Helminth products promote anti-inflammatory trained innate immunity by imprinting long-term hematopoietic stem cells

Kyle Cunningham

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Supervisor: Professor Kingston Mills

Immune Regulation Research Group

School of Biochemistry and Immunology

Trinity College Dublin

Declaration of Authorship

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Kyle Cunningham

School of Biochemistry and Immunology

Trinity College Dublin

Abstract

Recent research has shown that immunological memory is not confined to the cells of the adaptive immune system but can be imbued upon innate immune cells, including monocytes and macrophages, in a process known as trained innate immunity. While the exact mechanisms remain largely uncharacterized, it is thought to require epigenetic and metabolic reprogramming induced by pathogens that modify the innate immune cell to respond faster and more robustly to secondary challenge. Recently, our laboratory has demonstrated that mature macrophages can undergo anti-inflammatory trained innate immunity following *in vitro* and *in vivo* exposure to helminth-derived products. Macrophages trained with helminth products secreted more anti-inflammatory but less pro-inflammatory cytokines, which resulted in modulated T cell responses that mediated the autoimmune disease experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. However, macrophages and monocytes are often short-lived cells, and this has led to uncertainty over the longevity of trained innate immunity in mature innate immune cells found in the periphery. There is emerging evidence to suggest that hematopoietic stem cells (HSCs) in the bone marrow (BM) can be modified to produce mature innate immune cells with enhanced pro-inflammatory effector functions in a process now known as central trained innate immunity.

This study demonstrated that excretory-secretory products of the helminth *Fasciola hepatica* (FHES) can imprint an anti-inflammatory phenotype on long-term HSCs (LT-HSCs) and monocyte precursor populations, enhancing their proliferation and differentiation into anti-inflammatory Ly6C^{low} monocytes. These monocytes expand and populate multiple compartments in mice, conferring hyporesponsiveness to pro-inflammatory stimuli and reduced susceptibility to induction of EAE. Furthermore, treatment of mice with FHES enhanced myelopoiesis, and reduced inflammatory innate immune responses and suppressed migration of pathogenic Th1 and Th17 cells into the central nervous system (CNS) during EAE.

Anti-inflammatory modifications to the BM persisted for up to 8 months. Furthermore, transplantation of whole BM from mice treated with FHES

transferred a bias for myelopoiesis and promoted the generation of anti-inflammatory macrophages in recipient mice that reduced susceptibility to the induction of EAE. Moreover, FACS-sorted LT-HSCs from mice treated with FHES were also capable of transplanting anti-inflammatory central trained innate immunity to recipient mice. Inhibition of epigenetic or metabolic reprogramming during FHES stimulation of BM cells blocked the development of anti-inflammatory central training *in vitro*.

The findings demonstrate that helminth-derived products can epigenetically and metabolically reprogram HSCs to promote development of anti-inflammatory myeloid cells in response to secondary challenge, resulting in suppressed pathogenic T cells that mediate EAE. The demonstration that helminths and their products can induce long-lasting modifications to BM-resident HSCs to produce anti-inflammatory innate immune cells sheds new light on innate immune memory and has the potential to identify novel therapeutic approaches for autoimmune diseases.

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Abbreviations

AAI	Allergic airway inflammation
AIP	Anti-inflammatory protein
ANOVA	Analysis of variance
aP	Acellular pertussis
APC	Antigen presenting cell
AREG	Amphiregulin
BBB	Blood-brain barrier
BCA	Bicinchoninic Acid
BCG	Bacillus Calmette–Guérin
BCR	B cell receptor
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cells
BMDM	Bone marrow-derived macrophages
BMECF	Bone marrow extracellular fluid
BSA	Bovine serum albumin
C/EBP	CCAAT-Enhancer-Binding Protein
CDC	Center for Disease Control
CFA	Complete Freund's Adjuvant
CFU	Colony forming units
CLP	Common lymphoid progenitor
CLR	C-type lectin receptor
cMoP	Common monocyte precursor
CMP	Common myeloid progenitor
CNS	Central nervous system
cRPMI	Complete Freund's Adjuvant
Ct	Complete RPMI
DAMP	Damage-associated molecular patten
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EBAO	Ethidium Bromide/Acridine Orange
EDTA	Ethylenediaminetetraacetic acid

EGR2	Early growth response protein 2
ELISA	Enzyme-linked immunosorbent assay
ES	Excretory/Secretory
FaBP	Fatty acid binding protein
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FHES	Fasciola hepatica excretory/secretory products
FhHDM	Fasciola hepatica helminth defence molecule
FhKTM	Fasciola hepatica Kunits-type molecule
FHTE	Fasciola hepatica total extract
FhTLM	Fasciola hepatica transforming growth factor-like molecule
FIZZ1	Found in inflammatory zone 1
FMO	Fluorescence minus one
FPR2	Formyl peptide receptor 2
FSc	Forward scatter
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte monocyte-colony stimulating factor
GMP	Granulocyte/macrophage progenitor
HDAC	Histone deacetylase
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1
HSC	Hematopoietic stem cell
HSP	Heat shock protein
I.p.	Intraperitoneal
i.v.	Intravenous
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-1RA	Interleukin 1 receptor antagonist
ILC2	Type-2 innate lymphoid cell
IRF	Interferon response factor

KLF	Kruppel-like factor
LKS	Lineage-cKit+Sca-1+
LMIC	Low- and middle-income countries
LN	Lymph node
LPM	Large peritoneal macrophages
LPS	Lipopolysaccharide
LT-HSC	Long-term hematopoietic stem cell
M1	Classically activated macrophages
M2	Alternatively activated macrophages
MACS	Magnetic activated cell sorting
MBP	Myelin basic protein
M-CSF	Monocyte-colony stimulating factor
MDP	Macrophage/dendritic cell precursor
MEP	Megakaryocyte/erythrocyte progenitor
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MPP	Multipotent progenitor
mRNA	Messenger RNA
MS	Multiple sclerosis
MTA	5′-Deoxy-5′-(methylthio)adenosine
Mtb	Mycobacterium tuberculosis
mTOR	Mechanistic target of rapamycin
NK	Natural killer
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PD-L1	Programmed cell death protein 1 ligand 1
PEC	Peritoneal exudate cells
PFA	Paraformaldehyde
PGE2	Prostaglandin E2

PRR	Pathogen recognition receptor
PT	Pertussis toxin
RA	Rheumatoid arthritis
RELM-α	Resistin-like molecule alpha
RORγt	RAR-related orphan receptor gamma transcription
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT-qPCR	Reverse Transcriptase-quantitative polymerase chain reaction
s.c.	Subcutaneous
SAR	Systemic acquired resistance
Sca-1	Stem cell antigen1
SCF	Stem cell factor
SLE	Systemic lupus erythematosus
SPM	Small peritoneal macrophages
SSc	Side scatter
ST-HSC	Short-term hematopoietic stem cell
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	T regulatory
TSLP	Thymic stromal lymphopoietin
t-SNE	t-distributed stochastic neighbour embedding
WHO	World Health Organisation
wP	Whole-cell pertussis
YM1	Chitinase 3-like 3

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Chapter 1: General Introduction

1.1 Hematopoiesis

The mammalian immune system has been classically divided into the innate and the adaptive arms. While the adaptive immune response, consisting primarily of T and B cells, is thought to convey long-term, specific memory against pathogens, the innate immune response is the first line of defence against invading pathogens. Innate immune cells mediate non-specific responses upon recognition of pathogen-associated molecular patterns (PAMPs) and damage-associate molecular patterns (DAMPs). The innate immune system is composed of a plethora of cell types that circulate in the body and populate organs to facilitate a rapid response to infection or injury. The primary effector cells of the innate immune system are neutrophils, eosinophils, dendritic cells and monocytes, the latter of which can differentiate into macrophages.

Innate immune cells require constant maintenance and renewal, and cells are short-lived. For example, monocytes, a major component of the innate immune system, have a steady-state half-life of approximately 2 days [1]. Innate immune cells are maintained in the circulation, ready to sense inflammation or damage, and then initiating hematopoiesis in the bone marrow (BM), which can produce large numbers of new immune cells quickly and efficiently. There is continuous renewal of red and white blood cells through the differentiation of hematopoietic stem cells (HSCs) in the BM. HSCs are initially oligopotent, before becoming lineage-committed and finally single-lineage-committed mature immune cell precursors. Hematopoiesis is dependent on numerous factors, including, transcription factors, cytokines, cell-cell interactions, and metabolic changes. A brief summary of classical lineage hematopoiesis is shown in Figure 1.1.

1.1.1 Hematopoietic stem cells

Hematopoiesis largely occurs in the BM in a specialized niche containing a relatively small pool of oligopotent, multipotent and unipotent progenitors. The HSC niche is tightly controlled and primarily consists of HSCs, mesenchymal stem cell populations, endothelial cells, megakaryocytes and neuronal synapses that work together to suppress aberrant activation of HSCs and permit hematopoiesis in the context of inflammation [2]. In adult mammals, the long-

term (LT)-HSC reside at the top of the hematopoiesis hierarchy. LT-HSCs can be broadly defined by their ability to self-renew and their ability to differentiate into all cells of the hematopoietic cell system [3–6]. Notably, self-renewal allows for LT-HSC to completely reconstitute the entire immune system after HSC transplantation to irradiated mice in 12 weeks [7]. During homeostasis, LT-HSCs are quiescent, with markedly long half-lives. In mice, renewal only occurs approximately every few months [8]. However, the rate of renewal can be significantly longer, with many LT-HSCs having renewal cycles up to 200 days in mice [9]. LT-HSCs express multiple receptors in order to respond to the environment and potential inflammation, including receptors for interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF), interferon (IFN)-γ and granulocyte-colony stimulating factor (G-CSF) [9, 10]. LT-HSCs can be activated in response to these cytokines, cell-cell interactions and microenvironmental factors, rapidly expanding and differentiating into downstream multipotent and lineage-committed progenitors to fulfil the increased cell requirements of inflammation. Additionally, stimulation with CSFs promote long-term survival and quiescence of HSCs [11]. LT-HSC reside in the strictly hypoxic HSC niche and have been demonstrated to rely on oxidative phosphorylation as its primary source of metabolism [12].

Upon activation, LT-HSCs differentiate into short-term (ST)-HSCs and multipotent progenitors (MPPs). This differentiation is key because LT-HSCs and ST-HSCs are the only progenitor cells that maintain the ability to restore the hematopoietic system after total BM irradiation [13]. ST-HSC have been classically distinguished from LT-HSC due to their shorter self-renewal time, which occurs approximately over 1 month. In mice, LT-HSC, ST-HSC and MPPs are identified by their surface expression of the receptor for stem cell factor (SCF) cKit (CD117), stem cell antigen (Sca-1) and lack of lineage markers, jointly earning the title of LKS$^+$ cells (Lineage$^-$cKit$^+$Sca-1$^+$).
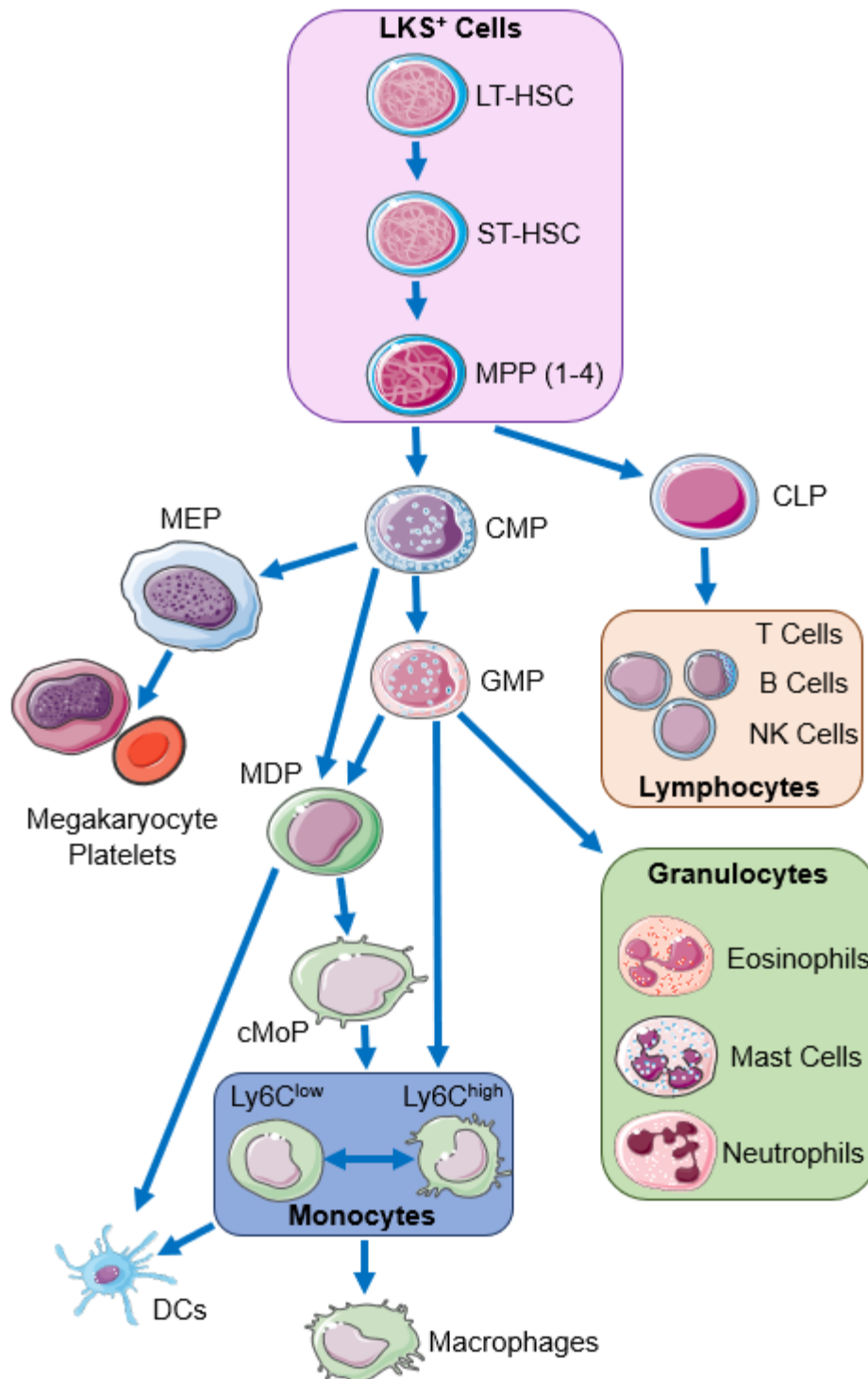
Figure 1.1 Classical model of Hematopoiesis: LT-HSC to Monocytes

Long-Term Hematopoietic Stem Cell (LT-HSC), Short-Term HSC (ST-HSC), Multipotent Progenitor (MPP), Common Lymphoid Progenitor (CLP), Common Myeloid Progenitor (CMP), Megakaryocyte-Erythrocyte Progenitor (MEP), Granulocyte-Macrophage Progenitor (GMP), Macrophage-DC Precursor (MDP), common Monocyte Precursor (cMoP).

1.1.2 Multipotent progenitors

MPP are the only cell type currently known to maintain multipotency, yet lack the abilities of previous HSCs, such as self-renewal or reconstitution of myeloablative BM. MPPs have a comparatively higher frequency of cell cycle progression, more differentiation activity and no self-renewal capacity [7]. Under both steady-state and stress conditions, MPPs are currently considered the primary source of oligopotency; in maintaining hematopoiesis, while HSC populations play little to no role in repopulation or response to stress [9, 14]. However, this role is contested, as several conflicting studies using single cell mapping have shown that LT-HSC, ST-HSC and MPP can promote steady-state hematopoiesis [15–17]. This highlights the complexity and heterogeneity of HSCs.

Until recently, HSC populations were strictly categorized into discreet populations, however, the advent of single cell transcriptomic and epigenomic sequencing has revealed remarkable heterogeneity. Analyses of various HSC populations in mice and humans have revealed that, on a transcriptional level, there is no clear delineation between HSC populations during steady-state or inflammation-induced hematopoiesis [18, 19]. MPPs were once considered to be a large, homogenous population of HSCs, however, it is now clear that MPPs are a heterogenous population of smaller subtypes (MPP1-4). Each subset of MPP has the ability to differentiate into all cells of the hematopoietic lineage. However, transcriptomic and single-cell analyses have revealed that individual MPP subpopulations have different propensities to differentiate into certain lineages. For example, MPP4, also known as lymphoid MPPs, have a higher probability of differentiating into cells of the lymphoid lineage (B cells, T cells and natural killer cells), whereas MPP2/3 are significantly more likely to develop into cells of the myeloid lineage [14]. Single-cell genomic sequencing revealed that MPP2 express significantly higher levels of myeloid-lineage-committed genes, including: *Csf2*, *Cebpe*, Irf4 and *Tirap*. In contrast, MPP4, express higher levels of lymphoid genes, such as, *Flt3*, *Bcl6* and *Cd24a* [14]. However, these genes indicate that while a subpopulation is likely to differentiate into a distinct lineage, they still retain the ability to differentiate into any cell type. This is best illustrated by the fact that MPP2, the "myeloid" MPP, and MPP4, the "lymphoid" MPP,

express genes required for granulocyte and myeloid differentiation, including, *Cebpa*, *Irf8* and *Spi1* [14].

Despite the heterogeneity of HSCs and MPPs, these cells have been extensively studied for their abilities to differentiate during inflammation. Both HSCs and MPPs express receptors for cytokines, growth factors, chemokines and neurotransmitters, including IL-1β, macrophage (M)-CSF, G-CSF and IFN-γ. Furthermore, Nagai et al. demonstrated that HSCs and MPPs express pattern recognition receptors (PRRs) and are capable of responding to directly to PAMPs, such as lipopolysaccharide (LPS) binding to toll-like receptor (TLR) 4 to induce myelopoiesis [20]. Activation of HSCs by PAMPs or DAMPs has been shown to promote transcription of various hematopoiesis-associated genes, and induce the release of cytokines, including IL-6, which acts in a paracrine manner to induce proliferation and promote myeloid differentiation [21]. During emergency hematopoiesis, LPS stimulation activates endothelial cells in the HSC niche to release G-CSF, which acts on downstream myeloid progenitors to induce differentiation into granulocytes [22]. Recent research has highlighted the importance of IFNs in promoting HSC proliferation and differentiation. For example, during infection of mice with *Mycobacterium avium,* HSCs respond to IFN-γ signalling to proliferate and increase myelopoiesis [23]. HSCs are a heterogeneous population of multipotent cells that readily respond to infection and inflammation to expand the production of effector immune cells to suit the context. It is likely that the definition of HSC populations will constantly change through continued innovations in methods of analysis. Current efforts to understand HSCs in the context of inflammation rely on a simplified model of hierarchical differentiation as assessed by cell surface markers.

1.1.3   Lineage-committed progenitors

After activation with various factors, MPPs differentiate into the lineage-committed progenitors: the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). CMPs and CLPs are the progenitor populations that will eventually give rise to cells of the innate and the adaptive immune systems, respectively. Furthermore, CMP also give rise to erythrocytes and megakaryocytes [7]. Interestingly, recent studies have highlighted that MPPs

preferentially differentiate towards the myeloid pathway, providing a rationale for the greater abundance of innate immune cells [16]. This is consistent with the fact that the innate immune cells are short-lived and needed for a robust, early response to emergency signals.

Like HSCs, lineage-committed progenitor cells express receptors for various cytokines, notably, receptors for the various colony stimulating factors: G-CSF, granulocyte-macrophage (GM)-CSF and M-CSF. These factors help promote survival and indirect activation of HSCs. However, ligand-binding on CMPs results in the rapid proliferation and differentiation into the various innate immune cells. G-CSF and M-CSF are important for the differentiation of progenitors through the myeloid lineage. Activation of HSCs and CMPs with M-CSF results in the activation of PU.1, a transcription factor and a key myelopoiesis regulator [24].

CSFs are very effective at enhancing proliferation of HSCs and progenitors and have been used as a therapy for multiple disorders. For example, G-CSF is widely used as prophylactic treatment for patients with cancer-associated leukopenia or fever, and is also administered to HSC transplant donors to expand HSCs before donation [11, 25]. In addition to growth factors, CMP and CLP express various receptors for cytokines, such as IL-1β, IL-6 and IFN-γ, and PRRs, including TLR2, TLR4 and TLR7 [20, 21, 26, 27]. During inflammation, HSCs and lineage-committed progenitors sense PAMPs and DAMPS along with changes in cytokine concentrations in the BM microenvironment that direct differentiation of HSCs into myeloid and lymphoid progenitors that mature into immune cells that mediate inflammation. For example, a potent eosinophil response is necessary during many helminth infections. Progenitor cells respond to helminth products and type-2 cytokines, such as IL-5, in the BM niche and selectively promote eosinophil production [28]. Conversely, during infection with bacterial pathogens, such as *Listeria monocytogenes*, there is highly selective proliferation and expansion of monocytes [29].

1.1.4 Monocyte and macrophage precursors

CMP differentiate into either granulocyte-macrophage progenitors (GMPs) or megakaryocyte-erythrocyte progenitors (MEPs). GMP give rise to various granulocyte precursors and macrophage-dendritic cell precursors (MDPs). Traditionally, it was thought that the MDP directly differentiated into either dendritic cells (DCs) or monocytes. Upregulation of a myriad of transcription factors direct differentiation into DCs and monocytes. For example, upregulation of *Irf8* and *Klf4* in MDPs has been demonstrated to be essential for development of monocytes; mice with defects in *Irf8* fail to develop monocytes [30]. Furthermore, several HSC-related transcription factors, including, CCAAT enhancer-binding protein (C/EBP)-α, C/EBP-β, PU.1 and Kruppel-like factor 4 (KLF4), are required for differentiation of progenitors to monocytes [31–34].

The model of myelopoiesis that describes MDP differentiating directly into monocytes has recently been challenged. The newly discovered intermediate precursor, the common monocyte precursor (cMoP), has been characterized in mice and humans to give rise to monocytes [35, 36]. The cMoP is similar to MDP and functional monocytes in marker expression, however, unlike monocytes, they are incapable of phagocytosis, and primarily express proteins and transcription factors associated with cell cycle and division [35]. Interestingly, the lines between these late-stage progenitors/precursors has become increasingly blurred. For example, Yáñez et al. recently showed that, depending on the stimuli, monocytes can be derived directly from either GMP or MDP [37]. Mice that received LPS promoted the differentiation of monocytes directly from GMP, however, administration of CpG induced MDP and cMoP differentiation into monocytes. The source of monocytes, either GMP or MDP/cMoP, was shown to produce monocytes with differing functions, with GMP-derived monocytes exhibiting a 'neutrophil-like' inflammatory profile, while cMoP-derived monocytes acted as patrolling cells that were prone to differentiation into DC. Much work still needs to be done to understand monocyte differentiation fully. A simplified model of hierarchical hematopoiesis is shown in Figure 1.1.

1.1.5 Monocytes

Murine monocytes can be divided into Ly6C^{high} 'pro-inflammatory' or Ly6C^{low} 'anti-inflammatory' monocytes. In humans, CD14^{high} CD16$^-$ and CD14$^+$ CD16^{high} monocytes correlate with the Ly6C^{high} and Ly6C^{low} populations, respectively. Monocytes are activated via ligation of various PAMPs and DAMPs, such as LPS or amyloid-β binding to TLR4 [38, 39]. Furthermore, monocyte-derived macrophages are crucial in the defence against *Mycobacterium tuberculosis* (Mtb), the pathogen which causes tuberculosis. Monocytes express multiple TLRs (TLR4, TLR1/2, TLR1/6), NOD-like receptors (NLRs) (NOD2), C-type lectin receptors (CLRs) (Dectin-1), complement receptors and other scavenger receptors. Following innate sensing through PRRs, monocytes play an important role in initiating the adaptive immune response.

1.1.5.1 Ly6C^{high} monocytes

In response to infection or stress, Ly6C^{high} monocytes are rapidly produced in the BM and recruited to the site of infection through their expression of the chemokine receptor CCR2. Indeed, mice deficient in CCR2 fail to recruit Ly6C^{high} monocytes to sites of inflammation, though monocyte development is not impaired [40]. At the site of infection, Ly6C^{high} monocytes further differentiate into macrophages and DCs, which then produce reactive oxygen species (ROS) and inflammatory cytokines, such as IL-1β, TNF and type-I IFNs [41]. In high-dose infections of mice with Mtb, Ly6C^{high} monocyte-derived classical macrophages are essential for clearance of the infection via induction of an appropriate T cell response [42, 43]. Loss of Ly6C^{high} monocytes impairs the ability of mice to mount a successful IFN-γ-secreting CD4 T helper 1 (Th1) priming and recruitment of immune cells to the lung [44]. Ly6C^{high} monocytes are also essential in host defence against *L. monocytogenes* infection in mice. Monocyte-derived DCs are essential for host defence against *L. monocytogenes* through production of TNF and inducible nitric oxide synthase (iNOS) [45].

1.1.5.2 Ly6C^{low} monocytes

Ly6C^{low} monocytes are characterized by high expression of fractalkine receptor (CX3CR1), and act as patrolling, anti-inflammatory cells [46]. Ly6C^{low} monocytes

constantly patrol the vasculature and extravasate into tissues under non-inflammatory conditions, whereas Ly6C^{high} monocytes selectively travel to sites of active inflammation [47]. Not only does CX3CR1 promote Ly6C^{low} monocytes chemotaxis through the vasculature and organs, it plays a significant role in Ly6C^{low} monocyte cell survival [48]. In disease models, Ly6C^{low} monocytes secrete IL-10 in response to bacterial infections [49]. Importantly, Ly6C^{low} monocytes help mediate resolution of inflammation by facilitating the clearance of necrotic tissue [50]. Moreover, Ly6C^{low} monocytes play a key role in tolerizing T cells through expression of programmed death-ligand 1 (PD-L1) [51].

Early studies suggested that Ly6C^{low} monocytes originated solely from Ly6C^{high} monocytes – a process known as class-switching - through various factors, most notably upregulation of the transcription factor, NR4A1 [52, 53]. Adoptive transfer of Ly6C^{high} monocytes into mice lacking inflammation resulted in homing of monocytes to the BM and a loss of Ly6C expression [54, 55]. Furthermore, treatment of mice with a NOD2-agonist, can directly induce the class-switching in both circulating and BM-resident monocytes, resulting in an abundance of Ly6C^{low} monocytes [56]. However, it has also been shown that genetic defects that result in a loss of Ly6C^{high} monocytes do not affect the generation of Ly6C^{low} monocytes, nor their infiltration into tissues [57–59].

1.1.6 Macrophages

Macrophages play an essential role in sensing PAMPs and DAMPs, directing induction of adaptive immune responses, and eventual resolution of inflammation. Tissue-resident macrophages are primarily embryonic-derived, and include alveolar macrophages in the lungs, microglia in the central nervous system (CNS) and Kupffer cells in the liver. Although initially embryonically-derived and capable of self-renewal, tissue-resident macrophages are replenished by BM-derived monocytes [60]. Furthermore, during radiation or chemotherapy-induced myeloablation, tissue-resident macrophages are rapidly replenished by donor-derived HSCs. During homeostasis, tissue-resident macrophages are anti-inflammatory and act to inhibit the development of aberrant inflammation. For example, IL-10-producing intestinal macrophages

maintain gut integrity and limit inflammation to the commensal bacteria residing in the gut by inhibiting production of IL-23 [61].

During infection or injury, circulating and BM-derived monocytes are rapidly recruited to the site of inflammation and differentiate into effector macrophages and DC. Both Ly6C^{high} and Ly6C^{low} monocytes are capable of differentiating into macrophages [62]. During bacterial or viral infection, tissue-resident macrophages and tissue-infiltrating monocytes become classically activated. These classically activated macrophages, known as M1 macrophages, produce IFN-γ, IL-1β, IL-12 and TNF [63]. In addition to secreting pro-inflammatory cytokines, M1 macrophages phagocytose and present antigen, and promote Th1 responses. LPS is a key PAMP for stimulation to M1 macrophages, and its effect can be enhanced by IFN-γ [64].

During resolution of the immune response, M1 cells phenotypically switch to alternatively activated or M2 macrophages, which act to prevent chronic, potentially immunopathogenic, inflammation. M2 macrophages are typically anti-inflammatory and secrete anti-inflammatory mediators, such as IL-1 receptor antagonist (IL-1RA) and IL-10, transforming growth factor (TGF)-β and YM1 [64]. IL-4 and IL-13 play key roles in promoting M2 macrophage activation [65]. In addition, M2 activation is induced during parasitic infection. For example, the liver fluke, *Fasciola hepatica*, induces a potent M2 phenotype *in vivo* [66]. The simplistic model of macrophages as strictly M1 or M2 obscures the fact that macrophages are a far more heterogeneous population and utilize an array of classical and alternative activation cytokines and chemokines simultaneously. Like HSCs, while there is considerable heterogeneity within 'defined' populations, the M1/M2 classification of macrophages (Fig. 1.2) remains a useful tool for understanding the innate immune response to infection and inflammation.
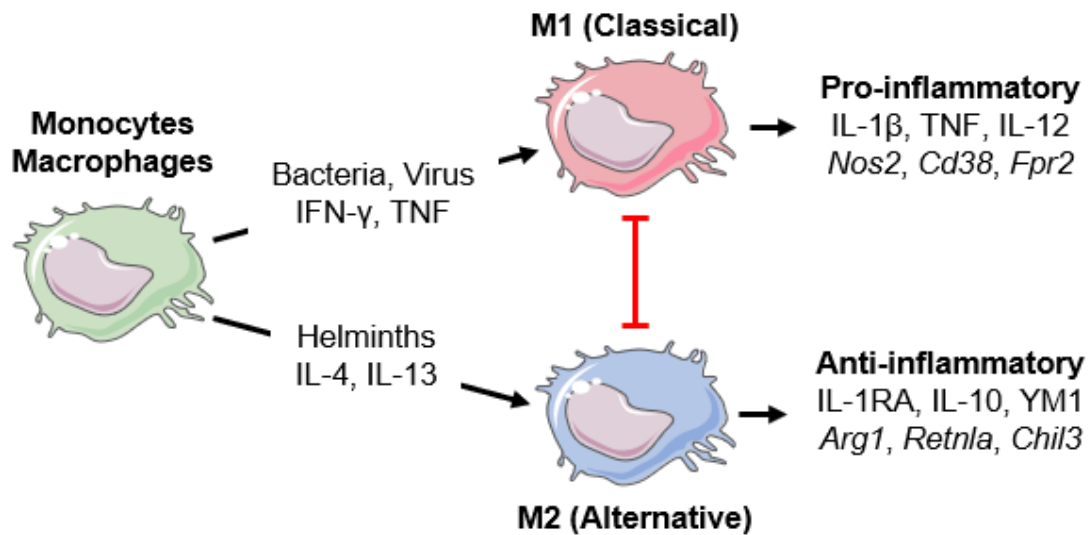
Figure 1.2 Classical (M1) and alternative (M2) activation of macrophages

Monocytes, tissue-resident and infiltrating macrophages are activated by bacterial and viral infection or in response to stimulation with IFN-γ or TNF. M1 macrophages produce pro-inflammatory cytokines and express pro-inflammatory markers. In contrast, M2 macrophages are activated by helminth products or by IL-4/IL-13 and produce anti-inflammatory cytokines and express anti-inflammatory markers.

1.2 Immunological memory

The immune system is typically divided into the innate and adaptive immune responses. The innate immune system has traditionally been characterized by short-lived, non-specific immune cells, including monocytes, eosinophils, and neutrophils. Conversely, the adaptive immune response, consisting of B and T cells, is characterized by highly specific and long-lasting memory against specific antigens. However, recent studies have shown that innate immune cells can be 'trained,' 'tolerized' or 'primed' in response to various stimuli [67]. This new form of immunological memory has become known as trained innate immunity, or innate immune memory.

1.2.1 Adaptive immune memory

The adaptive immune response is primed by the innate immune system. Initial activation of T and B cells typically requires several days to develop fully. T and B cells are capable of responding to a plethora of antigens and have highly specific receptors for antigens: T cell receptors (TCR) and B cell receptors (BCR) or antibodies. After activation, T and B cells undergo genetic recombination of their respective receptor genes, rapidly expand, migrate to the site of infection, and efficiently clear the invasion. Upon clearance, the vast majority of cells undergo a form of regulated cell death known as apoptosis. The remaining T and B cells known as memory cells that are quiescent until reinfection with the same antigen, when they expand and initiate a faster response to help prevent re-infection.

1.2.1.1 B Cells

B cells, named after the bursa organ, found in birds, arise from HSCs in the BM, before moving to the lymph nodes to await activation. Unlike T cells, B cells can be directly activated by an antigen [68]. B cells recognize antigen using immunoglobulin (Ig) molecules, and the primary role of B cells is to produce Ig, also known as antibodies. Antibodies are highly specific for an epitope on an antigen and provide one of three major roles in combatting infection: (1) inhibit microbes from entering cells, (2) provide a receptor for innate immune cells to recognize and phagocytose through opsonisation, and (3) aid in complement activation [69]. B cells produce 5 distinct classes of Ig in mammals: IgM, IgG, IgD, IgA, and IgE. Naïve B cells express IgM on their surface, which acts to recognize the initial unprocessed antigens and promote activation. Upon activation, B cells mature into plasma cells and secrete antigen-specific IgG, IgA and IgE [70]. The function of each class of Ig can be broadly divided by their role in the host: IgG subtypes are essential for opsonisation and neutralization, IgA has been shown to play a role in mucosal barrier immunity, and IgE plays a role in anti-helminth and allergic inflammation.

1.2.1.2 T cells

T cells, named after their initial discovery in the thymus, recognizes antigen after it is processed and presented by major histocompatibility complex (MHC) molecules in mice, or human leukocyte antigen (HLA) in humans. Upon infection, antigen-presenting cells (APCs), such as macrophages and DC, phagocytose and process pathogens. The activated APCs in the tissue migrate to the lymph nodes and form an immunological synapse and present the antigen to T cells through MHC, a specialized antigen-presenting surface protein. MHC Class I (MHC-I) is expressed by all nucleated cells in the body and activate CD8$^+$ T cells, which are responsible for anti-viral and anti-tumoral immunity. Virally-infected and tumour cells enhance expression of MHC-I on their surface and upon recognition by CD8$^+$ T cells, the aberrant cells are destroyed through the release of perforin and granzyme into the immunological synapse.

In contrast, MHC Class II (MHC-II) molecules, which are only expressed by APCs, such as DC and macrophages, present antigen to T cell receptor on CD4$^+$ T cells. In addition to antigen presentation, activation of CD4$^+$ T cells requires a secondary stimulation provided by co-stimulatory molecules expressed on APCs. The most studied co-stimulatory molecules include CD40, CD80, CD86 and PD-L1, which have corresponding receptors on the surface of T cells [71]. Co-stimulation with one or more of these molecules is essential for induction of T cell responses. In the absence of co-stimulation, presentation of the antigen by APCs will result in T cell anergy or apoptosis [72]. The third and final signal required for the induction of T cell responses is the production of polarizing cytokines into the immunological synapse that promotes the differentiation of the T cell into the appropriate T helper (Th) cell subtype [73].

CD4$^+$ Th cells can be divided into a number of subgroups which are suited to respond to the type of infection or inflammation. Th cell differentiation is determined by the cytokines secreted by myeloid cells during antigen presentation (Fig. 1.3). Th1 cells are important in immunity against infection with intracellular bacteria, viruses and small parasites [74]. Activation of Th1 cells is promoted by IL-12 secretion from, which induces expression T-bet, the master transcription factor for Th1 cells.
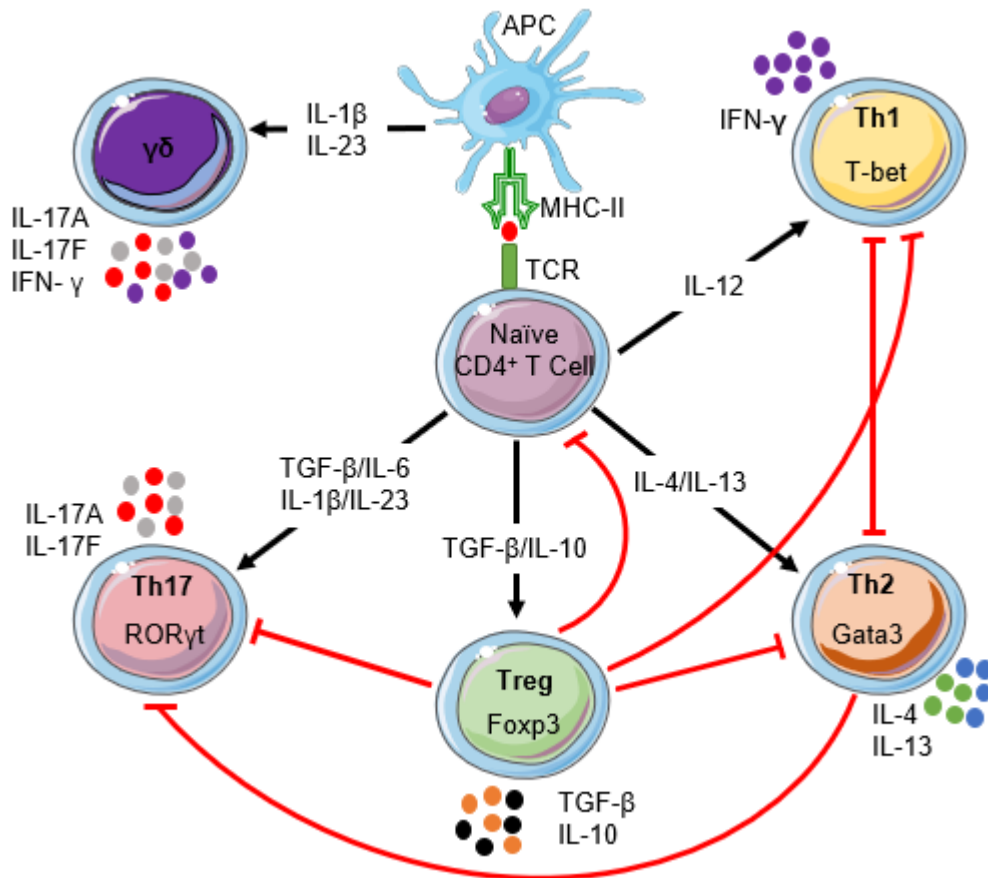
Figure 1.3 T cell differentiation, activation and suppression

Naïve T helper cells migrate to the lymph node and are activated following interaction of TCR with antigen presented by MHC-II and by interaction with co-stimulatory molecules expressed pm APCs. The subset of Th cell induced is dependent on the release of specific cytokine combinations that commit T cells to different lineages. IL-12 induces Th1 cells, IL-4 and IL-13 induce Th2 cells, TGF-β and IL-6 induce Th17 cells, which are expanded by IL-1β and IL-23, and Treg cells are induced by IL-10 and TGF-β. Each subset produces a distinct combination of cytokines: Th1 cells produce IFN-γ, Th2 cells secrete IL-4 and IL-13, Th17 cells secrete IL-17 and GM-CSF, and Treg cells produce IL-10 and TGF-β. The differentiation into a particular subset induces the suppression of the other subsets. Furthermore, a specialized group of T cells, known as γδ T cells do not require antigen presentation and are activated by secretion of IL-1β and IL-23. γδ T cells can secrete IL-17 and IFN-γ.

Upon activation, Th1 cells secrete IFN-γ and IL-2 [75]. IFN-γ is a member of the IFN family of cytokines that can interfere with viral replication, and induces activation of M1 macrophages, which produce pro-inflammatory cytokines and have anti-microbial killing capacity [76].

Th2 cells are induced by the production of IL-4 and IL-13 by innate immune cells, which upregulate the transcription factor GATA3. The primary function of Th2 is to combat extracellular parasitic infections [74]. Th2 cells secrete of a myriad of type-2 cytokines, including IL-4, IL-5, IL-10, and IL-13. IL-4 and IL-13 promote alternative activation of macrophages, while IL-5 is responsible for activation, expansion and mobilisation of eosinophils [77]. Furthermore, IL-4 secretion from Th2 cells induces class switching of B cells to IgE [78]. The resulting IgE acts on granulocytes, including mast cells, to secrete anti-helminthic molecules such as histamine. Additionally, IL-10 is a potent anti-inflammatory cytokine that can inhibit Th1 responses by suppressing their proliferation and cytokine production. IL-13 plays a key role in activating the mucous membrane and induces the 'weep and sweep' response for the expulsion of parasites [79]. This response is characterized by goblet cell hyperplasia, including enhanced mucus secretion, smooth muscle contraction and epithelial cell turnover.

A recently discovered Th subtype, Th17 cells, are characterized by production of IL-17A, IL-17F, GM-CSF and IL-22 [80]. The differentiation of Th17 cells is mediated by myeloid cell secretion of TGF-β and IL-6, which promote the master Th17 transcription factor, RAR-related orphan receptor gamma transcription (RORγt), while IL-1β and IL-23 signalling aid in survival [81–83]. Th17 cells are essential for the protective immune response against extracellular bacterial and fungal infections, and have been shown to play a detrimental role in many T cell-mediated autoimmune disorders [74]. IL-17 promotes inflammation and recruits neutrophil infiltration to the site of infection.

Amplified production of IFN-γ and IL-17 are also potently secreted by γδ T cells. These cells are induced through IL-1β and IL-23 by myeloid cells, however they do not require TCR activation [84]. γδ T cells are found throughout the body, but are enriched at mucosal barrier surface, such as the gut and lungs, where they contribute to early inflammatory processes. IL-17 and IFN-γ production by γδ T

cells helps to maintain a strong, early response to infection. Additionally, they have been shown to play critical pathogenic roles in many autoimmune diseases [85, 86].

T regulatory (Treg) cells are the anti-inflammatory arm of the T cell response. Secretion of TGF-β and IL-10 by innate cells induces transcription of FoxP3 and differentiation of naïve T cells into Treg cells. These cells are essential for dampening immune responses after clearance of infection, as well as inhibiting auto-reactivity [87]. Treg cells produce IL-10 and TGF-β, the latter of which aids in wound repair. Treg cells suppress Th1-, Th2- and Th17-mediated inflammation, including autoimmunity [88].

1.2.2 Trained innate immunity

Immunological memory was originally thought to be a feature exclusive to B and T cells. During infection with a pathogen, effector T cells are induced and help to fight the infection. If the pathogen is cleared, most effector T cells die, but a small percentage remain in the tissues for months or years as memory T cells. This type of memory is a feature of the adaptive immune system, and the T and B cells are specific for antigens on the invading pathogen. However, recent studies have shown that innate immune cells can be 'trained' or 'tolerized' in response to various stimuli, suggesting an alternative form of immune memory, often called trained innate immunity or innate immune memory [67].

The field of trained innate immunity began to emerge following the seminal study in 2012 by Kleinnijenhuis et al. that demonstrated that circulating monocytes from healthy volunteers vaccinated with Bacillus Calmette–Guérin (BCG), a vaccine against Mtb, secreted higher concentrations of pro-inflammatory cytokines in response to various PAMPs [89]. Trained responses were seen up to 3 months post-vaccination. Interestingly it has been known for some time that BCG has non-specific protective effects against a range of malignancies, including bladder cancer, melanoma and leukemia [90–92]. Furthermore, immunization with BCG can confer non-specific protection against infection with unrelated pathogens [93, 94]. Importantly, studies using RAG1-deficient mice, which lack T and B cells, have demonstrated that protection mediated by trained immunity is independent

of adaptive immunity, and is mediated by cells of the innate immune system [95–97]. In the past 8 years, the ability of various stimuli to induce trained innate immunity have been rigorously studied in mature monocytes, macrophages, DCs and NK cells [98].

1.2.2.1 Innate immune training in invertebrates

Trained innate immunity was first characterized in plants, a class of organisms that lack an adaptive immune system. When plants are infected with a pathogen, epigenetic changes result in the priming of genes encoding host defence molecules. Upon reinfection, the plants can respond faster and stronger to the pathogen, in a process known as systemic acquired resistance [99, 100]. This resistance seen after exposure to a pathogen is not specific, rather it is effective against a diverse range of pathogens. It is also known that invertebrates, such as mosquitos and crustaceans, protect against invading pathogens through trained innate immunity, suggesting that this is an evolutionarily conserved process [101, 102]

1.2.2.2 Mouse models for the study of trained innate immunity

Studies in mice have generated considerable evidence of trained innate immunity *in vivo*. For example, injection of mice with β-glucan, a component of many bacterial and fungal cells walls and an agonist for Dectin-1, results in enhanced bacterial clearance and lower mortality following challenge with *Staphylococcus aureus* [103, 104]. Interestingly, the protective effects of β-glucan were found to be independent of its receptor, dectin-1, but dependent on IL-1β [104, 105]. Similarly, treatment with the TLR5-agonist flagellin protected mice against both *S. aureus* and rotavirus infections [106, 107]. Furthermore, treatment with unmethylated cytosine-guanidine motifs (CpG), agonists for TLR9, enhanced survival of neutropenic mice in a model of *Escherichia coli*-induced sepsis and meningitis [108]. Protection was associated with enhanced inflammatory monocytes, IL-12 and IFN-γ production, typical type-1 immune responses. Furthermore, latent infection of mice with murine gamma-herpesvirus 68 or murine cytomegalovirus confers protection against *L. monocytogenes* and *Yersinia pestis* [109]. Moreover, induction of trained innate immunity is not

confined to PRR signalling by PAMPs. Injection of mice with low doses of IL-1β three days before bacterial challenge enhanced protection against *Pseudomonas aeruginosa* infection [110].

Trained innate immunity can also be seen in type-2 immune responses. For example, infection of mice with the parasitic nematode, *Nippostrongylus brasiliensis*, resulted in priming of alternatively activated neutrophils which prime long-lived effector macrophages that mediate rapid expulsion of helminths following re-challenge with the parasite [111]. Interestingly, infection of humans with parasitic worms during pregnancy can result in reduced Th1 and enhanced Th2 responses in offspring following immunization with the BCG vaccine at birth [112]. This suggests that trained innate immunity is long-lived, can be maternally transferred and can influence type-1 and type-2 arms of the immune response. We have recently provided evidence that products from a helminth pathogen that promote type-2 immune responses can induce anti-inflammatory trained innate immunity [113]. Macrophages treated with total extract from *F. hepatica* (liver fluke) had heightened production of the anti-inflammatory cytokine IL-10 but reduced production of TNF following exposure to pro-inflammatory stimuli. Furthermore, treatment of mice with the helminth products induced anti-inflammatory trained immunity in macrophages *in vivo* and this suppressed activation of pathogenic Th17 cells that mediated experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS).

1.2.2.3 HSCs and progenitors in training

While there is now convincing evidence that innate immune cells can be trained to respond differently following re-exposure to a pathogen or its products, innate immune cells are short-lived, especially monocytes, with circulating lifespans of less than 1 week [1, 114]. However, the beneficial non-specific effects of BCG-induced trained innate immunity can last for months to years [89, 115]. A potential explanation for this paradox comes in the form of HSCs, which are long-lived, self-renewing cells that can differentiate into every cell of the innate and adaptive immune systems. Consequently, recent research on trained innate immunity has focused on the role of HSCs and their possible modulation by pro- or anti-inflammatory mediators [116]. This phenomenon is known as central trained

innate immunity, while peripheral trained innate immunity refers to training of circulating and tissue-resident mature innate immune cells.

HSCs express various PRRs and cytokine receptors, enabling them to respond directly and indirectly to inflammation via changes to the BM microenvironment. For example, macrophages derived from HSCs that have been directly stimulated with PAM_3CSK_4, a TLR2 agonist, or LPS, a TLR4 agonist, secreted significantly less of the pro-inflammatory cytokines IL-6 and TNF in response to re-stimulation with either TLR2 or TLR4 agonists [117]. In contrast, HSCs treated with *Candida albicans* resulted in the development of macrophages that secreted significantly higher concentrations of IL-6 and TNF in response to re-stimulation with PAM_3CSK_4 or LPS. Treatment of mice with BCG significantly expands MPPs and modifies their transcriptional activity to induce genes associated with cell cycle and replication, and imprints a distinct bias towards the myeloid lineage via upregulation of C/EBP-α and IRF8 [118]. Furthermore, macrophages derived from mice treated with BCG mediate enhanced protection against Mtb challenge through enhanced antimicrobial elimination, and upregulated expression of *Il1b*, *Tnf* and *Ifng*. It has recently been demonstrated that treatment of mice with β-glucan similarly expands myeloid progenitors, including MPPs and GMPs in the BM. This effect persisted up to 4-weeks post-treatment and the increased myelopoiesis from HSCs resulted in enhanced response to secondary stimulation with LPS and conferred protection against chemotherapy-induced myelosuppression [119]. The expansion of HSCs and protection mediated by β-glucan was dependent on IL-1β signaling in the BM, with significantly increased GM-CSF and IL-1β in the BM extracellular fluid.

Recent studies showed that mice treated with LPS expanded MPPs and LT-HSCs, biasing their transcriptional landscape towards a myeloid-lineage, which conferred long-lasting protection against infection with *P. aeruginosa* [120]. It has also been demonstrated that LPS-mediated septic shock in mice was associated with enhanced myeloid-biased HSCs and trained BM-resident monocytes, which secreted higher concentrations of IL-6 and TNF in response to re-challenge with LPS 12 weeks later [121].

1.2.2.4 Mechanisms of trained innate immunity

While antigen-specific memory in T and B cells is maintained by genetic restructuring of specific loci in T and B cell receptors, the exact mechanism of trained innate immunity is still unclear. It appears that non-specific memory or trained innate immunity involves metabolic and epigenetic changes in cells of the innate immune system [96, 122].

Studies on epigenetics, heritable changes in gene expression, as the basis for trained innate immunity have focused on chromatin remodeling, which enables more robust transcription through easier access of specific loci on the genome by transcription factors. Histone modifications, including acetylation and methylation can weaken or tighten the bond between histones and DNA, modifying access to transcription factors that are essential to metabolism and activation of innate immune cells [123]. Histone modifications influence trained innate immunity through epigenetic regulation of genes coding for inflammatory or anti-inflammatory cytokines and other molecules. For example, infection of mice with *C. albicans* or treatment with β-glucan, which resulted in enhanced cytokine production by monocytes, resulted in stable changes in histone trimethylation at H3K4 in various promoter regions of genes coding for inflammatory cytokines and chemokines. Quintin et al. found H3K4me3 at promoters for myeloid differentiation primary response 88 (MyD88), TNF, IL-6 and various TLRs and CLRs [124]. Further analysis revealed changes in positive histone regulatory marks for H3K4me1 and H3K27ac, resulting in the enhanced ability to produce pro-inflammatory cytokines [125]. These epigenetic modifications appear consistently in the heightened response to pro-inflammatory stimuli associated with trained innate immunity induced by a variety of different mediators (Figure 1.4). Furthermore, we have reported that helminth product-induced anti-inflammatory trained innate immunity is reversed by pre-treatment of macrophages with a histone methyltransferase inhibitor [113].
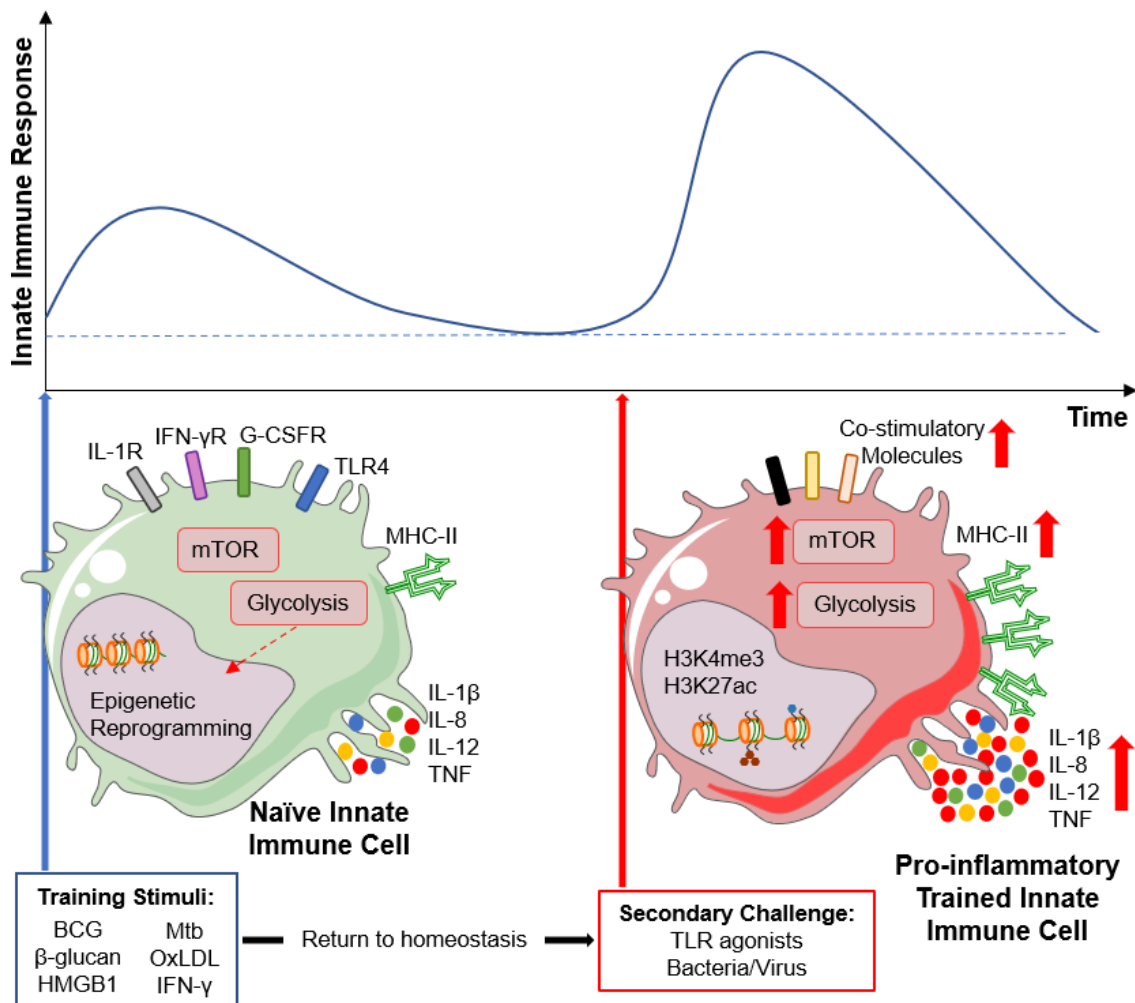
Figure 1.4 Mechanisms of pro-inflammatory trained innate immunity

Transient exposure of mature innate immune cells to certain pro-inflammatory stimuli from bacteria, viruses or fungi or endogenous danger molecules, such as HMGB1, induces metabolic activation and epigenetic reprogramming. After the initial training signal has dissipated, the innate immune cell returns to basal levels of activation. Upon secondary challenge with the same or a novel stimulus, chromatin marks allow for faster and more robust responses to challenge. In pro-inflammatory peripheral trained immunity, this results in increased pro-inflammatory cytokine secretion, and enhanced MHC-II and co-stimulatory molecule expression on the cells surface. The resulting trained response, which is distinct from activation or differentiation, confers on the cells an improved capacity to kill pathogens and present antigens to T cells.

Cellular metabolism has been shown to play a crucial role in the development of trained innate immunity. While there is yet to be a centrally defined mechanism for induction of trained innate immunity, several factors have been established, including mechanistic target of rapamycin (mTOR) and a preference for glycolysis. For example, induction of glycolysis has been shown to be essential for β-glucan-induced trained innate immunity through the Akt-mTOR-HIF-1α pathway [126]. Furthermore, monocytes from BCG-vaccinated patients have increased activation of mTOR and a bias towards aerobic glycolysis [127]. β-glucan training of mice also leads to the enhancement of cholesterol biosynthesis and glycolysis in BM myeloid progenitor populations [119]. Additionally, there is a tight link between metabolic and epigenetic changes that lead to trained innate immunity [128]. Indeed, blockade of glycolysis reversed epigenetic changes and induction of trained immunity [126, 127]. Furthermore, there is evidence of a nuanced interplay between metabolic intermediates and epigenetics in trained innate immunity. For example, the metabolic intermediate, acetyl coenzyme A, is increased in trained monocytes and is required for histone acetylation at loci responsible for transcription of glycolytic enzymes [129]. In addition, fumarate, a byproduct of the tricarboxylic acid cycle, modifies epigenetic modifications leading to trained innate immunity by downregulating histone demethylases [130].

1.3 Transplantation

Organ transplantation is a life-saving procedure that extends and improves the quality of life for patients with end-stage organ failure. In 2015, over 125,000 solid organ transplantations were performed around the world, primarily in North America and Europe [131]. The success of organ transplantation is dependent on establishing immune tolerance in the recipient to the donor organ. The adaptive immune system plays a key role in graft rejection by responding to allo-antigens of the graft. However, while the widespread use of immunosuppressive drugs has improved the first-year outcome of many patients, the long-term (5-10 year) success of transplantations has progressed little over the past few decades [132–134]. Activation of anti-graft T cell responses is mediated by APCs that respond to PAMPs and DAMPs released during transplantation. DAMPs, such as heat shock protein (HSP) 60, HSP70, surfactant protein-A, and high mobility

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group box 1 (HMGB1) are released during transplantation and induce pro-inflammatory responses in innate immune cells [135, 136]. HMGB1-TLR4 and inflammasome activation may contribute to enhanced inflammatory responses in low-quality kidneys and may affect transplant outcomes [137].

1.3.1 Trained innate immunity in solid organ transplantation

Understanding how innate immune cells respond to transplanted allo-grafts may prove beneficial for improving graft survival and patient outcomes and this has led to recent studies investigating a potential role for trained innate immunity in transplantation [138]. Macrophages from mice treated with BCG were found to secrete significantly higher concentrations of pro-fibrotic cytokines, and exacerbated immune-mediated fibrosis in a mouse model of systemic sclerosis [139, 140]. Moreover, Schreurs et al., found that macrophages derived from peripheral blood mononuclear cells of lung transplant patients preferentially differentiate into pro-fibrotic alternatively activated macrophages, potentially contributing to chronic lung allograft dysfunction [141].

Further evidence of a role for monocyte training in transplant rejection came from studies by Braza and colleagues, who found that HMGB1 and vimentin were capable of inducing epigenetic and metabolic modifications in transplant-infiltrating monocytes and macrophages [136]. The trained myeloid cells had significantly enhanced TNF and IL-6 secretion in response to re-stimulation with LPS *ex vivo*, an effect that was lost in Dectin-1- or TLR4-deficient mice. Furthermore, inhibition of myeloid-specific mTOR using a nanobiologic, inhibited this allo-training, which allowed expansion of regulatory macrophage populations [136]. Inhibition of pro-inflammatory training led to Treg cell infiltration and promotion of long-term tolerance to heart transplants. Importantly, while the nanobiologic was concentrated in myeloid cells in the periphery, it also inhibited mTOR in multiple HSC populations, implicating central trained innate immunity in transplant tolerance and rejection.

1.3.2 Trained innate immunity in HSC transplantation

Transplantation of HSCs is an effective therapeutic approach for a number of chronic diseases, including primary immunodeficiency diseases, hematological

cancers and autoimmune diseases, such as MS [142]. Many HSC transplants utilize autologous HSCs reintroduced to the body after chemotherapy or radiation therapy [143]. Conversely, allogeneic HSC transplantation has the advantage of graft-vs-leukemia response in treating hematological cancers [144]. However, allogeneic HSC transplantation is associated with the risks of developing graft-versus-host disease. Unfortunately, the possibility of disease relapse or development of a secondary autoimmune disease is a common adverse effect of autologous HSC transplantation [142]. Due to these potential adverse events associated with HSC transplantation, novel therapies which suppress inflammation and improve HSC engraftment are needed.

While central training of HSCs is a recently recognized aspect of trained innate immunity, the priming of HSCs to differentiate into cells of the innate and adaptive immune system that improve engraftment may be an attractive therapeutic approach to enhance the success of HSC transplantation. Studies in mice demonstrated that a single intraperitoneal injection of flagellin, an agonist for TLR5 and NLR4, induced proliferation of HSCs [145]. Furthermore, transplantation of HSCs from flagellin-treated mice significantly improved survival in irradiated recipient mice. The flagellin-modified HSCs were shown to be myeloid-lineage biased, providing further evidence that training of HSCs could provide a novel method of enhancing graft tolerance through induction of trained innate immunity. There is some evidence that these effects on HSC can last for months. Injection of mice with Mtb induced epigenetic modifications in HSCs, which persisted for up to 1 year [146]. Training of HSCs by vaccination of donor mice with BCG was able to be passed to multiple recipient mice. Furthermore, human HSCs exhibited changes that persisted for at least 90 days after vaccination with BCG [147, 148].

A common method for isolating HSCs for transplantations involves the injection of G-CSF to donors, which induces the mobilization of HSCs into the peripheral blood for relatively pain-free extraction [144]. However, in addition to mobilization of HSCs, G-CSF and GM-CSF alone have already been identified to confer trained innate immunity to HSCs, which differentiate into pro-inflammatory myeloid cells with improved responses to infection [119, 149]. Novel approaches

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to train HSCs to further enhance engraftment, fight infection/cancer, and promote long-term survival are the logical next step in HSC transplantation research.

1.4 *Bordetella pertussis*

Whooping cough, also known as pertussis, is a vaccine-preventable extracellular bacterial infection caused by the Gram-negative bacterium *Bordetella pertussis*. Normally, infections with *B. pertussis* are restricted to colonisation of the airways, including nasal tissue, trachea, and lungs. Though rare, systemic infections can occur. Infection induces a characteristic persistent coughing fits and difficulty breathing. Although treatable with antibiotics such as erythromycin, there are emerging antibiotic resistant strains, and therefore vaccine usage and improvements are necessary [150]. Originally, vaccines against pertussis were formulated using heat-killed bacteria, known as a whole-cell pertussis (wP) vaccines [151]. Due to their association with significant adverse effects, including swelling, fever, and occasionally seizures, wP vaccines were slowly phased out in favour of acellular pertussis (aP) vaccines. According to the World Health Organisation (WHO), there is approximately 85% global coverage with pertussis vaccines [152]. However, in 2019 there were more than 132,000 cases and in 2008 there were an estimated 89,000 deaths. There has been a measurable resurgence in the number of pertussis cases in countries with readily available, safe vaccines, prompting investigations into the potential causes for this increase [153, 154].

The immune response to pertussis infection is primarily mediated by phagocytic cells, such as neutrophils, macrophages and NK cells, along with robust activation of Th1/Th17 cell responses and B cell activation [155]. Innate immune, like neutrophils and M1 macrophages are important for clearance of pathogens, through phagocytosis and release of anti-microbial factors such as nitric oxide (NO) [156, 157]. Studies in animal models have demonstrated that Th1 and Th17 cells are essential for clearance of *B. pertussis* infection. Th1 cells secrete IFN-γ, which promotes classical activation of macrophages and enhances microbial killing [158]. Indeed, infection of mice deficient for the receptor for IFN-γ develop lethal, systemic infection. Furthermore, Th17 cells play a vital role in recruiting anti-bacterial neutrophils [159]. One potential explanation for the increase in

pertussis cases globally despite strong vaccination efforts is that aP vaccination preferentially induces a Th2 cell response, while the more effective, but more dangerous, wP produces Th1 and Th17 responses [160–162]. Indeed recent work from our lab has demonstrated that differences in aP and wP efficacy in mouse models of infection can be partially attributed to an inability of aP to produce long-lasting tissue-resident memory Th17 cells in the lung [163, 164]. Furthermore, vaccination of mice with aP in combination with a novel adjuvant capable of inducing Th17 cell responses was able to provide long-lasting protection in mice [165]. Trained innate immunity provides beneficial effects against bacterial pathogens and its incorporation into aP has been proposed to enhance vaccination efficacy [166, 167]. Indeed, there is some evidence to suggest that vaccination of mice with the live pertussis vaccine BPZE1 is protective against infections with other *Bordetella* species, influenza virus and respiratory syncytial virus [168–170]. Understanding how trained innate immunity plays a role in the efficacy of BPZE1 may prove beneficial for the development of improved aP vaccine combinations.

1.5 Allergy

Allergies can result from dysregulated or overactive immune responses to various environmental antigens, such as pollen, house dust mite and food allergens [171]. While many allergic disorders only cause minor irritation, some allergies can result in potentially fatal diseases. The immune response to allergens is largely characterized by increased IgE, which binds to receptors on granulocytes, including mast cells, to induce degranulation [172, 173]. Degranulation of mast cells results in acute release of histamines, cytokines, and prostaglandins, leading to the development of symptoms, including inflammation, itching, blood vessel dilation and smooth muscle contraction. Asthma can result in bronchoconstriction and the increased production of mucus, resulting in obstruction of the airways [174]. Cytokines and chemokines produced during allergic/asthmatic responses attract cells typically associated with type-2 inflammation, including Th2 cells, eosinophils, and basophils, which further exacerbate inflammation. Th2 cells are the predominant mediator of allergic inflammation, though exact mechanisms for their induction remain unclear.

During allergic inflammation, Th2 cells secrete IL-4, IL-5, IL-10, and IL-13, which mediate immunopathology, through induction of eosinophil recruitment, alternative activation of macrophages, and tissue remodelling [175]. In addition to Th2 cells, eosinophils are major contributors to immunopathology of allergic reactions, due to the many molecules released upon activation, such as major basic protein (MBP) [176].

1.6 Autoimmunity

Autoimmunity develops as a result of aberrant immune responses to self-antigens leading to the induction of immunopathology and destruction of tissues. Autoimmune diseases include rheumatoid arthritis, system lupus erythematosus (SLE) and MS. These diseases affect different organs and are mediated by different cell types. For example, in rheumatoid arthritis, auto-reactive T cells and macrophages infiltrate the synovial fluid of joints and break down cartilage and bone [122]. In SLE, patients have multiple organs affected, in which auto-reactive T cells and inflammatory low-density neutrophils infiltrate and break down the tissue [177].

1.6.1 Multiple sclerosis

MS is one of the most common neurological disorders, with over 2.3 million sufferers worldwide, and roughly 8,000 in Ireland alone [178]. Although the exact cause of MS is unknown, various studies have identified certain risk factors, including genetic factors (roughly 30%) and non-genetic, such as smoking [179]. MS is a chronic autoimmune disease of the CNS, caused by infiltrating immune cells. Though the exact pathomechanisms of disease are still enigmatic, it is known to involve the periodic infiltration of self-reactive Th1 and Th17 cells into the CNS, resulting in demyelination and a resultant myriad of symptoms, most notably the loss of muscle function [180]. Interestingly, monocytes are also a key mediator of disease. Recent evidence has shown that there is an increase in inflammatory monocytes in the circulation of MS patients [181]. While their role in disease still needs to be fully established, it was recently revealed that fingolimod, a first line drug in the treatment of MS, suppresses TNF output from human monocytes and induces a tolerized response to LPS [182].

MS is characterized by the infiltration of both myeloid and lymphoid immune cells, including monocytes, macrophages, T cells and B cells, across the blood-brain barrier (BBB) into the CNS. Once in the CNS, auto-reactive T cells respond to self-antigens including myelin oligodendrocyte protein (MOG), myelin basic protein and proteolipid protein, to break down the myelin sheath and destroy synapses between neurons [183]. Relapsing-remitting MS is the most common form of MS, which occurs in approximately 85% of patients. Relapsing-remitting MS is characterized by unpredictable episodes of demyelination and symptoms, followed by periods of remission. In secondary progressive MS, patients no longer experience periods of remission, and the disease simply worsens without a break. Primary progressive MS, which affects 10% of patients, is characterized solely by worsening symptoms, where patients have no period of recovery.

1.6.2 Experimental autoimmune encephalomyelitis

EAE is an experimental mouse model of MS. It shares many clinical and pathological features with MS, making it a useful model for studying CNS inflammation. Similar to the immunopathology in MS, EAE is a demyelinating CNS disorder, associated with CNS-infiltrating neutrophils, monocytes and lymphocytes which cross the BBB. EAE is induced by immunization of mice with a myelin antigen, such as MOG or PLP, emulsified in complete Freund's adjuvant (CFA) followed by injection with pertussis toxin (Pt) [184]. The clinical signs of EAE include ascending paralysis, beginning with tail weakness/paralysis, before hind limb weakness emerges, followed by hind limb paralysis, then front limb weakness, before final tetraplegia.

The pathology of EAE develops when innate immune cells in the periphery respond to the Mtb in the CFA and migrate to draining lymph nodes to present antigen to and activate T cells [184]. The activated myeloid cells promote induction of Th1 and Th17 response, through secretion of IL-1β, IL-12 and IL-23, as well as activating IL-17-producing γδ T cells [185, 186]. Upon activation, pathogenic T cells infiltrate the BBB, where they mediate pathology.

1.6.2.1 Monocytes in EAE

While pathogenic T cells play a crucial role in the effector phase of EAE, inflammatory Ly6C^{high} monocytes have also been found to play an important pathogenic role in EAE. For example, CCR2-deficient mice are entirely resistant to EAE, and CCL2 expression correlates with disease severity [187, 188]. Furthermore, Ly6C^{high} CCR2^{+} monocytes have been found to infiltrate into the diseased CNS, where they proliferate and contribute the effector phase of disease [189]. Ly6C^{high} monocytes infiltrate the CNS and express pro-inflammatory cytokines, such as IL-1β and TNF, which are involved in pathology [190–192]. Recent research has revealed that Ly6C^{low} monocytes may play a role in suppression of the effector phase of EAE and act as mediators of immune resolution. In mice deficient in NR4A1, a transcription factor essential for the generation of Ly6C^{low} monocytes, EAE develops significantly sooner than in wildtype controls [193].

1.7 Helminths

Helminths, also known as parasitic worms, are large, multicellular organisms that are grouped into two separate phyla: Nematoda and Platyhelminthes. Nematodes, or roundworms, include intestinal and filarial worm species. Nematodes are often referred to as soil-transmitted helminths, as they are primarily transmitted through contaminated soil. Two examples of roundworms are *Ascariasis lumbricoides* and *Brugia malayi*, the worms responsible for ascariasis and lymphatic filariasis (also known as elephantiasis), respectively [194]. Platyhelminthes, or flatworms, include the trematodes (flukes) and cestodes (tapeworms). *Schistosoma mansoni* and *F. hepatica* are two of the most common flukes, whereas *Taenia solium*, the causative agent of cysticercosis, is a widely prevalent tapeworm [194].

Helminth species often have complex life cycles that involved multiple hosts and stages of maturity, including egg, larval and adult stages [195]. The definitive host is infected by adult, sexually mature helminths. Most helminths require an intermediate host, in which egg or larval stages of maturity must occur. However, some helminth species do not require an intermediate host, such as hookworms,

which can survive independently in the soil. Parasites primarily infect definitive hosts via ingestion of contaminated food or water, insect bites and the soil.

Helminths infect approximately one-third of the human population, with many sufferers contracting multiple parasitic infections at once, a phenomenon known as polyparasitism. According to the Centre for Disease Control (CDC), ascariasis is the most common helminth infection, and is estimated to infect between 800 million and 1.2 billion people globally. The majority of helminth infections occur in sub-Saharan Africa, Latin America, China and East Asia (Figure 1.2). Helminth infections rarely cause death; however, they are often long-lasting and frequently debilitating. The competition within the definitive host for nutrients results in malnutrition, anaemia and fatigue [196]. Children, especially young children, typically maintain a higher burden of parasites within the body. While the exact reasons for this remain unclear, this results in growth stunting, diminished physical fitness, impaired memory and cognition, as well as lower school performance/attendance, which often results in reduced future wage-capacity [195, 197–199]. Helminths can also be directly debilitating. For example, onchocerciasis (river blindness) is the leading cause of blindness and skin disease, in the adult populations of low- and middle-income countries (LMIC), while *B. malayi* is responsible for major limb/genital deformities (elephantiasis) [194].

1.7.1 Immune response to helminths

Helminths have probably parasitized humans for as long as we have existed. Helminth eggs have been found in mummified remains, and symptoms have been clearly defined by some of our earliest written works, including the bible. Helminths have exerted major evolutionary influence on the immune system. For example, in areas with regular parasitic infections, local human populations exhibit a greater diversity in the number of polymorphisms in genes encoding for cytokines [200]. Infection with helminths typically induces a type-2 immune response in the host, which, in some cases, can eliminate the parasite. This response is primarily mediated by the CD4 Th2 cells and the type-2 cytokines IL-4, IL-5, IL-10, and IL-13. Epidemiological studies have revealed that polymorphisms in genes encoding for type-2 immune responses, including *Il13*

and *Stat6*, are associated with increased susceptibility to helminth infections [201–204]. Further evidence for the role of type-2 immune responses have been found in mouse models of infection.

Epithelial cells are the first cells to recognize and respond to helminth infection. Upon infection with a helminth, epithelial cells release alarmin cytokines such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). These cytokines signal to and activate mature innate immune cells at the site of infection, including type-2 innate lymphoid cells (ILC2s), mast cells, eosinophils, macrophages and DC [205]. IL-25 and IL-33 have proven to be essential in the clearance of multiple helminth infects, including *N. brasiliensis*, *T. muris*, and *S. mansoni* [206–209]. TSLP has been demonstrated to modify the activation of local DC populations, inducing surface expression of OX40L and suppressing IL-12p40 secretion, resulting in enhanced Th2 cell polarisation [205].

ILC2s respond quickly to alarmin signalling from epithelial cells. Upon recognition of IL-25 or IL-33, ILC2s produce IL-5 and IL-13, which promote further anti-helminthic responses by activating and recruiting eosinophils and M2 macrophages, as well as stimulating mucus production by goblet cells and maintaining epithelial barrier integrity [210, 211]. Previous work from our lab has shown that mast cells act as early responders to IL-33 by secreting IL-6 and IL-13, which enhance M2 macrophage polarisation [212]. Similarly, TSLP promotes the production of IL-4 by basophils, which promotes the induction of Th2 cells [213, 214]. IL-5 promotes the differentiation, survival and recruitment of eosinophils to the site of infection [215]. Once activated and recruited to the site of infection, eosinophils degranulate and secrete major basic protein, eosinophil peroxidase, and eosinophil cationic protein, are toxic to helminths and aid in worm clearance [216, 217]. In addition to granulocytic proteins, eosinophils also secrete type-2 cytokines, such as IL-4, IL-10 and IL-13, as well as TGF-β. Secretion of IL-13 by multiple cells is essential for effective worm expulsion through the 'weep and sweep' response, which includes epithelial shedding, mucus secretion and smooth muscle contraction [218].

IL-4 and IL-13 drive the alternative activation of macrophages. Furthermore, IL-4 expands local tissue-resident macrophages populations [219]. During infection,

infiltrating monocyte-derived macrophages have been shown to adopt the alternative activation of tissue-resident macrophages [220]. Macrophages that are alternatively activated during helminth infection can be characterized by their production of resistin-like molecule-α (RELM-α, also known as FIZZ1) and chitinase 3-like 3 (YM1), which directly damage the worm [79, 206]. Furthermore, M2 macrophages actively produce Arginase-1, which induces smooth muscle contraction to expel helminths and functionally metabolises arginine, effectively starving helminths of the essential amino acid [221, 222]. Together with eosinophils, M2 macrophages form granulomas, which limit translocation of helminths [79]. In addition to combatting helminths, M2 macrophages and their secreted factors contribute to wound repair mechanisms. For example, TGF-β, RELM-α and YM1 mediate deposition of extracellular matrix [222].

Although the exact mechanisms for Th2 cell induction are still unclear, it is thought to occur through recognition of helminth-derived products by DCs or other innate immune cells through ligation of PRRs. For example, DCs recognize multiple excretory/secretory (ES) proteins from *S. mansoni* through TLR2 and TLR4 [223]. Furthermore, the glycoprotein omega-1 secreted by *S. mansoni* has been shown to condition DCs to induce Th2 cells [224] In addition to DCs, IL-4 from eosinophils and ILC2s are essential in driving the Th2 response. Depletion of CD4 T cells in mice infected with *N. brasiliensis* resulted in failure to clear the infection [225]. Binding of IL-4 and IL-13 to IL-4Rα induces antibody class switching in B cells, to parasite-specific IgG1 and IgE, and induces alternative activation of macrophages [79].

Importantly, while immune responses to helminths may be similar across all species, they are not identical. Indeed, eosinophil-deficient mice exhibit enhanced worm burdens during primary infection with *Heligmosomoides polygyrus*, however, expulsion is not impaired upon primary infection with *N. brasiliensis*, *T. muris*, or *S. mansoni* [226–229]. This suggests diverse and novel roles for innate immune cells that vary between infections.

1.7.2 Immune modulation by helminths

Despite the robust type-2 responses induced during infection with helminths, these responses often fail to expel the parasite, leading to chronic infections.

While a co-evolution of humans and helminths has allowed for sophisticated immune responses to helminths, helminths have similarly sophisticated methods for immunomodulation. Antigens located on the surface of helminths, as well as ES derived from the helminths induce immunoregulation. During helminth infection, suppression of immune responses are beneficial to host and parasite as they promote wound healing and suppress potentially harmful Th1/Th17-mediated immunopathology [79]. Helminths induce Treg cells, which suppress the function of Th1, Th2 and Th17 cells, prolonging helminth survival. Treg cells produce anti-inflammatory cytokines, including IL-10 and TGF-β, suppressing anti-helminthic Th2 responses and potentially harmful Th1/17 cells. Indeed, depletion of Treg cells in helminth infection leads to the clearance of the worms in mice. Additionally, the M2 macrophages present during infection play a key role in maintaining and expanding Treg cells, while also aiding in wound repair mechanisms [230]. Furthermore, helminths secrete various proteins that actively promote Treg cell development. For example, during infection, *Heligmosomoides polygyrus bakeri* secrete a family of TGF-β mimics, resulting in the preferential activation of Treg cells [230–232].

Helminths and their ES products also promote regulatory responses through innate immune cells. Molecules derived from the ES of *Trichinella spiralis* have been shown to inhibit maturation of DC, and promote tolerogenic IL-10-secreting DCs that preferentially induces Treg cells [233–235]. In another example, lysophosphatidylserine found in the ES of *S. mansoni* binds to TLR2 expressed on DCs, resulting in the induction of IL-10-secreting Treg cells [236]. In mice deficient in TLR2, infection with *S. mansoni* leads to heightened Th1 responses and associated immunopathology, with a commensurate decrease in Treg cells [237]. Many immunomodulatory proteins found in ES are conserved across species and induce similar modifications to host responses. For example, cystatins found in the ES of multiple helminths, including *B. malayi*, *A. lumbricoides*, and *Litomosomoides sigmodontis*, induce the expansion of regulatory macrophages with reduced APC function and the induction of hyporesponsive T cells [238–240].

