Modulation of innate and adaptive immunity by *Fasciola hepatica*

Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

Shauna Quinn

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Supervisor: Professor Kingston Mills

Immune Regulation Research Group

School of Biochemistry and Immunology

Declaration of authorship

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Shauna Quinn
School of Biochemistry and Immunology
Trinity College Dublin

Abstract

Helminth parasites have developed highly effective mechanisms of immune subversion that helps prolong their survival in the host, resulting in the development of chronic infections. A bystander effect of this immune modulation is the simultaneous suppression of immune responses that are pathogenic in autoimmune diseases. This forms the basis of the hygiene hypothesis, which has attributed the rise in allergy and autoimmune diseases in developed countries to the simultaneous decrease in the incidence of infectious disease, in particular helminth infection. The experimental evidence to support the hygiene hypothesis has inevitably led to the study of live helminth therapy for the treatment of autoimmune diseases in the clinic, however, most studies have failed to show significant beneficial effects in patients. Furthermore, the logistical and ethical obstacles associated with the use of live infection has motivated the search for helminth-derived immunomodulatory molecules. Helminth-derived products provide an invaluable tool for the study of helminth-induced immune modulation, without the complication of pathological responses associated with helminth infection.

This study focussed on the immunomodulatory properties of total extract from *Fasciola hepatica* (FHTE). The results revealed that FHTE enhanced IL-10 and IL-1RA production, but suppressed LPS-induced IL-1β, IL-23 and IL-12p40 production by dendritic cells (DCs). Interestingly, FHTE also trained macrophages to be more anti-inflammatory. Bone-marrow derived macrophages trained *in vitro* with FHTE produced more IL-10 and less TNF in response to restimulation with LPS and Pam3Cys. Furthermore, training of mice with FHTE *in vivo* polarised M2 macrophages and suppressed neutrophil recruitment, resulting in subsequent impairment of pathogenic T cell responses in the periphery and protection against the T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis (EAE). This was associated with a significant reduction of IL-17-producing γδ and CD4 T cells infiltrating the central nervous system (CNS). Furthermore, attenuation of EAE by innate immune training was mediated by components that segregated in the high molecular weight fraction of FHTE and the immunomodulatory effects were found to be protein-mediated.

Although most research to date on parasite-mediated immune subversion has focused on modulation of innate immune responses and induction of regulatory T cells, this study demonstrated that helminth products can directly supress effector and pathogenic T cells. FHTE was found to have a potent suppressive effect on IL-1R1 and IL-23R expression on γδ T cells, resulting in reduced receptor signalling and suppressed IL-17A production. Further investigation revealed that the immunomodulatory activity was present in the fraction that contained low molecular weight non-protein components. FHTE also suppressed activation of autoantigen-specific T cells, impairing their ability to proliferate and to induce EAE upon transfer to naive mice. This was accompanied by suppressed infiltration of IL-17A-producing CD4 T cells and γδ T cells into the CNS. Furthermore, this is the first demonstration of direct immunosuppressive effect of helminths on T cells.

The findings demonstrate that helminth-derived products provide useful tools to study the mechanisms of helminth immunomodulation and the bystander effect of suppressing pathogenic T cells responses that mediate allergy and autoimmunity. As well as providing further validation of the hygiene hypothesis, the study has identified two novel mechanisms of helminth-mediated subversion strategies that directly target innate or adaptive immune responses.

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Publications

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In loving memory of Mary Miskelly,
My Grandmother and Godmother.

Abbreviations

2-ME	2-mercaptoethanol
ANOVA	Analysis of variance
AP1	Activator protein 1
APC	Antigen presenting cell
Arg1	Arginase 1
ASC	Apoptosis-associated speak-like protein containing a card
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCA	Bicinchonic acid
BCG	bacille Calmette-Guerin
BMDCs	Bone marrow derived dendritic cells
BMDMs	Bone marrow derived macrophages
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
cDCs	Conventional DCs
CFA	Complete Freund's Adjuvant
Chi3l3	Chitinase 3-like proteins
CNS	Central nervous system
cRPMI	Complete RPMI
Ct	Cycle threshold
CTLA4	Cytotoxic T-lymphocyte antigen 4
CTV	Cell trace violet
DAMPs	Danger associated molecule patterns
DCs	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
EB/AO	Ethidium bromide/acridine orange
EDTA	Ethylenediaminetetraacetic acid
Egr2	Early growth response protein 2
ELISA	Enzyme-linked immunosorbent assay

F. hepatica	*Fasciola Hepatica*
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FHES	*F. hepatica* excretory secretory
FHTE	*F. hepatica* total extract
FHTE-H	High molecular fraction FHTE
FHTE-L	Low molecular fraction FHTE
Fig	Figure
FMO	Fluorescence Minus One
Foxp3	Factor forkhead box P3
Fpr2	Formyl peptide receptor 2
GM-CSF	Granulocyte monocyte-colony stimulating factor
HLA	human leukocyte antigen
HRP	Horseradish peroxidase
i.p	Intraperitoneal
IBD	Inflammatory Bowel Disease
iDC	Immature DC
IFI16	IFN-inducible protein 16
IFN-γ	Interferon-gamma
IL	Interleukin
IL-1RA	IL-1 receptor antagonist
IL-1α	IL-1 alpha
IL-1β	IL-1 beta
ILC2	Innate lymphoid cells 2
iTreg	Inducible Treg
JAK2	janus kinase 2
LAL	Limulus amebocyte lysate
LAP	Latency associated peptide
LD	Live-dead
LN	Lymph node
LPM	Large peritoneal macrophages
LPS	Lipopolysccharide
M1	Classically activated macrophages

M2	Alternatively activated macrophages
MACS	Magnetic activated cell sorting
MBP	Myelin basic protein
M-CSF	Monocyte-colony stimulating factor
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MNCs	Mononuclear cells
MOG	Myelin oligodendrocyte protein
MRC1	Mannose receptor, C type 1
MS	Multiple Sclerosis
Mtb	*Mycobacterium tuberculosis*
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
nTreg	Natural Treg
PAMP	Pathogen associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PBS/PS	Penicillin/streptomycin in PBS
PD-L2	programmed cell death protein 1 ligand 1
PEC	Peritoneal exudate cells
PLP	Proteolipid protein
PMA	12-o-tetra decanoylphorbol 13 acetate
PRR	Pattern recognition receptor
PT	Pertussis toxin
qRT-PCR	Quantitative real time polymerase chain reaction
Relm-α	Resistin-like molecule alpha
RIG	Retinoic acid-inducible gene 1
RLR	RIG-I like receptor
RORγt	RAR-related orphan receptor gamma transcription

ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT-PCR	Real time polymerase chain reaction
s.c	Subcutaneous
SAR	Systemic acquired resistance
SD	Standard deviation
SEM	Standard error of the mean
SPF	Specific pathogen free
SPM	Small peritoneal macrophages
TCR	T cell receptor
Tfh	Follicular helper T
TGF-β	Transforming growth factor-β
Th	T helper
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TSLP	thymic stromal lymphopoietin
γδ	Gamma-delta

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Chapter 1.
General Introduction

1.1 The innate immune system

The innate immune system is the first line of defence against invading pathogens and plays a vital role in the early recognition of pathogens, initiation of the inflammatory response and activation of the adaptive immune system. The innate immune system relies on the recognition of conserved motifs on the pathogen via specific receptors, known as pattern-recognition receptors (PRRs) [1]. PRRs are found on the surface of innate immune cells and recognise pathogen associated molecular patterns (PAMPs), which are considered to be invariable among entire classes of pathogens, are often virulence factors and are vital to the survival of the microorganism [1, 2]. Recognition of invading microorganisms is achieved through different families of PRRs, including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG)-I-like receptors (RLRs) [3]. Furthermore, damaged host cells are capable of releasing damage-associated molecular patterns (DAMPS) in times of stress or damage following infection or injury and these DAMPS also activate innate immune cells by binding to PRRs [4].

TLRs are transmembrane receptors that contain leucine-rich repeating in their extracellular domains [5]. TLRs interact with PAMPs on the surface of pathogens and trigger the induction of proinflammatory cytokines, which orchestrate early host immune responses, as well as activating and shaping the adaptive immune response. Examples of cell surface TLRs found on innate immune cells include TLR4 and TLR5, which recognise lipopolysaccharide (LPS) and flagellin respectively. The TLR family also includes intracellular TLRs, expressed on the endosomal membrane, which recognise foreign nucleic acids of viral and bacterial origin [3]. An example of an intracellular TLR is TLR9 that recognises unmethylated ssDNA-containing CpG motifs found in bacteria and viruses [1]. Two of the main cells within the innate immune system that recognise PAMPs and orchestrate the adaptive immune system are macrophages and dendritic cells (DCs).

1.1.1 *Macrophages*

Macrophages are vital cells of the innate immune system and play an important role in organ development, tissue turnover, and regeneration. PRR activation initiates phagocytosis, an important innate defence mechanism involving the internalization of particles and infectious agents. This process is actin dependent and usually involves the uptake of large particles [6]. Furthermore, activation of these PRRs leads to a signalling cascade through MYD88 causing induction and secretion of inflammatory mediators, such as tumour necrosis factor alpha (TNF), interleukin (IL)-1β and IL-6 [1], which are required for T cell differentiation and activation. Tissue resident macrophages can be embryonically or bone marrow derived depending on the tissue and are maintained through lifelong self-renewal [7]. During steady state conditions, tissue resident macrophages are anti-inflammatory and protect against indiscriminate inflammation. For example, the production of IL-10 in the gut maintains macrophages in an anti-inflammatory state, however, in the absence of IL-10, macrophages can cause gut inflammation by reacting to commensal bacteria [8]. During infection or injury, circulating blood-derived monocytes are recruited into the tissues and differentiate into distinct types of effector macrophages depending on the microenvironment [9]. The diversity and overlap of cues in response to different stimuli generate either classically activated macrophages (M1), which induce inflammation or alternatively activated macrophage (M2), which decrease inflammation and encourage tissue repair.

1.1.2 *M1 and M2 responses*

During an inflammatory response, resting macrophages are converted into potent inflammatory cells with an enhanced ability to phagocytose and produce nitric oxide (NO). Classical activation occurs primarily during bacterial or viral infection, where release of PAMPS and DAMPS or production of IFN-γ during infection or injury, promotes M1 activation and ensures tissue sterility [10, 11]. In contrast, alternatively activated macrophages are induced in response to helminth infection. Classically activated macrophages produce large amounts of IL-6, IL-1β and TNF as well as IL-12 required for Th1 polarisation, leading to initiation of adaptive immune responses.

However, since M1 macrophages responses are vital for ensuring resistance to bacterial infection, many pathogens such as *Salmonella Typhimurium* and *Mycobacterium tuberculosis* have evolved mechanisms to interfere with M1 polarization [12–14]. Conversely, excessive or unresolved M1 activation can cause chronic inflammation and tissue damage [15]. As such, M1 macrophages have been implicated in the pathogenesis of many inflammatory diseases, including atherosclerosis and diabetes [16, 17]. Furthermore, M1 macrophages are known to play a role in the development of multiple sclerosis (MS), as well as spinal cord injury and traumatic brain injury [18, 19]. During the resolution of inflammation, M1 macrophages switch to alternatively activated or M2 macrophages to prevent immunopathology. As a result, M2 macrophages are often described as having anti-inflammatory or reparative functions and release anti-inflammatory cytokines such as IL-10 and Transforming growth factor-β (TGF-β). Furthermore, in the EAE model, polarising macrophages from M1 to M2 has shown protective effects in the modulation of disease [20]. Furthermore, IL-4 is essential in promoting M2 macrophage activation as mice lacking IL-4 or IL-4Rα fail to induce alternatively activated microglia and have significantly worse EAE pathology [21]. As a result, the identification and targeting of specific macrophage phenotypes in disease may be useful diagnostically or therapeutically. However, until recently, it has not been clear that distinct M1 and M2-type macrophages exist *in vivo*, due to a shared set of genes involved in basic macrophage functions. A recent study by Jablonski et al. has identified markers specific for classically and alternatively activated macrophages [22]. Using gene expression profiling, they have identified expression of cluster of differentiation (CD)38 and Formyl peptide receptor 2 (Fpr2) specific for M1 macrophages, whereas the expression of early growth response protein 2 (Egr2) and mannose receptor, C type 1 (MRC1/CD206) was M2-specific (Figure 1.1). The identification of these genes is not only important for distinguishing macrophage populations, but also provide further information on macrophage function and modulation during infection and disease.

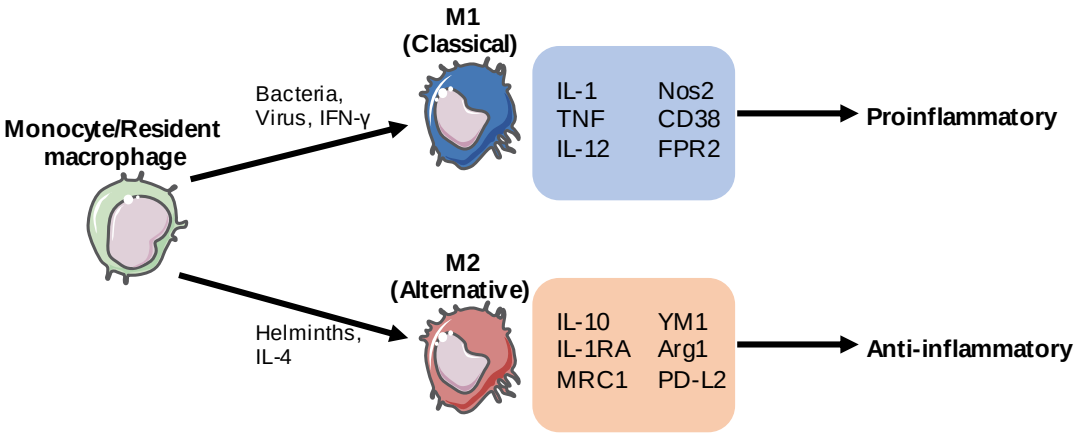

Figure 1.1 The differentiation pathways of classically activated M1 macrophages and alternatively activated M2 macrophages.
Classical activation occurs primarily during bacterial and viral infection or in response to IFN-γ production, leading to release of proinflammatory mediators that promote the differentiation of Th1 cells. In contrast, macrophages are polarized towards alternatively activated macrophages during helminth infection, which is important for limiting Th1 and Th17 responses and preventing immunopathology.

1.1.3 *DCs*

One of the main roles of the innate immune system is the presentation of antigens to T cells by antigen presenting cells (APCs), such as DCs. DCs were first visualized in the skin as langerhan cells [23, 24] and are essential in driving T cell-mediated responses, thereby linking innate and adaptive immunity. DCs arise from hematopoietic stem cells produced in the bone marrow. They are initially transformed into immature DCs (iDCs) that can be found in all tissue exposed to the external environment. These cells are highly phagocytic but have poor antigen presenting capacity [25]. They constantly sample the surrounding environment and are capable of recognizing microbial structures via PRRs [1]. Upon entry of a pathogen, iDCs effectively recognize, ingest and break down microbes. However, unlike other cells of the innate immune system, recognition of a pathogen leads to the activation of transcription genes encoding for major histocompatibility complex (MHC) and costimulatory molecules CD40, CD80 and CD86. Pathogen recognition triggers upregulation of C-C chemokine receptor type 7 (CCR7) on DCs, allowing migration to the local lymph nodes (LNs) where they function as APCs to activate naïve T cells [26]. Here, the processing of

peptides derived from pathogens and the loading onto MHC molecules allow DCs to effectively present antigens and activate T cells.

1.2 The adaptive immune system

The adaptive immune system consists of T and B lymphocytes that have an ability to recognise specific antigens through a large repertoire of antigen-specific recognition receptors. The adaptive immune response is primed by the innate immune system and usually takes days to be fully activated and to initiate effective responses against invading pathogens. T and B cells can respond to a huge range of antigens and the specificity of the adaptive immune response is due to somatic hypermutation and genomic recombination of receptor genes. Furthermore, these cells have the ability to develop immunological memory that can initiate a faster response to re-infection and prevent chronic disease [27]. The primary role of B cells is to produce antibodies, which inhibit the binding of microbes to tissues in addition to enhancing the ability of innate immune cells to phagocytise pathogens, via a process known as opsonisation. T cells also have an ability to recognise antigens, however, the antigen must be presented via MHC on the surface of an APC. MHC I can be found on the surface of all nucleated cells and engage with CD8 T cells, which can recognise and kill virally infected cells. In contrast, MHC II molecules are only found on the surface of antigen presenting cells and are required for presentation of antigens to CD4 T cells. The TCR itself does not transmit signals upon binding the ligand, but it is covalently associated with CD3 signalling molecules, forming a multisubunit TCR complex [28].

1.2.1 *T cell activation*

Unlike B cells, T cells usually fail to recognise antigens in the absence of antigen presentation. T cells migrate from the bone marrow to the thymus, where they undergo positive and negative selection and their affinity for foreign peptide is determined. Before leaving the thymus, T cells are committed to either a CD4 or CD8 lineage, depending on their affinity for MHC I/II. However, T cells have the potential to become αβ or γδ T cells, where β or δ TCR chain selection is determined by recombination of the gene encoding the TCR chain [29]. T cells then travel to the periphery where they can be activated by antigen presented on

MHC molecules expressed on DCs and other APCs. During migration, DCs lose the ability to phagocytise material and become highly efficient at presenting antigen to T cells via TCR and MHC interaction. Binding of the TCR on CD4 T cells to MHC II bound peptide on the surface of the APC provides signal one and leads to activation of the TCR, causing downstream signalling culminating in the activation of transcription factors, nuclear factor of activated T-cells (NFAT), activator protein 1 (AP1) and NFκB, which are required for induction of the IL-2 gene. However, interaction between the TCR and the MHC bound peptide is not sufficient to fully activate T cells. An additional signal is required though the interaction of the co-stimulatory molecule CD28 on the surface of the T cell, with CD80/86 on the surface of the APC [30]. Furthermore, activated CD4 T cells require polarizing cytokines produced by other immune cells to differentiate into the distinct T helper (Th) subtypes (Figure 1.2). The newly activated T cell can then clonally expand and elicit effect functions against invading pathogens.

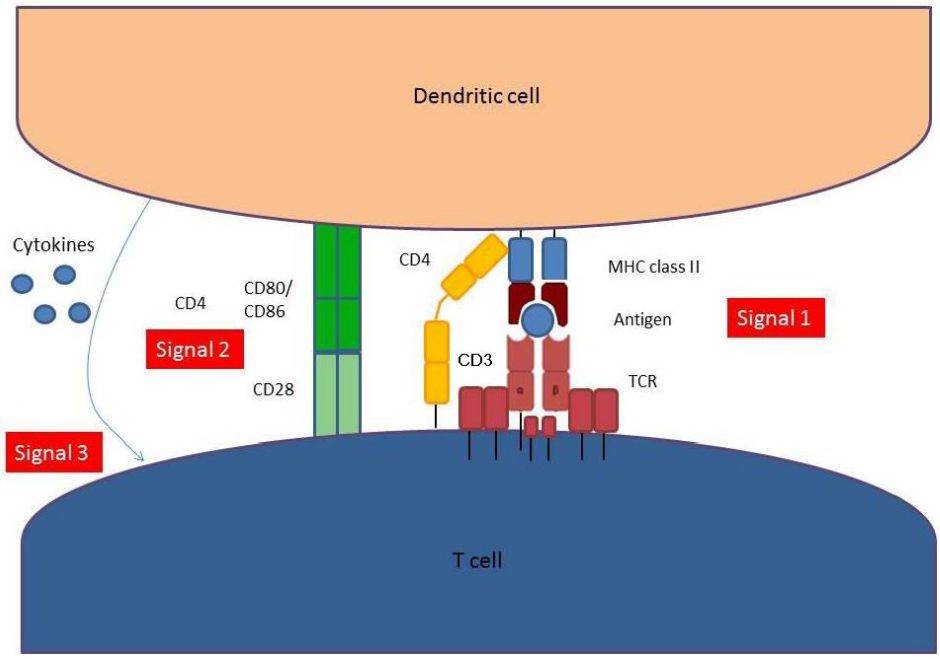

Figure 1.2 Antigen presentation and activation of naïve CD4 T cells.
Upon encountering a pathogen, iDCs migrate to the LNs where they present antigen to a naïve CD4 T cells. Full activation of CD4 T cells requires three signals: signal one is the interaction between the peptide bound MHC II and the TCR; signal two is co-stimulation involving the interaction of CD80/CD86 on the surface of the APC and CD28 on the T cell and; signal three involves cytokine production leading to the differentiation into different Th lineages.

1.2.2 *T cell differentiation*

Upon activation, naïve CD4 T cells differentiate into effector T cell subgroups depending on the cytokine production and signals they receive during activation. Mossman and Coffman in 1986 demonstrated that long-term CD4 T cell clone could be divided into two dominant groups, Th1 and Th2, and that the nature of the pathogen is considered to be fundamental in driving their induction [31]. Th1 cells produce IFN-γ and play a key role in M1 activation and host defence against intracellular pathogens, including *M. tuberculosis* [32]. In contrast, Th2 cells are involved in humoral immunity and are vital to the control of helminth infection. Another more recently described subset, Th17, are important in the clearance of certain fungal and extracellular bacterial infection, but also play a role in T cell-mediated autoimmunity [33]. Follicular helper T (Tfh) cells are specialized

providers of T cell help to B cells. Tfh cells trigger the formation and maintenance of germinal centres, as well as being vital for the development of high affinity antibodies and memory B cells. Lastly, Treg cells are key regulators of the immune system and can suppress immune responses via cell-cell contact or through the production of anti-inflammation cytokines [34]. Unlike Th cells, γδ T cells emerge for the thymus as mature T cells that respond rapidly to cytokines (Figure 1.3).

1.2.3 *Th1 cells*

The induction of Th1 responses is essential for the clearance of intracellular bacteria [32], as well as being implicated in the pathogenesis of many autoimmune disorders, such as MS [35]. IL-12 production by DCs is vital for the development and activation of Th1 cells. Once activated, they move to the outer area of B cell follicles where the Th1 cells are presented with antigen by antigen-specific B cells [36]. This results in further B cell activation and class switching towards IgG2a and IgG2b through the interaction with CD154 and soluble cytokines, in particular IFN-γ [37–39]. IFN-γ functions to upregulate T-bet, the master regulatory transcription factor of Th1 cells. IFN-γ is a dimerized cytokine and a member of the interferon family that was originally identified as interfering with viral replication [40]. Furthermore, IFN-γ is important in activating M1 macrophages to increase their microbicidal activity. Classical activation leads to the expression of inducible NO synthase and induction of reactive oxygen species (ROS) and nitrogen mediators that act to kill microbes. The role of IFN-γ in autoimmunity remains unclear, however, a study revealed that 7 out of 18 MS patients treated with IFN-γ resulted in exacerbation of disease [35]. Furthermore, Willenborg et al. demonstrated that IFN-γ was not required for the induction of myelin oligodendrocyte protein (MOG)-specific effector T cells in EAE but plays a protective important role during the effector phase of disease [41, 42].

1.2.4 *Th2 cells*

Th2 cells are vital for control of extracellular parasites including helminths infection, however, they also play a key role in promoting wound repair following infection or injury [43]. Furthermore, Th2 cells also mediate the pathogenic immune responses that cause asthma and other allergic diseases [44, 45]. Th2

cells produce a wide variety of cytokines including IL-4, IL-5, IL-13, IL-33 and IL-10. IL-4 is important in driving alternatively activated macrophages, as well as being involved in T cell activation. By signalling through STAT6, IL-4 from Th2 cells drives isotype switching in B cells to IgG1 and then IgE [46]. IgE then binds to FcεR1 on basophils and mast cells. This leads to antigen recognition, resulting in release of granulocytes and secretion of active mediators including histamine, which are required for control of helminth infection. Furthermore, IL-5 from Th2 cells is required for eosinophil recruitment [47, 48], as well as enhancing antibody secretion by B cells. In addition, production of IL-13 is required for the “weep and sweep” process during helminth infection, which is required for elimination of the parasite by inducing changes in the macroscopic nature of mucous membranes.

The role for IL-4 in driving Th2 responses has been under debate. *In vitro* studies have shown that IL-4 binds to IL-4Rα, leading to activation of the transcription factor STAT6, which controls GATA3, the master transcription factor for Th2 responses [49]. However, studies *in vivo* have demonstrated that Th2 differentiation takes place even in the absence of IL-4. Infection with *Nippostrongylus brasiliensis* induces a robust Th2 response in mice that lack the IL-4Rα [50]. Furthermore, IL-4-independent Th2 differentiation was shown to be mediated by other type 2 cytokines including IL-25, thymic stromal lymphopoietin (TSLP) and IL-33 [49]. In the absence of IL-4, TSLP produced by epithelial cells can act on DCs to induce a type-2 APC phenotype. In addition, TSLP has been implicated in Th2-mediated models of airway hypersensitivity and asthma [51].

1.2.5 *Th17 cells*

Th17 cells are an inflammatory CD4 T cell subset that secrete a plethora of cytokines, including IL-17A, IL-17F, GM-CSF, IFN-γ and IL-22 [52]. IL-6 and TGF-β were originally described as the cytokines that promoted Th17 differentiation, as they were crucial for the activation of STAT3, which regulates transcription of RAR-related orphan receptor gamma transcription (RORγt), the master regulatory transcription factor of Th17 cells. However, it was later shown that IL-1β and IL-23 were also vital for the differentiation of human Th17 cells and expansion of mouse Th17 cells [53–55]. Furthermore, IL-23 was shown to be required for the maturation and pathogenic function of Th17 cells [56, 57]. IL-23

is composed of two subunits, p40 and p19 and is a member of the IL-12 family [58]. The receptor for IL-23 is composed of IL-12R subunit, IL-12Rβ1 but uses IL-23R for signal transduction [59]. IL-23 predominately associates with janus kinase 2 (JAK2) in a ligand dependent manner with STAT3, unlike IL-12, which predominantly induces STAT4 [60]. Naïve T cells do not express IL-23R, therefore IL-23 acts as a survival signal for differentiating Th17 cells. STAT3 activation leads to upregulation of IL-23R on the surface of Th17, which is critical for IL-17A production. Furthermore, IL-1β is a vital cytokine required for Th17 differentiation. IL-23 stimulation alone was shown to be a poor driver of IL-17A production by CD4 T cells, however, this was significantly enhanced in the presence of IL-1β [61]. Th17 cells express receptors for IL-1 and IL-23 and there is now evidence that IL-18 can also synergise with IL-23 to produce IL-17A from CD4 cells, even in the absence of IL-1 [62]. Furthermore, γδ T cells are known to produce early IL-17 in response to IL-1β and IL-23, leading to feedback activation of Th17 cells [33].

Th17 cells are important for the clearance of extracellular bacteria and fungi and IL-17A production from Th17 cells promotes inflammation as well as recruitment of neutrophils to the site of infection [63, 64]. During *Bordetella pertussis* infection, the production of IL-17A from Th17 cells promotes neutrophil recruitment to the lung and clearance of the infection [65]. However, Th17 cells have also been shown to play a vital role in T-cell mediated autoimmunity. Originally autoimmunity was thought to be mediated exclusively by Th1 cells, however, a number of groups have demonstrated a pathogenic role for IL-17 and Th17 cells in different mouse models of autoimmunity. Furthermore, Th17 cells were sufficient to induce EAE when transferred to recipient mice [66, 67].

1.2.6 *Treg cells*

Treg cells are the master regulators of the immune system and play a vital role in limiting pathology during inflammation [68]. Furthermore, Treg cells prevent the onset of autoimmunity by maintaining peripheral tolerance through suppression of autoreactive T and B cells that recognise self-antigen [69]. Suppressive T cells that inhibit Th1 and Th2 responses have been extensively investigated by many groups and to date several distinct T cell subsets with regulatory activity have

been described. Naturally occurring Treg (nTreg) cells develop from thymic TCR high affinity T cells selection and are known to be important in controlling autoantigen-specific immune responses, thereby preventing the development of autoimmune diseases [70]. nTeg cells differ from other conventional T cells in that they constitutively express CD25. Transfer of T cell suspensions depleted of $CD25^+$ T cells from wild type (WT) mice resulted in development of autoimmune disease in athymic nude mice, demonstrating the importance of these cells in controlling autoimmunity [71].

Inducible Treg (iTreg) cells develop in the periphery from conventional CD4 T cells and they can express CD25 with TCR engagement under tolerogenic conditions [72]. The conversion of naïve CD4 T cells to iTreg cells is dependent on IL-10, TGF-β and retinoic acid, which inhibits the differentiation of Th17 cells [73]. Factor forkhead box P3 (Foxp3) is a master transcription factor of Treg cells and is required for the development and function of nTreg cells, as well as for the conversion and maintenance of iTreg cells [74]. Furthermore, IL-2 is another molecule critical for the function of Treg cells. The Treg cell marker CD25 is a component of the high-affinity IL-2 receptor (IL-2R) and is functionally essential for development into Treg cells [75].

iTreg cells are potent producers of IL-10 and TGF-β, which are required for suppressing effector T cell responses [76, 77]. TGF-β specifically inhibits Th1 response by inhibiting the Th1-specific transcription factor, Tbet [78]. Furthermore, IL-10 is capable of inhibiting Th1 and Th17 responses directly by suppressing cytokine production or indirectly through inhibition of APC function, resulting in downregulation of MHC II and co-stimulatory molecules on their surface [79]. However, the mechanism of suppressive action of nTreg cells is thought to be cytokine independent. nTreg cells are known to express the inhibitory molecule cytotoxic T-lymphocyte antigen 4 (CTLA4), which suppresses effector T cell responses directly through cell-cell contact [80]. Furthermore, nTreg cells can induce anergy in T cells, demonstrating their importance in immune homeostasis [81].

Besides iTreg cells, there are other types of Treg cells that can be induced from naive CD4 T cells in the periphery. IL-10-producing Tr1 cells or TGF-β-

producing Th3 cells are induced in response to pathogens and differentiate in the periphery under the direction of DCs. In mice, Tr1 cells can be defined based on secretion of IL-10 with no IL-5, TGF-β or IFN-γ production. They also differ significantly from the other Treg cell subsets, largely due to the Foxp3$^-$ phenotype [82]. Furthermore, Tr1 cells are antigen-specific regulatory T cells, which can be generated both *in vivo* and *in vitro*. In addition, there is growing evidence that Tr1 cells are important for maintaining immunological tolerance and preventing the development of autoimmunity [77]. Adoptive transfer of Tr1 clones suppressed Th1-mediated colitis induced in SCID mice by pathogenic splenic T cells [77]. Moreover, parasite specific Th3 clones are found in humans during helminth infection [83]. Latency associated peptide (LAP) marker is found on the surface of Th3 cells and promotes TGF-β production, leading to suppression of pathogenic T cells *in vivo* [84].

1.2.7 *γδ T cells*

γδ T cells are a unique population of T cells that develop in the thymus alongside conventional αβ T cells. However, γδ T cells leave the thymus as mature γδ T cells and are not restricted to the recognition of peptides complexed to MHC, thus distinguishing them from the majority of αβ T cells [85]. Unlike conventional T cells, γδ T cells constitute a very small fraction of T cells in the thymus, spleen, LNs and peripheral blood [86]. They are enriched in tissues associated with mucosal surfaces of the body and thus play an important role in the initiation of the early immune response to infection. Here they can recognise both self and benign antigenic peptides, which is consistent with their role as a bridge between innate and adaptive immunity.

γδ T cells are polyfunctional as they can produce an array of cytokines including IL-17A, IL-17F, IL-22 and IFN-γ. A number of studies have shown that γδ T cells are programmed into distinct IFN-γ–producing CD27$^+$ (γδ27$^+$ cells) and IL-17–producing CD27$^-$ (γδ27$^-$ cells) subsets [87]. IL-17A-producing γδ T cells share many features with Th17 cells, including the expression of IL-1R, IL-23R and RORγt [88]. Moreover, the Th1 transcription factor T-bet, is critical for IFN-γ production by γδ27$^+$ cells [89–91]. In addition, murine γδ T cells consist of various subtypes characterized by their anatomic location and their functional properties.

Vγ4 and Vγ6 γδ T cells are the main IL-17-producing γδ T cell, however, Vγ1 have also been shown to produce IL-17A. In contrast, IFN-γ production is specific to the Vγ1, Vγ5 and Vγ7 γδ T cells [92–94].

Early IL-17 production by γδ T cells is vital in containing bacterial and fungal infection by recruiting neutrophils to the site of infection [63, 64]. However, IL-17-producing γδ T cells are also known to play an important role in autoimmunity, including EAE. Our lab has demonstrated that γδ T cells are an important source of IL-17 production early in the development of autoimmune disease [95]. These cells constitutively express IL-23R and secrete IL-17 in response to IL-1β and IL-23. IL-17 production by γδ T cells in response to IL-1, IL-18, and IL-23, without TCR engagement, also promotes the induction of Th1 and Th17 cells, amplifying their encephalitogenic function during the development of EAE [96]. Naive mice have a low frequency of γδ T cells in the CNS, however, during EAE IL-17 and IFN-γ-producing γδ T cells infiltrate the brain and spinal cord [95]. Furthermore, a reduction in EAE disease severity was observed in TCRδ$^{-/-}$ mice, demonstrating the importance of γδ T cells in the pathology of this autoimmune disease [97].

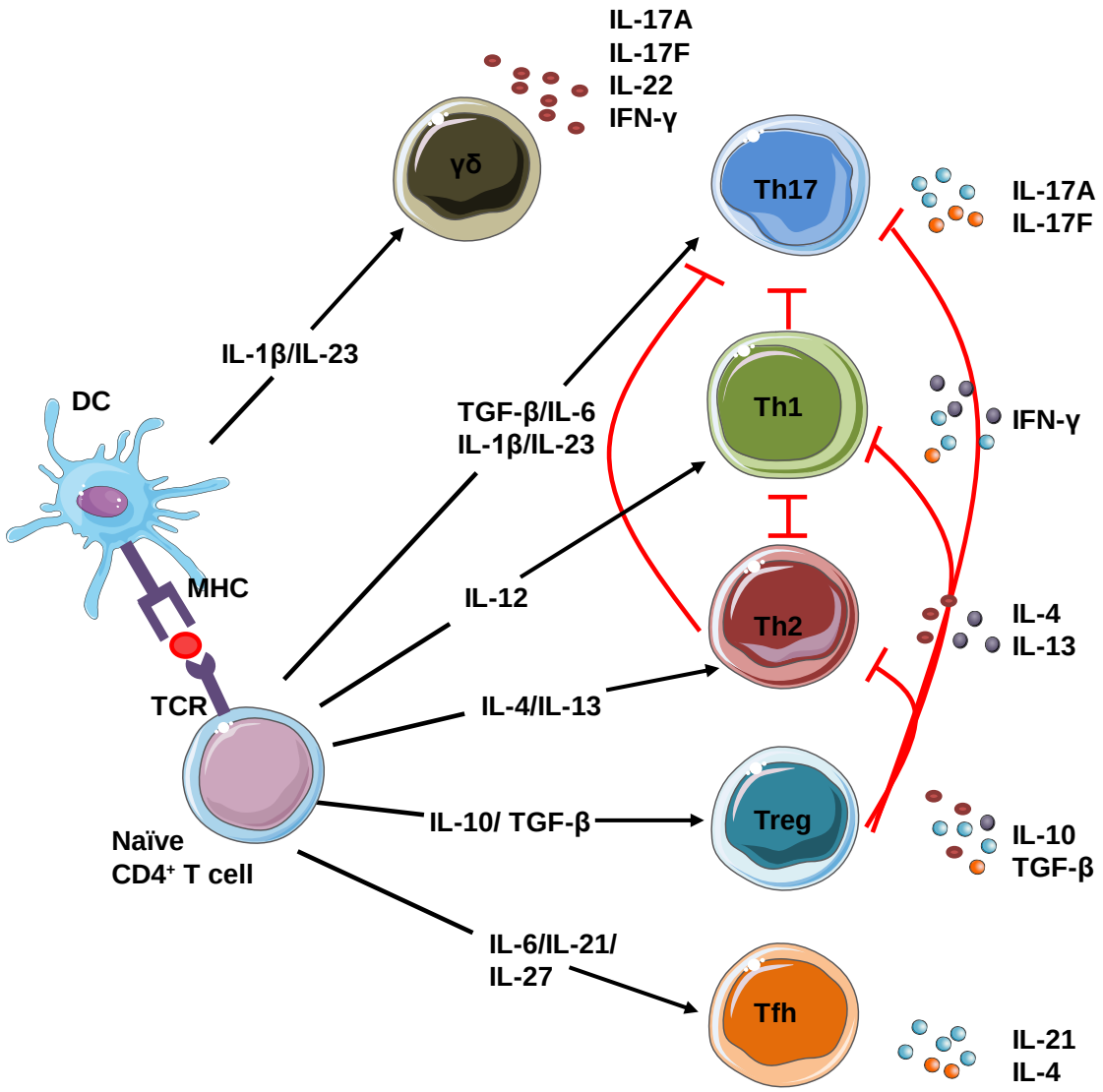

Figure 1.3 T cell differentiation.
Upon TCR activation triggered by interaction between T cells and APCs, CD4 T cells differentiate into effector Th lineages, depending on the signals they receive. IL-12 promotes the development of Th1 cells that secrete IFN-γ. IL-4 promotes development of Th2 cells, which secrete IL-4, IL-5 and IL-13. IL-10 and TGF-β promote the induction of Treg cells, which produce IL-10 and TGF-β. TGF-β with IL-6 promote the development of Th17 cells and their expansion is enhanced by IL-1β and IL-23. These cells secrete IL-17A and IL-17F. Concurrently, mature γδ T cells emerge from the thymus and are not restricted to the recognition of peptides complexed to MHC. These cells constitutively express IL-23R and secrete IL-17 in response to IL-1β and IL-23.

1.3 Immunological memory and innate immune training

Immunological memory is considered to be one of the cardinal features of the adaptive immune system. During the resolution of infection, most of the antigen-specific effector T and B cells die. However, even in the absence of a stimulus, a small percentage of T and B cells remain in the tissues and ultimately persist for life. Adaptive immunological memory is specific to the pathogen or antigens and upon reinfection with the same pathogen, clonal expansion occurs and memory lymphocytes confer immediate protection in peripheral tissues and mount recall responses to antigens in secondary lymphoid organs (Figure 1.4A) [98].

Until recently immunology memory was thought to be confined to antigen-specific T and B cells, however, recent studies have demonstrated that cells of the innate immune system have the ability to be trained or tolerized in response to different stimuli. The dogma that only the adaptive immune system develops immunological memory was first challenged by studies showing that innate immune responses in organisms lacking an adaptive immune system can mount resistance to reinfection. In plants, epigenetic changes lead to the priming of genes encoding host defence molecules. Upon re-exposure, these cells respond more rapidly and robustly, a process known as systemic acquired resistance (SAR) [99, 100]. Due to the development of SAR, plants inoculated with attenuated microorganisms acquire long term protection from a wide variety of pathogens including bacteria, viruses and fungi [101].

Furthermore, numerous studies have demonstrated that invertebrates also mount resistance non-specifically to secondary infection induced by the first encounter with a pathogen. The induction of cross-protection can be observed in mealworm beetles, who mount resistance to fungi after an initial exposure to LPS [102]. More recently, innate immune memory has been described in *Anopheles gambiae* mosquitoes infected with *Plasmodium falciparum*, which elicit heightened immune response upon reinfection [103].

In addition, there is increasing evidence to suggest that training of the innate immune system is also found in vertebrates that display adaptive characteristics. Early observation in West Africa showed that the Bacille Calmette-Guerin (BCG) vaccine protects children from infections other than *M. tuberculosis*, resulting in

a lower overall mortality rate [104]. This observation was confirmed in experimental mouse models, which showed that administration of BCG protected mice against secondary infections with *Candida albicans*, in a T cell-independent manner [105]. Furthermore, mice challenged with CPG were found to mount resistance to infection with *Listeria monocytogenes* [106, 107]. In addition, β-glucan induced innate immune training that protected mice against infection with *Staphylococcus aureus* [108, 109]. Moreover, proinflammatory cytokines have been shown to induce innate immune memory. Injection of mice with IL-1 induced innate immune training and protected mice from mortality associated with *Pseudomonas aeruginosa* infection [110].

The key mechanisms that mediate the effects of innate immune training are only partly defined. Epigenetic reprogramming induced by fungal structures such as β-glucan was shown to induce changes in positive histone regulatory marks, including H3K4me1, H3K4me3 and H3K27ac, resulting in heighten immune response, characterised by enhanced proinflammatory cytokine production [111]. Other genes that are modified by β-glucan encode glycolytic enzymes that cause a shift in glucose metabolism required for the training of cells. Inhibition of glycolysis inhibits training of innate cells, indicating that the induction of aerobic glycolysis through an Akt-mTOR-HIF-1α pathway represents the metabolic basis of trained immunity (Figure 1.4B) [112].

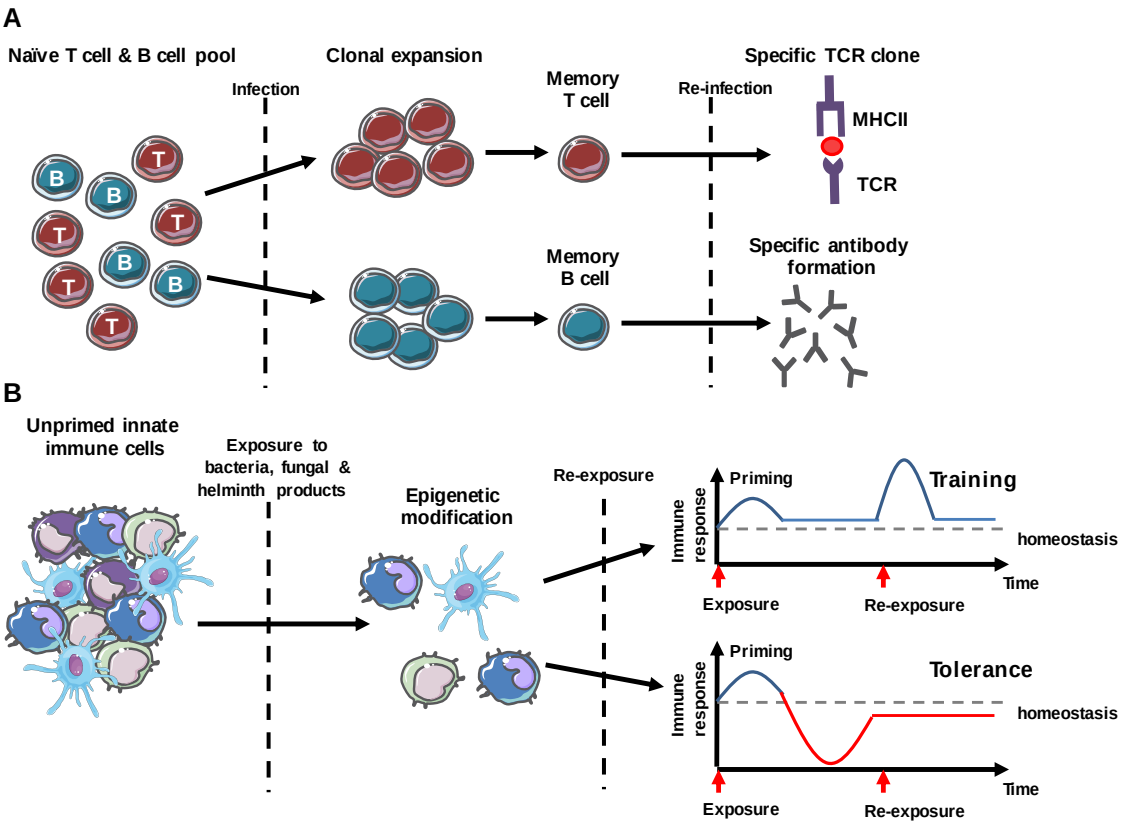

Figure 1.4 Adaptive vs innate immunological memory.
(A) During classical immunological memory, naïve T and B cells clonally expand in response to infection and upon resolution a small percentage of these cells remain in the tissues and ultimately persist for life. Memory T and B cells confers high specificity upon reinfection and respond more rapidly and robustly. (B) During exposure to bacteria, fungal or helminth products, innate immune cells undergo immunological imprinting of either tolerance or trained immunity through epigenetic modifications that influences the capacity of the immune system to respond to re-exposure. Part A adapted from Netea et al., Science. 2016 April; Vol. 352, Issue 6284 [113].

1.4 Autoimmunity

Autoimmunity is recognised as an abnormal immune response to self-antigens leading to immune-mediated destruction of the tissues. A healthy immune system is tolerized to self-antigen. However, a loss of tolerance and failure to discriminate from foreign antigens results in immune recognition of self-antigens. There are two types of tolerance: central tolerance and peripheral tolerance. Central tolerance can be described as the physical removal of autoreactive T and B cells from the thymus and the bone marrow. In doing so, autoreactive lymphocytes are prevented from entering the peripheral tissues, becoming activated and producing an immune response against the host. Even though T cells are generated in the bone marrow, central tolerance occurs in the thymus where T cells develop and mature by undergoing positive and negative selection. Positive selection involves the elimination of cells with TCRs that are unable to bind to self-MHC molecules. These thymocytes receive a 'death signal' and undergo apoptosis. Cells that have received a 'survival signal' then begin the process of negative selection. 20-50% of the TCRs created bind with a potentially dangerous affinity for self-antigen and these thymocytes with too high an affinity for self-antigens are eliminated by apoptosis. This is a key feature in maintaining central tolerance [114]. However, some autoreactive cells can escape central tolerance and as a result, peripherial tolerance mechanisms are required. Peripheral tolerance involves the suppression of autoreactive cells either by Treg cells or the inactivation of lymphocytes by rendering them anergic. T cells become anergic when they fail to respond to activation through the TCR via an MHC-peptide complex in the absence of co-stimulation [115]. A balance between the effector and regulatory elements of the adaptive immune response is critical for maintaining self-tolerance. A breakdown of tolerance results in activation of autoreactive T cells, leading to a variety of autoimmune diseases, including MS. The inevitable outcome is a chronic immune response against self-antigen accompanied by the accumulation of inflammatory mediators and autoreactive cells into a tissue leading to immunopathology.

1.4.1 *IL-1 in autoimmunity*

IL-1 is a key mediator of innate immunity and inflammation. IL-1β production is vital for protection against bacterial infection, however, dysregulation of IL-1 leads to monogenic autoinflammatory diseases, as well as autoimmunity [116]. IL-1β production by innate cells, together with IL-23, promotes IL-17 production from γδ T cells and the expansion of Th17 cells. Our lab has previously shown that caspase-1–processed cytokines, IL-1β and IL-18, promote IL-17 production by γδ and CD4 T cells that mediate autoimmunity [62]. This study also demonstrated that inhibition of caspase-1 suppressed IL-1β and IL-18 production by DCs and suppressed IL-17 expression by γδ T cells and CD4 T cells, attenuating experimental autoimmune encephalomyelitis (EAE). Moreover, neutrophils and inflammatory monocytes are an important source of IL-1β required for the initiation phase of EAE but also important during the effector phase in the CNS [117, 118]. In addition, Sutton et al. demonstrated that EAE was significantly attenuated in IL-1RI$^{-/-}$ compared with WT mice [61], suggesting a vital role for IL-1 in the development of T cell-driven autoimmunity.

The interleukin 1 family comprises eleven members including IL-1β, IL-1 receptor antagonist (IL-1RA) and IL-1α [119]. IL-1 is found in virtually all cell types and has a protective role against invading pathogens but also plays a detrimental role in many autoinflammatory and autoimmune diseases [116, 120] . Although IL-1α and IL-1β are both encoded by different genes, they bind to the same receptor, IL-1 receptor (IL-1R1). IL-1α and IL-1β signalling can induce mRNA expression of many genes including IL-1R1 and, as a result, IL-1RA is required in order to prevent excess inflammation by competitively binding to IL-1R1 and demonstrating no agonist activity. IL-1α is usually associated with the plasma membrane of the cell and acts locally, whereas IL-1β is released and circulates systemically. Furthermore, IL-1α precursor is fully activated and can rapidly initiate a cascade of inflammatory cytokines and chemokines. In contrast, the IL-1β precursor contains a pro-domain at its amino terminus and, as a result, usually requires activation of the inflammasome and cleavage by caspase-1 [121]. Caspases are a family of evolutionary conserved cysteine proteases, which play a crucial role in inflammation as well as apoptosis and necrosis. Capase-1 is

abundant in many cell types, however, its precursor, pro-caspase-1, must also be cleaved by activation of the inflammasome complex.

The inflammasome is a multiprotein complex that is activated in response to cellular infection or stress and is required for the maturation of IL-1β and IL-18 [122]. The complex is activated by a wide variety of pathogenic microbes and is required for restriction of pathogen replication and for the generation of the adaptive immune system. Although the complete mechanism of activation of the inflammasome remains unclear, its formation is initiated by the recognition of pathogens via NLRs and nucleic acid receptors AIMS and IFN-inducible protein 16 (IFI16). NLRs are a group of intracellular PRRs that recognise foreign material as well as DAMPs. A subset of NLRs, named NLRPs, are activated in response to DAMPs and PAMPs and consist of NLPR1, NLRP2 and NLRP3. The NLRP3 inflammasome is currently the most fully characterized inflammasome and has a protein structure consisting of an N-terminal pyrin domain, a central NACHT, and a C-terminal leucine-rich region. Three components, NLRP3, Apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1, compile to form the NALP3 inflammasome complex in response to certain stimuli. The adaptor protein, ASC, plays a crucial role in the assembly of the NALP3 inflammasome via its interaction with the N-terminal pyrin domain of NLRP3 [123]. TLR activation in response to PAMPs leads to downstream signalling, causing transcription of pro-IL-1β and NLRP3 via degradation of IκB and the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Activation of the inflammasome leads to the activation of caspase-1, resulting in the cleavage of 31-kDa inactive IL-1β precursor to the mature bioactive 17-kDa IL-1β form (Figure 1.5).

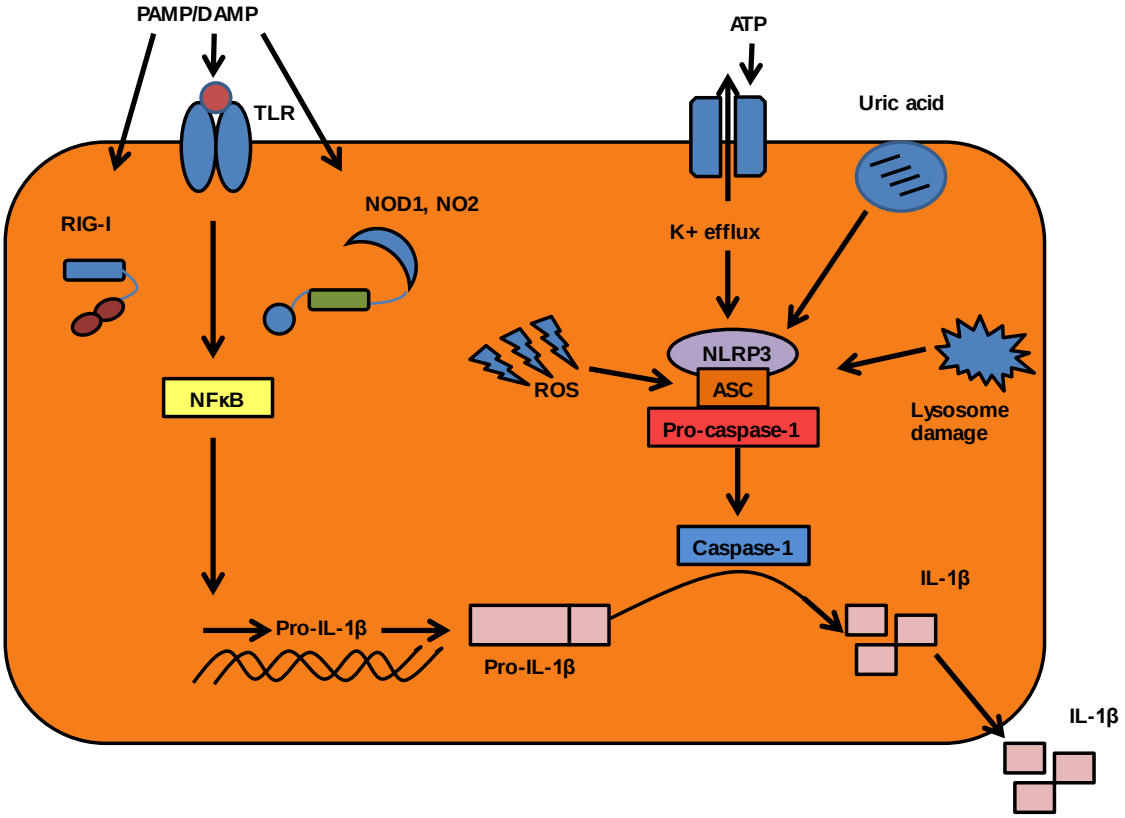

Figure 1.5 Inflammasome activation and release of IL-1β
Effective release of IL-1β from innate cells usually requires two signals. Signal one involves the activation of TLRs and NLRs in response to PAMPs and DAMPs. This leads to the activation of NFκB to generate pro-IL-1β and prime the inflammasome. Signal two involves the activation of the inflammasome by a number of inducers, including adenosine triphosphate **(**ATP), ROS and lysosomal damage. This leads to the recruitment of ASC protein and pro-caspase-1 to the inflammasome complex and generation of caspase-1. The activation of caspase-1 leads to the cleavage of pro-IL-1β to mature IL-1β and subsequent release from the cell.

Until recently, it was thought that secretion of mature IL-1β was dependent on two signals. However, recent studies have demonstrated mechanisms for IL-1β secretion independent of NLRP3 and inflammasome activation. Fantuzzi et al. demonstrated the first evidence that pro-IL-1β could be processed independently of caspase-1 [124]. Subsequent studies identified neutrophil and macrophage-derived neutral serine proteases, such as proteinase 3, elastase, and cathepsin-G, as enzymes that can process pro-IL-1β into mature IL-1β [125]. Caspase-1-independent processing of IL-1β was also found to occurs in arthritis by serine

proteinases [126]. Furthermore, human monocytes secrete IL-1β in response to LPS, independently of classical inflammasome stimuli [127]. In addition, IL-1β processing during *P. aeruginosa* infection was found to be mediated by neutrophil serine proteases but independent of NLRP4 and caspase-1 [128]. Therefore, it is possible that caspase-1-independent maturation of IL-1β may play a role in the initiation or progression of a number of autoimmune diseases, including MS.

1.4.2 *MS*

MS is an autoimmune disease of the central nervous system (CNS) affecting over two million people worldwide, mostly young adults. MS affects approximately twice as many women as men, with the onset of disease usually occurring between 20 and 40 years of age. The pathology of MS is characterised by inflammation of the CNS and immune-mediated destruction of the myelin sheath. The damage to oligodendrocytes, which cover the nerve fibres, leads to a deficiency in sensation, as well as impaired motor and autonomic functions. Patients with MS also suffer from neurodegeneration, axonal loss and cumulative disability. During MS, a breakdown of tolerance results in autoreactive T cells crossing the blood brain barrier (BBB) where they are reactivated by proteins of the myelin sheath, such as MOG, myelin basic protein (MBP), and proteolipid protein (PLP). This results in demyelination and the formation of plaques in the white matter of the brain, a hallmark of MS [129]. The myelin sheath insulates the neuron, increasing electrical resistance and thus preventing the loss of electrical currents from the axon. The location of these plaques correlates with the type of dysfunction elicited during MS (Figure 1.4) [130].

Relapsing remitting MS is the most common form of the disease and is characterised by episodes of acute neurological deterioration (relapse). During remission, inflammation in the CNS resolves and it is believed that there is restoration of CNS conduction due to glial ensheathment and remyelination. However, the symptoms worsen after each attack during which the patient's overall health gradually declines. Frequently this progresses into secondary progressive MS, which involves a continuing deterioration in health status.

Although the aetiology of MS is not well defined, there are many factors known to have contributing roles in disease development and progression.

Studies have shown a 30% genetic concordance among monozygotic twins in the development of MS [131]. Furthermore, human leukocyte antigen (HLA)-DR15 has been associated with early onset of MS, suggesting HLA molecules may influence thymic selection and antigen presentation [132]. Along with the genetic predispositions, there is also evidence to suggest that environmental factors have a role in development of MS. The highest environmental risk factor is smoking, which causes citrullination of proteins, with the relative risk for the development of MS approximately 1.5 higher for smokers compared with non-smokers [133]. Furthermore, higher latitude is known to be associated with a higher prevalence of MS, with incidences increasing during months with less sunshine [134]. Moreover, vitamin D is one of the factors that can regulate Treg cell differentiation and activity [135]. In Iran, lower serum vitamin D level were also seen in MS patients than that in normal population, in spite of sufficient sun exposure [136]. In addition, the prevalence of MS and severity of disease is significantly lower in areas chronically exposed to helminth infection. This is due to the dampening of the immune system by the parasite as well as the immune response being skewed towards a type two or regulatory response [137].

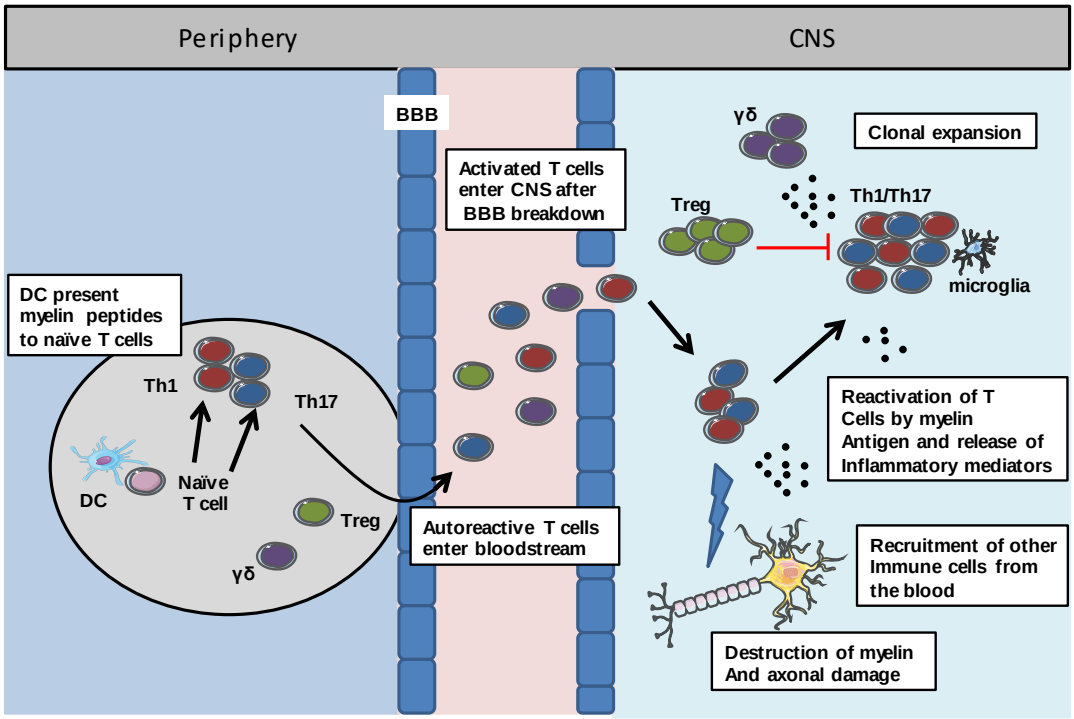

Figure 1.6 Migration and function of autoreactive T cells during MS.
After priming with myelin antigens, DCs are activated in the LNs where they present antigen to naïve T cells. Myelin-specific T cells then enter the bloodstream and transmigrate across the BBB to the CNS. In the CNS, T cells encounter their specific antigens and become reactivated by microglia. The release of inflammatory mediators leads to further cell recruitment into the CNS. Activated microglia release proteases and other cytotoxic agents that lead to the destruction of the myelin sheath, thus leading to axonal damage and impaired neurological function. Figure adapted from Fletcher et al., Clin. Exp. Immunol. 2010 Oct; 162(1): 1–11 [138].

1.4.3 *EAE*

EAE is a T cell-mediated autoimmune disease and is the most commonly used experimental model for the human inflammatory demyelinating disease, MS. EAE clinically manifests as ascending paralysis beginning at the tail, followed by hind limb paralysis and forelimb paralysis and is assessed using a 5-point scale. The myelin-antigens used to induce EAE range from single polypeptides, such as MOG and PLP to whole spinal cord homogenates. Immunization of different mouse strains with various myelin antigens results in different forms of the disease: acute, chronic or relapsing remitting. C57BL/6 mice develop chronic disease following immunization with MOG peptide emulsified in Complete Freund's Adjuvant (CFA) followed by injection with pertussis toxin (PT) [139]. PT increases the permeability of the BBB in EAE, allowing entry of autoreactive lymphocytes into the CNS. PT has also been shown to enhance IL-1β and IL-23 production by myeloid cells, which is required for priming and enhancing encephalitogenic γδ T cells and Th17 cells [117].

EAE shares similar features to MS, including induction of pathogenic Th1 and Th17 cells, infiltration of cells into the CNS and damage to myelin sheaths, making it a useful model for human disease. However, a major difference between MS and EAE is the requirement of an external immunization step in EAE, whereas sensitization to autoantigens in MS occurs through loss of tolerance. Furthermore, antigens that initiate EAE are known, whereas the exact antigenic targets of autoreactive T cells in MS are not clear.

The passively induced model of EAE involves the adoptive transfer of myelin-specific T cells from mice with EAE into naïve recipient mice. Donor mice are injected with MOG peptide emulsified in CFA without PT. Spleen and LN cells are isolated 10 days later and stimulated with MOG, IL-1β and IL-23 before being transferred to recipient mice [140]. The ability to induce adoptive transfer EAE was first demonstrated in naïve rats that developed disease when cells were transferred from the LNs of donor rats with EAE [141]. One advantage of adoptive transfer EAE is that different subset of T cells can be labelled prior to transfer to examine their location, migration and activity [142]. Furthermore, manipulation of T cells in the culture stage of disease allows for an examination of different T cells subset and their pathogenic functions.

1.4.4 *T cells in MS and EAE*

The pathogenesis of MS and EAE is primarily dependent on autoreactive T cells. CD4 T cells are greatly enriched in the blood and CNS lesions of MS patients [47]. Furthermore, passive or adoptive–transfer EAE can be induced in recipient animals by transferring pathogenic, myelin-specific CD4 T cells generated in donor animals by active immunisation [140]. Macrophages and CD4 T cells are the main cell types in the inflammatory infiltrate. Once in the CNS, antigen-specific T cells are restimulated by endogenous myelin antigens and APCs and initiate their pathogenic effects. These include production of proinflammatory cytokines and chemokines leading to further cell recruitment, resulting in tissue damage and paralysis.

Before the discovery of Th17 cells, MS and EAE was originally thought to be mediated by Th1 cells. This theory was supported by studies that demonstrated resistance to EAE in IL-12p40$^{-/-}$ mice [143]. Further evidence showed that over expression of IFN-γ in the CNS induced spontaneous demyelination, similar to that observed in MS and EAE [35]. However, the concept of Th1 driven autoimmunity was challenged by the fact that IFN-γ$^{-/-}$, IFN-γR$^{-/-}$ or IL-12p35$^{-/-}$ mice were more susceptible to EAE [143]. The discovery of a novel cytokine IL-23, which shares a second common chain p40 with IL-12, led to the discovery that IL-23p19$^{-/-}$, but not IL-12p35$^{-/-}$ mice, were resistant to the induction of EAE, confirming a vital role for IL-23 in the pathogenesis of EAE [144]. It was subsequently shown that IL-23 is a crucial differential factor for the development of Th17 cells and these cells were found to induced disease when adoptively transferred into recipient mice [145].

1.5 Helminths

Helminths are eukaryotic parasitic worms that infect approximately 2 billion people worldwide, with the vast majority infected living in developing countries. These parasites are divided into two phyla: platyhelminthes (flatworms), including trematodes (flukes) and cestodes (tapeworms), and nemathelminths also known as nematodes (roundworms) [146]. Infection with helminth represents one of the greatest challenges to our immune system and causes significant debility in children, including malnutrition and fatigue. Helminths reach sexual maturity and

reproduce in the definitive host, however, an intermediate host houses one or multiple larvae stages during the life cycle [147]. Furthermore, there are certain helminth species that do not require an intermediate house and exist as independent soil living worms in their larval stage [146]. Helminths reside mainly in the gastrointestinal tract of the definitive host but also colonize other organs. They are highly successful parasite that establish long lasting infections, causing mechanical and enzymatic damage during their migration through the tissue [148]. Furthermore, certain helminths cause significant morbidity and disability. For example, *Brugia malayi* is the causative agent lymphatic filariasis (elephantiasis), which results in chronic inflammation and tissue damage even after the worm is expelled [149].

1.5.1 *Immune response to helminths*

Originally it was thought that helminth infections did not elicit an immune response, mainly due to the observation that adult helminths were rarely eliminated from human hosts. However, in the 1920's Stoll et al. demonstrated that sheep previously infected with gastrointestinal nematodes were resistant to re-infection [150]. Since then, several studies have highlighted the complex mechanisms by which helminths modulate and subvert the immune system in order to induce long lasting infections [151, 152]. Following the discovery of Th1 and Th2 cells, it was reported that protective immunity to helminths was mediated by Th2 cells and their associated cytokines. Adult helminths are rarely killed by the host's immune system, however, the induction of Th2 and Treg cells limits infection and the capacity of helminths to reproduce. The role of Th2 responses in helminth infection has been detailed in a study showing that depleting of CD4 T cells from rats infected with *N. brasiliensis* resulted in chronic infection [153], however, this affect appears to be species specific. Although studies have suggested that children infected with helminths have higher worm burdens when compared to adults due to weaker Th2 responses, other studies in filarial or hookworm infections have shown a higher prevalence in adults, highlighting the complexity and diversity of each helminth and the immune responses they evoke [154, 155].

Although number of studies have detailed the importance of Th2 responses in protective immunity to helminths, it has been demonstrated that production of the alarmin cytokines IL-33 and IL-25, as well as TSLP, produced by epithelial cells, is the first immune response to helminth infection. Alarmins rapidly activate type 2 innate lymphoid cells (ILC2) and induce early production of IL-13 and IL-5. TSLP induces IL-4 production by basophils, which is required for Th2 differentiation. IL-4 is also important in IgE class switching of B cells, which causes mast cells to release inflammatory mediators. This along with IL-13 induce the “weep and sweep” response that results in epithelial shedding and mucus secretion. In parallel, helminth products trigger Th2 and Treg differentiation and the release of IL-4, IL-5, IL-13 and IL-10 through induction of tolerogenic DCs, which suppress pathogenic Th1 and Th17 responses [137]. M2 macrophages are polarized in response to IL-4 and release effector proteins, such as resistin-like molecule-α (RELM-α) and chitinase 3-like proteins (Chi3l3), which directly damage the parasite. They also produce programme death ligand (PD-L2), which suppress T cell activation via ligation of PD-1 on T cell receptors. Furthermore, arginase 1 starves helminths by depleting L-arginine from the tissue microenvironment [156]. In addition, M2 macrophages along with eosinophils, recruited in response to IL-5 production, form granulomas that limit parasite translocation (Figure 1.7).

Protective immunity to helminth infection is associated with type 2 immune responses, however, many infections remain chronic due to the induction of regulatory responses [157, 158]. This reflects a combination of parasite immune suppression strategies as well as host immunoregulatory mechanisms that limit pathology during infection. Several helminths exploit the function of Treg cells in order to evade host immune responses. Our lab has previously shown that infection with live *Fasciola hepatica* enhanced TGF-β production by DC and macrophages, leading to the expansion of Treg cells [159]. Furthermore infection with *Heligmosomoides polygyrus* is associated with enhanced Treg cell responses [158]. However, one of the advantages of helminth-induced immunosuppression is the bystander effect of attenuating the pathogenic immune responses to allergens and autoantigens that mediate allergy and autoimmune diseases [157].

