The immunomodulatory properties of helminth-derived products on dendritic cell maturation and function

David J. Dowling

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A thesis submitted for the degree of Ph.D.

by

David J. Dowling, B.Sc.

Supervisor:

Dr. Sandra O'Neill, School of Nursing, Faculty of Science and Health, Dublin City University, Ireland.

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Abstract:

Parasitic worms and molecules derived from them drive Th2/Treg immune responses in mammalian hosts and have powerful anti-inflammatory properties which are shown to have therapeutic effects on inflammatory diseases. Using products derived from the helminth parasites *Fasciola hepatic*a and *Ascaris lumbricoides* we investigated the mechanism by which these responses are induced. Dendritic cells (DC's) play a crucial role in linking the innate to adaptive arms of the immune system and directing the subsequent immune responses. Here, we report that *Fasciola hepatic*a tegumental antigens (FhTeg) can suppress DC maturation and function. While FhTeg alone did not induce cytokine production or cell surface marker expression on DCs, it can maintain the DCs in an immature state, suppressing their function to Toll-Like-Receptor (TLR) stimulation and impairing the subsequent development of adaptive immunity. Furthermore, we selected recombinant forms of two major *F. hepatica* secreted molecules, the protease cathepsin L (rFhCL1) and anti-oxidant, sigma class glutathione S-transferase (rFhGST-si), to examine their interactions with DCs. Despite enzymatic and functional differences between these antigens they both induced IL-6, IL-12p40 and chemokine secretion from DCs in a TLR4 dependent manner. While neither helminth enzyme induced Th2/Treg immune responses both could instruct DCs that suppress IL-17 secretion *in vivo*. This study also demonstrates that another helminth-derived heterogeneous extract, *Ascaris lumbricoides* pseudocoelomic fluid (AlPCF), induced partial maturated of DCs as characterised by the increased production of IL-6, IL-12p40 and MIP-2. These DCs were capable of inducing Th2 responses. Overall the data suggests that helminth parasites secrete multiple molecules each possessing a unique mechanism of modulation, which can either suppress inflammatory Th1/Th17 responses or induce/permit the uninhibited development of modified Th2 responses.

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Abbreviations

µg:	Microgram (only with numbers)
µl:	Microliter (only with numbers)
0-3hRP:	Schistosoma larvae derived products
2-ME:	2-mercaptoethanol
AgB:	Antigen B
AlPCF:	*Ascaris lumbricoides* pseudocoelomic fluid
APC:	Ag-presenting cell
AsES:	*Ascaris suum* excretory/secretory antigens
BCA:	Bicinchoninic acid
BCR:	B cell receptor
BES:	*Brugia malayi* ES antigens
BMDC:	Bone-marrow derived dendritic cells
BSA:	Bovine serum albumin
CCR:	CC chemokine receptor
CD:	Cluster of differentiation
CpG:	Cytosine guanine dinucleotide
CTL:	C-type lectin
d:	Distilled (as in dH2O)
Da:	Dalton (only with numbers)
DC:	Dendritic cell
DiAg:	Dirofilaria immitis polyproteins
DMSO:	Dimethylsulfoxide
DNA:	Deoxyribonucleic acid
Ds:	Double-stranded (as dsDNA)
DTT:	Dithiothreitol

ECL:	Enhanced chemiluminescence
EDTA:	Ethylenediaminetetraacetic acid
ELISA:	Enzyme-linked immunosorbent assay
ERK:	Extracellular signal-regulated kinase
FACS:	Fluorescence-activated cell sorter
FBS:	Fetal bovine serum
FCS:	Fetal calf serum
FhES:	*Fasciola Hepatica* excretion-secretion
FhTeg:	*Fasciola Hepatica* tegumental coat
FITC:	Fluorescein isothiocyanate
g:	Gram (only with numbers)
G:	Gauge
GM-CSF:	granulocyte-macrophage CSF
GST:	glutathione S-transferase
h:	hours (use only with numbers)
HBV:	Hepatitis B virus
HCV:	Hepatitis C virus
HIV:	human immunodeficiency virus
HPBM:	Human peripheral blood mononuclear
HpES:	Heligmosomoides polygyrus ES antigens
HPLC:	High performance liquid chromatography
HRP:	Horseradish peroxidase
i.p.:	Intraperitoneal
IFN:	Interferon (e.g., IFN-g)
IκB:	Inhibitory NF-κB
IL:	Interleukin (e.g., IL-12)

ITAM:	Immunoreceptor tyrosine-based activation motif
ITIM:	Iimmunoreceptor tyrosine-based inhibitory motif
JNK:	c-Jun N-terminal kinase
LNFPIII:	Lacto-N-fucopentaose III
LPS:	Lipopolysaccharide
m.w:	Molecular weight
mAb:	Monoclonal Ab
MAPK:	Mitogen-activated protein kinase
MCP:	Monocyte Chemotactic Protein
Mg:	Milligram (only with numbers)
MHC:	Major histocompatibility complex
Min:	Minute (only with numbers)
MIP:	Macrophage-inflammatory protein
ml:	Milliliter (only with numbers)
mRNA:	Messenger RNA
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide
MyD88:	Myeloid differentiating factor 88
NaES:	Necator americanus ES antigens
ND:	Not determined
NES:	Nippostrongylus brasiliensis excretory/secretory antigens
NF:	Nuclear factor
NF-κB:	Nuclear factor kappa B
NO:	Nitric oxide
NS:	Not significant
OD:	Optical density
OVA:	Ovalbumin

P38:	P38 MAPK
PAGE:	Polyacrylamide gel electrophoresis
PBS:	Phosphate-buffered saline
PC:	Phosphorylcholine
PCF:	Pseudocoelomic fluid
PCR:	Polymerase chain reaction
PE:	Phycoerythrin
PerCP,	Peridinin chlorophyll protein
PG,	Prostaglandin
PGN:	Peptidoglycan
PI,	*Ascaris suum* high-molecular-weight components
PMA, acetate	Phorbol myristate
Poly (I:C):	Polyinosinsic:cytidylic acid
PRR:	Pattern recognition receptor
Prx:	Peroxiredoxin
PS:	Phosphatidylserine
r:	Recombinant, (e.g., rIFN-g)
RBC:	Red blood cell
rFC:	Recombinant Factor C
RNA:	Ribonucleic acid
rpm:	Revolutions per minute
s:	Second (use only with numbers)
s.c.:	Subcutaneous
SD:	Standard deviation
SDS:	Sodium dodecyl sulfate
SE:	Standard error

SEA:	*Schistosoma Mansoni* water-soluble egg antigen
SEM:	Standard error of the mean
TCR:	T cell receptor for Ag
TGF:	transforming growth factor
Th:	T helper cell
TLR:	Toll-like receptor
TNF:	Tumor necrosis factor
TNP:	trinitrophenyl
TPX:	Thioredoxin peroxidase
Tris:	Tris(hydroxymethyl)aminomethane
TsES:	*Trichinella spiralis* ES antigens
TsMLCr:	*Trichinella spiralis* crude muscle larvae antigen
Zym:	Zymosan A

Chapter 1: General Introduction

Helminth parasites infect 3 billion people worldwide (Hotez *et al*., 2008) and while associated with a low mortality rate, if one considers the high number of infections, they still cause millions of deaths worldwide (Hotez *et al*., 2008, Pullan and Brooker, 2008). Helminth infections are associated with a polarised T helper 2 (Th2)/T regulatory cell (Treg) host response and it is well documented that such polarisation leaves the host more susceptible to bystander infections (Maizels and Yazdanbakhsh, 2003). This bystander effect has been demonstrated by the ability of helminths to suppress hosts protective Th1 responses to bacterial (Brady *et al*., 1999, Sacco *et al*., 2002) viral (Wolday *et al*., 2002) and protozoa (Hartgers and Yazdanbakhsh, 2006) pathogens.

Current knowledge of the mechanism by which helminths influence host immune responses is limited, particularly their role in driving adaptive immunity (Maizels *et al*., 2004). Since dendritic cells (DCs) are crucial for bridging innate and adaptive immunity by controlling the initiation and differentiation of T helper cells (Banchereau and Steinman, 1998), there is a growing interest in understanding and defining the interactions between helminths and DCs (MacDonald and Maizels, 2008). Since helminth-derived molecules have been shown to mimic the immune response to helminth infections, the focus has turned to understanding the mechanisms of innate recognition of helminth antigens, particularly by DCs. In this thesis the interactions between molecules derived from *Fasciola hepatica* and *Ascaris lumbricoides* with DCs is studied.

1.6. What are parasitic helminths

The term ‘helminth’ is use to describe a group of wide ranging parasitic worm-like organisms mainly belonging to the phylum’s Platyhelminthes (flatworms) and Nematoda (round worms) (Roberts, 1996). Helminths are estimated to infect upwards of 3 billion people worldwide (Hotez *et al*., 2008). While ostensibly associated with high

morbidity and low mortality, helminth infections are still a causative effect of millions of deaths worldwide mainly in developing countries (Hotez *et al*., 2008, Pullan and Brooker, 2008). Trematodes, commonly referred to as "flukes", are a class within the phylum Platyhelminthes and contain the family Fasciolidae, which are large, leaf shaped parasites of mammals causing Fascioliasis (Dalton, 1999). Parasitical nematodes on the other hand conform to a slender cylindrical shape with bilaterally symmetrical, tapering at either end and are often referred to as "round worms" (Bradley and Jackson, 2004). Parasitical nematodes are usually small but some species can grow above 40 cm in length (Taylor, 2007). The following section gives a brief introduction and background to the trematode *F. hepatica* and the nematode *A. Lumbricoides,* as these parasitic helminths are the origin of the antigens of study in this thesis.

1.6.1. Helminth infections and life cycle

Fascioliasis is an important public health problem with increasingly prevalence in humans (WHO, 1995, Mas-Coma, 1999). Estimates of 2.4 - 17 million people are infected worldwide (Mas-Coma *et al*., 2005). The adult *F. hepatica* occupies the liver and bile duct of the host and has a life span of 9 to 13.5 years (Behm and Sangster, 1999). In both human and animals, pathogenesis is related to parasitic load (Mas-Coma *et al*., 2005). *F. hepatica* is also of major veterinary importance, causing economic losses of up to €2 billion globally due to infection of domestic animals such as cattle and sheep (Taylor, 2007). Two hosts are needed for the completion of *F. hepatica's* life cycle (Fig. 1.1). The intermediate hosts are predominantly freshwater snail species of the family Lymnaeidae (Gastropoda: Basommatophora) (Andrews, 1999, Taylor, 2007).

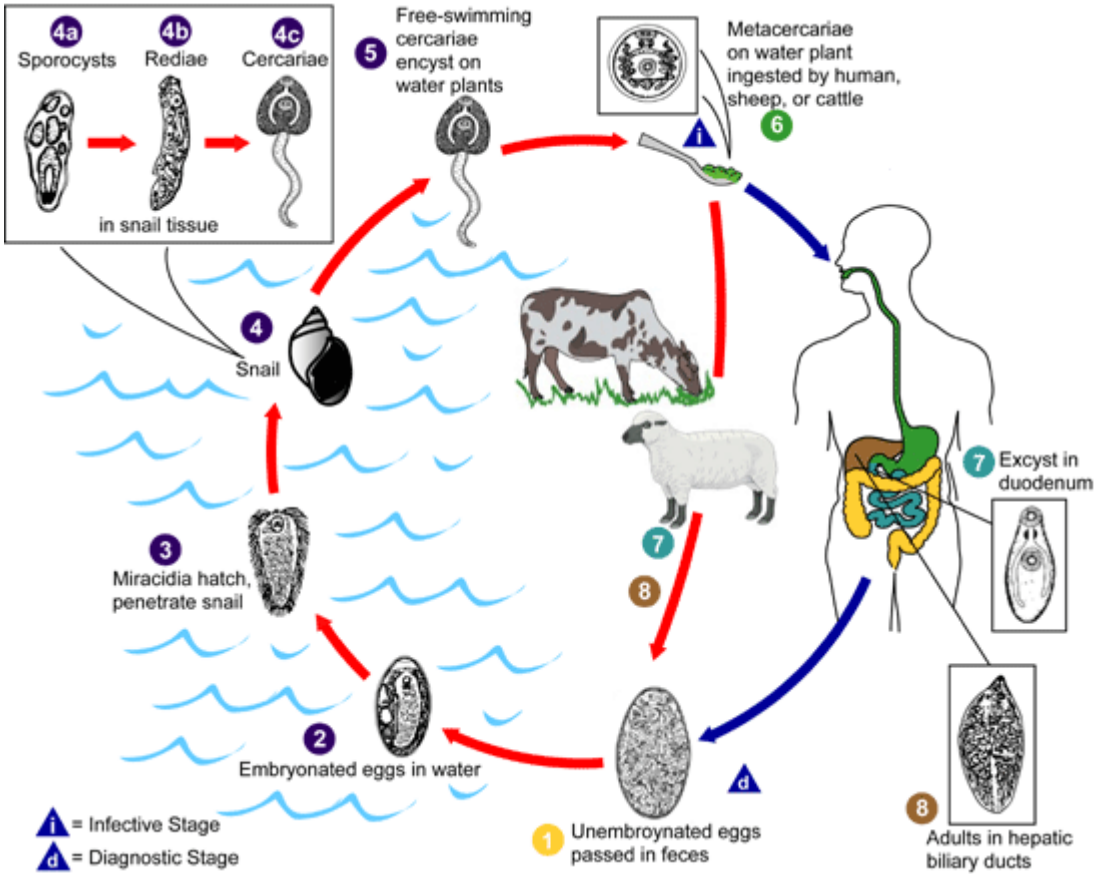

Figure 1.1: The life cycle of *Fasciola hepatica*. Immature eggs are discharged in the biliary ducts and in the stool 1. Eggs become embryonated in water 2, eggs release miracidia 3, which invade a suitable snail intermediate host 4. In the snail the parasites undergo several developmental stages (sporocysts 4a, rediae 4b, and cercariae 4c). The cercariae are released from the snail 5 and encyst as metacercariae on aquatic vegetation or other surfaces. Mammals acquire the infection by eating vegetation containing metacercariae. Humans can become infected by ingesting metacercariae-containing freshwater plants, especially watercress 6. After ingestion, the metacercariae excyst in the duodenum 7 and migrate through the intestinal wall, the peritoneal cavity, and the liver parenchyma into the biliary ducts, where they develop into adults 8. In humans, maturation from metacercariae into adult flukes takes approximately 3 to 4 months. The adult flukes reside in the large biliary ducts of the mammalian host. *F. hepatica* infect various animal species, mostly herbivores (Andrews, 1999, Taylor, 2007).

Nematodes are the most abundant parasitic worms on the planet (Bradley and Jackson, 2004) infecting almost a quarter of the worlds population. The nematode *A. lumbricoides* is of major medical importance infecting 1.5 billion people worldwide (Harnett and Harnett, 2008). At heavier loads symptoms include abdominal pains,

diarrhea, malnutrition, obstruct of the intestines or bile ducts. Migrating larvae can also cause pneumonitis and eosinophilia (Baron, 1996). Adult worms primarily infect the gastrointestinal tract. From here larval worms released from eggs penetrate the wall of the duodenum enter the bloodstream and migrate to the lungs were they invade the pulmonary tissues and alveoli before being coughed up and swallowed, to return to the the small intestine where they mature to adults (Wossene *et al*., 2002). Unlike *F. hepatica*, *A. lumbricoides* has direct life cycle (Baron, 1996, Das *et al*., 2007) meaning there is no intermediate host (Fig. 1.2).

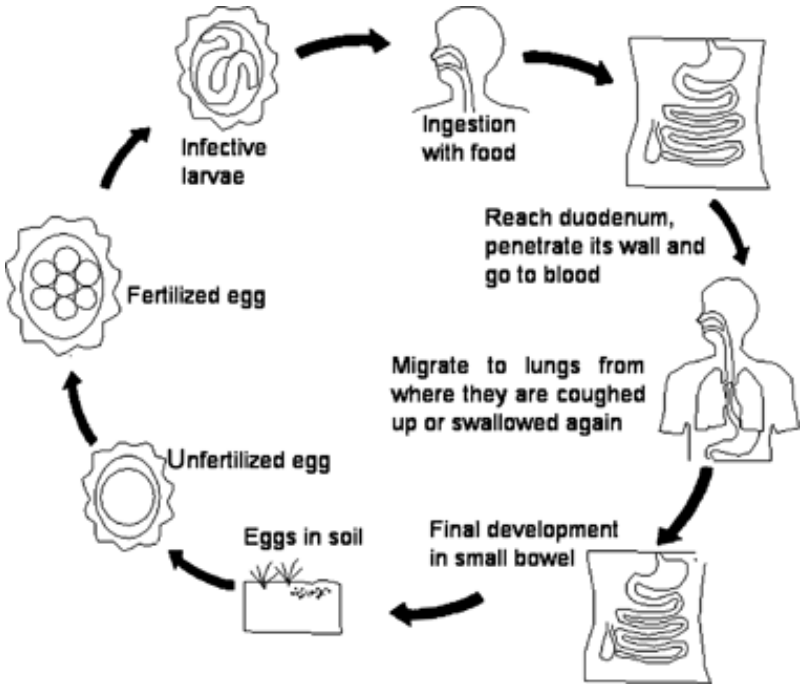

Figure 1.2: Life cycle of *A. lumbricoides*. Adult worms occupy the small intestines of the host. The females produce eggs that are excreted with faeces were the infective larvae develop. Infection occurs by the accidental ingestion of mature infective larvae typically in food or on fingers. After oral ingestion by the host, the eggs reach the intestine and hatch. The hatched larvae then migrate through the intestinal wall where they migrate thought several organs and tissues before returning to the intestine as adult worms (Das *et al*., 2007).

1.6.2. Characteristics of helminth infections

Epidemiological studies show that effective immunity against helminth infections is highly dependent upon the hosts ability to mount an adaptive immune response, characterised by a CD4+ T helper (Th) type 2 response (Maizels and Yazdanbakhsh, 2003). Most helminths are too large to be ingested by phagocytes, so the anti-worm response induced by Th2 cells is mediated by the production of immunoglobulin E (IgE) antibodies and the activation of eosinophils (Anthony *et al*., 2007). Th2 responses are primarily achieved by high levels of the cytokine IL-4 and low levels of interferon (IFN)-γ. Other Th2 related cytokines, including IL-5, IL-9, IL-13 and IL-21 are also highly produced (Maizels *et al*., 1993). In combination with a Th2 cell responses helminth infections are also associated with skewing of the host immune response towards a T regulatory cell (Treg) response (Diaz and Allen, 2007). This dominant Th2/Treg cell profile induced by helminths is sometimes called a "modified Th2" response (Maizels and Yazdanbakhsh, 2003).

During helminth infection CD4+ Th2 plasma cells (B-lymphocytes) switch to secreting IgE (Hagan *et al*., 1991). IgE antibodies mediate the activation of eosinophils. IgE antibodies bind to the surface of helminths allowing eosinophils to attach through Fcε receptors (Hogan *et al*., 2008). Eosinophils can then locate and kill helminth parasites by the secretion of granule enzymes (degranulation) (Rothenberg and Hogan, 2006). In a similar way, IgE initiated immune responses also recruit other effector cells such as mast cells and basophils for the eradication of helminth parasites (Wedemeyer *et al*., 2000). In addition, alternatively activated macrophages (AAMΦ) have also been implicated with the promoting of Th2 responses and suppressing Th1-driven inflammatory pathology during helminth infections (Kreider *et al*., 2007). Therefore, Th2/Treg associated cytokines, IgE antibodies and eosinophilia are the hallmarks of the immune response to helminth infection (Maizels *et al*., 2004).

1.6.3. Helminths and concurrent/bystander infections

There is strong evidence that concurrent helminth infections and helminth co-infections with protozoa, bacterial and viral diseases are common in developing countries (Petney and Andrews, 1998). As has been noted, helminth infections are synonymous with inducing a polarised Th2/Treg host response (See section 1.1.2). It has been well documented that such polarisation can facilitate host hyporesponsiveness to co-infections by suppressing Th1-Th17 responses or by inducing anti-inflammatory responses (van Riet *et al*., 2007). Co-infection with helminths and malaria, often observed due to the prevalence of both in tropical areas, best highlights this down regulation of pro-inflammatory responses (Hartgers and Yazdanbakhsh, 2006). Epidemiological studies show that helminth co-infection can increase the incidence of *Plasmodium falciparum* malaria infection (Nacher *et al*., 2002, Spiegel *et al*., 2003). In a murine model of infection with the blood-stage malaria parasite *Plasmodium chabaudi*, protective immunity which is associated with Th1 immune responses is impaired by concurrent gastrointestinal nematode *Heligmosomoides polygyrus* infection (Su *et al*., 2005). Whilst there are some incidences of improved malaria protection (Nacher *et al*., 2000), the majority of studies point to the detrimental effect of helminth infections on malaria outcome (Druilhe *et al*., 2005, Hartgers and Yazdanbakhsh, 2006).

Other murine models and human studies highlight the ability of helminth co-infections to suppress protective Th1 responses against bacterial respiratory infections such as with *Mycobacterium avium* (Sacco *et al*., 2002) or *Bordetella pertussis* (Brady *et al*., 1999). Although there are few epidemiological studies on the effects of helminth-tuberculosis co-infections, one study has shown that helminth induced Th2 responses in patients with newly diagnosed *Mycobacterium tuberculosis* (MTB) help favour a persistent MTB infection (Resende Co *et al*., 2007). *F. hepatica* infection has also been shown to have down regulatory effects on *Mycobacterium bovis* BCG-specific immune

responses in cattle (Flynn *et al*., 2007). *In vitro* studies also indicate that helminths can suppress the immune response to mycobacterial infections (Talaat *et al*., 2006).

Helminth infections may also impair immunological responses to viral pathogens (Kamal and El Sayed Khalifa, 2006). Humans with acute or chronic hepatitis C virus (HCV) and schistosomiasis co-infection cannot generate an efficient, broad HCV-specific Th1 response, which might play a role in the persistence and severity of HCV infection (Kamal *et al*., 2001). Similarly, helminth induced modified Th2 responses can accelerate the progression of human immunodeficiency virus (HIV) infection in people co-infected with helminths and the virus (Wolday *et al*., 2002). While cells from persons with helminth infections are more susceptible to HIV infection than cells from persons without helminth infection (Gopinath *et al*., 2000). In addition, helminth infections may also be detrimental for effective vaccination programs. Many modern vaccines are designed to induce a Th1 response for optimal efficiency, and Th2 responses induced by helminths can reduce vaccine effectiveness (van Riet *et al*., 2007, Helmby, 2009). In humans, *A. lumbricoides* infection can impair effectiveness of an oral cholera vaccine (Cooper *et al*., 2002), while in a mouse model of *Schistosoma mansoni* infection, the protective efficacy of BCG vaccination against MTB is reduced (Elias *et al*., 2005).

Overall, helminth co-infections studies are characterised by a downregulation of pro-inflammatory responses, leading to the exacerbation of concurrent infections (Maizels *et al*., 2004). Conversely, a reduction in pro-inflammatory responses may also be beneficial to the host in situations of atopy, allergy or autoimmune disease (Flohr *et al*., 2009). Similarly strong evidence also exists that helminth infections reduced host reactivity to bystander allergens (Elliott *et al*., 2007). For example, epidemiological studies have shown that allergic diseases are rare in areas with high helminth exposure, but high in urban/industrialised countries where helminth infections are low (Fallon and

Mangan, 2007). This observation has been explained by a decline in infections during childhood (Yazdanbakhsh *et al*., 2002), particularly the lack of exposure to helminth infections in developed countries (Dunne and Cooke, 2005). It has been dubbed the 'Hygiene Hypothesis' (Strachan, 1989, Smits and Yazdanbakhsh, 2007). By inducing a modified Th2 response, helminths infections may co-opt host regulatory cells to control the magnitude and phenotype of Th effector cell populations, possibly through the promotion of immunosuppressive cytokines (Diaz and Allen, 2007, Maizels and Yazdanbakhsh, 2008). Therefore evidence exists that under some conditions helminth parasites may be therapeutically beneficially (Zaccone *et al*., 2006, Erb, 2009, McKay, 2009). Some animal and human models support the use helminths as therapeutic agents in ameliorating autoimmune and atopic disorders such as inflammatory bowel diseases (IBDs) (Weinstock and Elliott, 2009) and Crohn's disease (Croese *et al*., 2006).

1.7. Helminths and adaptive immune responses

Activation of PRRs, displayed on antigen presenting cells (APCs), by PAMPs initiates the development of adaptive immune responses (Pasare and Medzhitov, 2004, Iwasaki and Medzhitov, 2004). Following interaction with APCs, naïve CD4+ T cells receive signals through their T-cell receptors (TCRs) that recognise peptide antigens presented to them on major histocompatibility complex (MHC) II complexes in conjunction with co-stimulatory receptors signals, that initiate differentiation towards functionally distinct CD4+ T helper (Th) cell subtypes (Kapsenberg, 2003). In 1986 Mosmann and Coffman reported two Th cell subsets; Th1 and Th2 cells (Mosmann *et al*., 1986), that could be distinguished from each other by the signature cytokines that the differentiated cells secreted.

At present, linage commitment is defined by the cytokines; IFN-γ, IL-4 and IL-17 for Th1, Th2 and Th17 cells respectively (Korn *et al*., 2009). Th1 cells direct the

elimination of intracellular pathogens, viruses, tumours and some bacteria (Trinchieri, 2003); Th17 cells promote resistance to extracellular bacteria and fungi; and Th2 cells are important for humoral immunity and the control of helminth infections (Anthony *et al*., 2007). Excessive or detrimental (e.g., autoimmune) responses by these effector T cells are controlled by regulatory T cells (de Jong *et al*., 2005).

Instructions for the development of adaptive immune responses, specifically the control and initiation of differentiation of T helper cells, are largely orchestrated by DCs (Banchereau *et al*., 2000, Banchereau and Steinman, 1998). Therefore, variations in DC maturation status, in conjunction with the cytokine environment, can influence the subsequent polarisation of T helper cell responses, and that this alteration can be at the point of DC activation (Pulendran, 2005, de Jong *et al*., 2002). The most important Th1 polarizing cytokine is IL-12 (bioactive IL-12p70). Transforming growth factor (TGF)-beta and IL-6 acting in concert induce differentiation of mouse naive T cells into the Th17 lineage (Ouyang *et al*., 2008).

But much less is known about the mechanisms that result in Th2-cell-inducing DCs (Kapsenberg, 2003). Both IL-4 and IL-10 from DCs have been identified as possible polarizing candidates for Th2-driving, but both IL-4- (MacDonald and Pearce, 2002) and IL-10-deficient (Perona-Wright *et al*., 2006a) DCs can still drive Th2 responses. Therefore without any obvious candidates or mechanisms, a number of hypotheses have been put forward to explain Th2 induction by DCs. These fall into three groups; (A) the default mechanism, (B) the inhibition mechanism or (C) the alternative pathway hypothesis (MacDonald and Maizels, 2008).

Firstly, the maturation or default model proposes that unless triggers for Th1 responses are received by the DC (i.e. high IL-12 (Kalinski *et al*., 1999)) the T cell will automatically ‘default’ into a phenotype that will initial Th2 responses (Trinchieri, 2003). Examples of helminth-derived molecules can be used to highlight each

hypothesis. The best example of this model is the ability of *S. mansoni* egg antigen (SEA) to induce Th2-cell-inducing DCs (MacDonald *et al*., 2001, de Jong *et al*., 2002, Agrawal *et al*., 2003) in a absence of overt DC activation. The type of DC induced by SEA is sometimes called a DC 2 phenotype (see section 1.4). Similar results have been observed for the filarial nematode *Acanthocheilonema viteae* secreted ES 62 kDa glycoprotein (ES-62) product (Whelan *et al*., 2000), *S. mansoni* derived Lyso-phosphorylcholine (PC) (van der Kleij *et al*., 2002), *N. brasiliensis* excretory/secretory antigens (NES) (Balic *et al*., 2004), Schistosoma larvae products (0-3hRP) (Jenkins and Mountford, 2005), *E. granulosus* antigen B (AgB) and sheep hydatid fluid (SHF) (Rigano *et al*., 2007) and the glycoconjugate lacto-N-fucopentaose III (LNFPIII) derived from *S. mansoni* (Thomas *et al*., 2003).

The second model is a system of competition and inhibition, in which activated signalling pathways may intersect and inhibit one another (Kapsenberg, 2003). The best examples of this include helminth products that block or suppress the production of key Th1 polarising cytokines, such as IL-12. Lipopolysaccharide (LPS) induced IL-12 from DCs can be suppressed by pre-treatment with SEA (Jankovic *et al*., 2004, Cervi *et al*., 2004, Kane *et al*., 2004, van Liempt *et al*., 2007), NES (Balic *et al*., 2004) or *E. granulosus* AgB (Rigano *et al*., 2007). DC pre-treatment with SEA has also been shown to modulate LPS induced gene expression (Kane *et al*., 2004).

The last hypothesis conceives alternative pathways associated with the activation of DCs through specific signals that induce specific Th responses (Reis e Sousa, 2006). In this model various PAMPs can be theorized to be either Th1 or Th2 stimuli, linked to distinct sets of PRRs, such as C-type lectin receptors (CTLs) (MacDonald and Maizels, 2008). For example, toll-like receptors (TLR) 4 binding LPS have been shown to activate DC to prime for Th1-type responses (MacDonald *et al*., 2001) as compared to CTL binding SEA products that prime Th2-type responses (van

Liempt *et al*., 2007). As can be seen by the overlapping diversity of the helminth-derived products in the varying circumstances highlighted in each scenario, the possibility arises that no single mechanism fully explains the mechanisms of Th2 induction by DCs. This bring forth the possibility that a combination of some or all the possible mechanisms is the most likely determinate of Th2 induction by DCs (MacDonald and Maizels, 2008).

1.8. Helminths and innate immune responses

While anti-helminth immune responses are mainly characterised by Th2/Treg cells, the innate arm of the immune system plays two very important roles in frontline host defence against helminths (Janeway and Medzhitov, 2002). Firstly, the innate system reduces infection through mechanisms such as physical barriers (Male *et al*., 2006). Secondly, the innate system plays a crucial role in pathogen detection (Kawai and Akira, 2009). This is achieved through a limited number of germ-line encoded receptors; the PRRs (Mogensen, 2009). PRRs mainly recognise PAMPs produced by pathogens and that are not naturally found on host cells or tissues (Medzhitov and Janeway, 2000). The best characterised family PRRs are the TLRs (Medzhitov, 2001, Takeda and Akira, 2005, West *et al*., 2006). There are 13 known mammalian TLRs, 10 in humans and 13 in mice (Kumagai *et al*., 2008). TLRs are expressed mostly by APCs; including macrophages and B lymphocytes, but most importantly DCs (McGettrick and O'Neill, 2007) (see section 1.5). Along with TLRs other PRRs also play important roles in innate PAMPs recognition. C-type lectin receptors (CLRs) are transmembrane lectins (sugar-binding proteins) that in contrast to TLRs, recognize carbohydrate glycan structures present on the pathogens (Geijtenbeek *et al*., 2004).

Helminth-derived molecules are recognised by the host in an effort to generate an immune response to eliminate the parasite. But the current knowledge how helminths

are recognised by the innate immune is limited by the few PRR ligands of parasite origin that had been identified (McGuinness *et al*., 2003, Zaccone *et al*., 2006). In a number of cases recognition of helminth-derived molecules has been demonstrated to be dependent various TLRs, including TLR2, 3 and most prominently TLR4 (Table. 1). The best described helminth-derived product is *A. viteae* ES-62, which is recognised by DCs in a TLR4 dependent manner (Goodridge *et al*., 2001, Goodridge *et al*., 2005). Similarly, LNFPIII is also recognised in a TLR4 dependent fashion (Perona-Wright *et al*., 2006b). Studies on knockout and mutant mice have shown Schistosoma larval preparation (0-3hRP) to be partially dependent on TLR4 (Jenkins *et al*., 2005). While Schistosomal derived lipids (lyso-PS) (van der Kleij *et al*., 2002) and dsRNA from schistosomal eggs (Aksoy *et al*., 2005) bind TLR2 and TLR3 respectively.

CTL receptors are also important for the recognition of helminth products (van Kooyk and Rabinovich, 2008). The CTL receptors DC-SIGN (van Die *et al*., 2003), MGL and MR (van Liempt *et al*., 2007) have all be implicated in SEA recognition, while TLR2 nor TLR4 play a role (Kane *et al*., 2008). Similarly, MBL has been shown to be involved in the detection of *Trichinella spiralis* ES products (TsES) (Gruden-Movsesijan *et al*., 2003). There is also a wide range of helminth-derived products with immunological properties to which no recognition mechanism has yet been attributed (Harnett and Harnett, 2008, Johnston *et al*., 2009) (Table 1). At the signalling level, many of the helminth-derived molecules in Table 1 have been associated with the activation of a number of pathways. Ligand binding of PRRs such as TLRs activate signal transduction pathways that initiated gene expression (Mogensen, 2009). Structurally, TLR proteins possess a extracellular domain made up mainly of leucine-rich repeats, a single transmembrane segment and a intracellular (cytoplasmic) domain called TIR (Toll/interleukin-1 (IL-1) receptor) (Jin and Lee, 2008).

	Worm product	Species or origin	PRR(s)	Activation status	Th	Reference
Helminth-derived molecules and DCs	**Heterogeneous**					
	SEA	*Schistosoma mansoni*	DC-SIGN, MGL, MR, L-SIGN	DC↔(**Y**)	Th2	(van Die *et al*., 2003), (van Liempt *et al*., 2007), (Meyer *et al*., 2007)
	0-3hRP	*Schistosoma mansoni*	TLR4	DC↗/MΦ↗	Th2	(Jenkins *et al*., 2005)
	NES	*Nippostrongylus brasiliensis*	ND	DC↗	Th2	(Balic *et al*., 2004).
	HpES	*Heligmosomoides polygyrus*	ND	DC↔(**Y**)	Th2/Treg	(Segura *et al*., 2007)
	TsES	*Trichinella spiralis*	MBL	DC↗	Th2/Treg	(Gruden-Movsesijan *et al*., 2003), (Ilic *et al*., 2008)
	TsMLCr	*Trichinella spiralis*	MR	DC↔	Th2/Treg	(Gruden-Movsesijan and Milosavljevic Lj, 2006), (Ilic *et al*., 2008)
	PCF	*Ascaris suum*	ND	DC↔(**Y**)	ND	(McConchie *et al*., 2006)
	PI	*Ascaris suum*	ND	DC↔ (**Y**)	ND	(Silva *et al*., 2006)
	Defined					
	ES-62	*Acanthocheilonema viteae*	TLR4	DC↗(**Y**)/MC(**Y**)/MΦ	Th2	(Goodridge *et al*., 2005), (Whelan *et al*., 2000)
	LNFPIII	*Schistosoma. mansoni*	TLR4	DC↗	Th2	(Thomas *et al*., 2003)
	AgB	*Echinococcus granulosa*	ND	DC↗	Th2	(Rigano *et al*., 2007)
	lyso-PS	*Schistosoma. Mansoni*	TLR2	DC↗	Th2/Treg	(van der Kleij *et al*., 2002)
	Egg-derived dsRNA	*Schistosoma. mansoni*	TLR3	DC↗	ND	(Aksoy *et al*., 2005).
	PC-glycosphingolipids	*Ascaris suum*	ND	DC↗/MΦ↗ (**Y**)	ND	(Kean *et al*., 2006), (Deehan *et al*., 2002)
Other selected helminth-derived molecules	**Heterogeneous**					
	NaES	*Necator americanus*	ND	NKc↑	ND	(Hsieh *et al*., 2004), (Teixeira-Carvalho *et al*., 2008)
	BES	*Brugia malayi*	ND	AAMΦ↑	ND	(Allen and MacDonald, 1998)
	FhES	*Fasciola hepatica*	ND	Eo↓, AAMΦ↑(**Y**)	ND	(Serradell *et al*., 2007), (Flynn and Mulcahy, 2008)
	DiAg	*Dirofilaria immitis*	ND	B cell↑	Th2	(Tezuka *et al*., 2003)
	AsES	*Ascaris suum*	ND	MΦ↗	ND	(Andrade *et al*., 2005)
	Defined					
	FhTPx	*Fasciola hepatica*	ND	AAMΦ↑	ND	(Donnelly *et al*., 2005)
	FhPrx	*Fasciola hepatica*	ND	AAMΦ↑	Th2	(Donnelly *et al*., 2008)
	SmPrx	*Schistosoma mansoni*	ND	AAMΦ↑	Th2	(Donnelly *et al*., 2008)
	Sm16	*Schistosoma mansoni*	ND	HPBM↔ (**Y**)	ND	(Brannstrom *et al*., 2009)
	Alpha-1/Omega 1	*Schistosoma mansoni*	ND	Basophil↑	Th2	(Cass *et al*., 2007), (Schramm *et al*., 2007)

Table 1.1: Helminth-derived products; a full list of helminth-derived immunomodulators documented and DC interaction, plus a selection of molecules of interest. *Abbreviations*: ND, Not determined; TLR, Toll-like receptor; PS, phosphatidylserine; SEA, water-soluble egg antigen; HPBM, Human peripheral blood mononuclear; GST, glutathione-*S* transferase; PC, phosphorylcholine; TPX, thioredoxin peroxidase; 0-3hRP, Schistosoma larvae derived products; NES, *N. brasiliensis* excretory/secretory antigens; HpES, *H. polygyrus* ES antigens; TsES, *T. spiralis* ES antigens; TsMLCr, *T. spiralis* crude muscle larvae antigen; PCF, pseudocoelomic fluid; PI, high-molecular-weight components; LNFPIII, lacto-N-fucopentaose III; AgB, antigen B; NaES, *N. americanus* ES antigens; BES, *B. malayi* ES antigens; FhES, *F. hepatica* ES antigens; DiAg, Dirofilaria immitis polyproteins; AsES, *A. suum* excretory/secretory antigens; Prx, peroxiredoxin; Sm16, Alpha-1 and Omega 1 is a non-acronymic designation. ↗; partial DC activation (Dowling *et al*., 2008).; ↔, no change, ↓, suppression, ↑ induction; (Y), suppression of secondary activation; DC, dendritic cell; MΦ, macrophage; MC, mast cell, NKc, natural killer cell; AAMΦ, alternatively activated macrophage; Eo, eosinophil.

