Immunomodulatory role of helminth-derived antigens and C-type lectin receptors on inflammatory mast cells

A thesis submitted for the degree of Ph.D.

by

Krisztina Visnovitzné Vukman (M.Sc.)

Supervisor: Dr Sandra M O'Neill

School of Nursing and Human Sciences

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Declaration

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Krisztina V. Vukman: The immunomodulatory properties of helminth-derived antigens on inflammatory mast cells

Abstract

Mast cells are normally associated with T-helper (Th)2 driven IgE mediated immune responses in helminths infection, however it was recently shown that they have an important role to play in promoting Th1 immunity during infectious and non-infectious diseases. *Fasciola hepatica*, a parasitic worm of humans and domestic animals, induces strong Th2/T-regulatory immune responses during infection while simultaneously suppressing Th1 immune responses that are associated with immunity. The Th1 suppressive properties of helminths can leave the host more susceptible to bystander bacterial and viral infections while protecting the host from developing Th1 mediated autoimmune diseases. It was previously shown that *Fasciola* tegumental antigen (FhTeg) directly targets dendritic cells impairing their ability to drive Th1 inflammatory responses. Here we show that *F. hepatica* infection and FhTeg enhance mast cell numbers *in vivo* in a STAT6 independent manner. The increased mast cell number is not the result of FhTeg induced proliferation; rather FhTeg indirectly induces mast cell migration by dendritic cell derived chemokines. FhTeg-activated mast cells do not produce Th2 cytokines, drive Th2 immune responses or inhibit IgE degranulation of mast cells. Instead FhTeg inhibits mast cells ability to drive Th1 immunity suppressing pro-inflammatory cytokines and ICAM1 expression in mast cells. FhTeg suppresses the activation of transcription factors in the TLR signalling pathway through the up-regulation of SOCS3 inhibitory molecule which explains its suppressive effect. This study also demonstrated for the first time that the C-type lectin receptors, mannose receptor and macrophage galactose lectin, are expressed by mast cells and that they have an important role to play in the induction of SOCS3 by FhTeg and the promotion of mast cells inflammatory responses during *Bordetella pertussis* infection. This study advances our knowledge on mast cell biology, in particular the identification of potential targets on pathogenic mast cells.

Abbreviations

2-ME	2-mercaptoethanol
Ab	antibody
Ag	antigen
APC	antigen presenting cell
APC	allophycocyanin
APS	ammoniumpersulphate
BCA	bicinchoninic acid
BCR	B-cell receptor
BMDC	bone marrow-derived cultured dendritic cells
BMMC	bone marrow-derived cultured mast cells
bp	basis pair
BP	*Bordetella pertussis* antigen
BSA	bovine serum albumin
CD	cluster of differentiation
CpG	Cytosine guanine dinucleotide
CLR	C-type lectin receptor
d	distilled
Da	Dalton
DC	dendritic cell
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNP	dinitrophenol

ds	double-stranded
E	efficiency
EDTA	ethylenediamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FCS	Foetal calf serum
FhES	*Fasciola hepatica* excretory-secretory product
FhTeg	*Fasciola hepatica* tegumental coat antigen
FITC	fluorescein isothyocyanate
FSC	forward scatter
g	gram
GalNac	N-acetil-D-galactoseamine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
GST	glutathione S-transferase
Gusb	Glucuronidase
h	hours
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
i.p.	intraperitoneal
i.v.	intravenous
IBD	Inflammatory bowel disease
ICAM	intracellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin

IκB	Inhibitory-kappa B
IKK	IκB kinase
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IRAK	IL-1-receptor associated kinase
IRF	Interferon regulatory factor
JNK	c-JUN w-ternal kinase
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
mAb	monoclonal Ab
MAPK	mitogen-activated protein kinase
MC	mast cell
MFI	mean fluorescence density
MIP	macrophage inflammatory protein
μg	microgram
mg	milligram
MGL	macrophage galactose lectin
MHC	Major histocompatibility complex
min	minutes
μl	microliter
ml	milliliter
MR	mannose receptor
mRNA	messenger RNA
MTT	3(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetratrazolin bromide

MyD88	Myeloid differentiation factor 88
ND	not determined
NF-κB	nuclear factor kappa B
NS	not significant
OD	optical density
p-	phosphorylated
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCA	Passive Cutaneous Anaphylaxis
PCMC	peritoneum-derived cultured mast cells
PCR	Polymerase chain reaction
PE	phyycoerythrin
PGD	Prostaglandin D
PGN	peptidoglycan
PIAS	protein inhibitor of activated STAT
PMA	phorbal myristate
pNAG	p-Nitrophenyl-N-acetyl-β-D-glucosaminide
Poli(I:C)	polyinosinsic:cytidylic acid
PRR	pattern recognition receptor
PSA	Passive Systemic Anaphylaxis
qPCR	quantitative PCR
r	recombinant
RIPA	radioimmunoprecipitation
RNA	ribonucleic acid

RPMI	revolutions per minute
s	second
SCF	stem cell factor
SD	Standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SignR1	SIGN related gene 1
SOCS	suppressor of cytokine signalling
SSC	side scatter
STAT	signal transducer and activator of transcription
Syk	spleen tyrosine kinase
TBA	TRIS base boric acid EDTA
TBK	TANK-binding kinase
TBS	TRIS buffered saline
TCR	T-cell receptor
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
Th	T-helper
TLR	Toll like receptor
TMB	tetramethyl benzidine
TNF	Tumour necrosis factor
TRAF	TNF receptor associated kinase
Treg	T-regulatory
TRIF	TIR-domain-containing adapter-inducing interferon
VCAM	vascular cell adhesion molecule

ZA	zoledronic acid

1 Chapter 1 – Introduction

Helminths are parasitic worms that cause a wide variety of infectious disease in animals and humans (McSorley and Maizels, 2012). Helminths and the products they release exert strong anti-inflammatory T-helper (Th) 2/T regulatory (Treg) immune responses. Th2 immune response is important in allergy, against toxins and extracellular pathogens, such as helminths. It is characterised by increased number and activity of eosinophils (eosinophilia) and mast cells (mastocitosys) and high level of immunoglobulin (Ig)E (Anthony et al., 2007; Maizels and Yazdanbakhsh, 2003). Parasitic worm infection is usually associated with a suppressive regulatory T-cells population that can switch off inflammatory and protective immune responses leading to increased worm survival (Anthony et al., 2007; Maizels et al., 2004; Maizels and Yazdanbakhsh, 2003).

Helminth infection also effects the incidence and progression of other diseases by suppressing protective pro-inflammatory (Th1) immune response to bystander bacterial, viral and protozoan infections, making individuals more susceptible to malaria (Nacher, 2011), HIV (Carod-Artal, 2008) and tuberculosis (Resende Co et al., 2007). In human populations helminths are also thought to prevent the initiation of inflammatory disorders like arthritis, multiple sclerosis and Crohn's disease (Song et al., 2011). To this end we wish to understand the immune modulatory properties of helminths in order to understand host-pathogen interactions and to identify novel therapeutic targets to treat inflammatory conditions.

Studies examining the bystander effects of *Fasciola hepatica* (liver fluke) infection have predominantly examined the role of dendritic cells (Hamilton et al., 2009) and macrophages (Donnelly et al., 2009). However, recent studies have shown that mast cells have a critical role in the protection against microbes like *Bordetella pertussis* or *Escherichia coli* (Mielcarek et al., 2001; Wierzbicki and Brzezinska-Blaszczyk, 2009). Mast cells were also shown to be involved in inflammatory disorders such as arthritis, multiple sclerosis and Crohn's disease (De Winter et al., 2011; Theoharides and Kalogeromitros, 2006), as they are key players of the initiation and maintenance of the inflammation associated with these diseases. As helminth infection is thought to prevent the development of these diseases it is also important to study the immunomodulatory effect of *F. hepatica* and their products on mast cells.

1.1 Characteristics of helminth infections

The word helminth is originated from the Greek "hɛlmɪnθs", which means worm. This term is used to describe various types of parasitic worms that cause a wide variety of infectious disease in animals and humans. Around third of world's population (~3 billion people) are infected with helminths (Anthony et al., 2007). Helminths are responsible for many diseases in wild and domestic animals and cause significant economic losses. Helminth infection of cattle and sheep causes reduced grow-rates, weight loss, reproductive loss, and also can affect lactation (Bamaiyi, 2012; Tisdell et al., 1999). Estimating the global economic impact of helminth infection is difficult, but for example in South America the loss in swine production because of porcine cysticercosis is about the half of the national investment and is responsible for a loss of more than $160 million every year (Murrell, 1991). Parasitic worms also can make sheep or cattle more susceptible to other bacterial or viral

infections like *Salmonella dublin*, or *Mycobacterium tuberculosis* (Aitken et al., 1979; Barger, 1993), as they suppress protective immune responses normally controlling these infection. Liver fluke infection also has negative effect on the diagnosis of tuberculosis (*Mycobacterium bovis*) in cattle resulting in false negative results (Claridge et al., 2012).

Although helminth infections are generally non-fatal in humans; they are associated with high rates of morbidity and chronic inflammation (Anthony et al., 2007). We can get more accurate picture of disease burden by estimating the disability-adjusted life year (DALY) lost due to helminth infection (WHO, 2012); the estimates of DALYs for soil transmitted helminth infections (*Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*, etc.) is between 4.7 and 39 million (Bethony et al., 2006; Uneke, 2010) and for food-borne trematoidiases (*Opisthorchis viverrini*, *Fasciola hepatica*, *Clonorchis sinensis*, etc.) is 0.5-0.9 million (WHO, 2012). Gastrointestinal worm infection can cause vomiting, insomnia, weakness, stomach pains (Wang et al., 2008). In children heavy infections can result in growth retardation, apathy and learning difficulties (Levinger, 1992; Oberhelman et al., 1998). In some rare cases blood helminths can cause granulomatous disease, which can lead to organ failure. Worms in the bile ducts can cause inflammation and oedema resulting in the atrophy of the liver parenchyma (Grimaud, 1987).

Helminth infections generally do not cause severe disease relative to number of people infected; however, the suppression of the immune responses can make bystander bacterial, viral or other helminth infections more severe (Maizels and Yazdanbakhsh, 2003). Women with urinary or genital schistomiasis have an

increased risk of HIV (Carod-Artal, 2008; Ndhlovu et al., 2007), although the effect of schistosoma infection on HIV has not been studied. Patients with *Mycobacterium tuberculosis* infection developed more severe radiological pulmonary disease when co-infected with intestinal helminths (Resende Co et al., 2007). Helminth infection can also be responsible for decreased effectiveness of vaccination such as tetanus and diphtheria in humans (Nookala et al., 2004).

Studies in mouse model system showed that the number of *Tuberculosis* bacteria was higher in the lungs of mice infected with *Nippostrongylus brasiliensis* compared to the control group (Potian et al., 2011). Malaria parasitemias was significantly lower in mice infected with *Plasmodium chabaudi* only compared to mice with *Schistosoma mansoni* co-infection (Helmby et al., 1998) although in children *Schistosoma hamatobium* infection can be protective against malaria (*Plasmodium falciparium*) (Briand et al., 2005; Lyke et al., 2006; Lyke et al., 2005).

Despite its morbidity worm infections have beneficial effects on the host suffering from diseases including allergy, which are linked to overactive immune system (Carvalho et al., 2006; Harnett and Harnett, 2008; Liu et al., 2009). There are increasing evidences that the lack of exposure to parasitic worms is responsible for the prevalence of certain diseases in developed countries where helminth infection is not widespread ("hygiene hypothesis"). Inflammatory disorders caused by infections can be less severe with helminth co-infection (Wilson and Maizels, 2004). The presence of helminth infection affects the likelihood of the development of Th1 mediated immune disorders, like autoimmune disease, such as ulcerative colitis,

inflammatory bowel disease (IBD) or Crohn's disease (Carvalho et al., 2006; MacDonald et al., 2002; Reddy and Fried, 2009).

There are well defined animal model systems to study autoimmunity. Studies with helminths such as *Schistosoma mansoni* showed that parasitic worm infections can reduce incidence of autoimmune diseases by 50% (Wilson and Maizels, 2004) in mice. Gastrointestinal nematodes can suppress innate and adaptive pro-inflammatory immune responses and may be capable of suppressing unrelated inflammation in disorders such as IBD (Whelan et al., 2011). A tapeworm, *Hymenolepsis diminuta* was shown to have beneficial effect in a murine model of oxazolone-induced colitis (Reddy and Fried, 2009). In mice *Trichinella spiralis* infection was shown to be protective against colitis following intrarectal challenge with dinitrobenzene sulphate (Ruyssers et al., 2008).

There is increasing interest of parasitic worms as therapeutic agents ("helminthic therapy"). Within the past few years parasitic worms have been used in clinical trials against allergy, IBD, Crohn's disease, rheumatoid arthritis or multiple sclerosis (Harnett and Harnett, 2008; Reddy and Fried, 2009; Ruyssers et al., 2008), however, there are only a few successful examples from human trials. For example, experimental hookworm infection in patients with allergic asthma did not improve bronchial responsiveness (Feary et al., 2009). While treatment with the porcine whipworm, *Trichuris suis* had beneficial effects on patients with Crohn's disease or ulcerative colitis in clinical trials (Ruyssers et al., 2008). This worm seems to be a good candidate in IBD treatment, as it persists for limited period in the human host. However, treating patients with living helminths can have unpredicted pathological

effects like invasion into other tissues and development of serious diseases. Consideration must also be given that helminth therapy cannot be used in some patient groups such as children, elderly people, immunocompromised individuals or pregnant women (Johnston et al., 2009).

Identification and characterisation of helminth-derived molecules could lead to new therapeutic approaches. Studies in different animal models investigated broad spectrums of immunomodulatory products of parasitic worms, for example, excretory-secretory products (ES) of *Nippostrongylus brasiliensis* was shown to prevent the development of asthma and *Ascaris suum* extracts (PAS1 and APAS3) inhibit lung inflammation in a mouse model for allergy (Harnett and Harnett, 2008). *Brugia malay* antigens can mimic mammalian anti-inflammatory cytokines, like the transforming growth factor (TGF)-β and may be used in chronic inflammatory diseases (Ruyssers et al., 2008). *Dirofilaria immitis* derived antigen was shown to prevent autoimmune diabetes (Imai et al., 2001) in mice. Using parasite antigens or synthetic analogues allows developing specific and more effective drugs to cure inflammatory disorders. Understanding how helminth antigens interact with host immunity will help us to identify immune modulatory products.

1.2 Immunology of helminths

Helminth infections are usually associated with polarized Th2/Treg immune responses (Anthony et al., 2007; Maizels and Yazdanbakhsh, 2003; Mulcahy et al., 2004). The protective immune response against helminths has been referred as Th2 immune response (Anthony et al., 2007) and is characterised by increased level of Th2 cytokines, IgE, eosinophilia and mastocytosis (Seidl et al., 2011; Svensson et

al., 2010). The major sources of Th2 cytokines (interleukin (IL)-4, IL-5, IL-9 and IL-13) are $CD4^+$ T-lymphocytes; these lymphocytes are involved in killing parasites and perpetuating immune responses. Innate immune cells (mast cells, granulocytes, macrophages and dendritic cells) are important in priming the adaptive immune response and also in the effector phase in the defence against helminth infection (Flynn et al., 2010).

Mast cells and granulocytes (eosinophils, basophils and neutrophils) can react directly to the presence of helminths, but mainly became activated after the Th2 immune response developed. Cytokines secreted by $CD4^+$ T-cells direct B-cells to produce helminth specific IgE (Anthony et al., 2007; Erb, 2007). Mast cells and basophils express the high affinity FcεRI receptor, which in the presence of helminth antigens and the specific IgE antibody induce cell degranulation and the release of inflammatory mediators such as histamine, cytokines or chemokines. These mediators can promote Th2 immune responses (Melendez et al., 2007; Pearce, 2007) For example, *Heligmosomoides polygyrus* infection results in mast cell activation and induces tissue derived IL-25, IL-33 and TSLP cytokines (Hepworth et al., 2012a), which drive Th2 cell priming by dendritic cells (Hepworth et al., 2012b).

During helminth infection the number of eosinophils and basophils increases in the blood and they migrate to the site of infection. Eosinophils degranulate and release secondary granule proteins (ESGP) and may be involved in tissue remodelling and wound healing (Lee and Lee, 2005). With basophils, eosinophils also contribute to the secretion of IL-4 and IL-13 in the early stages of infection (Anthony et al., 2007).

Eosonophils also can act as antigen presenting cells (APC) to T-cells (Padigel et al., 2006).

Although Th2 immune responses are usually associated with eosonophils and mast cells, the number of alternatively activated macrophages (M2) also increases during Th2 type response against helminth parasites (Allen and Maizels, 2011). They express high level of arginase1 and IL-4 receptor but no iNOS (inducible isoform of nitric oxide synthase, usually associated with classically activated macrophages, M1) and important in regulation of the immune response, wound healing and resistance against helminth invasion (Anthony et al., 2007; Flynn et al., 2007).

Dendritic cells are orchestrated to Th2/Treg immune responses during worm infection and their pro-inflammatory function is down-regulated (Anthony et al., 2007; Jackson et al., 2009). They are important antigen representing cells and are involved in $CD4^+$ T-cell activation and regulation (Mulcahy et al., 2004). They can interpret helminth signals to T-cells and induce Th2 responses. For example Th2 immune responses can be induced by exposing dendritic cells to helminth products and inject them into recipients (Balic et al., 2004). Helminths and their products were also shown to inhibit dendritic cell maturation by bacterial ligands (Carvalho et al., 2009; Kane et al., 2004).

Interaction of helminths with the host immune system is the result of a co-evolution. As a part of the adaptation to a parasitic life, helminths had to develop a strategy to deal with the host's immune system (MacDonald et al., 2002). The intension of the host is to clear the parasite and reduce its harmful effects and the helminth wants to

be invisible to the immune system and live in the host as long as possible without killing it (Allen and Maizels, 2011). There are various mechanisms for helminth immunomodulation resulting in down-regulation of pro-inflammatory Th1 immune responses, like intense stimulation of Th2/Treg immune responses, secretion of anti-effector molecules, inhibition of effector cells or complement components and inhibition of immune cell proliferation (Allen and Maizels, 2011; Mulcahy et al., 2004).

Helminth infection is usually associated with the presence of a parasite-specific suppressive T-cell population in a regulated environment (Maizels and Yazdanbakhsh, 2003). These regulatory T-cells (Treg) secrete regulatory cytokines, IL-10 and TGF-β and can switch off inflammatory and protective immune responses (Flynn and Mulcahy, 2008; Maizels et al., 2004). This immunosuppression can lead to increased worm survival (Anthony et al., 2007) and also important in the suppression of potentially pathogenic immune responses. For example the mortality of IL-10 knockout mice are higher during *Trichinella muris* (Schopf et al., 2002) or *Schistosoma mansoni* (Wynn et al., 1998) infection compared to wild type mice with intact Treg immune responses. Dendritic cells and macrophages also play role in regulatory immune responses. As antigen presenting cells they are important in the polarisation of T-cells, and alternatively activated macrophages (M2) can prevent T-cell proliferation and Th2 differentiation (Maizels and Yazdanbakhsh, 2003).

Th1 immune responses are characterised by the secretion of interferon (IFN)-γ and associated with Th1 $CD4^+$ T-cells, cytotoxic $CD8^+$ T-cells, neutrophils and classically activated macrophages (M1). This type of immune response is typically

evoked by microbial infections including viruses and bacteria. Unrestricted Th1 immune response is harmful for the helminth and can cause pathology in the host as well, and it is down-regulated by helminth parasites (Anthony et al., 2007). In some cases host can develop Th1 immune responses against parasites, such as *Schistosoma mansoni*, which provoke Th1 associated pro-inflammatory cytokine secretion in the early larval stages of infection and induce Th2 immune response latter when the parasite starts to produce eggs (Stadecker et al., 2004).

1.3 Receptors in helminth infection

Pathogens share similar conserved structures known as pathogen-associated molecular patterns (PAMP). Immune cells express many pattern recognition receptors (PRR), which enable them to recognise PAMPs. PRRs include TLRs, intracellular nucleotide-binding domain and leucine-rich-repeat-containing family (NLR), retinoic acid-inducible gene I (RIG-I)-like receptors and c-type lectin receptors (CLR) (Geijtenbeek and Gringhuis, 2009). The complexity and crosstalk between the different PRR pathways allows cells to recognised wide range of pathogens and their ligands.

Helminth parasites can elicit immune response through TLRs (Venugopal et al., 2009). *Schistosome mansoni* was shown to activate dendritic cells through TLR2 and TLR3 (Ludwig-Portugall and Layland, 2012; Semnani et al., 2008), although its egg antigen act independently of these receptors (Kane et al., 2008). *Brigia malayi* alter the expression of TLR3 TLR5 and TLR7 on innate immune cells (Venugopal et al., 2009). TLR2 and TLR4 was shown to contribute to host immune response during *Schistosoma japonicum* infection (Zhang et al., 2011)

1.3.1 TLR signalling

TLR4 can be activated by gram negative bacterial ligand such as LPS and *Bordetella pertussis* (Lu et al., 2008; Mann et al., 2005; Wolfe et al., 2009). Upon activation with the contribution of the CD14 co-stimulatory molecule TLR4 undergoes oligomerization. The downstream signalling pathway divides into two distinct pathways, the MyD88 independent and dependent pathway (Figure 1.1). Based on studies using MyD88 macrophages it is known that MyD88-dependent pathway mediates pro-inflammatory cytokine expression, while MyD88-independent pathway is responsible for the production of Type I interferon (Lu et al., 2008). The Myd88-independent pathway involve the activation of three molecules: TIR-domain-containing adapter-inducing interferon-β (TRIF) and two IKK-related kinases, TRAF family member-associated NF-κB activator (TANK)-binding kinase (TBK1) and IKKε (IκB kinase-ε), and terminated in the phosphorylation and translocation of interferon regulatory factor (IRF) 3 (Mogensen, 2009) and the late-phase activation of NF-κB and mitogen-activated protein kinases (MAPK) (Lu et al., 2008). Studies on bone marrow mast cells reported the absence of the activation MyD88-independent pathway after LPS stimulation (Keck et al., 2011), which may be due to the low expression of cell surface CD14 (Sandig and Bulfone-Paus, 2012).

The MyD88-dependent signalling is activated by all TLRs except TLR3 (Takeda and Akira, 2004). Upon activation MyD88 associates with the cytoplasmic part of the TLR and recruits members of the IL-1-receptor-associated kinase (IRAK) family. IRAK4 and IRAK1/2 undergo phosphorylation and IRAK1 associates with an ubiquitin protein ligase, TRAF6 (TNF receptor associated factor 6), that leads to the

activation of a transforming growth factor-activated protein kinase, TAK1. TAK1 then stimulates two distinct pathways IKK involving NF-κB and the MAPK pathway (Mogensen, 2009). IKKα, IKKβ and IKKγ form a complex to phosphorylate IκB proteins. IκB degrades which leads to the subsequent translocation of NF-κB. MAPK pathways activation resulted in the induction of another transcription factor, AP-1 through the phosphorylation of ERK (extracellular signal-regulated kinase), JNK (c-JUN N-terminal kinase) and p38 (Guha and Mackman, 2001). Activation of both transcription factors induces the expression of pro-inflammatory cytokines and other inflammation related gens. In addition to NF-κB and MAPK, IκBζ and IRF5 are two important factors downstream of MyD88 and could be activated through unknown mechanism (Lu et al., 2008).

1.3.2 Regulation of TLR signalling

Proper immune responses are crucial in the clearance of pathogen infection, although extensive or inappropriate inflammation may be harmful and result in immunopathology or autoimmunity (Lu et al., 2008). The innate immune system needs to be able to control and down-regulate the signalling after the infection has been resolved. So the pathogen induced signalling pathways such as TLR4 signalling is highly regulated. The innate immune system has numerous molecules involved in these regulatory processes and TLR4 signalling can be regulated in multiple levels (Lu et al., 2008; Mogensen, 2009). Some of the most important inhibitory molecules of TLR4 signalling pathway are listed in Table 1.3.

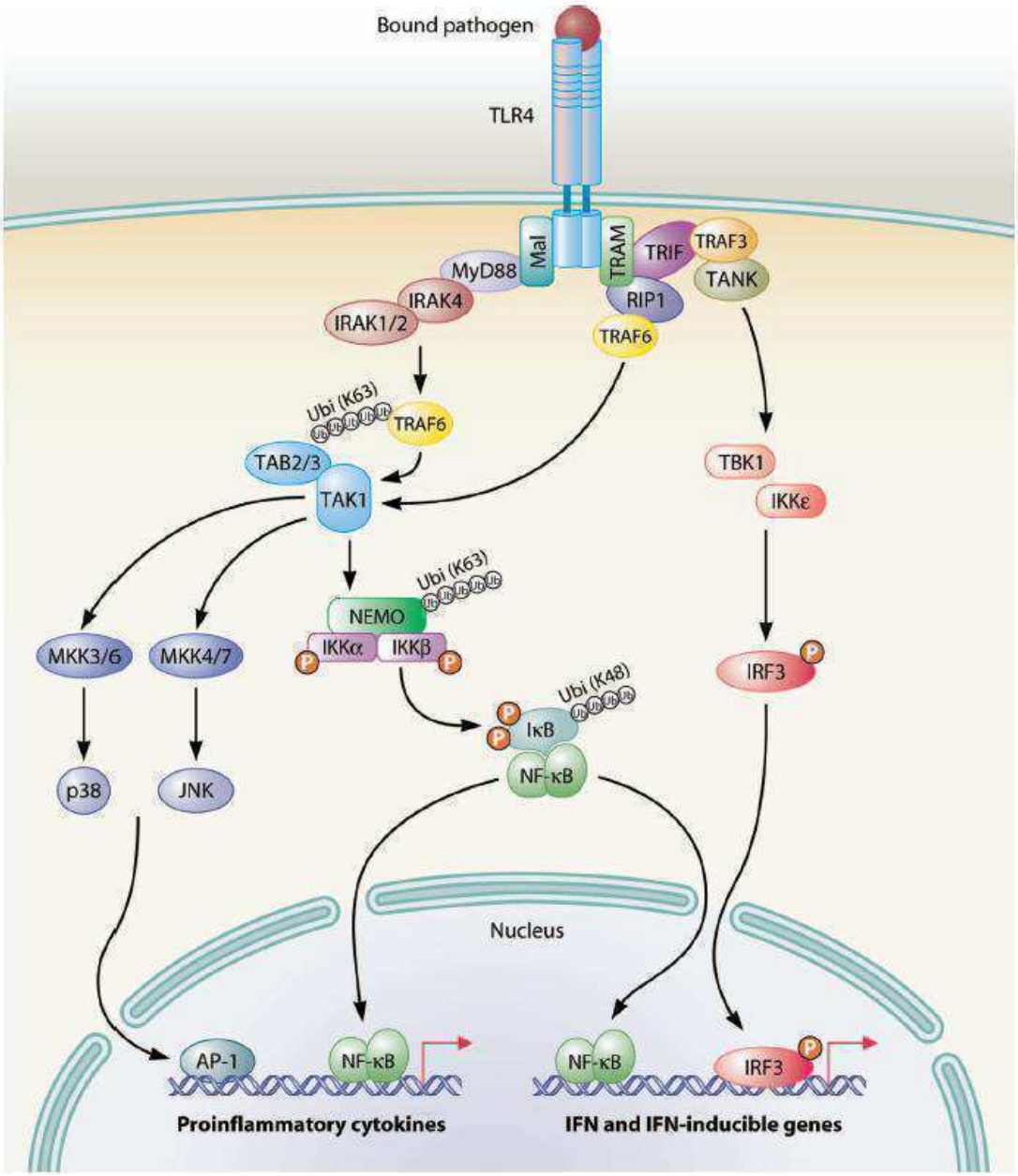

Figure 1.1. Principles in TLR signalling. TLR4 activates both the MyD88-dependent and MyD88-independent, TRIF-dependent pathways. The MyD88-dependent pathway is responsible for early-phase NF-κB and MAPK activation, which control the induction of pro-inflammatory cytokines. The MyD88-independent, TRIF-dependent pathway activates IRF3, which is required for the induction of IFN-β- and IFN-inducible genes. In addition, this pathway mediates late-phase NF-κB as well as MAPK activation, also contributing to inflammatory responses (Mogensen, 2009). *ASM authorizes an advanced degree candidate to republish the requested material in his/her doctoral thesis or dissertation.*

Table 1.1. Some inhibitors of TLR4 signalling pathways

Inhibitor	Function	Ref
SARM	Inhibition of TRIF dependent signalling. The mechanism is unknown	1
CYLD	Denaturation of TRAF6	2
TRIM30α	Degradation of TAB2/3 (required for TAK1 activation)	3, 4
IκB	Auto-regulation of NF-κB	4
RP105	Associates with MD/2 homologue MD/1 and inhibits the association between LPS and TLR4/MD-2	5,6
ST2, ST2L, IL1R1	Homologue of IL-1 receptor. Interacts with MyD88 and TIRAP and inhibits their function by sequestration	6, 7
SIGIRR	Inhibit TLR4 interaction with MyD88	8
TRIAD3A	E3 ubiquitin protein ligase. Interacts with TIR domain (TIRAP, TRIF, RIP1) what leads to the degradation of these proteins	6, 9
SOCS	E3 ubiquitin protein ligase. Ubiquitination of TIRAP1. Inhibit Jak/STAT signalling pathway as well	6, 10
IRAK/M	Belongs to IRAK family but without kinase activity. Prevents dissociation of IRAKs from MyD88	4, 11
IRAK2C	Variant of IRAK-2, but without functional domain	4
MyD88s	Variant of MyD88. Binds to IRAK4 and inhibit IRAK1/2 phosphorylation	4
TRAF1 TRAF4	Belong to TRAF family. After caspase cleavage, interact with TRIF and TRAF6	6
A20	De-ubiquitinating enzyme. Removes ubiquitin moieties from TRAF6	6

Ref: [1](Peng et al., 2010); [2](Into et al., 2008); [3](Shi et al., 2008); [4](Mogensen, 2009); [5](Divanovic et al., 2007); [6](Lu et al., 2008); [7](Brint et al., 2004); [8](Gong et al., 2010); [9](Chuang and Ulevitch, 2004); [10](Yoshimura et al., 2012); [11](Takeda and Akira, 2004)

SOCS (suppressor of cytokine signalling) molecule family is one the main inhibitory groups that plays important role in auto-regulation of pathogen induced inflammatory responses (Yoshimura et al., 2012). SOCS was first described to interfere with the cytokine induced Jak/STAT pathway, and now it is known that it is inducible by both cytokines and TLR signals resulted in down-regulation of TRAF6 activation and inhibition of both JAK/STAT and TLR pathways. The mechanism of

SOCS action is not clearly defined yet. It could be both a paracrine effect IFN-β or direct inhibition of the TLR pathway (Mogensen, 2009).

1.3.3 C-type lectins

CLRs as an important member of the PRR family are involved in the specific induction of gene expression in response to pathogens. They can modulate TLR signalling or can directly induce gene expression. CLRs are expressed by most cell types including macrophages and dendritic cell (Geijtenbeek and Gringhuis, 2009). They have conserved carbohydrate-recognition domains (CRD) and they can bind carbohydrates in a calcium dependent manner. The CRD is a compact structural module which determines the specificity of the receptor.

1.3.4 C-type lectin receptors in immune responses against helminths

Helminths express glycans, glycoproteins and glycolipids on their surfaces and on their excretory/secretory products (van Die and Cummings, 2009). Most antibodies isolated from *Schistosoma* infected murine host's serum recognised carbohydrate epitopes (Levis^{x}-containing structure) (Thomas and Harn, 2004). The glycans from helminths can be host-like glycans and pathogen-associated glycans (van Die and Cummings, 2009). There is increasing number of evidences that these glycans have key role in the induction of Th2-type (van Die and Cummings, 2006) and anti-inflammatory immune responses (Harn et al., 2009; Thomas and Harn, 2004; van Die and Cummings, 2010).

Glycans from helminth parasites were shown to be important in the induction of alternatively activated M2 macrophages and in dendritic cell's maturation and

activation (Thomas and Harn, 2004). Dendritic cell function can be modulated by carbohydrates from *S. mansoni* and its soluble egg antigen. These glycans were shown to bind to CLRs like DC-SIGN (homologue in mouse: SignR1), mannose receptor (MR) and macrophage galactose lectin (MGL), which can contribute to the suppression of LPS induced activation (Kuijk et al., 2012; Kuijk and van Die, 2010; van Liempt et al., 2006; van Liempt et al., 2007). Although most of the studies are investigating the carbohydrates from *Schistosoma* species (Klabunde et al., 2000; Paveley et al., 2011; Thomas and Harn, 2004; van Vliet et al., 2005) information is available about other parasites, like *Ascaris suum*, *Trichuris suis* (Kuijk et al., 2012) or *F. hepatica* (Guasconi et al., 2011) and their immunomodulatory properties by their glycans (Table 1.2).

Table 1.2. C-type lectins, their ligands and function important in helminth infection (Modified table from Geijtenbeek and Gringhuis, 2009)

CLR	Glycan PAMPs	Expression	Helminths	Function	Ref
DC-SIGN (CD209, mouse: SignR1)	high mannose and fucose (Le_x, Le_y, Le_A, Le_B)	Mieloid DCs	*D. viviparous, S. Japonicum, S. mansoni,*	↑ TLR-induced IL-10 production; ↑ Th1, Th2, Th17 and Treg T-cell differentiation; ↓ Th1 cell differentiation; ↑ Treg T-cells; antigen presentation	1, 2, 3, 4
MGL (CLEC10A, CD301)	Terminal GlcNAc	Myeloid DCs and macrophages	*T. spiralis* *S. japonicum* *S. mansoni* *T. canis* *F. hepatica* *E. granulosus* *M. vogae*	↓ TLR induced DC activation	1, 3, 4, 5
Dectin 1 (CLEC7A)	b-1.3-glucan	Myeloid DCs, monocytes, macrophages, B cells	*S. mansoni*	↑ Th1 and Th17 cell differentiation; ↑ TNF-α and CXCL2 production, phagocytosis	1, 3, 6
MR (CD206)	high mannose, fucose and sulphated sugars (sLe_x)	Myeloid DCs and macrophages	*T. spiralis* *A. suum* *A. vitae* *S mansoni* *F. hepatica*	Phagocytosis and antigen presentation	1, 3, 4, 7

Ref: [1](Geijtenbeek and Gringhuis, 2009); [2](Saunders et al., 2010); [3](van Liempt et al., 2007); [4](Kuijk and van Die, 2010); [5](van Vliet et al., 2007); [6](Guasconi et al., 2011); [7](Paveley et al., 2011)

Our model helminth, *F. hepatica* was also shown to express wide range of glycans and glycoproteins on their surface and in their FhES (Wilson et al., 2011; Wuhrer and Geyer, 2006) with possible immunomodulatory effects. It was shown that FhES failed to induce alternative activation of macrophages in the presence of mannan, laminarin or the blocking antibodies of MR or Dectin1, suggesting that these CLRs are important in the recognition of *F. hepatica* and their products (Guasconi et al., 2011). However, it was shown that TLR2 is also involved in the inhibition of *Mycobacterium bovis* activated macrophages by FhES (Flynn and Mulcahy, 2008).

The major components of FhTeg are carbohydrates, so CLRs may be important in its immunomodulatory effect on immune cells (Wilson and Maizels, 2004; Wilson et al., 2011; Wuhrer and Geyer, 2006).

1.3.5 C-type lectin receptors in immune responses against bacteria

CLRs are not only important in helminth host interaction, but they also play role in immunity against bacterial and fungal infections. Bacterial carbohydrate CLR interactions have important roles in innate immunity as they are involved in pathogen recognition and in the cellular interactions that lead to the modulation of immune responses (Weis et al., 1998). On the bacterial surface there are complex oligosaccharide structures, and as CLRs have specificity for glycans (like mannos, Lewis, GalNAc and β-glucan) they can mediate specific interactions with pathogens (Lloyd et al., 2007). Some pathogens can interact with more than one CLR and cells express different CLRs on their surfaces that can contribute to their different function at different locations. CLRs play role in antigen internalization and presentation onto major histocompatibility complex (MHC) molecule. As CLRs possess signalling properties they can modify TLR mediated immune reactions (van Vliet et al., 2005).

1.4 Fasciola hepatica

Our model helminth, *Fasciola hepatica* (liver fluke) is a parasitic worm causing liver disease (fascioliasis) in mammals, including cattle and sheep. Heavy liver fluke infection can cause weight loss, anaemia, wool break or even sudden death in domestic animals (Behm and Sangster, 1999; Taylor, 2012). It is an important zoonotic disease in human with approximately 2.4 million people infected

worldwide (McManus and Dalton, 2006). The life cycle of the parasite in the mammalian host is complex. The definitive host is infected following ingestion of metacercariae shed from snails (*Lymnaeidae*). These metacercariae excyst in the duodenum to form newly excysted juvenile flukes, those penetrate of the intestinal wall. They migrate through the abdominal cavity and liver for several weeks and then finally the adult worm resides in the bile ducts releasing eggs (Figure 1.2). Penetration of the small intestine generally not associated with clinical disease, as only a few cells are disrupted. But migration through the peritoneal cavity into the liver can cause haemorrhage or fibrosis. Also the larvae occasionally penetrate other organs than liver, including lymph nodes, lung or pancreas (Andrews, 1999; Behm and Sangster, 1999).

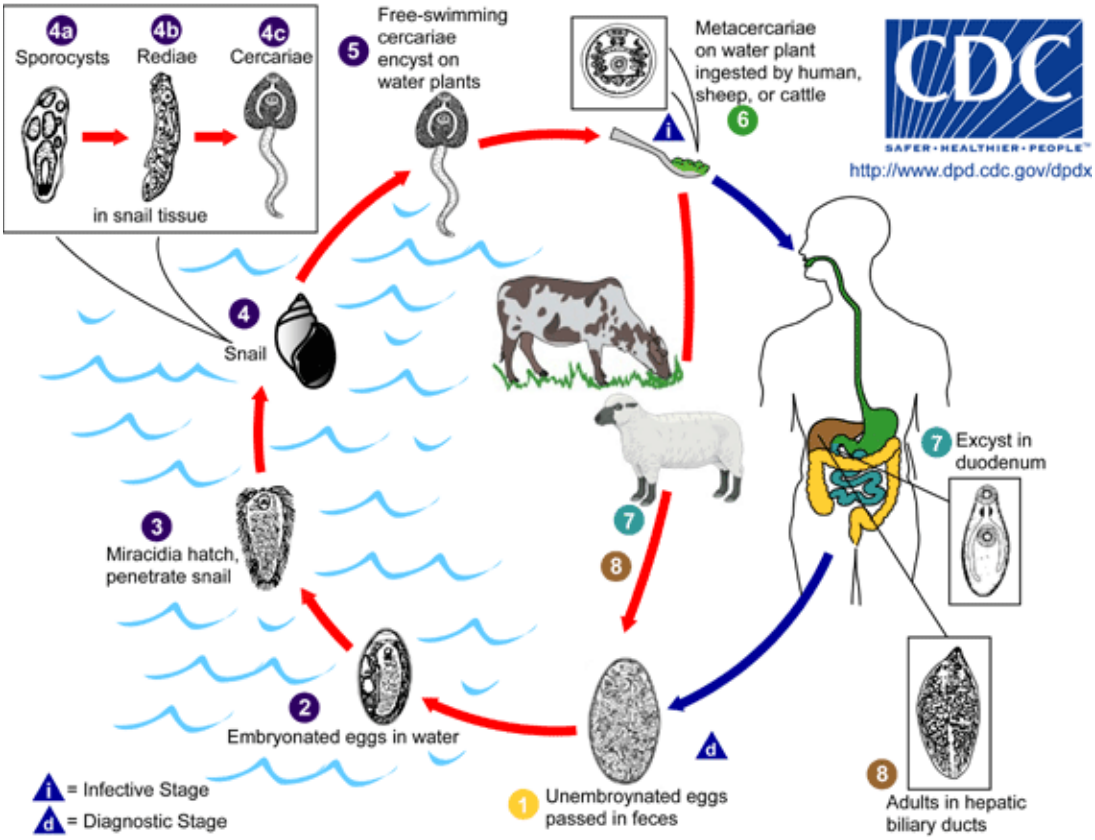

Figure 1.2. Life cycle of Fasciola hepatica. Immature eggs are discharged in the biliary ducts and in the stool 1. Eggs become embryonated in water 2, eggs release miracidia 3, which invade a

suitable snail intermediate host 4, including the genera *Galba, Fossaria* and *Pseudosuccinea*. 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 (*Fasciola hepatica*: up to 30 mm by 13 mm) reside in the large biliary ducts of the mammalian host. *Fasciola hepatica* infects various animal species, mostly herbivores (Public Health Image Library, http://www.dpd.cdc.gov/dpdx).

Fasciola hepatica is able to survive in the liver bile duct for many years, although it is exposed to the immune system. The fluke promotes strong Th2/Treg T-cell mediated immune responses and induce the production of IL-4, IL-5 and IL-10 (Miller et al., 2009; O'Neill et al., 2000). Innate and adaptive immune responses are both important in the defence against *F. hepatica*. The number of eosinophils increases during liver fluke infection in cattle (Bossaert et al., 2000) and sheep (Zhang et al., 2005). *Fasciola hepatica* antigens were shown to cause apoptosis of these cells (Serradell et al., 2007). The fluke is capable of inducing alternatively activated macrophages (M2) with up-regulated arginase1 expression and activity (Donnelly et al., 2005). These macrophages are involved in priming Th2 T-cell differentiation and also has inhibitory effect as they secrete the regulatory cytokine, IL-10 (Flynn and Mulcahy, 2008; Hacariz et al., 2010). Macrophages and dendritic cells act as antigen presenting cells and has role in the activation of Th2 T-cells (Flynn et al., 2010; Wynn, 2009). Adaptive B-cells are responsible for the generation

of parasite-specific antibodies during *F. hepatica* infection (Clery et al., 1996; O'Neill et al., 2000).

In order for *F. hepatica* to survive within its host it must suppress Th1 inflammatory responses as immunity against *Fasciola* infection requires strong Th1 immunity (Moreau and Chauvin, 2010). IL-4 knockout mice, which were predisposed towards a Th1 immune response, are less susceptible to *F. hepatica* infection than IFN-γ receptor knockout mice with only Th2 immune responses (O'Neill et al., 2000). Studies in mouse (O'Neill et al., 2000) cattle (Flynn and Mulcahy, 2008) and sheep (Moreau et al., 1998) suggest that early Th1 immune responses (characterised by IFN-γ secretion) change to Th2 (characterised by IL-4 production) as infection established. Treg cells (secreting IL-10 and TGF-β) are capable of suppressing these Th1 and Th2 immune responses (Flynn et al., 2010).

Liver fluke is known to suppress the protective Th1 immune response against bacterial infections. It was shown that cattle with fascioliasis had delayed immune response against *Mycobacterium bovis* (Flynn et al., 2009). In mouse model, *F. hepatica* infection reduced *Bordetella pertussis*-specific IFN-γ production (O'Neill et al., 2001). Liver fluke infection could also prevent the development of Th1 autoimmune diseases. It was showed to suppress antigen specific Th1 and Th2 immune responses that mediate experimental autoimmune encephalomyelitis (Walsh et al., 2009).

1.5 Antigens from *Fasciola hepatica*

Molecules secreted or shed by *Fasciola hepatica* play central role in manipulating the host's immune system (Caffrey, 2012). There are three groups of antigens derived from *F. hepatica*: the excretory/secretory products (FhES), the whole liver fluke homogenate (LFH) and the tegumental coat antigen (FhTeg). The most intensively studied molecule group is the excretory/secretory product of the fluke (FhES). It can be isolated from the media *F. hepatica* is maintained (Robinson et al., 2012a). There are secretory vesicles in the gastrodermal cells of the fluke; FhES is secreted into the lumen of the helminth's gut and then externally into the host tissues (Collins et al., 2004). The major secreted molecules and their immunomodulatory effects are listed in Table 1.3 (Robinson et al., 2012a).

Two protein fractions of FhES can be separated by gel permeation chromatography, PI and PII. PI contain peroxiredoxin (FhPrx) and helminth defence molecule-1 (FhHDM-1) and can induce antigen-specific Th2 responses *in vivo* in mice, while PII fraction consist of cathepsin L cysteine protease (FhCL) and does not induce Th2 immune responses but suppresses Th1 cytokine production in T-cells (Donnelly et al., 2008; Robinson et al., 2011; Robinson et al., 2010). These molecules have immune-therapeutic potential (Robinson et al., 2012b).

Table 1.3. Major secreted molecules of Fasciola hepatica (Robinson et al., 2012a)

Molecule	Effect on the immune system
Cathepsin L1 and L2	Degrades endosomal TLR3 Cleaves hinge region of IgG Inhibits the differentiation of Th17 cells by dendritic cells Cleaves hinge region of IgG
Peroxiredoxin	Induces M2 macrophages
Helminth defence molecule	Modulates LPS-mediated response in macrophages
Leucine aminopeptidase	Unknown
Glutathione S-transferase	Inhibits proliferation of spleen cells and production of nitric oxide by macrophages Inhibits differentiation of Th17 cells by dendritic cells
Fatty acid-binding protein	Unknown

Peroxiredoxin is not only important in reducing oxidative stress on flukes (Caffrey, 2012), but also induce M2 alternative activated macrophages, associated with Th2 cytokines in parasitic inflammation. These macrophages are involved in controlling Th1 type immune responses (Donnelly et al., 2009; Donnelly et al., 2008) and in murine model of human auto-inflammatory disease these M2 macrophages were shown to suppress the progression EAE (Mikita et al., 2011).

Cathepsin L Cysteine peptidases are the major components of FhES and play role in the nutrition of the parasite by degrading host proteins into peptides (Robinson et al., 2012b) These enzymes do not induce Th2 immune responses rather prevent the development of protective Th1 immune responses. FhCL inhibits the release of inflammatory mediators, such as IL-6, IL-12, TNF or nitric oxide from macrophages and also suppress IL-23 secretion from dendritic cells and enhances co-stimulatory molecule expression (Dowling et al., 2009). FhCL treatment was shown to suppress *Bordetella pertussis* induced IFN-γ production in mice (O'Neill et al., 2001).

F. hepatica also secretes molecules which can mimic the function of the host defence peptides (cathelicidin-like helminth defence molecule, FhHDM) (Robinson et al., 2011). They act as antimicrobial peptides but also important in the activation of wound healing and angiogenesis. These molecules can modulate innate cell activation by TLR ligands like LPS. They can bind to LPS preventing its interaction with macrophages (Robinson et al., 2011). They might be important in the survival of the host and therefore of the parasite as well (Robinson et al., 2012a; Robinson et al., 2012b). FhHDM can protect mice against LPS-induced inflammation by reducing the release of inflammatory mediators from macrophages (Robinson et al., 2011).

There are few studies examining the immunomodulatory effect of the whole liver fluke antigen. LFH is a complex mixture of antigens that has shown cross reactivity with proteins in FhES or components of FhTeg (Falcon et al., 2012), but also contains proteins that are unique for this preparation. LFH was studied in vaccine trials in different animal models (Cervi et al., 2004; McCole et al., 1999). *In vitro* in dendritic cells and *in vivo* in mice it was shown to induce the production of Th2 and anti-inflammatory cytokines, but suppress pro-inflammatory responses (Carranza et al., 2012; Falcon et al., 2012).

1.6 FhTeg

A major interface between the parasite and its environment is the tegument. It is a syncytial cytoplasmic layer bounded by plasma membrane and covered by glycocalyx (Wilson et al., 2011) and connected by cytoplasmatic strands to nucleated cell bodies located in the parenchyma below the muscle layers (Threadgold, 1963).