Direct suppression of innate and adaptive immune responses is vital to helminth survival and allows for chronic infection. However, type-2 and regulatory

responses actively inhibit the induction of type-1 and type-3 immune responses. While this is beneficial to the helminth, it can suppress the necessary inflammation required for clearance of bacterial, viral, and fungal pathogens, as well as suppressing immune responses to vaccination. Osborne et al. recently demonstrated that infection of mice with *T. spiralis* impaired the protective CD8 T cell response to norovirus via induction of M2 macrophages and secretion of YM1 [241]. Introduction of an antibody against YM1 ameliorated the impaired CD8 T cell responses and improved viral clearance. Mice infected with *N. brasiliensis* have a reduced ability to clear *M. tuberculosis* infection from the lungs due to impaired bacterial killing in alveolar macrophages [242].

A number of studies have found that helminth infection during vaccination with BCG inhibits induction of Th1 cell responses, and increases TGF-β, resulting in reduced efficacy and increased bacterial load when infected with Mtb [243, 244]. Furthermore, mice infected with *L. sigmodontis* had impaired immune responses to seasonal influenza virus vaccination, and this was associated with elevated IL-10 [245]. Suppression of vaccine efficacy lasted up to 100 days after clearance of the parasite.

1.8 Hygiene hypothesis

Since the early 20th century, the incidence of helminth infections in high-income countries has declined. Simultaneously, the incidence of autoimmune and allergic diseases has risen at a commensurate rate [246, 247]. In 1989, David Strachan observed a correlation between the incidence of hay fever, and smaller family sizes, with a lower incidence in the youngest children. He posited that infections and antigens from "unhygienic" older sibling acted to protect younger siblings from developing allergic rhinitis or hay fever [247]. This has given rise to the modern 'hygiene hypothesis,' which proposes that exposure to various infectious diseases early in life protects against the development of autoimmune and allergic disease later in life (Figure 1.5).

Recent work has shown that individuals infected with helminths exhibit a diminished propensity for developing allergies. For example, schistosomiasis, has been shown to reduce the risk of development of asthma and atopic

dermatitis [248, 249]. Additionally, mouse studies have shown that helminth-induced Treg cells and IL-10 can suppress allergic airway inflammation [250]. Administration of anti-inflammatory protein (AIP)-2 derived from the ES of the hookworm *Necator americanus* to mice induced tolerogenic DCs, which promoted induction of Treg cells and resulted in suppression of immunopathology in mouse models of asthma and ovalbumin (OVA)-induced allergic airway inflammation (AAI) [251].
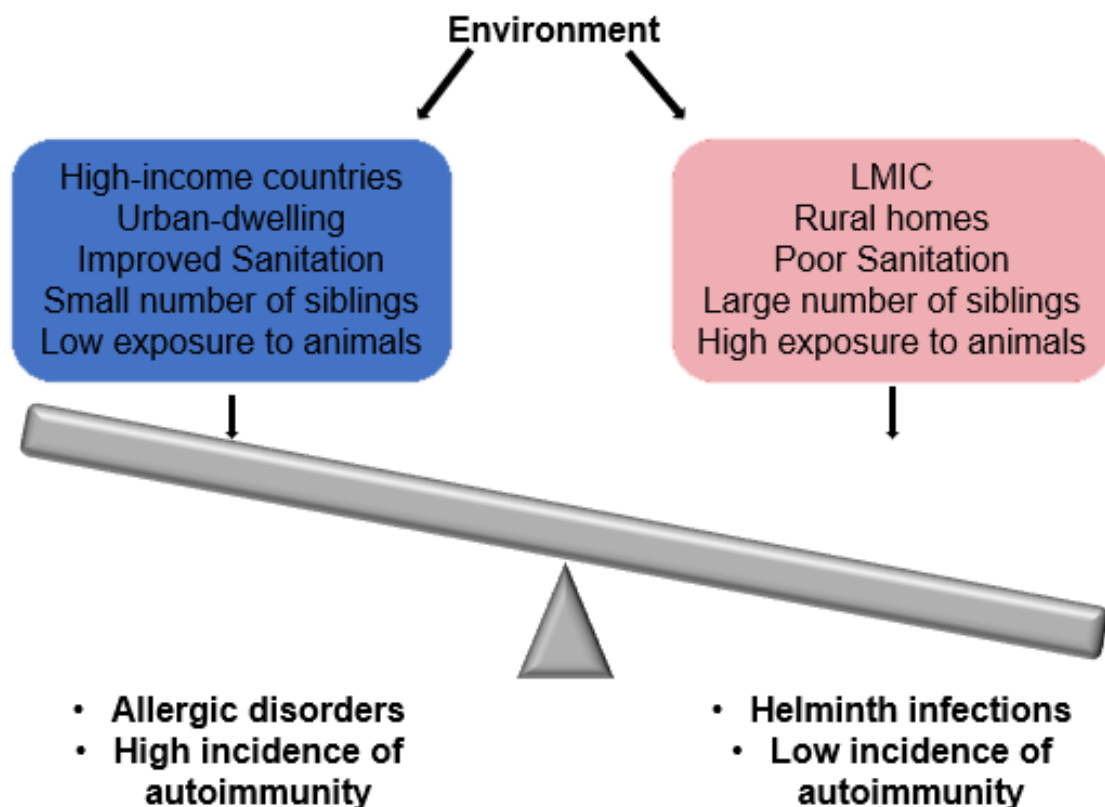
Figure 1.5 Simplified hygiene hypothesis

Comparison of environmental factors associated with high-income countries versus low- and middle-income countries (LMIC). Improved sanitation, fewer siblings and low exposure to animals and pathogens correlates with an increase in the incidence of allergic and autoimmune disorders. Conversely, LMIC with higher exposure to pathogens and helminths, exhibit low frequencies of allergic and autoimmune disorders. Adapted from Wills-Karp, 2001 [253].

While the hygiene hypothesis originally only dealt with allergic inflammation, it has since been updated to include autoimmunity (Figure 1.6). For example, a plethora of studies have demonstrated a decreased risk for type-1 diabetes in humans previously exposed to a helminth infection [252]. In a study in 2007, Correale and Farez found that parasite-infected MS patients had significantly lower numbers of symptoms and relapses compared with uninfected MS patients across 5 years. In addition, assessment of *ex vivo* immune responses revealed that peripheral blood mononuclear cells (PBMCs) from MS patients infected with parasites produced significantly higher IL-10 and TGF-β, and less IL-12 and IFN-γ after stimulation with MBP [254]. Furthermore, a study by Ponsonby et al. found that within the first 6 years of life, infants with higher numbers of siblings had a reduced risk of developing MS later in life [255].

Animal models have shown that infection with helminth parasites can attenuate autoimmune disease. Previous work from our lab has demonstrated that infection of mice with *F. hepatica* attenuated EAE via production of TGF-β and induction of Treg cells [256]. Infection with the tapeworm *Taenia crassiceps* attenuated EAE through the induction of M2 macrophages, differentiated from Ly6C^{high} monocytes [257]. Attenuation of autoimmunity by helminths can be replicated though administration of helminth-derived products, such as eggs or ES. For example, treatment of mice with eggs from *S. mansoni* resulted in a decrease in the severity of EAE, and this was associated with an increase in the production of TGF-β, IL-4, and IL-10, and a decrease in IFN-γ [258]. Furthermore, treatment of mice with soluble products derived from the ES of *T. suis* or *T. spiralis* have been shown to induce tolerogenic DCs, which inhibit Th1 and Th17 cells, resulting in attenuation of EAE [259]. Previous work from our lab has demonstrated that ES from *F. hepatica* can attenuate EAE, when administered before and after disease onset, through IL-5 and IL-33 production [260].

Following the promising data from animal models, there have been multiple clinical trials investigating the use of live helminth infection as treatment for autoimmune disorders. These early-phase clinical trials examined the capacity of infection with live helminth or helminth-derived products to treat a range of autoimmune diseases, including metabolic syndrome, Crohn's disease, secondary progressive MS and type-2 diabetes [261–264].
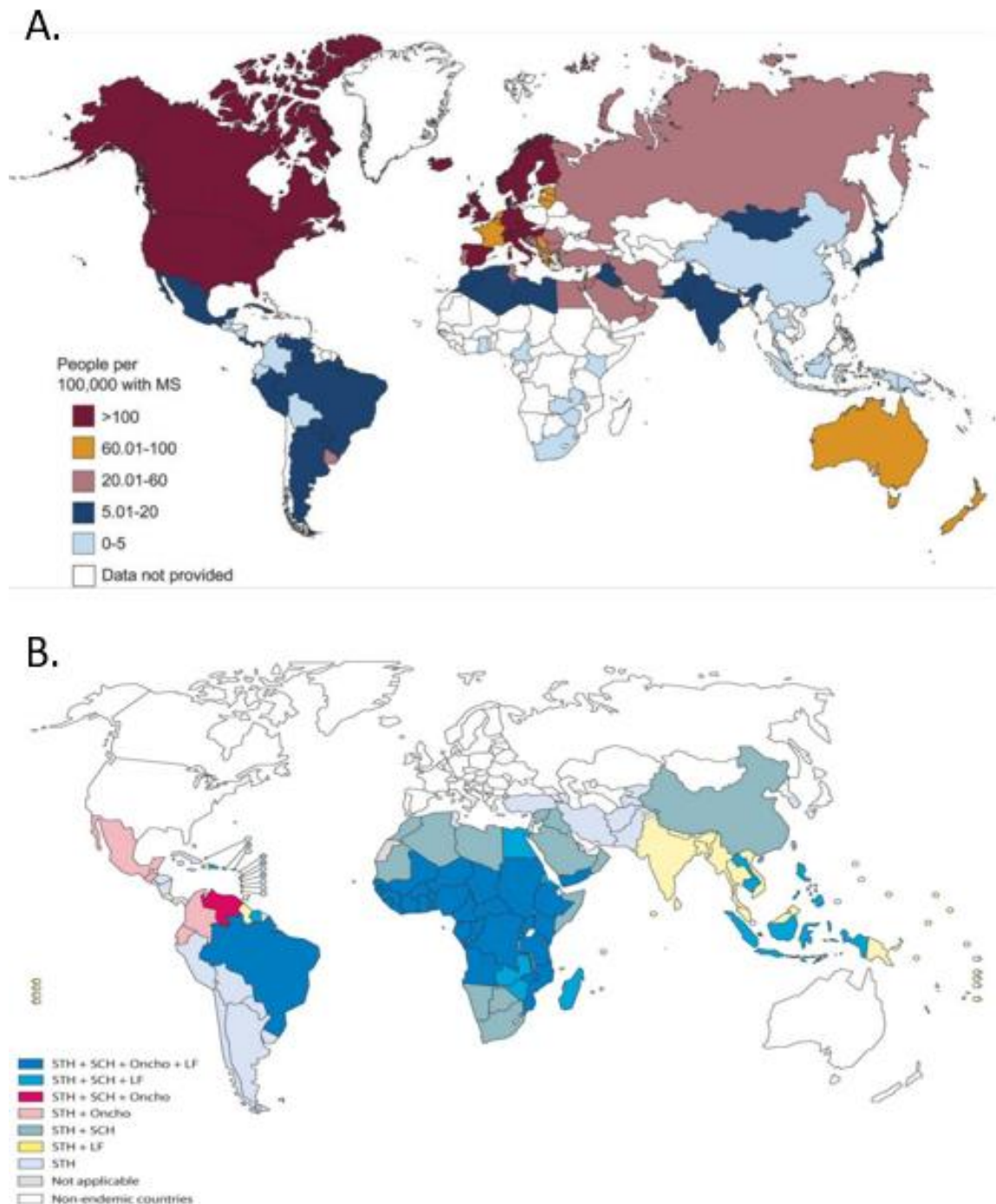
Figure 1.6 Inverse relationship between the incidence of MS and various helminth infections globally

Global map of MS incidence; adapted from Browne et al (2014) (A). Global map detailing distribution of various helminth parasites and detailing co-infection. Helminths listed include: Soil-Transmitted Helminths (STH), Schistosomiasis (SCH), Onchocerciasis (Oncho) and Lymphatic Filariasis (LF); adapted from Lustigman et al (2012) (B) [178, 266].

Recent reports from a phase 2 clinical trial involving infection of relapsing-remitting MS patients with *N. americanus* showed that the infection was well tolerated and increased the number of circulating Treg cells [265]. However, there was no significant change in the number of disease relapses between helminth-treated and placebo-treated patients. Importantly, the authors noted that due to restrictions in eligibility criteria, MS patients in the trial were in early stages of diseases, when relapses are not yet common.

In a systematic review of 28 ongoing live helminth clinical trials in MS patients, Fleming and Weinstock reported that disease progression was neither worsened nor improved, and that there were no serious adverse effects associated with helminth infection [267]. Due to the many regulatory and practical concerns with the use of live helminth infections, there have been several early-stage clinical studies investigating the ability of helminth-derived products to attenuate autoimmune disease. OVA peptide isolated from the ES of *T. suis* has had mixed results as a therapeutic intervention for relapsing-remitting MS, with the small number of patients either marginally improving or showing no change in symptoms [268, 269]. More research is required before helminths or their products can be used as therapeutic interventions for autoimmune diseases. Therefore, further understanding the immunomodulatory mechanisms of helminths and helminth-derived products may provide novel avenues to induce better protection against autoimmune diseases.

1.9 *F. hepatica*

F. hepatica and its close relative *Fasciola gigantica* are the primary causative parasites for fascioliasis. *F. hepatica* is a flatworm trematode, of the phyla Platyhelminthes, and is commonly known as liver fluke. The WHO estimates that over 2.4 million people are currently infected with *F. hepatica* across 70 countries spanning all continents, with millions more at risk of infection [270]. Despite relatively low numbers of infected humans when compared to other helminths, *F. hepatica* is an important parasite of cattle, goats, sheep, and other ruminants. An estimated $3 billion is lost worldwide per year due to losses from mortalities, resulting in loss of meat, milk, and wool [271].

The life cycle of *F. hepatica* is considerably complex, requiring an intermediate and definitive host. The entire process usually takes 14-23 weeks and is comprised of four stages. A mammalian definitive host harbours the liver fluke in the bile ducts, where it grows to sexual maturity for approximately 3-4 months. Mature flukes release eggs though the ducts, which travel to the intestines and are passed out through the definite host's faeces and into fresh water. This allows the eggs to enter a water supply, develop into larvae and infect an intermediate host. For the liver fluke, the intermediary host is typically a freshwater gastropod snail of the family *Lymnaeid*. The larvae then develop into cercariae in the snail. Finally, upon expulsion from the snail, the cercariae will travel through water until it can encyst as metacercariae (infectious larvae) on a plant near the water's surface. Within 24 h the larvae are infectious to mammals again and infect another definitive host when ingested. While this typically is how livestock are infected, humans tend to get infected through the consumption of infected foods. Upon ingestion by the definitive host, the immature flukes travel from the gastrointestinal tract to the liver. Once inside the liver, the adult worm will feed on erythrocytes and hepatocytes until finally reaching the bile duct and going through sexual maturity, whereupon the cycle begins anew (Figure 1.7) [272].

Symptoms of fascioliasis come from either the invasive or chronic phases of infection. During the invasive phase, the larvae travel through the body to reach the liver. This causes significant tissue damage in addition to fever, abdominal pain, gastrointestinal disturbances (decreased appetite, nausea, diarrhoea), cough, and many more symptoms. Conversely, during the chronic stage, when the fluke is resident in the liver, symptoms arise primarily due to blockages, which includes abdominal pain, hepatomegaly, fever, epigastric pain, jaundice, and more [272]. For much of the chronic phase, people infected with *F. hepatica* are asymptomatic, suggesting that co-evolution with humans has yielded robust mechanisms of immunosuppression to promote its successful invasion [273]. Currently, the WHO recommend Triclabendazole for treatment against infection with *F. hepatica* as it is efficacious against both immature and mature flukes. However, there is emerging evidence of drug-resistance to triclabendazole in livestock and humans, prompting the need to develop new anthelmintic drugs and potential vaccines against *F. hepatica* antigens [274–277].
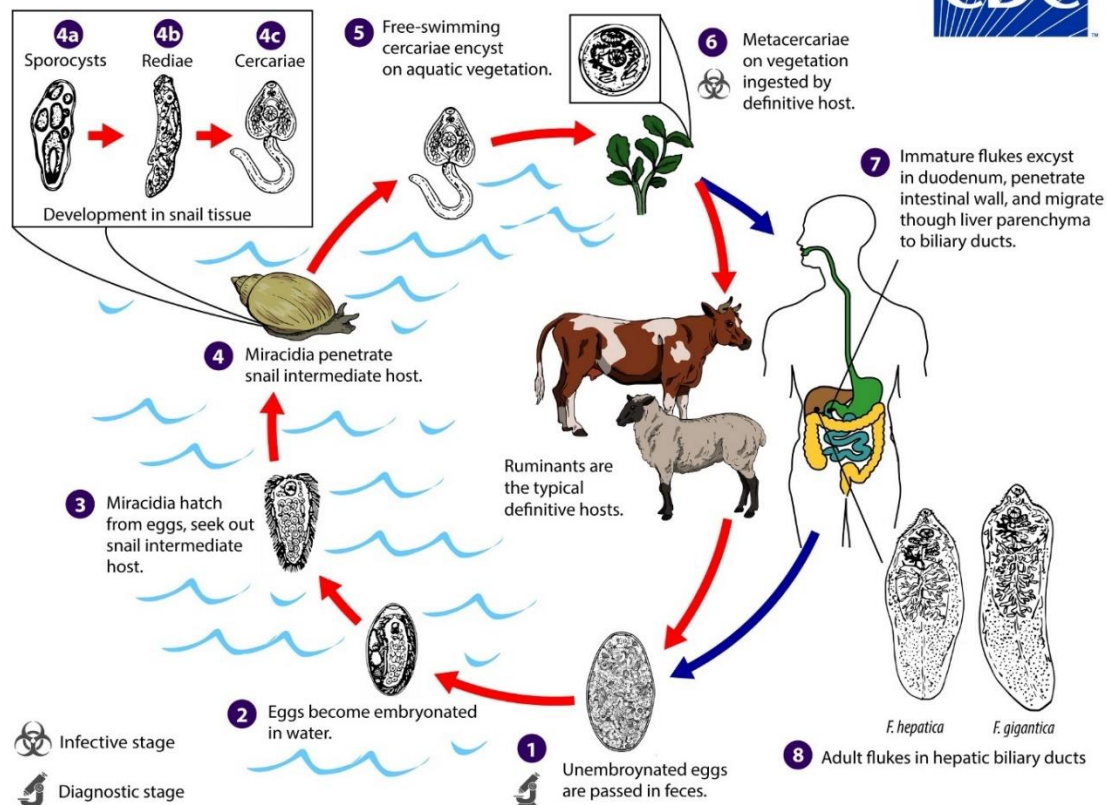
Figure 1.7 The life cycle of _F. hepatica_

Eggs are released from infected sheep, cattle or humans (1), becoming embryonated in water (2), before developing further into a miracidium (3) and infecting the snail intermediate host (4). Upon maturation in the intermediate host (4a-c), cercariae burrow out of the snail and into the surrounding water (5), encysting onto aquatic vegetation or other surfaces as metacercariae (6). The definitive mammal host then acquire infection through ingestion of infected plants or water (7). Once inside the definitive host, the metacercariae encyst in the intestine before burrowing into the peritoneal cavity, liver, and finally biliary ducts (8). The flukes then sexually mature across several months and produce thousands of eggs per day, which are then released into the environment through the digestive tract of the definitive host to begin the cycle anew (1). Figure provided by CDC [279].

1.9.1 Immune response to *F. hepatica*

Helminth infections are typically defined by combined type-2/regulatory immune responses. Indeed, the immune response to *F. hepatica* early in infection in cattle appears to be type-2, as circulating PBMCs isolated from infected cattle show robust IL-4, and diminished IFN-γ upon stimulation with parasite antigen [278]. However, this response was found to be largely inefficient at clearing the infection in cattle, which then persisted until deemed chronic, after approximately 12 weeks. At this time, PBMCs could no longer mount robust IL-4 responses, instead producing robust IL-10 and TGF-β responses. Interestingly, depletion of IL-10 is able to restore the IL-4 response [278].

Studies in sheep and cattle have found a strong increase in parasite-specific IgG1, significant enhancement of circulating eosinophils and M2 macrophages, and increased IL-4, IL-10, IL-13 and TGF-β in response to infection [280–283]. Experimental infection in mice has also been shown to promote eosinophilia and robust alternative activation of macrophages, resulting in the development of Th2 cell and Treg cells [256, 284].

1.9.2 Immune modulation by *F. hepatica* and ES products

Helminths have evolved different methods to modify host innate and adaptive immune response. During natural infection, *F. hepatica* potently induces type-2 and regulatory immune responses, which are associated with suppression of Th1 and Th17 cells. Previous work from our lab has shown that infection of mice with *F. hepatica* attenuated the clinical signs of EAE through the induction of Th2 cells and inhibition of autoantigen-specific Th1 and Th17 cells [256]. Conversely, type-1 and type-3 immune responses are required for clearance of bacterial, viral, and fungal pathogens. Suppression of type-1 and type-3 responses during helminth infection results in an inability to mount protective immune responses against these pathogens. For example, our lab has previously demonstrated that co-infection of mice with *F. hepatica* and *B. pertussis*, resulted in expansion of Th2 cells, suppression of Th1 cells, and delayed clearance of the *B. pertussis* from the lungs [285]. Additionally, it has been shown that cattle infected with *F.*

hepatica had reduced Th1 responses and pro-inflammatory cytokine production during co-infection with *M. tuberculosis* [286].

Infection with *F. hepatica* is potential beneficial in the treatment of autoimmune disease, however it is associated with high mortality in mice [287]. Therefore, research has begun to focus on helminth products and their mechanisms of immunomodulation. The *F. hepatica* excretory/secretory product (FHES) modifies the innate and adaptive immune responses, directly and through induction of cytokines. For example, ES and tegument antigens from *F. hepatica* induce tolerogenic, immature DCs, which inhibit Th17 cells [288–290]. Furthermore, FHES was capable of preventing onset, and attenuating disease severity in EAE through suppression of auto-reactive Th1 and Th17 cells [260].

FHES is composed of a myriad of immunomodulatory molecules that can suppress the host immune responses [291]. These include families of fatty acid binding proteins (FaBPs), helminth defence molecules (FhHDMs), TGF-like molecules (FhTLMs), Kunitz-type molecules (FhKTMs), glutathione s-transferases and thioredoxin peroxidase. Fh15, a FaBP, was found to inhibit TLR4 activation, resulting in a significant decrease in circulating pro-inflammatory cells in murine models of sepsis [292]. Furthermore, Fh12, another FaBP, has been shown to directly induce alternative activation of human macrophages [293]. FhKTMs are a family of Kunitz-type inhibitors that were shown to regulate inflammation and mediate tissue repair mechanisms by inhibiting proteases [294–296]. It was recently demonstrated that FhKTM inhibits LPS-induced secretion of IL-12 and TNF from DCs [297]. Importantly, DCs pre-treated with FhKTM were also found to inhibit LPS-induced secretion of IFN-γ and IL-17 by spleen cells.

FhHDM-1 is an extensively studied cathelicidin-like molecule found in FHES [298, 299]. FhHDM-1 has been shown to directly inhibit NLRP3 inflammasome activation, resulting in reduced IL-1β secretion by macrophages [300]. Furthermore, Liu et al. discovered that FhHDM-1 is capable of directly binding, and potentially inhibiting, IL-2, IL-17 and IFN-γ [301]. Treatment of mice with FhHDM-1 has shown promise in suppressing pathogenic immune responses in several animal models of autoimmunity. Tanaka et al. demonstrated that

treatment of mice with FhHDM-1 inhibited neutrophil and eosinophil trafficking to the lung during house dust mite-induced asthma [302]. Furthermore, Lund et al. showed that treatment of mice with FhHDM-1 improved clinical signs of disease in a mouse model of type-1 diabetes and EAE [298]. Interestingly, treatment of mice with FhHDM-1 did not affect autoantigen-specific T cell secretion of IFN-γ or IL-17, rather attenuation of EAE clinical signs was attributed to suppression of TNF and IL-6 secretion by macrophages. In addition to individual compounds, treatment of mice with extracellular vesicles from *F. hepatica* are able to decrease disease severity in a model of ulcerative colitis, independent of T cells, via inhibition of TNF, IL-6 and IL-17 [303]. Previous work in our lab has found that treatment of mice with *F. hepatica* exosomes induces eosinophilia, M2 macrophages and potent production of IL-1RA, resulting in suppression of EAE (Walsh and Mills, unpublished).

Helminths and helminth-derived products are potent immunomodulators, especially in the context of autoimmunity, a study of their mechanisms should provide key insights into potential therapeutic strategies. Recently, our lab has demonstrated that treatment of mice with *F. hepatica* total extract (FHTE) induced peripheral anti-inflammatory trained innate immunity [113]. Pre-treatment of BMDM with FHTE induced trained immunity in macrophages, which produced less TNF and more IL-10 upon stimulation with LPS or Pam3Cys. Treatment of mice with FHTE enhanced M2 macrophages in peripheral tissues of mice, resulting in delayed onset and reduced severity of EAE. FHES contains a plethora of immunomodulatory proteins and may induce trained innate immunity as a mechanism of host immune evasion. The present study focuses on investigating how FHES modulates innate immune cells and HSCs to induce peripheral and central anti-inflammatory trained innate immunity.

1.10 Aims

- To characterise the immunomodulatory properties of FHES on innate immune cells

- To assess if FHES can induce trained innate immunity in macrophages

- To examine the effect of FHES on innate immune cells *in vivo*

- To examine the effect of FHES on HSCs

- To examine the role of FHES treatment on models of type-1, type-2 and type-3 inflammation

- To examine the innate immune and HSC response to FHES

- To examine mechanisms and cells involved in FHES-induced central trained immunity

- To investigate the role of FHES-induced central training can be transferred by BM transplantation

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, filtered fetal calf serum (FCS), 100 mM L-Glutamine (Gibco), and 100 µg/ml penicillin/streptomycin (Biowest). RPMI was used to culture murine BMDC and BMDM, while RPMI supplemented with 50 µM β-mercaptoethanol was used during culture of purified T cells, spleen cells and lymph node cells *ex vivo*.

2.1.2 ELISA Blocking Buffer

1% w/v Bovine Serum Albumin (BSA; Sigma) dissolved in 1X PBS.

2.1.3 ELISA Wash Buffer

0.5% Tween (Sigma)

Prepared in 1X PBS

2.1.4 ELISA Developing Solution

1X TMB Substrate Solutions (eBioscience)

2.1.5 ELISA Stop Solution

1 M sulphuric acid (H_2SO_4; Sigma)

2.1.6 Ethidium Bromide/Acridine Orange

2.5 g ethidium bromide ($C_{21}H_{20}BrN_3$; Sigma)

2.5 g acridine orange ($C_{17}H_{19}N_3$; Sigma)

Dissolved in 50 ml 1X PBS

2.1.7 Fluorescence-Activated Cell Sorting (FACS) Buffer

2% FCS

1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Thermo-Fisher)

0.1 M Ethylenediaminetetraacetic acid (EDTA; Sigma)

Prepared in 1X PBS, stored at 4 °C

2.1.8 Fixation/Permeabilization Buffer

Fixation/Permeabilization Concentrate (Invitrogen)

Diluted 1:3 in Fixation/Permeabilization Diluent (Invitrogen)

2.1.9 Hematopoietic Stem Cell (HSC) Isolation Buffer

2% FCS

1mM EDTA (Sigma)

Prepared in 1X PBS, stored at 4 °C

2.1.10 Isotonic Percoll Solution

Percoll reagent (GE Healthcare) was diluted 9:1 with sterile 10x PBS (Sigma) to prepare

an isotonic Percoll solution with a density of 1.123 g/ml.

40% Percoll (diluted in cRPMI) and 70% Percoll (diluted in PBS) solutions were then prepared.

2.1.11 Red Blood Cell Lysis Buffer

0.88% ammonium chloride (NH_4Cl; Merck)

Dissolved in dH_2O

2.1.12 2% Paraformaldehyde (PFA)

1 ml of 16% PFA (Pierce) diluted in 7 ml 1X PBS

2.1.13 Permeabilization Buffer

Permeabilization Buffer 10X (Invitrogen)

Diluted 1:10 in dH_2O

2.1.14 Phosphate-buffered saline (PBS) 10x

400 g sodium chloride (NaCl; Sigma)

58 g sodium hydrogen phosphate (Na_2HPO_4; Sigma)

10 g potassium dihydrogen phosphate (KH_2PO_4; Sigma)

10 g potassium chloride (KCl; Sigma)

Dissolved in 5 L of dH_2O, pH 7

2.1.15 Stainer-Scholte (S & S) liquid medium

L-Glutamic acid (monosodium salt) ($C_5H_8NNaO_4.xH_2O$)

L-Proline ($C_5H_9NO_2$)

Sodium Chloride (NaCl)

Potassium phosphate monobasic (KH_2PO_4)

Potassium chloride (KCl)

Magnesium chloride hexahydrate ($CaCl_2.2H_2O$)

Tris (Trizma base; $C_{14}H_{11}NO_3$)

Made up to 1 litre with ddH_2O (pH 7.3-7.4) and sterilized by autoclaving

(All reagents obtained by Sigma)

2.1.16 Supplement (200X)

0.4 g L-cystine ($C_6H_{12}N_2O_4S_2$) – Dissolved in 1 ml 37% Hydrogen chloride (HCl)

0.1 g Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$)

0.2 g L-ascorbic acid ($C_6H_8O_6$)

0.04 g Nicotinic acid ($C_6H_5NO_2$)

1 g L-glutathione (reduced; $C_{10}H_{17}N_3O_6S$)

Made up to 50 ml with ddH_2O and filter sterilized

(All reagents obtained by Sigma)

2.1.17 1% Casein salt solution

6 g NaCl (Sigma)

10 g BactoCasamino acids (Becton Dickinson)

Made up to 1 L with ddH_2O (pH 7.0-7.2) and autoclaved at 115 °C for 20 min.

2.1.18 Bordet-Gengou blood agar plates

5 ml glycerol (C3H8O3; Fisher Chemical)

15 g Bordet-Gengou agar (Becton Dickinson)

Made up to 500 ml with dd H20 and autoclaved at 121 °C for 20 min

2 ml Cephalexin (10 mg/ml; Sigma)

100 ml horse blood (pre-warmed to 37 °C; Cruinn)

2.1.15 ELISA Kits

Cytokine	Capture Antibody	Top Working Standard	Detection Antibody	Supplier
GM-CSF	1:60	1000 pg/ml	1:60	R&D
IL-1β	1:180	1000 pg/ml	1:180	R&D
IL-1RA	1:180	10,000 pg/ml	1:180	R&D
IL-4	1:500	2500 pg/ml	1:500	BD Biosciences
IL-5	1:500	2500 pg/ml	1:500	BD Biosciences
IL-6	1:500	5000 pg/ml	1:500	BD Biosciences
IL-10	1:120	2000 pg/ml	1:60	R&D
IL-12p70	1:180	1500 pg/ml	1:180	R&D
IL-13	1:120	4000 pg/ml	1:60	R&D
IL-17A	1:180	1000 pg/ml	1:180	R&D
IL-33	1:180	1000 pg/ml	1:180	R&D
IFN-γ	1:1000	10,000 pg/ml	1:500	BD Biosciences
TGF-β	1:180	2000 pg/ml	1:180	R&D
TNF	1:125	2000 pg/ml	1:60	R&D
YM1	1:180	5000 pg/ml	1:180	R&D

2.1.16 TLR ligands used *in vitro*

Agonist	Supplier	Working Concentration
Ultrapure LPS	Invivogen	100 ng/ml
CpG	Sigma	5 µg/ml

2.1.17 Antigens

Antigen	Supplier	Concentration *In vitro*	Concentration *In vivo*
FHES	See Section 2.2.7	0-100 µg/ml	100 µg/mouse
β-1,3-Glucan (Curdlan; from *Alcaligenes faecalis*)	Invivogen	n/a	100 µg/mouse
Heat-Killed *Mycobacterium tuberculosis* (Mtb)	Invivogen	10 ng/ml	n/a
MOG$_{35-55}$	Genscript	100 µg/ml	100 µg/mouse
Ovalbumin (OVA)	Sigma	12.5-50 µg/ml	6% w/v aerosolized
Pertussis toxin (PT)	Kaketsuken	n/a	125 ng/mouse

2.1.18 Primers used in RT-qPCR

Gene	Protein	Product Code	Ref. Seq	Supplier
18S	18S RNA	4319413E	X03205.1	ABI
Areg	Amphiregulin	Mm01354339_m1	NM_009704.3	ABI
Arg1	Arginase-1	Mm00475988_m1	NM_007482.3	ABI
Cd38	CD38	311558	NM_007646.4	Roche
Cebpa	C/EBP-α	Mm00514283_s1	NM_001287514.1	ABI
Cebpb	C/EBP-β	Mm00843434_s1	NM_001287738.1	ABI
Chil3	YM1	312249	NM_009892	Roche
Csf1	CSF-1	Mm00432686_m1	NM_001113529.1	ABI
Csf1r	CSF-1R	Mm01266652_m1	NM_001037859.2	ABI
Csf2	CSF-2	Mm01290062_m1	NM_009969.4	ABI
Csf2ra	CSF-2Rα	Mm00438331_g1	NM_009970.2	ABI
Csf3	CSF-3	Mm00438334_m1	NM_009971.1	ABI
Csf3r	CSF-3R	Mm00432738_m1	NM_007782.3	ABI
Cx3cr1	CX3CR1	Mm02620111_s1	NM_009987.4	ABI
Egr2	EGR2	310303	NM_010118	Roche
Foxp3	FOXP3	Mm00475162_m1	NM_001199347.1	ABI
Fpr2	FPR2	317870	NM_008039	Roche
Gata3	GATA3	Mm00484683_m1	NM_008091.3	ABI
Ifng	IFN-γ	Mm01168134_m1	NM_008337.3	ABI
Il10	IL-10	Mm00439614_m1	NM_010548.2	ABI
Il13	IL-13	Mm00434204_m1	NM_008355.3	ABI
Il1b	IL-1β	Mm00434228_m1	NM_008361.3	ABI
Il1rl1	ST2	Mm00516117_m1	NM_001025602.3	ABI
Il1rn	IL-1RA	Mm00446186_m1	NM_001039701.3	ABI
Il23a	IL-23	Mm00519943_m1	NM_144548.1	ABI
Il4	IL-4	Mm00445259_m1	NM_021283.2	ABI
Il5	IL-5	Mm00439646_m1	NM_010558.1	ABI
Irf4	IRF4	Mm00516431_m1	NM_013674.1	ABI
Irf8	IRF8	Mm00492567_m1	n/a	ABI
Junb	Jun-B	Mm04243546_s1	NM_008416.3	ABI

Kitl	SCF	Mm00442972_m1	NM_013598.2	ABI
Klf2	KLF2	Mm00500486_g1	NM_008452.2	ABI
Mrc1	CD206	313688	NM_008625	Roche
Nos2	iNOS	Mm00440502_m1	NM_010927.3	ABI
Nr4a1	NR4A1	Mm01300401_m1	NM_010444.2	ABI
Ptgs2	PTGS2	Mm00478374_m1	NM_011198.3	ABI
Retnla	FIZZ1	Mm00445109_m1	NM_020509.3	ABI
Rorc	RORγt	Mm01261022_m1	NM_001293734.1	ABI
Socs2	SOCS2	Mm05820064_g1	NM_001168655.1	ABI
Spi1	PU.1	Mm00488140_m1	NM_011355.1	ABI
Tbx21	T-bet	Mm00450960_m1	NM_019507.2	ABI

2.1.19 Antibodies used in flow cytometry

Specificity	Fluorochrome	Clone	Isotype	Supplier
CD3	PE/Dazzle594	17A2	Rat IgG2b	Biolegend
CD4	AF700	RM4-5	Rat IgG2a, κ	Biolegend
CD4	BV650	RM4-5	Rat IgG2a, κ	Biolegend
CD11b	APC/eFluor780	M1/70	Rat IgG2b	eBioscience
CD11b	PE/Dazzle594	M1/70	Rat IgG2b	Biolegend
CD11b	PE-Cy5	M1/70	Rat IgG2b	eBioscience
CD11c	eFluor450	N418	Armenian Hamster IgG	eBioscience
CD16/32	APC/Cy7	93	Rat IgG2a	Biolegend
CD19	PE/CF594	1D3	Rat IgG2a	BD
CD34	FITC	RAM34	Rat IgG2a	BD
CD34	BV421	RAM34	Rat IgG2a	BD
CD40	FITC	3/230	Rat IgG2a	BD
CD41	BB700	MWReg30	Rat IgG1, κ	BD

CD45	AF700	30-F11	Rat IgG2b	Biolegend
CD45	BV711	30-F11	Rat IgG2b	Biolegend
CD45	BV785	30-F11	Rat IgG2b	Biolegend
CD45	PE	30-F11	Rat IgG2b	eBioscience
CD45.1	AF700	A20	Mouse IgG2a, κ	Biolegend
CD45.2	PE	104	Mouse IgG2a, κ	Biolegend
CD48	APC	HM48-1	Armenian Hamster IgG	Biolegend
CD80	PE/CF594	16-10A1	Armenian Hamster IgG	BD
CD86	FITC	GL-1	Rat IgG2a	BD
CD86	APC/Cy7	GL-1	Rat IgG2a	Biolegend
CD86	PE/Cy7	GL-1	Rat IgG2a	Biolegend
CD115	BV711	AF598	Rat IgG2a	Biolegend
CD117	APC/Cy7	2B8	Rat IgG2b	Biolegend
CD117	PE/Cy7	2B8	Rat IgG2b	eBioscience
CD127	PE/Cy5	A7R34	Rat IgG2a	eBioscience
CD150	BV785	TC15-12F12.2	Rat IgG2a	Biolegend
CCR2	AF647	SA203G11	Rat IgG2b	Biolegend
CCR2	PE/Cy7	SA203G11	Rat IgG2b	Biolegend
CX3CR1	APC	SA011F11	Mouse IgG2a	Biolegend
CX3CR1	BV650	SA011F11	Mouse IgG2a	Biolegend

CX3CR1	BV711	SA011F11	Mouse IgG2a	Biolegend
F4/80	PerCP/Cy5.5	BM8	Rat IgG2a	Biolegend
F4/80	AF700	BM8	Rat IgG2a	Biolegend
F4/80	PE/Cy5	BM8	Rat IgG2a	eBioscience
F4/80	PE/Cy7	BM8	Rat IgG2a	eBioscience
IFN-γ	BV711	XMG1.2	Rat IgG1, κ	BD
IL-4	FITC	11B11	Rat IgG1, κ	Biolegend
IL-5	APC	TRFK5	Rat IgG1, κ	Biolegend
IL-10	BV421	JES5-16E3	Rat IgG2b, κ	Biolegend
IL-13	PE	W17010B	Rat IgG1, κ	Biolegend
IL-17	PerCP/Cy5.5	TC11-18H10.	Rat IgG1, κ	Biolegend
FLT3/CD135	PE	A2F10	Rat IgG2a	Biolegend
Ki67	eFluor450	SolA15	Rat IgG2a	eBioscience
Ly6C	FITC	AL-21	Rat IgM	BD
Ly6C	PerCP/Cy5.5	HK1.4	Rat IgG2c	eBioscience
Ly6C	BV605	AL-21	Rat IgM	BD
Ly6G	PE/Dazzle594	IA8	Rat IgG2a	Biolegend
MHC Class II	FITC	M5/114.41.2	Rat IgG2b	eBioscience
MHC Class II	APC	M5/114.41.2	Rat IgG2b	eBioscience
MHC Class II	BV711	M5/114.41.2	Rat IgG2b	BD
MHC Class II	PE	M5/114.41.2	Rat IgG2b	eBioscience
NK1.1	PE-Dazzle 584	PK136	Mouse IgG2a, κ	Biolegend

Phospho-S6	PE	D57.2.2E	Rabbit IgG	Cell Signaling Technology
Sca-1	FITC	D7	Rat IgG2a	Biolegend
Sca-1	eFluor605NC	D7	Rat IgG2a	eBioscience
Sca-1	PE/Dazzle594	D7	Rat IgG2a	Biolegend
Siglec-F	PE	E50-2440	Rat IgG2a	BD
Siglec-F	PE/Dazzle594	E50-2440	Rat IgG2a	BD
TCRγδ	PerCP/Cy5.5	GL3	Armenian Hamster IgG	BIolegend
Ter119	PE/Dazzle594	TER-119	Rat IgG2b	Biolegend
TNF	PE/Cy7	MP6-XT22	Rat IgG1, κ	Biolegend

2.1.20 Mice

Female specific pathogen-free C57BL/6 were purchased from Harlan UK Ltd (Bicester, UK). Mice were 6-12 weeks old at the initiation of each experiment and were sacrificed by cervical dislocation or by asphyxiation with CO_2. Animals were maintained according to the regulations and guidelines of the European Union and the Irish Medicines board. Experiments were conducted under license from the Irish Medicines board and with the approval from the Trinity College Dublin comparative medicine department. Mice were age-matched at the time of initiation of treatments, 4-7 weeks old.

2.2 Methods

2.2.1 Cell Counting

Viable cells were counted by diluting 10 µl of cells in 90 µl EBAO solution. The mixture was then placed in a haemocytometer (Hycor Biomedical). The numbers of viable cells (green) and numbers of dead cells (orange) in the nine squares were counted using a UV-fluorescent microscope.

No. Cells/ml= Count x Dilution Factor (10) x 10^4

2.2.2 Generation of bone marrow-derived macrophages (BMDM)

BMDM were generated from C57Bl/6 mice. Naïve mice were sacrificed by asphyxiation with CO_2. The tibia and femur were removed, followed by the connective and muscle tissue surrounding. The bones were then left in cold, fresh media until ready to be processed. The tibias and femurs then had the tips of the epiphysis removed by a sterile scissors exposing the marrow. The bone marrow was then flushed out using a 25-gauge needle and a 20ml syringe filled with complete RPMI into a petri dish. The flecks of bone marrow were then homogenised using a 19-gauge needle bevel facing down and an empty 20ml syringe. The single cell suspension was centrifuged at 500g for 5 min. Cells were resuspended in 10 ml media, counted as described below, and resuspended to 1 x 10^6 cells/ml supplemented in 30% L929 media. 10 x 10^6 cells were then cultured in petri dishes at 37°C (5% CO_2). After 3-4 days, fresh media was added. Cells were harvested and plated for experiments on day 6 by washing with 1x PBS onto the cells to remove any non-adherent cells. Adherent cells were removed using a cell scraper and were transferred to a fresh culture vessel. BMDMs were allowed to adhere for 1.5-2 h before the addition of treatments.

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

Mice were killed and bone marrow harvested as described above in Section 2.2.2 and cultured in petri dishes at 1 x 10^6 cells/ml. Media used was cRPMI, supplemented with 20 ng/ml GM-CSF supernatant from the J559 cell line. After 3 days of culture, fresh medium containing 40 ng/ml GM-CSF was added to all petri dishes. On day 6, the supernatant was carefully removed, and with it the

loosely adherent cells (e.g. granulocytes). Warm PBS was then added to the plates and gently swirled and transferred to a 50 ml tube with fresh cRPMI. The remaining cells were detached via incubation with EDTA (Sigma) for 10 min at 37°C. This was then transferred to the aforementioned tube and centrifuged for 5 min at 500 g. Cells were then resuspended and counted. Cells were then cultured in fresh petri dishes in cRPMI supplemented with 20 ng/ml GM-CSF. 2 days later, a further 20 ng/ml of GM-CSF was added to cells. On day 10, loosely adherent cells were then harvested, counted, and plated at the desired concentration. Immature BMDC were allowed to rest for a minimum of 1h at 37°C before use.

2.2.4 Isolation of spleen cells

Spleens were isolated from freshly killed mice and collected in tubes containing ice-cold RPMI. Spleens were then dissociated through a sterile 40 µm cell strainer using the plunger of a sterile 5 ml syringe. Cells were washed through with RPMI and centrifuged at 500g for 5 min. This pellet was drained of all media by blotting on a dry towel. The pellet was then resuspended in 1 ml RBC lysis solution for 90-120 seconds to lyse the red blood cells. Cells were then washed in warm 1x PBS and centrifuged at 500g for 5 min. The pellet was then dried and resuspended in RPMI at 1×10^6 cells/ml. Cells were then left on ice until ready to be stimulated or stained for FACS.

2.2.5 Isolation of lymph node (LN) cells

Inguinal, brachial, and axillary lymph nodes were excised from freshly killed mice and collected in tubes containing ice-cold RPMI. LN were then dissociated through a sterile 70 µm cell strainer using the plunger of a sterile 5 ml syringe. Cells were then washed with RPMI and centrifuged at 500g for 5 min. The pellet was then resuspended in RPMI, counted and resuspended at 1×10^6 cells/ml. Cells were then left on ice until ready to be stimulated or stained for FACS.

2.2.5 Isolation of peritoneal exudate cells (PEC) and peritoneal lavage

The abdominal skin of mice was cut away to expose the intact peritoneal sack. A 25-gauge needle was inserted through the peritoneum and, taking care not to

pierce any internal organs, 6 ml of ice cold sterile PBS was injected into the cavity. An air bubble was then injected as a reference point for re-insertion of the needle and also to prevent the regress of liquid through the injection site. The body of the mouse was gently shaken to aid the removal of the peritoneal exudate into the PBS. The needle was then re-inserted and the maximum possible volume of PBS was removed. The cells were placed on ice before being pelleted by centrifugation at 1200 rpm for 5 min. The resulting pellet, the peritoneal-exudate cells (PEC), was resuspended in RPMI; counted and used in culture or analyzed by flow cytometry.

2.2.6 Isolation of blood serum

Blood was collected following the cardiac puncture of freshly killed mice and allowed to clot in a 1.5 ml tube at 4°C. After 24 hours cells, were removed by centrifugation at 13,000 g in a micro-centrifuge. The serum was collected and frozen at -20 °C or used immediately.

2.2.7 Preparation of *Fasciola hepatica* excretory/secretory product (FHES)

FHES was purified from *F. hepatica* (liver flukes) collected from the livers of infected cows slaughtered in an abattoir (Kildare Chilling Ltd.). Livers from freshly killed cows were inspected by on-site veterinarians and identified as exhibiting hallmarks of *F. hepatica* infection. Liver fluke were then removed from the livers using forceps and placed in tubes containing PBS supplemented with penicillin/streptomycin (100 µg/ml; Biowest). Flukes were then transported to the laboratory and washed copiously with the aforementioned supplemented PBS to remove large debris. Live flukes were then placed in 6-well plates, 3-6 flukes/well. The wells were filled with 3 ml PBS each. These flukes were then incubated at 37°C for 24h (Figure 2.1).
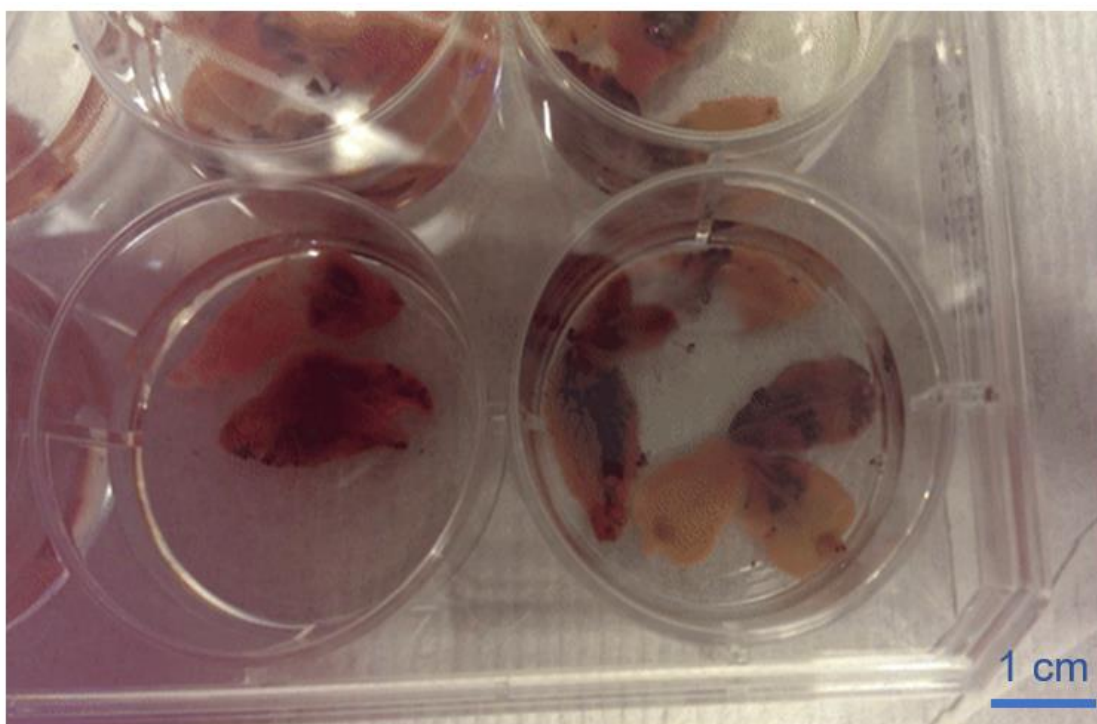
Figure 2.1 *Fasciola hepatica* in culture

Live adult *F. hepatica* cultured in PBS, supplemented with penicillin/streptomycin, in 6-well plates. Photographed after initial washing stages, before overnight incubation at 37 °C.

After incubation, flukes were removed using forceps and the media pooled and filtered through a 0.45 µm filter (Millipore) to remove fluke tissue and eggs. To remove further contaminants, the FHES went through various spins. After each centrifugation, pellets were discarded. First, FHES was spun 3 times at 400 g (2x 5 min, 1x 20 min). Next, supernatant was spun down in a series of 2000 g spins (2x 5 min, 1x 20 min). Finally, the remaining supernatants were spun at 15,000 g for 30 min. The remaining supernatants, henceforth known as FHES, were then aliquoted and stored at -80 °C until use (Figure 2.2). The concentration of FHES was determined by BCA assay (Section 2.2.8) and endotoxin contamination determined by LAL assay (Section 2.2.9).
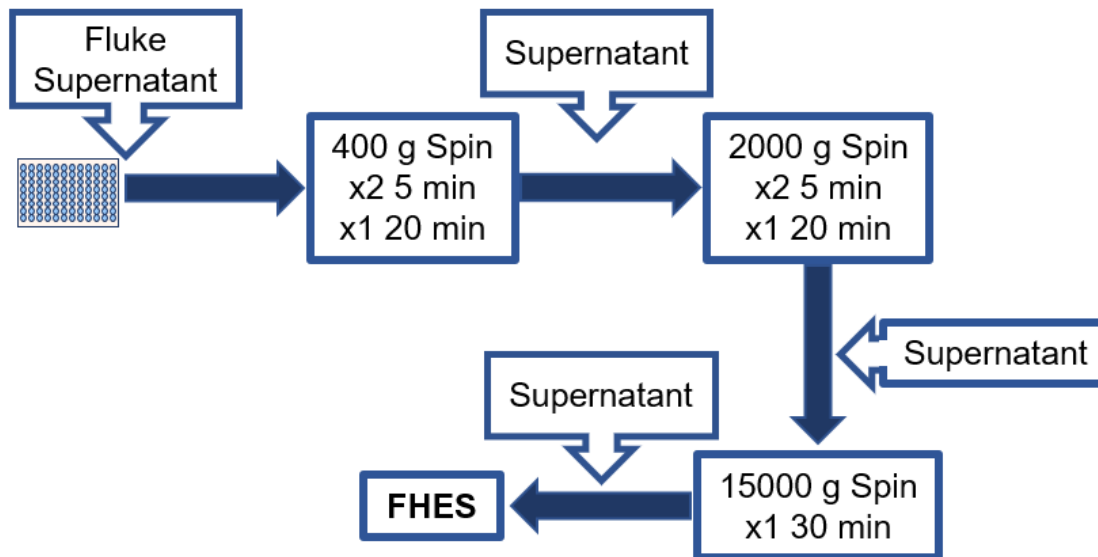
Figure 2.2 Centrifugation schematic for isolation of FHES from *F. hepatica* supernatant

F. hepatica were incubated overnight at 37 °C. The supernatants were then collected, pooled and centrifuged as detailed. Supernatant after the 15000 g spin was designated FHES.

2.2.8 Bicinchoninic acid (BCA) protein assay

Total protein concentration within the FHES was assessed by BCA protein assay kit (Pierce Thermo Scientific). FHES, diluted 1:1, 1:2, 1:5, 1:10, 1:50 and 1:100 (in triplicate) were placed in a clear 96-well plate, together with an 8-point standard curve (0-2000 µg/ml). BCA working solution was then added to all wells. The plate was then covered and incubated for 30 min at 37°C. Absorbance was measured at 562 nm and protein concentration determined via the standard curve.

2.2.9 Detection of endotoxin by limulus amebocyte lysate (LAL) assay

The endotoxin contaminant concentration of FHES was determined by LAL assay (LONZA). FHES was diluted 1:100, 1:1,000 and 1:10,000 in triplicate along with a 4-point standard curve (0.1 EU/ml, 0.25 EU/ml, 0.5 EU/ml and 1 EU/ml). Samples and standards were then mixed with the LAL reagent and incubated at

37°C. After 10 minutes, chromogenic substrate was added to all wells and incubated at 37°C for a further 6 minutes. The presence of endotoxin triggers an enzymatic reaction resulting in a yellow-colored project from the colorless substrate. The reaction is then stopped with 0.25% acetic acid. Absorbance was then measured at 405 nm and endotoxin concentration determined via the standard curve.

2.2.10 *In vitro* stimulation of BMDM, BMDC and total PEC

BMDC, BMDM or PEC were cultured in round or flat-bottomed, 96-well plates, at a concentration of 1×10^6 cells/ml. Cells were cultured with various agonists (LPS, CpG, Mtb, etc.). After 24h, supernatants were removed and the concentration of cytokines determined by ELISA. Concurrently, total RNA was isolated by addition of trizol to the cells and mRNA expression levels assessed by RT-qPCR.

2.2.11 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of murine IL-1β, IL1-1ra, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-17a, IFN-γ, TGF-β, TNF and YM-1 were quantified using the pairs of appropriate antibodies listed above. High binding 96-well microtitre plates (Greiner Bio-one) were coated, wrapped in cling film, and left overnight at 4 °C with 50 µl/well of capture antibody diluted in 1x PBS. On the following day, the coating antibody was washed off using ELISA washing buffer and non-specific binding sites were blocked by adding 150 µl of ELISA blocking solution to each well for 2 h at RT. Plates were then washed in washing buffer, dried, and had sample supernatants added (50 µl/well). In some cases, supernatants were diluted with sterile PBS before being put onto the coated plates. Cytokine standards were added in triplicate (50 µl/well) at the appropriate top working standard and serially diluted. Supernatants and standards were incubated at 4 °C overnight in cling film. Sample supernatants to be assessed for TGF-β were first heated using a PCR thermocycler to 80 °C for 10 min followed by a cool down to 4 °C. Samples were then centrifuged for 1 min at 500 g. The remaining supernatant was then added to the coated plate overnight. After washing with wash buffer and drying, 50 µl of biotinylated detection antibody, diluted using blocking buffer to manufacturer's specifications, was added and the plates then incubated for 2 hours at RT in the dark. Plates were washed again with washing buffer, dried, and incubated in 50

µl/well of horseradish peroxidase (HRP)-conjugated streptavidin (1:1000 dilution BD Pharmigen, 1:200 or 1:40 dilution R&D) for 20 min at RT in the dark. Plates were washed vigorously and 50 µl/well of TMB was added. The reaction was stopped when deemed appropriate through the addition of ELISA stopping solution (25 µl/well). Absorbance was read at 492 nm on a Versamax Tunable Microplate Reader (Molecular Devices) using SoftMax Pro software. Concentration of the cytokine was determined using the standard curve.

2.2.12 Flow Cytometry

2.2.12.1 Surface staining

Cells were isolated from organs or *in vitro* cultures and counted. Cells were then placed in polystyrene FACS tubes in varying concentrations depending on the experiments (0.5-5x10^6 cells/ml). Cells were then washed with FACS buffer (see section 2.1.9) and spun down at 500 g for 5 min. Cells were then resuspended in 50 µl PBS before adding LIVE/DEAD fixable aqua dead cell stain (Invitrogen) and Mouse BD Fc block (FcγR III/II; BD Pharmingen). The Fc block is designed to stop non-specific binding of antibodies to the Fc receptors on cells. However, if cells were being stained for CD16/32 (FcγR III/II), the Fc block was not used and replaced with the conjugated antibody. Tubes were vortexed and then left at room temperature for 15 min, in the dark. Following this, cells were washed with 2 ml FACS buffer and spun down (as before) and resuspended in 50 µl of FACS buffer. Fluorochrome-conjugated antibodies were then added to the cells at the desired concentration (1:200 or 1:100). Cells were mixed by vortex and left in the dark for 15 min at room temperature. Cells were then washed and pelleted. If they were to be analyzed immediately, the cells were resuspended in 500 µl of FACS buffer and kept at 4 °C, on ice until read.

2.2.12.2 Intranuclear Staining

Cell surface staining does not require any additional steps. However, in order to access desired targets for staining within the cells, one must fix and permeabilize them. After initial cell surface staining (Section 2.2.13.1), cells were then resuspended in 100 µl fixation/permeabilization buffer (Invitrogen). Cells were then left at 4°C in the dark overnight. The following day, cells were washed with

permeabilization buffer (Invitrogen) diluted 1:10 in dH$_2$O. Cells were pelleted by centrifugation and then resuspended in 50 µl permeabilization buffer and fluorochrome-conjugated antibodies added (1:200; eBioscience). Cells were then left in the dark at 4 °C for 1 h. Cells were then washed twice with permeabilization buffer, then a further two times with FACS buffer. Finally, cells were resuspended in 500 µl and kept at 4 °C until ready to acquire (1-3 h).

2.2.12.3 Intracellular Cytokine Staining

For samples requiring staining for intracellular cytokines, prior to staining, cells were stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma), 500 ng/ml ionomycin (Sigma), and 5 µg/ml brefeldin A (Sigma) in capped, sterile FACS tubes (BD Biosciences) for 4 hours at 37 °C in cRMPI. Following incubation, cells were washed twice with FACS buffer, pelleted by centrifugation (1300 rpm, 5 min, 4 °C), supernatants poured off and cells resuspended in 50 µl FACS buffer/tube. Cells were then stained with Live/Dead, Fc block and surface antigens stained as previously described (2.2.12.1). Cells were then fixed, permeabilized and stained for intracellular/intranuclear proteins as described previously (2.2.12.2).

2.2.12.4 Acquisition and analysis of FACS data

Analysis of cells was done through either the BD Canto-II or BD LSRFortessa flow cytometers according to manufacturer's instructions. Voltages were calibrated via compensation by fluorochrome-tagged Comp Beads (BD Biosciences) on FacsDiva Software (BD Biosciences). Results were analyzed by FloJo (Stanford University) software. Debris was gated out using Forward Scatter (FSc) Area and Side Scatter (SSc) Area, along with doublets discrimination using FSc Area and FSc Height. Analysis included back-gating to ensure cell populations matched their requisite FSc/SSc profiles.

2.2.13 Isolation of bone marrow extracellular fluid (BMECF)

Femurs and tibias of mice were collected as described above. After cleaning, the tips of the epiphysis were cut off using forceps and scissors. Following this, the bone marrow was flushed out using minimal amounts of cRPMI (700 µl/leg). This cell suspension was then collected into a sterile Eppendorf and spun down at

1300 rpm for 5 min at 4°C. The supernatants were collected and frozen for later analysis. Cytokine concentrations were determined by ELISA.

2.2.14 Injections

2.2.14.1 Intraperitoneal Injections

Female C57Bl/6 mice were injected intraperitoneally (i.p.) with various reagents, diluted in 200 µl PBS/mouse. Injections were done using 27 G x ½" needles (BD) attached to 1 ml syringes (Braun). Mice were scruffed, inverted and injected into the right side of the abdomen to avoid hitting the gut with the needle. I.P. injections were used to determine site-specific or non-specific reactions to reagents. Site-specific effects are easily measured by isolation of PEC.

2.2.14.2 Subcutaneous Injections

Female C57Bl/6 mice were subcutaneously (s.c.) injected with various reagents diluted in 200 µl PBS/mouse, or, in the case of MOG, emulsified in CFA (Section 2.2.16). Injections were done using 23 G 1" needles (BD) attached to 1 ml syringes (Braun). Mice were gently placed in specialized tubes with the tops cut off to allow room for the tail and scruffing. The lid was then closed to disallow movement and the mouse scruffed along the back. The needle was then injected, bevel up, into the scruffed portion of the mouse, between the fingers. After injection, the needle was left in place for several seconds and then slowly removed. This reduces the amount of reagent leaking out of the injection site.

2.2.14.3 Intravenous Injections

Female C57Bl/6 mice were intravenously (i.v.) injected with either FHES or cells, diluted in 200 µl PBS/mouse. Injections were done using a 27 G x ½" needles (BD) attached to 1 ml syringes (Braun). Each syringe and needle were only used for a maximum of three injections, to ensure needle integrity. Mice were first placed in a for 5-10 min at 38 °C to induce mild dehydration, thereby causing veins within the tail to dilate and be easier to locate and inject. Mice were then scruffed and placed within a specialized tube with an opening for the tail and single hind leg to remain outside the tube. The lid was then placed firmly to avoid any unnecessary movement by the mouse. The lateral veins were then located

and, with the bevel facing up, the needle injected with as small an angle towards the mouse's body as possible. After the injection, the needle was then removed and a gauze held firmly on the injection site to minimize blood loss. Once the mouse has stopped bleeding, it is then placed back into its cage and monitored for wellbeing.

2.2.15 Total body, sub-lethal irradiation of mice

Mice were irradiated 24 h before bone marrow reconstitution/transfers. 5 mice at a time were placed into the irradiation chamber of a Nordian Gammacell 3000 Elan irradiator and were irradiated at a dose of 6 Gy (600 rad). Mice were then placed back into their respective cages and monitored for potential weight loss and dehydration. Bone marrow was reconstituted 24 h post-irradiation by i.v. injection of cells, and further experiments carried out 2 weeks later. This allowed for both myeloid and lymphoid immune populations to begin replenishment and provided time to monitor mice for any adverse reactions to the irradiations, such as opportunistic infection.

2.2.16 Hematopoietic Stem Cell (HSC) Enrichment by Manual Magnetic-Activated Cell Sorting (MACS)

HSC were enriched using the EasySep™ Mouse Hematopoietic Progenitor Cell Isolation Kit. The cells were isolated per the manufacturer's instructions, which were as follows. whole BM (BM) was isolated as described previously (Section 2.2.2) and resuspended in a sterile, capped 5 ml FACS tube, in HSC isolation buffer (Section 2.1.9) at 1×10^8 cells/ml, in a volume between 0.5-2 ml. Immediately after this, 50 µl/ml of provided rat serum was added to the sample. Next, 50 µl/ml of Isolation Cocktail was added to the samples, mixed and incubated on ice (4 °C) fir 15 min. Isolation Cocktail contained biotinylated antibodies specific for lineage-specific, non-HSC markers (CD5, CD11b, CD19, CD45R, Gr-1, Ter119). After the 15 min incubation, streptavidin-coated magnetic particles (RapidSpheres™) were added to each sample (75 µl/ml), mixed well and incubated on ice for a further 10 min. Following this, cells were then topped up to 2.5 ml with HSC isolation buffer and the tube (with the cap removed) placed within the magnet at room temperature for 3 min. After this incubation, the tube/magnet were lifted and, in one, smooth motion, the enriched cell suspension

was poured into a new 15 ml tube. The original 5 ml FACS tube was then removed from the magnetic and washed with 2.5 ml of HSC isolation buffer. The tube was then placed back in the magnetic for a further 3 min. As before, the cell-enriched suspension was then poured into the 15 ml tube and combined with the first poured-off fraction. Cells were then counted and left on ice until ready to be stained or stimulated.

2.2.15 Gene Expression Analysis

2.2.15.1 RNA Isolation

RNA from BMDM, PEC or whole BM was isolated from cells via the Trizol/chloroform method. After isolation or treatment of cells, they were spun down, supernatants removed and resuspended in 1 ml Trizol (Life Technologies) in RNAse-free tubes (Thermo Fisher). First, 200 µl of chloroform (Sigma) was added to each tube, which were then shaken vigorously for 30 seconds. The tubes were then allowed to stand at room temperature for 10 minutes. The mixture was then centrifuged at 13,000 g for 15 minutes at 4°C. With care, the top, aqueous layer containing the RNA was then removed via pipette and added to a fresh RNAse-free tube. These tubes then had 500 µl of isopropanol (2-propanol; Sigma) added. The tubes were then mixed by gentle inversion for 30 seconds and allowed to sit at room temperature for 10 minutes. The tubes were then centrifuged at 13,000 g for 10 minutes at 4°C. Carefully, the supernatants were poured off and 1 ml of 75% ethanol () was added and vortexed. Tubes were then spun down at 8,000 g for 10 minutes at 4°C. Supernatants were then removed entirely and the pellets left to dry for approximately 10-20 minutes. Once all ethanol was evaporated, the pellet was then resuspended in 30 µl of RNAse-free water (Life Technologies). RNA concentration was determined by Nanodrop spectrophotometer (Applied Biosystems) according to the manufacturer's instructions. Purity of RNA was assessed by the 260/280 and 260/230 ratios which are measurements for the protein/DNA and Trizol contaminants, respectively. This purified RNA was then stored at -80°C until conversion to complementary DNA (cDNA).

2.2.15.2 cDNA Synthesis

Concentrations of RNA up to 1 µg were reverse transcribed into cDNA by using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) according to manufacturer's instructions. A master mix was made using the kit (Table 2.1) For each cDNA reaction, 10 µl of master mix was added to a new PCR tube. Water and RNA were then added for a final volume of 20 µl/tube/sample. The cDNA was then synthesized using a DNA Engine Dyad Cycler under the conditions indicated in Table 2.2. Resultant cDNA was then diluted with RNAse-free H_2O (1:8). Samples were then stored at -80°C before further analysis.

Table 2.1 cDNA Reverse Transcription Master Mix

Reagent	Volume/Reaction (µl)
10X RT Buffer	2
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2
Multiscribe RT	1
RNAse-free H_2O	0 – 14.2 (Dependent on RNA)

Table 2.2 cDNA Reverse Transcription Cycle

	Temperature (°C)	Time (min)
Step 1	25	10
Step 2	37	120
Step 3	85	5
Step 4	4	∞

2.2.15.3 Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Transcript levels of nucleic acid were then quantified using our cDNA as template DNA for a PCR reaction. First, a pre-made master mix was combined with a reaction probe/18S and 5.5 µl added to each well on a PCR plate (Table 2.3). cDNA was then added to each well (4.5 µl). The reaction plate was then sealed using a clear, adhesive seal and promptly centrifuged for 1 minute at 500 g. The reaction was then carried out on Applied Biosystem 7500 fast Real-Time PCR machine using Real-time PCR cycle (Table 2.4).

Table 2.3 RT-qPCR Master Mix

Reagent	Volume/Reaction (µl)
SensiFAST Probe Lo-ROX Mix 2x (Bioline)	4.5
Reaction Probe	0.5
Endogenous Primer (18S)	0.5

Table 2.4 RT-qPCR Cycle

Temperature (°C)	Time (s)	Cycles
95	120	1
95	3	40
60	30	40

Data were analyzed using the relative quantification on the ABI 7500 software. The software provided cycle threshold (Ct) values, which are the number of cycles required for the fluorescent signal to cross a threshold which exceeds background fluorescence. Change in expression was first normalized to endogenous 18S expression from the corresponding sample ($Ct_{probe} - Ct_{18S} = dCt$). Next, the control samples were averaged and subtracted from the dCt of each sample ($dCt - dCt_{controls} = ddCt$). Finally, fold-induction change was calculated as 2^{-ddCt}.

2.2.16 Induction and Assessment of Experimental Autoimmune Encephalomyelitis (EAE)

2.2.16.1 Active Induction of EAE

EAE was induced in female C57Bl/6 mice by s.c. injection of MOG_{35-55} (100 µg/mouse) emulsified in complete Freund's adjuvant (CFA; Chondrex Inc.) containing 4 mg/ml (400 µg/mouse) of heat-killed H37 RA *M. tuberculosis* (Chondrex Inc.). On the same day, mice were also i.p. injected with 125 ng of PT (Kaketsuken). Two days later, mice were again i.p. injected with 125 ng of PT. In training experiments, mice were first injected s.c. with training reagents 21 and 7 days before induction of EAE.

2.2.16.2 Passive Induction of EAE

EAE was induced in female C57Bl/6 mice by s.c. injection of MOG_{35-55} (100 µg/mouse) and CFA without administration of PT. On day 10, donor mice were sacrificed and their spleens and inguinal, axillary and brachial lymph nodes (LN) excised. These cells were then counted and pooled (30% LN, 70% spleen cells). Cells were then spun down and resuspended at 10×10^6 cells/ml. Cells were then cultured in the presence of MOG_{35-55} (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml). After 72h, non-adherent cells were removed from the culture, washed, resuspended in cRPMI and counted. Cells were then washed with PBS and their concentration adjusted to 15×10^6 cells/200 µl. These cells were then injected into naïve, recipient mice (200 µl/mouse) via i.p. injection. For training experiment, donor mice were first treated 21 and 7 days before induction of EAE.

2.2.16.3 Assessment of clinical symptoms of EAE

EAE was induced in female C57Bl/6 mice through either active or passive induction. Animals were monitored daily for clinical symptoms and weight measured. Disease severity was graded as seen in Table 2.5 Animals were sacrificed if they reached a score above 4.

Table 2.5 Clinical EAE Scoring

Symptom	Score
Normal	0
Limp Tail	1
Altered Gait	2
Hind Limb Weakness	3
Hind Limb Paralysis	4
Tetraparalysis/Death	5

2.2.16.4 Isolation of Single Cells from Central Nervous System (CNS) Tissues

Mice were sacrificed and immediately perfused, intracardially with 10-20 ml of ice-cold PBS to remove peripheral blood from the CNS tissues. PBS was injected intracardially, through the left ventricle, while a cut was made in the superior vena cava to allow excess blood and PBS to flush from the body. Once perfused, the brains were isolated by removing the head, and cutting into the skull along the sides, taking care the avoid deep cuts into the brain. Once the skull cap was removed, the brain was removed using forceps and placed into 1 ml cRPMI in RNAse-free tubes with a single ball bearing inside. The spinal cord was then cut away from all organs and bones. Once sufficiently cleaned, the spinal column was cut in the cervical section, as high as possible. Then, using two forceps, the spinal cord was pushed through the column and out through the hole in the cervical section. The spinal cord was then placed into 1 ml cRPMI in RNAse-free tubes with a single ball bearing inside. The CNS tissues were then homogenized in a tissue lyser (Tissuelyser II, Qiagen) at 27,5 rpm for 5 min. This homogenate was then passed through a 70 μm cell strainer. The homogenized and strained cells were then transferred to 5 ml 40% isotonic Percoll solution in 15 ml tubes using a Pasteur pipette. This mixture was then centrifuged at 1600 rpm, 20 min, room temperature, with no breaks. After centrifugation, the upper layer of myelin sheath was removed and discarded. The mononuclear cells were found pelleted

on the bottom of the tube. The remaining supernatant was poured off and the cells were washed twice with cRPMI and left on ice until ready to be stained or stimulated.

2.2.16 Isolation of Single Cells from Lungs for Flow Cytometry

Lungs were placed within a petri dish and chopped finely using a sterile razor blade. Using a Pasteur pipette, lungs were then transferred into a 1.5 ml, sterile Eppendorf tube containing 1ml PBS and the enzymes Collagenase D (1 mg/ml; Sigma) and DNAse I (20 U/ml; Sigma). Tubes were placed incubated for 1 h at 37 °C. After incubation, cells were filtered through a 70 µm strainer using the plunger of a 5 ml syringe. Cells were then centrifuged (1300 rpm, 5 min, 4 °C). The supernatant was poured off and the remaining pellet was resuspended in RBC lysis buffer. After 1 min, 19 ml of cRPMI was added to the cells to halt further lysis. Cells were then centrifuged (1300 rpm, 5 min, 4 °C). The resultant pellet was then assessed for remaining RBC. If deemed to still contain a significant amount of RBC, cells were resuspended in RBC lysis for a further minute and washed as before. Once all RBC were lysed, cells were resuspended in 2 ml cRPMI and left on ice until ready to be stained or stimulated.

2.2.17 Induction of Airway Hypersensitivity

C57Bl/6 were sensitized by i.p. administration of ovalbumin protein (Ova; 50 µg; Sigma) emulsified in alum (1 mg). Mice were then left for 7 days. On days 7 and 8 mice were challenged with aerosolized Ova (6%, 30 min) via a nebulizer. Mice were sacrificed on day 9, 24 h after the second aerosol challenge, to assess airway inflammation and bone marrow populations.

2.2.18 Infection with *Bordetella pertussis*

2.2.18.1 Culturing of *B. pertussis*

Frozen glycerol stocks of *B. pertussis* (BP338) were thawed and 100 µl lawned onto Bordet-Gengou agar plates. Bacteria were allowed to grow on these blood agar plates for 3-4 days. After that, liquid cultures were established by inoculating conical flasks containing 100 ml S&S liquid medium and supplement (1X) with *B. pertussis* (half a Bordet-Gengou plate per flask). The liquid cultures were then

incubated at 37 °C in a shaking incubator (220 rpm) for 24 h. The next day, the bacteria were centrifuged (10500 rpm, 20 min, 4-20 °C, no brake). The remaining bacterial pellets were resuspended in 10 ml 1% casein salts/flask. The concentration of the bacteria was measured via OD values and referred back to previously achieved OD values with known concentrations of bacteria.

2.2.18.2 *B. pertussis* Aerosol Challenge

Respiratory challenge was achieved by aerosolizing 1×10^9 colony forming units (CFU)/ml using a Pari TurboBoy SX nebulizer. Within a biosafety cabinet in the Cat. 2 facility, mice were placed into a grated chamber within a larger, airtight container, and the nebulizer inserted. Parafilm was used to ensure an effective seal around the tubing and container. Mice were exposed to the aerosolized bacteria for 10 min, followed by 10 min of rest in the chamber. The challenge dose was confirmed by isolating the lungs from 2 mice/group, 2 hours post-challenge and performing CFU counts, as outlined in Section 2.2.18.3. The course of disease was followed by performing CFU counts on lungs from groups of 4 mice/group on days 7, 21 and 35 post-challenge.

2.2.18.3 CFU Counts from Lungs

The lungs were aseptically removed and homogenized in 1 ml of 1% casein solution. 100 µl of undiluted and serially diluted homogenates from individual lungs were spotted in duplicate onto Bordet-Gengou agar plates and left to incubate at 37 °C for 5-6 days. The number of CFU were then calculated. The counts were converted into $Log_{10}CFU$ for graphical representation of lung bacterial load.

2.2.17 Statistical and Graphical Analysis

Graphs were prepared using GraphPad Prism 7. Furthermore, statistical analyses were carried out using the GraphPad 7 software. Unpaired two-tailed student t-tests were used to compare the statistical difference between the mean values of two groups. One-way analysis of variance (ANOVA) test followed by Bonferroni post-hoc test was applied to determine statistical differences between more than two groups. Two-way ANOVA followed by Sidak's multiple comparison's test was used post-hoc to determine statistical differences between

2-3 groups treated in multiple ways. Statistical significance was considered for p-values less than 0.05.

Chapter 3: FHES modifies HSCs generating anti-inflammatory macrophages

3.1 Introduction

Helminths are a diverse range of large, multi-cellular parasitic worms, which infect an estimated 1.5 billion people around the world, primarily in LMIC [304]. It is only recently, with the introduction of improved sanitation and effective anthelmintic drugs, such as albendazole and mebendazole, that the human populations in high income countries have been relatively free from the burden of helminth infections. Helminth infections characteristically induce a combination of type 2 immune responses, along with potent anti-inflammatory immune responses in the host. This combined immunoregulation allows the helminth to promote and maintain chronic infection.

While helminth infections occur in approximately one third of the human population, the frequency of both co-infections and re-infections means that understanding the immune response to a singular pathogen remains relatively unclear [194, 304, 305]. This has led to the reliance of animal models of infection to elucidate the immune response to specific pathogens, resulting in several well-established live infection models, including, but not limited to, *H. polygyrus* (intestinal roundworm), *N. brasiliensis* (gastrointestinal roundworm), *T. muris* (whipworm) and *S. mansoni* (blood flukes) [206]. These and other helminths mimic human helminth infection during their lifecycle and in the immune response against the parasite. Studies from these models have shown that expulsion of the parasites are mediated by type-2 immune responses, primarily alternatively activated macrophages, eosinophils and Th2 cells.

Helminths secrete potent immunomodulatory proteins and enzymes in their ES product, which are capable of both activating type 2 responses and inhibiting the immune response in a myriad of ways. For example, *H. polygyrus* have been shown to induce the expansion of Treg cells, thus inhibiting the anti-helminthic Th2 response and promoting chronic infections [306]. During the innate phase of the immune response, helminths and their ES have been demonstrated to induce NLRP3 activation, inducing the secretion of pro-inflammatory, type 1 cytokines IL-1β and IL-18, thereby inhibiting the type 2 immune response. Indeed, treatment of mice with the soluble IL-1 receptor antagonist Anakinra during helminth infection results in elevated type 2 responses, correlating with faster

worm expulsion [307, 308]. Furthermore, infection of mice with low doses of *T. muris* induces a Th1 cell response characterized by chronic infection, whereas high-dose infection results in Th2 responses and worm clearance. Low-dose infection resulted in IFN-γ-mediated alterations to the HSC compartment, including enhanced proliferation of multipotent progenitors, leading to enhanced neutrophilia [309]. Though not fully understood, helminths have recently been identified to modify HSC populations in the bone marrow BM. Babayan and colleagues demonstrated that infections with *L. sigmodontis* enhanced multipotent HSCs in the BM of aged compared to young mice, and this correlated with an increase in worm burden [310]. Additionally, stimulation of HSCs with filarial nematode promoted the differentiation of dendritic cells (DCs), which were hyporesponsive to LPS stimulation and promoted Th2 cell differentiation [311].

Like most helminths, *F. hepatica* has evolved strategies for regulating immune responses which enable them to maintain long-term infections. Infection of mice with *F. hepatica* has shown to induce anti-inflammatory innate immune infiltration to the peritoneal cavity and spleens, resulting in alternatively activated macrophages that express both FIZZ1 and IL-10 [312]. However, mice are not a natural host of *F. hepatica*, and indeed De Paula and colleagues showed that infection of the C57BL/6 mice with *F. hepatica* resulted in 73% mortality by 5 weeks [287]. Therefore, immunomodulatory FHES was used in this project.

