley et al. 2008) while *Schistosoma haematobium* infection in humans has also been associated with FoxP3⁺ T-reg cells with the highest percentage of this cell population observed in younger patients (Nausch et al. 2011).

Few studies have examined the role of anergic T-cells during helminth infection. The induction of this cells population is thought to be the result of the persistent contact of parasite antigen with immune cells during chronic helminth infection (Borkow et al. 2000). Anergic T-cells differ from Treg cells in that they do not express FoxP3 or produce IL-10 or TGF- β . Instead anergy is defined as a hyporesponsive state with the failure of T-cells to proliferate or produce cytokines when re-stimulated with helminth antigens *in vitro* (Schwartz 2003). Anergic T-cells do not secrete IL-2, a factor important for T-cell proliferation and effector responses. However, the addition of IL-2 can overcome this hyporesponsiveness, restoring helminth driven T-cell responses. Gene analysis studies have

identified a number of genes including RNF128 (GRAIL), ITCH, Egr2 and 3 and CblB that are involved in the induction and maintenance of T-cell anergy (Zheng, Zha and Gajewski 2008, Harris 2004). The expression of PD1 and CTLA4 by T-cells are also defining markers of anergy. T-cell anergy is one of the main causes of T-cell unresponsiveness in *Schistosoma mansoni* infection, mediated by the up regulation of PD-L1 on the surface of macrophages (Smith et al. 2004) and also with the up regulation of RNF128 (GRAIL) in T-cells isolated from chronic infection (Taylor et al. 2009).

Fasciola secreted molecules can mimic the immune responses observed during infection, in particular, its excretory/secretory products (FhES) which comprise mainly of enzymes induce strong Th2/Treg immune responses with enhanced M2 macrophage and mast cell populations (Adams et al. 2014). The tegumental coat antigens (FhTeg), *Fasciola's* second major antigen source are shed every two to three hours which have been demonstrated to impair TLR driven immune responses in both dendritic cells and mast cells by suppressing TLR induced cytokines secretion and co-stimulatory marker expression. FhTeg induces SOCS3 a negative regulator of the TLR and STAT3 pathway which can explain its immune modulatory properties (Vukman, Adams and O'Neill 2013). Little is known about T-cell responses following *F. hepatica* infection or FhTeg stimulation, here we will characterise the T-cell responses to FhTeg during infection and following injection over the sternum.

4.2 Experimental Design

This chapter investigates the T-cell responses in mice following infection and if the injection of FhTeg can mimic this. It also shows a role of FhTeg treated dendritic cells in the induction of anergy in CD4⁺ cells. Firstly mice (Balb/c) were infected by pipetting 20µl of water containing 20 metacercariae orally. After two weeks mice were sacrificed by cervical dislocation and spleens were removed and re-stimulated with FhTeg (10µg/ml) in the presence or absence of IL-2(20ng/ml) to see if an antigen specific immune response could be restored, PMA (20ng/ml) and anti-CD3 (10µg/ml) were also used as a positive control. The levels of IL-2, IL-5, IL-4, IL-10 and IFN-γ were measured. To investigate if spleenocytes and CD4⁺ cells could proliferate, CFSE was used to measure proliferation after re-stimulation with FhTeg (10µg/ml) and IL-2 (20ng/ml), annexin V and PI staining was used to ensure apoptosis was not the cause of T-cell unresponsiveness.

Studies have shown helminths can induce both anergy and T-reg cells we used a qPCR gene array to investigate if genes relating to anergy induction and maintenance were up-regulated in spleenocytes isolated from infected mice. To investigate if the fold increases seen were significant individual qPCR assays were performed on triplicate samples isolated from control and infected mice for a number of genes which were highly expressed on the qPCR gene array. Protein levels were also measured to test if the protein levels were elevated. Flow cytometric analysis of cell surface markers were also analysed to see if any increase in PD1 or CTLA4 expression was seen. As lymph nodes are very important in the induction of tolerance to food proteins and are an early point of contact for immune cells after the ingestion of metacercariae, the levels of PD1 were assessed on cells isolated from lymph nodes from 24, 48, 72 hours and 1 week infection.

To investigate if FhTeg can mimic infection it was injected over the sternum of mice and mice were sacrificed after 72 hours and 1 week time points. Spleenocytes were isolated from spleens and re-stimulated with FhTeg (10µg/ml) and IL-2 (20ng/ml) and also PMA (20ng/ml) and anti-CD3 (10µg/ml). The levels of PD1 were also analysed by flow cytometry on CD4⁺ T-cells. Protein levels of RNF128 were also analysed to see if its expression was enhanced.

We have previously shown that FhTeg induces an immature dendritic cells phenotype with no enhancement of co-stimulatory markers CD80, CD86 or MHC II, and no production of cytokines. To investigate if these DCs could drive anergy in CD4⁺ cells, FhTeg treated DCs were co-cultured for 72 hours with CD4⁺ cells isolated from naïve mice in the presence of anti-CD3 (1µg/ml). Supernatants were analysed for the production of IL-2, IL-4, IL-5 and IFN-γ and mRNA levels of EGR2 and 3, RNF128, ICOS, CTLA4 and Zap70 were also analysed. As FhTeg is rich in mannose rich glycoproteins, the involvement of the mannose receptor was investigated. Co-cultures were performed using FhTeg treated DC's generated from both background (C57bl/6) and MR knockout mice and cytokine production and qPCR was performed as above.

4.3 Results

4.3.1. Splenocytes isolated from *F. hepatica* infected mice do not produce FhTeg-specific immune responses; however these responses are restored following the addition of IL-2.

We have demonstrated that FhES induces strong antigen specific-Th2/Treg immune responses in splenocytes isolated from *F. hepatica* infected mice (Adams et al. 2014). Here, antigen specific immune responses to FhTeg were examined in mice following *F. hepatica* infection. Splenocytes isolated from control or infected mice failed to induce FhTeg-specific IL-5, IL-4, IL-10 (Figure 4.3.1 B-D) and IFN- γ (Figure 4.3.1 E). The lack of antigen specific immune responses could be explained by a lack of antigen specific IL-2 as splenocytes from control and infected mice failed to produce significant levels of IL-2 upon FhTeg re-challenge while stimulation of splenocytes with PMA/anti-CD3 induced significant levels of IL-2 in both groups ($p<0.0001$; Figure 4.3.1 A). The levels of IL-5 ($p<0.001$), IL-4 ($p<0.01$) and IL-10 ($p<0.05$) were significantly increased when splenocytes from infected but not control mice were challenged with FhTeg in the presence of IL-2 compared to FhTeg alone (Fig 4.3.1 B-D). However, the levels of IFN- γ remained unchanged (Figure 4.3.1 E). Splenocytes isolated in mice from all groups produce significant levels of cytokines when treated with PMA/anti-CD3 (Figure 4.3.1 A-D) and no significant differences were observed between groups except IFN- γ where significant difference was observed between control and infected groups (Figure 4.3.1 E, $p<0.0001$) which supports previously published findings (O'Neill et al. 2000).

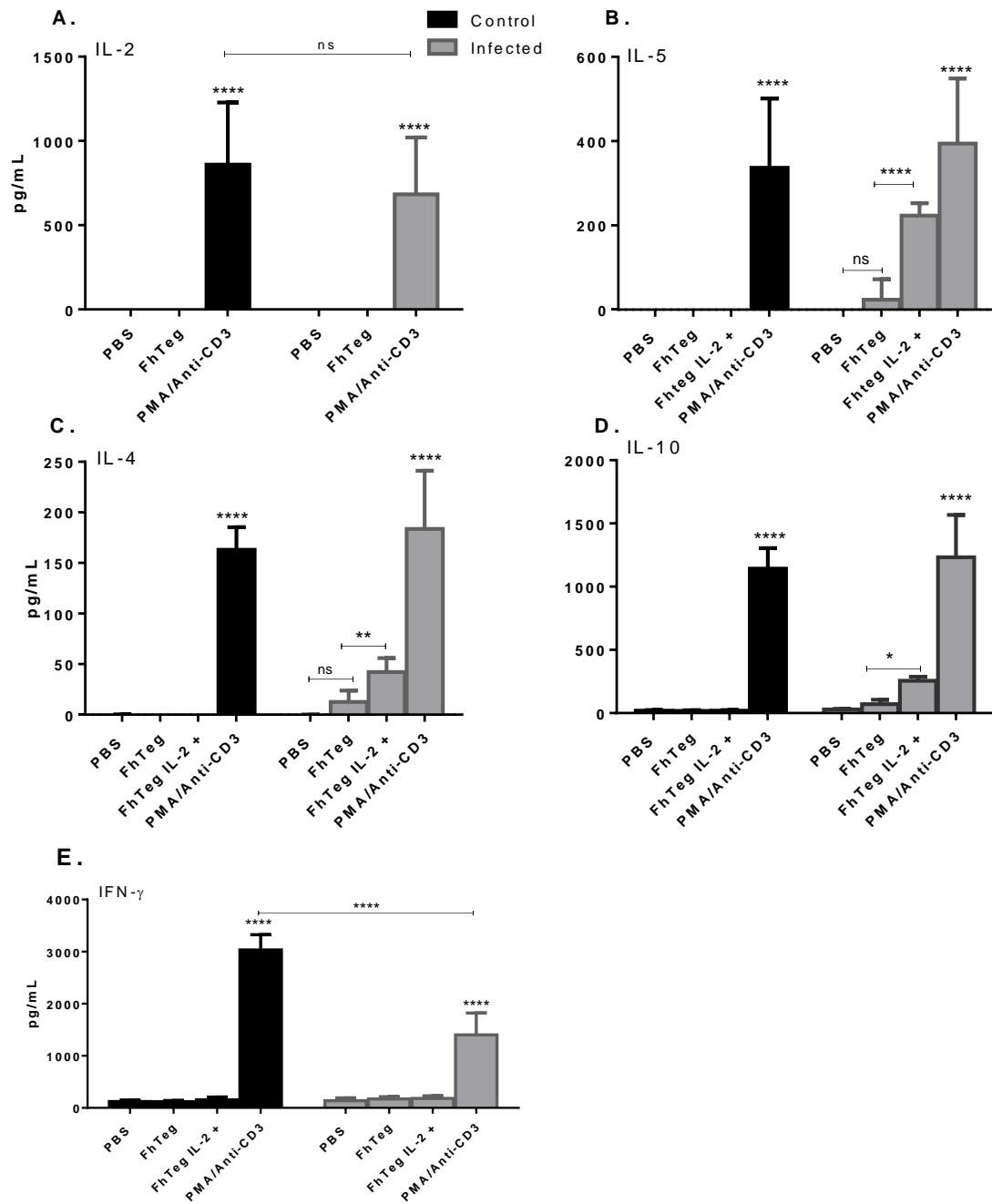


Figure 4.3.1. Splenocytes isolated from *F. hepatica* infected mice do not produce FhTeg-specific immune responses; however these responses are partially restored following the addition of IL-2. A. PMA and anti-CD3 significantly enhanced IL-2 production in both control and infected groups ($p < 0.0001$) but FhTeg did not. B. The addition of IL-2 significantly enhanced IL-5 ($p < 0.0001$), IL-4 ($p < 0.01$) and IL-10 production ($p < 0.05$) but not IFN- γ compared to FhTeg alone. Data are presented as the mean \pm SD of three independent

experiments, with three to four mice in each group. Data analysed using one-way anova for differences within groups, for differences between control and infected groups, two-way anova was used.

4.3.2. The lack of IL-2 in CD4⁺ T-cells from *F. hepatica* infected mice alters proliferation but not apoptosis.

IL-2 is essential for the differentiation of effector T-cells into Th-subsets (Liao et al. 2011) and is involved in numerous other important roles such as T-cell proliferation or programmed cell death (Malek and Castro 2010). Splenocytes isolated from *F. hepatica* infected mice failed to produce significant levels of IL-2 after re-stimulation with FhTeg and therefore it is possible that this lack of IL-2 could inhibit CD4⁺ T-cell proliferation or induce programmed cell death. To examine the effects of FhTeg on T-cell proliferation splenocytes were isolated from infected mice and labelled with CFSE prior to culturing with PBS, FhTeg or PMA/anti-CD3. IL-2 was also added to examine if this altered the proliferative activity of these cells. After 72 hours cells were washed and analysed by flow cytometry with a focus upon the number of undivided CD4⁺ cells.

In the control mice the number of undivided CD4⁺ T-cells was 75.5% and while FhTeg in the presence of IL-2 enhanced proliferation the difference was not statistically significant compared to the PBS group. In contrast CD4⁺ cells isolated from infected mice had significantly fewer undivided cells; however a comparable level of proliferation was observed between CD4⁺ T-cells stimulated with PBS (28.85%) and FhTeg (26.15%). The addition of IL-2 resulted in the number of undivided cells decreasing significantly ($p < 0.05$) and this was also less than the positive control (PMA/anti-CD3 group). CD4⁺ T-cells from infected and control mice when stimulated with PMA/anti-CD3 showed a similar level of undivided CD4⁺ T-cells (Fig 4.3.2 A).

To determine if the lack of IL-2 is inducing cell death or apoptosis, CD4⁺ T-cells were stained with annexin V and PI staining for analysis by flow cytometry. Cells treated with 4% formaldehyde were used as a positive control and PBS cells were used as a normal indicator of cell death. There was no significant difference between FhTeg treated cells or PMA/anti-CD3 for double staining for annexin V and PI or single PI staining alone (Figure 4.3.2 B) confirming that in this model the lack of IL-2 does not induce apoptosis in CD4⁺ T-cells.

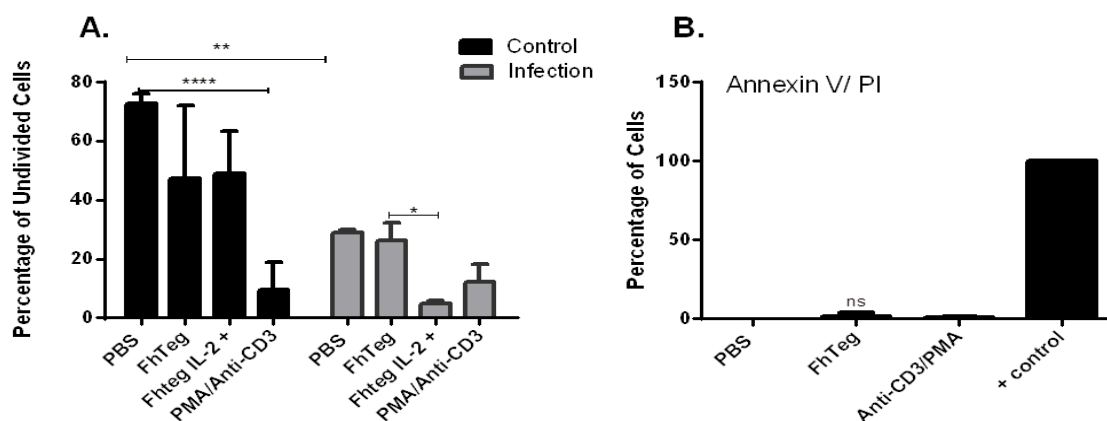


Figure 4.3.2. The lack of IL-2 in CD4⁺ T-cells from *F. hepatica* infected mice alters proliferation but not apoptosis. A. Control cells treated with PBS had undivided cells at 75% the addition of FhTeg +/- IL-2 did not significantly alter this. The addition of PMA and anti-CD3 significantly lowered this to less than 20%. Cells isolated from infected mice showed a lower percentage of undivided cells treated with PBS and FhTeg at 20% but IL-2 significantly lowered this ($p < 0.05$). **B.** There was no significant difference in annexin V or PI staining in PBS or FhTeg treated cells. Data are presented as the mean \pm SD of 2 independent experiments, with three mice in each group. Data analysed using one-way anova, for differences between groups, two-way anova was used.

4.3.3. FhTeg induces markers of anergy in splenocytes isolated from *F. hepatica* infected mice

Splenocytes isolated from infected mice were examined for markers of anergy by initially performing a qPCR gene array using pooled control (n=4) and infected samples (n=4) (Figure 4.3.3A). The results demonstrated enhanced expression of RNF128 (GRAIL), CTLA4 and IL-10 (> 5 fold increase) while EGR2 and ICOS had a fold increase of 3.66 and 3.53 respectively. To confirm these findings the most characterised anergic genes were selected and gene expression of RNF128, ITCH, EGR2 and ICOS was measured by qPCR. For control genes beta-actin, GusB and GAPDH were used. The gene expression of RNF128, ITCH, EGR2 and ICOS were significantly enhanced ($p < 0.05$) with a 70, 5, 60 and 3.5 fold increase respectively (Fig 4.3.3B-E). Protein levels of RNF128 were also analysed in control and infected splenocytes and a fourfold increase in RNF128 was observed compared to uninfected controls ($p < 0.0001$) (Fig 4.3.3F).

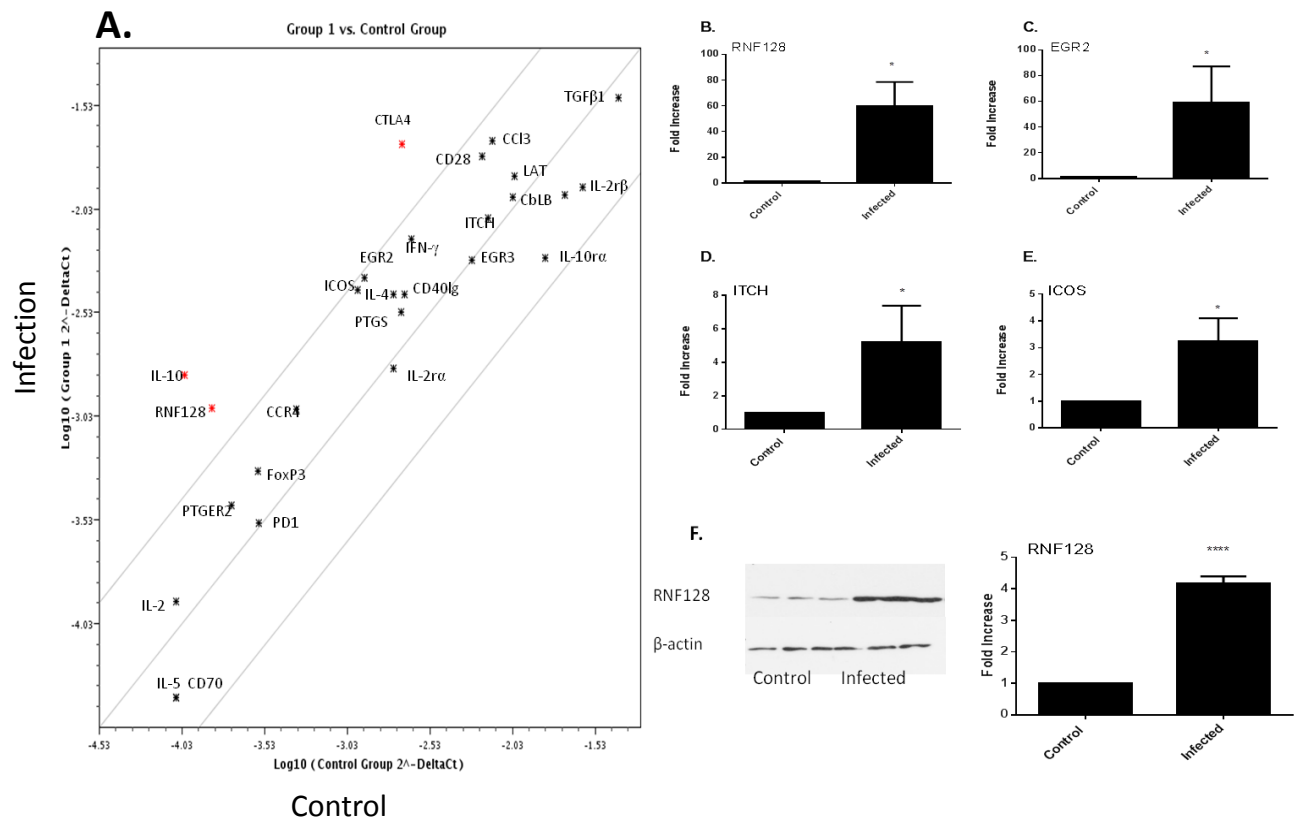


Figure 4.3.3. FhTeg induces markers of anergy in splenocytes isolated from *F. hepatica* infected mice. A.

A qPCR gene array with genes relating to anergy and tolerance was used with four pooled control and four pooled infection samples. The x-axis represents the control group and the y-axis represents the infection group.

B-E. Individual expression of RNF128, ERG2, ITCH and ICOS genes were shown to be significantly enhanced in splenocytes from infection samples compared to controls, using qPCR ($p < 0.05$). The data shown is the mean \pm SD of three independent experiments with 3-4 mice in each experiment. **F.** Protein expression of RNF128 in splenocytes isolated from control and infected mice ($p < 0.0001$). Data shown is the mean \pm SD of 1 independent experiment, with three mice in the group, * $p < 0.05$, **** $p < 0.001$. Student t-test used for differences between control and infection.

4.3.4. The expression of PD1, CTLA4 and RNF128 were also enhanced in CD4⁺ T-cells isolated from *F. hepatica* infected mice

CD4⁺ T-cells from control and infected mice were analysed for PD1, CTLA4 and RNF128 expression using flow cytometry (PD1, CTLA4) and western blot (RNF128). CD4⁺ T-cells isolated from infected mice had a significant increase in the expression of PD1 ($P < 0.0001$), and CTLA4 (Figure 4.3.4 A,B), ($P < 0.05$) by flow cytometry while RNF128 protein expression was higher in CD4⁺ T-cells with an 120 fold increase compared to non-infected controls (Figure 4.3.4C), ($p < 0.05$).

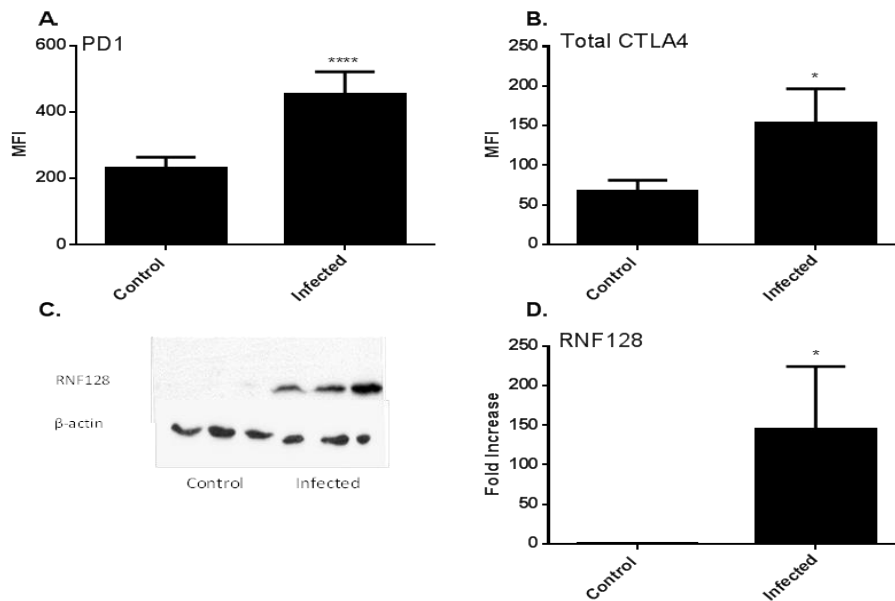


Figure 4.3.4. The expression of PD1, CTLA4 and RNF128 were also enhanced in CD4⁺ T-cells isolated from *F. hepatica* infected mice. A-B. Both PD1 (p<0.0001) and CTLA4 (p<0.05) were enhanced on CD4⁺ T-cells from infection compared to control cells. **C-D.** CD4⁺ T-cells isolated from spleens had a fold increase of 150 of RNF128 compared to non-infected controls (p<0.05). Data shown is the mean ±SD of 3 independent experiments, with three mice in each group, in **C** one representative experiment shown. Student T-test used for differences between control and infection.

4.3.5. PD1 is up-regulated on mesenteric lymphnodes after 72 hours of infection

Lymph nodes are critical in initiating and mounting an immune response against foreign pathogens (Buettner and Bode 2012). Mesenteric lymph nodes are one of the first areas in contact with a pathogen after oral infection and can either mount a response or induce tolerance. As our route of infection is oral, mesenteric lymph nodes from control and infected mice were pooled and analysed by flow cytometry for PD1 expression after 24 hours, 72 hours, 1 and 2 weeks. After 24 hours there is no significant change in the levels of PD1

expression (Figure 4.3.5 A). But after 72 hours, 1 week and 2 weeks of infection PD1 is up regulated with a peak of MFI after 1 week (Figure 4.3.5 B-D).

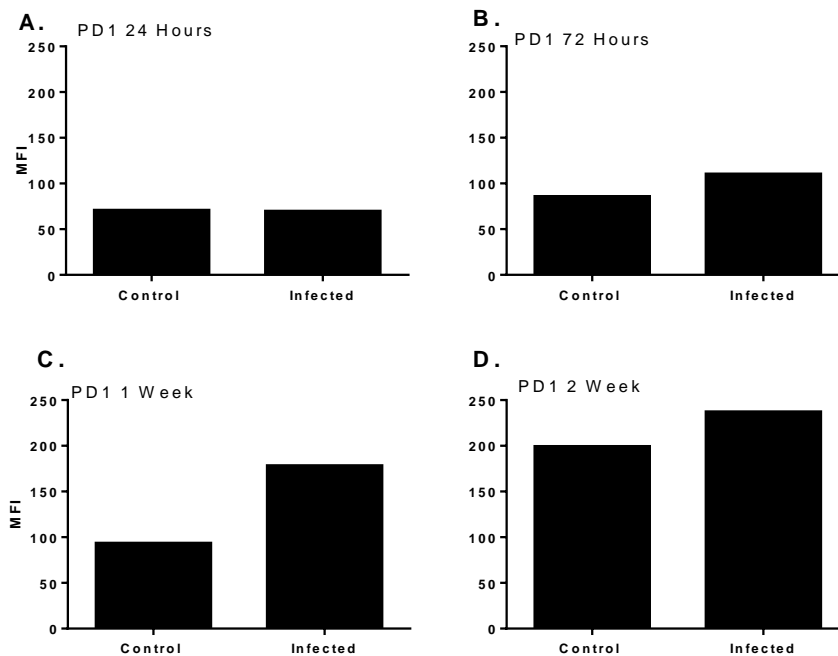


Figure 4.3.5. PD1 is up-regulated on mesenteric lymph nodes after 72 hours of infection. Mesenteric lymph nodes from control and infected mice were pooled and analysed for PD1 expression by MFI by flow cytometry. PD1 is enhanced from 72 hours to two weeks with a peak at one week of infection. Data is shown from three control pooled and three infected pooled samples.

4.3.6. FhTeg can directly induce anergy *in vivo*

In previous studies we have shown that when FhTeg is injected into the peritoneum of mice, it can mimic the immune response associated with infection by recruiting mast cells and M2-like macrophages into the peritoneal cavity (Adams et al. 2014). To investigate if FhTeg can mimic infection by inducing anergy directly, PBS or FhTeg was injected over the sternum of

mice and 1 week later spleens were removed. The splenocytes were re-stimulated as outlined in previous assays to determine if cytokine production could be restored with the addition of rIL-2. After 72 hours supernatants were removed and analysed for the production of IL-4, IL-5, IFN- γ and IL-2. Similar to infection no levels of IL-2 were observed in splenocytes from injected mice after stimulation with FhTeg (Fig 4.3.6 A). After 1 weeks significant levels of IL-5 and IL-4 ($P<0.0001$) and also IFN- γ ($P<0.05$) were detected after the addition of rIL-2 (Fig 4.3.6 B-D). Splenocytes were also examined for markers of anergy and the protein expression of RNF128 (Fig 4.3.6 E) and cell surface expression of PD1 (Fig 4.3.6 F) were significantly enhanced ($p<0.05$) in cells from FhTeg injected mice compared to controls.

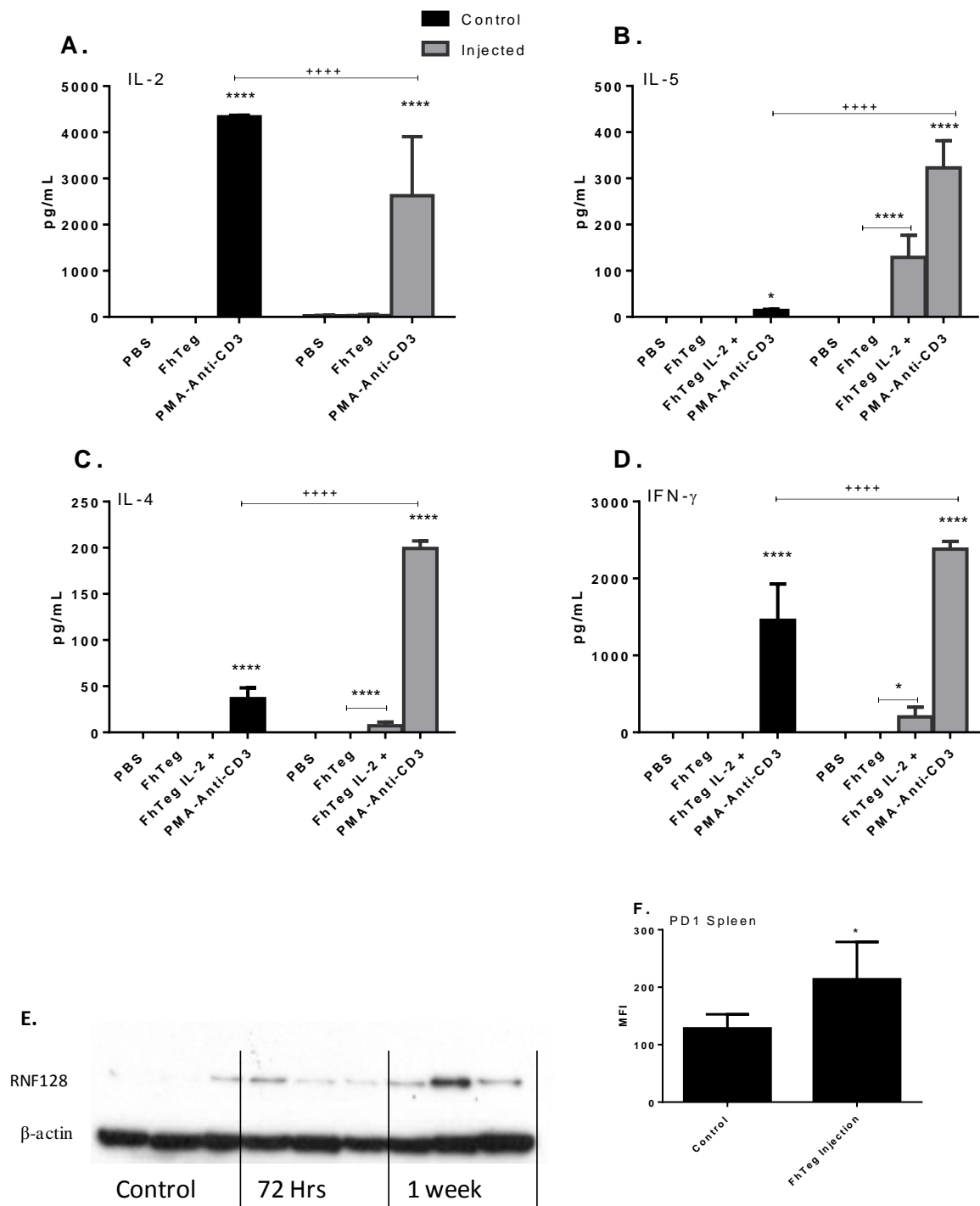


Figure 4.3.6. FhTeg can directly induce anergy *in vivo*. A-D. In splenocytes re-stimulated with FhTeg, no IL-2 production was observed but significant enhancement of IL-2 was seen after PMA and anti-CD3 stimulation ($p < 0.0001$) in both control and injected samples. The levels of IL-5 ($p < 0.0001$), IL-4 ($p < 0.0001$) and IFN- γ ($p < 0.05$) were all partially reversed after the addition of IL-2 compared to FhTeg alone. FhTeg alone did not

enhance the production of any cytokine measured. **E.** RNF128 was found to be enhanced after 72 hours and 1 week in splenocytes isolated from 72 hours and 1 week samples. **F.** Splenocytes isolated after 1 week were analysed by flow cytometry for the expression of PD1 and it was found to significantly enhanced ($p<0.05$). The data shown is the \pm SD of two independent experiments with three mice per group. Student T-test used for differences between control and infection groups in (**F**). One way anova used for differences within groups in **A-D**, two way anova used for differences between groups.

4.3.7. FhTeg activated dendritic cells induce markers of anergy in CD4⁺ T-cells

We have previously shown that FhTeg stimulated dendritic cells induce a novel cell population that are characterised by SOCS3^{high}, CD80^{low} and CD86^{low} (Vukman, Adams and O'Neill 2013, Hamilton et al. June 2009). It would be interesting to determine if this population could induce anergic T-cells. DCs were treated with PBS or FhTeg overnight, washed and co-cultured in a 1:10 ratio with CD4⁺ T-cells in the presence of plate bound anti-CD3. CD4⁺ T-cells were cultured with anti-CD3 on their own as a negative control. Following a 72 hour co-culture, supernatants were removed and the levels of IL-2, IL-5, IL-4 and IFN- γ were measured and FhTeg DC's significantly suppressed the production of all cytokines tested from CD4⁺ T-cells, ($p<0.05$, $p<0.0001$) (Fig 4.3.7 A-D). The expression of RNF128 and CTLA4 were measured using qPCR. The analysis of genes found RNF128, ($p<0.001$) and CTLA4 ($p<0.01$) were enhanced in CD4⁺ T-cells co-cultured with FhTeg treated DCs compared to PBS controls (Fig 4.3.7 D-F).

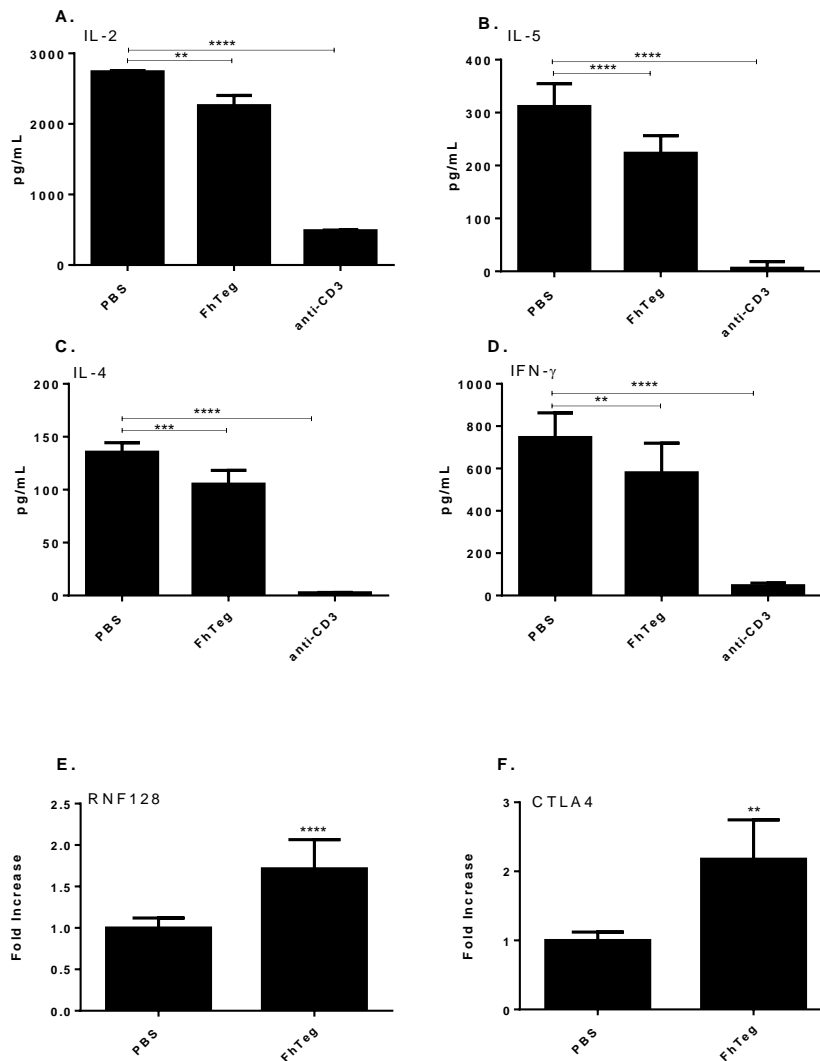


Figure 4.3.7. FhTeg treated dendritic cells induce anergy in CD4⁺ T-cells. **A-D** PBS treated DC's promoted the production of IL-2 ($p < 0.0001$), IL-5 ($p < 0.0001$), IL-4 ($p < 0.0001$) and IFN- γ ($p < 0.0001$) during a co-culture with CD4⁺ T-cells. FhTeg treated DC's significantly suppressed the production of IL-2 ($p < 0.01$), IL-5 ($p < 0.0001$), IL-4 ($p < 0.001$) and IFN- γ ($p < 0.01$). **E-F**. Cells were analysed for the enhancement of RNF128 and CTLA4 by qPCR. Both RNF128 ($p < 0.0001$) and CTLA4 ($p < 0.01$) were enhanced following co-culture with FhTeg treated DC's. The data is the mean \pm SD of three independent experiments. One way anova was used for analysis in **A-D**, students t-test was used in **E-F**.

4.3.8. The Mannose receptor is enhanced on dendritic cells, following activation with FhTeg and is important in the induction of anergy by DCs.

We know that C-type lectin receptors are involved in helminth induced immune responses and we have shown that the tegument of *F. hepatica* comprises of high mannose glycoproteins (manuscript in preparation). Here we show that MR expression is enhanced on DCs following stimulation with FhTeg (Fig 4.3.8 A) and that CD11c expression is decreased (Fig 4.3.8 B). To investigate if enhancement of MR may be involved in cell to cell communication between dendritic cells and CD4⁺ T-cells in the induction of anergy, DCs were treated with PBS or FhTeg overnight, washed and exposed to Mannan and GalNac-4S (two sugars that bind to the binding domains of the MR receptor), prior to co-culturing in a 1:10 ratio with CD4⁺ cells in the presence of plate bound anti-CD3. CD4⁺ cells were cultured with anti-CD3 on their own as a negative control. The expression of RNF128 and CTLA4 were measured using qPCR. The analysis of genes found RNF128, and CTLA4 were enhanced in CD4⁺ cells co-cultured with FhTeg treated DCs compared to PBS controls (Fig 4.3.8 A-B) and this expression was inhibited in the presence of mannan (p<0.001) and GalNac-4s (p<0.01).

DCs were also generated from MR knockout mice and stimulated with PBS or FhTeg (10µg/ml) overnight before being washed and co-culturing with CD4⁺ T-cells in a 1:10 ratio with plate bound anti-CD3. After 72 hours, RNA was isolated from cells and analysed for RNF128 and CTLA4 expression by qPCR. The absence of MR inhibited the induction of both genes in CD4⁺ T-cells and significantly decreased the expression compared to background cells (Figure 4.3.8 D-E) (p<0.05). The suppression of IL-2 was also significantly reversed in knockout compared to background controls (Figure 4.3.8 F), (p<0.0001).