In the cytoplasm there are mitochondria and secretory bodies. There are three types of secretory bodies: T0, T1 and T2, produced by T1 and T2 cells (species specific). The content of secretory bodies is age dependent and they are important in the adaption to the environment. T0 type secretory bodies build the glycocalix during invasion while T1 and T2 type antigens are less immunogenic than the juvenile antigens (Sobhon et al., 2000). In the adult fluke the tegument is 15-20 μm thick and the surface is increased by intuckings of the plasma membrane (apical invagination). The apical membrane is covered by thick (30+25 nm) glycocalix (Fairweather et al., 1999). The glycocalix contains oligosaccharides with negative charge and may provide the first line of defence, as it is thick and insoluble (Sobhon et al., 2000).

The tegument has multiple functions like absorption of exogenous nutrients, synthesis and secretion of various substances, osmoregulation and protection against enzymes, the bile and host's immune responses (Dalton, 1999; Halton, 2004). The tegument is in interaction with the immune system during the migration from the gut to the peritoneal cavity and from the peritoneal cavity to the bile duct. The tegument is shed every 3 hours and it is in direct contact with the host's immune cells. An intact glycocalix and syncytium is essential to the survival of the fluke in the host (Fairweather et al., 1999).

The most successful anthelminthics drugs (benzimidazoles) usually target the tegument in order to kill the fluke. The effect of triclabendazole (TCBZ), the most popular and effective drug of this group, on the tegument is well investigated *in vitro* and *in vivo* as well. They disrupt the tegumental coat within 96 hours, and by that time the flukes are usually dead (Toner et al., 2010a; Toner et al., 2010b; Toner et

al., 2010c). Other benzimidasole drugs like albendazole or mebendazole act in a similar way (Keiser and Morson, 2008).

Isolation of tegumental antigen has difficulties, because it contains different proteins and an insoluble glycocalix (Hillyer, 1980; Wilson et al., 2011). Use of detergents like Nonidet P40 is required. Most of the isolated proteins from FhTeg (Hacariz et al., 2012; Morphew et al., 2012; Wilson et al., 2011) and the contents of the glycocalix (Wuhrer and Geyer, 2006) are identified. Over 50 different proteins are present in the preparation and the biological active component(s) of FhTeg has yet to be identified. While FhES mainly consist of proteins, and has extensive enzyme activity, FhTeg contain more carbohydrates than proteins without much enzyme activity (Wilson et al., 2011; Wuhrer and Geyer, 2006).

It was demonstrated before that the isolated tegumental antigen of *F. hepatica* suppresses the secretion of pro-inflammatory cytokines (IFN-γ and IL-12p70) *in vivo* in the mouse model of septic shock (Hamilton et al., 2009). Dendritic cells play central role in driving Th1 inflammatory responses. They mature and became activated by conserved microbial molecules, such as lipopolysaccharide (LPS) of *E. coli,* and express co-stimulatory molecules on their surface (CD40, CD80 and CD86) and secrete pro-inflammatory cytokines as IL-12p70 and TNF-α (Murphy, 2011). FhTeg impairs dendritic cell maturation and function *in vitro* by inhibiting IL-6, tumour necrosis factor (TNF)-α, IL-10 and IL-12 production and CD80, CD86 and CD40 cell surface marker expression in Toll-like receptor (TLR)4 stimulated dendritic cells.

FhTeg also targeted other TLR and non-TLR signalling pathways, as peptidoglycan (PGN - TLR2), flagellin (TLR5), polyinosinic-polycytidylic acid (poli(I:C) - TLR3), dinucleotide from bacterial DNA (CpG - TLR9), zymozan (TLR6) and phorbol-myrsitate-acetate (PMA - non-TLR) induced Th1-type cytokine secretion and cell surface marker up-regulation was also inhibited by FhTeg in these cells. FhTeg impaired dendritic cell function by inhibiting its phagocytic capacity and its ability to prime T-cells. It was shown that FhTeg suppress cytokine production through the inhibition of the phosphorylation of nuclear factor kappa-light-enhancer of activated B-cells (NF-κB) p65, transcription factor in the TLR signalling pathway (Hamilton et al., 2009).

Although there are numerous studies of the interaction of the tegumental antigen of *Schistosoma* sp. and different cell types (Braschi et al., 2006; Caldas et al., 2000; Duraes et al., 2009) there is less to know about the effect of FhTeg on immune cells like macrophages and mast cells. Tegumental extraction of *F. hepatica* was shown to up-regulate arginase1 transcription in macrophages *in vitro*. Arginase1 activity is usually associated with M2 macrophages, although other M2 markers were not tested in this study (Hacariz et al., 2010). To our best knowledge, there is only one study about mast cell - tegumental coat antigen interaction; rat peritoneal mast cells were shown to produce histamine upon the stimulation with synthesized short peptides with homologue sequences as found in tegument of *F. hepatica* (Trudgett et al., 2003). As mast cells have a key role in the immune system, it is important to involve these cells in FhTeg research.

1.7 Mast cells

Mast cells are bone marrow derived haematopoietic immune cells. They are resident in tissues, but most common at sites which exposed to the environment like skin, intestine or airways (Galli and Tsai, 2010). Mast cells are a major component of protective immunity against microbial infections as bacteria and parasites, such as helminths. Mast cells are intensively studied in the context of Th2 immune responses. They express high affinity receptors on their surface like FcεRI, which allows these cells to be activated by IgE specific antigens and secrete various mediators like histamine, cytokines or chemokines and mediate Th2 immune responses (Melendez et al., 2007; Pearce, 2007). Mast cells also play key role in the development of Th1 immune responses and they are involved in Th1 immune disorders (Marshall et al., 2003).

1.8 Mast cells in parasitic infections

Parasitic infections are often associated with increased mast cell numbers and redistribution (Weller et al., 2011), although the importance of these cells in the clearance of helminth infections vary greatly (Anthony et al., 2007). During intestinal helminth infection the role of mast cells is well defined. Nematode fecundity is higher in mast cell-deficient mice when infected with *Heligmosomoides polygyrus* compared to normal or mast cell-reconstituted mice (Hashimoto et al., 2009). Studies in mouse and rat models showed that the clearance of *Strongloides ratti, Trichinella spiralis*, *N. brasiliensis* and *Strongyloides ratti* is mast cell number dependent (Abe and Nawa, 1988; Marshall, 2004; McDermott et al., 2003). Although mast cells do not seem to be crucial in the clearance of *Trichuris muris*, as ablation of mast cells in murine model has no effect on the development of immune

response (Betts and Else, 1999). The activation of mast cells during infection is usually dependent on Th2 cytokines secreted by $CD4^+$ T-cells (Anthony et al., 2007; Erb, 2007). Activated mast cells secrete serine proteases, chymase, tryptase and can directly protect against parasites (Metz and Maurer, 2007). They also contribute to the expulsion of nematodes by increasing intestinal permeability and inducing neutrophil influx (McDermott et al., 2003). Mast cells are important in tissue remodelling as they induce fibronectin turn-over and angiogenesis in order to protect the host from trauma caused by helminths (Maurer et al., 2003). While mast cells are mostly crucial in the clearance of gut helminths, they do not appear to a have key role in the clearance of other tissue dwelling helminths such as schistosomiasis, although they are present in the early stages of *Schistosoma* infection (Gerken et al., 1990).

1.8.1 C-type lectin receptors on mast cells

There are only few studies about CLRs on mast cell surface. They express an identical CLR, mast cells function associated antigen (MAFA) (Guthmann et al., 1995a; Guthmann et al., 1995b). The activation of MAFA leads to the inhibition of FcεRI-induced activation of mast cells (Abramson et al., 2002). This protein was found on mouse natural killer (NK) cells and human basophils (Katz, 2002). Mincle and Dectin 1 were also shown to be expressed by mast cells, although the expression level is very low (Ribbing et al., 2011). Upon mast cell activation by the fungal antigen zymosan the Dectin1 level increases and zymosan induced leukotriene production seems to be Dectin1 dependent (Yang and Marshall, 2009).

1.9 Mast cells in autoimmune diseases

Mast cells are thought to have protective role during sepsis and are associated with many Th1-mediated inflammatory disorders such as autoimmune disease and cancer where TNF-α secreting mast cells contribute to pathology (Metz and Maurer, 2007; Sutherland et al., 2011; Weller et al., 2011). Evidences have been published that mast cells play key role in IBD, the inflammatory autoimmune disorder of the gut. Experiments in animal models and in human studies showed that IBD is associated with high number of mast cells in the intestine and these cells degranulated and secrete histamine and pro-inflammatory cytokines like IL-6 and TNF-α (De Winter et al., 2011). Mast cell inhibitors have therapeutic potential in the treatment of IBD (He, 2004). Studies in mast cell-deficient mice ($Kit^{-/-}$) showed that mast cells derived pro-inflammatory cytokines, such as TNF-α are important in the development of EAE (the murine model for multiple sclerosis) (Christy et al., 2012; Costanza et al., 2012). As helminth derived molecules can prevent the initiation of Th1 mediated inflammatory diseases, it is important to investigate FhTeg and mast cells interactions.

1.10 Mast cells in bacterial infections

Similar to dendritic cells and macrophages, mast cells express pathogen recognition receptor such as TLRs (TLR2, TLR3, TLR4, TLR6, TLR7 and TLR9) that recognise invading pathogens (Marshall et al., 2003). Mast cells in response to bacterial ligands express co-stimulatory molecules and secrete cytokines that promote pro-inflammatory Th1 immune responses (Abraham and St John, 2010; Dawicki and Marshall, 2007; Galli and Tsai, 2010; Metcalfe et al., 1997; Weller et al., 2011). They secrete TNF-α, IL-5, IL-6 and IL-13 but not IL-1β after TLR2 activation with

bacterial peptidoglycan (Supajatura et al., 2001). TLR4 dependent stimulation with lipopolysaccharide (LPS) induces TNF-α, IL-1β, IL-6 and IL-13 but not IL-4 or IL-5 secretion (Supajatura et al., 2002).

Mast cell induced Th1 immune response is crucial in the clearance of protozoan and bacterial infection, such as *Escherichia coli* (Wierzbicki and Brzezinska-Blaszczyk, 2009), *Leishmania major* (Maurer et al., 2006) or *Pseudomonas aeruginosa* (Siebenhaar et al., 2007) infections. *Bordetella pertussis* is a highly infectious respiratory pathogen which causes whooping cough. Mast cells together with dendritic cells and macrophages are supposed to play role in the mediation of bacterial clearance from the lung. *In vitro B. pertussis* induces IL-6 and TNF-α release from mast cells and *in vivo* the early TNF-α production is delayed in mast cell depleted, *B. pertussis* infected mice (Mielcarek et al., 2001). TLR4 was shown to be critical in the recognition of this pathogen (Mann et al., 2005).

1.11 Mast cell - T-cell cross talk

$CD4^+$ T-cell dependent mast cell activation is well investigated during T-cell mediated inflammatory responses. T lymphocytes can induce degranulation, secretion of β-hexosaminidase, several inflammatory cytokines (TNF-α, IL-4 and IL-6) and chemokines (CXCL2, CCL5) following direct contact with mast cells (Gri et al., 2012). Mast cell-secreted mediators also can activate B and T-cells and are known to have immune-regulatory and immune modulatory effect on T-cell-dependent responses (Metcalfe et al., 1997). Mast cells can act as APCs and can process T-cell activation. They can be found in most of the tissues, also in the secondary lymphoid organs, so have potential to prime naïve T-cells or directly alter

T-cell function (Brown et al., 2008) via cell surface molecules or secreted mediators. In some studies, mast cell-deficient mice show delayed type hypersensitivity (Metcalfe et al., 1997), although in these mouse models the lack of c-kit signalling may have impact on T-cell development (Brown et al., 2008).

Mast cells express cell surface markers, adhesion molecules, co-stimulatory and co-inhibitory molecules which allows them to interact with other immune cells. MHC class I and II play central role in antigen presentation. Mast cells express high levels of MHCI but very low levels of MHCII, although this can be up-regulated during pathogen infection, LPS or IFN-γ stimulation (Kambayashi et al., 2009). Expression of intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, OX40L, CD40L, LFA-1 and many other molecules suggest broad ability to directly mediate T-cell activation, although the mechanism is unclear yet (Kashiwakura et al., 2004; Sayed and Brown, 2007).

Mast cells secrete cytokines and chemokines which can be important in cell to cell communication (Galli and Tsai, 2010). Mast cell derived IL-4 was shown to be important in Th2 immune responses (Sayed and Brown, 2007), while IL-12 and TNF-α induce Th1 polarisation and TGF-β and IL-6 are important in Th17 immune responses (Brown et al., 2008; Nakae et al., 2006). In a rodent model of multiple sclerosis, T-cell activation (IFN-γ production, CD44 and CD11a up-regulation) was shown to be mast cell dependent (Gregory et al., 2005). Bacterial and viral infections can induce cytokine secretion and adhesion molecule up-regulation in mast cells that leads to the activation of protective Th1 immune responses. Mast cells can regulate T-cell migration directly by producing chemotactic factors (IL-16, CCL2/MCP-1,

CCL3/MIP-1α, CCL4/MIP-1β or TGF-β). Exosomes may also be involved in mast cell T-cell communication. They were shown to contain molecules, like MHCII, CD86, CD40L, LFA-1, and ICAM-1 during infection (Galli et al., 2005). Mast cells mediate the up-regulation of the expression of surface adhesion molecules on endothelial cells and induce T-cell activation indirectly (Galli et al., 2005).

Mast cells communicate with other cells types like dendritic cells or B-cells. Mast cell-derived IL-4 initiates the allergic response by inducing IgE production by B-cells (Metcalfe et al., 1997; Sayed and Brown, 2007). CD40 and CD40L co-stimulatory molecule interaction was shown to be involved in mast cell induced IgE production by B-cells (Galli et al., 2005; Sayed and Brown, 2007). There are many evidences that mast cells can regulate dendritic cells migration, regulation and function by the production of TNF-α, IL-1, IL-16, IL-18 or PGD_2 (Galli et al., 2005). Mast cell - dendritic cell interactions can be important in the APC function of dendritic cells (Bryce et al., 2004; Mazzoni et al., 2006). Both soluble mediators and direct cross-talk are important in these processes (Gri et al., 2012). Mast cells were also shown to induce natural killer cell, eosinophil and neutrophil accumulation and activation in different animal models for bacterial infection, allergy and autoimmune disease (Gri et al., 2012).

1.12 Summary

Helminths and the products they release exert strong Th2/Treg immune responses (Anthony et al., 2007) that can suppress the Th1 driven immune response to bystander infections making malaria (Nacher, 2011) and HIV (Carod-Artal, 2008) infections more severe. Helminths are also thought to prevent the initiation of Th1

mediated inflammatory disorders like arthritis, multiple sclerosis and Crohn's disease (Song et al., 2011). Studies examining the bystander effects of helminths have predominantly examined the role of dendritic cells (Carvalho et al., 2006; Carvalho et al., 2009; Kane et al., 2004) and macrophages (Allen and Maizels, 2011), however, recent studies have shown that mast cells have a critical role in Th1 mediated immune responses against microbes, such as *E. coli* (Marshall, 2004) and in inflammatory disorders, such as arthritis, multiple sclerosis and Crohn's disease (Metz and Maurer, 2007; Sutherland et al., 2011; Weller et al., 2011). In this study we examine the interaction of the tegumental coat antigen of *F. hepatica* with mast cells. Better understanding the mechanism involved in the immunomodulation by *F. hepatica* may lead to potential identification of novel anti-inflammatory therapeutic targets.

1.13 Aims of the project:

The aim of the project is to understand interactions of mast cells with *F. hepatica* infection antigens derived from the tegumental coat (FhTeg) by answering the following questions:

- Are mast cells observed during *F. hepatica* infection and also following treatment with FhTeg?
- Are mast cells involved in FhTeg driven Th2 immune responses?
- Does FhTeg suppress Th1 mediated immune responses in mast cells?
- Can FhTeg treated mast cells modulate T-cells *in vitro*?
- What immune modulatory mechanisms does FhTeg use to modulate innate immune cells?
- Are C-type lectin receptors involved in FhTeg - mast cell interactions?

2 Chapter 2 – Material and Methods

2.1 Materials

2.1.1 Reagents

2.1.1.1 Animals and cell lines

Name	Catalog #	Company
BALB/C mice (female)		Harlan UK Ltd (UK) / Charles River (Ireland)
C57BL/6 mice (female)		Harlan UK Ltd (UK) / Charles River (Ireland)
STAT6$^{-/-}$ mice (female)		Charles River (Ireland)
Liver flukes (*Fasciola hepatica*)		abattoir in Ballyjamesduff, Ireland
Metacercariae (*F. hepatica*)		Baldwin Aquatics Ltd, USA
MC/9	CRL-8309	LGC Standards, Teddington, UK
RAW 264.7	ATCC: TIB-71	LGC Standards, Teddington, UK
WEHI-3	ATCC: TIB-68	LGC Standards, Teddington, UK

2.1.1.2 Cell culture

Product	Catalog #	Company
Foetal Calf Serum (FCS)	10270-106	Gibco, Invitrogen, Paisley, UK
IL-3	407631	Calbiochem, Merck, Darmstadt, Germany
IMDM	12440-048	Gibco, Invitrogen, Paisley, UK
L-Glutamine	G7513	Sigma-Aldrich, Dublin, Ireland
Phosphate Buffer Saline (PBS)	14190	Gibco, Invitrogen, Paisley, UK
Penicillin/Streptomycin	1570-063	Gibco, Invitrogen, Paisley, UK
RPMI 1640	31870-074	Invitrogen, Paisley, UK
Stem Cell Factor	S9915	Sigma-Aldrich, Wicklow, Ireland
Trypan blue	T8154	Sigma-Aldrich, Wicklow, Ireland
X VIVO 15	BE04-48Q	Lonza, Walkersville, USA

2.1.1.3 Stimulants and blocking antibodies

Product	Catalog #	Company
Anti- mouse ICAM1	ab25375	Abcam, Cambridge, UK
Anti-mouse mannose receptor	ab8918	Abcam, Cambridge, UK
Anti-mouse MGL	HM1081	Hycult biotech, Uden, The Netherlands
Anti-mouse SignR1	AF1836	R&D, Abingdon, UK
Mannan (from *Saccharomyces cerevisae*)	M7504	Sigma-Aldrich, Wicklow, Ireland
Laminarin (from *Laminaria digitata*)	L9634	Sigma-Aldrich, Wicklow, Ireland
N-Acetil-D-galactosamine (GalNac)	A2795	Sigma-Aldrich, Wicklow, Ireland
Bordetella pertussis antigen	kind gift of Bernie Mahon	
Calcium ionophore (A23187)	C7522	Sigma-Aldrich, Wicklow, Ireland
Flagellin (S. typhimurium)	tlrl-stfla	InvivoGen, CA, USA
Lipopolysaccharide (LPS) (E. coli)	ALX-581-007	Alexis Biochemicals, Lausanne, Switzerland
CpG ODN 1826	tlrl-modn	InvivoGen, CA, USA
Peptidoglycan (PGN) (S. aureus)	tlrl-pgnsa	InvivoGen, CA, USA
Phorbal myristate acetate (PMA)	P8139	Sigma-Aldrich, Wicklow, Ireland
Piceatannol (Syk inhibitor)	ALX-270-2002-M001	Enzo Life Sciences, Exeter, UK
Poli (I:C)	tlrl-pic	InvivoGen, CA, USA
Zoledronic acid	SML0223	Sigma-Aldrich, Wicklow, Ireland
Zymosan (S. cerevisiae)	Z4250	Sigma-Aldrich, Wicklow, Ireland

2.1.1.4 Kits

Product	Catalog #	Company
Annexin V-FITC apoptosis detection kit	556547	BD pharmingen, Oxford, UK
BCA protein kit	23288, 23224	Promega, Medison, USA
CD4+ Isolation kit	130-095-248	Milltenyi Biotech, UK
Cell Growth Determination Kit, MTT based	CG-D1	Sigma-Aldrich, Wicklow, Ireland
DNase kit	18068-015	Invitrogen, Paisley, UK
Greiss reagent system (nitric oxide)	G2930	Promega, Madison, USA
Maxima SYBR Green/ROX qPCR Master Mix (2x)	K0221	Fermentas, Fisher, Dublin, Ireland
Mouse IFN-γ ELISA Set	555138	BD Biosciences, Oxford, UK
Mouse IL-10 ELISA Set	555252	BD Biosciences, Oxford, UK
Mouse IL-3 ELISA	432101	Biolegend, Cambridge, UK

Mouse IL-4 ELISA Set	555232	BD Biosciences, Oxford, UK
Mouse IL-5 ELISA Set	555236	BD Biosciences, Oxford, UK
Mouse IL-6 ELISA Set	555240	BD Biosciences, Oxford, UK
Mouse TNF-α ELISA Set	558534	BD Biosciences, Oxford, UK
PCR Mastermix + DNAse free water	M7505	Promega, Madison, USA
PGD2 EIA kit	CAY512031	Cayman Chemical. USA
Pyrogene Recombinant Factor C Endotoxin Detection System	50-658U	Lonza, Walkersville, USA
rt-PCR kit	A5001	Promega, Madison, USA
TGF-β Platinum ELISA	BMS608/3	eBiosciences, Hatfield, UK
Trypan blue	T8154	Sigma-Aldrich, Wicklow, Ireland

2.1.1.5 Reagents

Product	Catalog #	Company
2-Mercaptoethanol	63689	Sigma-Aldrich, Wicklow, Ireland
2-Propanol	24137	Sigma-Aldrich, Wicklow, Ireland
3,3′,5,5′-Tetramethylbenzidine dihydrochloride hydrate	T8768	Sigma-Aldrich, Wicklow, Ireland
Acetone	27023	VWR, East Grinstead, UK
Acrylamide/Bis-acrylamide, 30% solution	A3699	Sigma-Aldrich, Wicklow, Ireland
Agarose	BIO-4125	Bioline, London, UK
Albumin from bovine serum	A7030	Sigma-Aldrich, Wicklow, Ireland
Alcian blue	A5268	Sigma-Aldrich, Wicklow, Ireland
Ammonium persulfate	A7460	Sigma-Aldrich, Wicklow, Ireland
Bovine serum albumin (BSA)	A7906	Sigma-Aldrich, Wicklow, Ireland
Bromophenol Blue	18030	Sigma-Aldrich, Wicklow, Ireland
Buffer solution pH 10.0 (20 °C)	33668	Sigma-Aldrich, Wicklow, Ireland
Buffer solution pH 4.0 (20 °C)	33665	Sigma-Aldrich, Wicklow, Ireland
Buffer solution pH 7.0 (20 °C)	33666	Sigma-Aldrich, Wicklow, Ireland
Calcium chloride	383147	Sigma-Aldrich, Wicklow, Ireland
Chloroform	3505	Fisher, Dublin, Ireland
Citric Acid	C1857	Sigma-Aldrich, Wicklow, Ireland
Dimethyl sulfoxide	D2650	Sigma-Aldrich, Wicklow, Ireland
DNAse I	D0018	Sigma-Aldrich, Wicklow, Ireland
DNP	D198501	Sigma-Aldrich, Wicklow, Ireland
Entellan	1079600500	Merck, Darmstadt, Germany
Eosin	E4009	Sigma-Aldrich, Wicklow, Ireland
Ethanol	E7023	Sigma-Aldrich, Wicklow, Ireland
Ethylenediamine-tetraacetic acid (EDTA)	E9884, E3889	Sigma-Aldrich, Wicklow, Ireland
Ferric sulphate	215422	Sigma-Aldrich, Wicklow, Ireland
Formaldehyde solution	F8775	Sigma-Aldrich, Wicklow, Ireland
Glucose	G8270	Sigma-Aldrich, Wicklow, Ireland
Glycerol	G8773	Sigma-Aldrich, Wicklow, Ireland

Glycine	G/0800	Fisher, Dublin, Ireland
Hematoxylin	98057-844	BDH, VWR, East Grinstead, UK
HEPES	H3375	Sigma-Aldrich, Wicklow, Ireland
Hydrochloric acid	H1758	Sigma-Aldrich, Wicklow, Ireland
HyperLadder IV	BIO33029	Bioline, London, UK
Immobilon Western Chemiluminescent HRP Substrate	WBKLS0100	Millipore, MA, USA
Indomethacin	I7378	Sigma-Aldrich, Wicklow, Ireland
Magnesium chloride	M8266	Sigma-Aldrich, Wicklow, Ireland
Mannan from Saccharomyces cerevisiae	M7504	Sigma-Aldrich, Wicklow, Ireland
Methanol	65543	Sigma-Aldrich, Wicklow, Ireland
N,N,N′,N′-Tetramethylethylenediamine	T9281	Sigma-Aldrich, Wicklow, Ireland
Neoclear	109843	Merck, Darmstadt, Germany
Nonidet™ P 40 Substitute	74385	Sigma-Aldrich, Wicklow, Ireland
Paraffin wax	107250	Merck, Darmstadt, Germany
Odyssey molecular weight marker (9928-40000)	3100 10899	Li-Cor, Lincoln, USA
Phosphatase Inhibitor Cocktail 1	P2850	Sigma-Aldrich, Wicklow, Ireland
Phosphatase Inhibitor Cocktail 2	P5726	Sigma-Aldrich, Wicklow, Ireland
Phosphatase Inhibitor Cocktail 3	P0044	Sigma-Aldrich, Wicklow, Ireland
Phosphate-Citrate Buffer with Sodium Perborate	P4922	Sigma-Aldrich, Wicklow, Ireland
p-Nitrophenyl-N-acetyl-β-D-glucosaminide (p-NAG)	N9003	Sigma-Aldrich, Wicklow, Ireland
Potassium chloride	P4504	Sigma-Aldrich, Wicklow, Ireland
Potassium dihydrogen phosphate	30407	Riedel de Haën, Sigma-Aldrich, Wicklow, Irelnad
Protease inhibitor Cocktail	P8340	Sigma-Aldrich, Wicklow, Ireland
Resazurin	R7017	Sigma-Aldrich, Wicklow, Ireland
RIPA Buffer	R0278	Sigma-Aldrich, Wicklow, Ireland
Safranin	S2255	Sigma-Aldrich, Wicklow, Ireland
Saponin	47036	Sigma-Aldrich, Wicklow, Ireland
SeeBlue Plus2 Prestained Standard	LC592	Invitrogen, Paisley, UK
Skim Milk Powder	70166	Sigma-Aldrich, Wicklow, Ireland
Sodium Acetate	S2889	Sigma-Aldrich, Wicklow, Ireland
Sodium azide	13412	Sigma-Aldrich, Wicklow, Ireland
Sodium bicarbonate	S5761	Sigma-Aldrich, Wicklow, Ireland
Sodium carbonate	S7795	Sigma-Aldrich, Wicklow, Ireland
Sodium Chloride	S/3160	Fisher, Dublin, Ireland
Sodium dodecyl sulphate (SDS)	L4390	Sigma-Aldrich, Wicklow, Ireland
Sodium hydroxide	S5881	Sigma-Aldrich, Wicklow, Ireland
Sodium phosphate	S8282	Sigma-Aldrich, Wicklow, Ireland

Sodium pyrophosphate	106580	Merck, Darmstadt, Germany
Sulphuric acid	435589	Sigma-Aldrich, Wicklow, Ireland
SYBRSafe DNA gel stain	S33102	Invitrogen, Paisley, UK
TEMED	T9281	Sigma-Aldrich, Wicklow, Ireland
Tetramethylbenzidine (TMB) dihydrochloride (tablets)	T3405	Sigma-Aldrich, Wicklow, Ireland
TMB Substrate Reagent Set	421101	Biolegend, Cambridge, UK
Toluidine blue	89640	Sigma-Aldrich, Wicklow, Ireland
Trizma base	93352	Sigma-Aldrich, Wicklow, Ireland
Trisure	BIO-38033	Bioline, London, UK
Triton X-100	BDH306324	VWR, East Grinstead, UK
Tween 20	P1379	Sigma-Aldrich, Wicklow, Ireland

2.1.2 Equipment

2.1.2.1 Equipment

Product	Catalog #	Company
Analogue Stirred Water bath	NE4-22T	VWR, East Grinstead, UK
Benchtop microcentrifuge	4214	MSC Co. Ltd. Dublin, Ireland
BIOQUEL Microflow Class II ABS Cabinet	ABS1200F	VWR, East Grinstead, UK
Block Heater	BBA series	MSC Co. Ltd. Dublin, Ireland
Consort nv electrophoresis power supply	AE-6450	Belgium
Dual Mini Slab Kit-gel electrophoresis apparatus	AE-6450	ATTO Bio-Instrument, Bunkyo, Japan
FacsAria flow cytometer		Becton Dickinson, Oxford, UK
FacsCalibur flow cytometer		Becton Dickinson, Oxford, UK
Fuji medical film processor	FPM 100A	Fuji photo film, Tokyo, Japan
Fuji film cassette (18x24)	EC-DW	Fuji photo film, Tokyo, Japan
G-Box Gel Imaging system		Syngene, Cambridge, UK
Haemocytometer, Neubauer, Double cell		MSC Co. Ltd. Dublin, Ireland
Hotplate Stirrer	AGB1000	Jenway, Stone, UK
Leica Inverted microscope	DMIL	Leica Microsystems, Wetzlar, Germany
MicroTec rotary microtome	4060	MicroTec Laborgeräte GmbH, Germany
Midi horizontal electrophoresis unit 692	Z338818	Sigma-Aldrich, Wicklow, Ireland
Mini horizontal electrophoresis unit 658	Z338796	Sigma-Aldrich, Wicklow, Ireland
Nanodrop 1000		Thermo Fischer Scientific, Dublin, Ireland
Olympus transmitted-reflected	BX60	Olympus, Hamburg, Germany

light microscope wit BF/DF/DIC/Polarised light		
Real time thermal cycler	ABI7900FH	Applied Biosciences, Paisley, UK
Sigma 4K15 Benchtop Refrigerated Centrifuge	10740	Sigma Centrifuges, Merringtn, UK
Stuart Scientific combined incubator and orbital shaker	S150	MSC Co. Ltd. Dublin, Ireland
TECAN Genios Microplate Reader		Tecan Group, Mannedorf, Switzerland
TECAN Safire2 UV/VIS/IR and fluorescence plate reader		MSC Co. Ltd. Dublin, Ireland
Thermo Scientific CO2 Water Jacketed Incubator	Model 3111	MSC Co. Ltd. Dublin, Ireland
Vortex mixer	SA8	Stuart, Stone, UK
West balance	BL120S	Sartons, Goettingen, Germany

2.1.2.2 Software

Product	Company
Alpha View SA	Cell Bioscience Inc., St Clara, USA
BD CellQuest Pro software	Becton Dickinson, Oxford, UK
Cyflogic	CyFlo Ltd., Turku, Finland
FlowJo	Tree Star, Ashland, USA
imageJ	National Institute of Health, USA
Origin	Origin Lab Corp., Northampton, USA

2.2 Methods

2.2.1 Animals

BALB/C, C57BL/6 and STAT6$^{-/-}$ mice 6-8 weeks old were purchased from Harlan UK Ltd (Bicester, UK) and Charles River (Carrentrila, Ireland). Mice were kept under specific pathogen-free conditions at the Bioresource Unit, Faculty of Health and Science, Dublin City University, Ireland. All mice were maintained according to the guidelines of the Irish Department of Children and Health. Ethical approval for mice experiments was obtained from DCU ethics committee and the Irish Department of Children and Health.

2.2.2 IL-3 preparation

IL-3 was obtained from WEHI-3-conditioned medium following culture of WEHI-3 cells (ATCC: TIB-68; LGC Standards, Teddington, United Kingdom) in Iscove's Modified Dulbecco's medium (IMDM; Invitrogen Ltd, Paisley, UK) supplemented with 10% foetal bovine serum (FCS; Invitrogen Ltd, Paisley, UK) 1% penicillin/streptomycin (Bio-Sciences, Dun Laoghaire, Ireland) at 37°C, supernatant was collected every 4th day. The cell-free conditioned medium was used in bone marrow derived mast cell cultures or concentrated by ultrafiltration (cut off point 10 kDa; Amicon ultra-15 centrifugal filter device, Millipore, Ireland). The concentration of IL-3 was determined by ELISA (BioLegend UK Ltd., Cambridge, UK).

2.2.3 FhTeg/FhES preparation

F. hepatica excretory-secretory products (FhES) and tegumental antigen (FhTeg) was prepared by adapting a previously published method (Hamilton et al., 2009). In brief, live worms were obtained from infected cattle and placed in RPMI (Roswell

Park Memorial Institute culture medium) supplemented with penicillin/streptomycin (100 u/ml) 2 mM L-glutamine and 10% FCS at 37°C and 5% CO_2. Culture medium containing FhES was collected after 8 hours. Worms were washed in sterile phosphate-buffered saline (PBS), incubated in 1% Nonidet P-40 (NP-40) for 30 min at 37°C, and supernatant was collected. NP-40 was removed using Bio-Beads™ (Bio-Rad, Herts, UK). Protein concentrations were measured using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Leicestershire, UK), and endotoxin levels were assessed using the Pyrogene endotoxin detection system (as described in manufacturer's protocol, Cambrex, Lonza, Walkersville. USA). FhES and FhTeg gave endotoxin levels similar to background levels. Toxicity of antigen (0, 1, 5, 10, 20 µg/ml) were tested on bone marrow-derived dendritic cells (section 2.2.10) with trypan blue staining (2.2.5) and resasurine (2.2.16). Viability of antigen treated (10 µg/ml) cells were also tested with flow cytometer with Annexin V-FITC apoptosis test (2.2.18)

2.2.4 *Fasciola hepatica* infection and treatment of mice with *F. hepatica* antigens

BALB/C, C57BL/6 or STAT6$^{-/-}$ mice were infected with 20 *F. hepatica* metacercariae orally (metacercariae were purchased from Baldwin Aquatics Ltd, USA) and were sacrificed 2 weeks post infection. Peritoneal cells were obtained from the mice by injecting 10 ml PBS into the peritoneal cavity and livers were removed for histological studies (Section 2.2.7).

BALB/C, C57BL/6 or STAT6$^{-/-}$ mice were injected intraperitoneally (i.p.) three times per week for three weeks with PBS, FhTeg (10 µg) or FhES (20 µg) or

injected with BP in the presence of absence of FhTeg. Peritoneal cells were harvested on day 21 and used to measure intracellular TNF-α and membrane c-type lectin expression (Section 2.2.18). Cell numbers and viability were monitored using Trypan blue staining. Percentages of mast cells were measured by cell surface expression of c-kit and FcεRI (flow cytometry, Section 2.2.17) and by Kimura staining (Section 2.2.5).

2.2.5 Trypan blue and Kimura staining

Cell viability was measured by trypan blue staining. Cells were resuspended in trypan blue dye (Sigma, Wicklow, Ireland) and PBS (1:5) and examined with Olympus phase contrast light microscope (BX60, Olympus, Hamburg, Germany) immediately. Dead cells are stained blue while viable cells are white against the light blue background.

Cells were incubated for 5 minutes at room temperature in 10 µl medium or PBS and 10 µl Kimura dye (Table 2.1) than examined with Olympus light microscope (BX60, Olympus, Hamburg, Germany). Mast cells were identified by their ability to exhibit purple staining (Kimura et al., 1973).

Table 2.1. Kimura staining

1. Toluidine blue solution		
Reagent	**Amount**	**Concentration**
Toluidine blue	25 mg	0.5 mg/ml
1.8% NaCl (18 g/L)	25 ml	9 g/L
Ethanol (100%)	11 ml	22%
Total	**50 ml**	
2. Saturated saponin		
Reagent	**Amount**	**Concentration**
Saponin	200 mg	4 mg/ml
Ethanol (50 %)	50 ml	
Total	**50 ml**	**Heat and vortex for 30 min**
3. NaH_2PO_4 solution		
Reagent	**Amount**	**Concentration**
NaH_2PO_4	1.03 g	60 mM
Total	**100 ml (pH 6.4)**	
Kimura staining		
Toluidine blue solution	50 ml	
Saturated saponin	2.27 ml	
NaH_2PO_4 solution	22.7 ml	

2.2.6 Passive Cutaneous and Systemic Anaphylaxis model

Passive Cutaneous and Systemic Anaphylaxis experiments were done at Charité Univesitätmedizin in Berlin by Dr Sandra O'Neill. Briefly, C57BL/6 mice were injected with FhTeg (10 µg) three times before administering dinitrophenol (DNP)-specific IgE prior to challenge with DNP either systemically in the Passive Systemic Anaphylaxis model (PSA) by intra venous (i.v.) injection or subcutaneously in the Passive Cutaneous Anaphylaxis model (PCA) by injecting DNP into the ear (Siebenhaar et al., 2007). In the PSA rectal temperature was recorded every 10 minutes for two hours. In the PCA model ear swelling was measured by micrometer for 1, 2, 4 and 8 hours.

2.2.7 Histological studies

The livers were gently removed from sacrificed mice and fixed in 4% formalin and stored at room temperature. After dehydration by ethanol liver tissues were

embedded into paraffin wax and 5 µm thick sections were cut. Sections were rehydrated and stained with toluidine blue, alcian blue/safranin or standard hematoxylin/eosin staining (Table 2.2). After staining sections were washed in water and dehydrated, cleared in Neoclear and mounted in Entellan (Merck, Darmstadt, Germany). Mast cells were identified by their ability to exhibit metachromatic staining. Quantitative analysis of mast cell density was performed by counting the number of stained cells. Mast cell density was expressed as cells/mm^2.

Table 2.2. Sample preparation for histological studies and dyes

	Solution	Time / temperature
1. Dehydration	30% Ethanol	1 h; RT
	50% Ethanol	1 h; RT
	70% Ethanol	1 h; RT
	90% Ethanol	1 h; RT
	96% Ethanol	1 h; RT
	ABS Ethanol	2x1 h; RT
2. Clearing and embedding solution	50% Ethanol, 50% Neoclear	1 h; RT
	100% Neoclear 1	1 h; RT
	100% Neoclear 2	1 h; RT
	Neoclear + wax	30 min; RT
	More wax	1 h; 65°C
	100% wax	1 h; 65°C
	Molding	RT
Sectioning	5 µm	
	On slides	Overnight; 37°C
3. Rehydrating, staining and dehydrating	Neoclear A	10 min; RT
	Neoclear B	10 min; RT
	ABS Ethanol	5 min; RT
	96% Ethanol	5 min; RT
	70% Ethanol	2 min; RT
	Running tap water	5 min; RT
	Dye*	
	Running tap water	5 min; RT
	70% Ethanol	2x1 sec; RT
	96% Ethanol	4x1 sec; RT
	ABS Ethanol 1	5 min; RT
	ABS Ethanol 2	5 min; RT
	50% ABS Ethanol 50% Neoclear	5 min; RT
	Neoclear C	5 min; RT
	Neoclear D	5 min; RT
4. Mounting	Entellan	Cover with cover slip

	* Dyes:	
	Ragent	**concentration**
5. Toluidine staining	Toloudine blue	0.5%
	HCl	0.5 N
	Incubation:	*30 sec; RT*
	Washing with running tap water	*5 min; RT*
	Eozin	1%
	Incubation:	*1 min; RT*
6. Alcian blue/Safranin staining	Alcian blue	0.36%
	Safranin	0.012%
	Ferric sulphate	0.48%
	Sodium acetate	0.2 M
	Incubation:	*20 min*
7. Hematoxilin/Eosin staining	Hematoxilin	1%
	Incubation:	*15 min; RT*
	Washing with running tap water	*5 min; RT*
	Eosin	1%
	Incubation:	*1 min; RT*

2.2.8 Bone marrow-derived mast cells

Bone marrow-derived cultured mast cells (BMMCs) were generated from the femoral bone marrow cells of C57BL/6 mice and maintained in complete IMDM (Table 2.3) in the presence of 10% heat-inactivated FCS, 100 u/ml penicillin/streptomycin, L-Glutamine (2 mM) and 30% WEHI-3 conditioned IMDM-medium (ATTC TIB-68) as a source of the murine growth-factor IL-3 for 4 weeks (Mrabet-Dahbi et al., 2009). >95% of the total cells were identified as mast cells on the basis of c-kit and FceRI cell surface expression or Kimura staining (Kimura et al., 1973). Cell number and viability were monitored using trypan blue staining (Section 2.2.5) (Figure 2.1).

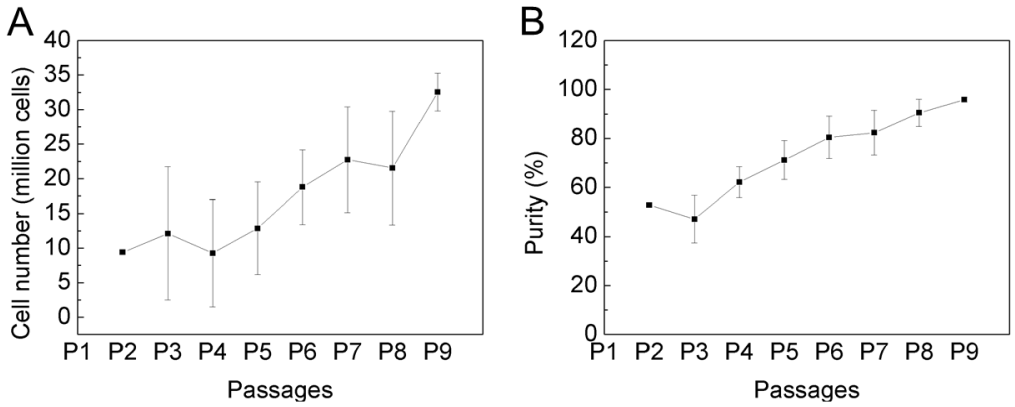

Figure 2.1. The purity of bone marrow-derived cultured mast cells is over 95% after 4 weeks of cultivation. Bone marrow cells from C57BL/6 mouse were cultured for 4 weeks in IMDM (with 10% FCS, 100 u/ml penicillin/streptomycin and 50 µM 2-mercaptoethanol) in the presence of 30% WEHI-3 conditioned medium. Cell number (**A**) and purity (**B**) was determined by every passage, twice a week with trypan blue and Kimura staining. Data are presented as the mean ± SD of nine independent experiments.

2.2.9 Peritoneum-derived cultured mast cells

Peritoneal cells were obtained from C57BL/6 mice after i.p. injection of 10 ml sterile PBS and then cultured in RPMI 1640 medium supplemented with 10% FCS and 100 u/ml penicillin⁄streptomycin, L-glutamine (2 mM) 10 ng⁄ml recombinant mouse IL-3 (Calbiochem, Merck, Darmstadt, Germany) and 30 ng⁄ml recombinant mouse stem cell factor (SCF) at 37°C as previously described (Mrabet-Dahbi et al., 2009) (Table 2.3). 48 h later, non-adherent cells were removed and replaced by fresh culture medium for a further 7 days. >95% of the total cells were identified as mast cells on the basis of c-kit and FceRI cell surface expression or Kimura staining. Cells expressing low level of c-kit on their surface are supposed to be immature mast cells. Cell number and viability were monitored using trypan blue staining (Section 2.2.5) (Figure 2.2).

With this method from one mouse only 1-2 million PCMCs can be obtained. We tried to develop a method to reduce the number of animal required. Mice were injected i.p. with the growth factor IL-3 for 5 days with 2 injections per day prior to the peritoneal lavage. Although the mast cell number increased in the IL-3 treated group the mast cells' ability to respond for bacterial stimulation was impaired. Our finding are summarised in an article (Appendix D)

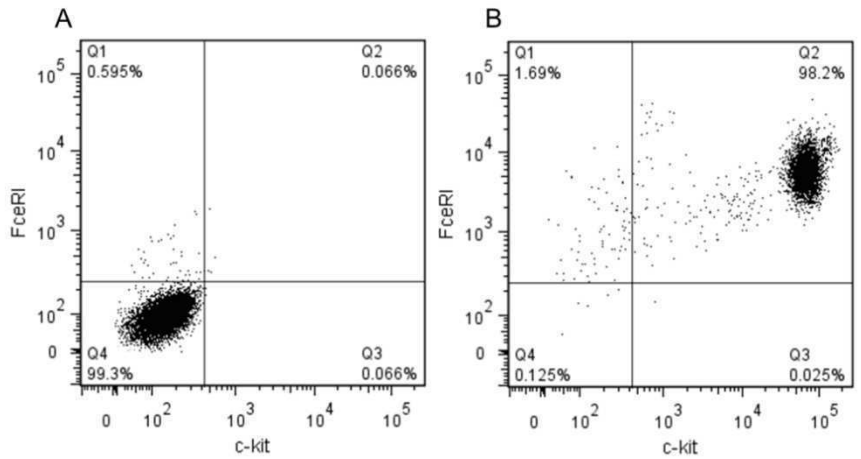

Figure 2.2. The purity of peritoneal-derived cultured mast cells is over 95% after 9 days of cultivation. Peritoneal cells were cultured for 9 days in complete RPMI medium in the presence of 10 ng/ml IL-3 and 30 ng/ml SCF. Purity was assessed on the basis of c-kit and FcεRI cell surface expression measured by flow cytometry. A: unstained control; B: double stained sample

2.2.10 Generation of bone marrow-derived dendritic cells (BMDC)

BMDCs were prepared using a modified protocol originally described by (Lutz et al., 1999). Bone marrow was harvested from the femurs and tibias of female BALB/C mice. Cells were seeded at $2x10^5$ cells/ml density in RPMI supplemented with 10% FCS, 1% L-glutamine, 100 u/ml penicillin/streptomycin, 50 μM 2-mercaptoethanol and 20 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) (Table 2.3). GM-CSF was obtained from a GM-CSF producing cell line, a kind gift from

Prof. Ton Rolink, University of Basel, Switzerland. Cells were fed with fresh media at every second day. Cells were harvested at day 10 giving >95% pure BMDC population. Cell number was monitored by trypan blue staining (Section 2.2.5) and purity was determined on the basis of CD11c expression measured by flow cytometry (Section 2.2.18).

Table 2.3. Contents of media used in cell culture

Complete RPMI		
Reagent	**Amount**	**Concentration**
RPMI 1640	500 ml	
FCS	50 ml	10%
Penicillin/Streptomycin	5 ml	100 u/ml; 100 µg/ml
L-Glutamine	5 ml	2 mM
2-mercaptoethanol (1 M)	25 µl	50 µM
Complete IMDM		
Reagent	**Amount**	**Concentration**
IMDM	1000 ml	
FCS	100 ml	10%
Penicillin/Streptomycin	10 ml	100 U/ml; 100 µg/ml
2-mercaptoethanol (1 M)	50 µl	50 µM
Complete ex vivo media		
Reagent	**Amount**	**Concentration**
X vivo 15	50 ml	
2-mercaptoethanol (1 M)	50 µl	1 mM
L-glutamine	0.5 ml	2 mM
Growth factors		
Reagent	**Amount**	**Concentration**
Mouse SCF		30 µg/ml
Mouse IL-3		10 µg/ml

2.2.11 BMDC stimulation

For cytokine stimulation experiments BMDC were stimulated with or without 10 µg/ml FhTeg 2.5 h before stimulation with PBS, recombinant IL-6, TNF-α (5, 10, 50, 100 pg/ml) or IFN-γ (50, 100, 500, 1000 pg/ml). Supernatant and cells were harvested after 18 h. Supernatant were analysed for IL-12 p70 and TNF-α with commercial ELISA (BD, Bioscience, Oxford, UK) and nitrite level were determined

by Griess reagent system (Promega, Madison, USA). Cells were analysed by two colour flow cytometry for CD80, CD86, CD40 and CD11c expression (Section 2.2.18).

2.2.12 BMMC and PCMC activation and measurement of cytokine secretion

To find good candidates to activate mast cells and promote Th1 but not Th2 immune response we tried a panel of TLR-ligands (Appendix A) and we found that lipopolysaccharide (LPS) (100 ng/ml; Alexis, San Diego United States) and heat inactivated *Bordetella pertussis* whole cell antigen (BP; was a kind gift from Dr Bernie Mahon, NUI, Maynooth, Ireland) (100 bacteria/cell) gave the strongest response.

BMMCs and PCMCs were stimulated with LPS (100 ng/ml) heat inactivated BP (100 bacteria/cell), phorbol myristate acetate (PMA; 25 ng/ml), A23187 (1 μM), FhTeg (10 μg/ml) or PBS at 37°C for 24 hours. Cells were also treated with FhTeg (10 μg/ml) 2.5 hour before stimulation with BP or LPS. Supernatants assessed for levels of TNF-α, IL-6, IL-4, IL-5, IL-10 and IFN-γ by commercial ELISA (BD Biosciences, Oxford, UK).

