Modulation of immune responses by *Fasciola hepatica*-derived products

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Declaration of Authorship

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Abstract

Helminths are successful pathogens that infect 25% of the world's population. They cause chronic infections that are associated with type-2 and regulatory immune responses that suppress host anti-parasite immune responses. However, helminths can also inhibit immune responses that mediate inflammatory pathology in autoimmune diseases. Indeed, a high prevalence of helminth infections in certain regions has been associated with low incidences of autoimmune diseases. Consequently, helminths are being trialed as potential therapies for immune-mediated diseases. However, the regulatory and practical concerns around their use has led to a search for specific helminth-derived immunoregulatory molecules. Our laboratory has demonstrated that the helminth *Fasciola hepatica* excretory-secretory products (FHES) can attenuate the development of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Protection was mediated by eosinophils and the type-2 cytokines IL-33 and IL-5. FHES is heterogeneous preparation but provides the basis for identifying individual immunomodulatory molecules. In this project, small extracellular vesicles (exosomes) were isolated from FHES by sequential centrifugation and examined for immunomodulatory activity. FH exosomes induced IL-1RA production by macrophages and eosinophils and when injected intraperitoneally into mice promoted recruitment or expansion of eosinophils and alternatively activated macrophages, and suppressed infiltration of pro-inflammatory cells into the peritoneal cavity. Treatment with two single injections of FH exosomes induced anti-inflammatory trained immunity, which rendered the mice less susceptible to the induction of EAE. This was associated with a reduction in the infiltration of pathogenic Th1 and Th17 into the CNS. In order to distinguish individual immunomodulatory proteins, *F. hepatica* Kunitz Type Molecule (FHKTM), identified in a mass spectrometry screen and was cloned, transformed into yeast, expressed, and purified. FHKTM promoted production of the anti-inflammatory cytokines IL-1RA and IL-10 and the alternative activation of macrophages *in vitro*. Finally, functional studies demonstrated that FHKTM had regulatory activity *in vivo*, attenuating the clinical severity of EAE. The findings demonstrate that *F. hepatica* exosomes and FHKTM promote regulatory immune responses and may be useful in the design of new approaches for the treatment of human inflammatory diseases.

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Publications

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In memory of my sister Eimear

Who I miss ever day

Everything I do in life is done in your honour

x

“The rain falls on the just and the unjust alike…”

Matthew 5:45

“…but joy comes with the morning”

Psalm 30:5

Abbreviations

2-ME	2-mercaptoethanol
ABI	Applied Biosystems
αCD3	anti-CD3ε
AIP-2	Anti-inflammatory protein-2
APCs	Antigen-presenting cells
APS	Ammonium persulfate
ASC	Apoptosis-associated speck-like proteins containing a CARD
BBB	Blood-brain-barrier
BCA	Bicinchoinic Acid
BCG	bacille Calmette–Guérin
BMDC	Bone marrow-derived dendritic cells
BMDM	Bone marrow-derived macrophages
CARD	Caspase activation and recruitment domains
CCR3	C-C chemokine receptor type 3
cDCs	Conventional DCs
CFA	Complete Freund's adjuvant
Chi3l3	Chitinase-3-like protein 3
CNS	Central Nervous System
cRPMI	Complete RPMI
DAMP	Damage-associated molecular patterns
DCs	Dendritic cells
DMARD	Disease-modifying anti-rheumatic drugs
DMSO	Dimethyl sulfoxide
E/S products	Excretory/secretory products
EAE	Experimental autoimmune encephalomyelitis
EB/AO	Ethidium bromide/acridine orange
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked immunosorbent assay,
EtOH	Ethanol
EU	Endotoxin units

FABP	Fatty acid binding protein
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FcεR	Fc epsilon receptor
FHES	*Fasciola hepatica* excretory/secretory Products
FHKTM	*Fasciola hepatica* Kunitz-type molecule
FHTE	*Fasciola hepatica* total extract
FMO	Fluorescence Minus One
FPR2	Formyl peptide receptor 2
f.s.	filter-sterilised
G-CSF	Macrophage colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HpARI	*H. polygyrus* alarmin release inhibitor
HRP	Horseradish Peroxidase
HSD	Honestly significant difference
i.p.	Intraperitoneal
Id2	Inhibitor of DNA binding 2
IL-1RAcP	IL-1 receptor accessory protein
ILC1	Group 1 innate lymphoid cell
ILC2	Group 2 innate lymphoid cell
ILC3	Group 3 innate lymphoid cell
ILCs	Innate lymphoid cells
iNKT	Invariant natural killer T cell
iTreg cell	Inducible Treg cell
LDL	Limulus amebocyte lysate
LNs	Lymph nodes
LPM	Large peritoneal macrophage
MBP	Major basic protein
MC	Mast Cell
MD	Minimum dextrose

MDA	Mass drug administration
MFI	Mean fluorescence intensity
miRNA	microRNA
MK	Megakaryocyte
MOG	Myelin oligodendrocyte glycoprotein
Mono	Monocytes
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSU	Monosodium Urate
NALP3	NACHT, LRR and PYD domains-containing protein 3
n.d.	None detected
n.s.	No significance
NETs	Neutrophil extracellular traps
NF-HEV	Nuclear factor of high endothelial venules
NK cells	Natural Killer Cells
NLRP3	Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3
NLRs	NOD-like receptors
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization-domain protein
NTDs	Neglected tropical diseases
nTreg cell	Natural T reg cell
O/N	Overnight
OD_{600}	Optical density
OPD	o-Phenylenediamine dihydrochloride
P/S	Penicillin/Streptomycin
PBMCs	Peripheral blood mononuclear Cells
PCB	Phosphate citrate buffer
PCR	Polymerase chain reaction
pDCs	Plasmacytoid DCs
PEC	Peritoneal exudate cells

PFA	Paraformaldehyde
pHSCs	Pluripotent hematopoietic stem cells
PLP	Proteolipid protein
PMA	Phorbol 12-myristate 13-acetate
PRR	Pathogen recognition receptor
RA	Rheumatoid arthritis
RIG	Retinoic acid-inducible gene
RLRs	RIG-I-like receptors
RPMI medium	Roswell Park Memorial Institute medium
rRNA	Ribosomal RNA
RT	Reverse transcriptase
rtPCR	Reverse transcriptase polymerase chain reaction
s.c.	Subcutaneous
SAR	Systemic acquired resistance
SCN	Severe congenital neutropenia
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEA	Soluble egg antigens
SPM	Small peritoneal macrophage
T1D	Type 1 diabetes mellitus
TB	Tuberculosis
TBS	Tris buffered saline
TBS-T	TBS-tween
TCRs	T cell receptors
TEMED	Tetramethylethylenediamine
TGM	TGF-β mimic
TIMP	Tissue inhibitor of metalloproteases
TLRs	Toll-like receptors
tPEC	Total peritoneal exudate cells
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TSLP	Thymic stromal lymphopoietin

WHO	World Health Organisation
YNB	Yeast nitrogen base

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Chapter 1

General Introduction

1.1 The Immune System

Classically, inflammation was defined by the Roman scholar Celsus to comprise of the four 'cardinal' symptoms of calor (heat), dolor (pain), rubor (redness), and tumor (swelling) [1]. The symptoms recognised by Celsus are a consequence of increased blood flow and vascular permeability into infected tissue permitting the influx of immune cells to fight infection [2]. Since this observation, almost two millennia of research have increased our understanding of the cells and molecules that mediate inflammation.

Inflammation is an adaptive response that has evolved over time to protect organisms from pathogens, infectious agents which cause harm and disease, and to facilitate the repair of damaged tissues [3]. A controlled inflammatory response is beneficial, removing infections and helping to maintain homeostasis. However excessive inflammation is pathological and can lead to serious morbidity and death. Therefore, immune activation must be carefully regulated to ensure dysfunctional responses are not generated.

The immune system comprises white blood cells (leukocytes) and soluble mediators and is divided into innate and adaptive components. Innate immunity is the body's initial response to infection, and provides non-specific defence against infection [4]. Adaptive immunity involves specific responses to pathogens mediated by T and B cells. It develops more slowly than innate immunity but provides resistance to reinfection with the same pathogen.

1.2 Innate Immunity

The ability to generate an innate immune response is common to all animals [5]. Innate defences are comprised of constitutive and inducible elements. Epithelial cells, which line the skin and the membranes of the gastrointestinal, respiratory, and urogenital tracts, provide the constitutive element of innate defence by acting as a barrier, preventing the entry of pathogens [4]. Internal membrane epithelial cells are known as mucosal epithelia as they secrete mucus, which aids in the expulsion of pathogens. Cells of mucosal surfaces also secrete antimicrobial proteins. If a pathogen successfully

breaches these barriers, then the inducible elements of the innate immune system provide defence. This consists of innate immune cells that fight infection by phagocytosis and the release of antimicrobial molecules [5]. Phagocytosis is the uptake of particulates (>0.5 μm in size) within a plasma-membrane envelope by cells [6]. It is a highly evolutionary-conserved process important in innate immunity. Phagocytic cells (phagocytes) of the immune system ingest pathogens. This serves two functions. Firstly, phagocytosis induces pathogen death by directing ingested microbes to lysosomes, which contain many hydrolytic enzymes. Secondly, phagocytes, in particular dendritic cells (DCs), use phagocytosis to deliver antigens to both MHC I and II molecules and stimulate an adaptive T cells response [7].

In 1989, Charles Janeway proposed a mechanism by which the innate immune system recognises the presence of pathogens and activates an appropriate response [8]. He argued that innate immune recognition was mediated by pattern recognition receptors (PRRs) that detect conserved microbial products, known as pathogen-associated molecular patterns (PAMPs). This was a theoretical proposal which was later confirmed when experimental evidence demonstrated the existence of several families of germline-encoded PRRs [9].

PRRs can be expressed on the cell surface or intracellularly and have been classified into four families: Toll-like receptors (TLRs), NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and C-type lectin receptors [2]. TLRs were the first PRRs to be discovered and they are the most widely characterised [10]. In response to the detection of PAMPs by PRRs, the transcription of inflammatory genes is induced, leading to the production of inflammatory mediators including cytokines, chemokines and antimicrobial proteins. PAMPs are synthesised by different pathogens but not by the host. This provides the mechanism for distinguishing between self and non-self. PAMPs can be derived from components of bacterial cell walls including LPS and peptidoglycan, unmethylated CpG DNA found in bacteria and viruses but not in mammalian cells, and viral double stranded RNA [10, 11]. Transmembrane PRRs detect PAMPs derived from extracellular pathogens, while cytosolic PRRs detect intracellular bacterial and viral infections.

1.2.1 Cells of the Innate Immune System

All blood cells including leukocytes arise from pluripotent hematopoietic stem cells (pHSCs) in the bone marrow in a process known as haematopoiesis (Figure 1.1) [12, 13]. pHSCs have two possible fates. The can develop into common lymphoid progenitors giving rise to the lymphoid lineage of T and B cells, natural killer (NK) cells, and innate lymphoid cells (ILCs). Some DCs (a minority) arise from common lymphoid progenitors. The other fate for pHSCs is to become common myeloid progenitors, developing into macrophages, monocytes, DCs, mast cells, or granulocytes (neutrophils, eosinophils, and basophils). Erythrocytes and platelets also differentiate from common myeloid progenitors.

1.2.1.1 Macrophages

Macrophages are tissue-resident immune cells with various homeostatic functions specific for the organs and tissues they populate. Macrophages from different tissues were historically given different names e.g. microglia in the central nervous system (CNS) and Kupffer cells in the liver[14]. Tissue resident macrophages are typically seeded early in embryogenesis, independent of haematopoiesis, however, as animals age, these macrophage populations are replenished by bone marrow-derived macrophages (at rates that differ between tissues). Monocytes develop in the bone marrow and circulate in the blood for several days before entering tissues and differentiating into tissue macrophage (and monocyte-derived, 'myeloid' DC) populations [15]. However, monocytes do not contribute significantly to tissue macrophages at steady state except in the gut, skin and heart (where monocytes constantly migrate and differentiate into macrophages) [16–18]. Instead, tissue macrophages are derived from progenitor precursors that seed tissues during embryonic development and maintain themselves by local self-renewal[19].

In the tissues, macrophages function as first responders if pathogens successfully evade the barriers of the innate immune system and enter the body. They detect damage-associated molecular patterns (DAMPs) and PAMPs via their PRRs. Receptor activation induces phagocytosis by the macrophages to eliminate pathogens and the production of cytokines, e.g. IL-1β and TNF, and chemokines, e.g. CXCL8 and CCL2/4, which

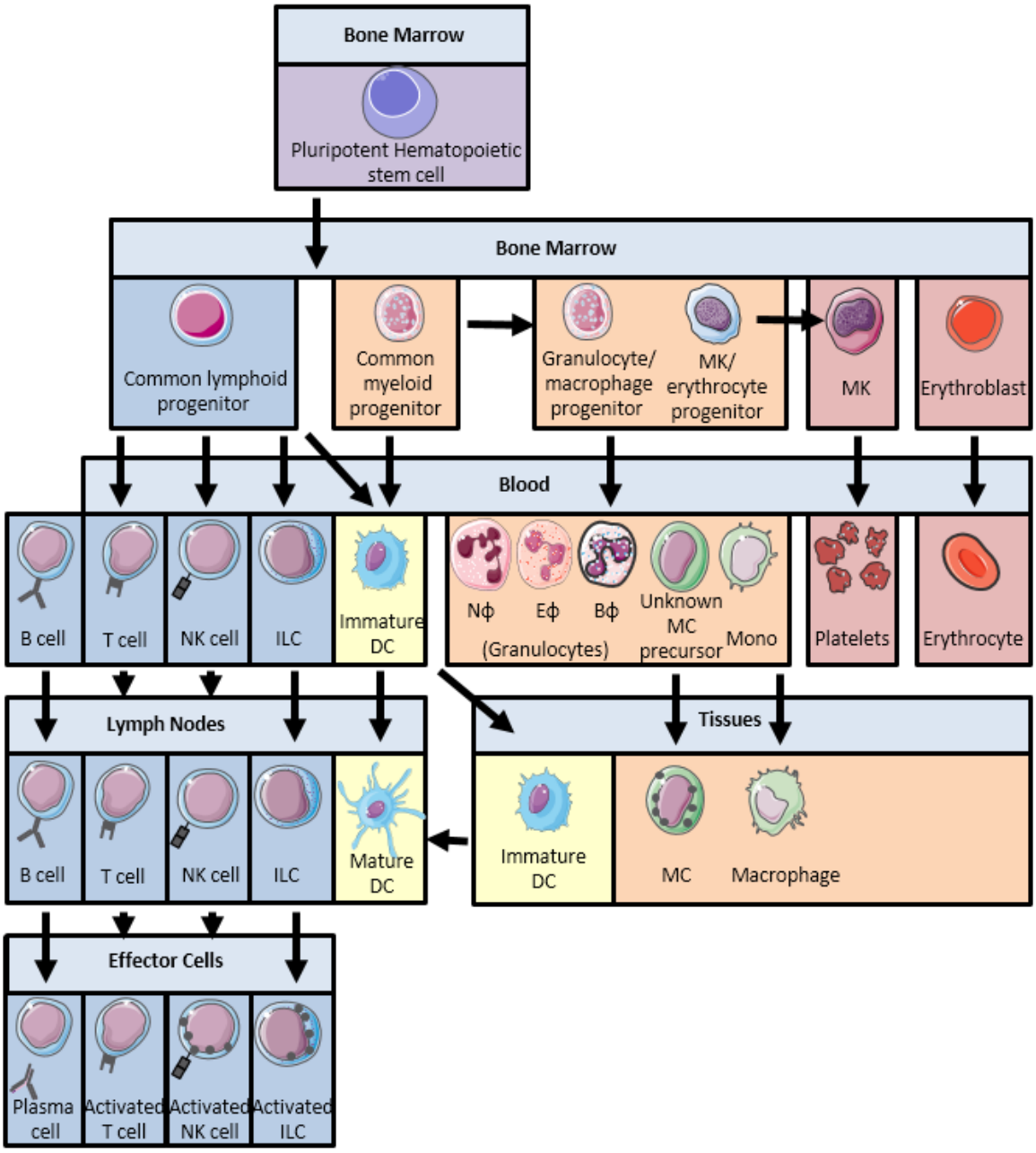


Figure 1.1: Hematopoiesis.

Following birth, cells in the immune system (and red blood cells and platelets) arise from pluripotent hematopoietic stem cells in the bone marrow. Myeloid cells develop from common myeloid progenitors and lymphocytes from common lymphoid progenitors. DCs are unique in that they can develop from either. ILCs (including NK cells) are of a lymphoid lineage but function as elements of innate immunity. Abbreviations: Bϕ (Basophil), Eϕ (Eosinophil), ILC (Innate lymphoid cell), MC (Mast cell), MK (Megakaryocyte), Mono (Monocyte), Nϕ (Neutrophil), NK (Natural Killer Cell). Figure adapted from Janeway's Immunobiology, 9th Edition (2016).

mediate the generation of an inflammatory response, recruiting cells, including neutrophils and monocytes, and helping to activate adaptive immunity [11].

At rest, tissue-resident macrophages are typically anti-inflammatory and prevent the development of excessive inflammation. For example, macrophages in the gut respond to IL-10 and prevent the development of unnecessary inflammation in response to harmless commensal bacteria [20]. Gut macrophages unable to respond to IL-10 become pro-inflammatory and mediate the spontaneous development of severe colitis [21]. Following immune activation, the numbers of tissue resident macrophages may be insufficient to generate an adequate response. Circulatory monocytes will be recruited to the tissue, differentiate into macrophages, and become activated to eliminate pathogens [22].

Macrophages can undergo radically different activation states that depend on the molecular and cellular stimuli present in their microenvironment. The best-known classification of these activation states is the M1/M2 paradigm (Figure 1.2). M1 and M2 macrophages have divergent phenotypes and function. The M1/M2 nomenclature was proposed by Mills (2000) and mimics the Th1/Th2 classification of T helper cells[23]. M1 macrophages produce factors required to destroy small, rapidly replicating bacterial infections, while M2 macrophages are more important in limiting the activity of slow-growing parasitic worms and promoting tissue repair. Mills demonstrated that the metabolism of arginine differed between macrophages from C57BL/6 and BALB/c mice. The difference correlated with differences T helper cell responses in two strains. C57BL/6 mice are prototypical Th1 strains and their macrophages produced NO from arginine while macrophages from BALB/c mice, prototypical Th2 strains, metabolised arginine to ornithine. The differential metabolism of arginine was originally used as a method of identifying M1 and M2 macrophages. The M1 marker Nos2 is the gene for iNOS, the protein responsible for the production of NO. Arg1 is the gene for the enzyme arginase-1, which catalyses the reaction of arginine to ornithine. Arg1 is a M2 marker in mice.

M1 macrophages are associated with type 1 immune responses. The major M1 stimuli are IFN-y, TNF, and bacterial products such as LPS [24]. M1 macrophages have potent

anti-microbial properties. They produce TNF and reactive oxygen and nitrogen species and kill intracellular pathogens and cancerous cells. M2 macrophages are induced by IL-4 and IL-13 produced by Th2 cells and basophils as part of a type 2 immune response [25]. They aid in the expulsion of extracellular parasites and also promote wound healing and tissue repair.

Martinez and Gordon (2014) have argued that macrophages dynamically respond to stimuli and can rarely be classified simply as either in an M1 or an M2 state; rather they will be skewed towards an M1 or M2 phenotype [26]. Within M2 macrophages there can be a high degree of heterogeneous phenotypes with different macrophages having elevated expression of different markers at different times. There are also issues with using markers that have classically been thought as M1 or M2. Arginase-1 is the standard M2 marker but its expression can also be induced in M1 macrophages[27]. Jablonski et al recently described novel, more robust markers that can be used to distinguish between murine M1 and M2 macrophages; CD38, Gpr18 and Fpr2 were identified as novel M1 markers and Egr2 and c-Myc as M2 markers [25]. Chi3l3 (Ym1) and Retnla (Fizz) are also robust M2 markers. These points notwithstanding, the M1/M2 classification of macrophages is a useful model that can help to demonstrate what type of immune response has been generated.

“All models are wrong but some are useful” – George Box, statistician [28].

1.2.1.2 DCs

DCs can behave similarly to macrophages and eliminate pathogens through phagocytosis [5]. However, their main function is as immune surveillance and antigen presenting cells (APCs). DCs act as sensors throughout the body sampling the environment for the presence of pathogens. Immature DC express low levels of MHC II molecules [29]. When a pathogen is detected, the activation of PRRs on the surface of DCs by PAMPs upregulates the expression of MHC II molecules and co-stimulatory molecules including CD86, CD80, and CD40 that are necessary to promote T cell activation [30]. Pathogens are phagocytosed and internally processed, generating T cell epitopes [31]. MHC II molecules deliver the antigens to the DC surface where they can be presented to $CD4^+$ T cells [32].

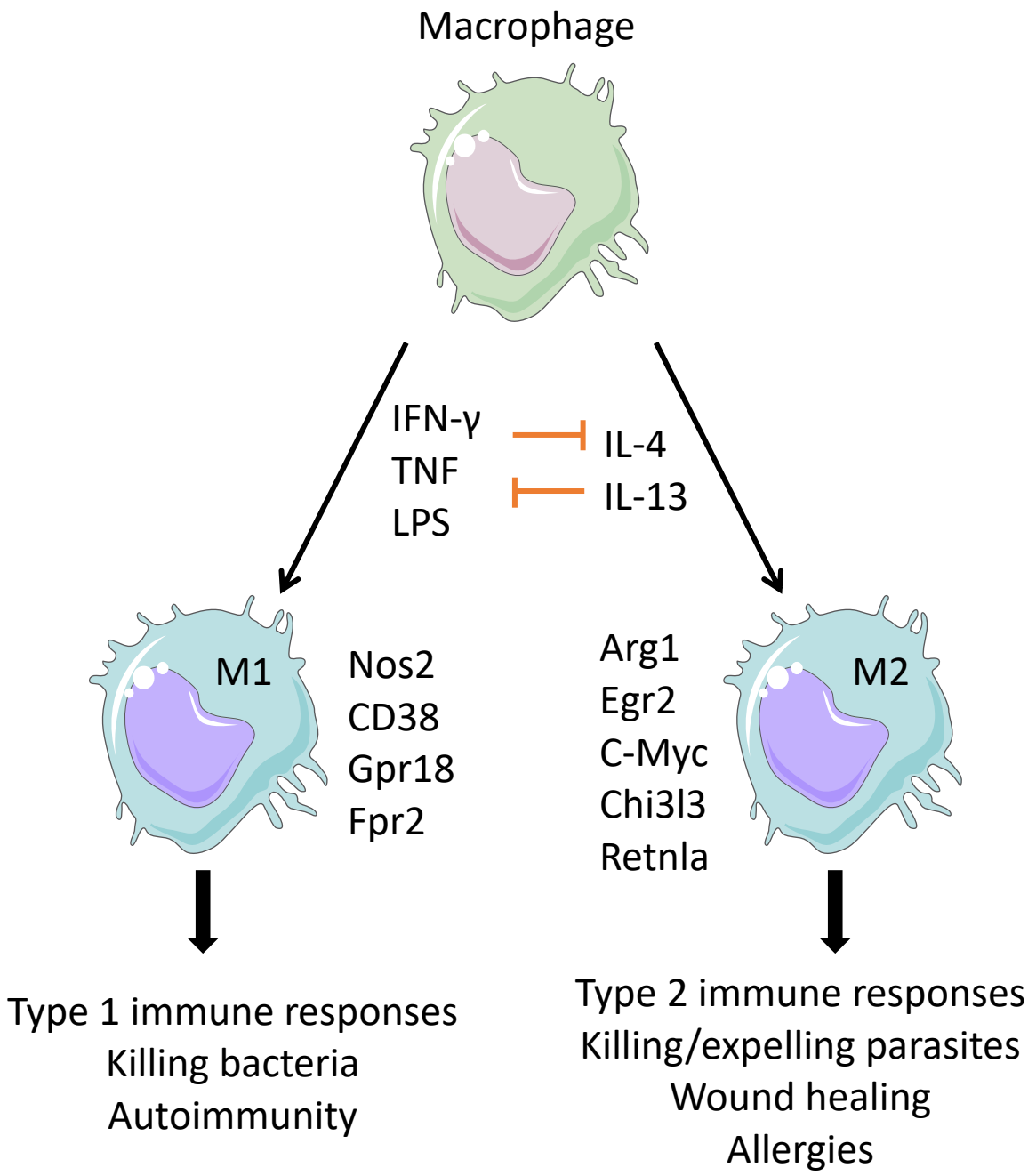


Figure 1.2: Macrophage activation states.

Depending on environmental cues, macrophages can become classically (M1) or alternatively (M2) activated. M1 activation is induced by IFN-γ, and LPS or TNF. M1 macrophages express Nos2, CD38, Gpr18, and Fpr2 markers and contribute to type 1 immune responses to bacterial infections. M2 activation is induced by IL-4 and IL-13, promoting the expression of Arg1, Egr2, c-Myc, Chi3l3, and Retnla markers. M2 macrophages are activated during infection by extracellular parasites. Inappropriate M1 and M2 activation are a feature of autoimmunity and allergies respectively.

Naive T cells are stimulated by immature DCs that have captured antigen migrate toward T cell zones either within their lymphoid organ of residence or to draining lymph nodes (LNs) where they mature and encounter large numbers of naïve T cells [33]. PRR activation by PAMPs induces the expression of chemokine receptor CCR7 in DCs [34]. CCL19 and CCL21 are CCR7 ligands produced in lymphoid organs [35]. The binding of ligand to CCR7 enhances DC chemotaxis and migratory activity. CCR7 is integral in facilitating DC migration to primary and secondary lymphoid organs. In this way, DCs can prime naïve T cells, (which also express CCR7, thus responding to the same cues in the lymph node), inducing their activation and differentiation and are a bridge between innate and adaptive immunity.

DCs develop from pluripotent stem cells into two major classes: $CD11c^{hi}$ conventional DCs (cDCs) and $CD11c^{low}$ MHC II^{low} plasmacytoid DCs (pDCs)[36]. Although the main function of DCs is in T cell activation, they also play a role in innate immunity; pDCs are major type 1 interferon producers and important in anti-viral defence [37]. pDC type 1 interferon production is induced by viral, but not bacterial, PAMPs binding to the pDC PRRs TLR7 and TLR9 [38].

1.2.1.3 Neutrophils

Neutrophils are the most abundant leukocytes in the blood but are generally absent in healthy tissue [39]. They are early mediators of immune responses, being the first cells recruited from the blood to the site of infection. They are present within minutes and neutrophil numbers peak at approximately 6 hours post infection [4]. Neutrophils eliminate pathogens by phagocytosis and the release of toxic products stored in pre-formed granules including reactive oxygen and nitrogen species, and anti-microbial proteins [6, 40]. Neutrophil serine proteases directly kill pathogens and inactivate toxic microbial products [41]. Neutrophils can also mediate the removal of pathogens with the formation of neutrophil extracellular traps (NETs) [42]. NETS comprise of neutrophil granule proteins and DNA material. They are released by neutrophils and form extracellular fibres that bind bacteria, inhibiting their virulence.

Genetic defects that cause neutropenia (a reduction in circulating neutrophil numbers) are often fatal when untreated, demonstrating the crucial role of neutrophils in

controlling bacterial and fungal infections. For example, in severe congenital neutropenia (SCN), neutrophil development is inhibited. Patients are predisposed to recurrent bacterial and fungal infections and require continuous treatment with G-CSF to stimulate neutrophil production of sufficient magnitude to fight infection [39].

1.2.1.4 Eosinophils

Eosinophils originate in the bone marrow from pluripotent hematopoietic stem cells, developing first into hybrid eosinophil/basophil granulocyte precursors before differentiating into mature eosinophils [43]. Eosinophils can phagocytose pathogens but have low phagocytic activity compared with other phagocytes like neutrophils [44]. Instead, eosinophils are important in the defence against multicellular pathogens, including parasites, that are too large to be phagocytosed [45]. They release toxic granule proteins and free radicles that kill microorganisms and parasites [46]. Major basic protein (MBP) is the most prominent toxic eosinophil granule protein [47]. MBP can kill parasites *in vitro*. Its toxicity is believed to be mediated by an enhancement of membrane permeability. Eosinophils also release cytokines and other inflammatory mediators. The evidence for *in vivo* killing of parasites by eosinophils is so far lacking [47].

Molecules released by eosinophils can cause immunopathology in the host e.g. when released during an allergic reaction [48]. Therefore, their activation is tightly regulated. Eosinophil numbers are low under normal conditions [49]. Following a parasitic infection and the generation of a type 2 immune response, IL-3, IL-5 and GM-CSF stimulate eosinophil differentiation and development from hematopoietic progenitor cells in the bone marrow [47]. IL-5 is necessary for the induction of eosinophils in the bone marrow and their release into the circulation, but it is the chemokine eotaxin which is responsible for the migration of eosinophils to infected tissues [50, 51].

1.2.1.5 Basophils

Basophils mature in the bone marrow and reside in the circulation in healthy individuals [52]. They constitute <1% of circulating leukocytes and have a typical lifespan of only a few days. Basophils numbers increase during inflammatory responses and migrate to

tissues from the blood. They are activated by IgE antibodies binding to basophil Fc epsilon receptors (FcεRs) within their constant region. The IgE antibodies are specific for parasite antigens or allergens that bind the IgE variable regions. Binding of antigen/allergen causes cross-linking of the antibodies and activates the basophil [52]. Basophils elicit a type-2 immune response that contributes to allergic reactions or fighting parasite infections. When activated, basophils release histamine and other mediators that can contribute to inflammation. For example, activated basophils release IL-4 and IL-13 [4]. IL-4 production following activation is rapid and high [47]. Basophils express the TSLP cytokine receptor [53]. TSLP can activate basophils and induce IL-4 production in an IgE-independent manner as part of an innate response [54, 55]. Basophils are an early source of IL-4 in type 2 immune responses before Th2 cell differentiation has occurred [56].

1.2.1.6 Mast Cells

Mast cells are found in primarily in tissues [47]. They are the main effector cells of allergic responses [57]. Upon exposure to an allergen, mast cells are activated in a similar manner to basophils via cross-linked allergen-specific IgE antibodies on their surface. Allergic activation of mast cells leads to degranulation and the release of vasoactive and pro-inflammatory mediators that lead to the development of symptoms associated with allergic reactions. Like basophils, mast cells can also be activated in an IgE-independent manner by IL-33 and possibly TSLP [58].

Mast cells produce mediators that are pre-formed and stored in granules [47]. Upon activation, granules fuse with the cell membrane and its contents are released into the extracellular space. Histamine is a major component of the granules. While mast cells play a central role in allergy, they mediate other immune responses, including in response to pathogens, autoimmunity, fibrosis, and wound healing.

1.2.1.7 Innate Lymphoid Cells

Innate lymphoid cells are descended from common lymphoid progenitor cells. However, they lack antigen specificity and are cells of the innate rather than adaptive immune system [4]. During development from stem cells the expression of the transcription

factor Id2 (inhibitor of DNA binding 2) supresses T and B cell differentiation and is required for ILC fates [59]. ILCs mature in the bone marrow and populate lymphoid and peripheral organs.

ILCs respond to cytokine produced following infection and serve to mediate and amplify immune recognition signals. ILCs reside in barrier tissues and quickly respond to pathogen detection to limit the spread of infection [60]. Three major groups of ILCs have been characterised, delineated based on their cytokine product profiles [61]. Group 1 ILCs (ILC1s and NK cells) produce IFN-γ in response to cytokines released from DCs and macrophages, particularly IL-12 and IL-18 [62]. They protect against intracellular bacteria and viruses. Group 2 ILCs (ILC2s) are involved in type 2 immune responses to parasites [60]. They produce IL-4, IL-5, and IL-13 in response to IL-25, IL-33, and TSLP cytokine production [63–65]. Finally, group 3 ILCs (ILC3s) produce IL-17 and IL-22 following stimulation by IL-1β and IL-23 and are important in promoting immunity against extracellular bacteria and fungal infections [66].

1.2.1.8 Natural Killer Cells

NK cells were the first innate lymphoid cells to be described [67]. They are lymphoid-like cells of the innate immune system that protect against intercellular infection. They are similar to but distinct from ILC1s. They resemble $CD8^+$ T cells in function while ILC1s resemble Th1 $CD4^+$ T cells [61, 68]. NK cells are important in the initial immune response to viral infections before an adaptive response has been generated and they mediate immune responses against tumour cells [61]. The specific role for NK cells in viral immunity is seen in patients with selective NK cell deficiencies who are susceptible to certain viral infections, particularly herpesvirus [69]. NK cells also have a role in anti-bacterial protection. NK cells are an early source of IFN-γ during *Bordetella pertussis* infection, enhancing the anti-bacterial activity of macrophages and promoting the differentiation of Th1 cells, thus helping to eliminate the bacteria from the host [70].

NK cells express receptors that sense pathological changes to self-cells. These receptors are a mix of activating and inhibitory, thus allowing NK cells to integrate many signals and determine whether to carry out their cytotoxic functions or not [71]. Activating receptors recognise the presence of cell surface proteins induced by metabolic stress

(due to malignant transformations or intracellular infections). The binding of MHC I, present on the surface of all healthy non-nucleated cells in the body to NK cell inhibitory receptors prevents NK cell activation [72]. Virally-infected and cancerous cells downregulate MHC class I surface expression to evade cytotoxic $CD8^+$ T cells [73, 74]. However, the lack of an inhibitory signal from MHC I molecules favours NK cell activation, providing the immune system with a mechanism for recognising and eliminating the infected or cancerous cells.

NK cells kill virus-infected or tumour cells by releasing cytotoxic granules from their cytoplasm that contain granzymes and performin that penetrate the cell membrane and induce apoptosis [75]. Antibodies binding to Fc receptors on NK cells also activate the release of the cytotoxic granules[4]. NK cells can initiate cell death in infected cells by activating the death receptors DR4 and DR5 on their surface with TRAIL (tumour necrosis factor-related apoptosis-inducing ligand), a member of the TNF family. DR4 and DR5 stimulation activates caspase-8 which leads to apoptosis [76].

1.2.2 Innate Immune Memory

Immunological memory is a key feature of the adaptive immune system (Section 1.3). However, recent studies have suggested that the innate immune system may also have a type of memory or can be trained. Most of the focus has been on enhanced responses of NK cells or macrophages to subsequent inflammation stimuli. However, there is also some evidence of unresponsiveness, including LPS tolerance.

The phenomenon of innate training first came to prominence through the observation that certain organisms could develop resistance to re-infection even though their primitive immune systems lacked an adaptive component. In plants, infection induced epigenetic changes priming genes which encoded host defence molecules so that, upon re-infection, a more rapid and stronger response was generated [77]. This was termed systemic acquired resistance (SAR). A key feature of SAR was that exposure to one particular pathogen conferred non-specific protection against a broad range of other unrelated pathogens, including bacteria, viruses, and fungi [78]. Innate memory has also been seen in invertebrates (also lacking adaptive immunity) including the mealworm

beetle (*Tenebrio molitor*) and *Anopheles gambiae* mosquitoes, where non-specific protection was induced following initial infection [79, 80].

Training of the innate immune system has also been documented in species which possess adaptive immune systems. This suggests that this is a highly conserved process through evolution and therefore is very important in helping generate an appropriate immune response to fight infection. In humans, the bacille Calmette–Guérin (BCG) vaccine is administered to protect against tuberculosis (TB). BCG-vaccinated children were shown to be protected not just against TB but against other infections [81]. Innate training in this case manifested as an enhanced production of pro-inflammatory cytokines including TNF and IL-1β from monocytes in response to bacterial and fungal pathogens in addition to *Mycobacterium tuberculosis*, the causative agent of TB[82]. This enhanced cytokine production persisted for at least three months after vaccination. As monocytes are short lived, living typically for only 1-2 days in circulation, it is thought that the training effect was mediated through long lived immune progenitor cells (hematopoietic stem cells and multipotent progenitors), which have an extended lifespan and give rise to all mature, functional immune cells including monocytes [83].

Innate memory is thought to be mediated via epigenetic changes through histone modification. Epigenetic changes to the genes encoding proteins of the innate immune system are thought to mediate trained immunity or tolerance [84]. In trained cells, stimuli such as β-glucan promote changes in cellular metabolism, inducing a metabolic shift toward aerobic glycolysis and enable immune cells to generate heightened immune responses [85]. Tolerance, on the other hand, is the priming of cells by stimuli including LPS to produce less pro-inflammatory cytokines to subsequent activation.

1.3 Adaptive Immunity

A feature of immunity in some species is an enhanced response following secondary infection with a previously experienced pathogen. This phenomenon is known as immunological memory and it is mediated by the adaptive immune system. The ancient Greeks were aware of immune memory. 2,400 years ago, the historian Thucydides

observed that recovering plague patients (including himself) could care for infected individuals without risk of death from reinfection [86]:

"…the ones who felt more pity for the sick and the dying were those who had had the plague themselves and had recovered from it. They knew what it was like and at the same time felt themselves to be safe, for no one caught the disease twice, or, if he did, the second attack was never fatal"

Adaptive immunity is found in vertebrates only. It comprises of B and T lymphocytes, named after their sites of maturation, in the bone marrow and thymus respectively [87]. B and T cells have receptors specific for one particular foreign antigen. An antigen is a foreign molecule contained on or within a pathogen. Lymphocyte receptors bind to small portions of antigens known as epitopes. The receptors on any single B or T cell are the same but there are millions of lymphocytes with different receptors [88]. When a specific lymphocyte receptor recognises an antigen, the cell is activated and divides. Some T cells activate other lymphocytes while others kill infected host cells. B cells secrete antibodies that promote phagocytosis or the neutralise viruses and prevent reinfection.

Lymphocyte receptors contain variable and constant regions. The constant portion contains transmembrane and cytoplasmic regions while the variable region is extracellular and contains the antigen binding sites. B cell receptors contain two light and two heavy chains [89]. Antibodies are similar to B cell receptors except they lack the transmembrane and cytoplasmic regions. The majority of T cell receptors consist of one α and one β chain [90].

There are billions of different T and B cells with unique antigen receptors [91, 92]. There are an insufficient number of genes in the genome to account for this extensive diversity. Receptor diversity is due to somatic mutations in and recombinant rearrangement of the lymphocyte receptor gene leading to a pool of lymphocyte receptors many orders of magnitude greater than the genes that encode them [93]. The variable region of antigen receptors is constructed from three segments, a variable (V), diversity (D), and joining (J) segment. Different versions of V, D, and J segments are arranged linearly along

the one receptor gene. One of each segment is randomly combined by recombination leading to enhanced receptor variety.

When lymphocytes bind their antigen they rapidly divide forming clones [4]. This is termed clonal selection, a concept first proposed by Burnet in 1957 [94]. Clones are either short lasting effector cells that fight infection or long-lasting memory cells. The immune response to a second infection is quicker, stronger, and longer lasting due to the presence of a reservoir of pathogen-specific memory cells that can persist for decades.

T and B cell generally lack reactivity against self-antigens. However, due to the vast diversity of lymphocyte receptors, some T and B cells will be specific for self-antigens (estimated to be between 20 and 75% of all lymphocytes) [95, 96]. Nevertheless, only 3-8% of the population suffer from autoimmune diseases, as the body has an efficient mechanism for eliminating these cells [97]. Cells with self-reactivity undergo negative selection during development. As lymphocytes mature in the bone marrow or thymus their receptors are tested for self-reactivity. APCs present self-antigen on MHC molecules to lymphocytes. If cells are found to be positive they undergo apoptosis or are made non-functional [88].

The presence of many diverse receptors, immunological memory, and a lack of reactivity versus self are three processes common to T and B cells even though their activation and effector functions are different.

1.3.1 T cells

T cells develop in the thymus, exiting as mature, 'naïve' T cells [98]. Early in development, two distinct T cell lineages are established: αβ and γδ T cells. Subsequently αβ T cells develop into either $CD8^+$ or $CD4^+$ T cells [4]. Naïve T cells translocate among the body, migrating between tissue and secondary lymphoid organs including LNs. Unlike B cell receptors and antibodies, T cell receptors (TCRs) cannot bind free antigen; they bind to antigens presented on the surface of a host cell by MHC molecules [99]. Once a pathogen enters a cell, its proteins are cleaved, and antigens bind to MHC molecules which shuttle to the cell surface [100]. MHC I molecules are

found on all cells except non-nucleated cells and they activate cytotoxic $CD8^+$ T cells [74]. MHC II molecules are expressed only on DCs, macrophages, and B cells. These cells can act as APCs [101]. They typically bind antigen after a pathogen had been internalised by phagocytosis or endocytosis and activate helper $CD4^+$ T cells.

Cytotoxic $CD8^+$ T cells are activated by interactions with MHC I presenting processed antigen, with help from $CD4^+$ T cells. TCRs on the surface of cytotoxic T cells recognise antigen presented by MHC I on infected or cancerous host cells. CD8 enhances the interaction of antigen-MHC I molecules and the TCR, ensuring the cells are in contact [102]. Once activated, $CD8^+$ T cells destroy infected and cancerous cells, releasing perforin, which forms pores on the cells' surface, and granzymes, which enter the cell and destroy proteins initiating apoptosis [103]. When the cell has been killed the T cell is released to kill further cells.

Helper $CD4^+$ T cells are activated with the help of APCs. APCs presenting antigen-MHC class II complexes on their surface encounter T cells when they migrate into secondary lymphoid organs [98]. The binding of the TCR to antigen is insufficient to induce T cell activation. Naïve T cell activation requires three signals, all provided by APCs. The first signal is the antigen-MHC II molecule interaction with the TCR. The second signal is provided by co-stimulatory molecules expressed on the surface of APCs. Without co-stimulation TCR activation causes apoptosis. CD80 and CD86 are costimulatory molecules expressed on the surface of APCs which interact with CD28 and CTLA-4 on the T cell surface. CD28 provides a positive co-stimulatory signal to T cells upon binding to CD80 or CD86 [104]. CTLA-4 expression is induced 2 to 3 days following T cell activation and is negatively regulates T cell activation by antagonising CD28 co-stimulation [105, 106]. The final signal is provided by cytokines. Before this signal, T cells are activated as Th0 cells. Cytokines from APCs determine the fate of the T cell; they differentiate into the helper T cell type appropriate for the specific pathogen encountered by the APC. When activated, helper $CD4^+$ T cells secrete cytokines that activate cytotoxic T cells and B cells.

Some $CD4^+$ T cell populations are fully developed upon exiting the thymus. These include "natural" Treg (nTreg) cells that function similarly to inducible Treg (iTreg) cells. nTreg

cells function to promote self-tolerance [107]. On the other hand, naïve T cells have several different potential fates after they leave the thymus (Figure 1.3). Mosmann and Coffman demonstrated that naïve T cells could mature into two distinct populations defined by their different products and functions [108]. IL-12 and IFN-y were responsible for the induction of IFN-y-producing Th1 cells which had pro-inflammatory functionality. Th2 cells were induced by IL-4 and then produced IL-4 themselves, which was important in Th2 effector function and in a positive feedback loop for Th2 cell differentiation [109]. Initially Th1 cells were thought to be the cell type responsible for mediating pathological inflammation in autoimmune diseases. However studies with knockout mice and the discovery of the cytokine IL-23 led to the identification of new IL-17-producing T helper cell lineage: Th17 cells [110, 111]. In the absence of pro-inflammatory cytokines, naïve T cells can develop into iTreg cells, cells which regulate immune responses [109, 112].

1.3.1.1 Th1 Cells

Th1 cells are important in immunity against intracellular bacteria, viruses, and small parasites [113]. Following antigen presentation, the cells are induced by innate immune cell IL-12 and IL-18, which promote the transcription of T-bet [109, 114]. Th1 cells produce IFN-y and IL-2. IFN-y induces the activation of M1 macrophages, which kill microbes [115]. IL-2 is a T cell growth factor and is required for the formation of Th1 $CD4^+$ memory T cells [116]. Th1 cell activation is linked to the development of certain autoimmune diseases.

1.3.1.2 Th2 Cells

IL-4 is produced in response to infection by extracellular parasites and induces Th2 cell differentiation [113]. Stat6 is the major signal transducer of Th2 cell differentiation [117]. Th2 cells produce IL-4, IL-5, IL-9, IL-10, and IL-13 [109]. IL-4 mediates IgE class switching in B cells; IgE antibodies are responsible for the activation of basophils and mast cells [118]. IL-9 induces mucin production in epithelial cells and along with IL-13 mediates the physical expulsion of parasites. IL-4 and IL-13 promote the alternate activation of macrophages [25]. IL-5 is critical in the expansion and recruitment of eosinophils [119]. IL-10 attenuates Th1 activity, suppressing proliferation and cytokine

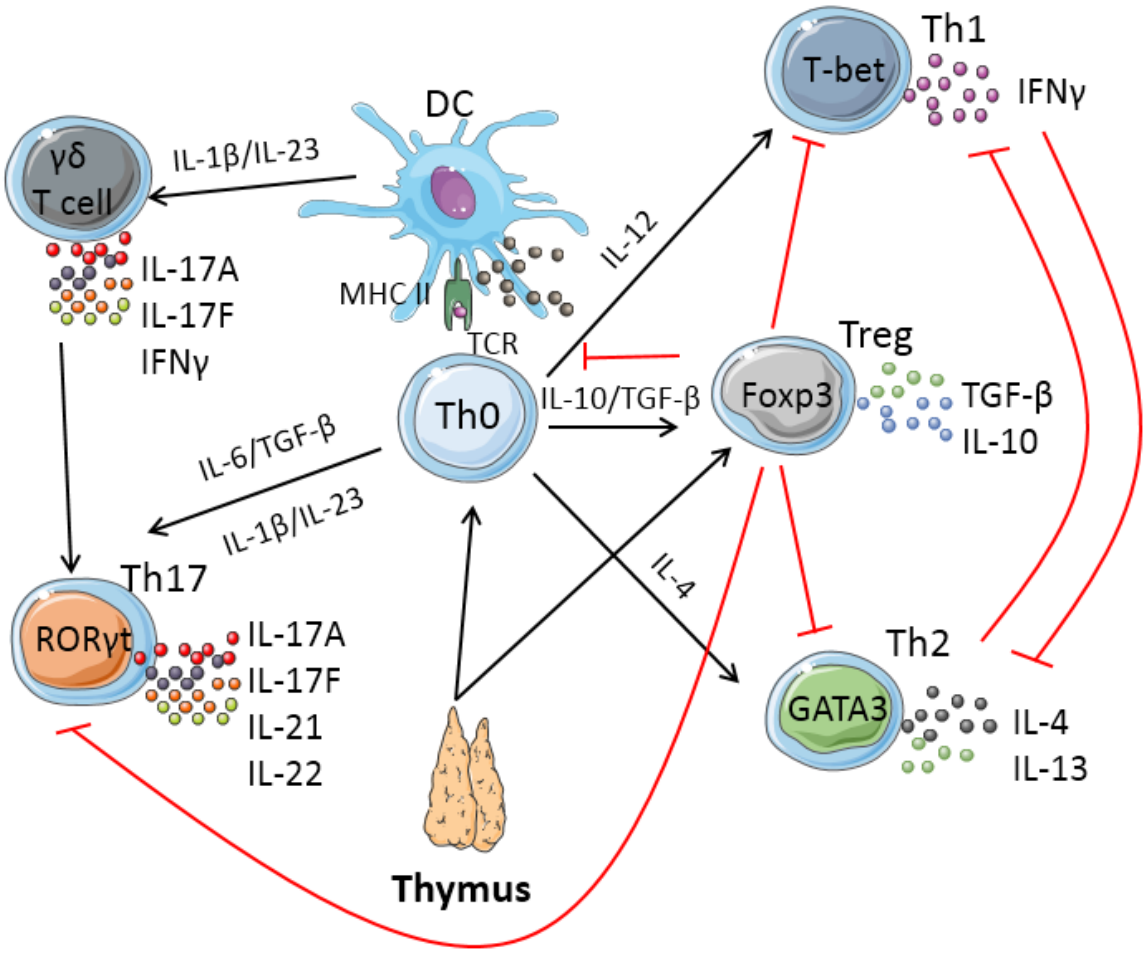


Figure 1.3: T helper cell differentiation.

Naïve T helper cells (Th0 cells) exit the thymus and are activated by antigen presented by MHC II and co-stimulatory molecules on the surface of APCs. Depending on the cytokine(s) produced by the APC, naïve T cells are committed to a particular T helper cell fate: Th1, Th2, Th17, or iTreg cells. Mature Treg cells can also emerge from the thymus (nTreg cells). The various T helper cells inhibit the differentiation of each other. γδ T cells do not require antigen presentation to be activated, IL-1β and IL-23 produced by innate immune cell are sufficient to promote differentiation. γδ T cells promote the development of Th17 cells.

production [120]. Aberrant Th2 cell activity contributes to allergic responses and asthma.

1.3.1.3 Th17 Cells

Th17 cells were discovered more recently than Th1 and Th2 cells. They mediate immunity to extracellular bacteria and fungi[121]. Like Th1 cells, Th17 cells have a pathogenic role in many autoimmune diseases. They are induced by IL-1β, IL-23, IL-6, and TGF-β, which promote the expression of RORγT, the master Th17 regulator, in the T cells [122]. Th17 cells were named through their production of IL-17; they secrete IL-17A, IL-17F, IL-21, and IL-22. IL-17A and IL-17F recruit neutrophils to the site of infection. IL-21 stimulates Th17 development in the positive feedback mechanism, as IFN- γ and IL-4 do for Th1 and Th2 development respectively [123].

1.3.1.4 Treg Cells

Treg cells are central to preventing immune self-reactivity and regulating immune responses [124]. They are generated from naïve T cells. Foxp3 expression induction is sufficient to convert naïve T cell into Tregs [125].The production of TGF-β, IL-10, and IL-35 mediate the immunosuppressive effects of Treg cells [109]. IL-10 is critical in the prevention of autoimmunity. Spontaneous colitis develops in mice with a selected deletion of IL-10 in Treg cells [126].

1.3.1.5 γδ T cells

A minority of T cells have TCRs consisting of one γ and one δ chain instead of an αβ receptor. γδ T cells are not restricted to recognising antigens presented by MHC and are part of the innate as well as adaptive immune system [127]. They express the IL-23 receptor and are activated by IL-1β and IL-23 from produced by APCs [128]. γδ T cells are enriched in the gut and other mucosal surfaces where they contribute to early immune responses against infection, producing IL-17A, IL-17F, and IFN-γ. In autoimmunity, γδ T cells are important early mediators of disease.

1.3.2 B cells

B cell receptors can recognise and bind free antigen. Some antigen is internalised and ultimately presented on the cell surface by MHC II [129]. This antigen presentation activates a helper T cell, specific for the same antigen, response which is usually critical to B cell activation. The T cells express surface molecules and cytokines that aid B cell proliferation and differentiation [130]. When activated, B cells proliferate and differentiate in to effector antibody-producing plasma cells and memory B cells [4].

1.3.2.1 Humoral response

Adaptive humoral immunity is mediated by antibodies produced by plasma cells [101]. The humoral response is robust; a single B cell gives rise to 1000s of plasma cells which each secrete approximately 2,000 antibodies per second for 4-5 day duration that they are active [5].

Antibodies perform 3 main functions [4]. Firstly, they bind to pathogens and their toxic products, neutralising their effects. Secondly antibodies bound to pathogens are recognised by macrophages promoting phagocytosis in a process known as opsonisation. Finally, antibodies activate the complement system, triggering a cascade that forms a pore in infected cells causing water and ions to rush in, ultimately lysing the cell. Additionally, antibodies bind to viral antigens presented on the surface of virally-infected cells. The antibodies recruit NK cells to the infected cell and apoptosis is induced by NK release of proteins.

1.4 Inflammatory Mediators

The activation of immune cells induces the production of inflammatory mediators which function to communicate between different cells of the immune system. Intercellular communication helps to propagate and amplify an immune response. Cytokines are important mediators with wide-ranging functions. The effect of a cytokine on a target cell is generally to promote or inhibit its effector functions.

1.4.1 IL-1β

The interleukin-1 family of cytokines comprises 11 members including IL-1α, IL-1β, IL-18, and IL-1RA [131]. IL-1β has a broad range of biological functions and target cells and so protects against infection by mediating and amplifying immune responses; IL-1β promotes the activity of innate cells and also lymphocyte activation and proliferation [132]. Most IL-1 family members are synthesised as inactive precursor proteins that are activated by cleavage. IL-1β and IL-18 are cleaved by caspase-1 in response to TLR signalling and inflammasome activation.

The inflammasome is a multiprotein complex required for IL-1β (and IL-18) maturation [133]. Inflammasome formation is initiated by NLRs. The most characterised inflammasome contains NLRP3. There are three components of the NLRP3 inflammasome: NLRP3, apoptosis-associated speck-like proteins containing a CARD (ASC), and pro-caspase-1 [132]. The inflammasome is activated by a variety of signals including ATP, uric acid, reactive oxygen species, and lysosomal damage. Formation of the inflammasome complex activates caspase-1, which in turn processes 31 kDa pro-IL-1β to active 17 kDa IL-1β. In this way, two signals are required for IL-1β activation and release: TLR activation and inflammasome assembly. Recently, inflammasome-independent mechanisms of IL-1β maturation and secretion have been identified. Caspase1 knockout mice could still process IL-1β in a model of arthritis [134]. Serine proteases can cleave pro-IL-1β in the place of caspase-1 [135].

IL-1β and the inflammasome are implicated in autoimmunity. The caspase-1-processed cytokines IL-1β and IL-18 promoted pathological inflammation in EAE and the suppression of caspase-1 or IL-1β attenuated disease development t[136, 137]. Furthermore, MCC950, a small-molecule inhibitor of NLRP3 inflammasome activation, attenuated the severity of EAE [138].

1.4.2 IL-1RA

IL-1RA is an anti-inflammatory member of the IL-1 cytokine family. It is produced by monocytes, macrophages, neutrophils, eosinophils, and other non-immune cells [139].

IL-1RA functions as an endogenous inhibitor of IL-1β, binding to IL-1 receptors but not inducing an intracellular signal [140].

IL-1RA is important in supressing pathological inflammation. Gout is an inflammatory disease caused by monosodium urate (MSU) crystal-activation of the inflammasome leading to IL-1β maturation and release. Administration of IL-1RA (generic name: anakinra) suppressed MSU crystal-induced inflammation in mice and is therapeutic in patients with acute gouty arthritis, suppressing symptoms [141].

IL-1RA has an important role in the development and has the potential to treat certain autoimmune diseases. IL-1RA deficient mice spontaneously develop an autoimmune rheumatoid arthritis–like disease due to a failure to inhibit IL-1β-induced Th17 cells [142]. In the treatment of moderate-to-severe Rheumatoid arthritis (RA), anakinra is licenced by the FDA for patients who have not responded to initial disease-modifying anti-rheumatic drugs (DMARD) therapy [143]. Clinical trials have demonstrated that anakinra is a relatively safe and modestly efficacious RA therapy. IL-1RA can cross the blood-brain-barrier (BBB) and so has the potential to suppress CNS autoimmunity [144]. Administration of IL-1RA reduced EAE clinical signs in rats and IL-1RA gene therapy delayed EAE onset and reduced disease severity [145, 146]. Individuals with endogenously high IL-1β and low IL-1RA production are more susceptible to develop relapse-onset MS [147].

1.4.3 IL-6

IL-6 was first identified as a factor that promotes T cell activation and expansion, B cell differentiation, and the acute-phase response to infection [148]. IL-6 production is induced rapidly following the generation of an immune response. The major promoters of production are IL-1β and TNF. In the acute phase of infection, IL-6 activates lymphocytes and induces antibody production. Together with IL-23, IL-6 activates STAT3 signalling in Th0 cells inducing RORγT expression and the differentiation of IL-17A producing Th17 cells. IL-6 also inhibits Treg cell activation. B cells exposed to IL-6 will differentiate into plasma cells and secrete antibodies. IL-$6^{-/-}$ mice display reduced antibody responses and increased susceptibility to infection from different types of pathogens [149].

IL-6 was first described as pro-inflammatory cytokine but it is now recognised to have immunoregulatory activity [150]. IL-6 potentiates IL-4-induced alternate activation of macrophages polarisation. *In vitro*, IL-6 induced the expression of the IL-4 receptor (IL-4R) on macrophages, priming them for IL-4 stimulation and leading to enhanced IL-4-promoted M2 gene expression [151].

1.4.4 IL-4

IL-4 is a pleiotropic cytokine produced by ILC2s, basophils and Th2 cells that promotes Th2 cell differentiation, B cell activation, and IgE class switching in B cells [152]. IL-4 shares sequence homology, receptors, intracellular signalling pathways, and some functions with IL-13 [153]. IL-4 polarises macrophages toward an alternatively activated M2 state and is used *in vitro* as a tool to induce the expression of alternate macrophage activation [154].

1.4.5 IL-33

IL-33 is a member of the IL-1 cytokine family [155]. Initially discovered as NF-HEV in 2003 [156], it is a cytokine associated with type 2 immune responses [157]. IL-33 is abundantly expressed in endothelial and epithelial cells during homeostasis and expression is enhanced following inflammation [155, 156]. It is generally not expressed in leukocytes under resting conditions but can be induced in macrophages and DCs under inflammatory conditions [155]. However expression is 10-fold lower than in endothelial or epithelial cells. Therefore most IL-33 in the body is tissue, rather than immune cell, derived.

Under basal conditions, IL-33 is localised to the nucleus. IL-33 associates with chromatin in the nucleus via a chromatin-binding motif in its N-terminal region [158]. Deletion of this region in mice led to the release of IL-33, the induction of severe, non-resolving inflammation, and ultimately death [159]. This indicates that the binding of IL-33 to chromatin is required to prevent the harmful constitutive release of the cytokine. Full length IL-33 is released from cells following necrotic injury and thus acts as an alarmin (an intracellular molecule released following cell injury that elicits an immune response) [160]. IL-33 is also released from cells following exposure to allergens or infection by

viruses or parasites. Following its release, IL-33 signals through the ST2/IL -1 receptor accessory protein (IL-1RAcP) receptor complex and induces type-2 cytokine production in ILC2s, mast cells, basophils, eosinophils, Th2 cells, and NK cells [155, 161, 162].

Proteases including neutrophil serine proteases, cathepsin G, and elastase are activated during inflammation and cleave full length IL-33 into mature forms (IL-$33_{95\text{-}270}$, IL-$33_{99\text{-}270}$, IL-$33_{109\text{-}270}$) with IL-1-like domains that have greater biological activity than the full length cytokine [162]. IL-33 can also be inactivated by cleavage. It is important that the cells undergoing programmed cell death do not release any molecules that induce unnecessary inflammation. To prevent IL-33 being released from apoptotic cells and promoting an immune response, caspases activated during apoptosis (caspase-3 and -7) cleave the cytokine into 2 inactivated products [163].

1.4.6 IL-5

IL-5 is a cytokine involved in type 2 immune responses induced by extracellular parasites. The major IL-5-producing cells during a type 2 immune response are first ILC2s and subsequently Th2 cells. IL-5 promotes the proliferation of eosinophil precursors in the bone marrow and the activation and survival of mature eosinophils in the blood [50]. Eosinophils express the IL-5 receptor subunit α on their surface [164]. IL-5 knockout or neutralisation with antibodies prevents parasite-induced eosinophil expansion and infiltration [165, 166]. IL-5 induces eosinophil differentiation and release into circulation but it is eotaxin which functions to recruit eosinophils from the blood to sites of infection [51].

1.4.7 Eotaxin

Eotaxins are chemokines responsible for the migration of eosinophils to infected tissues. There are three human eotaxins, eotaxin 1 (CCL11), eotaxin 2 (CCL24) and eotaxin 3 (CCL26) [167]. Mice express only eotaxin 1 and eotaxin 2. Eosinophils selectively and in great numbers express C-C chemokine receptor 3 (CCR3), the receptor for eotaxin ligands [168]. Epithelial cells, macrophages, and eosinophils themselves can produce eotaxin in response to parasitic infections or in allergic responses [169–171]. IL-4 production by ILC2s, basophils and Th2 cells induces eotaxin production in cells. Eotaxin-

activation of CCR3 promotes eosinophil chemoattraction and induces eosinophil degranulation[172].

1.4.8 IL-10

IL-10 is an anti-inflammatory cytokine. It was initially discovered as cytokine synthesis inhibitory factor (CSIF), which suppressed Th1 cell cytokine production [120]. IL-10 binds to two receptors, IL-10R1 and IL-10R2 and both receptors are required as a complex for IL-10 signalling [173–175]. IL-10R2 expression is widespread but IL-10R1 expression is mainly restricted to leukocytes. This reflects the major function of IL-10, which is to inhibit innate and adaptive immune cells. The pro-inflammatory activity of Th1 cells, NK cells, and macrophages is important for the clearance of infection. However excessive activity can cause immunopathology and tissue damage so the role of IL-10 is to limit this. IL-10 supresses the production of pro-inflammatory cytokines, and the expression of MHC-II and co-stimulatory molecules on APCs, thus attenuating T helper cell activation [176, 177].