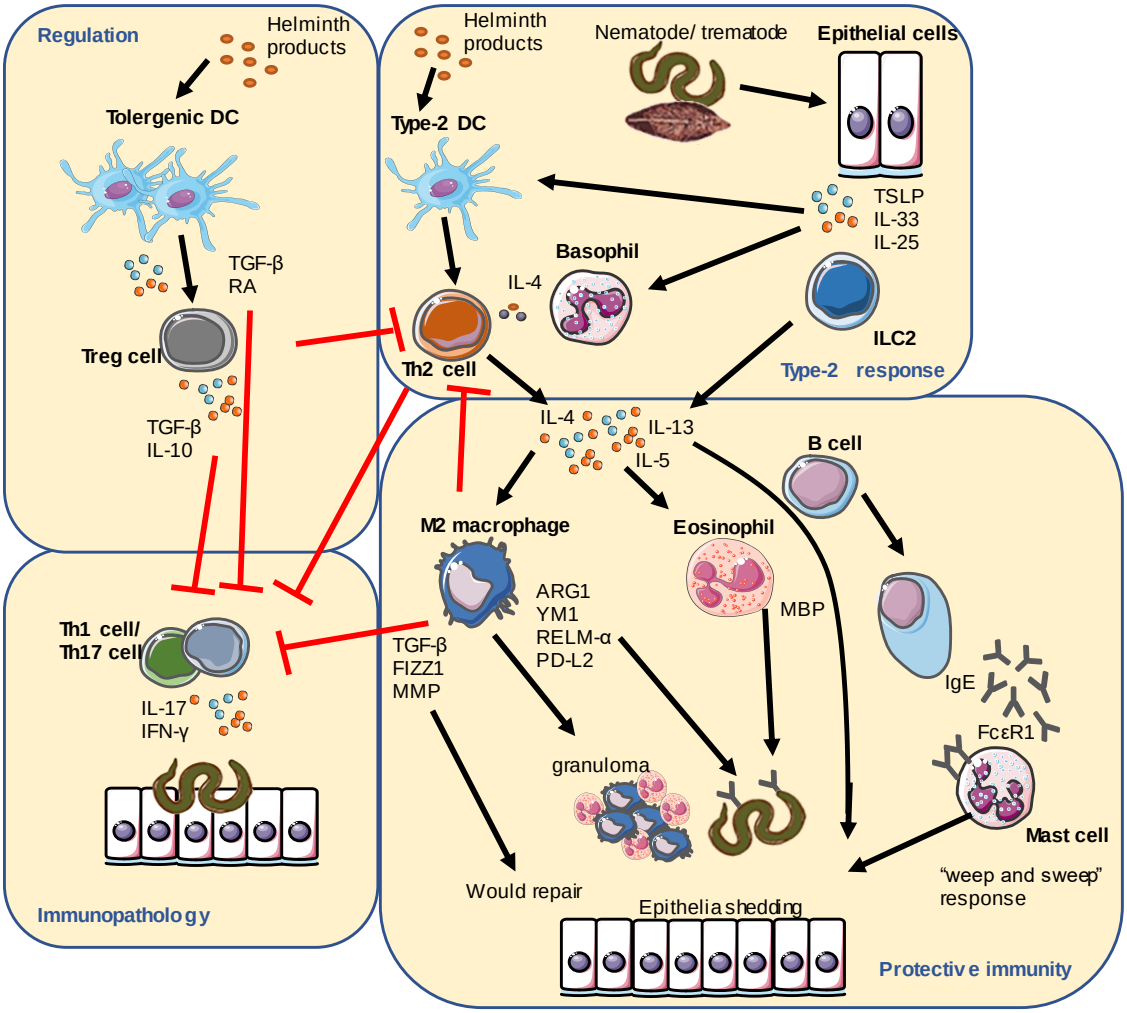

Figure 1.7 Immune response to helminths.

Type-2 induction: Epithelial cells are the first line of defence in response to helminth infection. They release the alarmins TSLP, IL-33 and IL-25 that activate ILC2s to produce protective type-2 cytokines, IL-5 and IL-13. TSLP induces IL-4 production by basophils, which is critical for the differentiation of Th2 responses. *Protective immunity*: Th2 cells produce IL-4 that activates M2 macrophages and is important for class switching of B cells, resulting in activation of mast cells and release of inflammatory mediators. IL-5 is important for recruitment of eosinophils that cause granulomas, along with M2 macrophages, which limit translocation of the helminth. IL-13 promotes epithelial shedding and mucus secretion. *Immunopathology and regulation*: The induction of Th2 response during helminth infection is required for limiting immunopathology of Th1 and Th17 cells. Helminth-derived products also induce tolerogenic DCs that activate Treg cells, which suppress Th1 and Th17 cells. Figure adapted from Finlay et al., Immunol. Rev. 2014; 259 (1):206-230 [146].

1.5.2 *Hygiene hypothesis*

In the twentieth century, life style changes in industrialized countries and the increase in antibiotic use, vaccinations and improved hygiene, led to a decrease in the incidence of infectious diseases. Concurrently, epidemiologic data provide strong evidence of a steady rise in the incidence of allergic and autoimmune diseases in developed countries over the past three decades. Furthermore approximately one in five children now suffer from allergic diseases, such as asthma, allergic rhinitis or atopic dermatitis [160]. This inverse correlation between exposure to pathogens and incidence of allergy was first described by Strachan and coined the “hygiene hypothesis”. Strachan observed that the risk of allergic rhinitis was inversely linked to birth order and the size of the family [161, 162]. Since then, a number of environmental factors have been linked to the susceptibility of atopic diseases (Figure 1.8). Exposure to animals, both domesticated and farmed, early in life confers protection against allergy [163, 164]. Furthermore, early exposure to probiotics including lactobacillus GG resulted in reduced incidence of atopic dermatitis as well as eczema [165, 166]. The hypothesis that infectious agents that co-evolved with humans have the ability to protect against a large spectrum of immune related disorders was extended to autoimmune diseases, including type 1 diabetes and MS. A study by Kilpatrick et al. found that higher infant sibling exposure in the first 6 years of life was associated with a reduced risk of MS, possibly by altering childhood infection patterns and related immune responses [167]. Similarly, epidemiological studies in Sardinia link malaria eradication with the concomitant increase in the incidence of MS [168, 169].

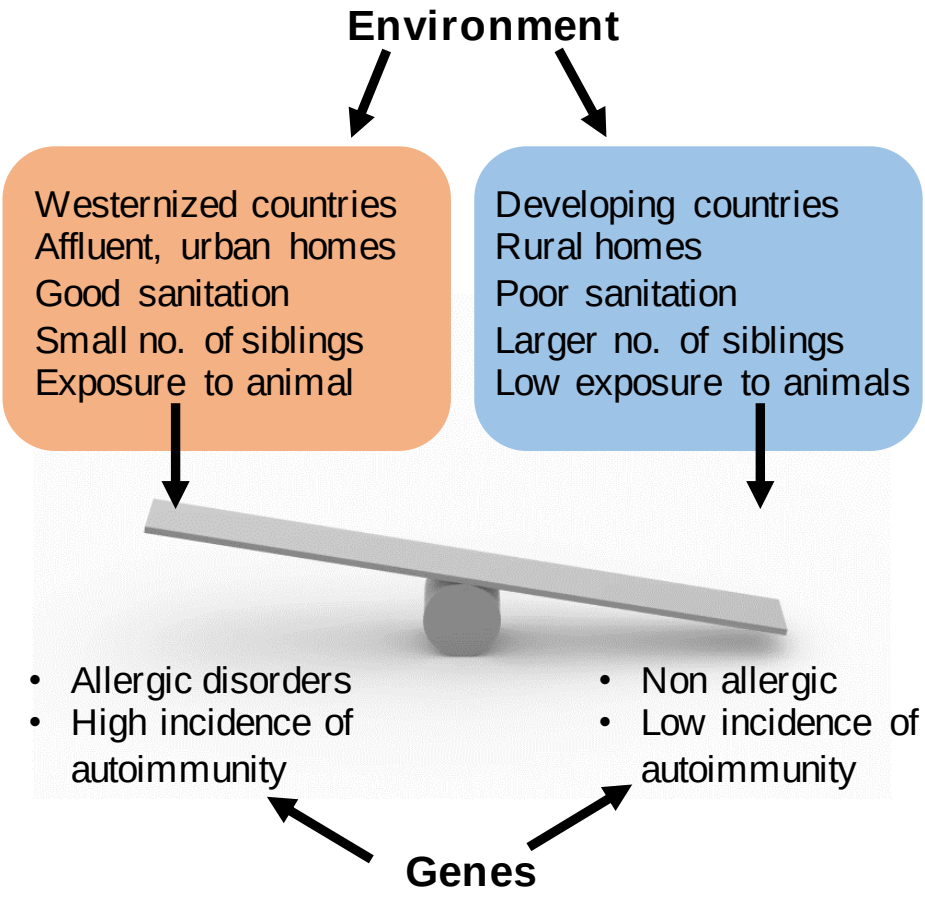

Figure 1.8 Environmental factors associated with an increase in allergy and autoimmune diseases in westernized societies over the past few decades. A comparison of environmental conditions in developed and underdeveloped countries has provided insight into possible causes. Exposure to animals, poor sanitation and a larger sibling size confers protection against the development of allergy and autoimmune diseases. Adapted from Wills-Karp et al., Nature Reviews Immunology 2001; 1, 69–75 [170].

A number of studies have found an inverse relationship between the incidence of helminth infections and the risk of developing allergic and autoimmune diseases [171, 172]. A study by Wilson et al. reported that helminth infection resulted in the suppression of allergic airway inflammation in a mouse model through induction of Treg cells [173]. Moreover, in Gabon, helminth infections were shown to have a protective effect against allergic responses and anti-helminthic treatment of infected children resulted in increased atopic reactivity [174]. Furthermore, Correal and Farez reported that helminth infection attenuated the severity of MS in South American patients [175]. Over a five-year period, parasite-infected patients displayed a significantly lower number of relapses as well as reduced

disease severity. Moreover, peripheral blood from infected patients produced significantly lower levels of IL-12 and IFN-γ but more IL-10 and TGF-β upon restimulation with MBP. In mouse models, infection with a helminth parasite *F. hepatica* was shown to attenuate EAE through TGF-mediated suppression of Th17 and Th1 responses [159]. Furthermore, infection with *Schistosoma mansoni* prevented insulin-dependent diabetes mellitus in non-obese diabetic mice (Figure 1.9) [176]. Although the use of *T. suis* has shown promising effects in the treatment of ulcerative colitis [177], most studies to date have been unsuccessful in larger human trials, suggesting more research is required to fully understand helminth immunomodulation.

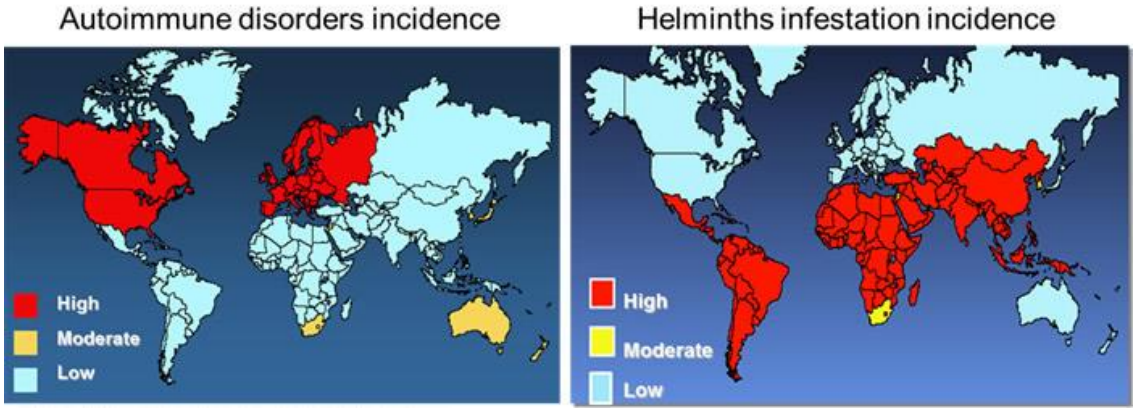

Figure 1.9 Incidence of helminth infection and autoimmune disorders worldwide. Figure taken from Hill and Williams, Int J Tryptophan Res. 2017 [178].

1.5.3 *F. hepatica*

F. hepatica, the causative agent of fascioliasis, is a parasitic flatworm of the class trematode that is commonly known as liver fluke. It infects mainly ruminants such as sheep and cattle, causing great economic losses in animal farming. *F. hepatica* is one of the largest parasites to infect humans, with over 3 million thought to be infected worldwide.

The complex life cycle of *F. hepatica* involves two distinct stages within two different hosts, the primary or definitive host and the secondary or intermediate host. The secondary host is usually the snail species *Lymnea truncatula,* which live in marshy areas and standing water [179, 180]. In the snail, the parasites

develop into cercariae, which are released and encyst as metacercariae on aquatic vegetation. Sheep and cattle, the definitive hosts, are infected through ingestion of contaminated vegetation. After ingestion, the metacercariae encyst in the duodenum and migrate through the intestinal wall and the peritoneal cavity before entering the liver [181]. The juvenile liver fluke spends between 8-12 weeks feeding on the parenchymal cells of the liver, causing extensive haemorrhaging and perforation. The parasites move into the biliary ducts, where it achieves sexual maturity and develops into an adult. The adult flukes live in the large biliary ducts of the mammalian host and feed on blood to obtain nutrients necessary for egg production. Unembryonated eggs are dispatched into the duodenum and are subsequently passed onto pastures in the faeces. The eggs are deposited in fresh water where they complete their development, hatch and the ciliated miracidium actively swim in search of the intermediate host [182].

1.5.4 *Host immune response to F. hepatica*

Exposure to *F. hepatica* results in long lasting infection and as a result the parasite is a target of the host immune system. Studies from experimentally infected cattle have shown that the host initially mounts a robust Th2 response against *F. hepatica*, which is characterised by eosinophilia, IL-4 production and IgG1 antibodies [183, 184]. Peripheral blood mononuclear cells (PBMCs) isolated from cattle 10 days post infection show high levels of IL-4 but no IFN-γ upon restimulation with parasitic antigen [185]. However, this response is ineffective at clearing the parasite and therefore a chronic infection develops by 12 weeks. PBMCs taken from chronically infected animals have reduced levels of IL-4 and produce high levels of IL-10 and TGF-β in response to parasite antigens, suggesting that chronic infection dampens the protective Th2 response in favour of Treg cells, which ensures parasite survival [185]. It remains unclear whether Th2 responses provide protective immunity against the invading parasite, or are responsible for immune related pathology, or both.

1.5.5 *Immunomodulation by F. hepatica and its products*

Helminth parasites have developed different mechanisms to subvert host immune response through immunomodulation of both the innate and adaptive immune system. *F. hepatica* induces Th2 responses within the host and this is

associated with suppression of Th1 and Th17 responses [146]. Furthermore, immunosuppression associated with *F. hepatica* infections impairs control of concurrent microbial infections. Our lab has previously reported that infection with *F. hepatica* induced polarized Th2 responses in mice, suppressed Th1 responses, and delayed clearance of the bacterial pathogen *B. pertussis* [186]. Furthermore, it has been shown that calves infected with *F. hepatica* had reduced Th1 responses when co-infected with *Mycobacterium bovis* [187]. *F. hepatica* infection of mice is associated with the expansion of alternatively activated macrophages in infected animals. These macrophages produce high levels of IL-10 and TGF-β [188]. Furthermore, recent studies have also shown that *F. hepatica* infection attenuates EAE through TGF-β-mediated suppression of T cell responses [47].

As infection with live *F. hepatica* is associated with significant mortality in mice [189], efforts are now focusing on exploring the potential of parasite-derived products. Previous studies have shown that *F. hepatica* glycoconjugates modulated DC responses to TLR stimulation by suppressing the expression of IL-12p70, IL-27, IL-10 and TNF [190]. Furthermore, tegumental antigens from *F. hepatica* were found to inhibit maturation of DCs [191]. This study also demonstrated suppression of TLR induced proinflammatory cytokines and suppressed levels of IFN-γ and IL-12 in the serum of mice with septic shock. Fatty acid binding protein from *F. hepatica* was also found to induce the alternative activation of human macrophages [192]. Interestingly, treatment with ES products of *F. hepatica* attenuated autoimmune disease by suppressing the induction of autoantigen-specific Th1 and Th17 cells [47]. The present study focused on the soluble total extract from *F. hepatica* (FHTE) and the ability of FHTE to modulate immune responses that mediate autoimmune diseases.

1.6 Aims

Helminth parasites have evolved highly successful mechanisms of immune evasion that helps prolong their survival in the host, resulting in the development of chronic infections. Furthermore, immune subversion by helminths is associated with simultaneous suppression of pathogenic immune responses that mediate allergy and autoimmunity. The experimental evidence to support the hygiene hypothesis has inevitably led to the study of live helminth therapy for the treatment of autoimmune diseases in the clinic, however, most studies have failed to show significant beneficial effects in patients. Furthermore, there are practical and ethical obstacles to the use of live infection as a therapy. However, the use of helminth-derived products provides an invaluable tool in identifying and examining helminth immunomodulation without the obscuring pathological responses associated with infection. Therefore, the aims of this study were:

- To characterise the immunomodulatory effects of FHTE on cells of the innate immune system
- To examine the possibility that FHTE may exert a training effect on innate immune cells *in vitro* and *in vivo*
- To examine the effect of FHTE-trained innate immunity on a T cell-mediated autoimmune disease
- To examine if FHTE had a direct immunomodulatory effect on T cells
- To examine the immune responses associated with attenuation of autoimmunity by FHTE
- To attempt to identify immunomodulatory components mediating the immunosubversive effects of FHTE through fractionation based on molecular weight

Chapter 2.
Materials and methods

2.1 Materials

2.1.1 *Cell culture medium*

Roswell Park Memorial Institute-1640 medium (RPMI; Sigma) was supplemented with 10% heat-inactivated foetal calf serum (FCS; Sigma), 100 mM L-Glutamine (Gibco) and 100 µg/ml penicillin/streptomycin (PS; Biowest). Complete RPMI (cRPMI) was used to culture murine innate immune cells, while cRPMI supplemented with 50 µM β-mercaptoethanol (2-ME) was used to culture purified T cells, spleen cells and LN cells *ex vivo.*

2.1.2 *Ammonium chloride lysis solution*

0.87% ammonium chloride (NH4CL)

Dissolved in 100 ml ddH_2O

2.1.3 *Ethidium bromide/acridine orange (EB/AO)*

2.5 g ethidium bromide (Sigma)

2.5 g acridine orange (sigma)

Dissolved in 50 ml 1xPBS (Sigma)

2.1.4 *Enzyme-linked immunosorbent assay (ELISA) reagents*

Phosphate-buffered saline (PBS) 20X

320 g sodium chloride (NaCL; Sigma)

46 g sodium phosphate dibasic ($Na_2HPO_4.12\ H_2O$; Sigma)

8 g potassium phosphate monobasic (KH_2PO_4; Sigma)

8 g potassium chloride (KCL; Sigma)

Dissolved in 2 L of dH_2O, pH 7.2

ELISA wash buffer (PBS-T)

0.5% Tween

Prepared in 1xPBS

ELISA blocking buffer

10% (w/v) skimmed milk (Sigma)

Prepared in 1xPBS

Or

1% (w/v) Bovine serum albumin (BSA; Sigma)

Prepared in 1xPBS

ELISA developing solution

3,3',5,5'-Tetramethylbenzidine (TMB)

ELISA stop solution

1 M sulphuric acid (H_2SO_4; Sigma)

2.1.5 *FACS buffer*

2% FCS (Sigma)

2mM Ethylenediaminetetraacetic acid (EDTA)

Prepared in 500 ml sterile 1xPBS (Sigma)

2.1.6 *Automated MACs*

Running buffer

2% FCS (Sigma)

2 mM EDTA (Sigma)

Prepared in 1xPBS

Rinsing buffer

2 mM EDTA (Sigma)

Prepared in 1xPBS

2.1.7 *Manual MACS*

3% FCS (Sigma)

10 mM EDTA (Sigma)

Prepared in 1xPBS

2.1.8 *Isotonic Percoll solution*

Isotonic Percoll solution was prepared by diluting Percoll reagent 9:1 with sterile 10X PBS (Sigma). Isotonic Percoll solution prepared this way had a density of 1.123 g/ml. 40% and 70% isotonic Percoll solution (made in cRPMI and 1XPBS respectively) was used for the isolation of mononuclear cells (MNCs) from brain and spinal cord tissue.

2.1.9 *General reagents*

Percoll	GE healthcare
High binding ELISA kit	Griene
CD4 T cell isolation kit	Miltenyi Biotec
γδ T cell isolation kit	Miltenyi Biotec
EDTA	Sigma Aldrich
TRIzol	Thermo Fisher Scientific
High capacity cDNA reverse transcription kit	Applied Biosystems
BSA	Sigma Aldrich
Saponin	Sigma Aldrich
Foxp3 Transcription Factor staining buffer set	eBioscience
Dimethyl sulfoxide	Sigma Aldrich
Bicinchonic acid	Pierce Thermo Scientific
Limulus amebocyte lysate assay	LONZA
GM-CSF	J559
M-CSF	L929
Chloroform	Honeywell
Isoprophanol	Sigma
18s rRNA	Applied Biosystems
TMB	Life Technologies
Arginase assay Kit	Bioassay Systems
CompBeads	BD Biosciences
LS columns	Miltenyi Biotec
autoMACS columns	Miltenyi Biotec
Amicon Ultra4- 3,000 MWCO	Milippore
Trypan blue	Sigma
Hemocytometer	Hycor Biomedical, UK

2.1.10 *TLR ligands used in vitro and in vivo*

Ligand	*Receptor*	*Supplier*	*Conc. in vitro*	*Dose in vivo*
Ultrapure LPS	TLR4	Invivogen	100 ng/ml	-
LPS	TLR4	Enzo	-	10 µg
Pam3Cys	TLR2	Invivogen	100 ng/ml	-

β-glucan	Dectin-1	Sigma	5 µg/ml	

2.1.11 *Antigens and bacteria-derived molecules*

Reagent	*Supplier*	*Conc. in vitro*	*Conc. in vivo*
MOG_{35-55}	Genscript	12.5-100 µg/ml	100 µg/mouse
FHTE, FHTE-H, FHTE-L	-	0.625-5% v/v	2.5% v/v in 200 µl PBS
Heat killed Mtb	Chondrex	25 µg/ml	-
Mtb in CFA	Chondrex	-	400 µg/mouse
PT	Kaketsuken		100 ng/mouse

2.1.12 *Antibodies used for in vitro culture*

Antibody	*Clone*	*Supplier*	*Concentration*
Anti-CD3	145-2C11	BD Biosciences	1 µg/ml
Anti-CD28	37.51	BD Biosciences	5 µg/ml

2.1.13 *Primers used in quantitative real-time polymerase chain reaction (qRT-PCR)*

Protein	*Gene*	*Product code*	*Ref. Seq*	*Supplier*
18S rRNA	18S	4319413E	X03205.1	ABI
Arginase	*arg1*	Mm00475988_m1	NM_007482.3	ABI
Relm-α	*retnla*	Mm00445109_m1	NM_020509.3	ABI
iNOS	*Nos2*	Mm00440502_m1	NM_010927.3	ABI
IL-1β	*il1β*	Mm00434228_m1	NM_008361.3	ABI
IL-23	*il23*	Mm01160011_g1	NM_031252.2	ABI
IL-17A	*il17a*	Mm00439618_m1	NM_010552.3	ABI
IFN-γ	*ifnγ*	Mm01168134_m1	NM_008337.3	ABI
YMI	*chi3l3*	312249	NM_009892	Roche
CD206	*mrc1*	313688	NM_008625	Roche
Fpr2	*fpr2*	317870	NM_008039	Roche
IL-1R1	*il1r1*	Mm00434237_m1	NM_001123382.1	ABI
IL-23R	*il23r*	Mm00519942_m1	NM_144548.1	ABI
Rorγt	*rorc*	Mm01261022_m1	NM_001293734.1	ABI
T-bet	*tbx21*	Mm00450960_m1	NM_019507.2	ABI

2.1.14 *ELISA kits*

Cytokine	*Block*	*Top working standard*	*Supplier*
IL-1β	1% BSA	1000 pg/ml	R&D
IL-23	1% BSA	2500 pg/ml	R&D
IL-10	1% BSA	2000 pg/ml	R&D
TNF	1% BSA	2000 pg/ml	R&D
IFN-γ	10% milk	10 ng/ml	BD Biosciences
IL-17A	1% BSA	1000 pg/ml	R&D
IL-17F	1% BSA	1500 pg/ml	R&D
IL-22	1% BSA	2000 pg/ml	R&D
GM-CSF	1% BSA	1000 pg/ml	R&D
IL-12p40	1% BSA	2000 pg/ml	R&D

2.1.15 *Antibodies used in FACS*

Specificity	*Fluorochrome*	*Clone*	*Isotype*	*Supplier*
CD3	APC	145-2C11	Armenian hamster / IgG	eBiosciences
CD45	BV785	30-F11	Rat IgG2b	Biolegend
CD4	BV785	RM4-5	Rat IgG2a	Biolegend
CD4	APC-eFluor780	RM4-5	Rat IgG2b	Thermofisher
Vγ4	PECy7	UC3-10A6	Armenian hamster IgG	eBiosciences
Ki67	PerCP-eFluor710	SolA15	Rat IgG2a	eBiosciences
Ki67	e450	SolA15	Rat IgG2a	Thermofisher
TCR-δ	FITC	GL-3	Armenian Hamster IgG2,	BD biosciences

TCR-δ	BV605	GL-3	Armenian Hamster IgG	Biolegend
IL-17A	BV650	TC11-18H10.1	Rat IgG1	Biolegend
IFN-γ	PeCF594	XMG1.2	Rat IgG1	BD Biosciences
IL-1R1	PE	35F5	Rat IgG1	BD biosciences
MHC II	FITC	M5/114.41.2	Rat IgG2b	eBiosciences
MHC II	BV711	M5/114.15.2	Rat IgG2b	BD bioscience
MHC II	APC	M5/114.15.2	Rat IgG2b	Biolegend
PD-L2	PE	TY25	Rat IgG2a	Biolegend
CD45	AF700	30-F11	Rat IgG2b	BD Biosciences
NK1.1	AF700	PK136	Rat IgG2a	BD Biosciences
CD11b	APC-eFluor780	M1/70	Rat IgG2b	eBioscience
F4/80	PerCP-Cy5.5	BM8	Rat IgG2a	Biolegend
F4/80	PECy5	BM8	Rat IgG2a	eBioscience
CD206	BV605	C068C2	Rat IgG2a	Biolegend
Siglec F	PeCF594	E50-2440	Rat IgG2a	BD biosciences
CD40	FITC	3/23	Rat IgG2a	BD biosciences
CD86	APC-Cy7	GL-1	Rat IgG2a	Biolegend
LY6C	FITC	AL-21	Rat IgM	BD biosciences
LY6G	BV650	IA8	Lewis IgG2a	BD biosciences
B220	A700	RA3-6B2	Rat IgG2a	Biolegend
CD3	A700	17A2	Rat IgG2b	Biolegend

2.1.16 *Other reagents used in flow cytometry*

Chemical	*Description*	*Supplier*	*Concentration*
Live/ dead (LD) aqua	Fixable cell stain (viability)	Invitrogen	1 in 500 dilution
Paraformaldehyde	Mitogen	Sigma	5 ng/ml
Ionomycin	Ionophore	Sigma	500 ng/ml

Brefeldin A	Inhibitor of intracellular protein transport	Sigma	5 µg/ml
PMA	Stimulation	Sigma	10 ng/ml
Cell trace violet	Dicycle violet	Thermo fisher	1 µM
Fc Block	Block non-specific binding	BD Biosciences	12.5 µl/2.5 ml

2.2 Mice

Specific pathogen-free (SPF) female C57BL/6 mice were purchased from Harlan UK Ltd (Bicester, Olac, UK). SPF female mice were also bred in-house in the BioResources Unit in Trinity College Dublin. All animals were maintained according to the regulation and guidelines of the Irish Department of Health and Children. All experiments were performed under license from Health Products Regulatory Authority and with the approval from Trinity College Dublin Comparative Medicine Department. Mice were age-matched for each experiment and at the time of initiation of each experiment mice were between 6-10 weeks old.

2.3 Methods

2.3.1 *Preparation of FHTE*

Adult flukes were collected from infected bovine livers at a local abattoir (Kildare Chilling Ltd). Freshly isolated flukes were washed several times in PBS containing 100 µg/ml PS (PBS/PS) to remove contaminants and cellular debris and transported to the lab. Live flukes were incubated at 5-6 worms per 3 ml in PBS/PS overnight in a cell culture incubator at 37°C and 5% CO_2. Supernatants, containing FHES, were removed and the flukes were washed three times in PBS/PS before being washed twice with PBS. Supernatants were decanted after the last wash and flukes were mechanically homogenised for 5 min. The homogenate was centrifuged for 5 min at 2,000 x g to remove large debris followed by centrifugation for 30 min at 15,000 x g. The total soluble fraction (FHTE) was filtered through a 5 mm filter and then a 0.2 µm filter. The sterile homogenate was harvested, aliquoted and stored at -80°C.

2.3.2 *Size fractionation using centrifugal filter units*

FHTE was fractionated into high molecular weight fraction (FHTE-H) and low molecular weight fraction (FHTE-L) using centrifugal filter unit with a 3 kDa cut off membrane (Amicon Ultra-4, 3,000 MWCO, Milippore). FHTE (1 ml) was loaded onto the filter device and centrifuged at 3,000 x g for 2 hours at 4°C. The fraction that passed through the filter was collected without diluting (FHTE-L). The remaining fraction on the top of the filter was replenished up to the original volume of 1 ml with sterile PBS. FHTE-H and FHTE-L were aliquoted and stored at -80°C.

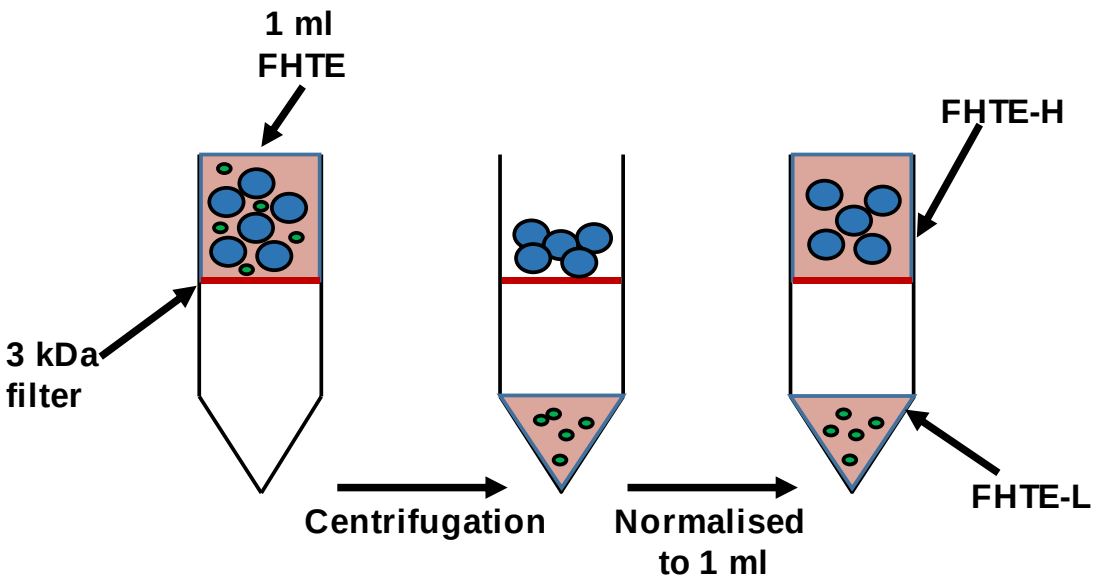

Figure 2.1 Overview of FHTE-H and FHTE-L fractionation via centrifugation.

2.3.3 *Bicinchoininic acid (BCA) protein assay*

Protein concentration of FHTE was determined using BCA protein assay kit. This method combines a reduction of Cu^{2+} to Cu^{1+} cation using a reagent containing BCA. Reduced Cu^{1+} cation chelates molecules of BCA forming purple-coloured, water-soluble complex, which absorbance can be read at 562 nm. FHTE was diluted 1:10, 1:20 and 1:40 in triplicates and together with an 8-point standard curve (25-2000 µg/ml), samples were incubated on a 96-well plate with BCA working solution for 30 min at 37°C in the dark. The absorbance was measured at 562 nm and the concentration of protein in FHTE was calculated based on the standard curve.

2.3.4 *Detection of endotoxin by limulus amebocyte lysate (LAL) assay*

The endotoxin concentration of FHTE was determined using LAL assay according to the manufacturer's protocol. FHTE was diluted 1:100, 1:1000, 1:10,000 in triplicate and together with a 4-point standard curve (0.1 EU/ml, 0.25 EU/ml, 0.5 EU/ml and 1 EU/ml), samples were mixed with LAL reagent and incubated on a 37°C block for 10 min. Chromogenic substrate solution was added to the reaction vessels and the samples were incubated on a 37°C heating block for 6 min. In the LAL assay, endotoxin triggers the enzymatic reaction releasing a yellow-coloured product from colourless substrate, which can be measured photometrically at 405

nm, after the reaction is stopped by 0.25 % acetic acid solution. The endotoxin concentration in FHTE was calculated based on the standard curve.

2.3.5 *Cell count*

Cell counts were performed by diluting cells 1:10 in trypan blue. 10 µl of the cell suspension was then loaded onto a disposable hemocytometer. The number of viable cells (appearing as white in contrast to dead cells that stain blue) was counted using a light microscope. In the case where discrimination of live and dead cells was hampered by substantial amount of debris, cell counts were performed in EB/AO at a 1:10 dilution. Using this method, viable cells fluoresce green when viewed under a UV fluorescent microscope, while dead cells appear orange. The number of cells/ml was calculated using the following formula:

Number of cells per ml= average cell number x dilution factor x 10^4

2.3.6 *Generation of BMDCs*

Bone marrow-derived dendritic cells (BMDCs) were generated from C57BL/6 mice. Naïve mice were sacrificed by asphyxiation with CO_2 and their femurs and tibiae were removed and cleaned from the surrounding muscle. The bone marrow was then flushed from the bones using a 25G needle attached to a 20 ml syringe containing cRPMI. Cell clusters were disrupted by aspirating the cell suspension through a 19G needle. The single cell suspension was centrifuged at 300 x g for 5 min before being resuspended in 2 ml of ammonium chloride lysis solution for 2 min in order to lyse the red blood cells. Cells were washed in cRPMI and centrifuged at 300 x g for 5 min. Cells were then resuspended, counted and cultured in T175 flasks at 0.7 x 10^6 cells/ml in cRPMI supplemented with 20 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) supernatant obtained from the J559 cell line. The J558 cell line was transfected with plasma encoding for GM-CSF and obtained from BALC/b B myeloma cells. After 3 days of culture at 37°C and 5% CO_2, 20 ml of fresh medium containing 20 ng/ml of GM-CSF was added to each T175 flask. On day 6, the supernatant was carefully aspirated, removing loosely adherent contaminating cells such as granulocytes from the culture. 20 ml of warm sterile PBS was added to each flask and gently agitated before being transferred to 50 ml tube containing 10 ml of fresh cRPMI.

Remaining cells were detached from the flask by incubation of 20 ml of sterile EDTA at 37°C for 10 min. Cells were transferred to 50 ml tube containing 10 ml fresh cRPMI. Total cells were centrifuged at 300 x g for 5 min, resuspended in fresh cRPMI, pooled together and counted. The cells were cultured in fresh T175 flasks at 0.7 x 10^6 cells/ml in cRPMI supplemented with 20 ng/ml GM-CSF. ON day 8, 20 ml of fresh cRPMI containing 20 ng/ml GM-CSF was added to each flask. On day 10, loosely adherent cells were harvested, counted and seeded at the required concentration in tissue culture plates. BMDCs were allowed to rest for at least 1 hr at 37°C before use.

2.3.7 *Generations of BMDMs*

Bone marrow-derived macrophages (BMDMs) were generated from C57BL/6 mice. Bone marrow cells were isolated as described in 2.3.6 and cultured in petri dishes at 1x10^6 cells/ml in cRPMI supplemented with 20% v/v of macrophage-colony stimulating factor (M-CSF) in the form of supernatants obtained from culture of the L929 cell line. The origin of the L929 cell line is mouse C3H/An connective tissue. M-CSF-producing L929 cells were obtained by transfection with a plasmid encoding murine M-CSF. On day 3, additional 2 ml of L929 medium was added to each petri dish. On day 6, loosely adherent wells were removed by washing with PBS and adherent macrophages were gently scraped in cRPMI, counted and seeded at the required concentration in tissue culture plates. BMDMs were allowed to adhere and rest for at least 3 hr at 37°C before use.

2.3.8 *Isolation of peritoneal exudate cells (PEC) by peritoneal lavage*

Mice were sacrificed by asphyxiation with CO_2 and the peritoneum was exposed by removing the skin in the abdomen. Cold PBS (6 ml) was injected into the peritoneal cavity using a 27G needle. The mouse was gently shaken to detach any adherent peritoneal cells, and 5 mls were removed with a 19G needle. PEC were placed on ice to prevent adherence of macrophages to the plastic. Cells were counted and seeded at the require concentration in tissue culture plates.

2.3.9 *In vitro stimulation of BMDCs, BMDMs and PEC*

BMDCs, BMDMs and PEC were seeded at the concentration of 1x10^6 cells/ml in tissue culture plates. Cells were cultured with TLR ligands in the presence or

absence of increasing doses of FHTE, FHTE-H or FHTE-L (0.625-5% v/v). After 24 hr, supernatants were removed, and the concentration of cytokines was quantified by ELISA. Total RNA was also isolated and gene mRNA expression was evaluated by qRT-PCR. For BMDCs and BMDMs studies, bone marrow from 2 mice were pooled before the generation of either BMDCs or BMDMs and each experiment was repeated 2 or 3 times as detailed in the individual figure legends. For PEC studies, PEC was isolated and pooled from 4 mice before plating and each experiment was repeated 2 or 3 times as detailed in the individual figure legends.

2.3.10 *Arginase activity assay*

After 24 hr stimulations, cells were incubated in 80 µl of passive lysis buffer (Promega) containing protease inhibitors. Arginase activity of cell lysate was tested using the Quntichrom Arginase Assay Kit (Bioassay Systems). A urea working standard (1 mM) was made and 25 µl of the urea working standard solution was then added to a well. Next, 20 µl of each sample was then added to each appropriate well, with one well containing H_2O and another as a blank well to be left substrate free. 5 µl of arginase substrate buffer was added to each sample, bar the blank well. Arginase activity was determined by the quantity of urea produced in a 2 hr period. 80 µl of urea reagent containing 1:1 of reagent A and reagent B was added to all wells. Finally, the substrate buffer was added to the blank well. This method utilises a chromogen that forms a coloured complex specifically with urea produced in the arginase reaction. The intensity of the colour is directly proportional to the arginase activity in the sample. The OD values were determined by measuring the absorbance at 430 nm using a microtiter plate reader. Arginase activity in units/L was calculated using the following formula:

$$\textit{Arginase Activity (units/L)} = \frac{A_{430}(Sample) - A_{430}(Blank)}{A_{430}(Urea\ Standard) - A_{430}(H2O)}\ x\ \frac{1mM\ x\ 25\ \mu l\ x\ 10^3}{20\ \mu l\ x\ 120\ min}$$

2.3.11 *In vitro training of PEC and BMDMs*

PEC or BMDMs from naïve C57BL/6 mice were incubated with 2.5% v/v FHTE, LPS (100 ng/ml) or β-glucan (5 µg/ml) (1st stim). Cells were washed after 24 hr and left to rest. After three days, cells were restimulated with 2.5% v/v FHTE, LPS (100

ng/ml) or Pam3cys (10 µg/ml) (2^{nd} stim). Supernatants were collected 24 hr after restimulation and the concentration of cytokines quantified by ELISA. *In vitro* training was optimised before final experiments for both the concentration of FHTE and resting periods prior to restimulation. PEC was isolated and pooled from 4 mice before plating and each experiment was repeated 3 times. Bone marrow from 2 mice was pooled before the generation of BMDMs and each experiment was repeated 3 times.

2.3.12 *Purification of large and small peritoneal macrophages by fluorescence-activated cell sorting (FACS)*

Freshly isolated PEC were washed and resuspended in a volume of 1 ml. The cells were incubated with Fc block (12.5 µl/2.5 ml) for 10 min to prevent non-specific binding. Cells were surface stained with fluorescent antibodies directed against CD11b, F4/80 and MHC II (1 in 200 dilution) for 15 min at room temperature (RT) in the dark. Cells were washed and resuspended in 1 ml of FACS buffer and sorted by flow cytometry. Large peritoneal macrophages (LPM) were identified as $CD11b^{+}F4/80^{high}$ $MHCII^{low}$ and small peritoneal macrophages (SPM) identified as $CD11b^{+}F4/80^{low}MHCII^{high}$.

2.3.13 *CTV staining*

Spleen cells were stained with CTV using CellTrace Violet Cell Proliferation Kit according to the manufacturer's protocol. Cells were washed and resuspended in PBS at a concentration up to $5x10^{6}$ cells/ml. CTV was added while vortexing to obtain a final volume of 1 µM. Cells were incubated at 37°C in the dark for 20 min before being washed with 5 times the original volume in pre- warmed cRPMI and incubated for 5 min at RT. Cells were centrifuged, counted and used as required.

2.3.14 *CD4 T cell isolation by manual Magnetic-activated cell sorting (MACS)*

A CD4 T cell isolation kit containing CD4 T cell biotin antibody cocktail and anti-biotin microbeads was used to isolate CD4 T cells. The cell number was determined and the cells were then centrifuged at 300 x g for 10 min. The supernatant was removed and 40 µl of MACS running buffer and 10 µl of antibody cocktail per $1x10^{7}$ cells was added to the cell pellet (as stipulated by the

manufacturer's instructions). The cells were incubated for 5 min on ice. After 5 min, another 30 µl of buffer and 20 µl of anti-Biotin MicroBeads (per $1x10^7$ cells) were added and incubated for 10 min on ice. An LS column was placed in the MACS separator and rinsed with 3 ml of buffer. Cell suspension was applied, and all effluent was collected as enriched CD4 T cells. Cellular purity was determined by flow cytometry and was consistently ~ 97%.

2.3.15 *γδ T cell isolation by automated MACS*

γδ T cells were purified from pooled inguinal, auxiliary, brachial, cervical and mesenteric LN cells by sequential automated MACS using TCR γδ T cell isolation kit. 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: rate IgG2b) while γδ T cells were labelled with biotin-conjugated monoclonal antibody against mouse TCR γδ. Non-T cells were depleted on an autoMACS machine using the "depl05" program. γδ T cells in remaining T cells enriched fraction were indirectly labelled with Anti-Biotin MicroBeads and purified by positive selection on an autoMACs machine using the "posseld2" program. Cellular purity of separated γδ T cells was determined by flow cytometry to be ~96%.

2.3.16 *In vivo training of mice*

C57BL/6 mice were injected subcutaneously (s.c) with PBS, 2.5% v/v FHTE in 200 µl PBS, or LPS (10 µg) on day -21 and day -7. On day 0 cells were either isolated from the peritoneal cavity or the LNs and used as required. 2.5% v/v FHTE (in 200 µl PBS) *in vivo* is the equivalent of 50 µg/mouse and the concentration was optimised and tested *in vitro* prior to *in vivo* studies. EAE was also induced in mice on day 0 and mice were assessed for clinical signs of disease and weighed. 5-6 mice per group was used in each *in vivo* training experiment.

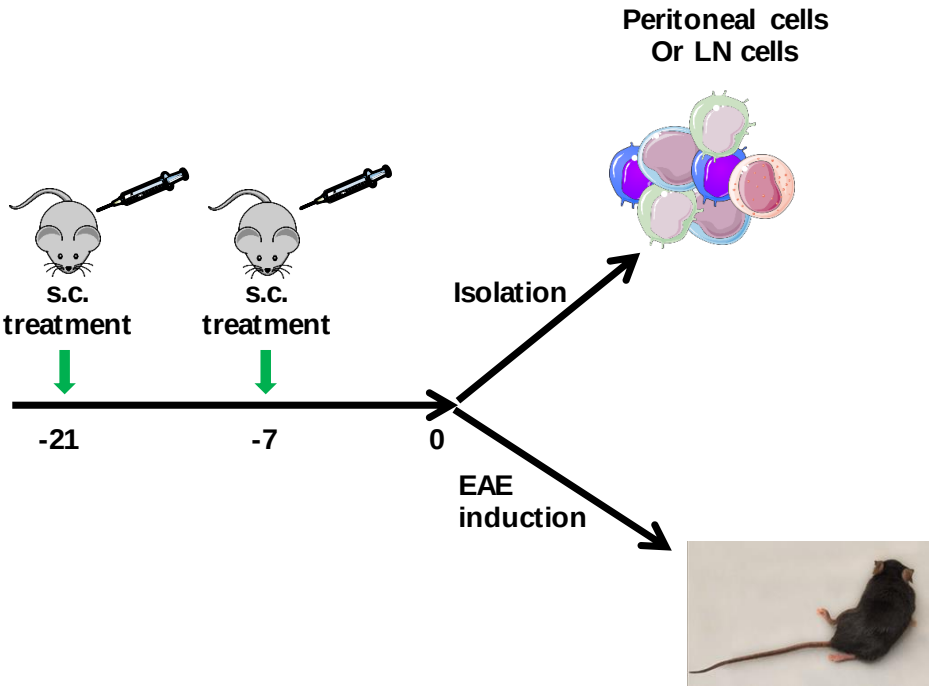

Figure 2.2 Overview of *in vivo* training of mice.

2.3.17 *Induction, treatment and assessment of EAE*

Active induction of EAE

EAE was induced in C57BL/6 mice by s.c injection of 100 µg/mouse MOG emulsified in CFA containing 400 µg/mouse of heat killed *M. tuberculosis* (Mtb). Mice were injected intraperitoneally (i.p) with 100 ng of PT on day 0 and day 2. Disease severity was assessed according to percentage weight change and EAE clinical scores as follows:

0- No clinical signs
1- Limp tail
2- Ataxic gait
3- Hind limb weakness
4- Hind limb paralysis
5- Tetra-paralysis/moribund

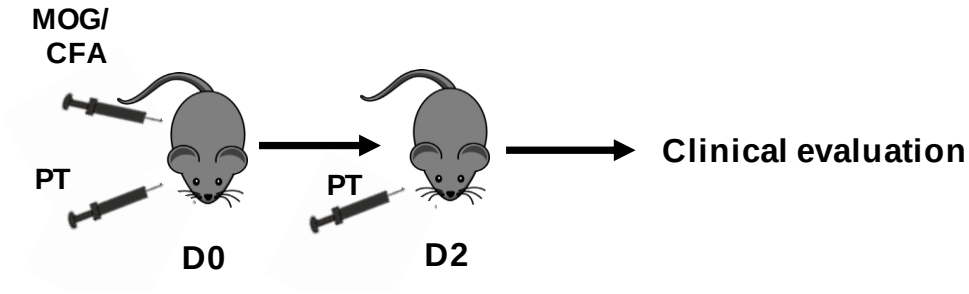

Figure 2.3 Active EAE model.

Induction of EAE by adoptive transfer

EAE was induced in donor C57BL/6 mice by immunization with MOG (100 µg) and CFA without the administration of PT. Donor mice were scarified on day 10 post-induction and their spleen and LN cells were cultured at $10x10^6$ cells/ml in cRPMI supplemented with 2-ME in the presence of MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml). After 72 hr, non-adherent cells were collected from the culture vessel, washed and resuspended in PBS and counted. Supernatants were taken at this point and cytokine production quantified by ELISA. Cells were then washed and transferred into naïve recipient mice at a concentration of $10x10^6$ live cells in 200 µl PBS via i.p injection. Animals were monitored daily for clinical signs of disease as described in the previous section.

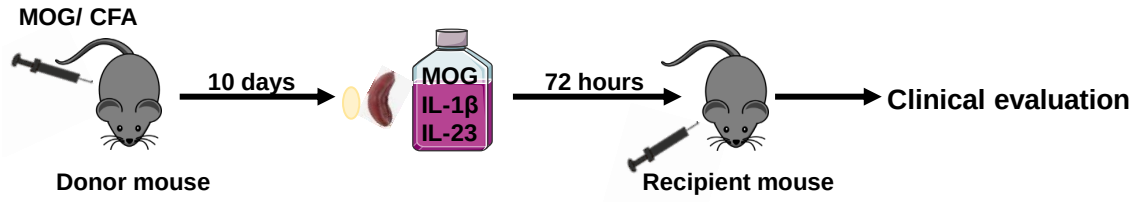

Figure 2.4 Overview of passively induced EAE.

Isolation of MNCs from the spleen and LNs

Mice were sacrificed by asphyxiation with CO_2 and the spleen and inguinal, brachial and axillary LNs were removed. Spleen and LNs were process by pushing them through a sterile cell strainer (70 or 40 µm respectively) using a plunger from a sterile 5 ml syringe. Cells were pelleted by centrifugation at 300 x g for 5 min and resuspended in cRPMI and used as required.

Isolation of MNCs from CNS tissue

Mice were sacrificed by asphyxiation with CO_2 and perfused intra-cardinally with 20 ml of ice-cold PBS to remove peripheral blood from CNS tissue. Brain and spinal cord tissue was isolated, homogenised in cRPMI and passed through a 70 µm cell strainer. Cells were centrifuged at 300 x g and MNCs were resuspended in 5 ml of 40% isotonic Percoll solution. This was carefully overlaid on 5 ml 70% isotonic Percoll solution with a plastic Pasteur pipette. The Percoll gradients were centrifuged at 1600 rpm for 20 minutes without the break at RT. After centrifugation, the upper myelin layer was removed and discarded. MNCs were found on the interface of the Percoll gradients and were isolated using a Pasteur pipette. Cells were washed twice with cRPMI, counted using EB/AO and used as required.

2.3.18 *qRT-PCR*

RNA isolation

Total RNA was extracted from cells using the TRIzol/chloroform method. Cells were resuspended in 200-500 µl of TRIzol and incubated at RT for 5 min, allowing complete dissociation of the nucleoprotein complex. Chloroform (100-200 µl) was added to each sample and the tubes were capped securely before shaking vigorously by hand for 15 seconds. Samples were incubated for 2-3 min at RT and then centrifuged at 12,000 x g for 15 min at 4˚C. The mixture separated into a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase was removed from each sample by angling the tube at 45˚ and pipetting the solution out into newly labelled RNase-free tube containing 250 µl Isopropanol. The samples were then incubated for 10 min at RT and centrifuged at 12,000 x g for 10 min at 4˚C. Supernatants were carefully removed and the pellet was washed with 500 µl of 75% ethanol and vortexed briefly before being centrifuged at 7,500 x g for 5 min at 4˚C. The RNA pellet was air dried for 10 min before being resuspended in 20 µl of RNase-free water. RNA yield was determined using the NanoDrop® Spectrophotometer and the purity of the sample was determined by 260/280 nm ratio. Samples were stored at -80˚C.

cDNA synthesis

RNA (1-2 µg) was used as a template for reverse transcription reaction. A master mix was made from a high capacity cDNA reverse transcriptase kit (table 2.1) and for each cDNA reaction in a PCR tube, 10 µl of 2X RT master mix was required for 10 µl of RNA dilution (20 µl total/reaction). RNA was reverse transcribed as indicated in table 2.2.

Table 2.1

Reagent	Volume/ reaction (µl)
10X RT Buffer	2
25X dNTPs mix (100mM)	0.8
10X RT Random primers	2
MultiScribe RT	1
RNase free H_2O	4.2
Total per reaction	10 µl

Table 2.2

	Temperature [˚C]	Time [min]
Step 1	25	10
Step 2	37	120
Step 3	85	5
Step 4	4	∞

RT-PCR reaction step

cDNA was diluted in nuclease-free water and used as a template for PCR reaction. Eukaryotic 18S rRNA was used as an endogenous control for the PT-PCR. The reaction mix contained 5.5 µl of the Probe/18S master mix (table 2.3) and 4.5 µl of the cDNA sample. The PCR reaction plate was sealed using an adhesion seal and the plate was centrifuged at 500 x g for 1 min. The reaction was carried out on Applied Biosystem 7500 fast Real-Time PCR machine using Real-time PCR Cycle (table 2.4).

Table 2.3

Reagent	Volume required per reaction
TaqMan Fast Universal PCR Master mix (2X)(applied BioSystems)	4.5 µl
Reaction probe	0.5 µl
Endogenous primer (18S)	0.5 µl

Table 2.4

Temperature [˚C]	Time	Cycle
95	2 min	1
55 72	3 sec 30 sec	40

Data was analysed using relative quantification on Applied BioSystems 7500 software, which provided cycle threshold (Ct) values. Ct is defined as the number of cycles required for the fluorescent signal to cross a threshold, exceeding background fluorescence. The change in gene expression was normalised to 18S expression from the corresponding samples (CT_{gene}-CT_{endo}=dCT). Furthermore, Ct values from control samples were averaged and subtracted from the dCT value of each sample (dCT-$dCT_{control}$=ddct). Fold induction was calculated as $2^{(-ddct)}$.

2.3.19 *ELISA*

ELISA were performed to access protein levels of mouse IL-1β, TNF, IL-10, IL-23, IL-17, GM-CSF, IL-12p40 and IFN-γ in cell supernatants. DuoSet ELISA kits from R&D systems (mouse) were used, except for mouse IFN-γ BD Pharmingen ELISA kit, and were performed according to the manufacturer's specifications.

High-binding 96-well ELISA plates were coated with 50 µl of diluted primary capture antibody to a working concentration in PBS (at concentrations stipulated in the manufacturer's protocol). Plates were sealed and incubated at 4˚C overnight. Each plate was aspired and washed 4x with PBS-T before being blocked in 150 µl of appropriate blocking buffer and incubated at RT for 2 hr. The plates were washed and 50 µl of standards and supernatant samples were added to each well. The standards were serial diluted in 1% BSA and plates were incubated overnight at 4˚C. After washing, 50 µl of recommended concentration of detection antibody was

added and incubated for 2 hr at RT, with the exception of mouse IFN-γ, which was incubated for 1 hr. The plates were then washed before the addition of 50 µl of appropriate concentration of Streptavidin HRP to each well and incubated in the dark for 20 min at RT. After washing, 50 µl of TMB substrate solution was added to each well and incubated for a further 20 min in the dark or until a colour change occurred. In order to stop the reaction, 25 µl of stop solution was added. The optical density of each well was determined immediately using a microplate reader at 490 nm and the concentration of each cytokine calculated based on the standard curve.

2.3.20 *Flow cytometry*

Surface staining

Cell suspensions, prepared from organs isolated from *in vivo* studies or obtained from *in vitro* cultures were added to sterile FACS tubes and washed in PBS. Cells were stained in 50 µl of LD aqua (1:500 dilution) and incubated on ice for 15 min. The cells were then washed in PBS before being centrifuged at 300 x g for 5 min. Cells were then incubated in 50 µl of the appropriate FACS antibody cocktail directed against surface antigens as well as FC block (12.5 µl/2.5 ml) to prevent non-specific binding for 15 min at RT in the dark. In parallel, compensation controls were prepared in order to calibrate the FACS machine before use. Compensation was performed using CompBead, which contain a mixture of negative beads and positive beads that directly bind Ig non-specifically. Fluorescence Minus One (FMO) controls were used as a guide for gating strategy for populations that were not well defined. Excess straining was removed by washing each tube in PBS. Cells were centrifuged and resuspended in 150 µl of PBS and analysed by flow cytometry.

Intracellular staining

For intracellular staining samples were first restimulated with 5 ng/ml 12-o-tetra decanoylphorbol 13 acetate (PMA), 500 µg/ml ionomycin and 5 µg/ml brefeldin A for 4-5 hrs in cRPMI supplemented with 50 µM 2-ME. After incubation, cells were washed with PBS, pelleted by centrifugation at 300 x g for 5 min and stained with LD aqua and surface marker antibodies. Cells were then fixed and permeabilised using 2% paraformaldehyde in PBS and incubated for 15 min at 4°C in the dark. After washing, cells were centrifuged in 1 ml of saponin buffer (0.5% saponin in

PBS) and pelleted by centrifugation for 5 min. Cells were stained with appropriate intracellular antibody dilutions in saponin buffer for 25 min at 4°C in the dark. Cells were washed in saponin to remove unbound antibody and then washed in PBS for membrane closure and analysed by flow cytometry.

Intranuclear staining

For intranuclear staining of Ki67, samples were stained with LD aqua and surface stain as described above before being incubated with 200 µl of Fixation/Permeabilization from the Foxp3/Transcription Factor Staining Buffer Kit according to the manufacturer's protocol overnight at 4°C. Cell were then washed in 1 ml of 1X Permeabilization buffer (diluted in Baxter water) and stained intranuclearly for 40 min in the dark at RT. Cell were washed using 1X Permeabilization buffer followed by FACS buffer and analysed by flow cytometry.

2.3.21 *Statistical analysis*

Statistical analysis was carried out using GraphPad prism software. Unpaired student t-test was used to compare the statistical difference between the mean values of two groups. One-way analysis of variance (ANOVA) test followed by Dunnett or Tukey post-test was applied to determine the statistical difference between more than two groups. Two-way or three-way ANOVA followed by Sidak or Tukey post-test was used to determine statistical significance between groups that have been split on two or three independent variables respectively. Repeated measures ANOVA followed by Sidak or Tukey post-test was used to determine the statistical differences between groups in a time course. Error bars indicate the mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) as indicated in the individual figure legends. Statistical significance was considered for p values less than 0.05.

Chapter 3.

F. hepatica TE attenuates the induction of T cell-mediated autoimmunity via innate immune training.

3.1 Introduction

Helminths are large multicellular organisms that infect approximately 2 billion people worldwide, mainly in the developing world. A number of studies have demonstrated that infection with helminths induces type 2 immune responses, however, many parasites have developed highly effective mechanisms of immune subversion that result in chronic infections. Much of our understanding of the immune response to helminths comes from the study of mouse models of infection, where immunity and expulsion is mediated by M2 macrophages, eosinophils and Th2 cells. However, helminths have evolved a wide variety of approaches for immune suppression, especially anti-inflammatory cytokine production and the generation of Treg cells. *H. polygygrus* has been shown to enhance the frequency of Treg cells in the host during infection to establish long-term persistence [193]. Likewise, Taylor et al. have shown that infection with *Litomosoides sigmodontis* induces hyporesponsiveness in mice that can be reversed by the depletion of Treg cells *in vivo* [194].

Immunosuppression associated with helminth infections impairs control of concurrent microbial infections. For example, effective immunity against malaria is dependent on a strong Th1 response [195] and epidemiological studies have demonstrated that helminth infection is associated with increased incidence of malaria [196]. Furthermore, our lab has previously reported that infection with *F. hepatica* induced polarized Th2 responses in mice, suppressed Th1 responses and delayed clearance of the bacterial pathogen *B. pertussis* [186]. However, suppression of T cell responses may be beneficial to the host in certain immune-mediated conditions. Indeed, epidemiology studies have shown that developing countries with a high prevalence of parasite infections have a lower incidence of allergy and autoimmune diseases. The inverse correlation between exposure to pathogen and incidence of allergy was first described by Strachan and coined the term “the hygiene hypothesis” [161]. A lack of early childhood exposure to infectious agents, symbiotic microorganisms and parasites increases susceptibility to allergic or autoimmune diseases by suppressing the natural development of the immune system. A study by Cooke et al. demonstrated that infection with *S. mansoni* prevents insulin dependent diabetes mellitus in non-obese diabetic mice [176]. Intestinal nematode Infection was also found to

ameliorate experimental colitis in mice [197]. Furthermore, our lab has shown that infection with live *F. hepatica* attenuated the clinical symptoms of EAE through immunomodulatory mechanisms that suppressed autoantigen-specific pathogenic Th1 and Th17 responses [159].

Although a lot of studies have focused on the induction of Th2 and Treg cell responses during helminth infection, more recent research has shown direct effect of helminths on innate immune cells. DCs that are primed during helminth infection often fail to show signs of classical maturation. Our lab has demonstrated that infection of mice with *F. hepatica* resulted in recruitment and activation of regulatory DCs that had low surface expression of costimulatory molecules and increased IL-10 production [159]. Furthermore, in a mouse model of *S. mansoni* infection, alternatively activated macrophages are essential for parasite survival in the host [198]. *F. hepatica* infection is also associated with the polarisation of alternatively activated macrophages in infected animals that produce increased levels of IL-10 and TGF-β [159]. However, the mouse is not a natural host of *F. hepatica*, as recognised by the significant mortality associated with infection [189]. As a result, efforts are now focusing on exploring the immune targets of parasite-derived products. Hamilton et al. demonstrated that tegumental antigens from *F. hepatica* inhibit maturation of DCs [191]. This study also demonstrated that *F. hepatica* products suppressed TLR-induced proinflammatory cytokines and suppressed the concentration of IFN-γ and IL-12 in the serum of mice with septic shock. Moreover, glycoconjugates from *F. hepatica* were shown to enhance TLR-driven IL-10 production by DCs [190]. Furthermore, fatty acid binding protein from *F. hepatica* was also found to induce the alternative activation of human macrophages [192].

Until recently, immunological memory was believed to be confined to antigen-specific T and B cells. However, this dogma has recently been challenged by studies showing that innate immune responses in plants and invertebrates, that lack an adaptive immune response, mount resistance to reinfection. In plants, epigenetic changes lead to the priming of genes encoding host defence molecules. Upon re-exposure, these cells respond more rapidly and robustly, a process known as SAR. More recently, training of the innate immune

system has also been described in vertebrates that display adaptive characteristics. Studies by Mihai Netea's group have demonstrated that human monocytes exposed to β-glucan or the BCG vaccine have higher capacity to respond and produce cytokine upon restimulation, when compared with non-trained cells [199]. In contrast, stimulation with LPS induced long-term tolerant cells, resulting in reduced production of proinflammatory mediators [111]. Furthermore, there is also some evidence of innate immune modulation and memory by parasites. A recent study by Schrum et al. demonstrated innate immune training by *P. falciparum*, where hyperresponsiveness to subsequent ligation of TLR2 was observed in infected PBMCs [200].

The present study investigated the hypothesis that training of innate immune cells can induce an anti-inflammatory phenotype in response to helminths both, *in vitro* and *in vivo*. Furthermore, the link between innate immune training and the hygiene hypothesis was examined using a mouse model of MS. The data revealed that T cell responses that mediate autoimmune disease were indirectly attenuated by training of the innate immune system in response to FHTE.

3.2 Results

3.2.1 *FHTE induces IL-1RA and IL-10 production but suppresses LPS-induced TNF, IL-12p40, IL-1β and IL-23 production by innate immune cells*

Our lab has previously demonstrated that infection of mice with *F. hepatica* resulted in recruitment and activation of regulatory DCs and macrophages that produced IL-10 [159]. As a result, this study examined the effect of FHTE on cytokine production by innate immune cells. PEC, BMDMs and BMDCs were stimulated with LPS (100 ng/ml), FHTE (1.25 or 2.5% v/v) or a combination of both. After 24 hours, supernatants were removed and the concentration of IL-1RA, IL-10, TNF, IL-12p40, IL-1β and IL-23 was quantified by ELISA.

Stimulation of PEC with FHTE significantly induced IL-1RA and IL-10 production, however, FHTE did not enhance LPS-induced IL-1RA and IL-10 production. In addition, FHTE significantly suppressed LPS-induced TNF and IL-12p40 production by PEC, in a dose dependent response (Fig 3.1). A similar trend was observed in BMDMs, with FHTE stimulation significantly enhancing IL-1RA and IL-10 in addition to suppression of LPS-induced TNF and IL-12p40 secretion (Fig 3.2). Furthermore, the results revealed that FHTE induced IL-1RA production by BMDCs but also enhanced LPS-induced IL-10 production. Furthermore, stimulation of BMDCs with FHTE significantly suppressed LPS-induced TNF and IL-12p40 production, in a dose dependent manner (Fig 3.3).

IL-1β and IL-23 released from DCs play an important role in the activation of γδ T cells and expansion of Th17 cells, which are crucial in autoimmune diseases [201]. Unlike macrophages, DCs produce IL-1β and IL-23 in response to LPS and this was significantly suppressed by FHTE (Fig 3.3). Furthermore, FHTE significantly suppressed LPS-induced *il1β* and *il23* mRNA expression, at both concentrations tested (Fig 3.4). Previous studies in human monocytes have demonstrated that IL-10 production inhibits LPS-induced production of inflammatory cytokines, including IL-1β, by blocking gene transcription [202]. However, this study demonstrated that stimulation of BMDCs with FHTE suppressed LPS-induced *il1β* and *il23* mRNA expression in the presence of anti-IL-10R antibody, demonstrating that suppression is independent of IL-10 (Fig

3.5). Taken together these results indicate that treatment of innate immune cells with FHTE induced anti-inflammatory cytokine production while suppressing proinflammatory responses.

3.2.2 *FHTE enhances arginase activity in IL-4-activated BMDMs*

Macrophages adopt an alternatively activated phenotype when activated in response to helminth infection through IL-10 production [203]. Having shown FHTE induces IL-10 production by BMDMs, this study examined the ability of FHTE to polarise M2 macrophages responses in BMDMs. BMDMs did not induce arginase activity in response to FHTE stimulation alone, however, FHTE significantly enhanced IL-4-induced arginase activity, in a dose dependent manner (Fig 3.6).

To confirm the finding that FHTE promotes M2 macrophage activation, total RNA was isolated from FHTE and IL-4 stimulated cells and the expression of *arg1* and *mrc1* determined by qRT-PCR. FHTE significantly enhanced expression of *arg1* in IL-4-activated BMDMs. Furthermore, FHTE stimulation enhanced IL-4-induced mRNA expression of the M2-specific gene, *mrc1* (Fig 3.7). These results confirm that FHTE polarise M2 macrophage markers in IL-4-activated BMDMs.

3.2.3 *FHTE trains innate immune cells to be more anti-inflammatory*

Until recently, immunological memory was believed to be confined to antigen-specific T and B cells, however, recent studies from Netea and colleagues have shown that innate immune cells can be non-specifically trained in response to certain stimuli and that inflammatory responses are hyperresponsive (trained) or hyporesponsive (tolerant) after second exposure to immunomodulatory molecules [111]. Having shown that FHTE significantly enhances anti-inflammatory responses, while also polarising M2 macrophages, the ability of FHTE to train innate immune cells was examined. PEC or BMDMs were stimulated with either 2.5% v/v FHTE, LPS (100 ng/ml) or β-glucan (5 µg/ml). After 24 hours, cells were washed and rested for three days before restimulation with 2.5% v/v FHTE, LPS (100 ng/ml) or Pam3cys (10 µg/ml). After 24 hours, supernatants were removed and the concentration of IL-10 and TNF determined by ELISA.

Training of PEC with FHTE reduced production of the proinflammatory cytokine, TNF, while enhancing IL-10 production upon restimulation with LPS. β-glucan-trained PEC had increased production of TNF and IL-10 after second stimulation with LPS, whereas LPS-trained PEC had reduced TNF production but no change in IL-10 production (Fig 3.8). This study was then extended to examine the effect of FHTE-training in response to restimulation with different TLR ligands. FHTE-trained PEC significantly enhanced IL-10 production when restimulated with FHTE and Pam3cys, however, the levels of TNF were unchanged in PEC trained with FHTE upon stimulation with Pam3cys. Training of cells with LPS did not modulate IL-10 or TNF production upon restimulation with FHTE or Pam3cys. Furthermore, β-glucan trained cells had enhanced IL-10 production upon restimulation with FHTE and enhanced IL-10 and TNF production upon secondary stimulation with Pam3cys (Fig 3.9).

Since peritoneal exudate contains many cell types, this study examined the effect of FHTE-training on a more clearly defined population of macrophages. FHTE-trained BMDMs enhanced IL-10 production upon restimulation with FHTE or LPS, while suppressing TNF production upon secondary stimulation with LPS and Pam3cys. In contrast, BMDMs trained with LPS did not modulate IL-10, however, significantly suppressed TNF production upon restimulation with FHTE, LPS or Pam3cys. Furthermore, β-glucan-trained BMDMs did not induce IL-10 production upon restimulation with FHTE, LPS or Pam3cy, however, enhanced TNF production after secondary stimulation with Pam3cys (Fig 3.10). These findings suggest that FHTE can train innate immune cells non-specifically to be more anti-inflammatory.

3.2.4 *Training of mice with FHTE polarises an M2 phenotype*

The *in vitro* studies described above suggest that FHTE can train innate immune cells to be more anti-inflammatory. In order to test if innate immune cells can be trained *in vivo*, C57BL/6 mice were injected with PBS, FHTE (2.5% v/v in 200 µl PBS) or LPS (10 µg) at day 0 and day 14. 7 days after the last injection, PEC were isolated by peritoneal lavage and stained with antibodies specific for NK1.1, CD40, CD3, CD11b, Siglec F, F4/80, MHC II (Fig 3.11). FHTE-trained mice had a significant increase in the number of cells in the peritoneal cavity when compare with PBS or LPS-trained mice (Fig 3.12A). Furthermore, the number of

macrophages was also significantly enhanced in FHTE-trained mice, indicating expansion or recruitment of macrophages to the peritoneal cavity (Fig 3.12B).

It has been previously shown that eosinophilia is a key feature of *F. hepatica* infection in mice [159]. This study found that the relative frequency of Siglec F^+ eosinophils was increased from 4% in PBS-treated mice to 30% in mice trained with FHTE (Fig. 3.13A). Furthermore, training of mice with FHTE led to significant increase in the absolute numbers of eosinophils in the peritoneal cavity, when compared with PBS or LPS-trained mice (Fig. 3.13B). This contrasts with previous work in our lab demonstrating that treatment of mice with FHES does not induce eosinophilia in mice 7 days later after one injection (Walsh and Mills, Unpublished), indicating that training of mice induces long lasting infiltration of eosinophils into the peritoneal cavity.

The peritoneal cavity contains two distinct macrophage populations, large peritoneal macrophages (LPM) and small peritoneal macrophages (SPM). LPM are tissue resident cells that make up approximately 90% of macrophages in naïve mice and express high levels of the macrophage surface markers, CD11b and F4/80. SPM are derived from blood monocytes and rapidly enter the peritoneal cavity upon stimulation, and differentiate into mature SPM expressing lower levels of CD11b and F4/80 but high levels of MHC II [204]. Training of mice with FHTE did not enhance the frequency of SPM or LPM in the peritoneal cavity, however, FHTE-trained mice had an increase in the number of both SPM and LPM, when compared with PBS mice, indicating possible expansion and infiltration of these macrophages. In contrast, training of mice with LPS led to a small decrease in the frequency of LPM, when compared with PBS mice (Fig 3.14). These results indicate that training of mice with FHTE results in recruitment of eosinophils to the site of injection as well as inducing expansion of tissue resident macrophages and recruitment of circulating monocytes.