Adapter molecules binding to the intracellular TIR domain can initiate the transductional signalling (Akira and Takeda, 2004, O'Neill, 2006). There are five TIR domain-containing adaptor proteins involved in signalling by TLRs. They are known as MyD88 (myeloid differentiation primary-response gene 88), MAL (MyD88-adaptor-like protein), TRIF (TIR-domain-containing adapter-inducing interferon-β), TRAM (TRIF-related adaptor molecule) and SARM (Sterile alpha and HEAT/Armadillo motif) (O'Neill and Bowie, 2007). MyD88 is the key signalling adaptor for all TLRs with the exception of TLR3 and some TLR4 signals. Its main role is the activation of nuclear factor kappa B (NF-κB) (Kenny and O'Neill, 2008). NF-κB transcription factors are a family of DNA-binding proteins are most the important signal transducers (Hayden and Ghosh, 2008). NF-κB is expressed in all cell types has multiple critical roles in the regulation the large number of genes that involved in multiple stages of immune responses, including DC activation (Li and Verma, 2002). The mammalian NF-κB family of transcription factors consists of five members; p50 (NF-*v*B1), p52 (NF-κB2), p65 (RelA), c-Rel, and RelB. IκB family proteins, of which there are several, bind and sequester NF-κB in the cytoplasm (Beinke and Ley, 2004, Sun and Ley, 2008). Consequently, inhibitory NF-κB (IκB) degradation must occur for NF-κB to be translocated into the nucleus to function. (Hayden and Ghosh, 2004).

Limited data is available on the signal transduction pathways induced by many helminth-derived molecules but NF-κB activation is a common trait of Th2 inducing DCs, suggesting its importance (Perrigoue *et al*., 2008). While LPS induces the persistent activation of NF-κB, LNFPIII can induce rapid but transient NF-κB nuclear translocation and activity in the nucleus (Thomas *et al*., 2005). *E. granulosus* AgB (Rigano *et al*., 2007) can activated NF-κB in stimulated DC, while there is also evidence that SEA pulsed DCs may activate NF-κB1 and not NF-κB2 (Artis *et al*., 2005).

MAPKs (mitogen-activated protein kinases) and IRFs (interferon regulatory factors) work in conjunction with NF-κB to initiate the regulation and termination of immune responses (Krishna and Narang, 2008, Tamura *et al*., 2008). The MAPK signalling pathways are among the most ancient signal transduction pathways and are highly evolutionary conserved intracellular signalling enzymes that are activated in response to infectious agents and innate stimulators (Dong *et al*., 2002, Zhang and Dong, 2005). They play an important role in immune responses. There are three main families of MAPKs in mammals: p38 MAPK (p38), extracellular signal-regulated kinases (ERKs) and the c-Jun NH_2-terminal kinases (JNKs) (Nakahara *et al*., 2006, Dowling *et al*., 2008). LNFPIII, which has already been discussed above, also has the ability to phosphorylate ERK. Likewise, SEA can induce the phosphorylation of ERK and p38 but not JNK (Perona-Wright *et al*., 2006b). While ES-62 suppresses activation of p38 and JNK, induces ERK dependent inhibition of IL-12 production (Goodridge *et al*., 2003).

Many IRF members play central roles in the cellular differentiation of hematopoietic cells and in the regulation of gene expression in response to pathogen-derived danger signals (Tamura *et al*., 2008). IRF family members are important in two innate signal transduction pathways; TLR signalling and the regulation of DC development (Honda and Taniguchi, 2006). A total of nine members have been identified in humans and mice (Mamane *et al*., 1999, Taniguchi *et al*., 2001). There is no current knowledge as to the role of IRF family transcription factors in signal transduction following DC activation by helminth parasite molecules.

1.9. Helminths and DCs

As mentioned earlier DCs are crucial for bridging innate and adaptive immune responses to infection, specifically the control and initiation of the differentiation of T

helper cells (Banchereau *et al*., 2000, Banchereau and Steinman, 1998). But our knowledge of how helminths are recognised by DCs is limited and until relatively recently few PRR ligands of parasite origin had been identified (Zaccone *et al*., 2006). DCs where discovered and identified in the early 1970's by Steinman *et al*. (Steinman and Cohn, 1973, Steinman and Cohn, 1974, Steinman *et al*., 1974, Steinman *et al*., 1975) and shown to be seeded throughout the peripheral tissues. A heterogeneous population of bone-marrow derived cells, the main function of DCs is to continuously sample their local environment for pathogen antigens (Sher *et al*., 2003). These are then processed and presented as antigenic fragments on MHC class I or II molecules to regulate naïve T cells with TCR specificity for the MHC:peptide complex, leading to the initialisation and determination of adaptive immune responses (Banchereau and Steinman, 1998, Wu and Liu, 2007). There are two main categories of DCs: conventional (myeloid) DCs (cDCs) and plasmacytoid (lymphoid) DCs (pDCs) ((Shortman and Liu, 2002, Villadangos and Schnorrer, 2007). All cDCs lineages express high levels of CD11c, which is considered a definitive pan marker (Villadangos and Heath, 2005). cDCs can be divided two into categories, and further subdivided into five subset DC populations (Bonasio and von Andrian, 2006); characterised by their path taking to access the lymph organs (i.e. conform or not to Langerhans cell paradigm) and their varying cell surface marker expression profiles (Randolph *et al*., 2008).

Before encountering PAMPs DCs exist in an 'immature' phenotype. Immature DCs (iDCs) are characterised by low expression of the co-stimulatory molecules such as CD80, CD86 and CD40 and low MHC expression (Banchereau and Steinman, 1998, Banchereau *et al*., 2000). DCs stimulated with PAMPs such as LPS take up a "classical" activation status, defined by the elevated expression of cell surface co-stimulatory markers, MHC and the secretion various cytokines (Kapsenberg, 2003, Dowling *et al*.,

2008). Signalling thought NF-κB and MAPK pathways are highly involved in the initiation of this DC activation (Nakahara *et al*., 2006, Dowling *et al*., 2008).

Helminth-matured DCs can be often characterised by lack many of these classical maturation markers which are often absent or expressed at low levels or by the secretion of a select panel of cytokines such as IL-12p40 (Johnston *et al*., 2009). While helminths-matured DCs are classed as being less phenotypically mature they are still capable of priming naïve T-cells to activate Th2/Tregs associated with helminth infection (Maizels *et al*., 2004) (See section 1.2).

Helminth modulation of DC phenotype is most likely through the release of soluble mediators from various life stages of the parasites. Therefore some of the best characterised helminth-matured DCs involve heterogeneous mixes of soluble preparation from whole or partial parasites. There have been a number of independent studies on SEA-DC interactions. In all cases DCs pulsed with SEA do not show an increase in expression of co-stimulatory molecules or cytokines, indicating that no conventional maturation was induced (MacDonald *et al*., 2001, Kane *et al*., 2004, van Liempt *et al*., 2007). Furthermore in all cases SEA-matured DCs are hyporesponsive to subsequent TLR activation. In one case SEA was shown to suppress LPS-induced activation of murine DCs, including MHC class II, co-stimulatory molecule expression, and IL-12 production (Kane *et al*., 2004). In another study, SEA could suppress both polyinosinsic:cytidylic acid (poly-(I:C)) and LPS induced DCs maturation, as indicated by a decrease in co-stimulatory molecule expression and production of IL-12, IL-6 and tumor necrosis factor (TNF)-α (van Liempt *et al*., 2007). In addition, SEA suppressed Th1 responses induced by the poly-(I:C)-pulsed DCs, and skewed LPS-induced mixed responses towards a Th2 response (van Liempt *et al*., 2007). SEA is also of particular interest since it fails to activate DCs yet these antigens are capable of driving Th2 immune responses (See Table 1).

From the same species but different life stage, Schistosoma larvae derived products (0-3hRP) can to induce partial maturation of DCs (Jenkins and Mountford, 2005). These DCs are characterised by low IL-6 and IL-12p40, partial costimulatory marker expression and when co-cultured with naive T-cells *in vitro* or injected into mice can polarise T-cell responses towards a Th2 phenotype. 0-3hRP could also stimulate IL-6, IL-10 and IL-12p40 from macrophages, where the secretion of IL-12p40 and IL-10 are reduced in TLR4 knockout and mutant mice. However in that study the secretion of IL-6 was not dependent upon TLR4 suggesting that other TLRs are involved (Jenkins *et al*., 2005).

Similarly, NES have been shown to induce partial maturation of DCs characterised by IL-6, IL-12p40, partial costimulatory marker expression and to have Th2 priming abilities (Balic *et al*., 2004). Another study has shown that the gastrointestinal nematode *Heligmosomoides polygyrus*-ES products alone failed to induce DC activation, but further had the ability to impair DC function and suppressed both Th1 and Th2 adaptive immune responses (Segura *et al*., 2007). The same DCs promoted the differentiation of T-cells with a regulatory phenotype and an ability to suppress effector CD4+ cell proliferation and cytokine secretion. TsES but not TsMLCr could partially up regulate DC costimulatory molecules; both *T. spiralis* preparations increased amounts of secreted IL-10 and decreased amounts of IL-12p70. TsES and TsMLCr primed DC could also instruct the *in vitro* production of IL-4 by T cells purified from of *T. spiralis* infected rats, indicating a polarization to a modified Th2 response (Ilic *et al*., 2008). While neither pseudocoelomic fluid (PCF) (McConchie *et al*., 2006) or high-molecular-weight components (PI) (Silva *et al*., 2006) of *A. suum* did not activate DCs, both could suppress costimulatory molecule expression and cytokine production in response to third party antigens.

While the above mentioned preparations are heterogeneous mixes of molecules, the goal remains to identify the exact antigens from these preparations that interact with DCs (Hewitson *et al*., 2009). The best characterised such case is the PC-containing single filarial nematode-secreted product ES-62, (Goodridge *et al*., 2001, Goodridge *et al*., 2005) which induces the low level production of IL-12p40, TNF-α and IL-6 from DCs in a TLR4 dependent manner. Pre-treatment of DCs and macrophage with ES-62 also inhibits their ability to produce LPS induced IL-12 (Goodridge *et al*., 2003). LNFPIII derived from *S. mansoni* acts in a TLR4 dependent fashion to partially up-regulate costimulatory molecule expression on DCs which can then drive Th2 immune responses (Thomas *et al*., 2003). Similarly, purified antigens from *Echinococcus granulosa* (Rigano *et al*., 2007) partially activate DCs that can subsequently prime Th2 immune responses.

Schistosome lyso-PS can condition DCs to secrete IL-10 in a TLR2 dependent manner, thus swaying the immune system response towards a modified Th2 form (van der Kleij *et al*., 2002). While dsRNA from schistosomal eggs can activate TLR3 in DCs to produce IL-12p40 and TNF-α (Aksoy *et al*., 2005). In the same way, the PC containing native glycophingolipid from *A. suum* have been shown to increase DC and macrophage co-stimulatory molecule expression and secretion of IL-12p40 and TNF-α. On the other hand, pre-treatment can suppress LPS induced IL-12p70 in both cells (Kean *et al*., 2006).

1.10. Project background

In this study we set out to understand how helminth parasites instruct DCs to induce Th2 responses in their hosts. The main focus of this thesis is to understand the immunomodulatory properties of *F. hepatica* and *A. lumbricoides* derived molecules with a focus on their ability to modulate immature DCs and subsequent adaptive

immunity. Understanding the immunomodulatory properties of *F. hepatica* and *A. lumbricoides* derived molecules may also lead to the development of improved vaccines and/or novel therapeutics to treat autoimmune and atopic disorders (Carvalho *et al*., 2009).

1.10.1. *F. hepatica* tegument (FhTeg)

The tegumental coat of *F. hepatica* is a metabolically active layer that is in intimate contact with the host tissues and body fluids (Threadgold, 1976). It is here that much of the immune interplay between the fluke and host takes place. The tegument is shed every 2 to 3 hrs during the course of infection, thus representing a constant source of antigen in direct contact with the host's immune cells. The tegument has a number of functions, which includes protection against the host immune response. Studies have shown that by shedding its tegument, the fluke can also shed immune complexes that have formed on the surface, thereby evading a damaging immune response, and the shed antigen acts as a decoy "mopping up" host immune cells (Mulcahy, 1999, Halton, 2004). While a number of studies have investigated the proteome (Morphew *et al*., 2007) and the secretome (Robinson *et al*., 2009) of *F. hepatica*, none have exclusively examined the tegument. Only one study has attributed any immunomodulatory properties of the *F. hepatica* tegument (Trudgett *et al*., 2003). Synthetically produced short peptides, with sequences homologous to tegumental proteins, were incubated with rat peritoneal mast cells resulting in their degranulation.

1.10.2. *F. hepatica* excretory/secretory antigens (FhES)

ES products from adult *F. hepatica* (FhES) have been shown to contain carbohydrate components that are cytotoxic to eosinophils (Serradell *et al*., 2007, Serradell *et al*., 2009). Whereas *in vitro* treatment of bovine macrophages with FhES products inhibited

IFN-γ and NO production in response to bacterial products and skewed the macrophage population towards an AAM phenotype (Flynn and Mulcahy, 2008) (Table 1.1). During all developmental stages, *F. hepatica* secretes an abundance proteases and antioxidant molecules that are critical to the survival of the parasite within the host. Fasciola cathepsin L proteases (CLs) and glutathione S-transferases (GSTs) are families of proteases and antioxidant respectively and are of particular interest in helminth biology. Both CLs and GSTs are dominant molecules in FhES, with both found in relatively large abundance (Jefferies *et al*., 2001). Both the GST superfamily and cathepsins have been extensively studied as potential vaccine candidates against *F. hepatica* (Hillyer, 2005, McManus and Dalton, 2006).

1.10.3. *F. hepatica*-derived glutathione transferases (FhGSTs)

GSTs are an ancient protein superfamily with multiple roles in all eukaryotic organisms, but within parasites represent the main detoxification mechanism of hydrophobic and electrophilic compounds. GSTs are a versatile enzyme superfamily whose main role is to inactivate a wide range of exogenous/endogenous toxic molecules enzymes in helminths and to turn them into water-soluble compounds (Torres-Rivera and Landa, 2008). GSTs are one of many antioxidant molecules secreted by *F. hepatica,* including peroxiredoxin and thioredoxin. GSTs are very versatile enzymes and apart from their major role as detoxification enzymes are also involved in a number of functions including the metabolism of drugs, pesticides and other xenobiotics, signal modulation, transport of molecules and may have a function in prostaglandin and steroid hormone synthesis (Hayes *et al*., 2005). GSTs feature extensively in the secretome of *F. hepatica*, and can be found in ES products but also associated with the tegument (Robinson *et al*., 2009). They account to up too 4% of the total soluble protein in *F. hepatica* (Chemale *et al*., 2006).

GSTs can be grouped in three subfamilies according to their cellular location: mitochondrial GSTs; microsomal (membrane-bound) GSTs; and cytosolic GSTs (cGSTs) (Frova, 2006). The soluble or cGSTs are the most numerous and well described group and are subdivided into several classes. The group members are have ubiquitous classes and can occur in any organism including the mu (M), alpha (A), pi (P), theta (T), sigma (S), zeta (Z) and omega (O) classes. The main cGSTs classes identified in parasites, including *F. hepatica,* are M, P and S, along with some in the A and O classes (Torres-Rivera and Landa, 2008). While there are few instances in the current literature of helminth-derived GSTs exerting immunomodulatory properties, there are some examples of related proteins (Ouaissi *et al*., 2002). One, a structurally related molecule to GST derived from FhES, could inhibition of NO production from rat peritoneal macrophages and T cell proliferation *in vitro* (Cervi *et al*., 1999).

1.10.4. *F. hepatica*-derived cathepsin L (FhCLs)

F. hepatica expresses papain-like cysteine peptidases termed cathepsins (Smith *et al*., 1993b). Cathepsins are expressed by all organisms from bacteria to mammals, but helminth parasites have exploited cathepsins to perform a wide range of functions that enable them to reside in their mammalian hosts. Cathepsins are involved in a variety of pathogen-specific functions which enhance virulence, including modulation or suppression of host immune defences by cleaving immunoglobulin (Smith *et al*., 1993a), altering the activity of immune effector cells, penetration migration through host tissues, and catabolism of host proteins to peptides and amino acids (Robinson *et al*., 2008). Fasciola cathepsins have varying but overlapping substrate specificities. The family can be grouped into five distinct genetically similar groups named FhCL1-5. FhCL1, FhCL2 and FhCL5 are required for liver penetration and tissue and blood feeding.

Experiments using purified native enzymes demonstrated that FhCL1 and FhCL2 can degrade haemoglobin, immunoglobulin, and interstitial matrix proteins (Morphew *et al*., 2007). Helminth cathepsins are predominantly associated with the cells lining the gut lumen and reproductive structures, the sites at which rapid protein degradation and synthesis takes place. *F. hepatica* cathepsins are synthesized within the gastrodermal epithelial cells of the parasite and are stored in specialized secretory vesicles as inactive zymogens (Collins *et al*., 2004). Cathepsins make up a large proportion of the total transcriptome of many trematode parasites (Robinson *et al*., 2009), and nearly 15% of the transcripts derived from adult *F. hepatica*. Mature *F. hepatica*s only secrete cathepsin L cysteine proteases, highlighting the importance of these molecules in the parasite pathology (Dalton *et al*., 2003). The importance of FhCL1 and FhCL2 in *F. hepatica* survival and adaptation can be shown by the fact that FhCL1 and FhCL2 are by far the most predominantly expressed cathepsins by adult parasites and can account for approximately 67% and 28% of total secreted cathepsin Ls respectively (Stack *et al*., 2008). *F. hepatica* CL has also been shown to suppresses *Bordetella pertussis*-specific IFN-γ production *in vivo* (O'Neill *et al*., 2001).

1.10.5. *A. Lumbricoides*-derived pseudocoelomic fluid (PCF)

Antigens derived from *A. lumbricoides* and *A. suum*, which infect humans and pigs respectively, have been of growing interest due to the potential immunosuppressive effects of ascarids on their hosts (Johnston *et al*., 2009). Previous studies have mainly focused on *A. Suum*-derived antigens (Table 1.1). *A. Suum* ES (Andrade *et al*., 2005) and lipid products (Kean *et al*., 2006) have been shown to trigger *in vitro* nitric oxide from macrophage. While both PCF (McConchie *et al*., 2006) and PI (Silva *et al*., 2006) derived from *A. suum* can suppress DCs responses to secondary antigens. Also, various *A. Suum* products can alter expression of allergic diseases (Rocha *et al*., 2008, Araujo *et*

al., 2008, Lima *et al*., 2002, Itami *et al*., 2005, Matera *et al*., 2008). But our understanding of the interaction of *A. lumbricoides* antigens on DCs function is limited.

1.10.6. Aim of thesis

The aims of the thesis were to:

A) Study the ability of *F. hepatic*a tegumental antigen to suppress DC maturation and function.

B) Investigate the ability of *F. hepatic*a-derived enzymes to induce and/or modulate DC maturation and alter T helper cell priming ability *in vivo*.

C) Examine the capacity of an *A. lumbricoides* extract to induce and/or modulate DC maturation and alter T helper cell priming ability *in vivo*.

Chapter 2: Materials and Methods

2.5 Materials

2.5.1 Reagents

Table 2.1 list of reagents used in methods

Product	Catalog #	Company
Compressed industrial air	---	BOC Gases Ireland, Dublin 12, Ireland.
Compressed carbon dioxide (CO_2) industrial	40-VK	BOC Gases Ireland, Dublin 12, Ireland.
Medical oxygen	---	BOC Gases Ireland, Dublin 12, Ireland.
Halotane	---	BOC Gases Ireland, Dublin 12, Ireland.
C57BL/6j mice, female	---	Harlan UK Limited, Oxfordshire, UK
Balb/cj mice, female	---	Harlan UK Limited, Oxfordshire, UK
CeH/HeN	---	Harlan UK Limited, Oxfordshire, UK
CeH/HeJ	---	Harlan UK Limited, Oxfordshire, UK
DO11.10 mice, female	003303	The Jackson Lab, ME, USA
Balb/cj mice, female	000651	The Jackson Lab, ME, USA
RPMI 1640 medium	31870-025	GIBCO BRL, Paisley, Scotland
Foetal calf serum (FCS)	10270-106	GIBCO BRL, Paisley, Scotland
Sterile phosphate saline buffer (PBS)	14190-094	GIBCO BRL, Paisley, Scotland
L-glutamine	25030-024	GIBCO BRL, Paisley, Scotland
Penicillin-streptomycin	15070-063	GIBCO BRL, Paisley, Scotland
Mouse CD40 (PE)	553791	Becton Dickinson, Oxford, England
Mouse CD80 (PE)	553769	Becton Dickinson, Oxford, England
Mouse CD86 (FITC)	553691	Becton Dickinson, Oxford, England
PE Rat IgG2a κ Isotype Control (For CD40)	553930	Becton Dickinson, Oxford, England
PE Hamster IgG2 κ Isotype Control (For CD86)	550085	Becton Dickinson, Oxford, England
FITC Rat IgG2a, κ Isotype Control (For CD80)	553929	Becton Dickinson, Oxford, England
Mouse CD4 (FITC)	553650/ 553651	Becton Dickinson, Oxford, England
Mouse CD3 (PerCP)	553067	Becton Dickinson, Oxford, England
Mouse CD3 (PE)	555275	Becton Dickinson, Oxford, England
Mouse CD11c (APC)	550261	Becton Dickinson, Oxford, England
Mouse CD25 (PE)	553075	Becton Dickinson, Oxford, England
Hamster Anti-Mouse CD11c (PE-Cy5.5)	MCD11c18	Caltag-Medsystems Ltd, Buckingham, UK.
PE-Cy5.5 Hamster IgG Isotype Control (For CD11c)	HM18	Caltag-Medsystems Ltd, Buckingham, UK.
Hamster Anti-Mouse CD11c (APC)	MCD11c05	Caltag-Medsystems Ltd, Buckingham, UK.
CD28	553297	Becton Dickinson, Oxford, England
CTLA-4	553720	Becton Dickinson, Oxford, England
Mouse IL-5 ELISA Set	555236	Becton Dickinson, Oxford, England
Mouse IL-10 ELISA Set	555252	Becton Dickinson, Oxford, England
Mouse IL-12 (p70) ELISA Set	555256	Becton Dickinson, Oxford, England
Mouse TNF-α ELISA Set	555268	Becton Dickinson, Oxford, England
Mouse IL-6 ELISA Set	555240	Becton Dickinson, Oxford, England
Mouse IL-12 (p40) ELISA Set	555165	Becton Dickinson, Oxford, England
Mouse IL-23 (p19/p40) ELISA Ready-SET-Go	88-7234	eBioscience Ltd, Hatfield, UK
Mouse IFN-γ DuoSet ELISA kit	DY485	R&D Systems Europe Ltd., Oxon, UK

Mouse IL-4 DuoSet ELISA kit	DY404	R&D Systems Europe Ltd., Oxon, UK
Mouse IL-10 DuoSet ELISA kit	DY417	R&D Systems Europe Ltd., Oxon, UK
Mouse IL-17 DuoSetELISA kit	DY421	R&D Systems Europe Ltd., Oxon, UK
Mouse MIP-1α DuoSet ELISA kit	DY450	R&D Systems Europe Ltd., Oxon, UK
Mouse MIP-2 DuoSet ELISA kit	DY452	R&D Systems Europe Ltd., Oxon, UK
Mouse MCP DuoSet ELISA kit	DY479	R&D Systems Europe Ltd., Oxon, UK
Prostaglandin E2 ELISA Kit	514010	Cayman Chemicals, MI, USA
Griess reagent system (Nitric oxide)	G2930	Promega, Madison, USA
Pipette Tips-Eppendorf (200µl)	70.760.001/70.760.002	Sarstedt, Wexford, Ireland
Pipette Tips-Eppendorf (1000µl)	70.762	Sarstedt, Wexford, Ireland
96-well plate (flat bottom)	82.1581	Sarstedt, Wexford, Ireland
96-well plate (round bottom)	82.1582	Sarstedt, Wexford, Ireland
Sterile 24-well plate (round bottom)	83.1836	Sarstedt, Wexford, Ireland
Sterile petri dishes (92/16mm)	82.1472.001	Sarstedt, Wexford, Ireland
Sterile 10 ml pipettes	86.1245.001	Sarstedt, Wexford, Ireland
Sterile 25 ml pipettes	86.1685.001	Sarstedt, Wexford, Ireland
Sterile 1.5 ml micro tubes	72.690.001	Sarstedt, Wexford, Ireland
Sterile 5 ml tubes	62.558.201	Sarstedt, Wexford, Ireland
Sterile 15 ml centrifuge tubes	62.554.002/62.554.502	Sarstedt, Wexford, Ireland
Sterile 50 ml centrifuge tubes	62.559.001	Sarstedt, Wexford, Ireland
Sterile 3.5 ml transfer pipettes	86.1171/86.1171	Sarstedt, Wexford, Ireland
Filtropur S 0.2µm	83.1826.001	Sarstedt, Wexford, Ireland
Sterile Nunc F96 MicroWell™ Plates	137101	Nunc, Roskilde, Denmark
Sterile 6-well plate (round bottom)	83.1839	Sarstedt, Wexford, Ireland
tissue culture flask (75cm2)	83.1813.002	Sarstedt, Wexford, Ireland
Finnpipette® Multistepper® pipette tips	Z353507	Sigma-Aldrich, Dublin 24, Ireland
Corning Costar High Binding Capacity 96-well plate (flat bottom)	44-2504	eBioscience Ltd, Hatfield, UK
Sterile Nunclon Surface 12-well plate (flat bottom)	1-50628A	Nunc, Roskilde, Denmark
FluoroNunc 96 Well Black Plates F96 MaxiSorp	4-37111A	Nunc, Roskilde, Denmark
Sterile tissue culture plate 35mm plate (flat bottom)	1-50318A	Nunc, Roskilde, Denmark
40mm cell strainer	352340	Unitech/BD, Dublin 24, Ireland
70mm cell strainer	352350	Unitech/BD, Dublin 24, Ireland
19G needles	301500	Unitech/BD, Dublin 24, Ireland
25G needles	300600	Unitech/BD, Dublin 24, Ireland
27G needles	300635	Unitech/BD, Dublin 24, Ireland
1 ml syringes	300013	Unitech/BD, Dublin 24, Ireland
10 ml syringes	ASY225-40BX	Lennox Ltd, Dublin 12, Ireland
Parafilm-M	ASE165-15	Lennox Ltd, Dublin 12, Ireland
FACS tubes	352054	Unitech/BD, Dublin 24, Ireland
FACS flow sheath	342003	Becton Dickinson, Oxford, England
FACS clean	340345	Becton Dickinson, Oxford, England
FACS rinse	340346	Becton Dickinson, Oxford, England
The CellTiter 96® AQ_{ueous} Cell Proliferation Assay (MTS based)	G3582	Promega, Madison, USA
Cell Growth Determination Kit (MTT based)	CGB-1	Sigma-Aldrich, Dublin 24, Ireland
Annexin V-FITC apoptosis detection kit I	556547	BD pharmingen, Oxford, England
Dialysis tubing	D-9777	Sigma-Aldrich, Dublin 24, Ireland

Hydrogen peroxide 30% (H_2O_2)	H1009	Sigma-Aldrich, Dublin 24, Ireland
Trizma® hydrochloride ($NH_2C(CH_2OH)_3 \cdot HCl$)	T5941	Sigma-Aldrich, Dublin 24, Ireland
Calcium chloride ($CaCl_2$)	383147	Sigma-Aldrich, Dublin 24, Ireland
Glycine (NH_2CH_2COOH)	G8898	Sigma-Aldrich, Dublin 24, Ireland
Sulfuric acid (H_2SO_4)	435589	Sigma-Aldrich, Dublin 24, Ireland
Phenylmethylsulfonyl fluoride (PMSF) ($C_7H_7FO_2S$)	78830	Sigma-Aldrich, Dublin 24, Ireland
HEPES ($C_8H_{18}N_2O_4S$)	H3375	Sigma-Aldrich, Dublin 24, Ireland
Triton® X-114 (non-ionic detergent)	93421	Sigma-Aldrich, Dublin 24, Ireland
Chloroform ($CHCl_3$)	C2432	Sigma-Aldrich, Dublin 24, Ireland
Sodium dodecyl sulphate (SDS)	L4390	Sigma-Aldrich, Dublin 24, Ireland
DMSO (Dimethyl sulfoxide) ($(CH_3)2SO$)	D2650	Sigma-Aldrich, Dublin 24, Ireland
TRI Reagent®	T9424	Sigma-Aldrich, Dublin 24, Ireland
Sodium hydroxide (NaOH)	S5881	Sigma-Aldrich, Dublin 24, Ireland
Sodium chloride (NaCl)	71378	Sigma-Aldrich, Dublin 24, Ireland
Sodium phosphate dibasic (NaH_2PO_4)	S9763	Sigma-Aldrich, Dublin 24, Ireland
Ethanol absolute (CH_3CH_2OH)	E7023	Sigma-Aldrich, Dublin 24, Ireland
Tetramethylbenzidine (TMB) dihydrochloride (tablets)	T3405	Sigma-Aldrich, Dublin 24, Ireland
Paraformaldehyde (PFA)	P6148	Sigma-Aldrich, Dublin 24, Ireland
Temed	T9281	Sigma-Aldrich, Dublin 24, Ireland
Methanol (CH_3OH)	179337	Sigma-Aldrich, Dublin 24, Ireland
Hydrochloric acid (HCL)	07102	Sigma-Aldrich, Dublin 24, Ireland
Phorbal myristate acetate (PMA)	P8139	Sigma-Aldrich, Dublin 24, Ireland
Bovine serum albumin (BSA)	A7906	Sigma-Aldrich, Dublin 24, Ireland
Skim Milk Powder	70166	Sigma-Aldrich, Dublin 24, Ireland
Trypan Blue solution (sterile-filtered, cell culture tested)	T8154	Sigma-Aldrich, Dublin 24, Ireland
Phosphate-Citrate Buffer with Sodium Perborate (for ELISA)	P4922	Sigma-Aldrich, Dublin 24, Ireland
Acetone (CH_3COCH_3)	27023	AGB Scientific/VWR Int. Limited, Dublin 15, Ireland
PyroGene® Recombinant Factor C Endotoxin Detection System	50-658U	Lonza, Walkersville, MD, USA
X-ray developer Solution	120100	Fuji photo film Co Ltd, Toyko, Japan
X-ray fixer Solution	120204	Fuji photo film Co Ltd, Toyko, Japan
X-ray starter Solution	120401	Fuji photo film Co Ltd, Toyko, Japan
Fuji medical X-ray film (18x24cm)	100 NIF	Fuji photo film Co Ltd, Toyko, Japan
c-Jun N-terminal kinase (JNK) inhibitor	420116	Calbiochem, La Jolla, CA, USA
p38 MAP kinase inhibitor	S7067	Sigma-Aldrich, Dublin 24, Ireland
extracellular signal-regulated kinase (ERK) inhibitor	U0126	Sigma-Aldrich, Dublin 24, Ireland
CytoSelect 96-well Phagocytosis Assay, Colorimetric (RBC)	CBA-220	Cell Biolabs Inc., CA, USA
Sheep Red Blood Cells (Suspended 100%)	0855876	Innovative Reseach, MI, USA
Triton® X-114	93421	Sigma-Aldrich, Dublin 24, Ireland
Tween® 20, Molecular Grade	H5151	Promega, Madison, USA
Coverslips	MLS17-20	Lennox Ltd, Dublin 12, Ireland
Cellulose Acetate Membrane Filters	11104-047N	Sartorius, Goettingen, Germany
OVA Peptide (323-339, ISQAVHAAHAEINEAGR)	RP10610	GenScript Corp., NJ, USA
Extracti-Gel D detergent removing gel	20208	Pierce, Rockford, IL, USA
Recombinant mouse GM-CSF	415-ML-050	R&D Systems Europe Ltd., Oxon, UK

Microcon-YM-30 Filter Unit, 30 kDa	42409/42422	Millipore, MA, USA
Purified anti-mouse CD3	553057	BD pharmingen, Oxford, England
Nonident® P40 substitute detergent	74385	Sigma-Aldrich, Dublin 24, Ireland
[3H] thymidine	TRA310	GE Healthcare UK Ltd., Buckinghamshire, UK
Mouse CpG ODN 1826	tlrl-modn	InvivoGen, CA, USA
Flagellin (*S. thyphimurium*)	tlrl-stfla	InvivoGen, CA, USA
Peptidoglycan (PGN) (*Staphylococcus aureus*)	tlrl-pgnsa	InvivoGen, CA, USA
Poly (I:C) (polyinosinsic:cytidylic acid)	tlrl-pic	InvivoGen, CA, USA
Lipopolysaccharide (LPS) (*E. coli*, Serotype R515)	ALX-581-007	Alexis Biochemicals, Lausanne, Switzerland
Zymosan A (*Saccharomyces cerevisiae*)	Z4250	Sigma-Aldrich, Dublin 24, Ireland
Anti-mouse CD40/TNFRSF5 Antibody	MAB440	R&D Systems Europe Ltd., Oxon, UK
RIPA Buffer	R0278	Sigma-Aldrich, Dublin 24, Ireland
Protease Inhibitor Cocktail	P8340	Sigma-Aldrich, Dublin 24, Ireland
Phosphatase Inhibitor Cocktail 1	P2850	Sigma-Aldrich, Dublin 24, Ireland
Phosphatase Inhibitor Cocktail 2	P5726	Sigma-Aldrich, Dublin 24, Ireland
Bicinchoninic acid (BCA) reagent A	23228	Promega, Madison, USA
Bicinchoninic acid (BCA) reagent B	23224	Promega, Madison, USA
Precision Plus Protein All Blue Standards	161-0373	Biorad, Hercules, USA
Immobilon™ chemiluminescent HRP substrate	WBKLS0050	Millipore, MA, USA
Immobilon®-P Polyvinylidene Difluoride (DVDF) membranes	P2938	Sigma-Aldrich, Dublin 24, Ireland
Whatman 3MM Chromatography Paper 20X20CM	3030/861	Lennox Ltd, Dublin 12, Ireland
Phospho-SAPK/JNK (Thr183/Tyr185) Rabbit mAb	4668	Cell Signaling Technology Inc., MA, USA
Phospho-p38 MAPK (Thr180/Tyr182) Antibody	9211	Cell Signaling Technology Inc., MA, USA
Phospho-NF-κB p65 (Ser276) Antibody	3037	Cell Signaling Technology Inc., MA, USA
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	4370	Cell Signaling Technology Inc., MA, USA
Anti-rabbit IgG, HRP-linked Antibody	7074	Cell Signaling Technology Inc., MA, USA
p44/42 MAPK (total Erk1/2) Antibody	9102	Cell Signaling Technology Inc., MA, USA
IRF-5 Antibody	3257	Cell Signaling Technology Inc., MA, USA

2.6 Solutions and Media

2.6.1 Solutions

10X Phosphate buffered saline (PBS)

400g NaCl

10g KCl

58g Na_2HPO_4

10g KH_2PO_4

Solution adjusted to pH 7.3 with HCL or NaOH and brought to 1 L with dH2O. Stored at room temperature. 1X PBS was prepared by diluting 10X PBS at a ratio of 1:9 with dH_2O.