Due to the anti-inflammatory properties of IL-10, it has an important role in the promotion of self-tolerance and prevention of autoimmunity. T cell-produced IL-10 maintains homeostasis in the gut by preventing reactivity against the microbiota [126, 178]. IL-10 deficient mice develop chronic enterocolitis, pathological inflammation of the digestive tract [179]. In EAE, a mouse model of multiple sclerosis mediated by Th1 and Th17 cells directed against self-antigen in the brain, IL-10 knockout mice are more susceptible to development of EAE and the disease is more severe [180]. The induction of IL-10 production in the CNS supressed the development of EAE and the transfer of IL-10-producing Treg cells from naïve mice into mice with EAE decreased disease severity [181, 182].

1.5 Type 1, Type 2, and Type 3 Immune Responses

The cells and molecules of innate and adaptive immunity ultimately need to co-ordinate their actions to efficiently eliminate infection. Depending on the nature of an infection, their activities are integrated in different manners, leading to the induction of distinct

overall immune responses. There are three major separate types of immune responses that have been described: type 1, type 2, and type 3.

1.5.1 Type 1 Immune Responses

The immune system controls intracellular bacterial, viral, and unicellular parasitic infections using type 1 immune responses [183]. Intracellular pathogens activate PRR on DCs inducing the production of IL-12 and IL-18, which promote the differentiation of Th1 cells and ILC1s and the production of IFN-γ. IFN-γ from ILC1s and Th1 cells induces classically activated M1 macrophages. NK cells are activated during viral infections and have a specific role in killing virus-infected cells. Type 1 immune responses, along with type 3 responses, are implicated in several autoimmune diseases [60].

1.5.2 Type 2 Immune Responses

Type 2 immune responses are generated to fight multicellular parasites infections as type 1 immunity is unsuited to killing large extracellular parasites [184]. Instead, a type 2 response fights parasitic infections by improving barrier functions and promoting the expulsion of the parasites [185]. Elements of the type 2 immune response also help to maintain metabolic homeostasis, promote tissue remodelling following injury, and supress type 1 and 3 responses, which are important in preventing autoimmunity.

Type 2 immune responses involve ILC2s, Th2 cells, eosinophils, basophils, mast cells, IgE antibodies and IL-4, IL-5, IL-9, IL-13, IL-25, IL-33, and TSLP cytokines. The response is required for controlling extracellular parasite infections, but it is also responsible for the immunopathology that develops in patients with allergic diseases [60].

1.5.3 Type 3 Immune Responses

Type 3 immunity protects against extracellular bacteria and fungi. DCs detection of extracellular bacteria and fungi induces IL-1β and IL-23 production. These cytokines promote Th17 cell differentiation and IL-17 and IL-22 production by ILC3s. CXCL8 (the neutrophil recruiting chemokine) production is induced and neutrophils are recruited to the site of infection. IL-17 knockout mice have increased susceptibility to bacterial and fungal infection [186]. This is mirrored in humans with deficiencies in IL-17-mediated

immune responses. For example, patients with an autosomal loss-of-function mutation in the IL-17 receptor suffer from chronic *Candida albicans* fungal infections [187].

1.6 Immune-mediated Diseases

The immune system has evolved mechanisms to remove potentially harmful lymphocytes that are reactive to harmless environmental or self-antigens. However sometimes these cells can persist and generate the unnecessary and damaging immune responses that underpin allergic and autoimmune diseases.

1.6.1 Allergy

An allergic reaction is a hypersensitive immune response to certain non-toxic environmental antigens called allergens. Allergens include pollen, house dust mite, and food allergens [188]. The prevalence of allergic diseases has been increasing worldwide; one in three people are now thought to suffer from allergies [189]. There are several causes of allergies but the most common forms involve IgG antibodies specific for allergens [190]. Antibodies are released in response to allergen exposure and they attach their base to mast cells and basophils [189]. Following subsequent exposure, allergens bind to the antigen-binding sites of the antibodies attached to mast cells/basophils. Adjacent antibodies are cross linked and this induces mast cells and basophils to release histamine and other inflammatory agents from granules (degranulation). Histamine causes blood vessel dilution and increased permeability leading to recognisable symptoms, some of which are tissue specific e.g. airway narrowing in asthma. In the early phase of the allergic reaction, inflammation is accompanied by pruritus (itching sensation), oedema, urticarial (hives) and, in severe cases, blood vessel dilation causing a drop in blood pressure that can often be fatal if untreated (anaphylaxis). Subsequent recruitment of cells associated with a type-2 response (Th2 cells and eosinophils) mediates the late phase of an allergic reaction. Late reactions feature oedema, pain, warmth and erythema (redness).

1.6.2 Autoimmunity

Autoimmunity is a phenomenon caused by cells of the adaptive immune system responding to self-antigen and inducing pathological inflammation. Examples include rheumatoid arthritis where the self-antigens are found in joints and multiple sclerosis where T cells are reactive to cells in the CNS.

1.6.2.1 Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of CNS, caused by the immune system damaging the myelin that sheaths and insulates neuronal axons, leading to the destruction of grey and white matter. Approximately 2.5 million people worldwide suffer from MS [191]. The precise disease aetiology is unknown, but it appears to involve a combination of genetic susceptibility and non-genetic triggers, including different pathogens and smoke constituents, that together cause MS. 30% of the overall risk is accounted for by genetic variation.

MS is characterised by the infiltration of macrophages, and autoreactive T and B cells across the BBB into the CNS, causing astrocyte and microglial activation, myelin breakdown and axonal damage. The demyelination of axons weakens signal transduction within the CNS causing the manifestation of neurological symptoms. The disease is diagnosed in most patients based on clinical manifestations involving motor, sensory, visual, and/or autonomic dysfunction. In cases where clinical observations are ambiguous, supporting evidence is provided by magnetic resonance imaging (MRI) that can detect white matter damage [192].

While the disease course and symptomatology of MS can vary, in most patients, the disease is characterized by initial periods of reversible neurological deficits, with accompanying progressive neurological deterioration over time. MS generally manifests itself in one of three ways (although rarer forms are seen also) [191]:

1) Relapsing–remitting MS: The most common form, affecting approximately 85% of MS patients. It is marked by episodes (relapses) of symptoms followed by periods of remission, when symptoms ameliorate or are absent.

2) Secondary progressive MS: Some relapsing–remitting patients may progress to secondary progressive MS wherein disease course eventually continues to worsen without periods of remission.
3) Primary progressive MS: Affects approximately 10% of MS patients. Symptoms progressively worsen from the onset of disease with no periods of relapse or remission.

1.6.2.2 Experimental autoimmune encephalomyelitis

The most commonly used animal model of MS is EAE. EAE is induced by promoting the generation of an auto-immune response to myelin protein in the CNS [193]. Myelin antigens used for EAE induction include myelin basic protein, proteolipid protein (PLP), myelin-associated glycoprotein, and myelin oligodendrocyte glycoprotein (MOG). Animals are injected with a combination of myelin antigen, complete Freund's adjuvant (CFA), and pertussis toxin (PT).

DCs are activated by PAMPs contained in *Mycobacterium tuberculosis* component of CFA and present myelin antigen to naive myelin-specific T cells in LNs [194]. The activated myelin-specific T cells exit the LNs and traffic to the CNS crossing the BBB, which is broken down by PT. T cells entering the CNS encounter their myelin antigens presented by CNS-resident APCs and are reactivated, releasing cytokines and recruiting other immune cells to the site of inflammation. Microglial cells and recruited cells release cytotoxic factors including proteases and reactive oxygen species which facilitate myelin destruction and ultimately axonal damage and neurological dysfunction. Initially thought to be mediated by Th1 cells, it is now known that Th17 cells are the predominant cell type responsible for mediating pathology in EAE [195, 196].

There are differences between MS and EAE and some treatments that are beneficial in EAE were deleterious in MS like IFN-γ and TNF [194]. However, EAE is an effective tool to help better understand the cellular and molecular mechanisms of CNS autoimmunity and has led to the discovery of novel, effective MS therapies, including natalizumab.

1.7 Parasitism and Helminths

In 1857 the German physician Friedrich Küchenmeister defined parasites as "independent organised beings, descended from a particular animal or vegetable parents, which require, in order that they may be enabled to complete their development, growth, or reproduction, to take up their abode either constantly or temporarily in or upon a second animal or vegetable organism of a different kind, from which they also derive their nourishment" [197]. This definition holds today: parasitism is a non-mutual symbiotic relationship between two species, parasite and host, in which the parasite benefits at the expense of the host [198]. Parasites have complex, multi-staged life cycles and live on or within their hosts [5].

1.7.1 Helminths

There are three major classes of parasites that infect humans: protozoa, helminths, and ectoparasites [199]. Helminths are large, multicellular parasitic worms. Like protozoa, helminths can be either free-living or parasitic in nature. Knowledge of helminths has existed for millennia: ancient Egyptian medical papyri refer to intestinal worms and the ancient Greek physician Hippocrates described worms that were discovered in fish, domestic animals, and humans [200].

There are two phyla of helminths: Nematoda and Platyhelminthes [201]. Nematodes, or round worms, can be intestinal e.g. *Necator americanus* or filarial e.g. *Brugia malayi* [202, 203]. They are elongated and cylindrical organisms containing tubular digestive systems with openings at both ends [201]. There are two classes of Platyhelminthes (flat worms): cestodes e.g. *Taenia solium* and trematodes (flukes) e.g. *Fasciola hepatica* [204, 205]. Unlike nematodes, platyhelminthes do not possess a body cavity or specialized circulatory or respiratory organs. Thus they are required to have compressed, flattened shapes to allow for oxygen and nutrients to be delivered throughout the entire individual organism by diffusion [201].

Many helminth species have complex life cycles comprising of egg, larval and adult stages and require more than one host [201]. The host which the adult helminth infects is defined as a definite host. The host in which one or more egg or larval life cycle stages

are present is an intermediate host. Some helminth species, soil-transmitted helminths, do not require an intermediate host as they can live independently in their larval stage.

Helminth infections are widespread worldwide; According to the WHO, approximately 1.5 billion people (or 24% of the global population) are infected by soil-transmitted helminths alone [206]. Infections are widely distributed in tropical and subtropical areas of the developing world, with prevalence being the highest in sub-Saharan Africa, Central and South America, and East Asia. Helminth infections are examples of neglected tropical diseases (NTDs), infectious diseases strongly associated with poverty that predominantly affect individuals in low- and middle-income countries [207].

There are high medical, educational, and economic burdens with helminth infections if they are left untreated [208]. Common symptoms of soil-transmitted long lasting helminth infections include diarrhoea, abdominal pain, malnutrition, and impaired growth, development, and physical fitness [206]. Helminth infections also cause more serious morbidities. Individuals infected with filarial nematodes like *B. malayi* develop lymphatic filariasis (elephantiasis) [209]. Infection with the *Onchocerca volvulus* causes river blindness (onchocerciasis). Worm burdens are higher in school-aged children than in any other age group. The adverse health implications of helminth infection lead to impaired school performance which ultimately reduces the future earning potential of infected adolescents [210, 211]. In underdeveloped regions morbidity arising from helminth infections leads to reduced productivity in school or in the workplace which in turn leads to increased poverty causing a downward spiral [208].

Campaigns have been established to eliminate the helminth infections that are widespread in populations from economically disadvantaged regions. The WHO is aiming to eradicate soil-transmitted helminth infections in children and lymphatic filariasis by 2020 [206, 212]. Elimination is achievable through mass drug administration (MDA) [212]. MDA involves the systematic administration of anti-helminthic drugs on the entire population of endemic areas whether individuals are infected or not. As helminth infections manifest mainly in some of the world's poorest areas, there is little investment from the pharmaceutical companies in the development of new anthelminthic drugs due to the lack of an economic incentive [208]. Thankfully

parasitism is largely preventable without the need for therapeutic intervention. Helminth infection is linked to poverty and untreated water supplies. Therefore, cases can be prevented with basic water treatment and education around best practice hygiene.

1.7.2 Immune Response to Helminths

Helminth infection elicits a protective type 2 immune response in the host that is mediated by Th2 cells and the type 2 cytokines IL-4, IL-5, and IL-13 [213] (Figure 1.4). This immune response rarely kills the helminths but instead infection is limited and the worms physically expelled [201]. The importance of a Th2 response to clear infection was revealed in epidemiological studies. Loci that control Th2 responses or their effector cytokines, e.g. polymorphisms in the *IL13* and *STAT6* genes, are associated with susceptibility to helminth infection [214–216]. Children are more susceptible to helminth infection than adults [208]. Juvenile rhesus macaque monkeys and juvenile mice infected with the helminths *Schistosoma mansoni* and *Nippostrongylus brasiliensis* respectively developed reduced type 2 immune responses with decreased Th2 cytokine production compared to adult animals [217, 218].

Epithelial cells produce the alarmin cytokines IL-25, IL-33, and TSLP in response to helminth-induced cell damage [201]. Alarmins are endogenous danger signals released from damaged cells [219]. IL-25, IL-33, and TSLP all promote type 2 responses. ILC2s respond to IL-25 and IL-33 and produce an early source of the type-2 cytokines IL-5 and IL-13 that protect against infection before an adaptive response has been generated [220]. TSLP promotes IL-4 production by basophils, which induces the differentiation of Th2 cells [53]. Basophils are the initial source of IL-4 following helminth infection before Th2 cells have been induced [221]. Th2 cells produce the type 2 cytokines IL-4, IL-5, and IL-13 which play a central role in inducing various innate and adaptive immune cells involved in clearing the infection [185]. IL-4 and IL-5 act on B cells to induce parasite-specific IgG1 and IgE antibody production. Anti-parasite IgE binds FcεR on mast cells and the cells are activated upon IgE cross linking, triggering mast-cell degranulation and the release of soluble inflammatory mediators involved in the physical expulsion of helminths [185, 201].

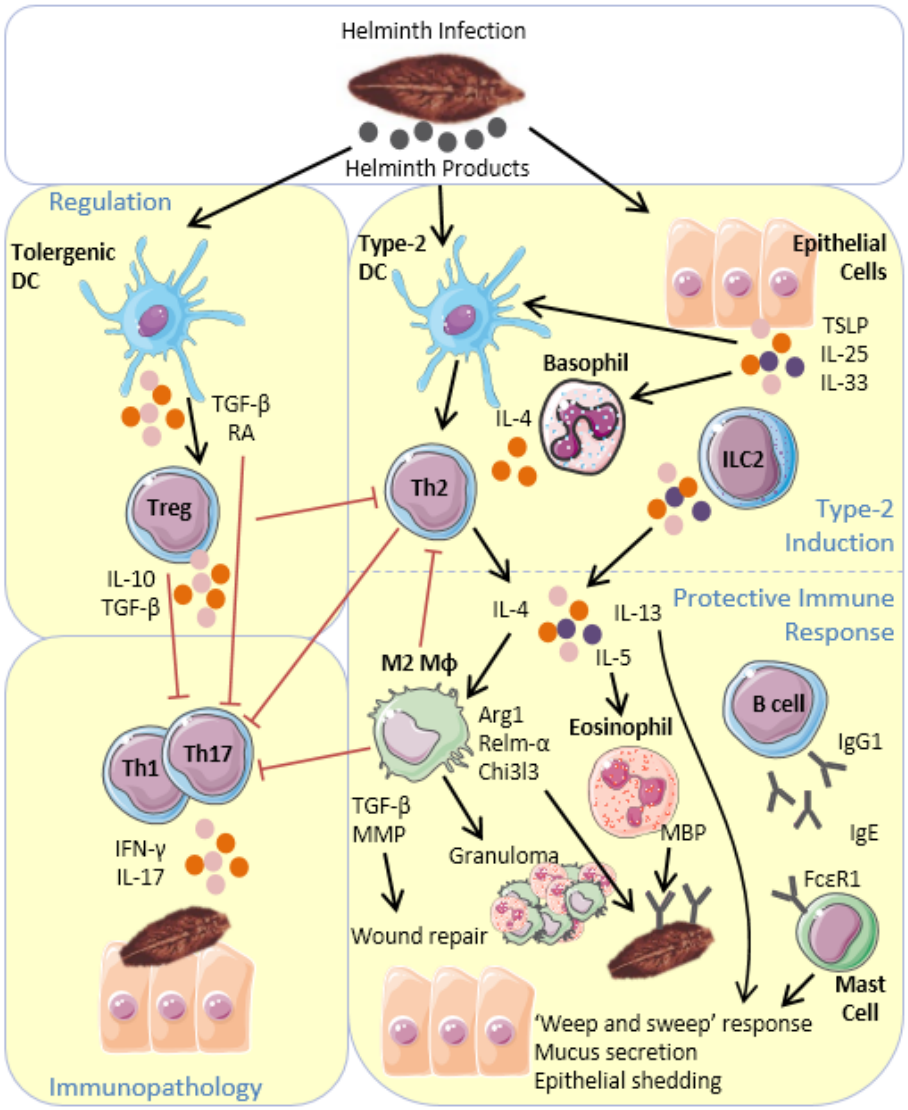


Figure 1.4: The immune responses induced by helminths.

Epithelial cells produce TSLP, IL-25, and IL-33 inducing the release of the type-2 cytokines IL-5 and IL-13 from ILC2s. and IL-4 production by basophils, promoting the differentiation of Th2 cells. Th2 cells produce IL-4, IL-5, and IL-13. IL-4 and IL-5 induce B cell class switching, promoting mast cell activation. IL-4 and IL-13 induce M2 macrophage activation, promoting wound healing. IL-5 induces eosinophil expansion and, with M2 macrophages, they enclose helminths in granulomas. IL-13 promotes the 'weep and sweep' response. Inappropriate Th1/Th17 response to a helminth or excessive Th2 responses induce immunopathology. Helminth products also induce tolerogenic DCs, activating Treg cells that suppress Th1/Th17/Th2 cells thus limiting immunopathology. Abbreviations: ILC2 (Group 2 innate lymphoid cell), RA (Retinoic acid). Figure adapted from Finlay et al (2014).

IL-5 promotes the development, survival and recruitment of eosinophils [119]. Eosinophils produce granule proteins e.g. major basic protein, which are toxic *in vitro* against helminths [47]. However, it has been difficult to replicate this toxicity *in vivo* and as helminth infections are chronic it is unlikely eosinophils are capable of killing the parasites during infection. However by releasing toxic granule proteins, eosinophils aid in the ability of the immune system to reduce helminth fitness and reproduction [201].

IL-4 and IL-13 induce the alternative activation of macrophages. M2 macrophages produce molecules including RELM-α and chitinases that target chitin, a glycan expressed by helminths but not mammals, thus damaging the helminths [185]. M2 macrophages are high expressers of Arginase-1. Arginase-1 converts L-arginine into urea and ornithine, thus starving helminths of the essential amino acid [201, 222]. Together with eosinophils, M2 macrophages are major components of type-2 granulomas in which antibody-bound helminths are encapsulated. Type-2 granulomas protect the host by sequestering the toxic products of helminths [223].

IL-13 has direct effect on mucosal tissues to promote helminth expulsion. It induces epithelial shedding, mucus secretion by goblet cells, and smooth muscle contraction promoting the physical removal of helminths in what is termed the 'weep and sweep' response [185]. IL-4 is not required for worm clearance [224].

Helminth-induced type 2 immune responses inhibit the generation of a type 1 immune response. An inappropriate Th1 or Th17 response to a helminth can be induced in the event that an insufficiently potent type 2 response is generated. The induction of IFN-γ producing Th1 cells is linked to chronic helminth infection [225]. In macrophage-specific IL-4 receptor knockout mice, *S. mansoni* infection could not induce M2 macrophages. Th1 cytokine production was elevated and significant immunopathology developed, which was sufficiently severe to kill all the infected knockout mice [226]. *Schistosoma haematobium*-infected humans presenting with immunopathologic hepatic lesions had elevated numbers of Th17 cells in peripheral blood when compared with pathology–free patients [227].

1.7.3 Immune Regulation induced by Helminths

The type 2 immune response induced by helminth infection is protective and will expel the parasites from the host. Despite this, most helminth infections are chronic, asymptomatic, and without significant immunopathology [228]. This is due to the induction of a regulatory immune response by helminths (Figure 1.4). Helminth-induced regulatory responses are ultimately beneficial to the host as well as the parasite, as they not only to suppress the protective Th2, responses but also prevent Th1/Th17 immunopathology that is deleterious for both host and parasite [201]. A prolonged or excessive Th2 response can also induce immunopathology e.g. Schistosomiasis is a disease caused by a strong Th2 response to schistosome eggs which induces a granulomatous response and pathological fibrogenesis [229, 230]. Therefore, helminth induced-regulation prevents the elimination of infection but also limits immune pathology.

Helminths promote the differentiation of Treg cells, which suppress the function of effector Th1 and Th2 cells activated following helminth infection. They produce the anti-inflammatory cytokines IL-10 and TGF-β that regulate both anti-parasite Th2 cells and inflammatory Th1/Th17 cells that mediate immunopathology. Hyper-reactive onchocerciasis (or sowda) is a complication in patients infected with *O. volvulus* where the worm is killed but the dead parasite induces a strong, harmful Th2 response leading to severe dermatitis and lymphadenitis [231]. *O. volvulus*-infected patients who do not display hyper-reactive onchocerciasis avoid pathological Th2 responses via the induction of TGF-β production. Type 1 responses induced by helminth infections are also attenuated by Treg cells. Patients infected with *Wuchereria bancrofti* developed elephantiasis due to the induction of high Th1 and Th17 responses [232]. Pathology correlated negatively with Treg numbers but not with Th2 responses.

Helminth-derived molecules promote the differentiation of tolerogenic DCs that preferentially induce Treg cells [233, 234]. Activation of TLR2 by phosphatidylserine from *S. mansoni* promoted the ability of DCs to induce Treg cell differentiation [235]. The importance of TLR2 in helminth-induced immune regulation was demonstrated in $TLR2^{-/-}$ mice infected with *S. mansoni*. Knockout mice demonstrated elevated Th1

responses and immunopathology, and reduced Treg expansion compared to wildtype mice [236].

Helminths induce effector T-cell hyporesponsiveness via T cell inhibitory molecules including PD-1 and CTLA-4. Following chronic helminth infection in mice with the filarial nematode *Litomosoides sigmodontis*, Th2 cells became hyporesponsive, displaying reduced proliferation and cytokine production [237]. Th2 cell hyporesponsiveness was a crucial determinant of susceptibility to *L. sigmodontis* infection. Hyporesponsiveness was reversed *in vivo* by blockade of PD-1. CTLA4 is a negative regulator of T cell activation. Neutralisation of CTLA4 enhanced the T cell immune response to *N. brasiliensis* infection, promoting type 2 cytokine production and reducing worm burden [238]. Treg cells are crucial in mediating helminth-induce effector T cell hyporesponsiveness and maintaining the chronicity of infection. The hyporesponsiveness displayed by Th2 cells following *L. sigmodontis* infection in mice was attenuated following the depletion of Treg cells and infection was subsequently cleared [239].

Helminth-induced M2 macrophages upregulate arginase-1 expression leading to the depletion of L-arginine from the tissue. This not only starves helminths of a vital nutrient but also T cells, particularly effector T cells, which have a high metabolic turnover [201]. L-arginine-depletion halts T cell cell-cycle progression and impairs T cell proliferation. Expression of the TCR ζ-chain (CD3ζ) is reduced leading to reduced T cell receptor signalling and cytokine production [240, 241]. M2 macrophages also suppresses type-1 inflammation via the production of IL-10 [24].

The feeding and migratory behaviour of helminths can cause tissue damage, particularly in the gut and lung [242, 243]. M2 macrophages facilitate tissue repair as they produce many factors associated with wound repair [244]. Metabolites of the arginase-1 substrate l-arginine (polyamines and proline) are used in cell proliferation and collagen production respectively [245]. RELM-α and YM1 mediate the deposition of extracellular matrix, a process involved in wound repair [246].

Due to the induction of Treg cells and M2 macrophages and the concurrent inhibition

of effector T cell responses, helminth infection will often remain chronic with no expulsion of the parasite.

1.7.4 Hygiene Hypothesis

In 1989 David Strachan proposed the 'hygiene hypothesis' as a potential explanation for the apparent rise in the prevalence of allergic diseases in Britain, an industrialised, first world country [247]. He commented that the increased prevalence of hay fever could "be explained if allergic diseases were prevented by infection in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally from a mother infected by contact with her older children…Over the past century declining family size, improvements in household amenities, and higher standards of personal cleanliness have reduced the opportunity for cross infection in young families." The hygiene hypothesis was initially thought to be explained by lifestyle changes that occur in a region as it becomes more economically developed; that improved general hygiene conditions, widespread vaccination, and easy and cheap access to antibiotics will reduce the incidence of bacterial and viral infection in childhood (Figure 1.5). A lack of exposure to infectious agents early in life leads to insufficient stimulation of the Th1/Th17 response. This in turn cannot attenuate Th2 cell expansion and so enhances susceptibility to allergic diseases [248]. However the observation that the was a strong positive association between the incidence of asthma and type 1 diabetes mellitus, an autoimmune disease mediated by Th1 responses, argued against the explanation that increased prevalence of allergic diseases was due to an imbalance in the Th1/Th2 axis [249].

Autoimmune diseases are also more prevalent in areas with low levels of infections and higher sanitation (Figure 1.6). This suggested that there were common factors which are responsible for the rise in allergy and autoimmunity [249–251]. The hygiene hypothesis has now been updated to reflect this. The rise in the prevalence of autoimmune as well as allergic diseases in developed countries has been linked with reduced exposure to infectious organisms, especially helminths: specifically it is linked to reduced regulatory immune responses that infections invoke in the host [252, 253]. While both allergic diseases and helminth infection induce strong Th2 responses, there is a significant

regulatory proportion of the immune response to helminths which is protective. It is this which is thought to be protective against autoimmune and allergic disease as it leads to global immune suppression of Th1/Th17 and Th2 responses.

Helminth infected MS patients had a dramatically improved disease outcome with reduced relapse rates, less lesion formation and reduced disability scores compared to uninfected patients [254]. Infected patients had an enhanced frequency of Treg cells. The administration of anti-helminthic therapy reversed the improvements seen in infected patients [255]. Similarly, it has been demonstrated that helminth infection is protective against the development of allergic disorders. A smaller proportion of children infected with *S. haematobium* tested positive for skin-prick tests for house dust mite allergy compared with uninfected children [256].

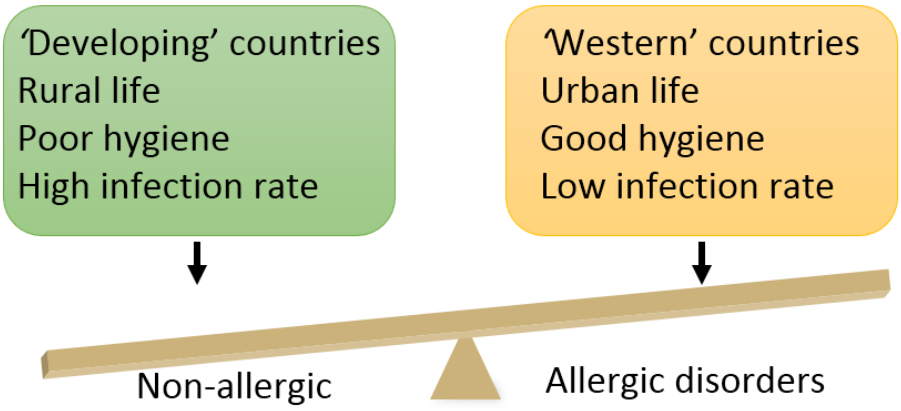


Figure 1.5: The Hygiene Hypothesis, initially.

In 'Western' societies, allergic diseases became more prevalent This was initially explained by the environmental changes in developed countries; that reduced exposure to animals, better hygiene, and lower infection rates in turn lead to increased susceptibility to allergies. Adapted from Wills-Karp et al (2001) [257].

Data from epidemiological studies suggesting that helminth infections are protective in autoimmunity and allergy is supported by strong experimental evidence in animal models. Different helminth infections including *L. sigmodontis, N. brasiliensis*, and *S. mansoni* reduced pathological type 2 inflammation and inhibited the development of airway hyper-responsiveness in mouse model of asthma [258–260]. Helminth infections are also protective in mouse models of different autoimmune diseases also. *Schistosoma japonicum* protected against collagen-induced arthritis by inhibiting the production of pro-inflammatory cytokines, including IFN- γ [261]. In a trinitrobenzenesulfonic acid (TNBS)-induced mouse model of colitis, *S. mansoni* promoted protection by decreasing IFN-γ and inducing IL-10 production [262]. EAE, the mouse model of MS, is mediated by myelin-specific Th1 and Th17 cells. Infection with *S. mansoni* and *F. hepatica* attenuated the development of EAE through suppression of pathogenic Th1 and Th17 cells in the brain and spinal cord [205, 263]

Following on from the promising data seen in animal models, clinical trials involving live helminths in humans have been completed or are currently underway. Fleming and Weinstock (2015) reviewed clinical trials of live helminth therapy in autoimmune diseases [264]. In all they reviewed 28 clinical trials of helminth therapy in ten autoimmune, allergic or similar conditions in which immunopathology is implicated. The trials were enrolling, recruiting, ongoing, or completed and varied from phase 0 to phase 2. Trials were conducted using one of two helminths: *Trichuris suis* or *N. americanus*. The helminths were chosen due to the perception that they would be safe and tolerable. Results from the trials completed so far bore this out; disease progression was not worsened in infected patients and no serious adverse effects were reported. Many of the trials did not test efficacy and those that did showed only modest improvements at best. Often the methodologies of the trials were imperfect and they require optimisation before more meaningful conclusions can be drawn [265].

There are many regulatory and practical concerns with the use of live helminths as a therapy for human diseases. Even helminth species non-pathogenic to humans will cause tissue damage as they cross mucosal barriers [201]. Limiting the number of parasites administered ensures there is less of a safety risk, but the resulting dose of

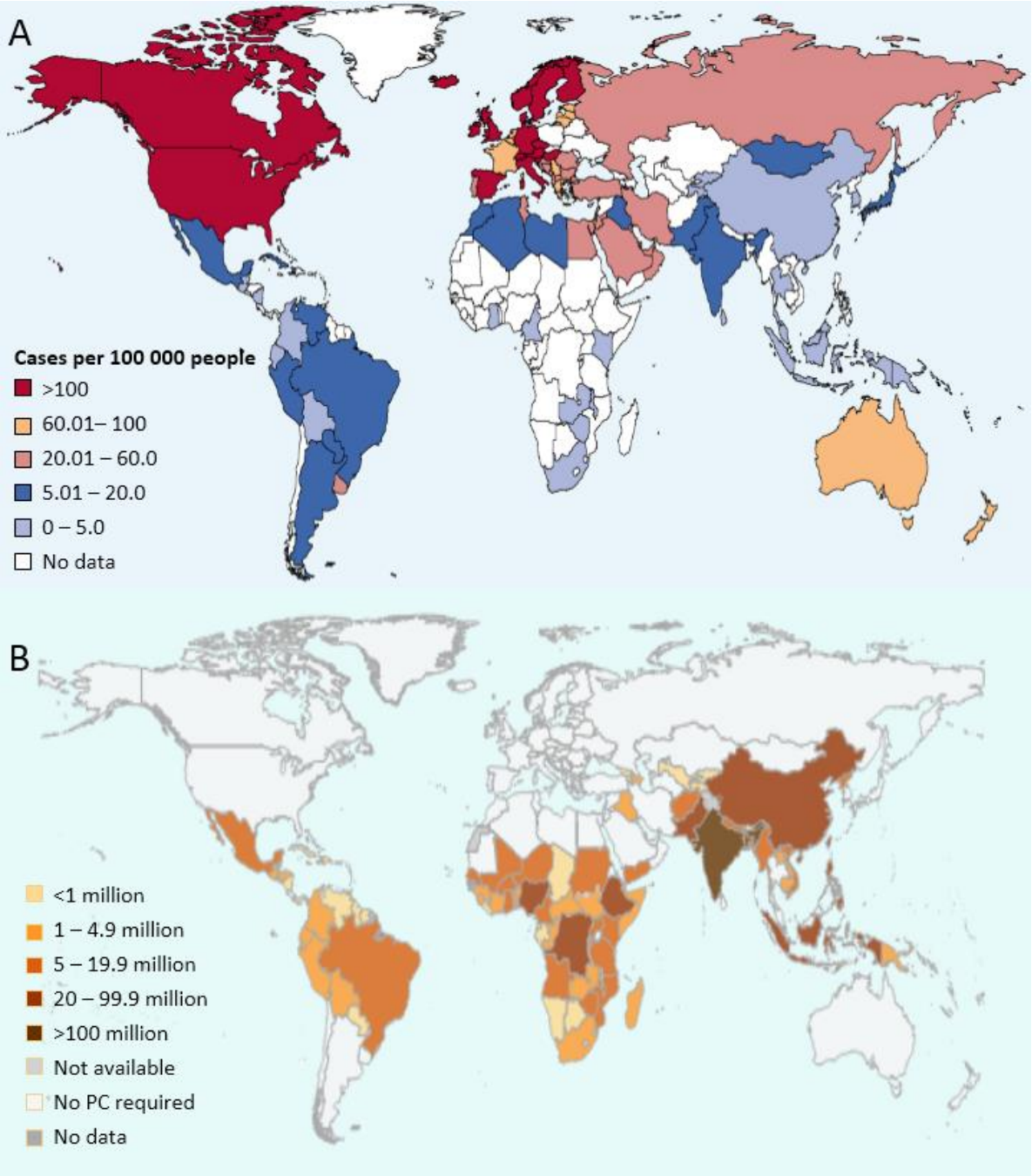


Figure 1.6: The Hygiene Hypothesis, updated.

Areas with high incidences of autoimmune disease (and allergies) have low levels of exposure to infectious agents. (A) Global prevalence of MS; adapted from Thompson et al (2018). (B) Number of children requiring preventative chemotherapy (PC) for soil-transmitted helminthiases; adapted from the WHO's Global Health Observatory (GHO) data (2016) [266, 267].

helminths may then be insufficient to generate a therapeutic effect. Optimising clinical trial conditions is challenging to achieve as it is very difficult to ensure the patients are blinded; it will often be obvious to an individual whether they have been infected with a worm or not. It is preferable instead to identify and characterise immunomodulatory molecules produced by helminths as this would eliminate many of the outstanding issues with using live parasites. Work is ongoing in our lab to isolate and better understand immunomodulatory factors produced by the helminth *Fasciola hepatica*.

1.7.5 *Fasciola hepatica*

F. hepatica is a trematode platyhelminth known colloquially as liver fluke. It causes the disease fascioliasis, a zoonosis (an animal disease that can be transmitted to humans) [268]. *F. hepatica* is a parasite common to cattle and sheep livestock and cause significant economic damage to the agriculture industry, costing an estimated £23 million annually to the UK cattle industry alone [269]. *F. hepatica* also infects humans in large numbers. The WHO estimates that more than 2.4 million people are infected across at least 70 countries worldwide, with several million at risk [268]. *F. hepatica* infection is more widespread around the world than that of any other parasite [270].

F. hepatica possesses a complex life-cycle involving a definite host (in which the adult worm lives) and an intermediate host (in which the larval stages of the worm develop). Infected animals contain *F. hepatica* eggs in their faeces so when they defecate in fresh-water the eggs are released. They hatch into larvae and enter into water snails, their intermediate hosts. Once in the snail, the larvae develop and are released as cercariae into the water. The cercariae encyst as metacercariae (infectious larvae) and attach to aquatic plants. When the plants with the metacercariae attached are ingested by cattle and other animals, the larvae enter their definite host. Upon ingestion the immature worms travel from the gut, penetrating the intestinal wall through to the peritoneum. Then they enter the liver feeding on blood and hepatocytes until they eventually reach the bile ducts. This translocation leads to significant tissue damage. In the bile ducts the worms mature and produce eggs. These eggs are released into the bile, reaching the intestine via the gall bladder and bile duct, and they are eventually deposited in faeces. Therein the life cycle is completed [268, 271].

1.7.5.1 *Fasciola hepatica* products

As *F. hepatica* translocate through their host they release different excretory-secretory products collectively known as *F. hepatica* excretory-secretory product or FHES [233]. In a laboratory setting, FHES is isolated following overnight incubation of liver fluke at 37°C in PBS [157]. The fluke supernatant fluid contains FHES. FHES induces regulatory responses in the host aiding the fluke in avoiding immune detection and expulsion [233, 272]. FHES administration was protective in EAE, mimicking like infection [157]. As FHES is a mixture of many molecule including proteins and nucleic acids, there is the potential to discover specific helminth-derived immunomodulatory factors, including individual proteins that are contained within FHES.

There are currently no ongoing clinical trials of purified helminth products or molecules in autoimmune diseases but the potential benefit of individual immunomodulatory factors produced by helminths is clear [264]. These factors include exosomes; small, membrane-bound vesicles 40-100nm in diameter that contain proteins, mRNA, and miRNA, and other potential signalling molecules [273–275]. Exosomes were initially described as vesicles secreted by maturing mammalian red blood cells [276]. Now, it has been shown that exosomes are found in hematopoietic and non-hematopoietic cells as well as in extracellular environments including serum, saliva, and urine [277]. The functions of exosomes are varied, can be complex, and depend on the cell type from which they are derived [278]. The secretion of extracellular vesicles like exosomes for cell signalling is important as it allows for the safe delivery of molecules which would otherwise be degraded in the extracellular environment [279]. This can explain why we see cytosolic previously thought as 'intracellular' proteins when we examine the helminth secretome [280].

Initially, the function of exosomes is thought to have been in the removal of undegraded endosomal and lysosomal proteins and membranes from the cell but now it is thought that they play a role in intercellular communication, especially in immune responses and cancer [281]. Mast cell secrete exosomes that induce mitogenesis in T and B cells [282]. Exosomes derived from DCs can both activate T effector cells and also promote tolerance to self-antigens [283]. Indeed, DC-derived exosomes have potential as anti-

cancer therapies by activating cytotoxic T cells to remove tumour cells [284]. While there is potential to use exosomes to treat cancer, tumour-derived exosomes can inhibit anti-tumour immune effector cells and also help to promote tumour escape from immune control [285].

Many parasite species including nematodes [286], trematodes [287], and protozoa [288] release exosomes and helminth-produced exosomes have been shown to contribute to parasite immune modulation. The gastrointestinal nematode *Heligmosomoides polygyrus* secretes exosomes containing micro RNAs (miRNAs) that suppress the type 2 innate immune response thus helping the helminth persist in the host [286]. Exosome-like vesicles from *S. japonicum* adult worms promote M1 macrophage activation [289]. *F. hepatica* was demonstrated to produce exosome-like vesicles in 2012 and, in 2015, Cwiklinski et al. identified a subpopulation of extracellular vesicles released by *F. hepatica* that were 30-100 nm in diameter and expressed several exosome markers including Hsp70, ALIX, and tetraspanin [287, 290]. This strongly suggested that *F. hepatica* secrete exosomes. *F. hepatica* secrete exosomes as a component of FHES from which they can be isolated by ultracentrifugation amongst other methods [291] (Figure 1.7) . Their immunomodulatory potential has yet to be examined.

The identification of individual proteins derived from helminths is another promising route for the discovery of novel helminth-derived immunomodulatory molecules with the potential to treat autoimmunity. As described, helminths can establish chronic infections in their hosts. Unsurprisingly, many helminth-derived immunomodulatory molecules have been identified. The field is now very well advanced and was recently extensively reviewed by Maizels et al (2018) [292]. Helminth-derived molecules have been shown to modulate the host immune response at every stage, from induction to effector, and finally resolution. DAMPs and PAMPs, including IL-33, initiate immune responses and are released early following helminth infection. The murine intestinal nematode *H. polygyrus* suppresses IL-33 release through HpARI (*H. polygyrus* alarmin release inhibitor) contained within its excretory-secretory (ES) products [293]. HpARI binds both DNA and IL-33 in the nucleus, thus inhibiting IL-33 release. DAMPs and PAMPs are detected on the surface of APCs by PRRs, leading to antigen recognition, internalisation, processing, and presentation. These processes of immune response

propagation are extensively targeted by helminth molecules. *F. hepatica*-produced FABP (fatty acid binding protein) suppresses the activations of TLRs on the surface of macrophages, inhibited activation [294]. Th2 cell differentiation induced by APCs is crucial to help fight helminth infection. Helminth-derived effector molecules can modulate the host's adaptive immune response to avoid expulsion. *H. polygyrus* produces a TGF-β mimic (TGM) that can skew the adaptive response away from Th2 towards a more anti-inflammatory Treg response [295]. Cells including eosinophils contribute to the effector phase in immune responses to helminths, helping to directly harm the parasites, limit their toxicity, and promote expulsion. Human hookworm-derived metalloproteinases cleave eotaxin, inhibiting eosinophil recruitment [296].

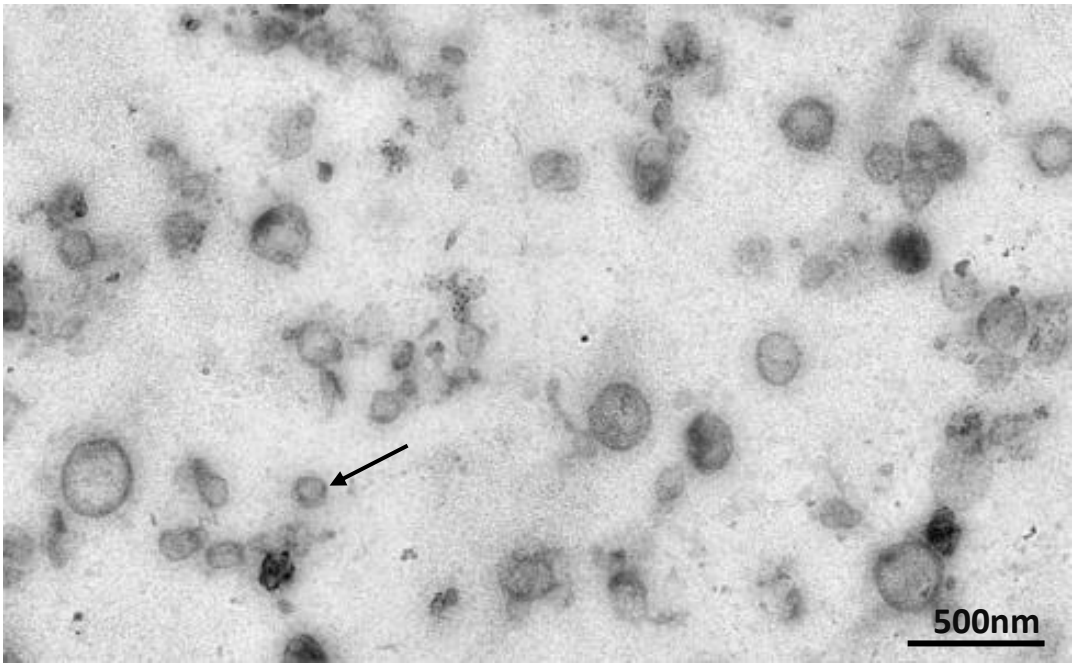


Figure 1.7 *F hepatica* secrete small extracellular vesicles called exosomes.

Adult *F. hepatica* fluke were cultured in PBS with P/S at 37 °C. Extracellular vesicles were isolated from the PBS + P/S (*F. hepatica* supernatant) by differential centrifugation; exosomes were contained in the 120,000 g pellet fraction. The above is a transmission electron microscopy image of the exosome-like 120,000 g vesicle pellet with an example of an exosome indicated by the arrow. Figure adapted from Cwiklinski et al. (2015).

Therefore, individual molecules derived from helminths can modulate all aspects of the immune response from the earliest to the end effector responses. This is promising in the search for new molecules capable of supressing pathological inflammation in autoimmunity. Indeed, the hookworm helminth *Ancylostoma caninum* secretes excretory/secretory products (ES), similar to FHES, which supressed pathological inflammation in a mouse model of colitis [297]. Anti-inflammatory protein-2 (AIP-2) was identified in *A. caninum* ES and was protective in a mouse model of asthma [298, 299]. *F. hepatica* helminth defence molecule (FhHDM-1), isolated from FHES, improved clinical signs in a relapse remitting model of EAE [243].

Previously in our lab, a study was designed to identify individual proteins in FHES with immune-modulating activity. FHES was isolated and divided into different fractions by size-exclusion chromatography. Intraperitoneal (i.p). injection of FHES induces eosinophil infiltration and expansion in the peritoneum and significantly enhances the serum concentration of eotaxin, an eosinophil-recruiting chemokine [157]. Therefore, each FHES fraction was injected i.p. into mice and the concentration of in the serum 2 hours post injection was measured. Two fractions were shown to strongly induce eotaxin production (Figure 1.7). Fractions positive for eotaxin induction were sent for analysis by mass spectrometry to identify candidate individual FHES proteins, which were identified by comparing peptides detected in the two fractions with two *F. hepatica* organism databases (UniProt Swiss-Prot and UniProt TrEMBL). Among the proteins identified was FHKTM (*F. hepatica* Kunitz-type molecule), a relatively novel *F. hepatica*-derived protein

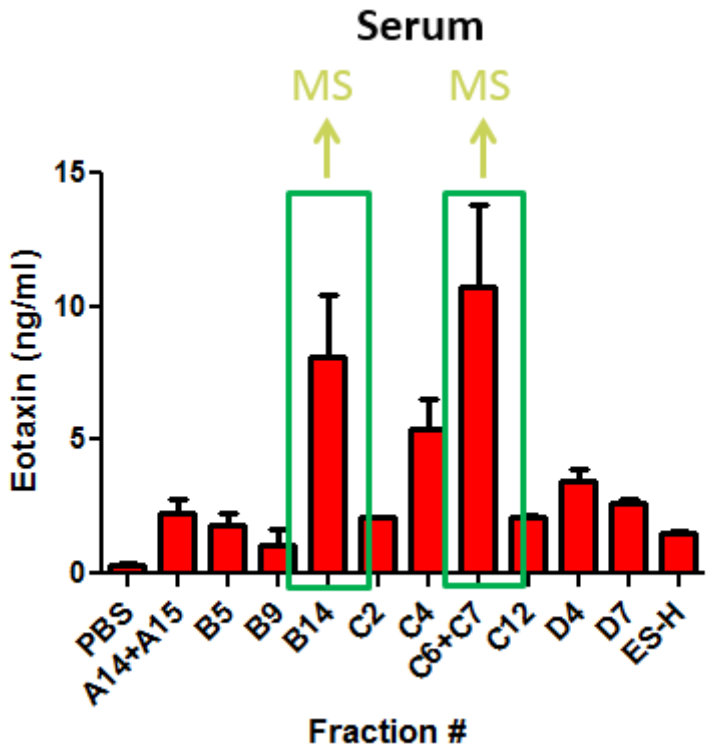


Figure 1.8: Two FHES fractions enhanced serum concentration of eotaxin.

Different fractions were prepared from FHES by size-exclusion chromatography (A14+15, B5, B9, B14, C2, C4, C6+7, C12, D4, D7, ES-H). The fractions were assayed *in vivo* for the ability to promote type-2 responses (eotaxin production), a characteristic of FHES injection. Positive fractions were analysed by mass spectrometry and several candidate proteins were identified by comparing peptides detected in the fractions with two *F. hepatica* organism databases.

Aims of the project:

- To purify and compare the activity of *F. hepatica* exosomes from FHES by differential centrifugation and by using the commercially available Exospin Exosome Purification Kit from Cell Guidance Systems.
- To examine the immunomodulatory activity of *F. hepatica* exosomes.
- To express and purify the individual recombinant *F. hepatica* protein FHKTM from *P. pastoris*.
- To examine the immunomodulatory effects of *F. hepatica* exosomes and FHKTM on macrophages and other immune cells and to investigate whether they can promote type-2 and/or anti-inflammatory immune responses *in vitro* and *in vivo*.
- To investigate if *F. hepatica* exosomes and FHKTM can suppress pro-inflammatory responses *in vitro* and *in vivo*.
- To identify the mechanisms by which *F. hepatica* exosomes, and FHKTM induce their immunomodulatory effects.
- To examine how the type-2 and anti-inflammatory responses induced by IL-33 might supress pathological inflammation mediated by pro-inflammatory stimuli.

Chapter 2

Materials and Methods

2.1 Materials

Ammonium chloride lysis solution (0.88 %)

0.77 g NH_4Cl (Merck) was dissolved in 100 mL Baxter's H_2O, filter-sterilised (fs), and stored at room temperature.

Cell culture medium

Complete RPMI (Roswell Park Memorial Institute medium) was prepared by supplementing 500 mL RPMI (Sigma) with 50 mL fs heat-inactivated foetal calf serum (FCS; Biosera or Sigma), 5mL fs L-Glutamine (200 mM; Sigma), and 5mL fs penicillin/streptomycin (10,000 units penicillin and 10 mg streptomycin/mL; Sigma) and stored at 4 °C. An additional supplement of 50 µM 2-mercaptoethanol (2-ME; Sigma) was added to cRPMI for the culture of spleen and/or lymph node cells.

Ethidium bromide/acridine orange (EB/AO)

2.5 g ethidium bromide (Sigma) and 2.5 g of acridine orange (Sigma) were dissolved in 50mL PBS (company) and stored at 4 °C.

ELISA blocking solution

5% Milk (5 g Skim Milk Powder (Sigma) was dissolved in 100 mL PBS)

Or

1% BSA.

ELISA developing solution

1 OPD (o-Phenylenediamine dihydrochloride) tablet (Sigma) was developed in 25 mL PCB with 7 µL H_2O_2 (company)

Or

TMB solution (Thermo Fisher).

ELISA stopping solution (1 M H_2SO_4)

473.26 mL dH_2O was added to 26.47 mL H_2SO_4 (18 M; Sigma).

ELISA washing buffer (PBS-Tween)

500 mL 20x PBS and 5 mL Tween 20 (Sigma) were topped up to 10 L with dH_2O and stored at room temperature.

Fluorescence-activated cell sorting (FACs) Buffer

500 mL sterile PBS (Sigma) was supplemented with 5 mL fs FBS, 5 mL fs HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 1 M; Thermo Fisher), and 10 mL fs EDTA (Ethylenediaminetetraacetic acid; 0.1 M; Sigma) and stored at 4 °C.

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 supplemented RPMI) and 70% Percoll solutions (diluted in PBS) were then prepared.

MACS buffer

500 mL sterile PBS was supplemented with 5 mL fs FBS and 10 mL fs EDTA (0.1 M) and stored at 4 °C.

Phosphate citrate buffer (PCB)

10.19 g anhydrous citric acid (ICN Biomedicals) and 15.77 g Na_2HPO_4 (Sigma) were dissolved in 1 L of dH_2O. pH was adjusted to 5 and the solution stored at 4 °C.

1% Bovine serum albumin (BSA) solution

5 g bovine serum albumin (Sigma) was dissolved in 500 mL PBS and stored at 4 °C.

1x Passive lysis buffer

A protease inhibitor cocktail tablet (Roche) was added to 2 mL 5x passive lysis buffer (Promega) combined with 8 mL MilliQ H_2O and the solution stored at 4 °C.

1M Hydrochloric acid (HCl)

8.3 mL HCl 37% (Sigma) was topped up to 100 mL with dH_2O and stored at room temperature.

2% Paraformaldehyde (PFA)

16% v/v Pierce PFA (Fischer) was diluted in PBS 1 in 8.

20x PBS

800 g NaCl (Sigma), 116 g Na_2HPO_4, 20 g KH_2PO_4 (Sigma), and 10 g KCl (Sigma) were dissolved in 5 L of dH_2O. pH was adjusted to 7 and the solution stored at room temperature

75% Ethanol (EtOH)

37.5 mL 100% EtOH (in house) was added to 12.5 mL dH_2O, filter-sterilised, and stored at room temperature.

2.1.1 *Pichia pastoris* yeast expression buffers (mainly from *Pichia* Expression Kit User Guide from Invitrogen by Life Technologies™).

Buffer I (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole)

3.55 g Na_2HPO_4, 8.77 g NaCl (Sigma), and 340 mg imidazole (Sigma) were dissolved in 500 mL dH_2O, pH adjusted to 8 (using HCl/KOH), filter-sterilised, and stored at 4 °C.

Buffer II (50 mM Na_2HPO_4, 300 mM NaCl, 250 mM imidazole)

710 mg Na_2HPO_4, 1.75 g NaCl, and 1.7 g imidazole were dissolved in 100 mL dH_2O, pH adjusted to 8 (using HCl/KOH), filter-sterilised, and stored at 4 °C.

Buffered glycerol-complex medium (BMGY) and buffered methanol-complex medium (BMMY)

10 g of yeast extract (Sigma) and 20 g of peptone (Fluxa) were dissolved in 700 mL dH_2O and autoclaved. 100 mL 10x YNB, 100 mL 10x Sodium Hexametaphosphate

buffer or 1 M potassium phosphate buffer (pH 6.0), 2 mL 500x B, and 100 mL 10x GY were added to give BMGY. For BMMY, 100 mL of 10x M instead of 10x GY was added. Both were stored at 4 °C.

Minimal dextrose (MD) medium plates

15 g agar (Oxoid) was added to 800 mL dH_2O and autoclaved. When cooled to approximately 60 °C, 100 mL 10x YNB, 100 mL 10x D, and 2 mL 500x B were added and plates were poured immediately.

NiNTA beads

4 mL NiNTA beads (Thermo Fisher) were centrifuged at 600 g for 1 minute. The upper 20% ethanol layer was removed, and the beads resuspended in 10 mL Milli-Q H_2O. They were centrifuged again at 600 g for 1 minute. The top H_2O layer was removed, and the beads suspended in 10 mL Buffer I. The beads were rolled at 4 °C until required.

1 M K_2HPO_4

43.5g K_2HPO_4 (Sigma) was dissolved in 250 mL dH_2O.

1 M KH_2PO_4

136 g KH_2PO_4 (Sigma) was dissolved in 1 L dH_2O.

1 M potassium phosphate buffer (pH 6.0)

132 mL of 1 M K_2HPO_4 was combined with 868 mL of 1 M KH_2PO_4 and pH was adjusted to 6. The solution was autoclaved and stored at room temperature.

10x D (20% dextrose)

200g D-glucose (Sigma) was dissolved in 1L dH2O, autoclaved and stored at room temperature.

10x GY (10% glycerol)

100 mL glycerol (Sigma) was mixed with 900 mL of dH_2O, autoclaved and stored at room temperature.

10x M (5% methanol)

5 mL 100% methanol (in house) was mixed with 95 mL of dH_2O, filter-sterilised and stored at 4 °C.

10x Sodium hexametaphosphate buffer

125 g sodium hexametaphosphate (Sigma) was dissolved in 500 mL dH_2O, pH was adjusted to 6, the solution filter-sterilised, and stored at room temperature.

10x YNB (13.4% yeast nitrogen base with ammonium sulphate without amino acids)

134 g YNB with ammonium sulphate and without amino acids (Sigma) was dissolved in 1 L of H_2O, filter-sterilised and stored at 4 °C.

500x B (0.02% biotin)

20 mg biotin (Sigma) was dissolved in 100 mL dH_2O, filter-sterilised and stored at 4 °C.

2.1.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) related solutions.

Coomassie stain

2.5 g Coomassie Brilliant Blue k250 (0.25%; Sigma), 420 mL 100% methanol, and 170 mL 100% acetic acid (Sigma) were topped up to 1 L with dH_2O, filter-sterilised, and stored at room temperature.

Coumaric acid (90 mM)

4.104 g coomaric acid (Sigma) was dissolved in 100 mL Dimethyl Sulfoxide (DMSO; Sigma) and stored at -20 °C.

Destain

150 mL 100% methanol and 50 mL 100% acetic acid were added to 350 mL dH_2O, filter-sterilised, and stored at room temperature.

HisProbe-HRP (5 mg/mL)

400 µL MilliQ H_2O was added to 2 mg lyophilised HisProbe-HRP (Thermo Fisher), aliquoted, and frozen at -20 °C.

Luminol (250 mM)

4.429 g luminol (Sigma) was dissolved in 100 mL DMSO and stored at -20 °C.

Running gel (15%)

8.05 mL Acryl-Bis (30%; Biorad), 2.8 mL dH_2O, 3.8 mL 1.5M Tris-HCl (pH8.8), 150 µL 10% SDS, 150 µL 10% APS, and 6 µL TEMED (Sigma) were combined and poured immediately.

Solution A

1 mL luminol (250 mM), 220 µL coumaric acid (90 mM), and 5 mL 1 M Tris (pH 8.6) were topped up to 50 mL with dH_2O and stored in darkness at 4 °C.

Solution B

60 µL H_2O_2 (Sigma) and 5 mL 1M Tris (pH 8.6) were topped up to 50 mL with dH_2O and stored in darkness at 4 °C.

Stacking gel

1 mL Acryl-Bis, 4.1 mL dH_2O, 750 µL 1 M Tris-HCl (pH6.8), 60 µL 10% SDS, 60 µL 10% APS, and 6 µL TEMED (Sigma) were combined and poured immediately.

TBS-tween (TBS-T)

100 mL of 10x TBS was combined with 900 mL dH_2O and 1 mL Tween 20 was added.

1x Running buffer

100 mL 10x Running Buffer was combined with 900 mL dH_2O.

1x Transfer buffer

10 mL 10x Transfer Buffer, 20 mL 100% methanol, and 70 mL dH_2O.

1 M Tris-HCl (pH6.8)

121.14 g Tris (Sigma) was dissolved in 800 mL dH_2O, pH adjusted to 6.8 with HCl, topped up to 1 L with dH_2O, and stored at room temperature.

1 M Tris (pH8.6)

12.114 g Tris was dissolved in 100 mL dH_2O, pH adjusted to 8.6, and stored at room temperature.

1.5 M Tris-HCl (pH8.8)

181.71 g Tris was dissolved in 800mL dH_2O, pH adjusted to 8.8 with HCl, topped up to 1L with dH_2O, and stored at room temperature.

5x SDS Sample buffer

5 mL glycerol, 10 mL 10% SDS, 6.25 mL 1M Tris-HCl (pH6.8), and 10 mg Bromophenol blue (Sigma) were topped up to 50 mL using dH_2O. Note, before use added 50 µL β-mercaptoethanol (Sigma) and 950 µl 5xSDS Sample Buffer.

10x Running buffer

144 g glycine (Sigma), 30.3 g Tris, and 10 g SDS were dissolved in 1 L of dH_2O and stored at room temperature.

10x Transfer buffer

144 g glycine and 30.3 g Tris were dissolved in 1 L of dH_2O and stored at room temperature.

10% APS (Ammonium persulphate)

1 g of APS (Sigma) was dissolved in 10 mL dH_2O and stored at -20 °C.

10% SDS

10 g SDS was dissolved in 100 mL dH_2O and stored at room temperature.

10x Tris buffered saline (TBS)

24 g Tris and 88 g NaCl were dissolved in 1 L dH_2O, pH was adjusted to 7.6 and stored at room temperature.