For TGF-β and prostaglandin D2 (PGD_2) measurements mast cells were treated with 10 μg/ml FhTeg or PBS for 6 or 24 hours. Supernatant was collected and used immediately or stored at -80°C until ELISAs were performed.

In c-type lectin experiments mannose receptor, MGL, SignR1 and Dectin1 was blocked using neutralizing antibodies (α-MR, 1 μg/ml; α-MGL 1 μg/ml, α-SignR1 1

μg/ml, mannan 50 μg/ml, GalNac 50 mM, laminarin 100 μg/ml and EDTA 10mM) and Syk was blocked by Piceatannaol 30 min prior to stimulation. Neutralizing antibodies were used in concentrations recommended by manufacturer. Integrity and specificity was tested with flow cytometer following staining of BMMCs with anti-MR, anti-MGL, anti-Dectin1 and anti-SignR1 (2.2.18). All tested antibodies fully blocked the binding of the labelled antibodies only on their target CLR. Piceatannaol concentration (1, 10, 20 μM) was validated using TNF-α/IFN-γ treated BMDCs (2.2.11). 10 μM of Pinceatannaol was used in blocking experiments as it showed the strongest suppression on CD40 and CD80 up-regulation by the cytokine treatment. In SOCS3 blocking experiments cells were treated with zoledranic acid (0.5, 1 or 5 μg/ml) for 5 days prior to stimulation with FhTeg and bacterial ligands.

2.2.13 SOCS3 blocking experiments

SOCS3 inhibiting experiments were performed by a modification of a previously described method (Bringmann et al., 2007). BMDCs and BCMCs were cultured with 0.5 μg/ml zoledronic acid (Sigma, Wicklow, Ireland). Zoledronic acid was added every other day for one week before experiments.

2.2.14 Measurement of β-hexosaminidase release from BMMCs and PCMCs

BMMCs or PCMCs were washed twice with PBS, resuspended in tyrodes buffer (Table 2.4) and seeded in a 96-well round-bottom plate at a density of 4 x 10^5cells/200 μl followed by stimulation with stimulants, A23187 (1 μM) or PBS for 1 hour at 37°C. Cell pellets were solubilised in tyrodes buffer supplemented with 1% Triton-X, and cell lysates and supernatants were incubated separately with p-nitrophenyl-N-acetil-β-D-glucosamide (p-NAG) substrate (Table 2.4) for 1 h at

37°C. The production of β-hexosaminidase-mediated p-nitrophenol was terminated by adding 150 µl 0.2 M glycine (pH10.7) and quantified by absorbance at 405 nm with an ELISA plate reader (Tecan Genios, Mannedorf, Switzerland). The percentage of β-hexosaminidase release indicating the extent of degranulation was calculated as the ratio of p-nitrophenol absorbance in the supernatants and the sum of absorbance in the supernatant and cell lysates (Figure 2.3). Degranulation was measured following stimulation of mast cells with various antigens or with DNP/anti-DNP immunoglobulin (Ig)E in the presence or absence of FhTeg.

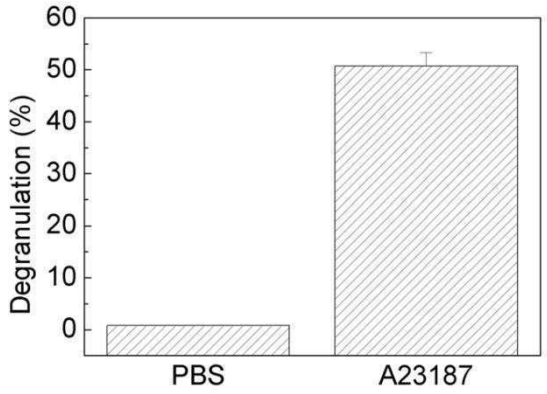

Figure 2.3. Mast cells usually show 50% of degranulation when stimulated with A23187. Mast cells were stimulated with 1 µM A23187 for 1 h and β-hexosaminidase release was measured. Graph shows the percentages of degranulation of the cells. Data are represented as the mean ± SD of three independent experiments.

Table 2.4. Components of buffers for β-Hexosaminidase assay

Tyrodes buffer **Reagent**	**Amount**	**Concentration**
1 M HEPES (238.3 g/L; pH7.4)	10 ml	10 mM
NaCl	7.54 g	130 mM
KCl	0.37 g	5 mM
$CaCl_2$ (•$2H_2O$)	0.206 g	1.4 mM
$MgCl_2$ (•$6H_2O$)	0.203 g	1 mM
Glucose	1.008 g	5.6 mM
BSA	1 g	1 mg/ml
Total	**1000 ml**	
p-Nitrophenyl-N-acetyl-β-D-glucosaminide (p-NAG) substrate		

Reagent	Amount	Concentration
0.2 M Na_2HPO_4 (28.4 g/L)	70 ml	0.2 M
Citric acid (84.1 g/L)	20 ml	0.4 M
p-NAG	300 mg	1.3 mg/ml
Total	**225 ml pH4.5**	

2.2.15 Preparing serum samples for β-hexosaminidase assay

F. hepatica infected and control BALB/C mice were sacrificed after 3 weeks of infection by cervical dislocation. The abdominal cavity was opened and the vena cava posterior was cut. Using a 1 ml syringe with a pipette tip approximately 1 ml blood was collected from each mouse. Blood samples were incubated for 1 h at room temperature and centrifuged for 15 min at 1500 g at 4°C. Serum (the top yellow phase) was collected and stored at -20°C until tested without dilution on BMMCs in β-hexosaminidase assay.

2.2.16 Proliferation

Proliferation was measured by MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide) test (as described in manufacturer's protocol, Sigma Aldrich, Wicklow, Ireland) or 0.9 mM resazurin. SCF (10 ng/ml) was used as positive control.

2.2.17 Chemotaxis assay

Mast cell migration was measured in 8 µm trans-well migration plates. BMMCs and PCMCs were plated in the upper wells in density of 1 million cells in 200 µl in the presence of 10 ng/ml IL-3. For controls FhTeg (10 µg/ml), SCF (10 ng/ml) was used in the bottom wells. RAW macrophages, BMMCs and BMDCs were stimulated with FhTeg or PBS for 18 hours prior transfer to trans-well plates with density of 0.6 million cells in 600 µl. In some experiments cells were centrifuged and only

supernatants were used. Total cell number and mast cell number was monitored with trypan blue and Kimura staining in the first 3 hours taking samples in every hour from the bottom wells. After 3 hours cell numbers were determined by flow cytometry. Mast cells were identified on the basis of c-kit cell surface expression.

2.2.18 Flow cytometric analysis

Cells were harvested, washed twice in Facs buffer (Table 2.5) and pre-incubated for 15 min with CD16/CD32 (FcγIII/II, BD Biosciences, Oxford, UK) to block FcR. After washing, 0.25 x 10^6 cells were incubated with the appropriate antibodies (Table 2.6) or the appropriate immunoglobuline (IgG) isotype control for 30 min at 4°C in the dark. In multicolour experiments unstained, single stained and fluorescence minus one (FMO) controls were also used. After washing twice the monoclonal antibody (mAb) binding was analysed by flow cytometry (FACSAria and FACSCalibur, BD, Oxford, UK), in the presence of DNase to avoid aggregation, using FacsDiva (BD, Oxford, UK) and FlowJo Software (Tree Star, Ashland, USA).

For intracellular staining cells were fixed in 4% formaldehyde in Facs buffer for 5 min at room temperature and membranes were permeabilize with 0.5% Tween20® in Facs buffer for 15 min.

Table 2.5. Contents of Facs buffer

Facs buffer **Reagent**	**Amount**	**Concentration**
PBS	100 ml	
FCS	2 ml	2%
NaH3	0.05 g	0.05%
EDTA	0.019 g	1 mM
DNAse (1u/µl)	Add prior to use	10 u/ml

Table 2.6. Antibodies used in flow cytometry

Name	Fluorochrome	Clone	Catalog #	Company
FcεRI	PE	MAR1	12-5898	Ebioscience, UK
FcεRI	FITC	MAR1	11-5898	Ebioscience, UK
CD117 (c-kit)	PE	2B8	12-1171	Ebioscience, UK
CD117 (c-kit)	FITC	2B8	11-1171	Ebioscience, UK
CD117 (c-kit)	APC	2B8	17-1171	Ebioscience, UK
CD282 (TLR2)	FITC	6C2	11-9021	Ebioscience, UK
CD54 (ICAM1)	PE	YN1/1.7.4	12-0541	Ebioscience, UK
CD252 (OX40L)	PE	MRI	12-5905	Ebioscience, UK
CD40L	PE	MRI	12-1541	Ebioscience, UK
CD28	PE	37.51	553297	BD Bioscience, UK
TLR4/MD-2	PE	MTS510	12-9924	Ebioscience, UK
TNF-α	APC	MP6-XT22	17-7321	Ebioscience, UK
CD206(MMR)	Alexa fluor 488	MR5D3	123005	Biolegend, UK
MGL	Alexa fluor 488	ER-MP23	MCA2392A	AbD serotec, UK
Dectin1	Alexa fluor 488	GE2	MCA4661A	AbD serotec, UK
SignR1	Alexa fluor 688	eBio22D1	51-2093	Ebioscience, UK

PE: phycoerythrin; FITC: fluorescein isothyocyanin; APC: allophycocyanin

2.2.19 Co-culture of BMMCs or PCMCs with CD^+ T-cells:

$CD4^+$ T-cells were isolated using MACS $CD4^+$ T-Cell Isolation Kit (Miltenyi Biotec, Surrey, UK) and were used if the purity were 95% or above as determined by flow cytometry (FITC conjugated CD4, clone L3T4, BD Biosciences, Oxford, UK). BMMCs and PCMCs were cultured in appropriate medium (Table 2.7) the presence of FhTeg (10 μg/ml) 2.5 hour before stimulation LPS (100 ng/ml) or BP (100 bacteria/cell) for 24 hours. In c-type lectin blocking experiments cells were treated with α-SignR1, α-MR or α-MGL (1 μg/ml) neutralizing antibodies 30 min prior to stimulation. Washed BMMCs and PCMCs or supernatant was added to $CD4^+$ T-cells (1:1) in plates coated with anti CD3 (1 μg/ml; BD Biosciences, Oxford, UK) for 72 hours and supernatants were tested for IFN-γ, IL-4, IL-5 and IL-10 by commercial ELISA (BD Biosciences, Oxford, UK). In some experiments intracellular adhesion molecule 1 (ICAM-1) blocking antibody (clone YN1/1.4;

Abcam, Cambridge, UK) was given to mast cells 30 min before co-culturing with $CD4^+$ cells.

Table 2.7. Different variations of media used in co-culture studies

Media	Result
100 % X vivo	Mast cells died
100 % c. RPMI	Too high background in ELISA
50-50% X vivo and c. RPMI	Used in PCMC studies
100 % c. IMDM	Used in BMMC studies

2.2.20 Protein extraction

BMDCs, BMMCs and PCMCs were stimulated with LPS (100 ng/ml) or BP (100 bacteria/cell) for 5, 15, 30, 60 or 120 minutes with and without FhTeg (10 µg/ml) treatment. In c-type lectin blocking experiments cells were treated with α-MR or α-MGL (1 µg/ml) neutralizing antibodies 30 min prior to stimulation. Cells were washed in PBS twice and total protein was extracted from cell lysates by use of radioimmunoprecipitation (RIPA) buffer (100 µl/1 million cells) containing protease and phosphatase inhibitor cocktails (1%; Table 2.8).

Table 2.8. RIPA buffer

Reagent	Amount	Concentration
RIPA	1 ml	100 µl/cell
Protease inhibitor cocktail	2 ml	1%
Phosphatase inhibitor cocktail 1/3	0.05 g	1%
Phosphatase inhibitor cocktail 2	0.019 g	1%

Samples were incubated at least 5 minutes on ice and lysates were cleared by centrifugation at 8000xg for 10 minutes. Supernatants were transferred to new tubes and protein concentrations were determined using the BCA protein assay kit (Pierce, Thermo Scientific, Leicestershire, UK) following the protocol provided by the company.

2.2.21 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed based on a previously established method (Laemmli, 1970) using a mini-gel system (ATTO Bio-Instrument, Bunkyo, Japan). 10 % acrylamide resolving gels with 5 % stacking gels were prepared (Table 2.9).

Table 2.9. Gel for SDS-PAGE

10% Separating gel (2 gels)		5% Stacking gels (2 gels)	
Reagent	**Volume (ml)**	**Reagent**	**Volume (ml)**
40 % acrylamide	3.8	40 % acrylamide	0.625
1M Tris-HCl, pH 8.8	5.6	1M Tris-HCl, pH 6.8	0.625
d. H_2O	5.4	d. H_2O	3.625
10 % SDS	0.15	10 % SDS	0.05
10 % APS (fresh)	0.075	10 % APS (fresh)	0.05
TEMED	0.025	TEMED	0.025
Total	**15**	**Total**	**5**

Protein samples were prepared in SDS-PAGE reducing dye, boiled for 2 min on a heating plate and then incubated on room temperature for at least 5 min. Samples and pre-stained protein markers (See Blue Plus 2, Invitrogen) were loaded on the gels and electrophoresis was performed between 150-200 V for 1 hour in ice cold SDS running buffer (Table 2.10).

Table 2.10. Contents of solutions for SDS PAGE

	Reagent	**Amount**	**Concentration**
Running buffer	Tris	3 g	25 mM
	Glycine	14.4 g	192 mM
	SDS	1 g	0.2 %
	Total	**1000 ml, pH 8.3**	
Reducing dye (5x)	Tris	0.7571 g	0.625 M
	Glycerol	5 ml	50 %
	SDS	1 g	10%
	Bromphenol blue	0.01 g	0.1%
	2-mercaptoethanol	0.5 ml	5%
	Total	**10 ml; pH6.8**	

2.2.22 Western blot analysis

Protein samples (10 to 40 μg) were separated by SDS-PAGE and blotted onto 0.45-μm Immobilon-P polyvinylidene fluoride membranes (Millipore, Carrigtwohill, Ireland) using Semi-dry blotter (Z340502, Sigma-Aldrich) with fresh ice cold transfer buffer (Table 2.11).

Table 2.11. Components of buffers for western blot

	Reagent	Amount	Concentration
1. Transfer buffer	Tris	6.06 g	25 mM
	Glycine	28.8 g	192 mM
	Methanol	400 ml	20 %
	Total	**2000 ml, pH 8.3**	
2. Tris buffer saline (PBS)	Tris	2.4228 g	20 mM
	NaCl	8.766 g	150 mM
	Total	**1000 ml, pH 7.5**	
3. Washing buffer (TBS-T)	Tween 20®	0.5 ml	0.05 %
	TBS	1000 ml	
4. Blocking/Antibody buffer	Marvel (skim)	0.5 g	5 %
	TBS-T	10 ml	

Membranes were stained using standard western blot protocol (Table 2.12) with antibodies listed in Table 2.13. Proteins were visualized with a Chemiluminescent HRP substrate (Millipore, Carrigtwohill, Ireland), exposed to film (1-60 sec) and processed using an FPM 100A processor (Fuji Film). Protein bands were quantified using ImageJ analysis software (imagej.nih.gov). Levels of proteins were normalized to total protein controls or β-actin and expressed in arbitrary units as percentage increases over the medium control levels.

Table 2.12. Western blot staining protocol

Solution	Incubation time	Temperature
Blocking buffer	1 h	RT
1st Antibody (in Ab buffer)	overnight	4°C
Washing buffer	30 min	RT
2nd Antibody (in Ab buffer)	1 h	RT
Washing buffer	1 h	RT

Table 2.13. Antibodies and their concentration

Primary Antibody	Concentration	Catalog #	Company
Phospho-ERK	1:1000	9106	Cell Signalling, USA
Total-ERK	1:1000	9107	Cell Signalling, USA
NF-κB (p65)	1:2500	ST1047/3375	Millipore, USA
Phospho-p38	1:1000	9211	Cell Signalling, USA
Total-p38	1:1000	9212	Cell Signalling, USA
Phospho-JNK	1:1000	4668	Cell Signalling, USA
Total-JNK	1:1000	9252	Cell Signalling, USA
Phospho-IRF3	1:1000	4947	Cell Signalling, USA
Total-IRF3	1:1000	4302	Cell Signalling, USA
Phospho-cFos	1:1000	5348	Cell Signalling, USA
Total-cFos	1:1000	2250	Cell Signalling, USA
SOCS3	1:1000	2932	Cell Signalling, USA
β-actin	1:500	643802	Biolegend, UK
Secondary Antibody	**Concentration**	**Catalog #**	**Company**
Anti-Rabbit IgG	1:2500	A1949	Sigma-Aldrich, Ireland

2.2.23 RNA extraction and reverse-transcriptase polymerase chain reaction (RT-PCR)

BMDCs/BMMCs were stimulated with or without FhTeg (10 μg/ml) prior to stimulation with LPS or PBS. Cells were harvested and washed after 5, 15, 30, 60 min, 6 and 18 hours. Total RNA was extracted from homogenised samples by using TRISure (Bioline, London, UK), and following the manufacturer's instructions. RNA quality and quantity were monitored by Nanodrop® (Thermo Fischer Scientific, Dublin, Ireland). To eliminate genomic DNA contamination, RNA samples were treated with RNase free DNase (Invitrogen, Paisley, UK) 0.4 μg DNase treated total RNA was reverse-transcribed using the reverse transcriptase kit (Promega, Madison, USA) according to manufacturer protocol.

Sequences of target DNAs were selected from the database of the National Center for Biotechnology Information (www.ncbi.nih.gow). Primers were designed using Primer3 program (frodo.wi.mit.edu) with the criteria that the product must be less

than 250 basis pairs and the melting temperature has to be around 60-61°C. Primers were tested *in silico* by the free online program Oligo Analyser (eu.idtdna.com) if there was any hairpin, self-dimer or hetero-dimer which could form a secondary product during the PCR analysis. Finally primer specificity was checked using BLAST (blast.ncbi.nlm.nih.gov).

Prior to quantitative PCR (qPCR) analysis primers (Table 2.14.) were tested in conventional PCR reaction with the same settings as were used in qPCR using 20 ng cDNA templates. PCR was performed using GoTaq PCR master mix (Promega, Madison, USA) in Thermo-Hybrid PCR express PCR thermocycler (MSC, Dublin, Ireland) (Table 2.15). PCR products were separated on 1% agarose gels in 0.5 strength TRIS base boric acid EDTA (TBA) buffer containing SYBRSafe (Invitrogen, Paisley, UK) and acquired under UV light (G-Box Gel imaging system, Syngene, Cambridge, UK). Primers were excluded from further experiments if they gave more than one single band when PCR products were run on 1% agarose gel or if they form primer dimers. In addition melting curves analysis was performed by a real time thermal cycler ABI7900FH (Applied Biosciences, Paisley, UK) to check product size and homogeneity.

Table 2.14. Primers

Name	Sequence	Product size (bp)	Tm (^{o}C)
β-actin	AGTCCCTCACCCTCCCAAA	266	61.83
	CTGCCTCAACACCTCAACC		59.25
GAPDH	CAAGAAGGTGGTGAAGCAG	112	56.92
	GAAGGTGGAAGAGTGGGAGTT		59.59
Gusb	ACCAGCCACTATCCCTACTCA	200	58.68
	GCCACAGACCACATCACAAC		60.01
TRAF6	CGGGTTGTGTGTGTCTGTGT	58	60.54
	CCTTCTTCTGGCTTTCGTG		58.58
MyD88	AAATACAAGGCGATGAAGAAGG	279	59.63
	TGGGAGGAAAGGCAGTC		57.07
TLR4	GAGCCGTTGGTGTATCTTTGA	274	60.13
	TGCCGTTTCTTGTTCTTCCT		59.85
RelA (NFκB)	CCCTCATCTTTCCCTCA	155	54.14
	TGTCTTGGTGGTATCTGTGCT		59.66
SOCS1	GAGTGGTTGTGGAGGGTGAG	61	60.56
	TGAGAGGTGGGATGAGGTC		58.51
SOCS2	GCTGAAGGAGGGAAAGACC	159	58.80
	GCATAAGACGAGGGATAAATACAAG		59.49
SOCS3	GGTTCTGCTTTGTCTCTCCTATGT	95	60.19
	TGTGTTTGGCTCCTTGTGTG		60.79
SOCS4	GCCCTTCCCATTCCTTC	115	57.91
	TTCTAACCTCTCCGCATCACT		58.95
SOCS5	CGAGAACCAGTCAAAGCAAA	222	59.05
	TCACAGCCCACCGAACA		60.87
PIAS1	ACGAGCAAAGGGAATAAGGAA	174	60.08
	TGAAGGTGGGAGCAGGTAAG		60.25
PIAS3	AACAAGAGTGGGCGGAAAC	159	60.10
	GGGCAAAGAAAGCAAGGAG		59.94
PIAS4	TCGGCAGGTGGAGATGA	235	59.83
	GAGGTGGGTAAGGTTGATGG		59.26

Table 2.15. Conventional PCR reaction

Temperature	Time	Cycle number
95°C	30 sec	x 35
95°C	30 sec	
60°C	60 sec	
72°C	120 sec	

2.2.24 Quantitative RT-PCR analysis

The qPCR expression analysis was carried out on a real time thermal cycler ABI7900FH (Applied Biosciences Paisley, UK), using Maxima SYBRgreen master mix according to manufacturer's protocol (Table 2.16.) (Fermentas, Fischer Scientific, Dublin, Ireland) in triplicate on 96 well Microamp optical plates (Applied Biosystems, Paisley, UK). Three internal standards were used: β-actin (NM_007393.3), GAPDH (NM_008084.2), Gusb (β-glucuronidase) (NM_010368.1) (Table 2.14.).

Table 2.16. Working solution for q-PCR

Reagent	Amount
SYBRgreen	12 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Template cDNA	20 ng
Total	**25 µl**

Samples were maintained for 10 s at 95°C as initial step, then 15 s at 95°C and 60 s at 60°C through 40 cycles. After amplification, melting curves were recorded (from 50 to 95°C) to check product size and homogeneity. Pfaffl's method was used to calculate relative changes in gene expression determined from real-time quantitative revers transcription PCR experiments (Pfaffl, 2001). Real-time PCR efficiencies were calculated from the given and averaged Ct values of the dilution series for each

primers determined with RQ Manager 1.2 software (Applied Biosystems, Paisley, UK). The corresponding real time PCR efficiency (E) was calculated according to the equation: $E=10^{(-1/slope)}$. The investigated range was from 0.3 to 20 ng total cDNA input (n = 3) using 8 step dilution series (Figure 2.4.).

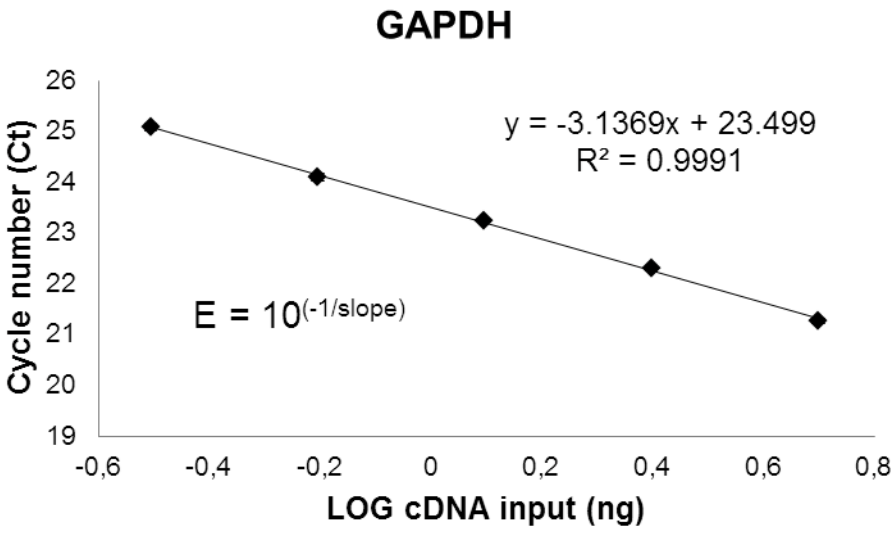

Figure 2.4. Determination of real-time PCR efficiencies of reference gene (GAPDH). Ct cycles versus cDNA concentration input were plotted to calculate the slope (mean ± SD; n=3). The corresponding real time PCR efficiency (E) was calculated according to the equation: $E=10^{(-1/slope)}$. The investigated range was from 0.3 to 20 ng total cDNA input (n = 3) using 8 step dilution series.

To determine the relative quantification of a target gene in comparison to a reference gene a mathematical model was used described by (Pfaffl, 2001). The relative expression ratio of a target gene was calculated based on the efficiency (E) and the Ct deviation of a treated sample versus a control, and expressed in comparison to a reference gene.

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct_{target}(\text{control-sample})}}{(E_{ref})^{\Delta Ct_{ref}(\text{control-sample})}}$$

E_{target}: real-time PCR efficiency of a target gene transcript

E_{ref}: real-time PCR efficiency of a reference gene transcript

ΔCt_{target}: Ct deviation of control – sample of target gene transcript

$\Delta Ct_{reference}$: Ct deviation of control – sample of reference gene transcript

2.2.25 Isolation of *Bordetella pertussis* Lipooligosaccharide (LOS)

Isolation of BP LOS was performed according to a previously described protocol (Schaeffer et al., 2004) and performed by Dr Alessandra Ravidà (DCU, Dublin, Ireland). Briefly, $1x10^{11}$ cells of heat inactivated BP were suspended in 3 ml of 0.1 mg/ml proteinase K (Roche, Manheim, Germany) in PBS and incubated for 1.5 h at 56°C. After incubation the mixture was subjected to acetone precipitation and the pellet solubilised in 1 ml of sterile PBS. Reaction completion was evaluated by 16% SDS-PAGE followed by silver staining (Figure 2.5.A) (Kittelberger and Hilbink, 1993). The contaminating presence of Proteinase K in the crude LOS preparation was removed by centrifugation (Inzana et al., 2002) (Avanti®J-26 XP centrifuge, Beckman Coulter, Fullerton, USA) at 40,000 x g for 4 h at 4°C in a fixed angle rotor (Beckman JA-20, Fullerton, USA). Both the LOS-containing pellet and the acetone precipitated supernatant were dissolved in 300 µl of sterile PBS. Purified LOS was separated on 16% SDS-PAGE and stained by the silver nitrate method (Figure 2.5.B).

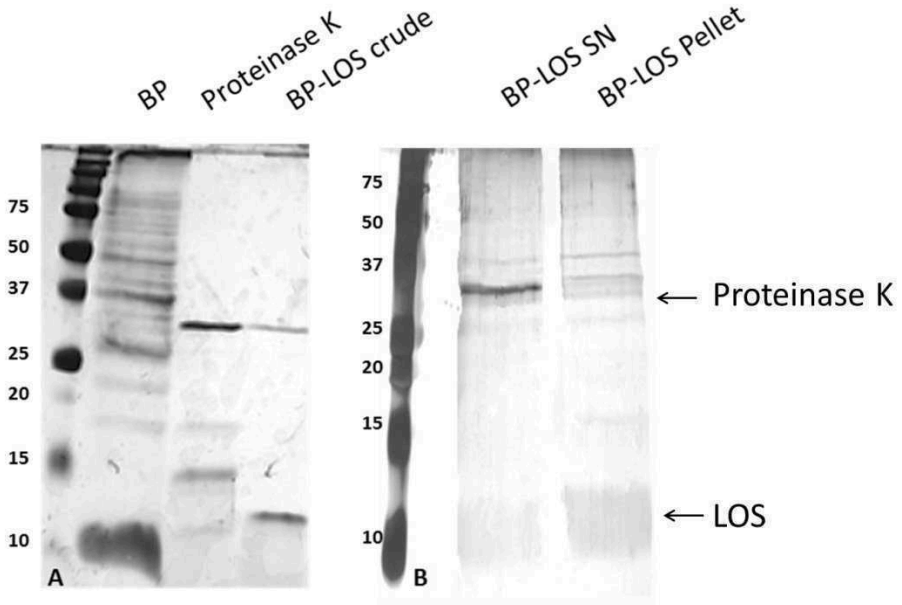

Figure 2.5. Silver stained SDS-PAGE. A, BP: Whole *B. pertussis* cell lysate (10 μg), Proteinase K (5 μg), *B. pertussis* LOS crude preparation (10 μl); **B**, *B. pertussis* LOS supernatant (SN), *B. pertussis* LOS pellet

BMMCs were stimulated with blocking anti-MR and anti-MGL neutralisation antibodies (1 μg/ml) 30 min prior to stimulation with this LOS preparation (1 u/cell, equal to 100 bacteria/cell) for 24 h. Supernatant were tested for TNF-α and IL-6 cytokines. Proteinase K was used as a control to see if it has any effect on cytokine secretion.

2.2.26 Statistics

All data were analysed for normality prior to statistical testing by Origin® 6.1 (OriginLab Corporation) software ($n \geq 3$, exact numbers of origin data points are indicated below each figures). Where multiple group comparisons were made, data were analysed using one-way ANOVA to asses if data belong to the same group. For comparisons between two groups, the Student's *t* test was used. In all tests, $p < 0.05$

was deemed significant. All results (exept results from ELISA) were presented as the mean ± SD of at least three independent experiments. Flow cytometry and western blot results were normalised to isotype control or untreated control before statistic analysis. Figures from ELISA results are representatives of three independent experiments and presented as the mean ± SD of three replicate samples from the same experiment.

3 Chapter 3 – *Fasciola hepatica* tegumental antigens enhance mast cell numbers *in vivo* in a STAT6 independent manner

3.1 Introduction

Helminth infections are often associated with increased mast cell numbers (mastocytosis) and redistribution of these cells to the site of infection (McCole et al., 1998; Maizels et al., 2004; Weller et al., 2011). Mast cells are associated with helminth induced Th2 immune responses (Dawicki and Marshall, 2007) where Th2 cytokines secreted by $CD4^+$ T-cells direct B-cells to produce helminth specific IgE (Anthony et al., 2007; Erb, 2007). Mast cells express the high affinity FcεRI receptor, which in the presence of helminth antigens and the specific IgE antibody induce mast cell degranulation and the release of inflammatory mediators such as histamine, cytokines or chemokines. These mediators can promote Th2 immune responses (Melendez et al., 2007; Pearce, 2007), for example during *Heligmosomoides polygyrus* infection results in mast cell activation and induces tissue derived IL-25, IL-33 and TSLP cytokines (Hepworth et al., 2012a) which drive Th2 cell priming by dendritic cells (Hepworth et al., 2012b).

Mast cells contribute to the clearance of helminth infection by gut parasites (Anthony et al., 2007; Klementowicz et al., 2012). For example, studies in mouse and rat models demonstrated that the clearance of *Trichuris muris*, *Nippostrogylus brasiliensis* or *Strongyloides ratti* is mast cell dependent (Abe and Nawa, 1988; McDermott et al., 2003; Marshall, 2004). Nematode fecundity is higher in mast cell-deficient mice when infected by *Heligmosomoides polygyrus* compared to normal or mast cell-reconstituted mice (Hashimoto et al., 2009), and mast cells were shown to

contribute to the development of Th2 responses towards this gastrointestinal helminths by activating other cells involved in Th2 immunity, like epithelial cells or dendritic cells (Hepworth et al., 2012b). While mast cells are crucial in the clearance of gut helminths, they do not appear to have a key role in the clearance of tissue dwelling helminths such as *schistosomiasis*, although they are present in the early stages of *Schistosoma* infection (Gerken et al., 1990).

Fasciola hepatica causes a chronic liver infection (fascioliasis) in mammals, including cattle and sheep (Collins et al., 2004) and is an important zoonotic disease in humans with approximately 2.4 million people infected worldwide (McManus and Dalton, 2006). The life cycle of the parasite is complex with an intermediate snail host and definitive mammalian host. The definitive host is infected following ingestion of metacercariae shed from snails. These metacercariae excyst in the duodenum to form newly excysted juvenile flukes that penetrate the intestinal wall. They migrate through the peritoneal cavity and liver for several weeks and then finally the adult worm resides in the bile ducts releasing eggs for up to 30 years.

Fasciola promotes strong Th2/Treg mediated immune responses characterised by production of IL-4, IL-5 and IL-10 (O'Neill et al., 2000; Miller et al., 2009). Previous studies have shown mast cell number to be increased in the liver of cattle and sheep infected with *F. hepatica* (Rahko, 1969; Ferreras et al., 2000). An increase in mast cell number was also reported in the Peyer's patches (O'Brien et al., 2008) and mucosa of the small intestine (Charbon et al., 1991; Van Milligen et al., 1998) in *F. hepatica* infected rats. We know that injection of FhES mimics the Th2 immune response observed during *Fasciola* infection; however, it does not enhance mast cell

numbers in rats (Trudgett et al., 2003). Other studies have shown that *F. hepatica* derived peptides from the tegumental coat induce degranulation of rat peritoneal mast cells and histamine release (Trudgett et al., 2003).

Fasciola tegumental coat is shed from the parasite every 2-3 hours and is in constant contact with host immune cells (Halton, 2004). The isolated tegumental antigen of *F. hepatica* (FhTeg) suppresses dendritic cell maturation and function by inhibiting IL-6, tumour necrosis factor (TNF)-α, IL-10 and IL-12 production and CD80, CD86 and CD40 cell surface marker expression in TLR4 stimulated dendritic cells. FhTeg also impaired dendritic cell function by inhibiting its phagocytic capacity and its ability to prime T-cells (Hamilton et al., 2009; Hacariz et al., 2010). In this study we examined the interaction between FhTeg and mast cells in order to help us to understand the function of mast cells in the immunity against *F. hepatica*.

3.2 Experimental design

This chapter investigates the role of mast cells during *Fasciola hepatica* infection in mice and investigates if FhTeg can mimic these responses *in vivo*. FhTeg's interaction with mast cells was also examined to determine if FhTeg can enhance or suppress Th2 immune responses. Firstly, BALB/C mice were infected with *F. hepatica* metacercariae (2.2.4) to determine if mast cells numbers increase in the peritoneal cavity (2.2.18) and liver (2.2.7) of mice infected with *F. hepatica.* The initial experiments used BALB/C mice as these are model mice for Th2 immune responses. Since STAT6 is important to the establishment of Th2 immune responses, STAT6$^{-/-}$ mice and background C57BL/6 control mice were infected with *F. hepatica* metacecariae to determine if mast cell numbers were enhanced during infection in the absence of Th2 immune responses. We have previously established a model system were *Fasciola* antigens injected three time per week for three weeks can mimic the immune responses during *F. hepatica* infection. Using this system we determined if one of two fasciola antigen sources (FhES and FhTeg) could induce an increase in peritoneal mast cell number following treatment and the role of STAT6 was also determined.

Since FhTeg can induce mast cell numbers *in vivo* in a STA6 independent manner it was also determined if FhTeg can activate mast cells to promote Th2 immune responses. *In vitro* experiments were designed to determine if mast cells treated with FhTeg for 24 hours *in vitro* degranulate (2.2.14), produce Th2 promoting cytokines (2.2.12) or undergo proliferation (2.2.16). In these experiments BMMCs (2.2.8) were used for all experiments. However, PCMCs (2.2.9) are more mature and a better model to study mast cell – antigen interactions, although the isolation of these cells

require a substantial number of mice (1 mouse for 1 million cells) and therefore for ethical reasons only some positive results were confirmed with these cells. Cells were isolated from C57BL/6 mice as these mice are considered the model system for mast cell research. FhTeg treated BMMCs and PCMCs were also co-cultured with naïve T-cells (2.2.19) to determine if they can promote Th2 immune responses. Since IgE-dependent degranulaiton of mast cells is associated with helminth infection it was also tested if FhTeg can suppress IgE driven mast cell degranulation in two *in vivo* (PSA and PCA models, 2.2.6) and one *in vitro* (BMMCs, 2.2.14, 2.2.15) model systems.

Since FhTeg can enhance mast cells numbers *in vivo* it was investigated if FhTeg can induce proliferation and/or migration of mast cells. BMMCs and PCMCs were treated with FhTeg or FhTeg in the presence of stem cell factor which induces mast cell proliferation and proliferation measured after 24 hours (2.2.18). Migration of BMMCs and PCMCs were also tested on trans-well plates against FhTeg (10 µg/ml), IL-4, SCF (10 ng/ml) or FhTeg treated/untreated mast cells, macrophages and dendritic cells or their supernatants.

3.3 Results

3.3.1 Mast cell numbers are significantly enhanced during *F. hepatica* infection and this is mimicked by intraperitoneal injection of FhTeg.

BALB/C mice were infected with 20 *F. hepatica* metacecariae and after two weeks peritoneal exudate cells and whole livers were obtained to determine mast cell numbers. The ratio and number of mast cells increased in the peritoneal cavity from 0.5 ± 0.2% ((0.89 ± 0.51) x 10^4) control mice) to 17.5 ± 9.1% ($p \leq 0.05$) ((265.64 ± 177.54) x 10^4) infected mice) (Figure 3.1A) with similar increases observed in the liver; 1.5 ± 2.2/mm^2 (control mice) to 7.9 ± 6.6/mm^2 ($p \leq 0.01$) (infected mice) (Figure 3.1B, C, E). Hematoxylin/eosin staining showed tumour like cells in infected liver by the bile duct (Figure 3.1D, F). This is a symptom of fluke infection (Rahko, 1969).

It was previously shown that *F. hepatica* derived antigens when injected into BALB/C mice can mimic the immune response associated with *F. hepatica* infection (Donnelly et al., 2005). Using this model system we found that both FhTeg (19.6 ± 3.5%; $p \leq 0.01$; (35.39 ± 3.9) x 10^4) and FhES (5.4 ± 2.2 %; $p \leq 0.05$; (5.79 ± 3.5) x 10^4) when injected into mice increased mast cell numbers in the peritoneal cavity (control, 0.6 ± 0.2% (0.31 ± 0.13) x 10^4)) (Figure 3.1A). However, the increase in mast cell ratio in response to FhTeg was significantly greater when compared to FhES ($p \leq 0.01$) and was similar to that observed during natural infection. Given that the total cell number in the peritoneal cavity was higher during infection than after FhTeg injection, increase in mast cell number also appear to be higher in the infected group compared to FhTeg group, although the values are not significantly different ($p = 0.1$).

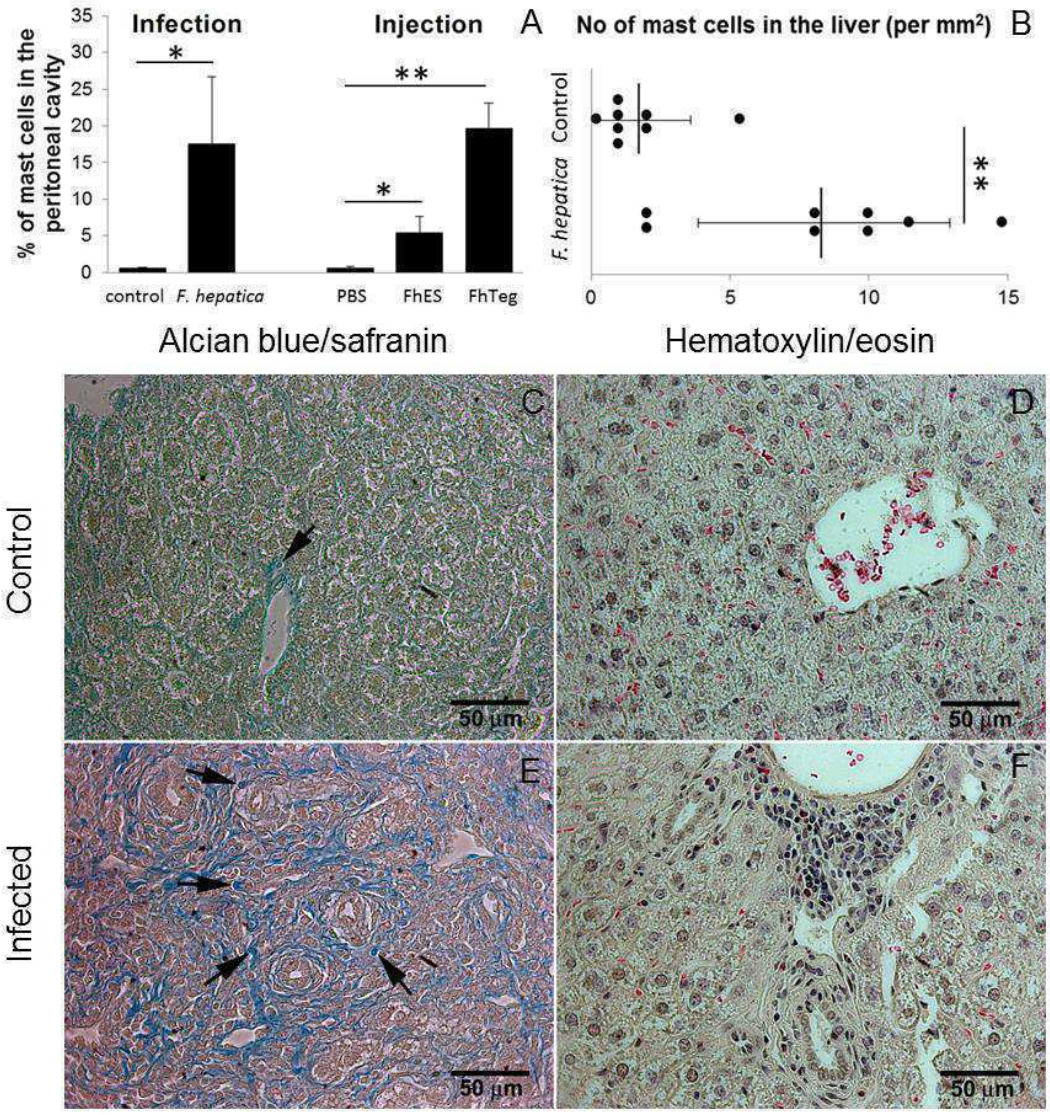

Figure 3.1. Mast cell number increased in the peritoneal cavity and in the liver after *Fasciola* infection. Injection of FhTeg but not ES mimicked the effect of Fasciolosis. Peritoneal exudate cells were harvested from un-infected (control), *F. hepatica* metacercariae (infected); PBS, FhES and FhTeg (10 µg) injected BALB/C mice. Percentages of mast cells were determined by two-colour flow cytometry for c-kit and FcεRI (**A**). Flow cytometry data were normalised to isotype controls and are presented as the mean ± SD of three independent experiments with 3 or 5 mice (n=9). *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control group. 5 µm sections of liver from control and *F. hepatica* infected mice were stained with alcian blue/safranin dye (**C, E**) and mast cells were counted (**, $p \leq 0.05$, **B**). Sections were also stained with standard hematoxiliyn/eosin dye (**D, F**).

3.3.2 Mast cells treated with FhTeg do not degranulate and exhibit reduced FcεRI expression but this does not alter IgE mediated activation of mast cells *in vivo* or *in vitro*.

As mast cell number in the peritoneal cavity increased after FhTeg injection mast cell – FhTeg interaction was investigated in more detail. Previous studies have shown that *F. hepatica* derived peptides induce degranulation of rat peritoneal mast cells and histamine release (Trudgett et al., 2003). In contrast, we found that bone marrow and peritoneum-derived mast cells do not degranulate in the presence of FhTeg (Figure 3.2A, B), even in the presence of sera from *F. hepatica* infected mice (Figure 3.3D).

Mast cells treated with FhTeg exhibited consistent and significant decrease in FcεRI expression (Figure 3.2C, D; $p \leq 0.01$). FcεRI is the receptor for IgE and is critical in IgE mediated degranulation of mast cells. We therefore investigated if FhTeg could alter IgE mediated degranulation in two *in vivo* model systems; passive systemic anaphylaxis model (PSA) or passive cutaneous anaphylaxis (PCA). We found that FhTeg does not markedly alter the IgE mediated responses in either model systems. However in the PCA model there was significant decrease in ear swelling at 4 and 6 hours (Figure 3.3A, B; $p \leq 0.05$). Similarly FhTeg did not alter DNP-specific IgE degranulation in BMMCs *in vitro* (Figure 3.3C).

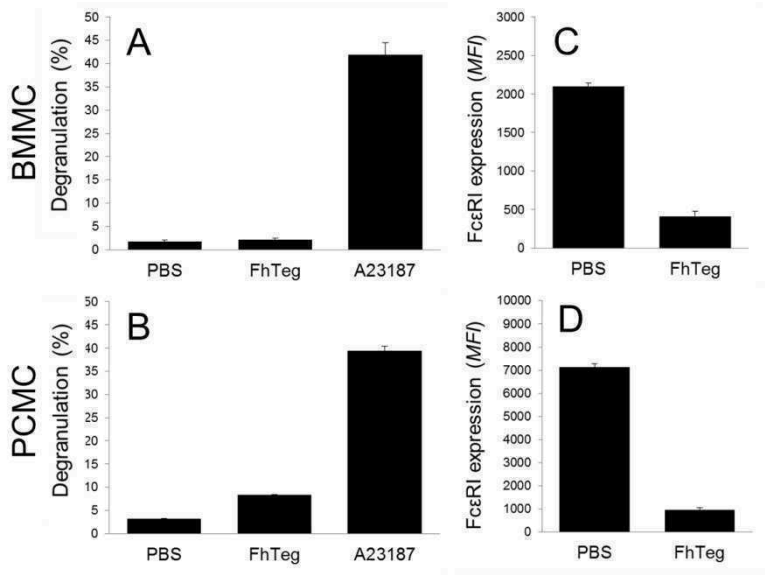

Figure 3.2. Mast cells treated with FhTeg do not degranulate and exhibit reduced FcεRI expression. BMMCs and PCMCs were treated with FhTeg (10 µg/ml) for 24 hours. Degranulation was tested by measuring β-hexosaminidase release (**A, B**). FcεRI expression was measured by two colour flow cytometry (FcεRI and c-kit) (**C, D**). Data are presented as the mean ± SD of at least three independent experiments (n=3).

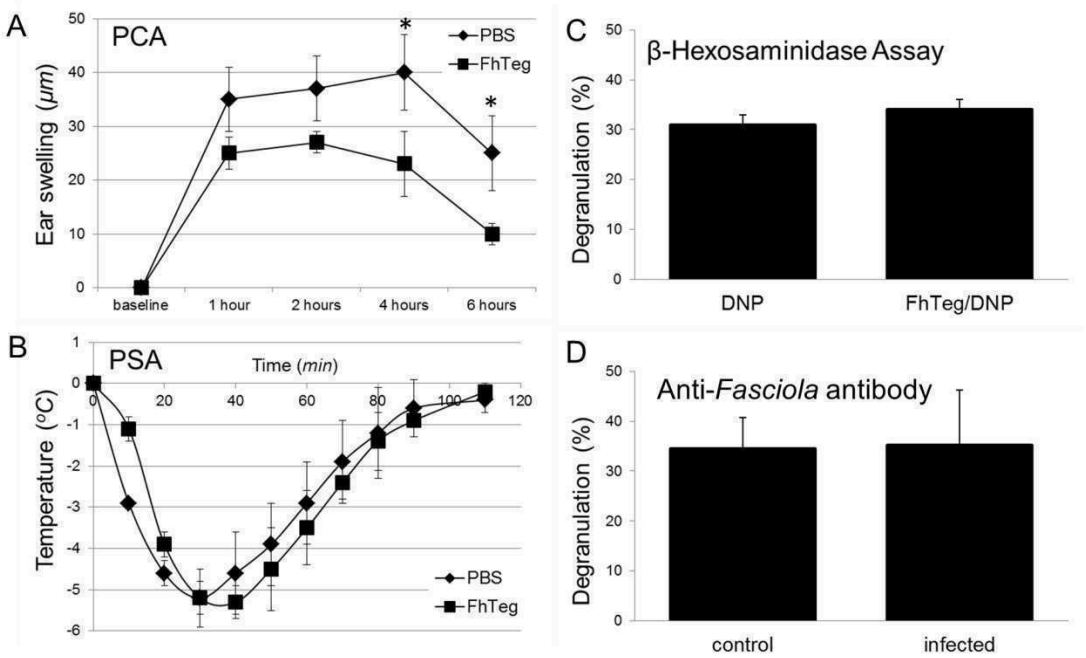

Figure 3.3. FhTeg does not alter IgE mediated activation of mast cells *in vivo* or *in vitro*. C57BL/6 mice were injected with FhTeg (10 µg/ml) three times before administering DNP-specific

IgE prior to challenge with DNP subcutaneously (**A**; Passive cutaneous anaphylaxis model – PCA) or systemically (**B**; Passive systemic anaphylaxis model – PSA). DNP-specific IgE mediated degranulation was measured by β-hexosaminidase assay *in vitro* on FhTeg treated and untreated BMMCs (**C**). Data are presented as the mean ± SD of three independent experiments (n=3). *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control group. These experiments (except D) were done at Charité Universitätmedizin in Berlin by Dr Sandra O'Neill.