Citric acid buffer

2.941g Citric Acid

Citric acid buffer and adjusted to pH 5.5 with HCL or NaOH made up to 100mls with dH_2O. Stored at room temperature.

TMB substrate solution

9.8ml Citric Acid buffer

2mg TMB

200µl DMSO

3µl H_2O_2

2mg TMB powder added to 200µl DMSO. TMB/DMSO solution added to 9.8mls citric acid buffer make TMB Substrate Solution. Hydrogen peroxide added and TMB/DMSO solution added just before use.

10X electrophoresis buffer

250 mM tris

1.92 M glycine

1% SDS

Solution adjusted to pH 8.3 with HCL and stored at room temperature. 1X electrophoresis buffer was prepared by diluting 10X electrophoresis buffer at a ratio of 1:9 with dH_2O.

5X SDS-PAGE reducing buffer

0.625 M tris/HCl pH 6.8

50% glycerol

10% SDS

0.1% bromophenol blue

5% 2-mercaptoethanol

FACS Buffer (PBA)

100mls PBS

2mls (2%) FCS

0.05 g (0.05%) NaH_3

Fixation buffer: 1% paraformaldehyde (PFA)

Paraformaldehyde: 1.0g (1%)

Make up to 1000 ml with dH_2O, at 70^oC. Stored at 4^oC

0.2 M Sodium phosphate ELISA buffer

11.8g Na_2HPO_4

16.1g NaH_2PO_4

Solution adjusted to pH 6.5 with HCL or NaOH and brought to 1 L with dH2O. Stored at 4^oC.

0.1 M Sodium carbonate ELISA buffer

7.3g $NaHCO_3$

1.59g Na_2CO_3

Solution adjusted to pH 9.5 with HCL or NaOH and brought to 1 L with dH2O. Stored at 4°C.

0.1 M Ammonium bicarbonate buffer

7.906g Ammonium bicarbonate.

Solution brought to 1 L with dH_2O. Stored at 4°C.

Western Blot Transfer Buffer (one blot)

Tris: 0.9g (25mM)

Glycine: 4.5g (200mM)

Methanol: 60ml (20%)

Make up to 300 ml with dH_2O

Western Blot 10X Running Buffer

Tris: 30g (250mM)

Glycine: 144g (1920mM)

SDS: 10g

Make up to 1L with dH_2O, pH 8.3. Store at RT. 1X was prepared by diluting 10X Running Buffer at a ratio of 1:9 with dH_2O.

Western Blot Blocking Buffer

Marvel 5g (5%) (0.75g in 15mls; 1.5g in 30mls)

Make up to 100ml with PBS.

Western Blot Antibody Buffer

5% blocking buffer with 0.05% Tween-20 (50µl)

0.75g in 15mls, 7.5µl Tween-20.

2.6.2 Media

Complete RPMI (cRPMI) 5%:

RPMI 1640 medium with 5% (or 2% for FhCL1 antigens) heat inactivated FCS (30 mins at 60^{o}C), 0.05 mM 2-ME, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 50µM 2-mercaptoethanol (All products supplied by Invitrogen-Gibco).

2.7 Equipment

Table 2.2: list of equipment used in methods

Equipment	Catalog #	Company
Stirred ultra-filtration cell model 8220	5123	Millipore
Merit water still, 240V	W4000	Lennox Ltd, Dublin 12, Ireland
HiPrep 26/60 Sephacryl S-200 HR gel filtration column	17-1195-01	Amersham Biosciences Limited, Amersham, UK
Perista pump	SJ-1211	ATTO Bio-Instrument, Bunkyo, Tokyo
BioCollector Fraction Collector	AC-5750	ATTO Bio-Instrument, Bunkyo, Tokyo
Microcentrifuge Benchtop model	4214	Medical Supply Co Ltd., Dublin 15, Ireland
Stuart Scientific Combined Incubator and Orbital Shaker	S150	Medical Supply Co Ltd., Dublin 15, Ireland
Lightwave Diode Array UV/Vis Spectrophotometer	S2000	Medical Supply Co Ltd., Dublin 15, Ireland
Bloch Heater - BBA series - Grant Boekel		Medical Supply Co Ltd., Dublin 15, Ireland
Analogue Stirred Water Baths	NE4-22T	AGB Scientific/VWR Int. Limited, Dublin 15, Ireland
Consort nv electrophoresis power supply	E844	Belgium
Dual Mini Slab Kit-gel electrophoresis apparatus	AE-6450	ATTO Bio-Instrument, Bunkyo, Tokyo
Hemacytometer, Neubauer, Double cell	---	Medical Supply Co Ltd., Dublin 15, Ireland
Tecan Genios Microplate Reader	---	Tecan Group Ltd., Männedorf, Switzerland
Tecan Safire2 UV-VIS-IR and fluorescence plate reader	---	Tecan Group Ltd., Männedorf, Switzerland
Thermo / Forma Scientific CO2 Water Jacketed Incubator	Model 3111	Medical Supply Co Ltd., Dublin 15, Ireland
Leica Inverted microscope	DMIL	Leica Microsystems, Wetzlar, Germany
SIGMA 4K15 Benchtop Refrigerated Centrifuge	10740	Sigma Laboratory Centrifuges
BIOQUELL Microflow Class II ABS Cabinet	ABS1200F	AGB Scientific/VWR Int. Limited, Dublin 15, Ireland
FACSCalibur flow cytometer	---	Becton Dickinson, Oxford, England
Flowjo software	---	Tree Star, Inc, Ashland, OR, USA
BD CellQuest™ Pro software	---	Becton Dickinson, Oxford, England
ENDNOTE	---	ISI ResearchSoft, Berkeley, USA

GeneGenius Gel Documentation and Analysis System	---	Syngene, Cambridge, UK
Fuji medical film processor	FPM 100A	Fuji photo film Co Ltd, Toyko, Japan
Fuji film cassette (18x24cm)	EC-DW	Fuji photo film Co Ltd, Toyko, Japan
Vortex mixer	TT52-9008200	Lennox Laboratory Supplies Ltd, Dublin 12, Ireland.

2.8 Methods

2.4.21 *In-vitro* cultivation of parasites and preparation of *F. hepatica* excretory-secretory (FhES) and tegumental coat (FhTeg)

Adult *F. Hepatica* worms were collected from infected cattle from a local abattoir. Excretion-secretion (FhES) products were collected from live *F. Hepatica*. Briefly, *F. Hepatica* worms were washed three times in sterile PBS. 20-25 worms were placed in 40 mls cRPMI at 37^{o}C and 5% CO_2 for 24h. This culture medium was removed and designated FhES. FhES extracts were clarified by centrifugation at 14,000 x g for 30 mins at 4^{o}C (to remove any shed eggs). *F. Hepatica* tegument (FhTeg) was obtained by washing 20-25 worms *F. hepatica* adult worms (live) in sterile PBS twice and then incubating them in 1% Nonidet P-40 detergent (NP-40 (Sigma-Aldrich), in PBS) for 30 min at 4^{o}C with gentle shaking. Supernatant was collected and FhTeg were clarified by centrifugation at 14,000 x g for 30 mins at 4^{o}C (to remove any shed eggs). NP-40 was removed from FhTeg preparation using an Extracti-Gel D detergent-removing gel column (Pierce) (Hillyer, 1980). The FhTeg preparation was then and dialysis against sterile PBS overnight.

Both FhTeg and FhES were filter concentrated using a stirred ultra-filtration cell (Millipore) and compressed air. Briefly, Ultra-filtration membranes (Millipore) were first prepared by washing in sterile dH_2O for 90 min in agitated petri dish. dH_2O was changed every 30 mins and the membrane inverted. Each membrane was stored overnight in 10% ethanol/sterile dH_2O and washed in dH_2O for 30 mins in an agitated petri dish before use. Either FhTeg or FhES were added to the stirred ultra-filtration cell

under 25 psi of compressed air, with samples kept on ice at all times. Once concentrated, FhTeg and FhES were stored at -20^{o}C. Protein concentrations were measured using the BCA protein assay kit (Pierce) (see 2.4.3) and endotoxin levels were assessed using the Pyrogene® endotoxin detection system (Cambrex) (see 2.4.4). Both FhES and FhTeg gave endotoxin levels similar to background control levels and to complete RPMI 1640 medium so was taken to be endotoxin-free.

2.4.22 Recombinant protein production and native Ascaris antigen isolation

Active and variant inactive *F. hepatica* cathepsin L antigen rFhCL1 and rvFhCL1 (accession No. AAB41670), respectively and *F. hepatica* GST sigma and mu class (rFhGST-si (accessions No. ABI79450) and rFhGST-mu (accession No. ACF59730)) were prepared in *Pichia pastoris* and *Escherichia coli* recombinant expression systems as previously described in the laboratories of Professors John Dalton and Peter Brophy (Roche *et al.*, 1999, LaCourse *et al.*, 2009), respectively. *A. lumbricoides* extracts was prepared by obtaining adult worms (female) from ascaris infected individuals. Worms were washed with sterile PBS and a sterile needed was injected into the pseudocoelom of the worm to remove *A. lumbricoides* pseudocoelomic fluid (AlPCF). PCF is a metabolically active fluid that maintains hemostatic pressure in the worm and provides precursor molecules for membrane and cuticular synthesis and heme containing proteins for oxidative metabolism (Kennedy and Qureshi, 1986). Protein concentrations were measured using the BCA protein assay kit (Pierce) (see 2.4.3) and endotoxin levels were assessed using the Pyrogene® endotoxin detection system (Cambrex) (see 2.4.4). AlPCF tested negative for endotoxin levels since levels were similar to background control levels and to complete RPMI 1640 medium so was taken to be endotoxin-free.

2.4.23 Determination of Protein concentration

Determination of protein concentrations was done using the bicinchoninic acid (BCA) protein assay in a clear 96-well micro-titre plate. Standards were prepared using in 0.25 mg/ml increment between 0 and 2.0 mg/ml with bovine serum albumin (BSA) in an appropriate buffer. Samples or BCA standards were pipetted in 10µl aliquots in duplicate or triplicate into a clear 96-well micro-titre plate. BCA working reagent was prepared by mixing BCA Reagent A: BCA Reagent B in the ratio 50:1. 200µl BCA working reagent was then added to all standard and sample wells. Plates were covered and incubate at 37^{o}C for 30 mins, and absorbance was read at 562nm in a micro-titre plate reader. Duplicate or triplicate values were averaged and standard curves were generated and protein concentrations determined using Microsoft Excel software.

2.4.24 Endotoxin detection assay

Endotoxin levels were assessed using the Pyrogene® endotoxin detection system (Cambrex) which utilizes recombinant Factor C (rFC), an endotoxin sensitive protein, which is activated by endotoxin binding. When activated by endotoxin binding, recombinant Factor C acts upon a fluorogenic substrate in the assay mixture to produce a fluorescent signal in proportion to the endotoxin concentration in the sample. Reactions were run in steile 96-well microplate fluorescent plates (Nunc F96 MicroWell™ Plates). Samples were plated in triplicate in the presence of rFC and the fluorogenic substrate. Fluorescence was measured in a fluorescent microplate reader (Tecan Safire2) at time zero and then measured again after one-hour incubation at 37^{o}C. Excitation/emission wavelengths of 380/440 nm were used. The log net fluorescence is proportional to the log endotoxin concentration and is linear in the 0.01-10 EU/ml range.

2.4.25 Endotoxin removal procedure

Removal of detectable endotoxin was preformed by phase separation using Triton X-114 detergent (Aida and Pabst, 1990). This phase separation technique provides a rapid and gentle method for removing endotoxin from protein solutions. Since both rFhGST-si and rFhGST-mu were prepared in *E. coli* recombinant expression systems (see section see 2.4.2), each were treated to remove any detectable endotoxin. Briefly, protein solutions containing endotoxin were treated with 1% Triton X-114, vortexed vigorously and placed on ice for 5 mins. The solutions were vortexed again and warmed to 37^{o}C for 5 mins then 56^{o}C for 1 further min in a water bath. Two separation phases are subsequently formed; an endotoxin-Triton X-114 containing pellet and an endotoxin-detergent free supernatant. The Triton X-114 phase, containing the endotoxin, can be further clarified by is precipitated by brief centrifugation (approx 10 sec) in a micro centrifuge. Normally, phase separation can lead to a 1000-fold reduction of endotoxin levels in contaminated preparations. It has been reported that proteins treated by this procedure retained normal functions and that complete removal of endotoxin can be achieved by repetition of phase separation. All antigens that gave endotoxin levels similar to background control levels and to cRPMI 1640 medium as tested in the Pyrogene® endotoxin detection system (see 2.4.5) were taken to be endotoxin-free.

2.4.26 Isolation and culture of murine bone marrow derived DCs (BMDCs)

Mice were killed by cervical dislocation. Bone marrow-derived immature DCs were prepared by culturing bone marrow cells, isolated from the femurs and tibia of mice using sterile surgical equipment, 70% alcohol to sterilise the dissection area and performed in a microflow class II cabinet (AGB). Cells were re-suspended in cRPMI 1640 with recombinant mouse GM-CSF (20 ng/ml; R&D Systems Ltd, UK) and were

cultured in humidified incubator at 37^{o}C and 5% CO^{2} in sterile petri dishes (92/16mm, Sarstedt). On days 3 fresh medium with GM-CSF (20 ng/ml) was added to the cells. On day 6 of culture half of total volume of supernatant was carefully removed without disturbing the cell monolayer and replaced with fresh cRPMI medium containing GM-CSF (20 ng/ml). On day 8, cells were collected by removing adherent cells with gentle "scrapping" with a sterile 1 ml syringe butt, counted (see section 2.4.14) and used for assays (Lutz *et al*., 1999). DC purity was >90% positive for expression of the pan marker CD11c (APC) (Caltag Laboratories). Appropriately labelled isotype-matched antibodies were used as controls. Acquisition was performed using a fluorescence-activated cell sorter (FACS) Calibur flow cytometer (BD Biosciences), and analysis of results performed using FlowJo software (Tree Star) (see section 2.4.23).

2.4.27 Determination of cell yield

Trypan blue stains can be used in dye exclusion procedures for viable cell counting. Viable cells do not take up the dye whereas non-viable cells do. A Neubauer haemocytometer slide and an inverted microscope (Leica) were used to determine the number of cells harvested. Cell to be counted were added to a cell suspensions usually at a dilution factor = 3, containing Trypan Blue (Sigma-Aldrich) and allowed to stand for 5-10 mins. With a cover-slip in place the cell suspension in Trypan were pipetted onto the slide beneath the cover slip ensuring that the solution covered the entire surface of the counting chamber/grid. The microscopic slide contains a grid etched on its surface. The volume of a solution in any give part of the grid can be calculated from the area of the grid and the height between the grid and the cover-slip. Each square of the haemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. This is the conversion factor for the haemocytometer. Since 1 cm^3 is

approximately 1 ml, the subsequent cell concentration/ml (and total cell number) can be determined using the following calculations:

Cell No. =

(Volume (mls)) x (average count) x (dilution factor) x 10^4 [chamber conversion factor]

Example:

(10mls) x (45 cells) x (3) x (10^4) = 13.5 x 10^6 cells/ml

2.4.28 Determination of cell viability and apoptosis: MTT, MTS and annxein

The CellTiter 96 AQ_{ueous} Non-Reactive Cell Proliferation Assay (the key component; 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium, inner salt or MTS) (Promega) and the Cell Growth Determination Kit (the key component; 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide or MTT) (Sigma) were used to determine cells viability of DCs *in vitro*. MTS and MTT are bio-reduced by cells into a formazan product that is soluble in tissue culture medium, and that can be read at an absorbance of 490 nm and 562 nm respectively. The conversion of MTS and MTT into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by absorbance is directly proportional to the number of living cells in culture. Briefly, for the MTS assay cells were plated on sterile 96 well plates at a density of 10,000 cells/well in 200 µl cRPMI. For the MTT assay cells were plated on 96 well plates at a density of 200,000 cells/well in 200µl cRPMI. Cells were treated with antigens and TLR ligands as in section 2.4.10 and were incubated for a further 18 hours 37^{o}C and 5% CO^2 in a humidified incubator. For the MTS assay, MTS solution was added (40 µl/well) and cells were incubated for 3 further hours at 37^{o}C and 5% CO^2 and the absorbance of 490nm measured with a microplate reader (Genios, Tecan). For

the MTT assay, MTT solution was added (22 µl/well) and cells were incubated for 3 hours in a humidified incubator at 37°C and 5% CO^2. Culture medium was carefully removed as not to disturb cells and 200 µl MTT solvent was added to each well and pipette multiple times to dissolve MTT formazan crystals. The absorbance at 562 nm was measured with a microplate reader. Duplicate or triplicate values were averaged, standard curves were generated and protein concentrations determined using Microsoft Excel software.

Similarly, cell viability was also measured using the Annexin V-FITC apoptosis detection kit I (BD Biosciences). Briefly, 1 x 10^6 cells/ml were seeded into a 24-well plate and treated with respective antigens and each TLR ligand, either separately or together, or medium alone. Cells were harvested 24 h later, washed twice in cold PBS and incubated in binding buffer (0.1 M Hepes/NaOH, 1.4 M NaCl, 25mM $CaCl_2$) with Annexin V-FITC and propidium iodide (PI) for 15 min. Following addition of more binding buffer, cells were immediately analysed by flow cytometry.

2.4.29 Preparation of MAPK inhibitions studies and use in cell cultures

For MAPK inhibition studies, highly selective cell permeable inhibitors were used to block the functions of p38, JNK and ERK MAPKs. DCs where incubated with commercially available inhibitors at 20 µM/ml; c-Jun N-terminal kinase (JNK) (420116) (Calbiochem, La Jolla, CA, USA), P38 (S7067) and extracellular signal-regulated kinase (ERK) (U0126) (both Sigma-Aldrich) for 1h before addition of stimulus (Dowling *et al*., 2008). Eighteen hours after TLR ligand activation, supernatants were removed for quantification of cytokine and nitrite levels, and FACS analysis was performed to determine cell surface marker expression. JNK inhibitor was dissolved in sterile PBS (Invitrogen) and stored at -20°C in the dark. ERK and P38 inhibitor were dissolved in sterile DMSO (Sigma-Aldrich) and stored at 4°C in the dark.

2.4.30 *In vitro* DCs TLR and non-TLR -ligand activation

DCs were usually seeded into 24-well plates (Nunc) at $1x10^6$/ml in complete RPMI 1640 plus 5 ng/ml GM-CSF. The cells were treated with the respective antigens (FhTeg, FhES, rFhGST-si, rFhCL1 or AlPCF) typically at 10 µg/ml for 2.5 h prior to stimulation with LPS (*Escherichia coli* 0111:B4, Alexis and Sigma Aldrich; 100 ng/ml), zymosan A (*Saccharomyces cerevisiae*; 5 µg/ml), PGN (*Staphylococcus aureus* peptidoglycan; 5 µg/ml), poly (I:C) (polyinosinsic:cytidylic acid, synthetic analogue of double stranded RNA 2.5 µg/ml), flagellin (*Salmonella. typhimurium* 0.5 µg/ml), CpG (stimulatory oligonucleotide (ODN) 1826 5 µg/ml) (Invivogen, France) or anti-CD40 Ligand (clone 1C10; R&D Systems) for 18 h. In time course experiments, DCs were treated with respective antigens 2.5 h before, at the same time as, or 2.5 h after LPS stimulation. Control cells were treated with medium, respective antigens or TLR ligands alone. For dose response experiments DCs were usually treated with antigens at concentration of 15-1 µg/ml for 2.5 hours before stimulation with ligands for a further 18 h. For all experiments control DC's were treated with medium, respective antigens or the respective agonist alone.

2.4.31 ELISA (enzyme linked immunosorbent assay)

ELISAs are used to quantify cytokine levels in a given liquid solution such as culture medium or serum. The principles of ELISAs are as follows. A monoclonal Capture antibody (mAb) specific for the cytokine of interest is bound to a 96 well plate by incubation overnight (usually at 4^oC) at a defined concentration in a buffer such as PBS. Excess or unbound antibodies are removed by washing with an appropriate buffer such as 1x PBS with 0.05% Tween-20, BCA or FCS blocking buffers added prevents non-specific binding, washed again before the addition of samples and known standards (usually for 2 h at room temperature). The plate are washed again to remove unbound

cytokine and a biotinylated detection mAb specific for the cytokine of interest in the presence of streptavidin horseradish peroxidase (HRP) usually for 2 h at RT. Streptavidin has high binding affinity for biotin and catalyses the oxidation of tetramethylbenzidine (TMB). After washing again TMB is added which forms a blue colour that increases intensity in coloration with the level of captured cytokine. The reaction is stopped by the addition sulfuric acid and absorbance read at 450 nm. To calculate concentrations Absorbance reading at 450 nm of unknown samples can be plotted against the optical density (OD) absorbance readings of known standard curve (Male *et al*., 2006).

Supernatant sample from cell cultures were collected at set time points as outlined in section 2.4.16 and cytokine, chemokines and prostaglandin production was measured by commercially available ELISA kits. Supernatants were tested for IL-6, IL-10, IL-12p40, IL-12p70, IL-23, TNF-α, IL-4, IL-5, IL-17 and IFN-γ levels with BD OptEIA™ ELISA sets; (BD Biosciences) and Duoset™ ELISA kits (R&D systems). Production of MIP-1α and MIP-2 Duoset™ ELISA kits (R&D systems). Prostaglandin E_2 (PGE_2) and levels were also measured by ELISA (Cayman Chemical EIA Kits, USA).

2.4.32 Nitric oxide assay

Nitric oxide levels were detected using the Griess Reagent System (Promega). Nitric oxide (NO) is an important physiological messenger and effector molecule in immunoloigical systems (Bredt and Snyder, 1994). The Griess Reagent System is based on the chemical reaction detect NO_2- by its use of sulfanilamide solution and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. Sulfanilamide and NED were added sequentially each for 5-10 minutes and

the sample the absorbance of 565 nm measured with a microplate reader (Genios, Tecan).

2.4.33 TLR screening: InvivoGen

This method is based on TLR-induced activation of the transcription factor NF-κB in HEK293 clones and takes advantage of the fact that TLR ligands are typically recognized by a single TLR. Ag-induced stimulation of TLRs 2-9 (see table 2.3) was tested in HEK-293 cells expressing a given TLR protein as well as a reporter gene driven by NF-κB promoter (InvivoGen). Each 293-TLR cell line was induced with a known specific ligand as a positive control, and a recombinant HEK-293 cell line for the reporter gene only was used as a negative control. TLR activation is represented as activity of the secreted alkaline phosphatase in arbitrary units read at an optical density of 650nm. TNF-α was used to stimulate HEK-293 cells as a non-TLR ligand positive control (TLR-). Respective antigens were added at same concentrations as reported in section 2.4.16.

TLR	Ligands	Concentration
TLR2	PAM2	10 ng /ml
TLR3	Poly(I:C)	25 μg/ml
TLR4	*E.coli* K12 LPS	100 ng/ml
TLR5	*S. typhimurium* flagellin	1 μg/ml
TLR7	Imidazoquinoline/R848	3 μg/ml
TLR8	Imidazoquinoline/R848	10 μg/ml
TLR9	CpG ODN 2006	10 μg/ml

Table 2.3: TLR positive control ligands used during TLR screening. (InvivoGen). http://www.invivogen.com/family.php?ID=175&ID_cat=10&ID_sscat=94

2.4.34 Flow cytometry

Expression of cell surface markers on cells was quantified by four-colour flow cytometry. Briefly, adherent cells were recovered from culture plates or flasks after supernatant removal and gentle “scrapping” with a sterile 1 ml syringe butt and re-suspended in PBA (1X sterile PBS, 2% FCS and 0.02% sodium azide). FCS prevents

non-specific binding of fluorescently conjugated antibodies while the sodium azide minimises their endocytosis by the cells. Where suspension cells were used, they were taken direct from culture and buffer exchanged into PBA. Cell suspensions in PBA were transferred to 5ml FACS tube and centrifuged at 300 x g for 5 mins at 4^{o}C with an acceleration speed of 5 x g and a deceleration speed of 3 x g. Centrifugation adheres the cells to the bottom of the FACS tube allowing the supernatant to be carefully removed from each FACS tube without disturbing the cell pellet. Typically, 2.5 µl fluorescently conjugated antibody (BD Biosciences and Caltag) specific for the protein of interest usually, was added to each FACS tubes containing a cells of interest. 100 µl PBA was added, the cells re-suspended by voxtexing and the subsequent cell suspension incubated for 30 mins at 4^{o}C in the dark. Cells were washed in 2 ml PBA and centrifuged as outlined above and supernatant again carefully removed from each FACS tube as not to disturb the cell pellet. Finally, cells were fixed with 1-5% paraformaldehyde (PFA) solution in FACS flow sheath for a minimum of 10 mins and acquired with in 24 hrs.

DCs and T cells were tested for various cell surface markers as outlined in section 2.1.1. Multiple fluorescently conjugated antibodies were used in tandem ensuring that their flourochromes differed. All antibodies were used at suppliers recommended concentrations, incubation lengths and temperatures (which can vary from product to product according to manufacturer's specifications). Unlabelled cells or appropriately labelled isotype-matched antibodies were used as controls for all experiments. Surface marker expression was analysed on a FACSCalibur flow cytometer using CellQuest (BD Biosciences) and FlowJo Software (Tree Star).

2.4.35 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed based on a previously established method (Laemmli, 1970) and carried out using the mini-gel system (ATTO Bio-Instrument). Plates were first arranged in the relevant casting rig and the system was checked for leaks. Based on the molecular weight range of proteins within FhTeg 12% acrylamide resolving gels (10kDa-200kDa) were used. For all cases western blots 10% resolving gels (20kDa-300kDa) were used. Resolving gels were prepared based on the recipes given in table 2.4 TEMED and 10% ammonium persulphate were added last just prior to pouring to prevent the gel polymerisation before being poured. The resolving gel was poured in a way to leave room for the stacking gel and topped with isopropanol to ensure a flat surface, prevent drying and air bubbles forming. The resolving gel was let set for between 5-30 mins.

5% stacking gel were prepared based on the recipes given in table 2.1. Once again, TEMED and 10% ammonium persulphate were added last just prior to pouring to prevent the gel solidifying before being poured. Once the resolving gel had polymerised, the isopropanol was decanted from the gels. The stacking gel was then poured into place quickly followed by the placing of the appropriate spacer combs to form the loading wells and the stacking gel was allowed to polymerise for between 5-30 mins. The combs were then removed from the gels and the gels transferred from the casting rig to the electrophoresis chamber. The chamber was then filled with 1X electrophoresis buffer (see 2.2.1). For protein sample with sufficiently high concentrations, such as FhTeg, sample preparation was preformed by mixing 20 µl of neat sample stock with 5 µl 5X SDS-PAGE reducing buffer. This 25µl mixture was boiled for 2 mins on a heading plate. Prepared samples, usually 12.5 µl aliquots (10µg) and 10 µl aliquots standards (Precision Plus Protein™ Standards; Bio-Rad) were loaded onto wells of the gel. For protein samples with low concentrations such as those form

protein extrations, samples were concentrated by incubation for 2 h at -20°C in acetone at a 1:5 dilution. After 2 h samples were centrifuged at 8,000 x g for 10 mins to pellet the proteins and the acetone supernatant discarded. Protein samples (10 µg for pNF-κBp65, and 40 µg for all others signal transduction proteins) were loaded with pre-stained protein markers into wells as above. Electrophoresis was performed between 150 V and 200 V for 60-90 mins or until samples began to run off end of gel. Gels were carefully removed from electrophoresis chamber and casting rig, washed in H_2O and the 5% stacking gel was cut away and discarded.

No. of Resolving Gel	**15ml - 2 Atto Mini-Gels**		**7.5ml - 1 Atto Mini-Gels**		**Stacking (2 gels)**
Resolving Gel % Acrylamide	**10%**	**12%**	**10%**	**12%**	**5%**
40% Acrylamide/Bisacrylamide	3.75ml	4.5ml	1.875ml	2.25ml	0.625ml
1M Tris/HCl pH 8.8	5.6ml	5.6ml	2.8ml	2.8ml	---
1M Tris/HCl pH 6.8	---	---	---	---	0.625ml
dH_2O	5.4ml	4.65ml	2.7ml	2.325ml	3.625ml
10% SDS	150µl	150µl	75µl	75µl	50µl
10% APS	75µl	75µl	37.5µl	37.5µl	50µl
TEMED	25µl	25µl	12.5µl	12.5µl	25µl

Table 2.4: Components of SDS-PAGE gels. Resolving and stacking gels, with preparation volumes for one and four gels (ATTO Bio-Instrument Mini-Gels).

2.4.36 Protein extraction and western blot analysis (Immunoblots)

Total protein was extracted from cell lysates using RIPA buffer (containing 50mM Tris-HCl, 150mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, and protease and phosphatase inhibitor cocktails, pH 8.0 (Sigma-Aldrich)). Cells were incubated in the extraction buffer on ice for 5 min before being centrifuged at 8,000 x g for 10 min at 4°C**.** Supernatants were transferred to clean tubes, and protein concentrations were determined using the BCA protein assay kit (Pierce) (see 2.4.3).

Where necessary, protein samples were concentrated by incubation for 2 h at -20^{o}C in acetone at a 1:5 dilution. Protein samples (10-40 µg) and pre-stained protein markers (Precision Plus Protein™ Standards; Bio-Rad) were separated by SDS-PAGE (10% resolving gel) (section 2.4.15) and blotted onto 0.45 µM Immobilon™-P polyvinylidene difluoride membrane (Sigma-Aldrich). Membranes were blocked for 1 h at room temperature in 5% nonfat dried milk in PBS, and incubated overnight at 4^{o}C with the respective antibody selective for the protein of interest (see table 2.1). Membranes were washed in PBS with 0.05% Tween-20 (PBS-T) and incubated for 1 h at room temperature with peroxide-conjugated anti-rabbit IgG (Sigma-Aldrich; 1:2000). After further washing, proteins were visualized with SuperSignal West Pico chemiluminescent substrate (Pierce) or Immobilon™ chemiluminescent HRP substrate, exposed to film for 1-30 mins, and processed using an FPM 100A Processor (FujiFilm). Protein bands were quantified using the GeneSnap acquisition and GeneTools analysis software (GeneGenius Gel Documentation and Analysis System; Syngene).

2.4.37 Phagocytosis assay

The phagocytic ability of DCs was measured using the CytoSelect™ 96-well Phagocytosis Assay (Cell Biolabs Inc.). Briefly, DCs from C57BL/6 mice were plated at 0.5 x 10^{6}/ml in complete RPMI and incubated overnight at 37^{o}C to allow adherence to plate. On day two, DCs were treated with antigens (FhTeg, rFhGST-si, rFhCL1 or AlPCF; all 10 µg/ml) or TLR ligands (LPS (100 ng/ml) or zymosan A (5 µg/ml)) alone, or with each antigen and TLR ligands at the same time, for 2.5hrs before the addition of sheep erythrocytes opsonised by IgG at a ratio of 50:1 (Innovative Reseach). Supernatants were aspirated after 1h and adherent cells washed with sterile PBS to remove non-phagocytosed erythrocytes. Adherent DCs were then lysed, substrate solution added and the amount of engulfed erythrocytes determined by colorimetric

assay at absorbance of 610nm and quantified by a 2 fold serial dilution standard curve of lysed erythrocytes. Negative control cells where treated with 2 µM Cytochalasin D to block phagocytosis.

2.4.38 *In vivo* T-cell priming assay

For assays of *in vivo* T-cell priming, DCs isolated from BALB/c mice (as per 2.4.11) were stimulated *in vitro* with medium or one of each antigens; FhTeg, rFhGST-si, rFhCL1 or AlPCF; (all 10µg/ml) in the presence of ovalbumin (OVA) peptide (323-ISQAVHAAHAEINEAGR-329; 100 nM; GenScript Corp.). After 24 h, the cells were washed with sterile endotoxin free PBS and DCs (3 x 10^5) were delivered over the sternum of naive DO11.10 mice under halothane anaesthesia by subcutaneous injection. After 7 days, mice were killed by cervical dislocation, skin draining lymph nodes and spleens were removed and a single cell suspension of cells plated with medium, OVA peptide (100-1000 nM) or with PMA (25 ng/ml; Sigma-Aldrich) and anti-CD3ε monoclonal antibody (1 µg/ml; clone 145-2C11; BD Biosciences). After 72 h incubation period at 37^0C and 5% CO^2, supernatants were removed for measurement of IL-4, IL-5, IFN-γ, IL-17 and IL-10 by commercial assay (R&D Systems). Flow cytometry analysis of CD4, CD28 and T-lymphocyte-associated antigen 4 (CTLA-4) (BD Biosciences) expression and sdLN cells was done as described above.

2.4.39 T-cell Proliferation assay

Lymph node cells were prepared as described above and plated on 96 well plates. After 72 h 1 µCi of [^{3}H] thymidine was added per well. After four additional hours cells were harvested onto filter plates and [^{3}H] thymidine uptake determined via a liquid scintillation counter (NUI Maynooth).

2.4.40 Statistics

All data were analysed for normality prior to statistical testing. Where multiple group comparisons were made, data were analysed using one-way ANOVA. For comparisons between two groups, the Student's *t* test was used or non parametric Mann-Whitey U test was used analysis used for analysis when two experimental samples were compared. In all tests, $p < 0.05$ was deemed significant.

Chapter 3: FhTeg suppresses DC maturation and function

3.1. Introduction

Controlling immune pathology, in particular inflammatory responses, prolongs parasitic worm's survival within the host and thus increases its chances of completing its life cycle. Helminths classically induce highly polarized Th2 and T-regulatory immune responses, correlating with chronic infection in the host but with little associated immune pathology (Maizels and Yazdanbakhsh, 2003). *Fasciola* infection (O'Neill *et al*., 2000) and *Fasciola*-derived antigens (O'Neill *et al*., 2001) have also been shown to suppress antigen-specific Th1 immune responses both *in vitro* and *in-vivo*, or modulate the function of innate immune cells by inducing alternative activation of macrophages (AAMø) (Donnelly *et al*., 2005). But in contrast to bacteria and viruses, little is known about how helminths induce DC maturation. A limited number of studies demonstrate that helminths can induce a less phenotypically mature DC that differs predominantly by their activation status, which lacks many of the classical maturation markers such as co-stimulatory molecules (CD80, CD86, MHC II, CD40) and pro-inflammatory cytokines (IL-12p70 and TNFα) (MacDonald and Maizels, 2008). However, the effect of *Fasciola* antigens on DC function has yet to be determined.

In this study, we have isolated the tegumental coat from *F. hepatica* and are the first to investigate the effects of *F. hepatica* tegumental antigen on DC maturation and function. Preliminary studies on the immunomodulatory properties of FhTeg had taken place. Since parasitic helminth infections have been reported to ameliorate symptoms of pro-inflammatory diseases (Elliott *et al*., 2003, La Flamme *et al*., 2003, Elliott *et al*., 2004, Summers *et al*., 2005), it was we assessed whether FhTeg could suppress pro-inflammatory cytokine production *in vivo* using a model of septic shock. Intraperitoneal (i.p.) injection of LPS alone induced significantly higher levels of serum IFN-γ Injection of FhTeg alone induced similar cytokine responses to PBS. But injection of mice with

FhTeg either 2.5h prior to, or after, LPS injection resulted in significantly reduced serum levels of IFN-γ and IL-12p70 but not serum levels of IL-12p40. Therefore, it was shown that FhTeg could suppresses pro-inflammatory cytokines *in vivo* in a model of septic shock (Hamilton *et al*., 2009). TLR activation is associated with inflammatory disorders such as inflammatory bowel disease and septic shock (Abreu and Arditi, 2004), thus mechanisms of suppressing TLR maturation of DCs may lead to the development of potential therapeutics (Kobayashi *et al*., 2003, Daubeuf *et al*., 2007). It was subsequently been shown that LPS-stimulated DC activation could also be modulated by FhTeg. Treatment of LPS-stimulated DCs with FhTeg resulted in the reduced cytokine production of IL-12p70 IL-10, TNF-a, NO in addition to the reduced expression of IL-12p35 RNA. Levels of supernatant IL-12p40, IL-6 and IL-23 were unaffected. FhTeg was also shown to down regulate the LPS-stimulated cell surface co-stimulatory molecules CD40, CD80 and CD86 as well as MHC II. Similar to the suppression of pro-inflammatory cytokines *in vivo* in a model of septic shock this modulation was found to not to be dependent of time of exposure. Finally, the modulatory effects of FhTeg were shown to work independently of TLR4, since the suppressive effects outlined above was still evident in TLR4KO or C3H/HeJ (defective LPS response) mice. It was also shown that FhTeg did not interfere with DC cell growth or viability.