Table 2.1 Antigens

Antigen	Supplier	Conc. *in vitro*	Dose *in vivo*
FHES	See Section 2.3.2	-	100 µg/mouse/day
MOG_{35-55}	Cambridge	10–100 µg/mL	150 µg/mouse

Table 2.2 Bacterially-derived molecules

Molecule	Supplier	Conc. *in vitro*	Dose *in vivo*
Pertussis Toxin	Katetsuken	-	100-200 ng/mouse/day

Table 2.3 ELISA antibodies, standards, and blocking solutions (murine)

Cytokine	Block	Top Working Standard	Supplier
IL-1RA	1% BSA	10,000 pg/mL	R&D
IL-1β	1% BSA	1000 pg/mL	R&D
IL-4	5% Milk	2500 pg/mL	BD Pharmigen
IL-6	5% Milk	5000 pg/mL	BD Pharmigen

Cytokine	Block	Top Working Standard	Supplier
IL-10	1% BSA	2000 pg/mL	R&D
IL-12p40	5% Milk	5000 pg/mL	BD Pharmigen
IL-13	1% BSA	4000 pg/mL	R&D
IL-25	1% BSA	4000 pg/mL	R&D
IL-33	1% BSA	1000 pg/mL	R&D
GM-CSF	1% BSA	1000 pg/mL	R&D
TGF-β	1% BSA	2000 pg/mL	R&D
TNF	1% BSA	2000 pg/mL	R&D
TSLP	1% BSA	1000 pg/mL	R&D
YM1	1% BSA	5000 pg/mL	R&D

Table 2.4 Fluor-conjugated FACS antibodies

Specificity	Clone	Isotype	Fluorochrome	µL/10^6 cells	Supplier
CCR2	SA203 G11	Rat/IgG_{2b}, κ	PE	0.25	Biolegend
CD11b	M1/70	Rat/IgG_{2b}, κ	APC-eFluor 780	0.25	eBioscience
CD11b	M1/70	Rat/IgG_{2b}, κ	PE-Cy5	0.25	eBioscience
CD11b	M1/70	Rat/IgG_{2b}, κ	PerCP-Cy5.5	0.25	eBioscience
CD11c	N418	Armenian Hamster IgG	BV785	0.25	Biolegend

Specificity	Clone	Isotype	Fluorochrome	µL/10^6 cells	Supplier
CD19	1D3	Lewis IgG_{2a}, κ	PE-CF594	0.25	BD Biosciences
CD19	1D3	Rat/IgG_{2a}, κ	PE-Cy7	0.25	eBioscience
CD45	30-F11	Rat/IgG_{2b}, κ	APC-eFluor 780	0.5	eBioscience
CD206	PC61	Rat/IgG_{2a}, κ	BV605	0.5	Biolegend
CD3	145-2C11	Armenian Hamster IgG	APC	0.25	eBioscience
CD3	145-2C11	Armenian Hamster IgG	APC-eFluor 780	0.25	eBioscience
CD3	17A2	Rat/IgG_{2b}, κ	BV650	0.25	Biolegend
CD4	RM4.5	Rat/IgG_{2a}, κ	BV785	0.25	Biolegend
CD4	GK1.5	Rat/IgG_{2b}, κ	FITC	0.25	eBioscience
CD45	30-F11	LOU/M IgG2b, κ	BV711	0.25	BD Biosciences
cKit	2B8	Rat/IgG_{2b}, κ	PE-Cy7	0.125	Biolegend
F4/80	BM8	Rat/IgG_{2a}, κ	PerCP-Cy5.5	0.25	Biolegend
GM-CSF	MP1-22E9	Rat/IgG_{2a}, κ	PE	0.25	eBioscience
IFN-γ	XMG1.2	Rat/IgG_1, κ	PE-Cy7	0.25	Biolegend
IL-17A	eBio 17B7	Rat/IgG_{2a}, κ	PerCP-Cy5.5	0.25	eBioscience

Specificity	Clone	Isotype	Fluorochrome	µL/10^6 cells	Supplier
Ly6C	AL-21	Rat/IgM, κ	FITC	0.25	BD Biosciences
Ly6G	RB6-8C5	Rat/IgG_{2b}, κ	APC-eFluor 780	0.25	eBioscience
Ly6G	1A8	Rat/IgG_{2a}, κ	Pac Blue	0.25	Biolegend
Ly6G	RB6-8C5	Rat/IgG_{2b}, κ	PE	0.25	eBioscience
MHCII	M5/114.15.2	Rat/IgG_{2b}, κ	APC	0.125	eBioscience
MHCII	M5/114.15.2	Rat/IgG_{2b}, κ	FITC	0.25	eBioscience
NKp46	29A1.4	Rat/IgG_{2a}, κ	BV711	0.25	Biolegend
Siglec F	E50-2440	LOU/M IgG_{2a}, κ	PE	0.25	BD Biosciences
Siglec F	E50-2440	LOU/M IgG_{2a}, κ	PE-CF594	0.25	BD Biosciences
ST2	RMST2-2	Rat/IgG_{2a}, κ	APC	0.25	eBioscience
TCRγ/δ	GL3	Armenian Hamster IgG	BV605	0.25	Biolegend

Table 2.5 Other flow cytometry reagents

Chemical	Description	Supplier	Conc. *in vitro*
Brefeldin A	Inhibitor of intracellular protein transport	Sigma	5 µg/ml
Fc Block	Block non-specific binding	BD Biosciences	0.5 µL/sample
Ionomycin	Ionophore	Sigma	500 ng/mL

Chemical	Description	Supplier	Conc. *in vitro*
Live/ dead (LD) aqua	Fixable cell stain (viability)	Invitrogen	0.1 µL/sample
PFA	Cell Fixative	Thermo Fisher	50 µL/sample (2% PFA)
PMA	Stimulation	Sigma	10 ng/mL

Table 2.6 NA/LE antibodies

Antibody	Clone	Supplier	Conc. *in vitro*	Dose *in vivo*
Anti-CD3ε	145-2C11	BD Biosciences	1 µg/mL	-
Anti-CD28	37.51	BD Biosciences	5 µg/mL	-
Anti-IL-5	TRFK-5	Bioceros BV	-	80 µg/mouse/day

Table 2.7 PRR ligands

PRR Ligand	PRR	Supplier	Conc. *in vitro*	Dose *in vivo*
LPS	TLR4	Invivogen	100 or 500 ng/mL	50 µg/mouse
MSU crystals	NALP3	Invivogen	250 µg/mL	250 µg/mouse

Table 2.8 Real-time PCR primers

Protein	Gene	Product Code	Ref. Seq.	Supplier
Arginase	Arg1	Mm00475991_m1	Nm_007482.3	ABI
CD206	Mrc1	313688	Nm_008625	Roche
CD38	CD38	311558	Np_031672	Roche
Chitinase-like protein 3	Chi3l3	312249	Nm_009892	Roche
Early growth response protein 2	Egr2	310303	Nm_010118	Roche
Formyl peptide receptor 2	Fpr2	317870	Nm_008039	Roche
IL-1RA	IL-1RN	Mm00446186_m1	Nm_031167.5	ABI
Relm-α	Retnla	Mm00445109_m1	Nm_020509.3	ABI
18S rRNA	18S	4310893E	X03205.1	ABI

Table 2.9 Murine cytokines

Cytokine	Type	Supplier	Conc. *in vitro*	Dose *in vivo*
IL-1β	Recombinant	Immunotools	10 pg/mL–10 ng/mL	-
IL-23	Recombinant	Immunotools	2.5 or 10 ng/mL	-
IL-4	Recombinant	Miltenyi	10-80 ng/mL	-
IL-33	Recombinant	Immunotools	10–250 ng/mL	200 ng/mouse/day

Cytokine	Type	Supplier	Conc. *in vitro*	Dose *in vivo*
GM-CSF	J558 Supernatants	In house	20-100 ng/mL	-
M-CSF	L929 Supernatants	In house	20% v/v	-

2.2 Mice

Specific pathogen-free C57BL/6 mice were purchased from Envigo (Bicester, UK) or were bred in house in the CMU Unit in Trinity College Dublin. The mice were maintained according to the guidelines and regulations of the Health Products Regulatory Authority and experiments were carried out under licence with the approval of Trinity College Dublin Bioresources Ethics Committee. Mice were sacrificed by cervical dislocation or by asphyxiation with CO_2. All animals used were age and sex matched at 8-12 weeks old at the initiation of experiments.

2.3 Methods

2.3.1 Cell Culture

2.3.1.1 Preparation of a single cell suspension

All organs were harvested from mice collected in ice-cold RPMI. Organs were processed by pushing them through a sterile cell strainer (40 µm or 70 µm; Greiner Bio-One) using the plunger from a sterile 5 mL syringe (Greiner Bio-One). The cells were washed through the strainer with RPMI and pelleted by centrifugation (1300rpm, 5 min; Eppendorf 5810R) prior to cell counting and/or red blood lysis.

2.3.1.2 Red blood cell lysis

Red blood cells from single cell suspensions were lysed using ammonium chloride lysis solution. The cell pellet was resuspended in 2 mL ammonium chloride lysis solution (0.88 %) for 2 min before being neutralised with 18 mL of RPMI and pelleted by centrifugation (1300 g, 5 min). The pellet was then resuspended in an appropriate volume of RPMI for counting (1-10 mL).

2.3.1.3 Cell counting

Live cells were counted by diluting the cells in EB/AO solution (1 in 2 to 1 – 20 dilution). The diluted cells were loaded onto a plastic disposable haemocytometer (Hycor Biomedical) and the slide placed under a UV-fluorescent microscope. Viable cells fluoresce green while dead cells fluoresce orange. Cells in the central square of the haemocytometer were counted.

Count x Dilution Factor x 10^4 = No. Cells/mL

2.3.1.4 Cell culture

Single cell suspensions were cultured in RPMI in cell culture plates (48-well or 96-well; Greiner Bio-One) or cell culture flasks (T75 or T175; Greiner Bio-One) in an incubator maintained at a constant temperature and atmosphere (37 °C, 5% CO_2).

2.3.1.5 Generation of murine bone marrow derived dendritic cells (BMDCs)

BMDCs were generated from C57BL/6 mice; the femurs and tibiae of freshly killed mice were removed and separated from the surrounding muscle and tissue. The bone marrow was flushed using a 27G needle (BD) attached to a 20 mL syringe (BD) containing RPMI. Any cell aggregates were broken down by syringing the cells up and down using a 19 G needle (BD). The red blood cells were lysed and then the cells were counted. The cells were seeded at a concentration of $1x10^6$ cells/mL in T175 flasks (15 mL/flask) in RPMI supplemented with 20 ng/mL of GM-CSF. After three days of culture an additional 20 mL of GM-CSF-supplemented (20 ng/mL) RPMI was added to the flasks. On day 6 the supernatant was gently removed from the flasks, discarded, and 20 mL of sterile PBS heated to 37 °C was added. The flasks were agitated to disrupt loosely adherent cells.

The cells in PBS were removed and added to 10 mL of RPMI in 50 mL tubes (Greiner Bio-One). Remaining cells were removed by incubation for 10 minutes (37 °C, 5% CO_2) with 20 mL of EDTA (Sigma) was added to the flasks. Following incubation, the EDTA was pipetted up and down the flasks before being collected and centrifuged at 1300 rpm for 5 mins. The PBS tubes were also centrifuged (1300 rpm, 5 min), both pellets were pooled, and the cells counted. The cells were seeded at a concentration of $1x10^6$ cells/mL in T175 flasks (15 mL/flask) in GM-CSF-supplemented (20 ng/mL) RPMI. 20 mL of RPMI supplemented with 20 ng/mL of GM-CSF was added two days later. On day 10 loosely adherent cells were removed by gentle, repeat pipetting the supernatant over the bottom of the flask before being removed and centrifuged (1300 rpm, 5 min). Cell pellets were pooled and resuspended in 10 mL of RPMI, counted and re-seeded in cell culture plates at the appropriate concentration. The immature DCs were rested for at least 3 hours before stimulation.

2.3.1.6 Generation of murine bone marrow derived macrophages (BMDMs)

Bone marrow from the legs C57BL/6 mice was flushed using a 27 G needle (BD) attached to a 20 mL syringe (BD) containing RPMI. Any cell aggregates were broken down by syringing the cells up and down using a 19 G needle (BD). The red blood cells were lysed and then the cells were counted. The cells were cultured at a concentration of $1x10^6$ cells in 10 mL RPMI supplemented with 20% v/v of M-CSF/L929 in petri dishes (company). After three days of culture an additional 2 mL of M-CSF/L929 was added to each dish. On day 6 the medium was gently poured off each flask and discarded. 10 mL of sterile PBS was added to the flasks, gently swirled, and poured off to remove any non-adherent cells remaining. 10 mL fresh RPMI was then added and the adherent macrophages gently scraped into the medium. The cells were collected, centrifuged (1300 rpm, 5 mins), counted and re-seeded in cell culture plates at the appropriate concentration. The macrophages were rested for at least 3 hours before stimulation.

2.3.1.7 Peritoneal lavage

The abdominal skin of C57BL/6 mice was carefully dissected to expose the intact peritoneal sack. A 27 G needle was inserted through the peritoneum and, taking care not to pierce any internal organs, 5 mL of ice cold PBS was injected into the cavity. An

air bubble was then injected to prevent the regress of liquid through the injection site. The body of the mouse was gently shaken to encourage the detachment of the peritoneal exudate cells. A 19G needle was then inserted and the maximum possible volume (≈4 mL) of PBS was removed. The cells in PBS were added to 0.5 mL ice cold RPMI before being pelleted by centrifugation (1300 rpm, 5 min). The cytokine concentration in the supernatant (PEC fluid) was when indicated determined by ELISA whilst the pellet, containing total peritoneal exudate cells (PEC), was resuspended in RPMI, counted and seeded in cell culture plates (2 hour rest followed by stimulation), added to Trizol (for RNA isolation) or analysed by flow cytometry. Alternatively, PEC were let rest for 2 hours at 37 °C in a 6-well plate (Greiner Bio-One) before removing the media containing non-adherent cells, then scraping the adherent cells in fresh media, pelleting by centrifugation (1300 rpm, 5 min). The resulting pellet contained 'peritoneal exudate macrophages' which were worked with in the same manner as total PEC.

2.3.1.8 Isolation of spleen cells

A single cell suspension was prepared from the whole spleen of a C57BL/6 mouse as described in 2.3.1.1. The splenocytes were counted and kept on ice until being plated out and treated or stained. To treat splenocytes from naïve mice, 96-well cell culture plates were coated with anti-CD3 (1 µg/mL in 100 µL PBS/well) and incubated for 2 hours at 37°C. The PBS was then removed using a pipette and the wells cleaned with fresh PBS pipetted up and down. The spleen cells were then added to the wells at the required concentration and treated.

2.3.1.9 Isolation of lymph nodes (LNs)

The inguinal, brachial and axillary LNs were removed from mice with EAE (Section 2.3.12.5) and a single cell suspension prepared as described in 2.3.1.1. LN cells were kept on ice until being stained or treated.

2.3.1.10 Cell Stimulation

After the various cell types were isolated and allowed to rest, treatments were added to each well at a final volume of 200 µL in 96-well plates and 500 µL in 48 well plates. The plates were incubated at 37 °C and 5% CO_2 for the required time. The cells were

then centrifuged, and the supernatants removed for analysis by ELISA. The cells were kept for gene expression or arginase activity analysis.

2.3.2 Exosome Preparation

Adult *F. hepatica* parasites were collected from infected bovine livers at a local abattoir (Kildare Chilling Ltd). Livers from freshly killed adult cows were identified by an on-duty veterinarian as exhibiting the morphological changes associated with *F. hepatica* infection. The bile ducts of the liver were dissected to examine for the presence of adult fluke. Live flukes were collected with sterile forceps and transferred into PBS containing penicillin/streptomycin (P/S; 100 µg/mL). In the lab, the flukes were washed 5 times with PBS with P/S to remove debris and cellular contaminants. Dead flukes were removed, and live flukes incubated O/N at 37 °C and 5% CO_2 in 6 well plates at 6 flukes per well in 3 mL PBS with P/S. The following day the flukes were discarded and the supernatant collected for exosome isolation

2.3.2.1 Differential Centrifugation

To prepare FHES and *F. hepatica* exosomes, the *F. hepatica* supernatant was differentially centrifuged at various speeds as outlined in Figure 2.2. The supernatants were centrifuged in sterile 50mL tubes at 400 g in an Eppendorf 5810R centrifuge (4 °C) and at 2000 g in a Sorvall RT7 tabletop centrifuge (4 °C). For the 15,000 and 120,000 g centrifugation steps, the supernatants were centrifuged in Sorvall X25 PA 36mL tubes (Thermo Fisher; sterilised by autoclave) in a Thermo Scientific Sorvall WX 100+ ultracentrifuge (4 °C) using an AH-629 swinging bucket rotor (sterilised with 70% ethanol). The supernatant from the 15,000 g centrifugation stage was FHES (excretory/secretory products of *Fasciola hepatica*). To prepare FHES, the 15,000 g supernatant fraction was aliquoted and frozen down at -80°C until use. The pellet from the 120,000 g centrifugation were the exosomes. The 120,000 g pellet fraction was subsequently washed by resuspension in PBS and centrifugation at 120,000 g. The pellet was resuspended in 2mL PBS, filtered with a Minisart RC4 0.45 µm syringe filter (Sartorius; sterilised by autoclave), aliquoted and frozen down. at -80 °C until use.

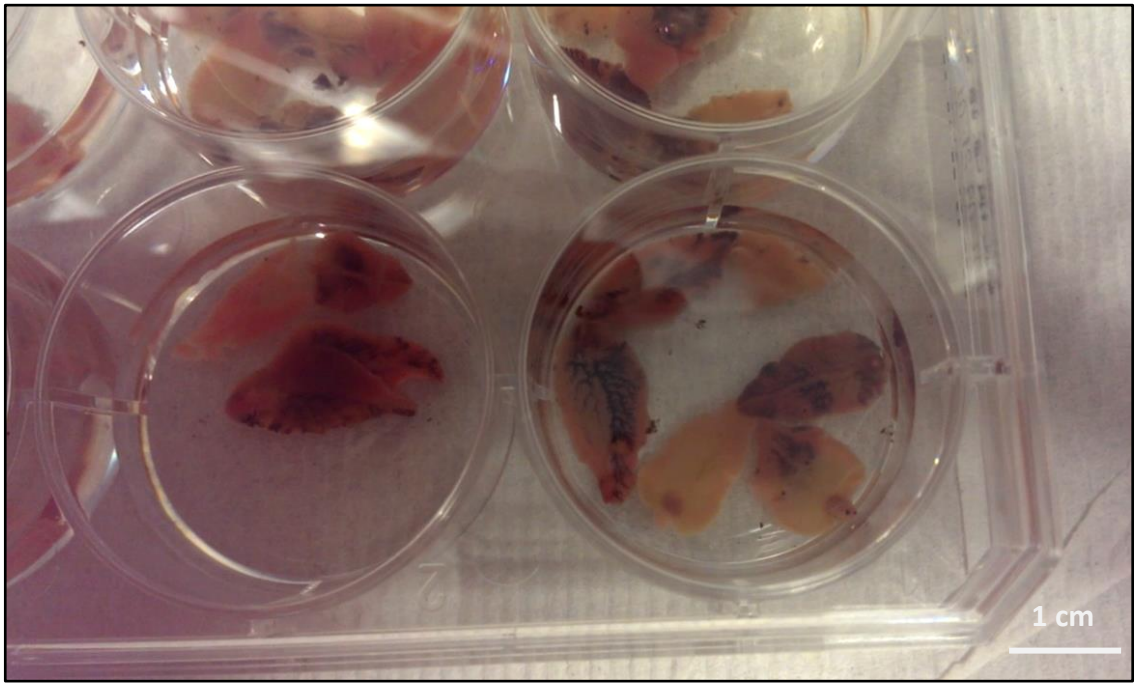


Figure 2.1: *F. hepatica* in culture.

Close up of live adult *F. hepatica* cultured in PBS with P/S in 6 well plates at 37 °C. Having been incubated overnight, the culture plate was removed and photographed.

The protein concentration of FHES and the exosomes preparations were determined by BCA Assay. The typical protein yield of FHES and the exosomes were 1.2 mg and 15 µg per fluke respectively.

2.3.2.2 Exospin Exosome Purification Kit

Alternatively, exosome preparation was undertaken using the commercially available Exospin exosome purification kit (Cell Guidance Systems) according to the manufacturer's protocol. Briefly, the *F. hepatica* supernatant was poured into a sterile 50 mL tube centrifuged at 300 g for 10 minutes in an Eppendorf 5810R centrifuge (4 °C) to remove cells. The supernatant was transferred to a Nalgene centrifuge bottle (Thermo Fisher; sterilised with NaOH and then by autoclave) and centrifuged at 15,000 g for 30 minutes in a Sorvall RC-5C Plus Superspeed Centrifuge (4 °C) using a GSA fixed-angle rotor to remove any remaining cell debris. The supernatant was poured into a fresh, sterile Nalgene centrifuge bottle and ½ the volume of the supernatant of Exospin-

Buffer (provided in the kit) was added. The tube was mixed and incubated at 4 °C for 1 hour. The mixture was then centrifuged at 15,000 g for 1 hour. The supernatant was discarded, and the exosome-containing pellet resuspended in 100 µL of PBS. The Exo-spin column (provided with the kit) was prepared according to the protocol prior to application of the sample. All centrifugation steps with the columns were carried out

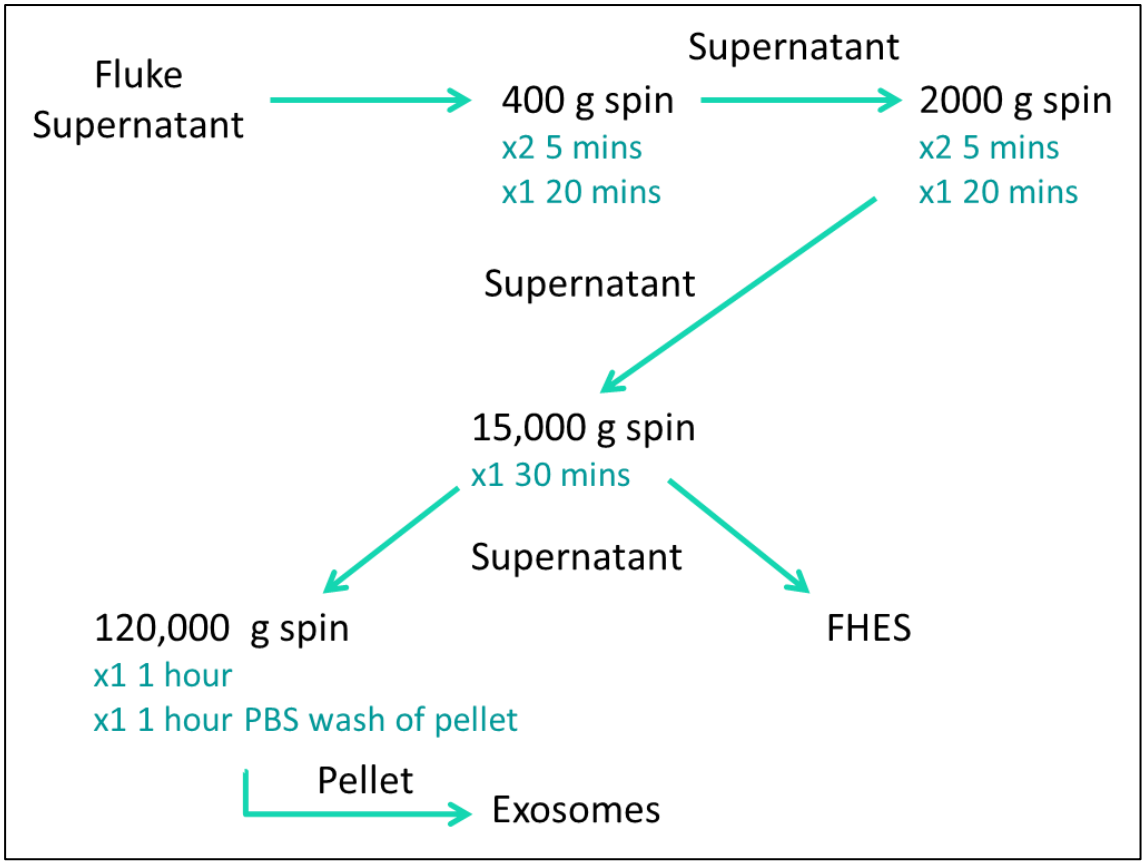


Figure 2.2: Centrifugation plan for the isolation of FHES and exosomes from *F. hepatica* supernatant.

F. hepatica liver flukes were incubated overnight. Their supernatant was collected and centrifuged at various speeds as shown. The supernatant from the 15,000 g centrifugation step was FHES and the pellet from the 120,000 g centrifugation step was the exosomes.

using an Eppendorf 5424 Microcentrifuge. The preservative buffer from the top of the column was discarded and the column equilibrated by adding 200 µL of PBS and spinning at 50 g for 10 s (4 °C). If any PBS remained above the top filter, the spin was repeated at the same speed in 5 s increments. The exosome-containing pellet in 100 µL PBS was added to the top of the column, centrifuged at 50 g for 60 s, and the eluate discarded. The column was placed into a 1.5 mL Eppendorf tube and 200µl PBS added to the top of the column. It was centrifuged at 50 g for 60 seconds. The 200 µL eluate contained the purified exosomes. It was diluted in PBS, filtered with a Minisart RC4 0.45 µm syringe filter (sterilised by autoclave), aliquoted and frozen down at -80 °C until use. The protein concentration was determined by BCA Assay.

2.3.3 FH Protein Expression and Purification

Previous research in the Mills laboratory identified FHKTM as an individual component of FHES. The protein was cloned and amplified in *E. coli*, transformed into *Pichia pastoris* yeast, and expression and purification of the protein was optimised.

2.3.3.1 FH Protein Expression

The yeast stocks were streaked out onto MD medium plates using an inoculating loop (Sigma) and incubated at 26°C for 72 hours. The yeast were scraped off an individual agar plate (whether they were colonies or lawns) and resuspended in 25 mL BMGY by breaking the yeast against the inside of a sterile 250 mL Erlenmeyer flask (Corning) using a loop. Briefly, this was achieved by dragging the loop containing the yeast along the plastic of the inside of the flask and dipping the loop into the medium to aid the break-up of the colony. The colony became 'milky' and floated off into the medium as the flask was swirled. The culture was incubated O/N at 150 rpm, 26 °C in a 311DS Labnet shaking incubator. The next day the culture was scaled up by adding the 25 mL cultures to 400 mL BMGY in sterile 2 L baffled flasks (Thermo Fisher). The flasks were incubated O/N at 250 rpm, 26°C in an Innova 4330 refrigerated incubator shaker. On the following day, the BMGY medium was exchanged by spinning the cultures in sterile 50 mL flasks at 3,000 rpm for 10 minutes in a Sorvall RT7 tabletop centrifuge (4 °C) and resuspending

the pellets containing the yeast cells in 400 mL BMMY. Before exchanging the medium, the OD_{600} values of the yeast cultures were calculated using an Eppendorf BioPhotometer spectrophotometer to confirm they were approximately 10 (1 OD_{600} ~ 5 × 10^7 cells/mL). The BMMY cultures were incubated for two nights at 250 rpm, 26 °C, adding 2 mL 100% f.s. methanol (in house) at the halfway stage to replace the methanol lost by evaporation. Finally, the cultures were centrifuged (10 minutes, 4 °C) and the supernatants taken to purify FHKTM.

2.3.3.2 FH Protein Purification

NaCl and imidazole were added to the yeast cell supernatants before purification. Supernatant volume was measured, and the appropriate amount of the salts added to give a NaCl concentration of 200 mM and an imidazole concentration of 10 mM. The supernatants were then pH'd to 8 using NaOH. FHKTM were purified by column chromatography using a CrystalCruz chromatography column, 2.5 cm x 10 cm (Santa Cruz Biotechnology). First the column was washed with 1 M HCl and then both the column and the associated tubing were washed with dH_2O. The 4 mL NiNTA beads were added to the column and washed with 10 mL Buffer I. After the column ran dry, the supernatants were added, and a gravity flow was set up. Having run the supernatants twice through the column, the beads were washed with 200 mL Buffer I. 12 mL Buffer II was then added to eluate FHKTM, which was collected as 1.5 mL aliquots. The protein samples were dissolved in Buffer II so had a high imidazole concentration; 250 mM. To exchange this buffer for PBS, the samples were run through PD-10 desalting columns (GE Healthcare Life Sciences) according to the manufacturer's instructions. The now desalted protein samples were concentrated using Amicon Ultra-4 3 K centrifugal filters (Merck), filter-sterilised, aliquoted, and frozen at -80 °C. FHKTM concentration was determined by BCA assay and endotoxin levels by LAL assay. A typical yield of FHKTM was 0.5 mg protein per 400 mL culture of yeast cells.

2.3.4 Gel Electrophoresis

FHKTM samples were run on gels to confirm its presence by size (Coomassie stain) and His-tag (Western blot).

2.3.4.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were diluted with 5x loading buffer (30 µL sample and 9 µL buffer/gel) and boiled at 95 °C on a heating block for 5 min to denature the proteins. Samples were then resolved by SDS-PAGE using a Bio-Rad Mini-PROTEAN electrophoresis cell, in 1x running buffer. Samples were run on a 15% polyacrylamide gel (≈70% running gel and 30% stacking gel) at 30 mA/gel with 10 µL/well protein markers (SeeBlue Plus2 Pre-stained Protein Standard; Thermo Fisher) as molecule weight standards.

2.3.4.2 Coomassie Stain

Coomassie Brilliant Blue R-250 can be used to visualise proteins on a gel. SDS-PAGE gels were removed from the cell and incubated at room temperature with Coomassie stain for 20 minutes. The gels were then washed with destain, changing regularly, until background staining of the gel was removed, leaving only protein bands stained.

2.3.4.3 Transfer of Proteins to PVDF Membrane

Proteins were transferred from SDS-PAGE gels to 0.4 µm polyvinylidene fluoride (PVDF) membrane (Whatman) using a semi-dry transfer system. PVDF was activated by immersion in 100% methanol before being rinsed in 1x transfer buffer. The gel and 8 sheets of blotting paper were also soaked in 1x transfer buffer. Both the membrane and the sheets of blotting paper were cut to be the same size as the gel. The membrane was placed on top of 4 pieces of the blotting paper followed by the gel and then four more pieces of the paper on top inside an EBU-4000: Semi-Dry Blotting System CE, 20 cm x 20 cm (CBS Scientific) with both lids having been washed with 1x transfer buffer. The transfer was run at 40 mA/blot for 90 minutes and the membrane removed for Western blotting.

2.3.4.4 Western Blot

Following transfer, the membranes were placed into 50 mL falcon tubes and blocked O/N with 10 mL Western blocking solution for HRP detection systems (Sigma) at 4 °C, rolling. The following day 2.5 µL HisProbe-HRP (5mg/mL) was added directly to the 10 mL blocking agent (1:4000 dilution) and the tubes were rolled for 90 minutes at RT. The HisProbe-HRP is a nickel (Ni^{2+})-activated derivative of horseradish peroxidase (HRP) that enables direct detection of His-tagged proteins, like FHKTM, via detection of HRP activity. The membrane was then washed three times in TBS-T, 10 mins/wash on a roller. Blots were developed in a dark room. 5 mL Solution A and 5 mL Solution B were combined and added directly to the membrane. The membrane was then placed in a Kodak BioMax exposure cassette and exposed to Kodak X-Omat LS film (Sigma). The film was developed in Mediphot x-ray processor machine.

2.3.5 Bicinchoninic acid (BCA) Protein Assay

The Protein concentrations of FHKTM, FHES, and each exosome preparation were determined using a Pierce BCA protein assay kit (Thermo Fisher) following the manufacturer's instructions. In the assay, any protein present will reduce Cu^{2+} to Cu^{1+}. Reduced Cu^{1+} cations chelate molecules of BCA forming purple-coloured, water-soluble complexes, whose absorbance can be read at 562 nm. Samples are added neat as well as diluted 1:5, 1:10, and 1:50 triplicates together with 8-point standard curve samples (BSA; 25-2000 µg/mL) to a 96-well plate with BCA working solution (from the kit) and incubated for 30 minutes at 37 °C in dark. The absorbance at 562 nm was measured and the concentration of protein was calculated based off the standard curve calculated using SoftMax Pro software; the range for the assay is 20-2000 µg/mL of protein.

2.3.6 Limulus Amebocyte Lysate (LAL) Chromogenic Endotoxin Assay

The endotoxin concentrations of FHKTM and each exosome preparation were determined using a Pierce LAL chromogenic endotoxin quantitation kit (Thermo Fisher) according to the manufacturer's instructions. The samples were diluted 1:10, 1:100, and

1:1000 in triplicate and together with a 4-point standard curve samples (0.1 EU/mL, 0.25 EU/mL, 0.5 EU/mL and 1 EU/mL) were added (50 µL/well) to a 96-well plate preheated on a heating block to 37 °C. 50 µL LAL reagent (from the kit) was added to each well and the plate was incubated on a 37 °C block. After 10 minutes, 100 µL chromogenic substrate solution (from the kit) was added to the wells and the plate was gently shaken and incubated at 37 °C for 6 minutes. In a LAL assay, endotoxin trigger an enzymatic reaction releasing a coloured product from a colourless substrate which can be measured photometrically at 405 nm, after the reaction is stopped by 50 µL 0.25% acetic acid solution. Absorbance at 405 nm and endotoxin concentration is linear in the range 0.1-1 EU/mL. Endotoxin concentration was calculated based on the standard curve.

2.3.7 Enzyme linked immunosorbent assay (ELISA)

The concentrations of murine IL-1RA, IL-1β, IL-4, IL-6, IL-10, IL-18, IL-12p40, IL-13, IL-25, IL-33, GM-CSF, TGF-β, TNF, TSLP, and YM1 were measured using pairs of rat anti-mouse capture and detection antibodies specific for the cytokine in question (Table 2.3). High-binding 96-well microtiter plates (Greiner Bio-one) were coated overnight at 4°C with 50µL/well of the appropriate capture antibody diluted in PBS according to the manufacturers guidelines (batch-specific). The plates were washed in PBS-Tween and blocked with 200µL/well of either 1% BSA (R&D kits) or 5% Milk (BD kits) for 2 hours at RT to prevent non-specific binding. After blocking, plates were washed, and sample supernatant was added (50µL/well). Samples to be assayed for the presence of latent TGF-β were heated using a PCR thermocycler to 80°C for 10 minutes prior to addition. In some cases, supernatants were diluted in 1% BSA in the plate. Cytokine standards were added in triplicate (50µL/well) at the appropriate top working standard and serially diluted in 1% BSA (x6). Blank wells containing only 1% BSA were also included in triplicate. The supernatants and standards were incubated at 4°C overnight. After washing, 50µL of the appropriate biotinylated detection antibody diluted in 1% BSA according the manufacturers guidelines (batch-specific) was added per well and the plates incubated for 2 hours at RT in the dark. The plates were washed again and incubated in the dark for 20 minutes with 50µL/well of horse radish peroxidase-

conjugated streptavidin (1:1000 dilution for BD, batch-specific dilution for R&D; in 1% BSA). The plates were washed with PBS -Tween and 50µL/well of ELISA developing solution was added. The reaction was stopped by adding 25µL/well of 1M H_2SO_4. The ELISA should be stopped when the top working standard looks like it has an absorbance value around 1 and before the appearance of any colour in the blank wells. Absorbance was read at 492nm on a Versamax Tunable Microplate Reader (Molecular Devices) using SoftMax Pro software. Concentration of the cytokine was determined from the standard curve, calculated using the software.

Absorbance was read at 492 nm on a Versamax Tunable Microplate Reader (Molecular Devices) using SoftMax Pro software. Concentration of the cytokine was determined from the standard curve, calculated using the software.

2.3.8 Gene Expression Analysis

2.3.8.1 RNA Isolation

RNA was isolated from cells using the Trizol/chloroform method. Cells of interest were centrifuged, supernatants removed, and then resuspended in Trizol (Life Technologies; 1 mL/1x10^6 cells) in RNAse-free tubes (Thermo Fisher). 200 µL of chloroform (Sigma) was added per 1 mL Trizol and the tubes were inverted vigorously for 30 s. The tubes were allowed to stand for 5 minutes before being centrifuged (13000 g, 15 min, 4 °C; Eppendorf 5424 Microcentrifuge). The top aqueous layer containing the RNA was removed from the lower red phenol-chloroform layer and added to a fresh RNAse-free tube containing 500 µL of 2-Propanol (Sigma) per 1 mL TRIzol added to the cells. The solution was mixed by inversion for 30 s then left at RT for 10 minutes. The tubes were centrifuged (13000 g, 10 min, 4 °C), the supernatant was removed, and 1 mL of 75% ethanol was added. The tubes were vortexed before being centrifuged (8000 g, 10 min, 4 °C). After removing the supernatant, the pellets were air-dried for approximately 15 minutes and then resuspended in 20 µL of RNAse-free water (Life Technologies). RNA concentration was determined spectrophotometrically using a Nanodrop spectrophotometer (Thermo Scientific) according to the manufacturer's instructions.

The purity of the RNA was determined by the 260/280 absorbance ratio. RNA was stored at -80 °C until being converted to cDNA.

2.3.8.2 cDNA Synthesis

Up to 1 µg of RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) according to the manufacturer's instructions. A master mix was made using the kit (Table 2.10). For each cDNA reaction in a PCR tube, 10 µL of master mix was added for 10µL of the RNA (which may have been diluted in nuclease-free H_2O). RNA was reverse transcribed as indicated in Table 2.11. The cDNA synthesis reaction was carried out in a DNA Engine Dyad cycler. The cDNA was diluted 1:8 with nuclease–free water and stored at -20 °C until performing rtPCR analysis.

Table 2.10 cDNA Synthesis Master Mix Composition

Component	Volume/reaction (µL)
10x RT Buffer	2
25x dNTPs mix (100mM)	0.8
10x RT Random primers	2
MultiScribe Reverse Transcriptase	1
RNase free H_2O	4.2
Total per reaction	10 µl

Table 2.11 Reverse Transcription cDNA Synthesis Cycle

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

2.3.8.3 Real Time Quantitative rtPCR

Transcript concentrations were quantified by real-time quantitative rtPCR carried out on an ABI 7500 Fast Real Time PCR System using Real-time PCR Cycle (Table 2.12) with pre-made Taqman Gene Expression Assays (Table 2.8) and reagents from Applied Biosystems according to the manufacturer's instructions. The reaction mix contained 5.5µL of the Probe/18S master mix (Table 2.13) and 4.5 µL of cDNA sample. The PCR reaction plate was sealed using an adhesion seal and the plate was centrifuged at 500 g for 1 minute. Data was analysed using relative quantification on Applied BioSystem 7500 software, which provided cycle threshold (Ct) values. Ct is defined as the number of cycles required for the fluorescent signal to cross a threshold, exceeding background fluorescence. The abundance of mRNA in each sample was normalised by the measurement of expression of the endogenous control 18S ribosomal RNA (rRNA) from the same sample. (CT_{gene}-CT_{endo} = dCT). Furthermore, Ct from control samples were averaged and were subtracted from the dCT value of each sample (dCT-$dCT_{control}$ = ddct). Fold induction was calculated as $2^{(-ddct)}$. The relative expression of each gene was then normalised to an experimental control, for example medium only-treated cells.

Table 2.12 Real-time PCR Cycle

Temperature (°C)	Time	Cycles
95	2 min	1
95 60	3 s 30 s	40

Table 2.13 Real-time quantitative rtPCR Master Mix Composition

Reagent	Volume required per reaction (µL)
SensiFAST Probe Lo-ROX Mix (2x) (Bioline)	4.5
Reaction probe	0.5
18S rRNA Endogenous primer	0.5

2.3.9 Arginase Activity Assay

Arginase activity was measured in cells using the Arginase Activity Assay Kit from Sigma following the manufacture's procedure. Up to $1x10^6$ cells were suspended with 70 µL of 1x passive-lysis buffer, left on ice for 30 minutes and then stored at -20 °C until the assay was performed. 20 µL sample (sometimes requiring prior dilution in MilliQ H_2O) were incubated with the arginase substrate L-arginine (4 µL 5x Substrate Buffer; made as per the Sigma protocol) for 2 hours at 37 °C in a 96-well flat-bottomed plate. A urea standard, a water control (receiving the substrate), and a sample without substrate (blank) were also added to the plate. The arginase reaction was stopped by adding 70 µL Urea Reagent (made as per the Sigma protocol) per well and the plate read at several time points post addition from 5 minutes to 30 minutes. Arginase activity was quantified by measuring the production urea, a product of arginase activity. The kit utilises a chromogen that forms a coloured complex (A_{max} = 430 nm) specifically with urea. The intensity of the colour is directly proportional to the arginase activity in the sample.

Absorbance was measured at 430 nm and compared to a urea standard using the following equation:

$$\text{Activity (Units/L)} = \frac{A_{430}\,(\text{sample}) - A_{430}\,(\text{blank})}{A_{430}\,(\text{urea standard}) - A_{430}\,(\text{water})} \times \frac{(1\ \text{mM} \times 25\ \mu\text{L} \times 10^3)}{(V \times T)}$$

T = Reaction time in minutes (120 m in this case)

V = sample volume (µL) added to well (20 µL in this case)

1 mM = concentration of Urea Standard

25 = reaction volume (µL)

10^3= mM to µM conversion factor

2.3.10 Proteinase K digestion and heat inactivation

Proteinase K is a broad-spectrum serine protease used in molecular studies to digest proteins [300]. Heat inactivation is another method for inactivating proteins via denaturation. Proteinase K digestion and heat inactivation were carried out to try and identify the composition of the immunomodulatory components of *F. hepatica* exosomes. A 20 mg/mL Proteinase K stock (Sigma) was diluted to 100 µg/mL by addition directly to a sample of *F. hepatica* exosomes. The sample containing Proteinase K was then incubated for 3 hours at 55°C before the Proteinase K in the sample was heat inactivated for 10 minutes at 95°C. Separately, a sample of *F. hepatica* exosomes were heat inactivated through incubation for 10 minutes at 95°C. Cell were then treated as required with the Proteinase K digested or heat inactivated exosome samples.

2.3.11 Flow cytometry

Cells suspensions prepared from *in vivo* studies or from *in vitro* cultures were analysed by multi-colour flow cytometry in FACS tubes (BD Biosciences) in a volume of $1x10^6$ cells/50 µl. Cells were counted, added to the tubes, washed with FACs buffer, pelleted

by centrifugation (1300 rpm, 5 mins, RT), the supernatants poured off and the tubes blotted before resuspending the cells in the liquid remaining in the tubes (≈ 50 µL).

2.3.11.1 Discrimination of Live and Dead Cells and Fc block

Live and dead cells were discriminated using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher). LIVE/DEAD Aqua is detected in the V500/Am-Cyan channel (excitation/emission maxima = 375/512 nm). LIVE/DEAD Aqua binds dead cells with a higher affinity than with live cells and therefore they can be separated during acquisition. Cells were incubated with 0.1 µL LIVE/DEAD Aqua reagent as well as 0.5 µL Fc block (BD Biosciences) diluted in 10 µL of PBS per tube for 30 minutes at RT in the dark. Fc Block helps to prevent the non-specific binding of antibodies to cells via the somatic Fc region to cellular Fcγ receptors. The cells were then washed with 3 mL FACS buffer/tube, pelleted by centrifugation (1300 rpm, 5 mins, RT), the supernatants poured off and the tubes blotted before resuspending the cells in the liquid remaining in the tubes.

2.3.11.2 Surface marker staining

Following Live/Dead and Fc Block incubation, surface staining was carried out at RT for 30 minutes in the dark using the appropriate concentration of fluor-conjugated antibody (Table 2.4). The antibodies were added as a master mix diluted in PBS at 50 µL/tube. The cells were then washed with 3 mL FACS buffer/tube, pelleted by centrifugation (1300 rpm, 5 mins, RT), the supernatants poured off and the tubes blotted before resuspending the cells in the liquid remaining in the tubes.

2.3.11.3 Cell Fixing

After surface staining cells were either read immediately on the flow cytometers or fixed and read later. If fixing was required, the cells were resuspended in 50 µL of 2% PFA (16% PFA diluted 1:8 in PBS; Thermo Fisher) and incubated for 15 minutes at RT in the dark. After washing and pelleting the cells once more, they were stored at 4 °C until being acquired.

2.3.11.4 Intracellular staining

For samples requiring intracellular staining, prior to staining, the 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 lidded sterile FACs tubes (BD Biosciences) for 4-5 hrs at 37°C in cRPMI supplemented with 50 µM 2-ME. Following incubation, the cells were washed twice in 3 mL FACs buffer/tube, pelleted by centrifugation (1300 rpm, 5 mins, RT), the supernatants poured off and the tubes blotted before resuspending the cells in the liquid remaining in the tubes. The cells were then stained with Live/Dead Aqua and Fc Block, and surface stained (Sections 2.3.11.1-2).

To fix and permeabilise cells being stained intracellularly, they were incubated for 20 minutes at room temperature with 100 µL/sample of Foxp3 Transcription Factor Fixation/Permeabilization Concentrate and Diluent solutions (combined in 1:4 ratio; Invitrogen) then washed with FACs buffer, pelleted by centrifugation (1300 rpm, 5 mins, RT), the supernatants poured off and the tubes blotted before resuspending the cells in 100 µL FACs buffer and left O/N at 4°C.

The next day, prior to intracellular staining, the cells were washed with 3 mL 1x Permeabilization Buffer/tube (10x diluted in sterile Baxter's H_20; Invitrogen) and resuspended in the liquid remaining in the tubes. Intracellular staining was carried out at RT for 30 minutes in the dark using the appropriate concentration of fluor-conjugated antibody (Table 2.4). The antibodies were added as a master mix diluted in 1x Permeabilization Buffer and added at 50 µL/tube. The cells were then washed once with 3 mL/tube 1x Permeabilization Buffer and once with 3 mL/tube FACS buffer and stored at 4 °C until being acquired.

2.3.11.5 Acquisition and analysis of flow cytometry data

Flow cytometric acquisition was carried out on a BD CANTO-II or BD LSRFortessa flow cytometers according to the manufactures instructions. Compensation was calibrated using individually stained Comp Beads (BD Biosciences) on the FacsDiva Software (BD Biosciences). Results were analysed using FloJo (Stanford University) software. Debris was excluded from analysis by applying a FSc and SSc gate. Dead cells and doublet cells

were excluded by their LIVE/DEAD staining and pulse width profile, respectively. Percentages were calculated based on live single cells. Analysis included back-gating to ensure cell populations matched with their requisite FSc and SSc profiles.

2.3.11.6 Purification of eosinophils by FACS

Eosinophils were induced by injection of exosomes (2 µg/mouse/day) i.p. on days 0, 2, 4, and 6. On day 7, peritoneal exudate cells were isolated by lavage. The cells were incubated with Fc block (Section 2.3.11.1) to prevent non-specific binding and then surface stained (Section 2.3.11.2) with antibodies directed against CD11b, Ly6G, and CD19. Eosinophils were identified as $CD11b^{int}$, $CD19^{-}$, $Ly6G^{-}$, SSc^{hi}, cells and purified by FACS in a BD FACSAria Fusion flow cytometer. Purified eosinophils were centrifuged, counted and cultured in cRPMI supplemented with 2-ME (50 µM) and HEPES (20 mM).

2.3.12 *In vivo* Experiments

2.3.12.1 Intraperitoneal injections

Mice were injected intraperitoneally (i.p.) with various reagents diluted in PBS to a total volume of 100-300 µL/mouse/day. Injections were carried out either with BD 1/2 mL Tuberculin Syringe with Permanently Attached Needle (27 G x 1/2") or with 27 G x 1/2" needles (BD) attached to Injekt®-F Luer Solo syringes (Braun). I.p. injections were used predominantly in this study because of the ease of use compared with other parenteral methods and because cells from the target organ, the peritoneal cavity, can be easily isolated by lavage.

2.3.12.2 Subcutaneous injections

Mice were injected subcutaneously (s.c.) using a 23 G x 1/2" needles (BD) attached to Injekt®-Fluer Solo syringes. Reagents were diluted in PBS and a total volume of 100-200 µL/mouse was injected.

2.3.12.3 Isolation of blood serum

Blood was collected by the cardiac puncture of freshly killed mice and allowed to clot in a 1.5 mL Eppendorf tube. After 24 hours, cells were removed by centrifugation at 13,000 g for 10 minutes at 4 °C in a micro-centrifuge. The serum-containing supernatant was collected and frozen at -20 °C until being analysed.

2.3.12.4 Monosodium Urate (MSU) Crystal Challenge

Mice were injected with MSU crystals to induce the infiltration of inflammatory cells into the peritoneum. 5 mg MSU crystals (Invitrogen) were dissolved in 1 mL of PBS to prepare a MSU crystal stock solution (5 mg/mL). MSU crystals were injected as 250 µg/300 µL/mouse (833.33 µg/mL) so the MSU crystal stock was diluted 1 in 6 in PBS before being injected i.p. Mice were sacrificed 4 hours post injection and cells taken for analysis.

2.3.12.5 EAE

2.3.12.5.1 Active induction of EAE

EAE was induced in C57BL/6 mice by s.c. injection of MOG_{35-55} (100 µg/mouse; Genscript) emulsified in CFA (Chondrex Inc.) containing 4 mg/mL (400 µg/mouse) of heat killed H37 RA Mycobacterium tuberculosis (Chondrex Inc.). Mice were also injected i.p with 100 ng of PT (Kaketsuken, Japan) on day 0 and day 2. Animals were weighed and monitored for signs of clinical disease daily. Clinical score was measured every day as follows: 0 – normal; 1 – limp tail; 2 – altered gait; 3 – hind limb weakness; 4 – hind limb paralysis and 5 – tetraparalysis/death. Animals were sacrificed if they reached a score of 4 or above.

2.3.12.5.2 Passive induction of EAE by adoptive transfer

Donor mice were immunized by s.c. injection of 100 µg/mouse of MOG emulsified in CFA (See section 1.1.15.1) without administration of PT. On day 10, the spleens and inguinal, brachial and axillary LNs of immunized mice were isolated and single cell suspensions prepared (Section 2.3.1.9). Spleen cells and LN cells were cultured (70:30)

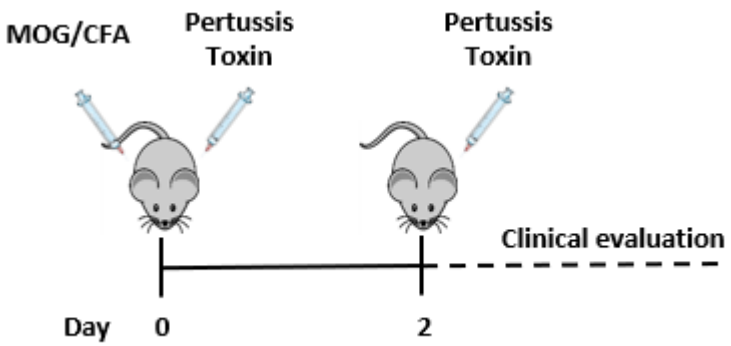


Figure 2.3: Overview of active EAE induction

at 1 x 10^7 cells/ml in cRPMI.supplemented with 2-ME in the presence of MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml). After 24 hours, a small volume (60 µL) was removed and cytokine production quantified by ELISA. After 72 hours, non-adherent cells were collected from the culture flask, washed and resuspended in RPMI. Cell supernatants were removed, and cytokine production quantified by ELISA. The live cells were resuspended in PBS and injected i.p. (200 µL/mouse) into naïve recipient mice at a concentration of 1 x 10^7 cells/200 µl. Mice were monitored daily for clinical signs of disease as with active induction.

2.3.12.5.3 Isolation of single cells from CNS tissue

Mice were sacrificed and immediately perfused intra-cardinally with 20 ml of ice-cold PBS to remove peripheral blood from CNS tissue. Brain and spinal cord tissues were isolated, placed into 1 mL cRPMI in RNAse-free tubes, homogenised in a tissue lyser (TissueLyser II, Qiagen), before being passed through a 70 µm cell strainer. The homogenised cells were then added to 5 mL 40% isotonic Percoll solution in a 15 mL tube using a Pasteur pipette (Sigma). This mixture was then carefully layered on top of

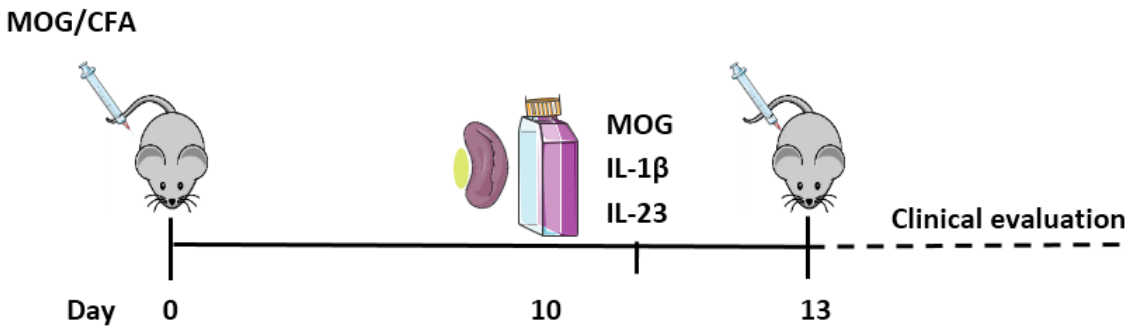


Figure 2.4: Overview of passive EAE induction

5 ml 70% isotonic Percoll solution in a separate 15 mL tube with a Pasteur pipette. The Percoll gradients were centrifuged at 1600 rpm for 20 minutes without the break at RT (Sorvall RT7 tabletop centrifuge). After centrifugation, the upper myelin layer was removed and discarded. Mononuclear cells were found on the interface of the Percoll gradients and were isolated using a Pasteur pipette (figure 2.5). The cells were washed twice with cRPMI, counted and used as required.

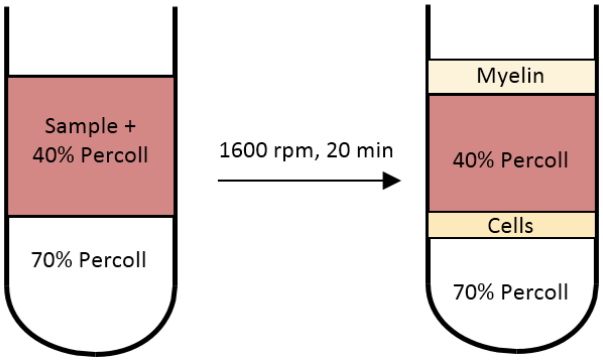


Figure 2.5: Percoll Density Layers

2.3.13 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 7.00. Unpaired two-tailed Student’s t-test was used to compare the statistical difference between the mean values of two groups. One-way or two-way analysis of variance (ANOVA) test followed by Dunnett’s or Tukey's honestly significant difference (HSD) post-hoc test was applied to determine the statistical difference between more than two groups. Repeated measures ANOVA followed by Tukey's HSD post-hoc test was used to determine statistical significance between groups in a time course. Statistical significance was considered for p values less than 0.05. All data presented are means +/- SEM or SD.

Chapter 3

Fasciola hepatica exosomes induce an anti-inflammatory response and suppress autoimmunity

3.1 Introduction

Helminths like *F. hepatica* characteristically promote type-2 and anti-inflammatory immune responses in the host that maintain the chronicity of infection [201]. Helminth-induced immune suppression can also attenuate inflammation unrelated to the anti-parasite immune response, but instead resulting from bacterial [301] and viral [302] infection as well as autoimmunity [254]. The original hygiene hypothesis has been updated to account for the observation that autoimmune diseases as well as allergies are more prevalent in areas with low levels of helminth. One possible explanation for this observation is that the regulatory immune response, involving T reg cells, M2 macrophages and other cells, induced by helminths can confer protection from the development of autoimmunity. Evidence from animal models supports the idea that helminth-induced immunosuppression can inhibit pro-inflammatory Th1 and Th17 responses, which mediate pathology in a number of autoimmune diseases. Our lab has previously demonstrated that infection with *F. hepatica* suppressed the Th1 response normally generated in response to *Bordetella pertussis* infection, leading to an exacerbation of the course of infection [301]. In humans, helminth infections correlate with the prevalence of other infections that are controlled by Th1 and/or Th17 cells. For example, humans with soil-transmitted helminth infections have higher incidences of concurrent human papillomavirus infections [303].

While the suppression of effector T cells responses can disadvantageous in fighting bacterial and viral infections, it can be beneficial to the host in the case of autoimmunity. MS patients with helminth infections had improved disease outcomes compared to uninfected patients and the benefits seen were reversed upon the administration of anti-helminthic therapy [254, 255]. In animal models of autoimmunity, mice with helminth infections were protected against collagen-induced arthritis, TNBS-induced of colitis, and EAE [261–263]. In our lab, we demonstrated that *F. hepatica* attenuated the development of EAE through suppression of pathogenic autoantigen-specific Th1 and Th17 cells in the brain and spinal cord [205]. This project is focused on the investigation of the anti-inflammatory properties of helminth-derived products. We have previously shown that the administration of FHES attenuated the development of EAE [205]. In

these experiments, FHES was injected therapeutically (every second day beginning one day before disease induction) thereby mimicking live infection.

Helminth infections can influence effector T cell function but they also can modulate the function of innate immune cells, including macrophages and DCs [201]. Recently in our lab has also demonstrated that helminth products can induce anti-inflammatory trained immunity. Immune memory, long believed to be solely a feature of T and B cells, has been described in the immune cells of organisms lacking adaptive immunity and in the innate immune cells of vertebrates including humans [79, 81]. Innate immune cells, upon re-stimulation, can be trained to generate a stronger pro-inflammatory response or tolerised to generate weaker pro-inflammatory responses. The murine parasitic nematode *Nippostrongylus brasiliensis* trained macrophages to be more efficient at damaging other helminth larvae [304]. Our lab has demonstrated that innate immune cells can be trained by *F. hepatica* products (FHTE) *in vitr*o and *in vivo* to be more anti-inflammatory, rather than pro-inflammatory, and to protect against pathological inflammation (Quinn et al, In review). Innate training in this manner may provide an alternate explanation for the hygiene hypothesis; Strachan initially described the hypothesis in terms of early infections being protective against the development of allergies [247]. In support of Strachan's initial hypothesis, it has been reported that children infected early in life with the helminth *Trichuris trichiura* had a significantly reduced incidence of allergic reactions [305]. Early exposure to helminths may train the immune system to have a more anti-inflammatory phenotype and thus be able to inhibit any potential pathological autoimmune (or allergic) responses which could develop later in life.

As there are many regulatory and practical concerns over the use of live helminths in the treatment of autoimmune diseases, the search is ongoing to identify specific helminth immunoregulatory molecules that could be used as therapeutics as an alternative. Our lab has demonstrated that like live infection, treatment of mice with *F. hepatica* excretory-secretory product (FHES) attenuates the development of EAE [157]. This is associated with the suppression of the infiltration of disease-mediating Th1 and Th17 cells into the CNS. Protection was dependent upon eosinophils and the type-2 cytokines IL-5 and IL-33. FHES is a mixture of many proteins, nucleic acids, and small

molecules. The aim of this project was to identify individual components of FHES and to explore in detail how they influence the host immune response.

Exosomes are small extracellular membrane-bound vesicles of homogenous shape and size being 40-100 nm in diameter [273, 274]. They contain proteins, mRNA, and miRNA and have a role in intercellular communication [274, 275]. Many parasite species including nematodes [286], trematodes [287], and protozoa [288] are known to release exosomes and other extracellular vesicles. Helminth-produced exosomes have been shown to contribute to parasite immune modulation [286, 288, 289]. The discovery that parasites communicate using extracellular vesicles changed the understanding of host-parasite interactions; parasites utilise extracellular vesicles for the safe delivery of molecules which would otherwise be degraded in the extracellular environment [279]. This provides an explanation for the presence of cytosolic "intracellular" proteins in the helminth secretome [280]. Marcilla et al. described *F. hepatica* producing exosome-like vesicles in 2012 [287]. Cwiklinski et al. (2015) identified two subpopulations of extracellular vesicles released by *F. hepatica* [290]. One subpopulation of extracellular vesicles were 30-100 nm in diameter and expressed several exosome markers including Hsp70, ALIX, and tetraspanin that have been identified in exosomes from other helminths. This strongly suggests that *F. hepatica* secrete "true" exosomes [279].

There are different methods for purifying exosomes from parasite or mammalian cells. These include (1) differential centrifugation, (2) commercially available kits which facilitate the sedimentation of exosomes, or (3) density gradient centrifugation [275]. In this study exosomes were isolated from FHES by both differential centrifugation as described by Cwiklinski et al. (2015) and also by using the commercially available ExoSpin kit (Section 2.3.2.2).

The specific questions to be addressed were:

- Do exosomes purified from *F. hepatica* have immunomodulatory activity?
- Can *F. hepatica* exosomes promote type-2 and/or anti-inflammatory immune responses *in vitro* and *in vivo*?
- Can *F. hepatica* exosomes suppress pro-inflammatory responses?
- Which cell types are responsible for mediating the anti-inflammatory effects of *F. hepatica* exosomes
- What components of *F. hepatica* exosomes are responsible for the immunomodulatory activity demonstrated *in vivo* and *in vitro*?
- Can *F. hepatica* exosomes train the immune system to be more anti-inflammatory?
- Does administration of *F. hepatica* exosomes, either therapeutically or prophylactically (to potentially induce innate training), inhibit the development of EAE?
- What are the mechanisms of any *F. hepatica* exosome-mediated attenuation of disease in the EAE model?
- Can *F. hepatica* exosomes inhibit cytokine production of by MOG-specific T cells, either directly or via the modulation of immune cells?
- Do *F. hepatica* exosomes suppress the ability of pathogenic T cells to infiltrate into the CNS?

3.2 Results

3.2.1 *F. hepatica* exosomes are potent inducers of IL-1RA production and other anti-inflammatory markers in immune cells and supress pro-inflammatory cytokine production.

IL-1RA is an anti-inflammatory cytokine and a member of the IL-1 cytokine family. It is produced by different immune cells and binds to IL-1 receptors, blocking the binding of IL-1β and IL-1α, but does not induce an intracellular signal [140]. Helminth infection has been shown to promote IL-1RA production [306, 307]. *F. hepatica* infection induces the expression of IL-1R2 in the liver tissue of mice [308]. IL-1R2, along with IL-1RA, is an endogenous inhibitor of IL-1 signalling [309]. Therefore, the potential for *F. hepatica* exosomes to induce IL-1RA production was examined. Different immune cells were cultured *in vitro* to examine if *F. hepatica* exosomes could promote IL-1RA production. Peritoneal exudate cells, BMDMs and BMDCs were treated with *F. hepatica* exosomes (10 μg/ml) or medium for 24 hours. Similarly, spleen cells from naïve C57BL/6 mice were stimulated with anti-CD3ε (1 μg/ml) or medium in the presence of *F. hepatica* exosomes (10 μg/ml) or medium for 72 hours. The concentration of IL-1RA in the supernatants was determined by ELISA. *F. hepatica* exosomes promoted IL-1RA production by peritoneal exudate cells, BMDMs, and BMDCs (Figure 3.1A-C). *F. hepatica* exosomes also induced IL-1RA production by spleen cells cultured with and without anti-CD3ε (Figure 3.1D).