Helminth infections are known to trigger highly polarized type 2 immune responses typically associated with increased numbers of alternatively activated macrophages [205]. Furthermore, recent studies have showed that alternatively activated macrophages can be derived from either proliferation of tissue resident macrophages (LPM) or recruitment of SPM to the site of injection [203]. Cells

from FHTE-trained mice were isolated as described above and LPM and SPM were stained with antibodies against the M2 macrophage markers, CD206 and PD-L2. LPM from FHTE-trained mice had similar expression of CD206 and PD-L2 compared with LPM from PBS and LPS-trained mice (Fig 3.15A). However, FHTE-trained mice had a significant increase in the frequency and number of infiltrating $CD206^+$ $PD\text{-}L2^+$ SPM (Fig 3.15B). These results are consistent with research showing that blood derived monocytes are reprogrammed into $PD\text{-}L2^+$ expressing macrophages during *Taenia crassiceps* and *N. brasiliensis* helminth infection [206, 207].

To confirm the finding that training of mice with FHTE polarises M2 macrophages and to examine the expression of other markers specific for classically and alternatively activated macrophages, total RNA was isolated from FACS purified LPM and SPM on day 21 from mice treated on day 0 and 14 with PBS, FHTE (2.5% v/v in 200 µl PBS) or LPS (10 µg). mRNA expression of *retlna, arg1*, and *chi3l3* was significantly increased in LPM from FHTE-trained mice. There was also a marginal increase in *mrc1* mRNA expression, when compared with PBS or LPS-trained mice. Moreover, *fpr2* expression was higher in LPS-trained mice, when compared with FHTE-trained mice (Fig 3.16).

mRNA expression of M2-associated genes, *retlna* and *arg1* and M2-specific genes, *chi3l3* and *mrc1* was significantly increased in SPM from FHTE-trained mice, when compared with PBS or LPS-trained mice. Furthermore, FHTE-trained mice had lower *nos2* expression in SPM, when compared with PBS or LPS-trained mice. In addition, expression of *frp2* was enhanced in LPS-trained mice, when compared with FHTE-trained mice (Fig. 3.17). Taken together, these results indicate that FHTE trains both tissue resident LPM as well as monocyte-derived SPM to be anti-inflammatory and polarised towards an M2 phenotype.

3.2.5 *$F4/80^+CD11b^+$ macrophages from FHTE-trained mice suppress MOG-specific T cell responses*

Having shown that FHTE-trained mice polarise towards an M2 phenotype *in vivo*, this study next examined the ability of these cells to suppress antigen-specific T cell responses. MOG-specific T cells were generated in mice by immunization with the autoantigen MOG (100 µg) emulsified in CFA. IL-17A and IFN-γ was

produced by MOG-specific T cells restimulated with MOG and this was significantly enhanced by the addition of macrophages from naïve mice injected with PBS (ratio 5:1). In contrast, macrophages from FHTE-trained mice significantly suppressed MOG-specific IL-17A and IFN-γ production by spleen and LN cells, in a ratio dependent manner (Fig 3.18). These results confirm that FHTE-trained mice polarise towards M2 macrophages that can suppress MOG-specific T cells responses.

3.2.6 *Training of mice with FHTE attenuates EAE*

In the EAE model, M1 macrophages have been shown to play a pathogenic role in initiation of disease [208] and polarising macrophages from M1 to M2 has shown protective effects in the modulation of EAE [20]. The results so far have demonstrated that training of mice with FHTE polarises an M2 macrophage phenotype that suppresses MOG-specific T cell responses. Here, the EAE model was used to examine the effect of innate immune training on a model of T cell-mediated autoimmunity. The onset and clinical course of EAE was significantly attenuated in FHTE-trained mice compared with mice injected with PBS. By day 12 after the induction of EAE, 60% of control mice had already developed moderate to severe paralysis, whereas 86% of FHTE-trained mice had no clinical signs of disease (Fig 3.19A). Training of mice with FHTE also decreased disease severity, significantly reducing cumulative score on day 30 (termination of experiment) from 4.3 to 1.9 ($p<0.001$) (Fig 3.19C). Furthermore, PBS mice lost a significant amount of weight during the effector phase of disease, whereas no weight loss occurred in FHTE-trained mice, reflecting the less severe disease outcome in these mice (Fig 3.19B).

3.2.7 *Reduced peripheral effector T cell responses following induction of EAE in FHTE-trained mice*

Before the induction of EAE, FHTE-trained mice were found to have significantly less IL-17A-producing Vγ4 cells in the LNs, when compared with PBS-injected mice (Fig 3.20). Furthermore, peripheral T cell expansion was significantly reduced in FHTE-trained mice after the induction of EAE. FHTE-trained mice had significantly less CD3, CD4 and γδ T cells in the LNs, when compared with PBS-injected mice 7 days after the induction of EAE, indicating reduced cell proliferation in FHTE-trained mice (Fig 3.21). Proliferation of CD4 T cells was

examined using intranuclear straining for ki67, a protein that is strictly associated with cellular proliferation, and is not expressed in resting cells. The frequency and absolute number of $Ki67^+$ proliferating CD4 T cells was significantly decreased in the LNs of FHTE-trained mice compared with PBS-treated mice on day 7 of EAE (Fig 3.22). In addition, the number of IL-17A-producing CD4 T cells was also significantly reduced (Fig 3.23). Interestingly, there was no change in the number of IFN-γ-producing CD4 T cells in FHTE-trained mice, when compared with PBS-injected mice. Reactivation of spleen and LN cells on day 7 of EAE from PBS-treated mice with MOG resulted in IL-17A, IFN-γ and GM-CSF production, in a dose dependent manner. However, MOG-specific production of IL-17A, IFN-γ and GM-CSF was significantly reduced in FHTE-trained mice (Fig 3.24). Furthermore, the frequency and absolute number of IL-17A-producing Vγ4 T cells in the spleen on day 12 was significantly reduced in FHTE-trained mice, when compared with PBS-treated mice (Fig 3.25). These results indicate that training of mice with FHTE polarized M2 macrophages, resulting in suppressed induction and expansion of autoantigen-specific pathogenic T cells, even during the effector phase of disease.

3.2.8 *Reduced T cells infiltration into the CNS of FHTE-trained mice following induction of EAE*

Infiltration of encephalitogenic T cells into the CNS leads to demyelination and ascending paralysis of mice [209]. Assessment of infiltration and cytokine production by CD4 and γδ T cells into the brain and spinal cord of PBS or FHTE-trained mice on day 12 of EAE revealed that training of mice with FHTE suppress T cell migration into the CNS.

Compared with PBS-treated mice, FHTE-trained mice had significantly reduced numbers of infiltrating CD4 T cells in the brain (Fig 3.26 B) and spinal cord (Fig 3.26 C) 12 days after the induction of EAE. FHTE-trained mice also had a reduction in the number of IL-17A as well as IFN-γ-secreting CD4 T cells in the brain and spinal cord on day 12 (Fig 3.26B,C). Furthermore, frequency of double positive IL-17A and IFN-γ-producing CD4 T cells was also significantly reduced in FHTE-trained mice, when compared with PBS mice (Fig 3.26A).

Previous work in our lab has shown that Vγ4 cells are the pathogenic subset of γδ T cells that infiltrate the CNS during EAE (Mc. Ginley et al., Unpublished). FHTE-trained mice had a significant reduction in the frequency and absolute number of IL-17-producing Vγ4 T cells in the brain (Fig 3.27B) and spinal cord (Fig 3.27C) on day 12 of EAE, when compared with PBS-injected mice. This is consistent with data shown above demonstrating reduced Vγ4 responses in the periphery, before the induction and during the effector phase of EAE.

In addition, FHTE-trained mice had a significant reduction in the frequency and number of $Ki67^+$ proliferating CD4 in the brain (Fig 3.28B) and spinal cord (Fig 3.28C). Furthermore, FHTE-trained mice had significantly less proliferating Vγ4 T cells in the brain (Fig 3.29B) and spinal cord (Fig 3.29C) on day 12 of EAE, when compared with PBS-treated mice. These results suggest that although a small portion of pathogenic T cells in FHTE-trained mice traffic to the CNS, these cells have less proliferative capacity and may account for decreased effector responses.

3.2.9 *$F4/80^+CD11b^+$ macrophages from FHTE-trained mice delay the onset of EAE*

Initial studies have demonstrated that FHTE-trained macrophages suppress MOG-specific T cells responses *in vitro*. Furthermore, training of mice with FHTE attenuates EAE. To study the contribution of FHTE-trained macrophages in attenuation of EAE, purified macrophages from FHTE-trained mice were transferred to mice with EAE. Treatment of mice induced with EAE with macrophages from FHTE-trained mice significantly delayed the onset of disease (Fig 3.30A). Furthermore, transfer of macrophages significantly delayed the weight loss associated with EAE (Fig 3.30B). These results confirm that M2 macrophages from FHTE-trained mice have suppressive effect of MOG-specific T cell responses both *in vitro* and *in vivo*.

3.3 Discussion

Helminth parasites have evolved several mechanisms to successfully evade and suppress the immune system, and these are known to be associated with simultaneous subversion of immune responses that mediate allergy and autoimmune diseases [210]. The significant new findings of this study are that helminth products can protect against an experimental autoimmune disease mediated by pathogenic Th1 and Th17 cells through training of the innate immune system.

DCs act as professional APCs for naïve T cells and play a vital role in the initiation and control of T cell responses [30]. As a result, they are a major target for immune modulating and suppressive effects of parasite products. IL-1RA is a naturally occurring antagonist of IL-1 that inhibits IL-1 signalling by competitively binding to IL-1 receptor and demonstrating no agonist activity. Although IL-1RA has been shown to be protective in models of inflammatory and autoimmune diseases [211, 212], very little research has focused on the induction of IL-1RA during helminth infection. One paper reported that human keratinocytes produce IL-1RA in response to *S. mansoni* infection [213]. However, this study is the first to demonstrate that helminth products can induce IL-1RA production by innate immune cells. Another striking finding of this study was the observation that FHTE suppressed LPS-induced IL-1β and IL-23 production by BMDCs. Although it has been demonstrated that tegumental antigen *F. hepatica* can suppress LPS-induced TNF production [191], this study is the first to observe suppression of both IL-1β and IL-23. Induction of IL-1RA and simultaneous suppression of IL-1β and IL-23 production by FHTE likely reflects a successful immune subversion strategy used to prevent parasite expulsion. Furthermore, IL-1β and IL-23 play an important role in the activation of γδ T cells and the expansion of Th17 cells and our lab has previously demonstrated that IL-1R1$^{-/-}$ mice are resistant to EAE [95]. Therefore, inhibition of TLR-induced IL-1β and IL-23 secretion by FHTE may in part account for suppression of autoreactive Th17 responses observed during helminth infection.

IL-10 plays a major role in the regulation of Th1 and Th2 responses during helminth infections and is vital in minimizing immunopathology. Furthermore, many studies have demonstrated that helminth infection can attenuate the

symptoms of autoimmunity through the induction of IL-10. A study by Matisz et al. demonstrated that suppression of colitis by adoptive transfer of helminth antigen-treated DCs is mediated by IL-10 [214]. The present study demonstrated that FHTE induced IL-10 production by BMDMs and enhanced LPS-induced IL-10 production by BMDMs and BMDCs. This data is consistent with studies showing that BMDMs produce IL-10 in response to FHES [215]. Although previous studies in human monocytes have demonstrated that IL-10 inhibits LPS-induced production of inflammatory cytokines by blocking gene transcription [216], the present study found that suppression of LPS-induced IL-1β and IL-23 expression was independent of IL-10. It is therefore possible that active components of FHTE target signalling molecules in TLR pathways or directly inhibit signal transduction leading to suppression of IL-1β and IL-23, thus preventing the development of pathogenic Th17 cells.

Until recently, immunological memory was thought to be confined to the adaptive immune system, however, recent studies have shown that the innate immune system has a form of memory and can be trained [111, 112]. Immunological imprinting through tolerance or trained immunity influences the capacity of the immune system to respond to reinfection. The current study, using different TLR ligands, demonstrated that training of BMDMs with LPS induced long-term tolerant cells that produce less TNF in response to Pam3cys. In contrast, priming of BMDMs with β-glucan trained cells that had enhanced inflammatory status and higher levels of TNF production following restimulation with Pam3cys. These results are consistent with a study by Saeed et al. demonstrating that exposure to certain microbial stimuli increases long-term responsiveness of monocytes to secondary infection [111]. Furthermore, the results of the present study revealed that FHTE trains innate immune cells to be more anti-inflammatory. Training of BMDMs with FHTE increased secretion of IL-10 while inhibiting TNF production following restimulation with LPS. This is the first example of anti-inflammatory innate immune training where restimulation leads to enhanced anti-inflammatory status, as well as decreased ability to produce proinflammatory cytokines. The observation that FHTE can non-specifically train innate immune cells to be more anti-inflammatory may in part explain the non-specific immunomodulatory effect of helminth parasites on host

immune responses and consequently, the lower incidence of autoimmune diseases in areas with high helminth burden.

A recent study by Schrum et al. demonstrated that PBMCs can be proinflammatory trained in response to the parasite *P. falciparum* [200]. However, the present study is the first demonstration of anti-inflammatory training of cells in response to helminths or helminth-derived products. Training of mice with FHTE 21 and 7 days before sacrifice led to the accumulation of M2 macrophages in the peritoneal cavity. This is consistent with a study by Adams et al. demonstrating that infection with *F. hepatica* lead to the accumulation of macrophages in the peritoneum, which exhibit an M2-like phenotype [215]. Alternatively activated macrophages in the peritoneal are known to be derived either from proliferation of tissue resident macrophages as well as recruited blood-derived SPM [204]. The present study demonstrated that training of mice with FHTE polarised M2 responses accompanied with enhanced expression of CD206 and PD-L2 on SPM. This is consistent with previous data demonstrating that monocyte-derived alternatively activated macrophages express CD206 and PD-L2 [203]. Upregulation of PD-L2 on the surface of SPM may be an important mechanism of immune evasion used by helminths, as PD-L2 can suppress T cell activation via ligation of PD-1 on T cell receptors, thus damping the host immune response. Moreover, the mannose receptor, CD206 is expressed at high levels during the resolution of inflammation to ensure inflammatory agents are removed from the circulation and therefore protect against inappropriate T cells activation in the periphery.

Classical activation of macrophages occurs primarily during bacterial or viral infection, whereas alternatively activated macrophages are induced in response to helminth infection [205] . However, it has not been clear that distinct M1 and M2-type macrophages exist *in vivo*, where a spectrum of macrophage phenotypes exists. A recent study identified novel M1 and M2-specific genes using gene expression profiling [22]. The present study demonstrated that macrophages from FHTE-trained mice expressed high levels of M2-associated genes *arg1* (encoding arginase 1) and *retlna* (encoding relmα) in both LPM and SPM. Relmα has been shown to suppress inflammation and helminth-induced

Th2-type immunity. The induction of arginase expression likely reflects wound repair mechanisms during prolonged helminth infection [217]. Furthermore, arginase produced by alternatively activated macrophages depletes L-arginine from the tissue microenvironment, which inhibits T-cell function. In addition, FHTE-trained mice not only had enhanced expression of M2-associated genes, but also increased expression of M2-specific genes, *mrc1* (encoding MRC1/CD206) and *chi3l3* (encoding YM1) in SPM and LPM. This is consistent with a report demonstrating enhanced expression of *chi3l3* in response to *S. mansoni* infection, which limited parasite survival [218]. The findings reveal that training of mice with FHTE polarise an M2 macrophage phenotype in both tissue resident macrophages as well as infiltrating blood-derived monocytes.

M1 macrophages have been identified as key regulators in demyelinating diseases of the CNS and are thought to contribute to axonal loss in MS and EAE [219]. Furthermore, infiltrating blood-derived M2 macrophages have been shown to play a vital anti-inflammatory role in recovery from spinal cord injury [220]. This study demonstrated that polarised M2 macrophages from FHTE-trained mice have the ability to suppress MOG-specific T cell responses. Furthermore, suppression of IL-17A and IFN-γ production by CNS infiltrating T cells was associated with attenuation of clinical signs of EAE and reduced weight loss. This is consistent with a recent study demonstrating that long term infection with the helminth parasite *T. crassiceps* induces monocyte-derived alternatively activated macrophages that attenuate EAE [206]. However, this study is the first to demonstrate anti-inflammatory training of macrophages in response to helminth-derived products, which have the capacity to suppress MOG-specific T cells responses when transferred to mice during the effector phase of EAE. Therefore, the polarisation of blood-derived M2 macrophages in FHTE-trained mice may account for the protective mechanism of helminth infection, which prevents the development of autoimmune diseases in the developing world. Furthermore, polarisation of M1 macrophages through the induction of innate immune training may be exploited in the context of vaccine adjuvant development or cancer immunotherapies.

In addition to inducing alternatively activated macrophages *in vivo*, this study demonstrated that innate immune training of mice with FHTE can modulate a disease mediated by adaptive immune responses. The pathology of EAE is mediated by activation of Th1 and Th17 cells in the periphery induced by immunisation with MOG antigen, followed by infiltration into the CNS leading to disease pathology [33]. This study demonstrated that training of mice with FHTE delayed the onset and decreased disease severity in the EAE model. This was associated with decreased activation and expansion of T cells in the periphery. Furthermore, MOG-specific IL-17A, IFN-γ and GM-CSF production by CD4 T cells was impaired in FHTE-trained mice. This conclusion was consistent with the observation that FHTE-trained mice had significantly less infiltration of cytokine secreting CD4 and Vγ4 T cells into the CNS during the effector phase of disease.

Several studies in mouse models of autoimmunity have shown that infection with helminth parasites can attenuate autoimmune disease [159, 176, 197]. However, since helminth infection is a very complex process associated with significant mortality obscuring accompanying immune responses [189], these findings provide a cleaner model in which to study parasite related immunosuppression. Collectively, the current study has established a novel finding that the innate immune system can be anti-inflammatory trained with the ability to suppress pathogenic T cell responses. Furthermore, parasitic products may attenuate T cell-mediated autoimmune disease indirectly by modulating innate immune responses. The findings of this study not only shed new light on the hygiene hypothesis but also improve the overall view of trained immunity as being a fundamental part of mammalian immune responses. Furthermore, these results provide alternative avenues in the field and suggest considerable potential for aiding the design of novel therapeutic approaches for autoimmune diseases.

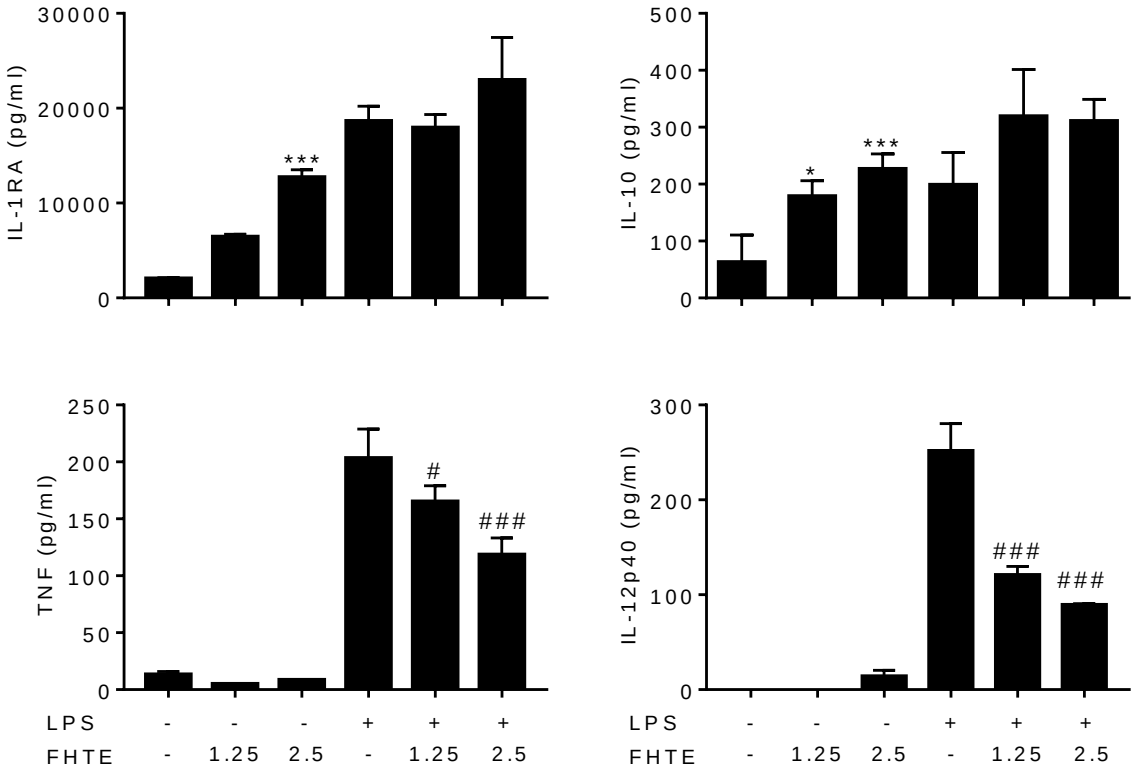

Figure 3.1 FHTE induced IL-1RA and IL-10 but suppressed LPS-induced TNF and IL-12p40 production by PEC cells. PEC from naïve C57BL/6 mice were stimulated with FHTE (1.25 or 2.5% v/v), LPS (100 ng/ml), or a combination of both. Supernatants were collected after 24 hr and concentration of IL-1RA, IL-10, TNF and IL-12p40 determined by ELISA. Results are mean ±SD for triplicate culture and are combined from two separate experiments. *p<0.05, ***p<0.001 vs medium control or #p<0.05, ###p<0.001 vs LPS by two-way ANOVA with Tukey post-test.

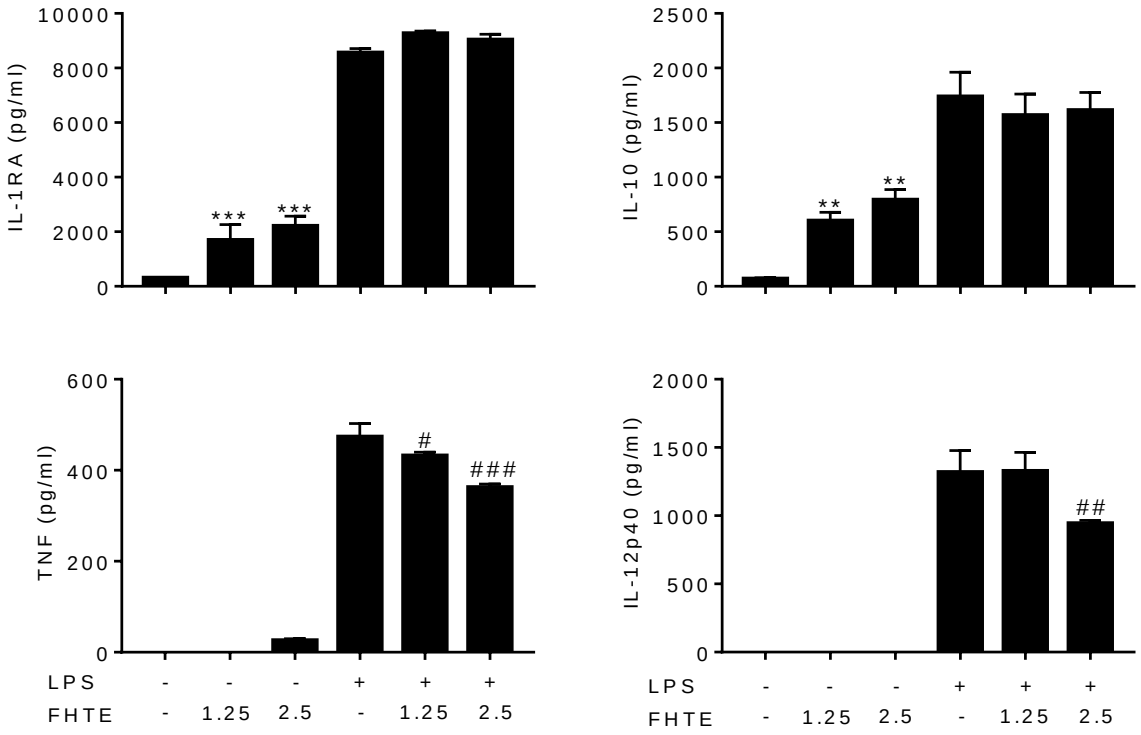

Figure 3.2 FHTE induced IL-1RA and IL-10 but suppressed LPS-induced TNF and IL-12p40 production by BMDMs. BMDMs from naïve C57BL/6 mice were stimulated with FHTE (1.25 or 2.5% v/v), LPS (100 ng/ml), or a combination of both. Supernatants were collected after 24 hr and concentration of IL-1RA, IL-10, TNF and IL-12p40 determined by ELISA. Results are mean ±SD for triplicate culture and are combined from two separate experiments. **p<0.01, ***p<0.001 vs medium control or #p<0.05, ##p<0.01, ###p<0.001 vs LPS by two-way ANOVA with Tukey post-test.

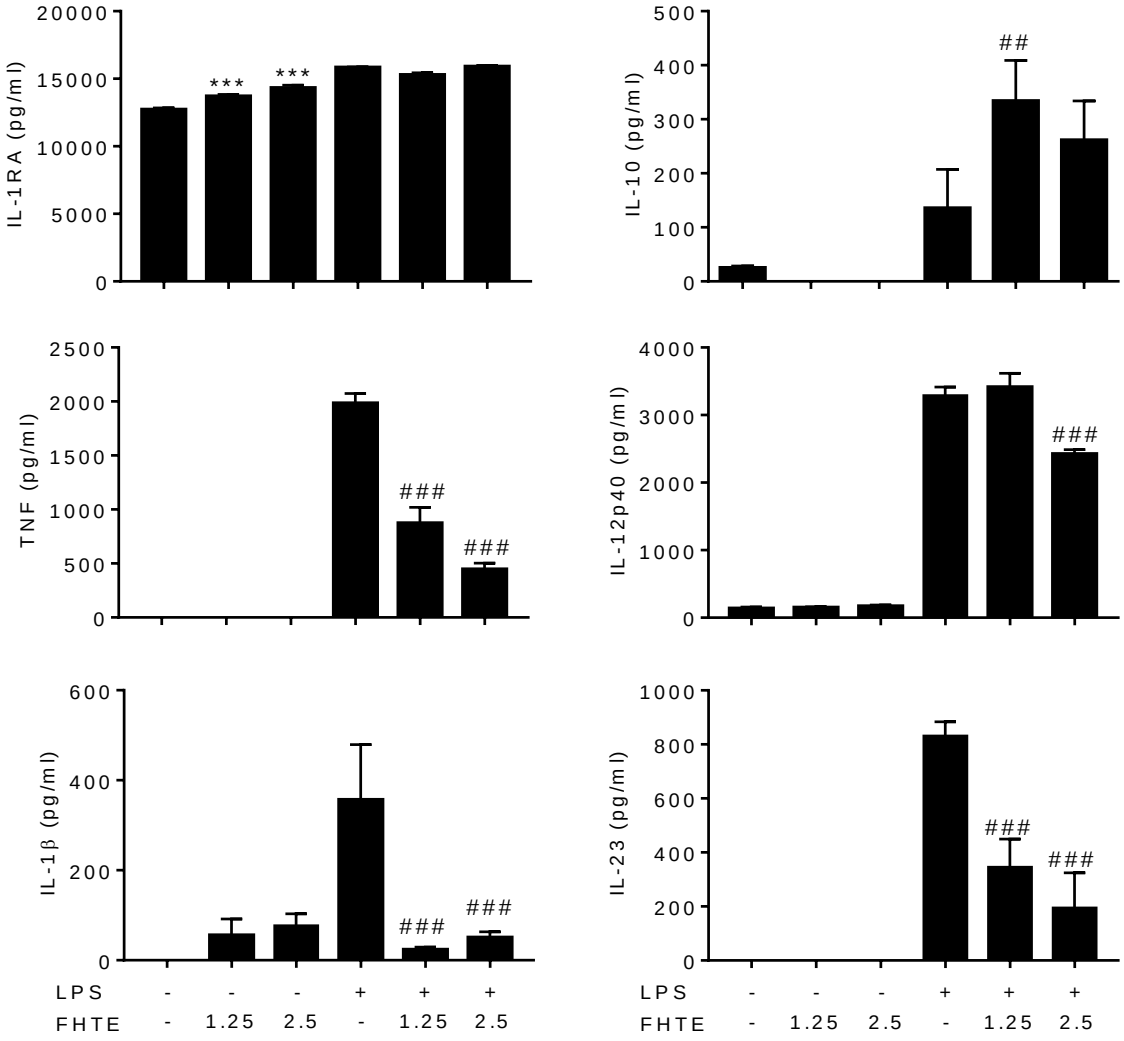

Figure 3.3 FHTE enhanced LPS-induced IL-10 production but suppressed LPS-induced TNF, IL-12p40, IL-1β and IL-23 production by BMDCs. BMDCs from naïve C57BL/6 mice were stimulated with FHTE (1.25 or 2.5% v/v), LPS (100 ng/ml), or a combination of both. Supernatants were collected after 24 hr and concentration of IL-1RA, IL-10, TNF, IL-12p40, IL-1β and IL-23 determined by ELISA. Results are mean ±SD for triplicate culture and are combined from two separate experiments. ***p<0.001 vs medium control or ##p<0.01, ###p<0.001 vs LPS by two-way ANOVA with Tukey post-test.

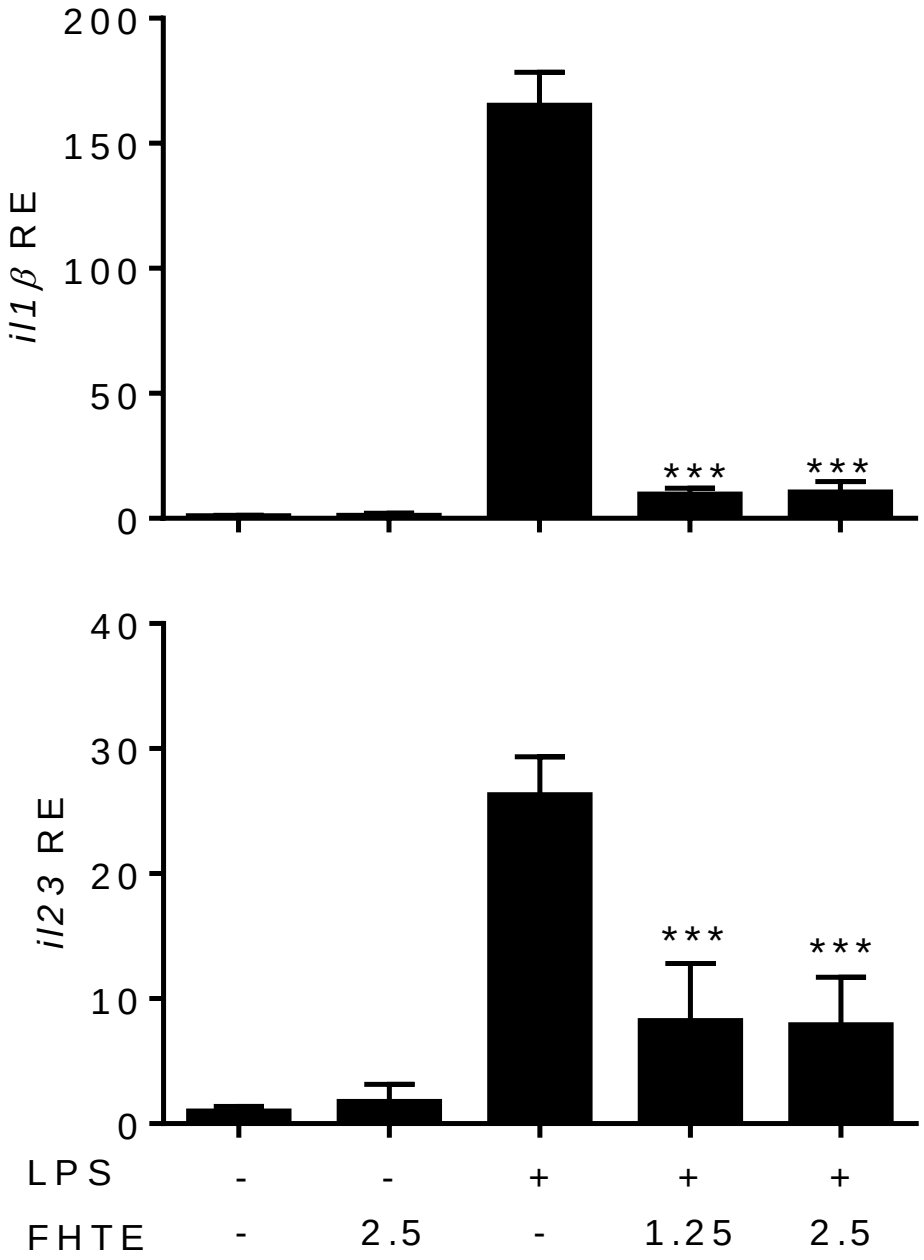

Figure 3.4 FHTE suppressed LPS-induced *il1β* and *il23* mRNA expression in BMDCs. BMDCs from naïve C57BL/6 mice were stimulated with FHTE (1.25 or 2.5% v/v), LPS (100 ng/ml), or a combination of both. Cells were lysed after 24 hr, total RNA was isolated and expression of *il1β* and *il23* was evaluated by qRT-PCR relative to medium cultured cells following normalisation by the endogenous control 18s rRNA. Results are mean ±SD for triplicate culture and are representative of two independent experiments. ***p<0.001 vs LPS by two-way ANOVA with Tukey post-test.

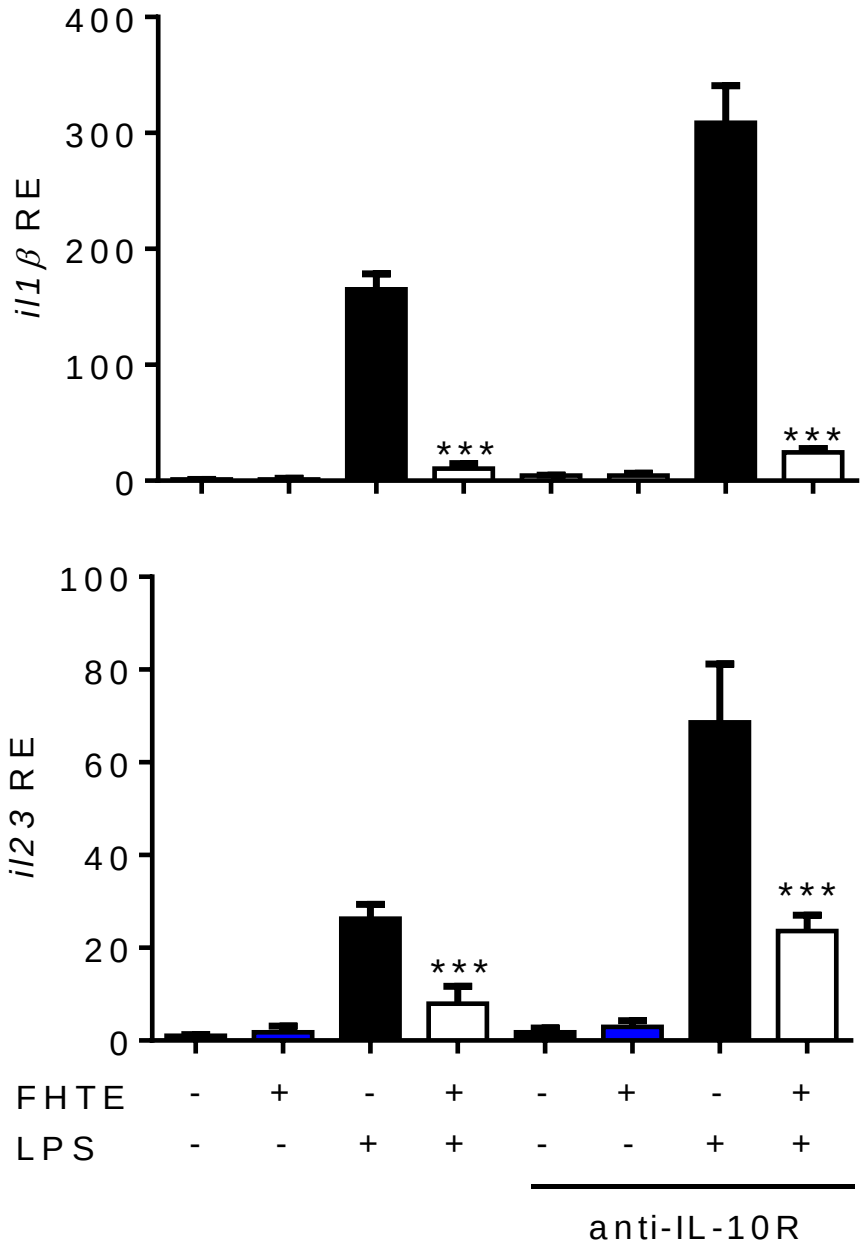

Figure 3.5 Suppression of LPS-induced *il1β* and *il23* mRNA expression by FHTE is independent of IL-10. BMDCs from naïve C57BL/6 mice were stimulated with LPS (100 ng/ml), FHTE (2.5% v/v) or a combination of both in the presence or absence of the anti-IL-10R antibody (10 µg/ml). After 24 hr, cells were lysed, total RNA was isolated and expression of *il1β* and *il23* was evaluated by qRT-PCR relative to medium cultured cells following normalisation by the endogenous control 18s rRNA. Results are mean ±SD for triplicate culture and are representative of two independent experiments. ***p<0.001 vs LPS by three-way ANOVA with Tukey post-test.

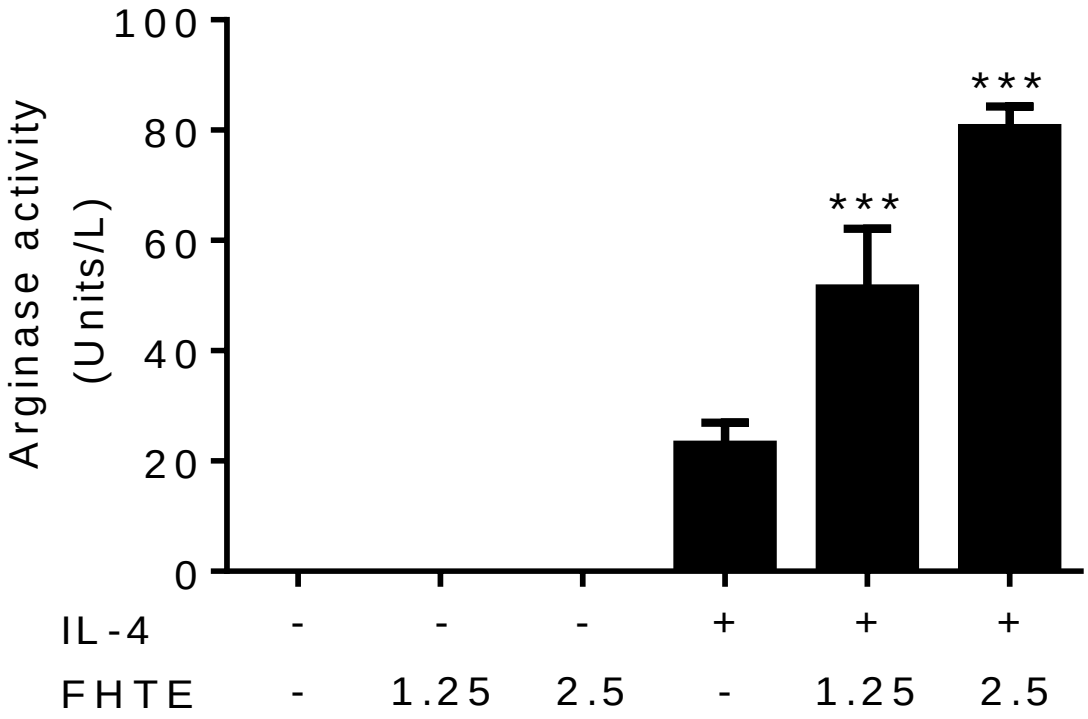

Figure 3.6 FHTE enhanced arginase activity in IL-4-activated BMDMs. BMDMs from naïve C57BL/6 mice were cultured with 1.25 or 2.5% v/v FHTE in the presence or absence of IL-4 (10 ng/ml). After 24 hr, lysates were tested for arginase activity. Results are mean ±SD for triplicate culture and are representative of three independent experiments. ***p<0.001 vs IL-4 by two-way ANOVA with Tukey post-test.

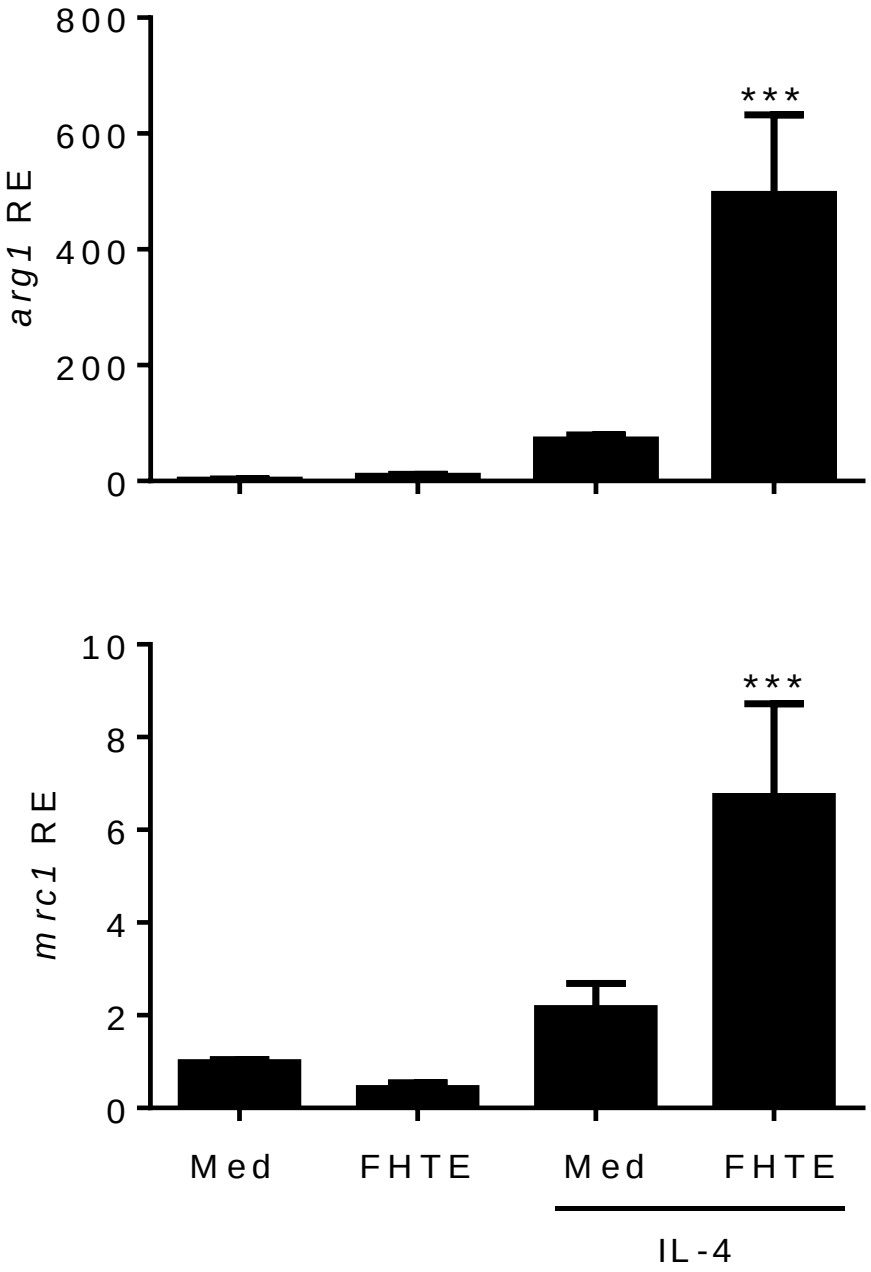

Figure 3.7 FHTE enhanced expression of regulatory macrophages markers in IL-4-activated BMDMs. BMDMs from naïve C57BL/6 mice were cultured with 2.5% v/v FHTE, IL-4 (10 ng/ml) or a combination of both. After 24 hr, total RNA was isolated from cells and the expression of *arg1* and *mrc1* was analysed by qRT-PCR relative to medium cultured cells following normalisation by the endogenous control 18s rRNA. Results are mean ±SD for triplicate culture and are representative of two independent experiments. ***p<0.001 vs IL-4 by two-way ANOVA with Tukey post-test.

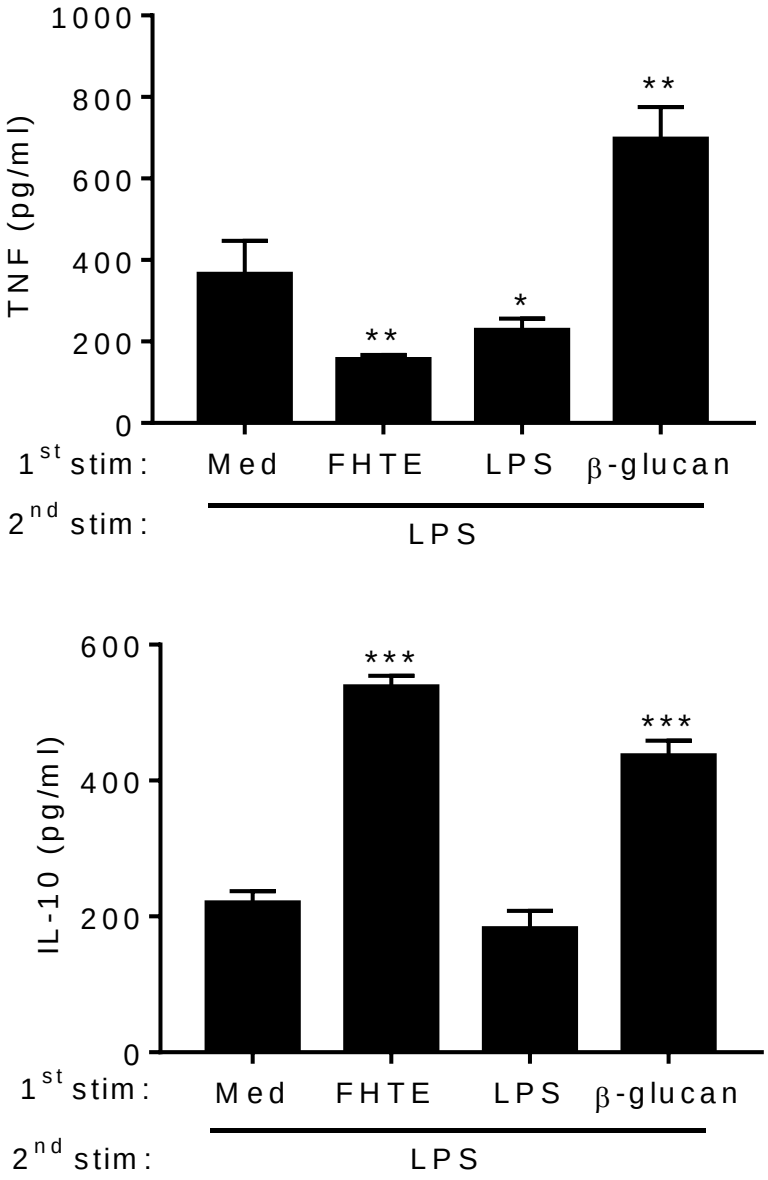

Figure 3.8 Enhancement of IL-10 and suppression of TNF production in FHTE-trained PEC. PEC from naïve C57BL/6 mice were incubated with 2.5% v/v FHTE, β-glucan (5 µg/ml) or LPS (100 ng/ml). Cells were washed after 24 hr and left to rest. After three days, cells were restimulated with LPS (100 ng/ml). Supernatants were collected 24 hr after restimulation and the concentration of TNF and IL-10 determined by ELISA. Results are mean ±SD for triplicate culture and are representative of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs control by one-way ANOVA with Dunnett post-test.

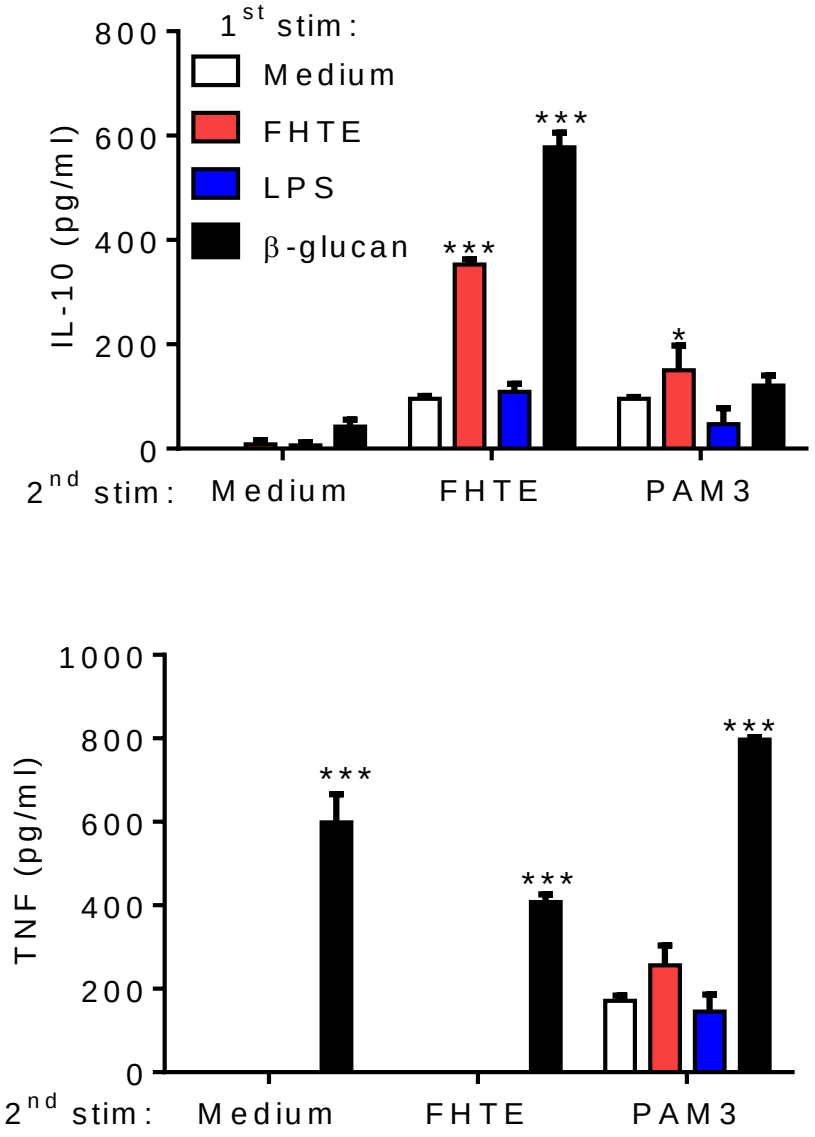

Figure 3.9 FHTE trains PEC to be more anti-inflammatory. PEC from naïve C57BL/6 mice were incubated with 2.5% v/v FHTE, LPS (100 ng/ml) or β-glucan (5 µg/ml). Cells were washed after 24 hr and left to rest. After three days, cells were restimulated with 2.5% v/v FHTE or Pam3cys (10 µg/ml). Supernatants were collected 24 hr after restimulation and the concentration of IL-10 and TNF determined by ELISA. Results are mean ±SD and are representative of three independent experiments. *p<0.05, ***p<0.001 vs medium controls by two-way ANOVA and Tukey post-test.

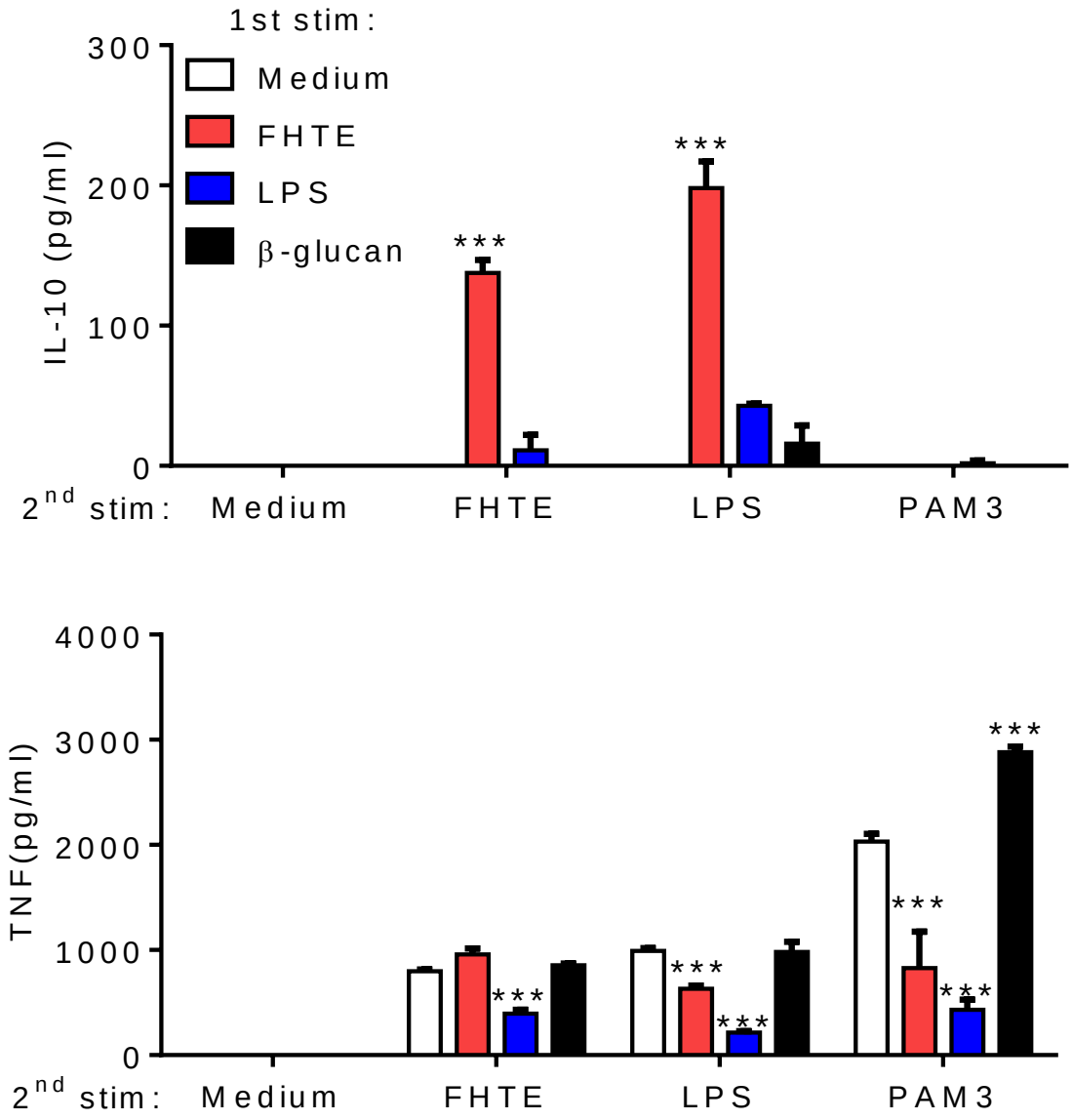

Figure 3.10 FHTE trains BMDMs to be more anti-inflammatory. BMDMs from naïve C57BL/6 mice were incubated with 2.5% v/v FHTE, LPS (100 ng/ml) or β-glucan (5 µg/ml). Cells were washed after 24 hr and left to rest. After three days, cells were restimulated with either 2.5% v/v FHTE, LPS (100 ng/ml) or Pam3cys (10 µg/ml). Supernatants were collected 24 hr after restimulation and the concentration of IL-10 and TNF determined by ELISA. Results are mean ±SD and are representative of three independent experiments. ***p<0.001 vs medium controls by two-way ANOVA and Tukey post-test.

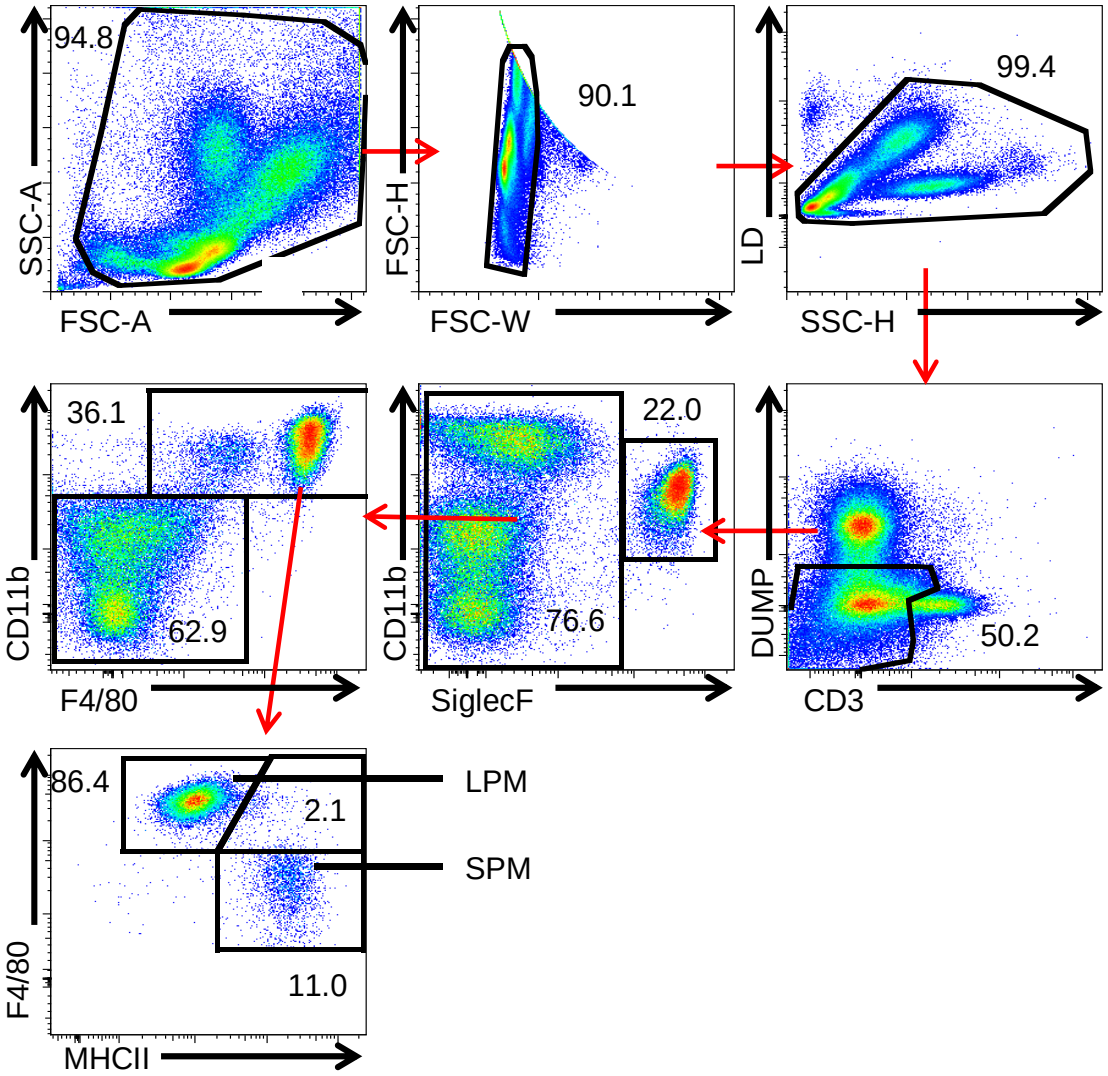

Figure 3.11 Gating strategy for analysing innate cells in the peritoneal cavity. The peritoneal cavity of C57BL/6 mice was lavaged to obtain PEC. Cells were surfaced stained with antibodies specific for NK1.1, CD40, CD3, CD11b, Siglec F, F4/80, MHC II. LPMs were identified as $CD11b^{+}F4/80^{high}$ $MHCII^{low}$ and SPMs identified as $CD11b^{+}F4/80^{low}MHCII^{high}$.

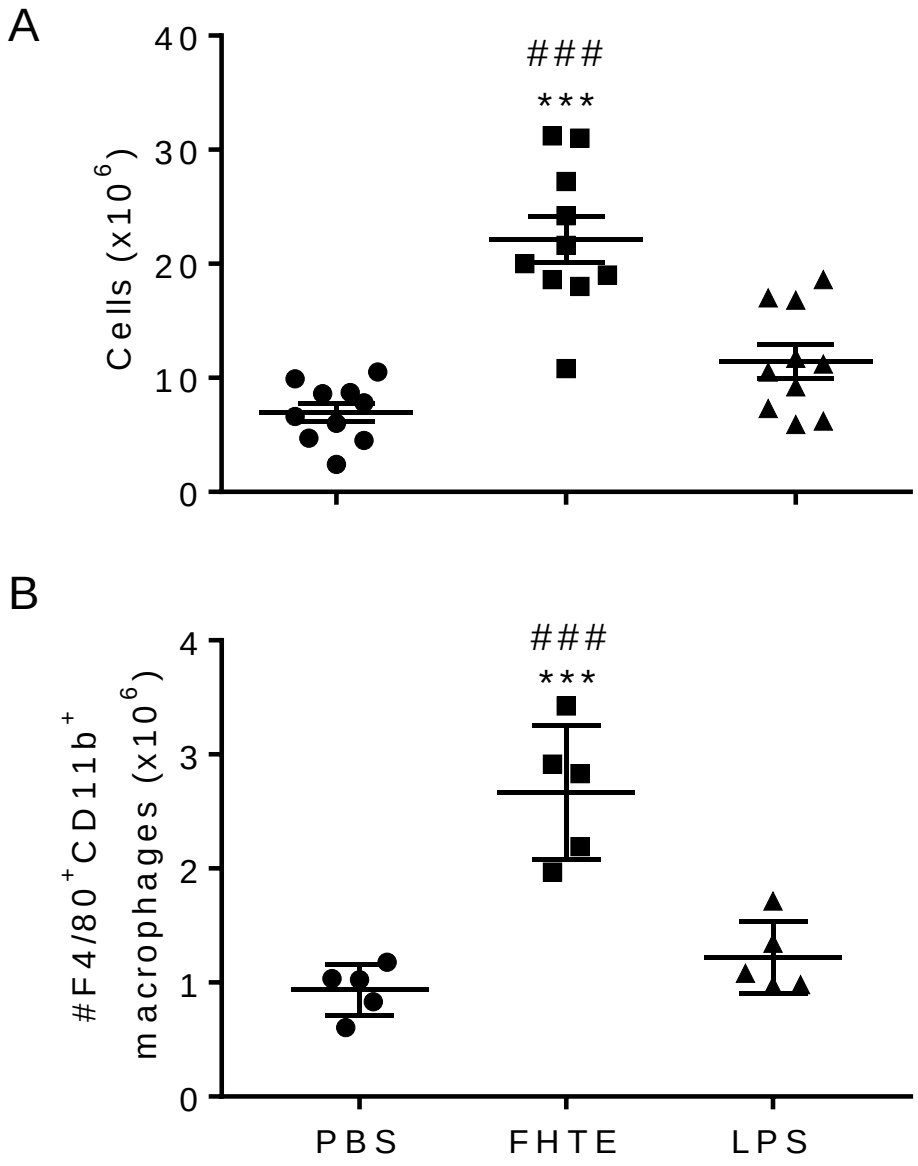

Figure 3.12 Training of mice with FHTE leads to the accumulation of macrophages in the peritoneal cavity. C57BL/6 mice were injected i.p with PBS, FHTE (2.5% v/v in 200 µl PBS) or LPS (10 µg) at day 0 and day 14. On day 21, PEC were isolated by peritoneal lavage, stained for F4/80 and CD11b and analysed by flow cytometry. Results shown are total cells (n=10) (A) and $F4/80^{+}CD11b^{+}$ cells (n=5) (B). Data are mean ± SEM and each symbol represents an individual mouse. ***p<0.001 vs PBS or ###p<0.001 vs LPS by one-way ANOVA with Dunnett post-test.

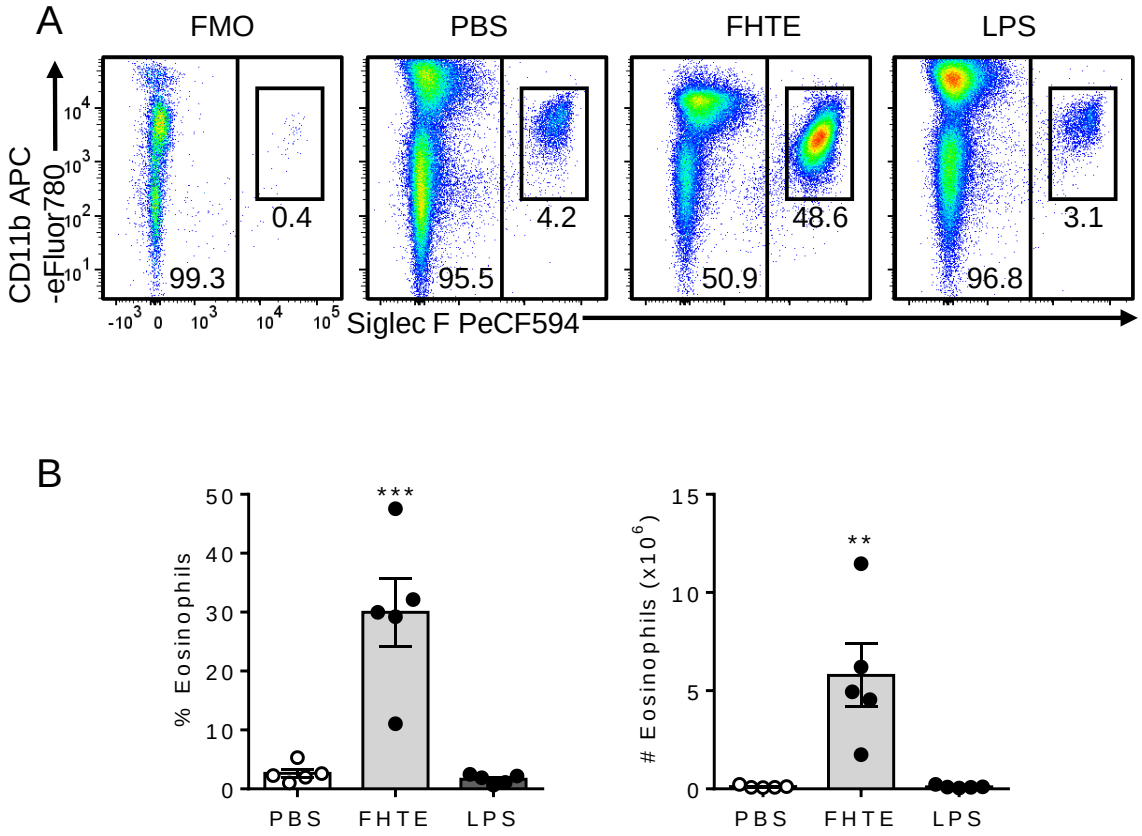

Figure 3.13 FHTE-trained mice have increased numbers of eosinophils in the peritoneal cavity. C57BL/6 mice were trained as described in figure 3.12. On day 21, PEC were isolated by peritoneal lavage and stained for CD11b and Siglec F. Results are representative flow cytometry plots for each treatment group (A) and percentage and absolute numbers of eosinophils (B). Data are mean ± SEM (n=5) and each symbol represents an individual mouse. **p<0.01, ***p<0.001 vs PBS by one-way ANOVA with Dunnett post-test.