3.3.3 FhTeg treated mast cells do not produce Th2 cytokines or promote a Th2 immune response in $CD4^+$ cells.

Mast cells are potent source of pro-inflammatory mediators, such as IL-4, that play key role in the development of Th2 immune responses against helminth parasites (Maizels et al., 2004). We, therefore, assessed if FhTeg stimulated mast cells promote Th2 immune responses. BMMCs and PCMCs stimulated with different concentrations (5, 10, 20 μg/ml) of FhTeg for 6 (data not shown) or 24 hours did not release significant amounts of IL-6, TNF-α (Figure 3.4A-B), IFN-γ, IL-13, IL-10, IL-5 or IL-4 (data not shown). Given that PCMCs are more mature and were shown previously to give a different activation profile when stimulated with pathogen antigens (Mrabet-Dahbi et al., 2009) we repeated these experiments with PCMCs and a similar result were observed (Figure 3.4C-D). All positive control responded appropriately and responses were statistically significant ($p \leq 0.01$).

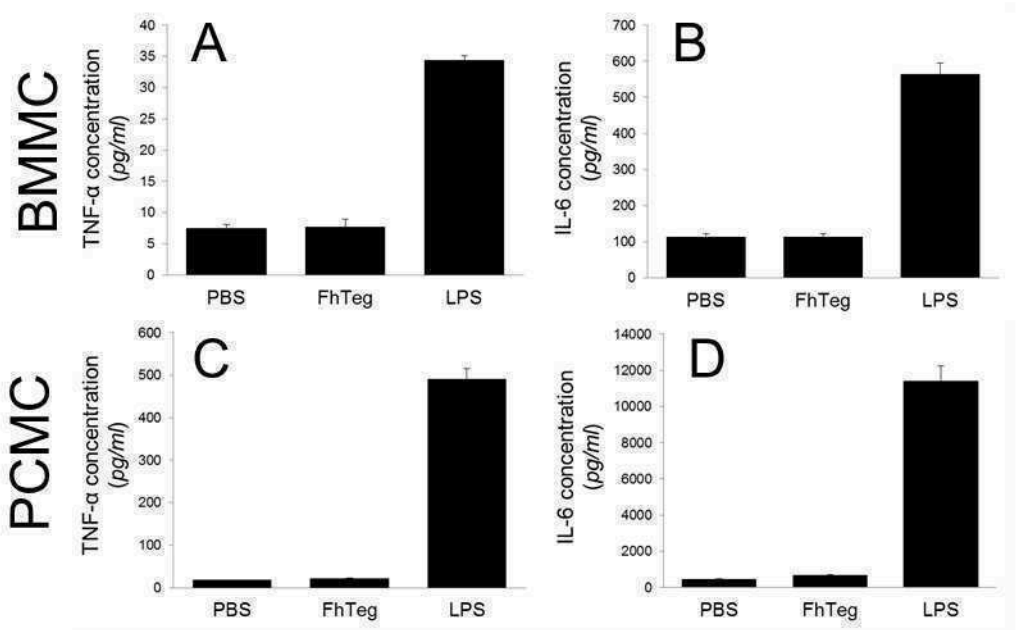

Figure 3.4. FhTeg treated mast cells do not produce cytokines. BMMCs and PCMCs were stimulated with FhTeg (10 µg/ml) or PBS for 24 hours. TNF-α (**A, C**) and IL-6 (**B, D**) secretion was determined from supernatant using ELISA. A23187, or LPS was used as positive controls and responds appropriately and statistically significant ($p \leq 0.01$). Data are presented as the mean ± SD of at least three independent experiments (n=3).

Previous studies have shown that BMMCs and PCMCs co-cultured with $CD4^+$ T-cells can promote cytokine secretion (Huels et al., 1995; Nakae et al., 2005) and this is enhanced when mast cells first exposed to TLR ligands (Nie et al., 2011). Co-culturing of FhTeg treated mast cells with naïve $CD4^+$ T-cell did not promote Th1 or Th2/Treg responses in mast cells (Figure 3.5). Next we investigated if Th2 cytokines are involved in the increase of mast cell number during *F. hepatica* infection.

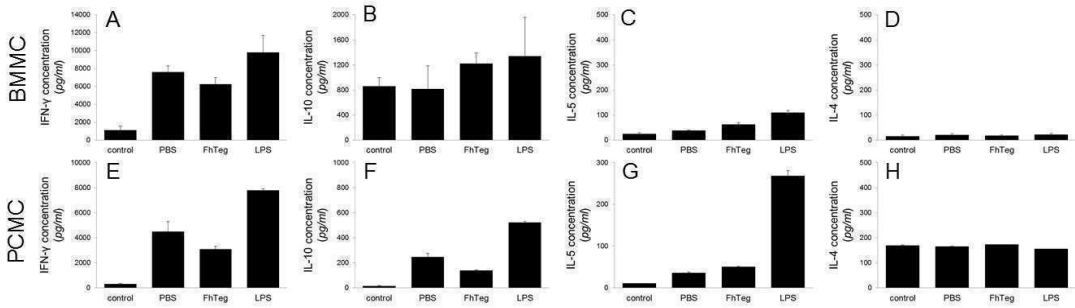

Figure 3.5. Mast cells stimulated with FhTeg do not promote Th1 or Th2 immune responses when co-cultured with anti-CD3 stimulated naïve CD4+ T-cells. BMMCs and PCMCs were stimulated with PBS, FhTeg (10 µg/ml) or LPS (100 ng/ml) for 24 hours. Cells were washed and co-cultured with anti-CD3 stimulated naïve $CD4^+$ cells (1:1) and after 72 hours supernatant were analysed for IFN-γ (**A, E**), IL-10 (**B, F**), IL-5 (**C, G**) and IL-4 (**D, H**) cytokines. T-cells cultured without mast cells were used as negative controls. Figures are representative of at least three independent experiments and presented as the mean ± SD of three replicate samples from the same experiment.

3.3.4 Enhancement of mast cell numbers *in vivo* is not dependent upon STAT6.

Since FhTeg stimulated mast cells did not promote Th2 immunity *in vitro* we wanted to determine if Th2 cytokines are involved in the increase in mast cells ratio and number during infection and following injection of FhTeg. STAT6 activation is critical in driving Th2 immune responses associated with helminth infection (Finkelman et al., 2004). When $STAT6^{-/-}$ mice were infected with *F. hepatica* metacercariae or injected with FhTeg, we found increases in mast cell numbers in control background mice (C57BL/6, from uninfected 2.9 ± 1.4%; (1.53 ± 2.51) x 10^4 to infected 24.6 ± 11.3%; (385.07 ± 143.40) x 10^4 ($p \leq 0.05$)) to be similar to those in $STAT6^{-/-}$ mice (from uninfected 2.3 ± 0.8%; (1.87 ± 2.03) x 10^4 to infected 19.2 ± 7%; (297.04 ± 149.37) x 10^4 ($p \leq 0.01$)). $STAT6^{-/-}$ mice injected with FhTeg

also demonstrated a similar increase in mast cells numbers (from 5.3 ± 3.4%; (1.89 ± 2.64) x 10^4 to 21.7 ± 6.7%; (45.81 ± 17.26) x 10^4 ($p \leq 0.05$) as controls (C57BL/6: from 4.1 ± 2.9%; (1.46 ± 1.35) x 10^4 to 22.7 ± 1.9%; (31.26 ± 2.70) x 10^4 ($p \leq 0.01$)) (Figure 3.6).

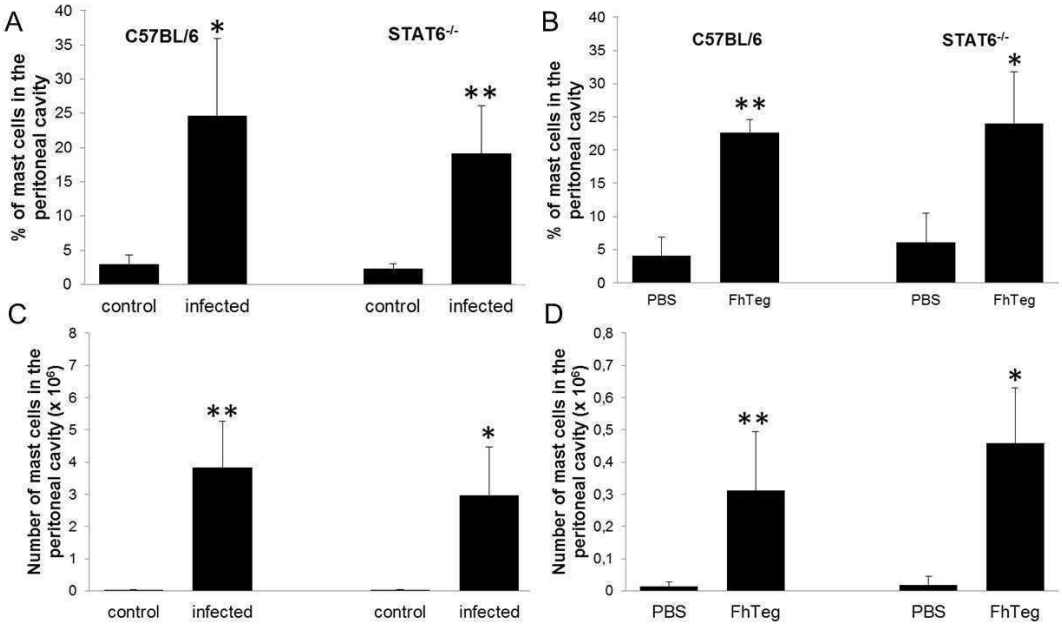

Figure 3.6. Mast cell numbers are significantly enhanced during *F. hepatica* infection and after FhTeg injection in a STAT6 independent manner. Peritoneal cells were harvested from un-infected (control), *F. hepatica* infected (infected) (**A**); PBS and FhTeg injected (**B**) STAT6$^{-/-}$ or its control C57BL/6 mice Percentages and numbers (**C**, **D**) of mast cells were determined by two colour flow cytometry for c-kit and FcεRI. Flow cytometry data were normalised to isotype controls and are presented as the mean ± SD of three independent experiments (**A, C**: n=10; **B, D**: n=8). *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control group.

3.3.5 Mast cells treated with FhTeg exhibit decreased expression of c-kit and this is associated with reduced cell proliferation in response to stem cell factor.

To determine if FhTeg-induced mast cell hyperplasia is due to mast cell proliferation, peritoneal mast cells were treated with FhTeg and found that they do

not proliferate (5, 10, 20 µg/ml) (Figure 3.7A-B). However, these cells exhibited a consistent and significant decrease in c-kit expression (Figure 3.6C-D; $p \leq 0.01$). When mast cells were stimulated with stem cells factor (that can induce proliferation in mast cells) in the presence of FhTeg a significant dose dependent decrease in proliferation was observed (Figure 3.8).

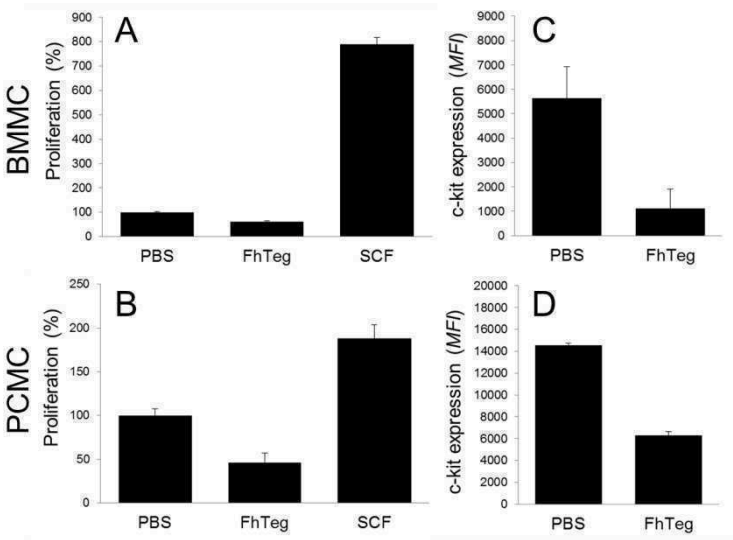

Figure 3.7. Mast cells treated with FhTeg does not proliferate and exhibit decreased expression of c-kit. BMMCs and PCMCs were treated with FhTeg (10 µg/ml) for 24 hours. Proliferation was measured with MTT test (**A, B**) and c-kit expression was determined by two colour flow cytometry (c-kit and FcεRI) (**C, D**). Flow cytometry results were normalised to isotype controls. Data are presented as the mean ± SD of three independent experiments (n=3).

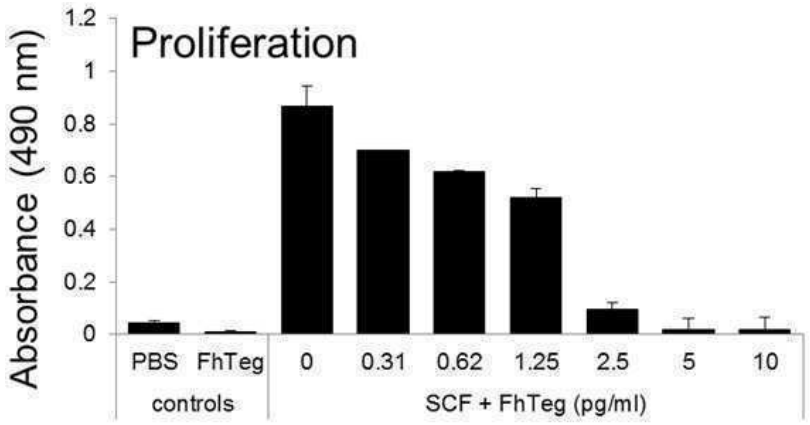

Figure 3.8. FhTeg suppresses SCF induced mast cell proliferation. SCF (10 ng/ml) induced proliferation was measured by MTT test on BMMCs following FhTeg treatment. Concentrations of FhTeg are indicated on the graph. Figure is representative of three independent experiments (n=3).

3.3.6 Mast cell migration is induced by FhTeg stimulated dendritic cells but not mast cells.

Since mast cells do not proliferate in response to FhTeg we asked if mast cells, stimulated *in vitro* with FhTeg, can induce mast cell migration which could explain the increase of mast cells *in vivo*. FhTeg, IL-4 and FhTeg stimulated macrophages or mast cells did not induce mast cell migration (Figure 3.9). FhTeg treated dendritic cells or their supernatants induced increased migration rate of both BMMCs and PCMCs. Migration rate increased significantly compared to control PBS treated cells after 3 hours tested with trypan blue and Kimura staining ($p \leq .0.01$). Results were confirmed with flow cytometry at 3 hour time point ($p \leq .0.05$) (Figure 3.10).

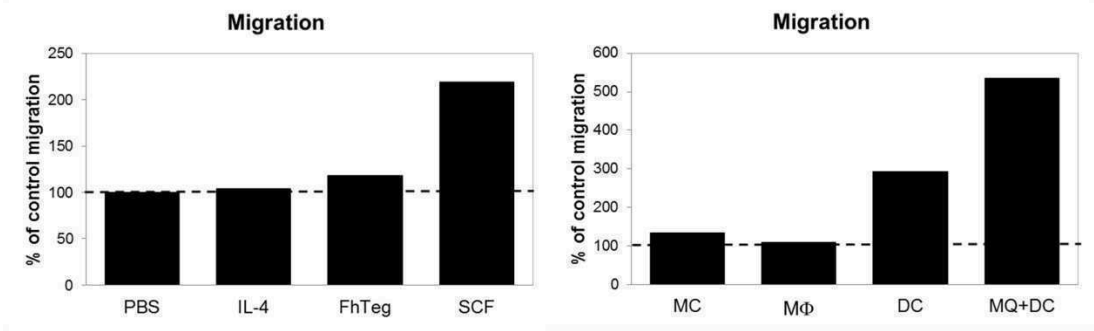

Figure 3.9. Chemotaxis of mast cells is induced by FhTeg activated dendritic cells, but not by IL-4, FhTeg or FhTeg treated mast cells or macrophages. Cemothaxis was measured against IL-4, FhTeg or SCF for 3 hours (**A**). BMMCs (MC), RAW macrophages (MΦ) and BMDCs (DC) were treated with FhTeg (10 µg/ml) or PBS for 18/24 h. Chemotaxis of mast cells were measured for 3 hours against the treated cells (**B**). Mast cell number was assessed by Kimura staining or by flow cytometric analysis on the basis of c-kit expression. Graphs are representative of two independent experiments.

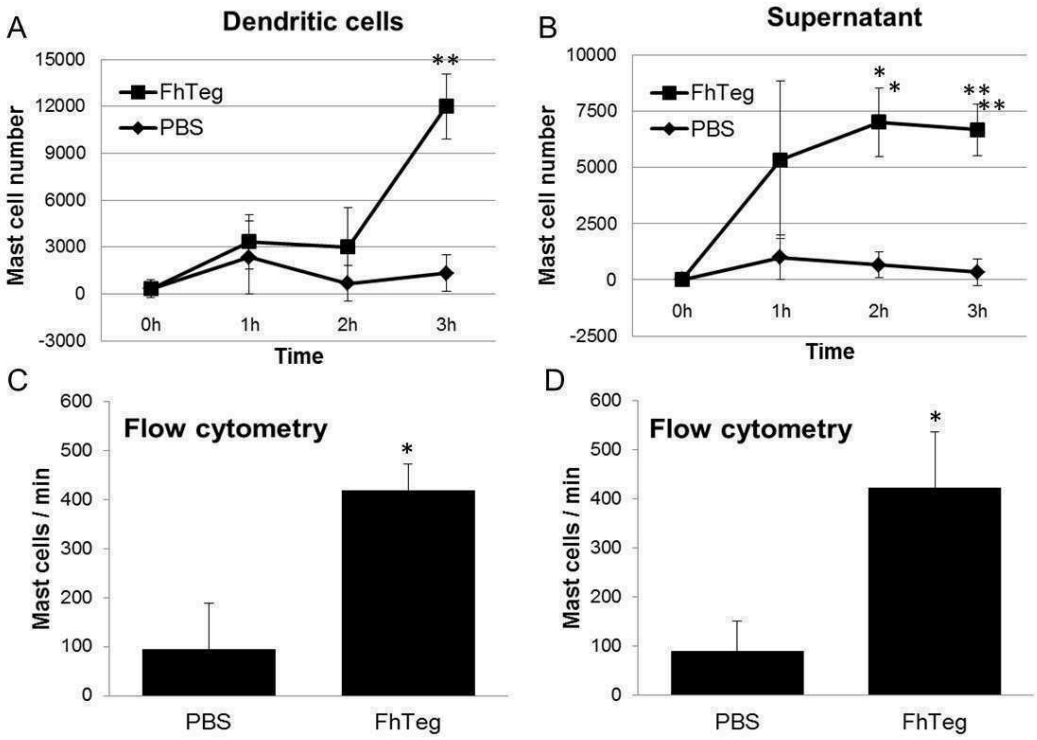

Figure 3.10. Chemotaxis of mast cells is induced by FhTeg activated dendritic cells or their supernatant. BMDCs were treated with FhTeg (10 µg/ml) or PBS for 18 h. Chemotaxis of PCMCs were measured against dendritic cells or their supernatants. Mast cell number was assessed by Kimura

staining at 0, 1, 2, and 3 h time points (**A, B**) or flow cytometric analysis on the basis of c-kit expression after 3 h (**C, D**). Flow cytometry data were normalised to isotype controls and data are presented as the mean ± SD. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

3.4 Discussion

Increased mast cell numbers in the liver is one of the key features of fascioliasis (Rahko, 1969; Ferreras et al., 2000), and increased mast cell numbers were observed in the gut mucosa of rats experimentally infected with *F. hepatica* (Van Milligen et al., 1998). To our knowledge this study is the first to demonstrate an increase in mast cell numbers *in vivo* in the liver and the peritoneal cavity of *F. hepatica* infected mice. We also show that this increase in mast cell numbers can be mimicked by treatment of mice with *F. hepatica* tegumental antigen (FhTeg). During *F. hepatica* infection the worm first migrates through the peritoneal cavity before residing in the bile ducts for many years. Increased mast cell numbers are first observed in the early stages of infection, which suggests that these cells may have an important role to play in shaping the adaptive immune response during infection.

FhTeg stimulated mast cells do not secrete key Th2 promoting cytokines and fail to promote Th2 responses in naïve T-cells. This suggests that FhTeg interaction with mast cells may not be important in driving Th2 immunity. This is supported by our studies in STAT6 knockout mice as the increase in mast cell number in the peritoneal cavity following *F. hepatica* infection and FhTeg injection is STAT6 independent. IL-13 and IL-4 signal through the STAT6 signalling pathway and activation of this pathway is important in Th2 immune responses (Finkelman et al., 2004). Our finding contrasts other studies that demonstrate the involvement of mast cells in Th2 immune responses against multiple nematodes and other parasitic infections (Dawicki and Marshall, 2007). Activation of STAT6 signalling pathway is important in Th2 immune responses required for the protection against helminths like *Trichinella spiralis* (Bian et al., 2005; Finkelman et al., 2004) and *N.*

brasiliensis (Finkelman et al., 2004; Sakamoto et al., 2004) in murine models, although the mechanism is unclear (Urban et al., 2000). These studies suggest that mast cells react to the presence of helminths by generating cytokines like IL-4 that shapes the development of Th2 immune responses (Maizels et al., 2004; Allen and Maizels, 2011).

During *F. hepatica* infection Th1 immune responses are more important for protection (O'Neill et al., 2000; Mulcahy et al., 2004; Moreau and Chauvin, 2010) as IL-4$^{-/-}$ mice, which are predisposed towards a Th1 immune response, are less susceptible to *F. hepatica* infection with reduced liver damage (O'Neill et al., 2000). Similar studies with *Taenia crassiceps* showed that STAT6$^{-/-}$ mice that exhibited polarized Th1-type responses were more effective in worm clearance (Rodriguez-Sosa et al., 2002). Mast cells co-cultured with CD4^{+} T-cells can promote the secretion of IFN-γ (Huels et al., 1995; Nakae et al., 2005) and this is enhanced when mast cells are first exposed to TLR ligands (Nie et al., 2011). Mast cells are important in driving Th1 immunity; perhaps during *F. hepatica* infection mast cell contribute to Th1 immunity which is important for immune protection. This hypothesis requires further investigation.

FhTeg does not induce mast cell proliferation but rather inhibits stem cell factor (SCF) driven proliferation of mast cells through the inhibition of c-kit expression, a pan marker for mast cells and a receptor for SCF (Metcalfe et al., 1997). This suggests that the increase in mast cell numbers in the peritoneal cavity may be the result of mast cell migration into the peritoneal cavity. This contrasts studies in *N. brasiliensis* and *Strongyloides venezulensis* infected rats where intensive

proliferation of these cells is observed in the intestinal mucosa, Peyer's patches and mesenteric lymph nodes (Kasugai et al., 1995; Tegoshi et al., 1997). In another study, nematode-induced proliferation was reported in the gut but not in the peritoneal cavity, although mast cell numbers increased at both sites (Arizono et al., 1994) suggesting that mast cell migration is also important.

FhTeg stimulated mast cells did not induce mast cell migration *in vitro*, but FhTeg-activated dendritic cells induced mast cell migration. In our laboratory it was shown previously that FhTeg stimulated dendritic cells secrete MIP2 (CXCL2) and MIP1α (CCL3) chemokines (Appendix F) and these cytokines might be involved in mast cell migration (Gilfillan et al., 2011; Kunii et al., 2011; Taub et al., 1995). MIP1α was shown to induce migration but not degranulation of BMMCs *in vitro* (Taub et al., 1995). Mast cells express chemokine receptors on their surface including CXCR2 and CCR3, the receptors for MIP2 and MIP1α (Juremalm and Nilsson, 2005). This result is supported by other studies underlying the importance of mast cell – dendritic cell cross talk in the development of immune responses (Bryce et al., 2004; Galli et al., 2005; Mazzoni et al., 2006). Peritoneal mast cells and dendritic cell crosstalk was shown to cause dendritic cell maturation resulting in cytokine (IFN-γ, IL-2, IL-6 and TGF-β) secretion that can modulate T-cells (Dudeck et al 2011). In contact hypersensitivity mouse models, activated dendritic cells bind to mast cells and promote Ca^{2+} influx and TNF-α production in these cells. These activated mast cells further promote additional activation of dendritic cells inducing migration (Otsuka et al., 2011).

In the early stages of infection, *F. hepatica* larvae penetrate trough the gut wall into the peritoneum and then into the liver, causing extensive tissue damage (Robinson et al., 2010). One explanation, for the increased mast cell number in the peritoneal cavity in the early stage of infection, may be that mast cells are involved in wound healing. Mast cells represent a heterogeneous population and have various functions (Metcalfe et al., 1997), and they play a key role in wound healing and tissue remodelling. In mast cell-deficient mice wound closure is significantly impaired compared to normal or mast cell reconstructed mice (Weller et al., 2006). Mast cells secrete mediators, such as histamine, serotonin, enzymes and cytokines that are important in inducing fibroblast proliferation, increased vascular permeability (marker for wound healing responses) and neutrophil recruitment (Maurer et al., 2003; Weller et al., 2006). These findings suggest that mast cell functions at sites of infection may not be limited to promoting Th1 or Th2 immune responses and further studies are required to investigate this.

Mast cells do not degranulate in response to FhTeg. Mast cells became activated by IgE through their high affinity FcεRI receptor (Melendez et al., 2007; Pearce, 2007) in the presence of IgE-specific antibody. In our experiments FhTeg treated mast cells express lower level of FcεRI compared to control cells. This down-regulation of FcεRI by FhTeg does not have an impact on IgE dependent mast cell degranulation *in vitro* or *in vivo*, although there is a significant decrease in ear swelling at 4 and 6 hours in the passive cutaneous anaphylaxis model. This is in keeping with FhTeg's ability to suppress inflammatory responses *in vivo.* Previous studies have shown that mast cell-derived TNF-α is protective during septic peritonitis (Echtenacher et al., 1996) and FhTeg can suppress the release of pro-inflammatory cytokines in a

mouse model of septic shock (Hamilton et al., 2009). Similarly, FhTeg can impair the release of inflammatory mediators from TLR activated dendritic cells (Hamilton et al., 2009). During anaphylaxis, mast cell degranulation and the immediate release of mediators, such as histamine and tryptase, is followed by a delayed phase (2-6 hours) when mast cells generate cytokines like TNF-α (Finkelman, 2007; Metcalfe et al., 2009). As FhTeg suppresses TNF-α secretion in TLR activated immune cells, it may also suppress TNF-α secretion in mast cells in the passive cutaneous anaphylaxis model and this might be responsible for the decreased ear swelling in its late phase. Further investigation is warranted to identify the mechanism of suppression at later time points.

In this study we showed that mast cells number increased in the peritoneal cavity and liver of *F. hepatica* infected mice, and this can be mimicked by injection of FhTeg. FhTeg activated mast cells express reduced levels of c-kit and FcεR1 but do not secrete a panel of cytokines or drive Th2 or Treg immune responses. Our study suggests that the increase in mast cell numbers is not the result of FhTeg induced proliferation; rather FhTeg indirectly induces mast cell migration by dendritic cell derived chemokines. The increase in mast cells numbers occurs in the absence of Th2 cytokines and independent of STAT6 and our finding is that FhTeg interactions with mast cells do not drive Th2 or Treg immune responses. While it is clear that mast cells have role in the expulsion of gut dwelling helminths, our finding contrast studies examining the interaction of gut dwelling helminths with mast cells. This highlights the need to revisit the role of mast cells in tissue dwelling parasitic infections in order to advance our understanding of mast cells in host immunity.

4 Chapter 4 - *Fasciola hepatica* tegumental coat antigens supress Th1 immune responses in mast cells

4.1 Introduction

Mast cells are associated with helminth infection but are mainly studied in the context of IgE mediated responses and Th2 immunity. Mast cells express pathogen recognition receptors such as TLRs and when activated with TLR ligands are a potent source of Th1 pro-inflammatory mediators, such as TNF-α and IFN-γ. Mast cells promote Th1 driven host defence against bacterial and protozoan infections (Galli and Tsai, 2010; Marshall et al., 2003; Mielcarek et al., 2001; Wierzbicki and Brzezinska-Blaszczyk, 2009), and in the absence of mast cells these infections are significantly exacerbated (Maurer et al., 2006; Siebenhaar et al., 2007) demonstrating a critical role for mast cells. Mast cells are also associated with many Th1-mediated inflammatory disorders such as multiple sclerosis and Crohn's disease where TNF-α secreting mast cells are important contributors to immune pathology (Metz and Maurer, 2007; Sutherland et al., 2011; Weller et al., 2011).

Helminths and the products they release exert effects that impair Th1 immunity to bystander microbial infections, such as malaria and tuberculosis (Mulcahy et al., 2004; Nacher, 2011) and this effect significantly impacts population in the developing world (Anthony et al., 2007). However they can prevent the initiation and perpetuation of many Th1 immune disorders such as arthritis and Crohn's disease (De Winter et al., 2011; Song et al., 2011) in these populations. Helminth driven regulatory networks through the induction of regulatory cytokines, TGF-β and IL-10 can suppress these pro-inflammatory Th1 immune response (Song et al.,

2011; Whelan et al., 2011) or helminths release molecules that directly impair the ability of innate immune cells to drive Th1/Th17 immune responses (Allen and Maizels, 2011; Mulcahy et al., 2004). Understanding these mechanisms could lead to the development of novel anti-inflammatory therapeutic targets and expand our understanding of helminth – microbial interactions in human population.

Fasciola hepatica can suppress protective Th1 driven immunity against bystander infections such as tuberculosis and *Bordetella pertussis* (Brady et al., 1999; Claridge et al., 2012; Flynn et al., 2009). It can also attenuate experimental autoimmune encephalitis (EAE) through the suppression of Th17 and Th1 immune responses (Walsh et al., 2009). The isolated *F. hepatica* coat antigen (FhTeg) has suppressive effect *in vivo* by inhibiting the release of pro-inflammatory cytokines in the mouse model of septic shock (Hamilton et al., 2009) FhTeg alters the ability of dendritic cells to prime Th1 immune responses by rendering them hypo-responsive to TLR activation suppressing the production of a selected panel of inflammatory cytokines and co-stimulatory molecules. The suppressive effect is not mediated trough the common MAPKs found in the TLR pathway and is dependent of MyD88 and TLR4. FhTeg suppresses the transcription factor NF-κB p65 which is involved in the transcription of many Th1 promoting factors (Hamilton et al., 2009). This study examines FhTeg's interaction with mast cells to determine if its modulatory effect can impair mast cell ability to promote Th1 immunity and the possible mechanism involved in this process will be examined.

4.2 Experimental design

Since FhTeg does not enhance or suppress Th2 immune responses in mast cells this chapter investigates the effect of FhTeg on Th1 promoting mast cells. The effect of FhTeg on bacterial ligand-induced cytokine production of mast cells was tested *in vivo* and *in vitro*. *In vivo* C57BL/6 mice were treated with FhTeg prior to i.p. injection of *Bordetella pertussis* antigen (BP) (2.2.4) to determine if FhTeg treatment has any effect on BP-induced TNF-α expression in peritoneal mast cells (2.2.18). In this experiment C57BL/6 mice were used as these mice are good models to study Th1 immune responses. These *in vivo* results were confirmed *in vitro* with LPS or BP activated BMMCs (2.2.8) and with the more mature PCMCs (2.2.9, 2.2.12). Since it was shown that the mast cell number increase in the peritoneal cavity during *F. hepatica* infection is STAT6 independent, BMMCs from STAT6$^{-/-}$ mice was also investigated to determine the role of STAT-6 on FhTeg's immunomodulatory effect. Since mast cells can activate T-cells *in vitro* FhTeg treated and LPS/BP stimulated BMMCs and PCMCs were co-cultured with naïve T-cells (2.2.19) to examine if FhTeg can suppress mast cell induced cytokine secretion (IFN-γ, IL-10, IL-5 and IL-4) in T-cells. Involvement of ICAM1 in mast cell – T-cell communication was also examined (2.2.18-19).

FhTeg – mast cell interaction was further examined to find a potential mechanism. LPS and BP are TLR4 ligands that induce transcription factors of TLR4 signalling pathway. The modulatory effect of FhTeg on this signalling pathway was investigated in BMMCs by western blot analysis. (2.2.20-22). These experiments required high amount of cells, so only some positive results were confirmed with the more mature PCMCs. Dendritic cells (BMDCs) (2.2.10) were also examined in this

study as the activation of transcription factors is restricted in mast cells compared to dendritic cells. FhTeg effect on the members and inhibitory molecules of the TLR4 signalling pathway (RelA, MyD88, TLR4, TRAF6, PIAS1/3/4, SOCS1/2/3/4/5) were examined with qPCR (2.2.23-24) and western blot (2.2.20-22). It was also investigated if FhTeg can modulate cytokine secretion and cell surface marker up-regulation by cytokine signalling pathways (TNF-α, IFN-γ or IL-10) in BMDCs (2.2.10-11, 2.2.18).

4.3 Results

4.3.1 FhTeg inhibits TNF-α expression in mast cells *in vivo.*

F. hepatica suppresses protective Th1 driven immunity against *B. pertussis* (Flynn et al., 2009) and since mast cells have an important role to play in protection against bacterial infection we wanted to determine if FhTeg can inhibit the production of pro-inflammatory cytokines by mast cells *in vivo*. Here we firstly demonstrated that *B. pertussis* antigen (BP) when injected into the peritoneal cavity of C57BL/6 mice induced high numbers of TNF-α expressing mast cells (Figure 4.1). When these mice were co-injected with FhTeg, mast cells expressed significantly less TNF-α when compared to BP injected mice only, confirming that FhTeg can inhibit the production of pro-inflammatory cytokines by mast cells *in vivo* (Figure 4.1).

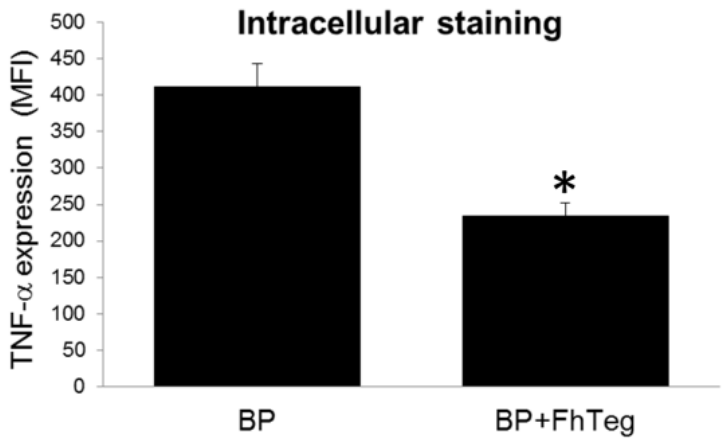

Figure 4.1. FhTeg inhibit TNF-α expression in mast cells *in vivo*. C57BL/6 mice were injected with FhTeg (10 μg) or PBS prior to be immunised by BP (10 μg) 4 times for 10 days. Peritoneal mast cells were intracellular stained for flow cytometry with TNF-α. Mast cells were identified on the basis of c-kit and FcεRI expression. Data are presented as the mean ± SD of two independent experiments with 3-5 mice per group (n=8). *, $p \leq 0.05$ compared to control group.

4.3.2 FhTeg suppresses cytokine secretion from LPS or BP stimulated mast cells.

To confirm our *in vivo* findings we stimulated BMMCs and PCMCs *in vitro* with BP or LPS as a positive control in the presence of absence of FhTeg and cytokine secretion was measured in supernatant after 24 hours. Both BMMCs (IL-6 and TNF-α) and PCMCs (IL-6, TNF-α, IL-10 and IFNγ) secreted significant levels of cytokines however in the presence of FhTeg cytokine secretion was significantly suppressed (Figure 4.2; $p \leq 0.05$ to $p \leq 0.001$).

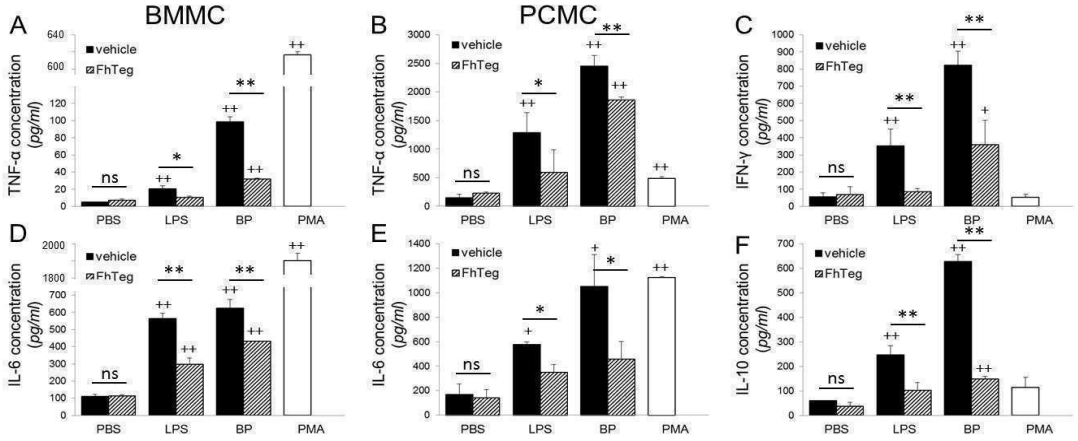

Figure 4.2. FhTeg suppressed cytokine expression in TLR4 stimulated BMMCs and PCMCs. Mast cells were stimulated with and without FhTeg (10 µg/ml) for 2.5 hours following with PBS, LPS (100 ng/ml) or BP (100 bacteria/cell) for 24 hours. PMA (phorbal myristate acetate, 20 ng/ml) was used as positive control. Supernatants were tested for TNF-α (**A, B**), IFN-γ (**C**), IL-6 (**D, E**) and IL-10 (**F**) ELISA. Figures are representatives of three independent experiments (n=3) and presented as the mean ± SD of three replicate samples from the same experiment.. $^{+}$, $p \leq 0.05$; $^{++}$, $p \leq 0.01$ compared to vehicle (PBS) group; *, $p \leq 0.05$; **, $p \leq 0.01$ compared to LPS and BP group.

4.3.3 Mast cells activation by BP and LPS is dependent upon STAT6, however this had no role to play in the suppressive effect of FhTeg.

To test if STAT6 pathway is involved in the inhibitory effect of FhTeg we cultured BMMCs from C57BL/6 (control) and STAT6$^{-/-}$ mice and stimulated them with LPS and BP with or without FhTeg treatment. The absence of STAT6 maintains FhTeg's suppressive effect on TNF-α and IL-6 secretion and cell surface marker (c-kit and FcεRI) expression of mast cells. However STAT6$^{-/-}$ mast cells secreted more Th1 cytokines after BP stimulation ($p \leq 0.05$, Figure 4.3).

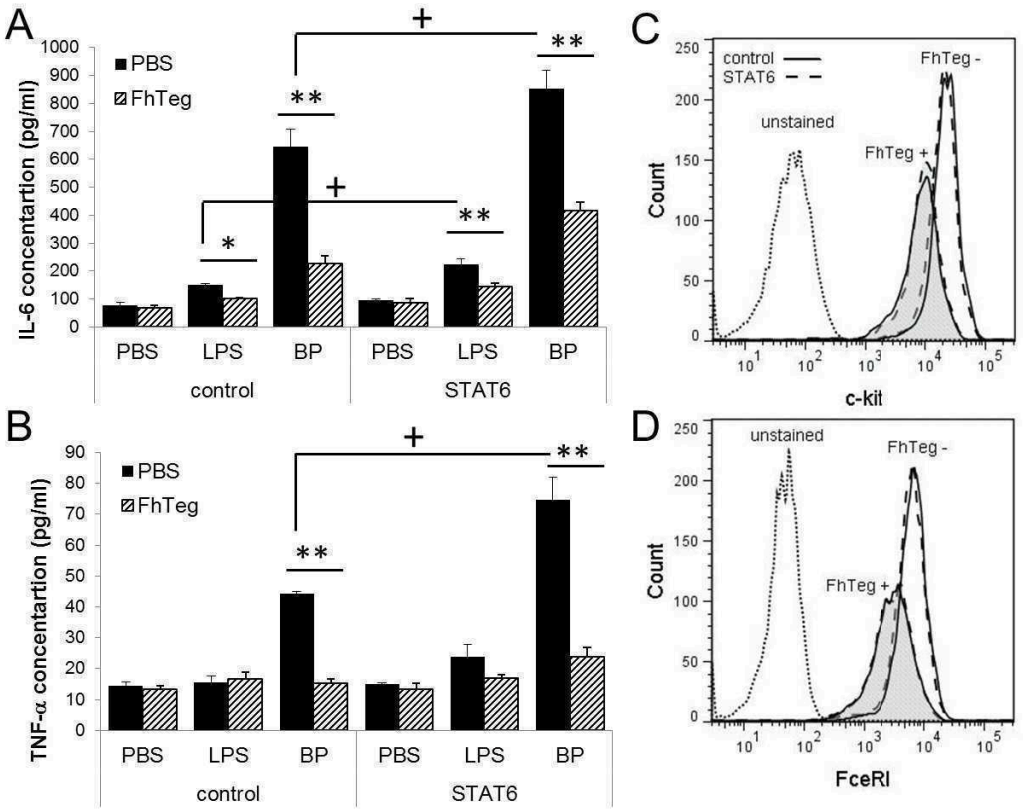

Figure 4.3. Mast cells activation by BP and LPS was dependent upon STAT6, however this had no role to play in the suppressive of FhTeg. BMMCs from STAT6$^{-/-}$ and control mice were stimulated with and without FhTeg (10 µg/ml) for 2.5 hours following with PBS, LPS (100 ng/ml) or BP (100 bacteria/cell) for 24 hours. Supernatants were tested for IL-6 (**A**) and TNF-α (**B**) ELISA. Figures are representatives of three independent experiments (n=3) and presented as the mean ± SD of

three replicate samples from the same experiment.; *, $p \leq 0.05$; **, $p \leq 0.01$ when FhTeg/LP and FhTeg/BP compared to LPS and BP group. $^{+}$, $p \leq 0.05$ when mast cells from STAT6$^{-/-}$ mice compared to control C57BL/6 group Cells were stained for cytometry with c-kit (**C**) and FcεRI (**D**). Results are represented on histograms: STAT6$^{-/-}$ BMMCs are indicated with dashed line and curves of FhTeg treated cells are tinted.

4.3.4 CD4^{+} T-cells co-cultured with FhTeg treated mast cells secrete less IL-10, IFN-γ and IL-5.

Mast cells when co-cultured with naïve CD4^{+} cells in the presence of anti-CD3 can promote Th1 immune responses and this can be enhanced when mast cells are pre-treated with bacterial antigens or TLR ligands (Huels et al., 1995; Nakae et al., 2005; Nakae et al, 2006). BMMCs and PCMCs were stimulated with BP and LPS for 24 hours to determine if mast cells ability to promote cytokine production from naïve T-cell can be inhibited. After 24 hours supernatants were removed and cells were washed. Supernatant was added to CD4^{+} T-cells or CD4^{+} T-cells were co-cultured with washed mast cells. CD4^{+} T-cells co-cultured with BP- and LPS-stimulated BMMCs/PCMCs or when cultured with supernatant from these cells secreted significant levels of IFN-γ, IL-10 and IL-5 (Figure 4.4A, F, I, N) but not IL-4 (Figure 4.4G, H, O, P). Significantly less IFN-γ, IL-10 and IL-5 cytokines were obtained when T-cells were co-cultured with BP- and LPS-stimulated BMMCs/PCMCs in the presence of FhTeg or when cultured with the supernatant from these cells (Figure 4.4). BMMCs, PCMCs and T-cells alone were used as negative controls and levels of IFN-γ, IL-10, IL-4 or IL-5 were not significantly higher than background and were significantly less than co-cultured samples when measured after 72 hours.

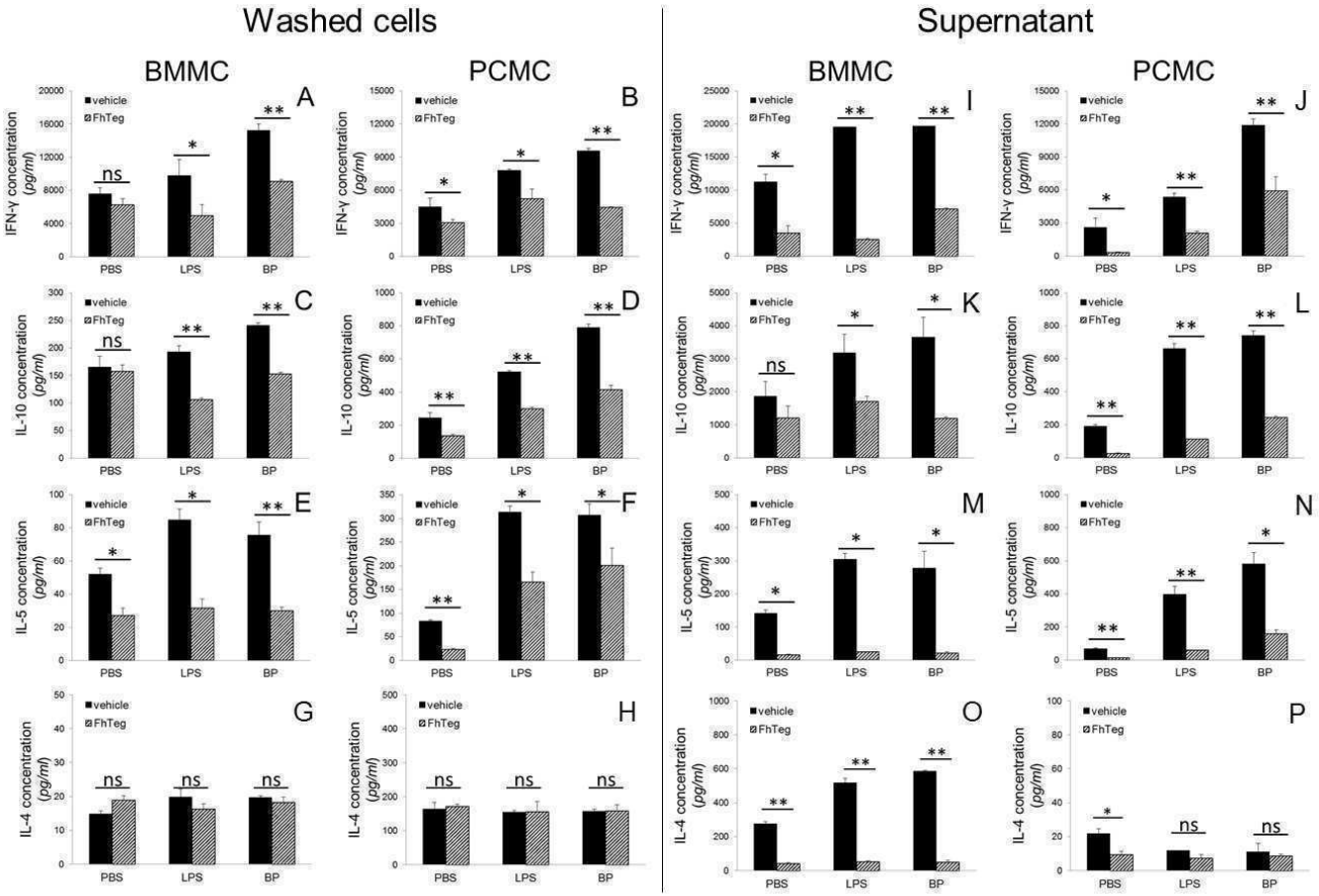

Figure 4.4. FhTeg suppressed the activation of T-cells by LPS or BP stimulated mast cells. BMMCs and PCMCs were stimulated with and without FhTeg (10 µg/ml) for 2.5 hours following with PBS, LPS (100 ng/ml) or BP (100 bacteria/cell) for 24 hours. anti-CD3 stimulated naïve $CD4^+$ cells were co-cultured with the washed cells (**A-H**) (1:1) and with the supernatants (**I-P**) for 72 hours. Supernatants were collected and tested for IFN-γ (**A-B, G-H**), IL-10 (**C-D, I-J**), IL-5 (**E-F, K-L**) and IL-4 (**G-H, O-P**) ELISA. Figures are representatives of three independent experiments (n=3) and presented as the mean ± SD of three replicate samples from the same experiment.. *, $p \leq 0.05$; **, $p \leq 0.01$ compared to LPS and BP group.