Having shown that *F. hepatica* exosomes at a concentration of 10 μg/ml were capable of inducing IL-1RA production in peritoneal exudate cells, macrophages, DCs, and spleen cells, it was investigated whether smaller concentrations could have a similar effect. Spleen cells were treated with increasing concentrations of *F. hepatica* exosomes (0.75, 1.5, 2.5, 3, 5, 10 μg/ml), LPS (100 ng/ml), or medium in the presence or absence of anti-CD3ε (1 μg/ml). Cytokine concentration after 72 hours was determined by ELISA. In the presence of anti-CD3ε 10μg/ml of *F. hepatica* exosomes induced significant production of IL-1RA (Figure 3.2). IL-1RA was not induced by any of the lower concentrations of exosomes. Peritoneal exudate cells were treated for 24 hours with increasing concentrations of *F. hepatica* exosomes (0.03, 0.1, 0.3, 1, 3, 10 μg/ml), LPS (100 ng/ml), or medium. All concentrations of *F. hepatica* exosomes promoted IL-1RA and IL-6

production (Figure 3.3A-B). The cells were lysed and arginase activity in the cells was detected. Each exosome concentration induced arginase activity (Figure 3.3C).

To test whether *F. hepatica* exosomes could supress the induction of pro-inflammatory cytokine production by LPS, peritoneal exudate cells and BMDMs were treated with *F. hepatica* exosomes (10 μg/ml) or medium +/- LPS (100 ng/ml). After 24 hours, the concentrations of TNF, IL-6, IL-12p70, IL-23, and GM-CSF in the supernatants were determined by ELISA. In peritoneal exudate cells, *F. hepatica* exosomes significantly inhibited LPS-induced production of TNF, IL-6, and IL-23 (Figure 3.4A). No suppressive effect of *F. hepatica* exosomes was seen on LPS-induced TNF, IL-6, and IL-12p70 production in BMDMs (Figure 3.4B).

3.2.2 Different preparations of *F. hepatica* exosomes have distinct activities *in vitro*.

In order to optimise the possible immunomodulatory effects of *F. hepatica* exosomes, other exosome purification approaches were explored (Section 2.3.2). The commercially available Exospin Exosome Purification Kit from Cell Guidance Systems was used to purify a batch of *F. hepatica* exosomes (Section 2.3.2.2) The *in vitro* properties of the batch were examined and compared with the activity of *F. hepatica* exosomes purified by ultracentrifugation. BMDMs were treated with exosomes prepared using the Exospin kit (10 μg/ml) or medium. After 24 hours supernatants were removed to quantify IL-1RA, IL-6, and GM-CSF concentrations. The exosomes purified using the kit induced IL-1RA but not IL-6 or GM-CSF production (Figure 3.5A). RNA was isolated from the BMDMs to examine the expression of Arg1, IL-1RN, and Mrc1. The exosomes induced the expression of all three genes (Figure 3.5B). Next it was examined whether *F. hepatica* exosomes purified using the kit could suppress LPS-induced pro-inflammatory activity. Peritoneal exudate cells were treated with the exosomes (10 μg/ml) or medium +/- LPS (100 ng/ml). After 24 hours supernatants were harvested. The exosomes promoted IL-1RA and IL-6 production in the cells and inhibited LPS-induced IL-6 and GM-CSF production (Figure 3.6A). The cells were harvested to investigate IL-1RN, Arg1, and Mrc1 expression by rtPCR. As well as inducing IL-1RA production, *F. hepatica* exosomes purified using the kit induced IL-1RN expression. Arg1 and Mrc1 expression in peritoneal exudate cells was also enhanced by exosome treatment (Figure 3.6B).

Exosomes prepared using the Exospin kit had similar *in vitro* activity to those isolated by ultracentrifugation, namely the induction of IL-1RA and anti-inflammatory markers. Therefore, an experiment was designed to directly compare the two exosome preparations. BMDMs, peritoneal exudate cells, and peritoneal exudate macrophages were treated with the two different preparations of *F. hepatica* exosomes (10 μg/ml) or medium +/- LPS (100 ng/ml). After 24 hours supernatants and cells were harvested for ELISA and rtPCR analysis respectively. *F. hepatica* exosomes prepared by ultracentrifugation, Ex(UC), were the more active of the two exosome preparations in the cells examined, inducing IL-1RA and IL-6 production in all three cell types and GM-CSF production in peritoneal exudate cells and peritoneal exudate macrophages (Figure 3.7). *F. hepatica* exosomes prepared using the Exospin kit, Ex(Kit), promoted IL-1RA production in BMDMs, IL-1RA and IL-6 production in peritoneal exudate cells, and IL-6 production in peritoneal exudate macrophages but cytokine production was not induced as strongly as with exosomes prepared by ultracentrifugation. Both exosome preparations attenuated LPS-induced GM-CSF production in peritoneal exudate cells and peritoneal exudate macrophages and LPS-induced IL-1RA and IL-6 production in peritoneal exudate macrophages.

Both preparations promoted IL-1RN and Arg1 expression in all cell types examined (Figure 3.8). Consistent with the data on cytokine production, exosomes prepared by ultracentrifugation were more potent inducers of expression of IL-1RN and Arg1. LPS-induced IL-1RN was inhibited by both exosome preparations in peritoneal exudate cells and peritoneal exudate macrophages. The effect of the different exosome preparations on LPS-induced Arg1 expression was more varied. In BMDMs, exosomes prepared using the kit inhibited LPS-induced Arg1 expression, but exosomes prepared by ultracentrifugation did not. Both exosome preparations inhibited LPS-induced Arg1 expression in peritoneal exudate cells and peritoneal exudate macrophages. A striking difference between the two preparations is that in many of the output assays, the presence or absence of LPS makes no difference when using Exo(UC) and the effect of LPS is only seen when using Exo(Kit). In conclusion and based on these observations, it was decided to prepare all future *F. hepatica* exosome samples by ultracentrifugation.

3.2.3 *F. hepatica* exosomes promote the alternative activation of macrophages and eosinophilia *in vivo*.

F. hepatica infection leads to the expansion and infiltration of eosinophils and other type-2 cells [201]. *F. hepatica* exosomes promoted the development of type-2-associated M2 macrophages *in vitro* and previous work in our lab has demonstrated that the exosomes promoted eosinophil differentiation from bone marrow (Finlay and Mills, Unpublished). It was investigated if *F. hepatica* exosomes could behave similarly *in vivo*. C57BL/6 mice were injected i.p. with *F. hepatica* exosomes (2 µg/mouse/day) or PBS on days 0, 2, 4, and 6. On day 7, peritoneal exudate cells were collected by lavage (Figure 3.9A). The cells were stained with antibodies specific for CD11b, Siglec-F, Ly6G, Ly6C, CD19, F4/80, MHCII, and CD206 and analysed by flow cytometry; expression of MHCII was used as an M1 macrophage-associated marker [310] and expression of CD206 (mannose receptor) as an M2 macrophage-associated marker [311]. LPMs, SPMs and inflammatory monocytes were identified by a sequential gating strategy. First, $CD19^+$ B cells and then $Siglec\text{-}F^+$ eosinophils were excluded. Then all CD11b-expressing cells, high and intermediate, were chosen. LPMs were distinguished as $F4/80^+$, SPMs as $F4/80^-$, and inflammatory monocytes as $Ly6C^+$ (Figure 3.9B). The results revealed that injection of *F. hepatica* exosomes injection reduced MHCII expression, based on MFI, on all three cell types (Figure 3.10A). Exosomes also promoted CD206 expression on SPMs and inflammatory monocytes (Figure 3.10B).

Since *F. hepatica* exosomes were found to promote the alternate activation of macrophages, their effect on other innate cell populations was investigated, namely eosinophils and neutrophils. Eosinophils were identified as $Siglec\text{-}F^+$ cells and confirmed by their characteristic high side scatter and low forward scatter profile (Figures 3.9B and 3.11A). The results revealed that *F. hepatica* exosomes induced an increase in the frequency and absolute numbers of eosinophils in the peritoneal cavity. Neutrophils were distinguished as $Ly6G^+$ cells with low side scatter and forward scatter profiles (Figures 3.9B and 3.11B). *F. hepatica* exosome injection resulted in reduced numbers of neutrophils in the peritoneal cavity.

In some of the peritoneal exudate cells collected by lavage, gene expression was analysed by rtPCR and arginase activity was examined using an Arginase Activity Assay Kit from Sigma. *F. hepatica* exosome injection induced arginase activity and expression, and Retnla, Mrc1, and IL-1RN expression in the peritoneal cavity (Figure 3.12A-E). All five of these effects correlated positively with the frequency of eosinophils induced by *F. hepatica* exosome injection (Figures 3.12F-J). Finally, after the peritoneal exudate cells were pelleted by centrifugation and isolated for flow cytometry and rtPCR analysis, the cell supernatants (PEC fluid) were isolated and the concentration of IL-1RA was determined by ELISA. There was a significant enhancement of the concentration of IL-1RA in the PEC fluid isolated from *F. hepatica* exosome-injected mice compared to PBS-injected mice (Figure 3.13).

An experiment was devised to test whether a series of *F. hepatica* exosome injections was required to promote eosinophilia or if only one injection seven days earlier was sufficient. This timing has been chosen from previous work in the lab, which demonstrated that eosinophil numbers in the peritoneal cavity peak 7 days after helminth challenge or injection of helminth products [312]. C57BL/6 mice were injected i.p. with *F. hepatica* exosomes (2 µg/mouse/day) either on day 0 only or on days 0, 2, 4, and 6. A control group was injected with PBS. On day 7, peritoneal exudate cells were isolated by lavage (Figure 3.14A). Some cells were harvested for rtPCR analysis; the rest were stained with antibodies specific for CD11b and Siglec-F and analysed by flow cytometry to identify eosinophils. The results show that a single injection of *F. hepatica* exosomes was insufficient to induce eosinophilia; a series of injections was required to increase the frequency and absolute numbers of eosinophils present in the peritoneal cavity (Figure 3.14B). Multiple injections were also needed for the induction of Arg1 expression and the series of injections promoted IL-1RN expression more than a single injection (Figure 3.14C).

F. hepatica exosomes promoted IL-1RA production in peritoneal exudate cells, macrophages, DCs, and spleen cells *in vitro* and *F. hepatica* exosome injection *in vivo* induced IL-1RA production. Eosinophils can produce IL-1RA [313]. As *F. hepatica* exosome injection also promoted a significant increase in eosinophil numbers and IL-1RN expression correlated positively with the frequency of eosinophils induced, it was

investigated whether *F. hepatica* exosomes could promote IL-1RA production by eosinophils. The number of eosinophils in the peritoneal cavity of naïve mice is very low (Figures 3.11A and 3.14B) and so it will be difficult to obtain a sufficient number of cells to be treated in vitro with *F. hepatica* exosomes. Therefore, it was decided to induce eosinophils *in vivo* using the *F. hepatica* exosomes, isolate them by FACS, and investigate their ability to produce IL-1RA in culture. As previously, C57BL/6 mice were injected i.p. with *F. hepatica* exosomes (2 µg/mouse/day) on days 0, 2, 4, and 6. On day 7, peritoneal exudate cells were collected by lavage. The cells were stained with antibodies specific for CD11b and CD19 and sorted through a BD FACSAria Fusion flow cytometer (Figure 3.15A). Eosinophils were identified as SSc^{hi} $CD11b^{int}$ $CD19^{-}$ $Ly6G^{-}$ cells (Figure 3.15B). The FACS-sorted eosinophils were plated out in a 96-well plate ($2x10^5$ cells/well), incubated at 37°C for 0, 24 or 72 h and the concentration of IL-1RA and YM1 in the supernatants was determined by ELISA. Eosinophils incubated for 72 hours produced IL-1RA and YM-1 (Figure 3.15C). The induction of YM-1 (also known as chitinase-3-like protein 3 or Chi3l3) production is characteristic of helminth infections and, more broadly, type 2 immune responses [246]. YM-1 is an eosinophilic chemotactic factor and is generally thought to be produced by APCs, especially macrophages. Here it was demonstrated that eosinophils can also produce YM-1.

3.2.4 *F. hepatica* exosomes do not induce IL-33 production in macrophages or DCs.

Together with IL-1RA induction, a characteristic property of *F. hepatica* exosomes has been the mobilisation and expansion of eosinophils. A key early step in the induction of type-2 responses, including eosinophil recruitment, is the production of the alarmins IL-33, IL-25, and TSLP. These alarmins act on ILC2 cells to promote the production of the type-2 cytokines IL-5 and IL-13 [201]. Macrophages and DCs can both produce IL-33 [314]. BMDMs and BMDCs were treated with different concentrations of *F. hepatica* exosomes (1, 3, and 10 µg/ml) and the supernatants were collected after 15min, 30min, 1h, 2h, and 6h or medium only for 6h. The concentrations of IL-13, IL-25, TSLP, and IL-1RA were determined by ELISA. No IL-33, IL-25, or TSLP production was detectable at any time point or concentration of exosomes in either cell type (Figure 3.16). In BMDMs IL-1RA was detected 6 hours after treatment with all three concentrations of *F. hepatica* exosomes.

3.2.5 *F. hepatica* exosome-induced IL-1RA production is likely mediated by protein but eosinophil induction is not.

Exosomes contain proteins and signalling nucleic acids, including miRNAs. Proteinase K incubation and heat inactivation are two methods of inactivating the protein content of a sample, by digestion and denaturation respectively. An experiment was designed to investigate whether proteins contained within *F. hepatica* exosomes mediate their characteristic activity; namely their ability to induce IL-1RA production *in vitro* and to promote eosinophil recruitment *in vivo*. To test this hypothesis, samples of *F. hepatica* exosomes had their protein content depleted by Proteinase K incubation or heat inactivation (Section 2.3.10). BMDMs were treated with *F. hepatica* exosomes (control, Proteinase K-treated, or heat inactivated; 10 μg/ml) or medium control for 24 h and the concentrations of IL-1RA in the supernatants were determined by ELISA. Both Proteinase K-treatment and heat inactivation suppressed the ability of *F. hepatica* exosomes to induce IL-1RA production suggesting this is a protein-mediated effect (Figure 3.17).

In parallel, C57BL/6 mice were injected i.p. with *F. hepatica* exosomes (control, Proteinase K-treated, or heat inactivated; 2 μg/mouse/day) or PBS on days 0, 2, 4, and 6. On day 7, peritoneal exudate cells were collected by lavage, stained with antibodies specific for CD11b and Siglec-F, and analysed by flow cytometry (Figure 3.18A). The results demonstrate that protein inactivation does not inhibit the ability of *F. hepatica* exosomes to promote eosinophilia; all three exosome preparations induced an increase in the frequency and absolute numbers of eosinophils present in the peritoneal cavity (Figure 3.18B). Thus, the induction of IL-1RA production and the recruitment of eosinophils are probably mediated by distinct protein and non-protein fractions of *F. hepatica* exosomes.

3.2.6 *F. hepatica* exosomes supress MSU crystal induced inflammation *in vivo*.

F. hepatica exosomes generated type-2 and regulatory responses *in vitro* and attenuated LPS-induced pro-inflammatory responses. In healthy unchallenged mice, *F. hepatica* exosomes promoted type-2 and regulatory responses. They also promote IL-1RA production, which suppresses IL-1 cell signalling via IL-1RI. Since *F. hepatica* exosomes were found to be capable of inducing IL-1RA production, their ability to

reverse the IL-1β-mediated pro-inflammatory effects of MSU crystals was investigated. MSU crystals are pro-inflammatory DAMPs that activate the inflammasome leading to the release of active IL-1β and IL-18. I.p. injection of MSU crystals will lead to neutrophil infiltration into the peritoneal cavity in an IL-1β-dependent manner [315]. C57BL/6 mice were injected i.p. with *F. hepatica* exosomes (2 μg/mouse/day) or PBS vehicle only as a control on days 0, 2, 4, and 6 followed by i.p. injection with MSU crystals (250 μg/mouse) or PBS on day 7. After 4 hours, peritoneal exudate cells were isolated by lavage (Figure 3.19A). The cells were stained with antibodies specific for CD11b, Siglec F, Ly6G, and Ly6C and analysed by flow cytometry to investigate eosinophil, neutrophil and inflammatory monocyte populations (Figure 3.19B). *F. hepatica* exosomes recruited eosinophils to the peritoneal cavity (Figure 3.20A). MSU crystal injection lead to neutrophil induction which was supressed by the exosomes (Figure 3.20B). In mice treated with *F. hepatica* exosomes and MSU crystals, the recruitment of eosinophils negatively correlated with the recruitment of neutrophils (Figure 3.21). I.p. injection of *F. hepatica* exosomes did not inhibit MSU crystal-induced inflammatory monocytes recruitment into the peritoneal cavity (Figure 3.22).

RNA was isolated from peritoneal exudate cells to analyse gene expression by rtPCR. I.p. injection of *F. hepatica* exosomes promoted expression of Arg1 and IL-1RN in the peritoneal cavity (Figure 3.23A-B). In mice treated with *F. hepatica* exosomes, Arg1 expression closely correlated with the frequency of eosinophils (Figure 3.23C). There was no correlation between IL-1RN expression and eosinophils (Figure 3.23D). As seen in mice injected with *F. hepatica* exosomes (Figure 3.13), there was an enhancement in the concentration of IL-1RA in the PEC fluid isolated from F. hepatica exosome-injected mice compared to PBS-injected mice (Figure 3.24).

In vitro, MSU crystal treatment, in combination with LPS, will induce NLRP3 inflammasome activation and subsequent mature IL-1β and IL-18 release from macrophages and DCs [316]. Having demonstrated that *F. hepatica* exosomes inhibit MSU crystal-mediated inflammation *in vivo*, it was investigated whether they could suppress MSU crystal-induced IL-1β and IL-18 release in macrophages and DCs *in vitro*. BMDMs and BMDCs were treated with *F. hepatica* exosomes (10 μg/ml) or medium +/- LPS (100 ng/ml) and MSU crystals (250 μg/ml) for 24 hours. LPS and MSU crystals

induced IL-1β and IL-18 release from BMDMs and BMDMs and *F. hepatica* exosomes were unable to supress this effect (Figure 3.25). This suggests that *F. hepatica* exosome-inhibition of MSU crystal-induced inflammation occurs downstream of inflammasome activation.

There was a negative correlation between the frequencies of eosinophils and neutrophils recruited into the peritoneal cavity in MSU crystal-challenged mice treated with the *F. hepatica* exosomes (Figure 3.20C), suggesting that it is eosinophils that mediate the anti-inflammatory effect of the exosomes in this model of sterile inflammation. Anti-IL-5 administration inhibits eosinophil recruitment[317]. Therefore, an experiment was designed to investigate whether eosinophils are required for *F. hepatica* exosomes to suppress MSU crystal-induced neutrophil recruitment. C57BL/6 mice were injected i.p. with anti-IL-5 (80 µg/mouse/day) or PBS on days -2, 0, 2, 4, and 6 and *F. hepatica* exosomes (2 µg/mouse/day) or PBS on days 0, 2, 4, and 6 followed by MSU crystal i.p. injection (250 µg/mouse) or PBS on day 7. After 4 hours, peritoneal exudate cells were isolated by lavage (Figure 3.26A). The cells were stained with antibodies specific for CD11b, Siglec F, and Ly6G and analysed by flow cytometry analysis to investigate the recruitment of eosinophils and neutrophils (Figure 3.26B).

F. hepatica exosomes induced a significant increase in the frequency of eosinophils and total numbers in the peritoneal cavity, and this was supressed by the administration of anti-IL-5 (Figure 3.27A). MSU crystal injection induced an increase in the frequency and absolute numbers of neutrophils in the peritoneal cavity. *F. hepatica* exosome treatment suppressed the infiltration of the pro-inflammatory neutrophils. Inhibition of *F. hepatica* exosome-induced eosinophilia with anti-IL-5 did not suppress the ability of IL-33 to attenuate MSU crystal-induced inflammation (Figure 3.27B). These findings demonstrate that eosinophils do not mediate *F. hepatica* exosome-inhibition of MSU crystal-induced inflammation.

3.2.7 Innate immune cells from mice treated with *F. hepatica* exosomes inhibit pro-inflammatory cytokine production by MOG-specific cells.

F. hepatica exosomes induced IL-1RA production and inhibited pro-inflammatory responses *in vitro* and *in vivo*. It was investigated whether *F. hepatica* exosomes could attenuate inflammation which mediates autoimmune diseases. EAE is the most commonly used animal model for MS and is characterised by the infiltration of autoreactive T-cells into the CNS leading to myelin destruction and the development of progressive paralysis [193]. In the passively induced model or adoptive transfer model of EAE, myelin-specific T cells from mice immunised with MOG are injected into naïve recipient mice who then develop EAE-like disease. As in active EAE, donor mice are injected with MOG and CFA, without PT. 10 days following immunisation, spleen and LN cells are isolated and restimulated with MOG along with IL-1β and IL-23 in culture for 72 hours before being transferred to recipient mice [318]. As IL-1β is one of the factors required to prime the MOG-specific culture and as it has been shown that *F. hepatica* exosomes induce IL-1RA production and attenuate IL-1β-mediated inflammation, an experiment was designed to investigate whether the exosomes could inhibit the generation of pathogenic MOG-specific cells in culture. A common readout of the pathogenicity of the MOG-specific cells is to measure the concentrations of the pro-inflammatory cytokines IL-17A, IFN-γ, and GM-CSF produced by the cells. As our lab previously demonstrated that recombinant IL-4 inhibited IL-17A production by MOG-specific cells (McGuinness and Mills, Unpublished), IL-4 treatment was used as a positive control.

C57BL/6 mice were injected s.c. with MOG emulsified in CFA (100 μg/mouse). 10 days following injection, spleens and LN cells were isolated and cultured for 72 hours in a 70:30 ratio (Spleen:LN cell) with MOG (100 μg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence of medium, IL-4 (20 ng/ml), or *F. hepatica* exosomes (10 μg/ml) (Figure 3.28A). Supernatants were collected and the concentrations of IL-17A, IFN-γ, GM-CSF, and YM-1 were determined by ELISA. Administration of IL-4 to the MOG-specific induced YM-1 production and inhibited IL-17A production as we had previously demonstrated (Figure 3.28B). *F. hepatica* exosome administration did not suppress pro-inflammatory cytokine production by MOG-specific cells.

Having shown no inhibitory effect of direct administration of *F. hepatica* exosomes to the culture, it was decided next to co-culture the MOG-specific cells with immune cells induced *in vivo* by *F. hepatica* exosome injection, specifically eosinophils. The rationale for this was based on the data that *F. hepatica* exosomes are potent inducers of eosinophilia *in vivo* and that these cells produce IL-1RA. Therefore, C57BL/6 mice were injected i.p. with *F. hepatica* exosomes (2 μg/mouse/day) on days 0, 2, 4, and 6. On day 7, peritoneal exudate cells were collected by lavage and eosinophils were purified by FACS (Section 3.2.3 and Figure 3.15). As before, mice were injected s.c. with MOG and CFA and 10 days later, spleens and LN cells were isolated and cultured in a 70:30 ratio (Spleen:LN cell) with MOG (100 μg/ml), IL-1β (1 pg/ml) and IL-23 (2.5 ng/ml) (Figure 3.29A). The spleen/LN cells were co-cultured with the *F. hepatica* exosomes-induced eosinophils at a ratio of 0.2:1 (eosinophil:spleen+LN). After 72 hours the supernatants were harvested and the concentrations of GM-CSF, IFN-γ, IL-1RA, IL-17A, and YM-1 determined by ELISA. Co-culture with eosinophils significantly supressed IL-17A production by the MOG-specific cells and induced IL-1RA production (Figure 3.29B).

Prior to FACS purification of eosinophils from the peritoneal exudate cells (tPEC) isolated from mice injected with *F. hepatica* exosomes, some of the tPEC were cocultured for 72 hours with the spleen and LN cells at ratios of 0.05:1, 0.1:1, 0.2:1, 0.5:1, and 1:1 (tPEC:spleen+LN). The cells were treated with MOG (100 μg/ml), IL-1β (one of two concentrations; 1 or 10 pg/ml) and IL-23 (2.5 ng/ml). (Figure 3.30A). The concentrations of GM-CSF, IFN-γ, IL-1RA, IL-17A, and YM-1 in the supernatants were determined by ELISA. tPEC isolated from mice injected with F. hepatica exosomes inhibited GM-CSF and IFN-γ production by the MOG-specific spleen and LN cells and induced IL-1RA and YM-1 production (Figure 3.30B).

3.2.8 Therapeutic administration of and pre-treatment with *F. hepatica* exosomes delays EAE disease onset.

Injections of *F. hepatica* exosomes 4 times over 6 days induced an anti-inflammatory response *in vivo* which could supress the generation of a subsequent immune response to pro-inflammatory stimuli. An experiment was designed to determine whether this anti-inflammatory effect is transient or whether it can be longer lasting and suppress

the development of EAE development. C57BL/6 mice were injected i.p. with *F. hepatica* exosomes (2 μg/mouse/day) or PBS on days -7, -5, -3, and -1. On day 0 the mice were injected with MOG emulsified in CFA (100 μg/mouse) and PT (200 ng/mouse) and with PT again on day 2 to induce disease (Figure 3.31A). Animals were monitored daily for clinical signs and weight loss (Figure 3.31B). Injection of *F. hepatica* exosomes for a week prior to the induction of EAE significantly delayed disease onset by delaying the onset of clinical signs.

Our lab has demonstrated that the therapeutic administration of FHES attenuated the development of EAE. In order to assess the potential immune modulating effect of exosomes, mice were treated therapeutically with *F. hepatica* exosomes prior to induction of EAE. C57BL/6 mice were injected i.p. with *F. hepatica* exosomes (2 μg/mouse/day) or PBS vehicle on days -1, and every subsequent second day until the end of the experiment. EAE was induced on days 0 and 2 and monitored as before (Figure 3.32A). Treatment of mice with *F. hepatica* exosomes delayed disease onset (Figure 3.32B).

I next assessed the possibility that anti-inflammatory trained innate immunity induced by *F. hepatica* exosomes could attenuate EAE. Work in the Mills lab has shown that two s.c. injections of FHTE 21 and 7 days before the induction of EAE trained monocytes to be more anti-inflammatory resulting in the suppression of disease (Quinn et al, in review). C57BL/6 mice were injected s.c. with *F. hepatica* exosomes (2 μg/mouse/day) or PBS on days -21 and -7 (Figure 3.33A). EAE was induced and monitored as before. Pre-treatment of mice with *F. hepatica* exosomes significantly attenuated the development of EAE (Figure 3.33B).

3.2.9 *F. hepatica* exosome pre-treatment does not inhibit cytokine production by T cells in EAE.

Training mice with *F. hepatica* exosomes resulted in the inhibition of EAE disease progression. In order to assay the possibility that *F. hepatica* exosomes induce innate training, mice were injected s.c. with *F. hepatica* exosomes (2 μg/mouse/day) or PBS on days -21 and -7 and on day 0, spleen cells were isolated and cultured with Mtb (100 ng/ml), LPS (100 ng/ml), LPS (100 ng/mL) and MSU crystals (250 μg/ml), or medium

control (Figure 3.34A). After 24 hours the concentrations of GM-CSF and TNF in the supernatants were determined by ELISA. Training with *F. hepatica* exosomes suppressed Mtb-induced TNF production but did not significantly inhibit the production of GM-CSF induced by the pro-inflammatory stimuli (Figure 3.34B).

In EAE, DCs are activated by Mtb in CFA and present myelin to naive myelin-specific T cells in LNs [194]. These activated myelin-specific T cells will then exit the LNs and traffic to the CNS, crossing the BBB, where they encounter myelin antigens presented by APCs in the CNS. This reactivates the T cells, which produce cytokines including IL-17A, IFN-γ, and GM-CSF and recruit other immune cells ultimately leading to myelin destruction [195, 196]. Activated γδ T cells and later Th1 and Th17 cell mediate pathology in EAE [319]. The effect of training with *F. hepatica* exosomes on effector T cell function was examined in the spleen and LNs of mice with EAE

C57BL/6 mice were injected s.c. with *F. hepatica* exosomes (2 µg/mouse/day) or PBS on days -21 and -7 and EAE was induced on Day 0 as previously (Figure 3.35A). On day 3, LNs were isolated. Single cell suspensions were prepared from the LNs and surface stained with antibodies specific for CD3, CD4, Ly6C, Ly6G, and TCRγδ, then stimulated with PMA, ionomycin and brefeldin A and stained intracellularly for IL-17A before being analysed by flow cytometry (Figure 3.35B). γδ T cells were identified as $CD3^+$ $TCR\gamma\delta^+$ (Figure 3.36A) and CD4 T cells as $CD3^+$ $CD4^+$(Figure 3.36B). No significant differences in the frequency of total or IL-$17A^+$ γδ or CD4 T cells between PBS and exosome-treated mice were found.

Following MOG/CFA immunisation, antigen-specific cytokine production can be detected by spleen and LN cells re-stimulated *in vitro* with MOG. C57BL/6 mice were injected s.c. with *F. hepatica* exosomes (2 µg/mouse/day) or PBS on days -21 and -7 and EAE was induced as before. On day 7, spleen and LNs were isolated and restimulated with increasing concentrations of MOG (10, 30, or 100 µg/ml) or medium control (Figure 3.37A). After 72 hours, the concentrations of GM-CSF and IL-17A in the supernatants were determined by ELISA. MOG induced cytokine production from spleen and LN cells in a dose-dependent manner but training with *F. hepatica* exosomes did not significantly attenuate cytokine production (Figure 3.37B).

3.2.10 Training with *F. hepatica* exosomes suppresses T cell infiltration into the CNS during EAE.

The development of EAE is associated with the infiltration of pathogenic autoreactive T cells into the CNS where they activate innate cells to initiate neuronal destruction. An experiment was designed to investigate whether training with *F. hepatica* exosomes could inhibit the trafficking of T cells to the CNS. C57BL/6 mice were s.c. with *F. hepatica* exosomes (2.5 µg/mouse/day) or PBS on days -21 and -7. EAE was induced on day 0 and on day 11 the mice were sacrificed, perfused, and the brains and spinal cords isolated (Figure 3.38A). Single cell suspensions were prepared from the brains or spinal cords and surface stained with antibodies specific for CD3 and CD4. Cells were then stimulated with PMA, ionomycin and brefeldin A and stained intracellularly for GM-CSF, IFN-γ, and IL-17A before being analysed by flow cytometry (Figure 3.39B). By day 11, the PBS-treated group had developed disease as demonstrated by a significant drop in body weight and the development of clinical signs, while the mice trained with exosomes were disease free (Figures 3.39A and 3.40A). Compared with PBS-treated mice, mice treated with *F. hepatica* exosomes had significantly reduced numbers of total $CD4^+$ cells and reduced numbers of $GM\text{-}CSF^+$, $IFN\text{-}\gamma^+$, and $IL\text{-}17A^+$ $CD4^+$ cells in both the brain (Figure 3.39B-E) and the spinal cord (Figure 3.40B-E). These findings suggest that training of mice with *F. hepatica* exosomes suppresses the ability of pathogenic T cells to infiltrate into the CNS.

3.3 Discussion

The most exciting findings of the study was the observation that *F. hepatica* exosomes induced IL-1RA production in multiple cell types and that the exosome attenuate EAE, a disease mediated by IL-1β. *F. hepatica* exosomes were capable of inducing IL-1RA production detectable in supernatants as little as 6 hours post treatment. While BMDMs, BMDCs, and peritoneal exudate cells were capable of the constitutive production of IL-1RA, *F. hepatica* exosomes significantly enhanced IL-1RA production by each of these cell types as well as by spleen cells. In spleen cells, a high concentration (10 μg/ml) of *F. hepatica* exosomes was required to induce IL-1RA production but in peritoneal exudate cells, concentrations in the nanogram range were able to promote IL-1RA production.

Eosinophils constitutively produce IL-1RA and elevated IL-1RA production has been seen in different eosinophil-mediated diseases [313, 320, 321]. Injection of *F. hepatica* exosomes i.p. in mice promoted eosinophil infiltration and the induction of IL-1RN expression in the peritoneal cavity, effects which positively correlated with each other. This suggested that *F. hepatica* exosomes promoted the expression of IL-1RN in eosinophils. In mice challenged with MSU crystals, *F. hepatica* exosome injection promoted eosinophil infiltration and the induction of IL-1RN expression in peritoneal exudate cells, but the effects were not correlated with each other. A potential explanation for this is that as MSU crystals induce IL-1RA *in vivo* [322], they are at least partially obscuring the effect of the exosomes. Cultured eosinophils induced *in vivo* by *F. hepatica* exosome injection produced IL-1RA *in vitro*, confirming that the exosomes promote IL-1RA production in eosinophils.

F. hepatica exosomes induced were capable of inducing IL-1RA production *in vivo* as seen in PEC fluid as well as *in vitro*. The flow cytometry data and the rtPCR analysis demonstrated that *F. hepatica* exosomes favoured the alternate activation of macrophages in the peritoneal cavity and *in vitro F. hepatica* exosomes strongly induced IL-1RA production in macrophages. Alternatively activated macrophages produce IL-1RA [323]. It is still to be determined if *F. hepatica* exosomes induce IL-1RA production from M2 macrophages *in vivo* as they do with eosinophils. Flow cytometry analysis of

peritoneal exudate cells following *F. hepatica* exosome injection with an antibody specific for IL-1RA would help determine in which immune cell (or cells) the exosome induce IL-1RA production *in vivo*. This could help elucidate which cell type is required to mediate the anti-inflammatory activity of *F. hepatica* exosomes shown in the MSU crystal model of sterile inflammation.

F. hepatica exosomes inhibited MSU crystal-induced neutrophil infiltration and there was a negative correlation between eosinophil and neutrophil recruitment. Eosinophils are a characteristic feature of type 2 responses while neutrophils are associated with a type 1 phenotype [201]. Type 1 and type 2 immune responses reciprocally inhibit each another [324]. Soluble egg antigens (SEA) from the helminth *Schistosoma mansoni* were shown to be protective in a mouse model of atherosclerosis, a chronic inflammatory disease of blood vessel walls. Injection of SEA reduced the number of circulating neutrophils [325]. An inverse or antagonistic relationship between eosinophils and neutrophils in the MSU crystal challenge model was suggested from the negative correlation. However, suppressing the induction of eosinophilia using anti-IL-5 did not reverse the inhibitory activity of *F. hepatica* exosomes in MSU crystal-mediated inflammation. This demonstrates that eosinophil recruitment to the peritoneal cavity is not required for the exosomes to suppress neutrophil infiltration. Like eosinophils, M2 macrophages are key features of a type-2 response induced by helminth infection. As *F. hepatica* exosomes induce M2 macrophage activation, it is possible that the production of IL-1RA by these cells mediates the suppressive activity of the exosomes and not eosinophils. The use of anti-IL-4 and/or anti-IL-13 antibodies to suppress M2 macrophage activation by *F. hepatica* exosomes prior to MSU crystal challenge might resolve this possibility. While *F. hepatica* exosomes suppressed MSU crystal inflammation *in vivo*, the exosomes did not inhibit MSU crystal-induced inflammasome activation and mature IL-1β and IL-18 release. Suppression of inflammasome activation is only one mechanism by which to inhibit MSU crystal-mediated inflammation. Results from the studies of *F. hepatica* exosomes *in vitro* and *in vivo* suggest that the anti-inflammatory activity of the exosomes demonstrated in the MSU crystal model of sterile inflammation is mediated downstream of inflammasome activation via it is the induction of IL-1RA production. Indeed, patient improvements seen in both a proof-of-

concept, open labelled, pilot study [141] and a retroactive study [326] provide evidence that anakinra (the generic drug name for IL-1RA) is effective at treating gout, an inflammatory disease caused by MSU crystals.

There are different methods for isolating exosomes, with different strengths and weaknesses [275]. The *F. hepatica* exosome samples used in this study were prepared one of two ways: (1) differential ultracentrifugation or (2) the Exospin commercial kit. Both exosome preparations induced IL-1RA production in innate immune cells. Comparing the two preparations *in vitro*, *F. hepatica* exosomes prepared by ultracentrifugation were more active than exosomes prepared using the kit. *F. hepatica* exosomes prepared by ultracentrifugation were also more effective at inducing eosinophilia than exosomes isolated using the kit. Based on these observations, it was decided to prepare future *F. hepatica* exosome samples by ultracentrifugation.

IL-33 is a key cytokine involved early in the recruitment of eosinophils and other type-2 responses. ILC2s respond to IL-33 and produce IL-5 which will promote eosinophil recruitment from the blood [155]. Macrophages and DCs are known to produce IL-33 [327]. However, when BMDMs and BMDCs were treated with *F. hepatica* exosomes no IL-33 production could be detected. While both macrophages and DCs can produce IL-33, secretion of the cytokine is low [314] and the primary IL-33-releasing cells in the body are epithelial and endothelial cells [327]. Therefore, future studies will examine if *F. hepatica* exosomes can induce IL-33 production from epithelial/endothelial cells and/or IL-33 production *in vivo* following injection of *F. hepatica* exosomes.

The results of this study demonstrated that *F. hepatica* exosomes had immunomodulatory effects comparable to live helminth infection [201] and FHES administration [157] in mice. They induced an infiltration of eosinophils into the peritoneal cavity. Previous work in our lab has demonstrated that *F. hepatica* exosomes promoted eosinophil differentiation from bone marrow *in vitro* (Finlay and Mills, Unpublished). This data demonstrates that it is possible to mimic some of the biological activity of helminth infection using isolated products of *F. hepatica*. A series of *F. hepatica* exosome injections was required to promote eosinophil recruitment. A single injection of exosomes did not promote eosinophilia in the peritoneal cavity. Falcone et

al (2001) reported similar findings with *Bm*-MIF-1 (*Bm* macrophage migration inhibitory factor-1), a protein secreted from the helminth *Brugia malayi* [312]. Three *Bm*-MIF-1 injections over a week promoted the induction of M2 macrophages. Importantly, a single injection of *Bm*-MIF-1 had no effect, even though the single injection was at a concentration larger than the total amount of protein administered in the series of three injections. A potential explanation for this is that a series of injections more closely mimics live infection, during which exosomes or an individual protein would be continuously produced and released by the helminth.

Proteinase K is a broad-spectrum serine protease used in molecular biology to digest proteins. After the protein content of a sample has been digested by Proteinase K, the protease is inactivated by heat (95°C for 10 minutes) so it cannot digest any proteins when the sample is subsequently tested. Therefore, the attenuation of IL-1RA induction activity demonstrated may be due to the heat inactivation of exosome-contained proteins (or other components including lipids and small molecules) and not the Proteinase K treatment and so the only strict conclusion that can be drawn is that the induction of IL-1RA production is heat sensitive. To investigate whether *F. hepatica* exosome induction of IL-1RA production is sensitive to the digestion of protein, Proteinase K could be inactivated in a more specific manner e.g. with the use of chemical inhibitors [328].

While *F. hepatica* exosomes induction of IL-1RA production *in vitro* may be protein-mediated, eosinophil induction *in vivo* is not. Therefore, it must be a non-protein component of *F. hepatica* exosomes that is responsible for promoting eosinophilia. Potentially it is a small molecule or metabolite. Another possibility is that it is mediated by microRNAs (miRNAs). miRNAs are 20-26 nucleotide long non-coding RNAs that regulate gene expression by binding to messenger RNAs (mRNAs) and inhibiting their translation [329]. Work on the helminth *H polygyrus* demonstrated that helminth-derived extracellular vesicles contain miRNAs that are taken up by host cells where they regulate the expression of genes related to innate immunity [286]. miRNAs have also been identified in *F. hepatica* exosomes [291]. It is possible that that eosinophil induction by *F. hepatica* exosomes is mediated by miRNAs.

Previously in the lab, we demonstrated that adding IL-4 to an adoptive transfer EAE culture of spleen cells, LN cells, MOG, IL-1β, and IL-23 induced a more anti-inflammatory environment such that when the cells were injected into mice to induce EAE, no disease developed (McGuinness and Mills, Unpublished). Therefore, *F. hepatica* exosomes were added to the culture to test whether they could inhibit cytokine production from the MOG-specific cells; it was thought that exosome-induced IL-1RA production might inhibit IL-1β-mediated inflammation. No inhibition was shown. This may be due to a stoichiometric issue as the concentration of IL-1β in the culture was 10 ng/ml while the concentration of IL-1RA production induced by *F. hepatica* exosomes is typically 5-10 ng/ml (from Chapter 3). Therefore, there may simply have been a sufficient concentration IL-1β in the culture to overcome competitive inhibition from IL-1RA. In our lab, the concentration of IL-1β needed for the culture was successfully titrated down; IL-1β at a concentration of 1 pg/ml induced cytokine production in conjunction with MOG and IL-23 (the concentration of which was titrated down from 10 to 2.5 ng/ml). Purified eosinophils and tPEC from mice injected with *F. hepatica* exosomes induced the production of IL-1RA at a concentration that was 500-1000-fold greater than the concentration of IL-1β added (1 or 10 pg/ml).

Thus, it more likely to observe IL-1RA-mediated inhibition of IL-1β inflammation. This was observed upon the addition; As purified eosinophils and tPEC supressed pro-inflammatory cytokine production as IL-4 was observed to do, it would be interesting to inject the spleen/LN and eosinophil or tPEC cocultures into mice and see could the induction of EAE be inhibited. Unfortunately, especially in the case of the eosinophils, this requires a large number of purified cells to give a sufficient number for injection into the recipient mice. While Siglec F is a specific marker for eosinophils, antibody-mediated ligation of Siglec F induces eosinophil apoptosis [330]. Therefore, the use of antibodies specific to Siglec F is not preferred for the FACS sorting of live, healthy eosinophils required for functional studies. For this reason, we chose a FACS isolation protocol that, rather than targeting Siglec F^+, identified eosinophils as SSc^{hi} $CD11b^{int}$ $CD19^-$ $Ly6G^-$ cells. However, even using this approach in attempt to minimise the death of eosinophils during the sorting process, the yield of live eosinophils was very low. From an initial count of over 145 million total peritoneal exudate cells, the final count of purified

eosinophils was only 2.6 million. That is a yield of less than 1.8%. As demonstrated in Chapter 3, *F. hepatica* exosome injection typically induces the expansion of the frequency of eosinophils in the peritoneal cavity up to approximately 15% of total live cells. Therefore, it is clear that a significant proportion, a large majority even, of *F. hepatica* exosome-induced eosinophils were dying in the sorting process. A new approach to purify eosinophils is required to ensure a sufficient number of viable cells can be procured for the coculture with MOG-specific cells with the ultimate aim of injecting into mice to see is disease induction attenuated. Human eosinophils can be purified by density gradient centrifugation followed by immunomagnetic selection[331]. Density gradient centrifugation isolates eosinophils and neutrophils together and then the former are purified by negative selection with the use of anti-CD16 antibody-coated magnetic beads (neutrophils express high levels of CD16 compared with eosinophils)[332]. Miltenyi produce microbeads for the isolation of murine eosinophils so this could be utilised as an alternate source of purified *F. hepatica* exosome-induced eosinophils *in vivo*. Another approach is to work with the tPEC isolated following *F. hepatica* exosome injection. As there are no isolation steps needed that will adversely affect cell viability, cell numbers will not be an issue. At higher concentrations of tPEC, inhibition of pro-inflammatory cytokine production by MOG-specific cells was demonstrated. The highest concentration of tPEC added to the culture was where the tPEC:spleen+LN ratio was 1:1. At even higher tPEC:spleen+LN ratios, greater inhibition may be possible. It would be beneficial to also have a population of tPEC from naïve mice as a control.

Evidence for the hygiene hypothesis is provided for by epidemiological studies demonstrating negative correlations between helminth infections and autoimmune (and allergic) disease incidence [252]. Supporting these observations, helminth infections in animals induce protection against the development of experimental models of autoimmunity including EAE. While a course of 4 injections of *F. hepatica* exosomes over 6 days induced an anti-inflammatory response and suppressed the generation of a response to subsequent pro-inflammatory stimulation, only a slight protective effect against EAE was seen in mice administered the same course of exosome injections. This may be because the anti-inflammatory response induced by

this course of *F. hepatica* exosome injections is only transient and requires the constant administration of the exosomes. This would support the findings of Falcone et al (2001) discussed above and the observation that in this project that a series of *F. hepatica* exosome injections was required to promote eosinophil recruitment[312]. This may be due to the fact that a series of injections more closely mimics live infection, in which helminth products are being continuously produced and released [312].

The therapeutic administration (every second day) of *F. hepatica* exosomes more closely mimics live infection, which our lab has previous shown to protect against EAE development[205]. However, unlike the therapeutic administration of FHES [157], injection of *F. hepatica* exosomes did not attenuate the severity of disease in the mice and only delayed the onset of disease by 1 or 2 days. FHES is made up of very many components including proteins and nucleic acids so it cannot be assumed that one of these components, like *F. hepatica* exosomes, can mimic all activities of FHES that have been previously demonstrated. For example, while *F. hepatica* exosomes administered therapeutically cannot suppress the development of EAE as FHES has been shown to do, both *F. hepatica* exosomes and FHES induce eosinophilia. This suggests that it is a component other than exosomes which mediates the protective effect of FHES against EAE when administered therapeutically.

Innate immune cells are now known to be capable of developing memory, long believed to be a feature only of adaptive immunity. Innate cells can develop memory such that they can generate a stronger (training) or weaker (tolerance) immune response upon subsequent exposure to pathogens [84]. Chenet al (2014) demonstrated that helminths induce innate immune training; macrophages trained by the nematode *Nippostrongylus brasiliensis* had enhanced worm killing activity, by decreasing metabolism and increasing mortality [304]. This is an example of pro-inflammatory training by helminths. Our lab has demonstrated anti-inflammatory training by helminths for the first time (Quinn et al, In review). Training with the total extract from *Fasciola hepatica* (FHTE) induced an anti-inflammatory environment and suppressed the development of EAE. Here it was shown that *F. hepatica* exosomes also can induce anti-inflammatory training. Injecting the exosomes on days -21 and -7 delayed EAE disease onset.

EAE progression is mediated by pathological Th1 and Th17 cells specific for MOG antigen. They are activated in the periphery upon presentation of MOG by APCs and then infiltrate into the CNS where they interact with innate populations leading to the release inflammatory mediators causing myelin destruction and disease pathology [333]. EAE can be prevented or at least attenuated by impairing MOG-specific production of IL-17A, IFN-γ, and GM-CSF by γδ and CD4 T cells. Training with *F. hepatica* exosomes did not suppress pathological T cell cytokine production. However, protection from disease was still demonstrated and this was accompanied by suppression of the recruitment of IL-17A, IFN-γ, and GM-CSF-producing CD4 cells into the brain and spinal cord. This suggests that *F. hepatica* exosome pre-treatment attenuates the development of EAE via the inhibition of the migration rather than function of pathological T cells. Recruitment of myelin-specific T cells to the CNS requires the expression of trafficking molecules on their cell surface. T cells express the adhesion molecule α4β1-integrin (VLA-4) on their surface and it is important as a trafficking molecule for pathological cells in EAE. Anti-VLA-4 neutralizing antibodies attenuate EAE, inhibiting T cell migration, and Natalizumab, a drug successful in treating MS, is a humanized antibody which binds to VLA4, inhibiting its interaction with vascular cell adhesion molecule-1 (VCAM-1) expressed by endothelial cells lining the BBB[334, 335]. This interaction is required for T cell migration into the CNS. Training with *F. hepatica* exosomes may inhibit T cell migration by interfering with adhesion molecules like VLA-4. Regardless of the mechanism, this study provides evidence that exosomes can train the innate immune system to be anti-inflammatory, inducing IL-1RA production, and this can suppress the development of autoimmune disease.

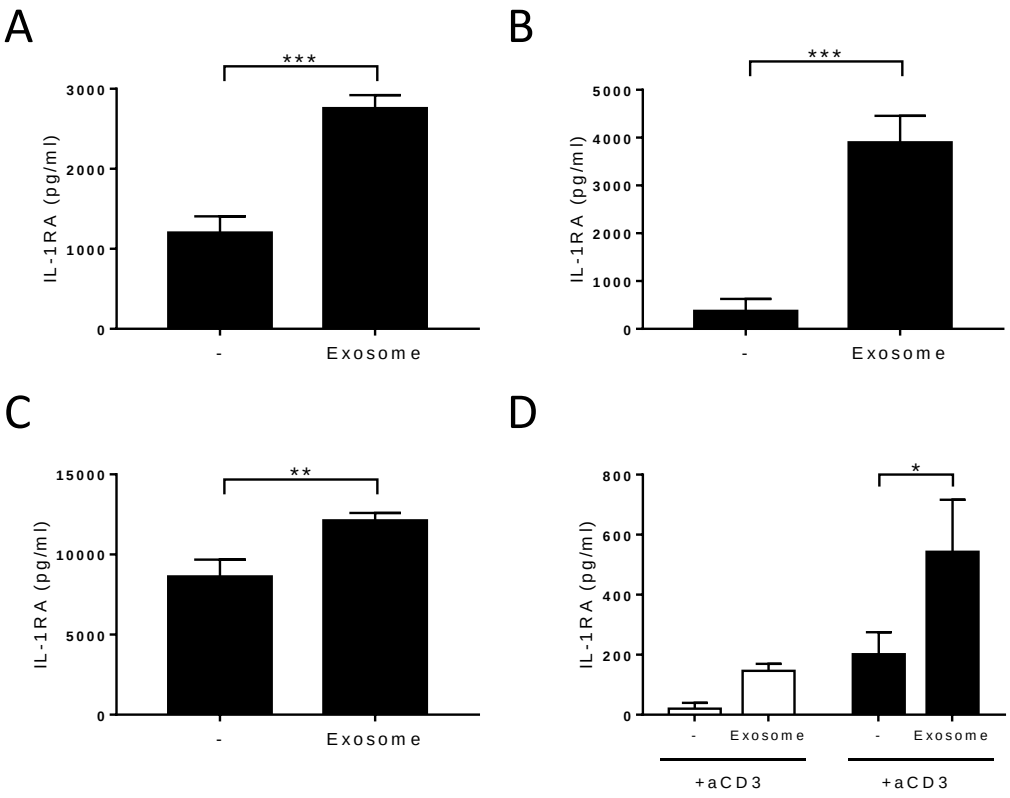


Figure 3.1: *F. hepatica* exosomes induce the production of IL-1RA by immune cells. Peritoneal exudate cells (A), M-CSF-expanded bone marrow macrophages (B) and GM-CSF-expanded bone marrow dendritic cells (C) from C57BL/6 mice were treated with *F. hepatica* exosomes (10 µg/ml) or medium control for 24 h. The concentrations of IL-1RA in the supernatants were determined by ELISA. (D) Spleen cells isolated from C57BL/6 mice were stimulated with for 72 h with anti-CD3ε (1 µg/ml) in the presence or absence of *F. hepatica* exosomes (10 µg/ml) or RPMI-medium control. Concentration of IL-1RA was determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by Student's t-test v medium control or two-way ANOVA with Tukey's honestly significant difference (HSD) post hoc test. *P<0.05, ** P<0.01, ***P<0.001

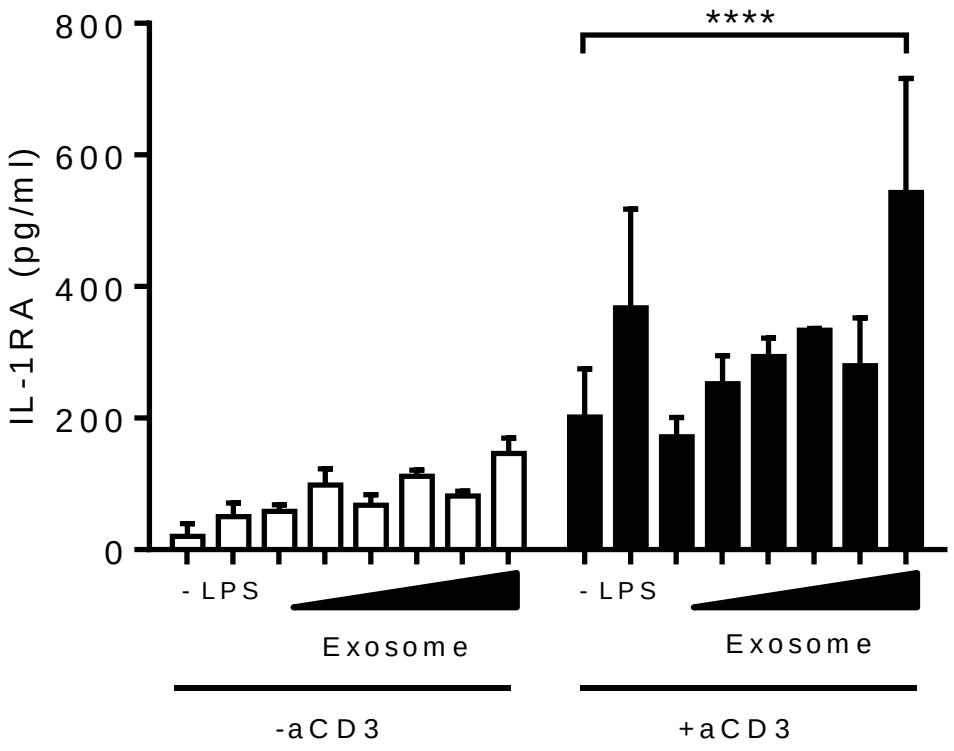


Figure 3.2: Dose response of *F. hepatica* exosome-induced IL-1RA production by spleen cells.

Spleen cells isolated from C57BL/6 mice were stimulated for 72h +/- anti-CD3ε (1 µg/ml) in the presence of *F. hepatica* exosomes (0.75, 1.5, 2.5, 3, 5, 10 µg/ml), LPS (100 ng/ml) or medium control. The concentrations of IL-1RA in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test. *P<0.05, ****P<0.0001.

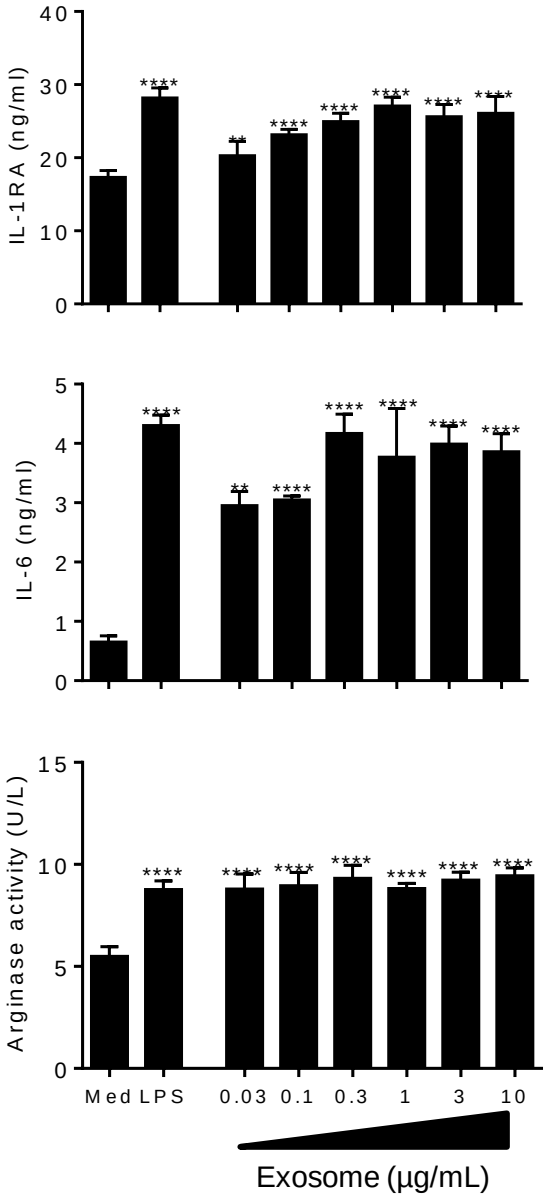


Figure 3.3: Dose response of *F. hepatica* exosome-induced IL-1RA and IL-6 production and arginase activity in peritoneal exudate cells.

Peritoneal exudate cells isolated from C57BL/6 mice were treated for 72h with *F. hepatica* exosomes (0.03, 0.1, 0.3, 1, 3, 10 µg/ml), LPS (100 ng/ml), or medium control. The concentrations of IL-1RA and IL-6 in the supernatants were determined by ELISA. Arginase activity was determined using an Arginase Activity Assay Kit from Sigma. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by one-way ANOVA with Dunnett's post hoc test vs. medium control. ** P<0.01, **** P<0.0001 vs medium control.

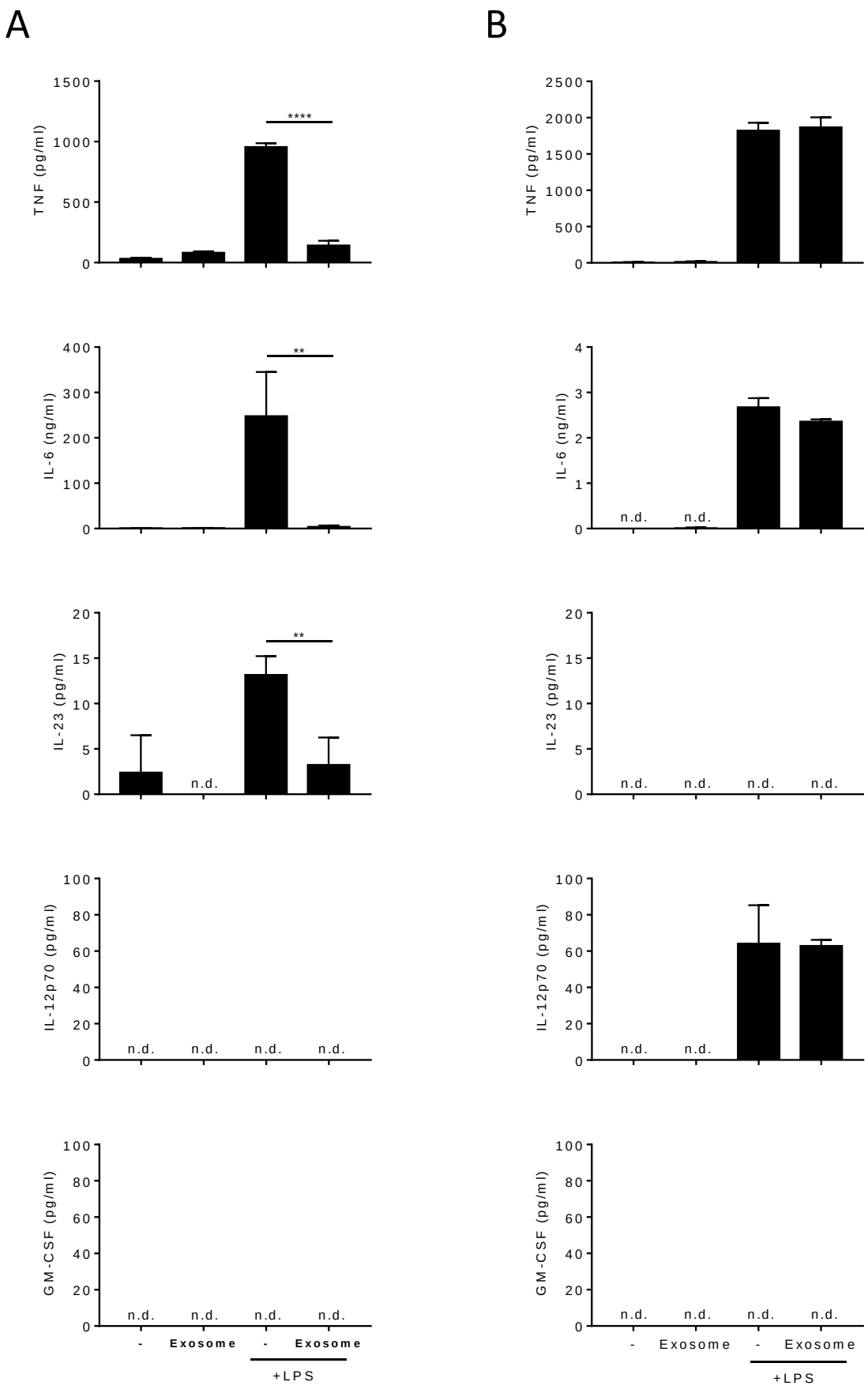

A
B
TNF (pg/ml)
IL-6 (ng/ml)
IL-23 (pg/ml)
IL-12p70 (pg/ml)
GM-CSF (pg/ml)
****
**
**
n.d.
- Exosome - Exosome
+LPS

Figure 3.4: *F. hepatica* exosomes suppress LPS-induced pro-inflammatory cytokine production in peritoneal exudate cells but not in M-CSF-expanded bone marrow macrophages.

Peritoneal exudate cells (A) and M-CSF-expanded bone marrow macrophages (B) generated from C57BL/6 mice were treated for 24h with *F. hepatica* exosomes (10 µg/ml) or medium control +/- LPS (100 ng/mL). The concentrations of TNF, IL-6, IL-23, IL-12p70, and GM-CSF in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by Student's t-test vs. medium control. ** P<0.01, **** P<0.0001. n.d. = not detected.