Since DCs are a good source of IL-12p70 and critical in driving adaptive immunity, a complete comparative analysis of the DC phenotypes induce by a range of TLR ligands was preformed (Dowling *et al*., 2008). Given that FhES has been shown to have immunomodulatory properties (see Table 1.1), and studies of the proteome (Morphew *et al*., 2007) and the secretome (Robinson *et al*., 2009) of *F. hepatica* have highlighted some similarities, we firstly compared the ability of FhTeg and FhES to modulate cytokine production in DCs matured with a range of TLR ligands. We further

investigated the effect of FhTeg on DC cell surface marker expression, its ability to bind PRRs and modulate signal transduction pathways. Lastly, the effect of FhTeg on DC function was characterised by its ability to alter their phagocytic and T cells priming capacity.

3.2. Results

3.2.1. FhTeg does not reduce DC viability or proliferation

DC viability and proliferation was assessed after FhTeg, FhES and TLR-ligand stimulation. Cell viability was measured using the CellTiter 96 AQ_{ueous} Non-Reactive Cell Proliferation assay (MTS; Promega) and cell growth/proliferation was measured using the Cell Growth Determination Kit (MTT; Sigma). MTS is bio-reduced by cells into a formazan product that is soluble in tissue colour medium, and that can be read at an absorbance of 490nm for the MTS assay and 562nm for the MTT assay. Absorbance is proportional to formazan, with is proportional to viable living cells (MTS assay) or cell growth (MTT assay). Blank wells containing no cells were used to determine background and each absorbance result was divided by the absorbance of the negative control cells (medium only) to determine the relative cell viability. Cell viability and proliferation was determined for FhTeg and FhES treated DCs alone and after stimulation with multiple TLR agonists. FhTeg or FhES did not affect cell viability for any ligands (see Fig. 3.1).

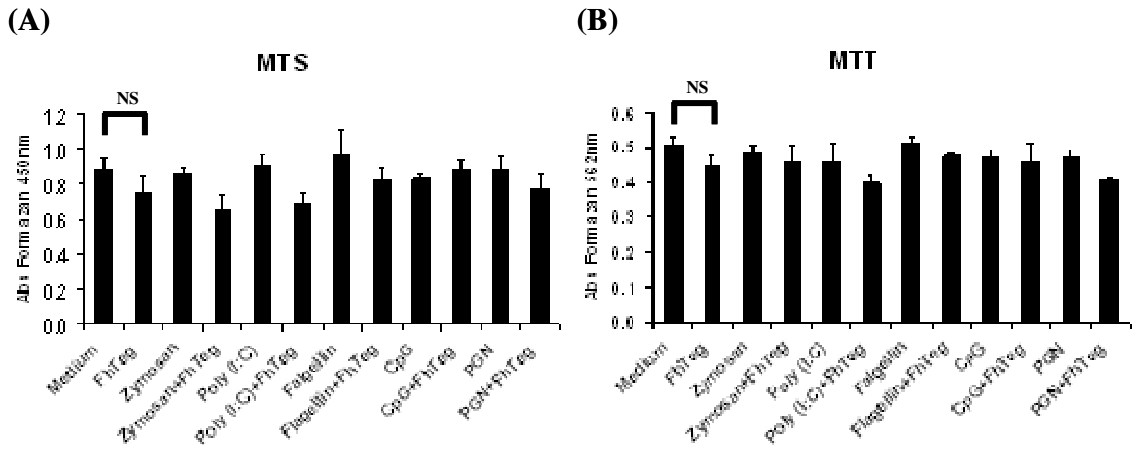

Figure 3.1: FhTeg does not affect cell viability and cell growth. (A) MTS and (B) MTT assays preformed on DCs from C57BL/6 mice shows FhTeg has no affect cell viability and cell growth of dendritic cells at a concentration of 10 μg/ml. DCs were treated with FhTeg (10 μg/ml) for 2.5 hours before stimulation with zymosan A (Zym, 5μg/ml), PGN (5 μg/ml), flagellin (Flag, 0.5 μg/ml), poly (I:C) (2.5 μg/ml), or CpG-ODN1826 (2.5 μg/ml) in the presence or absence of FhTeg (10 μg/ml), added 2.5h before TLR stimulation, or with medium alone as a control. Data are the mean values of 3 individual wells (±SEM), and a representative of at least 2 individual experiments. NS; not significant.

3.2.2. FhTeg not FhES suppresses cytokines in TLR-stimulated DCs

The effect of FhTeg and FhES on cytokine production of DC stimulated with multiple agonists was investigated. The 11 known TLRs can be and placed into groups according to the ligands they recognise (O'Neill, 2006). Five TLR agonists where chosen which broadly represent each group and which are triggered by a distinct set of microbial components. These include a variety of microbe-derived molecules including gram-positive and negative bacteria, fungi and viruses, all of which have been shown to unregulated cytokine production and cell surface marker expression. TLR3 is triggered by Poly (I:C), a synthetic analogue of double-stranded RNA (Tsujimoto *et al*., 2006); Flagellin, which is a major component of flagellar filament, activates TLR5 (Agrawal *et al*., 2003). TLR9 is trigger by the synthetic oligonucleotide CpG (ODN 1826) that contains unmethylated CpG dinucleotides (Napolitani *et al*., 2005); and Peptidoglycan (PGN), major surface component of gram-positive bacteria activates TLR2 (Michelsen *et al*., 2001). Finally, Zymosan A from *Saccharomyces cerevisiae* binds TLR2/6 (Dillon

et al., 2006). The concentrations of TLR ligands were determined by previous published data, manufacturer's recommendations and by *in vitro* test in our laboratory. The concentration employed were as follows: Poly (I:C) -100 μg/ml, Flagellin -0.5 μg/ml, CpG (ODN 1826) -2.5 μg/ml, PGN -5 μg/ml (all from Invivogen) and Zymosan A from *S. cerevisiae* -5 μg/ml (Sigma-Aldrich).

DCs were treated with FhTeg or FhES (both 10 μg/ml) 2.5 h before stimulating DCs with each TLR ligand. As controls DCs were also treated with medium (negative), FhTeg (negative), FhES (negative) or each TLR-Ligand alone (positive controls). Cells were incubated for a further 18 hours and culture supernatant where assayed for cytokine production by ELISA and nitrite was measured using Griess reagent system. Medium, FhTeg or FhES alone did not induce any of the cytokines tested, therefore are not shown. FhTeg significantly inhibited the production of IL-6, IL-10, IL12p70, TNF-α and nitrite in zymosan-stimulated DCs (see Fig. 3.2). PGN induced IL-6, IL-12p40, IL12p70, TNF-α and nitrite were suppressed after FhTeg treatment. FhTeg did not influence cytokine secretion in response to flagellin. Low cytokines levels were induced in response to flagellin, which may be explained by the fact that murine BMDC have previously been shown to express much lower levels of TLR5 (10 fold less) compared to splenic DCs (Didierlaurent *et al*., 2004). Poly (I:C) did not induce all cytokines but suppression in response to FhTeg was seen for IL-6 and nitrite. CpG induce all cytokines but suppression in response to FhTeg was observed for IL-10, IL-12p70 and nitrite. FhES, apart from a limited suppressive affect on PGN-induced TNF-α, did not suppress any cytokines induced by any of the 5 TLR ligands (see Fig. 3.3). Thus, only FhTeg has the ability to suppress the production of multiple cytokines induce by various TLR ligands with the greatest down regulation seen for IL-6, IL-10, IL-12p70 and nitrite from DCs.

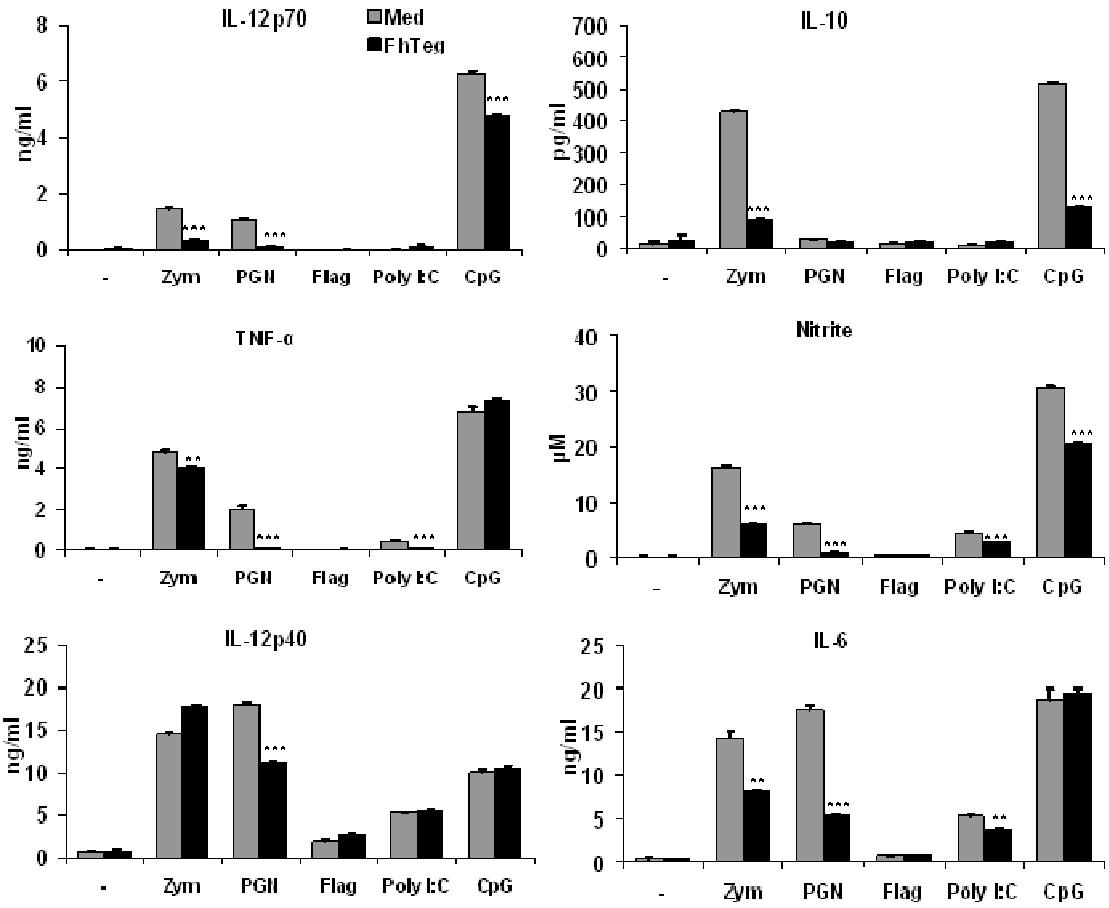

Figure 3.2: FhTeg targets multiple TLR pathways in DCs. DCs from C57BL/6 mice were cultured with zymosan A (Zym, 5μg/ml), PGN (5 μg/ml), flagellin (Flag, 0.5 μg/ml), poly (I:C) (2.5 μg/ml), or CpG-ODN1826 (2.5 μg/ml) in the presence or absence of FhTeg (10 μg/ml), added 2.5h before TLR stimulation, or with medium alone as a control. Supernatants were harvested 18 h after TLR-ligand stimulation and IL-12p70, IL-10, TNF-α, IL-12p40 and IL-6 were measured using sandwich ELISA, and nitrite was measured using medium Griess reagent. Data are the mean values (±SEM) of at least 3 individual experiments. ***, $p < 0.001$. **, $p < 0.01$. *, $p < 0.1$ compared with TLR-ligand stimulated only group.

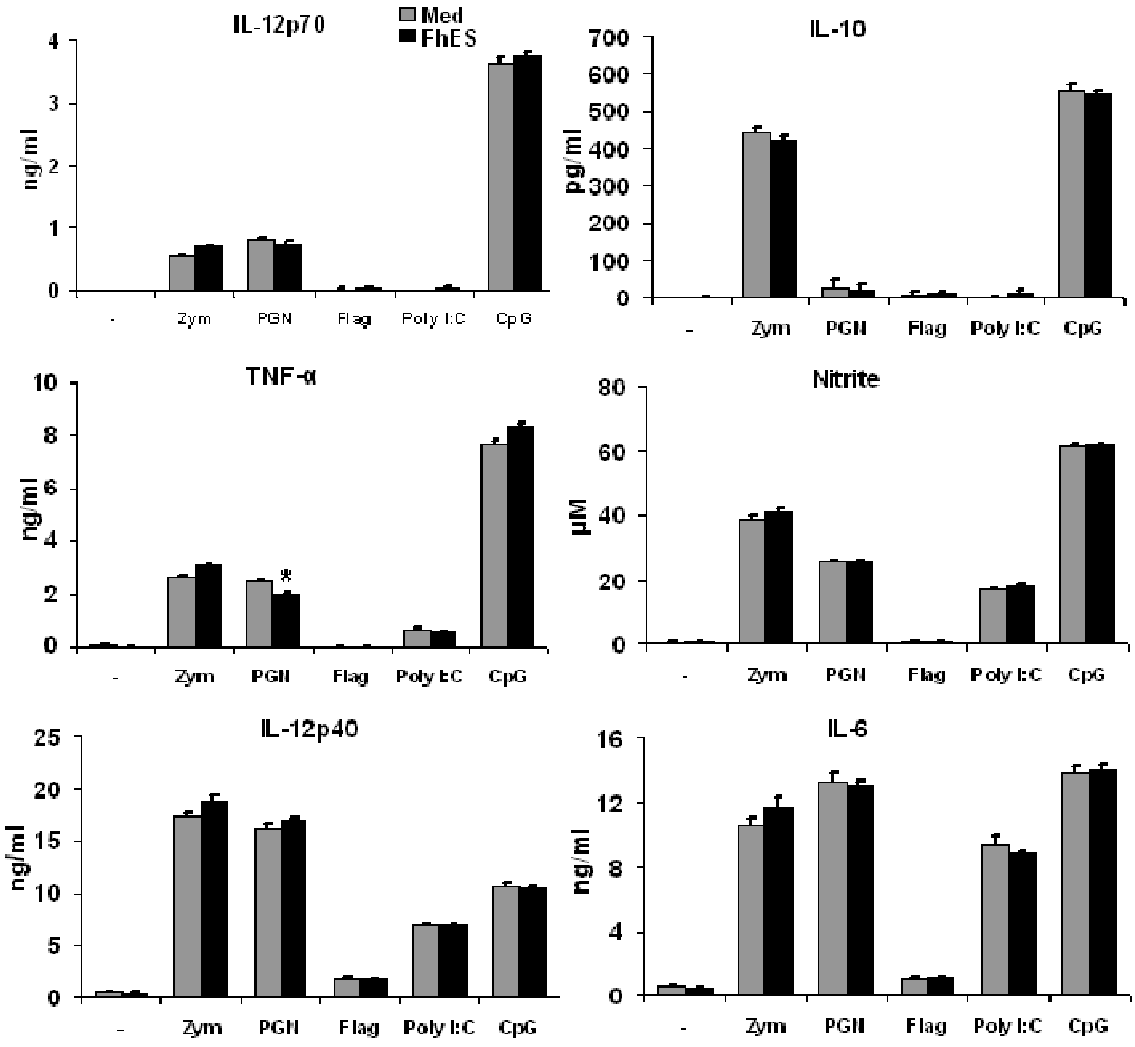

Figure 3.3: Cytokine production of stimulated DCs is unaffected by FhES. DCs from C57BL/6 mice were cultured as in Fig 3.2 in the presence or absence of FhES (10 μg/ml), added 2.5h before TLR stimulation, or with medium alone as a control. Supernatants were harvested 18 h after TLR-ligand stimulation and IL-12p70, IL-10, TNF-α, IL-12p40 and IL-6 were measured using sandwich ELISA, and nitrite was measured using medium Griess reagent. Data are the mean values (±SEM) of at least 2 individual experiments. *, $p < 0.1$ compared with TLR-ligand stimulated only group.

3.2.3. FhTeg suppresses costimulatory marker expression

The effect of FhTeg on cell surface expression of DC stimulated with various TLR ligands was investigated. We have previously established that expression of all three costimulatory marker; CD40, CD80 and CD86 are significantly up regulated by all 5 TLR ligands tested (Dowling *et al.*, 2008). DCs were treated with TLR ligands in the presence or absence of FhTeg as in Fig 3.2, and flow cytometric analysis was preformed. Pre treatment at 2.5 hr with FhTeg was shown to suppress increased the

expression all three markers in response to TLR ligand DC activation, except for zymosan induced CD40 and CD80 (Fig. 3.4).

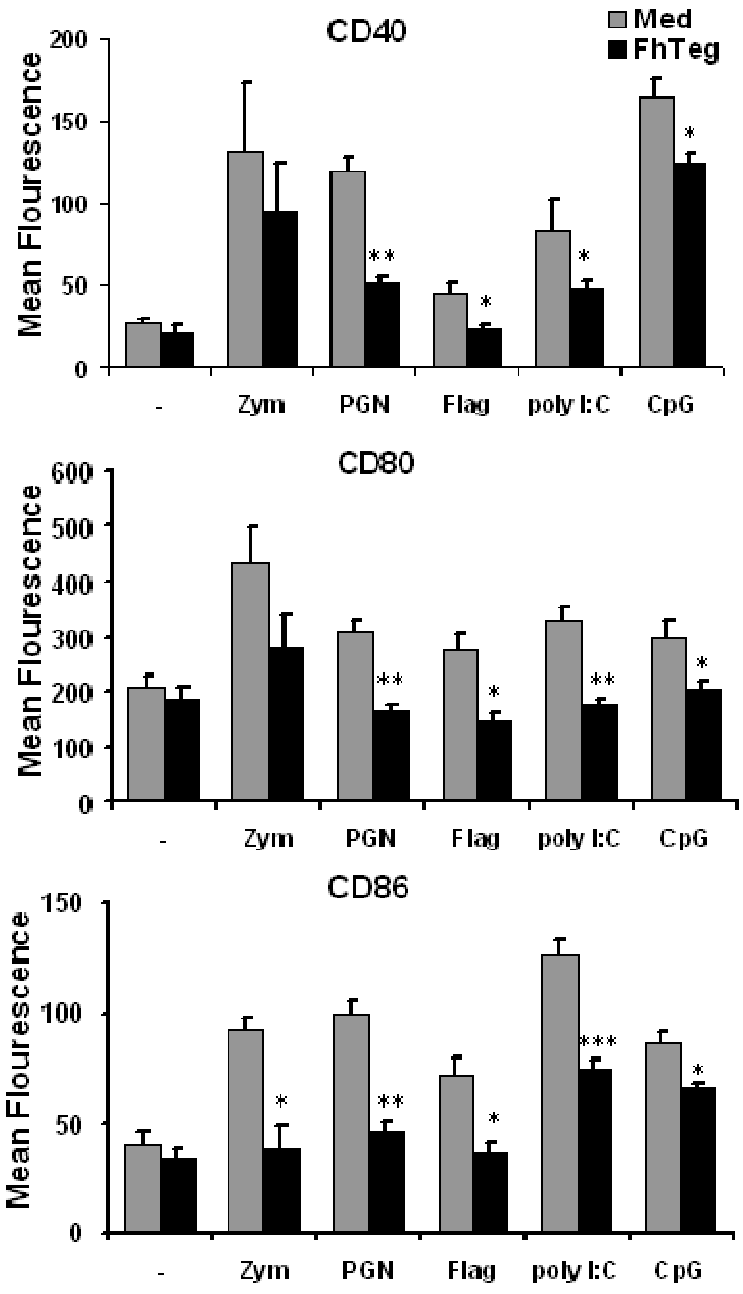

Figure 3.4: FhTeg suppresses co stimulatory marker expression in TLR stimulated DCs. DCs from C57BL/6 mice were matured with TLR-ligands as in Fig. 3.2 and cell surface expression of CD40, CD80 and CD86 were measured by flow cytometry after 18h. DCs cultured in medium alone and gated on a live $CD11c^+$ population were used as controls. Data are the mean values (±SEM) of 3 independent experiments. ***, $p < 0.001$. **, $p < 0.01$. *, $p < 0.1$ compared with TLR-ligand stimulated only group.

3.2.4. FhTeg suppression of DC TLR activation can be time dependent or time independent

As shown previously, FhTeg suppression of LPS-stimulated DC maturation was independent of time of exposure. To investigate if this was the same for all TLR ligands, DCs were treated with FhTeg (10 μg/ml) for 2.5 hours before, at same time as, or 2.5 hours after stimulation with the previously stated panel of TLR-ligands. DCs were incubated for a further 18 hours from time of DC stimulation and culture supernatants where assayed for cytokine production by ELISA and nitrite was measured using medium and Griess Reagent (Fig. 3.5). FhTeg suppressed cytokine production of zymosan stimulated DCs when added simultaneously (0hr) or after zymosan (+2.5 hrs). The greatest suppression is seen at -2.5 hrs and suppression decreases inversely in line with time after DC zymosan stimulation. This trend is largely followed for poly (I:C) and CpG, with the greatest cytokine suppression observed with FhTeg treatment at -2.5hrs before stimulation and the least suppression with FhTeg treatment +2.5hrs after stimulation. Therefore, FhTeg suppression of cytokines induced by these ligands shows a dependence on time of exposure. The suppression of PGN induced cytokines on the other hand is time independent in all cases (Fig. 3.5).

Again, the cell surface expressions of co-stimulatory molecules CD40, CD80 and CD86 were measured by flow cytometry, with FhTeg down regulating cell surface expression of CD40, CD80 and CD86 for all time points measured. In line with the suppression seen for the cytokine results, the greatest down regulation of co-stimulatory markers was observed at -2.5 hrs (Fig. 3.6). Once more as with cytokine production, down regulation of co-stimulatory markers decreases inversely with time to DC stimulation. Therefore, addition of FhTeg at +2.5 hrs had the least effect of decreasing co-stimulatory marker expression, which indicates that the suppressive effects of FhTeg

are time dependent in some instances. Medium or FhTeg alone did not induce any effects on DCs (data not shown).

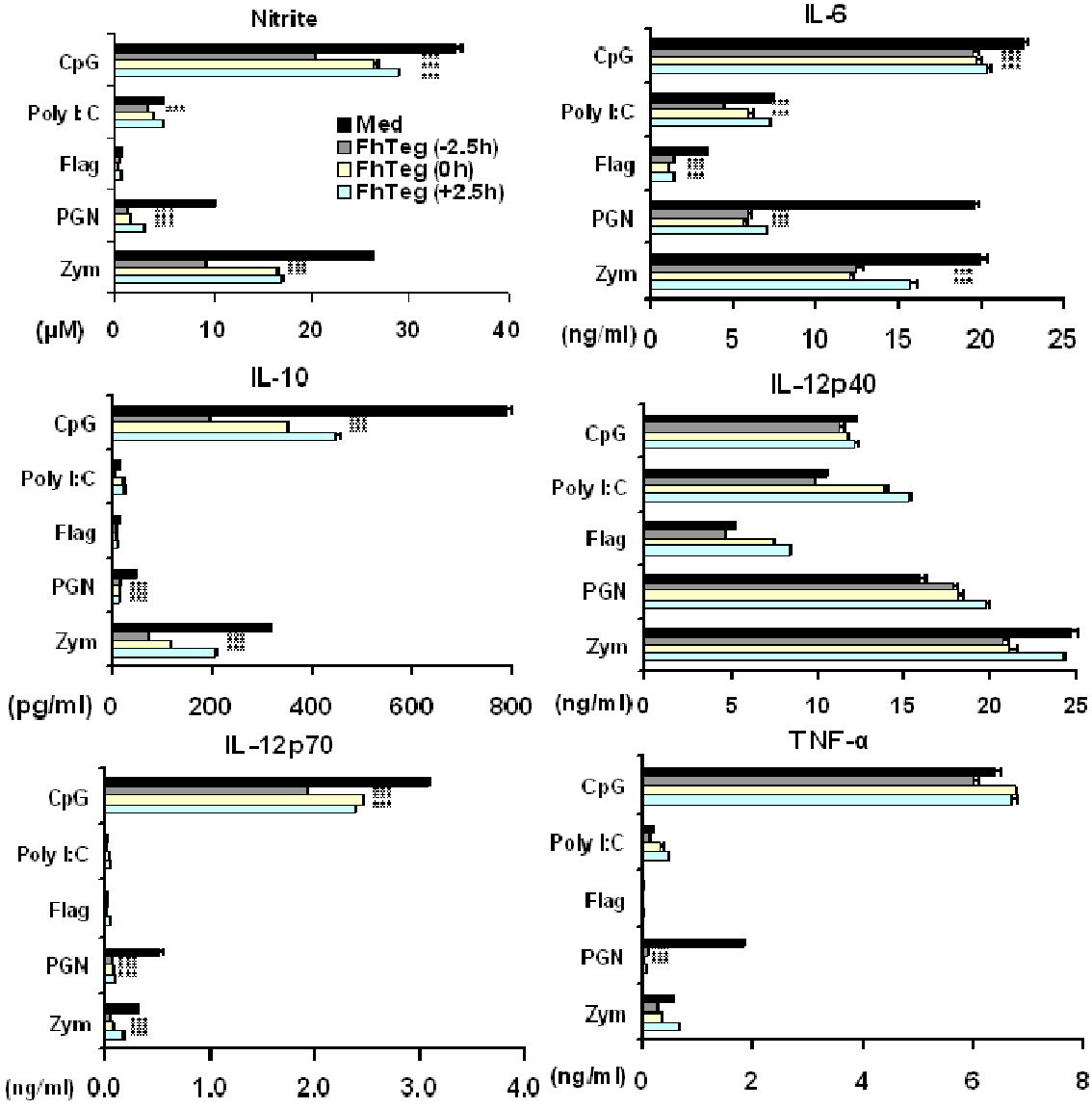

Figure 3.5: FhTeg inhibits TLR-induced DC cytokine production in a time dependent or independent manner. DCs from C57BL/6 mice were matured as in Fig. 3.2. DCs were also treated with FhTeg (10 μg/ml) 2.5 hours before, same time as, or 2.5 hours after TLR-ligand stimulation. Data are the mean values of 3 individual wells (±SEM), and a representative of at least 2 individual experiments. ***, $p < 0.001$. **, $p < 0.01$. *, $p < 0.05$ compared with TLR-stimulated group.

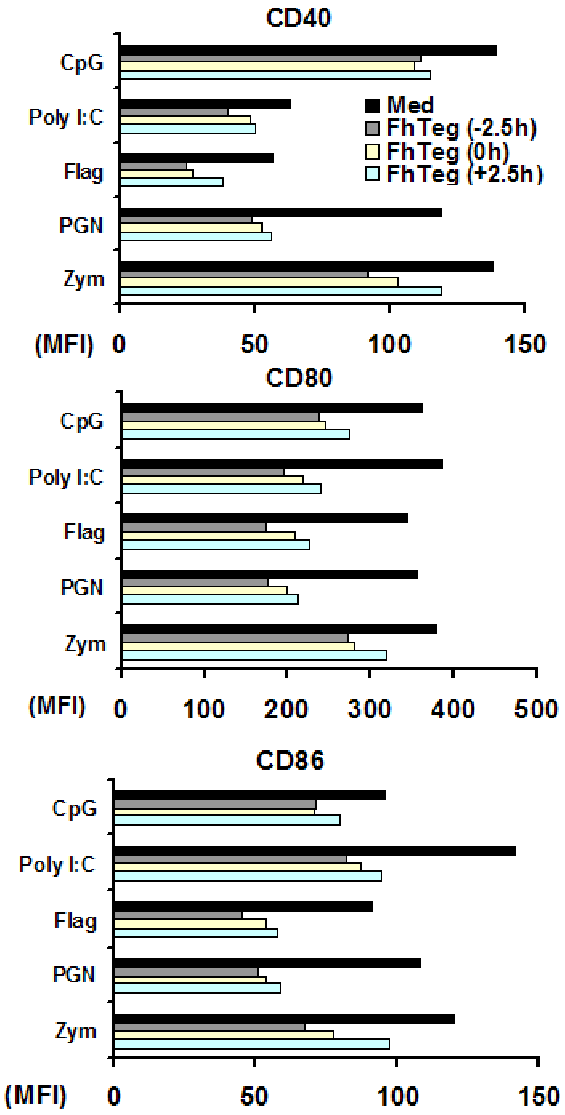

Figure 3.6: FhTeg inhibits TLR-induced DC surface marker expression in a time dependent or independent manner. Cell surface expression of CD40, CD80 and CD86 were measured by flow cytometry on TLR-stimulated DCs for 18h or medium alone. DCs from C57BL/6 mice were treated as in (Fig 3.5), and are gated on a live $CD11c^{+}$ population. Data are the mean values of 3 individual wells (±SEM), and a representative of at least 2 individual experiments.

3.2.5. FhTeg reduces DC phagocytotic ability

Phagocytosis is an integral part of DC biology, and like macrophages and neutrophils, DCs are considered professional phagocytes. However, DCs are distinguished from these cells in that their primary function is not to destroy phagocytised particles but rather to present them to naive T cells thus initiating adaptive immune responses (Savina and Amigorena, 2007). The phagocytic ability of DCs was measured using the CytoSelect™ 96-well Phagocytosis Assay (Cell Biolabs Inc.). Briefly, DCs from C57BL/6 mice were plated at 0.5 x 10^6/ml in complete RPMI and incubated overnight at 37^{o}C to allow adherence to plate. DCs were then treated with FhTeg (10 μg/ml), LPS (100 ng/ml) or zymosan A (5 μg/ml) alone, or with FhTeg and LPS or FhTeg and zymosan at the same time, for 2.5 h before the addition of sheep erythrocytes opsonised by IgG at a ratio of 50:1. Supernatants were aspirated after 1 h and adherent cells washed with sterile PBS to remove non-phagocytosed erythrocytes. Adherent DCs were then lysed, substrate solution added and the amount of engulfed erythrocytes determined by colorimetric assay at absorbance of 610nm. Negative control cells where treated with 2μM Cytochalasin D to block phagocytosis.

Both LPS- and zymosan-stimulated DCs showed a significant increase ($p \leq 0.01$) in engulfed erythrocytes compared with medium controls (Fig. 3.7). To determine whether FhTeg was interfering with phagocytic ability, DCs were cultured with medium, LPS or zymosan in the presence or absence of FhTeg prior to exposure to opsonised erythrocytes. Results demonstrate that incubation with FhTeg alone did not induce phagocytosis of RBCs by DCs (Fig. 3.7). Furthermore, incubation with FhTeg significantly suppressed the phagocytic ability of DCs in response to both LPS and zymosan (Fig.. 3.7; $p \leq 0.01$).

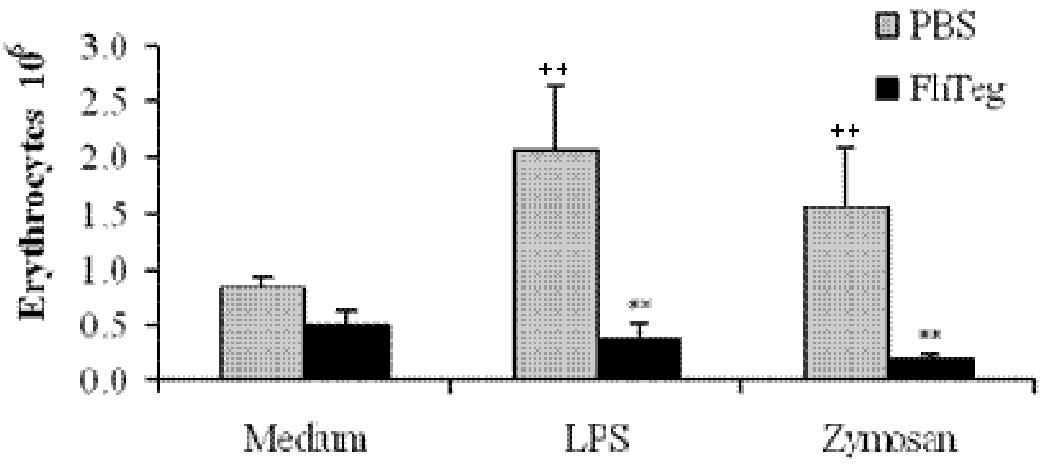

Figure 3.7: Exposure of TLR ligand-activated DCs to FhTeg significantly reduces their ability to phagocytose erythrocytes. DCs from C57BL/6 mice mice were cultured with medium only, FhTeg (10 µg/ml), LPS (100 ng/ml) or zymosan A (5 µg/ml) for 2.5hrs before the addition of opsonised sheep erythrocytes to DCs at a ratio of 50:1. After 1 hr phagocytosis was stopped, cells washed to remove non-opsinised erythrocytes, lysed and amount of engulfed erythrocytes determined by colorimetric assay. Negative control cells where treated with 2µM Cytochalasin D to block phagocytosis (data not shown). Data are the mean values (±SEM) of at least 3 individual experiments. **, $p \leq 0.01$ compared to non-FhTeg treated control group. ++, $p \leq 0.01$ compared to medium control group.

3.2.6. FhTeg and NF-κB signalling

3.2.6.1. FhTeg does not activate the transcription factor NF-κB in DCs by TLRs

The transcription factor NF-κB, is commonly referred to as the master switch for the immune system (Hayden and Ghosh, 2008). NF-κB is expressed in all cell types has multiple critical roles in the regulation the large number of genes that involved in multiple stages of immune responses, including innate immune cell activation, inflammation, lymphocyte activation and DC maturation (Li and Verma, 2002). The mammalian NF-κB family of transcription factors consists of five members; with p65 being one of the most important. With the exception of TLR3 all TLRs work through the MyD88 adapter to activate NF-κB and MAP kinases (O'Neill, 2006). FhTeg mediated activation of the transcription factor NF-κB was investigated in HEK-293

cells which functionally express TLR proteins. FhTeg failed to induce NF-κB when exposed to a panel of TLR expressing cell lines.

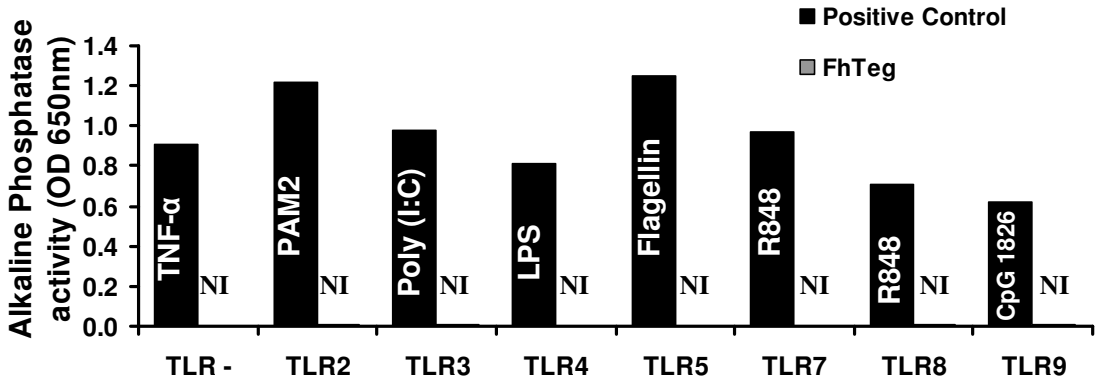

Figure 3.8: FhTeg does not induce activation of TLR-2, 3, 4, 5, 7, 8 and 9, as tested in HEK-293 cell line expression one of each TLR. The HEK-293 cells functionally express a given TLR protein as well as a reporter gene driven by NF-κB promoter which controls secretion of alkaline phosphatase. TLR activity results are given as optical density (OD). Screening was performed in duplicate by challenging the TLR clones with 10μg/ml FhTeg and comparing its ability to induce alkaline phosphatase activity to that of negative controls (non-induced TLR clones) and positive controls (TLR-induced TLR clones). The ligands used for each HEK-293-TLR cell line positive controls were: PAM2 (10 ng/ml) for TLR-2, Poly I:C (100 ng/ml) for TLR-3, LPS K12 (100 ng/ml) for TLR-4, Flagellin (1 μg/ml) for TLR-5, R848 (10 μg/ml) for both TLR-7 and TLR-8, ODN 2006 (10 μg/ml) and ODN 1826 (10 μg/ml) for TLR-9. An HEK293 cell line expressing only the receptor gene was used as a control (TNF-α is a NF-κB activator) (InvivoGen). NI; not induced.