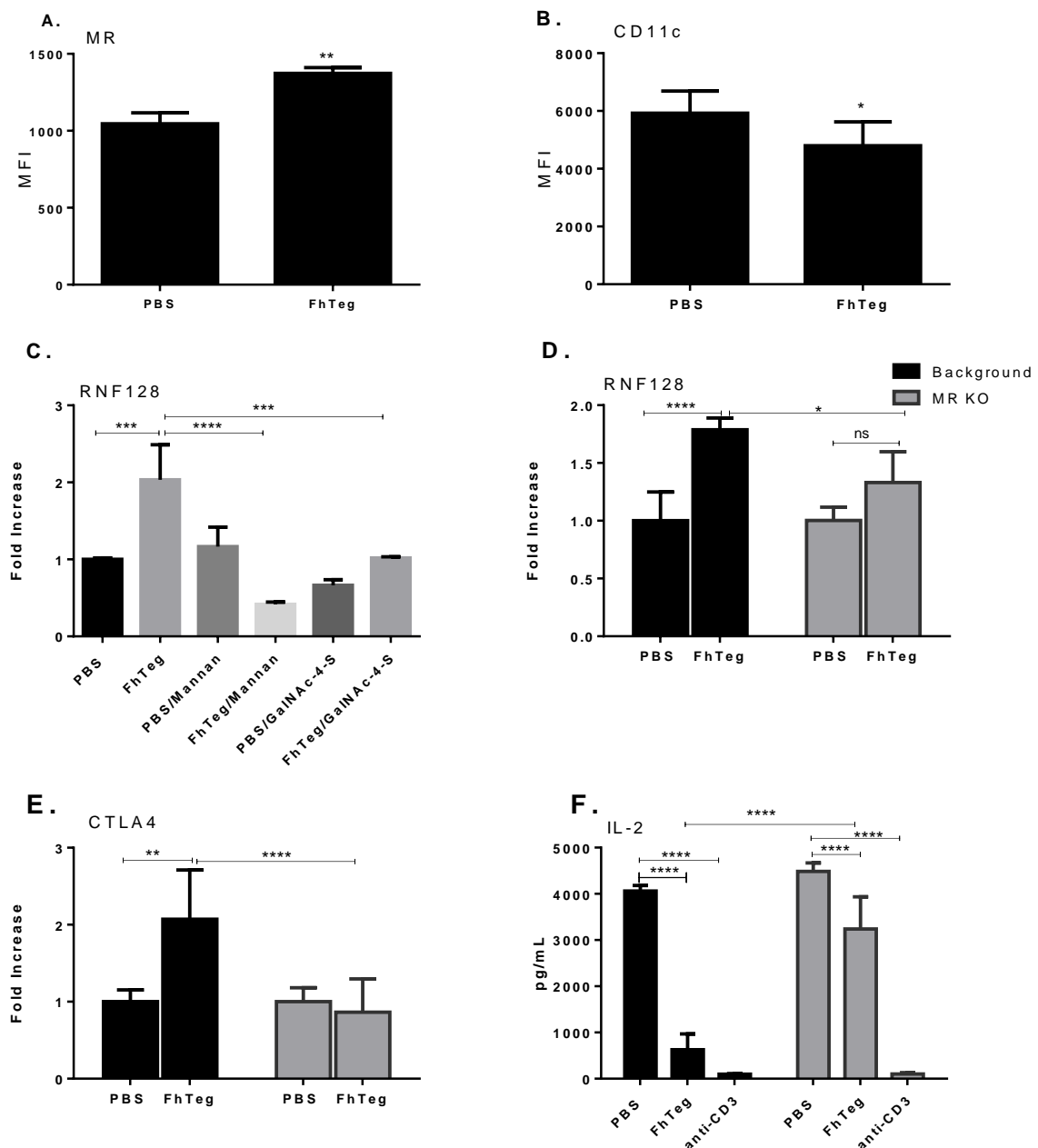


Figure 4.3.8. The MR receptor is enhanced following activation with FhTeg and is important in the induction of anergy by DCs. A-B. FhTeg significantly enhances the expression of MR ($p < 0.01$) and significantly decreases the expression of CD11c compared to PBS ($p < 0.05$) on DCs. **C.** RNF128 was significantly enhanced following co-culture with FhTeg treated DC's compared to PBS ($p < 0.001$) but incubation with mannan ($p < 0.0001$) and Gal-4-S ($p < 0.001$) significantly suppressed this. **D-E.** FhTeg treated DC's from background mice significantly enhanced RNF128 and CTLA4 ($p < 0.0001$, $p < 0.01$) during a co-

culture with CD4⁺ T-cells but FhTeg treated DC's from MR knockout mice failed to enhance either RNF128 or CTLA4. **F.** In background mice PBS treated DC's enhanced IL-2 production ($p<0.0001$) and FhTeg treated DC's significantly inhibited this ($p<0.0001$). In MR knockout mice, PBS treated DC's significantly enhanced IL-2 production ($p<0.0001$) and FhTeg significantly decreased this ($p<0.0001$) but it was significantly enhanced compared to background mice ($p<0.0001$). The data shown is the mean \pm SD of three independent experiments, with four mice in background and four mice in knockout group. Students t-test used in **A-B**, one way anova used for comparisons within groups in **C-F** and two way anova used for comparisons between background and knockout mice.

4.4. Discussion

Studies examining T-cell responses in other helminth infections report two main regulatory phenotypes, FoxP3⁺ T-reg cells or anergic T-cells. FoxP3⁺ T-reg cells are the major cause of T-cell unresponsiveness in *Brugia malayi* infection (McSorley et al. 2008) while chronic *Schistosoma mansoni* infection can lead to T-cell anergy (Smith et al. 2004). *F. hepatica* infection is typically associated with a polarised Th2/T-reg immune response with a decrease in protective Th1 immune responses within hours post infection. To our knowledge this is the first study to show that *F. hepatica* tegumental antigens induce T-cell anergy during *F. hepatica* infection which can be mimicked by the injection of FhTeg. Splenocytes isolated from *F. hepatica* infected or FhTeg injected mice fail to produce IL-2 which can explain this lack of antigen specific cytokine responses and the decrease in CD4⁺ T-cell proliferation. These responses are re-stored following the addition of IL-2 to cultures. T-cell responses when characterised further exhibit phenotypic extracellular and intracellular markers typically associated with anergy. However CD4⁺ T-cells isolated from infected mice showed an increase in proliferation which is not generally associated with anergy, although the levels of proliferation of PBS and FhTeg from infected groups were significantly lower than the positive controls of PMA and anti-CD3. This may be due to the length of infections used and further experiments would need to be performed to understand this fully.

CTLA4 and PD1 co-signalling is involved in the induction of anergy (Okazaki and Honjo 2006, Wells et al. 2001, Butte et al. 2007) and these cell surface markers are enhanced on splenocytes and CD4⁺ T-cells during *F. hepatica* infection and following injection with FhTeg. Signalling through the CTLA4 receptor induces negative regulation of T-cell activation which can lead to an un-responsive state in these cells (Greenwald et al. 2001). During normal activation CD28 and TCR engagement leads to CD4⁺ T-cell activation

promoting IL-2 secretion, proliferation and progress through the cell cycle. Alternatively, activation through the CTLA4 pathway results in poor production of IL-2, the loss of proliferation and the induction of anergy (Wells et al. 2001). CD4⁺ T-cells isolated from *F. hepatica* infected mice lose their proliferative ability which can be reversed with the addition of IL-2. CTLA4 has been implicated in other helminth infections, studies using lymphatic filariasis showed an increase in expression of CTLA4 (Babu et al. 2006, Steel and Nutman. 2003) and also during both acute and chronic infection of *S. mansoni* a CTLA4 positive population of CD4⁺ T-cells is enhanced (Walsh, Smith and Fallon 2007).

PD1 is also a negative regulator of T-cell responsiveness leading to diminished proliferation and cytokine secretion (Okazaki and Honjo 2006). Similar to CTLA4, its enhanced expression supports our key finding that FhTeg induces anergic T-cells. In other studies PD1 has been shown to play a role in T cell hypo-responsiveness seen during *S. mansoni* infection (Van et al. 2013) and also was elevated during filariasis (Babu et al. 2006). PD1 expression is also associated with T-cell exhaustion (Wherry 2011) which is mostly centred on CD8⁺ T-cells. Exhausted T-cells cannot produce cytokines but can produce some IFN- γ . In our studies, IFN- γ production is not observed when splenocytes from infected or injected mice are stimulated with FhTeg and in the case of infection even after the addition of IL-2. Similarly, FhTeg re-stimulated cells do not exhibit other characteristics associated with exhausted T-cells such as high deletion of cells and apoptosis which further supports its role in the induction of anergy.

Studies on anergic T-cells have uncovered a complex mechanism involving a number of negative regulators (Lechner 2001) including EGR2, EGR3 and the E3-ubiquitin ligases, RNF128, ITCH and CblB (Mueller 2004a). Both EGR2 and EGR3 are involved in the

induction and maintenance of T-cell anergy (Safford et al. 2005). EGR2 inhibits IFN- γ and IL-2 production and enhances the expression of CblB. Studies have shown that a full anergic state cannot be achieved without this latter gene (Harris 2004) as studies in CblB knockout mice have shown that anergy cannot be induced *in vivo* or *in vitro* in the absence of CblB (Jeon 2004). In our study we have observed a 60 fold increase of EGR2 in splenocytes isolated from a two week infection. EGR3 was not increased at this time point; however this was not surprising given that it is involved in the early signalling events. Similarly, an increase in CblB or ITCH expression was not observed at later time points (Venuprasad 2010). RNF128 was highly expressed at the mRNA and protein level following *F. hepatica* infection and injection with FhTeg. RNF128 has been implicated in helminth infections previously, with an increase in expression seen after chronic *S.mansoni* infection (Taylor et al. 2009) and high expression of this gene alone is enough to convert a naïve CD4⁺ T-cell into an anergic phenotype (Seroogy et al. 2004).

This is the first study to show that *F. hepatica* released antigens can directly induce anergy, although a recent paper reported FhES to induce anergic T-cells, however the definition was based upon IL-10 secreting CD4⁺ cells that express CTLA4 (Guasconi, Chiapello and Masih). From our definition we would consider this to be a T-reg rather than an anergic T-cell. It is not surprising that FhTeg and FhES induce different immune responses during infection as proteomic analysis demonstrates that FhTeg mainly comprises of a rich source of glycoproteins while FhES comprises of released gut enzymes (Wilson et al. 2011, van Rossum, Barrett and Brophy 2001). It was previously shown that these antigens induce different M2 like macrophage subsets *in vitro* and *in vivo* (Adams et al. 2014) while studies in mast cells show that FhTeg *in vivo* induces significantly higher masocytosis compared to

FhES (Vukman et al. 2013a). Further studies are required to investigate the interaction of these antigens on immune cells.

FhTeg modulates dendritic cell function by inducing a novel DC phenotype characterised by SOCS3^{high}, CD80^{low}, CD86^{low} (Vukman, Adams and O'Neill 2013, Hamilton et al. June 2009). Studies during *Heligmosomoides polygyrus* infection have found a dendritic cell population which has a low expression of CD11c and were defined as being tolerogenic and enhancing regulatory T-cells (Smith et al. 2011) . FhTeg stimulated DC's phenotype was further characterised as having an enhanced expression of MR with decreased expression of CD11c observed. However, neither PGE2 nor PGD2 were secreted by DCs following stimulation with FhTeg. This immature phenotype that fails to respond to TLR and non-TLR activation (Vukman et al. 2013a, Dowling et al. February 2010) drives anergic T-cells *in vitro* as measured by enhanced RNF128 and CTLA4 by RNA and suppressed cytokine expression in anti-CD3 stimulated CD4⁺ T-cells. These findings support the hypothesis that FhTeg induces an anergic like DC given its effect upon CD4⁺ T-cells. To our knowledge there are few examples of anergic DC's characterised with the exception of DC's isolated from children with severe malnutrition and endotoxemia which were found to be anergic and inhibited T-cell proliferation (Hughes et al. 2009). Immature DC's have also been implicated in the induction of anergy, BMDC's cultured in GM-CSF with high levels of LPS induced anergy *in vitro* (Lutz et al. 2000) and also DC's treated with IL-10 to induce semi-mature DC's have been shown to induce anergy in both CD4⁺ and CD8⁺ T-cells (Sato, Yamashita and Matsuyama 2002).

These anergy enhancing DCs highly express MR, a type-I membrane protein with a cytoplasmic domain involved in antigen processing, receptor internalisation and three

different types of binding domains. Its binding domain consists of multiple C-type lectin-like carbohydrate-recognition domains (CRDs) responsible for Ca^{2+} -dependent binding to terminal mannose, fucose or *N*-acetyl glucosamine, a fibronectin type II (FNII) domain involved in collagen binding and an *N*-terminal cysteine-rich (Cys-MR) domain that mediates Ca^{2+} -independent binding to sulfated sugars such as SO_4 -3-Gal or SO_4 -3/4-GalNAc (Martinez-Pomares 2012). We have shown that the action of FhTeg is independent of MR so the lack of anergy induction in DC's generated from knockout mice is not from the inhibition of binding or engagement of the receptor on the DC (manuscript in preparation). CTRs also have other important functions, including cell to cell communication, DC-SIGN which binds through its ligand ICAM3, mediates initial binding of DC's with T-cells and helps stabilise binding through the TCR (Geijtenbeek et al. 2000). MR has been shown to bind through CD45 on CD4^+ cells (Martínez-Pomares et al. 1999), and here we show that blocking MR with specific sugars before co-culture with CD4^+ T-cells inhibits the induction of anergy. This was confirmed using DCs generated from MR knockout mice, as the absence of MR led to a significant decrease in the induction of RNF128 and CTLA4 compared to background mice. This is the first study to show that MR is involved in anergy induction in CD4^+ cells through communication between dendritic cells and CD4^+ T-cells. Other studies have shown MR's involvement in anergy but this has been through binding of antigens through MR and cross presentation to CD4^+ T-cells (Chieppa et al. 2003) and not through the binding of MR to its counter receptor on the CD4^+ T-cell.

This study demonstrated a novel mechanism for the T-cell unresponsiveness observed during *F. hepatica* infection and after injection with FhTeg. During infection, anergy is the main T-cell response observed when examining adaptive immune responses to FhTeg as a lack of cytokine responses and proliferation and can be reversed with the addition of IL-2 to cultures.

Markers of T-cell anergy such as CTLA4 and PD1 are also increased and we also have an up-regulation of genes relating to anergy induction and maintenance, RNF128, EGR2, ICOS and ITCH. The injection of FhTeg over the sternum can mimic the effect of infection and dendritic cells treated with FhTeg can induce anergy associated genes and cytokine suppression in CD4⁺ cells *in-vitro* which is dependent on the mannose receptor.

Chapter 5

FhTeg induces anergy directly in CD4⁺ cells

5.1. Introduction

CD4⁺ T-cells are an important group of cells which control the outcome of the immune response. Activation of CD4⁺ T-cells requires three signals from antigen presenting cells and this includes presentation of peptides through MHC class II, signals via co-stimulatory molecules and releases of cytokines. These signals influence the differentiation of naïve CD4⁺ cells into different subsets such as Th1, Th2, Th17, T-regs or anergic T-cells (Yamane and Paul 2013). Following this, most CD4⁺ cell subsets proliferate and produce cytokines which in concert with the environment already created by the APCs, further supports the differentiation of CD4⁺ and other immune cell subsets. However, anergic T-cells, induce a state of hypo-responsiveness where cytokine secretion and proliferation is suppressed but this can be reversed by the addition of recombinant or exogenous IL-2 (Schwartz 2003).

Anergic T-cells are often found following chronic infection or after multiple exposures to antigens (Taylor, van der Werf and Maizels 2012a). The signalling mechanism for anergy is usually started by insufficient signals from APCs which are either not fully matured or cannot present antigens correctly. Two signals are needed for normal activation, receiving signal one only can lead to anergy (Sadegh-Nasseri et al. 2010). The characteristics of anergic T-cells are a lack of production of IL-2 which in turn leads to a suppression of proliferation and a block in the cell cycle at the G1 phase (Powell, Bruniquel and Schwartz 2001). A number of genes are also involved in the induction and maintenance of T-cell anergy, the E3 ubiquitin ligases play a major role, EGR2 and 3 have been found to be involved in the induction of anergy and the levels of EGR2 stay elevated after the initial stimuli (Harris 2004), RNF128 or GRAIL is also crucial for anergy induction (Seroogy et al. 2004).

Anergy can also be directly induced in CD4⁺ T-cells using a number of different methods. Stimulation with anti-CD3 antibody followed by a brief resting period and subsequent re-stimulation with PMA (Schwartz 1990) or by the addition of Ionomycin directly to cells (Heissmeyer 2004). Few antigens have been shown to directly interact with CD4⁺ T-cells to elicit an immune response; however super-antigens are produced by pathogenic microbes like bacteria which can cause non-specific activation of T-cells. This occurs through binding to both MHC class II molecules on APCs and to the TCR receptor on CD4⁺ T-cells simultaneously, causing uncontrolled proliferation and production of cytokines from CD4⁺ T-cells. Following this a number of cells undergo apoptosis and the remaining cells are functionally unresponsive (Seth et al. 1994). They also can induce anergy in CD4⁺ memory cells directly by binding the TCR and inducing negative signalling pathways and are used by pathogens as a defence mechanism to the hosts' immune response (Watson, Mittler and Lee 2003).

In the previous chapter we have shown that FhTeg (either during infection or following injection) is associated with the induction of anergy *in vivo* as measured by a lack of cytokine responses and proliferation following re-stimulation which can be reversed with the addition of IL-2 to culture. Markers of T-cell anergy such as CTLA4 and PD1 were enhanced on the surface of CD4⁺ T-cells and these cells expressed genes relating to anergy induction and maintenance (RNF128, EGR2, ICOS and ITCH). Dendritic cells treated with FhTeg can induce anergy associated genes and cytokine suppression in CD4⁺ T-cells *in-vitro*. As there are antigens which can influence T-cell activation directly the aim of this study was to investigate the direct interaction of FhTeg with CD4⁺ T-cells.

5.2. Experimental Design

The aim of this study was to investigate if FhTeg directly interacts with naïve CD4⁺ T-cells and if the mannose receptor plays a role in this interaction. Firstly we investigated if FhTeg can directly induce anergic CD4⁺ T-cells, CD4⁺ T-cells were isolated from naïve (Balb/c) mice. These cells were stimulated with FhTeg (10µg/ml) for 2 and 24 hours and RNA was isolated from cells, reverse transcribed to cDNA and used in qPCR gene array assays for the expression of genes relating to tolerance and anergy. From these results there were a number of genes highly expressed after two hours and to check if these were significantly expressed, individual qPCR assays were performed. The expression of PD1 and CTLA4 were also measured by flow cytometry by directly stimulating CD4⁺ T-cells with FhTeg overnight. The phosphorylation of PKC-θ at 30mins, 1 hour and 2 hours was also investigated. To examine if FhTeg could inhibit cytokine secretion from CD4⁺ cells, they were incubated with FhTeg (10µg/ml) for 2.5 hours before the addition of PMA (20ng/ml) and anti-CD3 (10µg/ml). After 72 hours supernatants were removed and analysed for IL-5 and IFN-γ production. To investigate if FhTeg would have an effect on proliferation, CD4⁺ cells were labelled with CFSE and stimulated with PBS, FhTeg (10µg/ml) or PMA (20ng/ml) and anti-CD3 (10µg/ml) for 72 hours, cells were then analysed by flow cytometry. As some genes that were highly expressed on the qPCR gene arrays were related to the production of GM-CSF and PGE2 the levels of these cytokines were measured following PBS, FhTeg (10µg/ml) or PMA (20ng/ml) and anti-CD3 (10µg/ml) following a 24 and 72 hour stimulation.

The binding efficiency of FhTeg on CD4⁺ cells was measured, FhTeg and BSA (a non-specific binding control) was fluorescently labelled with a FITC-488 label. CD4⁺ cells were stimulated with 1 and 5 (µg/ml) of FhTeg and analysed by flow cytometry. To check if the

binding was calcium dependent and if MR was involved, cells were pre-incubated for 30 mins with EDTA (10mM) and Mannan (50µg/ml), GalNAc-4-sulphate (1mM) and anti-MR (10µg/ml) before the addition of FhTeg (1 and 5µg/ml). Finally to show the mannose receptor is on CD4⁺ cells, they were analysed by flow cytometry.

To investigate if MR plays a role in the immune-suppressive effect of FhTeg on CD4⁺ cells, cells were stimulated with PBS or FhTeg (10µg/ml) with or without Mannan (50µg/ml) for 24 hours, spun down and washed twice before the addition of fluorescently labelled antibodies for PD1 and CTLA4 or their isotype controls. Cells were then analysed by flow cytometry. Studies in MR knockout and background mice (C57bl/6) were carried out to investigate the effect of FhTeg in the absence of MR. Binding was measured by stimulating CD4⁺ cells from C56BL6 background and MR knockout mice were stimulated with BSA (10µg/ml) or FITC-labelled FhTeg (10µg/ml) for 45 mins before being analysed by flow cytometry. The expression of and PD1 was also measured following a 24 hour stimulation with PBS or FhTeg (10µg/ml). The levels of CblB and EGR3 were also measured in knockout and background CD4⁺ cells following a 2 hour stimulation with FhTeg (10µg/ml).

Finally the role of FhTeg treated CD4⁺ cells on dendritic cells ability to secrete IL-12p70 were examined. CD4⁺ cells were stimulated with PBS or FhTeg for 24 hours, before being washed twice and co-cultured with dendritic cells for a further 24 hours. The dendritic cells were re-isolated and rested before being re-challenged with LPS (100ng/ml). Supernatants were removed after 18 hours and analysed for IL-12p70 production.

5.3. Results

5.3.1. FhTeg can directly induce the expression of anergic markers on CD4⁺ T-cells

As we have previously shown that infection with *F. hepatica* and the injection of the tegumental antigen can cause anergy we wanted to investigate if FhTeg could induce anergy directly in CD4⁺T- cells. CD4⁺ T-cells isolated from spleens of naive mice were stimulated with PBS or FhTeg (10µg/ml) for 2 and 24 hours. After the indicated time points cells were washed and lysed for RNA analysis. A qPCR gene array with genes relating to anergy was used to analyse cDNA from 2 and 24 hour treated cells. After two hours the array showed an increase in a number of genes which are involved in anergy induction which are listed in Table 1, and no increase in FoxP3 expression which is commonly seen in conventional T-reg cells (Figure 5.3.1A).

Table 5.3.1. The fold changes seen in the 2 hour qPCR gene array for genes associated with anergy induction and maintenance.

Gene	Fold change
CblB	3.4
Ccl3	4.3
CD40	-2.53
CSF	368
Egr2	4.3
Egr3	8.2
ICOS	2.8
PD1	3.22
Ptgs2	5.11

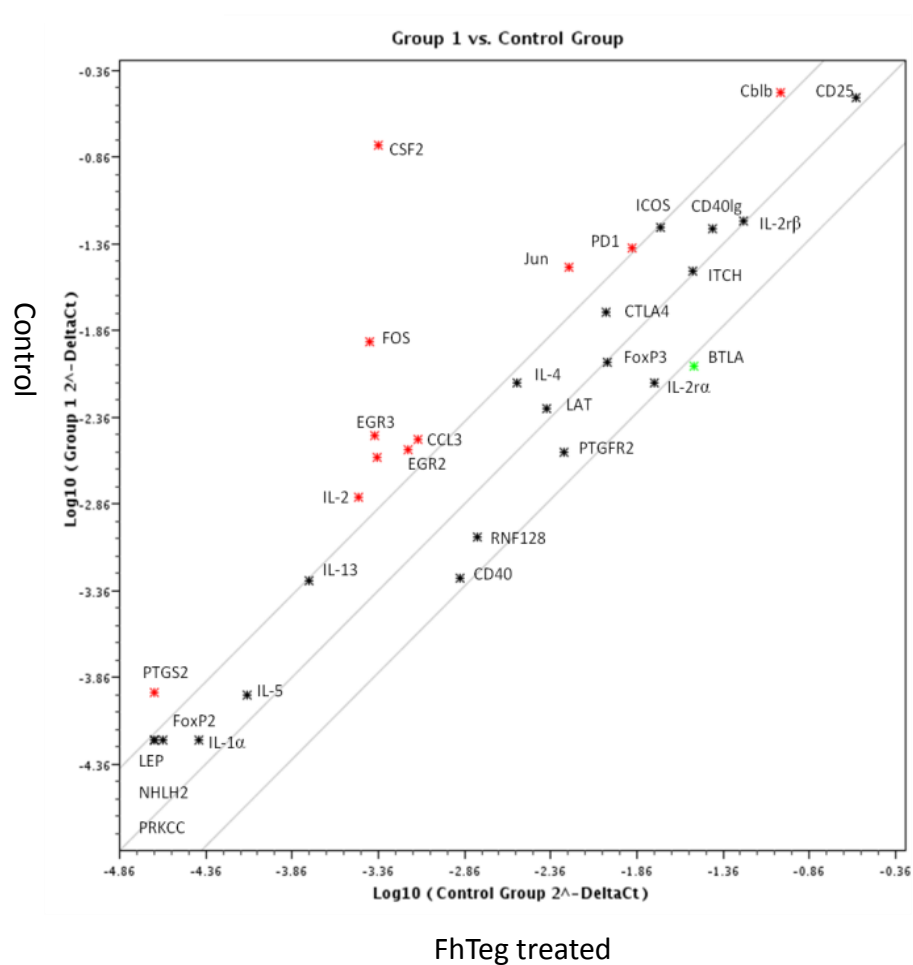
After 24 hours the gene expression is quite different with only a small number of genes having a high expression including CSF2 (14.5), LEP (6.7), Early growth response gene 3 (EGR3) (2.26) and PTGS2 (4.9). IL-2 was down-regulated with a fold regulation of -2.2 (Figure 5.3.1B). To determine if the genes are significantly enhanced, individual qPCR analysis of a number of genes was performed following a 2 hour stimulation. Egr2 and 3,

CTLA4, ICOS, ITCH and CblB were analysed (Figure 5.3.1C-H) and their fold increases are summarised in Table 5.3.1B.

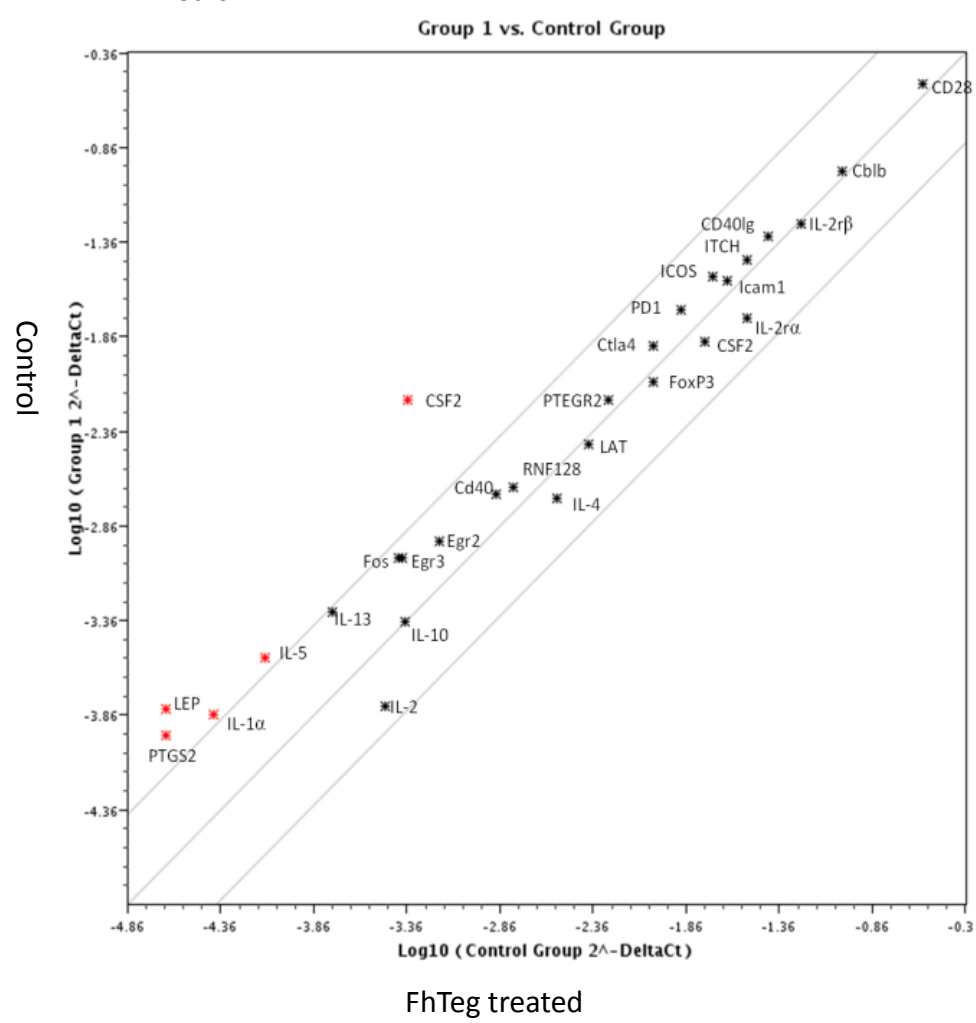
Table 5.3.1B The fold changes seen in the 24 hour qPCR gene array for genes associated with anergy induction and maintenance.

Gene	Fold Change
Egr2	2.5
Egr3	3.3
CTLA4	3.0
ICOS	2.4
ITCH	1.7
CblB	1.6

A. 2 hours



B. 24 hours



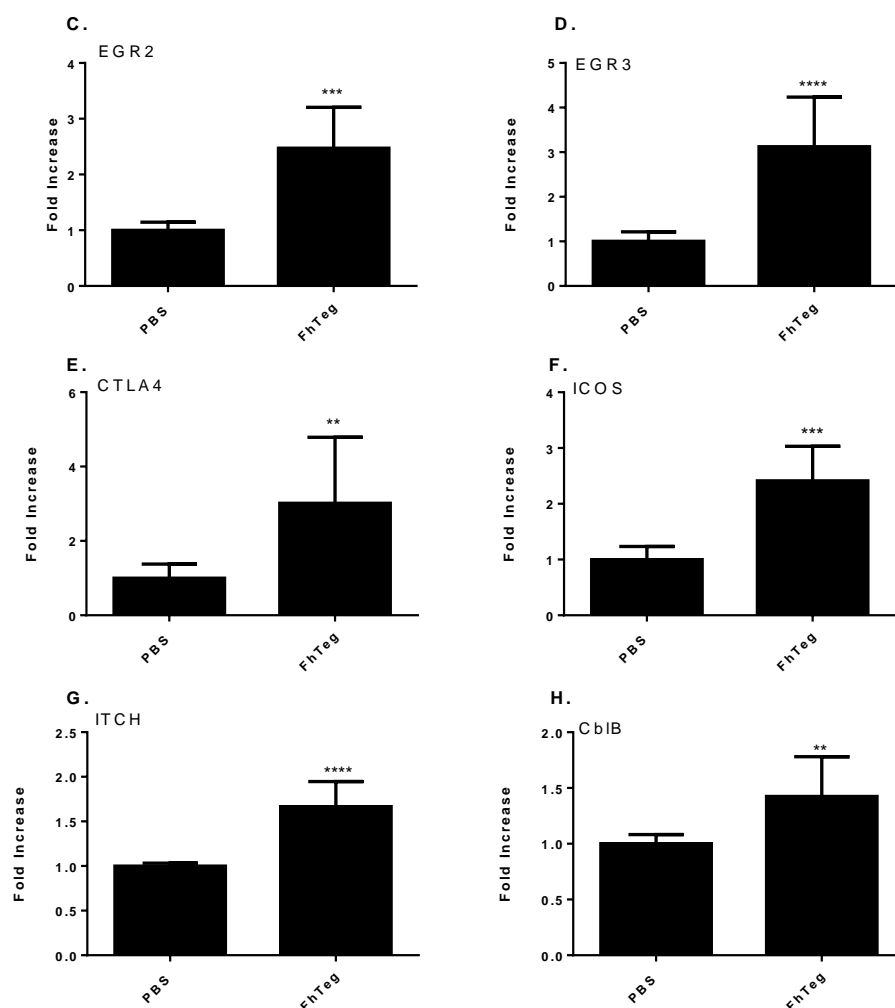


Figure 5.3.1. FhTeg causes the expression of anergic genes in CD4⁺ T-cells following stimulation. CD4⁺ T-cells were stimulated with PBS or FhTeg (10 μg/ml) for 2 and 24 hours. A qPCR array was used to analyse a number of genes. The data is presented as the result of one independent experiment. **C-D.** Individual genes were analysed after 2hrs. FhTeg significantly enhanced the expression of EGR2 (p<0.001), EGR3 (p<0.0001), CTLA4 (p<0.01), ICOS (p<0.001), ITCH (p<0.0001) and CblB (p<0.01) compared to PBS. The data is presented as the mean ±SD for three independent experiments. Data was analysed using the student t-test.

5.3.2. FhTeg causes the up-regulation of PD1 and CTLA4 on CD4⁺ T-cells

PD1 and CTLA4 were shown to be enhanced on the 2 hour qPCR gene array. To investigate if the cell surface expression of these markers was significant, CD4⁺ T-cells were incubated

with PBS or FhTeg (10 μ g/ml) to cultures. After 24 hours the cells were washed and stained for PD1 or CTLA4 (total). Cells which received FhTeg alone showed a significant increase in expression of PD1 ($P<0.001$) (Figure 5.3.2A). CTLA4 total, surface and intracellular expression, was also up-regulated on CD4⁺ T-cells after treatment with FhTeg ($P<0.01$) (Figure 5.3.2B).

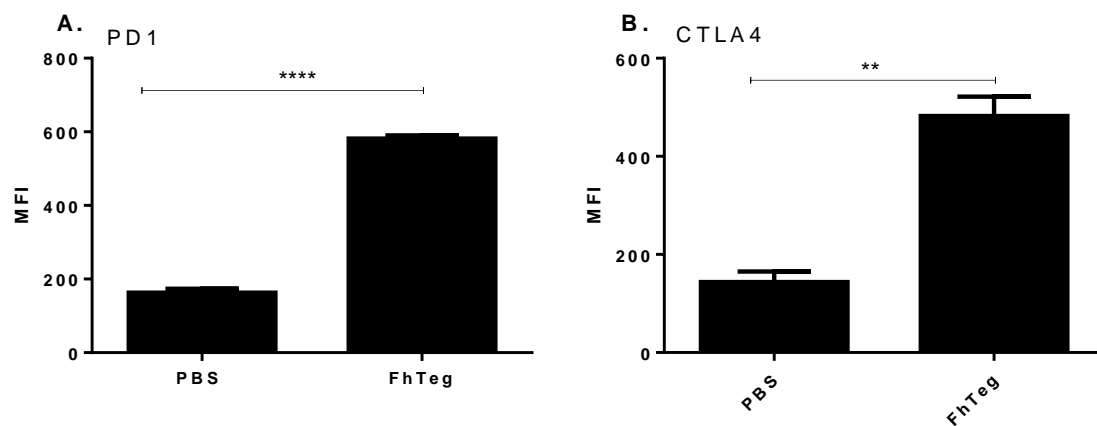


Figure 5.3.2. FhTeg enhances the expression of PD1 and CTLA4 on CD4⁺ T-cells A-B FhTeg significantly enhances the expression of PD1 ($p<0.0001$) and CTLA4 ($p<0.01$) on CD4⁺ T-cells following an overnight stimulation. The data shown is the mean \pm SD of three independent experiments. Data analysed using the student t-test.

5.3.3. FhTeg does not induce the phosphorylation of PKC-theta in CD4⁺ T-cells

The activation of CD4⁺ T-cells requires the initiation of a number of signalling pathways which in turn lead to the activation of the transcription factor NF κ B and a T-cell specific immune response. Important signalling modulators which lead to the activation of NF κ B are PKC-theta, AP1 and in conjunction with calcium signalling NFAT. To determine if FhTeg would induce any of these transcription factors, CD4⁺ cells were stimulated with PBS, FhTeg (10 μ g/ml) or PMA (20ng/ml) and anti-CD3 (10 μ g/ml) for 30 mins, 1 and 2 hours. Protein

levels of PKC- θ were measured. The phosphorylation or activated state of PKC- θ was seen for samples which received both PMA and anti-CD3 (Figure 5.3.3 3,6,9) but not in FhTeg samples (Figure 5.3.3 2,5,8).

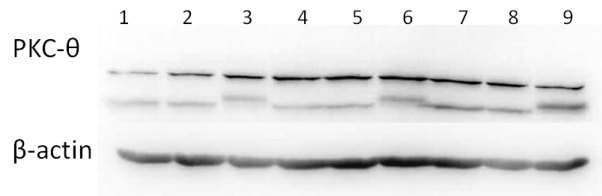


Figure 5.3.3. FhTeg does not induce the phosphorylation of PKC-theta in CD4⁺ T-cells. Protein levels of PKC- θ were measured. FhTeg fails to induce the phosphorylation of PKC-theta in CD4⁺ T-cells following a 30 min, 1 hour and 2 hour stimulation. Lane 1,4,7 PBS, Lane 2,5,8 FhTeg, Lane 3,6,9 PMA/anti-CD3.

5.3.4. FhTeg inhibits the PMA/Anti-CD3 activation of CD4⁺ T-cells

CD4⁺ T-cells were incubated with FhTeg (10 μ g/ml) for 2.5 hours before the addition of PMA (20ng/ml) and anti-CD3 (10 μ g/ml). After 72 hours supernatants were removed and the levels of IL-5 and IFN- γ were measured using ELISA. Viability was measured using trypan blue staining. Cells which received PMA and anti-CD3 produced high levels of IFN- γ and IL-5 but co-stimulation with FhTeg significantly reduced the production of IFN- γ ($p < 0.0001$) and IL-5 ($p < 0.001$) FhTeg did not cause the production of either cytokine (Figure 5.3.4 A-B).

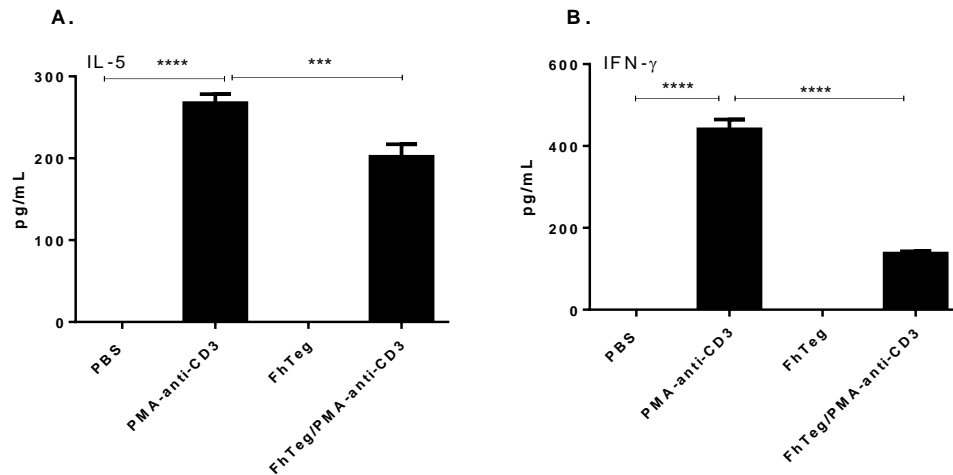


Figure 5.3.4. FhTeg inhibits the PMA/anti-CD3 activation of CD4⁺ T-cells. A-B. PMA and anti-CD3 significantly enhanced the production of IL-5 and IFN- γ from CD4⁺ T-cells compared to PBS ($p < 0.0001$), FhTeg significantly inhibited the production of IFN- γ ($p < 0.0001$) and IL-5 ($p < 0.001$) when co-stimulated with PMA/anti-CD3. The data is presented as the mean \pm SD of one experiment, this experiment was repeated three times and one representative experiment is shown. Data analysed using one-way anova.

5.3.5. FhTeg inhibits the PMA/anti-CD3 induced proliferation in CD4⁺ T-cells

FhTeg inhibits the production of cytokines from CD4⁺ cells following a 72 hour stimulation; this may impact the cells ability to proliferate. To investigate if FhTeg would inhibit proliferation in CD4⁺ T-cells, cells were labelled with CFSE (10 μ M) and incubated with PBS, FhTeg (10 μ g/ml) in the presence or absence of PMA (20ng/ml) and anti-CD3 (10 μ g/ml). Following a 72 hour incubation, cells were washed and analysed by flow cytometry. Cells which had received PMA and anti-CD3 showed a high level of proliferation with only 6% of cells un-divided; although FhTeg and PMA/anti-CD3 cells had undivided cells of 13% the following generation had a very high percentage at 59% (Figure 5.3.5 A).

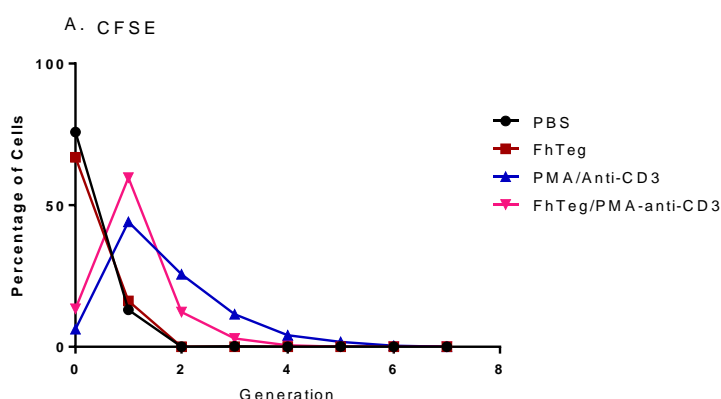


Figure 5.3.5. FhTeg inhibits the proliferation of CD4⁺ T-cells. FhTeg inhibits CD4⁺ T-cell proliferation but this was not significant. The data shown is representative of one experiment which was repeated three times.