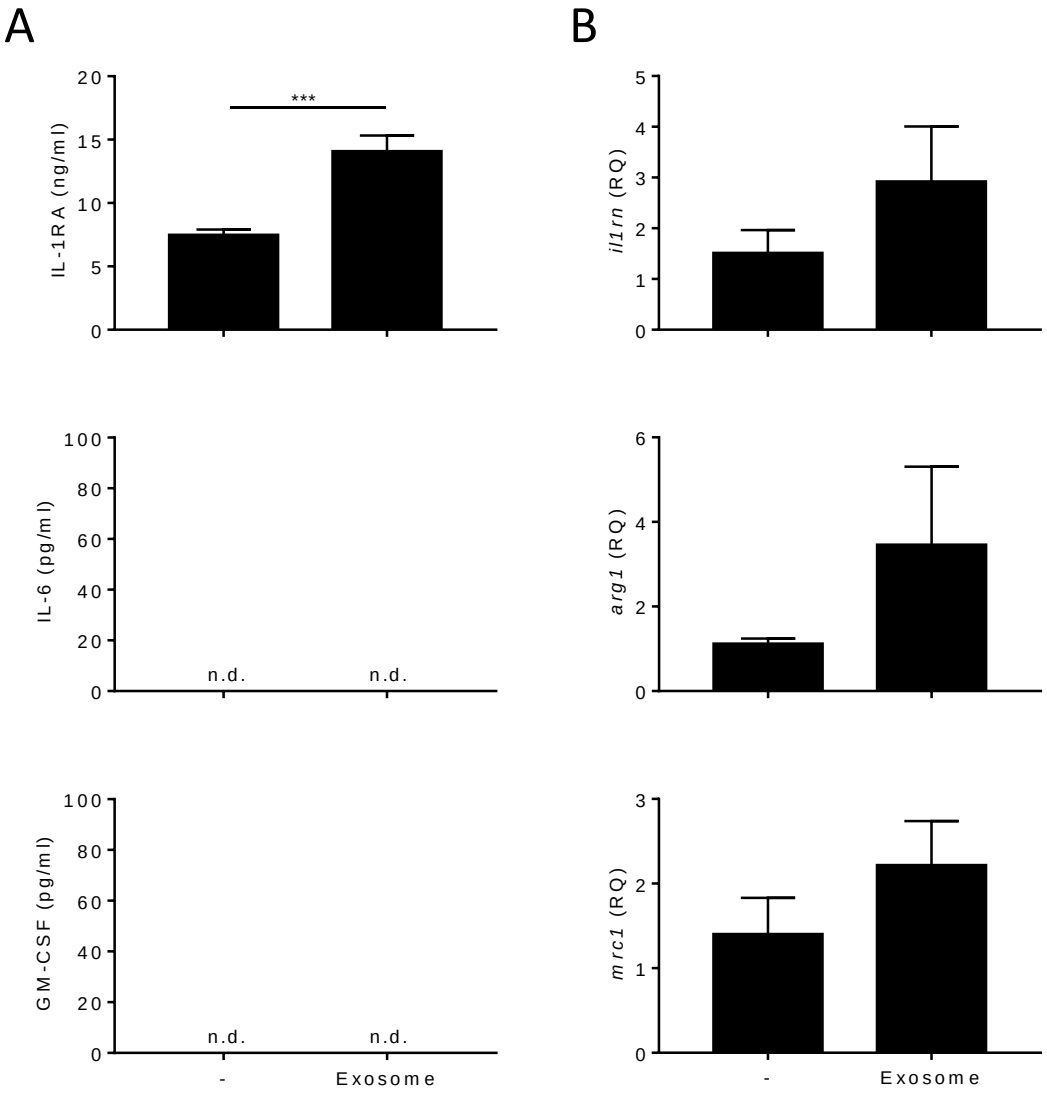


Figure 3.5: *F. hepatica* exosomes prepared using the Exospin kit induce anti-inflammatory cytokine production and gene expression in M-CSF-expanded bone marrow macrophages.

M-CSF-expanded bone marrow macrophages generated from C57BL/6 mice were treated for 24h with *F. hepatica* exosomes prepared using the Exospin kit (10 µg/ml) or medium control. The concentrations of IL-1RA, IL-6, and GM-CSF in the supernatants were determined by ELISA (A). The expression levels of IL-1RN, Arg1, and Mrc1 were determined by rtPCR (B). Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by Student's t-test vs. medium control. *** P<0.001.

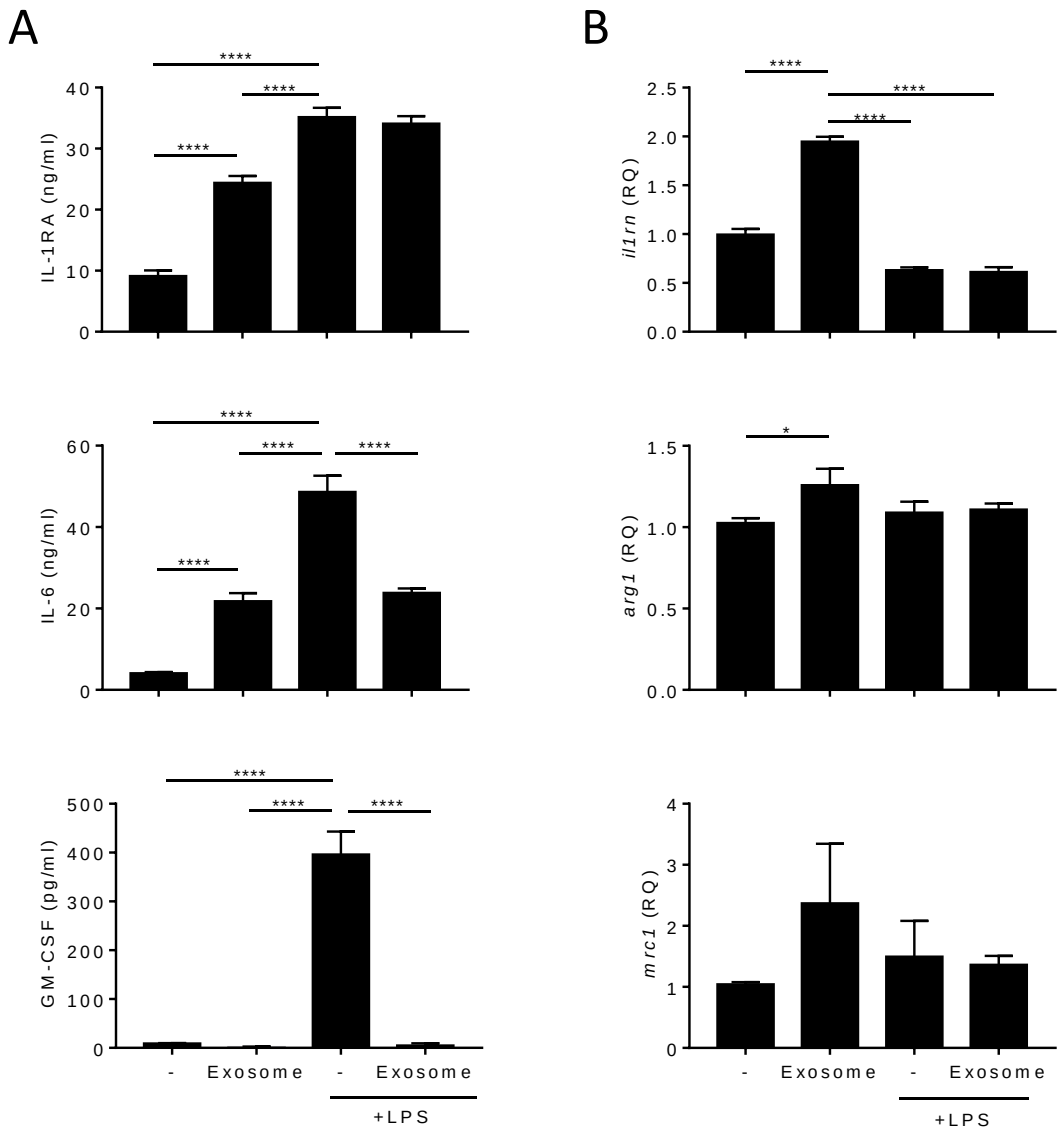


Figure 3.6: *F. hepatica* exosomes prepared using the Exospin kit inhibit LPS-induced pro-inflammatory cytokine production in peritoneal exudate cells.

Peritoneal exudate cells isolated from C57BL/6 mice were treated for 24h with *F. hepatica* exosomes prepared using the Exospin kit (10 µg/ml) or medium control +/- LPS (100 ng/mL). The concentrations of IL-1RA, IL-6, and GM-CSF in the supernatants were determined by ELISA (A). The expression levels of IL-1RN, Arg1, and Mrc1 were determined by rtPCR (B). Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test. * P<0.05, **** P<0.0001.

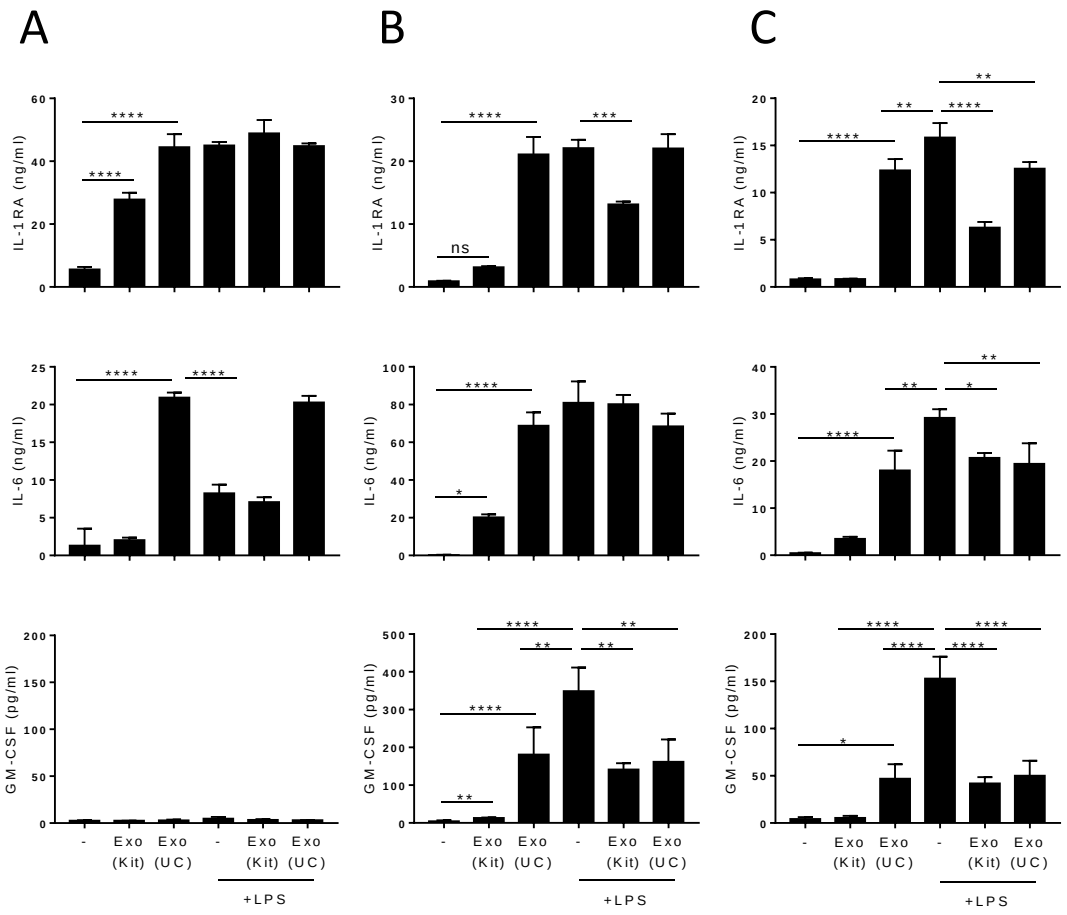


Figure 3.7: Comparison of the effects of two different *F. hepatica* exosome purification methods.

M-CSF-expanded bone marrow macrophages (A), peritoneal exudate cells (B), and peritoneal exudate cells macrophages (C) isolated from C57BL/6 mice were treated for 24h with two different exosome preparations (Ex(Kit) or Ex(UC); 10 µg/ml) or medium control +/- LPS (100 ng/ml). The concentrations of IL-1RA, IL-6, and GM-CSF in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test. *P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001. Ex(Kit) – exosomes prepared using the Exospin Exosome Purification Kit from Cell Guidance Systems; Ex(UC) – exosomes prepared from the ultracentrifugation pellet fraction.

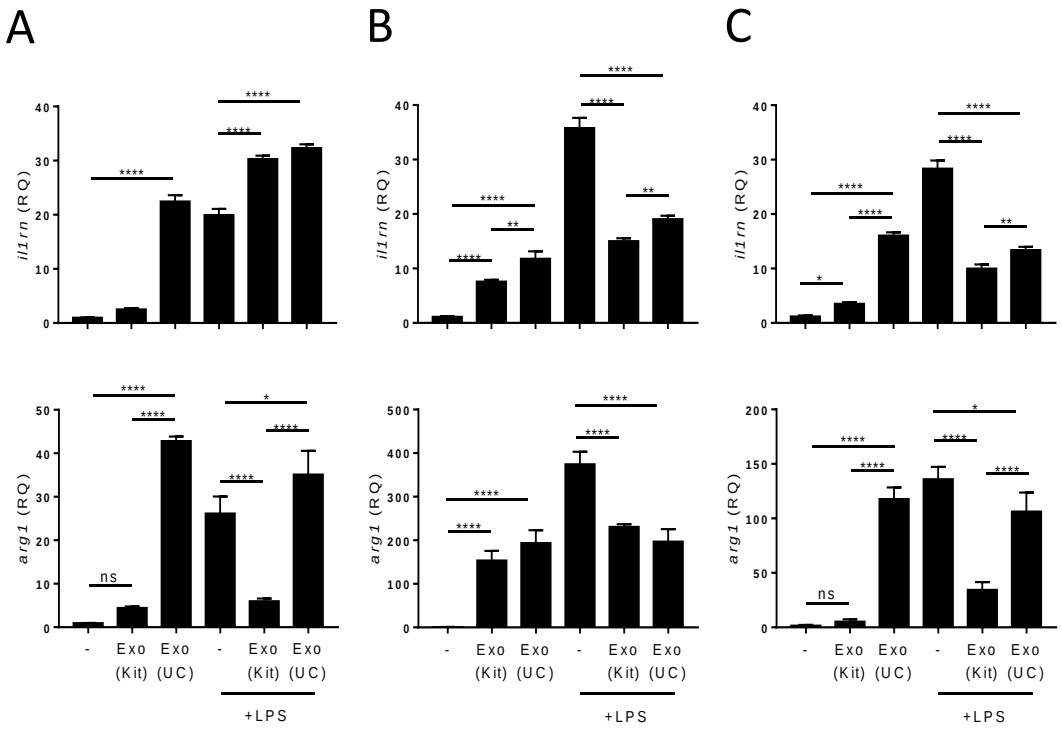


Figure 3.8: Comparison of the effects of two different *F. hepatica* exosome purification methods.

M-CSF-expanded bone marrow macrophages (A), peritoneal exudate cells (B), and peritoneal exudate cells macrophages (C) isolated from C57BL/6 mice were treated for 24h with two different exosome preparations (Ex(Kit) or Ex(UC); 10 µg/ml) or medium control +/- LPS (100 ng/ml). The expression levels of IL-1RN and Arg1 were determined by rtPCR. Data presented are means +/- SEM of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test. *P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Ex(Kit) – exosomes prepared using the Exospin Exosome Purification Kit from Cell Guidance Systems; Ex(UC) – exosomes prepared from the ultracentrifugation pellet fraction

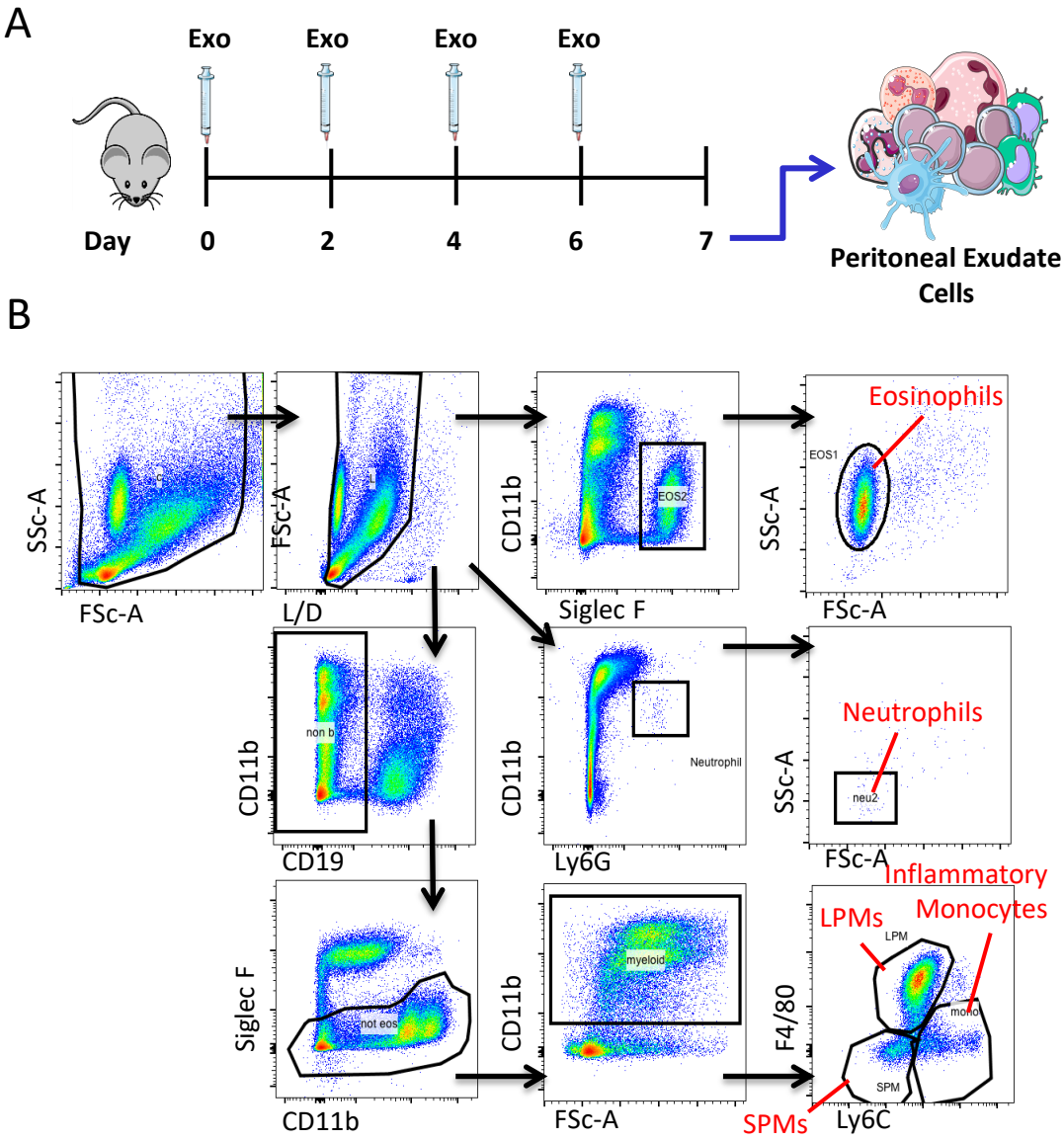


Figure 3.9: Experimental design and flow cytometric gating strategy to examine the *in vivo* activity of *F. hepatica* exosomes.

(A) C57BL/6 mice were injected i.p. with *F. hepatica* exosomes (2 µg/mouse/day) or PBS on days 0, 2, 4, and 6 (n = 5). On day 7, peritoneal exudate cells were isolated for flow cytometry and rtPCR analysis. (B) Gating strategy to identify eosinophils, neutrophils, LPMs, SPMs, and inflammatory monocytes.

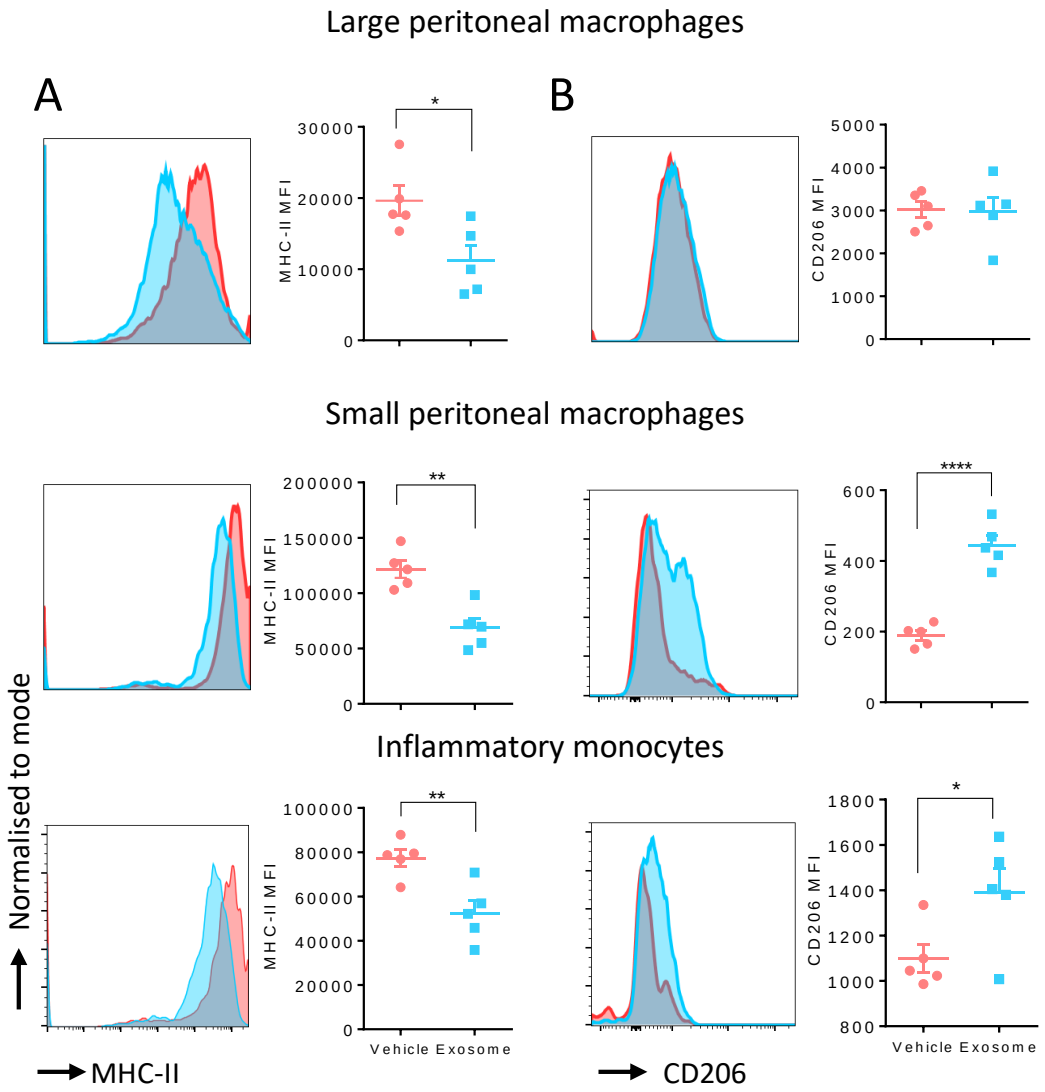


Figure 3.10: *F. hepatica* exosomes promote the alternate activation of macrophages.

C57BL/6 mice injected with *F. hepatica* exosomes or PBS (Figure 3.9). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Large and small peritoneal macrophages and inflammatory monocytes were identified and the surface expression levels of MHC-II (A) and CD206 (B) were examined using histogram plots. Data from the mice is presented as means +/- SEM (n=5). Statistical assessment was performed by Student's t-test vs. vehicle control. * P<0.05, ** P<0.01, **** P<0.0001.

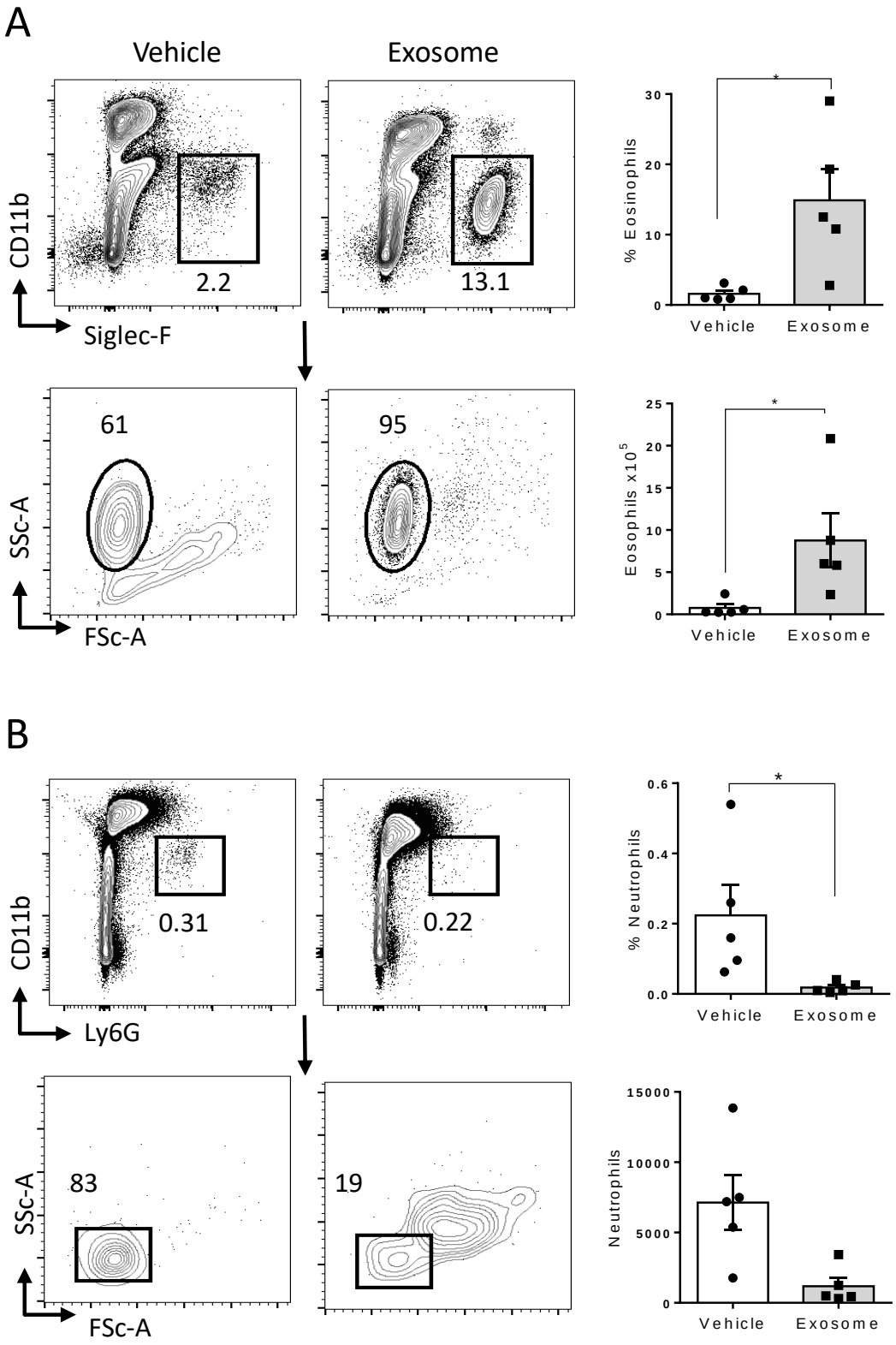

A
Vehicle
Exosome
CD11b
Siglec-F
2.2
13.1
% Eosinophils
Vehicle
Exosome
61
95
SSc-A
FSc-A
Eosophils x10^5
B
0.31
0.22
Ly6G
% Neutrophils
83
19
Neutrophils

Figure 3.11: I.p. injection of mice with *F. hepatica* exosomes induces eosinophil recruitment and reduces neutrophil numbers in the peritoneal cavity.

C57BL/6 mice injected with *F. hepatica* exosomes or PBS (Figure 3.9). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile (A). Neutrophils were identified by the expression of the surface marker Ly6-G and confirmed by their low SSc-A and FSc-A profile (B). Data from the mice is presented as means +/- SEM (n=5). Statistical assessment was performed by Student's t-test vs. vehicle control. * $P<0.05$.

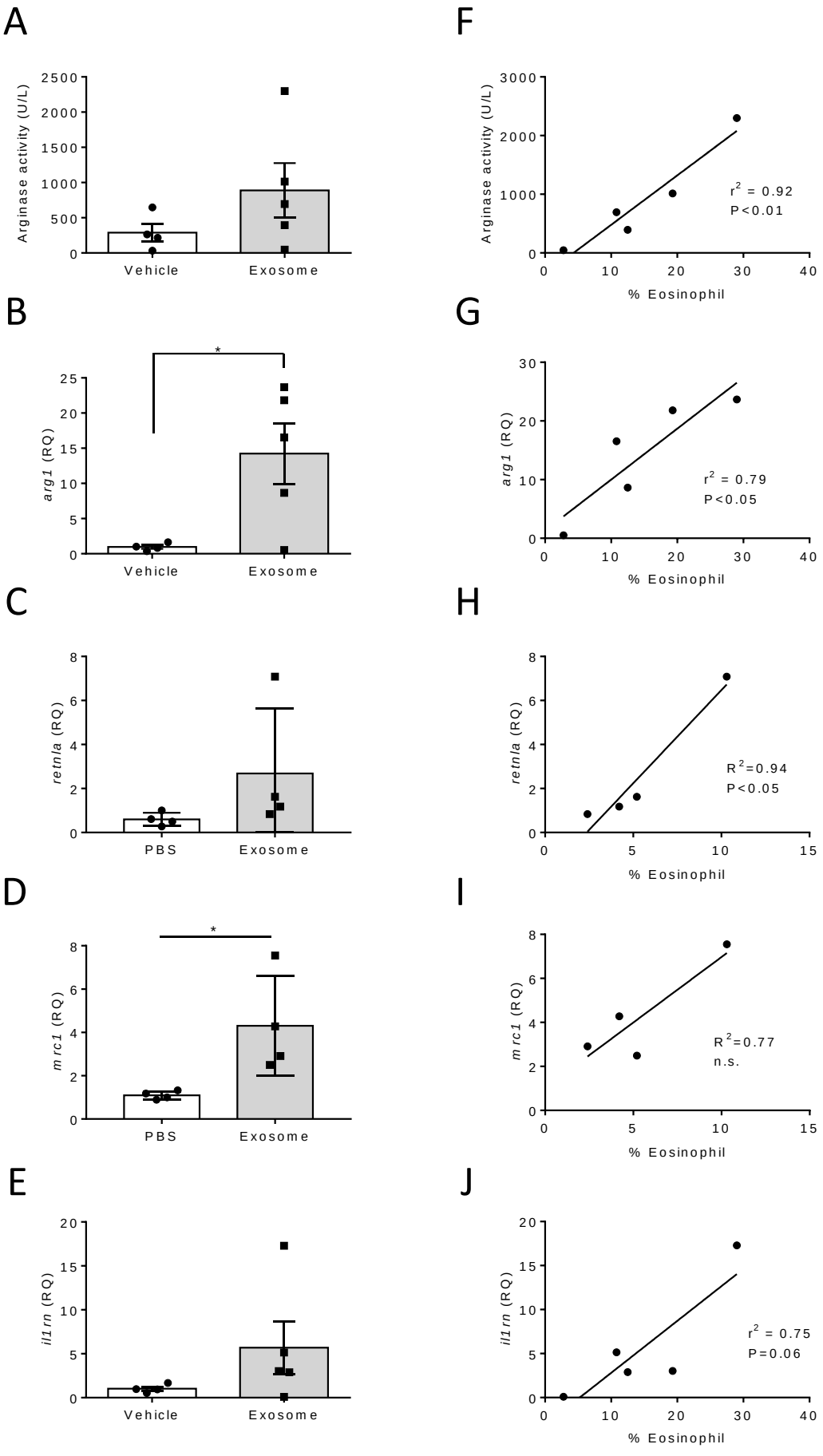

A
Arginase activity (U/L)
2500
2000
1500
1000
500
0
Vehicle
Exosome
F
Arginase activity (U/L)
3000
2000
1000
0
r2 = 0.92
P<0.01
0
10
20
30
40
% Eosinophil
B
*
arg1 (RQ)
25
20
15
10
5
0
Vehicle
Exosome
G
arg1 (RQ)
30
20
10
0
r2 = 0.79
P<0.05
0
10
20
30
40
% Eosinophil
C
retnla (RQ)
8
6
4
2
0
PBS
Exosome
H
retnla (RQ)
8
6
4
2
0
R2=0.94
P<0.05
0
5
10
15
% Eosinophil
D
*
mrc1 (RQ)
8
6
4
2
0
PBS
Exosome
I
mrc1 (RQ)
8
6
4
2
0
R2=0.77
n.s.
0
5
10
15
% Eosinophil
E
il1rn (RQ)
20
15
10
5
0
Vehicle
Exosome
J
il1rn (RQ)
20
15
10
5
0
r2 = 0.75
P=0.06
0
10
20
30
40
% Eosinophil

Figure 3.12: *F. hepatica* exosomes induce the alternative activation of macrophages and enhance IL-1RA expression in peritoneal exudate cells, an effect that correlates with eosinophils recruited.

C57BL/6 mice injected with *F. hepatica* exosomes or PBS (Figure 3.9). Peritoneal exudate cells were isolated. Arginase activity was determined using an Arginase Activity Assay Kit from Sigma (A). Alternatively, RNA was isolated for cDNA synthesis and the expression levels of Arg1, Retnla, Mrc1, and IL-1RN were determined by rtPCR (B-E). Correlation plots between the percentage of eosinophils recruited (Figure 3.11A) and arginase activity (F), Arg1 (G), Retnla (H), Mrc1 (I), and IL-1RN (J) expression for the exosome-treated group were produced. Data from the mice is presented as means +/- SEM (n=5). Statistical assessment was performed by Student's t-test vs. vehicle control. * $P<0.05$. Curves were fitted by linear regression using GraphPad Prism 7.00.

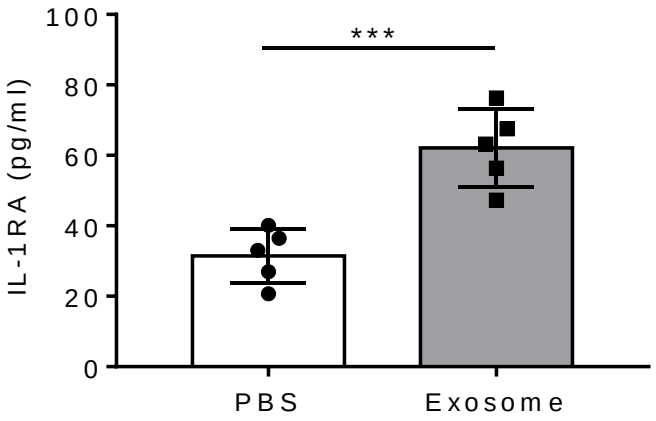
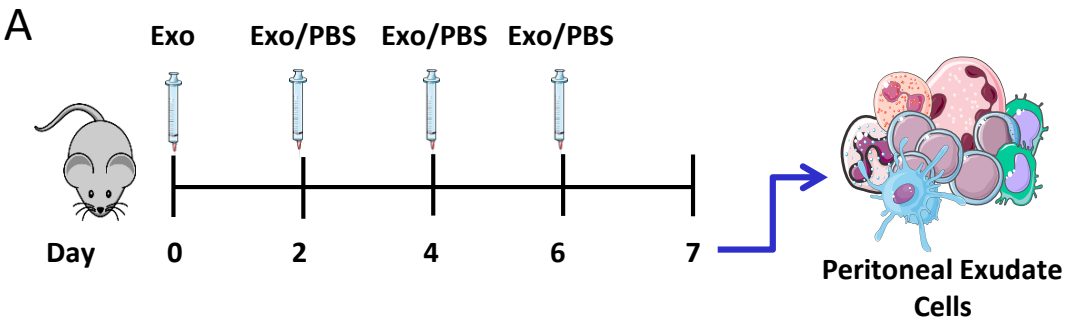


Figure 3.13: *F. hepatica* exosomes induce IL-1RA production *in vivo.*

C57BL/6 mice were injected with *F. hepatica* exosomes or PBS (Figure 3.9). Peritoneal exudate cells were isolated and pelleted by centrifugation. The concentration of IL-1RA in the supernatants ('PEC fluid') was determined by ELISA. Data from the mice is presented as means +/- SEM (n=5). Statistical assessment was performed by Student's t-test vs. vehicle control. *** P<0.001.


A
Exo
Exo/PBS
Exo/PBS
Exo/PBS
Day
0
2
4
6
7
Peritoneal Exudate Cells

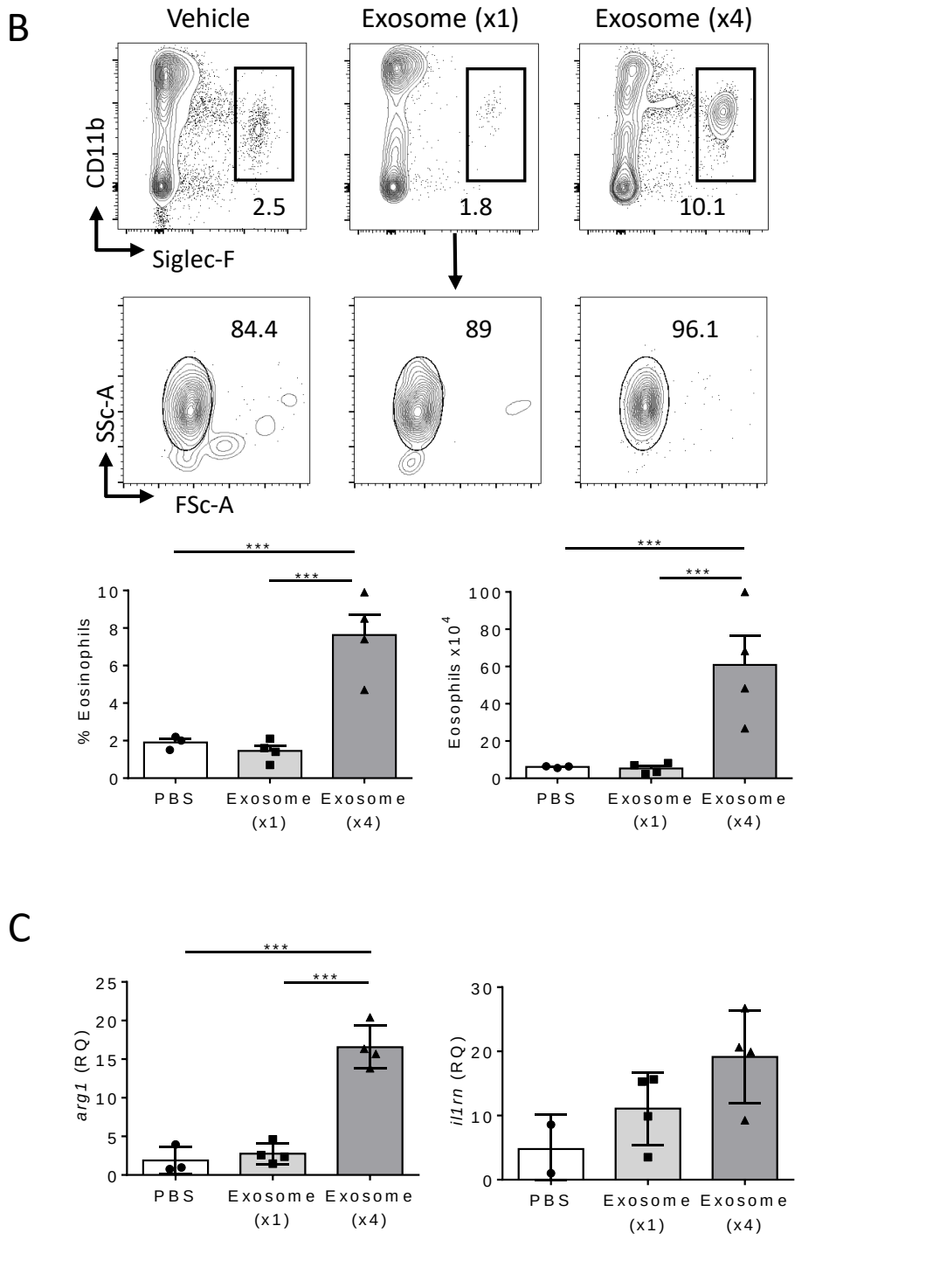
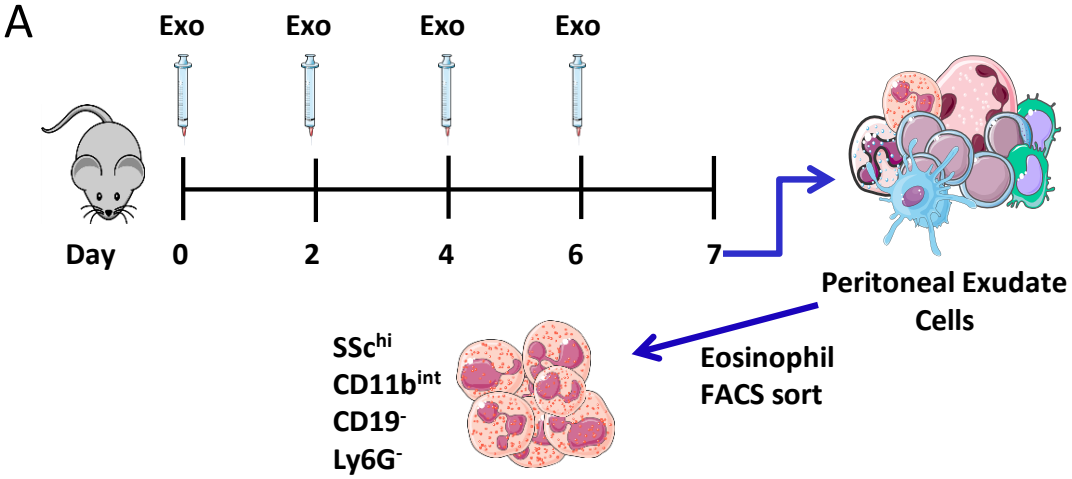
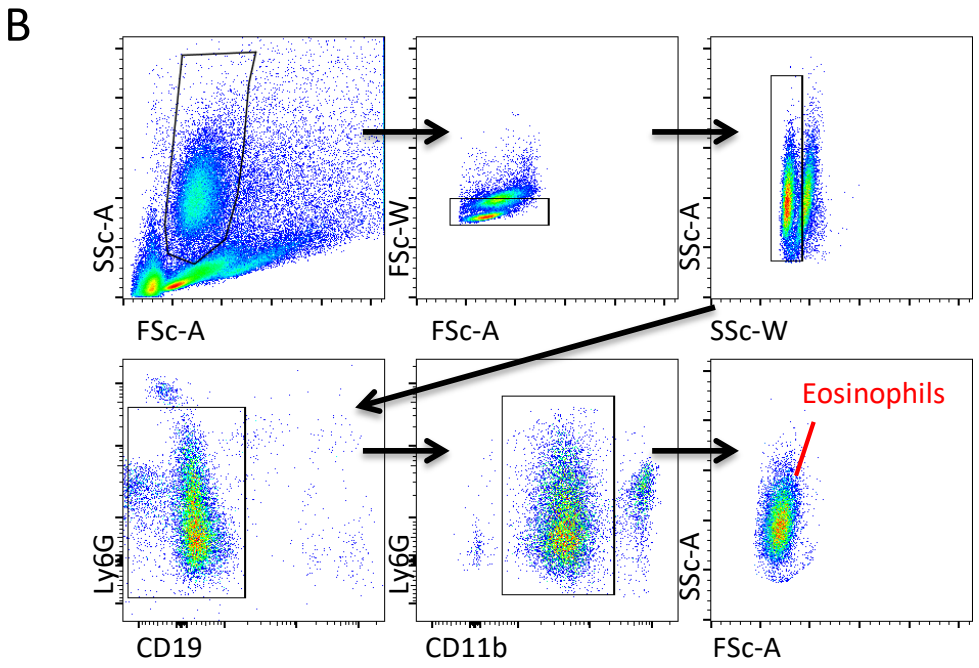

B
Vehicle
Exosome (x1)
Exosome (x4)
CD11b
Siglec-F
2.5
1.8
10.1
SSc-A
FSc-A
84.4
89
96.1
% Eosinophils
Eosophils x10^4
PBS
Exosome (x1)
Exosome (x4)
C
arg1 (RQ)
il1rn (RQ)

Figure 3.14: Multiple i.p. injections of mice with *F. hepatica* exosomes are required to drive eosinophil recruitment and anti-inflammatory effects.

C57BL/6 mice were injected i.p. with either *F. hepatica* exosomes (2 µg/mouse/day) on day 0 and PBS on days 2, 4, and 6 (Exosome x1), *F. hepatica* exosomes (2 µg/mouse/day) on days 0, 2, 4, and 6 (Exosome x4 or PBS (n = 3-4). On day 7, peritoneal exudate cells were isolated for flow cytometry and rtPCR analysis (A). Eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile (B). RNA was isolated from the peritoneal exudate cells for cDNA synthesis. The expression levels of Arg1 and IL-1RN were determined by rtPCR (C). Data from the mice is presented as means +/- SEM (n=3-4). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test. *** P<0.001.


A
Exo
Exo
Exo
Exo
Day
0
2
4
6
7
Peritoneal Exudate Cells
Eosinophil FACS sort
SSc^hi
CD11b^int
CD19^-
Ly6G^-


B
SSc-A
FSc-A
FSc-W
FSc-A
SSc-A
SSc-W
Eosinophils
Ly6G
CD19
Ly6G
CD11b
SSc-A
FSc-A

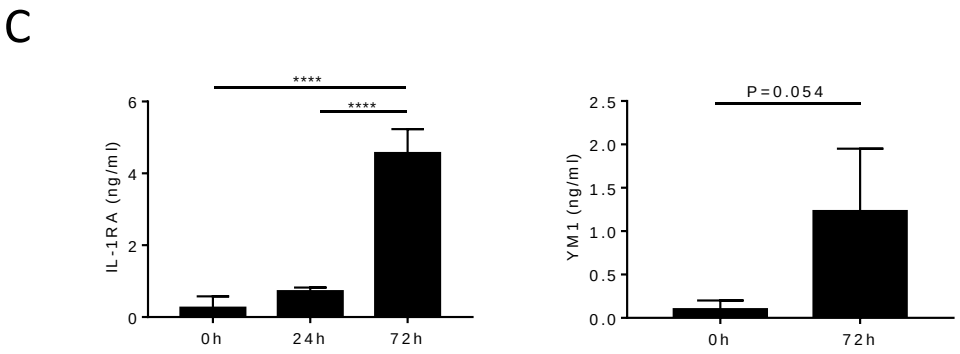

C
IL-1RA (ng/ml)
****
****
0h
24h
72h
YM1 (ng/ml)
P=0.054
0h
72h

Figure 3.15: Eosinophils induced by *F. hepatica* exosomes injection produce IL-1RA.

(A) C57BL/6 mice were injected i.p. with *F. hepatica* exosomes *(*2 µg/mouse/day) on days 0, 2, 4, and 6 (n = 3). On day 7, peritoneal exudate cells were isolated, and eosinophils were FACS sorted (Ssc^{hi} $CD11b^{int}$ $CD19^{-}$ $Ly6G^{-}$ cells). (B) Gating strategy to purify eosinophils. (C) FACSs sorted eosinophils ($2x10^{5}$ cells/well) were incubated at 37°C for 0, 24 or 72 h (n=3). The concentration of IL-1RA and YM1 in the supernatants was determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by one-way ANOVA with Dunnett's post hoc test vs. vehicle control ****P<0.0001.

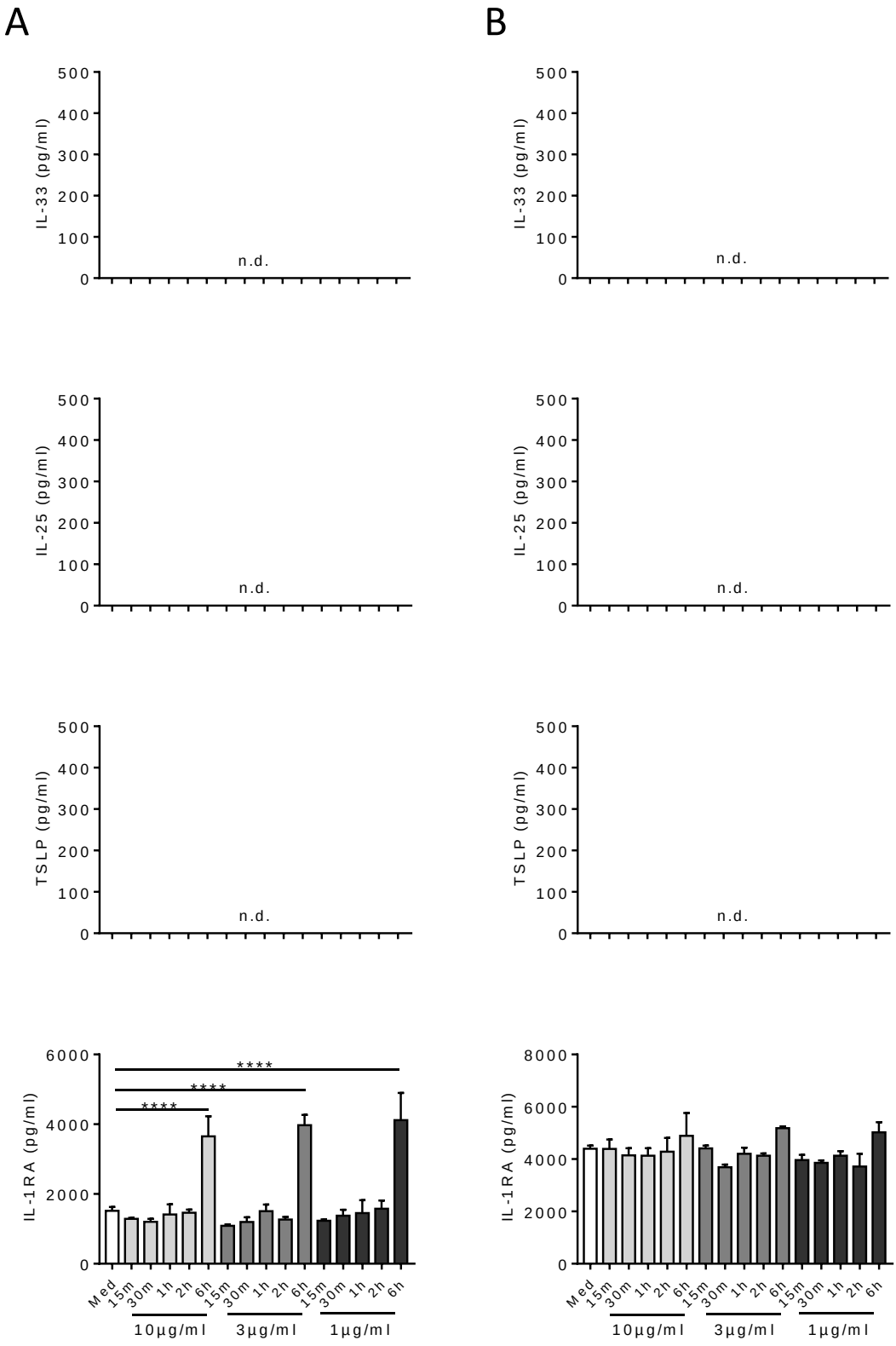

A
B
IL-33 (pg/ml)
IL-25 (pg/ml)
TSLP (pg/ml)
IL-1RA (pg/ml)
n.d.
****
Med
15m
30m
1h
2h
6h
10µg/ml
3µg/ml
1µg/ml

Figure 3.16: Time course of exosomes treatment on M-CSF-expanded bone marrow macrophages and GM-CSF-expanded bone marrow dendritic cells.

M-CSF-expanded bone marrow macrophages (A) and GM-CSF-expanded bone marrow dendritic cells (B) isolated from C57BL/6 mice were treated with *F. hepatica* exosomes (1, 3, and 10 µg/ml) for 15min, 30min, 1h, 2h, and 6h or medium control for 6h. The concentrations of IL-13, IL-25, TSLP, and IL-1RA in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by one-way ANOVA with Dunnett's post hoc test vs. medium control. ****P<0.0001.

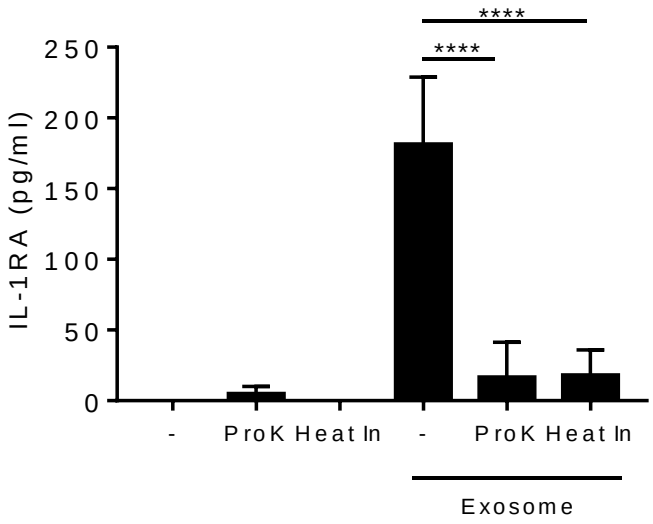
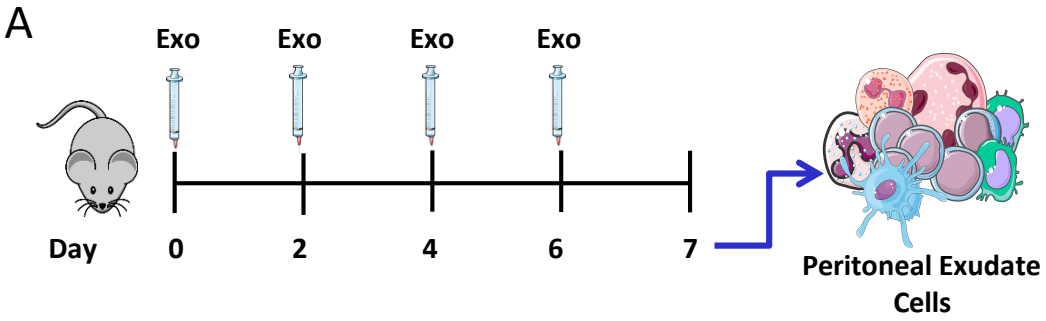


Figure 3.17: *F. hepatica* exosome induction of IL-1RA production is mediated by protein fraction of the exosomes.

M-CSF-expanded bone marrow macrophages from C57BL/6 mice were treated with *F. hepatica* exosomes (control, Proteinase K-treated, or heat inactivated; 10 µg/ml) or medium control for 24 h. The concentrations of IL-1RA in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference (HSD) post hoc test. ****P<0.0001


A
Exo
Exo
Exo
Exo
Day
0
2
4
6
7
Peritoneal Exudate Cells

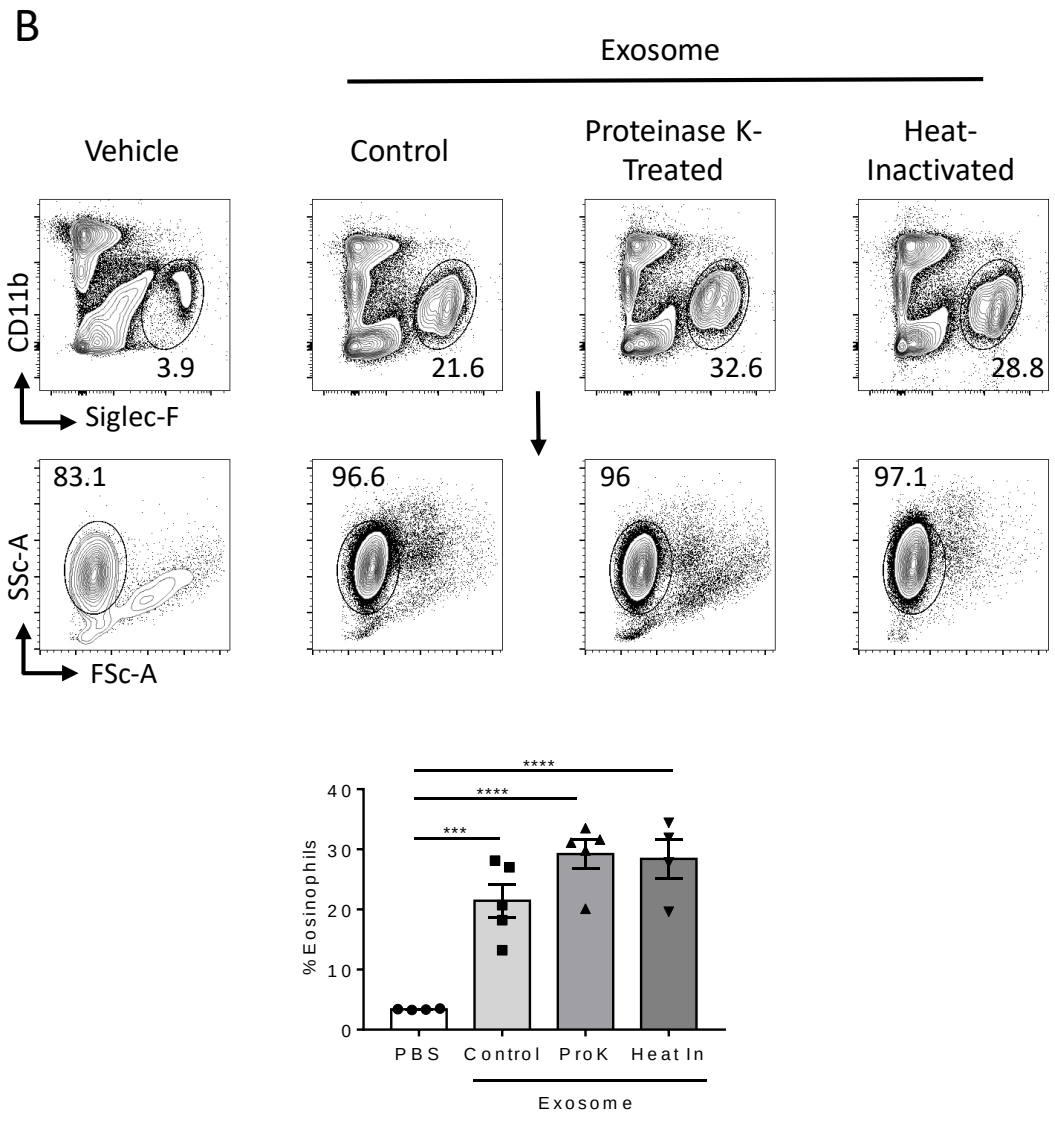

B
Exosome
Vehicle
Control
Proteinase K-Treated
Heat-Inactivated
CD11b
Siglec-F
3.9
21.6
32.6
28.8
SSc-A
FSc-A
83.1
96.6
96
97.1
% Eosinophils
PBS
Control
ProK
Heat In
Exosome
****
****
***

Figure 3.18: Digestion of the protein contents of *F. hepatica* exosomes does not reverse their ability to induce eosinophil recruitment.

C57BL/6 mice injected with i.p. *F. hepatica* exosomes (control, Proteinase K-treated, or heat inactivated; 2 µg/mouse/day) or PBS. Peritoneal exudate cells were isolated and stained for flow cytometry analysis (A). Eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile (B). Data from the mice is presented as means +/- SEM (n=4-5). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test. *** P<0.001, **** P<0.0001.