FHES is a potent mix of immunomodulatory molecules and proteinases, which are capable of acting directly and indirectly on immune cells to suppress and regulate the immune response. For example, *F. hepatica* helminth defence molecule-1 (FhHDM-1) binds to and inhibits recognition of LPS by TLR4 on immune cells, while simultaneously reducing antigen presentation and lysosomal-dependent NLRP3 activation in macrophages [313, 314]. Additionally, *F. hepatica* fatty acid binding protein has been demonstrated to inhibit LPS-induced pro-inflammatory cytokine secretion from macrophages both *in vitro* and in an *in vivo* model of septic shock [315]. Other immunomodulatory proteins, such as cathepsin L-proteinases, and a family of TGF-like molecules combine to promote the modulation of innate immune responses.

Classically, the innate immune system was not considered to have any form of memory, a trait reserved for T and B lymphocytes, cells of the adaptive immune system. However, this dogma has now been overturned, and recent research has demonstrated that innate immune cells can have non-specific memory phenotype in a range of hosts, including invertebrates, plants, mice and humans. Innate immune memory, also referred to as trained innate immunity, occurs when a stimulus induces epigenetic and metabolic reprogramming in innate immune cells. Upon non-specific re-stimulation with pathogens or their products, trained innate immune cells respond faster and with a more robust inflammatory output (cytokines, chemokines, ROS) compared with untrained cells. This was first established in plants during a process called systemic acquired resistance (SAR). When plants are infected with a pathogen, epigenetic changes result in the priming of genes encoding host defence molecules, and following reinfection the plants respond faster and stronger to clear the secondary infection [99, 100]. In humans, circulating monocytes from individuals vaccinated with BCG secrete higher levels of pro-inflammatory cytokines in response to various, non-specific stimulants, including TLR agonists [89]. Recently, studies have suggested that parasites have an ability to induce trained innate immunity. Work from our lab has demonstrated that helminth products from *F. hepatica* induce training of macrophages both *in vitro* and *in vivo*, which results in an anti-inflammatory phenotype capable of inhibiting EAE in mice [113].

The present study investigated the capacity of FHES to mediate an anti-inflammatory phenotype in innate immune cells in the context of trained immunity. The study also addressed the ability of FHES to alter the innate immune system following addition of FHES by different routes of administration was simultaneously addressed. Furthermore, the link between anti-inflammatory activation of innate immune cells and their development from HSC in the BM was examined using our previously established model of innate priming in mice.

The specific aims to be addressed were:

- Can FHES induce trained innate immunity *in vitro*?
- Can FHES-induced priming of innate immune cells be induced *in vivo*?
- Which route of administration of FHES induces robust, systemic trained innate immunity?
- Which innate immune cells are altered by *in vivo* treatment of mice with FHES?
- Does FHES-mediated priming of peripheral innate immune cells result from modifications to the BM *in vivo*?
- Which HSC(s) are modified by administration of FHES?
- Can macrophages derived from FHES-primed HSC respond to TLR ligation with an anti-inflammatory phenotype?

3.2 Results

3.2.1 *F. hepatica* ES pre-treatment of innate immune cells mediates an anti-inflammatory response to inflammatory stimuli

Our lab has previously demonstrated that T cells, macrophages and DC recruited during *F. hepatica* infection potently produce IL-10 [256]. Furthermore, helminth infections have also been shown to promote IL-1RA production in the duodenum in a mouse model of infection with *H. polygyrus bakeri* [316, 317]. In addition, FHES administration *in vivo* induces production of the type-2 cytokine IL-5 from CD4 T cells, and the release of the alarmin IL-33 [260]. These two cytokines are essential in the immune response to helminth infection; IL-5 is widely known to attract eosinophils, while IL-33 enhances alternative activation of macrophages. This study aimed to determine the effect of FHES on innate immune cells. Macrophages and DC were cultured with FHES and spontaneous or LPS-induced anti-inflammatory cytokine production was assessed by ELISA. BMDC were stimulated with LPS (100 ng/ml), FHES (100 µg/ml) or a combination of both. After 24 h, supernatants were collected, and the concentration of cytokines determined by ELISA. Direct stimulation of BMDC with FHES did not induce expression of any measured cytokine, including IL-10. In contrast, stimulation of BMDC with LPS induced expression of IL-1β, IL-10, TNF and IL-12p70. Co-treatment of BMDC with FHES and LPS significantly reduced the expression of IL-1β, TNF and IL-12p70, but significantly enhanced production of IL-10 (Fig. 3.1).

In a parallel experiment, BMDM were cultured with PBS, FHES (50 or 100 µg/ml), LPS (100 ng/ml) or a combination for 24h, after which supernatants were collected and analysed for cytokines by ELISA (Fig. 3.2). Treatment of BMDM with FHES alone did not induce production of any cytokines examined. However, FHES significantly reduced LPS-induced secretion of IL-1β, IL-6, and TNF in a dose-dependent manner. Furthermore, the higher concentration of FHES (100 µg/ml) inhibited the secretion of LPS-induced IL-1RA. Conversely, both low and high concentrations of FHES significantly enhanced LPS-induced IL-10 secretion.

3.2.2 FHES induces anti-inflammatory trained innate immunity in BMDM

Having demonstrated that FHES is capable of altering the innate response to classical activation via LPS, it was next investigated whether FHES could promote trained innate immunity. Our lab has recently reported that FHTE can induce anti-inflammatory trained immunity in macrophages [113]. We previously demonstrated that pre-treatment, or training, of macrophages *in vitro* with FHTE modifies the macrophages to produce more IL-1RA and IL-10, and less TNF when stimulated with TLR agonists LPS and Pam3CSK4 [113]. Furthermore, the modified response could be inhibited if the cells were first incubated with an inhibitor of epigenetic modifications. Here, an experiment was designed to examine the possibility that FHES induced trained immunity in macrophages (Fig. 3.3A). Mature BMDM from naïve mice were incubated with either FHES (100 µg/ml) or PBS. After 24 h, supernatants were removed, and cells washed thoroughly, resuspended in medium and left to rest for 72 h. BMDM were stimulated with LPS (100 ng/ml), CpG (5 µg/ml) heat-killed Mtb (10 µg/ml), or PBS. After 24 h supernatants were collected and analysed for cytokines by ELISA. IL-1RA and IL-10 production in response to LPS was significantly enhanced in BMDM that had been pre-treated (trained) for three days with FHES. Similarly, IL-10 was significantly elevated in supernatants of FHES-trained BMDM in response to the TLR9 agonist CpG, whereas IL-1RA, though increased, this was not-significant. IL-1RA and IL-6 were both significantly enhanced in the supernatants of FHES-trained stimulated with Mtb, with a slight increase in IL-10 secretion. Furthermore, FHES-trained BMDM also secreted significantly less TNF in response to CpG (Fig. 3.3B).

Our lab has recently demonstrated that the related helminth product FHTE can induce anti-inflammatory trained immunity via epigenetic modifications. Inhibition of histone methyltransferases with 5′-Deoxy-5′-(methylthio)adenosine (MTA) during the primary stimulus (training) resulted in loss of the anti-inflammatory phenotype during secondary stimulation with LPS [113]. To demonstrate that the FHES-induced training of BMDM was similarly dependent on epigenetic modifications, training was induced with and without the presence of histone deacetylase (HDAC) inhibitor givinostat (25 nM). FHES training did not enhance IL-1RA or IL-10 in response to LPS, CpG and Mtb (Fig. 3.3B). This finding

demonstrated that FHES training of BMDM was dependent on epigenetic modifications.

3.2.3 FHES induces eosinophilia and macrophage recruitment to the peritoneal cavity *in vivo.*

The results described above showed that FHES can induce trained immunity in macrophages *in vitro*. It was next assessed whether FHES was also able to modify innate immune responses *in vivo*. Previous studies on the induction of trained innate immunity *in vivo* have included various routes of administration, dosages and compounds. Therefore, experiments were designed to examine the effect of different routes of administration (i.p., s.c., and i.v.) of the FHES, to best understand its ability to induce trained immunity.

The protocol used to examine trained immunity *in vivo* was based on one previously utilized in a study from this lab on FHTE, that involved two s.c. injections of helminth products two weeks apart [113]. C57BL/6 mice were injected with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, seven days after the last injection, mice were euthanized, and various tissues recovered for analysis of changes in innate immune profiles (Figure 3.4A).

Flow cytometric analyses of PEC revealed that FHES administered by any of the routes enhanced infiltration of immune cells into the peritoneal cavity (Fig. 3.5A). Both i.p. and s.c. injections significantly enhanced recruitment of $CD45^+$ cells into in the peritoneal cavity. However, treatment of mice by i.v. route was unable to induce significant infiltration of immune cells, though there was a trend towards increased cell numbers. Visualization of the total $CD45^+$ cell populations were achieved through t-distributed stochastic neighbour embedding (t-SNE), an algorithm designed to arrange multi-dimensional data on a two-dimensional space. In a t-SNE plot, events (cells) are grouped by how similar they are in their marker expression, with highly relatable events clustered together. This provides a clear, unbiased visualization of populations which can then be identified through traditional gating strategies. The PEC of mice treated s.c. with FHES were assessed using t-SNE (Fig. 3.5B). Though not a measure of cell numbers, the t-SNE plots confirmed that FHES treatment enhanced cell numbers in the peritoneal cavity. In addition to cell numbers, t-SNE provides a rudimentary

estimate of reduction or enhancement of specific populations (Figure 3.5B). The fraction of eosinophils (magenta), large peritoneal macrophages (LPM; royal blue), and small peritoneal macrophages (SPM; red) are markedly increased in FHES-treated mice. In contrast, the relative number of non-eosinophil granulocytes, such as neutrophils (lime green) and mast cells (black) appear to be markedly reduced compared with that seen in PBS-treated mice. The percentage differences of individual populations were then confirmed by traditional flow cytometric analyses.

The analysis shown in Figure 3.5B demonstrated that FHES increased the frequency of eosinophils; therefore, I assessed the effect of FHES on expansion of eosinophils *in vivo* by conventional flow cytometry. Injection of mice with FHES by s.c., i.p., or i.v. routes induced eosinophil infiltration to the peritoneal cavity, and this was most significant following i.p. or s.c. injection of mice with FHES. I.P. injection of FHES elevated the number of eosinophils and increased their frequency from 2.5% in PBS-treated mice to 33%. This study similarly found that s.c. administration of FHES resulted in an elevated number of eosinophils, with mice treated s.c. with FHES exhibiting a frequency of 26% (Fig. 3.6B/C). Combined, these data are consistent with previously published work in the lab which identified the ability of FHTE and *F. hepatica* exosomes to induce eosinophilia [113]. Interestingly, i.v. administration of FHES did not result in significant changes to frequency or absolute numbers of eosinophils in the PEC (Fig. 3.6B/C).

In addition to eosinophilia, helminth infection induces the expansion of tissue-resident macrophages and infiltration of circulating monocytes and macrophages [318]. The peritoneal cavity contains both of these populations. LPM are embryonically derived, tissue-resident cells, which comprise approximately 90% of the macrophage population in the peritoneal cavity. Conversely, SPM are derived from circulating monocytes that infiltrate the peritoneal cavity and rapidly differentiate into macrophages. SPM and LPM are distinguished through differential size and surface expression of F4/80 and MHC class II (MHC-II). LPM are identified by high expression of CD11b and F4/80, while SPM can be identified by high surface expression of MHC-II and low/intermediate CD11b and F4/80 (Fig. 3.6A). Administration of FHES to mice by the s.c. route significantly

increased the frequency of LPM from 15% in the control PBS-treated mice to 24%. Neither i.p., nor i.v. administration of FHES increased the percentage of LPM in the peritoneal cavity (Fig. 3.7B). In contrast, mice treated i.v. with FHES resulted in a non-significant decrease in frequency of LPM. Interestingly, all routes of FHES administration resulted in significantly increased absolute numbers of LPM (Fig. 3.7C). No route of FHES administration was capable of expanding the frequency of SPM in the PEC. I.v. administration of FHES resulted in significant reduction of SPM, from 3% in PBS-treated mice, to 1.9% (Fig. 3.8A). However, i.p. and s.c. treatment of mice with FHES led to a significant increase in the absolute numbers of SPM, while i.v. treatment did not (Fig. 3.8B). These data indicate that FHES is capable of expanding tissue-resident macrophage populations both at the site of injection (i.p.) and after s.c. and i.v. injection in mice.

Helminth infection induces potent alternative activation of macrophages. This is caused by a myriad of factors, including the helminth itself, mechanical damage to tissue, and secretion of immunomodulatory enzymes and signalling proteins [318]. Injection of helminth-derived products in mice is capable of inducing robust alternative activation of macrophages [7]. Furthermore, previously published data from our lab demonstrated that i.p. administration of FHTE generates alternatively activated SPM and LPM, which express enhanced M2 markers on the cell surface and specifically upregulate mRNA for M2 markers [113]. To confirm that the macrophage populations in FHES-treated mice were alternatively activated, total RNA was isolated on day 21 from mice treated with FHES (100 µg/mouse) or PBS on days 0 and 14. Intraperitoneal treatment of mice with FHES enhanced mRNA expression of the alternative activation markers *Retnla* (FIZZ1) and *Chil3* (YM1). Furthermore, PEC expressed significantly higher levels of *Il1rn* (IL-1RA), *Il4* (IL-4) and *Il5* (IL-5), and elevated, but not significant, levels of *Arg1* (Arginase-1) (Fig. 3.9A). Moreover, i.p. treatment of mice with FHES resulted in downregulation of expression of M1-specific markers *Nos2* (iNOS), *Ifng* (IFN-γ), and *Fpr2* (FPR2) when compared to PBS-treated mice (Fig. 3.9B). Interestingly, i.p. injection of FHES did not alter M2-specific *Mrc1* (CD206) mRNA expression, nor M1-specific *Ptgs2* (PTGS2). Furthermore, s.c. injection of FHES resulted in enhances expression of *Arg1*.

Retnla, *Chil3*, *Egr2*, *Mrc1*, *Il4*, *Cx3cr1* and *Il1rl1* (ST2) (Fig. 3.12A). Interestingly, s.c. treatment with FHES enhanced expression of classical activation marker, *Fpr2* (Fig. 3.10B). Taken together, these data indicate that i.p. injection of FHES induces alternative activation and direct downregulation of classical activation of macrophages, whereas s.c. administration of FHES may result in a mixed population of classically and alternatively activated macrophages.

To confirm that s.c. treatment of mice with FHES does not polarise all macrophage populations in the peritoneal cavity, total RNA was isolated from FACS-purified LPM and SPM on day 21 from mice treated s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. mRNA expression of M2 markers *Arg1*, *Retnla*, *Chil3*, *Egr2*, *Mrc1* and *Cx3cr1* in LPM were not significantly altered in FHES-treated mice when compared with PBS-treated mice (Fig. 3.11A). Furthermore, mRNA of *Nos2* and *Fpr2* remained unchanged in LPM (Fig. 3.11B). mRNA expression of M2-associated genes *Arg1* and *Retnla* was significantly increased in SPM from FHES-treated when compared with PBS-treated mice (Fig. 3.12A). Furthermore, FHES-treated mice had lower mRNA expression of M1-associated gene *Ptgs2* in SPM, when compared with PBS-treated mice (Fig. 3.12B). In addition, expression of *Fpr2* and *Nos2* remained unchanged in SPM of FHES-treated when compared to PBS-treated mice (Fig. 3.12B).

As eosinophilia was induced by treatment with FHES following both routes of administration, mRNA expression of eosinophils from the PEC was next assessed. Though eosinophils are notably transcriptionally quiescent in steady-state, previous unpublished data in the lab has shown that eosinophils isolated by fluorescence-activated cell sorting from the peritoneal cavity of helminth product-treated mice secrete significantly more IL-1RA. To test whether FHES treatment similarly resulted in transcriptional modifications in the eosinophil population in the PEC, eosinophils were purified and assessed for mRNA expression of *Il5* and *Il1rn* by RT-qPCR. FHES treatment (s.c.) did not alter the mRNA expression of either *Il1rn* or *Il5* when compared to peritoneal eosinophils from PBS-treated mice (Fig. 3.13).

Finally, the PEC supernatants (PEC fluid) were isolated and concentrations of type-2 cytokines IL-1RA and YM1 were determined by ELISA. There was a

significant enhancement of IL-1RA and YM1 in the PEC fluid of mice treated i.p. with FHES compared with PBS-injected mice (Fig. 3.15). Furthermore, IL-1RA and YM1 were significantly enhanced in the PEC fluid of mice treated s.c. with FHES when compared with PBS-treated mice. IL-10 and IL-33 were found in higher concentrations, though not significantly, in the PEC fluid of s.c. FHES-treated when compared with PBS-treated mice (Fig. 3.16). Taken together, these data indicate that FHES treatment induces a preferential switch to alternative activation *in vivo*. Furthermore, s.c. administration of FHES affects circulating monocytes and monocyte-derived macrophages but does not modify tissue-resident macrophages or infiltrating eosinophils.

3.2.4 Treatment of mice with FHES induces eosinophil and anti-inflammatory monocyte expansion in the BM

FHES treatment induces the development and mobilization of eosinophils and monocytes in the peritoneal cavity. SPM, unlike steady-state LPM, are monocyte-derived and therefore originate in the BM niche. Monocyte recruitment is essential in mediating host response to helminth infections [318]. Innate immune cells, such as monocytes, originate from in the BM from progenitors during both normal and emergency haematopoiesis. Furthermore, circulating monocytes have been shown to respond to peripheral activation by homing to the BM niche and directing further monocyte differentiation in response to the NOD2 agonist MDP [56].

C57BL/6 mice were treated with FHES or PBS, as described previously (Figure 3.4A). On day 21, BM cells were stained with antibodies for monocytes and analysed by flow cytometry. Treatment of mice with FHES s.c. induced significant increases in total immune cell numbers in the BM compared to PBS-treated mice (Fig. 3.17). Interestingly, treatment of mice with FHES i.v. resulted in significantly fewer immune cells in the BM. Treatment of mice i.p. with FHES did not enhance or decrease to cell numbers. Treatment of mice with FHES, both i.p. and s.c., induced an increase in the frequency and absolute numbers of eosinophils present in the BM (Fig. 3.18). Treatment of mice with FHES i.v. did not result in changes to the eosinophil population in the BM (Fig. 3.18).

The data in Figure 3.8 showed that monocyte-derived macrophages (SPM) in the peritoneal cavity were significantly enhanced in mice treated either i.p. or s.c. with FHES. Monocytes patrol the circulatory system and are continuously being produced in the BM during normal and emergency haematopoiesis [1]. Ly6C^{high} monocytes are characteristically pro-inflammatory, while Ly6C^{low} are typically anti-inflammatory [320]. The BM cells from mice treated with FHES was stained with antibodies specific for CD11b and Ly6C and analysed by flow cytometry. The results demonstrate that s.c. treatment with FHES reduced the fraction of Ly6C^{high} monocytes in the BM when compared with BM from PBS-treated mice (Fig. 3.19B/C). Furthermore, treatment of mice with FHES i.v. led to a significant reduction in the absolute number of Ly6C^{high} monocytes in the BM. Additionally, s.c. treatment of mice with FHES significantly enhanced the absolute numbers of Ly6C^{low} monocytes in the BM (Fig. 3.20B). Furthermore, i.v. treatment with FHES resulted in a significant reduction in Ly6C^{low} monocytes (Fig. 3.20B). Ly6C^{low} monocytes can also be characterized by their surface expression of CX3CR1, also known as fractalkine receptor, and changes in expression of CX3CR1 may indicate enhanced capacity of Ly6C^{low} monocytes to leave the BM niche or to sense pathogens [48]. Additionally, CX3CR1 has recently been shown to play a pivotal role in anti-inflammatory and wound repair functions in monocyte-derived macrophages [321, 322]. Treatment of mice with FHES s.c. enhanced CX3CR1 expression on the surface of Ly6C^{low} monocytes in the BM (Figure 3.21). Taken together, these data demonstrate that s.c. treatment with FHES resulted in enhancement of anti-inflammatory Ly6C^{low} monocytes in the BM.

The data indicate that mice treated s.c. with FHES have a potent type 2 response in the BM. To confirm that FHES induces type 2 cytokines in the BM milieu, mice were treated with FHES, and on day 21 the supernatant from BM, termed BM extracellular fluid (BMECF), was collected and assayed for cytokines by ELISA. The results revealed that mice treated with FHES had significantly more YM1 in the BMECF compared to mice treated with PBS (Fig. 3.22). The remaining cells collected by lavage of BM were assed for gene expression of M2 markers by RT-qPCR. Expression of *Retnla* was significantly enhanced, while expression of *Chil3* was simultaneously reduced in mice treated with FHES compared to mice treated with PBS. Expression of M2 factors such as *Arg1*, *Mrc1*, *Egr2* and *Il1rn*

remained unchanged in mice treated with FHES compared with mice treated with PBS (Fig. 3.23A). The results show that M1 markers *Nos2*, *Fpr2* and *Cd38* gene expressions remained unchanged after FHES treatment (Fig. 3.23B). The induction of YM1 production is a hallmark of helminth infection and the type 2 immune response, with high expression in the peritoneal cavity, spleen and BM throughout the lifespan of mice in steady-state and during helminth infection [323]. YM1 is a eosinophil chemoattractant and is capable of inducing the expression of FIZZ1 during innate and adaptive immune responses to *N. brasiliensis* infection, in which both are required for clearance of the parasite and effective wound repair [324]. The role of promoting type 2 immune responses is highly time-dependent, as FIZZ1 has been shown to suppress Th2 cells during the later stages of infection [325]. Here it was demonstrated that s.c. treatment of mice with FHES promotes YM1 protein production in the BM milieu, while simultaneously enhancing *Retnla* expression and decreasing *Chil3* expression in the BM (Figure 3.23).

3.2.5 Treatment of mice with FHES directs development of anti-inflammatory BMDM

Treatment of BMDM with FHES significantly alters cytokine production, with increased anti-inflammatory and decreased pro-inflammatory cytokines. Furthermore, *in vivo* treatment with FHES promoted the development of Ly6C^{low} monocytes, which are typically classified as anti-inflammatory [49]. Therefore, it was investigated whether monocytes and macrophages derived from mice treated with FHES showed an anti-inflammatory phenotype in response to pro-inflammatory stimulation. Mice were injected s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7. On day 0, BM was harvested and cultured in CSF-1-containing L929 media. After 7 days, BMDM were harvested and stimulated with LPS (100 ng/ml) CpG (5 µg/ml), or PBS and subsequently analysed by flow cytometry, RT-qPCR and ELISA (Fig. 3.24A). Any alterations in the response of the BMDM from *in vivo*-trained mice is likely to have resulted from the effect on BM-resident monocytes and macrophage progenitors.

The results revealed that BMDM differentiated from BM of mice treated with FHES secreted significantly less IL-1β, while simultaneously secreting more IL-

1RA, IL-10 and TGF-β in response to LPS (Figure 3.24B). A similar effect was seen in response to CpG, a TLR9 agonist, with significantly enhanced secretion of IL-1RA, IL-10 and TGF-β by BMDM from mice treated with FHES compared to BMDM from mice treated with PBS (Figure 3.24B). The effect of FHES-treatment on gene expression in response to LPS was next assessed. BMDM from PBS or FHES-treated mice were stimulated with LPS (100 ng/ml) for 24 h. Total RNA was isolated and the expression of *Il1rn* and *Il10* assessed by RT-qPCR relative to medium cultured PBS-trained BMDM. LPS activation increased the gene expression of *Il1rn* and *Il10* in BMDM from FHES- and PBS-treated mice. However, cells from mice treated with FHES exhibited significantly greater expression of *Il10*, and a marked increase in *Il1rn* expression when compared with PBS-trained BMDM (Figure 3.25).

Innate immune cells express MHC-II and various costimulatory activation molecules on their surface which bind to and activate T cells. Therefore, LPS-induced expression of MHC-II, CD40 and CD80 was assessed by flow cytometry. FACS analysis revealed that BMDM from FHES-treated mice had reduced constitutive surface marker expressions of MHC-II and CD80. Additionally, BMDM from FHES-treated mice had lower LPS-induced MHC- II expression when compared with BMDM from PBS-treated mice (Figure 3.26). Interestingly, the constitutive expression of CD40 is not affected by FHES treatment, however, upon stimulation with LPS for 24 h, BMDM from FHES-trained mice express significantly less CD40 on their surface compared with BMDM from untreated mice (Figure 3.26). These findings suggest that *in vivo* treatment of mice with FHES results in the development of macrophages and monocytes which respond to inflammation with an anti-inflammatory phenotype.

3.2.6 Treatment of mice with FHES expands long term hematopoietic stem cells and the common monocyte precursor

As FHES treatment can affect the development of macrophages and monocytes, it was next addressed whether FHES could modulate HSCs. Innate immune cells are not long-lived cells and after differentiation, monocytes typically survive for approximately 4-7 days which would not be long enough to mediate long-lasting innate memory [114]. Evidence has begun to emerge indicating that progenitor

cells in the BM are affected by external stimuli. This may provide a clue to how innate immune memory can last longer than the peripheral cells that initially encountered the pathogen or pathogen-derived molecule.

To determine the *in vivo* effects of FHES treatment on HSCs, I first assessed genes associated with haematopoiesis. Throughout haematopoiesis, progenitor cells respond to the environment by upregulating or downregulating the expression of various genes which drive differentiation into effector immune cells. For example, during differentiation transcription of C/EBP-α, PU.1, and IRF8, among others, are enhanced in HSCs to direct them towards the myeloid lineage of monocytes and macrophages. β-Glucan was chosen as a positive control, due to its established ability to train HSCs to develop into myeloid-lineage immune cell [119, 326]. Mice were treated with two s.c. injections of FHES (100 μg/mouse), β-glucan (50 μg/mouse) or PBS on days 0 and 14. On day 21 BM was isolated and total RNA extracted and analysed for transcriptional regulators of haematopoiesis. Expression of transcription factors in BM from FHES-treated mice was not altered when compared with BM from PBS- or β-glucan-treated mice (Fig. 3.27). Next, I used flow cytometry to examine potential changes in phenotype of the earliest progenitor cell populations in adult mice (Fig. 3.28). This subgroup of cells, identifiable as CD45$^+$**L**ineage$^-$c**K**it(CD117)$^+$**S**ca-1$^+$ (LKS$^+$) cells, include long-term HSCs (LT-HSCs), short-term HSCs (ST-HSC) and multipotent progenitors (MPP). LKS$^+$ cells give rise to all mature immune cells and are capable of restoring the entire hematopoietic cell system when transferred into a recipient which has had its immune system depleted. FHES training did not result in significant changes in the percent or absolute numbers of LKS$^+$ cells (Fig. 3.29A-C). However, the data revealed a marked increase in the percentage and total numbers of LT-HSC (3.29A). Indeed, the number of LT-HSC in FHES-treated mice were elevated from a mean of 21.5 x 10^5 cells/leg, to approximately 33.9 x 10^5 cells/leg, an increase of 58%. In contrast, β-glucan treatment resulted in a significant increase in MPP (Figure 3.29C). This is consistent with the literature regarding β-glucan-induced HPC alterations in LKS$^+$ cells. Despite increases in myeloid and granulocyte mature immune cells in the BM and periphery, mice treated s.c. with FHES showed no significant differences in frequency and absolute number of the common myeloid progenitor (CMP) or

the granulocyte/macrophage progenitor (GMP) (Fig. 3.30A/B). In contrast, treatment of mice with β-glucan did enhance the absolute number of CMP and GMP (Fig. 3.30A/B). While no significant alterations to LKS, CMP or GMP populations were found, FHES treatment significantly enhanced the absolute number of common monocyte precursors (cMoP) and monocyte/DC precursors (MDP) in the BM when compared to PBS-treated mice (Fig. 3.30B).

cMoP is considered to be the last progenitor in the chain of cell differentiation before fully mature monocytes [24, 25]. Typically, cMoP are thought to differentiate into Ly6C^{high} monocytes which can then convert to Ly6C^{low} monocytes [162]. However, there is evidence to suggest that cMoP or MDP can differentiate into either Ly6C^{high} or Ly6C^{low} monocytes [46–48]. Therefore, I examined the possibility that treatment of mice with FHES may affect the precursors directly responsible for the differentiation of Ly6C^{low} monocytes. Using Ki67 labelling, I examined the effect of FHES on proliferation of the progenitors (Figure 3.31A). FHES treatment markedly enhanced the proliferation of MDP and cMoP when compared to treatment with either PBS or β-glucan (Figure 3.31B/C). Taken together, these data suggest that treatment of mice with FHES alters the HSC niche to favour differentiation and development of monocytes and macrophages, in a manner distinct from β-glucan-induced modifications to HSCs.

3.2.7 FHES directs cMoP to express the Ly6C^{low} monocyte marker CX3CR1

Ly6C^{low} monocytes are thought to primarily differentiate from conversion of Ly6C^{high} monocytes. However, this has recently come under scrutiny and there is evidence to suggest that cMoP may be capable of direct differentiation into Ly6C^{low} monocytes, without first becoming Ly6C^{high} monocytes. However, it is still unknown how cMoP cells can differentiate directly into Ly6C^{low} monocytes. Analyses of secondary markers expressed on the monocyte progenitors can potentially elucidate the type of monocyte the progenitor will develop into. In addition to Ly6C expression, fractalkine receptor, also known as CX3CR1, is a common marker for monocyte activation. Expression of CX3CR1 is higher on the surface Ly6C^{low} monocytes compared with Ly6C^{high} monocytes and aids with their function as patrolling, anti-inflammatory cells. Therefore, I examined the expression of the chemokine receptor on the surface of cMoP. The results

revealed that treatment of mice with FHES significantly enhanced CX3CR1 expression on the surface of cMoP cells in the BM when compared with treatment with PBS or β-glucan (Fig. 3.32). Collectively, these data indicate that treatment with FHES modifies MDP and cMoP to proliferate and differentiate into anti-inflammatory Ly6C^{low}CX3CR1$^+$ monocytes. These cells then patrol the periphery and provide protection against classical activation.

3.2.8 S.C. FHES treatment of mice enhances Ly6C^{low} monocytes in the spleen

Treatment of mice with FHES s.c. induces the proliferation of HSCs directly responsible for monocyte development. Indeed, Ly6C^{low} monocytes are selectively enhanced in the BM of mice treated with FHES compared to PBS-treated mice. However, it was not known if these Ly6C^{low} monocytes trafficked out of the BM to peripheral tissues. Therefore, the next avenue of investigation was to determine the circulatory effects of treatment with FHES. The peritoneal cavity and BM are distinct and relatively distant reservoirs and would therefore require mobilisation through the circulatory and lymphatic system. Mice were treated s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, spleens were isolated, and cells stained for flow cytometric analyses. FHES treatment increased total immune cell numbers in the spleen, however this increase was not significant (Fig. 3.33A). FHES treatment of mice induced eosinophilia and increased the percentage of immune cells and absolute numbers when compared to PBS-treated mice was shown (Fig. 3.33B/C). While the proportion of eosinophils in PBS-treated mice remained low at 0.38% of the total immune cell numbers, FHES-treatment more than tripled this percentage to a mean of 1.19% of total immune cells. Furthermore, FHES treatment significantly increased the monocyte fraction from 1.7% in PBS-treated mice, to approximately 2.5% of total immune cells (Fig. 3.34A). This resulted in a significant increase in the absolute numbers of monocytes in the spleen (Fig. 3.34B). Moreover, treatment of mice with FHES significantly increased the percentage and the absolute numbers of Ly6C^{low} monocytes in the spleen (Fig. 3.34C-E). These results confirm that treatment of mice with FHES induces HSCs to produce Ly6C^{low} monocytes, which migrate from the BM and populate peripheral organs.

Helminths and their products are known inducers of the type-2 immune response, in particular T helper 2 (Th2) and T regulatory (Treg) cells [79]. Therefore, the potential for an induction of the adaptive immune response was a potential factor in the development of the innate treatment protocol. Mice were treated with FHES and spleens were isolated on day 21 and subsequently analysed by RT-qPCR for alterations in adaptive mRNA expression. FHES treatment resulted in a significant increase in mRNA expression of gata3, the Th2 master transcription factor (Fig. 3.35A). FHES treatment did not significantly alter any other Th transcription factor measured (Fig. 3.35A). However, the data revealed that treatment of mice with FHES did not significantly enhance the mRNA expression of key Th2 cytokine IL-4 (Fig. 3.27B). Furthermore, treatment of mice with FHES significantly enhanced expression of mRNA for the type 2 innate signalling molecules IL-1RA, Amphiregulin (AREG) and IL-5 (Fig. 3.35B). Taken together, these data reveal that the immune response in the spleen is primarily characterised by type 2, anti-inflammatory innate immune cells.

3.3 Discussion

Helminths have evolved sophisticated methods for manipulating the innate and adaptive immune responses in their hosts to maintain chronic infections. During infection, helminths induce potent type-2/regulatory immune responses, which dampen type-1 and type-17 immune responses [79]. Suppression of pro-inflammatory immune activation during helminth infections or administration with ES products has recently been demonstrated to play a beneficial role in suppressing type-1/type-17 autoimmune diseases [327]. The key findings of the current study were the identification of novel ways in which helminths and their ES products can modify innate immune responses in the periphery and in the BM.

Immunological memory was thought to be confined to T and B cells of the adaptive immune system. However, recent studies have demonstrated that the innate immune system can be trained in response to various stimuli, suggesting an alternative form of immunological memory, now referred to as trained innate immunity [67, 96]. Most reported findings have demonstrated that trained innate immune cells have enhanced pro-inflammatory responses to secondary challenge, including increased metabolism and production of cytokines and chemokines, resulting in enhanced clearance of bacterial or viral infections [98]. Recently, our lab has identified that innate immune cells can be trained using helminth products to develop enhanced anti-inflammatory responses to secondary challenge [113]. BMDM trained with FHTE secrete more IL-10 and less TNF in response to stimulation with TLRs. FHTE-induced anti-inflammatory training was reversible if BMDM were pre-treated with an inhibitor to block, methyltransferase activity, an epigenetic modification common to the induction of trained immunity. The current study has shown that treatment of BMDM with another helminth-derived product, FHES, induced anti-inflammatory trained innate immunity. BMDM primed with FHES secreted higher concentrations of IL-1RA, IL-6 and IL-10 in response to LPS, CpG or Mtb, while simultaneously suppressing TLR-induced TNF secretion. FHES-induced anti-inflammatory training increased production of anti-inflammatory cytokines, and therefore differs from LPS-induced immunological tolerance. During innate immune tolerance cells are unable to undergo gene transcription required for activation [67].

Although there is no centrally-defined mechanism for the induction of trained innate immunity, several factors have been shown to play a key role. Innate cell activation with a primary stimulus induces metabolic and epigenetic modifications at loci along the genome responsible for encoding pro-inflammatory cytokines and cell cycle proteins. For example, training with β-glucan or BCG induces trimethylation of H3K4 and acetylation of H3K27 in the genome at promoter regions for cell cycle proteins and inflammatory cytokines, resulting in enhanced transcription upon re-challenge [98]. In the present study, training of BMDM with FHES was inhibited by pre-treatment of cells with givinostat, an HDAC inhibitor. Treatment with givinostat suppressed FHES-induced IL-1RA and IL-10 production, while not affecting IL-6 or TNF secretion. This suggested that, while training of mature macrophages with FHES partially requires HDAC activity, the process of epigenetic reprogramming with FHES may require more than one method of histone modification. FHES is composed of multiple proteins, enzymes and extracellular vesicles, and its method of action is likely to be multi-factorial. This is consistent with research that has found that methylation/demethylation, acetylation/deacetylation and metabolic reprogramming all coalesce to induce trained innate immunity [98, 328, 329]. Future studies using additional epigenetic inhibitors, such as the methyltransferase inhibitor MTA, may help elucidate the mechanism of anti-inflammatory trained innate immunity.

It is well-established that helminth infections induce potent mobilization and activation of eosinophils, M2 macrophages and monocytes [79]. Previous work from our lab has shown that infection of mice with *F. hepatica* and administration of FHES or FHTE induces recruitment of eosinophils and M2 macrophages to the peritoneal cavity [113, 256, 260]. The data presented here is consistent with these observations and demonstrate that i.p. and s.c. administration of FHES promoted eosinophilia and alternative activation of macrophages. Interestingly, gene expression data on FACS-sorted PEC found that s.c. administration of FHES induced M2-associated genes in SPM, but did not affect mRNA expression in LPM or eosinophils isolated from the peritoneal cavity. A potential explanation for this is that s.c. administration of mice with FHES acts on circulating monocytes, which activate, expand and traffic to several sites throughout the mouse, resulting in the expansion of more innate immune cell populations. This

is evident in the BM and spleen, where mice treated s.c. with FHES have increased frequency and number of Ly6C^{low} monocytes. Patrolling Ly6C^{low} monocytes are found throughout the circulatory and lymphatic vessels of mice, and, upon activation, cycle through the BM to promote hematopoiesis and production of more Ly6C^{low} monocytes [46, 49, 56]. Notably s.c. treatment of mice with FHES was the only route of administration which suppressed Ly6C^{high} monocytes, decreased Ly6C^{low} monocytes, resulting in M2 mRNA expression and type-2 cytokine YM1 in the BMECF. Due to the effect on circulating monocytes and its delivery to the BM, s.c. administration was chosen to be the best route of delivery of FHES for all future experiments.

In addition to peripheral trained immunity, treatment of mice with FHES can induce central trained innate immunity. Recent advances in the field of trained innate immunity have demonstrated that priming progenitor populations in the BM alters the innate immune cells produced upon secondary challenge. Kaufmann et al., demonstrated that administration of BCG to mice expanded LKS$^+$ cells, specifically MPP populations, in the BM, which were epigenetically and metabolically reprogrammed to preferentially produce macrophages with enhanced anti-microbial capabilities [118]. Furthermore, they showed that BMDM generated from mice treated with BCG exhibited a pro-inflammatory trained phenotype, resulting in increased secretion of cytokines in response to TLR stimulation, and enhanced microbial killing capacity. In the present study, we found that BMDM generated from mice treated with FHES produced more anti-inflammatory and less pro-inflammatory cytokines in response to stimulation with LPS and CpG. BMDM from mice treated with FHES did not constitutively express anti-inflammatory markers but required a secondary stimulus to exhibit a trained response. This suggests that progenitor cells that give rise to the generation of macrophages was modified to differentiate into anti-inflammatory BMDM. Furthermore, the macrophages derived from the progenitor cells did not exhibit sustained activation following treatment with FHES, but rather responded to TLR stimulation with increased anti-inflammatory cytokine production.

Mitroulis et al., found that treatment of mice with β-glucan induces expansion of the LKS$^+$ MPP and mediated central trained immunity through IL-1β and GM-CSF in the BM [119]. In the present study, we found that treatment of mice with

FHES expands the LKS$^+$ LT-HSC and the myeloid precursor populations of MDP and cMoP. This confirmed that treatment of mice with FHES modified HSCs, resulting in the production of anti-inflammatory myeloid cells. It is highly probable that treatment of mice with FHES modifies circulating monocytes that then cycle to the BM, secreting type-2 cytokines, such as IL-4, and growth factors to modify the BM microenvironment. However, it is possible that FHES enters the BM and directly activates BM-resident immune cells, stromal cells or HSCs in the BM niche. The ability of FHES to act directly on HSCs and hematopoiesis will be addressed in future chapters. Regardless of the exact mechanism, the data presented here reveal that FHES can induce peripheral and central trained innate immunity.
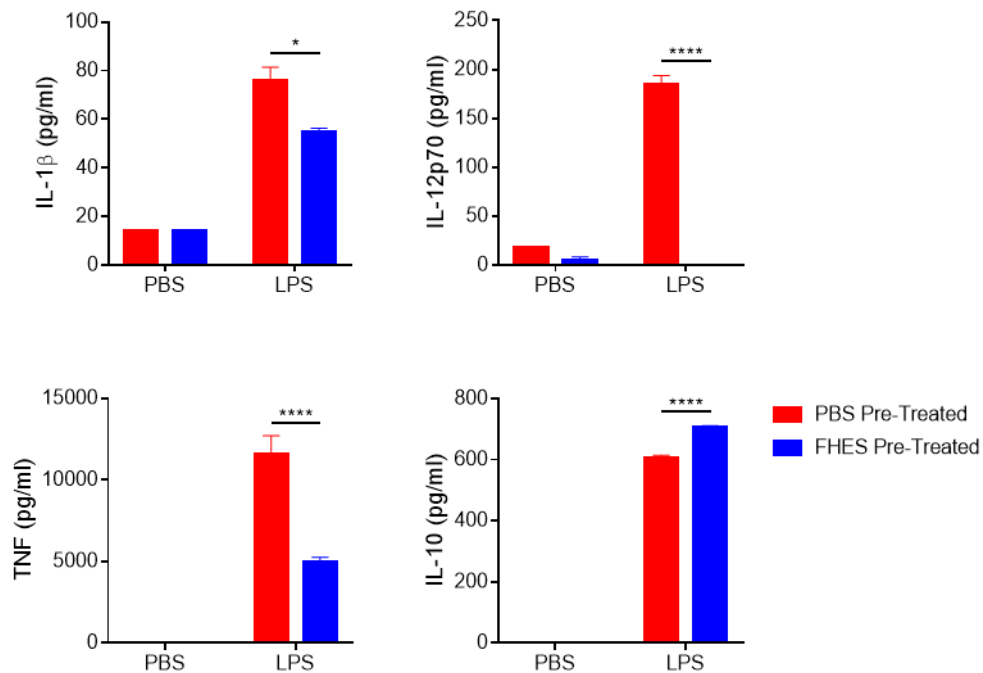
Figure 3.1 *F. hepatica* **ES (FHES) inhibits LPS-induced IL-1β, IL-12p70 and TNF, while enhancing IL-10 production by BMDC.** BMDC ($1x10^6$/ml) expanded from C57BL/6 mice were pre-treated for 30 min with FHES (100 μg/ml) or PBS and then treated with either LPS (100 ng/ml) or PBS. After 24 h, the concentrations of various cytokines in the supernatant were determined by ELISA. Results are mean ± SEM for triplicate culture and are representative of four independent experiments. *$p<0.05$, ***$p<0.001$, ****$p<0.0001$ vs PBS pre-treated LPS by two-way ANOVA with Sidak's multiple comparisons post-test.
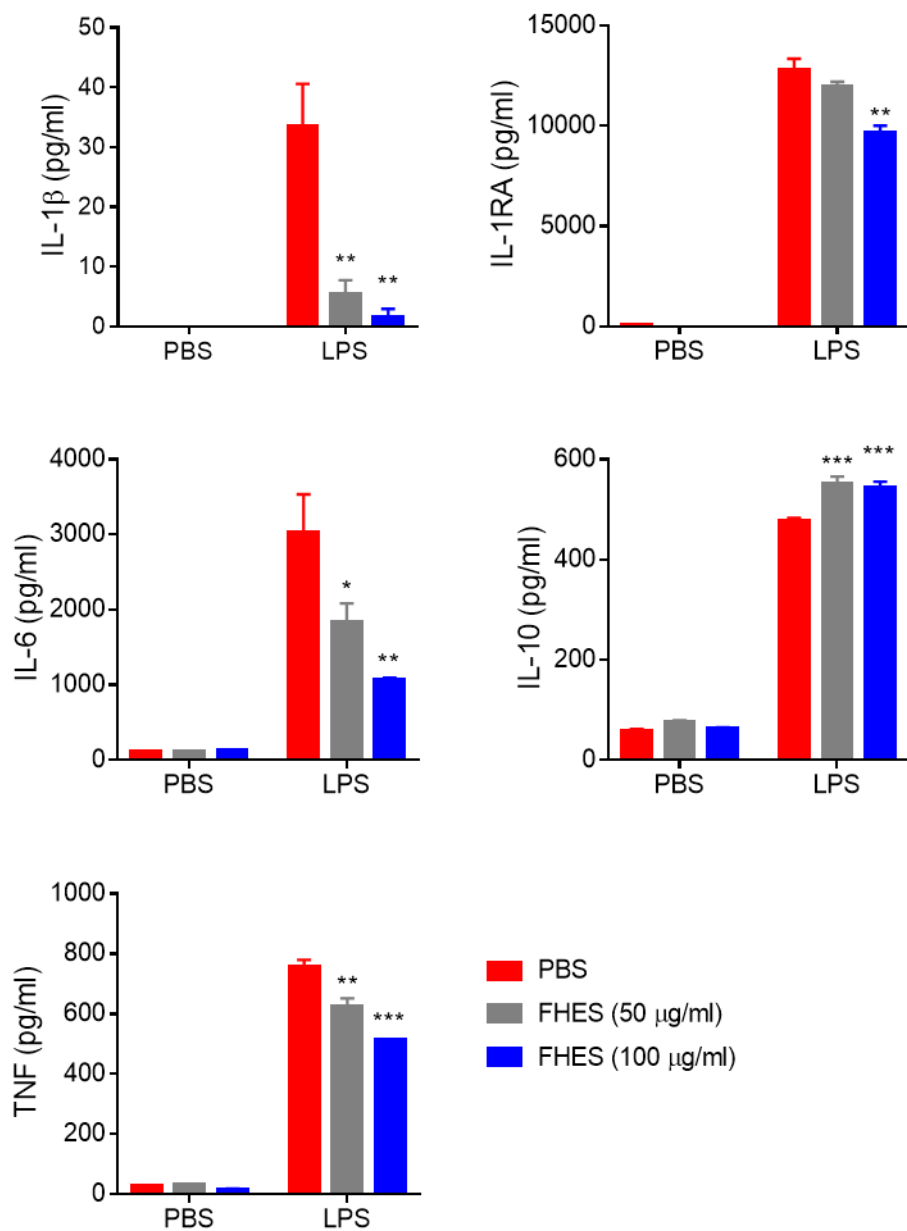
Figure 3.2 FHES inhibits LPS-induced production of IL-1β, IL-1RA, IL-6 and TNF, and enhances secretion of IL-10 by BMDM. BMDM ($1x10^6$/ml) expanded from C57BL/6 mice were pre-treated for 30 min with FHES (50 or 100 µg/ml) or PBS and then treated with either LPS (100 ng/ml) or PBS. After 24 h, the concentrations of various cytokines in the supernatant were determined by ELISA. Results are mean ± SEM for triplicate culture. ***$p<0.001$ vs PBS Pre-treated LPS by two-way ANOVA with Tukey's multiple comparisons post-test.
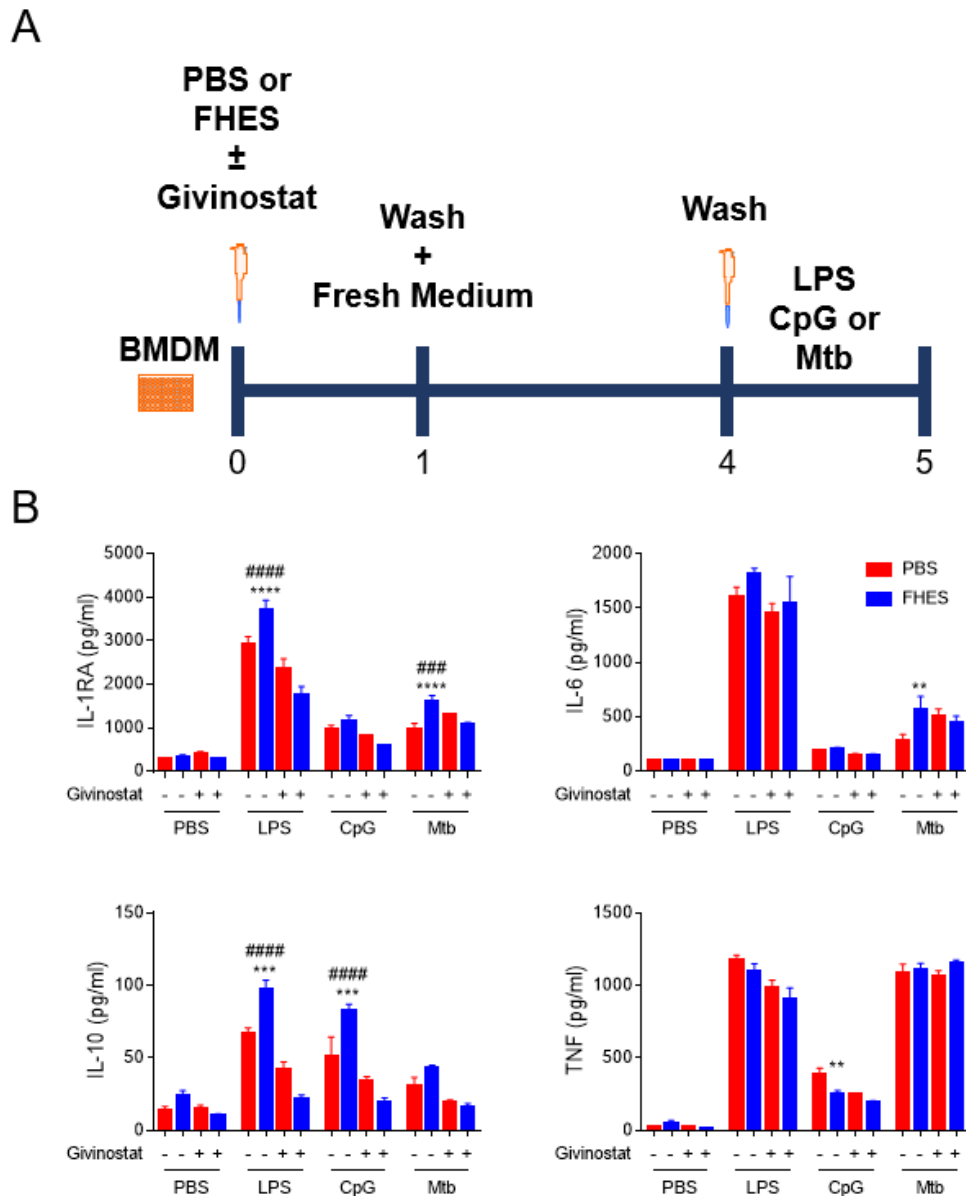
Figure 3.3 FHES-trained BMDM secrete significantly more IL-1RA and IL-10 in response to various PAMPs in an HDAC-dependent process. (A) Schematic detailing BMDM training. BMDM (1×10^6/ml) expanded from C57BL/6 mice were trained with either FHES (100 µg/ml) or PBS with or without the pan-HDAC inhibitor, givinostat (25 nM). After 24 h, cells were washed and rested for 72 h, and restimulated with LPS (100 ng/ml) CpG (5 µg/ml) Mtb (10 µg/ml) or PBS. (B) After 24 h, the concentrations of various cytokines in the supernatant were determined by ELISA. Results are mean ± SEM for triplicate culture. **p<0.01, ***p<0.001 ****p<0.0001 vs PBS Pre-treated control or #### p<0.0001 vs FHES Pre-Treated + givinostat by two-way ANOVA with Tukey's multiple comparisons post-test.

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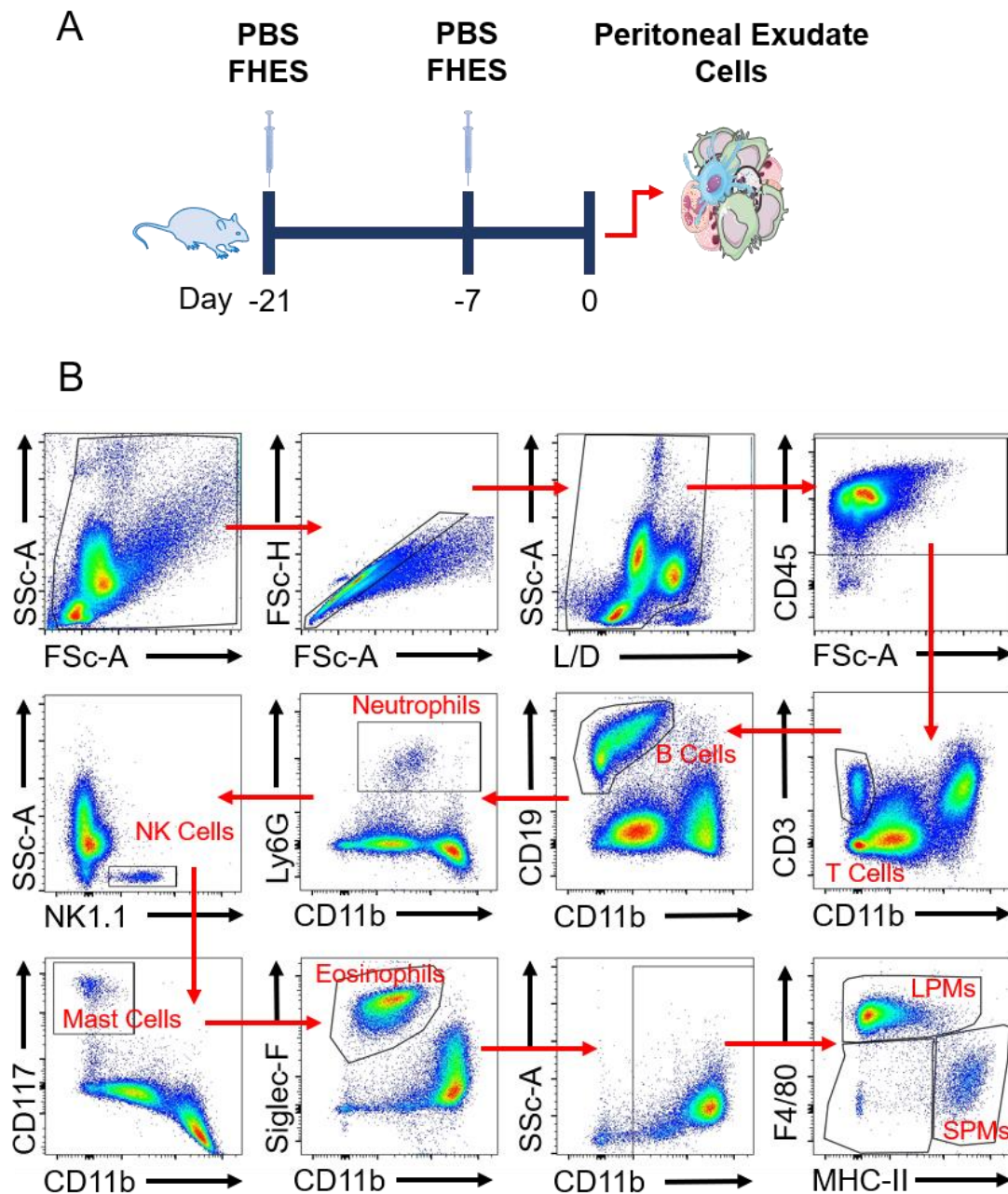
Figure 3.4 *In vivo* treatment scheme and PEC gating strategy to induce trained immunity *in vivo*. (A) Schematic for *in vivo* treatment. C57BL/6 female mice, aged 4-7 w, were trained with FHES (100 µg/mouse) or PBS i.p., s.c. or i.v.. on day 0 and 14. On day 21, mice were euthanized, and various organs/compartments assayed. (B) Gating strategy to identify immune populations of peritoneal exudate cells (PEC) within the peritoneal cavity on day 21.
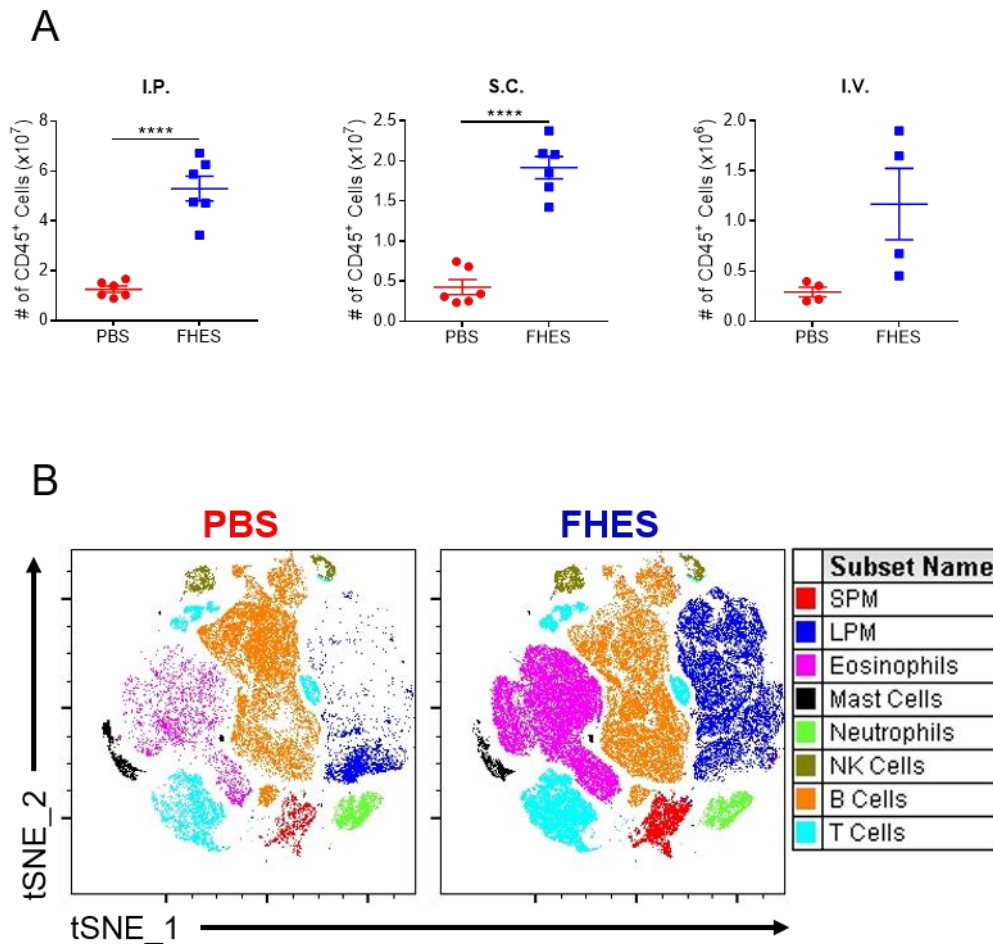
Figure 3.5 Treatment of mice with FHES enhances immune cell infiltration to peritoneal cavity. C57BL/6 female mice were treated with FHES (100 μg/mouse) or PBS i.p., s.c. or i.v. on days 0 and 14. On day 21, mice were sacrificed, and cells were isolated from the peritoneal cavity. Cells were surface stained and assessed for immune cell populations by flow cytometry. (A) Total number of immune cells (CD45$^+$) within the peritoneal cavity (n=4-6/group). Data are mean ± SEM and each symbol represents an individual mouse. ****p<0.0001 vs PBS by unpaired t test. (B) tSNE representative plot of cell populations in the PEC after s.c. injection of FHES (100 μg/mouse) or PBS.
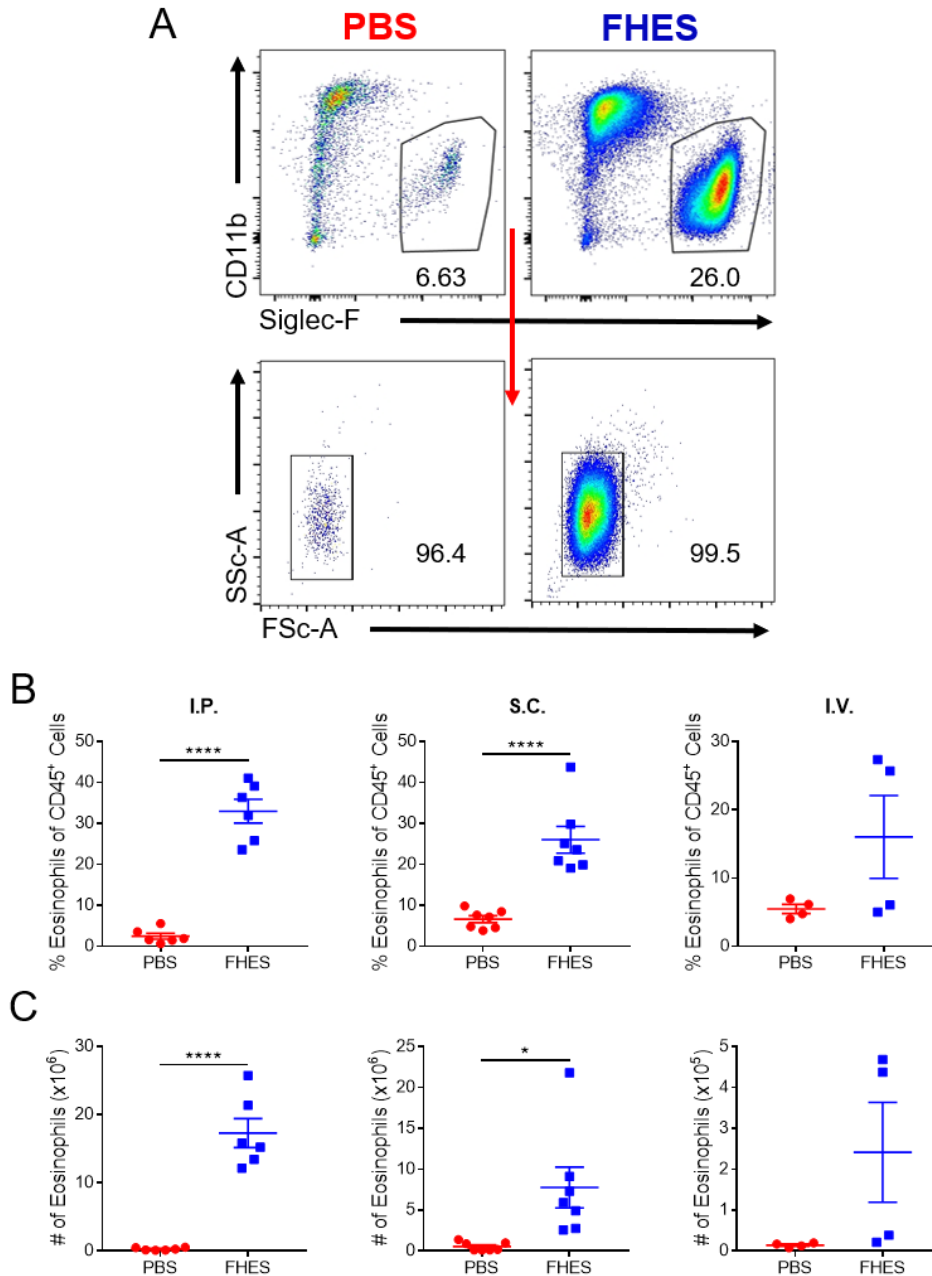
Figure 3.6 Mice treated with FHES have significantly more eosinophils in the peritoneal cavity. C57BL/6 female mice were treated with FHES (100 µg/mouse) or PBS i.p., s.c. or i.v. on days 0 and 14. On day 21, PEC were isolated by peritoneal lavage and eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile. Results are representative flow cytometry plots (A) and percentage (B) and absolute numbers of eosinophils (C). Data are mean ± SEM (n=4-6/group) and each symbol represents an individual mouse. *$p<0.05$, ****$p<0.0001$ vs PBS by unpaired t test.
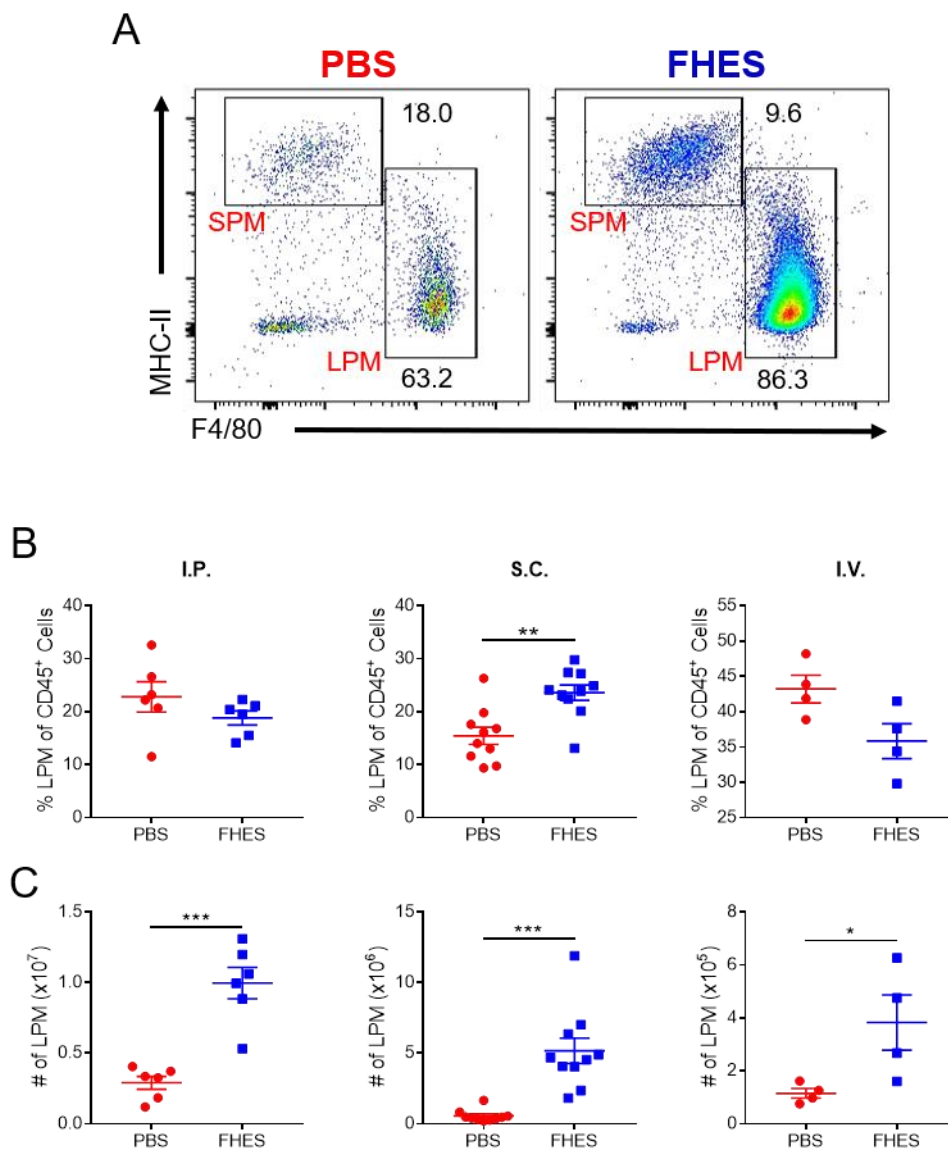
Figure 3.7 Mice treated with FHES have increased numbers of LPM in the peritoneal cavity. C57BL/6 female mice were treated with FHES (100 µg/mouse) or PBS i.p., s.c. or i.v. on days 0 and 14. On day 21, PEC were isolated by peritoneal lavage and LPM were identified by the expression of the surface marker CD11b, F4/80 and MHC-II. (A) Results are representative flow cytometry plots for LPM and SPM (gated on CD11b$^+$ cells). Percentage (B) and absolute number of LPM (C). Data are mean ± SEM (n=4-10/group) (B). *p<0.05, **p<0.01, ***p<0.001 vs PBS by unpaired t test.
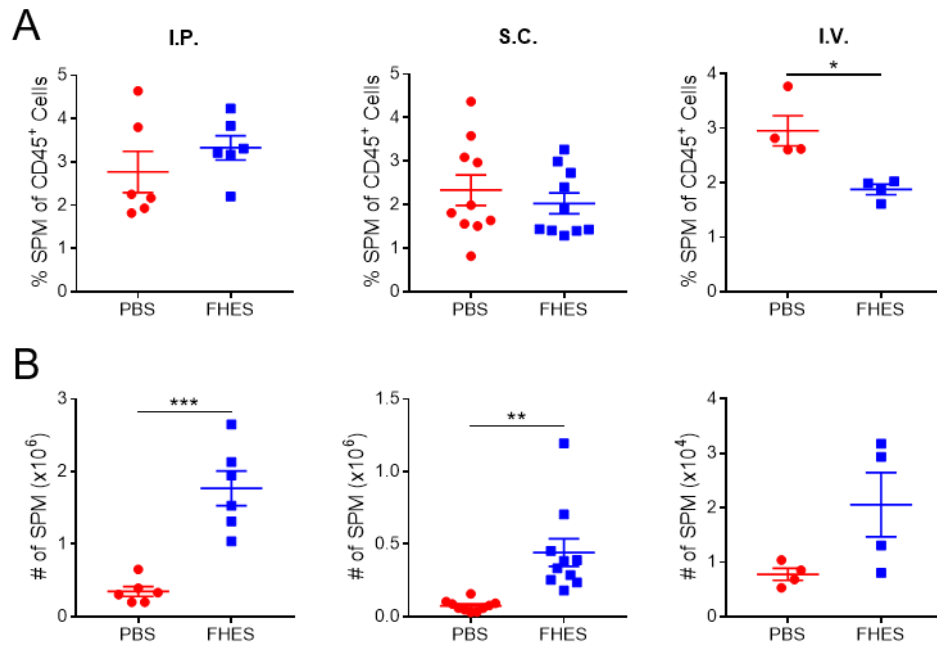
Figure 3.8 Mice treated with FHES have significantly more SPM in the peritoneal cavity. C57BL/6 female mice were treated with FHES (100 µg/mouse) or PBS i.p., s.c. or i.v. on days 0 and 14. On day 21, PEC were isolated by peritoneal lavage and SPM were identified by the expression of the surface marker CD11b, F4/80 and MHC-II. (A) Results are representative flow cytometry plots for LPM and SPM (gated on CD11b$^+$ cells). Percentage (B) and absolute number of LPM (C). Data are mean ± SEM (n=4-10/group). *p<0.05, **p<0.01, ***p<0.001 vs PBS by unpaired t test.
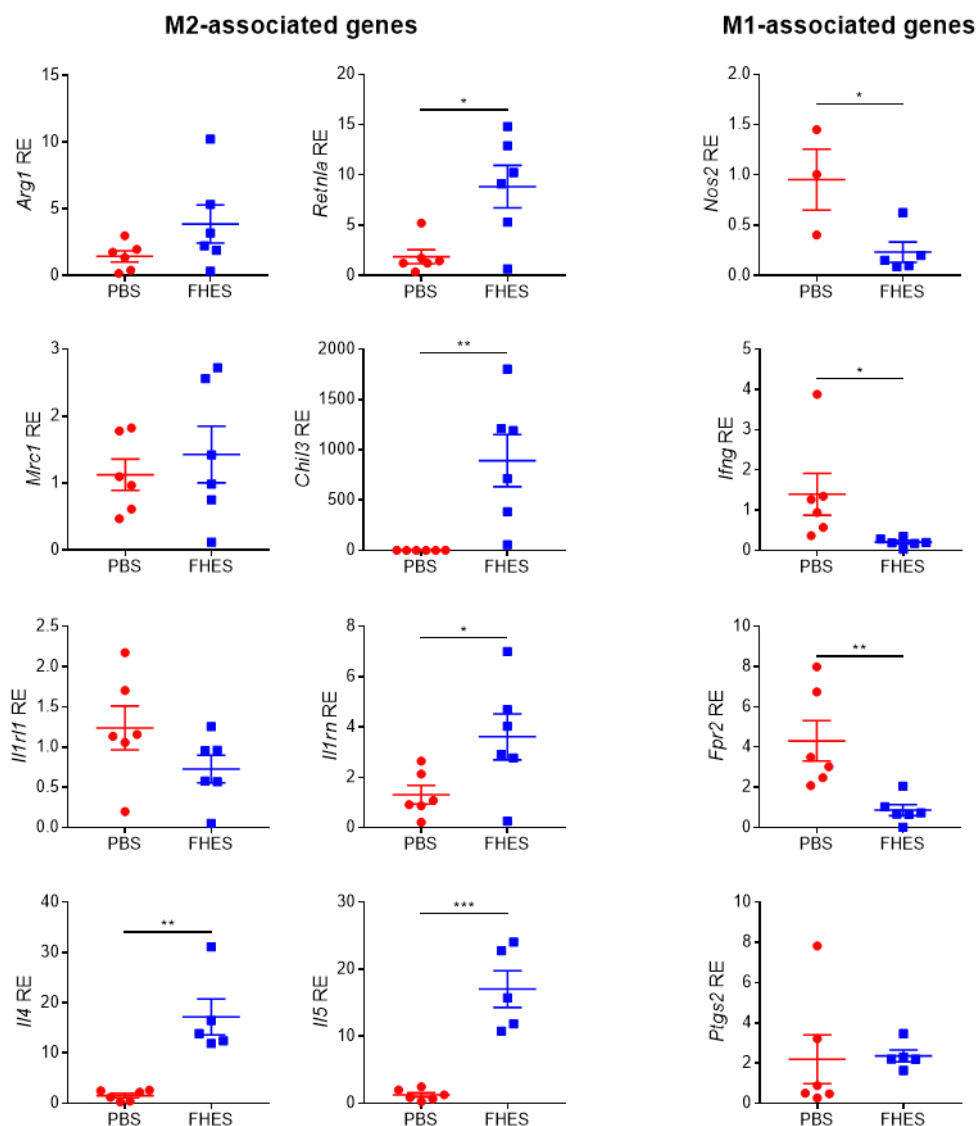
Figure 3.9 Treatment of mice with FHES i.p. significantly upregulates M2 markers and downregulates M1 markers in PEC. C57BL/6 mice were injected i.p. with FHES (100 μg/mouse) or PBS on days 0 and 14. On day 21, mice were sacrificed, and PEC isolated and pelleted by centrifugation. Total RNA was extracted and expression of *Arg1*, *Retnla*, *Chil3*, *Mrc1*, *Il1rl1*, *Il1rn*, *Il4*, *Il5*, *Nos2*, *Fpr2*, *Ifng* and *Ptgs2* was evaluated by RT-qPCR relative to PBS-injected mice following normalisation by the endogenous control 18s rRNA. Results are mean ± SEM (n=3-6/group) and each symbol represents an individual mouse. *p<0.05, **p<0.01, ***p<0.001 vs PBS by unpaired t test.

Figure 3.10 Treatment of mice with FHES s.c. significantly upregulates M2 markers in PEC. C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, mice were sacrificed, and PEC isolated and pelleted by centrifugation. Total RNA was extracted and expression of *Arg1*, *Retnla*, *Chil3*, *Egr2*, *Mrc1*, *Il1rl1*, *Il1rn*, *Il4*, *Il5*, *Nos2*, *Fpr2*, and *Ifng* was evaluated by RT-qPCR relative to PBS-injected mice following normalisation by the endogenous control 18s rRNA. Results are mean ± SEM (n=6-9/group) and each symbol represents an individual mouse. *p<0.05, **p<0.01, ***p<0.001 vs PBS by unpaired t test.
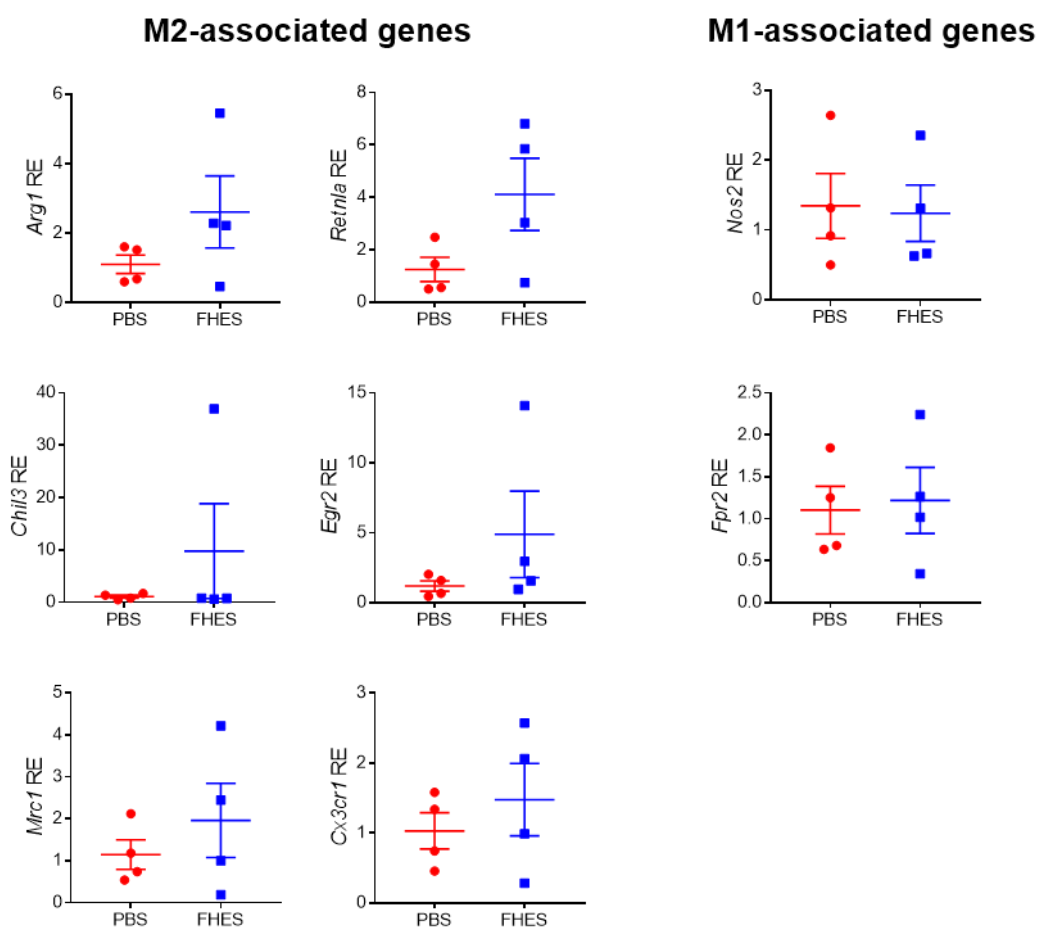
Figure 3.11 No changes in M1 or M2 markers in LPM from mice treated s.c. with FHES. C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, mice were sacrificed, and PEC isolated and stained for CD11b, F4/80 and MHC-II. LPM were FAC-sorted as CD11b⁺F4/80^High^MHC-II^Low^. Total RNA was extracted and expression of *Arg1*, *Retnla*, *Chil3*, *Egr2*, *Mrc1*, *Nos2*, and *Fpr2* was evaluated by RT-qPCR relative to PBS-injected mice following normalisation by the endogenous control 18s rRNA. Results are mean ± SEM (n=4/group) and each symbol represents an individual mouse.
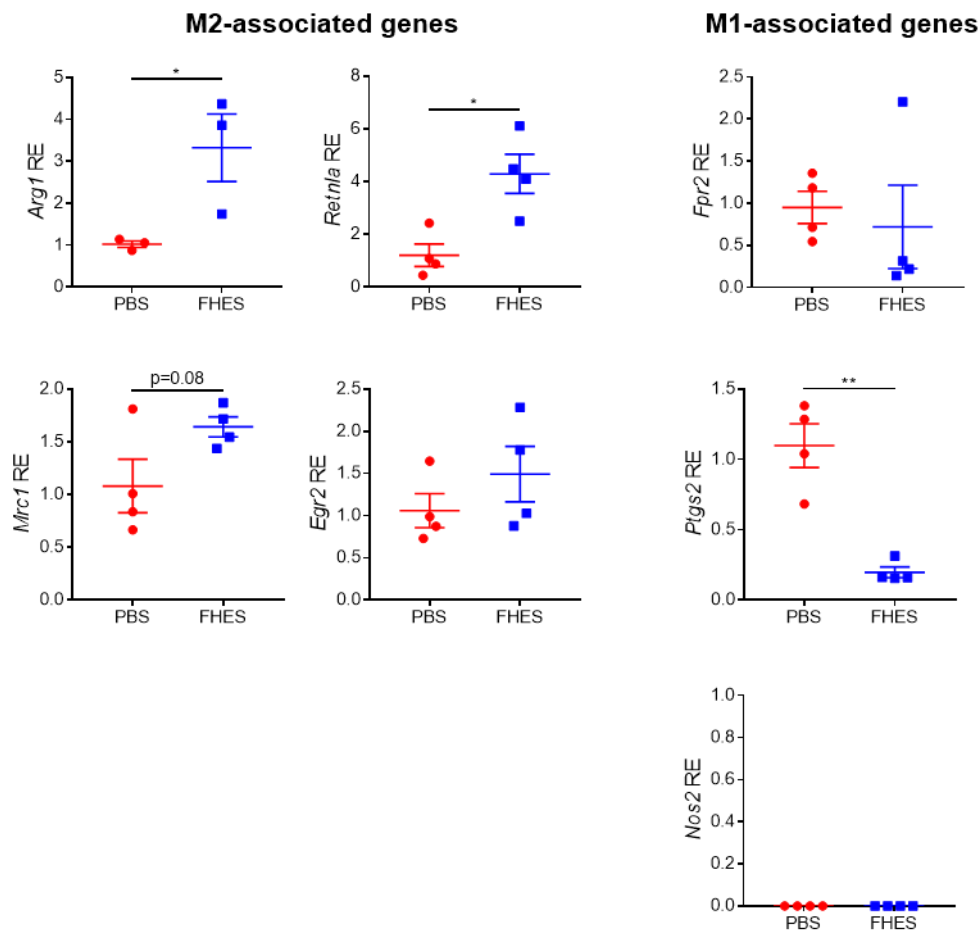
Figure 3.12 SPM from mice treated s.c. with FHES exhibit significantly higher expression of M2-specific markers and less M1 markers. C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, mice were sacrificed, and PEC isolated and stained for CD11b, F4/80 and MHC-II. SPM were FAC-sorted as CD11b+F4/80LowMHC-IIHigh. Total RNA was extracted and expression of *Arg1*, *Retnla*, *Chil3*, *Egr2*, *Mrc1*, *Nos2*, *Fpr2*, and *Ptgs2* was evaluated by RT-qPCR relative to PBS-injected mice following normalisation by the endogenous control 18s rRNA. Results are mean ± SEM (n=4/group) and each symbol represents an individual mouse. *p<0.05, **p<0.01 vs PBS by unpaired t test.
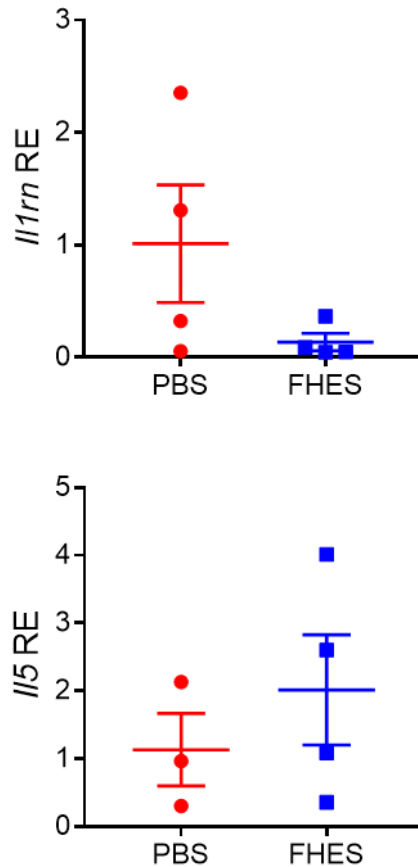
Figure 3.13 FHES treatment of mice does not affect eosinophil expression of IL-1RA or IL-5. C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, mice were sacrificed, and PEC isolated and stained for CD11b, Siglec-F. Eosinophils were FAC-sorted as CD11b⁺Siglec-F⁺Ly6G⁻F4/80⁻. Total RNA was extracted and expression of *il1rn* and *il5* was evaluated by RT-qPCR relative to PBS-injected mice following normalisation by the endogenous control 18s rRNA. Results are mean ± SEM (n=4/group) and each symbol represents an individual mouse.