5.3.6. FhTeg does not induce the production of GMCSF or PGD2 from CD4⁺ cells, but enhances the production of PGE2

FhTeg induces a very significant enhancement of the expression of CSF2 after 2 and 24 hours, with fold increases of 368 and 14.9 respectively. The gene CSF codes for the production of GMCSF, to investigate if the high expression of CSF2 at mRNA level induced high levels of GMCSF after 24 and 72 hours, CD4⁺ T-cells were stimulated with FhTeg (10µg/ml) in the presence or absence of PMA (20ng/ml) and anti-CD3 (10µg/ml). Supernatants were removed after 24 and 72 hours and assessed for GMCSF production. Cells which received PBS or FhTeg alone did not produce significant levels of GMCSF after both 24 or 72 hours but cells which received PMA and anti-CD3 did ($p < 0.0001$) and interestingly FhTeg significantly inhibited the production at both time points. (Figure 5.3.6 A-B)

The gene PTGS2, which codes for the enzyme Cox2, was also highly expressed following FhTeg stimulation after 2 and 24 hours with fold increases of 5.11 and 4.87. To investigate if the high expression of PTGS2 would lead to either PGD2 or PGE2 to be produced, CD4⁺

cells were stimulated for 24 and 72 hours with PBS and FhTeg (10 μ g/ml) or PBS and FhTeg (10 μ g/ml) in the presence or absence of PMA (20ng/ml) and anti-CD3 (10 μ g/ml). Supernatants were analysed for the production of PGD2 and PGE2 using commercially available EIA Elisa kits. After 24 and 72 hours there were no levels of PGD2 expressed in any samples but there was a significant increase in the levels of PGE2 produced after 24 hours in FhTeg samples and after 72 hours in FhTeg/PMA-antiCD3 samples ($P < 0.0001$ and $p < 0.01$) (Figure 5.3.6 C-D).

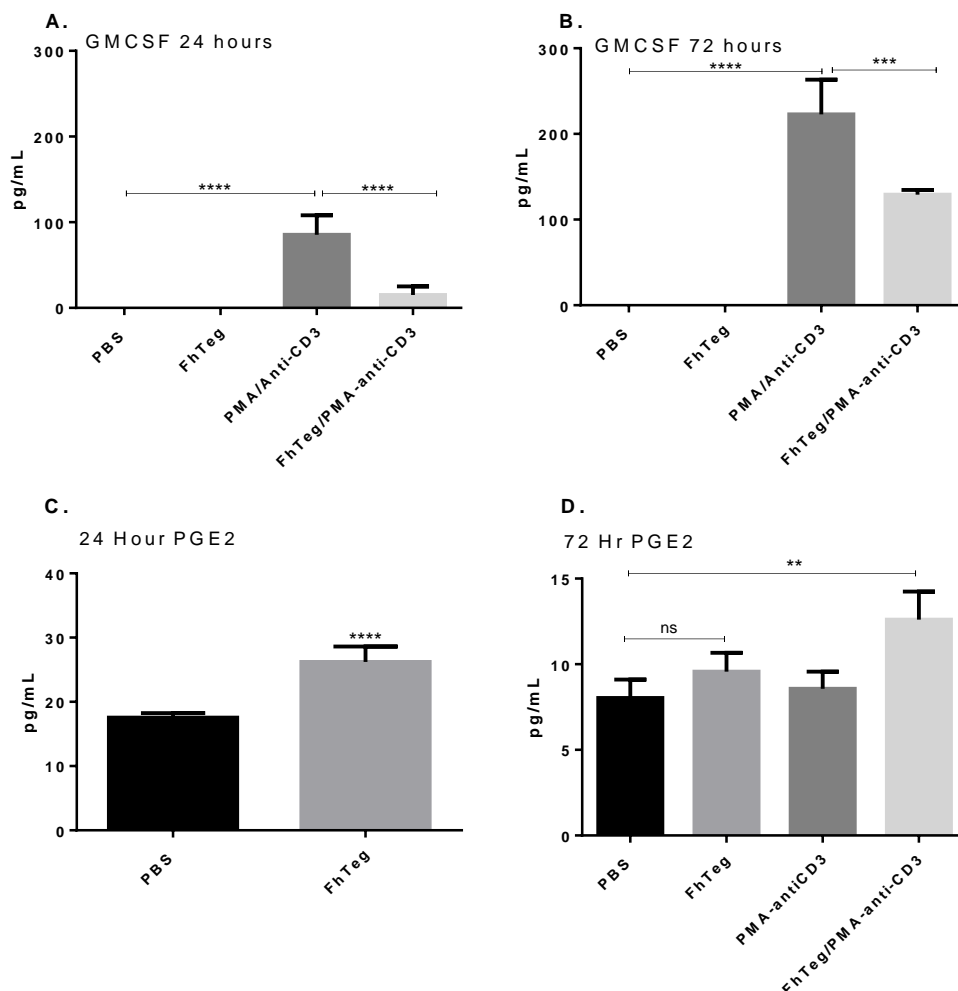


Figure 5.3.6. FhTeg does not induce the production of GM-CSF or PGD2 from CD4⁺ T-cells, but enhances the production of PGE2. A. PMA and anti-CD3 enhanced the production of GM-CSF from CD4⁺ T-cells after

24 (p<0.0001) and 72 hours (p<0.0001) compared to PBS and FhTeg significantly inhibited this at both time points (p<0.0001, p<0.001). **C-D.** PGE2 levels were enhanced following stimulation with FhTeg after 24 and 72 hours compared to PBS controls (p<0.0001, p<0.01). The data shown is the mean \pm SD of three independent experiments. Data analysed using one-way anova.

5.3.7. FhTeg binds to CD4⁺ T-cells and this binding can be suppressed by the addition of mannan and GalNAc-4-Sulphate to cultures.

FhTeg was labelled with a FITC 488nm fluorophore label which can be used to detect binding by flow cytometry and by fluorescent microscopy. As a control BSA was also labelled with the FITC fluorophore to check for unspecific binding. Cells were incubated with PBS, the sugar mannan (10ug/ml) or GalNAc-4-Sulphate (1ug/ml) for 45 minutes and then PBS, 488-BSA or 488-FhTeg at two different concentrations, 1ug/ml and 5ug/ml, was added to the cells for a further 45 minutes. The cells were then washed, counter stained for CD4⁺ APC and analysed by flow cytometry. 488-BSA did not show any specific binding to CD4⁺ T-cells but the 1 and 5ug of 488-FhTeg showed significant binding to CD4⁺ T-cells, (Figure 5.3.7 A) (P<0.05 and 0.005) . The percentage of cells that showed binding to FhTeg was 7% for 1 μ g and 15% for 5 μ g (Figure 5.3.7 C-E). This binding was suppressed in the 1ug FhTeg cultures by the addition of mannan (P<0.01), GalNAc-4-sulphate (P<0.05) and a combination of both to cells (Figure 5.3.7 B -D) (P<0.01).

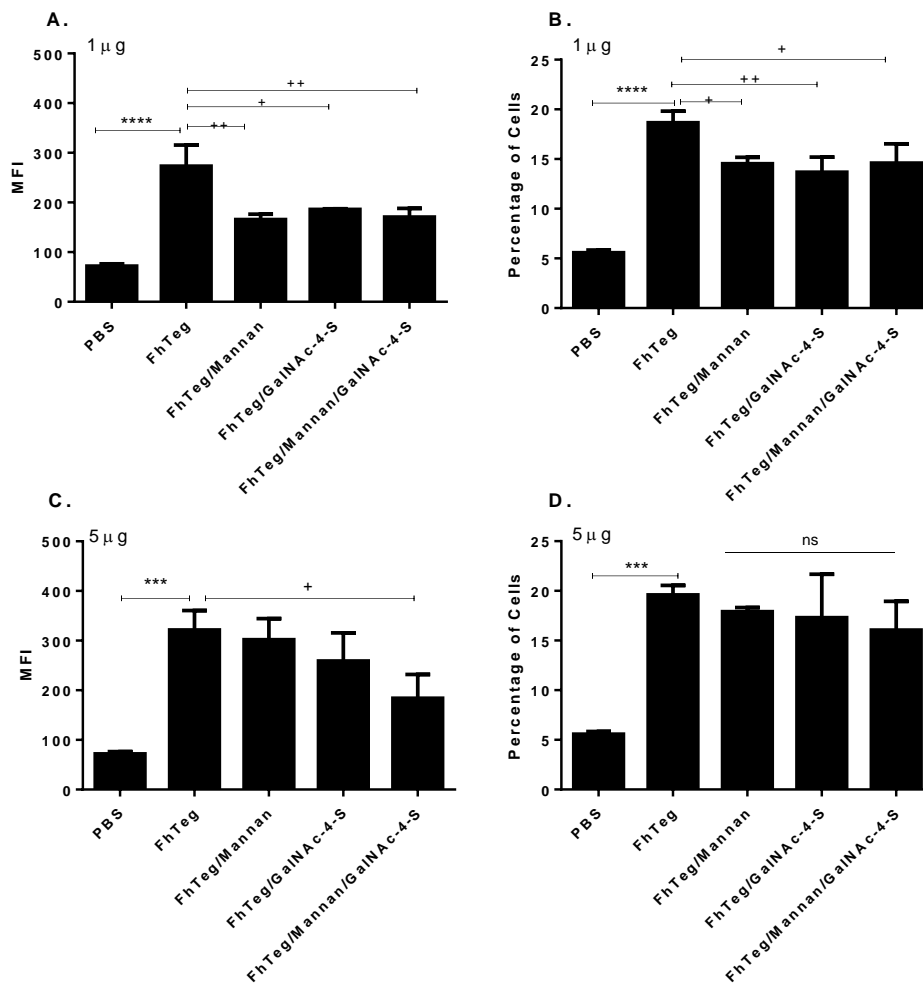


Figure 5.3.7. FhTeg binds to CD4⁺ cells and this binding can be reversed using mannan and Gal-4-Sulphate. **A-B.** FhTeg significantly binds to CD4⁺ T-cells at 1μg/ml using both MFI and percentage of cells measured ($p<0.0001$), the addition of mannan ($p<0.01$), GalNAc-4-S ($p<0.05$) and a combination of both ($p<0.01$) can inhibit the binding. **C-D.** FhTeg binds at 5μg/ml to CD4⁺ T-cells ($p<0.001$), this can be inhibited by the addition of Mannan and GalNAc-4-S combined ($p<0.05$) but not on their own. The data shown is the mean \pm SD of two independent experiments which were done in triplicate. Data analysed using one-way anova.

5.3.8. The C-type lectin MR is on the surface of CD4⁺ T-cells

Mannan and GalNAc-4-S have binding specificities to the C-Type lectin, the mannose receptor. As both of these compounds have inhibited binding of FhTeg to CD4⁺ cells, it was

decided to investigate if the MR receptor is on the surface of CD4⁺ T-cells. CD4⁺ T-cells were isolated from spleens of naive mice and stained for MR, counterstained for CD4 and analysed by flow cytometry. The MR receptor was found to be on a small percentage of CD4⁺ T-cells by flow cytometry (Figure 5.3.8A), with a significant increase in MFI ($p<0.05$) and in the percentage of cells (Figure 5.3.8B) ($p<0.0001$). The percentage of cells which expressed the MR receptor was small at only 4%.

To investigate if the MR receptor was involved in the signalling events following FhTeg stimulation, CD4⁺ T-cells were stimulated with Mannan (10 μ g/ml) for 30 minutes before the addition of FhTeg (10 μ g/ml) for 24 hours. CD4⁺ T-cells were then washed and counterstained for CD4 and analysed for the expression of PD1 and CTLA4. FhTeg significantly enhances the production of PD1 and CTLA4 ($p<0.01$) and mannan significantly inhibits this ($p<0.05$, 0.01), (Figure 5.3.8 C-D). As FhTeg inhibits the production of IL-5 from CD4⁺ T-cells when stimulated with PMA and anti-CD3, the ability of Mannan and GalNAc-4-Sulphate was examined. CD4⁺ T-cells were stimulated with Mannan (10 μ g/ml) or GalNAc-4-Sulphate (1 μ g/ml) for 30 minutes before the addition of FhTeg (10 μ g/ml) for 2.5 hours. Following this, PMA (20ng/ml) and anti-CD3 (10 μ g/ml) was added and the cells were incubated for 72 hours, supernatants were then removed and analysed for the production of IL-5 by commercial ELISA. PMA and anti-CD3 significantly enhanced the production of IL-5 ($p<0.0001$) and FhTeg inhibited this ($p<0.0001$). The addition of Mannan and GalNAc-4-Sulphate did not reverse this (Figure 5.3.8 E).

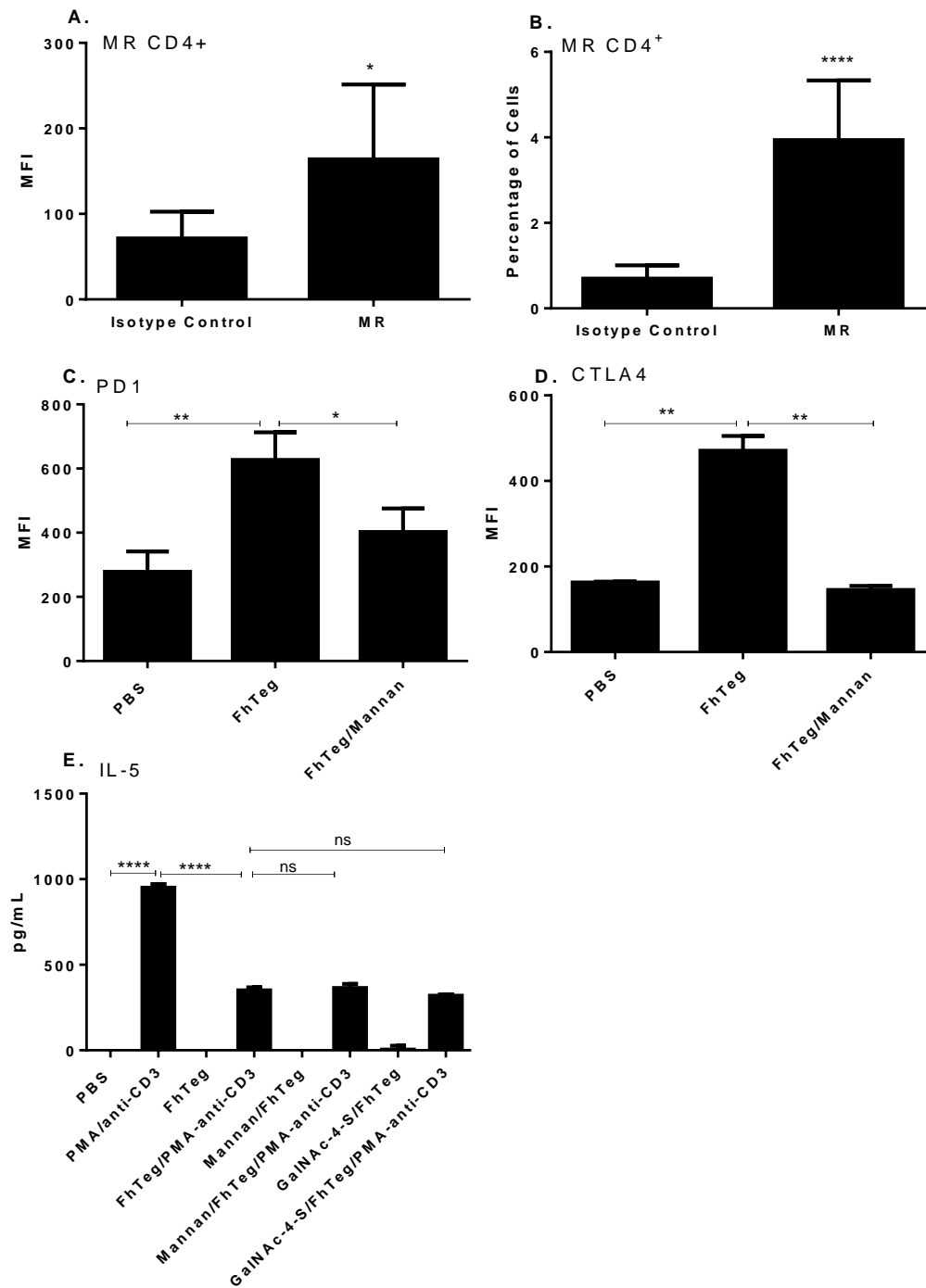


Figure 5.3.8. The C-type lectin Mannose Receptor is on the surface of CD4⁺ T-cells. A-B. MR was found to be significantly expressed on CD4⁺ T-cells compared to isotype controls ($p < 0.05$, $p < 0.0001$). Data analysed using student t-test. C-D. FhTeg significantly enhanced the expression of PD1 on CD4⁺ T-cells ($p < 0.01$) and

Mannan significantly inhibits the expression ($p<0.05$). FhTeg also enhanced the expression of CTLA4 ($p<0.01$) and mannan significantly inhibited this ($p<0.01$). **E.** PMA and anti-CD3 significantly enhanced the production of IL-5 from CD4⁺ T-cells ($p<0.0001$) and FhTeg significantly inhibited this ($p<0.0001$). Mannan does not reverse the suppressive effect of FhTeg on IL-5 production. The data shown is the mean \pm SD of three independent experiments. Data analysed using one way anova.

5.3.9. The Mannose receptor is not involved in the immune-modulatory effect of FhTeg on CD4⁺ T-cells

We have previously shown that FhTeg is rich in glycoproteins and has a high percentage of mannose rich glycans which may have a high affinity to bind to the mannose receptor (Manuscript in prep). We also showed that binding of FhTeg to CD4⁺ cells can be blocked using anti-MR and mannan. To evaluate if the mannose receptor is playing a part in FhTeg and CD4⁺ cell interaction, cells were isolated from background and knockout mice. To investigate if FhTeg can still bind to CD4⁺ cells in the absence of MR, binding assays were carried out using fluorescently labelled FhTeg. Cells were stimulated for 45 mins with PBS, FITC labelled BSA or FhTeg (10 μ g/ml), cells were then washed and binding was determined by flow cytometry. Both background and MR knockout mice showed comparable levels of binding to FhTeg in terms of the percentage of cells that bound (Figure 5.3.9A), there was no statistical difference between the two groups.

PD1 was highly expressed following a 24 hour stimulation with FhTeg on CD4⁺ cells. To measure PD1 expression, CD4⁺ cells isolated from background and MR knockout were stimulated with PBS or FhTeg (10 μ g/ml) for 24 hours, washed and stained with both CD4 and PD1 antibodies, cells were then analysed for PD1 expression by flow cytometry. The expression of PD1 was enhanced in both background and knockout mice but this was not statistically significant (Figure 5.3.9 B).

To investigate if we still get an up-regulation of genes related to anergy in CD4⁺ cells isolated from MR knockout mice, CD4⁺ cells from background and knockout mice were stimulated for 2 hours and RNA was isolated from the cell pellets. QPCR was used to measure the levels of CblB and EGR3, as these genes have been found to be highly expressed after FhTeg stimulation in Balb/C mice. FhTeg induced both CblB and EGR3 in both background and knockout mice, with a significant difference between the two groups for CblB (Figure 5.3.9 C-D) (*p<0.05, ****p<0.0001).

We had enhanced expression of PGE2 in CD4⁺ cells following a 24 hour FhTeg stimulation, to evaluate if MR knockout mice would still produce PGE2, cells were stimulated from both background and MR knockout for 24 hours and the levels of PGE2 were measured in the supernatant. In background mice there were significant levels of PGE2 but in the absence of MR, there were no significant levels of PGE2 produced (Figure 5.3.9 E) (****p<0.0001).

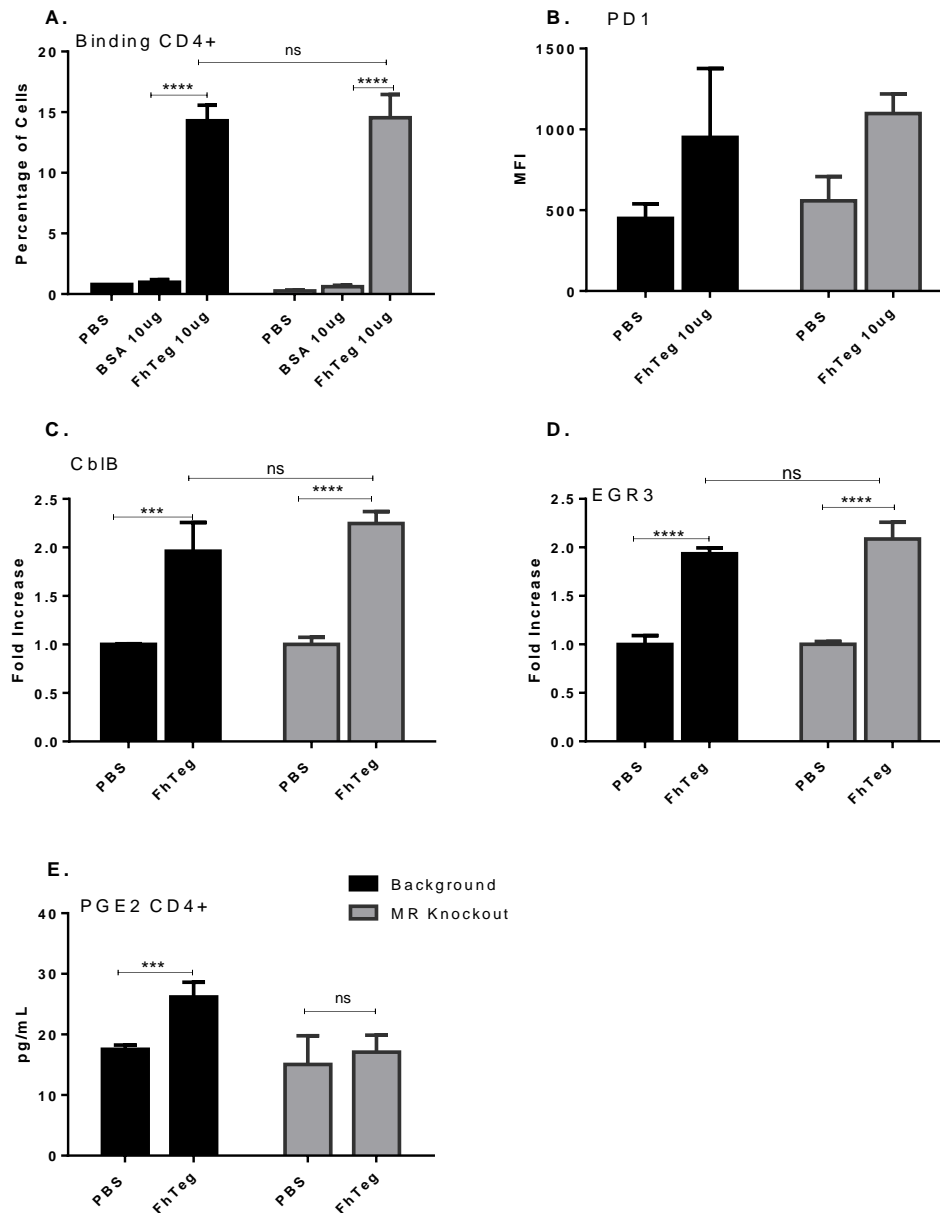


Figure 5.3.9 The Mannose receptor is not involved in the immune-modulatory effect of FhTeg on CD4⁺ T-cells. **A.** FhTeg binds significantly to CD4⁺ T-cells isolated from background and MR knockout mice ($p < 0.0001$). **B.** There was no significant enhancement of PD1 on CD4⁺ T-cells isolated from background and MR knockout cells; there was a trend of enhancement seen in both groups. **C-D.** Both Cb1B and EGR3 were significantly enhanced from CD4⁺ T-cells isolated from both background ($p < 0.001$, $p < 0.0001$) and MR knockout mice ($p < 0.0001$). **E.** FhTeg significantly enhanced the production of PGE2 from CD4⁺ T-cells isolated

from background mice compared to PBS ($p<0.001$) but this was diminished in MR knockout mice. The data shown is the mean \pm SD of two independent experiments. Data analysed using one way anova.

5.3.10. FhTeg treated CD4⁺ cells modulate dendritic cells ability to produce IL-12p70

CD4⁺ T-cells have been shown to influence DC's and other T-cells directly. T-reg cells can directly down-regulate co-stimulatory markers on APCs and inhibit their ability to present antigen (Cederbom, Hall and Ivars 2000, Misra et al. 2004, Tadokoro et al. 2006, Oderup et al. 2006). To investigate if FhTeg treated CD4⁺ T-cells can directly modulate DC's, CD4⁺ cells were treated with PBS or PMA (20ng/ml) and anti-CD3 (10 μ g/ml) in the presence or absence of FhTeg (10 μ g/ml) overnight before being washed and co-cultured with DC's for a further 18 hours. DC's were then separated from CD4⁺ T-cells and rested before being re-challenged with LPS (100ng/ml). Supernatants were analysed for the production of IL-12p70 by commercial ELISA. DC's which had been co-cultured with FhTeg treated CD4⁺ cells showed impaired ability to produce IL-12p70 when re-challenged with LPS, cells which had received PMA and anti-CD3 alone produced significant levels of IL-12p70 ($p<0.01$) but FhTeg significantly decreased this ($p<0.01$) (Figure 5.3.10 A).

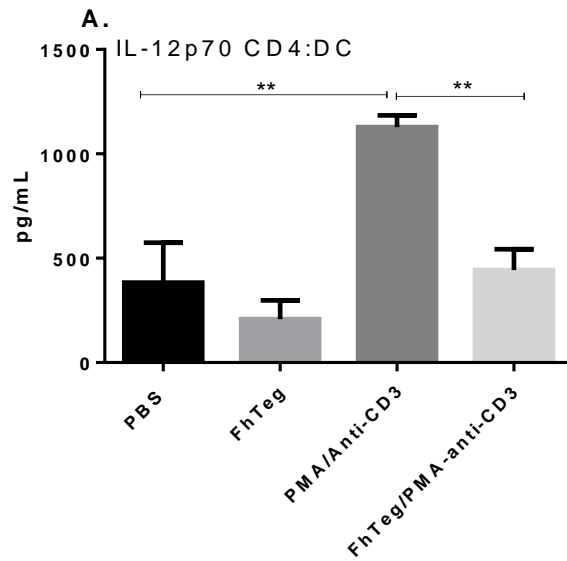


Figure 5.3.10 FhTeg treated CD4⁺ cells modulate dendritic cells ability to produce IL-12p70. PMA and anti-CD3 stimulated CD4⁺ T-cells significantly increase the production of IL-12p70 from DC's ($p < 0.01$) but FhTeg significantly inhibits this ($p < 0.01$). The data shown is the mean \pm SD of three independent experiments, (** $p < 0.01$). Data analysed using one way anova.

5.4. Discussion

Fasciola hepatica infection leads to a Th2/T-reg response in the host with an inhibition of a protective Th1 response. The major antigens of *F. hepatica*, the excretory secretory products and the tegumental coat have been shown to have a wide range of effects on different cell types. FhES causes the alternative activation of macrophages (Donnelly et al. 2005) and the priming of a Th2 immune response from T-cells. We are interested in the tegumental coat and have demonstrated that it induces a number of novel cell populations including dendritic cells, macrophages and mast cell. To our knowledge this is the first study to demonstrate that FhTeg has a direct effect on CD4⁺ cells, inducing an anergic like CD4⁺ cell population.

FhTeg induces an anergic like CD4⁺ cell that expresses Early growth response gene 2 and 3 (EGR2/3), CblB, and ITCH, genes that are associated with anergy. EGR2 is an important regulator of the induction of anergy (Harris 2004) as studies on EGR2 knockout mice have shown that full induction of anergy cannot be achieved without the full expression of the gene (Zheng et al. 2012). EGR3 is also involved in the induction and maintenance of the anergic phenotype and both genes lead to diminished IL-2 production and an increase in CblB expression which leads to negative regulation of T-cell activation (Harris 2004, Safford et al. 2005).

The E3 ubiquitin ligases have a major role to play in the maintenance of anergy, RNF128, CblB and ITCH promote the ubiquitination and degradation of the proteins PKC- θ and phospholipase C- γ and this leads to an inhibition of the calcium signalling pathway which in turn promotes anergy in the cell (Heissmeyer 2004). While we have shown an up-regulation

of this gene during infection and also in CD4⁺ cells following stimulation with FhTeg stimulated dendritic cells, we did not observe an up-regulation of RNF128 in our cells at the 2 hour time point but we did observe it at an earlier time point of 1 hr (Appendix C). Here we also have an increase in CblB expression; CblB inhibits actin re-organisation which impairs the formation of an active immunological synapse (Doherty et al. 2010).

We have found the up-regulation of several genes which may impair T-cell responses by inhibiting the activation of NFκB and its signalling molecules PKC-θ and AP1. PKC-θ is a protein kinase which is involved in the maturation and activation of CD4⁺ cells and the subsequent proliferation and initiation of a T-cell specific immune response. Here we have shown that FhTeg does not induce the activation of PKC-θ, which may explain the lack of proliferation and production of cytokines seen in CD4⁺ cells following stimulation. NFAT is also critical in the activation of cells, in conjunction with calcium influx, it travels to the nucleus and upregulates the transcription of a number of genes needed for activation (Soto-Nieves et al. 2009), but in the absence of AP1, NFκB transcription cannot proceed and instead negative regulators are transcribed.

Activation and differentiation of CD4⁺ cells is typically induced by APCs through binding of MHC II or other co-stimulatory markers like CD80 or CD86 to receptors on the T-cell like the T-Cell Receptor (TCR) or CD28 and a second signalling cascade through CD3 is also needed for activation. During anergy induction, insufficient signals from APCs consisting of signal one only or signalling through receptors like PD1 or CTLA4 can cause negative signalling pathways in the cell. CD80 (B7-1) and CD86 (B7-2) can bind to CTLA4 and have a 20 to 50 fold higher specificity for CTLA4 than CD28 delivering negative signals to T-cells

(Linsley et al.). Signalling through CTLA4 can predominantly happen if the expression of the co-stimulatory markers is low on the APC rather than after activation with antigens which positively enhance the expression (Greenwald et al. 2001). Here we have induced high CTLA4 expression directly by stimulation with FhTeg rather than through APC co-stimulation. The enhanced expression inhibits proliferation and IL-2 production and may help to keep the cell in a hypo-responsive state. PD1 is another cell surface marker which is used to create negative signals in T-cells. Signalling through PD1 by its receptors PD-L1 or PD-L2 creates unresponsiveness in cells by affecting IL-2 production and proliferation (Carter et al. 2002, Fife et al. 2009).

Incubation with the sugar mannan which binds the MR receptor before stimulation with FhTeg inhibited this enhanced expression of CTLA4 and PD1 on CD4⁺ cells. MR is a type-I membrane protein with a cytoplasmic domain involved in antigen processing and receptor internalisation and three different types of binding domains at its extracellular region. It features multiple C-type lectin-like carbohydrate-recognition domains (CRDs) responsible for calcium dependent binding to terminal mannose, fucose or *N*-acetyl glucosamine (Gazi and Martinez-Pomares 2009) a fibronectin type II (FNII) domain involved in collagen binding (Martinez-Pomares et al. 2006) and an *N*-terminal cysteine-rich (Cys-MR) domain that mediates calcium-independent binding to sulfated sugars (Leteux et al. 2000). Previously we have shown that MR is on the surface of mast cells and it plays a role in *B. pertussis* signalling (Vukman et al. 2013c) and similarly there have not been any studies to date to our knowledge on investigating if MR is on the surface of CD4⁺ cells. As we have been able to inhibit the binding of FhTeg to cells using sugars which bind to MR and also to inhibit the up-regulation of CTLA4 and PD1 we investigated if MR was on the surface of CD4⁺ cells. Using flow cytometry we have found that MR is on a small percentage of CD4⁺ T-cells.

FhTeg directly induces genes relating to anergy induction and maintenance and inhibits the expression of PKC- θ in CD4⁺ cells and here we have shown this impairs the cells ability to proliferate and to produce cytokines. The levels of IL-5 and IFN- γ were unchanged in FhTeg samples compared to PBS but following pre-incubation with FhTeg, the levels of cytokines produced is significantly decreased compared to PMA and anti-CD3. This is in keeping with our previous studies as dendritic cells when pre-incubated with FhTeg before LPS stimulation they have diminished production of inflammatory cytokines like IL-12p70 and TNF- α (Hamilton et al. 2009b) and FhTeg stimulated cells either following *F. hepatica* infection or immunisation fail to secrete cytokines. FhTeg also inhibited the cells ability to proliferate which lower percentages of cells in all generations seen following pre-incubation with FhTeg.

FhTeg can induce the expression of genes relating to anergy induction, promote the enhancement of negative cell surface markers and inhibit cytokine secretion, we have shown previously that FhTeg binds to dendritic cells and this can be blocked with the addition of antibodies and sugars for the mannose receptor but the mannose receptor did not play a role in the signalling events following binding. Here by fluorescently labelling FhTeg, we have shown that it can also bind directly to a percentage of CD4⁺ cells. We also show that using antibodies and sugars that bind the mannose receptor, we can inhibit this binding but studies using MR knockout mice have shown that binding still occurs to CD4⁺ cells isolated from knockout mice and that in the absence of the receptor the inhibitory effect of FhTeg is still seen. Further studies would need to be completed to identify the receptor involved on CD4⁺ cells which is involved in the signalling events following FhTeg stimulation.

In this study we have demonstrated that FhTeg has a direct effect on CD4⁺ cells, with an increase in expression of PD1 and CTLA4 and an up-regulation of a number of genes involved with anergy. Using the sugar mannan the binding of FhTeg and the up-regulation of CTLA4 and PD1 can be inhibited. To our knowledge this is the first study to show the presence of MR on the surface of CD4⁺ cells but it does not play a role in the induction of anergy in these cells. This study opens up a new area of study in helminth biology on CD4⁺ cells and the effect of glycan helminth products which may have a direct effect on cells through unknown receptors.

Chapter 6.

FhTeg modulates human PBMC's via CD4⁺ cells

6.1. Introduction

Helminth infections affect up to a third of the world's population with prevalence in the developing world, infection is rarely fatal but can lead to anaemia, weight loss and a susceptibility to bystander infections (Hotez et al. 2008). We have previous studies on mouse models using the helminth parasite *F. hepatica*. Infection leads to anergic T-cell responses and this can be mimicked by the injection of the tegumental antigens. FhTeg also causes the alternative activation of macrophages in vivo which impair cytokine secretion from CD4⁺ cells (Adams et al. 2014) and inhibits Th1 immune responses in mast cells (Vukman et al. 2013b). Studies on dendritic cells also show an inhibition of dendritic cells maturation and function following stimulation with bacterial ligands (Hamilton et al. June 2009) with an increase in SOCS3 expression (Vukman, Adams and O'Neill 2013). These FhTeg treated DCs induce anergy in CD4⁺ T-cells via the mannose receptor.

But infection with helminths parasites have some advantages and studies are ongoing into the use of whole worm and helminths products on inflammatory disorders such as multiple sclerosis (MS), inflammatory bowel disease (IBD), allergy, psoriasis and rheumatoid arthritis. Studies using whole worm therapy have shown potential such as multiple sclerosis and irritable bowel syndrome (Finlay, Walsh and Mills 2014) but whole worm therapy isn't always an option as complications can occur such as invasion of the parasite into other organs and when treatment is considered for immune-compromised individuals, pregnant women or for children.

There have been studies on helminths and the effect their antigens have on immune cells isolated from PBMCs. Studies in lymphatic filariasis using CD14⁺ cells shown an increase in IL-1 β when cells were re-challenged with LPS from control individuals but cells from infected patients showed a decrease in IL-1 β production, the cells ability to adhere was also diminished (Sasisekhar et al. 2004). Studies investigating lymphocyte responses from *Brugia malayi* infected individuals show a decrease in cytokine secretion and an increase in anergy factors (Babu et al. 2006) and studies using ES-62 from filarial nematodes induced anergy in Jurkatt T-cells (Harnett et al. 1998). To our knowledge there are no studies on the effect of FhTeg on PBMCs and here we will investigate the response of PBMCs, CD14⁺ and CD4⁺ cells to FhTeg.

6.2. Experimental Design

PBMCs were isolated from human buffy coat blood packs sourced from the Irish Blood Transfusion Service, St James' Hospital, Dublin, using density gradient centrifugation. A range of blood types were used for these studies. For binding studies cells were plated in a 96 well plate at 50,000 cells per 50 μ l, 1,5 or 10 μ g/ml of 488 labelled FhTeg was added to cells at 37 °C for forty five minutes and cells were subsequently washed in ice cold PBS before analysis using flow cytometry. As control for non-specific binding, cells were incubated with 10 μ g/ml of FITC-488 labelled BSA. As FhTeg bound to human PBMCs and in particular to both CD14 and CD4⁺ cells, the modulatory role of FhTeg on cells was measured. PBMCs were plated at 1x10⁶ cells per ml and stimulated with FhTeg (10 μ g/ml) for 2.5 hours before the addition of LPS (100ng/ml) or PMA (20ng/ml) and Ionomycin (1 μ M). Brefaldin A was added to inhibit the excretion of cytokines for analysis by intracellular staining. Following a 4 hour stimulation cells were labelled with FITC labelled CD11c and CD14, fixed and lysed for staining with APC labelled TNF- α antibody. As FhTeg inhibited the production of TNF- α from cells following a short stimulation PBMCs were stimulated as above but with no Brefaldin A overnight and supernatants were removed, the levels of TNF- α were measured using commercially available ELISA.

As FhTeg inhibited the production of TNF- α both intra-cellular and following an overnight incubation the effect of FhTeg was tested on populations isolated from PBMCs, CD14 and CD4⁺ cells were isolated and stimulated with FhTeg (10 μ g/ml) for 2.5 hours before the addition of LPS (100ng/ml) to CD14⁺ cells and PMA (20ng/ml)/Ionomycin

(1 μ M) to CD4⁺ cells. Following incubations of 18 and 72 hours respectively supernatants were removed and cytokines were analysed by ELISA.

From studies conducted in mouse models, we have shown that dendritic cells treated with FhTeg induce anergy in a mannose dependent manner and to investigate if this could be replicated with CD14⁺ cells, they were incubated with PBS or FhTeg overnight before being washed and co-cultured with CD4⁺ T-cells in a 1:10 ratio with plate bound anti-CD3 (1 μ g/ml). Following a 72 hour culture cells were removed, washed and lysed for analysis of CTLA4 expression and supernatants were analysed for the production of IFN- γ by ELISA.

The effect of FhTeg on CD4⁺ cells was also investigated by stimulating cells for 2 hours with FhTeg (10 μ g/ml) and analysing the expression of CTLA4, RNF128 and EGR2. The modulatory capacity of these cells was also analysed by treating CD4⁺ cells with FhTeg (10 μ g/ml) for 24 hours before washing and co-culturing with CD14⁺ cells for a further 24 hours. The CD14⁺ cells were then re-isolated and challenged with LPS (100ng/ml) for a further 24 hours, supernatants were collected and analysed for the production of TNF- α .

6.3. Results

6.3.1. FhTeg binds to PBMCs and inhibits the production of TNF- α

We have previously shown that FhTeg binds to dendritic cells generated from mouse but to our knowledge no studies have shown the effect of FhTeg on PBMCs. Cells were isolated from whole blood by gradient centrifugation and plated at 50,000 cells per well in a 96 well plate. Cells were treated with PBS, FITC labelled FhTeg (1, 5 and 10 μ g/ml) or FITC labelled BSA (10 μ g/ml) for 45 mins before the binding of FhTeg was analysed using flow cytometry. The binding of FhTeg to the whole PBMC population was measured. FhTeg binds significantly to PBMCs as a whole population (Figure 6.3.1 A-B) ($P < 0.01$, $P < 0.0001$), compared to BSA.