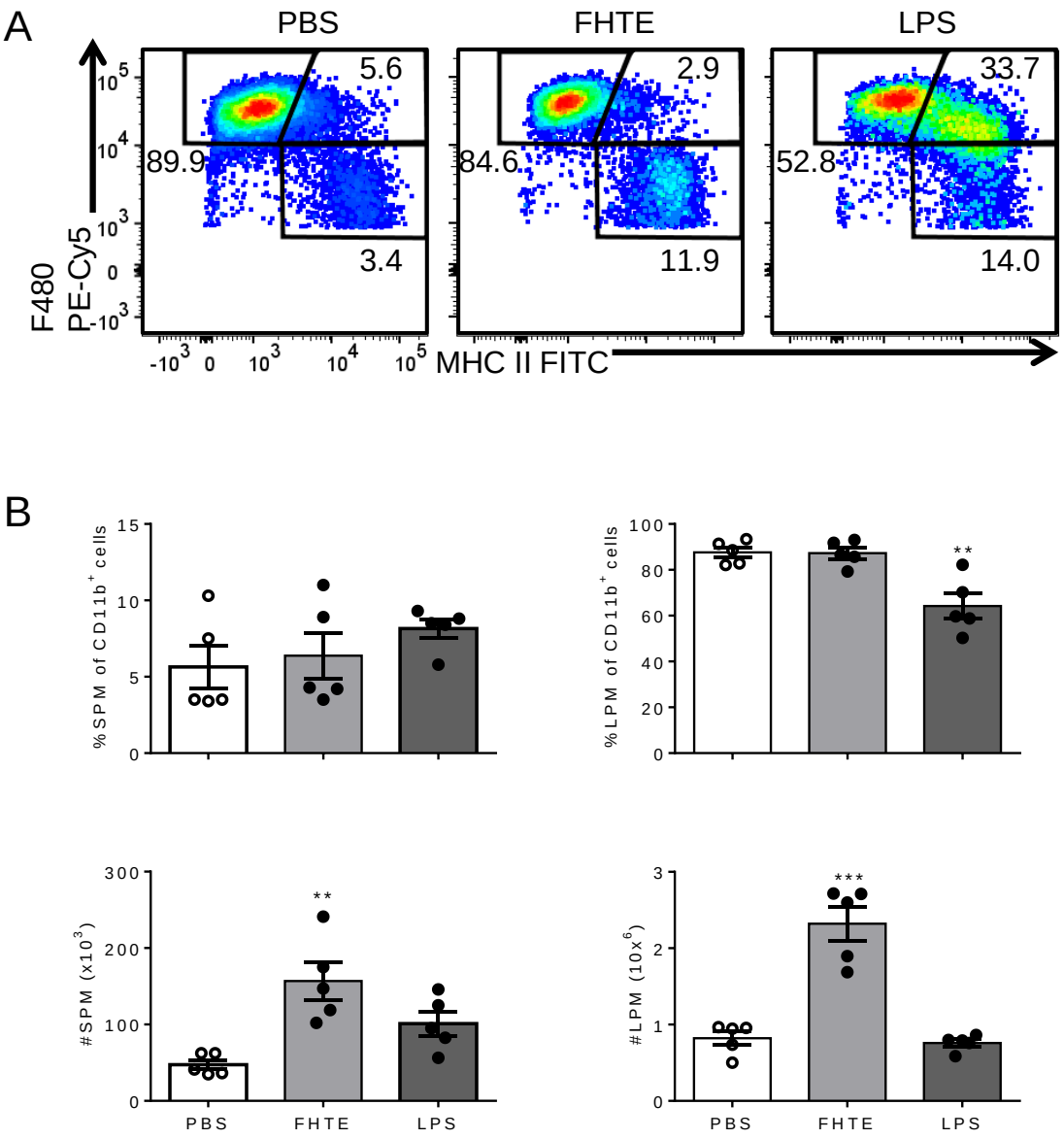

Figure 3.14 FHTE-trained mice have increased numbers of SPM and LPM in the peritoneal cavity. C57BL/6 mice were trained as described in figure 3.12. On day 21, PEC were isolated by peritoneal lavage and stained for CD11b, F4/80, and MHC II. Results are representative flow cytometry plots for LPM and SPM (gated on CD11b$^+$cells) (A) and percentage and absolute number of SPM and LPM (B). Data are mean ± SEM (n=5) and each symbol represents an individual mouse. **p<0.01, ***p<0.001 vs PBS by one-way ANOVA with Dunnett post-test.

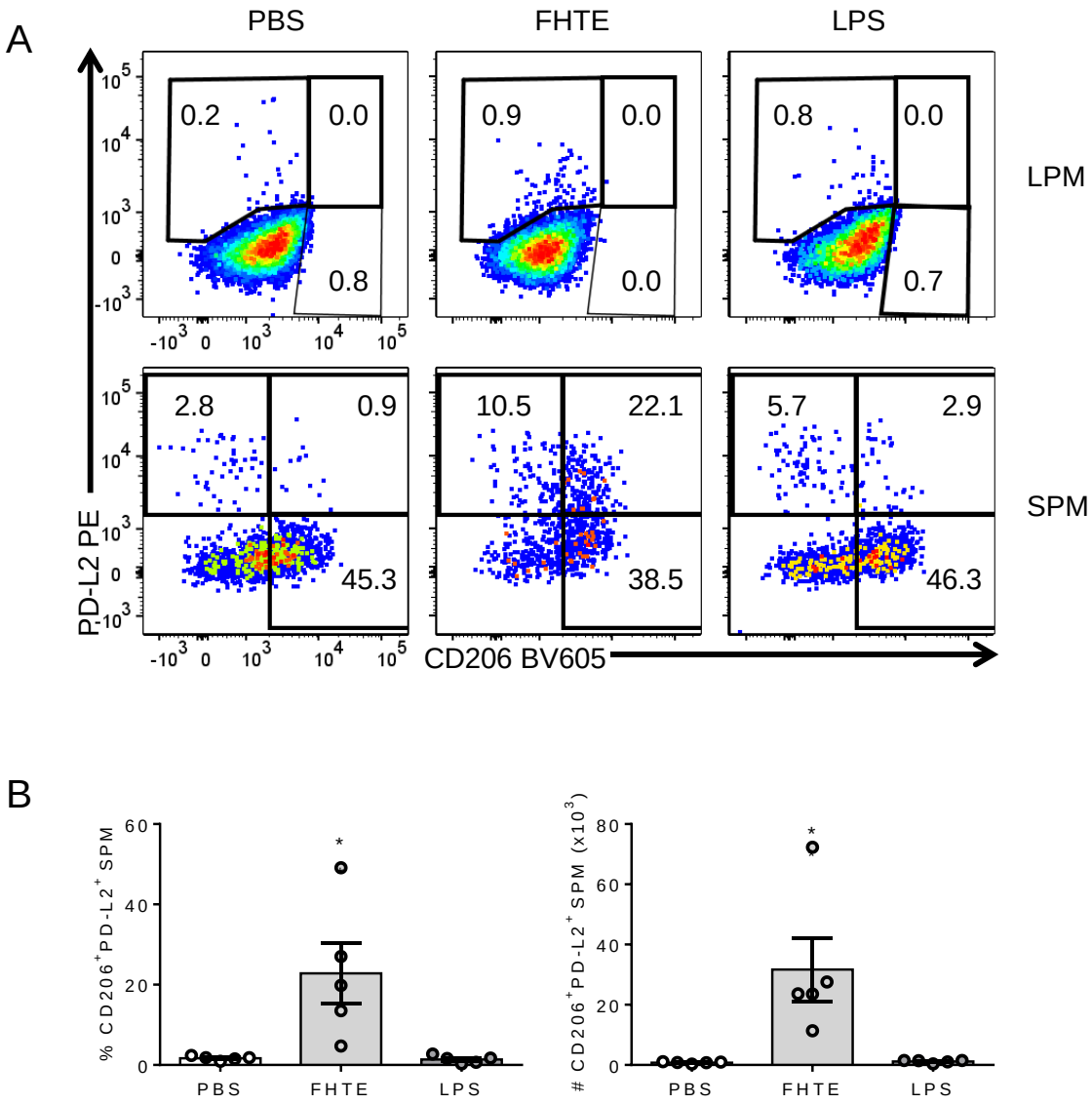

Figure 3.15 Mice trained with FHTE have increased frequency and number of CD206⁺PD-L2⁺ expressing SPM. C57BL/6 mice were trained as described in figure 3.12. On day 21, PEC were isolated by peritoneal lavage and stained for CD11b, F4/80, MHC II, CD206 and PD-L2. Results are representative flow cytometry plots for CD206⁺ PD-L2⁺ (gated on LPM or SPM) (A) and percentage and absolute numbers of CD206⁺ PD-L2⁺ SPM (B). Data are mean ± SEM (n=5) and each symbol represents an individual mouse. *p<0.05 vs PBS by one-way ANOVA with Dunnett post-test.

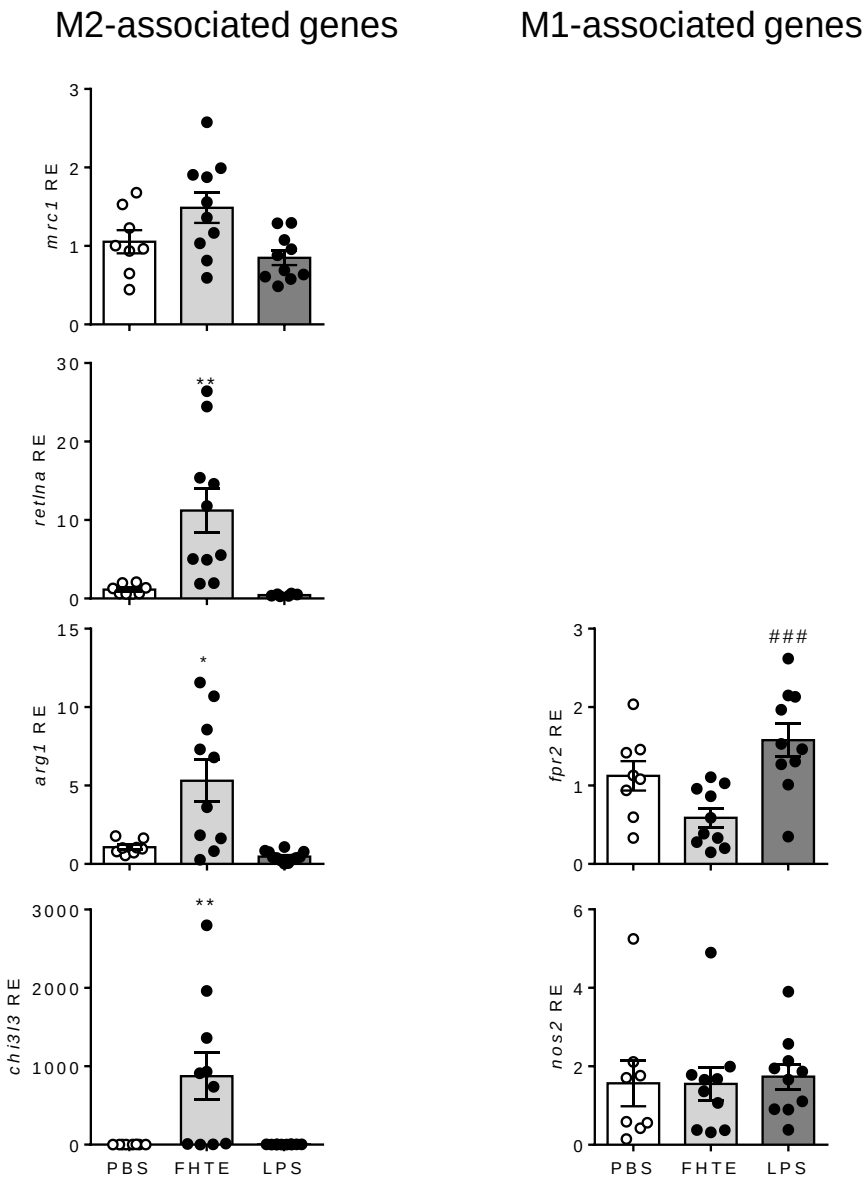

Figure 3.16 FHTE-trained mice have increased expression of M2-associated genes in LPM. C57BL/6 mice were trained as described in figure 3.12. On day 21, PEC were isolated by peritoneal lavage and stained for CD11b, F4/80 and MHC II. LPM were FACS sorted as $CD11b^{+}F4/80^{high}MHCII^{low}$. Total RNA was extracted and expression of *mrc1, retlna, arg1, chi3l3, fpr2 and nos2* was evaluated by qRT-PCR relative to PBS-injected mice following normalisation by the endogenous control 18s rRNA. Results are mean ±SEM (n=8-10 per group) and each symbol represents an individual mouse. Results are combined from two separate experiments. *p<0.05, **p<0.01 vs PBS, ###p<0.001 vs LPS by one-way ANOVA with Dunnett post-test.

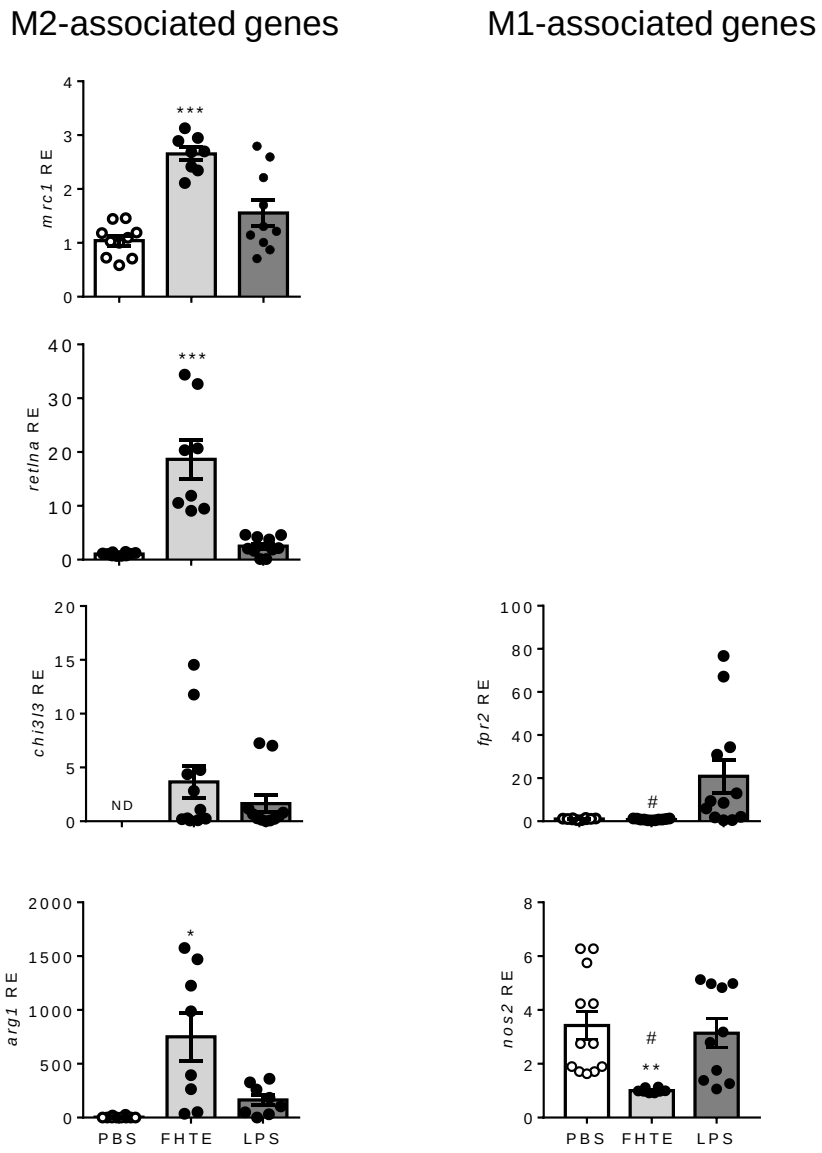

Figure 3.17 FHTE-trained mice have increased expression of M2-associated genes in SPM. C57BL/6 mice were trained as described in figure 3.12. On day 21, PEC were isolated by peritoneal lavage and stained for CD11b, F4/80 and MHC II. SPM were FACS sorted as $CD11b^{+}F4/80^{low}MHCII^{high}$. Total RNA was extracted and expression of *mrc1, retlna, chi3l3, arg1 and fpr2* was evaluated by qRT-PCR relative to PBS mice, whereas *nos2 was* evaluated relative to FHTE-trained mice following normalisation by the endogenous control 18s rRNA. Results are mean ±SEM (n=8-10 per group) and each symbol represents an individual mouse. Results are combined from two separate experiments. *p<0.05, **p<0.01 ***p<0.001 vs PBS or #p<0.05 vs LPS by one-way ANOVA with Dunnett post-test.

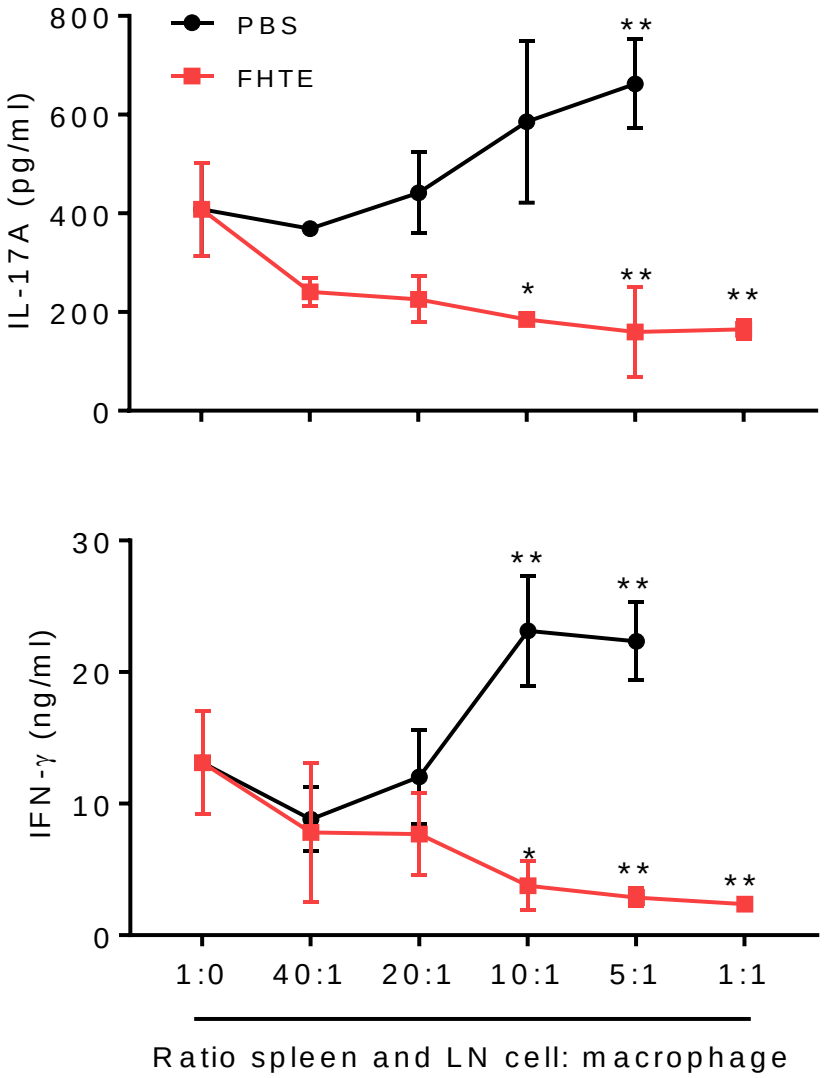

Figure 3.18 F4/80⁺CD11b⁺ macrophages from FHTE-trained mice suppressed MOG-specific T cell responses. C57BL/6 mice were trained as described in figure 3.12. On day 21, PEC were isolated by peritoneal lavage, stained and FACS sorted for CD11b⁺F4/80⁺ cells. Different ratios of F4/80⁺CD11b⁺ cells were co-cultured in the presence of MOG (100 µg/ml) with spleen and LN cells (70:30) from mice injected with MOG (100 µg) emulsified in CFA 10 days earlier. After 72 hr, supernatants were collected and the concentration of IL-17A and IFN-γ determined by ELISA. Results are mean ±SD and are representative of two independent experiments. *p<0.05, **p<0.01 vs control (ratio 1:0) by two-way ANOVA with Sidak post-test.

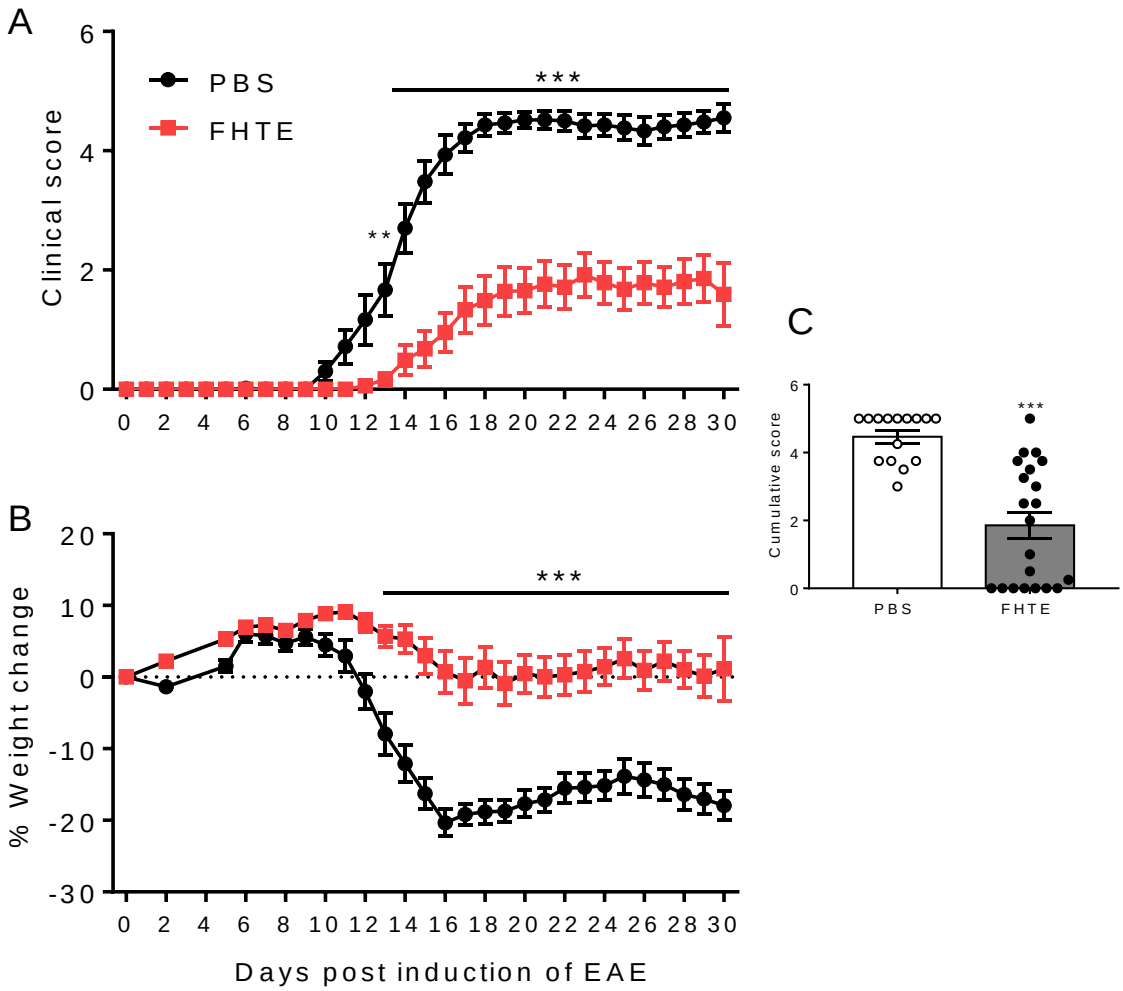

Figure 3.19 Training of mice with FHTE attenuates EAE. C57BL/6 mice were injected s.c with PBS or FHTE (2.5% v/v in 200 µl PBS) 21 days and 7 days before the induction of EAE. EAE was induced by s.c injection of MOG (100 µg) peptide emulsified in CFA, followed by i.p injection of PT (100 ng) on day 0 and day 2. Mice were assessed daily for clinical signs of the disease and weighed. Results are mean clinical score (A), percentage body weight change (B) and cumulative score on day 30 (C). Data are mean ± SEM (n=15 PBS, n=21 FHTE) and are combined from three different experiments. ***p<0.001 vs PBS by repeated measures ANOVA with Sidak post-test (clinical score and weight change) or unpaired t test (cumulative score).

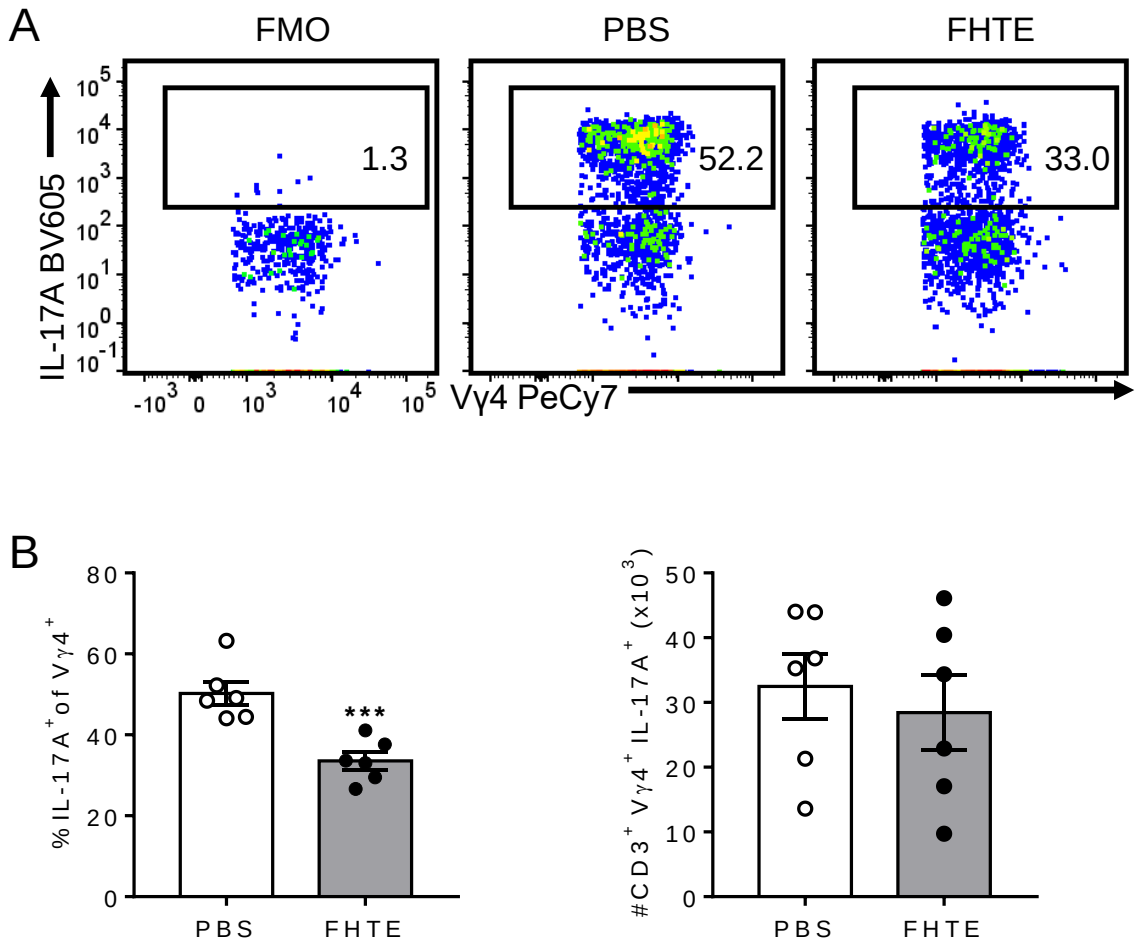

Figure 3.20 FHTE-trained mice have suppressed IL-17A production by Vγ4 T cells in the LNs before the induction of EAE. C57BL/6 mice were trained as described in figure 3.19. On day 21, mice were sacrificed and cells were isolated from the inguinal, brachial and axillary LNs. Cells were stained for surface CD3, Vγ4 and intracellular IL-17A and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+Vγ4^+$ cells) (A) and frequency and absolute number of IL-17A-producing $Vγ4^+$ T cells in the LNs (B). Data are mean ± SEM (n=6) and each symbol represents an individual mouse. ***p<0.001 vs PBS by unpaired t test.

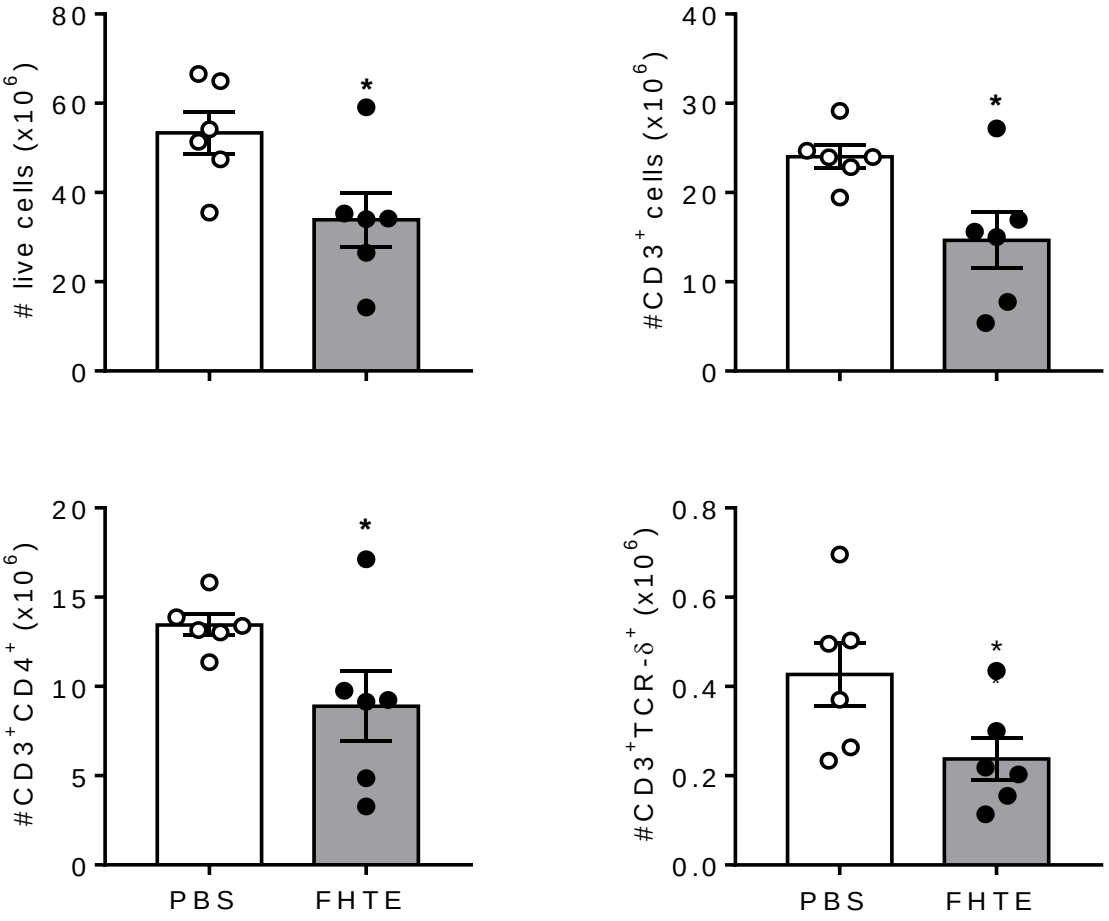

Figure 3.21 Absolute numbers of CD3, CD4 and γδ T cells are reduced in the LNs of FHTE-trained mice 7 days after the induction of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 3.19. On day 7 after induction, mice were sacrificed and cells were isolated from the inguinal, brachial and axillary LNs. Cells were stained for surface CD3, CD4 and TCR-δ and analysed by flow cytometry. Results are absolute number of total live cells, CD3, CD4 and γδ T cells in the LNs. Data are mean ± SEM (n=6) and each symbol represents an individual mouse. *p<0.05 vs PBS by unpaired t test.

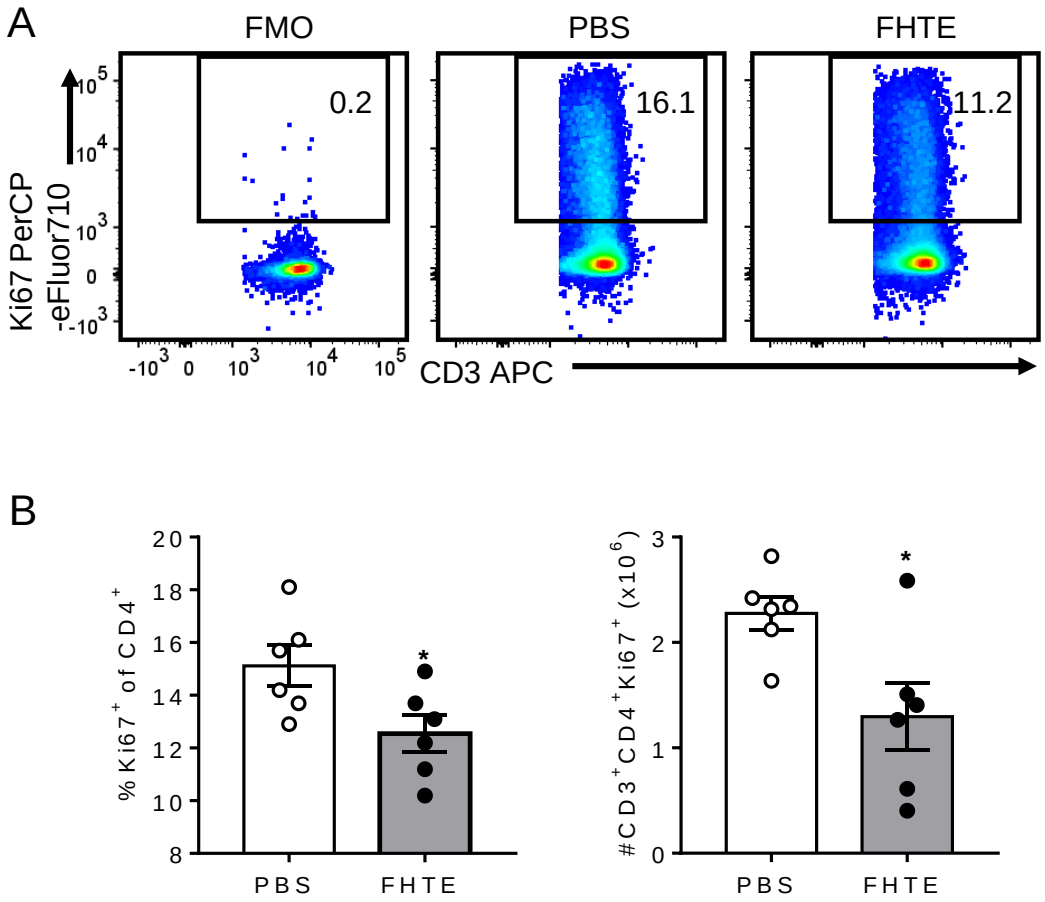

Figure 3.22 Training of mice with FHTE suppressed proliferation of CD4 T cells on day 7 after the induction of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 3.19. On day 7, mice were sacrificed, and cells were isolated from the inguinal, brachial and axillary LNs. Cells were stained for surface CD3 and CD4 and intranuclear Ki67 and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+CD4^+$ T cells) (A) and frequency and absolute number of $Ki67^+$ proliferating $CD4^+$ T cells in the LNs (B). Data are mean ± SEM (n=6) and each symbol represents an individual mouse. *p<0.05 vs PBS by unpaired t test.

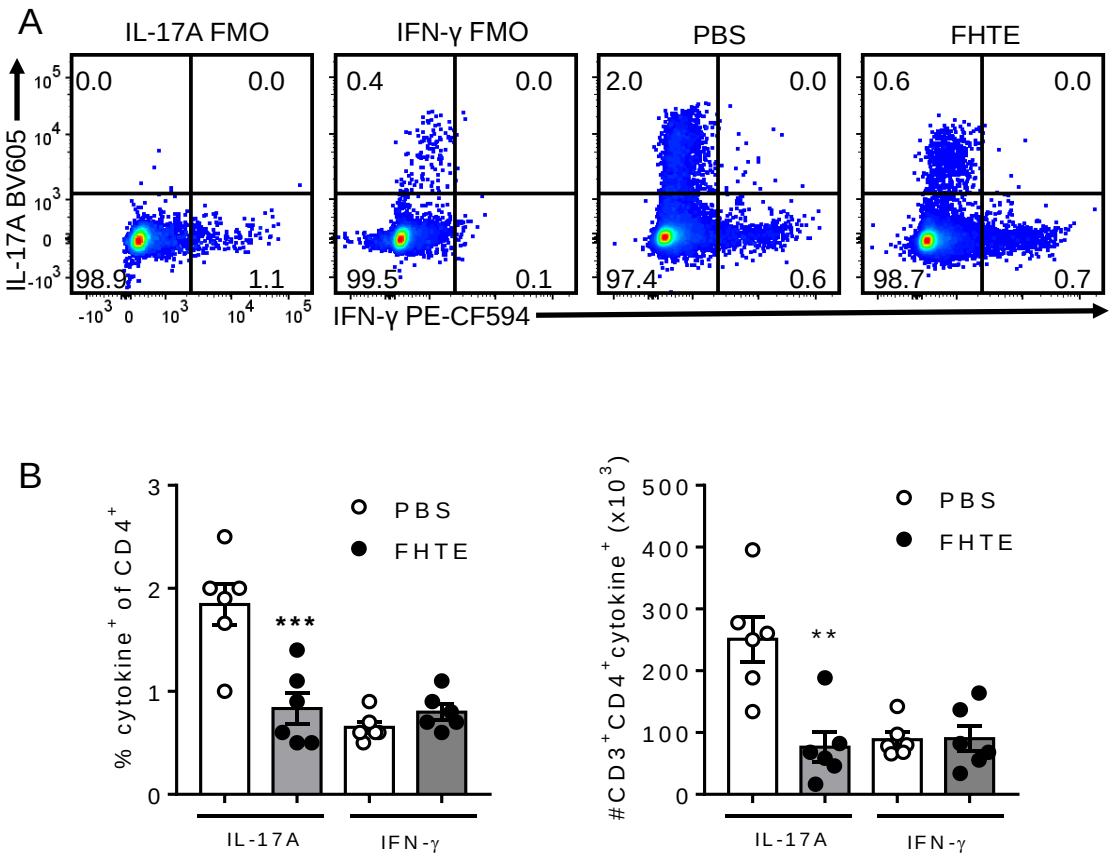

Figure 3.23 Training of mice with FHTE suppressed IL-17A production by CD4 T cells in the LNs on day 7 after the induction of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 3.19. On day 7, mice were sacrificed and cells were isolated from the inguinal, brachial and axillary LNs. Cells were stained for surface CD3 and CD4 and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+CD4^+$ cells) (A) and frequency and absolute number of IL-17A-producing or IFN-γ-producing $CD4^+$ T cells in the LNs (B). Data are mean ± SEM (n=6) and each symbol represents an individual mouse. Results are representative of two independent experiments. **p<0.01, ***p<0.001 vs PBS by unpaired t test.

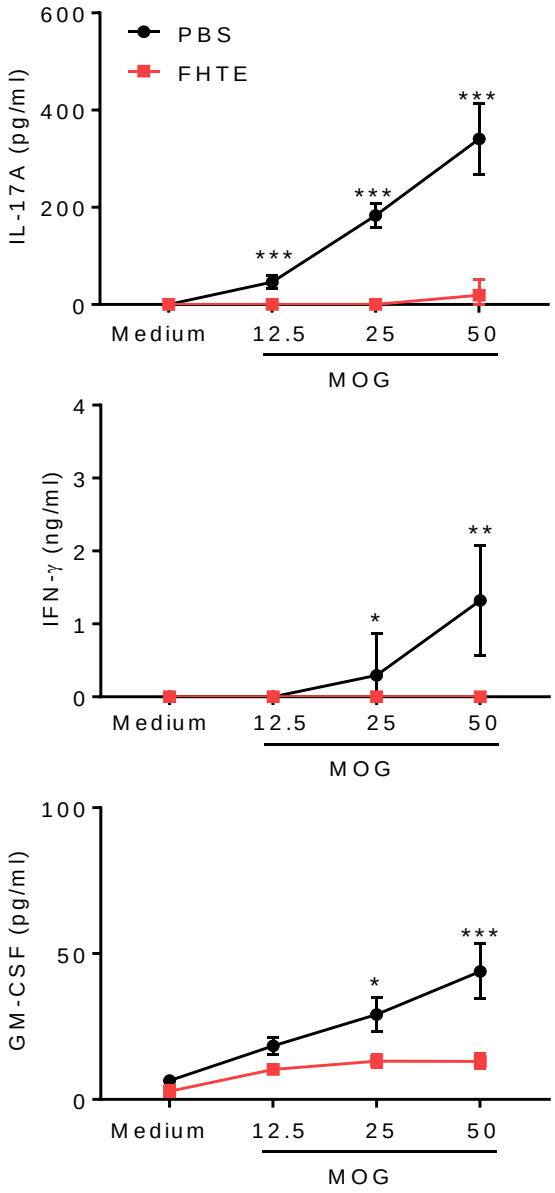

Figure 3.24 MOG-specific production of IL-17A, GM-CSF and IFN-γ by spleen and LN cells is reduced in FHTE-trained mice. C57BL/6 mice were trained and EAE was induced as described in figure 3.19. On day 7 after induction, mice were sacrificed and cells were isolated from the spleen and the inguinal, brachial and axillary LNs. Cells were restimulated with increasing concentrations of MOG (12.5, 25 and 50 µg/ml). After 72 hr, supernatants were collected and the concentrations of IL-17A, IFN-γ and GM-CSF quantified by ELISA. Data are mean ± SEM (n=6) and are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs PBS by two-way ANOVA with Sidak post-test.

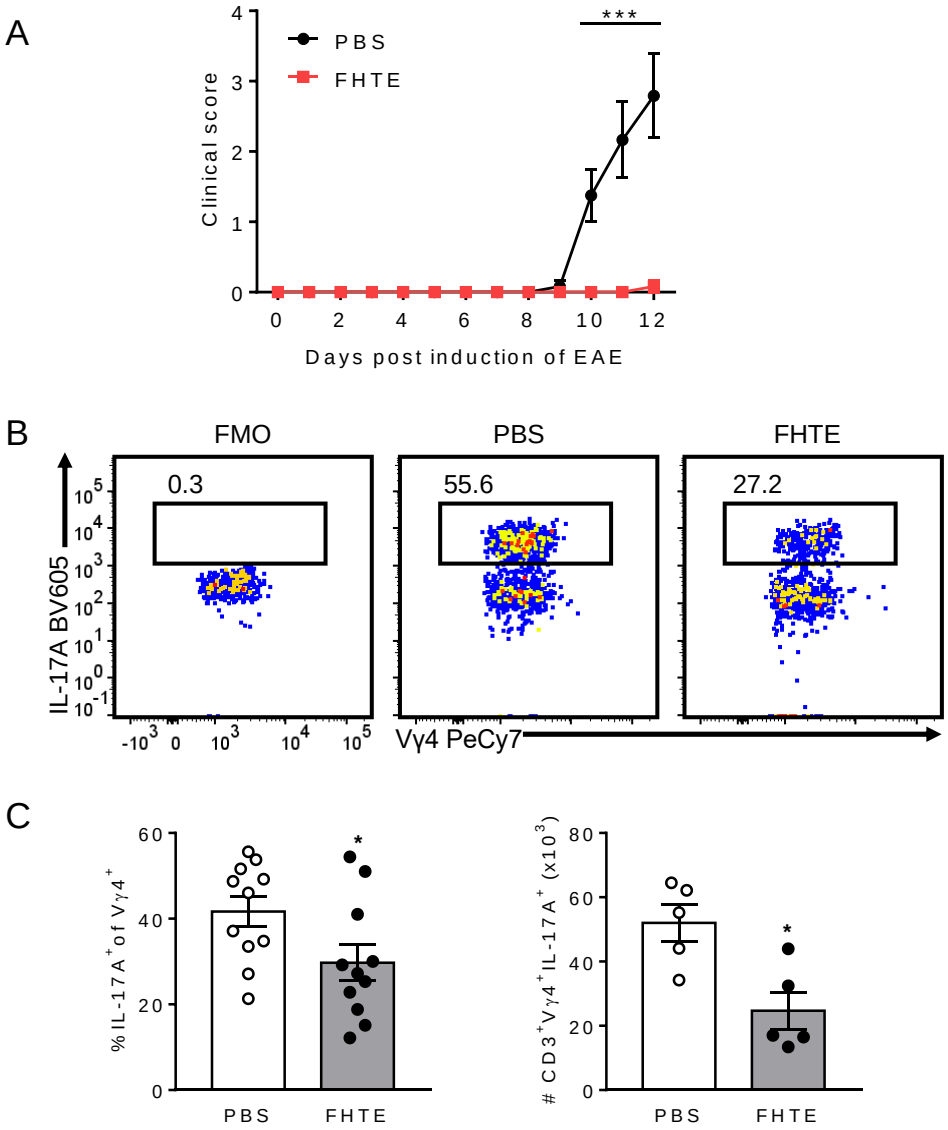

Figure 3.25 Training of mice with FHTE suppressed IL-17A production from Vγ4 T cells in the spleen 12 days after the induction of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 3.19. On day 12 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the spleen and stained for surface CD3 and Vγ4 and intracellular IL-17A and analysed by flow cytometry. Results are mean clinical score (A), representative FACS plots (gated on $CD3^+V\gamma4^+$ T cells) (B) and percentage and absolute number of IL-17A-producing $V\gamma4^+$ T cells (C). Data are mean ± SEM (n=11 or 5 per group) and each symbol represents an individual mouse. *p<0.05, ***p<0.001 vs PBS by repeated measures ANOVA with Sidak post-test (clinical score) or unpaired t test (percentage and absolute number).

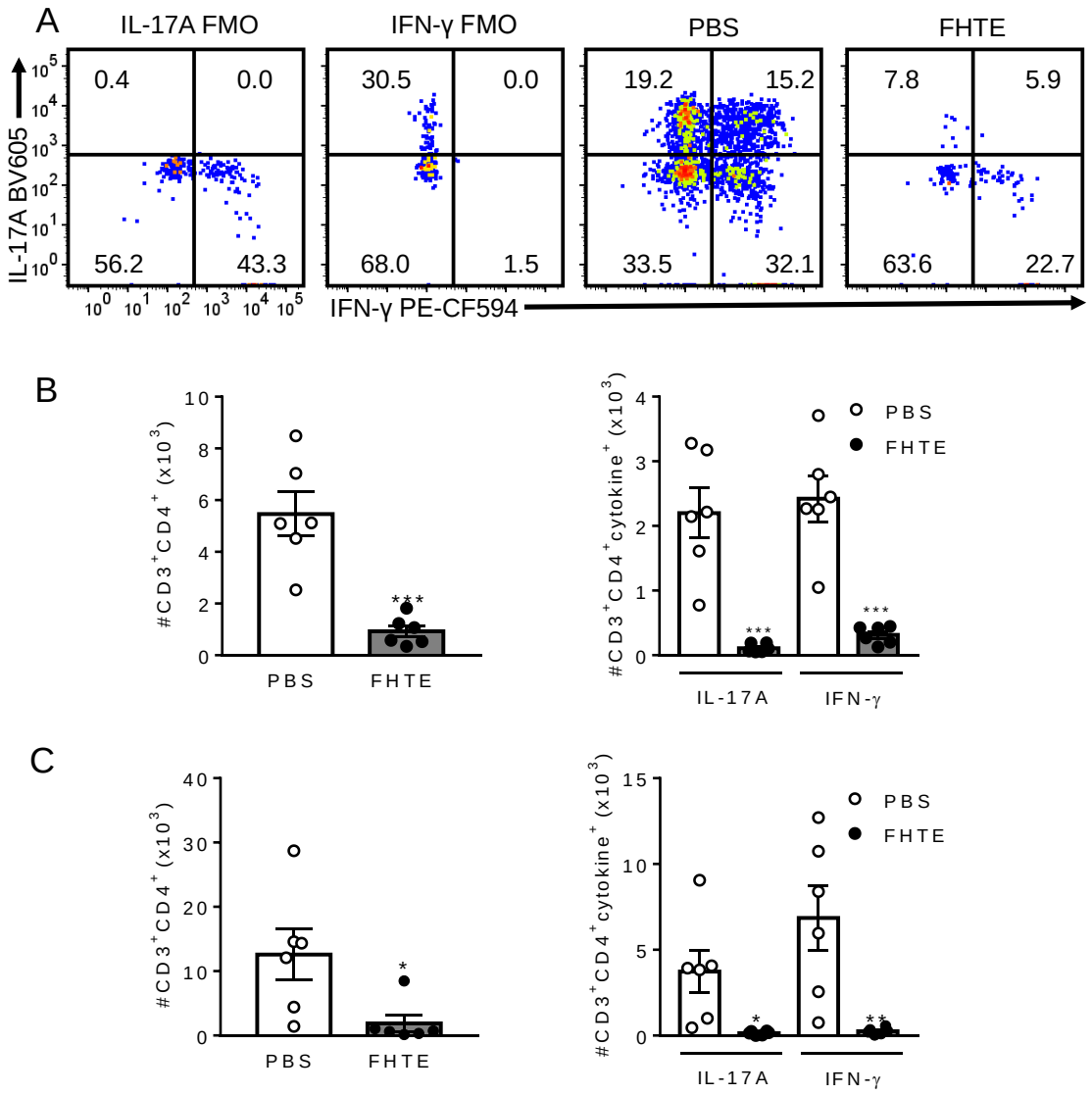

Figure 3.26 FHTE-trained mice have significantly reduced numbers of IL-17A and IFN-γ-producing CD4 T cells in the brain and spinal cord on day 12 of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 3.19. On day 12 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the brain and spinal cord and stained for surface CD3 and CD4 and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are representative FACS plots from the brain (gated on $CD3^+CD4^+$ T cells) (A), absolute number of $CD4^+$ T cells and $cytokine^+$ $CD4^+$ T cells in the brain (B) and absolute number of $CD4^+$ T cells and $cytokine^+$ $CD4^+$ T cells in the spinal cord (C). Data are mean ± SEM (n=6) and each symbol represents an individual animal. Results are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs PBS by unpaired t test.

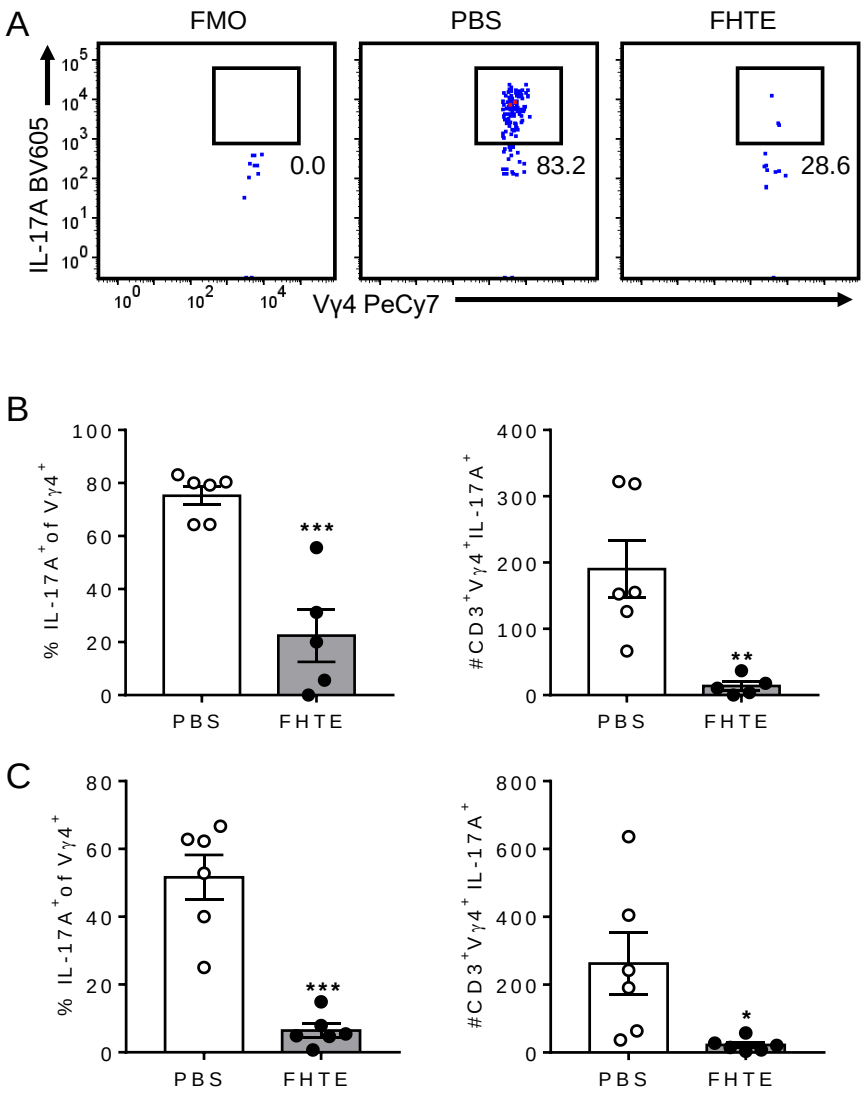

Figure 3.27 FHTE-trained mice have significantly reduced numbers of IL-17A-producing Vγ4 T cells in the brain and spinal cord on day 12 of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 3.19. On day 12 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the brain and spinal cord and stained for surface CD3 and Vγ4 and intracellular IL-17A and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+Vγ4^+$ T cells) in the brain (A), frequency and absolute number of IL-17A-producing $Vγ4^+$ T cells in the brain (B) and frequency and absolute number of IL-17A-producing $Vγ4^+$ T cells in the spinal cord (C). Data are mean ± SEM (n=6) and each symbol represents an individual mouse. Results are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs PBS by unpaired t test.

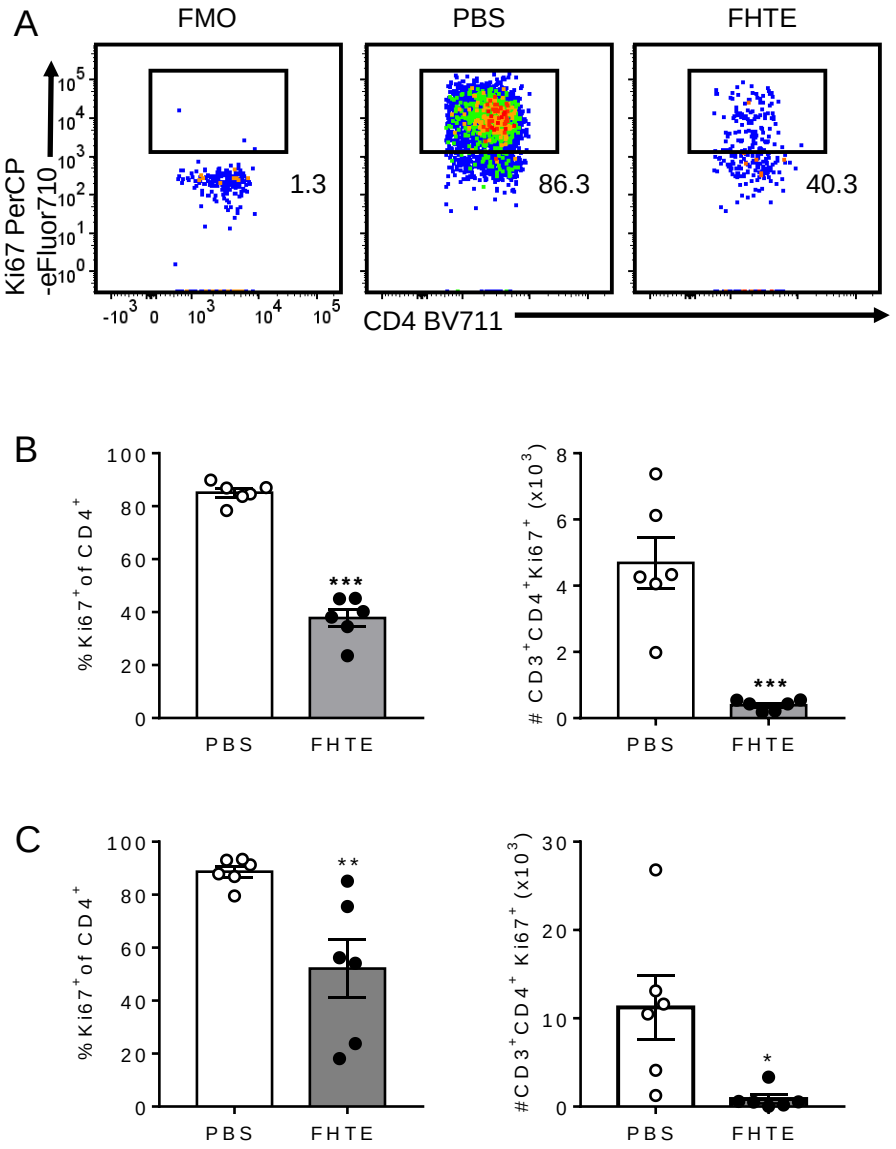

Figure 3.28 FHTE-trained mice have reduced proliferating CD4 T cells in the brain and spinal cord on day 12 of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 3.19. On day 12 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the brain and spinal cord and stained for surface CD3 and intranuclear Ki67 and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+CD4^+$ cells) in the brain (A) frequency and absolute number of proliferating $CD4^+$ T cells in the brain (B) and frequency and absolute number of proliferating $CD4^+$ T cells in the spinal cord (C). Data are mean ± SEM (n=6 per group) and each symbol represents an individual animal. Results are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs PBS by unpaired t test.

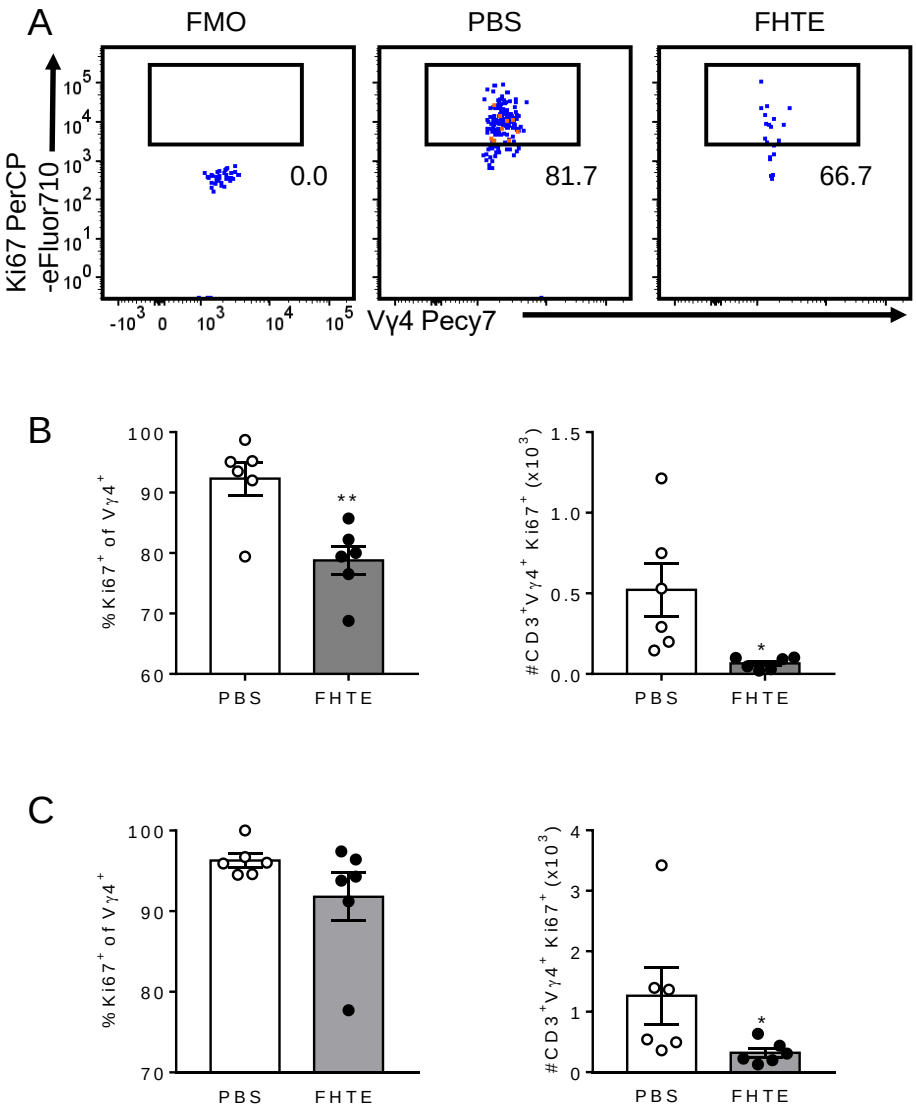

Figure 3.29 FHTE-trained mice have reduced proliferation of Vγ4 T cells in the brain and spinal cord on day 12 of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 3.19. On day 12 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the brain and spinal cord and stained for surface CD3, Vγ4 and intranuclear Ki67 and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+V\gamma4^+$ cells) in the brain (A), frequency and absolute number of proliferating $V\gamma4^+$ T cells in the brain (B) and frequency and absolute number of proliferating $V\gamma4^+$ T cells in the spinal cord (C). Data are mean ± SEM (n=6) and each symbol represents an individual mouse. Results are representative of two independent experiments. *p<0.05, **p<0.01 vs PBS by unpaired t test (brain) or Mann-Whitney test (spinal cord).

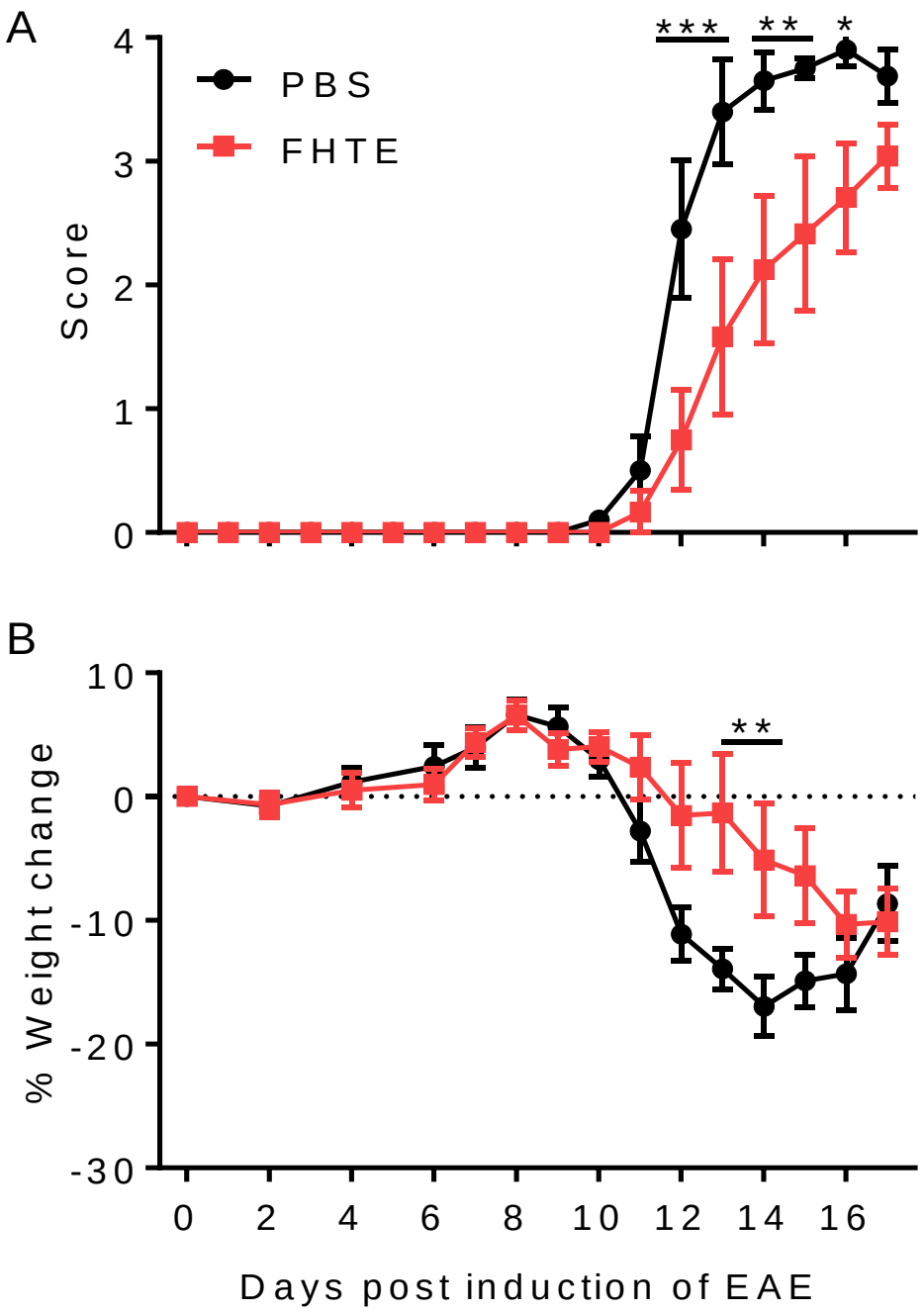

Figure 3.30 F4/80^{+}CD11b^{+} macrophages from FHTE-trained mice delay the onset of EAE. EAE was induced in naïve mice by s.c injection of MOG peptide (100 µg) emulsified in CFA, followed by i.p injection of PT (100 ng) on day 0 and day 2. Mice were subsequently injected intravenously with PBS or F4/80^{+}CD11b^{+} macrophages FACS sorted from the PEC of FHTE-trained mice (injected with 2.5% v/v in 200 µl PBS 21 and 7 days prior) on days 8 and 15 of EAE. Results are mean clinical score (A) and percentage body weight loss (B). Data are mean ± SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 vs PBS by repeated measures ANOVA with Sidak post-test.

Chapter 4.

F. hepatica TE directly modulates T cell responses.

4.1 Introduction

Helminths have evolved several mechanisms of immune subversion that been shown to modulate pathological inflammatory responses associated with allergy and autoimmune diseases. Furthermore, MS patients that are infected with helminths have reduced severity of disease, as well as a lower relapse rate, when compared with uninfected patients [175]. The protective mechanism of helminth infection that leads to a lower incidence of autoimmunity is thought to be mediated by the modulation of innate immune responses that direct the induction of Th2 or Treg cells. Our lab has previously shown that infection with live *F. hepatica* enhanced TGF-β production by DC and macrophages, leading to the expansion of Treg cells and attenuation of EAE [159]. Furthermore, infection with *H. polygyrus* induced tolerogenic DCs in the gut [152] that protected against colitis in mice [221, 222]. Although treatment of live helminths is under clinical evaluation for autoimmune diseases, ethical and practical implications may hamper advances of this approach. Alternatively, helminth-derived products could assist in the identification and targeting of pathways in the pathogenic immune responses that mediate allergy and autoimmune diseases. Helminth products such as ES-62 from *Acanthocheilonema viteae* have been shown to modulate DC activation, promoting Th2 cells, inhibiting the induction of Th1 responses, leading to the attenuation of collagen-induced arthritis [223]. In addition, helminth-derived products were shown to promote tolerogenic DCs that fail to prime effector T cell responses but promote the differentiation of Treg cells [224]. For example, Lysophosphatidylserine from *S. mansoni* induced tolergenic DCs that promoted the differentiation of IL-10-secreting Treg cells [225]. While there is compelling evidence that helminth products can modulate innate immunity, which suppresses pathogenic T cells responses in autoimmunity, there is little evidence of a direct effect on T cells.

Autoreactive T cells play a vital role in the development of autoimmune diseases and transfer of these cells is sufficient to induce disease in murine models of autoimmunity, including EAE [140, 226]. Furthermore, CD4 T cells are found at high levels in CNS lesions of MS patients [227], as well as in the brain and spinal cord of mice with EAE [47]. Before the discovery of Th17 cells, the pathology in EAE was thought to be mediated by Th1 cells. This theory was

partly supported by the observation that IL-12p40$^{-/-}$ mice were resistant to EAE [143]. However, while Th1-deficient mice did not develop EAE, transfer of autoantigen-specific Th1 cells induced inflammation in the CNS [228]. Following the discovery of the novel cytokine IL-23, which shares the common p40 chain with IL-12, IL-23p19$^{-/-}$ and not IL-12p35$^{-/-}$ mice were shown to be resistant to EAE [229]. Furthermore, IL-23 was shown to be a crucial differentiating factor for the development of Th17 cells and these cells were found to induce disease when adoptively transferred to naïve recipient mice [66, 67].

Although Th17 cells are considered to be a major source of IL-17 in autoimmune diseases, in the earlier phase of disease other IL-17-producing T cell populations play a critical role in the initiation of inflammation. γδ T cells are at the interface between innate and adaptive responses [230] and respond rapidly following infection or an inflammatory response providing an early source of IL-17A as well as IFN-γ and IL-22 [231]. γδ T cells have been implicated in the development of a number of autoimmune diseases, including EAE [33]. Our lab has previously demonstrated that γδ T cells constitutively express IL-23R and produce IL-17A in response to IL-1β and IL-23, without the requirement for TCR engagement [61]. In addition, IL-17A production by γδ T cells and in particular the Vγ4 subset, promotes the induction of Th1 and Th17 responses, amplifying their encephalitogenic function during EAE (Mc Ginley et al, Unpublished). Furthermore, the importance of γδ T cells in initiation of disease was confirmed using TCR-δ$^{-/-}$ mice, where delayed and reduced clinical signs of disease was observed, as well as a reduced IL-17A production from autoantigen-specific CD4 T cells, when compared with WT mice [61]. While there is little evidence to date of a direct effect of helminth products on γδ T cell responses, a study by Pineda et al. demonstrated that ES-62 from the helminth parasite *A. viteae* can target γδ T cells responses *in vivo* suppressing IL-17A production [232]. However, they did not examine the direct effect of ES-62 on γδ T cells and reduced IL-17A production by γδ T cells may be an indirect effect of suppressing IL-1β secretion by innate immune cells.

There is growing evidence that a number of helminth products can protect mice from autoimmunity, mainly through the induction of regulatory responses including Treg cells. However, the effect of FHTE on T cells in a model of autoimmunity has not been addressed to date. Given the importance of T cells and in particular γδ T cells in the development of EAE, this study investigated whether FHTE could directly modulate the induction of pathogenic CD4 and γδ T cells leading to attenuation of EAE. This chapter focused mainly on the modulation of cytokines that are crucial in the initiation and progression of autoimmune diseases. The data revealed that FHTE can directly suppress γδ T cells through downregulation of IL-1R1 and IL-23R expression, leading to decreased cytokine production and effector responses. Furthermore, FHTE directly inhibited cytokine production and proliferation of autoantigen-specific T cells, impairing their ability to transfer EAE to naïve mice. This was associated with reduced infiltration of pathogenic cells into the CNS and reduced clinical signs of disease.