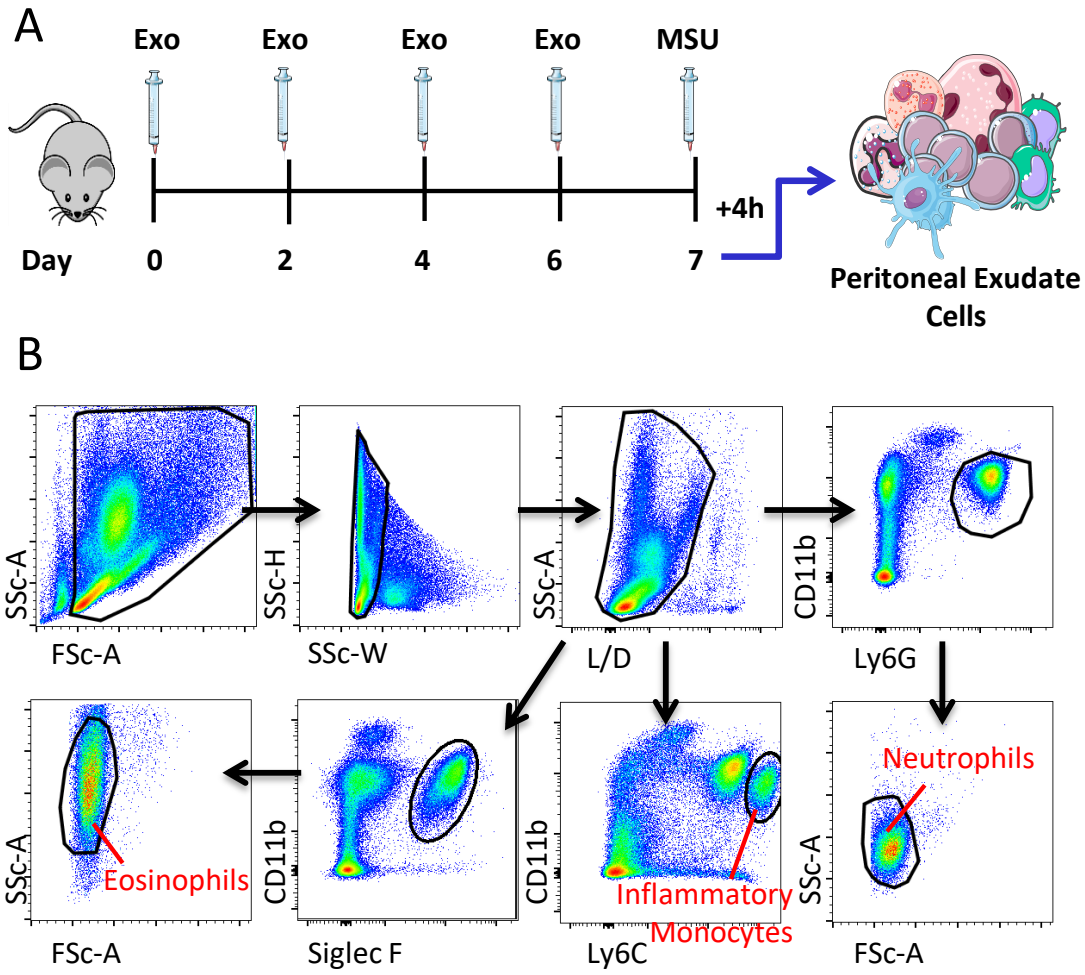


Figure 3.19: Experimental design and flow cytometric gating strategy to examine the ability of *F. hepatica* exosomes to suppress MSU- crystal induced inflammation. (A) C57BL/6 mice were injected i.p. with *F. hepatica* exosomes (2 µg/mouse/day) or PBS on days 0, 2, 4, and 6 (n = 5). On day 7 the mice were injected with MSU crystals (250 µg/mouse). Naïve mice were also injected with PBS as a control (n = 2). Four hours post-MSU crystal/PBS injection, peritoneal exudate cells were isolated for flow cytometry analysis. (B) Gating strategy to identify eosinophils, neutrophils, and inflammatory monocytes.

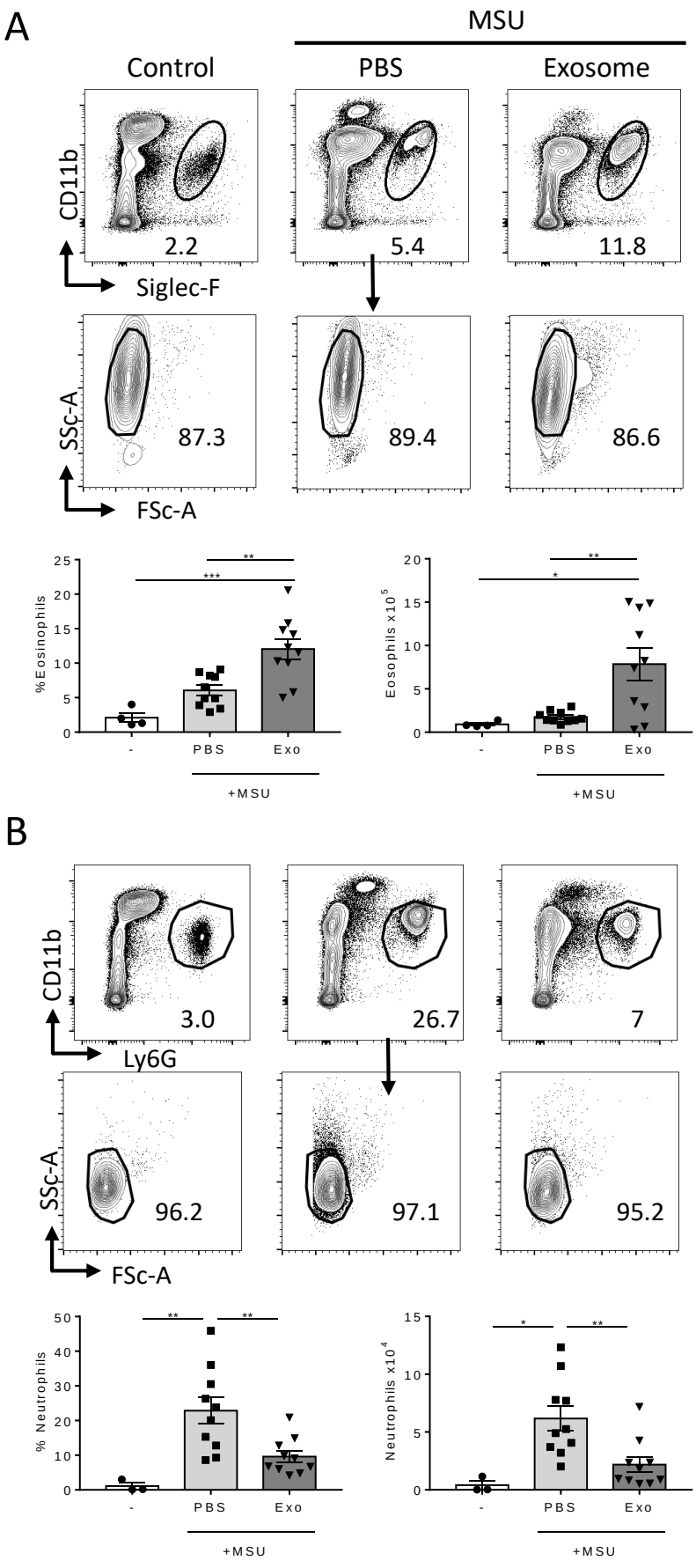

A
MSU
Control
PBS
Exosome
CD11b
Siglec-F
2.2
5.4
11.8
SSc-A
FSc-A
87.3
89.4
86.6
% Eosinophils
Eosophils x10^5
-
PBS
Exo
+MSU
B
CD11b
Ly6G
3.0
26.7
7
SSc-A
FSc-A
96.2
97.1
95.2
% Neutrophils
Neutrophils x10^4

Figure 3.20: I.p. injection of mice with *F. hepatica* exosomes induces eosinophil recruitment and inhibits MSU crystal -induced neutrophil infiltration in the peritoneal cavity.

C57BL/6 mice were injected with *F. hepatica* exosomes or PBS and challenged with MSU crystals (Figure 3.19). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile (A). Neutrophils were identified by the expression of the surface marker Ly6-G and confirmed by their low SSc-A and FSc-A profile (B). Data from the mice is presented as means +/- SEM (n=4-10). Results are combined from 2 separate experiments. Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test.

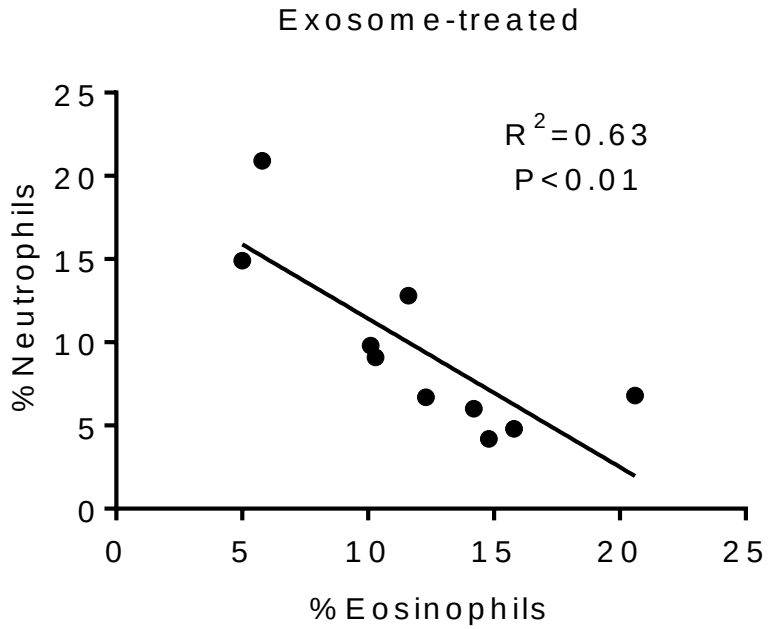


Figure 3.21: The frequency of neutrophils in the peritoneal cavity following MSU crystal injection correlates negatively with the frequency of eosinophils in *F. hepatica* exosome-treated mice.

A correlation plot between the percentage of eosinophils and neutrophils recruited for the exosome-treated group (Figure 3.20) was produced. Results are combined from 2 separate experiments (n=10). Curves were fitted by linear regression using GraphPad Prism 7.00.

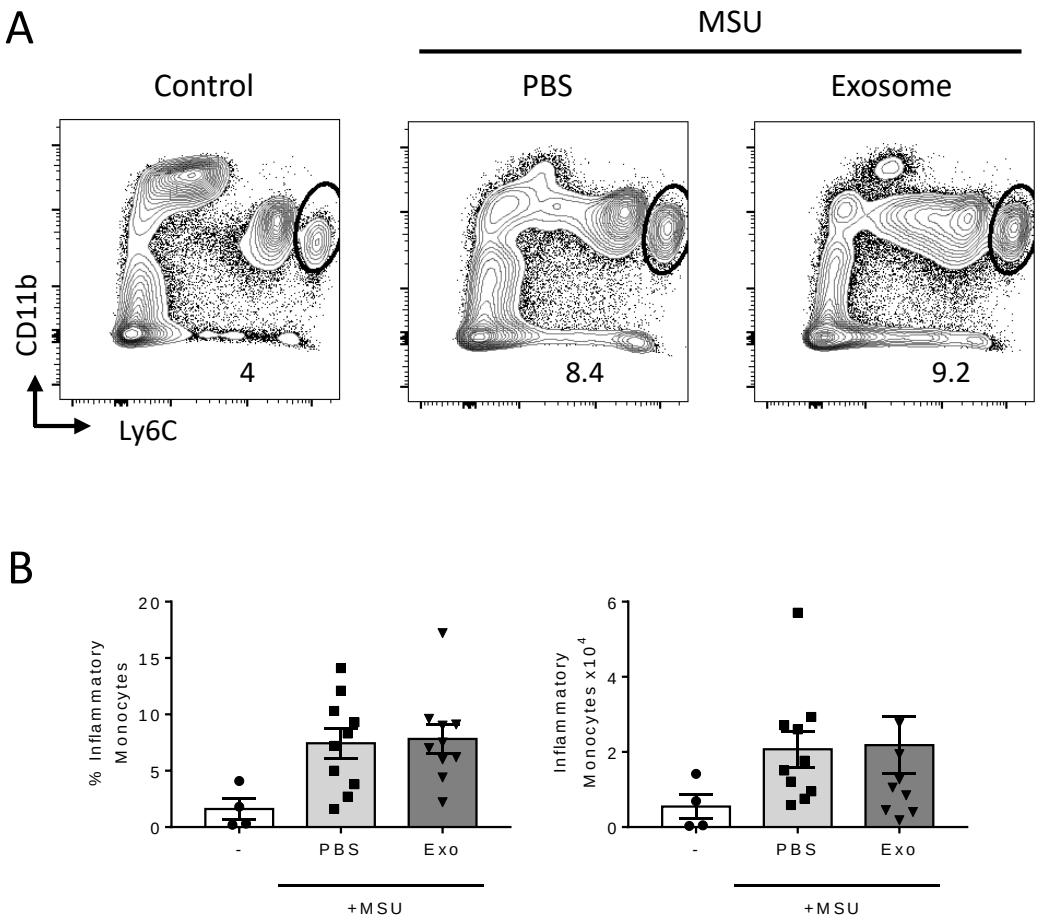


Figure 3.22: I.p. injection of mice with *F. hepatica* exosomes did not affect MSU-crystal-induced inflammatory monocyte recruitment in the peritoneal cavity.

C57BL/6 mice were injected with *F. hepatica* exosomes or PBS and challenged with MSU crystals (Figure 3.19). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Inflammatory monocytes were identified by the very high expression of the surface marker Ly6C (A). Data from the mice is presented as means +/- SEM (n=4-10). Results are combined from 2 separate experiments (B). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test.

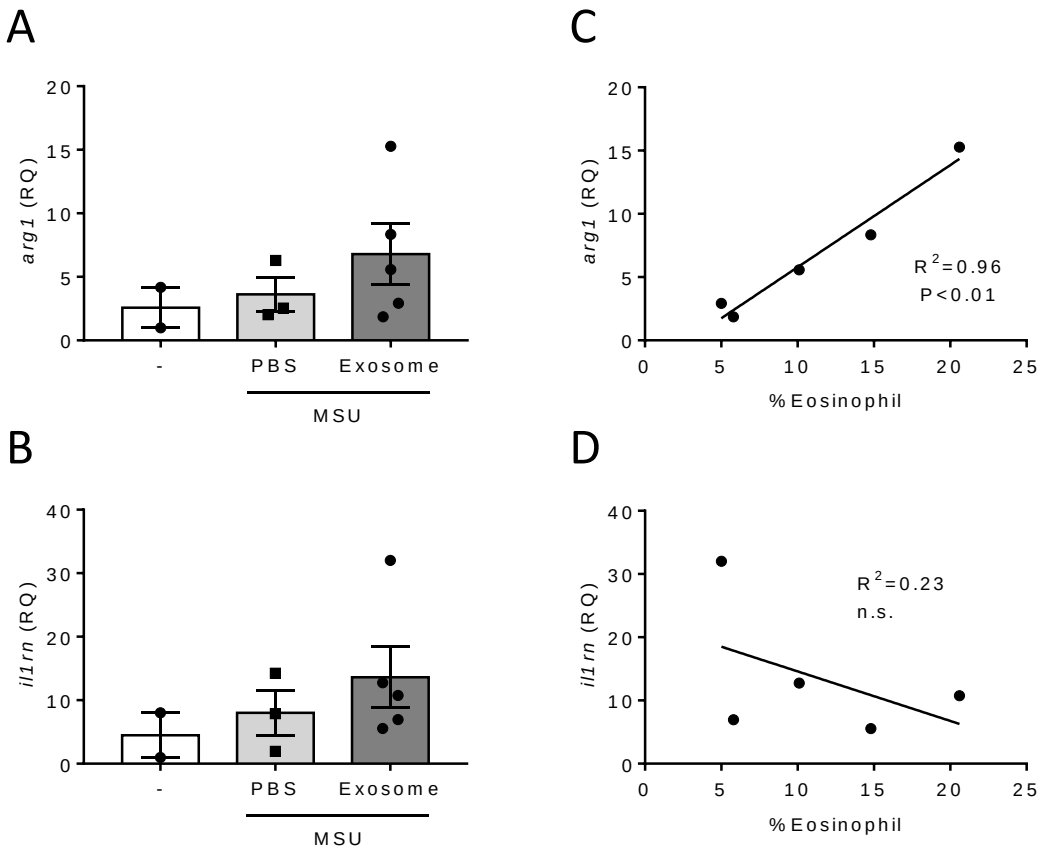


Figure 3.23: I.p. injection of mice with *F. hepatica* exosomes induces the expression of anti-inflammatory markers in peritoneal exudate cells.

C57BL/6 mice were injected with *F. hepatica* exosomes or PBS and challenged with MSU crystals (Figure 3.19). Peritoneal exudate cells were isolated, and RNA was extracted for cDNA synthesis. The expression levels of Arg1 and IL-1RN were determined by rtPCR (A-B). Correlation plots between the percentage of eosinophils recruited (Figure 3.16A) and Arg1 (C) and IL-1RN (D) expression for the exosome-treated group were produced. Data from the mice is presented as means +/- SEM (n=2-5). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test. Curves were fitted by linear regression using GraphPad Prism 7.00.

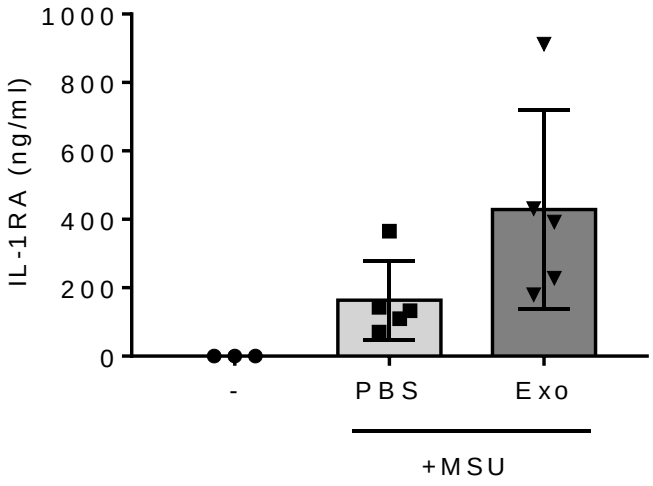


Figure 3.24: *F. hepatica* exosomes induce IL-1RA production *in vivo.*

C57BL/6 mice were injected with *F. hepatica* exosomes or PBS and challenged with MSU crystals (Figure 3.19). Peritoneal exudate cells were isolated and pelleted by centrifugation. The concentration of IL-1RA in the supernatants ('PEC fluid') was determined by ELISA. Data from the mice is presented as means +/- SEM (n=3-5). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test.

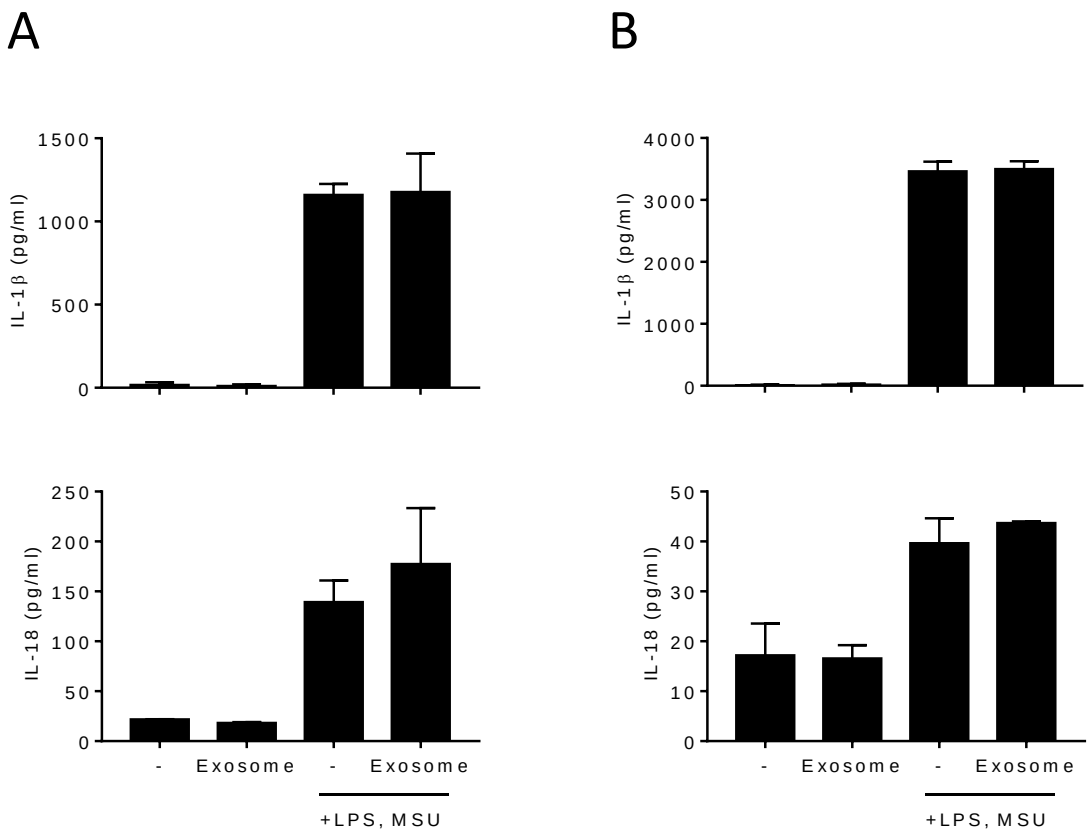


Figure 3.25: *F. hepatica* exosomes do not suppress MSU crystal -induced IL-1β and IL-18 production in macrophages and DCs *in vitro*.

M-CSF-expanded bone marrow macrophages (A) and GM-CSF-expanded bone marrow dendritic cells (B) generated from C57BL/6 mice were treated for 24h with exosomes (10 µg/ml) or medium control +/- LPS (100 ng/mL) and MSU crystals (250 µg/ml). The concentrations of IL-1β, and IL-18 in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test.

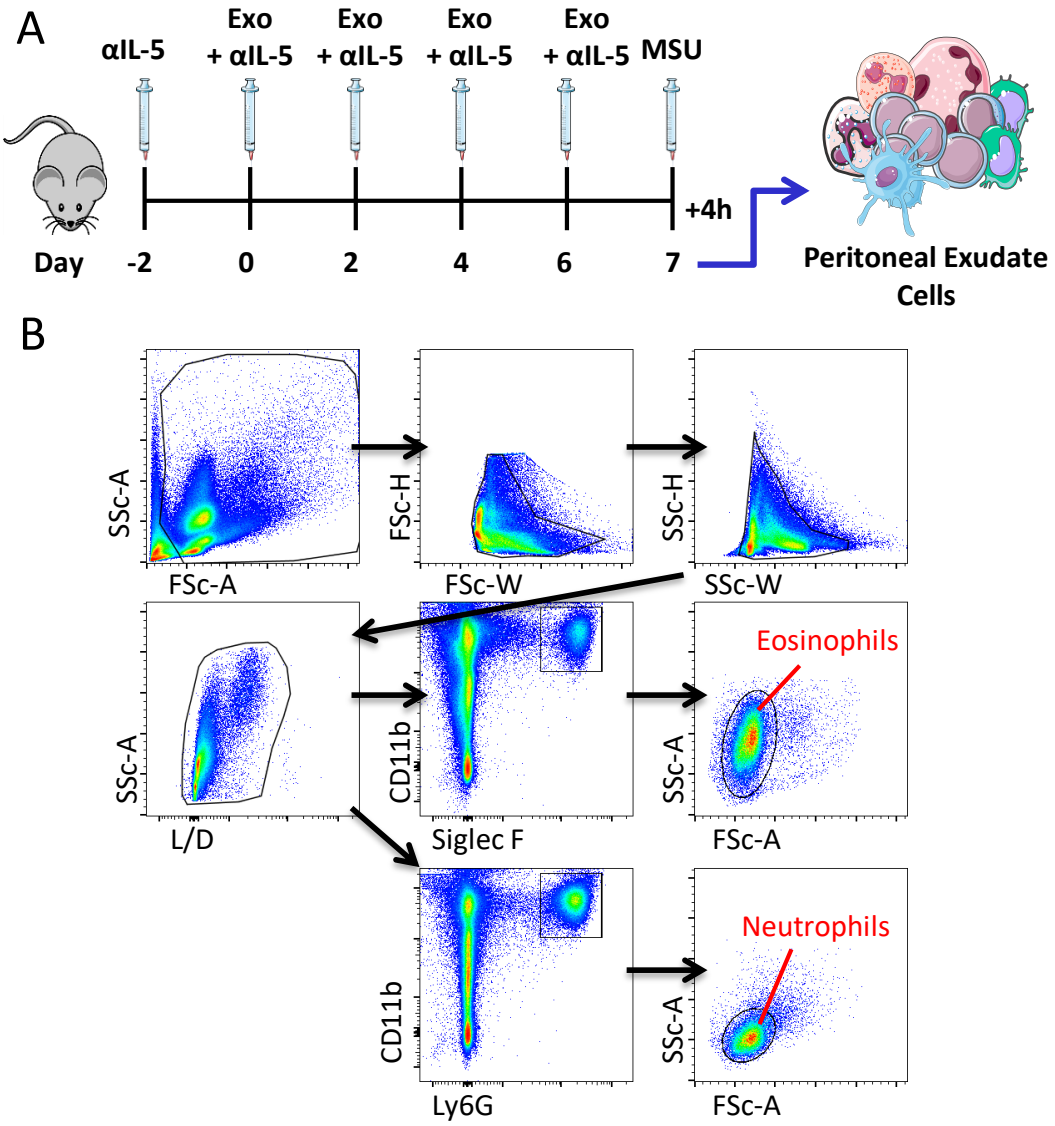


Figure 3.26: Experimental design and flow cytometric gating strategy to examine the ability of *F. hepatica* exosomes to suppress MSU crystal-induced inflammation.

(A) C57BL/6 mice were injected i.p. with PBS or anti-IL-5 (80 µg/mouse/day) on days -2, 0, 2, 4, and 6 and *F. hepatica* exosomes (2.5 µg/mouse/day) or PBS on days 0, 2, 4, and 6 (n=5). On day 7 the mice were injected with MSU crystals (250 µg/mouse). Naïve mice were also injected with PBS as a control (n = 2). Four hours post-MSU crystal/PBS injection, peritoneal exudate cells were isolated for flow cytometry analysis. (B) Gating strategy to identify eosinophils, neutrophils, and inflammatory monocytes.

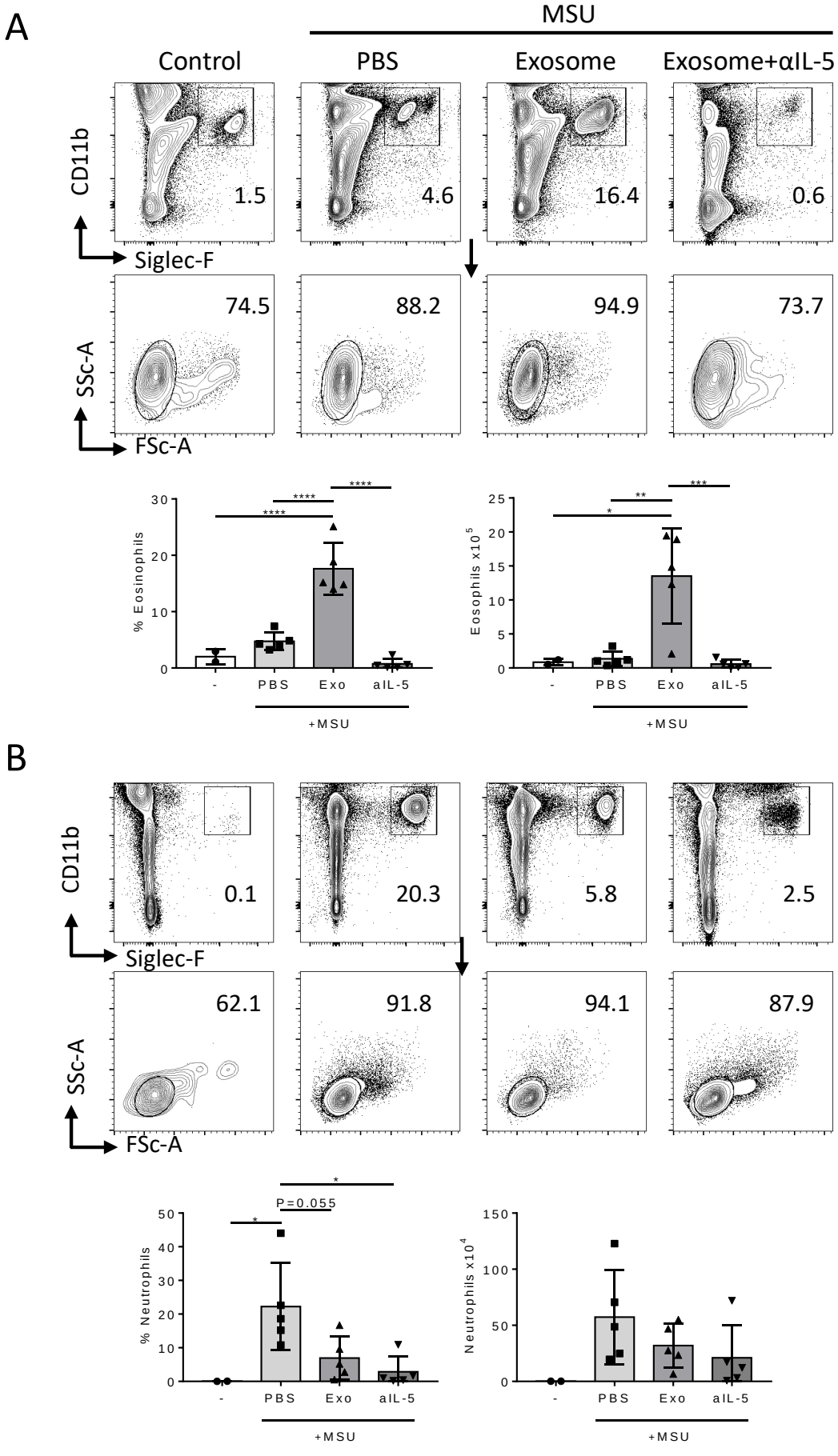

A
MSU
Control
PBS
Exosome
Exosome+αIL-5
CD11b
Siglec-F
1.5
4.6
16.4
0.6
SSc-A
FSc-A
74.5
88.2
94.9
73.7
% Eosinophils
Eosophils x10^5
- PBS Exo aIL-5
+MSU
B
CD11b
Siglec-F
0.1
20.3
5.8
2.5
SSc-A
FSc-A
62.1
91.8
94.1
87.9
P=0.055
% Neutrophils
Neutrophils x10^4
- PBS Exo aIL-5
+MSU

Figure 3.27: Anti-IL-5 suppresses *F. hepatica* exosome-induced eosinophil recruitment but not *F. hepatica* exosome-mediated attenuation of neutrophil infiltration in the peritoneal cavity

C57BL/6 mice were injected with F. *hepatica* exosomes +/- anti-IL-5 or PBS and challenged with MSU crystals (Figure 3.26). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile (A). Neutrophils were identified by the expression of the surface marker Ly6-G and confirmed by their low SSc-A and FSc-A profile (B). Data is presented as means +/- SEM (n=2-5). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

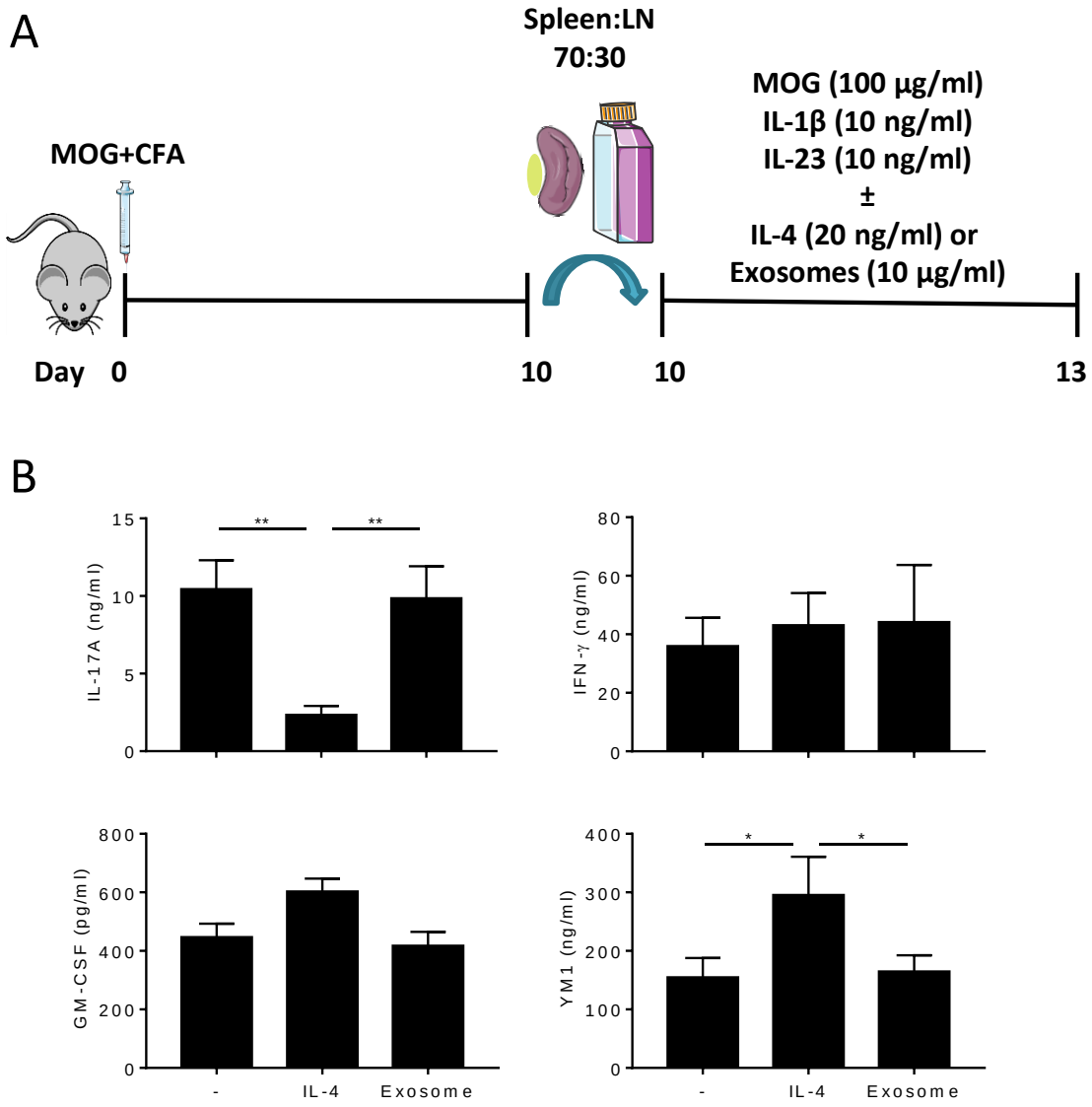
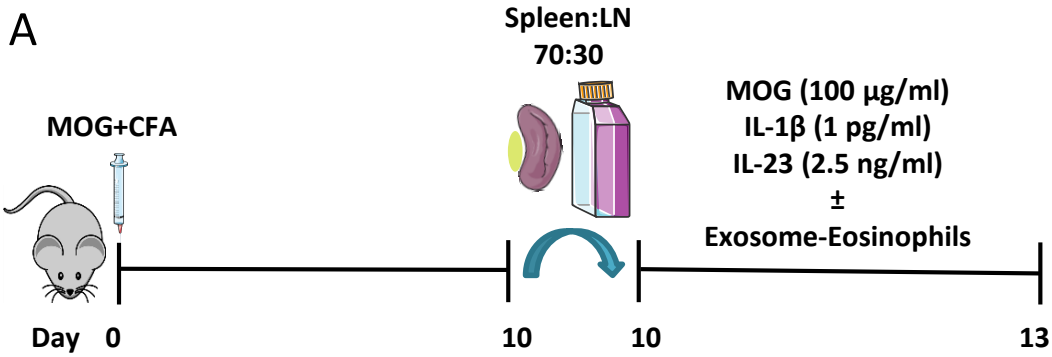


Figure 3.28: IL-4, but not *F. hepatica* exosomes, inhibits IL-17A production by a MOG-specific spleen and LN cells.

(A) Donor mice were injected with MOG and CFA. After 10 days, spleen and LN cells were cultured for 72 h in a 70:30 ratio (Spleen:LN cell) with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10ng/ml) in the presence of medium, IL-4 (20 ng/ml), or *F. hepatica* exosomes (10 µg/ml). (B) The concentrations of IL-17A, IFN-γ, GM-CSF, and YM1 in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test. * P<0.05, ** P<0.01.


A
Spleen:LN
70:30
MOG+CFA
MOG (100 µg/ml)
IL-1β (1 pg/ml)
IL-23 (2.5 ng/ml)
±
Exosome-Eosinophils
Day 0
10
10
13

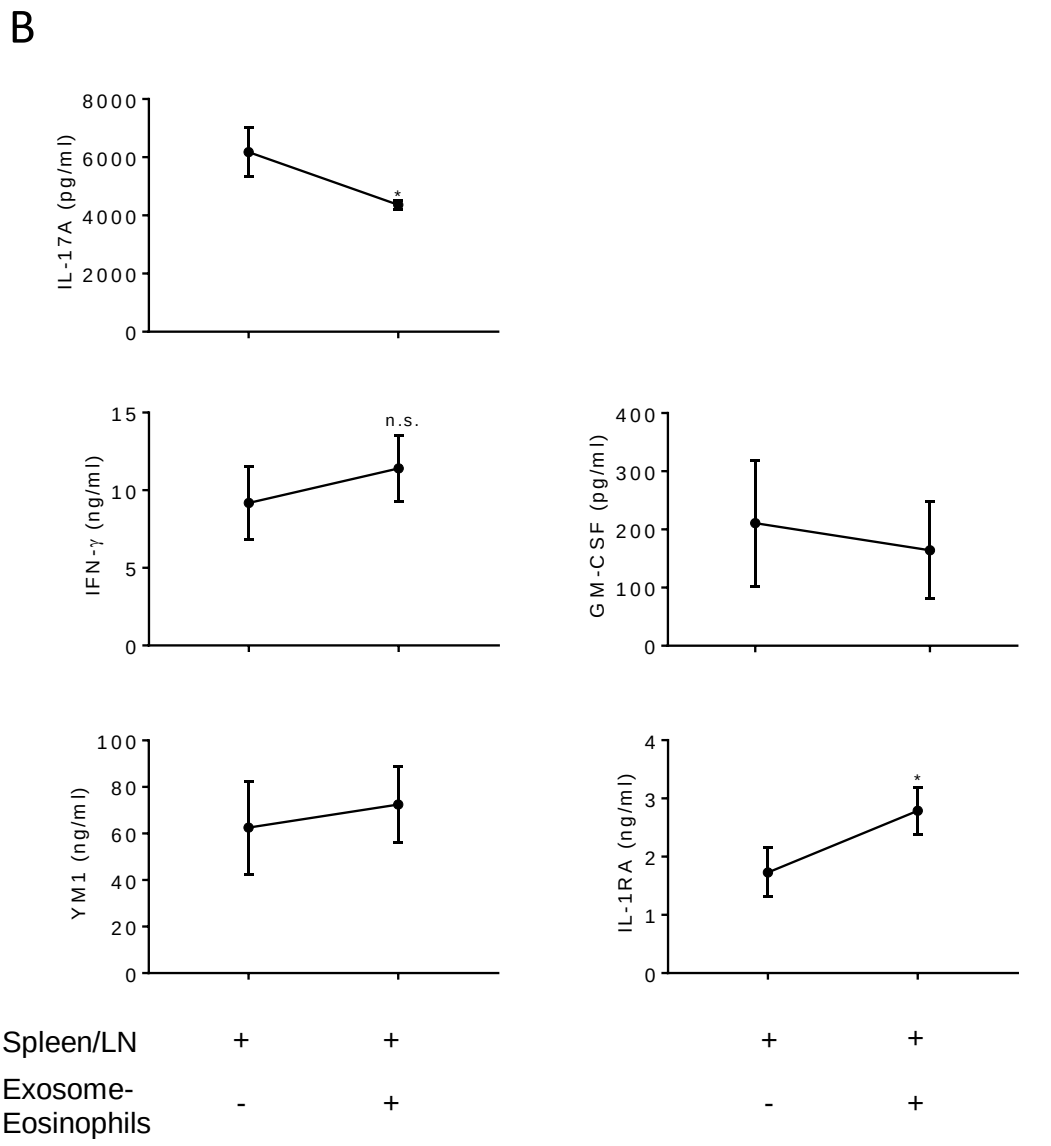
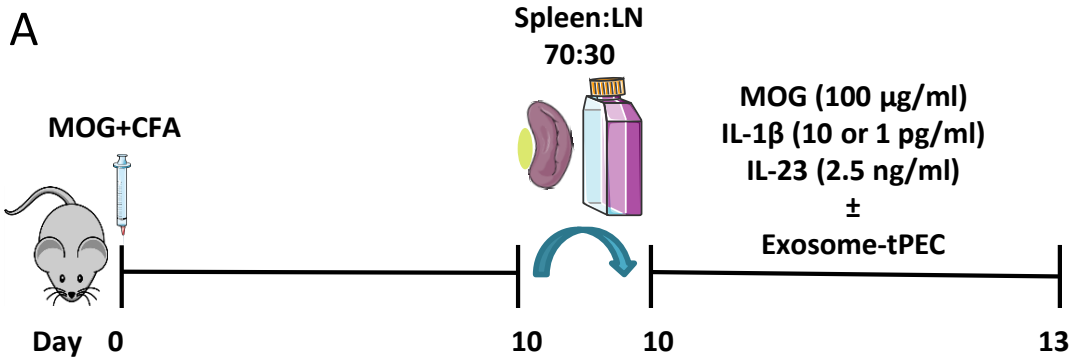

B
IL-17A (pg/ml)
8000
6000
4000
2000
0
*
IFN-γ (ng/ml)
15
10
5
0
n.s.
GM-CSF (pg/ml)
400
300
200
100
0
YM1 (ng/ml)
100
80
60
40
20
0
IL-1RA (ng/ml)
4
3
2
1
0
*
Spleen/LN + + + +
Exosome-Eosinophils - + - +

Figure 3.29: *F. hepatica* exosome-induced eosinophils promotes IL-1RA production and supresses IL-17A production by MOG-specific cells.

(A) Donor mice were injected with MOG and CFA. After 10 days, spleen and LN cells were cultured for 72 h in a 70:30 ratio (Spleen:LN cell) with MOG (100 μg/ml), IL-1β (1 ng/ml) and IL-23 (2.5 ng/ml) in the presence of medium or FACS sorted *F. hepatica* exosome-induced eosinophils (at a ratio of 0.2:1, eosinophil:spleen+LN). (B) After 72 hours, the concentrations of IL-17A, IFN-γ, GM-CSF, YM1, and IL-1RA in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by Student's t-test v medium control. *P<0.05.


A
Spleen:LN
70:30
MOG+CFA
MOG (100 µg/ml)
IL-1β (10 or 1 pg/ml)
IL-23 (2.5 ng/ml)
±
Exosome-tPEC
Day 0
10
10
13

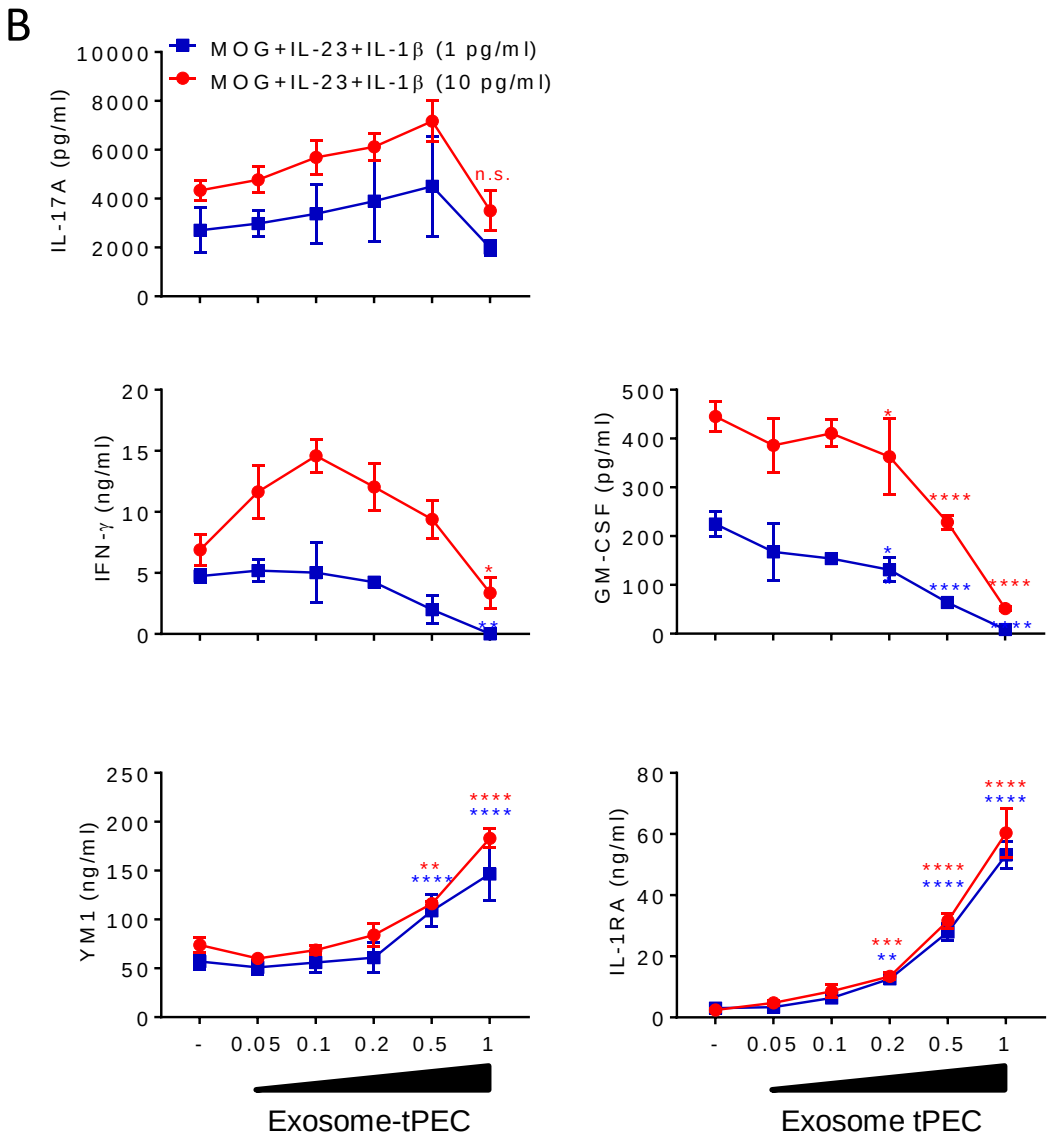

B
MOG+IL-23+IL-1β (1 pg/ml)
MOG+IL-23+IL-1β (10 pg/ml)
IL-17A (pg/ml)
n.s.
IFN-γ (ng/ml)
GM-CSF (pg/ml)
YM1 (ng/ml)
IL-1RA (ng/ml)
- 0.05 0.1 0.2 0.5 1
Exosome-tPEC
Exosome tPEC

Figure 3.30: PEC from mice injected with *F. hepatica* exosomes induce IL-1RA production and supress pro-inflammatory cytokine production by MOG-specific cells.

(A) Donor mice were injected with MOG and CFA. After 10 days, spleen and LN cells were cultured for 72 h in a 70:30 ratio (Spleen:LN cell) with MOG (100 µg/ml) +/- IL-1β (one of two concentrations, 1 or 10 pg/ml) and IL-23 (2.5 ng/ml) in the presence of medium or PEC from mice injected i.p. with *F. hepatica* exosomes (at ratios varying from 0.05:1 to 1:1, tPEC:spleen+LN). (B) After 72 hours, the concentrations of IL-17A, IFN-γ, GM-CSF, IL-6, YM1, and IL-1RA in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference (HSD) post hoc test. *P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 vs. medium control. n.s. = no significance

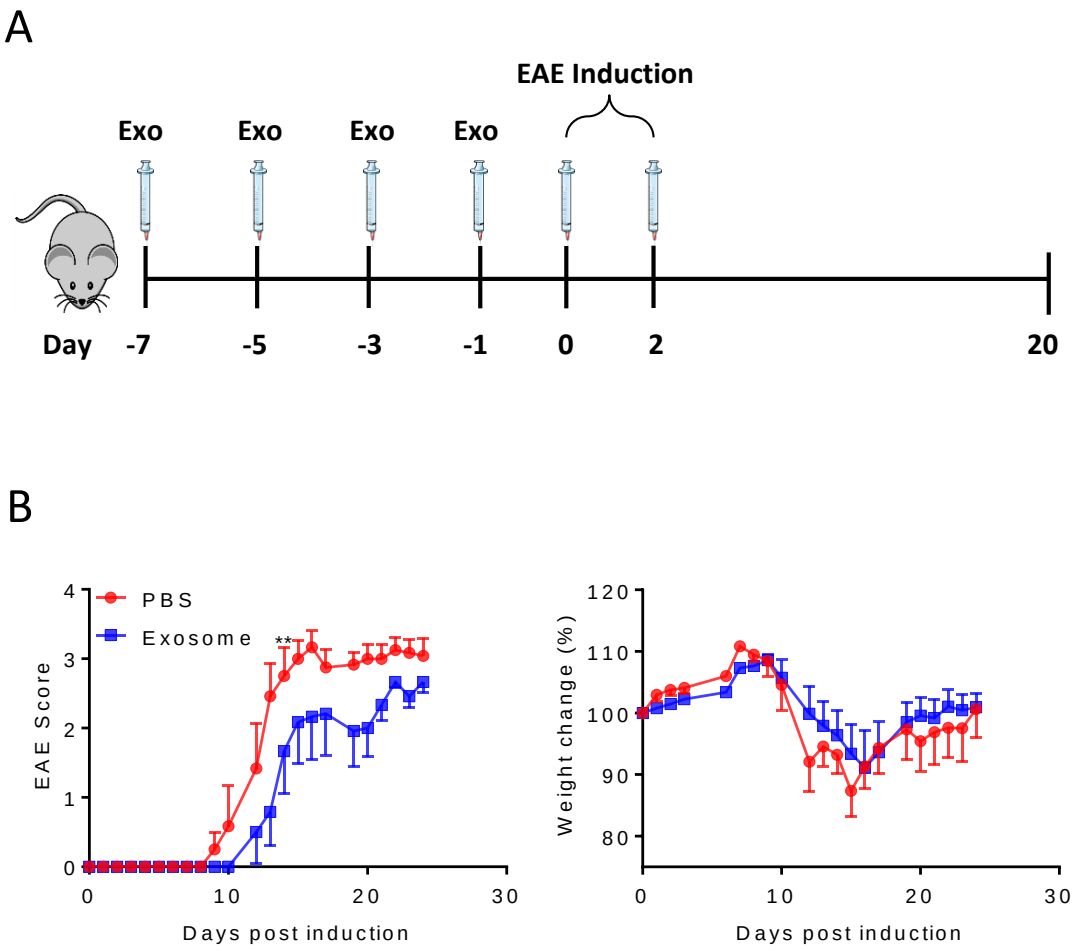


Figure 3.31: Administration of *F. hepatica* exosomes for a week prior to the induction of EAE delays disease onset.

(A) C57BL/6 mice were injected i.p. with PBS (vehicle control) or *F. hepatica* exosomes (2.5 µg/mouse/day) on days -7, -5, -3, and -1 (n=6). EAE was induced on day 0 by injection of MOG/CFA and PT (200 ng/mouse) and on day 2 by injection of PT (200 ng/mouse). (B) Disease progression was monitored by measuring body weight and clinical score daily. Data presented are means +/- SEM (n=6). Statistical assessment was performed by repeated measures ANOVA with Sidak's post hoc test. ** P<0.01.

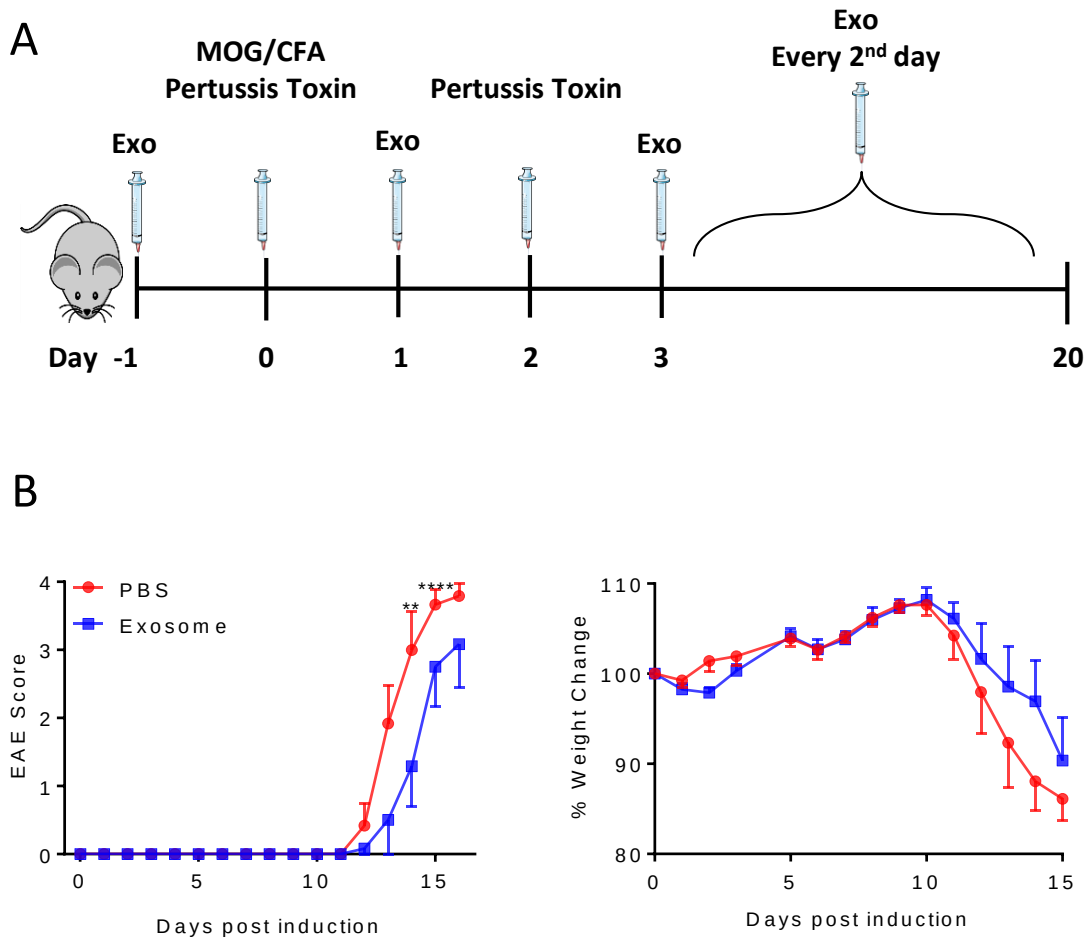


Figure 3.32: Therapeutic administration of *F. hepatica* exosomes delays the onset of EAE.

(A) C57BL/6 mice were injected i.p. with PBS (vehicle control) or *F. hepatica* exosomes (2.5 µg/mouse/day) on days -1, and every subsequent second day until the end of the experiment (n=6). EAE was induced on day 0 by injection of MOG/CFA and PT (200 ng/mouse) and on day 2 by injection of PT (200 ng/mouse). (B) Disease progression was monitored by measuring body weight and clinical score daily. Data presented are means +/- SEM (n=6). Statistical assessment was performed by repeated measures ANOVA with Sidak's post hoc test. ** P<0.01, **** P<0.0001.

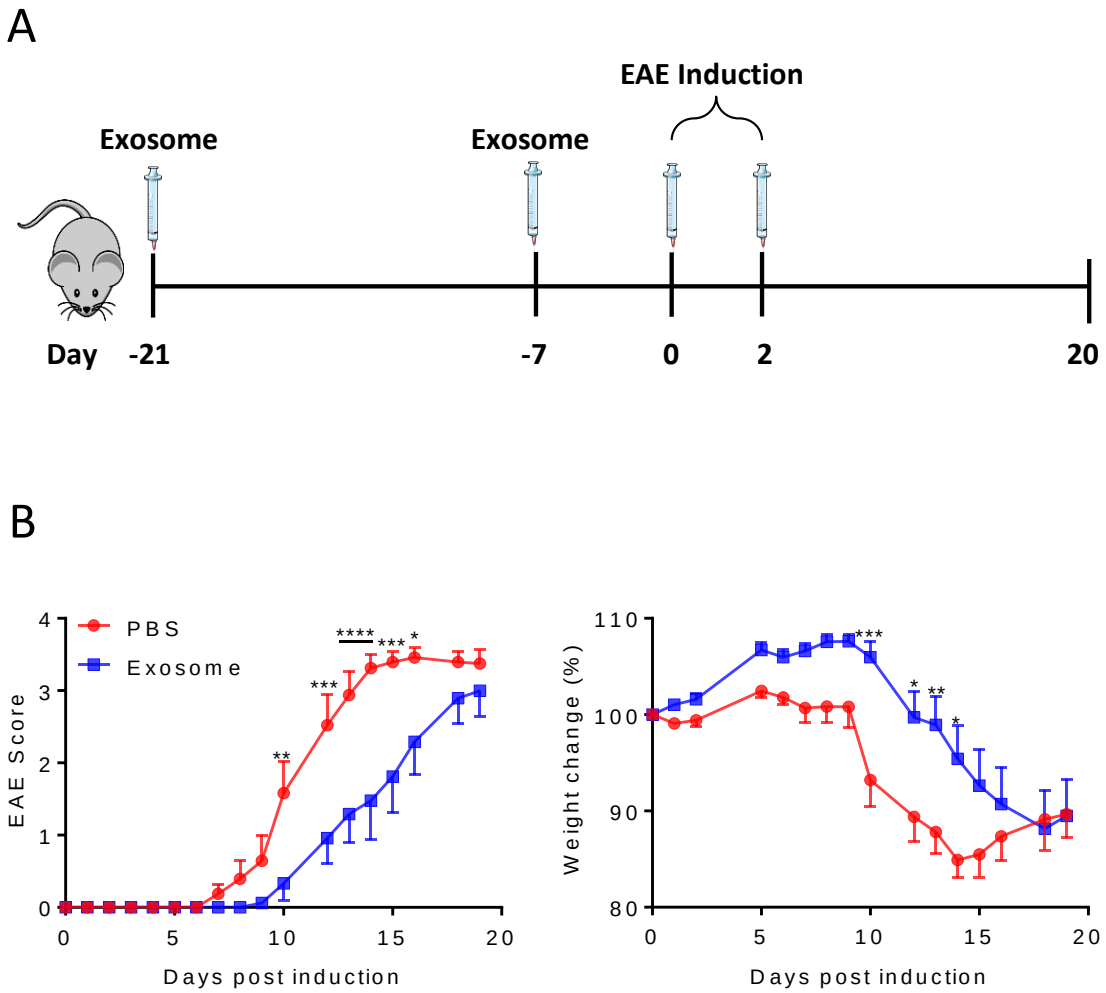


Figure 3.33: Training of mice with *F. hepatica* exosomes attenuates EAE.

(A) C57BL/6 mice were injected i.p. with PBS (vehicle control) or *F. hepatica* exosomes (2.5 µg/mouse/day) on days -21 and -7 (n=6). EAE was induced on day 0 by injection of MOG/CFA and PT (200 ng/mouse) and on day 2 by injection of PT (200 ng/mouse). (B) Disease progression was monitored by measuring body weight and clinical score daily. Data presented are means +/- SEM (n=12) and are combined from two different experiments. Statistical assessment was performed by repeated measures ANOVA with Sidak's post hoc test. ** P<0.01, ***P<0.001, **** P<0.0001.

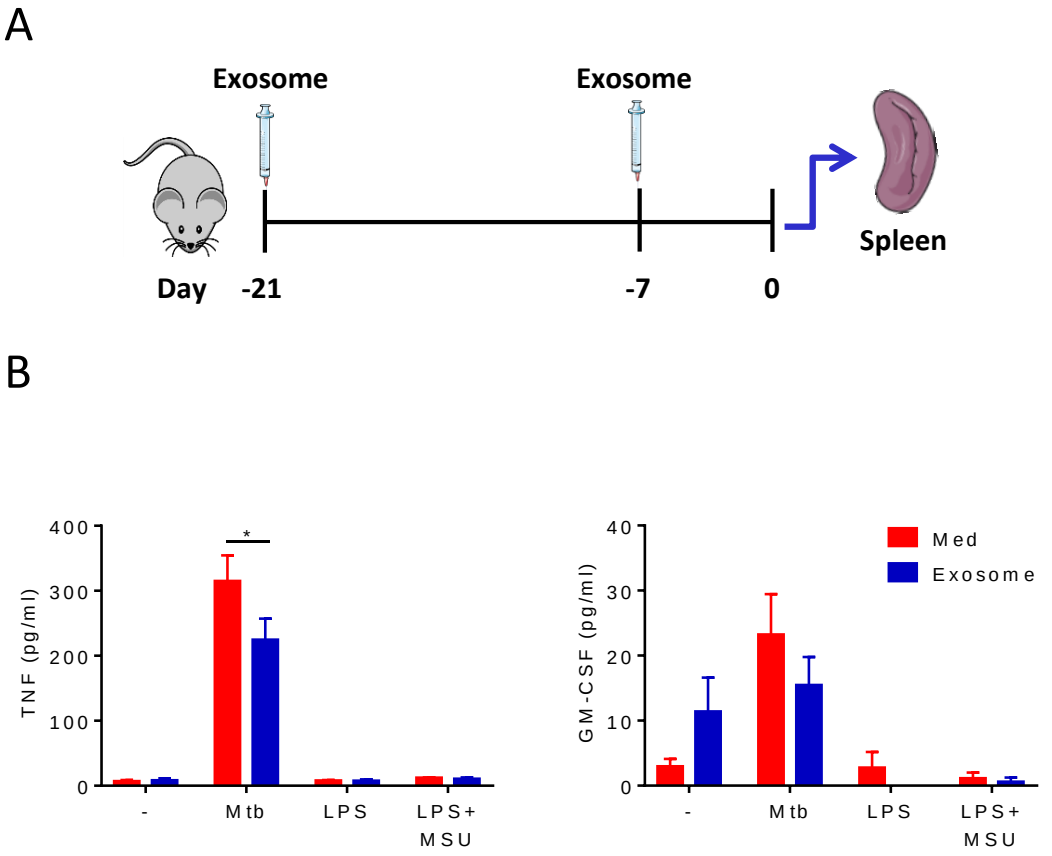


Figure 3.34: Pre-treatment with *F. hepatica* exosomes suppressed Mtb-induced TNF production but did not significantly inhibit the production of GM-CSF induced by the pro-inflammatory stimuli.