FhTeg can inhibit cytokine production from dendritic cells from mouse which have been pre-treated with FhTeg before being challenged with bacterial ligands (Hamilton et al. June 2009). To investigate if FhTeg would have similar effects on human PBMCs, cells were stimulated with FhTeg (10 μ g/ml) 2.5 hours prior to the addition of LPS (100 ng/ml) or PMA (20 ng/ml) and Ionomycin (1 mM). Brefeldin A was added to inhibit the excretion of cytokines to enable intracellular staining for TNF- α . The addition of FhTeg before LPS inhibited the production of TNF- α from both LPS and PMA/Ionomycin stimulated cells (Figure 6.3.1 C-D).

To investigate the modulatory capacity of FhTeg on cytokine secretion following an overnight stimulation, cells were stimulated with FhTeg (10 μ g/ml) for 2.5 hours before the addition of LPS (100 ng/ml) or PMA (20 ng/ml) and Ionomycin (1 μ g/ml) for 24 hours, supernatants were removed and analysed for TNF- α production by commercially available ELISA. Both LPS and PMA/Ionomycin significantly enhanced the production of TNF- α from

PBMCs ($p < 0.0001$) but FhTeg significantly inhibited the production of TNF- α from both sets of cells ($p < 0.05$, $p < 0.0001$) (Figure 6.3.1 E-F).

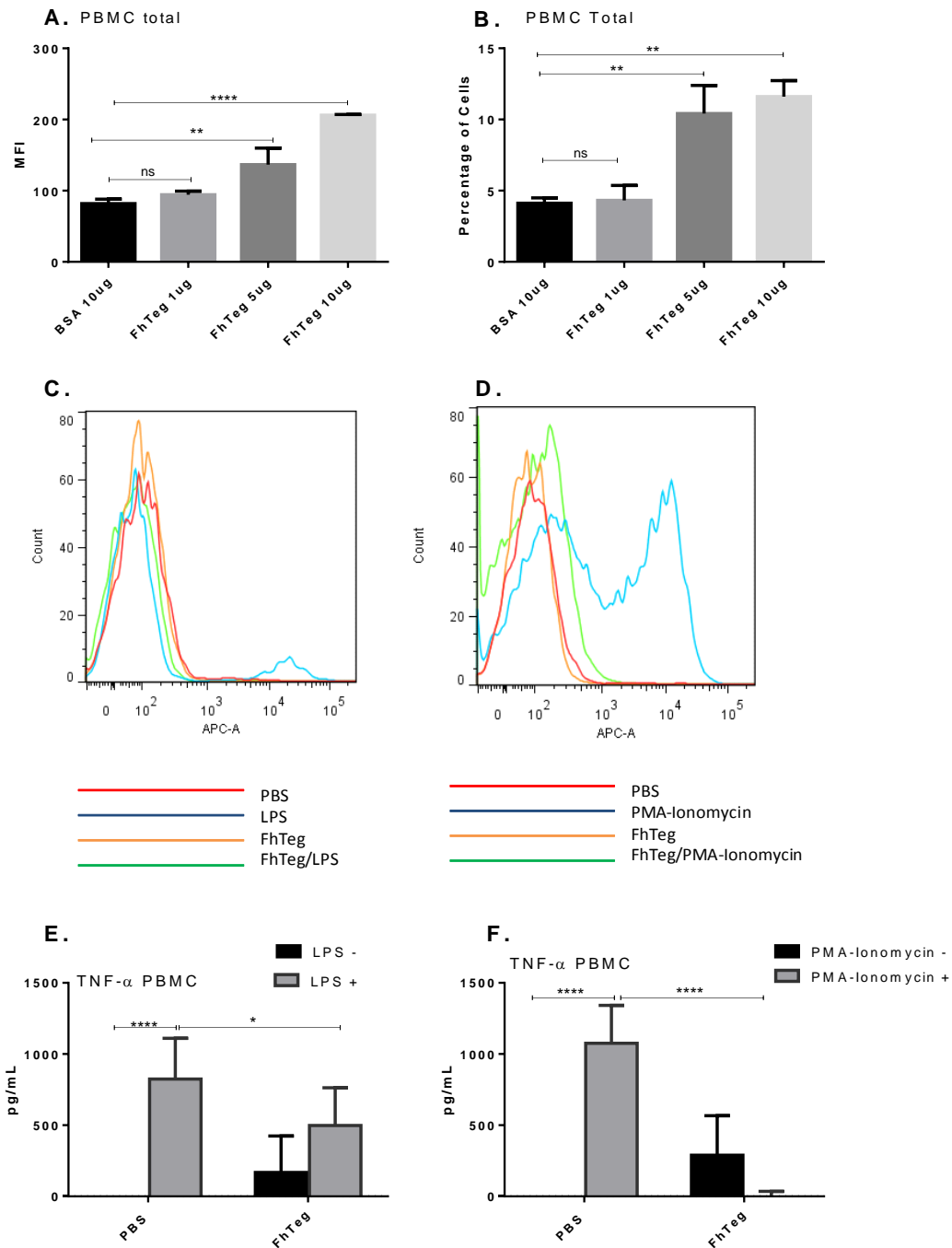


Figure 6.3.1. FhTeg binds to human PBMCs and inhibits the production of TNF- α . **A-B.** FhTeg binds significantly to PBMCs as a whole population at 5 μ g/ml ($p<0.01$) and 10 μ g/ml ($p<0.0001$) compared to BSA. **C-D.** Both LPS and PMA/Ionomycin enhanced the production of TNF- α from PBMCs compared to PBS but the addition of FhTeg inhibited this using intra-cellular staining. **E-F.** Both LPS and PMA/Ionomycin significantly enhanced the production of TNF- α from PBMCs compared to PBS ($p<0.0001$) but FhTeg significantly inhibited this ($p<0.05$, $p<0.0001$). The data shown is the mean \pm SD of four independent experiments, donor number $n=4$. Data analysed using one way anova.

6.3.2. FhTeg does not inhibit the production of TNF- α from CD14⁺ cells

As FhTeg targets multiple cell types in mouse including inhibiting dendritic cells (Hamilton et al. June 2009), mast cells (Vukman et al. 2013a) and macrophages, CD14⁺ cells were isolated from PBMCs and the ability of FhTeg to bind to CD14⁺ cells was investigated. Cells were treated with PBS, FITC labelled FhTeg (1, 5 and 10 μ g/ml) or FITC labelled BSA (10 μ g/ml) for 45 mins before the binding of FhTeg was analysed using flow cytometry. FhTeg significantly binds to CD14⁺ cells stimulated with 10 μ g of FITC-labelled FhTeg ($p<0.01$) (Figure 6.3.2 A) and binds significantly to a small percentage of less than 10% of cells at both 5 and 10 μ g/ml (Figure 6.3.2.B) ($p<0.01$).

We have previously shown that FhTeg enhances the expression of SOCS3 in dendritic cells, to examine if FhTeg would induce SOCS3 in CD14⁺ cells; cells were isolated from PBMCs and stimulated with PBS or FhTeg (10 μ g/ml) for 2 hours. FhTeg significantly enhances the expression of SOCS3 ($p<0.0001$) (Figure 6.3.2 C). To examine cytokine responses from CD14⁺ cells following FhTeg stimulation, PBMCs were stimulated with FhTeg (10 μ g/ml) 2.5 hours before the addition of LPS (100ng/ml), Brefeldin A was also added to enable intracellular staining. Following a 4 hour stimulation, cells were fixed, lysed and the levels of TNF- α were measured using flow cytometry. CD14⁺ cells gated on from a whole PBMC

population were inhibited following pre-incubation with FhTeg (Figure 6.3.2 D). To examine cytokine responses following a 24 hours stimulation, CD14⁺ cells were stimulated with PBS or FhTeg (10µg/ml) 2.5 hours before the addition of LPS (100ng/ml), supernatants were removed from cells and the levels of TNF-α and IL-1β were measured. LPS induced significant levels of IL-1β (p<0.0001) but not TNF-α from CD14⁺ cells. FhTeg did not inhibit the production of either cytokine from CD14⁺ cells but significantly increased the production of TNF-α (p<0.01) and IL-1β (p<0.0001) from cells (Figure 6.3.3A-B).

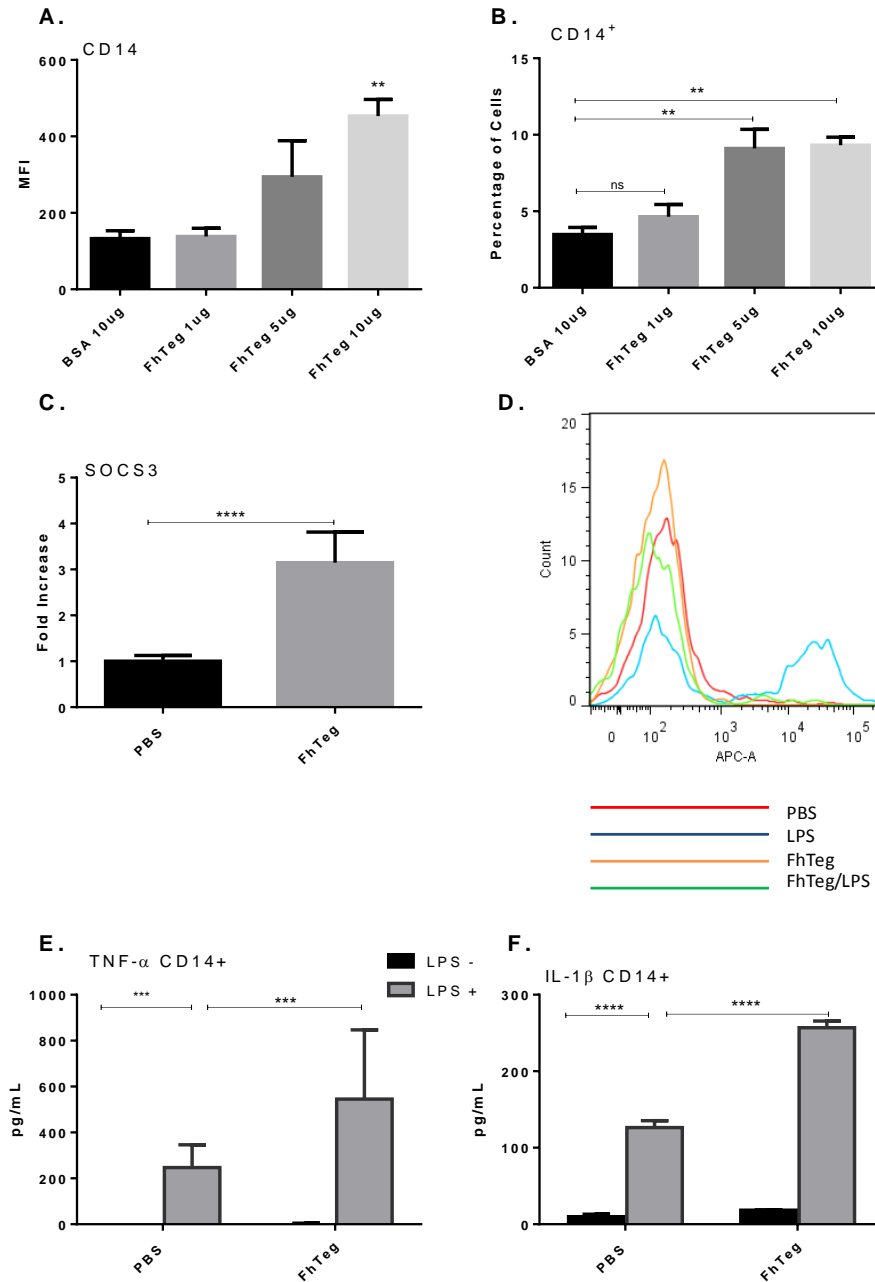


Figure 6.3.2. FhTeg does not inhibit cytokine secretion from human CD14⁺ cells. **A-B.** FhTeg significantly binds to a small percentage of CD14⁺ cells at 5 and 10μg compared to BSA ($p < 0.01$). **C.** FhTeg significantly enhanced the production of SOCS3 ($p < 0.0001$) from CD14⁺ cells compared to PBS. **D.** LPS enhances TNF-α production but FhTeg inhibits this from CD14⁺ cells when in the larger PBMC population. **E-F.** FhTeg significantly increased the production of TNF-α ($p < 0.001$) and IL-1β ($p < 0.0001$) compared to both PBS and LPS. The data is presented as the mean \pm SD of three independent experiments, donor number $n=3$, ** $p < 0.01$, **** $p < 0.0001$.

6.3.3. FhTeg inhibits the production of cytokines from CD4⁺ T-cells

FhTeg binds to PBMCs as a whole and to CD14⁺ cells. To investigate if FhTeg would bind to CD4⁺ T-cells, PBMCs were incubated with 1, 5 and 10µg of FITC-labelled FhTeg. CD4⁺ T-cells were gated on and the binding of FhTeg to the CD4⁺ population was measured. There was significant binding of FhTeg to CD4⁺ cells by both MFI and percentage of cells ($p < 0.01$, $P < 0.0001$) (Figure 6.3.3 A-B). FhTeg can inhibit the production of TNF- α from PBMCs as a whole population but not from CD14⁺ cells, to examine if FhTeg would inhibit CD4⁺ cells ability to produce cytokines, CD4⁺ cells were isolated from PBMCs and stimulated with FhTeg (10µg/ml) 2.5 hours before the addition of PMA (20ng/ml) and Ionomycin (1mM). 72 hours later supernatants were removed and analysed for the production of TNF- α and IL-2. PMA and Ionomycin induced significant levels of the two cytokines ($p < 0.0001$), the addition of FhTeg significantly inhibited this, (Figure 6.3.3 C-D) ($p < 0.0001$).

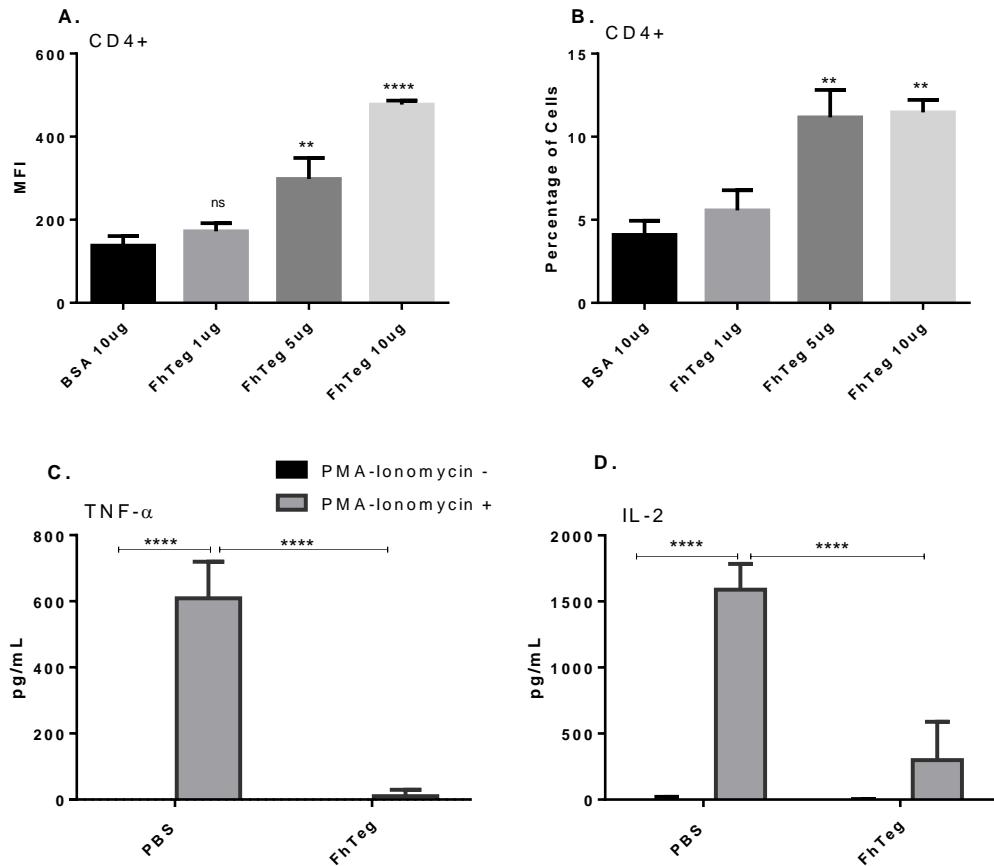


Figure 6.3.3. FhTeg inhibits the production of cytokines from CD4⁺ cells. **A-B.** FhTeg binds significantly to CD4⁺ T-cells at 5 and 10µg/ml ($p<0.01$, $p<0.0001$). **C-D.** PMA/Ionomycin stimulation significantly enhanced the production of TNF-α ($p<0.0001$) and IL-2 ($p<0.0001$) production compared to PBS but FhTeg significantly inhibited this ($p<0.0001$). The data is presented as the mean \pm SD of three independent experiments, donor number $n=3$, ** $p<0.01$, **** $p<0.0001$. Data analysed using one way anova.

6.3.4. FhTeg induces genes relating to anergy in CD4⁺ T-cells

CD4⁺ T-cells isolated from PBMCs were stimulated with PBS or FhTeg (10µg/ml) for two hours. Cells were then washed and RNA was isolated from cell pellets. The levels of CTLA4 and RNF128 were measured. The level of both CTLA4 and RNF128 were significantly enhanced with a fold increase of 1.5 and 3.5 respectively ($p<0.001$, $p<0.05$).

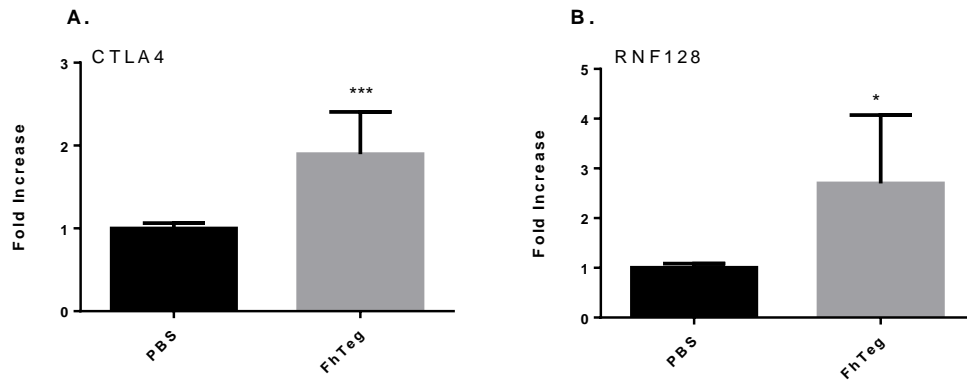


Figure 6.3.4. FhTeg induces genes relating to anergy in CD4⁺ T-cells. FhTeg significantly enhances the expression of both CTLA4 and RNF128 genes on CD4⁺ T-cells compared to PBS, $p < 0.001$, $p < 0.05$. The data shown is the mean \pm SD of three independent experiments, donor number $n=3$. Data analysed using student t-test.

6.3.5. FhTeg treated CD14⁺ cells do not negatively modulate CD4⁺ cells

As FhTeg does not inhibit TNF- α or IL-1 β production from CD14⁺ cells isolated from PBMCs, their ability to modulate CD4⁺ cells was investigated. CD14⁺ cells were isolated by positive selection from PBMCs and stimulated with PBS or FhTeg (10 μ g/ml) overnight. Cells were then washed twice and co-cultured in a 1:10 ratio with CD4⁺ cells from both the same and different donors with plate bound anti-CD3 (1 μ g/ml). Following a 72 hour stimulation, supernatants were removed and RNA was isolated from cell pellets. The levels of IFN- γ were measured using commercially available ELISA. CD14⁺ cells pre-treated with FhTeg do not inhibit the production of IFN- γ from CD4⁺ cells, isolated from either the same or different donors (Figure 6.3.5 A-B). The levels of CTLA4 were measured in CD4⁺ cells following co-culture and there was no significant difference in the levels of CTLA4 in either group (Figure 6.3.5 C-D)

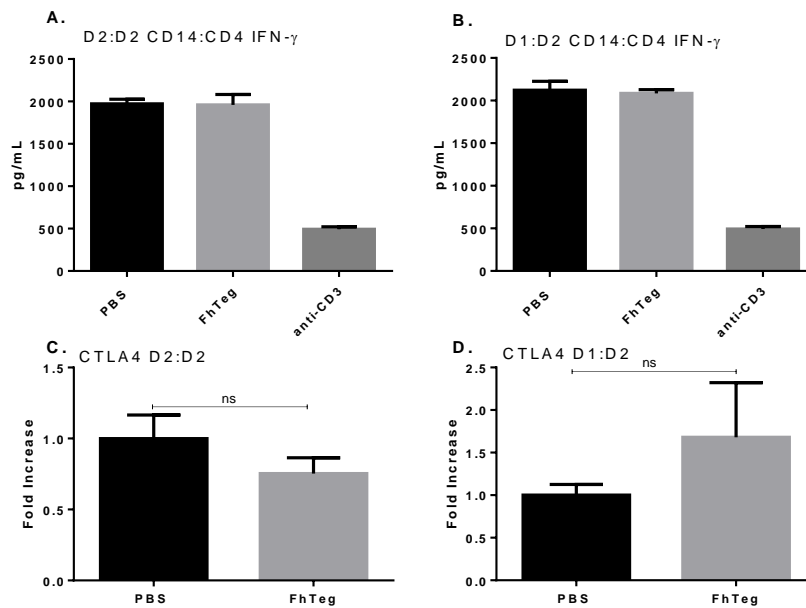


Figure 6.3.5. FhTeg treated CD14⁺ cells do not negatively modulate CD4⁺ cells. **A-B.** Co-culture of CD14⁺ cells with CD4⁺ cells showed no significant differences in the levels of IFN- γ compared to PBS controls. In A, the same donor was used for both CD4 and CD14⁺ cells, in B a different donor was used for CD4⁺ and CD14⁺ cells. **C-D.** No significant differences in the expression of CTLA4 were seen in either group compared to PBS controls. The data shown is the mean \pm SD of three independent experiments, donor number n=3.

6.3.6. FhTeg treated CD4⁺ cells modulate CD14⁺ cells and inhibit cytokine secretion upon re-stimulation with LPS

CD14⁺ cells ability to produce TNF- α is inhibited while part of a larger PBMC population once pre-incubated with FhTeg but isolated CD14⁺ cells are not inhibited by FhTeg upon LPS stimulation, these CD14⁺ cells also do not modulate CD4⁺ cells so the ability of CD4⁺ cells to modulate CD14⁺ cells directly was investigated. CD4⁺ cells were stimulated with PBS or FhTeg (10 μ g/ml) overnight, washed and co-cultured with CD14⁺ cells in a 1:1 ratio for 24 hours, CD14⁺ cells were then re-isolated and rested in fresh medium overnight before being re-challenged with LPS (100ng/ml). Supernatants were then removed after 24 hours and the

production of TNF- α was measured. FhTeg treated CD4⁺ cells significantly inhibited the production of TNF- α from CD14⁺ cells when re-stimulated with LPS ($p<0.05$).

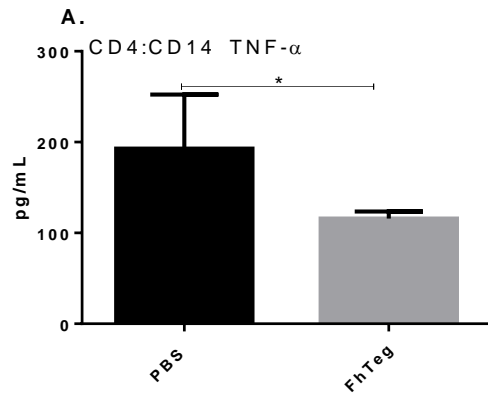


Figure 6.3.6 FhTeg treated CD4⁺ cells modulate CD14⁺ cells and inhibit cytokine secretion upon re-stimulation with LPS. Co-culture with FhTeg treated CD4⁺ cells significantly inhibited the production of TNF- α from re-stimulated CD14⁺ cells, ($p<0.05$). The data shown is the mean \pm SD of three independent experiments, donor number $n=3$. Data analysed using student t-test.

6.4. Discussion

Helminth parasites infect up to a third of the world's population, with the majority of these people in the developing world. Infection is rarely fatal but can lead to anaemia, weight loss and a susceptibility to bystander infections such as malaria. But infection with helminths can have advantages and studies are underway into whole worm therapy into multiple sclerosis and irritable bowel syndrome (Finlay, Walsh and Mills 2014) and studies are also ongoing into using helminth products as therapeutics in the treatment of MS (Zheng et al. 2008, Kuijk et al. 2012a), IBD, allergy, psoriasis and rheumatoid arthritis. (Heylen et al. 2014, Whary et al. 2001). To further advance these studies, the mechanism of immune modulation by helminths needs to be investigated. Here we showed how the tegumental antigens of *F. hepatica* interact with human PBMCs and modulate cytokine secretion and cell activation.

We have previously shown in mouse studies that FhTeg binds to dendritic cells and to CD4⁺ cells, here we have also shown that FhTeg can bind to PBMCs as a whole population and also to both CD14 and CD4⁺ cells. The effect of FhTeg on DC's, macrophages and mast cells from mice has been well studied (Adams et al. 2014, Vukman et al. 2013b, Vukman et al. 2013a, Vukman, Adams and O'Neill 2013). FhTeg inhibits dendritic cell maturation and cytokine secretion when challenged with bacterial ligands (Hamilton et al. June 2009) and here FhTeg inhibited TNF- α secretion from PBMCs as a whole population using both intracellular staining and ELISA to show the suppression.

PBMCs consist of a number of cell types including monocytes and CD4⁺ cells (Geissmann et al. 2010), we have shown that FhTeg induces the alternative activation of macrophages in

vivo (Adams et al. 2014) and induces anergy in CD4⁺ cells both *in vivo* and *in vitro*. As FhTeg inhibited cytokine secretion in both PBMCs as a whole and from CD14⁺ cells intracellular, we examined responses of both CD14 and CD4⁺ cells to FhTeg. Interestingly, CD14⁺ cells were not inhibited by pre-treatment with FhTeg, their production of TNF- α and IL-1 β was enhanced, whereas CD4⁺ cells were inhibited upon pre-treatment with FhTeg with significantly less cytokines produced in response to PMA and Ionomycin activation. The response of PBMCs to *F. hepatica* tegumental antigens in experimental infections of cattle showed a loss of proliferation and IL-2 production from the fifth week of infection and the authors concluded that this was not due to the macrophage population (Oldham and Williams 1985). Other studies in helminth infections have shown roles for CD14⁺ cells, in lymphatic filariasis, CD14⁺ cells isolated from control and infected individuals showed markedly different responses, cells isolated from control and endemic normal patients showed an increase in IL-1 β when cells were re-challenged with LPS but cells from infected patients showed a decrease in IL-1 β production and inhibited the adherence of CD14⁺ monocytes compared to controls (Sasisekhar et al. 2004). Further studies would need to be completed to investigate the effects of *F. hepatica* infection on monocytes *in vivo*.

We have shown previously that FhTeg treated dendritic cells can induce anergy in CD4⁺ cells, inhibiting cytokine secretion and inducing RNF128 and CTLA4 in cells. Macrophages isolated from FhTeg injected mice also inhibit cytokine responses from CD4⁺ cells (Adams et al. 2014). The inhibitory capacity of FhTeg treated CD14⁺ was measured by co-culturing with CD4⁺ cells isolated from the same or a mismatched donor. The secretion of IFN- γ was unchanged in both PBS and FhTeg treated cells and neither group had a significantly higher

level of CTLA4 expression. This may point towards the CD4⁺ cells population playing a role in cytokine suppression seen in PBMCs as a whole and not CD14⁺ cells.

The suppression of CD4⁺ cells cytokine secretion is similar to results we have previously shown in mouse studies. To investigate if FhTeg could induce anergy directly in CD4⁺ cells isolated from PBMCs, cells were stimulated with FhTeg directly and the levels of CTLA4 and RNF128 were measured and showed an increase for both genes. CTLA4 is a receptor found on the surface of T-cells which binds its ligands CD80 and CD86 on APCs (Linsley et al.). Signalling through CTLA4 induces negative regulators including SHP1/2 which inhibit CD28 and TCR co-signalling and IL-2 production (Greenwald et al. 2001). RNF128 is also involved in anergy induction (Seroogy et al. 2004). A loss of IL-2 production leads to no phosphorylation of Akt and activation of mTOR. This leads to subsequent GRAIL expression inhibiting proliferation (Whiting et al. 2011). Studies investigating lymphocyte responses from *Brugia malayi* infected individuals show an impairment in cytokine secretion and an increase in anergy factors such as CblB and ITCH (Babu et al. 2006) and studies using ES-62 from filarial nematodes induced anergy in Jurkatt T-cells via the TCR receptor (Harnett et al. 1998) but to our knowledge this is the first study to show that *F. hepatica* tegumental antigens can directly induce anergy in CD4⁺ T-cells isolated from PBMCs without prior priming of cells *in vivo* by infection.

As FhTeg inhibits cytokine secretion from CD14⁺ cells when they are in the whole PBMC population but not when isolated as a single population, and they do not inhibit T-cell responses from CD4⁺ cells, we investigated if it was the CD4⁺ population modulating the CD14⁺ cells. CD4⁺ cells were stimulated with PBS or FhTeg overnight before co-culturing

with CD14⁺ cells for a further 24 hours. CD14⁺ cells were then re-isolated and re-challenged with LPS to see if the CD4⁺ cells had inhibited their ability to produce TNF- α . Interestingly, following culture with FhTeg CD4⁺ cells, CD14⁺ cells ability to produce TNF- α was significantly inhibited. T-cells have been shown to modulate APCs, in previous studies T-reg cells down-regulate co-stimulatory markers on APCs and inhibited their ability to present antigen successfully to naive CD4⁺ T-cells (Cederbom, Hall and Ivars 2000, Misra et al. 2004, Tadokoro et al. 2006, Oderup et al. 2006). Anergic CD4⁺ cells have also been shown to modulate APCs by affecting their ability to present antigens or by inducing apoptosis in DC's. The effect of FhTeg on CD4⁺ cells is important as there are a number of diseases which are T-cell mediated and FhTeg or molecules isolated from it may be used as therapeutics in the treatment of these conditions.

Graft versus host disease is a complication encountered following an allogenic transplant, usually of bone marrow or stem-cells. T-cells which are present in the transplanted graft recognise the hosts tissues and cells as antigens and mount an immune response. This can lead to damage to liver, skin and can cause vomiting, diarrhoea and severe abdominal pain (Przepiorka et al. 1995). The engraftment of donor T-cells is needed for most transplants to be successful, this is to prevent host versus graft occurring and it can also be beneficial for some cancer treatments as the donor T-cells can have an anti-tumour effect (Ringdén et al. 2009). Using helminths products may be helpful in the treatment of cases of GVHD as in the case of FhTeg, it would target the whole PBMC population through the donor CD4⁺ cells and may reduce the occurrence and severity of the disease. Some studies have shown that helminths can be protective in GVHD, studies using *Heligmosomoides polygyrus* showed that infection reduced GVHD related mortality and inflammatory cytokine secretion while also maintaining graft versus tumour (Li et al. 2015). Studies with our collaborators have shown a

reduction in weight loss and disease score in GVHD once donor PBMCs were pre-treated with FhTeg before given to mice compared to mice which received PBMCs alone (see appendix 9.4). The mechanism of FhTeg in preventing GVHD seems to be by stopping donor CD4⁺ cells from engrafting in the host which would not be favourable for the treatment of GVHD as donor CD4⁺ T-cells are needed to stop the hosts CD4⁺ T-cells from attacking the graft; but points towards FhTeg possibly being useful in cases like organ transplantation.

This study is the first to show the modulatory effects of *F. hepaticas* tegumental antigens on human PBMCs. FhTeg inhibits the secretion of TNF- α from PBMCs as a whole population and from CD4⁺ cells but not from CD14⁺ cells. The FhTeg treated CD14⁺ cells also do not negatively regulate CD4⁺ cells, with no inhibition of IFN- γ or enhancement of CTLA4. FhTeg modulates PBMCs by targeting CD4⁺ cells with an increase in the expression of the negative regulators CTLA4 and RNF128 and these cells can negatively regulate CD14⁺ cells by inhibiting their ability to secrete TNF- α following re-challenge with LPS. This study will help further our understanding of how *F. hepatica* modulates the immune system and how it may be used as a therapeutic in the future.

Chapter 7- Final Discussion

This study sheds new light on the immune-modulatory role of *Fasciola hepatica* infection and its tegumental antigens on both dendritic cells and CD4⁺ T-cells from both mouse and human PBMCs. The study has shown a number of novel findings including characterisation of the T-cell responses during infection as anergic and following injection of FhTeg. The effect of FhTeg on dendritic cells is independent of the mannose receptor and the effect of FhTeg on CD4⁺ T-cells from both mouse and human origin. The following discussion will highlight key findings from the study and discuss future studies which are required.

7.1. *F. hepatica* induces anergy in CD4⁺ T-cells following infection and injection of FhTeg

T-cells are crucial players in both the innate and adaptive immune response. Once they recognise antigens being presented by APCs through MHC class I and II along with environment factors, they can differentiate into a number of subsets including Th1, Th2, T-reg and anergic to name a few (Adam, Schweitzer and Sharpe 1998, Gamper and Powell 2010). Th1 cells are characterised by the production of inflammatory cytokines, IFN- γ and TNF- α and drive inflammation, whereas Th2 cells produce anti-inflammatory cytokines such as IL-5 and IL-4. T-reg's "turn off" or dampen the immune response by producing cytokines like IL-10 and TGF- β but anergic cells do not produce any cytokines or proliferate upon re-stimulation but are still key mediators in self-versus non-self-recognition and help to control auto-immunity (Schwartz 2005, Sakaguchi 2004).

During helminth infections, T-reg cells are the most prominent response observed. Studies using *Brugia malayi* have shown T-reg cells to express enhanced FoxP3 and

increased cell surface expression of CTLA4, a negative regulator of T-cell function (McSorley et al. 2008). Another helminth, *Schistosoma haematobium* has also been associated with T-reg cells (Nausch et al. 2011). Helminth infections are also associated with anergic T-cells that are most commonly associated with chronic infection which may be due to the persistence of parasite antigen and the constant contact of the cells with the antigens. During *S. mansoni* infection, anergy is the main T-cell response seen, mediated by the up-regulation of PD-L1 on the surface of macrophages (Smith et al. 2004) and RNF128 (GRAIL) in T-cells isolated from chronic infection. Here they show that T-cell anergy following chronic infection is driven by RNF128 expression (Taylor et al. 2009). This demonstrates that during *F. hepatica* infection, T-cell anergy is the main T-cell response observed with the rescue of cytokine secretion with IL-2 and the up-regulation of a number of genes involved in anergy induction and maintenance like RNF128, CblB and ITCH and no enhancement of FoxP3, IL-10 or TGF- β . Further studies would need to be carried out to investigate if T-cell anergy is observed in natural models of infection in sheep/cattle or in patient samples from endemic areas of infection.

Fasciola hepatica has two main sources of antigen, its excretory/secretory molecules (FhES), which it releases constantly which consists of mostly enzymes, and its tegumental antigens. FhTeg is a complex mix of glycoproteins and is in constant contact with the hosts' immune cells. We have previously shown that injection of FhTeg into the peritoneal cavity of mice mimics infection by recruiting mast cells and indirectly inducing alternative activation of macrophages (Adams et al. 2014, Vukman et al. 2013a). Here we have shown that injecting FhTeg over the sternum also induces anergy with an increase in PD1 and RNF128 seen in splenocytes and a reversal of cytokine suppression with IL-2.

FhTeg was also injected into the peritoneal cavity and anergy was also seen, although the response was not as strong as seen with infection or after injection over the sternum (Appendix A). The effect of FhTeg differs to that of FhES (Figure 7.1), the injection of FhES into the peritoneal cavity induces the alternative activation of macrophages and this can be mimicked by thioredoxin peroxidase, this also resulted in the alternative activation of Raw 264.7 macrophages with high levels of IL-10 and PGE2 (Donnelly et al. 2005) and another component of FhES, peroxiredoxin, also induced alternative activation of macrophages (Donnelly et al. 2008). The injection of FhES also recruits mast cells to the peritoneal cavity as does FhTeg but the response to FhTeg was significantly higher than FhES (Vukman et al. 2013a). We know from proteomic studies that there antigen sources vary hugely in the antigenic make-up while much is published on FhES further studies are required to identify what are the bioactive molecules that drive anergy.

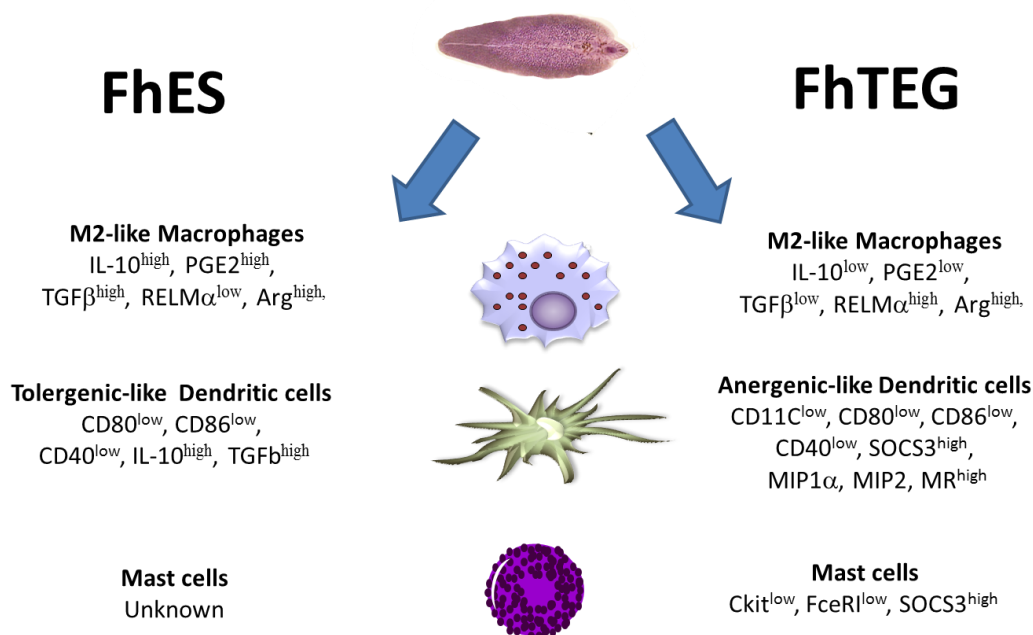


Figure 7.1. The difference between FhES and FhTeg on immune cells. FhES and FhTeg differ in their actions on immune cells. Both FhES and FhTeg induce M2-like macrophages with Arg expression enhanced but differ in cytokine expression, FhES induces IL-10, PGE2 and TGF-β but FhTeg does not. FhTeg also induces the expression of RELMα but FhTeg does not. The action of FhTeg differs to FhTeg as

FhTeg induces an MR high and SOCS3 high population. The effect of FhES has yet to be determined on mast cells.

7.2. FhTeg induces a novel dendritic cell population

We have previously shown that dendritic cells when treated with FhTeg do not respond to LPS stimulation, with a decrease in both IL-12p70 and TNF- α production and no enhancement of co-stimulatory markers (Hamilton et al. June 2009). These dendritic cells remain immature despite LPS stimulation. Here we have further investigated the mechanism of action of FhTeg on dendritic cells generated from bone marrow. Following FhTeg stimulation, DC's have an enhancement of the expression of the mannose receptor high and a decrease in CD11c expression. Other studies have also shown a change in expression of CD11c following interaction with helminth antigens. During *H. polygyrus* infection, a change in CD11c expression in dendritic cells was observed (Smith et al. 2011). We have shown that FhTeg is rich in high mannose glycans (manuscript in prep). As MR is enhanced following FhTeg stimulation, the binding efficiency of FhTeg was examined after pre-treatment with a number of sugars and antibodies which would bind both MR and MGL. Pre-incubation with anti-MR, mannan and GalNAc-4-Sulphate significantly inhibited the binding of FhTeg to DC's and this was also in a calcium dependent manner as EDTA also inhibited the binding of FhTeg but using sugars for MGL, GalNAc and anti-MGL the binding could not be inhibited.