4.2 Results

4.2.1 *FHTE suppresses IL-17A and IFN-γ production by spleen cells*

The results in chapter 3 have shown that FHTE can induce anti-inflammatory responses in innate immune cells, however, here I examined the direct effect of helminth products on T cells. Stimulation of spleen cells with anti-CD3 induced IL-17A and IFN-γ production and this was significantly suppressed by the addition of FHTE (Fig 4.1A). Furthermore, FHTE suppressed anti-CD3 and anti-CD28-induced IL-17A and IFN-γ production by CD4 T cells (Fig 4.1B).

Having established that FHTE is a potent suppressor of anti-CD3-induced IL-17A and IFN-γ production, this study examined the effect of FHTE stimulation on TCR-independent T cell responses. IL-1β in combination with IL-23 is capable of inducing IL-17A and IFN-γ production by CD3 T cells, without the requirement for TCR engagement [61]. As a result, spleen cells were stimulated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of increasing concentration of FHTE (0.625-2.5% v/v). IL-17A and IFN-γ production was induced in spleen cells stimulated with IL-1β and IL-23 and this was significantly suppressed by stimulation with FHTE, in a dose dependent manner (Fig 4.2). These results demonstrate that FHTE suppresses TCR-independent responses, which are vital in the initiation of inflammation.

4.2.2 *FHTE suppresses IL-1β and IL-23-induced IL-17A and IFN-γ production by γδ T cells*

Since γδ T cells are known to produce IL-17 and IFN-γ in response to IL-1β and IL-23 and play a crucial role in the pathogenesis of autoimmunity [95], this study next examined the direct effect of FHTE on γδ T cells. γδ T cells were purified from the LNs of naïve mice and stimulated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 1.25 or 2.5% v/v FHTE. Supernatants were collected after 72 hours and the concentration of IL-17A, IL-17F, IFN-γ and IL-22 quantified by ELISA. Stimulation of γδ T cells with IL-1β and IL-23 induced high concentration of IL-17A, IL-17F, IL-22 and IFN-γ, whereas addition of FHTE significantly suppressed IL-1β and IL-23-induced cytokine production, in a dose dependent manner (Fig 4.3).

Total RNA was isolated from γδ T cells 72 hours after stimulation with IL-1β and IL-23 in the presence or absence of FHTE and the mRNA expression of *il17a*, *il17f*, *il22*, *rorc* and *tbx21* was analysed by qRT-PCR. Stimulation of γδ T cells with IL-1β and IL-23 induced potent mRNA expression of *il17a*, *il17f* and *il22*, and this was significantly suppressed by treatment with FHTE, in a dose dependent manner. Furthermore, FHTE suppressed IL-1β and IL-23-induced mRNA expression of *rorc* and *tbx21*, with 2.5% v/v FHTE completely inhibiting transcription of these genes (Fig. 4.4).

To examine whether FHTE can suppress cytokine activated γδ T cell responses *in vivo*, mice were injected into the footpad with PBS or IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of FHTE (50 µg). After 4 hours the popliteal LNs were isolated and stained for CD3, Vγ4 and IL-17A. The results revealed that treatment of mice with IL-1β and IL-23 induced IL-17A production by Vγ4 T cells, and this was significantly suppressed by treatment of mice with FHTE (Fig 4.5). These findings suggest that FHTE directly inhibits γδ T cell activation by suppressing IL-1β and IL-23-induced gene expression of *rorc* and *tbx21*, thus leading to reduced production of cytokines that are crucial in the initiation of autoimmunity.

4.2.3 *FHTE suppresses the expression of IL-1R1 and IL-23R on γδ T cells*

γδ T cells respond to IL-1β and IL-23 through expression of receptor for their cytokines. Furthermore, γδ T cells are known to constitutively express IL-23R on their surface [201]. In order to examine the effect of FHTE on expression of IL-1R1 and IL-23, γδ T cells were purified from the LNs of naïve mice and stimulated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 1.25 or 2.5% v/v FHTE. After 72 hours, total RNA was isolated and mRNA expression of *il1r1* and *il23r* mRNA was evaluated by qRT-PCR. Stimulation with IL-1β and IL-23 increased *il1r* and *il23r* expression 25 fold and 20 fold respectively, however, stimulation with 2.5% v/v FHTE reduced *il1* and *il23* receptor expression to levels comparable with medium cultured cells. Furthermore, stimulation of γδ T cells with FHTE alone suppressed basal expression of *il1r1* and *il23r* mRNA (Fig 4.6A).

Direct suppression of receptor expression by FHTE was confirmed by flow cytometry. Culture of γδ T cells with FHTE suppressed IL-1β and IL-23-induced

surface expression of IL-1R1. In addition, stimulation with FHTE in the absence of IL-1β and IL-23 downregulated IL-1R1 expression on the surface of γδ T cells (Fig 4.6B). These results demonstrate that even under stead state conditions, FHTE can suppress γδ T cell activation through downregulation of receptor expression.

To examine the effect of FHTE on IL-1 receptor expression on the surface of γδ T cells *in vivo*, WT mice were injected i.p with PBS or FHTE (2.5% v/v in 200 µl PBS) and as a control IL-1R1$^{-/-}$ mice were injected with PBS. After 18 hours, PEC were isolated by peritoneal lavage and stained for CD3, TCR-δ and IL-1R1. Although the total number of γδ T cells (Fig 4.7B) and Vγ4 T cells (Fig 4.8B) in the peritoneal cavity was unchanged, mice treated with FHTE had a significant reduction in the percentage and absolute number of IL-1R1-expressing γδ T cells (Fig 4.7C) as well as reduced IL-1R1-expressing Vγ4 T cells, when compared with PBS-injected mice (Fig 4.8C). Collectively, these results demonstrate that FHTE directly inhibits the activation of γδ T cells by suppressing *il1r1* and *il23r* mRNA, which leads to downregulated receptor expression on the cell surface. This results in decreased receptor signalling and suppression of IL-1β and IL-23-induced IL-17A production, which is crucial in driving pathogenic γδ T cells and Th17 cell responses in EAE.

4.2.4 *Early treatment of mice with FHTE delays the onset of EAE*

Early IL-17A production by γδ T cells is crucial in the initiation of inflammation in response to infection. During *Escherichia coli* infection, γδ T cell provide an early source of IL-17, leading to recruitment of neutrophils and the clearance of infection [63]. Furthermore, IL-17A-producing γδ T cells are detected within a few hours of infection of mice with *B. pertussiss* [64]. In the context of autoimmunity, γδ are rapidly activated in response to IL-1β and IL-23 and play a major role in the development of pathogenic Th17 cells, amplifying their encephalitogenic function [201]. The current study demonstrated that early IL-17A production by γδ T cells peaks 3 days post induction of EAE (Fig 4.9). Having shown that treatment with FHTE directly suppressed γδ T cells activation in response to IL-1β and IL-23, the EAE model was used to examine the effect of early FHTE treatment on γδ T cells in a model of autoimmunity.

EAE was induced by s.c injection of MOG peptide (100 µg) emulsified in CFA, followed by i.p injection of PT (100 ng) on day 0 and day 2. Mice were injected s.c with FHTE (2.5% v/v in 200 µl PBS) 2 hours before EAE induction, day 1 and day 2 post induction. Early treatment of mice with FHTE significantly delayed the onset of clinical signs of EAE (Fig 4.10A) and weight loss associated with disease (Fig 4.10B), when compared with PBS-injected mice. Furthermore, FHTE-treated mice had suppressed T cell responses in the periphery. Spleen and LN cells recovered from mice 7 days post induction of EAE secrete IL-17A and IFN-γ in response to *in vitro* stimulation with MOG and this MOG-specific cytokine production was significantly suppressed in mice treated with FHTE (Fig 4.11). In addition, treatment of mice with FHTE resulting in reduced infiltration of CD3 T cells into the brain (Fig 4.12B) and spinal cord (Fig 4.12C) on day 13 of EAE. Treatment of mice with FHTE was also associated with a significant reduction in the percentage and absolute number of IL-17A-producing CD4 T cells in the brain (Fig 4.13B) and spinal cord (Fig 4.13C), when compared with PBS-treated mice. Furthermore, infiltration of IL-17A and IFN-γ-producing γδ T cells into the spinal cord on day 13 was significantly suppressed in FHTE-treated mice (Fig 4.14) as well as reduced IL-17A-producing Vγ4 T cells, when compared with PBS-injected mice (Fig 4.15). These results suggest that treatment of mice with FHTE suppresses early IL-17A and IFN-γ production, which is required for the initiation of inflammation and development of Th17 responses, resulting in delayed onset of EAE.

4.2.5 *FHTE inhibits IL-17 production by CD4 and γδ T cells from mice with EAE*

IL-17A-producing γδ and CD4 T cells have been shown to play a pathogenic role in many autoimmune diseases, including MS and EAE [61]. Having shown that FHTE can directly modulate T cell responses, this study next examined the effect of FHTE on the passive transfer model of EAE and in particular the effect on CD4 and γδ T cells during the adoptive transfer culture phase. Mice were immunized with MOG (100 µg/ml) emulsified in CFA. After 10 days, spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of FHTE (2.5% v/v). After 24 or 72 hours cells were lysed, total RNA was extracted and expression of *arg1*, *nos2*, *il17a, ifnγ, rorc, tbx21* was

evaluated by qRT-PCR. Treatment of cells with FHTE significantly enhanced mRNA expression of *arg1* while suppressing *nos2* (Fig 4.16). Furthermore, culture of cells with FHTE significantly suppressed mRNA expression of *il17a*, *ifnγ* and *il10* as well as the genes encoding the transcription factors RORγt and T-bet (Fig 4.17). In addition, treatment of encephalitogenic cells from donor mice with FHTE suppressed MOG, IL-1β and IL-23-induced *il1r1* and *il23r* expression but did not modulate expression of trafficking molecules *mcam* and *itga4* (Fig 4.18). Moreover, MOG-specific IL-17A and IFN-γ production quantified in the culture by ELISA was significantly suppressed by the addition of FHTE. In contrast, FHTE enhanced IL-1RA production (Fig 4.19). This effect was specific for IL-1β and IL-23-induced cytokines, as culture with FHTE did not modulate IL-12 and IL-18-driven IL-17A and IFN-γ production.

Using flow cytometry, this study next examined the effect of FHTE on IL-17A and IFN-γ production by CD4 and γδ T cells from spleen and LN cells at the end of the adoptive transfer culture stage. Stimulation with MOG, IL-1β and IL-23 induced IL-17A production from CD4 T cells and this was significantly suppressed by the addition of FHTE (Fig 4.20B). Furthermore, FHTE suppressed IL-17A from γδ T cells (Fig 4.21B) and particularly Vγ4 T cells (Fig 4.21C). In addition, IFN-γ production from CD4 (Fig 4.20) and γδ T cells (Fig 4.21) was unchanged in cells from donor mice that were stimulated with FHTE.

In addition to modulation of IL-17A and IFN-γ, treatment with FHTE significantly suppressed proliferation of γδ and Vγ4 T cells but did not modulate proliferation of CD4 T cells (Fig 4.22). Furthermore, culture of encephalitogenic cells with FHTE selectively suppressed expression of IL-1R1 on the surface of γδ and Vγ4 T cells, but not on CD4 T cells (Fig 4.23). These results demonstrate that FHTE directly inhibits T cells responses from mice immunized with MOG and CFA, in particular γδ T cells, leading to reduced proliferation and production of cytokines that known to be involved in the pathogenesis of EAE.

4.2.6 *FHTE suppresses the pathogenic function of CD4 and γδ T cells in the EAE model*

Having shown that FHTE suppresses IL-17A production by CD4 and γδ T cells from MOG-immunized mice, the study examined the effect of FHTE on the

effector function of these cells by adoptive transfer to recipient mice. Mice were immunized with MOG and CFA and spleen and LN cells were recovered on day 10 and cultured with MOG, IL-1β and IL-23 in the presence or absence of FHTE. After 72 hours, the cells were transferred to recipient mice.

Culture of spleen and LN cells from MOG-immunized mice with FHTE *in vitro* prior to transfer delayed the onset of disease. By day 12 after transfer, 100% of control mice had developed moderate to severe paralysis, whereas 50% of FHTE-treated mice had no clinical signs of disease. Furthermore, treatment of mice with FHTE led to a reduction in the severity of EAE, with mice showing minimal clinical signs of disease by day 24 (Fig 4.24A). In addition, body weight loss reached on average 24% by day 13 in mice transferred with cells cultured with MOG, IL-1β and IL-23, however, weight loss was significantly reduced when cells were cultured in the presence of FHTE (Fig 4.24B).

An examination of infiltrating lymphocytes in the brain of mice 10 days after transfer revealed that the number of CD4 and γδ T cells infiltrating the brain was significantly reduced in the recipients of FHTE-treated cells, when compared with the mice injected with cells that had been cultured without FHTE (Fig 4.25). Furthermore, treatment of cells with FHTE prior to transfer led to a significant reduction in the percentage and number of IL-17A-producing CD4 T cell infiltrating the brain (Fig 4.26). Moreover, the percentage and number of IL-17A-producing γδ T cells were significantly lower in the brain of mice injected with FHTE-treated cells, when compared with cells that had been cultured without FHTE (Fig 4.27). However, no change in IFN-γ-producing CD4 T cells (Fig 4.26) or IFN-γ production by γδ T cells (Fig 4.27) was observed. These results demonstrate that FHTE suppressed IL-17 production by CD4 and γδ T cells induced by IL-1β and IL-23 *in vitro*, resulting in decreased pathogenic T cell responses and attenuation of clinical signs of EAE.

4.2.7 *Selective treatment of CD3$^+$ T cells from MOG-immunized mice with FHTE alters their capacity to induce EAE by cell transfer*

In order to confirm that FHTE was directly acting on T cells, an EAE transfer experiment was performed where only T cells were cultured with FHTE. Spleen and LN cells from MOG-immunized mice were separated into a CD3$^+$ T cell

fraction and a CD3-depleted fraction. $CD3^+$ T cells were stimulated for 18 hours with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE before being washed and combined with the CD3-depleted fraction. The combined cells were cultured with MOG (100 µg/ml), IL-1β and IL-23 for 48 hours before transfer to naïve mice. The cytokine profile in the supernatant was examined at the end of the culture prior to transfer. Consistent with the data on the treatment of total spleen and LN cells with FHTE, selective treatment of $CD3^+$ cells with FHTE significantly suppressed MOG, IL-1β and IL-23-induced IL-17A and IFN-γ production (Fig 4.28). Transfer of cells that were stimulated with MOG, IL-1β and IL-23 to recipient mice resulted in clinical signs of EAE by day 8, however, delayed onset of disease and a reduction in disease severity was observed in recipients injected with $CD3^+$ T cells cultured with FHTE (Fig 4.29). This was accompanied by reduced infiltration of CD4 (Fig 4.30) and γδ T cells (Fig 4.31) into the brain. Furthermore, there was a significant reduction in the number of IL-17A-producing CD4 T cells (Fig 4.30), as well as IL-17A-producing γδ T cells in $CD3^+$ T cells cultured with FHTE prior to transfer (Fig 4.31). Taken together, these results demonstrate that selective treatment of $CD3^+$ T cells with FHTE prior to transfer results in significant attenuation of their pathogenic function in a model of autoimmunity.

4.3 Discussion

Immune evasion strategies that parasites have evolved to ensure their survival are associated with simultaneous subversion of immune responses that mediate allergy and autoimmune diseases [146]. This phenomenon forms the basis of the hygiene hypothesis, which attempts to explain the lower incidence of allergy and autoimmunity in rural areas of developing countries that have a higher incidence of helminth infection. However, most studies have shown that helminths alter pathogenic T cell responses indirectly through modulation of the innate immune system [159, 225]. This study showed that under steady state conditions as well as during inflammation, FHTE inhibits IL-1R1 expression on the surface of γδ T cells, thus inhibiting IL-1-induced cytokine production and effector responses by these cells. In addition, this study revealed that FHTE directly suppresses encephalitogenic T cells, impairing their ability to proliferate and produce cytokine, thus reducing their pathogenic activity in a transfer model of CNS autoimmunity.

In the present study, FHTE was found to inhibit IL-17A and IFN-γ production by spleen cells stimulated through the TCR. This suppressive effect was not mediated through APCs, as FHTE also significantly reduced IFN-γ production by purified CD4 T cells. Furthermore, treatment of spleen cells with FHTE suppressed IL-1β and IL-23-induced IL-17A and IFN-γ production. Previous work from the our lab has demonstrated that CD3 T cells secrete IL-17 and IFN-γ production in response to IL-1β and IL-23 without the requirement for TCR stimulation [201]. Furthermore, IL-17 and IFN-γ production are crucial effector cytokines of Th1 and Th17 cells, which are pathogenic in many autoimmune diseases, including EAE.

γδ T cells play a crucial role in early defences against bacterial and fungal infection [63, 64]. They constitutively express receptors for IL-1 and IL-23 [201] and are known to produce innate IL-17 and IFN-γ in response to IL-1β and IL-23 secreted by DCs and macrophages [233]. In addition to their role in infection, γδ T cells have also been implicated in the development of IL-17-mediated autoimmunity [234, 235]. Previous work in our lab has shown that stimulation of γδ T cells with IL-1β and IL-23 enhances expression of *il23r* as well as *rorc*, a signature transcription factor of Th17 cells [201]. The present study also

demonstrated that treatment of γδ T cells with IL-1β and IL-23 enhanced *rorc* as well as *tbx21* mRNA expression and this was suppressed by treatment with FHTE. A number of studies have shown that γδ T cells are programmed into distinct IFN-γ–producing CD27$^+$ (γδ 27$^+$ cells) and IL-17–producing CD27$^-$ (γδ 27$^-$ cells) subsets [87]. Furthermore, the transcription factor T-bet is critical for IFN-γ production by γδ 27$^+$ cells, whereas RORγt deficiency completely abolished the production of IL-17 by γδ 27$^-$ cells. The results from this study have demonstrated that FHTE suppression of *rorc* and *tbx21* mRNA expression was associated with reduced IL-17A and IFN-γ production, suggesting that suppression of γδ T cell responses by FHTE is not limited to one subset.

The ability of γδ T cells to produce IL-17A in response to IL-1β and IL-23 is dependent on expression of the receptor for their cytokine on the surface of the cell. Furthermore, IL-17A production and development of CD4 T cells is compromised in mice deficient for the IL-1 receptor [201]. Our lab has previously shown that γδ T cells constitutively express IL-23R, which is upregulated in response to IL-1β and IL-23. The present study revealed that FHTE not only suppressed IL-1β and IL-23-induced IL-1 and IL-23 receptor expression, but also downregulated basal expression of IL-1R1 and IL-23R on the surface of γδ T cells. Suppression of IL-1R1 and IL-23R, along with downregulation of *rorc* and *tbx21*, resulted in potent inhibition of IL-17A and IFN-γ production by γδ T cells. Given the importance of early IL-17 production in response to infection, these findings could in part explain the impaired ability of parasite bearing hosts to control bacterial infection [186]. Furthermore, inhibition of γδ T cell-derived cytokine production by parasitic products may also be a useful potential therapeutic approach for control of autoimmune diseases.

In addition to suppressing γδ T cell responses both *in vitro* and *in vivo*, this study demonstrated that treatment of mice with FHTE during the induction phase of EAE, suppressed pathogenic T cell responses and delayed the onset of disease. The pathology of EAE is mediated by activation of γδ T cells early in disease and Th1 and Th17 cells later in disease [236]. The present study demonstrated that the differentiation of MOG-specific Th17 and Th1 cells, and the production of their associated cytokines IL-17A and IFN-γ, were impaired in

mice that were treated with FHTE during the induction phase of disease. Furthermore, early treatment of mice with FHTE reduced the infiltration of pathogenic CD4 T cells into the CNS. In addition, this study demonstrated that treatment of mice with FHTE had a potent inhibitory effect on IL-17A-producting γδ T cells in the brain and in particular the Vγ4 subtype, known to be pathogenic in EAE [237]. However, although early treatment of mice with FHTE delayed the onset of EAE, FHTE-treated mice eventually developed clinical signs and weight loss associated with disease. Our lab has previously shown that a high proportion of γδ T cells were found to constitute express IL-18R as well as the IL-23R. Furthermore, IL-18 and IL-18R were found to play a key role in promoting IL-17A production from CD4 T cells, even in IL-1R1$^{-/-}$ mice [62], suggesting that IL-18 production can overcome the need for early IL-1 signalling in the development of autoimmune diseases, including EAE. Having shown that the suppression effect of FHTE on T cells is specific to the IL-1β and IL-23 pathway, these results suggest that suppression of IL-1-specific T cell activation may have been overcome during the effector phase of EAE, leading to infiltration of pathogenic T cells and development of clinical signs of disease.

EAE can be induced by transfer of pathogenic T cells to naïve recipient mice [140, 226]. The passive EAE model can be used to manipulate the effector functions of T cells *in vitro* before transfer, therefore allowing investigation of particular T cell functions in the pathogenesis of disease [140]. Culture of cells with FHTE from MOG-immunized mice suppressed the genes encoding the transcription factors RORγt and Tbet, which are essential for the differentiation of Th17 and Th1 cells. mRNA expression of *il17a* and *ifnγ* was also significantly suppressed in reactivated MOG-specific T cells cultured with FHTE. This is consistent with a study demonstrating that inhibitors specific for RORγt effectively suppress Th17 differentiation and attenuate arthritis [238]. In addition, *il10* mRNA expression was also reduced by treatment with FHTE. The production of IL-10 and the induction of regulatory T cells have been shown to be one of the most common mechanisms whereby parasites subvert host immune responses to prevent expulsion, while also preventing excessive inflammation [146]. These observations suggest that other mechanisms besides the induction of regulatory cells may be involved in modulation and suppression of T cell responses by

FHTE. Furthermore, FHTE stimulation did not modulate mRNA expression of the genes encoding the trafficking molecules CD146 and the CD49d subunit of VLA4. T cell–associated integrins are known to be required for the adhesion and diapedesis of encephalitogenic T cells into the CNS [239]. Moreover, studies in the adoptive transfer model of EAE demonstrated that neutralization of VLA-4 can suppress the ability of antigen-specific T cells to induce EAE [240]. However, the results in the present study suggests that FHTE suppresses the activation of pathogenic Th17 responses, rather than inhibiting trafficking of these cells into the CNS during EAE.

Treatment of MOG-specific T cells with MOG, IL-1β and IL-23 *in vitro* in the presence of FHTE prior to transfer reduced the expression of IL-1R1 on γδ and Vγ4 T cells. This is consistent with the data presented earlier in this chapter, demonstrating that FHTE targets the IL-1 pathway by suppressing receptor expression leading to reduced γδ T cell responses. Furthermore, transfer of T cells that were treated with FHTE attenuated the effect of transfer EAE. This was accompanied by reduced infiltration of IL-17-producing CD4 and γδ T cells into the CNS, however, there was no change in the production of IFN-γ from CD4 and γδ T cells upon treatment with FHTE prior to transfer. To date, there have been conflicting reports regarding the role of IFN-γ in the pathogenesis of autoimmune diseases. However, the results from the current study are consistent with data from our lab, demonstrating that IFN-γ is required for initiation of EAE but has a protective role during effector phase of disease, through suppression of Th17 responses [41]. Another important finding of this study was that selective treatment of $CD3^+$ T cell with FHTE prior to transfer, significantly attenuated EAE and reduced infiltration of pathogenic T cells into the CNS. These finding demonstrate that direct treatment of $CD3^+$ T cells with helminth products can inhibit reactivation of CD4 and γδ cells and suppress their ability to elicit their pathogenic function in the development of CNS autoimmunity.

Several studies in mouse models of autoimmunity have demonstrated that infection with helminth parasite can attenuate autoimmune diseases through regulatory mechanisms involving the induction of Treg cells. However, direct targeting and suppression of T cell responses may be an alternative mechanism

of immunomodulation by helminth products, which may explain the lower incidence of allergy and autoimmunity in areas of high helminth burden. Collectively, this study provides the first evidence that helminths products can directly target T cell activation, in particular, γδ T cells, suppressing their ability to drive pathogenic responses in autoimmune diseases. A major focus in the development of new anti-inflammatory drugs for autoimmune diseases has been on the IL-23/IL-17 axis. Indeed, it is generally accepted that Th17 cells provide a vital source of IL-17A and IL-17F in MS and EAE. However, there is growing evidence that γδ T cells not only contribute to early production of these cytokines but also amplify Th17 responses [61]. As a result, antibodies that specifically target IL-1β and lL-23 receptor expression may not only target Th17 cells but also suppress the activation of IL-17A-secreting γδ T cells and therefore have protective effects in treating autoimmune diseases.

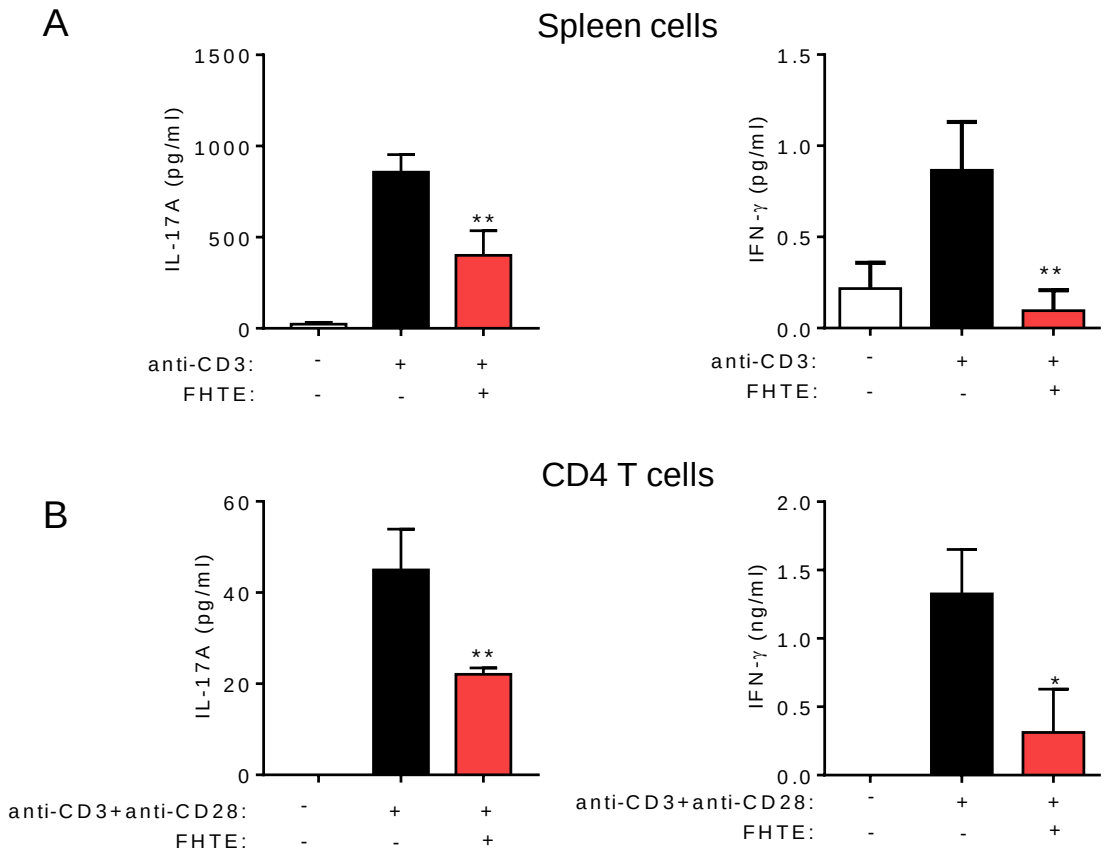

Figure 4.1 FHTE suppressed anti-CD3-induced or anti-CD3+anti-CD28-induced IL-17A and IFN-γ production by spleen cells and CD4 T cells. (A) Spleen cells from naïve C57BL/6 mice were stimulated with anti-CD3 (1 µg/ml) in the presence or absence of 2.5% v/v FHTE. After 72 hr, the supernatants were removed and the concentration of IL-17A and IFN-γ production quantified by ELISA. (B) Purified CD4 T cells were incubated with plate bound anti-CD3 and soluble anti-CD28 (5 µg/ml) in the presence or absence of 2.5% v/v FHTE for 72 hr before IL-17A and IFN-γ was quantified by ELISA. Results are mean ±SD for triplicate culture and are combined from two independent experiments. *p<0.05, **p<0.01 vs anti-CD3 or anti-CD3+anti-CD28 by one-way ANOVA with Dunnett post-test.

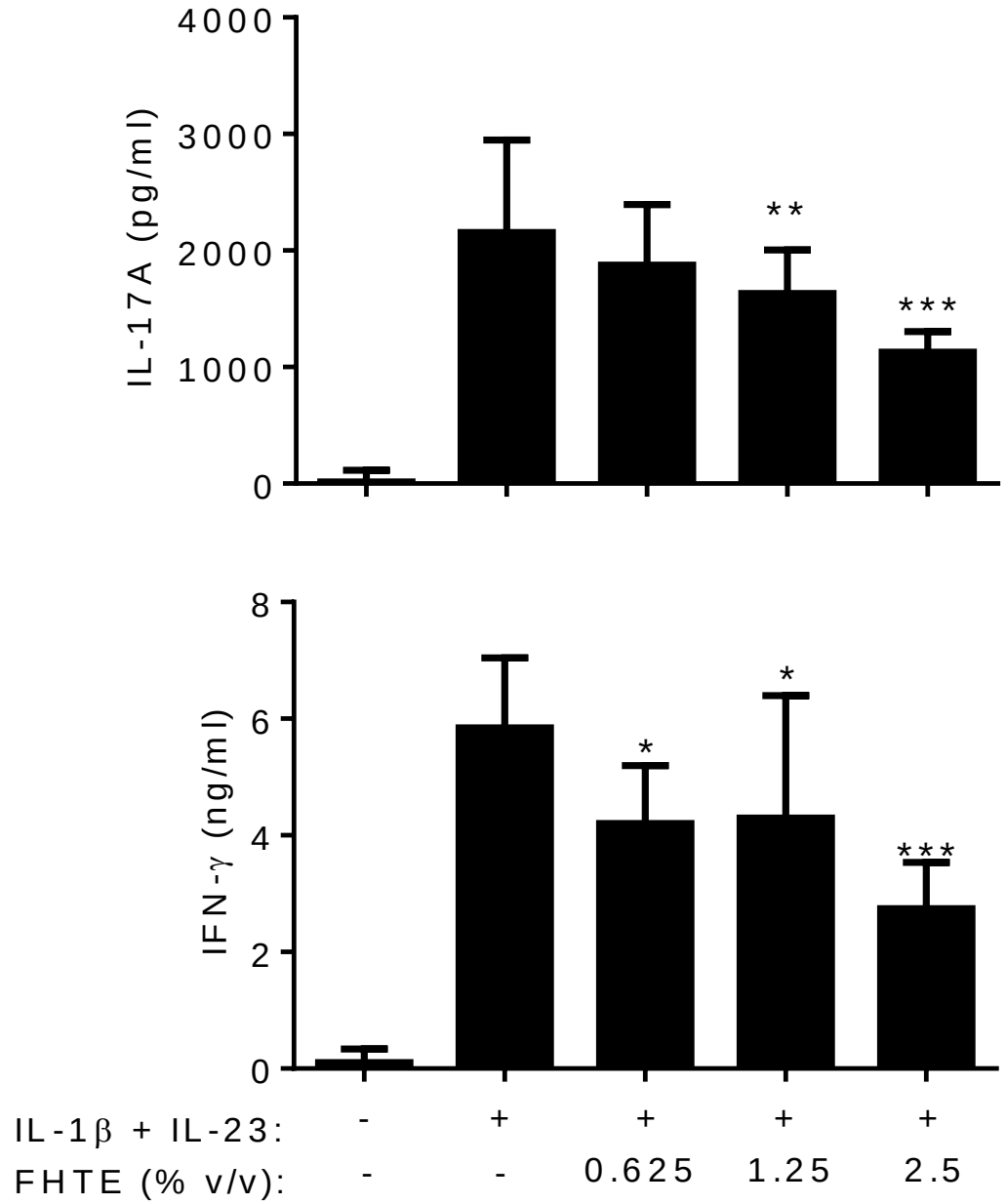

Figure 4.2 FHTE suppressed IL-1β and IL-23-induced IL-17A and IFN-γ production by spleen cells. Spleen cells from naïve C57BL/6 mice were incubated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) with or without increasing concentration of FHTE (0.625-2.5% v/v). Supernatants were collected after 72 hr and the concentrations of IL-17A and IFN-γ determined by ELISA. Results are mean ±SD for triplicate culture and are combined from three independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

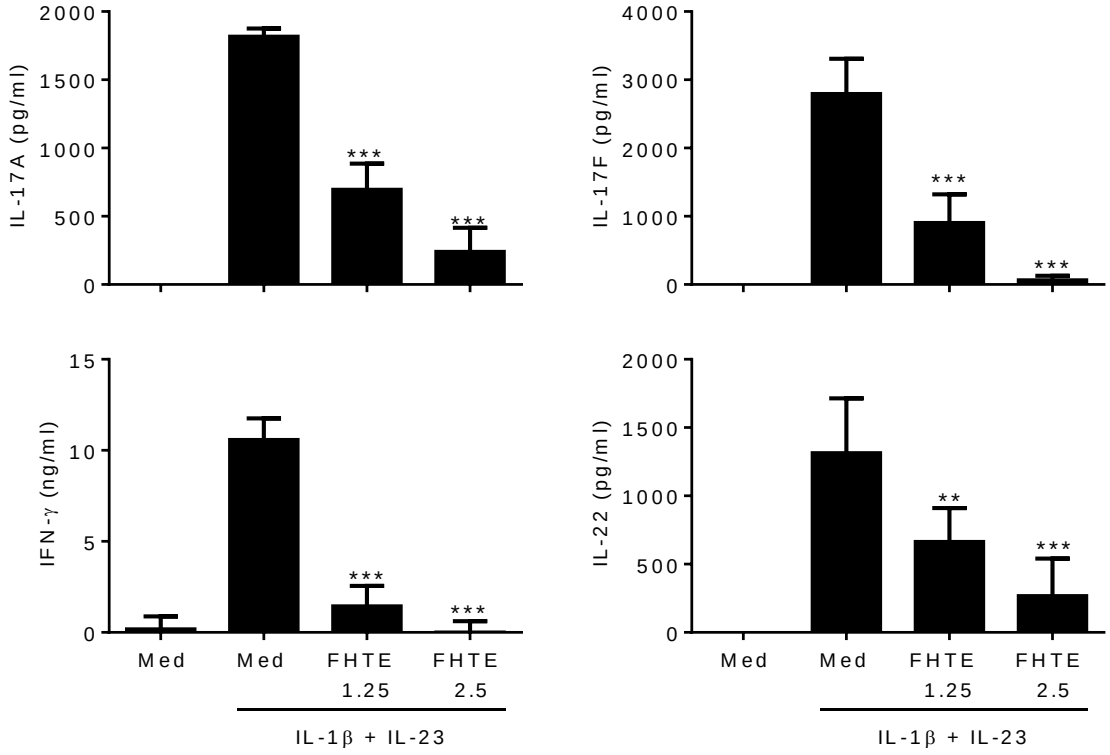

Figure 4.3 FHTE suppressed IL-1β and IL-23-induced IL-17A and IFN-γ production by purified γδ T cells. γδ T cells were MACS purified from the LNs of naïve C57BL/6 mice and incubated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence of absence of 1.25 or 2.5% v/v FHTE. Supernatants were collected after 72 hr and the concentration of IL-17A, IL-17F, IFN-γ and IL-22 determined by ELISA. Results are mean ±SD for triplicate culture and combined from two independent experiments. **p<0.01, ***p<0.001 vs IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

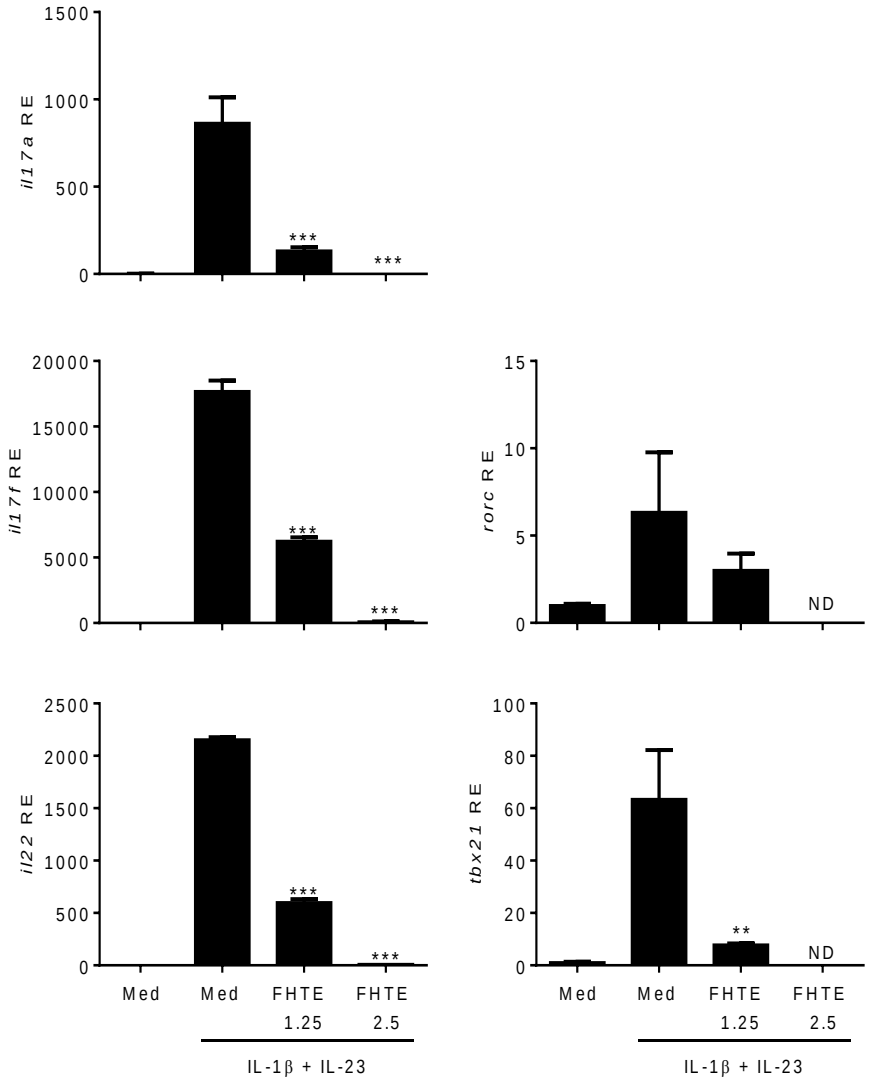

Figure 4.4 FHTE suppressed IL-1β and IL-23-induced expression of IL-17 family member genes by purified γδ T cells. Purified γδ T cells from the LNs of naïve C57BL/6 mice were incubated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 1.25 or 2.5% v/v FHTE. After 72 hr cells were lysed, total RNA was extracted and the expression of *il17a, il17f, il22, rorc* and *tbx21* was evaluated by qRT-PCR relative to medium cultured cells following normalisation by the endogenous control 18s rRNA. Results are mean ±SD for triplicate culture and are representative of three independent experiments. **p<0.01, ***p<0.001 vs IL-1β and IL-23 by one-way ANOVA with Dunnett post-test. ND: not detected.

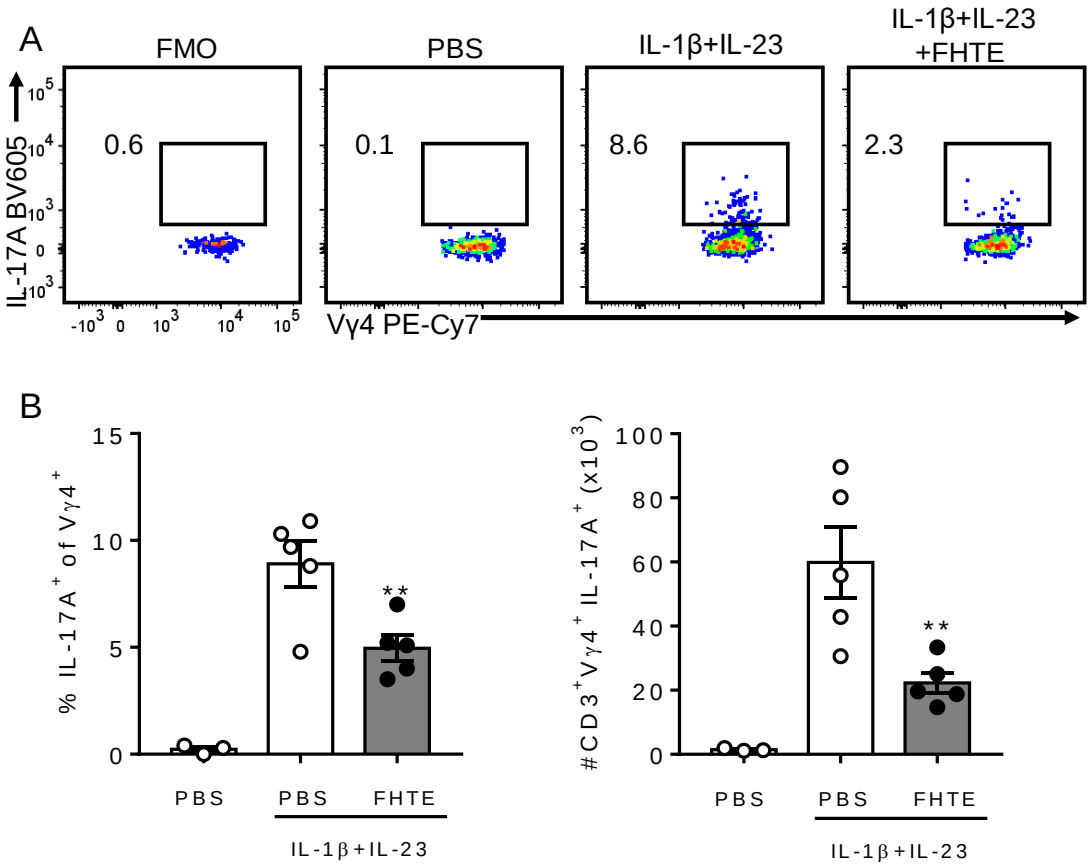

Figure 4.5 FHTE suppressed IL-1β and IL-23-induced IL-17A production by Vγ4 T cells *in vivo.* C57BL/6 mice were injected into the footpad with PBS, IL-1β (10 ng/ml) and IL-23 (10 ng/ml) or IL-1β, IL-23 and FHTE (2.5% v/v in 200 µl PBS). After 4 hr, the popliteal LNs were isolated and stained for surface CD3, Vγ4 and intracellular IL-17A. Results are representative flow cytometry plots for each treatment group (gated on $CD3^+V\gamma4^+$ cells) (A) and percentage and absolute numbers of IL-17A-producing $V\gamma4^+$ T cells (B). Data are mean ± SEM (n=5) and each symbol represents an individual mouse. **p<0.01 vs IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

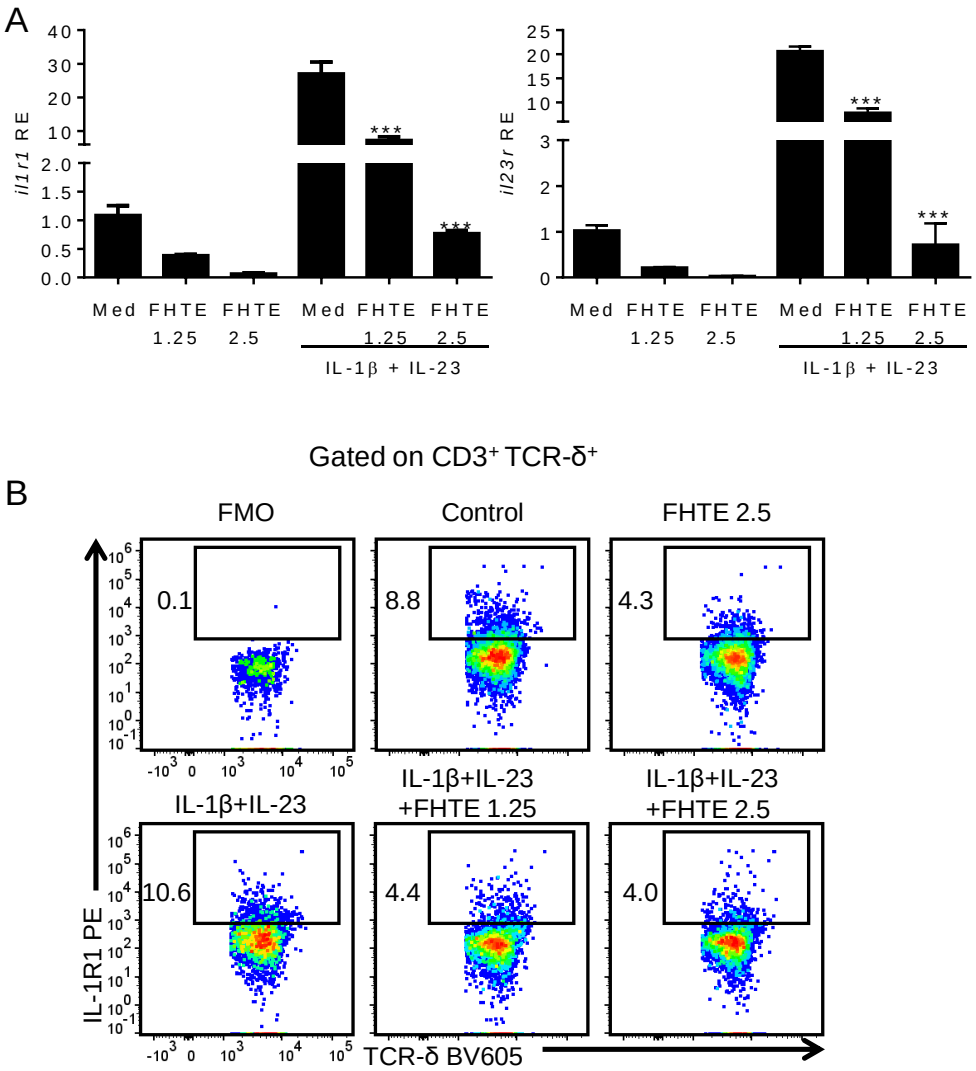

Figure 4.6 FHTE suppressed IL-1β and IL-23-induced *il1r1* and *il23r* expression on purified γδ T cells. γδ T cells were MACS purified from the LNs of naïve C57BL/6 mice and were incubated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 1.25 or 2.5% v/v FHTE. (A) After 72 hr, cells were lysed, total RNA was extracted and expression of *il1r1* and *il23r* mRNA was evaluated by qRT-PCR relative to medium cultured cells following normalisation by the endogenous control 18s rRNA. (B) Representative plots (gated on $CD3^+TCR\text{-}\delta^+$) of cells stained for surface CD3, TCR-δ and IL-1R1 and analysed by flow cytometry. Results are mean ±SD for triplicate culture and are representative of two independent experiments. ***p<0.001 vs IL-1β and IL-23 by two-way ANOVA with Tukey post-test.

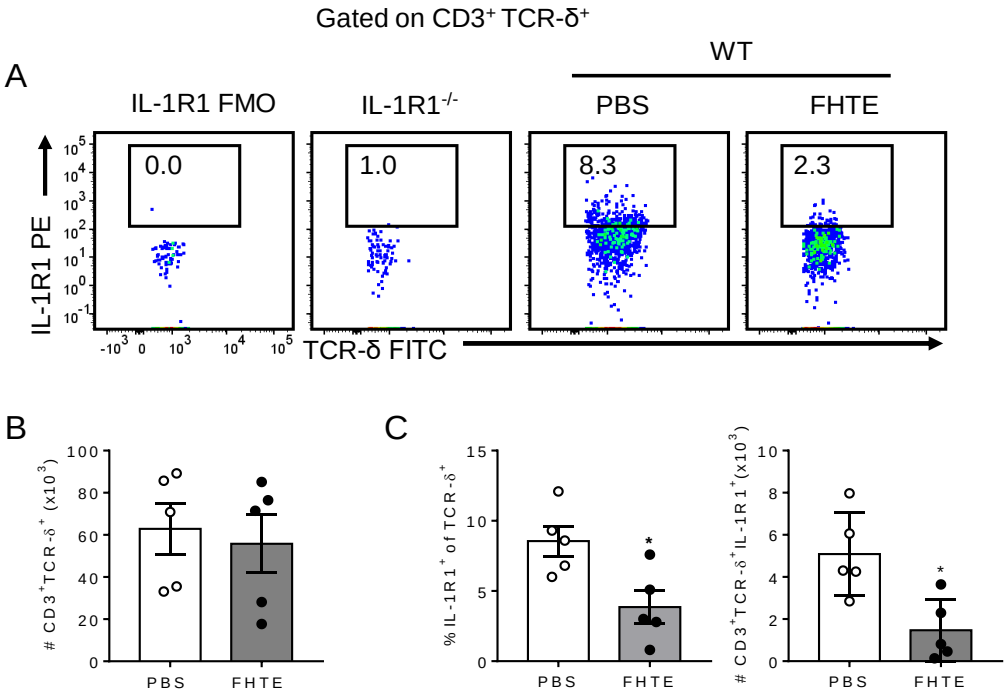

Figure 4.7 Treatment of mice with FHTE suppressed endogenous IL-1R1 expression on the surface of γδ T cells. WT C57BL/6 mice were injected i.p with PBS or FHTE (2.5% v/v in 200 µl PBS) and as a control IL-1R1$^{-/-}$ mice were injected with PBS. After 18 hr, PEC were isolated by peritoneal lavage and stained for CD3, TCR-δ and IL-1R1. Results are representative flow cytometry plots for each treatment group (gated on CD3^{+}TCR-δ^{+} cells) (A), absolute number of TCR-δ^{+} T cells (B) and percentage and absolute numbers of IL-1R1-expressing TCR-δ^{+} T cells (C). Data are mean ± SEM (n=5) and each symbol represents an individual mouse. *p<0.05 vs PBS by unpaired t test.

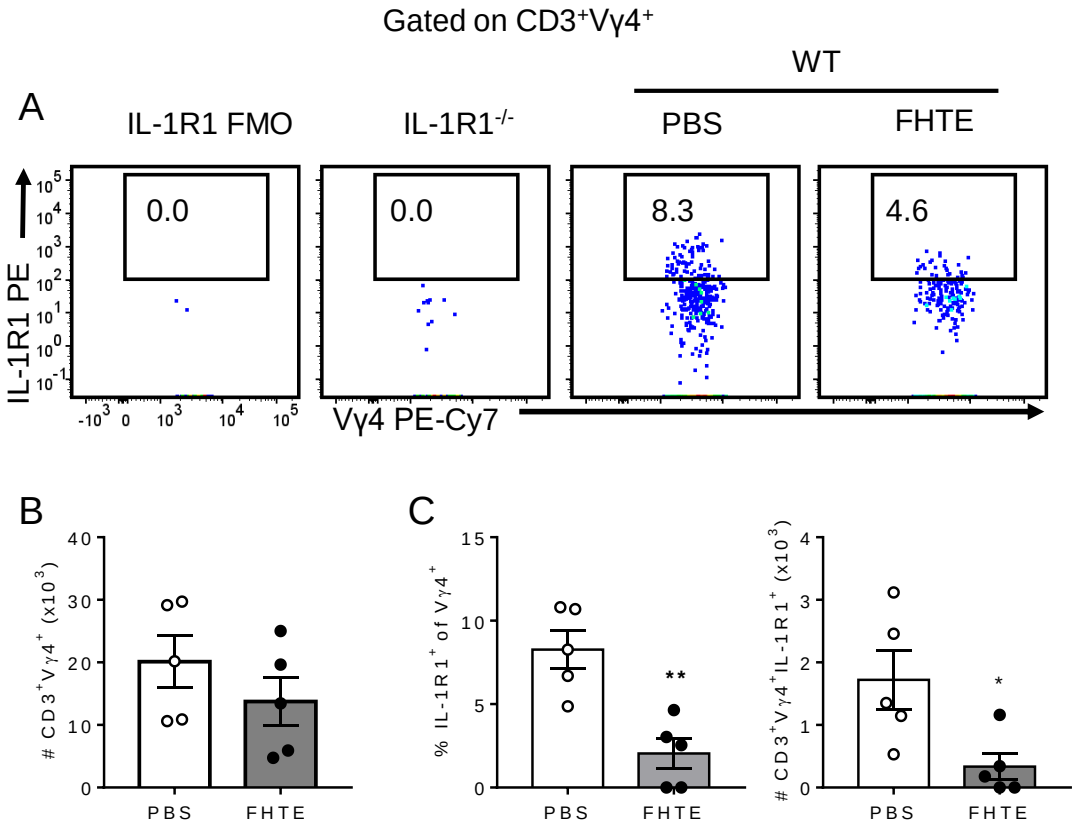

Figure 4.8 Treatment of mice with FHTE suppressed endogenous IL-1R1 expression on the surface of Vγ4 T cells. WT C57BL/6 mice were injected i.p with PBS or FHTE (2.5% v/v in 200 µl PBS) and as a control IL-1R1$^{-/-}$ mice were injected with PBS. After 18 hr, PEC were isolated by peritoneal lavage and stained for CD3, Vγ4 and IL-1R1. Results are representative flow cytometry plots for each treatment group (gated on CD3^{+}Vγ4^{+} cells) (A), absolute number of Vγ4^{+} T cells (B) and percentage and absolute numbers of IL-1R1-expressing Vγ4^{+} T cells (C). Data are mean ± SEM (n=5) and each symbol represents an individual mouse. *p<0.05, **p<0.01 vs PBS by unpaired t test.

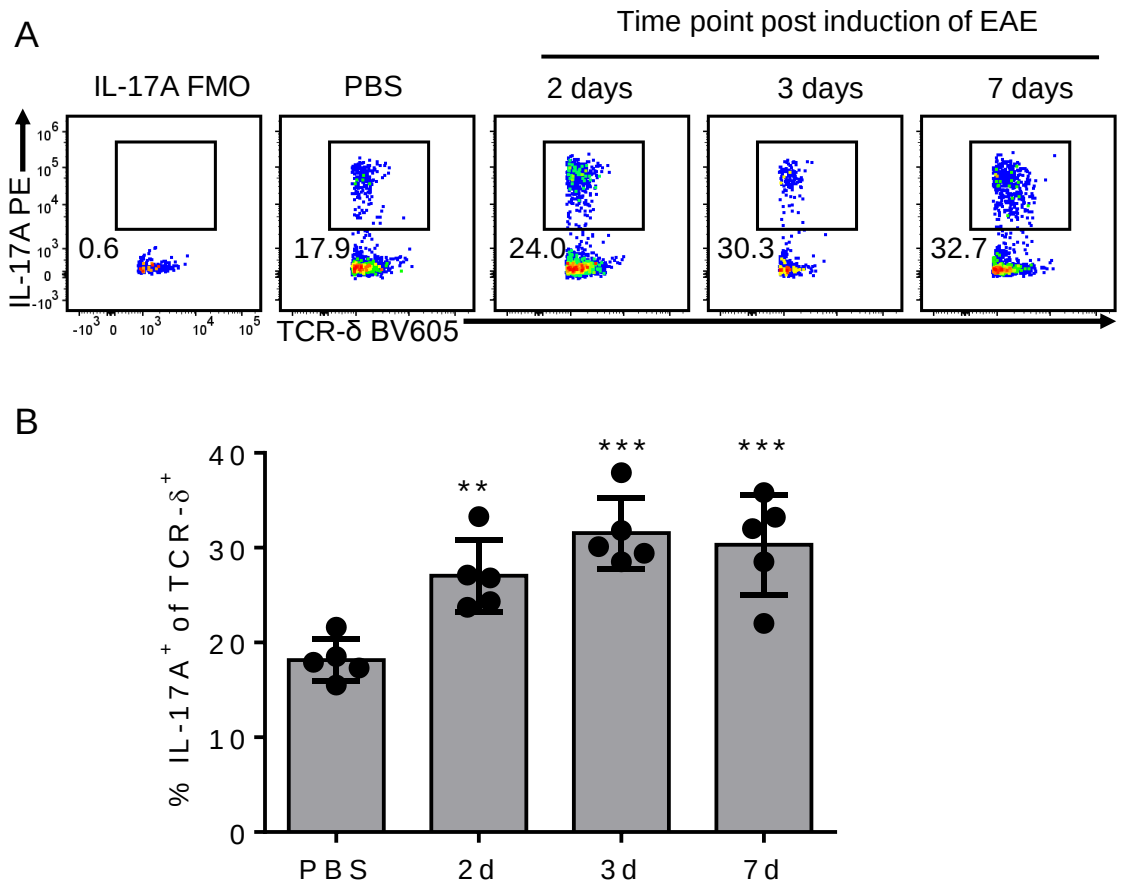

Figure 4.9 IL-17A production by γδ T cells peaks on day 3 post induction of EAE. C57BL/6 mice were injected s.c with PBS, or MOG (100 µg) emulsified in CFA followed by i.p injection of PT (100 ng) on day 0 and day 2. Mice were sacrificed 2, 3 or 7 days post induction and their LNs were stained for CD3 and TCR-δ and intracellular IL-17A. Results are representative flow cytometry plots for each time point (gated on $CD3^+TCR\text{-}\delta^+$ cells) (A) and percentage and absolute numbers of IL-17A-producing TCR-δ^+ T cells (B). Data are mean ± SEM (n=5) and each symbol represents an individual mouse. **p<0.01, ***p<0.001 vs PBS by one-way ANOVA with Dunnett post-test.

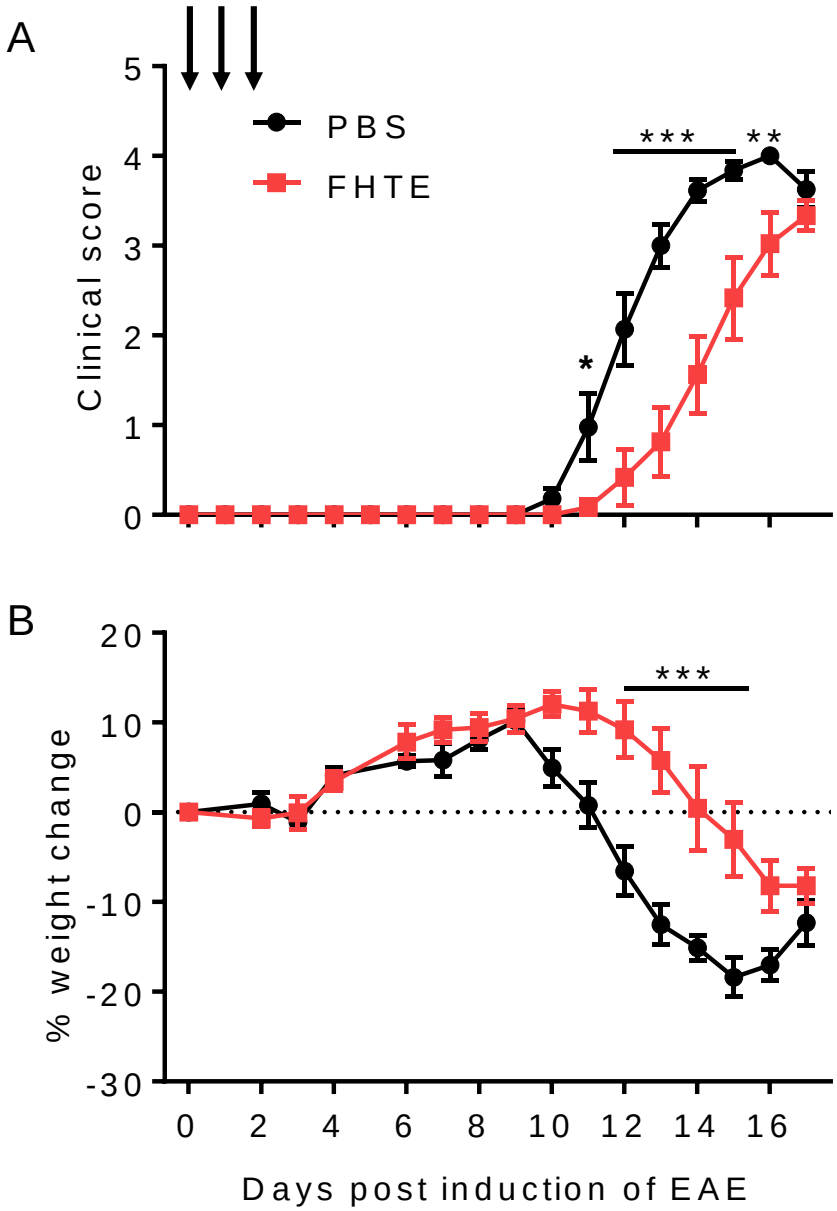

Figure 4.10 Early administration of mice with FHTE delays the onset of EAE. EAE was induced by s.c injection of MOG (100 µg) peptide emulsified in CFA, followed by i.p injection of PT (100 ng) on day 0 and day 2. Mice were injected s.c with FHTE (2.5% v/v in 200 µl PBS) 2 hours before induction, day 1 and day 2 post induction. Mice were assessed daily for clinical signs of the disease and weighed. Results are mean clinical score (A) and percentage body weight change (B). Data are mean ± SEM (n=12). Results are combined from two separate experiments. *p<0.05, **p<0.01, ***p<0.001 vs PBS by repeated measures ANOVA with Sidak post-test.

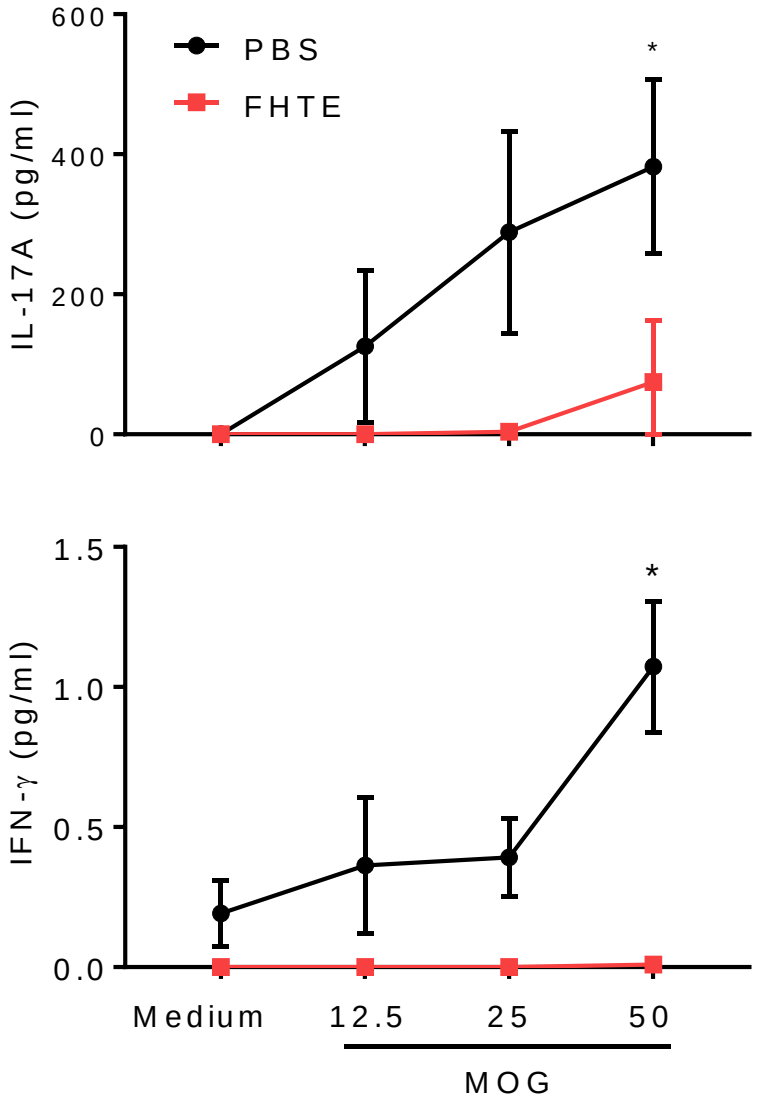

Figure 4.11 MOG-specific production of IL-17A and IFN-γ from spleen and LN cells is reduced in FHTE-treated mice with EAE. EAE was induced as describe in Fig 4.10 and mice were injected s.c with FHTE (2.5% v/v in 200 µl PBS) 2 hours before induction, day 1 and day 2 post induction. On day 7 after induction, mice were sacrificed and cells were isolated from the spleen and the inguinal, brachial and axillary LNs. Cells were restimulated with increasing concentrations of MOG (12.5, 25 and 50 µg/ml). After 72 hr, supernatants were collected and the concentrations of IL-17A and IFN-γ quantified by ELISA. Data are mean ± SEM (n=6). *p<0.05 vs PBS by two-way ANOVA with Sidak post-test.

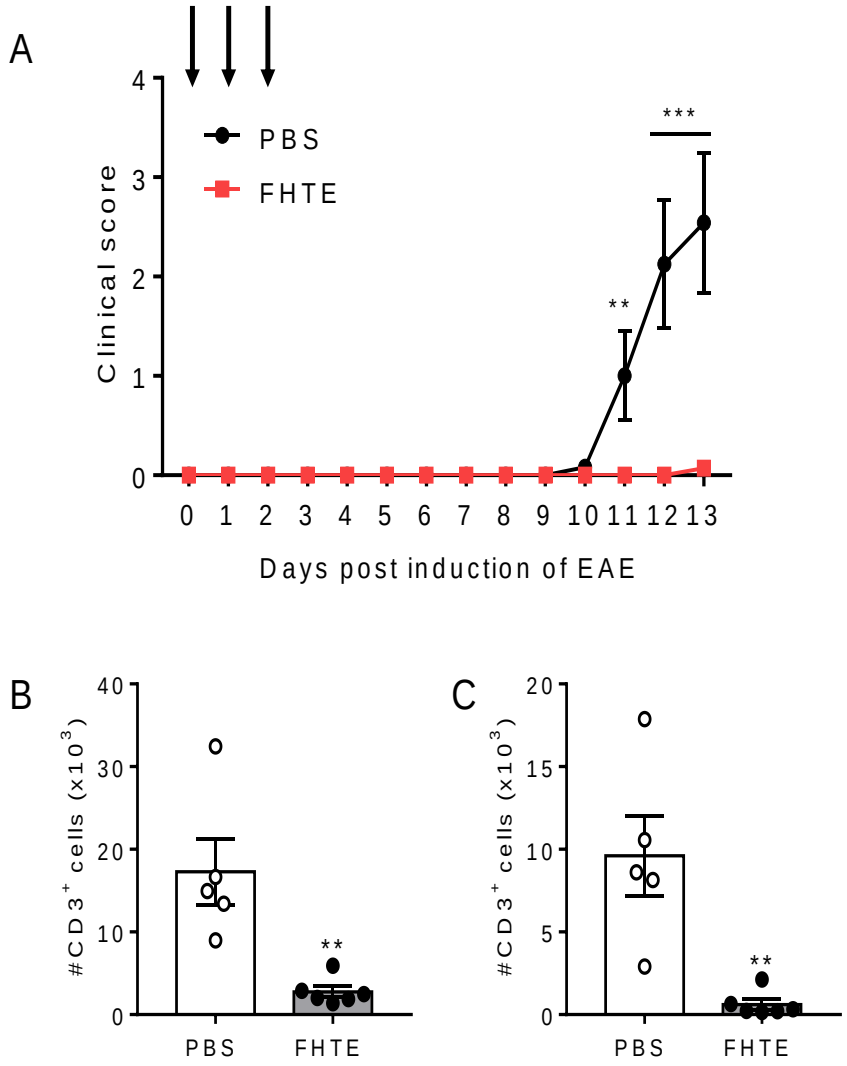

Figure 4.12 Early administration with FHTE suppressed the number of CD3 T cells in the brain and spinal cord in mice with EAE on day 13. EAE was induced as described in Fig 4.10 and mice were injected s.c with FHTE (2.5% v/v in 200 µl PBS) 2 hours before induction, day 1 and day 2 post induction. On day 13 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the brain and spinal cord and stained for surface CD3 and analysed by flow cytometry. Results are mean clinical score (A), absolute number of $CD3^+$ T cells in the brain (B) and spinal cord (C). Data are mean ± SEM (n=5 (PBS), n=6 (FHTE)) and each symbol represents an individual mouse. **p<0.01, ***p<0.001 vs PBS by repeated measures ANOVA with Sidak post-test (clinical score) or unpaired t test (absolute number).