3.2.6.2. FhTeg suppresses NF-κBp65 activation

The transcription factor NF-κB is primarily associated with TLR activation and is associated with regulating the expression of a large number of genes associated with pro-inflammatory processes and Th1 immune responses. The active nuclear form of the NF-κB transcription factor complex is composed of two DNA binding subunits, p65 and p50, both of which are critical for DNA binding (Hayden and Ghosh, 2008). Here, we investigated if FhTeg could modulate NF-κBp65 expression in resting DCs and in DCs matured with LPS. FhTeg alone did not significant increase in NF-κBp65 at any time point tested (data not shown). As anticipated, treatment of DCs with LPS led to a

significant increase in NF-κBp65 at 15 min which gradually decreased over time (Fig. 3.9 A, representative blot). However, densitometric analysis on blots revealed that this was only significant at 15 min (Fig. 3.9 B; $p \leq 0.01$). Pre-treatment of DCs with FhTeg suppressed LPS-induced NF-κBp65 at all time points but suppression was only significant at 15 min (Fig. 3.9 A and 3.9 B; $p \leq 0.01$).

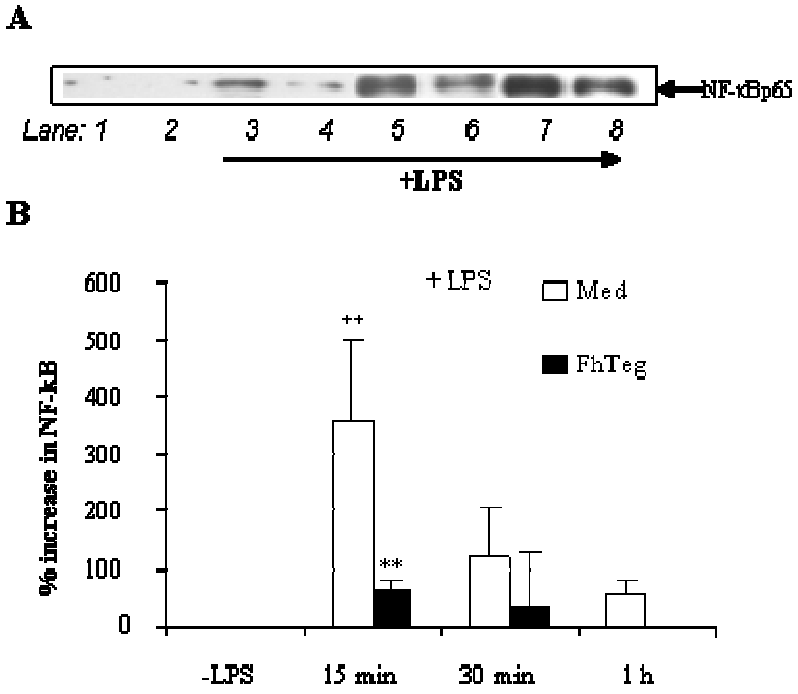

Figure 3.9: FhTeg suppresses NF-κBp65 activation. DCs from C57BL/6 mice were treated with FhTeg (10 µg/ml) or medium 2.5 h prior to stimulation with LPS (100 ng/ml) for 18 h. Control DCs were treated with FhTeg or medium alone. (A) Cells were harvested 0 to 2 h after LPS stimulation, and NF-κBp65 was determined in whole-cell lysates by Western blot analysis. A representative blot is shown. The cells were treated with medium (lane 1), FhTeg (lane 2), LPS for 15 min (lane 3), FhTeg plus LPS for 15 min (lane 4), LPS for 30 min (lane 5), FhTeg plus LPS for 30 min (lane 6), LPS for 1 h (lane 7), and FhTeg plus LPS for 1 h (lane 8). (B) Densitometric analysis was performed on all immunoblots, and NF-κBp65 was expressed in arbitrary units as a percentage increase over the group treated with medium only (control) group. Values that were significantly different ($P \leq 0.01$) from the value for the LPS-treated group are indicated (**). Values that were significantly different ($P \leq 0.01$) from the value for the group treated with medium (control) are indicated (++).

3.2.7. No uniform suppression of MAPKs by FhTeg

Since the suppressive effects of FhTeg are not mediated through TLR4 binding we investigated whether it is targeting a common component of TLR pathways, such as the MAPKs. To address this question, DCs were treated with MAPK inhibitors specific for ERK, p38 and JNK following exposure to FhTeg but prior to stimulation with 5 TLR ligands. Although specific MAPK inhibitors abrogated the FhTeg-mediated suppression for a limited number of cytokines, the effect was not uniform (Table 3.1 (a), (b), (c)). Furthermore, no abrogation of the suppressive effect of FhTeg on cell surface marker expression was detected following the use of inhibitor for ERK, JNK or p38 (Table 3.2).

Ligand	IL-6 (ng/ml)	IL-10 (pg/ml)	IL-12p40 (ng/ml)	IL-12p70 (ng/ml)	TNF-α (ng/ml)	Nitrite (μM/ml)
(a) The effect of JNK inhibitor on cytokine production from TLR ligand-matured DCs						
PGN	19.5 ± 0.3 6.6 ± 1.4***	47.0 ± 3.1 25.3 ± 1.1***	15.8 ± 0.4 19.6 ± 0.2***	0.5 ± 0.0 0.4 ± 0.0***	1.8 ± 0.0 1.9 ± 0.1	10.1 ± 0.1 7.0 ± 0.0***
	5.9 ± 0.2 3.1 ± 0.3	14.9 ± 4.0 11.6 ± 2.4	17.9 ± 0.3 14.4 ± 0.5	0.1 ± 0.0 0.0 ± 0.0	0.1 ± 0.0 0.1 ± 0.0	1.3 ± 0.1 1.6 ± 0.1
Zymosan	15.3 ± 0.1 14.7 ± 0.4	336.1 ± 7.7 285.8 ± 0.7***	15.7 ± 0.1 14.8 ± 0.3***	0.2 ± 0.0 0.2 ± 0.0	6.0 ± 0.1 8.6 ± 0.1***	15.4 ± 0.2 9.7 ± 0.0***
	10.5 ± 0.3 12.5 ± 1.3	72.7 ± 3.5 80.1 ± 2.4	13.9 ± 0.1 11.5 ± 0.1	0.0 ± 0.0 0.1 ± 0.0	2.5 ± 0.1 7.1 ± 0.1	6.5 ± 0.1 3.6 ± 0.1
Poly (I:C)	7.3 ± 0.2 6.4 ± 0.1***	-	10.9 ± 0.1 8.3 ± 0.2***	-	0.2 ± 0.0 0.1 ± 0.0***	5.0 ± 0.1 6.9 ± 0.1***
	4.3 ± 0.1 4.6 ± 0.1		9.7 ± 0.2 7.8 ± 0.2		0.1 ± 0.0 0.1 ± 0.0	3.4 ± 0.0 4.6 ± 0.0
Flagellin	3.5 ± 0.1 2.9 ± 0.2**	-	5.2 ± 0.1 7.8 ± 0.1***	-	-	-
	1.4 ± 0.1 0.3 ± 0.2		4.5 ± 0.2 5.9 ± 0.2			
CpG	22.5 ± 0.4 6.8 ± 0.5***	786.0 ± 13.1 887.2 ± 34.6***	12.1 ± 0.2 11.0 ± 0.4**	3.1 ± 0.0 3.4 ± 0.1***	6.4 ± 0.1 7.2 ± 0.1***	34.6 ± 0.6 33.2 ± 0.1**
	19.4 ± 0.4 7.0 ± 1.5	195.3 ± 1.6 270.7 ± 7.8	11.3 ± 0.3 10.5 ± 0.3	1.9 ± 0.0 2.8 ± 0.0	6.0 ± 0.1 8.6 ± 0.1	20.3 ± 0.2 24.2 ± 0.1

Table 3.1 (a): The effect of JNK inhibitors on cytokine production by DCs induced with a panel TLR ligands with or without FhTeg. DCs from C57BL/6 mice were stimulated with TLR ligand for 18h, with inhibitors added 90 mins before stimulation, FhTeg 2.5 h after and cytokines were measured in supernatants using sandwich ELISA, and nitrite was measured using medium Griess Reagent. Data are the mean values (±SEM) of at least 3 individual experiments. ***, $p < 0.001$. **, $p < 0.01$ compared with TLR ligand stimulated group. Top group: TLR Ligand (top) / TLR Ligand + MAPK Inhibitor (bottom). Bottom group: FhTeg + TLR Ligand (top) / FhTeg + TLR Ligand + MAPK Inhibitor (bottom).

Ligand	IL-6 (ng/ml)	IL-10 (pg/ml)	IL-12p40 (ng/ml)	IL-12p70 (ng/ml)	TNF-α (ng/ml)	Nitrite (μM/ml)
(b) The effect of ERK inhibitor on cytokine production from TLR ligand-matured DCs						
PGN	13.9 ± 0.4 8.9 ± 0.5***	55.3 ± 1.9 11.9 ± 1.4***	29.0 ± 0.8 32.7 ± 1.0***	1.4 ± 0.1 0.6 ± 0.0***	1.4 ± 0.1 0.0 ± 0.0***	18.1 ± 0.2 2.9 ± 0.5***
	8.6 ± 0.3 1.4 ± 0.1	22.8 ± 1.3 12.5 ± 2.1	37.8 ± 2.0 13.6 ± 0.2	0.0 ± 0.1 0.8 ± 0.1	0.0 ± 0.0 0.0 ± 0.0	4.0 ± 0.1 1.4 ± 0.0
Zymosan	15.6 ± 0.3 7.8 ± 0.2***	334.7 ± 9.9 34.0 ± 1.2***	10.5 ± 0.5 10.1 ± 0.3	0.3 ± 0.0 0.0 ± 0.0***	3.6 ± 0.2 0.1 ± 0.0***	23.0 ± 0.2 3.7 ± 0.1***
	11.7 ± 0.4 4.3 ± 0.1	112.5 ± 4.0 28.9 ± 3.4	11.4 ± 0.3 7.2 ± 0.0	0.1 ± 0.0 0.0 ± 0.0	2.7 ± 0.0 0.1 ± 0.1	12.3 ± 0.1 2.0 ± 0.3
Poly (I:C)	7.3 ± 0.5 2.2 ± 0.1***	-	28.3 ± 0.4 5.0 ± 0.0***	-	0.3 ± 0.0 0.0 ± 0.0***	9.6 ± 0.1 1.1 ± 0.0***
	5.7 ± 0.3 1.9 ± 0.1		24.4 ± 0.1 3.5 ± 0.0		0.2 ± 0.0 0.0 ± 0.0	6.4 ± 0.2 1.6 ± 0.5
Flagellin	0.3 ± 0.0 0.0 ± 0.0***	-	5.8 ± 0.1 2.4 ± 0.1***	0.2 ± 0.2 1.7 ± 0.1***	-	-
	0.4 ± 0.0 0.0 ± 0.0		8.5 ± 0.1 2.6 ± 0.1	0.6 ± 0.1 0.5 ± 0.0		
CpG	13.2 ± 0.2 12.6 ± 0.4	479.6 ± 8.2 11.0 ± 0.8***	24.2 ± 0.2 16.8 ± 0.6***	5.4 ± 0.2 1.1 ± 0.1***	4.3 ± 0.1 0.0 ± 0.0***	42.8 ± 0.4 8.1 ± 0.1***
	13.1 ± 0.4 7.8 ± 0.1	183.2 ± 3.6 9.6 ± 1.5	24.7 ± 0.7 25.4 ± 0.2	3.8 ± 0.3 0.9 ± 0.1	4.4 ± 0.1 0.1 ± 0.0	28.9 ± 0.2 3.1 ± 0.1

Table 3.1 (b): The effect of ERK inhibitors on cytokine production by DCs induced with a panel TLR ligands with or without FhTeg. DCs from C57BL/6 mice were stimulated with TLR ligand for 18h, with inhibitors added 90 mins before stimulation, FhTeg 2.5 h after and cytokines were measured in supernatants using sandwich ELISA, and nitrite was measured using medium Griess Reagent. Data are the mean values (±SEM) of at least 3 individual experiments. ***, $p < 0.001$. **, $p < 0.01$ compared with TLR ligand stimulated group. Top group: TLR Ligand (top) / TLR Ligand + MAPK Inhibitor (bottom). Bottom group: FhTeg + TLR Ligand (top) / FhTeg + TLR Ligand + MAPK Inhibitor (bottom).

Ligand	IL-6 (ng/ml)	IL-10 (pg/ml)	IL-12p40 (ng/ml)	IL-12p70 (ng/ml)	TNF-α (ng/ml)	Nitrite (μM/ml)
(c) The effect of p38 inhibitor on cytokine production from TLR ligand-matured DCs						
PGN	13.9 ± 0.4 0.1 ± 0.0***	55.3 ± 1.9 15.1 ± 0.7***	29.0 ± 0.8 16.8 ± 0.6***	1.4 ± 0.1 0.0 ± 0.2***	1.4 ± 0.1 0.0 ± 0.0***	18.1 ± 0.2 0.7 ± 0.1***
	8.6 ± 0.3 0.2 ± 0.0	22.8 ± 1.3 18.6 ± 1.5	37.8 ± 2.0 8.3 ± 0.3	0.0 ± 0.1 0.0 ± 0.6	0.0 ± 0.0 0.0 ± 0.0	4.0 ± 0.1 0.6 ± 0.1
Zymosan	17.6 ± 0.2 0.6 ± 0.0***	739.1 ± 20.7 34.3 ± 3.4***	17.9 ± 0.4 12.9 ± 0.2***	0.2 ± 0.0 0.0 ± 0.0***	3.5 ± 0.1 0.0 ± 0.0***	19.4 ± 0.3 1.4 ± 0.0***
	12.3 ± 0.3 0.4 ± 0.0	139.0 ± 5.3 31.5 ± 9.6	16.5 ± 0.3 2.7 ± 0.1	0.1 ± 0.0 0.0 ± 0.0	2.3 ± 0.2 0.0 ± 0.0	8.5 ± 0.1 0.5 ± 0.1
Poly (I:C)	7.3 ± 0.5 1.3 ± 0.0***	-	28.3 ± 0.4 12.8 ± 0.3***	0.0 ± 0.2 0.3 ± 0.1**	0.3 ± 0.0 0.0 ± 0.0***	9.6 ± 0.1 2.1 ± 0.0***
	5.7 ± 0.3 0.7 ± 0.0		24.4 ± 0.1 2.2 ± 0.1	0.4 ± 0.2 0.0 ± 0.1	0.2 ± 0.0 0.0 ± 0.0	6.4 ± 0.2 0.6 ± 0.2
Flagellin	0.3 ± 0.2 0.0 ± 0.0***	-	5.8 ± 0.1 4.5 ± 0.1***	-	-	-
	0.4 ± 0.0 0.0 ± 0.0		8.5 ± 0.1 3.4 ± 0.1			
CpG	13.2 ± 0.2 3.6 ± 0.2***	479.6 ± 8.2 13.1 ± 3.1***	24.2 ± 0.2 23.8 ± 0.4	5.4 ± 0.2 0.0 ± 0.1***	4.3 ± 0.1 0.0 ± 0.0***	42.8 ± 0.4 12.9 ± 0.1***
	13.1 ± 0.4 1.1 ± 0.1	183.2 ± 3.6 14.1 ± 3.3	24.7 ± 0.7 30.0 ± 0.1	3.8 ± 0.3 0.0 ± 0.3	4.4 ± 0.1 0.1 ± 0.0	28.9 ± 0.2 1.3 ± 0.0

Table 3.1 (c): The effect of p38 inhibitors on cytokine production by DCs induced with a panel TLR ligands with or without FhTeg. DCs from C57BL/6 mice were stimulated with TLR ligand for 18h, with inhibitors added 90 mins before stimulation, FhTeg 2.5 h after and cytokines were measured in supernatants using sandwich ELISA, and nitrite was measured using medium Griess Reagent. Data are the mean values (±SEM) of at least 3 individual experiments. ***, $p < 0.001$. **, $p < 0.01$ compared with TLR ligand stimulated group. Top group: TLR Ligand (top) / TLR Ligand + MAPK Inhibitor (bottom). Bottom group: FhTeg + TLR Ligand (top) / FhTeg + TLR Ligand + MAPK Inhibitor (bottom).

Ligand	CD40	CD80	CD86
(a) The effect of JNK inhibitor on mean fluorescence intensity from TLR ligand-matured DCs			
PGN	119/98 - ↓ 18%	355/345 - ↓ 31%	108/91 - ↓ 23%
	49/46 - ↓ 6 %	176/162 - ↓ 8 %	51/39 - ↓ 24 %
Zymosan	195/130 - ↓ 33%	416/460 - ↑ 11%	118/139 - ↑ 18%
	114/73 - ↓ 34%	214/255 - ↑ 16%	61/68 - ↑ 10%
Poly (I:C)	63/77 - ↑ 22%	387/283 - ↓ 27%	142/111 - ↓ 22%
	40/50 - ↑ 20%	196/164 - ↓ 16%	61/68 - ↑ 12%
Flagellin	57/58 - ↔	343/326 - ↓ 27%	91/60 - ↓ 34%
	24/28 - ↑ 17%	174/140 - ↓ 20%	46/31 - ↓ 33 %
CpG	139/110 - ↓ 21%	361/326 - ↓ 10%	96/78 - ↓ 19%
	111/93 - ↓ 16 %	237/234 - ↓ 1%	71/64 - ↓ 10%
(b) The effect of ERK inhibitor on mean fluorescence intensity from TLR ligand-matured DCs			
PGN	134/111 - ↓ 17%	288/185 - ↓ 36%	103/84 - ↓ 18%
	60/32 - ↓ %	177/87 ↓ 51%	52/45 - ↓ 14%
Zymosan	230/207 - ↓ 10%	517/341 - ↓ 34%	100/55 - ↓ 45%
	158/80 - ↓ 49%	365/157 - ↓ 57%	56/17 - ↓ 70%
Poly (I:C)	129/79 - ↓ 39%	296/170 - ↓ 43%	125/102 - ↓ 18%
	57/32 - ↓ 44%	176/73 - ↓ 59%	77/54 - ↓ 30%
Flagellin	45/38 - ↓ 23%	245/175 - ↓ 29%	63/59 - ↓ 6%
	27/22 - ↓ 19%	148/79 - ↓ 47%	36/38 - ↑ 6% ¥
CpG	179/137 - ↓ 23%	251/218 - ↓ 13%	76/75 - ↔
	136/78 - ↓ 43%	184/151 - ↓ 18%	62/53 - ↓ 15%
(c) The effect of p38 inhibitor on mean fluorescence intensity from TLR ligand-matured DCs			
PGN	136/137 - ↓ 72%	288/126 - ↓ 64%	103/39 - ↓ 61%
	60/19 - ↓ 68%	177/100 - ↓ 44%	51/28 - ↓ 45%
Zymosan	120/127 - ↑ 6%	342/239 - ↓ 30%	80/60 - ↓ 25%
	86/62 - ↓ 28%	297/196 - ↓ 34%	47/25 - ↓ 47%
Poly (I:C)	134/37 - ↓ 72%	296/156 - ↓ 47%	128/80 - ↓ 36%
	57/20 - ↓ 65%	176/84 - ↓ 52%	77/37 - ↓ 52%
Flagellin	45/31 - ↓ 31%	245/126 - ↓ 49%	63/38 - ↓ 40%
	27/17 - ↓ 37%	148/93 - ↓ 37%	36/29 - ↓ 19%
CpG	179/56 - ↓ 69%	251/215 - ↓ 13%	76/60 - ↓ 21%
	136/27 - ↓ 80%	184/120 - ↓ 35%	61/29 - ↓ 52%

Notation:

TLR Ligand / TLR Ligand + MAPK Inh
FhTeg + TLR Ligand / FhTeg + TLR Ligand + MAPK Inh

Table 3.2: The effect of MAPK inhibitors on cell surface marker expression by DCs induced with a panel TLR ligands with or without FhTeg. DCs from C57BL/6 mice were stimulated with TLR ligands for 18h; with inhibitors and FhTeg added 90 mins before and 2.5 h before stimulation respectively. Cell surface expression of CD40, CD86, and CD80 were measured by flow cytometry. Data are the mean values (±SEM) of at least 3 individual experiments, showing mean fluorescent intensity percentage decrease/increase from TLR ligand stimulated DCs. ↓: Significantly down regulated, ↑: Significantly up-regulated, ↔: No significant change. Value considered significant when $p < 0.05$ compared to stimulated DCs with no inhibitor.

3.2.8. *In vivo* T-Cell priming

3.2.8.1. T-cell cytokine profiles and cell proliferation

Since FhTeg significantly suppressed cytokine production and costimulatory marker expression from stimulated DCs, its effects on the ability of differentially activated DCs to prime T-cell responses in sdLN was investigated in DO11.10 mice. DCs were cultured with OVA peptide in the presence or absence of FhTeg prior to their inoculation into naive DO11.10 mice. T-cell priming was measured 7 days later by re-stimulation of sdLN cells with OVA peptide and PMA/anti-CD3. DCs primed with medium induced IFN-γ, IL-10 and IL-4 production from sdLN cells in response to OVA stimulation (Fig. 3.10). Although the levels of IL-4 and IL-10 were low in comparison to PMA/anti-CD3-stimulated cells, they were comparable to that published in the literature (Jenkins and Mountford, 2005). DCs primed with FhTeg induced significantly less OVA-specific IFN-γ from sdLN cells than control DCs primed with medium (Fig. 3.10; $p \leq 0.05$) indicating that DCs exposed to FhTeg suppress local Th1 responses *in vivo*. Unlike other helminth antigens such as *S. mansoni* 0-3hRP (Jenkins and Mountford, 2005), in this model system FhTeg did not appear to drive local Th2 responses, with IL-10 and IL-4 levels from DC-FhTeg recipients similar to those of DC-Med recipients.

Interestingly, sdLN cells from DC-FhTeg recipients, stimulated with PMA/anti-CD3, produced significantly decreased levels of all cytokines (Fig. 3.10; $p \leq 0.05$) when compared to cells from DC-medium recipients indicating that FhTeg had a general suppressive effect on the non specific T-cell responses. This suppressive effect was also observed when we examined proliferation, since sdLN cells from DC-FhTeg recipients proliferated significantly less in response to OVA stimulation than cells from DC-Med recipients (Fig. 3.10; $p \leq 0.01$). However, there was no significant difference in proliferation of cells stimulated with PMA/anti-CD3 (Fig. 3.10).

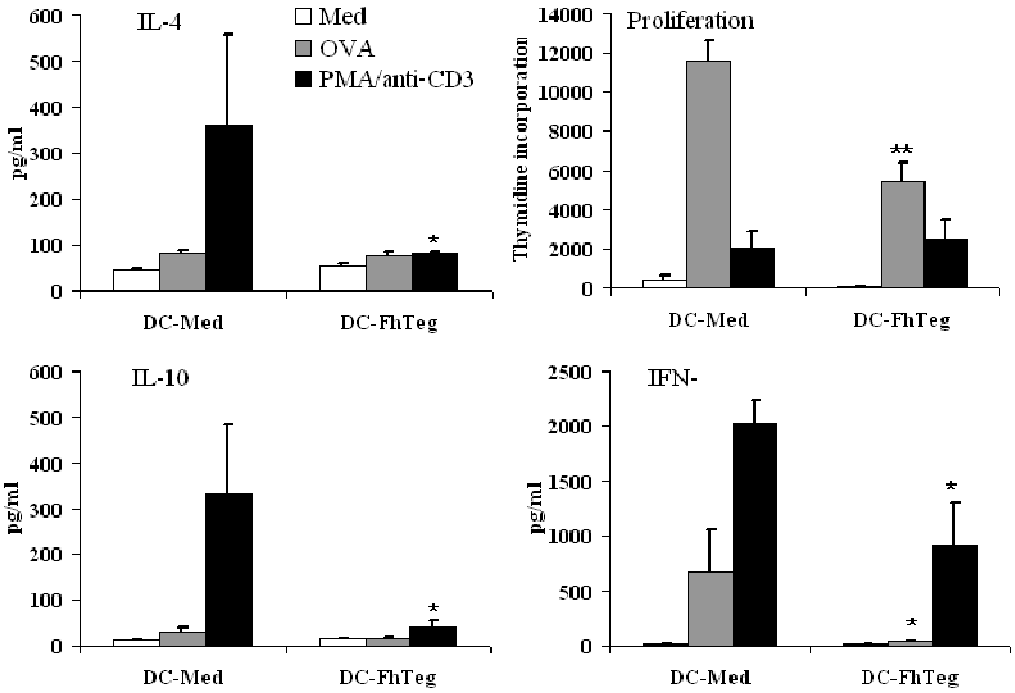

Figure 3.10: FhTeg alters the ability of DCs to prime T-cell responses. DCs from DO11.10 mice were cultured with OVA (100 nM) in the presence or absence of FhTeg (10 µg/ml) overnight at 37°C. Stimulated DCs were thoroughly washed and subcutaneously injected over the sternum of naïve DO11.10 mice. After 7 days, sdLN cells were removed for re-stimulation *in vitro* with OVA (500 nM) or with PMA (25 ng/ml) and anti-CD3 (1 µg/ml). After 72 h, supernatants were analyzed by ELISAs for IFN-γ, IL-10, and IL-4. Data are the mean values (±SEM) of three individual wells from four individual mice and are representative of three experiments. Values that were significantly different ($P \leq 0.01$) from the value for the appropriate group of DCs treated with medium (DC-Med) are indicated as follows: *, $P \leq 0.05$; **, $P \leq 0.01$.

3.2.8.2. Flow cytometry analysis

CD28 and CTLA-4 are related receptors that differentially regulate T-cell activation. While CD28 enhances T-cell proliferation and survival, in contrast CTLA-4 inhibits T-cell responses (Green, 2000). We measured the expression of CD28 and CTLA-4 on CD4 positive cells and found that FhTeg did not impact on the expression of these molecules (Fig. 3.11). The suppression of cell proliferation or cytokine production can therefore not be attributed to changes in the expression of these cell surface markers.

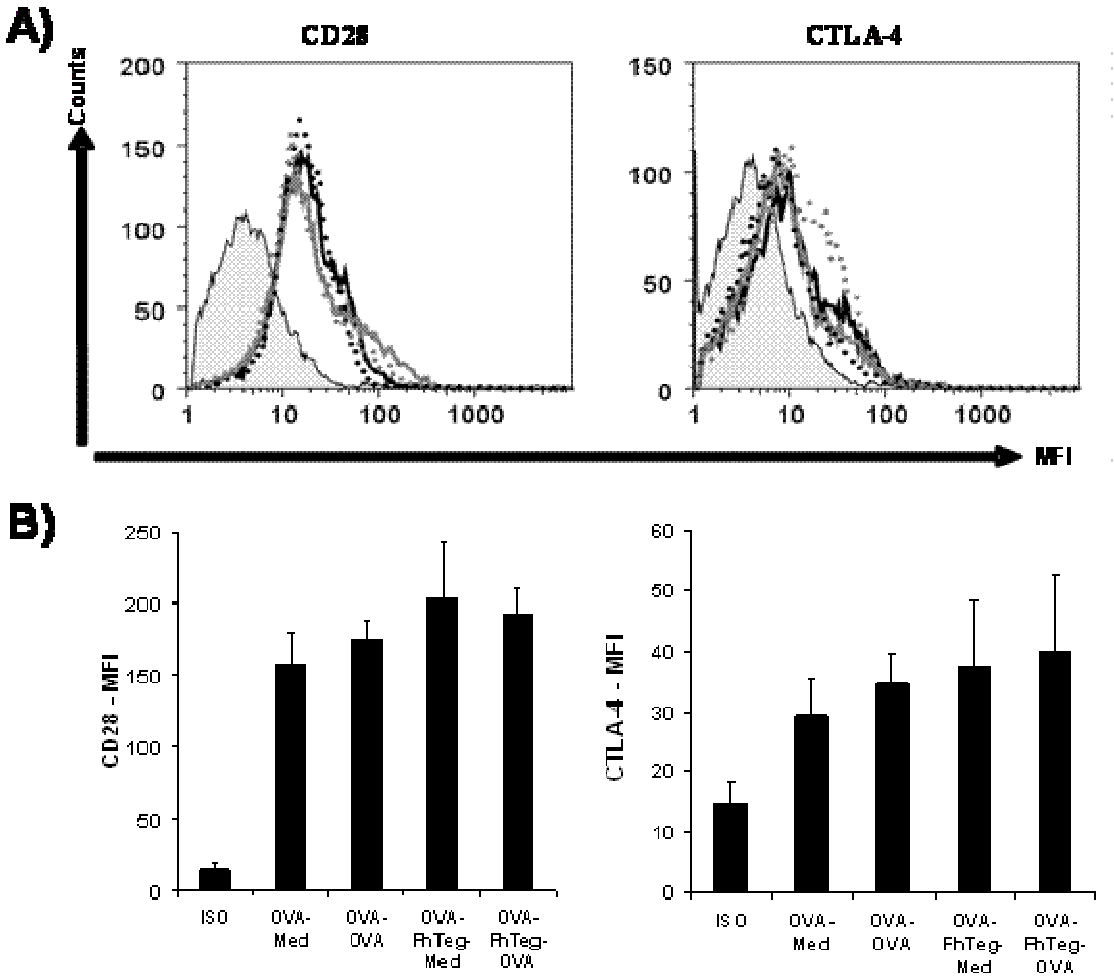

Figure. 3.11: The expression levels of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) on CD4 T cells is unchanged following OVA antigen specific activation. Cells from DO11.10 mice were harvested and analysed by three-colour flow cytometry for CD4, CD28 and CTLA-4. (A) The histograms show representative data of the surface expression of CD28 and CLTA-4 on CD4+ cells from sdLN after 72 h *in vitro* culture; OVA-med (black line), OVA-OVA (grey line), OVA-FhTeg-med (black dotted line) OVA-FhTeg-OVA, (grey dotted line), and isotype controls (solid histogram). (B) Combined data from 3 independent experiments showing the mean fluorescent intensity (MFI) for surface expression of CD28 and CLTA-4 on CD4+ cells from sdLN after 72 h *in vitro* culture.

3.3. Discussion

This present study demonstrates the suppressive effect of *F. hepatica* tegument on DC maturation and function. As discussed in the introduction helminth infections are associated with a modified Th2 response. Studies have shown that helminth-derived antigens, such as the filaria-derived antigen, ES-62, which induces IL-12p40, IL-6, and TNF-α production from DCs, can partially induce DC maturation (Goodridge *et al*.,

2005) and *S. mansoni* larva antigen, which can induce low, yet significant, increases in IL-12p40 and IL-6 from DCs (Jenkins and Mountford, 2005). Despite the inactivation or partial activation of DCs, many helminth-matured DCs are still capable of driving potent Th2 immune responses (Jenkins and Mountford, 2005, Whelan *et al*., 2000).

The tegumental coat of *F. hepatica* is a metabolically active layer that is in intimate contact with the host tissues and body fluids. It is here that much of the immune interplay between the fluke and host takes place. The tegument is shed every 2 to 3 h during the course of infection, thus representing a constant source of antigen in direct contact with the host's immune cells. Studies have shown that by shedding its tegument, the fluke can also shed immune complexes that have formed on the surface, thereby evading a damaging immune response, and the shed antigen acts as a decoy "mopping up" host immune cells (Halton, 2004, Mulcahy, 1999).

Previous work had highlighted the modulatory potential of FhTeg. Firstly, the role of FhTeg *in vivo* in a model of septic shock was examined. Mice injected with FhTeg either prior to or following LPS exposure demonstrated significantly suppressed levels of serum IL-12p70 and IFN-γ, but not IL-12p40 (Hamilton *et al*., 2009). The suppression of proinflammatory cytokines *in vivo* carries implications for the control of concurrent infections with *F. hepatica*, as studies have shown that it suppresses IFN-γ, altering the immune response to tuberculosis (O'Neill *et al*., 2001) and delaying bacterial clearance in concurrent infections with *Salmonella enterica* serovar Dublin or *Bordetella pertussis* (Aitken *et al*., 1981, Brady *et al*., 1999). Secondly, due to their central role in priming immune adaptive responses the effect of FhTeg on DCs was examined. Previously published studies have demonstrate that exposure of immature DCs to helminth-derived antigens failed to induce DC maturation (Balic *et al*., 2004, Perona-Wright *et al*., 2006b). Similarly, FhTeg alone did not have any effect on DC maturation as these cells did not secrete cytokines or increase costimulatory marker

expression in response to FhTeg. Also, FhTeg was shown not to affect the viability or proliferation of DCs as shown by Annexin staining and MTT/MTS assays. Thirdly, FhTeg treated DCs exhibited marked hypo-responsive to LPS stimulation, both in a reduction in a selected number of cytokines and a down regulation of costimulatory markers.

So Could FhTeg interfere with activation of DCs to a wider range of TLR ligands? FhTeg was shown to render DCs hyporesponsive to a range of TLR ligands with significant decreases in cytokine production of IL-12p70, IL-10, IL-6, TNF-α, and nitrite. Similar to FhTeg/LPS-treated DCs there was a decrease in costimulatory marker expression (CD80, CD86, and CD40). As with LPS, It was shown that cytokine suppression by FhTeg was selective, given that in general IL-12p40 and IL-23 production were not affected. This was reflected in zymosan treated DCs (data not shown). RNA analysis of LPS activated DCs treated with FhTeg showed reduced levels of IL-12p35 subunit is down regulated but no the IL-12p40 subunit compared to LPS treated controls (Hamilton *et al*., 2009). This is of interest given that the p40 subunit is shared by IL-12 and IL-23 and both cytokines belong to the same family of proinflammatory cytokines (Waibler *et al*., 2007). Also, the timing of exposure of DCs to FhTeg did not affect the significant suppressive effects in response to activation to a range of TLR ligands. But the suppressive effects of FhTeg on zymosan, CpG and poly-(I:C)-activated DCs decreased inversely with increasing time after TLR activation. This effect was not observed for PGN-induced cytokine production. This indicates that the suppression of DC TLR activation by FhTeg is time dependent or time independent depending on the TLR ligand. But since suppression always remains significantly reduced, the results suggest that FhTeg does not compete with LPS or any other TLR ligand tested for binding, and that FhTeg is not exerting its effects through any TLR.

Similarly to FhTeg, FhES failed to induce maturation of DCs. The ability of FhES to interfere with TLR activation of DCs was also investigated. Unlike FhTeg, FhES had no effect on maturation of cytokine production induced by TLR ligands. Even thought FhTeg and FhES have been shown to share commonality in the presence of some helminth-derived antigens ((Robinson *et al*., 2009) and R. M. Morphew and P. M. Brophy, unpublished data), the immunomodulatory property to suppress DC activation in response to TLR ligands is unique to FhTeg.

The ability of FhTeg to decrease proinflammatory cytokines is in keeping with a helminths need to suppress inflammatory processes (Goodridge *et al*., 2005), and since IL-12p70 is an important polarizing cytokine known to drive Th1 differentiation (Trinchieri, 2003), this suppression may result in the Th1 suppression observed during *F. hepatica* coinfections. The decrease in IL-10 observed was not anticipated, since this cytokine is traditionally associated with an anti-inflammatory or regulatory response (Moore *et al*., 2001). However, recent studies show IL-10 production by Th1 cells, emphasizing the versatile role that IL-10 plays during infection (O'Garra and Vieira, 2007).