To investigate if inhibiting binding of FhTeg to DC's using mannan and GalNAc-4-Sulphate would reverse the effect of FhTeg, both inhibitors were used and the levels of SOCS3 and IL-12p70 were measured. GalNAc-4-Sulphate inhibited the enhancement of SOCS3 in DC's but mannan did not, interestingly GalNAc-4-Sulphate did not reverse IL-

12p70 suppression but Mannan did. Mannan is a simple polysaccharide which is isolated from *Saccharomyces cerevisiae*. It has binding specificities for MR but also any other high mannose binding receptors which include SIGNR1 and Dectin 2. MR has been shown to be involved in the induction of negative signalling pathways following helminth infection, studies with products excreted/secreted by *Taenia crassiceps* triggered cRAF phosphorylation through MGL, MR, and TLR2 (Terrazas et al. 2013) also during *S. mansoni* infection MR has been shown to play a role in IFN- γ production (Paveley et al. 2011). To confirm that MR had a role to play in the signalling response seen after FhTeg treatment, MR knockout mice were used. The knocking out of MR did not have any significant effect on the induction of SOCS3 or IL-12p70 production but did have an effect on binding. FhTeg is a complex mix of over 70 proteins, many of which are glycoproteins so the signalling may not be due to one CTR, it may be due to a number of receptors working together. It may not be possible to find one receptor which FhTeg may bind through. Further studies would need to be completed to find bioactive components of FhTeg and what receptors they may signal through, rather than looking at FhTeg as a whole. Other studies also need to be carried out on other high mannose binding receptors such as SIGN-R1, as FhTeg is mannose rich to fully understand the mechanism of action of FhTeg on DC's.

7.3. C-type lectin receptors influence CD4⁺ T-cell differentiation

To prime an effective immune response, crosstalk must occur between different cell types. This can be in the form of cell to cell contact or via soluble mediators released from cells. The communication between dendritic cells and CD4⁺ cells is crucial in initiating a response against antigens. Generally dendritic cells encounter antigens and present them to CD4⁺ cells on MHC class I or II molecules and also use secondary signals

like binding of the ligands CD80 or 86 to their respective receptors. This along with cytokines released can push CD4⁺ cells towards a number of different subsets including Th1, Th2, Th17 or T-reg (Yamane and Paul 2013).

We have previously shown that mast cells treated with FhTeg and co-cultured with CD4⁺ cells, impair their ability to produce cytokines and this is due to signalling through ICAM1 (Vukman et al. 2013b), macrophages isolated following injection of FhTeg also impair CD4⁺ cells ability to produce cytokines (Adams et al. 2014). Dendritic cells treated with FhTeg and LPS produce significantly less IL-12p70 and TNF- α than with LPS alone, and these cells drive T-cell anergy by a reduction in IL-2, IL-5, IL-4 and IFN- γ production and an enhancement of the expression of RNF128 and CTLA4 in CD4⁺ cells following co-culture. DC's have been implicated in driving anergy as DC's isolated from children with severe malnutrition and endotoxemia were found to be anergic and inhibited T-cell proliferation (Hughes et al. 2009) but most studies have focused on immature DC's driving anergy, studies using BMDC's cultured in GM-CSF with high levels of LPS induced anergy *in vitro* (Lutz et al. 2000). Further studies are required to investigate if dendritic cells isolated from *F. hepatica* infected mice would induce anergy in naive CD4⁺ cells.

The induction of anergy by FhTeg treated DC's was also found to be in an MR dependent manner as DC's generated from MR knockout mice inhibited the induction of anergy (Figure 7.1). MR is a type-I membrane protein with a cytoplasmic domain involved in antigen processing and receptor internalisation and three different types of binding domains at its extracellular region (Martinez-Pomares 2012). Most studies on MR have focused on its ability as an antigen recognition receptor but few have looked at its ability

to promote immune responses through communication with its counter ligand or receptor CD45, (Martínez-Pomares et al. 1999) which has numerous roles in T-cell development and is both a positive and negative regulator of T-cell function (Saunders and Johnson 2010). Studies examining if engagement of CD45 by MR is responsible for inducing anergy in CD4⁺ cells would need to be performed. Preliminary results have shown that using a blocking anti-body for CD45 inhibited the cells ability to produce cytokines and had a significant decrease in the expression of CTLA4 and RNF128 in controls (Appendix B), further investigation is needed to examine this further as studies using silencing RNA or studies in CD45 knockout mice may give us further insights into how MR modulates CD4⁺ cells through DCs.

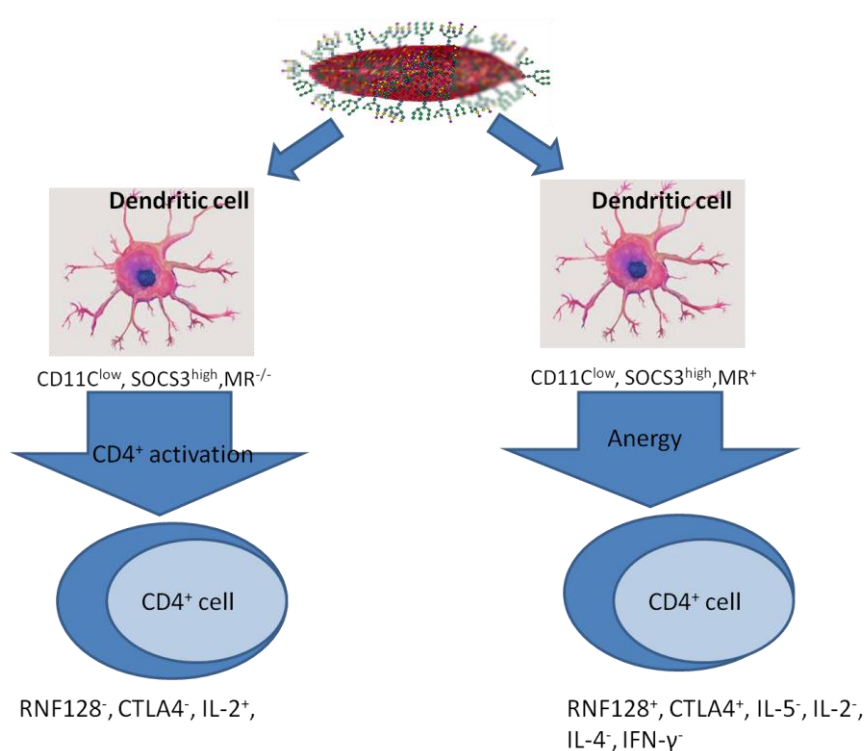


Figure 7.2 Mechanism by which MR expression influences CD4⁺ cells differentiation. Dendritic cells treated with FhTeg have an enhanced expression of MR, and subsequent co-culture with CD4⁺ cells induces anergy with an increase in RNF128, CTLA4 and a decrease in production of IL-5, IL-2, IL-4 and IFN-γ. The loss of MR inhibits the induction of anergy.

7.4. FhTeg induces anergy directly in CD4⁺ T-cells

Studies on the effects of FhTeg on immune cells have mostly concentrated on its effects on dendritic cells (Vukman, Adams and O'Neill 2013, Hamilton et al. June 2009), macrophages (Adams et al. 2014) and mast cells (Vukman et al. 2013b, Vukman et al. 2013a) and their subsequent effects on CD4⁺ cells, but to our knowledge no studies have been completed on the effect of FhTeg on CD4⁺ cells directly. In general, activation of CD4⁺ cells requires three signals, signal one from antigen presenting cells and this includes presentation of peptides through MHC class II, signal two via co-stimulatory molecules and signal three either inflammatory or anti-inflammatory cytokines. These signals influence the differentiation of naïve CD4⁺ cells into different subsets such as Th1, Th2, Th17, T-regs or anergic T-cells (Yamane and Paul 2013). Following this, most CD4⁺ cells proliferate and produce cytokines which can further help other cells to differentiate and to mount an immune response.

There are instances where CD4⁺ cells can be directly influenced to differentiate without the help of an APC. Studies using super-antigens have found that binding of the antigen directly to the TCR can induce negative signalling pathways in memory cells; this is used by pathogens as a defence mechanism to the hosts' immune response (Watson, Mittler and Lee 2003). Binding of the super-antigen to the TCR and to MHC II receptors on an APC simultaneously can also cause non-specific activation of T-cell, causing uncontrolled proliferation and production of cytokines. Following this a number of cells undergo apoptosis and the remaining cells are functionally unresponsive (Seth et al. 1994).

Here we have shown that FhTeg has a direct effect on CD4⁺ cells, inducing an anergic like cell with the up-regulation of CblB, EGR2 and 3, ITCH and RNF128, FhTeg also inhibits the production of cytokines. These genes were also enhanced during infection and following injection of FhTeg. The ability of FhTeg to directly interact with CD4⁺ cells may be important in understanding how *F.hepatica* modulates the hosts' immune response. FhES is another antigen secreted by *F.hepatica*; it would be interesting to examine the effect it would have on CD4⁺ cells directly. Other studies have shown that FhES inhibits the proliferation of sheep PBMCs and down-regulates the expression of CD4 on T-cells, this was found to be due in part to the action of cathepsin L cleaving the CD4 receptor, further studies to examine its action on CD4⁺ cells are required (Prowse et al. 2002).

To understand the mechanism by which FhTeg induces anergy in CD4⁺ cells, the presence of MR on CD4⁺ cells was investigated. Although MR was found to be expressed on CD4⁺ cells, knocking out the receptor did not reverse the suppressive effect of FhTeg. Studies on CLRs mainly focus on APCs like dendritic cells and macrophages, further studies would need to be completed to investigate other possible receptors which may be on CD4⁺ cells and which may be contributing to the induction of anergy or other possible mechanisms such as FhTeg binding to the TCR receptor and inducing signal one alone to T-cells and inducing anergy.

7.5. FhTeg modulates human PBMCs via CD4⁺ T-cells

Helminth infections affects up to a third of the world's population with a high percentage of those affected in the developing world. Infection is rarely fatal but can lead to anaemia, weight loss and a susceptibility to bystander infections (Hotez et al. 2008). Studies using FhTeg and its immune-modulatory role have mainly focused on its interactions with cells from murine origin; here we investigated the effect of FhTeg on different cell types isolated from PBMCs and on PBMCs as a whole population. In our mouse studies, dendritic cells stimulated with FhTeg before being challenged with LPS produced significantly lower levels of IL-12p70 and TNF- α than cells which had received LPS alone (Hamilton et al. June 2009), it also inhibits mast cells ability to drive Th1 responses (Vukman et al. 2013b) and in chapter 5 we showed that FhTeg inhibits the PMA and anti-CD3 activation of CD4⁺ cells directly from naive mice. Here we show that FhTeg inhibits the LPS and PMA/Ionomycin activation of PBMCs, with an inhibition of TNF- α production.

As PBMCs consist of a wide range of cell types including, CD4⁺ lymphocytes and CD14⁺ monocytes, the effect of FhTeg on both cell types was investigated. CD14⁺ is expressed mainly by a subset of monocytes, but it can also be expressed by dendritic cells and is involved in the binding of LPS (Schütt 1999). Studies involving lymphatic filariasis have shown CD14⁺ cells isolated from control and endemic normal patients showed an increase in IL-1 β when cells were re-challenged with LPS but cells from infected patients showed a decrease in IL-1 β production. The adherence of CD14⁺ monocytes compared to controls was also inhibited (Sasisekhar et al. 2004). Here we have shown that FhTeg does not inhibit CD14⁺ cells ability to produce TNF- α or IL-1 β , but it did inhibit CD4⁺ cells ability to produce TNF- α , IL-2 and enhanced the expression of CTLA4 and RNF128.

FhTeg negatively regulated PBMCs as a whole population but not CD14⁺ cells, we have previously shown that dendritic cells treated with FhTeg induces anergy in CD4⁺ cells and macrophages isolated from the peritoneal cavity of mice injected with FhTeg impair CD4⁺ cells (Adams et al. 2014). CD14⁺ cells do not induce CTLA4 in CD4⁺ cells isolated from the same donor or impair their ability to produce IFN- γ . The ability of FhTeg treated CD4⁺ cells to modulate CD14⁺ cells was examined and they impair the cells ability to produce TNF- α when re-challenged with LPS. This result may lead to FhTeg being used as a possible therapeutic for a number of CD4⁺ mediated diseases. Studies are ongoing into the treatment of a number of conditions with helminths molecules, studies on MS (Zheng et al. 2008, Kuijk et al. 2012a) , IBD, allergy, psoriasis and rheumatoid arthritis. (Heylen et al. 2014, Whary et al. 2001) are all ongoing.

Another CD4⁺ cell mediated disease is graft versus host disease; GVHD is a condition which occurs after an allogenic transplant. T-cells which are present in the transplanted graft recognise the hosts tissues and cells as foreign and mount an immune response. This can lead to damage to organs and can cause vomiting, diarrhoea and severe abdominal pain (Przepiorka et al. 1995). The engraftment of donor T-cells is needed for most transplants to be successful, this is to prevent host versus graft occurring and to ensure graft rejection is not seen and so finding ways to combat both GVHD and HVGD and to extend transplant longevity is important. Some treatments to prevent GVHD are pharmacological agents but these require long term use and suppress the immune system, leaving patients with compromised immunity. Other areas under investigation are trying to induce T-cell anergy or T-reg cells that have impaired responses to host cells only and do not impair other T-cell responses. A study inducing GVHD in control and

Heligmosomoides polygyrus infected animals showed that infection suppressed recipient T-cell inflammatory responses and inhibited GVHD (Li et al. 2015), this study used infection as a source of antigen and only was successful when host T-reg cells were generated, this may not always be possible as using whole worm treatment may not always be a viable option.

Work completed by our collaborators to investigate if FhTeg could be used to prevent GVHD has shown that mice which received PBMCs alone developed GVHD but pre-treatment of PBMCs significantly prolonged survival and lowered the pathological score of GVHD (Appendix D). Although this is promising as a treatment for GVHD, the study showed that FhTeg impaired the donor cells engraftment and this would be needed for the impairment of recipient cells, and so would not be suitable as a possible treatment. It would be interesting to see what role FhTeg would play in a model of organ transplant such as a skin transplant, as FhTeg may prevent the rejection of the graft.

Studies using mouse models have given great insight into mechanisms of helminth infections and other models of disease. Although we have gained knowledge using mouse models, there are differences between mice and human immune systems. Studies have shown that generally mice show similar results to human studies and genomic studies have shown approximately 300 unique genes for one species or the other (Initial sequencing and comparative analysis of the mouse genome. 2002). Experimental autoimmune (allergic) encephalomyelitis (EAE) is a widely used model for MS in mice. This mimics the demyelination seen in central and peripheral nerves in MS. Studies using this model in mice showed promising results using IFN- γ and a decrease in symptoms and using blocking antibodies to IFN- γ exacerbated the disease. Translating this to human

models, the addition of IFN- γ to human patients exacerbated the disease and trials had to be stopped (Panitch et al. 1987). Mouse models are also generally conducted in pathogen free areas and as such do not mimic the everyday pathogens that humans would encounter which could also alter trials. Further work needs to be carried out to investigate if what we see in mouse models of infection, injection and *in vitro* is comparable with what is seen naturally in sheep, cattle and humans.

7.6. Final Conclusion

This thesis has presented novel mechanisms by which *F. hepatica* modulates the host immune response both during infection and by its tegumental antigens. It has also shown how CD4⁺ T-cells play a role in modulating other cells and how this may impact on investigations into new therapies using helminths molecules. From these studies, it is hoped additional investigations will be carried out to further our knowledge on how *F. hepatica* modifies cells of the immune system to survive within the host and on how its tegumental antigens may be used as therapeutics or vaccines.

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Appendix

9.1. Appendix A. Injection of FhTeg into the peritoneal cavity induces anergy

We have previously shown that FhTeg injected into the peritoneal cavity recruits mast cells and macrophages (Adams et al. 2014, Vukman et al. 2013a). To investigate if FhTeg would mimic infection and induce anergy, PBS or FhTeg (10 μ g in 100 μ l) was injected three times a week for three weeks. Splenocytes were re-stimulated with PBS or FhTeg (10 μ g/ml) in the presence or absence of IL-2 or PMA (20ng/ml) and anti-CD3 (10 μ g/ml). Following a 72 hour stimulation, supernatants were removed and the levels of IL-5, IL-4 and IFN- γ were measured using ELISA. No significant levels of IL-5 were produced in controls, but significant levels of IL-4 and IFN- γ were produced (Figure 9.1 A-C)($p < 0.0001$). FhTeg induced significant levels of IL-5 but not IL-4 or IFN- γ in splenocytes isolated from injected mice ($p < 0.01$) (Figure 9.1A-C). The addition of IL-2 significantly reversed the levels of IL-5 and IL-4 but not IFN- γ , (Figure 9.1 A-C) ($p < 0.0001$). RNA was also isolated from splenocytes and a qPCR array was performed to look for genes relating to anergy. Table 9.1 summarises the fold changes,

Table 9.1 Fold changes seen in splenocytes isolated from FhTeg injection compared to controls

Gene Name	Fold Change
CTLA4	1.84
EGR2	1.68
FoxP3	1.52
ICOS	1.54
ITCH	1.21
RNF128	1.88

The injection of FhTeg did induce small fold changes in expression of genes that were seen to be highly expressed during infection but they were much smaller changes. The injection of FhTeg into the sternum to mimic infection is not a good method, injection over the sternum gives a higher response.

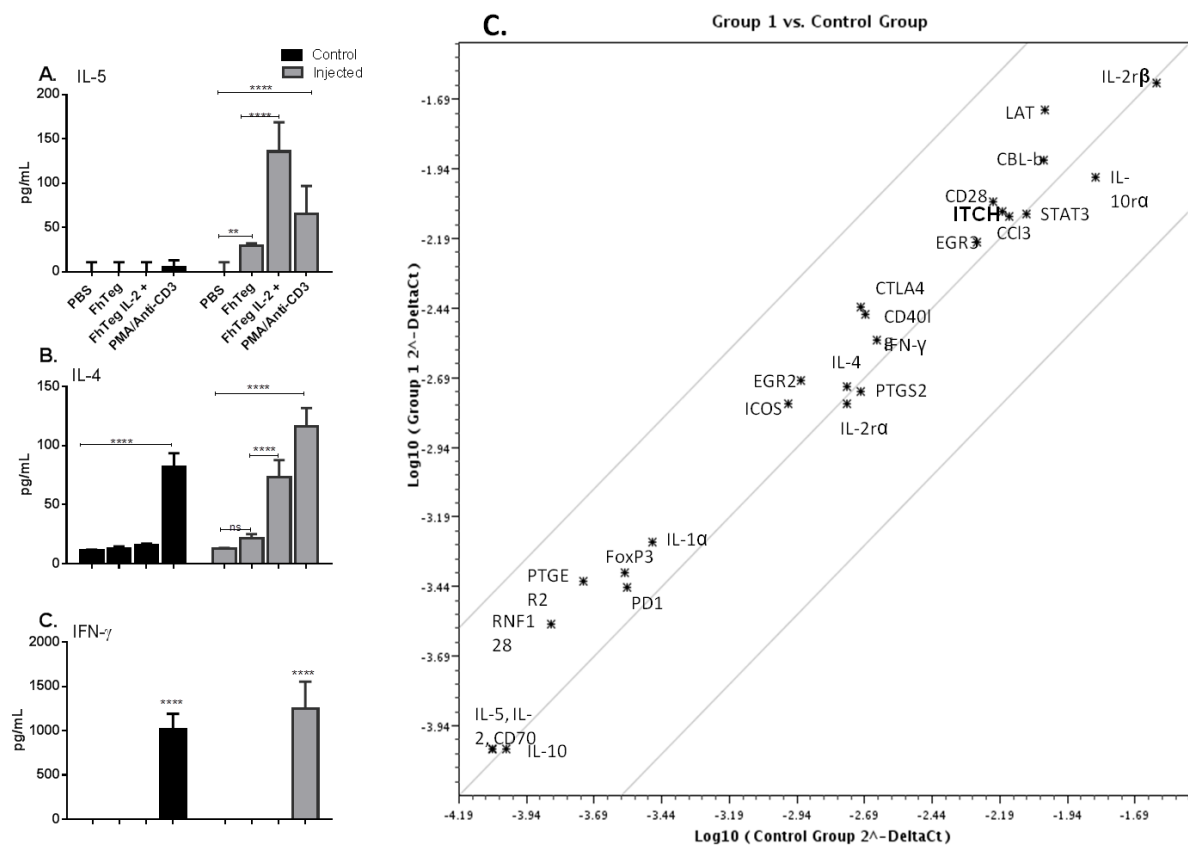


Figure 9.1 Injection of FhTeg into the peritoneal cavity induces anergy. PBS or FhTeg was injected into the peritoneal cavity three times a week for three weeks, splenocytes were isolated and re-stimulated with PBS or FhTeg (10µg/ml) with or without IL-2 (20ng/ml) or PMA (20ng/ml) and anti-CD3 (10µg/ml). A-C. Supernatants were analysed for the presence of IL-5, IL-4 and IFN-γ. D. A qPCR gene array shows the difference in gene expression in splenocytes. The data shown is the mean ±SD of four independent experiments, (**p<0.01, ***p<0.0001).

9.2. Appendix B. Anti-CD45 inhibits CD4⁺ cells ability to produce cytokines

BMDCs generated from mannose receptor knockout mice fail to induce anergy in CD4⁺ cells by inhibiting IL-2 suppression and RNF128 and CTLA4 enhancement. MR has been shown to bind to CD45 on T-cells (Martínez-Pomares et al. 1999), to investigate if blocking CD45 would have any effect on anergy induction, CD4⁺ cells were incubated with PBS or anti-CD45 (10µg/ml) for 30 mins before being washed and co-cultured with anti-CD3 (1µg/ml) and dendritic cells which had been cultured with PBS or FhTeg over night. Following a 72 hour stimulation, supernatants were removed and analysed for the production of IL-5 and IFN-γ and RNA was extracted from cells and the level of RNF128 was measured using qPCR. Cells which received anti-CD45 blocking anti-body and were subsequently cultured with PBS treated cells produced significantly less IL-5 and IFN-γ than controls (Figure 9.2 A-B) (p<0.0001), FhTeg treated cells produced significantly higher levels of IL-5 and IFN-γ than PBS cells (Figure 9.2 A-B) (p<0.01, p<0.0001).

The level of RNF128 was measured and compared to PBS control cells which had received no anti-CD45, the levels of RNF128 in anti-CD45 treated cells was significantly lower than

in untreated PBS controls (Figure 9.2 C) ($p < 0.0001$). Anti-CD45 is involved in both positive and negative regulation of $CD4^+$ cells function (Saunders and Johnson 2010), the blocking antibody may have inhibited normal crosstalk between DC's and $CD4^+$ cells, it also could have triggered negative signalling pathways by binding the receptor. Further studies would need to be completed to investigate this further.

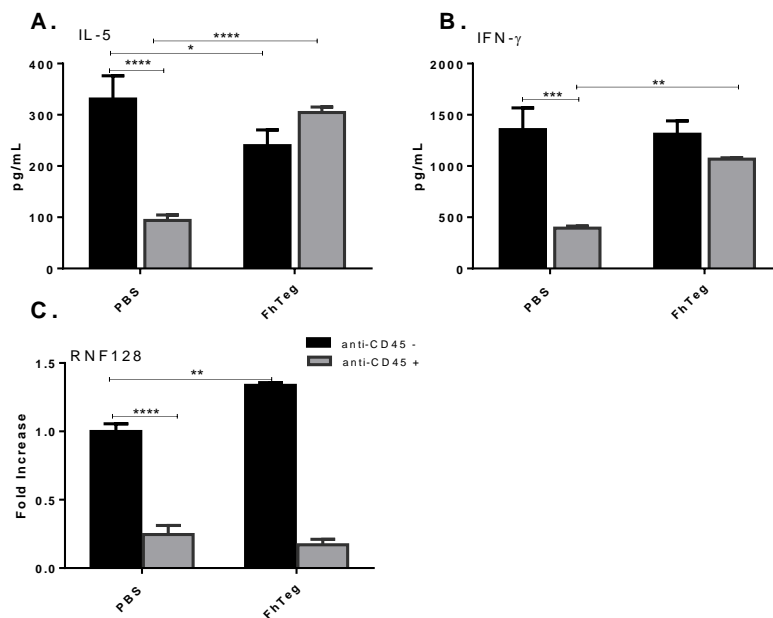


Figure 9.2 Anti-CD45 inhibits $CD4^+$ cells ability to produce cytokines. $CD4^+$ cells were cultured with anti-CD45 antibody (10 μ g/ml) before being washed and co-cultured with anti-CD3 (1 μ g/ml) and dendritic cells stimulated with either PBS or FhTeg. Following a 72 hour co-culture, supernatants were removed and analysed for the production of IL-5 and IFN- γ , the levels of RNF128 was also measured using qPCR. The data shown is the mean \pm SD of one independent experiment, (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

9.3. Appendix C. FhTeg enhances the expression of genes relating to anergy from 1 hour stimulation but not at earlier time points.

To investigate if FhTeg would induce genes relating to anergy at an earlier time point than two hours, CD4⁺ cells were stimulated with PBS or FhTeg (10µg/ml) for 30 mins and 1 hour. RNA was isolated from cell pellets and qPCR was used to determine the expression of EGR2, 3 and RNF128. FhTeg did not induce EGR2, 3 or RNF128 following a 30 min stimulation (Figure 9.3 A,C,E) but all genes were significantly enhanced following a 1 hour stimulation (Figure 9.3 B,D,F) ($p<0.05$, $p<0.001$).

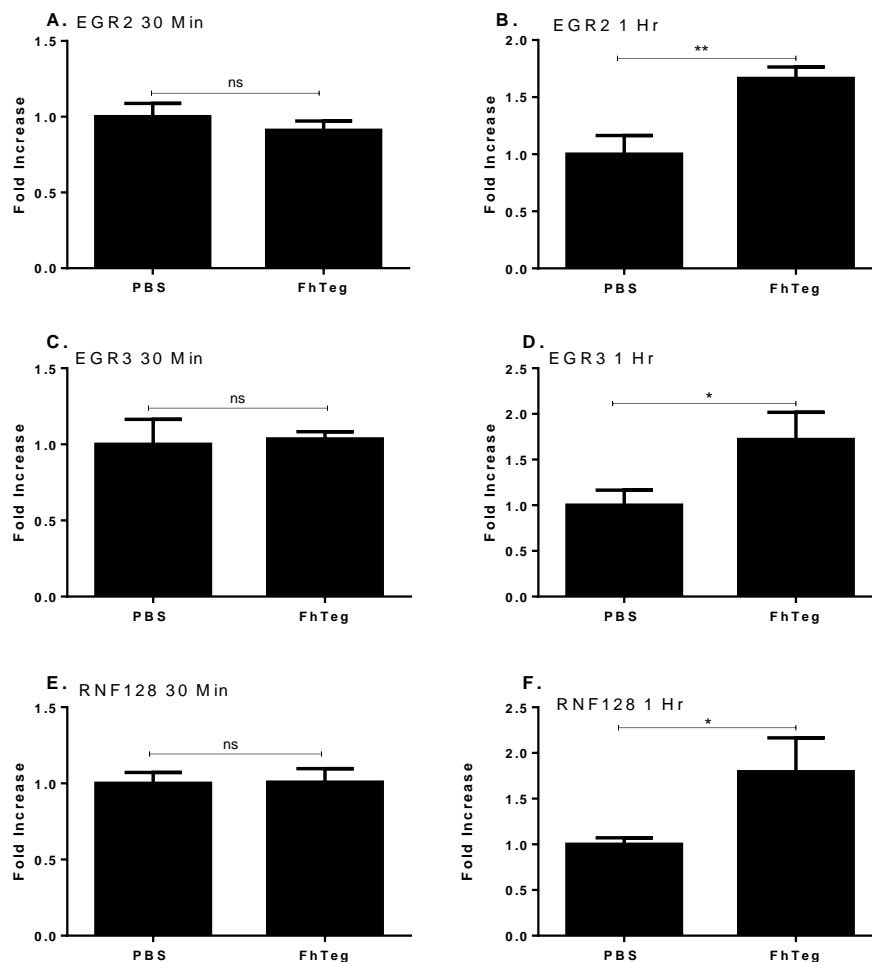


Figure 9.3. FhTeg enhances the expression of genes relating to anergy from 1 hour stimulation but not at earlier time points. CD4⁺ cells were stimulated with PBS or FhTeg for 30 mins or 1 hour and the levels of EGR2,3 and RNF128 were measured using qPCR. FhTeg does not induce any gene following a 30 min stimulation but significantly enhances all genes following a 1 hour stimulation. The data shown is the mean \pm SD of one independent experiment. (*p<0.05, **p<0.01).

9.4. Appendix D. FhTeg is protective against GVHD

To investigate if FhTeg would be protective in a humanised model of graft versus host disease, PBMCs were isolated from whole blood and stimulated over night with FhTeg (10 μ g/ml). The PBMCs were then injected into the tail vein of whole body irradiated humanised NSG mice. As controls, mice received PBMCs which had been stimulated with PBS and a group received PBS only. The mice were monitored over a period of one month for signs of GVHD which included weight changes and dermatitis. Animals which received PBS treated PBMCs had a significantly higher loss of body weight up to 15 days post-transplant (p<0.05, p<0.01, p<0.001) and had a higher pathological score for the entire study compared to FhTeg treated PBMCs. FhTeg also prolonged the survival of recipients compared to PBS treated PBMCs (p<0.001). PBS controls had no significant changes in body weight or pathological score (Figure 9.4 A-C).

High levels of TNF- α in serum of patients is a marker of GVHD {{550 Korngold,Robert 2003}}, to investigate if FhTeg would reduce the levels of TNF- α , serum from mice from controls and FhTeg treated PBMCs were measured for TNF- α production. Mice which received PBS alone had no significant levels of TNF- α , mice which had received PBMCs treated with PBS had significant levels of TNF- α and FhTeg significantly suppressed this (Figure 9.4 D) (p<0.01).

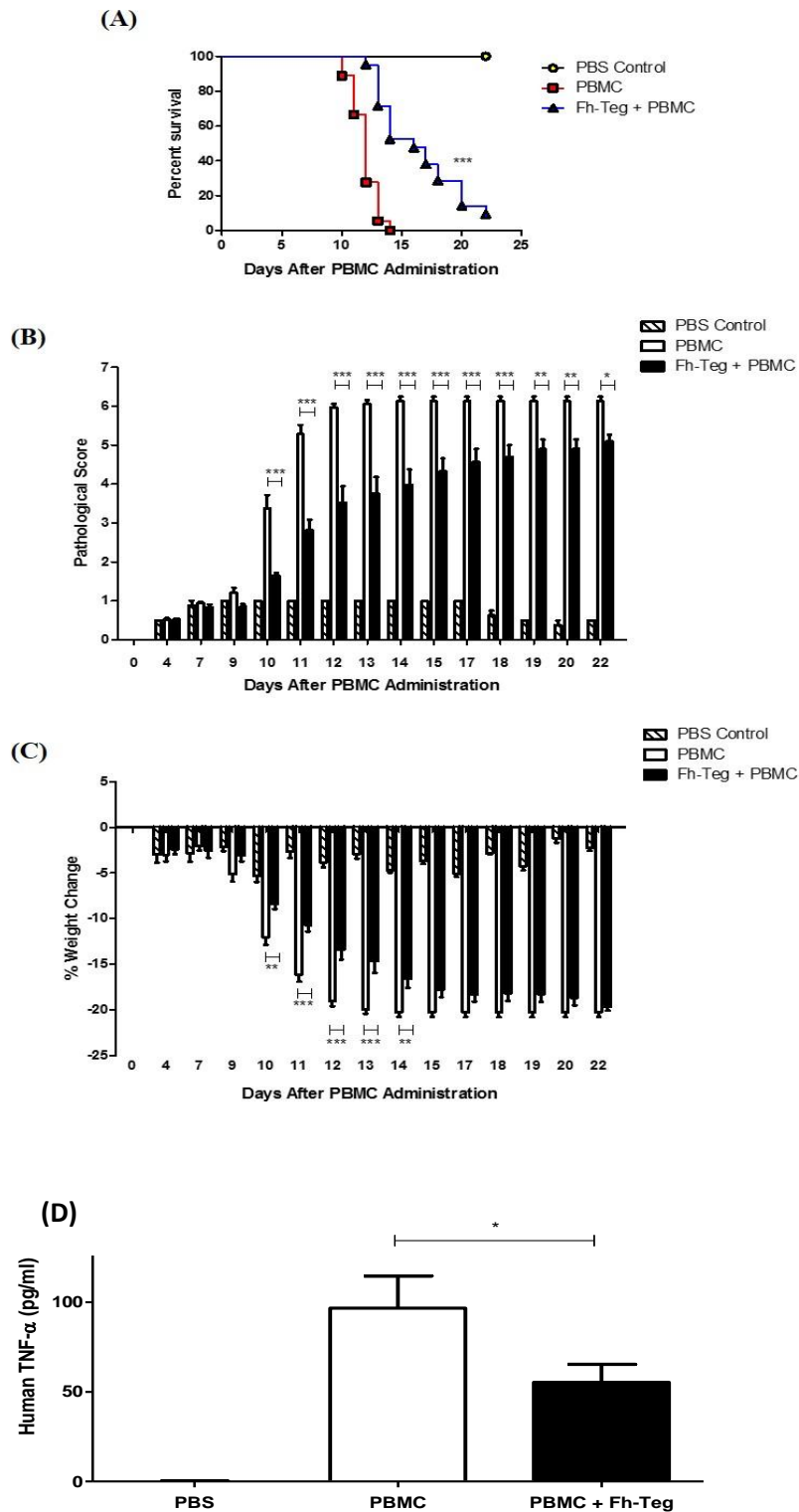


Figure 9.4. Fh-Teg PBMC significantly prolonged survival and reduced pathological score in GvHD mice.

PBS control mice are represented by diagonally lined bars, mice which received PBMC are represented by white bars and mice which received Fh-Teg PBMC are represented by black bars. Statistical significance was

determined using student t test where $*$ < 0.05 , $**$ < 0.005 and $***$ < 0.001 . $n=5$, $n=18$ for PBMC, $n=21$ for Fh-Teg PBMC.

10. Publications

RESEARCH

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Plasma cytokines, chemokines and cellular immune responses in pre-school Nigerian children infected with *Plasmodium falciparum*

Cariosa Noone¹, Michael Parkinson¹, David J Dowling¹, Allison Aldridge¹, Patrick Kirwan², Síle F Molloy², Samuel O Asaolu³, Celia Holland² and Sandra M O'Neill^{1*}

Abstract

Background: Malaria is a major cause of morbidity and mortality worldwide with over one million deaths annually, particularly in children under five years. This study was the first to examine plasma cytokines, chemokines and cellular immune responses in pre-school Nigerian children infected with *Plasmodium falciparum* from four semi-urban villages near Ile-Ife, Osun State, Nigeria.

Methods: Blood was obtained from 231 children (aged 39–73 months) who were classified according to mean *P. falciparum* density per μ l of blood (uninfected ($n = 89$), low density ($<1,000$, $n = 51$), medium density ($1,000$ – $10,000$, $n = 65$) and high density ($>10,000$, $n = 22$)). IL-12p70, IL-10, Nitric oxide, IFN- γ , TNF, IL-17, IL-4 and TGF- β , C-C chemokine RANTES, MMP-8 and TIMP-1 were measured in plasma. Peripheral blood mononuclear cells were obtained and examined markers of innate immune cells (CD14, CD36, CD56, CD54, CD11c AND HLA-DR). T-cell sub-populations (CD4, CD3 and $\gamma\delta$ TCR) were intracellularly stained for IL-10, IFN- γ and TNF following polyclonal stimulation or stimulated with malaria parasites. *Ascaris lumbricoides* was endemic in these villages and all data were analysed taking into account the potential impact of bystander helminth infection. All data were analysed using SPSS 15 for windows and in all tests, $p < 0.05$ was deemed significant.

Results: The level of *P. falciparum* parasitaemia was positively associated with plasma IL-10 and negatively associated with IL-12p70. The percentage of monocytes was significantly decreased in malaria-infected individuals while malaria parasitaemia was positively associated with increasing percentages of CD54⁺, CD11c⁺ and CD56⁺ cell populations. No association was observed in cytokine expression in mitogen-activated T-cell populations between groups and no malaria specific immune responses were detected. Although *A. lumbricoides* is endemic in these villages, an analysis of the data showed no impact of this helminth infection on *P. falciparum* parasitaemia or on immune responses associated with *P. falciparum* infection.

Conclusions: These findings indicate that Nigerian children infected with *P. falciparum* exhibit immune responses associated with active malaria infection and these responses were positively associated with increased *P. falciparum* parasitaemia.

Keywords: Cytokines, Chemokines, Cellular responses, Plasmodium falciparum, Children, Nigeria, Ascaris lumbricoides

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Background

Plasmodium falciparum malaria accounts for approximately 250–300 billion clinical cases of malaria world-wide and is highly endemic in Africa [1]. Approximately one in every five child deaths in Africa are due to malaria with the risk of cerebral malaria being highest in children aged two to four years. Natural acquired immunity rarely occurs before two years and its development is associated with increasing age, which correlates with a reduction in mortality rates due to the more severe forms of *P. falciparum* infection [2]. It is, therefore, important that immune responses in young children are examined in order to further define immunological association with *P. falciparum* infection.

Malaria infection is predominantly characterized by a T helper 1 (Th1) response and the production of pro-inflammatory cytokines such as IL-12-p70, interferon gamma (IFN- γ) and tumour necrosis factor (TNF). These inflammatory cytokines are considered critical in controlling parasitaemia, especially during the early stages of *P. falciparum* infection [3,4]. Conversely during chronic malaria infection, if these robust inflammatory responses are not tightly regulated, they can lead to immunopathology and severe forms of malaria [5,6]. Regulatory cytokines, including interleukin (IL)-10 and transforming growth factor beta (TGF- β) were shown to be important in dampening down T helper (Th) 1 inflammatory responses associated with immune pathology in the more severe forms of *P. falciparum* infection [5,6]. There also a range of other mediators,

such as IL-17, IL-4, nitric oxide, C-C chemokine RANTES, matrix metalloproteinases 8 (MMP8s) and tissue inhibitor of metalloproteinases 1 (TIMP1) that have been linked to disease severity in malaria-infected individuals [7-9].

Malaria infection is strongly influenced by the release of inflammatory mediators from innate immune cells where early interactions between blood-stage parasites and these are critical in controlling parasitaemia and the subsequent elimination of infection [5,6]. Innate immune cells including antigen presenting cells, such as dendritic cells, and macrophages are an early source of pro-inflammatory cytokines, such as IL-12 and TNF. Other innate immune cells such as natural killer cells and $\gamma\delta$ -T-cells are an early source of IFN- γ . These cells, through the release of inflammatory mediators and from cell-to-cell contact with naïve T-cells, also shape the adaptive immune response.

This is the first study to assess immune responses during uncomplicated malaria infection in Nigerian pre-school children in four semi-urban villages near Ile-Ife, Osun State, Nigeria [10]. *Plasmodium falciparum* infection is endemic in this region with a high prevalence in pre-school children [11]. Recent studies have also shown a 25% prevalence of *Ascaris lumbricoides* in this cohort [12]. Since helminth infection can impact upon the outcome of malaria infection [13-15] the potential impact of *A. lumbricoides* upon *P. falciparum* parasitaemia and its associated immune responses were examined.

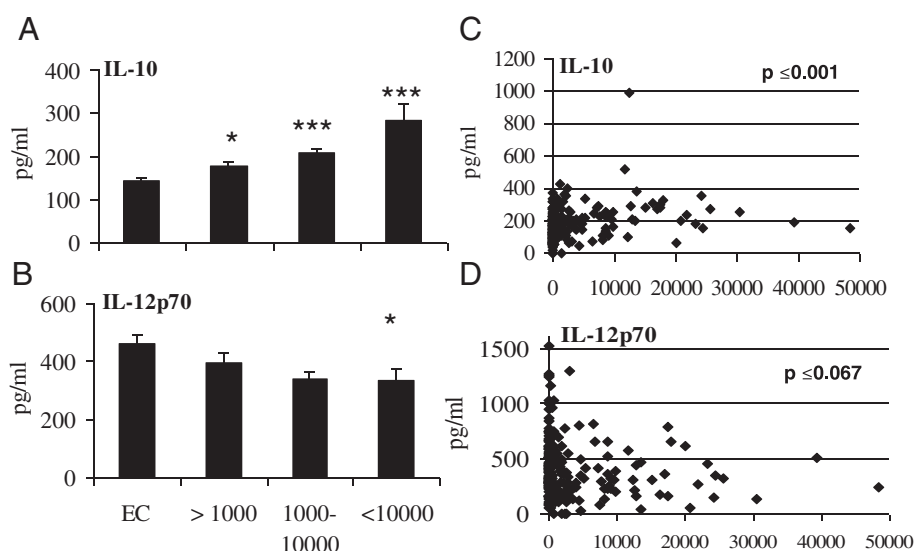


Figure 1 *Plasmodium falciparum* parasitaemia was positively associated with IL-10 and negatively associated with IL-12p70 levels in the plasma of infected children. Mean plasma levels of (A) IL-10 and (B) IL-12p70 were determined by ELISA for each group (low (<1,000; n=51), medium (1,000-10,000; n=65) and high (>10,000; n=22) mean parasite density (per μ l of blood) and compared to endemic controls (EC) (n=89). *, $p \leq 0.05$; **, $p \leq 0.01$ and ***, $p \leq 0.001$ (ANOVA). Parasitaemia was plotted against (C) IL-10 and (D) IL-12p70 and association between variables was assessed using regression analysis. $p < 0.05$ was deemed significant.