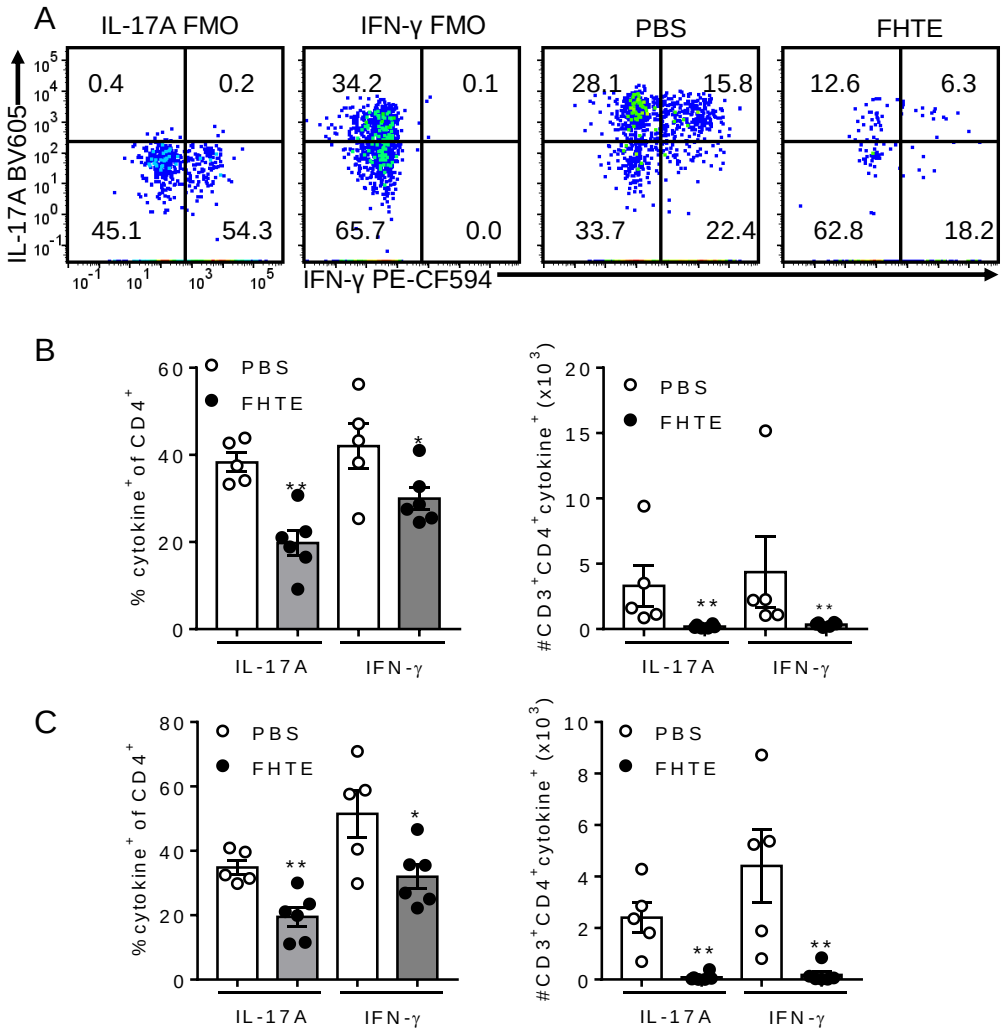

Figure 4.13 FHTE-treated mice have significantly reduced numbers of IL-17A and IFN-γ-producing CD4 T cells in the brain and spinal cord on day 13 after the induction of EAE. EAE was induced as described in Fig 4.10 and mice were injected s.c with FHTE (2.5% v/v in 200 µl PBS) 2 hours before induction, day 1 and day 2 post induction. On day 13 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the brain and spinal cord and stained for surface CD3 and CD4 and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are representative FACS plots from the brain (gated on $CD3^+CD4^+$ T cells) (A), percentage and absolute number of IL-17A and IFN-γ-producing $CD4^+$ T cells in the brain (B) and spinal cord (C). Data are mean ± SEM (n=5 (PBS) n=6 (FHTE)) and each symbol represents an individual mouse. *p<0.05, **p<0.01 vs PBS by unpaired t test.

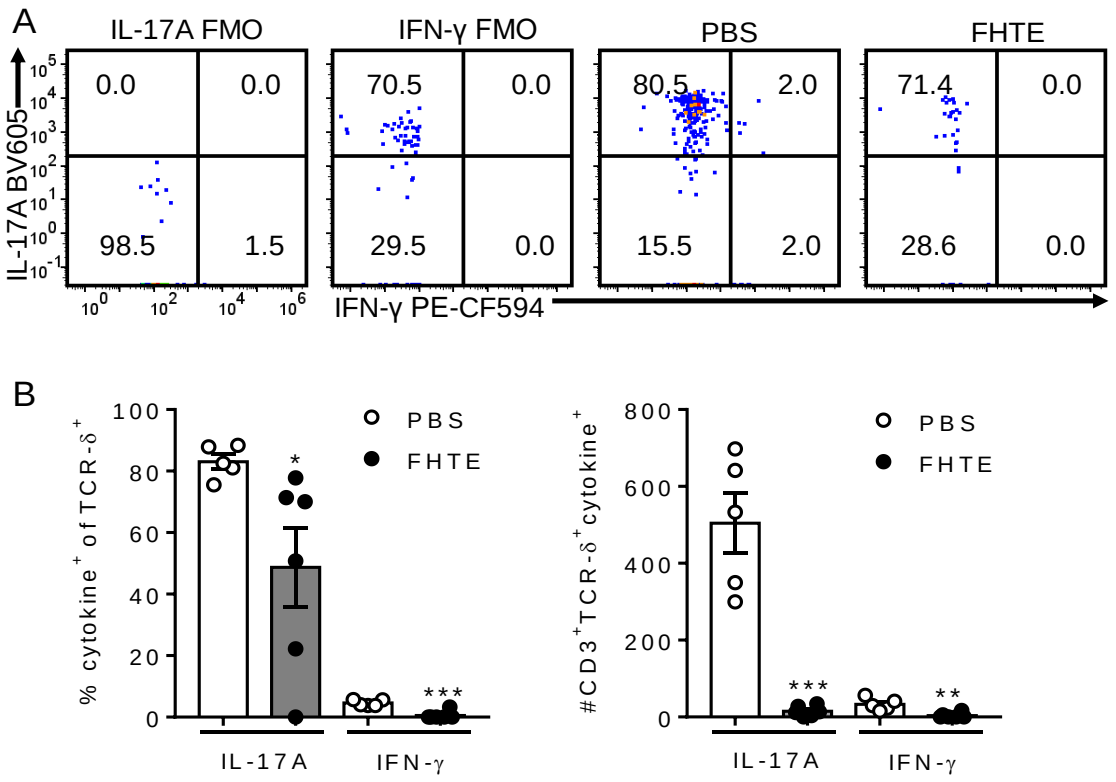

Figure 4.14 FHTE-treated mice have significantly reduced numbers of IL-17A and IFN-γ-producing γδ T cells in the spinal cord on day 13 of EAE. EAE was induced as described in Fig 4.10 and mice were injected s.c with FHTE (2.5% v/v in 200 µl PBS) 2 hours before induction, day 1 and day 2 post induction. On day 13 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the spinal cord and stained for surface CD3 and TCR-δ and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+TCR\text{-}\delta^+$ cells) (A) and percentage and absolute number of IL-17A and IFN-γ-producing $TCR\text{-}\delta^+$ T cells (B). Data are mean ± SEM (n=5 (PBS) n=6 (FHTE)) and each symbol represents an individual mouse. *p<0.05, **p<0.01, ***p<0.001 vs PBS by unpaired t test.

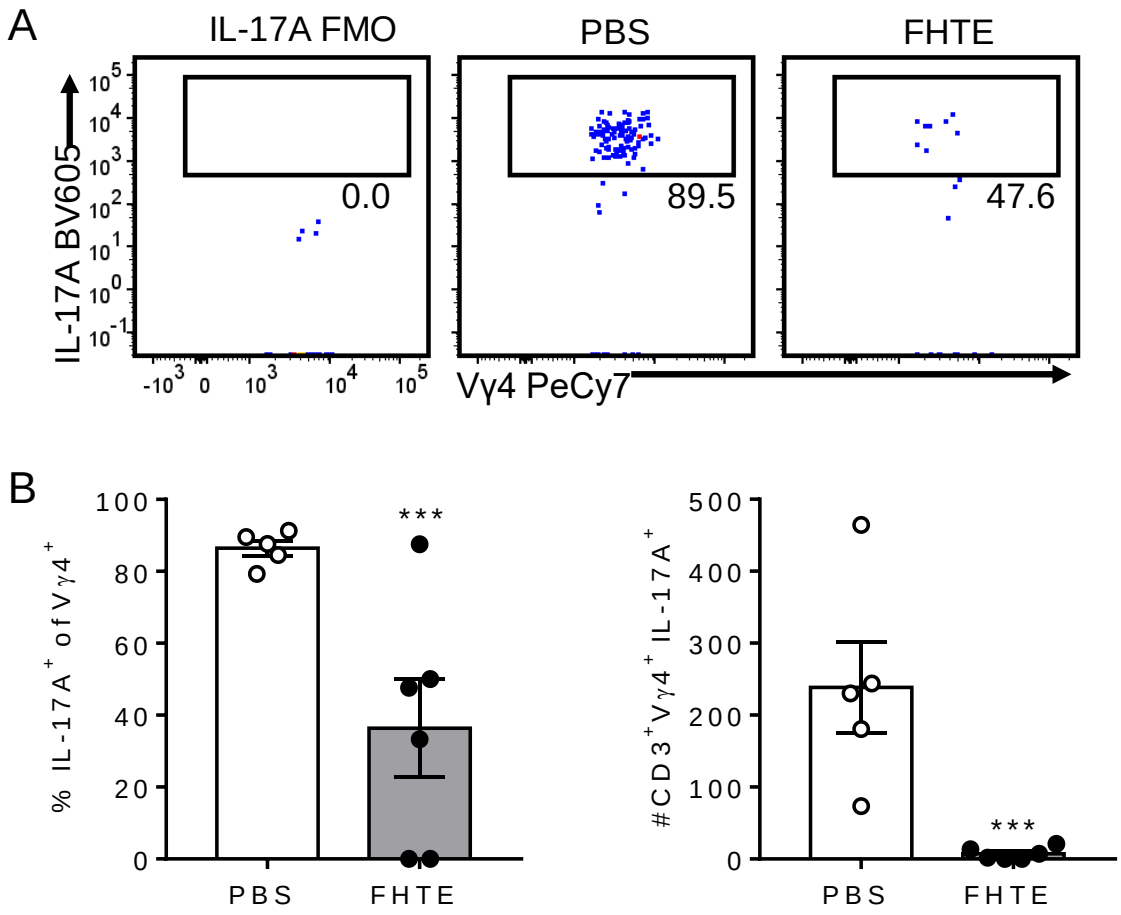

Figure 4.15 FHTE-treated mice have significantly reduced numbers of IL-17A-producing Vγ4 T cells in the spinal cord on day 13 of EAE. EAE was induced as described in Fig 4.10 and mice were injected s.c with FHTE (2.5% v/v in 200 µl PBS) 2 hours before induction, day 1 and day 2 post induction. On day 13 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the spinal cord and stained for surface CD3 and Vγ4 and intracellular IL-17A and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+Vγ4^+$ cells) (A) and percentage and absolute number of IL-17A-producing $Vγ4^+$ T cells (B). Data are mean ± SEM (n=5 (PBS) n=6 (FHTE)) and each symbol represents an individual mouse. ***p<0.001 vs PBS by unpaired t test.

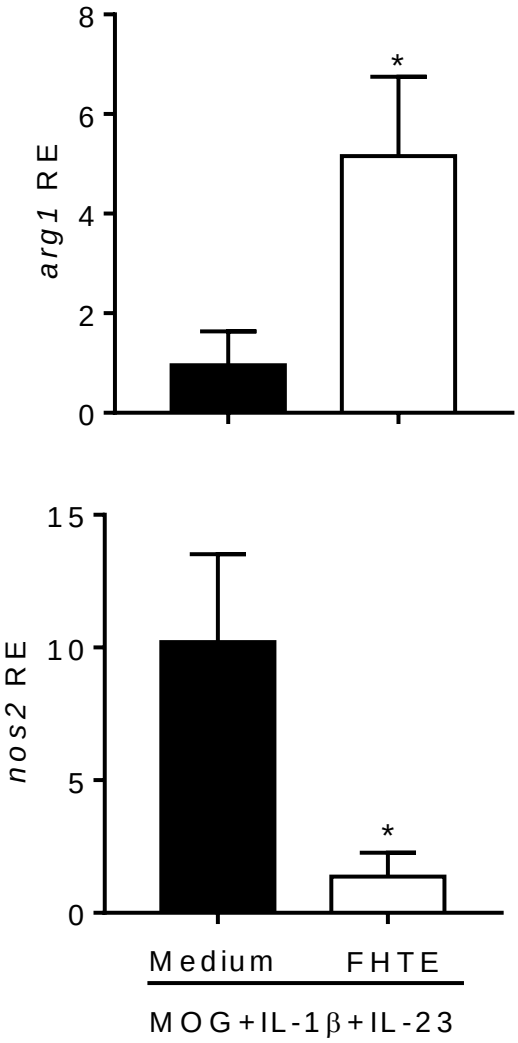

Figure 4.16 FHTE-stimulation of spleen and LN cells from mice with EAE *in vitro* enhanced *arg1* and reduced *nos2* expression. EAE was induced in donor C57BL/6 mice by immunization with MOG (100 µg) emulsified in CFA. Donor mice were scarified on day 10 post-induction and their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of FHTE (2.5% v/v). After 24 hr cells were lysed, total RNA was extracted and expression of *arg1* was evaluated by qRT-PCR relative to MOG, IL-1β and IL-23 stimulated cells, whereas *nos2* expression was evaluated relative to FHTE stimulation following normalisation by the endogenous control 18s rRNA. Results are mean ±SD for triplicate culture and are representative of two independent experiments. *p<0.05 vs MOG, Il-1β and IL-23 by unpaired t test.

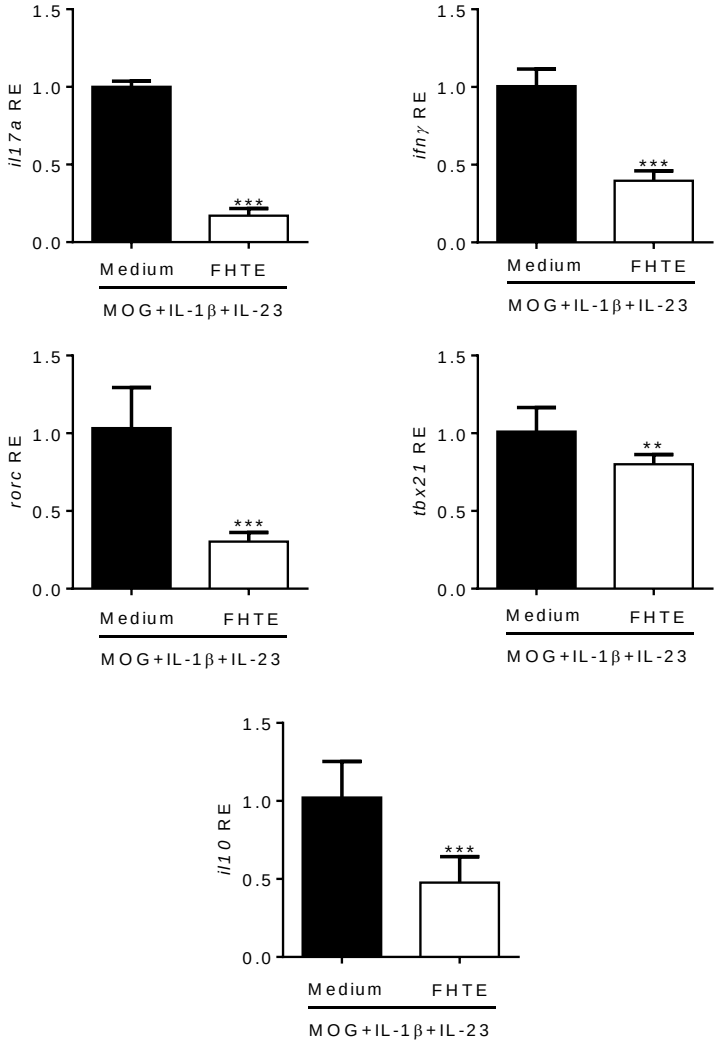

Figure 4.17 FHTE-stimulation of spleen and LN cells from mice with EAE *in vitro* reduced *il17a* and *ifnγ* mRNA expression. EAE was induced in donor C57BL/6 mice as described in Fig 4.16 and after 10 days their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE. After 72 hr cells were lysed, total RNA was extracted and expression of *il17a, ifnγ, rorc, tbx21* and *il10* was evaluated by qRT-PCR relative to MOG, IL-1β and IL-23 stimulated cells following normalisation by the endogenous control 18s rRNA. Results are mean ±SD for triplicate culture and are representative of three independent experiments. **p<0.01, ***p<0.001 vs MOG, IL-1β and IL-23 by unpaired t test.

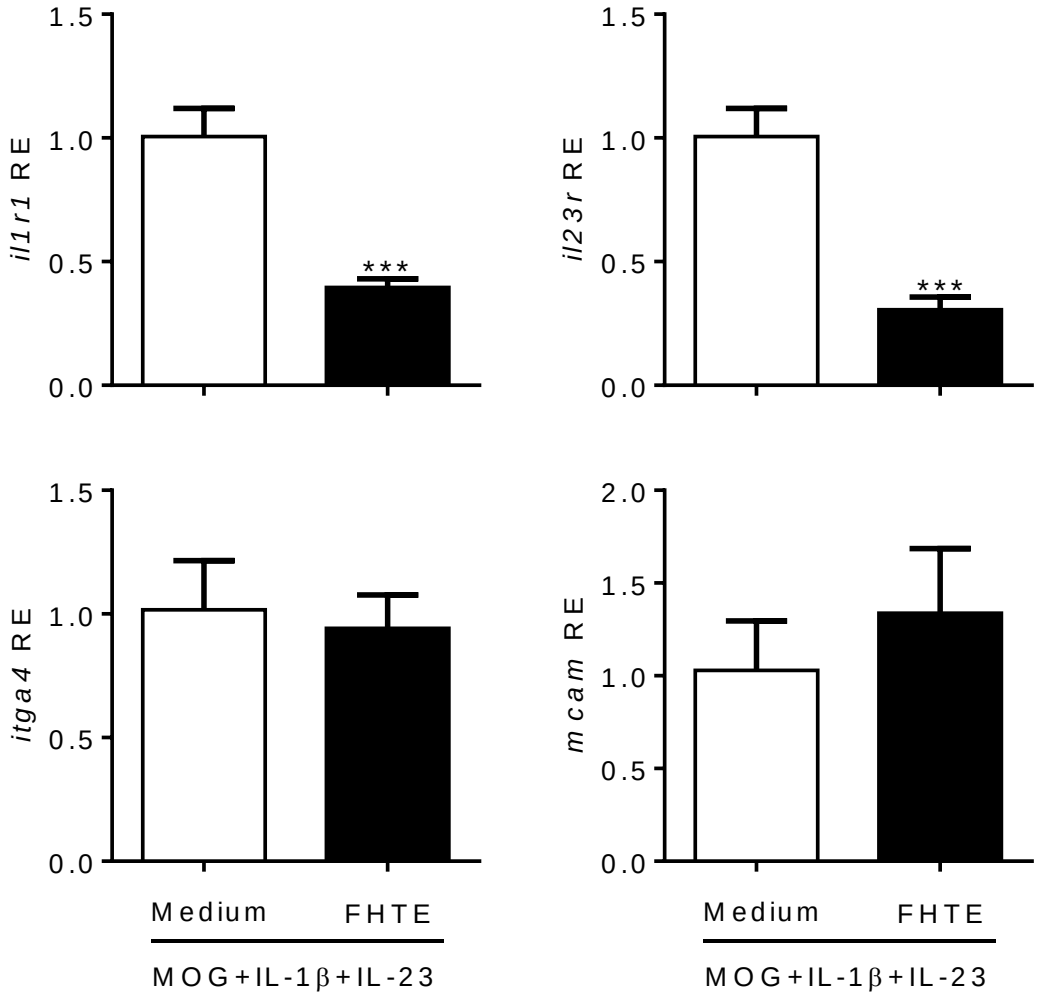

Figure 4.18 FHTE-stimulation of spleen and LN cells from mice with EAE *in vitro* suppressed mRNA expression of *il1r1* and *il23r* expression but not *mcam* and *itga4.* EAE was induced in donor C57BL/6 mice as described in Fig 4.16 and after 10 days their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE. After 72 hr cells were lysed, total RNA was extracted and expression of *il1r1*, *il23r, mcam* and *itag4* was evaluated by qRT-PCR relative to MOG, IL-1β and IL-23 stimulated cells following normalisation by the endogenous control 18s rRNA. Results are mean ±SD for triplicate culture and are representative of three independent experiments. ***p<0.001 vs MOG, IL-1β and IL-23 by unpaired t test.

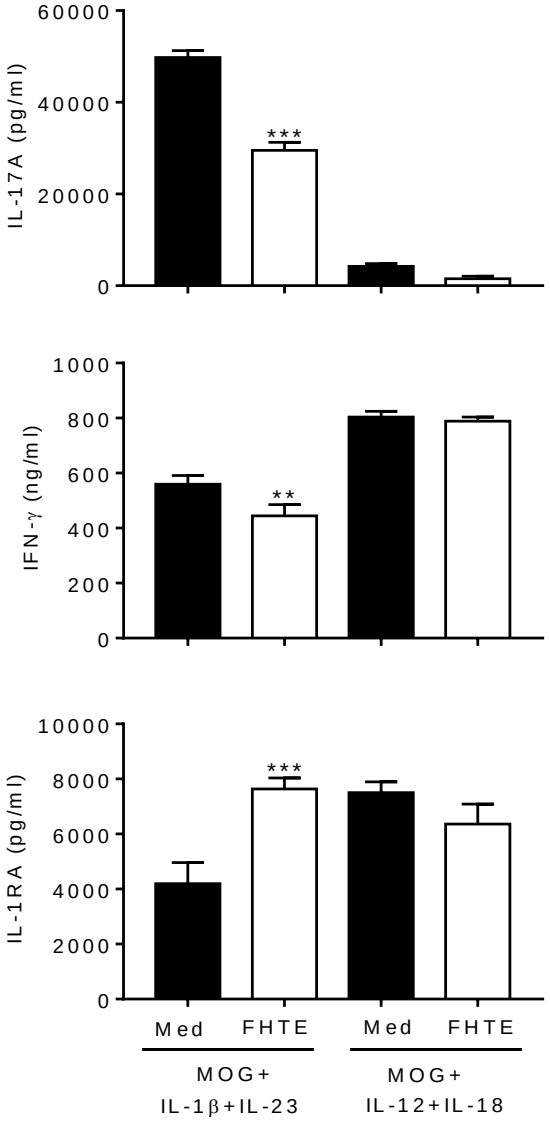

Figure 4.19 FHTE inhibits IL-1β and IL-23, but not IL-12 and IL-18-induced IL-17A and IFN-γ production by spleen and LN cells from mice with EAE. EAE was induced in donor C57BL/6 mice as described in Fig 4.16 and after 10 days their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) or IL-12 (10 ng/ml) and IL-18 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE. After 72 hr the concentration of IL-17A, IFN-γ and IL-1RA was determined by ELISA. Results are mean ±SD for triplicate culture and are combined from two separate experiments. **p<0.01, ***p<0.001 vs MOG, IL-1β and IL-23 by unpaired t test.

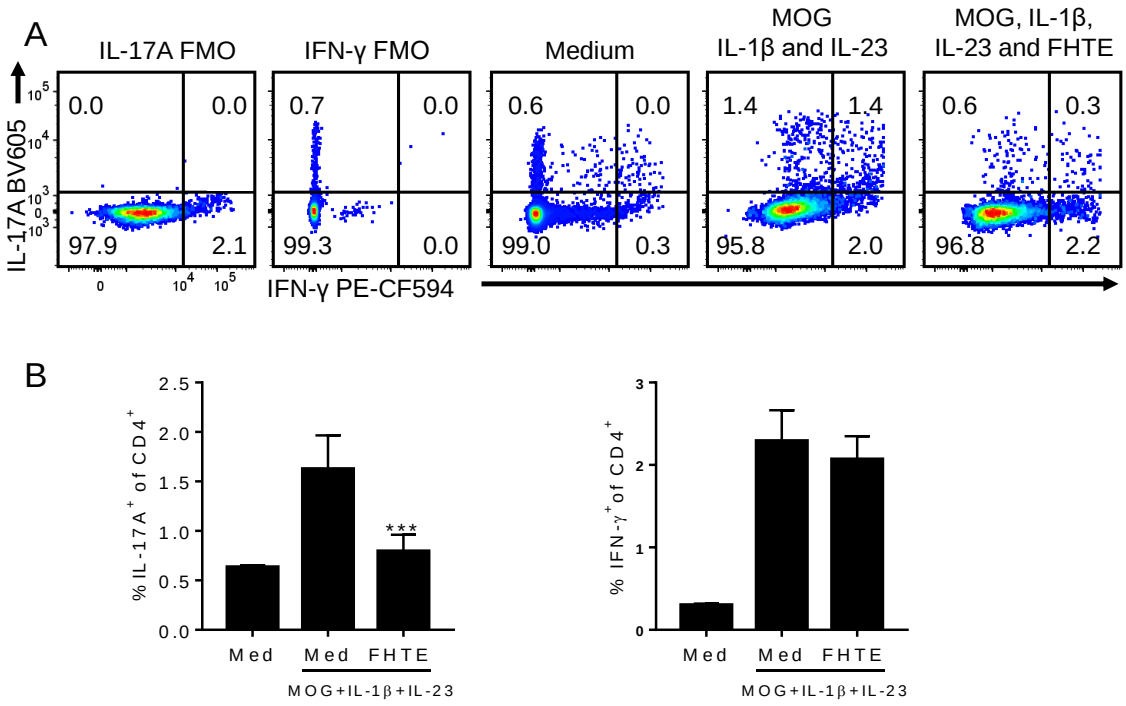

Figure 4.20 Culture of spleen and LN cells from mice with EAE with FHTE *in vitro* suppressed IL-17A production by CD4 T cells. EAE was induced in donor C57BL/6 mice as described in Fig 4.16 and after 10 days their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE. After 72 hr cells were stained for surface CD3 and CD4 and intracellular IL-17A and IFN-γ. Results are representative FACS plots (gated on $CD3^+CD4^+$ cells) (A) and percentage of IL-17A and IFN-γ-producing $CD4^+$ T cells (B). Data are mean ±SD for triplicate culture and are combined from three separate experiments. ***p<0.001 vs MOG, IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

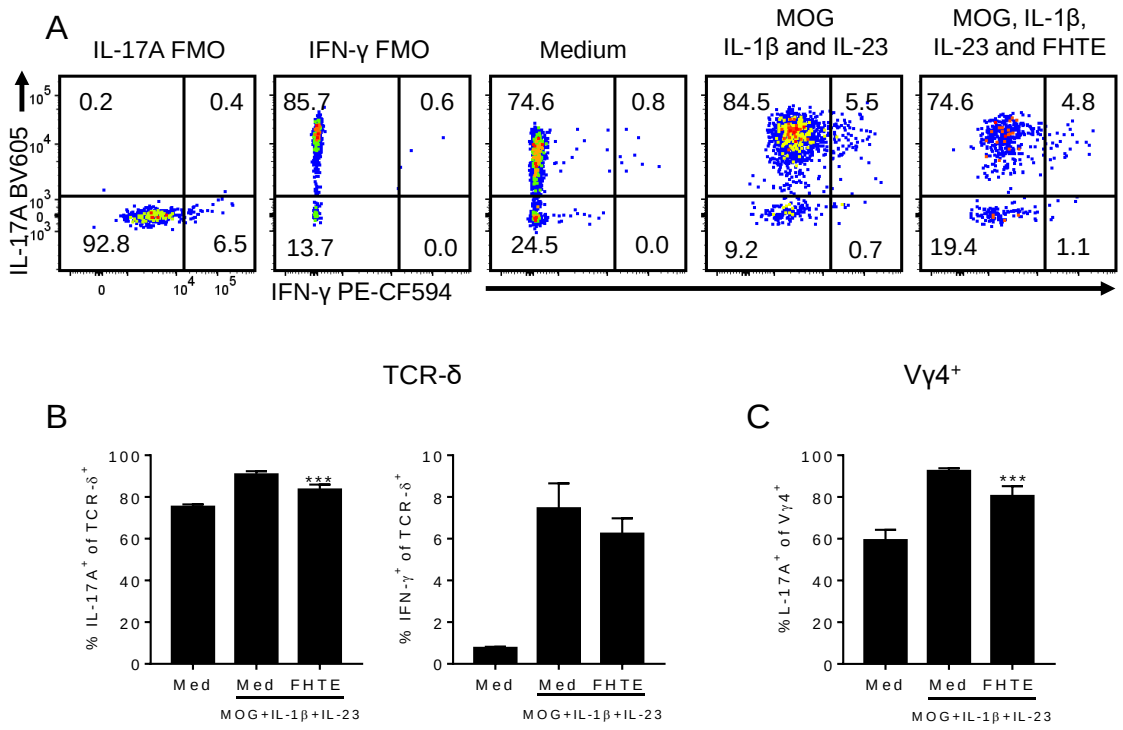

Figure 4.21 Culture of spleen and LN cells from mice with EAE with FHTE *in vitro* suppressed IL-17A production by γδ and Vγ4 T cells. EAE was induced in donor C57BL/6 mice as described in Fig 4.16 and after 10 days their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE. After 72 hr cells were stained for surface CD3, TCR-δ and Vγ4 and intracellular IL-17A and IFN-γ. Results are representative FACS plots (gated on $CD3^+TCR\text{-}\delta^+$ cells) (A), percentage of IL-17A and IFN-γ-producing $TCR\text{-}\delta^+$ T cells (B) and percentage of IL-17A-producing $V\gamma4^+$ T cells (C). Data are mean ±SD for triplicate culture and are combined from three separate experiments. ***p<0.001 vs MOG, IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

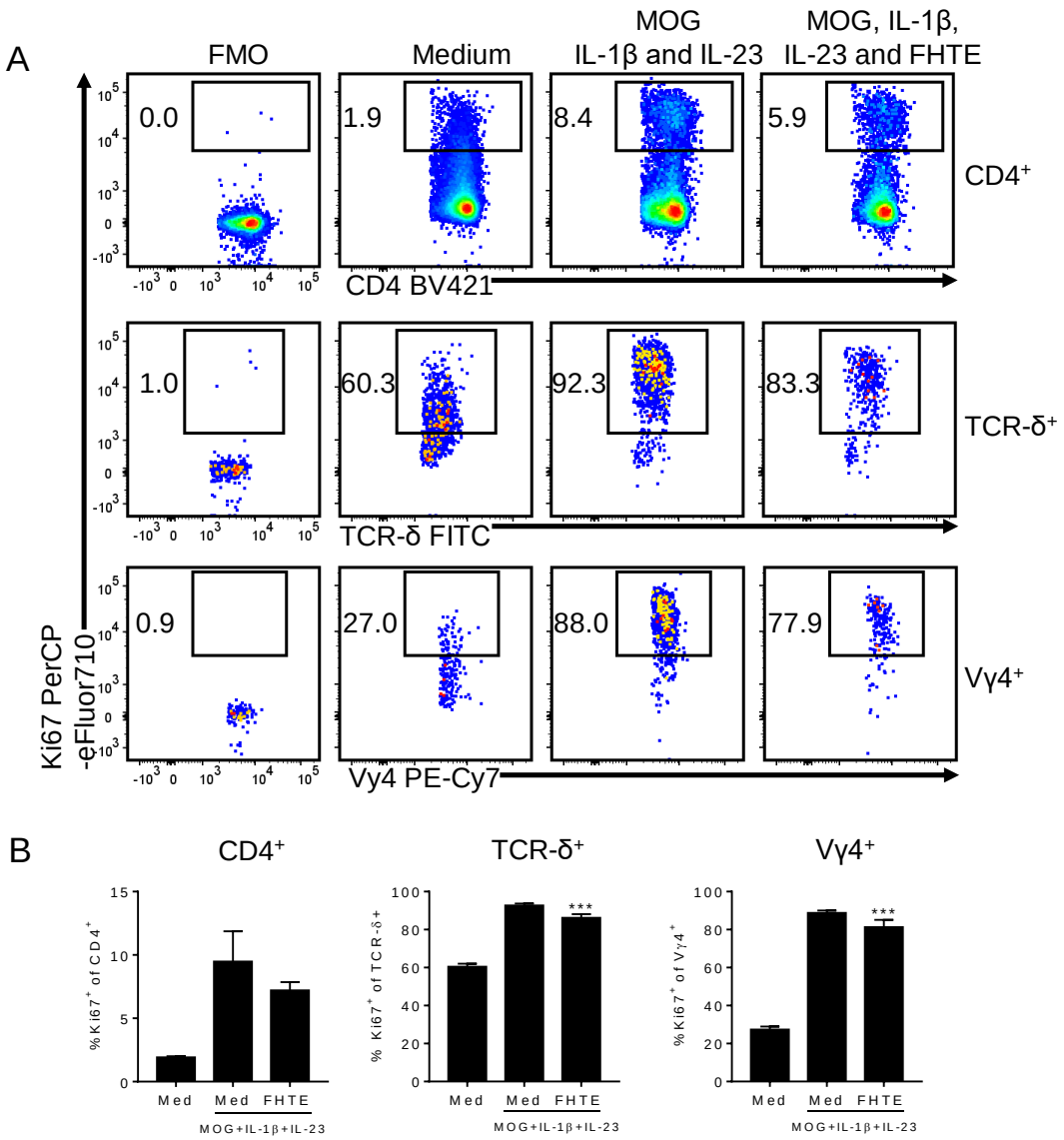

Figure 4.22 Culture of spleen and LN cells from mice with EAE with FHTE *in vitro* suppressed proliferation of γδ and Vγ4 T cells. EAE was induced in donor C57BL/6 mice as described in Fig 4.16 and after 10 days their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE. After 72 hr cells were stained for surface CD3, CD4, TCR-δ and Vγ4 and intranuclear Ki67. Results are representative FACS plots (A) and percentage Ki67$^+$ of CD4$^+$, TCR-δ$^+$ or Vγ4$^+$ T cells (B). Data are mean ±SD for triplicate culture and are combined from two separate experiments. ***p<0.001 vs MOG, IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

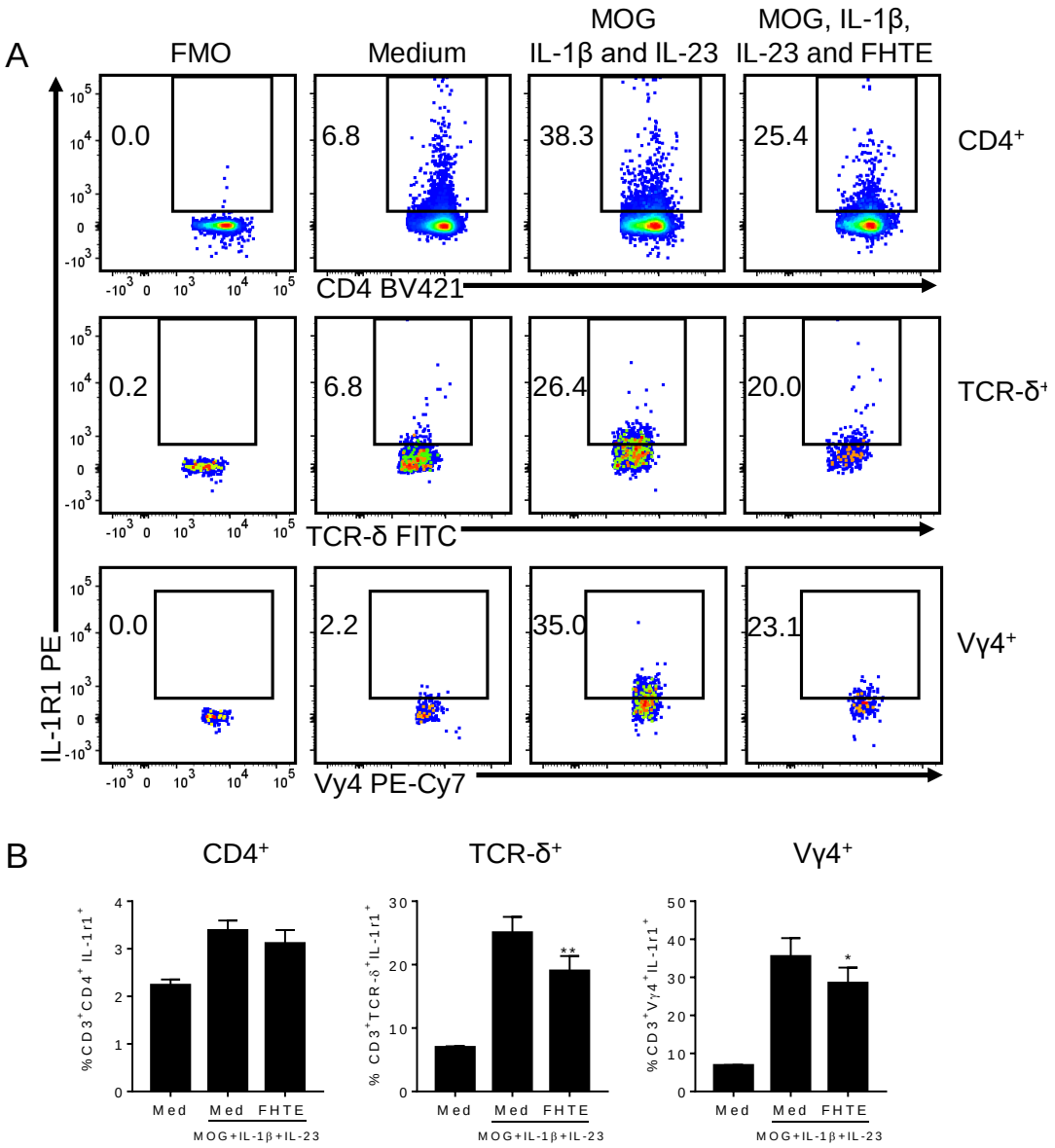

Figure 4.23 Culture of spleen and LN cells from mice with EAE with FHTE *in vitro* suppressed expression of IL-1R1 on γδ and Vγ4 T cells. EAE was induced in donor C57BL/6 mice as described in Fig 4.16 and after 10 days their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE. After 72 hr cells were stained for surface CD3, CD4, TCR-δ, Vγ4 and IL-1R1. Results are representative FACS plots (A) and percentage IL-1R1$^+$ of CD4$^+$, TCR δ$^+$ or Vγ4$^+$ T cells (B). Data are mean ±SD for triplicate culture and are combined from two separate experiments. *p<0.05, **p<0.01 vs MOG, IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

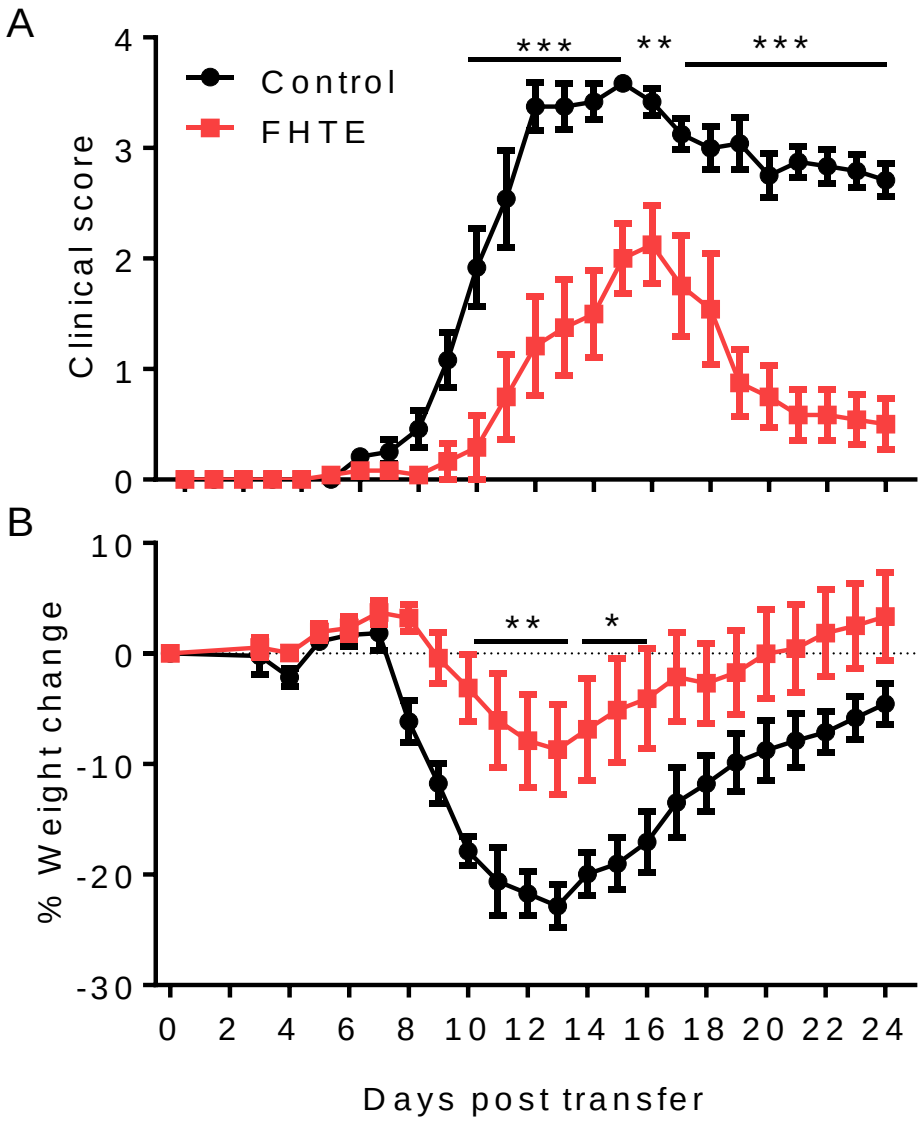

Figure 4.24 FHTE attenuates EAE induced by T cell transfer. EAE was induced in donor C57BL/6 mice as described in Fig 4.16 and after 10 days their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE. After 72 hr, cells were washed and injected i.p into naïve C57BL/6 recipient mice ($10x10^6$ cells per mouse). Mice were assessed daily for clinical signs of disease and weighed. Results are mean clinical score (A) and percentage body weight change (B). Data are mean ± SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 vs control by repeated measures ANOVA with Sidak post-test.

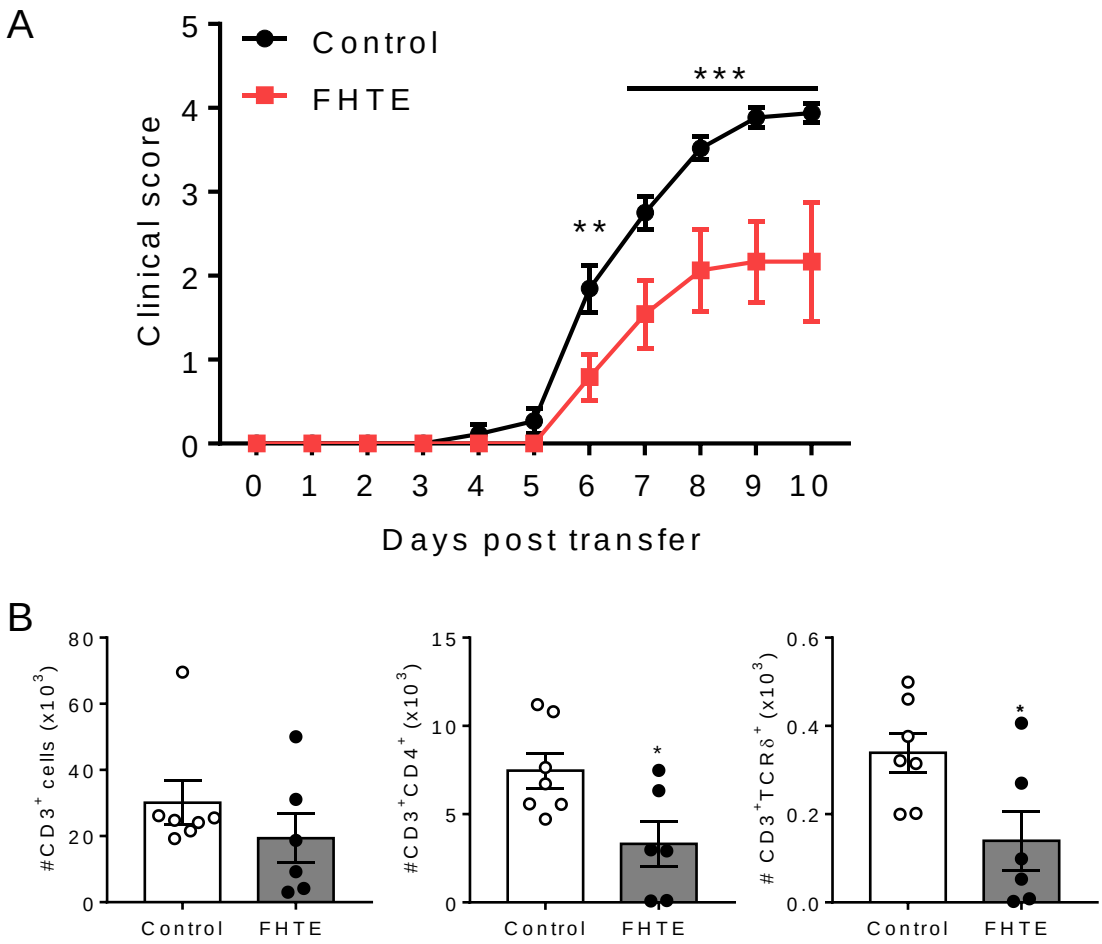

Figure 4.25 FHTE-treatment of donor spleen and LN cells prevents the induction of EAE by passive transfer and suppresses infiltration of CD4 and γδ T cells into the brain. Passive EAE was induced in mice as described in Fig 4.24. After 10 days their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE for 72 hr before being transferred to naïve C57BL/6 recipient mice ($10x10^6$ cells per mouse). On day 10 after transfer, mice were sacrificed and perfused. Cells were isolated from the brain and stained for surface CD3, CD4 and TCR-δ and analysed by flow cytometry. (A) Results are mean clinical score ± SEM (n=13 (control), n=12 (FHTE)). **p<0.01, ***p<0.001 vs control by repeated measures ANOVA with Sidak post-test. (B) Absolute number of $CD3^+$, $CD4^+$ and TCR-$δ^+$ T cells in the brain, mean ± SEM (n=7 (control), n=6 (FHTE)). Each symbol represents an individual mouse. *p<0.05 vs control unpaired t test.

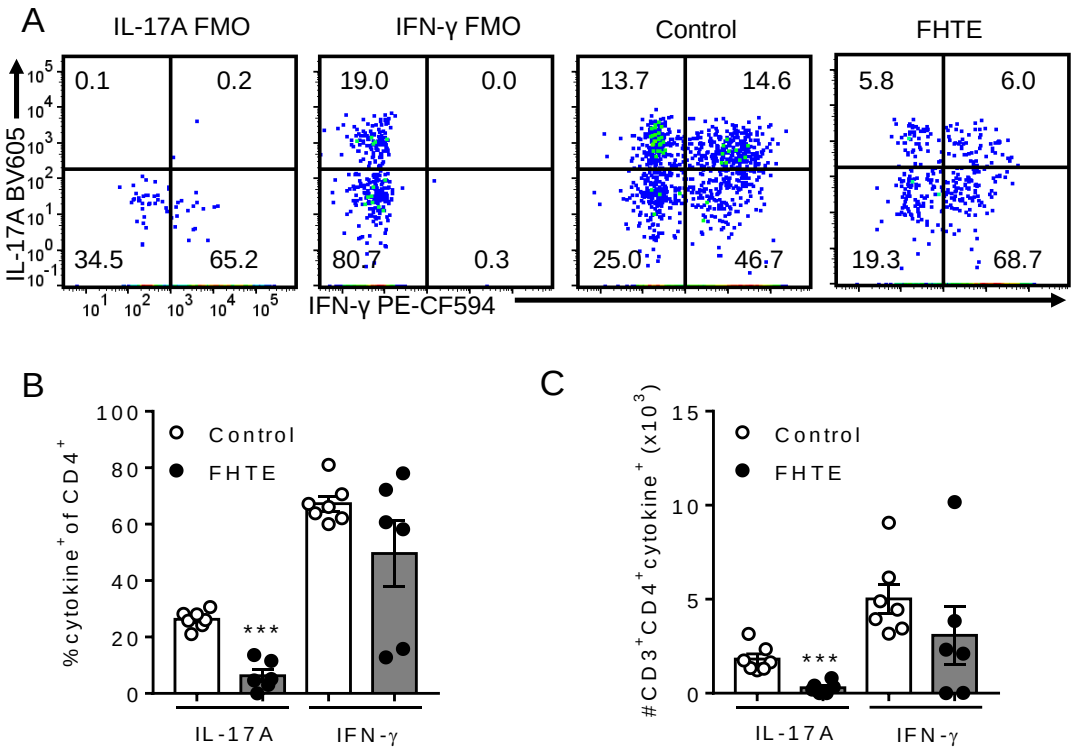

Figure 4.26 FHTE-stimulation of spleen and LN cells from EAE mice *in vitro* suppressed the percentage and absolute number of IL-17A-producing CD4 T cells in the brain on day 10 post transfer. Passive EAE was induced in mice as described in Fig 4.24. After 10 days their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE for 72 hr before being transferred to naïve C57BL/6 recipient mice (10x10^6 cells per mouse). On day 10 after transfer, mice were sacrificed and perfused. Cells were isolated from the brain and stained for surface CD3 and CD4 and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are representative FACS plots (gated on CD3$^+$CD4$^+$ cells) (A), percentage of IL-17A and IFN-γ-producing CD4$^+$ T cells (B) and absolute number of IL-17A and IFN-γ-producing CD4$^+$ T cells (C). Data are mean ± SEM (n=7 (control), n=6 (FHTE)) and each symbol represents an individual mouse. ***p<0.001 vs control by unpaired t test**.**

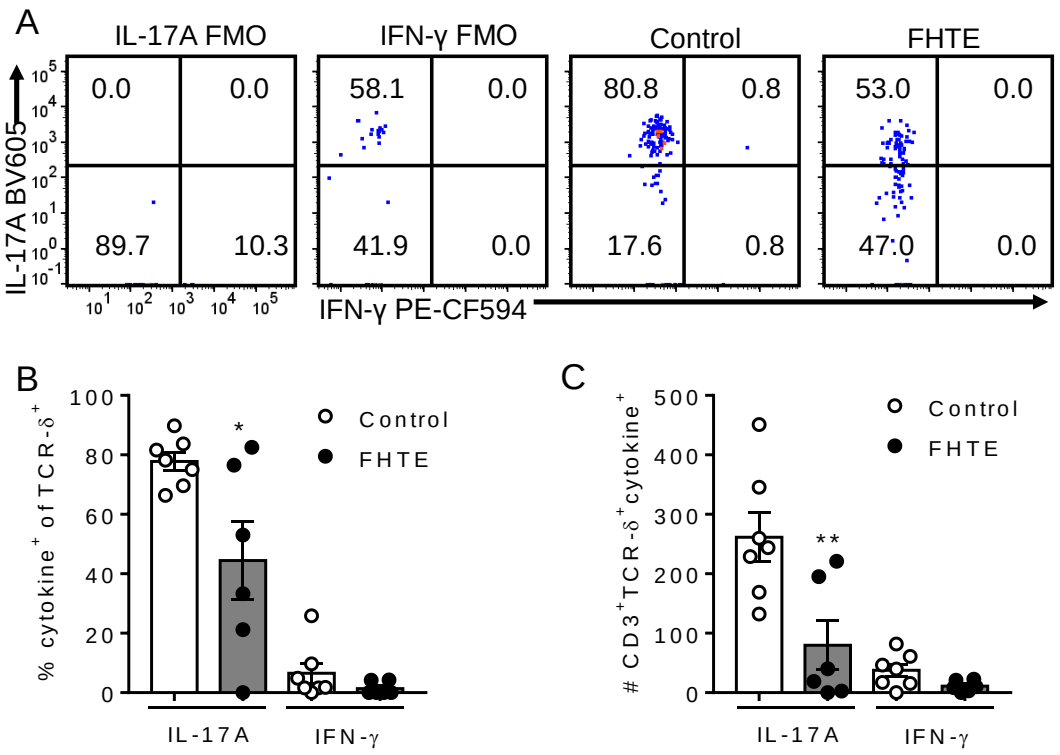

Figure 4.27 FHTE-stimulation of spleen and LN cells from EAE mice *in vitro* suppressed the percentage and absolute number of IL-17A-producing γδ T cells in the brain on day 10 post transfer. Passive EAE was induced in mice as described in Fig 4.24. After 10 days their spleen and LN cells were reactivated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE for 72 hr before being transferred to naïve C57BL/6 recipient mice ($10x10^6$ cells per mouse). On day 10 after transfer, mice were sacrificed and perfused. Cells were isolated from the brain and stained for surface CD3 and TCR-δ and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+TCR\text{-}\delta^+$ cells) (A), percentage of IL-17A and IFN-γ-producing $TCR\text{-}\delta^+$ T cells (B) and absolute number of IL-17A and IFN-γ-producing $TCR\text{-}\delta^+$ T cells (C). Data are mean ± SEM (n=7 (control), n=6 (FHTE)) and each symbol represents an individual mouse. *p<0.05, **p<0.001 vs control by unpaired t test**.**

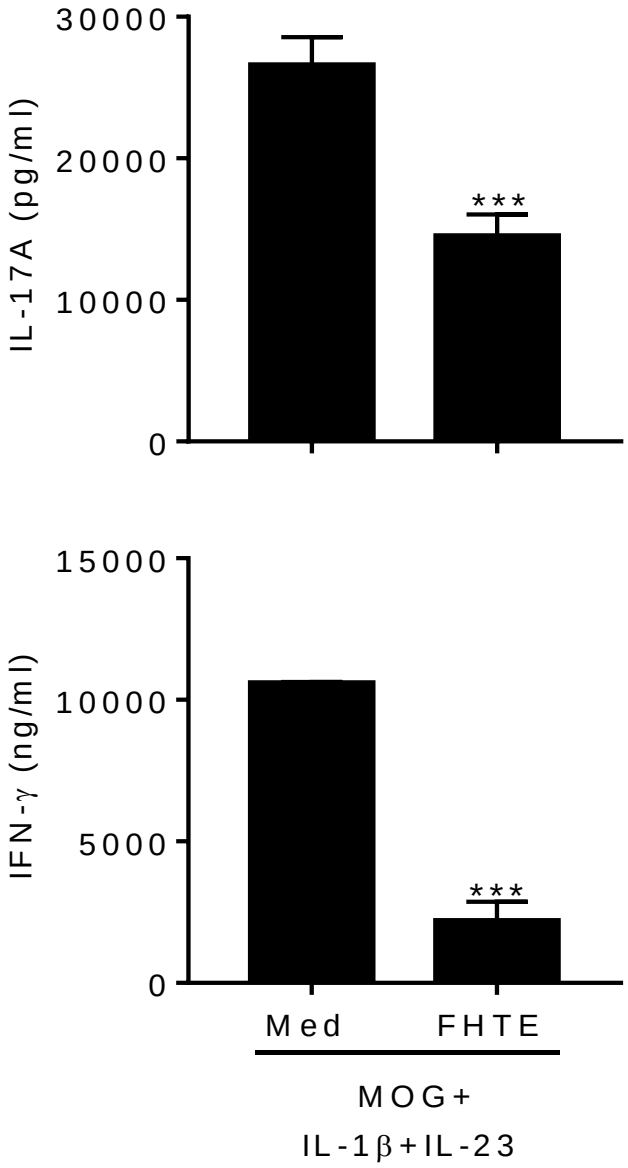

Figure 4.28 FHTE suppresses IL-17A and IFN-γ production by CD3 T cells from MOG-immunized mice. EAE was induced in donor C57BL/6 mice by s.c immunization with MOG (100 µg) emulsified in CFA and 10 days later, spleen and LN cells were isolated. $CD3^+$ T cells were MACS purified and cultured separately with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence of absence of 2.5% v/v FHTE for 18 hr before being put back in culture with the non-T cells. The combined cells were then cultured with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) for 48 hr before the concentration of IL-17A and IFN-γ was quantified by ELISA. Results are mean ±SD for triplicate culture. ***$p<0.001$ vs MOG, IL-1β and IL-23 by unpaired t test.

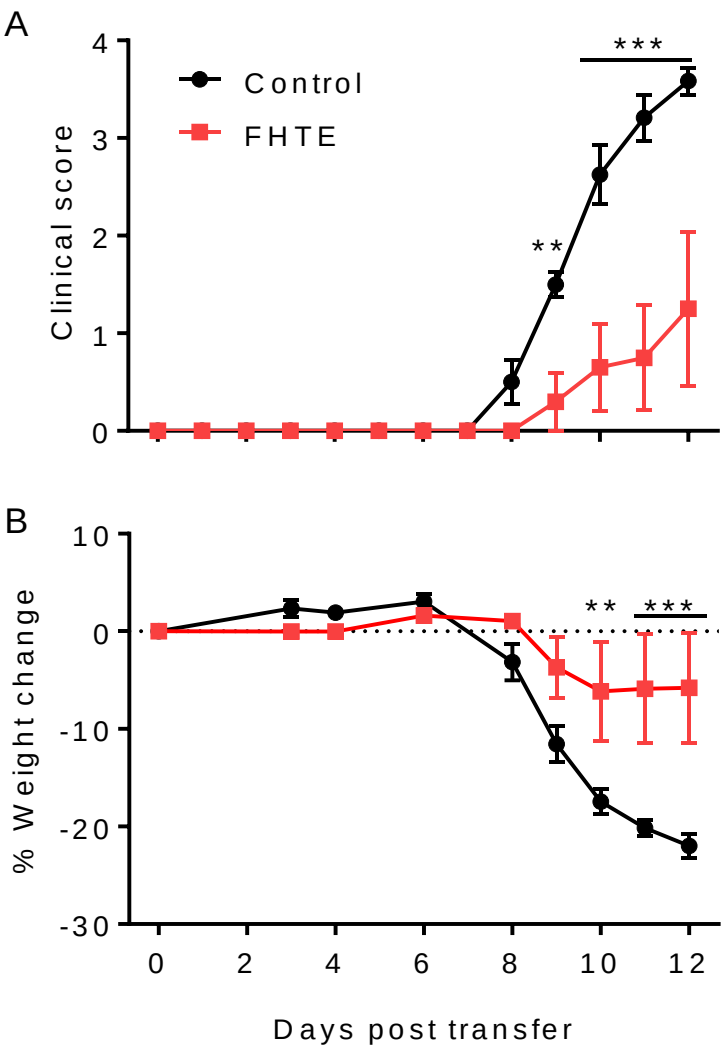

Figure 4.29 Treatment of CD3 T cells from MOG-immunized mice with FHTE suppresses their ability to induce EAE by cell transfer. EAE was induced in donor C57BL/6 mice by s.c immunization with MOG (100 µg) emulsified in CFA. 10 days later, spleen and LN cells were isolated and $CD3^+$ T cells were MACS purified and cultured with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence of absence of 2.5% v/v FHTE for 18 hr before being put back in culture with the non-T cells. The combined cells were then cultured for 48 hr with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) before being transferred to naïve mice ($7x10^6$ cells per mouse). Mice were assessed daily for clinical signs of disease and weighed. Results are mean clinical score (A) and percentage body weight change (B). Data are mean ± SEM (n=6). **p<0.01, ***p<0.001 vs control by repeated measures ANOVA with Sidak post-test.

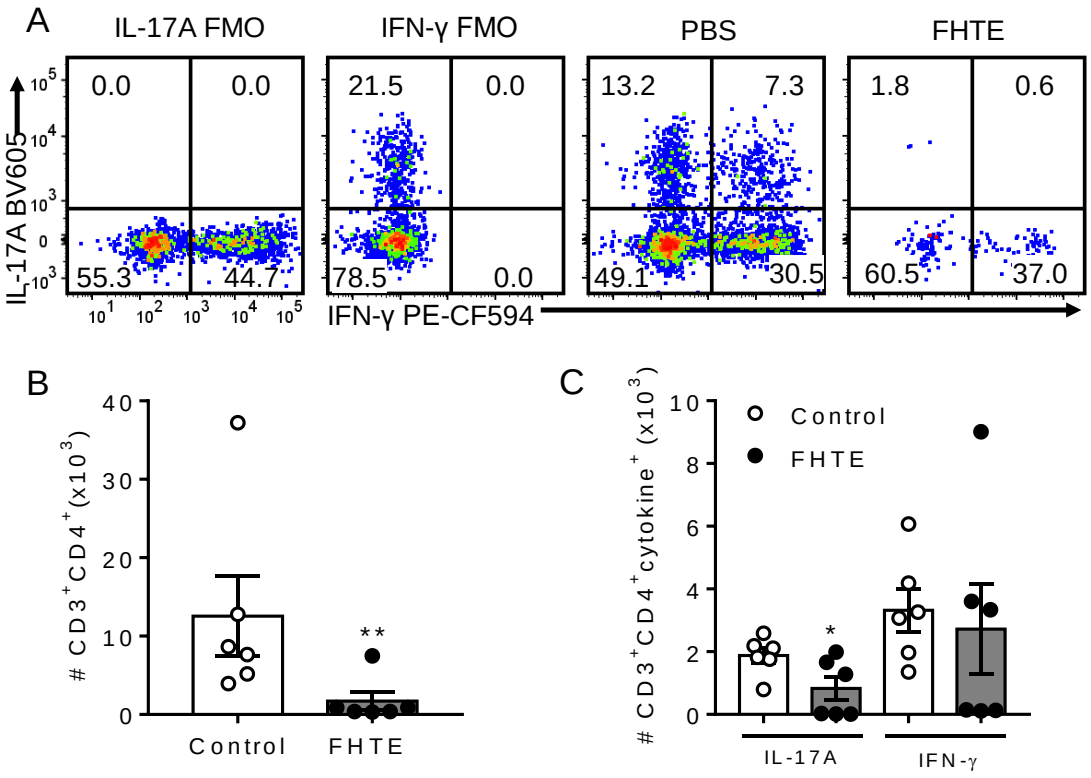

Figure 4.30 Treatment of CD3 T cells from MOG-immunized mice with FHTE reduced the infiltration of IL-17A-producing CD4 T cells in the brain on day 12 post transfer. EAE was induced in donor C57BL/6 mice and 10 days later $CD3^+$ T cells were MACS sorted from spleen and LN cells and culture as described in Fig 4.29. After 18 hr $CD3^+$ T cells were put back in culture with non-T cells and cultured for 48 hr with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) before being transferred to naïve mice ($7x10^6$ cells per mouse). On day 12 after transfer, mice were sacrificed and perfused. Cells were isolated from the brain and stained for surface CD3 and CD4 and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+CD4^+$ cells) (A), absolute number of $CD4^+$ T cells (B) and absolute number of IL-17A and IFN-γ-producing $CD4^+$ T cells (C). Data are mean ± SEM (n=6) and each symbol represents an individual mouse. *p<0.05, **p<0.01 vs control by unpaired t test.

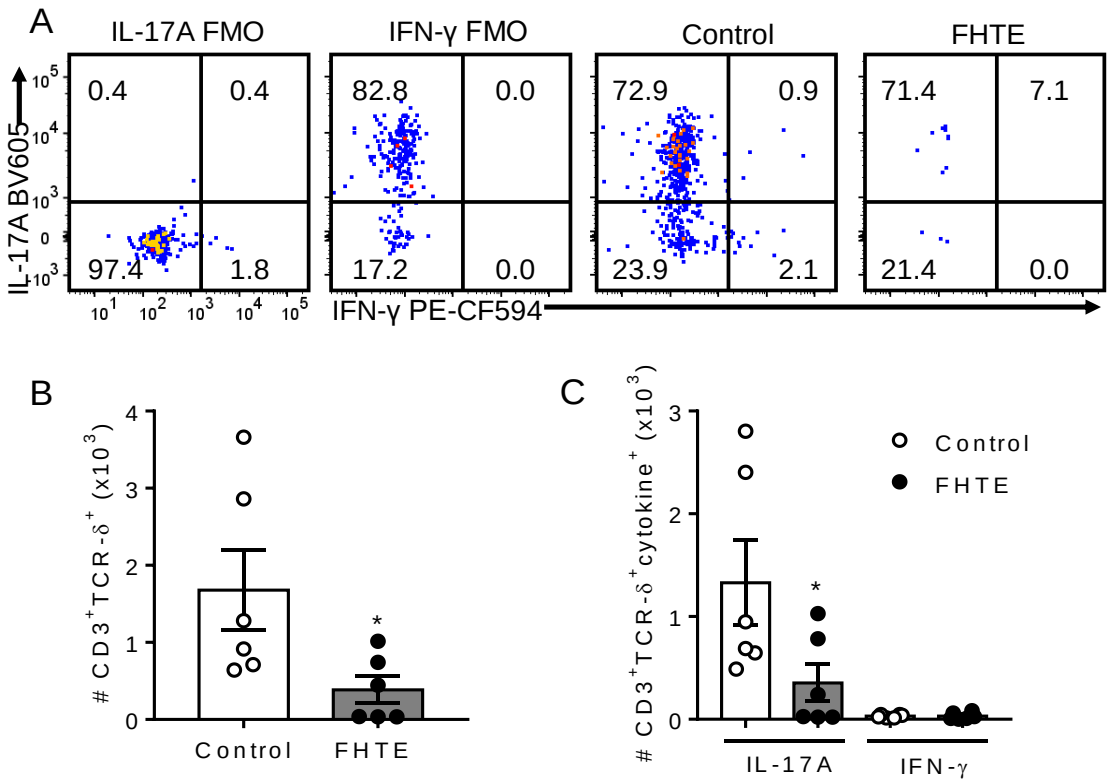

Figure 4.31 Treatment of CD3⁺ T cells from MOG-immunized mice with FHTE reduced the infiltration of IL-17A-producing γδ T cells in the brain on day 12 post transfer. EAE was induced in donor C57BL/6 mice and 10 days later CD3⁺ T cells were MACS sorted from spleen and LN cells and culture as described in Fig 4.29. After 18 hr CD3⁺ T cells were put back in culture with non-T cells and cultured for 48 hr with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) before being transferred to naïve mice ($7x10^6$ cells per mouse). On day 12 after transfer, mice were sacrificed and perfused. Cells were isolated from the brain and stained for surface CD3 and TCR-δ and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are representative FACS plots (gated on CD3⁺TCR-δ⁺ cells) (A), absolute number of TCR-δ⁺ T cells (B) and absolute number of IL-17A and IFN-γ-producing TCR-δ⁺ T cells (C). Data are mean ± SEM (n=6) and each symbol represents an individual mouse. *p<0.05, vs control by unpaired t test.

Chapter 5.

Distinct immunomodulatory activity of high and low molecular weight fractions of *F. hepatica* TE.

5.1 Introduction

Infection with helminths has been shown to be associated with subversion of pathogenic immune responses associated with allergy and autoimmunity [175, 210, 241]. However, infection is accompanied with significant tissue damage and destruction during migration of the parasite throughout the body [148]. Furthermore, in mouse models, infection with live helminths results in translocation of the parasite through the gut wall and liver, causing widespread pathology and significantly mortality [189]. As a result, studies are also focusing on helminth-derived products in order to examine helminth immunomodulation, without the unwanted pathological responses associated with infection. This study so far has demonstrated that FHTE can train innate immune cells to be anti-inflammatory, thereby preventing the development of a T cell-mediated autoimmune disease in mice. Furthermore, direct treatment of pathogenic T cells with FHTE suppresses their activation and proliferation, thus inhibiting their ability to transfer EAE to naïve mice. However, FHTE is a complex mixture of proteins, metabolites and enzymes, as well as structural elements from the parasite surface. These molecules include a number of different classes compounds that can be distinguished in both size and biochemical nature. Many components may be able to interact with the immune system, however, not all can subvert host pathogenic immune responses that are associated with allergy and autoimmunity. This study was designed to evaluate the immunomodulatory components of FHTE, in particular their ability to suppress immune responses that mediate autoimmune diseases.

To date, many studies have examined and identified immunomodulatory components of different helminth species that have the ability to modulate host immune responses. One of the main focuses has been on helminth-derived proteins. Omega-1, a glycoprotein secreted from *S. mansoni* was found to polarize Th2 responses via suppression of protein synthesis, following internalization by the mannose receptor [242, 243]. IPSE/α-1, another glycoprotein from *S. mansoni*, induced IgE-dependent IL-4 production by basophils, which is required for polarization of Th2 responses. Furthermore, ES-62 from *A. viteae* was shown to mimic the immunomodulation observed during natural infection [244]. ES-62, a phosphorylcholine-containing glycoprotein, was

also found to induce Th2 responses by inhibiting the maturation of DCs and their ability to prime naïve T cells [245]. In addition, a number of proteins from *F. hepatica* have been found to have immunomodulatory properties. Tegumental antigen *from F. hepatica* was shown to suppress DC maturation and function [191], as well as inhibiting mast cell-dependent Th1 responses [246]. Cathepsin L1 was shown to inhibit the TRIF-dependent signalling pathway in macrophages via cleavage of TLR3 in the endosome [247]. Cathepsin L1 was subsequently shown to suppress LPS-induced cytokine production *in vivo*. Moreover, helminth-defence molecule-1, which share biochemical, structural, and functional characteristics with mammalian cathelicidin-like host defence peptides, prevented acidification of endolysosomes, leading to inhibition of antigen presentation by DCs [248].

