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Immunomodulatory activity of products from the helminth parasite *Fasciola hepatica*

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M.Sc. Biotechnology

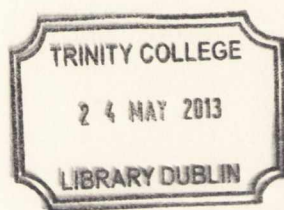


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of
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ABSTRACT

Helminths have evolved strategies to evade host immune responses and these have been shown to be associated with simultaneous subversion of immune responses towards bystander antigens. This phenomenon forms the basis of the hygiene hypothesis and partially explains the lower incidence of allergy and autoimmune disease observed in areas of developing countries where helminth infections are endemic. Moreover, evidence from human clinical trials has demonstrated that helminth infections can attenuate disease symptoms in patients with allergy, inflammatory bowel disease and multiple sclerosis (MS). Therefore a greater understanding of how helminth parasites modulate the immune response could give invaluable insight into the host regulatory mechanisms that control allergy and autoimmunity. Furthermore, the immunomodulatory molecules secreted by helminth parasites, or their synthetic analogues, could provide novel therapeutics against immune-mediated diseases such as MS.

This study focused on characterisation of potential immunomodulatory properties of excretory/secretory (ES) products of the helminth parasite *Fasciola hepatica* and on an evaluation of *F. hepatica* ES-derived immunomodulatory factors as therapeutics in experimental autoimmune encephalomyelitis (EAE), an animal model for MS. The study found that the administration of *F. hepatica* ES delayed the onset and reduced the incidence of EAE in mice, demonstrating that live parasites are not necessary for helminth-induced attenuation of autoimmunity. Furthermore, *F. hepatica* ES was also shown to suppress development of dextran sodium sulphate-induced (DSS)-colitis in mice. Collectively these findings demonstrate that *F. hepatica*-induced immunosuppression was mediated by soluble and stable factors produced by the parasite *in vitro* which is important for future identification and isolation of immunomodulatory molecules.

In order to dissect the immunomodulatory mechanisms triggered by the helminth products, this study examined direct effects of *F. hepatica* ES on activation of DCs and T cell responses *in vitro*. *F. hepatica* ES suppressed TLR-ligand induced maturation and production of IL-12p70, IL-6 and IL-23 by DCs, while promoting the release of regulatory cytokine TGF- β . *F. hepatica* ES significantly suppressed polyclonal activation and cytokine production by CD4⁺ T cells and inhibited IL-17 production by $\gamma\delta$ T cells stimulated with IL-1 β and IL-23. The factors mediating these immunomodulatory effects segregated into a low molecular fraction of *F. hepatica* ES (< 3kDa; ES-L). Although ES-L showed strong immunosuppressive effect on activation of DCs and T cells *in vitro* this fraction had no effect on the attenuation of EAE. The suppression of MOG-induced EAE was mediated by proteins segregating to high molecular weight fraction of *F. hepatica* ES (> 3 kDa; ES-H) which appeared to have no modulatory effects on T cells and DCs *in vitro*. The attenuation of EAE by ES-H was associated with a reduced frequency of Th1 and Th17 cells and an increased frequency of Th2 cells infiltrating brains of treated mice. ES-H also suppressed expression of pro-inflammatory cytokines and chemokines in CNS and significantly decreased the total numbers of infiltrating T cells. In contrast, ES-H treatment substantially increased the absolute numbers of IFN- γ , or IL4 and IL-13 producing T cells in the peripheral lymph nodes. Furthermore, ES-H reduced the ability of lymph node cells to produce IL-17 in response to MOG but did not affect production of MOG-specific IL-4 or IFN- γ .

The current study further identified fraction Fr.1 that was responsible for immunosuppressive effect of ES-H in EAE. Mass spectrometry analysis revealed that Fr.1 contained FhHDM-1, FhTPx and Cys-1. These proteins, or their homologues from other helminth species, have known immunomodulatory properties, but none have been evaluated in models of autoimmunity. Therefore these *F. hepatica*-derived proteins are interesting and novel candidates for potential therapeutics.

The data presented in this work constitute a solid foundation for further investigation into specific immunomodulatory factors secreted by *F. hepatica* and could contribute to the development of novel and effective treatments against autoimmune disorders.

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cell
BBB	Blood-brain barrier
BFA	Brefeldin A
BMDMs	Bone marrow derived macrophages
BSA	Bovine serum albumin
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen-induced arthritis
CLR	C-type lectin receptors
CNS	Central nervous system
CpG	Cytosine-guanine dinucleotide
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CXCL	Chemokine (C-X-C motif) ligand
DCs	Dendritic cells
DC-SIGN	DC-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin
DMSO	Dimethyl sulfoxide
DSS	Dextran sodium sulphate
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ES	Excretory-secretory products
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum

FhCatL	<i>Fasciola hepatica</i> cathepsin L
FhHDM-1	<i>Fasciola hepatica</i> helminth defence molecule-1
FhTPx	<i>Fasciola hepatica</i> thioredoxin peroxidase
Foxp3	Forkhead box P3
GA	Glatiramer acetate
GM-CSF	Grnulocyte-macrophage colony stimulating factor
IBD	Inflammatory bowel disease
IFN- β	Interferon- β
IFN- γ	Interferon- γ
IL	Interleukin
IRF	Interferon regulatory factor
LDH	Lactate dehydrogenase
LN	Lymph node
LPS	Lipopolisaccharide
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NF- κ B	Nuclear factor κ B
NK cell	Natural killer cell
NOD	Non-obese diabetic
OVA	Ovalbumin
PAMPs	Pattogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PC	Phosphorylcholine
PLP	Proteolipid protein
PMA	Phorbol myristate acetate
PML	Progressive multifocal leukoencephalopathy
PRRs	Pattern recognition receptors
PS	Phosphatidylserine

PT	<i>Pertussis</i> toxin
ROR γ t	Retinoic acid receptor-related orphan receptor γ t
RR-MS	Relapsing-remitting MS
S1P	Sphingosine-1-phosphate
SEA	<i>S. masoni</i> egg antigen
STAT	Signal transduced and activator of transcription
T1D	Type 1 diabetes
TCR	T cell receptor
TGF- β	Transforming growth factor- β
Th cell	T helper cell
TLR	Toll-like receptor
Treg cells	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
VLA-4	Very late antigen-4

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General Introduction

Chapter 1

1.1 *Innate and adaptive immune responses in infection*

The mammalian immune system is a complex network of cells, tissues and organs that has evolved to protect the body against infectious pathogens. Its ability to distinguish self from non-self or abnormal tissues enables it to successfully defend against invasion by various foreign organisms and cancer. In order to maintain the body's homeostasis, immunoregulatory mechanisms have simultaneously evolved to ensure tolerance to the body's own tissues, symbiotic microflora and other innocuous ingested or inhaled antigens (Janeway, 2001).

The immune system consists of innate and adaptive arms. The innate immune system constitutes the first line of defence. It recognises threats on the basis of conserved microbial components termed pathogen associated molecular patterns (PAMPs) and induces a rapid but unspecific and short-lived response to the invading pathogens. In contrast, the adaptive immune system acts at a later phase of infection and provides a pathogen-tailored, antigen-specific response followed by life-long immunological memory. Both arms of the immune system cooperate and complement one another; the adaptive immune response is directly shaped by signals delivered by the innate immune system (Janeway, 2001).

1.1.1 Innate immunity

The effector mechanisms of the innate immune system are mediated by phagocytic cells such as macrophages and granulocytes, including neutrophils, and eosinophils that are very efficient at killing extracellular bacteria, fungi and parasites. While these cell types have their own distinct effector functions, they can also influence the induction of adaptive responses through production of cytokines and chemokines. A direct link between the innate and adaptive immune responses is provided by antigen presenting cells (APC) especially macrophages and dendritic cells (DCs) that engulf and process pathogens and present peptide antigens to T cells to initiate adaptive immune responses.

Dendritic cells

DCs are a heterogeneous class of professional APC that have a potent capacity to initiate and direct adaptive immune responses. As powerful orchestrators of immunity, DCs are distributed throughout the tissues of the entire body, particularly in the skin and mucosal surfaces which are directly exposed to contact with pathogens. Immature DCs constantly sample the surrounding environment and internalise encountered pathogens and antigens via several mechanisms including phagocytosis and receptor-mediated pinocytosis. These cells process and display fragments of antigen as peptide complexes, bound to MHC class I or II molecules. In their immature state, DCs express low levels of MHC class II and co-stimulatory molecules on their surface and are therefore unable to prime naive T cells. Activation and maturation are triggered by recognition of pathogens through specific intra and extra cellular pattern recognition receptors (PRR), including Toll-like receptors (TLR) and C-type lectin receptors (CLR) (Geijtenbeek and Gringhuis, 2009; Kawai and Akira, 2010). Ligation of these receptors allows migration of DCs and transport of antigens from the periphery to lymphoid tissues. Activated DCs shut off their antigen capturing and processing capacity and increase surface expression of co-stimulatory molecules, such as CD40, CD80 or CD86 that allow them to effectively present the antigen and provide T cells with signals required for their activation. Depending on the PAMP stimulation, DCs produce cytokines that help to shape the phenotypic lineage of differentiating T cells (Kapsenberg, 2003). Presentation of antigens by DCs without PRR activation or up-regulation of necessary co-stimulatory molecules can lead to the induction of tolerance in the immune system (Mahnke et al., 2007).

Innate immune recognition

TLRs are the most studied family of PRRs that recognise PAMPs derived from viruses, bacteria, fungi, and parasites. These include lipoproteins (recognised by TLR1, TLR2, and TLR6), double-stranded RNA (TLR3), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), single-stranded RNA (TLR7 and TLR8), and cytosine-guanine dinucleotide (CpG) motifs from bacterial DNA (TLR9) (Akira et al., 2006). Activation of TLRs by their ligands results in the

initiation of downstream signalling pathways, such as nuclear factor κ B (NF- κ B), interferon regulatory factor (IRF) and mitogen-activated protein kinase (MAPK) pathways culminating in the induction of genes involved in inflammatory responses (Akira et al., 2006).

TLR-induced MAPK signalling pathways have been shown to regulate many aspects of maturation and cytokine production by DCs (Nakahara et al., 2006). It has been demonstrated that distinct TLR agonists differentially modulate signalling pathways leading to phosphorylation of extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK and therefore induce different maturation and cytokine secretion profiles of DCs (Agrawal 2003). ERK and p38 MAPK in particular play a role in regulation of LPS-induced production of IL12p70, IL-23 and IL-10 by DCs (Brereton et al., 2009; Goodridge et al., 2003; Jarnicki et al., 2008).

1.1.2 Adaptive immune response

To ensure pathogen clearance followed by life-long immunity, the immune system has to develop an appropriate set of adaptive effector and memory responses that are tailored to the specific type of pathogen. Although DCs are the initiators of the immune response, B and T cells drive protective immune responses following recognition of antigens via their antigen-specific surface receptors. B cells mediate humoral immune responses producing antibodies that neutralise toxins or viruses and opsonise extracellular pathogens to facilitate their destruction by other cells of immune system. In contrast T cells are responsible for generation of cell-mediated immunity. This involves recruitment and activation of phagocytes and direct killing of infected cells (Janeway, 2001).

T cells recognise MHC-antigen complexes through the cell surface expressed heterodimeric T cell receptor (TCR). In the majority of T cells the receptor is composed of two trans-membrane glycoprotein α and β chains, however some, so called $\gamma\delta$ T cells, express an alternative but structurally similar receptor consisting of γ and δ chains (Kaufmann, 1996).

Classically $\alpha\beta$ T cells can be subdivided into several functional subpopulations. Cytotoxic CD8⁺ T cells (CTLs) are responsible for direct killing of abnormal and infected cells while CD4⁺ T helper cells (Th) play a major role in mediating and regulating immune responses through the secretion of specific cytokines. The T helper cells can be further divided into subtypes based on their cytokine expression profile and associated functions (Janeway, 2001).

T helper cell differentiation

Differentiation of naive T cells requires three signals provided by APCs and other cells of the immune system. Signal 1 is delivered to the T cell by the APC through activation of the TCR by recognition of specific MHC-antigen complex. Signal 2 is generated by ligation of co-stimulatory molecules displayed by T cells, such as CD28, CTLA-4, with CD80 and CD86 on the surface of APC. In the absence of this second signal, T cells become anergic, failing to induce an antigen-specific immune response, which can lead to immune tolerance. Finally, cytokines produced mainly by APC and other cells of innate immune system act as a third signal directing naive T cell into one of a number of functionally distinct Th subtypes (Kapsenberg, 2003). Th cells have been divided into 4 distinct subsets termed Th1, Th2, Th17 and regulatory Treg cells.

Th1 and Th2 cells

Mosmann and Coffman first proposed a model whereby CD4⁺ Th cells, emerging in the course of an adaptive response, could be divided into two subsets representing different biological properties (Mosmann et al., 1986). These subsets, referred to as Th1 and Th2 cells, were distinguished on the basis of helper functions mediated through the cytokines they secrete. The activity of Th1 cells depends on the secretion of interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) that allows them to activate macrophages and natural killer (NK) cells and thus augment protection against intracellular bacterial and viral pathogens. In contrast Th2 cells secrete IL-4, IL-5, and IL-13, the cytokines responsible for the induction of B cell expansion, antibody isotype class switching, eosinophil recruitment and mast cell degranulation all of which aid

the development of a humoral response that protects against parasites and extracellular bacteria (Kapsenberg, 2003).

Differentiation of naive T cells into one of the effector Th cell subsets depends primarily on the signals provided by DCs through co-stimulatory molecules and secreted cytokines. The activation status of DCs and the cytokines they produce are influenced by factors that induced their maturation. Therefore the profile of developing effector T cells can be tailored to the specific pathogen.

IL-12p70, a pro-inflammatory cytokine produced by APC, together with IFN- γ produced by pathogen-activated lymphocytes, such as NK cells, are the main polarizing cytokines in the differentiation of naive T cells towards a Th1 subset (Kapsenberg, 2003). Other cytokines, such as IL-27 and type I interferons produced by DCs may also be involved (de Jong et al., 2005). The T-box transcription factor (T-bet) induced in differentiating T cells by polarising cytokines is critical for the development of Th1 cells (Flavell et al., 1999). T-bet not only significantly enhances the production of IFN- γ and expression of IL12R β 2, thus ensuring expansion of Th1 cells but also suppresses signalling that could result in production of IL-4. *Tbx21*^{-/-} (T-bet knockout) cells have severe defects in Th1 cell differentiation both *in vitro* and *in vivo* (Zhu et al., 2010)

Differentiation of Th2 cells is dependent on IL-4 induced signalling that promotes downstream activation of STAT6 and expression of Th2 signature transcription factor GATA3. It is believed that basophils may provide the early source of IL-4 required for Th2 cell differentiation (Sokol et al., 2008). DCs activated with Th2 promoting factors also provide signals such as CCL2 and OX40L, that are involved in polarisation of naive T cells towards the Th2 phenotype (Ohshima et al., 1998) Induction of Th2 cells *in vivo* is augmented by epithelial cell-derived mediators such as thymic stromal lymphopoietin (TSLP), IL-33 or IL-25 (Pulendran et al., 2010).

IFN- γ and IL-4 produced by Th1 and Th2 cells can further promote the development of their own lineage stabilising expression of respective cytokines

and transcription factors through positive feedback. However, these polarising cytokines can also negatively regulate differentiation and growth of opposite lineages. IFN- γ , produced by Th1 cells, suppresses transcription of GATA3 in developing T cells and inhibits their commitment to Th2 phenotype. Likewise, IL-4 from Th2 cells enhances the expression of GATA3 and suppresses the development Th1 cells (Zhu et al., 2010).

Allergy and asthma are common Th2-mediated inflammatory conditions, while autoimmune diseases have long been thought to depend on Th1-mediated responses to auto-antigens. More recent discoveries in the field of T cell immunology have demonstrated that a novel IL-17-producing T cell subset designated Th17 cells, may be the main mediator of pathology in many autoimmune disorders (Aggarwal et al., 2003; Langrish et al., 2005).

Th17 cells

Th17 cells are characterised by production of IL-17 and exhibit effector functions distinct from Th1 and Th2 cells. These cells are abundant at mucosal surfaces, where they combat infections caused by pathogenic bacteria and fungi (Weaver et al., 2007). In addition to the signature cytokine IL-17A (often referred to as IL-17), these cells also produce IL-17F, IL-21, GM-CSF and IL-22. Cytokines produced by Th17 cells act on a broad range of cell types to induce coordinated expression of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6,) and chemokines (CXCL1, CXCL8, CXCL10), that recruit and activate macrophages and neutrophils involved in pathogen clearance (Weaver et al., 2007). Th17 cells have also been implicated in a range of autoimmune and inflammatory diseases in mice and humans. Many conditions previously thought to be associated with Th1 cells, such as, experimental autoimmune encephalomyelitis (EAE, a murine model for multiple sclerosis) (Langrish et al., 2005), collagen-induced arthritis (CIA) (Bush et al., 2002), and some forms of colitis (Zhang et al., 2006), have now been shown to be mediated, at least in part by Th17 cells.

Initial studies leading to the discovery of Th17 lineage have demonstrated that IL-23 is critical for the development of Th17 cells, as mice deficient in IL-23

have reduced Th17 responses and are resistant to Th17-mediated autoimmune diseases. However, it is now believed that IL-23 is necessary for expansion of Th17 cells but not for the initial differentiation from naive precursors, as naive CD4⁺ T cells do not express the IL23R (McGeachy et al., 2009).

Several groups have reported that a combination of the pro-inflammatory cytokine IL-6 along with the TGF- β is required for the differentiation of Th17 cells from naive T cells (Mangan et al., 2006). Mechanistic studies by Das *et al.* demonstrated that TGF- β indirectly promotes differentiation of Th17 cells by inhibiting expression of STAT4 and GATA3, thus blocking development of Th1 and Th2 cells while IL-6 activates STAT3 leading to the expression of transcription factor ROR γ t (Das et al., 2009). ROR γ t is the signature transcription factor of Th17 cells required for the production of IL-17. Mice deficient in ROR γ t have reduced numbers of Th17 cells and are resistant to EAE (Ivanov et al., 2006).

IL-1 is another pro-inflammatory cytokine implicated in the development of Th17 cells. Studies by Sutton *et al.* showed that mice deficient in the IL-1 type I receptor (IL-1RI) had reduced antigen-specific Th17 responses, thus were resistant to actively induced EAE. However, these mice were susceptible to disease induced by transfer of antigen-specific IL-17-producing T cells indicating that IL-1 signalling is required for expansion of Th17 cells (Sutton et al., 2006).

Differentiation of Th17 cells can be inhibited by signalling induced by IFN- γ and IL-4 therefore Th1 and Th2 cells can suppress development of Th17 cells. Moreover, Th17 phenotype is unstable and under certain conditions these cells can become IL-17 and IFN- γ double producers or can completely switch off production of IL-17 in favour of IFN- γ (Hirota et al., 2011). The mechanism of Th17 cell plasticity is not fully understood and some reports suggest that Th2 cells may also be susceptible to similar phenotypic switch (Hegazy et al., 2010). Nevertheless, it has been demonstrated that redirecting the Th cell balance

towards a Th2 response can suppress autoimmunity (Sewell et al., 2003; Shaw et al., 1997).

Regulatory T cells

In contrast to the pro-inflammatory T cell subsets described above, regulatory T cells (Treg) play a central role in maintaining self tolerance and restraining excessive inflammatory responses in the mammalian immune system. Although a large number of distinct T cell subsets with regulatory activity have been described in recent years, the current convention broadly segregates these cells on the basis of their origin into two pre-eminent subsets, natural Treg (nTreg) cells and inducible Treg (iTreg) cells (Curotto de Lafaille and Lafaille, 2009). Natural Treg cells are generated in the thymus along with conventional naive CD4⁺ T cells through a tightly regulated homeostatic process of TCR affinity selection (Sakaguchi, 2005). In contrast, inducible Treg cells adaptively develop in the periphery from naive or central memory T cells under the influence of immunoregulatory signals in a process known as peripheral conversion (Feuerer et al., 2009).

The discovery that deficiency in the expression of transcription factor forkhead box P3 (Foxp3) resulted in the multiorgan autoimmunity observed in *scurfy* mice led to the identification of Foxp3 as a firm molecular signature of Treg cells (Zheng and Rudensky, 2007). Foxp3 controls the transcription of several Treg cell associated genes and is essential to establish a functional regulatory T cell lineage although some iTreg, such as Tr1 cells, do not express Foxp3 (Curotto de Lafaille and Lafaille, 2009).

Inducible Treg cells

In contrast to thymically derived nTreg, iTreg cells differentiate in the periphery from antigen-committed conventional T cells under conditions of suboptimal antigenic stimulation or in the presence of an immunoregulatory cytokine milieu. A variety of experimental approaches have been used to generate suppressive iTreg cells, with somewhat variable properties and as

such they represent a heterogeneous group of cells. Most subsets of iTreg described to date inhibit pro-inflammatory responses primarily through the secretion of immunosuppressive cytokines. As such, IL-10 secreting Tr1 cells (O'Garra and Vieira, 2004) and TGF- β secreting Th3 cells (Weiner, 2001) are classified by their cytokine production rather than by surface or intracellular markers.

Antigen-specific IL-10 producing Tr1 cells have been experimentally generated via an IL-10-dependent process *in vivo* by repetitive administration of suboptimal doses of specific antigen and *in vitro* by priming naive T cells with antigen in the presence of IL-10 and immunosuppressive drugs (Roncarolo et al., 2006; Vieira et al., 2004). Although Tr1 cells do not express Foxp3 they are functionally similar to CD25⁺Foxp3⁺ nTreg cells as they also fail to produce IL-2, but respond to specific antigens by the secretion of IL-10 and are capable of inhibiting inflammatory diseases *in vivo* (Barrat et al., 2002).

Weiner and colleagues have shown that induction of oral tolerance to myelin antigens *in vivo*, by repeated administration of antigenic peptides, resulted in suppression of EAE by generation of antigen specific Th3 cells that secreted TGF- β , IL-4 and IL-10 (Weiner, 2001). Th3 cells play a crucial role in inducing and maintaining peripheral tolerance by driving the differentiation of antigen-specific Foxp3⁺ regulatory cells in the periphery most likely through the TGF- β they secrete (Carrier et al., 2007).

It is now well established that iTreg cells can develop in the periphery as a result of conversion from naive CD4⁺CD25⁻ T cells into fully functional CD4⁺CD25⁺Foxp3⁺ suppressor cells in a TGF- β dependent process (Chen et al., 2003; Zheng et al., 2007) and are phenotypically indistinguishable from nTreg cells (Chen and Konkel, 2010). TGF- β is a key cytokine involved in the conversion process; it induces the expression of Foxp3 in TCR-stimulated naive CD4⁺ T cells via a mechanism involving activation of STAT3 and nuclear factor of activated T cells (NFAT) (Fantini et al., 2004).

Although DCs are not essential for induction of Foxp3 expression in naive T cells (Chen et al., 2003), several studies have demonstrated that DCs can participate in the process of conversion. Wang *et al.* have shown that splenic DC were capable of promoting the conversion of naive CD4⁺ T cells to inducible

CD4⁺Foxp3⁺ regulatory T cells in the presence of exogenous TGF- β , (Wang et al., 2008). Similarly, CD103⁺ DCs, isolated from the small intestine and the mesenteric LN, were shown to efficiently mediate conversion of naive CD4⁺ T cells into Foxp3⁺ Treg cells through the production of endogenous TGF- β (Coombes et al., 2007; Sun et al., 2007).

$\gamma\delta$ T cells

While most CD3⁺ T cells express classical $\alpha\beta$ TCR, about 5% of T cells bear alternative TCR consisting of γ and δ chains. These $\gamma\delta$ T cells are found in relatively low numbers in tissues of adult mice and humans, however, they constitute the majority of T cells in newborn and young animals (Hayday, 2000). $\gamma\delta$ TCR recognises antigen independent of MHC presentation and, at least in mice, $\gamma\delta$ T cell subsets display limited TCR diversity, suggesting that perhaps these cells represent a conserved, primitive form of immunity (Hayday, 2000; Modlin and Sieling, 2005). It has been proposed that $\gamma\delta$ TCRs functionally resemble danger-sensing receptors and recognise host molecules induced by cellular stress and infection rather than a diversity of microbial antigens (Bonneville et al., 2010). Moreover, these cells respond rapidly to TCR independent stimulation via PRRs and pro-inflammatory cytokines (Martin et al., 2009; Sutton et al., 2009) thus they display some characteristics of innate immune cells.

$\gamma\delta$ T cells form a part of the early defence system against bacterial (Shibata et al., 2007) and fungal (Dejima et al., 2011) infections. Some subsets of $\gamma\delta$ T cells rapidly produce IL-17 following stimulation with pathogens or pro-inflammatory cytokines contributing towards protective immune responses days before the appearance of antigen-specific IL-17⁺ $\alpha\beta$ T cells (Jensen et al., 2008). The importance of $\gamma\delta$ T cell-derived IL-17 is underscored by the fact that mice deficient in these cells are more susceptible to infections where pathogen clearance is highly dependent on IL-17-mediated neutrophil recruitment (King et al., 1999; Moore et al., 2000).

Although their activation resembles that of innate cells, $\gamma\delta$ T cells produce cytokines that control the function of other innate or adaptive immune cells and therefore perform similar cellular functions as conventional $\alpha\beta$ T cells (Modlin

and Sieling, 2005; Sutton et al., 2009). Importantly, it has been shown by Sutton *et al.* that resting $\gamma\delta$ T cells, like differentiated Th17 cells, constitutively express IL23R and can produce IL-17 *in vitro* and *in vivo* after stimulation with IL-23 produced by DCs activated with Th17-promoting pathogen such as *Mycobacterium tuberculosis*. This effect is further enhanced by IL-1 β (Sutton et al., 2009). Moreover, IL-17 and IL-21 produced by $\gamma\delta$ T cells promote IL-17 production by Th17 cells (Sutton et al., 2009). Consistent with these findings IL-17-producing $\gamma\delta$ T cells, similar to Th17 cells, have been implicated in pathogenesis of several IL-17 mediated inflammatory diseases, such as EAE (Spahn et al., 1999) and CIA (Roark et al., 2007), where they may contribute to the pathology directly or by amplifying Th17 responses. Mice deficient in $\gamma\delta$ T cells develop attenuated EAE, highlighting the need for this amplifying loop in the induction and expansion of pathogenic Th17 cells (Sutton et al., 2009).

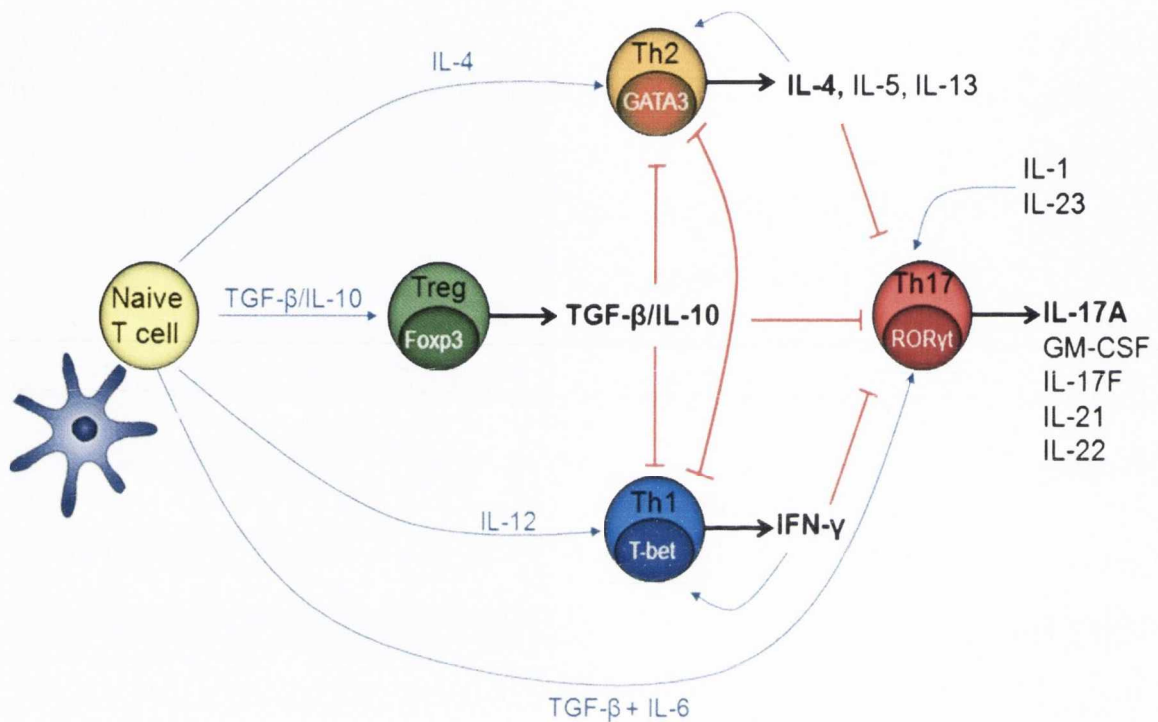


Fig. 1.1 The differentiation and regulation of CD4⁺ Th subsets.

Naive T cells primed by APC, such as DCs can differentiate into Th1, Th2, Th17 and Treg cell subsets depending on the cytokine environment. Priming in presence of IL-4 promotes differentiation of Th2 cells, while IL-12 promotes development of Th1 cells. Th17 cells differentiate in presence of TGF- β and IL-6 while IL-10 and TGF- β are involved in differentiation of inducible Treg cells. Cytokines produced by committed Th cells promote development of cells of their own lineage but suppress differentiation of other lineages. Blue arrows indicate factors promoting the differentiation. Red lines indicate antagonisms between the developing subsets.

1.2 Immune responses to helminths

Helminth parasites are estimated to infect over 1 billion people worldwide. While most of these infections are not life-threatening, they can have a serious impact on human health, causing impoverishment and malnutrition that can increase susceptibility to other diseases, impair children's development and reduce an adult's work performance contributing to the poor socio-economic situation of infected people (Hotez et al., 2008).

Helminths are a diverse group of multicellular eukaryotic organisms. However helminth infection of animals and humans are predominantly caused by species belonging to phyla Platyhelminthes, including classes Cestoda (tapeworms) and Trematoda (flukes) and phyla Nematodes (roundworms). Parasitic worms vary in their biology and many have complicated multistage life cycles including at least two hosts, an intermediate host where asexual divisions occur and a definitive host where parasites reach sexual maturity and reproduce.

Infections with parasitic helminths induce Th2 type of immune response which in general is protective and results in elimination of the pathogen (Allen and Maizels, 2011; Anthony et al., 2007). However, many parasites developed highly effective mechanisms of immune evasion that result in long lasting, chronic infection. The development of protective Th2 responses and strategies employed by the host immune system to eradicate parasitic worms have been extensively studied in experimental murine infections with model pathogens such as *Nippostrongylus brasiliensis*, *Heligmosomoides polygyrus*, or *Trichuris muris*. IL-4-producing CD4⁺ T cells are central mediators of protective responses against intestinal worms. It has been demonstrated that mice depleted of CD4⁺ T cells were no longer able to effectively expel *N. brasiliensis* worms from the intestines (Katona et al., 1988). Adoptive transfer of IL-4-expressing CD4⁺ T cells from infected wild type mice resulted in worm expulsion in normally susceptible T cell-deficient mice (Mohrs et al., 2001). Furthermore, deficiencies in key receptors and signalling molecules associated with development of Th2 cells, such as IL-4 receptor α -chain (IL4R α) and STAT6 increase the susceptibility of mice to infections with helminths (Urban et al., 1998).

IL-4, IL-5 and IL-13 produced by parasite-induced Th2 cells mobilize a broad range of downstream effector mechanisms aimed at elimination of parasites. In the gut IL-4 and IL-13 promote epithelial turnover, mucus production and secretion of resistin-like molecule- β (RELM β) by intestinal epithelial cells, which directly affects helminth survival (Herbert et al., 2009). Other Th2-related cytokines promote tight junction disruption, thereby increasing fluid flow into the intestine. This together with IL-13 induced intestinal muscle hypercontractility helps to expel worms from the gut (Allen and Maizels, 2011).

In tissues other than gut, where parasites cannot be physically ejected, helminths are subjected to attacks by the host innate immune effector cells, including macrophages, neutrophils and eosinophils. Because of their large size, helminths are not vulnerable to phagocytic killing by these cells, which makes the worms difficult to eradicate. However, cytotoxic substances released by macrophages, neutrophils and eosinophils can significantly compromise fitness and fecundity of the pathogen (MacDonald et al., 2002). The action of innate effector cells is often dependent on Th2-promoted antibody production. Parasite structures can be opsonised by specific IgE as a part of antibody dependent cellular cytotoxicity (ADCC) defence mechanism. Effector cells such as eosinophils degranulate upon recognition of antibodies, releasing substances toxic to the worm, such as major basic protein, eosinophil cationic protein or eosinophil-derived neurotoxin (Allen and Maizels, 2011). Similarly reactive oxygen species released by phagocytic cells can contribute to the defence mechanisms employed by the host.

Many tissue-dwelling parasites have developed several mechanisms to escape effector immune responses. For instance, metalloprotease secreted by the human hookworm *Necator americanus*, cleaves eotaxin, a chemotactic factor for eosinophils, while *Schistosoma mansoni* eggs were found to secrete a chemokine binding protein (smCKP) that blocks IL-8-induced neutrophil migration (Culley et al., 2000; Smith et al., 2005). *Fasciola hepatica* has been shown to secrete cysteine proteases that not only facilitate migration through the tissues, but also cleave host immunoglobulins (Smith et al., 1993) potentially involved in ADCC. Moreover, thioredoxin peroxidase and superoxide dismutase expressed by liver flukes constitute a mechanism likely to protect

them from damage imposed by reactive oxygen species released by hosts phagocytic cells during immune attack (Adisakwattana et al., 2009).

Despite induction of Th2-mediated immune responses, some helminth infections are chronic in nature. This is thought to reflect the parasites ability to evade immunity by induction of systemic immunosuppression and generation of regulatory responses. Down regulation of anti-helminthic immune responses not only facilitates parasite survival, but may also be beneficial to the host as it results in reduction of collateral damage associated with attempts to contain large, often migrating parasites. Evidence from experimental infection of mice has demonstrated that number of helminth species induce production of anti-inflammatory cytokines and promote expansion of Treg cells. *H. polygyrus* infection is associated with a dramatic increase in the frequency of Foxp3⁺ Treg cells in the gut and draining lymph nodes. Interestingly, *H. polygyrus* was found to produce a TGF- β -like molecule that is responsible for Treg cell induction *in vitro* and *in vivo*. Consequently, inhibition of TGF- β receptor signalling *in vivo* reduced helminth associated expansion of Treg cells and resulted in expulsion of worms (Grainger et al., 2010).

In chronic infection with the murine filarial pathogen *Litomosoides sigmodontis* expansion of Treg cells is associated with induction of hyporesponsive Th2 effector cells expressing the co-inhibitory receptor CTLA-4 (Taylor et al., 2007) that has been implicated in the down-regulation of T cell function. Protective responses leading to parasite killing were restored by the depletion of CD25⁺ Treg cells, but only in combination with neutralization of CTLA-4 (Taylor et al., 2007). This indicates that *L. sigmodontis* induces immunosuppression not only by induction of Treg cells, but also by subversion of parasite-specific Th2 responses. During *Trichinella spiralis* infection CD4⁺CD25⁻ effector T cells, rather than CD4⁺CD25⁺ regulatory T cells, suppressed inflammation and promoted parasite survival by an IL-10-dependent mechanism. TGF- β and IL-10 had an additive effect in the suppression of protective responses in this model and inhibition of both resulted in efficient parasite killing, but also increased pathology (Beiting et al., 2007).

1.2.1 Helminths and co-infections

Treg cell mediated immune suppression, which facilitates parasite survival, can affect immune response to other pathogens including protozoa, bacteria and viruses that infect with high frequencies in helminth-endemic areas. Moreover robust Th2 responses induced by the parasite may impair development of protective effector responses required for clearance of viruses and bacteria (Potian et al., 2011). Effective immunity against primary malaria infection is dependent on the development of a strong Th1 response. Epidemiological studies have demonstrated that intestinal helminth infections with hookworms are associated with increased incidence of malaria (Nacher et al., 2002). This observation was confirmed in an experimental murine model of malaria where co-infection with *H. polygyrus* resulted in impaired Th1 responses and severe mortality. The protective anti-malaria responses were restored by treatment with anti-helminth drugs (Su et al., 2005).

Similarly, protective Th1/Th17 responses against bacterial pathogens can be suppressed by co-infection with helminth. It was demonstrated that infection with *F. hepatica* significantly impaired protective Th1 immune responses against *Bordetella pertussis* and exacerbated pathology in mice infected with this bacteria (Brady et al., 1999; Chen et al., 2006). *F. hepatica* infection was also shown to have suppressive effect on *Mycobacterium bovis* BCG specific IFN- γ production in cattle (Flynn et al., 2007).

Helminth infections may also impair immune responses to viral pathogens. *Schistosomiasis* has detrimental effects on patients infected with hepatitis C virus (HCV) (Kamal et al., 2001) or human immunodeficiency virus (HIV) (Walson et al., 2008). The immune bias towards Th2 responses associated with helminth infection results in exacerbation of clinical symptoms of viral infection, accelerated disease progression and poor prognosis (Walson et al., 2009).

1.2.2 Helminths and immune-mediated disorders

Epidemiological data strongly suggest that increase in incidence of allergy-related and autoimmune disorders in developed countries inversely correlates with a decrease in prevalence of infectious diseases (Bach, 2002). This observation supports the concept of the *hygiene hypothesis* proposed over 20

years ago by Strachan (Strachan, 1989). This hypothesis, modified according to current understanding of immunological processes, postulates that the limited exposure to microorganisms in childhood may lead to discordant regulation of the immune system, and hence may increase the risk of certain inflammatory disorders. Therefore infections with certain pathogens such as helminths could favour normal development of regulatory mechanisms within immune system (Rook, 2009).

While immunosuppression associated with helminth infections clearly impairs control of concurrent microbial infections, it may be beneficial to the host in certain immune-mediated conditions, such as allergy or chronic inflammatory and autoimmune diseases (van Riet et al., 2007). A number of studies conducted in areas where parasite infections are endemic have shown that individuals naturally infected with *S. masoni* or *S. haematobium* have a reduced risk of positive skin reactivity to the house-dust mite (van den Biggelaar et al., 2000) or have a lower incidence of asthma related symptoms (Scrivener et al., 2001) when compared with non-infected subjects. In some studies, eradication of the parasitic infection by a treatment with an antihelmintic drug reversed the observed beneficial effects of infection and increased the risk of atopy (van den Biggelaar et al., 2004).

A few epidemiological investigations have demonstrated a beneficial effect of intestinal worm infection on disease activity in multiple sclerosis (MS) and inflammatory bowel disease patients (IBD). Correale and Farrez have reported that MS patients who had parasitic infection experienced a significantly lower number of relapses and less disease progression than uninfected MS patients (Correale and Farez, 2007). Patients with a helminth-associated suppression of disease had elevated levels of serum IL-10 and TGF- β and expanded populations of both regulatory B and T cells compared with uninfected controls (Correale and Farez, 2011). Remarkably, treatment with anti-helmintic drugs substantially increased the activity of the disease (Correale and Farez, 2011).

Infection with intestinal parasites also proved beneficial for patients with inflammatory bowel disease. The protective efficacy of infection with a porcine whipworm *Trichuris suis* in the treatment for Crohn's disease (Summers et al., 2005b) and active ulcerative colitis (Summers et al., 2005a) have been tested in

a number of clinical trials. These trials have demonstrated that transient colonisation by the parasite markedly ameliorated the disease severity in participating patients.

Although human epidemiological studies have provided convincing evidence that helminth infection can suppress immune-mediated diseases, much of what is currently known about the molecular mechanisms of parasite induced immunomodulation stems from experimental infections in animal models of human diseases. *H. polygyrus* infection was shown to reduce airway eosinophilia and lung pathology in experimentally induced airway allergy to ovalbumin (OVA) (Wilson et al., 2005). This attenuation of disease was associated with a substantial increase in numbers of functionally suppressive CD4⁺CD25⁺ Treg cells (Finney et al., 2007). The therapeutic effect of *H. polygyrus* infection was reversed by treatment with depleting antibodies against CD25 (Finney et al., 2007). Furthermore adoptive transfer of mesenteric lymph node cells from infected animals to uninfected OVA-sensitized mice prevented development of allergic response to OVA, demonstrating that already established allergic responses could be suppressed by transfer of parasite induced CD4⁺CD25⁺ Treg cells (Wilson et al., 2005).

The cross-regulation of immunity by parasite-induced Treg and Th2 responses has also been observed in models of autoimmune diseases, where pathological inflammatory responses to host tissues are mediated by effector Th1 and Th17 cells (Table 1.1). In EAE, a murine model of MS, immunization with myelin oligodendrocyte (MOG) peptide leads to a Th1/Th17 mediated disease that shares clinical and neuropathological characteristics of MS (Steinman, 1999). A range of helminthic parasites have been shown to suppress clinical manifestations of EAE. Experimental infections with *S. mansoni* (La Flamme et al., 2003), *F. hepatica* (Walsh et al., 2009) or *Trypanosoma brucei* (Wållberg and Harris, 2005) protected from autoimmunity or markedly delayed onset and attenuated clinical symptoms of the disease. Protected mice displayed a markedly diminished MOG-specific proliferation and expression of IL-17 and IFN- γ by lymph node and spleen cells compared to uninfected control mice. Analysis of the cellular infiltration of the spinal cords and brains revealed

that a pre-established helminth infection decreased CNS inflammation by reducing macrophage and CD4⁺ T cell infiltration (La Flamme et al., 2003). *F. hepatica* infection was shown to induce expansion of Foxp3⁺ Treg cells and IL-10 secreting Ag-specific Treg cells (Walsh et al., 2009). Notably, the protective effect of *F. hepatica* infection on development of EAE could be reversed by neutralization of TGF- β indicating that *F. hepatica*-induced TGF- β expression plays a critical role in the bystander suppression of autoantigen-specific Th1 and Th17 responses (Walsh et al., 2009).

Type 1 diabetes (T1D) is a chronic condition diagnosed in children and young adults characterized by loss of insulin-dependent control of blood glucose levels, which results from selective autoimmune destruction of insulin-producing beta cells of the pancreas (Knip and Siljander, 2008). The non-obese diabetic (NOD) mice provide an excellent model to study T1D as these animals spontaneously develop insulin-dependent diabetes with inflammation mediated by IFN- γ producing Th1 effector cells reactive against beta cell antigens (Cooke, 2009). The incidence of spontaneous T1D in NOD mice was shown to be significantly reduced by experimental infection with a human trematode *S. mansoni*. This was associated with suppressed infiltration of beta-islet by pathogenic lymphocytes and a failure to develop anti-insulin autoantibodies (Cooke et al., 1999). Protection from T1D was associated with dramatic expansion of CD4⁺Foxp3⁺ T cells and statistically significant increases in expression of TGF- β , IL-10, IL-4, IL-35, and galectin 3 in the pancreas of treated NOD mice. The effect of *S. mansoni* infection was mirrored by administration of *S. mansoni* egg antigen (SEA). Importantly SEA-induced generation of Foxp3⁺ Treg cells *in vitro* was dependent on TGF- β , which suggests that parasite products may induce peripheral conversion of inducible Tregs *in vivo* (Zaccone et al., 2009).

Helminth infections were also found to be protective in several models of inflammatory bowel disease (Elliott et al., 2003; Elliott et al., 2004; Reardon et al., 2001). Khan and co-workers have demonstrated that infection with *Trichinella spiralis* decreased mortality and reduced severity of

dinitrobenzenesulfonic acid (DNBS)-induced colitis (Khan et al., 2002). Suppression of inflammatory response was associated with a reduction in Th1-type responses, measured by expression of the Th1 signature cytokine IFN- γ in colonic tissue. Administration of ES products of *H. diminuta* also attenuated symptoms of DNBS-colitis however the effect was found to be dependent on the induction of alternatively activated macrophages (Hunter et al., 2010).

<i>Disease</i>	<i>Helminth</i>	<i>Treatment</i>	<i>Outcome</i>	<i>Reference</i>
Human				
Crohn's disease	Human	<i>Trichuris suis</i>	Amelioration of Crohn's disease	(Summers et al., 2005b)
Ulcerative colitis	Human	<i>Trichuris suis</i>	Reduced ulcerative colitis	(Summers et al., 2005a)
Multiple sclerosis	Human	<i>Trichuris suis</i>	Reduced number of new lesions	(Fleming et al., 2011)
Multiple sclerosis	Human	various	Reduced number of relapses, reduced number of new lesions	(Correale and Farez, 2007)
Mouse				
T1D	NOD	<i>Schistosoma mansoni</i>	Inhibition of development of T1D	(Cooke et al., 1999)
T1D	NOD	<i>Schistosoma mansoni</i>	Inhibition of development of T1D	(Zaccane et al., 2003)
T1D	NOD	<i>Diriofilaria immitis</i>	Inhibition of development of T1D	(Zaccane et al., 2009)
T1D	NOD	<i>Litomosoides sigmodontis</i>	Inhibition of development of T1D	(Hübner et al., 2009)
Colitis	IL10 KO C57BL/6	<i>Heligmosomoides polygyrus</i>	Suppression of established colitis	(Elliott et al., 2004)
Colitis	BALB/c	<i>Schistosoma mansoni</i>	Inhibition of development of colitis	(Elliott et al., 2003)
Colitis	BALB/c	<i>Hymenolepis diminuta</i>	Suppression of symptoms	(Reardon et al., 2001)
Colitis	C57BL/6	<i>Trichinella spiralis</i>	Reduction of severity of colitis	(Khan et al., 2002)
EAE	C57BL/6J	<i>Schistosoma mansoni</i>	Attenuation of clinical symptoms	(La Flamme et al., 2003)
EAE	SJL, C57BL/6	<i>Schistosoma mansoni</i>	Attenuation of clinical symptoms	(La Flamme et al., 2003)
EAE	C57BL/6J	<i>Fasciola hepatica</i>	Attenuation of clinical symptoms	(Walsh et al., 2009)
EAE	DBA/1	<i>Trypanosoma brucei</i>	Attenuation of clinical symptoms	(Wållberg and Harris, 2005)
EAE	C57BL/6J	<i>Trichuris suis</i>	Attenuation of clinical symptoms	(Kuijk et al., 2012)
EAE	C57BL/6J	<i>Trichinella spiralis</i>	Attenuation of clinical symptoms	(Kuijk et al., 2012)
CIA	DBA/1	<i>Acanthocheilonema viteae</i>	Delayed onset, reduced severity	(McInnes et al., 2003)

Table 1.1 Selected studies that have examined the effect of helminth infection on the development of autoimmune disease.

1.3 Molecular mechanisms of immune modulation by helminths

The factors promoting development of Th1 and Th17 responses are currently well established, however, the pathways involved in Th2 polarisation by helminths are poorly understood. Despite their capacity to induce robust Th2 responses to helminth antigens *in vitro* and *in vivo*, DCs primed by parasite products often fail to show signs of classical maturation such as up-regulation of co-stimulatory molecules or cytokine production (Everts et al., 2010) (Carvalho et al., 2009). However, some studies have reported that Th2 promoting DCs stimulated with helminth products undergo partial maturation. For instance, DCs incubated with products released by *S. masoni* larvae showed limited expression of MHC class II, CD40, and CD86 and increased production of IL-12p40 and IL-6, albeit at much lower levels than observed in response to Th1 stimuli, such as LPS (Jenkins and Mountford, 2005). Similarly incubation of DCs with ES products from *N. brasiliensis* (NES) resulted in up-regulation of CD86 and OX40L, which is associated with Th2 responses, while little change was observed to CD80 and MHC class II. Moreover, DCs exposed to NES produced IL-6 and IL-12p40, but not IL-12p70 (Balic et al., 2004). Although TLR signalling is mostly associated with induction of Th1/Th17 responses, some parasite derived products prime Th2-inducing DCs via TLR pathways. For instance glycoconjugate lacto-N-fucopentaose III (LNFP III) from *S. masoni*, as well as the filarial phosphorylcholine (PC)-containing glycoprotein ES-62, were both shown to induce Th2 responses in a TLR4 dependent fashion (Goodridge et al., 2005; Thomas et al., 2003).

1.3.1 Immunomodulatory strategies

Numerous studies have reported that helminth-derived products have a potent capacity to suppress TLR-mediated activation of DCs induced by microbial stimuli. Preincubation of bone marrow derived DCs with *S. mansoni* SEA or ES products from *H. polygyrus* prior to stimulation with TLR9 ligand CpG resulted in a dramatic reduction in expression of MHC class II and co-stimulatory molecules, CD40, CD80 and CD86 (Kane et al., 2004) (Segura et al., 2007). Importantly, CpG-induced secretion of IL-12p70, which is central for the induction of Th1 responses against bacteria, was significantly inhibited

by SEA (Segura et al., 2007) while IL-10 was enhanced (Kane et al., 2004). Parasite induced immunomodulation is not restricted to one signaling pathway as similar inhibitory effects were observed when cells pretreated with helminth products were stimulated with other TLR ligands, such as LPS or polyinosinic:polycytidylic acid (poly I:C) which activate TLR4 and TLR3 respectively (Kane et al., 2004). Similarly, the cysteine proteases from *S. mansoni* and *F. hepatica* were able to prevent TLR3 and TLR4 mediated production of nitric oxide, $\text{TNF}\alpha$, IL-6 and IL-12p70 from APCs and therefore protect mice from endotoxic shock. Interestingly the mechanism of cytokine suppression was associated with endosomal degradation of the TLR3 signalling complex, resulting in the disruption of the TRIF-dependent pathway shared by TLR3 and TLR4 (Donnelly et al., 2010).

The cytokines secreted by DCs and other innate immune cells upon activation with PAMPs are essential for the development of appropriate effector T cell populations (Kapsenberg, 2003). Various studies have demonstrated that parasite products can target cytokine secretion pathways to generate aberrant or impaired adaptive immune responses both *in vitro* and *in vivo* (Chen et al., 2006; Schönemeyer et al., 2001; Segura et al., 2007). Although the mechanisms underlying this immunomodulatory activity are still poorly understood, it has been demonstrated that SEA can affect activation of the intracellular signalling pathway leading to phosphorylation of ERK and p38 MAP kinases following stimulation with TLR ligands. Activation of p38 by LPS stimulation in DCs is important for expression of maturation markers and cytokine production, whereas LPS-induced phosphorylation of ERK is linked to dampening of these responses (Nakahara 2006). SEA affected MAPK signalling in DCs by suppressing phosphorylation of MAPK p38 but enhancing activation of ERK which resulted in the inhibition of LPS-induced IL-12 and increased IL-10 expression (Everts et al., 2010). Similarly ES62, a glycoprotein from ES products of *Acanthocheilonema vitae* suppresses LPS and CpG induced IL-6, IL-12p70 and $\text{TNF}\alpha$, in a TLR4 dependent manner, by modulating activation of the MAP kinases ERK, p38 and JNK (Goodridge et al., 2005). Similar to SEA the down-regulation of IL-12p70 seemed to be due to increased activation of ERK that suppressed expression of IL-12p40. Notably production of IL-12p70

was restored by pre-treatment of cells with a specific inhibitor of the ERK pathway (Goodridge et al., 2003).

Parasite products can also affect the host response by exploiting other pattern recognition receptors, such as CLRs expressed on the surface of DCs. DC-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN), an innate lectin receptor on human DCs, was shown to bind glycolipids derived from *S. mansoni* cercariae (Meyer et al., 2005). Concurrent triggering of DC-SIGN with *S. masoni* glycolipids and TLR4 with LPS resulted in induction of signalling pathways leading to enhanced IL-10, but suppressed IL-12p70 secretion by DCs (Everts et al., 2010; van Die and Cummings, 2010; van Liempt et al., 2007).

Evidence is emerging that helminth-driven modulation of DC function can also be mediated by TLR/CLR-independent mechanisms. Notably, cysteine protease inhibitors (cystatins) secreted by various parasitic species such as *A. vitae*, *B. malayi* and *O. vulvus* have been shown to inhibit the activity of mammalian intracellular enzymes that are required for antigen processing and for peptide loading onto MHC class II (Everts et al., 2010). Interference with antigen processing and presentation suppressed APC function and resulted in diminished capacity of those cells to prime and polarize naïve T cells *in vitro* and *in vivo* (Dainichi et al., 2001; Manoury et al., 2001; Schönemeyer et al., 2001).

Finally, parasites and their products have been shown to indirectly modulate DC function through induction of host-derived mediators secreted by various non-immune cells that come in contact with the pathogen (Everts et al., 2010). In this respect TSLP and IL-33 have been reported to induce partial activation of DCs through up-regulation of co-stimulatory molecules but without IL-12 production which, enables those cells to initiate protective immunity against helminths (Humphreys et al., 2008; Ramalingam et al., 2009; Rank et al., 2009).

1.3.2 Helminth-derived molecules as immunomodulators

There is a large body of evidence demonstrating that regulatory networks triggered by helminths may protect against allergy and autoimmunity. This has resulted in a major effort to exploit parasites or parasite derived molecules as therapeutics and to evaluate these in a clinical setting. Although infection with live worms has been tested, this approach is often controversial for reasons of safety. Therefore efforts have mainly focused on exploring the potential of purified parasite-derived excretory/secretory (ES) products or their synthetic analogues which could be safely used as therapeutic agents. In recent years a large number of ES product-derived immunomodulators have been identified and characterized in terms of their molecular composition and bioactivity. Structurally, these components fall into diverse biochemical classes and regulate various pathways of the host immune response (Hewitson et al., 2009). Identification and molecular characterization of immunomodulatory helminth-derived compounds could be not only used as new drugs or drug templates but also serve as unique tools for defining key events in the development of immune-mediated diseases (Harnett and Harnett, 2010).

Lipids

Several studies have demonstrated that helminth parasites secrete immunomodulatory bioactive lipids and lipid conjugates (van der Kleij and Yazdanbakhsh, 2003). van der Kleij *et al.* have shown that *S. mansoni* specific phosphatidylserines (PS) were responsible for the modulation of DCs function observed during infection with this parasite. PS primed and CD40 stimulated DCs produced significantly less IL-12p70 and polarized naive T cells towards Th2 cells and IL-10 producing Treg cells. In contrast, DCs matured with CD40 ligand only induced Th1 type responses. Molecular characterization of these PS fractions demonstrated that T cell polarization towards a Th2 subset was induced by DCs stimulated with schistosomal di-acylated PS while development of IL-10 producing Treg cells was dependent on PS molecules containing only a single acyl chain (so called lyso PS) (van der Kleij et al., 2002).

ES62, a glycoprotein from *A. vitae* that has been shown to exhibit a wide range of immunomodulatory properties contains a phosphorylcholine (PC)

attached through an N-type glycan. (Goodridge et al., 2005; Harnett and Harnett, 2010). Experiments involving PC-free ES-62 and PC conjugated with an irrelevant protein (ovalbumin) have revealed that the anti-inflammatory actions of ES-62 depend solely on this PC moiety (Goodridge et al., 2005).

The best characterized immunomodulatory lipids are endogenous eicosanoids, such as prostaglandins that are potent inhibitors of cellular proliferation and regulators of cytokine synthesis (van der Kleij and Yazdanbakhsh, 2003). *Trypanosoma cruzi* has been shown to induce the production of high levels of PGE₂ from spleen cells of infected mice, which was responsible for lymphocyte hyporesponsiveness (Pinge-Filho et al., 1999). As well as inducing prostaglandin synthesis by the host, some parasites can also secrete immunomodulatory prostaglandins. For example schistosomes have been shown to synthesize and secrete PGD₂ thereby inhibiting the maturation and function of DCs (Angeli et al., 2001).

Carbohydrates and glycans

The unusual repertoire of glycans displayed by helminths has long been regarded as a potential mechanism to subvert recognition by the host immune system (van Die and Cummings, 2010). A number of studies on immunomodulatory helminth glycans have focused on Lacto-N-fucopentaose III glycoconjugate containing Lewis^X (LNFPIII) isolated from various live stages of *S. mansoni*. Using a synthetically produced version of LNFPIII polyvalently linked to human serum albumin as a carrier molecule, Vellupillai *et al.* demonstrated that this carbohydrate alone could induce B cell proliferation and secretion of IL-10 and PGE₂ (Velupillai and Harn, 1994). Moreover, DCs activated with LNFPIII had properties similar to Th2 promoting DCs activated with schistosome SEA showing that the glycoconjugate compound plays a role in driving Th2 responses (Thomas et al., 2003). In agreement with these findings, it has been shown that SEAs induce anti-inflammatory responses in human DCs via three distinct glycan binding C-type lectins, DC-SIGN, the mannose receptor (MR), and macrophage galactose lectin-1 (MGL-1). Separate studies have demonstrated that conjugation of Lewis^X containing LNFPIII to protein antigens significantly enhanced their immunogenicity, promoting

responses to the antigen in a Th2-directed manner. These studies suggested that parasite derived glycoconjugates can function as adjuvants for third party protein antigens (Harn et al., 2009).

Proteins

Several parasite derived proteins have been shown to have immunomodulatory function. Among these, omega-1, a glycoprotein with a ribonuclease activity secreted by *S. mansoni* eggs and present in SEA is capable of conditioning human monocyte-derived DCs to drive Th2 polarization, with similar characteristics as whole SEA. Omega-1-depleted SEA had reduced capacity to induce Th2 responses *in vitro*, but not *in vivo*, suggesting that additional factors within SEA can compensate in the absence of omega-1-mediated effects (Everts et al., 2009).

The production of the reactive oxygen species by phagocytes is a primary mechanism of innate defence against parasitic pathogens. However, parasites like *B. malayi* and *F. hepatica* secrete significant amounts of antioxidant enzymes including superoxide dismutase, glutathione peroxidase and thioredoxin peroxidase as a strategy of immune evasion (Hewitson et al., 2009). In addition to restricting inflammation by quenching free radicals, the thioredoxin peroxidase from *F. hepatica* has been shown to induce alternatively activated macrophages, characterized by the production of high levels of IL-10, PGE₂, and low levels of IL-12 (Donnelly et al., 2005).

As well as secreting bioactive enzymes, parasites can also produce specific enzyme inhibitors that interfere with signalling pathways within the host. Various parasitic species including *A. vitae*, *B. malayi* and *O. vulvus* (Everts et al., 2010) have been shown to secrete cysteine protease inhibitors (cystatins) that are able to inhibit the activity of mammalian papain-like cathepsins B, L and S and the legumain-type asparagine endopeptidase. These enzymes are required for efficient antigen processing and for peptide loading onto MHC class II molecules. Interference with antigen processing and presentation by parasite-derived cystatins can suppress APC function, resulting in a diminished capacity to activate naive T cells *in vitro* and *in vivo* (Dainichi et al., 2001; Manoury et al., 2001; Schönemeyer et al., 2001).

Since cytokines and chemokines play a central role in the development and regulation of immune responses, it is not surprising that parasites have evolved mechanisms to interfere with their function in order to subvert host immunity (Maizels and Yazdanbakhsh, 2003). In addition to suppressing the release of pro-inflammatory cytokines from DCs, the filarial worm *B. malayi* has been shown to actively secrete functional homologues of the mammalian regulatory cytokine TGF- β (Gomez-Escobar et al., 1998). Interestingly, various families of helminths, including filarial worms and schistosomes, encode members of the TGF- β receptor family and TGF- β ligand-like molecules which could play important roles in exploiting TGF- β -mediated mechanisms of immune suppression (Maizels and Yazdanbakhsh, 2003). Recently a TGF- β -like molecule that binds to TGF- β receptor and mimics biological effects of this important regulatory cytokine was identified in ES products of *H. polygyrus*. It was demonstrated that this TGF- β homolog was responsible for induction of Foxp3⁺ Treg cells associated with the immunosuppressive effect of *H. polygyrus* ES.

1.4 *Fasciola hepatica*

The specific focus of this study centres on *F. hepatica*, a parasitic flatworm of the class Trematoda, commonly known as a liver fluke. *F. hepatica* infects mainly ruminants, such as sheep and cattle causing significant economic losses in farming. However the host preference is not restricted to ruminants and a wide variety of vertebrates including mice and humans can become infected with *F. hepatica* (Esteban et al., 1997). In order to complete its complex life cycle, the parasite requires an intermediate host, such as the lymnaeid snail *Galba truncatula*, from which asexually reproducing encysted metacercariae are shed and infect the vertebrate host (Hurtrez-Boussès et al., 2001). After ingestion by the definitive host, the metacercariae excyst in the duodenum and penetrate the intestinal wall. The juvenile fluke then migrates across the peritoneal cavity to the liver where it spends up to 8 weeks (in cattle) feeding on the parenchyma cells before finally progressing to the bile duct to reach sexual maturity and begins to produce eggs (Dalton et al., 2003).

The infected host initially mounts a robust Th2 response against the parasite, which is characterised by eosinophilia and the production of IL-4 and IgG1 antibodies (Brown et al., 1994). However, this Th2 response is ineffective at clearing the parasite and the chronic infection that develops is associated with significant IL-10 and TGF- β production by peripheral blood mononuclear cells (PBMCs) in infected cattle (Flynn and Mulcahy, 2008). Interestingly, the *ex vivo* neutralization of TGF- β enhanced production of parasite-specific IL-4 and IFN- γ by PBMCs indicating that TGF- β plays a key role in suppressing Th1 and Th2 responses against the parasite in cattle (Flynn and Mulcahy, 2008).

Studies in a mouse model of *F. hepatica* infection gave invaluable insight into mechanisms of immune evasion developed by this helminth. Strikingly experimental infection with *F. hepatica* was associated with significant expansion of TGF- β -producing Th3 cells, Foxp3⁺CD4⁺ Tregs and parasite-specific IL-10 producing CD4⁺CD25⁺CTLA-4⁺ Tr1 cells. Induction of IL-10 from Tregs and innate cells was an important component of the immunosuppressive mechanism employed by *F. hepatica* in this model, since effector Th1 and Th2 responses were markedly enhanced in IL-10^{-/-} mice (Walsh et al., 2009). *F. hepatica* infection was shown to compromise not only host effector immune responses to the parasite, but also to other pathogens and bystander antigens. Infection with *F. hepatica* as well as administration of *F. hepatica* ES suppressed Th1 responses, and impaired pathogen clearance in mice infected with *B. pertussis* (Brady et al., 1999; O'Neill et al., 2001). Furthermore, a recent study by Walsh *et al.* demonstrated that infection with *F. hepatica* suppressed the development of EAE and reduced auto-antigen specific Th1 and Th17 responses. Attenuation of autoimmunity was associated with high frequencies of IL-10 and TGF- β -producing Treg cells and was mediated through TGF- β but not IL-10-dependant mechanisms (Walsh et al., 2009). Similarly *F. hepatica* ES products were found to be immunosuppressive in a murine model of autoimmune arthritis. Cervi and colleagues demonstrated that antigen pulsed, *F. hepatica* ES treated DCs were able to protect mice from CIA via mechanism involving CD4⁺CD25⁺ Treg cells (Carranza et al., 2012).