Methods

Study design and participants

231 blood samples were obtained from children at the final time point in a double-blind placebo-controlled randomized trial on children aged 39–73 months in four semi-urban villages, Akinlalu, Ipetumodu, Moro and Edunabon, near Ile-Ife, Osun State, Nigeria. Details of the study area, design and participants were published previously [10]. This current sub-study was to examine immune responses associated with *P. falciparum* infection and examine the impact of *A. lumbricoides*. Data were available on children's age and infection status for *P. falciparum* and *A. lumbricoides* infection. Children who suffered from severe malaria were treated and excluded from the study

and therefore only individuals with uncomplicated malaria were included (malaria parasitaemia and fever $>37.5^{\circ}\text{C}$) [10]. The study protocol was approved by the Ethics and Research Committee, Obafemi Awolowo University Teaching Hospitals' Complex, Ile-Ife, Nigeria.

Isolation of peripheral blood mononuclear cells

Ten ml of blood (in tubes containing heparin) was obtained from 231 children, ranging in age from 39–73 months. Peripheral blood mononuclear cells (PBMCs) and plasma were obtained following histopaque (Sigma-Aldrich, St Louis, MO, USA) density gradient centrifugation. PBMCs were collected and stored in liquid nitrogen and plasma samples were stored at -80°C .

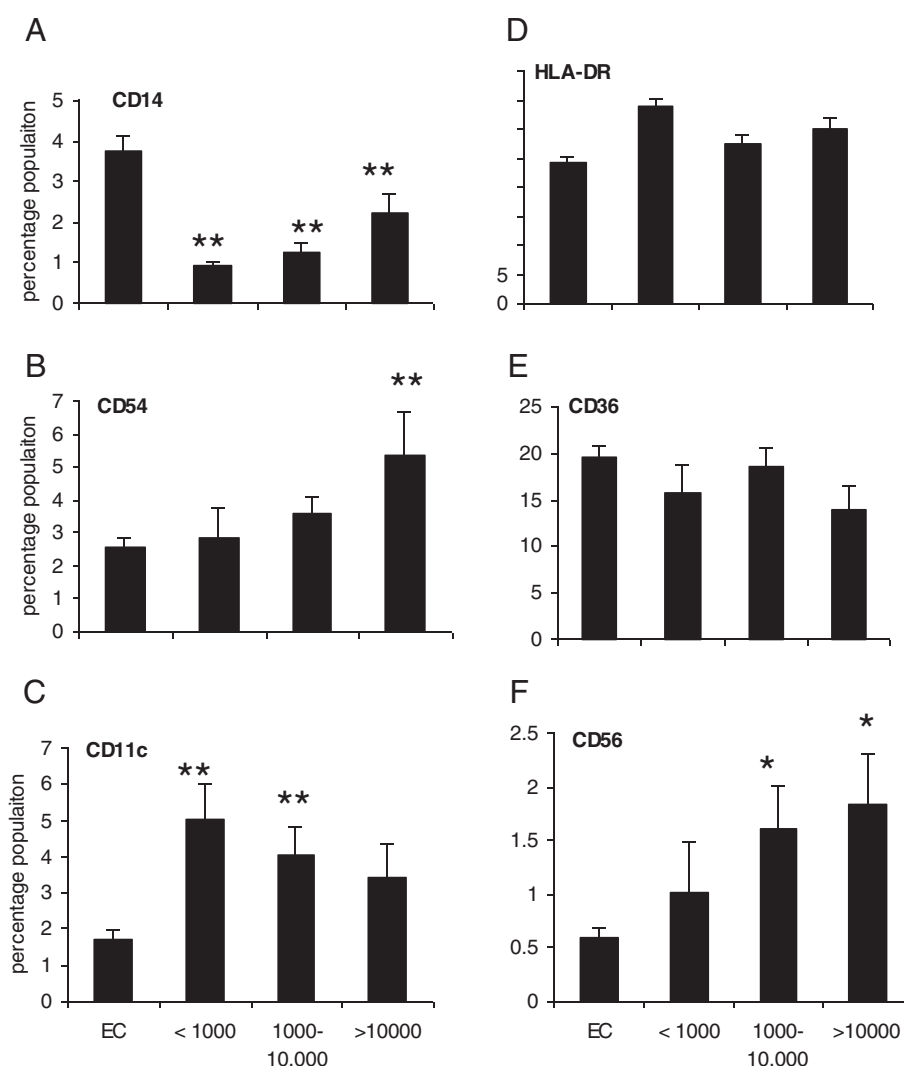


Figure 2 Malaria infection was associated with decreased CD14⁺ percentages and enhanced percentages of CD11c⁺, CD54⁺, CD56 but not HLA-DR⁺ and CD36⁺ cell populations. PBMCs were stained extracellularly for CD14 (A), CD54 (B), CD11c (C), HLA-DR (D), CD36 (E) and CD56 (F) cell expression for each group (low (<1,000; n=51), medium (1,000-10,000; n=65) and high (>10,000; n=22) mean parasite density (per µl of blood)) was determined by flow cytometry and compared to endemic controls (EC). ** $p \leq 0.01$ (ANOVA).

ELISA

Human IFN- γ , IL-10, TGF- β , TNF, IL-4 and IL-12p70 Opti-EIA kits (BD Biosciences) and human IL-17, RANTES, MMP-8 and TIMP-1 DuoSet ELISA Developmental Kits (R&D, Minneapolis, MN, USA) were used to quantify cytokine, chemokine and metalloproteinase levels in plasma samples as described per manufacturers instructions. NO levels were also measured in plasma using the Greiss Reagent System (Promega, Madison, WI, USA).

Flow cytometry and *in vitro* culture

The following mAbs were used for cells surface staining and intracellular cytokine staining: FITC-conjugated anti- CD4, CD14, CD36, CD56, IFN- γ ; PE-conjugated anti- $\gamma\delta$ TCR, CD54, IL-10; and APC-conjugated anti-CD3, CD11c, HLA-DR, IL-2 and TNF (eBiosciences, San Diego, CA, USA). Isotype controls included FITC-conjugated mouse IgG1, IgM, IgG2a, IgG2b; PE-conjugated mouse IgG1, IgG2b and APC-conjugated mouse IgG1, IgG2b and rat IgG2a (eBiosciences). PBMCs were thawed and cultured in complete RPMI containing 10% FCS (foetal calf serum), 1% L-glutamine and 1% penicillin/streptomycin solution (Bio-sciences Ltd, Co. Dublin, Ireland). For intracellular cytokine staining, PBMCs were stimulated with 50 ng/ml PMA (Phorbol 12-myristate 13-acetate) and 1 mg/ml ionomycin for 4 h and to block cytokine secretion, 10 mg/ml Brefeldin A (Sigma) was added to the culture media. Cells were then washed and stained with cell surface mAbs, fixed with 4% PFA and permeabilized with 0.2% saponin (Sigma), before incubation with antibodies for IL-12, IFN- γ , IL-10 and TNF cytokines. Appropriately labelled isotype-matched antibodies were used as controls. Acquisition was performed using a FACSCalibur

flow cytometer (BD Biosciences), and analysis of results performed using FlowJo software (Tree Star). A sample of gating strategy for T Cells is shown in Additional file 1.

PBMCs (1×10^6 cells/ml) from a cohort of children were also cultured on a 24-well plate with mycoplasma free *P. falciparum* parasites (at a ratio of 1:5), which were extracted from cell culture by saponin lysis (0.15%), were kindly provided by Dr Alison Creasey, University of Edinburgh, Scotland. After three days, cell culture supernatants were harvested and frozen for subsequent measurement of IFN- γ , IL-10, and IL-5 by commercial ELISA.

Statistical analysis

All data were analysed using SPSS 15 for windows. Percentage data were normalized prior to analysis by Arcsin transformation. Skewed data were normalized prior to analysis by log transformation. For differences between multiple groups, one-way ANOVA with Post-hoc testing by Tukey's HSD test was used. For data with more than one factor, factorial ANOVA was used. Association between variables was assessed using regression analysis. For differences between two treatments 2-tailed Student *t*-test was used. In all tests, $p < 0.05$ was deemed significant.

Results

Plasmodium falciparum parasitaemia was positively associated with IL-10 and negatively associated with IL-12p70 levels in the plasma of infected children

Children infected with *P. falciparum* were divided into groups based upon the mean *P. falciparum* density per μ l of blood (uninfected ($n = 89$), low density ($<1,000$, $n = 51$), medium density (1,000-10,000, $n = 65$) and high density ($>10,000$, $n = 22$)) to determine if an association exists between parasitaemia and plasma immune factors

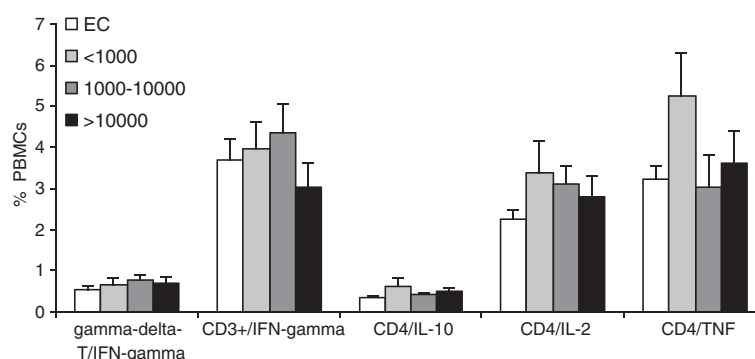
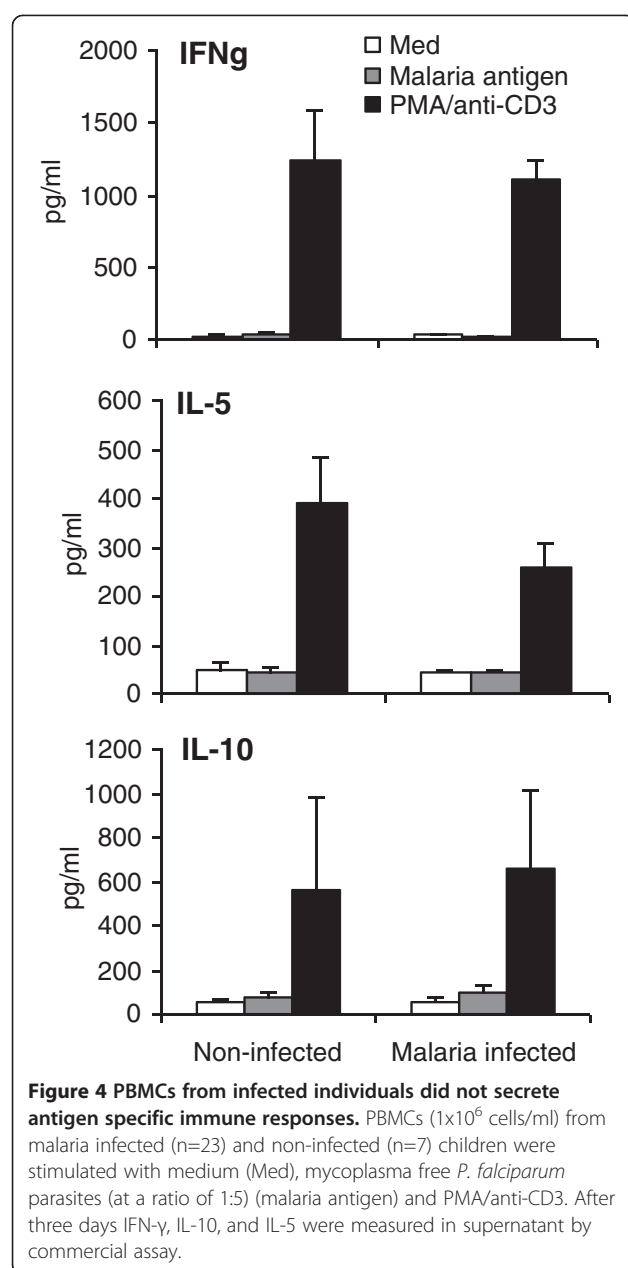
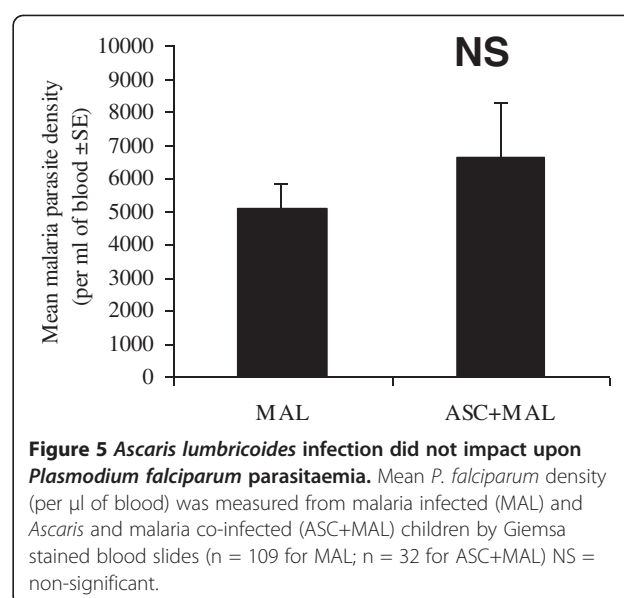


Figure 3 Malaria infection was not correlated with an increase in the secretion of IFN- γ , TNF, IL-10 and IL-2 in mitogen-activated T-cell populations. PBMCs were stimulated for 4 h with PMA and ionomycin. Cells were washed and stained for cell surface expression of $\gamma\delta$ TCR, CD3 and CD4 and were intracellularly stained for IFN- γ , IL-2, IL-10 or TNF. Appropriate isotype controls were included to define parameter gates. PBMCs were gated on the lymphocyte population and the percentage of positive cells for each group (low ($<1,000$; $n = 51$), medium (1,000-10,000; $n = 65$) and high ($>10,000$; $n = 22$) mean parasite density (per μ l of blood) were also compared to uninfected endemic controls (EC).



(Figure 1A-B, Additional file 2). A reciprocal relationship between IL-10 (low density ($p \leq 0.01$), medium ($p \leq 0.001$) and high density ($p \leq 0.001$) (Figure 1A) and IL-12p70 levels (Figure 1B; high density $p \leq 0.05$) was observed. Regression analysis revealed that increases in IL-10 were significantly associated with increased *P. falciparum* parasitaemia ($P \leq 0.001$, Figure 1C), however, IL-12p70 was not negatively associated with *P. falciparum* parasitaemia ($P \leq 0.067$, Figure 1D). Nitric oxide, IFN- γ , TNF IL-17, IL-4 and TGF- β , RANTES, MMP-8 and TIMP-1 in plasma was not associated with *P. falciparum* parasitaemia (Additional file 2).



Malaria infection was associated with a decrease in the percentage of monocytes and enhanced percentages of CD11c $^+$, CD54 $^+$, CD56 $^+$ but not HLA-DR $^+$ and CD36 $^+$ cell populations

Previous studies have shown that *P. falciparum* infection is associated with changes in antigen presenting cell populations (APCs) [16]. Here, the percentage of CD14 $^+$ monocytes (precursor dendritic cells (DCs)/macrophage) in PBMCs for each group were examined. All groups displayed significantly lower percentages of CD14 $^+$ cells ($<4\%$) compared to endemic controls (5-7%) (Figure 2A; $P \leq 0.01$ for all infected groups). To dissect the APC subsets associated with malaria infection specific surface markers including HLA-DR (indicative of an active infection), CD11c (myeloid marker found on DCs), CD54 (also known as ICAM-1) (Intercellular Adhesion Molecule 1, is important for lymphocyte-APC binding and has been shown to be upregulated on APC during malaria infection) and CD36 (involved in phagocytosis of malaria infected red blood cells) were examined [17-21]. The percentage of CD54 $^+$ populations were associated with high density *P. falciparum* parasitaemia only (Figure 2B; $P \leq 0.01$) while the percentage of CD11c $^+$ populations were associated with medium ($P \leq 0.01$) and high ($P \leq 0.01$) but not low density *P. falciparum* parasitaemia (Figure 2C). No association was observed between HLA-DR $^+$ (Figure 2D) and CD36 $^+$ cells (Figure 2E) and parasitaemia.

Since an increase in IFN- γ was not detected in plasma cells that are known to produce IFN- γ were examined to determine if there was a decrease in this population. Early IFN- γ is important in the control of *P. falciparum* parasitaemia and studies have shown

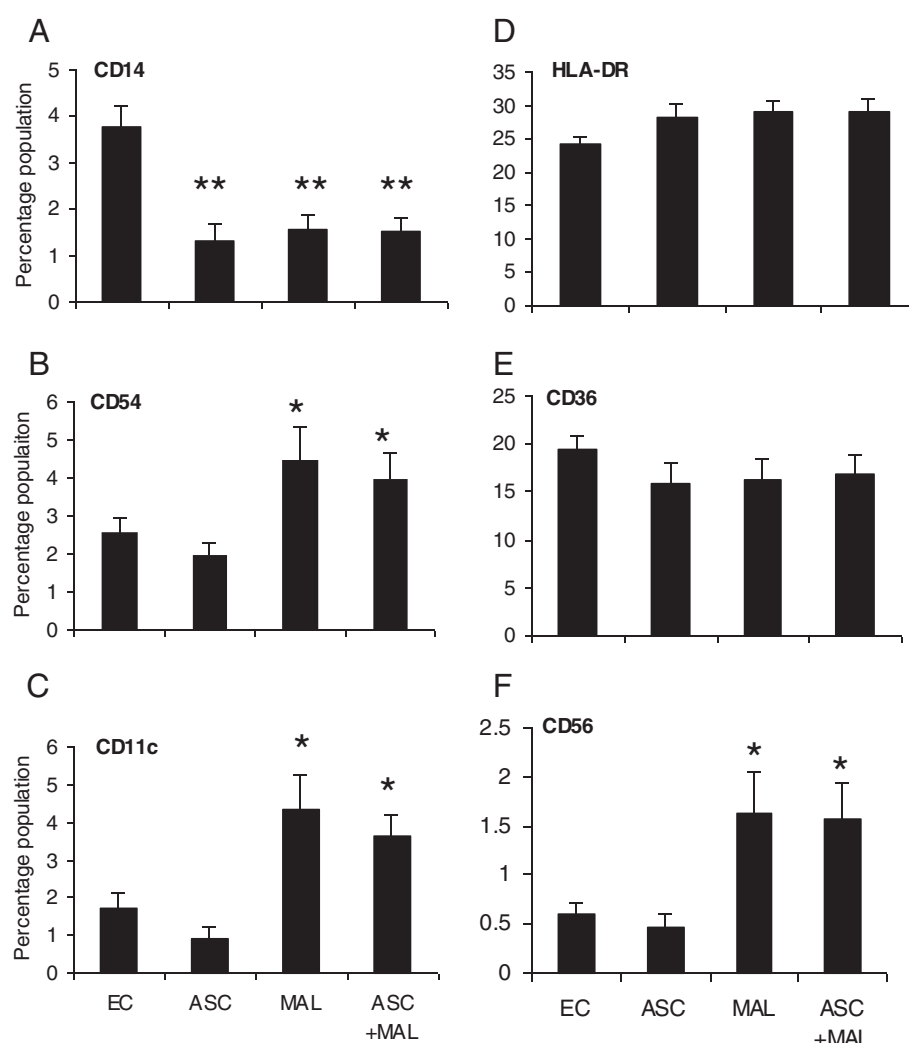


Figure 6 *Ascaris lumbricoides* infection did not alter the percentage of CD14⁺ CD11c⁺, CD54⁺, CD56⁺ HLA-DR⁺ and CD36⁺ cell populations in-co-infected individuals. PBMCs were stained extracellularly for CD14 (A), CD54 (B) CD11c (C), HLA-DR (D), CD36 (E) and CD56 (F) cell expression for each group *Ascaris* infection only (ASC), Malaria infection only (MAL) and co-infected (ASC + MAL) was determined by flow cytometry and compared to endemic controls (EC). * $p \leq 0.05$; ** $p \leq 0.01$ (ANOVA) and MAL group was compared to the ASC + MAL group (no significant differences).

that in the early stages of infection CD56⁺ natural killer cells (NK) and other leukocytes expressing the CD56 marker are good sources of IFN- γ [22]. The percentage of CD56⁺ cells in PBMCs were measured and these cells were positively associated with *P. falciparum* parasitaemia with significant increases in cell percentages for medium ($P \leq 0.05$) and high density *P. falciparum* parasitaemia ($P \leq 0.05$) (Figure 2F).

Malaria infection was not associated with increased secretion of IFN- γ , TNF, IL-10 and IL-2 in mitogen-activated T-cell populations and no parasite specific immune responses were detected

PBMCs were polyclonally activated with PMA and ionomycin and intracellular cytokine staining for IFN- γ ,

IL-10, IL-2 and TNF was performed. In order to evaluate the phenotype of the T cells present, cells were also stained for CD3, CD4, and $\gamma\delta$ T-cells. All groups expressed significantly high levels of IFN- γ and TNF and low, but significant, levels of IL-10 and IL-2 following stimulation. There were no differences observed between the groups (Figure 3).

PBMCs (1×10^6 cells/ml) from malaria infected ($n=23$) and non-infected ($n=7$) individuals were stimulated with mycoplasma free *P. falciparum* parasites (at a ratio of 1:5) and after three days IFN- γ , IL-10, and IL-5 were measured in supernatant. No parasite specific immune responses were detected while cPBMCs were capable of secreting all cytokines tested in response to our positive control PMA/anti-CD3 (Figure 4).

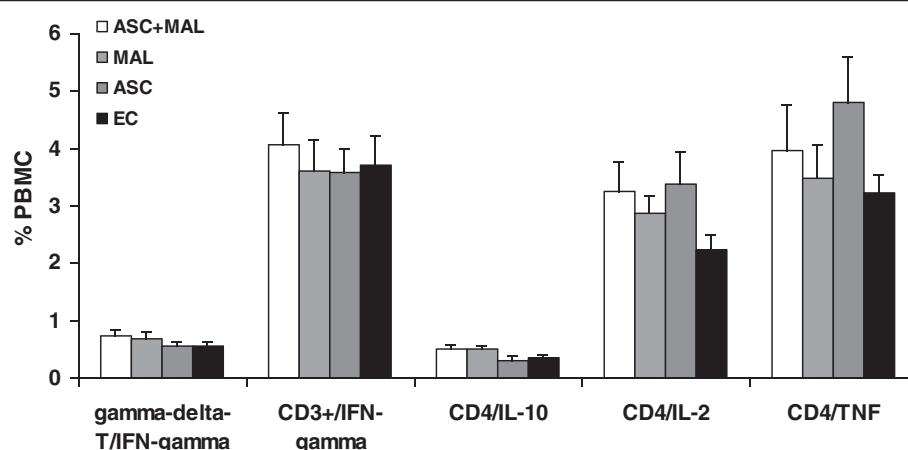


Figure 7 *Ascaris lumbricoides* infection did not alter IFN- γ , TNF, IL-10 and IL-2 expression in mitogen-activated T-cell populations.

PBMCs were stimulated for 4 h with PMA and ionomycin. Cells were washed and stained for cell surface expression of $\gamma\delta$ TCR, CD3 and CD4 and were intracellularly stained for IFN- γ , IL-2, IL-10 or TNF- α . Appropriate isotype controls were included to define parameter gates. PBMCs were gated on the lymphocyte population and the percentage of positive cells for each group *Ascaris* infection only (ASC), Malaria infection only (MAL) and co-infected (ASC + MAL) was determined by flow cytometry and compared to uninfected endemic controls (EC) and MAL group was compared to the (ASC + MAL).

Low intensity *Ascaris lumbricoides* infection did not impact upon *Plasmodium falciparum* parasitaemia or its associated immune responses

The data was reanalysed taking into account *Ascaris* infection (uninfected (n=69), *Ascaris* only (n=21), malaria only (n=109), *Ascaris* and malaria (n=32)). The prevalence of *A. lumbricoides* was 23% and all *A. lumbricoides*-infected children had a low intensity infection (<3,700 eggs per gram (epg)). *Plasmodium falciparum* percentages in the blood were calculated in the malaria-infected and co-infected individuals and no differences in *P. falciparum* parasitaemia were observed between the malaria-infected and co-infected groups (Figure 5). Furthermore, no differences were observed in immune responses between the malaria-infected and co-infected groups for all parameters tested (Additional file 3; Figures 6 and 7).

Discussion

This study provides valuable insights into the immune responses associated with malaria infection in pre-school Nigerian children from 39–73 months. Studies looking at the immunological parameters in this age group are important given that the highest malaria mortality rates occur in children under five years [1]. Studies have shown that immune responses to malaria infection are established early in life and furthermore different ethnic groups respond differently to malaria infection and this is also established early in life [23]. There is little evidence of natural acquired immunity to malaria in children under two years as neonates less than 30 days old and children up to one year have reduced IFN- γ producing capacity [1]. No increase in IFN- γ levels was

observed in the plasma of these children and this contradicts previous studies in children where increased levels of plasma IFN- γ were observed [1]. In addition, a number of other factors associated with active malaria infection were not observed in these children.

Significantly high levels of IL-10 in plasma was observed from malaria-infected children and this corresponded with a reciprocal decrease in IL-12p70 levels, similar to that reported in other studies [24]. Regression analysis revealed a positive association between *P. falciparum* parasitaemia and IL-10. IL-10 is a known antagonist of this pro-inflammatory cytokine [25] and recurrent malaria infection can induce an immunosuppressive environment through secretion of high levels of IL-10, thus inhibiting Th1 responses and facilitating parasite persistence [26]. Intracellular IL-10 was not detected in polyclonally stimulated T-cell subsets from malaria-infected children when compared to endemic controls. PBMCs from malaria infected children when stimulated with *P. falciparum* parasites did not exhibit antigen specific immune responses. Previous studies have reported that T-cell responses can be weak in young children infected with malaria and this lack of responses could explain why this age group is most susceptible to infection [2].

The significant reduction in monocyte percentages in the infected groups compared to endemic controls was observed. Other reports have also shown a decrease in monocyte percentages during active parasitic infection [27]. During infection, circulating monocytes may be required at a higher rate to replenish resident macrophages and DCs. Alternatively, parasitic infections may induce monocyte apoptosis which could explain the

observed decrease in monocyte percentages [28]. Despite the decreases in monocytes there was an increase in CD11c⁺ and CD54⁺ DCs in the infected individuals. The presence of predominantly mature CD11c⁺ DCs population may explain the lack of T-cell responses observed in young children as mature CD11c⁺ DC population lose its ability to phagocytose antigens which is necessary for presentation to effector and memory T-cells [17,18]. The increases in the percentage of CD54⁺ cells supports previous findings which demonstrated a link between increased CD54 expression and disease severity [28]. CD54 was previously shown to be upregulated on activated DCs, monocytes and other APCs during malaria infection in children and it is a known receptor that can bind *P. falciparum* erythrocyte membrane protein 1 [21]. CD11c and CD54 are expressed by many cell types and further analysis would shed light on the specific cell subset observed during infection.

Both $\gamma\delta$ T cells and NK cell express CD56⁺ and this surface marker increased in the malaria-infected children. NK cells and $\gamma\delta$ T cells are thought to be critical in protection from malaria infection [29,30]. Depletion of these cells in murine malaria models has led to increased parasitaemia and delayed resolution of infection, emphasising their importance in early IFN- γ production [30,31]. While these cells are more likely to act as accessory IFN- γ secreting cells to effector T cells there was no increase in IFN- γ detected in the plasma of infected children. However, recently, these cells have also been shown to act as APCs and compensate for DCs in certain situations and further studies would be required to determine the role of this subset [31,32]. While an increase in CD56 cell population was observed, further studies are required to determine the CD56 population subset and examine if these cells secrete IFN- γ .

While the study region is endemic for *A. lumbricoides*, no differences in immune parameters were observed between the co-infected groups compared to the *P. falciparum*-infected group only. Perhaps no differences were observed because children had a low intensity *A. lumbricoides* infection, as was demonstrated in a previous report study by Nacher *et al.* (2000). Moreover, since infection resides in the gut, perhaps only the medium and high intensity infection can alter the immune response systemically. *Ascaris lumbricoides* infection can be protective in the more severe forms of *P. falciparum* infection [33] and since individuals with severe malaria infection were excluded from the study an association could not be determined.

Conclusion

This data corroborates previous reports examining immune responses in malaria-infected children by showing that increases in IL-10 were positively associated with

increased *P. falciparum* parasitaemia. Increases in innate immune cell populations that were previously associated with disease severity in malaria-infected individuals was also demonstrated [19,21,33]. Given that there are so few immunological studies in this age group, these findings could be useful in defining immune responses associated with increasing malaria parasitaemia in young children and therefore markers of disease susceptibility. It is estimated that 87% of children below the age of five are infected with malaria in the Osun State in south-western Nigeria [34], and validating these methodologies is important for future studies in this area. For example, birth to age five is an important range for the administration and study of many prophylactic paediatric vaccines and World Health Organization recommendations for routine immunization within this age group in Nigeria include both measles and yellow fever vaccines [35].

Additional files

Additional file 1: Sample of gating strategy for T Cells. Shown is a PBMC sample stimulated with 50 ng/ml PMA and 1 mg/ml ionomycin for 4 h in the presence of BFA (10 mg/ml). Cell subsets were identified as CD3 cells and then analysed for expression of cytokines and markers $\gamma\delta$ cells.

Additional file 2: The average age, parasitemia, cytokine, nitric oxide (NO), RANTES, metalloproteinase (MMP) type 8 and tissue inhibitor of metalloproteinase (TIMP) type 1 plasma concentrations from the study cohort classified according to infection status (uninfected, *Ascaris* only, malaria only, *Ascaris* and malaria).

Additional file 3: The average age, cytokine, nitric oxide (NO), RANTES, metalloproteinase (MMP) type 8 and tissue inhibitor of metalloproteinase (TIMP) type 1 plasma concentrations from the study cohort classified according to *P. falciparum* parasitaemia.

Competing interests

GlaxoSmithKline sponsored the drug albendazole, which was used in larger clinical trial. The authors declare that they have no competing interests. The authors also declare that they have no financial competing interests.

Authors' contributions

CN carried out all immunological experiments and drafted the manuscript. MP is our biostatistician and performed the statistical analysis. DJD provided assistance in the immunological assays, AA provided assistance in the immunological assays, PK collected clinical samples and provided the parasitological data, SFM collected clinical samples, SOA participated in the design of the study, CH conceived the study, and participated in its design and coordination. SMO'N conceived the immunological aspects of study, participated in its design and edited the manuscript. All authors read and approved the final manuscript.

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Mannose receptor and macrophage galactose-type lectin are involved in *Bordetella pertussis* mast cell interaction

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ABSTRACT

Mast cells are crucial in the development of immunity against *Bordetella pertussis*, and the function of TLRs in this process has been investigated. Here, the interaction between mast cells and *B. pertussis* with an emphasis on the role of CLRs is examined. In this study, two CLRs, MGL and MR, were detected for the first time on the surface of mast cells. The involvement of MR and MGL in the stimulation of mast cells by heat-inactivated BP was investigated by the use of blocking antibodies and specific carbohydrate ligands. The cell wall LOS of BP was also isolated to explore its role in this interaction. Mast cells stimulated with heat-inactivated BP or BP LOS induced TNF- α , IL-6, and IFN- γ secretion, which was suppressed by blocking MR or MGL. Inhibition of CLRs signaling during BP stimulation affected the ability of mast cells to promote cytokine secretion in T cells but had no effect on the cell-surface expression of ICAM1. Blocking MR or MGL suppressed BP-induced NF- κ B expression but not ERK phosphorylation. Syk was involved in the CLR-mediated activation of mast cells by BP. Bacterial recognition by immune cells has been predominantly attributed to TLRs; in this study, the novel role of CLRs in the BP-mast cell interaction is highlighted. *J. Leukoc. Biol.* 94: 439–448; 2013.

Introduction

B. pertussis is a Gram-negative coccobacillus that causes the respiratory infection, whooping cough [1]. Despite being preventable by vaccination, pertussis causes nearly 300,000 deaths in children every year, mainly in developing countries [2]. The incidence of whooping cough in Europe has increased as a result of the change in national immunization programs from the whole-cell

vaccine to acellular vaccine [1]. Vaccination can induce protection against infection by inducing strong Th1/Th2 immune responses; however, the acellular vaccine induces a polarized Th2 immune response [2, 3] and confers protection for a limited time only [3]. Better understanding of the interaction of *B. pertussis* with innate immune cells may lead to the development of a more effective vaccine that induces lifelong immunity.

Mast cell-induced Th1 immune responses are crucial in the clearance of protozoan and bacterial infection, such as *Escherichia coli* [4], *Leishmania major* [5], and *Pseudomonas aeruginosa* [6]. Activation of mast cells during vaccination contributes to the development of protective Th1 immunity [7], and these cells are crucial in the effectiveness of vaccination against anthrax [8] and *Helicobacter* infection [9]. In vitro *B. pertussis* induces IL-6 and TNF- α release from mast cells, while in vivo, early TNF- α production is delayed in mast cell-depleted mice infected with *B. pertussis* [10]. Despite the crucial role of mast cells in the clearance of bacterial infection from the lung and in mediating protective Th1 immune responses [11, 12], only few studies have examined the interaction of mast cells with *B. pertussis* [10].

TLRs were shown to be critical in the recognition of the *B. pertussis* outer membrane. The strain-specific endotoxin component of the bacteria's outer membrane, the LOS, is the key TLR4 initiator of inflammatory immune responses [13, 14]. The thick bacterial coat of glycoconjugate structures in the form of polysaccharidic capsules, peptidoglycans, LPS, and other glycolipidic moieties [15] are PAMPs that can activate other PPRs, such as [16] CLRs [17]. The signaling pathways of TLRs and CLRs are complex, and the crosstalk between the receptors allows immune cells to develop pathogen-specific immune responses [18].

CLRs play a key role in immunity against bacterial infection as a result of their calcium-dependent interaction with pathogen-associated glycan epitopes [19]. MR binds to carbohydrates, such as mannose, *N*-acetylglucosamine, and fucose. These saccharides can be found on the surfaces of microorganisms, including *E. coli*, *P. aeruginosa*, and *Mycobacterium tu-*

Abbreviations: BM=bone marrow, BP=*Bordetella pertussis* antigen, CLR=C-type lectin receptor, CRD=carbohydrate recognition domain, DCU=Dublin City University, GalNAc=N-acetyl-D-galactosamine, GlcNAc=N-acetyl-D-glucosamine, LOS=lipooligosaccharide, MAFA=mast cell function-associated antigen, MGL=macrophage galactose-type lectin, Mincle=macrophage-inducible C-type lectin, MR=mannose receptor, Ng=Nisseria gonorrhoea, PCMC=peritoneal cell-derived mast cells, Syk=spleen tyrosine kinase

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berculosis [20]. Another important CLR, MGL, has been reported to bind to GalNAc motifs on filoviruses, *Campylobacter jejuni* and *Schistosoma mansoni* [17, 21]. Some bacteria can interact with more than one CLR, and the restricted expression of CLRs on immune cell subsets can contribute to the different immune responses [22].

Upon carbohydrate binding, CLRs have the ability to trigger the immune machinery by antigen internalization and presentation onto the MHC molecule or by modulation of the TLR-mediated signaling pathway [20]. Differences in sugar-binding ability and ligand specificity of the various CLRs are conferred by highly conserved CRDs. The ubiquitous expression of CLRs by most cell types, including macrophages and DCs [17], together with organism-specific differences in glycosylation among pathogens, make the CLRs a central defense system in innate immunity.

To date, the study of CLRs expressed on mast cells is in its infancy. Mast cells express a specific CLR, MAFA [23], later found also in NK cells and human basophils [24]. Stimulation of MAFA leads to the inhibition of FcεRI-induced activation of mast cells [25]. Other CLRs, such as Mincle and Dectin-1, were also shown to be expressed by mast cells, although expression levels are very low [26, 27]. These two CLRs, also present on macrophages and DCs, play important roles in anti-fungal immunity [28]. Upon CLR ligand binding, ITAM and ITAM-like signaling pathways can be activated, promoting the recruitment of Syk, which results in the activation of cellular proliferation, differentiation, and migration [29].

We reported recently that mast cells stimulated with heat-inactivated BP secrete proinflammatory cytokines, such as TNF-α and IL-6, and display enhanced expression of ICAM1. BP-stimulated mast cells promote cytokine secretion in T cells. BP induces the activation of two members of the TLR4 signaling pathway, ERK and NF-κB [30]. In this study, we investigated the interaction between mast cells and *B. pertussis* with an emphasis on the role of CLRs. Better understanding of this interaction may lead to the development of a more efficient *B. pertussis* vaccine and may advance our understanding of the complex interactions between PPR crosstalk in activating innate immune responses.

MATERIALS AND METHODS

Animals and antigens

C57BL/6 mice, 6–8 weeks old, were purchased from Charles River (Carretrilla, Ireland). Mice were kept under specific pathogen-free conditions at the Bioresource Unit, Faculty of Health and Science, DCU (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 the DCU Ethics Committee and the Irish Department of Children and Health.

Heat-inactivated BP (Tohama I) was a kind gift from Professor Bernie Mahon (National University of Ireland Maynooth, Ireland) [31]. Bacteria number was determined by measuring OD, and protein concentrations were determined using a BCA protein assay kit (Pierce, Thermo Fisher Scientific, Dublin, Ireland).

Isolation, maturation, and characterization of BM and PCMCs

BMMCs were generated from the femoral and tibia BM cells of C57BL/6 mice and maintained in complete IMDM (Gibco, Life Technologies, Carlsbad, CA, USA) in the presence of 10% heat-inactivated FCS (Gibco, Life Technologies), 100 u/ml penicillin/streptomycin (Sigma-Aldrich, Arklow, Ireland), and 30% WEHI-3 conditioned IMDM medium (TIB-68; American Type Culture Collection, Manassas, VA, USA) as a source of the murine growth factor IL-3 for 4 weeks [32]. PCMCs were obtained using a protocol described previously [33]. Briefly, C57BL/6 mice were i.p.-injected with 10 ml sterile PBS, and then the obtained peritoneal cells were cultured in RPMI-1640 medium, supplemented with 10% FCS and 100 u/ml penicillin/streptomycin, L-glutamine (2 mM; Sigma-Aldrich), 10 ng/ml mouse rIL-3 (Calbiochem, Merck, Darmstadt, Germany), and 30 ng/ml recombinant mouse stem cell factor (Sigma-Aldrich) at 37°C. Forty-eight hours later, nonadherent cells were discarded and replaced by fresh culture medium for a further 7 days. In both preparations, >95% of the total cells were identified as mast cells on the basis of the c-kit (Clone 2B8; eBioscience, Hatfield, UK) and FcεRI (Clone MARI; eBioscience) cell-surface expression or Kimura staining [34]. Cell number and viability were monitored using trypan blue staining (Sigma-Aldrich). β-Hexosaminidase release from mast cells was measured as described previously [33] to test functionality.

Stimulation of mast cells for ELISA and flow cytometry

BMMCs or PCMCs were cultured with anti-MR (Abcam, Cambridge, UK) or anti-MGL (Hycult Biotech, Uden, The Netherlands)-blocking antibodies (1 μg/ml), EGTA (10 mM), mannan (50 μg/ml), and GalNAc (50 mM; all from Sigma-Aldrich) or Syk inhibitor (10 μM; piceatannol; Enzo Life Sciences, Exeter, UK), 30 min before stimulation with PBS or BP (100 bacteria/cell) for 24 h. Levels of TNF-α, IL-6, IFN-γ, and IL-10 cytokines were measured in supernatant by commercial ELISA (BD Biosciences, Oxford, UK).