In addition to proteins and peptides, lipid-based components from helminths have also been shown to modulate host immune responses. Lysophophatidylserine from *S. mansoni* promotes the induction of IL-10-secreting Treg cells, by interacting with TLR2 on the surface of DCs [225]. Cytokine homologues and molecules that mimic cytokine activity from helminths have also been identified. Macrophage migration inhibitory factor (MIF) homologues from *B. malayi* enhance IL-4R expression and polarize alternatively activated macrophages induced by IL-4 [249]. Furthermore, Johnston et al. identified TGF-β mimic*,* a member of the complement control protein superfamily, from *H. polygyrus* that replicates the biological and functional properties of TGF-β [250].

Helminth-derived components have also shown protective effects in murine models of autoimmunity. For example, soluble egg antigens from *Trichinella spiralis* and *Trichuris suis* were shown to protect mice against EAE [251]. Furthermore, ES-62 from *A. viteae* suppressed the development of collagen-induced arthritis when administered both at the induction phase of disease and also after the onset of disease [252]. Moreover, cystatin, a secreted protease inhibitor of filarial nematodes, was found to inhibit Th2-induced inflammation, leading to reduced allergic airway hyperresponsiveness in mice [253]. Cystatin was also shown to inhibit colitis in a murine model by enhancing the induction of

Treg responses. In addition, our lab has identified *F. hepatica* kumitz type molecule, a protein found in FHES, which was found to have protective effects in EAE (Walsh and Mills, Unpublished).

The aim of this study was to evaluate the activity of high and low molecular weight fractions of FHTE (FHTE-H and FHTE-L) on IL-10 production and suppression of LPS-induced IL-12p40 and IL-1β by DCs. Furthermore, the effects and contributions of FHTE-H and FHTE-L on innate immune training was examined, in a model of T cell-mediated autoimmunity. In addition, having shown that FHTE can inhibit IL-17A and IFN-γ production by T cells in response to IL-1β and IL-23, this study examined the direct effect of FHTE-H and FHTE-L on T cell activation and proliferation. In order to identify and characterise the immunomodulatory components of FHTE, fractionation on the basis of molecular mass was performed using centrifugal units. This method allowed rough separation of FHTE into a fraction lower than 3 kDA (FHTE-L) and the remaining fraction termed FHTE-H. Fractionation via centrifugation did not alter the molar concentration of molecules in both fractions, allowing direct comparison of activity with FHTE based on volume. Furthermore, as the biochemical nature of these molecules is unknown, this approach was more suitable than evaluating immunomodulatory effects based on protein content. The results obtained demonstrated that FHTE-H contains proteins that inhibit innate immune responses and protects mice against EAE via innate immune training, whereas FHTE-L directly suppresses T cell activation, in particular γδ T cells.

5.2 Results

5.2.1 *FHTE-H induces IL-1RA and IL-10 production by BMDMs*

The results in chapter 3 demonstrated that FHTE can enhance IL-1RA and IL-10 production but also suppress LPS-induced TNF, IL-1β and IL-23 production by innate immune cells. In order to test the contribution of high (FHTE-H) and low (FHTE-L) molecular weight fractions of *F. hepatica* in modulation of innate immune responses, BMDMs were stimulated with 2.5% v/v FHTE, FHTE-H or FHTE-L for 24 hours. Stimulation with FHTE and FHTE-H significantly induced IL-10 and IL-1RA production by BMDMs, however, the production of IL-10 or IL-1RA was unchanged in cells treated with FHTE-L (Fig 5.1).

Proteinase K is a broad-spectrum serine protease, which can be used in biological studies to digest proteins [254]. Furthermore, heat inactivation is an effective method for inactivating proteins via denaturation. In order to biochemically characterize the immunomodulatory factors in FHTE-H, BMDMs were stimulated with 2.5% v/v FHTE, FHTE-H or proteinase-treated FHTE and FHTE-H for 24 hours. Treatment of BMDMs with FHTE and FHTE-H induced production of IL-1RA and IL-10, while stimulation with proteinase-treated FHTE and FHTE-H induced low levels of IL-1RA and IL-10 production (Fig 5.2A). Similar to the effects observed with the addition of proteinase K-treated FHTE, heat inactivation significantly suppressed FHTE and FHTE-H-induced IL-1RA and IL-10 production by BMDMs (Fig 5.2B). These results indicate that immunomodulation of innate immune cells by FHTE-H is predominantly mediated by a protein.

5.2.2 *FHTE-H inhibits LPS-induced cytokine production and co-stimulatory molecule expression by BMDCs*

To examine the contribution of FHTE-H and FHTE-L molecular weight fractions on suppression of LPS-induced cytokine production by FHTE, BMDCs were stimulated with LPS (100 ng/ml) in the presence or absence of 2.5% v/v FHTE, FHTE-H or FHTE-L for 24 hours. Consistent with the data from chapter 3, stimulation with FHTE significantly enhanced LPS-induced IL-10 production, while suppressing LPS-induced IL-1β and IL-12p40. Treatment of BMDCs with FHTE-H and FHTE-L also enhanced LPS-induced IL-10 production, however,

suppression of LPS-induced IL-1β and IL-12p40 was only observed upon stimulation with the FHTE-H fraction (Fig 5.3). These results suggest that the suppressive effects of FHTE on LPS-induced proinflammatory cytokine production is specific to the FHTE-H fraction.

Cytokine production by DCs is a central part of directing lineage commitment of effector T cells. However, direct DC-T cell contact and expression of co-stimulatory molecules on DCs is required for effective antigen presentation and activation of T cells [255, 256]. This study demonstrated that treatment of BMDCs with FHTE suppressed LPS-induced expression of CD40 (Fig 5.4) and MHC II (Fig 5.5), at each concentration tested. Furthermore, FHTE-H and FHTE-L mirrored the effect of FHTE, suppressing expression of both CD40 (Fig 5.4) and MHC II (Fig 5.5), induced by LPS stimulation. Moreover, FHTE, FHTE-H and FHTE-L treatment did not modulate LPS-induced expression of CD80 and CD86 co-stimulatory molecules.

5.2.3 *FHTE-H and FHTE-L suppresses proliferation and cytokine production by CD3 T cells*

The results in chapter 4 demonstrated that FHTE inhibits the activation of naïve T cells through TCR stimulation or in response to IL-1β and IL-23. Having shown the immunomodulatory factors that affect macrophages and DCs segregate into the high molecular weight fraction FHTE-H, this study next examined the fraction that contains compounds that directly modulate T cell responses. Spleen cells from naïve mice were labelled for 20 mins with CTV (1 µm), a dye that enables the quantification of proliferating cells by flow cytometry, before being incubated with anti-CD3 (1 µg/ml) in the presence or absence of 2.5% v/v FHTE, FHTE-H or FHTE-L. Cells were recovered after 72 hours and analysed by flow cytometry. Treatment of spleen cells with anti-CD3 induced proliferation of CD3 T cells and this was significantly suppressed by the addition of FHTE. Furthermore, stimulation of cells with FHTE-H significantly suppressed CD3 T cell proliferation in response to anti-CD3, while suppression was more pronounced in FHTE-L-treated cells (Fig 5.6).

Previous work from our lab has shown that T cells produce IL-17A and IFN-γ in response to IL-1β and IL-23, without the requirement for TCR engagement

[95]. Consistent with the results from chapter 4, treatment of spleen cells with FHTE significantly suppressed IL-1β and IL-23-induced IL-17A and IFN-γ production. Furthermore, stimulation with FHTE-H and FHTE-L also suppressed production of IL-17A and IFN-γ, while FHTE-L was a more potent inhibitor of IFN-γ (Fig 5.7). In addition, suppression of IL-1β and IL-23-induced IL-17A and IFN-γ production by FHTE-L was dose dependent (Fig 5.8). Inhibition of proliferation in combination with reduced cytokine production by T cells may in part account for the protective effect of FHTE observed in the adoptive transfer model of EAE.

5.2.4 *FHTE-L suppresses cytokine production by γδ T cells*

Having shown in chapter 4 that γδ T cells respond rapidly and produce IL-17A and IFN-γ in response to IL-1β and IL-23, it was important to evaluate the direct effect of FHTE-L treatment on IL-1β and IL-23-induced γδ T cell activation. Purified γδ T cells treated with IL-1β and IL-23 induced potent mRNA expression of *il1r1* and *il23r* and this was significantly suppressed by the addition of FHTE-L, in a dose dependent manner (Fig 5.9). Furthermore, the results demonstrated that FHTE-L suppressed IL-1β and IL-23-induced production of IL-17A, IFN-γ, GM-CSF and IL-22 by γδ T cells, at all concentrations tested (Fig 5.10). This is consistent with results from chapter 4 demonstrating that unfractionated FHTE can inhibit IL-1β and IL-23-induced receptor expression for their cytokines, leading to reduced IL-1β and IL-23 signalling that are crucial in the initiation of pathogenic Th17 responses in autoimmunity.

Having shown the immunomodulatory effect of FHTE-H on cells of the innate immune system is protein mediated, this study next examined whether the suppressive components of FHTE-L on γδ T cells was also mediated by a protein complex. Purified γδ T cells were stimulated with IL-1β and IL-23 in the presence or absence of FHTE-L that had been pre-incubated with proteinase K to digest any proteins in the fraction. Consistent with the results in figure 5.10, treatment of γδ T cells with FHTE-L suppressed IL-1β and IL-23-induced IL-17A, IFN-γ and GM-CSF and suppression was also observed in cells that were stimulated with proteinase K-treated FHTE-L fraction (Fig 5.11). These results suggest that FHTE-L contains immunomodulatory components other than protein complexes that have the ability to suppress γδ T cell responses.

IL-17A production by γδ and Vγ4 T cells is important in driving pathogenic T cell responses in EAE. Therefore, FHTE-L was examined for its effect on IL-17A by γδ and Vγ4 T cells *in vivo*. Mice were injected into the footpad with PBS or IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5 % v/v FHTE-L. After 4 hours, the popliteal LNs were isolated and stained for CD3, TCR-δ, Vγ4 and IL-17A. The results revealed that treatment of mice with IL-1β and IL-23 promoted the production of IL-17A by γδ T cells *in vivo*, and this was significantly suppressed by treatment of mice with FHTE-L (Fig 5.12B). Furthermore, treatment of mice with FHTE-L also significantly suppressed the percentage and absolute number of IL-17A-producing Vγ4 T cells induced in response to IL-1β and IL-23 (Fig 5.12C). This is consistent with results from chapter 4 demonstrating that unfractionated FHTE supresses γδ T cells responses *in vivo*. Together, these findings demonstrate that the immunomodulatory components of FHTE, which directly inhibit γδ T cell activation was retained in the lower molecular weight fraction. Furthermore, suppressing IL-1β and IL-23-induced gene expression of *il1r1* and *il23r* by FHTE-L results in reduced production of IL-17A and IFN-γ, which are crucial in the initiation of autoimmunity.

5.2.5 *FHTE-H attenuates EAE*

Having shown that training of mice with FHTE attenuates EAE through innate immune training, this study next evaluated the contribution of FHTE-H and FHTE-L on the suppressive effect of FHTE in development of this T cell-mediated autoimmune disease. Mice were trained with PBS or 2.5% v/v FHTE, FHTE-H or FHTE-L 21 days and 7 days before the induction of EAE. Consistent with results from chapter 3, training of mice with FHTE attenuated EAE, with onset and severity of disease reduced. The results also demonstrated that the clinical course of EAE was significantly attenuated in FHTE-H-trained mice, however, the effect was less pronounced than in FHTE-trained mice (Fig 5.13A). In contrast, training of mice with FHTE-L did not modulate the course of EAE, however, FHTE-L-trained mice recovered from disease with percentage weight loss returning to zero by day 28 (Fig 5.13B). These results suggest that FHTE-H-induced modulation of innate cells protects mice from EAE via innate immune training, whereas the effect of FHTE-L on T cells does not translate to attenuation

of disease via this mechanism, as FHTE-L treatment does not inhibit innate immunity.

5.2.6 *Training of mice with FHTE and FHTE-H results in reduced recruitment of neutrophils to the spleen after the induction of EAE*

EAE is initiated in the periphery where innate immune cells, in particular DCs and macrophages, produce IL-1β and IL-23 that activates early IL-17A production by γδ T cells. This leads to recruitment of neutrophils and inflammatory monocytes that activate the cascade leading to the induction of encephalitogenic γδ and Th17 cells (Mc Ginley et al., Unpublished). Having shown in chapter 3 that FHTE-trained mice have reduced T cell responses during the induction phase of EAE, flow cytometry was used to examine the innate immune cell populations in the spleen of mice trained with FHTE, FHTE-H and FHTE-L. Cells were isolated on day 3 post induction of EAE and stained with antibodies specific for B220, CD3, CD11b, Siglec F, Ly6G, Ly6C, CD11c and MHC II (Fig 5.14).

On day 3 after the induction of EAE, neutrophils were the most abundant innate immune cell type in the spleen of mice in all treatment groups (Fig 5.15A). This is consistent with data published by Rumble et al. demonstrating that neutrophils accumulate in the spleen after the induction of EAE [257]. However, the percentage and absolute numbers of neutrophils was significantly reduced in FHTE-trained mice, when compared with PBS-injected mice. Reduced neutrophil recruitment was also observed in the spleen of FHTE-H-trained mice, but not in FHTE-L-trained mice, when compared with PBS-injected mice on day 3 after the induction of EAE (Fig 5.15C). In addition, there was a significant increase in the number of eosinophils in the spleen of FHTE and FHTE-H-trained mice, when compared with PBS-injected mice on day 3. However, infiltration of eosinophils was unchanged in FHTE-L-trained mice (Fig 5.16). Depletion of neutrophils prior to disease has been shown to attenuate EAE, demonstrating a vital role for neutrophil function during the initial formation of lesions [258]. These results suggest that decreased neutrophils recruitment to the spleen after the induction of EAE may in part account for attenuation of disease in FHTE and FHTE-H-trained mice.

5.2.7 *FHTE-H-trained mice have reduced peripheral T cell responses*

Neutrophils are an important source of IL-1β required for the initiation phase of EAE but also important during the effector phase in the CNS [117, 118]. Furthermore, it is thought that neutrophils may contribute to APC maturation and subsequent induction of pathogenic T cell responses [258]. Having shown that FHTE and FHTE-H-trained mice have reduced neutrophil recruitment into the spleen after the induction of EAE, this study next investigated peripheral T cell responses in mice trained with FHTE, FHTE-H and FHTE-L after the induction of EAE. The frequency and absolute number of IL-17A-producing Vγ4 T cells in the LNs on day 3 after the induction of EAE was significantly reduced in FHTE and FHTE-H-trained mice, when compared with PBS-treated mice. In contrast, training of mice with FHTE-L did not alter IL-17A production by Vγ4 T cells (Fig 5.17). Furthermore, training of mice with FHTE inhibited proliferation of Vγ4 T cells in the LNs on day 3, when compared with PBS-injected mice and this effect was mirrored in FHTE-H-trained mice, but not FHTE-L-trained mice (Fig 5.18). This is consistent with results from chapter 3, demonstrating that FHTE-trained mice have reduced Vγ4 T cells responses in the periphery before the induction of EAE.

The number of IL-17A-producing CD4 T cells was also significantly reduced in FHTE, FHTE-H and FHTE-L-trained mice on day 7 after the induction of EAE, when compared with PBS-injected mice. However, similar to results observed in chapter 3, there was no change in the number of IFN-γ-producing CD4 T cells in mice trained with FHTE, FHTE-H or FHTE-L (Fig 5.19). Reactivation of spleen and LN cells on day 7 from PBS-injected mice with MOG resulted in IL-17A and IFN-γ production, in a dose dependent manner and a similar effect was observed in FHTE-L-trained mice. However, MOG-specific IL-17A and IFN-γ production by CD4 T cells was significantly suppressed in FHTE-H-trained mice (Fig 5.20). These results suggest that training of mice with FHTE-H indirectly suppresses γδ T cell responses, resulting in reduced neutrophil recruitment and subsequent suppression of IL-17-producing T cells in the periphery during the induction phase of EAE.

5.2.8 *Reduced infiltration of neutrophils, inflammatory monocytes and CD4 T cells into the brain of FHTE-H-trained mice*

Infiltration of encephalitogenic T cells and myeloid cells into the CNS initiates the effector phase of EAE and results in the clinical course of disease [209]. Furthermore, infiltration of neutrophils and inflammatory monocytes and IL-1β production by these cells enhances inflammation in the brain by further activating γδ T cells and Th17 responses [95] (Mc Ginley et al., Unpublished). An examination of myeloid cell infiltration into the CNS revealed that FHTE and FHTE-H-trained mice had a significant reduction in the total number of infiltrating immune cells ($CD45^{high}$) into the brain on day 13, when compared with PBS-injected mice, whereas the effect was less pronounced in FHTE-L-trained mice (Fig 5.21). Furthermore, the absolute number of neutrophils and inflammatory monocytes into the brain on day 13 after the induction of EAE was significantly reduced in FHTE and FHTE-H-trained mice, but not in FHTE-L-trained mice, when compared with PBS-injected mice (Fig 5.22).

An investigation of lymphocytes in the brain of mice 13 days after the induction of EAE demonstrated that the infiltration of IL-17A and IFN-γ-producing CD4 T cells was significantly suppressed in FHTE-H-trained, but not FHTE-L-trained mice (Fig 5.23). This is consistent with results from chapter 3 demonstrating that training of mice with unfractionated FHTE resulted in impaired cytokine production by CD4 and γδ T cells that infiltrated the brain during EAE. Taken together, the results from this chapter demonstrate that the immunomodulatory components of FHTE that are responsible for inducing innate immune memory, resulting in attenuation of EAE was retained in the high molecular weight fraction. Furthermore, FHTE-H-trained mice had suppressed neutrophil and inflammatory monocyte recruitment, resulting in reduced activation and infiltration of pathogenic T cells into the CNS.

5.3 Discussion

Helminth parasites have developed highly effective mechanisms of immune subversion that helps prolong their survival in the host, resulting in the development of chronic infections. A bystander effect of this immune subversion is the simultaneous suppression of pathogenic immune responses associated with allergy and autoimmunity. Live helminth therapy in both mouse models and clinical trials have shown promising effects for the treatment of autoimmune diseases [159, 175, 259]. However, live therapy is associated with significant immunopathology, as well as having practical and ethical implications. As a result, efforts are now focusing on helminth-derived products to examine helminth immunomodulation, without the unwanted pathological responses associated with infection. The current study has demonstrated that separation of FHTE via fractionation based on molecular size, resulted in two fractions with distinct immunomodulatory activities. FHTE-L was found to inhibit T cells responses, in particular γδ T cells, but had little effect on cytokine production by macrophages and DCs. In contrast, FHTE-H displayed potent immunosuppressive effect on DCs. Furthermore, FHTE-H-trained mice had reduced neutrophil and inflammatory monocyte recruitment, resulting in suppression of pathogenic T cell responses and the attenuation of EAE.

IL-10 production is crucial for limiting immunopathology during helminth infection [260]. Moreover, many studies have demonstrated that helminth-derived products can induce IL-10 production by innate immune cells [151, 191, 261]. However, little research has focused on the production of IL-1RA in response to helminths. The present study demonstrated that IL-10 and IL-1RA induction in response to FHTE treatment was mediated by compounds with a molecular weight of more than 3 kDa (FHTE-H). Furthermore, the induction of IL-10 and IL-1RA by BMDMs in response to FHTE-H was reversed in protein-digested FHTE-H stimulation, suggesting that immunomodulation of innate immune responses by FHTE-H is protein mediated. Although helminth infection and helminth-derived proteins have been shown to induce Treg cell responses through the induction of IL-10 from innate immune cells [151, 262, 263], this is the first demonstration of helminth-derived proteins directly inducing IL-1RA secretion by macrophages. These regulatory mechanisms may be important for ensuring survival of the

parasite within the host and consequently, may also be responsible for limiting pathogenic T cell responses associated with allergy and autoimmune diseases.

DCs are innate immune cells that are vital in the initiation and control of T cell responses. iDCs are poor antigen presenting cells that continuously sample the surrounding area for invading pathogens. Upon recognition of PAMPs and DAMPs, DCs mature to become fully immunogenic [264]. Maturation leads to down regulation of certain chemokine receptors as well as the uptake of antigens. Furthermore, DC activation results in upregulation of co-stimulatory molecules, including CD40, CD80, CD86, MHC II and polarising cytokines that promote the differentiation of distinct Th cell subsets [256]. However, in the absence of co-stimulation, DCs become tolerogenic leading to suppressed T cell responses. The present study demonstrated that treatment of DCs with FHTE suppressed LPS-induced co-stimulatory expression of CD40 and MHC II. Furthermore, suppression of CD40 and MHC II induced by LPS was retained in both the FHTE-H and FHTE-L fractions. This is consistent with a report demonstrating that *F. hepatica* tegumental protein suppresses TLR-induced cell surface marker expression (CD80, CD86, and CD40) on DCs [191].

In the context of MS and EAE, expression of MHC II is highly upregulated on APCs [265–267]. Furthermore, down-regulation of MHC II was shown to ameliorate the clinical severity of EAE [268]. In addition, CD40 expression plays a vital role in the initiation of many autoimmune diseases. One study demonstrated that over-expression of CD40 resulted in disease exacerbation of experimental autoimmune Graves' disease, whereas deletion of thyroidal CD40 suppressed disease [269]. Furthermore, CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in non-obese diabetic mice [270]. The use of the antagonistic anti-CD154, which disrupts the CD40-CD154 interactions, delayed diabetes in NOD mice [271]. Moreover, Ruplizumab and Toralizumab, drugs which target CD40L, have demonstrated biological activity in phase I/II trials of systemic lupus erythematosus [272, 273]. Suppression of co-stimulatory molecule expression on the surface of DCs results in tolerance, an important mechanism used by helminth to ensure survival within a host. A bystander effect of inducing tolerogenic DCs is the induction of regulatory

mechanisms that limit pathogenic immune responses and attenuate autoimmune diseases. These results suggest that direct blockage of CD40 may be another promising target in the treatment of autoimmune disease.

Upon activation of CD4 T cells in response to MHC II and TCR interaction, as well as co-stimulation, the fate of naive Th cells is determined by polarising cytokine released by innate immune cells including DCs. IL-12 promotes the development of Th1 cells, whereas IL-1β and IL-23 is important in the expansion of Th17 cells [40, 61]. The current study demonstrated that the suppressive effect of FHTE on LPS-induced IL-1β and IL-12p40 production is mediated by the FHTE-H fraction, but not FHTE-L. This is consistent with studies demonstrating that helminth-derived proteins can suppress Th1 responses through inhibition of IL-12 production by DCs [191, 242]. Furthermore, inhibition of IL-1β and IL-12 by DCs may account for suppression of Th1 and Th17 responses that are vital during the effector phase of EAE. Moreover, this study demonstrated that enhanced IL-10 production in response to LPS was retained in both the FHTE-H and FHTE-L fraction, indicating that distinct immunomodulatory molecules in FHTE enhance the production of IL-10. This is consistent with reports demonstrating that not only proteins but also small molecules from helminths can enhance Treg responses by inducing IL-10 production by DCs [191, 225]. Inhibition of TLR-induced co-stimulatory molecule expression and cytokine production, by different fractions of FHTE, may in part account for inhibition of autoreactive Th1 and Th17 responses, resulting in a lower incidence of allergy and autoimmune diseases in helminth-infected individuals.

A common feature of helminth infection is T cell hyporesponsiveness. Furthermore, the induction of anergic or Treg cells is usually dependent on IL-10 and TGF-β production by DCs. The results in chapter 4 demonstrated that FHTE directly inhibits the activation of naïve T cells through TCR stimulation or in response to IL-1β and IL-23. The current study has revealed that FHTE-induced suppression of TCR-independent T cell activation was more potent with factors that segregate into the low molecular fraction of FHTE (FHTE-L). Our lab has previously demonstrated that IL-1β and IL-23 activate memory CD4 T cell and γδ T cells that rapidly produce IL-17 and IFN-γ [61]. Furthermore, stimulation with

IL-1β and IL-23 is known to enhance IL-23R on the surface of γδ T cells and stabilises their effector function [95]. The present study demonstrated that FHTE contains low molecular weight products that suppress IL-1β and IL-23-induced cytokine production by spleen cells. Furthermore, the immunomodulatory components of FHTE-L were found to suppress upregulation of *il1r1* and *il23r* on the surface of γδ T cells in response to IL-1β and IL-23, resulting in decreased signalling and reduced IL-17A, IFN-γ and GM-CSF production. This is consistent with results from chapter 4 demonstrating that treatment with unfractionated FHTE suppresses γδ T cell responses. It was also established that inhibition of IL-1β and IL-23-induced cytokine production in response to FHTE-L was not mediated by a protein, as inhibition was still observed in response to protein-digested FHTE-L treatment. This suggests that components other than proteins, possibly small molecules or metabolites, are responsible for modulation of γδ T cell responses both *in vitro* and *in vivo*.

In chapter 3, FHTE was found to induce innate immune training of M2 macrophages *in vivo*, resulting in suppression of MOG-specific T cell responses and the attenuation of EAE. Similar to unfractionated FHTE, FHTE-H-trained mice had reduced disease severity and delayed onset of EAE. Although FHTE-L was a potent immunomodulator of γδ T cell responses *in vitro* and *in vivo*, training of mice with FHTE-L was not effective in delaying onset and reducing clinical signs of EAE. The observation that FHTE-L treatment did not attenuate EAE is consistent with previous results from this study, demonstrating that FHTE-L does not modulate innate immune cells. FHTE-H, and not FHTE-L, modulates innate immune responses and attenuates EAE, further demonstrating that protection of mice from EAE in response to FHTE is mediated by innate immune training mechanisms. However, FHTE-L-trained mice eventually recovered from EAE and weight loss associated with disease, indicating that components of FHTE-L may have some immunomodulatory effects later in disease. Collectively, this study confirms that protection from EAE in FHTE-trained mice is mediated through innate immune training mechanisms, as attenuation is observed only in the fraction that modulates innate immunity.

Our lab has previously demonstrated that Vγ4 T cells are the principle source of IL-17A during the induction phase of EAE (Mc Ginley et al., Unpublished). Furthermore, early IL-17A production by Vγ4 T cells initiates inflammation, which promotes infiltration of myeloid cells and the production of innate IL-1β and IL-6 [95, 274]. The current study demonstrated that training of mice with FHTE-H resulted in suppression of IL-17A-producing Vγ4 T cells in the spleen on day 3 after the induction of EAE, however, this effect was not observed in FHTE-L-trained mice. This finding is consistent with results from chapter 3 demonstrating training of mice with FHTE resulted in suppressed Vγ4 T cell responses before the induction of EAE.

Neutrophils are one of the first immune cells to respond to infection [275]. They are recruited and expand in response to early IL-17A by Vγ4 T cells and are an important source of cytokines [276]. They secrete large quantities of IL-1, which play a critical role in the further activation of γδ T cells and the expansion of Th17 (Mc Ginley et al., Unpublished). Furthermore, neutrophils are not only required for the initiation of EAE but are one of the most abundant cell types found in CNS lesions [257, 277, 278]. The current study demonstrated that neutrophils are the main innate immune cell that infiltrate the spleen, 3 days after the induction of EAE. This is consistent with data from Rumble et al. demonstrating that neutrophils accumulate in the bone marrow and spleen after the induction of EAE [257]. However, training of mice with FHTE led to a significant reduction in neutrophil recruitment into the spleen on day 3 after the induction of EAE. Furthermore, reduced neutrophil infiltration was also observed in FHTE-H-trained mice, however, this effect was not seen in mice trained with FHTE-L. In addition, mice trained with FHTE and FHTE-H, but not FHTE-L, had significantly reduced numbers of neutrophils and inflammatory monocytes infiltrating the brain on day 13 of EAE. A number of studies have demonstrated that depletion of neutrophils leads to attenuation of EAE, suggesting a critical role for these cells in disease pathogenesis [279–281]. Furthermore, neutrophils have been found to play a role in the breakdown of the BBB [282]. However, the role of neutrophils in helminth infection remains to be fully elucidated. Al-Qaoud et al. demonstrated that neutrophils are the main cell type required for the formation of inflammatory nodules during the control of *L. sigmodontis* infection [283]. Furthermore, studies

in patients infected with *Trichuris trichiura* a higher number of neutrophils present at worm sites [284, 285]. These results suggest that helminths may have development mechanisms in order to suppress neutrophil recruitment in order to avoid detection and expulsion.

Our lab has demonstrated that infiltration of neutrophils and inflammatory monocytes into the spleen after the induction of EAE is impaired in IL-17$^{-/-}$ mice, resulting in decreased Th17 responses and the attenuation of EAE. The current study demonstrated that FHTE-trained mice suppressed IL-17A-producing Vγ4 T cells and neutrophils in the periphery on day 3 of EAE, but also had reduced IL-17 production by CD4 T cells in the LN on day 7 after induction. Suppression of IL-17A-producing CD4 T cells was observed in both the FHTE-H and FHTE-L fractions. Moreover, MOG-specific IL-17A and IFN-γ production by T cells was impaired in FHTE-H-trained mice, but not FHTE-L-trained mice. Collectively, these results suggest that training of mice with FHTE-H indirectly suppresses IL-17A production by Vγ4 T cells via innate immune training, which results in reduced infiltration of neutrophils, inhibition of pathogenic Th17 and γδ T cell responses and the attenuation of EAE. In contrast, training of mice with FHTE-L did not suppress Vγ4 T cell responses or neutrophil and inflammatory monocyte recruitment and the expansion of Th17 cells that mediate EAE.

During the effector phase of EAE, T cells traffic to the CNS as MOG-specific encephalitogenic T cells that promote demyelination and axonal damage [286]. This study has demonstrated that FHTE-H-trained mice had suppressed T cell responses in the periphery during the induction phase of EAE. This study further demonstrated that training of mice with FHTE-H not only reduced infiltration of cells into the CNS, but also modulated cytokine production by T cells observed in the brain during EAE. Similar to unfractionated FHTE, FHTE-H-trained mice had a significantly reduction in IL-17A and IFN-γ-producing CD4 T cells in the brain on day 13. In contrast, training of mice with FHTE-L resulted in similar infiltration of IL-17A-producing T cells into the brain, to that observed in PBS-injected mice.

Helminths have evolved multiple mechanisms to subvert and evade host immune responses resulting in the development of chronic infections. Collectively, this study has identified two fractions of FHTE with distinct

immunomodulatory activities. Fractionation, based on molecular size, resulted in the identification of a protein complex, greater than 3 kDa, which suppressed proinflammatory cytokine production by DCs and protected mice against a T cell-mediated model of autoimmunity via innate immune training. These findings add significant weight to the hygiene hypothesis and provide an alternative mechanism of subverting host immune responses via targeting or reprogramming the innate immune cells to be more anti-inflammatory. Further analysis is required to identify the specific molecules of FHTE that induce innate immune training and directly modify T cell responses. However, this study has identified new methods of immunomodulation by helminth-derived products that could be exploited for the treatment of autoimmune diseases in humans.

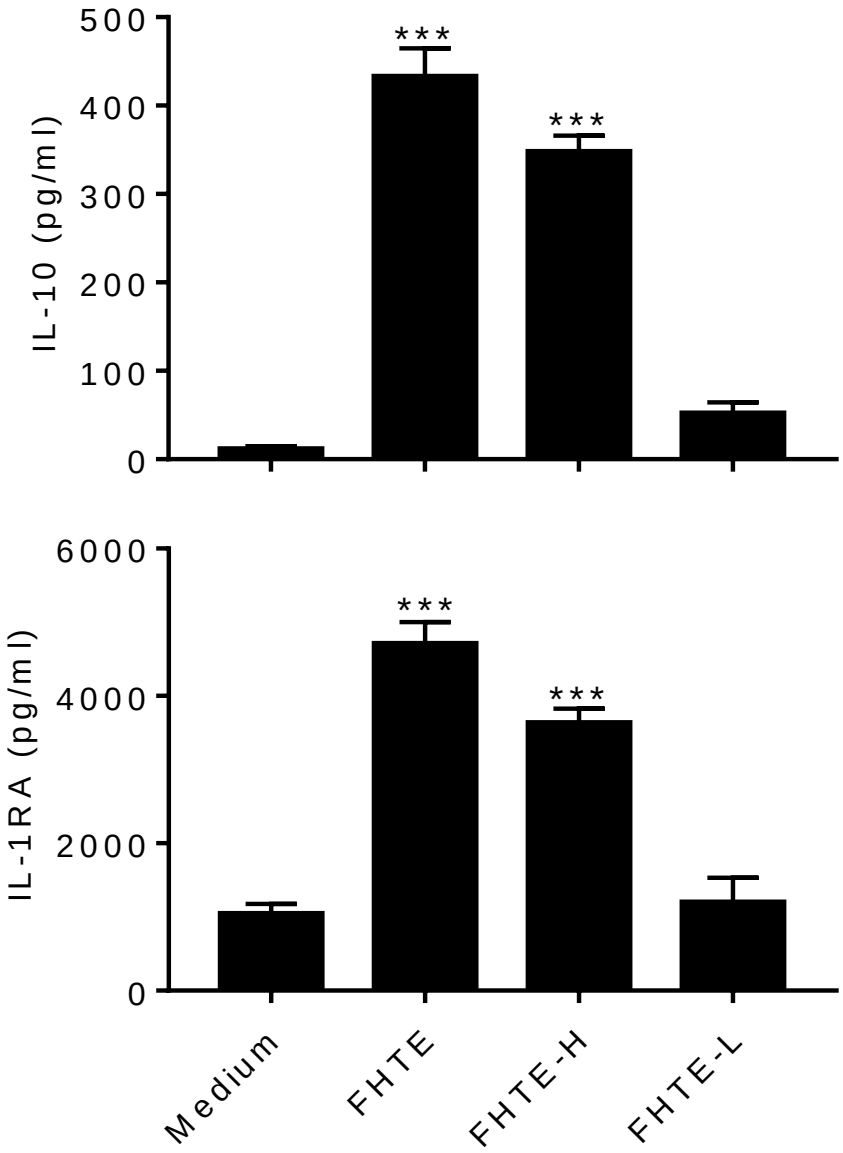

Figure 5.1 FHTE-H induced IL-1RA and IL-10 production by BMDMs. BMDMs from naïve C57BL/6 mice were stimulated with 2.5% v/v FHTE, FHTE-H or FHTE-L. Supernatants were collected after 24 hr and the concentration of IL-10 and IL-1RA determined by ELISA. Results are mean ±SD for triplicate culture and are combined from two separate experiments. ***p<0.001 vs medium control by one-way ANOVA with Dunnett post-test.

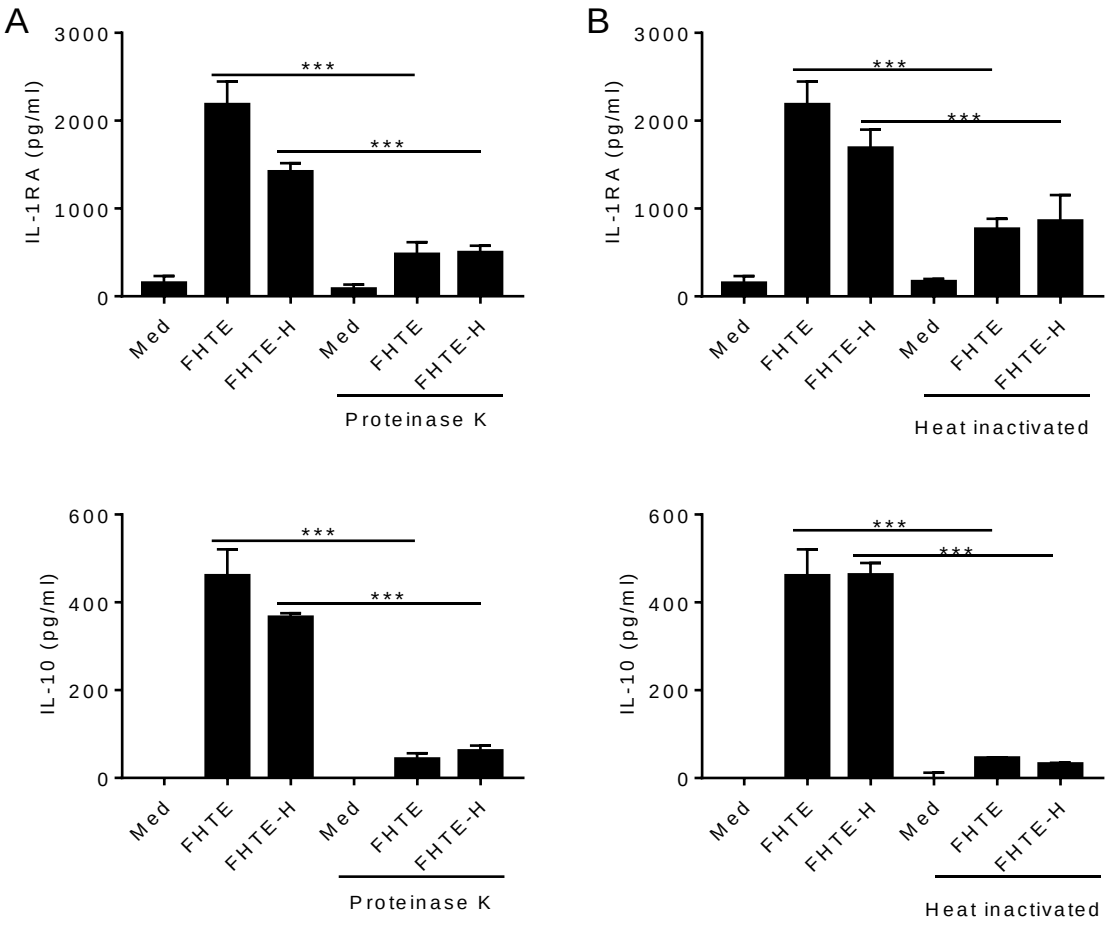

Figure 5.2 Proteinase K treatment and heat inactivation reverses the immunomodulatory effects of FHTE-H on BMDMs. (A) BMDMs from naïve C57BL/6 mice were stimulated with 2.5% v/v FHTE and FHTE-H or FHTE and FHTE-H treated with proteinase K (50 µg/ml) for 10 mins, followed by heat inactivation (95°C) prior to use. Supernatants were collected after 24 hr and the concentration of IL-1RA and IL-10 determined by ELISA. (B) BMDMs were stimulated with 2.5% v/v FHTE or FHTE-H or heat inactivated (95°C) FHTE and FHTE-H and the concentration of IL-1RA and IL-10 quantified by ELISA after 24 hr. Results are mean ±SD for triplicate culture and are combined from two separate experiments. ***p<0.001 vs proteinase k treated FHTE or FHTE-H by two-way ANOVA with Sidak post-test.

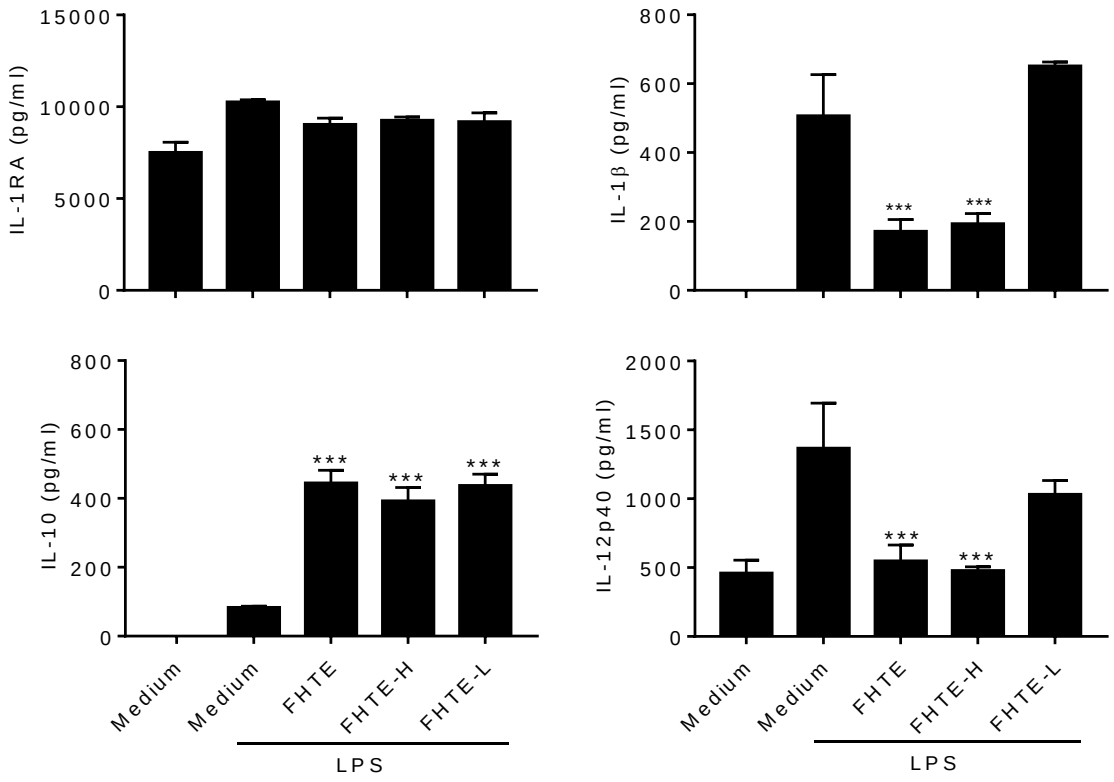

Figure 5.3 FHTE-H enhanced IL-10 but suppressed LPS-induced IL-1β and IL-12p40 production by BMDCs. BMDCs from naïve C57BL/6 mice were stimulated with LPS (100 ng/ml) in the presence or absence of 2.5% v/v FHTE, FHTE-H or FHTE-L. Supernatants were collected after 24 hr and the concentration of IL-1RA, IL-10, IL-1β and IL-12p40 determined by ELISA. Results are mean ±SD for triplicate culture and are combined from two separate experiments. ***p<0.001 vs LPS by one-way ANOVA with Dunnett post-test.

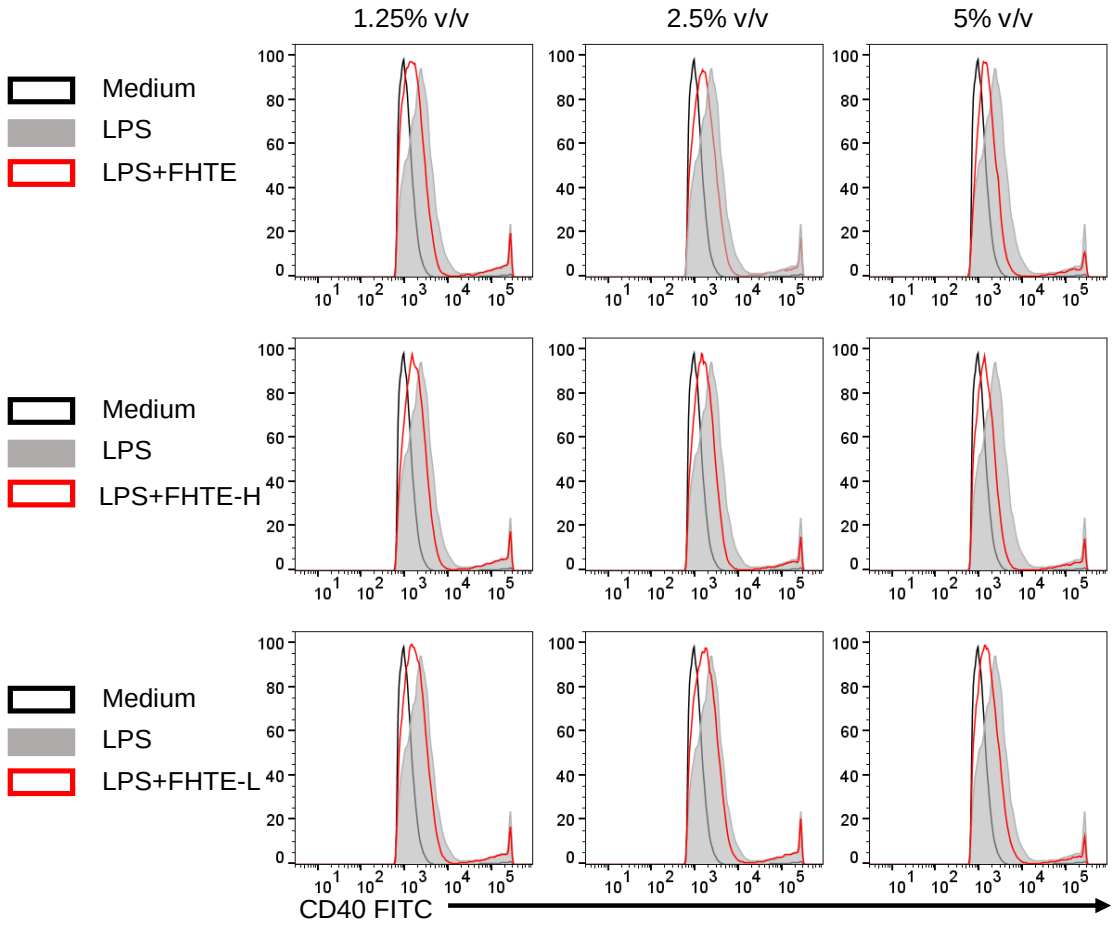

Figure 5.4 FHTE-H and FHTE-L suppressed LPS-induced expression of CD40 on BMDCs. BMDCs from naïve C57BL/6 mice were stimulated with LPS (100 ng/ml) in the presence or absence of 2.5% v/v FHTE, FHTE-H or FHTE-L. After 24 hr, the expression of CD40 was analysed by surface staining and flow cytometry, gating on $CD11c^{+}$ cells. Data are shown as overlay histograms of fluorescence intensities.

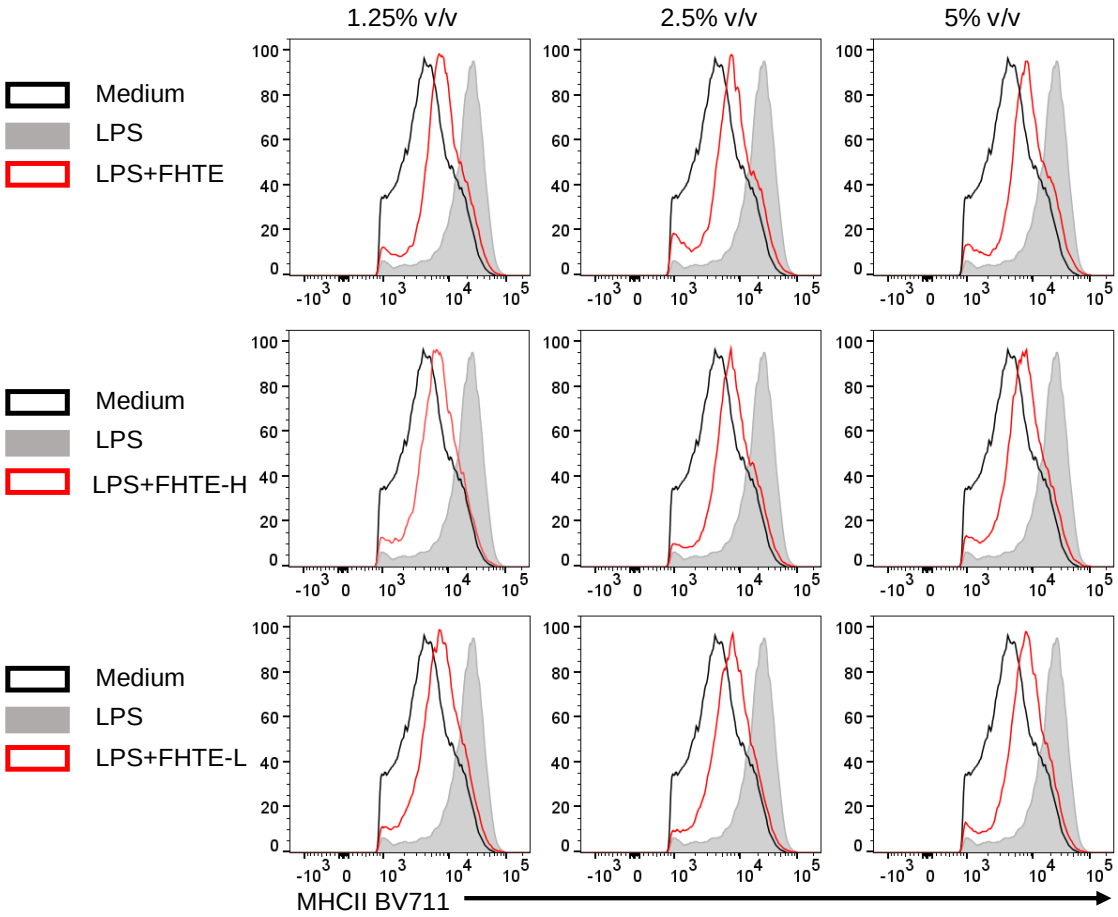

Figure 5.5 FHTE-H and FHTE-L suppressed LPS-induced expression of MHC II on BMDCs. BMDCs from naïve C57BL/6 mice were stimulated with LPS (100 ng/ml) in the presence or absence of 2.5% v/v FHTE, FHTE-H or FHTE-L. After 24 hr, the expression of MHC II was analysed by surface staining and flow cytometry, gating on $CD11c^{+}$ cells. Data are shown as overlay histograms of fluorescence intensities.

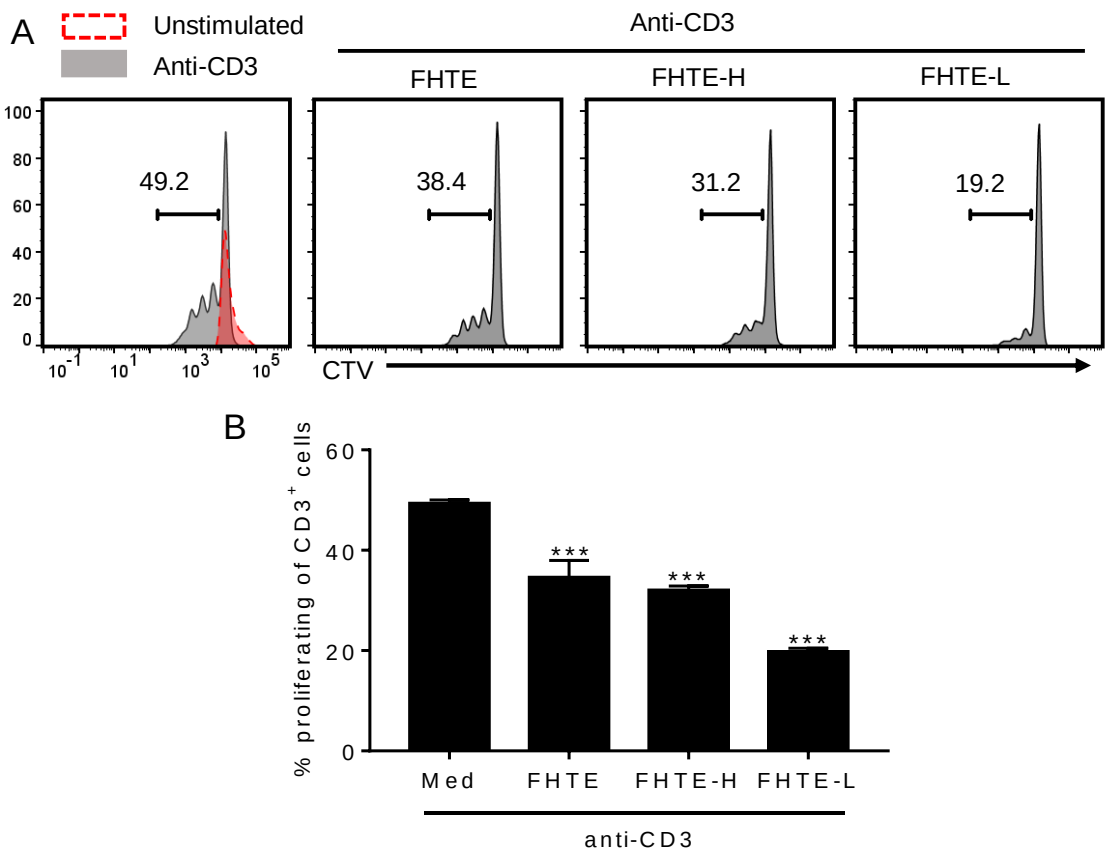

Figure 5.6 FHTE-H and FHTE-L suppressed anti-CD3-induced proliferation of CD3 T cells. Naïve spleen cells from C57BL/6 mice were CTV labelled (1 µM) for 20 mins before being stimulated with anti-CD3 (1 µg/ml) in the presence or absence of 2.5% v/v FHTE, FHTE-H or FHTE-L. Cells were harvested after 72 hr and cells were stained for surface CD3. CD3 T cells were analysed by flow cytometry for CTV dilution. Results shown as histograms for each treatment group (A) and percentage proliferating of $CD3^+$ T cells (B). Data are mean ±SD for triplicate culture and is representative of two independent experiments. ***p<0.001 vs anti-CD3 by one-way ANOVA with Dunnett post-test.

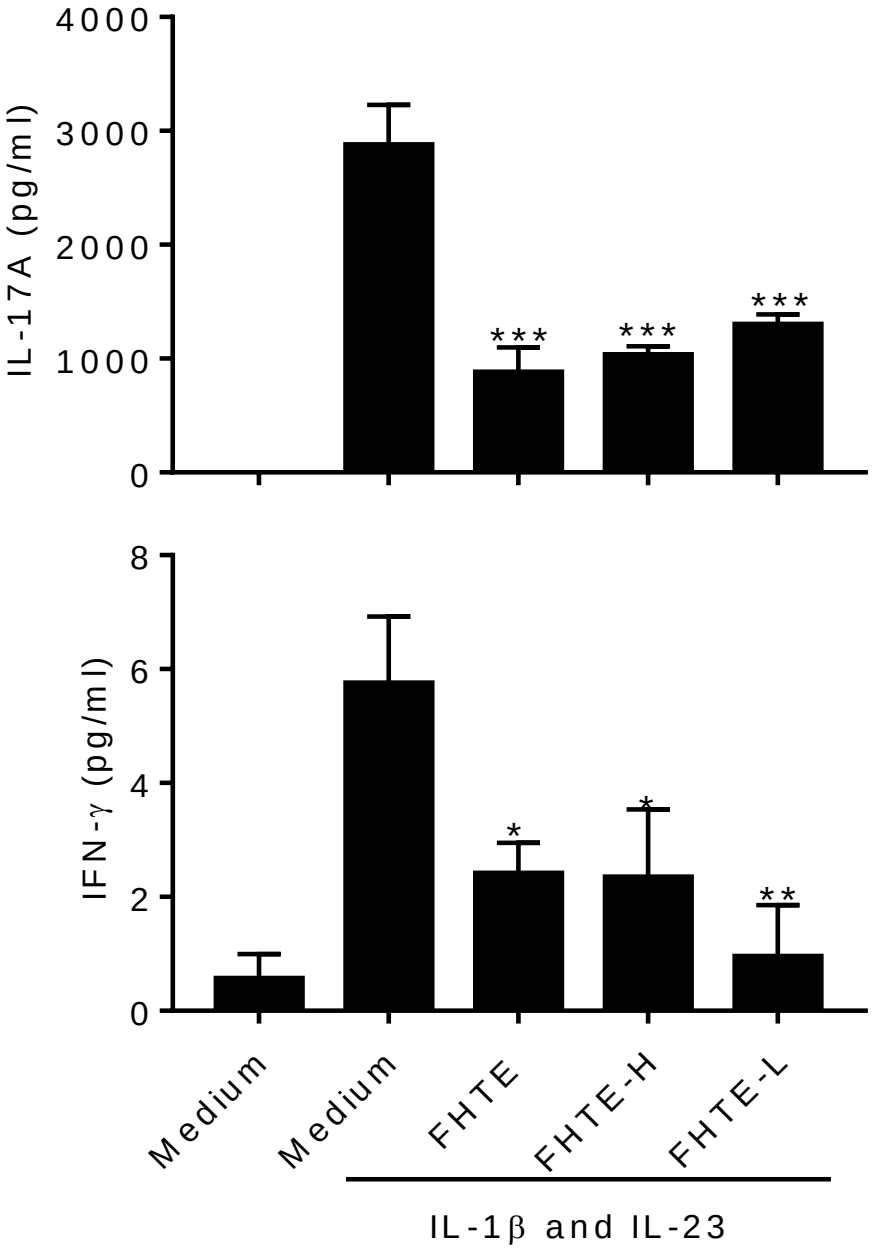

Figure 5.7 FHTE-H and FHTE-L inhibited IL-1β and IL-23-induced IL-17A and IFN-γ production by spleen cells. Spleen cells from naïve C57BL/6 mice were incubated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE, FHTE-H or FHTE-L. Supernatants were collected after 72 hr and the concentrations of IL-17A and IFN-γ determined by ELISA. Results are mean ±SD for triplicate culture and are combined from two separate experiments. *p<0.05, **p<0.01, ***p<0.001 vs IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

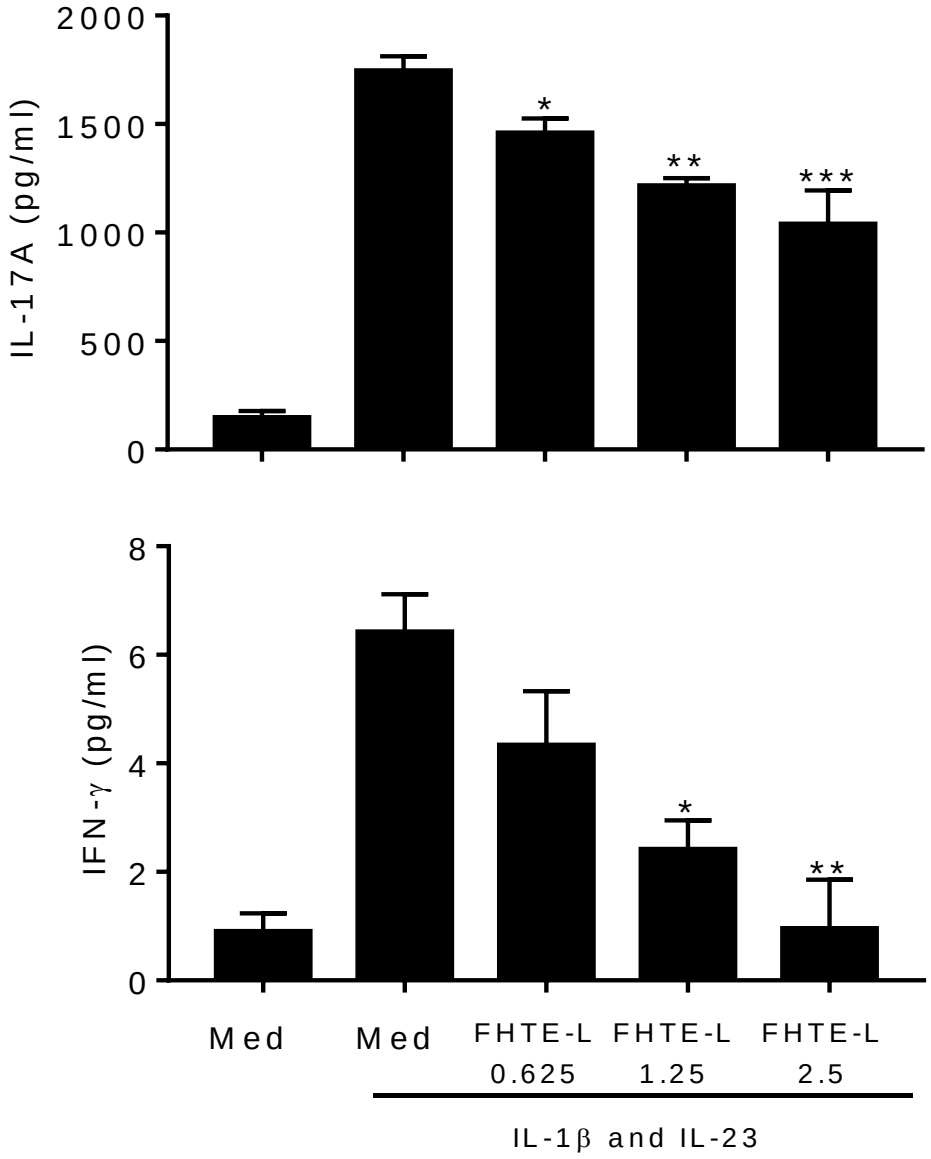

Figure 5.8 FHTE-L suppressed IL-1β and IL-23-induced IL-17A and IFN-γ production by spleen cells in a dose dependent manner. Spleen cells from naïve C57BL/6 mice were incubated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) with or without increasing concentration of FHTE-L (0.625-2.5% v/v). Supernatants were collected after 72 hr and the concentrations of IL-17A and IFN-γ determined by ELISA. Results are mean ±SD for triplicate culture and are combined from two separate experiments. *p<0.05, **p<0.01, ***p<0.001 vs IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

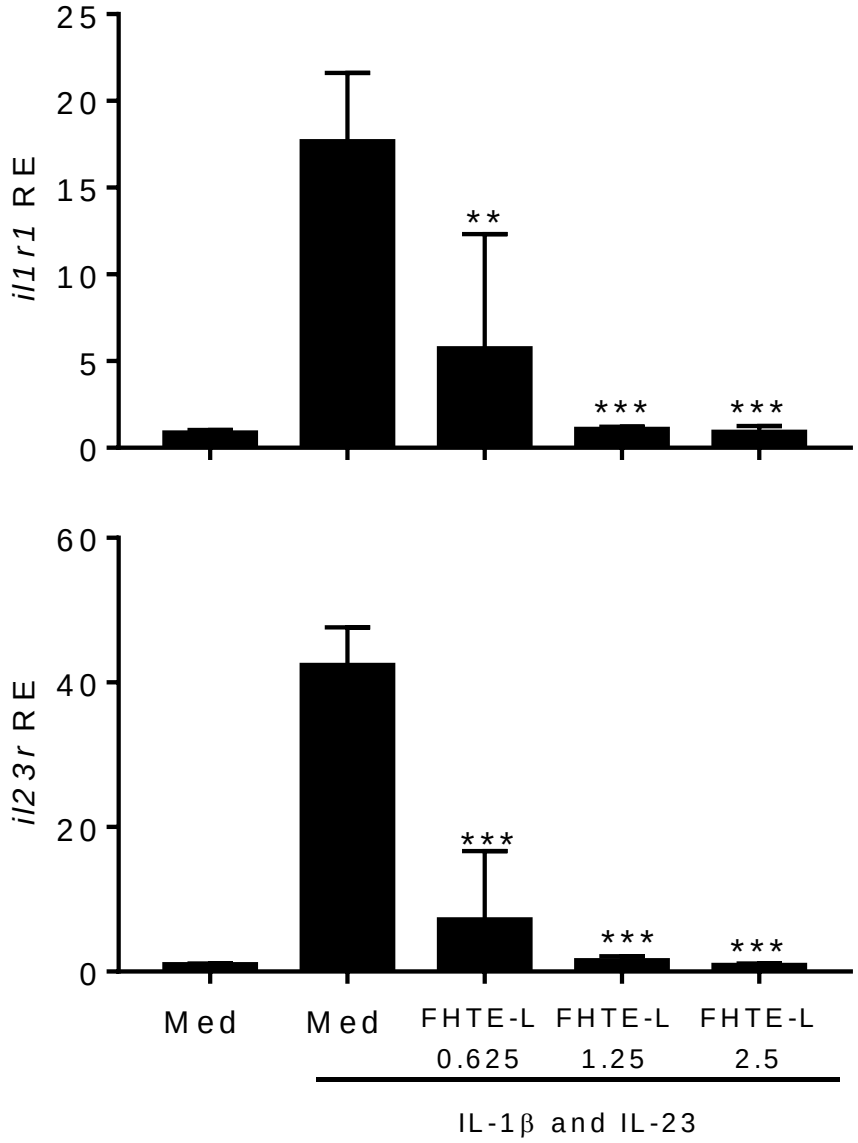

Figure 5.9 FHTE-L suppressed IL-1β and IL-23-induced *il1r1* and *il23r* expression on γδ T cells. γδ T cells were MACS purified from the LNs of naïve C57BL/6 mice and incubated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of increasing concentrations of FHTE-L (0.625-2.5% v/v). After 72 hr, cells were lysed, total RNA was extracted and the expression of *il1r1* and *il23r* was evaluated by qRT-PCR relative to medium cultured cells following normalisation by the endogenous control 18s rRNA. Results are mean ±SD for triplicate culture and are representative of two independent experiments. **p<0.01, ***p<0.001 vs IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

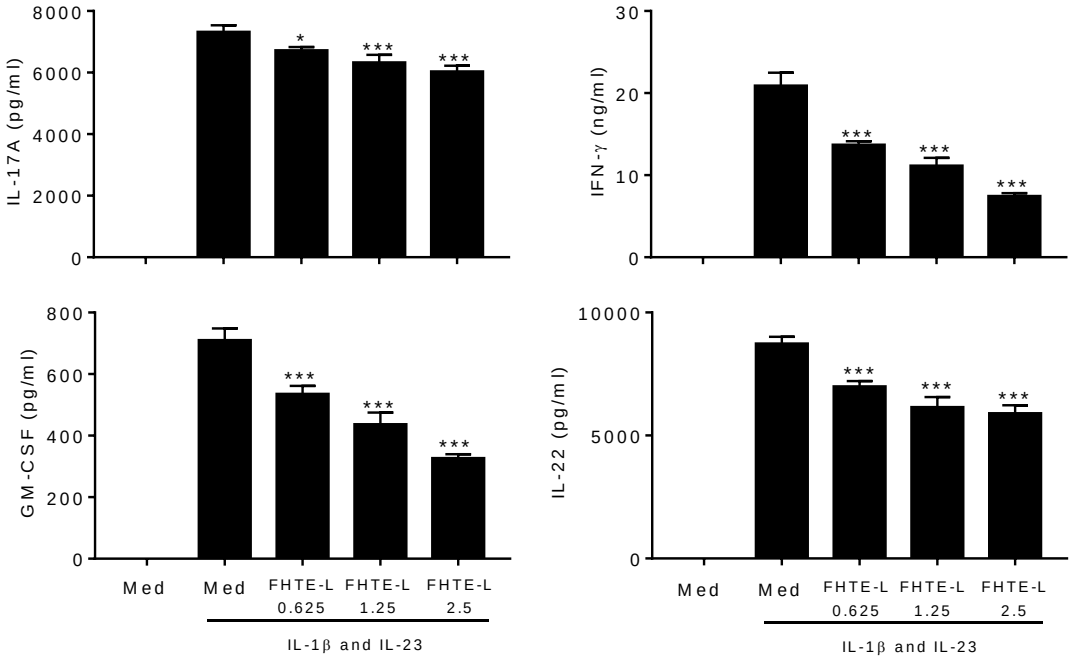

Figure 5.10 FHTE-L suppressed IL-1β and IL-23-induced IL-17A and IFN-γ production by γδ T cells. γδ T cells were MACS purified from the LNs of naïve C57BL/6 mice and incubated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of increasing concentrations of FHTE-L (0.625-2.5% v/v). Supernatants were collected after 72 hr and the concentration of IL-17A, IFN-γ, GM-CSF and IL-22 determined by ELISA. Results are mean ±SD for triplicate culture and are combined from two separate experiments. *p<0.05, ***p<0.001 vs IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

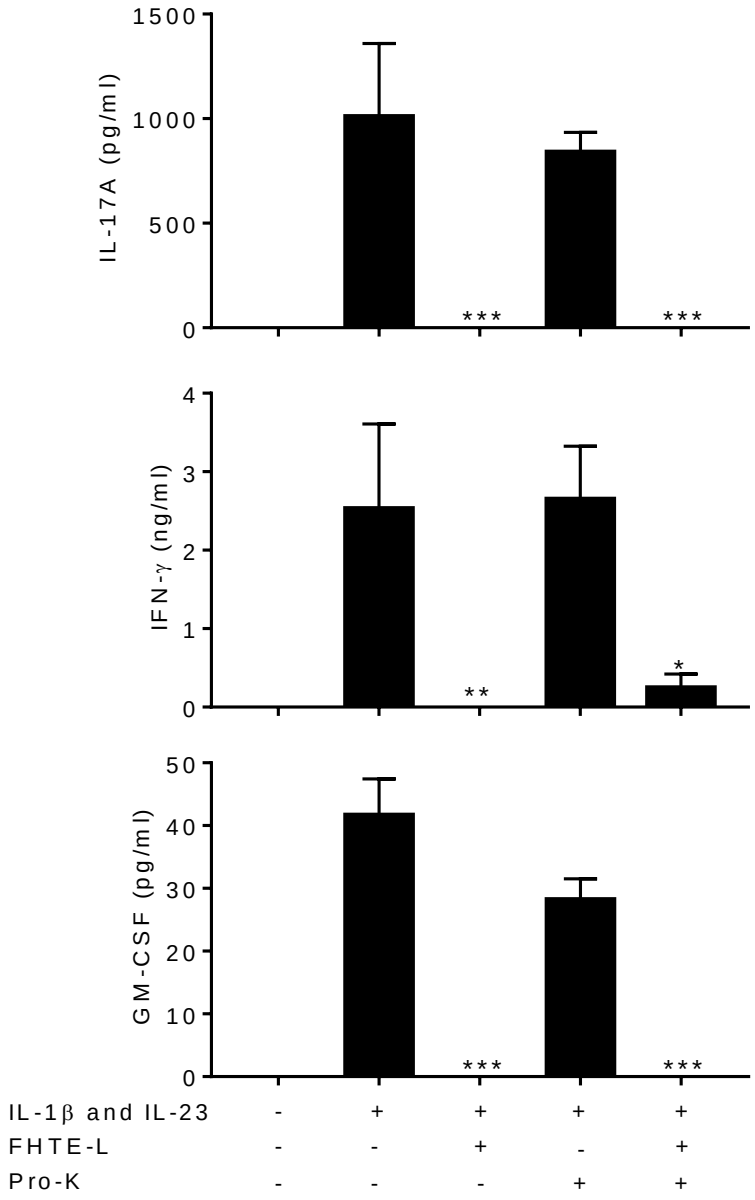

Figure 5.11 Proteinase K does not reverse the immunomodulatory effects of FHTE-L on γδ T cells. γδ T cells were MACS purified from the LNs of naïve C57BL/6 mice and incubated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of 2.5% v/v FHTE-L that had been preincubated with proteinase K (50 µg/ml) for 10 mins, followed by heat inactivation (95°C) prior to use. Supernatants were collected after 72 hr and the concentration of IL-17A, IFN-γ and GM-CSF determined by ELISA. Results are mean ±SD for triplicate culture and are combined from two separate experiments. *p<0.05, **p<0.01, ***p<0.001 vs IL-1β and IL-23 by three-way ANOVA with Tukey post-test.