M2-associated genes

M1-associated genes
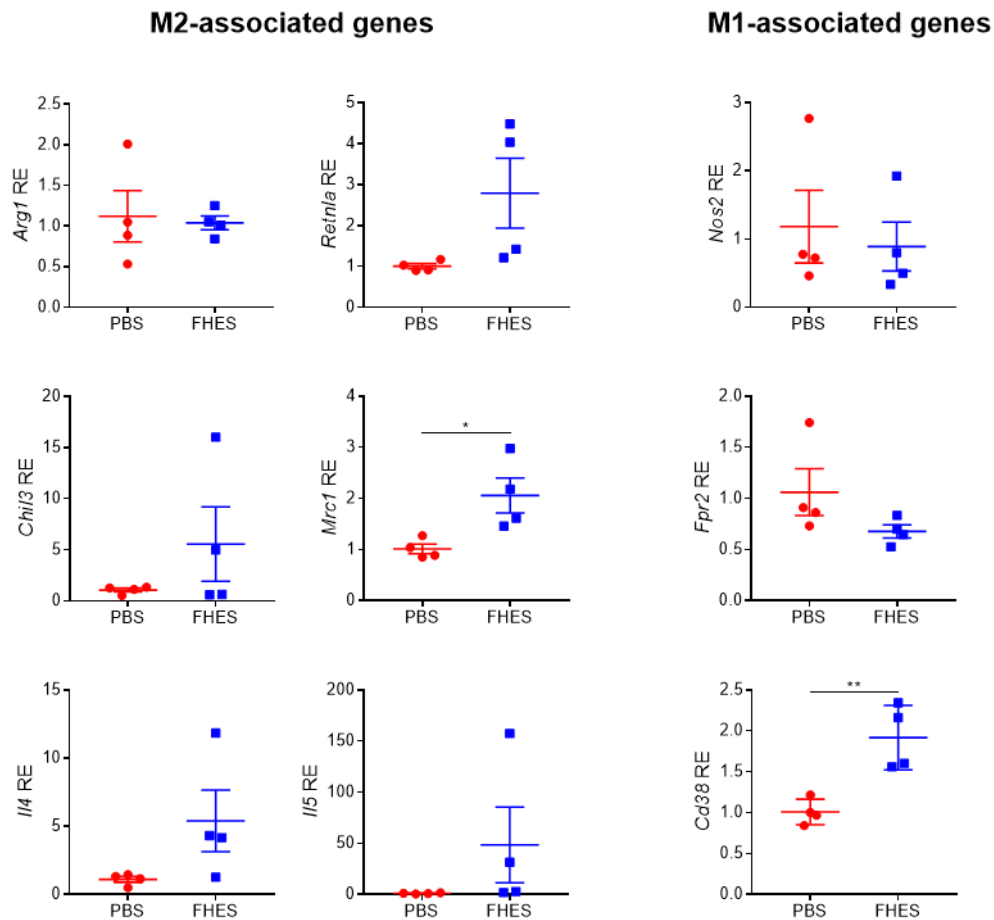
Figure 3.14 FHES treatment by the i.v. route significantly enhances both M2 and M1 mRNA expression in PEC. C57BL/6 mice were injected i.v. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, mice were sacrificed, and PEC isolated and pelleted by centrifugation. Total RNA was extracted and expression of *Arg1*, *Retnla*, *Chil3*, *Mrc1*, *Il4*, *Il5*, *Nos2*, *Fpr2*, and *Cd38* was evaluated by RT-qPCR relative to PBS-injected mice following normalisation by the endogenous control 18s rRNA. Results are mean ± SEM (n=6-9/group) and each symbol represents an individual mouse. *p<0.05, **p<0.01 vs PBS by unpaired t test.
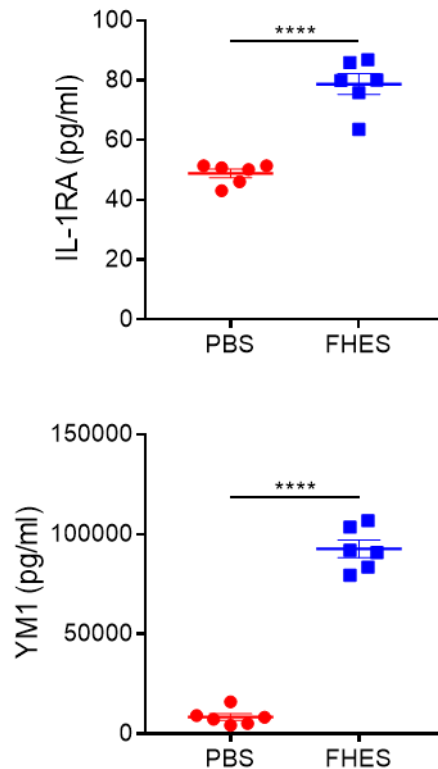
**Figure 3.15 Treatment of mice with FHES i.p. significantly enhances IL-1RA and YM1 in the peritoneal cavity.** C57BL/6 mice were injected i.p. with FHES (100 µg/mouse) or PBS on days 0 and 14 (n=6). On day 21, mice were sacrificed, and PEC isolated and pelleted by centrifugation. The concentration of cytokines in the supernatants ('PEC fluid') was determined by ELISA. Data are mean ± SEM (n=6/group) and each symbol represents an individual mouse. ****$p<0.0001$ vs PBS by unpaired t test.
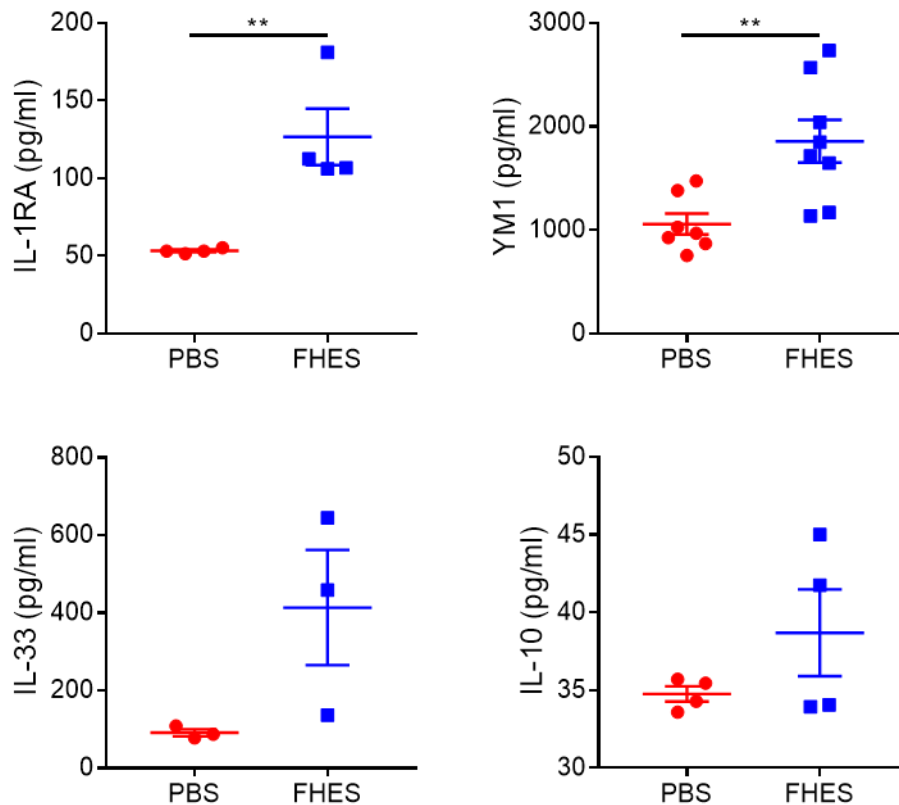
Figure 3.16 Treatment of mice with FHES s.c. significantly enhances IL-1RA and YM1 and increases other M2 markers IL-10 and IL-33. C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14 (n=6). On day 21, mice were sacrificed, and PEC isolated and pelleted by centrifugation. The concentration of cytokines in the PEC fluid was determined by ELISA. Data are mean ± SEM (n=6/group) and each symbol represents an individual mouse. **p<0.01 vs PBS by unpaired t test.
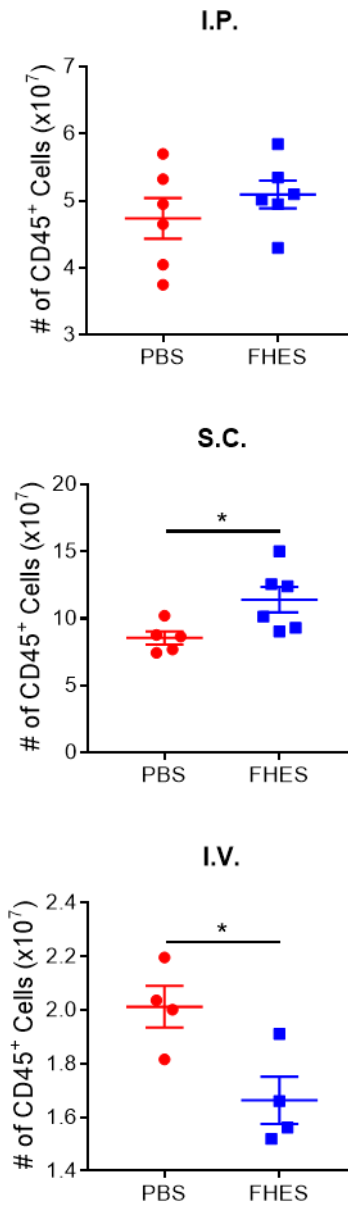
Figure 3.17 Treatment of mice with FHES s.c. significantly enhances the number of immune cells in the BM. C57BL/6 mice were treated with FHES (100 μg/mouse) or PBS i.p., s.c. or i.v. on day 0 and 14. On day 21, mice were sacrificed, and the bone marrow cells isolated. Cells were assessed for immune cell populations by flow cytometry. Total number of immune cells (CD45^{+}) in the bone marrow (n=4-6/group). Data are mean ± SEM and each symbol represents an individual mouse. *p<0.05 vs PBS by unpaired t test.
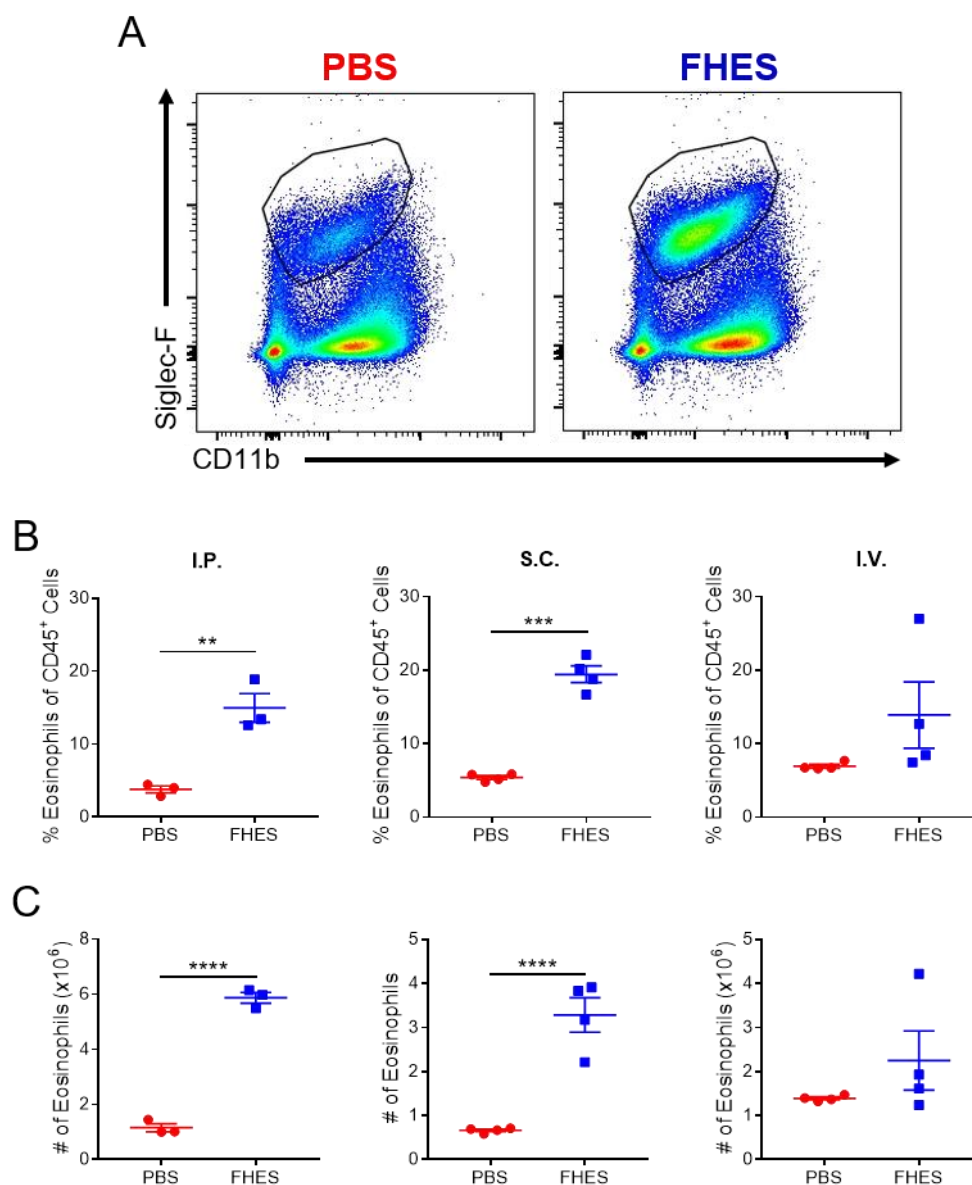
Figure 3.18 Treatment of mice with FHES induces eosinophil production within the bone marrow. C57BL/6 female mice were treated with FHES (100 µg/mouse) or PBS i.p., s.c. or i.v. on days 0 and 14. On day 21, whole BM was isolated, and eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile. Results are representative flow cytometry plots (A) and percentage (B) and absolute numbers of eosinophils (C). Data are mean ± SEM (n=4/group) and each symbol represents an individual mouse. *p<0.05, ****p<0.0001 vs PBS by unpaired t test.
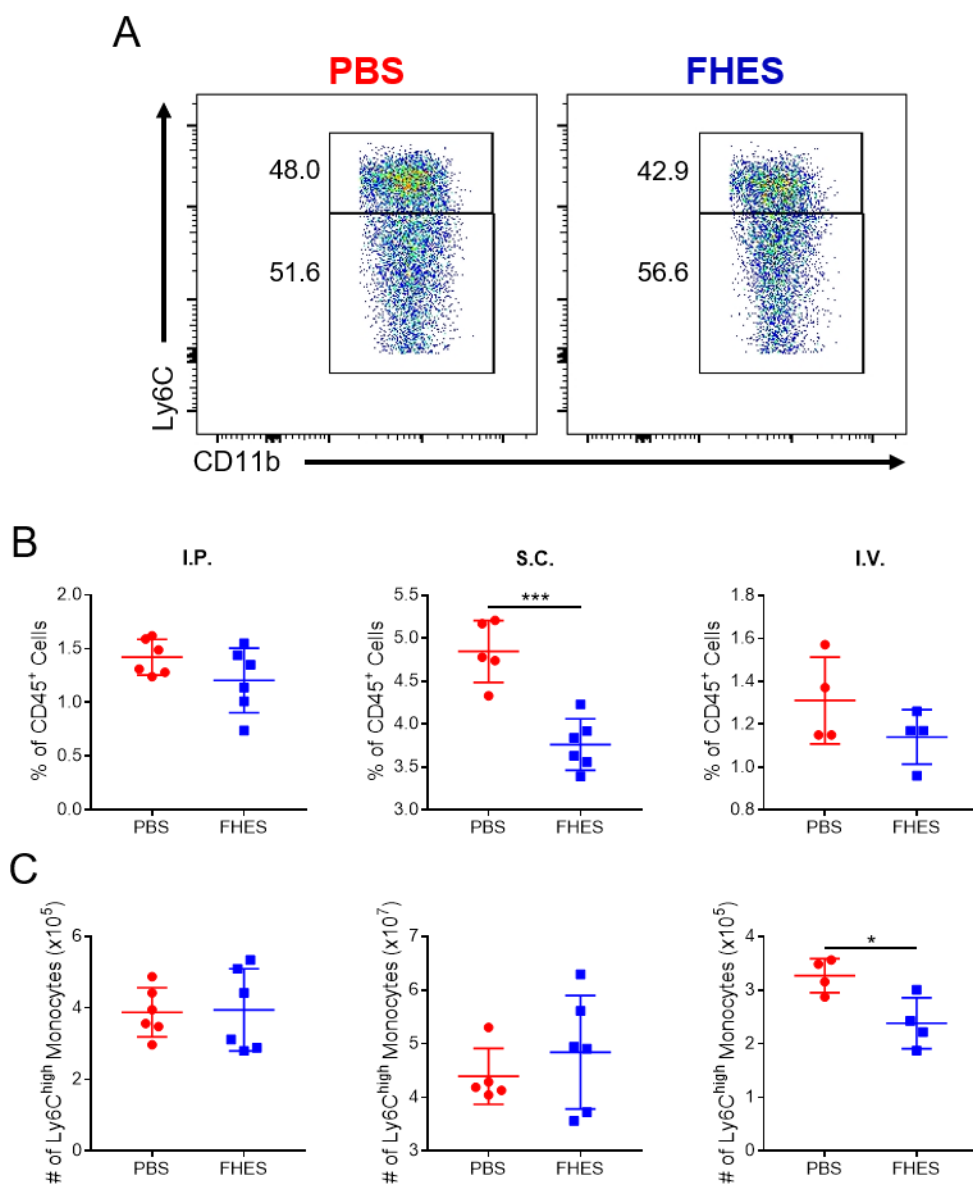
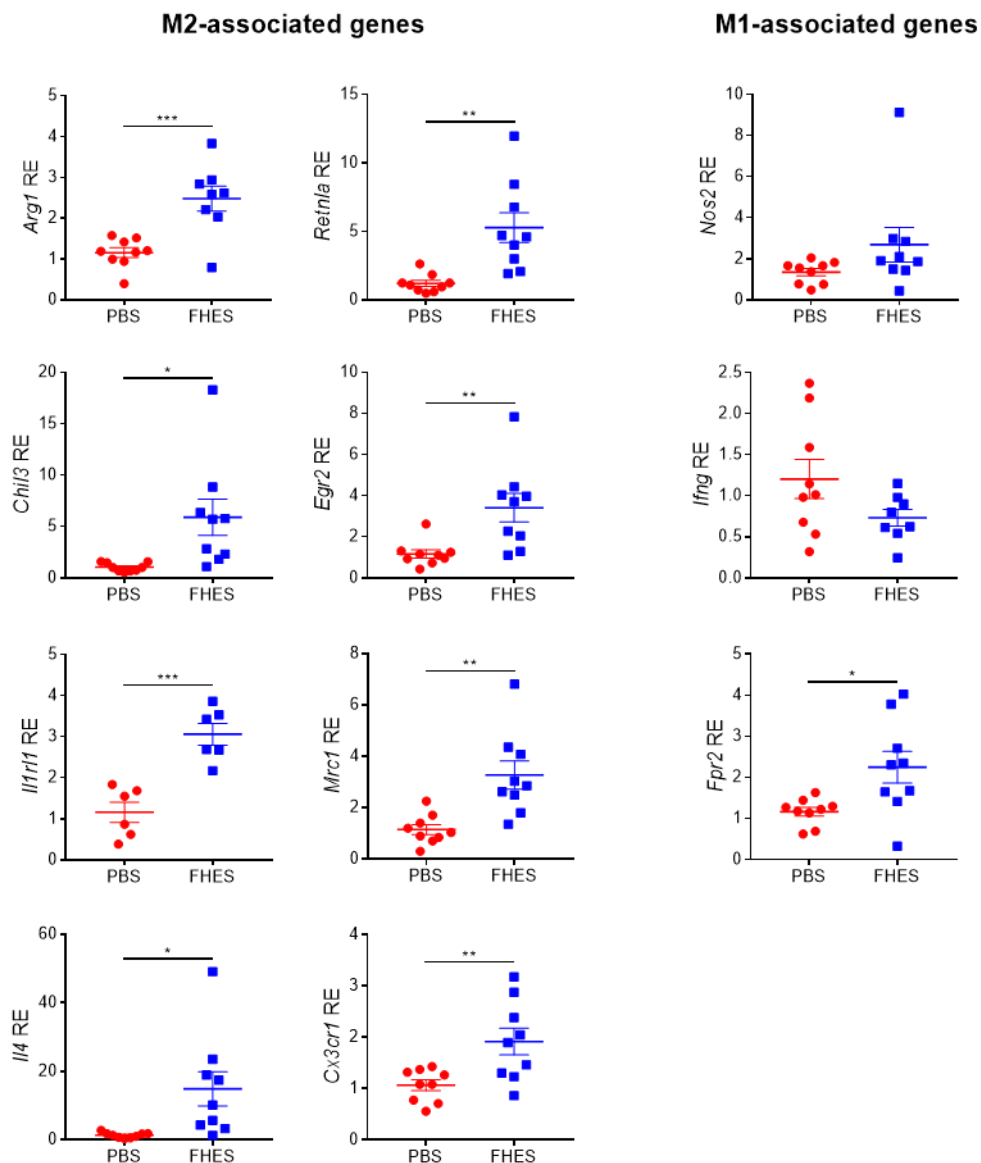**Figure 3.19 Treatment of mice with FHES reduces inflammatory Ly6C^high monocytes in the bone marrow.** C57BL/6 female mice were treated with FHES (100 µg/mouse) or PBS i.p., s.c. or i.v. on days 0 and 14. On day 21, whole BM was isolated and monocytes subsets assessed by surface expression of Ly6C by flow cytometry. Results are representative flow cytometry plots (A) and percentage (B) and absolute numbers of Ly6C^high monocytes (C). Data are mean ± SEM (n=4-6/group) and each symbol represents an individual mouse. *p<0.05, ***p<0.001 vs PBS by unpaired t test.
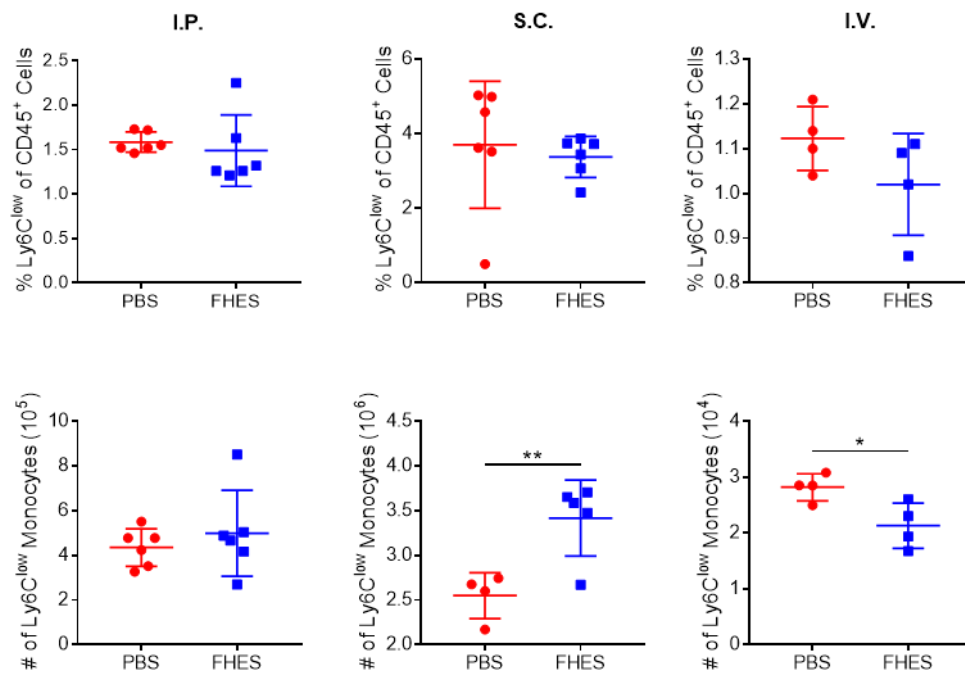
**Figure 3.20 Treatment of mice with FHES s.c. drives monocyte differentiation towards anti-inflammatory Ly6C^{low} monocytes.** C57BL/6 female mice were treated with FHES (100 µg/mouse) or PBS i.p., s.c. or i.v. on days 0 and 14. On day 21, whole BM was isolated and monocytes subsets were stratified by the expression of the surface marker Ly6C. Percentages (A) and absolute numbers of Ly6C^{low} monocytes (B). Data are mean ± SEM (n=4-6/group) and each symbol represents an individual mouse. *p<0.05, **p<0.01 vs PBS by unpaired t test.
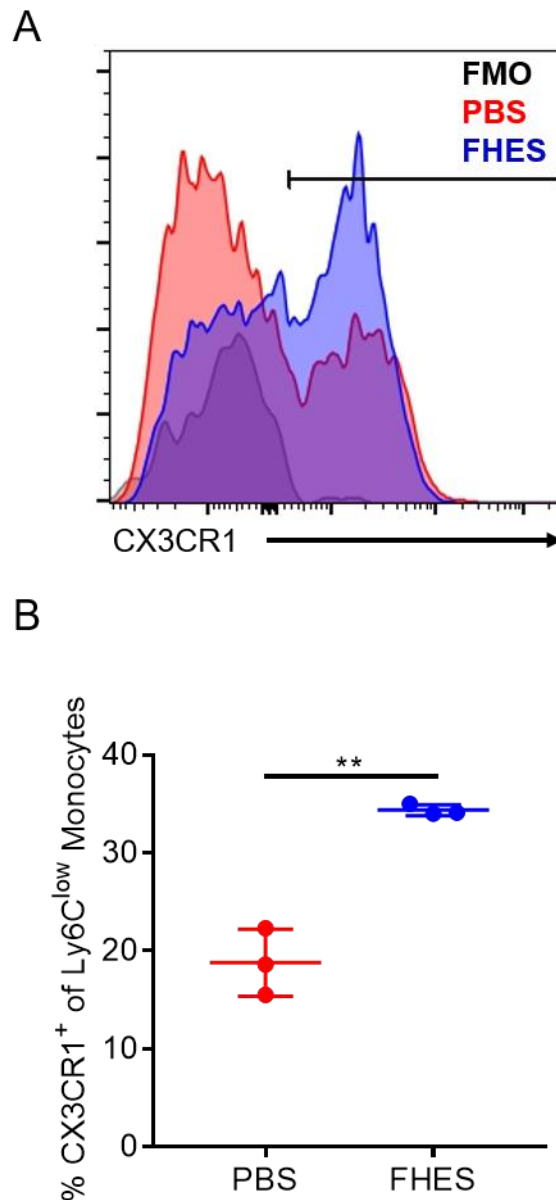
Figure 3.21 Treatment of mice with FHES s.c. enhances surface expression of CX3CR1 on Ly6C^{low} monocytes. C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, BM was isolated and stained for CD11b, CD115, Ly6C and CX3CR1. Results are representative flow cytometry plots for CX3CR1 expression on Ly6C^{low} monocytes (A) and percentage of CX3CR1+ LY6C^{low} monocytes (B). Results are mean ± SEM (n=3/group) and each symbol represents an individual mouse. **p<0.01 vs PBS by unpaired t test.
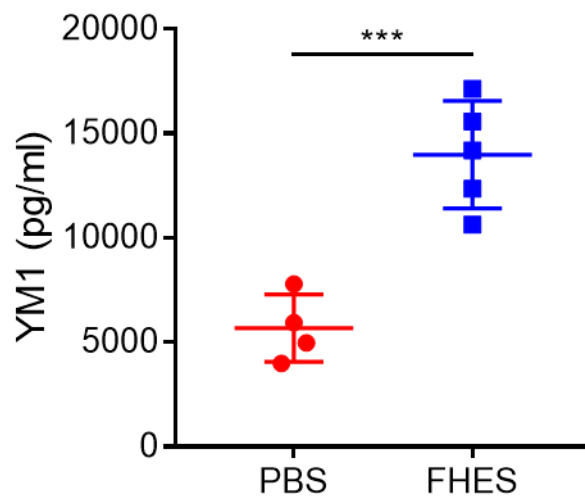
**Figure 3.22 The type-2 cytokine YM1 is significantly enhanced in the BMECF of mice treated with FHES.** C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, BM of mice were isolated by perfusion with cRPMI (600 µl/leg), the cells pelleted and the resultant supernatant (BMECF) analysed for Ym1 by ELISA. ***p<0.001 vs PBS by unpaired t test.
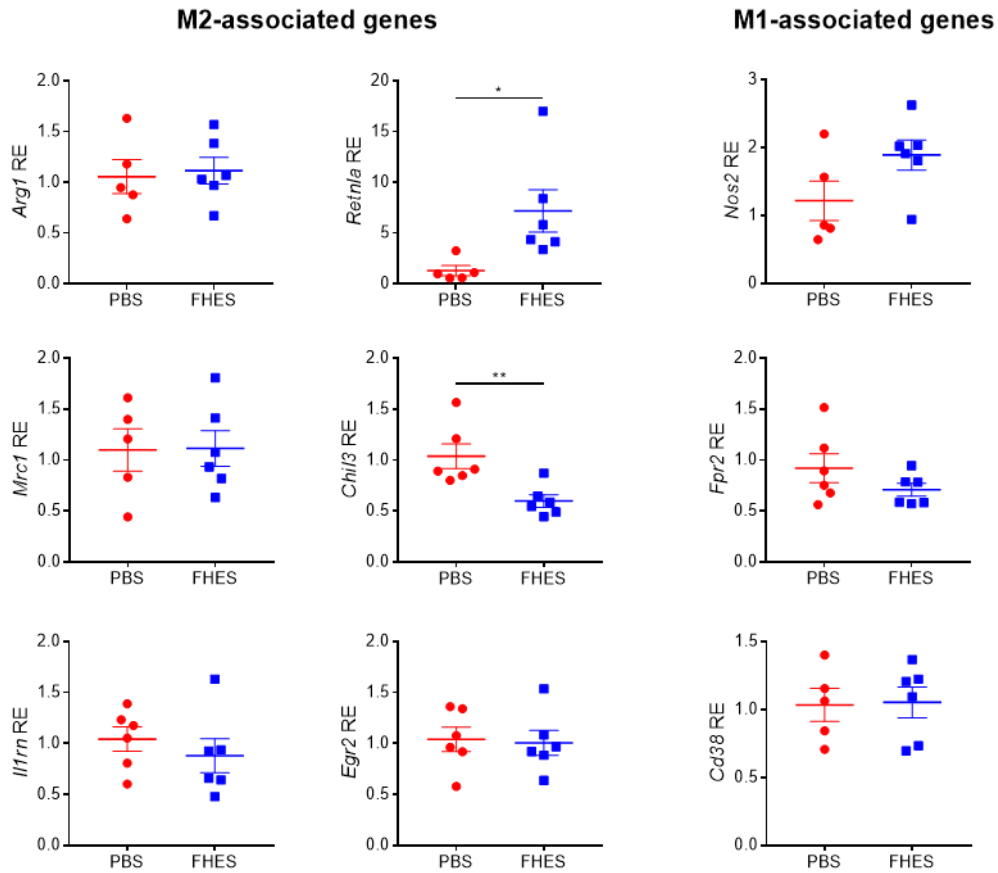
Figure 3.23 Treatment of mice with FHES s.c. significantly enhances *Retnla* in BM. C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, mice were sacrificed, and BM isolated and pelleted by centrifugation. Total RNA was extracted and expression of *Arg1*, *Retnla*, *Chil3*, *Mrc1*, *Il1rn*, *Nos2*, *Fpr2*, and *Ifng* was evaluated by RT-qPCR relative to PBS-injected mice following normalisation by the endogenous control 18s rRNA. Results are mean ± SEM (n=6/group) and each symbol represents an individual mouse. *p<0.05, **p<0.01, ***p<0.001 vs PBS by unpaired t test.
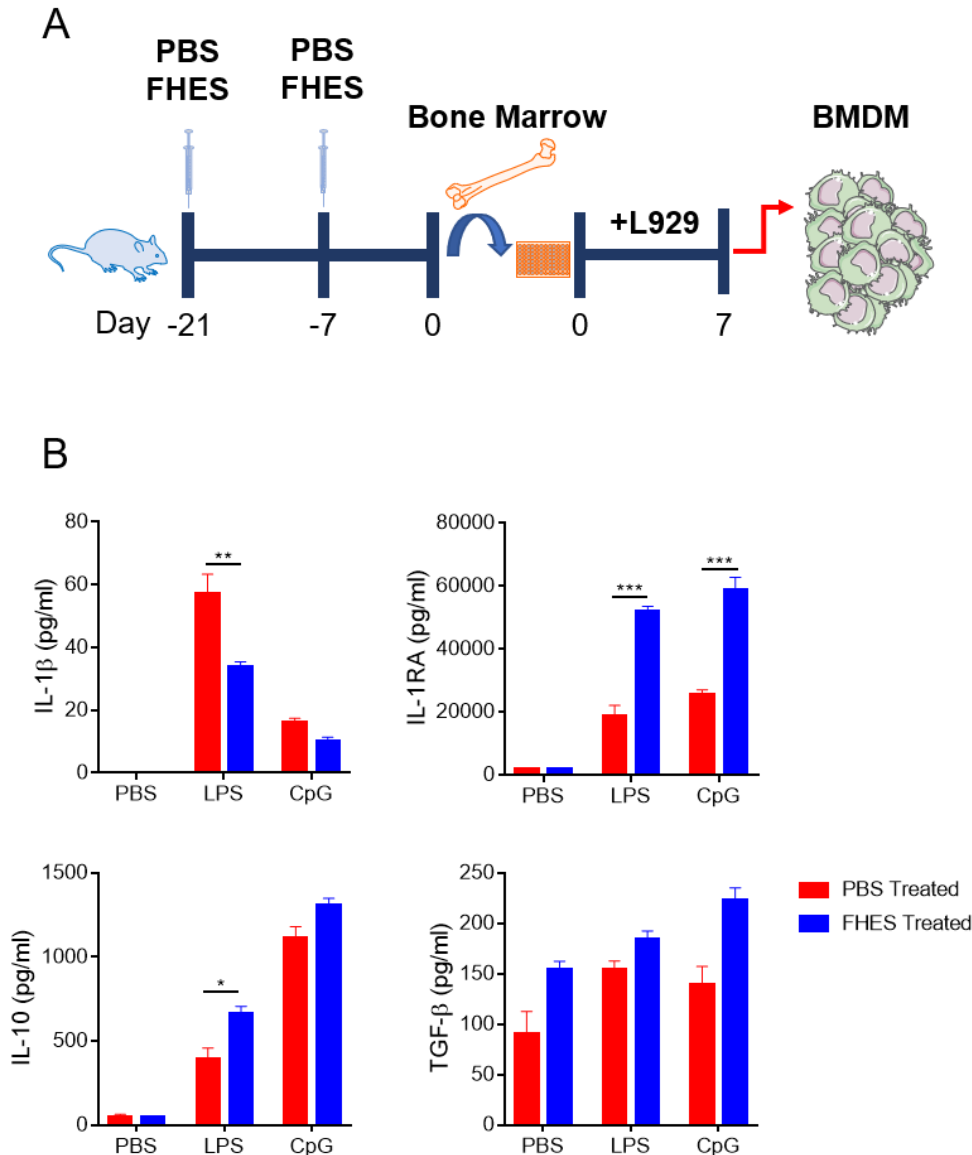
Figure 3.24 BMDM from mice treated with FHES secrete higher levels of IL-1RA, IL-10 and TGF-β and less IL-1β in response to TLR agonists. (A) Schematic detailing *ex vivo* differentiation of BMDM from BM of mice treated with FHES (100 µg/mouse) or PBS. (B) BMDM grown from untrained/trained mice via addition of L929 supernatants containing CSF-1. On day 7, BMDM were washed, re-plated ($2x10^5$/well) and treated with LPS (100 ng/ml), CpG (5 µg/ml) or PBS. Supernatants were collected after 24 h. The concentration of cytokines was determined by ELISA. Results are mean ± SEM for triplicate culture and are representative of four independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, vs PBS pre-treated control by two-way ANOVA with Sidak's multiple comparisons post-test.

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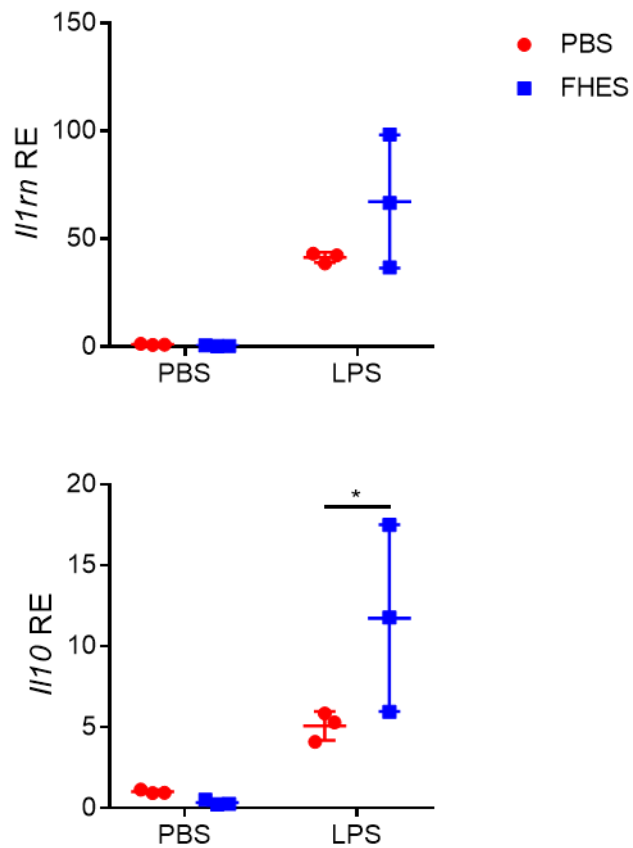
Figure 3.25 BMDM from FHES-treated mice exhibit higher expression of mRNA for *Il10* and *Il1rn* in response to LPS. BMDM from FHES-treated and untreated mice were stimulated with LPS (100 ng/ml) or PBS. After 24 h, cells were lysed, total RNA were isolated and expression of Il10 and Il1rn were evaluated by RT-qPCR relative to medium cultured cells following normalization by the endogenous control 18S mRNA. $*p<0.05$, $**p<0.01$, $***p<0.001$, vs PBS pre-treated control by two-way ANOVA with Sidak's multiple comparisons post-test.
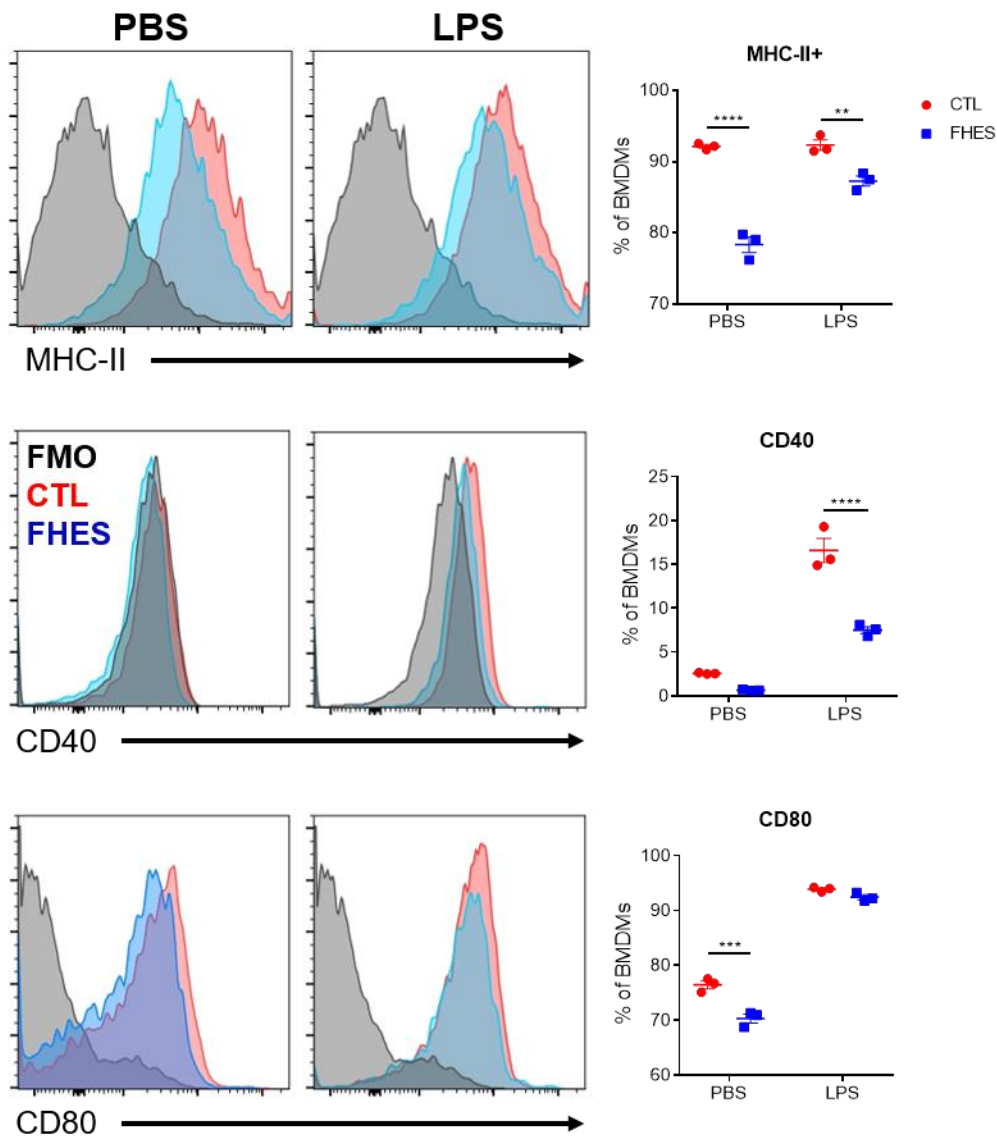
Figure 3.26 Reduced surface expression of MHC Class II and CD40 by BMDM generated from mice pre-treated with FHES in response to LPS. BMDM were differentiated from FHES-treated and untreated mice (CTL) via addition of L929 supernatants containing CSF-1. On day 7, BMDM were washed, and cultured (2×10^5/well) with LPS (100 ng/ml) or PBS for 24 h. Cells were washed and stained for flow cytometry, PBS-trained in red, FHES in blue, FMO in grey. **p<0.01, ***p<0.001, ****p<0.0001, vs PBS pre-treated control by two-way ANOVA with Sidak's multiple comparisons post-test.
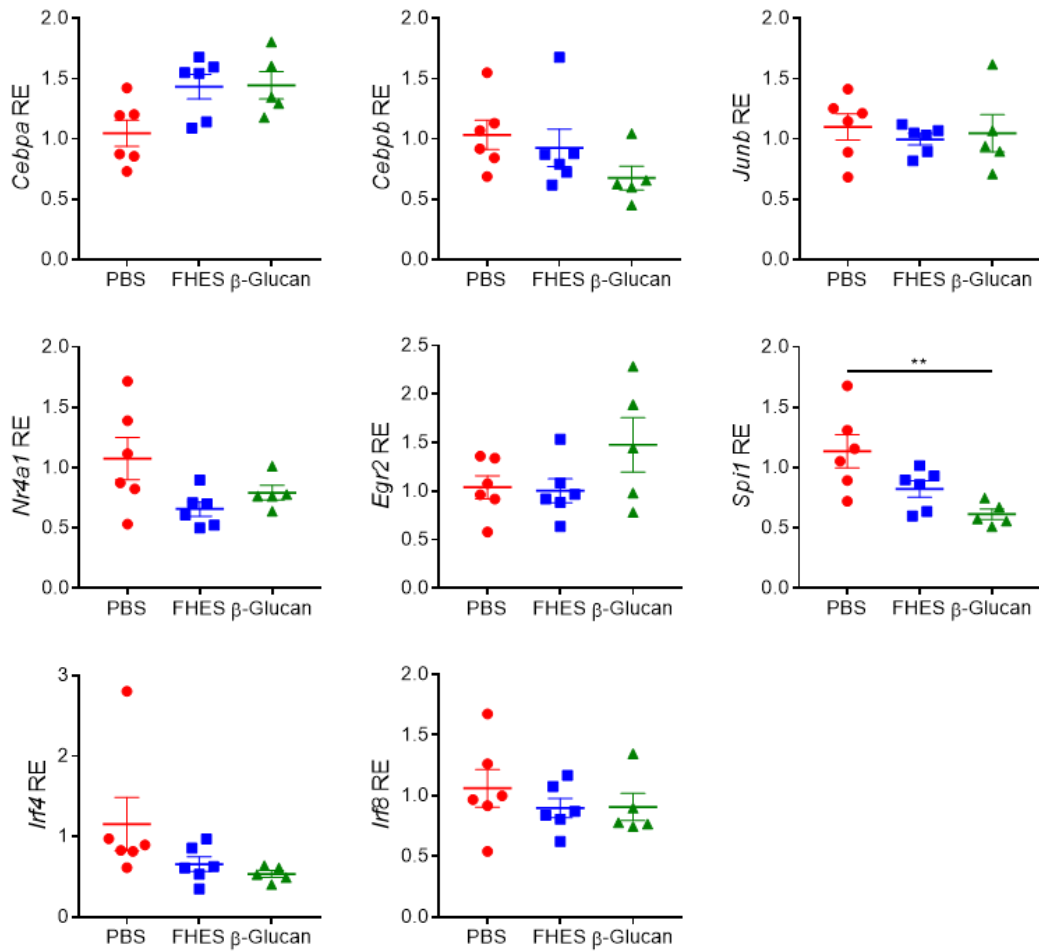
Figure 3.27 Gene expression of myelopoiesis-associated transcription factors in BM is not significantly altered by FHES treatment. C57BL/6 mice were s.c. injected twice with FHES (100 μg/mouse) or PBS , once on day 0 and 14. On day 21, mRNA from whole BM was isolated and analysed by RT-qPCR. Results are mean ± SEM (n=5-6 mice/group). Each dot represents an individual mouse. **p<0.01 vs control by one-way ANOVA with Tukey post-test.
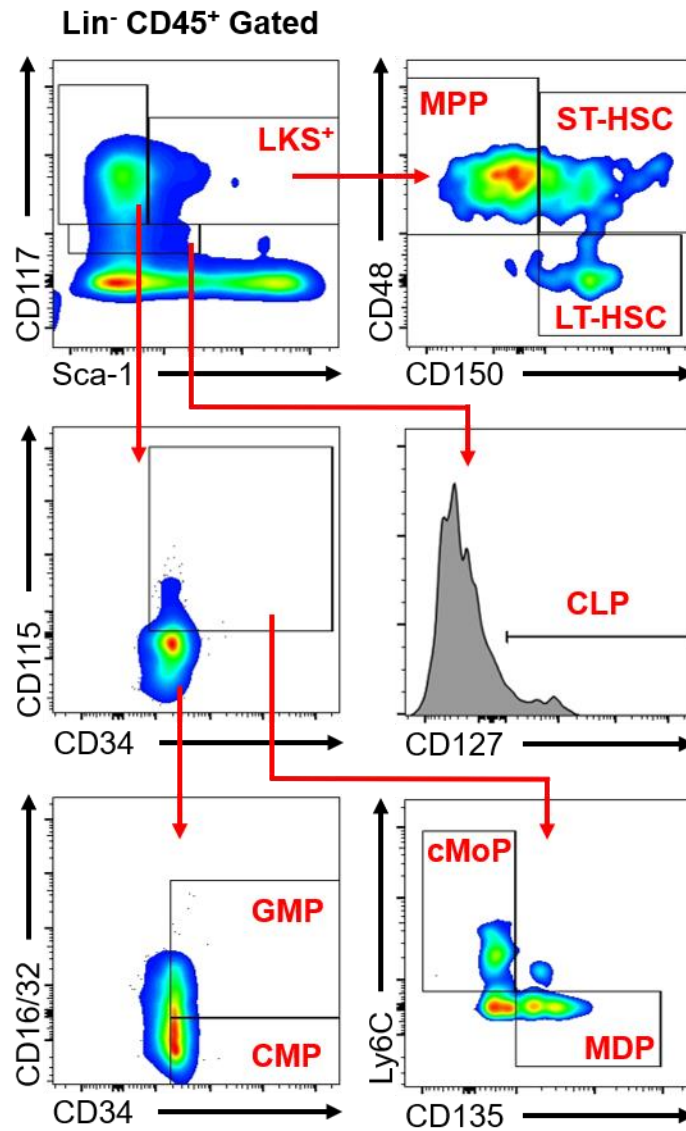
Figure 3.28 Gating strategy for identification of multipotent hematopoietic stem and progenitor cells (HSCs) in the BM of mice. BM was isolated and stained with antibodies against various surface markers. Cells we first gated on Singlets, CD45+Lineage-. Lineage gating includes CD19, CD3, Siglec-F, Ly6G, CD11b, and Ter119. Populations shown include: Lineage-cKit(CD117)+Sca-1+ (LKS+), Long-Term Hematopoietic Stem Cell (LT-HSC), Short-Term HSC (ST-HSC), Multipotent Progenitors (MPP), Granulocyte-Macrophage Progenitors (GMP), Common Myeloid Progenitor (CMP), Common Lymphoid Progenitor (CLP), Monocyte-Dendritic Cell Precursor (MDP) and Common Monocyte Precursor (cMoP).
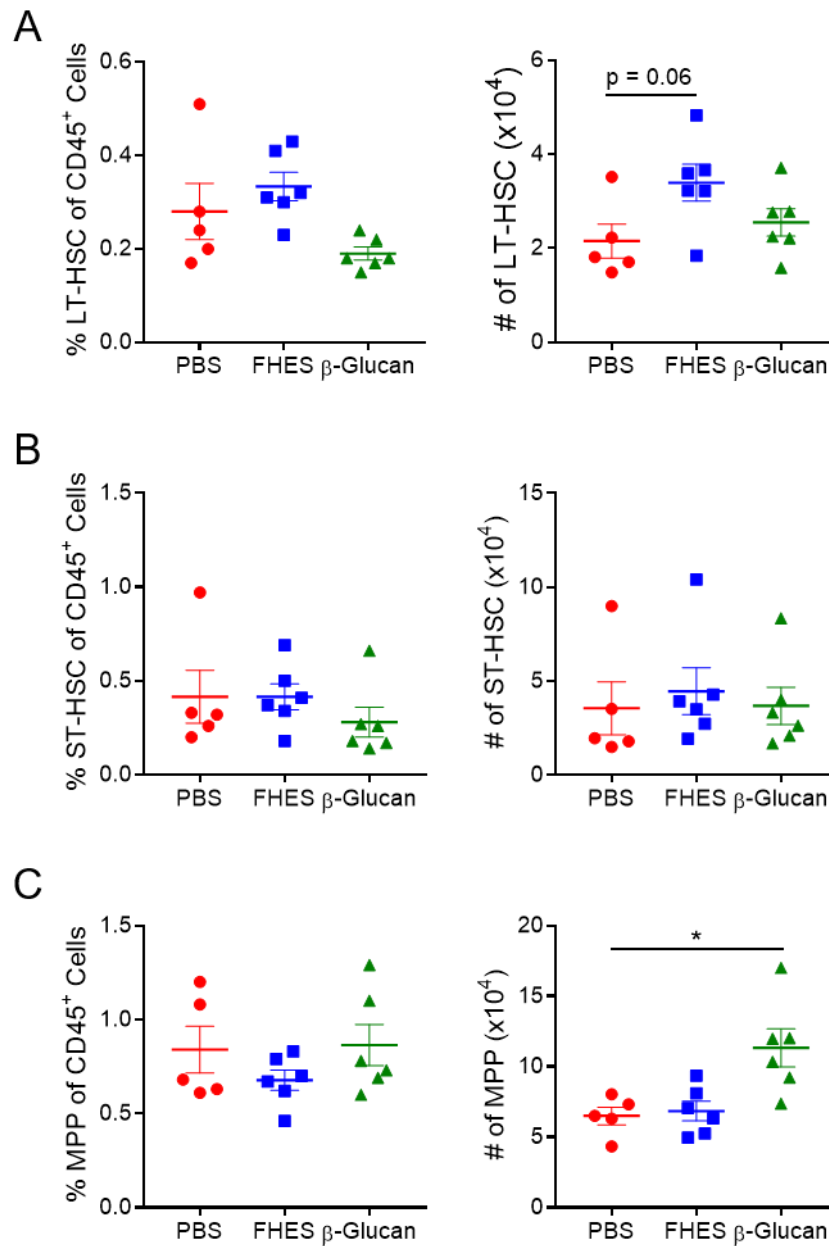
Figure 3.29 FHES treatment marginally increases the number of LT-HSC in the BM. C57Bl/6 mice were treated s.c. with either FHES (100 µg/mouse) β-glucan (50 µg/mouse) or PBS on days 0 and 14. On day 21, BM was collected and assessed by flow cytometry. The frequency and total cell numbers of LT-HSC (A), ST-HSC (B) and MPP (C). Results are mean ± SEM (n=6 mice/group). Each dot represents an individual mouse. *p<0.05 vs control by one-way ANOVA with Tukey post-test.
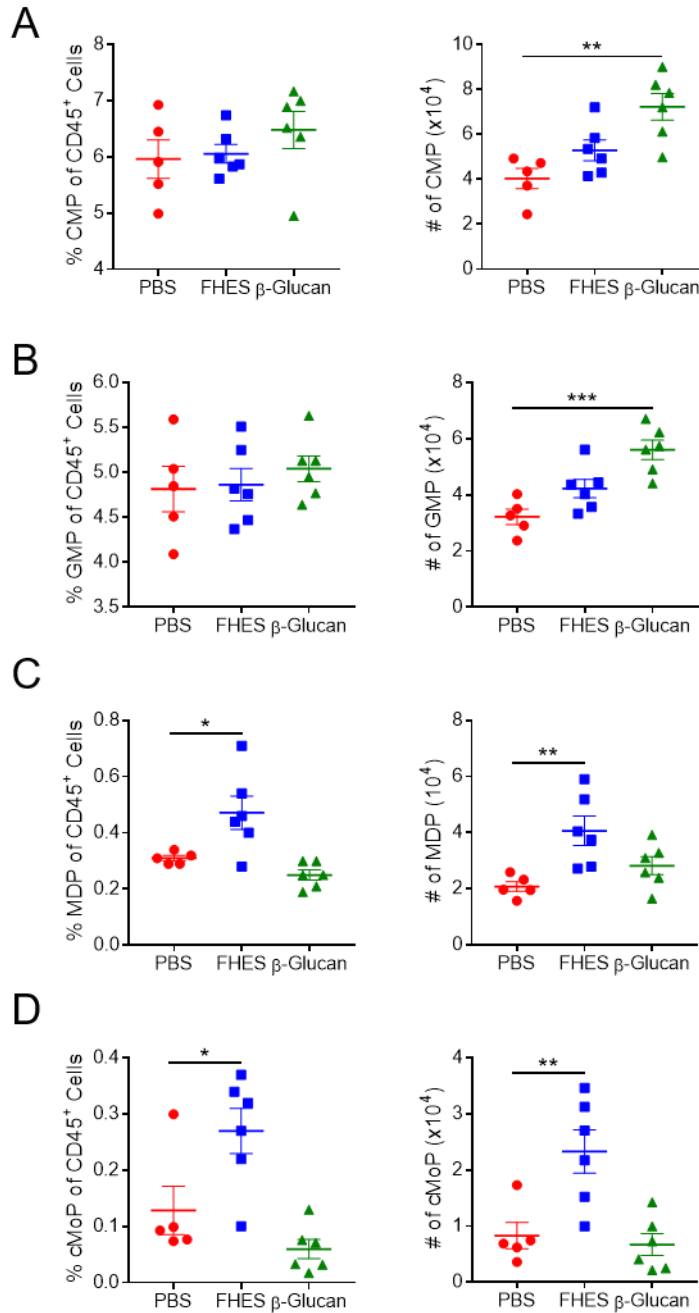
Figure 3.30 Treatment of mice with FHES s.c. significantly increases the number of MDP and cMoP in the BM. C57Bl/6 mice were treated s.c. with FHES (100 μg/mouse) β-glucan (50 μg/mouse) or PBS on days 0 and 14. On day 21, BM was collected and assessed by flow cytometry. The frequency and absolute numbers of CMP (A), GMP (B), MDP (C) and cMoP (D). Results are mean ± SEM (n=6 mice/group). Each dot represents an individual mouse. **p<0.01 vs control by one-way ANOVA with Tukey post-test.
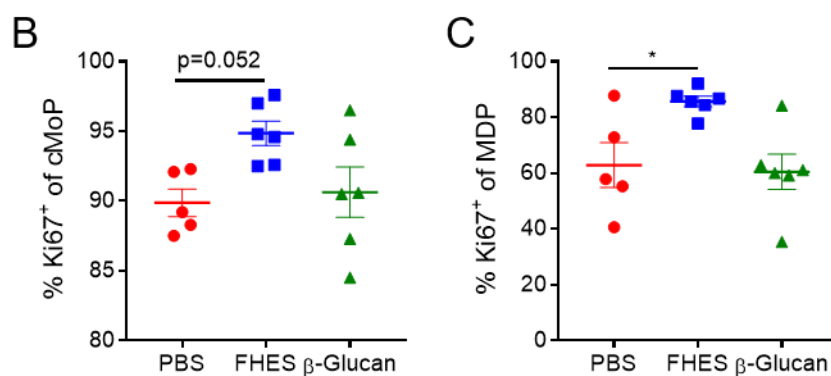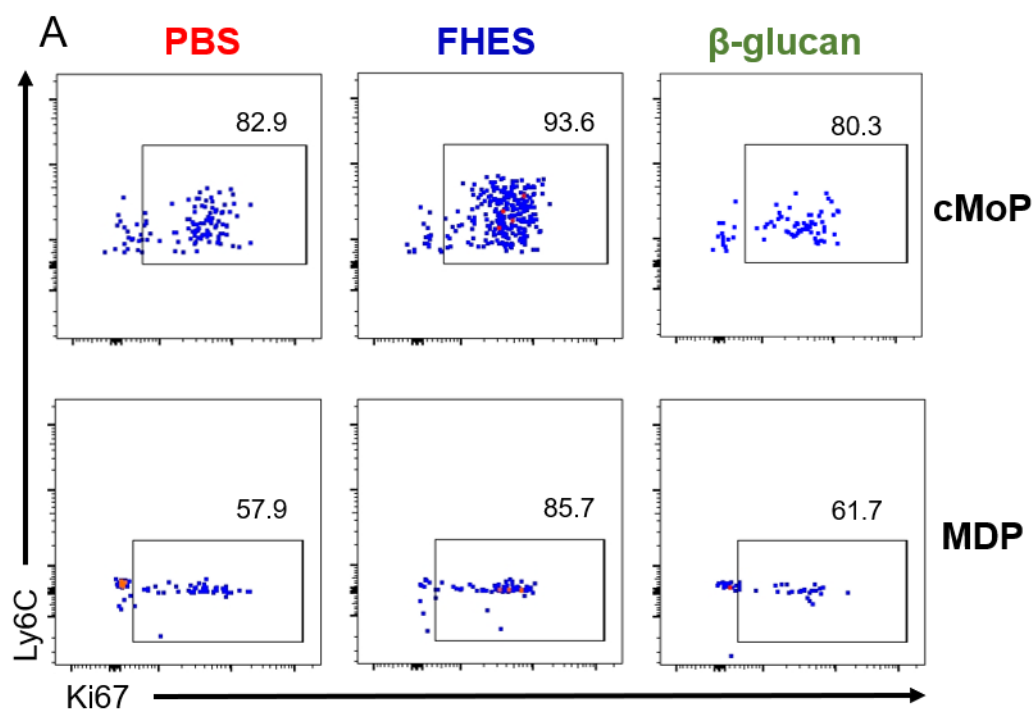
Figure 3.31 Treatment of mice with FHES s.c. specifically induces the proliferation of direct monocyte precursors MDP and cMoP. C57Bl/6 mice were treated s.c. with FHES (100 µg/mouse) β-glucan (50 µg/mouse) or PBS on days 0 and 14. On day 21, BM was collected and assessed by flow cytometry for intranuclear Ki67 expression. Representative FACS plots for Ki67 staining in MDP and cMoP (A) Percentage Ki67+ of cMoP (B) and MDP (C). Results are mean ± SEM (n=5-6 mice/group). Each dot represents an individual mouse. **p<0.01, ***p<0.001 vs control by one-way ANOVA with Tukey post-test.
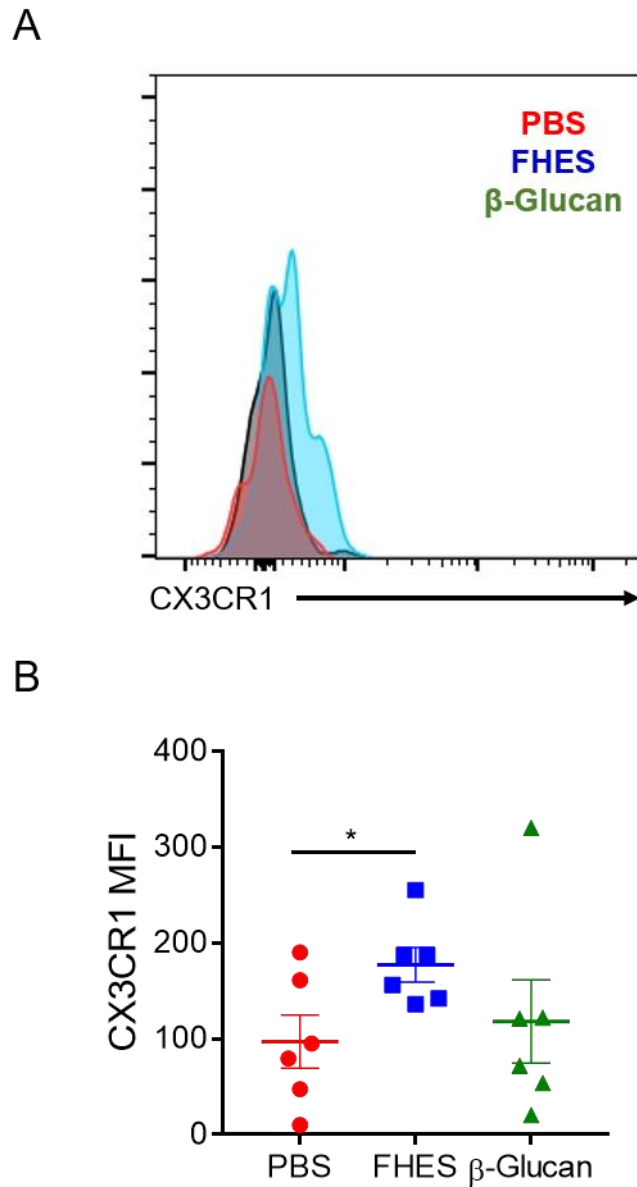
Figure 3.32 FHES treatment enhances the surface expression of Ly6C^{low} monocyte marker CX3CR1 on cMoP. C57Bl/6 mice were treated s.c. with FHES (100 μg/mouse) β-glucan (50 μg/mouse) or PBS on days 0 and 14. On day 21, BM was collected and assessed by flow cytometry for CX3CR1. Representative FACS plot for CX3CR1 expression on cMoP (A). Surface expression intensity of CX3CR1, as represented by Median Fluorescence Intensity (MFI), on cMoP (B). Results are mean ± SEM (n=5-6 mice/group). Each dot represents an individual mouse. *p<0.05 vs control by one-way ANOVA with Tukey post-test.
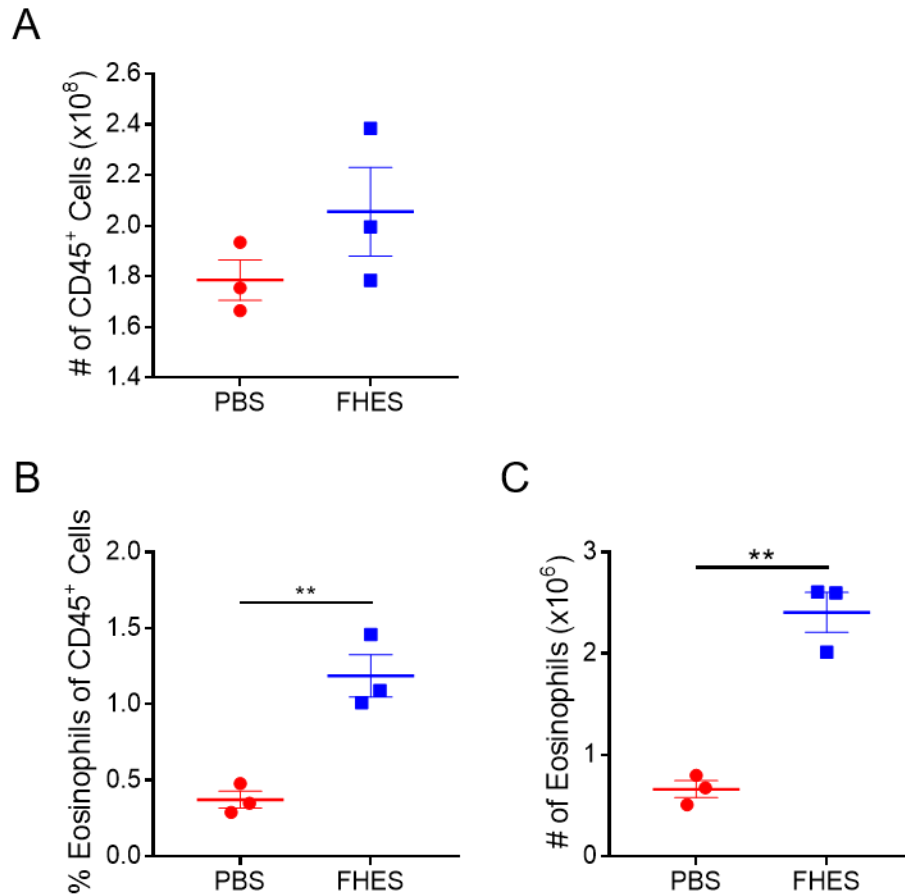
Figure 3.33 Treatment of mice with FHES s.c. induces eosinophilia in the spleen. C57BL/6 female mice were treated s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, whole BM was isolated and total immune cells and eosinophils were identified by the expression of the surface marker CD45 and Siglec-F and confirmed by their high SSc-A/low FSc-A profile, respectively. Total number of immune cells (CD45$^+$) within the peritoneal cavity (n=3/group) (A) and percentage (B) and absolute numbers of eosinophils (C). Data are mean ± SEM (n=3/group) and each symbol represents an individual mouse. **p<0.01 vs PBS by unpaired t test.
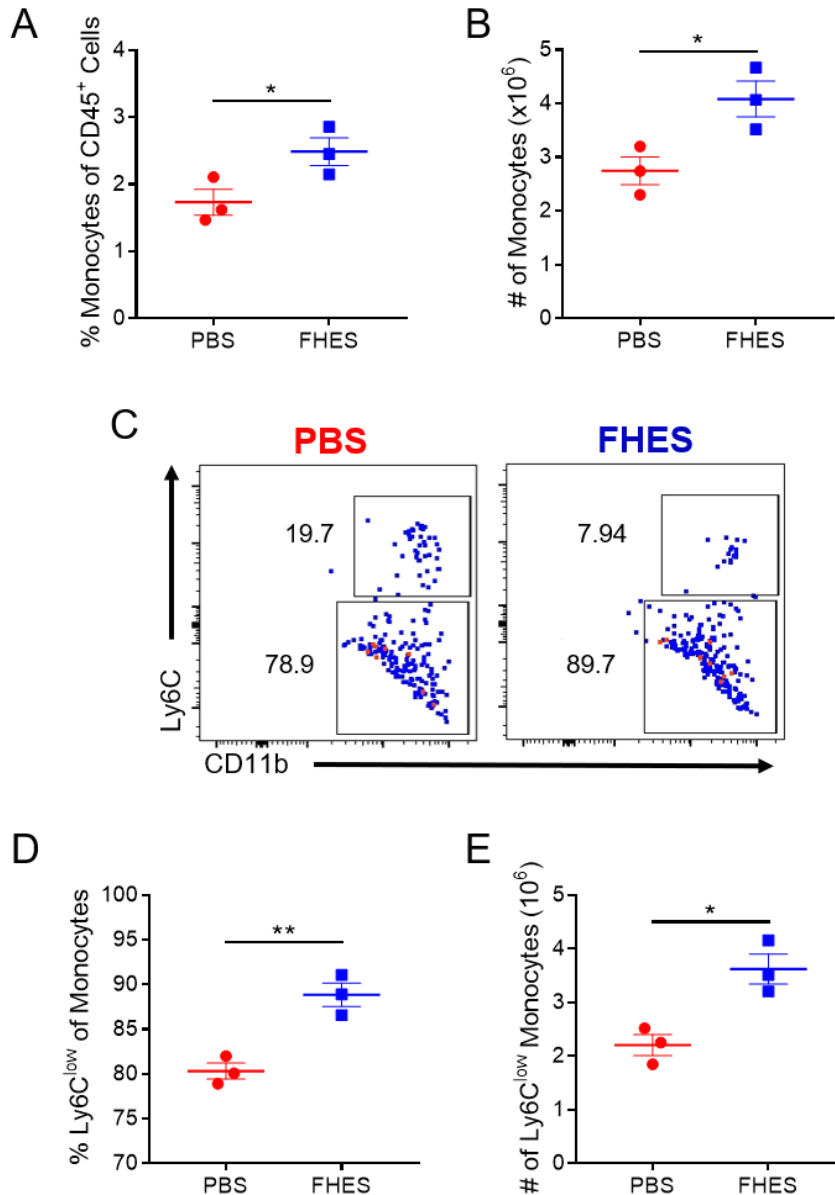
Figure 3.34 S.C. treatment with FHES induces expansion of anti-inflammatory Ly6C^{low} monocyte in the spleen. C57BL/6 female mice were treated s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, whole BM was isolated and monocytes subsets were stratified by the expression of the surface marker Ly6C. Frequency (A) and total number of all monocytes (B) within the spleen. Monocytes were stratified by Ly6C surface expression (C). Percent increase in Ly6C^{low} monocytes as a percent of the splenic monocyte population (D). Total Ly6C^{low} monocyte cell numbers (E). Data are mean ± SEM (n=3/group) and each symbol represents an individual mouse. *p<0.05, **p<0.01 vs PBS by unpaired t test.
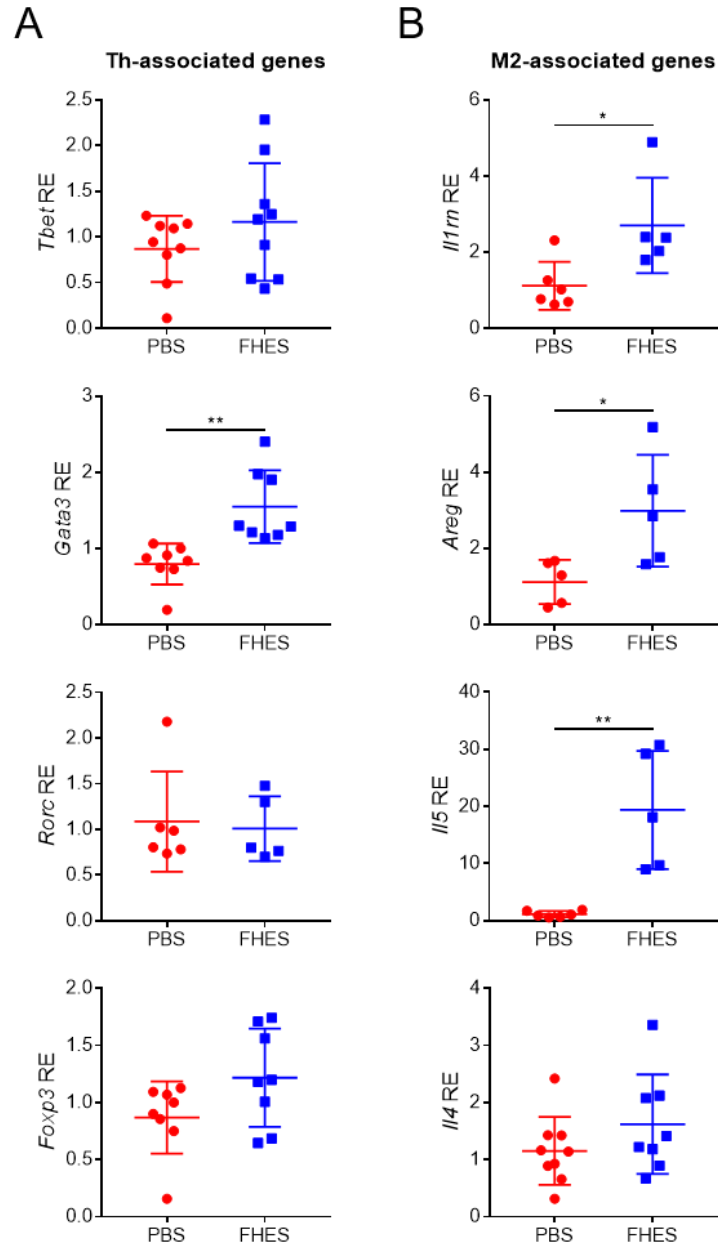
**Figure 3.35 Spleens of mice treated s.c. with FHES express significantly higher mRNA of *Gata3*, *Il1rn*, *Il5* and *Areg*, but not *Il4*.** C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. On day 21, mice were sacrificed, and spleens isolated. Total RNA was extracted and expression of T cell transcription factors *Tbet*, *Gata3*, *Rorc* and *Foxp3* (A), along with *Il1rn*, *Il4*, *Il5*, and *Areg* (B) was evaluated by RT-qPCR relative to PBS-injected mice following normalisation by the endogenous control 18s rRNA. Results are mean ± SEM (n=6-9/group) and each symbol represents an individual mouse. *$p<0.05$, **$p<0.01$ vs PBS by unpaired t test.

Chapter 4: Consequences of FHES-induced trained innate immunity in models of bacterial infection, allergy and autoimmune disease

4.1 Introduction

Parasitic infections occur worldwide, though predominantly in low- and middle-income countries. The estimated number of infected individuals ranges from 1-2 billion [194, 304]. Infection with helminths, while largely non-fatal, results in significant reduction in quality of life and occasionally debilitating adverse effects. For example, characteristic swelling of limbs in lymphatic filariasis (elephantiasis) caused by *Brugia malayi* and river blindness caused by *Onchocerca volvulus*. These adverse effects, along with other factors, including malnutrition and anaemia caused by helminth infection, result in overall reduction in quality of life and productivity, measured as disability-adjusted life years (DALYs). Soil-transmitted helminths alone are responsible for 5.2 million DALYs, the majority (62%) due to hookworm, signifying a substantial burden on endemic countries [304]. Recently, improved sanitation methods, water filtration and the advent of cheap anthelmintic drugs has allowed for the large-scale removal of many helminth infections from the populations of high-income countries. However, the reduction in helminth burden has coincided with a commensurate increase in the incidence of autoimmune and allergic diseases.