Cells were incubated for 15 min with CD16/CD32 (FcγIII/II; BD Biosciences) to block FcR before flow cytometric analysis and were stained with PE/FITC/allophycocyanin-conjugated anti-FcεRI, anti-CD117, and anti-CD206 (MR; Clone MR5D3; BioLegend, London, UK) and anti-MGL (Clone ER-MP23; AbD Serotec, Kidlington, UK) or the appropriate IgG isotype control for 30 min at 4°C in the dark. mAb binding was analyzed by flow cytometry (FACS Aria; BD Biosciences) in the presence of DNase (Roche, Mannheim, Germany) to avoid aggregation, using FACSDiva (BD Biosciences), FlowJo (TreeStar, Ashland, OR, USA), and Flowing Software (Cell Imaging Core, Turku Centre for Biotechnology, Finland).

Coculture studies and cytokine measurements

CD4⁺ T cells were isolated using the MACS CD4⁺ T cell isolation kit (Miltenyi Biotec, Surrey, UK). Purity of isolates was determined by flow cytometry (FITC-conjugated CD4; Clone L3T4; BD Biosciences); only preparations with ≥95% CD4⁺ T cells purity were used in this study. PCMCs were cultured in the presence of anti-MR- or anti-MGL-blocking antibodies (1 μg/ml) for 30 min before stimulation with PBS or BP (100 bacteria/cell) for 24 h. Washed, stimulated PCMCs were added to CD4⁺ cells (in a 1:1 ratio) in plates coated with anti-CD3 (1 μg/ml; BD Biosciences), cocultured for 72 h, and supernatants were tested for IFN-γ, IL-4, IL-5, and IL-10 by commercial ELISA (BD Biosciences).

Protein extraction and Western blot analysis

BMMCs were cultured with anti-MR- or anti-MGL-blocking antibodies for 30 min and then stimulated with BP (100 bacteria/cell) for 15 min. Total protein was extracted from cell lysates using RIPA buffer containing protease and phosphatase inhibitor cocktails (both from Sigma-Aldrich). Protein samples (10–40 μg) and prestained protein markers (SeeBlue Plus2; Invitrogen, Paisley, UK) were separated by 10% SDS-PAGE and blotted onto 0.45 μm Immobilon-P polyvinylidene fluoride membranes (Millipore,

Carrigtwohill, Ireland). Membranes were probed, according to standard Western blot protocol, using anti-phospho-ERK (Cell Signaling Technology, Danvers, MA, USA), anti-total-ERK (Cell Signaling Technology), and anti-phospho-NF- κ B p65 (Ser 276), and anti- β -actin (BioLegend) primary antibodies and peroxidase-conjugated anti-rabbit IgG secondary antibody (Sigma-Aldrich). Proteins were visualized by incubation with a chemiluminescent HRP substrate (Millipore), exposed to film, and processed using an FPM 100A processor (Fuji Film, Tokyo, Japan). Protein bands were quantified using ImageJ analysis software (imagej.nih.gov). Levels of phospho-ERK and NF- κ B p65 were normalized to total-ERK and β -actin and expressed in arbitrary units as percentage increases over the medium control levels.

Isolation of BP LOS

Isolation of BP LOS was performed according to a protocol described previously [13]. Briefly, 1×10^{11} cells of heat-inactivated BP were suspended in 3 ml 0.1 mg/ml proteinase K (Roche) in PBS and incubated for 1.5 h at 56°C. After incubation, the mixture was subjected to acetone precipitation and the pellet solubilized in 1 ml sterile PBS. Reaction completion was evaluated by 16% SDS-PAGE, followed by silver staining [35]. The contaminating presence of proteinase K in the crude LOS preparation was removed by centrifugation [36] (Avanti J-26 XP centrifuge; Beckman Coulter, Fullerton, CA, USA) at 40,000 *g* for 4 h at 4°C in a fixed angle rotor (Beckman JA-20). The LOS-containing pellet and the acetone-precipitated supernatant were dissolved in 300 μ l sterile PBS. Purified LOS was separated on 16% SDS-PAGE and stained by the silver nitrate method.

Stimulation of mast cells by LOS

BMMCs were stimulated with anti-MR- and anti-MGL-blocking antibodies (1 μ g/ml), 30 min before stimulation with this LOS preparation (1 u/ml; equal to 100 bacteria/cell) for 24 h. Supernatants were tested for TNF- α and IL-6 cytokines, as described above. As a result of the presence of traces of proteinase K in LOS preparation, the effect of proteinase K on cytokine secretion was also evaluated as an additional control.

Statistics

All data were analyzed for normality before statistical testing by Origin 6.1 (OriginLab, Northampton, MA, USA) software. Where multiple group comparisons were made, data were analyzed 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.

RESULTS

MR and MGL are expressed by mast cells

Previous studies have reported the expression of MAFA [37], Dectin-1, and MinCLE [27] on mast cells. Here, the expression of MGL and MR on BMMCs (Fig. 1A–C; MR: *P* ≤ 0.05; MGL: *P* ≤ 0.01) and PCMCs (Fig. 1D–F; MR: *P* ≤ 0.01; MGL: *P* ≤ 0.01) was examined by flow cytometry, revealing that these CLRs are expressed significantly by mast cells when compared with isotype controls. However, BMMCs and PCMCs, when stimulated with heat-inactivated BP for 24 h, did not display enhanced expression of these receptors (MR: *P* = 0.12; MGL: *P* = 0.91; data not shown).

BP-induced TNF- α , IL-6, and IFN- γ secretion in mast cells is reduced in the presence of anti-MR- or anti-MGL-blocking antibodies

As MR and MGL are expressed by mast cells, CLR involvement in the interaction of BP with mast cells was investigated. Stimulation of BMMCs induced TNF- α and IL-6 secretion (Fig. 2A and B; *P* ≤ 0.05). The levels of TNF- α (MR: $74.6 \pm 10.5\%$, *P* ≤ 0.05; MGL: $29.0 \pm 4.5\%$, *P* ≤ 0.01) and IL-6 (MR: 88.0 ± 1.4 ; MGL: $91.1 \pm 3.4\%$, *P* ≤ 0.01) were reduced significantly when BMMCs were treated with anti-MR- or anti-MGL-blocking antibodies before BP stimula-

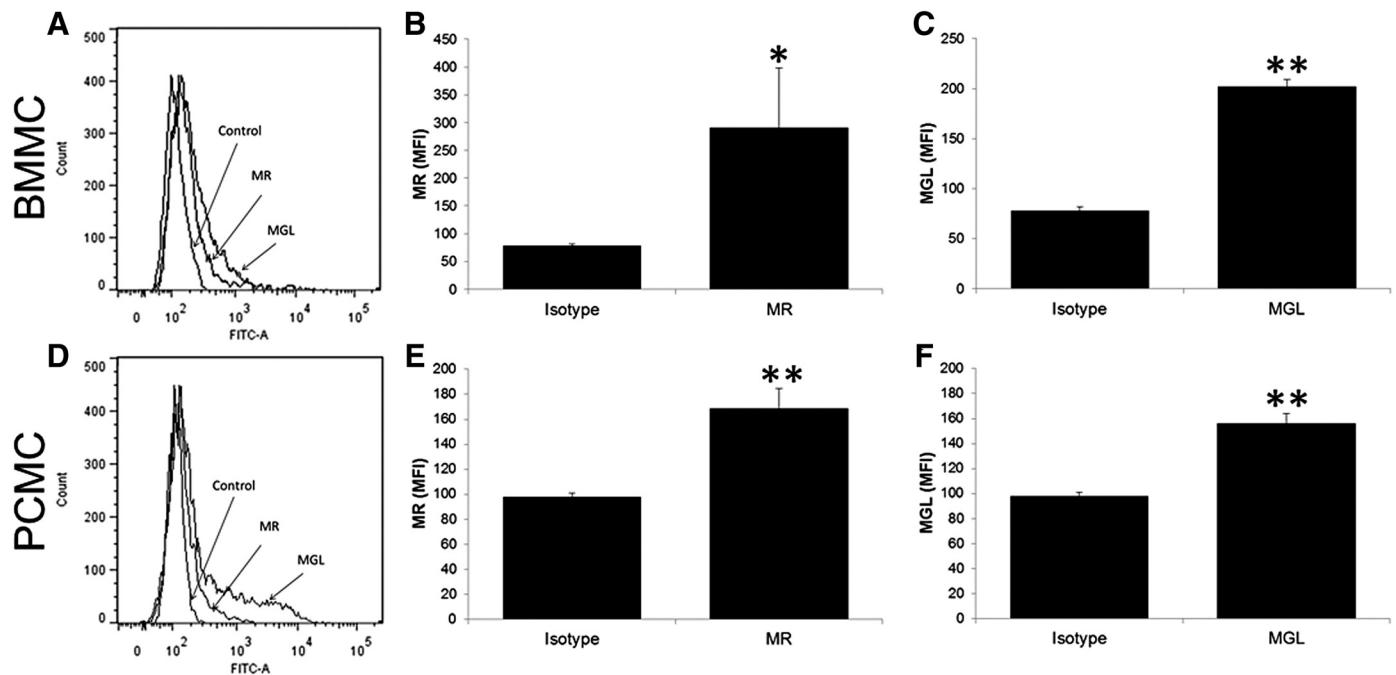


Figure 1. MR and MGL are expressed by mast cells. Cell-surface expression of MR (B and E) and MGL (C and F) was measured by three-color (Fc ϵ RI, c-kit, and CLR) flow cytometry on BMMCs (A–C) and PCMCs (D–F). Data are presented on histograms (A and D) and as the mean \pm SD of three independent experiments. ***P* ≤ 0.01 compared with isotype control group. FITC-A, FITC-area; MFI, mean fluorescence intensity.

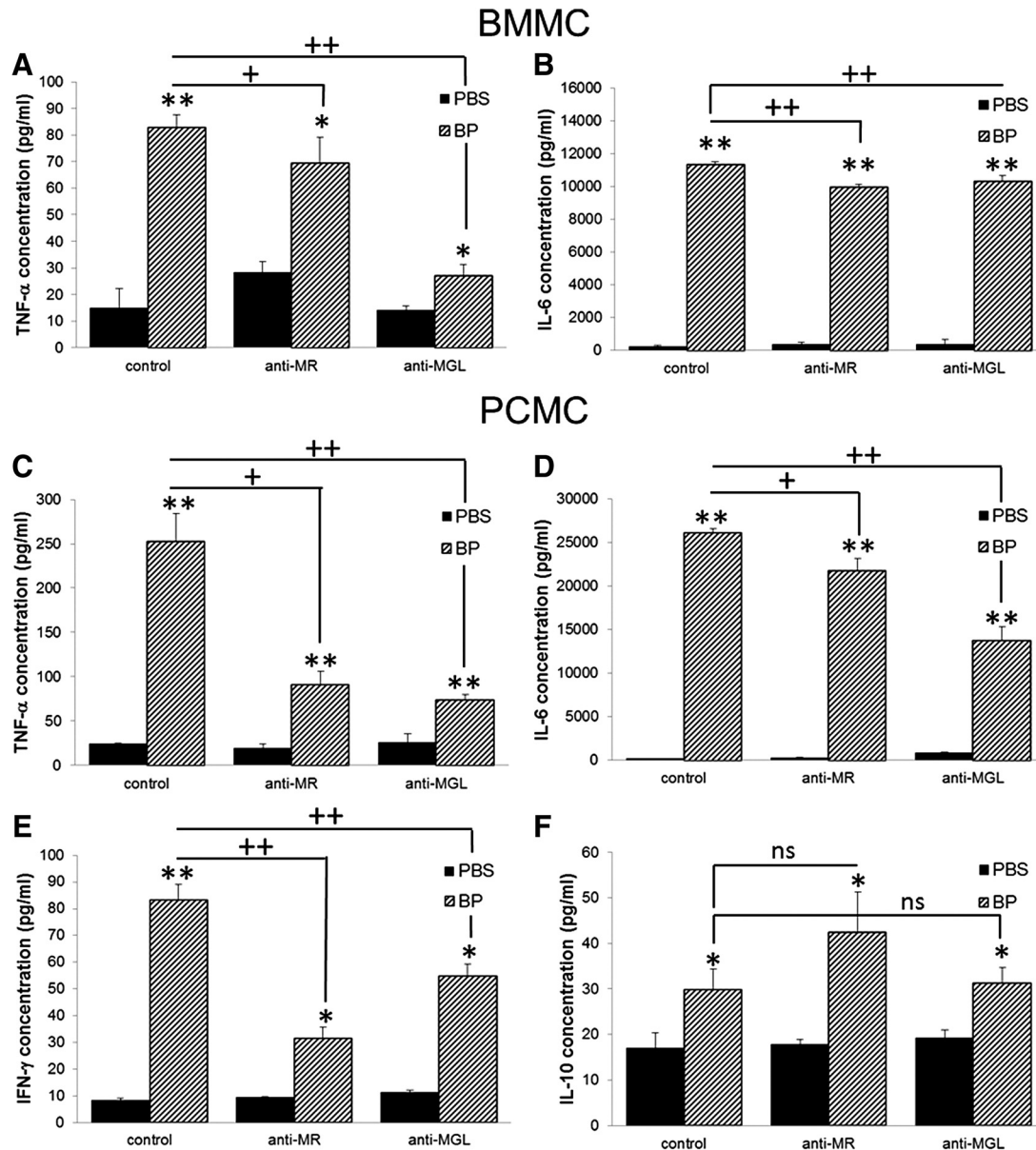


Figure 2. Blocking of MR and MGL suppresses BP-induced cytokine secretion in mast cells. BMMCs (A and B) and PCMCs (C–F) were treated with anti-MR- or anti-MGL-blocking antibody (1 μ g/ml), 30 min before stimulation with BP (100 bacteria/cell) for 24 h. TNF- α (A and C), IL-6 (B and D), IFN- γ (E), and IL-10 (F) secretion was measured with commercial ELISA. Data are presented as the mean \pm SD of three independent experiments. * $P \leq 0.05$ and ** $P \leq 0.01$ compared with control group; + $P \leq 0.05$ and ++ $P \leq 0.01$ compared with control BP-stimulated group.

tion (Fig. 2A and B). MR- and MGL-blocking antibodies were added together, 30 min before treatment of BMMCs with BP. BP-induced TNF- α ($12.1 \pm 1.6\%$, $P \leq 0.05$), and IL-6 ($52.4 \pm 31.7\%$, $P \leq 0.05$) secretion was suppressed significantly by the blocking antibodies; however, further suppression was not observed in the presence of the two antibodies (data not shown).

As PCMCs are more mature mast cells than BMMCs, studies were repeated to demonstrate that similar results would be obtained. Stimulation of PCMCs with BP for 24 h induced secretion of TNF- α , IL-6, IFN- γ ($P \leq 0.01$), and IL-10 ($P \leq 0.05$) cytokines (Fig. 2C–F). The levels of TNF- α , IL-6, and IFN- γ were reduced significantly when PCMCs were treated with anti-MR (TNF- α and

IL-6: $P \leq 0.05$; IFN- γ : $P \leq 0.01$)- or anti-MGL-blocking antibody for 30 min before BP stimulation (Fig. 2C–E). IL-10 secretion by PCMCs was not impaired in the presence of either of the antibodies (anti-MR: $P = 0.18$; anti-MGL: $P = 0.80$; Fig. 2F).

Anti-MR- and anti-MGL-blocking antibodies reverse T cell activation by BP-primed mast cells

BMMCs and PCMCs were stimulated with BP in the presence or absence of anti-MR- or anti-MGL-blocking antibodies for 24 h, as described above. BP-activated mast cells were cocultured with naïve CD4⁺ T cells, and cytokine secretion was measured. As coculture of mast cells with naïve CD4⁺ cells in the presence of

anti-CD3 is reported to promote cytokine secretion per se [38–41], PBS-primed BMMCs/PCMCs were also used in the coculture experiments as controls. CD4⁺ cells, cocultured with BMMCs, secreted significant levels of IL-10 and IFN- γ ($P \leq 0.05$; Fig. 3A and B). IFN- γ secretion was suppressed significantly when BMMCs were treated with anti-MR ($68.3 \pm 3.3\%$, $P \leq 0.01$)- or anti-MGL ($18.0 \pm 5.1\%$, $P \leq 0.01$)-blocking antibodies before BP stimulation and coculture. Levels of IL-10 did not change significantly (Fig. 3A and B).

As per the previous experiment, the results were confirmed with PCMCs (Fig. 3C–F). CD4⁺ T cells cocultured with BP-stimulated PCMCs secreted higher levels of IFN- γ ($P \leq 0.05$), IL-10 ($P \leq 0.05$), and IL-5 ($P \leq 0.05$) than the control group (Fig. 3C–E),

whereas IL-4 levels remained unchanged ($P = 0.55$; Fig. 3F). When PCMCs were pretreated with anti-MR or anti-MGL before BP stimulation, they no longer secreted significant levels of IFN- γ (anti-MR: $P = 0.41$; anti-MGL: $P = 0.12$) and IL-5 (anti-MR: $P = 0.13$; anti-MGL: $P = 0.12$) in coculture compared with controls (Fig. 3C and E). However only PCMCs treated with anti-MGL no longer secreted IL-10 compared with controls (Fig. 3D).

ICAM1 expression on mast cells is not altered by anti-MR- and anti-MGL-blocking antibodies

As ICAM1 expression is important in T cell–mast cell communication and was previously shown to be enhanced on mast

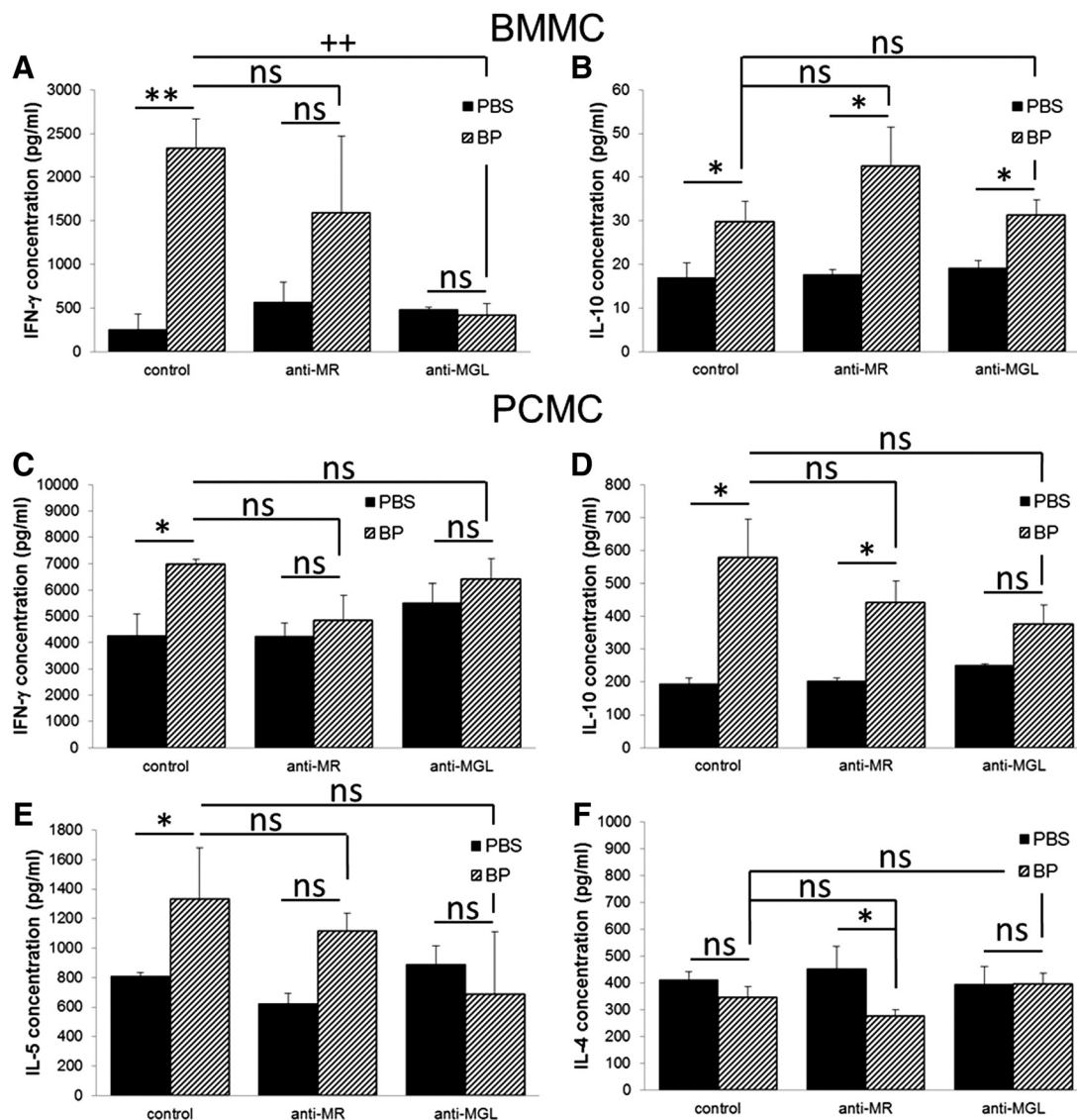


Figure 3. Anti-MR- and anti-MGL-blocking antibodies interfere with BP-induced T-cell activation by PCMCs. BMMCs (A and B) and PCMCs (C–F) were treated with anti-MR- and anti-MGL-blocking antibody (1 $\mu\text{g/ml}$), 30 min before stimulation with BP (100 bacteria/cell) for 24 h. Cells were washed and cocultured with naïve CD4⁺ T cells (1:1) for 72 h. IFN- γ (A and C), IL-10 (B and D), IL-5 (E), and IL-4 (F) secretion was measured with commercial ELISA. Data are presented as the mean \pm SD of three independent experiments. * $P \leq 0.05$ and ** $P \leq 0.01$ compared with control group; ++ $P \leq 0.01$ compared with control BP-stimulated group.

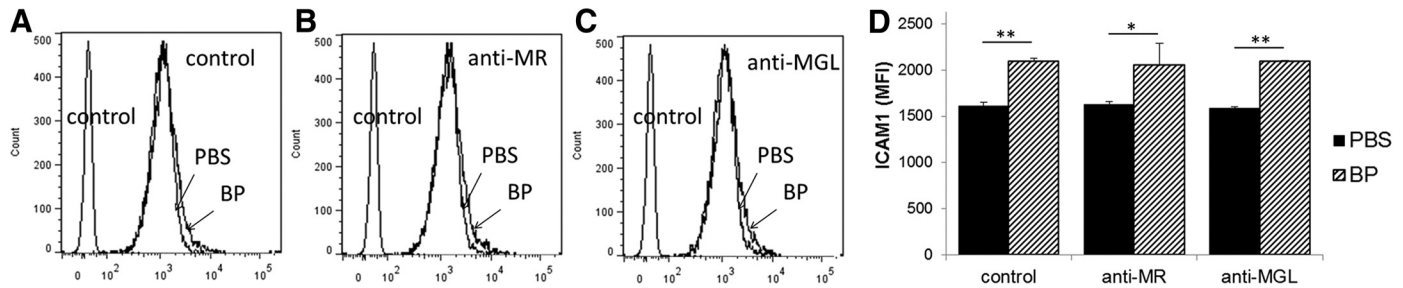


Figure 4. Anti-MR- and anti-MGL-blocking antibodies do not suppress BP-induced up-regulation of ICAM1. PCMCs were treated with PBS (control; A) or anti-MR (B)- or anti-MGL (C)-blocking antibody (1 μ g/ml), 30 min before stimulation with BP (100 bacteria/cell) for 24 h. Cell-surface marker expression of ICAM1 was measured by flow cytometry. Data are presented as the mean \pm SD of three independent experiments (D). * $P \leq 0.05$ and ** $P \leq 0.01$ compared with control group.

cells following BP stimulation [30] (Fig. 4), the involvement of CLR in ICAM1 expression was investigated. PCMCs were treated with anti-MR- or anti-MGL-blocking antibodies before stimulation with BP for 24 h. BP enhanced ICAM1 expression

in all groups, with no significant effect as a result of blocking of MR or MGL ($P=0.97$; Fig. 4).

BP-induced TNF- α and IL-6 secretion by mast cells is reduced in the presence of glycans (mannan and GalNAc) and EGTA

To fully validate that the observed suppression of cytokine secretion is a result of effective blocking of Ca^{2+} -dependent CRDs on CLR, BMMCs were treated with mannan (50 μ g/ml, MR ligand), GalNAc (50 mM, MGL ligand), or EGTA (10 mM, Ca^{2+} ligand) for 30 min before stimulation with BP for 24 h. All glycans and EGTA suppressed BP-induced TNF- α and IL-6 secretion significantly (EGTA and mannan: $P \leq 0.01$; GalNAc: $P \leq 0.05$; Fig. 5). Notably, EGTA treatment reduced the cytokine secretion ability, not only of BP-stimulated mast cells but also of PBS-primed (control) mast cells ($P \leq 0.01$; Fig. 5). The effect of laminarin, a non-MR and -MGL ligand, on BP-induced cytokine secretion was also tested on BMMCs. TNF- α (to $63.8 \pm 1.4\%$, $P \leq 0.01$) and IL-6 (to $45.0 \pm 2.4\%$, $P \leq 2.4$) secretion was suppressed significantly when cells were pretreated with laminarin, which also suggests a role for Dectin-1 in the BP-mast cell interaction (data not shown).

Syk inhibition suppresses TNF- α and enhances IL-6 secretion by BP-stimulated mast cells

Downstream CLR signaling was investigated by assessing the involvement of Syk kinase [29] in cytokine secretion by BP-stimulated mast cells. BMMCs were treated with Syk inhibitor (piceatannol) before stimulation with BP for 24 h. TNF- α secretion was reverted to basal level by Syk inhibition (Fig. 6A), whereas IL-6 secretion levels appeared enhanced dramatically in BP-stimulated and control groups (Fig. 6B).

Blocking of MR and MGL suppresses BP-induced NF- κ B activation but not ERK phosphorylation

To further understand the signaling pathways involved in CLR-mediated BP stimulation of mast cells, the effect of anti-MR- and anti-MGL-blocking antibodies on NF- κ B p65 and ERK phosphorylation was investigated. As reported previously, BP stimulation of mast cells enhances ERK and NF- κ B p65 activation [30]. NF- κ B p65 activation could be reversed by blocking MR or MGL ($P \leq 0.01$; Fig. 7A and C), whereas anti-MGL- or anti-MR-blocking antibody

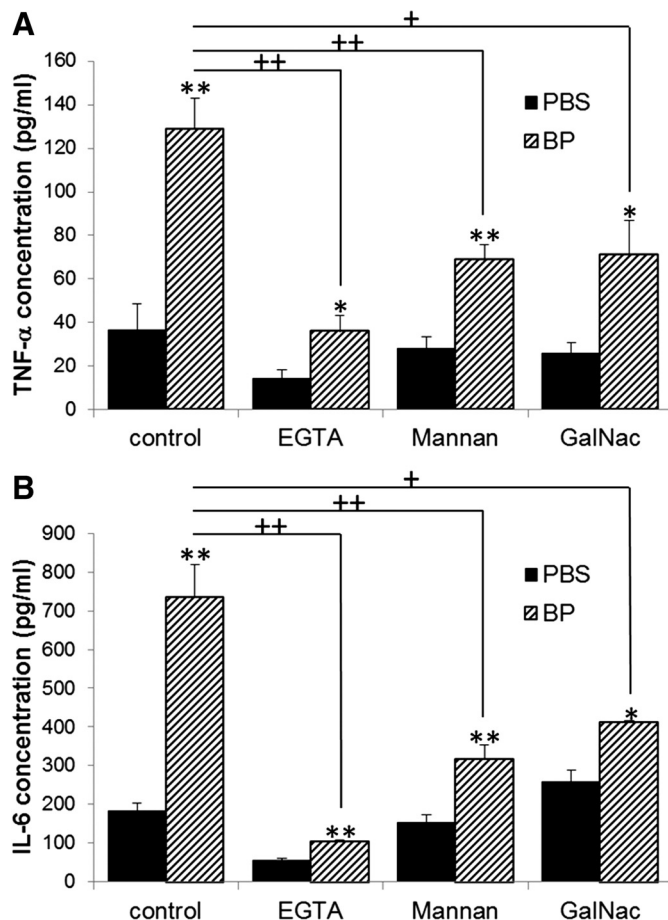


Figure 5. Carbohydrate treatment impairs BP-induced cytokine secretion in mast cells. BMMCs were treated with EGTA (10 mM), mannan (50 μ g/ml), and GalNAc (50 mM), 30 min before stimulation with BP (100 bacteria/cell) for 24 h. TNF- α (A) and IL-6 (B) secretion was measured with commercial ELISA. Data are presented as the mean \pm SD of three independent experiments. * $P \leq 0.05$ and ** $P \leq 0.01$ compared with control group; + $P \leq 0.05$ and ++ $P \leq 0.01$ compared with control BP-stimulated group.

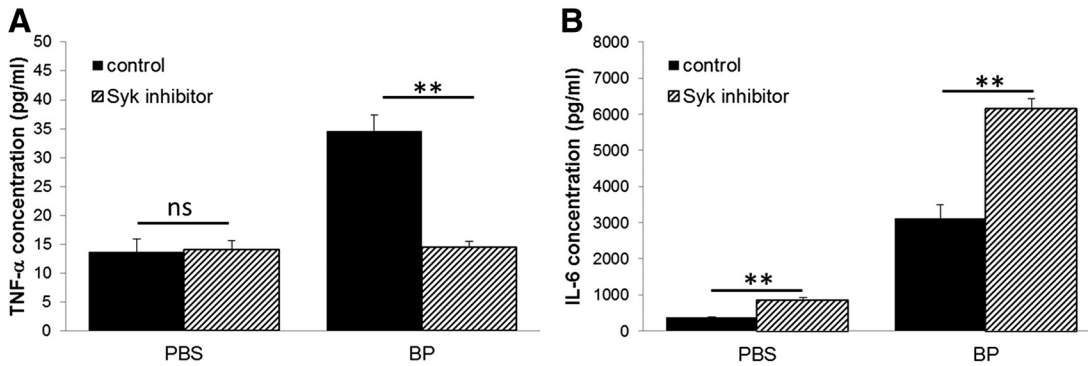


Figure 6. Inhibition of Syk suppresses BP-induced TNF- α secretion and enhances IL-6 secretion in mast cells. BMMCs were treated with PBS (control) or Syk inhibitor (10 μ M), 30 min before stimulation with BP (100 bacteria/cell) for 24 h. TNF- α (A) and IL-6 (B) secretion was measured with commercial ELISA. Data are presented as the mean \pm SD of three independent experiments. ** $P \leq 0.01$ compared with control (PBS) group.

treatment, before BP stimulation, had no statistical effect on ERK phosphorylation (Fig. 7A and B).

Blocking of MR and MGL suppresses LOS-induced cytokine secretion in mast cells

Whole-cell vaccine studies suggest that BP-derived LOS is crucial to activate innate immune cells to generate proinflammatory cytokines in *Bordetella* infections [42]. The role of *B. pertussis* LOS in CLR-mediated mast cell activation was investigated. LOS from BP heat-inactivated cells was isolated by digestion of the cell lysate with proteinase K, followed by high-speed centrifugation (Fig. 8A). BMMCs were stimulated with blocking anti-MR and anti-MGL antibodies, 30 min before stimulation with this LOS preparation for 24 h. As a result of the presence of traces of proteinase K in LOS preparation, the effect of proteinase K on mast cell was also tested, resulting in negligible cytokine secretion. The blocking of MR and MGL on LOS-stimulated mast cells inhibited cytokine secretion (Fig. 8B and C), as observed previously in the case of BP-stimulated cells (Fig. 1A and B).

DISCUSSION

Mast cells are a major component of protective immunity against microbial infection. These cells are a potent source of

proinflammatory mediators, such as TNF- α and IFN- γ , that can promote Th1-driven host defense against bacteria and protozoa [7, 43–46]. They play a crucial role in the clearance of bacterial infection, such as *B. pertussis* and *E. coli* [4, 10]. Here, we show for the first time that MR and MGL are expressed by BMMCs and PCMCs. Furthermore, MR and MGL proved to be functionally involved in the induction of cytokine secretion of *B. pertussis*-challenged mast cells and reversed the ability of these cells to prime naïve CD4⁺ T cells to secrete cytokines. MR and MGL have been reported to play a role in T cell activation by APCs, such as DCs and macrophages [47].

In the case of MR, our findings correlate with studies in other immune cells, as MR was shown to be involved in the activation of macrophages by *M. tuberculosis* in vitro [48]. In contrast, MGL was associated previously with the suppression rather than induction of cytokine secretion by pathogen-stimulated immune cells. In DCs, a *C. jejuni* mutant lacking a functional MGL ligand induced increased IL-6 production compared with WT [21]. Similarly, modulation of MGL on DCs by cestode antigens leads to inhibition of LPS-induced proinflammatory cytokine secretion [49]. However, other studies have shown that activation of MGL leads to maturation of DCs and IFN- γ secretion in CD8⁺ T cell cocultures [50]. Our results reveal a new stimulatory function of MGL in pathogen–mast cell interaction. Furthermore, MR and MGL play a role

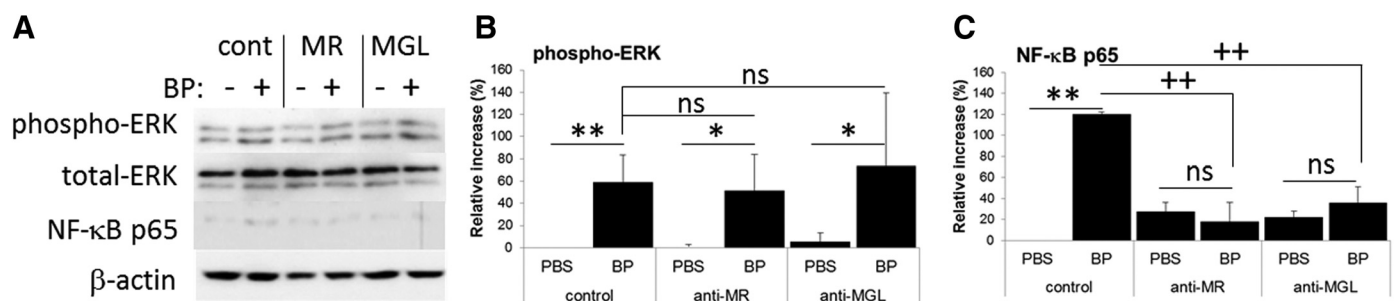


Figure 7. Blocking of MR and MGL suppresses BP-induced activation of NF- κ B p65 but not ERK phosphorylation. BMMCs were treated with anti-MR- and anti-MGL-blocking antibodies before stimulation with BP for 15 min. Protein samples were analyzed by Western blot for phosphorylated-ERK (phospho-ERK), total ERK, phosphorylated-NF- κ B p65, and β -actin expression. cont, Control (A). Values of phosphorylated-ERK (B) and NF- κ B p65 (C) were normalized to total ERK and β -actin and are expressed in arbitrary units as percent-fold increases over PBS control. Densitometry data are presented as the mean \pm SD of three independent experiments. * $P \leq 0.05$ and ** $P \leq 0.01$ compared with control (PBS) group; ++ $P \leq 0.01$ compared with control BP-stimulated group.

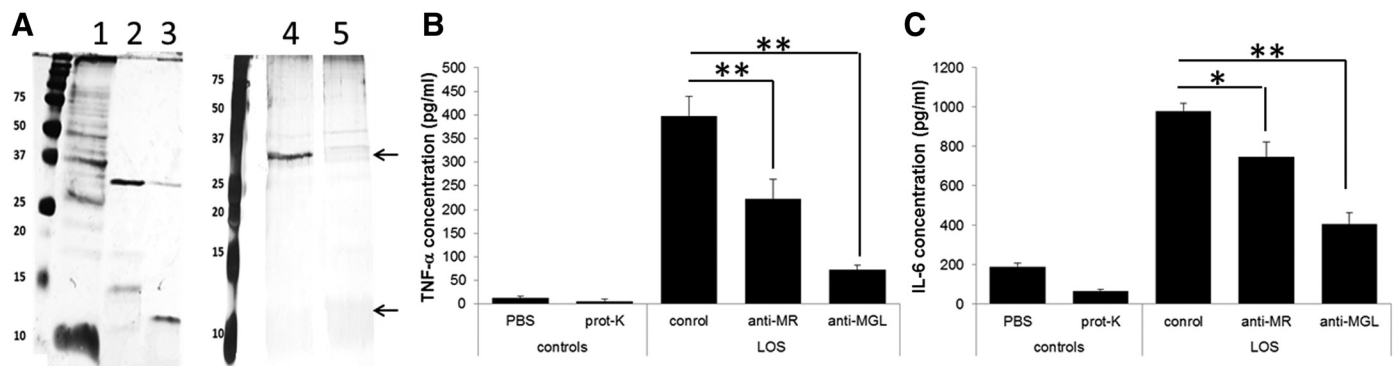


Figure 8. Blocking MR and MGL suppresses LOS-induced cytokine secretion in mast cells. LOS was isolated from BP antigen and analyzed by silver-stained SDS-PAGE (A). 1: Whole *B. pertussis* cell lysate (10 μg); 2: proteinase K (prot-K; 5 μg); 3: *B. pertussis* LOS crude preparation (10 μl); 4: *B. pertussis* LOS supernatant; 5: *B. pertussis* LOS pellet. Proteinase K (28.9 kDa) and LOS (~10 kDa) are indicated by arrows. BMMCs were stimulated with 1 u/ml (equivalent to 100 bacteria/cell) LOS for 24 h in the presence and absence of anti-MR- or anti-MGL-blocking antibodies. TNF-α (B) and IL-6 (C) secretion was measured by commercial ELISAs. Data are presented as the mean ± SD of three independent experiments. * $P \leq 0.05$ and ** $P \leq 0.01$ compared with the control LOS group.

in the internalization of pathogens, as observed for *M. tuberculosis* [51, 52], and *B. pertussis* can adhere and be phagocytised by mast cells [10]. It would be interesting in future studies to investigate the role of MR and MGL in the binding and uptake of BP antigens.

Similar to DCs and macrophages, mast cells express PRRs, such as TLR2, TLR3, TLR4, TLR6, TLR7, and TLR9, that recognize invading pathogens [53, 54]. BP stimulation has been reported to cause ERK phosphorylation on mast cells via activation of the TLR4 signaling pathway [55, 56] and activation of MAPK and NF-κB pathways inducing cytokine secretion and cell-surface marker up-regulation in immune cells [30]. Blocking MR and MGL on mast cells suppressed NF-κB p65 but not ERK phosphorylation. A similar trend has also been observed in LPS-stimulated macrophages, where blocking the CLR signaling pathway resulted in the suppression of the phosphorylation of JNK but not p38 or ERK [57]. Furthermore, the recently observed link between ERK phosphorylation and ICAM1 up-regulation [58] can be used to better understand the absence of any effect of CLR blocking on the expression level of ICAM1. CLR modulation has been associated recently with impairment of NF-κB activation in LPS-stimulated DCs, suggesting the involvement of alternative signaling pathways to TLR4 in LPS challenge [59].

Syk is a ubiquitous nonreceptor tyrosine kinase with diverse biological activities [60]. It is involved in the signaling from BCRs and TCRs, FcRs, and CLRs, including Dectin-1 [29, 61]. Syk binds to the phosphorylated intracellular domain of the CLR and mediates signaling that results in transcription factor activation of cytokine secretion in DCs [62]. In macrophages, blocking of Syk suppressed LPS-induced activation of the MAPK pathway [57]. Interestingly, in our experiments, blocking of Syk had conflicting effects on TNF-α and IL-6 secretion by BP-stimulated mast cells. These results could be explained with the diverse function of Syk in immune cells. Recently, it was reported that Syk binds to TLR4 and plays a role in the response to bacterial pathogens or LPS in DCs and macro-

phages [60, 63, 64], leading to proinflammatory cytokine secretion. On the other side, Syk is also activated at different stages of the CLR signaling pathway, resulting in activation or inhibition of the TLR signaling pathway, and affects cellular responses, such as migration, adhesion, differentiation, or inflammatory and regulatory mediator release. The interaction of Syk with different receptors has yet to be identified [29].