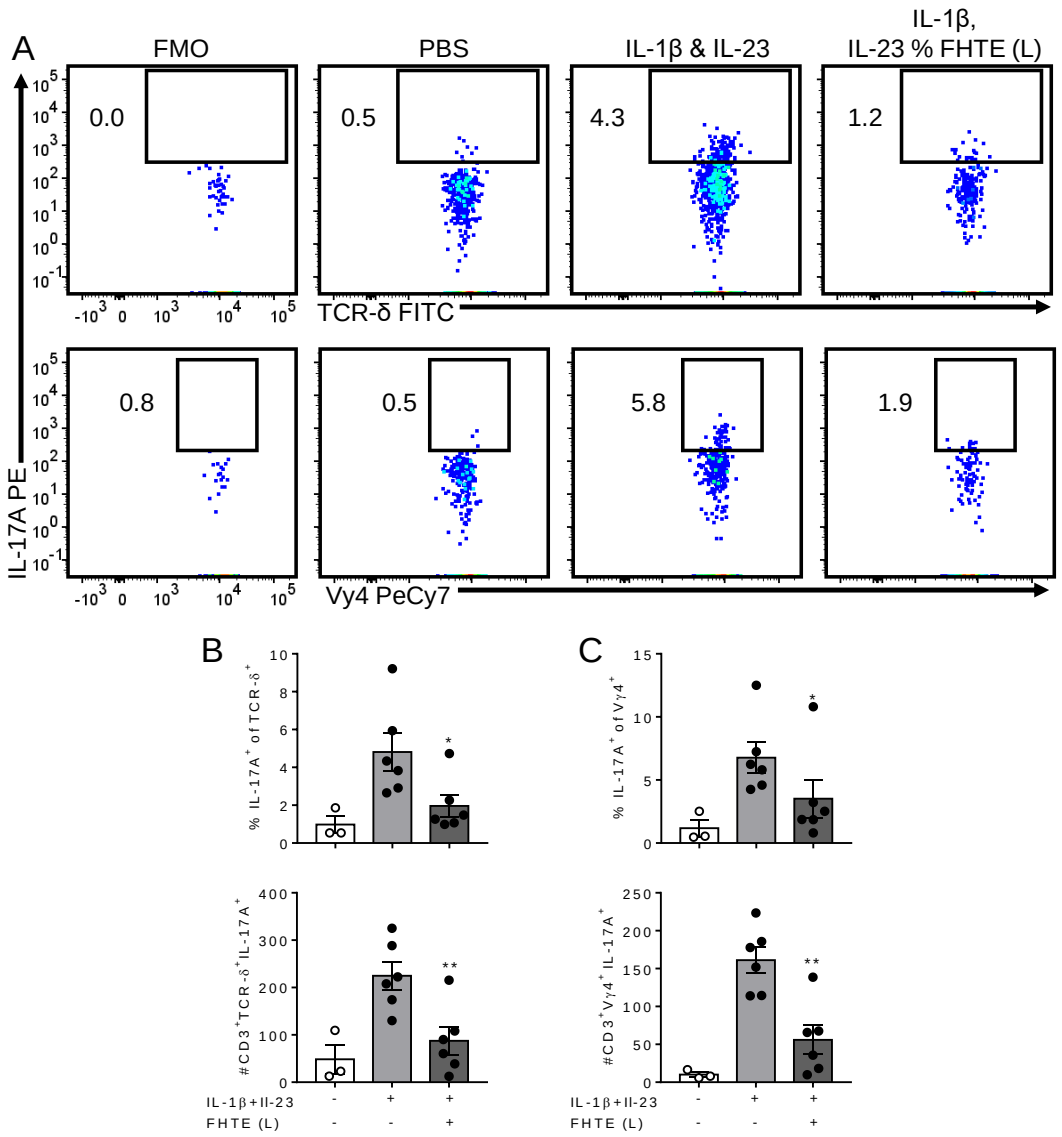

Figure 5.12 FHTE-L inhibited IL-1β and IL-23-induced IL-17A production by γδ T cells *in vivo.* C57BL/6 mice were injected into the footpad with PBS, IL-1β (10 ng/ml) and IL-23 (10 ng/ml) or IL-1β, IL-23 and FHTE-L (2.5% v/v in 200 µl PBS). After 4 hr, the popliteal LNs were isolated and stained for surface CD3, TCR-δ and Vγ4 and intracellular IL-17A. Results are representative flow cytometry plots for IL-17A-producing TCR-δ$^+$ or IL-17A-producing Vγ4$^+$ T cells (A), percentage and absolute numbers of IL-17A-producing TCR-δ$^+$ T cells (B) and percentage and absolute numbers of IL-17A-producing Vγ4$^+$ T cells. Data are mean ± SEM (n=5) and each symbol represents an individual mouse. *p<0.05, **p<0.01 vs IL-1β and IL-23 by one-way ANOVA with Dunnett post-test.

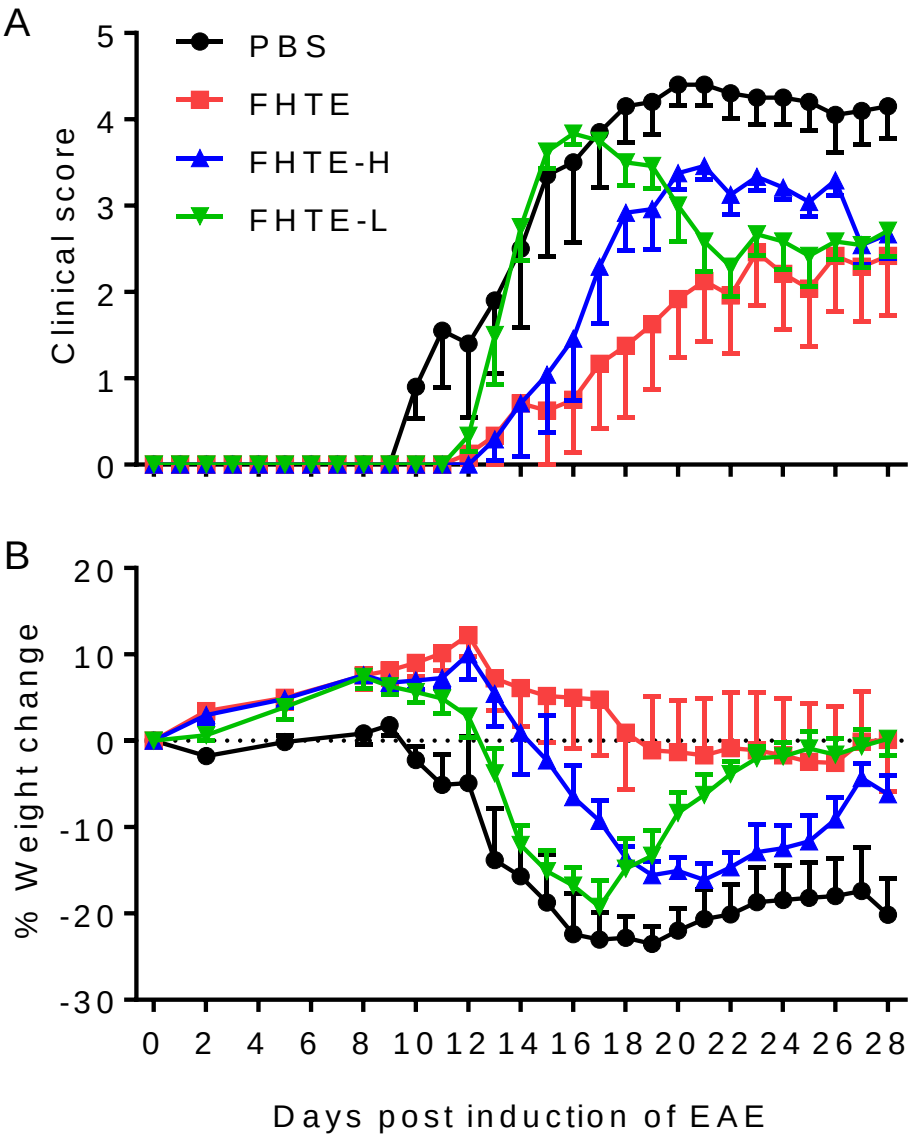

Figure 5.13 Training of mice with FHTE-H attenuates EAE. C57BL/6 mice were injected s.c with PBS or FHTE, FHTE-H or FHTE-L (2.5% v/v in 200 µl PBS) 21 days and 7 days before the induction of EAE. EAE was induced by s.c injection of MOG peptide (100 µg) emulsified in CFA, followed by i.p injection of PT (100 ng) on day 0 and day 2. Mice were assessed daily for clinical signs of the disease and weighed. Results are mean ± SEM (n=6) for clinical score (A) and percentage body weight change (B).

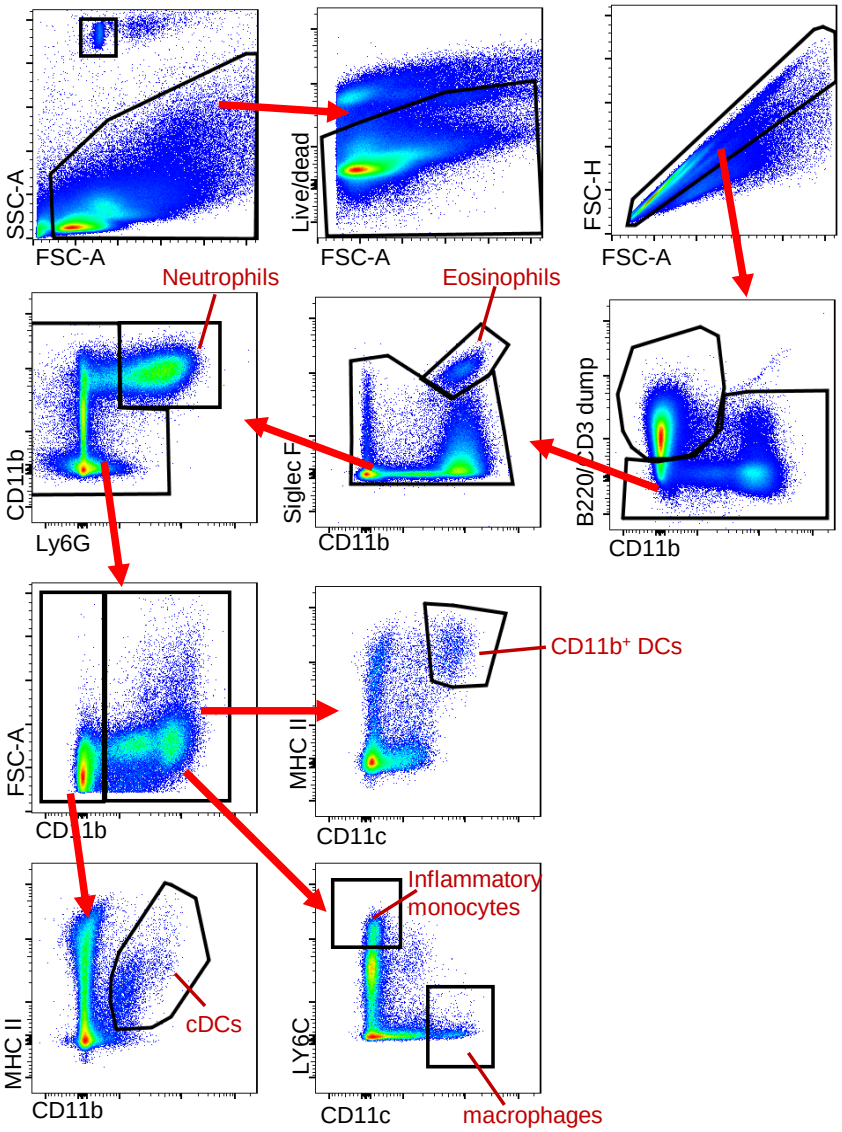

Figure 5.14 Gating strategy for analysing the innate immune populations in the spleen after the induction of EAE. Mice were trained and EAE was induced in mice as described in figure 5.13. On day 3 after induction, mice were sacrificed and cells were isolated from the spleen. Cells were stained for viability and surface B220, CD3, CD11b, Ly6G, Siglec F, Ly6C, CD11c and MHC II in the presence of the Fc receptor blocking antibody. Neutrophils were identified based on their expression of CD11b and Ly6G. Eosinophils express high expression of Siglec F. Conventional DCs (cDCs) were identified as $CD11b^{-}CD11c^{+}MHCII^{+}$. Furthermore, $CD11b^{+}$ cells were divided in three populations: $CD11b^{+}CD11c^{+}MHCII^{+}$ DCs, $CD11b^{+}Ly6C^{+}CD11c^{-}$ inflammatory monocytes and $CD11b^{+}Ly6C^{-}CD11c^{+}$ macrophages.

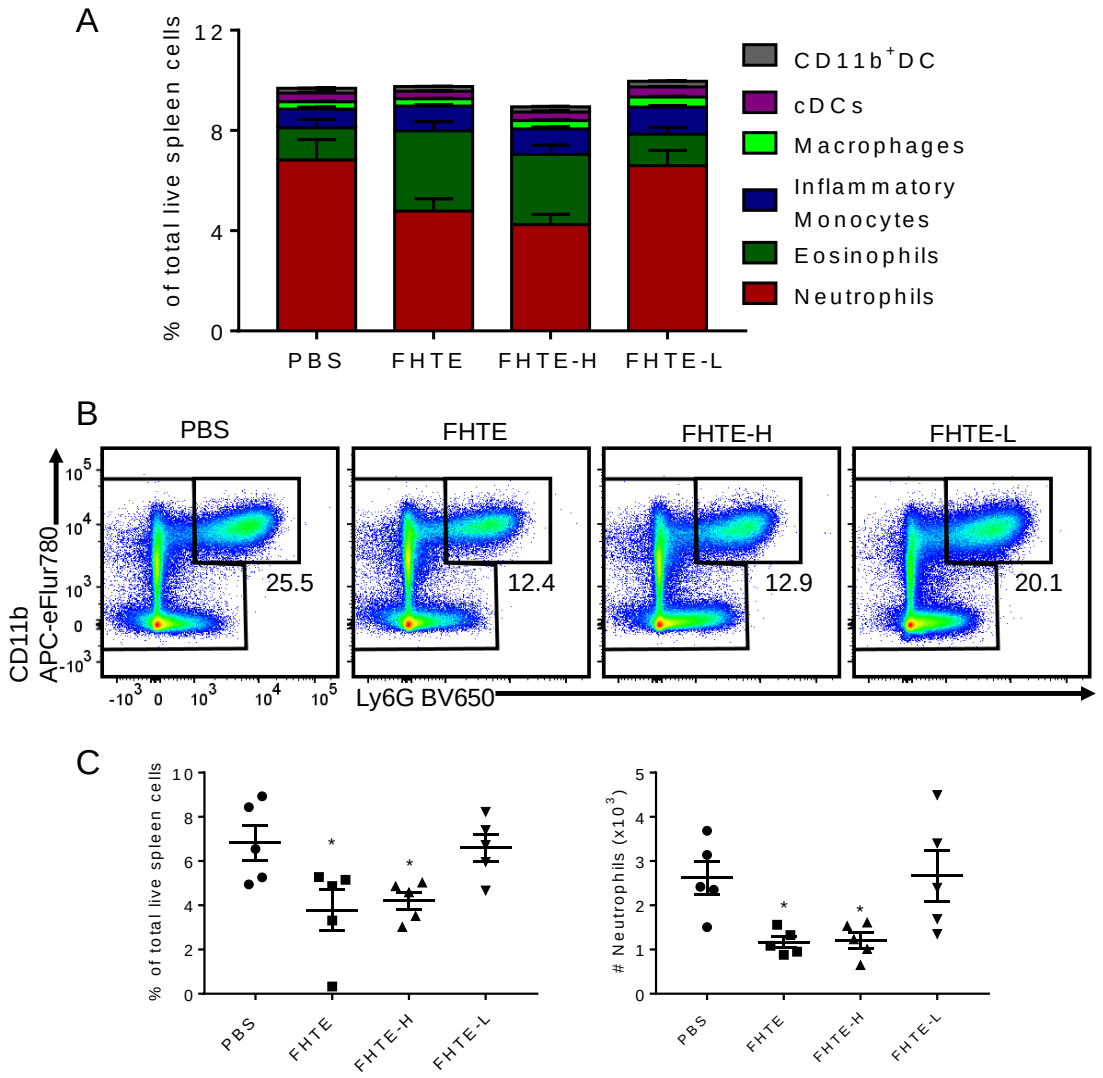

Figure 5.15 Training of mice with FHTE and FHTE-H suppressed neutrophil recruitment to the spleen on day 3 after the induction of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 5.13. On day 3 after induction, mice were sacrificed and cells were isolated from the spleen and stained for CD11b, Ly6G, Siglec F, Ly6C, CD11c and MHC II and analysed by flow cytometry. (A) Different innate cell populations as a percentage of total live cells in the spleen of each treatment group. (B) Representative FACS plots of neutrophils in the spleen ($CD11b^+$ $Ly6G^+$ as % of parent). (C) Percentage (of total live cells) and absolute number of neutrophils in the spleen. Data are mean ± SEM (n=5) and each symbol represents an individual mouse. *p<0.05 vs PBS by one-way ANOVA with Dunnett post-test.

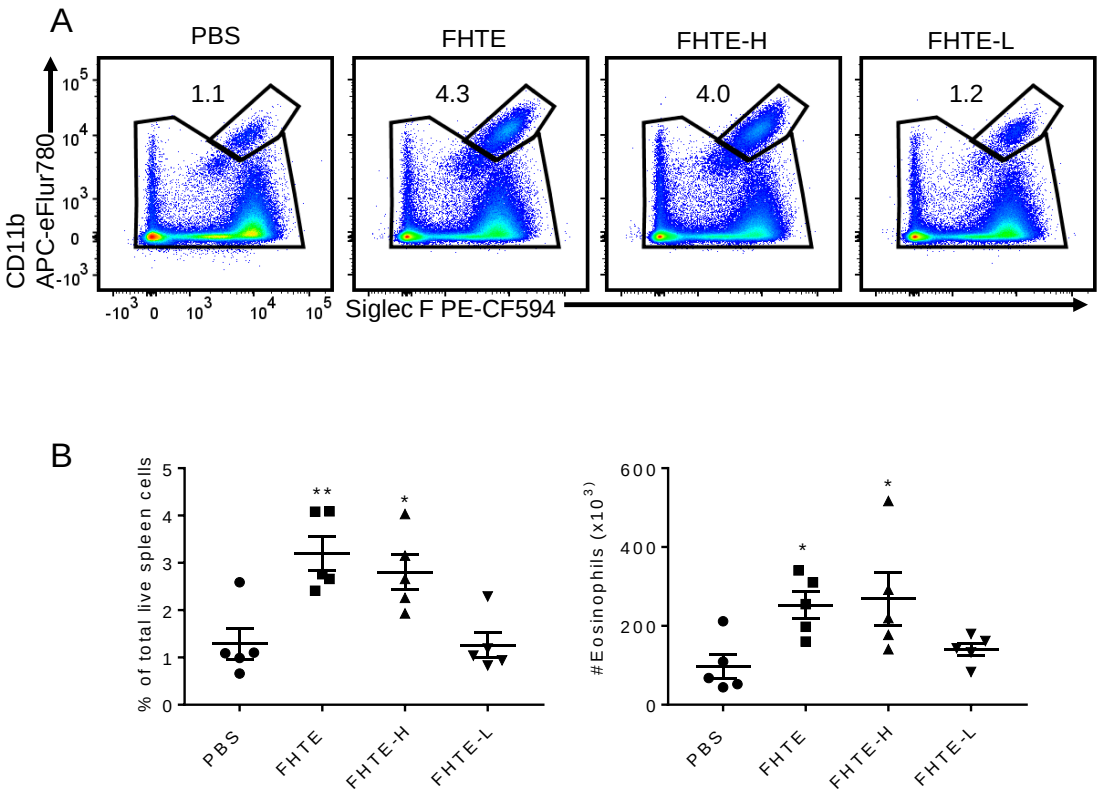

Figure 5.16 Training of mice with FHTE and FHTE-H enhanced eosinophil recruitment to the spleen on day 3 after the induction of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 5.13. On day 3 after induction, mice were sacrificed and cells were isolated from the spleen and stained for CD11b and Siglec F and analysed by flow cytometry. (A) Representative FACS plots of eosinophils in the spleen ($CD11b^+$ $SiglecF^+$ as % of parent). (B) Percentage (of total live cells) and absolute number of eosinophils in the spleen. Data are mean ± SEM (n=5) and each symbol represents an individual mouse. *p<0.05, **p<0.01 vs PBS by one-way ANOVA with Dunnett post-test.

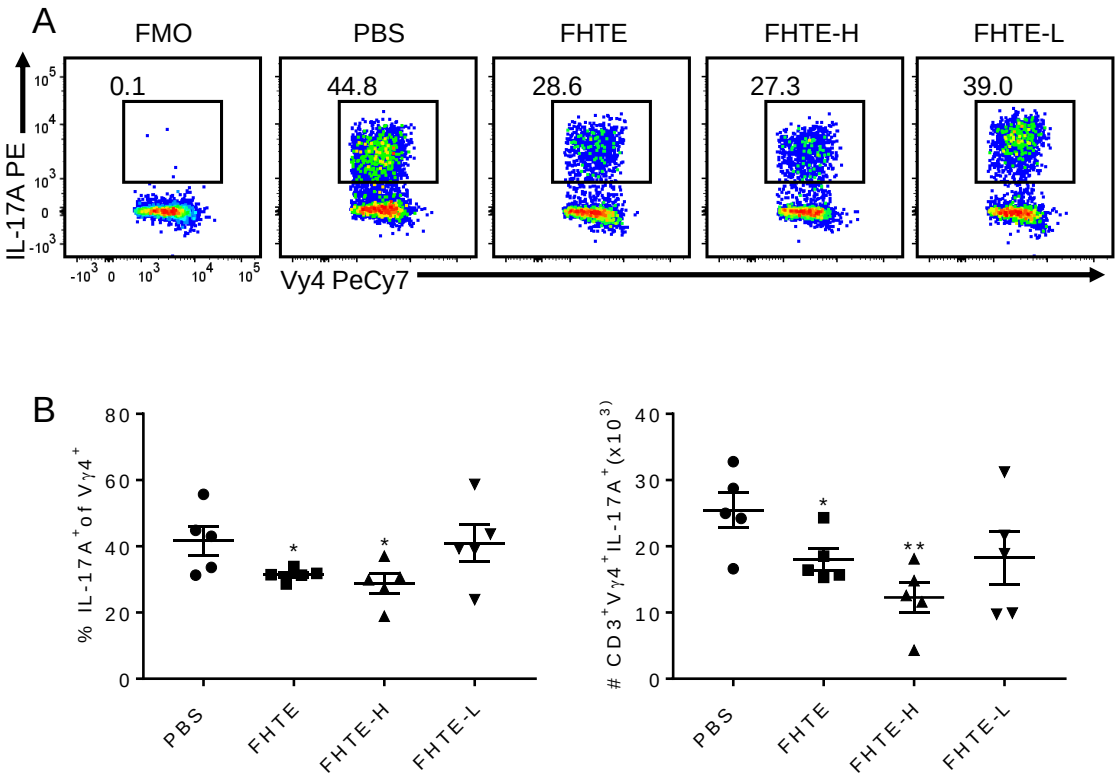

Figure 5.17 Training of mice with FHTE-H suppressed IL-17A production by Vγ4 T cells in the LNs on day 3 after EAE induction. C57BL/6 mice were trained and EAE was induced as described in figure 5.13. On day 3 after induction, mice were sacrificed and cells were isolated from the inguinal, brachial and axillary LNs. Cells were stained for surface CD3, Vγ4 and intracellular IL-17A and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+Vγ4^+$ T cells) (A) and percentage and absolute number of IL-17A-producing $Vγ4^+$ T cells in the LNs (B). Data are mean ± SEM (n=5) and each symbol represents an individual mouse. *p<0.05, **p<0.01 vs PBS by one-way ANOVA with Dunnett post-test.

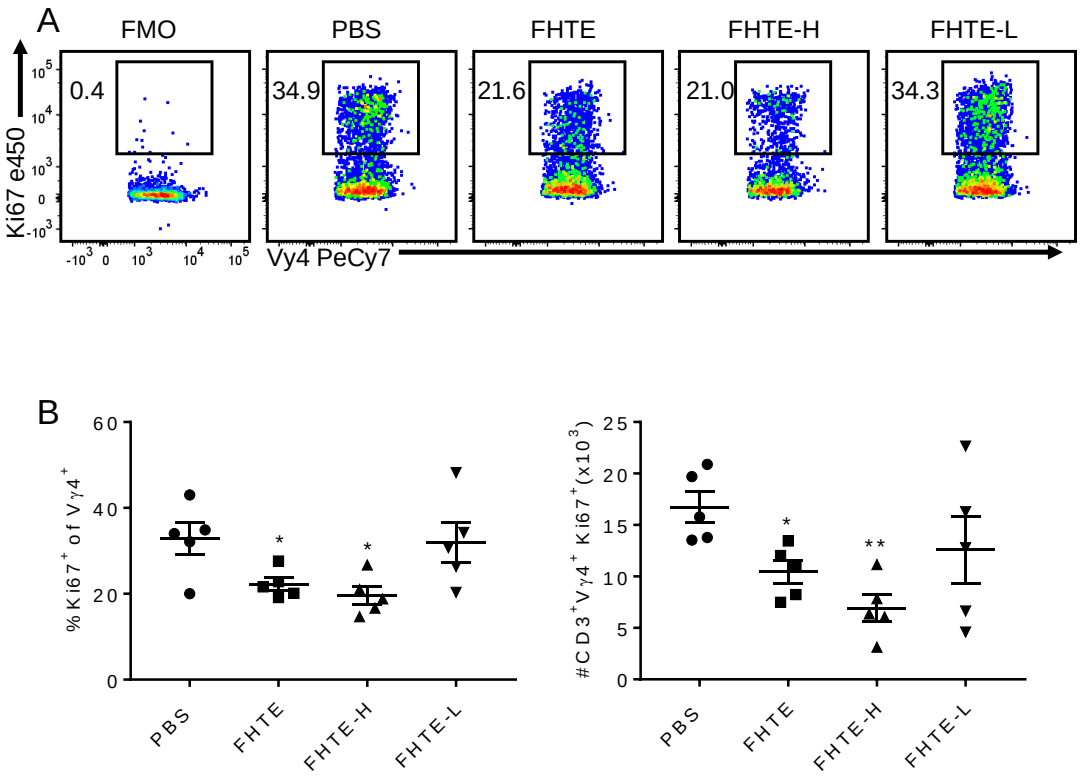

Figure 5.18 Training of mice with FHTE-H suppressed proliferation of Vγ4 T cells in the LNs on day 3 after EAE induction. C57BL/6 mice were trained and EAE was induced as described in figure 5.13. On day 3 after induction, mice were sacrificed and cells were isolated from the inguinal, brachial and axillary LNs. Cells were stained for surface CD3, Vγ4 and intracellular IL-17A and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+Vγ4^+$ T cells) (A) and percentage and absolute number of $Ki67^+$ proliferating $Vγ4^+$ T cells in the LNs. Data are mean ± SEM (n=5) and each symbol represents an individual mouse. *p<0.05, **p<0.01 vs PBS by one-way ANOVA with Dunnett post-test.

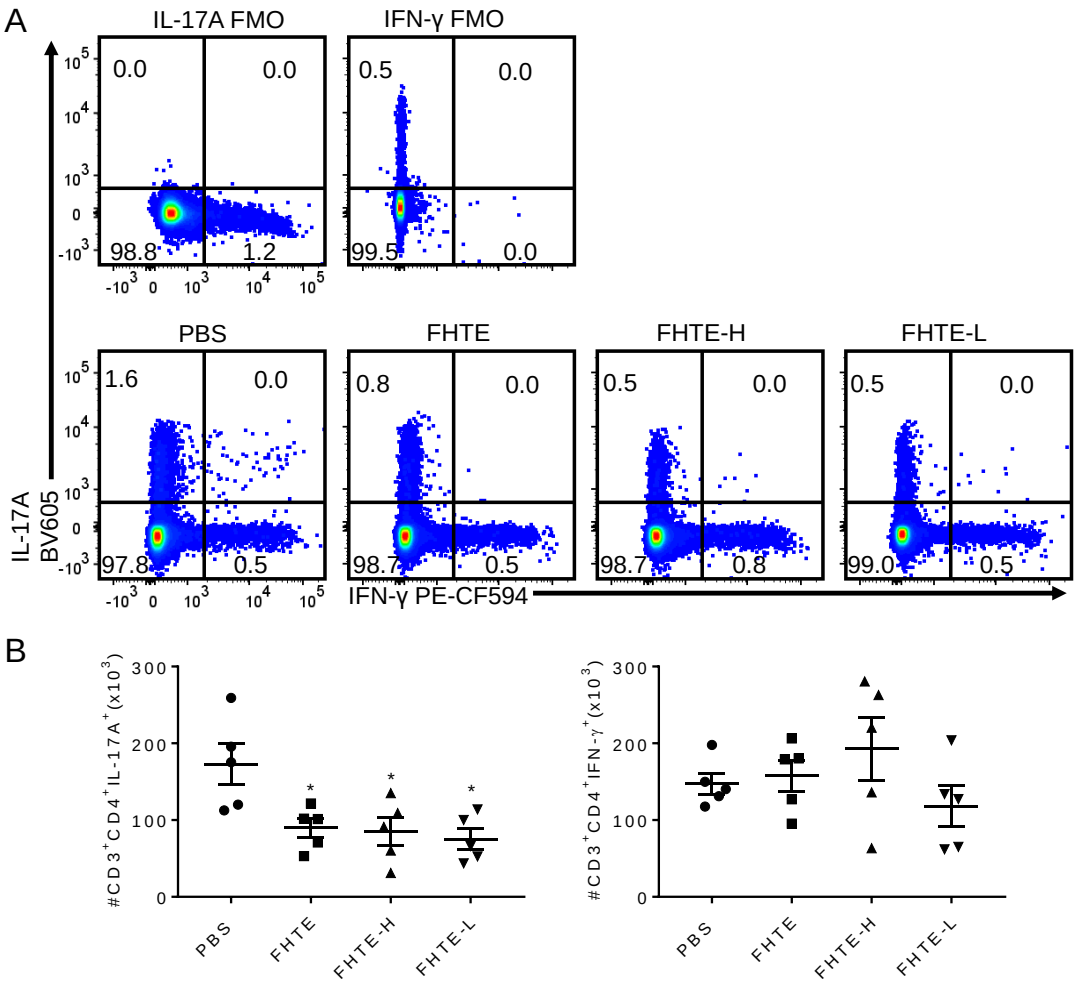

Figure 5.19 Training of mice with FHTE-H and FHTE-L suppressed IL-17A production by CD4 T cells in the LNs on day 7 after EAE induction. C57BL/6 mice were trained and EAE was induced as described in figure 5.13. On day 7, mice were sacrificed and cells were isolated from the inguinal, brachial and axillary LNs. Cells were stained for surface CD3 and CD4 and intracellular IL-17A and analysed by flow cytometry. Results are representative FACS plots for each treatment group (gated on $CD3^+CD4^+$ cells) (A) and absolute number of IL-17A-producing or IFN-γ-producing $CD4^+$ T cells in the LNs (B). Data are mean ± SEM (n=5) and each symbol represents an individual mouse. *p<0.05 vs PBS by one-way ANOVA with Dunnett post-test.

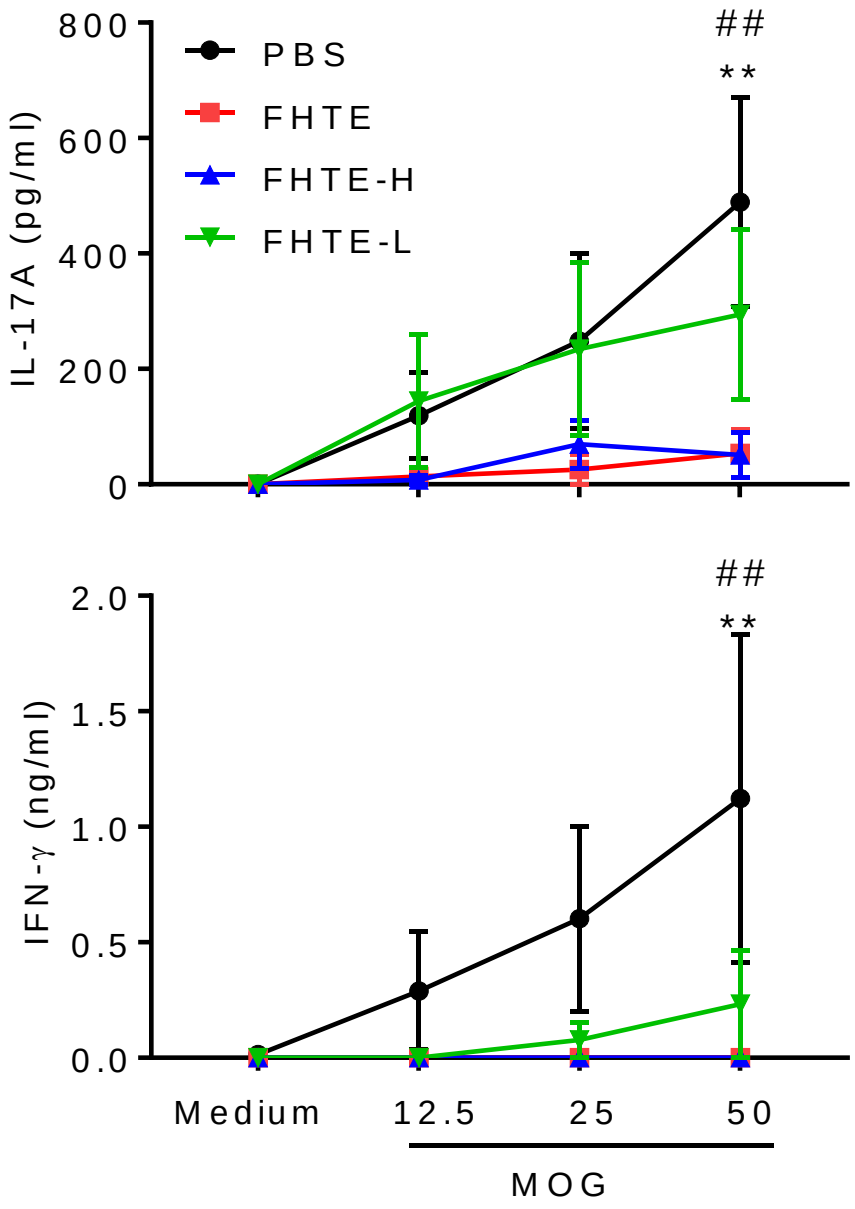

Figure 5.20 MOG-specific IL-17A and IFN-γ production by spleen and LN cells is reduced in FHTE-H-trained mice. C57BL/6 mice were trained and EAE was induced as described in figure 5.13. On day 7 after induction, mice were sacrificed and cells were isolated from the spleen and the inguinal, brachial and axillary LNs. Cells were restimulated with increasing concentrations of MOG (12.5, 25 and 50 µg/ml). After 72 hr, supernatants were collected and the concentrations of IL-17A and IFN-γ were quantified by ELISA. Data are mean ± SEM (n=5). **p<0.01 PBS vs FHTE, ##p<0.01 PBS vs FHTE-H by two-way ANOVA with Tukey post-test.

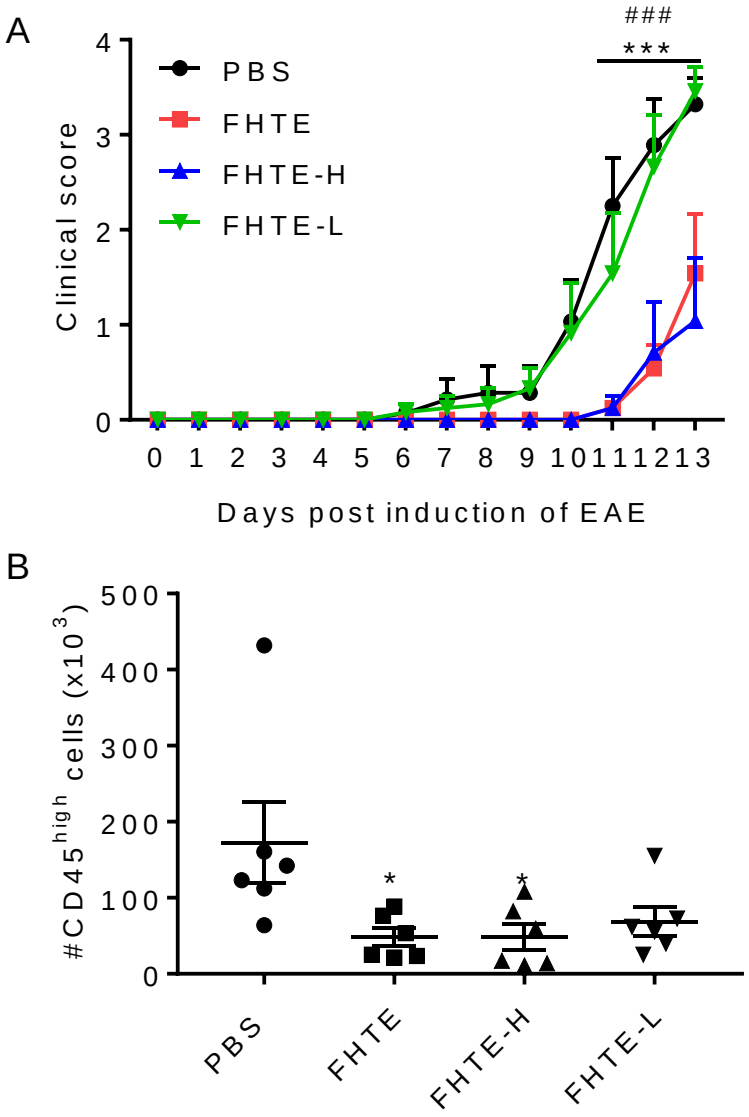

Figure 5.21 FHTE-H-trained mice have significantly reduced numbers of infiltrating immune cells in the brain on day 13 of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 5.13. On day 13 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the brain, stained with surface CD45 and analysed by flow cytometry. (A) Results are mean clinical score, mean ± SEM (n=6) and each symbol represents an individual mouse. ***p<0.001 PBS vs FHTE, ###p<0.001 PBS vs FHTE-H by repeated measures ANOVA with Tukey post-test. (B) Absolute number of infiltrating immune cells in the brain. Data are mean ± SEM (n=6) and each symbol represents an individual mouse. *p<0.05 vs PBS by one-way ANOVA with Dunnett post-test.

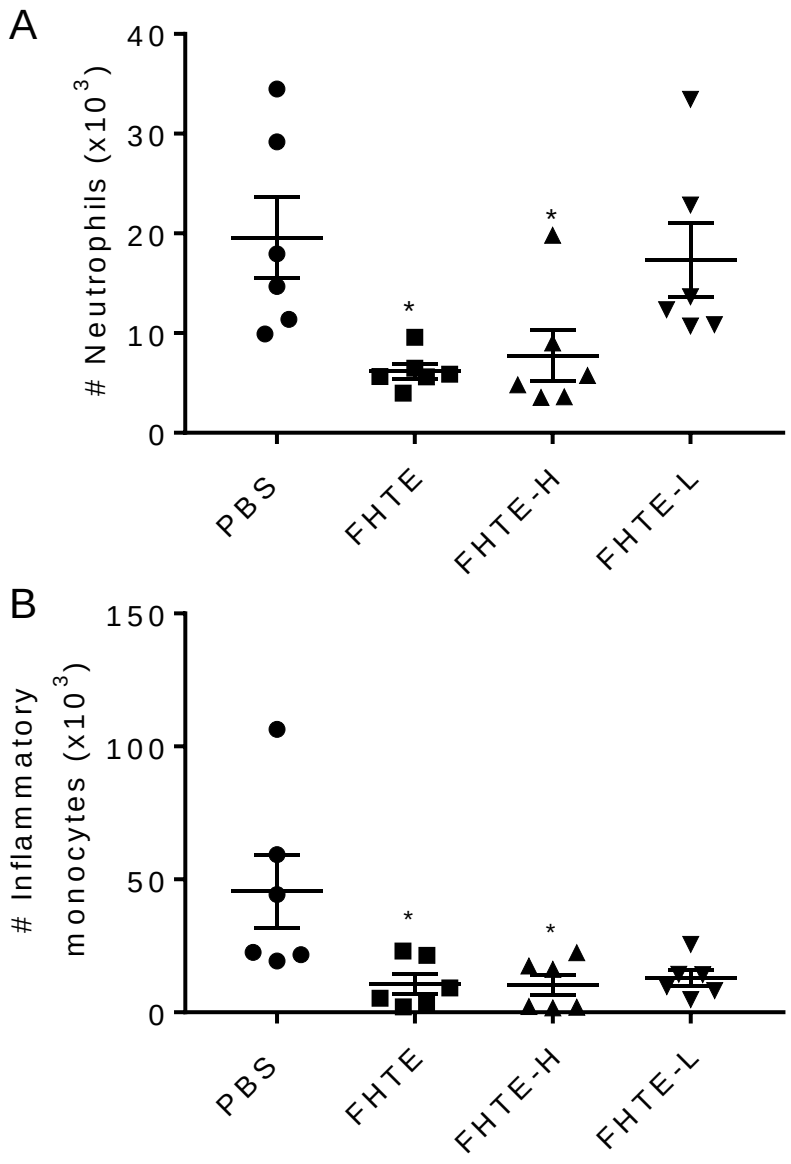

Figure 5.22 Reduced numbers of neutrophils and inflammatory monocytes in the brain of FHTE and FHTE-H-trained mice on day 13 of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 5.13. On day 13 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the brain and stained for surface CD45, CD11b, LY6G and LY6C. Data are mean ± SEM (n=6) and each symbol represents an individual mouse. *p<0.05 vs PBS by one-way ANOVA with Dunnett post-test.

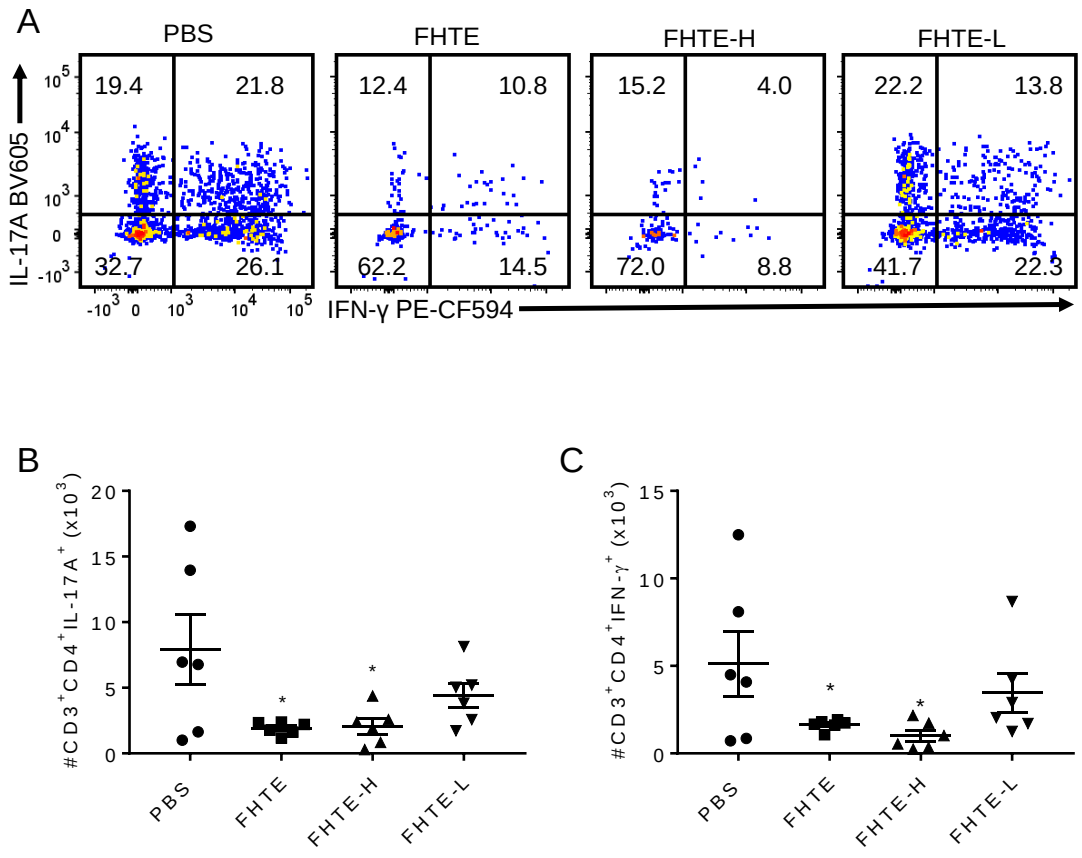

Figure 5.23 FHTE-H-trained mice have significantly reduced numbers of IL-17A and IFN-γ-producing CD4 T cells in the brain on day 13 of EAE. C57BL/6 mice were trained and EAE was induced as described in figure 5.13. On day 13 after EAE induction, mice were sacrificed and perfused. Cells were isolated from the brain and stained for surface CD3 and CD4 and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are representative FACS plots (gated on $CD3^+CD4^+$ T cells) (A), absolute number IL-17A-producing $CD4^+$ T cells (B) and absolute number IFN-γ-producing $CD4^+$ T cells (C) Data are mean ± SEM (n=6) and each symbol represents an individual mouse. *$p<0.05$ vs PBS by one-way ANOVA with Dunnett post-test.

Chapter 6.
General Discussion

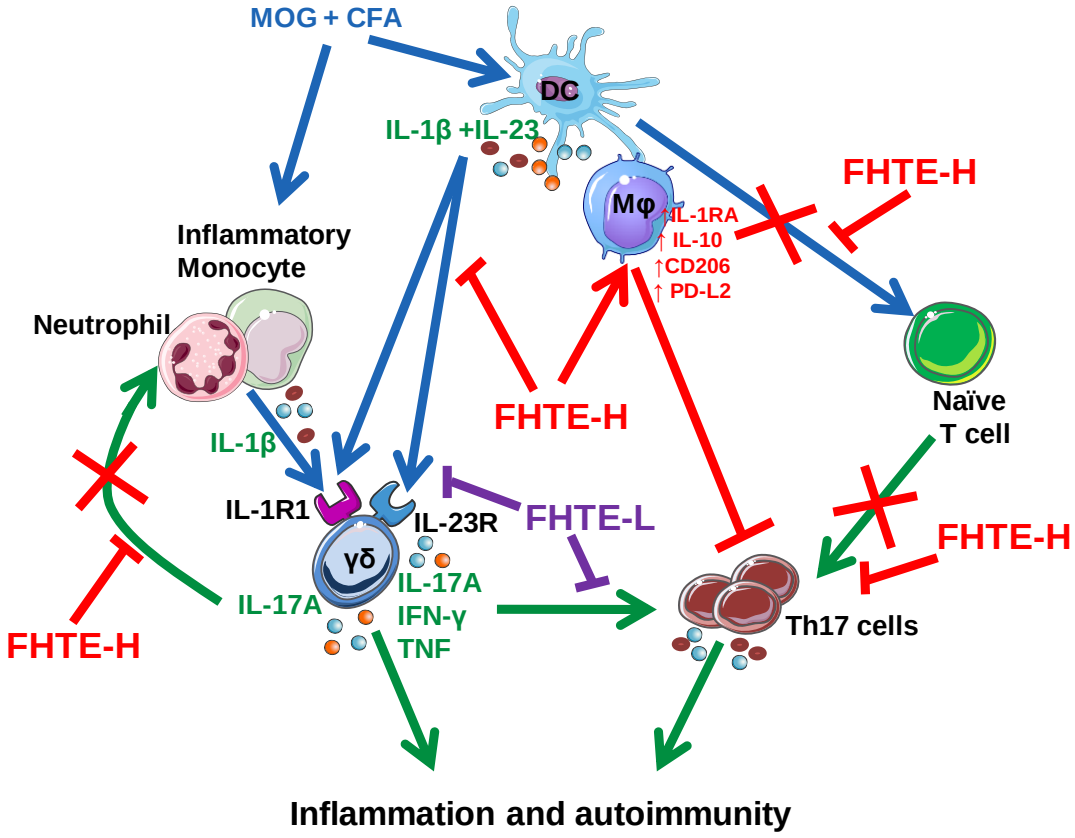

Figure 6.1 Immunomodulatory effects of FHTE

EAE is initiated in the periphery where DCs and other innate immune cells produce IL-1β and IL-23 in response to MOG and CFA. IL-1β and IL-23 drives early IL-17A production by γδ T cells, which initiates inflammation and promotes infiltration of neutrophils and inflammatory monocytes and the production of innate IL-1β and IL-6. Simultaneously, IL-1β and IL-23 production by DCs, along with presentation of MOG antigen to naïve T cells, results in the differentiation and expansion of Th17 cells. The encephalitogenic activity of these cells is further enhanced by IL-17A-producing γδ T cells. FHTE-H inhibits IL-1β and IL-23 production by DCs resulting in suppressed effector function of these cells and inhibition of pathogenic Th17 cells. Furthermore, training of mice with FHTE-H polarizes M2 macrophages that results in reduced neutrophil recruitment and suppression of MOG-specific T cells responses. In addition, FHTE-L directly suppresses IL-1β and IL-23 receptor expression on the surface of γδ T cells, resulting in potent inhibition of IL-17A and IFN-γ.

This study has identified novel mechanisms through which products of the helminth parasite *F. hepatica* can modulate immune responses that mediate inflammation, in a murine model of autoimmune disease. The most significant finding was that treatment of mice with FHTE could train macrophages to be more anti-inflammatory, thereby suppressing the induction of innate and adaptive pathogenic inflammatory responses in the CNS autoimmune disease, EAE. Previous studies have reported that human monocytes could be trained to be more pro-inflammatory [111, 112]. However, this study demonstrated for the first time that the innate immune system can be trained with helminth products to be more anti-inflammatory and suggests priming of the innate immune system with the appropriate environmental cues can influence the development of autoimmunity. In addition, the findings revealed that FHTE directly suppresses encephalitogenic T cells, in particular γδ T cells, impairing their ability to proliferate and produce IL-17A, IFN-γ and GM-CSF, thus reducing their pathogenic activity in a cell transfer model of EAE. These findings provide evidence of a novel approach evolved by helminths for targeting γδ T cells through downregulation of IL-1 and IL-23 receptor expression. Furthermore, this suggests that IL-17A-seceting γδ T cells, in particular the Vγ4 subset, may be important in protective immunity against helminth parasites.

Changes in life style, improved sanitation and medical interventions in industrialized countries have led to a decrease in infectious diseases. Concurrently, the incidence in allergy and autoimmune diseases have increased over the last few decades. This forms the basis of the hygiene hypothesis, which proposes that a lack of childhood exposure to pathogens increases the tendency to develop allergy and autoimmune diseases [170]. Originally described in the 1980s, Strachan observed an inverse correlation between birth order, sibling size and the rate of hay fever [161]. Moreover, a report from the US showed that the incidence of asthma increased by 75% from 1980 to 1994 [287]. A number of environmental factors have been implicated in the risk of developing immune-mediated diseases, including exposure to farm livestock and antibiotic use [288–291]. Furthermore, a report on identical twins demonstrated that 77% of immunological parameters are determined by non-inheritable influences, highlighting the importance of environmental factors in immune-mediated

phenotypes [292]. In addition, the hygiene hypothesis was later extended from the field of allergy to autoimmune diseases. Kilpatrick et al. demonstrated that children with a higher number of siblings had a reduced risk of developing MS [167]. The authors suggested that this was due to increased exposure to varying pathogens. In addition, cigarette smoking is a well characterised risk factor for MS and increased exposure to sunlight and vitamin D is associated with reduced risk of developing MS, due to enhanced Treg responses [293–295].

Since the first observations by Godfrey and colleagues [210], a number of studies have reported that infection with helminths, mainly in the developing world, is associated with a reduced frequency of allergy and autoimmune diseases [175, 241]. Helminths are successful pathogens that have evolved several mechanisms of immune subversion, which not only prevent parasite expulsion but also limit immunopathology during infection. In the context of allergy, Hagel et al. demonstrated that helminth infection of children in urban slums in Venezuela resulted in reduced allergic responses to environmental allergens [296]. A similar observation was reported in The Gambia, where the risk of asthma was lower in adults infected with intestinal helminths [297]. Moreover, a milder course of asthma was observed in patients infected with *S. mansoni*, when compared to uninfected controls [241]. In addition, studies in autoimmunity have shown that helminth-infection of MS patients is associated with reduced number of relapses, as well as an overall better disease outcome [175]. Furthermore, helminth infection has been implicated as an environmental factor associated with a lower risk of developing inflammatory bowel disease (IBD) [168, 298].

The epidemiological and experimental evidence to support the hygiene hypothesis has inevitably led to the suggestion that helminths could be exploited as potential therapeutics for inflammatory diseases. A number of studies have demonstrated effective treatment with live helminth in murine models of autoimmunity, including colitis [259], EAE [159], type 1 diabetes [176] and arthritis [299]. Furthermore, two species of helminths, *T. suis* and *Necator americanus,* have shown promising results in the treatment for Crohn's disease and ulcerative colitis [177, 300–304]. Moreover, live helminths have been tested as a therapy

for MS. Correale and Farez monitored MS patients that were infected with helminths over a 5-year period and reported significantly lower disease scores and relapse rates in helminth-infected patients [175]. Reduced disease severity in helminth-infected MS patients was associated with decreased IL-12 and IFN-γ production in peripheral blood. Furthermore, a small clinical study involving live infection with the helminth *T. suis* in 5 MS patients showed that repeated oral doses of pig whipworm over 12 weeks was well tolerated by the patients, but was without significant beneficial effect [301, 302]. These results indicate that more research is required to fully explore the safety, protective effects and mechanisms of helminth immunomodulation. Furthermore, ethical and practical considerations in the delivery and treatment of patients with live helminths suggest that this approach will not be a licensed therapeutic for allergy or autoimmune diseases in humans. However, helminth-derived products provide an invaluable tool to exploit helminth immunomodulation, without the unwanted pathological responses associated with live infection. They may also assist in the identification and targeting of pathways involved in pathogenic immune responses that mediate allergy and autoimmune diseases.

During the induction phase of autoimmune diseases, DCs and other innate immune cells become activated in response to PAMPs and DAMPs and co-stimulatory molecule expression is enhanced [305]. Furthermore, presentation of antigens to T cells via TCR-MHC II interactions and the production of T cell polarizing cytokines results in development of distinct effector T cell populations [30]. Previous research that focused on the immunomodulatory components of helminths has shown that FHES products can suppress TLR-activated co-stimulatory molecule expression and IL-12p70 production by DCs [191]. The current study demonstrated that FHTE can suppress LPS-induced CD40 and MHC II expression on DCs. Although MHC II has a well characterised role in a number of autoimmune diseases [265–267], targeting MHC II would not be an appropriate therapeutic approach due to the side effects associated with suppressing all CD4 T cell responses. However, Ruplizumab and Toralizumab, drugs which target CD40L, have demonstrated therapeutic efficacy in phase I/II clinical trials in systemic lupus erythematosus patients [272, 273]. The present study suggests that another effective approach of targeting DC-T cells

interactions may be through direct blockade of CD40, as blocking CD40 may lead to the induction of anergic T cells and prevent the development of autoantigen-specific Th1 and Th17 responses. Indeed, a number of studies in mice have shown protective effects of CD40 neutralization in allograft rejection [306, 307], however, this requires further investigation in the context of autoimmunity.

This study also revealed that FHTE induced IL-10 and IL-1RA, while suppressing TLR-induced production of IL-1β, IL-23 and IL-12p40 by DCs. Further investigation revealed that a high molecular weight fraction of FHTE suppressed IL-1β and IL-12p40 production and the immunomodulatory activity of FHTE-H was found to be protein-mediated. IL-1β is vital in the pathogenesis of EAE and works in synergy with IL-23 to promote the expansion of Th17 cells. Furthermore, IL-1β and IL-23 bind to IL-1R1 and IL-23R respectively on the surface of γδ T cells, resulting in early IL-17A production, which also promotes IL-17A expression by CD4 T cells [95]. Therefore, inhibition of TLR-induced IL-12p40, IL-1β and IL-23 secretion in response to FHTE may in part account for the suppression of autoreactive Th1 and Th17 cells observed during helminth infection [159]. In addition, the development of Treg cell responses through the induction of IL-10 from innate immune cells is a common feature of helminth infection [262, 263, 308], however, this is the first demonstration of helminth-derived proteins directly inducing IL-1RA secretion by immune cells. Although the IL-1RA based biological drug Anakinra (Kinerit) has been a target for the treatment of autoimmune and autoinflammatory diseases and was licenced for the treatment of rheumatoid arthritis (RA) in 2011, the benefits of Anakinra in other autoimmune diseases is less convincing. The results from the present study reveal that molecules that suppress IL-1β and IL-23, as well as promoting IL-1RA production may be a more effective target in treating autoimmune diseases.

One of the key findings of this study was the observation that helminth-derived products can protect mice against autoimmunity through innate immune training mechanisms. Until recently, immunological memory was thought to be confined to the adaptive immune system, were upon reinfection, memory T and B cells respond more rapidly and robustly. However, the observation that organisms that lack adaptive immune systems can still mount an immune

response to reinfection, led to the discovery that the innate immune system had memory or can be trained. Plants undergo a version of trained immunity called SAR, whereby epigenetic reprogramming of host defence molecules allows them to respond more effectively to reinfection [99, 101]. A study in tobacco plants revealed that exposure to tobacco mosaic virus led to resistance against subsequent infection [309]. Furthermore, invertebrates elicit non-specific innate immune memory that confers long-lasting protection against a broad spectrum of microorganisms. For example, the microbiota protects mosquitoes against *P. falciparum* via innate immune training [103]. Furthermore, initial exposure to LPS induces cross-protection in mealworm beetles, resulting in resistance to fungi [102]. In addition, copepod crustacean show features of trained immunity in response to the tapeworm *Schistocephalus solidus* [310].

The evidence of innate immune memory in vertebrates is expanding significantly, with a number of studies showing priming of innate immune cells through epigenetic modification or changes in glucose uptake and metabolism [111, 199, 200]. However, no studies to date have detailed innate immune memory in response to helminths or helminth-derived products. During exposure to bacterial or fungal products, innate immune cells undergo immunological imprinting of either tolerance or trained immunity that influences the capacity of the innate immune system to respond to re-exposure. LPS was shown to induce tolerance in human monocytes, characterised by suppressed pro-inflammatory responses, whereas β-glucan induced trained immunity accompanied by increased production of TNF and IL-6 [111]. This effect was found to be mediated by epigenetic changes in positive histone regulatory marks, for example H3K4me1, H3K4me3 and H3K27ac, resulting in heightened immune responses. Furthermore, activation of the cholesterol synthesis pathway was recently identified as being crucial for innate immune memory in myeloid cells [311]. Moreover, inhibition of the metabolite mevalonate was sufficient to prevent the induction of trained immunity, revealing that metabolism plays a fundamental role in innate immune memory [311].

The results presented here identified a new mode of innate immune training involving heightened anti-inflammatory responses generated by helminth-derived

products. Training of mice with FHTE polarised M2 macrophages and suppressed neutrophil recruitment, resulting in subsequent impairment of pathogenic T cell responses in the periphery and attenuation of the autoimmune disease, EAE. Furthermore, attenuation of CNS inflammation was mediated by components that where segregated in the high molecular weight fraction of FHTE. The results from this study not only add considerable weight to the hygiene hypothesis, but also provide a new mechanism of immunomodulation that could be exploited for the treatment of allergy and autoimmunity via targeting and reprogramming innate immune cells to be more anti-inflammatory. However, the role of innate immune training in inflammation that leads to the development of autoimmune diseases, and how this could be modulated, is only starting to be examined. A recent study by Wendeln et al. demonstrated that innate immune memory can be induced in the brain in response to peripheral LPS stimulation [312]. They demonstrated that immunological tolerance induced in macrophages in responses to LPS was characterised by a decrease in IL-1β, TNF and IL-6 but no change in IL-10 production, and led to suppressed pro-inflammatory cytokine production by microglia. They also showed, using a mouse model of Alzheimer's disease, that training of APP23 mice with LPS resulted in increased plaque burden, whereas the induction of innate immune tolerance decreased amyloid-β levels and reduced plaque formation [312]. The apparent adaptive capabilities of innate immune cells primed in response to changing environment appears to be an important feature of the immune system to protect against pathogens [102, 103]. However, trained immunity in response to environmental triggers may also result in disease-associated phenotypes.

The results presented in this thesis suggest that appropriate priming of the innate immune system may prevent aberrant T cell responses that lead to the development of autoimmune diseases. These findings also suggest that trained immunity that leads to a more anti-inflammatory or immunosuppressive response could be exploited in the design of novel therapeutic approaches for autoimmune diseases. Furthermore, another approach that could be explored is the design of novel and more effective vaccines that combine trained innate immunity with adaptive memory. Innate immune memory has already been implicated in the mechanisms of protection induced by the BCG vaccine. Vaccination with BCG

was found to increase survival beyond the burden of the target disease *M. tuberculosis* and improved overall survival of infants [104, 313]. More recently, BCG was found to induce innate immune training that protected mice from candida, confirming an non-specific effect mediated by epigenetic modifications of innate immune cells [314]. The discovery of non-specific trained immunity may help in the development of novel and more effective vaccination approaches but could also help to boost the efficiency of existing poorly protective vaccines. Moreover, innate immune training has been suggested as a possible adjuvant for immunotherapy in cancer [315]. Indeed, BCG is the main immunotherapy for treating early-stage bladder cancer [316, 317] and a recent study revealed that the induction of innate immune training and non-specific protective effects of BCG were autophagy-dependant [318]. In addition, β-glucan administration was shown to improve the overall quality of life in women with breast cancer that were undergoing chemotherapy [319]. Furthermore, promising results have been reported from clinical trials of chronic lymphatic leukaemia involving β-glucan and the monoclonal antibody against CD52 [320]. The results in the present study have demonstrated that training of mice with FHTE led to polarization of M2 macrophages that suppressed autoreactive T cell responses and protected against the development of EAE. It is therefore possible that polarising M1 macrophages through innate immune training might be a suitable and effective approach for cancer by interfering with the tolerant state of T cells observed during the development of tumours. Although preliminary results have suggested that immunotherapy for cancer can be more effective if epigenetic reprogramming of the innate immune system is incorporated into the treatment [321], this avenue of research requires further investigation.

Although most research on helminth-induced immunomodulation to date has focused on subversion of innate immune cells, the present study provides evidence of a novel method of immunomodulation evolved by helminths to directly target effector T cells. EAE can be induced by direct transfer of pathogenic CD4 T cells [66, 67]. Furthermore, TCR-$\delta^{-/-}$ mice had suppressed IL-17A production by CD4 T cells and were resistant to EAE, revealing a key pathogenic role for γδ T cells in the initiation of disease in the EAE model [61]. The results of the present study revealed that FHTE had a potent suppressive

effect on surface expression of IL-1R1 and IL-23R on γδ T cells, resulting in reduced receptor signalling and suppressed IL-17A and IFN-γ production. Further examination revealed that the immunomodulatory activity was not mediated by a protein complex and was present in the fraction that contained low molecular weight components. These results suggest that small molecules or indeed metabolites in helminth-derived products may be responsible for the immunosuppressive activity on γδ T cells. However, further research is required to fully identify the individual molecule(s) that mediate the direct immunosuppressive effects of FHTE on these cells.

γδ T cell activation is dependent on IL-1β and IL-23 signalling through their specific receptors, leading to production of IL-17A in the absence of TCR engagement [201]. As well as their role in infection, γδ T cells have been implicated in the development of a number of autoimmune diseases, including EAE [33]. IL-17A production by γδ T cells, in particular the Vγ4 subset, promotes the induction of Th1 and Th17 responses, amplifying their encephalitogenic function during EAE (McGinley et al., Unpublished). To date, modulation of γδ T cells has not been explored in the treatment of autoimmune diseases, however, targeting γδ T cell responses was shown to have promising effects in the treatment of cancer [322]. Wilhelm et al. reported that *in vivo* activation of γδ T cells by pamidronate and low dose IL-2 had anti-tumour activity in patients with low-grade non-Hodgkin lymphoma [323]. Furthermore, ongoing phase I/II clinical trials are targeting γδ T cells for the treatment of cancer, including the combination of *in vivo* activation plus adoptive transfer of γδ T cells [324, 325].

The findings of this study not only suggest that direct targeting of γδ T cell responses may be a useful approach for treating autoimmune diseases, but also adds weight to the idea that γδ T cells may be involved in protective immunity against helminth infection. The importance of γδ T cells in response to bacterial infection is well characterised [63, 64], however, only a few studies to date have demonstrated a role of γδ T cells in helminth infection. One study found that expulsion of adult worms from the intestine during *N. brasiliensis* infection was depend on γδ T cells, and more severe microscopic lesions were observed in

infected TCR-δ$^{-/-}$ mice [326]. Moreover, Božić et al. demonstrated that the frequency of γδ T cells increased significantly in the intestines of mice infected with *T. spiralis* [327]. They concluded that expulsion of adult worms from the small intestines of mice was mainly dependent on γδ T cells. In addition, early IL-17A production by γδ T cells may be required for the recruitment of neutrophils that are found in abundance in granulomas during *L. sigmodontis* infection and help limit worm translocation [283]. In humans and livestock, adult helminth worms are generally found in the gastrointestinal tract, where they exert physical and mechanical damage to the host. The majority of γδ T cells are found in the skin and at mucosal sites, including the intestines [328]. Therefore, it is not surprising that helminth-derived products influence γδ T cell function. The results presented in this thesis provide indirect evidence that limiting the activation of γδ T cells, in particular the Vγ4 subset, could be an important mechanism of subversion by helminths that may help to limit their expulsion from the host.

There is still an unmet medical need for new, effective and safe drugs to treat autoimmune diseases. A number of studies, including the current study, have demonstrated the importance of IL-1β and IL-23 signalling in the development of autoimmunity [95, 329]. Much of the research to date has focused on the IL-23/IL-17A axis for the treatment of autoimmune disease. Indeed blocking IL-17 is effective in psoriasis and ankylosing spondylitis, but not in colitis [330–332]. The current study revealed that targeting the receptor of the pathogenic cytokines IL-1β and IL-23 by helminth-derived products, suppressed activation of IL-17A-producing γδ and CD4 T cells that mediate EAE. Furthermore, suppression was specific for IL-1β and IL-23 as FHTE did not modulate T cell-mediated cytokine production induced in response to IL-12 and IL-18. These results indicate that IL-1β and IL-23 signalling may be critical cytokines in protective immunity to helminths and as a result suppression of the receptor for these cytokines may be a mechanism of immune evasion used by these parasites to avoid detection.

Before the discovery of Th17 cells, the pathology of EAE was thought to be mediated by Th1 cells. However, IL-23 was identified as a crucial differentiating factor for the development of Th17 cells and IL-23p19$^{-/-}$ mice

were found to be resistant to EAE [229]. IL-23 along with IL-1β is required for IL-17A production by γδ T cells which acts in a feedback loop to further enhance Th17 responses [201]. Ustekinumab, which targets IL-12p40, was the first drug to be approved for the treatment of psoriasis [333]. However, the anti-IL-17A drugs, Secukinumab and Brodalumab have shown to be more effective [334–336]. On the other hand, targeting IL-1 has proven more of a challenge. Unlike IL-23, which is only required for the initiation of EAE, IL-1 plays a role throughout this disease. Anakinra currently dominates the field of IL-1 therapeutics and has been in use for the treatment of RA since 2001 [337–339], however, its effects on other autoimmune diseases is less convincing. Further understanding of how helminths and helminth-derived products modulate IL-1 signalling will allow development of more appropriate and safe drugs for the treatment of autoimmune diseases.

Although helminth infection is associated with suppression of host pathogenic T cell responses, both directly and indirectly, the use of live helminth infection as a therapy for autoimmune diseases has many practical, ethical and safety concerns. However, the results from this thesis allow a greater understanding of immunity to helminths and how these parasites modulate and evade the immune system but also provide an invaluable insight into host regulatory mechanisms that control allergy and autoimmune diseases.

Chapter 7.
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