This phenomenon was initially described by David Strachan in 1989 to underscore how children raised in rural areas, with larger families, were found to be less susceptible to the development of allergies later in a life, a phenomenon now known as the 'hygiene hypothesis' [247]. While initially this hypothesis was restricted to allergies, epidemiological evidence over the years led to the inclusion of autoimmune diseases. Autoimmunity is caused by immune dysregulation, where immune responses to auto-antigen immunosuppression or detection. However, in 2007, Correale and Farez demonstrated that MS patients who developed concurrent helminth infections exhibited no MS disease exacerbations across 5 years [254]. Evidence from animal models supports this; helminth-induced type-2 and regulatory immune responses have been shown to inhibit auto-specific pathogenic type-1 and type-17 responses that mediate many autoimmune diseases. Our lab has shown that infection of mice with *F. hepatica* significantly delays induction and reduces severity of EAE through increased Treg and anti-inflammatory innate immune cell activation, resulting in reduced infiltration of pathogenic Th1 and Th17 into the CNS [256]. In other animal models

of autoimmune disease this connection has been confirmed, as helminth infections were protective against collagen-induced arthritis, diabetes mellitus in non-obese diabetic mice, and colitis [330–333]. Similarly, helminth-derived products are capable of protecting against autoimmunity in animal models. Our lab has previously demonstrated that treatment of mice with FHES inhibits induction of EAE in an IL-5- and IL-33-dependent manner [260]. Protection mediated by FHES-induced anti-inflammatory responses was found to be independent of Th2 and Treg cells, but dependent on eosinophilia and anti-inflammatory innate immune cell activation. Immune modulation using ES from other parasitic worms, including hookworms and filarial nematodes, has also been proven effective in dampening auto-inflammation by suppressing pathogenic inflammatory cells and the induction of regulatory T cells in animal models of colitis and systemic lupus erythematosus [334–337].

Helminth-induced immunosuppression of inflammation is also capable of attenuating immune responses to unrelated pathogens. Indeed, areas in which helminth infections are endemic exhibit higher incidence in malaria infection [338–340]. Additionally, epidemiological evidence from patients with various bacterial and viral pathogens reveals that helminth co-infection results in higher mortality, particularly in children [341, 342]. Our lab has previously demonstrated that infection of mice with *F. hepatica* results in a potent Th2 response, which suppresses the Th1 response to co-infection with *Bordetella pertussis*. Co-infection resulted in enhanced IL-4 and diminished IFN-γ secretion, thereby delaying clearance of *B. pertussis* [285]. Immune suppression by helminths during co-infection with *Mycobacterium tuberculosis* impairs detection of latent tuberculosis (TB) and increased worm burden is associated with active TB infections [342]. Notably, many studies have found that infection with a helminth, and its associated immunomodulation has adverse effects on the efficacy of BCG, the vaccine against TB [244, 343–345]. This is thought to largely be attributed to the potent anti-inflammatory immune response mediated by helminths, in particular the induction of TGF-β and the inhibition of IFN-γ.

Helminth-induced modulation of disease outcome is not restricted to ongoing infections. In humans, infection with helminths early in life has been associated with a decreased risk of development of various autoimmune diseases later in

life, including MS [255]. Furthermore, in a study conducted on Brazilian children, infection with the whipworm *Trichuris trichiura* in the first 5 years of life resulted in lower incidence in the development of allergy in later years, even in children with no active infection [346]. In a mouse model of *S. mansoni* infection, offspring born to infected mothers display altered immune responses during ovalbumin (OVA)-induced allergic airway inflammation (AAI) [347]. Interestingly, protection from AAI in offspring was dependent on helminth infection burden. During the course of *S. mansoni* infection, mice initially develop an acute Th1 response, followed by Th2 and finally a regulatory phase, characterized by Treg cell expansion. Offspring conceived when the mother was in the regulatory/late-stage of infection developed significantly less eosinophilia and associated lung inflammation. However, pups conceived during the Th2 phase of infection, which occurs before the regulatory phase, exhibited enhanced eosinophilia and airway inflammation during AAI. These studies suggest that helminth-mediated immune modulation lasts beyond the clinical course of infection, imprinting a type-2 or regulatory phenotype on the immune system.

It is possible that trained innate immunity plays a key role in the response to infection and autoimmune inflammation in later life. For example, infection of mice with the parasitic nematode, *Nippostrongylus brasiliensis*, resulted in modification of alternatively activated neutrophils which prime long-lived effector macrophages that mediate rapid expulsion of helminths following re-challenge with the parasite [111]. Furthermore, we have recently provided evidence that FHTE induces anti-inflammatory trained innate immunity, thereby reducing susceptibility to the induction of autoimmune disease [113]. Macrophages treated with FHTE had heightened production of the anti-inflammatory cytokine IL-10 but reduced production of TNF following exposure to pro-inflammatory stimuli. Furthermore, treatment of mice with FHTE induced anti-inflammatory trained immunity in macrophages *in vivo* and this suppressed activation of pathogenic Th17 cells that mediated EAE.

It is still unclear how helminths induce trained innate immunity. Additionally, while current work in the lab has highlighted an ability for FHES and FHTE to induce anti-inflammatory trained innate immunity, there is little known regarding the effect of this training regime in the context of infection and allergy. Inhibition of

M1 macrophages, Th1 and Th17 cells is beneficial in the context of T cell-mediated autoimmune diseases, including MS. However, a rapid and robust innate immune response is required during infection with bacteria and viruses. Furthermore, induction of type-2 cytokines, such as TGF-β and IL-33, has the potential to exacerbate type-2-mediated diseases, such as AAI.

The specific questions to be addressed were:

- Can pre-treatment of mice with FHES prime an anti-inflammatory phenotype and mediate protection against the Th1/Th17-mediated autoimmune disease EAE?

- Can FHES attenuate T cell responses directly or via the modulation of innate immune cells?

- Does FHES suppress MOG-specific infiltration of immune cells to the CNS during EAE?

- How persistent is the FHES-mediated attenuation of EAE?

- How are innate immune cells altered during EAE?

- Does pre-treatment with FHES alter haematopoiesis during EAE?

- Does pre-treatment with FHES inhibit the Th1/Th17-mediated clearance of bacteria during infection of mice with *B. pertussis*?

- Does pre-treatment with FHES exacerbate immune-mediated inflammation during type-2-mediated allergy model, OVA-induced AAI?

4.2 Results

4.2.1 Treatment of mice with FHES produces macrophages which inhibit T cell activation

FHES is a potent modulator of macrophage/monocyte and DC function *in vivo* and *in vitro.* Treatment of mice with FHES induces significant increases in alternatively activated M2 macrophages in the peritoneal cavity and inhibits LPS-induced secretion of pro-inflammatory cytokines [260]. Moreover, pre-treatment of mice with FHTE delays induction of EAE, and attenuates disease severity [113]. EAE is mediated by pathogenic T cell infiltration to the CNS, resulting in progressive demyelination and reduced mobility. Pathogenic autoreactive T cells require activation by macrophages and DCs through MHC-II and co-stimulatory molecule expression, and production of polarising cytokines [348, 349]. Having shown that s.c. injection of FHES induces anti-inflammatory macrophage production from the BM, an experiment was designed to investigate whether pre-treatment of mice with FHES induces differentiation of macrophages that modify T cell activation. C57BL/6 mice were injected with FHES (s.c.; 100 µg/mouse) or PBS on days -21 and -7, and on day 0 BM was isolated and incubated with CSF-1-containing L929 media. After 7 days, BMDM were harvested and co-cultured with αCD3-activated spleen/LN cells (70:30) in increasing concentrations. Spleen/LN cells secreted IFN-γ, IL-17, IL-10 and IL-4 in response to αCD3 stimulation, and this was significantly enhanced by the addition of BMDM from mice treated with PBS (Fig. 4.1). In contrast, BMDM from mice treated with FHES significantly suppressed IFN-γ, IL-17, and IL-10 secretion by αCD3-stimulated T cells (Fig. 4.1A). Interestingly, BMDM from FHES and PBS treated mice did not affect the production of IL-4 from spleen/LN cells (Fig. 4.1).

4.2.2 Pre-treatment of mice with FHES inhibits Th1 responses during infection with *B. pertussis*

IFN- γ and IL-17 are the characteristic cytokines secreted by Th1 and Th17 cells, respectively. A combined Th1 and Th17 responses is essential for clearance of bacterial infections, including TB and *B. pertussis,* and is required for effective long-term immunity from disease after vaccination [350]. Training of mice with β-

glucan has been demonstrated to enhance survival and improve clearance of TB through alterations to the HSC compartment and elevated IL-1β signalling [326]. Having demonstrated that BMDM from mice pre-treated with FHES inhibit non-specific secretion of IL-17 and IFN-γ by T cells, I next investigated the ability of mice treated with FHES to inhibit beneficial Th1/17 responses during infection with the bacteria *B. pertussis*. Mice were treated s.c. with FHES (100 µg/mouse), β-glucan (50 µg/mouse) or PBS on days -21 and -7. On day 0, mice were challenged with an aerosol of live *B. pertussis.* On day 35, spleens were isolated and spleen cells re-stimulated with sonicated *B. pertussis* (sBp; Fig. 4.2A). After 72 h, supernatants were collected and concentrations of IFN-γ, IL-17, IL-5 and IL-10 measured by ELISA. Spleen cells from mice pre-treated with FHES secreted significantly less IFN-γ in response to sBp when compared with spleens from mice treated with PBS (Fig. 4.2B). FHES pre-treatment did not modify spleen cell secretion of antigen-specific IL-17, IL-5 or IL-10. Interestingly, spleen cells isolated from mice treated with β-glucan secreted significantly less Bp-specific IFN- γ and IL-17 when compared with PBS-treated mice (Fig. 4.2B). Taken together, these data indicate that pre-treatment of mice with FHES inhibits Th1 response but does not affect Th17 or Th2 responses in mice during infection with *B. pertussis.*

4.2.3 Pre-treatment of mice with FHES does not alter clinical course of disease in mice infected with *B. pertussis*

Th1 cells are essential in clearance of *B. pertussis* from the lungs of mice*.* Having demonstrated that pre-treatment of mice with FHES reduces *B.* pertussis-specific IFN-γ production, it was next investigated whether pre-treatment with FHES could inhibit clearance of *B. pertussis* from the lungs of infected mice. Mice were treated with FHES (100 ug), B-glucan (50 ug) or PBS on days -21 and -7 and infected with live *B. pertussis* on day 0. Lungs were harvested on days 7, 21 and 35 and the number of colony-forming units (CFUs) measured. Mice treated with FHES did not significantly change the number of CFUs in the lungs post-challenge when compared with mice treated with PBS (Fig. 4.3). Interestingly, treatment of mice with β-glucan non-significantly delayed clearance of *B. pertussis* on day 35 when compared with PBS-treated mice. However, lungs of

mice pre-treated with β-glucan did have significantly more CFU counts on day 35 post-challenge when compared with lungs of FHES-treated mice (Fig. 4.3).

The immune response to *B. pertussis* is mediated by tissue-resident immune cells and mobilization and infiltration of circulating immune cells [155, 351, 352]. To determine the relative expansion or recruitment of immune cell populations, lungs were harvested and stained for flow cytometric analyses on days 7, 21 and 35. There was no significant difference in total immune cell numbers through the course of disease (Fig. 4.3A). While there were more CD45$^+$ cells in PBS-treated mice on day 35 when compared with FHES- and β-glucan-treated mice, this was not significant. Similarly, there was no difference in the total number of T cells, neutrophils, monocyte-derived macrophages, and monocytes in the lungs of mice (Fig. 4.3B). Interestingly, when compared with mice treated with PBS, mice treated with FHES had significantly elevated numbers of eosinophils in lungs on day 21 post *B. pertussis* challenge. However, there was no significant difference in the absolute number of eosinophils on days 7 and 35 post-challenge (Fig. 4.3B).

4.2.4 FHES pre-treatment of mice delays mobilization of immune cells in the circulation but does not affect immune populations in the lungs of mice infected with *B. pertussis*

To determine the contribution of circulating and tissue-infiltrating immune cells, I next investigated whether pre-treatment of mice altered the infiltration of circulating immune cells into lungs of mice infected with *B. pertussis.* To achieve this, mice were injected i.v. with a fluorescently conjugated antibody specific for CD45 (a pan-immune cell marker) to live mice 10 min before harvesting the lungs for flow cytometric analyses of populations. Circulating (CD45 i.v.^{high}), infiltrating (CD45 i.v.^{med}), and tissue-infiltrating (CD45 i.v.^{neg}) immune cells were then determined by positivity of the CD45 i.v. stain (Fig. 4.5A).

Treatment of mice with FHES and β-glucan did not alter the frequency immune cells present in the lung tissue at the time of sacrifice (Fig. 4.5B). Interestingly, on day 7, there was a significantly higher frequency of T cells and monocyte-derived macrophages actively infiltrating the lungs when compared with lungs of PBS-treated mice (Fig. 4.5C). On day 21, during infection with control (PBS)

mice, there was marked increases in innate cell populations in the interstitial space. However, mice treated with FHES had significantly lower frequency of eosinophils and monocyte-derived macrophages in the interstitial space when compared with PBS-treated mice. By day 35, the frequency of infiltrating T cells, neutrophils and monocyte-derived macrophages was comparable to PBS-treated mice. However, there were significantly more interstitial eosinophils in mice treated with FHES compared with PBS-treated mice (Fig. 4.5C). Conversely, on day 7, there was a significant reduction in the frequency of circulating T cells and neutrophils in mice treated with FHES when compared with mice treated with PBS (Fig. 4.5D). However, on day 21, mice treated with FHES had a significantly higher proportion of circulating T cells, neutrophils, eosinophils and monocyte-derived macrophages when compared with mice treated with PBS (Fig. 4.5D). By day 35, there were no significant differences in the frequency of circulating immune populations in FHES-treated mice when compared with PBS-treated mice. Interestingly, treatment of mice with β-glucan resulted in the same trends in circulating cell population ratios as seen in mice treated with FHES (Fig. 4.5B/C/D). Taken together, these data indicate that treatment of mice with FHES does not affect the ability of lung-resident immune cells to mediate clearance of *B. pertussis* infection. However, FHES treatment significantly delays mobilization of circulating immune cells and delays cell infiltration to the tissues.

4.2.5 Pre-treatment of mice with FHES enhances OVA-specific secretion of IL-4 by spleen cells

Type-2 immune responses are largely responsible for the pathology in allergic inflammation. Live infection with helminths or administration of their products induces a combined type-2 and regulatory immune response, characterized by Th2 cells, Tregs, eosinophils and anti-inflammatory monocytes/macrophages [79]. Having demonstrated that administration of FHES induces potent type-2 responses *in vivo*, including eosinophils and anti-inflammatory myeloid cells, it was next investigated whether FHES could enhance pathology in allergic airway inflammation (AAI), a type-2-mediated mouse model of lung allergy. Mice were treated s.c. with FHES on days -21 and -7. On day 0, mice were immunized i.p. with OVA peptide emulsified in alum (Fig. 4.6A). On day 7 spleen cells were isolated and re-stimulated with increasing concentrations of OVA peptide (0,

12.5, 25, 50 μg/ml) and type-2 cytokines measured by ELISA. Spleen cells from mice pre-treated with FHES produced significantly more OVA-specific IL-4 when compared with spleen cells from mice treated with PBS (Fig. 4.6B). Interestingly, FHES pre-treatment did not affect the secretion of other Th2-associated cytokines, including IL-5, IL-13 and IL-10. Moreover, FHES pre-treatment did not significantly alter OVA-induced IFN-γ or IL-17 secretion (Fig. 4.6B). However, there was a decrease in IL-17 secretion after stimulation with the highest concentration of OVA peptide used (50 μg/ml). These data demonstrate that FHES pre-treatment enhances antigen-specific Th2 cytokine secretion.

4.2.6 Mice treated with FHES expand anti-inflammatory myeloid populations and Th2 cells in the lung during AAI

OVA-induced AAI is primarily mediated by pathogenic Th2 cells, eosinophils and inflammatory Ly6C^{high} monocytes [353]. FHES treatment of mice increases eosinophils and Th2 cells systemically and may therefore exacerbate inflammation during AAI. When compared with mice treated with PBS, treatment of mice with FHES increased the absolute number of alveolar macrophages and anti-inflammatory Ly6C^{low} monocytes after OVA-immunization (Fig. 4.7). Pre-treatment of mice with FHES did not increase the absolute number of T cells or eosinophils in lungs of mice sensitized with OVA (Fig. 4.7).

Having demonstrated that mice treated with FHES have enhanced anti-inflammatory myeloid populations in the lungs after OVA-sensitization, but before airway challenge, the response to secondary challenge to the lungs was next investigated. Mice were treated s.c. with FHES (100 μg) or PBS on days -21 and -7 before sensitization with OVA peptide (20 μg) mixed in alum on day 0. One week later, mice were challenged with two doses of aerosolized OVA on days 7 and 8 and lungs harvested on day 9 (Fig. 4.8A). Mice treated with FHES had significantly fewer pro-inflammatory Ly6C^{high} monocytes and neutrophils in the lungs after airway challenge when compared with PBS-treated mice (Fig. 4.8B). Interestingly, the results show no difference in the number of alveolar macrophages or eosinophils in the lungs of FHES-treated when compared with PBS-treated mice (Fig. 4.8B).

Pre-treatment of mice with FHES did not enhance AAI-induced eosinophilia. Next it was examined whether FHES pre-treatment modified Th2 cell infiltration to the lungs. The results show that there was no change in the absolute number of CD4 T cells in the lungs during AAI in mice treated with FHES (Fig. 4.9A). To determine cytokine secretion of CD4 T cells, single cell suspensions were prepared from lungs at day 9 of AAI and surface stained for CD3 and CD4. Cells were then stimulated with PMA, ionomycin and brefeldin A and stained intracellularly for IL-4, IL-5, IL-13 and IL-10 before analysis by flow cytometry. Compared with PBS-treated mice, mice treated with FHES had significantly enhanced numbers of IL-4$^+$, IL-13$^+$, IL-5$^+$ and IL-10$^+$ CD4 T cells in the lungs (Fig. 4.9B/C). Taken together, these data suggest that treatment with FHES inhibits activation of pro-inflammatory myeloid cells, while enhancing Th2 responses during AAI, a model of type-2 inflammation.

4.2.7 Treatment of mice with FHES reduces the number of myeloid and granulocyte progenitors during AAI

During allergic inflammation, there is an increased demand for myelopoiesis and granulopoiesis. The CMP and GMP expand and differentiate in response to allergens and type-2 cytokines in the circulation, including IL-4 and IL-33 [354, 355]. Enhanced numbers of CMP and GMP allow for robust myeloid responses to allergens. Having demonstrated that FHES treatment suppressed the number of innate immune cells in the lungs of mice during AAI, it was next investigated whether these changes were also seen in the HSC compartment in the BM. Mice were treated s.c. with FHES (100 µg) or PBS on days -21 and -7 before sensitization with OVA peptide (20 µg) mixed in alum on day 0. One week later, mice were challenged with two doses of aerosolized OVA on days 7 and 8. On day 9, BM was isolated and analysed by flow cytometry. The results show that when compared to pre-treatment with PBS, pre-treatment of mice with FHES before induction of AAI reduced the frequency and absolute number of CMP (Fig. 4.10A/B) and GMP (Fig. 4.10A/C) after airway challenge. These findings suggest that pre-treatment of mice with FHES supresses OVA-induced myelopoiesis and granulopoiesis, which reduces the number of inflammatory monocytes and eosinophils, which mediate inflammation in AAI.

4.2.8 Macrophages derived from mice treated with FHES inhibit pro-inflammatory cytokine production by MOG-specific T cells

EAE, a mouse model of MS, is mediated by pathogenic, auto-antigen-specific Th1 and Th17 cells which infiltrate the CNS and activates local and infiltrating inflammatory innate immune cells to degrade the myelin sheath surrounding neuronal axons [184]. Demyelination induces a progressive ascending paralysis. Inflammatory innate immune cells, such as macrophages and neutrophils, are essential to induce Th1 and Th17 cell polarisation [349]. Additionally, while Ly6C^{high} monocytes and M1 macrophages are essential for pathogenesis in EAE, alternative activation of macrophages to M2 phenotype has been shown to suppress EAE clinical signs [356, 357]. Our lab has previously demonstrated that therapeutic administration of mice with FHES attenuates the onset of disease and delays severity of EAE [260]. Furthermore, pre-treatment of mice with FHTE, another product derived from *F. hepatica*, resulted in trained anti-inflammatory macrophages which suppressed EAE [113].

Having shown that BMDM from mice treated with FHES inhibit the secretion of IFN-γ and IL-17, two pathogenic cytokines in EAE, I next investigated the ability of these BMDM to suppress auto-specific T cell responses. MOG-specific T cells were generated by immunisation of mice with autoantigen MOG emulsified in CFA. 10 days after immunization, spleen/LN cells were re-stimulated with MOG, and cytokine production was assessed. IL-17, IFN-γ, and IL-4 were secreted by T cells restimulated with MOG and IL-17 secretion was significantly enhanced by the addition of BMDM from mice treated with PBS (Fig. 4.11). In contrast, BMDM generated from mice treated with FHES significantly suppressed secretion of MOG-specific IL-17 (Fig. 4.11). Furthermore, increasing frequency of BMDM from mice treated with PBS and FHES inhibited secretion of IFN-γ and IL-4. Interestingly, the inhibition of MOG-specific IFN-γ and IL-4 was no dependent on the source of BMDM (Fig. 4.11).

4.2.9 Pre-treatment of mice with FHES attenuates EAE

The results so far have shown that multiple routes of administration with FHES induces alternative activation of macrophage in the peripheral tissues of mice. Furthermore, s.c. administration of FHES induces changes to HSC populations,

promoting increased absolute numbers of anti-inflammatory Ly6C^{low} monocytes in the BM. Macrophages derived from mice treated s.c. with FHES secrete pro-inflammatory cytokines, express less MHC-II and CD40 on their surface, and inhibit MOG-specific T cell responses. The autoimmune disease EAE is mediated by pathogenic Th1 and Th17 cells which are directed by inflammatory monocytes, macrophages and neutrophils. Here, the EAE model was used to examine the effect of FHES pre-treatment on T cell-mediated autoimmune disease. C57BL/6 mice were injected with FHES (100 µg/mouse) or PBS on days -21 and -7, followed by induction of EAE on day 0 (Fig. 4.12A). The onset and clinical course of disease was significantly attenuated in mice treated with FHES s.c. compared with mice treated with PBS (Fig. 4.12B). By day 12 post-induction of EAE, 66.67% of mice treated with PBS had developed severe disease (clinical score ≥ 3), compared with 11.11% of mice treated with FHES (Fig. 4.12B). The onset of EAE was significantly delayed by 3 days in mice treated with FHES i.v. (Fig. 4.12B). Interestingly, unlike s.c. administration of FHES, i.v. administration did not attenuate the clinical course of EAE. Mice treated with PBS lost significant weight during the course of EAE, whereas this weight loss was delayed in mice treated s.c. and i.v. with FHES (Fig. 4.12C). Finally, no effect was observed in the clinical course of EAE when FHES was administered by i.p. injection (Fig. 4.12B/C).

4.2.10 Treatment of mice with FHES reduces T cell responses in peripheral lymphoid organs following induction of EAE

In EAE, APCs are activated by Mtb in CFA and present myelin peptide to T cells in lymph nodes, which expand before trafficking to the CNS. Having shown that FHES induces anti-inflammatory macrophages and monocytes, which suppress T cell activation *in vitro*, an experiment was designed to examine the ability of FHES pre-treatment to inhibit T cell activation *in vivo* using the EAE model. The effect of FHES treatment on effector T cell function was first examined in the spleen and LNs of mice with EAE.

C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7, with EAE induced on day 0 (Fig. 4.13A). On day 3, LNs were isolated and stained for CD45, CD3, CD4, TCRδ and IL-17 before being analysed by flow

cytometry (Fig. 4.13B). No significant difference was seen in frequency or absolute numbers of CD4 T cells (CD45$^+$CD3$^+$CD4$^+$SSc^{low}FSc^{low}) (Fig. 4.14A/B). Furthermore, there was no change in the frequency and absolute number of IL-17$^+$ CD4 T cells in LNs of FHES-treated mice when compared with LN of mice treated with PBS. Interestingly, while there was no reduction in frequency and absolute number of total γδ T cells in the LNs of mice treated with FHES, there was a non-significant (p=0.06) decrease in the frequency of IL-17$^+$ γδ T cells (10.2%) compared with LNs from mice treated with PBS (14.3%) (Fig. 4.14C/D).

Induction of pathogenic Th17 and IL-17$^+$ γδ T cells in the LN during EAE requires pro-inflammatory DCs and macrophages to traffic to the LN, where they actively promote induction of pathogenic T cell responses by secretion of pro-inflammatory cytokines, such as IL-1β and IL-23 [349, 358]. Having demonstrated that FHES did not alter IL-17-producing T cell responses in the LN, I next assessed innate immune cell activation in the LN by RT-qPCR on day 3 of EAE. Compared with LN of mice treated with PBS, FHES significantly enhanced mRNA expression of *Il1RN* and *Chil3* in the LN of mice on day 3 of EAE (Fig. 4.15). Moreover, there was a marked increase in the mRNA expression of *Il10* and *Retnla*, two factors commonly associated with M2 macrophages, in the LN of mice treated with FHES. No significant differences in the expression of mRNA for pro-inflammatory cytokines *Il1b* and *Il23a* was observed between PBS- and FHES-treated mice were found. Taken together, these data indicate that while T cell responses remain unchanged on day 3, there is a significant increase in anti-inflammatory myeloid cell activity in the LN.

On day 3 of EAE, effector T cells may be found in the circulation and peripheral lymphoid organs including the spleen. Pre-treatment of mice with FHES before EAE was associated with significantly decreased frequency of CD4 T cells on day 3 post-induction (Fig. 4.16A). Interestingly, there was no difference in the absolute numbers of CD4 T cells between groups. Furthermore, spleens of FHES-treated mice had significantly lower frequency and absolute number of IL-17-producing CD4 T cells compared with PBS-treated mice (Fig. 4.16A/B). Pathogenic IL-17$^+$ γδ T cells are induced early during EAE and are essential to produce early responses and promote disease. Pre-treatment of mice with FHES significantly reduced the frequency and absolute number of γδ T cells in the

spleens of mice 3 days after induction of EAE when compared with PBS pre-treatment (Fig. 4.16C/D). Moreover, FHES significantly reduced the number of IL-17$^+$γδ T cells in the spleen (Fig. 4.16C/D).

During the early phase of EAE, circulating pathogenic Th1 and Th17 cells can be found in the spleen. Previous work from our lab has demonstrated that FHTE can directly inhibit the induction of pathogenic IL-17-producing T cells [359]. Having demonstrated a significant decrease in the number of IL-17-secreting CD4 and γδ T cells in mice treated with FHES, I next investigated Th1/2/17 cell responses in the spleen. Spleen cells were analysed for gene expression of markers specific to Th subsets and M2 macrophages by RT-qPCR. Treatment of mice with FHES did not influence mRNA expression of Th subset markers for Th1 (*Tbet*), Th2 (*Gata3*), Th17 (*Rorc*) or Treg (*Foxp3*) cells (Fig. 4.17A). Assessment of M2 macrophage markers showed that FHES pre-treatment enhanced expression of mRNA for *Il1rn, Chil3, Retnla,* and *Arg1* (Fig 4.17B). These data indicate that FHES pre-treatment does not alter the development of Th17 cells but may alter the early phase of EAE through the production and alternative activation of anti-inflammatory macrophage populations in the circulation.

4.2.11 FHES pre-treatment expands oligopotent and myeloid-specific HSCs in EAE

Induction of EAE expands and induces the proliferation of HSC populations to promote the hyper inflammatory conditions required for pathogenesis of disease [360]. Administration of BCG and β-glucan have recently been demonstrated to imprint epigenetic and metabolic modifications in HSC subsets [118, 119, 326]. HSCs modified by these products preferentially expand and produce trained innate immune cells in a process known as central trained innate immunity. In a recent model of central trained innate immunity, LPS administration modified LT-HSC to promote myelopoiesis and inhibition of inflammation in a septic shock model [120]. Having demonstrated that FHES could train peripheral innate immune cells to be anti-inflammatory and expanded LT-HSCs, it was next investigated whether FHES could induce central trained innate immunity. Mice were injected s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7, followed by the induction of EAE on day 0. On day 3, bone marrow cells were

isolated and stained with fluorescent antibodies specific for HSCs and analysed by flow cytometry. Treatment of mice with FHES significantly expanded the LKS⁺ cell population of oligopotent progenitors in the BM on day 3 of EAE (Fig. 4.18A). Furthermore, the results showed that FHES-induced expansion of LKS cells occurred due to a significant increase in the frequency and absolute number of LT-HSC (LKS⁺CD150⁺CD48⁻) (Fig. 4.18B/C). Indeed, treatment of mice with FHES increased the total number of LT-HSC by 58%, from an average of $5.25x10^3$ cells/leg in PBS-treated mice, to $8.31x10^3$ cells/leg in FHES-treated mice.

LKS cells include all oligopotent progenitors, therefore modifications to this population may result in modifications to any cell in the hematopoietic system downstream. In previous studies on central trained innate immunity, the priming stimulus modified LKS cells to preferentially differentiate into myeloid cells upon secondary challenge[118–120, 326]. Having demonstrated that FHES treatment (primary stimulus) expands LT-HSC and myeloid populations on day 3 of EAE (secondary challenge), it was next investigated whether modifications could be traced through progenitors specific to the myeloid lineage. FHES pre-treatment resulted in a moderate expansion in the frequency of CMP (LK⁺Sca-1⁻CD34⁺CD16/32⁻), with FHES treatment increasing the frequency of CMP by 16% of all Lineage⁻CD45⁺ cells (Fig. 4.19A/B). Moreover, this resulted in significantly higher absolute number of CMP in the BM of mice treated with FHES when compared with BM of mice treated with PBS (Fig. 4.19B). Furthermore, treatment of mice with FHES markedly increased the frequency and absolute number of cMoP on day 3 of EAE (Fig. 4.19C/D). Taken together, these results suggest that FHES treatment of mice modified LT-HSC to preferentially induce myelopoiesis during active EAE.

4.2.12 FHES treatment of mice alters the transcriptional landscape of BM to preferentially induce myelopoiesis and type-2 immunity

Hematopoiesis is an ongoing process during steady-state to maintain an adequate number of circulating and resident immune cells ready to respond to infection. During inflammation, immune cells, PAMPs, cytokines, and growth factors are released into the circulation and cycle to the BM. In response, HSCs

induce rapid upregulation of transcription factors, which work synergistically to guide differentiation to the most effective response. This cascade of transcription factors can guide the differentiation of HSCs toward myelopoiesis, granulopoiesis or lymphopoiesis. Identifying the differences in transcription factor expression can aid in identifying the cellular response being mediated. As treatment of mice with FHES induces an expansion in the number of myeloid-specific HSCs during EAE, it was next investigated whether enhanced myelopoiesis was due to upregulation of myelopoiesis transcription factors. Mice were treated with FHES (100 μg/mouse) or PBS on day -21 and -7, and EAE induced on day 0. Three days later, RNA was isolated from whole BM to analyse gene expression by RT-qPCR. BM from FHES-treated mice expressed significantly more mRNA for genes responsible for myelopoiesis, including *Cebpa* (C/EBP-α), *Cebpb* (C/EBP-β), *Junb* (JUNB), *Spi1* (PU.1) and *Klf2* (KLF2) (Fig. 4.20). Furthermore, BM of mice treated with FHES did not have altered gene expression for transcription factors associated with lymphopoiesis, such as *Irf8* (IRF8) and *Socs2* (SOCS2). These data suggest that treatment of mice with FHES preferentially induces the differentiation of HSCs toward myeloid cells in response to induction of autoimmune disease.

Having demonstrated that treatment of mice with FHES prior to the induction of EAE induces myelopoiesis and expansion of myeloid cells early in EAE, the activation state of myeloid cells was investigated. The active induction of EAE requires early expansion and pro-inflammatory activation of innate immune cells, including macrophages and monocytes, in order to promote the polarisation of pathogenic T cells [349]. Furthermore, classically activated monocytes/macrophages provide pro-inflammatory cytokines and growth factors which direct hematopoiesis and maintain T cell populations throughout disease. FHES pre-treatment did not alter mRNA expression for any measured growth factors, including *Csf1* (CSF-1), *Csf2* (CSF-2), *Csf3* (CSF-3) and *Kitl* (SCF) (Fig. 4.20). However, BM from mice pre-treated with FHES had increased expression of mRNA for growth factor receptors: *Csf2ra* (GM-CSFRα), *Csf3r* (G-CSFR) and *Egr2* (EGR2) on day 3 of EAE when compared with BM from mice pre-treated with PBS (Fig. 4.20). These data indicate that treatment of mice with FHES does

not alter growth factor production during EAE but enhances responsiveness of BM cells to growth factors present in the BMECF.

Macrophage transcription factors were analysed by RT-qPCR. Expression of M2-associated marker *Retnla* and *Il1rl1* (ST2) were significantly upregulated in the BM of mice treated with FHES compared with BM of mice treated with PBS (Fig. 4.20). Expression of *Mrc1* and *Chil3* remained unchanged in FHES-treated mice when compared with PBS-treated mice. mRNA expression for type-2 cytokines, IL-1RA (*Il1rn*), IL-4 (*Il4*), IL-5 (*Il5*) and IL-13 (*Il13*) were all significantly enhanced in the BM of mice treated with FHES when compared with BM from PBS-treated mice on day 3 of EAE (Fig. 4.20).

Taken together, these results suggest the preferential development of type-2 macrophages during EAE, which act to inhibit the development of pro-inflammatory immune responses in the BM niche. To test whether the BM niche contained anti-inflammatory cytokines, the BMECF was next analysed. Briefly, after s.c. treatment of mice with FHES (100 µg/mouse) or PBS on days -21 and -7, EAE was induced on day 0 and BMECF isolated on day 3. The results revealed that mice treated with FHES had significantly higher concentrations of IL-1RA in the BMECF on day 3 of EAE when compared with BMECF from PBS-treated mice (Fig. 4.21). Indeed, five of six mice (83.3%) treated with PBS did not have any measurable IL-1RA in the BMECF. Furthermore, there was a non-significant increase in the concentration of IL-10 in the BMECF of FHES-treated mice compared with BMECF of PBS-treated mice on day 3 of EAE (Fig. 4.21). Collectively, the data suggest that FHES pre-treatment modifies the transcriptional landscape of the BM niche to respond to secondary challenge with an enhanced anti-inflammatory, myeloid bias.

4.2.13 Pre-treatment of mice with FHES suppresses T cell infiltration into the CNS during EAE

The development of symptoms in EAE is largely due to infiltration of pathogenic auto-antigen-specific T cells into the CNS, where they activate inflammatory innate immune cells, leading to the destruction of neurons. To investigate whether pre-treatment of mice with FHES could inhibit T cell infiltration, mice were injected s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7. EAE

was induced on day 0 and mice were sacrificed when control (PBS-treated) mice reached peak disease on day 12. Mice were sacrificed, circulating blood perfused, and brains isolated (Fig. 4.22A). Single cell suspensions were prepared from the brains and stained with antibodies specific for CD45, CD3 and CD4. Cells were then stimulated for 4 h with PMA, ionomycin and brefeldin A and stained intracellularly for IL-17, IFN-γ, IL-4, and TNF, and intranuclearly for proliferation marker Ki67 before being analysed by flow cytometry (Fig. 4.22B).

On day 12, 66.66% of PBS-treated mice had moderate/severe EAE (clinical score>2), while mice treated with FHES had delayed EAE onset, with 83.33% exhibiting no clinical signs (Fig. 4.23A). Mice treated with FHES had significantly reduced numbers of total CD4$^+$ T cells in the brains on day 12 when compared with PBS-treated mice (Fig. 4.23B/C). Compared with PBS-treated mice, brains of FHES-treated mice had significantly fewer IL-17-producing CD4 T cells on day 12 of EAE (Fig. 4.24A/B). Furthermore, FHES pre-treatment markedly suppressed infiltration of IFN-γ-producing CD4 T cells when compared with pre-treatment with PBS (Fig. 4.24A/B). FHES pre-treatment also significantly reduced the absolute number of brain-infiltrating TNF-secreting CD4 T cells on day 12 of EAE (Fig. 4.25A). Moreover, when compared to PBS-treatment, FHES-treatment significantly reduced the total number of proliferating CD4 T cells in the brain on day 12, as measured by Ki67 staining (Fig. 4.25B). Interestingly, FHES pre-treatment did not affect the absolute number of IL-4+ CD4 T cells in the brains of mice on day 12 of EAE (Fig. 4.25C).

Apart from pathogenic auto-antigen-specific CD4 T cell infiltration to the CNS, brain-infiltrating inflammatory innate immune cells are essential in maintenance of T cells and pathogenicity during EAE [187, 189, 190, 192, 361, 362]. CNS-infiltrating neutrophils and inflammatory Ly6C^{high} monocytes secrete inflammatory cytokines, including IL-1β and TNF, and directly contribute to neurodegeneration. Compared to PBS-treated mice, FHES-treated mice had significantly fewer absolute numbers of brain-infiltrating neutrophils and Ly6C^{high} monocytes on day 12 (Fig. 4.26A/B). Importantly, there was a marked decrease in the absolute number of eosinophils in the brains of mice treated with FHES compared with PBS-treated mice (Fig. 4.26C). Taken together, these findings

suggest that treatment of mice with FHES suppresses the ability of pathogenic T cells, neutrophils and inflammatory monocytes to infiltrate the CNS in EAE.

4.2.14 Pre-treatment of mice with FHES does not attenuate passive induction of EAE

Despite expanding Th2 cells and eosinophils in AAI, the results reveal that inhibition of CNS-infiltrating pathogenic immune cells was not due to the preferential induction of Th2 cells or eosinophilia. As EAE is primarily mediated by T cell populations in the mouse, attenuation of disease could be due to a direct effect on T cells. To assess whether attenuation of EAE in mice treated with FHES was due to direct inhibition of the induction of pathogenic Th1 and Th17 cells, an experiment was designed to stimulate pathogenic MOG-specific T cells in culture. In response to re-stimulation with MOG, pathogenic T cells in the spleen and lymph nodes secrete high concentrations of pro-inflammatory cytokines IL-17A, IFN-γ and GM-CSF. Our lab has previously demonstrated that recombinant IL-4 inhibited IL-17A production by MOG-specific T cells in culture (McGuinness and Mills, Unpublished). Therefore, IL-4 was used as a positive control.

C57BL/6 mice were injected s.c. with MOG (100 µg/mouse) emulsified in CFA. After 10 days, mice were euthanized and spleen and lymph node cells were co-cultured for 72 hours in a ratio of 70% spleen cells:30% lymph node cells and stimulated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence of IL-4 (20 ng/ml) or FHES (100 µg/ml) (Fig. 4.27A). Supernatants were collected after 72 h and concentrations of IL-17A, IFN-γ, IL-6 and GM-CSF were determined by ELISA. IL-4 significantly suppressed MOG-specific secretion of IL-17A and enhanced production of IL-6 (Fig. 4.27B). Administration of FHES did not suppress pro-inflammatory cytokine secretion by MOG-specific T cells.

Having demonstrated that FHES does not impact MOG-specific secretion of pathogenic cytokines *in* vitro, it was next decided to investigate the effect of pathogenic T cells in mice. Passive induction of EAE involves the transfer of MOG-specific lymphocytes from spleens and lymph nodes of MOG-immunized donor mice into naïve recipient mice. Mice were treated with FHES (100 µg/mouse) or PBS on days -21 and -7. On day 0, mice were then immunized s.c.

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with MOG emulsified in CFA. Ten days later, mice were sacrificed, and spleen and lymph node cells were co-cultured (70% spleen cell:30% lymph node) and stimulated with MOG (100 μg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml). After 72 hours, cells were harvested, counted and injected i.p. into naïve recipient mice (Fig. 4.28A). FHES pre-treatment did not affect the clinical score of EAE when compared with pre-treatment with PBS (Fig. 4.28B). All recipient mice developed disease irrespective of the source of donor T cells. As disease could still be transferred via lymphocytes alone, these data clearly indicate that treatment of mice with FHES does not attenuate disease severity in EAE through direct inhibition of T cell functions.

4.2.15 Attenuation of EAE by FHES can be transferred to naïve mice through BM transplantation

FHES-mediated attenuation of EAE is not facilitated by direct suppression of pathogenic autoreactive T cells. The results have shown that treatment of mice with FHES induced modifications to the HSC compartment, resulting in expanded HSCs and increased myelopoiesis in response to EAE induction. FHES pre-treatment expand the LT-HSC, a uniquely long-lived HSC which is capable of reconstituting the entire hematopoietic system when transplanted into radio- or chemo-therapy-induced immune cell depletion [363]. Modifications to the HSC compartment can produce trained innate immune cells which respond to infection with enhanced bacterial clearing [118]. In order to test whether the attenuation of EAE by treatment with FHES resulted from modifications to the HSC compartment, an experiment was designed to reconstitute the hematopoietic with HSCs from FHES-treated mice. C57BL/6 mice were injected s.c. with FHES (100 μg/mouse) or PBS on days -21 and -7, followed by isolation of whole BM on day 0. Separately, on day -1, naïve mice were sub-lethally irradiated (6 Gy) to deplete resident and circulating mature immune cells and HSCs. On day 0, whole BM from FHES- or PBS-pre-treated mice were counted and injected i.v. (7.5×10^6 cells) into the irradiated mice, which were then left to rest for 2 weeks to allow for immune cell reconstitution. EAE was induced on day 14 and mice were monitored daily for clinical signs and weight loss (Fig. 4.29A). Transplantation of whole BM from FHES-treated donor mice significantly delayed the onset and reduced the severity of EAE in recipient mice when compared with mice which received BM

from PBS-treated mice (Fig. 4.29B). These data show that FHES-mediated modifications to HSC attenuates disease severity in EAE, which can be transferred through BM transplantation.

4.3 Discussion

Immune evasion methods used by helminth parasites have evolved to suppress pro-inflammatory immune responses, allowing for prolonged survival and the development of chronic infections. Simultaneously, this suppression of type-1 and type-3 immune response is associated with attenuation of autoimmune diseases. This has provided the foundation for expansion of the 'hygiene hypothesis,' where it has been proposed that exposure of children to helminths early in life induces type-2/regulatory immune responses which indirectly suppress harmful auto-reactive immune responses mediated by Th1 or Th17 cells [327]. This has led to an increase in research and clinical trials attempting to use helminth infection or administration of their product as a therapeutic intervention for autoimmune diseases [336]. However, Th1 and Th17 cell responses also play an essential role in the successful clearance of bacterial, viral and fungal infections.

In chapter 3, it was demonstrated that direct stimulation of BMDC and BMDM with FHES inhibited the secretion of pro-inflammatory cytokines, including IL-1β, IL-12p40 and TNF. Furthermore, BMDM generated from mice treated with FHES produced higher concentrations of pro-inflammatory cytokines and lower concentrations of pro-inflammatory cytokines after PRR stimulation. Pro-inflammatory cytokines, such as IL-1β are essential for effective immune responses to and clearance of pathogens such as *B. pertussis*. Furthermore, effective clearance of *B. pertussis* from the respiratory tract and establishment of long-term immune memory requires robust induction of Th1 and Th17 responses [350, 364]. Previous work from our lab has shown that mice infected with *F. hepatica* had a suppressed Th1 response against *B. pertussis*, resulting in delayed clearance of the bacteria from the lungs [285]. Consistent with this, the results from the current study demonstrate that macrophages generated from FHES-treated mice significantly IFN- γ and IL-17 production by αCD3-activated T cells. Furthermore, antigen-specific IFN-γ secretion was similarly decreased after stimulation of splenic/LN T cells with *B. pertussis* antigen. Interestingly, unlike the study by Brady et al., this study found no commensurate increase in Th2 responses, as measured by IL-4 and IL-5 [285].

Treatment of mice with FHES did not alter the clearance of *B. pertussis* from the lungs of infected mice. Furthermore, flow cytometry data revealed that FHES did not affect the absolute number of immune cells in the lungs on days 7, 21 and 35 post-infection. Having demonstrated the suppression of *B. pertussis*-specific secretion of IFN-γ from peripheral T cells, it was surprising that CFUs remained unchanged. A potential explanation for this may be that pre-treatment of mice with FHES did not affect *B. pertussis*-specific IL-17A production by spleen and lymph node cells isolated from mice infected with *B. pertussis*. Recent research in our lab has highlighted the essential role for IL-17A in clearance of infection with *B. pertussis* [164].

Alveolar macrophages, the resident macrophage of the lungs, is a long-lived, embryonically-derived macrophage population that preferentially undergoes self-renewal [365]. Alveolar macrophages are capable of being trained in a pro-inflammatory context. Recently, it was demonstrated that intranasal vaccination of mice with an adenoviral vaccine vector, primed alveolar macrophages to maintain a high glycolytic capacity through CD8 T cell production of IFN-γ [366]. These pro-inflammatory trained alveolar macrophages exhibited an enhanced ability to activate and produce neutrophil-attracting chemokines upon subsequent infection with *S. pneumoniae* or *E. coli*. The present study found that the frequency of circulating T cells and neutrophils were significantly reduced in mice treated with FHES and β-glucan early in disease, suggesting that s.c. administration of FHES resulted in systemic modifications to the immune response which were readily compensated for by tissue-resident immune cells. It is possible that intranasal administration of FHES could have altered disease further.

Of particular interest in this study, was the observation that treatment of mice with FHES or β-glucan were unable to alter clearance of *B. pertussis* from the lungs. Pre-treatment of mice with β-glucan has been shown to provide protection against multiple infectious bacteria, including *M. tuberculosis* and *S. aureus* [103, 104, 326]. A potential explanation for this difference may be the route of administration. In previous studies, the protective effects of pre-treatment with β-glucan were induced following i.p. and i.v. injection. As demonstrated in chapter 3, the route of administration significantly alters the training effect in the

periphery. However, in a similar experiment, Boehm et al. inoculated mice intranasally with curdlan, the same β-glucan used in these experiments, and found that at day-3 post-infection, mice had higher CFUs in the lungs [367]. Further studies will need to assess the ability of β-glucan and FHES to induce modifications of local inflammatory responses in the lungs and nasal tissue.

There is growing epidemiological evidence to support the hygiene hypothesis in the context of allergies and asthma [346]. However, there are several conflicting studies indicating that helminth infections can decrease, increase or have no effect on the prevalence of allergic reactivity [368]. In the present study, treatment of mice with FHES resulted in enhanced OVA-specific production of IL-4, however IL-5, IL-10 and IL-13 secretion remained unchanged when compared with spleen cells from control mice. Flow cytometric analyses revealed that IL-4-secreting CD4 T cells were only marginally increased in the lungs of mice treated with FHES. Indeed, the largest differences in cytokine-secreting CD4 T cell populations in the lung were in IL-5$^+$ and IL-13$^+$ CD4 T cells. Importantly, the spleen cell responses were based on antigen-specific stimulation, whereas the flow cytometric analysis of cytokine secretion was based on stimulation with PMA. It is therefore likely that antigen-specific secretion of IL-5, IL-10 and IL-13 in mice treated with FHES is not affected. Notably, regardless of pre-treatment, there was no change in the absolute number of total CD4 T cells in the lungs.

Treatment of mice with FHES did alter the innate immune cell populations in the lungs before and after OVA airway challenge. Most notably, mice treated with FHES had significantly elevated numbers of alveolar macrophages and anti-inflammatory monocytes in the lungs. Furthermore, post-airway challenge, lungs of mice treated with FHES had significantly fewer inflammatory monocytes and neutrophils. This correlates well with a reduction in myeloid/granulocyte precursors, CMP and GMP, in the BM. Interestingly, despite significantly higher numbers of IL-5-secreting T cells, mice treated with FHES did not have significantly altered numbers of eosinophils pre- or post-airway challenge. Eosinophils have a well-established role in promoting pathogenic inflammation in the lungs during AAI [369]. Taken together, these data indicate that treatment of mice with FHES modifies the immune response of circulating and central monocytes/macrophages during secondary challenge, not eosinophils.

Furthermore, recent research has highlighted the role of recruited inflammatory monocytes in mediating inflammation in experimental AAI [353]. This suggests that treatment of mice with FHES maintained circulating anti-inflammatory monocyte and macrophage populations throughout the course of disease, however these may have contributed to increased Th2 cell cytokine production during non-specific activation. Assessment of cell populations found in the lung suggest that inflammation was not exacerbated by treatment with FHES, though the extent of fibrosis was not examined. It is possible that the decrease in inflammatory monocytes and neutrophils may have reduced fibrosis in the lungs. In future studies FHES should be administered intranasally or intratracheally to understand how FHES modifies lung-resident innate and adaptive immune cells in the context of type-2 inflammation in the airways.

Epidemiological data has proved strong evidence for a correlation between the lower prevalence of autoimmune disease in countries with higher incidence of endemic helminth infections [336]. Infection with live parasites has been demonstrated to ameliorate MS symptoms, including decreased development of new lesions in the CNS, for a period of up to 5 years [254]. However, studies involving live helminth therapy have seen mixed results [370]. Furthermore, health complications arising from live helminth infection and ethical issues has limited the use of live helminth infection as a viable treatment for autoimmune disorders. Helminth-derived products are an attractive target to modulate the immune system, and the study of their mechanisms may provide key insights into future novel therapeutics. Previous work from our lab has demonstrated that pre-treatment of mice with FHTE significantly delays onset and reduces severity of EAE [113]. The present study has demonstrated that pre-treatment of mice with FHES can delay onset and reduce severity of EAE. Therapeutic i.p. administration of FHES has been demonstrated to attenuate EAE via IL-33 and IL-5-mediated recruitment of eosinophils [260]. This was achieved through a series of injections of FHES, which were designed to mimic helminth infection and continuously promote type-2 immune responses locally, which would lead to inhibition of immune responses required to mediate EAE.

In a further study, Quinn et al. demonstrated that FHTE directly inhibits the activation of IL-17-producing γδ T cells, which have been shown to be essential

in initiating EAE [349, 359]. The present study found that pre-treatment of mice with FHES did not alter the frequency or absolute number of IL-17-producing CD4 T cells or γδ T cells in the lymph nodes. However, analysis of the lymph nodes by RT-qPCR did reveal an increase in mRNA expression of genes encoding for IL-1RA, YM1 and IL-10, with no change to mRNA expression for pro-inflammatory cytokines IL-1β and IL-23. This is likely due to the increase in anti-inflammatory effector innate immune cells in the periphery which dampen the immune activation. However, FHES treatment did reduce IL-17 producing CD4 and γδ T cells, though this was not correlated with a decrease in mRNA expression of *Rorc*, the key lineage determinant in Th17 cells. The decrease in frequency and number of IL-17-producing T cells may be due to the enhanced expression of mRNA encoding for IL-1RA and YM1 in the tissue. These are specific markers for M2 activation and could act to inhibit innate IL-1β signalling and pathogenic Th cell trafficking during the early stage of disease in secondary lymphoid tissues. Furthermore, Arginase-1 is one of two enzymes which process L-arginine for use during metabolism and induction of T cell responses [371]. Break down of L-arginine may impede T cell metabolism, thereby reducing effective IL-17 production and secretion. Indeed, in mouse models of cancer, arginase-induced depletion of L-arginine inhibits effective anti-tumour T cell responses, while recruiting further anti-inflammatory myeloid cells to the tumour microenvironment [371, 372]. Recently, arginase-1 expression in anti-inflammatory macrophages was found to be essential for spermidine-mediated attenuation of EAE [373]. Spermidine was shown to induce potent arginase-1 expression in macrophages which promoted alternative activation and inhibition of pathogenic T cells. It is likely that pre-treatment of mice with FHES did not directly suppress induction of Th1 and Th17 cells, but rather induced the production of anti-inflammatory monocyte and macrophage populations, secrete IL-1RA and arginase-1 in response to the induction of EAE, which suppressed effector T cell function in the periphery and migration of T cells into the CNS.

The present study revealed that pre-treatment of mice with FHES induced persistent anti-inflammatory monocyte/macrophage activation after the induction of EAE. The early immune response after the induction of EAE involves mobilization and activation of IL-1-secreting neutrophils, M1 macrophages, and

Ly6C[high] monocytes, culminating in the development of potent Th1 and Th17 responses [349, 374]. Expansion of innate immune cell populations occurs locally and centrally within the BM. In previous studies on the role of central trained innate immunity, expansion of LKS^{+} cells were measured after a priming signal, but before a secondary challenge [118–120, 146]. Furthermore, central trained immunity has been demonstrated to promote a myeloid bias in progenitor populations. HSCs are expanded during inflammation in response to cytokines, growth factors, neurotransmitters and PRR signalling. The flow cytometric and RT-qPCR data from the current study demonstrated that priming mice with FHES induced an expansion of LT-HSC and preferentially induced transcription of myelopoiesis markers, resulting in expansion of CMP and cMoP during EAE. Interestingly, despite increases in myeloid-specific lineage markers and corresponding HSCs during EAE, there was no change in expression of mRNA encoding for CSF-1,2,3 or SCF. However, priming of mice with FHES did increase the expression of growth factor receptors in the BM during EAE. Furthermore, this study found a significant enrichment of mRNA expression for genes specific to type-2 immune responses, including IL-4, IL-5, IL-13 and the receptor for IL-33 (ST2). It is possible that these cytokines play a role in the anti-inflammatory response mediated due to modifications to HSCs in this context. It is unclear if these cytokines play a role in the development of FHES-induced training as stype-2 cytokine were not found significantly changed in the BMECF of mice treated with FHES. However, the receptor for IL-33 was upregulated in the BM of mice treated with FHES during EAE. ST2 is highly expressed on HSCs, including LT-HSC and CMP, and IL-33 has previously been demonstrated to play a protective role against development of EAE [260, 375]. Furthermore, IL-33 can act synergistically with IL-4 and IL-13 to enhance M2 polarisation [376]. It is possible that IL-33, or increased responsiveness to its secretion, is the driving force behind anti-inflammatory modifications in the HSC compartment. Therefore, future studies should examine if IL-4 and IL-33 can induce anti-inflammatory central training.

It is highly likely that FHES-induced modifications to HSCs work through a combinatorial mechanism. While type-2 cytokines likely play an essential role in the induction of FHES-mediated central training, it is also possible that FHES

enters the BM and modulates HSCs directly. FHES contains multiple proteins, enzymes, exosomes and peptides which may reach the BMECF or the HSC niche. In a recent study by Coakley et al., it was demonstrated that macrophages readily engulf extracellular vesicles in the ES of *H. polygyrus* [377]. Interestingly, uptake of these extracellular vesicles inhibited type-1 and type-2 activation in the macrophage, including reduction in ST2 expression. Furthermore, monocytes trained in the circulation with NOD agonists have been demonstrated to circulate the blood and return to the BM, passing on a trained phenotype to naïve monocytes in the BM [56]. Previously unpublished data from our lab has found that treatment of mice with *F. hepatica* exosomes had comparable immunomodulatory effects to FHES administration, including induction of eosinophilia and attenuation of EAE (Walsh and Mills, unpublished data). Therefore, it is possible that activation of circulating monocytes/macrophages with FHES, or exosomes, return to the BM and signal through the BMECF to induce anti-inflammatory central training.

CNS demyelination in the MOG-induced model of EAE is mediated by pathogenic MOG-specific Th1 and Th17 cells. Induction of pathogenic T cell responses requires presentation of MOG antigen and polarizing pro-inflammatory cytokine secretion by myeloid cells. EAE can be attenuated through direct inhibition of pathogenic T cell responses, or through expansion of anti-inflammatory APCs. The present study has demonstrated that FHES does not directly inhibit the production of MOG-specific IL-17A, GM-CSF or IFN-γ by spleen and lymph node cells. However, treatment of mice with FHES appeared to protect mice from development of severe EAE by suppressing the recruitment of IL-17A-, IFN- γ- and TNF-secreting CD4 T cells to the CNS. The study found that there was no change to IL-17-producing T cells in the lymph nodes early in EAE, suggesting that treatment of mice with FHES attenuates the development of EAE through the induction of anti-inflammatory innate cells, which may suppress their egress from the lymph nodes. Suppression of pathogenic T cell mobilization from the lymph nodes is a well-established treatment for MS patients. For example, fingolimod is a sphingosine-1-phosphate receptor modulator that suppresses T cell egress from the lymph node [378, 379]. Natalizumab is another successful therapeutic that inhibits T cell migration to the CNS via the BBB [380].

During development of EAE through pathogenic, CNS-infiltrating T cells require pro-inflammatory immune cells in the CNS to maintain inflammatory processes [190, 361]. BMDM generated from mice treated with FHES expressed less MHC-II and co-stimulatory markers and produce anti-inflammatory cytokines in response to pro-inflammatory stimuli, such as LPS and Mtb. Therefore, it is possible that mice pre-treated with FHES have more anti-inflammatory myeloid cells that contribute to diminished disease severity following induction of EAE. Indeed, monocytes isolated from mice infected with *T. crassiceps* can confer some protection against EAE following adoptive transfer [257]. Taken together, these findings suggest that FHES induces anti-inflammatory trained innate immunity, which inhibits T cell-induced inflammation that mediates EAE.

A significant finding of this study was the observation that modulation of BM by FHES could be transferred to recipient mice by BM transplantation, resulting in attenuation of EAE in recipient mice. To our knowledge, this is the first study to demonstrate that helminth products can train the BM microenvironment to transfer an anti-inflammatory immune phenotype to naïve mice. The application of trained innate immunity in the context of transplantation is a relatively new. To date, very few studies have characterized innate immune training mechanisms during transplantation. A study by Braza et al., found that, during heart transplantation in mice, release of DAMPS HMGB1 and vimentin induced pro-inflammatory trained immunity in local macrophages [136]. Inhibition of mTOR using a nanobiologic specific for myeloid cells in the periphery, resulted in a suppression of the trained phenotype and long-term acceptance of the allograft. While this and other studies may prove useful in the context of solid organ transplantation, there is little research regarding the ability to train, or prime, HSC before transplantation. For example, a single injection of mice with flagellin, a TLR5 and NLR4 agonist, enhanced proliferation in the HSC compartment. Transplantation of HSCs from flagellin-treated mice significantly improved recipient survival in lethally-irradiated recipient mice [145]. The present study has demonstrated that pre-treatment of mice with FHES enhances proliferation in the HSC compartment and can transfer some protection against induction of EAE through transplantation of BM to irradiated mice. These experiments do not rule out the possibility of protection being mediated by BM-resident mature innate or

adaptive immune cells. These will be addressed in the following chapter. Regardless of a singular mechanism, it is likely that protection against induction of EAE in mice treated with FHES arises through modifications in the BM, which preferentially produce anti-inflammatory myeloid cells that inhibit circulating and CNS-infiltrating pathogenic T cells.
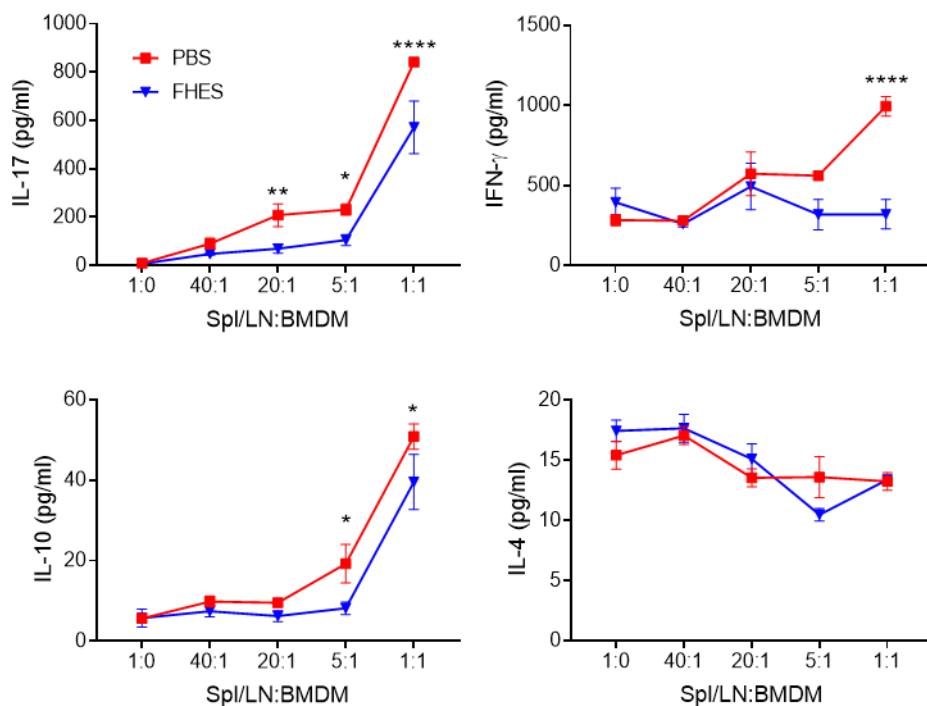
Figure 4.1 BMDM from FHES-treated mice significantly reduce αCD3-induced secretion of IL-17, IFN-γ and IL-10 by T cells. BMDM were generated from FHES-treated and untreated mice. Increasing ratios of BMDM were co-cultured with spleen and lymph node cells (70:30) from naïve mice in the presence of αCD3 (1 µg/ml). After 72 h, supernatants were collected and the concentration of IL-17A, IFN-γ and IL-4 determined by ELISA. Results are mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001, vs PBS ratio-matched PBS-treated BMDM control by two-way ANOVA with Sidak's multiple comparisons post-test.
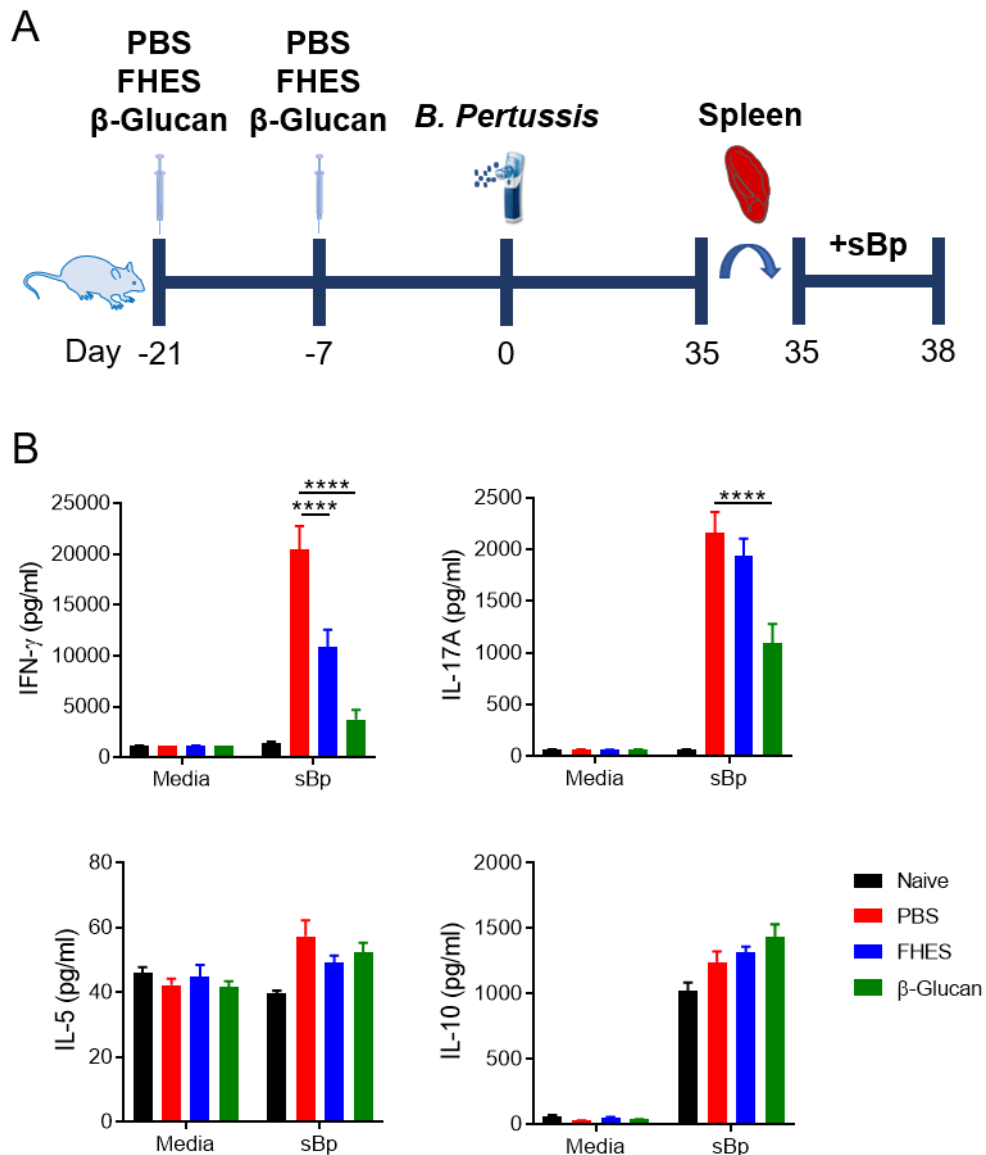
Figure 4.2 FHES pre-treatment of mice significantly reduces pertussis-specific secretion of IFN-γ by spleen cells. (A) C57BL/6 mice were injected s.c. with FHES (100 µg/mouse), β-glucan (β-G; 50 µg/mouse) or PBS on days -21 and -7. On day 0 mice were challenged by exposure to live *B. pertussis* and spleens harvested on day 35. (B) Spleen cells were then re-stimulated with sBP (5 µg/ml) or media and after 72 h IFN-γ, IL-17A, IL-5 and IL-10 concentrations in the supernatants were quantified by ELISA. Results are mean ± SEM for triplicate culture (n=2-4 mice). ****$p < 0.0001$ vs PBS pre-treated control by two-way ANOVA with Dunnett's multiple comparisons test.
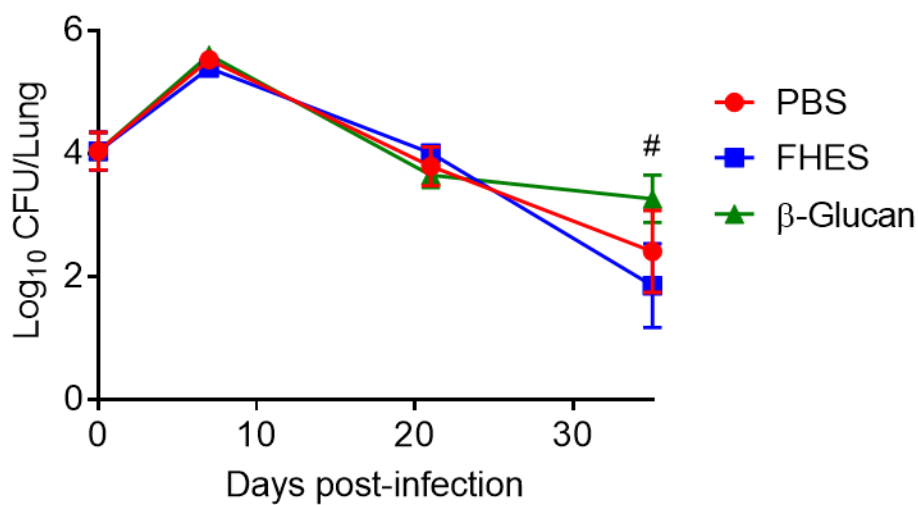
Figure 4.3 Pre-treatment of mice with FHES does not affect clearance of *B. pertussis* in the lungs. C57BL/6 mice were injected s.c. twice (days -21 and -7) with FHES (100 µg/mouse), β-glucan (50 µg/mouse) or PBS. On day 0, mice were challenged by exposure to live *B. pertussis* and lungs were taken from 4 mice/group on days 7, 21, 35 to determine bacterial colonisation. Results are mean ± SEM CFU counts (n=4). #$p < 0.05$ vs FHES-treated by two-way ANOVA with Bonferroni post test.
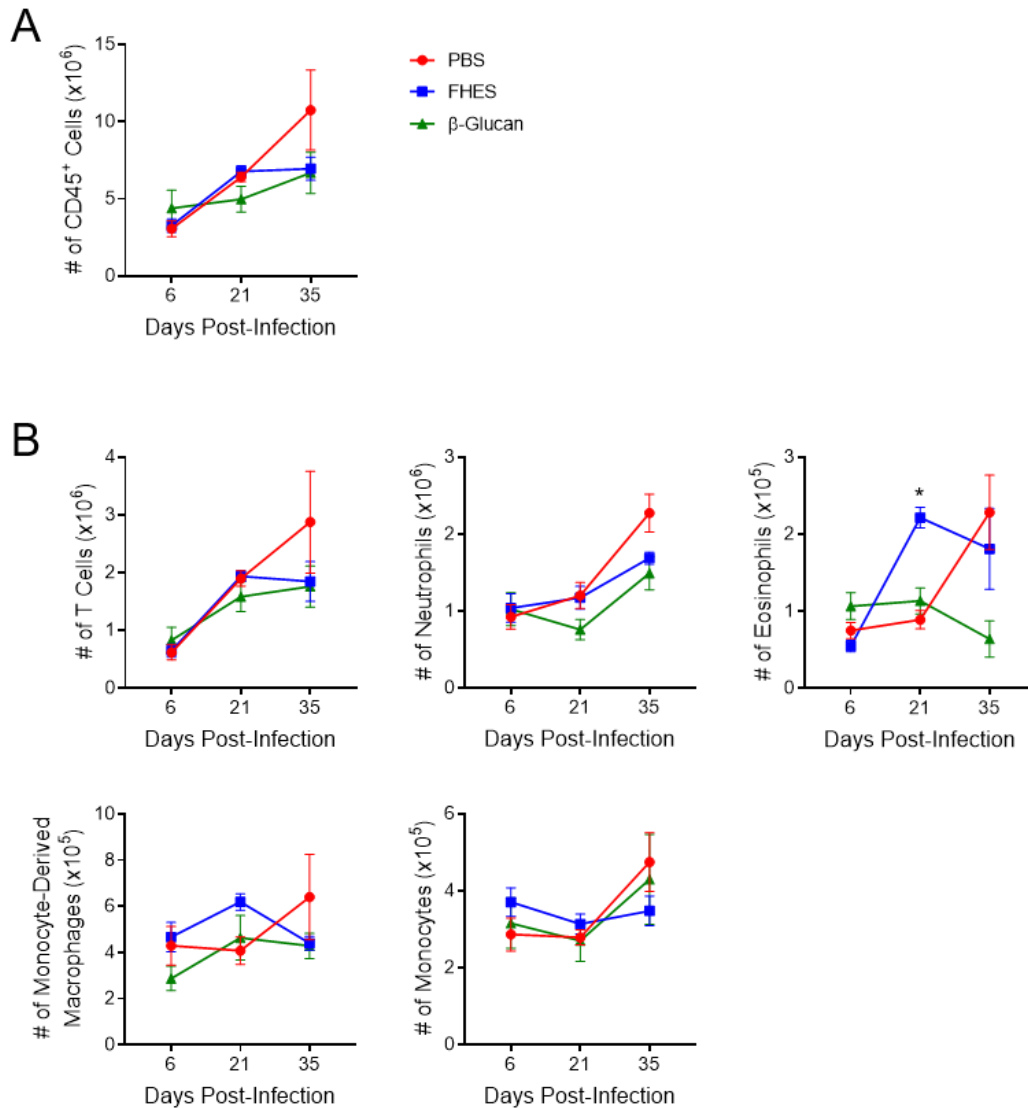
Figure 4.4 FHES pre-treatment of mice increases eosinophils on day 21 but does not alter total T cell or myeloid cells in the lungs post-infection. Mice were treated as described in figure 4.3A. On days 7, 21 and 35 lungs were isolated, and lung mononuclear cells stained with markers for CD45, CD3, CD11b, CD11c, Ly6G, Siglec-F, F4/80 and MHC-II for FACS analysis. (A) Absolute number of total CD45$^+$ immune cells in the lungs. (B) Absolute number of T cells, neutrophils, eosinophils, monocyte-derived macrophages, and monocytes in the lungs on days 7, 21 and 35. Results shown are mean ± SEM (n=3-4/group/day). *p<0.05 vs PBS-treated control by two-way ANOVA with Sidak's post hoc test.
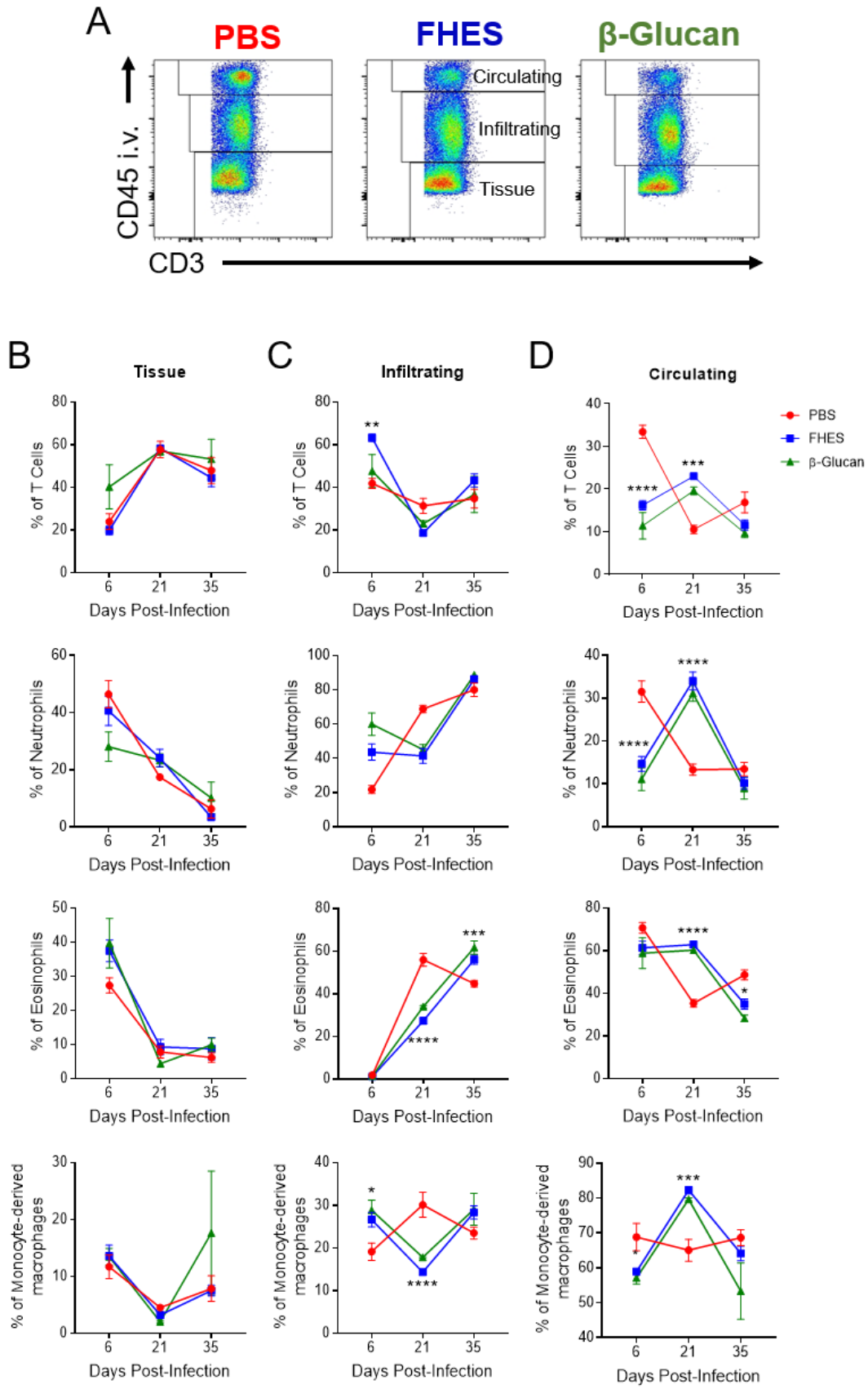
Figure 4.4 FHES pre-treatment of mice reduces circulating T cells, neutrophils and macrophages early in infection, but does not alter tissue infiltration of any immune cell population. Mice were treated as described in figure 4.3A. On days 7, 21 and 35, mice were injected i.v. with fluorescent antibody specific for CD45 (CD45 i.v.) 10 min before sacrifice. Lungs were isolated, and cells stained with markers for CD45, CD3, CD11b, CD11c, Ly6G, Siglec-F, F4/80 and MHC-II for FACS analysis. (A) FACS plots representing CD45 i.v. staining for CD3 T cells in the lungs. Absolute number of tissue (B), infiltrating (C), and circulating (D) T cells, neutrophils, eosinophils and monocyte-derived macrophages in the lungs. Data are mean ± SEM (n=3-4/group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs PBS by two-way ANOVA with Sidak's post hoc test.
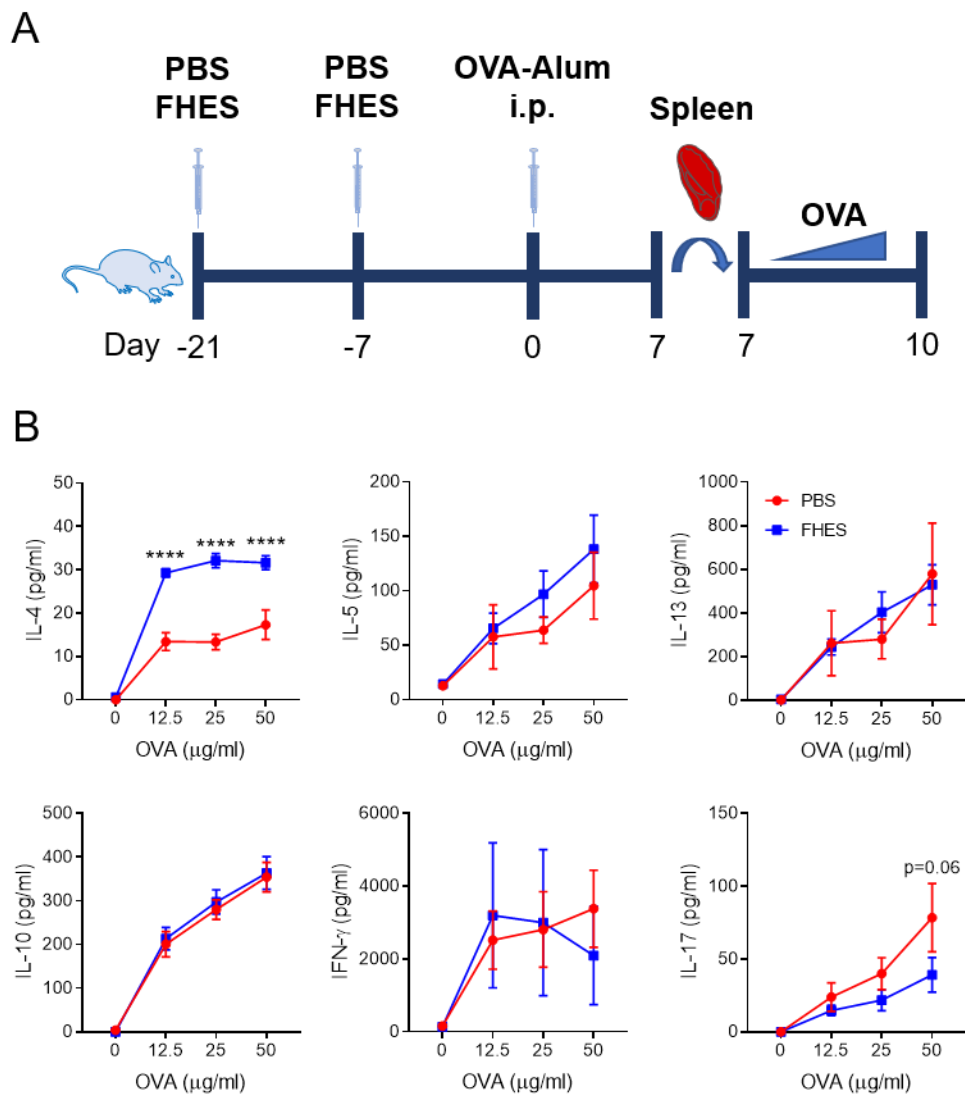
Figure 4.6 FHES pre-treatment enhances Ova-specific secretion of IL-4 and decreased secretion of IL-17 from spleen cells. (A) C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7, followed by immunisation with OVA (20 µg/mouse) mixed with alum (2 mg/mouse). Seven days later, spleens were harvested and re-stimulated with OVA peptide (0, 12.5, 25, 50 µg/ml). (B) After 72 h IL-4, IL-5, IL-13, IL-10, IFN-γ, and IL-17A concentrations in the supernatants were quantified by ELISA. Results are mean ± SEM for triplicate culture (n=6 mice). ****$p < 0.0001$ vs PBS pre-treated control by two-way ANOVA with Sidak's multiple comparisons test.
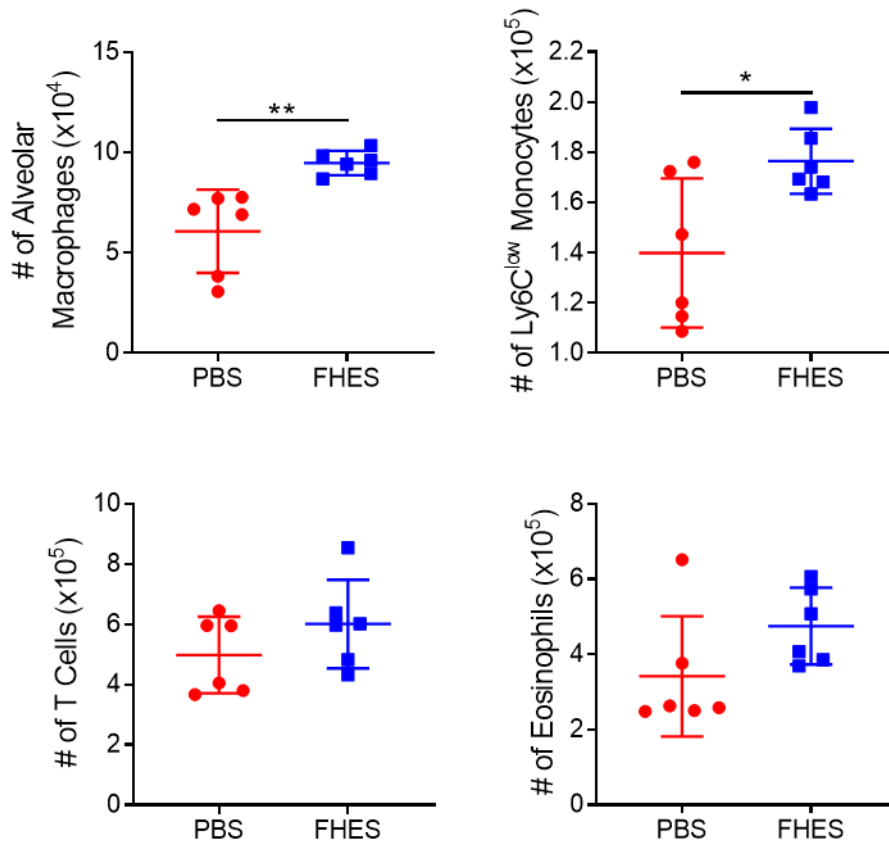
Figure 4.7 Pre-treatment of mice with FHES enhances alveolar macrophages and Ly6C^{low} monocytes in the lungs of mice pre-airway challenge in AAI. Mice were treated as described in Figure 4.6A. On day 7, lungs were isolated, and cells stained with markers for CD45, CD3, CD11b, CD11c, Ly6G, Siglec-F, F4/80 and MHC-II for FACS analysis. Absolute number of alveolar macrophages, Ly6C^{low} monocytes, T cells and neutrophils in the lungs on days 7. Results shown are mean ± SEM (n=6 mice/group) and each dot represents an individual mouse. *p<0.05, **p<0.01 vs PBS-treated control by unpaired t test.
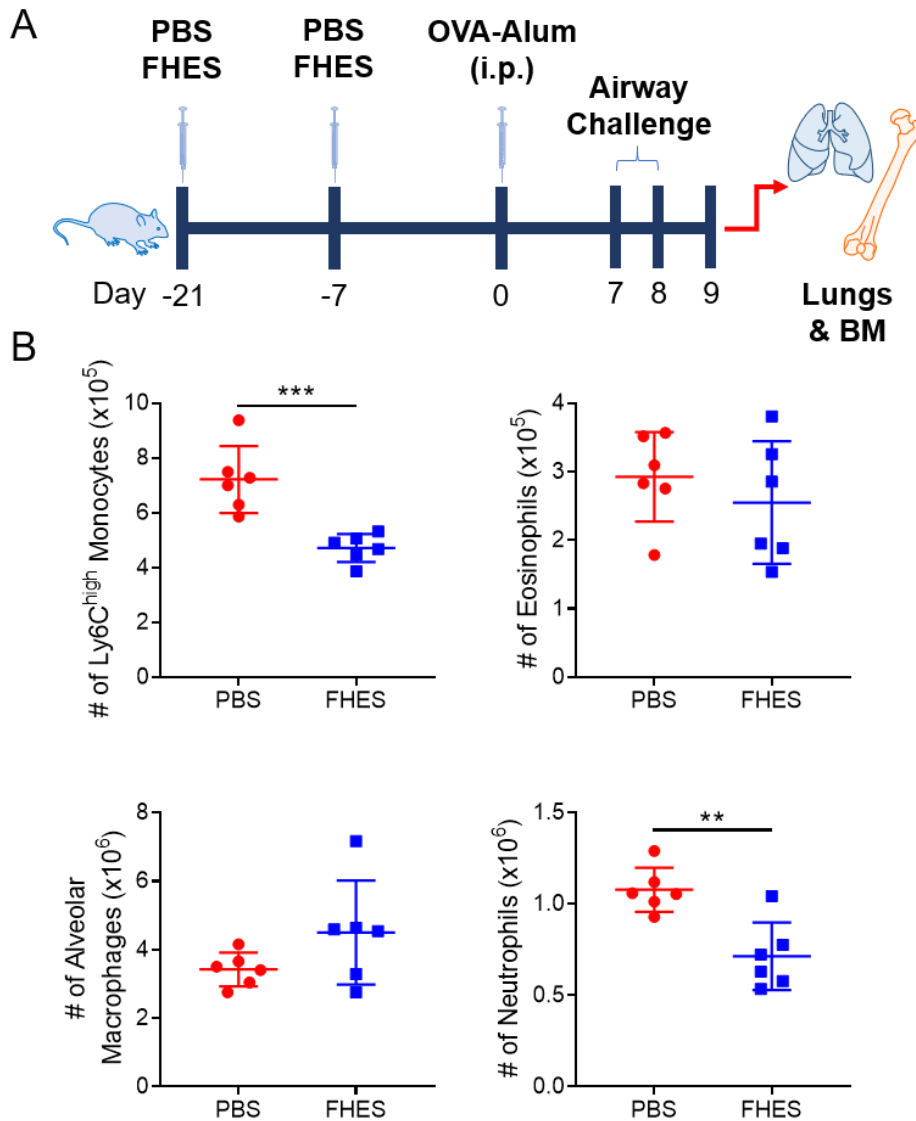
Figure 4.8 Reduced inflammatory monocytes and neutrophils in the lungs of mice pre-treated with FHES post-airway challenge during AAI. (A) C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7, followed by immunisation with OVA (20 µg/mouse) mixed with alum (2 mg/mouse). In days 7 and 8, mice were challenged with aerosolized OVA (1% v/v). On day 9, lungs were isolated, and cells stained with markers for CD45, CD3, CD11b, CD11c, Ly6G, Siglec-F, F4/80 and MHC-II for FACS analysis. Absolute number of alveolar macrophages, Ly6C^{high} monocytes, eosinophils and neutrophils in the lungs on days 7. Results shown are mean ± SEM (n=6 mice/group) and each dot represents an individual mouse. **p<0.01, ***p<0.001 vs PBS-treated control by unpaired t test.