4.3.5 ICAM1 is important in mast cell - T-cell communication and the expression of this receptor is suppressed by FhTeg.

OX40L, CD40L, CD28 and ICAM1 are thought to have role in mast cell – T-cell communication (Nakae et al., 2006; Henz et al., 2001; Brill et al., 2004). BMMCs express low levels OX40L, CD40L and CD28 and this was not significantly enhanced in the presence of LPS and BP. In contrast ICAM1 was highly expressed on these cells and this was enhanced in the presence of LPS (Figure 4.5) or BP (data not shown). FhTeg did not enhance OX40L, CD40l, CD28 and ICAM1 expression on mast cells.

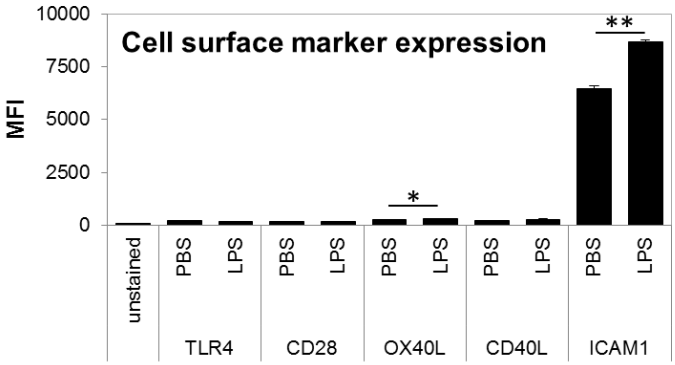

Figure 4.5. BMMCs express low levels of TLR4, CD28, OX40L and CD40L but high level of ICAM1. BMMCs were stimulated with PBS of LPS (100 ng/ml) for 24 h and cell surface expression of TLR4, CD28, OX40L, CD40L and ICAM1 was measured by two colour (double stained with FcεRI) flow cytometry. Figure is representative of three independent experiments (n=3). *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control group.

Since ICAM1 was highly expressed we stimulated mast cells with BP or LPS in the presence of FhTeg and demonstrated that FhTeg significantly inhibited ICAM1 expression on BMMCs and PCMCs (Figure 4.6; $p \leq 0.05$). We confirmed that ICAM1 is important in mast cells – $CD4^{+}$ T-cells communication by adding anti-

ICAM1 blocking antibody to activated mast cells half hour prior to washing and co-culturing with CD4$^+$ T-cells. Similar to FhTeg it caused significant decrease in IFN-γ, IL-10 and IL-5 secretion (Figure 4.7).

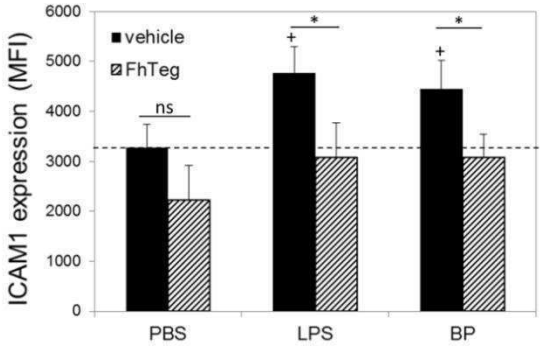

Figure 4.6. FhTeg suppress the up-regulation of ICAM1 expression on LPS/BP stimulated mast cells. PCMCs were stimulated with and without FhTeg (10 µg/ml) for 2.5 hours following activation with PBS, LPS (100 ng/ml) or BP (100 bacteria/cell) for 24 hours. ICAM1 expression was measured by Flow cytometry. Data are presented as the mean ± SD of three independent experiments (n=3). *, $p \leq 0.05$; compared to control group.

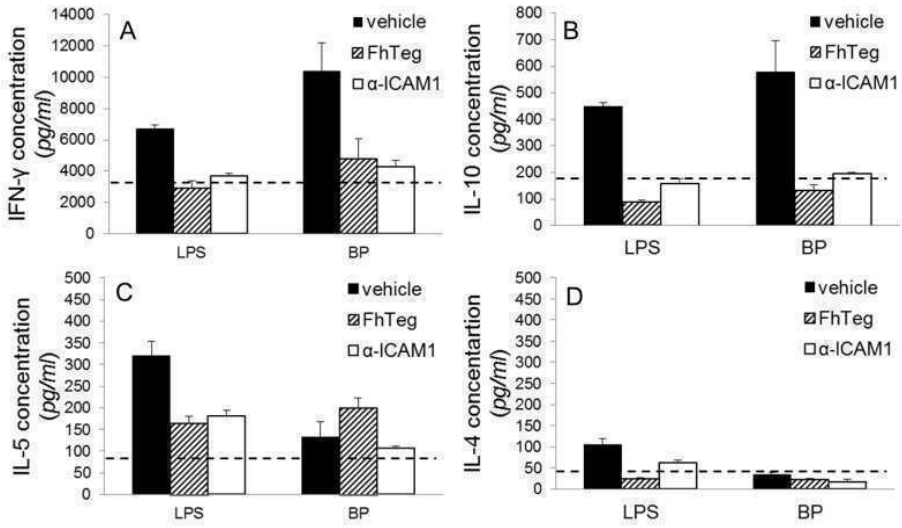

Figure 4.7. Effect of FhTeg on TLR stimulated mast cells can be mimicked by anti-ICAM1 antibody when mast cells are co-cultured with anti-CD3 stimulated naïve CD4$^+$ cells. PCMCs were stimulated with and without FhTeg (10 µg/ml) for 2.5 hours following with PBS, LPS (100

ng/ml) or BP (100 bacteria/cell) for 24 hours. anti-ICAM1 antibody (1 μg/ml) was added to mast cells 30 minutes before co-culturing. Then, washed cells were co-cultured with anti-CD3 stimulated naïve $CD4^+$ cells (1:1) for 72 hours. Supernatants were tested for IFN-γ (**A**), IL-10 (**B**), IL-5 (**C**) and IL-4 (**D**) ELISA. Figures are representatives of three independent experiments and presented as the mean ± SD of three replicate samples from the same experiment.

4.3.6 FhTeg suppresses phospho-ERK and NF-κB p65 expression in BP and LPS stimulated mast cells.

It was previously shown that FhTeg suppresses the activation of NF-κB in LPS stimulated dendritic cells (Hamilton et al., 2009) and this was examined in mast cells. Firstly we confirmed that BP can induce p-NF-κB p65 expression (at 15 and 30 min) in BMMCs (Figure 4.8A, C). FhTeg suppressed the activation of NF-κB in LPS and BP stimulated mast cells (Figure 4.8A, C) and this was confirmed in PCMCs at 15 and 30 minutes (Figure 4.8D, F).

To test if this effect was specific to NF-κB, the phosphorylation of ERK, JNK and p38 was measured. BP significantly enhanced phospho-ERK but not phospho-JNK and p38. We also demonstrated that FhTeg suppressed the activation of phospho-ERK in LPS and BP stimulated BMMCs at 5, 30 and 60 minutes (Figure 4.8A, B). These results were confirmed in PCMCs at 15 and 30 minutes (Figure 4.8D, E).

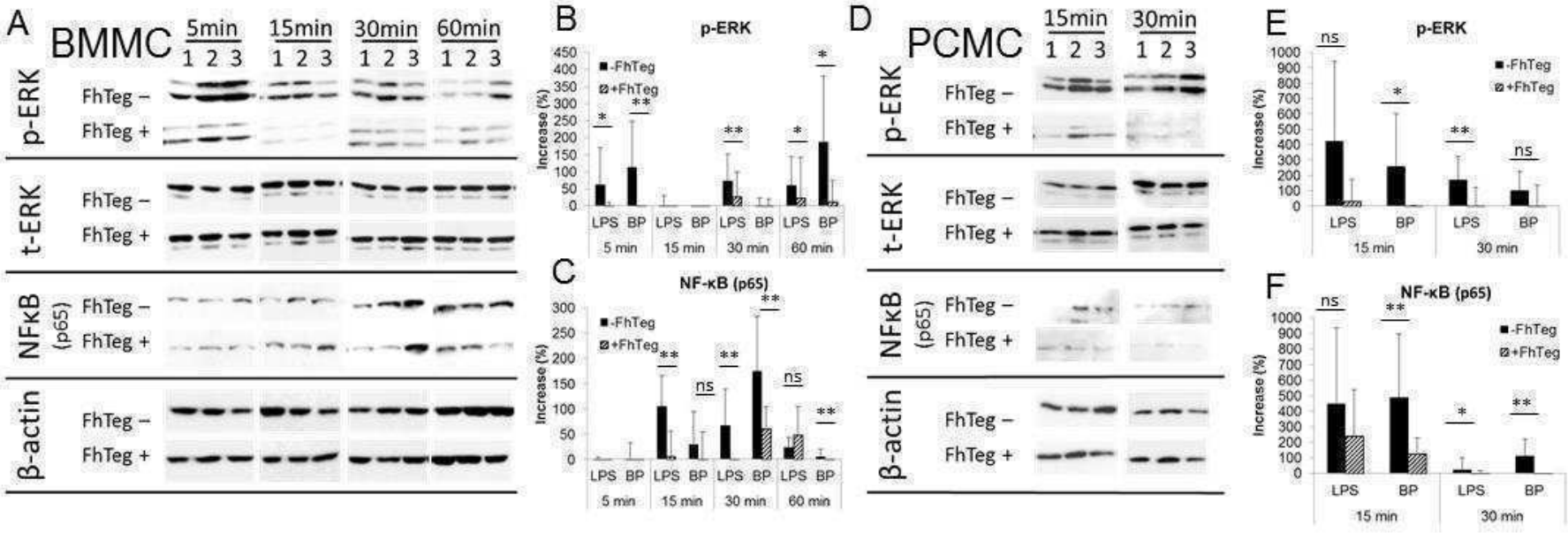

Figure 4.8. BP and LPS stimulated ERK and NF-κB expression in BMMCs and PCMCs was suppressed by FhTeg. PCMCs were stimulated with and without FhTeg (10 µg/ml) for 2.5 hours following with PBS, LPS (100 ng/ml) or BP (100 bacteria/cell) for 24 hours. Protein samples were analysed by western blot for phosphorylated-ERK (p-ERK), total-ERK (t-ERK), p-NF-κB p65 and β-actin expression (**A, D**). Values of p-ERK and p-NF-κB p65 were normalised to t-ERK and β-actin and are expressed in arbitrary units as fold increases over PBS control (**B-C, E-F**). Densitometry data are presented as the mean ± SD of three independent experiments (n=3). *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control group.

4.3.7 FhTeg suppressive effect is not specific to NF-κB p65 expression as it inhibits MAPK activation in dendritic cells.

Since we could not test if FhTeg has the same inhibitory effect on other members of the MAPK pathway (p38, JNK) we decided to involve dendritic cells in our study. Firstly we confirmed that FhTeg inhibitory effect is not limited to NF-κB we stimulated dendritic cells with LPS for 5, 15, 30, 60 min in the presence and absence of FhTeg. LPS can induce phospho-ERK, -JNK and -p38 expression in dendritic cells. We demonstrated that FhTeg suppressed the activation of these molecules. Phospho-ERK and phospho-p38 protein expression was suppressed in all time points (5, 15 and 30 min, $p \leq 0.01$- $p \leq 0.05$), although it was not significant after 60 minutes (Figure 4.9A-C), while JNK was only activated at 30 min and it could be inhibited by FhTeg ($p \leq 0.05$) (Figure 4.9A, D).

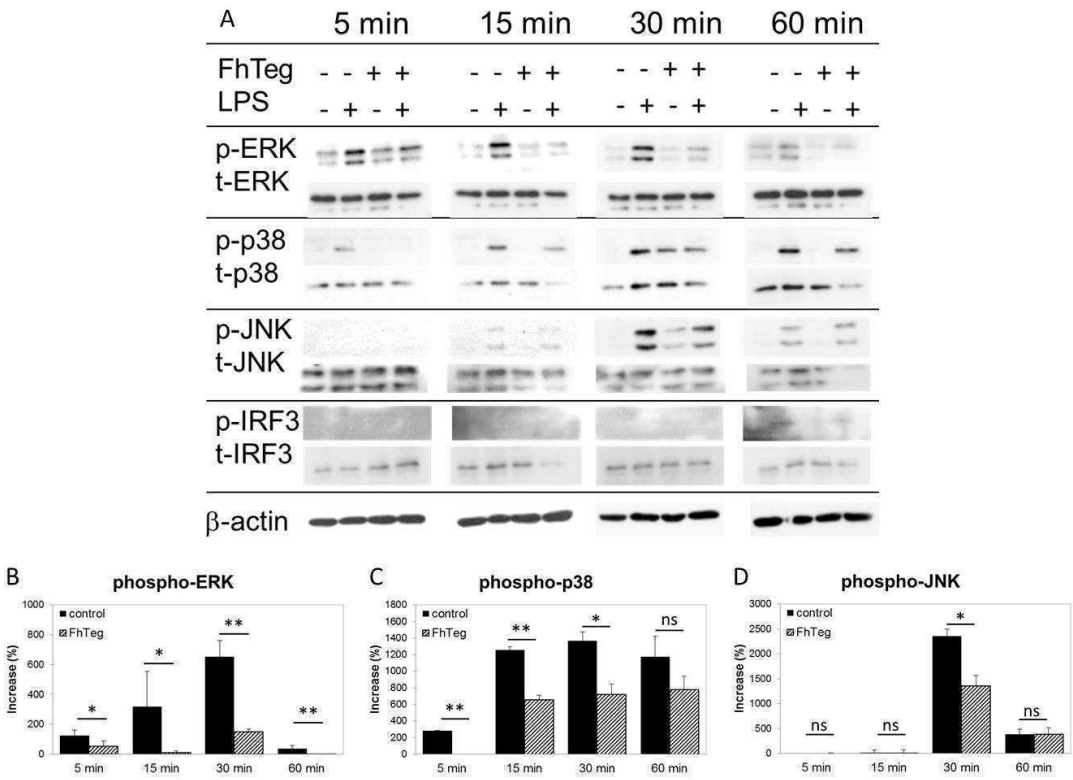

Figure 4.9. FhTeg suppressed the phosphorylation of ERK, p38 and JNK in LPS stimulated dendritic cells. BMDCs were stimulated with and without FhTeg (10 µg/ml) for 2.5 hours prior to stimulation with PBS or LPS (100 ng/ml) for 5, 15, 30, 60 min. Protein samples were analysed by western blot for phosphorylated-ERK (p-ERK), total-ERK (t-ERK), p-38, t-38, p-JNK, t-JNK, p-IRF, t-IRF and β-actin expression (**A**). Values of phosphorylated proteins were normalised to total target proteins and β-actin and are expressed in arbitrary units as percentage increases over PBS control value (**B-D**). Densitometry data are presented as the mean ± SD of three independent experiments (n=3). *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control group.

We included IRF-3 in our analysis as IRF-3 is the central protein of the alternative TLR4 signalling pathway. IRF-3 was only slightly activated after 60 min in LPS stimulated dendritic cells (Figure 4.9) and FhTeg did not suppress this activation (even in later time points after 2 and 4 h; data not shown).

4.3.8 The primers for qPCR analysis of TLR4 signalling showed adequate specificity and efficiency in traditional and qPCR analysis.

As both NF-κB and MAPK pathway is suppressed by FhTeg we hypothesize that this antigen targets signalling molecules further up the TLR4 signalling pathway or FhTeg may induce negative regulators of the TLR pathway. Based on the western blot results we decided to use LPS activated dendritic cells as a model system to investigate FhTeg's effect on TLR4 signalling and its inhibitors with qPCR before mast cell experiments. We chose 3 different genes as controls to use in qPCR analysis. In the literature the most intensively used control is β-actin, although studies suggest that it can only be used with some consideration as upon activation the RNA level may vary (Watson et al., 2007). The other two controls were Gusb (Glucuronidase) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), other housekeeping genes. As targets we chose 4 proteins from different level of the TLR4 signalling (TLR4, MyD88, TRAF6 and RelA) and 7 inhibitors (SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, PIAS1, PIAS3 and PIAS4).

Primers were designed for qPCR analysis with product size lower than 250 bp and melting temperature around 60°C. Specificity and efficiency were tested with conventional PCR and with qPCR. All tested primers gave one single bend PCR products when run on agarose gel without primer dimers and gave one peak after melting curves analysis. Efficiency was calculated from Ct values of solution series for every primer according to the equation: $E=10^{(-1/slope)}$. All efficiency values were in the range of 1.8-2.2 so primers were used in further analysis (Figure 4.10).

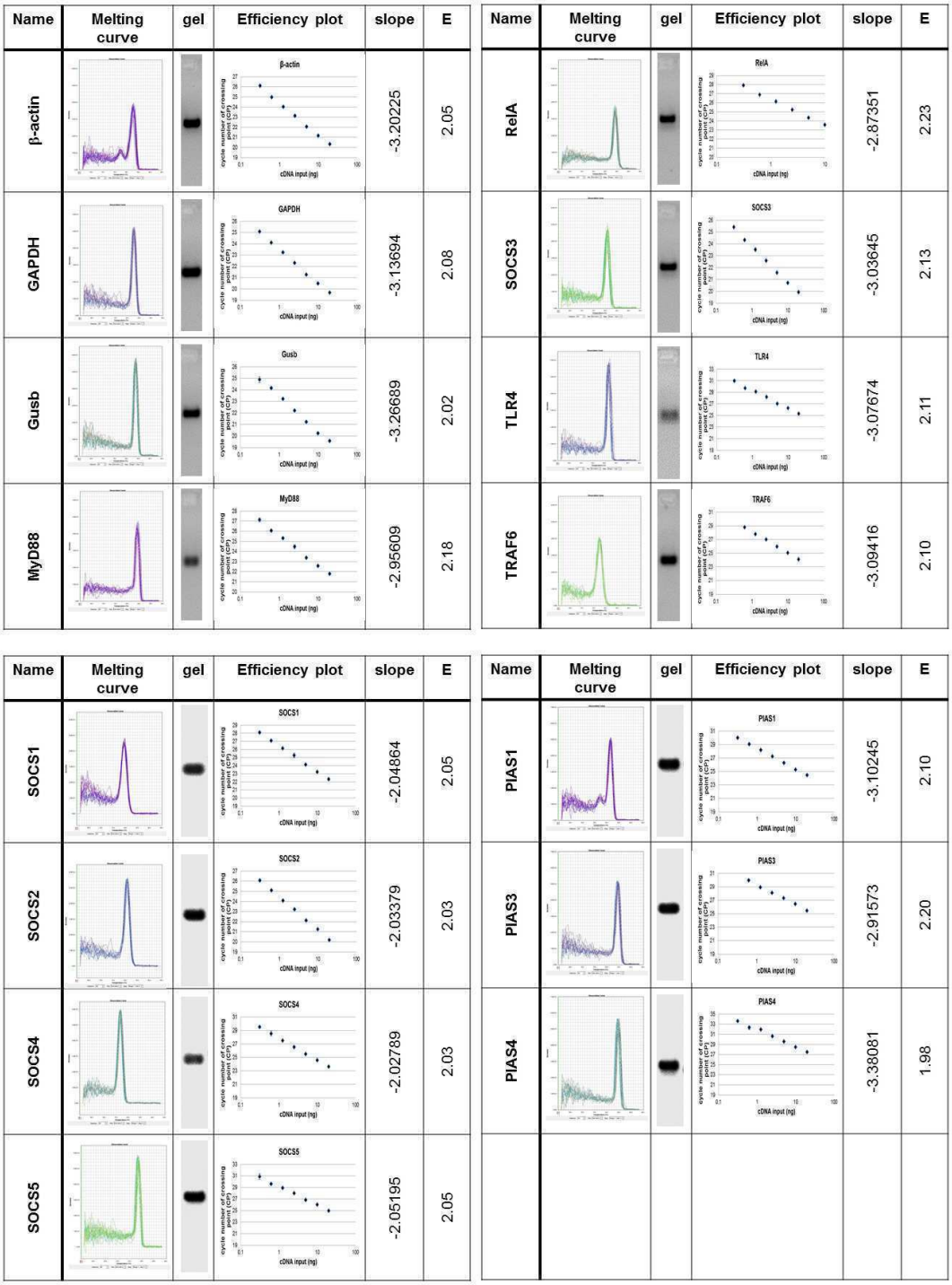

Figure 4.10. Determination of real-time PCR specificity and efficiencies of reference and target genes. Primers were tested in conventional PCR reaction using 20 ng cDNA templates and were excluded from latter experiments if they gave more than one single band when PCR products were run on 1% agarose gel or if they form primer dimers. Melting curves analysis was performed to check product size and homogeneity. Ct versus cDNA (reverse transcripted total RNA) concentration input

were plotted to calculate the slope (mean ± SD, n=3). The corresponding real-time PCR efficiencies were calculated according to the equation: $E = 10^{[-1/slope]}$.

4.3.9 FhTeg up-regulates the transcription and expression SOCS3.

To test the effect of FhTeg on members of the TLR4 signalling pathways BMDCs were treated with FhTeg prior to LPS stimulation. As we did not know the optimal time point when the transcription is highly up- or down-regulated (that can be different for the tested genes) we tested 6 incubation times: 5, 15, 30, 60 minutes and 6, 18 hours.

During analysis we tested the integrity of the used control genes. Within the first hour all three control genes showed the same pattern within the samples, although in the last two time points (at 6 and 18 hours) β-actin and Gusb expression seemed to change upon LPS stimulation. Later we used GAPDH as control by latter time points in further analysis of dendritic cells as it showed stable expression in all time points.

First we tested the effect of FhTeg on 4 members of the TLR4 signalling pathway. TLR4 as the main receptor for LPS stimulation was one of our target. Interestingly TLR4 RNA transcription was up-regulated in FhTeg treated samples after 1 hour (Figure 4.11A). MyD88 was slightly down regulated by FhTeg in the first 30 minutes and although it was up-regulated later by LPS, this up-regulation was supressed by FhTeg, but only at 1 hour (Figure 4.11B). TRAF6 was up-regulated by both FhTeg and LPS but only in later time points and there was no difference between LPS and LPS - FhTeg treated samples (Figure 4.11C). RelA (Nf-κB) is

known to be regulated on protein level and not on transcription level, so it was used as a negative control. Interestingly RelA was slightly up-regulated by FhTeg even in early time points and by LPS later after 6 hours (Figure 4.11D) although this transcription factor is known to be regulated on protein level by phosphorylation instead of by transcription/expression.

We also tested some possible inhibitors of the TLR4 signalling pathway. The expression of suppressor of cytokine signalling (SOCS)2, SOCS4, protein inhibitor of activated STAT (PIAS)1 and PIAS4 did not change after FhTeg and LPS treatment so they seem to not play important role in FhTeg inhibition. SOCS1 (Figure 4.11E) and PIAS3 (Figure 4.11G) was up-regulated only by LPS after 6 and 18 h with no significant difference between LPS and LPS/FhTeg treated samples and FhTeg had no effect on this up-regulation. There was no increase in the transcription level of SOCS5 (Figure 4.11F). The most promising results came from the SOCS3 analysis as it was highly up-regulated by FhTeg (10 fold after 5 min) and this up-regulation was even higher after LPS stimulation (90 fold after 60 min) (Figure 4.11H).

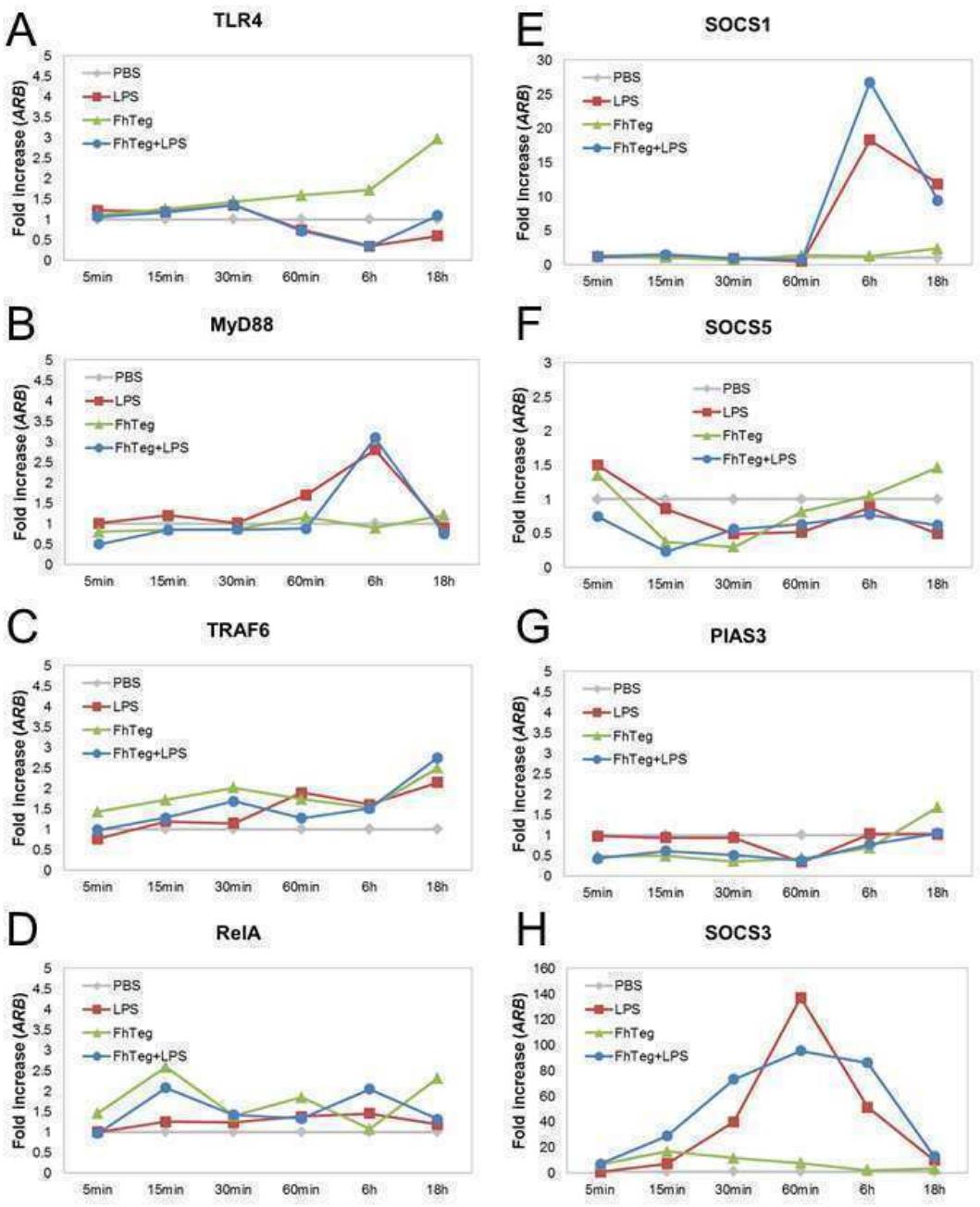

Figure 4.11. Time dependence of the expression of 8 target genes. BMDCs were stimulated with and without FhTeg (10 µg/ml) for 2.5 hours prior to stimulation with PBS or LPS (100 ng/ml) for 5, 15, 30, 60 min, 6h or 18h. Total RNA was extracted and after reverse transcription cDNA was analysed with qPCR for RelA, TLR4, TRAF6, MyD88, SOCS1, SOCS3, SOCS5 and PIAS3. RNA expression was normalized to GAPDH control gene expression and showed relative to PBS control. Figures are representative of two independent experiments.

4.3.10 SOCS3 transcription and expression is up-regulated by FhTeg.

As SOCS3 was up-regulated by FhTeg in early time points and it is known to be one of the most important inhibitory molecules of the TLR4 signalling pathway, we confirmed the qPCR results with western blot analysis. We repeated the FhTeg/LPS treatment experiment and extracted the proteins in the first 4 time points (5, 15, 30, 60 min), as FhTeg seems to act in early stage of LPS stimulation (Figure 4.12). SOCS3 up-regulation nicely correlated in RNA and protein levels at different time points (Figure 4.13). After 15 min LPS treated samples exhibited increased SOCS3 expression although in FhTeg and FhTeg/LPS treated samples SOCS3 up-regulation was still higher (Figure 4.13). At 30 and 60 minutes both LPS and LPS/FhTeg treated samples showed increased SOCS3 RNA and protein levels (Figure 4.11H, Figure 4.12, Figure 4.13)

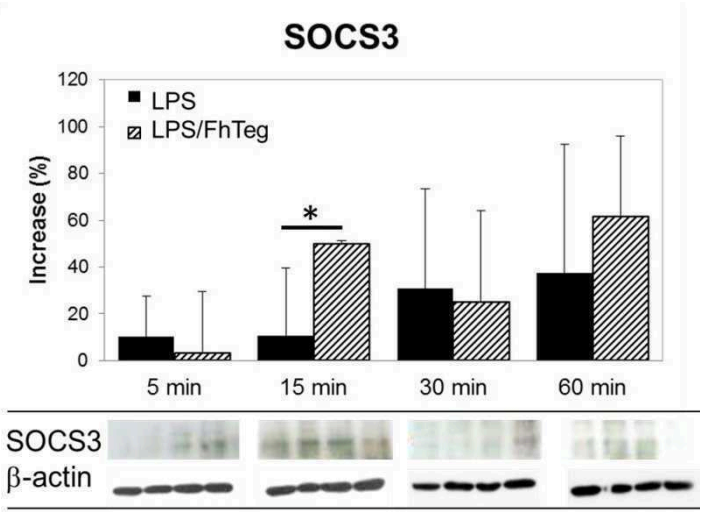

Figure 4.12. SOCS3 up-regulation was confirmed on protein level. BMDCs were stimulated with and without FhTeg (10 µg/ml) for 2.5 hours prior to stimulation with PBS or LPS (100 ng/ml) for 5, 15, 30, 60 min. Protein samples were analysed by western blot for SOCS3 and β-actin expression (**A**). Values of proteins were normalised to β-actin and are expressed in arbitrary units as percentage increases over PBS control value (**B-D**). Densitometry data are presented as the mean ± SD of three independent experiments (n=3). *$p \leq 0.05$ when compared to LPS control.

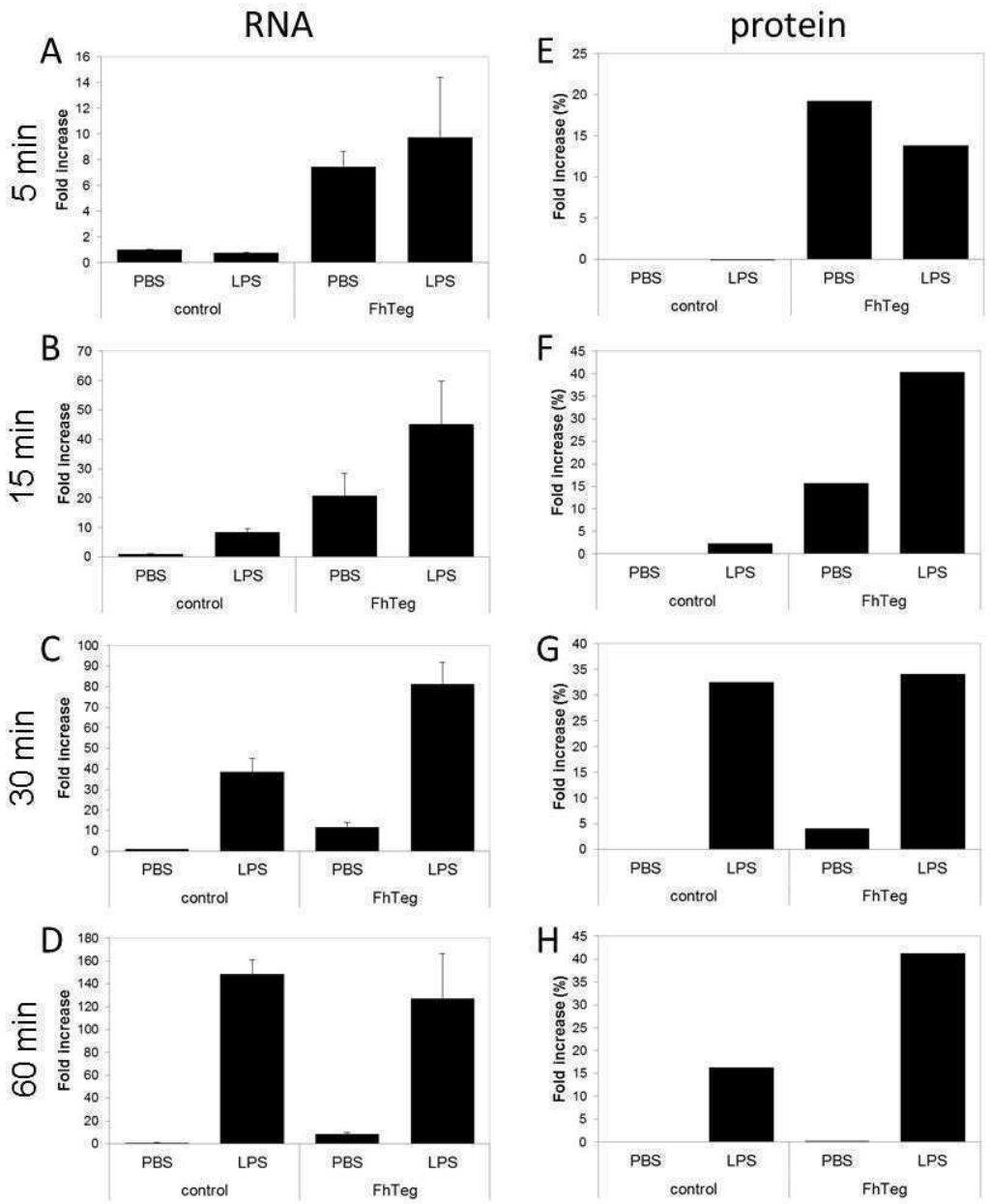

Figure 4.13. Pattern of SOCS3 expression was similar on RNA and protein level. BMDCs were stimulated with and without FhTeg (10 µg/ml) for 2.5 hours prior to stimulation with PBS or LPS (100 ng/ml) for 5, 15, 30 and 60 min. Total RNA and proteins were extracted. After the reverse transcription of RNA cDNA was analysed with qPCR for SOCS3 expression. RNA expression was normalized to GAPDH control gene expression and showed relative to PBS control (**A-D**). RNA data are presented as the mean ± SD of three independent experiments (n=3). Protein samples were analysed by western blot for SOCS3 and β-actin expression. Values of proteins were normalised to β-actin and are expressed in arbitrary units as percentage increases over PBS control value (**E-H**). Figures are representatives of three independent experiments.

4.3.11 SOCS3 transcription and expression is up-regulated in mast cells as well by FhTeg.

We were interested in whether FhTeg has the same effect on SOCS3 transcription and expression in mast cells as in dendritic cells, so we repeated the qPCR analysis with BMMCs at 5, 15, 30 and 60 minutes as FhTeg seems to act in early stage of bacterial activation. SOCS3 RNA level was significantly higher at 5 and15 minutes after LPS stimulation and 5 minutes after BP stimulation in FhTeg treated BMMCs than in control cells (Figure 4.14A, B), although this up-regulation was much lower than was seen in dendritic cells. There is a reversal of FhTeg up-regulation at the 15 min time point in both LPS and BP experiment although the decrease in SOCS3 level is not significant.

We confirmed the qPCR results with western blot analysis, so we repeated the experiment and extracted the proteins. Similarly to qPCR results, in the early time points SOCS3 was up-regulated in FhTeg treated samples and it was followed by LPS and BP induced SOCS3 expression in latter time points (Figure 4.14C).

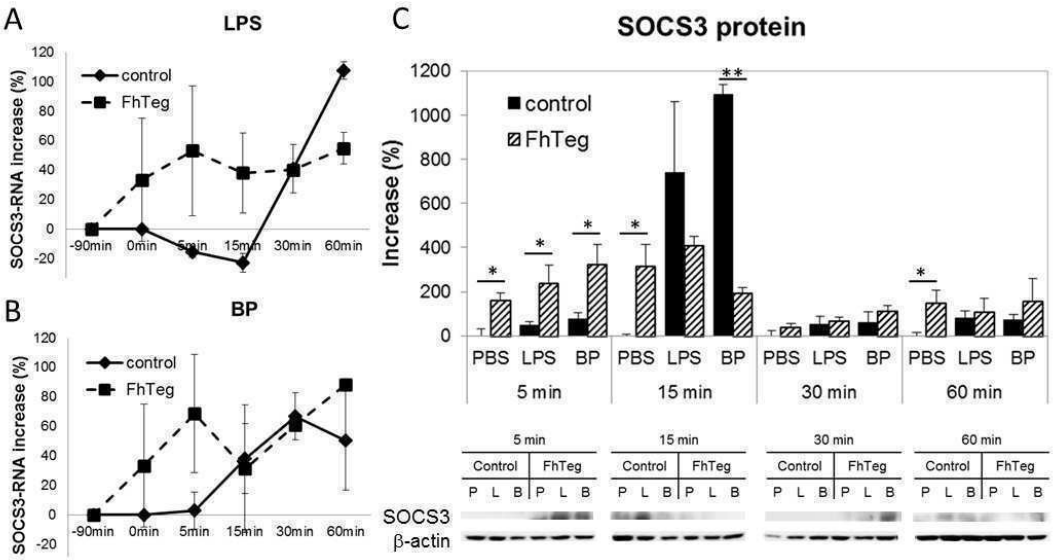

Figure 4.14. SOCS3 transcription and expression was up-regulated by FhTeg in mast cells. BMMCs were stimulated with and without FhTeg (10 µg/ml) for 2.5 hours prior to stimulation with PBS (P), LPS (L; 100 ng/ml) or BP (B; 100 bacteria/cell) for 5, 15, 30 and 60 min. Total RNA was extracted and after reverse transcription cDNA was analysed with qPCR for SOCS3 (**A-B**). RNA expression was normalized to GAPDH, β-actin and Gusb control gene expression and showed relative to PBS control using Pfaffl's method. Protein samples were analysed by western blot for SOCS3 and β-actin expression (**C**). Values of proteins were normalised to β-actin and are expressed in arbitrary units as percentage increases over PBS control value. Densitometry data are presented as the mean ± SD of three independent experiments (n=3). *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control group.

4.3.12 FhTeg suppresses cytokine induced cell surface marker up-regulation in dendritic cells.

In order to see if FhTeg has effect on other pathways than TLR4, we tried to develop a model system where we stimulated BMDCs with different cytokines (IL-6, IFN-γ or TNF-α). When we stimulated the cells with the individual cytokine we saw only nitrite secretion upon IFN-γ treatment and FhTeg did not inhibit rather it enhanced it in a dose dependent manner (data not shown). Cell surface expression of CD40, CD80 and CD86 were also measured (Figure 4.15). Although we got significant up-

regulation of CD40 by TNF-α ($p \leq 0.05$), FhTeg suppressed the cell surface expression of all markers in all groups ($p \leq 0.05$ to $p \leq 0.01$; Figure 4.15). We tried if the combination of IFN-γ and TNF-α induce dendritic cells stimulation and we found only nitrite secretion when the concentrations of the cytokines were 1000 times more than the recommended values (The ED_{50} values was determined by producer (Biolegend, Cambridge, UK) corresponding to a specific activity of 5-10 x 10^7 u/mg) and FhTeg had no effect on this up-regulation (data not shown).

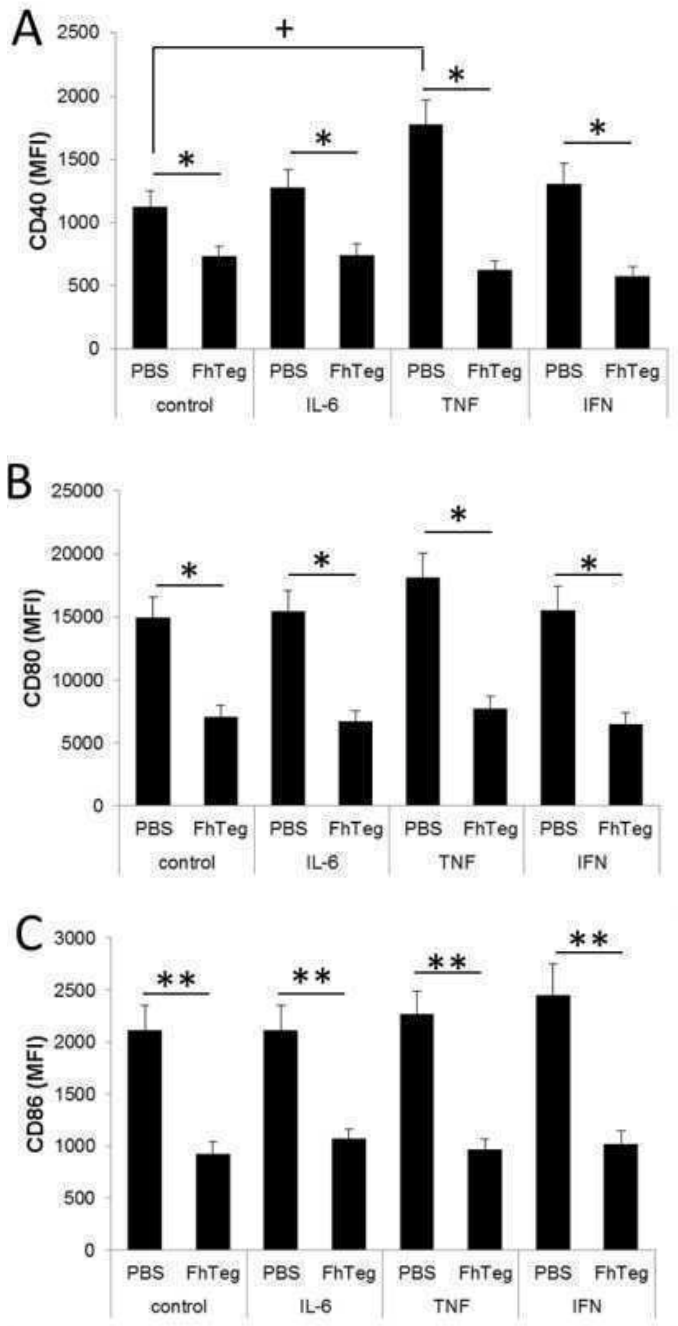

Figure 4.15. FhTeg suppressed cytokine induced cell surface marker up-regulation in dendritic cells. BMDCs were treated with FhTeg (10 μg/ml) 2.5 h before stimulation with IL-6 (50 pg/ml or as indicated), TNF-α (20 pg/ml or as indicated) or IFN-γ (500 pg/ml or as indicated) for 18 h. TNF-α, IL-12 and nitrite secretion and CD40, CD80 and CD86 expression was measured with commercial ELISAs, Griess reagent system or flow cytometry. Figures are representatives of three independent experiments (n=3). *, $p \leq 0.05$; **, $p \leq 0.01$ compared to PBS (non-FhTeg) group. $^{+}$, $p \leq 0.05$ compared to control PBS group

4.4 Discussion

In the previous chapter we showed that FhTeg does not induce Th2/Treg immune responses in mast cells and our hypothesis is that mast cells are recruited during *F. hepatica* infection to promote a Th1 environment where *F. hepatica* tegumental antigen switches off the activity of these cells to influence this process. Here we shed new light on the anti-inflammatory effects of helminth antigens on the Th1 pro-inflammatory properties of mast cells. FhTeg impairs mast cells ability to drive Th1 immune responses by inhibiting the release of key mediators such as, TNF-α, IL-6, IFN-γ and IL-10. It also suppresses the expression of ICAM1, a cell surface molecule that has an important role in mast cell/T-cell crosstalk stabilizing cell to cell interaction and has function in signal transduction (Brill et al., 2004). While studies have shown the importance of ICAM1 expression on T-cells in mast cell/T-cell communication here we show for the first time that ICAM1 expression on mast cells is also critical in this process. Our results suggest that in T-cell – mast cell communication both the mast cell derived cytokines and cell to cell contact are crucial to induce sufficient immune responses as we showed that the inhibition of cytokine secretion and cell surface molecule up-regulation by FhTeg both impaired mast cells ability to prime T-cells. This study supports previous findings demonstrating that FhTeg can suppress Th1 immunity *in vivo* by directly rendering innate immune cells such as dendritic cells hypo-responsive to TLR activation (Hamilton et al., 2009) a characteristic of helminth activated dendritic cells (MacDonald et al., 2002).

The suppression of cytokine secretion by FhTeg was STAT6 independent, this supports our previous finding that FhTeg injection induced increase of mast cell

number in the peritoneal cavity was independent of STAT6. STAT6 signalling pathway is important in driving Th2 immune responses and promoting Th2 cytokine secretion (Finkelman et al., 2004), supporting our theory that FhTeg does not play role in the development of Th2 immune responses from mast cells. It also correlates with a study on STAT6$^{-/-}$ mice where the immunity against *Taenia crassiceps* was shown to be STAT6 independent (Rodriguez-Sosa et al., 2002).

The suppression of Th1 immune response is critical for *F. hepatica* to survive within its host as immunity to *Fasciola* infection requires strong Th1 inflammatory responses (Moreau and Chauvin, 2010). IL-4$^{-/-}$ mice, which are predisposed towards a Th1 immune response, are less susceptible to *F. hepatica* infection with reduced liver damage compared to IFN-γR$^{-/-}$ mice that elicited strong Th2 responses and are highly susceptible to infection (O'Neill et al., 2000). Vaccine efficacy using *F. hepatica* excretory-secretory molecules or using recombinant *F. hepatica* secretory enzymes is associated with enhanced lymphocyte proliferation and IFN-γ expression (Mulcahy et al., 1999; Mulcahy et al., 2004).

We know that *F. hepatica* infection is sufficiently potent to impact upon bystander microbial infections (Brady et al., 1999; Flynn et al., 2009) where in humans *Fasciola* infection is associated with susceptibility to *Cryptosporidium* and in cattle to increased incidence of *Salmonella* and *Tuberculosis* (Claridge et al., 2012; Flynn et al., 2009). Mice co-infected with *Fasciola* exhibit delayed clearance of *Bordetella pertussis* from their lungs which is associated with a decrease in antigen specific Th1 immune responses (Brady et al., 1999). This was also observed in mice co-injected with *B. pertussis* and *Fasciola* antigens (O'Neill et al., 2000). Here we demonstrate

that FhTeg injected into mice suppresses *B. pertussis* induced TNF-α expression in mast cells, suggesting that mast cells may be targeted by helminth antigens during bystander infections. This could explain the failure of *F. hepatica* co-infected mice to clear *B. pertussis* infection since mast cells secrete pro-inflammatory mediators like TNF-α and IFN-γ that mediate host defence against *B. pertussis* (Mielcarek et al., 2001; Wierzbicki and Brzezinska-Blaszczyk, 2009). Similarly mast cells have a critical role to play during experimental autoimmune encephalitis (EAE) as mast cell knockout mice are protected from developing this disease (Piconese et al., 2011) and this could explain how *Fasciola* could attenuate EAE in mice (Walsh et al., 2009). Further investigation is required to determine the role of mast cells in these processes which would shed further light on the impact of helminths on Th1 mediated autoimmune diseases.