(A) C57BL/6 mice were injected i.p. with PBS (vehicle control) or *F. hepatica* exosomes (2.5 µg/mouse/day) on days -21 and -7 (n=5-6). On day 0, mice were sacrificed, spleens isolated and cultured for 24 hours with Mtb (100 ng/ml), LPS (100 ng/ml), LPS (100 ng/mL) and MSU crystals (250 µg/ml), or medium control. The concentrations of GM-CSF and TNF in the supernatants were determined by ELISA. Data from the mice is presented as means +/- SD (n=5-6). Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test. * P<0.05.

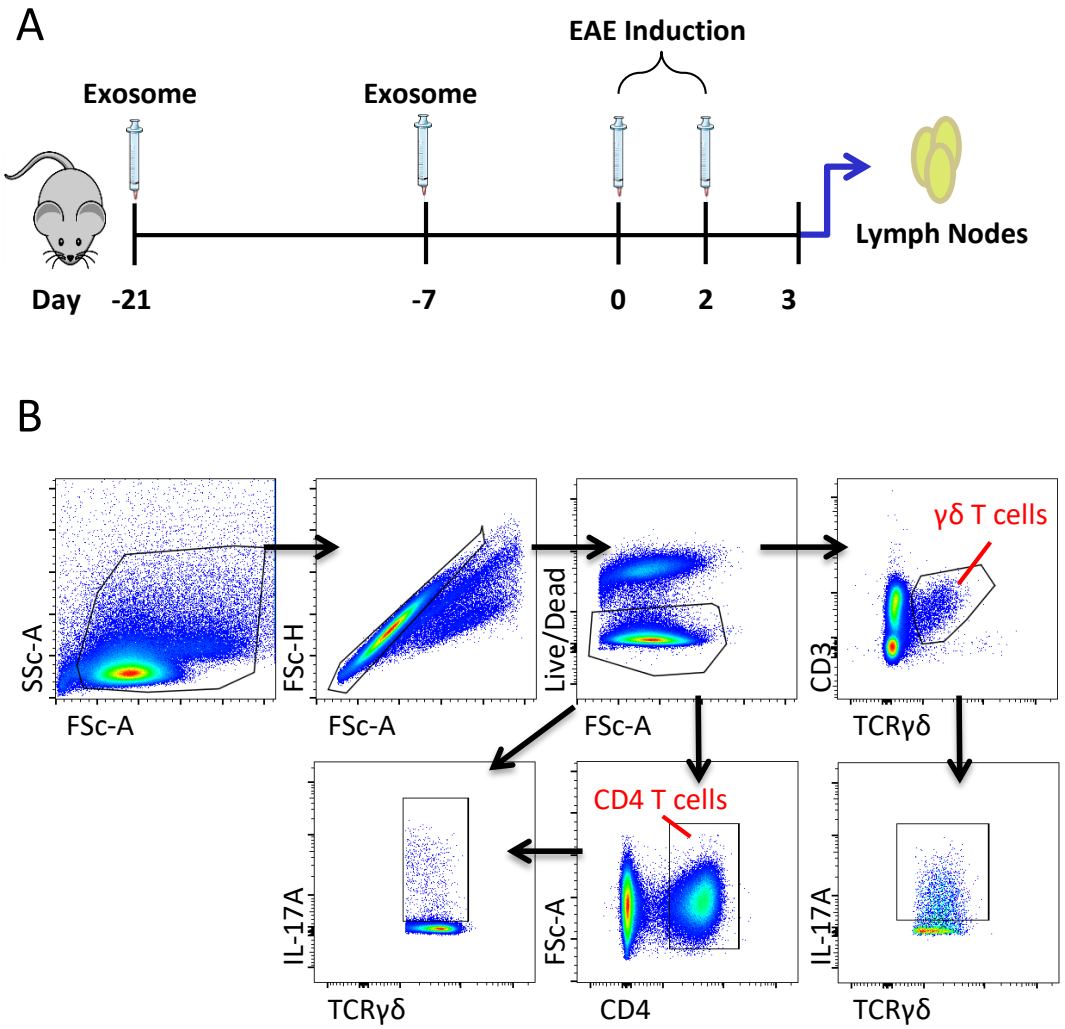
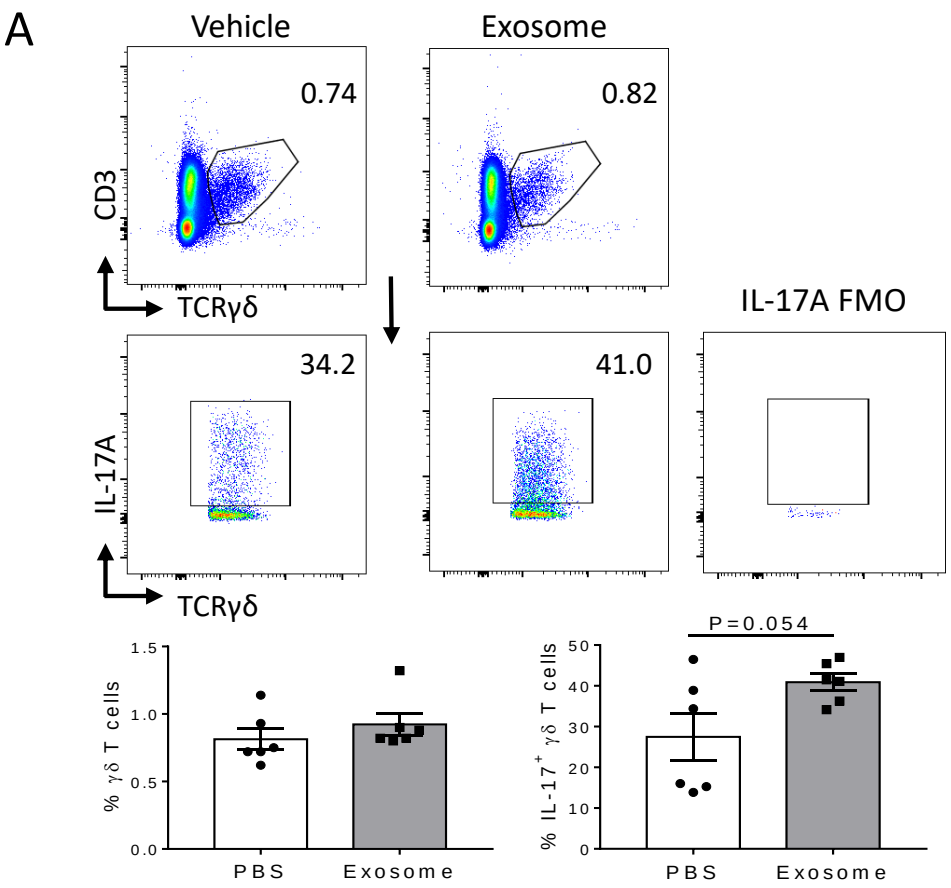


Figure 3.35: Experimental design to examine the effect of training with *F. hepatica* exosomes on immune cell populations in lymph nodes following EAE induction.

(A) C57BL/6 mice were injected s.c. with PBS (vehicle control) or *F. hepatica* exosomes (2.5 µg/mouse/day) on days -21 and -7 (n=6). EAE was induced on day 0 by injection of MOG/CFA and PT (200 ng/mouse) and on day 2 by injection of PT (200 ng/mouse). On day 3, mice were sacrificed, and lymph nodes were isolated for flow cytometry analysis. (B) Gating strategy to identify γδ T cells, CD4 t cells, neutrophils, and inflammatory monocytes.


A
Vehicle
Exosome
0.74
0.82
CD3
TCRγδ
IL-17A FMO
34.2
41.0
IL-17A
TCRγδ
P=0.054
% γδ T cells
% IL-17+ γδ T cells
PBS
Exosome
PBS
Exosome

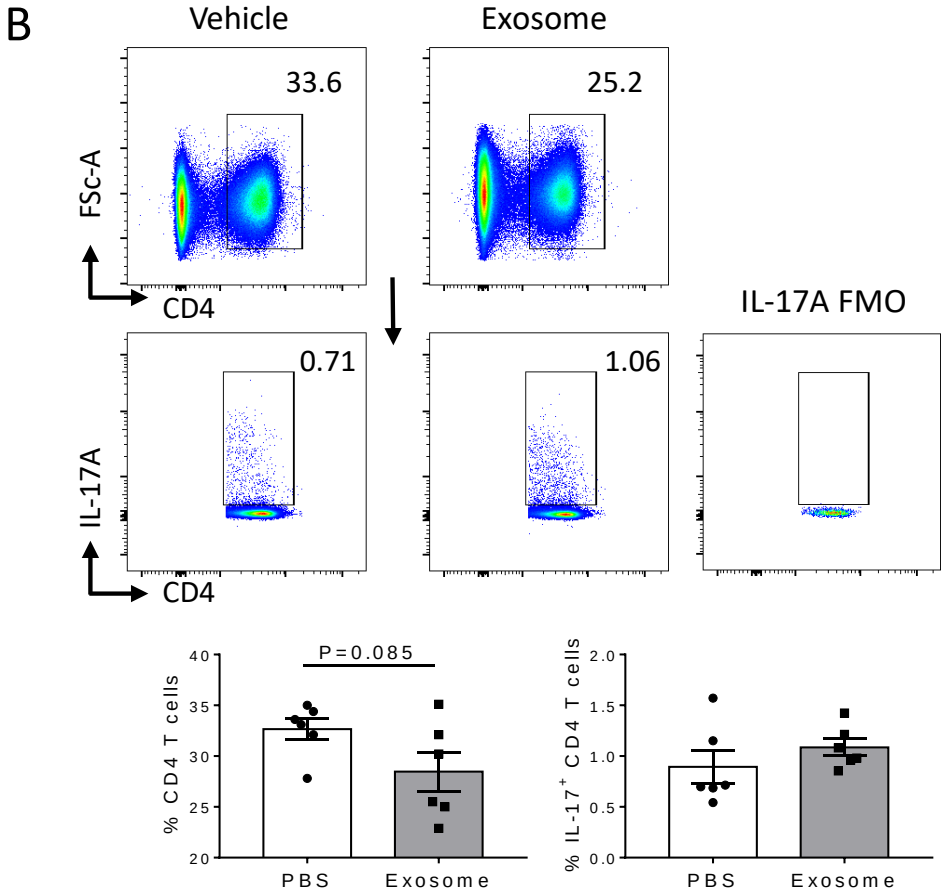

B
Vehicle
Exosome
33.6
25.2
FSc-A
CD4
IL-17A FMO
0.71
1.06
IL-17A
CD4
P=0.085
% CD4 T cells
% IL-17+ CD4 T cells
PBS
Exosome
PBS
Exosome

Figure 3.36: IL-17A production by γδ and CD4 T cells in lymph nodes is not reduced in *F. hepatica* exosomes-trained mice following the induction of EAE.

C57BL/6 mice were trained with *F. hepatica* exosomes and EAE was induced (Figure 3.35) On day 4, the mice were sacrificed, perfused, and LN cells were isolated and flow cytometry analysis carried out. (A) The percentage of total γδ cells and IL-17A$^+$ γδ cells in the LNs including representative FACS plots and an IL-17A FMO. (B) The percentage of total CD4$^+$ cells and IL-17A$^+$ CD4$^+$ cells in the LNs including representative FACS plots and an IL-17A FMO. Data presented are means +/- SEM (n=6). Statistical assessment was performed by Student's t-test vs. vehicle control.

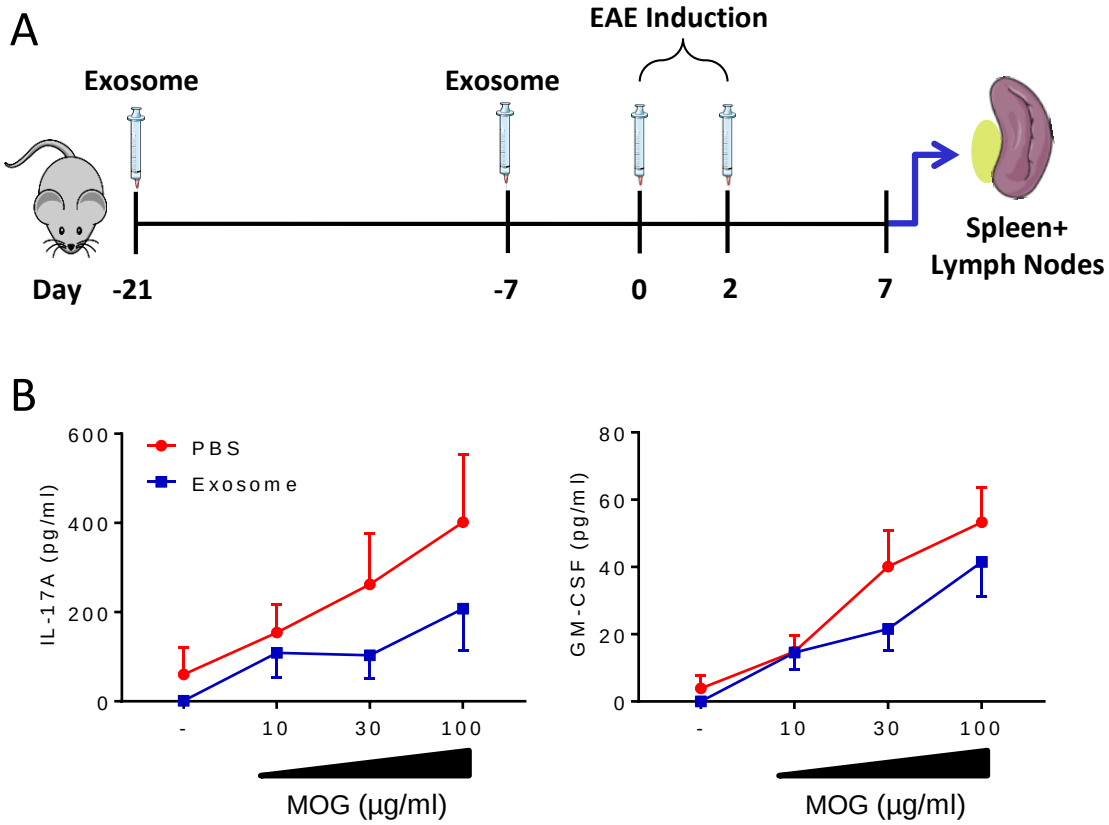
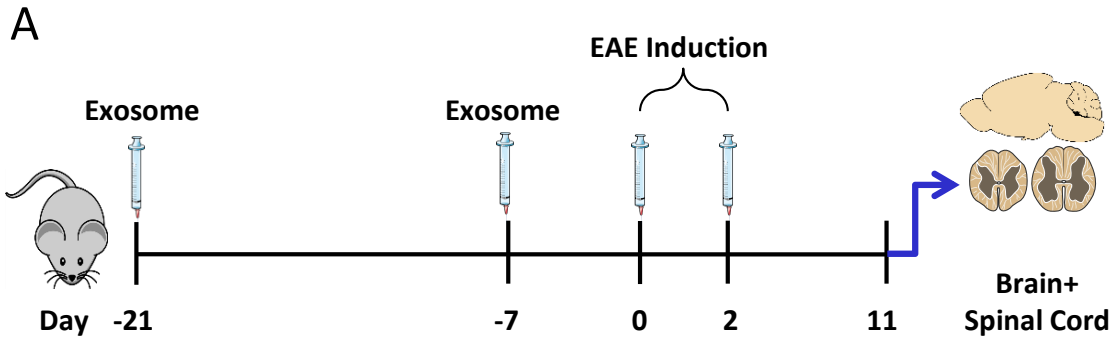
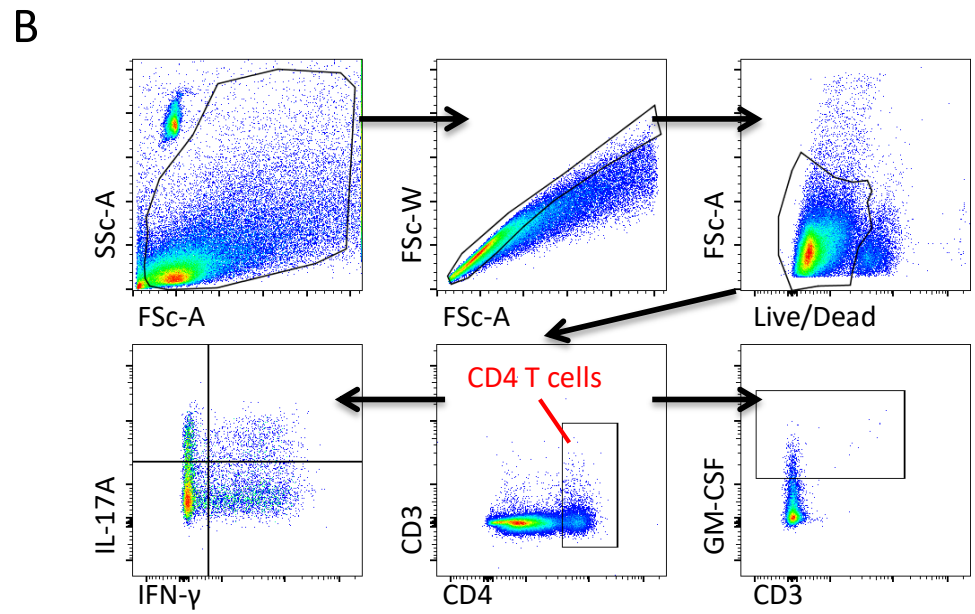


Figure 3.37: MOG-specific production of IL-17A and GM-CSF from spleen and LN cells is not significantly reduced in *F. hepatica* exosomes-trained mice with EAE.

(A) C57BL/6 mice were injected i.p. with PBS (vehicle control) or *F. hepatica* exosomes (2.5 µg/mouse/day) on days -21 and -7 (n=6). EAE was induced on day 0 by injection of MOG/CFA and PT (200 ng/mouse) and on day 2 by injection of PT (200 ng/mouse). On day 7, mice were sacrificed, and spleen and LN cells were isolated and restimulated for 72 hours with increasing concentrations of MOG (10, 30, or 100 µg/ml) or medium control. The concentrations of GM-CSF and IL-17A, in the supernatants were determined by ELISA. Data from the mice is presented as means +/- SD (n=6). Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test.


A
EAE Induction
Exosome
Exosome
Day -21
-7
0
2
11
Brain+
Spinal Cord


B
SSc-A
FSc-A
FSc-W
FSc-A
FSc-A
Live/Dead
CD4 T cells
IL-17A
IFN-γ
CD3
CD4
GM-CSF
CD3

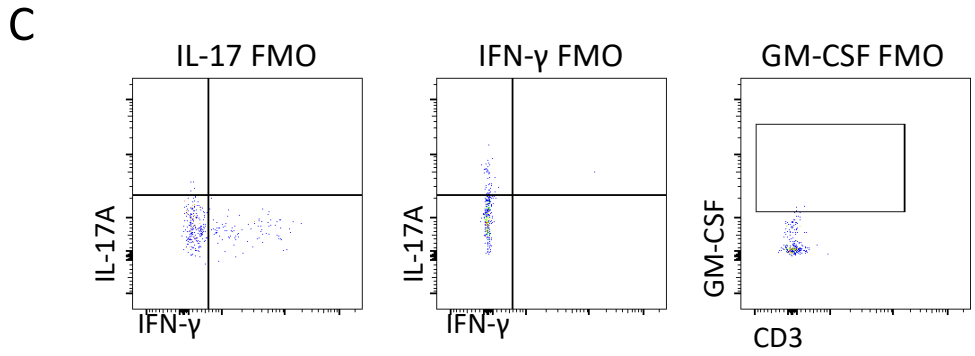

C
IL-17 FMO
IFN-γ FMO
GM-CSF FMO
IL-17A
IFN-γ
IL-17A
IFN-γ
GM-CSF
CD3

Figure 3.38: Experimental design and flow cytometric gating strategy to examine the effect of training with *F. hepatica* exosomes on immune cell populations in the CNS following the induction of EAE.

(A) C57BL/6 mice were injected i.p. with PBS (vehicle control) or *F. hepatica* exosomes (2.5 µg/mouse/day) on days -21 and -7 (n=6). EAE was subsequently induced by injection of MOG/CFA and PT (200 ng/mouse) on day 0 and injection of PT (200 ng/mouse) on day 2. On day 11, mice were sacrificed, perfused, and brains and spinal cords isolated. (B) Gating strategy to identify eosinophils and neutrophils. (C) FMO controls to help identify IL-17A, IFN-γ, and GM-CSF-expressing populations in the brain and spinal cord.

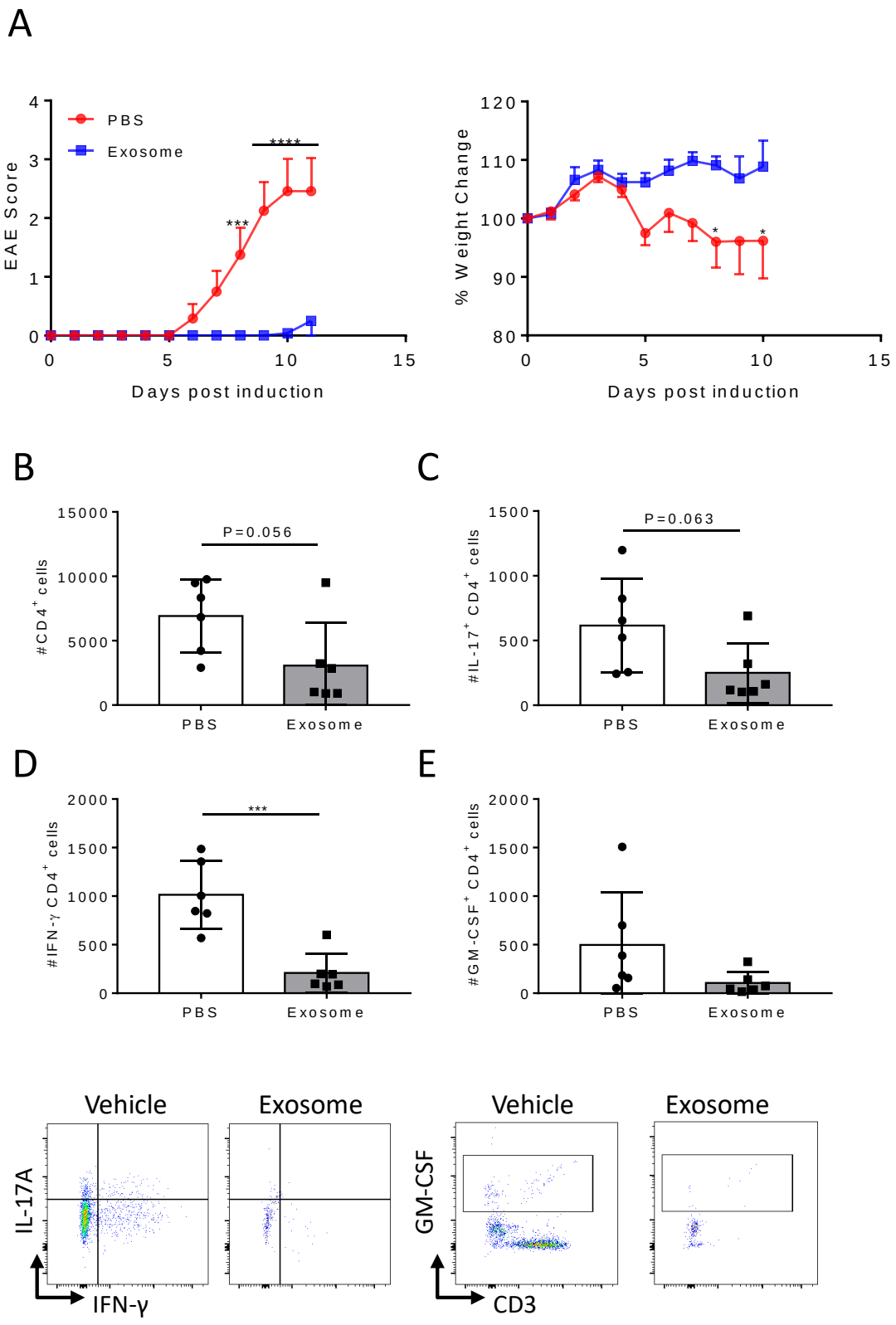

A
PBS
Exosome
EAE Score
Days post induction
% Weight Change
Days post induction
B
P=0.056
#CD4+ cells
PBS
Exosome
C
P=0.063
#IL-17+ CD4+ cells
PBS
Exosome
D
#IFN-γ CD4+ cells
PBS
Exosome
E
#GM-CSF+ CD4+ cells
PBS
Exosome
Vehicle
Exosome
IL-17A
IFN-γ
Vehicle
Exosome
GM-CSF
CD3

Figure 3.39: *F. hepatica* exosome-trained mice have reduced numbers of IL-17A, IFN-γ, and GM-CSF-producing CD4 cells in the brain in mice with EAE.

C57BL/6 mice were trained with *F. hepatica* exosomes and EAE was induced (Figure 3.38). Disease progression was monitored by measuring body weight and clinical score daily. Data presented are means +/- SEM (n=6) (A). On day 11, the mice were sacrificed, perfused, and brain cells were isolated and flow cytometry analysis carried out. Training with the exosomes reduced the absolute number of $CD4^+$ cells in the brain (B) and the absolute number of IL-17A (C), IFN-γ (D), and GM-CSF-producing (E) $CD4^+$ cells in the brain. Representative FACS plots ae included. Data presented are means +/- SEM (n=6). Statistical assessment was performed by Student's t-test vs. vehicle control. *** $P<0.001$.

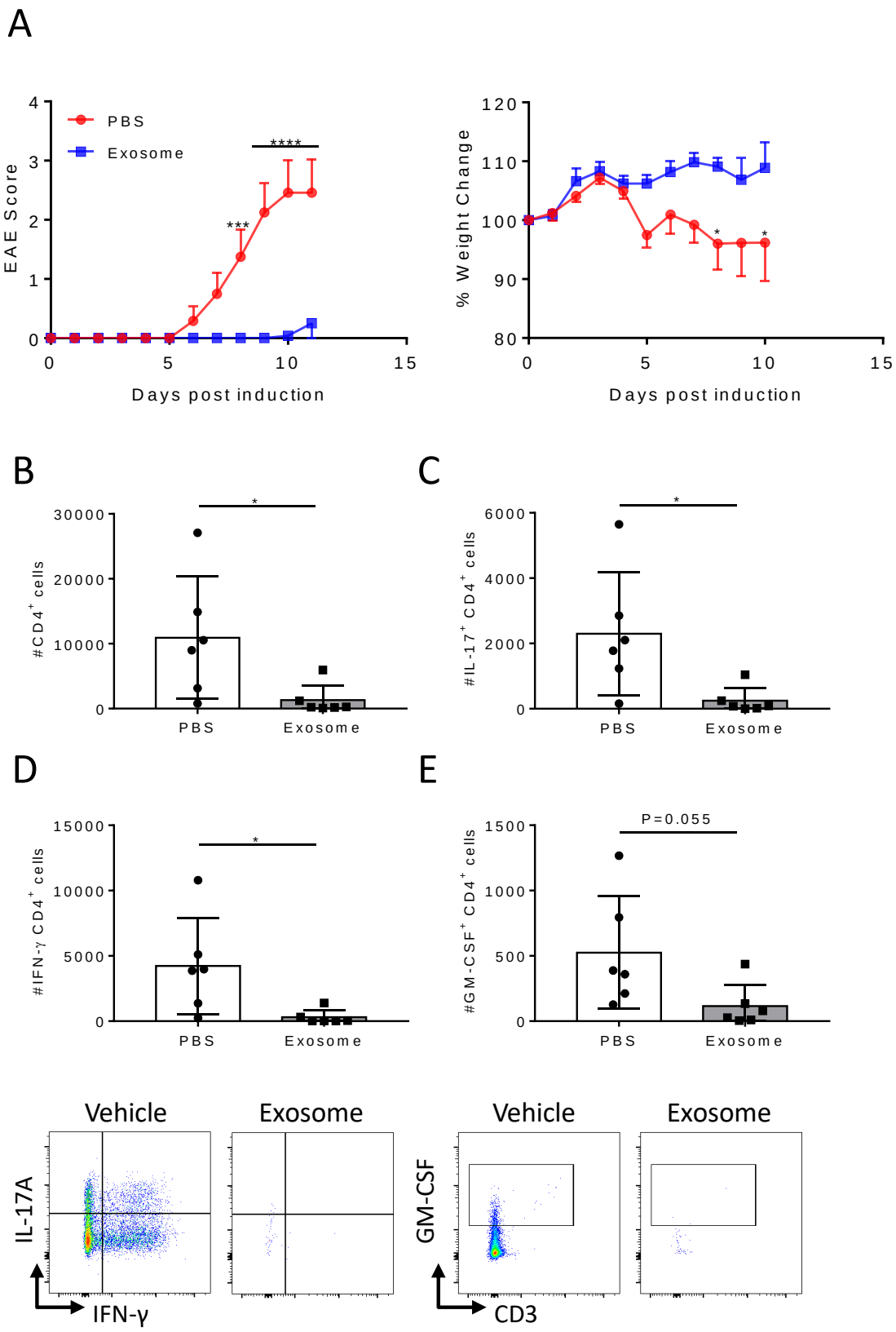

A
PBS
Exosome
EAE Score
Days post induction
% Weight Change
Days post induction
B
#CD4+ cells
PBS
Exosome
C
#IL-17+ CD4+ cells
PBS
Exosome
D
#IFN-γ CD4+ cells
PBS
Exosome
E
P=0.055
#GM-CSF+ CD4+ cells
PBS
Exosome
Vehicle
Exosome
IL-17A
IFN-γ
Vehicle
Exosome
GM-CSF
CD3

Figure 3.40: *F. hepatica* exosome-trained mice have reduced numbers of IL-17A, IFN-γ, and GM-CSF-producing CD4 cells in the spinal cord in mice with EAE.

C57BL/6 mice were trained with *F. hepatica* exosomes and EAE was induced (Figure 3.38). Disease progression was monitored by measuring body weight and clinical score daily. Data presented are means +/- SEM (n=6) (A). On day 11, the mice were sacrificed, perfused, and spinal cord cells were isolated and flow cytometry analysis carried out. Training with the exosomes reduced the absolute number of $CD4^+$ cells in the spinal cord (B) and the absolute number of IL-17A (C), IFN-γ (D), and GM-CSF-producing (E) $CD4^+$ cells in the spinal cord. Representative FACS plots ae included. Data presented are means +/- SEM (n=6). Statistical assessment was performed by Student's t-test vs. vehicle control. *** P<0.001.

Chapter 4

The anti-inflammatory effects of IL-33 *in vivo*

4.1 Introduction

IL-33 is a cytokine from the IL-1 family produced predominantly by epithelial and endothelial cells as well as by immune cells [327]. Since its discovery in 2003 as Nuclear factor of high endothelial venules (NF-HEV), IL-33 has been shown promote IL-5 and IL-13 production by ILC2 cells, which mediate type-2 immune responses including eosinophilia [155, 156]. IL-33 production is induced by helminths; *F. hepatica* infection and FHES injection promoted IL-33 production and subsequent eosinophil infiltration in mice [157, 205].

Injection of mice with MSU crystals induces inflammatory cell infiltration and is used as a model of sterile inflammation [336]. During sterile inflammation, innate immune cells are activated due to the release of DAMPs rather than by infection [337]. Most frequently this is due to necrotic cells releasing intracellular DAMPs into the extracellular environment. During necrosis, IL-33 is released as an alarmin and induces a healing response [336]. IL-33 has been shown to be protective in atherosclerosis and obesity models in mice, diseases caused by sterile inflammation [337]. Gout is the most common metabolic disorder in humans and results from MSU crystal formation leading to the development of symptoms, including arthritis and kidney damage [338]. Serum levels of IL-33 were shown to be elevated in gout patients compared with healthy controls. IL-33 concentrations were highest in patients without kidney injury. MSU crystals are an endogenous factor for kidney damage [339]. Data from the gout patients suggests a protective role for IL-33 in MSU crystal-induced inflammation.

In Chapter 3 it was demonstrated that injection of mice with *F. hepatica* exosomes induced eosinophil infiltration and inhibited MSU crystal-induced recruitment of neutrophils into the peritoneal cavity. The frequency of eosinophils present negatively correlated with the frequency of neutrophils in the peritoneal cavity, suggesting an antagonistic relationship between the two cell types. An antagonistic relationship between eosinophils and neutrophils has been suggested from investigations into inflammatory diseases. Patients with type 1 diabetes mellitus, a chronic autoimmune disease [340], were shown to have elevated neutrophil and reduced eosinophil numbers in plasma compared with healthy controls [341]. However, suppressing eosinophil

induction by *F. hepatica* exosomes did not reverse the ability of the exosomes to inhibit neutrophil infiltration. *F. hepatica* exosomes also induced M2 macrophage activation and it is possible that these cells mediate the suppressive activity of the exosomes rather than eosinophils.

IL-33 is a potent inducer of M2 macrophage activation as well as eosinophilia [327]. Alternatively activated M2 macrophages play a role in initiating tissue repair and resolving inflammation [342]. In response to sterile inflammation, monocytes are recruited from the circulation and are classically activated by M1-associated cytokines. The monocytes persist in the wound and transition into alternatively activated macrophages to repair injury and maintain homeostasis [343, 344].

Previous research in the Mills laboratory has demonstrated that i.p. injection of IL-33 suppresses MSU crystal-induced neutrophil infiltration into the peritoneum similar to injection of *F. hepatica* exosomes. It is still unclear whether eosinophils, macrophages, and/or other immune cell types are required to mediate the anti-inflammatory effect of IL-33.

The specific questions to be addressed were:

- Do *F. hepatica* products induce IL-33 production?
- Does IL-33 induce IL-1RA production?
- Can IL-33 supress MSU crystal-induced inflammation *in vitro* and *in vivo*?
- Are eosinophils or macrophages required to mediate IL-33-attenuation of MSU crystal inflammation?
- Do eosinophils play a role in the induction of the alternative activation of macrophages by IL-33?

4.2 Results

4.2.1 Pre-treatment with FHES induces IL-33 production *in vivo*.

IL-33 is a potent inducer of eosinophilia and other type-2 responses when injected into mice and both live helminth infection and FHES injection induce IL-33 production [157, 345]. An experiment was designed to investigate whether pre-treatment with *F. hepatica* products induces IL-33 production. C57BL/6 mice were injected s.c. with FHES (100 µg/mouse/day) or PBS on days -21 and -7 and on day 0, peritoneal exudate cells were isolated and pelleted by centrifugation. The concentration of IL-33 in the supernatants of the peritoneal exudate cells was determined by ELISA. Pre-treatment with FHES induced IL-33 production in the peritoneal cavity (Figure 4.1).

Previous research in the Mills laboratory has demonstrated that the injection of IL-33 will suppress MSU crystal-induced neutrophil infiltration into the peritoneal cavity similar to FHES and *F. hepatica* exosomes. As IL-33 production is promoted by *F. hepatica* products and both IL-33 and *F. hepatica* products induce anti-inflammatory responses mediated by eosinophils and M2 macrophages, a series of experiments were designed to investigate the mechanism by which IL-33 inhibits pathological inflammation.

4.2.2 IL-33 induces IL-1RA production from macrophages but does not inhibit MSU crystal activation of the inflammasome *in vitro*.

MSU crystals activate the inflammasome, inducing the release of mature IL-1β and IL-18. Injection of MSU crystals into the peritoneal cavity of mice promotes neutrophil infiltration into the peritoneal cavity in an IL-1β-dependent manner [315]. MSU crystal-induced inflammation can be inhibited in different ways, including by supressing IL-1β and IL-18 release or by the induction of endogenous inhibitors of IL-1 signalling including IL-1RA. Therefore, I investigated whether IL-33 treatment could induce IL-1RA production and/or inhibit IL-1β and IL-18 production *in vitro*.

Previous studies with IL-33 had employed concentrations in the range of 20-100 ng/ml [314, 346]. Therefore, it was decided to treat macrophages with increasing concentrations of IL-33 around this range and to investigate whether IL-33 could induce the production of IL-1RA and the type 2 cytokines IL-4, IL-5, and IL-13. BMDMs were

treated for 24h with IL-33 (10, 25, 100, 250 ng/ml) or medium only as a control and the concentrations of IL-1RA, IL-4, IL-5, and IL-13 in the supernatants determined by ELISA. 250 ng/ml of IL-33 induced significant production of IL-1RA (Figure 4.2). No production of IL-4, IL-5, or IL-13 was detected. Since IL-33 induced IL-1RA production at a concentration of 250 ng/ml, this concentration was then chosen to determine whether IL-33 could inhibit MSU crystal inflammasome activation. BMDMs were treated with IL-33 (250 ng/ml) or medium +/- LPS (100 ng/ml) and MSU crystals (250 µg/ml) for 24 hours. IL-33 did not supress LPS and MSU crystal-induced IL-1β release (Figure 4.3).

4.2.3 IL-33 inhibits MSU crystal-induced inflammation, independent of eosinophil recruitment.

The results presented in Chapter 3 demonstrated that injection of mice with *F. hepatica* exosomes injection attenuated MSU crystal-induced inflammation. There was a negative correlation between the frequencies of eosinophils and neutrophils recruited into the peritoneal cavity in mice treated with the exosomes (Figure 3.20). A key function of IL-33 is to mobilise and expand eosinophils [314]. Work in the Mills lab has previously demonstrated that i.p. injection of mice with IL-33 every 2nd day over a course of six days mobilised and expanded eosinophils in the peritoneal cavity (Finlay and Mills, Unpublished Data). IL-33-induced eosinophilia is mediated by IL-5 [155] and anti-IL-5 administration will block eosinophil recruitment [317]. An experiment was designed to investigate whether IL-33 injection can inhibit MSU crystal-induced inflammation and to examine if eosinophils are required for any potential anti-inflammatory activity of IL-33. C57BL/6 mice were injected i.p. with anti-IL-5 (80 µg/mouse/day) or PBS on days -2, 0, 2, 4, and 6 and IL-33 (200 ng/mouse/day) or PBS on days 0, 2, 4, and 6 followed by MSU crystal i.p. injection (250 µg/mouse) or PBS on day 7. After 4 hours, peritoneal exudate cells were isolated by lavage (Figure 4.4S). The cells were stained with antibodies specific for CD11b, Siglec F, Ly6G, Ly6C, CD19, and F4/80 and analysed by flow cytometry analysis to investigate the recruitment of eosinophils (Siglec-F^{+}, SSc^{hi}, FSc^{lo}), neutrophils (CD19^{-}, F4/80^{-}, CD11b^{+}, Ly6G^{+}) and inflammatory monocytes (CD19^{-}, F4/80^{-}, CD11b^{+}, Ly6C^{+} Ly6G^{-}) (Figure 4.4B).

IL-33 induced a large increase in the frequency and total numbers of eosinophils in the peritoneal cavity, and this was supressed by the administration of anti-IL-5 (Figure 4.5). Mice injected with MSU crystals had an increased frequency and absolute numbers of neutrophils and inflammatory monocytes in the peritoneal cavity (Figure 4.6A). IL-33 treatment suppressed the infiltration of the pro-inflammatory cells. Inhibition of IL-33-induced eosinophil mobilisation and expansion with anti-IL-5 did not suppress the ability of IL-33 to attenuate MSU crystal-induced inflammation. While eosinophils were not required for the anti-inflammatory effects of IL-33 administration in MSU crystal-induced sterile peritonitis, there was a strong negative correlation between the frequency of eosinophils and neutrophils in the peritoneal cavity of mice treated with IL-33 (Figure 4.6B). There was no correlation found between the frequency of eosinophils and inflammatory monocytes (Figure 4.6C).

4.2.4 IL-33 induces M2 macrophage activation, an effect enhanced by blocking IL-33-mediated eosinophilia.

Having demonstrated that eosinophils were not the mediators of IL-33-induced anti-inflammatory activity in the MSU crystal sterile peritonitis model, the possible role of macrophages was investigated. IL-33 can promote the alternate activation of macrophages via the induction of the Th2 cytokines IL-4 and IL-13 [327, 347]. An experiment was designed to investigate whether the ability of IL-33 to promote M2 macrophage activation is affected by the presence of eosinophils. C57BL/6 mice were injected i.p. with anti-IL-5 (80 µg/mouse/day) or PBS on days -2, 0, 2, 4, and 6 and IL-33 (200 ng/mouse/day) or PBS on days 0, 2, 4, and 6. On day 7 peritoneal exudate cells were isolated by lavage for RNA isolation or for staining with antibodies specific for CD11b, Siglec F, Ly6G, and F4/80 and analysed by flow cytometry analysis to investigate eosinophil, neutrophil and macrophage populations (Figure 4.7). IL-33-induction of eosinophils was suppressed by anti-IL-5 injection (Figure 4.8A). The numbers of neutrophils in the peritoneal cavity were low across all the groups (Figure 4.8B).

The expression of the M2 markers Arg1, Chi3l, Retnla, Egr2, and Mrc1 was examined by rtPCR in the peritoneal exudate cells. Injection of mice with IL-33 induced the expression of all the M2 markers examined except for Mrc1. Treatment with anti-IL-5, which

suppressed eosinophil recruitment, significantly enhanced IL-33-induced expression of M2 macrophage markers (Figure 4.9A). As anti-IL-5 suppresses eosinophil infiltration and expansion, mice treated with both IL-33 and anti-IL-5 had a higher frequency of $CD11b^{+}$, $F4/80^{hi}$ LPMs in the peritoneal cavity compared to mice treated with IL-33 only (Figure 4.9B). Finally, after the peritoneal exudate cells were pelleted by centrifugation and isolated for rtPCR and flow cytometry analysis, the concentrations of IL-1RA and IL-6 in the supernatants of the peritoneal exudate cells were determined by ELISA. IL-1RA was detected in the supernatants from the IL-33 group and the IL-33 and anti-IL-5 treated group but not in the control group. IL-6 was not induced by IL-33 (Figure 4.10)

4.2.5 Macrophages may mediate IL-33 inhibition of MSU crystal-induced inflammation.

As IL-33 can promote M2 macrophage activation, it was addressed whether the anti-inflammatory activity of IL-33 in MSU crystal-induced inflammation is dependent on macrophages. Macrophages can be depleted *in vivo* using clodronate liposomes, which contain *bis*phosphonate clodronate. Following injection, the liposomes are ingested by macrophages and degraded intracellularly releasing clodronate. When the intracellular concentration of clodronate is sufficiently high, the macrophages will undergo apoptosis [348]. C57BL/6 mice were injected i.p. with clodronate liposomes (200 µl/mouse) or PBS on day -2 and clodronate liposomes (100 µl/mouse) or PBS on day 2 to deplete macrophages from the peritoneal cavity. The mice were also injected with IL-33 (200 ng/mouse/day) or PBS on days 0, 2, and 4. On day 7, the mice were injected with MSU crystals (250 µg/mouse) or PBS and after four hours, peritoneal exudate cells were removed and stained with antibodies specific for CD11b, Siglec F, Ly6G, and F4/80 and analysed by flow cytometry (Figure 4.11). The injection of the clodronate liposomes resulted in a complete depletion of $F4/80^{+}$ macrophages in the peritoneal cavity (Figure 4.12). IL-33 mobilised and expanded eosinophils (Siglec F^{+}, SSc^{hi}, FSc^{lo}) in the peritoneal cavity (Figure 4.13) and resulted in a partial but non-significant suppression of MSU crystal-induced neutrophil recruitment. In mice treated with clodronate liposomes, the mean percentage of neutrophils present in the peritoneal cavity was similar to in control mice, although the results were extremely variable (Figure 4.14).

4.3 Discussion

IL-33 is a recently discovered member of the IL-1 cytokine family. It promotes type-2-associated cytokine production and tissue repair processes [347]. Injection of mice with MSU crystals into the peritoneal cavity induces the infiltration of neutrophils and the differentiation of monocytes into classically activated M1 macrophages [201, 349]. Type-1 and type-2 immune responses cross-regulate each another [324]. Secretion of the type-1 cytokine IFN-γ by Th1 cells suppresses IL-4 secretion, which inhibits the ability of naïve Th cells to polarize into Th2 cells [350]. Reciprocally, the type-2 associated cytokines IL-4 and IL-10 inhibit the secretion and activity of IL-12 and IFN-γ, thereby suppressing Th1 cell differentiation and function [351–353]. The present study showed that injection of IL-33 into the peritoneal cavity of mice suppressed MSU crystal-induced influx of neutrophils and inflammatory monocytes. The results demonstrated that suppression was independent of eosinophil recruitment. However, IL-33 may require the presence of macrophages to mediate its anti-inflammatory effect.

Clodronate liposomes were used to investigate macrophage function. When injected they will deplete macrophages *in vivo*, whether they are classically M1 or alternatively M2 activated. The use of clodronate liposomes in the MSU crystal-induced sterile peritonitis model is complicated by the fact that macrophages mediate the pro-inflammatory effects induced by MSU crystals. The crystals are taken up by macrophages leading to NLRP3 activation and IL-1β production. Murine primary and bone marrow-derived macrophages produced IL-1β following exposure to MSU crystals *in vitro* [354]. Martin et al. (2009) demonstrated that macrophages are required to mediate the inflammatory effect of MSU crystal-induced inflammation *in vivo* [355]. The study found that injection of mice with MSU crystals induced neutrophil and inflammatory monocyte infiltration and IL-1β, TNF and IL-6 production in the peritoneal cavity. Following injection of MSU crystals, resident macrophages were activated and expressed IL-1β, TNF, and IL-6. Depletion of resident macrophages in the peritoneal cavity using clodronate liposomes attenuated MSU crystal-induced inflammation, reducing neutrophil recruitment and IL-1β and IL-6 concentrations. Therefore, the use of clodronate liposomes to deplete macrophages in this experiment has limitations. In mice treated with the liposomes and MSU crystals there was very large variability in the

frequency of neutrophils when compared with the mice not treated with clodronate liposomes. Unlike the report of Martin et al., a large frequency of neutrophils was observed in the peritoneal cavity of some of the mice treated with clodronate liposomes and MSU crystals. Other mononuclear phagocytes, such as monocytes, can mediate MSU crystal-induced inflammation. Therefore, they may have mediated the neutrophil influx demonstrated in these mice [354, 356].

Instead of using clodronate liposomes to deplete the entire population of resident macrophage, future experiments could be designed to examine the effect of specific suppression of macrophages activated by IL-33. The major effect of IL-33 on macrophages *in vivo* is to promote their polarization to an alternatively activated M2 state. IL-33 enhanced IL-4 and IL-13-induced expression of the M2 markers Arg1 and YM1 in BMDMs [357]. ST2 is the IL-33 receptor and is expressed on macrophages [327]. BMDMs generated from the bone marrow of $ST2^{-/-}$ mice treated with IL-33 failed to demonstrate the synergised effect of IL-4 or IL-13 and IL-33 administration on M2 marker expression [357]. This effect of IL-33 on macrophages *in vivo* is indirect and involves the induction of IL-4 and IL-13 production, cytokines which induce alternate activation of macrophages [311]. IL-33 promotes IL-4 production by basophils [358] and IL-13 by ILC2s [155, 359]. The present study demonstrated that IL-33 induced alternatively activated M2 macrophages. M2 macrophages ameliorate type-1 inflammatory responses like those induced by MSU crystals [357]. Future studies should examine if IL-4 or IL-13 mediates IL-33 attenuation of MSU crystal-induced inflammation by inducing M2 macrophage differentiation.

Finally, it was shown that eosinophils are not required for IL-33 induction of M2 macrophages. Administration of anti-IL-5 lead to enhanced M2 marker expression. This suggests an inhibitory role for eosinophils on M2 macrophages. This is surprising as eosinophils have previously been shown to promote and sustain M2 macrophage activation [360, 361]. As there was no eosinophil recruitment in the mice treated with anti-IL-5, the frequency of $F4/80^{+}$ LPMs in the peritoneal cavity was greater so the enhanced expression of the M2 markers may simply be due to the presence of more macrophages in the peritoneal cavity. The relationship between the two cell types has to be further investigated.

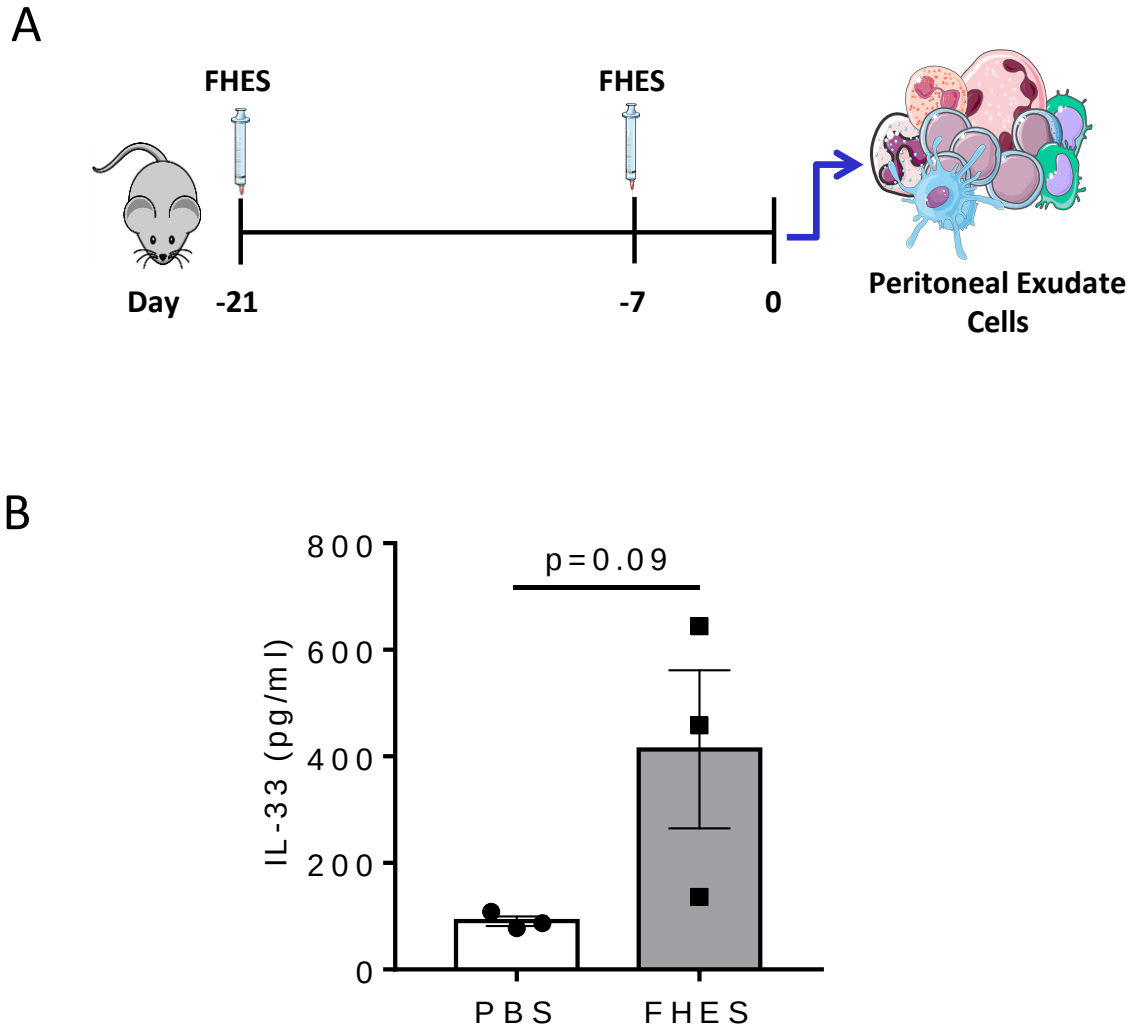


Figure 4.1: FHES induces IL-33 production *in vivo*.

C57BL/6 mice were injected s.c. with PBS (vehicle control) or FHES (100 µg/mouse/day) on days -21 and -7 (n=3). On day 0, mice were sacrificed, and peritoneal exudate cells isolated and pelleted by centrifugation (A). The concentration of IL-33 in the supernatants ('PEC fluid') was determined by ELISA. Data from the mice is presented as means +/- SEM (n=3). Statistical assessment was performed by Student's t-test vs. vehicle control.

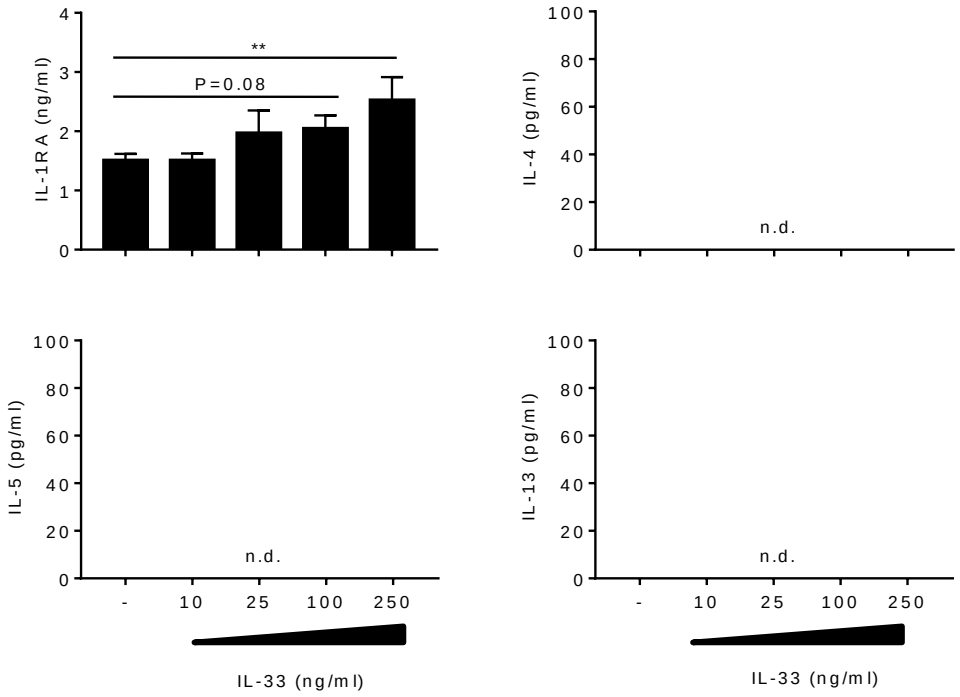


Figure 4.2: IL-33 induces IL-1RA production by bone marrow-derived macrophages. M-CSF-expanded bone marrow macrophages generated from C57BL/6 mice were stimulated for 24h with IL-33 (10, 25, 100, 250 ng/ml) or medium control. The concentrations of IL-1RA, IL-4, IL-5, and IL-13 in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test. **P<0.01.

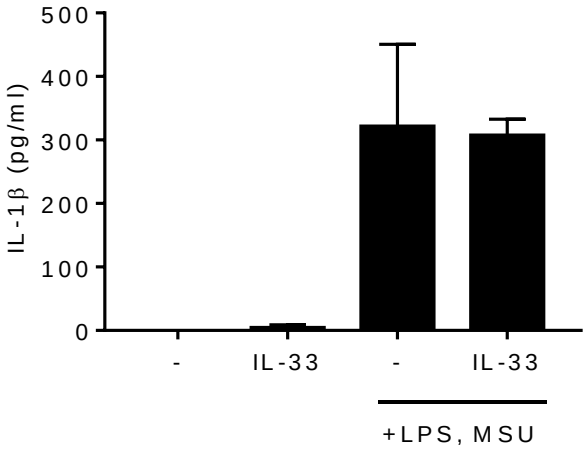


Figure 4.3: IL-33 does not suppress MSU-induced inflammasome activation in macrophages.

M-CSF-expanded bone marrow macrophages generated from C57BL/6 mice were treated for 24h with IL-33 (250 ng/ml) or medium control +/- LPS (100 ng/mL) and MSU (250 µg/ml). The concentration of IL-1β in the supernatant was determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test.

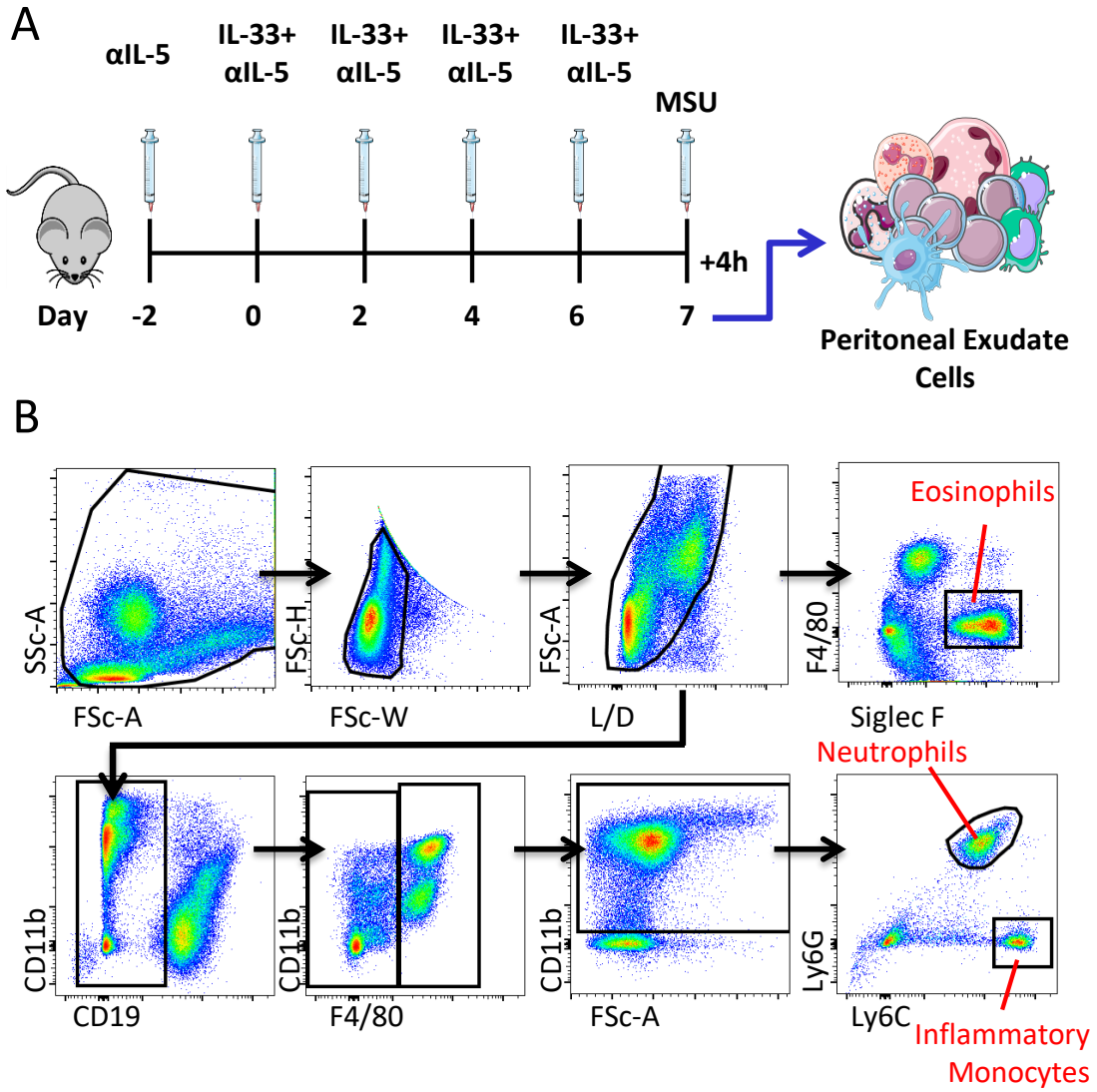


Figure 4.4: Experimental design and flow cytometric gating strategy to examine the ability of IL-33 to modulate MSU crystal-induced inflammation.

(A) C57BL/6 mice were injected i.p. with PBS or anti-IL-5 (80 µg/mouse/day) on days -2, 0, 2, 4, and 6 and IL-33 (200 ng/mouse/day) or PBS on days 0, 2, 4, and 6 (n = 5). On day 7 the mice were injected with MSU crystals (250 µg/mouse). Naïve mice were also injected with PBS as a control (n = 3). Four hours post-MSU crystal/PBS injection, peritoneal exudate cells were isolated for flow cytometry analysis. (B) Gating strategy to identify eosinophils, neutrophils, and inflammatory monocytes.