Figure 4.9 Pre-treatment of mice with FHES increases the number of T cells secreting IL-4, IL-5, IL-13 and IL-10 in the lungs during AAI. C57BL/6 mice treated as described in Figure 4.8A. On day 9, lungs were isolated, and cells stained with markers for surface CD45, CD3, CD4 and intracellular IL-4, IL-13, IL-5, and IL-10 and analysed by flow cytometry. (A) Absolute number of CD4 T cells in the lungs on day 9. (B) Representative FACS plots of type-2 cytokine secreting CD4 T cells from the lungs (gated on CD45+CD3+CD4+ T cells). (C) Absolute number of cytokine+ CD4+ T cells in the lungs on day 9. Results shown are mean ± SEM (n=6 mice/group) and each dot represents an individual mouse. *p<0.05, ***p<0.001, ****p<0.0001 vs PBS-treated control by unpaired t test.

Figure 4.10 Pre-treatment of mice with FHES decreases the frequency and number of CMP and GMP in response to airway challenge in AAI. C57BL/6 mice were treated as described in Figure 4.8A. On day 9, BM was collected and assessed by flow cytometry. (A) Representative FACS plots for CMP (Lineage⁻ CD45⁺cKit⁺Sca-1⁻CD34⁺CD16/32⁻) and GMP (Lineage⁻CD45⁺cKit⁺Sca-1⁻ CD34⁺CD16/32⁺). Frequency and absolute number of CMP (B) and GMP (C) in the BM on day 9. Data presented as mean ± SEM (n=6/group) and each symbol represents an individual mouse. *p<0.05, vs PBS-treated control by unpaired t test.
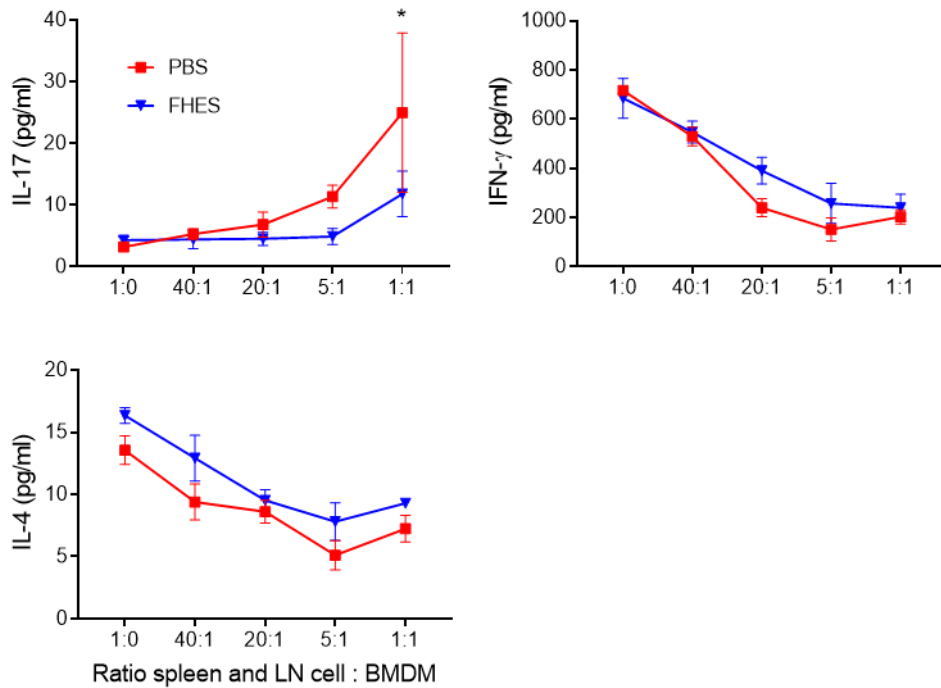
Figure 4.11 BMDM from mice treated with FHES significantly reduce MOG-specific secretion of IL-17 by T cells, with no change to IFN-γ and IL-4. C57BL/6 mice were treated as described in Figure 3.24A. BMDM were generated from FHES-treated and untreated mice. Different ratios of BMDM were co-cultured with spleen and lymph node cells (70:30) from mice injected with MOG (100 µg) emulsified in CFA 10 days earlier and stimulated with MOG (100 µg/ml). After 72 h, supernatants were collected and the concentration of IL-17A, IFN- γ and IL-4 determined by ELISA. Results are mean ± SEM. *p<0.05, vs PBS ratio-matched PBS-treated BMDM control by two-way ANOVA with Sidak's multiple comparisons post-test.
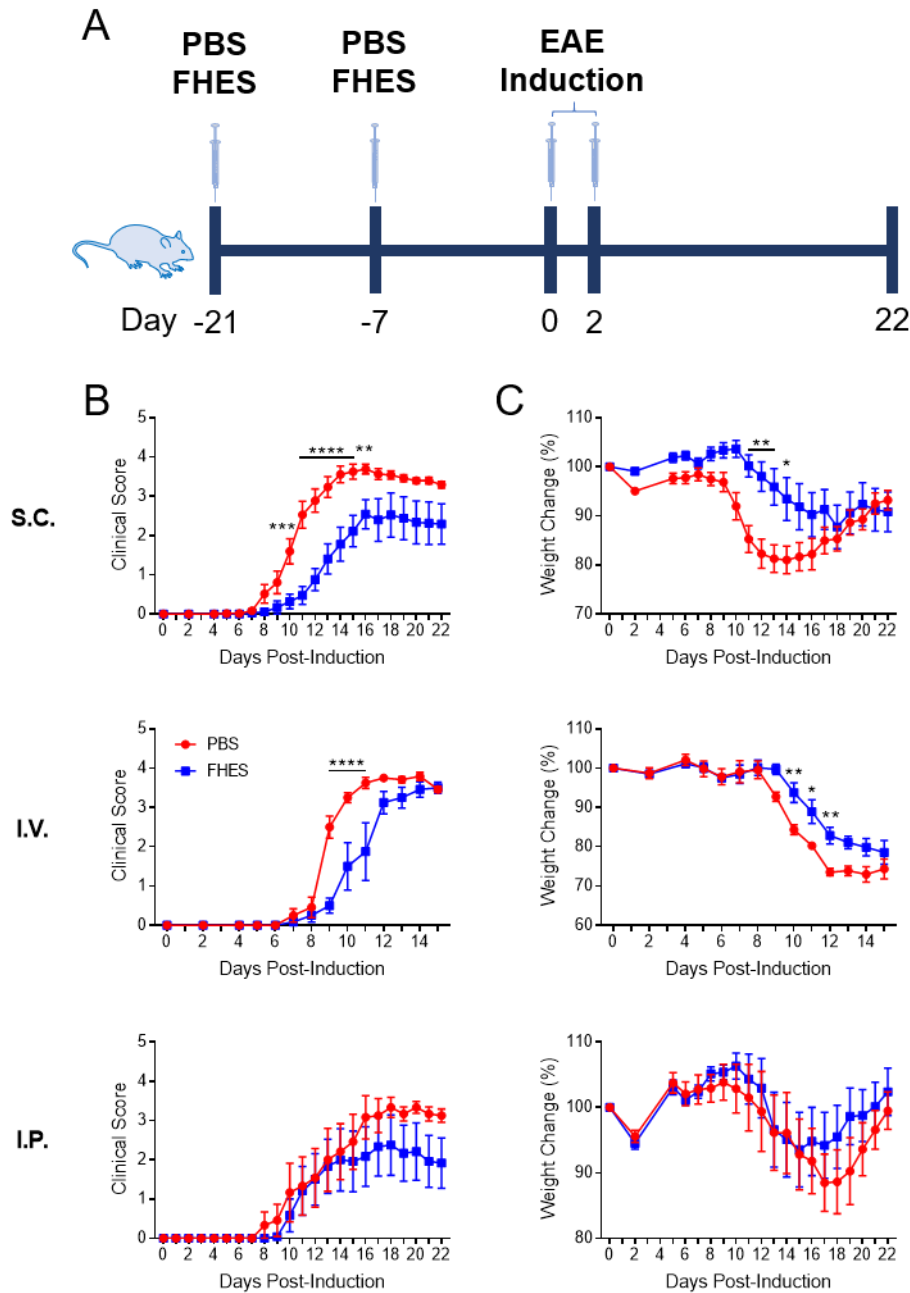
Figure 4.12 Pre-treatment of mice with FHES significantly delays onset of active EAE. (A) C57BL/6 mice were treated with FHES (100 µg/mouse) or PBS s.c., i.v. or i.p. on days -21 and -7. On day 0, EAE was induced by s.c. injection with 100 µg of MOG emulsified in CFA, followed by i.p. injection with 125 ng of PT on days 0 and 2. Mean clinical scores (B) and percent body weight changes (C) were assessed daily and mice were euthanized at peak of clinical score. Data are mean ± SEM (n=6-18/group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs PBS-treated control by two-way ANOVA with Sidak post-test.
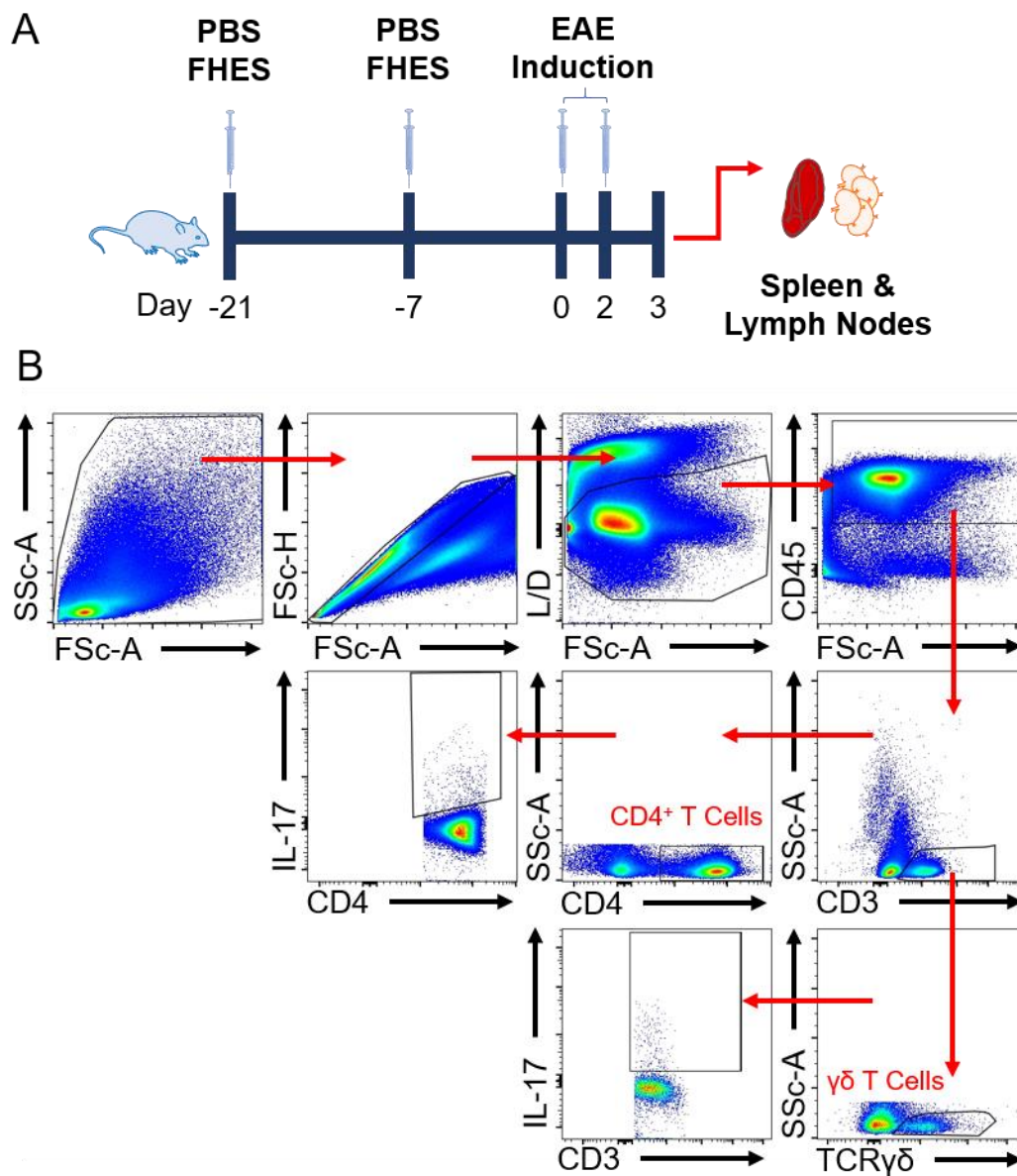
Figure 4.13 Induction of EAE and gating scheme to examine the effect of treatment with FHES on IL-17+ T cells in the spleens and lymph nodes. (A) C57BL/6 were injected s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7, and EAE induced on days 0 and 2. On day 3, mice were euthanized and spleens, lymph nodes and BM were isolated for flow cytometric analyses. (B) Gating strategy to identify IL-17-producing CD4 T cells and γδ T cells.
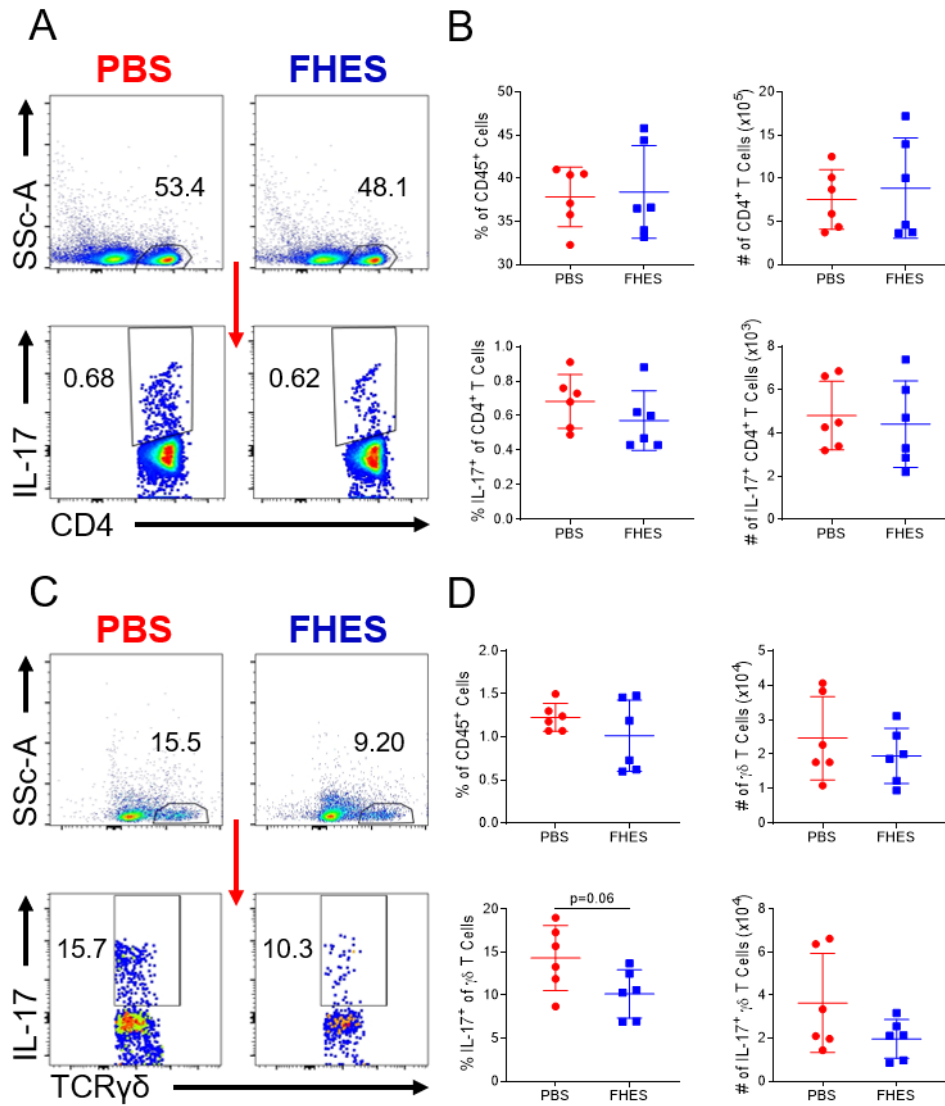
Figure 4.14 IL-17A production by CD4 and γδ T cells in lymph nodes is not reduced in FHES-treated mice following induction of EAE. C57BL/6 mice treated as described in Figure 4.13A. On day 3, lymph nodes were isolated, and cells stained with markers for surface CD45, CD3, CD4, TCRγδ and intracellular IL-17 and analysed by flow cytometry. (A) Representative FACS for IL-17+ CD4+ T cells. (B) Frequency and absolute number of total CD4 and IL-17-secreting CD4 T cells. (C) Representative FACS for IL-17+ γδ T cells. (D) Frequency and absolute number of total CD4 and IL-17-secreting γδ T cells. Results shown are mean ± SEM (n=6 mice/group) and each dot represents an individual mouse. Statistical assessment was performed by Student's t-test vs PBS-treated control.

Figure 4.15 FHES treatment enhances M2-associated mRNA expression of *Il1rn, Chil3, Il10* and *Retnla* in lymph nodes after induction of EAE. C57BL/6 mice treated as described in Figure 4.13A. On day 3, lymph nodes were isolated, and total RNA was extracted and the expression of *Ilrrn*, *Chil3*, *Il10*, *Retnla*, *Il1b*, and *Il23a* was evaluated by RT-qPCR relative to PBS-treated controls following normalisation by the endogenous control 18s rRNA. Data presented as mean ± SD (n=5-6/group) and each symbol represents an individual mouse. *p<0.05, vs PBS-treated control by unpaired t test.
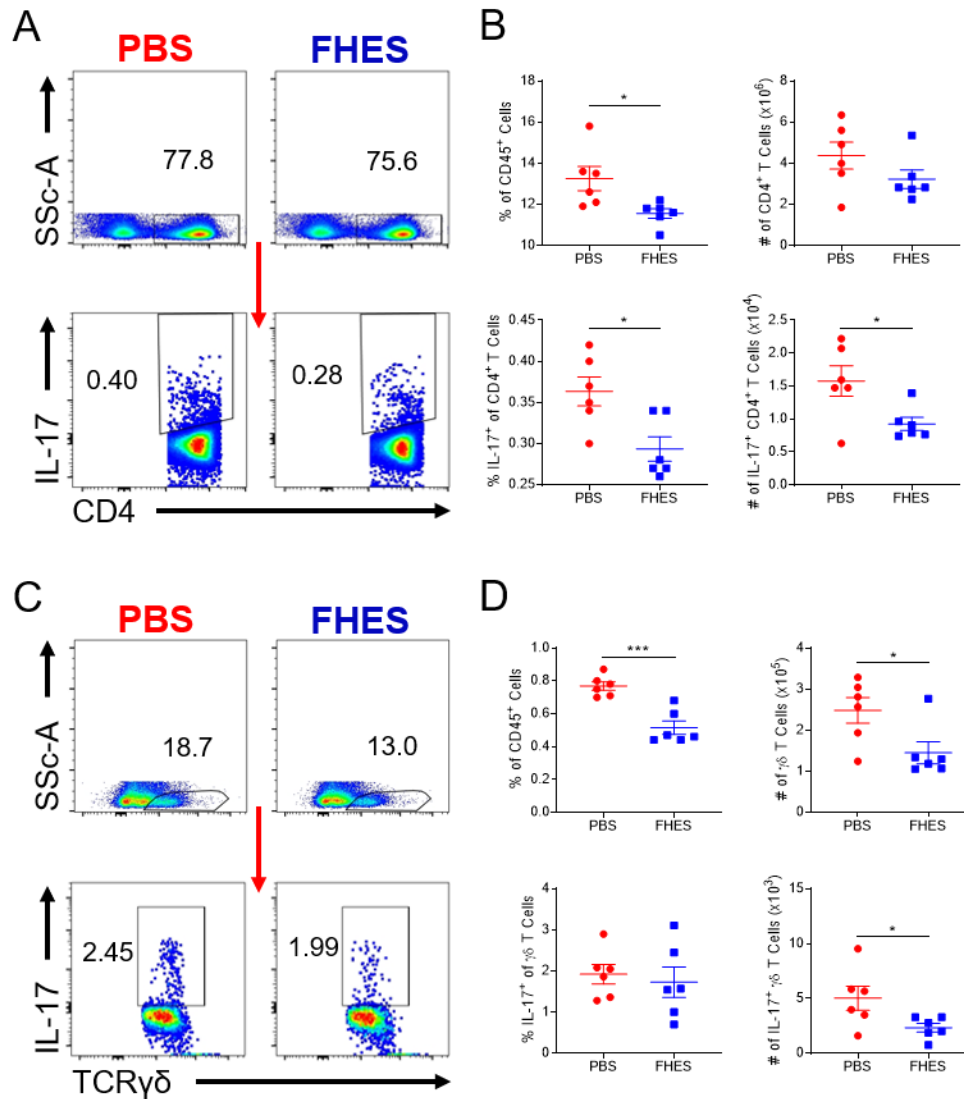
Figure 4.16 Treatment with FHES results in decreased IL-17⁺ CD4 and γδ T cells in the spleen after EAE induction. C57BL/6 mice treated as described in Figure 4.13A. On day 3, spleens were isolated, and cells stained with markers for surface CD45, CD3, CD4, TCRγδ and intracellular IL-17 and analysed by flow cytometry. (A) Representative FACS for IL-17⁺ CD4⁺ T cells. (B) Frequency and absolute number of total CD4 and IL-17-secreting CD4 T cells. (C) Representative FACS for IL-17⁺ γδ T cells. (D) Frequency and absolute number of total CD4 and IL-17-secreting γδ T cells. . Results shown are mean ± SEM (n=6 mice/group) and each dot represents an individual mouse. Statistical assessment was performed by Student's t-test vs PBS-treated control.

Figure 4.17 FHES treatment does not alter Th cell-associated transcription factors while increasing M2-associated mRNA expression in the spleen on day 3 of EAE. C57BL/6 mice treated as described in Figure 4.13A. On day 3, spleens were isolated, and total RNA was extracted and the expression of *Tbet*, *Gata3*, *Rorc*, *Foxp3*, *Ilrrn*, *Chil3*, *Il10*, *Retnla*, *Il1b*, and *Il23a* was evaluated by RT-qPCR relative to PBS-treated controls following normalisation by the endogenous control 18s rRNA. Data presented as mean ± SD (n=6/group) and each symbol represents an individual mouse. *p<0.05, **p<0.01, ***p<0.001 vs PBS-treated control by unpaired t test.
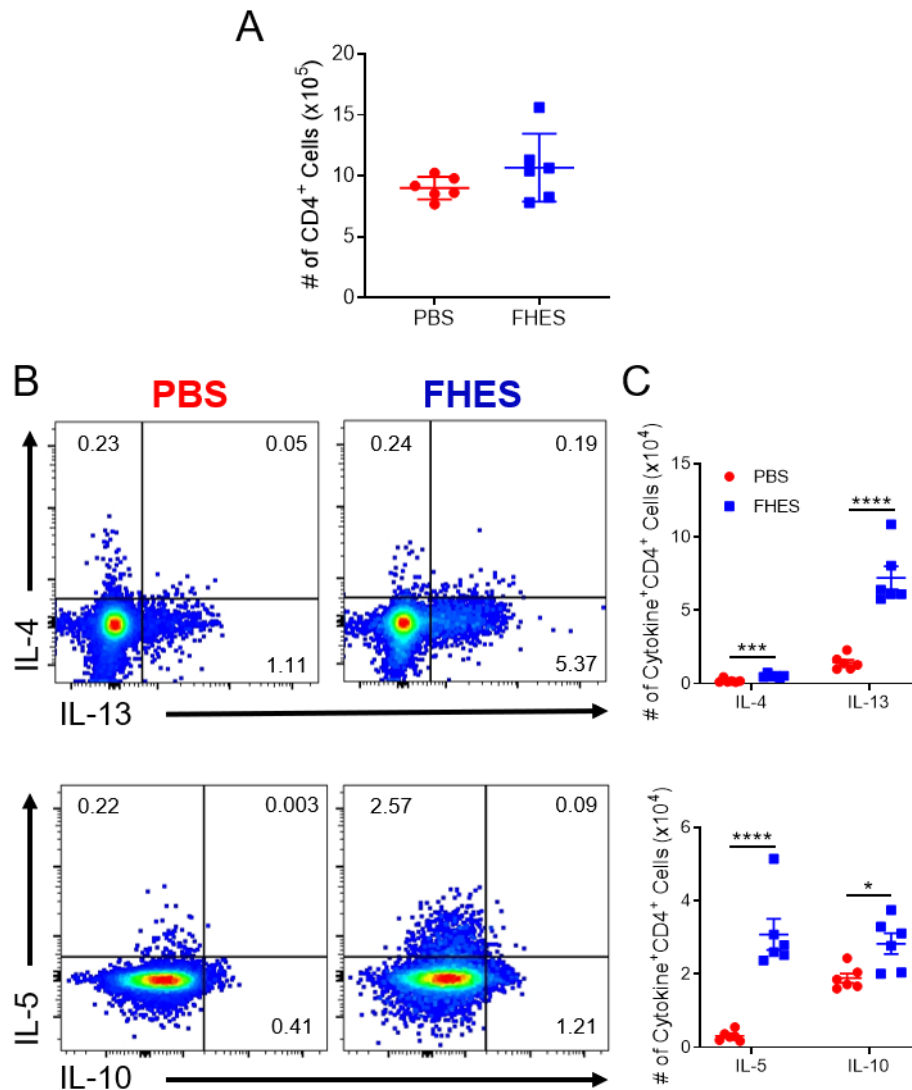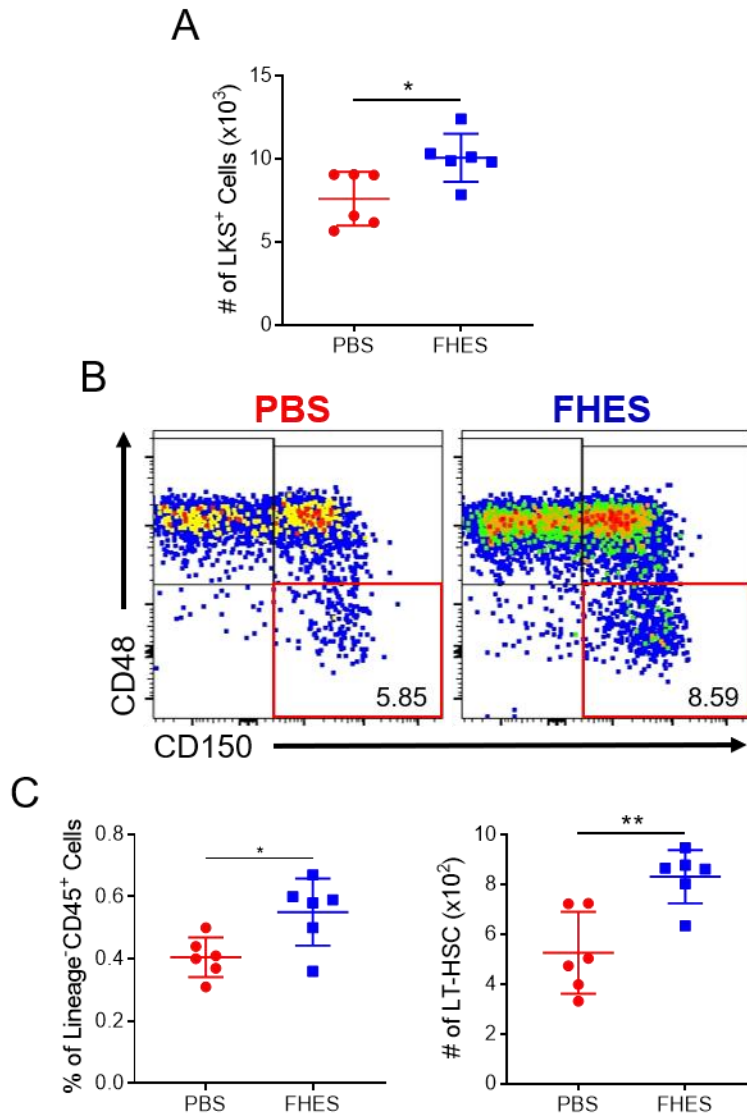
Figure 4.18 Treatment of mice with FHES expands LT-HSC after induction of EAE. C57BL/6 mice treated as described in Figure 4.13A. On day 3, BM cells were isolated and stained with HSC surface markers and analysed by flow cytometry. (A) Absolute number of Lineage⁻cKit⁺Sca-1⁺ (LKS⁺) oligopotent progenitor cells. (B) Representative FACS for LKS cells, with LT-HSC (LKS⁺CD150⁺CD48⁻) indicated by a red box. (C) Frequency and absolute number of total LT-HSC in the BM. Results shown are mean ± SEM (n=6 mice/group) and each dot represents an individual mouse. *p<0.05, **p<0.01, vs PBS-treated control by unpaired t test.
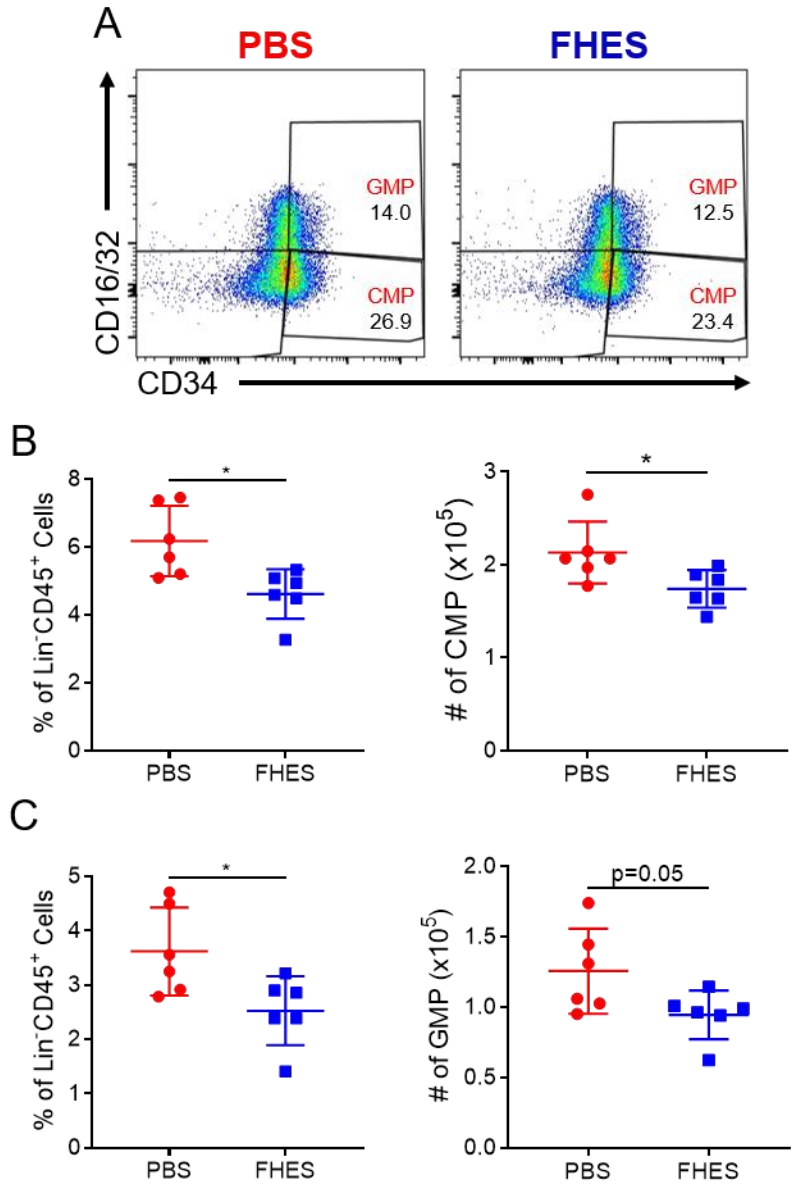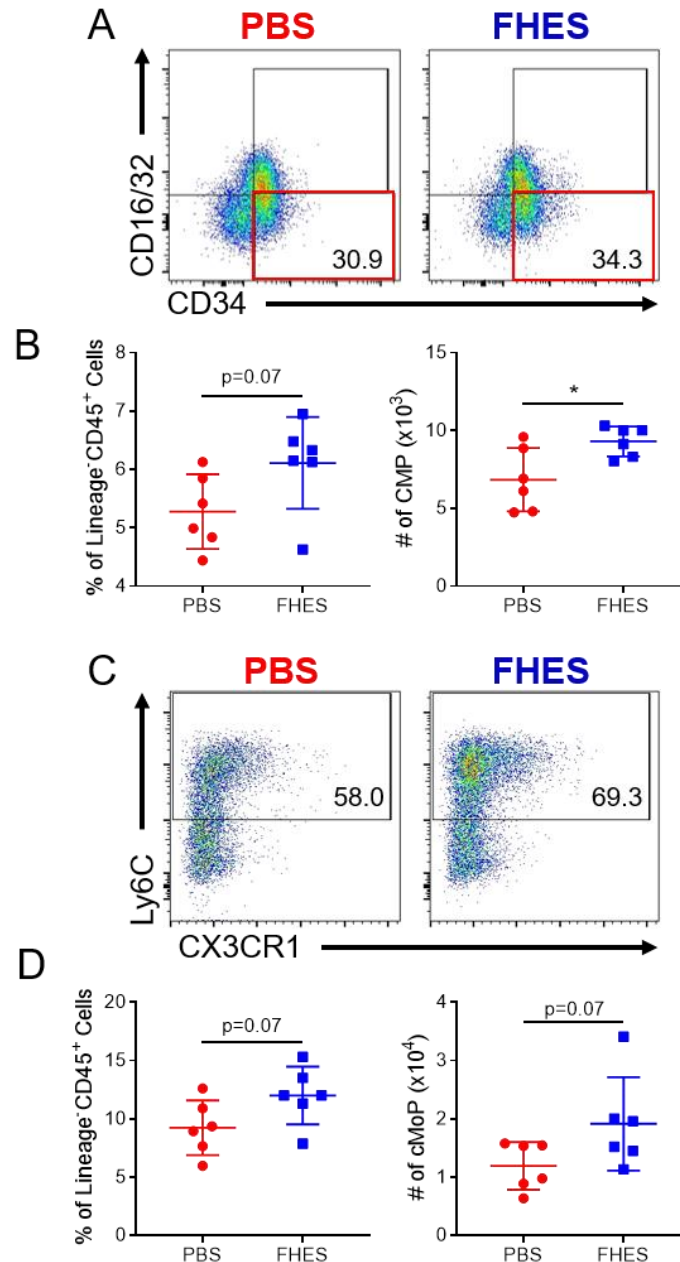
Figure 4.19 Treatment of mice with FHES expands the myeloid-specific progenitors CMP and cMoP in the BM during active EAE. C57BL/6 mice treated as described in Figure 4.13A. On day 3, BM cells were isolated and stained with HSC surface markers and analysed by flow cytometry. (A) Representative FACS for CMP (LK$^+$CD34$^+$CD16/32$^-$) indicated by a red box. (B) Frequency and absolute number of CMP in the BM. (C) Representative FACS for cMoP. (D) Frequency and absolute number of total cMoP in the BM. Results shown are mean ± SEM (n=6 mice/group) and each dot represents an individual mouse. *p<0.05 vs PBS-treated control by unpaired t test.
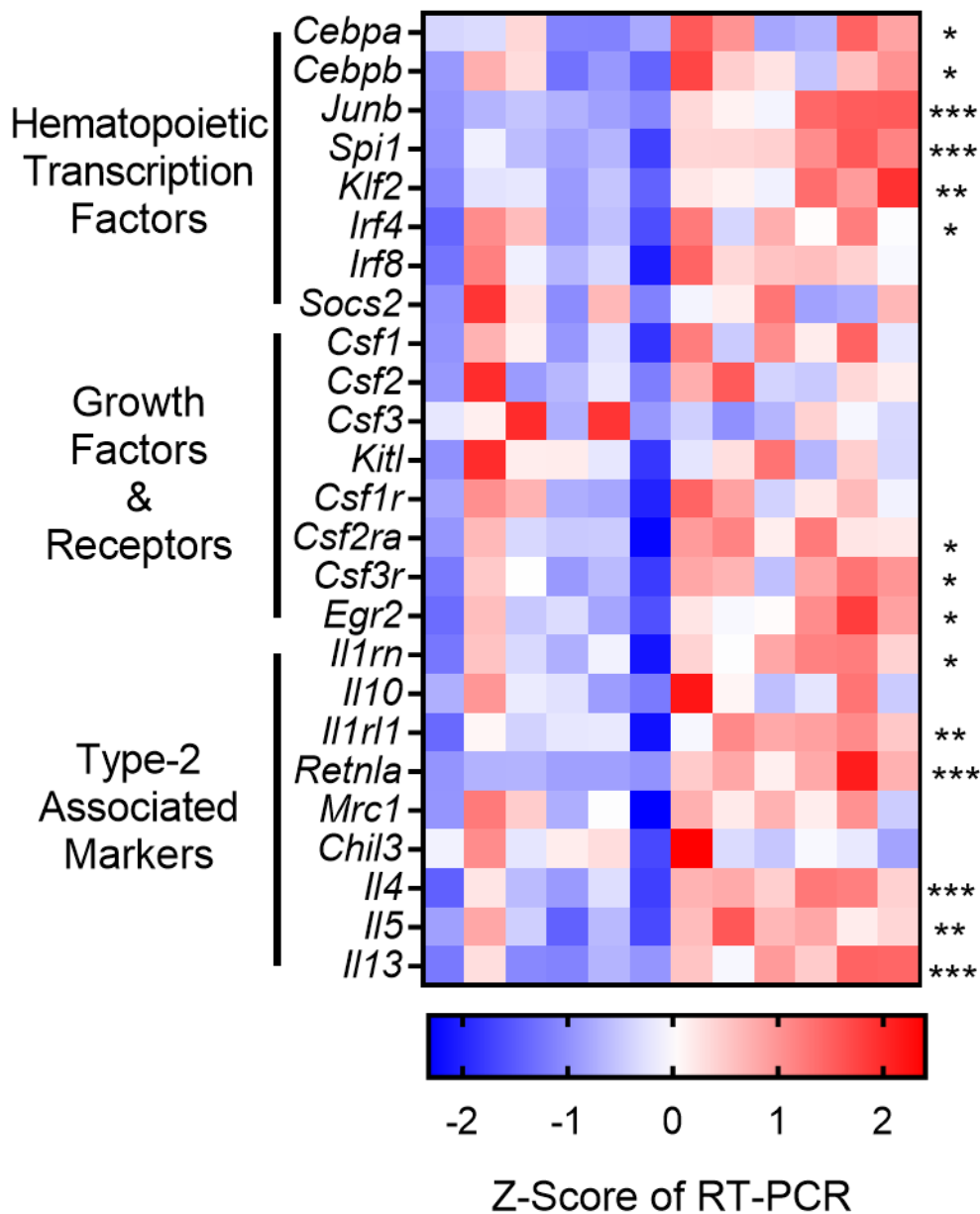
Figure 4.20 BM of mice treated with FHES show upregulated mRNA expression for genes associated with myelopoiesis and type-2 immunity on day 3 of EAE. C57BL/6 mice treated as described in Figure 4.13A. On day 3, BM cells were isolated, and total RNA was extracted and the expression of transcription factors for hematopoiesis, growth factors and type-2-associated markers was evaluated by RT-qPCR relative to PBS-treated controls following normalisation by the endogenous control 18s rRNA. Results are displayed as a heatmap of Z-Scores with corresponding p-values. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ vs PBS-treated control by unpaired t test.
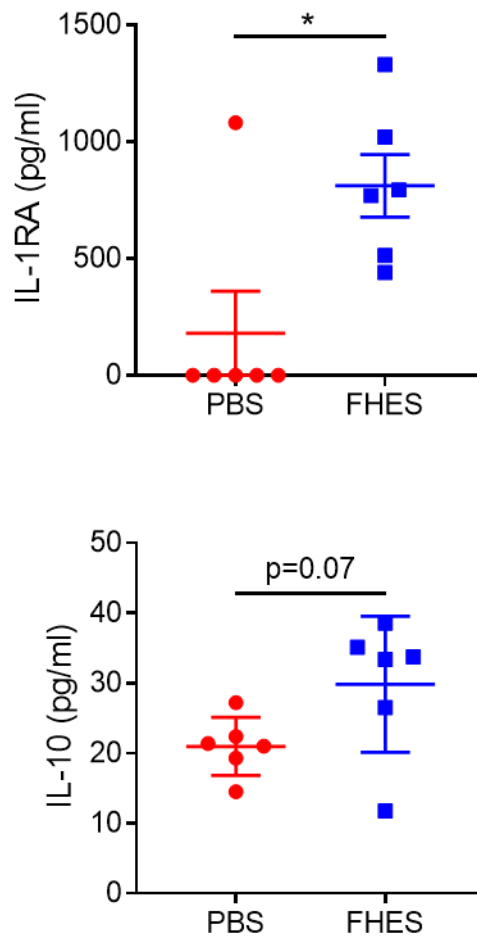
Figure 4.21 BM of mice treated with FHES have higher concentrations of IL-1RA and IL-10 in the BMECF on day 3 of EAE. C57BL/6 mice were injected s.c. with PBS or FHES (100 µg/mouse) on days -21 and -7, and on days 0 and 2, EAE was induced. On day 3, BMECF was collected from each mouse and analysed for IL-1RA and IL-10 by ELISA. Data presented as mean ± SEM (n=6/group) and each symbol represents an individual mouse. *p<0.05 vs PBS-treated control by unpaired t test.
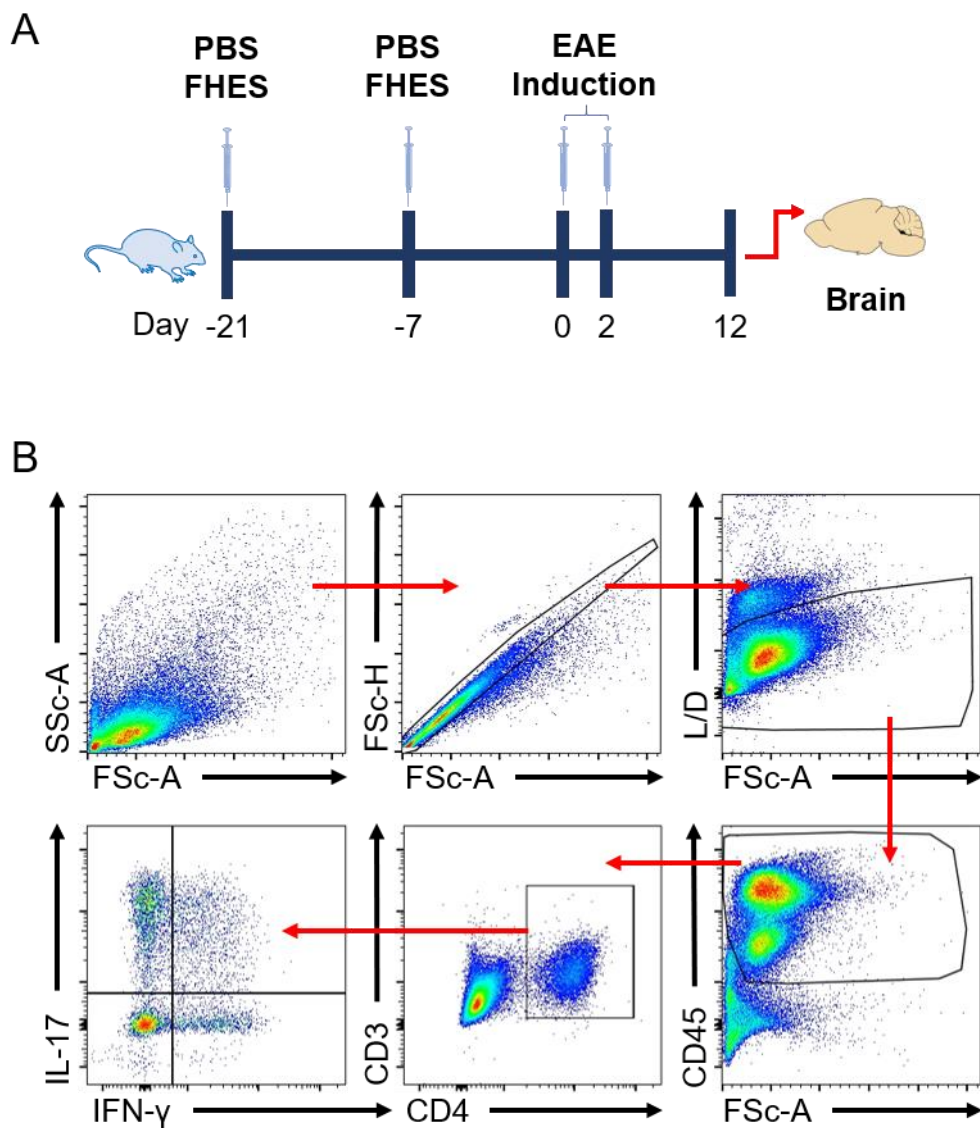
Figure 4.22 Induction of EAE and gating scheme to examine the effect of treatment with FHES on immune cell populations in the CNS following induction of EAE. (A) C57BL/6 were injected s.c. with FHES (100 μg/mouse) or PBS on days -21 and -7, and EAE induced on days 0 and 2. On day 12, mice were euthanized, and brains were isolated for flow cytometric analyses. (B) Gating strategy to identify pathogenic CD4 T cells.
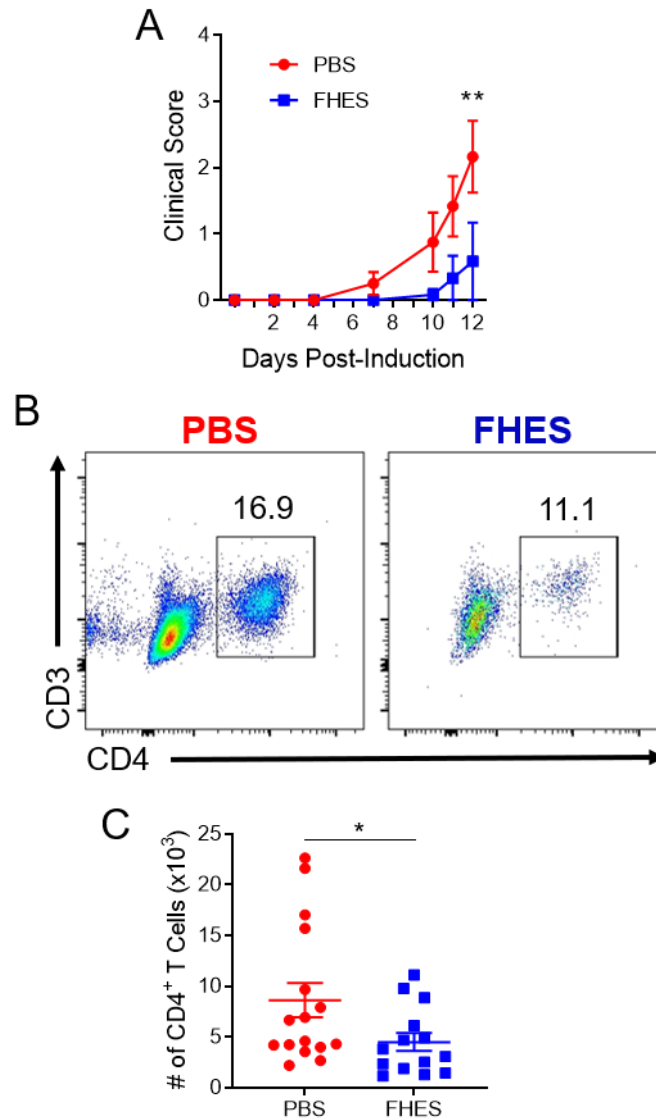
Figure 4.23 Treatment of mice with FHES reduces the number of infiltrating T cells in the brain and spinal cord. C57BL/6 mice treated and EAE induced on day 0 as described in Figure 4.22A. (A) Disease progression was monitored by clinical scores. Data presented as mean ± SEM (n=6/group). On day 12 mice were euthanized and cells were isolated from the brains and stained for surface CD45, CD3, CD4 and analysed by flow cytometry. Results are displayed as representative FACS plots from the brain (gated on CD45+ cells) (B), and absolute number CD3+ CD4+ T cells in the brain (C). Data are expressed as mean ± SEM (n=14-16 mice/group) and each symbol represents an individual mouse. Results are representative of two independent experiments. *p<0.05, **p<0.01 vs PBS-treated control by two-way ANOVA with Sidak post-test (clinical scores) or unpaired t test (absolute number of CD4 T cells).
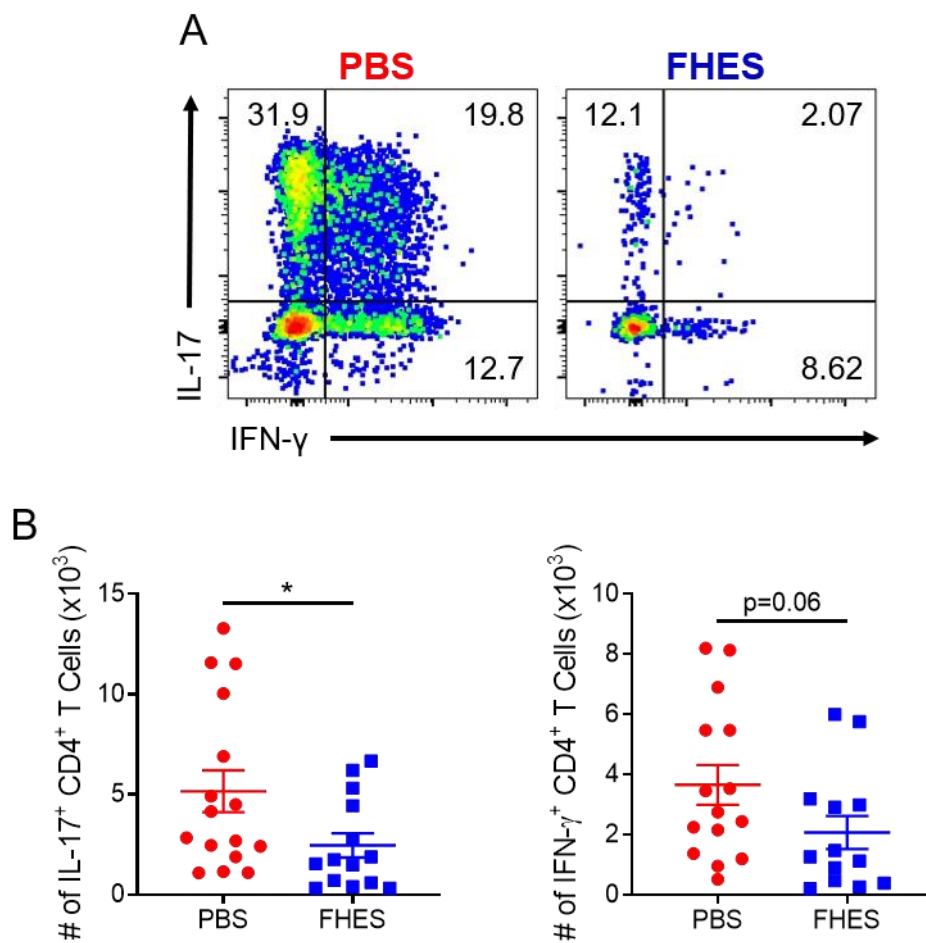
Figure 4.24 Pre-treatment of mice with FHES reduces the number of infiltrating, pathogenic Th1 and Th17 cells in the brains of mice at peak of EAE. C57BL/6 mice treated as before and EAE induced on day 0 as described in Figure 4.22A. On day 12 mice were euthanized and cells were isolated from the brains and stained for surface CD45, CD3, CD4 and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are displayed as representative FACS plots from the brain (gated on CD45⁺CD3⁺CD4⁺ T cells) (A), and absolute number of IL-17⁺ and IFN-γ⁺ CD4⁺ T cells in the brain (B). Data are expressed as mean ± SEM (n=14-16 mice/group) and each symbol represents an individual mouse. Results are representative of two independent experiments. *p<0.05 vs PBS-treated control by unpaired t test.
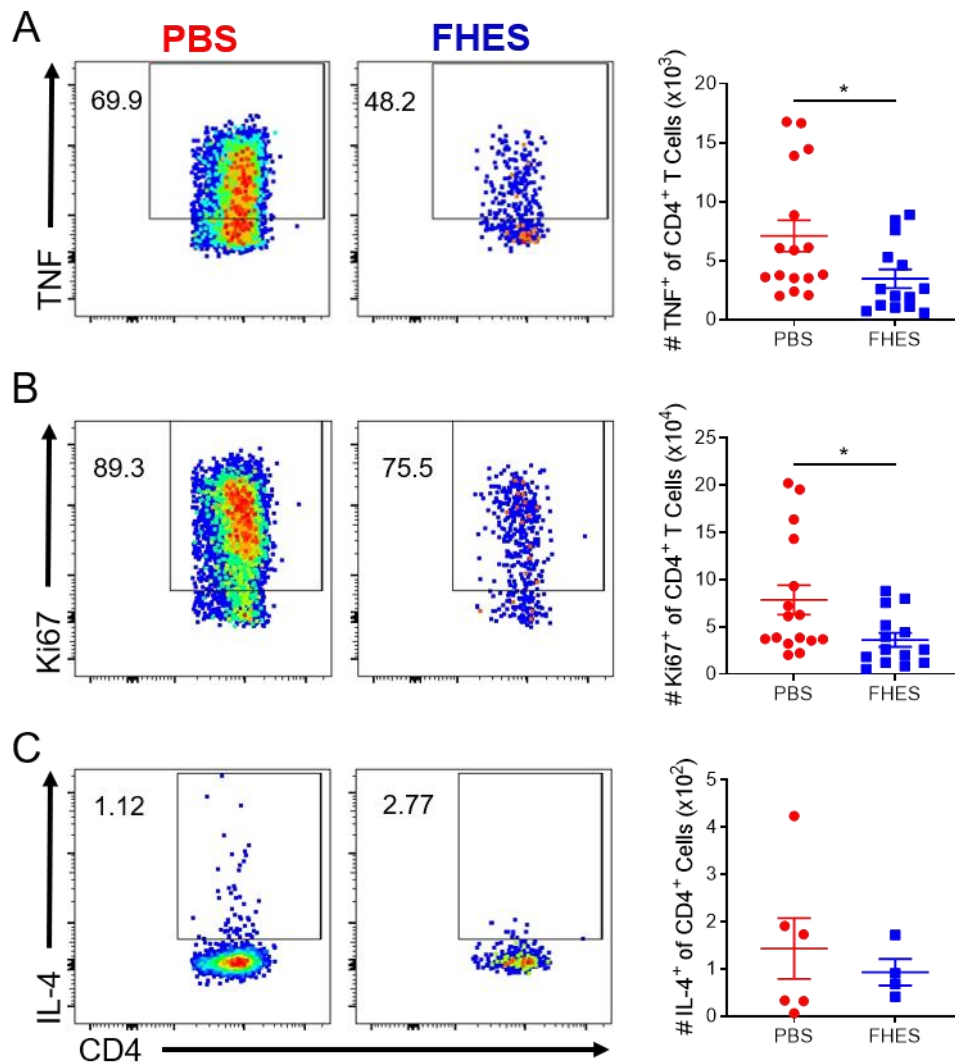
Figure 4.25 Pre-treatment of mice with FHES significantly reduces infiltrating TNF-secreting and proliferating CD4 T cells in the brain during EAE. C57BL/6 mice treated and EAE induced on day 0 as described in Figure 4.22A. On day 12 mice were euthanized and cells were isolated from the brains and stained for surface CD45, CD3, CD4 and intracellular TNF, Ki67 and IL-4 and analysed by flow cytometry. Representative FACS plots and absolute number of TNF+ (A), Ki67+ (B), and IL-4+ (C) CD4+ T cells in the brain. Data are expressed as mean ± SEM (n=6-16 mice/group) and each symbol represents an individual mouse. *p<0.05 vs PBS-treated control by unpaired t test.
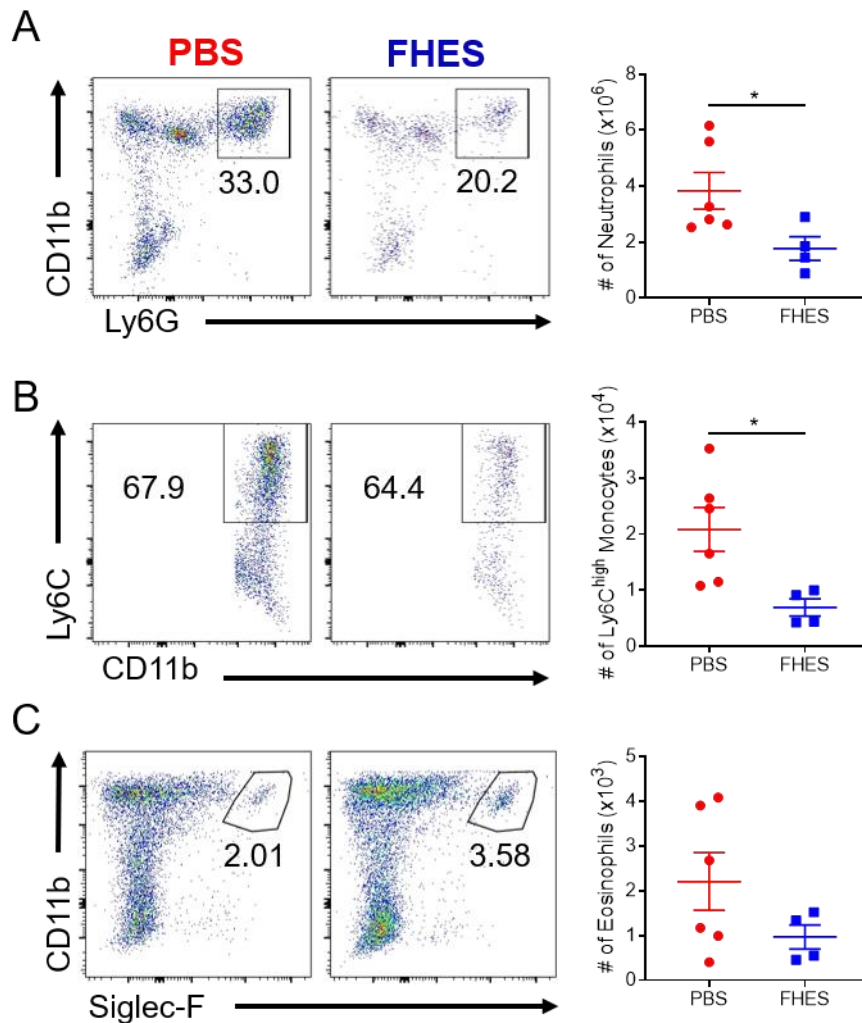
Figure 4.26 FHES pre-treatment reduces neutrophil and inflammatory monocyte infiltration of brains at peak EAE. C57BL/6 mice treated and EAE induced on day 0 as described in Figure 4.22A. On day 12 mice were euthanized and cells were isolated from the brains and stained for surface CD45, CD11b, F4/80, Ly6C, and Siglec-F analysed by flow cytometry. Representative FACS plots and absolute number of neutrophils (CD45+CD11b+Ly6G+) (A), inflammatory Ly6C^{high} monocytes (CD45+Ly6G-Siglec-F-F4/80-CD11b+Ly6C^{high}) (B), and eosinophils (CD45+CD11b+Siglec-F+SSc^{high}) (C) in the brain. Data are expressed as mean ± SEM (n=4-6 mice/group) and each symbol represents an individual mouse. *p<0.05 vs PBS-treated control by unpaired t test.
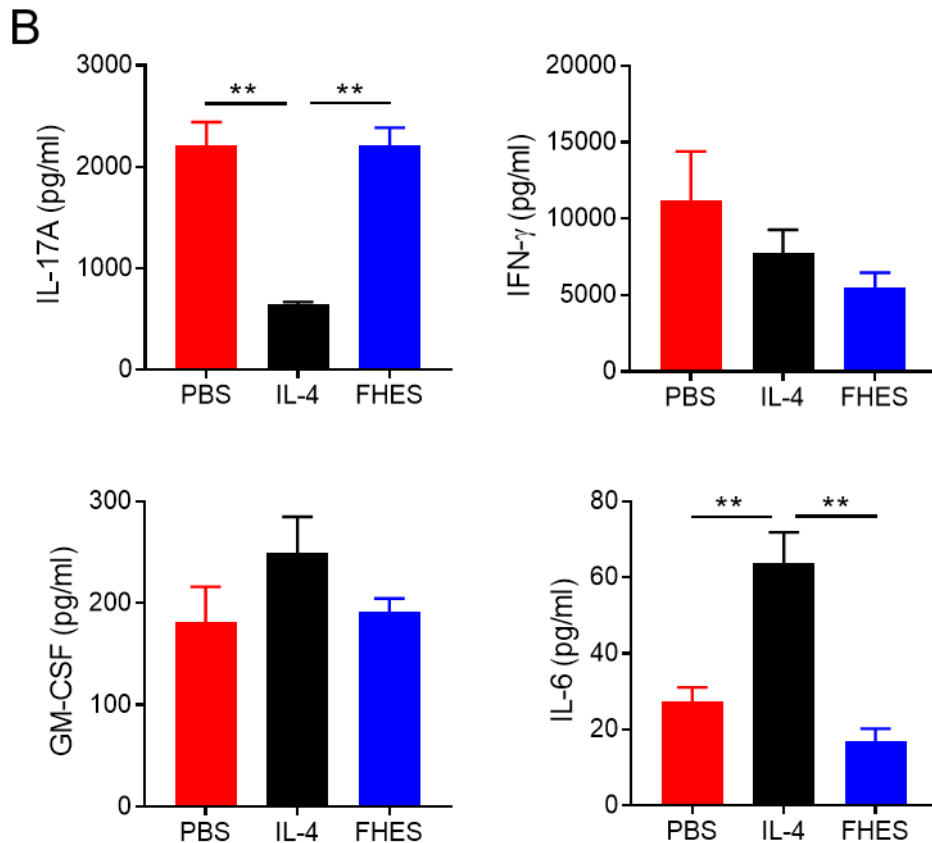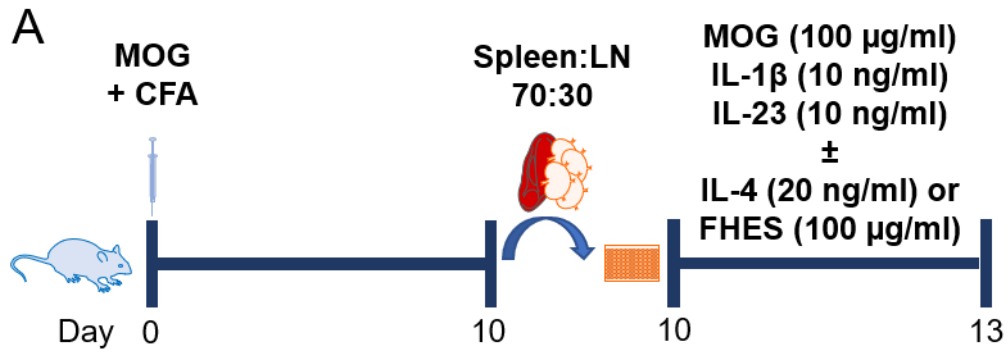
**Figure 4.27 IL-4, but not FHES, inhibits IL-17A production by MOG-specific spleen and LN cells.** (A) C57BL6/J mice were injected s.c. with MOG (100 µg/mouse) emulsified in CFA. After 10 days, spleen and LN cells were cultured for 72 h in a 70:30 (spleen:LN cell) ratio and stimulated with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence of IL-4 (20 ng/ml), FHES (100 µg/ml) or PBS. (B) The concentrations of IL-17A, IFN-γ, GM-CSF and IL-6 in the supernatants were assessed by ELISA. Data presented as mean ± SEM of triplicate assays. **p<0.01. Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test.
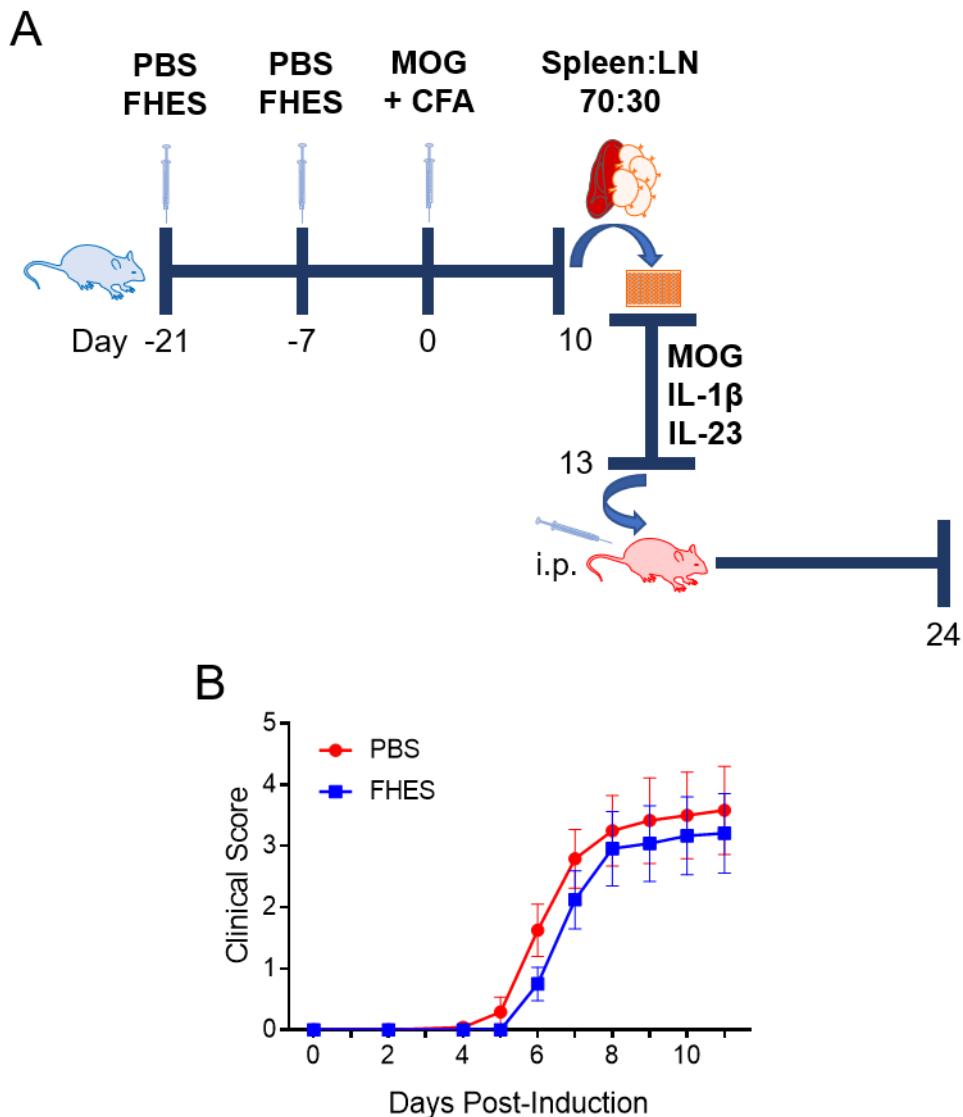
Figure 4.28 Pre-treatment of donor mice with FHES does not prevent recipient mice from developing clinical symptoms in passive transfer EAE. C57BL/6 mice were trained with PBS or FHES via s.c. injections on days -21 and -7. On day 0, mice were immunized with MOG (100 μg/mouse) emulsified in CFA, injected s.c.. On day 10, spleens and inguinal and axillary lymph nodes were excised. The excised cells were incubated with MOG, IL-1β, and IL-23 for 3 days. On the third day, the cells were isolated, washed, counted and i.p. injected into naïve C57BL/6 mice (15×10^6 cells/mouse). (B) Clinical scores were assessed daily, and mice were euthanized at peak of clinical score. Statistical assessment was performed by 2-way ANOVA vs PBS.
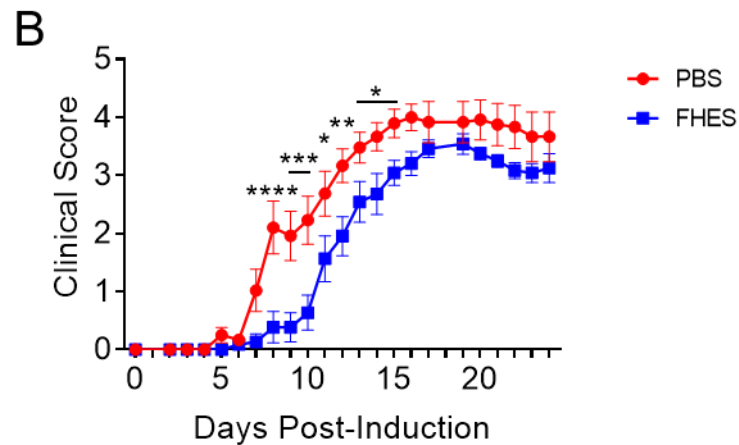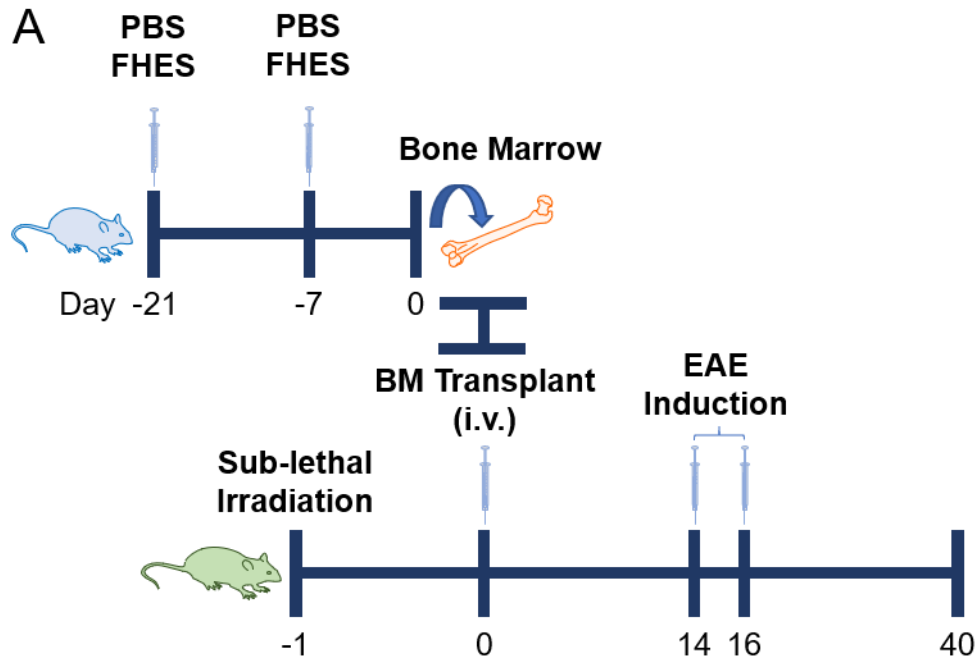
Figure 4.29 Transplantation of BM from mice pre-treated with FHES transfers protection against EAE. (A) C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7 and BM isolated on day 0. Separately, on day -1, naïve C57BL/6 mice were non-lethally irradiated (6 Gy) to deplete mature immune cells and HSCs. On day 0, BM from FHES- or PBS-treated mice were injected i.v. into irradiated mice (7.5×10^6 cells/mouse) to reconstitute the hematopoietic system. On days 14 and 16, EAE was induced, and mice monitored for clinicals signs and body weight loss. (B) Results are mean clinical score. Data are expressed as mean ± SEM (n=6-12 mice/group) and are combined from two separate experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ vs PBS by two-way ANOVA with Sidak's post hoc test.