Because of the potent immunosuppression and significant losses that *F. hepatica* infection generates in farming there is a need for a better

understanding of the parasite-host interaction and identification of factors promoting immune evasion. Therefore the proteome of *F. hepatica* is well studied and many tegumental (Morphew et al., 2012; Wilson et al., 2011) and secretory (Jefferies et al., 2001; Robinson et al., 2009) proteins specific to various life stages of *F. hepatica* have been identified and some of them have shown immunomodulatory properties.

F. hepatica secretes numerous components that facilitate the survival and directly protect the parasite from clearance by the host. It has been demonstrated that *F. hepatica* ES contains carbohydrate components that induce apoptosis in peritoneal macrophages and eosinophils, which could potentially constitute a mechanism protecting from antibody dependent cellular cytotoxicity (ADCC) (Serradell et al., 2007; Serradell et al., 2009).

Cysteine protease cathepsin L (FhCatL) is a major virulence factor that allows the parasites migration through host tissues. The enzymatic activity of FhCatL may provide a mechanism to protect the parasite from killing through ADCC, since it was shown that FhCatL can cleave host immunoglobulins (Smith et al., 1993). Recent reports have provided convincing evidence that FhCatL may interfere with many aspects of host's immune response. It was demonstrated that FhCatL was able to prevent TLR3 and TLR4 mediated induction of nitric oxide, $\text{TNF}\alpha$, IL-6 and IL-12p70 from APCs and therefore protect mice from endotoxic shock (Donnelly et al., 2010). Interestingly the mechanism of cytokine suppression was associated with endosomal degradation of the TLR3 signalling complex, resulting in the disruption of the TRIF-dependent pathway shared by TLR3 and TLR4. Interestingly FhCatL was also found to play a role in parasite mediated suppression of protective Th1 responses during infection with *B. pertussis* (O'Neill et al., 2001) and recently it was shown that FhCatL primed DCs are less able to prime Th17 responses *in vivo* (Dowling et al., 2010).

Thioredoxin peroxidase (FhTPx), an antioxidant enzyme secreted by *F. hepatica* is believed to be a part of defence mechanism protecting helminth tissues from reactive oxygen species released by the host's phagocytes during the immune attack on the parasite. However, similar to FhCatL this protein was also shown to modulate immune responses independently of its primary

function as an antioxidant. Donnelly *et al.* demonstrated that FhTPx is a potent driver of Th2 responses in infected mice and is responsible for induction of alternatively activated macrophages that could potentially contribute to the immunosuppression induced by the parasite (Donnelly *et al.*, 2005; Donnelly *et al.*, 2008). Moreover heterogeneous preparations of *F. hepatica* ES (Falcón *et al.*, 2010) and tegumental (Hamilton *et al.*, 2009) proteins were previously shown to inhibit activation and cytokine production by DCs and to induce expansion of Th2 and Treg cells, but specific components were not identified.

Although many *F. hepatica*-derived immunomodulatory molecules have been discovered and characterised *in vitro* and *in vivo*, thus far none has been evaluated as potential therapeutic in allergy or autoimmunity. Given the significant protective effect of *F. hepatica* infection on the development of EAE the identification of immunosuppressive component(s) mediating the protection could potentially lead to the discovery of novel therapies for MS and other debilitating autoimmune diseases.

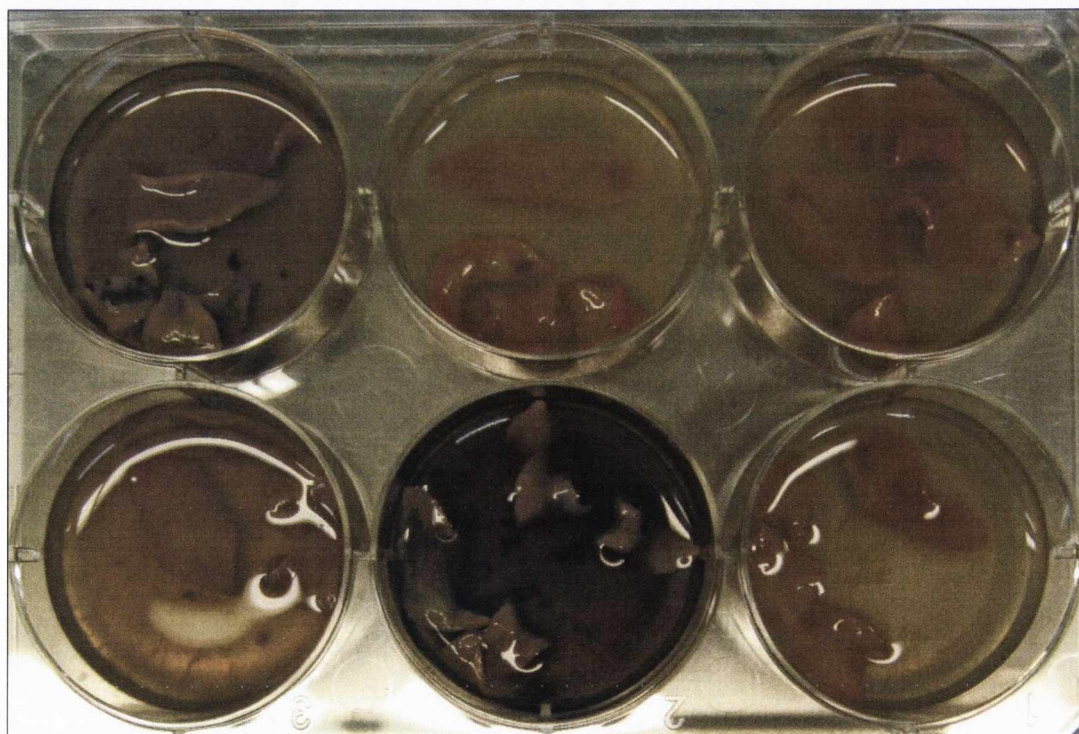


Fig. 1.2 *Fasciola hepatica* in culture.

Adult worms were incubated for 18 h in PBS at 37°C in order to obtain ES products.

1.5 Autoimmunity

1.5.1 Multiple sclerosis

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) that causes substantial disability through deficits of sensory, motor, autonomic and cognitive function. The disease typically presents between the ages of 20 and 40 and is the leading cause of nontraumatic neurological disability in young people (Sospedra and Martin, 2005). In MS, pathology is driven by autoimmune T cell attack on the myelin sheath that normally surrounds nerve axons and potentiates neurotransmission along the axons. This inflammatory response causes focal demyelination of axons and subsequent axonal damage, impairing signal conduction and ultimately leading to the neurological symptoms characteristic of MS. Demyelinating plaques within the CNS are a distinctive histopathological feature of MS and contain massive inflammatory cell infiltrates, demonstrating the central role of inflammation in the development of MS. However, the mechanisms that lead to initiation and progression of MS are poorly defined. Moreover the available disease modifying therapies are very expensive and often have limited efficacy or are associated with severe side effects (Miller and Rhoades, 2012)

More than 80% of MS patients present with relapsing-remitting form of the disease (RR-MS) that is characterised by episodes of symptomatic disease interspersed by periods of relative improvement. The recovery from each episode is incomplete and persistent symptoms accumulate with time. Most patients that initially present with RR-MS will later develop secondary progressive MS (SP-MS), where the steady neurologic decline occurs without clinical signs of remission. A minority of patients (>15%) have primary progressive MS (PP-MS) characterized by a progressive course from the onset in the absence of clinically evident relapses (Keegan and Noseworthy 2002).

MS is clinically and pathologically heterogeneous and disease manifestations depend on the area of CNS affected by demyelinating plaques. Inflammation can occur in the brain, spinal cord and optic nerve, and resulting

symptoms may either affect single neurological functions or a combination of them. The most common symptoms of MS include monocular visual loss or blurry vision (optic neuritis) due to involvement of optical nerves; sensory and motor changes including paraesthesia, hemiparesis or paraparesis resulting from spinal cord involvement; and diplopia, facial sensory disturbance and ataxia due to brain stem involvement. Bladder and bowel dysfunction as well as memory and cognitive impairment can also occur (Goldman et al., 2006).

Although the aetiology of MS is poorly defined, there is substantial evidence implicating both genetic and environmental factors in the development of disease. The strong contribution of genetic predispositions to the development of MS is supported by the 5% concordance rate among same-sex dizygotic twins and 20%–30% concordance observed among monozygotic twins compared with an incidence of 0.1%–0.4% among the general population in high risk areas (Keegan and Noseworthy, 2002; Willer et al., 2003). While multiple genes appear to be involved, human leukocyte antigen (HLA) class II molecules are particularly strongly associated with the risk of MS. In Caucasian populations of Northern European descent, the HLA-DR15 haplotype (DRB1*1501-DQA1*0102-DQB1*0602) has been hypothesized to be the primary HLA genetic susceptibility factor (Schmidt et al., 2007). Moreover, the significantly higher prevalence of MS among females indicates that sex hormones may increase the risk of the disease.

Despite the clear association, genetic factors account only partially for MS susceptibility. The low concordance rate between identical twins highlights the role of environmental factors in precipitating the disease (Ontaneda et al., 2012). Infection, diminished exposure to sunlight and vitamin D deficiency have all been implicated in the aetiology of MS, and the prevalence of MS has been long acknowledged to increase with the distance from the equator. While it is difficult to separate latitude and diminished exposure to UV light from vitamin D insufficiency, lower levels of vitamin D in serum are common in MS patients and inversely correlate with relapse rate and disability (Simpson et al., 2010). In addition, the geographical distribution of MS inversely correlates with socio-economic factors indicative of less developed areas, including poor sanitation

and high prevalence of infectious diseases, particularly helminth infections. It has been proposed that as countries develop improved hygienic standards, MS prevalence increases (Cabre et al., 2005; Fleming and Cook, 2006). Interestingly, people that move from areas of high MS risk to low risk areas at young age (<15y) have a lower chance of developing MS than is observed in the population of origin. How environmental factors shape the autoimmune response that drives MS pathology is unclear and warrants thorough investigation.

Histopathological examination of inflammatory lesions in brains of patients with RR-MS, together with evidence from animal models of the disease, has led to the generally accepted hypothesis that MS is an autoimmune disease where T cells mount a pathogenic response against myelin sheath antigens. Although myelin specific T cells can be found in peripheral blood of healthy individuals, in MS patients these cells have markers indicative of activated or memory status, suggestive of a recent or ongoing myelin-specific inflammatory response (Lovett-Racke et al., 1998). Genetic associations between MS and specific T cell associated molecules, including MHC and the IL-2 and IL-7 receptor α chains, highlight the important role of T cells in the pathogenesis of MS (Hafler et al., 2007).

1.5.2 Experimental autoimmune encephalomyelitis

The current understanding of immunological events leading to the development of MS comes largely from studies of EAE in mice. EAE is an autoimmune demyelinating disease that shares clinical and pathological similarities with MS and serves as experimental model of the human disease. EAE can be induced in several species of non-human primates and rodents by immunisation with whole brain or spinal cord extracts, myelin proteins or synthetic peptides from such proteins, emulsified in complete Freund's adjuvant (CFA) (Baxter, 2007). Depending on the specific species and antigen used, the ensuing disease may take either an acute, chronic or relapsing-remitting course. Several strains of laboratory mice are susceptible to EAE. C57BL/6 mice develop moderate chronic disease when immunised with myelin

oligodendrocyte glycoprotein (MOG) peptide 35-55. Conversely, SJL mice, when immunised with proteolipid protein (PLP) peptide 139-151, develop a severe relapsing-remitting form of EAE that more closely resembles the clinical course of human MS (Miller and Karpus, 2007).

The pathology in EAE is primarily mediated by T cells and the disease can be induced by adoptive transfer of myelin-reactive CD4⁺ T cells into naive recipients (termed passive induction) (Pettinelli and McFarlin, 1981). Naive CD4 T cells are excluded from the CNS, but following immunisation or T cell adoptive transfer, activated and memory CD4⁺ T cells are able to gain access to the CNS. This is true regardless of the antigen specificity of the T cell; however, antigen specificity is important for T cell restimulation and activation within the CNS. Restimulation of T cells in the CNS results in the release of proinflammatory cytokines and chemokines, opening of the blood-brain barrier (BBB) and further recruitment of inflammatory cells into the CNS. This drives the tissue damage, demyelination and axonal injury that manifest clinically in mice as ascending tail flaccidity and hind limb paralysis.

Until the discovery of Th17 cells, EAE and MS were thought to be mediated solely by IFN- γ producing Th1 cells. This notion was supported by several observations. Firstly, mice deficient in the p40 subunit of IL-12p70, a Th1 polarising cytokine, were resistant to EAE (Segal et al., 1998). Horwitz *et al.* demonstrated that over-expression of IFN- γ in the CNS caused spontaneous inflammation and demyelination similar to that observed in EAE and MS (Horwitz et al., 1997). In addition, it was reported that intravenous administration of IFN- γ to MS patients in a clinical trial induced relapses in a substantial number of participants (Panitch et al., 1987). On the other hand, systemic treatment with exogenous IFN- γ suppressed disease development in the EAE model, while its blockade exacerbated the symptoms (Billiau et al., 1988). Thus, the role of IFN- γ in the pathogenesis of EAE and MS remains unclear (Hu and Ivashkiv, 2009; Kelchtermans et al., 2008).

These discrepancies were partially resolved following the discovery of IL-23, which was found to drive the development and maintenance of Th17 cells (Langrish et al., 2005). IL-23 belongs to IL-12 family of cytokines and shares the p40 subunit with IL-12. An elegant study by Cua *et al.* demonstrated that mice deficient in IL-12p40 or IL-23p19 and therefore lacking IL-23 and IL-12 or IL-23 respectively were resistant to EAE. In contrast IL-12p35^{-/-} mice were susceptible which demonstrated that IL-23, not IL-12, was critical for the induction of EAE (Cua et al., 2003). Further studies showed that IL-23 was necessary for the expansion of PLP-specific IL-17-producing cells that were able to induce EAE upon transfer to naive recipients (Langrish et al., 2005). IL-17 producing cells have also been found in inflammatory lesions and the cerebrospinal fluid of MS patients (Matusevicius et al., 1999)(Lock et al., 2002). Some reports suggest that IL-17 may be involved in promoting the opening of the BBB in MS, as exposure of human BBB-endothelial cells to IL-17 *in vitro* led to a decrease in the expression of the tight junction proteins occludin and zonula occludens (ZO)-1, which are associated with BBB integrity (Kebir et al., 2007).

The indispensable role of IL-23 in the induction of EAE underscores the involvement of IL-23-dependent Th17 cells in the development of the autoimmunity. However, the notion that Th17 cells are the only mediators of CNS inflammation in EAE is an oversimplification. It has been demonstrated that upon transfer, PLP-specific Th1 or Th17 cells were able to induce EAE, although these effector T cell populations triggered immunologically distinct pathogenic processes. Th1 cells induced expression of monocyte-attracting chemokines and triggered macrophage infiltration, while Th17 cells promoted neutrophil recruitment. Regardless, the inflammation induced by these two populations of effector T cells resulted in demyelination and similar clinical outcomes (Kroenke et al., 2008). Another study also reported that EAE could be induced by adoptive transfer of MOG-specific Th1 or Th17 cells, although with clinically distinct disease and different localization of inflammatory lesions within the CNS. In contrast to the work by Kroenke *et al.*, this study used sublethally irradiated mice as recipients to exclude the involvement of host T cells. Transfer of Th1 cells induced classical EAE with only spinal cord involvement, whereas EAE induced by Th17 cells was characterized by infiltration of the brain and

atypical clinical symptoms (Stromnes et al., 2008). Collectively these reports suggest that both Th1 and Th17 cells are involved in the development of EAE, and possibly MS, and play distinct but complementary roles in the pathogenesis of the disease.

Interestingly, IFN- γ signalling inhibits the development of Th17 cells (Park et al., 2005) and it has been suggested that IFN- γ limits EAE pathology by inhibiting Th17 differentiation and activity (Kelchtermans et al., 2008). This hypothesis could explain the exacerbation of the disease observed when IFN- γ is neutralised (Ferber et al., 1996; Lublin et al., 1993).

1.5.3 MS therapies

Studies in the EAE model provide invaluable insight into the immunology and pathology of MS. Many therapies against human disease, including glatiramer acetate and natalizumab were developed in this model. Currently, several disease modifying treatments are available for RR-MS, although there is no cure for neuronal damage and thus the drugs are aimed at suppressing inflammation, reducing the number of relapses and extending remission periods.

Interferon- β (IFN- β ; marketed as Betaferon or Avonex) is an established, first-line treatment for RR-MS. IFN- β was shown to have various immunomodulatory effects including inhibition of pro-inflammatory mediators and indirect protection of BBB integrity. While the exact mechanism of action is unclear, the therapeutic effect of IFN- β may be mediated, at least in part, through the induction of IL-27, which inhibits Th17 cells both directly and through the inhibition of cytokines that promote Th17 differentiation (Sweeney et al., 2011). The therapy reduces relapse rate by approximately 30% (Panitch and Bever, 1993); however, not all patients respond to the treatment and many develop neutralising anti-IFN- β antibodies (Sorensen et al., 2003). Efficacy of the treatment is evaluated on the basis of relapse frequency and non-responding patients may be subjected to this expensive and ineffective therapy for long periods before resistance is discovered.

An alternative first-line MS therapeutic, glatiramer acetate (GA) (Copoxane), is a synthetic random polymer of the amino acids alanine, glutamate, lysine and tyrosine (Teitelbaum et al., 1971). It has been suggested that the therapeutic effects of GA in MS are due to its ability to bind promiscuously to MHC Class II molecules, preventing the interaction of MHC with TCR and thus inhibiting activation of T cells (Ruggieri et al., 2007). However, growing evidence from studies of MS and EAE suggests that GA may have broader immunomodulatory effects including the induction of anti-inflammatory cytokines, Treg cells and neurotrophic factors (Racke and Lovett-Racke, 2011). Several studies that examined the cytokine production of myelin-reactive T cells directly *ex vivo* from GA-treated patients have suggested that GA may induce immune deviation from Th1 to a Th2/Th3 phenotype (Miller et al., 1998; Racke and Lovett-Racke, 2011). Moreover, it was demonstrated that in EAE, the GA-induced Th2 cells infiltrated brains of protected mice and promoted bystander expression of IL-10 and TGF- β by resident astrocytes and microglia (Aharoni et al., 2003). Treatment with GA may therefore promote active suppression of ongoing immune responses, although the poor understanding of the mechanism of action of GA highlights the paucity of knowledge surrounding the immune processes at play in MS.

Other, novel MS treatments target T cell migration to inhibit T cell entry into the CNS and therefore prevent MS pathology. Natalizumab, a monoclonal antibody against very late antigen (VLA)-4 integrin, blocks leukocyte adhesion to the inflamed epithelium thus preventing entry of activated T cells into the CNS. This treatment was developed in the EAE model (Yednock et al., 1992) and is currently the most potent licensed drug for treatment of MS, reducing relapse rate by up to 70% (Saidha et al., 2012). Despite its efficiency, natalizumab, distributed under brand name Tysabri, is recommended as a monotherapy only for MS patients that have failed other disease-modifying therapies or those with aggressive disease. This is because natalizumab is associated with a significant risk of developing potentially fatal progressive multifocal leukoencephalopathy (PML) (Clifford et al., 2010). PML is an opportunistic viral infection of the CNS caused by reactivation of common latent

JC virus that may occur as a result of limited leukocyte infiltration and impaired immune surveillance within the CNS (Becher, 2008; Stüve et al., 2006). Importantly, impaired T cell access to the CNS may be observed for months after stopping the therapy with natalizumab (Stüve et al., 2009).

Recently the FDA has approved the first oral disease-modifying drug for RR-MS. Fingolimod is a small molecule inhibitor that interacts with sphingosine-1-phosphate (S1P) receptor, blocking lymphocyte egress from secondary lymphoid organs and preventing lymphocyte recruitment to sites of inflammation, such as the CNS (Cyster and Schwab, 2012). Interestingly, the drug does not prevent EAE in conditional knockout mice lacking S1P1 receptors on astrocytes, suggesting that fingolimod acts also on cells of the CNS (Choi et al., 2011). The results of phase 3 clinical trials showed that therapy with fingolimod reduced the annual relapse rate of patients by 54%, making this drug more effective than either GA or IFN- β (Kappos et al., 2010). However, due to potential side effects associated with immunosuppression, fingolimod remains a second-line treatment for relapsing-remitting MS in Europe.

GA and IFN- β have comparable efficacy in RR-MS and reduce annual relapse rate by only 30 - 40%. Although the therapeutic effect is modest both treatments are well tolerated. Moreover the relatively long global experience with these treatments has enabled detailed characterization of their safety profiles. Conversely, natalizumab and fingolimod, although very effective, pose significant risk of side effects associated with immunosuppression (Saidha et al., 2012). The price and inconvenience of frequent self-administered injections (GA and IFN- β) or intravenous infusions (natalizumab) constitute additional disadvantage of these therapies. Several other treatments for MS including antibodies such as anti-CD52 and anti-CD20 or oral drugs such as cladribine, dimethyl fumarate and teriflunomide, are currently being tested in clinical trials. However, like existing medication, many of these emerging MS treatments are known to be associated with serious adverse events (Saidha et al., 2012). Therefore there is a great need for inexpensive MS therapy that would be

convenient for the patient, have the advantage of improved efficacy and a good safety profile.

Recent epidemiological and experimental data support an exciting hypothesis that infection with live helminths could serve as an alternative probiotic therapy against MS. Controlled helminth infections were shown to be relatively safe and efficient in clinical trials involving patients with Crohn's disease and ulcerative colitis (Summers et al., 2005a; Summers et al., 2005b). Employing immunomodulation provided by helminth infection could prove to be inexpensive and associated with only mild and perhaps manageable side effects.

Interest in helminth therapy for MS increased considerably following publication of a report in 2007 showing that MS patients that were naturally infected with intestinal parasites had milder neurological symptoms and significantly less relapses than matched uninfected MS patients (Correale and Farez, 2007). Furthermore, PBMCs from infected patients when stimulated with myelin basic protein produced significantly more IL-10 and TGF- β , and less IL-12 and IFN- γ , than cells from uninfected patients. At least three small phase I studies have been conducted to date examining the effect of deliberate helminth infection in MS patients. The recently completed phase I trial of *Trichuris suis* ova in RR-MS showed promise for this alternative MS therapy, and no significant safety concerns were identified in the trial (Fleming et al., 2011). This preliminary study showed that helminth infection reduced the number of new active lesions in 4 out of 5 enrolled patients and following this remarkable result, two larger phase II trials testing infection with *T. suis* or *N. americanus* in MS patients are underway at the University of Wisconsin, Madison, USA and the University of Nottingham, UK (U.S. NIH ClinicalTrials.gov, study identifier NCT00645749 and NCT01470521).

Although probiotic helminth therapy seems to have many advantages there are certain drawbacks to a live worm approach. The infection with *T. suis* appears to be safe, as this porcine whipworm causes only transient colonisation of non-permissible human host, however, other helminth species may not be safe to use. Parasitic helminths are pathogens that benefit at the expense of the host and long term exposure to infectious helminths is likely to result in side

effects associated with accumulating tissue damage and inflammation. Therefore the characterisation of defined helminth-derived molecules should prove more beneficial. In addition the use of helminth products or their synthetic analogues could allow for better control and fine tuning of immunoregulatory effects.

1.6 Aim of the study.

Evidence from epidemiological studies and experimental infections suggest that immunomodulation induced by helminths could be beneficial for patients with autoimmune diseases. Clinical trials investigating the use of helminth infections as a therapy for MS are ongoing; however, the concept of deliberate inoculation with live worms is controversial. The identification of specific parasite products responsible for helminth-induced immunoregulation could eliminate the need for live worms and make potential helminth-based therapies safer and more convenient.

Infection with *F. hepatica* was shown to significantly attenuate clinical symptoms of EAE (Walsh et al., 2009). The use of live *F. hepatica* as probiotic human therapy is not possible since this parasite causes significant damage to the liver tissue. However it should be possible to reproduce immunomodulatory effect of infection using ES products from *F. hepatica*. The effect of *F. hepatica* ES on the development of immune-mediated disease have not yet been examined. Although several *F. hepatica*-derived products such as FhCatL were shown to have immunomodulatory properties, none have been evaluated in the EAE or other models of autoimmunity. Therefore this study aimed to:

- characterise immunomodulatory effects of *F. hepatica* ES on DC and T cell activation *in vitro*;
- determine if administration of *F. hepatica* ES can reproduce the suppressive effect of *F. hepatica* infection on the development of EAE;
- examine immune responses associated with attenuation of EAE by *F. hepatica* ES;
- attempt to identify putative immunomodulatory molecules mediating the immunomodulatory effect of *F. hepatica* ES.

Materials and Methods

Chapter 2

2.1 Materials

2.1.1 Ammonium chloride lysis solution

0.87% Ammonium chloride (NH_4Cl)

Dissolved in 100 ml ddH₂O

2.1.2 Cell culture medium

Roswell Park Memorial Institute-1640 medium (RPMI) or Dulbecco's Modified Eagle Medium (DMEM) (both from Sigma) were supplemented with 10% heat inactivated foetal calf serum (FCS; Biosera), 100 mM L-Glutamine (Gibco) and 100 µg/ml penicillin/streptomycin (Biowest). Complete RPMI (cRPMI) was used to culture murine bone marrow derived DCs while cRPMI supplemented with β-mercaptoethanol (2-ME 50 µM) was used to culture T cells, splenocytes and lymph node cells *ex-vivo*. Complete DMEM (cDMEM) was used to culture MFB-11 cell line.

2.1.3 ELISA reagents

Phosphate citrate buffer

10.19 g Anhydrous citric acid

36.69 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$

Made up in 1L of dH₂O, pH 5

ELISA developing solution

1 x 10 mg OPD tablet

25 ml Phosphate citrate buffer

7 µl 30% H₂O₂

ELISA stopping solution

1 M H₂SO₄

ELISA washing buffer

1x PBS

0.5% Tween

ELISA blocking buffer

5 % w/v milk (Marvell)

Or

1% w/v Bovine serum albumin (BSA)

Dissolved in 1 X PBS

Table 2.1 ELISA kits used in the study

Cytokine	Supplier	Top Working Standard
IFN- γ	BD Biosciences	10 ng/ml
IL-4	BD Biosciences	4000 pg/ml
IL-6	BD Biosciences	5000 pg/ml
IL-2	BD Biosciences	2000 pg/ml
IL-17A	R&D Systems	1000 pg/ml
IL-1 β	R&D Systems	1000 pg/ml
IL-12p70	R&D Systems	1000 pg/ml
TGF- β	R&D Systems	1000 pg/ml
IL-23	R&D Systems	2000 pg/ml
IL-10	R&D Systems	2000 pg/ml
KC	R&D Systems	4000 pg/ml

2.1.4 FACS buffer

2% FCS

0.1% Sodium azide

Made up in sterile PBS

2.1.5 MACS buffer

2% FCS

2 mM EDTA

Made up in sterile PBS

2.1.6 Phosphate-buffered saline (PBS) 20X

800 g NaCl

92 g Na₂HPO₄

20 g KH₂PO₄

20 g KCL

Dissolved in 5L of dH₂O, pH 7

2.1.7 Western blot reagents

RIPA buffer

50 mM Tris

150 mM NaCl

0.1% SDS

0.5% Sodium deoxycholate

1% Triton X100

Dissolved in dH₂O

Electrophoresis running buffer

125 mM Tris base

0.96 M Glycine

17 mM Sodium dodeca sulphate

Dissolved in dH₂O

Transfer buffer

25.5 mM Tris-HCl pH 8.3

0.2 M Glycine

20% v/v Methanol

0.05% w/v SDS

Dissolved in dH₂O

1x Sample Buffer

2.5 mM Tris-HCl pH 6.8

11% Glycerol

2% SDS

0.1% bromophenol blue
dH₂O
50 mM dithiothreitol (DTT) - only added before use

2.1.8 Isotonic percoll solution

Isotonic percoll solution was prepared by diluting Percoll reagent (GE Healthcare) 9:1 with sterile 10X PBS (Sigma-Aldrich). Isotonic percoll solution prepared this way had density of 1.123 g/ml. 40% and 70% isotonic percoll solution (made in sterile PBS) was used for isolation of mononuclear cells from brain tissue.

Table 2.2 TLR ligands used in the study

Ligand	TLR	Supplier	Conc. <i>in vitro</i>	Conc. <i>in vivo</i>
Pam ₃ CSK ₄	2	Invivogen	100 ng/ml	n/a
Poly I:C	2	Invivogen	25 µg/ml	n/a
Ultrapure LPS	4	Invivogen	100 ng/ml	10 µg/mouse
CpG	9	Sigma-Genosys	5 µg/ml	n/a

Table 2.3 Inhibitors used in the study

Reagent	Description	Supplier	Concentration
Complete Mini	Protease inhibitor cocktail	Roche	1 tablet/25 ml
Phosphatase inhibitor cocktail 3	Phosphatase inhibitors	Sigma	1/1000
E-64	Cysteine protease inhibitor	Sigma	2-50 nM
Z-FA-DMK	Cysteine protease inhibitor	Enzo	0.05-5 mM
Aprotinin	Serine protease inhibitor	Sigma	20-500 µM
GM6001	Inhibitor of MMP-1, 2, 3, 7, 8, 9,	Enzo	2.5-10 µM
U0126	ERK pathway inhibitor	Calbiochem	5 µM
SB-431542	ALK5 (TGF-βR II) inhibitor	Calbiochem	10 µM
Cycloheximide	Translation inhibitor	Sigma	1.6-200 µM

Table 2.4 Recombinant cytokines used in the study

Cytokine	Type	Supplier	Concentration used <i>in vitro</i>	Concentration used <i>in vivo</i>
GMCSF	J558 Supernatant	In-house	40 ng/ml	n/a
IL-1 β	Recombinant	R&D	10 ng/ml	100 ng/mouse
IL-23	Recombinant	R&D	10 ng/ml	100 ng/mouse
TGF- β	Recombinant	R&D	25 - 600 pg/ml	n/a

Table 2.5 Reagents used in animal studies

Reagent	Supplier	Concentration used <i>in vitro</i>	Dose used <i>in vivo</i>
DSS	MP Biomedicals, USA	n/a	2% in H ₂ O
<i>F. hepatica</i> ES	See section 2.2.1	0.32-10% (v/v)	20-40 μ l/mouse
MOG ₃₅₋₅₅	Genscript	2-100 μ g/ml	100 μ g/mouse
PT	Katetsuken	n/a	500 ng/mouse
CFA	Chondrex	n/a	100 μ l/mouse

Table 2.6 Antibodies used in cell cultures and *in vivo*

Antibody	Clone	Supplier	Concentration used <i>in vitro</i>	Dose used <i>in vivo</i>
Anti-CD3e	145-2C11	BD Biosciences	1 μ g/ml	n/a
Anti-CD28	37.51	BD Biosciences	5 μ g/ml	n/a
Anti-TGF- β	1D11	Bioceros BV	n/a	100 μ g/mouse
Unspecified IgG ₁	n/a	BioXcell	n/a	100 μ g/mouse

Table 2.7 Antibodies used for FACS

Antigen Specificity	Fluorochrome	Clone	Isotype	Supplier
CD11c	APC	N418	Hamster IgG	eBioscience
CD25	PE	PC61.5	Rat IgG1	eBioscience
CD3	A780	145-2C11	Hamster IgG	eBioscience
CD3	V450	17A2	Hamster IgG	eBioscience
CD3	V500	17A2	Rat IgG2a	eBioscience
CD4	FITC	GK1.5	Rat IgG2b	eBioscience
CD4	PE-Cy7	GK1.5	Rat IgG2b	eBioscience
CD4	V450	GK1.5	Rat IgG2a	eBioscience
CD40	FITC	HM40-3	Hamster IgM	BD Pharmingen
CD45	PE	30-F11	Rat IgG2b	BD Pharmingen
CD69	FITC	H1.2F3	Hamster IgG1	BD Pharmingen
CD8	A780	53-6.7	Rat IgG2a	eBioscience
CD80	PE	16-10A1	Hamster IgG2k	BD Pharmingen
CD86	PE-Cy5	B7.2	Rat IgG2ak	BD Pharmingen
FOXP3	APC	FJK-16S	Rat IgG2ak	eBioscience
Gr-1	PE-Cy7	RB68-C5	Rat IgG2bk	eBioscience
I-A/I-E	Pacific-Blue	M5/114.15.2	Rat IgG2ak	eBioscience
IFN- γ	PerCp Cy5.5	XMG1.2	Rat IgG1	eBioscience
IFN- γ	Pe-Cy-7	XMG1.2	Rat IgG1	BD Pharmingen
IL-13	PE	eBio13A	Rat IgG2a	eBioscience
IL-17 A	PE	TC11-18H10	Rat IgG1	BD Pharmingen
IL-17A	V450	TC11-18H10	Rat IgG2a	eBioscience
IL-4	PE-Cy7	BVD6-24G2	Rat IgG2a	eBioscience
TGF- β	PE	1D11	Rat IgG2a	IQ Products

Table 2.8 Other reagents used in FACS analysis

Chemical	Description	Supplier	Concentration
CFSE	Cell membrane dye (FL-1)	Invitrogen	2.5 µg/ml
7-AAD	Viability dye (FL-3)	Miltenyi	1/100
LIVE/DEAD	Viability dye compatible with ICF, (Aqua FL-8)	Invitrogen	1/600
PMA	Mitogen	Sigma	10 ng/ml
Ionomycin	Ionophore	Sigma	500 ng/ml
BFA	Golgi inhibitor	Sigma	5 µg/ml

Table 2.9 Primers used in quantitative real time PCR

Gene	Protein	Product Code	Supplier
<i>18S rRNA</i>	18S rRNA	4319413E	ABI
<i>lfng</i>	IFN-γ	Mm00801778_m1	ABI
<i>il10</i>	IL-10	Mm00439616_m1	ABI
<i>il17</i>	IL-17	Mm00439619_m1	ABI
<i>Rorc</i>	RORγt	Mm01261022_m1	ABI
<i>il4</i>	IL-4	Mm00445259_m1	ABI
<i>il23</i>	IL-23	Mm00518984_m1	ABI
<i>Il1r1</i>	IL-1R1	Mm00434237_m1	ABI
<i>il1b</i>	IL-1β	Mm00434228_m1	ABI
<i>tgfb1</i>	TGF-β	Mm00441724_m1	ABI
<i>il22</i>	IL-22	Mm00444241_m1	ABI
<i>homox1</i>	HO-1	Mm00516005_m1	ABI
<i>cd40</i>	CD40	Mm00441891_m1	ABI
<i>cd68</i>	CD68	Mm03047340_m1	ABI
<i>ccl2</i>	CCL2	Mm00441242_m1	ABI

<i>ccl3</i>	CCL3	Mm00441258_m1	ABI
<i>ccl5</i>	CCL5	Mm01302427_m1	ABI
<i>ccl20</i>	CCL20	Mm01268754_m1	ABI
<i>cxcl10</i>	CXCL10	Mm00445235_m1	ABI

2.1.9 Animals

Specific pathogen-free (SPF) female C57BL/6 and C3H/HeJ mice were purchased from Harlan UK Ltd (Bicester, Olac, UK). All animals were maintained according to the regulations and guidelines of the Irish Department of Health and Children. All experiments were performed under license from the Department of Health and with the approval from the Trinity College Dublin BioResources Ethics Committee. Mice were age matched for every experiment and were 12-20 weeks old at the initiation of the experiments.

2.2 Methods

2.2.1 Preparation of *F. hepatica* ES

Adult flukes were collected from infected bovine livers at a local abattoir, washed in several changes of PBS containing 100 µg/ml penicillin/streptomycin (PBS/PS) and transported to the lab. Live flukes were incubated overnight (18 h) in PBS/PS in cell culture incubator at 37°C / 5%CO₂. Supernatant fluid was harvested, cleared by centrifugation at 13,000 g for 30 min, sterilized by filtration through 0.22 µm filter attached to sterile filter unit, aliquoted and stored at -80°C.

2.2.2 Size fractionation using centrifugal filter units

Sterile *F. hepatica* ES was fractionated into high molecular weight fraction (ES-H) and low molecular fraction (ES-L) using centrifugal filter unit with 3 kDa molecular weight cut off membrane (Amicon Ultra-4, 3,000 MWCO, Milipore). 4 ml of *F. hepatica* ES were loaded onto filter device and centrifuged at 3000 g in 4°C for 1.5 h. The fraction retained over the filter (ES-H), which was containing concentrated compounds larger than 3 kDa was replenished to the original volume with sterile PBS. The fraction that passed through the filter (ES-

L) was collected without diluting. This approach did not change molar concentrations of molecules in any of the fractions allowing direct comparison of activity with the original *F. hepatica* ES preparation on a basis of volume. ES-H and ES-L were aliquoted and stored in -80°C.

2.2.3 Protein depletion using BindPro

BindPro (Biotech Support Group) is a polymeric, protein binding reagent designed for the removal of proteins from solution and is supplied as a 50% slurry. Before use, BindPro slurry was washed in PBS and centrifuged at 10,000 g for 5 min a number of times to remove the storage buffer. ES-L, ES-H or Pam₃CSK₄ were incubated at a 1 in 2 dilution (v/v) with BindPro slurry in 1.5 ml tube for 10 min in room temperature and constantly mixed by inverting the tube. Tubes were centrifuged at 10,000 g for 5 min and protein free supernatants were collected and used immediately or stored at -20. In some experiments proteins were eluted from BindPro resin by incubation in elution buffer (Tris-HCL, pH 10) for 5 min. Tubes were centrifuged at 10,000 g for 5 min and protein containing supernatant was collected. Incubation with elution buffer was repeated twice more, supernatants were collected and pooled. Protein containing supernatant was desalted and concentrated using centrifugal filter unit Amicon Ultra-4 with 3 kDa MWCO membrane. Concentrated sample was replenished to original volume with PBS.

2.2.4 Cell counts

Cell counts were performed by diluting cells (usually 1/10 dilution) with trypan blue (Sigma). A 10 µl volume of the cell suspension was then loaded onto a disposable hemocytometer (Hycor Biomedical, UK). The number of viable cells (appearing as white in contrast to dead cells, which stain blue) was counted using a light microscope. In case of samples where discrimination of live and dead cells was hampered by substantial amount of debris, or when cell suspensions contained a large proportion of red blood cells (RBCs), cell counts were performed in ethidium-bromide/acridine-orange solution (Sigma-Aldrich) at a 1 in 2 dilution. Using this method viable cells fluoresce green when viewed under a UV fluorescent microscope, while dead cells appear orange. The

number of cells per ml, regardless from the method, was calculated using the following formula: number of cells per ml = cell number $\times 10^4 \times$ dilution factor.

2.2.5 Generation of bone marrow derived dendritic cells

Bone marrow derived DCs were generated from C3H/HeJ or C57Bl/6 mice. Naive mice were sacrificed by cervical dislocation and their femurs and tibiae were removed and cleaned from the surrounding muscle and tissue. The bone marrow was then flushed from the bones using a 25 gauge needle attached to a 20 ml syringe containing cRPMI. Cell clusters were disrupted by aspirating the cell suspension through 19 gauge needle. The single cell suspension was centrifuged at 300 g for 5 min and cells were resuspended in 1 ml of warm 0.87% ammonium chloride for 2 min, to lyse contaminating red blood cells. Cells were washed in cRPMI and pelleted by centrifugation at 300 g for 5 min. The washed mononuclear cells were then resuspended in 10 ml cRPMI, cell viability and a total cell count was performed using trypan blue. The immature DCs were cultured at 1×10^6 cells/ml (C3H/HeJ) or 0.5×10^6 cells/ml (C57Bl/6) of complete RPMI with no more than 30 ml of cell suspension in a T175 tissue culture flask (Greiner). 40 ng/ml of granulocyte macrophage-colony stimulating factor (GM-CSF) in the form of supernatant obtained from the J558 cell line was added. After culture for 3 days at 37°C and 5% CO₂, 25 ml of fresh medium containing 40 ng/ml of GM-CSF was added to each T175 flask. On day 6, the supernatant was carefully removed so as not to dislodge the loosely adherent immature DCs. This step removed contaminating cells, such as granulocytes from the culture. Removed supernatant was immediately replaced with 25 ml of warm fresh cRPMI containing 40 ng/ml of GM-CSF. After a further 2 days at 37°C, (day 8) 20 ml of fresh medium containing GM-CSF (40 ng/ml) was added to each of the flasks. On day 9 the loosely adherent cells were harvested by gentle pipetting. The viability of DCs was assessed and the cells were cultured at the required concentration in tissue culture plates. The immature DCs were allowed to rest for at least 2 h at 37°C before use.

2.2.6 Stimulation of DCs with TLR ligands

Bone marrow derived DCs were cultured at the concentration of 1×10^6 in 96-well U-bottom tissue culture plates (Greiner). DCs were pre-treated with indicated concentration of *F. hepatica* ES for 1-2 h and then stimulated with various TLR agonists at the concentrations indicated in the Table 2.2 or with medium only. In some experiments DCs were pre-treated with specific inhibitors (Table 2.3) for 30 min prior to treatment with *F. hepatica* ES. After 24 h, supernatants were harvested and concentrations of cytokines were determined by ELISA.

2.2.7 Western blotting

Preparation of samples for western blotting

DCs were seeded at a density of 1×10^6 cells/ml, 2 ml per well in 6 well tissue culture plates (Greiner). After the appropriate stimulation, the supernatant was removed and the cells were washed with PBS and lysed in 100 μ l of RIPA buffer supplemented with Complete Mini protease inhibitor cocktail (Roche) and Phosphatase Inhibitor Cocktail 3 (Sigma). Samples were cleared by centrifugation at 10,000 g for 10 min at 4°C and then stored at -20°C until used.

SDS polyacrylamide gel electrophoresis

Directly before electrophoresis samples were diluted with 5x loading buffer and boiled at 95°C for 5 min. Samples were then resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad Mini-PROTEAN electrophoresis cell. Samples were run on 10% polyacrylamide gel at 120V alongside with pre-stained protein markers (Precision Plus Standards; Bio-Rad) as molecular weight standards.

Table 2.10 Polyacrylamide gel components

10% Resolving gel		Stacking gel	
dH ₂ O	5.9 ml	dH ₂ O	4.1 ml
1.5 M Tris-HCl (pH 8.8)	3.8 ml	1 M Tris-HCl (pH 6.8)	0.75 ml
Acrylamide:bisacrylamide (30% ProtoGel, National Diagnostics)	5 ml	Acrylamide:bisacrylamide (30% ProtoGel, National Diagnostics)	1 ml
10% SDS	0.15 ml	10% SDS	60 µl
10% ammonium persulfate (APS; Sigma)	0.15 ml	10% ammonium persulfate (APS; Sigma)	60 µl
TEMED (Fluka)	6 µl	TEMED (Fluka)	6 µl

Transfer of proteins to membrane

Directly after SDS-PAGE proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Whatman) using a wet transfer system. PVDF was activated by immersion in MeOH before rinsing in transfer buffer. The gel, and filter paper were soaked in transfer buffer. Membrane was placed on top of two pieces of filter paper followed by the gel and then two other sheets of filter paper to make a layered sandwich. Air bubbles were then removed and the sandwich was placed between soft pads in the transfer cassette. The cassette was placed in the transfer cell which was filled with transfer buffer. The transfer was carried out at 200 mA for 60 min.

Antibody blotting

Membranes were blocked to prevent non-specific binding by incubation in blocking buffer (5% BSA in PBS) in wash buffer (PBS/0.05% Tween 20) for 1 h in room temperature on a rocker. The membrane was rinsed in wash buffer and incubated with the primary antibody (p-ERK1/2, ERK1/2, p-p38, all 1/1000, Cell Signalling) on a rocker, at 4°C overnight. The membrane was washed 4 x 5 min on a rocker and incubated with the secondary horseradish peroxidase (HRP) linked antibody (goat anti-rabbit 1/5000, Cell Signalling) for 1 h in room temperature. The membrane was washed several times with wash buffer. Blots were developed by ECL Chemiluminescent Substrate (GE Healthcare). The membrane was placed between two sheets of transparent acetate in a cassette;

photographic film was placed on the top of the membrane and exposed for the required time. The film was processed using a Fuji X-ray developer.

2.2.8 NF- κ B Reporter gene assay

HEK293 cells stably transfected with TLR4, MD2 and CD14 (HEK293-TLR4) were a gift from Prof. A. Bowie and were maintained in cDMEM supplemented with 10 μ g/ml ciproflaxin, 10 μ g/ml blasticidin (Sigma) and 50 μ g/ml of HygroGold (Invivogen). For the NF- κ B reporter assay experiments cells were seeded in 96 well plates (1×10^5 cells/well) and transfected 24 h later using Genejuice. Genejuice mix was prepared by adding Genejuice (Novagen) to the serum free DMEM (0.8 μ l/10 μ l medium) and incubating for 5 min at room temperature. Then 60 ng/well of firefly luciferase reporter expression vector pPK-NF- κ B-luc and 20 ng/well of phRL-TK reporter gene (renilla luciferase, Promega) as the transfection efficiency control was added to the mixture and incubated for further 15 min at room temperature. Cells were then transfected by adding the mixture to the cells (10 μ l/well of a 96 well plate) and incubated at 37°C. After 24 h cells were treated with *F. hepatica* ES for 1 h following stimulation with LPS for 24 h. Then cells were lysed with the Passive Lysis Buffer (Promega). Samples were divided in two and 40 μ l of luciferase substrate (Luciferase mix, for the firefly luciferase or 2 μ g/ml Colenterazine in 1xPBS for the control renilla luciferase) were added to 20 μ l of cell lysates. Reporter gene activity was measured spectrophotometrically using the Luminoskan machine (Thermo) and Ascent Software v2.6. The final reporter activity was normalized to the activity of the renilla luciferase transfection efficiency control within the sample and expressed relative to the corresponding reporter gene only control.

2.2.9 TGF- β reporter assay

MFB-11 cell line has been developed by (Tesseur et al., 2006) and was a gift from Dr. A. Dunne. MFB-11 cells are embryonic fibroblasts from *tgfb1*^{-/-} mice that have been stably transfected with a reporter plasmid consisting of TGF- β responsive Smad-binding elements coupled to a secreted alkaline phosphatase reporter gene (SBE-SEAP). These cells produce SEAP in response to TGF- β 1,

TGF- β 2 and TGF- β 3, but do not respond to related family members activin, nodal, BMP-2 and BMP-6 or trophic factors bFGF and BDNF. The MFB-11 cells were maintained in cDMED supplemented with 100 μ l/ml hygromycin B (Sigma). For the experiments, cells were seeded in 96-well, flat-bottom plates (4 x 10⁴ cells/well) and let to rest overnight. Cells were washed twice with warm PBS and incubated with 100 μ l of serum free DMEM for 2 h before recombinant TGF- β or DCs (in serum free DMEM) with or without ES-L were added. The concentration of SEAP in supernatants was quantified by enzymatic assay using commercially available chemiluminescent SEAP detection kit (Great EscAPe SEAP Chemiluminescence Kit 2.0, Clontech) according to manufacturer's instructions. Chemiluminescence intensity was measured spectrophotometrically using the Luminoskan machine (Thermo) and Ascent Software v2.6. The final reporter activity was normalized to untreated control.

2.2.10 LDH cytotoxicity assay

The release of LDH to cell culture supernatants was measured by the CytoTox Non-Radioactive Cytotoxicity Assay Kit (Promega) according to manufacturer instructions. 50 μ l of the supernatant from cultured cells were transferred to flat-bottom 96-well plate and incubated with 50 μ l of Substrate Solution at room temperature, protected from light, for 30 min. Reaction was stopped by adding 50 μ l of Stop Solution and absorbance was recorded at 490 nm.

2.2.11 Determination of spleen cells proliferation by CFSE staining

Carboxyfluorescein succinimidyl ester (CFSE, ex/em 492/517 nm Sigma-Aldrich) is a lipid-based fluorescent dye that binds to cell membrane without affecting cell function and is used to assess cell proliferation. When cells divide, CFSE labelling is distributed equally between the daughter cells, which therefore have half of the fluorescence of the parent cell. Proliferation can be monitored by flow cytometry (FL-1) and is seen as a decrease in fluorescence intensity. A single cell suspension was prepared from spleens of naive mice. Red blood cells were lysed with 1 ml of heated 0.87% ammonium chloride for 2 min, washed and pelleted by centrifugation at 300 g for 5 min. Cells were

resuspended in 5 ml of warm PBS and added to previously prepared 5 ml of 5 µg/ml CFSE (Invitrogen) in PBS. Cells were incubated with the dye for 2.5 min (final working concentration 2.5 µg/ml), constantly mixed by inverting the tube. At the end of the incubation time 3 ml of FCS (Biosera) was added to quench residual dye and cells were centrifuged at 300 g for 5 min. Cells were then resuspended in cRPMI, counted and diluted with medium to the concentration of 1×10^6 cells/ml. Anti-CD3 antibody at the final concentration of 0.25 µg/ml was added to the suspension, cells were plated onto 96-well plate, treated with *F. hepatica* ES or PBS as a control and left for 72 h in the cell culture incubator. After 72 h cells were recovered, stained with antibodies against CD3 and CD4 as described in the FACS staining section of this chapter and analysed by flow cytometry.

2.2.12 CD4⁺ T cell isolation by FACS

Freshly isolated spleen cells from naive mice were washed and resuspended in a maximum volume of 1 ml of RPMI. Cells were surface stained with fluorescent antibodies directed against CD3 and CD4 (1/100 dilution). Double positive cells were purified by FACS with a MoFlo cell sorter (Beckman Coulter). Cellular purity was determined by flow cytometry and was consistently > 98%

2.2.13 γδ T cell isolation by MACS

γδ T cell were purified from pooled inguinal, auxiliary, brachial, cervical and mesenteric lymph node cells by sequential MACS sorting using TCRγδ T cell isolation kit (Miltenyi Biotec) as follows: first non-T cells were directly labelled with MicroBeads conjugated to monoclonal antibodies against mouse CD45R (B220; isotype: rat IgG2a), and CD11b (Mac-1 α-chain; isotype: rat IgG2b) while γδ T cells were labelled with biotin-conjugated monoclonal antibody against mouse TCRγδ. Non-T cells were depleted on an autoMACS machine using the “depl05” program. γδ T cells in remaining T cell enriched fraction were indirectly labelled with Anti-Biotin MicroBeads and purified by positive selection on an autoMACS machine using the “possel2” program. Cellular purity of separated populations was determined by flow cytometric analysis and was routinely >90%. γδ T cells were seeded at 0.5×10^6 cells/ml and stimulated as required.

2.2.14 Detection of T cell cytokine production *in vitro*

Spleen cells isolated from naive mice or purified $\gamma\delta$ T cells were stimulated with combination of IL-1 β (10 ng/ml) and IL-23 (10 ng/ml) or with anti-mouse CD3e (1 μ g/ml, BD Biosciences) and incubated alone or with increasing concentrations of *F. hepatica* ES. The concentration of cytokines in the supernatant after 18 h or 72 h was determined by ELISA.

Purified CD4⁺ T cells were stimulated with plate bound anti-mouse CD3e (1 μ g/ml: BD Biosciences) and anti-mouse CD28 (5 μ g/ml, BD Biosciences) and incubated alone or with increasing concentrations of *F. hepatica* ES. Plates were coated with 50 μ l of antibodies for 2h in 37°C and thoroughly washed in PBS before use. The concentration of cytokines in the supernatant after 18 h or 72 h was determined by ELISA.

2.2.15 Detection of LPS-induced cytokine production in serum

Naive C57BL/6 mice were injected i.p. with PBS, *F. hepatica* ES (50 μ l), LPS (10 μ g) or LPS and *F. hepatica* ES. After 4h animals were euthanized by CO₂ exposure and blood was collected following the cardiac puncture. The blood was allowed to clot in a 1.5 ml tube for 2 h in room temperature. Cells were removed by centrifugation at 13,000 g in a micro-centrifuge. The serum was collected and frozen at -20 °C or used immediately.

2.2.16 Subcutaneous immunization into the footpad

Mice were injected subcutaneously (s.c.) into the footpad with reagents dissolved in PBS in a total volume of 25 μ l (50 μ l/mouse). The lymphatics of the foot drain preferentially to the easily isolated popliteal LN (pLN), located behind the knee. pLN were removed 4 h after immunisation and cells were used as required

2.2.17 Induction, treatment and assessment of DSS colitis

Experimental colitis was induced in C57BL/6 mice. Animals were given 2% (w/v) dextran sodium sulfate (DSS, MP Biomedicals, USA, MW 36,000-50,000) *ad libitum* in sterile water for 7 days. Fresh DSS solution was made every 3 days. Mice were treated with 40 μ l of *F. hepatica* ES or PBS (i.p.) on days: 0, 2,

4, and 6. Weight loss was monitored daily. Mice were sacrificed at day 7 and colons were removed and measured from the ceco-colonic junction to the rectum with a ruler. The colon was cleaned of fecal material and sections of the proximal colon (5 mm) were collected into RNeasy lysis reagent and stored in -20°C for gene expression analysis or were fixed in 10% buffered formalin for histology. Tissues were embedded in paraffin, sectioned, dehydrated, and stained with haematoxylin and eosin. Samples were processed under direction of Professor J. Cassidy, School of Veterinary Medicine, University College Dublin.

2.2.18 Induction, treatment and assessment of EAE

Active induction of EAE

EAE was induced in C57BL/6 mice s.c. injection of 100 $\mu\text{g}/\text{mouse}$ of myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ (GenScript) emulsified in complete Freund's adjuvant (CFA, Chondrex Inc.) containing 4 mg/ml (400 $\mu\text{g}/\text{mouse}$) of heat killed H37 Ra *Mycobacterium tuberculosis* (Chondrex Inc). Mice were injected i.p. with 500 ng of pertussis toxin (PT; Kaketsuken, Japan) on days 0 and 2.

Induction of EAE by adoptive transfer

A donor group of mice were immunized by s.c. injection of 100 $\mu\text{g}/\text{mouse}$ of MOG emulsified in CFA without administration of PT. On day 10 after immunisation, spleens and inguinal, auxiliary, brachial, cervical and mesenteric LN of immunized mice were excised and cultured at $5-8 \times 10^6$ cells/ml in cRPMI in the presence of MOG (100 $\mu\text{g}/\text{ml}$), IL-1 β (10 ng/ml) and IL-23 (10 ng/ml). After 96 h, non-adherent cells were aspirated from the culture vessel, washed, resuspended in cRPMI and counted. Cells were then washed in PBS and the concentration was adjusted to transfer 1×10^7 live cells in 200 μl PBS into naive recipient mice via i.p. injection.

Treatment and scoring

Mice were treated with 20-40 μl of *F. hepatica* ES or its fractions i.p on the day before the induction of EAE, by immunisation or transfer, and every second day thereafter. For some experiments neutralising antibodies were used and

were administered i.p on the same day as *F. hepatica* ES. Animals were monitored daily for clinical signs of disease. Disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis; grade 5 – tetraparalysis/death. Animals were sacrificed for humane reasons if they reached a score above 4.

Leukocyte isolation from CNS tissue

Mice were anesthetized by intra-peritoneal injection of 40 µl pentobarbital (Eutheathal) and perfused intra-cardially with 20 ml ice-cold PBS to remove peripheral blood from CNS tissue. Brain tissue was isolated, homogenised in ice-cold Hanks Balanced Salt Solution containing 3% FCS (HBSS/FCS), and passed through 100 µm cell strainer. Tissue homogenate was washed with HBSS/FCS and centrifuged at 170 g for 10 min. Cell pellet was then suspended in 2 ml of HBSS/FCS containing Collagenase D (1mg/ml; Calbiochem) and DNase I (10 µg/ml; Calbiochem) and incubated for 45 min in 37°C with gentle shaking. Cells were then washed in HBSS/FCS and resuspended in 5 ml of 40% isotonic percoll solution. This was carefully overlayed on 5 ml of 70% isotonic percoll solution with a plastic Pasteur pipette. Percoll gradients were centrifuged at 2,000 g for 20 min at 18°C, without brake. After centrifugation leukocytes were found on the interface between 40% and 70% percoll solutions, myelin debris were floating on the surface of the 40% percoll. Cells were aspirated with a Pasteur pipette, washed twice with cRPMI, counted in etidium bromide/acridine orange solution and used as required.

2.2.19 Antigen specific restimulation

Single cell suspensions were prepared by homogenizing spleen or inguinal lymph nodes through a 40 µm cell strainer. Cells were centrifuged at 300 g for 5 min and red blood cells were lysed by incubating loose cell pellets with heated 0.87% ammonium chloride. Cells were washed with cRPMI, centrifuged at 300 g for 5 min and counted using trypan blue solution. Cells were resuspended at 2×10^6 (spleens) or 1×10^6 (lymph nodes) and seeded in 200 µl in a 96-well U-bottomed plate. The MOG peptide solution was added to triplicate wells at final concentrations of 2-100 µg/ml. PMA at 10 ng/ml and anti-mouse CD3e

antibody at 1 µg/ml were used as a positive control. Cells were incubated for 72 h at 37°C/5% CO₂ and cytokine concentration in the supernatants was quantified by ELISA.

2.2.20 Flow cytometry

Surface staining

Single cell suspensions, prepared from organs isolated from *in vivo* studies or obtained from *in vitro* cultures were added to a FACS tube at 1 x 10⁶ cells in 100 µl per tube. The cells were then washed in FACS buffer and centrifuged at 300 x g for 5 min, non-specific binding was blocked by incubation with 1 µg/ml of anti-CD16/CD32 (Fcγ Block, BD Pharmingen) for 10 min at room temperature. Cells were then incubated with 100 µl of the appropriate FACS antibody (Table 2.7) directed against a surface antigen for 15 min at room temperature in the dark. In parallel, single stained compensation controls were prepared using FACS comp beads (BD). Fluorescence minus one (FMO) controls were also stained. Excess staining antibody was removed by washing each tube twice in FACS Buffer, 1 ml per tube, with centrifugation at 300 g for 5 min between each wash. Cells were resuspended in 0.5 ml FACS buffer for the analysis. If the discrimination of live and dead cells was required 7-AAD (1/200 Miltenyi Biotech), which is a fluorescent DNA dye that only stains dead cells (as intact membranes prevent the ingress of the dye into live cells), was added to the tubes directly before acquisition by flow cytometer.

Cell activation prior to the detection of cytokine production by FACS

Cell suspensions were added to a sterile FACS tube at 1-2 x 10⁶ cells in 500 µl per tube. Cells were activated with PMA (10 ng/ml) and Ionomycin (1 µg/ml) in the presence of Brefeldin A (BFA) (5 µg/ml) in cRPMI supplemented with 2-ME (50 µM) and incubated at 37°C and 5% CO₂ for 6 h. Alternatively, cells were incubated with BFA alone for 4 hours at 37°C. After incubation, cells were washed twice with 3 ml of FACS buffer, pelleted by centrifugation at 300 g for 5 min and stained as required.

Intracellular staining

In order to detect intracellular epitopes such, as cytokines or transcription factors, cells were first stained with surface antibodies, as described above. Before surface staining cells were washed in PBS and stained with LIVE/DEAD Fixable Aqua Dead Cell stain (Aqua-Live/dead, Molecular probes, Life Technologies) diluted 1/600 in PBS for 20 min at room temperature in the dark. Aqua-Live/dead is an amine-reactive fluorescent dye that stains dead cells and can be detected by FACS using a filter band for V500/Am-Cyan. In contrast to 7-AAD this dye is compatible with intracellular FACS staining. After the last wash the FACS buffer was completely removed and cells were resuspended in Fix/Perm buffer (eBioscience) and incubated for 30 min at 4°C in the dark. Cells were then washed twice with Permeabilization buffer (eBioscience) and stained with 100 µl of appropriate fluorochrome-conjugated antibody (eBioscience) diluted 1/20 in Permeabilization buffer. After 30 min cells were washed twice with Permeabilization buffer followed by centrifugation at 300 g for 5 min, resuspended in 0.5 ml FACS buffer and acquire as described above.

Stained cell suspensions were acquired using DAKO CyAN_{ADP}, (DakoCytomation) or CANTO-II (BD) flow cytometers according to the manufactures instructions. The flow cytometer was calibrated using single-stained suspensions of compensation beads (BD). FACS data analysis was performed using FlowJo (Treestar) software.

2.2.21 Measurement of cytokine concentration by ELISA

Cytokine concentrations were determined using commercially available ELISA kits according to the manufacturer guidelines. Antibody dilutions and incubation times were different between manufacturers and are detailed below. High binding microtitre plates (Greiner bio-one) were coated overnight at 4°C with 50 µl per well of capture antibody diluted in PBS. Plates were washed 5 times in ELISA wash buffer (PBS / 0.05% Tween 20) and non-specific binding sites were blocked by incubation with 150 µl of blocking buffer for 2 h at room temperature. Plates were again washed 5 times before the addition of 50 µl per well of sample supernatant or an eight point two-fold serial dilution from the top working standard recombinant protein for each cytokine diluted in reagent

diluent (1% BSA in PBS). For TGF- β ELISA samples were prepared by incubation in 80°C for 10 min before applying onto ELISA plate. Plates were incubated overnight at 4°C. After washing, 50 μ l of specific biotinylated detection antibody diluted in 1% BSA was added per well and incubated at room temperature. Plates were again washed 5 times and incubated with 50 μ l / well horseradish peroxidase (HRP)-conjugated streptavidin for 20 min at room temperature in the dark. Plates were washed 7 times and 50 μ l of OPD substrate in phosphate citrate buffer added to each well. ELISA plates were developed in the dark after which, the enzyme reaction was stopped by the addition of 25 μ l/well of 1 M H₂SO₄. The OD values were determined by measuring absorbance at 492 nm using a Versamax microplate reader (Molecular Devices) and cytokine concentrations for supernatant samples were calculated using the standard curve prepared using recombinant mouse cytokine.

ELISA assay details

R&D systems

Capture antibody dilution: 1/180;

Blocking reagent: 1% BSA in PBS;

Detection antibody dilution: 1/180, incubation time 2 h;

Streptavidin dilution: 1/200;

BD Bioscience

Capture antibody concentration: 1 μ g/ml;

Blocking reagent: 5% skimmed milk (Sigma) in PBS;

Detection antibody concentration: 1 μ g/ml incubation time 1 h;

Streptavidin dilution: 1/1000

2.2.22 Endotoxin detection by LAL assay

The endotoxin (LPS) levels in *F. hepatica* ES were determined using commercially available kinetic chromogenic limulus amoebocyte lysate (LAL) assay according to manufacturer's instructions. ES samples in triplicates and a 8-point standard curve comprising of 10-fold serial dilution of endotoxin

provided with a kit were mixed with reconstituted LAL reagent and placed in an incubating plate reader at 37°C (Versamax; Molecular Devices). The enzymatic reaction triggered by endotoxin released chromophore from the chromogenic substrate and resulted in accumulation of yellow coloured product. The changes in the absorbance of the samples were monitored at 405 nm over a 1 h. The time (onset time) required for each sample to reach a specified absorbance (OD = 200) was recorded and a standard curve showing the linear correlation between log onset time and the log concentration of endotoxin was generated. The concentration of endotoxin in tested samples was calculated on the basis of the standard curve.