The MAPK pathway is a highly conserved pathway involved in the initiation of DC maturation through all known TLR ligands (Dowling *et al*., 2008). Activation of the three main mammalian groups, JNK, ERK, and p38, culminates in the release of cytokines from DCs following the downstream activation of a signalling cascade involving adaptor proteins, such as MyD88 and Mal (Nakahara *et al*., 2006). Helminth-mediated suppression of cytokine production by antigen-presenting cells has been shown to be modulated by MAPK pathways. For example, ES-62-mediated suppression of LPS-induced IL-12p40 by macrophages can be reversed with the addition of the ERK inhibitor (Goodridge *et al*., 2003), and *S. mansoni* egg antigen dramatically reduces LPS-stimulated phosphorylation of p38 (Kane *et al*., 2004). However, in the present

study, we have shown that FhTeg does not target these pathways, since MAPK pathways are involved in the suppressive effect of only a small number of select cytokines. Instead, we found that FhTeg targeted the transcription factor NF-κBp65, one of the active subunits of the NF-κB complex involved in NF-κB binding to DNA. This effect on downstream signalling events could explain the observed decrease in proinflammatory cytokines demonstrated in this study. Activation of NF-κB is also inhibited by other parasites, such as *Brugia malayi* (Semnani *et al*., 2008) and *Toxoplasma gondii* (Butcher *et al*., 2001), where NF-κB translocation is blocked from entering the nucleus. We have yet to determine whether FhTeg can suppress other members of the NF-κB family (such as the p50 subunit) or other transcription factors, such as IRF3, which are also activated following LPS stimulation.

The effect of FhTeg on DC function was further confirmed by the demonstration of reduced phagocytic ability by FhTeg-primed DCs which would impact on the development of the adaptive immune response. Adaptive immune responses were then investigated by T-cell priming studies. In addition to cytokine production, co-stimulation is essential for the successful activation and differentiation of naïve $CD4^+$ T cells, and similarly, the suppression of costimulatory markers could influence Th1 cell differentiation. This was confirmed in the T-cell priming studies, which demonstrated that FhTeg-primed DCs significantly suppressed local Th1 immune responses. These results indicate that exposure to FhTeg, and the subsequent lack of cytokine production and costimulatory molecule expression, can interfere with the function of DCs and their ability to prime naïve T cells. Thus, from these results it was hypothesised that that FhTeg maintains the DCs in an immature state, impairing their function and ultimately modulating the development of adaptive T-cell responses. Our data support previous findings which demonstrate that *F. hepatica* infection and antigens suppress Th1 immune responses *in vivo* (Brady *et al*., 1999, Flynn *et al*., 2007). Furthermore, the

suppression of Th1 responses is likely to be a survival mechanism for the worm, since vaccine trials in domestic livestock have shown the importance of inducing strong Th1 immune responses in the host to protect against challenge infection (Mulcahy *et al*., 1998).

While the exact mechanism of FhTeg-mediated suppression remains to be elucidated, the timing of exposure of DCs to our antigen did not affect the suppressive effects, suggesting that it does not compete with LPS for binding and that it is not exerting its effects through TLR4 itself. This was confirmed by the fact that FhTeg is still effective in DCs from TLR4 mutant and knockout mice (Hamilton *et al*., 2009). Furthermore, FhTeg was able to suppress the effects of all TLR ligands tested (which included both MyD88-dependent and -independent pathways), in addition to non-TLR ligands. While we would not directly rule out the involvement of other TLRs, it seems likely that FhTeg utilizes a pathway common to these receptors, particularly since it did not activate HEK-296 cells functionally expressing a range of TLRs.

In summary, we report that *F. hepatica* tegumental antigen modulates DC maturation and function, which may go some way to explaining its reported immunomodulatory properties *in vivo*. FhTeg is a heterogeneous group of molecules, and preliminary proteomic studies have already pointed to a number of potential molecules that can render DCs hyporesponsive. These proteomic studies also show that the profile observed for the tegument differs from that of its *F. hepatica* ES products (R. M. Morphew and P. M. Brophy, unpublished data). Although the precise immunological scenario is not completely understood it is clear that FhTeg maintains the DCs in an immature state, impairing their function and the subsequent development of adaptive immunity. We have yet to determine whether FhTeg drives Th2 or T-regulatory immune responses, but the effect of FhTeg most closely aligns with the inhibition hypothesis, in which activated signalling pathways may intersect and inhibit one another (MacDonald

and Maizels, 2008). Given the powerful modulatory effect that FhTeg exhibits, understanding its exact mechanisms may lead to the development of novel immune therapeutics for the treatment of Th1-mediated inflammatory diseases (Elliott *et al*., 2003, Summers *et al*., 2005).

<u>Chapter 4:</u> Major secretory antigens of the helminth *Fasciola hepatica* activate a suppressive DC phenotype that suppresses Th17 cells but fails to activate Th2/Treg immune responses.

4.4. Introduction

Until relatively recently, few PAMPs of helminth origin have been identified (Zaccone *et al*., 2006) (Table 1.1). For this reason, it is important to investigate the interaction of defined helminth-derived molecules with DCs and elucidate the mechanism by which they alter DC function. The second reason for the use of defined helminth-derived molecules is the diverse range of DC phenotypes and responses that can be induced using complex mixtures of helminth antigens. DCs activated by helminths induce a modified form of DC maturation, lacking the classical activation markers. In some cases these markers can be absent (Kane *et al*., 2004), but most commonly are expressed at low levels (Johnston *et al*., 2009). Despite the limited maturation, helminth-primed DCs can nevertheless activate naïve T-cells (Maizels *et al*., 2004). DCs exposed to a soluble preparation of whole pathogen native antigens such as the Th2-priming Schistosoma larval preparation (0-3hRP) (Jenkins *et al*., 2005, Jenkins and Mountford, 2005) or SEA (MacDonald *et al*., 2001) and either co-cultured with navie T-cells *in vitro* or injected into mice can polarise T-cell responses towards a Th2 phenotype. SEA is of particular interest since fail to activate DCs yet these antigens are capable of driving Th2 immune responses (Cervi *et al*., 2004, MacDonald *et al*., 2001, van Liempt *et al*., 2007). Similar findings have been reported with and excretory/secretory (ES) material from *Nippostrongylus brasiliensis* (Balic *et al*., 2004). However, Segura *et al* (2007) found that adoptive transfer of DCs treated with ES from *Heligmosomoides polygyrus* resulted in suppression of both Th1 and Th2 responses in recipient mice (Segura *et al*., 2007). The same DCs promoted the differentiation of T-cells with a regulatory phenotype and an ability to suppress effector $CD4^+$ cell proliferation and cytokine secretion.

While, the above mentions preparations are heterogeneous mixes of molecules, the exact antigens from these preparations that interact with DCs have yet to be identified. However, single antigens from native helminth preparations have been shown to drive similar responses. The single filarial nematode-secreted product ES-62 from *Acanthocheilonema viteae* binds to TLR4 inducing low levels of IL-12p40, TNF-α and IL-6 from DCs (Goodridge *et al*., 2001). Similarly, purified antigens from *Echinococcus granulosa* (Rigano *et al*., 2007) and *A. suum* (Silva *et al*., 2006) partially activate DCs that can subsequently prime Th2 immune responses. In this study, we examined the interactions of recombinant *Fasciola* cathepsin Ls (CL) and a recombinant sigma class glutathione S transferase (GST) with DCs to determine if these are novel helminth PAMPs. ES products have been shown to modulate T cell responses in mice (Donnelly *et al*., 2008) and as stated previously, both are major components of *F. hepatica* ES. *F. hepatica* cathepsins are papain-like cysteine peptidases which facilitate the entry into and migration of the parasite through the host tissue, enhance virulence and suppress the host immune effector cell responses (Robinson *et al*., 2008). One of the predominant secreted products, a cathepsin L1 cysteine protease (FhCL1), suppressed the onset of protective Th1 immune responses in mice to bacterial infections and prevented the development of a Th1 response to vaccination (O'Neill *et al*., 2001, Collins *et al*., 2004). Mature *F. hepatica* only secrete cathepsin L cysteine proteases, highlighting the importance of these molecules in the parasite pathology. Another major antigen, comprising 4% of the ES of *F. hepatica*, is the anti-oxidant glutathione transferase (FhGST) (Chemale *et al*., 2006) which in dimeric form significantly inhibited the proliferation of rat spleen cells in response to ConA stimulation *in vitro* (Cervi *et al*., 1999). GST's neutralise oxygen radical intermediates released by innate immune cells thus protecting the parasite from host immune attack. They can be subdivided into seven species independent classes which include the sigma and mu class

(Torres-Rivera and Landa, 2008). We investigate the ability of rFhGST-si and rFhCL1 to induce an innate immune response in comparison with the archetypal TLR ligands such as LPS and ability of each in driving the immune responses.

4.5. Results

4.5.1. rFhCL1 and rFhGST-si but not rvFhCL1 or rFhGST-mu induced partial activation of DCs

To assess the immunomodulatory properties of the secreted enzymes of *F. hepatica,* functionally active recombinant sigma class GST (rFhGST-si), mu class GST (rFhGST-mu) and cathepsin L1 (rFhCL1) were synthesized (Collins *et al*., 2004, LaCourse *et al*., 2009). In addition, a conformationally intact proteolytically-inactive variant of cathepsin L1 (rvFhCL1), which has the active site cysteine replaced with a glycine, was also produced (Collins *et al*., 2004). All recombinant preparations were deemed to be endotoxin free as assessed using the Pyrogene® endotoxin detection system (Cambrex). Initially, a series of dose response analyses were completed to determine the optimum quantity of recombinant for subsequent analyses. A concentration of 10 μg/ml for each antigen was selected since it did not affect DC viability or growth and stimulated optimum cellular responses as compared to those induced by the positive control, LPS (Fig 4.1 B, C).

We demonstrated that DCs stimulated with rFhCL1 secreted significant levels of IL-12p40 and IL-6 not IL-12p70, TNF-α, nitric oxide, IL-23 or IL-10 (Fig. 4.1 A-C and data not shown) compared to unstimulated cells. This activation of DCs was dependent upon the dose of rFhCL1 delivered, with increasing doses showing correspondingly increased secretion of IL-12p40 and IL-6 (Fig. 4.1 D, E), indicating a requirement for enzymatic activity. Consistent with this, was the observation that stimulation of DCs with the enzymatically inactive variant rvFhCL1 did not induce the secretion of any

cytokines measured (Fig. 4.1 A-C and data not shown). Treatment of DCs with rFhGST-si induced an identical cytokine secretion profile to rFhCL1 (Fig 4.1 A-C; $p \leq 0.001$) although the quantity of IL-6 measured was far greater than that seen following exposure to rFhCL1. Similar to rFhCL1, the secretion of these cytokines was also dose-dependent (Fig. 4.1 F, G). However, rFhGST-mu did not induce the secretion of any cytokines (Fig. 4.1 A-C and data not shown) suggesting that the enzyme activity required for DC activation by Fasciola-GST may be specific to distinct sub-classes.

Following these results we went on to determined if these enzymes could induce elevated expression of co-stimulatory markers CD40, CD86, CD80 and MHC II on DCs. Both rFhCL1 and rFhGST-si stimulated a significant increase in CD40 expression compared to the isotype control (Fig. 4.1 H). The expression of CD80, CD86 (Fig. 4.1 H) or MHC II (data not shown) was not altered following exposure to these recombinant proteins. Both rvFhCL1 and rFhGST-mu failed to alter the expression levels of all cell surface markers measured (data not shown). We have given due consideration to the possibility that residual endotoxin (the major component of which is LPS) in the antigen preparations contributed to the activation of DCs described here. However, all the data indicates that the effects seen are due to the activity of the recombinant proteins and not the presence of endotoxin. For example, the phenotype of DC activation described for the rFhCL1 and rFh-GST-si is different to that induced by LPS (Fig. 4.1 A-C, H). These enzymes were produced from the same expression systems as rvFhCL1 and rFhGST-mu, respectively, both of which had no measurable effect on the activation status of DCs suggesting that any effect seen is unlikely to be due to endotoxin contamination. Finally, the effects of the rFhCL1 and rFhGST-si on DC activation were shown to be heat labile since they were no longer observed following boiling (Fig. 4.2; $p \leq 0.001$) which contraindicates the involvement of endotoxin.

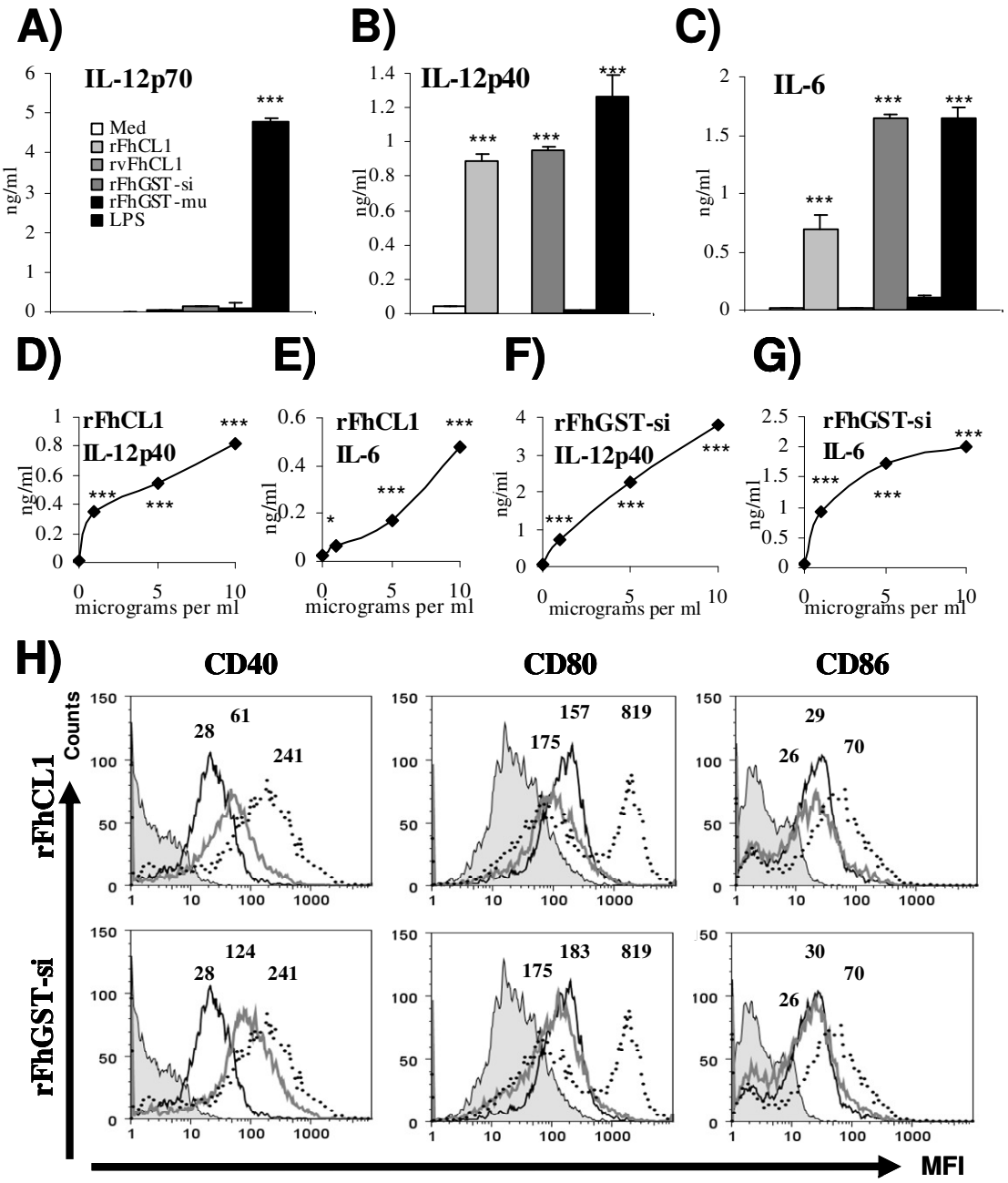

Figure 4.1: Exposure of DCs to rFhCL1 and rFhGST-si but not rvFhCL1 and rFhGST-mu induces partial activation of DCs. *(A-C)* DCs from C57BL/6 mice were cultured with medium (Med), rFhCL1 (10 µg/ml), rvFhCL1 (10 µg/ml), rFhGST-si (10 µg/ml), rFhGST-mu (10µg/ml) or LPS (100 ng/ml) for 24 h, and the production of IL-12p40, IL-6 and IL-12p70 in supernatants was determined by ELISA. *(D, E)* DCs were cultured with a range of concentrations of rFhCL1 or *(F, G)* rFhGST-si for 24h and the levels of IL-12p40 and IL-6 measured by ELISA. Data are the mean values (±SEM) of at least 3 individual experiments. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ compared to unstimulated cells (not shown). *(H)* Cells were harvested and analysed by four-colour flow cytometry for CD40, CD80 and CD86. Cells were gated on $CD11c^+$ cells. The histogram shows isotype control (filled histogram), unstimulated cells (black line), rFhCL1 or rFhGST-si-stimulated cells (grey line) and LPS (dotted line). Numbers represent mean fluorescence intensity from 5 different experiments.

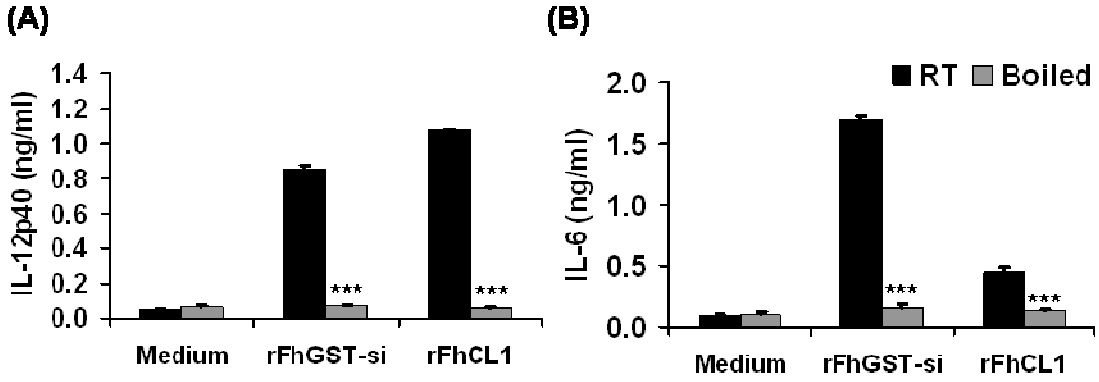

Figure 4.2: Induction of partial activation of DCs by rFhGST-si and rFhCL1 is heat labile. DCs from C57BL/6 mice were cultured with culture medium, rFhCL1 or rFhGST-si (10 μg/ml) brought to room temperature of 100^0C for 15 mins. 18h after the addition of antigens IL-6 and IL-12p40 were measured in the supernatants by sandwich ELISA. Data are the mean values of 3 individual wells (±SEM), and a representative of at least 2 individual experiments. ***, $p \leq 0.001$ compared with positive untreated control.

4.5.2. The partial activation of DCs by rFhCL1 and rFhGST-si is TLR4-dependent

Since previous reports have shown that helminth antigens can induce partial maturation in DCs through TLR4 activation (Jenkins *et al*., 2005, Goodridge *et al*., 2005), we went on to determine if the activation of DCs by rFhCL1 and rFhGST-si was dependent upon the TLR4 pathway. The secretion of IL-12p40 and IL-6 by rFhCL1 and rFhGST-si was significantly reduced in the absence of the TLR4 receptor (Fig. 4.3 A, B; $p \leq 0.001$). While a low level of IL-6 secretion was measured in response to rFhCL1 in the TLR4 deficient DCs, this was not significant compared to cells stimulated with culture medium. Similar to the cytokine results, rFhCL1, rFhGST-si and LPS failed to induce any alteration in CD40 expression in the absence of TLR4 (Fig. 4.3 C). As expected, TLR4 deficient DCs failed to respond to LPS.

In addition, zymosan A, a TLR4-independent, TLR2 dependent agonist, induced the secretion of comparable quantities of IL-12p40 and IL-6 in both wild type and TLR4 deficient strains of mice, confirming the integrity of TLR signalling in the knockout DCs.

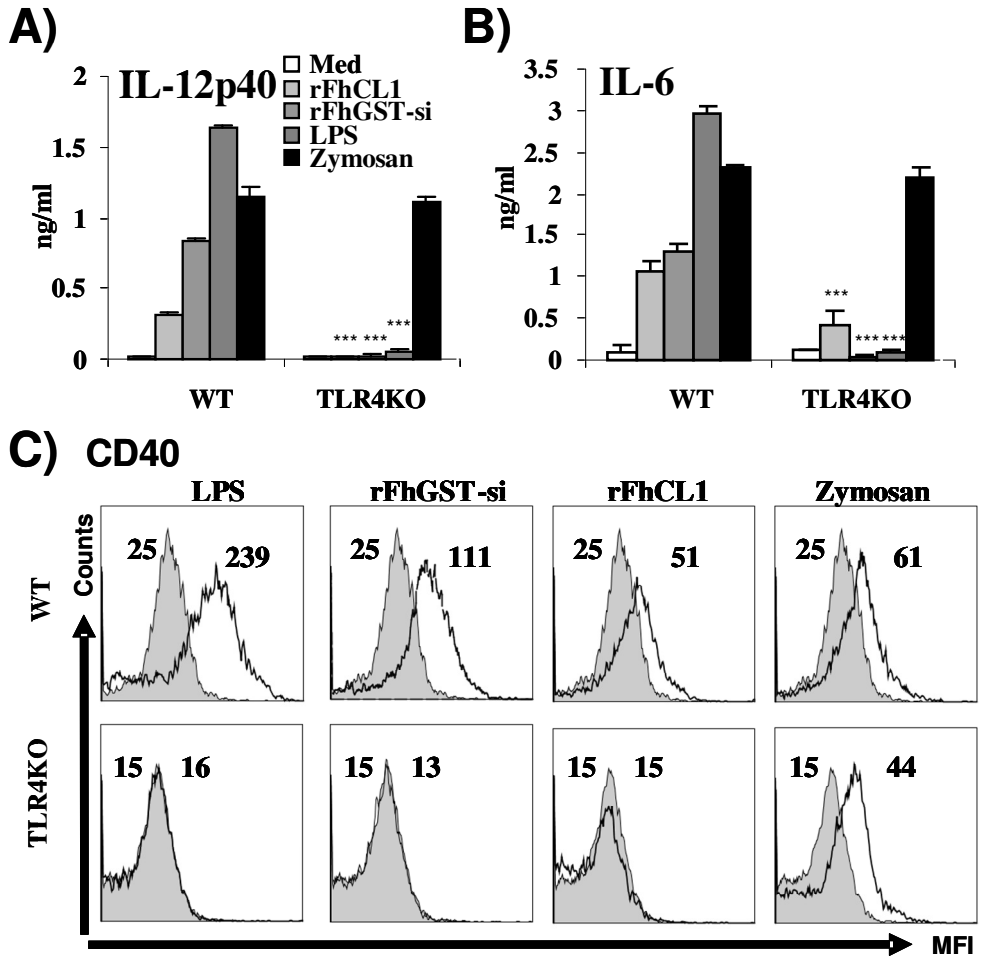

Figure 4.3: The partial activation of DCs by rFhCL1 and rFhGST-si is TLR4 dependent. DCs from C57BL/6 (wildtype background strain; WT) and TLR4 deficient (TLR4 KO) mice were cultured with medium (Med), rFhCL1 (10 μg/ml), rFhGST-si (10 μg/ml), zymosan (5 μg/ml) or LPS (100 ng/ml) for 24 h and the quantities of IL-12p40 (*A*) and (*B*) IL-6 in supernatants determined by ELISA. Data are the mean values (±SEM) of at least 3 individual experiments. ***, $p \leq 0.001$ compared to control WT mice. *(C)* DCs were harvested and analysed by four-colour flow cytometry, gated on $CD11c^+$ cells, for the expression of CD40. In each histogram, the stimulated cells are represented by the black line and the isotype control by the filled peak. Numbers represent mean fluorescence intensity from three separate experiments.

These results confirm that activation of DCs by rFhCL1 and rFhGST-si is dependent upon the presence of TLR4. To support this observation, the same analyses were repeated in C3H/HeN and C3H/HeJ mice which exhibit a point mutation in the TIR domain of TLR4 (Poltorak *et al*., 1998), so although they express the receptor, they fail to produce a response to LPS. DCs isolated from C3H/HeN mice gave similar results to DCs from C57BL/6 mice when stimulated with either rFhCL1 or rFhGST-si. DCs isolated from C3H/HeJ mice showed no increase in the expression of CD40 or secretion IL-6 or IL-12 p40 in response to rFhCL1, rFhGST-si or LPS (data not shown).

4.5.3. rFhCL1 and rFhGST-si induce the secretion of chemokines from DCs

Recently, it has been shown that Th1 promoting DCs selectively express elevated MIP1-α and promotes recruitment of Th1 cells (Gafa *et al*., 2007, Lebre *et al*., 2005) while MIP-2 a chemokine associated with helminth infection (Muller *et al*., 2003), promotes the recruitment of Th2 cells. Given the role played by these chemokines in the selective homing of immune cells, we sought to characterize the profile of MIP1-α (CCL3) and MIP-2 (CXCL2) chemokines produced by DCs following treatment with rFhCL1 and rFhGST-si. Results demonstrate that rFhCL1 or rFhGST-si induced both MIP1-α and MIP-2 from DCs (Fig. 4.4 A, B). However, these enzymes again showed a differential profile compared to LPS with both enzymes inducing significantly less MIP1-α than LPS but comparable levels of MIP-2. The polarised secretion of MIP-2 by these enzymes could influence a Th2 micro-environment by directly recruiting Th2 cells to the site of infection. The secretion of MIP1-α was TLR4 dependent since the production of this chemokine was absent in DCs isolated from TLR4 deficient mice. In contrast, the secretion of MIP-2 appeared to be partially dependent upon TLR4 (Fig. 4.4 A, B; $p \leq 0.05$) suggesting the involvement of a co-receptor. Further studies are required to identify this receptor and also characterise a role for MIP-2 in Fasciola infection.

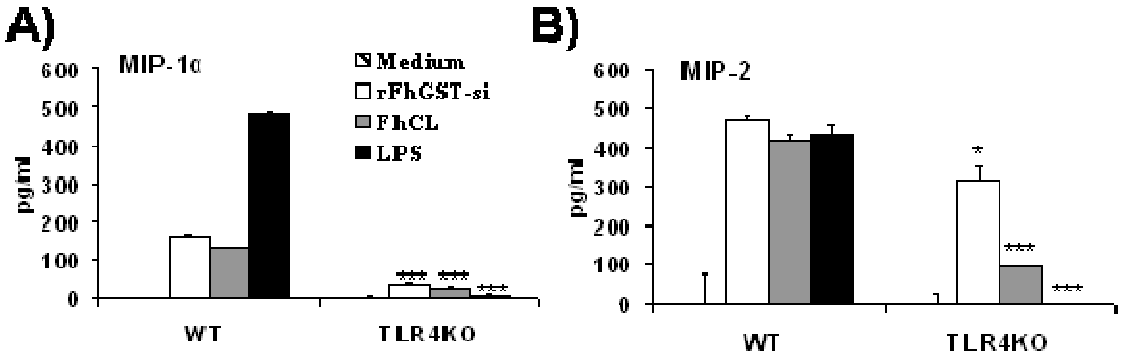

Figure 4.4: Chemokine secretion is observed in DCs matured with rFhCL1 and rFhGST-si. DCs from C57B/6L (wildtype background strain; WT) and TLR4 deficient (TLR4 KO) mice were cultured with rFhCL1 (10 μg/ml), rFhGST-si (10 μg/ml) and LPS (100 ng/ml) for 24 hours, and the production of (*A*) MIP-1α and (*B*) MIP-2 in supernatants determined by ELISA. Data are the mean values of 3 individual wells (±SEM), and a representative of at least 3 individual experiments. *, $p \leq 0.05$; ***, $p \leq 0.001$ compared to wildtype mice.

4.5.4. rFhCL1 and rFhGST-si differentially activate MAPKs (p38, ERK and JNK), NF-κB and IRF5 in DCs compared to LPS

Since the partial activation of DCs by rFhCL1 and rFhGST-si is mediated through TLR4, we investigated whether these antigens activated intracellular MAP Kinases associated with the TLR4 signalling pathway. To address this question, the level of phosphorylation of ERK, p38 and JNK in DCs following treatment with rFhCL1 and rFhGST-si was determined by western blot using LPS as a positive control. As expected, LPS caused the activation of all three signal transducers in comparison to medium with distinct kinetics similar to previously published data (Fig. 4.5 A, C) (Agrawal *et al*., 2003, Ardeshna *et al*., 2000, Arrighi *et al*., 2001). In contrast, only an increase in the activation of p38 was detected following treatment with rFhCL1. In addition, this was observed at a much later time point (60min) than that seen for LPS activation. Stimulation of DCs with rFhGST-si induced the phosphorylation of JNK and p38 but not ERK at most time points tested (Fig. 4.5 A, C). While the kinetic profile of

JNK and p38 activation was similar to that for LPS, peaking at 30min, the level of phosphorylation activated by rFhGST-si was significantly lower than LPS.

The transcription factor NF-κB is primarily associated with TLR innate immune cell activation, typically DC maturation, and is involved in regulating the expression of a large number of inflammatory mediators. The phosphorylation of the DNA binding subunits, p65 is an indicator of NF-κB activation (Hayden and Ghosh, 2008). As expected, LPS stimulation of DCs led to an increase in the presence of phosphorylated NF-κBp65 (p-NF-κBp65) at 15, 30 and 60 minutes (Fig. 4.5 A, C). Similarly, rFhGST-si induced a significant increase in the levels of p-NF-κBp65 15, 30 and 60 minutes. However, the extent of phosphorylation measured at 15 and 60 minutes was less than that seen for LPS (Fig. 4.5 C).

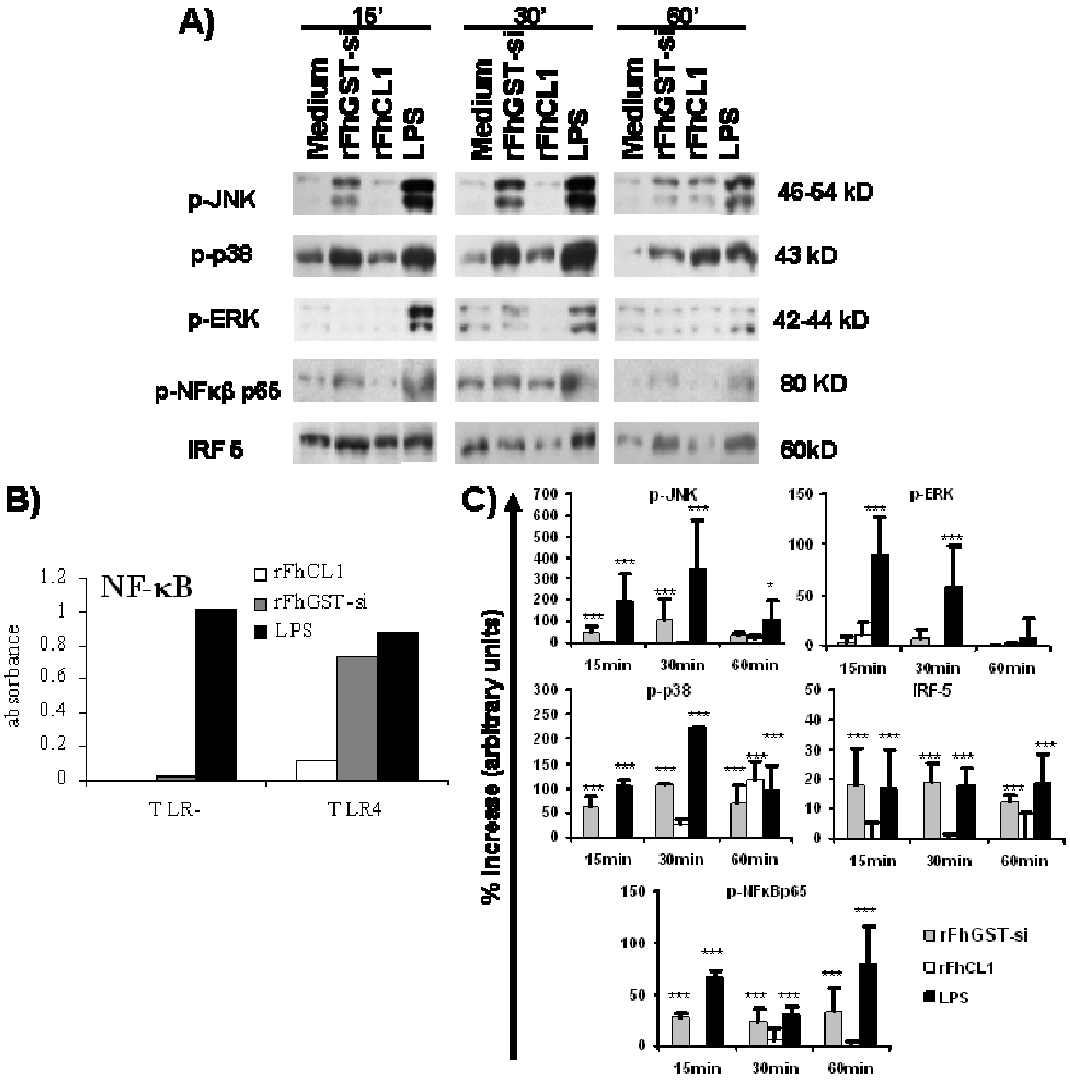

Figure 4.5: Differential activation of MAPKs, NF-κB and IRF5 in DCs is observed when treated with rFhCL1 and rFhGST-si compared to LPS. *(A)* DCs from C57B/6 mice were cultured with culture medium, rFhGST-si (10 μg/ml), rFhCL1 (10 μg/ml) and LPS (100 ng/ml). At 15, 30, and 60 minutes, cells were lysed and total cellular protein was extracted. 10μg/sample of protein was separated on 10% SDS-PAGE gel, transferred onto a PVDF membrane and sequentially probed for phosphorylated- (p) JNK, p-p38, p-ERK, p-NF-κBp65 and IRF5. Representative blots are shown from three individual experiments. (*B)* Recombinant HEK-293 cells, functionally expressing TLR4 protein linked to an NF-κB luciferase reporter gene, were stimulated with LPS, rFhGST-si (10 μg/ml) or rFhCL1 (10 μg/ml) for 24hrs. A HEK-293 cell line expressing the reporter gene only was used as a control (TLR-). (C) Densitometric analysis was performed on all immunoblots and values were expressed in arbitrary units as a percentage increase over the medium control group. *, $p \leq 0.05$; ***, $p \leq 0.001$ compared with medium control group.

Activation of NF-κB p65 was not detected in DCs stimulated by rFhCL1 at any time point examined. To confirm these findings, human recombinant HEK-293 cells, functionally expressing TLR4 linked to an NF-κB reporter gene, were stimulated with rFhGST-si, rFhCL1 and LPS (Fig. 4.5 B). Consistent with the western blot analyses, data indicated that rFhGST-si but not rFhCL1 activated NF-κB and demonstrated that this was dependent upon TLR4.

Since no phosphorylation of NF-κBp65 subunit was detected following treatment of DCs with rFhCL1, we investigated whether this enzyme activated the IRF5 transcription factor. Following activation of TLR4, IRF5 directly interacts with the TLR4 adaptor protein MyD88 and functions as a key transcription factor for TLR4-dependent induction of IL-6 and IL-12p40, independently of NF-κB and the MAPKs (Takaoka *et al*., 2005). Considering we have demonstrated that rFhCL1 induced secretion of IL-6 and IL-12p40 is dependent upon the presence of TLR4 (Fig. 4.3) we measured the levels of IRF5 by western blot in DCs activated with rFhCL1 and rFhGST-si (Fig 4.5 A, C). However, only rFhGST-si and not rFhCL1 significantly enhanced the activation of IRF5 compared to unstimulated cells at 15 and 30 minutes.

4.5.5. rFhCL1 and rFhGST-si treated DCs do not phagocytose antigen

As highlighted in section 3.2.5, DCs are considered professional phagocytes. The functional hallmark of 'classical' DC maturation is its ability to phagocytose antigen in combination with MHC II. Considering both enzymes failed to induce the expression of MHC II or to fully activate DCs, we investigated the ability of Fasciola enzyme activated DCs to phagocytose opsonised sheep erythrocytes. DCs treated with medium alone were used as a base control, while LPS was use as a positive activator of DC phagocytosis. DCs fully matured by LPS-stimulation, demonstrated an ability to efficiently phagocytose antigen with a significant increase ($p \leq 0.01$) in the number of

engulfed erythrocytes compared with medium controls (Fig. 4.6). In contrast, rFhCL1- and rFhGST-si-treated DCs engulfed the same number of erythrocytes as immature unstimulated DCs, indicating an inability to phagocytose antigen (Fig. 4.6).