Chapter 5: FHES induces central trained innate immunity

5.1 Introduction

Until recently, the dogma concerning immunological memory was that only cells of the adaptive immune response, T and B cells, were capable of long-lasting memory to pathogens. However, recent studies have demonstrated that innate immune cells can be 'trained' in response to various stimuli, giving rise to a form of immune memory now known as trained innate immunity. The evidence for trained innate immunity has long been characterized in plants and invertebrates, however recent epidemiological studies indicated that there may be a role for innate immune memory in humans and other vertebrates.

Initially characterized by priming with BCG, there are now many recognized mediators of trained innate immunity, including bacterial, viral and fungal components, cytokines, alarmins and microRNAs [98]. The two most studied mediators of trained innate immunity are BCG and β-glucan, a common component of fungal and bacterial cell walls. Upon priming with one of these mediators, mature innate immune cells are metabolically activated, which induces epigenetic modifications. These modifications, such as histone methylations and acetylation, occur at proximal and distal locations to genes encoding pro-inflammatory cytokines or transcription factors for activation. After the transient stimulus, the mature immune cells are then primed, or 'trained,' to respond faster and stronger to non-specific re-stimulation [98].

Trained innate immunity was first characterized in plants and invertebrates, classes of organisms which lack an adaptive immune system. In response to infection with a pathogen, plants acquire epigenetic alterations, which result in priming of genes responsible for host defence. Upon reinfection at distal sites, the plants can respond faster and more robustly to a new pathogen, a process known as systemic acquired resistance [99, 100]. Further studies in invertebrates, such as crustaceans and mosquitos, have shown that these organisms also utilize trained innate immunity, suggesting that innate immune memory is immunological conserved [101, 102]. In humans, immunization with BCG, a vaccine against *M. tuberculosis* (Mtb), can confer non-specific protection against infection with unrelated pathogens [93, 94]. In 2012, Netea and colleagues demonstrated that circulating peripheral blood mononuclear cells

(PBMCs) from healthy volunteers vaccinated with BCG, secreted higher concentrations of pro-inflammatory cytokines in response to re-stimulation with mycobacterial and non-mycobacterial stimuli [89].

Studies in mice have helped to further the understanding of how trained innate immunity occurs *in vivo,* and how it plays a role in the immune response to infection, cancers and transplantation. For example, injection of mice with β-glucan results in faster bacterial clearance and reduced mortality following subsequent challenge with *S. aureus* [103, 104]. Similarly, treatment of mice with the TLR9 agonist CpG enhanced survival of neutropenic mice in a model of *E. coli*-induced sepsis and meningitis. This protection was associated with an expansion of inflammatory monocytes, and increased IL-12 and IFN-γ production [108]. Furthermore, priming of monocytes and macrophages with HMGB1, a DAMP and TLR4 agonist, generated trained myeloid cells, which produced significantly higher concentrations of TNF and IL-6 in response to LPS re-stimulation *ex vivo* [136]. Inhibition of mTOR suppressed this training effect, leading to the expansion of anti-inflammatory macrophages and Tregs which promoted long-term tolerance to heart transplants.

Classical, antigen-specific immune memory in T and B cells is maintained via genetic restructuring of specific T and B cell receptor loci, while the exact mechanism of trained innate immunity remains unclear. However, recent studies have found that non-specific trained innate immunity involves metabolic and epigenetic modifications during the priming stage, which allows the innate immune cell to respond differently to re-stimulation [122, 129]. Studies on epigenetic modifications, heritable changes in gene expression, as a mediator in trained innate immunity have focused on chromatin remodelling, in which certain loci on the genome are made more or less accessible to transcriptions factors. Histone modifications include acetylation and methylation, which can weaken or tighten the bonds between histones and DNA. These modifications, if located at sites specific for metabolism or activation of innate immune cells, allows the cell to respond faster and more robust to re-stimulation with non-specific stimuli. For example, treatment of mice with β-glucan resulted in histone trimethylation at H3K4 in various promoter regions of genes encoding for inflammatory cytokines and chemokines, including TNF and IL-6 [124]. Furthermore, cellular metabolism

is essential in the development of innate memory responses. Though not yet fully understood, several factors have been implicated, including enhanced mTOR activation. For example, induction of glycolysis has been shown to be essential for β-glucan-induced trained innate immunity through the Akt-mTOR-HIF-1α pathway [126]. Additionally, monocytes from BCG-vaccinated patients exhibit a greater dependence on glycolysis, including enhanced mTOR and phosphorylated S6 (P-S6) [127].

While the majority of studies have focused on mature innate immune cells, a central question in the field is, how long can training last? In initial experiments detailing BCG-induced training, Kleinnijenhuis et al. reported that monocytes responded more robustly to re-stimulation when isolated up to 3 months post-vaccination [89]. Furthermore, the beneficial non-specific effects of BCG can last for years [115]. However, circulating monocytes have a short lifespan of several days. Recent studies have now demonstrated that long-lived, self-renewing HSCs in the BM can be modified to produce trained innate immune cells. This phenomenon is known as central trained innate immunity, while peripheral trained innate immunity refers to training of circulating and tissue-resident mature innate immune cells.

HSCs primarily reside in the BM and use PRRs and cytokine receptors to respond directly and indirectly to inflammation via changes in the BM microenvironment. Treatment of mice with BCG significantly expands MPPs and modifies their transcriptional landscape, imprinting a myeloid lineage bias by upregulation of transcription factors such as C/EBP-β and IRF8 [118]. Similarly, β-glucan-induced central trained innate immunity results in expansion of MPPs via increased IL-1β and GM-CSF in the BMECF [119]. In a recent study, de Laval and colleagues found that treatment of mice with LPS induced central trained immunity via upregulation of C/EBP-β in LT-HSC, promoting a myeloid bias during hematopoiesis and conferring long-term protection against infection with *P. aeruginosa* [120]. This form of immunological memory is now called central trained innate immunity.

In most studies to date, the models used to study trained innate immunity are specific to type-1, classical priming of immune cells (BCG, β-glucan), followed by

a further pro-inflammatory challenge, such as LPS or bacterial pathogens. However, innate immune cells can also be alternatively activated, producing anti-inflammatory cytokines and growth factors when activated with IL-4/IL-13 or with helminth-derived products. Recently, evidence has emerged indicating a role for type-2 responses in peripheral trained innate immunity. For example, infection of mice with the parasitic nematode *Nippostrongylus brasiliensis* resulted in priming of alternatively activated neutrophils, which prime long-lived effector macrophages that mediate rapid expulsion of helminths following re-challenge with the parasite [111]. Recently, our lab has demonstrated that induction of type-2 trained innate immunity can induce anti-inflammatory macrophages that suppress classical type-1 activation [113]. Macrophages treated *in vitro* with FHTE had increased secretion of IL-10 and decreased TNF in response to exposure to various PAMPs. Furthermore, treatment of mice with FHTE expanded these anti-inflammatory macrophages, which were able to suppress pathogenic Th17 cells that mediate EAE. Treatment of the macrophages with a methyltransferase inhibitor to block epigenetic modifications reversed the anti-inflammatory trained immunity.

There is evidence to suggest that helminths and their products play a role in modifying HSC differentiation into effector innate immune cells. For example, BMDM and BMDC derived from mice treated with filarial nematode glycoprotein ES-62 had reduced responsiveness to stimulation with LPS [311]. Additionally, age-related deficits in the clearance of helminths during infection in mice has been shown to correlate with a diminished frequency and number of HSCs in the BM in older mice [310]. It is possible that helminths and their products can mediate central trained innate immunity in addition to peripheral training. Epigenetic and metabolic modification of HSCs by helminth products, in particular FHES, may explain the persistence of chronic infections and life-long alterations in immune responses in previously infected individuals.

The specific aims to be addressed were:

- Does transplantation of FHES-primed BM transfer modifications to mature innate immune cells in recipient mice?
- Can FHES directly induce myelopoiesis in HSCs?
- Is FHES-induced central training mediated through epigenetic and/or metabolic modifications to HSCs?
- Is FHES-induced anti-inflammatory central training a result of type-2 cytokines IL-4 and/or IL-33?
- Which HSC is directly affected by stimulation with FHES?
- Can FHES-induced training of LT-HSC transfer protection from development of EAE to naïve mice?

5.2 Results

5.2.1 BM transplantation from mice that were treated with FHES engrafts HSCs and results in elevated numbers of macrophages in the periphery

The results in chapter 4 have shown that treatment of mice with FHES attenuates induction of EAE via modifications to the BM, which can be transferred to irradiated recipient mice. Here, I examine how FHES-induced BM modifications transfer protection during EAE. To investigate whether HSCs from donor mice were transferred to recipient mice during transplantation, an experiment was devised to examine the changes to myeloid populations in recipient mice after transplantation. Briefly, donor mice expressing CD45.1 were treated s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7 and BM (7.5×10^6 cells/mouse) cells were transplanted i.v. into sub-lethally irradiated CD45.2-expressing mice on day 0. On day 14, the frequency and number of donor-derived (CD45.1$^+$) or recipient (CD45.2$^+$) immune cells and HSCs (Lineage$^-$CD45$^+$) were assessed by flow cytometry using antibodies specific for CD45.1 and CD45.2 (Fig. 5.1A). Transplantation of BM from FHES-treated mice engrafted donor HSCs in the BM of recipient mice (Fig. 5.1B). Furthermore, treatment of donor mice with FHES did not improve or impair engraftment of HSCs in the BM of recipient mice. Interestingly, not all recipient HSCs had been ablated and there were marginally more recipient than donor HSCs in the BM on day 14. Importantly, there is substantial variation in the absolute number of recipient HSCs across different mice.

Irradiation induces cell death in tissue-resident and circulating immune cells throughout the body. After BM transplantation, donor HSCs rapidly differentiate and mobilize to repopulate tissue-resident innate immune cell populations, such as LPM in the PEC [381]. Having demonstrated that donor FHES treatment does not alter HSC engraftment in recipient BM after transplantation, I next investigated the ability of transplanted BM to produce, mobilize and repopulate innate immune cells in the periphery. To determine this, PEC were isolated and assessed by flow cytometry for donor-derived or recipient immune cells (Fig. 5.2A). Mice that received BM transplants from FHES-treated mice had significantly more donor-derived LPM in the PEC when compared with mice that

received BM from PBS-treated mice (Fig. 5.2B). Taken together, these data indicate that FHES treatment does not alter engraftment of HSCs, but rather improves monocyte/macrophage differentiation and repopulation after transplantation.

5.2.2 Transplantation of BM from FHES-treated mice transfers the anti-inflammatory innate phenotype

Early activation of innate immune cells is essential in mediating disease pathogenesis in EAE [349]. Macrophages and DC present antigen to MOG-specific T cells that infiltrate the CNS to mediate EAE disease pathology. Having demonstrated that FHES pre-treatment of mice modified the HSC compartment in the BM to preferentially produce anti-inflammatory monocytes and macrophages, an experiment was devised to investigate whether HSCs engrafted into recipient mice produced innate cells that respond to activation with an anti-inflammatory phenotype. Sub-lethally irradiated mice were reconstituted with BM from mice treated with FHES (100 µg/mouse) or PBS. Two weeks after BM transplantation, PEC were isolated and cultured with LPS (100 ng/ml) or PBS for 24 h and concentrations of IL-1RA, IL-6, IL-10 and TNF in the supernatant were determined by ELISA. PEC isolated from mice that received BM from FHES-treated mice secreted significantly higher concentrations of LPS-induced IL-1RA, IL-6 and IL-10 (Fig. 5.3). Furthermore, PEC isolated from mice that received BM from FHES-treated mice secreted significantly less LPS-induced TNF, when compared with PEC from mice that received BM from PBS-treated mice (Fig. 5.3).

Innate immune cells can activate the adaptive immune system through modulations of surface expression of MHC-II and co-stimulatory molecule. Having established that recipient mice expand anti-inflammatory macrophages in the periphery, it was next investigated whether suppression of co-stimulatory molecule expression was also transferred. Irradiated mice were reconstituted with BM (7.5 x 10^6 cells/mouse) from mice treated with FHES (100 µg/mouse) or PBS, and on day 14 post-transplantation, BM cells were collected and BMDM generated. On day 7, BMDM were harvested and stimulated with LPS (100 ng/ml) or PBS. After 24 h, cells were washed and stained with fluorescent

antibodies specific for CD11b, F4/80, MHC-II, CD40, CD80 and CD86 before analysis by flow cytometry. The results revealed that, BMDM generated from mice that received BM from FHES-treated mice have significantly less constitutive surface expression of co-stimulatory molecules CD80 and CD86 when compared with BMDM from control mice (Fig. 5.4C/D). Additionally, BMDM from FHES treated recipient mice had lower LPS-induced surface expression of MHC-II, CD40, CD80 and CD86 (Fig. 5.4A-D). Interestingly, while PBS-BM recipient BMDM enhance surface expression of CD86, BMDM generated from mice that received BM from FHES-treated mice had markedly decreased surface expression of CD86 after stimulation with LPS, from 17.3% to 11% (Fig. 5.4D). Importantly, LPS-enhanced surface expression of MHC-II on BMDM of control mice, however, LPS did not enhance expression of MHC-II on BMDM generated from mice that received BM from FHES-treated mice. Taken together, these data indicate that anti-inflammatory macrophage imprinting has been transferred to recipient mice through the transplantation of BM.

5.2.3 Transplantation of BM from FHES-treated mice transfers myeloid-biased response in HSCs during early-stage EAE

The results presented in Chapter 4 demonstrated that treatment of mice with FHES modified HSC populations to preferentially undergo myelopoiesis following induction of EAE. Having demonstrated that HSCs from donor mice have been engrafted successfully in irradiated recipients by two weeks post-transplantation and that donor-derived macrophages respond to LPS with an anti-inflammatory cytokine profile, I next investigated if transplanted HSCs preferentially undergo myelopoiesis during inflammation. The hematopoietic system of sub-lethally irradiated mice was reconstituted with BM transplanted from FHES (100 µg/mouse) or PBS. Two weeks after transplantation, EAE was induced, and after 3 days, BM cells were isolated and stained with fluorescent antibodies specific to HSCs and CD41 and were analysed by flow cytometry. CD41 is a surface antigen expressed on adult HSCs that has recently been identified as a marker for myeloid bias in the HSC compartment [382, 383]. Additionally, β-glucan-induced central trained immunity has been shown to increase CD41 surface expression on HSCs in mice [119]. The results revealed an increase in the frequency of CD41$^+$ LKS$^+$ ST-HSC (Fig. 5.5A) and MPP (Fig. 5.5B) in the BM of mice that

received a BM transplant from FHES-treated mice. Furthermore, there was a significant increase in the frequency of CD41^{+} GMP in the BM of mice that received BM from FHES-treated mice when compared with the HSCs of mice that received BM from PBS-treated mice (Fig. 5.5C). Taken together, these data reveal that treatment of mice with FHES induces modifications to LKS^{+} cells, which enables these long-lived, self-renewing cells to retain a myeloid bias.

5.2.4 Direct stimulation with FHES primes HSCs to produce anti-inflammatory macrophages and monocytes

Treatment of mice with helminths and their products expands anti-inflammatory monocytes and macrophages, eosinophils, Th2 cells and Tregs in the periphery [79]. Furthermore, infection of mice with low-dose *Trichuris muris* has been demonstrated to expand LKS^{+} MPP cells and GMPs in the BM [309]. This effect was shown to be dependent on IFN-γ-secreting T cells that are activated in the periphery and traffic to the BM. There is little evidence directly connecting helminths or their products to direct modulation of HSC function *in vivo.* Direct stimulation of naïve BM *in vitro* with filarial nematode glycoprotein ES-62 polarizes HSCs to develop into anti-inflammatory BMDM and BMDCs [311]. Recently, Megías and colleagues established that continuous or transient treatment of HSCs with PAMPs during differentiation significantly altered the phenotype of the innate immune cells derived *in vitro* [117]. Continuous exposure to PAMPs during differentiation with CSF-1 resulted in significantly enhanced secretion of IL-6 and TNF upon re-stimulation of the BMDM with TLR agonists.

Having shown that FHES expands myeloid-biased progenitors and induces production of macrophages/monocytes at steady-state and during EAE, it was next investigated whether FHES could directly modify differentiation of macrophages/monocytes. BM from naïve C57BL/6 mice were isolated and cultured in CSF-1-containing L929 media to induce macrophage differentiation in the presence of FHES (100 µg/ml) or PBS. After 7 days, BMDM were harvested and stained with antibodies specific for CD11b and F4/80 and analysed by flow cytometry. The results revealed that continuous exposure to FHES for 7 days significantly reduced the frequency of BMDM when compared with BMDM differentiated in the presence of PBS (Fig. 5.6A/B). Furthermore, the decrease in

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mature BMDM resulted in an expanded proportion of monocytes when compared with PBS-treated cells (Fig. 5.6C).

Having demonstrated that FHES directly impacts differentiation of BM cells, it was next investigated which HSC populations were affected. BM cells from naïve C57BL/6 mice were treated with increasing concentrations of FHES (0, 50, 100 µg/ml) or β-glucan (10 µg/ml) as a positive control. After 24 h, cells were collected and stained with antibodies specific for HSCs and analysed by flow cytometry. Direct stimulation of BM cells with FHES significantly expanded the frequency of MPP and MDP in a dose-dependent manner (Fig. 5.7A/B). Furthermore, treatment of whole BM with FHES significantly decreased the percentage of CLP after 24 h when compared with PBS- and β-glucan-treated BM (Fig. 5.7C). Interestingly, culture with β-glucan expanded the proportion of CLP and MPP, however treatment with β-glucan had no effect the frequency of MDP in the culture (Fig. 5.7A-C).

Since continuous exposure to FHES significantly reduced the differentiation of macrophages *in vitro* and having demonstrated that FHES expands HSCs within 24 h of treatment, it was next examined whether FHES could prime HSCs to differentiate into trained macrophages without inhibiting their differentiation. BM cells from naïve C57BL/6 mice were isolated and transiently treated with FHES (100 µg/ml) or PBS. After 24 h, cells were washed and resuspended in CSF-1-containing L929 media to induce macrophage differentiation. After 7 days, BMDM were harvested and stained with antibodies specific for CD11b and F4/80 and analysed by flow cytometry (Fig. 5.8A). Unlike continuous exposure, transient treatment of BM with FHES for 24 h did not affect the development of BMDM (Fig. 5.8B). Supernatants collected on day 7 were analysed by ELISA. Transient stimulation with FHES significantly elevated secretion of TGF-β and markedly increased IL-1RA secretion by BMDM (Fig. 5.8C). Furthermore, priming of BM with FHES before differentiation significantly increased expression of M2-associated markers *Chil3* and *Egr2* on day 7 when compared with PBS priming (Fig. 5.9). Taken together, these data reveal that FHES primes BM cells to produce macrophages with a constitutively anti-inflammatory phenotype.

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5.2.5 HDAC and mTOR inhibitors block FHES anti-inflammatory priming of HSCs

Trained innate immunity is induced when a priming stimulus modifies the metabolic and epigenetic landscape of a cell. In central trained innate immunity, the modifications to HSCs result in altered hematopoiesis and differentiation into mature monocytes/macrophages with trained responses to secondary inflammatory stimuli [118, 119]. Our lab has recently demonstrated that inhibition of methyltransferases blocks the development of FHTE-induced anti-inflammatory peripheral trained innate immunity [113]. Therefore, it was investigated whether addition of an epigenetic inhibitor during priming of BM cells with FHES could block alterations in HSCs that are passed to mature immune cells during hematopoiesis. BM cells were isolated from naïve C57BL/6 mice and transiently treated with FHES (100 µg/ml) or PBS. After 24 h, cells were washed and cultured with L929 media to generate BMDM. On day 7, BMDM were washed and re-stimulated with LPS (100 ng/ml), CpG (5 µg/ml) or PBS. After 24 h, supernatants were collected and analysed for IL-6 and IL-10 by ELISA. Priming of BM cells with FHES before differentiation significantly enhanced secretion of IL-6 and IL-10 in response to re-stimulation with LPS (Fig. 5.10). Furthermore, FHES pre-treatment significantly increased CpG-induced secretion of IL-6 and IL-10 when compared with PBS pre-treatment. The addition of an HDAC inhibitor, givinostat (25 nM), during priming with FHES significantly reversed LPS- and CpG-induced IL-6 secretion by BMDM to concentrations comparable of un-primed BMDM (Fig. 5.10). Interestingly, HDAC inhibition by givinostat did not affect FHES-enhanced secretion of IL-10 following CpG stimulation.

Metabolic reprogramming is essential for the induction of central and peripheral trained innate immunity [126, 127]. BCG and β-glucan utilize mechanistic target of rapamycin during priming, which mediates epigenetic shifts at various loci, resulting in trained innate immune cells. During homeostasis, HSCs are transcriptionally and metabolically quiescent, in order to expend little energy and maintain survival [8, 12]. However, upon activation with cytokines and other inflammatory stimuli, HSCs rapidly begin increasing metabolic activity to expand and differentiate. In particular, HSCs utilize the mTOR pathway to regulate glucose metabolism, maintain high levels of proliferation, and differentiate into

effector immune cells [384, 385]. To demonstrate that the FHES-induced training of HSCs was similarly dependent on epigenetic modifications, training was induced in the presence of absence of mTOR inhibitor rapamycin (5 nM). Treatment with FHES enhanced LPS- and CpG-induced IL-6 and IL-10, and this was reversed in the presence of rapamycin (Fig. 5.10). These findings demonstrated that FHES training of HSCs was dependent on metabolic signalling through the mTOR pathway and partially through HDAC activity.

5.2.5 FHES pre-treatment of mice modifies HSCs to increase metabolism during EAE-induced hematopoiesis

Cellular metabolism of individual HSCs can be measured after FACS sorting, or by analyses of the whole HSC compartment. However, these methods may negatively influence the metabolic activity of the cell and do not reveal cell-specific modifications, respectively [386]. Therefore, to quantitatively investigate the role of the mTOR pathway in individual HSCs during FHES-mediated attenuation of EAE, BM cells were stained for flow cytometric analysis of HSC populations, followed by intracellular staining using a fluorescently labelled antibody specific for phosphorylated S6 protein (phospho-S6). Ribosomal S6 protein is phosphorylated when the mTOR-HIF-1α pathway is activated during glycolysis. Therefore, phospho-S6 is an indirect marker for mTOR activation and can be quantitatively measured by flow cytometry in individual cells.

During trained innate immunity, cells are epigenetically and metabolically reprogrammed to respond faster and more robustly on re-challenge. An experiment was designed to investigate whether FHES mediates alterations to the HSC compartment that modifies their metabolic profile upon activation and differentiation. Mice were treated s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7. On day 0, EAE was induced and BM cells isolated on day 3. The cells were stained for HSC surface markers, followed by intracellular staining for phospho-S6, and analysed by flow cytometry. LT-HSC and MPP from FHES-treated mice significantly enhanced utilization of the mTOR pathway, as measured by MFI of phospo-S6, on day 3 of EAE (Fig. 5.11A/B). Indeed, FHES pre-treatment of mice increased the MFI of phospho-S6 by 12.4% when compared with LT-HSC from PBS-treated mice (Fig. 5.11A). Taken together,

these data suggest that treatment of mice with FHES modifies HSCs, which respond to induction of EAE with an enhanced mTOR activation, enhancing hematopoiesis, and the development of anti-inflammatory innate immune cells.

5.2.6 Anti-inflammatory training is not mediated by type-2 cytokines IL-4 or IL-33

Cytokines mediate a plethora of actions in the immune system, including activation of innate cells, induction of Th cell responses, and initiation of hematopoiesis. Recent research has now highlighted a role for cytokines to act as priming signals in the induction of peripheral and central trained innate immunity. For example, Kamada and colleagues demonstrated that IFN-β and IFN-γ can imprint trained phenotypes in mouse embryonic fibroblasts and BMDM via methylation of histones at promoter regions of IFN-stimulating genes [387]. The epigenetic modifications imprinted allowed for faster recruitment of RNA polymerase and subsequently more robust gene transcription after re-stimulation with IFNs. Additionally, β-glucan-induced central training of MPP and GMP cells was found to be dependent on IL-1β signalling in the BMECF [119]. Indeed, blocking IL-1β signalling using IL-1RA, resulted in inhibition in the development of trained phenotypes in HSCs and circulating myeloid cells. Infection of mice with *F. hepatica* or administration with FHES results in increased levels of type-2 cytokines IL-4 and IL-33 in the peritoneal cavity [256, 260]. IL-4 is largely responsible for mediating type-2 immune responses, including alternative activation of macrophages, induction and recruitment of Th2 cells, and decreases production of Th1 cells and type-1 cytokines [79]. IL-33 is an alarmin that is released in response to helminth infections and amplifies IL-4 signalling responses, further driving the alternative activation of macrophages [376].

Having demonstrated that FHES induces trained innate immunity, and that administration with FHES enhances type-2 cytokines and their receptors in the periphery and the BM, it was next investigated whether type-2 cytokines mediated these effects. C57BL/6 mice were injected with IL-4 (200 ng/mouse), IL-33 (200 ng/mouse) or a combination of IL-4 and IL-33 (200 ng/cytokine/mouse) on days -21 and -7. On day 0, mice were sacrificed, BM isolated and stained with fluorescent antibodies specific for HSCs and analysed by flow cytometry.

Treatment of mice with IL-33 alone had no effect on the frequency or absolute number of any HSC. However. treatment of mice with a combination of IL-4 and IL-33 resulted in a significant increase in the absolute number of CMP in the BM when compared with HSCs of PBS-treated mice (Fig. 5.12A). Additionally, treatment of mice with IL-4 or a combination of IL-4 and IL-33 resulted in a significant increase in the absolute number of CLP (Fig. 5.12B).

Having demonstrated a role in IL-4 in attenuating MOG-specific IL-17 secretion *in vitro*, and the ability of IL-4 and IL-33 to expand HSC populations, it was next investigated whether they could attenuate EAE similar to FHES priming. Mice were treated with IL-4 (200 ng/mouse), IL-33 (200 ng/mouse) or a combination of IL-4 and IL-33 (200 ng/cytokine/mouse) on days -21 and -7. On day 0, EAE was induced, and clinical scores measured daily. IL-4, IL-33 or a combination were incapable of altering disease scores during EAE when compared with PBS-treated control mice (Fig. 5.13). These data suggest that, while IL-4 and IL-33 play key roles in mediating classical type-2 immune responses, they are not responsible for the anti-inflammatory central trained immunity observed during treatment of mice with FHES.

5.2.7 Direct stimulation of HSCs with FHES expands LT-HSC and biases cells towards myelopoiesis *in vitro*

Oligopotent LKS$^+$ HSCs reside in a specialized niche in the BM that mature immune cells, stromal cells and neuronal synapses strictly regulate [2, 388]. While cytokines are potent mediators in inducing hematopoiesis during inflammation, HSCs also express receptors for growth factors and even TLRs, which allow them to sense the BM microenvironment and respond to infection and inflammatory stimuli [389, 390]. Having demonstrated that whole BM responds to treatment with FHES, and that type-2 cytokines IL-4 and IL-33 do not mediate anti-inflammatory central training, it was next investigated whether purified HSCs could respond directly to FHES. To achieve this, HSCs were purified from the BM of naïve mice and stimulated with FHES (100 µg/ml) or PBS. After 24 h, cells were harvested, and flow cytometry performed with antibodies specific for HSC populations. Direct stimulation of enriched HSCs with FHES significantly increased the absolute number of LT-HSC in the culture when

compared with stimulation with PBS (Fig. 5.14A). In contrast, FHES treatment significantly reduced the number of CLP (Fig. 5.14B). Total RNA was isolated from HSCs 24 hours after stimulation with FHES and the mRNA expression of myelopoiesis-associated transcription factors was analysed by RT-qPCR. Stimulation of HSCs with FHES significantly increased the expression of *Cebpa*, *Cebpb*, *Junb* and *Spi1* (Fig. 5.15). Taken together, these data affirm that FHES can act directly on HSCs to preferentially induce myelopoiesis at the expense of lymphopoiesis.

5.2.8 Treatment of mice with FHES protects against development of EAE symptoms for up to 8 months

There are many aspects of trained innate immunity that have yet to be elucidated, including the longevity of effect. LT-HSC are long-lived cells with a very low rate of turnover and give rise to every cell in the hematopoietic system [8, 9]. Having demonstrated that FHES modifies LT-HSC via direct stimulation *in vitro* and through s.c. administration *in vivo*, it was next investigated how long the anti-inflammatory central training effects persist for. Mice were treated s.c. with FHES (100 µg/mouse) or PBS two weeks apart. EAE was then induced 1 or 8 months after the final injection. Pre-treatment of mice with FHES significantly delayed disease, with average onset up to 4 days later than mice pre-treated with PBS (Fig. 5.16A). While modest, FHES pre-treatment 8 months earlier significantly delayed the onset of clinical signs of EAE when compared with PBS-treated control mice (Fig. 5.16B). Interestingly, 1-month and 8-month pre-treatment with FHES resulted in comparable clinical scores by day 12 post-induction of EAE. However, mice treated with FHES 8-months prior also developed less severe disease when compared with PBS treatment (Fig. 5.16B). Taken together, these data suggest that priming of HSCs with FHES results in modification of long-lived HSC populations, resulting in reduced susceptibility to development of clinical scores in EAE.

5.2.9 FHES pre-treatment increases the percentage of myeloid lineage-dependent progenitors in the BM for up to 8 months

Having demonstrated moderate protection from the onset of EAE 8 months post-treatment with FHES, it was next investigated whether HSC populations

remained altered in the BM of these mice. Mice were twice treated s.c. with FHES (100 µg/mouse) or PBS two weeks apart. 8 months later, BM cells were harvested and stained with antibodies specific for HSC populations before analysis by flow cytometry. FHES pre-treatment resulted in a non-significant increase in the frequency of LT-HSC in the BM 8 months post-treatment when compared to PBS-treated control mice (Fig. 5.17A). Indeed, the proportion of LT-HSC in the HSC compartment was elevated by 59%, rising from 10.49% to 16.65% of all LKS$^+$ cells. FHES treatment 8 months earlier significantly expanded the percentage of CMP in the HSC niche (Fig. 5.17B). Interestingly, this did not translate into a change in the absolute number of LT-HSC or CMP in the BM (Fig. 5.17). Taken together, these data indicate that FHES modifications to LT-HSC and myeloid progenitors lasts up to 8-months after treatment.

5.2.10 Innate immune cell activation is suppressed 8 months post-treatment with FHES

There is evidence to suggest that central trained innate immunity persists for months to years after priming. In a recent study, Khan et al. showed that HSCs could be reprogrammed by BCG and Mtb, and this lasted for up to one year post-vaccination [146]. In particular, mice trained with BCG were shown to have improved engraftment of LKS$^+$ cells and CMP in the BM up to one year after transplantation into irradiated mice. Having demonstrated that FHES-induced central training yields resulted in enhanced frequencies of LKS$^+$ cells and CMP in the BM 8 months after treatment, it was next investigated whether innate immune responses were altered. PEC were isolated 8 months after treatment with FHES (100 µg/mouse) or PBS. and stimulated with LPS (100 ng/ml) or PBS. After 24 h the concentrations of IL-1β, IL-1RA, IL-6, and IL-10 were determined by ELISA. Constitutive and LPS-induced secretion of IL-1β, IL-1RA and IL-10 were significantly lower in PEC from FHES-treated compared with PBS-treated mice (Fig. 5.18). Pre-treatment of mice with FHES did not significantly alter the secretion of IL-6 in response to stimulation with LPS or PBS.

Having demonstrated that total PEC from mice treated with FHES 8 months earlier exhibited a reduced inflammatory response to LPS stimulation, it was next investigated whether macrophages generated *de novo* from BM were similarly

altered. Mice were twice treated s.c. with FHES (100 µg/mouse) or PBS two weeks apart. 8 months following the final injection, BM cells were harvested and incubated with CSF1-containing L929 media to generate BMDM. On day 7, BMDM were harvested, washed and stimulated with LPS (100 ng/ml), CpG (5 µg/ml), Mtb (10 µg/ml) or PBS. After 24 h, supernatants were collected and assessed for IL-1β, IL-1RA and IL-10 by ELISA. FHES treatment did not significantly alter LPS- or CpG-induced secretion of IL-1β or IL-1RA by BMDM when compared with BMDM from PBS-treated mice (Fig. 5.19). However, LPS- and CpG-induced secretion of IL-10 was significantly suppressed in BMDM generated from FHES-treated mice. Interestingly, BMDM derived from FHES-treated mice secreted significantly less Mtb-induced IL-1β, however no change was observed in the secretion of IL-1RA or IL-10 (Fig. 5.19). Following stimulation with LPS and PBS, BMDM were harvested and stained with antibodies specific to CD11b, F4/80, CD86 and CCR2 before analysis by flow cytometry. Surface expression of co-stimulatory molecule CD86 and inflammatory chemokine CCR2 were markedly decreased in BMDM of mice treated with FHES when compared with PBS controls (Fig. 5.20A/B). Interestingly, BMDM from FHES-treated mice had higher expression of CD86 in response to LPS stimulation (Fig. 5.20A). Furthermore, BMDM from FHES-treated mice had a markedly impaired ability to increase surface expression of CCR2 in response to LPS stimulation (Fig. 5.20B). Taken together these data indicate that FHES reprogramming of HSCs is sustained for up to 8 months, however the effect is modest and characterized primarily by a tolerized response to inflammatory stimuli.

5.2.11 Transplantation of LT-HSC from FHES-treated mice transfers protection from development of EAE

LT-HSC are long-lived stem cells that give rise to all cells of the innate and adaptive immune system and are capable of reconstituting the entire hematopoietic system after chemical or radiation therapy-induced myeloablation [363]. Furthermore, HSC transplantation has recently emerged as an attractive therapeutic in the treatment of MS [142]. Preliminary research has highlighted a potential role for priming, or training, HSCs before transplantation to recipient patients. Indeed, pulsing of HSCs with prostaglandin E2 (PGE2) yields HSCs with enhanced ability to engraft irradiated recipients and a bias towards

myelopoiesis, generating anti-inflammatory mature immune cells [391, 392]. Having shown that FHES modifies LT-HSC to expand and bias towards anti-inflammatory myelopoiesis at steady-state and during EAE, the next study investigated whether transplantation of LT-HSC to myeloablative mice could reconstitute the immune system to attenuate the development of EAE.

To confirm that FHES-induced modifications to HSCs were passed through the myeloid lineage, CLP isolated from mice treated with FHES were also transplanted to a separate group of sub-lethally irradiated mice to reconstitute the lymphoid lineage. Mice were treated s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7. On day 0, BM cells were harvested, enriched for HSCs using a MACS sort kit, and stained with antibodies specific to CD45, Lineage (CD11b, CD19, CD3, NK1.1, Ter119), cKit, Sca-1, CD48, CD150 and CD127. Purified LT-HSC (CD45$^+$Lin$^-$cKit$^+$Sca-1$^+$CD48$^-$CD150$^+$) and CLP (CD45$^+$Lin$^-$cKit^{low}Sca-1^{low}CD127$^+$) were then FACS sorted. On day -1, a separate group of mice were sub-lethally irradiated (6 Gy). After a day of rest, purified LT-HSC (1000-3500/mouse) or CLP (8 x 10^4 cells/mouse) from FHES- and PBS-treated mice were injected i.v. to irradiated mice. Mice were then left to rest and reconstitute the immune system for two weeks before EAE was induced (Fig. 5.21). Mice that received LT-HSC from FHES-treated mice had significantly delayed onset and reduced severity of EAE when compared with mice that received LT-HSC transplant from PBS-treated mice (Fig. 5.22A). In contrast, treatment of donor mice with FHES had no effect on the outcome of EAE in recipient mice that received CLP when compared with PBS-treated donors (Fig. 5.22B). Taken together, these data reveal that FHES modifies LT-HSC to generate anti-inflammatory mature myeloid cells but does not impart alterations to the lymphoid lineage.

5.2.12 Reduced pathogenic immune cell infiltration into the CNS of mice that received FHES-primed LT-HSC following induction of EAE

Infiltration of pathogenic, auto-antigen-specific T cells and inflammatory myeloid cells into the CNS initiates the effector phase of EAE and results in an ascending paralysis [349, 393]. To investigate whether transplantation of FHES-primed LT-HSC could similarly inhibit trafficking of T cells to the CNS. Mice were treated

with FHES (100 μg/mouse) or PBS on days -21 and -7. On day 0, LT-HSC were isolated by FACS sorting and injected i.v. (1000 cells/mouse) to sub-lethally irradiated (6 Gy) mice. Two weeks later, EAE was induced, and clinical scores assessed regularly. By day 8, the mice that had received LT-HSC from PBS-treated group had developed disease, while those that received LT-HSC from the FHES-treated group had significantly lower disease scores (Fig. 5.23A). Mice were sacrificed on day 8 to perform flow cytometric analyses of brain-infiltrating mononuclear cells. Compared with LT-HSC from PBS-treated mice, mice that received LT-HSC from FHES-treated mice had significantly fewer CD4$^+$ T cells in the brain (Fig. 5.23B/C). Mice that received LT-HSC from FHES-treated mice had reduced absolute number of IFN-γ- or IL-17-secreting CD4$^+$ cells (Fig. 5.24A-C) and γδ T cells (Fig. 5.25A-C). Furthermore, there was a reduction in the absolute number of proliferating CD4$^+$ and γδ T cells in the brains on day 8 after induction of EAE in mice that received LT-HSC from FHES-treated mice when compared with mice that received LT-HSC from PBS-treated mice as measured by Ki67 staining (Fig. 5.26A/B).

An examination of myeloid cell infiltration to the brain on day 8 revealed that mice that received FHES-primed LT-HSC had a significant reduction in the total number of infiltrating neutrophils when compared with mice that received PBS-primed LT-HSC (Fig. 5.27A). Furthermore, the absolute number of infiltrating Ly6C^{high} monocytes and eosinophils in the brain on day 8 after the induction of EAE was significantly reduced in the FHES-treated when compared to the PBS-treated mice (Fig.5.27B/C). These results are consistent with results from chapter 4, demonstrating that treatment of mice with FHES results in a reduction in inflammatory innate and adaptive immune cells infiltrating the CNS during EAE. Taken together, the results from this chapter demonstrate that FHES modification of hematopoiesis is dependent upon epigenetic and metabolic reprogramming of progenitor cells, lasting for up to 8 months. Furthermore, FHES primes LT-HSC to respond more robustly and with an altered metabolic profile upon activation during EAE to produce anti-inflammatory myeloid cells, resulting in reduced activation and infiltration of auto-specific pathogenic T cells into the CNS.

5.3 Discussion

Helminth parasites have evolved an impressive array of immunomodulatory abilities which ensure their long-term survival in the host and are associated with suppression of immune responses that mediate autoimmunity. This connection has formed the basis of the hygiene hypothesis, which was based on the observation that areas of high helminth burden had lower incidence of autoimmune disease [336]. Infection with helminths, or administration of helminth-derived products, is known to directly inhibit the activation of pro-inflammatory innate immune cells and suppress the induction of Th1 and Th17 cells, while enhancing alternatively activated macrophages and promoting expansion of Th2 cell and Treg cells [79]. Recently, evidence has suggested that induction of peripheral and central trained innate immunity by viral and bacterial mediators may explain the hygiene hypothesis [146, 394, 395]. Previously, we have shown that FHTE induces peripheral training of macrophages, resulting in suppression of autoimmune inflammation in EAE [113]. The present study showed that FHES induces central trained immunity in the BM, leading to suppression of EAE. In addition, this study revealed that FHES imprints an anti-inflammatory myeloid bias in LT-HSC that can be transferred upon transplantation into irradiated mice.

FHES was found to reconstitute the immune system of irradiated mice with anti-inflammatory macrophages. PEC harvested from mice that received BM transplantation from FHES-treated mice secreted significantly higher concentrations of IL-1RA and IL-10, and less TNF after stimulation with LPS. This is consistent with data from previous chapters showing that BMDM generated from FHES-treated mice secrete higher concentrations of anti-inflammatory cytokines in response to stimulation with PAMPs. Furthermore, BMDM generated from mice that received BM from FHES-treated mice had similarly suppressed co-stimulatory molecule expression on their surface following stimulation with LPS. Taken together these results reveal that central training of mice with FHES transfers the ability of HSCs to differentiate into anti-inflammatory mature innate immune cells. This is consistent with recent literature in central training. Khan et al., reported that BM from mice treated with BCG conferred a myeloid bias on HSC populations through IFN-1 signalling, and this bias was transferrable to

irradiated mice [146]. Interestingly, the BCG-mediated myeloid bias was maintained in the BM of engrafted mice for up to 1-year post-transplantation. They further demonstrated that while BCG induced central training, infection with Mtb suppressed the development of central training. This suggests that pathogens inhibit central trained innate immunity as a form of immunomodulation. FHES did not enhance type-1 or type-2 IFN, however, BMDM generated from FHES-treated mice significantly reduce IFN-γ secretion from activated T cells. It is possible that FHES induces central training through a different mechanism than IFN signalling, either through direct activation of HSCs or through type-2 cytokines.

HSCs respond to inflammation through direct stimulation by PAMPs, cytokines or growth factors. It is possible that FHES acts directly on HSCs to induce anti-inflammatory central training. The data from the present study revealed that direct stimulation of HSCs with FHES modified myelopoiesis to promote the production of monocytes. Additionally, transient stimulation with FHES initiates myelopoiesis, leading to an expansion of myeloid and suppression of lymphoid progenitors. It is possible that FHES is able to enter the BM and signal to HSCs. In a study using the joint TLR5 and NLRC4 agonist, demonstrated that flagellin primed donor HSCs and improved engraftment in recipient mice, resulting in better survival after irradiation [145]. Megías et al. demonstrated that direct treatment of HSCs with TLR2, TLR4 and Dectin-1 agonists modified HSCs to produce mature effector innate immune cells that secrete lower concentrations of pro-inflammatory cytokines in response to stimulation with fungal antigens [396]. Importantly, priming of HSCs with TLR agonists appears to inhibit the pro-inflammatory capacity of the mature immune cells they generate. Importantly, while the present study confirms that priming HSCs directly with FHES inhibits pro-inflammatory cytokine secretion, FHES priming also increases LPS- and CpG-induced secretion of IL-6 and IL-10. It is possible that FHES, a concentrated mixture of proteins and exosomes, mediates central training through similar mechanisms, while simultaneously directing further changes to increase production of anti-inflammatory cytokines.

Previous work in the lab demonstrated that FHTE induces peripheral anti-inflammatory trained innate immunity through epigenetic modifications that could

be partially inhibited by the addition of MTA, a histone methyltransferase inhibitor [113]. Indeed, epigenetic and metabolic modifications has been shown to be essential for the development of peripheral and central trained innate immunity [98]. However, the use of epigenetic and metabolic inhibitors during differentiation is significantly different from their use in mature immune cells that have a robustly defined transcriptional landscape. For example, methyltransferase activity is essential to HSC cell-cycling and renewal. Indeed, specific deletion of methyltransferase activity in LKS$^+$ cells has been shown to ablate the development of myeloid cells *in vitro* [397]. In addition to methylation/demethylation, cells can be epigenetically modified via histone acetylation/deacetylation. Therefore, the pan-histone deacetylase inhibitor givinostat was used to assess potential epigenomic modifications induced by FHES stimulation. The data revealed that givinostat reversed FHES-induced elevated secretion of IL-6, however it did not affect IL-10 secretion in BMDM. This is likely due to the multitude of proteins and effector molecules in FHES acting on different sites along the genome in methods beyond deacetylase activity. This may be due to acetylation or methylation at the site encoding for IL-10. In future studies, the use of other inhibitors, including inhibitors for acetyltransferases and demethylases, such as ECGC and pargyline, respectively, may shed additional light on this finding. Sequencing of chromatin accessibility of the genome by ATAC-seq would provide the clearest insight into the modifications induced in HSCs by treatment of mice with FHES.

The present study demonstrated that FHES modification of HSCs was mediated by activation of cells along the mTOR pathway. The mTOR-HIF-1α plays a role in central and peripheral training, directing the epigenetic modifications essential for imprinting memory in the affected cell [126, 328]. The present study revealed that anti-inflammatory trained immunity is mediated through the mTOR pathway. Inhibition of mTOR ablated FHES-induced modifications to HSCs *in vitro*. Furthermore, flow cytometric analyses revealed that HSCs in mice treated with FHES increase mTOR activity in LKS$^+$ cells during EAE, as measured by increased phospho-S6. This suggests that FHES training is specific to LKS$^+$ cells that then pass down training through the myeloid lineage during hematopoiesis. Moreover, mTOR activation is required to induce FHES-mediated central training

by acting upstream of epigenetic modifications and directing acetylation and methylation. Furthermore, mTOR activity is increased in LKS$^+$ cells and transcription factors associated with myelopoiesis are enhanced during inflammation, suggesting enhanced myelopoiesis and activation in downstream mature innate immune cells.

In recent studies on the effect of β-glucan, central training was shown to be dependent on IL-1β and GM-CSF in the BMECF, implicating a role for pro-inflammatory cytokines in the induction of training [119]. Furthermore, type-1 IFN signalling has been shown to induce trained innate immunity, and essential for the training effects of BCG [118, 146, 387]. In the present study, BM from mice treated with FHES had reduced pro-inflammatory cytokines and increased anti-inflammatory cytokine production. Furthermore, a significant increase in the receptor for IL-33 (ST2) was observed in the BM during EAE. While IL-4 and IL-33 in combination was capable of increasing the absolute number of CMP and CLP in the BM of mice, neither cytokine alone, nor in combination, attenuated EAE. Furthermore, an increase in the CLP is in direct contrast to treatment with FHES, suggesting a different method for the activation and differentiation of HSCs through type-2 cytokines. These results suggest that FHES does not induce anti-inflammatory central training through type-2 cytokines, IL-4 and IL-33, rather training appears to be mediated by direct stimulation of HSCs with FHES.

This study revealed that FHES specifically modifies the LT-HSC to produce more anti-inflammatory myeloid cells that attenuate disease severity in EAE. FHES-modified LT-HSC were able to engraft into sub-lethally irradiated mice and transfer protection from development of EAE. In previous studies of central trained immunity, BCG and β-glucan were revealed to mediate effects through the MPP [118, 119]. However, the data from the present study is consistent with the finding by De Laval et al., that demonstrated LT-HSC were capable of maintaining immunological memory after stimulation with LPS [120]. This study found that LPS induced C/EBP-β-dependent modifications to LT-HSC that resulted in improved survival from sepsis in mice. It is possible that a similar mechanism is involved in the induction of anti-inflammatory central training by FHES. LT-HSC are long-lived and give rise to all cells of the innate and adaptive

immune system. Additionally, HSC transplantation has been shown to be an effective therapeutic for patients suffering from MS [398]. There is preliminary research which suggests that priming HSCs before transplantation improves engraftment and prolongs survival [145, 149]. The data presented here suggest that modification of donor HSCs with helminth products could similarly improve engraftment and attenuate disease severity in autoimmune disorders.

Modifications to LT-HSC could provide improved engraftment and anti-inflammatory protection from development of autoimmune disorders. Indeed, the beneficial effects of LT-HSC treatment in mice was shown to last for up to 8 months. Helminth-induced modification of LT-HSC could elucidate the long-term, non-specific anti-inflammatory effects seen in patients with previous helminth infections. The exact mechanism for training of LT-HSC is yet to determined. However, the results in the present study suggest that FHES activates LT-HSC through the mTOR pathway, inducing histone deacetylation at various loci, resulting in the generation of more myeloid precursors that produce anti-inflammatory innate immune cells.
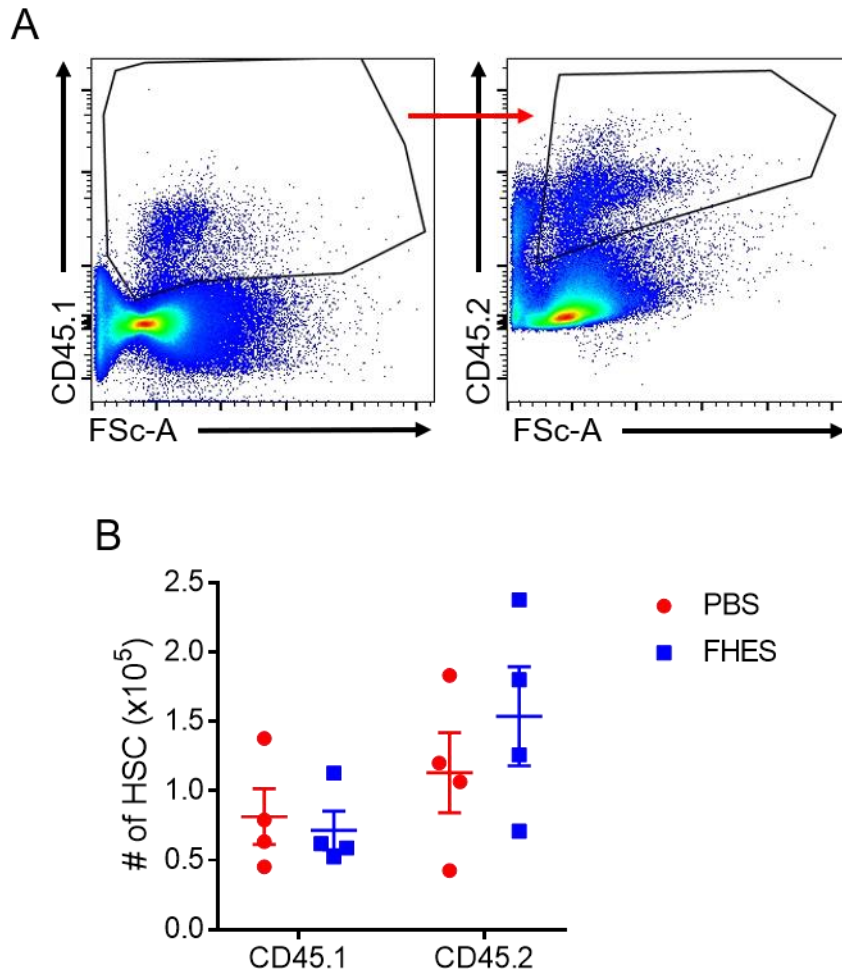
Figure 5.1 Transplantation of BM from FHES-treated mice successfully engrafts HSCs. CD45.1 C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7 and BM isolated on day 0. Separately, on day -1, naïve CD45.2 C57BL/6 mice were sub-lethally irradiated (6 Gy) to deplete mature immune cells and HSCs. On day 0, BM from FHES- or PBS-treated mice were injected i.v. into irradiated mice (7.5×10^6 cells/mouse) to reconstitute the hematopoietic system. On day 14, BM cells from recipient (CD45.2) mice were isolated and stained for CD45.1, CD45.2 and HSC surface markers and analysed by flow cytometry. (A) Representative FACS for Lineage⁻CD45.1⁺ and Lineage-CD45.2⁺ cells. (B) Absolute number of CD45.1⁺ (donor) and CD45.2⁺ (recipient) HSCs in the BM, Data are expressed as mean ± SEM. Each individual dot represents an individual mouse (n=4/group).
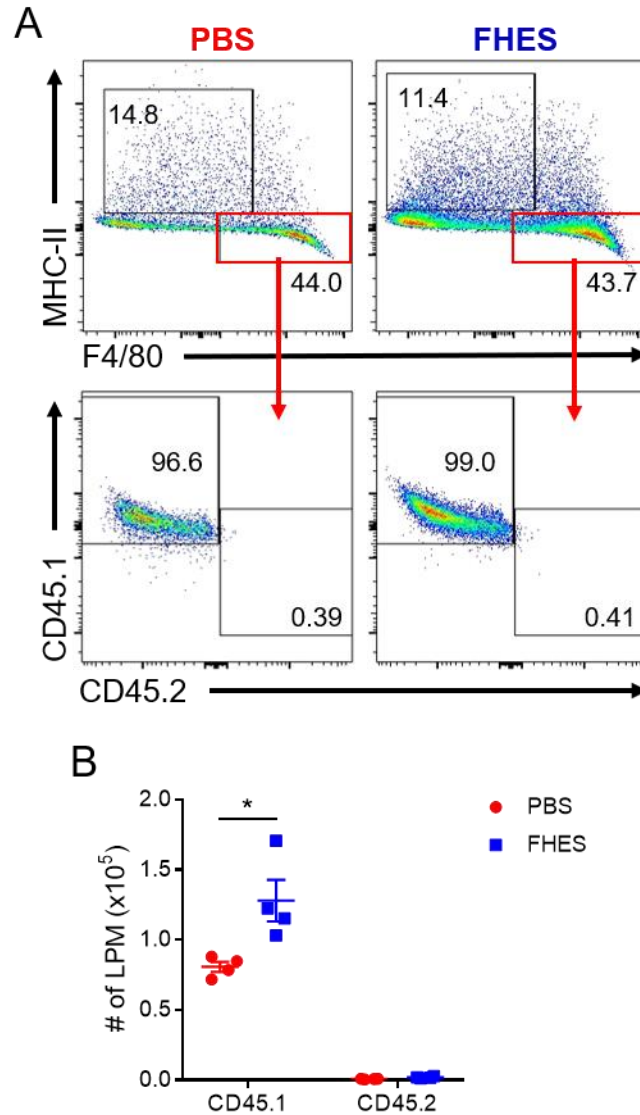
Figure 5.2 Transplanted BM from FHES-treated mice repopulates peritoneal cavity with LPM. Sub-lethally irradiated (6 Gy) CD45.2 C57BL6/J mice were reconstituted with BM (7.5 x 10^6 cells/mouse) from CD45.1 C57BL6/J mice treated with FHES (100 µg/mouse) or PBS as described in figure 5.1. On day 14, PEC were isolated and stained for CD45.1, CD45.2. CD11b, MHC-II and F4/80, and analysed by flow cytometry. (A) Representative FACS for CD45.1$^+$ and CD45.2$^+$ LPM. (B) Absolute number of CD45.1$^+$ (donor) and CD45.2$^+$ (recipient) LPM in the peritoneal cavity, Data are expressed as mean ± SEM. Each individual dot represents an individual mouse (n=4/group). *p<0.05 vs PBS-treated donors by two-way ANOVA with Sidak's post hoc test.
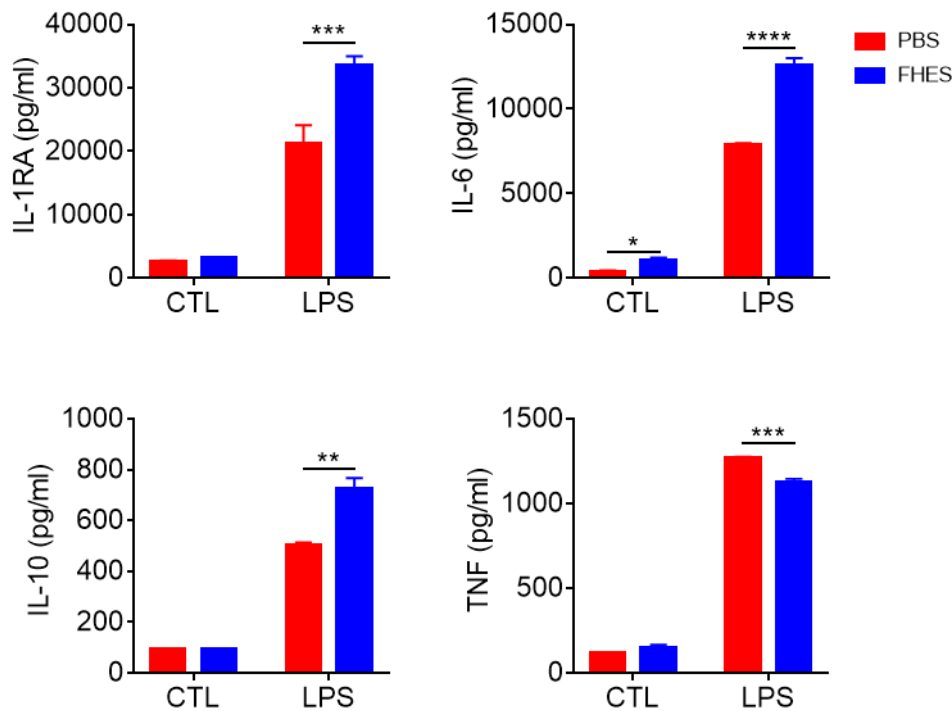
Figure 5.3 PEC from mice that received BM transplant from FHES-treated mice produced more IL-1RA, IL-6 and IL-10 but less LPS-induced TNF. Sub-lethally irradiated (6 Gy) CD45.2 C57BL6/J mice were reconstituted with BM (7.5 x 10^6 cells/mouse) from CD45.1 C57BL6/J mice treated with FHES (100 µg/mouse) or PBS as described in figure 5.1. On day 14, PEC (predominantly macrophages) were isolated and re-stimulated with LPS (100 ng/ml) or PBS. After 24 h, supernatants were collected and assayed for IL-1RA, IL-6, IL-10 and TNF by ELISA. Results are mean ± SEM for triplicate culture. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs PBS pre-treated controls by two-way ANOVA with Sidak's multiple comparisons post-test.
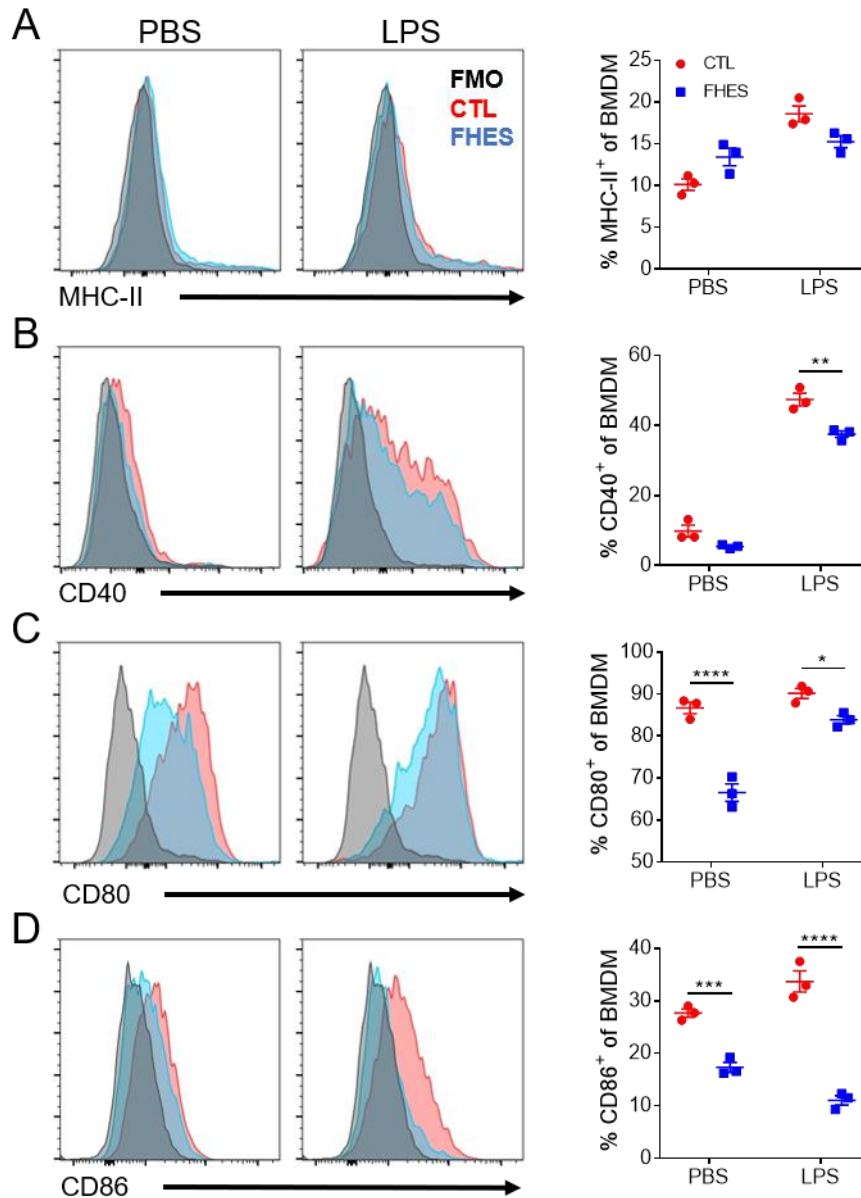
Figure 5.4 Reduced surface expression of MHC-II and co-stimulatory molecules on BMDM from recipient mice when re-stimulated with LPS. Sub-lethally irradiated (6 Gy) CD45.2 C57BL6/J mice were reconstituted with BM (7.5 x 10^6 cells/mouse) from CD45.1 C57BL6/J mice treated with FHES (100 µg/mouse) or PBS as described in figure 5.1. On day 14, BM cells were isolated and BMDM generated. On day 7, BMDM were washed, and cultured ($2x10^5$/well) with PBS or LPS (100 ng/ml) for 24 h. Cells were washed and stained for flow cytometry, PBS-trained in red, FHES in blue, FMO in grey. **p<0.01, ***p<0.001, ****p<0.0001, vs PBS pre-treated control or #p<0.05 vs PBS-treated FHES BMDM by two-way ANOVA with Sidak's multiple comparisons post-test.
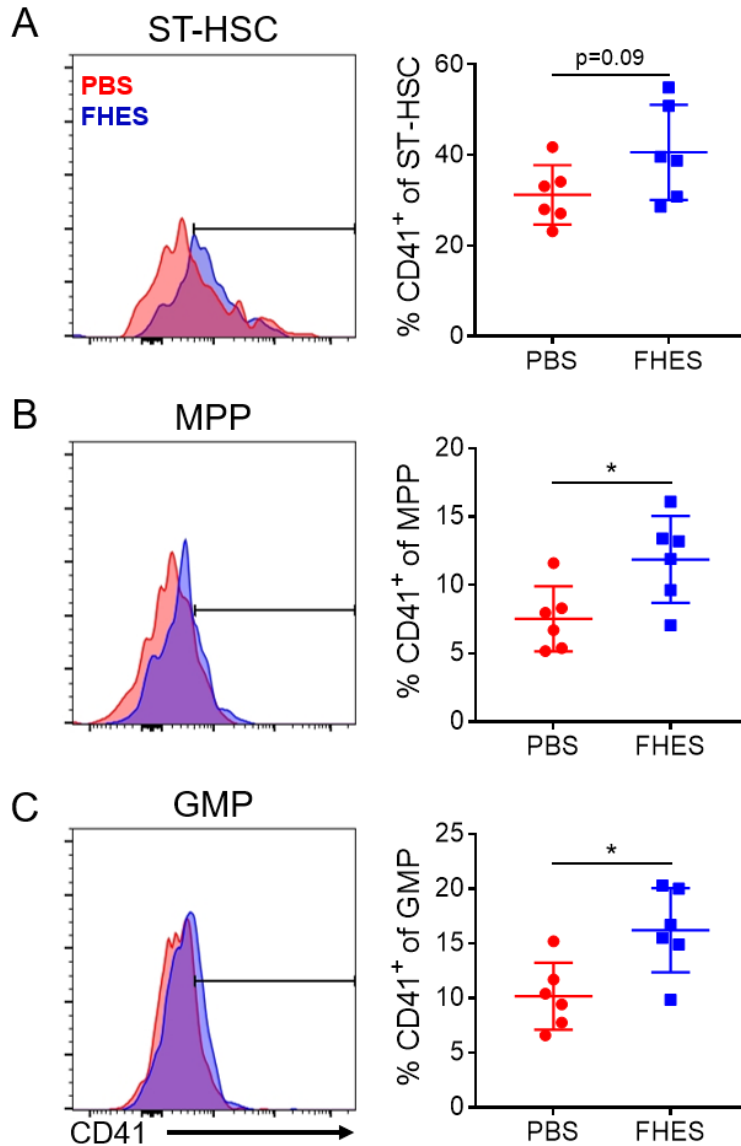
Figure 5.5 Transplanted BM from FHES-treated mice expands myeloid-biased CD41$^+$ HSCs in the BM of recipient mice during EAE. Sub-lethally irradiated (6 Gy) CD45.2 C57BL6/J mice were reconstituted with BM (7.5 x 10^6 cells/mouse) from CD45.1 C57BL6/J mice treated with FHES (100 µg/mouse) or PBS as described in figure 5.1. Two weeks after BM transplantation EAE was induced and on day 3, BM cells were isolated and stained with HSC surface markers and CD41 and analysed by flow cytometry. Representative FACS histograms and CD41 surface expression on ST-HSC (A), MPP (B) and GMP (C). Data are expressed as mean ± SEM (n=6 mice/group) and each symbol represents an individual mouse. *p<0.05 vs PBS-treated control by unpaired t test
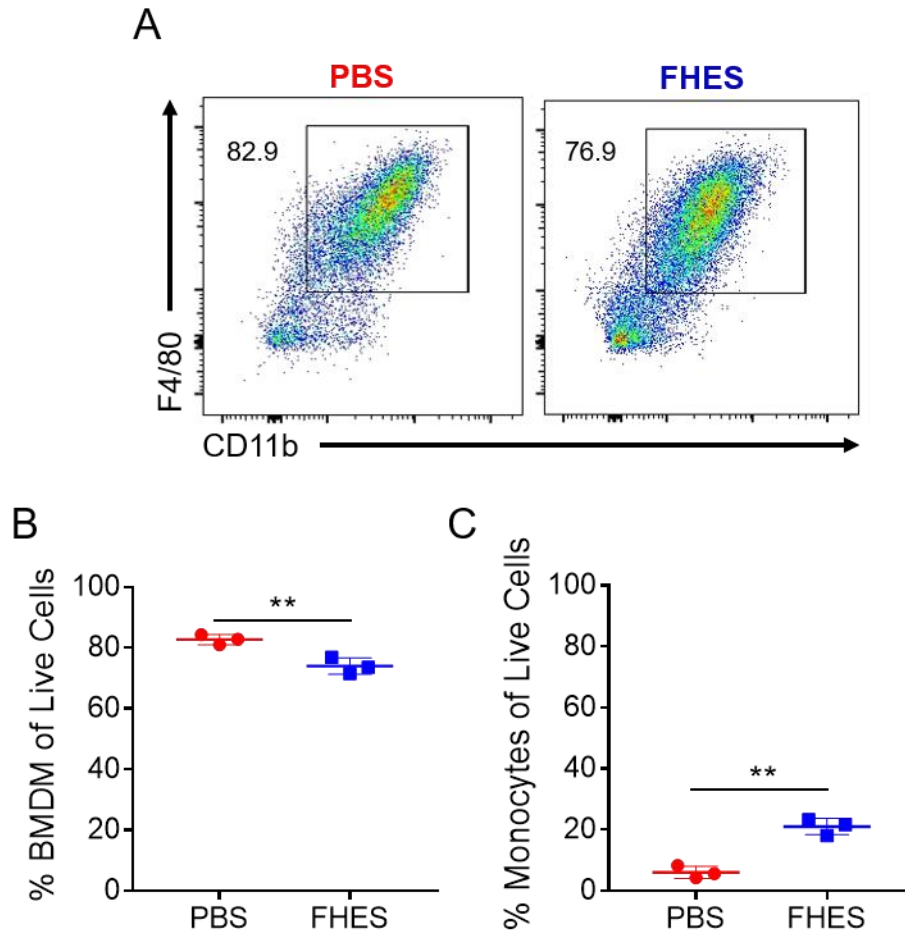
Figure 5.6 Culture of BM with FHES impairs development of BMDM but enhances development of monocytes. BMDM were differentiated from naïve C57BL/6 mice by culture with CSF1-containing L929 media. Cells were cultured with FHES (100 μg/ml) or PBS. On day 7, cells were stained with CD11b, F4/80, CD115 and Ly6C and purity was determined by flow cytometry. (A) Representative FACS plot of CD11b+F4/80+ BMDM. Frequency of BMDM (B) and monocytes (C) on day 7. Results are representative of 2 independent experiments. **p<0.01 vs PBS-treated control by unpaired t test.
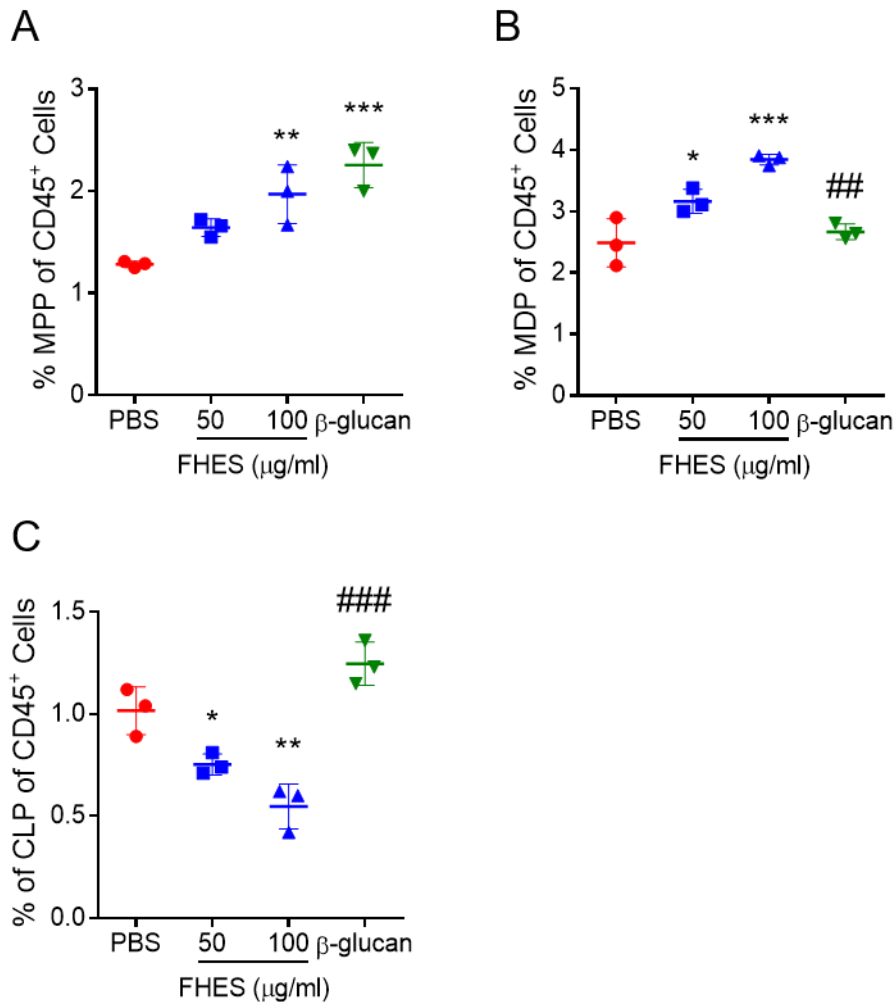
Figure 5.7 Stimulation of BM with FHES expands MPP and MDP cells and suppresses development of CLP *in vitro*. BM cells (1 x 10^6/ml) were cultured with FHES (50 or 100 μg/ml), β-glucan (10 μg/ml) or PBS. After 24 h, cells were isolated and stained for HSC surface markers and analysed by flow cytometry. Frequency of MPP (A), MDP (B) and CLP (C). Results are mean ± SEM for triplicate culture. *p<0.05, **p<0.01, ***p<0.001 vs PBS or ##p<0.01, ###p<0.001 vs FHES (100 μg/ml) by one-way ANOVA with Dunnett post hoc test.
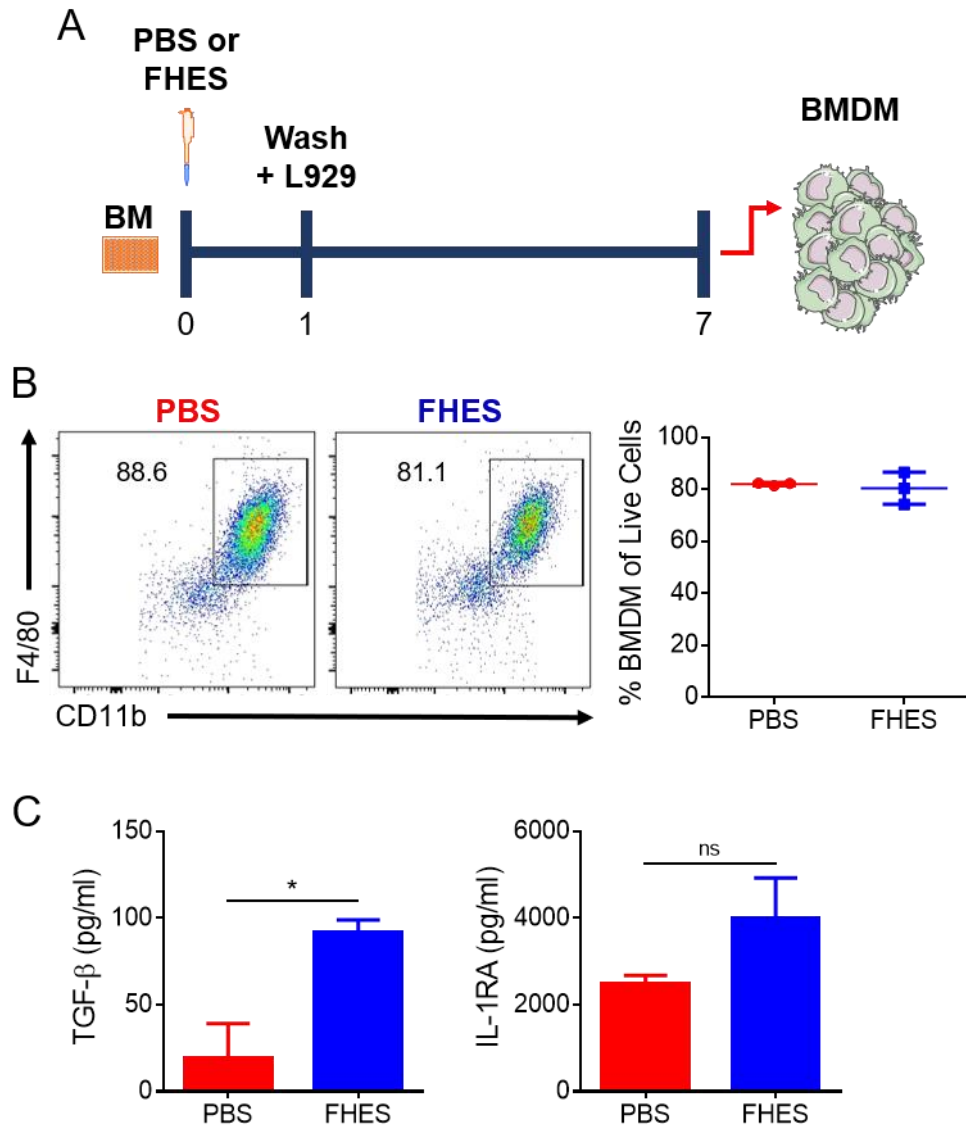
Figure 5.8 Priming of HSCs with FHES before generation of BMDM yields macrophages that secrete elevated IL-1RA and TGF-β. (A) Bone marrow from C57BL/6 mice was incubated with FHES (100 µg/ml) or PBS for 24 hours, cells were then washed with PBS and BMDM generated by addition of L929 supernatants containing CSF-1. On day 7, cells were harvested and stained for CD11b and F4/80 to assess purity. (B) Representative FACS plot and frequency of BMDM in culture. (C) Supernatant was collected and concentrations of TGF-β and IL-1RA quantified by ELISA. Results are representative of 2 independent experiments. Data are mean ± SEM. *p<0.05 vs PBS by unpaired t test.
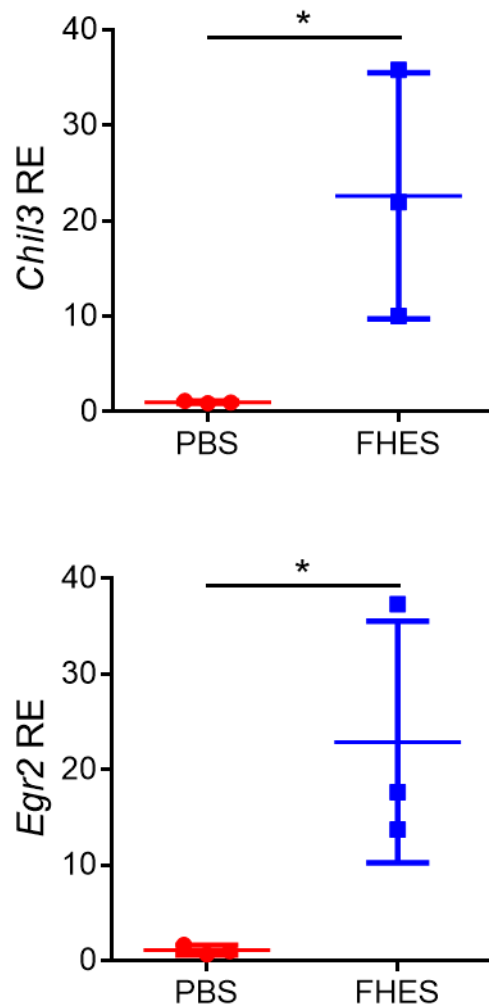
Figure 5.9 Transient treatment of BM with FHES enhances expansion of M2 macrophages. Bone marrow from C57BL/6 mice was cultured with FHES (100 µg/ml) or PBS. After 24 h, cells were washed with PBS and BMDM generated. On day 7, total RNA was extracted and expression of *Chil3* and *Egr2* evaluated by RT-qPCR relative to PBS-treated BMDM following normalization by the endogenous control 18s rRNA. Results are mean ± SEM (n=3). *p<0.05 vs PBS by unpaired t test.
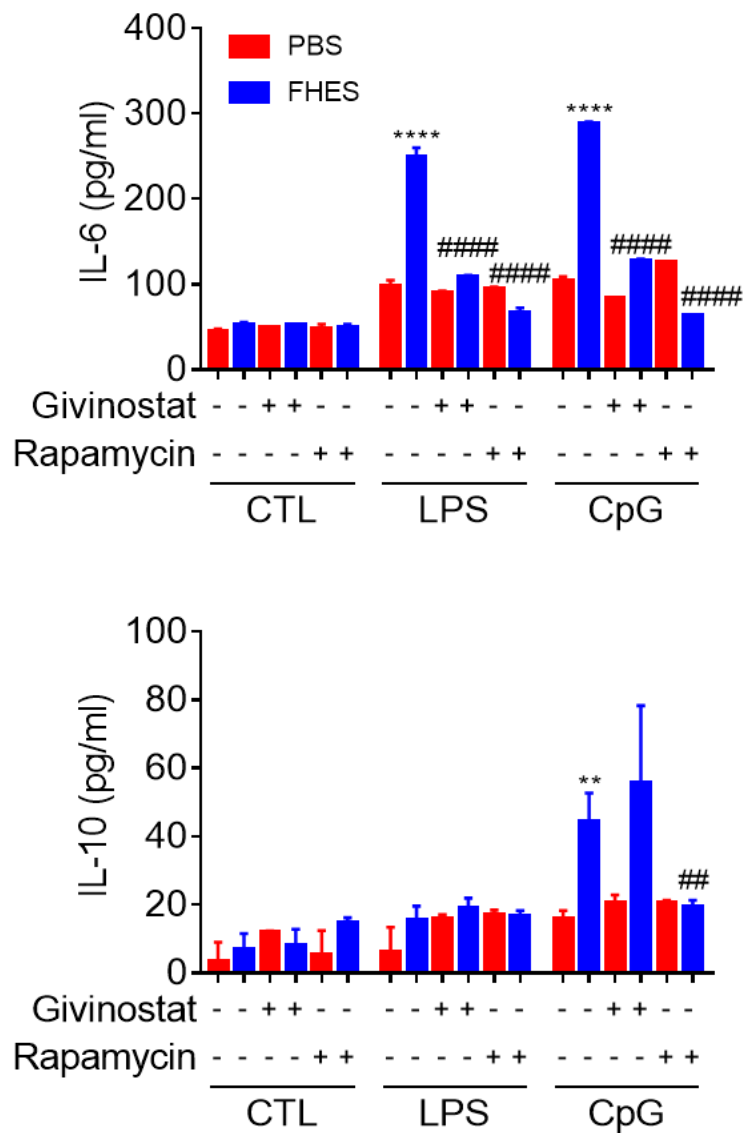
Figure 5.10 FHES modification of myelopoiesis can be inhibited through blockade of HDAC and mTOR activity. BM cells were primed with either FHES (100 μg/ml) or PBS with or without the pan-HDAC inhibitor, Givinostat (25 nM) or mTOR inhibitor rapamycin (5 nM). After 24 h, cells were washed and BMDM generated. On day 7, mature BMDM were harvested and re-stimulated with PBS, LPS (100 ng/ml) or CpG (5 μg/ml). After 24 h, the concentrations of IL-6 and IL-10 in the supernatant were determined by ELISA. Results are mean ± SEM for triplicate culture. **$p<0.01$, ****$p<0.0001$ vs PBS pre-treated control or ##$p<0.01$, ####$p<0.0001$ vs FHES pre-treated by two-way ANOVA with Tukey's multiple comparisons post-test.
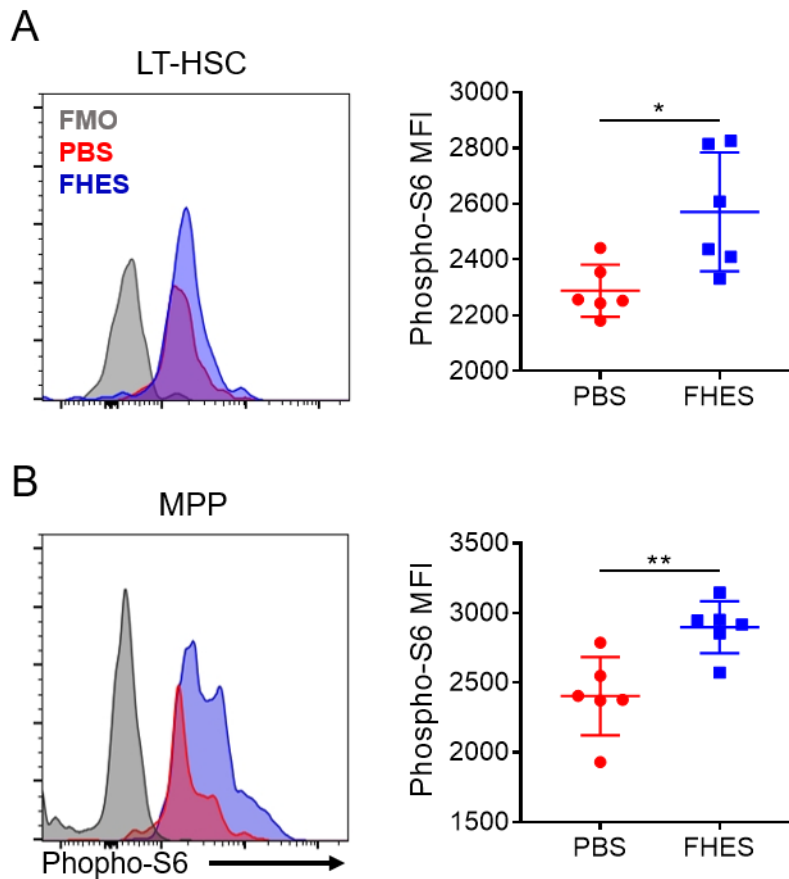
Figure 5.11 Treatment of mice with FHES prior to induction of EAE promotes activation of mTOR in LT-HSC and MPP. Mice were treated as described in figure 4.22. On day 3, BM cells were isolated and stained with HSC surface markers and intracellular Phospho-S6 and analysed by flow cytometry. Representative FACS histogram and MFI of Phospho-S6 intracellular expression in LT-HSC (A) and MPP (B). Data are expressed as mean ± SEM (n=6 mice/group) and each symbol represents an individual mouse. *p<0.05 vs PBS-treated control by unpaired t test