2.2.23 RNA isolation

RNA isolation from cells

RNA was isolated from *in vitro* cultured cells or lymph node cells *ex vivo* using RNeasy Mini Kit (Qiagen) according to manufacturer instruction. Cells ($<5 \times 10^6$) were lysed with 350 µl of RLT buffer and homogenised by aspirating through 23 gauge needle. 350 µl of 70% ethanol was added to the lysates and samples were transferred to the RNeasy spin columns and centrifuged for 30 sec at 10,000 g, the flow through was discharged. Column was washed once with 700 µl of RW1 buffer and then twice with RPE buffer. After each wash column was centrifuged for 30 sec at 10,000 g and flow through was discharged. Finally column was placed in a new collection tube and RNA was eluted with 20 µl of RNase-free H₂O by centrifugation for 2 min at 10 000 g. RNA concentration was determined spectrophotometrically using a Nanodrop (Thermo Scientific) according to the manufacturer's instructions.

RNA isolation from tissues

Total RNA was extracted from colon or brain tissue using the TRIzol/chloroform method and RNase-free plastic equipment. Colon sections were removed from RNAlater reagent and homogenised in 500 µl of TRIzol (Invitrogen) using a benchtop rotor-stator homogeniser. Brain tissue was homogenised in 3% HBSS, washed and resuspended in TRIzol (400 µl TRIzol/100 µl tissue homogenate). 200 µl of chloroform was added to each

tube, vortex mixed, left to stand at room temperature for 3 min and then centrifuged at 12,000 g for 15 min at 4°C. The resulting upper aqueous phase was removed to sterile RNase-free tubes, and 500 µl isopropanol was added. Samples were mixed and left to stand at room temperature for 10 min, before centrifugation at 12,000 g for 10 min at 4°C. Supernatants were decanted and the pellet washed in 75% ethanol in nuclease-free water at 7,600g for 5 min at 4°C. Finally, remaining ethanol was removed with a pipette and the tubes were inverted and allowed to air dry for 20 min. RNA was resuspended in 40 µl nuclease-free water. RNA concentration was determined spectrophotometrically using a Nanodrop (Thermo Scientific) according to the manufacturer's instructions.

mRNA purification from colon tissue

DSS, a chemical used to induce colitis in mice interferes with the analysis of gene expression by inhibiting polymerase activity at various stages of the process (Kerr et al., 2012). Therefore, to reduce the contamination with DSS, mRNA was purified from total RNA isolated from colon tissue using Dynabeads mRNA Purification Kit according to manufacturer instructions. The magnetic Dynabeads were coupled with Oligo(dT)₂₅ that specifically capture poly(A)-tails of mRNA molecules. The assay was performed on flat-bottom RNase-free 96-well plate, and during buffer changes the magnetic beads were immobilised with help of a magnet designed for the purpose of the assay. First beads were equilibrated with Binding Buffer, and then mixed with equal volume (25 µl) of purified total RNA (10 µg per sample). The beads and RNA were incubated with gentle mixing for 5 min in room temperature. The beads were then washed twice with Wash Buffer to remove contaminating RNA species. Finally the interaction between mRNA and the beads was disrupted by heating at 65°C for 2 min and purified mRNA was eluted into 15 µl of RNase-free H₂O. The samples processed immediately after isolation.

2.2.24 Reverse transcription and real time PCR reaction

1 µg (tissues) or 0.05 µg (γδ T cells) of each RNA sample was reverse-transcribed into cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems), according to the manufacturer's protocol, and the cDNA reverse transcriptase product was

diluted 1:8 with nuclease free water. Transcripts were quantified by real time quantitative PCR on an ABI 7500 Fast Real Time PCR System with Applied Biosystems predesigned Taqman Gene Expression Assays and reagents according to the manufacturer's instructions. For each sample, mRNA abundance was normalised to the amount of 18S ribosomal RNA (rRNA) and is expressed as fold difference compared to cells incubated in medium only or as fold difference compared to control mice.

2.2.25 Cysteine protease assay

ES-H was diluted in cathepsin buffer (100 mM sodium acetate pH 5.5, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, and 0.01% Tween) to a concentration of 0.2-5% (v/v) and added in duplicates to black flat-bottom 96 well plate (Nunc), 50 μ l per well. 50 μ l of fluorogenic substrate Z-FR-AMC (Sigma) was added to the wells at a concentration of 10 μ M in cathepsin buffer to start the reaction. The progress of the reaction was monitored by fluorescence spectroscopy, using a Molecular Devices Gemini XS system, with an excitation of 350 nm and emission wavelength of 460 nm, and the data were plotted versus time. In inhibition assays ES-H was directly incubated with cathepsin inhibitor Z-FA-DMK (Enzo) at a range of concentrations (0.05 – 5 mM) for 20 min at 37°C. Then ES-H/Z-FA-DMK was diluted in cathepsin buffer to make 0.2% solution and added in duplicates to black flat-bottom 96 well plate, 50 μ l per well. 50 μ l of substrate was added to each well and the reaction was monitored as described above.

2.2.26 Size exclusion chromatography

The size exclusion chromatography was performed using Akta Purifier system with a Superdex75 10/300 GL column (GE Healthcare). ES-H was concentrated 10X using Amicon Ultra-4 3kDa MCWO and was applied on the previously equilibrated column using 100 μ l sample loop and resolved in PBS at a flow rate of 0.5 ml/min in room temperature. The fractions (0.5 ml) were collected and stored in -20°C until required. Three separations were performed (3x100 μ l of 10x ES-H) and multiple fractions were pooled as indicated on the Fig. 5.21 and concentrated using Amicon Ultra-4 3kDa MCWO to 3 ml each to

match the molar concentration of compounds in ES-H. Fractions were stored at -80°C.

2.2.27 Protein identification by mass spectrometry

Mass spectrometry analysis was commercially performed in Laboratory of Mass Spectrometry, IBB PAN in Warsaw, Poland. In brief the sample fraction Fr.1 underwent reduction and alkylation of protein disulfide bonds, followed by tryptic digestion. Obtained peptide mixture was separated by liquid chromatography (LC) followed by MS measurement of peptides and their fragmentation spectra (tandem mass spectrometry). Mascot search engine was used to search and identify proteins on the basis of acquired spectra against the NCBI nr protein sequence database.

2.2.28 Statistical analysis

Statistical analysis was performed using the computer based mathematical package GraphPad Prism. Student's t test was used to compare the mean values between two groups. Statistical differences in mean values between more than two experimental groups were determined by One-way Analysis of Variance (ANOVA) followed by Tukeys post test or by Two-way ANOVA with repeated measures where appropriate. P values less than 0.05 were considered significant.

***Immunomodulatory properties of
F. hepatica ES***

Chapter 3

3.1 Introduction.

Therapeutic helminth infection has been shown to produce spectacular improvements in inflammatory conditions in both humans and mice. However, the infection itself can often lead to adverse side effects including extensive tissue damage or iron-deficiency anaemia, both resulting from the parasite's feeding behaviour (Satoskar et al., 2009). Therefore parasite-derived products rather than live worms have much more therapeutic potential and are the subject of extensive investigations aimed at identifying immunomodulatory components capable of blocking pathogenic T cell responses. Studies in murine models have already shown that parasitic excretory/secretory products (ES) or defined ES-derived immunomodulatory molecules can protect against allergy or autoimmunity as efficiently as parasite infection (Kuijk et al., 2012; McInnes et al., 2003).

The specific focus of this study centred on *F. hepatica*, a parasitic flatworm of the class *Trematoda*, commonly known as a liver fluke. Previous work by Walsh *et al.* (Walsh et al., 2009), investigating the interaction between *F. hepatica* and the host immune system, have shown that infection of mice with *F. hepatica* reduced the severity of EAE. Although the exact mechanism of *F. hepatica*-mediated suppression of pathogenic T cells was not determined, it was demonstrated that neutralisation of TGF- β reversed the protective effects of the parasite infection (Walsh et al., 2009).

Experimental models of infection have proved to be an invaluable tool in mechanistic studies on helminth-induced immune suppression *in vivo*, nonetheless they have to be employed with caution. The use of mice as hosts for experimental *F. hepatica* infection, albeit widely employed by researchers, is not ideal from a host-pathogen perspective. Fully developed adult liver fluke can measure up to 30mm. It naturally infects large ruminants such as cattle and sheep, causing chronic but not severe disease. In rodents however, parasites that burrow through the gut wall and liver cause widespread pathology that, depending on number of developing worms, can kill mice within 4-6 weeks from experimental inoculation (De Paula et al., 2010; López-Abán et al., 2007).

Therefore extensive tissue damage that indirectly affects the function of all organs and systems may obscure immune modulatory mechanisms that are normally triggered to subvert protective anti-helminth responses of the host. Moreover the *F. hepatica* metacercariae do not always develop into fully sexually matured adult worms in the murine host (De Paula et al., 2010), thus a complete understanding of an established, chronic interaction between the parasite and immune system is not possible in this model. Since experimental infection is a very complex model that has the limitation of causing death of the host, this study aimed to characterise the immunomodulatory properties of excretory/secretory products (*F. hepatica* ES) derived from adult *F. hepatica* and investigate their effects on the development of immune responses and attenuation of autoimmunity.

In experimental parasitology, excretory/secretory products are usually obtained by incubating adult worms *in vitro* in a protein-free medium and subsequently collecting the cell free supernatant remaining after several hours. The *Trematode* excretory/secretory products isolated in this way consist of a crude mixture of various types of biological material ranging from metabolites and structural elements, to dead cells and cecum contents. Many of these components can potentially exhibit immunomodulatory activity *in vitro* although only application in *in vivo* models can verify their value as potential therapeutics.

This part of the study focused on an examination of potential immunomodulatory properties of *F. hepatica* ES using a range of *in vitro* and *in vivo* models. The aim was to employ experimental systems that would generate highly reproducible and robust data on modulation of innate and adaptive immune responses allowing for further isolation and identification of specific immunomodulatory molecules.

3.2 Results

3.2.1 *F. hepatica* ES modulate TLR agonist induced cytokine production by dendritic cells

Recently, a number of studies have shown that helminth infection, as well as helminth derived products have suppressive immunomodulatory effects on DCs function and cytokine production (Everts et al., 2010; Hamilton et al., 2009; Kane et al., 2004). In order to examine effects of *F. hepatica* ES products on cytokine production by DCs, bone marrow derived DCs were treated with *F. hepatica* ES for 2 h prior to stimulation with Pam₃CSK₄, poly I:C, LPS or CpG-ODN (CpG) – ligands for TLR2, 3, 4 and 9 respectively. Concentration of the Th1 polarizing cytokine IL-12p70 in supernatants was determined 24 h later by ELISA. The result revealed that, *F. hepatica* ES suppressed LPS and poly I:C induced secretion of IL-12p70 but enhanced expression of this cytokine following CpG or Pam₃CSK₄ stimulation. Importantly, treatment of DCs with *F. hepatica* ES alone also induced low levels of IL-12p70 production (Fig. 3.1). This observation raised the possibility that the *F. hepatica* ES was contaminated with bacterial LPS, which could potentially synergize with CpG and Pam₃CSK₄ leading to enhanced IL-12p70 expression. Since adult worms were isolated from the livers of naturally infected cattle in a non-sterile environment, the *F. hepatica* ES preparation was exposed to environmental microorganisms. Indeed the *F. hepatica* ES preparation showed moderate contamination with endotoxin (45 EU/ml; Fig 3.2A). As a result various LPS removal techniques including Triton X114, Polymyxin B or Endotrap columns were employed in order to reduce endotoxin levels in ES products. Unfortunately these approaches resulted in significant loss of bioactive material. Therefore, to exclude the influence of contaminating LPS, the modulatory effect of *F. hepatica* ES on TLR2 and TLR9 signalling pathway was tested using DCs derived from bone marrow of C3H/HeJ mice stimulated with CpG and Pam₃CSK₄. C3H/HeJ mice bear a mutated version of TLR4 and are non-responsive to LPS. Pre-treatment of DCs from C3H/HeJ mice with *F. hepatica* ES significantly inhibited IL-12p70 production induced by CpG (Fig. 3.2B) or Pam₃CSK₄. These data

demonstrate that *F. hepatica* ES products are capable of inhibiting DCs activation induced by ligands of TLR2, TLR3, TLR4 and TLR9.

Having shown that *F. hepatica* ES inhibits TLR ligand induced secretion of IL-12p70, the effects of *F. hepatica* ES on the expression of other cytokines involved in T cell polarization by DCs were examined. DCs derived from C57BL/6 (Fig 3.3) or C3H/HeJ (Fig. 3.4) mice were pretreated with a range of concentrations of *F. hepatica* ES for 2 h prior to stimulation with LPS (100 ng/ml) or CpG (5 µg/ml). Following 24 h of incubation concentration of IL-12p70, IL-23 and IL-6 in supernatants was measured by ELISA. Pretreatment with *F. hepatica* ES inhibited IL-12p70 production induced by LPS (Fig. 3.3 A) or CpG (Fig. 3.4 A) in a dose dependent manner ($p < 0.001$). In addition *F. hepatica* ES also significantly suppressed LPS-induced production of IL-23 (Fig. 3.3 B) which is important for the development of Th17 cells and IL-17 production from $\gamma\delta$ T cells and NK cells (McGeachy and Cua, 2008; Sutton et al., 2009). *F. hepatica* ES induced similar dose dependent inhibition of CpG induced IL-23 production (Fig. 3.4 B). Secretion of IL-6 induced by both LPS (Fig. 3.3 C) and CpG (Fig. 3.4 C) was also inhibited by *F. hepatica* ES and this was significant at the highest concentration of *F. hepatica* ES. Pretreatment with *F. hepatica* ES without TLR stimulation induced negligible production of the cytokines tested. Collectively the data demonstrate that capacity of *F. hepatica* ES to inhibit production of multiple cytokines induced by TLR ligands is not limited to a pathway triggered by a specific TLR receptor.

Activation of TLR4 by LPS triggers signalling via MAP kinases and transcription factor NF- κ B, and culminates in the induction of pro-inflammatory cytokine expression. Since *F. hepatica* ES inhibits LPS induced IL-12p70 and IL-23, there was a possibility that this is mediated through modulation of NF- κ B signalling. To address this possibility the study employed a reporter system utilizing cells transfected with a firefly luciferase plasmid under control of NF- κ B. Activation of the NF- κ B pathway with LPS led to luciferase expression, the activity of which was detected in cell lysates. Incubation of transfected cells with *F. hepatica* ES for 1 h prior to stimulation with LPS did not impair luciferase

expression suggesting that *F. hepatica* ES induced suppression of cytokine production is not mediated by modulation of NF- κ B signalling (Fig. 3.5 A).

MAP kinases are also involved in IL-12p70 production and have been targeted by certain parasite products (Everts et al., 2010; Goodridge et al., 2003). A study by Goodridge and co-workers showed that ES-62, a product of filarial nematode *A.viteae* targets ERK and p38 pathways in order to suppress expression of IL-12p70 (Goodridge et al., 2005). Therefore it is possible that *F. hepatica* ES may target MAP kinases pathways in DCs. However, western blot analysis of cell lysates revealed that pretreatment of DCs with *F. hepatica* ES for 1 h did not affect LPS-induced phosphorylation of ERK or p38 (Fig. 3.5 B). Furthermore, inhibition of ERK with the MEK1/2 inhibitor U0126 for 30 min prior to treatment with *F. hepatica* ES and subsequent stimulation with LPS did not reverse the inhibition of IL-12p70 (Fig. 3.5 C).

To rule out a possibility that the observed inhibition of TLR-ligand induced cytokine production was due to *F. hepatica* ES related cytotoxicity, the lactate dehydrogenase (LDH) activity assay was employed to assess viability of DCs following *F. hepatica* ES treatment. LDH is a soluble cytosolic enzyme that is released into the culture medium following the loss of membrane integrity resulting from cell death. The activity of LDH can be quantified spectrophotometrically by measuring degradation of a specific chromogenic substrate. The supernatants from DCs, incubated for 24 h with a range of concentrations of *F. hepatica* ES, showed an activity of LDH that was comparable to that of untreated control cells (Fig.3.6) demonstrating that *F. hepatica* ES did not have any cytotoxic effect on DCs.

3.2.2 *F. hepatica* ES induces production of TGF- β from DCs

Having shown that *F. hepatica* ES exerts an inhibitory effect on the expression of pro-inflammatory cytokines, the role of *F. hepatica* ES as a potential modulator of regulatory cytokine expression was also examined. *F. hepatica* ES induced significant, dose dependent secretion of TGF- β from C57BL/6 DCs (Fig. 3.7 A). Similar results were observed with C3H/HeJ DCs suggesting that induction of TGF- β was independent of TLR4 stimulation (Fig.

3.7 B). *F. hepatica* ES also induced TGF- β production by other antigen presenting cells including bone marrow derived macrophages and primary glial cells that significantly up-regulated TGF- β secretion following 24 h incubation with *F. hepatica* ES (Fig. 3.7 C and D).

Having demonstrated that *F. hepatica* ES induced secretion of TGF- β from DCs at the protein level, it was important to determine whether it also induced transcription of the gene encoding for TGF- β . Total RNA was extracted from DCs incubated with 5% (v/v) *F. hepatica* ES for 2, 4, 6 or 18 h and the expression of the *tgfb1* gene was measured by quantitative, real-time PCR. Supernatants from these cultures were collected to examine the kinetics of TGF- β protein production. Despite the fact that significant amounts of TGF- β protein were detected in cell culture supernatants as soon as 2 h after stimulation (Fig 3.8A), no changes were observed in relative expression of *tgfb1* mRNA versus untreated control at any of the time points examined (Fig.3.8B). Furthermore, pretreatment of DCs with cycloheximide, a potent inhibitor of translation, did not affect *F. hepatica* induced TGF- β production by these cells. Taken together these results suggest that *F. hepatica* ES induced secretion of TGF- β does not involve transcription or translation and most likely results from a release of pre-formed TGF- β from cell surface.

It has been widely reported that TGF- β can suppress the function of DCs (Lyakh et al., 2005) (Mou et al., 2011) by inhibiting TLR ligand induced maturation and production of IL-12p70. Having demonstrated that *F. hepatica* ES inhibits LPS induced production of pro-inflammatory cytokines by DCs and induced release of TGF- β , an experiment was designed to determine whether this suppressive effect was mediated through TGF- β . However inhibition of TGF- β dependent signalling in DCs by targeting the TGF- β receptor kinase ALK5 with the specific inhibitor SB-431542 did not reverse suppression of IL-12p70 or IL-6 production by *F. hepatica* ES (Fig. 3.9).

3.2.3 Effect of *F.hepatica* ES on DC maturation

The cytokines produced by activated DCs play a central role in directing phenotypic lineage commitment of effector T cells. However, direct DC-T cell contact is necessary for initiation of adaptive immune responses. Upon activation, DCs up-regulate the expression of MHC II as well as co-stimulatory molecules such as CD80, CD86 and CD40. The interaction of these molecules with their corresponding ligands on the T cell surface provides bidirectional stimulatory signals that are important for the activation of the T cells and for stabilisation of the APC function of the DCs (Kapsenberg, 2003). Having shown that *F. hepatica* ES is a potent inhibitor of cytokine production, the effect of pretreatment with *F. hepatica* ES on LPS induced expression of CD80, CD86, CD40 and MHC II on the surface of DCs was examined (Fig.3.10). LPS activation of DCs enhanced surface expression of MHCII, CD40, CD80, and CD86. Pretreatment with a range of concentrations of *F. hepatica* ES markedly suppressed the expression of CD40 and MHC II induced by LPS. The suppressive effect of *F. hepatica* ES on the expression of CD80 and CD86 was less pronounced, nevertheless inhibition was observed at the highest concentration of *F. hepatica* ES used.

3.2.4 Effects of *F. hepatica* ES on T cell activation

Hyporesponsiveness of T cells is a well described feature of many chronic parasite infections (Sher et al., 2003). In order to investigate the effects of *F. hepatica* ES treatment on polyclonal T cell activation, spleen cells from naive mice were labelled with CFSE, a dye that enables monitoring of cell division by flow cytometry, and activated with anti-CD3 (0.25 µg/ml) in the presence of absence of *F. hepatica* ES. Supernatants were harvested after 3 days and concentrations of IFN-γ and IL-17 were measured by ELISA. Remaining cells were recovered and proliferation of the CD4⁺ population was analysed by flow cytometry. CFSE, the reagent used to assess T cell proliferation in this experiment, is a lipid-based fluorescent dye that binds to cell membrane without affecting cell function. When cells divide, CFSE labeling is distributed equally between the daughter cells, which therefore have half of the fluorescent

intensity of the parent cell. As a result, when cells proliferate, dilution of fluorescence intensity is seen as a shift of the monitored population from bright to dim. Treatment with *F. hepatica* ES markedly suppressed anti-CD3 induced dilution of CFSE on CD4⁺ T cells suggesting that *F. hepatica* ES inhibited proliferation of this population (Fig. 3.11 A). In agreement with this observation, a significantly lower concentration of IL-2 was detected in supernatants (after 18 h) from cells treated with *F. hepatica* ES when compared to controls (Fig. 3.11 B). Incubation with *F. hepatica* ES also substantially decreased IFN- γ (Fig. 3.11 C) and IL-17 (Fig. 3.11 D) production by spleen cells which directly reflects its suppressive effect on polyclonal T cell activation.

Having shown that *F. hepatica* ES can suppress activation of both DCs and CD4⁺ T cells from mixed spleen cell culture the next experiment was designed to establish whether the inhibition of T cell activation could be attributed to the direct effect of *F. hepatica* ES on CD4⁺ T cells. CD4⁺ T cells were FACS purified (99% pure, Fig. 3.12 A) from spleen cells and activated with plate bound anti-CD3e (1 μ g/ml) and anti-CD28 (5 μ g/ml) in the presence of 2.5% *F. hepatica* ES. Analysis of cytokine concentration in supernatants demonstrated that *F. hepatica* ES suppressed IL-2 and IFN- γ production by both total spleen cells and purified CD4⁺ cells (Fig. 3.12.B). This result demonstrates that *F. hepatica* ES also has a direct inhibitory effect on the T cell activation that is not mediated through or depends on the presence of other cell types such as APCs.

It is known that IL-1 β in combination with IL-23 is capable of inducing IL-17 and IFN- γ production from CD3⁺ T cells from naive mice without T cell receptor (TCR) engagement (Sutton et al., 2009). Having established that *F. hepatica* ES is a potent suppressor of anti-CD3e induced proliferation and cytokine production, the study was extended to examine the effect of *F. hepatica* ES on TCR independent T cell responses. Spleen cells were incubated with combination of IL-1 β and IL-23 with or without *F. hepatica* ES. Stimulation of spleen cells with IL-1 β and IL-23 without TCR engagement generated significant production of IL-17 and IFN- γ , which was suppressed by *F. hepatica*

ES in a dose dependent manner (Fig. 3.13). This provides further evidence that *F. hepatica* ES can suppress T cell responses independent of its effect on DCs.

3.2.5 *F. hepatica* ES modulates LPS-induced cytokine production *in vivo*

Having demonstrated that *F. hepatica* ES is a potent inhibitor of LPS-induced cytokine production by DCs *in vitro*, the capacity of parasite derived products to suppress pro-inflammatory responses *in vivo* was examined. Mice were injected i.p. with LPS (10 µg/mouse) in presence or absence of *F. hepatica* ES (50 µl/mouse), sacrificed after 4 h and concentrations of IFN-γ, IL-6, and KC were measured in the sera. The administration of *F. hepatica* ES significantly reduced serum concentration of LPS-induced IFN-γ, however it had no effect on the concentrations of IL-6 and KC (Fig. 3.14). This result demonstrates that *F. hepatica* ES is capable of exerting its suppressive effect on components of immune system *in vivo* as well as *in vitro*.

3.2.6 Administration of *F. hepatica* ES ameliorates symptoms of DSS-induced colitis

In order to further evaluate the immunosuppressive properties of *F. hepatica* ES *in vivo*, the dextran sodium sulphate (DSS) induced model of colitis was employed. The exact mechanism of DSS induced disease is unknown, however the literature suggests that the innate responses initiated by chemical disruption of the intestinal mucosal barrier constitute the major factor that contributes to the pathology. Animals were given 2% DSS in drinking water and disease severity was assessed by monitoring the weight loss and measuring of the degree of colon shortening post mortem. Administration of DSS resulted in the loss of 12.6% of initial body weight by day 7. Administration of *F. hepatica* ES via i.p. injection on days 0, 2, 4 and 6 reduced the weight loss by 47% ($p < 0.001$) with animals losing on average 6.7% of their initial body weight (Fig. 3.15A). Weight loss was associated with significant shortening of the colons of DSS fed mice which, when compared to control animals that were given

drinking water only, experienced more than 30% reduction of the colon length (6.2 mm +/-SEM vs. 9.0 mm +/- SEM). Mice that were treated with *F. hepatica* ES had markedly less severe changes in the colon length showing average 13.3% reduction vs. healthy animals (7.8 mm +/- SEM; $p < 0.01$) (Fig. 3.15 B and C).

The protective effect of *F. hepatica* ES on colitis was confirmed by histological analysis of colon tissue from treated and untreated mice with DSS colitis (Fig. 3.16). Untreated animals fed with DSS experienced widespread inflammation and destruction of the normal gut architecture, including severe erosion of the mucosa and partial loss of crypts. *F. hepatica* ES markedly diminished the signs of inflammation in the colon; tissue sections showed mild, DSS induced hyperplasia of mucosal cells but the infiltration of immune cells and erosion of the mucosal layer was significantly reduced (Fig. 3.16).

Analysis of gene expression in colons of DSS treated mice revealed that attenuation of clinical features of the disease by *F. hepatica* ES was associated with modest reduction in DSS induced expression of IL-1 β , IL-17 and IFN- γ (Fig. 3.17). Surprisingly, IL-22 which has been shown to have a beneficial effect on maintaining the integrity of the colonic mucosa (Zenewicz et al., 2008) was also reduced. However *F. hepatica* ES significantly increased expression of IL-10 and heme oxygenase 1 (HO-1), both of which have been reported to play protective roles during DSS induced colitis. Administration of *F. hepatica* ES was also associated with a very strong up-regulation of IL-4 (Fig. 3.17).

3.2.7 Administration of *F. hepatica* ES attenuates clinical symptoms of EAE

Having shown that *F. hepatica* ES suppressed the pathology driven by innate immunity in a model of DSS colitis the study was complemented by a investigation of the effect of *F. hepatica* ES on the development of inflammatory disease mediated by T cells. EAE is a model of T cell mediated demyelinating disease of the CNS that shares clinical and pathological features with MS. The disease is induced in C57BL/6 mice by immunisation with myelin derived MOG peptide emulsified in CFA followed by administration of pertussis toxin (PT).

The exact role of PT in the induction of EAE is not fully understood, however it is believed that PT aids the development of Th17 responses and helps to open BBB to facilitate leukocyte infiltration into the CNS. During the course of EAE, autoimmune responses to the components of nervous system manifest as a progressive paralysis that can be graded to evaluate disease severity.

EAE was induced by s.c. immunisation with MOG peptide in CFA and mice were treated with *F. hepatica* ES or vehicle on day -1, 2 and every second day thereafter. Animals, which were monitored daily for the clinical symptoms of the disease, started to show early signs of paralysis between days 6 and 10 (Fig. 3.18 A). The administration of *F. hepatica* ES markedly delayed the onset of EAE with 72% of treated mice with no signs of the disease on day 12, while at this stage 89% of control mice had already developed moderate or severe paralysis. The treatment also significantly attenuated the severity of the disease, reducing average clinical score on day 17 (the cessation of experiment) from 2.7 in control group to 1.7 ($p<0.01$) with only 62% mice developing the symptoms before termination of the experiment in striking contrast to 95% of mice in control group (Fig. 3.18 A). The general condition of the animals was assessed on the basis of changes in the average body mass (Fig. 3.18 B), since in this model mice gradually lose weight as the disease progresses. Treatment with *F. hepatica* ES prevented the weight loss throughout the course of the experiment reflecting the less severe disease outcome.

Attenuation of EAE was associated with suppression of antigen-specific T cell responses in mice treated with *F. hepatica* ES. Production of the Th1 and Th17 signature cytokines, IFN- γ and IL-17, by spleen cells in response to restimulation with increasing concentrations of MOG *in vitro* was significantly lower in *F. hepatica* ES-treated mice (Fig. 3.19 A and B). No differences in production of antigen specific IL-4 and IL-10 were detected between the two experimental groups of mice (Fig. 3.19 C and D).

To better understand *F. hepatica* ES induced attenuation of EAE, the study investigated the effect of *F. hepatica* ES on the development of antigen-specific responses in draining lymph nodes. Mice were immunized into the flank with

MOG peptide emulsified in CFA without PT and treated with *F. hepatica* ES or PBS on day -1, 2 and every second day thereafter. Ten days post immunisation cells from draining inguinal lymph nodes were recovered and analysed *ex vivo* (without any *in vitro* stimulation) for cytokine expression by quantitative real-time PCR. The result revealed that administration of *F. hepatica* ES suppressed the expression of IL-17A and the Th17 related gene RORc, which encodes ROR γ t - a signature transcription factor of IL-17 producing cells (Fig. 3.20). Expression of the IL-23 receptor, which is normally up-regulated on the surface of IL-17 producing cells, was also reduced. In contrast expression levels of IL-10 and TGF- β and interestingly IFN- γ were significantly enhanced (Fig. 3.20). FACS analysis of lymph node cells revealed that treatment with *F. hepatica* ES increased the frequency of CD4⁺ T cells expressing cell surface bound TGF- β (Fig. 3.21 A). Notably, these TGF- β ⁺ cells did not co-express Foxp3 (Fig. 3.21 C) and the frequency of Foxp3⁺ Treg cells did not increase following *F. hepatica* ES administration (Fig. 3.21 A). In order to examine antigen specific responses, lymph node cells from mice immunised with MOG and treated with *F. hepatica* ES or PBS were stimulated *in vitro* with a range of concentrations of MOG peptide. The result (Fig. 3.22) demonstrated that administration of *F. hepatica* ES reduced the capacity of LN cells to produce IL-17 in response to antigen restimulation, but had no effect on MOG specific IFN- γ secretion. Antigen restimulation did not induce expression of IL-10 or TGF- β above the level of medium alone regardless from *F. hepatica* ES treatment.

Taken together, these data demonstrate that administration of *F. hepatica* ES significantly suppressed development of Th17 responses induced by antigen and CFA. On the other hand *F. hepatica* ES treatment enhanced expression of IFN- γ and regulatory cytokines IL-10 and TGF- β in an antigen independent manner.

Having shown that prophylactic treatment with *F. hepatica* ES significantly suppressed the induction of EAE, this study was extended to evaluate the efficacy of *F. hepatica* ES administration in a model of passively induced EAE. The disease was initiated through adoptive transfer of pathogenic MOG specific T cells expanded *in vitro* into naive recipients. Mice started to develop paralysis

from day 10 post T cell transfer. Treatment with *F. hepatica* ES on the day of the transfer and every second day thereafter did not attenuate disease severity (Fig. 3.23). This result suggests that *F. hepatica* ES acts at the induction phase of the EAE, suppressing initiation of pathogenic responses but does not affect the function of already expanded pathogenic T cells.

3.2.8 The effect of TGF- β neutralisation on the protective effect of *F. hepatica* ES administration in EAE

It has been recently reported by Walsh and co-workers (Walsh et al., 2009) that suppression of EAE associated with *F. hepatica* infection was mediated through TGF- β as neutralization of this cytokine with a specific antibody reversed the protective effect of infection. Their observation indicates that the induction of TGF- β expression, which occurs as part of a subversive mechanism exploited by a parasite to establish tolerance and prolong survival within the host, also exerts bystander suppression of pathogenic T cells that mediate autoimmune responses.

This study had already demonstrated that administration of *F. hepatica* ES results in increased frequencies of TGF- β ⁺CD4⁺ T cells and up regulation of TGF- β expression in the lymph nodes of mice immunised with antigen in CFA. Therefore it was important to investigate the possible role of *F. hepatica* ES induced TGF- β in attenuation of EAE. Mice were immunised with MOG in CFA, followed by PT and treated with *F. hepatica* ES on day -1, 2 and every second day thereafter. A neutralising antibody against TGF- β (100 μ g/mouse) or isotype control was administered every two days and clinical scores were recorded daily (Fig. 3.24 A). The treatment with *F. hepatica* ES significantly attenuated EAE, with average clinical score of 0.91 recorded at the termination of experiment compared with 2.3 observed in control group. The disease incidence was reduced by 55.6%, from 75% control mice to 33.3% of *F. hepatica* treated animals eventually suffering from autoimmunity (Fig. 3.24 B). Neutralisation of TGF- β in untreated mice with EAE resulted in development of more severe symptoms with an average clinical score of 3.1. Treatment with the TGF- β neutralising antibody diminished the capacity of *F. hepatica* ES to attenuate EAE (Fig. 3.24 A), increasing the average clinical score of treated

mice and accelerating disease progression in relation to animals treated with *F. hepatica* ES and isotype control antibody. Moreover, co-administration of anti-TGF- β antibody with *F. hepatica* ES reduced the disease incidence by 14.3 % compared with 55.6% reduction in absence of neutralising antibody. Despite the fact that lack of TGF- β clearly exacerbated disease in *F. hepatica* ES treated mice these animals still experienced markedly less severe paralysis than mice that received only TGF- β neutralising antibody. Taken together this data suggests that, suppression of autoimmunity provided by *F. hepatica* ES could be partially attributed to TGF- β , however there are other unknown mechanisms that mediate protection.

3.3 Discussion

Several pathogens, including helminthic parasites, have evolved mechanisms to evade and suppress immune responses in order to survive and successfully populate the host. Immunosuppression induced by helminths is often systemic therefore can affect responses to bystander antigens unrelated to the parasite. This phenomenon, known as “linked” suppression could explain attenuation of inflammatory or autoimmune diseases co-existing with parasite infection. A better understanding of how parasites and parasite derived products modulate immune responses could contribute to the design of new therapeutic strategies to treat inflammatory and autoimmune disorders.

This study examined direct effects of *F. hepatica* ES on DCs activation and T cell responses *in vitro* in order to better understand immunomodulatory mechanisms employed by the helminthic parasite. The suppressive effect of *F. hepatica* ES on the development of colitis and EAE revealed that this effect has relevance in models of inflammatory and autoimmune diseases.

DCs, as professional antigen presenting cells, play a central role in the initiation and control of T cell responses and as such are a prime target cell for multiple immune modulating signals delivered by the parasite in order to subvert the development of protective immunity. The strength and the quality of the T cell response can be regulated by DC activation through surface expression of co-stimulatory molecules such as CD40, CD80 or CD86 and production of polarizing cytokines (Kapsenberg, 2003). Importantly, presentation of self antigens by DC without PPR activation and up-regulation of necessary co-stimulatory molecules can provide a mechanism for the induction of tolerance in the immune system.

This study has demonstrated that *F. hepatica* ES is a potent modulator of the phenotype and function of DCs. Pretreatment with *F. hepatica* ES impaired DCs responses to ligands of TLR2, 3, 4 and 9 by suppressing the expression of IL-12p70. Interestingly the inhibition of TLR2 or TLR9 induced IL-12p70 was not

initially clear since endotoxin contaminants in the *F. hepatica* ES preparation synergised with CpG and Pam₃CSK₄ to increase expression of IL-12p70. Nonetheless *F. hepatica* ES inhibited CpG and Pam₃CSK₄ induced production of IL-12p70 from LPS unresponsive DCs from C3H/HeJ mice, demonstrating that the suppressive effect of *F. hepatica* ES was not exclusive to specific TLRs. In addition to IL-12p70, treatment with *F. hepatica* ES also inhibited LPS or CpG-induced production of IL-23 and IL-6 by DCs. The cytokines play important roles in polarizing naive T cells towards Th1 and Th17 type responses and inhibition of TLR induced expression of these cytokines could impair the development of adaptive immune responses. Moreover *F. hepatica* ES negatively regulated LPS induced expression of co-stimulatory molecules on DCs surface preventing full maturation of these cells. In addition antigen presentation by immature DCs could lead to immune tolerance and the induction of regulatory cells (Belkaid and Oldenhove, 2008). The ability to inhibit maturation and expression of pro-inflammatory cytokines likely reflects a successful evasion strategy employed by the parasite.

Since *F. hepatica* ES inhibited cytokine secretion triggered by various TLR ligands, it is possible that active components of parasite products target signalling molecules shared between the TLR pathways. Parasite products have been reported to target the function of transcription factor NF- κ B (Hamilton et al., 2009) and components of MAP kinase (MAPK) cascades in both murine and human APCs (Everts et al., 2010; Feng et al., 1999). Activation of MAPK p38 downstream of TLR activation in DCs has been shown to be important for expression of maturation markers and cytokine production, whereas ERK phosphorylation is linked to dampening of these responses by a negative feedback mechanism (Nakahara et al., 2006). An elegant study investigating the mechanism of immune evasion by filarial nematode *A. viteae* explored differential regulation of IL-12p70 subunits p35 and p40 by MAP kinases p38 and ERK and demonstrated that the parasite derived molecule ES-62 targets both pathways in order to inhibit production of IL-12p70 (Goodridge et al., 2005). The present study established that *F. hepatica* ES affected neither LPS induced phosphorylation of ERK or p38, nor did the inhibition of ERK activation

with a MEK1/2 inhibitor, U0126 reverse the observed suppression of IL12p70. Moreover *F. hepatica* ES had no effect on the normal function of NF- κ B pathway. These results highlight the complexity of TLR triggered signalling pathways leading to cytokine production in immune cells. It is possible that *F. hepatica* ES targets other pathways involved in inducing IL-12p70 expression such as IRFs.

A striking finding of this study was the observation that *F. hepatica* ES could induce the production of the regulatory cytokine TGF- β by DCs. Interestingly *F. hepatica* ES induced secretion of TGF- β did not involve transcription or translation of *tgfb1* gene and therefore the increased concentration of TGF- β observed in the supernatants appears to result from release of pre-formed TGF- β from cell surface by an unknown mechanism. The crucial role of TGF- β in maintaining immunological homeostasis is emphasized by the fact that TGF- β null mice die within 3-4 weeks after birth due to a massive inflammatory response and tissue necrosis (Feng et al., 1999). Furthermore, disruption of TGF- β signalling in immune cells by a conditional knock out of TGF- β receptor II on either T cells (Marie et al., 2006) or DCs (Ramalingam et al., 2012) results in severe multiorgan autoimmune inflammation. TGF- β controls many facets of immune homeostasis but most remarkably it is critically required for differentiation and function of Treg cells (Liu et al., 2008). TGF- β derived from DCs or other sources can directly suppress T cell function or create a permissible environment for the generation of inducible Treg cells through a mechanism of peripheral conversion (Zaccone et al., 2009). Moreover several studies have shown that TGF- β can inhibit the activation and function of DCs *in vitro* (Mou et al., 2011; Strobl and Knapp, 1999) and *in vivo* (Imai et al., 2012). The inhibition of TLR-driven cytokines from DCs by *F. hepatica* ES is not associated with the secretion of TGF- β , since impairment of TGF- β signalling in DCs through inhibition of TGF- β receptor kinase ALK5 had no effect on *F. hepatica* ES induced suppression of IL-12p70 and IL-6. However it is possible that autocrine TGF- β could affect other aspects of DCs function hence contribute to the generation of cells with a tolerogenic/suppressive phenotype (Belladonna et al., 2008).

Together these data demonstrate that treatment with *F. hepatica* ES can significantly modulate the phenotype of DCs by suppressing the expression of pro-inflammatory cytokines and co-stimulatory molecules, while inducing the expression of TGF- β . Therefore DCs exposed to *F. hepatica* ES acquire a tolerogenic phenotype, which may favour the development of Treg cells.

This study has also demonstrated that ES products of a helminth parasite can have direct effect on activation of T cells. Treatment with *F. hepatica* ES inhibited proliferation and cytokine production by CD4⁺ T cells from spleen cell cultures activated with an anti-CD3e antibody. Reduced proliferation was also associated with a decreased expression of IL-17 and IFN- γ . Interestingly this suppressive effect was not mediated by APCs since *F. hepatica* ES strikingly reduced production of IL-2 and IFN- γ by purified CD4⁺ T-cells. Previous work from the Mills laboratory (Sutton et al., 2009) showed that CD3⁺ cells secrete IL-17 and IFN- γ in response to IL-1 β and IL-23 without stimulation through TCR engagement. Remarkably, *F. hepatica* ES was able to suppress this innate cytokine production by spleen cells stimulated with combination of IL-1 β and IL-23. Taken together these data suggest that *F. hepatica* ES-induced immunosuppression occurs by multiple mechanisms which target DCs to inhibit their maturation and cytokine production but also render T cells unresponsive to stimulation by innate cells.

Experiments in the mouse model confirmed that *F. hepatica* ES have the capacity to suppress immune responses *in vivo*. In a murine model of LPS-induced septic shock, the early stages of the immune response are controlled by peritoneal macrophages that produce a vast array of pro-inflammatory cytokines and chemokines, including TNF- α , IL-1 β , IL-6, KC and IL-12p70 that trigger further inflammatory cascades. IL-12p70 in particular elicits production of IFN- γ from innate T cells and NK cells (Varma et al., 2002), which in turn acts in a paracrine-positive feedback loop to enable further more efficient clearance of LPS-bearing pathogens (Schroder et al., 2004). This model is often used at initial stages of the drug discovery process, because it constitutes a relatively

inexpensive and quick way of determining the efficacy of certain immunosuppressive compounds *in vivo*. This study demonstrated that administration of *F. hepatica* ES significantly suppressed LPS-induced IFN- γ and this most likely reflects *F. hepatica* ES induced inhibition of IL-12p70. Interestingly other pro-inflammatory mediators such as IL-6 and KC were not affected, which may help to identify which pathways are targeted by *F. hepatica* ES *in vivo*. Hamilton *et al.* have previously shown that *F. hepatica* derived products were able to reduce serum concentrations of IL-12p70, IFN- γ and TNF- α in a similar LPS challenge model (Hamilton *et al.*, 2009). Independently, a group led by Dalton additionally identified cathepsin-L (FhCatL) and helminth defence molecule-1 (FhHDM-1) - two major components of *F. hepatica* secretions to be responsible for the suppressive effect observed in this model (Donnelly *et al.*, 2010; Robinson *et al.*, 2009).

F. hepatica ES was also able to attenuate the clinical symptoms of DSS-induced colitis. In this model of inflammatory bowel disease, oral administration of DSS for several days leads to colonic epithelial cell lesions and acute inflammation, characterized by the infiltration of neutrophils and macrophages. The exact mechanism of DSS induced inflammation is not well understood, however it is believed that damaged epithelium results in increased intestinal permeability that allows luminal bacteria to interact with immune cells and cause their unconstrained activation, thereby leading to an intense and sustained inflammatory response (Boismenu and Chen, 2000). SCID mice fed with DSS suffer from colitis, suggesting that T cells and B cells are not required for acute colitis to develop further highlighting the importance of innate immunity in the pathogenesis of this disease (Dieleman *et al.*, 1994).

This study demonstrated that administration of *F. hepatica* ES could attenuate the clinical manifestations of DSS colitis. Administration of *F. hepatica* ES during the course of the disease reduced the rate of weight loss and shortening of the colon which are hallmarks of severe inflammation in the gut. This clinical improvement was associated with milder histopathological changes in colon tissue, less damage to the mucosa and underlying crypts and reduced leukocyte infiltration. Treatment with *F. hepatica* ES had a modest suppressive effect on the expression of pro-inflammatory cytokines such as IL-1 β , IL-17, and

IFN- γ , whilst significantly enhancing expression of IL-4, IL-10 and HO-1 in the colon tissue. IL-10 and HO-1 have strong anti-inflammatory function and both have been shown to play protective roles against colitis (Sasaki et al., 2005; Zhong et al., 2010). Increased expression of IL-4 is perhaps not surprising since parasite products typically induce Th2 responses but the role of this cytokine in controlling intestinal inflammation remains obscure (Boismenu and Chen, 2000).

In addition to suppressing an inflammatory disease mediated by innate immunity the study demonstrated that *F. hepatica* ES can modulate a disease mediated by adaptive responses. In EAE, which is a murine model of MS, the pathology is mediated by antigen-specific Th1 and Th17 cells induced by immunisation with MOG peptide. Prophylactic administration of *F. hepatica* ES delayed onset and significantly attenuated the clinical symptoms of EAE. This was associated with reduced production of IFN- γ and IL-17 by spleen cells in response to MOG restimulation *in vitro* which suggests that treatment with *F. hepatica* ES suppressed development of MOG-specific Th1 and Th17 cells. In contrast administration of *F. hepatica* ES did not attenuate clinical score of adoptively transferred EAE which suggests that regulatory networks triggered by the parasite target the induction phase of the disease

Further investigation into the modulation of antigen-specific responses demonstrated that administration of *F. hepatica* ES following immunisation with MOG in CFA significantly reduced expression of IL-17A and Th17 associated molecules such as ROR γ t, a signature transcription factor of Th17 cells, and IL-23R in draining lymph node while enhancing expression of IFN- γ . *F. hepatica* ES also reduced the ability of lymph node cells to produce IL-17A in response to MOG restimulation *in vitro*, but had no effect on antigen-specific expression of IFN- γ . Treatment of MOG immunised mice with *F. hepatica* ES significantly enhanced expression of IL-10 and TGF- β in total lymph nodes, however cells restimulated *in vitro* did not produce these cytokines in response to MOG. This finding suggests that parasite products may dampen MOG

specific Th17 responses through a mechanism of linked suppression, whereby parasite induced production of IL-10 and TGF- β by various innate or adaptive cells cross-regulate rather than directly control responses to bystander antigens (Hartmann et al., 2011; Nakagome et al., 2007; Walsh et al., 2009; Wammes et al., 2010).

Induction of Treg cells and anti-inflammatory cytokines is a feature of many parasitic infections and TGF- β is a major host factor shown to mediate parasite induced immunosuppression (Anderson et al., 2008; Dittrich et al., 2008; Flynn and Mulcahy, 2008; Ince et al., 2009; Nakagome et al., 2007; Waghabi et al., 2007). It has been reported that SEA can prevent type 1 diabetes in NOD mice through the generation of Treg cells and, at least *in vitro*, induction of Foxp3 expression in naive T cells by SEA treated DCs is TGF- β -dependent (Zacone et al., 2009). Furthermore, studies by Ince *et al.* have also found a central role for TGF- β in mediating the immunosuppressive effects of *H. polygyrus* infection demonstrating that modulatory effect was lost in transgenic mice expressing a dominant negative form of the TGF- β II receptor (Ince et al., 2009). Interestingly in *H. polygyrus* infection, which is associated with significant expansion of Treg cells, induction of Foxp3⁺ cells has been ascribed to a novel TGF- β like-protein secreted by the parasite. This helminth derived homolog mimics TGF- β activity by engaging SMAD dependent signalling pathways thus inducing *de novo* expression of Foxp3 in naive T cells (Grainger et al., 2010).

The increase in TGF- β production in lymph nodes of *F. hepatica* ES treated mice was associated with higher frequency of TGF- β expressing CD4⁺ T cells. Interestingly these CD4⁺TGF- β ⁺ cells did not co-express Foxp3, the signature transcription factor which determines regulatory phenotype of Treg cells. This expansion of CD4⁺TGF- β ⁺ cells was consistent with an unpublished study by Galvin and Mills, which has demonstrated that subversion of anti-tumour responses and exacerbation of tumour growth by *F. hepatica* ES in murine melanoma model was associated with a dramatic increase in the frequency of tumour infiltrating CD4⁺TGF- β ⁺ but not CD4⁺Foxp3⁺ T cells (Galvin, 2011). Enhanced tumour growth induced by *F. hepatica* ES was mediated through TGF- β -dependent mechanisms and could be reversed by treatment with an anti-TGF- β antibody. Interestingly neutralization of TGF- β completely blocked

infiltration of *F. hepatica* ES induced CD4⁺TGF-β⁺ T cells into the tumour mass. In contrast, infection with *F. hepatica*, which also resulted in a significant increase in tumour growth, was associated with an expansion of CD4⁺Foxp3⁺ T cells (Galvin, 2011).

Correspondingly the importance of TGF-β signalling for *F. hepatica* mediated immunosuppression has also been demonstrated in autoimmunity (Walsh et al., 2009). Neutralisation of TGF-β completely reversed the protective effect of *F. hepatica* infection on development of EAE, providing further evidence that *F. hepatica*-induced TGF-β expression plays a critical part in the bystander suppression of autoantigen-specific Th1 and Th17 responses. In contrast to these findings treatment with anti-TGF-β antibody reduced but did not completely abolished the immunosuppressive effect of *F. hepatica* ES. Mice treated with *F. hepatica* ES in the presence of anti-TGF-β antibody developed more severe disease than those that received *F. hepatica* ES and control antibody. Furthermore, administration of the neutralising antibody strikingly limited the capacity of *F. hepatica* ES to reduce disease incidence which suggests that TGF-β is involved in mediating *F. hepatica* ES induced suppression. However, even though lack of TGF-β clearly worsens clinical scores of *F. hepatica* ES treated mice, these animals still experienced less severe EAE than mice that received only anti-TGF-β antibody. This in turn suggests that there are other mechanisms than TGF-β that contribute to the attenuation of autoimmunity by *F. hepatica* ES.

Taken together this work demonstrates that, suppression of autoimmunity provided by *F. hepatica* ES could be partially attributed to TGF-β, however it is clear that other unknown mechanisms are involved in protection.

Collectively the present study has established several novel findings on the immunomodulatory effects of *F. hepatica* derived ES products. Firstly, it demonstrates that *F. hepatica* ES can subvert innate immunity through modulation of DCs responses to activation with common PAMPs. Secondly, administration of *F. hepatica* ES can directly suppress T cell activation and reduce the clinical symptoms of a T cell mediated autoimmune disease.

Attenuation of autoimmunity was associated with a down-regulation of Th17 responses and the expansion of CD4⁺TGF-β⁺ cells that could potentially be involved in restraining disease through a mechanism of 'linked' suppression. Immunoregulation induced by *F. hepatica* ES is partially dependent on TGF-β but other mechanisms involved in this process are likely.

Experimental infections with a range of helminthic parasites such as *S. mansoni* (La Flamme et al., 2003), *Trypanosoma brucei* (Wållberg and Harris, 2005) and *F. hepatica* (Walsh et al., 2009) have been shown to suppress clinical manifestations of EAE. This study revealed that the attenuation of EAE associated with *F. hepatica* infection can be mimicked by administration of excretory/secretory products of the parasite. These results formally demonstrate that protection is mediated by specific worm-derived molecules and does not require the presence of live flukes. Since helminthic infection is a very complex process associated with extensive collateral damage obscuring accompanying immune responses, this finding provides a cleaner model in which to study parasite related immunosuppression. Among *in vivo* models employed in this study, EAE proved to be the most suitable for further identification of active components of *F. hepatica* ES, mainly because of its robustness and greater translational significance when compared to the endotoxin shock model or DSS -induced colitis.

The results presented in this part of the thesis constitute a solid foundation for further investigation into specific immunomodulatory components found in *F. hepatica* ES.

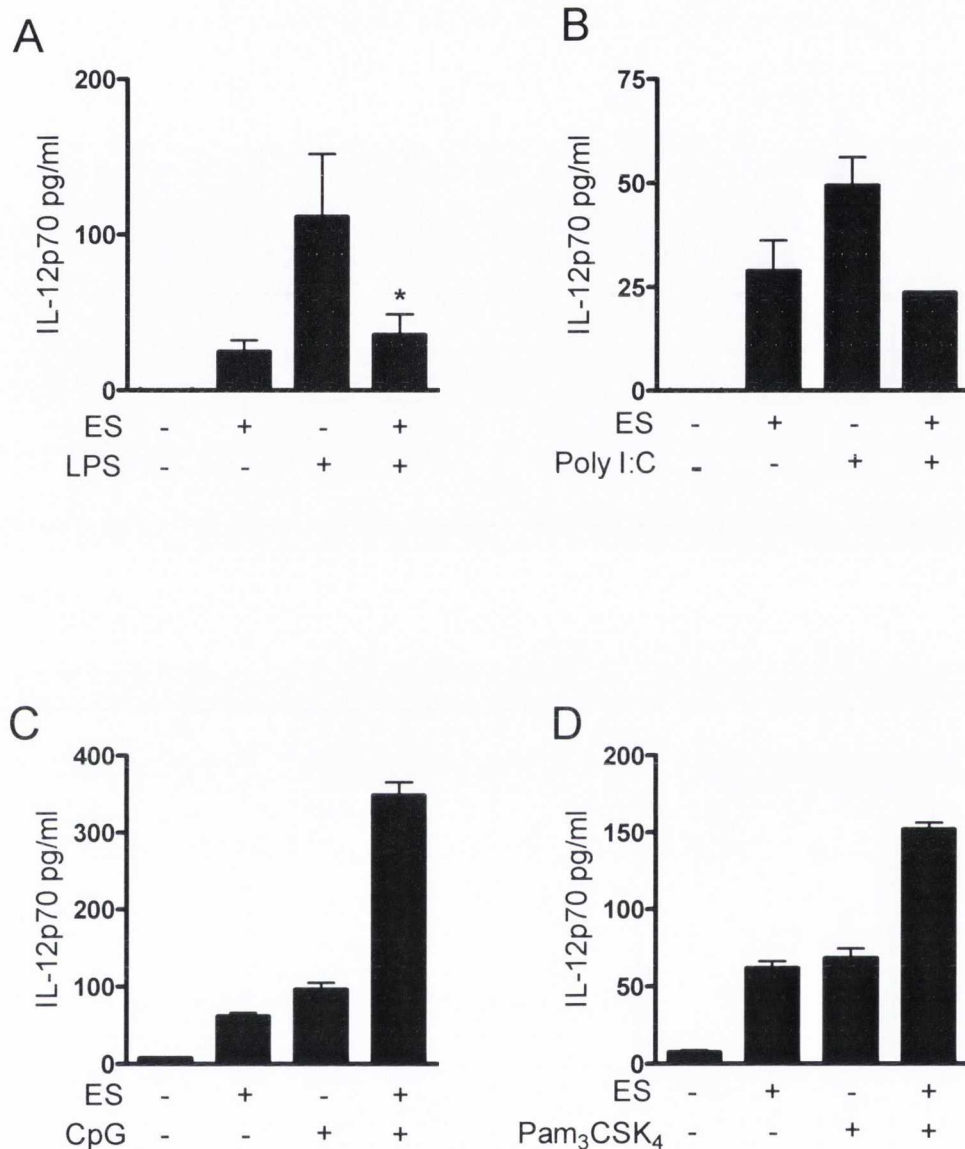


Fig. 3.1 *F. hepatica* ES modulate TLR agonist induced secretion of IL-12p70. DCs from C57BL/6 mice were pretreated with ES (5% v/v) for 2 h prior to stimulation with LPS (100 ng/ml) (A), poly I:C (25 µg/ml) (B), CpG (5 µg/ml) (C) or Pam₃CSK₄ (100 ng/ml) (D). The concentration of IL-12p70 in supernatants was quantified after 24 h by ELISA. Results shown are triplicate samples from a single experiment. At least 3 independent experiments were performed with similar results. * $P < 0.05$ vs. TLR ligand only.

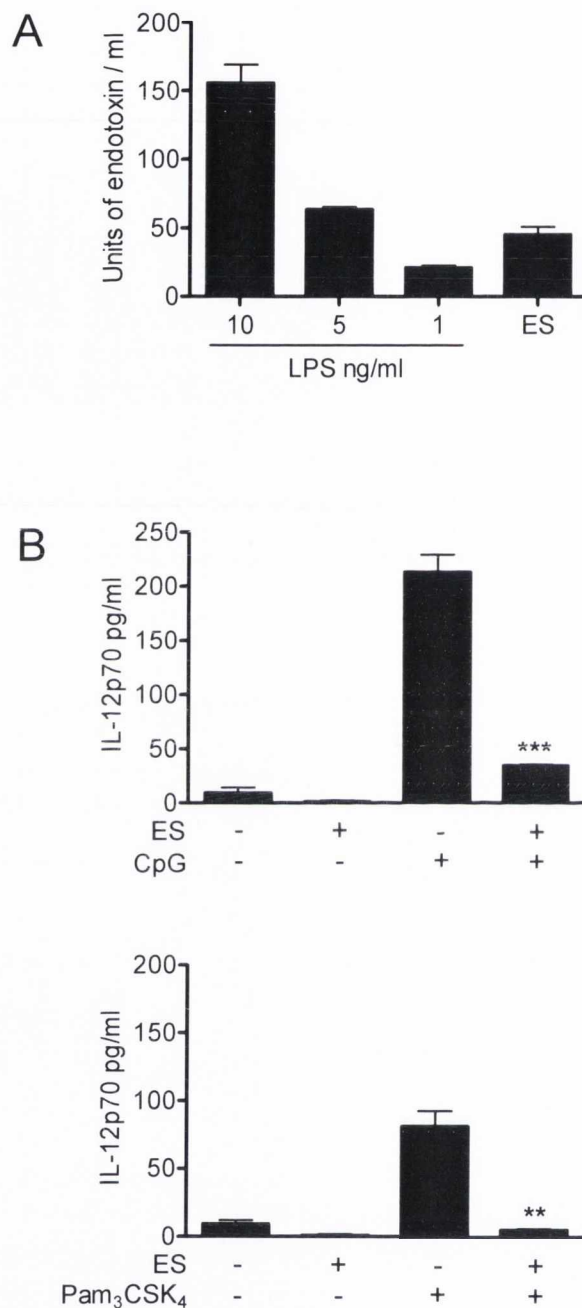


Fig. 3.2 *F. hepatica* ES suppress TLR-induced IL-12p70 production by DCs from C3H/HeJ mice. A) The concentration of endotoxin in *F. hepatica* ES preparations was measured by LAL assay. B) C3H/HeJ DCs were pretreated with *F. hepatica* ES (5% v/v) for 2 h prior to stimulation with CpG (5 μ g/ml) or Pam₃CSK₄ (100 ng/ml). The concentration of IL-12p70 in supernatants was quantified after 24 h by ELISA. Results shown are quantified from triplicate samples. ** P<0.01, *** P<0.001 vs. TLR ligand only.

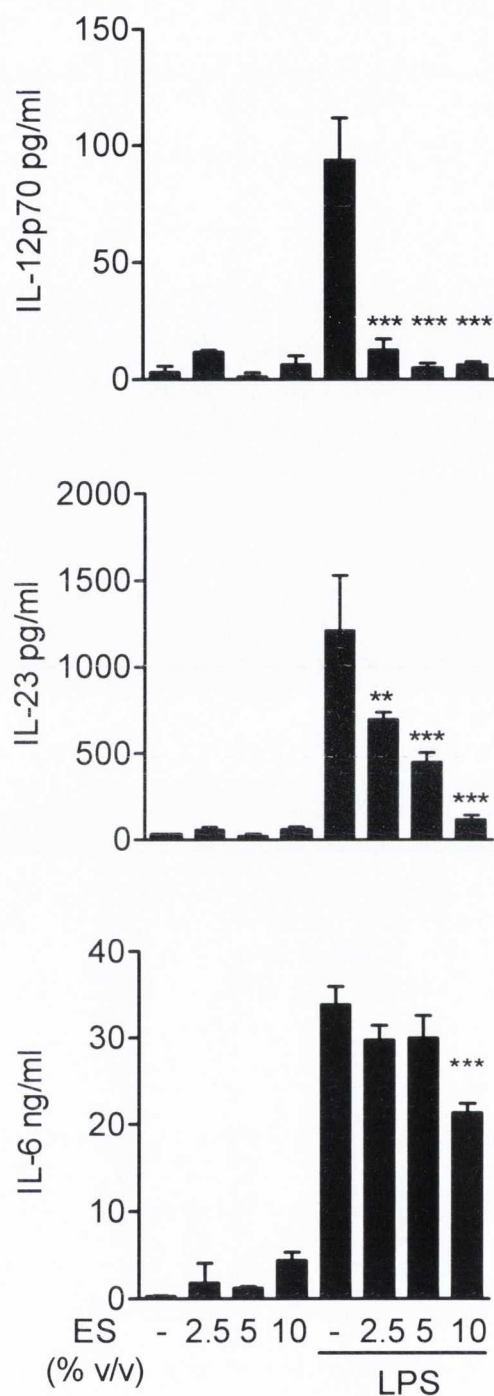


Fig. 3.3 *F. hepatica* ES modulate TLR agonist induced secretion of pro-inflammatory cytokines by DCs from C57BL/6 mice in a dose dependent manner. DCs derived from C57BL/6 mice were pretreated with *F. hepatica* ES as indicated (2.5-10% v/v) for 2 h prior to stimulation with LPS (100 ng/ml). The concentration of IL-12p70, IL-23 and IL-6 in supernatants was quantified after 24 h by ELISA. Results shown are means ($\pm$ SD) of triplicate assays and are representative of at least 3 independent experiments. * P<0.05; ** P<0.01; *** P<0.001 vs. LPS only control.