ERK and NF-κB are activated in mast cells after LPS and BP stimulation (Desai and Thurmond, 2011; Li et al., 2009) and here we found that FhTeg suppressed LPS induced NF-κB and MAPK pathway (ERK) activation in mast cells. It was shown in our laboratory that FhTeg suppresses LPS induced NF-κB activation in dendritic cells (Hamilton et al., 2009) and now we demonstrate that the MAPK pathway (ERK, JNK and p38) is also targeted by FhTeg in these cells. NF-κB and MAPKs are important signalling molecules in the expression of ICAM1 (Tsang et al., 2005) and secretion of pro-inflammatory cytokines. Our findings correlate with other studies in dendritic cells as *S. mansoni* egg antigens reduce LPS-stimulated phosphorylation of p38, JNK and ERK in murine dendritic cells (Kane et al., 2004). *S. haematobium* and *Ascaris lumbricuides* infection was shown to suppress the LPS induced p38 phosphorylation, but not ERK activation in dendritic cells (Everts et al.,

2010a; van Riet et al., 2009). Studies demonstrate that NF-κB$^{-/-}$ mice infected with *Trichuris muris* show increased susceptibility to infection with impaired Th2/Treg immune responses and the phosphorylation of NF-κB and ERK supposed to be crucial in helminth induced Th2 immune responses (Everts et al., 2010b; Mason et al., 2004). Given that FhTeg does not induce, rather suppresses the activation of these proteins supports our previous finding that FhTeg does not drive or suppress Th2 immunity (Figure 3.2, Figure 3.4, Figure 3.5), rather it has role in the suppression of Th1-type responses. As both Nf-κB and MAPK pathways are suppressed by FhTeg, the antigen should have its target further up in the TLR4 signalling pathway.

We decided to use dendritic cells to screen the possible targets of FhTeg by qPCR method. There was not much difference in the transcription level of the members of the TLR4 signalling pathway in early time points, suggesting that the suppression of immune response by FhTeg is not directly on the transcription of these proteins. Although in latter time points FhTeg alter the transcription of TLR4 and TRAF6, what correlates with a study where the expression of TLR4 is up-regulated in immune cells in mice with *Heligmosomoides polygyrus* infection (Ince et al., 2006). Innate immune cells have to respond quickly to bacterial infections; consequently the TLR pathway must be activated immediately after TLR4 activation. It takes time for the cells to make the proteins, so proteins downstream to TLR4 are activated on protein level by phosphorylation, ubiquitination or protein-protein interaction (Mogensen, 2009).

FhTeg induces negative regulators of the TLR pathway since FhTeg enhanced expression of SOCS3 in mast cells and in dendritic cells. SOCS3 is a member of a family of molecules that are one of the main inhibitory groups playing an important role in auto-regulation of pathogen induced inflammatory responses (Yoshimura et al., 2012). Previous studies showed increased SOCS3 RNA levels in blood cells from children with helminth (*Ascaris* sp, *Trichuris* sp. or *Schistosoma haematobium*) infection. They suggested that the higher level of SOCS3 might be responsible for the increased severity of bystander *Plasmodium* infection that requires Th1 immune responses (Hartgers et al., 2009). It is likely that *F. hepatica* has the same mechanism to suppress Th1 immune responses during bystander infection but further studies are required.

FhTeg treated dendritic cells did not alter the expression of SOCS1 or SOCS2 which contrast studies with other helminths since during *Brugia malay* infection SOCS1 up-regulation is important in the alternative activation of macrophages (Everts et al., 2010b, Whyte et al., 2011), and SOCS2 was shown to be essential in the regulation of Th1 immune responses in dendritic cells (Dalpke et al., 2001; Posselt et al., 2011). Since the main inhibitors of TLR4 pathway are induced by the Janus kinase/signal transducer and activators of transcription (JAK/STAT) pathway the down-regulation of PIAS3 can result in increased transcription of SOCS3 and the inhibition of TLR4 pathway. SOCS1 and PIAS3 were up-regulated by LPS in latter time points what is in conjunction with their auto-regulatory effect in TLR signalling during inflammation (Mogensen, 2009; Yagil et al., 2010).

The best way to prove that SOCS3 is responsible for the inhibition would be to block SOCS3 and see if FhTeg still keeps its effect. SOCS3-deficient mice die during embryotic development as a result of placental defect (Takahashi et al., 2006) and using zoledronic acid (ZA) (Scheller et al., 2011)., inhibitor of SOCS3, is not possible in our model system, as ZA impaired primary cell's ability to be activated by bacterial ligands (Appendix C) (Bringmann et al., 2007; Fiore et al., 2007; Su et al., 2010). Other method to suppress gene expression would be to use small interfering (si)-RNA; it has also some difficulties in primery cells as it may influence cell development. Involving cell lines in this study could solve this problem, considering the differences between mast cells in their inflammatory responses based on their origin (Hochdorfer et al., 2011; Matsushima et al., 2004; McCurdy et al., 2001; Supajatura et al., 2002).

It is likely that FhTeg do not only target the TLR signalling pathways but has effect on other processes in immune cells. The JAK/STAT pathway is the principal signalling mechanism for a wide array of cytokines (like IL-6 or IFN-γ) and growth factors (like TNF-α). The activation of this pathway can cause cell proliferation, differentiation, migration and apoptosis (Rawlings et al., 2004). This pathway can cross-talk with the TLR signalling pathway and cytokines were shown to alter TLR responses in macrophages *in vitro* (Hu et al., 2007) and in mice *in vivo* (Greenhill et al., 2011). In our study, although we did not manage to induce cytokine secretion from dendritic cells by cytokine treatment, we showed that FhTeg suppresses TNF-α induced cell surface marker up-regulation.

Mast cells were shown to contribute to the immune response against *Strongloides ratti*, *Trichinella spiralis* and *Nippostrongylus brasiliensis* in rats and mice models (Abe and Nawa, 1988; Marshall, 2004; McDermott et al., 2003). The fecundity of *Heligneosomoides polygyrus* was higher in mast cell-deficient mice than in the wild type controls (Hashimoto et al., 2009). These studies indicate that mast cell phenotype is important in driving Th2 immunity that is essential for clearing gut parasites (Pennock and Grencis, 2006). We previously showed that mast cell number increases markedly in the peritoneal cavity and liver of *F. hepatica* infected and FhTeg-injected mice, and that the interaction of FhTeg with mast cells does not promote Th2 immune response or suppress IgE mediated mast cell activation. Given that the Th1 immune responses are important in immune protection against *Fasciola* infection it is possible that in the early stages of infection mast cells are required to contribute to the overall Th1 milieu and this is inhibited by the release of FhTeg by *Fasciola* into the micro-environment. This is supported by FhTegs interaction with mast cells and dendritic cells as it directly inhibits their ability to drive Th1 immune responses.

In summary, FhTeg impairs mast cell ability to drive Th1 immune responses which sheds new light on how helminths leave population susceptible to bystander infections while simultaneously protecting them against Th1-mediated autoimmune disorders. Further studies will help us to determine how FhTeg enhances SOCS3 expression which may lead to potential identification of novel means by which helminths modulate innate immune cells. Since mast cells are involved in Th1 immune disorders like autoimmune diseases, inflammatory bowel disease or Crohn's disease where TNF-α secretion from these cells is critical to disease pathology

(Sutherland et al., 2011; Weller et al., 2011), it is therefore possible that the protective effect of helminths against autoimmunity may also be the result of its molecules direct blocking of pro-inflammatory cytokines from mast cells and dendritic cells. This finding might lead to the development of a therapeutic inhibitor for pathogenic cell phenotypes.

5 Chapter 5 – C-type lectin receptors of mast cells are up-regulated by *Fasciola hepatica* infection, although blocking of these C-type lectins does not reverse the inhibitory effect of FhTeg

5.1 Introduction

CLRs are important members of the pattern recognition receptor family and are important in the recognition of pathogen-associated molecular patterns (PAMPs). They can modulate TLR signalling or can directly induce gene expression. CLRs are expressed by most cell types including macrophages and dendritic cell (Geijtenbeek and Grinhuis, 2009; Ip et al., 2009). They have conserved carbohydrate-recognition domains (CRD) and they can bind carbohydrates in a calcium dependent manner. The CRD is a compact structural module which determines the specificity of the receptor (Geijtenbeek and Grinhuis, 2009).

CLRs are activated by glycans and helminths express glycans, glycoproteins and glycolipids on their surfaces and these molecules were shown to have key role in the induction of Th2-type (van Die and Cummings, 2006) and anti-inflammatory immune responses (Harn et al., 2009; van Die and Cumings, 2010; Thomas and Harn, 2004). Glycans from helminths are important in the induction of alternatively activated (M2) macrophages and dendritic cell's maturation and activation (Thomas and Harn, 2004). *Fasciola hepatica* expresses wide range of glycans and glycoproteins on its tegumental surface (Wilson et al 2011, Wuhrer and Geyer 2005) with possible immunomodulatory effects.

It was shown that *F. hepatica* antigens failed to induce alternative activation of macrophages in the presence of mannan, laminarin or the blocking antibodies of mannose receptor (MR) or Dectin1, suggesting that these CLRs are important in the recognition of *F. hepatica* and their products by immune cells (Guasconi et al 2011). *In vivo*, during *F. hepatica* infection the expression levels of macrophage galactose lectin (MGL) and MR are up-regulated on macrophages (Adams et al., unpublished data) suggesting that they are involved in the immunomodulatory effect of the fluke.

Immune cells express DC-SIGN (homologue in mouse: SignR1), MR and MGL on their surface and helminth glycans can bind to these CLRs and this can lead to the suppression of LPS induced activation (Kuijk et al., 2010; Kuijk et al., 2012; van Liempt et al., 2006). Mast cells express an identical CLR, mast cell function associated antigen (MAFA) (Guthmann et al., 1995a; Guthmann et al., 1995b) and the activation of MAFA leads to the inhibition of FcεRI-induced activation of mast cells (Abramson et al., 2002). This protein was subsequently found on mouse natural killer cells and human basophils (Katz, 2002). Mincle and Dectin 1 were also shown to be expressed by mast cells in low levels (Ribbing et al., 2011). Upon mast cell activation by the fungal antigen zymosan the Dectin1 level increases and zymosan induced leukotriene production seems to be Dectin 1 dependent (Yang and Marshall, 2009).

Schistosoma mansoni antigens were shown to bind to MR and Dectin1, and the blocking of these CLRs results in inhibition of immunomodulatory effect of the parasite (Guasconi et al., 2011) and decreased level of IL-4 secretion (Paveley et al., 2011). Carbohydrate components of *Trichinella spiralis* larvae were shown to

activate macrophages leading to pro-inflammatory cytokine expression (Gruden-Movsesijan et al., 2004). Helminth glycans have potential to interact with CLRs on dendritic cells. *Schistosoma mansoni* antigens were shown to bind to MGL (van Vliet et al., 2005), Dectin1 and DC-SIGN (van Kooyk et al., 2008) on dendritic cells and interfere with their APC function and impair T-cell polarisation (Kuijk et al., 2010). Since FhTeg may suppress Th1 immune responses in dendritic cells (Hamilton et al., 2009) and mast cells, and SOCS3 is involved in this process, in this chapter we are investigating the role of CLRs in the immune modulation of dendritic cells and mast cells.

5.2 Experimental design

Since C-type lectin receptors (CLR) have an important role to play during helminth infection, in this chapter we investigated if C-type lectin receptors are involved in FhTeg mast cell interaction. Firstly, BALB/C mice were infected with *F. hepatica* metacercariae (2.2.4) and CLR expression on mast cells from the peritoneal cavity was examined with flow cytometry. Since FhTeg treatment can mimic *F. hepatica* infection results were confirmed with mast cells from FhTeg treated mice (2.2.4). Dendritic cells represent a minor population in the peritoneal cavity, so CLR expression of these cells was measured *in vitro*. BMMCs and the more mature PCMCs were also examined *in vitro* to determine if FhTeg treatment can induce CLR up-regulation on these cells (2.2.12) by flow cytometry (2.2.18).

Since MR and MGL were up-regulated by FhTeg *in vitro* and *in vivo*, we examined if these CLRs are involved in FhTeg – immune cell interaction. It was determined if blocking of MR or MGL can reverse the inhibition of bacterial ligand induced cytokine secretion by FhTeg using specific blocking antibodies prior to FhTeg treatment and LPS/BP stimulation (2.2.12). Blocking antibody treated, stimulated BMMCs were also co-cultured with naïve T-cells (2.2.19) to test if blocking of CLRs can reverse FhTeg's inhibition on T-cell activation. Results were confirmed in dendritic cells (2.2.11-12). Involvement of MR and MGL in FhTeg induced SOCS3 transcription was also examined in this model system (2.2.23-24).

5.3 Results

5.3.1 *Fasciola hepatica* infection or its FhTeg up-regulates C-type lectin receptor expression on mast cells *in vivo*.

It was previously shown that *F. hepatica* infection and treatment of mice with *F. hepatica* antigen enhanced the expression of MR and MGL on murine peritoneal macrophages (Adams et al., unpublished data). In this study we examine if *F. hepatica* infection has the same effect on other immune cells. We investigated if Dectin1, MGL, MR and SignR1 are expressed by mast cells and if there is any change in the expression of these four CLRs during *F. hepatica* infection. Dendritic cells represent only a minor population in the peritoneal cavity, so we could not examine the expression of these CLRs on dendritic cells *in vivo*. The mice were orally infected with *F. hepatica* metacercariae and after 2 weeks of infection peritoneal exudate cells were collected and cell surface expression of CLRs on mast cells were measured by flow cytometry. After infection MR (to 125.0 ± 33.9%, $p \leq 0.05$) and MGL (to 774.6 ± 222.1%, $p = 0.014$) but not Dectin1 ($p = 0.34$) or SignR1 ($p = 0.77$) was significantly upregulted (Figure 5.1).

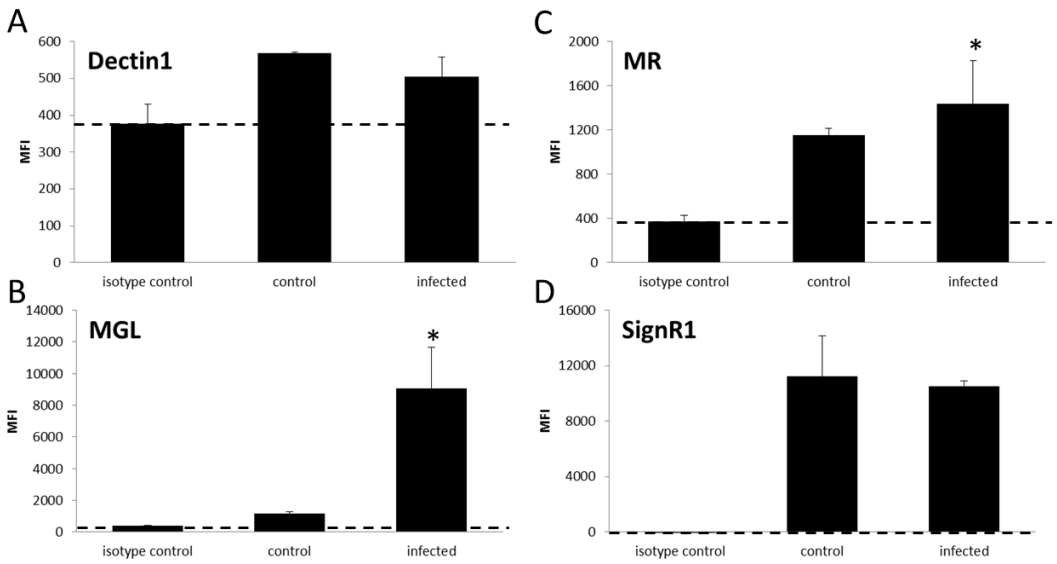

Figure 5.1. MGL and MR but not Dectin1 or SignR1 are up-regulated on peritoneal mast cells during *Fasciola hepatica* infection. BALB/C mice were orally infected with *Fasciola hepatica* metacercariae and after 2 weeks of infection peritoneal exudate were collected and Dectin1 (**A**), MGL (**B**), MR (**C**) and SignR1 (**D**) expression was measured by three colour (FcεRI, c-kit and CLRs) flow cytometry. Data are normalized to isotype controls and are presented as the mean ± SD of three independent experiments with 3-5 mice (n=13); *, $p \leq 0.05$; compared to control group.

Since the expression levels of MR and MGL, but not Dectin1 or SignR1 were up-regulated during *F. hepatica* infection we exclude the latter two from further experiments. Treatment of mice with FhTeg induces mast cells *in vivo* and here BALB/C mice were treated with FhTeg and CLR expression on mast cells was measured by three colour flow cytometry (c-kit, FcεRI and CLR). Mast cells from FhTeg treared mice exhibited significantly increased expression levels MGL (to 412.6 ± 77.1%, $p \leq 0.01$) (Figure 5.2A) although the increase of MR (to 138.5 ± 43.5%, $p = 0.21$) was not significant (Figure 5.2B).

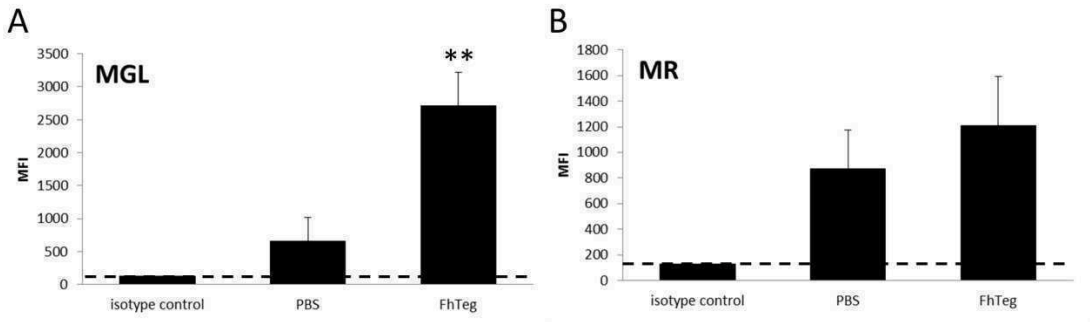

Figure 5.2. MGL but not MR was up-regulated on peritoneal mast cells after FhTeg injection. BALB/C mice were injected with FhTeg (10 μg) 5 times for 3 weeks After 3 weeks peritoneal exudate were collected and MGL (**A**) and MR (**B**) expression was measured by three colour flow cytometry (FcεRI, c-kit and CLR). Data are normalized to isotype controls and are presented as the mean ± SD of three independent experiments with three mice per group (n=9). *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control (PBS) group.

5.3.2 FhTeg up-regulates C-type lectin receptor expression on dendritic cells and mast cells *in vitro*.

To confirm our *in vivo* results, we wished to see if FhTeg can up-regulate CLR expression on dendritic cells and mast cells *in vitro*. First we confirmed by flow cytometry that MGL and MR are expressed by BMDCs, BMMCs and PCMCs. All examined cell populations expressed significant level of MR and MGL on their surface ($p \leq 0.01$; Figure 5.3). BMDCs, BMMCs and PCMCs were stimulated with FhTeg (10 μg/ml) for 24 hours and MGL and MR cell surface expression was measured by flow cytometry.

Expression level of MGL was down-regulated (to 67.45 ± 18.63% $p \leq 0.05$) and MR was up-regulated (to 131.37 ± 3.49% $p \leq 0.01$) in BMDCs after FhTeg treatment, (Figure 5.3A-B). In BMMCs the expression level of MR (to 160.7 ± 19.1%, $p \leq 0.05$) but not MGL (100.17 ± 23.27 $p = 0.99$) increased significantly after FhTeg

treatment compared to control group (Figure 5.3C-D). PCMCs showed higher expression of the tested CLRs and were more responsive when compared to the less mature BMMCs. In PCMCs all two tested CLRs (MGL: 155.6 ± 25.2%, $p \leq 0.01$; and MR: 289.2 ± 37.7%, $p \leq 0.05$) are significantly up-regulated by FhTeg (Figure 5.3E-F).

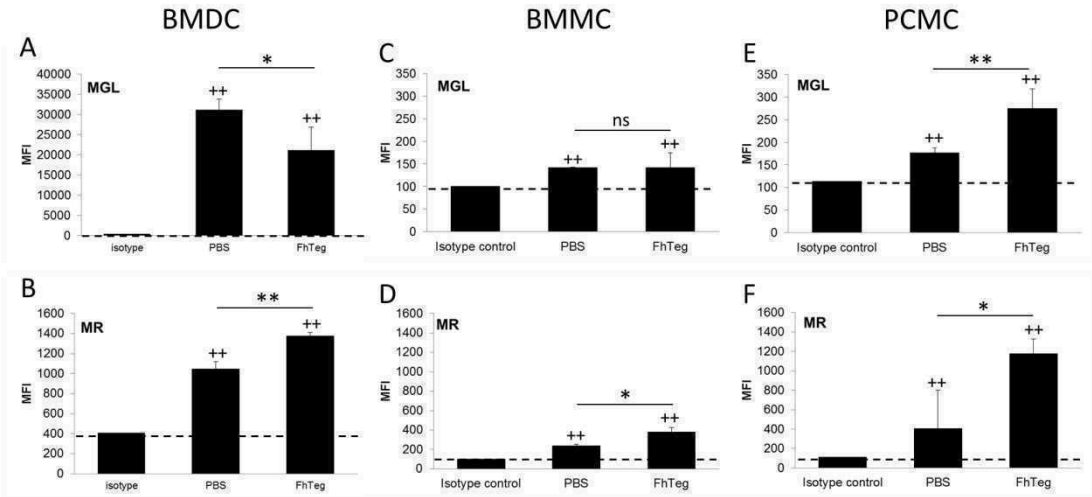

Figure 5.3. MGL and MR were up-regulated by FhTeg *in vitro*. BMDCS, BMMCs and PCMCs were treated with FhTeg (10 µg/ml) for 24 hours and MGL (**A, C, E**), and MR (**B, D, F**) membrane expression was measured by two colour (FcεRI/CD11c and CLR) flow cytometry. Data are normalized to isotype controls and presented as the mean ± SD of three independent experiments with triplicates (n=9); $^{++}$, $p \leq 0.01$ compared to isotype control *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control (PBS) group.

5.3.3 Blocking of MR and MGL does not reverse FhTeg's inhibition of LPS activated dendritic cells and mast cells.

MR and MGL are up-regulated *in vivo* during *F. hepatica* infection and after FhTeg treatment and there are commercially available neutralizing antibodies for these two CLRs. To see if the blocking of MR or MGL reverses the inhibitory effect of FhTeg on cytokine secretion of LPS stimulated dendritic cells BMDCs were treated with

anti-MR or anti-MGL blocking antibodies 30 min before FhTeg/LPS stimulation. Blocking antibody treatment had no effect on LPS stimulation and there was no significant difference between LPS/FhTeg treated groups either. Blocking of MR or MGL did not reverse the inhibitory effect of FhTeg on cytokine secretion (Figure 5.4).

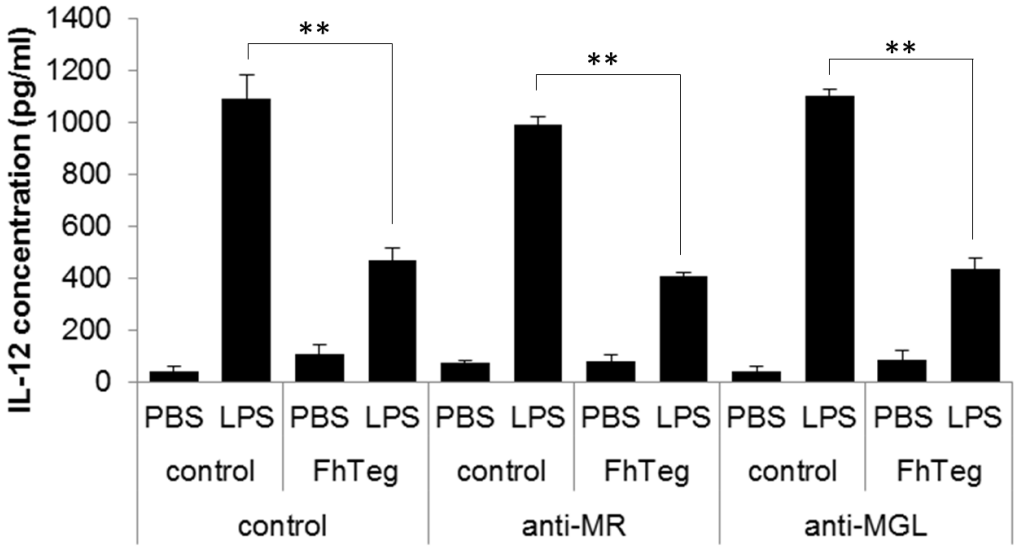

Figure 5.4 Blocking of CLRs did not reverse the effect of FhTeg on BMDC activation. BMDCs were treated with anti-MR or anti-MGL blocking antibody 30 min before treated with FhTeg (10 µg/ml) for 2.5 hours and after stimulated with LPS (100 ng/ml) for 18 hours. IL-12 (p70) was measured with commercial ELISA. Figure is representative of three independent experiments (n=3) and presented as the mean ± SD of three replicate samples from the same experiment; *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control (PBS) group.

The previous experiment in mast cells were repeated to see if we get the same results as in dendritic cells, and the blocking of MR or MGL did not reverse the inhibitory effect of FhTeg on BP-activated mast cells (Figure 5.5). In this experiment the less mature BMMCs were used so the LPS stimulation did not induce significant level of cytokines. There is an increase in cytokine output from controls by FhTeg but the difference is not significant and the levels are very low. Interestingly blocking of the

two CLRs impairs TNF-α secretion in BP activated mast cells ($p \leq 0.01$). Mast cells were co-cultured with $CD4^+$ naïve T-cells to see if the blocking of the CLRs has any effect on FhTeg inhibition and the blocking of CLRs did not reverse T-cell inhibition by FhTeg (Figure 5.6). In the case of IFN-γ blocking of MR and MGL inhibited BP-induced T-cell activation ($p \leq 0.01$; Figure 5.6A)

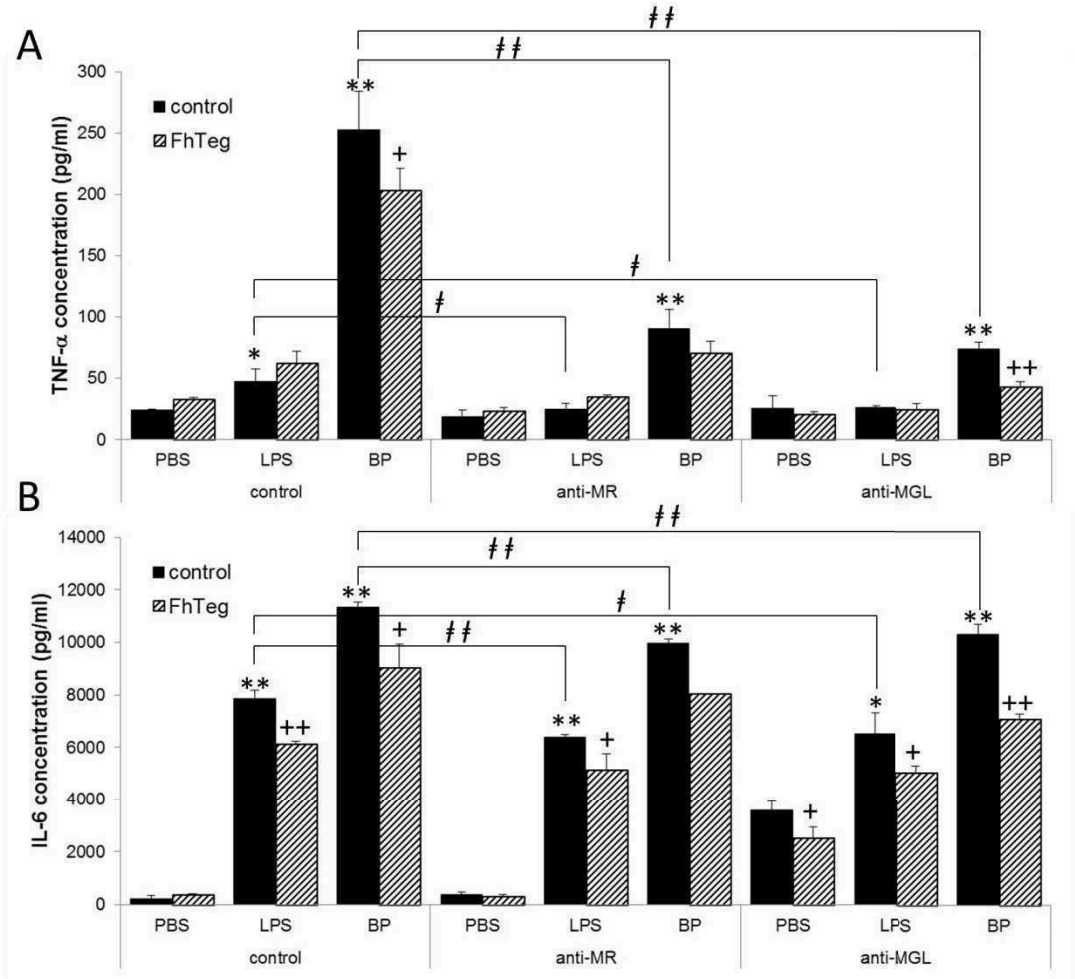

Figure 5.5. Blocking of MR or MGL did not reverse the effect of FhTeg on BMMC activation. BMMCs were treated with anti-MR and anti-MGL blocking antibody (1 μg/ml) 30 min before treated with FhTeg (10 μg/ml) for 2.5 hours and after stimulated with LPS (100 ng/ml) or BP (100 bacteria/cell) for 24 hours. TNF-α (**A**) and IL-6 (**B**) secretion was measured with commercial ELISA. Figures are representatives of three independent experiments (n=3) and presented as the mean ± SD of three replicate samples from the same experiment. *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control PBS

group. $^{+}$, $p \leq 0.05$; $^{++}$, $p \leq 0.01$ compared to control (non-FhTeg) group. $^{\sharp}$, $p \leq 0.05$; $^{\sharp\sharp}$, $p \leq 0.01$ compared to control LPS or BP treated group.

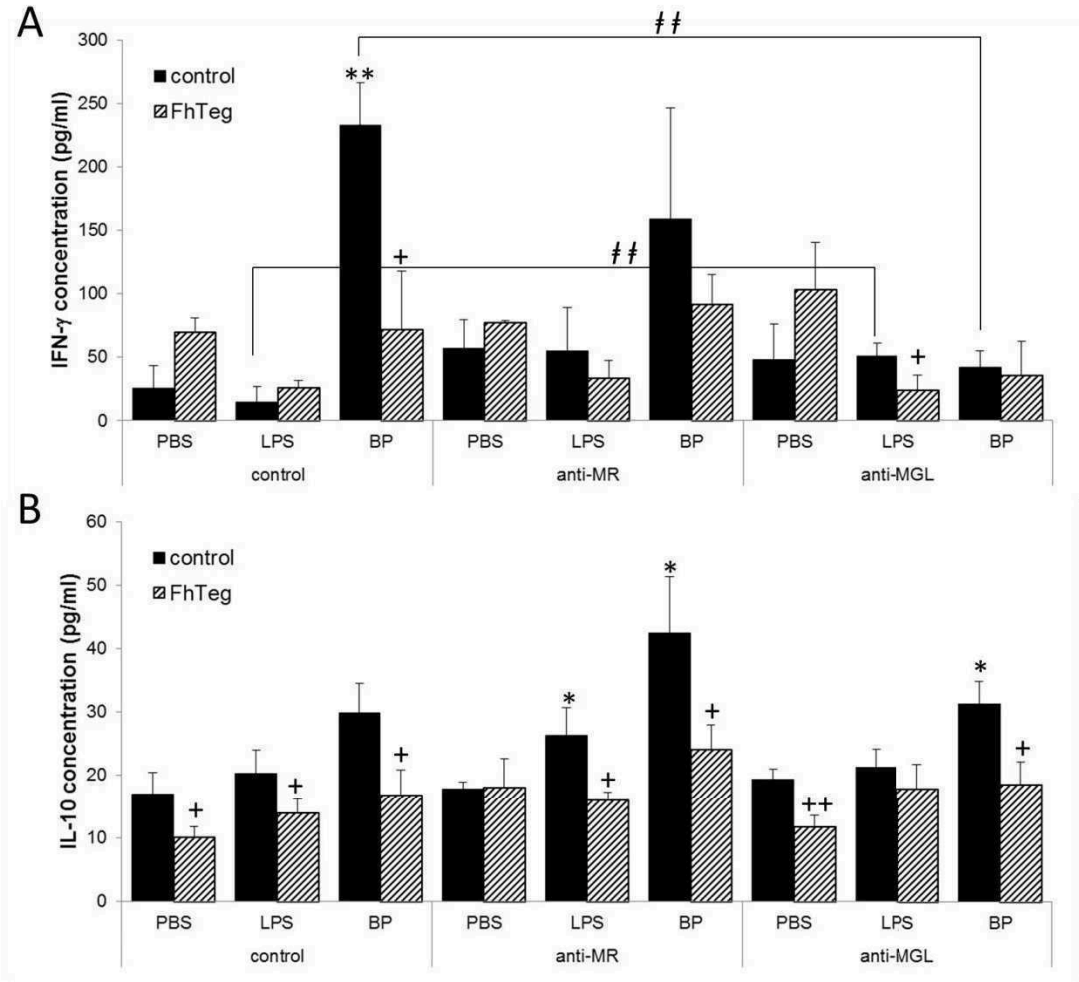

Figure 5.6. Blocking of MR or MGL did not reverse the effect of FhTeg on T-cell activation. BMMCs were treated with, anti-MR and anti-MGL blocking antibody (1 μg/ml) 30 min before treated with FhTeg (10 μg/ml) for 2.5 hours and after stimulated with LPS (100 ng/ml) or BP (100 bacteria/cell) for 24 hours. Cells were washed and co-cultured with anti CD3 activated naïve $CD4^{+}$ T-cells (1:1) for 72 h. IFN-γ (**A**) and IL-10 (**B**), secretion was measured with commercial ELISA. Figures are representatives of three independent experiments (n=3) and presented as the mean ± SD of three replicate samples from the same experiment. *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control PBS group. $^{+}$, $p \leq 0.05$; $^{++}$, $p \leq 0.01$ compared to control (non-FhTeg) group. $^{\sharp\sharp}$, $p \leq 0.01$ compared to control LPS or BP treated group.

5.3.4 Blocking of MGL suppresses FhTeg induced SOCS3 expression in dendritic cells, although it does not reverse FhTeg's inhibition of LPS activated dendritic cells.

In the previous chapter we showed that FhTeg induce SOCS3 transcription in dendritic cells and mast cells. We wished to see if the blocking of CLRs, possible receptors of FhTeg, could inhibit FhTeg induced SOCS3 up-regulation. We tested if the blocking of MR and MGL inhibits the up-regulation of SOCS3 by FhTeg. In this experiment dendritic cells were used as they are more reactive and showed higher SOCS3 transcription levels than mast cells in previous experiments. SOCS3 transcription level was up-regulated by FhTeg in control (3.7 ± 0.9, $p \leq 0.05$) and anti-MR treated (4.6 ± 0.8, $p \leq 0.05$) cells. Neutralisation of MGL but not MR inhibited FhTeg induced SOCS3 (Figure 5.7) suggesting the involvement of other inhibitory molecules in the immunostimulatory effect of FhTeg on immune cells.

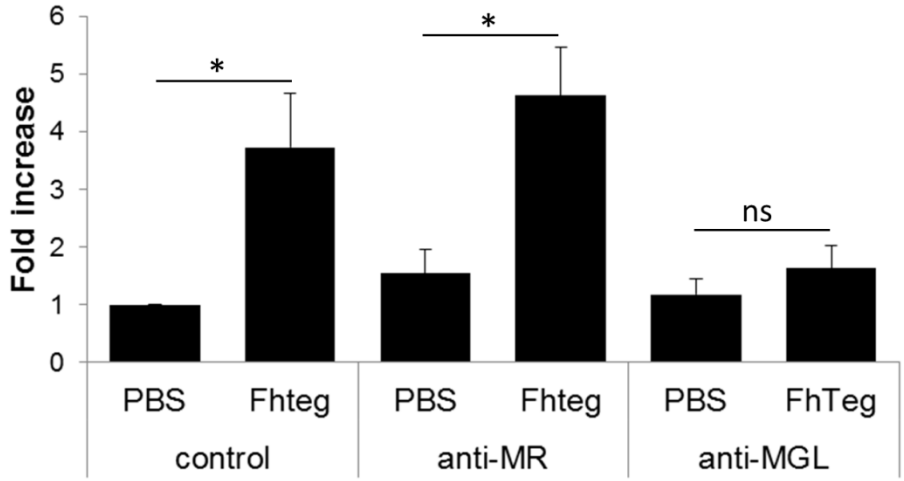

Figure 5.7. Blocking of MGL but not MR inhibit FhTeg induced SOCS3 transcription. BMDCs were stimulated with PBS (control), anti-MR and anti-MGL blocking antibody (1 μg/ml) 30 min before treated with FhTeg (10 μg/ml) for 2.5 hours. Total RNA was extracted and after reverse transcription cDNA was analysed with qPCR for SOCS3. RNA expression was normalized to

GAPDH, β-actin and Gusb control gene expression and showed relative to PBS control using Pfaffl's method; Figure is representative of two independent experiments with 3 control genes (n=3) *, $p \leq 0.05$; compared to control (PBS) group.

5.4 Discussion

Fasciola hepatica expresses a wide range of glycans and glycan conjugated molecules on their surface tegumental coat (Wilson et al., 2011; Wuhrer and Geyer, 2006), such as galactosylceramide or globotriasylceramide with a terminal GalNAc motif, a ligand of MGL (Kuijk and van Die, 2010; Wuhrer and Geyer, 2006). This study demonstrates that during *Fasciola hepatica* infection and after FhTeg treatment the expression of MR and MGL increased *in vivo* on mast cells. The cell surface levels of these CLRs are also modulated by FhTeg in mast cells and dendritic cells *in vitro*. MGL is involved in the up-regulation of SOCS3 transcription level in dendritic cells by FhTeg, although blocking of MGL or MR did not reverse the inhibitory effect of FhTeg inflammatory responses from bacterial ligand activated immune cells.

Immature dendritic cells express a large variety of CLRs, and these CLRs are often down-regulated upon maturation (van Vliet et al., 2008) and different subsets of dendritic cells express different CLRs (van Kooyk, 2008). Dendritic cell specific DC-SIGN (SignR1), Dectin1, MR and MGL can be found on the surface of tolerogenic and monocyte-derived dendritic cells. They were shown to be important in the internalization of glycosylated antigens and in the activation of dendritic cells during bacterial, viral, fungal and parasitic infections (van Vliet et al., 2008; Geijtenbeek and Gringhuis, 2009).

FhTeg induces down-regulation of MGL in dendritic cells *in vitro* and we demonstrated that MGL plays role in FhTeg induced SOCS3 up-regulation, although blocking of MGL did not reverse the inhibitory effect of FhTeg. CLRs activation can

inhibit TLR signalling (van Vliet et al., 2008; Geijtenbeek and Gringhuis, 2009), and up-regulation of negative regulatory molecules might play role in this process. Activation of CLRs was shown to induce SOCS expression in dendritic cells; however studies predominantly investigate SOCS1. SOCS1 is up-regulated in plasmocyloid dendritic cells after binding of hepatitis B antigen to a CLR, BDCA2 (Xu et al., 2009). Dendritic cells specific DC-SIGN mediated SOCS1 expression can suppress IL-12 secretion in mice with *Mycobacterium tuberculosis* infection (Srivastava et al., 2009). Dectin1 was shown to be important in SOCS1 up-regulation in zymosan treated BMDCs. Upon CLR-stimulation, IL-12 and IL-10 production and NF-κB activation is suppressed in TLR stimulated dendritic cells and these cells failed to prime T-cells (Eberle and Dalphe, 2012). Reg2 is a murine C-type lectin (HIP/PAP in humans) and was shown to be responsible for STAT3 activation and SOCS3 up-regulation (Lieu et al., 2006; Closa et al., 2007).

Our results suggest that FhTeg induces the expression of inhibitory molecules of the TLR signalling pathway, such as SOCS3, through MGL. CLR and TLR signalling crosstalk is very complicated and not fully understood (Geijtenbeek and Gringhuis, 2009). Probably the effect of FhTeg on immune cells is complex and it is not limited to the up-regulation of SOCS3. FhTeg contains lot of different glycoproteins so it is likely that these proteins activate more than one receptor and the activation of these receptors together may be responsible for the immunomodulatory effect of FhTeg. Further investigation would be to isolate the immunologically active component(s) of FhTeg and study its binding properties.

There are only few studies investigating CLR expression on mast cells. Mast cells were shown to express a specific CLR, mast cells function associated antigen (MAFA) (Guthmann et al., 1995a), involved in the inhibition of FcεRI-induced activation of mast cells (Abramson et al., 2001). Mincle (macrophage-inducible C-type lectin) and Dectin1 were also shown to be expressed by mast cells, although the expression level is very low (Ribbing et al., 2010; Yang and Marshall, 2008). These two CLRs are present on both macrophages and dendritic cells and play important role in antifungal immunity (Wells et al., 2008). Here we showed that MR, MGL and SignR1 can be found on the cell surface of BMMCs and PCMCs. The expression of these CLRs were higher on PCMCs which correlates with a study where other receptors such as TLR2 and TLR4 was highly expressed on PCMCs as they are more mature than BMMCs (Mrabet-Dahbi et al., 2009).

Helminth's glycans have the potential to interact with CLRs on immune cells and to interfere with their functions. SignR1, MR, MGL and Dectin1 have been shown to interact with helminths glycans (van Die et al., 2009) and it is likely that they play role in immunomodulation. Our results suggest that MR and MGL but not Dectin1 or SignR1 may be involved in the immunomodulation of immune cells (Table 7.1). MGL and MR are markers for M2 macrophages and were shown to be up-regulated in mice with *Taenia crassiceps* infection (Raes et al., 2005). MGL but not DC-SIGN was positively regulated in dendritic cells stimulated with the cestode antigens of *T. crassiceps* (Terrazas et al., 2011). MGL and MR induce internalisation of *Schistosoma mansoni* egg antigens, suppress TLR induced dendritic cell maturation and induce the development of Th2 phenotype (van Liemt et al., 2006). MGL was

also shown to be involved in the suppression of LPS induced cytokine secretion by the antigens of *T. crassiceps* (Terrazas et al 2011).

Our *in vivo* results correlate with a previous study where murine peritoneal macrophages exhibited increased expression levels of MGL and MR, but not Dectin1 or SignR1 during *F. hepatica* infection or FhTeg treatment (Adams et al., unpublished data), while FhES failed to up-regulate these CLRs. FhES mainly contains proteins and enzymes, while FhTeg derived from the glycocalix and its main components of FhTeg are carbohydrates and glyco-proteins, which can explain the differences between the two antigen preparations. It was shown previously that blocking MR with antibody or mannan partially inhibits the modulatory effect of the excretory-secretory product of *F. hepatica* in macrophages (Guasconi et al., 2011).

CLRs have multiple functions in pathogen recognition, although their function is not as well studied as TLR receptors. They can induce or inhibit cytokine secretion and modulate TLR4 signalling. Here we showed that MR and MGL play role in *F. hepatica* immune cells interaction, although we did not manage to reverse the inhibitory effect of FhTeg by blocking these two CLRs. Our results also suggest that beside TLR4 MR and MGL may be involved in the activation of mast cells by bacterial antigens. Further investigation of immune cell's CLR – pathogen interaction will help us to learn more about mast cell and dendritic cells function in innate immunity.

6 Chapter 6 - Mannose receptor and macrophage galactose lectin are involved in *Bordetella pertussis* mast cells interaction

6.1 Introduction

Although blocking of MR or MGL did not interfere with the immunomodulatory effect of FhTeg on mast cells, we found significant suppression of BP induced cytokine secretion in BMMCs. In this chapter we investigate the involvement of MR and MGL in *B. pertussis* mast cell interaction.

CLRs play a key role in immunity against bacterial infections due to their calcium-dependent interaction with pathogen-associated glycan epitopes (Lloyd et al., 2007). Upon carbohydrate binding, CLRs have the ability to trigger the immune machinery by antigen internalization and presentation onto major histocompatibility complex (MHC) molecule or by modulation of TLR-mediated signalling pathway (Weis et al., 1998). Differences in sugar-binding ability and ligand-specificity of the various CLRs are conferred by highly conserved carbohydrate-recognition domains (CRD). The ubiquitous expression of CLRs by most cell types, including macrophages and dendritic cells (Geijtenbeek and Grinhuis, 2009), together with organism-specific differences in glycosylation among pathogens make the CLRs a central defence system in the innate immunity.

Most bacteria exhibit a thick coat of glyco-conjugate structures in the form of polysaccharidic capsule, peptidoglycans, or lipopolysaccharides and other glycolipidic moieties (Kusumoto et al., 2010). This coat is usually composed of a number of PAMPs, which can activate multiple PRRs (Kawai and Akira, 2011).

Mannose receptor (MR) binds to carbohydrates such as mannose, N-acetylglucosamine and fucose. These saccharides can be found on the surfaces of microorganism, including *Escheria coli*, *Pseudomonas aeruginosa* or *Mycobacterium tuberculosis* (Weis et al., 1998). Another important CLR, macrophage galactose-like lectin (MGL) has been reported to bind to galactose and terminal N-acetylgalactosamine (GalNAc)-motifs on filoviruses and *Campylobacter jejuni* (Geijtenbeek and Gringhuis, 2009; van Sorge et al., 2009). Some bacteria can interact with more than one CLR, and the restricted expression of CLRs on immune cell subsets can contribute to the different immune responses (van Vliet et al., 2007).

To date, the study of CLRs expressed on mast cells is in its infancy. Mast cells express a specific CLR, mast cell function associated antigen (MAFA) (Guthmann et al., 1995b), later found also in natural killer cells and human basophils (Katz, 2002). Stimulation of MAFA leads to the inhibition of FcεRI-induced activation of mast cells (Abramson et al., 2002). Other CLRs such as macrophage-inducible C-type lectin (Mincle) and Dectin1 were also shown to be expressed by mast cells, although expression levels are very low (Ribbing et al., 2011; Yang and Marshall, 2009). These two CLRs, also present on macrophages and dendritic cells, play important roles in antifungal immunity (Wells et al., 2008). Upon CLR-ligand binding ITAM and ITAM-like signalling pathways can be activated, promoting the recruitment of spleen tyrosine kinase (Syk), which results in the activation of cellular proliferation, differentiation and migration (Kerrigan et al., 2010).

Bordetella pertussis is a Gram-negative coccobacillus that causes the respiratory infection, whooping cough (Gregory, 2006). Despite being preventable by

vaccination, pertussis causes nearly 300,000 deaths in children every year mainly in developing countries (Growcroft and Pebody, 2006), although the incidence of whooping cough in Europe has increased due to the change in national immunization schedules from the whole-cell vaccine to acellular vaccine (Growcroft and Pebody, 2006). Vaccination can induce protection against infection by inducing strong Th1/Th2 immune response; however, the acellular vaccine induces a polarised Th2 immune response (Growcroft and Pebody, 2006, von Konig et al., 2002) and confers protection for a limited time only (von Konig et al., 2002). Better understanding of the interaction of the bacteria with immune cells may lead to development of a more effective vaccine that induces lifelong immunity. *Bordetella* was shown to be a ligand for TLR4 (Mann et al., 2005), but the immunopathogenesis of *B. pertussis* is not fully understood. The key initiator of inflammatory and immune responses is the strain-specific endotoxin component of the bacteria outer membrane; the lipooligosaccharides (LOS) (Schaeffer et al., 2004; Marr et al., 2010), while actively released protein toxins, such as pertussis toxin, dermonecrotic toxin or adenylate cyclase (Locht, 1999; Locht et al., 2001), or other cell wall components, like adhesins might contribute to the development of disease (Le Blay et al., 1994).