A

PBS   IL-33   IL-4   IL-4 + IL-33

CD16/32

CD34

% CMP of CD45+ Cells

# of CMP (x10^5)

PBS IL-33 IL-4 IL-4+IL-33

B

PBS   IL-33   IL-4   IL-4 + IL-33

CD127

% CLP of CD45+ Cells

# of CLP (x10^4)

PBS IL-33 IL-4 IL-4+IL-33

241
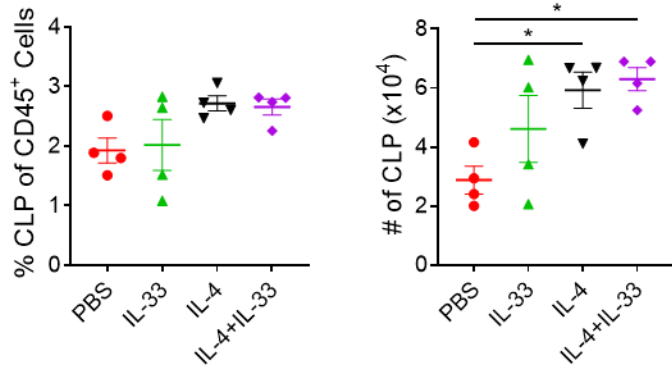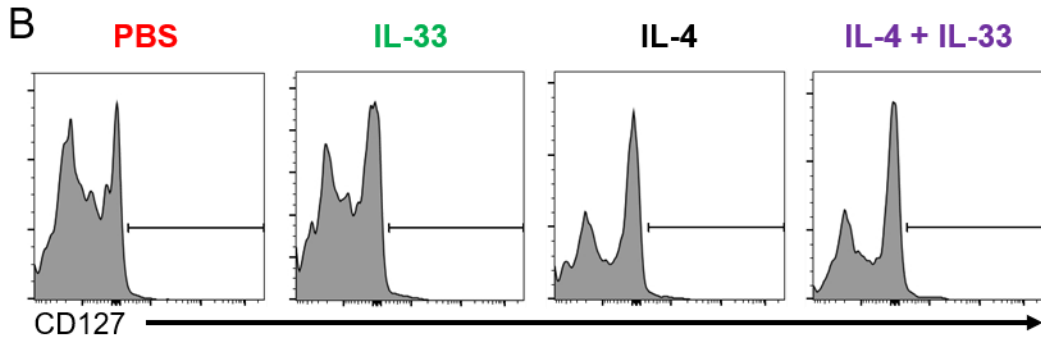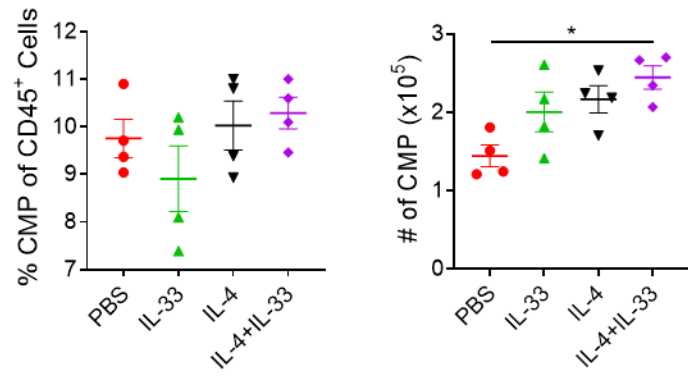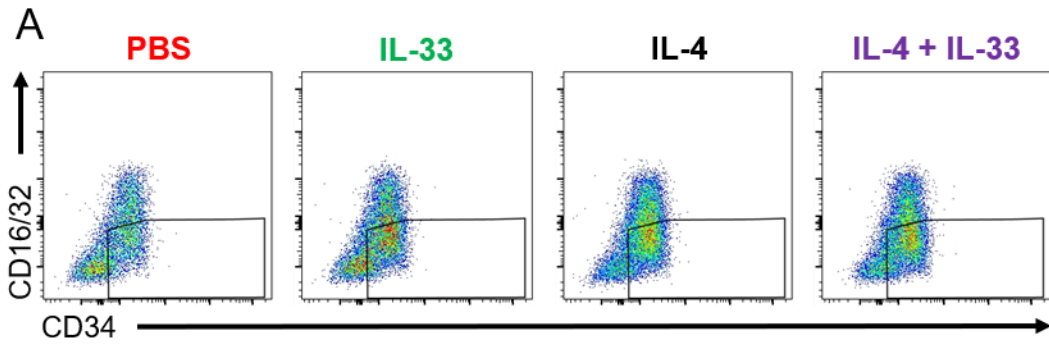
Figure 5.12 Treatment of mice with IL-4 and IL-33 expands CMP and CLP in the BM. C57BL/6 mice were treated s.c. with IL-33 (200 ng/mouse), IL-4 (200 ng/mouse), a combination of IL-4 and IL-33, or PBS on days -21 and -7. On day 0, BM cells were isolated and stained for HSC surface markers and analysed by flow cytometry. (A) Representative FACS plots, frequency and number of CMP (CD45$^+$Lineage$^-$cKit$^+$Sca-1$^-$CD34$^+$CD16/32$^-$). (B) Representative FACS plots, frequency and number of CLP (CD45$^+$Lineage$^-$cKit^{low}Sca-1^{low}CD127$^+$). Results are mean ± SEM (n=4 mice/group) and each dot represents and individual mouse. *p<0.05vs PBS-treated control by one-way ANOVA with Dunnett post hoc test.
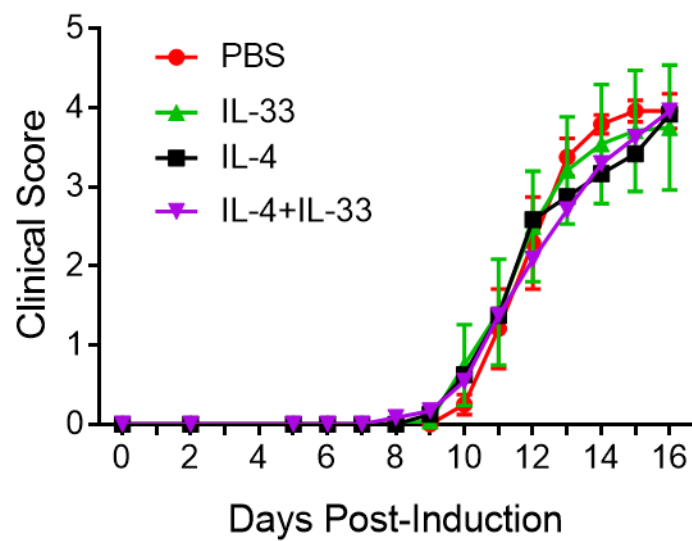
Figure 5.13 Treatment of mice with IL-4 and IL-33 does not alter the course of EAE. C57BL/6 mice were treated s.c. with IL-33 (200 ng/mouse; green), IL-4 (200 ng/mouse; black), a combination of IL-4 and IL-33 (purple), or PBS (red) on days -21 and -7 and EAE induced on days 0 and 2. Clinical scores were assessed daily. Data are mean ± SEM (n=6 mice/group).
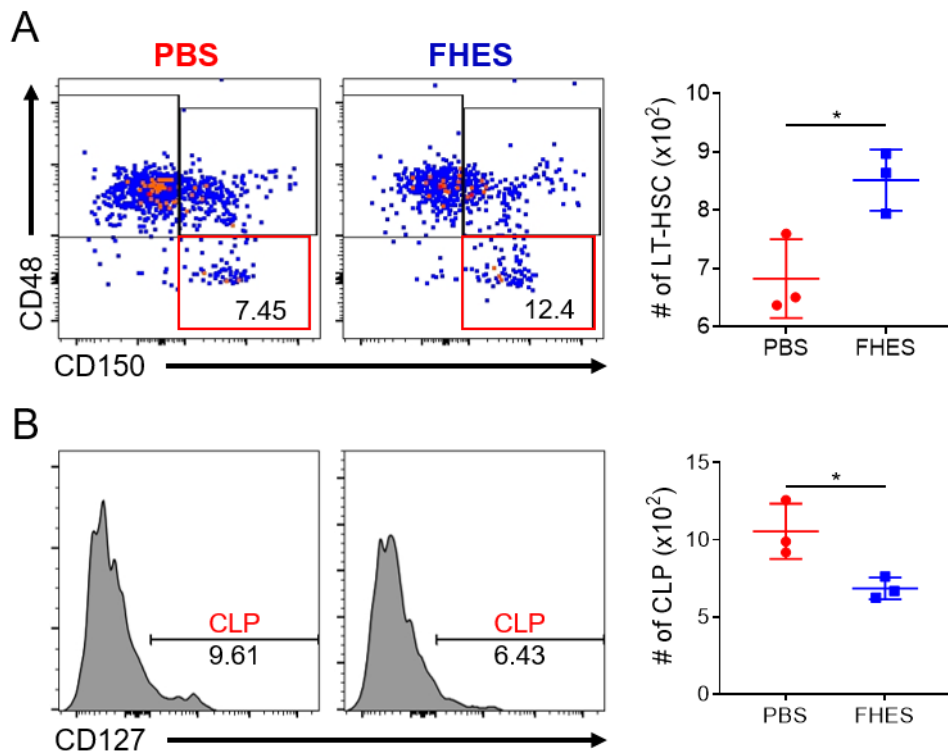
Figure 5.14 Direct stimulation of HSCs with FHES expands LT-HSC and decreases CLP cells *in vitro*. HSCs were enriched from naïve C57BL/6 mice and stimulated with FHES (100 µg/ml) or PBS. After 24 h, cells were harvested and stained for HSC surface markers and analysed by flow cytometry. (A) Representative FACS plots and absolute number of LT-HSC (CD45+Lineage-cKit+Sca-1+; red box). (B) Representative histogram and absolute numbers of CLP (CD45+Lineage-cKit^low^Sca-1^low^CD127+). Results shown are mean ± SEM (n=3). *p<0.05 vs PBS-treated control by unpaired t test.
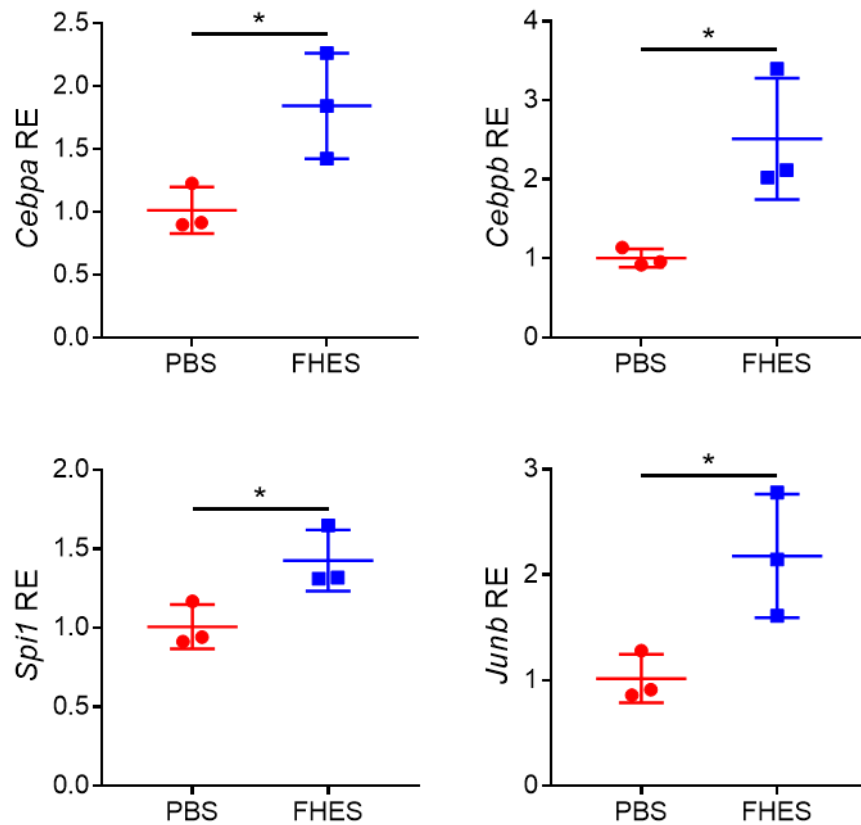
Figure 5.15 Direct stimulation HSCs with FHES significantly enhances expression of myelopoiesis-associated transcription factors. HSCs were enriched from naïve C57BL/6 mice and stimulated with FHES (100 µg/ml) or PBS. After 24 h, total RNA was extracted and expression of *Cebpa*, *Cebpb*, *Spi1* and *Junb* evaluated by RT-qPCR relative to PBS-treated Results shown are mean ± SEM (n=3). *p<0.05 vs PBS-treated control by unpaired t test.
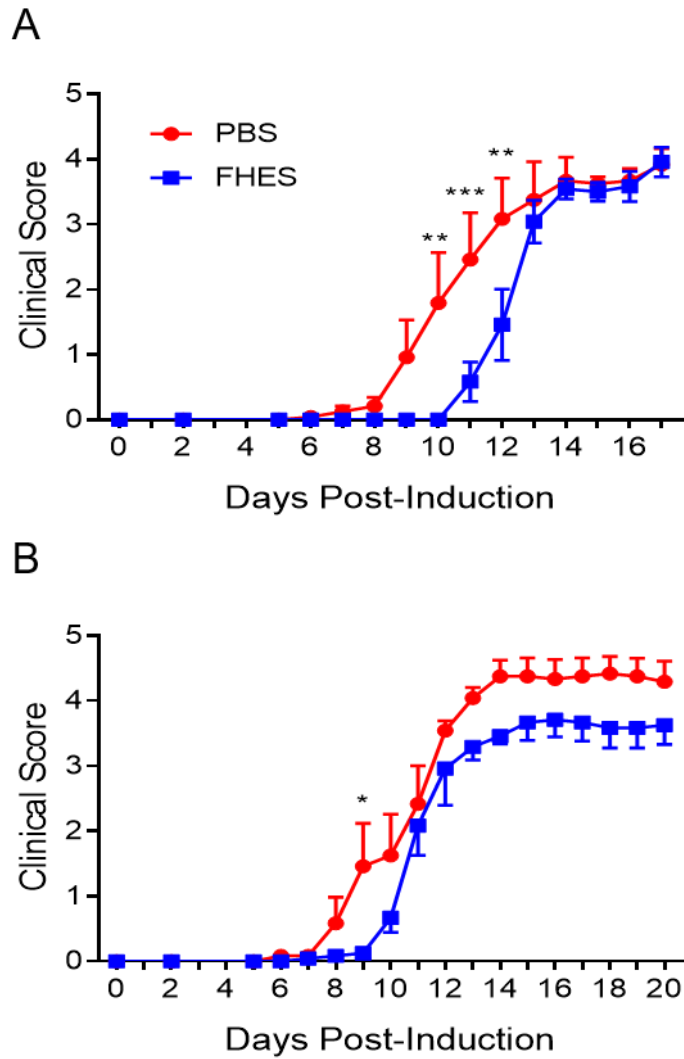
Figure 5.16 Long-term attenuation of EAE by treatment of mice with FHES.
C57BL/6 were injected s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14, and EAE induced 1 month (A) or 8 months (B) after the final injection. Mean clinical scores were assessed daily and mice were sacrificed at peak of clinical score. Data are mean ± SEM (n=6 mice/group). *p<0.05, **p<0.01, ***p<0.001 vs PBS-treated control by two-way ANOVA with Sidak post-test.
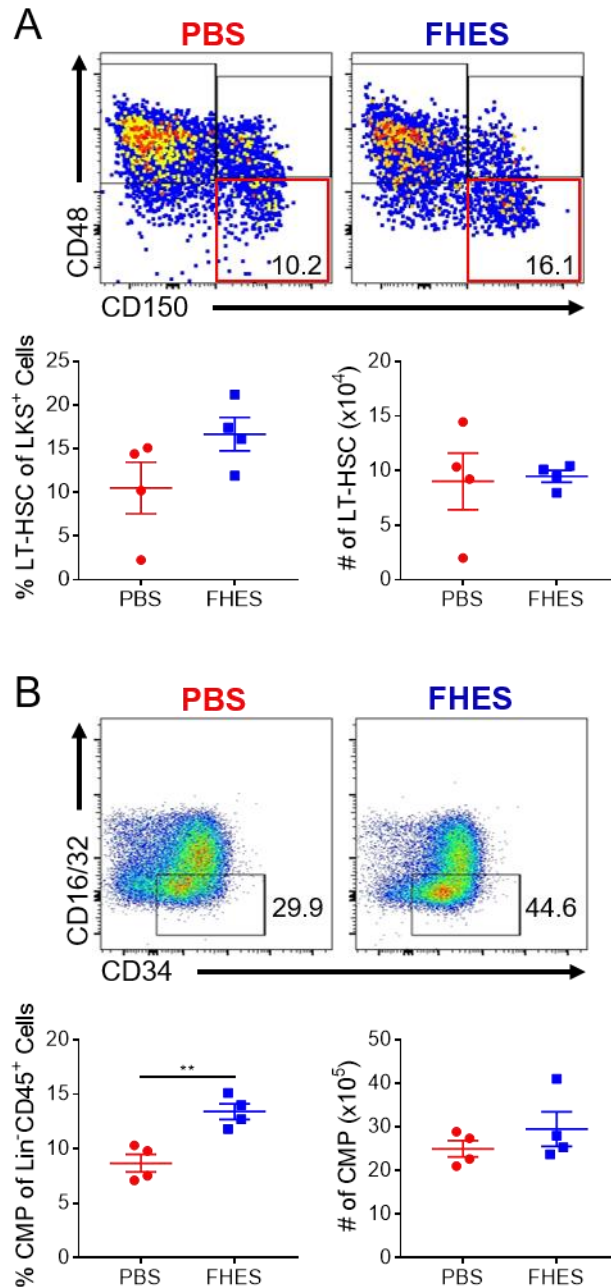
Figure 5.17 Increased LT-HSC and CMP in BM 8 months after treatment of mice with FHES. C57Bl/6 mice were treated s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. 8 months later, BM was collected and assessed for HSC markers by flow cytometry. Representative FACS plots, frequency and absolute numbers of LT-HSC (A; red box) and CMP (B). Results are mean ± SEM (n=4 mice/group). Each dot represents an individual mouse. *p<0.05 vs control by one-way ANOVA with Tukey post-test.
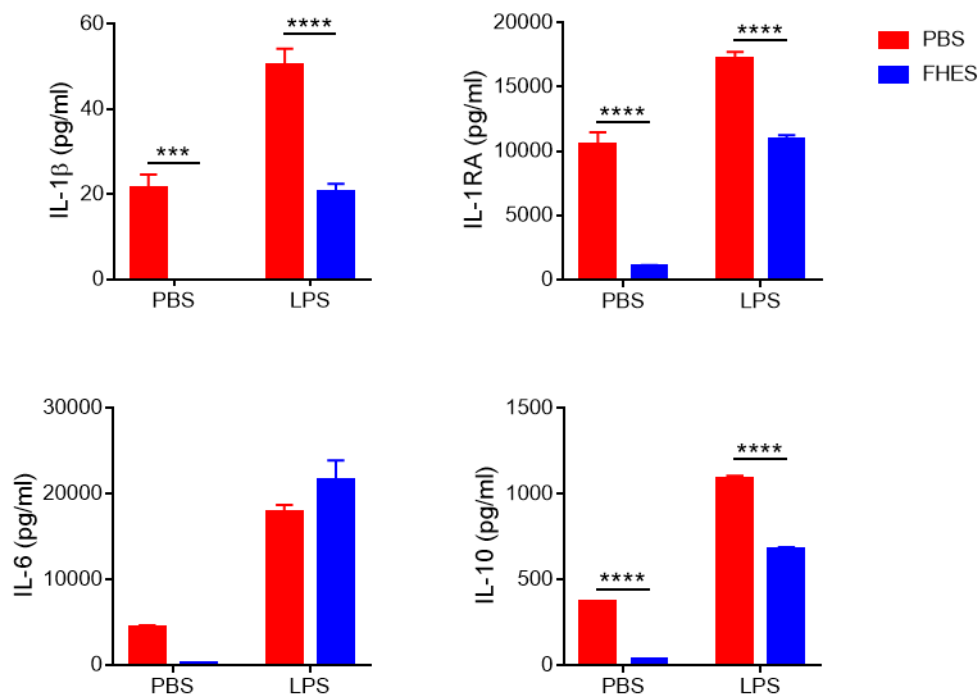
Figure 5.18 Reduced cytokine production by PEC 8 months after treatment with FHES. C57Bl/6 mice were treated s.c. with FHES (100 µg/mouse) or PBS on days 0 and 14. 8 months later, PEC were isolated and re-stimulated with LPS (100 ng/ml) or PBS. After 24 h, supernatants were collected and assayed for IL-1β, IL-1RA, IL-6, and IL-10 by ELISA. Results are mean ± SEM for triplicate culture. ***$p<0.001$, ****$p<0.0001$ vs PBS pre-treated controls by two-way ANOVA with Sidak's multiple comparisons post-test.
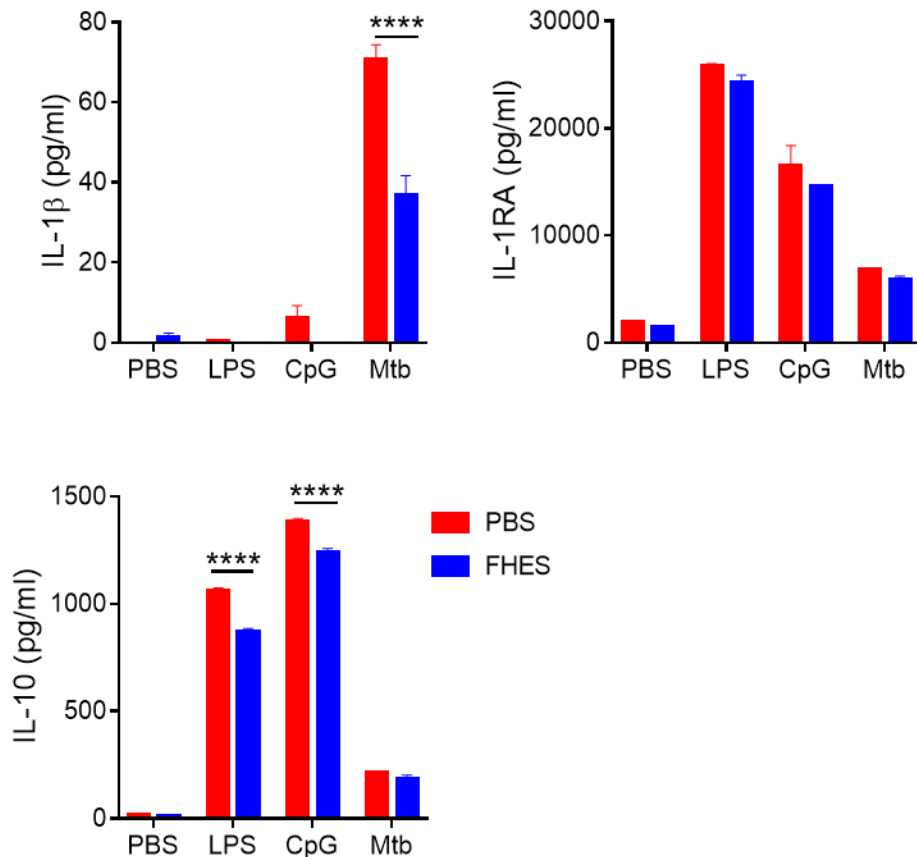
Figure 5.19 Reduced cytokine production by BMDM 8 months after treatment with FHES. C57BL/6 mice were treated s.c. with PBS or FHES (100 µg/mouse) on days 0 and 14. 8 months later, BM was collected and BMDM generated by culturing with L929 supernatants containing CSF-1. On day 7, BMDM were washed, cultured (2 x 10^5/well) and re-stimulated with LPS (100 ng/ml), CpG (5 µg/ml), Mtb (5 µg/ml) or PBS. Supernatants were collected after 24 h and the concentration of cytokines was determined by ELISA. Results are mean ± SEM for triplicate culture. ****p<0.0001 vs PBS pre-treated controls by two-way ANOVA with Sidak's multiple comparisons post-test.
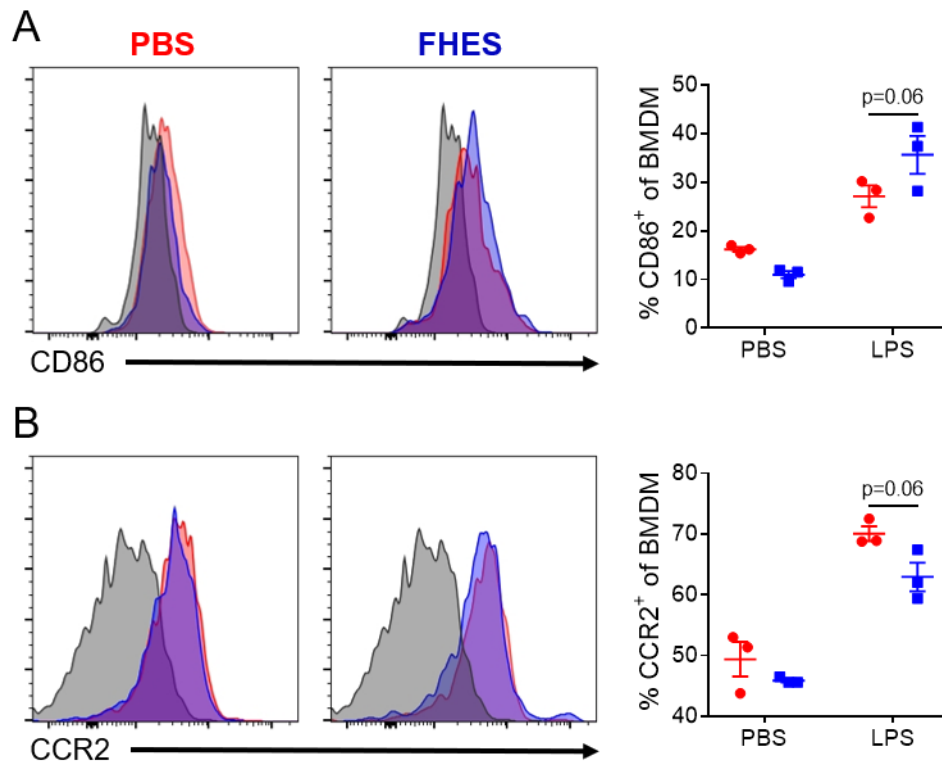
Figure 5.20 Altered surface expression of CD86 and CCR2 on BMDM 8 months after treatment with FHES. C57BL/6 mice were treated s.c. with PBS or FHES (100 μg/mouse) on days 0 and 14. 8 months later, BM was collected and BMDM generated by culturing with L929 supernatants containing CSF-1. On day 7, BMDM were washed, cultured (2 x 10^5/well) and re-stimulated with LPS (100 ng/ml) or PBS, On day 8, BMDM were washed, and stained for fluorescent antibodies specific for CD11b, F4/80, CD86 and CCR2. Representative FACS histograms and frequency of CD86^{+} (A) and CCR2^{+} (B) BMDM. Results are mean ± SEM for triplicate culture. Statistical analysis by two-way ANOVA with Sidak's multiple comparisons post-test.
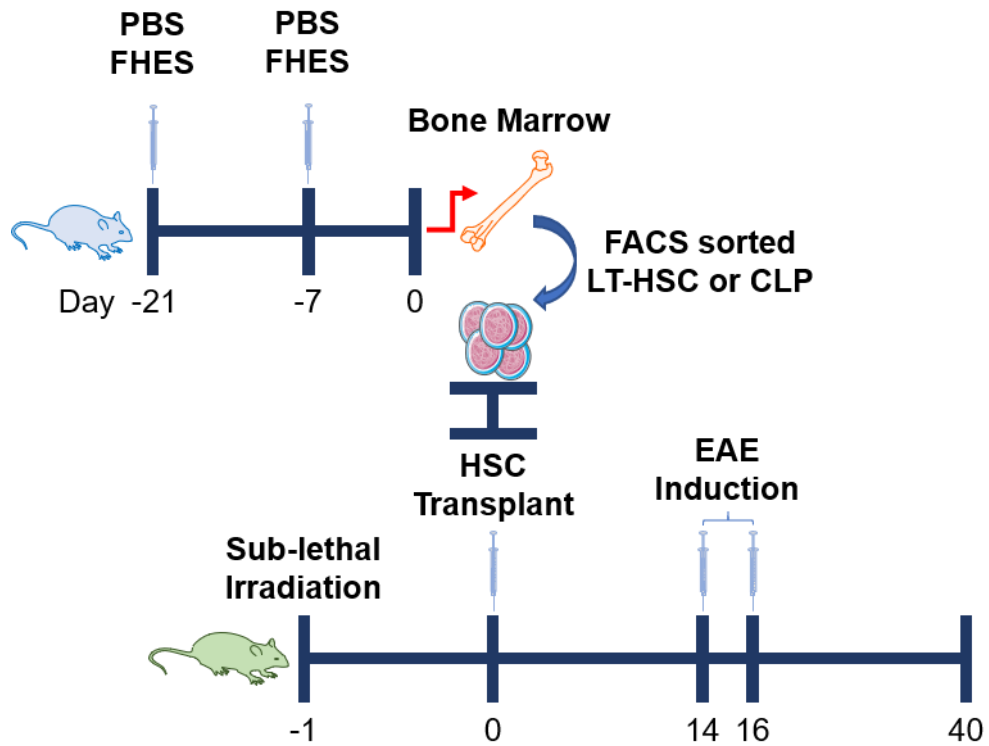
Figure 5.21 Schematic detailing transplantation of FACS sorted HSCs from FHES-treated mice to sub-lethally irradiated mice. C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7 and BM isolated on day 0. Separately, on day -1, naïve C57BL/6 mice were sub-lethally irradiated (6 Gy) to deplete mature immune cells and HSCs. On day 0, BM from FHES- or PBS-treated mice were isolated and LT-HSC (A; 1-3 x 10^4 cells/mouse) or CLP (B; 7.5 x 10^4 cells/mouse) were FACS sorted and injected i.v. into irradiated mice to reconstitute the hematopoietic system. On day 14, EAE was induced, and mice monitored for clinicals signs.
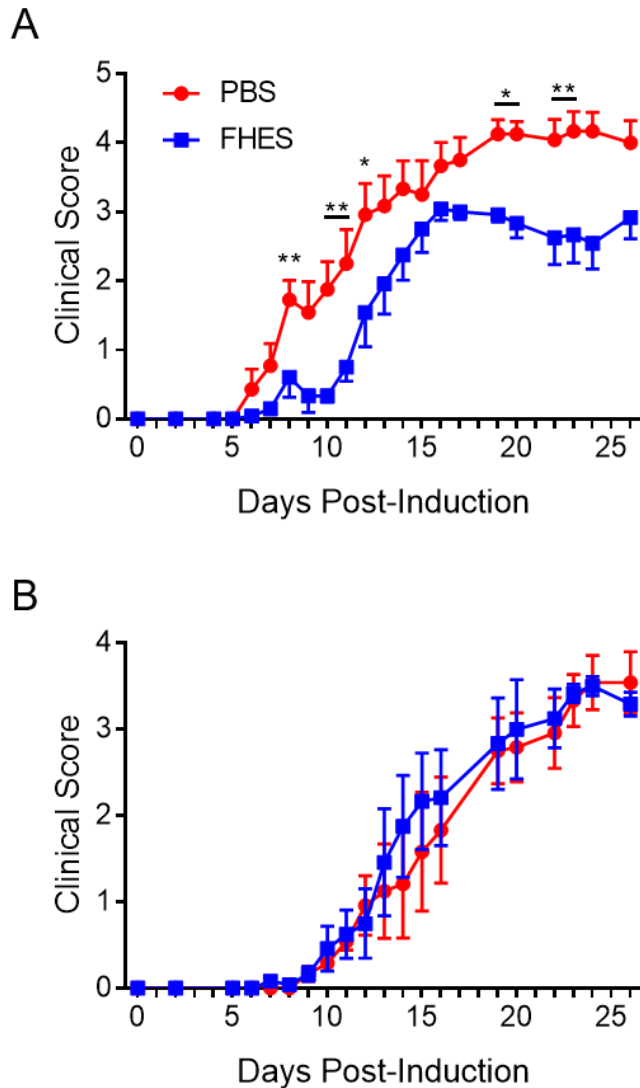
Figure 5.22 Transplantation of LT-HSC, but not CLP, from FHES-treated mice reduces susceptibility to induction of EAE. C57BL/6 mice were injected s.c. with FHES (100 µg/mouse) or PBS on days -21 and -7 and BM isolated on day 0. Separately, on day -1, naïve C57BL/6 mice were sub-lethally irradiated (6 Gy) to deplete mature immune cells and HSCs. On day 0, BM from FHES- or PBS-treated mice were isolated and LT-HSC (A; 1-3 x 10^4 cells/mouse) or CLP (B; 7.5 x 10^4 cells/mouse) were FACS sorted and injected i.v. into irradiated mice to reconstitute the hematopoietic system. On day 14, EAE was induced, and mice monitored for clinicals signs. Data are shown as mean clinical scores ± SEM (n=6-12 mice/group) and are combined from two separate experiments. *p<0.05, **p<0.01, vs PBS-primed LT-HSC recipient by two-way ANOVA with Sidak's post hoc test.
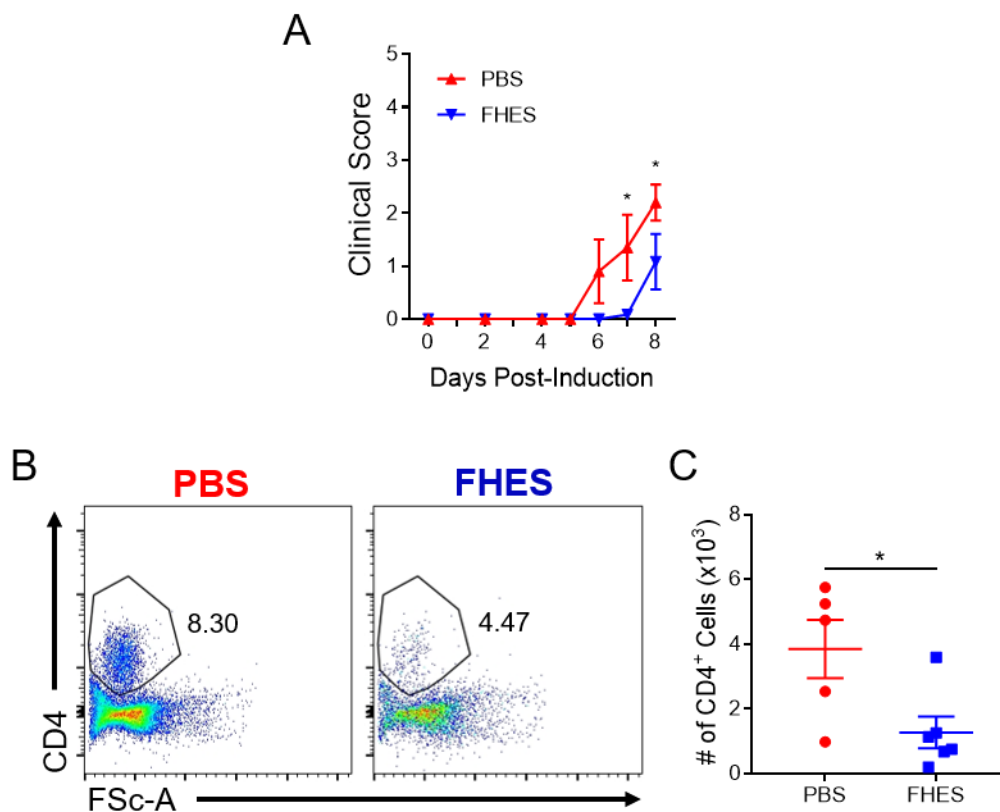
Figure 5.23 Reduced CD4 T cells in brains of mice that received LT-HSC from mice treated with FHES prior to the induction of EAE. Mice were reconstituted with LT-HSC from FHES- or PBS-treated mice as described in figure 5.21. (A) Disease progression was monitored by clinical scores. Data presented as mean ± SEM (n=5-6/group). On day 8 mice were euthanized and cells were isolated from the brains and stained for CD45, CD3, CD4 and analysed by flow cytometry. Results are displayed as representative FACS plots from the brain (gated on CD45$^+$ cells) (B), and absolute number CD3$^+$ CD4$^+$ T cells in the brain (C). Data are expressed as mean ± SEM (n=5-6 mice/group) and each symbol represents an individual mouse. Results are representative of two independent experiments. *p<0.05, **p<0.01 vs PBS-treated control by two-way ANOVA with Sidak post-test (clinical scores) or unpaired t test (absolute number of CD4 T cells).
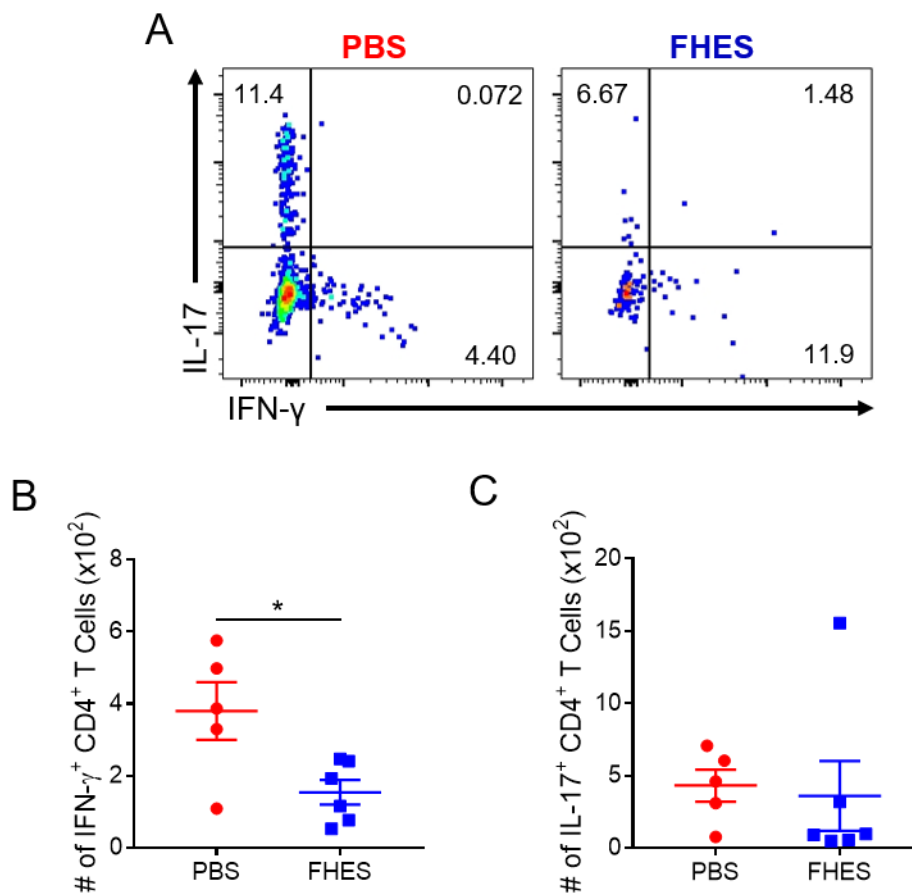
Figure 5.24 Reduced IFN-γ⁺ and IL-17⁺ CD4⁺ T cells in brains of mice that received LT-HSC from mice treated with FHES prior to the induction EAE. Mice were reconstituted with LT-HSC from FHES- or PBS-treated mice as described in figure 5.21. At peak disease on day 8 post-induction of EAE, mice were euthanized, and cells were isolated from the brains and stained for surface CD45, CD3, CD4 and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are displayed as representative FACS plots from the brain (gated on CD45⁺CD3⁺CD4⁺ T cells) (A), and absolute number of IFN-γ⁺ (B) and IL-17⁺ (C) CD4⁺ T cells in the brain. Data are expressed as mean ± SEM (n=5-6 mice/group) and each symbol represents an individual mouse. *p<0.05 vs PBS-treated control by unpaired t test.
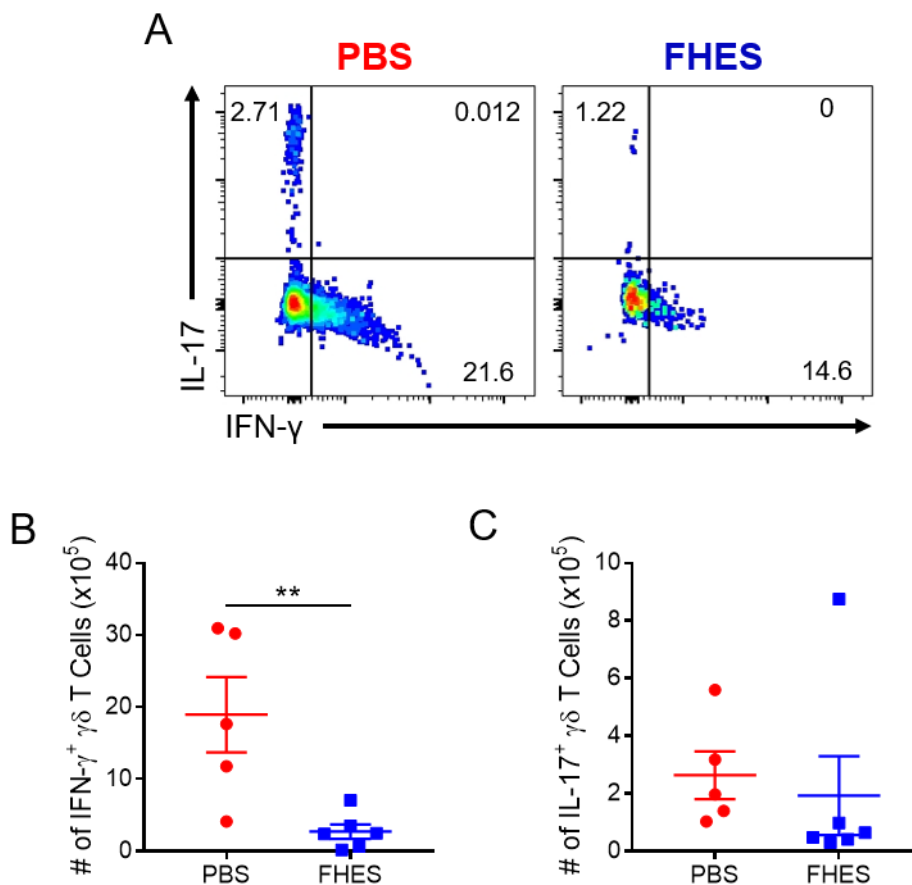
Figure 5.25 Reduced IFN-γ+ and IL-17+ γδ T cells in brains of mice which received LT-HSC from mice treated with FHES prior to the induction EAE. Mice were reconstituted with LT-HSC from FHES- or PBS-treated mice as described in figure 5.21. At peak disease on day 8 post-induction of EAE, mice were euthanized, and cells were isolated from the brains and stained for surface CD45, CD3, TCRγδ and intracellular IL-17A and IFN-γ and analysed by flow cytometry. Results are displayed as representative FACS plots from the brain (gated on CD45+CD3+TCRγδ+ T cells) (A), and absolute number of IFN-γ+ (B) and IL-17+ (C) γδ T cells in the brain. Data are expressed as mean ± SEM (n=5-6 mice/group) and each symbol represents an individual mouse. **p<0.01 vs PBS-treated control by unpaired t test.
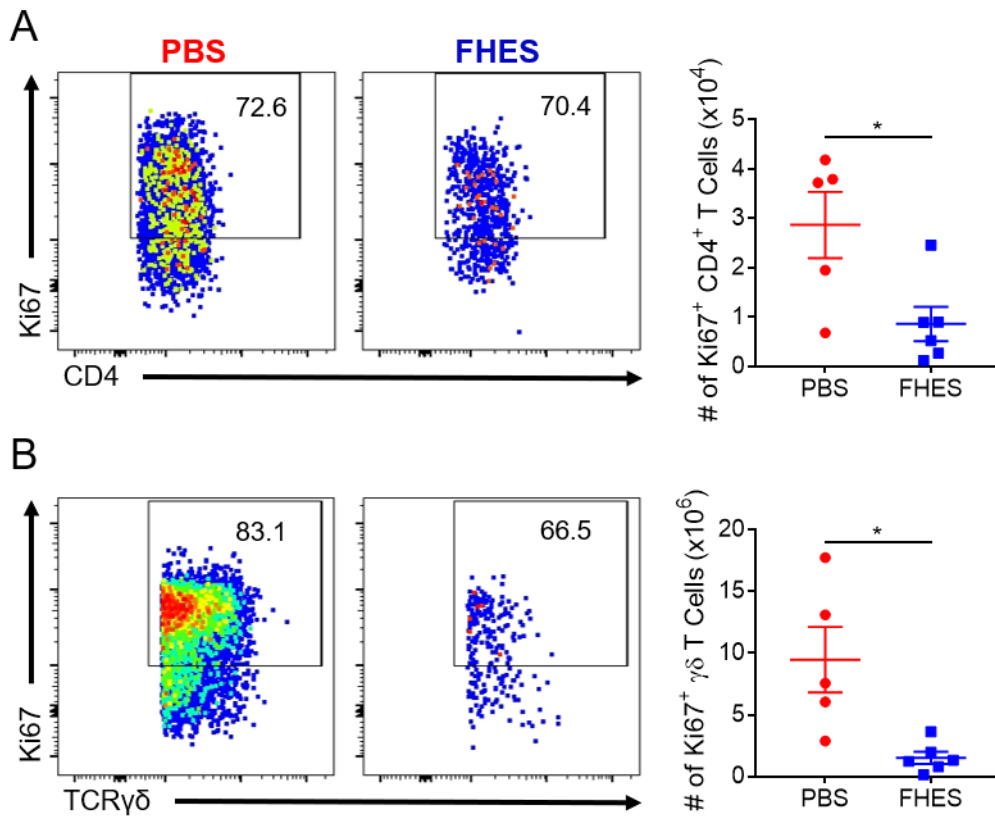
Figure 5.26 Reduced Ki67+ CD4+ and γδ T cells in brains of mice which received LT-HSC from mice treated with FHES prior to the induction EAE. Mice were reconstituted with LT-HSC from FHES- or PBS-treated mice as described in figure 5.21. At peak disease on day 8 post-induction of EAE, mice were euthanized, and cells were isolated from the brains and stained for surface CD45, CD3, CD4, TCRγδ, intranuclear Ki67 and analysed by flow cytometry. Results are displayed as representative FACS plots and absolute number of Ki67+ CD4+ T cells (A) and Ki67+ γδ T cells (B) in the brain. Data are expressed as mean ± SEM (n=5-6 mice/group) and each symbol represents an individual mouse. *p<0.05 vs PBS-treated control by unpaired t test.
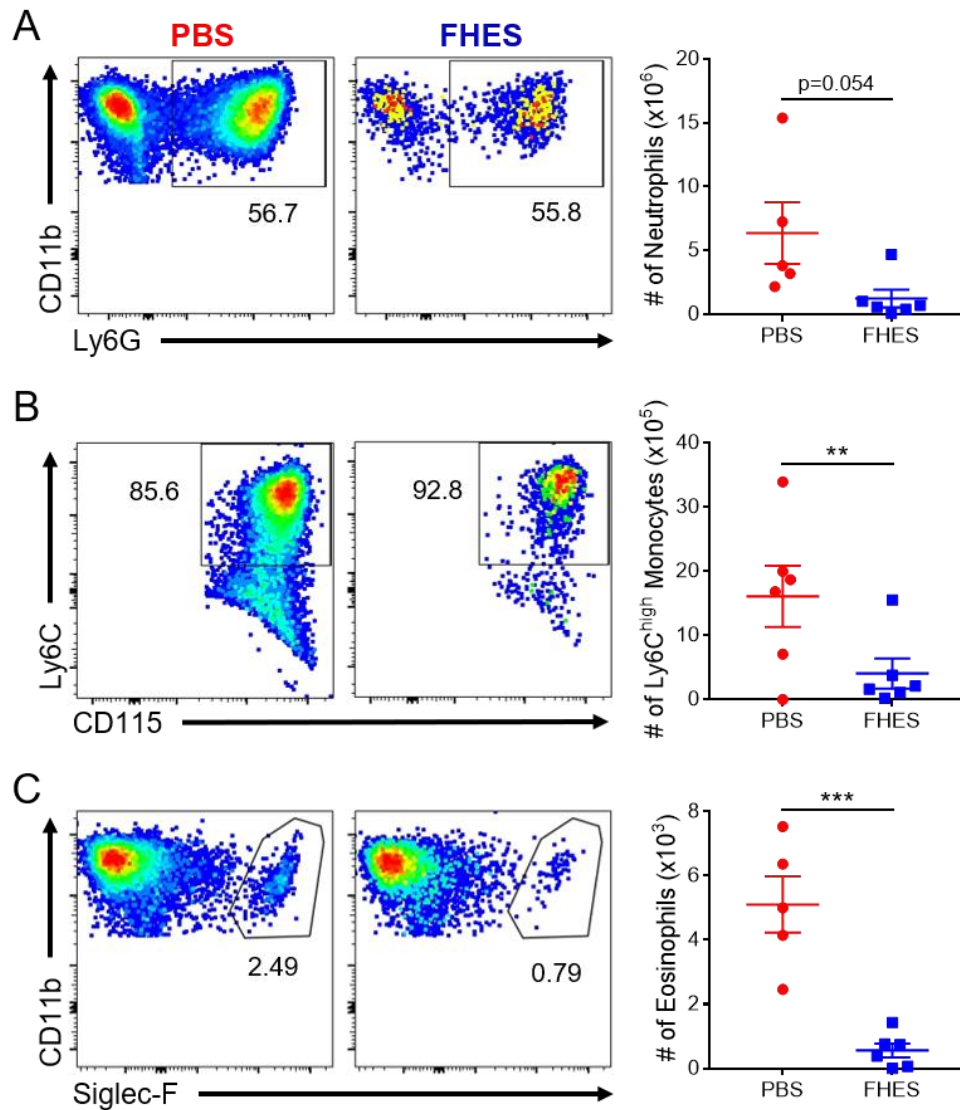
Figure 5.27 Reduced neutrophils, eosinophils and Ly6C^{high} monocytes in brains of mice which received LT-HSC from mice treated with FHES prior to the induction EAE. Mice were reconstituted with LT-HSC from FHES- or PBS-treated mice as described in figure 5.21. At peak disease on day 8 post-induction of EAE, mice were euthanized, and cells were isolated from the brains and stained for surface CD45, CD11b, F4/80, CD115, Ly6C, and Siglec-F analysed by flow cytometry. Representative FACS plots and absolute number of neutrophils (CD45$^+$CD11b$^+$Ly6G$^+$) (A), inflammatory Ly6C^{high} monocytes (CD45$^+$Ly6G$^-$Siglec-F$^-$F4/80$^-$CD11b$^+$CD115$^+$Ly6C^{high}) (B), and eosinophils (CD45$^+$CD11b$^+$Siglec-F$^+$SSc^{high}) (C) in the brain. Data are expressed as mean ± SEM (n=5-6 mice/group) and each symbol represents an individual mouse. *p<0.05 vs PBS-treated control by unpaired t test.

Chapter 6: General Discussion

6.1 General Discussion

The significant new finding of this study is the demonstration that the helminth *Fasciola hepatica* can sustain a state of immunosuppression by promoting long-lived anti-inflammatory trained innate immunity by modulating HSCs. The results showed that injection of mice with FHES induced modifications to HSCs, which altered hematopoiesis and resulted in the production of anti-inflammatory monocytes and macrophages. The most significant finding was that FHES could directly interact with oligopotent LT-HSC, modifying the epigenetic and metabolic profile to confer an anti-inflammatory myeloid bias that could be transferred to irradiated mice by bone marrow transplantation. Mice that received whole BM or LT-HSC from mice treated with FHES had suppressed immune responses to the induction of EAE, including fewer inflammatory innate and adaptive immune cells that infiltrated the CNS, resulting in delayed onset and reduced severity of disease. Our findings provide a novel explanation for long-lived attenuating effects of parasite products on the immune responses that mediate autoimmune diseases.

As infection rates in high-income countries have fallen over the 20th and 21st centuries, there has been a commensurate increase the rate of inflammatory disorders [399]. Epidemiological evidence for over forty years has shown that children born into environments favourable to the development of infections, such as rural homes with poor sanitation and an increased number of siblings, correlated with a decrease in the incidence of asthma and allergies [400]. Indeed, genetic studies on identical twins to identify risk factors have confirmed that heritable difference in gene expression do not account for the majority of asthma and allergy seen in high-income countries [401]. The original hygiene hypothesis proposed that a reduction in allergies mediated by Th2 responses reflected a reduction in the incidence of Th1-inducing infection [247]. However, this was revised following the recognition that helminth infection could also suppress autoimmune diseases that were driven by Th1 and Th17 responses [255]. This was explained on the basis of the fact that parasites had a propensity to induce Treg cells, which suppressed pathogenic Th1 or Th17 cells that mediate a variety of immune mediated diseases [248–250]. However, there is growing evidence

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that helminths and other pathogens can modulate innate as well as adaptive immune responses.

Helminths have evolved several mechanisms to modulate the innate and adaptive immune responses of the host to prevent their expulsion and minimize tissue damage and immunopathology. Due to their distinct immunomodulatory activity, helminths can exert potent immunosuppression on the immune responses that mediate allergic and autoimmune diseases. Many studies have highlighted this in human populations around the world. For example, in 2000, van den Biggelaar et al. demonstrated that Gabonese children with schistosomiasis were less prone to the development of allergy [402]. Further studies in Nigeria, Ecuador, Uganda and many more places have provided further evidence for the negative association between helminth infection and allergic and autoimmune diseases [403–406]. Correale and Farez found that relapsing-remitting MS patients that became infected with intestinal helminths exhibited no disease relapses across a 5 year period, and that disease attenuation was associate with enhanced IL-10 and TGF-β [254]. Helminth infection has also been implicated in playing a protective role against the development of IBD, ulcerative colitis, type 2 diabetes, rheumatoid arthritis, and Crohn's disease [319].

Animal models of allergic and autoimmune diseases have aided in further understanding helminth-mediated immunosuppression, however, there has been little progress in the use of live helminths or helminth-derived products as effective therapies in the clinical setting. Controlled infection with low doses of the hookworm *N. americanus* was shown to provide long-lasting protection against disease relapse in patients with Crohn's disease [262]. However, studies in MS patients have yielded mixed results. In a recent phase 2 clinical trial, infection of MS patients with low doses of *N. americanus* proved to be well-tolerated and able to enhance Treg cell responses, however, while there was a promising trend towards fewer relapses, there was no significant differences in relapse rates when compared with uninfected patients [265]. Furthermore, clinical trials with *T. suis* and *T. suis*-derived products have shown similarly negative results, indicating an inability of live helminth infection to significantly modify MS disease despite good tolerability [264, 268, 269]. Notably, these

studies were associated with increased regulatory and decreased inflammatory cytokines in the circulation, and found modest, but not significant improvements in disease outcome. These results indicate that more research must be conducted to understand how helminth infections confer protective effects against autoimmune diseases in humans.

The majority of studies on the hygiene hypothesis have assessed modification of adaptive immune cell responses [407]. However, a potential explanation for the inverse correlation between infection and asthma/allergy has been suggested to be partially the result of trained innate immunity [394]. It was recently demonstrated that infection of mice with murine gammaherpesvirus 68 inhibited the development of house dust mite-induced experimental asthma through replacement of alveolar macrophages with BM-derived regulatory monocytes [395]. Moreover, modified monocyte-derived tolerogenic macrophages persisted for several weeks after infection, indicating long-term functional training. Tissue-resident macrophages, such as microglia in the CNS and alveolar macrophages in the lungs, have also been shown to develop a trained phenotype that lasts for several months [366, 408].

Parasitic infections have recently been shown to confer trained innate immunity. Chen et al., demonstrated that infection of mice with *N. brasiliensis* resulted in the priming of macrophages, resulting in enhanced clearance of future parasitic infections [111]. Work from our lab has shown that treatment of mice with the helminth-derived product FHTE induced anti-inflammatory trained innate immunity in peripheral macrophages, resulting in suppressed pro-inflammatory responses to PAMPs, culminating in reduced susceptibility to induction of EAE [113]. Consistent with this finding, the present study revealed that FHES induced anti-inflammatory trained innate immunity in BMDM. However, in contrast with data underlying the role of peripheral mature macrophages in training, the present project found that treatment of mice with FHES did not modify tissue-resident macrophages, as seen by mRNA expression in FACS-sorted LPM. However, FHES did induce systemic innate activation and expansion of regulatory patrolling Ly6C^{low} monocyte populations. The circulating pool of Ly6C^{low} monocytes, which were found to fill the circulation and peripheral niches, exhibited enhanced M2 mRNA expression upon maturation into macrophages in

the peritoneal cavity and spleen. Under classical inflammatory conditions, monocytes mature into DCs or macrophages, which phagocytose and present antigen to T cells to induce adaptive immune responses. Therefore, modification of monocytes is a potentially useful method of inhibiting many effector responses to parasite infection. Indeed, individuals infected with *N. americanus* have elevated levels of circulating monocytes, which exhibit an increased frequency in IL-10 compared with IL-12 secretion [409]. Notably, there was no difference in IL-4 expression between hookworm-infected and non-infected individuals, suggesting this regulatory monocytic response was not due to systemic type-2 cytokines, but rather direct modification of monocytes by helminth-derived products. Furthermore, monocyte-derived DC from hookworm-infected individuals have been shown to express less HLA and co-stimulatory markers, such as CD86, which was shown to be partially due to ES products [410].

In the first study detailing trained innate immunity, Kleinnijenhuis et al. demonstrated that PBMC from patients vaccinated with BCG expressed more pro-inflammatory cytokines in response to various PAMPs up to 3 months post-vaccination [89]. However, circulating innate immune cells are characteristically short-lived, with monocytes being renewed every few days [1, 62]. In contrast, HSCs are long-lived and give rise to all immune cells, during steady-state and emergency haematopoiesis. BCG and β-glucan have been shown in mice and humans to induce epigenetic and metabolic modifications in HSCs, resulting in the differentiation of trained innate immune cells with an enhanced inflammatory profiles, in what is now referred to as central trained innate immunity [118, 119, 147, 148]. Treatment of mice with BCG or β-glucan was shown to expand MPP in the BM, conferring pro-inflammatory trained responses to downstream mature innate immune cells. These recent advances have highlighted the role of HSCs as pivotal determinants in providing immunological memory. Stimulation of HSCs with PAMPs, DAMPs, cytokines, and growth factors shape the initial immune response and imprint long-lasting epigenetic marks that modify the HSC response to future inflammatory challenge [116]. These modifications have been found to induce a myelopoiesis bias, enhance mycobacterial clearance, and improve innate responses to secondary challenges, ranging from treatment with LPS to radiation-induced myeloablation.

In the present study, we found that products of the helminth parasite *F. hepatica* could modify LT-HSC and downstream progenitors metabolically and epigenetically to imprint a memory-like phenotype and direct anti-inflammatory responses to secondary stimuli. BMDM generated from the BM of mice treated with FHES secreted less pro-inflammatory and more anti-inflammatory cytokines in response to stimulation with PAMPs. Furthermore, BMDM had diminished surface expression of MHC-II and co-stimulatory molecules, resulting in suppression of T cell activation. A recent study by de Laval et al., demonstrated that LPS can induce training in LT-HSC in response to LPS stimulation [120]. They showed that treatment of mice with LPS-primed LT-HSCs resulted in increased myelopoiesis and enhanced survival following subsequent infection with *P. aeruginosa* in a C/EBP-β-dependent mechanism. Furthermore, Khan et al. have shown that Mtb target and inhibit central training as a method of immune evasion [146]. Mtb induced the production of IFN-β that inhibited protective trained immunity in HSCs, and initiated necroptosis in myeloid progenitors and a commensurate expansion of CLP. This suggests that HSCs may act as adaptive hubs for immune evasion strategies by pathogens, and therefore may act as potentially viable targets for potential therapeutics for immune-mediated diseases. Indeed, vaccination with BCG induces type-1 IFN production and myelopoiesis, which has been demonstrated to improve monocyte and macrophage anti-bacterial responses, resulting in long-term protection against Mtb and other unrelated pathogens [148]. In contrast to these studies, the present study found no clear change in IFN signalling in mice treated with FHES, suggesting a novel mechanism for the induction of anti-inflammatory central training.

Our findings demonstrate that anti-inflammatory central trained innate immunity by helminth products can modulate T cell responses that mediate autoimmune disease. Most research on helminth-induced immunomodulation in autoimmunity has focused on direct suppression of classical M1 macrophage activation and the induction of type-2 or regulatory mature immune cells. Indeed, helminth infection or treatment of mice with helminth-derived products has been shown to inhibit EAE through M2 macrophages, eosinophils, Th2 cells and Treg cells [79]. The results in the present study revealed that treatment of mice with FHES

attenuated EAE through modifications to the BM niche. Direct stimulation of MOG-specific T cells with FHES was unable to inhibit IFN-γ, IL-17A or GM-CSF production. Furthermore, adoptive transfer of MOG-specific T cells from donor mice treated with FHES was capable of inducing EAE in recipient mice. However, the results presented here revealed that transplantation of BM from mice treated with FHES was associated with delayed onset of EAE in recipient mice. Furthermore, the anti-inflammatory trained profile of macrophages generated from HSCs was transferrable to recipient mice. Mice that received a BM transplant from mice treated with FHES expanded macrophages in the PEC that produced more IL-1RA and IL-10, and less TNF in response to LPS stimulation. Additionally, BMDM generated from recipient mice expressed less co-stimulatory molecules on their surface. These results suggest that anti-inflammatory modifications to HSCs is transferrable and could be exploited for the treatment of autoimmune disease or in conjunction with BM transplantation.

Transplantation of HSCs is an effective therapeutic approach for a number of chronic diseases, including primary immunodeficiencies, haematological cancers and autoimmune diseases, such as MS [142]. Many HSC transplants utilize autologous HSCs reintroduced to the body after chemotherapy or radiation therapy [143]. Alternatively, allogeneic transplantation of HSCs from a non-self donor has the advantage of graft-vs-leukemia response in treating hematological cancers, and a decreased susceptibility to development of further autoimmune diseases [144]. However, allogeneic HSC transplantation is associated with the risks of developing graft versus host disease. Conversely, the possibility of disease relapse or development of a secondary autoimmune disease is a common adverse effect of autologous HSC transplantation [142]. The present study found that transplantation of BM or LT-HSC transferred anti-inflammatory central training and delayed the onset and reduced the severity of EAE in recipient mice.

Classically activated macrophages are enhanced in the BM of patients with poor graft function after allogeneic HSC transplantation [411]. Furthermore, macrophages from the BM of patients with poor graft function secreted more TNF and IL-12 when co-cultured with HSCs *ex vivo*. HSCs co-cultured with macrophages from patients with poor graft function had enhanced reactive

oxygen species production and apoptosis, resulting in a reduced ability to differentiate into all cell types of the hematopoietic system. In contrast, this study revealed that treatment of donor mice with FHES transferred myeloid bias to recipient mice, with increased anti-inflammatory macrophages in the periphery and BM, culminating in reduced severity of disease during EAE (Fig. 6.1).

The findings of this study suggest that anti-inflammatory central training could be a potential target for therapies aimed at improving HSC transplant tolerance and its effectiveness in treating autoimmune disease. Currently, there is a dearth of effective, disease-modifying treatments for autoimmune diseases. Antibodies targeting pathogenic cytokines have seen considerable success in treating many autoimmune disorders, including RA, psoriasis and IBD, however there have been mixed results for their use in treating MS [412]. For example, the anti-IL-17A Secukinumab has shown efficacy in psoriasis, and has been shown to reduce the number of MS lesions [413, 414]. Ustekinumab, an antibody therapy targeted at IL-12p40, the common subunit of IL-12 and IL-23, has shown positive results in psoriasis, though it appears to be not efficacious in MS [415, 416]. Plozalizumab, an anti-CCR2 antibody, has been demonstrated to decrease synovial inflammation in RA patients and has been speculated to potentially inhibit inflammatory monocytes from entering the brain in MS [417, 418]. However, research has shown promising efficacy in using autologous HSC transplantation for the treatment of MS. The ability to epigenetically and metabolically prime HSCs to improve their transplantation engraftment has yet to be studied in full. However, recent advances have highlighted a potential role for training HSCs to improve transplant tolerance. Braza et al. recently reported that pro-inflammatory training of macrophages during heart transplantation impeded transplant tolerance [136]. However, treatment with a nanobiologic inhibitor of myeloid cell specific-mTOR was effective at inducing tolerogenic macrophages and improved recipient survival. Furthermore, although the nanobiologic was specific for mature myeloid cells in the periphery, the study showed that the nanobiologic was able to inhibit mTOR in HSCs *in vivo*. This suggested that, in addition to targeting peripheral training, HSC modifications may play a role in mediating transplant tolerance.
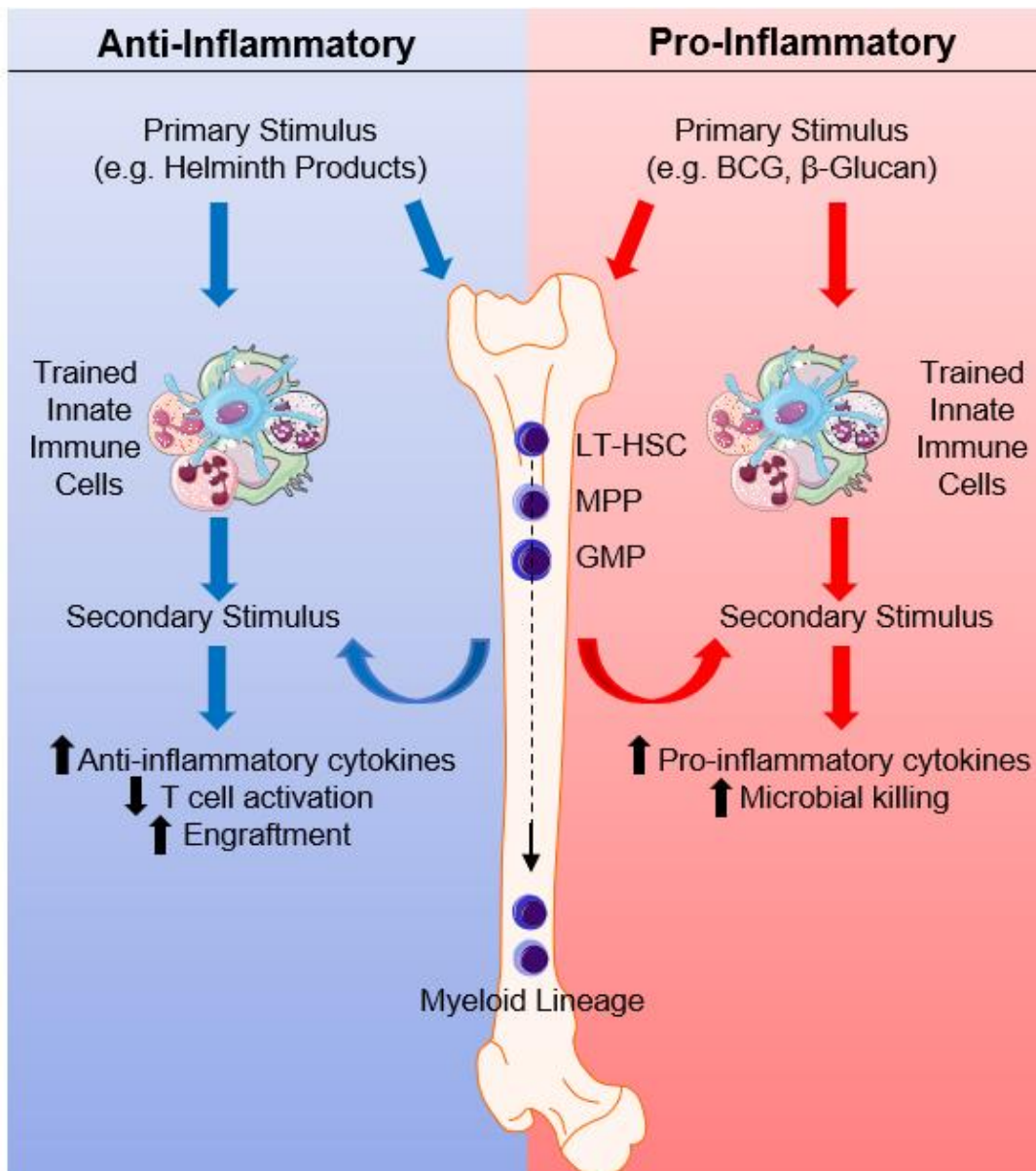
Figure 6.1 Anti- and pro-inflammatory central and peripheral trained innate immunity.

Pro-inflammatory mediators of trained innate immunity, such as β-glucan and BCG, act on peripheral innate immune cells and HSCs to imprint epigenetic modifications that produce trained cells that respond to secondary stimuli with enhanced pro-inflammatory responses. Anti-inflammatory trained innate immunity is mediated by stimulation of innate immune cells or HSCs with helminth-derived products. Epigenetic and metabolic reprogramming induces long-term anti-inflammatory responses in future generated innate immune cells, including suppression of immunopathology in autoimmune disease and improved engraftment after HSC transplantation.

In a phase 1 clinical trial, *ex vivo* priming of HSCs with prostaglandin E2 (PGE2) before HSC transplantation improved engraftment [419]. PGE2 modifies HSCs and imprints long-term changes, such as enhanced self-renewal [420]. Furthermore, priming of HSCs with PGE2 reduced pro-inflammatory responses to macrophages and DC derived from treated HSCs [391, 392, 421]. Meta analyses investigating the safety of HSC transplantation for treatment of MS have shown mixed results. One such study found a small but significant number of treatment-related mortalities in HSC transplant recipients (~2%), while another found no increase in mortality, but noted an increase in the rate of infections in the first few months after transplantation [422, 423]. Further research is required to fully characterize the engraftment of FHES-primed HSCs, and how this may affect long-term survival or response to infection. The findings of this study suggest that direct stimulation of HSCs *ex vivo* or administration of helminth-derived products to donors could improve engraftment of HSCs in recipients and suppress autoimmune disease.

IFN-β is a well-tolerated immunotherapy for MS and is capable of reducing annual disease relapses by 30-40% [412, 424, 425]. Currently, the mechanisms for IFN-β as a treatment for MS are currently understood to be multifactorial immunosuppression in the periphery, including downregulation of MHC-II, and increasing IL-10-producing T cells [426]. Recently, Khan et al., demonstrated that Mtb induced tolerogenic responses in HSCs and inhibited pro-inflammatory central trained innate immunity via induction of IFN-β signaling, while vaccination with BCG induced pro-inflammatory central training through IFN-γ production [146]. While the authors found this to be a successful evasion strategy for the mycobacteria, this immunomodulation could partially explain the effectiveness of IFN-β in MS. Interestingly, while IFN-β is a successful therapeutic to reduce relapses in early MS, a clinical trial using IFN-γ increased the frequency of relapses and was associated with increased circulating inflammatory monocytes and NK cells [427]. This further suggests that central training may play a role in the ongoing therapeutic success of IFN-β in MS. The results detailed in this thesis highlight that anti-inflammatory central training mediated by helminth products may constitute a similar avenue of therapeutic intervention in autoimmune

diseases, including MS. While the exact mechanism remains unclear, it is evident that helminths induce anti-inflammatory training to modify the immune system and promote long-term tolerance.

The incidence of autoimmune diseases has been rising in high-income countries with declining prevalence of helminth infections. Helminths have evolved mechanisms which have allowed them to maintain chronic infections through the induction of type-2 and regulatory immune responses. Animal models have confirmed this ability to suppress immunopathology in autoimmune diseases, however treatment of humans with live infections raises important ethical and viability concerns. Research into helminth-derived antigens found in ES has enabled better understanding of pathways and mechanisms of immune evasion and may provide potential keys to unlock novel therapeutic avenues for the treatment of autoimmune disorders. This study provides novel insight into how helminths can programme HSCs to give rise to progenitors with dampened inflammatory potential, and reduced ability to promote T cell-mediated autoimmune diseases.

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