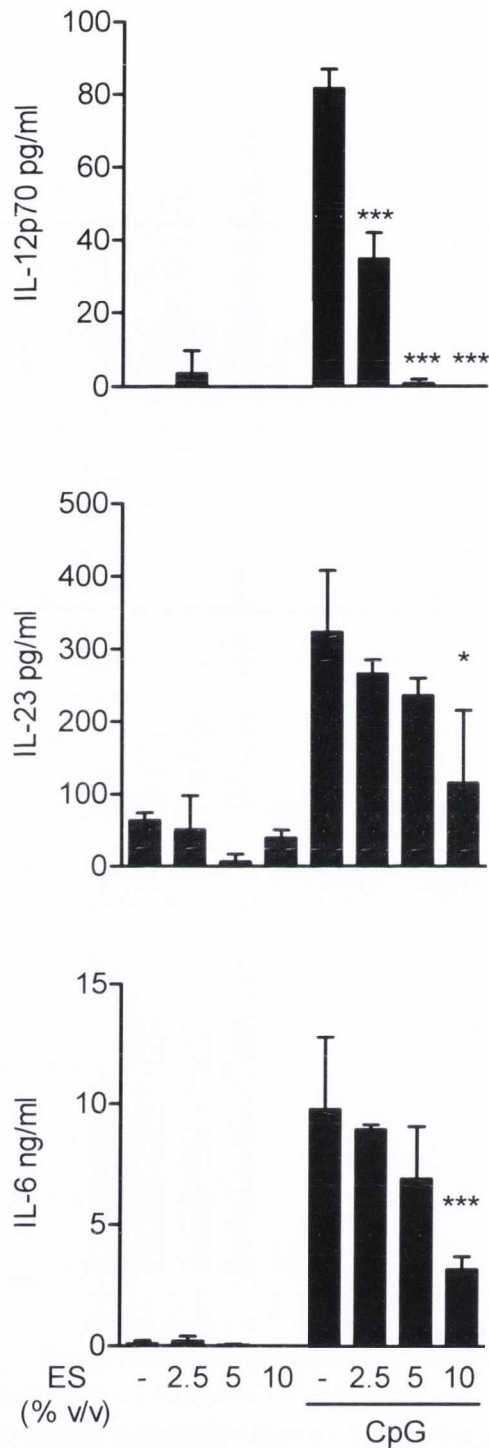
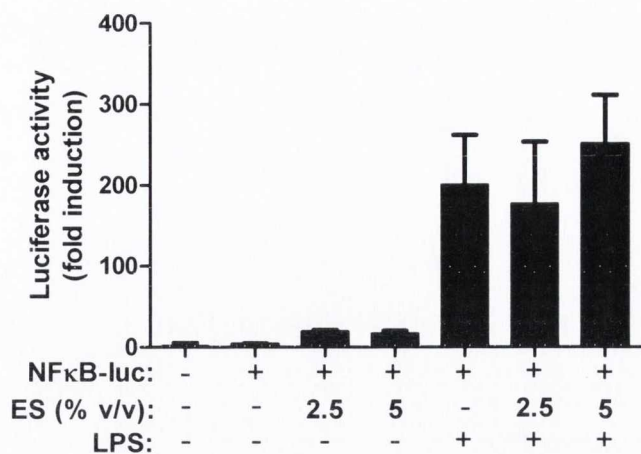
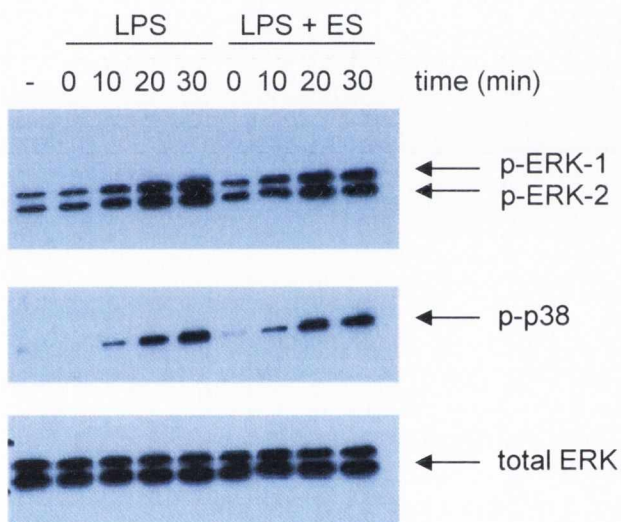


Fig. 3.4 *F. hepatica* ES modulate TLR agonist induced secretion of pro-inflammatory cytokines by DCs from C3H/HeJ mice in a dose dependent manner. DCs derived from C3H/HeJ mice were pretreated with *F. hepatica* ES as indicated (2.5-10% v/v) for 2 h prior to stimulation with CpG (5 µg/ml). The concentration of IL-12p70, IL-23 and IL-6 in supernatants was quantified after 24 h by ELISA. Results shown are means (+/- SD) of triplicate assays and are representative of at least 3 independent experiments. * P<0.05; ** P<0.01; *** P<0.001 vs. LPS only control.

A



B



C

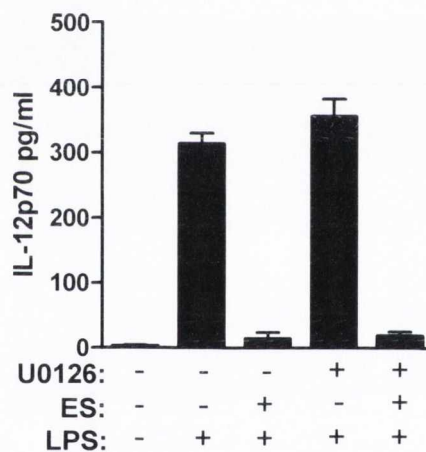


Fig. 3.5 *F. hepatica* ES does not modulate LPS-induced signal transduction through NF-κB or MAPK. A) HEK-TLR4 cells were transfected with luciferase plasmid expressing the NFκB binding sites. 24 h after transfection cells were pretreated with *F. hepatica* ES and stimulated with LPS for 24 h. Luciferase activity in total cell lysates was measured using chemiluminescent substrate. Results are mean ($\pm$ SD) fold induction, relative to control levels. B) DCs were pre treated with *F. hepatica* ES for 1 h prior to LPS stimulation. Cells were lysed at the time of adding LPS or after 10, 20 and 30 min. Cell lysates were resolved on SDS-PAGE gels and transferred to nitrocellulose membrane. Blots were probed with anti-phospho (p)-ERK1/2, anti-phospho (p)-p38 or anti-ERK. C) DCs were pre incubated with MEK1/2 inhibitor U0126 for 30 min prior to the treatment with *F. hepatica* ES and LPS (100 ng/ml). IL-12p70 was quantified in supernatants after 24 h by ELISA.

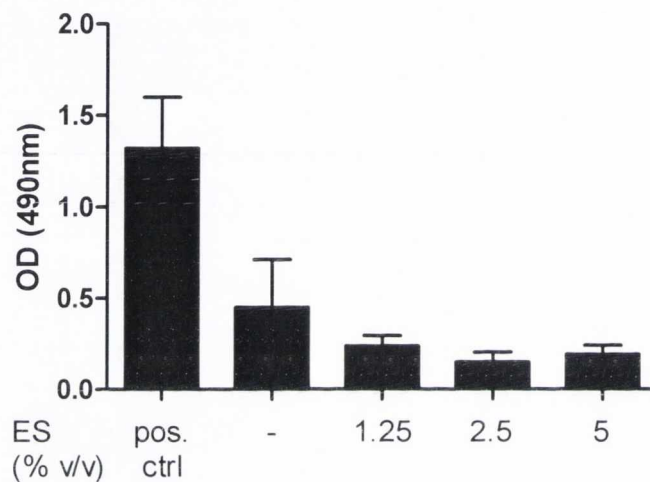


Fig. 3.6 Treatment with *F. hepatica* ES does not induce cell death. DCs from C57BL/6 mice were treated for 24 h with indicated range of concentrations of *F. hepatica* ES for 24 h. Supernatants were collected and the activity of lactate dehydrogenase (LDH), a surrogate indicator of cell death, was measured by the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). Results are shown as means ($\pm$ SD) of triplicate assays.

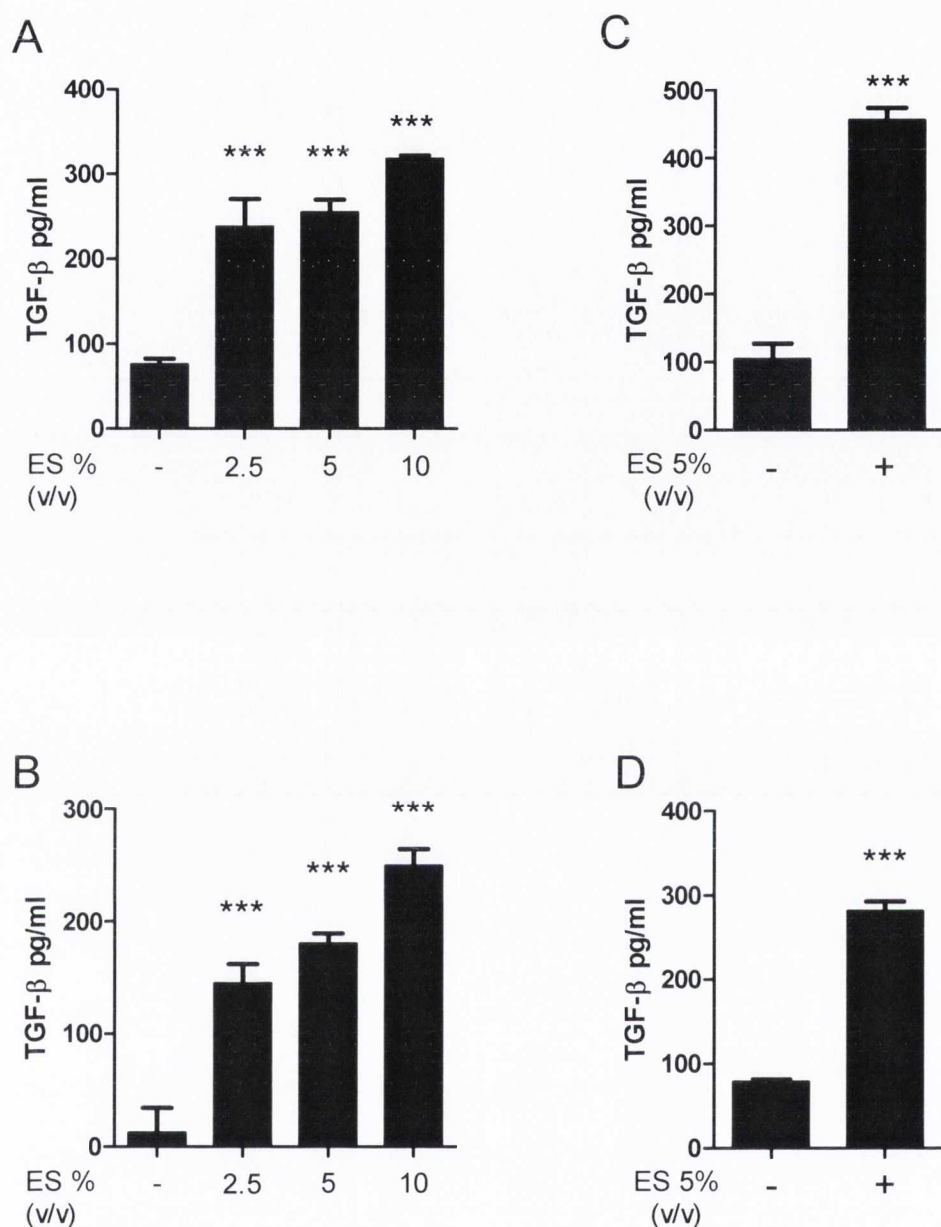


Fig. 3.7 *F. hepatica* ES promote TGF- β secretion by DCs, macrophages and glial cells. C57BL/6 DCs (A), C3H/HeJ DCs (B), C57BL/6 BMDMs (C) and primary murine glial cells (D) were treated for 24 h with indicated concentrations of *F. hepatica* ES. The concentration of total TGF- β in the supernatants was quantified by ELISA. Results shown are means ($\pm$ SD) of triplicate assays and are representative of at least 3 independent experiments. ** $P < 0.01$; *** $P < 0.001$ vs. untreated control.

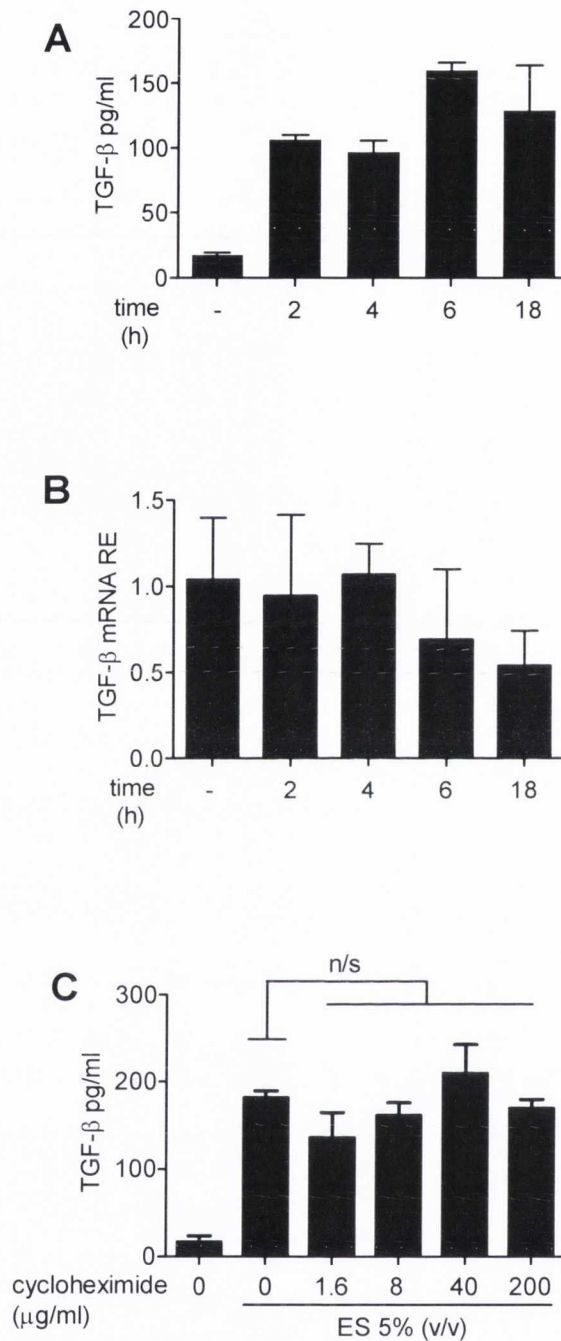


Fig. 3.8 *F. hepatica* ES treatment induces rapid release of TGF-β protein but does not trigger TGF-β gene expression. DCs from C57BL/6 mice were incubated with *F. hepatica* ES for indicated periods of time after which concentration of TGF-β in supernatants was quantified by ELISA (A) while cells were lysed and expression of *tgfb1* mRNA was analysed by quantitative real-time PCR (B). C) DCs from C57BL/6 mice were pre-incubated with increasing concentrations (1.6 – 200 μg/ml) of cycloheximide (translation inhibitor) for 1 h prior to treatment with *F. hepatica* ES. After 18 h concentration of TGF-β in supernatants was quantified by ELISA. Results are means (+/- SD) of triplicate assay. n/s – not significant vs. *F. hepatica* ES treated control. Results are representative of 2 independent experiments.

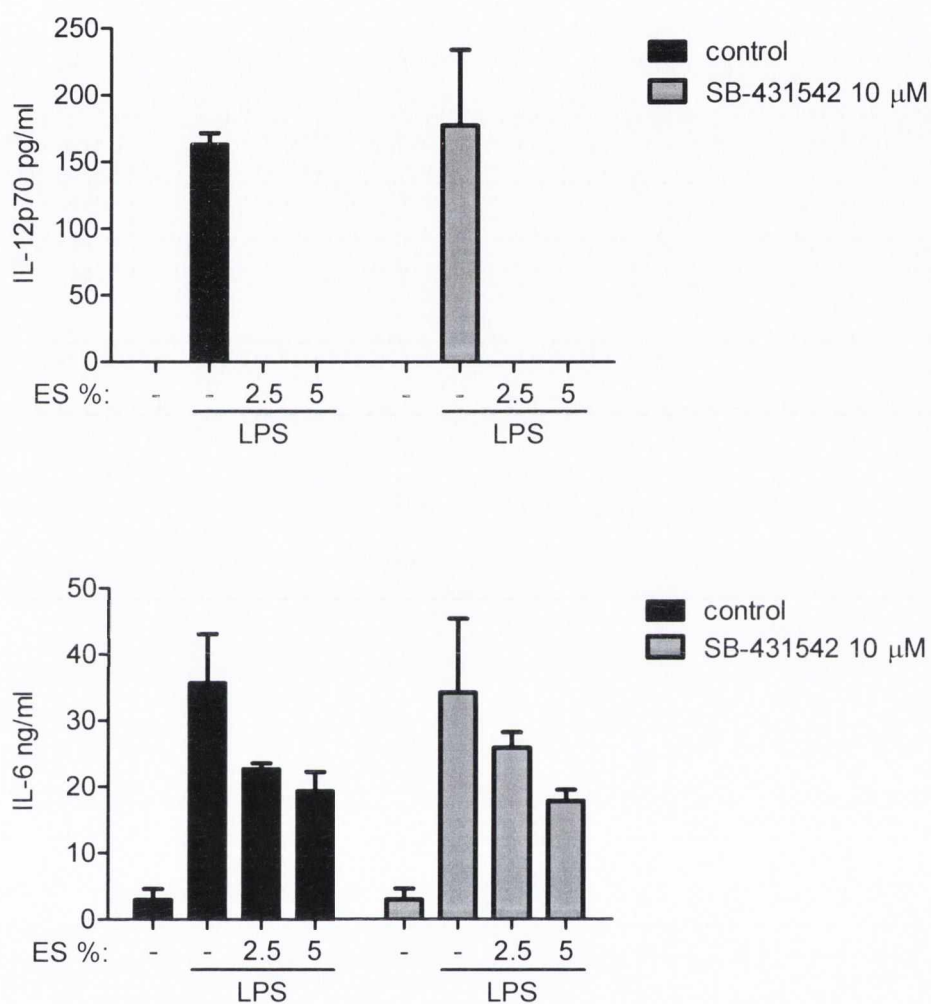


Fig. 3.9 Inhibition of cytokine production by *F. hepatica* ES is not mediated through TGF- β . DCs were pre-treated with the ALK5 inhibitor SB-431542 for 30 min before adding *F. hepatica* ES for 1 h followed by incubation with LPS for 24 h. Concentration of IL-12p70 and IL-6 in supernatants was measured by ELISA.

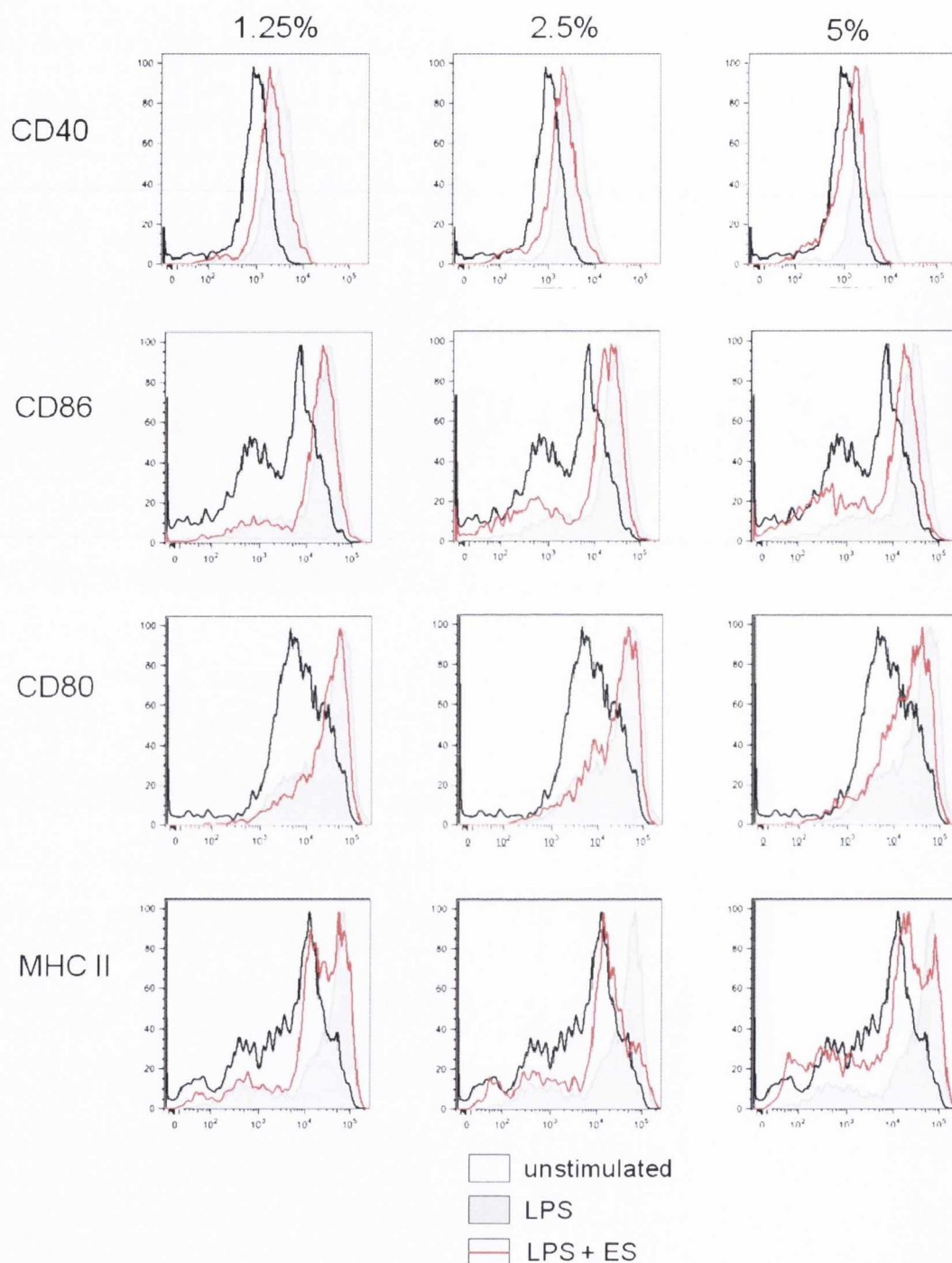


Fig. 3.10 *F. hepatica* ES products modulate TLR agonist induced maturation of DCs. DCs derived from C57BL/6 mice were pretreated with *F. hepatica* ES as indicated (1.25-5% v/v) for 2 h prior to stimulation with LPS (100 ng/ml). After 24 h cells were recovered, stained with antibodies against CD40, CD86, CD80 and MHC II (IA/IE) and analysed by flow cytometry.

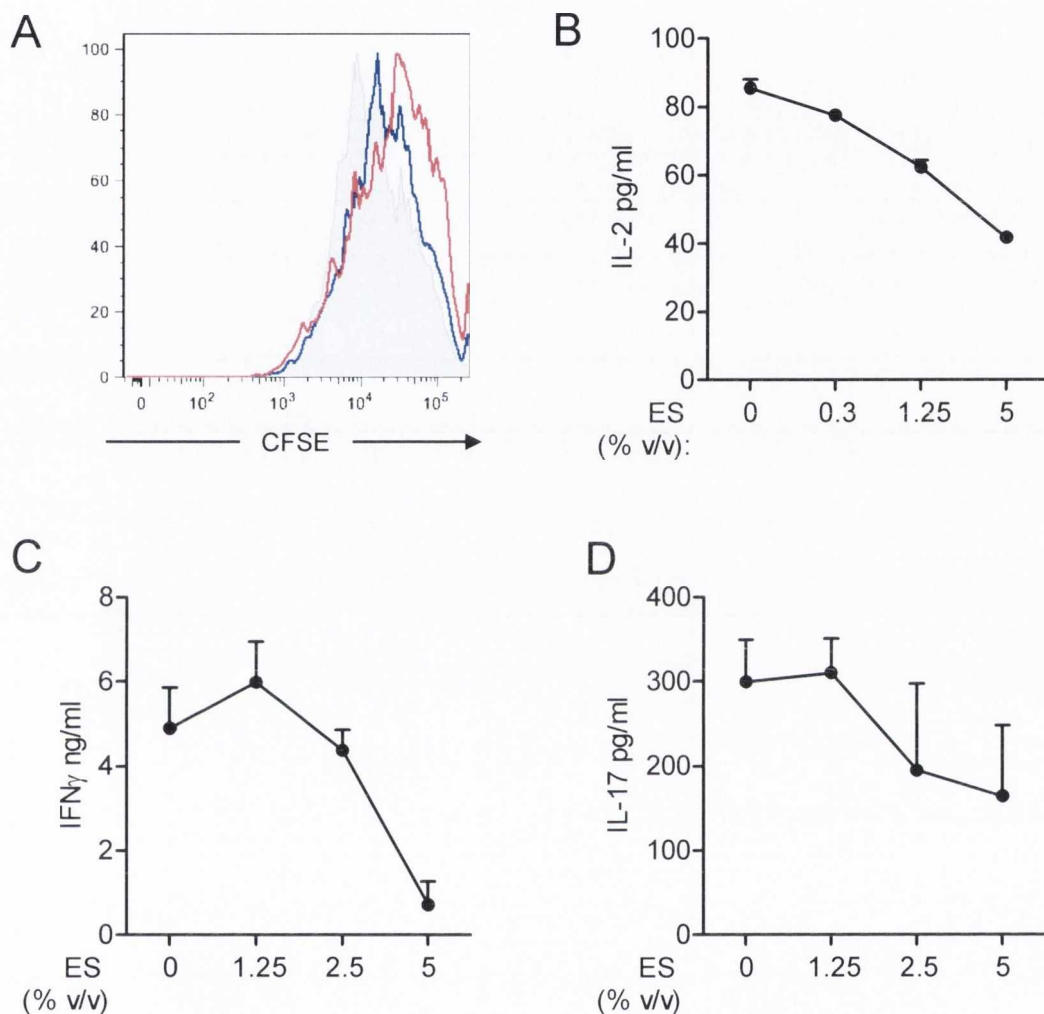


Fig. 3.11 *F. hepatica* ES inhibits spleen cell proliferation and cytokine production. Spleen cells from naive C57BL/6 mice were CFSE labeled, stimulated with anti-CD3e antibody (shaded histogram) and treated with 2.5% (blue histogram) or 5% (red histogram) v/v *F. hepatica* ES. Cells were harvested after 72 h, and CFSE dilution on CD4⁺ cells was analysed by flow cytometry (A). Naive spleen cells were stimulated with anti-CD3e (0.25 μ g/ml) and treated with *F. hepatica* ES at a range of concentrations. The concentration of IL-2 was quantified by ELISA after 18 h of incubation (B) and IFN- γ (C) and IL-17 (D) after 72 h. Data shown is quantified from triplicate samples and representative of at least 3 independent experiments with similar results.

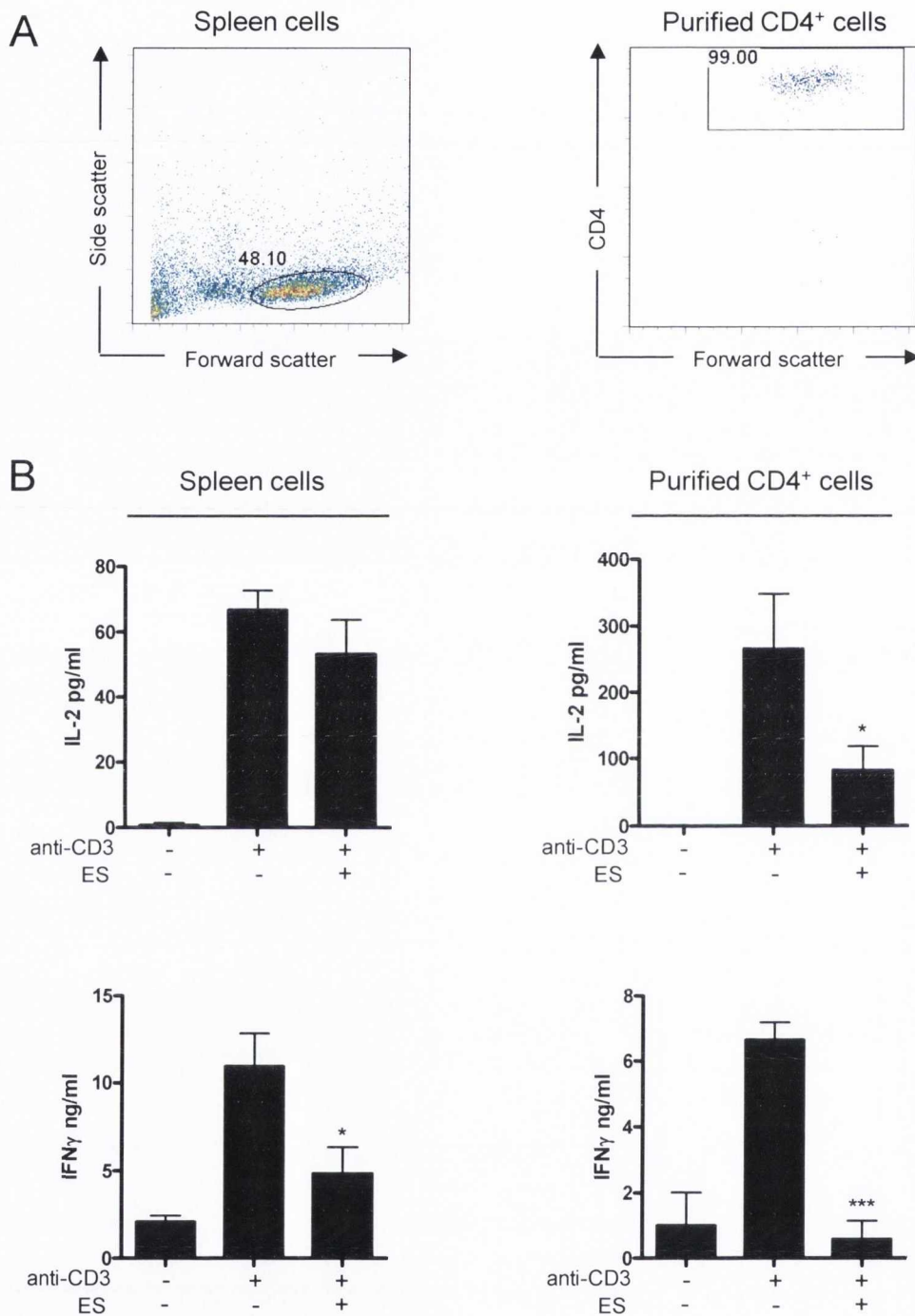


Fig. 3.12 Effect of *F. hepatica* ES treatment on cytokine production by purified CD4⁺ T cells. A) CD4⁺ T cells were purified from total spleen cells by FACS. B) Spleen cells and purified CD4⁺ cells were stimulated with soluble anti-CD3e (0.25 µg/ml) or plate bound anti-CD3 and anti-CD28 respectively. Cells were incubated for 72 h with or without *F. hepatica* ES (2.5% v/v). Supernatants were harvested after 18 or 72 h for determination of IL-2 or IFN-γ concentration respectively by ELISA. Results shown are quantified from triplicate samples and are representative of 2 independent experiments that produced similar results.

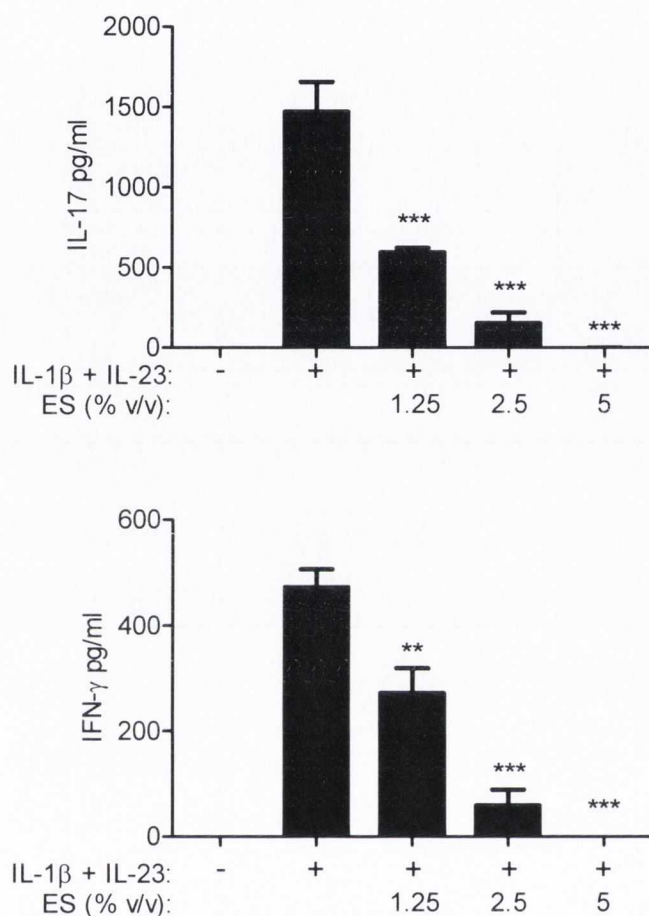


Fig. 3.13 *F. hepatica* ES suppress IL-17 and IFN- γ production by spleen cells stimulated *in vitro* with a combination of IL-1 β and IL-23. Spleen cells from a naïve C57BL/6 mice were incubated with combination of IL-1 β (10 ng/ml) and IL-23 (10 ng/ml) and with a range of concentrations of *F. hepatica* ES as indicated. Supernatants were collected after 18 or 72h to determine concentration of IL-2 or IL-17 and IFN- γ by ELISA. Results are means (+/- SD) of triplicate assay. ** P<0.01; *** P<0.001 vs. IL-1 β and IL-23 stimulated control.

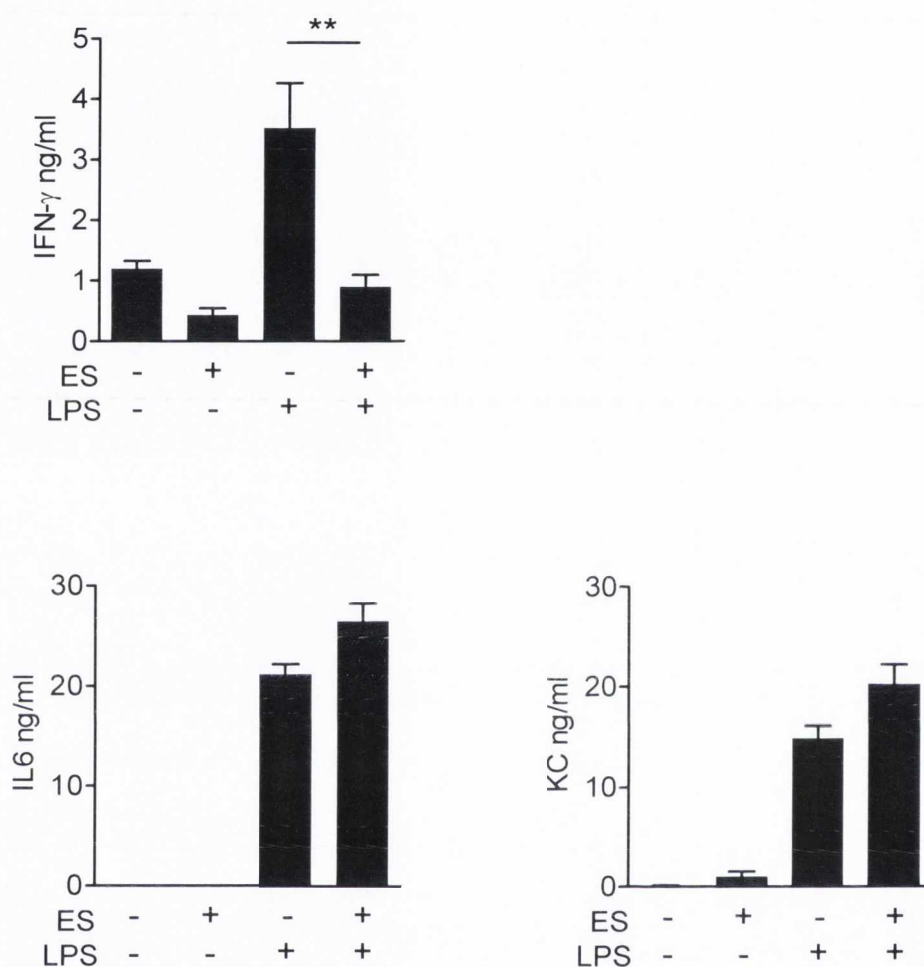


Fig. 3.14 *F. hepatica* ES inhibits LPS-induced production of IFN- γ *in vivo*. Naive C57BL/6 mice were injected i.p. with PBS, *F. hepatica* ES (50 μ l), LPS (10 μ g) or LPS and *F. hepatica* ES. After 4 h animals were sacrificed and IFN- γ , IL-6 and KC concentration in serum was determined by ELISA. Results are mean $\pm$ SEM of triplicate assay, n = 3 mice per group. ** P < 0.01. Data is representative of at least 4 independent experiments that produced similar results.

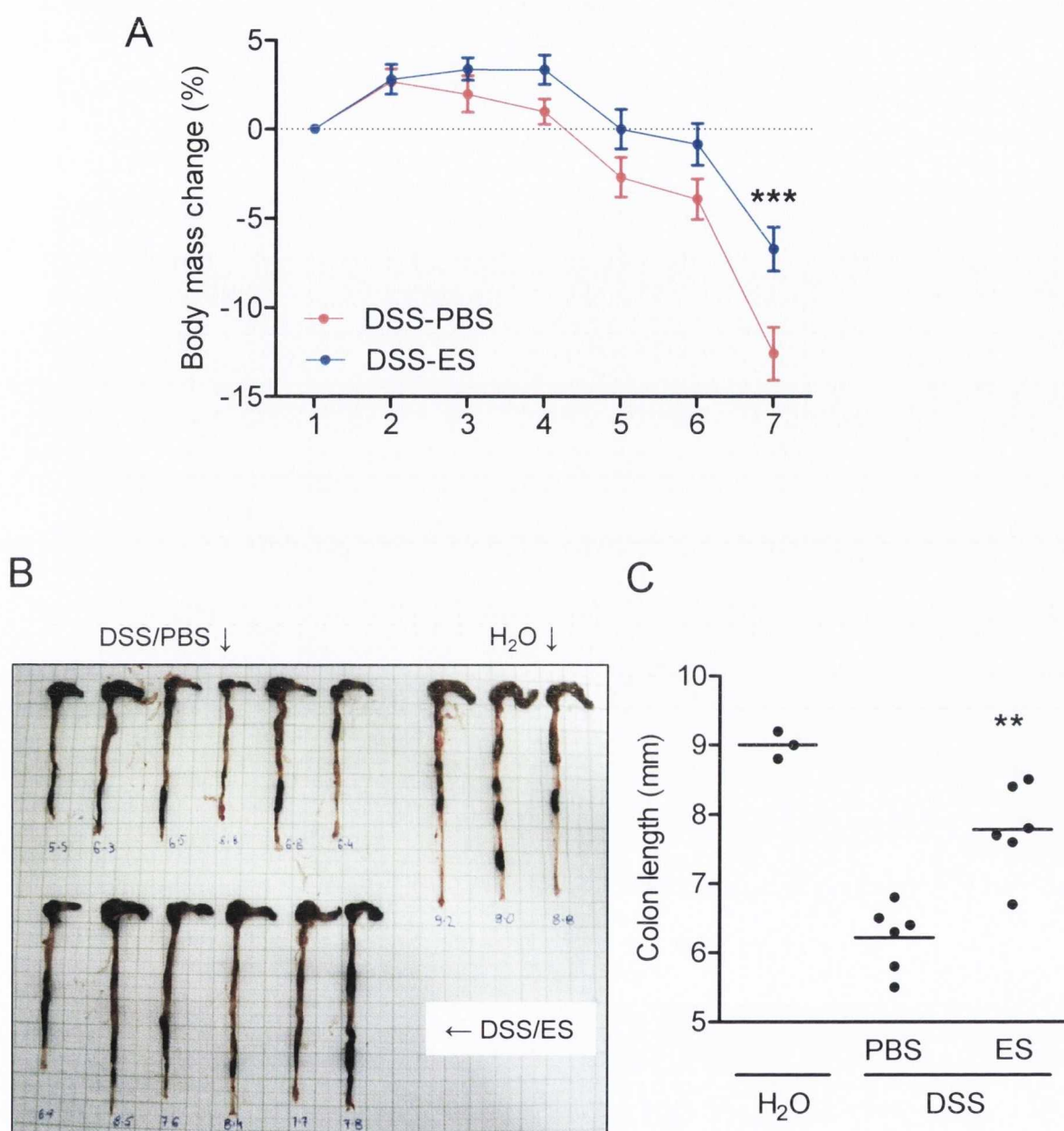
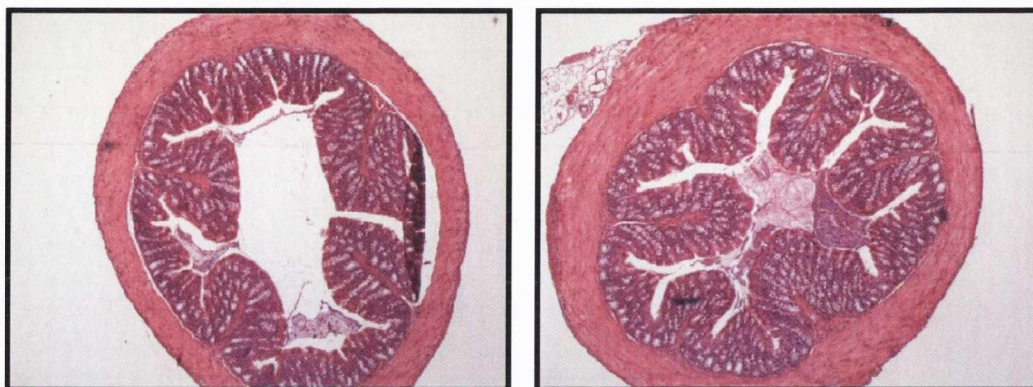
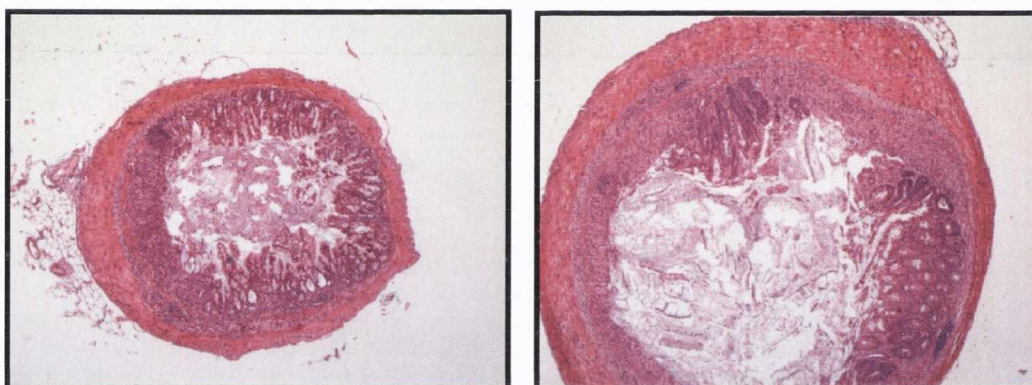


Fig. 3.15. *F. hepatica* ES attenuates clinical symptoms of DSS-induced colitis in C57BL/6 mice. Mice (n=6) were given 2% DSS *ad libitum* in drinking water for 7 days. Mice were treated with 40 μ l of *F. hepatica* ES or PBS (i.p.) on days: 0, 2, 4, and 6. A) Weight loss was monitored daily. B) Mice were sacrificed at day 7 and colon lengths were recorded (B). The results are summarized in C where each data point represents colon length of an individual animal. ** $P < 0.01$, *** $P < 0.001$. Data is from one of at least 3 separate experiments that produced similar results.

H₂O



DSS-PBS



DSS-ES

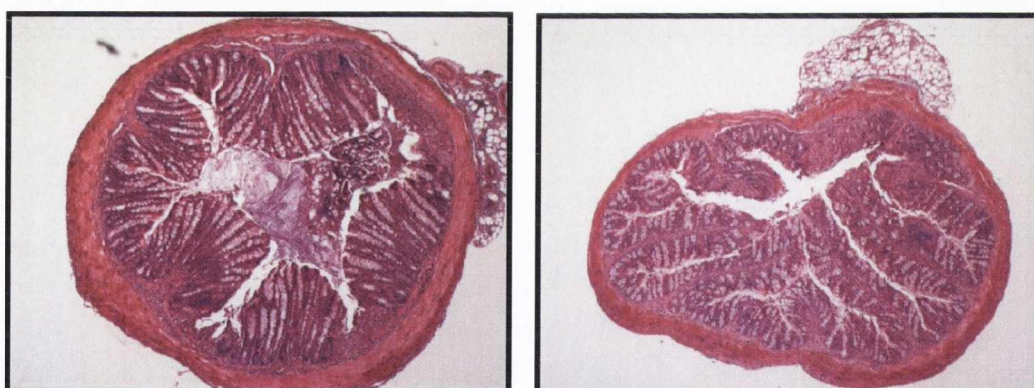


Fig. 3.16. *F. hepatica* ES attenuates pathological changes in colons of mice fed with DSS. Mice ($n = 3$) were given 2% DSS *ad libitum* in drinking water for 7 days. Mice were treated with 40 μ l of *F. hepatica* ES or PBS (i.p.) on days: 0, 2, 4, and 6. Animals were sacrificed at day 8, and sections of colons were collected into 10% buffered formalin. Samples were paraffin embedded, sections were stained with hematoxylin and eosin. Representative sections of 2 mice of 3 per group are shown.

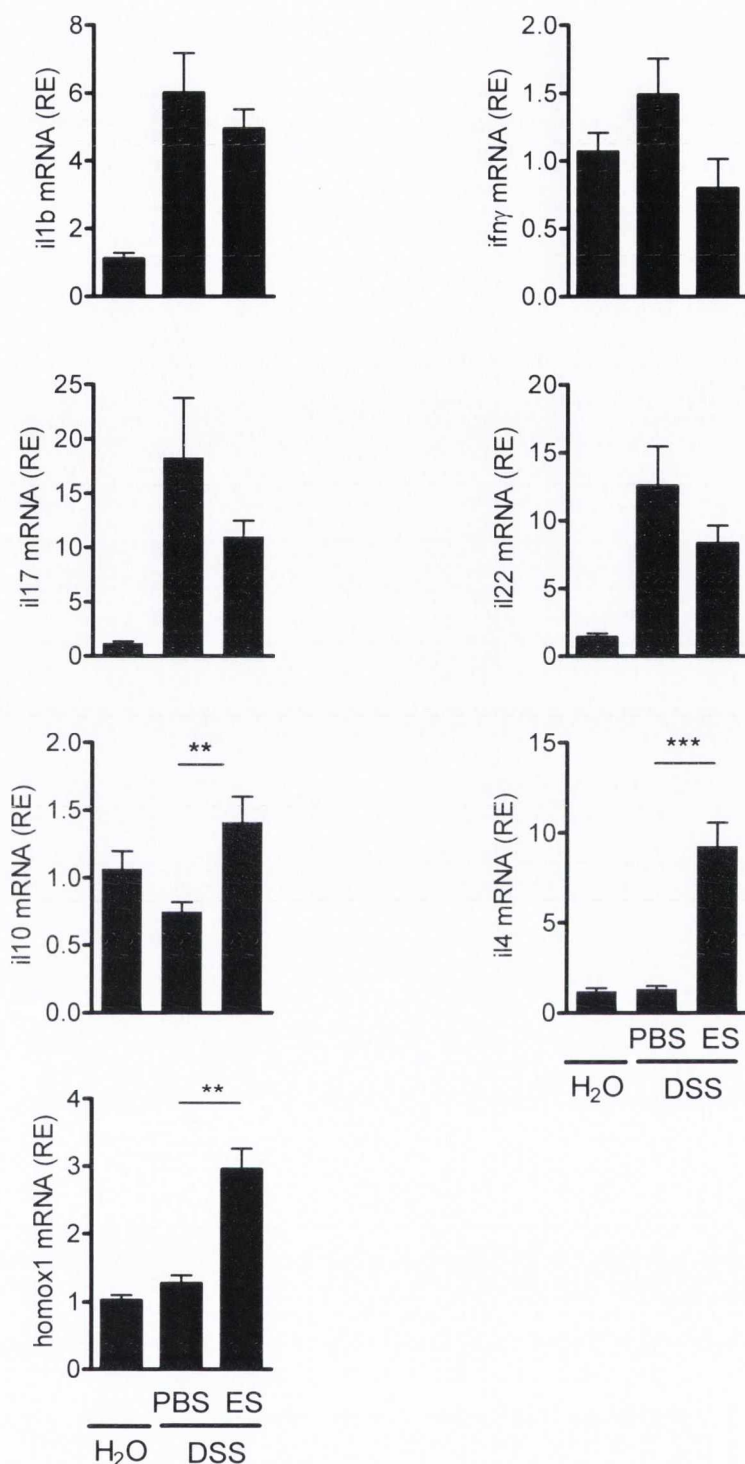


Fig. 3.17. The effect of *F. hepatica* ES administration on cytokine expression in colons of mice treated with DSS. Mice (n=4) were given 2% DSS *ad libitum* in drinking water for 7 days. Mice were treated with 40 μ l of *F. hepatica* ES or PBS (i.p.) on days: 0, 2, 4, and 6. Animals were sacrificed at day 7 and colon sections were collected into RNeasy lysis reagent. Total RNA was then extracted from the colon tissue and expression of selected genes was quantified by real-time PCR. * P < 0.05 ** P < 0.01 Results are from one experiment.

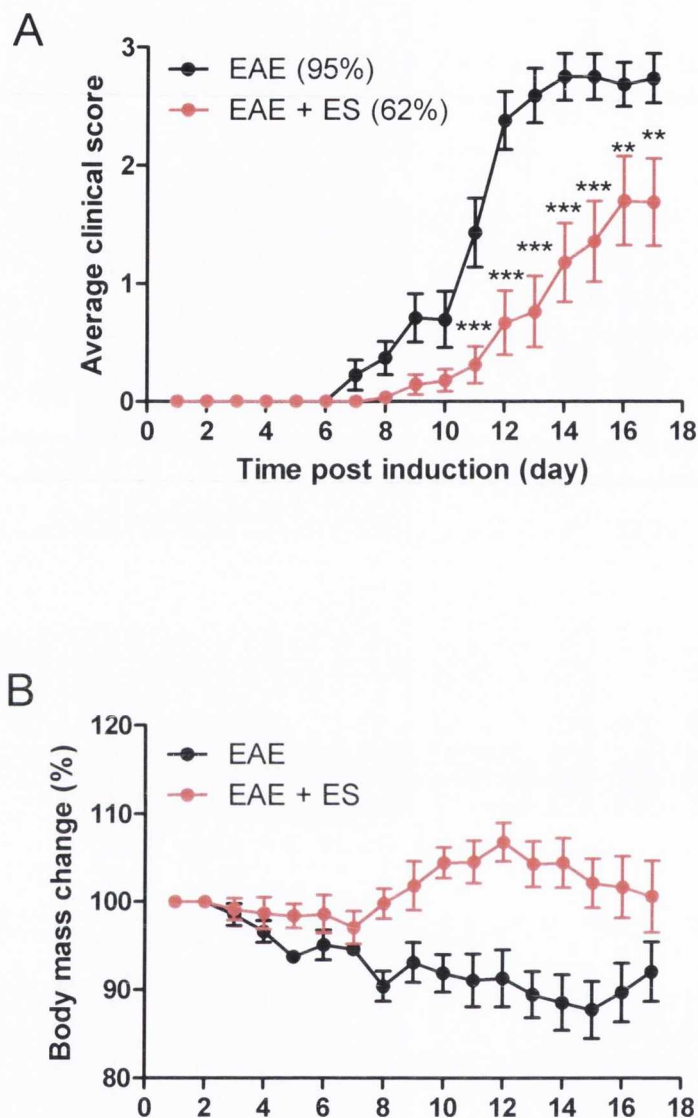


Fig. 3.18 Administration of *F. hepatica* ES throughout the course of the disease attenuates clinical symptoms of EAE. EAE was induced in C57BL/6 mice by immunisation with MOG emulsified in CFA. Mice were injected i.p. with 20 μ l of *F. hepatica* ES or PBS on days -1, 2 and every second day after. Animals were monitored daily for clinical signs of the disease (A) and body mass change (B). Disease severity was graded as follows: 0 – healthy, 1 – limb tail, 2 – wobbly gait, 3 – hind limb weakness, 4 – hind limb paralysis; 5 – tetraparalysis/death. Results are mean score $\pm$ SEM from 4 experiments (19–21 mice per group). ** $P < 0.01$; *** $P < 0.001$ vs. control group.

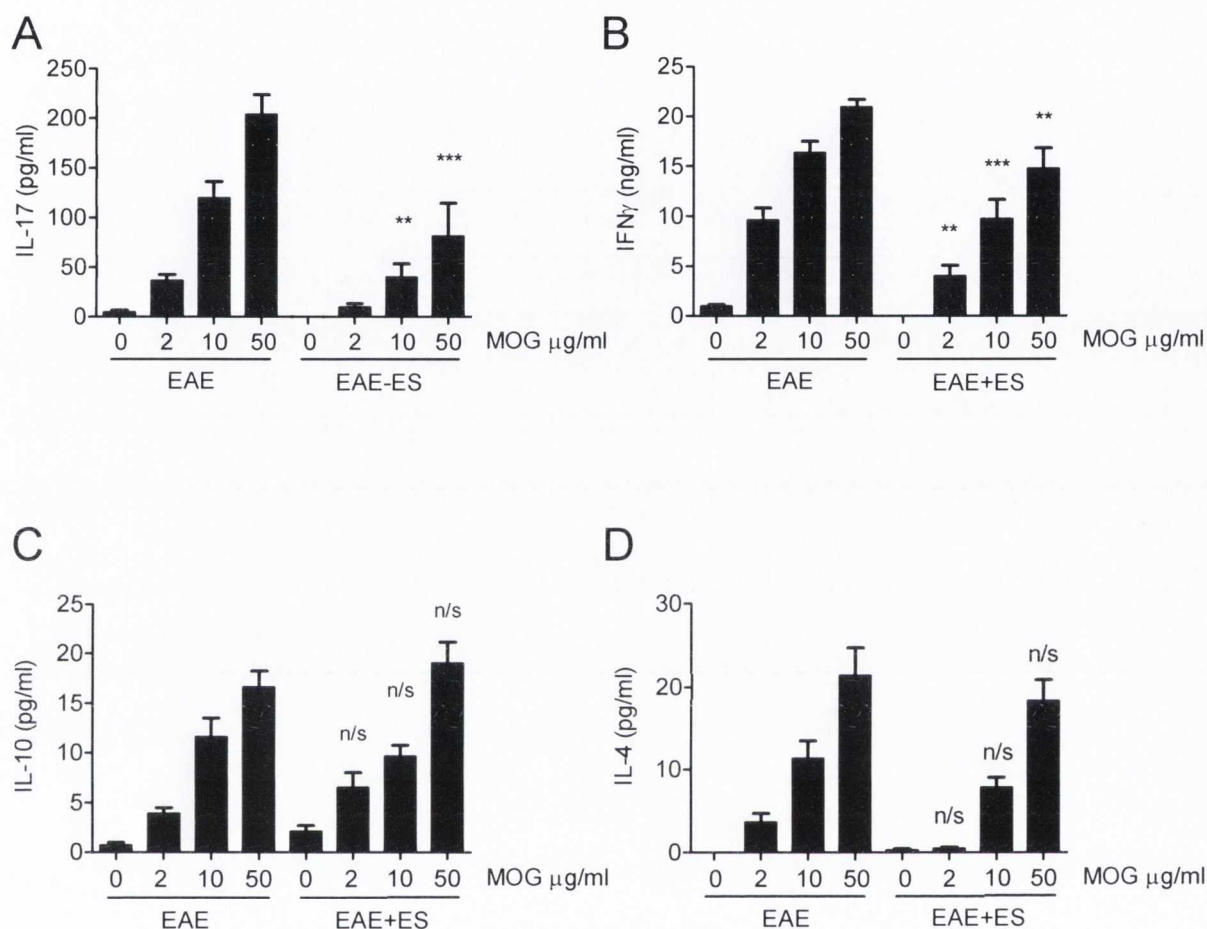


Fig. 3.19 Attenuation of EAE by *F. hepatica* ES is associated with suppression of MOG-specific Th1 and Th17 responses in spleens. EAE was induced in C57BL/6 mice by immunisation with MOG emulsified in CFA and mice were treated with *F. hepatica* ES as described in Fig 3.18. Eighteen days post induction of EAE spleen cells were stimulated for 72 h with MOG at 2, 10 or 50 $\mu\text{g/ml}$. Supernatants were collected for analysis of IL-17 (C), IFN- γ (D), IL-10 (E) and IL-4 (F) by ELISA * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control mice.

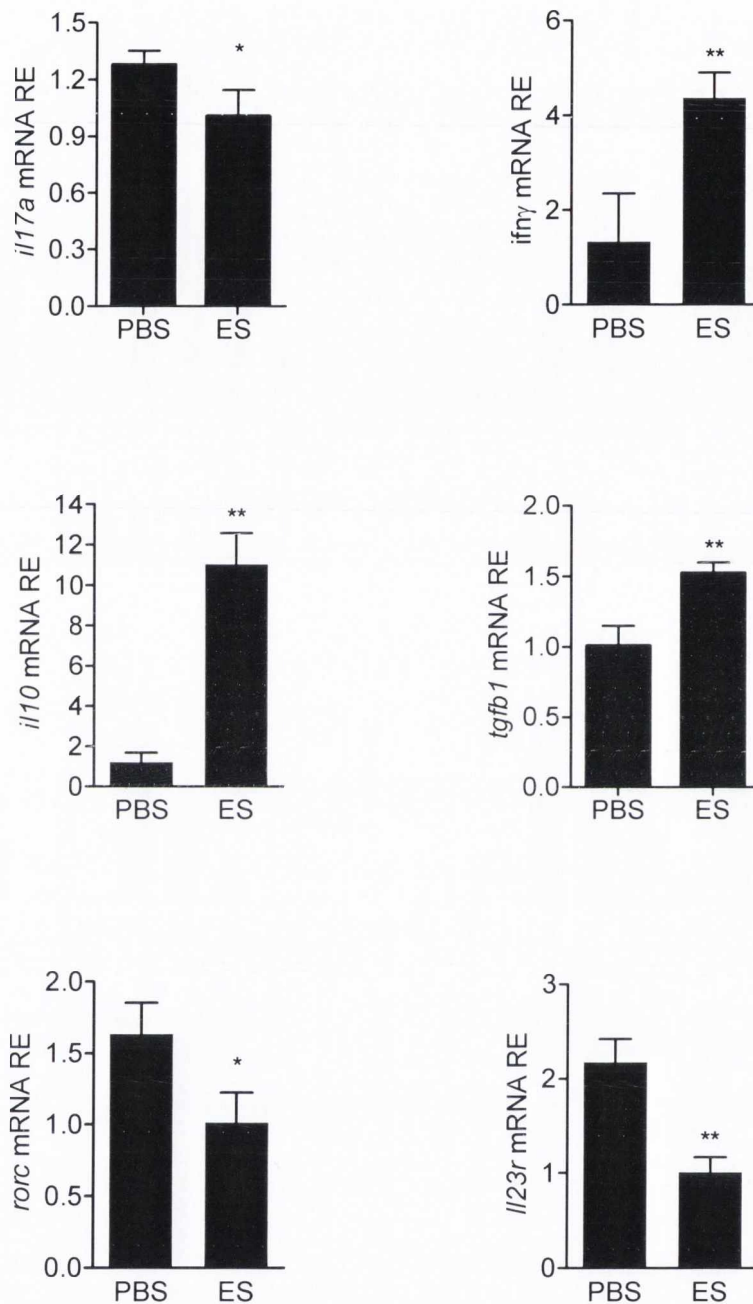


Fig. 3.20 *F. hepatica* ES administration suppresses IL-17, RORc and IL-23R but enhances IL-10, TGF- β and IFN- γ expression in draining lymph nodes of mice immunised with MOG/CFA. Mice were immunised into the flank with MOG peptide emulsified in CFA and injected s.c with *F. hepatica* ES on the day before MOG immunisation and every second day thereafter. Mice were sacrificed at day 10 post immunisation and inguinal lymph node cells were pooled from 10 mice per group. Total mRNA was extracted and gene expression was analysed by RT-PCR. Results are shown as mean ($\pm$ SD) from a triplicate assay. * $P < 0.05$; ** $P < 0.01$ vs untreated mice.

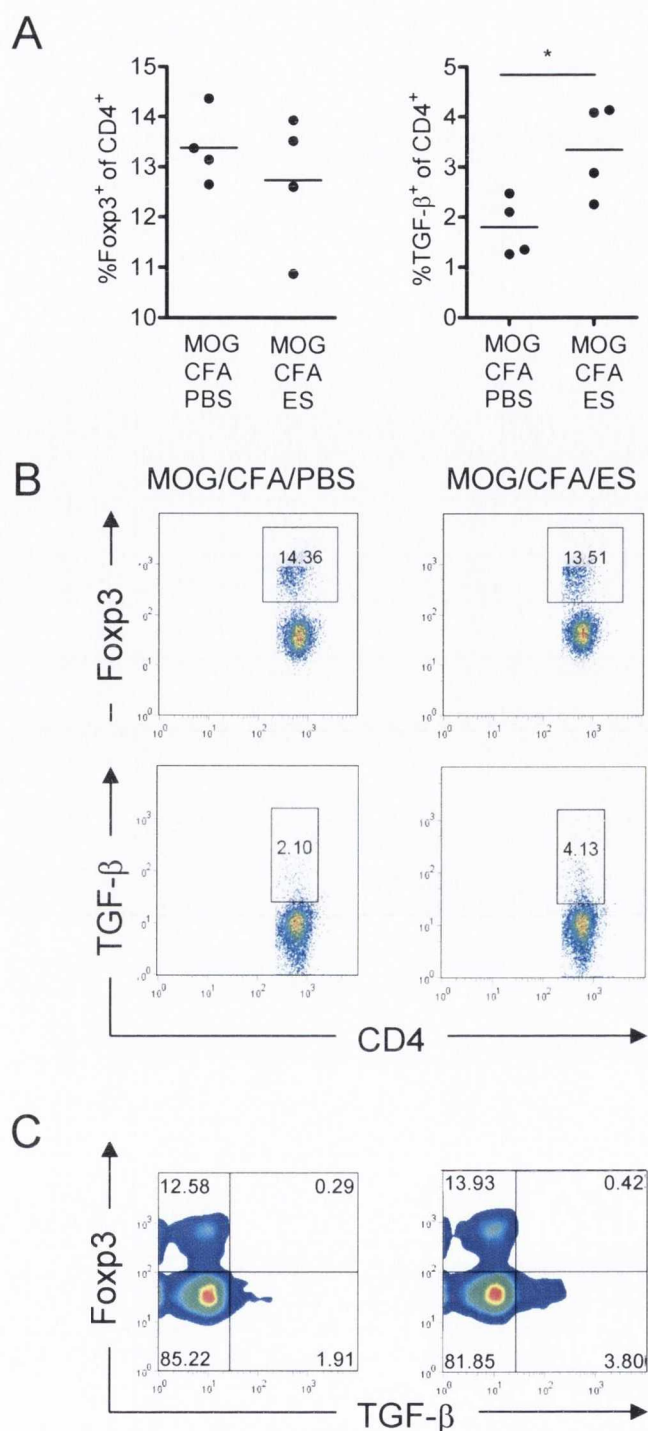


Fig. 3.21 Administration of *F. hepatica* ES *in vivo* results in induction of TGF-β expressing CD4⁺ cells. Mice were immunised into the flank with MOG peptide emulsified in CFA and injected s.c. with *F. hepatica* ES on the day before MOG immunisation and every second day after. Mice were sacrificed at day 10 post immunisation and expression of Foxp3 and TGF-β by CD4⁺ T cells from inguinal lymph nodes was analysed by flow cytometry. Mean frequencies of CD4⁺Foxp3⁺ and CD4⁺TGF-β⁺ cells are shown (A) along with representative dot plots (B) or (C) * P<0.05 vs. MOG/CFA/PBS. Results are representative of 2 independent experiments.