Despite the crucial role of mast cells in the clearance of bacterial infection from the lung and in mediating immune responses (Abraham et al., 1997; Moiseeva and Bradding, 2011), only few studies have examined the interaction of mast cells with *B. pertussis* (Mielcarek et al., 2001). In the previous chapters we showed that mast cells stimulated with the heat-inactivated *B. pertussis* antigen (BP) secrete pro-inflammatory cytokines, such as TNF-α and IL-6 and display enhanced expression of ICAM1. BP-stimulated mast cells promote cytokine secretion in T-cells. BP

induces the activation of two members of the TLR4 signalling pathway, ERK and NF-κB. In this study we investigated the interaction between mast cells and *B. pertussis* with an emphasis on the role of CLRs.

6.2 Experimental design

Since blocking MR and MGL suppresses BP-induced cytokine secretion in BMMCs, in this chapter *Bordetella pertussis* – mast cell interaction was investigated further to determine a role of C-type lectin receptors (CLRs). Firstly, it was confirmed that MR and MGL are expressed by BMMCs and PCMCs and it was tested if BP-stimulation can up-regulate this CLR expression (2.2.12, 2.2.18). PCMCs are more mature than BMMCs and they secrete more cytokines (IFN-γ and IL-10) after BP stimulation, so effect of anti-MR and anti-MGL blocking antibodies on BP-induced activation of mast cells were examined in PCMCs. To confirm that the effect of the blocking antibodies is specific for the binding sites of MR and MGL the results were confirmed with the specific carbohydrate ligand of these CLRs (mannan and GalNAc). EGTA (block all CLRs) and laminarin (ligand for Dectn1) were used as controls in these experiments (2.2.18).

BP-stimulated PCMCs were co-cultured with naïve T-cells (2.2.19) to test if blocking of MR and MGL on mast cells suppresses mast cell induced cytokine secretion in T-cells. Since ICAM1 has been shown to be important in mast cell – T-cell communication involvement of CLRs in BP-induced ICAM1 up-regulation was also tested (2.2.18). BP has been shown to activate NF-κB and ERK in mast cells and we tested the involvement of MR and MGL in BP-induced transcription factor activation using blocking antibodies (2.2.20-22). Involvement of Syk kinase (member of CLR signalling cascade) in cytokine secretion was also tested (2.2.12).

Since LOS is crucial to activate the immune system during *Bordetella* infection and the most probable ligand of CLRs, the role of *B. pertussis* LOS in CLR-mediated

mast cell activation was investigated. LOS from BP heat-inactivated cells was isolated by digestion of cell lysate with proteinase K followed by high-speed centrifugation (2.2.25). First we confirmed that LOS can mimic BP and promote cytokine secretion in mast cells than the effect of blocking anti-MR and anti-MGL antibodies on LOS-induced cytokine secretion in BMMCs was examined (2.2.25). Due to the presence of traces of proteinase K in LOS preparation, the effect of proteinase K on mast cell was also tested.

6.3 Results

6.3.1 MR and MGL are expressed by PCMCs and BP stimulation has no effect on this expression.

Previous studies have reported the expression of mast cell function associated antigen (MAFA) (Guthmann et al., 1995a), Dectin1 and Mincle (Yang and Marshall, 2009) on mast cells. Here the expression of MGL and MR on BMMCs and PCMCs was examined by flow cytometry revealing that these CLRs are highly expressed by mast cells (Figure 6.1).

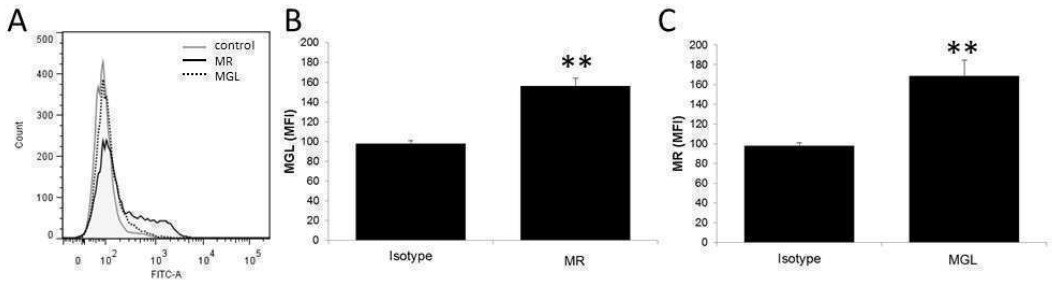

Figure 6.1. MR and MGL are expressed by mast cells. Cell surface expression of MR (**B**) and MGL (**C**) were measured by three colour (FcεRI, c-kit and CLR) flow cytometry on PCMCs. On histogram (**A**) isotype control is indicated with grey, MR and MGL are indicated with normal and dashed lines, respectively. Data were normalized to isotype controls and are presented as the mean ± SD of three independent experiments (n=3). *, $p \leq 0.05$; **, $p \leq 0.01$ compared to isotype control group.

Since neutralization of MR and MGL impairs TNF-α secretion in BP activated BMMCs (Figure 5.5) we tested if BP stimulation changes the expression levels of MR and MGL. BMMCs and PCMCs were stimulated with PBS or heat inactivated BP for 24 hours and expression levels of MR and MGL were measured by flow cytometry. There was no significant difference between the stimulated and control

groups in the expression of the investigated CLRs (MR: p = 0.12; MGL: p = 0.91) (Figure 6.2).

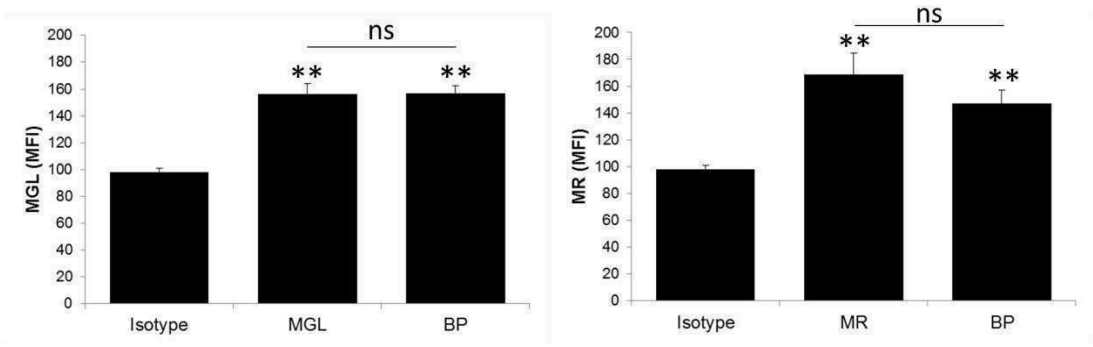

Figure 6.2. BP stimulation has no effect on MR and MGL expression. PCMCs were stimulated with BP (100 bacteria /cell) or PBS for 24 h. MR (**A**) and MGL (**B**) expression level was measured with flow cytometry. Data were normalised to isotype controls and are presented as the mean ± SD of three independent experiments (n=3). **, $p \leq 0.01$ compared to isotype control group.

6.3.2 Anti-MR and anti-MGL blocking antibodies suppress BP-induced TNF-α, IL-6 and IFN-γ secretion in mast cells.

As PCMCs are more mature compared to BMMCs and beside TNF-α and IL-6 secrete also IL-10 and IFN-γ after BP stimulation, consequently we repeated the CLR neutralization experiment with these cells. Stimulation of PCMCs with BP for 24 hours induced secretion of cytokines (TNF-α, IL-6, IFN-γ ($p \leq 0.01$), IL-10 ($p \leq 0.05$)) when measured by commercial ELISA (Figure 6.3). TNF-α, IL-6 and IFN-γ secretion levels were significantly suppressed when PCMCs were treated with anti-MR (TNF-α, IL-6: $p \leq 0.05$; IFN-γ: $p \leq 0.01$) or anti-MGL (TNF-α, IL-6, IFN-γ: $p \leq 0.01$) blocking antibody for 30 minutes prior to BP stimulation (Figure 6.3A-C). IL-10 secretion by PCMCs was not impaired by either of the antibodies (anti-MR: p = 0.18, anti-MGL: p = 0.80) (Figure 6.3D).

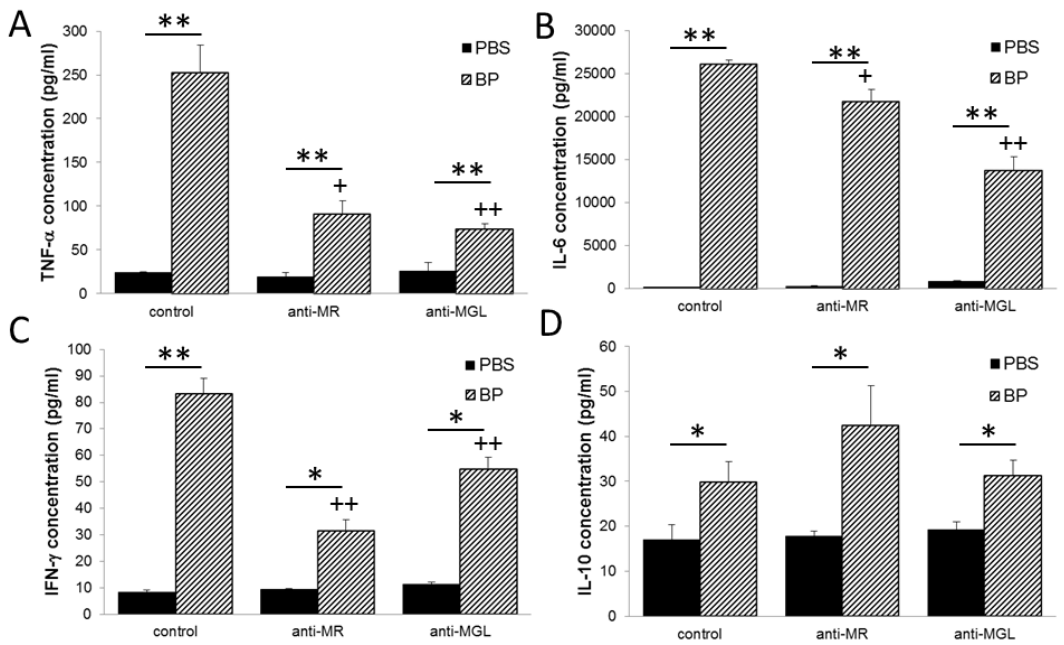

Figure 6.3. Blocking of MR and MGL suppressed BP induced cytokine secretion in mast cells. PCMCs were treated with anti-MR or anti-MGL blocking antibody (1 μg/ml) 30 min before stimulated with BP (100 bacteria/cell) for 24 hours. TNF-α (**A**), IL-6 (**B**), IFN-γ (**C**) and IL-10 (**D**) secretion was measured with commercial ELISA. Figures are representatives of three independent experiments (n=3) and presented as the mean ± SD of three replicate samples from the same experiment. *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control group; and $^{+}$, $p \leq 0.05$; $^{++}$, $p \leq 0.01$ compared to control BP stimulated group.

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

PCMCs were stimulated with BP in the presence or absence of anti-MR or anti-MGL blocking antibodies for 24 hours to determine if mast cell ability to promote cytokine production from naïve $CD4^{+}$ T-cells can be inhibited. BP-activated PCMCs were co-cultured with naïve $CD4^{+}$ T-cells and cytokine secretion was assessed. Since co-culture of mast cells with naïve $CD4^{+}$ cells in the presence of anti-CD3 is reported to promote cytokine secretion *per se* (Huels et al., 1995; Nakae et al., 2005; Nakae et al., 2006), PBS-primed PCMCs were also used in the co-culture experiments as

controls. CD4$^+$ T-cells co-cultured with BP-stimulated PCMCs secreted higher levels of IFN-γ ($p \leq 0.05$), IL-10 (, $p \leq 0.05$) and IL-5 ($p \leq 0.05$) than the control group (Figure 6.4A-C), while IL-4 levels remained unchanged ($p = 0.55$) (Figure 6.4D). When PCMCs were pre-treated with anti-MR or anti-MGL prior to BP-stimulation, mast cell no longer secreted significant levels of IFN-γ (anti-MR: $p = 0.41$, anti-MGL: $p = 0.12$) and IL-5 (anti-MR: $p = 0.13$, anti-MGL: $p = 0.12$) in co-culture compared to controls (Figure 6.4A, C). However only PCMCs treated with anti-MGLdid not secrete IL-10 compared to controls (Figure 6.4B).

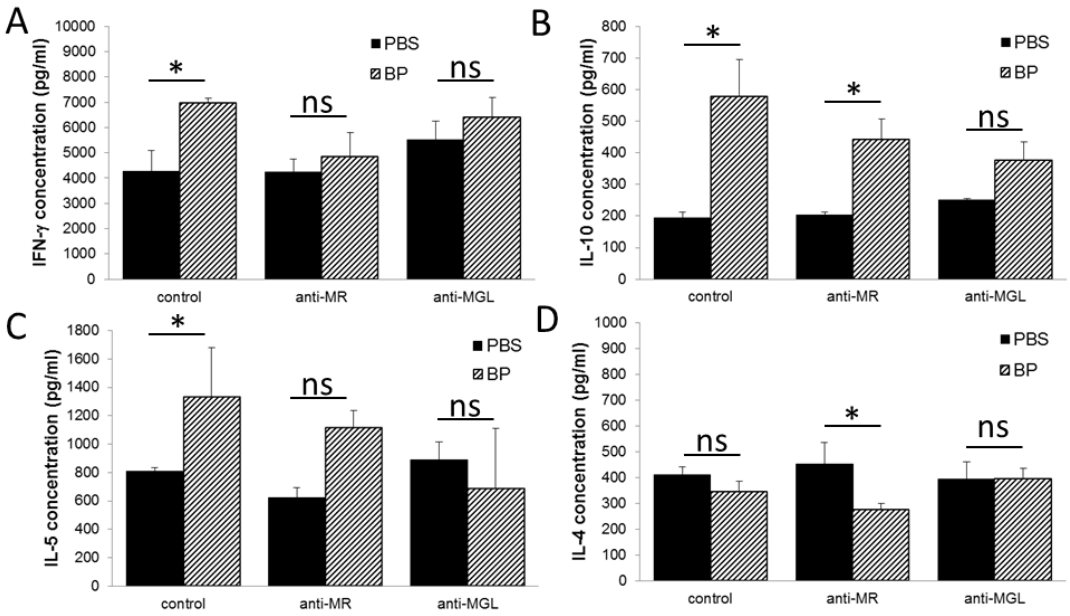

Figure 6.4. Anti-MR and anti-MGL blocking antibodies interfere with BP-induced T-cell activation by PCMCs. PCMCs were treated with anti-MR and anti-MGL blocking antibody (1 μg/ml) 30 min before stimulated with BP (100 bacteria/cell) for 24 hours. Cells were washed and co-cultured with naïve CD4$^+$ T-cells (1:1) for 72 h. IFN-γ (**A**), IL-10 (**B**), IL-5 (**C**) and IL-4 (**D**) secretion was measured with commercial ELISA. Figures are representatives of three independent experiments (n=3) and presented as the mean ± SD of three replicate samples from the same experiment; *, $p \leq 0.05$ compared to control group.

6.3.4 Anti-MR and anti-MGL blocking antibodies do not suppress BP induced up-regulation of ICAM1.

Since ICAM1 is important in T-cell – mast cell communication and was previously shown to be up-regulated on mast cells after BP stimulation (Figure 6.5), the involvement of CLRs in ICAM1 expression was investigated. PCMCs were treated with anti-MR or anti-MGL blocking antibodies prior to stimulation with BP for 24 h and ICAM1 expression was measured by flow cytometry. BP induced up-regulation of ICAM1 expression in all groups, with no significant effect due to blocking of MR or MGL (one-way ANOVA was used to asses if data belong to the same group; p = 0.97) (Figure 6.5).

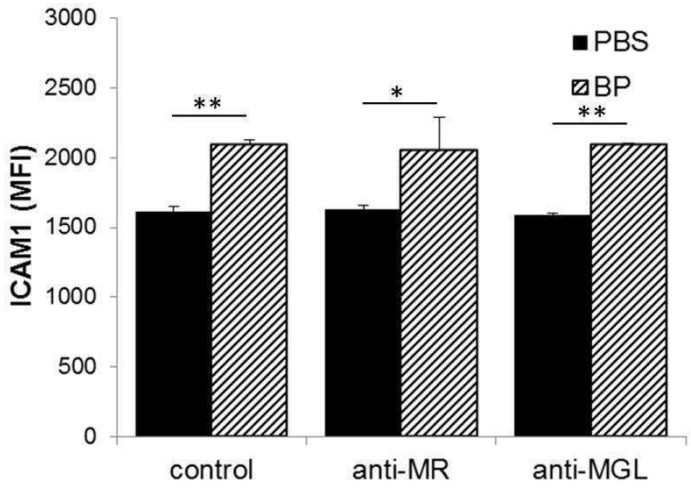

Figure 6.5. Anti-MR and anti-MGL blocking antibodies did not suppress BP induced up-regulation of ICAM1. PCMCs were treated with anti-MR or anti-MGL blocking antibody (1 μg/ml) 30 min before stimulated with BP (100 bacteria/cell) for 24 hours. Cell surface marker expression of ICAM1 was measured by Flow cytometry. Data are normalized to isotype control and presented as the mean ± SD of three independent experiments (n=3); *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control group.

6.3.5 Glycans (mannan, GalNac and laminarin) and EGTA suppress BP induced TNF-α and IL-6 secretion.

In order to fully validate that the observed suppression of cytokine secretion is due to effective blocking of Ca^{2+}-dependent CRDs on CLRs, BMMCs were treated with mannan (50 µg/ml, blocking SignR1 and MR), GalNAc (50 mM, blocking MGL), laminarin (100 µg/ml, blocking Dectin1) or EGTA (10 mM, Ca^{2+}-ligand) for 30 min before stimulated with BP for 24 h. All the glycans and EGTA suppressed BP induced TNF-α and IL-6 secretion significantly ($p \leq 0.01$ to $p \leq 0.05$) (Figure 6.6.). Notably, EGTA treatment reduced the cytokines secretion ability not only in BP-stimulated but also in PBS-primed (control) mast cells ($p \leq 0.01$) (Figure 6.6).

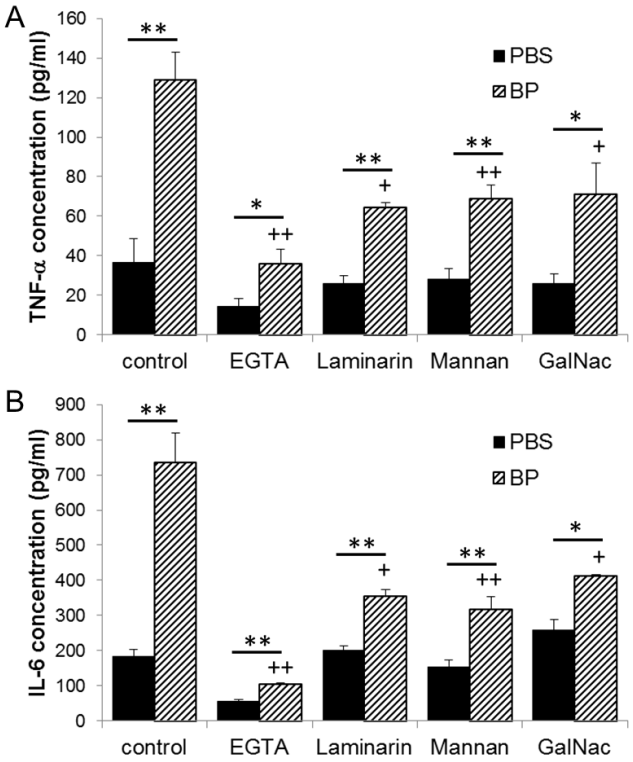

Figure 6.6. Carbohydrate treatment impaired BP induced cytokine secretion in mast cells. BMMCs were treated with EGTA (10 mM), laminarin (100 µg/ml), mannan (50 µg/ml), GalNAc (50mM) 30 min before stimulated with BP (100 bacteria/cell) for 24 hours. TNF-α (**A**) and IL-6 (**B**) secretion was measured with commercial ELISA. Figures are representatives of three independent

experiments (n=3) and presented as the mean ± SD of three replicate samples from the same experiment. *, $p \leq 0.05$; **, $p \leq 0.01$ compared to PBS groups; and $^{+}$, $p \leq 0.05$; $^{++}$, $p \leq 0.01$ compared to control BP stimulated group.

6.3.6 Syk inhibition supresses TNF-α and enhances IL-6 secretion by BP-stimulated mast cells.

Spleen tyrosine kinase is involved in CLR signalling; it binds to the phosphorylated intracellular domain of the CLR and mediates signalling resulting in transcription factor activation and cytokine secretion in dendritic cells (Lang et al., 2011). Downstream CLR signalling was investigated by assessing the involvement of Syk kinase (Kerrigan and Brown, 2010) in cytokine secretion by BP-stimulated mast cells. BMMCs were treated with Syk inhibitor (piceatannol) prior to stimulation with BP for 24 h. TNF-α secretion was reverted to basal level by Syk inhibition (Figure 6A), while IL-6 secretion levels appeared dramatically enhanced in both BP-stimulated and control groups (Figure 6B).

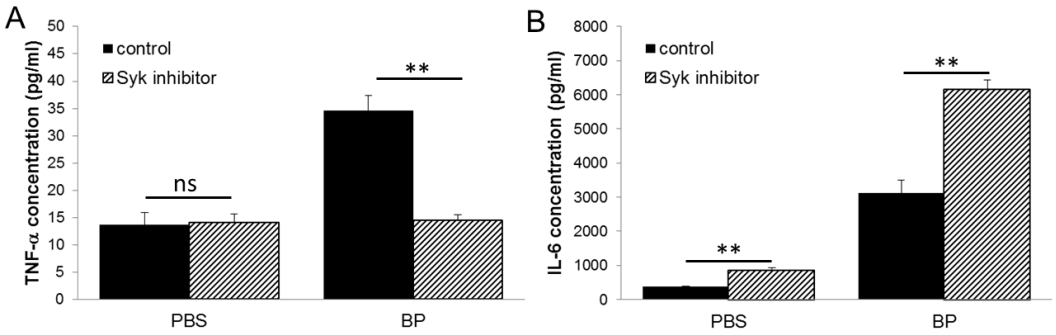

Figure 6.7. Inhibition of Syk suppressed BP-induced TNF-α secretion and enhances IL-6 secretion in mast cells. BMMCs were treated with PBS (control) or Syk inhibitor (10 μM) 30 min before stimulated with BP (100 bacteria/cell) for 24 hours. TNF-α (**A**) and IL-6 (**B**) secretion was measured with commercial ELISA. Figures are representatives of three independent experiments

(n=3) and presented as the mean ± SD of three replicate samples from the same experiment; *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control (PBS) group.

6.3.7 Blocking of MR and MGL has no effect on BP induced ERK phosphorylation.

To further understand the signalling pathways involved in CLR-mediated BP-stimulation of mast cells, the effect of CLRs blocking on NF-κB p65 and ERK phosphorylation was investigated. We previously reported that BP-stimulation of mast cells enhances ERK and NF-κB p65 activation. The latter effect could be reversed by blocking either MR or MGL ($p \leq 0.01$) (Figure 6.8A, B), whereas CLR-blocking had no statistical effect on ERK phosphorylation (Figure 6.8A, C).

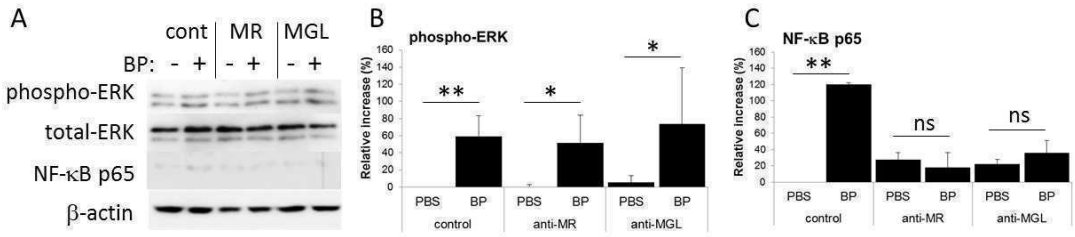

Figure 6.8. Neutralisation of MR and MGL did not interfere with BP induced ERK phosphorylation. BMMCs were treated with anti-MR and anti-MGL blocking antibodies prior to stimulation with BP for 15 min. Protein samples were analysed by western blot for phosphorylated-ERK (p-ERK), total-ERK (t-ERK), phosphorylated-NF-κB p65 and β-actin expression (**A**). Values of p-ERK (**B**), and NF-κB p65 (**C**) were normalised to t-ERK and β-actin and are expressed in arbitrary units as % fold increases over PBS control. Densitometry data are presented as the mean ± SD of three independent experiments (n=3); *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control (PBS) group.

6.3.8 Neutralisation of MR and MGL suppresses LOS induced cytokine secretion in mast cells

Whole cell vaccine studies suggest that BP derived lipooligosaccharide (LOS) is crucial to activate innate immune cells to generate pro-inflammatory cytokines in *Bordetella* infections (Higgins et al., 2006). The role of *B. pertussis* LOS in CLR-mediated mast cell activation was investigated. LOS from BP heat-inactivated cells was isolated by digestion of cell lysate with proteinase K followed by high-speed centrifugation (Figure 2.5). BMMCs were stimulated with blocking anti-MR and anti-MGL antibodies 30 min prior to stimulation with this LOS preparation for 24 h. Due to the presence of traces of proteinase K in LOS preparation, the effect of proteinase K on mast cell was also tested, resulting in negligible cytokine secretion. The neutralisation of MR and MGL on LOS-stimulated mast cells inhibited cytokine secretion (Figure 6.9) as previously observed in the case of BP-stimulated cells (Figure 6.3A, B).

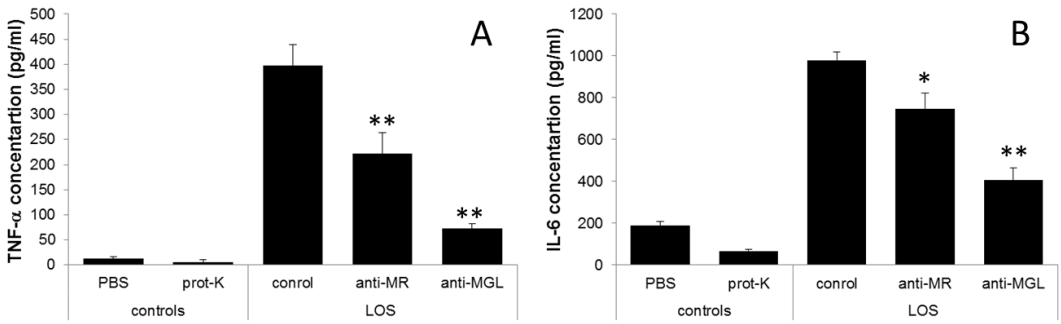

Figure 6.9. Neutralisation of MR and MGL suppressed LOS induced cytokine secretion in mast cells. LOS was isolated from BP antigen and BMMCs were stimulated with 2 u/ml (equivalent to 100 bacteria/cell) for 24 h in the presence and absence of anti-MR or anti-MGL neutralisation antibodies. TNF-α (**A**) and IL-6 (**B**) secretion was measured by commercial ELISAs. LOS isolation was done by Dr Alessandra Ravidà (Dublin City University, Ireland). Data are presented as the mean ± SD of three independent experiments; *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control-LOS group.

6.4 Discussion

Mast cells are a major component of protective immunity against microbial infection. These cells are a potent source of pro-inflammatory mediators, such as TNF-α and IFN-γ that can promote Th1 driven host defence against bacteria and protozoa (Abraham and John, 2010; Dawicki and Marshall, 2007; Galli and Tsai, 2010; Metcalfe et al., 1997; Weller et al, 2011). They have been shown to play a crucial role in the clearance of bacterial infection such as *Bordetella pertussis* or *Escherichia coli* (Mielcarek et al, 2001, Wierzbicki and Brzezinska-Blaszcyk, 2009). We showed for the first time that mannose receptor (MR) and macrophage galactose lectin (MGL) are also expressed by BMMCs and PCMCs. Furthermore, in *B. pertussis* challenged mast cells, MR and MGL were proven to be functionally involved in the induction of cytokine secretion with the use of blocking antibodies and CLR specific carbohydrate ligands. In this study, blocking of MR and MGL reversed the ability of mast cells to prime naïve $CD4^+$ T-cells and to induce cytokine secretion. Both MR and MGL have been reported to play a role in T-cell activation by antigen presenting cells as dendritic cells and macrophages (van Vliet at al., 2008). Activation of MGL leads to maturation of dendritic cells and IFN-γ secretion in $CD8^+$ T-cells co-cultures (Napoletano et al, 2012). CpG were shown to activate dendritic cells to induce IL-17 and IFN-γ production from $CD4^+$ T-cells and MGL is involved in this process (Denda-Nagai et al, 2010).

Similar to dendritic cells and macrophages, mast cells express pathogen recognition receptors such as toll like receptors ((TLR)-2, TLR3, TLR4, TLR6, TLR7 and TLR9) that recognise invading pathogens (Marshall et al., 2003; Galli et al., 2008). The function of TLRs in *B. pertussis* – immune cell interaction has been extensively

investigated (Mann et al., 2005), whereas less is known about C-type lectin's (CLR) involvement in this process, especially there is no study about the role of mast cell's CLRs in bacteria-mast cell interaction. On mast cells only few types of CLRs have been reported to date. Mast cell function associated antigen (MAFA) is specific for mast cells and has homology with several CLRs (Guthmann et al., 1995a; Guthmann et al., 1995b). Dectin1 and Mincle have been detected on mast cells, although they are expressed in very low levels (Yang and Marshall, 2009).

In the case of MR, our findings correlate with studies in other immune cells. MR was shown to be involved in the activation of macrophages by *Mycobacterium tuberculosis in vitro* (Rajaram et al., 2010). In macrophage-like placental trochoblast giant cells MR also proved to play a role in the uptake of various bacteria like *Listeria monocytogenes*, *Bacillus subtilis* and *Echerichia coli* (Hashino et al., 2012). On the contrary, in most literature MGL has been associated with the suppression rather than induction of cytokine secretion by bacteria-stimulated immune cells. In dendritic cells, a *Campylobacter jejunum* mutant lacking of functional MGL ligand induced increased IL-6 production compared to wild type (van Sorge et al., 2009). Similarly, modulation of MGL on dendritic cells by cestode antigens leads to inhibition of LPS induced pro-inflammatory cytokine secretion (Terrazas et al., 2011). Our results reveal a new stimulatory function of MGL in pathogen-immune cell interaction.

BP stimulation has been reported to cause ERK phosphorylation on mast cells *via* activation of TLR4 signalling pathway (Desai and Thurmond, 2011; Li et al., 2009) and activation of MAPK and NF-κB pathways inducing cytokine secretion and cell

surface marker up-regulation in immune cells (Tsang et al., 2005). Blocking of MR and MGL on mast cells suppressed NF-κB p65 but not ERK phosphorylation. A similar trend has been also observed in LPS-stimulated macrophages where blocking the CLR signalling pathway resulted in the suppression of the phosphorylation of JNK but not p38 or ERK (Lin et al., 2010). Furthermore, the recently observed link between ERK phosphorylation and ICAM1 up-regulation (Tsang et al., 2005) can be used to better understand the absence of any effect of CLR neutralization on the expression level of ICAM1. Notably, CLRs modulation has been associated with impairment of NF-κB activation in LPS-stimulated dendritic cells, suggesting the involvement of alternative signalling pathways to TLR4 in LPS challenge (Eberle and Dalpke, 2012).

Spleen tyrosine kinase (Syk) is a ubiquitous non-receptor tyrosine kinase with diverse biological activity (Miller et al., 2012). It is involved in the signalling from B- and T-cell receptors, Fc receptors and CLRs, including Dectin1 (Kerrigan and Brown, 2010; Olynych et al., 2006). In macrophages blocking of Syk suppressed LPS induced activation of the MAPK pathway (Lin et al., 2010). Interestingly in our experiments blocking of Syk had conflicting effects on TNF-α and IL-6 secretion by BP-stimulated mast cells. These results could be explained with the diverse function of Syk in immune cells. Recently it was reported that Syk binds to TLR4 and plays a role in the response to bacterial pathogens or LPS in dendritic cells and macrophages (Miller et al., 2012; Chaudhary et al., 2007; Ulanova et al., 2007), leading to pro-inflammatory cytokine secretion. On the other side, Syk is also activated at different stages of the CLRs signalling pathway, resulting in activation or inhibition of TLR signalling pathway and effects cellular responses like migration, adhesion,

differentiation or inflammatory and regulatory mediator release. The interaction of Syk with different receptors has yet to be identified (Kerrigan and Brown, 2010).

It has also to be highlighted that the heat-inactivated BP preparation used in this study is a source of heterogeneous antigens, like adhesins and toxins, high levels of LOS as well as residual active pertussis toxins (Mahon et al., 1996), interacting with many different receptors. In order to simplify our system focussing on the most probable ligands of CLRs and TLRs (Locht, 1999), LOS was isolated from heat-inactivated BP and used in mast cell stimulation assays. LOS is usually a potent inducer of TLR4-mediated signalling resulting in the release of pro-inflammatory cytokines (Marr et al., 2010; Higgins et al., 2006). In our study, the LOS-induced cytokine secretion by mast cells was suppressed by blocking of MR and MGL, thus supporting the hypothesis of CLRs involvement in LOS immune cell stimulation. Notably, LOS stimulation of mast cells resulted in lower cytokine levels compared to the levels obtained with heat-inactivated whole cell BP. This is in agreement with previously reported *in vitro* stimulation of macrophages with BP LOS, resulting in reduced levels of TNF-α and IL-6 secretion (Marr et al., 2010). All these data suggest the synergistic action of *B. pertussis* LOS on TLRs and CLRs together with other toxins during infection (Locht, 1999).

BP LOS characterisation has been pursued over the years (Chaby et al., 1977; Moreau et al., 1984; Martin et al, 1992) and recent analyses revealed strain-variability in both structure and endotoxicity of BP LOS (Marr et al., 2010; Caroff et al., 2001). The LOS of *B. pertussis* Tohama I has been recently investigated by Kubler-Kielb *et al* in an attempt to develop OS-conjugates with bactericidal

properties against *Bordetella* species (Kubler-Kielb et al., 2011). Human MGL has been reported to exhibit a narrow binding specificity towards terminal *N*-acetyl galactosamine (GalNAc) moieties (van Vliet et al., 2005). Notably the LOS structure of BP does not feature any Gal or GalNAc residues, but the presence of one terminal residue of 2-amino-2-deoxygalacturonic acid (GalNA) has to be noted on this complex oligosaccharide. Furthermore, several glucosamine (GlcN) residues are also present on the glycan backbone and branches of the LOS with one *N*-acetylated (GlcNAc) monosaccharide terminally located. To date, there are no reports of MGL affinity towards neither GalNA nor GlcNAc. It has to be speculated that either mast cell derived MGL exhibits alternative binding specificities or the LOS used in this study contains additional saccharidic epitopes not previously characterised.

The identification of the potential binding epitopes on BP-derived LOS for the MR is somewhat less challenging due to the broad range of ligands reported for this CLR. It can be hypothesized that MR recognises the terminal GlcNAc on BP LOS through its eight tandemly arranged C-type lectin-like domain (CTLDs) with reported affinity for mannose, fucose and GlcNAc (Gazi and Martinez-Pomares, 2009).

Whole cell vaccine studies suggest that BP-derived LOS plays a crucial role in the development of polarized Th1 immune responses; and signal trough TLR4 is critical in innate immune cells in the generation of inflammatory cytokines in *Bordetella pertussis* infection (Higgins et al., 2009; de Gouw et al., 2011). Our study suggests that beside TLR4, CLRs are also involved in BP activation mainly *via* the LOS antigen and in the development of new vaccines carbohydrate moieties could prove

to be useful in the activation of CTRs and the promotion of these inflammatory immune responses. In summary we showed that MR and MGL are involved in the interaction of *B. pertussis* antigen with mast cells, and the lipooligosaccharides in the cell wall are responsible for this activation. This sheds further light on mast cells biology and the role of mast cells in the defence system against bacterial infections. Bacterial-induced TLR signalling is well studied, although less is known about CLRs and their crosstalk with other signalling pathways. Further studies are required to examine CLRs involvement in bacteria recognition and their stimulatory/inhibitory effect on TLR signalling.

7 Chapter 7 - Final discussion

This study sheds new light on the role of mast cells during helminth infection as for the first time the anti-inflammatory properties of *Fasciola hepatica* antigens on mast cell pro-inflammatory immune responses was investigated. In particular, a mechanism was described, how *Fasciola hepatica* antigen suppressed TLR4 activated mast cells through the induction of negative regulators of the TLR pathway. This study also highlights the important role of C-type lectin receptors on mast cells during *Fasciola* and *Bordetella pertussis* infection. In particular, this study was the first to examine the role of MR and MGL. The following discussion highlights the key findings from this study and discusses future studies that are required to further advance the field of mast cell biology.

7.1 Mast cells are new players in *Fasciola hepatica* immune modulation

Mast cells are usually associated with Th2 immune responses during helminth infection (Dawicki and Marshall, 2007) and the important role of mast cells in intestinal helminth infection is well studied (Hepworth et al., 2012a; Hepworth et al., 2012b). Typically mast cells are activated by helminth-specific IgE antibody, which is secreted by B-cells (Anthony et al., 2007; Erb, 2007). Mast cells degranulate and release inflammatory mediators in an IgE dependent manner, releasing mediators such as histamine, serine proteases, chymase, tryptase that contribute to the expulsion of gut helminths (Metz and Maurer, 2007) by increasing intestinal permeability and inducing the influx of neutrophil granulocytes (McDermott et al., 2003). IgE secreting B-cells are activated by Th2 associated cytokines (IL-4, IL-5, IL-9, IL-13 TSLP and IL-33) that are secreted by innate immune cells and Th2 T-cells. Studies suggest that mast cells are an early source of these cytokines in

particular IL-4, TSLP and IL-33 in the early stages of infection and these cells are importance in the development of Th2 adaptive immunity (Anthony et al., 2007).

The role of mast cells during infection with tissue dwelling helminths such as *F. hepatica* is not clearly understood. Here the role of these cells was investigated during *F. hepatica* infection where, at least *in vitro,* they do not promote a Th2 immune response despite increase in mast cell number prior to the development of Th2 adaptive immunity (Appendix B, preliminary data). This suggests that there may be another role for mast cells during infection. Since host immunity to *F. hepatica* infection requires a strong Th1 immune response (Moreau and Chauvin, 2010), and in order for *Fasciola* to survive within the host, it must suppress this Th1 immune response. Further studies are required to investigate the role of mast cells during infection. Future studies could involve the role of mast cell knockout mice as this will confirm if mast cells are important for the development of Th2 immune responses, protection against helminth infection, wound healing or reduction in helminth burden.

The tegumental coat antigen of *F. hepatica* (FhTeg) was shown previously to suppress TLR ligand and non-TLR ligand stimulated dendritic cells by inhibiting their ability to secrete cytokines, express cell surface markers or prime T-cells (Hamilton et al., 2009; Vukman et al., 2013 – Appendix E). It was shown previously that FhTeg suppresses the phosphorylation of NF-κB in LPS activated dendritic cells (Hamilton et al., 2009). Here it was found that the MAPK pathway is also affected and a role for the inhibitory molecule SOCS3 in this process was determined. Therefore we hypothesize that SOCS3 induction inhibits the TLR signalling pathway

by suppressing the activation of NF-κB and MAPK transcription factors that lead to the inhibition of cytokine secretion, cell surface marker expression and ultimately T-cell priming (Figure 7.1).

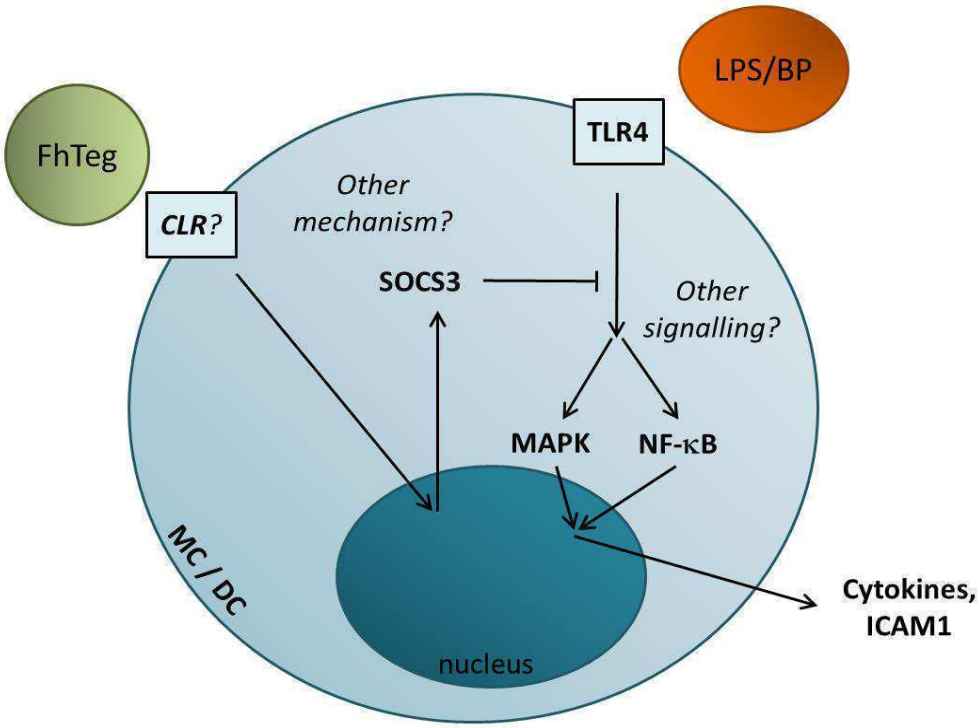

Figure 7.1. Possible mechanism how FhTeg suppress LPS/BP induced cytokine secretion in mast cells. FhTeg induce SOCS3 transcription. SOCS3 is an inhibitor of TLR4 signalling and suppress the activation of both MAPK and NF-κB pathways impairing pro-inflammatory cytokine secretion and ICAM1 up-regulation. There might be other mechanisms than SOCS3 how FhTeg suppress TLR4 signalling, and it is suggested that the effect of FhTeg is not limited to TLR4.

Here it was shown for the first time that similar to dendritic cells, mast cells activated by bacterial ligands in the presence of FhTeg cannot prime Th1 immune responses (Figure 7.2). The immunomodulatory effect of FhTeg on dendritic cells and mast cells is summarised in Figure 7.1. However, it is possible that FhTeg up-regulates inhibitory molecules other than SOCS3 and that these molecules act

together on the TLR and non-TLR signalling pathway in these cells. For example, other inhibitors such as A20 or MyD88s have been shown in knockout cell lines to directly target TLR signalling by suppressing TRAF6 activation and IRAK1/2 phosphorylation, important molecules of the TLR4 signalling pathway (Mogensen, 2009; Lu et al., 2008). Using specific inhibitors of these regulatory molecules or gene silencing techniques (si-RNA, sh-RNA) may help to define a role for these molecules in immunomodulation by FhTeg. However, currently the work presented in this thesis used primary immune cells. Si-RNA can modify the *in vitro* development of primary cells making them a poor *in vitro* model to investigate the role of SOCS3 .The development of models using cell lines therefore is required before further investigations can be performed. Preliminary stuies were performed with zoledronic acid, the only known inhibitor of SOCS3, (Scheller et al., 2011) following the *in vitro* method described by Bringmann et al. (2007). Using this drug is not possible in primary cells to investigate the role of SOCS3 as zoledronic acid impairs the ability of these cells to be stimulated with bacterial ligands (Appendix C).

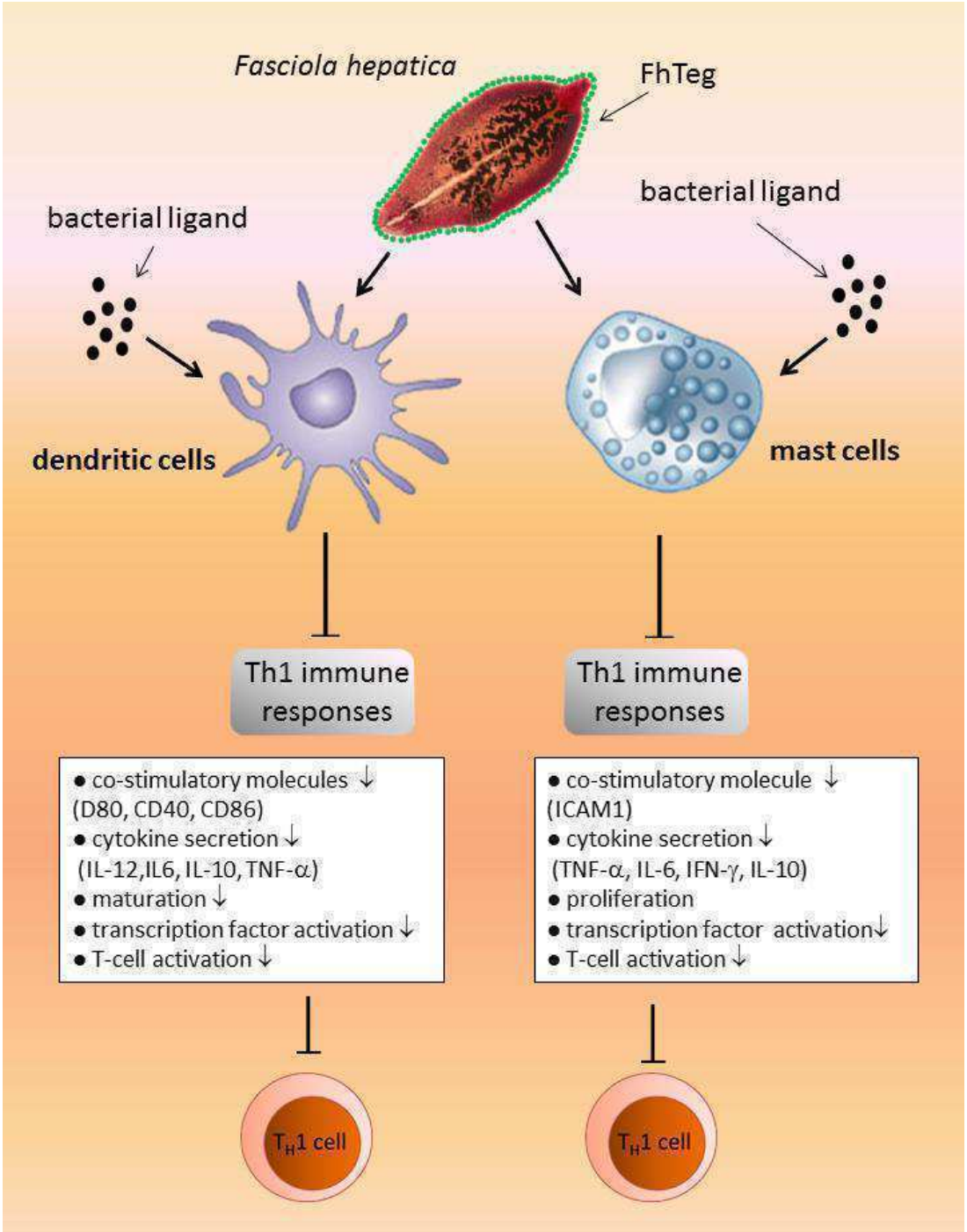

Figure 7.1. FhTeg directly modulates dendritic cells and mast cells by inhibiting their ability to drive Th1 immune responses. Bacterial ligand (LPS or BP) induced pro-inflammatory cytokine secretion, cell surface marker up-regulation, and transcription factor up-regulation is inhibited by FhTeg (indicated with green dots) from the tegumental coat of *F. hepatica* in dendritic cells and mast cells. These cells also fail to induce Th1-type immune responses in T-cells.