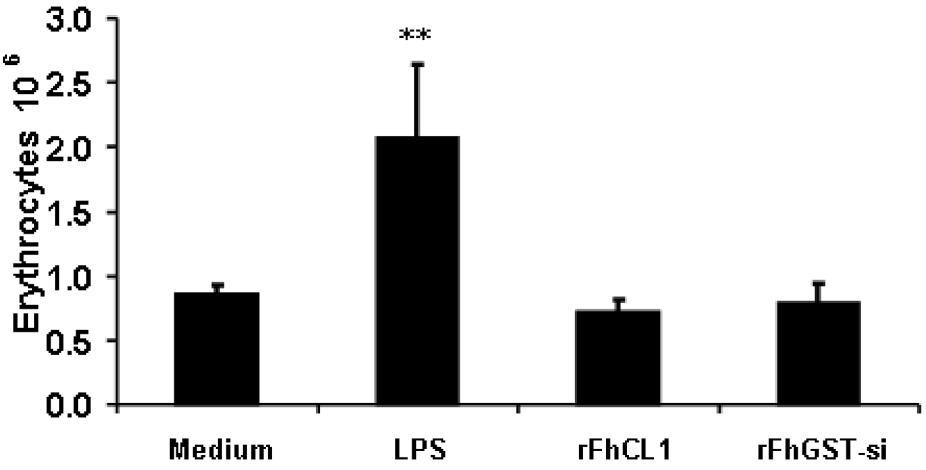

Figure 4.6: rFhCL1 and rFhGST-si treated DCs do not phagocytose antigen. DCs from C57BL/6 mice were cultured with medium, LPS (100 ng/ml), rFhCL1 or rFhGST-si (10 µg/ml) for 2.5 h before the addition of opsonised sheep erythrocytes at a ratio of 1:50. After 1 hour the extent of phagocytosis was assessed by measuring the number of engulfed erythrocytes using a colorimetric assay. Data are the mean values (±SEM) of at least 3 individual experiments. **, $p \leq 0.01$ compared to untreated (Med) control group.

4.5.6. rFhCL1 and rFhGST-si bias specific immune response by suppressing Th17 immune responses from OVA-specific T-cells

Following phagocytosis, the role of fully matured DCs is to present antigen to T cells thus inducing antigen specific immune responses. We examined the ability of subcutaneously injected differentially activated DCs pulsed with OVA, to prime T helper cell responses in the skin draining lymph nodes (sdLN) of DO11.10 transgenic mice, a strain expressing a T cell receptor specific for OVA peptide (Jenkins *et al*., 2005).

sdLN cells isolated from mice which received DCs, secreted antigen-specific IL-4, IL-10, IFN-γ and IL-17 (Fig. 4.7 A-D) in response to OVA peptide stimulation *ex vivo*. However, lymph node cells from mice that received DCs cultured with either

rFhCL1 or rFhGST-si and subsequently matured in the presence of OVA, had significantly reduced antigen-specific IL-17 production in response to stimulation with OVA peptide (Fig. 4.7 B; $p \leq 0.05$ and $p \leq 0.001$ respectively). No significant differences were observed in the antigen-specific production of IL-4, IL-10 and IFN-γ (Fig. 4.7 A, C, D)

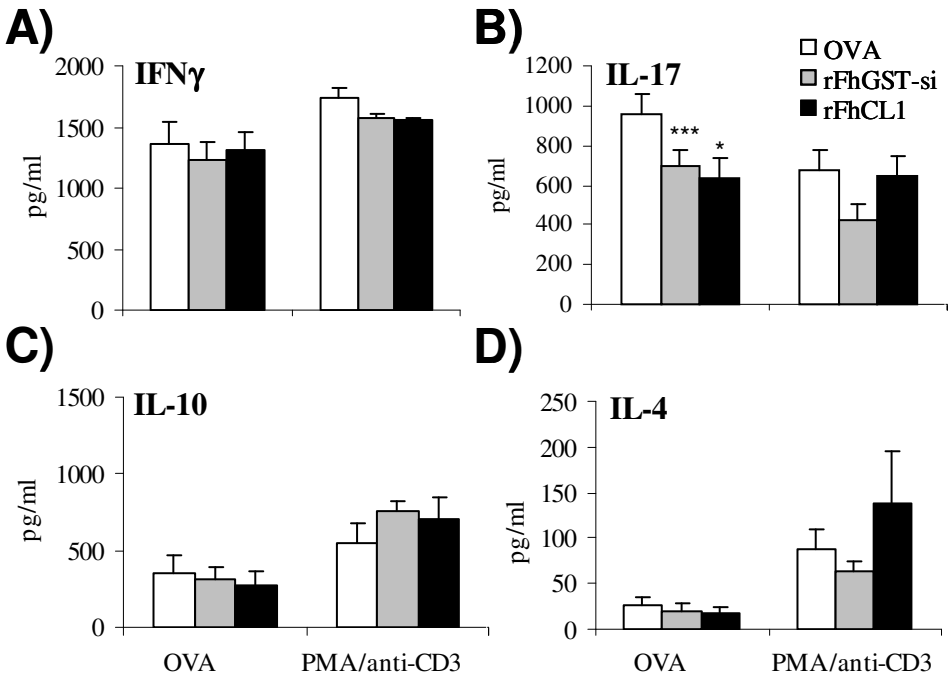

Figure 4.7: DCs activated with rFhCL1 and rFhGST-si suppress Th17 immune responses. DCs from DO11.10 mice were cultured with OVA (100 nM) following exposure to medium, rFhGST-si (10 µg/ml) or rFhCL1 (10 µg/ml) overnight at 37°C. *(A-D)* Stimulated DCs were subcutaneously injected over the sternum of naïve DO11.10 mice. After 7 days, local sdLN (skin draining lymph nodes) were removed for re-stimulation *in vitro* with OVA peptide (500 nM) or with PMA (25 ng/ml) and anti-CD3 (1 µg/ml). After 72 h, supernatants were analysed by ELISA for the presence of IFN-γ, IL-17, IL-10 and IL-4. Data are the mean (± SEM) of three individual wells from four individual mice, and representative of three experiments. *, $p \leq 0.05$; ***, $p \leq 0.001$ compared with OVA.

4.5.7. rFhGST-si and rFhCL1 suppress the ability of DCs to secrete IL-23 in response to LPS

Since IL-23 from DCs is important in driving the secretion of IL-17 from $CD4^+$ T cells (Aggarwal *et al*., 2003), we determined whether DCs exposed to rFhCL1 or rFhGST-si were less capable of producing IL-23. DCs were exposed to rFhCL1 and rFhGST-si 2.5 hrs before activation with LPS. After 18h incubation in the presence of LPS, the quantity of IL-23 in the supernatant was measured. Concurrent with the ability to suppress IL-17 specific responses, treatment of DCs with rFhCL1 and rFhGST-si prior to LPS stimulation resulted in significant suppression of IL-23 production (Fig. 4.8; $p \leq 0.001$).

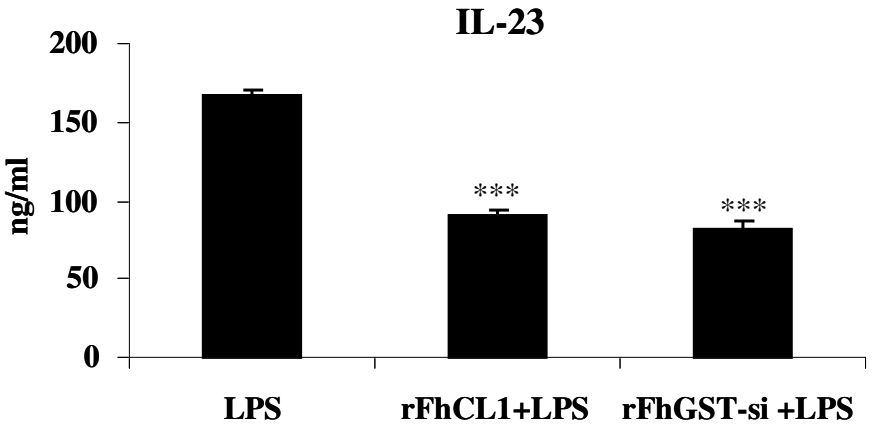

Figure 4.8: rFhGST-si and rFhCL1 suppress the ability of DCs to secrete IL-23 in response to LPS. DCs from C57BL/6 mice were cultured with culture medium, rFhCL1 or rFhGST-si (10 μg/ml) 2.5 h prior to LPS stimulation (100 ng/ml). 18h after the addition of LPS, IL-23 was measured in the supernatants by sandwich ELISA. Data are the mean values (±SEM) of at least 3 individual experiments. ***, $p \leq 0.001$ compared with LPS.

4.6. Discussion

Here we have selected recombinant forms of two major *F. hepatica* secreted molecules the protease rFhCL1 and anti-oxidant rFhGST-si, both of which are major components of the ES and examined their interactions with DCs. Both have the capacity to alter the function of innate immune cells and, consequently, the ensuing T-cell responses.

Despite enzymatic and functional differences between these antigens they both induced IL-6, IL-12p40 and MIP-2 secretion from DCs. This induction was mediated by TLR4 but the subsequent intracellular signalling pathway induced by each antigen differed; while rFhCL1 signalled through p38, rFhGST-si mediated its effect via JNK, p38, p-NF-κBp65 and IRF5. Neither rFhCL1 or rFhGST-si enhanced DC phagocytosis or induced Th2/Treg immune responses *in vivo*.

Partial activation of DCs and the development of Th2 responses are typical of helminth infections (O'Neill *et al*., 2001), however, we found that DCs stimulated with either rFhCL1 or rFhGST-si were unable to induce the differentiation of Th2 or Treg cells. In contrast, DCs matured in the presence of either enzyme suppressed IL-17 production from OVA-specific T-cells *in vivo*, inhibiting the development of a Th17 immune response, independently of Th2 cells. This function has never previously described for a helminth-activated DC. Therefore, despite sharing some phenotypic characteristics of other helminth-antigen activated DCs (Jenkins and Mountford, 2005, Goodridge *et al*., 2005, Balic *et al*., 2004), the Fasciola antigen-activated DCs exhibit a different activation state and effector function.

Regulation of IL-17 is critical to the control of inflammatory pathology associated with helminth infection, and has been particularly illustrated in the experimental model of *S. mansoni* where a strong correlation exists between the presence of Th1/Th17 immune responses and the development of severe inflammatory mediated pathology (Cheever *et al*., 1987, Rutitzky *et al*., 2008). Mice co-infected with *S. mansoni* and the intestinal hookworm *Heligmosomoides polygyrus*, showed reduced schistosome egg-induced hepatic immunopathology and a significant decrease in IL-17 secretion from both granuloma cells and CD4 T-cells suggesting that the strong polarized Th2 environment promoted by *H. polygyrus* prevented the development of Th1 and Th17 immune responses (Bazzone *et al*., 2008).

This hypothesis links in with the observation that DCs exposed to either rFhCL1 or rFhGST-si secreted reduced levels of IL-23, a cytokine known to be important in the expansion and survival of Th17 cells (Langrish *et al*., 2005). This is consistent with the studies of Shainheit *et al* (2008) showing that the *in vitro* differentiation of Th17 cells by schistosome-ligand activated DCs derived from CBA mice was dependent upon the secretion of IL-23 (Shainheit *et al*., 2008). The inhibition of Th17 development is clearly important for the success of *F. hepatica* infection considering that these two biochemically distinct enzymes perform this function via the modulation of DC activation. Moreover, rFhCL1 and rFhGST-si activated this suppressive population of DCs using different signalling pathways. While rFhCL1 activate p38 only amongst the transcription factors measured, rFhGST-si activated p38 and JNK, common MAPKs of the TLR4 pathway (Dowling *et al*., 2008), and the transcription factors NF-κB and IRF5.

The activation of IRF-5 by a helminth parasite molecules has not been reported previously and thus it's induction by rFhGST-si is of particular interest particularly because this transcription factor is expressed only in DC and B cells and is pivotally involved in the innate immune response (Honda and Taniguchi, 2006). IRF-5 was originally described as a transducer of virus-mediated signalling involved in the induction of anti-viral type 1 interferons (Barnes *et al*., 2002). However, despite functional similarity to the other IRFs, IRF5 appears to be induced in response to kinases activated by a range of TLR agonists rather than simply to viral antigens (O'Neill and Bowie, 2007). Therefore, while acting as an activator of type I interferon genes, IRF-5 also regulates the expression of proteins involved in cell growth regulation and immunomodulation (Barnes *et al*., 2003, Yasuda *et al*., 2007). While these functions are clearly important in host defence, there is also evidence that a deregulation

of IRF5 impacts on the development of a range of Th17 mediated autoimmune diseases (Gutierrez-Roelens and Lauwerys, 2008).

Activation of IRF5 by rFhGST-si may be required for the observed induction of IL-6 and IL-12p40 seen in stimulated DCs. In addition, the genes activated in response to IRF5 may be involved in the subsequent polarisation of T-cell responses by rFhGST-si-activated DCs. The activation of NF-κB has been shown to be characteristic of Th2 promoting DCs. *S. mansoni* LNFPIII induces rapid but transient NF-κB nuclear translocation and activity in the nucleus, in comparison with the persistent activation induced by Th1 promoting LPS (Thomas *et al*., 2005). Furthermore, studies suggest that the activation of NF-κB1 and not NF-κB2 is critical to the ability of DCs to prime Th2 responses following exposure to SEA, LNFPIII-dextran or *Trichuris muris* (Perrigoue *et al*., 2008). Here we found that rFhGST-si induced a more persistent activation of NF-κB in DCs than that observed for LNFPIII which may explain the distinct capacity of these DCs to suppress Th17 rather than promote Th2 cells. Assessment of the relative contributions of NF-κB1 and NF-κB2 in response to rFhGST-si would further characterise this phenotype of DC.

Studies suggest that ERK plays a central role in modulating the behaviour of DCs and promoting of Th2 responses, ERK deficient mice still retain the capacity to exhibit Th1 polarization but exhibit reduced Th2 immune responses (Agrawal *et al*., 2006). We found that rFhGST-si induced the phosphorylation of p38 and JNK, but not ERK in DCs. This contrasts with SEA, which conditions DCs to induce strong Th2 responses, induces the phosphorylation of ERK (for sustained periods) and p38 (weakly) but not JNK (Aggarwal *et al*., 2003). Similarly, schistosome egg LNFPIII which induce Th2 immune responses also stimulates the phosphorylation of ERK (Thomas *et al*., 2003). The absence of ERK phosphorylation in DCs stimulated by

rFhGST-si, and rFhCL1, may contribute to their lack of ability to promote the differentiation of Th2 cells.

Despite differences in the profile of transcription factors activated by rFhCL1 and rFhGST-si, induction of both phenotypes of DC was dependent on the presence of TLR4 receptor, thus identifying these two enzymes as potentially novel helminth PAMPs. TLRs are implicated in the recognition of helminth parasite products by DCs, but since helminth-associated Th2 responses are not abrogated in the absence of TLRs (Kane *et al*., 2008) activation of DCs through these receptors may not be critical in the development of Th2 immune responses. On the other hand, TLR ligation by helminth-derived antigens is recognized as a mechanism to limit the development of Th1 cytokine mediated inflammation. For example, the filarial nematode ES product, ES-62 interacts with TLR4 on DCs and results in the inhibition of IL-12 secretion in response to classic stimulators such as LPS/IFN-gamma (Goodridge *et al*., 2005).

Similarly, both rFhCL1 and rFhGST-si reduced the ability of DCs to respond to LPS, significantly inhibiting the secretion of IL-23. Therefore products secreted by *F. hepatica*, like other helminths, induce, via interaction with TLR4, a phenotype of DC that is hyporesponsive to classical stimulation. However, unlike that found in other helminth studies, the consequence of this in *Fasciola* is a reduction in the development of inflammatory Th1/Th17 responses. Considering that a biochemically diverse range of helminth products are recognised by TLR4, it is perhaps not surprising that both rFhCL1 and rFhGST-si may utilised the same receptor.

However, the variation in transcriptional activation of DCs may reflect differences in their enzyme activity. FhCL1 is a protease and a member of papain-like family of cysteine proteases (Stack *et al*., 2007, Stack *et al*., 2008). Proteases of this family are secreted from a range of pathogenic organisms have been shown to have the capacity to degrade physiologically significant proteins such as immunoglobulins and

collagen. rFhCL1 could feasibly digest peptides from the TLR4 receptor resulting in an altered conformational structure which could disrupt subsequent binding of co-receptors, ultimately leading to the inactivation of signalling pathways. In the context of the current study it is also interesting to note that gingipain, a protease from *Prevotella intermedia* the causative agent of periodontis, and bromelein, a protease extracted from pineapple, both cleave CD14 from the surface of monocytes rendering the cells hyporesponsive to LPS (Deschner *et al*., 2003). However, no change in the level of surface expression of CD14 in response to rFhCL1 was observed (data not shown).

Seven subclasses of GST have been identified in *F. hepatica* which can be distinguished from each other on the basis of substrate specificity and inhibitor sensitivity (Salvatore *et al*., 1995, Sheehan *et al*., 2001). In addition, the structure and binding interaction of the active site of sigma class-GST differs from that of the mu class (Sheehan *et al*., 2001). Therefore, fact the sigma class but not mu inhibited the activity of DCs would indicate that the modulatory effect is dependent on the specific enzymatic activity of the sigma class of GST.

The demonstration that two biologically distinct molecules, using different pathways, activate DCs with the same capacity to modulate developing adaptive responses supports the view that helminth parasites induce a heterogeneous population of innate immune cells to control the outcome of infection. As stated earlier the majority of research helminth antigen-DC interactions have used preparations of parasite secretions which represent a composite of many different protein, lipids and sugars. A number of those molecules, including CL1 and GST, are universally expressed by all helminth parasites and suggest the existence of common mechanism(s) of modulation. Understanding how each individual antigen from helminth antigenic preparations affect DC maturation and function may shed light on the importance of discrete immune

modulatory mechanisms that collectively lead to the immune responses associated with helminth infection.

There are a number of models put forward to define how DCs exposed to helminth antigens activate Th2/Treg immune responses associated with helminths (MacDonald and Maizels, 2008). In this study we have potentially identified two novel TLR PAMPs from the helminth *F. hepatica* which may give insights into a possible scenario for the induction of Th2/T regulatory responses cell associated with helminth infections. While they do not appear to promote a Th2/Treg immune response themselves, both rFhCL1 and rFhGST-si may promote an environment that is inductive to the generation of such a response by other secretory molecules. Overall the data suggests that helminth parasites secrete multiple molecules each possessing a unique mechanism of modulation, which can suppress inflammatory Th1/Th17 responses with redundancy thus permitting the uninhibited development of Th2/Treg by other secretory molecules.

<u>Chapter 5:</u> DCs stimulated with *Ascaris lumbricoides* extract induce a partially activated phenotype with Th2 inducing ability *in vivo*

5.4. Introduction

The human nematode parasite *A. lumbricoides* is of major medical importance and estimates put its prevalence at 1.5 billion people affected worldwide (Elliott *et al*., 2007). Ascaris infection causes acute effects such as intestinal obstruction and chronic infection impacts upon growth, appetite, physical fitness, work capacity and cognitive development (Bradley and Jackson, 2004). These impacts are concentrated in those children who are also compromised by poor nutrition, and other educational and health disadvantages. Ascaris infection is characterised by a predominant Th2/Treg immune response and protection from *A lumbricoides* infection has been associated with high levels of the Th2 cytokines, IL-4 and IL-5 and low levels of Th1 cytokines (Cooper *et al.*, 2000, Geiger *et al.*, 2002).

Antigens derived from *A. lumbricoides* and *A. suum*, which infect human and pigs respectively, have been of growing interest due to the potential immunosuppressive effects of ascarids on their hosts (Johnston *et al*., 2009). In both humans and animal studies, infection with parasitic nematodes such as *A. lumbricoides* has been associated with the protection from allergic and autoimmune disease (Harnett and Harnett, 2008). Previous studies have identified the ability of Ascaris derived antigens, mainly from *A. Suum*, to trigger *in vitro* nitric oxide from macrophage (Andrade *et al*., 2005), or alter expression of allegoric diseases (Rocha *et al*., 2008, Araujo *et al*., 2008, Lima *et al*., 2002, Itami *et al*., 2005, Matera *et al*., 2008).

While our understanding of the interaction of these Ascaris antigens on DCs function is limited, particularly those antigens derived from *A. lumbricoides*, a few studies have investigated the interaction of *A. suum* antigens and DCs (Table 1.1). Both

pseudocoelomic fluid (PCF) (McConchie *et al*., 2006) and high-molecular-weight components (PI) (Silva *et al*., 2006) of *A. suum* did not activate DCs. Pre-treatment of DC with PCF inhibited LPS-induced CD40 and CD86 as well as the production of IL-12 in a dose-dependent manner (McConchie *et al*., 2006). PI suppressed costimulatory molecule expression of purified $CD11c^+$ LN cells from OVA immunised mice. While compared to OVA immunised mice, purified $CD11c^+$ from OVA+PI immnunised mice have a reduced proliferation of OVA specific T cells *in vitro*. Both were shown to be dependent on IL-10 as the suppreson was reversed in IL-10KO mice (Silva *et al*., 2006). Alternatively, the PC containing native glycophingolipid from *A. suum* have been shown to increase DC and macrophage costimulatory molecule expression and secretion of IL-12p40 and TNF-α. Pre-treatment could suppress LPS induced IL-12p70 in both cells (Kean *et al*., 2006).

The focus of this chapter is to determine the interactions of native antigen preparation derived from the female form of *A. lumbricoides* since no previous studies have examined the effect of antigens upon DCs from this helminth. *A. lumbricoides* PCF antigens (AlPCF) was selected for use in this thesis because it is a metabolically active fluid which provides precursor molecules for membrane and cuticular synthesis (Kennedy and Qureshi, 1986). This makes it the closest possible approximation of FhTeg obtainable from *A. lumbricoides* for use as a comparison. Another factor for its selection is that AlPCF is easily obtained from the body cavity of adult worms. Other reasons for its use include results from studies that have shown PCF to contains a number of antigens that induce antibody responses in several different mammalian species, including mice (Kennedy and Qureshi, 1986, Urban *et al*., 1988). Some of these molecules are recognized as antigens by infected rabbits (Kennedy and Qureshi, 1986) and pigs (Urban *et al*., 1988). Notably, ABA-1 has been identified as a main protein constituent of PCF (Kennedy *et al*., 1991, Paterson *et al*., 2002), and is also the major

IgE-binding antigen in human patients that express resistance to ascariasis (Paterson *et al*., 2002).

A thorough analysis of the effect of AlPCF on DC phenotype was measured by examining cytokine secretion and cell surface marker expression. The effect of Ascaris antigen on DC function was also determined by phagocytosis assay and T-cell-polarizing function. Here we show that AlPCF extract can induce partial maturation of DCs as characterised by the increased production of IL-12 family related cytokines, and the chemokine MIP-2. No other inflammatory cytokines or maturation associated surface markers where induced. We also show that AlPCF-treated DCs can drive Th cells toward the Th2 pole. The present experiments shed further light on the DC activating properties of *A. lumbricoides* derived extracts. These results have generic implications for the understanding of DC-driven Th cell responses both directly on the host and the mechanisms of possible bystander effects.

5.5. Results

5.5.1. Ascaris antigen induces partial activation of murine BMDCs

A. suum derived antigens have previously shown to have cytokine-inducing and immunosuppressive abilities on bone marrow derived macrophage and DCs (Kean *et al*., 2006, Deehan *et al*., 2002). To determine if the same was true for native *A. lumbricoides* derived antigens, BMDCs were cultured with native AlPCF (10 μg/ml) medium as a negative control, LPS as a positive control (100 ng/ml) for 24h and the supernatants analysed for cytokines responses. DCs stimulated with AlPCF stimulated significant levels of the cytokines IL-6, IL-12p40 and the chemokine MIP-2 (CXCL2). As expected, treatment of DCs with LPS induced “classical” maturation of DCs, evident by highly elevated levels of all cytokines and cell surface markers measured (Dowling *et al*., 2008).

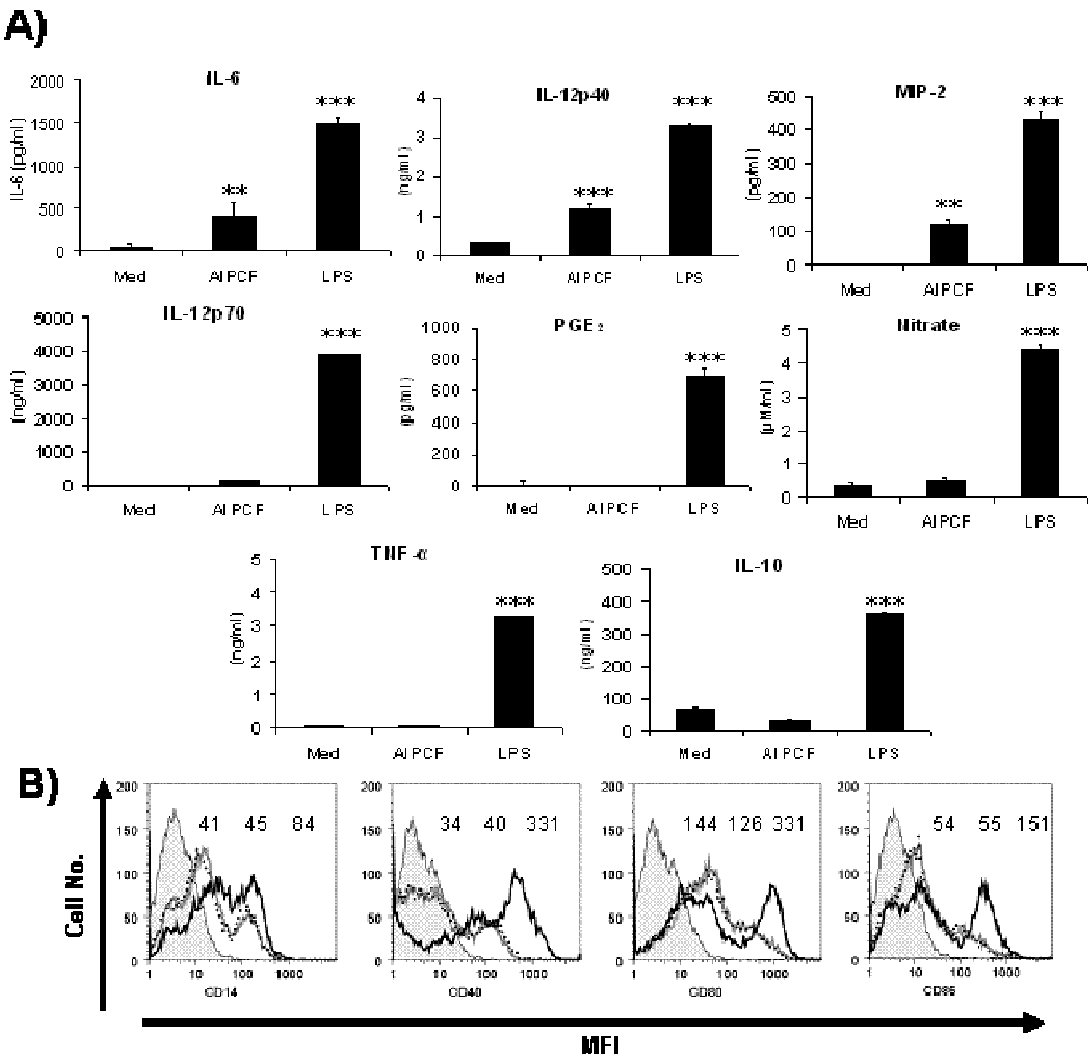

Figure 5.1: AlPCF induces partial activation of DCs. A) DCs from C57B/6 mice were cultured with medium (Med), AlPCF (10 µg/ml) or LPS (100 ng/ml) for 24 hours, and the production of IL-12p70, IL-10, TNF-α, IL-12p40, IL-6, MIP-2 and PGE_2 were measured using sandwich ELISA, and nitrite was measured using medium Griess reagent. Data are the mean values (±SEM) of at least 3 individual experiments. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ compared to Med. B) Cells were harvested and analysed by three-colour flow cytometry for CD14, CD40, CD80 and CD86. Mean fluorescent intensity (MFI); medium (left and grey line,), AlPCF (centre and dotted black line, 10 µg/ml), LPS (right and black line, 100 ng/ml) and Isotype (tinted).

The levels of cytokines induced by AlPCF were significantly higher than medium but less than those induced by the positive control, LPS (Fig. 5.1 A). We also measured IL-12p70, NO, PGE_2, TNF-α, IL-10, (Fig. 5.1 A), IL-23, MIP-1 and MCP (data not shown) and no up regulation of any of these cytokines was observed in response to AlPCF treatment. The ability of AlPCF to induce elevated expression of CD40, CD86 and CD80 and CD14 DCs was also investigated. Results demonstrated that AlPCF did not induce significant increased expression of any of the markers tested (Fig. 5.1 B) opposed to increased expression of all cell surface markers induced by the positive control LPS. AlPCF extracts used in these studies were deemed endotoxin free as assessed using the Pyrogene® endotoxin detection system (Cambrex) and did not effect DC viability Annexin V staining (data not shown).

5.5.2. AlPCF induced cytokine production by DCs is dependent upon MAPKs

To determine the possible signal transduction mechanism of AlPCF induced IL-6 and IL-12p40, we investigated whether it is targeting a common component of TLR pathways, such as the MAPKs. To address this question, DCs were pre-treated with MAPK inhibitors specific for ERK, p38 and JNK prior to AlPCF stimulation. As before, AlPCF induced IL-6 and IL-12p70 at significantly higher levels compared to untreated DCs. Pre-treatment with all three inhibitors resulted in a marked reducing in AlPCF-induced IL-6 (Fig. 5.2; $p \leq 0.001$). Similarly, inhibition of all MAPKs resulted in marked reduction in AlPCF-induced IL-12p70 (Fig. 5.2; $p \leq 0.001$). It should be noted that while pre-treatment of DCs with the JNK inhibitor significantly reduced AlPCF-induced IL-12p70, it was not totally abrogated to background control levels (untreated cells).

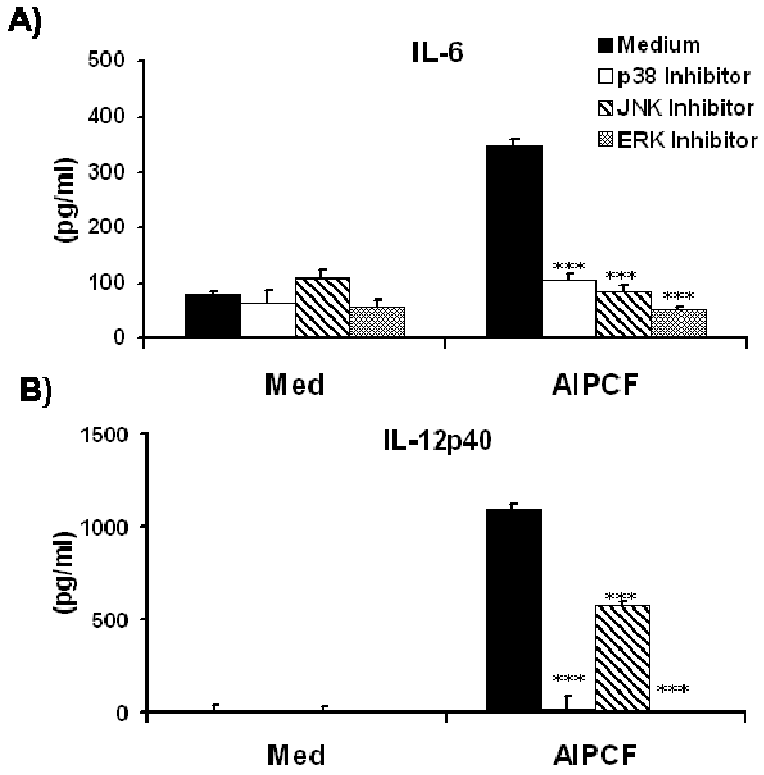

Figure 5.2: AlPCF induced cytokines expression is dependent upon MAPKs. IL-6 and IL-12p40 induced by AlPCF are partially dependent on all MAPKs tested. DCs from C57B/6 mice were cultured with medium (Med), AlPCF (10 μg/ml) and LPS (100 ng/ml) in the presence or absence of specific ERK (U0126; Sigma Aldrich), p38 (SB 202190; Sigma Aldrich) or JNK inhibitors (JNK inhibitor I (L)-Form; Calbiochem) (all 20 μM) which was added 1 h prior to antigen stimulation. Cells were incubated for a further 24 hours, and the production of IL-12p40 and IL-6 in supernatants determined by ELISA. Data are the mean values of 3 individual wells (±SEM), and a representative of at least 2 individual experiments. ***, $p \leq 0.001$ compared control mice.

5.5.3. The partial activation of DCs induced by AlPCF is not TLR4 dependent

Since TLRs has been shown to be important for helminth-derived antigen induction of IL-6 and IL-12 (Table 1.1), and that production of both cytokines is dependent on MAPK signalling pathways (Fig. 5.2), we went on to determine if the partial activation of DCs by AlPCF is dependent upon the TLR4. As expected, WT (wild type) DCs (on a C57BL background) produced significantly increased levels of IL-6, IL-12p40 and MIP-2 in response to LPS and zymosan compared with medium alone (Fig. 5.3; $p \leq 0.001$). Also as expected, TLR4KO DCs failed to respond to LPS, producing little or no IL-6, IL-12p40 and MIP-2, while stimulation with the TLR4-independent ligand zymosan, which signals through TLR2 (Gantner *et al*., 2003), remained unaffected.

AlPCF stimulated WT DCs produced increased levels of IL-6, IL-12p40 and MIP-2 as previously shown and no decrease was observed in TLR4KO DCs, which confirms that AlPCF partial activation of DCs is not TLR4 dependent.

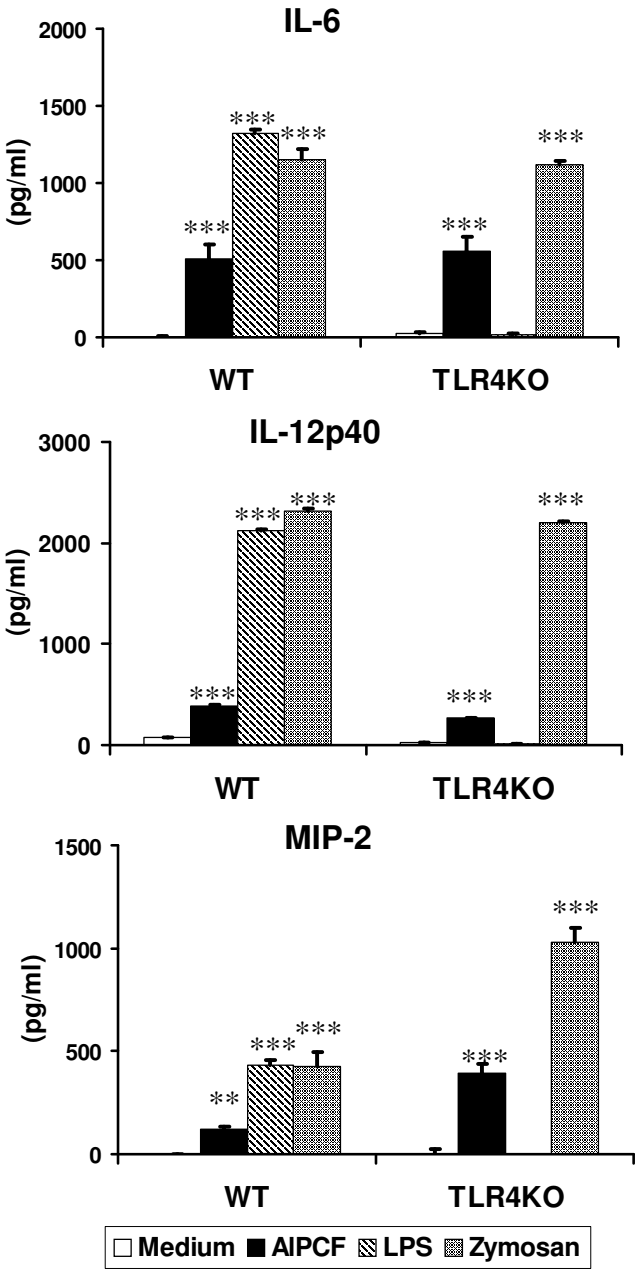

Figure 5.3: AlPCF induction of IL-6 and IL-12p40 and is not TLR4 dependent. DCs from C57BL/6 (wildtype background strain; WT) and TLR4 deficient (TLR4 KO) mice were cultured with medium only (Med), AlPCF (10 μg/ml), Zymosan (5 μg/ml) and LPS (100 ng/ml) for 24 hours, and the production of IL-12p40 and IL-6 in supernatants determined by ELISA. Data are the mean values (±SEM) of at least 3 individual experiments. ***, $p \leq 0.001$ compared control mice.