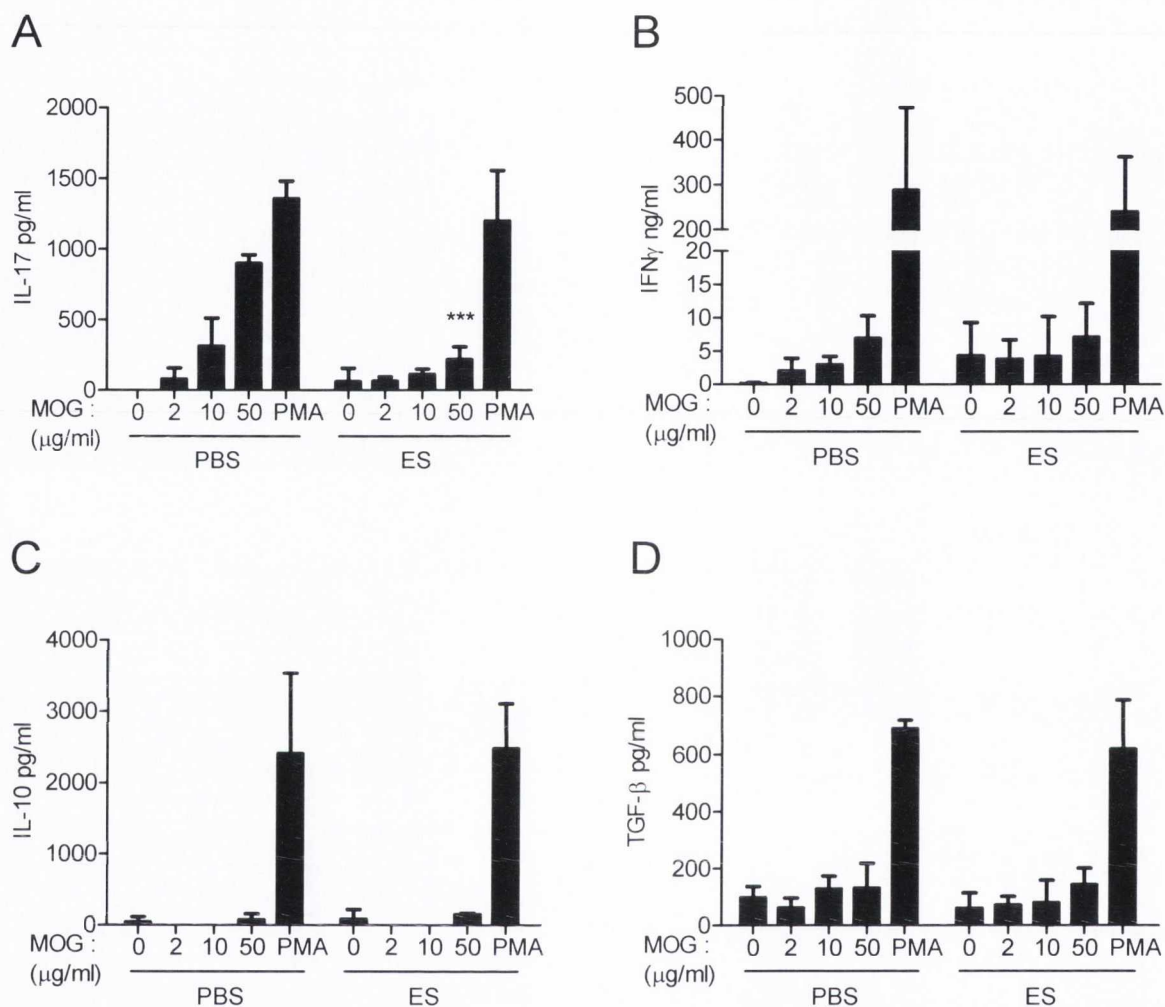


Fig. 3.22 The effect of *F. hepatica* ES administration on MOG-specific responses in lymph nodes. Mice were immunised with MOG peptide and treated with *F. hepatica* ES as described (Fig. 3.20) Mice were sacrificed at day 10 post immunisation and inguinal lymph node cells were pooled from 10 mice per group and re-stimulated *in vitro* with MOG peptide. After 72 h of culture the concentration of IL-17 (A), IFN- γ (B), TGF- β (C) and IL-10 (D) in supernatants was measured by ELISA. Results are mean (+/- SD) quantified from triplicate assay from one experiment out of two that produced similar results. * **P<0.001 vs PBS group.

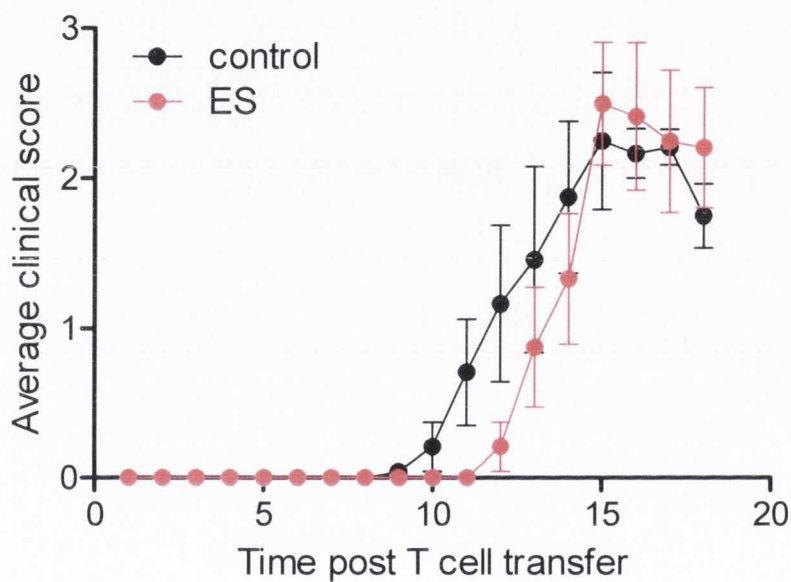


Fig. 3.23 The effect of *F. hepatica* ES administration on the clinical score of EAE induced by adoptive transfer. EAE was induced in C57BL/6 mice via adoptive transfer of MOG-specific T cells expanded *in vitro* in the presence of IL-1 β and IL-23. Experimental group was injected i.p with *F. hepatica* ES or PBS on the day of cell transfer and every second day thereafter. Results are mean score $\pm$ SEM for 6 mice per group and are representative of 2 independent experiments.

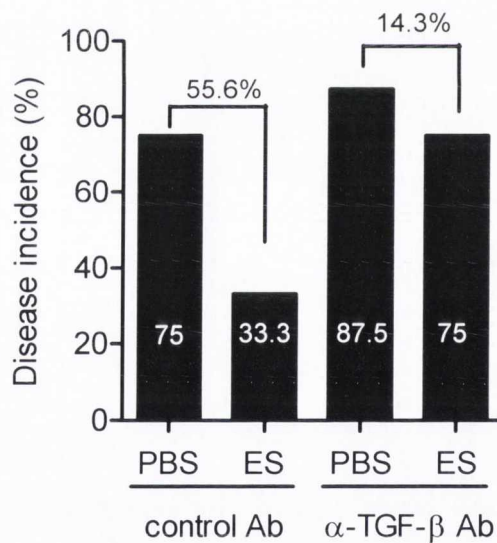
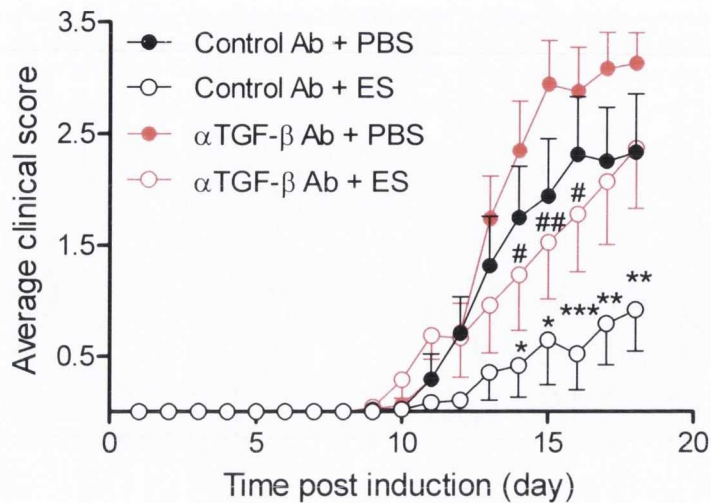


Fig. 3.24 The effect of TGF- β neutralisation on protective effect of *F. hepatica* ES administration in EAE. EAE was induced in C57BL/6 mice by immunisation with MOG emulsified in CFA, followed by PT. Mice were receiving α TGF- β Ab (100 μ g/mouse) or isotype control Ab i.p. from day -1 and every second day after through out the course of the disease. Animals treated with *F. hepatica* ES were injected with 20 μ l of ES on the same days as the antibody injection. Results are mean score $\pm$ SEM (A) and the disease incidence (B) from 2 independent experiments (12-18 mice per group). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ Control Ab + *F. hepatica* ES vs. Control Ab + PBS; # $P < 0.05$; ## $P < 0.01$ α TGF- β Ab + *F. hepatica* ES vs. α TGF- β Ab + PBS.

***Immunomodulatory properties of
high and low molecular weight
fractions of F. hepatica ES***

Chapter 4

4.1 Introduction.

Strategies of immune evasion that facilitate parasite survival within the host have been shown to be associated with simultaneous subversion of immune responses towards bystander antigens. This phenomenon forms the basis of the hygiene hypothesis, which has been used to explain the lower incidence of allergy and autoimmune disease observed in rural areas of developing countries. Therefore a greater understanding of how helminth parasites modulate the immune response could help to understand the mechanisms of immune dysregulation leading to allergy and autoimmunity and may contribute to the design of new therapeutic strategies.

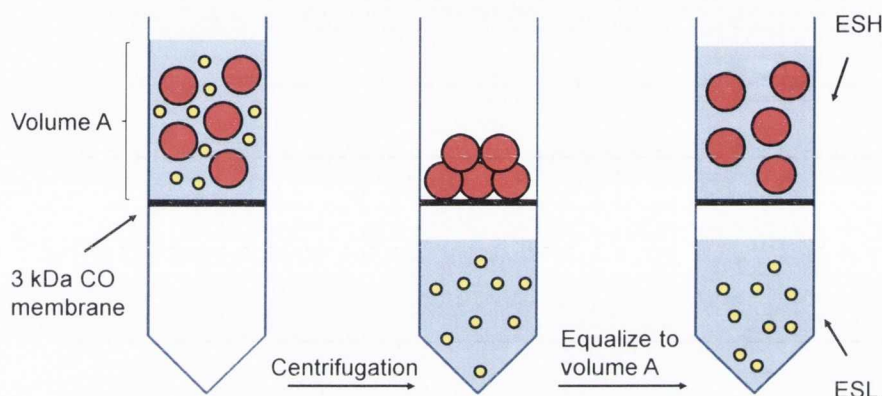
Parasitic excretory/secretory products contain a mixture of actively secreted immunomodulatory molecules, such as bioactive metabolites and enzymes as well as debris, cecum contents and structural elements shed from the parasite surface. These molecules fall into various classes of compounds which differ significantly both in size and biochemical nature. As such ES comprises a complex mix of biological material including proteins and their breakdown products, as well as various lipids and carbohydrates of undetermined composition. Many of those compounds may have the capacity to interact with components of the mammalian immune system, however not all are able to subvert the hosts immune defensive mechanisms and facilitate parasite survival. In recent years a number of ES product-derived immunomodulators have been identified and characterized in terms of their molecular composition and bioactivity. Structurally, these components fall into diverse biochemical classes and regulate various pathways in order to subvert the development of the host's immune response (Hewitson et al., 2009). Protein immunomodulatory components which have been described, including cysteine and serine protease inhibitors (cystatins and serpins), have been shown to block the activity of host enzymes playing key roles in antigen uptake, processing and presentation. Furthermore, worm-derived cytokine homologues, lectins and proteolytic enzymes can contribute to parasitic immune evasion

strategies (Maizels, 2009). In addition to proteins and peptides a number of oligo- and polysaccharides have been shown to influence T cell polarization and induce immunosuppression by modulating pathways leading to decreased pro-inflammatory cytokine expression by DCs (Johnston et al., 2009). Lipid-based components including prostaglandins, phosphorylserine (PS) and lyso-PS lipids have been shown to be produced by parasites and impair antigen presentation and T cell proliferation or promote the induction of Tregs (van der Kleij and Yazdanbakhsh, 2003). Taken together, these reports provide convincing evidence that parasitic helminths are a potent source of immunomodulatory compounds that, in pure form, could offer potential as therapeutics to treat inflammatory diseases.

The results described in Chapter 3 demonstrated that treatment of mice with *F. hepatica* attenuates the symptoms of EAE. Therefore from the perspective of further identification of active components it was important to establish the connection between the properties of ES observed *in vitro* with protection from autoimmunity *in vivo*.

In order to identify and characterize factors of *F. hepatica* ES which mediate the immunomodulatory properties described in Chapter 3 fractionation on the basis of molecular mass was performed using centrifugal units with a 3000 Da molecular weight cut off (MWCO) membrane. This basic approach allowed rough separation of *F. hepatica* ES into a low molecular weight fraction depleted of molecules larger than approximately 3 kDa (ES-L) and the remaining fraction termed high molecular weight fraction (ES-H). This fractionation approach was chosen over a literature based, proteome orientated screening of putative immunomodulatory proteins to avoid neglecting active components that do not belong to this class of compounds. The filtration procedure did not change molar concentrations of molecules in any of the fractions allowing direct comparison of activity with the original *F. hepatica* ES preparation on a basis of volume. This approach was chosen as more appropriate than evaluating immunosuppressive effects on the basis of protein content as firstly, the

biochemical nature of the immunosuppressive component(s) is unknown and secondly, the fractionation process may have distributed mass concentration of total protein unevenly between the ES-H and ES-L. An overview of the fractionation procedure is depicted in the cartoon below.



Picture 4.1 Overview of the fractionation procedure.

The aim of this part of the study was to evaluate distinct immunomodulatory effect of ES-L and ES-H on the activation and cytokine production by DCs and T cells *in vitro* and establish how the observed modulation correlates with the ability to attenuate EAE. Furthermore, the study continued to investigate the mechanisms involved in the secretion of TGF- β by *F. hepatica* treated DCs. Finally, having demonstrated that *F. hepatica* ES can inhibit innate IL-17 and IFN- γ production by spleen cells stimulated with IL-1 β and IL-23 (Fig. 3.13) this study further investigated the possibility that parasite products can have a direct impact on activation of IL-1 β and IL-23 responsive $\gamma\delta$ T cells.

4.2 Results

4.2.1 Immunomodulatory activity of *F. hepatica* ES segregates to a fraction containing molecules smaller than 3 kDa

This study has already demonstrated that *F. hepatica* ES has a capacity to modulate cytokine production and expression of co-stimulatory molecules by DCs stimulated with TLR ligands. Experiments were designed as described above to determine the contribution of low (ES-L) and high (ES-H) molecular weight fractions of *F. hepatica* ES towards inhibition of DC activation. DCs derived from or C3H/HeJ mice were pre treated with a range of concentrations of *F. hepatica* ES, ES-L and ES-H for 2 h prior to stimulation with CpG (5µg/ml). Following 24 h incubation, concentrations of IL-12p70, IL-23 and IL-6 in supernatants were measured by ELISA. Consistent with data presented in Chapter 3, pre-treatment with *F. hepatica* ES inhibited IL-12p70, IL-23 and IL-6 production induced by CpG in a dose dependent manner (Fig. 4.1). Both ES-L and ES-H were able to suppress IL-12p70, however, suppression with ES-L was more potent than that observed with ES-H. The capacity to suppress production of IL-23 was retained in low molecular fraction since ES-L provided comparable degree of inhibition as original *F. hepatica* ES preparation, while ES-H had no effect on CpG induced IL-23 produced by DCs. Similarly the suppressive effect of *F. hepatica* ES on IL-6 production was mirrored by ES-L, whereas in contrast, ES-H markedly increased CpG induced IL-6 expression.

Expression of co-stimulatory molecules on DCs is important for efficient antigen presentation and activation of T cells. This study examined the effect of treatment with ES-L and ES-H on the expression of MHC II and CD40 by DCs. DCs from or C57BL/6 mice were pre treated with a range of concentrations of *F. hepatica* ES, ES-L and ES-H for 2 h prior to stimulation with LPS (100 ng/ml). After 18 h recovered cells were stained with flouorochrome conjugated antibodies to MHC II and CD40 and analysed by FACS. *F. hepatica* ES suppressed LPS induced up-regulation of CD40 and MHC II. Treatment with ES-L but not ES-H mirrored the effect of total *F. hepatica* ES on the expression of both CD40 (Fig. 4.2) and MHC II (Fig. 4.3), confirming that factors modulating

responses of DCs segregate to the low molecular weight fraction of *F. hepatica* ES.

This study has already demonstrated that *F. hepatica* ES has a capacity to induce secretion of TGF- β from DCs. To determine which of the fractions contributes to this effect DCs were incubated with a range of concentrations of *F. hepatica* ES, ES-L and ES-H for 24 h. The concentration of total TGF- β in supernatants was examined by ELISA. The result shows that cells treated with *F. hepatica* ES and ES-L but not ES-H up-regulated TGF- β production in a dose dependent manner (Fig. 4.4). This demonstrated that TGF- β inducing factor also segregates to the low molecular weight fraction.

4.2.2 Immunomodulatory factor from low molecular weight fraction of *F. hepatica* ES is not a protein

A polymeric protein removal suspension reagent - BindPro, which non-covalently binds proteins, peptides and peptide-containing conjugates was employed to biochemically characterize the immunomodulatory factors in ES-L. Pam₃CSK₄, a synthetic triacylated lipopeptide containing a short amino-acid sequence was chosen to control for efficiency of protein removal. ES-L or an aqueous solution of Pam₃CSK₄ was incubated with BindPro for 10 min in room temperature, after which the protein/peptide depleted supernatant was recovered. DCs were stimulated with Pam₃CSK₄ or BinPro treated Pam₃CSK₄ for 24 h and concentration of IL-12p70 in supernatants was measured by ELISA. Pam₃CSK₄ induced moderate production of IL-12p70 from DCs while treatment with BindPro prevented production of this cytokine indicating that the procedure completely removed lipopeptide from the solution (Fig 4.5 A). DCs were treated with ES-L or protein depleted ES-L, alone or followed by LPS (100 ng/ml) and concentration of IL-12p70 (Fig. 4.5 B) and TGF- β (Fig. 4.5 C) was measured after 24 h. Protein depletion did not affect capacity of ES-L to inhibit LPS induced IL-12p70 or to elicit TGF- β production by DCs indicating that active components in ES-L are not peptide in nature.

4.2.3 ES-L induces release of latent TGF- β via protease-dependent mechanism

Experiments were designed to examine in more detail the mechanism of TGF- β induction by ES-L. Data presented in Chapter 3 demonstrated that *F. hepatica* ES did not trigger *de novo* production of TGF- β but rather induced a rapid release of pre-made cytokine. The production and bioavailability of TGF- β is tightly controlled. The cytokine is secreted as a cell surface bound latent complex and needs to be activated to allow binding to relevant receptors. Most of TGF- β released by cultured cells is latent and has to be activated by acid or heat treatment in order to be detected by commercially available ELISA. Active TGF- β can be measured in the medium directly, without any treatment, however, poor sensitivity of the TGF- β ELISA limits this approach. Therefore more accurate bioassay systems using various TGF- β responding cells are commonly employed. MFB-11 cells are stably transfected with a TGF- β -responsive alkaline phosphatase (SEAP) reporter construct which enables them to secrete soluble enzyme into the culture medium upon stimulation with active TGF- β in a dose dependent manner. In order to establish if ES-L induced TGF- β is released in active or latent form, DCs were co-cultured in serum free medium with MFB-11 reporter cells with or without ES-L and SEAP activity in the supernatants was measured after 48 h. A range of concentrations of recombinant, active TGF- β was added to co-cultures as a positive control. The presence of DCs did not induce SEAP activity above the levels detected when MFB-11 cells were cultured alone (Fig. 4.6). Recombinant TGF- β added to co-culture at 125 and 600 pg/ml significantly induced secretion of SEAP however addition of ES-L to co-cultured cells did not (Fig. 4.6). The result suggests that TGF- β induced from DCs by ES-L is not active.

LAP-associated latent TGF- β is usually anchored into cell membrane or extracellular matrix through covalent interaction between LAP and LTBP. Thus release of the soluble latent complex to cell culture medium requires proteolysis of LTBP or other components of large latency complex (Annes et al., 2003; Taipale et al., 1995). To determine whether ES-L may induce the release of latent TGF- β through activation of DC-derived proteolytic enzymes, DCs were

preincubated for 30 min with a range of concentrations of universal protease inhibitor cocktail before treatment with 5% ES-L (Fig. 4.7 A). Inhibition of proteases reduced concentration of ES-L induced TGF- β in a dose dependent manner, demonstrating that proteases are involved in this process. In order to identify the class of proteases mediating this activity of ES-L, DCs were pre incubated with a range of concentrations of aprotinin (Fig. 4.7 B), E64 (Fig. 4.7 C) or GM6001 (Fig. 4.7 D), inhibitors of serine proteases, cysteine proteases and metalloproteases respectively. The result shows that none of the inhibitors reduced ES-L induced release of TGF- β . Collectively, these data demonstrate that the release of latent TGF- β by ES-L is mediated by DCs-derived proteases however identification of exact mechanism and enzyme involved requires further investigation.

4.2.4 ES-L inhibits proliferation and cytokine production by CD4⁺ T cells

The results presented in Chapter 3 demonstrated that *F. hepatica* ES can inhibit polyclonal activation of naive T cells stimulated with anti-CD3 or with combination of IL-1 β and IL-23. Having shown that immunomodulatory factors that affect DCs responses segregate to the low molecular weight fraction ES-L, experiments were designed to identify the fraction that contains compounds that modulate T cell activation. Spleen cells from naive mice were labelled with CFSE, a dye that enables monitoring of cell division by flow cytometry, and activated with anti-CD3 (0.25 μ g/ml) in the presence or absence of *F. hepatica* ES, ES-L or ES-H. Cells were recovered after 72 h, stained with fluorochrome conjugated antibodies against CD3 and CD4 and proliferation of the CD4⁺ population was analysed by FACS. Incubation of spleen cells with 5% (v/v) *F. hepatica* ES completely suppressed anti-CD3 induced dilution of CFSE by CD4⁺ lymphocytes demonstrating that the treatment inhibited proliferation of these cells. However, this effect was insignificant at lower concentration (2.5 %) of *F. hepatica* ES (Fig. 4.8 A). ES-L robustly reduced CFSE dilution at both concentrations in a dose dependent manner (Fig. 4.8 B). In contrast ES-H had no effect on cell proliferation (Fig. 4.8 C). The reduction of proliferation was associated with a failure to up-regulate activation markers such as CD25, which is critical for T cell expansion, but was not associated with cell death. CD4⁺ T

cells from spleen cultures stimulated for 12 h with anti-CD3 in presence of *F. hepatica* ES or ES-L but not ES-H had reduced expression of CD69 and CD25 on the cell surface (Fig. 4.9 A). The frequency of 7-AAD positive dead CD4⁺ T cells did not differ between treated and the control (anti-CD3 only) cells (Fig 4.9 B).

Further experiments examined the effect of ES-L and ES-H on secretion of T cell cytokines. Spleen cells from a naive mouse were activated with anti-CD3 (0.25 µg/ml) in the presence or absence of *F. hepatica* ES, ES-L or ES-H. Supernatants were harvested to determine concentration of IL-2 (after 18 h), IFN-γ and IL-17 (after 72 h) by ELISA. In agreement with the reduced proliferation observed, both *F. hepatica* ES and ES-L, but not ES-H, significantly suppressed production of IL-2 - a cytokine necessary for expansion and survival of effector T cells (Fig. 4.10 A). Similarly, incubation with *F. hepatica* ES and ES-L substantially decreased IFN-γ (Fig. 4.10 B) and IL-17 (Fig. 4.10 C) production by spleen cells which directly reflects its suppressive effect on polyclonal T cell activation. Notably ES-L was a much more potent inhibitor of all three cytokines than the original preparation of *F. hepatica* ES. In contrast ES-H enhanced production of IFN-γ, but have no effect on IL-17. Collectively the data demonstrates that immunomodulatory factors of *F. hepatica* ES which inhibit anti-CD3 induced T cell proliferation and cytokine production segregate to the low molecular weight fraction.

T cells can also produce IL-17 and IFN-γ in response to stimulation with innate cytokines such as IL-1β and IL-23 without requirement for TCR engagement. The data presented in Chapter 3 demonstrated that treatment with *F. hepatica* ES inhibited production of IL-17 and IFN-γ elicited by IL-1β and IL-23 (Fig 3.13). To determine if ES-L can mirror suppressive effect of *F. hepatica* ES in this experimental system, spleen cells were incubated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence of *F. hepatica* ES, ES-L or ES-H for 72 h after which concentration of IL-17 and IFN-γ was measured by ELISA. Both *F. hepatica* ES and ES-L, but not ES-H, suppressed production of these cytokines in a dose dependent manner. Similar to the result obtained in the conditions involving TCR dependent stimulation of spleen cells, ES-L was a considerably more potent inhibitor of IL-17 and IFN-γ than *F. hepatica* ES.

4.2.5 ES-L inhibits activation of $\gamma\delta$ T cells *in vitro* and *in vivo*

Stimulation of spleen cells with IL-1 β and IL-23 can induce production of IL-17 and IFN- γ by memory CD4⁺ cells and unconventional T cells such as $\gamma\delta$ T cells. Since $\gamma\delta$ T cells play a crucial pathogenic role in autoimmunity and IL-1 β and IL-23 was shown to activate $\gamma\delta$ T cells and induce IL-17 secretion both *in vitro* and *in vivo* (Sutton et al., 2009), it was important to evaluate direct effect of ES-L on the ability of $\gamma\delta$ T cells to respond to IL-1 β and IL-23. The result showed that ES-L suppressed production of IL-17 and IFN- γ by purified $\gamma\delta$ T cells in a dose dependent manner (Fig. 4.12). Analysis of gene expression by real-time PCR further confirmed that ES-L inhibited these cytokines at mRNA level. In addition treatment with 2.5% ES-L completely inhibited transcription of genes encoding proteins strongly associated with IL-17 production such as: ROR γ t, IL-1R1 and IL-23R (Fig. 4.13).

The capacity of ES-L to inhibit IL-17 production by $\gamma\delta$ T cells *in vivo* was also examined. Mice were injected s.c. in the footpad with PBS, IL-1 β and IL-23 (both 100 ng/mouse) with or without ES-L (40 μ l/mouse) and popliteal lymph node cells were recovered 4 h later. Flow cytometric analysis of IL-17 production by CD3⁺ $\gamma\delta$ TCR⁺ revealed that IL-1 β and IL-23 considerably increased the frequency of $\gamma\delta$ TCR⁺IL-17⁺ cells in draining lymph nodes. Importantly co-administration of ES-L significantly reduced the percentage of IL-17-producing $\gamma\delta$ T cells. Inhibition of IL-17 expression in draining lymph nodes by ES-L was further confirmed by real-time PCR (Fig. 4.14).

Collectively this data demonstrates that the low molecular fraction of ES-L contains components with a capacity to inhibit activation of $\gamma\delta$ T cells by innate cytokines *in vitro* and *in vivo*.

4.2.6 ES-H attenuates EAE.

Having shown that factors responsible for the immunomodulatory effects of *F. hepatica* ES observed in *in vitro* models were retained in low molecular weight fraction, this study was extended to evaluate the contribution of ES-L and ES-H to the suppressive effect of *F. hepatica* on the development of autoimmunity *in vivo*. EAE was induced by s.c. immunisation with MOG peptide in CFA, followed by PT and mice were treated with *F. hepatica* ES, ES-L, ES-H

or vehicle on day -1, 2 and every second day thereafter. Animals were monitored daily for the clinical symptoms of the disease (Fig. 4.15 A) and their general condition was measured as changes in the body weight (Fig. 4.15 B). Consistent with the data in Chapter 3, the administration of *F. hepatica* ES markedly delayed the severity (Fig. 4.15 A) and incidence (Fig. 4.15 C) of EAE and prevented weight loss (Fig. 4.15 B). Surprisingly, and in contrast to the results of *in vitro* work, the suppressive effect of *F. hepatica* ES administration was mirrored by the high molecular weight fraction (ES-H). Interestingly ES-L marginally delayed disease onset and reduced the weight loss but the effect was much less pronounced than that observed with ES-H or *F. hepatica* ES.

Mice were sacrificed when untreated mice with EAE reached the peak of clinical symptoms (day 12). Analysis of the numbers of cells infiltrating the CNS revealed that suppression of autoimmunity was associated with considerable reduction in numbers of cells recovered from brains of mice treated with *F. hepatica* ES and ES-H (Fig. 4.16 A). To establish how administration of *F. hepatica* ES, ES-H and ES-L influence cytokine production by infiltrating CD4⁺ T cells, intracellular FACS staining was performed on recovered brain cells. The result demonstrates that *F. hepatica* ES and ES-H but not ES-L significantly reduced frequency of IL-17 and IFN- γ producing CD4⁺ T cells in the CNS during EAE. As a result of reduced leukocyte infiltration and low frequencies of IL-17 and IFN- γ producing CD4⁺ T cells absolute numbers of pathogenic T cells were markedly reduced in the brains of ES or ES-H treated mice when compared to control EAE mice (Fig. 4.16 C and D). Treatment with ES-L also reduced the numbers of infiltrating IL-17 and IFN- γ producing CD4⁺ T cells, however the effect was less pronounced than that observed with ES or ES-H.

Taken together, this data demonstrates that immunosuppressive factors responsible for the attenuation of EAE by *F. hepatica* ES segregate to high molecular fraction (ES-H).

4.3 Discussion

The current study has demonstrated that *F. hepatica* ES components, when separated on the basis of molecular size, segregated into two fractions with distinct immunomodulatory activities. The fraction containing low molecular weight components (ES-L) displayed potent immunosuppressive effect on activation of DCs and T cells but failed to attenuate EAE. In contrast the high molecular weight fraction (ES-H) did not display immunomodulatory properties *in vitro* but was a potent suppressor of autoimmunity.

The present study demonstrated that inhibition of maturation and cytokine production by DCs by *F. hepatica* ES is mediated by a small compound(s) with a molecular weight of less than 3 kDa. It was also established that inhibition of IL-12p70 and induction of TGF- β is mediated by a factor that is not a peptide since peptide-depleted ES-L retained its immunomodulatory activity. These findings are consistent with a growing body of literature describing parasite derived small molecules such as carbohydrates, glycolipids and lipids that are able to modulate immune responses (van der Kleij and Yazdanbakhsh, 2003). In particular lipid-based components including, schistosome-specific lysophosphatidylserine (van der Kleij et al., 2002) and phosphorylcholine moiety from *A. vitae* ES products (Goodridge et al., 2003) have been shown to directly inhibit production of IL-12p70 by DCs or macrophages. Small non-protein immunomodulators, including eicosanoids, such as PGE₂, have the capacity to inhibit production of IL-12p70 by monocytes and DCs. In fact it has been reported that *F. hepatica* expresses enzymes required to synthesize prostaglandins and is able to produce PGI₂ and PGE₂ (Ali et al., 1999).

In contrast to the inhibition of IL-12p70, this is one of the first studies to report that parasite products can directly induce secretion of TGF- β from DCs. Since attenuation of EAE by *F. hepatica* ES may partially be mediated through TGF- β , this study undertook more detailed investigation into the mechanism of TGF- β release from DCs. Rapid secretion of TGF- β induced by *F. hepatica* ES does not involve transcription or translation of *tgfb1* gene and therefore the increased concentration of TGF- β observed in the supernatants resulted from a

release of pre-formed TGF- β . Since TGF- β plays critical role in many biological processes its production is tightly controlled. TGF- β is secreted in an inactive form as a complex with latency associated peptide (LAP) that prevents this cytokine from binding to its receptors and exerting its function. Activation of the cytokine requires the release of TGF- β from LAP through various known mechanisms that disrupt this interaction. Notably many cell types, including DCs (Gandhi et al., 2007) secrete TGF- β associated with LAP as a cell surface bound complex. Little is known about anchorage of the TGF- β /LAP to the cell membranes of immune cells and on the role of soluble latent TGF- β in regulation of immunity. Nevertheless, the biology of TGF- β has been extensively studied in the context of wound healing and tissue remodelling. It has been shown that fibroblasts, chondrocytes and endothelial cells can produce TGF- β covalently bound through LAP to one of latent TGF- β -binding proteins (LTBPs), which associate with the cell membrane or the extracellular matrix (Koli et al., 2001). It has been proposed that liberation of latent TGF- β complex requires proteolytic cleavage of LTBP and this constitutes an important step in the activation cascade, allowing soluble latent TGF- β complexes to be available for further processing (Maeda et al., 2002; Saharinen et al., 1999). Serine proteases, such as plasmin (Pedrozo et al., 1999) and elastase (Taipale et al., 1995), as well as matrix metalloproteinases (Tatti et al., 2008) have been implicated in this process. The present study demonstrated that TGF- β produced by DCs in response to ES-L is released in a latent form, therefore it is possible that ES-L triggers a mechanism that allows dissociation of latent TGF- β /LAP complexes from the cell surface. This finding and the fact that human and mouse macrophages (Khalil et al., 2001; Squadrito et al., 2012) were reported to express LTBP-1 suggests that ES-L may perhaps activate DC-derived proteases, that could in turn release latent TGF- β . Figure 4.7A demonstrates that pre-incubation of DCs with a protease inhibitor cocktail reduced ES-L induced-TGF- β release from DCs suggesting that proteases may indeed be involved in this process. A broad panel of specific inhibitors will be required to identify the class of proteases responsible for liberation of TGF- β /LAP. Membrane type 1-matrix metalloproteinases, which are expressed by DCs (MT1-MMP) (Yang et al., 2006), but not sensitive to inhibition with GM6001 could possibly mediate this process. The result suggests that ES-L

could be a useful tool to study production and biology of DC-derived TGF- β , as conventional PAMPs do not induce expression of this cytokine and relatively little is known about the activation or the role of latent soluble TGF- β in the regulation of immunity.

T cell hyporesponsiveness is a common feature of many parasitic infections. This study has demonstrated that suppression of anti-CD3 induced T cell activation *in vitro* is associated with factors segregating to the low molecular fraction of *F. hepatica* ES. In fact ES-L was a more potent inhibitor of proliferation and cytokine production by T cells than unfractionated *F. hepatica* ES while ES-H modestly promoted cytokine production. The suppressive effect of *F. hepatica* ES and ES-L was directly associated with inhibition of IL-2 and failure to up-regulate CD25, both necessary for T cell growth and expansion. Although defects in proliferation of T cells isolated from parasite-infected mice have been described, there is a paucity of reports identifying parasite-derived small molecules that could directly affect T cell function *in vitro* or *in vivo*. A study by Deehan *et al.* has characterised *A. vitae* derived glycoprotein ES-62 that was able to disrupt signalling downstream from BCR thus inhibit B cell activation. The phosphorylcholine moiety of ES-62 was critical for this inhibition and free phosphorylcholine was able to mimic the effect (Deehan *et al.*, 1998). The same group of researchers described inhibition of anti-CD3 induced activation of Jurkat T cell line, but failed to reproduce this effect in primary murine T cells.

Importantly this study has demonstrated that ES-L was also able to inhibit T cell activation and IL-17 production induced by innate cytokines independently of TCR engagement. In contrast to the polyclonal activation with anti-CD3, stimulation with IL-1 β and IL-23 induces rapid production of IL-17 and IFN- γ from memory CD4⁺ T cells and innate lymphocytes such as NK cells or $\gamma\delta$ T cells but not from naive CD4⁺ T cells (Sutton *et al.*, 2009).

$\gamma\delta$ T cells constitute a part of early defence system against bacterial (Shibata *et al.*, 2007) and fungal (Dejima *et al.*, 2011) infections. These innate lymphocytes constitutively express receptors for IL-1 and IL-23 as well as a

signature transcription factor of Th17 cells ROR γ t (Sutton et al., 2009). Consequently, $\gamma\delta$ T cells are predisposed to rapidly produce IL-17 in response to IL-1 β and IL-23 secreted by innate cells, such as DCs and macrophages activated by infection. Thus $\gamma\delta$ T cells contribute to IL-17-dependent neutrophil recruitment and pathogen clearance (Lockhart et al., 2006; Shibata et al., 2007). Apart from their role in infection, $\gamma\delta$ T cells have been implicated in the development of autoimmune diseases such as EAE (Spahn et al., 1999) and CIA (Roark et al., 2007) where IL-17 mediates pathology.

Previous work from our laboratory showed that CD3⁺ cells stimulated *in vitro* with a combination of IL-1 β and IL-23 produce high concentrations of IL-17 and $\gamma\delta$ T cells are the main source of this cytokine. Stimulation with IL-1 β and IL-23 enhances expression of ROR γ t and IL-23R by $\gamma\delta$ T cells and further stabilise their effector function (Sutton et al., 2009). The present study demonstrated that *F. hepatica* ES contains low molecular weight components that can inhibit IL-1 β and IL-23 induced secretion of IL-17 and IFN- γ from spleen cells. Moreover parasite products appeared to act directly on $\gamma\delta$ T cells preventing up-regulation of ROR γ t, IL-1R, IL-23R and suppress production of IL-17 and IFN- γ . Administration of IL-1 β and IL-23 can elicit *in vivo* production of IL-17 which is detectable by RT-PCR in draining lymph nodes as early as 4 h after injection (Sutton et al., 2009). Notably expression of IL-17 was reduced when low molecular weight fraction of *F. hepatica* ES was co-administered with IL-1 β and IL-23. FACS analysis confirmed that $\gamma\delta$ T cells were main producers of IL-17 post IL-1 β and IL-23 stimulation. Strikingly, administration of ES-L together with IL-1 β and IL-23 reduced frequency of IL-17 producing $\gamma\delta$ T cells by 50%, formally demonstrating that parasite products can inhibit innate activation of $\gamma\delta$ T cells *in vivo*. Given the importance of innate IL-17 in supporting pathogen clearance (Shibata et al., 2007) the suppression of $\gamma\delta$ T cells could partially explain impaired ability of the parasite bearing host to control bacterial co-infection (Brady et al., 1999; Chen et al., 2005). Inhibition of $\gamma\delta$ T cell-derived cytokines may constitute yet another strategy contributing to the state of systemic hyporesponsiveness which is a feature of many parasite infections.

Despite the fact that it exerted potent immunosuppressive effects on DCs and T cells *in vitro*, the low molecular fraction of *F. hepatica* ES was not effective in attenuating EAE. Unexpectedly, it appeared that components responsible for the observed suppression of autoimmunity were retained in high molecular fraction of *F. hepatica* ES. Treatment of mice with ES-H, similar to unfractionated *F. hepatica* ES, delayed onset and reduced the incidence of EAE. Infiltration of immune cells into the brain is vital for the initiation and development of neuropathology associated with MS and EAE. Blocking migration of lymphocytes to CNS by administration of antibodies to VLA-4 – an integrin involved in transmigration across the blood brain barrier significantly suppressed the severity of EAE (Yednock et al., 1992). Interestingly, treatment with *F. hepatica* ES and ES-H reduced absolute numbers of cells recovered from brains of mice with EAE. In addition the frequency of IL-17- or IFN- γ -producing CD4⁺ T cells among infiltrating cells was significantly lower than that from untreated mice. This resulted in a dramatic reduction in the number of pathogenic CD4⁺ T cells in brains of mice treated with ES-H. ES-L also had only a modest suppressive effect on the disease score at the early stages of EAE and was able to decrease numbers of CNS infiltrating cells. However when compared with untreated mice, the reduction was not as prominent as that induced by ES-H or *F. hepatica* ES. Moreover ES-L had no influence on the frequency of IL-17 and IFN- γ producing CD4⁺ T cells in the brains of treated mice. This observation demonstrated that the capacity of low molecular weight components to modulate DC and T cell responses *in vitro* does not directly translate into suppression of adaptive immunity *in vivo*. These results suggest that induction of TGF- β and inhibition of DC and T cell responses either is not as dramatic *in vivo* or does not result in suppression of EAE. On the other hand the high molecular weight fraction that did not display any obvious suppressive effects when tested *in vitro*, with the exception of weak inhibition of IL-12p70, contains components capable of potentially attenuating autoimmunity. This discrepancy between simple *in vitro* systems and *in vivo* model highlights the importance of animal studies for identification and evaluation of novel immunomodulatory factors. The summary of immunomodulatory effects of ES-L and ES-H on activation of immune cells and attenuation of EAE is depicted in the table below.

	ES-T	ES-L	ES-H
DCs			
IL-12	↓↓↓	↓↓↓	↓
IL-23	↓↓↓	↓↓↓	—
IL-6	↓↓↓	↓↓↓	↑
TGF-β	↑↑↑	↑↑↑	—
maturation	↓↓↓	↓↓↓	—
T cells (αCD3)			
CD25/CD69	↓↓↓	↓↓	—
proliferation	↓↓↓	↓↓↓	—
IL-2	↓↓	↓↓↓	—
IL-17	↓↓	↓↓↓	—
IFN-γ	↓	↓↓↓	↑
T cells (IL-1/IL-23)			
IL-17	↓↓	↓↓↓	—
IFN-γ	↓↓	↓↓↓	↑
EAE			
active	↓↓↓	↓	↓↓↓

Table 4.1 Summary of immunomodulatory effects of ES, ES-L and ES-H.

↑ -increase; ↓- decrease; — - no change

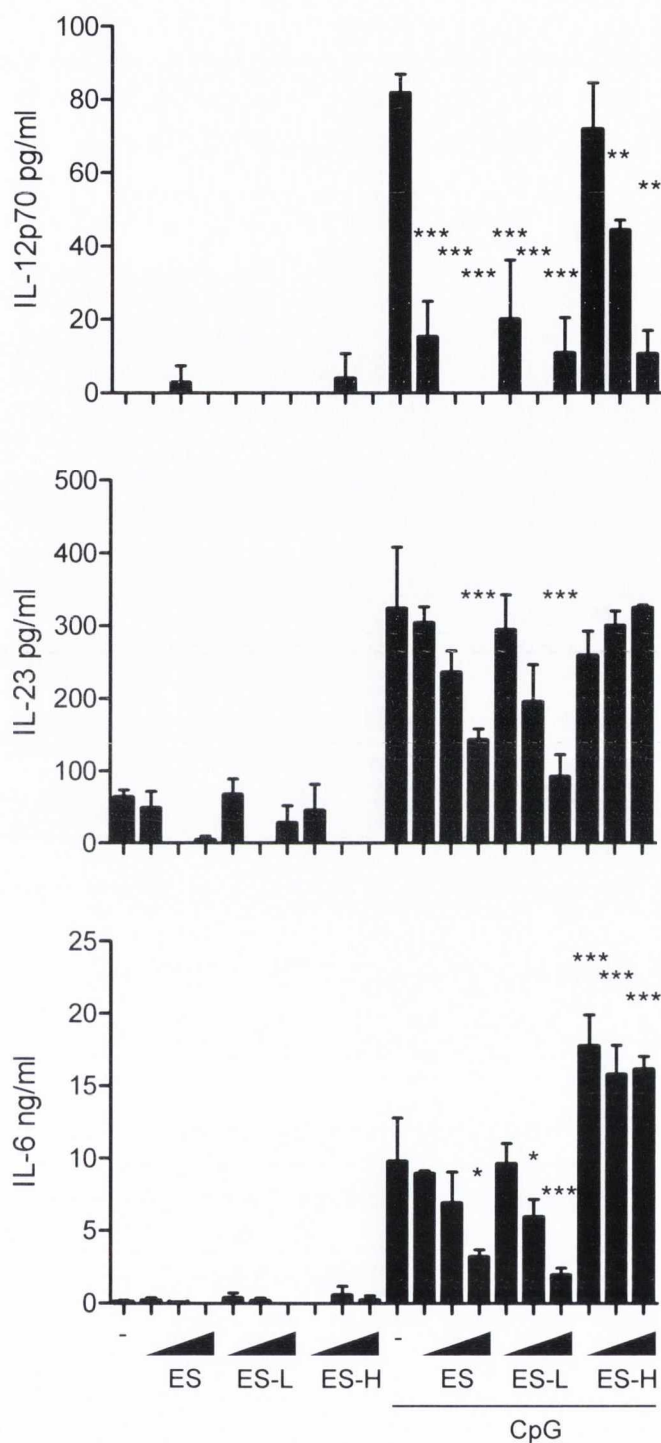


Fig. 4.1 Immunomodulatory activity of *F. hepatica* ES segregates to a fraction containing molecules smaller than 3 kDa (ES-L). DCs from C3H/HeJ mice were incubated with a range of *F. hepatica* ES concentrations (1.25 – 5% v/v) or equivalent volume of fractions containing molecules higher or lower than 3 kDa (ES-H and ES-L respectively) alone or followed by CpG (5 µg/ml). After 24 h concentration of IL-12p70 (A), IL-23 (B), and IL-6 (C) in supernatants was determined by ELISA. Results are means (+/- SD) of triplicate assay. * P<0.05; ** P<0.01; *** P<0.001 vs. untreated control. Results are representative of 2 independent experiments that gave similar results.

Gated on CD11c⁺ cells:

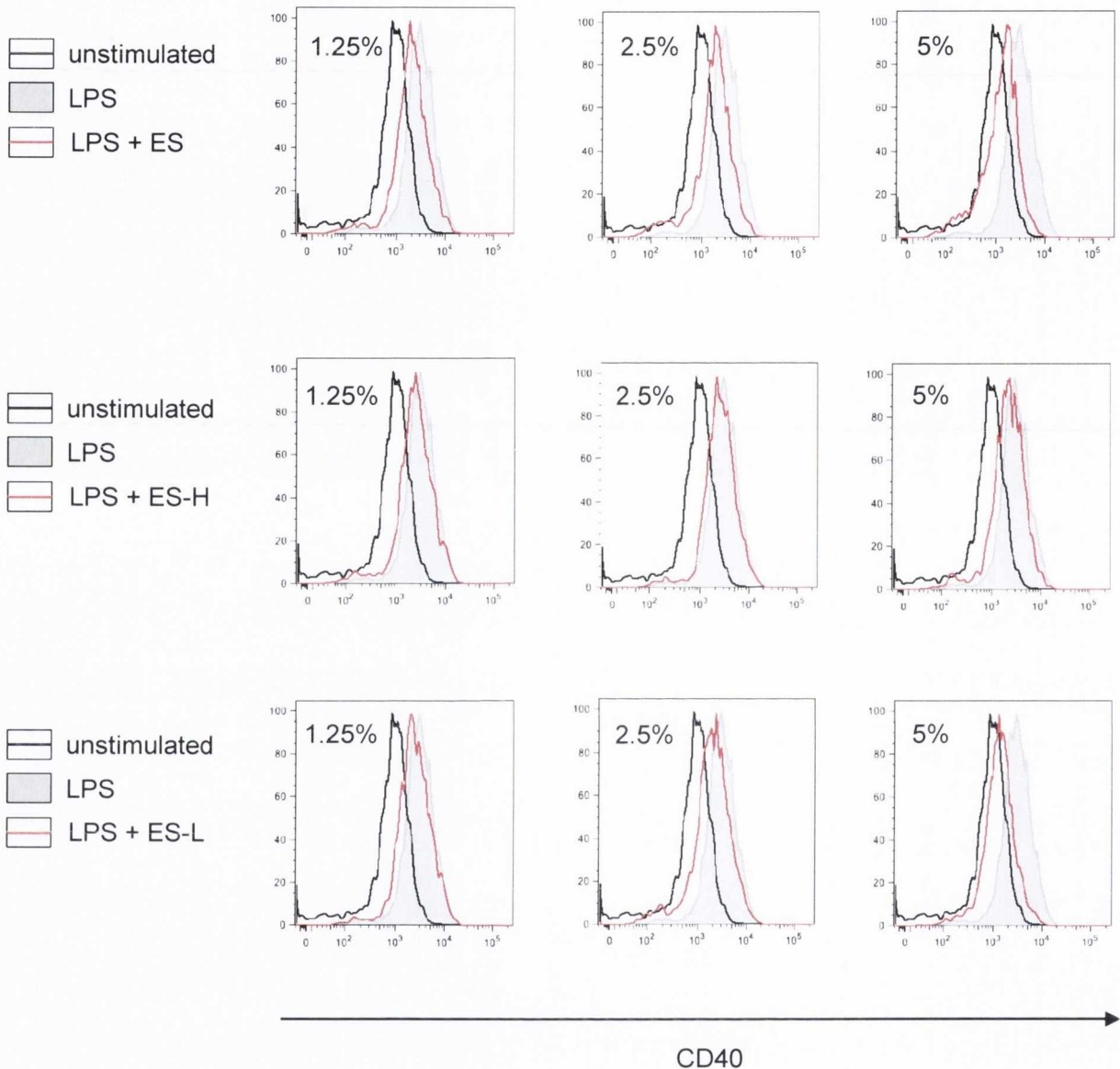


Fig. 4.2 ES-L inhibits LPS induced expression of CD40 on DCs. DCs derived from C57BL/6 mice were pretreated with *F. hepatica* ES, ES-H or ES-L as indicated (1.25-5% v/v) for 2 h prior to stimulation with LPS (100 ng/ml). After 24 h cells were recovered, stained with antibodies against CD11c and CD40 and the expression of CD40 on CD11c⁺ cells was analysed by flow cytometry.

Gated on CD11c⁺ cells:

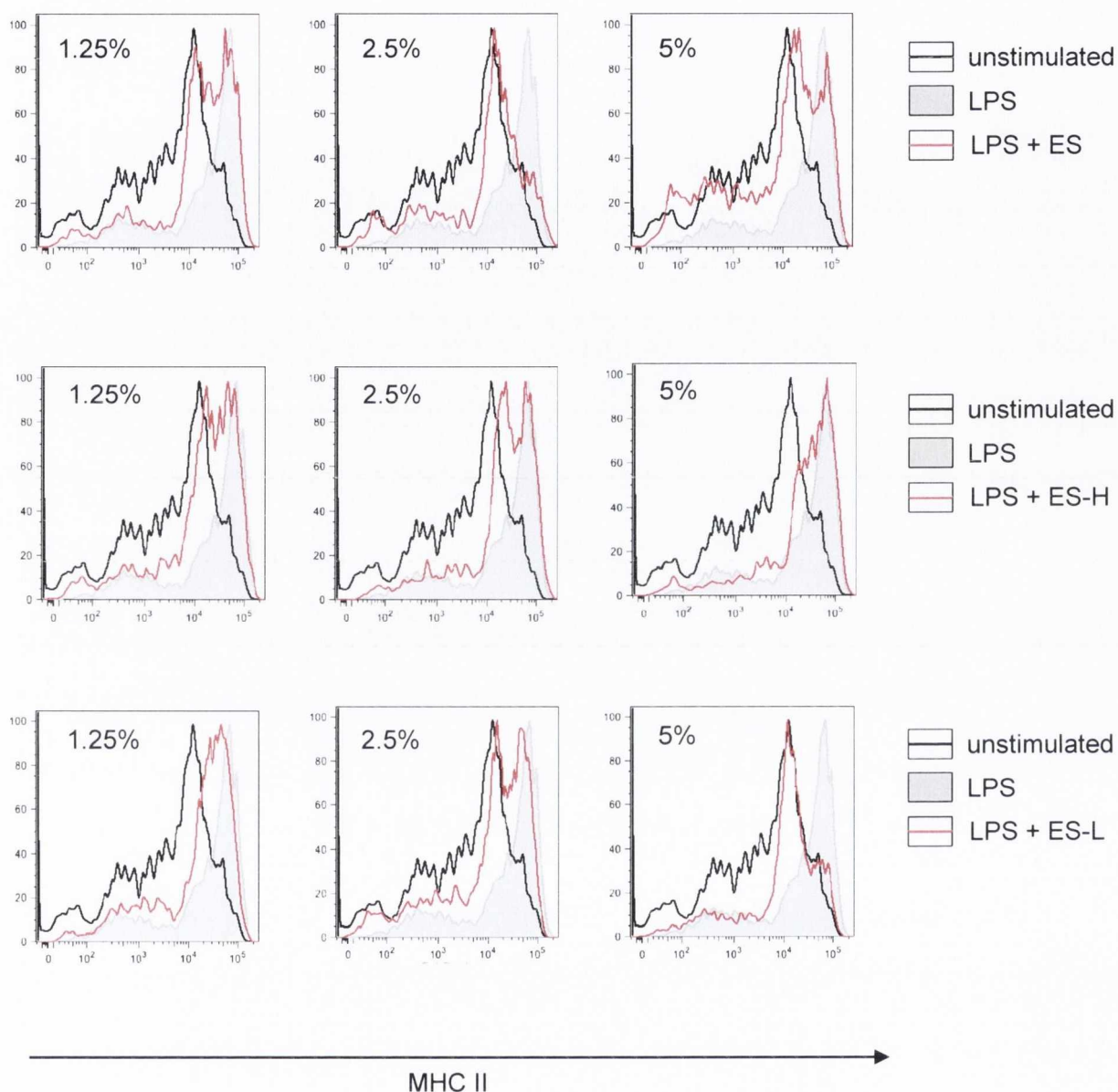


Fig. 4.3 ES-L inhibits LPS induced expression of MHC II on DCs. DCs derived from C57BL/6 mice were pre-treated with *F. hepatica* ES, ES-H or ES-L as indicated (2.5-5% v/v) for 2 h prior to stimulation with LPS (100 ng/ml). After 24 h cells were recovered, stained with antibodies against CD11c and MHC II and the expression of MHC II on CD11c⁺ cells was analysed by flow cytometry.

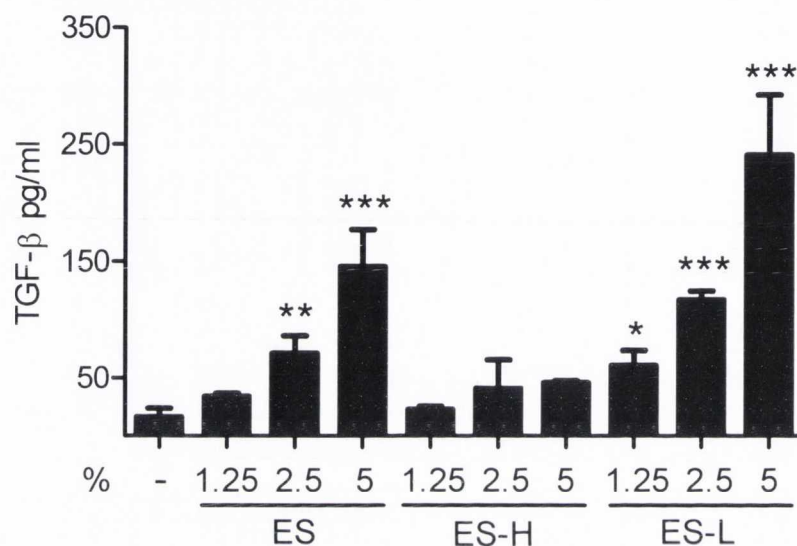


Fig. 4.4 TGF- β inducing activity of *F. hepatica* ES segregates to the fraction containing molecules smaller than 3 kDa. DCs from C57BL/6 mice were incubated with a range of *F. hepatica* ES concentrations (1.25 – 5% v/v) or equivalent volume of fractions ES-L and ES-H as indicated. After 18 h concentration of TGF- β in supernatants was determined by ELISA. Results are means ($\pm$ SD) of triplicate assay. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ vs. untreated control. Results are representative of at least 3 independent experiments.

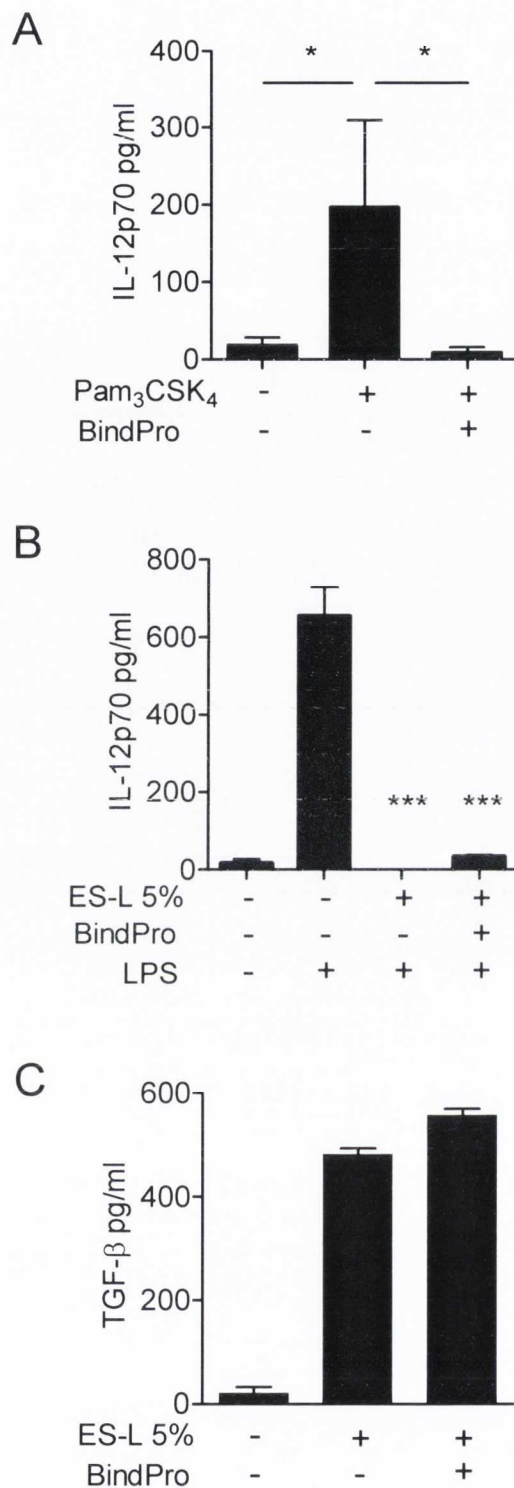


Fig. 4.5 Immunomodulatory factor from ES-L is not a peptide/protein component. Bone marrow derived DCs were incubated with Pam₃CSK₄ (100 ng/ml) or Pam₃CSK that was pre-treated with polymeric peptide/protein removal reagent BindPro (A), 5% ES-L or BindPro pre-treated ES-L followed by LPS (B) or 5% ES-L or BindPro pre-treated ES-L alone. After 24 h concentration of IL-12p70 (A and B) and TGF- β (C) in supernatants was measured by ELISA. Results are means ($\pm$ SD) of triplicate assay. * $P < 0.05$. vs Pam₃CSK₄; *** $P < 0.001$ vs LPS.

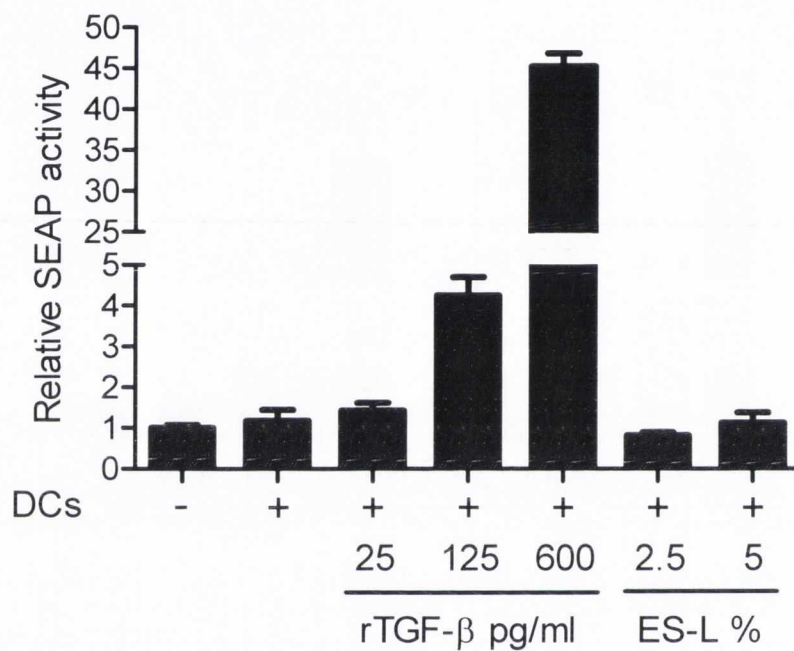


Fig. 4.6 ES-L induces release of TGF- β in a latent form. MFB-11 reporter cells were cultured alone or with DCs in serum free medium and treated with active recombinant TGF- β or ES-L at the indicated concentrations. After 48 h activity of soluble alkaline phosphatase (SEAP) in supernatants was measured by chemoluminescence based assay.

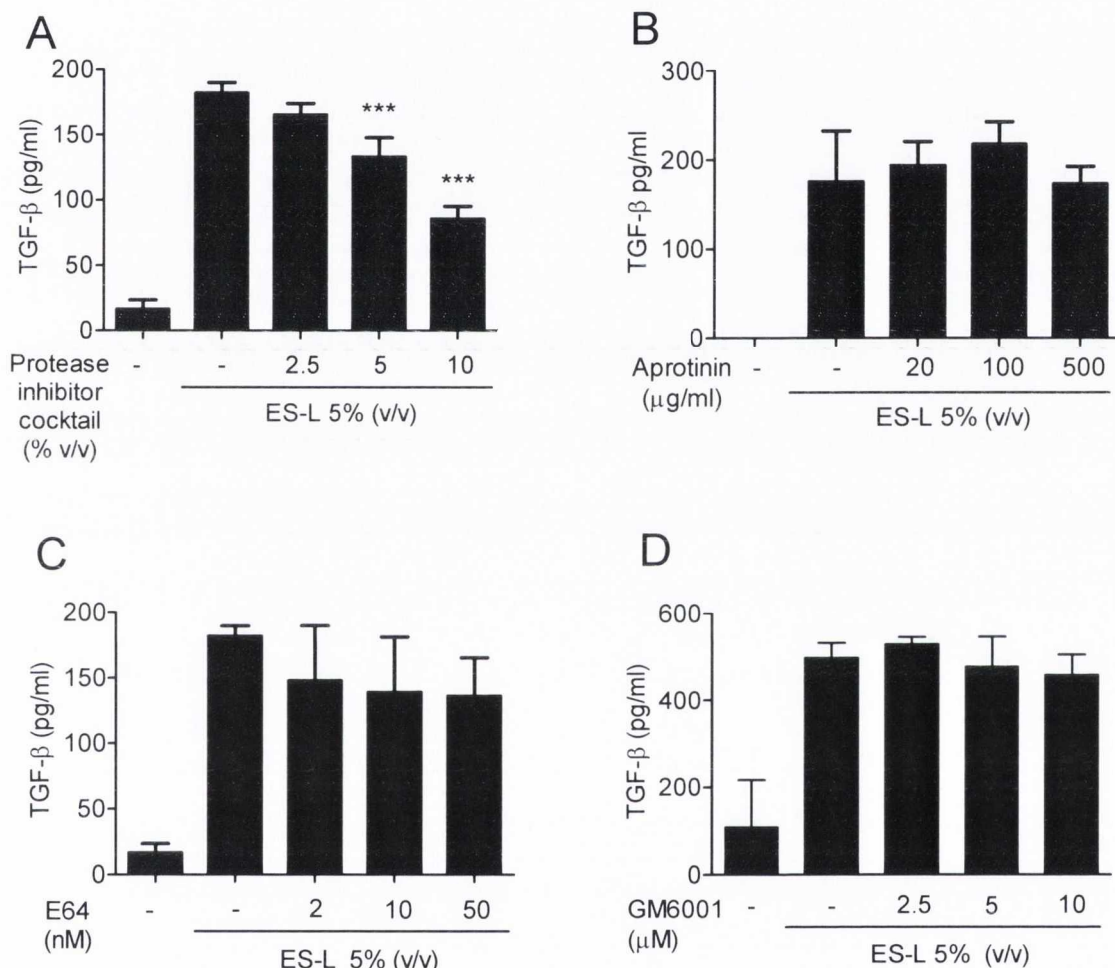


Fig. 4.7. ES-L-induced release of TGF- β from DCs is mediated by DC-derived proteases. DCs from C57BL/6 mice were preincubated with a range of concentrations of universal protease inhibitor cocktail (A), serine protease inhibitor aprotinin (B), cysteine protease inhibitor E64 (C) or matrix metalloprotease inhibitor GM6001 (D) as indicated for 30 min prior to treatment with ES-L. After 18 h concentration of TGF- β in supernatants was determined by ELISA. Results are means ($\pm$ SD) of triplicate assay. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. 5% ES-L without inhibitors. Result is representative of two experiments performed.