Mast cells play key role in the development of Th1 immune responses (Marshall et al., 2003) and mast cell-induced Th1 immune response is crucial in the clearance of protozoan and bacterial infection, such as *Escherichia coli* (Wierzbicki and Brzezinska-Blaszczyk, 2009) or *Pseudomonas aeruginosa* (Siebenhaar et al., 2007) infections. The immunomodulatory effect of helminths on macrophages and dendritic cells during bystander bacterial infection, such as *Mycobacterium tuberculosis* (Resende Co et al., 2007; Potian et al., 2011), is well studied, but before this study, to our best knowledge, nobody investigated the effect of *Fasciola hepatica* infection on pro-inflammatory mast cells. Given the important role of mast cells in the clearance of microbial infections future studies examining bystander response of helminths should also consider the role of mast cells in this process.

Mast cells are associated with many Th1-mediated inflammatory disorders such as autoimmune disease (Weller et al., 2011). Studies with mast cell-deficient mice demonstrated that mast cells-derived pro-inflammatory cytokines, such as TNF-α contributes to the pathology (Metz and Maurer, 2007; Weller et al., 2011). For example, mast cell knockout mice are resistant to rheumatoid arthritis where mast cell derived cytokines are involved in the development of inflammation (Metz and Maurer, 2007). Similarly, mast cells and mast cell-derived TNF-α is important in the development of EAE (the murine model for multiple sclerosis) (Christy et al., 2012; Costanza et al., 2012) and mast cells-deficient mice show delayed and decreased severity with less blood-brain-barrier damage.

Helminth derived antigens were shown to prevent the initiation of inflammatory diseases in animal models (Imai et al., 2001; Harnett and Harnett, 2008) and this study demonstrated that FhTeg suppresses TNF-α secretion in mast cells. It is possible that the bystander effect of helminth infection on autoimmune diseases is because they target mast cells and impair their inflammatory responses. Further studies required to investigate this using mast cell-knockout and mast cell-reconstructed mice. Targeting mast cells and identification of mast cell inhibitors have therapeutic potential in the treatment of inflammatory disorders such as IBD, the inflammatory autoimmune disorder of the gut (He, 2004). Identification and characterisation of helminth-derived molecules could lead to new therapeutic approaches.

7.2 The role of mast cells in tissue repair during helminth infection

Fasciola hepatica larvae penetrate through the gut wall into the peritoneum and then into the liver, causing extensive tissue damage (Robinson et al., 2010) and since mast cells together with macrophages are at the site of infection within days post infection it is possible that these cells work together to promote wound healing following the tissues destruction by the migrating flukes.

Mast cells are important in tissue remodelling as they induce fibronectin turn-over and angiogenesis (Metcalfe et al., 1997). In the acute phase of tissue damage endothelial cell-derived IL-3 and SCF induce migration, proliferation and local differentiation of mast cells (Noli and Milo, 2001). These activated mast cells secrete mediators, such as histamine, serotonin, enzymes and cytokines and induce the influx of neutrophil, basophil and eosinophil granulocytes and other leukocytes to

the site of the tissue damage (Maltby et al., 2009). These mediators are also important in inducing fibroblast proliferation, increased vascular permeability (marker for wound healing responses) and angiogenesis (Noli and Miolo, 2001; Maurer et al., 2003; Weller et al., 2006). Mast cells are also crucial in scar formation (Wilgus, 2008). The use of mast cell-deficient mice will determine whether the *F. hepatica*-infected mutant mice develop more severe disease as a result of the improper wound healing and if the activation of other cell types, involved in tissue remodelling (e.g. macrophages or granulocytes), is impaired.

7.3 Advances in mast cells' crosstalk with other immune cells

The study demonstrates new findings about mast cell - T-cell cross talk, as it was shown that ICAM1 is involved in T-cell activation. While studies have highlighted the importance of ICAM1 expression on T-cells (Brill et al., 2004), it was shown for the first time here that ICAM1 expression on mast cells is crucial in this process. Interaction of mast cells with T-cells requires further investigation as mast cells express many cell surface markers, adhesion molecules, co-stimulatory and co-inhibitory molecules on their surface (Kambayashi et al., 2009, Kashiwakura et al., 2004, Sayed and Brown, 2007) and the effect of FhTeg on the expression of these molecules, such as VCAM-1 or LFA-1, and their role in T-cell – mast cells communication should be examined. These results may help us to gain better understanding of the mechanism how mast cells are involved in the development of adaptive immune response and how helminths inhibit these responses.

In addition, mast cells – dendritic cell interaction seems to be critical in the development of appropriate adaptive immune response against pathogens (Bryce et

al., 2004; Mazzoni et al., 2006) and in this study it was demonstrated that during *F. hepatica* infection dendritic cell-derived chemokines may induce migration of mast cells to the site of infection. Mast cells may also modulate dendritic cell function during helminth infection as mast cells are known to modify the function of dendritic cells during antigen presentation to T-cells and are important in the development of adaptive immunity. For example, *in vitro* antigen stimulated mast cells were shown to activate dendritic cells to induce immune responses in $CD4^+$ T-cells (Dudeck et al., 2011) where mast cell-derived TNF-α is important in dendritic cell activation (Gri et al., 2012).

Pro-inflammatory responses in dendritic cells can be directly suppressed by FhTeg and it might be interesting to see whether indirectly, FhTeg stimulated mast cells could inhibit dendritic cell function during helminth infection. This can be investigated *in vitro* by co-culturing FhTeg-treated mast cells with dendritic cells to see if their pro-inflammatory responses are suppressed or not. *In vivo* Th2 immune responses can be induced by exposing immune cells to helminth products and inject them into recipients (Balic et al., 2004). This experiment can be repeated using FhTeg-treated mast cells, to decide if FhTeg-activated mast cells injected into wild type or mast cell-deficient mice can induce other immune cells types (e.g. dendritic cells and macrophages) or inhibit their Th1 immune responses.

7.4 Functional classification of mast cells

Given the expanding role of mast in the immune system we propose a new functional classification for mast cells based upon their ability to promote adaptive immune response. To develop this classification the functional characterisation of dendritic

cells and macrophages subsets should first be considered. Dendritic cells and macrophages are classified into different subsets based upon their different function in different environments (MacDonald and Maizels, 2008; Biswas and Mantovani, 2010). Dendritic cells have at least four subsets: conventionally mature, tolerogenic, Th17 and modulated dendritic cells. Conventionally mature dendritic cells are activated by bacterial, viral or protozoan antigens and secrete pro-inflammatory cytokines such as IL-12 and can induce Th1 immune response. They are important in the clearance of intracellular and extracellular pathogen infection and also in autoimmune diseases (Perona-Wright et al., 2006). Tolerogenic dendritic cells induce regulatory-type (Treg) immune responses through the secretion of IL-10 and TGF-β (MacDonald and Maizels, 2008) and are important in maintaining tolerance against apoptotic cells and commensal bacteria (Perona-Wright et al., 2006). Th17 dendritic cells induce $CD4^+$ T-cells to secrete TGF-β, IL-23 and IL-6. They take part in the immune defence against fungi and extracellular bacteria and also contribute to the pathogenesis of many autoimmune conditions. Modulated dendritic cells are orchestrated to Th2 immunity during helminth infections and their pro-inflammatory function is down-regulated (MacDonald and Maizels, 2008, Anthony et al., 2007; Jackson et al., 2009).

There are two different subsets of macrophages, M1 and M2 (Kreider et al., 2007). M1 or classically activated macrophage can stimulate Th1 T-cells and NK cells, inducing IFN-γ production (Biswas and Mantovani, 2010). Alternatively activated (M2) macrophages are characterised by arginase1 activity and IL-10 secretion (Biswas and Mantovani, 2010, Anthony et al., 2007; Flynn et al., 2007). During helminth infection these macrophages can induce Th2/Treg immune response,

suppress Th1 immunity and are important in tissue remodelling, wound healing and immunoregulation (Allen and Maizels, 2011).

Conventional classification of mast cells is based on phenotypical differences. Historically rodent mast cells were divided into two groups: connective tissue type mast cells (CTMC) and mucosal mast cells (MMC). CTMCs are in the skin and peritoneal cavity and MMCs can be found in the intestinal propria of mice. These subsets differ also in their origin and biochemistry (Metcalfe et al., 1997). For human mast cells there is another type of classification based on the different metachromatic staining properties of mast cells. These subpopulations differ in protease content of their vesicles in the cytoplasm. MC_{TC} mast cells contain trypase, chymase, chatepsin G-like protein and carboxypeptidase in their vesicles while MC_T-type mast cells contain only trypase (Irani et al., 1989). MC_T predominate in the lung and in the small intestine mucosa while MC_{TC} can be found in the skin and in the small intestine submucose. Localisation and staining similarities suggest that MC_T could be the human homologues of murine MMCs and human MC_{TC} corresponds most closely to CTMCs (Metcalfe et al., 1997). Although between these groups there are functional differences as well, functional studies of mast cells from different tissues suggest further heterogeneity, and a function-based classification, similar to macrophages and dendritic cells, may have some advantages.

Given the advances in mast cell biology there could be a new classification of mast cell based upon their ability to drive different adaptive immune responses. If we follow similar functional characterisation of macrophages and dendritic cells, at least two mast cells subsets can be distinguished as illustrated in Figure 7.3. The mast

cells subsets can be called as MC1 and MC2 based on their analogy to M1 and M2 macrophages. MC1 mast cells could be the subset that are important in the clearance of bacterial and viral infections (Marshall, 2004) and are able to activate the adaptive Th1 immune system by secreted cytokines, chemokines and can also act as antigen presenting cells. These mast cells are characterised by the lack of degranulation and the production pro-inflammatory mediators, like TNF-α. These cells also contribute to pathology in inflammatory disorders, such as autoimmune diseases (Metz and Maurer, 2007; Sutherland et al., 2011; Weller et al., 2011).

MC2 mast cells could be mast cells that are activated in the effector phase of the Th2 immune responses. They express the high affinity FcεRI receptor, which in the presence of antigens and the antigen-specific IgE antibody induce cell degranulation and the release of inflammatory mediators such as histamine, cytokines or chemokines (Melendez et al., 2007; Pearce, 2007). These mast cells have beneficial effect during gut helminth infection but also contribute to the pathology of Th2-type inflammatory diseases like allergy.

Mast cells can produce IL-33 and when co-cultured with dendritic cells they indirectly induce IL-17 secretion in T-cells (Dudeck et al., 2011) suggesting their involvement in the development of Th17 immune responses. Mast cells also play role in maintaining the homeostasis of the body. For example, these homeostatic mast cells are important in wound healing, tissue remodelling and angiogenesis (Metcalfe et al., 1997; Maurer et al., 2003; Weller et al., 2011) suggesting other possible mast cell subsets. There is no evidence in the literature about mast cells involved in the regulation of immune responses. During helminth infection mast

cells produce TGF-β and IL-10 and it is possible that with dendritic cells and macrophages they contribute to the activation of Treg-cells. Further studies with mast cells knockout mice could prove the existence of a MCreg subtype of mast cells.

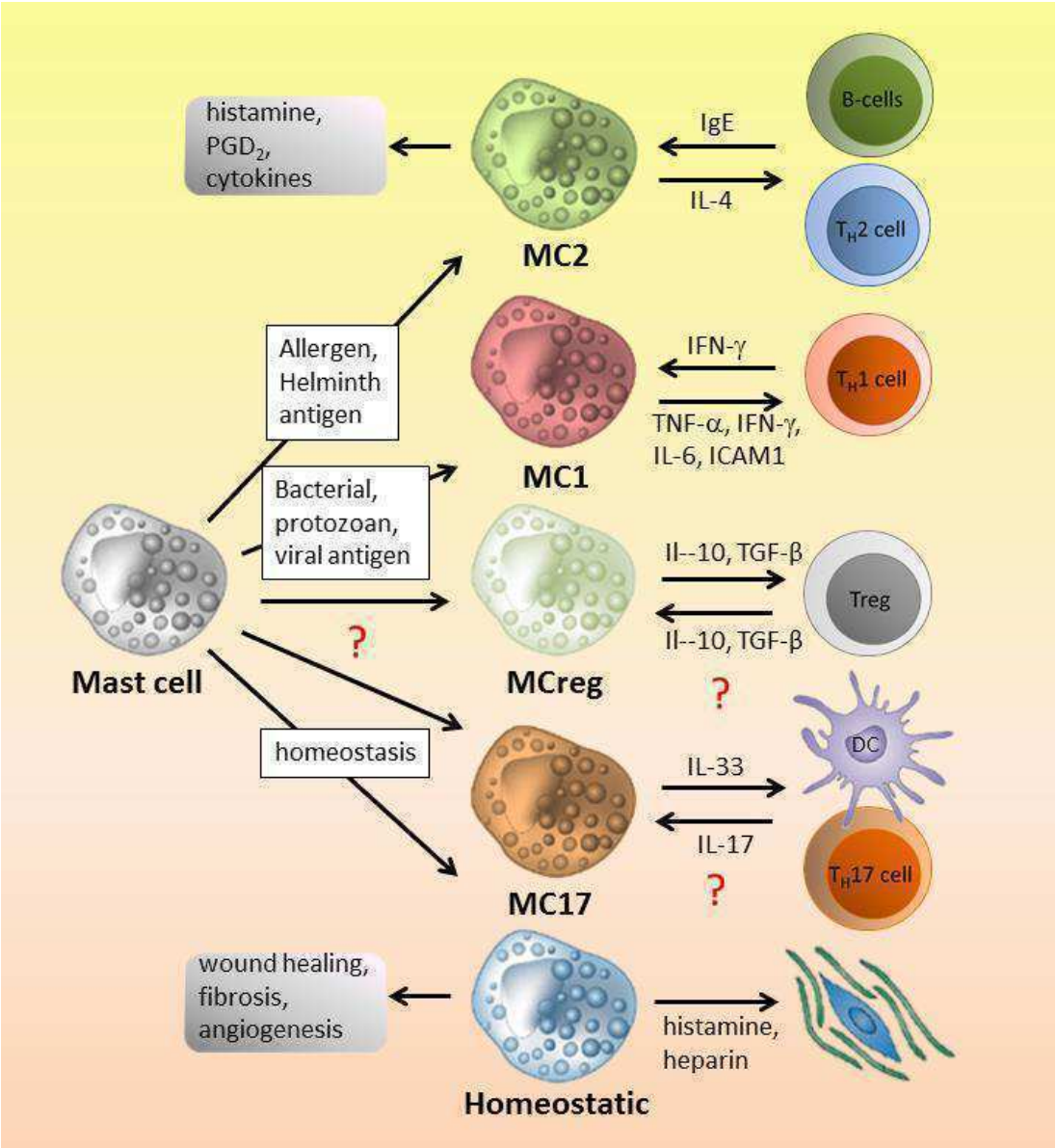

Figure 7.3. Suggested functional classification of mast cells. Allergy and helminth infection are associated with Th2-type mast cells (MC2) They express the high affinity FcεRI receptor, which in the presence of antigens and B-cell-derived specific IgE antibody induce cell degranulation and the release of inflammatory mediators such as histamine, cytokines or chemokines. During viral or bacterial infections Th1-type mast cells are able to activate the adaptive Th1 immune system by secreted pro-inflammatory cytokines, chemokines and can also act as antigen presenting cells (MC1).

Mast cells may also take part in the development of Treg (MCreg) and Th17 (MC17) immune responses and was showed to be important in the homeostasis of the body, and contribute to wound healing, tissue remodelling and angiogenesis.

7.5 New role of C-type lectin receptors on mast cells in immunity against pathogens

MR and MGL are expressed by subsets of dendritic cells and macrophages on their surface (van Vliet et al., 2008; Geijtenbeek and Gringhuis, 2009) and studies have shown that they are involved in the recognition of several pathogens such as *Leishmania donovani*, *Mycobacterium tuberculosis* or *Schistosoma mansoni* (Geijtenbeek and Gringhuis, 2009; Akilow et al., 2007) and play an important role during innate immune responses. This study demonstrates for the first time that these C-type lectin receptors (CLRs) are also expressed by mast cells and may play an important role in helminth and bacterial infection.

7.5.1 CTLs, mast cells and *Fasciola hepatica*

CLRs are known to play an important role in helminth infection as helminths release and express glycol-conjugates such as glycoproteins and glycolipids. CTLs are important in the induction of M2 macrophages and in the maturation and activation of dendritic cells during helminth infection (Thomas and Harn, 2004). In table 7.1 *in vivo* and *in vitro* data of MR, MGL, Dectin 1 and SignR1 expression on mast cells, dendritic cells and macrophages is summarised. The results suggest that MR and MGL but not Dectin 1 and SignR1 are expressed during *F. hepatica* infection and this can be mimicked *in vivo* and *in vitro* by its antigens. These findings are further supported as MGL activation is involved in FhTeg induced SOCS3 up-regulation in dendritic cells as blocking of MGL inhibited the FhTeg-dependent induction of

SOCS3 but supports our hypotheses that more than one inhibitor is important in infection as blocking this receptor could not reverse the suppressive effect of FhTeg.

Table 7.1 Effect of *F. hepatica* infection or FhTeg treatment on CLR expression of immune cells *in vitro* and *in vivo*

		Dectin1	MR	MGL	SignR1
***F. hepatica* infection**	mast cells	∅	↑	↑	∅
	macrophages[1]	∅	↑	↑	∅
FhTeg injection	mast cells	↑	∅	↑	∅
	macrophages[1]	∅	↑	↑	↑
***In vitro* FhTeg treatment**	BMMCs	∅	↑	∅	∅
	PCMCs	↑	↑	↑	∅
	BMDCs	∅	↑	↓	∅
	macrophages[1]	∅	↑	↑	∅

↑, up-regulated; ↓, down-regulated; ∅, no significant change, [1](Adams et al., unpublished data)

FhTeg contains many proteins with different carbohydrate moieties and identification of CLRs, that play role in the immunomodulation by FhTeg, may help to find the biological active component of this antigen. Bioassays with recombinant CHO cell lines, expressing only one CLR on their surfaces, are available to investigate CLR binding properties *in vitro*. When the possible receptors for FhTeg are identified, fluorescent labelling of FhTeg or its active component with the help of CLR-specific antibody could prove that FhTeg binds to this CLR. There are MGL or MR knockout mice commercially available, and these mice could be used in the next step of the investigation *in vivo*, or with primary cells isolated from these cells *in vitro*, to examine the role of these CTRs during *Fasciola* infections. CLRs are possible candidates as therapeutic targets in inflammatory autoimmune disease, targeting these receptors and identification and characterisation of the biological active component molecules of FhTeg could lead to new therapeutic approaches to cure inflammatory disorders.

7.5.2 CLRs on mast cells in *Bordetella pertussis* infection

Mast cells, together with dendritic cells and macrophages are crucial in the clearance of bacterial infection, such as *Bordetella pertussis*, from the lung (Mielcarek et al., 2001) and in mediating protective immune responses. TLRs on immune cells were shown to play key role in the activation of these cells (Geijtenbeek and Gringhuis, 2009, Mann et al., 2005). Here it was demonstrated for the first time that beside TLR4 (Mann et al., 2005) MR and MGL are also involved in the activation of mast cells with *B. pertussis* antigen by blocking CLRs with neutralising antibodies and carbohydrates. This study demonstrates novel results about mast cell-induced Th1 immune responses during bordetellosis (such as the involvement of ICAM1 in BP induced T-cell activation), and these findings may help us to gain better understanding of mast cell biology and the role of mast cells in the defence system against bacterial infection.

Further studies are required to investigate the role of mast cells during bordetellosis, for example, MR and MGL play role in the internalization of *Mycobacterium tuberculosis* (Kang and Schlesinger, 1998; Denda-Nagai et al., 2010) and *B. pertussis* can adhere and be phagocytised by mast cells (Miercarek et al., 2001). By blocking CLRs or using labelled BP antigens it could be examined whether MR and MGL are involved in the endocytosis of *B. pertussis* by mast cells and whether this endocytosis is crucial in the development of proper immune responses. It would be interesting to study if these CLRs are involved in mast cells – T-cell communication with the help of CLR-specific antibodies. Both MR and MGL were shown to play role in T-cell activation by antigen representing cells as dendritic cells and

macrophages (van Vliet et al., 2008; Napoletano et al., 2012), but, to our best knowledge, there is no study about mast cells.

In vaccine studies the role of mast cells are usually not considered however their importance in protective immunity against anthrax and *Heliobacter* infections following vaccination was demonstrated (McLachan et al., 2008; Velin et al., 2005). Successful acellular and cellular vaccines are available against *B. pertussis*, although acellular vaccination do not give life-long immunity and cellular vaccine has serious side effects (Crowcroft and Pebody, 2006; von Konig et al., 2002.), therefore, identifying novel targets in bordetellosis vaccine development may have some advantages. Targeting mast cells and receptors other than TLRs on immune cells may lead to development of novel and better vaccine; for example, by using adjuvants that activate mast cells or CLRs.

7.6 Summary

This studies presented in this thesis have shed new light on the role of mast cells during pathogen infections and these findings contribute to the better understanding of mast cell biology. It is hoped that the publications form this thesis will prompt further studies to investigate the expanding role of mast cells particular as therapeutic targets to inhibit inflammatory mast cells in autoimmune diseases and examine the importance of this cells in the promotion of protective immune responses to infectious diseases.

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9 Appendix

9.1 Appendix A – Optimization of methods

9.1.1 TLR ligands

To find good candidates to activate mast cells and promote Th1 but not Th2 immune response we tried a panel of TLR-ligands (Table 8.1). We measured TNF-α and IL-6 production as indicators of Th1 immune responses and β-hexosaminidase as indicator of Th2 immune response (Figure 8.1).

Table 8.1. List of stimulants, concentration and the type of TLR they bind to

Antigen	Concentration	Toll-like receptor
PGN	5 µg/ml	TLR2/1
Zymosan	10 µg/ml	TLR2/6
Poli(I:C)	10 µg/ml	TLR3
LPS	100 ng/ml	TLR4
BP	100 bacteria/cell	TLR4
Flagellin	25 µg/ml	TLR5

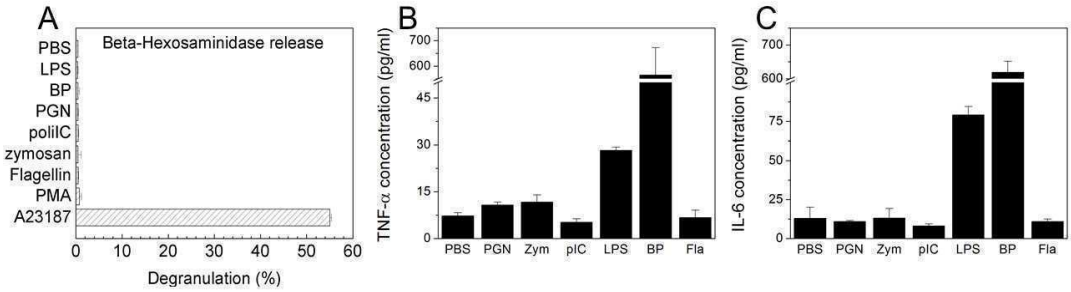

Figure 9.1. LPS and BP stimulated mast cells produced TNF-α and IL-6 but no β-hexosaminidase. BMMCs were cultured with LPS (100 ng/ml), BP (100 bacteria/cell), poli(I:C) (pIC; 10 µg/ml), zymosan (Zym; 10 µg/ml), flagellin (Fla; 25 µg/ml) and PGN (5 µg/ml) at 37°C and 5% CO_2 for 24 hours. PBS was used as negative and A23187 was used as positive control. Cells were harvested for analysis and supernatants were removed for measurement of TNF-α, IL-6 by commercial ELISA (BD Biosciences). Cells were stimulated with the same ligands and degranulation was determined by β-hexosaminidase assay.

None of the tested antigens caused degranulation. LPS and BP activated mast cells produced significant amount of TNF-α and IL-6. We decided to use these two TLR4 activators.

9.1.2 Validation of LPS and BP administration

To optimise our *in vitro* system concentration and time dependence studies were done. The concentrations of LPS were 25, 50, 100, 250, 500, 1000 and 1500 ng/ml. BP were used at 10, 25, 50, 100, 500 bacteria/cell. Time dependence was examined at four time points: 6, 12, 24 and 48 hours. Cells were harvested for analysis and supernatants were removed for measurement of TNF-α, IL-6 (Figure 8.2).

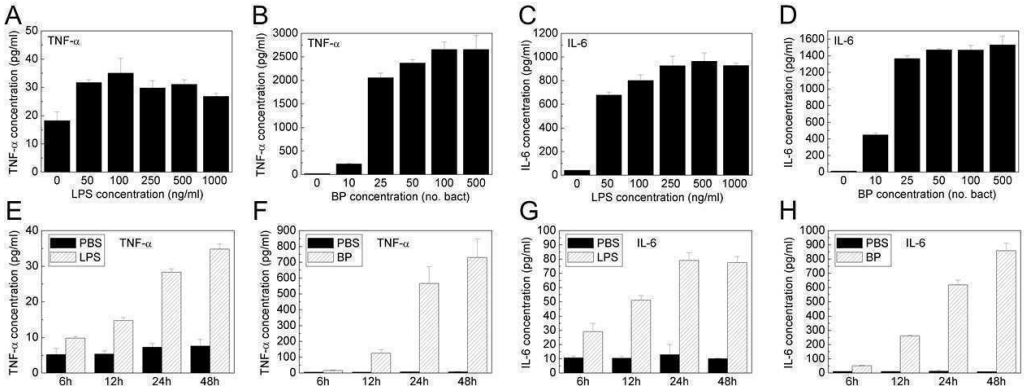

Figure 9.2. Concentration and time dependence of LPS and BP treatment. Mast cells were stimulated with LPS and BP with different concentration indicated on the graphs (**A-D**) for 24 hours. For time dependence studies 100 ng/ml LPS and 100 bacteria/cell BP was used for 0, 12, 24, 48 hours (**E-H**).

Based on the concentration and time dependence studies we chose 100 ng/ml and 100 bacteria/cell as a concentration for LPS and BP and 24 hours as incubation time.

9.2 Appendix B – Tim edependence of increase in mast cells ratio in the peritoneal cavity after FhTeg treatment

Mast cell ratio in the peritoneal cavity is increased after 2 weeks of *Fasciola hepatica* infection and it can be mimicked with FhTeg treatment. To investigate if mast cells are at the site of infection in early stages, before the adaptive Th2 immune response is developed, mice were treated with FhTeg and FhES and percentages of mast cells in the peritoneal cavity was measured by flow cytometry. Mast cell ratio increased in the peritoneal cavity in all tested time points (4 h: 25.8 ± 29.6%, $p = 0.45$; 48 h: 28.7 ± 15.2%, $p = 0.15$), although was only significant after 1 week (60.55 ± 11.3, $p \leq 0.05$) of treatment. FhES had no effect mast cell ratio at these time points (Figure 9.3).

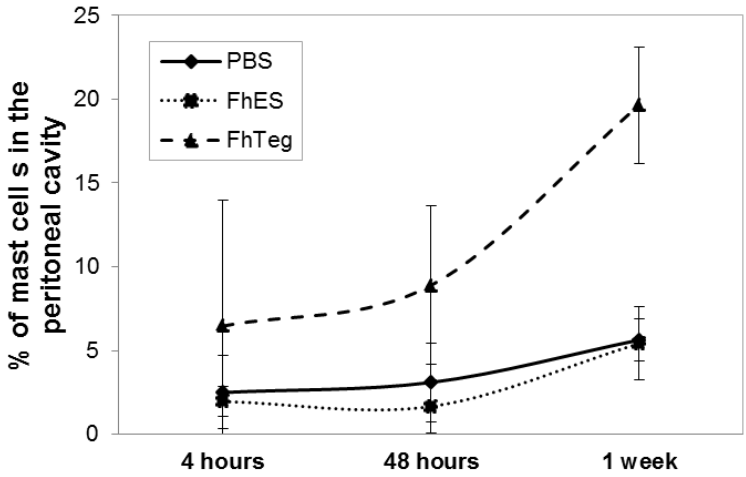

Figure 9.3. Mast cell ratio increased in the peritoneal cavity of mice after FhTeg but not FhES treatment. BALB/C mice were i.p. treated with 10 µg of FhTeg or FhES. Mice were sacrificed after 4 h, 48 h and 1 week and peritoneal lavage was performed. Mast cells ratio in the peritoneal cavity was measured by two colour flow cytometry (FcεRI and c-kit) and Kimura staining.

9.3 Appendix C – Experiment to inhibit SOCS3 in BMDCs and BMMCs by zoledronic acid

The best way to prove that SOCS3 is responsible for the inhibition would be to block SOCS3 and see if FhTeg still keeps its effect. As SOCS3-deficient mice die during embryotic development as a result of placental defect (Takahashi et al., 2006), we tried to block it with zoledronic acid (ZA). ZA is an amino-bisphosphonate used in the treatment of osteoarthritis and rheumatoid arthritis. It inhibits the SOCS3 expression in macrophages and one of the main risk factors in patients treated with ZA is the development of SOCS3-related inflammatory disorders, such as Chron's disease (Scheller et al., 2011).

We tried to repeat the experiments of Bringmann et al (Bringmann et al., 2007), where they treated monocyte derived dendritic cells with ZA for one week and showed inhibition of SOCS3. In our experiments BMDCs did not grow properly and they produce high background of nitrite but no IL-12, so it was not possible to compare FhTeg effect on ZA trated and untreated groups (Figure 4.16). We repeated the same experiment in BMMCs. Altough ZA had no effect on mast cells development, the cytokine secretion was impaired upon tratment. ZA had no effect on the suppression of bacterila ligand activation by FhTeg (Figure 4.16).

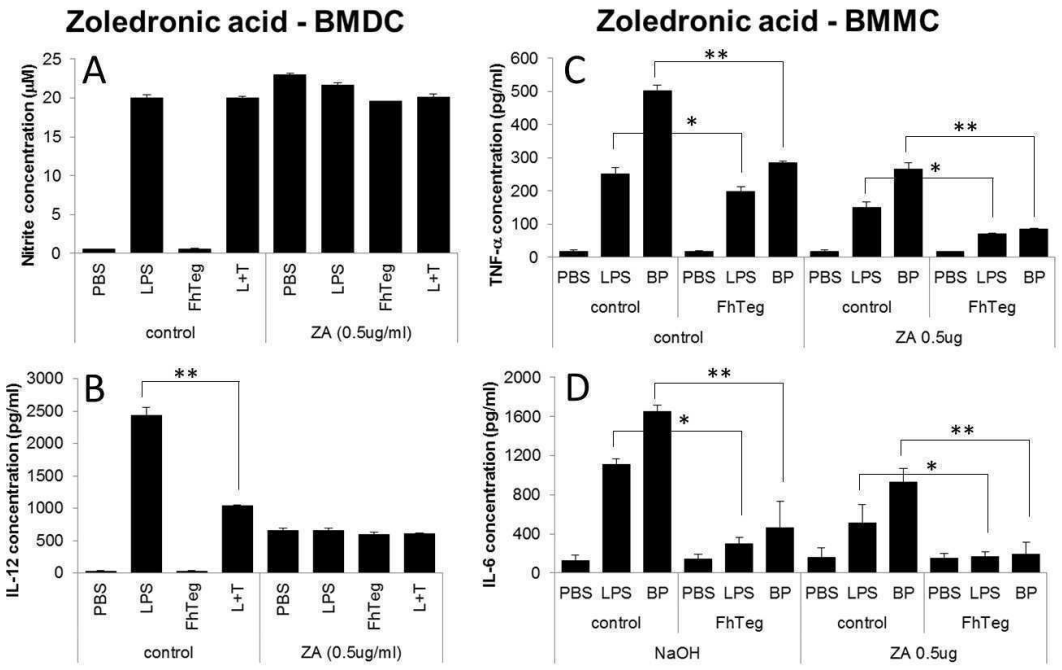

Figure 9.4. Zoledronic acid (ZA) impaired dendritic cell and mast cell function to secret cytokines after bacterial stimulation. BMDCs and BMMCs were treated with ZA (0.5 µg/ml) every second day for one week and then stimulated with LPS (100 ng/ml) or BP (100 bacteria/cell). Nitrite (**A**), IL-12 (**B**) (BMDCs), TNF-α (**C**) and IL-6 (**D**) (BMMCs) were measured after 18/24 h. Data are presented as the mean ± SD of three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$ compared to control group.

Protocols for *in vitro* use of ZA, advise a longer pre-treatment (one week) to gain sufficient inhibition of SOCS3 expression (Bringmann et al., 2007; Fiore et al., 2007; Su et al., 2010). We did not manage to use ZA in our experiments as primer cells were used and ZA impaired their ability to be activated by bacterial ligands. Other method to suppress gene expression, using small interfering (si)-RNA has also some difficulties in primer cells. Involving cell lines in this study could solve this problem, considering the differences between mast cells in their inflammatory responses based on their origin (Hochdorfer et al., 2011; Matsushima et al., 2004; McCurdy et al., 2001; Supajatura et al., 2002).

9.4 Appendix D

Vukman KV, Visnovitz T, Adams PN, Metz M, Maurer M and O'Neill SM

Mast cells cultured from IL-3-treated mice show impaired responses to bacterial antigen stimulation

Inflammation Research 2012, 61:79-85

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9.5 Appendix E

Vukman KV, Adams PN, Metz M, Maurer M and O'Neill SM

Fasciola hepatica tegumental coat impairs mast cell ability to drive Th1 immune responses

The Journal of Immunology 2013, 190(6): 2873-2879

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9.6 Appendix F

Vukman KV, Adams PN, Dowling D, Metz M, Maurer M and O'Neill S

The effect of *Fasciola hepatica* tegumental antigens on mast cell function

International Journal for Parasitology 2013, 43(7): 531-539

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9.7 Appendix G

Vukman KV, Adams PN and O'Neill S

Fasciola hepatica tegumental coat antigen suppresses MAPK signalling in dendritic cells and up-regulates the expression of SOCS3

Accepted for publication (Parasite Immunology)

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9.8 Appendix H

Vukman KV, Aldridge A, Ravidà A, Adams PN and O'Neill S
Mannose receptor and macrophage galactose lectin are involved in *Bordetella pertussis* mast cell interaction

Accepted for publication (Journal of Leukocyte Biology)

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Publications during PhD

Dowling DJ, Noone CM, Adams PN, Vukman KV, Molloy SF, Forde J, Asaolu S and O'Neill SM: *Ascaris lubricoides* pseudocoelomic body fluid induces a partially activated dendritic cell phenotype with Th2 promoting ability *in vivo*. *International Journal of Parasitology*, 2010, 41(2):255-61

Vukman KV, Adams PN, Metz M, Maurer M and O'Neill SM: *Fasciola hepatica* tegumental antigens suppress mast cell driven Th1 immune responses. *Immunology* 2012, 137(s1):133 (Conference paper)

Vukman KV, Visnovitz T, Adams PN, Metz M, Maurer M and O'Neill SM: Mast cells cultured from IL-3 treated mice show impaired responses to bacterial antigen stimulation. *Inflammation research* 2012, 61:79-85

Vukman KV, Adams PN, Metz M, Maurer M and O'Neill SM: *Fasciola hepatica* tegumental coat impairs mast cell ability to drive Th1 immune responses. *The Journal of Immunology* 2013, 190(6): 2873-2879

Vukman KV, Adams PN, Dowling D, Metz M, Maurer M and O'Neill S: The effects of *Fasciola hepatica* tegumental antigens on mast cell function. *International Journal for Parasitology* 2013, 43(7): 531-539

Vukman KV, Adams PN and O'Neill S: *Fasciola hepatica* tegumental coat antigen suppresses MAPK signalling in dendritic cells and up-regulates the expression of SOCS3. *Accepted for publication (Parasite Immunology)*

Vukman KV, Aldridge A, Ravidà A, Adams PN and O'Neill S: Mannose receptor and macrophage galactose lectin are involved in *Bordetella pertussis* mast cell interaction. *Accepted for publication (Journal of Leukocyte Biology)*

Adams PN, Aldridge A, Vukman KV, Donnelly S and O'Neill S: *Fasciola hepatica* tegumental antigens indirectly induce STAT6 dependent alternative activation of macrophages. *Manuscript in preparation*

Trainings and courses during PhD

2008 - 2009

– 1st year of T3 training program

Code	Title	Credits
BDI501	Introductory Biology, Chemistry and Physics 1-2	7.5
BDI505	Advances in Diagnostic Technology 1-2	5
BDI508	Introduction to Biology and Chemistry 1-2	5
BE518	Cell Recognition and Targeting 1-2	7.5
BE519	BPS Optional Module 1-2	5
BE520	Functional Definitions of Drug Targets 1-2	7.5
BE552	Advanced Technologies in Therapeutics* 1-2	10
BE598	Literature Review and Presentation 1-2	7.5
BE599	Lab Rotations** 1-2	30
CS508	Drug Design 1-2	5
XXX	Neurotherapeutics 1-2	5
XXX	Workshop in Commercialization/IP	2.5

– * Technical training
- Flow cytometry
- q-PCR

– ** Lab rotations (2x2 months)
- The role of SNARE s in dendritic cells following activation by Toll like receptor ligands (supervisor: Dr Christine Loscher)
- To develop an *in vitro* model system to examine differential activation of mast cells (supervisor: Dr Sandra O'Neill)

2009

– Animal Cell Culture Theory and Techniques (October 2009, NICB)
– MMI: Techniques and Strategies in Molecular Medicine (December 2009, UCD)

2010

– Health and Safety Module 1-2 (January 2010, DCU)
– LAST Ireland Animal Handling Course (January 2010, UCC)
– Out of Hours Access (March 2010, DCU)
– Genomics and Proteomics Technical road show (March 2010, NICB)

2011

– Molecular Biology Seminar (May 2011; DCU, Dublin)
– Experimental Design Workshop (February 2011; DCU, Dublin)

Research trips

- Charité Universitätmedizin Berlin, 1 week visit (November, 2009)
  - Mast cell culture techniques
- University College Dublin, Ireland, 2 days visit (February, 2011)
  - Histology
  - Microscopic techniques

Conferences, Meetings and workshops

2010

- School of Biotechnology Annual Research Day (January 2010, DCU)
- EMBL: Bring the Excellence of the EMBL to Ireland (February 2010, TCD)
- Innate Immunity: Mechanisms Linking with Adaptive Immunity, Keystone symposia (June 2010, TCD)
- Cell Symposia: Inflammation and Diseases (September 2010, Lisbon, Portugal): ***poster*** "Inflammatory Responses in Mast Cells to Different Th1 Stimuli"
- 25th Anniversary Symposium Annual Meeting "Autoimmunity" (Irish Society for Immunology) (October 2010; TCD, Dublin)
- VI. Meeting of the Irish Cytometry Society (November 2010; Dublin)

2011

- BPS Research Day (January 2011; DCU, Dublin): ***poster*** "SNARE Profiles in Proinflammatory Mast Cells"
- Biotechnology Research Day (January 2011; DCU, Dublin): ***poster*** "Inflammatory Responses in Mast Cells to Different Th1 Stimuli"
- BRS Postgraduate talks (March 2011; DCU, Dublin): ***talk*** "Strategies to inhibit inflammatory responses in mast cells"
- UK & Ireland NF-κB Workshop (June 2011; NUIM, Maynooth)
- International Mast Cell and basophil Meeting (November 2011, Southampton, United Kingdom): ***poster*** "Tegumental antigen of *Fasciola hepatica* (FhTeg) suppresses inflammatory immune responses in mast cells"

2012

- Irish Society for Parasitology 2012 Meeting (January 2012, TCD): ***talk*** "*Fasciola hepatica* tegumental antigens impair mast cell ability to drive Th1 immune responses"
- School of Biotechnology Annual Research Day (January 2012, DCU): ***talk*** *Fasciola hepatica* tegumental antigens impair Mast Cells ability to drive Th1 immune responses by blocking TLR-driven pro-inflammatory responses
- 75th Anniversary of Albert Szent-Györgyi's Nobel Prize Award (March 2012, Szeged, Hungary): ***talk*** "*Fasciola hepatica* tegumental antigens suppress Th1 promoting mast cells"
- Institute of Animal Technology Ireland Branch Symposium (May 2012, TCD, Dublin)
- Special Symposium of Immunology (July 2012, TCD, Dublin)

- European Congress of Immunology (September 2012, Glasgow, United Kingdom): ***talk*** "*Fasciola hepatica* tegumental antigens suppress mast cell driven Th1 immune responses"

Curriculum vitae

Personal information:

Name:	**Krisztina V. Vukman**
Address:	Jókai Mór utca 22. Dunaharaszti, H-2330
Mobil:	+36-20-437-1474
E-mail:	**sztitussz@gmail.com**

Marital status:	married
Nationality:	Hungarian
Date of birth:	21st October 1982

Education and Qualification:

2008 –	Targeted Therapeutics and Theranostics School of Nursing and Human Science, Dublin City University Dublin, Ireland 4th year PhD student
2011	Last Ireland animal handling course Licence to perform procedures on animals
2001 – 2006	Faculty of Science, Eötvös Loránd University Budapest, Hungary MSc Biologist
2001 – 2002	Course of Mycology Certificated Inspector of Mushrooms
1992 – 2001	Nagy László Secondary School Final examination at secondary school

Work experience:

2006 – 2008	SOLVO Biotechnology Budaörs, Hungary Scientist: Member of Toxicology Group
2006	Durham University Durham, United Kingdom 2 mounts internship

Topics of researches:

2009 –	To investigate Toll-like receptor activated mast cells (DCU, Ireland)
2008 – 2009	Training program in Immunology, Neurobiology and Chemistry (DCU, Ireland)
2006 – 2008	ABC transporters and toxicology (SOLVO, Hungary)
2006	Bacterial co-expression systems (Durham University, UK)
2003 – 2006	Chemotaxonomy of *Ganoderma* species (ELTE, Hungary)

Used methods:

Animal work (mouse):

Intraperitoneal injection
Cervical dislocation
Peritoneal and lung lavage
Bone marrow isolation
Blood and plasma collection

Cell culture techniques:

Cell line culture (WEHI-3, MC/9, raw MQ, Caco, MDCK, CHO etc.)
Primary cell culture (peritoneal and bone marrow derived mast cells and macrophages, bone marrow derived dendritic cells, and T-cells and B-cell isolation from spleen)
Co-culture

Cell culturing (animal, fungal and bacterial)
Cytotoxicity and sensitization tests
Monolayer techniques (Caco, MDCK)

Techniques of microscopy:

Conventional-, inverse-, polarization-, fluorescent-, DIC- microscopy

Methods of molecular biology:

PCR and qPCR techniques
Cloning
Agarose gel electrophoresis and DNA extraction from the gel
PAGE (native, SDS, gradient etc.)
Western blot

Biochemical methods:

ATPase activity measurements
Vesicular transport assays
Uptake assays
Cell based biochemical assays
ELISA/EIA

Other methods:

Flow cytometry (BD Facs Aria and Calibur)
Mating type identification by fungi
HPLC, TLC

Organisation memberships:

2011 – Irish Society for Parasitology (ISP; Ireland)
2011 – Federation of the Societies of Biochemistry (FEBS; Europe)
2011 – Hungarian Societies of Biochemistry (Hungary)
2010 – Irish Society of Immunology (Ireland)
2008 – Biological Research Society (Ireland)
2006 – Hungarian Biological Association (Hungary)

Congresses and Symposia:

2013:
ISP Scientific meeting, Dublin, Ireland (*talk*)

2012:
European Congress of Immunology, Glasgow, United Kingdom (*talk*)

Special Symposium on Immunology, Dublin, Ireland

Institute of Animal Technology Ireland Branch Symposium, Dublin, Ireland

75th Anniversary of Albert Szent-Györgyi's Nobel Prize Award, Szeged, Hungary (*talk)*

ISP Scientific Meeting, Dublin, Ireland (*talk*)
School of Biotechnology Annual Research Day, Dublin, Ireland (*talk*)

2011:
International Mast Cell and basophil Meeting, Southampton, United Kingdom (*poster*)
Biological Research Society Postgraduate Talks, Dublin, Ireland (*talk*)
UK & Ireland NF-kB Workshop, Maynooth, Ireland
Molecular Biology Seminar, Dublin, Ireland
Experimental Design Workshop Dublin, Ireland
Biotechnology Research Day, Dublin, Ireland (*poster*)
BPS Research Day, Dublin, Ireland (*poster*)

2010:
VI. Meeting of the Irish Cytometry Society, Dublin, Ireland
Cell Symposia: Inflammation and Diseases, Lisbon, Portugal (*poster*)
25th Anniversary Symposium Annual Meeting "Autoimmunity", Dublin, Ireland
Innate Immunity: Mechanisms Linking with Adaptive Immunity, Keystone symposia, Dublin, Ireland
Genomics and Proteomics Technical road show, Dublin, Ireland
EMBL: Bring the Excellence of the EMBL to Ireland, Dublin, Ireland
School of Biotechnology Annual Research Day, Dublin, Ireland

2009:
MMI: Techniques and Strategies in Molecular Medicine, Dublin, Ireland
Animal Cell Culture Theory and Techniques, Dublin, Ireland

2008:
2nd FEBS Special Meeting co-sponsored by FEMS on ATP binding cassette (ABC) transport proteins: From multidrug resistance to genetic diseases, Insbruck, Austria
SOLVO Transporter Symposium and Training, Budaörs, Hungary (*demonstration of assays*)

2005:
Congress of Scientific Students' Association of Eötvös University (TDK), Budapest, Hungary (*poster*)

Publications:

Vukman KV, Adams PN and O'Neill S: *Fasciola hepatica* tegumental coat antigen suppresses MAPK signalling in dendritic cells and up-regulates the expression of SOCS3. *Accepted for publication (Parasite Immunology)*

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Dowling DJ, Noone CM, Adams PN, Vukman KV, Molloy SF, Forde J, Asaolu S and O'Neill SM: *Ascaris lubricoides* pseudocoelomic body fluid induces a partially activated dendritic cell phenotype with Th2 promoting ability *in vivo*. *International Journal of Parasitology*, 2010, 41(2):255-61

Dreiseitel A, Oosterhuis B, Vukman KV, Schreier P, Oehme A, Locher S, Hajak G and Sand PG: Berry anthocyanins and anthocyanidins exhibit distinct affinities for the efflux transporters BCRP and MDR1. *British Journal of Pharmacology* 2009: 158(8): 1942-50

Oosterhuis B, Vukman K, Vagi E, Glavinas H, Jablonkai I and Krajcsi P: Specific interactions of chloroacetanilide herbicides with human ABC transporter proteins. *Toxicology* 2008, 248(1): 41-51

Conference papers:

Vukman KV, Adams PN, Metz M, Maurer M and O'Neill SM: *Fasciola hepatica* tegumental antigens suppress mast cell driven Th1 immune responses. *Immunology* 2012, 137(s1):133 (Conference paper)

Oosterhuis B, Vukman K, Vagi E, Glavinas H, Jablonkai I and Krajcsi P: Interaction of ABC transporters with environmental toxicants. *Toxicology Letters* 2009, 189: S114-5 (Conference paper)

Oosterhuis B, Vukman K, Vagi E, Glavinas H, Jablonkai I and Krajcsi P: Chloroacetanilide herbicides interact with human efflux transporters and modulate in vitro drug absorption. *Toxicology Letters* 2009, 189: S109 (Conference paper)

Oosterhuis B, Vukman K, Vagi E, Glavinas H, Krajcsi P and Jablonkai I: Pesticides and their metabolites interact with human efflux transporters. *Drug Metabolism Reviews* 2007, 39(1): 294 (Conference paper)

Personal Skills:

Good team player and can work independently as well, experience in writing scientific reports, solving scientific problems, etc.

Languages:

Mother language:	Hungarian
Other languages:	English (C1; Hungarian State Language Certification, intermediate level - C)
	German (B1; Hungarian State Language Certification, intermediate level - A)

Computer skills:

Microsoft Office (Word, Excel, Power-point), Photoshop, Origin, Flow Jo, Origin, ImageJ etc. software at user level and basic knowledge of Pascal/Delphi programming language

Driving Licence:

Driving Licence Category “B”