5.5.4. AlPCF activates partial DCs phagocytosis

The ability of DCs to phagocytose is a functional hallmark of their maturation. Both DCs and macrophages are considered professional phagocytes and are continuously sampling their environment for antigens. But DCs are distinguished in that their primary function is not to destroy phagocytised particles, but to process and express protein derived peptides on MHC class molecules leading to the initiation of adaptive immune responses (Savina and Amigorena, 2007). While maturation of DCs leads to the eventually down regulation of phagocytotic ability, studies have shown that phagocytosis is initially unregulated upon TLR stimulation (West *et al*., 2004). Here, DCs treated with medium alone were used as a base control, while LPS and zymosan treated DCs were use as positive activators of DC phagocytosis. DCs were cultured with AlPCF (10 µg/ml), LPS (100 ng/ml) or zymosan (5 µg/ml) for 2.5 hrs before the addition of opsonised sheep erythrocytes. After 1hr phagocytosis was stopped by the removal of supernatant and washing of cells with PBS to remove non-opsonised erythrocytes. Both LPS and zymosan stimulated DCs showed a significant increase ($p \leq 0.01$) in engulfed erythrocytes compared with untreated DCs (Fig. 5.4). AlPCF stimulated DCs showed increased phagocytosis of erythrocytes ($p \leq 0.01$) to that of untreated DCs but less than those induced by either positive control.

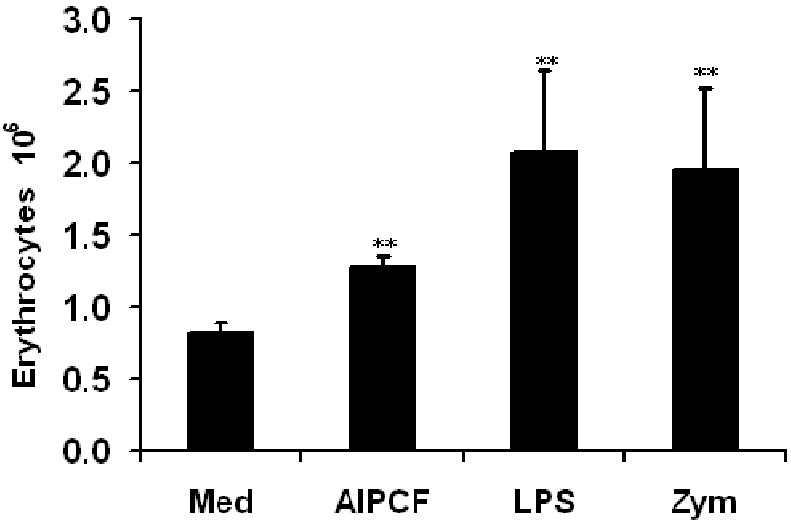

Figure 5.4: AlPCF activates partial DC phagocytosis. DCs from C57BL/6 mice were cultured with AlPCF (10 µg/ml), LPS (100 ng/ml) or zymosan for 2.5 hrs before the addition of opsonised sheep erythrocytes to DCs at a ratio of 50:1. After 1 hr phagocytosis was stopped, cells washed to remove non-opsonised erythrocytes, lysed and amount of engulfed erythrocytes determined by colorimetric assay. Negative control cells were treated with 2 µM Cytochalasin D to block phagocytosis (data not shown). Data are the mean values (±SEM) of at least 3 individual experiments. **, $p \leq 0.01$ compared to AlPCF treated control group.

5.5.5. AlPCF-stimulated DCs drive polarisation towards a Th2 phenotype *in vivo*

Next we investigated the priming ability of AlPCF treated DCs to prime Th cell responses in the skin draining lymph nodes (sdLN) of DO11.10, after subcutaneously delivery. DO11.10 mice express a T cell receptor specific for OVA peptide. DCs were cultured with OVA (100 nM) in the presence or absence of AlPCF (10 µg/ml) and subcutaneously injected over the sternum of naïve D10.11 mice. After 7 days sdLN cells were re-stimulated *in vitro* with OVA, (0, 100, or 1000 ng/ml) for 72 hours, to determine the in-vivo Th-cell priming outcome. Antigen-specific levels of IFN-γ and IL-4 in supernatants was measured by ELISA.

Analysis of the Th1 cytokine IFN-γ shown that sdLN cells from AlPCF DCs primed for increased production by sdLN cells (Fig. 5.5). This increase ranged from a 7.4 – fold increase using 100 nM OVA, to a 2.4 – fold increase using 1000 nM OVA (*p*

≤ 0.01) compared to control unstimulated DCs. sdLN cells from mice inoculated with AlPCF DCs were also primed for increased production of the Th2 cytokine IL-4 (8.1- to 5.6 – fold increase compared to medium control; $p \leq 0.001$ and $p \leq 0.01$, respectively). While IFN-γ and IL-4 were both stimulated by AlPCF primed DCs, IL-4 was induced at a much greater fold increase compared to that IFN-γ.

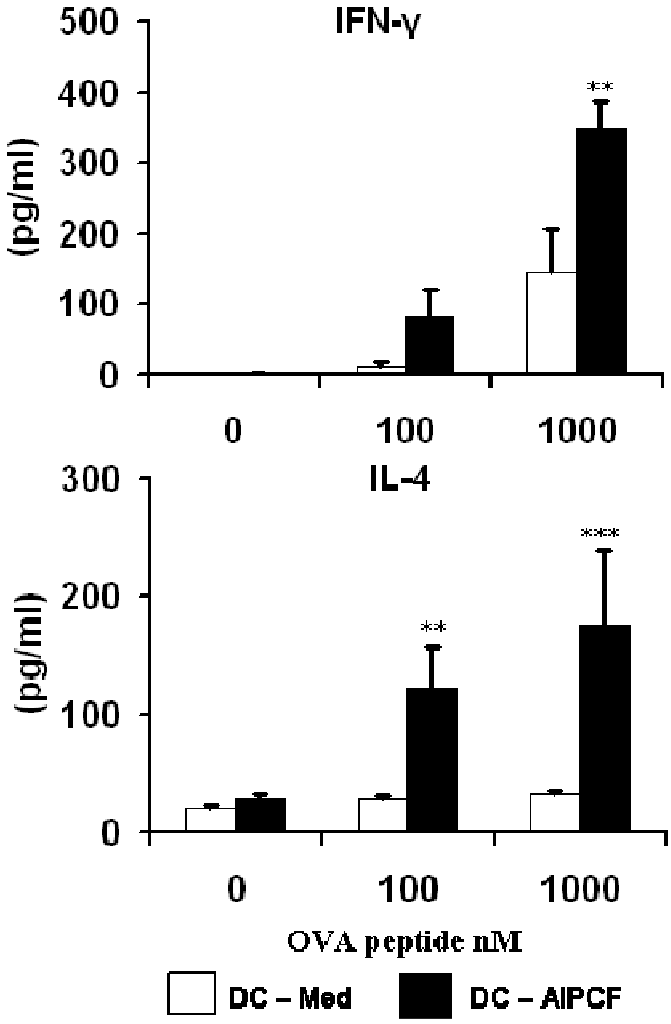

Figure 5.5: DCs activated with AlPCF drive polarisation towards a Th2 phenotype *in vivo*. DCs from DO11.10 mice were cultured with OVA (100 nM) in the presence or absence of AlPCF (10 µg/ml) overnight at 37°C. DCs were washed and injected s.c. over the sternum of naïve DO11.10 mice. After 7 days, sdLN cells were removed for re-stimulation *in vitro* with OVA (0 to 1000 nM) or PMA (25 ng/ml)/anti-CD3 (1 µg/ml). After 72 h, supernatants were analysed by ELISA for IFN-γ and IL-4. Data are the mean (± SEM) of three individual wells from four individual mice, and representative of two individual experiments. **, $p \leq 0.01$; ***, $p \leq 0.001$ compared with DC-Med.

5.6. Discussion

DCs bridge the innate and adaptive arms of the immune response by detecting PAMPs from pathogens and activating naïve T cells (Kapsenberg, 2003). Studies investigating the effects of *A. Lumbricoides* derived antigens on these vital immune responders are limited. This study demonstrates that AlPCF extract can induce partial maturation of DCs as characterised by the increased production of IL-6, IL-12p40 and MIP-2. Although AlPCF stimulated DCs produce the above cytokines in significant amounts, little or no other cytokines associated with classically induced LPS maturation (IL-12p70, TNF-α, NO, and IL-10 etc. (Dowling *et al*., 2008)) were observed. There is also a distinct lack maturation associated marker expression. This trait is also associated with FhTeg treated DCs.

The activation state induced by AlPCF resembles the DC phenotype identified in studies investigating other helminth-derived antigens. Both Schistosoma larvae derived products (0-3hRP) (Jenkins and Mountford, 2005), and *N. brasiliensis* excretory/secretory antigens (NES) (Balic *et al*., 2004) have been shown to induce partial maturation of DCs characterised by IL-6, IL-12p40 and to have Th2 priming abilities. 0-3hRP could also stimulate IL-6, IL-10 and IL-12p40 from macrophages where the secretion of IL-12p40 and IL-10 are reduced in TLR4 knockout and mutant mice. However in that study the secretion of IL-6 was not dependent upon TLR4 suggesting that other TLRs are involved (Jenkins *et al*., 2005). The filarial nematode phosphorylcholine (PC)-containing secreted product (ES-62), has been shown to be responsible for the immunomodulatory effect observed with native ES-62 (Goodridge *et al*., 2005). It induces the production of IL-12p40 and TNF-α in a TLR4 dependent manner. In contrast with these studies AlPCF antigen did not induce partial activation of costimulatory molecules or was it totally or partial dependent upon the TLR4 receptor. Most notably of all, apart from the lack of costimulatory marker activation, the DC

phenotype induced by AlPCF shows marked similarity to that of rFhGSTsi- and rFhCL1-stimulated DCs (Fig. 4.1).

To date, much of the immunological processes resulting in DC activated Th1 have been characterised (Kapsenberg, 2003). However, how Th2 responses are initiated is still poorly understood (MacDonald and Maizels, 2008). Our investigation into T helper cell priming capacity show that the DCs phenotype induced by AlPCF can prime for a mixed but Th2 polarised response *in vivo*. These observations would seem to support the DC maturation models, in which Th2 differentiation can occur through a default pathway, initiated by the absence of costimulatory molecule surface expression and strong to moderate cytokine production (MacDonald and Maizels, 2008). Also, AlPCF treated DCs could be seen to meet some of the criteria attributed to tolerogenic DCs (Morelli and Thomson, 2007), which while lacking archetypal maturation statues, remain immunogenic with Th2 polarising abilities (MacDonald *et al*., 2001, Whelan *et al*., 2000, Agrawal *et al*., 2003).

Furthermore, under our experimental conditions the induction of cytokines by AlPCF is heavily dependent on MAPK signal transduction. Inhibition of ERK and p38 lead to total reduction in IL-6 and IL-12p40. While inhibition of JNK results in total abrogation of IL-6, only partial reduction in IL-12p40 is observed. This inhibition profile differs from that that observed from LPS-matured DCs which shows an enhancement of IL-12p40 in the presence of each inhibitor (Dowling *et al*., 2008, Nakahara *et al*., 2006), indicating that MAPKs have varying roles in AlPCF-matured and LPS-matured DCs. Nonetheless, TLRs would seem to be the most likely PRR candidates for the detection of AlPCF. *S. mansoni* derived phosphatidylserine (PS) has been shown to have TLR2 activating capacity (van der Kleij *et al*., 2002). But while PS has been associated with inducing DC maturation (van Riet *et al*., 2009), limited data has been published on the effect PS has on DCs maturation statues. Our studies on

TLR4KO mice show that AlPCF is not dependent on TLR4 for the induction of cytokines, suggesting that other TLRs may be involved in the induction of the partial maturation statues. Kane *et al*. have shown that SEA can modulation both TLR-induced DC activation and induce Th2 responses in naïve mice independently of TLR2, TLR4, or MyD88 (Kane *et al*., 2008). Since the immunomodulatory characteristics of AlPCF differ from both PC and PS, further characterisation and qualification of both the ascaris extract and induced DC phenotype are needed. In summary we have shown the limited ability of AlPCF-treated DCs to phagocytose (Savina and Amigorena, 2007) and induce partial cytokine and chemokine production both of which may be an as yet undefined indicators of Th2 priming capacity, but further study on the effect of both functional capacities is warranted.

Chapter 6: Final Discussion

6.2. Discussion

Helminth parasites have evolved many mechanisms to suppress and/or modulate host immune responses. As a generalisation helminths induce a 'modified' Th2 immune response (Maizels *et al*., 2004). Experimental and epidemiological results also highlight the bystander effect of helminth infections on the outcome of co-current infections, in addition to atopic and autoimmune diseases (Elliott *et al*., 2007). But the mechanisms that induce these responses in the host are not fully understood. While much is known about how DCs respond to bacterial and viral pathogens that drive Th1/Th17 subsets, comparatively little is understood about how these cells respond to and influence the adaptive immune response to helminth parasites that drive Th2/Treg immune cells (Anthony *et al*., 2007).

Much of our knowledge on DC biology is based upon DCs exposed to LPS (Hoshino *et al*., 1999). DCs stimulated with LPS are said to drive a "classical" activation status which is defined as elevated expression of cell surface co-stimulatory markers (CD80, CD86, MHC II, CD40) and the production of pro-inflammatory cytokines (IL-6, IL-12 and TNF-α amongst others) (Kapsenberg, 2003, Dowling *et al*., 2008). Activation of TLRs not only initiates the innate immune response but also trigger the development of adaptive-specific immune responses (Takeda *et al*., 2003, Pasare and Medzhitov, 2004). Therefore understanding the role and/or effect of helminth infections or helminth derived products on DCs might further our knowledge of how helminths drive modified Th2 responses and induce such powerful anti-inflammatory effects.

But, the effect of helminths and/or helminth-derived products on DC activation and functionality is limited. One reason for this is the fact that helminths are a diverse group of extracellular organises with various morphologies, multiple life stages and

occupy numerous niches within their hosts. A number of approaches must be used to overcome these various problems. Firstly, the access and use of a relatively easily reproducible model system is needed. In this case, a murine model of DCs induced from bone marrow is used (Lutz *et al*., 1999) as this model is well established and DCs are critical in driving adaptive immune responses. Secondly, the use of living parasites outside of their host niche is difficult and therefore native antigens were obtained from the parasites and used to mimic infectious conditions. In this study we used the tegumental coat of *F. hepatica* and the PCF of *A. lumbricoides* because these are immune dominant antigens that come into contact with host immune system, and that both are produced in cellular layers which provide metabolically active precursor molecules for membrane and cuticular synthesis. Thirdly, helminth products, mostly derived from preparation directly from the parasites are predominantly heterogeneous in nature (See Table 1.1), making it hard to attribute signal molecules to PRRs to signal transduction pathways. Recombinant antigens or purified native antigens of helminths origin may provide a solution. In this study we had access to two recombinant Fasciola antigens, rFhGST-si and rFhCL1 since these are the predominant antigens secreted in ES. Lastly, the question of which helminth parasite to obtain antigens from arises. We selected *F. hepatica* because it is of major agricultural importance while *A. lumbricoides* is of major human significance. Furthermore, they represent two of the three helminth groups (trematodes and nematodes) and current knowledge into the effect of both on DCs has not been previously investigated.

We began with a comprehensive examination into the innate immune response to FhTeg by investigating its immune modulatory properties on bone marrow derived DCs. At the outset, a baseline of DC activation was needed from which the effect on *F. hepatica* antigens on DC function could be evaluated and compared. A comparative analysis of DC activation status matured against a panel of TLR ligands was used for

this purpose (Dowling *et al*., 2008). FhTeg alone could not induce any characteristic of a classical activated DC. But FhTeg could however down regulate the activation of DCs in response TLR and non-TLR ligands both prior to and following TLR and non-TLR stimulations. Our findings demonstrate FhTeg can act in an immunosuppressive manner on murine DCs *in vitro*. We then investigated the functional capacity of FhTeg treated DCs. When FhTeg-treated DCs were adoptively transferred into mice, mouse T cell responses were reduced and proliferation down when cultured ex vivo. T-cell priming studies demonstrated that FhTeg-primed DCs significantly suppressed local Th1 immune responses *in vivo*, but did not appear to drive local Th2 responses. In addition, DC-FhTeg recipients stimulated with PMA/anti-CD3 produced significantly decreased levels of all cytokines and proliferated significantly less in response to OVA stimulation than cells from DC-Med recipients. The observed suppression of T-cell proliferation or cytokine production in DC-FhTeg recipient mice could not attributed to changes in the expression of T-cell surface markers. Whereas no uniform suppression of MAPK could be ascribed to FhTeg, the phosphorylation of the transcription factor NF-κBp65 was significantly reducing in LPS-stimulated DCs.

Worm product	Species of origin	PRR(s)	Activation status	T helper
Heterogeneous				
FhTeg	*Fasciola hepatica*	ND	DC↔**(Y)**	Th1 ↓
AlPCF	*Ascaris lumbricoides*	ND	DC↗	Th2 ↑
Defined				
rFhCL1	*Fasciola hepatica*	TLR4	DC↗ **(Y)**	Th17 ↓
rFhGST-si	*Fasciola hepatica*	TLR4	DC↗ **(Y)**	Th17 ↓

Table 6.1: Helminth-derived products; a full list of helminth-derived products used in this thesis summarising their effect on DC phenotype and functionality. *Abbreviations*: ND, Not determined; TLR, Toll-like receptor; ↗; partial DC activation (Dowling *et al*., 2008); ↔, no change; ↑, induction; ↓, suppression; (Y), suppression of secondary activation; DC, dendritic cell.

In this study we also highlight the discovery of two potential TLR4 ligands derived from the helminth *F. hepatica*. Both rFhCL1 and rFhGST-si induce a partial activation

of DCs characterised by up regulation of IL-6, IL-12p40 and the cell surface marker CD40. In addition, DCs exposed to either antigen secreted reduced levels of IL-23. The partial activation of DCs by these molecules was dependant upon the presence of TLR4. Neither rFhCL1 nor rFhGST-si enhanced DC phagocytosis or induced Th2 immune responses *in vivo*. However, DCs matured in the presence of either enzyme attenuated IL-17 production from OVA-specific T-cells *in vivo*. Both rFhCL1 and rFhGST-si work through different signal transduction pathways. While rFhCL1 signalled through p38, rFhGST-si mediated its effect via JNK, p38, p-NF-κBp65 and IRF5.

Finally, we investigated the effect of AlPCF antigens on DC activation and functions. Similar to both rFhCL1 and rFhGST-si, AlPCF induced a partially activated DC phenotype characterised by the cytokines IL-6 and IL-12p40. Unlike rFhCL1 and rFhGST-si, no enhancement of cell surface marker expression was induced and only the chemokine MIP-2 is induced by AlPCF. DCs treated with AlPCF partially activated DC phagocytosis and polarisation T-cell responses towards a Th2 phenotype *in vivo*. AlPCF induced cytokine expression is dependent upon MAPKs signal transduction pathways but is not TLR4 dependent. The PRR responsible for AlPCF detection is yet to be elucidated.

Upon examining Table 1.1, a number of commonalities can be observed between the effects of various helminth-derived molecules on DCs. One such trend is that heterogeneous helminth derived-products, such as SEA, tend not to activate any signs of classical phenotypical DC activation. Therefore it is not unexpected that FhTeg alone does not activate DCs. Suppression of TLR-ligand induced DC activation by helminth products has also been an observed theme, with SEA (van Liempt *et al*., 2007) and HpES (Segura *et al*., 2007) being the best described examples. Our results show that FhTeg can also be grouped amongst these. However, it is unexpected that FhTeg does

not polarise modified Th2 responses, instead suppressing T-cell proliferation and cytokine production. The decrease in IL-10 observed was also not anticipated, since this cytokine is traditionally associated with an anti-inflammatory or regulatory response (Moore *et al*., 2001). But IL-10 may have a more versatile role in immune responses, with recent studies showing IL-10 production by Th1 cells (O'Garra and Vieira, 2007).

Similarly, where DC activation was shown as with rFhGST-si, rFhCL1, is was not unexpected that activation observed was lower than that observed with 'classical' activation of DCs. There is a growing diversity of ligands that TLR4s can specify for and while further studies are needed, both rFhGST-si and rFhCL1 may potentially be added to that list in the future. However, it is a little unexpected that rFhGST-si but not rFhCL1 activates NF-κBp65 and both can still activate phenotypically similar DCs with the same capacity to modulate adaptive responses. Also, the activation of IRF-5 by rFhGST-si is the first reported induction of this IRF by a helminth parasite molecule, with further study needed to quantify importance of this observation. It was however an unanticipated outcome that AlPCF does not induce co-stimulatory molecules while still inducing a cytokine secretion. While all three products induce a similar DC phenotype to that induced by Schistosoma larvae extracts (Jenkins and Mountford, 2005, Jenkins *et al*., 2005) or to a lesser extent ES-62 (Goodridge *et al*., 2005), only AlPCF-stimulated DCs could polarise a Th2 phenotype *in vivo*, while rFhGST-si and rFhCL1-stimulated DCs ameliorating Th17 responses. One major question to be answered from these studies is how AlPCF can induce polarised T helper responses in comparison to the suppression observed for FhGST-si and rFhCL1.

The anti-inflammatory properties of FhTeg is in keeping with helminths need to suppress potentially harmful host processes (Goodridge *et al*., 2005). The suppression of the Th1 polarizing cytokine IL-12p70 is important in this context since Th1 suppression has been observed during *F. hepatica* co-infections on a number of

occasions (Brady *et al*., 1999, Flynn *et al*., 2007). While the exact mechanism of FhTeg-mediated suppression remains to be elucidated, FhTeg was able to suppress the effects of all TLR ligands tested. This included both MyD88-dependent and -independent pathways in addition to non-TLR ligands. Therefore it seems likely that FhTeg utilizes a pathway common to these receptors. We demonstrated that FhTeg can suppress the phosphorylation of NF-κBp65, and the suppression of this and other such NF-κB family transcription factors may explain the broad suppressive ability of FhTeg.

When considering the various hypotheses put forward in the literature explaining the possible mechanisms of Th2 induction by DCs (MacDonald and Maizels, 2008), our results of the effect of FhTeg on DCs presented here correspond most closely to the inhibition model. These results suggested that FhTeg can maintain DCs in an immature state and the subsequent lack of cytokine production and costimulatory molecule expression can interfere with the function of DCs and their ability to prime naïve T cells. CTL receptors, which are important for the recognition of helminth products (van Kooyk and Rabinovich, 2008), have been highlighted as the most likely PRR to contribute to modulate TLR-ligand DC maturation. This study demonstrates a possible mechanism by which *F. hepatica* may use immunomodulatory products to its benefit by suppressing inflammatory cytokines.

Given that both rFhCL1 and rFhGST-si modulate DC activation resulting in the inhibition of the development of Th17 it is clear they play an important role in the survival of *F. hepatica* during infection. Furthermore, rFhCL1 and rFhGST-si activated this suppressive population of DCs utilising different signalling pathways. The persistent activation of NF-κB in rFhGST-si-stimulated DCs compared to that observed for other helminth-derived molecules such as LNFPIII may explain the distinct capacity of these DCs to suppress Th17 rather than promote Th2 cells. Alternatively, activation of IRF5 by rFhGST-si may be required for the observed induction of IL-6 and IL-12p40

seen in stimulated DCs. Assessment of the relative contributions of NF-κB1 and NF-κB2 in response to rFhGST-si would further characterise this phenotype of DC. Also, two of the best studied helminth products SEA (Aggarwal *et al*., 2003) and LNFPIII (Thomas *et al*., 2003), are strong induces of the phosphorylation of ERK. Both also conditions DCs to induce strong Th2 responses. Therefore the absence of ERK phosphorylation in DCs stimulated by rFhGST-si and rFhCL1, may contribute to their lack of ability to promote the differentiation of Th2 cells.

It could be argued that DCs stimulated by rFhGST-si or rFhCL1 may fit into either or both of the inhibition model or the alternative pathway model of induction of modified Th2 responses by helminth parasites (MacDonald and Maizels, 2008). While neither enzyme induced DCs capable of priming Th2 responses *in vivo* both suppress responses Th1 inducing ligands (i.e. LPS induced IL-23 from DCs). Conversely, both rFhGST-si and rFhCL1 may fit an alternative pathway model as both could be theorized to be neither Th1 nor Th2 stimuli, but rather a distinct set of PAMPs associated with the attenuation of Th17 responses which may then allow for the uninhibited induction of Th2/T regulatory responses cell associated with helminth infections. This theory supports broader observations that helminth parasites are in a continuous state of homeostasis with their hosts in an effort to enhance its rate of survival. Here we put forward one such mechanism were FhTeg may selectively suppress DC activation to other helminth products. For example, DCs exposed to FhTeg show suppression of IL-6 and IL-12p40 induced by rFhGST-si (Fig. 6.1; $p \leq 0.001$) but not rFhCL1. This may be part of an effectively effective system of helminths immunomodulation with a high level of redundancy.

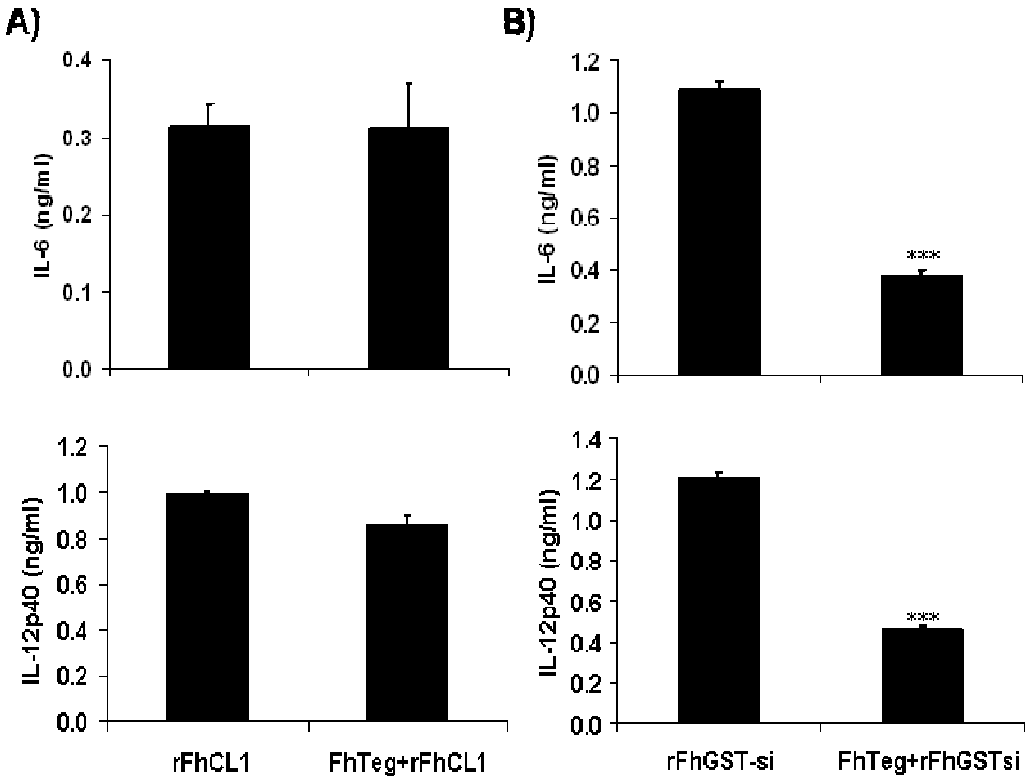

Figure 6.1: FhTeg suppresses rFhGSTsi but not rFhCL1-induced IL-6 and IL-12p40 production. DCs from C57BL/6 mice were cultured with FhTeg (10 µg/ml 2.5 h before) rFhCL1 or rFhGSTsi stimulation (10 µg/ml). Control DCs were treated with rFhCL1, rFhGST-si, LPS (100 ng/ml) or medium alone. After 18 h (*A*) IL-6 and (*B*) IL-12p40 were measured in the supernatants by sandwich ELISA. Data are the mean values of 3 individual wells (±SEM), and a representative of at least 2 individual experiments. ***, $p \leq 0.001$ compared with rFhCL1 or rFhGST-si groups.

Some TLRs, such as TLR4, recognizes ligand through accessory molecules like MD2 and CD14 (Kim *et al*., 2007). These variations in transcriptional activation of DCs may reflect differences in their enzyme activity of rFhCL1 and rFhGST-si. Either enzyme could feasibly digest peptides from PRRs resulting in an altered conformational structure disrupting subsequent binding of co-receptors such as MD2. Preliminary results indicated that rFhGST-si phosphorylation of NF-κB may be dependent on the presence of TLR4 co-receptors. HEK cells transfected with TLR4 alone without the co-receptors MD2 and CD14 exhibited reduced phosphorylation of NF-κB when treated with rFhGST-si compared to LPS stimulated cells in the same system (data not shown).

Further studies, including x-ray crystallography may be need to fully elucidate the specific binding mechanisms for each enzyme.

Unlike the possible mechanisms for Th2 induction attributed to FhTeg, rFhCL1 or rFhGST-si, AlPCF-stimulated DCs align most closely to the default mechanism. This model proposes that unless triggers for Th1 responses are received by the DC the T cell will automatically 'default' into a phenotype that will initial Th2 responses (Trinchieri, 2003). This model also applies to the Th2 polarising abilities of DCs stimulated with Schistosoma larvae products (0-3hRP) (Jenkins and Mountford, 2005), which whom AlPCF-stimulated DCs share some similarities. Overall, a comparison of both AlPCF and FhTeg shows that while both induce some similarities in treated DCs, most notably a lack of and induction of maturation associated cell surface marker expression, they drive diverging adaptive responses. AlPCF-treated DCs induced a skewed Th2 response while FhTeg-treated DCs suppress Th induced responses.

The firstly priority for future studies on FhTeg should be to identify any ligands contained within the tegument and the PRR used to detect them. Since FhTeg is a heterogeneous mix of molecules most likely compiled of proteins, lipids, carbohydrates and combinations of each, an extensive and systematic screening and identification process will need to be performed to detect the targets responsible for the results reported in this thesis. We have carried out a proteomic analysis on the FhTeg and have identified over 40 proteins. Initial fractionation using HPLC has identified a number of fractions and the proteomic analysis on each fraction is being performed to identify potential immune modulatory proteins.

As initial results from the analysis of SEA have revealed (van Liempt *et al*., 2007) multiple ligands may act in a synergistic fashion making such a process complex. It would also be interesting use confocal laser scanning techniques to track any

internalisation, compartmentalisation and subsequent presentation of any tegumental antigens. The effect FhTeg has on broader DC functions such as migration should also be studied. Extensive expression profiling of chemokine receptors (CCRs) following FhTeg exposure, particularly CCR5 and CCR7 which are down regulated and unregulated respectively during DC maturation (Luther and Cyster, 2001), would shed further light on broader effects on DC function.

We have yet to determine whether FhTeg can suppress other members of the NF-κB family (such as the p50 subunit) or any other IRF transcription factors, such as IRF3, which are also activated following TLR-ligand stimulation. Similarity, gene expression profiling of DCs exposed to all helminth products tested in this thesis could give insights into their broader effects on DC. SEA was shown to induced remarkably few genes in treated DCs but when mixed with LPS, SEA significantly affects the expression of >100 LPS-regulated genes (Kane *et al*., 2004). It would to interesting to compare the effect of FhTeg on a similar system.

When considering the results from chapters 4 and 5, the most obvious question to arise is how can AlPCF-stimulated DCs but not rFhCL1 or rFhGST-si-stimulated DCs lead to Th2 induction. Identifying the divergent characteristics of these DCs would be a priority. The use of helminth-derived immunomodulatory molecules which abrogate Th17 responses could lead to new therapeutic treatment for inflammatory disorders (Ruyssers *et al*., 2008). Studies have associated the abrogation of IL-23 to the reduction of IL-17 and ultimately attenuating the symptoms of experimental autoimmune uveitis (EAU) (Luger *et al*., 2008). It would be interesting to test rFhCL1 and rFhGST-si in this model system and indeed in other systems where IL-17 has a critical role, including rheumatoid arthritis, asthma and inflammatory bowel disease (IBD) (Zhang *et al*., 2007). Recent studies have shown that IL-27 can antagonise Th17

cell development (McGeachy and Cua, 2008), and its possible induction by both rFhCL1 and rFhGST-si may link with these studies.

While we have shown phosphorylation of one NF-κB family member by rFhGST-si it is possible that one or other member, such as the p50 subunit, many also be activated by AlPCF, rFhCL1 or rFhGST-si. The use of various transgenic mice, MyD88KO in the cases of rFhCL1 and rFhGST-si; TLR2KO mice in relation to AlPCF, would greatly enhance our understanding of the signal transduction pathways initiated by each ligand. If AlPCF is shown to contain a TLR2-activating ligand, mass spectrometry analysis would indicate the presence of phosphatidylserine (PS) which would be the most likely candidate.

In summary, we report that *F. hepatic*a tegumental maintains DCs in an immature state, impairing their function and the subsequent development of adaptive immunity, although the precise immunological scenario is not completely understood. Given the powerful modulatory effect that FhTeg exhibits, understanding its exact mechanisms may lead to the development of novel immune therapeutics for the treatment of Th1-mediated inflammatory diseases (Elliott *et al*., 2003, Summers *et al*., 2005). This study also demonstrates that another helminth-derived heterogeneous extract, *A. lumbricoides* PCF, can induce partial maturated of DCs as characterised by the increased production of IL-6, IL-12p40 and MIP-2 and that are capable of inducing Th2 responses. Lastly, two defined helminth enzymes have been shown to induce DCs capable of suppressing IL-17 secretion *in vivo*. Overall the data suggests that helminth parasites secrete multiple molecules each possessing a unique mechanism of modulation, which can either suppress inflammatory Th1/Th17 responses, or induce/permit the uninhibited development of modified Th2 responses.

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1. British Society of Parasitology (BSP) 2009, University of Edinburgh, Edinburgh, Scotland (Plenary session). Title: Helminth antigens induce distinct dendritic cell phenotype.

2. Innate Immunology Signalling Mechanisms (Feb 2008), Keystone CO, USA (Poster session). Title: *Fasciola Hepatica* tegumental antigens significantly reduce and suppress TLR-induced dendritic cell activation.

3. British Society of Parasitology (BSP) 2007, Queens University, Belfast, NI (Plenary session). Title: *Fasciola Hepatica* tegumental antigens suppress DC function irrespective of TLR stimulation.

4. Health 4 Life Conference 2007, Dublin City University, Dublin, Ireland (Plenary session). Title: Worming our way to a cure for asthma.

Publications:

1. DOWLING, D., HAMILTON, C. M. & O'NEILL, S. M. (2008) A comparative analysis of cytokine responses, cell surface marker expression and MAPKs in DCs matured with LPS compared with a panel of TLR ligands. *Cytokine*.

2. HAMILTON, C. M., DOWLING, D. J., LOSCHER, C. E., MORPHEW, R. M., BROPHY, P. M. & O'NEILL, S. M. (2009) Fasciola hepatica tegumental antigen suppresses dendritic cell maturation and function. *Infect Immun*.

3. DOWLING, D. J., HAMILTON, C. M., DONNELLY, S. M., LACOURSE, E. J., BROPHY, P. M., DALTON, J. P. & O'NEILL, S. M. (2009) Major secretory antigens of the helminth Fasciola hepatica activate a suppressive dendritic cell phenotype that suppresses Th17 cells but fails to activate Th2/Treg immune responses. *Submitted for review*.