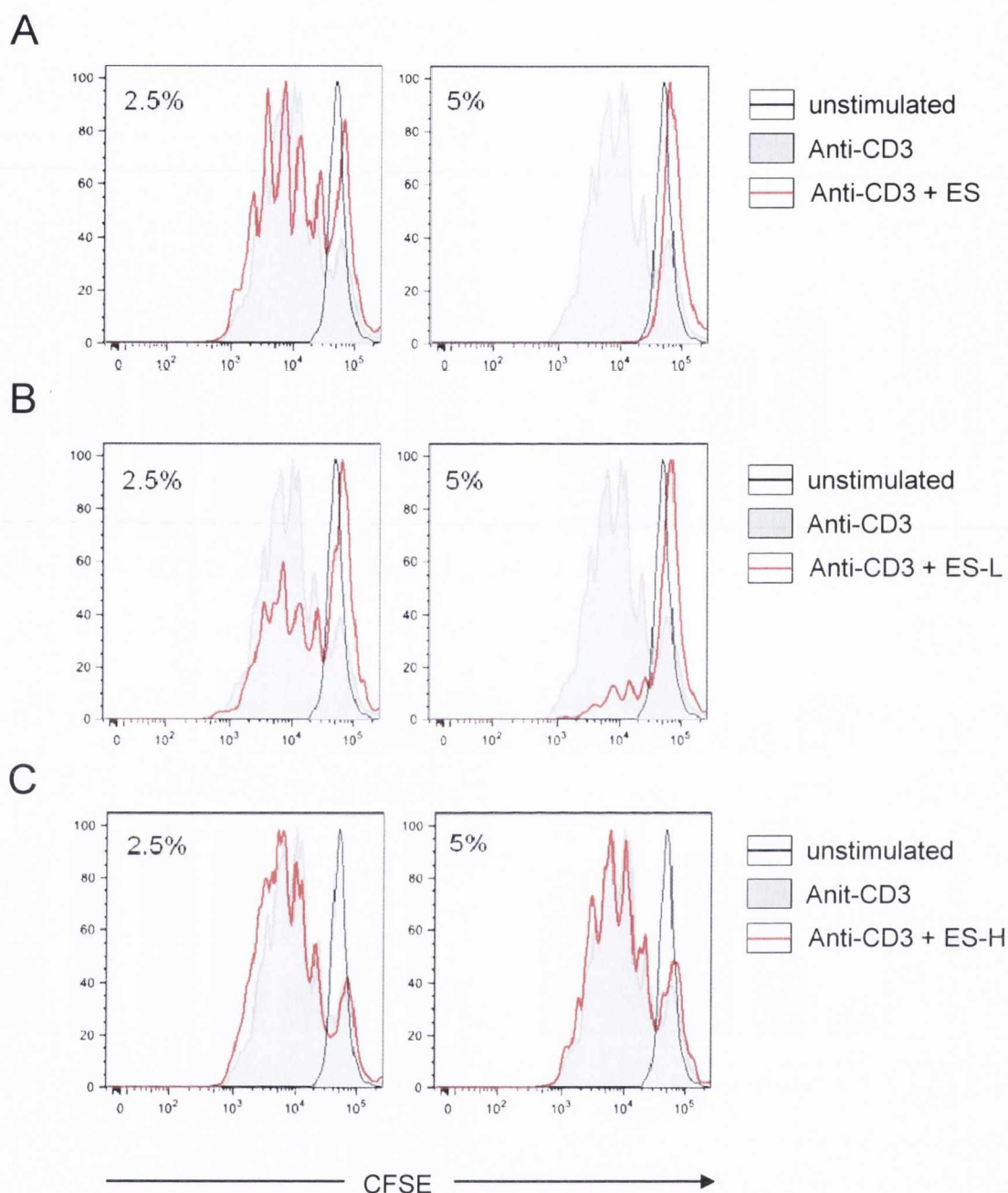


Fig. 4.8 Effect of ES-L and ES-H treatment on T cell proliferation. Naive spleen cells were CFSE labeled, stimulated with anti-CD3e (0.25 μ g/ml) antibody and treated with 2.5% or 5% v/v *F. hepatica* ES, ES-L or ES-H fractions. Cells were harvested after 72 h, and CFSE dilution on CD4⁺ T cells was analysed by flow cytometry.

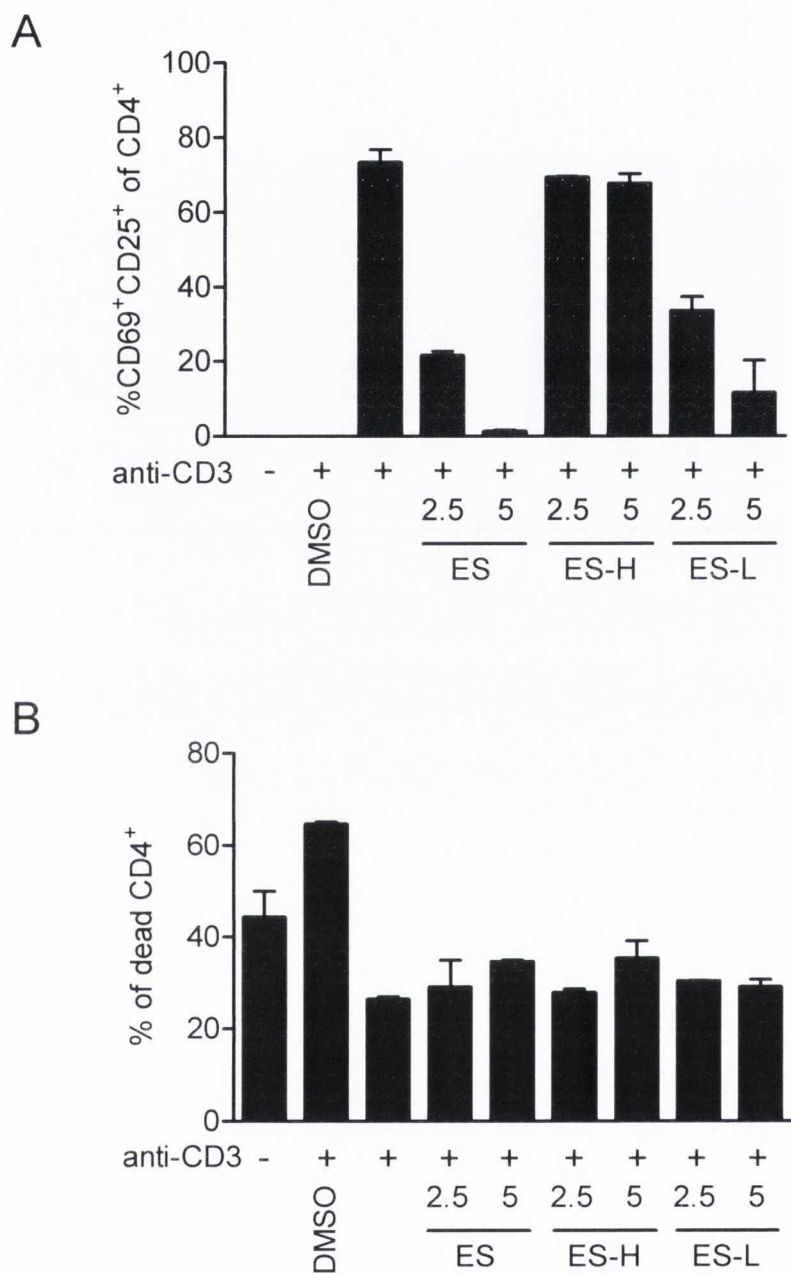


Fig. 4.9 ES-L inhibits early activation of anti-CD3 stimulated T cells. Spleen cells from naïve mice were stimulated for 12 h with anti-CD3e. Cells were stained with fluorochrome conjugated antibodies against CD4, CD69 and CD25 (A). Staining with 7-AAD was employed to assess cell death (B).

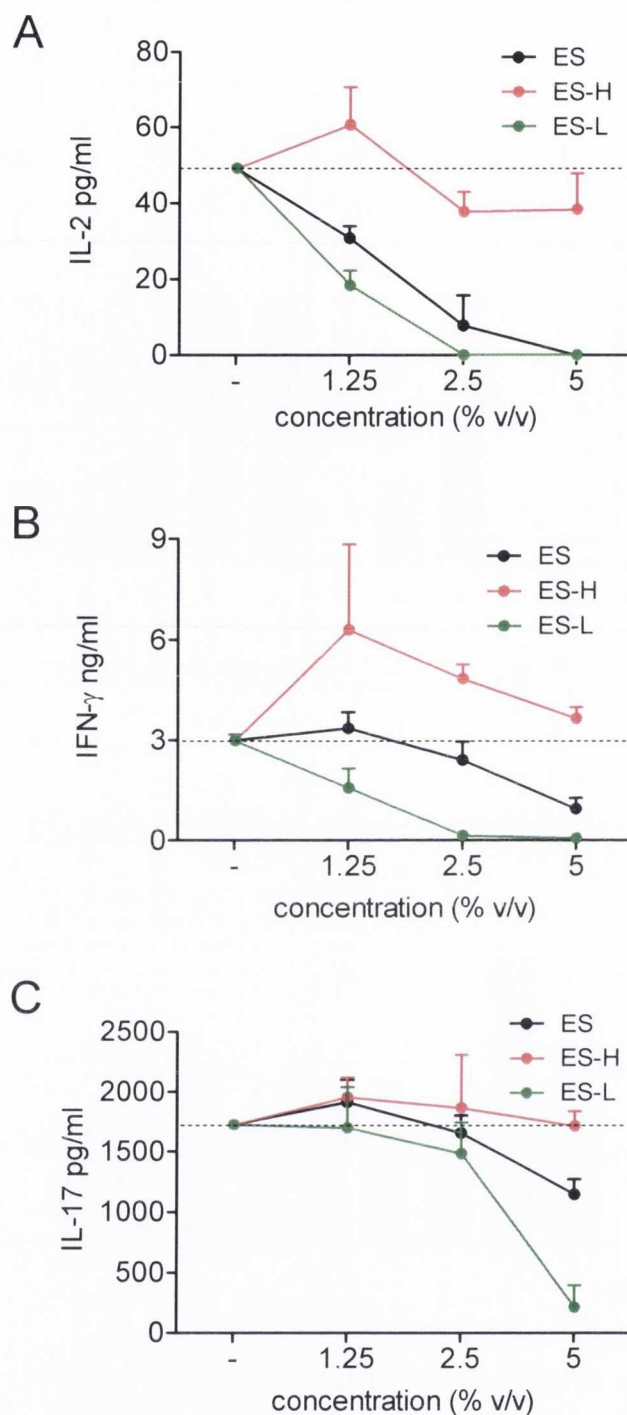


Fig. 4.10 ES-L inhibits anti-CD3 induced IL-2, IFN- γ and IL-17 production by spleen cells. Spleen cells from naive mouse were stimulated with anti-CD3 (0.25 μ g/ml) and treated with *F. hepatica* ES, ES-L and ES-H fractions at a range of concentrations. Concentration of IL-2 (A) was measured in supernatants after 18 h and IL-17 (B) and IFN- γ (C) after 72 h by ELISA. Results shown are quantified from triplicate samples and are representative of at least 3 independent experiments.

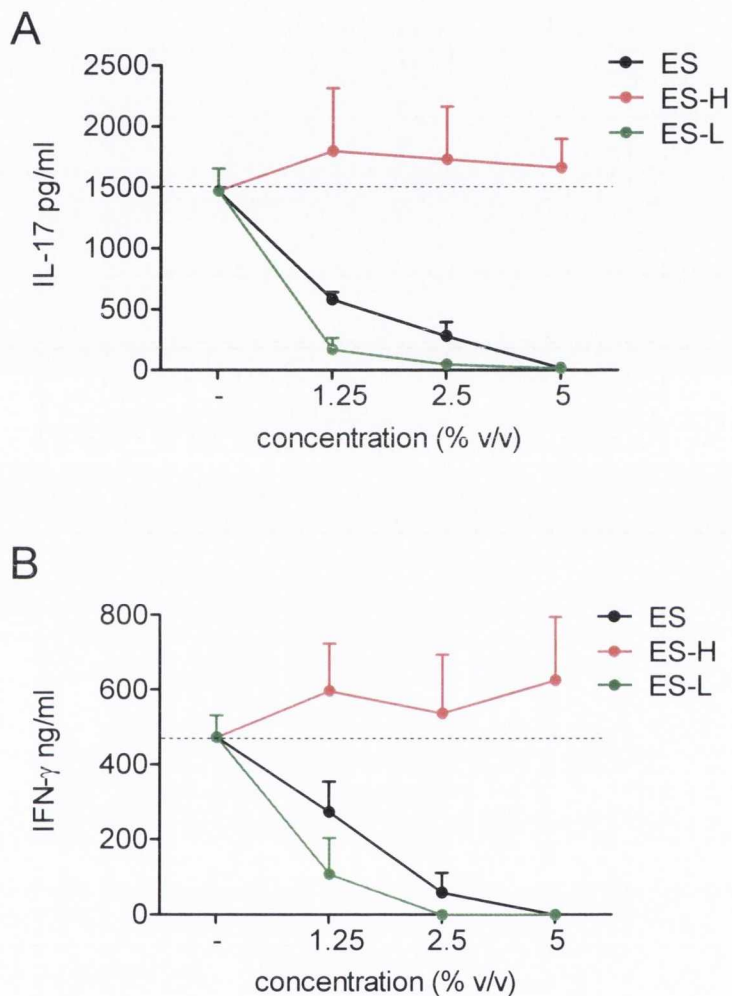


Fig. 4.11 ES-L inhibits IL-1 β and IL-23 induced production of IL-17 and IFN- γ by spleen cells. Spleen cells from naïve C57BL/6 mouse were incubated with IL-1 β (10 ng/ml) and IL-23 (10 ng/ml) and with a range of concentrations of *F. hepatica* ES, ES-L and ES-H as indicated. Supernatants were collected after 72 h to determine concentration of IL-17 and IFN- γ by ELISA. Results are means ($\pm$ SD) of triplicate assay.

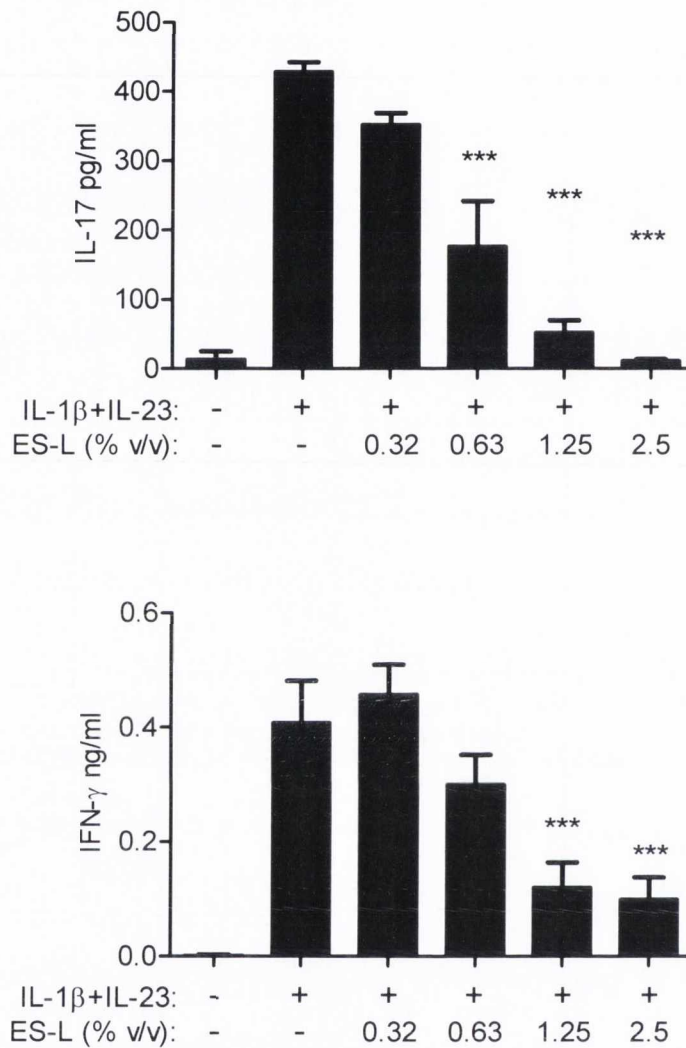


Fig. 4.12 ES-L inhibits IL-1 β and IL-23 induced activation and cytokine production by purified $\gamma\delta$ T cells. Purified $\gamma\delta$ T cells from naïve C57BL/6 mouse were incubated with IL-1 β (10 ng/ml) and IL-23 (10 ng/ml) and with a range of concentrations of ES-L as indicated. Supernatants were collected after 72 h to determine concentration of IL-17 and IFN- γ by ELISA * P<0.05; ** P<0.01; *** P<0.001 vs. IL-1 β and IL-23. Results are means (+/- SD) of triplicate assay.

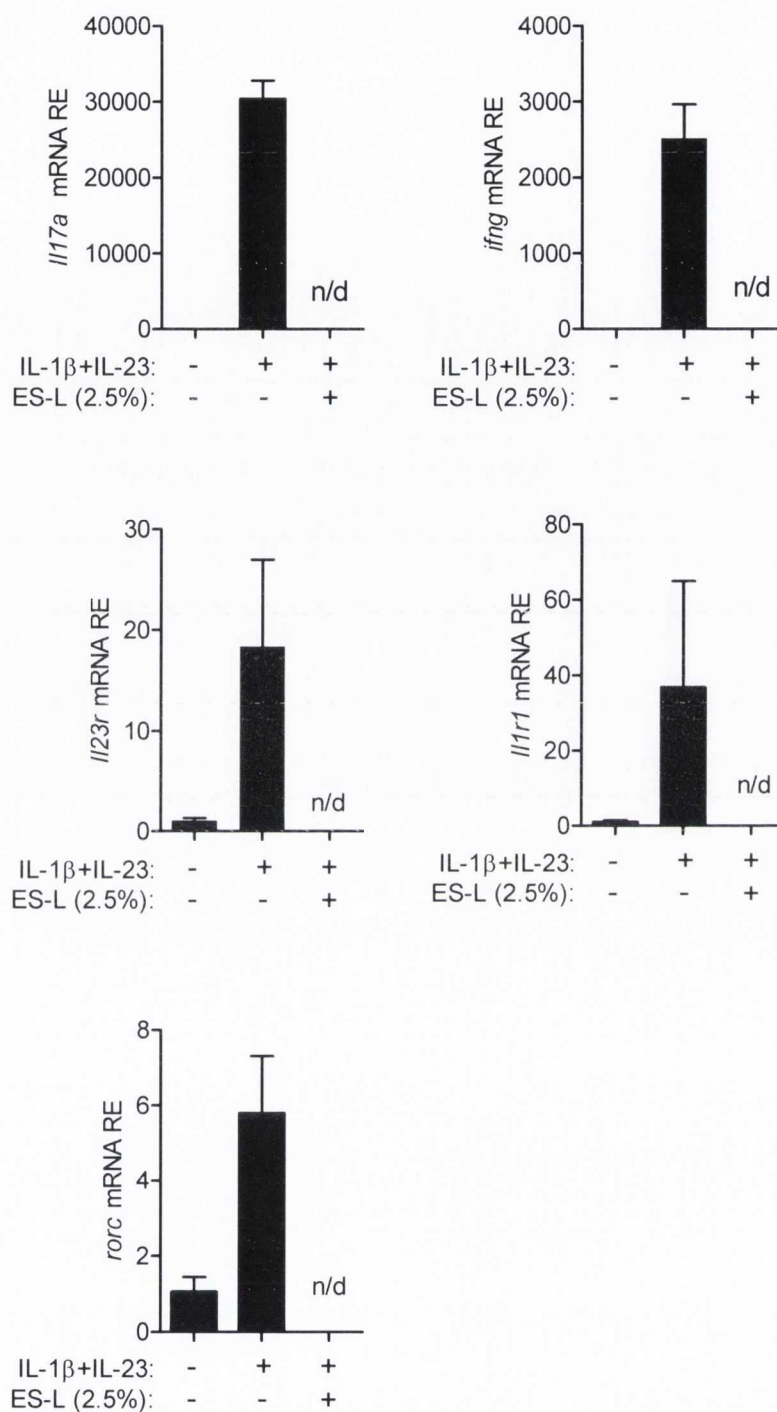


Fig. 4.13 ES-L inhibits IL-1 β and IL-23 induced gene expression by purified $\gamma\delta$ T cells. Purified $\gamma\delta$ T cells from naïve C57BL/6 mouse were incubated with IL-1 β (10 ng/ml) and IL-23 (10 ng/ml) with or without ES-L. After 72 h cells were lysed, total RNA was extracted and expression of *il17a*, *ifng*, *il23r*, *il1r* and *rorc* was evaluated by real-time PCR normalised to endogenous control, 18S rRNA and relative to unstimulated cells. n/d – not detected. Results are means (+/- SD) of triplicate assay.

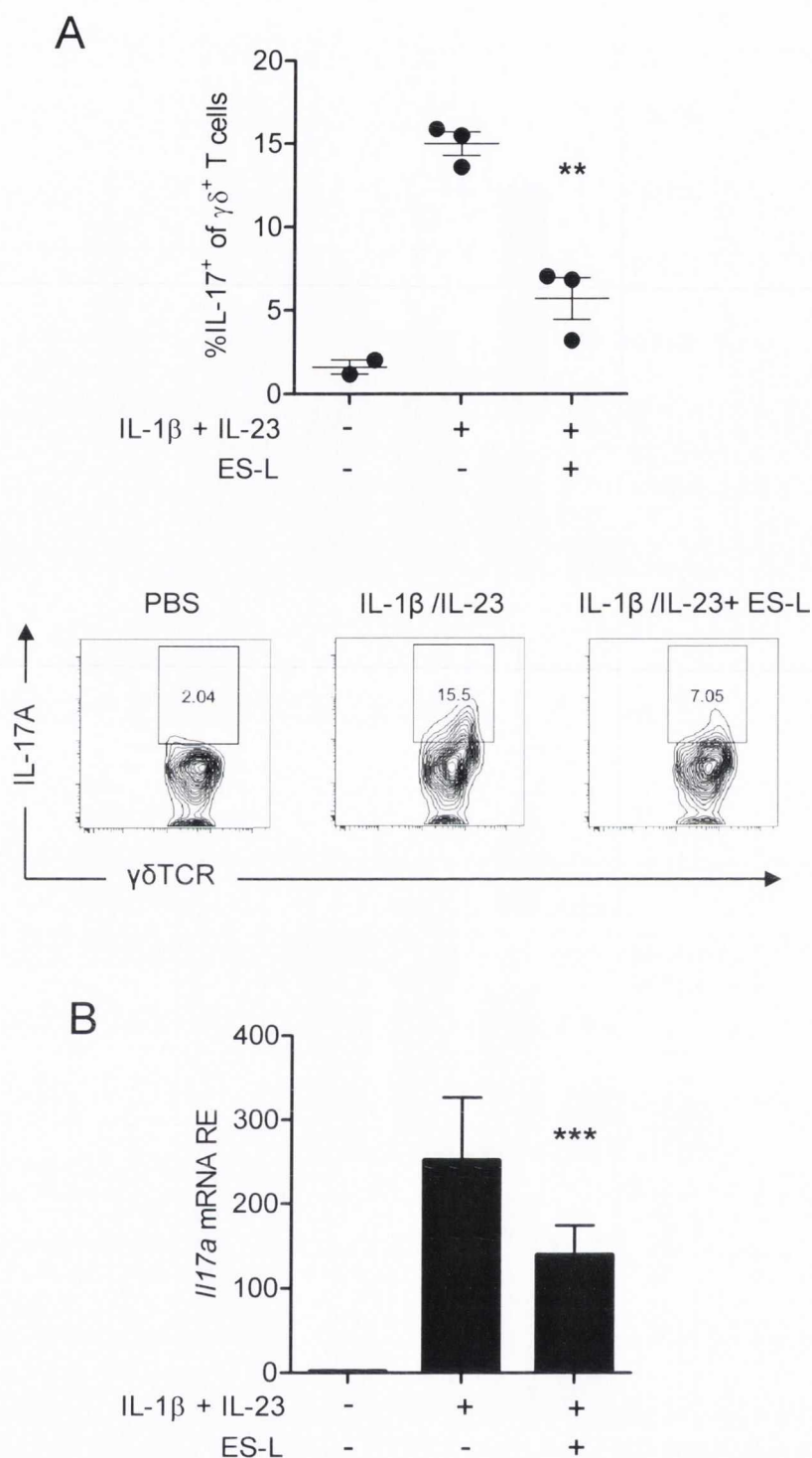


Fig. 4.14 ES-L inhibits IL-1 β and IL-23 induced production of IL-17 by $\gamma\delta$ T cells *in vivo*. C57BL/6 mice were injected s.c. into footpad with PBS, or IL-1 β and IL-23 (100 ng/mouse) with or without ES-L (40 μ l/mouse). Draining lymph nodes (popliteal) were harvested 4 h later. A) Cells were incubated for 4 h with brefeldin A and stained with antibodies against CD3, $\gamma\delta$ TCR and IL-17. Results are shown as frequencies of IL-17 producing CD3⁺ $\gamma\delta$ TCR⁺ cells with representative dot plots. B) Sample of total unpurified draining lymph node cells was collected from each animal to evaluate expression of *il17a* mRNA by real-time PCR 4 h after injection of cytokine cocktail and ES-L. Results are shown as mean (+/-) SD from triplicate assay. ** P<0.01; *** P<0.001 vs. IL-1 β and IL-23.

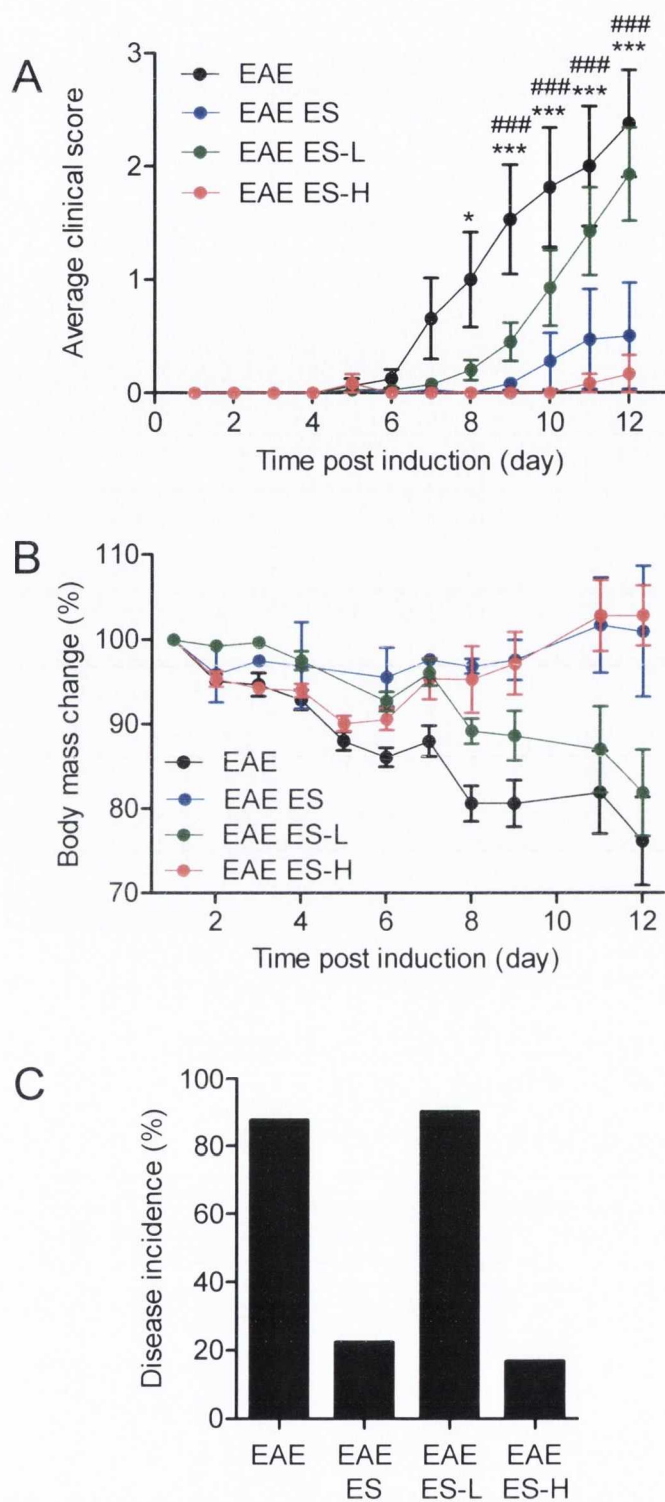


Fig. 4.15 High molecular weight fraction of ES products (ES-H) attenuates EAE. EAE was induced in C57BL/6 mice by immunisation with MOG emulsified in CFA. Mice were injected i.p with PBS, 40 μ l of *F. hepatica* ES, ES-L or ES-H on days -1, 2 and every second day thereafter. A) Animals were monitored daily for clinical signs of the disease and B) weight loss. C) Disease incidence was assessed at day 12. Results shown are from 2 independent experiments, mean score $\pm$ SEM for 6 – 10 mice per group. *** $P < 0.001$ ES vs. Control EAE group; ### $P < 0.001$ ES-H vs. Control EAE.

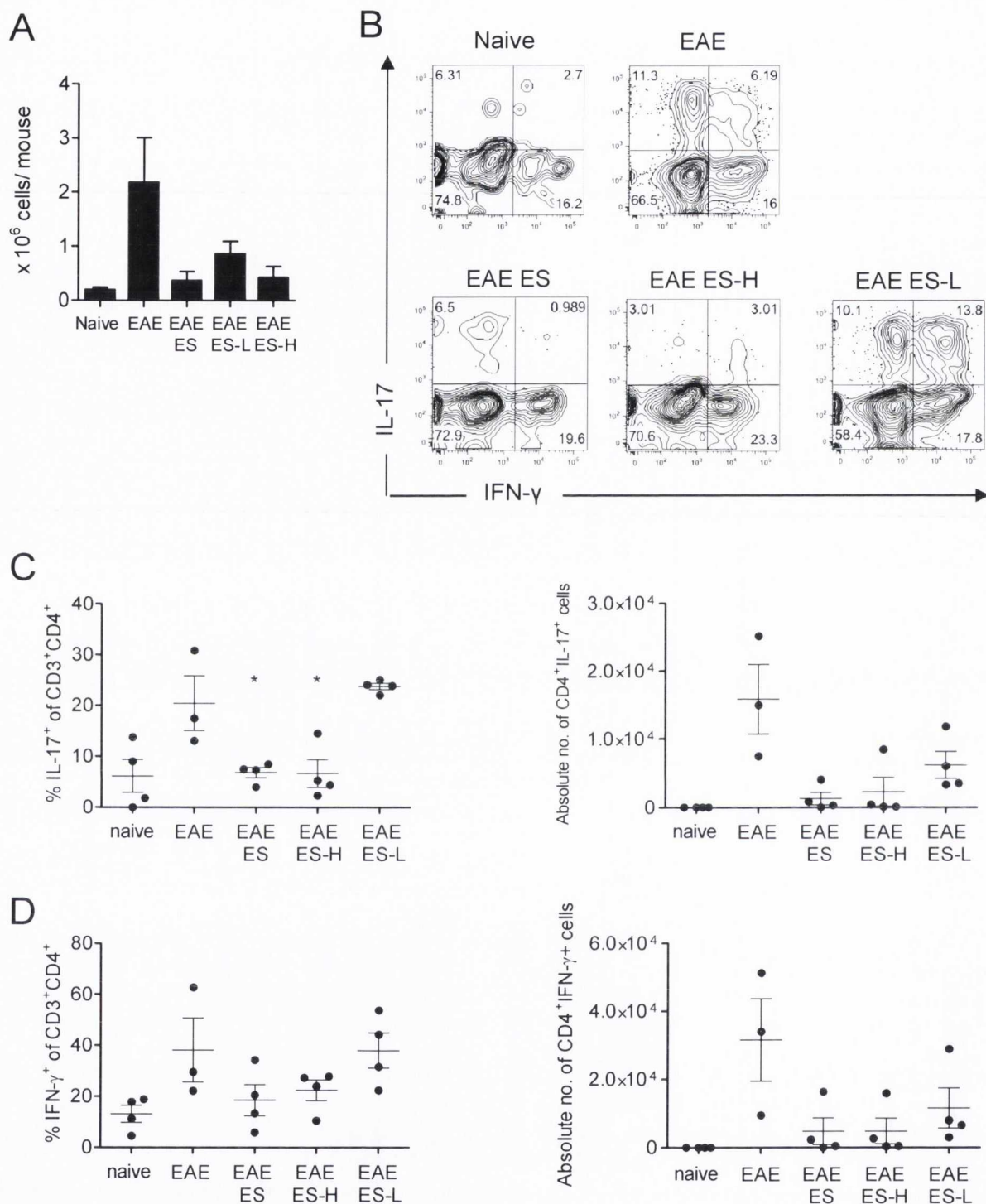


Fig. 4.16 Treatment with *F. hepatica* ES and ES-H reduces the frequency of IL-17 and IFN- γ producing CD4⁺ cells in CNS during EAE. EAE was induced in C57BL/6 mice by immunisation with MOG emulsified in CFA. Animals were injected i.p. with PBS, 40 μ l of *F. hepatica* ES, ES-L or ES-H on days -1, 2 and every second day thereafter. Infiltrating cells were recovered from brains (3-5 per group) on day 13 and counted (A). The frequency of IL-17 and IFN- γ producing CD4⁺ cells was analysed by flow cytometry. Results are representative dot plots (B) for each experimental group with the mean frequency and absolute numbers ($\pm$ SEM) of IL-17 (C) or IFN- γ (D) producing cells shown as the scatter plot where each data point represents an individual mouse. * $P < 0.05$ vs EAE.

***The effect of ES-H administration
on the immune responses during
EAE***

Chapter 5

5.1 Introduction.

It has been demonstrated that immunoregulation associated with helminth infection may attenuate clinical symptoms of inflammatory and autoimmune diseases, however, mechanisms of bystander suppression are dependent on the disease model and parasitic species. EAE is an organ specific autoimmune disease involving nervous system that shares pathological features with MS. Therefore EAE is often used as a model to study immunology of MS and evaluate the efficacy of potential therapies.

EAE is induced in mice by immunisation with defined myelin derived peptides, emulsified in complete Freud's adjuvant followed by administration of pertussis toxin (PT) on day 0 and 2. Depending on the specific mouse strain and antigen used the disease may take acute, chronic or relapsing-remitting course. C57BL/6 mice are susceptible to EAE and when immunised with MOG develop an acute monophasic disease characterised by progressive paralysis. Leukocyte recruitment to the CNS is crucial for the initiation and progression of EAE. It is believed that at the induction of EAE myelin-specific T cells must be first activated in the periphery to gain entry to the CNS as T cells must express specific adhesion molecules and chemokine receptors to cross the blood brain barrier (BBB). CNS infiltrating cells are reactivated by resident APCs presenting self antigen and can then trigger the production of chemokines and other mediators that disrupt blood-brain barrier and allow secondary influx of other inflammatory cells. Chemokine production in the brain is critical for the process of leukocyte recruitment and neutralisation of certain chemotactic proteins can attenuate or prevent EAE (Dogan and Karpus, 2004). The mechanisms of T cell migration are also targeted by certain therapeutics used for the management of MS. Natalizumab, a monoclonal antibody against VLA-4 integrin, blocks leukocyte adhesion to the inflamed epithelium thus prevents entry of activated T cells into the CNS. The treatment was developed using the EAE model (Yednock et al., 1992) and is currently the most potent licensed drug for MS, reducing annual relapse rate by up to 70% (Saidha et al., 2012). A small molecule inhibitor of sphingosine-1-phosphate receptor, fingolimod, also prevents lymphocyte recruitment to sites of inflammation by blocking

lymphocyte egress to the circulation from secondary lymphoid organs and has been recently approved as a therapy for RR-MS (Kappos et al., 2010).

IL-17 has recently emerged as a critical cytokine in T cell mediated autoimmune disease. Produced by Th17 cells and some subsets of $\gamma\delta$ T cells, IL-17 acts on various cells to induce expression of IL-1, TNF α and IL-6, upregulate a number of chemokines and matrix metalloproteinases that all contribute to the pathology in EAE (McGeachy and Cua, 2008). Importantly, IL-17 secreting T cells have been detected in CSF of MS patients. Th17 cells are also involved in the pathogenesis of a number of other models of autoimmune disease and symptoms of CIA (Nakae et al., 2003) colitis (Fujino et al., 2003), or experimental myocarditis (Rangachari et al., 2006) can be attenuated following depletion of or knockout of IL-17. The role of IFN- γ in the development of EAE is unclear. IFN- $\gamma^{-/-}$ mice are susceptible to EAE and in fact develop more severe disease than wild type mice, but IFN- γ -producing Th1 cells are capable of inducing EAE upon adoptive transfer (Billiau et al., 1988; Kroenke et al., 2008). IFN- γ signalling can suppress the development of Th17 cells (Park et al., 2005) and it has been suggested that IFN- γ may function to limit IL-17-dependent pathology during EAE (Kelchtermans et al., 2008).

It has been suggested that cytokines produced by parasite-induced Th2 cells can contribute to the protection against autoimmunity. Attenuation of EAE by immunisation with *S. masoni* ova was associated with increased frequency of IL-4 producing neuroantigen-specific T cells in the brain and with a reduction in frequency of pathogenic IFN- γ -producing T cells. Interestingly, the protective effect induced by helminth infection was lost in Th2 deficient STAT6 $^{-/-}$ mice suggesting that these cells play a crucial role in attenuation of EAE by *S. masoni* products (Sewell et al., 2003).

The immunosuppression induced by helminth infection can be associated with an expansion of Foxp3 $^{+}$ Treg cells. For instance, attenuation of OVA induced airway hypersensitivity by infection with *H. polygyrus* resulted in substantial increase in frequency of CD25 $^{\text{high}}$ CD4 $^{+}$ Tregs and upon adoptive transfer into uninfected, OVA challenged mice, these cells protected from the

symptoms of allergy (Wilson et al., 2005). Similarly, infection with *F. hepatica* induced the expansion of Foxp3⁺ Treg cells (Walsh et al., 2009).

The current study evaluated the effect of ES-H on the leukocyte recruitment into the CNS and cytokine production during EAE in order to better understand immune responses associated with the attenuation of the disease. Moreover, fractionation and mass spectrometry based analysis was performed in an attempt to identify putative ES-H derived immunomodulatory components.

5.2 Results

5.2.1 Administration of ES-H attenuates EAE

EAE was induced in C57BL/6 mice by s.c. injection of MOG emulsified in CFA, with PT administered i.p. on days 0 and 2. A control group of mice was injected with PBS emulsified in CFA with PT. Mice were treated with ES-H or PBS (40 µl) on day -1 and every second day after throughout the course of the disease. Animals were monitored daily for clinical symptoms of the disease (Fig. 5.1 A). Mice immunised with MOG and CFA started to show early signs of paralysis from day 6 post induction and 90% of them developed mild to severe EAE by day 14. By contrast, administration of ES-H markedly delayed onset of the clinical symptoms and reduced the incidence of the disease to 40%. This resulted in significant decrease ($p < 0.001$) in the average clinical score when compared to mice treated with PBS. None of control mice that were immunised with PBS and CFA without MOG developed any clinical symptoms. The general condition of the animals was assessed on the basis of changes in the average body mass (Fig. 5.1 B). Mice immunised with MOG and CFA showed gradual weight loss throughout the course of the experiment. Treatment with ES-H prevented weight loss reflecting the less severe disease outcome.

5.2.2 Treatment with ES-H reduces infiltration of immune cells into the CNS during EAE

In order to investigate immunological events associated with attenuation of EAE by ES-H, mice were sacrificed at the onset of the disease and at the peak of clinical symptoms 7 and 14 days after immunisation respectively (3-4 mice/group/time point). Brains removed from perfused animals were processed and subjected to a density gradient separation to recover infiltrating cells. Similarly, spleens and draining lymph nodes (inguinal) were also removed and examined in parallel with the brains. Naive mice were included in the experiment to provide a baseline for the analysis of cytokine production by selected populations of T cells.

The recruitment of immune cells into the CNS is one of the pathological features of EAE and is necessary for the disease development (Yednock et al.,

1992). Having shown that ES-H significantly attenuates EAE it was important to evaluate the effect of ES-H treatment on the degree of leukocyte infiltration over the course of the disease. 7 days after the induction of EAE total numbers of infiltrating cells in the brains of immunised mice were markedly elevated when compared to naive animals. Mice immunised with MOG and CFA or PBS and CFA had comparable numbers of infiltrating immune cells in their brains while treatment with ES-H significantly reduced cell infiltration levels (Fig. 5.2 A). On day 14, the numbers of cells recovered from brains of mice immunised with PBS and CFA or MOG and CFA and treated with ES-H dropped to the baseline levels in striking contrast with a massive infiltration observed in brains of mice immunised with MOG and CFA. At the peak of the clinical symptoms, the total number of cells recovered from CNS positively correlated with severity of the disease (Fig. 5.2 B), providing indirect evidence of the importance of leukocyte infiltration in the disease pathology.

Neutrophils are among the first leukocytes to infiltrate CNS in some models of EAE and it has been suggested that these cells facilitate further influx of immune cells to the CNS through their involvement in blood-brain barrier breakdown (Carlson et al., 2008; Veldhuis et al., 2003). To evaluate the effect of treatment with ES-H on neutrophil infiltration into the CNS during EAE, cells recovered from brains of experimental mice 7 and 14 days after the induction of the disease were stained for CD45, CD11b and GR-1. The result revealed that at day 7, neutrophils constituted approximately 30% of infiltrating leukocytes in the brains of mice immunised with MOG and CFA or PBS and CFA in contrast to just 1-2% in brains from naive mice. Treatment with ES-H significantly reduced the proportion of these cells to 17% on day 7. However, on day 14 there was no difference in the frequency of CD11b⁺GR-1⁺ cells in the CNS of ES-H treated and untreated mice with EAE (Fig. 5.3).

CD3⁺ T cells constituted nearly 3.5% of recovered live cells in brains of naive mice. There was only a little increase in frequency of these cells 7 days after the immunisation with PBS/CFA or MOG/CFA, but by day 14 the

frequency of CD3⁺ T cells in the CNS markedly increased in all three groups when compared to naive mice. Although there was no difference in frequency of CD3⁺ T cells between the groups, the immunisation significantly increased total numbers of CD3⁺ T cells infiltrating CNS at day 7 from 4×10^3 cells recovered from naive brains to approximately 8×10^4 in mice immunised with PBS and CFA or MOG and CFA (Fig. 5.4 A). Treatment with ES-H reduced the number of infiltrating CD3⁺ T cells to 3×10^4 . At day 14 there was a further increase in numbers of CD3⁺ T cells in brains of MOG and CFA immunised mice which at this stage had developed severe EAE. This was in a stark contrast with the total numbers of CD3⁺ T cells from ES-H treated mice ($P < 0.01$) that remained at the level observed at day 7. The number of CD3⁺ T cells in brains of control mice immunised with PBS and CFA dropped from 8×10^4 to 4.5×10^4 cells. A similar trend was observed in frequency and absolute numbers of CD4⁺ T cells.

5.2.3 Treatment of mice with ES-H increases absolute numbers of lymphocytes in draining lymph nodes and spleens

EAE is a model of organ specific autoimmune disease affecting CNS tissue but since the induction of MOG specific responses involves subcutaneous injection of MOG peptide into the flank of susceptible mice, the initiation of immune responses occurs in the inguinal lymph nodes. Analysis of T-cell populations in the spleen can also provide important information regarding immune responses in the periphery during EAE.

Immunisation with MOG and CFA or PBS and CFA did not affect total numbers of cells in lymph nodes or spleens at day 7, however a small increase in the lymph nodes was observed on day 14 after the induction of EAE. In contrast treatment with ES-H significantly increased the number of cells in lymph nodes (Fig. 5.5 B) and to a lesser extent in spleens (Fig. 5.5 C) at both time points. Remarkably spleens and lymph nodes of mice treated with ES-H were clearly enlarged in comparison these from untreated mice (Fig. 5.5 A).

CD3⁺ cells constituted 70% of all lymphocytes (gated by SSC and FSC) in inguinal lymph nodes of naive mice (Fig 5.6 A). Immunisation with MOG and CFA resulted in a moderate decrease in the frequency of CD3⁺ cells and treatment with ES-H transiently enhanced this effect on day 7. Despite the

changes in the frequency, the actual absolute numbers of CD3⁺ cells increased during the course of EAE and this was more pronounced in ES-H treated mice. There were no apparent changes in the frequency of CD4⁺ T cells following the induction of EAE but the treatment with ES-H resulted in substantially increased absolute numbers of CD4⁺ T cells in the lymph nodes when compared to untreated mice with EAE (Fig 5.6 B).

5.2.4 Lower frequency and absolute numbers of IL-17 and IFN- γ producing CD4⁺ T cells in CNS of ES-H-treated mice

Immunisation with both PBS and CFA or MOG and CFA resulted in 2-3 fold increase in the frequency of IL-17 producing CD4⁺ cells in CNS at day 7. The percentage of CD4⁺ T cells further increased with the progression of EAE to 20% at day 14 (Fig. 5.7). Treatment with ES-H resulted in striking reduction in frequency of IL-17-producing CD4⁺ T cells in CNS; at both time points the frequency remained at a level comparable to that found in brains of naive mice. Interestingly in control PBS and CFA immunised mice, which did not develop EAE, the frequency of these cells did not change by day 14. The absolute numbers of IL-17-producing T cells in the CNS were found to be directly associated with the disease outcome and increased over time in brains of mice immunised with MOG and CFA. Administration of ES-H resulted in a reduction in absolute numbers of IL-17⁺CD4⁺ cells by 5 and 10 fold on days 7 and 14 respectively. Control mice immunised with PBS and CFA had transiently elevated numbers of IL-17 producing CD4⁺ cells 7 days after the immunisation but these significantly dropped by day 14.

Immunisation with PBS and CFA or MOG and CFA did not result in any increase in the frequency of IFN- γ ⁺CD4⁺ cells over that observed in the brains of naive mice at day 7 but the frequency of these cells increased by day 14 in mice that developed EAE. Interestingly, treatment with ES-H transiently elevated the percentage of IFN- γ -producing cells 7 days after immunisation, but at day 14 the frequency of these cells was significantly lower when compared to untreated EAE mice. Similarly absolute numbers of IFN- γ producing CD4⁺ cells were markedly reduced in brains of ES-H treated mice at both time points (Fig. 5.7).

Analysis of cytokine production by CD8⁺ T cells in CNS revealed that CD8⁺ cells isolated from naive animals produced IFN- γ but not IL-17 (Fig. 5.8). The frequency of IL-17⁺CD8⁺ cells in CNS was elevated 7 days after immunisation with MOG and CFA or PBS and CFA and stayed at the same level until day 14. The increase in frequency of IFN- γ ⁺CD8⁺ was only noticeable on day 14. While there was some variation in percentages of IL-17- and IFN- γ -producing CD8⁺ cells, no differences between the three different experimental groups were apparent at any time point examined. The absolute numbers of IL-17⁺CD8⁺ in brains of mice with EAE increased with the progression of the disease and on day 14 approximately 1.5×10^4 IL-17⁺CD8⁺ T cells were detected in the CNS. Treatment with ES-H significantly ($P < 0.001$) reduced the numbers of IL-17⁺CD8⁺ cells. Similarly, the absolute numbers of IFN- γ -producing CD8⁺ cells were also lower in CNS of ES-H treated mice when compared to untreated mice immunised with MOG and CFA (Fig. 5.8). Collectively the data demonstrate that treatment with ES-H significantly reduces the absolute numbers of IL-17 and IFN- γ producing CD4⁺ and CD8⁺ T cells in the brains of mice with EAE.

The flow cytometry based examination of IL-17 and IFN- γ production by T cells was complemented by the analysis of gene expression. Total RNA was purified from CNS tissue of naive and immunised mice and changes in transcription of *il17a* and *ifng* were measured by RT-PCR (Fig. 5.9). Expression of IL-17 mRNA was significantly up-regulated in brains of mice with EAE at the onset of the disease, declining thereafter but remaining elevated 150 fold over baseline levels at the peak (Fig. 5.9 A). Treatment with ES-H suppressed the expression of IL-17 mRNA on day 7 and completely abolished it by day 14. 7 days after immunisation expression of IFN- γ mRNA in the CNS of mice immunised with MOG and CFA was significantly higher than that observed in naive mice and continued to increase as the disease progressed. The CNS tissue of ES-H treated mice was found to express low, but stable, levels of IFN- γ throughout the disease but these levels were significantly reduced in comparison to untreated mice with EAE (Fig. 5.9 B). Brain tissue of control mice immunised with PBS and CFA expressed high levels of IL-17 mRNA at day 7, but the transcripts were undetectable by day 14 (Fig. 5.9 A). Similar to ES-H treated mice, CNS tissue from PBS and CFA immunised mice expressed low levels of IFN- γ on day 7 and 14 (Fig. 5.9 B).

5.2.5 Higher frequency and absolute numbers of IFN- γ producing cells in draining lymph nodes of ES-H treated mice

The frequency of IL-17 producing CD4⁺ T cells increased moderately in the lymph nodes of mice with EAE 7 days following immunisation with MOG and CFA or CFA only (1.25% vs. 0.5% in naive mice) but was much lower than that observed in CNS at the same time point (Fig. 5.10 B). Treatment with ES-H resulted in a moderate reduction in the frequency but not absolute numbers of IL-17⁺CD4⁺ cells at day 7. At day 14 due to variation in percentages of IL-17 producing CD4⁺ cells no differences between the immunised groups were apparent.

Treatment with ES-H increased the frequency of IFN- γ producing CD4⁺ (Fig. 5.10 B) and CD8⁺ (Fig. 5.10 C) T cells at both time points when compared to untreated mice with EAE. This was reflected by a substantial increase in the absolute numbers of IFN- γ -producing T cells in the lymph nodes of ES-H treated mice, especially at day 14.

5.2.6 Administration of ES-H increases the frequency and absolute numbers of IL-4 and IL-13 producing CD4⁺ T cells in the CNS and in the periphery

The induction of Th2 type responses is a hallmark of helminth infection. Since Th1 and Th2 subsets have been reported to mutually suppress the development and function of each other it was important to investigate the effect of ES-H on Th2 responses during EAE.

IL-4 producing CD4⁺ cells constituted approximately 0.5% of all CD4⁺ T cells found in CNS of naive mice. Immunisation with CFA or MOG and CFA did not increase the frequency of these cells above the levels seen in naive mice. The treatment with ES-H expanded the population of IL-4-producing T cells to 1.1% on day 7 and to 4.8% by day 14, which represented a significant ($P < 0.01$) 8 fold increase over the frequency observed in untreated EAE mice (Fig. 5.11). Similarly, the frequency of IL-13-producing CD4⁺ T cells was much higher in the brains of ES-H treated mice when compared with untreated control on day 14 (5.9 vs. 0.25 %; $P < 0.001$). The increase in frequency of IL-4- and IL-13-producing cells was reflected by elevated absolute numbers of these cells in

brains of ES-H treated mice. Despite lower numbers of total infiltrating lymphocytes, mice treated with ES-H had more IL-4- and IL-13-producing cells in CNS than untreated mice with EAE (Fig. 5.11).

This expansion of IL-4⁺CD4⁺ T cell population was also observed in draining lymph nodes of ES-H-treated mice (Fig. 5.12). The increase in frequency of these cells was more pronounced than in the CNS and statistically significant at both time points ($P<0.05$ and $P<0.001$). Although there was no apparent difference in percentages of IL-13-producing CD4⁺ cells in the lymph nodes on day 7, administration of ES-H induced a significant increase in the frequency of this cells on day 14. The increase in frequency of IL-4- and IL-13-producing cells observed in periphery was reflected by a significant increase in absolute numbers of these cells.

5.2.7 Administration of ES-H inhibits auto-antigen specific cytokine production by lymph node and spleen cells

Having shown that treatment with ES-H induces substantial increase in frequencies of IFN- γ -and IL-4-producing T cells in the periphery this, study examined the effect of ES-H on the MOG specific IL-17, IFN- γ and IL-4 production by lymph node and spleen cells. Cells isolated from mice immunised with CFA or MOG and CFA treated with ES-H or PBS 14 days after immunisation were stimulated with or without MOG for 72 h and cytokines concentrations were quantified by ELISA.

Lymph node cells from mice with EAE stimulated with MOG produced IL-17, IFN- γ and IL-4 in a dose dependent manner. Treatment with ES-H significantly reduced concentration of IL-17 produced in response to the higher dose of MOG (100 μ g; $P<0.01$) but did not suppress MOG specific IFN- γ and IL-4 production. Lymph node cells from mice treated with ES-H produced low amounts of IL-4 independently of MOG. Cells from control mice immunised with PBS and CFA did not respond to stimulation with MOG (Fig. 5.13 A). Spleen cells from mice with EAE produced IL-17, IFN- γ and IL-4 in response to MOG. Treatment with ES-H significantly reduced the ability of these cells to produce MOG-specific cytokines.

5.2.8 Lower frequency of Foxp3⁺ T reg cells in CNS of ES-H treated mice

Treg cells play a central role in maintaining self tolerance and restraining excessive inflammatory responses. The expression of Foxp3 is specifically required for the development and function of Treg cells, and it provides a useful marker enabling identification of these cells in mice. Foxp3⁺ T cells constituted on average 3% of all CD4⁺ T cells present in the brains of naive mice (Fig. 5.14 A). The frequency of CD4⁺Foxp3⁺ T cells increased to 25% 7 days after immunisation with MOG and CFA. Treatment with ES-H reduced the frequency of these cells to 14%. As EAE progressed, the percentage of CNS infiltrating CD4⁺Foxp3⁺ T cells slightly decreased in MOG and CFA immunised mice, but it remained at the same level in the brains of ES-H treated mice. Mice with EAE had significantly higher absolute numbers of Foxp3⁺ CD4⁺ cells in their brains than ES-H treated mice at both time points (Fig. 5.14 A). In contrast, the frequency of these cells in the CNS of control PBS and CFA immunised mice increased over time to almost 30% on day 14 however the absolute numbers declined.

The frequency of Foxp3⁺ T cells in peripheral lymphoid organs was markedly higher than in the CNS and these cells constituted 7-9% of all CD4⁺ T cells in lymph nodes (Fig. 5.14 B) and spleens (Fig. 5.14 C) of naive mice. There was no increase in the frequency of CD4⁺Foxp3⁺ cells over the baseline levels either in the lymph nodes or the spleens of mice immunised with MOG and CFA with and without ES-H treatment. The increase in absolute numbers of CD4⁺Foxp3⁺ in lymph nodes of ES-H treated mice directly reflected the high absolute numbers of total CD4⁺ T cells in these mice.

5.2.9 Treatment with ES-H reduces expression of inflammatory markers in CNS

IL-1 β is an innate cytokine produced in CNS during inflammation by both infiltrating and resident immune cells. The expression of IL-1 β mRNA in the CNS was markedly up-regulated 7 days after immunisation with MOG and CFA and to a lesser extent following immunisation with PBS and CFA. Expression of

IL-1 β in the brains of mice treated with ES-H was significantly lower than observed in untreated mice with EAE ($P<0.001$). 14 days after the induction of EAE, IL-1 β mRNA expression was less pronounced than on day 7 but it was still significantly (500 fold) elevated over the baseline. In contrast the expression of IL-1 β mRNA in CNS tissue of ES-H treated mice was much lower (7 fold over baseline) than observed in untreated mice with EAE (Fig. 5.15 A)

IL-23 plays a role in development of Th17 cells and in the induction of IL-17 expression by innate and memory T cells. However the present study found little changes in IL-23 mRNA expression in the CNS during EAE. The expression of IL-23 was moderately higher 14 days post immunisation but there was no difference between the experimental groups (Fig. 5.15 B)

CD40 and CD68 are expressed on the surface of activated peripheral macrophages as well as on resident microglia during CNS inflammation. The expression of CD40 and CD68 mRNA in the brain tissue during EAE is an indirect measure of the severity of inflammation as well as the magnitude of macrophage infiltration into the CNS. The expression of both CD40 (Fig. 5.15 C) and CD68 (Fig. 5.15 D) was modestly up-regulated 7 days after immunisation with MOG and CFA and was further increased by day 14. In contrast, the expression of CD40 and CD68 was significantly ($P<0.001$) lower in mice treated with ES-H and remained at baseline level at all examined time points. Interestingly CD68 mRNA was up-regulated in brains of control mice immunised with PBS and CFA on day 7 but returned to the levels expressed in brains of naive mice by day 14.

5.2.10 Treatment with ES-H reduces expression of chemokines in CNS

It has been previously demonstrated that production of chemokines such as CCL2 or CCL5 is essential for macrophage infiltration into the CNS and development of EAE (Dogan and Karpus, 2004; dos Santos et al., 2005). 7 days after immunisation with MOG and CFA the expression of CCL2 mRNA in CNS of mice with EAE was significantly higher than in naive mice and continued

to increase as the disease progressed. Treatment with ES-H reduced expression of CCL2 by approximately 50% on day 7 and to the baseline levels by day 14. CCL3 (5.16 A), CCL5 (5.16 B) and CXCL10 (5.16 C) mRNA followed very similar expression pattern to that seen for CCL2 albeit the magnitude of induction varied between these chemokines. The expression of CCL20 mRNA in CNS of mice with EAE 7 days after immunisation was markedly higher than in naive mice and remained high throughout the course of EAE. In contrast the expression of CCL20 mRNA in CNS was reduced by treatment with ES-H and was significantly lower ($P<0.05$) than levels observed in untreated EAE mice on day 14 (Fig. 5.16 D).

5.2.11 Immunosuppressive component of ES-H is a protein

Having characterised immune responses associated with attenuation of EAE by ES-H the study evaluated components responsible for immunosuppressive properties of ES-H. In order to establish if suppression of EAE was induced by protein components of the ES preparation ES-H was incubated with a polymeric protein removal suspension reagent BindPro. The protein free supernatant (as confirmed by Bradford assay) and proteins eluted from the BindPro matrix were recovered and their concentrations were adjusted to that of initial ES-H preparation. EAE was induced in C57BL/6 mice by s.c. injection of MOG emulsified in CFA, with PT administered i.p. on days 0 and 2. Mice were injected with 40 µg of eluted protein or equivalent volume of protein depleted supernatant on day -1 and every second day thereafter. The result showed that protein components separated from ES-H completely prevented development of EAE, while the protein free fraction had no effect on the disease outcome. This clearly demonstrates that attenuation of EAE by ES-H is dependent on a protein component (Fig. 5.17).

5.2.12 Cysteine proteases from ES-H do not attenuate EAE

Previous work by Brady *et al.* demonstrated that infection with *F. hepatica* significantly impaired protective Th1 immune responses against *Bordetella pertussis* in mice co-infected with this bacterium or immunised with pertussis

whole cell vaccine (Brady et al., 1999). Cysteine protease cathepsin L (FhCatL), which is a major component of *F. hepatica* ES, was shown to be involved in the suppression of *B. pertussis*-specific responses. Immunosuppression induced by FhCatL was dependent on enzymatic activity of the protein and could be reversed by specific inhibitor of cysteine proteases ZFA-DMK (O'Neill et al., 2001). Therefore it is possible that cysteine proteases including FhCatL could be responsible for attenuation of EAE by ES-H.

First it was first established that ES-H contained active cysteine proteases that were able to convert specific fluorogenic peptide ZFR-AMC into a fluorescent product and the maximum initial velocity of the reaction increased with concentration of ES-H (Fig. 5.18). Enzymatic activity within ES-H was inhibited by preincubation with a specific inhibitor of cysteine proteases ZFA-DMK for 20 min prior the reaction with fluorogenic substrate (Fig. 5.19 A). ZFA-DMK at a concentration of 50 μ M reduced enzymatic activity by 65% and at concentration of 5 mM completely blocked ability of ES-H to hydrolyse ZFR-AMC (Fig. 5.19 B).

To evaluate the possible role of cysteine proteases in the ES-H preparation in attenuation of EAE, ES-H was incubated with 5 mM ZFA-DMK or vehicle (DMSO) for 20 min before it was used *in vivo*. EAE was induced in C57BL/6 mice by s.c. injection of MOG emulsified in CFA, with PT administered i.p. on days 0 and 2. Mice were injected with ES-H + ZFA-DMK, PBS + ZFA-DMK, ES-H + DMSO or PBS + DMSO on day -1 and every second day thereafter. As expected the administration of ES-H + DMSO significantly reduced the severity of EAE ($p < 0.001$ vs. PBS + DMSO). Preincubation of ES-H with the cysteine protease inhibitor did not reverse its suppressive effect ($p < 0.01$ vs. PBS + ZFA-DMK) suggesting that these enzymes do not contribute to the attenuation of EAE by ES-H (Fig. 5.20). Interestingly ZFA-DMK enhanced the attenuating effects of ES-H and injected on its own reduced severity of the disease when compared with PBS + DMSO but this was not significant (Fig. 5.20).

5.2.13 Immunomodulatory components of ES-H segregate to fraction

Fr.1 (>66 kDa)

Having shown that attenuation of EAE by ES-H was induced by a protein the study undertook further fractionation in an attempt to identify a fraction containing the active component.