To simplify our system focusing on the most probable ligands of CLRs and TLRs [65], LOS was isolated from heat-inactivated BP and used in mast cell stimulation assays. LOS is usually a potent inducer of TLR4-mediated signaling, resulting in the release of proinflammatory cytokines [14, 42]. In our study, the LOS-induced cytokine secretion by mast cells was suppressed by blocking MR and MGL, thus supporting the hypothesis of CLR involvement in LOS immune-cell stimulation. Notably, LOS stimulation of mast cells resulted in lower cytokine levels compared with the levels obtained with heat-inactivated, whole-cell BP. This is in agreement with previously reported in vitro stimulation of macrophages with BP LOS, resulting in reduced levels of TNF-α and IL-6 secretion [14]. All of these data suggest the synergistic action of *B. pertussis* LOS on TLRs and CLRs, together with other toxins during infection [65].

Human MGL has been reported to exhibit a narrow binding specificity toward terminal GalNAc moieties, whereas in mice, two distinct gene products, mMGL1 and mMGL2, show distinct carbohydrate-recognition profiles with affinity toward Lewis X [Galβ1-4(Fucα1-3)GlcNAc] and GalNAc, respectively [66]. To date, mMGL-1 and -2 have been reported to be expressed by mouse macrophages [67] and DCs [52]; this study represents the first report of MGL expression in PCMCs and BMMCs. As a result of the high sequence homology (91.5% amino acid identity) between mMGL-1 and -2, the ER-MP23 clone, used in this study, by binding to both lectins, does not allow for the identification of the specific lectin involved in the BP-mast cell interactions. However, the inhibitory effect

on cytokine secretion observed in the presence of GalNAc indicates a mMGL-2-type glycan-binding specificity.

Notably, the reported LOS structure of BP Tohama I, used in this study, does not feature any terminal GalNAc residues [68], and it has to be speculated that mast cell-derived MGL exhibits alternative glycan-binding specificities, or the LOS used in this study contains additional saccharidic epitopes not characterized previously. The latter hypothesis is supported by studies conducted on Ng LOS. Ng exhibits different LOS phenotypes, each targeting a different set of receptors on DCs; in particular, Ng LOS phenotype C carries a terminal GalNAc epitope and was the first bacterial ligand ever described for human MGL on DCs [67]. The identification of the potential binding epitopes on BP-derived LOS for the MR is somewhat less challenging as a result of the broad range of ligands reported for this CLR. MR can recognize the terminal GlcNAc on BP LOS through its eight tandemly arranged C-type lectin-like domains, with reported affinity for mannose, fucose, and GlcNAc [68].

Whole-cell vaccine studies suggest that BP-derived LOS plays a crucial role in the development of polarized Th1 immune responses, and signal through TLR4 is critical in innate immune cells in the generation of inflammatory cytokines in *B. pertussis* infection [42, 69]. Our study suggests that other than TLR4, CLRs are also involved in BP activation via the LOS antigen, and in the development of new vaccines, carbohydrate moieties could prove to be useful in the activation of innate immune cells and the promotion of these inflammatory immune responses. In summary, we showed that MR and MGL are involved in the interaction of BP with mast cells, and the LOS in the cell wall displays similar interactions. This sheds further light on mast cell biology and the role of mast cells in the defense system against bacterial infections. Bacterial-induced TLR signaling is well-studied, although less is known about CLRs and their crosstalk with other signaling pathways. Further studies are required to examine CLR involvement in bacteria recognition and its stimulatory/inhibitory effect on TLR signaling.

AUTHORSHIP

K.V.V. performed the experimental design, experiments (Figs. 1–8), interpretation of data, and writing and revising of the manuscript. A.R. provided the LOS preparation (Fig. 8A) and writing and revising of the manuscript. A.M.A. did the experiment (Fig. 5) and revised the manuscript. S.M.O. performed the experimental design, interpretation of data, and writing and revising of the manuscript.

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DISCLOSURES

The authors declare no conflict of interest.

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KEY WORDS:

C-type lectin · cytokine · ICAM1 · ERK · NF- κ B



Fasciola hepatica tegumental antigens indirectly induce an M2 macrophage-like phenotype *in vivo*

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SUMMARY

The M2 subset of macrophages has a critical role to play in host tissue repair, tissue fibrosis and modulation of adaptive immunity during helminth infection. Infection with the helminth, *Fasciola hepatica*, is associated with M2 macrophages in its mammalian host, and this response is mimicked by its excretory-secretory products (FhES). The tegumental coat of *F. hepatica* (FhTeg) is another major source of immune-modulatory molecules; we have previously shown that FhTeg can modulate the activity of both dendritic cells and mast cells inhibiting their ability to prime a Th1 immune response. Here, we report that FhTeg does not induce Th2 immune responses but can induce M2-like phenotype *in vivo* that modulates cytokine production from CD4⁺ cells in response to anti-CD3 stimulation. FhTeg induces a RELM α expressing macrophage population *in vitro*, while *in vivo*, the expression of Arg1 and Ym-1/2 but not RELM α in FhTeg-stimulated macrophages was STAT6 dependent. To support this finding, FhTeg induces RELM α expression *in vivo* prior to the induction of IL-13. FhTeg can induce IL-13-producing peritoneal macrophages following intraperitoneal injection. This study highlights the important role of FhTeg as an immune-modulatory source during *F. hepatica* infection and sheds further light on helminth–macrophage interactions.

Keywords dendritic cells, eosinophils, *Fasciola hepatica*, IL-13, M2 macrophages, STAT6

INTRODUCTION

The liver fluke, *Fasciola hepatica*, causes fascioliasis, a major global food-borne zoonosis infecting both humans and animals (1). The helminth can survive within its hosts for many years due to its ability to manipulate host immunity (2), in particular the suppression of Th1 immune responses as this is associated with immune protection in the definitive host (3). Infection gives rise to a polarized Th2 response that is characterized by the production of IL-4, IL-5 and IL-13 with the absence of IFN- γ (4, 5). During *F. hepatica* infection, the immediate effect on innate immune cells ultimately results in the polarization of Th2 immune response in concert with inhibiting the ability of these innate immune cells to promote Th1 immunity (5). For example, *F. hepatica* infection inhibits the development of classically activated macrophages (M1) which facilitates the switch to alternative activation (M2). In addition, dendritic cells and mast cells are hyporesponsive to activation by Th1 inducing microbial ligands (6, 7).

During *F. hepatica* infection, there are two major sources of modulatory antigens; flukes shed their outer tegumental coat (FhTeg) every 2–3 h and continuously release excretory/secretory products (FhES). Both of these antigenic mixes are in constant contact with cells of the hosts immune system and elicit immune-modulatory effects (5–7). We are particularly interested in examining the modulatory properties of FhTeg and have shown that FhTeg can inhibit LPS driven sepsis in mice by preventing the release of the pro-inflammatory mediators, nitric oxide, IL-6, TNF and IL-12 *in vivo* (5, 6). FhTeg impacts upon dendritic cell (DC) and mast cell maturation and function by inducing phenotypes that are hyporesponsive to TLR and non-TLR ligands, with decreased production of a panel of pro-inflammatory cytokines and reduced expression of costimulatory markers (6, 7), thus impairing both DCs and mast cells ability to prime T cells. FhTeg induces SOCS3 expression, a negative regulator of the

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TLR signalling pathway, which explains the suppression of NF κ B and the MAPKs, ERK, p38 and JNK in these cell populations (8). The interaction with mast cells occurs in a STAT6 independent manner suggesting that a Th2 environment is not required for mast cell–FhTeg interactions (9).

Like mast cells and DCs, macrophages are also an important component of the innate immune response and have the capacity to promote the differentiation of T cells. During infection with *F. hepatica* or exposure to FhES, peritoneal macrophages exhibit an M2-like phenotype as measured by the gene expression of Ym-1/2, Arg1 and RELM α while simultaneously demonstrating a lack of M1-associated genetic markers (5, 10). The induction of M2 macrophages is thought to be a necessary functional switch from killing to repair, ultimately benefiting both the parasite and host. Functionally, these macrophages are unable to support the differentiation of type 1 cells from naive CD4⁺ T cells and instead have been shown to promote the differentiation of Th2 and Treg cells (10, 11). Considering the modulatory effect that FhTeg displayed on mast cells and DCs, this paper examines whether FhTeg also alters the phenotype and biological function of macrophages.

MATERIALS AND METHODS

Animals and antigens

C57BL/6, BALB/c and STAT6^{−/−} mice 6–8 weeks old were purchased from Charles River (Carrentrilla, Ireland). 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.

BALB/c, C57BL/6 or STAT6^{−/−} mice were injected intraperitoneally (i.p.) three times per week for 1 or 3 weeks with *F. hepatica* excretory-secretory products [FhES: 20 μ g per mouse in PBS (200 μ L)] or tegumental coat antigen [FhTeg; 20 μ g per mouse in PBS (200 μ L)] was prepared as previously published (6). Prior to administration, endotoxin levels of antigens were determined using the Pyrogene endotoxin detection system (Cambrex). Antigen concentrations were determined by optimizations assays which are performed for each new antigen batch and the following concentrations were used for the *in vitro* experiments: FhES (20 μ g/mL) and FhTeg (10 μ g/mL). FhES and FhTeg gave endotoxin levels similar to background levels and were less than the lower limit of detection in this

assay (<0.01 EU/mL). All protein concentrations were determined using a BCA protein assay kit Thermo Scientific, Rockford, IL, USA.

Purification of macrophages from peritoneal exudate cells (PECs)

Peritoneal exudate cells (PECs) were obtained from each mouse by injecting 10 mL of ice cold sterile PBS into the peritoneal cavity. Cell numbers and viability were monitored using Trypan blue staining. Macrophages were isolated from PECs by culturing them in RPMI containing 10% FCS for 2 h. Adherent cells represented our macrophage population which were >95% positive for the expression of F4/80⁺ as determined by flow cytometry.

Coculturing of macrophages with CD4⁺ cells

Following stimulation with antigens, peritoneal macrophages were incubated with naive splenic CD4⁺ T cells at a ratio of 1 : 4 on a plate precoated with 0.5 μ g/well of anti-CD3 (e-Bioscience, Hatfield, UK). CD4⁺ T cells were isolated using MACS CD4⁺ T-Cell Isolation Kit (MiltenyiBiotec, Surrey, UK). Cocultures were maintained at 37°C and 5% CO₂ for 72 h when supernatants were taken, and IFN- γ , IL-4, IL-13 and IL-5 were then measured by commercial ELISA (BD Biosciences, Oxford, UK).

Determination of arginase activity

After lysis of 1×10^6 macrophages with Triton X-100 and with L-arginine as a substrate, the arginase activity was determined as previously described (5) (Donnelly 2008). One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 μ mol of urea per min.

RNA extraction and RT-PCR

Total RNA was extracted from cultured cells using TRI-sure (MyBio, Kilkenny, Ireland) as recommended by the manufacturers instructions. First strand cDNA was synthesized with random primers from 1 μ g total RNA using GoScript Reverse Transcription System (Promega, Madison, WI, USA.) 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. Each amplification step was preceded by a denaturation phase at 95°C for 5 min and preceded by a final extension phase of 72°C for 5 min. PCR products were electrophorized on 1% agarose gels with SYBRSafe (Invitrogen, Paisley, UK) as our gel stain. The primer sequences and amplification steps were as previously described (5, 10).

Cytokine assays

Spleen cells were removed for restimulation *in vitro* with PBS (negative control), FhTeg (10 µg/mL) or FhES (20 µg/mL) or PMA (25 ng/mL)/anti-CD3 (1 µg/mL; positive control). After 72 h, supernatants were collected and the levels of IFN-γ, IL-4, IL-13 and IL-5 quantified by ELISA (BD Biosciences). Supernatant was obtained from FhTeg-stimulated macrophages for measurement of IL-4 and IL-13 by ELISA (BD Biosciences).

Flow cytometry

Monoclonal antibodies with fluorescent tags were used which targeted the following cell surface markers: PE-conjugated CD11b (BD Biosciences), FITC-conjugated F4/80 (Biolegend, San Diego, CA, USA), efluor 660 conjugated IL-13 and PE-conjugated Siglec-f (e-Bioscience Ltd). Cells were blocked for 15 min with anti-CD16/CD32 (Fcγ III/II Receptor) prior to incubation with fluorescent antibodies. Antibody incubations were performed in washing buffer (PBS containing 2% FCS, 5 mM EDTA and 0.05% NaN₃). Isotype controls corresponding to the above antibodies were used to rule out nonspecific binding. Acquisition was performed using a FACSARIA cell sorter (BD Immunocytometry Systems, Oxford, UK), and data were analysed using FLOWJO software (Treestar, Ashland, OR, USA).

Statistics

All data were analysed for normality prior to statistical testing by ORIGIN[®] 6.1 software (OriginLab Corporation, Northampton, MA, USA). Where multiple group comparisons were made, data were analysed using one-way ANOVA. For comparisons between two groups, the Students *t*-test was used. In all tests, $P < 0.05$ was deemed significant. Where data was not normal, we normalized the data and measured significant differences based upon an analysis of variance using a *post hoc* Duggans test.

RESULTS

FhTeg induces a phenotype of macrophages *in vivo* that express M2 markers

Fasciola hepatica infection induces the expression of genetic markers within peritoneal macrophages that are characteristic of an M2 phenotype: Arg-1, RELMα and Ym-1/2. Furthermore, exposure of mice to the secretory products (FhES) of *F. hepatica* also results in enhanced expression of the same M2-associated genes (5). Consider-

ing the likely interaction that occurs between FhTeg and macrophages during infection, we asked whether, like FhES, FhTeg also contained molecules capable of inducing an M2 macrophage phenotype. Thus, BALB/c mice ($n = 4$) were injected three times per week over a 3 weeks period with either PBS, FhES (20 µg/mL), or FhTeg (10 µg/mL) and Arg-1, RELMα and Ym-1/2 gene expression measured by RT-PCR in isolated peritoneal macrophages. In comparison with the PBS-treated mice, macrophages isolated from both FhES- and FhTeg-treated mice expressed Arg-1, RELMα and Ym-1/2 while neither antigen induced iNOS expression, a marker of classically activated macrophages (Figure 1).

FhTeg-activated macrophages suppress cytokine secretion from CD4⁺ T cells

There have been many hypotheses to the role that helminth-induced M2 macrophages might play during infection such as modulating of T-cell function (12). To define a function for FhTeg-M2 macrophages, we assessed their ability to modulate the activation of T cells *in vitro*. Peritoneal macrophages were isolated from mice ($n = 4$) treated with either PBS, FhES or FhTeg as described above and then cocultured, in the presence of anti-CD3, with CD4⁺ spleen cells harvested from naïve mice. As expected, T cells cultured in the presence of macrophages isolated from PBS-treated mice secreted significant levels of IFN-γ ($P \leq 0.001$; Figure 2a), IL-13 ($P \leq 0.001$; Figure 2b), IL-4 ($P \leq 0.001$; Figure 2c) but not IL-5 (Figure 2d) in response to stimulation with anti-CD3 when compared to T cells stimulated with anti-CD3 only. In comparison with the PBS-control samples, the addition of macrophages isolated from both FhES- and FhTeg-treated mice caused

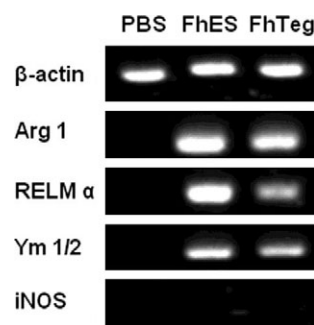


Figure 1 FhTeg induces a phenotype of macrophages *in vivo* that express M2 markers: Mice were injected three times per week for 3 weeks with PBS, FhES (20 µg per mouse) or FhTeg (10 µg per mouse). Total RNA was extracted from isolated peritoneal macrophages and RT-PCR performed for the gene markers Arg 1, RELM α, Ym-1/2 and iNOS. β-actin was measured as a reference gene. Data are representative of one mouse of four mice per group, and the experiment was repeated twice.

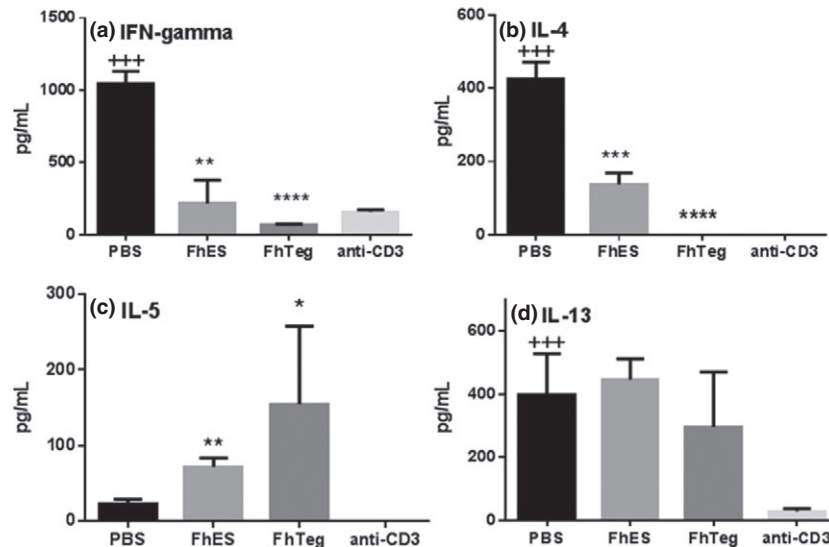


Figure 2 FhTeg-activated macrophages suppress cytokine secretion from CD4⁺ T cells. Mice were injected three times per week for 3 weeks with PBS, FhES (20 µg per mouse) or FhTeg (10 µg per mouse). Isolated peritoneal macrophages were cocultured with naïve CD4⁺ T cells at a ratio of 1 : 4 on plates precoated with anti-CD3 (0.5 µg/well). After 72 h, supernatants were taken and analysed by ELISA for IFN-γ (a), IL-4 (b), IL-5 (c) and IL-13 (d). Data are representative of four mice per group, and the experiment was repeated three times; +++ $P \leq 0.001$ compared with T cells stimulated with anti-CD3 only; * $p \leq 0.05$, ** $P \leq 0.01$; *** $P \leq 0.001$, **** $P \leq 0.0001$ compared with PBS controls.

significant decreases in the secretion of both IFN-γ (Figure 2a FhES $P \leq 0.01$, FhTeg $P \leq 0.0001$) and IL-4 (Figure 2b: (FhTegES $P \leq 0.001$, FhTeg $P \leq 0.0001$). T cells cocultured with macrophages isolated from FhES- and FhTeg-treated mice induced a significant increase in IL-5 (FhTegES $P \leq 0.01$, FhTeg $P \leq 0.05$), while no significant changes were observed from the mean production of IL-13 from T cells cocultured with macrophages from FhES- and FhTeg-treated mice compared with macrophages from PBS-treated mice.

FhTeg does not induce antigen-specific Th2 immune responses *in vivo*

Mice infected with *F. hepatica* metacercariae induce strong Th2 immune responses as characterized by the presence of IL-4, IL-5 and IL-13 with no antigen-specific IFNγ detected (O'Neill et al., 2000 (4)), and these responses can be mimicked by mice treated with FhES (10). Using FhES as a control, we injected BALB/c mice with PBS, FhES (20 µg) or FhTeg (10 µg) three times per week for 3 weeks and spleen cells were isolated for restimulation with PBS, FhTeg (10 µg/mL) or FhES (20 µg/mL) and PMA (25 ng/mL)/anti-CD3 (1 µg/mL). FhES injected mice produced significant levels of FhES-specific IL-4 ($P \leq 0.001$), IL-5 ($P \leq 0.01$) and IL-13 ($P \leq 0.001$) but not IFNγ cytokines (Figure 3), while spleen cells from FhTeg injected mice failed to produce IFN-γ, IL-4, IL-5 and IL-13 cytokines in response to FhTeg (Figure 3). PBS-stimulated spleen cells did not produce

cytokines, while cells stimulated with PMA/anti-CD3 secreted all cytokines tested.

FhTeg does not directly induce an M2-like macrophage phenotype

We then addressed the possibility that like FhES, FhTeg might directly interact with macrophages and induce the expression of M2 genetic markers including the secretion of IL-10 and PGE2. To investigate this, murine macrophages (RAW 264.7) were stimulated again with FhTeg (10 µg) and a positive and negative control for measurement of cytokine secretion and arginase activity. Here, FhTeg did not directly induce the secretion of IL-10 (Figure 4a), PGE2 (Figure 4b), the expression of Arg-1 (Figure 4c) or the production of arginase (Figure 4d).

FhTeg-activated macrophages are partially STAT6 dependent

Most typically, previous studies into the activation of M2 macrophages during helminth infection have suggested that they are primarily induced by the Th2-type cytokines IL-4 and IL-13 (10, 13, 14) and given that FhTeg does not induce Th2 cytokines we went on to determine a role for IL-13 and IL-4 in this process by performing these studies in STAT6^{-/-} mice. STAT6^{-/-} (or C57BL/6 background control) mice were given our standard regime of 3 × 3 intraperitoneal injections of FhES, FhTeg or PBS as a

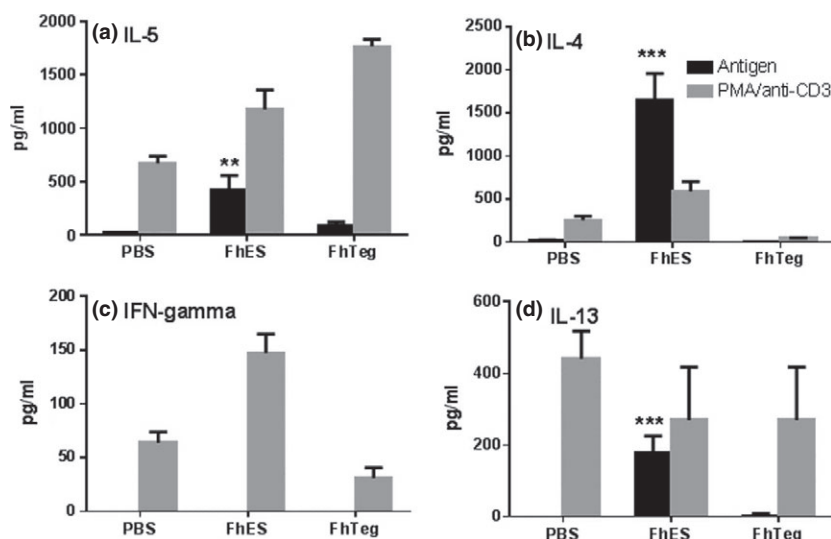


Figure 3 FhTeg does not induce antigen-specific Th2 immune responses *in vivo*: Balb/c mice were injected three times per week for 3 weeks with PBS, FhES (20 µg per mouse) or FhTeg (10 µg per mouse). Spleen cells were removed and plated at 2×10^6 /mL for restimulation *in vitro* with PBS, FhES (20 µg/mL) or FhTeg (10 µg/mL) and PMA (25 ng/mL)/anti-CD3 (1 µg/mL). After 72 h, spleen cell supernatants were analysed by ELISA (BD Biosciences) for IFN- γ (a), IL-4 (b), IL-5 (c) and IL-13 (d). Data are the mean (\pm SEM) of three individual wells for four individual mice and are representative of two experiments, **, $P \leq 0.01$; ***, $P \leq 0.001$ compared with controls.

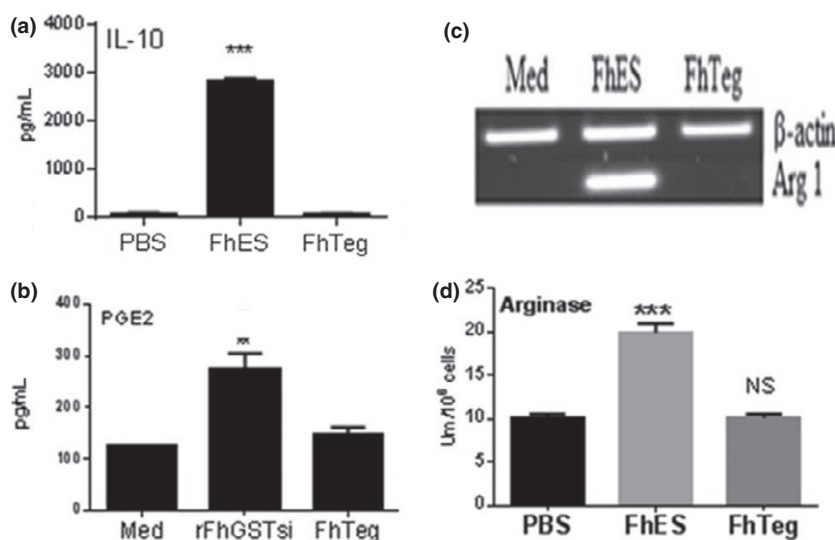


Figure 4 FhTeg does not directly induce an M2-like macrophage phenotype. RAW 264.7 macrophages and BMDMs were stimulated with PBS, FhES (20 µg/mL) or FhTeg (10 µg/mL). After 18 h, total RNA was extracted and Arg-1 and β -actin measured by RT-PCR and cells lysed for measurement of arginase activity. Supernatant was removed for measurement of IL-10 by ELISA (BD Biosciences). Data are the mean (\pm SEM) of three individual wells and are representative of two experiments, **, $P \leq 0.01$; ***, $P \leq 0.001$ compared with controls.

control. Both IL-4 and IL-13 activate M2 macrophages using STAT6 signalling pathways, and it has been shown that T cells from STAT6 $^{-/-}$ mice lack the ability to produce either IL-4 or IL-13. Analysis of peritoneal macrophages harvested after the final treatment of parasite antigens revealed that in the absence of STAT6, FhES failed to induce the expression of any of the M2 markers

examined (Figure 5a). In contrast, macrophages isolated from FhTeg-treated mice showed increased expression of RELM α but not Arg-1 or Ym-1/2 (Figure 5a). In agreement with this RT-PCR result, a decrease in arginase activity was observed in STAT6 $^{-/-}$ mice for both FhES ($P \leq 0.05$; Figure 5b) and FhTeg ($P \leq 0.001$; Figure 5d). These results indicate that each of the expression of M2

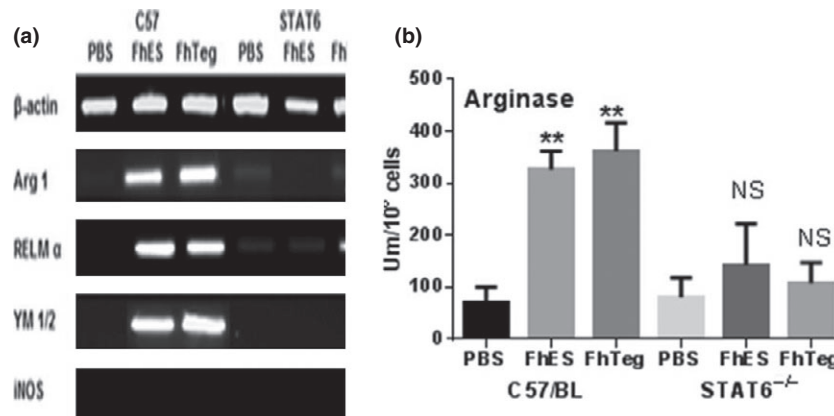


Figure 5 FhTeg-activated macrophages are partially STAT6 dependent: C57BL/6 (background strain) and STAT6^{-/-} mice were injected three times per week for 3 weeks with PBS, FhES (20 µg) or FhTeg (10 µg) and peritoneal macrophages isolated for measurement of Arg 1, Ym-1/2, RELMα, iNOS and β-actin gene expression by RT-PCR (a) or arginase activity (b). Data are representative of one of four mice per group, and the experiment was repeated twice; **, $P \leq 0.01$ compared with controls.

genotypic markers may be induced by different mechanisms, and at least, in the case of Arg-1 and Ym-1/2, the induction of expression by FhTeg is undoubtedly dependent upon STAT6 signalling mechanisms.

RELMα gene expression is observed prior to the expression of IL-13, while FhTeg can induce RELMα but not IL-13 directly from macrophages *in vitro*, *in vivo* it is also associated with IL-13 producing macrophages

As RELMα gene expression was observed in a stat/6 dependent manner, we went on to examine the expression of M2 markers and IL-4/IL-13 in the first 24 h following FhTeg injection. Here, mice were injected with FhTeg intraperitoneally and after 1, 6 and 24 h peritoneal exudate cells were collected for measurement of IL-4, IL-13, Arg-1, Ym-1/2 and RELMα gene expression by RT-PCR. Following FhTeg injection, RELMα was expressed at 1 h post-injection, while IL-13 and Arg-1 were detected at 6 h. FhES injection induced Arg1 expression at 1 h post-injection, while IL-13 and RELMα were expressed at 5 h. There was no expression of Ym-1/2 and IL-4 detected at any time point examined (Figure 6a).

As IL-13 is an early source of a Th2 cytokine following FhTeg injection, we examined the expression of IL-13 in peritoneal macrophages as these were previously shown to be good innate source of this cytokine (12). Peritoneal cavity cells were isolated following three i.p. injections of FhTeg over 7 days. On day 8, the total number of macrophages in the peritoneal cavity of FhTeg-treated mice was significantly increased ($P \leq 0.01$; Figure 6a) compared with mice receiving PBS. Furthermore, the number of these cells expressing intracellular IL-13 was also significantly enhanced ($P \leq 0.01$; Figure 6b). We then went on

to demonstrate that RAW macrophages (264.7) stimulated with FhTeg (10 µg/mL) induced RELMα gene expression, while it did not directly stimulate the secretion of IL-13 from these cells.

DISCUSSION

The tegumental coat of *F. hepatica* (FhTeg) is shed from the fluke every 2–3 h and is therefore a major interface between the parasite and its environment. In the adult fluke, the tegument is 15–20 µm thick and the surface is increased by intuckings of the plasma membrane (apical invagination). The apical membrane is covered by thick (30 + 25 nm) glycocalyx (15) which contains a rich source of glycoproteins. The tegument has multiple functions like absorption of exogenous nutrients, synthesis and secretion of various substances, osmoregulation and protection against enzymes, the bile and hosts immune responses (15). An intact glycocalyx and syncytium is essential to the survival of the fluke in the host (15). We have previously characterized FhTeg as a source of immune modulators. FhTeg-stimulated DCs and mast cells are both hyporesponsive to TLR ligand activation (6, 7), and FhTeg-treated DCs and mast cells are unable to prime T cells (6, 7). Consistently, this study demonstrates that FhTeg also modulates the activity of macrophages, inducing an M2-like phenotype that modulates cytokine secretion from CD4⁺ cells.

Both FhTeg- and FhES- activated macrophages suppress IFNγ from CD4⁺ cells which is one of the key modulatory properties displayed during *F. hepatica* infection. The importance of Th1 suppression during infection is critical to the parasites survival as these responses are associated with host protection as demonstrated in studies

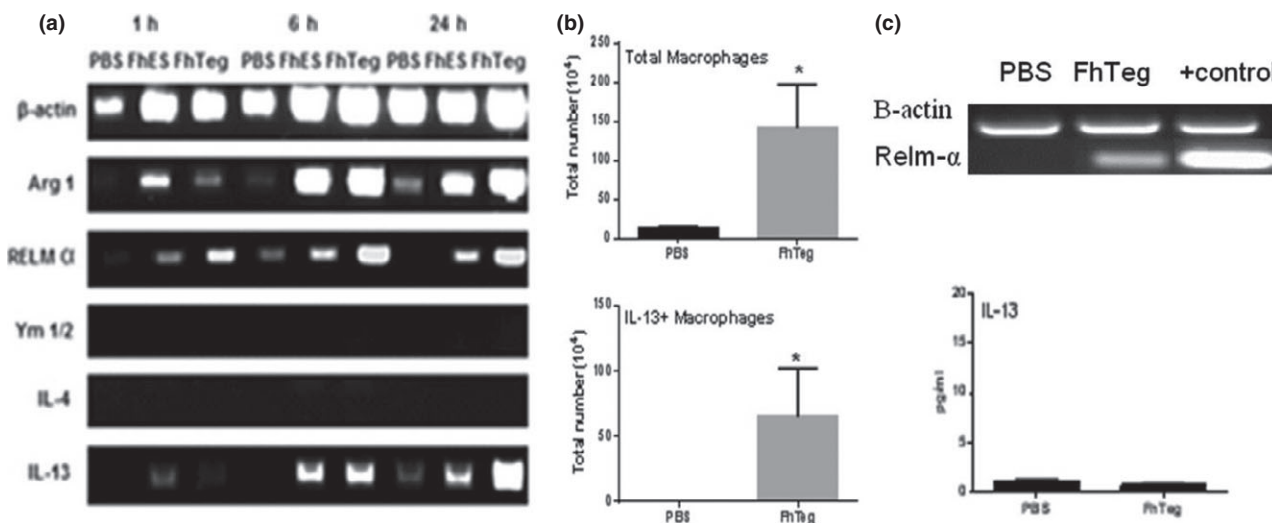


Figure 6 FhTeg induces RELM α gene expression prior to IL-13 expression and is associated with IL-13 producing macrophages, although it can induce RELM α but not IL-13 directly from macrophages *in vitro*. (a) BALB/c mice were i.p. injected with either PBS, FhES (20 μ g per mouse) or FhTeg (10 μ g per mouse) and peritoneal exudate cells (PECs) removed after 1, 6 and 24 h for RNA extraction. RT-PCR was performed for Arg1, Ym-1/2, RELM α , IL-4, IL-13 and β -actin as a reference gene. Data are representative of one of three mice per group and the experiment repeated twice. (b) BALB/c mice were i.p. injected with either PBS, FhES (20 μ g per mouse) or FhTeg (10 μ g per mouse). After 7 days, PECs were isolated and analysed by flow cytometry for intracellular IL-13 in macrophages. Data are representative of four mice per group, and the experiment was repeated twice; *, $P \leq 0.05$ compared with controls. (c) RAW 264.7 macrophages were stimulated with PBS, FhES (20 μ g/mL) or FhTeg (10 μ g/mL). After 18 h, total RNA was extracted and RELM α and B-actin measured by RT-PCR. Supernatant was removed for measurement of IL-13 by ELISA (BD Biosciences). Data are the mean (\pm SEM) of three individual wells and are representative of two experiments, * $P \leq 0.05$; compared with controls.

with cattle and sheep (3, 16). Studies in experimental models support this observation as IL-4 $^{-/-}$ mice, which are predisposed towards a Th1 immune response, are less susceptible to *F. hepatica* infection with reduced liver damage compared with IFN- γ R $^{-/-}$ mice that elicited strong Th2 responses and are highly susceptible to infection (4). The efficacy of *F. hepatica* vaccine is also associated with enhanced lymphocyte proliferation and IFN- γ expression (3). A number of *F. hepatica* secretory enzymes have been identified and recombinantly expressed, and these molecules mimic the Th1 inhibitory properties observed during *F. hepatica* infection (17, 18). The parasite through the release of these molecules suppress Th1 immune responses within hours post-infection, and this phenomenon can be seen in both innate and adaptive effector cells (5–7). Likewise, during *F. hepatica* infection, M2-like macrophages are observed and these cells are capable of suppressing IFN γ from CD4 $^{+}$ cells.

In conjunction with the suppression of Th1 immune cells, *F. hepatica* infection is associated with the early induction of Th2 immune responses. Here, we demonstrate that FhTeg-activated macrophages promote the secretion of IL-5 from CD4 $^{+}$ cells. In contrast to FhES, it fails to induce antigen-specific Th2 immune responses. FhES is an important antigen source in the promotion of a Th2 type immune response during infection; we believe that FhTeg

shares this property by supporting FhES in promoting a favourable environment for the promotion of Th2 immune responses. In support of this hypothesis, FhTeg induces the early expression of IL-13 and induces an IL-13 expressing macrophage population previously associated with Th2 mediated diseases (12). IL-13 mediates localized tissue effects such as chemokine secretion, goblet cell hyperplasia, mucus production and smooth muscle alterations (19, 20). IL-13 administered intranasally to mice promotes eosinophil activation and survival through a direct stimulation of eotaxin and RANTES production (21, 22). IL-13 is also critical for the expulsion of intestinal worms and for the induction of M2 macrophages that promote granuloma formation and tissue fibrosis during helminth infection. (23). It is possible that the early induction of IL-13 induced by FhTeg works locally promoting the differentiation of M2 macrophages which could contribute to the liver fibrosis observed during *F. hepatica* infection.

Our data indicate that signalling through STAT6 is required to induce the expression of Arg1 and Ym-1/2, as these markers were not observed in STAT6 $^{-/-}$ mice treated with FhTeg. Signal transducers and activators of transcription (STATs) are a family of transcription factors that activate gene transcription of factors such as cytokines and that IL-4/IL-13 both specifically activate STAT6 through the IL-4R (24). STAT6 $^{-/-}$ T cells are

unable to differentiate into IL-4 and IL-13 producing Th2 cells *in vitro* or *in vivo* and these cytokines are critical to the development of M2 macrophages (25). Therefore, while FhTeg can induce partial activation of M2 phenotype in the absence of IL-13/IL-4, like other helminths, they cannot induce the full complement of M2 markers (12). During *Schistosoma mansoni* infection, STAT6^{-/-} mice were unable to mount a Th2 immune response to soluble egg antigen (SEA) (26) and STAT6^{-/-} mice also fail to develop airway hyper-responsiveness after Th2-allergen provocation (27). Similarly, we did not observe antigen-specific Th2 immune responses in STAT6^{-/-} mice injected with FhES (unpublished data).

STAT6^{-/-} mice treated with FhTeg expressed RELM α gene, and FhTeg can also induce RELM α prior to IL-13 gene expression and directly from macrophages *in vitro* which supports this finding. RELM α is a family member of cysteine-rich secretory proteins that is highly expressed during helminth infection and allergy (19). RELM α has an important regulatory role during helminth infection protecting mice against gastrointestinal infection (28) and acting as a negative regulator of Th2 responses modulating granuloma formation, tissue fibrosis and exacerbated Th2 cytokine secretion during *S. mansoni* infection (29, 30). In support of our findings, a recent study in IL-13 receptor 1 deficient mice concluded that IL-13 is not required for gene expression of RELM α in the liver during *S. mansoni* infection (31). In this study, the appearance of RELM α in the absence of STAT6 and prior to IL-13 expression would support this finding, while the expression of RELM α within the peritoneal exudate cells could be attributed to a number of cellular sources such as eosinophils, B cells and macrophages. Our data clearly demonstrate that FhTeg induces expression of RELM α directly in macrophages and therefore represents an important source of this molecule. Furthermore, it would be interesting to examine FhTeg driven immune-modulatory

properties in the absence of RELM α as it is known to limit Th2 responses in the *Schistosoma* egg model of pulmonary inflammation (29), particularly because FhTeg does not drive antigen-specific Th2 immune responses.

In this study, while FhTeg and FhES both induce M2-like macrophages *in vivo*, there are a number of differences observed between the two antigenic sources. FhES can directly induce an Arg1⁺, IL-10⁺ and PGE2⁺ macrophage population, while FhTeg induces a RELM α ⁺, IL-10⁻ and PGE2⁻ macrophage population. The differences between the two antigen sources are not surprising as FhES consists predominantly of enzymes released from the parasite gut, whereas FhTeg is a rich source of glycoproteins from its glycocalyx coat (15). While FhTeg can induce RELM α in the absence of STAT6, FhES can induce Arg1 prior to the expression of Th2 cytokines. There is now a range of molecules identified as capable of directly inducing an M2 phenotype (32), some of which have been identified within the secretions and homogenates of helminth parasites (10). We have yet to identify this molecule in FhTeg; however, FhES contains a peroxiredoxin that can directly interact with macrophages to induce an M2-like phenotype (5, 10).

M2 macrophages are central to the immune response during helminth infection, and the findings in this study provide us with insight into the interaction between *F. hepatica* tegumental antigens and macrophages. While *F. hepatica* tegumental coat shares many properties with FhES, there are also differences in their interaction with macrophages which points to the fact that these antigen sources may utilize alternative pathways ensuring redundancy in the process of macrophage activation during infection. While this study sheds further light on the importance of FhTeg interaction with innate immune cells during *F. hepatica* infection, further work is needed to fully understand the role of FhTeg-macrophage interactions during *F. hepatica* infection.

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