ES-H was concentrated and further fractionated by size exclusion chromatography using a Fast Protein Liquid Chromatography (FPLC) system with Superdex 75 column designed for optimal separation of molecules between 3 - 70 kDa. Elution of the sample was monitored spectrophotometrically by absorbance at 210 nm (peptide bond) and 280 nm (aromatic rings) and separated material was collected in 0.5 ml fractions (Fig. 5.21). Standards of known molecular weight were run separately to allow for molecular weight determination of eluted proteins.

Fractionation of ES-H yielded three distinct peaks detected at 280 nm; a major peak containing molecules appearing larger than > 66 kDa and two smaller peaks at a range of 12–30 kDa and < 6.5 kDa respectively. On the basis of the chromatogram 0.5 ml fractions of eluted proteins were pooled into three working fractions: Fr.1 (>66 kDa), Fr.2 (6.5 – 66 kDa) and Fr.3 (<6.5 kDa). The fractions were then concentrated to the same volume as starting material in order to preserve the molar concentration of biochemicals equivalent to that of ES-H.

Further experiments were designed to evaluate the immunosuppressive capacity of fractions Fr.1 Fr.2 and Fr.3 in the EAE model. Mice immunised with MOG in CFA and PT were injected with 40 µl of ES-H, Fr.1 Fr.2, Fr.3 or PBS on a day -1, 1 and every second day thereafter and monitored for the signs of the disease and weight loss. None of the tested fractions was as effective in attenuating EAE as ES-H, however, Fr.1 clearly delayed disease onset by 3 days and prevented weight loss up until day 12 (Fig. 5.22 A and D). Fr.2 had marginal effect on the average clinical score and onset of EAE, but similar to Fr.1 improved general well being of the mice as they started to lose weight much later than the control animals (Fig. 5.22 B and D). In contrast, treatment with Fr.3 slightly exacerbated EAE, hastened the onset and did not have effect

on the weight change associated with EAE (Fig. 5.22 C and D). Analysis of cumulative disease score assessed for individual mice on day 17 supported the above findings (Fig. 5.22 E). Collectively these data demonstrate that although the fractionation procedure employed markedly reduced the efficacy of isolated fractions with respect to ES-H, Fr.1 and to lesser extent Fr.2 retained some of the capacity of ES-H to attenuate EAE.

In order to establish the effect of the tested fractions on innate responses DCs were pre treated with Fr.1 Fr.2, Fr.3 or PBS for 1h prior to stimulation with LPS for 24h. Analysis of cytokine concentrations in supernatants revealed that fraction Fr.1 was able to inhibit LPS-induced production of IL-1 β , IL-23 and IL-12p70 (Fig. 5.23).

5.2.14 Mass spectrometry based analysis reveals presence of potential immunomodulatory proteins in Fr.1

Identification of specific proteins within fraction Fr.1 was performed by mass spectrometry. Analysis was carried out on a commercial basis by Laboratory of Mass Spectrometry, IBB PAN in Warsaw, Poland. Sample Fr.1 underwent reduction and alkylation of protein disulfide bonds, followed by tryptic digestion. Resulting peptide mixture was separated by liquid chromatography (LC) followed by MS measurement of peptides and their fragmentation spectra (tandem mass spectrometry). The Mascot search engine was used to search and identify proteins on the basis of acquired spectra against the NCBI nr protein sequence database. The analysis returned 13 protein hits out of which three: helminth defence molecule-1 (FhHDM-1), thioredoxin peroxidase (FhTPx) and putative Cys-1 protein were identified with 3 or more matching sequences and were therefore considered a confident match. Leucyl aminopeptidase was identified with 2 matching sequences (Fig. 5.24).

5.3 Discussion

There is a large body of experimental evidence to suggest that regulatory networks triggered by helminths may protect against autoimmunity, however, mechanisms of immunosuppression are dependent on the disease model and parasitic species. In order to better understand how ES products of *F. hepatica* suppress autoimmune disease this study examined the kinetics of immune responses associated with attenuation of EAE by ES-H. The study found that administration of ES-H significantly reduced frequency of Th1 and Th17 cells in the CNS of mice with EAE and this was associated with a decrease in absolute numbers of immune cells infiltrating brains of treated mice when compared with untreated control mice. In contrast, ES-H treatment substantially increased the absolute numbers of IFN- γ , or IL-4 and IL-13 producing T cells in the peripheral lymph nodes. Furthermore, ES-H reduced the ability of lymph node cells to produce IL-17 in response to MOG but did not affect production of MOG-specific IL-4 or IFN- γ . This study further demonstrated that active components of ES-H segregated to a fraction Fr.1 that contains several molecules with immunomodulatory properties that could be responsible for the immunosuppressive effect of ES-H *in vivo*.

Although various immune cell subsets, including CNS resident cells, may play important roles in initiation and progression of EAE the infiltrating leucocytes are instrumental to the development of the disease. It has been demonstrated by Steinman and Yednock that blocking $\alpha 4\beta 1$ integrin (VLA-4), which is involved in extravasation, with a specific antibody impeded adhesion of activated leukocytes to inflamed endothelium effectively preventing the accumulation of leukocytes in the CNS and the development of EAE (Yednock et al., 1992). Indeed the humanised antibody against VLA-4, marketed under brand name Tysabri, is one of the most efficient treatments available currently for patients with highly active relapsing-remitting multiple sclerosis.

The present study has shown that attenuation of EAE by ES-H was associated with a significant reduction in total numbers of cells infiltrating CNS following immunisation with MOG in CFA and PT. The treatment significantly

decreased the influx of lymphocytes and frequency of infiltrating neutrophils. Interestingly, at day 7 control mice immunised with PBS and CFA showed substantial degree of leukocyte infiltration into CNS, which was comparable with that observed in brains of mice with EAE. The transient inflammation in the brain associated with leukocyte infiltration involved production of pro-inflammatory cytokines, chemokines and activation of resident APCs, but did not result in development of clinical symptoms of EAE and spontaneously resolved within 14 days. It is believed that activated leukocytes can cross the blood-brain barrier and enter the CNS regardless of their antigen specificity, however, only myelin-specific T cells can then be restimulated with a cognate antigen within the CNS. These proliferating reactivated T cells are sufficient to sustain the inflammatory status of microglia and the production of pro-inflammatory mediators, which facilitate further influx of pathogenic cells. (Goverman, 2009). The fact that treatment with ES-H resulted in reduction in numbers of infiltrating cells as early as 7 days after induction of the disease, when the influx seems to be independent from the antigen suggests that ES-H may interfere with either CFA induced activation and mobilisation of T cells or with migration across the BBB.

The present study demonstrated that treatment with ES-H not only reduced numbers of infiltrating cells but also modulated the cytokine expression pattern observed in the CNS during EAE. Although there is much debate regarding the exact role of Th1 cells in the initiation and regulation of EAE there is no doubt that both Th1 and Th17 subsets can be found in inflammatory lesions in CNS and both are able to induce the disease when adoptively transferred into naive recipients (Kroenke et al., 2008). Treatment with ES-H resulted in striking reduction in frequencies and absolute numbers of IL-17- and IFN- γ -producing CD4⁺ T cells. Interestingly ES-H did not affect the frequency of IL-17- and IFN- γ -producing CD8⁺ T cells but absolute numbers of these cells were significantly lower when compared to untreated mice with EAE. In addition the expression of IL-17 and IFN- γ mRNA was almost undetectable in CNS tissue of ES-H treated mice 14 day after induction of EAE.

The inhibition of IL-17 and IFN- γ production in the CNS was associated with a significant increase in frequency of IL-4 and IL-13 producing CD4⁺ T cells. Since parasite products are potent inducers on Th2 responses it is possible that suppression of pathogenic IL-17- and IFN- γ -producing T cells is a direct consequence of mutual cross-regulation between Th2, Th1 and Th17 subsets. It is well documented that redirecting the Th cell balance toward a Th2 response can suppress autoimmunity (Shaw et al., 1997) and both IL-13 and IL-4 have been reported to be protective in EAE (Cash et al., 1994; Falcone et al., 1998). Recent *in vitro* studies have suggested that the development of Th17 cells is negatively regulated by IL-4 signaling (Harrington et al., 2005) while IL-13 can suppress production of IL-17 through inhibition of Th17-promoting factors such as IL-1 β , IL-6, and IL-23 (Cash et al., 1994; Kleinschek et al., 2007).

In addition to modulation of cytokine production and cell entry into the CNS, treatment with ES-H affected the generation of immune responses in the periphery. ES-H induced a noticeable enlargement of spleens and draining lymph nodes that was associated with an increase in total numbers of CD3⁺ and CD4⁺ T cells in these organs. Similar to the CNS the frequencies and absolute numbers of IL-4- or IL-13-producing CD4⁺ T cells were also enhanced in lymph nodes of mice treated with ES-H. ES-H did not affect the frequency of IL-17⁺CD4⁺ T cells in the lymph nodes but significantly enhanced frequency and absolute numbers of IFN- γ producing CD4⁺ T cells. Interestingly high frequency of Th1 and Th2 cells detected by intracellular staining was not associated with enhanced capacity of lymph node cells to produce IFN- γ or IL-4 in response to MOG while MOG-specific IL-17 was reduced. It is possible that the expansion of IL-4 and IFN- γ producing cells was independent from MOG and could have been directed against parasite's antigens present within ES-H however this hypothesis was not tested in this study.

The high frequency of MOG independent IFN- γ associated with attenuation of EAE is interesting as the role of IFN- γ in the development of EAE is unclear. IFN- γ ^{-/-} mice are susceptible to EAE and in fact develop more severe disease than wild type mice (Ferber et al., 1996) and it has been proposed that this cytokine may play pathology limiting role during EAE. Moreover studies by Harrington *et al.* clearly demonstrated that IFN- γ signalling exerts a suppressive

effect on development of Th17 cells by down-regulating expression of IL-23R. Despite this potentially regulatory role of IFN- γ , purified MOG-specific Th1 cells are capable of inducing EAE when transferred to naive recipients (Kroenke et al., 2008).

The results of the study suggest that treatment with ES-H may cause retention of activated leukocytes in the draining lymph nodes. The increase in numbers of cells entrapped in lymph nodes correlated with reduced recruitment of immune cells to the CNS, which could potentially contribute to the attenuation of EAE by ES-H. In fact fingolimod, an approved therapy for MS, inhibits the function of S1P receptors, which blocks lymphocyte egress from secondary lymphoid organs thus prevents them from reaching the CNS.

Chronic helminth infections are often associated with significant expansion of Tregs which are believed to play a role in the generation of a suppressive environment favourable to parasite persistence. Studies in murine models of autoimmunity and allergy have shown that Treg cells can mediate bystander suppression induced by helminth infection. Several reports have demonstrated that parasite products could induce expression of Foxp3 in naive T cells either directly (Grainger et al., 2010) or through modulation of DCs (Zaccone et al., 2009). Attenuation of EAE by ES-H was not associated with an increase in frequency of Foxp3⁺ cells either in the periphery or in the CNS. In fact the frequency of Foxp3⁺ Treg cells in the brains of ES-H treated mice was lower than that observed in untreated mice and this did not change over the course of the disease. In contrast the frequency of Foxp3⁺ cells in mice that developed symptoms of EAE decreased as the disease progressed.

Chemokines are low molecular weight chemotactic cytokines that facilitate cellular migration. There are numerous reports in the literature that have demonstrated an association between chemokine mRNA or protein expression in the CNS and development of EAE (Dogan and Karpus, 2004). Although there is considerable redundancy of function amongst chemokine receptors and their ligands, several of them play critical role in leukocyte infiltration and initiation of

autoimmunity. The importance of specific chemokines has been explored with the help of neutralising antibodies and knockout mice. Treatment with anti-CCL3 (Karpus et al., 1995) and anti-CXCL10 (Fife et al., 2001) antibodies prevented acute clinical EAE, whereas CCL2 KO mice were markedly resistant to EAE after active immunization, with significantly impaired recruitment of macrophages to the CNS, yet were able to generate effector T cells that transferred severe disease to naive wild-type recipients (Huang et al., 2001). Similarly CCL20 was shown to regulate entry of CCR6 expressing Th17 cells into the CNS during EAE; interestingly expression of both CCL2 and CCL20 can be induced by IL-17 (Park et al., 2005; Reboldi et al., 2009). The present study demonstrated that treatment with ES-H substantially reduced the expression of CCL2, CCL3, CCL5, CXCL10 and CCL20 mRNA in the CNS tissue of mice with EAE. This inhibition of chemokine expression may explain the significant reduction in numbers of leukocytes infiltrating brains of ES-H-treated mice, however, it is difficult to interpret whether this suppression is the reason for reduced CNS infiltration or a consequence of it. Mice with EAE that were treated with ES-H also expressed lower levels of IL-1 β , CD40 and CD68 mRNA, which indicates that ES-H globally reduced inflammation observed in the CNS.

Taken together this data demonstrated that the attenuation of EAE by ES-H was associated with a reduced frequency of Th1 and Th17 cells and an increased frequency of Th2 cells infiltrating the brains of treated mice. ES-H also suppressed expression of pro-inflammatory cytokines and chemokines in CNS and significantly decreased the total numbers of infiltrating T cells. In contrast, treatment with ES-H during EAE resulted in accumulation of large numbers of cells in lymph nodes draining the site of immunisation. Frequency of IL-4, IL-13 and IFN- γ producing CD4⁺ T cells in the periphery was significantly higher than in untreated mice. ES-H did not affect capacity of lymph node cells to produce IL-4 and IFN- γ in response to MOG, however it inhibited production of antigen specific IL-17. Finally attenuation of EAE by ES-H was not associated with an increase in frequency of Foxp3⁺ Treg cells.

In an attempt to identify the active component within ES-H that is responsible for its immunosuppressive effect against EAE, the study examined the possible role of *F. hepatica*-derived cathepsins, which have been reported

to have immunomodulatory properties. *F. hepatica* secreted cysteine protease Catepsin L (FhCatL1) is a major virulence factor allowing migration of the parasite through host tissues during the infection. In addition to its enzymatic activity FhCatL1 has been reported to aid parasitic survival by modulating the host immune response. It has been demonstrated that administration of *F. hepatica* ES significantly impaired protective Th1 immune responses against *Bordetella pertussis* in mice infected with this bacterium or immunised with *pertussis* whole cell vaccine (Brady et al., 1999). Immunosuppression in that model was dependent on enzymatic activity of cysteine proteases and could be reversed by specific inhibitor ZFA-DMK. The study identified FhCatL as the key modulator involved in the suppression of *B. pertussis*-specific immune responses. Another study revealed that antigen-pulsed DCs matured in the presence of the functional, recombinant FhCatL had reduced capacity to support differentiation of Th17 cells, while antigen specific Th1 and Th2 immune responses were not affected (Dowling et al., 2010). These findings suggested that FhCatL could potentially be responsible for the attenuation of EAE by ES-H.

The present study demonstrated that ES-H indeed contained active cysteine proteases sensitive to inhibition with ZFA-DMK. However their enzymatic activity did not contribute to the immunosuppressive effect of ES-H in autoimmunity as treatment of ES-H with ZFA-DMK did not reduce its ability to attenuate EAE. Interestingly, administration of the ZFA-DMK reduced the severity of the disease and further enhanced the suppressive effect of ES-H. This may have been due to the inhibition of host cysteine proteases involved in numerous biological processes such as antigen processing and presentation that could hamper development of MOG-specific T cell responses. Collectively, the data demonstrate that despite of the reported immunomodulatory properties of FhCatL, cysteine proteases do not contribute to the attenuation of EAE by ES-H.

This study employed size exclusion chromatography to further fractionate the components of ES-H. The preparation was separated into three fractions Fr.1 (> 66 kDa), Fr.2 (6.5-66 kDa) and Fr.3 (< 6.5) and each was tested in the

EAE model. The fractionation procedure appeared to reduce the efficacy of isolated fractions when compared to ES-H and may be explained by inevitable loss of biological material during FPLC. Nevertheless, the result of the EAE experiment revealed that immunomodulatory proteins segregated to fraction Fr.1 (>66 kDa) but some activity was also observed in Fr.2 (6.5-66 kDa). The fact that both Fr.1 and Fr.2 showed immunosuppressive effects in EAE suggests that the active components may be a part of a multimeric protein complex and break down of this complex may result in various subunits segregating to separate fractions. Alternatively the active component may oligomerise to form aggregates of various sizes or the immunomodulatory effect of ES-H may be mediated by several independent molecules.

Mass spec analysis of Fr.1 returned 13 protein hits out of which only three, helminth defence molecule-1 (FhHDM-1), thioredoxin peroxidase (TPx) and putative Cys-1 protein, were identified by matching with 3 or more distinct sequences and received a score over 100. Leucyl aminopeptidase (FhLAP) was identified with 2 matching sequences. The remaining hits, many of which were matched to hypothetical proteins submitted for unrelated species scored too low to be considered a confident match. Interestingly FhHDM-1 and TPx were previously reported to exhibit some immunomodulatory properties whereas Cys-1 is a hypothetical cysteine protease inhibitor and this class of parasite derived proteins has been reported to interfere with host immune responses (Hewitson et al., 2009). There are no reports of immunomodulatory properties of FhLAP but the protein has been tested as potential vaccine candidate against fascioliasis in sheep (Maggioli et al., 2011).

FhHDM-1 is an 8 kDa protein that oligomerises in acidic pH of *F. hepatica* ES and can form large multimers which explains the presence of this protein in >66 kDa fraction Fr1. Immunomodulatory properties of FhHDM-1 are believed to stem from its unique structure that allows it to bind LPS thus prevents interaction of LPS with TLR4 complex and subsequent activation of TLR4 bearing cells (Robinson et al., 2011). Recombinant FhHDM-1 has been shown to protect mice from LPS induced inflammation in a septic shock model (Donnelly et al., 2010). Therefore the presence of FhHDM-1 in Fr.1 may explain

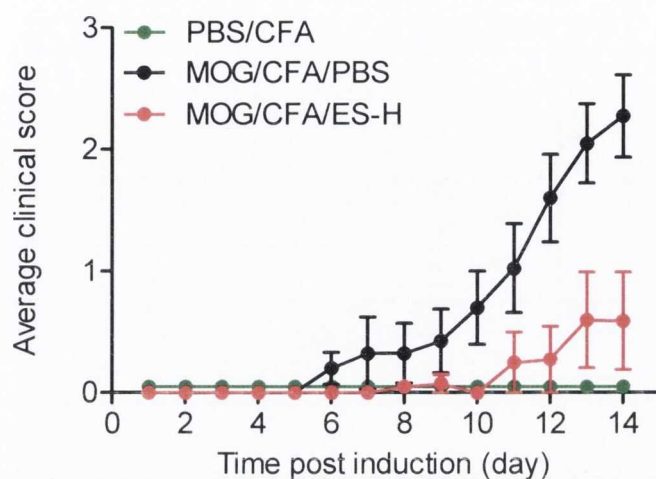
the suppressive effect of this fraction on cytokine production from LPS stimulated DCs. It's not clear why unfractionated ES-H did not suppress LPS-induced cytokine production of IL-6 and IL-23 (Fig. 4.1) but it is possible that ES-H contains molecules such as TLR agonists that also enhance cytokine production. It has also been demonstrated that immunomodulatory properties of FhHDM-1 expand beyond its function as LPS decoy and the protein can directly modulate cell function by interfering with antigen processing by macrophages. A C-terminal active peptide released from FhHDM-1 following internalisation by APC inhibits activity of vacuolar ATPase and prevents the acidification of the endolysosomal compartments necessary for normal antigen processing, thus impeding antigen presentation to CD4⁺ T cells (Robinson et al., 2012).

The mass spec analysis also identified FhTPx as one of the components of Fr.1. FhTPx belongs to an evolutionary conserved family of antioxidant enzymes that protect from damage associated with metabolic oxidation in aerobic cells. It has been proposed that in parasitic organisms the enzyme may play an additional role as a defence mechanism protecting from reactive oxygen species released by host's phagocytes during immune attack on the parasite (Hewitson et al., 2009). Independently from its function as an antioxidant, FhTPx was shown to directly modulate host immune responses by inducing araginase-1 and Ym-1 expressing alternatively activated macrophages (Donnelly et al., 2005; Donnelly et al., 2008). While these FhTPx induced cells are necessary for the development of Th2 responses during infection (Donnelly et al., 2008) alternatively activated macrophages were also shown to contribute to the parasite induced inhibition of T cell proliferation and IFN- γ production *in vitro* and *in vivo* (Taylor et al., 2006). Moreover Vaknin *et al.* reported that adoptive transfer of alternatively activated macrophages induced by IL-4 into mice with EAE significantly suppressed disease severity (Vaknin et al., 2011). Thus it is possible that alternatively activated macrophages induced by FhTPx could contribute to the immunosuppressive effect of ES-H in EAE. The immunomodulatory effect of FhTPx has not yet been tested in experimental models of autoimmunity, which makes it an interesting and novel candidate for therapeutic evaluation in future studies.

The cystatins are proteinase inhibitors regulating activity of cysteine proteases which play important role in many biological processes in homeostatic conditions and in pathology. The mass spec analysis of Fr.1 also identified a protein matching with a sequence of a putative multi-domain cysteine protease inhibitor Cys-1 described by Khaznadji *et al.* The hypothetical 680aa protein is predicted to have 6 cystatin domains; one of them has been cloned, expressed and shown to be a functional and potent inhibitor of papain and Cathepsin L (Khaznadji *et al.*, 2005). Cys-1 has not been purified and characterised so far, but cystatins derived from many parasites have been shown to be strong immunomodulators. A cysteine protease inhibitor from filarial parasite *A. viteae* targets macrophages and effectively suppressed pathological responses in murine models of asthma and colitis through an IL-10 dependent mechanism (Schnoeller *et al.*, 2008). Similarly, *Onchocerca vulvulus* derived cystatin was shown to down-regulate mitogen induced proliferation of human PBMC by directly inhibiting the co-stimulatory function of monocytes (Schönemeyer *et al.*, 2001). Parasite derived cystatins were also reported to directly inhibit the activity of host proteases involved in antigen processing and presentation as shown for cystatin (CPI-2) from *Brugia malayi* (Manoury *et al.*, 2001).

Taken together, this study demonstrated that the immunomodulatory effect of ES products of *F. hepatica* was mediated by a protein component suggesting that free polysaccharides, lipids and nucleic acids may not be involved. Despite the reported immunomodulatory properties of FhCatL, attenuation of EAE by ES-H was not dependent on cysteine proteases. The study further identified fraction Fr.1 of ES-H that contained active components responsible for the immunosuppressive effect of *F. hepatica* ES against autoimmunity. The proteins identified in Fr.1, FhHDM-1, FhTPx and Cys-1, or their homologs from other helminth species, have known immunomodulatory properties and could potentially mediate the immunosuppressive effects of ES-H. These molecules can be now cloned and expressed in suitable expression system to evaluate their immunomodulatory effect in EAE and other models of autoimmunity.

A



B

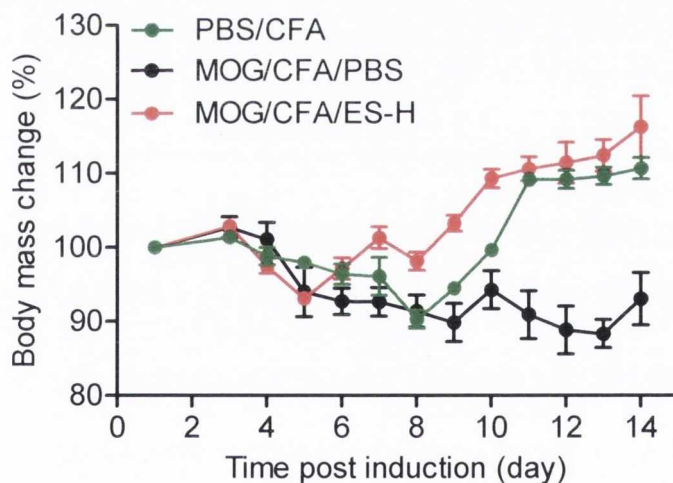
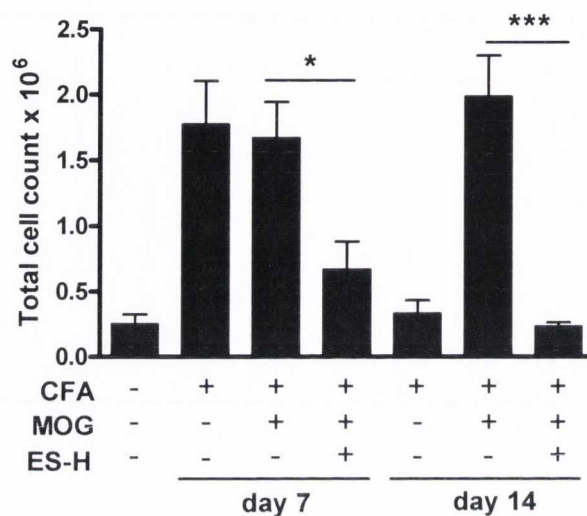


Fig 5.1 Administration of ES-H attenuates EAE. EAE was induced in C57BL/6 mice by s.c injection of 100 μ g/ml of MOG emulsified in CFA, followed by PT. A control group of mice was injected with PBS in CFA followed by PT. Mice immunised with MOG and CFA were injected with 40 μ l of ES-H or PBS on day -1 and every second day after throughout the course of the disease. Animals were monitored daily for clinical signs of the disease (A) and weight loss (B). Results are mean $\pm$ SEM from two independent experiments (n = 10 or n = 4 for PBS/CFA). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs MOG/CFA/PBS group.

A



B

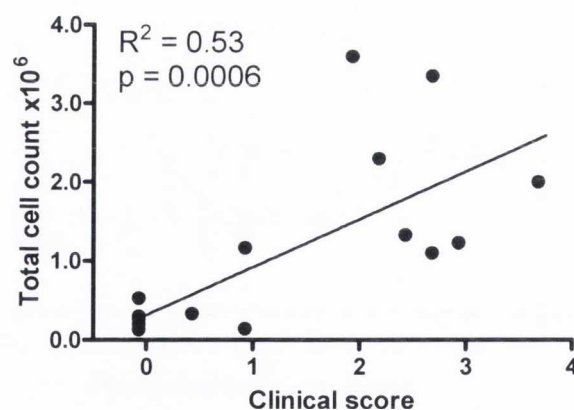


Fig. 5.2 Treatment with ES-H reduces leukocyte infiltration into CNS. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Leukocytes were isolated from brains of perfused mice at the onset of clinical symptoms (day 7) and at the peak of the disease (day 14) and counted (A). The severity of the disease correlates with high number of cells recovered from CNS on day 14 (B). Results are mean $\pm$ SEM from two independent experiments ($n = 7-9$ or $n=3-8$ for PBS/CFA). * $P < 0.05$; *** $P < 0.001$ vs MOG/CFA/PBS at relevant time point.

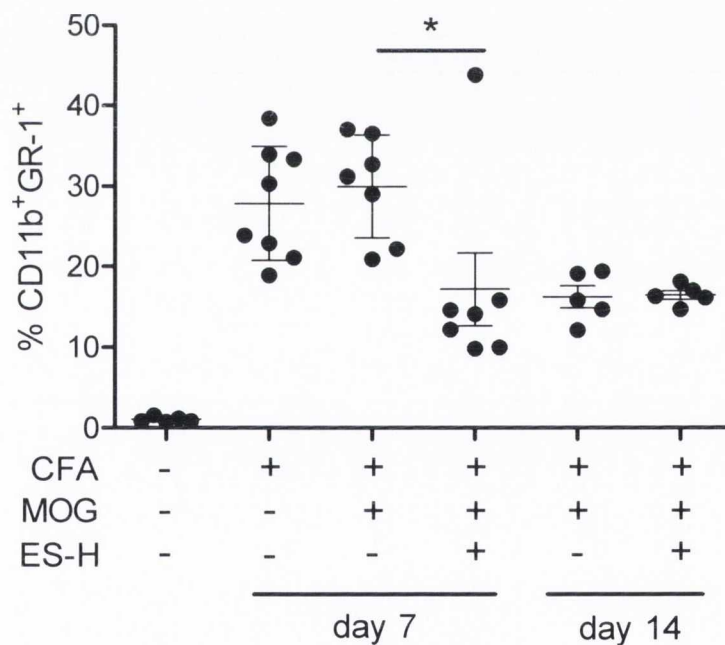
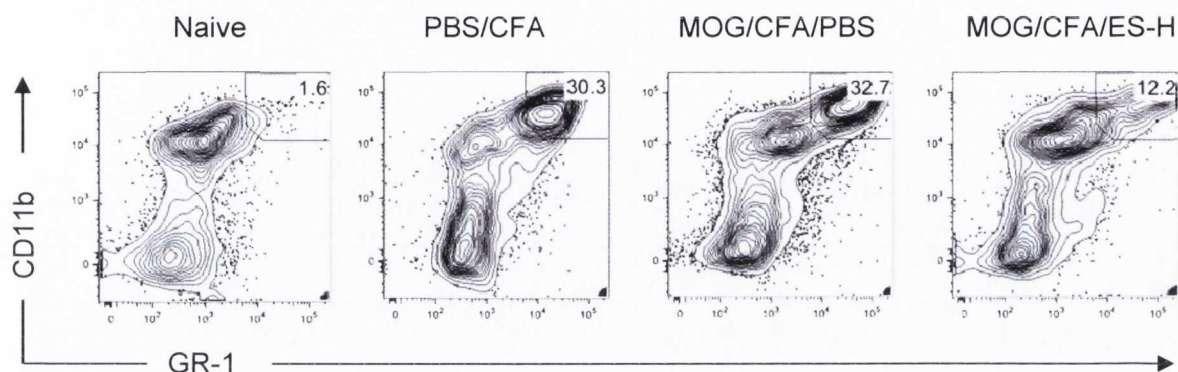


Fig. 5.3 Administration of ES-H prevents early neutrophil infiltration into the CNS during EAE. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Leukocytes were isolated from the CNS of naive and immunised mice 7 and 14 days post immunisation. Cells were stained with antibodies against CD11b and GR-1 and analysed by flow cytometry. Results are representative dot plots for each experimental group at day 7, with the mean frequency of CD11b⁺GR-1⁺ cells shown as the scatter plot where each data point represents an individual mouse. * P<0.05; vs MOG/CFA/PBS at relevant time point.

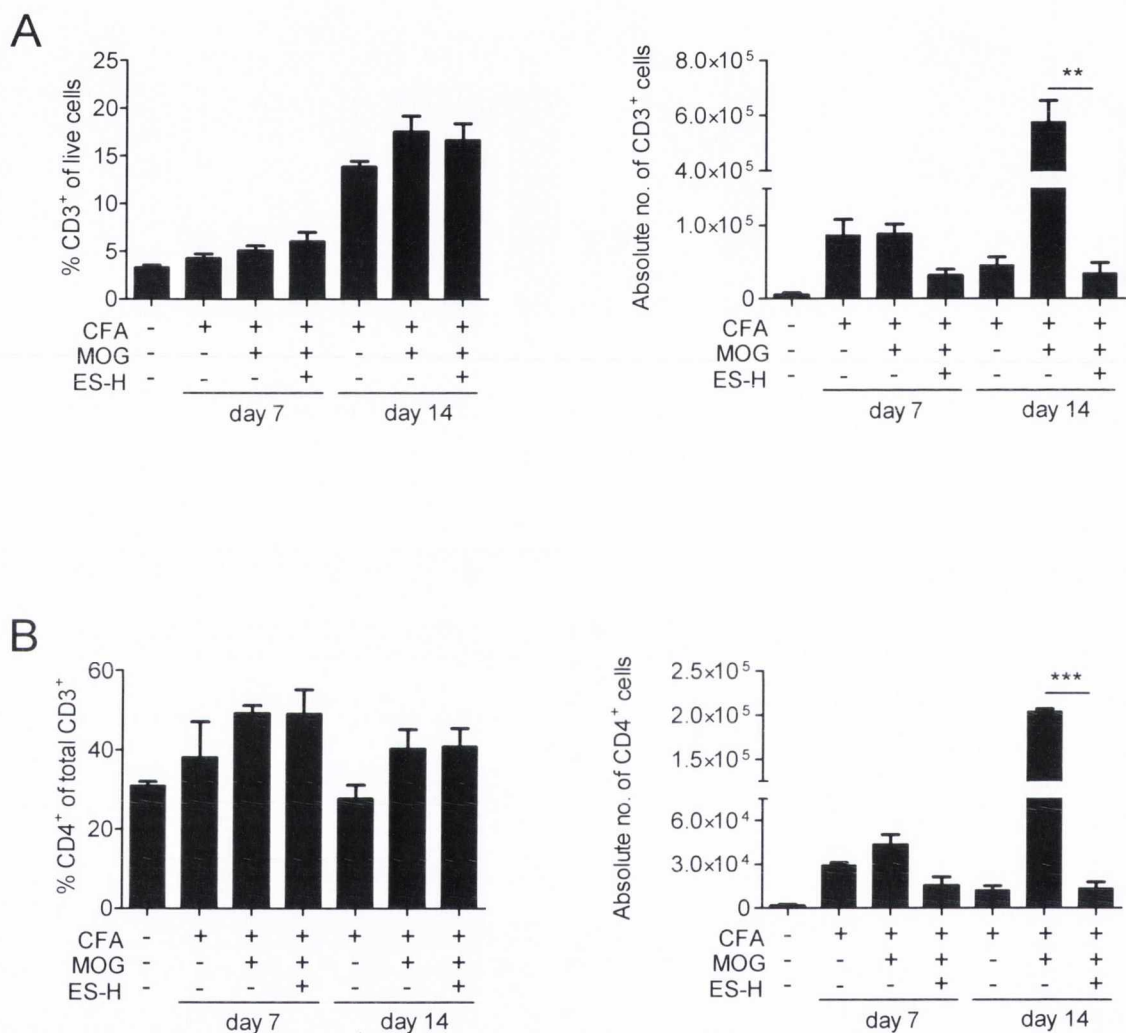


Fig. 5.4 Administration of ES-H reduces absolute numbers of CD3⁺ and CD4⁺ cells infiltrating CNS of mice with EAE. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Leukocytes were isolated from the CNS of naive and immunised mice 7 and 14 days post immunisation, stained with antibodies against CD3, and CD4. Frequency and absolute numbers of CD3⁺ (A) and CD3⁺CD4⁺ T cells (B) was analysed by flow cytometry. Results are mean (+/- SEM) ** P<0.01; *** P<0.001 versus MOG/CFA/PBS at relevant time point.

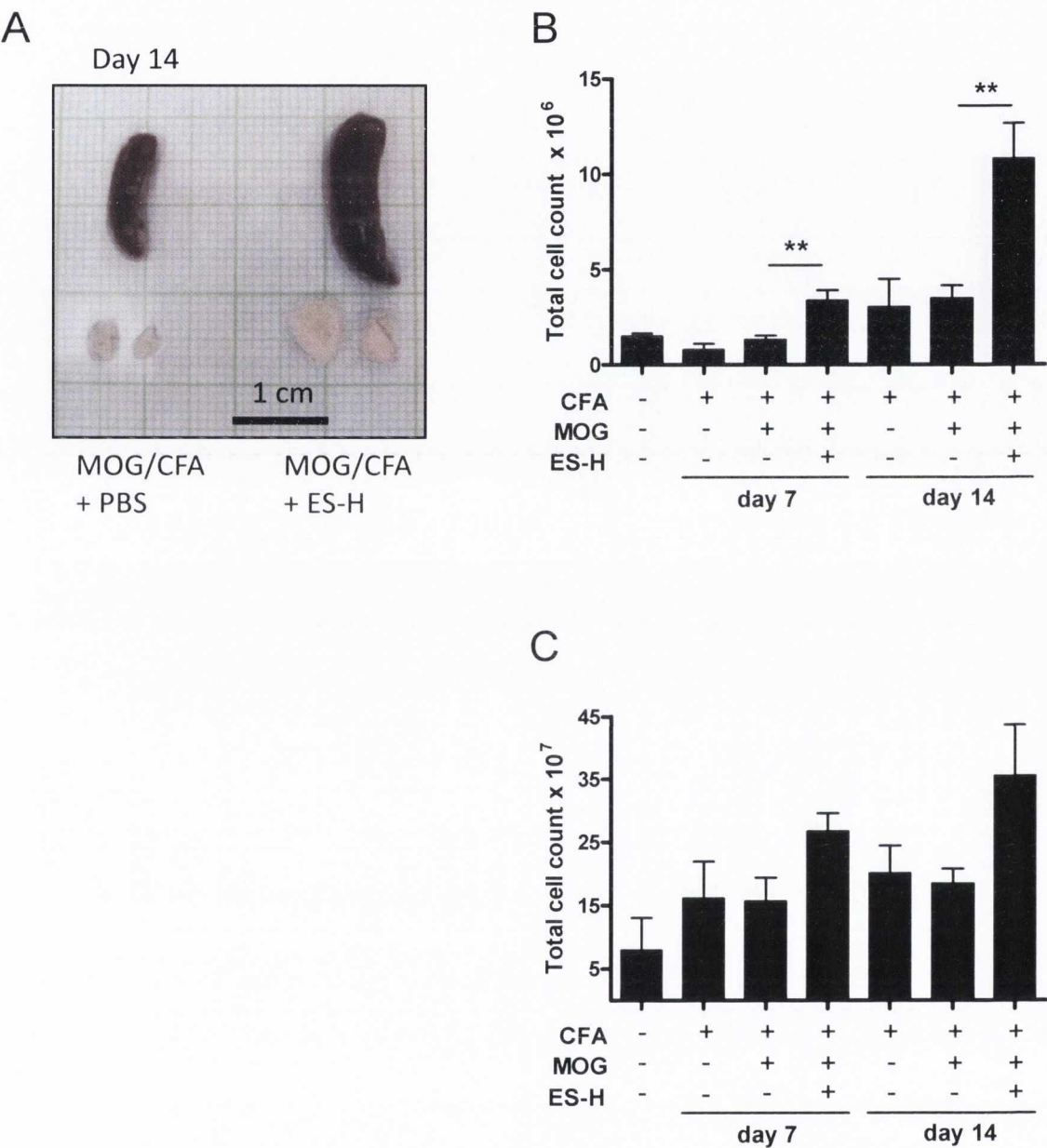


Fig. 5.5 Treatment of mice with ES-H results in significant enlargement of draining lymph nodes and spleens. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Mice were sacrificed at the onset of clinical symptoms (day 7) and at the peak of the disease (day 14) and inguinal lymph nodes and spleens were removed. Panel A shows representative organs from 1 MOG/CFA/PBS (left) and 1 MOG/CFA/ES-H (right) animal sacrificed at the day 14. Organs were processed and cells from inguinal lymph (B) nodes and spleens (C) were counted. Results are means $\pm$ SEM pooled from two independent experiments (n=7-9 or n=3-8 for PBS/CFA). ** P<0.01; versus MOG/CFA/PBS at relevant time point.

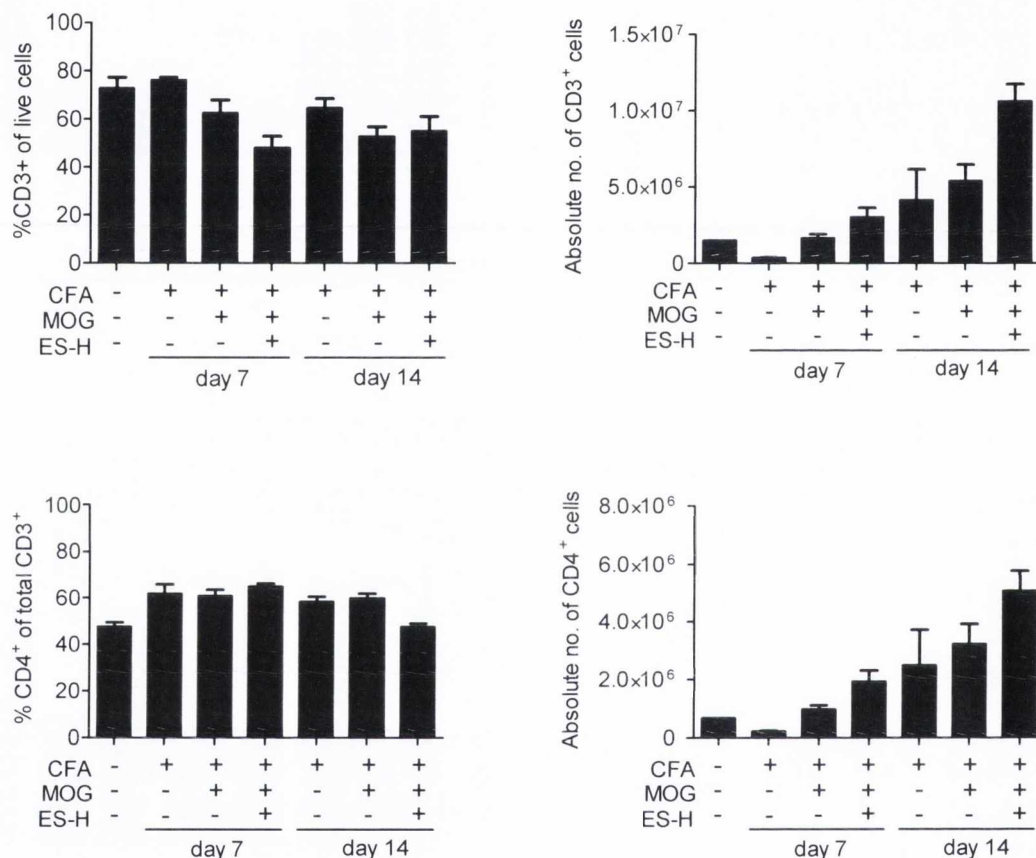


Fig. 5.6 Effect of ES-H administration on the frequency and absolute numbers of CD3⁺ and CD4⁺ cells in the draining lymph nodes. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Leukocytes were obtained from inguinal lymph nodes of naive and immunised animals 7 and 14 days post immunisation. Cells were stimulated with PMA and ionomycin in the presence of BFA for 6 h, stained with antibodies against CD3, CD4 and analysed by flow cytometry. Results are mean (+/-SEM) from one experiment representative of 2 independent experiments.

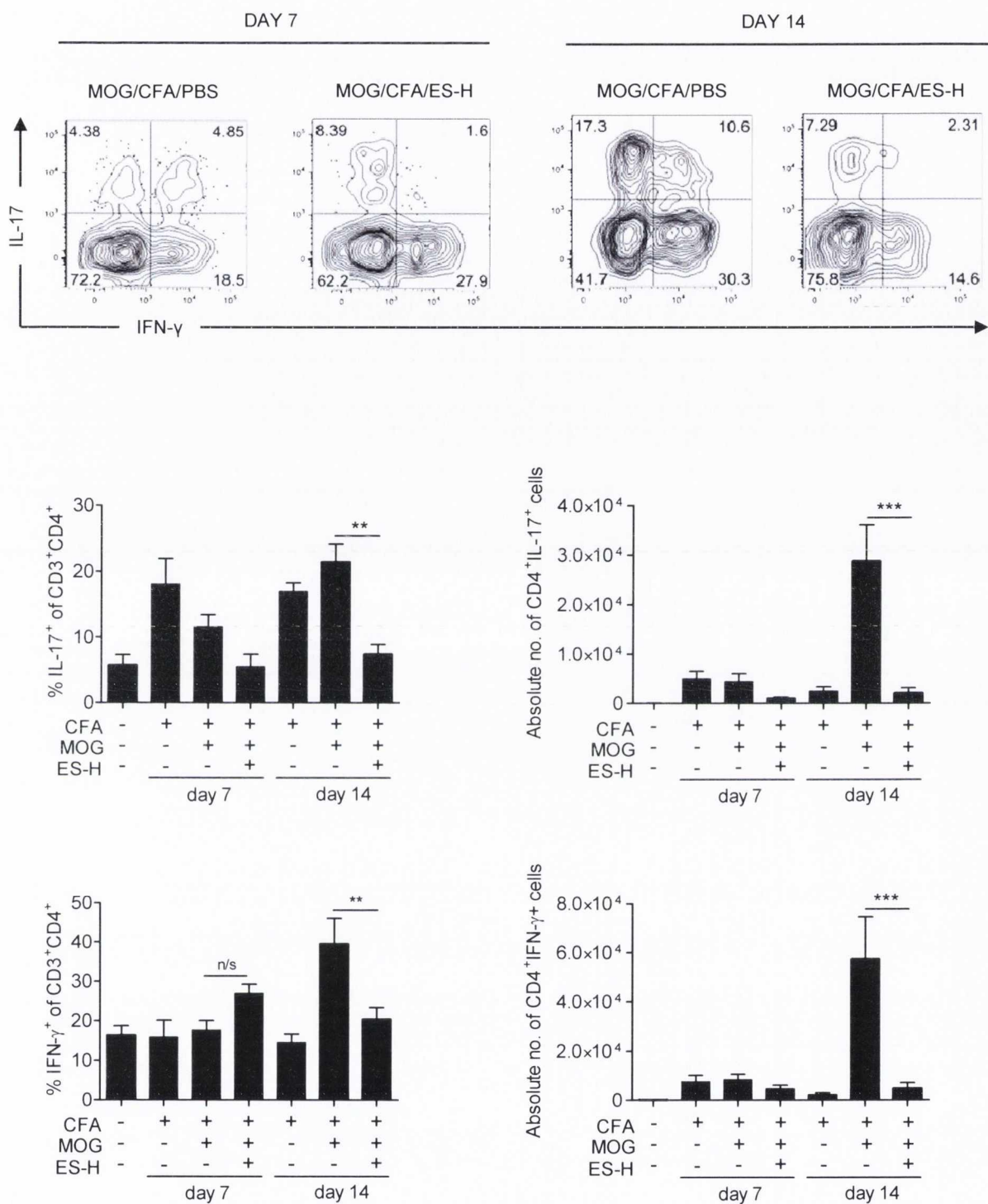


Fig. 5.7 Administration of ES-H reduces frequency and absolute numbers of IL-17 and IFN- γ -producing CD4⁺ cells in the CNS. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Leukocytes were isolated from the CNS of naive and immunised animals 7 and 14 days post immunisation. Cells were stimulated with PMA and ionomycin in the presence of BFA for 6 h, stained with antibodies against CD3, CD4, IL-17 and IFN- γ and analysed by flow cytometry. Results are representative dot plots for ES-H treated and control mice on each time point, with the mean frequency (+/-SEM) of cells in the CD4⁺ population in the bar chart. ** $P < 0.01$; *** $P < 0.01$ vs. MOG/CFA/PBS at relevant time point; n/s – not significant.

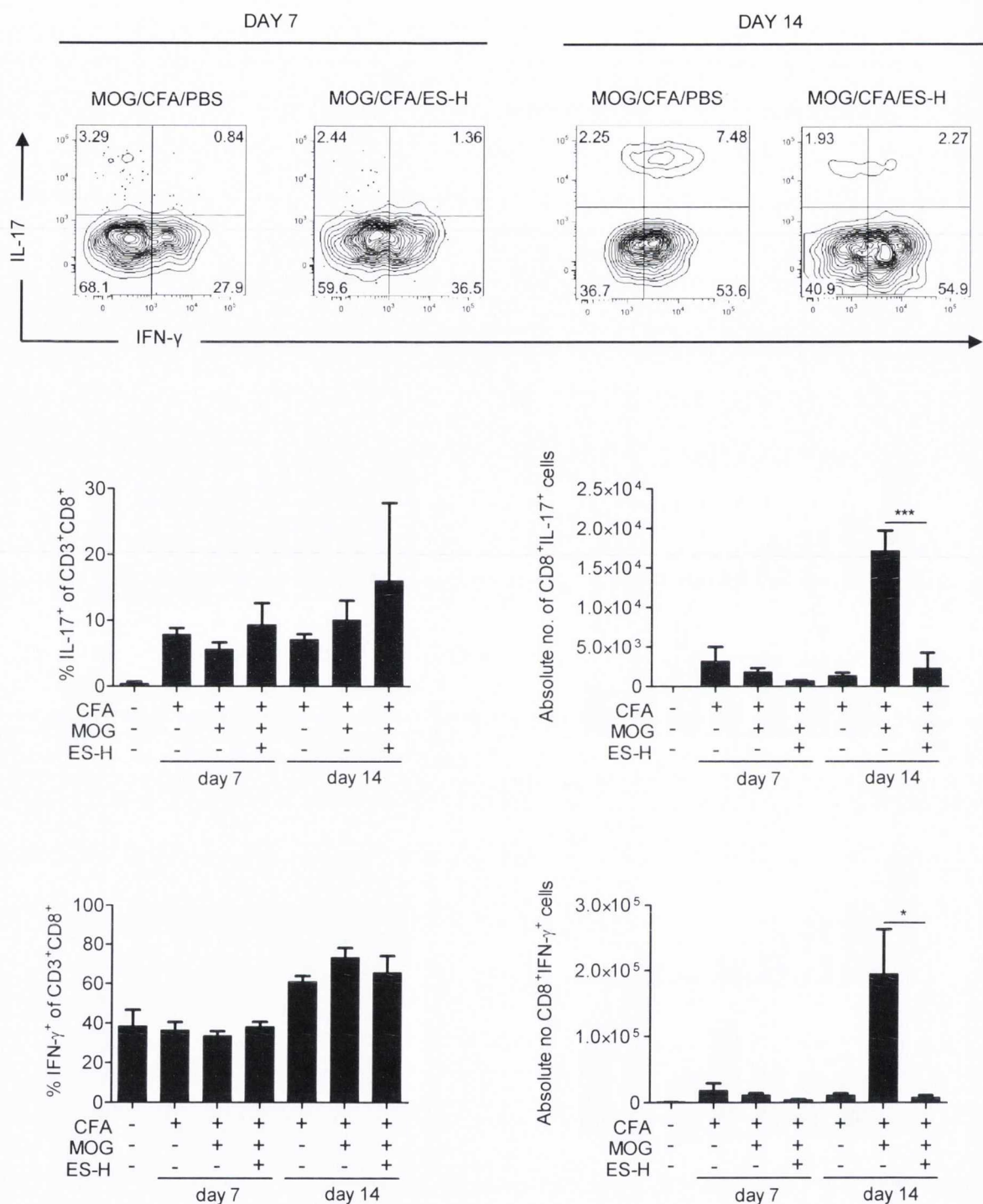


Fig. 5.8 Administration of ES-H reduces numbers of IL-17 and IFN-γ-producing CD8⁺ cells in the CNS. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Leukocytes were isolated from the CNS of naive and immunised mice 7 and 14 days post immunisation. Cells were stimulated with PMA and ionomycin in the presence of BFA for 6 h, stained with antibodies against CD3, CD8, IL-17 and IFN-γ and analysed by flow cytometry. Results are representative dot plots for ES-H treated and control mice on each time point, with the mean frequency (+/-SEM) of cells in the CD4⁺ population in the bar chart * P<0.05; *** P<0.001 vs. MOG/CFA/PBS at relevant time point.

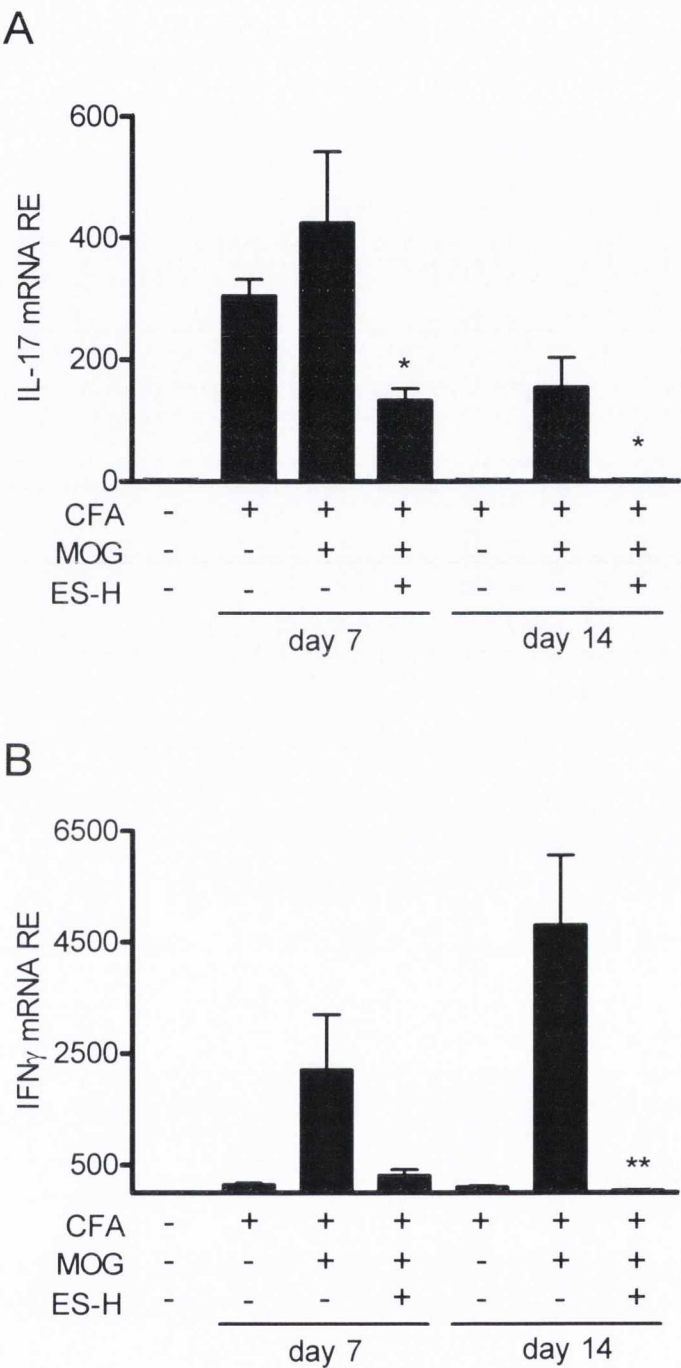


Fig. 5.9 Effect of ES-H treatment on the expression of IL-17 and IFN- γ in the CNS of mice with EAE. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Mice were sacrificed on day 7 and 14 post immunisation. Total RNA was purified from CNS tissue and expression of IL-17 (A) and IFN- γ (B) was analysed by quantitative RT-PCR. Data represent relative gene expression versus naive control following normalization to the endogenous control, 18S rRNA. Results are mean ($\pm$ SEM). * $P < 0.05$; ** $P < 0.01$ vs. MOG/CFA/PBS at relevant time point.

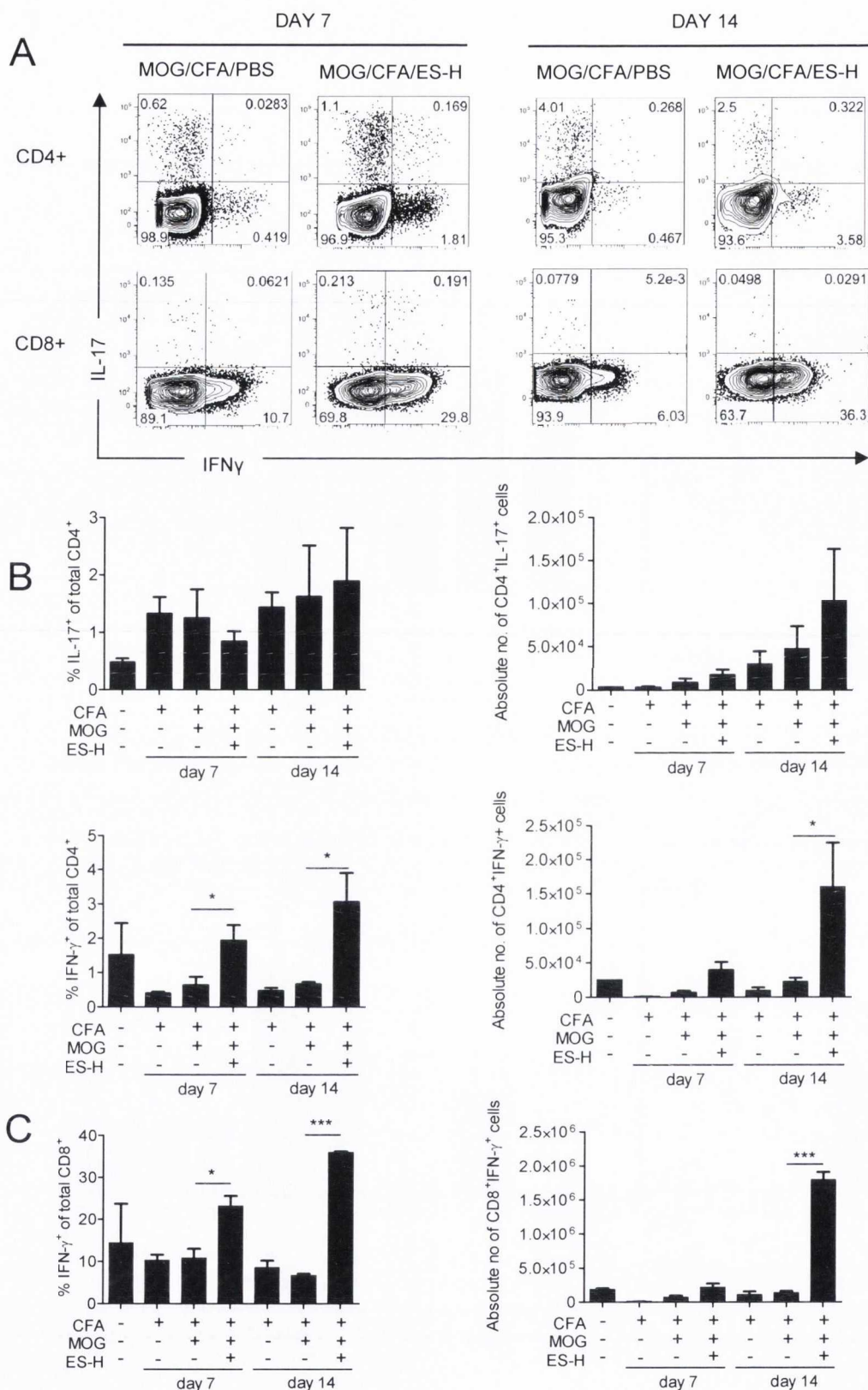


Fig. 5.10 Administration of ES-H increases the frequency and absolute numbers of IFN- γ -producing CD4⁺ and CD8⁺ cells in the draining lymph nodes during EAE. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Leukocytes were isolated from inguinal lymph nodes of naive and immunised animals 7 and 14 days post immunisation. Cells were stimulated with PMA and ionomycin in the presence of BFA for 6 h, stained with antibodies against CD3, CD4, CD8, IL-17 and IFN γ and analysed by flow cytometry. Results are representative dot plots for ES-H treated and control mice on each time point, with the mean frequency and absolute numbers (+/-SEM) of cells in the CD4⁺ or CD8⁺ population in the bar chart * $P < 0.05$, *** $P < 0.001$ vs. MOG/CFA/PBS at relevant time point.

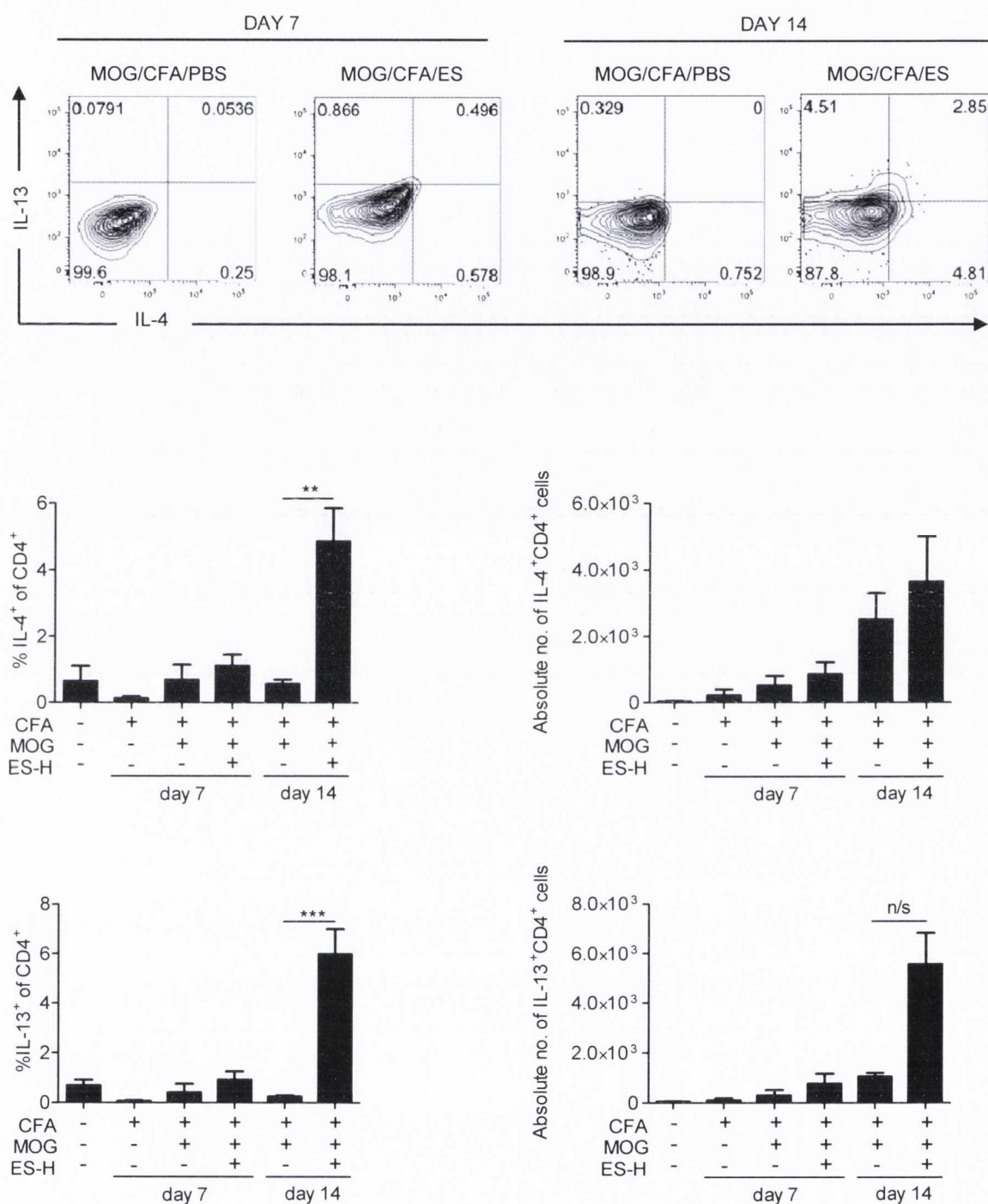


Fig. 5.11 Administration of ES-H increases the frequency of IL-4 and IL-13-producing CD4⁺ cells in the CNS. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Leukocytes were isolated from the CNS of naive and immunised animals 7 and 14 days post immunisation. Cells were stimulated with PMA and ionomycin in the presence of BFA for 6 h, stained with antibodies against CD3, CD4, IL-4 and IL-13 and analysed by flow cytometry. Results are representative dot plots for ES-H treated and control mice on each time point, with the mean frequency (+/-SEM) of cells in the CD4⁺ population in the bar chart. ** P<0.01; *** P<0.001 vs. MOG/CFA/PBS at relevant time point.

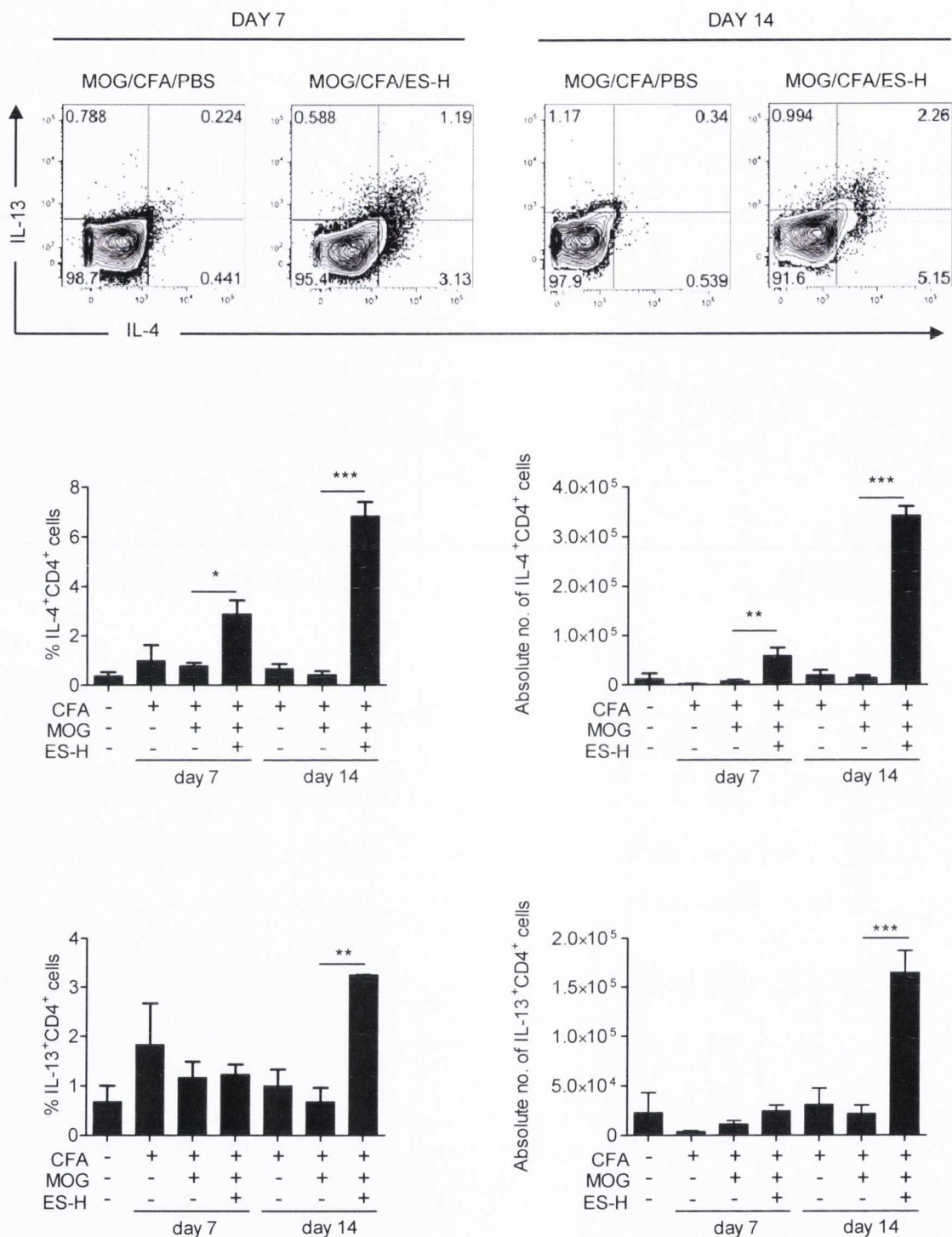


Fig. 5.12 Administration of ES-H increases the frequency of IL-4 and IL-13-producing CD4⁺ cells in the draining lymph nodes. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Leukocytes were isolated from lymph nodes of naive and immunised animals 7 and 14 days post immunisation. Cells were stimulated with PMA and ionomycin in the presence of BFA for 6 h, stained with antibodies against CD3, CD4, IL-4 and IL-13 and analysed by flow cytometry. Results are representative dot plots for ES-H treated and control mice on each time point, with the mean frequency (+/-SEM) of cells in the CD4⁺ population in the bar chart * P<0.05; ** P<0.01; *** P<0.001 vs. MOG/CFA/PBS at relevant time point.

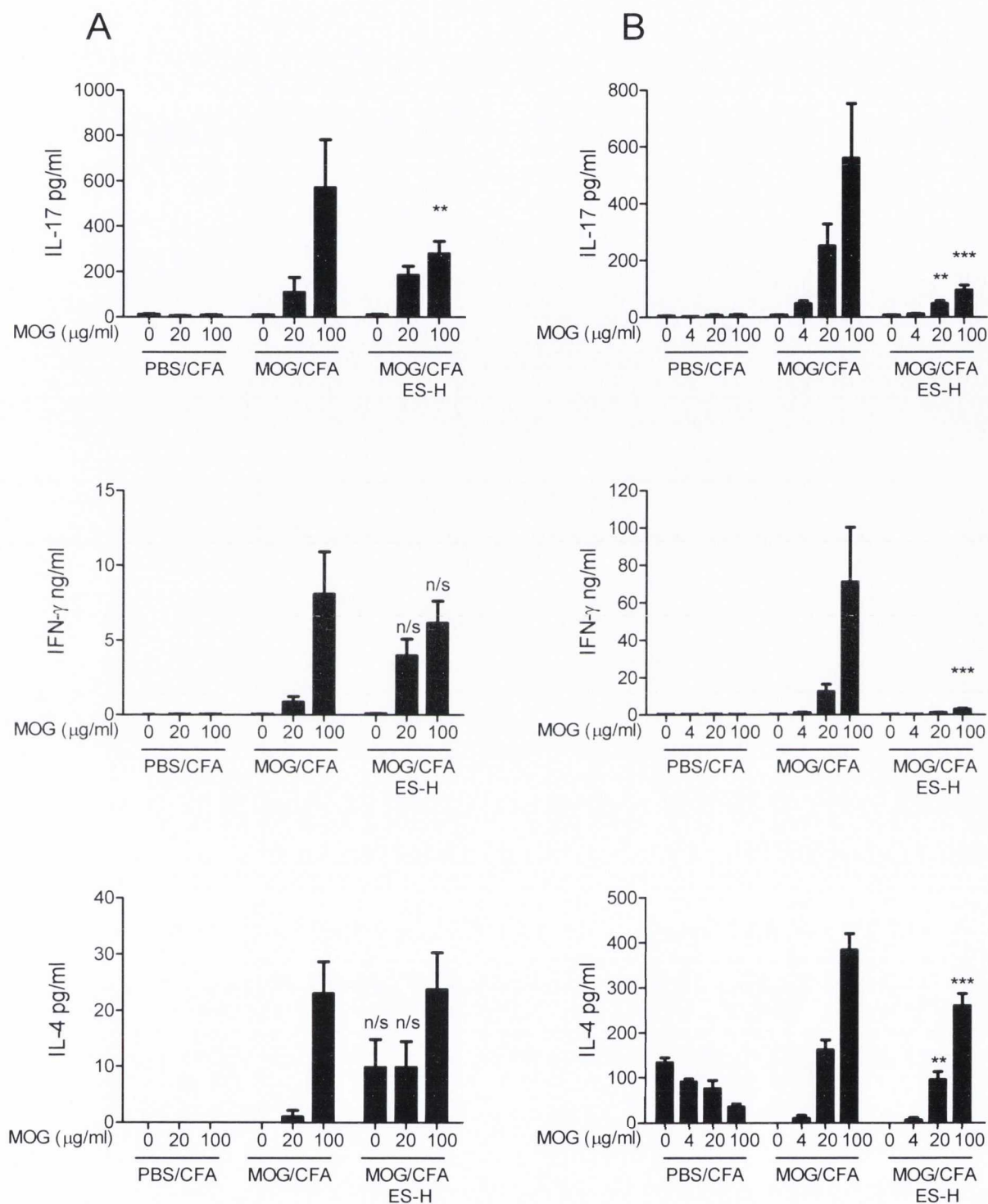


Fig. 5.13 The effect of ES-H administration on the production of MOG-specific IL-17, IFN- γ and IL-4 by lymph node and spleen cells. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Single cell suspensions were prepared from lymph nodes (A) and spleens (B) isolated from EAE mice, ES-H treated and control mice on day 14 post immunisation. Cells were stimulated with or without MOG as indicated and supernatants were collected after 72 h. Concentrations of IL-17, IFN- γ and IL-4 were quantified by ELISA, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control mice.

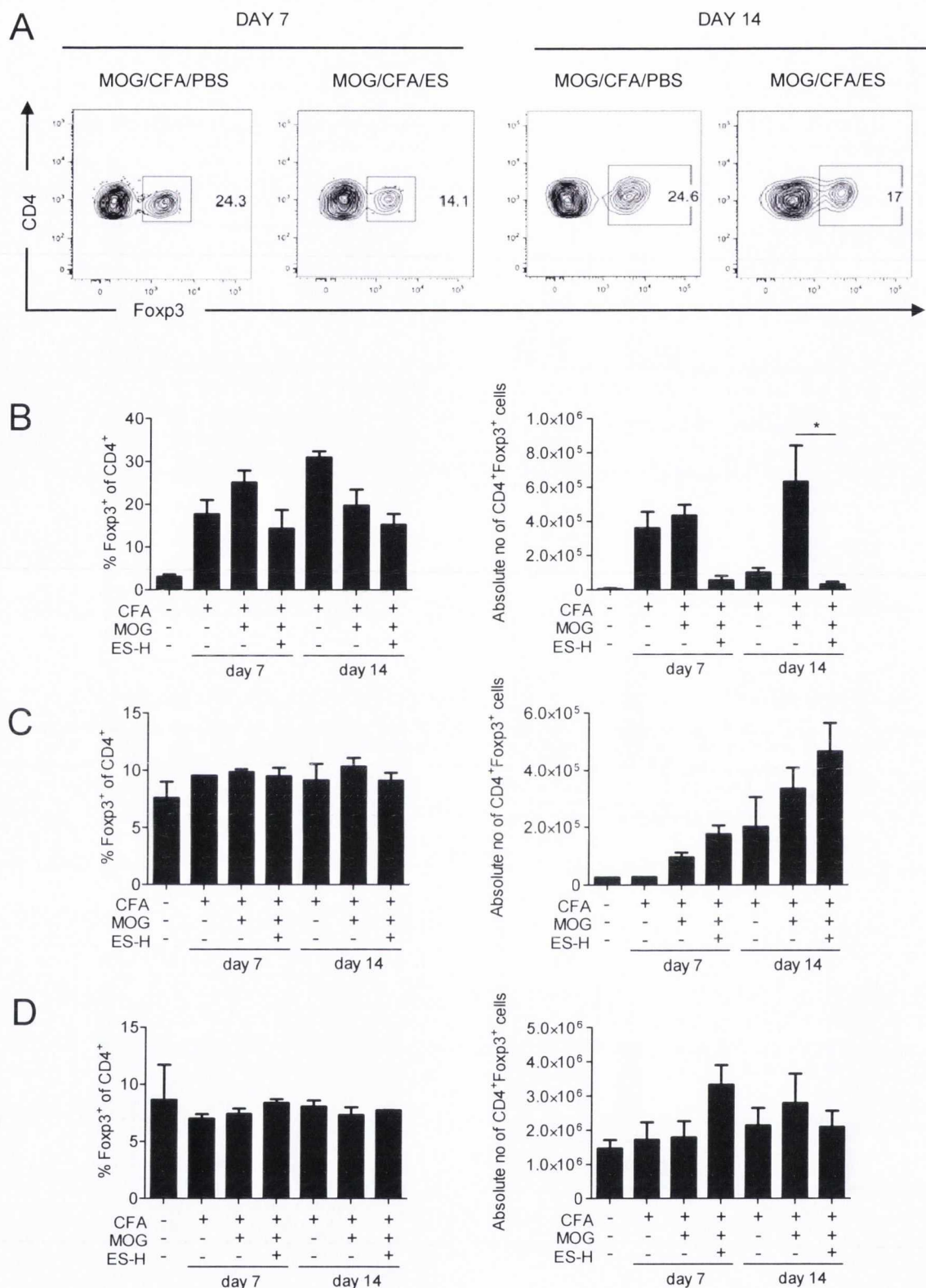


Fig. 5.14 The effect of ES-H administration on the frequency and absolute numbers of Foxp3⁺ CD4⁺ cells in CNS and the periphery during EAE. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Leukocytes were isolated from the CNS, draining lymph nodes and spleens of naive and immunised animals 7 and 14 days post immunisation. Cells were stained with antibodies against CD3, CD4 and Foxp3 and analysed by flow cytometry. Results are representative dot plots for brain cells of ES-H treated and control mice on each time point, with the mean frequency ($\pm$ SEM) of Foxp3⁺ cells in the CD4⁺ population in CNS (B), lymph node (C) and spleen (D) in the bar chart * $P < 0.05$ vs. MOG/CFA/PBS at relevant time point.

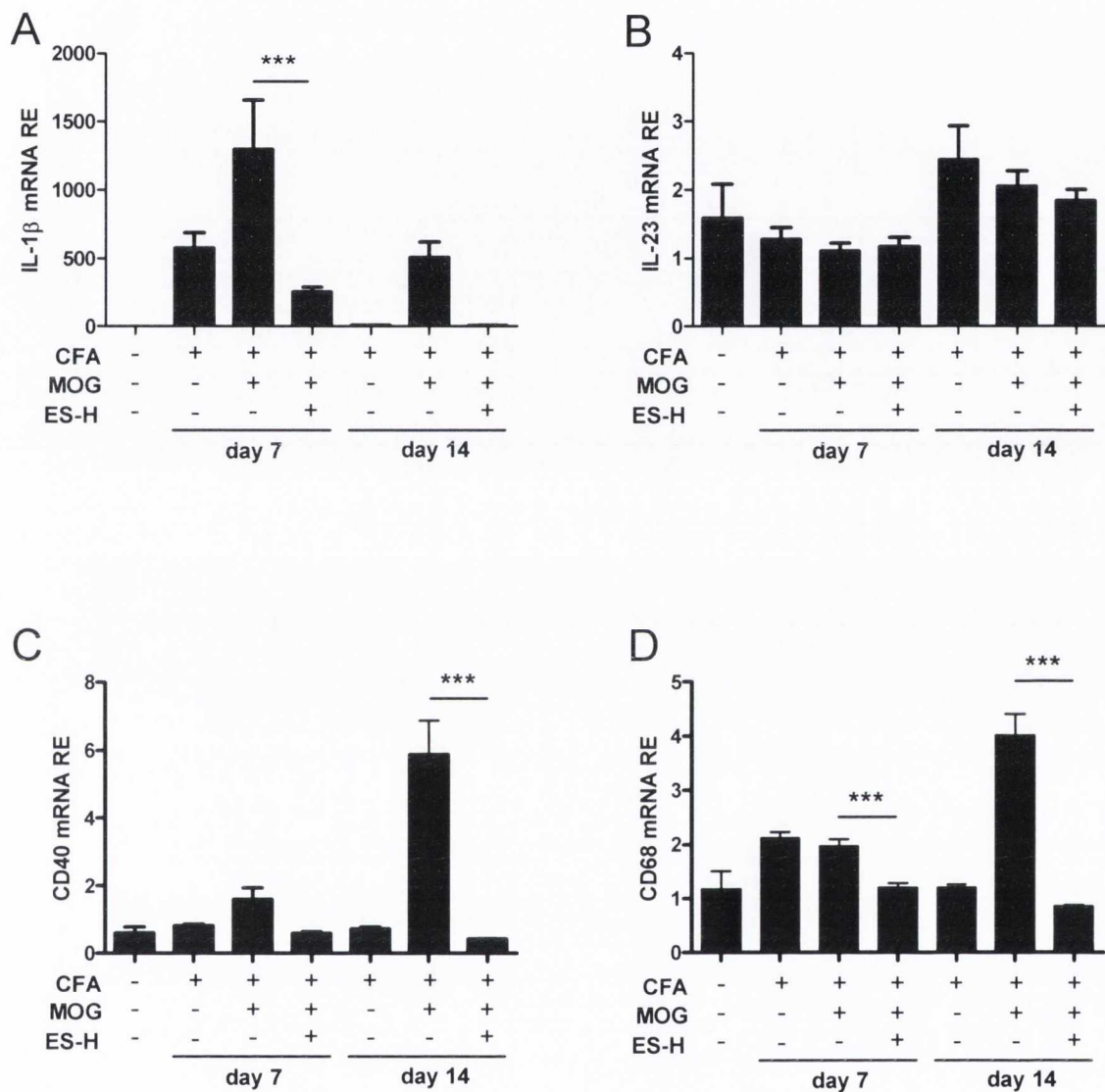


Fig. 5.15 Administration of ES-H inhibits expression of pro-inflammatory cytokines and microglia activation markers in the CNS. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Mice were sacrificed on day 7 and 14 post immunisation. Total RNA was purified from CNS tissue and expression of IL-1 β , IL-23, CD40 and CD 68 was analysed by quantitative RT-PCR. Data represent relative gene expression versus naive control following normalization to the endogenous control, 18S rRNA. Results are mean (+/- SEM); *** P<0.001 vs. MOG/CFA/PBS at relevant time point.

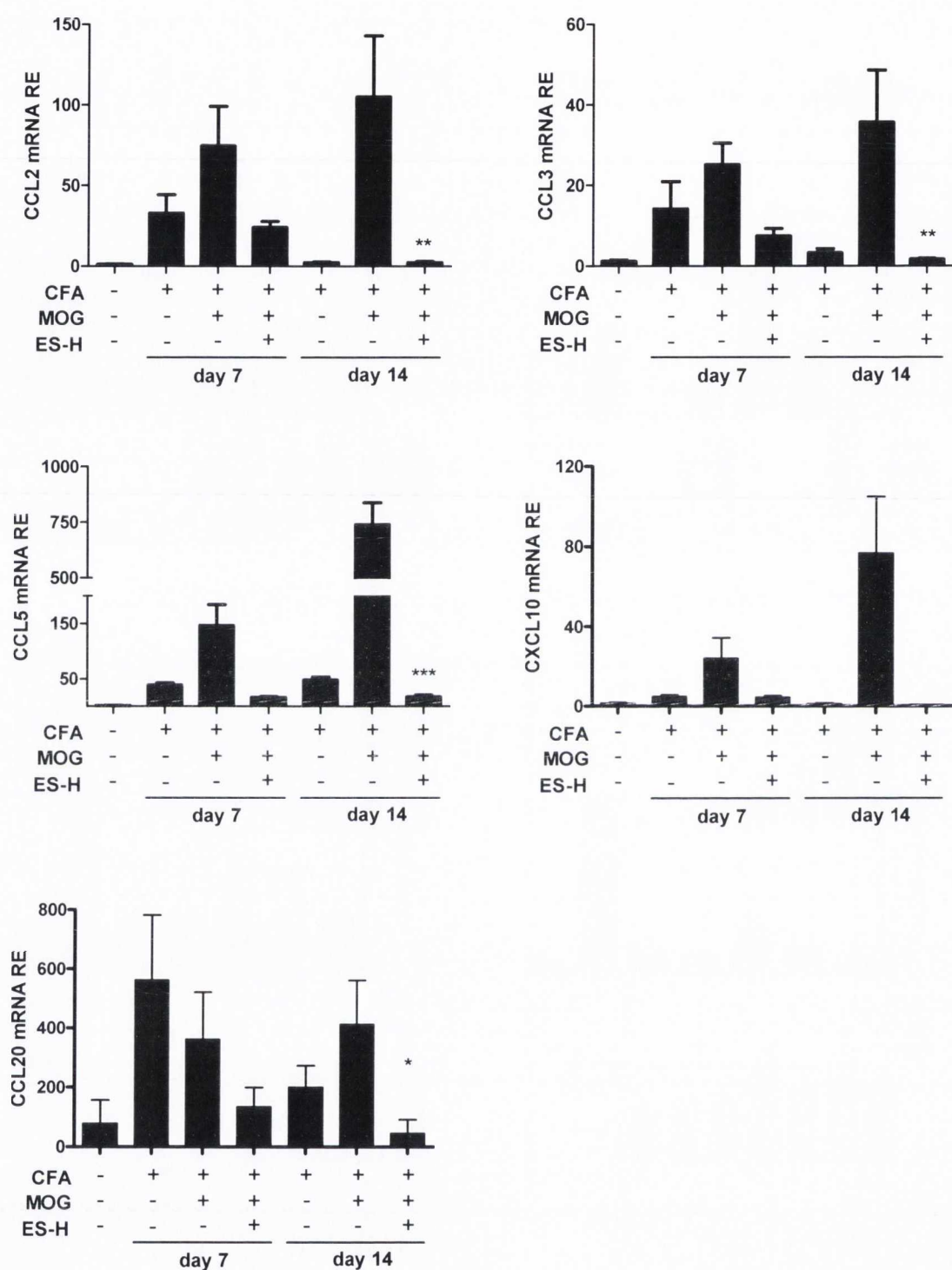


Fig. 5.16 Administration of ES-H inhibits expression of chemokines the CNS. EAE was induced in C57BL/6 mice and animals were treated with ES-H as described in Fig. 5.1. Mice were sacrificed on day 7 and 14 post immunisation. Total RNA was purified from CNS tissue and expression of selected chemokine transcripts was analysed by quantitative RT-PCR. Data represent relative gene expression versus naive control following normalization to the endogenous control, 18S rRNA. Results are mean ($\pm$ SEM); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. MOG/CFA/PBS at relevant time point.

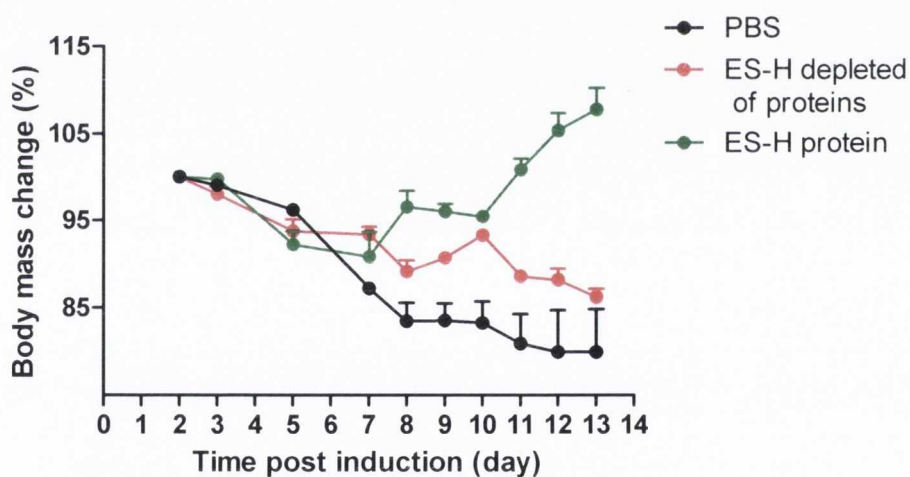
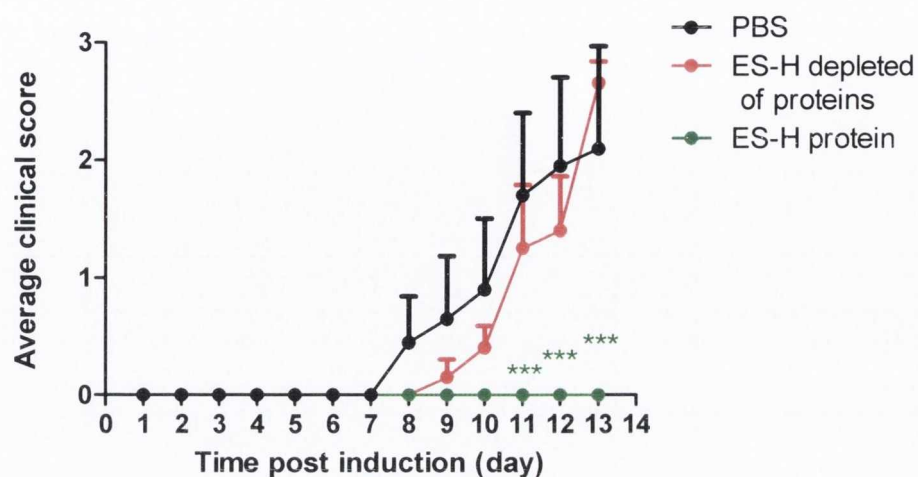


Fig. 5.17 Attenuation of EAE is mediated by a protein. EAE was induced in C57BL/6 mice by immunisation with MOG emulsified in CFA. Mice were injected i.p with protein depleted ES-H, or protein enriched ES-H or PBS on day -1 and every second day after. Animals were monitored daily for clinical signs of the disease (A) and body mass change (B). Results are shown as mean clinical score ($\pm$ SEM) (A), mean body mass change ($\pm$ SEM) (B) for 5 mice per group, *** $P < 0.001$ ES-H protein vs. PBS.

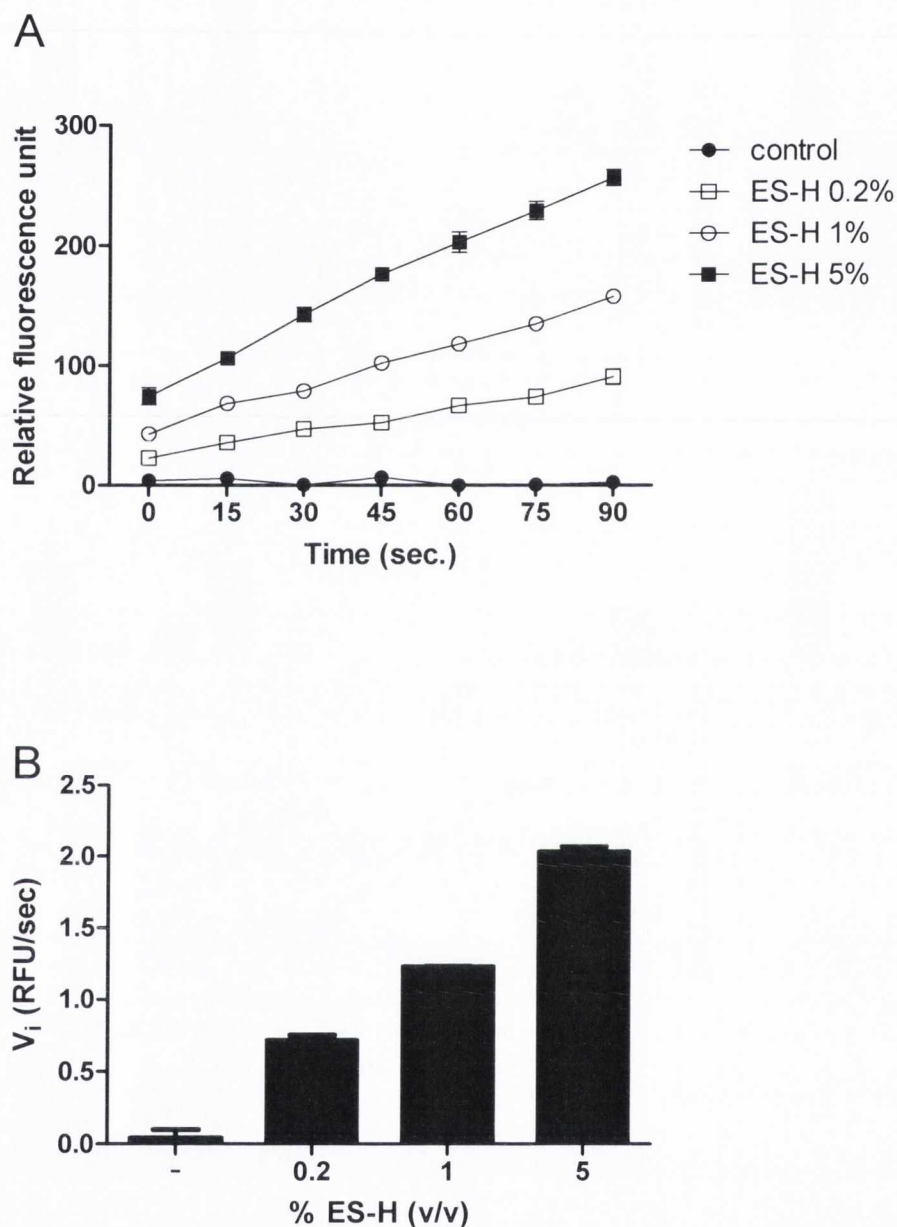
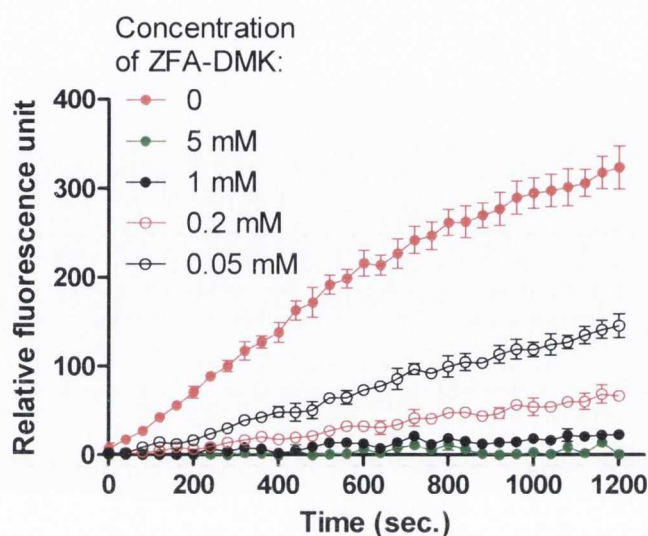


Fig. 5.18 ES-H contains active cysteine proteases. ES-H at a range of concentrations was incubated with fluorescent substrate for cysteine proteases Z-FR-AMC (10 μ M) in acetate buffer. The reaction was monitored over time and enzyme activity was measured as relative increase in fluorescence and plotted versus time (A) or as an enzymes maximum initial velocity (B). Results are shown as mean $\pm$ SD of duplicate assay.

A



B

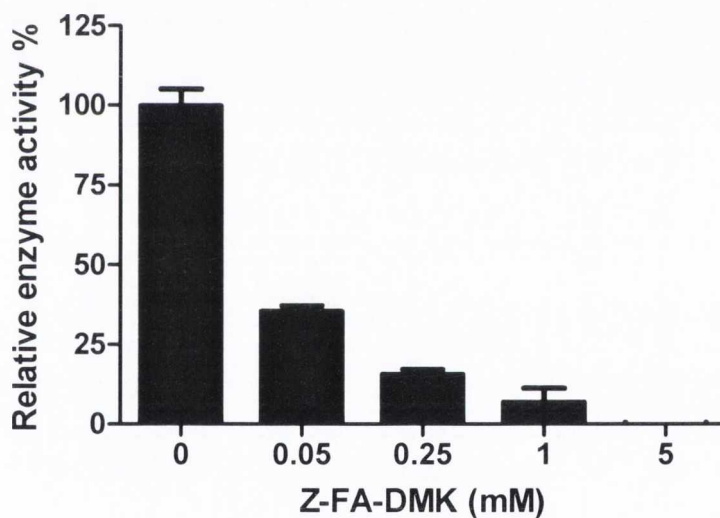


Fig. 5.19 Cysteine proteases from ES-H are inhibited by cathepsin inhibitor ZFA-DMK. ES-H was incubated with or without irreversible cathepsin inhibitor ZFA-DMK at a range of concentrations for 20 min at 37°C. ES-H was then adjusted to working concentration of 0.1% v/v in acetate buffer and incubated with fluorescent substrate for cysteine proteases Z-FR-AMC (10 μ M). Enzyme activity was measured as relative increase in fluorescence and plotted versus time (A). Dose dependent inhibition of enzyme activity with ZFA-DMK versus DMSO control is shown in (B). Results are shown as mean $\pm$ SD of duplicate assay.

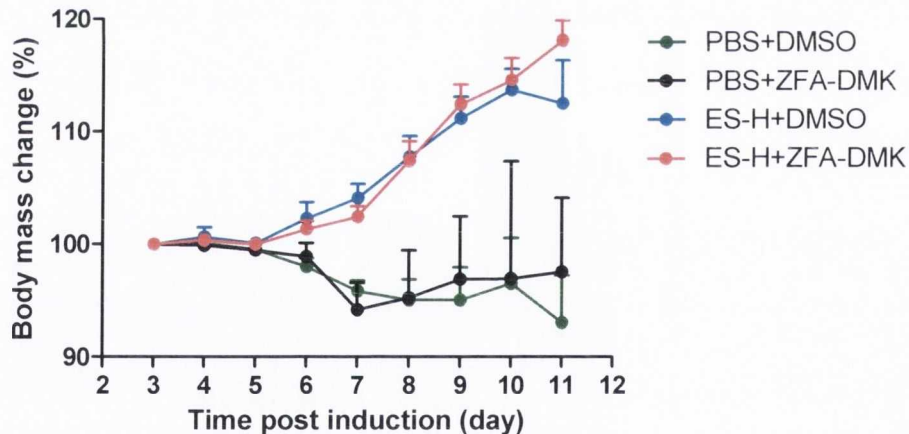
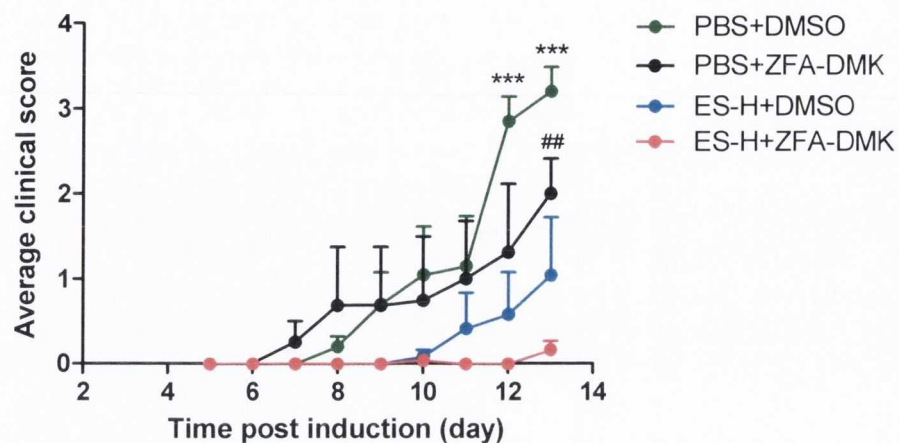
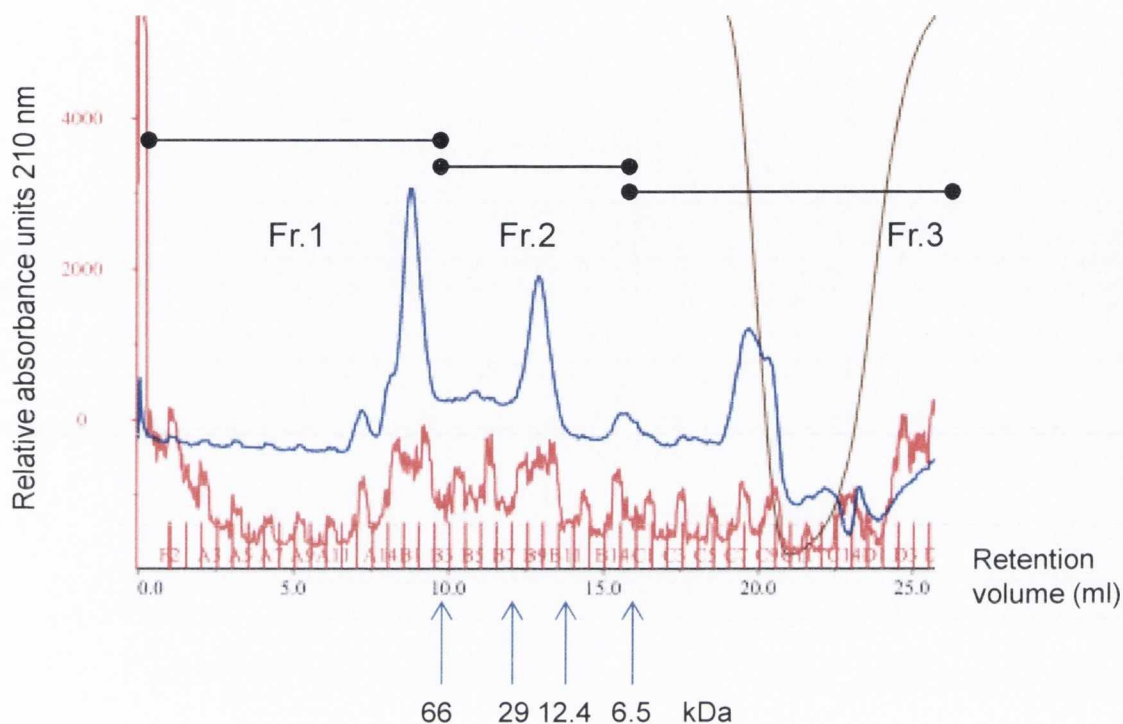


Fig. 5.20 Attenuation of EAE by ES-H is not mediated through active cysteine proteases. EAE was induced as described previously. Mice were injected every second day starting from day -1 with ES-H + DMSO, ES-H pre-incubated with ZFA-DMK (5 mM), PBS + DMSO or ZFA-DMK + DMSO as a control. Animals were monitored daily for clinical score of the disease (A) and weight loss (B). Results are mean score $\pm$ SEM for 5 mice per group.*** $P < 0.001$ ES+DMSO vs. PBS+DMSO; ## $P < 0.01$ ES+ZFA-DMK vs. PBS+ZFA-DMK.



Standard	V_e	M_r	Fraction
Aprotinin	15.81	6.5	B15
Cytochrom C	13.76	12.4	B11
Carbonic anhydrase	12.05	29	B7
BSA	9.73	66	B3

Fig. 5.21 Separation profile of ES-H using gel filtration chromatography. ES-H was subjected to further separation by gel filtration chromatography on a superdex 75 column with PBS as a solvent. Separation was monitored at 210 nm (red line) and 280 nm (blue line). 0.5 ml fractions were collected then pooled into fractions F1, F2, and F3 as indicated. Retention volume (V_e) for standards of known size (M_r) are shown in the table and indicated by arrows.

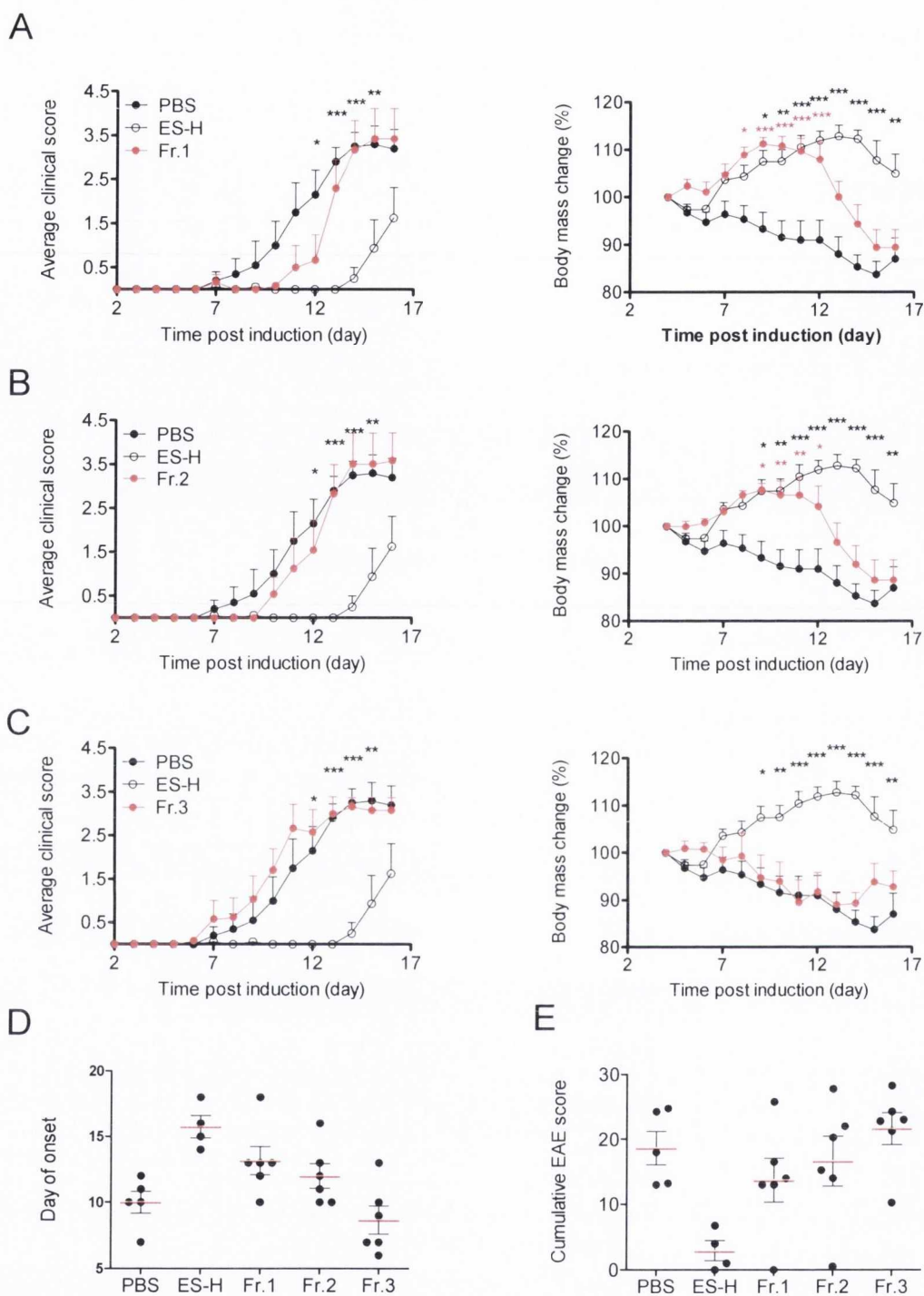


Fig. 5.22 Immunomodulatory activity of ES-H is retained in the fraction Fr.1. EAE was induced in C57BL/6 mice by immunisation with MOG emulsified in CFA. Mice were injected i.p with 40 μ l of ES-H or PBS and an equivalent volume of Fr.1 (A), Fr.2 (B), or Fr.3 (C) on day -1 and every second day after. Mice were monitored daily for clinical signs of the disease (left panel) and body mass change (right panel). Results are shown as mean clinical score ($\pm$ SEM), mean body mass change ($\pm$ SEM) for 4-6 mice per group. The day of onset of clinical symptoms (D) as well as the cumulative disease score (E) are summarised on scatter dot plots where each data point represents individual mouse. Results are from one experiment.

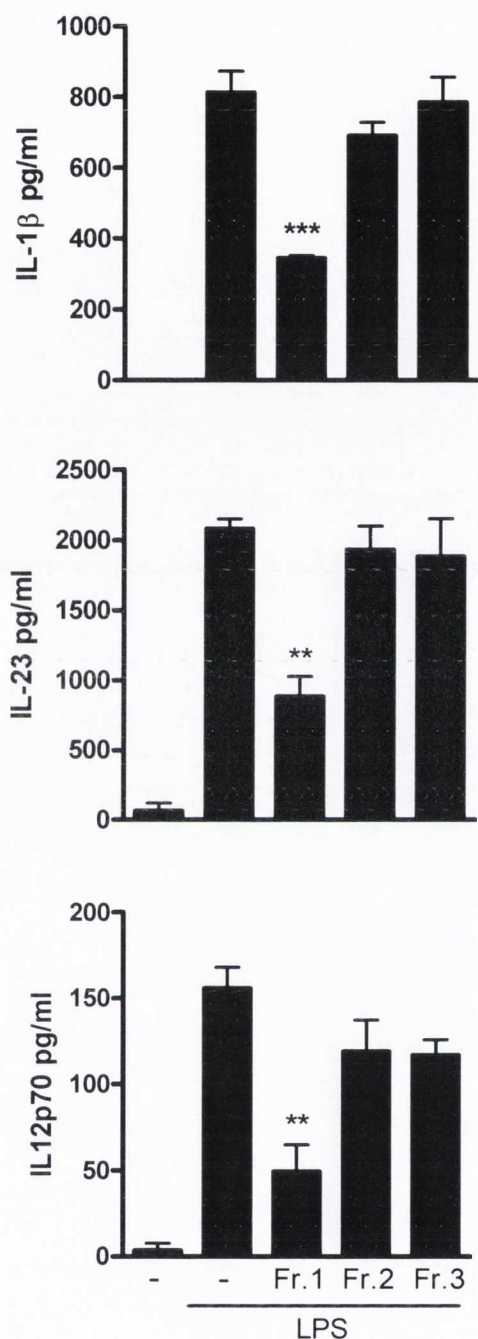


Fig. 5.23 Fraction Fr.1 inhibits LPS induced production of proinflammatory cytokines. DCs derived from C57BL/6 mice were pretreated with fractions Fr.1, Fr.2 and Fr.3 as indicated for 1 h prior to stimulation with LPS (100 ng/ml). The concentration of IL-1 β , IL-23, and IL-12p70 in supernatants was measured after 24 h by ELISA. Results are shown as means ($\pm$ SD) of triplicate assays. ** $P < 0.01$, *** $P < 0.001$ vs LPS only control.

No.	Score	Mass	Significant Matches	Significant Sequences	Identified Protein	Species	ACCESSION
1	1334	8076	32	5	helminth defence molecule-1	Fasciola hepatica	gi 325513923
2	245	24903	7	4	thioredoxin peroxidase	Fasciola hepatica	gi 166235906
3	164	80472	5	3	putative cys1 protein	Fasciola hepatica	gi 40643263
4	76	41537	1	1	fructose-bisphosphate aldolase class-I	Trichinella spiralis	gi 339250594
5	76	160803	7	1	GL14221	Drosophila persimilis	gi 195159972
6	71	56701	2	2	leucyl aminopeptidase	Fasciola hepatica	gi 55714631
7	55	18152	1	1	predicted: promethin-like	Saccoglossus kowalevskii	gi 291241635
8	43	66793	2	1	hypothetical protein BRAFLDRAFT_2475541	Branchiostoma floridae	gi 260792416
9	40	22552	1	1	predicted: sorting nexin-6-like, partial	Macaca mulatta	gi 109083285
10	37	15415	1	1	odorant-binding protein 56h	Drosophila melanogaster	gi 158703886
11	36	14474	1	1	NACP/alpha-synuclein	Homo sapiens	gi 1230575
12	34	50186	1	1	hypothetical protein CBG12261	Caenorhabditis briggsae	gi 268575396
13	33	99858	1	1	ATP-binding cassette sub-family F member 1	Culex quinquefasciatus	gi 170038583

Fig. 5.24 Analysis of mass spectrometry data from Fr.1 against NCBI nr database using Mascot software reveals several candidate immunomodulators. Mass spectrometry analysis was commercially performed by Laboratory of Mass Spectrometry, IBB PAN in Warsaw, Poland. In brief the sample fraction Fr.1 underwent reduction and alkylation of protein disulfide bonds, followed by tryptic digestion. Obtained peptide mixture was separated by liquid chromatography (LC) followed by MS measurement of peptides and their fragmentation spectra (tandem mass spectrometry). Mascot search engine was used to search and identify proteins on the basis of acquired spectra against the NCBI nr protein sequence database. Table shows all protein hits obtained for the sample. Proteins identified with 3 or more significant matched sequences are depicted in red.

General Discussion

Chapter 6.

The key finding of this study was the demonstration that products of the helminth parasite *F. hepatica* were capable of attenuating symptoms of inflammatory and autoimmune diseases in mice. Administration of *F. hepatica* ES suppressed the development of DSS-induced colitis and EAE, models for IBD and MS. The results showed that *F. hepatica* ES was a potent inhibitor of activation of DCs and T cells *in vitro* and these immunosuppressive effects were mediated by molecules segregating to low molecular weight fraction of *F. hepatica* ES (ES-L). However the attenuation of autoimmunity *in vivo* was independent of effects mediated by ES-L and was induced by proteins segregating into a high molecular weight fraction Fr.1. Mass spec analysis of Fr.1 revealed the presence of candidate immunomodulatory molecules FhHDM-1, FhTPx and Cys-1.

In 1966 Leibowitz *et al.* first suggested that the prevalence of MS was correlated with a childhood environment characterized by a high level of sanitation (Leibowitz *et al.*, 1966). Since then several epidemiological studies provided support for the notion that *hygiene hypothesis* could be relevant to MS and could partially explain growing incidence of this disease in developed countries (Cabre *et al.*, 2005; Fleming and Cook, 2006). In addition it was demonstrated that infection with certain helminth species can induce immunoregulatory responses that attenuate clinical symptoms of the EAE, an animal model of MS (Walsh *et al.*, 2009).

One of the most significant reports to suggest that helminth infections could attenuate disease in MS patients was the study carried out in the Raúl Carrea Institute for Neurological Research in Buenos Aires. Correale and Farez reported that among MS patients in their care those naturally infected with intestinal parasites had milder neurological symptoms and significantly less relapses than matched uninfected MS patients (Correale and Farez, 2007). Over a period of 4.5 years, regular MRI scans demonstrated that parasite infected patients had significantly lower number of new, active lesions in CNS when compared to uninfected patients. Remarkably, treatment with anti-helminthic drugs reversed beneficial effects of the infection and substantially increased the activity of the disease in these MS patients. However, the study was observational, not blinded, and involved only 12 infected patients.

Nevertheless these dramatic results encouraged further research into possible use of helminths as therapy for MS and at least three phase I clinical trials investigating safety and beneficial effects of helminth infection have been conducted to date. Although the studies were small, the results supported original suggestion that helminth infection can modulate pathogenic immune responses in MS. A three months treatment with porcine whipworm *Trichuris suis* ova resulted in lower than predicted incidence of new active lesions in brains of participating MS patients (Fleming et al., 2011). Importantly, the effect was lost when treatment was discontinued. No significant safety concerns were identified during that study and a larger phase II clinical trial is currently ongoing (U.S. NIH ClinicalTrials.gov, study identifier NCT00645749). In addition, another phase II study of long term effects of the infection with human hookworm *Necator americanus* on MS is conducted at the University of Nottingham, UK (U.S. NIH ClinicalTrials.gov, study identifier NCT01470521).

Despite the promising preliminary data from human and animal studies the use of live worms as a potential therapy should be considered with caution because of possible side effects and other limitations. Depending on the helminth biology, the migration pattern, the niche the parasite occupies and on the number of infecting worms, this approach has the potential to cause significant damage to host tissues. Light infestations are frequently asymptomatic and clinical trials involving transient colonisation with *T. suis* or infection with *N. americanus* proved to be generally safe with only minor side effects, such as gastrointestinal or flu-like symptoms. Nevertheless, heavy chronic helminth infections may be associated with malnutrition, anaemia and recurrent diarrhea (Satoskar et al., 2009). Infection with *F. hepatica* can cause significant damage to the liver and other tissues due to the migration of juvenile flukes, and may result in anaemia and obstruction of bile ducts at the chronic stage of the disease (Satoskar et al., 2009). Therefore helminth species chosen for deliberate infections must not be pathogenic in humans and cause minimal side effects. This requirement precludes the use of organisms such as *F. hepatica*, *S. masoni* or *A. lumbricoides* despite the excellent results in experimental infections with these parasites in attenuating autoimmunity in mouse models.

This parasite-induced pathology could be easily avoided by the use of immunomodulatory helminth-derived products instead of live worms. It has been demonstrated that crude preparations of ES from *T. spiralis*, *T. suis* and *S. masoni*, when administered to mice with EAE, can reproduce immunosuppressive effect of respective infections and attenuate symptoms of autoimmunity (Kuijk et al., 2012; Sewell et al., 2003). The identification and application of purified parasite-derived components would be far more desirable than infection especially on the grounds of safety. In addition, it is possible that the use of selected helminth products or their synthetic analogues may allow for a finer level of control over their immunomodulatory effects.

Following previous reports that infection with *F. hepatica* can attenuate EAE (Walsh et al., 2009), this study evaluated immunomodulatory properties of *F. hepatica* ES *in vitro* and *in vivo* aiming at providing proof-of-principle and data for future identification and isolation of immunomodulatory molecules that could be used as a therapy against MS or other immune-mediated diseases. Previous studies on the immunomodulatory properties of ES products from various helminth species have reported that these preparations have a potent capacity to suppress TLR-mediated activation and IL-12p70 production by DCs. The current study demonstrated that *F. hepatica* ES can suppress LPS-induced expression of co-stimulatory molecules and production of IL-12p70, IL-23 and IL-6 by DCs. This immunomodulatory effect was not exclusive to TLR4 pathway since pre-treatment with *F. hepatica* ES suppressed production of IL-12p70 by DCs stimulated with ligands of TLR2, TLR3, TLR4 or TLR9. Similar to these findings Hamilton *et al* have previously reported that *F. hepatica* tegument antigens (FhTeg) can inhibit TLR-ligand induced cytokine production by DCs (Hamilton et al., 2009). The present study extended these observations by showing that molecules suppressing production of IL-12p70, IL-23 and IL-6, were present in low molecular fractions (<3 kDa) of *F. hepatica* ES (ES-L) and the inhibition of LPS-induced IL-12p70 was not mediated by peptides. As IL-12p70, IL-23 and IL-6 play important role in polarisation and expansion of Th1 and Th17 cells, the treatment with ES-L could potentially impair the ability of DCs to initiate Th1 and Th17 responses.

In addition to its suppressive effect on pro-inflammatory cytokine production, ES-L also promoted the release of latent TGF- β protein from DCs and this is one of the first studies to demonstrate that parasite products can directly induce secretion of this immunoregulatory cytokine. The release of TGF- β protein was not associated with transcription and translation of *tgfb1* gene and could be partially suppressed by protease inhibitors. The process was mediated by a small molecule that was not a peptide, therefore it appears that the release of pre-made, latent TGF- β by ES-L was dependent on DC-derived proteases. Little is known about the mechanisms of storage and release of latent TGF- β from DCs and the role of these soluble complexes in immunity. Therefore, ES-L could be a useful tool to study production and biology of DC-derived TGF- β , as conventional PAMPs are poor inducers of this cytokine.

The results from this current study found that administration of *F. hepatica* ES was sufficient to delay the onset and reduce the incidence of EAE demonstrating that helminth products as well as live worms can attenuate the symptoms of the autoimmune disease in mice. In addition, *F. hepatica* ES suppressed the development of inflammation mediated by innate immune cells and reduced DSS-induced pathology in a murine model of colitis. These findings demonstrated that *F. hepatica*-induced immunomodulation is mediated by soluble and stable factors produced by the parasite *in vitro* and this enables identification and isolation of these molecules from *F. hepatica* ES by means of fractionation.

In the present study the attenuation of EAE by *F. hepatica* ES was mediated by proteins segregating to a high molecular weight fraction of ES (ES-H; > 3 kDa). Although ES-L showed strong immunosuppressive effects on TLR-mediated activation and cytokine production by DCs and T cells *in vitro*, this fraction had marginal effect on the attenuation of EAE *in vivo*. Conversely, ES-H, which appeared to have little modulatory effect on DCs and T cells *in vitro*, was a potent inhibitor of autoimmunity. These findings highlight the importance of appropriate animal models for characterising and identifying immunomodulatory factors from pathogens. Attenuation of EAE by ES-H was associated with significant reduction in frequency of pathogenic IL-17- or IFN- γ -producing CD4⁺ T cells and concurrent increase in the frequency of IL-4- or IL-

13-producing CD4⁺ T cells in the brains of treated mice. In addition, treatment with ES-H suppressed expression of pro-inflammatory cytokines and chemokines in CNS and significantly decreased the total numbers of infiltrating immune cells. At the same time the treatment substantially increased absolute numbers of IFN- γ -, or IL-4- and IL-13-producing T cells in the peripheral lymph nodes. Although this did not affect production of IL-4 or IFN- γ in response to MOG antigen, the treatment with ES-H reduced the ability of lymph node cells to produce MOG-specific IL-17. Collectively this data suggests that ES-H may attenuate autoimmunity by suppressing production of MOG-specific IL-17 and by promoting retention of activated cells in peripheral lymph nodes, thus limiting their access to the CNS.

A high frequency of cells isolated from lymph nodes and brains of ES-H treated mice secreted IL-4 and IL-13. It has previously been reported that induction of strong Th2 type responses can suppress autoimmunity (Shaw et al., 1997). Attenuation of EAE by immunisation with *S. masoni* ova is associated with increased frequency of IL-4 producing neuroantigen-specific T cells in the brain and the protective effect induced by helminth is lost in Th2 deficient STAT6^{-/-} mice (Sewell et al., 2003). In addition, GA, a disease modifying drug used to treat MS patients, was shown to induce a population of GA-specific Th2 cells that were able to infiltrate CNS and secrete neurotrophic factors that promoted oligodendrogenesis and remyelination (Skihar et al., 2009). The role of ES-H-induced Th2 responses in attenuation of EAE requires thorough investigation. However, unpublished findings from our laboratory suggest that the mechanism of suppression may be independent of Th2 responses, since the suppressive effect of treatment with ES-H was retained in IL-4^{-/-} mice (Finlay and Mills, personal communication).

Helminth infections are often associated with substantial expansion of Treg cells. These cells have been shown to mediate helminth-induced immune suppression and can inhibit immune responses to bystander antigens (Wilson et al., 2005). However, the results of the present study show that treatment with ES-H resulted in a reduced frequency of Foxp3⁺ Treg cells in CNS of mice with EAE, while the frequency of Treg cells in the spleens and draining lymph nodes was not affected by ES-H. Therefore it seems unlikely that attenuation of EAE

by ES-H is mediated by Foxp3⁺ Treg cells. Walsh *et al.* have shown that the protective role of *F. hepatica* infection in EAE was mediated by TGF- β (Walsh *et al.*, 2009). Although the administration of *F. hepatica* ES increased the expression of TGF- β in lymph nodes, the attenuation of EAE by *F. hepatica* ES was only partially dependent on TGF- β , demonstrating that there are other mechanisms mediating the immunosuppressive effect of the ES products that were not observed with the live helminth. It is possible that severe pathology associated with heavy infestation with live worms obscured the picture of regulatory responses in the infection model.

There is a significant unmet need for novel, low-cost, safe treatments for MS. Conventional first line disease modifying drugs are expensive and offer low efficacy for the additional price of inconvenient and painful daily injections. The more effective treatments such as natalizumab or fingolimod can cause rare but potentially fatal side effects associated with substantial immunosuppression and are recommended only as a second line treatment. Many patients do not respond well to available medications, cannot afford them or are reluctant to take them due to substantial safety concerns (Skihar *et al.*, 2009). Novel probiotic helminth therapy could serve as a cheaper and potentially more effective alternative to current therapies. Controlled helminth infections have already been shown to be relatively safe and efficient in successful clinical trials involving patients with Crohn's disease and ulcerative colitis (Summers *et al.*, 2005b) and also MS (Fleming *et al.*, 2011). Such therapy for MS could prove to be inexpensive and associated with only mild and perhaps manageable side effects. Importantly, the immunomodulation induced by helminth infections is associated with induction of host regulatory immune responses. Therefore this approach would have significant advantages over aggressive immunosuppressive treatments for MS, such as monoclonal antibodies, which target T cells, B cells (alemtuzumab), or lymphocyte adhesion molecules (natalizumab), for removal or inactivation causing impairment of immune surveillance. Although helminth infections have been previously associated with diminished immunity to bacterial and viral co-infections (Brady *et al.*, 1999; Walson *et al.*, 2009), it is unlikely that these could be life threatening. In

contrast, the treatment with natalizumab increases the risk of developing potentially fatal PML or other opportunistic viral infections of the CNS (Stüve et al., 2006). Importantly the immunosuppressive effects of the therapy with monoclonal antibodies can be observed months after cessation of the treatment, which significantly reduces the survival in cases of PML (Stüve et al., 2009).

Although infection with helminths can also be associated with immunosuppression, the use of purified immunomodulatory molecules or combinations thereof could give an advantage of better control over immunoregulatory effects and prevent impairment of host immunity to viruses, bacteria and protozoa. Infection with *F. hepatica* as well as treatment with *F. hepatica* ES was shown to reduce protective Th1 responses against the respiratory pathogen *B. pertussis* in mice immunised with whole cell pertussis vaccine (Pw) (Brady et al., 1999; O'Neill et al., 2001). The immune suppression observed was mediated by cysteine protease FhCatL and could be mimicked with recombinant FhCatL protein. Importantly enzymatic activity was crucial for the immunosuppressive effect of FhCatL as pre-treatment of *F. hepatica* ES with a specific inhibitor of cysteine proteases reversed the protective responses against *B. pertussis* in Pw-immunised mice (Brady et al., 1999). The present study demonstrated that active cysteine proteases were present in ES-H preparation, but they were not responsible for the attenuation of EAE. Therefore it is possible that therapies based on selected purified components of *F. hepatica* ES may not be associated with systemic immunosuppression that could be detrimental to the patient.

The current study further identified the fraction Fr.1 of ES-H, that contained molecules responsible for immunosuppressive effect of *F. hepatica* ES against autoimmunity *in vivo*. Mass spectrometry analysis of Fr.1 identified several proteins, including two prominent hits ascribed to molecules that had previously reported to exhibit some immunomodulatory properties. FhHDM-1 was shown to interfere with antigen presentation by macrophages, thus impeding initiation of adaptive responses (Robinson et al., 2012). FhTPx is a potent inducer of alternatively activated macrophages that are necessary for the development of

Th2 responses during *F. hepatica* infection, but can also be involved in helminth mediated immunosuppression (Donnelly et al., 2008; Taylor et al., 2006). The third identified protein Cys-1 (FhCys-1) is a putative cysteine protease inhibitor (cystatin) and this class of molecules have been associated with immunomodulation induced by various helminths such as *O. vulvus* or *B. malayi* (Johnston et al., 2009). Cystatins can interfere with plethora of immunologically important processes which are regulated by cysteine proteases. These processes include antigen processing, regulation of endosomal TLR signalling or activation of certain cytokines, chemoattractants and zymogens (Conus and Simon, 2010). However, there are no reports on the effect of FhHDM-1, FhTPx and FhCys-1 on the development of autoimmunity. The molecules identified in the present study can be now cloned and expressed in suitable expression system to evaluate their immunomodulatory effect in EAE and other models of autoimmunity as well as allergy. Recombinant helminth proteins will also enable the further study of cellular and molecular mechanisms involved in the immunity to parasites.

The data presented in this work constitute a solid foundation for further investigation into specific immunomodulatory factors secreted by *F. hepatica*. These findings have contributed to our understanding of immunomodulatory mechanisms induced by helminth-derived products and could help develop cheap, safe and effective treatments against autoimmune disorders.

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