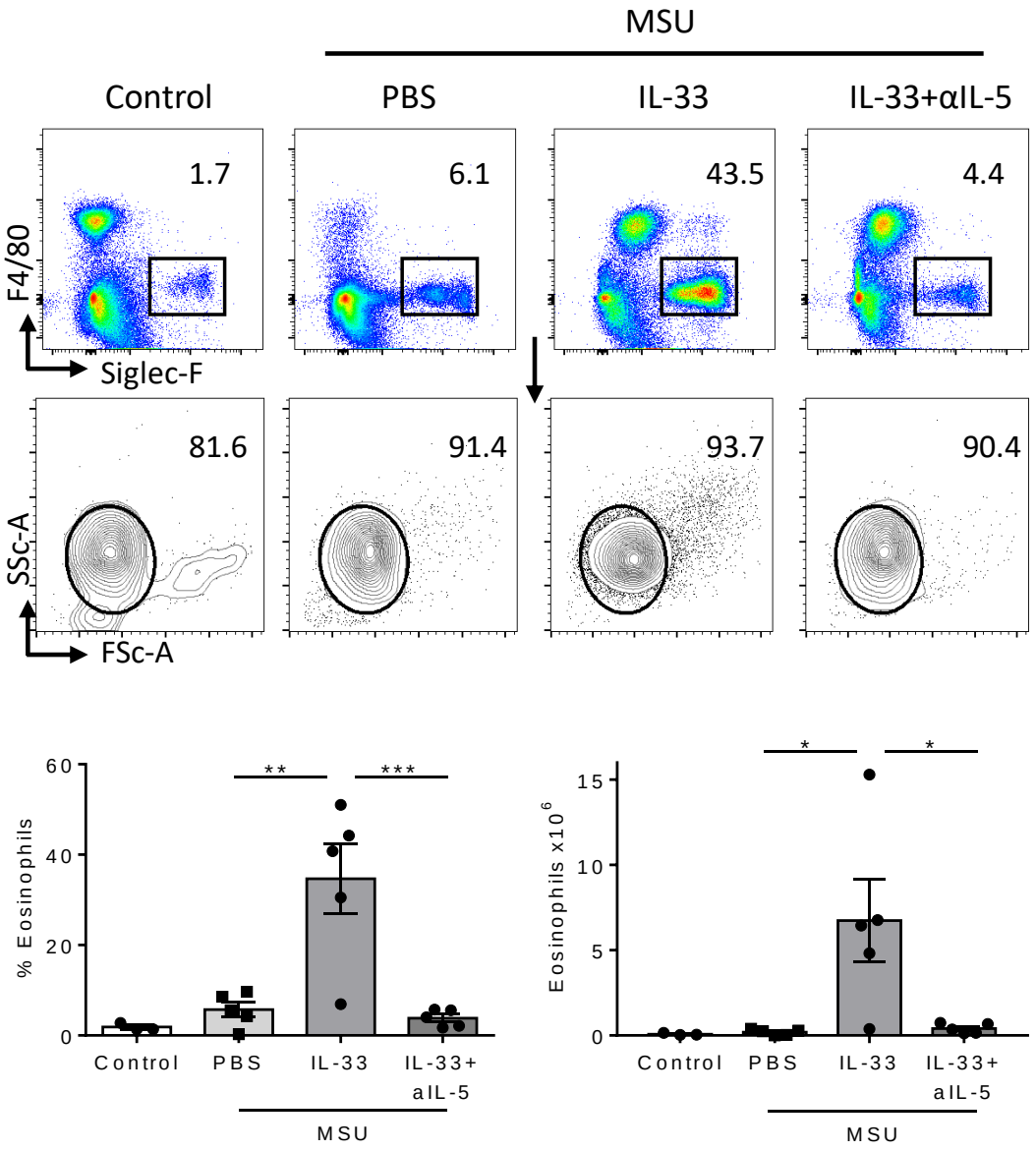


Figure 4.5: I.p. injection of mice with IL-33 induces eosinophil recruitment, which is blocked by anti-IL-5.

C57BL/6 mice were injected with IL-33 +/- anti-IL-5 or PBS and challenged with MSU crystals (as described in Figure 4.4). Peritoneal exudate cells were isolated and flow cytometry analysis was performed. Eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile. Data from the mice is presented as means +/- SEM (n=3-5). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test. * P<0.05, ** P<0.01, *** P<0.001.

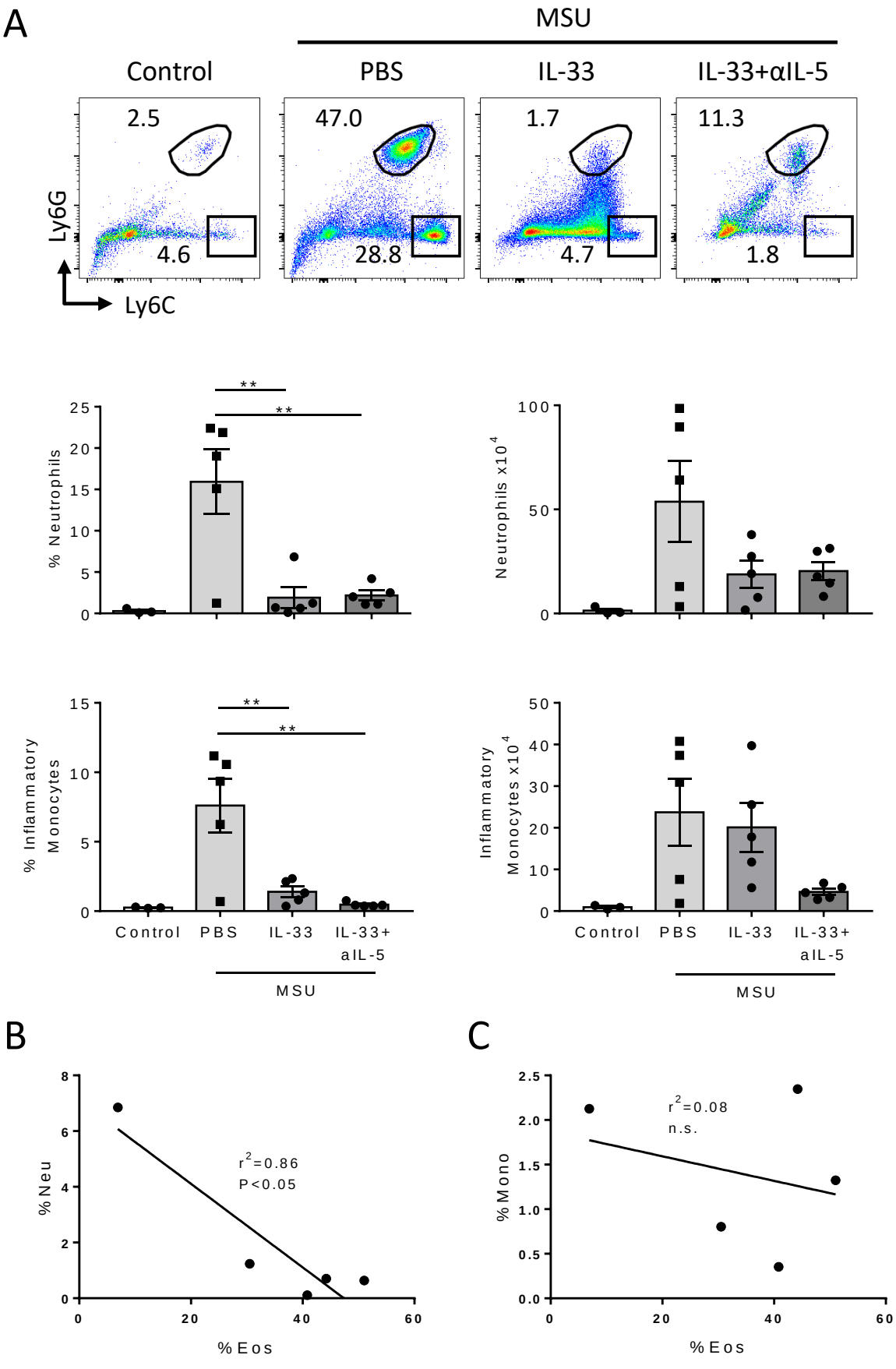

A
MSU
Control
PBS
IL-33
IL-33+αIL-5
2.5
47.0
1.7
11.3
4.6
28.8
4.7
1.8
Ly6G
Ly6C
% Neutrophils
Neutrophils x10^4
% Inflammatory Monocytes
Inflammatory Monocytes x10^4
**
**
Control
PBS
IL-33
IL-33+ aIL-5
MSU
B
% Neu
% Eos
r^2=0.86
P<0.05
C
% Mono
% Eos
r^2=0.08
n.s.

Figure 4.6: I.p. injection of mice with IL-33 inhibited the recruitment of neutrophils and inflammatory monocyte into the peritoneal cavity s and was not reversed by anti-IL-5.

C57BL/6 mice were injected with IL-33 +/- anti-IL-5 or PBS and challenged with MSU crystals (Figure 4.4). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Neutrophils and inflammatory monocytes were identified as $Ly6G^{+}$ and $Ly6C^{+}$, $CD19^{-}$, $F4/80^{-}$, $CD11b^{+}$ cells respectively (A). Data from the mice is presented as means +/- SEM (n=2-5). Correlation plots between the frequency of eosinophils (Figure 4.5) and neutrophils (Figure 4.5A) or inflammatory monocytes recruited (Figure 4.6B) for the IL-33-treated group were produced (B-C). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Curves were fitted by linear regression using GraphPad Prism 7.00.

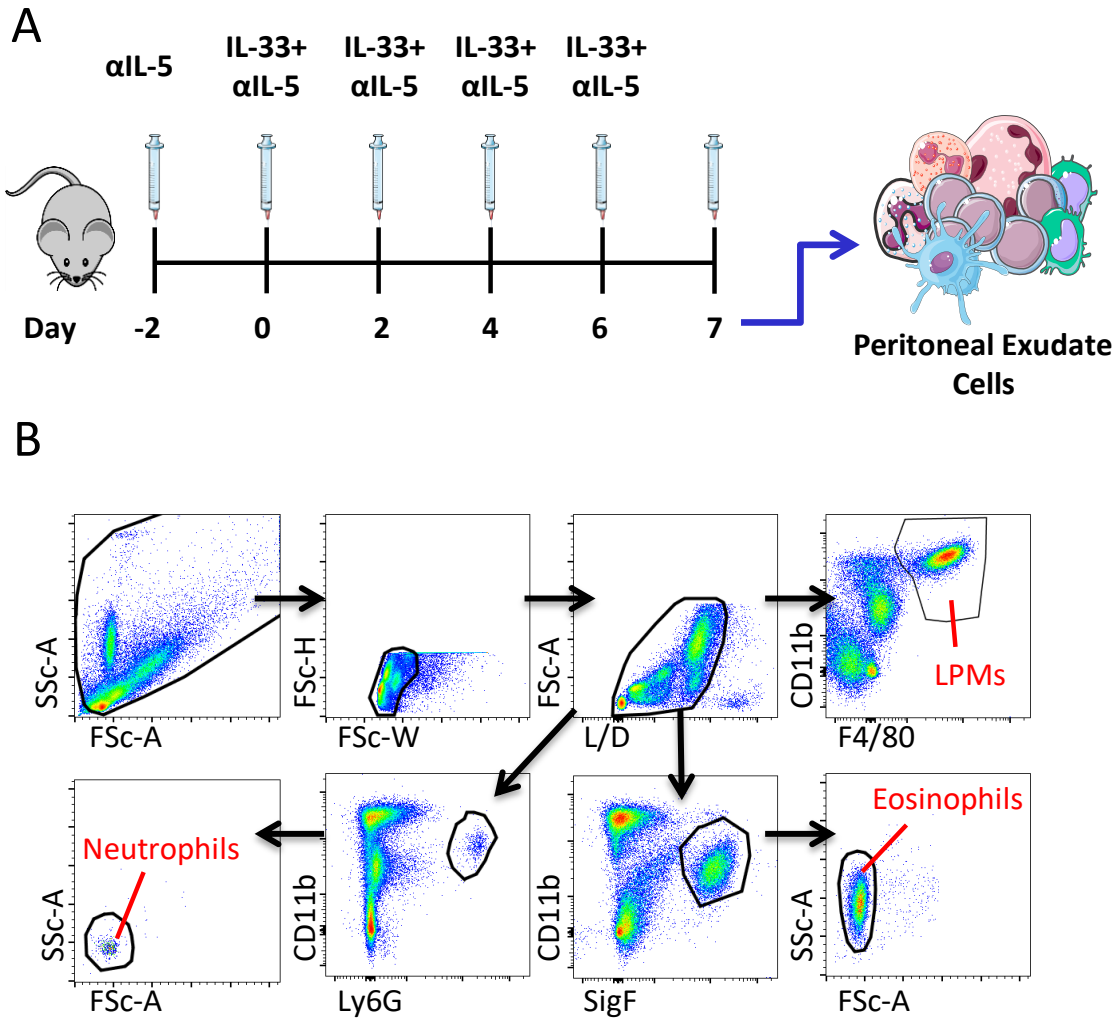


Figure 4.7: Experimental design and flow cytometric gating strategy to examine the ability of IL-33 to induce M2 macrophage activation.

(A) C57BL/6 mice were injected i.p. with PBS or anti-IL-5 (80 µg/mouse/day) on days -2, 0, 2, 4, and 6 and IL-33 (200 ng/mouse/day) or PBS on days 0, 2, 4, and 6 (n = 4-5). On day 7 peritoneal exudate cells were isolated for flow cytometry analysis. (B) Gating strategy to identify eosinophils, neutrophils, and LPMs.

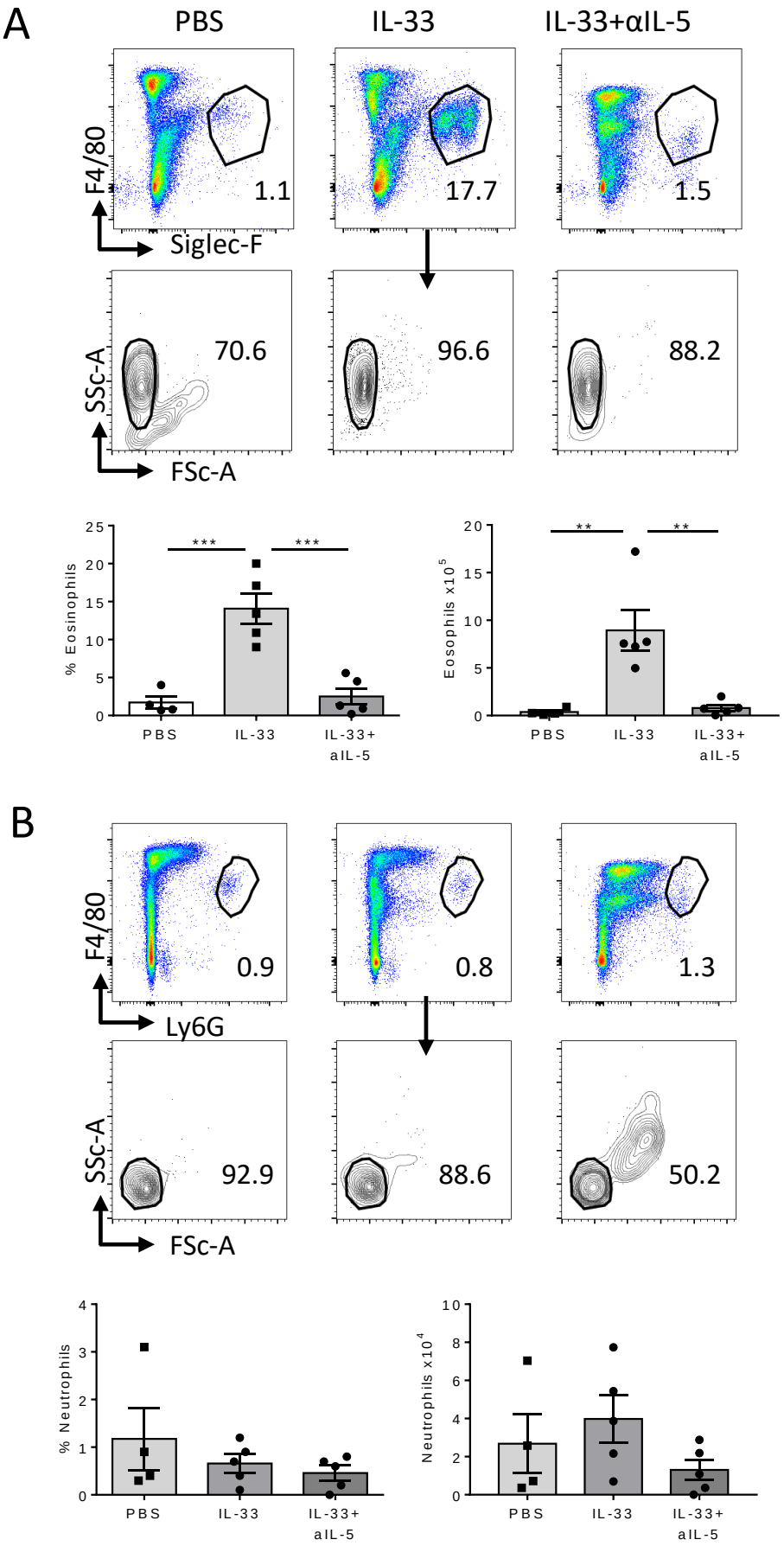
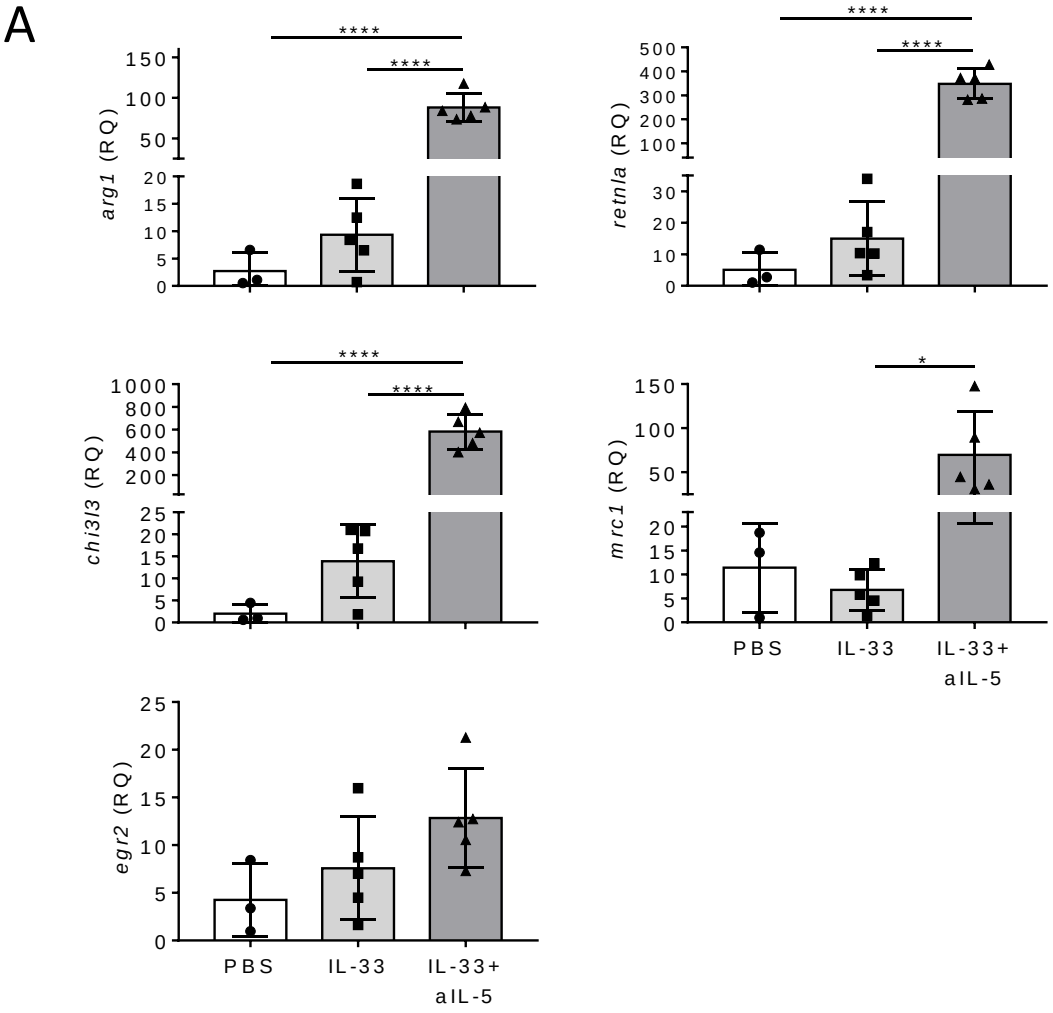

A
PBS
IL-33
IL-33+αIL-5
F4/80
Siglec-F
1.1
17.7
1.5
SSc-A
FSc-A
70.6
96.6
88.2
% Eosinophils
Eosophils x10^5
***
**
PBS
IL-33
IL-33+ aIL-5
B
F4/80
Ly6G
0.9
0.8
1.3
SSc-A
FSc-A
92.9
88.6
50.2
% Neutrophils
Neutrophils x10^4

Figure 4.8: I.p. injection of mice with IL-33 induces eosinophil recruitment, an effect blocked by anti-IL-5.

C57BL/6 mice injected with IL-33 +/- anti-IL-5 or PBS (Figure 4.7). Peritoneal exudate cells were isolated and flow cytometry analysis was performed. Eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile (A). Neutrophils were identified by the expression of the surface marker Ly6-G and confirmed by their low SSc-A and FSc-A profile (B). Data from the mice is presented as means +/- SEM (n=4-5). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.


A
arg1 (RQ)
retnla (RQ)
chi3l3 (RQ)
mrc1 (RQ)
egr2 (RQ)
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*
PBS
IL-33
IL-33+ aIL-5

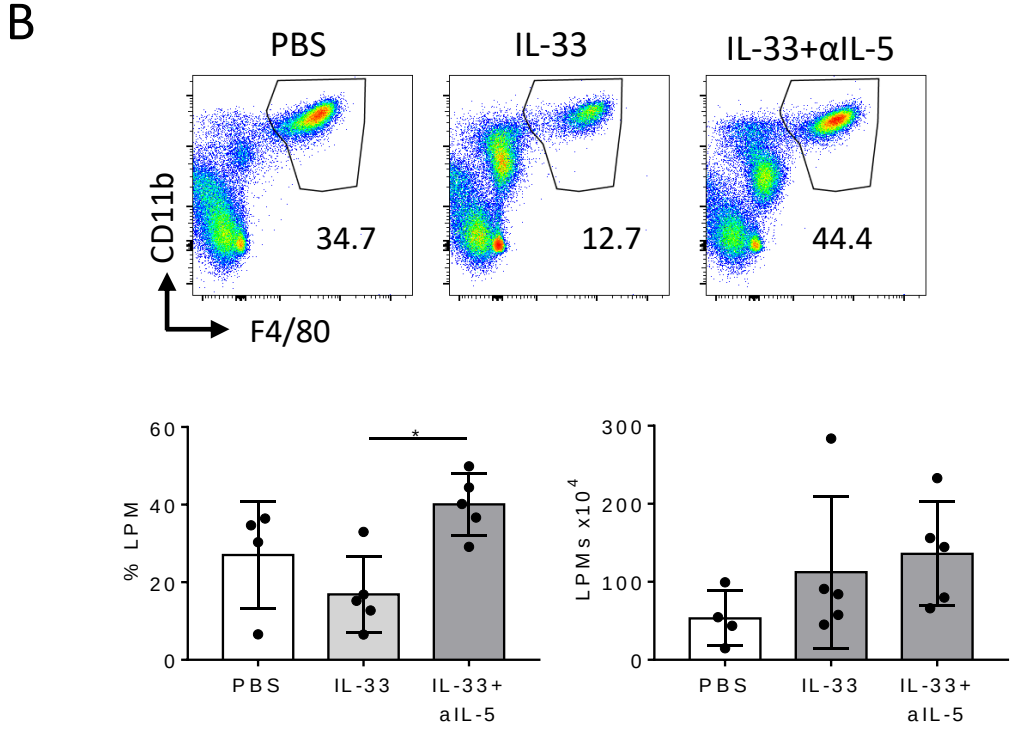

B
PBS
IL-33
IL-33+αIL-5
CD11b
F4/80
34.7
12.7
44.4
% LPM
LPMs x10^4
*
PBS
IL-33
IL-33+ aIL-5

Figure 4.9: Inhibition of eosinophil recruitment with anti-IL-5 enhances IL-33-induced M2 macrophage polarisation.

C57BL/6 mice injected with IL-33 +/- anti-IL-5 or PBS (Figure 4.7). Peritoneal exudate cells were isolated and flow cytometry and rtPCR analysis was carried out. RNA was extracted from some cells for cDNA synthesis. The expression of Arg1, Chi3l3, Retnla, Egr2, and Mrc1 was determined by rtPCR (A). Other cells were surface stained for flow cytometry analysis. LPMs were identified as $F4/80^{hi}$, $CD11b^{+}$ cells (B). Data from the mice is presented as means +/- SEM (n=3-5). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test. * $P<0.05$, **** $P<0.0001$.

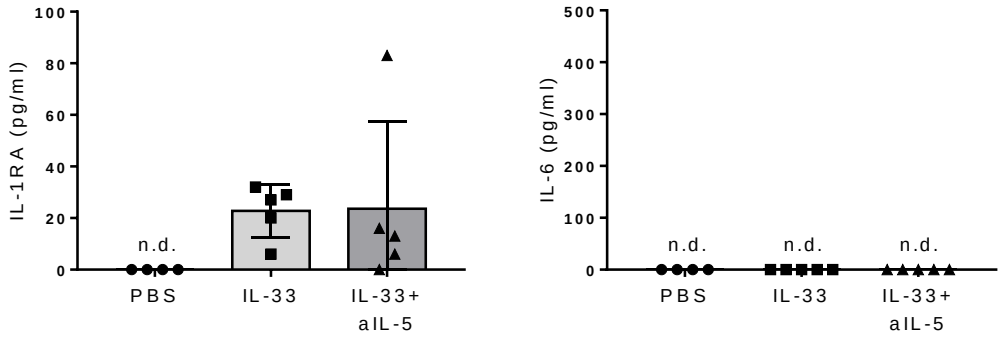


Figure 4.10: IL-33 induced IL-1RA production *in vivo*, which was not reversed by anti-IL-5.

C57BL/6 mice were injected with IL-33 +/- αIL-5 or PBS (Figure 4.7). Peritoneal exudate cells were isolated and pelleted by centrifugation. The concentration of IL-1RA and IL-6 in the supernatants ('PEC fluid') determined by ELISA. Data from the mice is presented as means +/- SEM (n=4-5). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test.

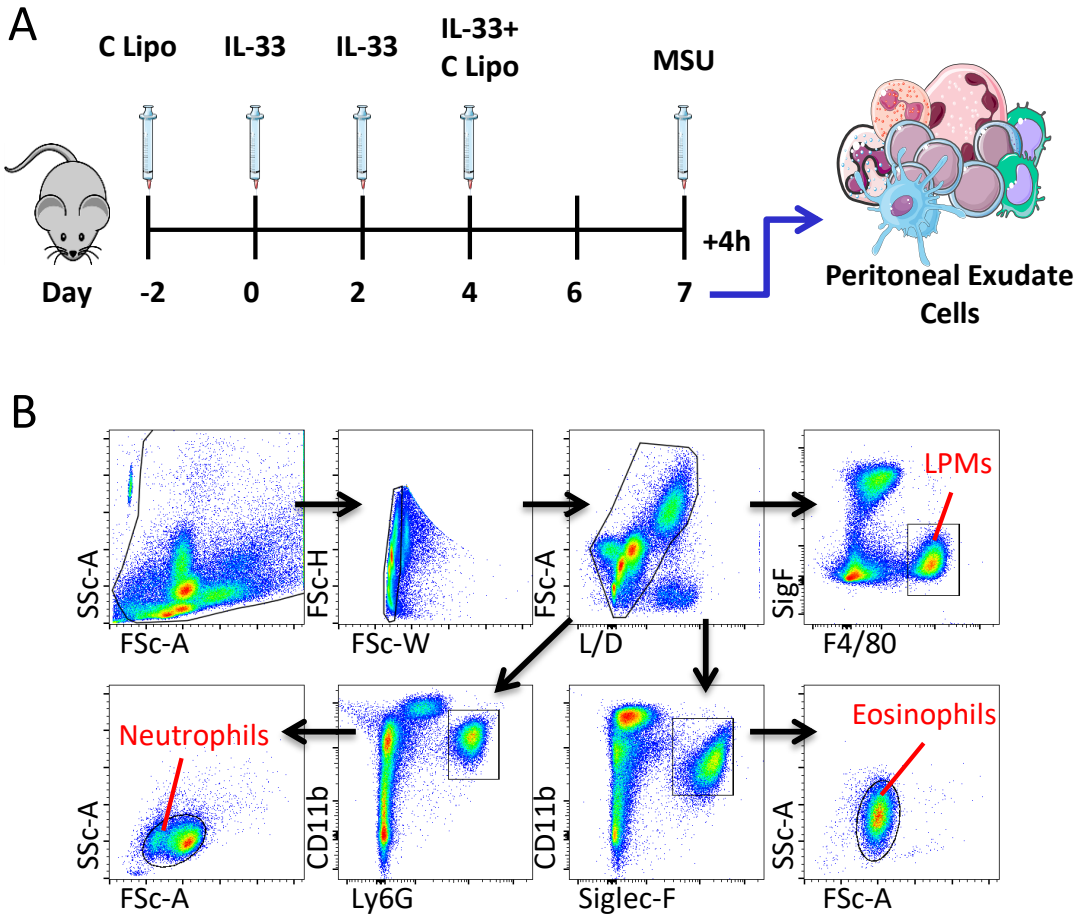


Figure 4.11: Experimental design and flow cytometric gating strategy to examine the role of macrophages in the ability of IL-33 to modulate MSU crystal-induced inflammation.

(A) C57BL/6 mice were injected i.p. with PBS or clodronate liposomes (200 µl/mouse) on day -2, PBS or clodronate liposomes (100 µl/mouse) day 2, and PBS or IL-33 (200 ng/mouse/day) on days 0, 2, and 4 (n=5). On day 7, the mice were injected with MSU (250 µg/mouse). Naïve mice were also injected with PBS as a control (n=2). Four hours post-MSU crystal/PBS injection, peritoneal exudate cells were isolated for flow cytometry analysis. (B) Gating strategy to identify eosinophils, neutrophils, and inflammatory monocytes.

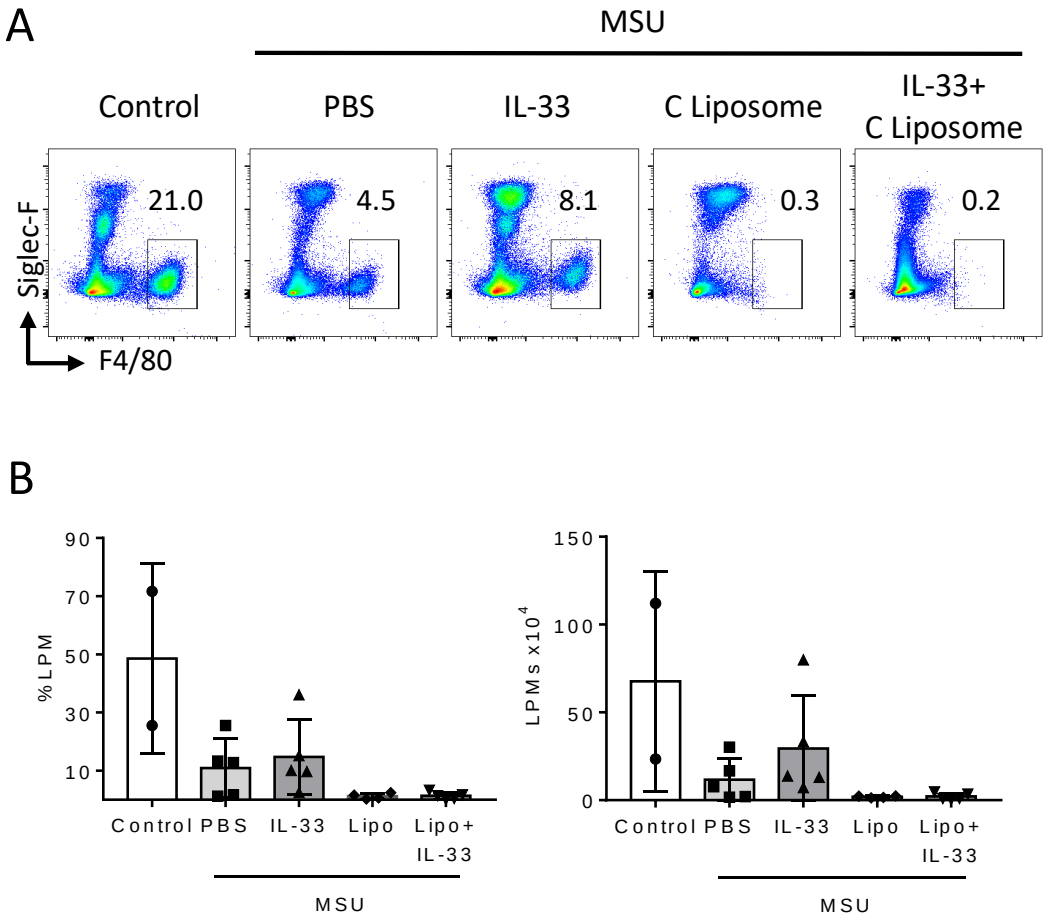


Figure 4.12: I.p. injection of mice with clodronate liposomes depletes macrophages in the peritoneal cavity.

C57BL/6 mice were injected with IL-33 +/- clodronate liposomes or PBS (Figure 4.11). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Macrophages were identified as F4/80^{+} cells (A). Data from the mice is presented as means +/- SEM (n=2-5) (B). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test.

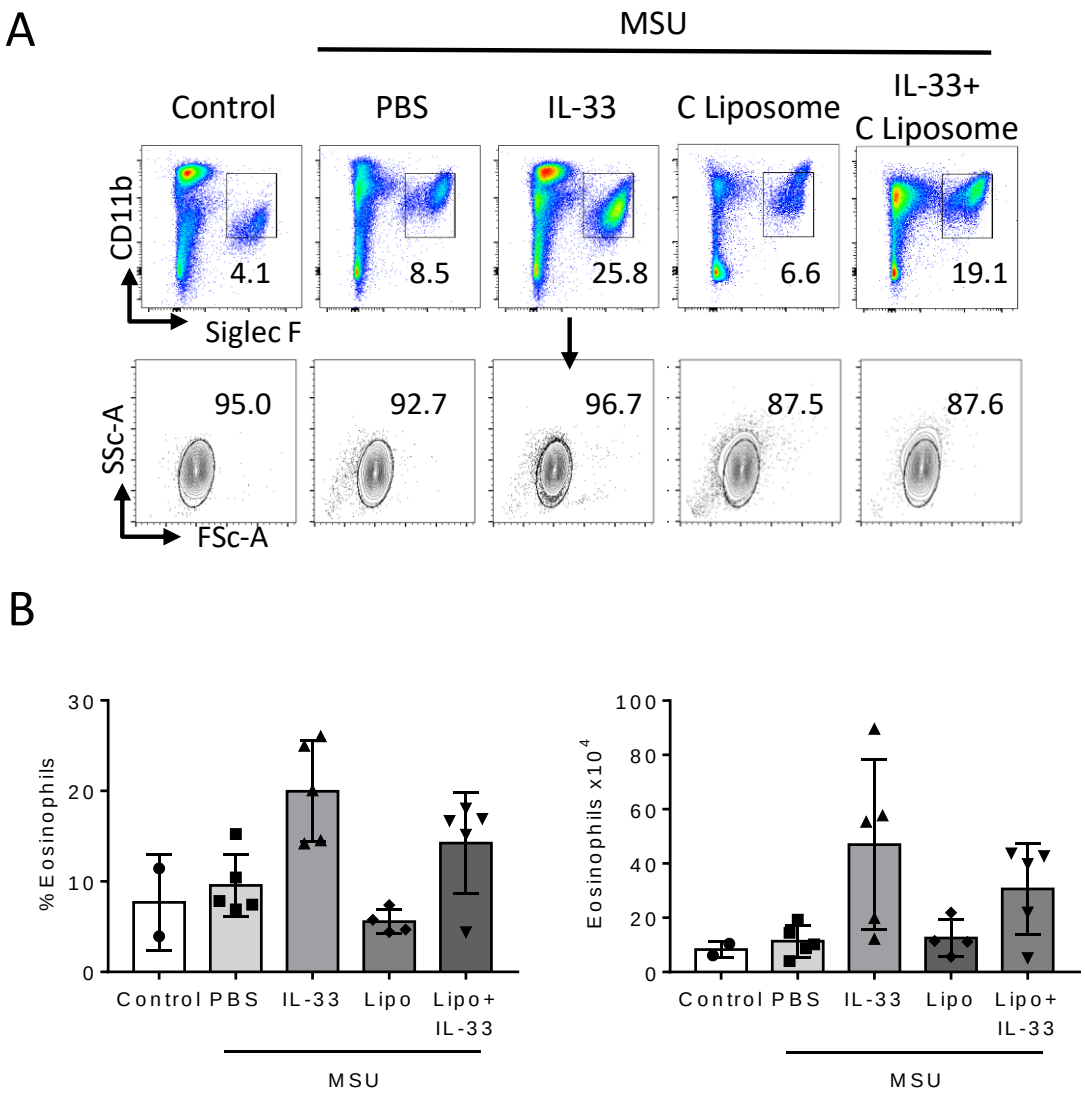


Figure 4.13: IL-33 induces eosinophil recruitment into the peritoneal cavity.

C57BL/6 mice injected with IL-33 +/- clodronate liposomes or PBS (Figure 4.11). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile (A). Data from the mice is presented as means +/- SEM (n=2-5). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test.

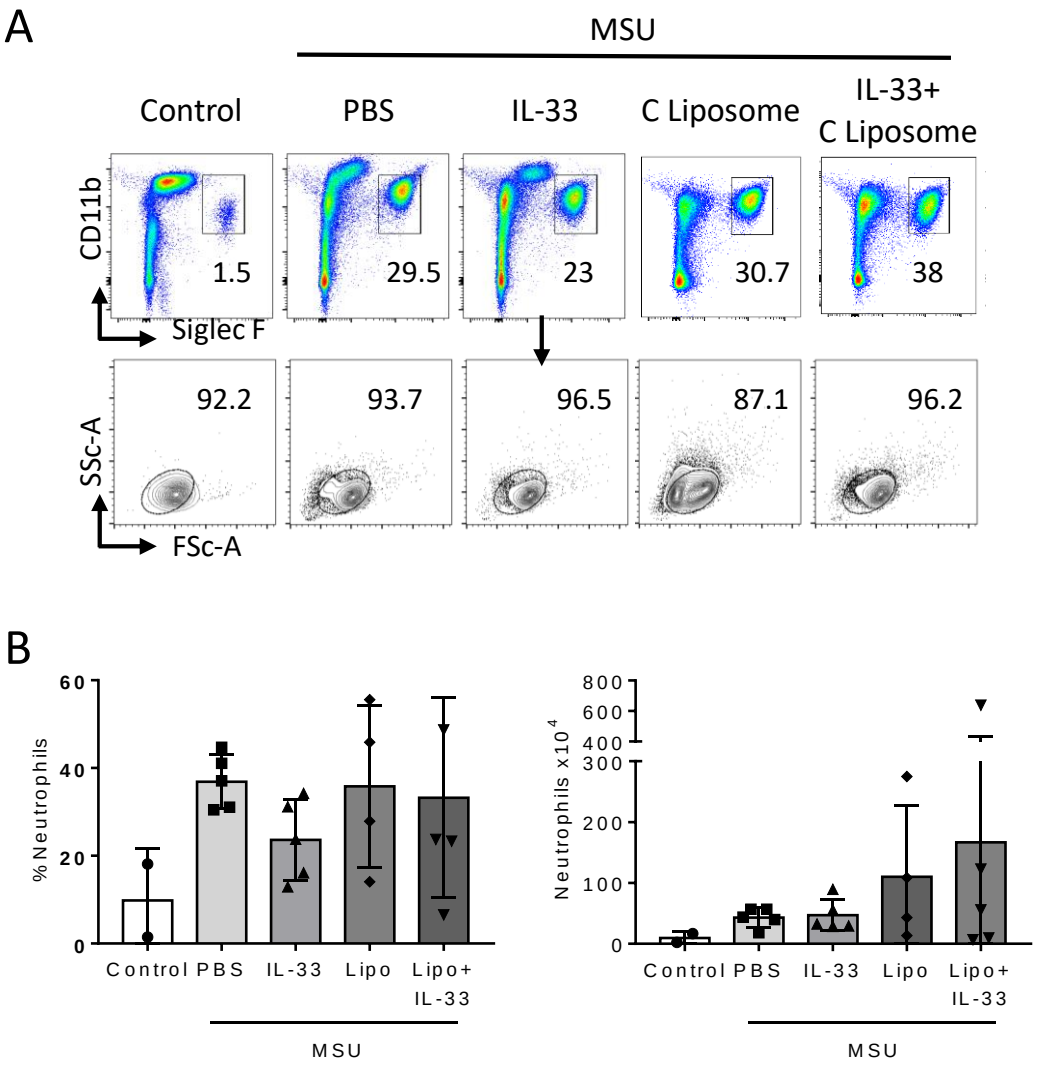


Figure 4.14: I.p. injection of mice with IL-33 attenuated the recruitment of neutrophils into the peritoneal cavity, an effect inhibited by clodronate liposomes injection.

C57BL/6 mice injected with IL-33 +/- clodronate liposomes or PBS (Figure 4.11). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Neutrophils were identified by the expression of the surface marker Ly6-G and confirmed by their low SSc-A and FSc-A profile (A). Data from the mice is presented as means +/- SEM (n=2-5) (B). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test.

Chapter 5

Isolation and characterisation of *Fasciola hepatica* protein FHKTM

5.1 Introduction

Infection with helminth parasites or treatment with helminth products, including FHES, can ameliorate the clinical signs and pathology in mouse models of autoimmune disease [157, 205]. The overall aim of my project was to identify individual helminth products with immunomodulatory activity that are capable of attenuating autoimmunity in mice. As FHES and *F. hepatica* exosomes are mixtures of many proteins and other molecules, they provide the basis for identifying and characterising individual immunomodulatory components. *F. hepatica* exosomes were isolated and their *in vitro* and *in vivo* profiles were examined (Chapter 3). Parallel to this, I have identified and characterised an individual protein from FHES, FHKTM (*F. hepatica* Kunitz-type molecule).

Several helminth-derived proteins have already been characterised. *Ancylostoma caninum* is a blood-feeding hookworm that releases excretory/secretory products (ES) similar to FHES. A mass spectrometry analysis of processed *Ancylostoma caninum* ES [298] revealed the presence of the tissue inhibitor of metalloproteases (TIMP) anti-inflammatory protein-2 (AIP-2) [362]. Recombinant AIP-2 protected mice against asthma via the expansion of mesenteric $CD103^+$ DCs leading to the development and migration of Treg cells to the mucosa [299]. Individual proteins produced by *F. hepatica* have also been previously isolated and characterised. FhHDM-1 (*F. hepatica* helminth defence molecule), a protein similar to mammalian cathelicidin-like host defence peptides [363], has been identified in FHES. FhHDM-1 inhibited the activation of pro-inflammatory macrophages, prevented the development of type 1 diabetes, and was protective in a relapse remitting model of EAE [243].

Previous research in the Mills lab aimed at identifying individual *F. hepatica* proteins involved the fractionation of FHES by size-exclusion chromatography. The fractions were assayed *in vivo* for the ability to mimic the ability of FHES injection to promote type-2 responses (eotaxin production). Positive fractions were analysed by mass spectrometry. Several candidate proteins were identified, cloned, transformed into *Pichia pastoris* yeast, expressed, and purified. One of the discovered proteins was FHKTM.

FHKTM is a relatively novel helminth-derived protein described in only a few publications to date. Bozas et al (1995) isolated FHKTM from *F. hepatica* describing it as

a 58 amino acids single polypeptide with a molecular mass of 6751 g/mol[364]. It has amino acid sequence homology to the Kunitz-type family of protease inhibitors, proteins which can regulate inflammation and tissue damage, and tissue repair by inhibiting proteases [365]. FHKTM has also been identified in *F. hepatica* exosome-like extracellular vesicles [290].

The Kunitz-type family are examples of serine protease inhibitors. They are found in almost living organisms, prokaryote and eukaryotes, from bacteria to plants [366]. Helminth-derived protease inhibitors are important for the development and survival of helminths as they can attenuate immune responses in the host [367]. The Kunitz-type serine protease inhibitor *Sm*KI-1 is produced by the helminth *Schistosoma mansoni* [368]. This protein inhibits neutrophil elastase, a serine protease produced by neutrophils after their activation. In several models of inflammation including gouty arthritis, *Sm*KI-1 inhibited neutrophil function and reduced tissue damage [366]. The identification of helminth-derived protease inhibitors may be important as diagnostic targets and in anti-parasite vaccine development. These inhibitors also have the potential to be utilised as novel therapies against inflammatory diseases, either as therapeutic recombinant proteins or in helping us to identify new drug targets.

To date there is has only been one functional description of FHKTM in the literature. Falcon et al (2014) identified FHKTM as a component of *F. hepatica* total extract (FHTE) rather than from FHES which was used in the present study. They demonstrated that FHKTM was capable of inhibiting LPS-induced pro-inflammatory cytokine production in DCs [369]. Inhibition of DCs could be of particular importance for *F. hepatica* as they are found in larger numbers in the liver compared with other organs, including the lungs and kidneys [370]. FHKTM also suppressed LPS-induced production of IFN-γ and IL-17 by spleen cells [369].

The specific aims were:

- To express and purify FHKTM from *P. pastoris* yeast cells.
- To examine the immunomodulatory effects of FHKTM on macrophages and other immune cells and to investigate whether they can promote an anti-inflammatory response.
- To assay the ability of the candidate protein to attenuate inflammatory pathology *in vivo* in mice.

5.2 Results

5.2.1 FHKTM was expressed and purified from *P. pastoris* yeast cells.

Work in the Mills lab had identified FHKTM as individual components of FHES. The gene encoding FHKTM was His-tagged and synthesised, cloned into a pPIC9 expression vector (Figure 5.1) and transformed into *P. pastoris* yeast. The protocol for expressing and purifying His-tagged FHKTM from the yeast stocks was optimised over several months and a method was developed for the reliable production of the protein (Figure 5.2). Initially the yeast cell culture supernatants (containing secreted FHKTM) were collected and purified by incubating the supernatants with NiNTA beads O/N at 4°C. As the FHKTM protein produced by the yeast is His-tagged, it will bind to the beads, which contain Ni^{2+}. Ni^{2+} ions have a high affinity for histidine residues [371]. Following incubation, the supernatants/beads were centrifuged to recover the beads with bound FHKTM. To release the protein, a buffer with a high concentration of imidazole was added to the bead pellet; the imidazole competed with FHKTM to bind to the Ni^{2+} and so the protein eluted and was collected in the supernatant following a further centrifugation step.

However, when attempting to confirm the presence of purified FHKTM post-NiNTA purification, Coomassie stain and Western blot revealed that FHKTM was not present in the 'purified' fraction (Figure 5.3A). This indicated that there was an issue either with the expression or the purification of FHTKM. A sample of the yeast cell supernatants (prior to any purification steps) was run on a gel and stained with Coomassie Brilliant Blue R-250. A band corresponding to FHKTM could be seen (Figure 5.3B). This confirmed that the cells were successfully expressing FHKTM suggesting the problem was with the protein purification method. After extensive troubleshooting, the purification protocol was altered. A 1 M potassium phosphate buffer was initially added as component of both the BMGY and BMMY media used to grow the yeast and induce protein expression. However, at high pHs potassium phosphate has a tendency to precipitate due to the very low solubility of orthophosphate (HPO_4^{2-}) [372]. The purification protocol calls for the adjustment of the pH of the yeast cell supernatants (containing the potassium phosphate buffer as part of the BMMY) to 8 to allow optimum binding to the NiNTA beads. At this pH a precipitate formed when the supernatants were incubated O/N at

4°C with the beads and this interfered with the ability to centrifuge the beads/protein complex the following day. It was decided to replace the potassium phosphate buffer with a sodium hexametaphosphate buffer, a non-phosphate-precipitate forming compound, as an alternate phosphate source [372]. It was also decided to purify the protein using the NiNTA beads by column chromatography rather than O/N incubation. Having made these alterations, a new batch of FHKTM was expressed and purification was attempted. Analysis of the new sample FHKTM post-NiNTA purification by Coomassie Brilliant Blue R-250 stain and by Western blot analysis using HisProbe-HRP (which binds His-tagged proteins) confirmed the presence of purified FHKTM (Figure 5.4).

5.2.2 FHKTM does not promote eosinophilia *in vivo*.

Having successfully prepared recombinant FHKTM, I investigated if FHKTM could induce eosinophil recruitment *in vivo* as *F. hepatica* infection leads to the mobilisation and expansion of eosinophils [201]. Furthermore, FHES [157] and *F. hepatica* exosomes (Chapter 3) induce eosinophilia. C57BL/6 mice were injected i.p. with FHKTM (2.5 μg/mouse/day), PBS or FHES (100 μg/mouse/day) as a positive control on days 0, 2, 4, and 6. On day 7 peritoneal exudate cells were collected by lavage and stained with antibodies specific for CD11b and Siglec F and analysed by flow cytometry (Figure 5.5A). Eosinophils were identified as Siglec-F^+ cells and confirmed by their characteristic high side scatter and low forward scatter profile (Figure 5.5B). FHES induced an increase in the frequency and absolute numbers of eosinophils in the peritoneal cavity. However, FHKTM injection did not promote the infiltration or expansion of eosinophils in the peritoneal cavity (Figure 5.6).

5.2.3 FHKTM does not suppress MSU-induced inflammation.

Data from Chapters 3 and 4 suggest that eosinophils do not mediate the suppression of neutrophil infiltration into the peritoneal cavity that was observed with *F. hepatica* exosomes and IL-33 in the MSU crystal model of sterile peritonitis (Figure 3.20). Therefore, while i.p. injection of FHKTM did not promote eosinophil recruitment, it was investigated whether the *F. hepatica* protein could still inhibit MSU-induced inflammation. C57BL/6 mice were injected i.p. with FHKTM (2.5 μg/mouse/day) or PBS

on days 0, 2, 4, and 6 followed by injection with MSU (250 μg/mouse) or PBS on day 7. After 4 hours, peritoneal exudate cells were isolated by lavage and stained with antibodies specific for CD11b, Siglec F, Ly6G, and Ly6C and analysed by flow cytometry (Figure 5.7). FHKTM did not induce an increase in the frequency or absolute number of eosinophils in the peritoneal cavity (Figure 5.8A). Neutrophils were identified as $Ly6G^+$ cells with low side scatter and forward scatter profiles. Injection of MSU enhanced the number of neutrophils in the peritoneal cavity. FHKTM injection was unable to attenuate this recruitment (Figure 5.8B). Inflammatory monocytes were identified by their high expression of Ly6C (Figure 5.9). MSU-induced inflammatory monocyte infiltration was not supressed by the injection of FHKTM.

5.2.4 FHKTM induces cytokine production and enhance IL-4-induced M2 macrophage polarisation.

FHKTM administration did not mimic the activity of FHES or *F. hepatica* exosomes *in vivo*. An experiment was designed to investigate the immunomodulatory activity of FHKTM *in vitro* and to see if it mimicked the results seen with *F. hepatica* exosomes in Chapter 3. BMDMs and peritoneal exudate cells were treated with FHKTM (2.5 μg/mL) or medium. After 24 hours the supernatants were removed, and the concentrations of cytokines determined by ELISA. Stimulation of BMDMs or peritoneal exudate cells with FHKTM induced IL-1RA, IL-10 and IL-6 production (Figure 5.10).

Having shown that FHKTM, like *F. hepatica* exosomes, induced IL-1RA production in peritoneal exudate cells and macrophages, a dose/response study was undertaken to investigate whether smaller concentrations could induce cytokine production. BMDMs and BMDCs were treated for 24 hours with increasing concentrations of FHKTM (0.1, 0.25, 1. 2.5 μg/ml) or medium. IL-1RA concentration was determined by ELISA. FHKTM at a concentration of 2.5 μg/ml induced significant production of IL-1RA by the macrophages and DCs (Figure 5.11). IL-1RA production was also induced to a lesser extent in BMDMs by FHKTM at a concentration of 1 μg/ml.

Helminth infection promotes the alternate activation of macrophages [201]. IL-4 polarises macrophages toward an alternatively activated M2 state and is used *in vitro* to induce the expression of alternate activation markers [154]. Therefore, it was

investigated if FHKTM could promote the expression of alternate activation markers and/or enhance IL-4-mediated alternate activation marker expression on macrophages. Treatment with FHKTM alone promoted arginase activity in peritoneal exudate cells (Figure 5.12). FHKTM also enhanced IL-4-induced arginase activity in BMDMs and peritoneal exudate cells. To further examine the ability of FHKTM to modulate IL-4-mediated gene expression, BMDMs were treated for with FHKTM (2.5 μg/mL) or medium +/- IL-4 (10 ng/mL). After 24 hours IL-4 induced Arg1, Mrc1, Retnla, and IL-1RN expression. Stimulation of BMDMs with FHKTM alone did not induce the expression of M2-associated genes but FHKTM did enhance IL-4-mediated Arg1, Mrc1 and IL-1RN expression, while suppressing IL-4-mediated Retnla expression (Figure 5.13). This is consistent with the findings that FHKTM enhanced IL-4-induced arginase activity in BMDMs and peritoneal exudate cells. These results demonstrate that FHKTM promotes anti-inflammatory type 2 macrophages.

5.2.5 Acute FHKTM administration does not induce IL-1RA production *in vivo*.

AJoosten et al (2016) described Alpha-1-anti-trypsin-Fc fusion protein (AAT-Fc), a novel recombinant form of human alpha-1-anti-trypsin (AAT) [373]. AAT-Fc, like FHKTM, is a serine protease inhibitor and induced IL-1RA production *in vitro*. AAT-Fc injection induced IL-1RA production *in vivo* and inhibited MSU-induced joint inflammation. AAT-Fc inhibition was fast-acting, shown when the protein was administered at the same time as MSU. However, AAT-Fc also induced more prolonged protection for up to two days; the protective effect was demonstrated when AAT-Fc was injected 48 hours prior to MSU injection but was lost when injected 72 hours prior to MSU. As FHKTM induced IL-1RA production in vitro and has similar properties to AAT-Fc, it was investigated whether acute administration of FHKTM could suppress MSU inflammation as AAT-Fc did. As AAT-Fc injection 2 hours prior to MSU administration induced a strong protective effect, it was decided to repeat this time course. C57BL/6 mice were injected i.p. with FHKTM (2.5 μg/mouse) or PBS and then with MSU (250 μg/mouse) 2 hours later. Four hours post-MSU injection, peritoneal exudate cells were isolated and stained with antibodies specific for CD11b, Ly6G, and Ly6C and analysed by flow cytometry (Figure 5.14). FHKTM injection 2 hours prior to MSU challenge did not attenuate neutrophil or inflammatory monocyte infiltration (Figure 5.15).

Having demonstrated that FHKTM does not suppress MSU-induced inflammation, an experiment was designed to investigate whether FHKTM injection could induce IL-1RA production *in vivo*. C57BL/6 mice were injected with FHKTM (at 6 hours, 4 hours, or 2 hours prior to sacrifice) or PBS, serum was collected immediately, and diluted as described (Figure 5.16A). For the 6 hours prior to serum collection group, insufficient volumes were collected to be able to do a 1 in 2 dilution of sample prior to analysis by ELISA. Therefore, two separate sample dilutions (1 in 2 and 1 in 4) were performed and the concentration of IL-1RA in the serum determined by ELISA (Figure 5.16B). No IL-1RA could be detected at any of the time points.

5.2.6 FHKTM attenuates LPS-induced inflammation.

In the only functional characterisation of FHKTM in the literature to date, the protein was shown to inhibit LPS-induced production of IL-12p70, TNF and IL-6 from DCs. Therefore, I examined the ability of recombinant FHKTM could inhibit pro-inflammatory cytokine production by DCs. BMDCs were treated with FHKTM (2.5 μg/ml) or medium +/- LPS (100 ng/ml). After 24 hours, the concentrations of IL-12p70, IL-1β, TNF, IL-6, and IL-23 in the supernatants were determined by ELISA. The results demonstrate that FHKTM suppressed LPS-induced production of IL-12p70, IL-1β, and TNF, whereas LPS-induced IL-6 and IL-23 production was not significantly reduced (Figure 5.18).

LPS is an inducer of classical M1 macrophage activation [24]. Having demonstrated that FHKTM can promote IL-4-mediated alternative activation of macrophages, an experiment was carried out to determine if the *F. hepatica* protein could modulate LPS-induced gene expression. BMDMs were treated with FHKTM (1 μg/ml) or medium +/- LPS (100 ng/ml). After 24 hours the cells were harvested and the expression the M1-associated genes CD38 and Frp2 [25] examined by rtPCR. Stimulation of the macrophages with FHKTM alone did not promote CD38 or Frp2 gene expression (Figure 5.18). LPS induced high CD38 and Fpr2 expression, which was significantly suppressed by FHKTM.

5.2.7 Therapeutic but not prophylactic administration of FHKTM delays EAE disease onset.

The results so far demonstrated that FHKTM induced anti-inflammatory responses and inhibited pro-inflammatory stimulation *in vitro.* An experiment was designed to examine whether FHKTM could inhibit inflammation in the mouse model EAE. EAE is demyelinating, T-cell-mediated autoimmune disease induced in mice by immunization with CNS tissue or myelin antigens. Work in the Mills lab has demonstrated that injection of FHTE can train monocytes to be more anti-inflammatory resulting in the attenuation of EAE in mice treated before the induction of disease (Quinn et al, In review). Furthermore, studies with *F. hepatica* exosomes demonstrated that administration of the exosomes 21 and 7 days prior to EAE induction attenuated disease progression (Chapter 3). Therefore, it was examined if prophylactic administration of FHKTM could suppress disease development. C57BL/6 mice were injected i.p. with FHKTM (2.5 µg/mouse/day) or PBS on days -21 and -7. On day 0 the mice were injected with MOG emulsified in CFA (100 µg/mouse) and PT (200 ng/mouse) and with PT again on day 2 to induce disease (Figure 5.19A). Mice were monitored daily for clinical signs and weight loss. Pre-treatment of mice with FHKTM did not attenuate the development of EAE (Figure 5.19B).

FHKTM promoted the alternate activation of macrophages *in vitro*. Alternate activated macrophages mediate anti-inflammatory responses *in vivo*. Since alternatively activated macrophages have been shown to suppress the development of CNS inflammation in the EAE model [374, 375], experiments were designed to examine the therapeutic effects of FHKTM in this autoimmune disease model. Our lab has demonstrated that infection with live *F. hepatica* and administration of FHES attenuated development of EAE through suppression of pathogenic Th1 and Th17 cell responses in the CNS [157, 205]. C57BL/6 mice were injected i.p. with FHKTM (2.5 µg/mouse/day) or PBS vehicle on days -1, and every subsequent second day until the end of the experiment (Figure 5.20A). EAE was induced and monitored as before. The untreated control mice began to develop clinical signs on approximately day 9 (Figure 5.20B). Treatment of mice with FHKTM delayed disease onset by 5-6 days. Modulation of the course of disease was

reflected in the weight changes; mice treated with FHKTM had significantly less weight loss than the untreated control mice.

5.3 Discussion

Helminths have evolved mechanisms to modulate the host immune response and promote their survival and persistence in the host. As well as suppressing the anti-parasite immune response, helminth-induced immune suppression can simultaneously suppress unrelated pathological inflammation, including autoimmunity [201]. This observation has led to an extension of the 'hygiene hypothesis' which was originally proposed to explain the reduced incidence of asthma/allergy in populations heavily infected with helminth parasites. The rise in the prevalence of autoimmune as well as allergic diseases in developed countries has been linked with increased levels of hygiene and thus reduced exposure to infectious organisms especially helminths which regulate the hosts' immune response [252]. While intentional infection of humans with non-pathogenic helminths has been assessed in clinical trials as a treatment for autoimmune diseases and shown some success, there are obvious concerns around the use of a live parasite as a therapy for human diseases. Even with the use of helminth species that are not pathogenic in humans the feeding and migratory behaviour of the parasite may lead to physiological side effects. As helminths are collected from a live mammalian host, there is also the risk of contamination with other pathogens [243]. It would be preferable to identify individual helminth products which could then be prepared in controlled laboratory conditions. FHKTM has been identified in this project as one such product.

In order to identify individual *F. hepatica* proteins, FHES was fractionated by size-exclusion chromatography and the individual fractions tested for their ability to promote eotaxin production *in vivo* as *F. hepatica* infection and FHES injection were found to induce eosinophilia via eotaxin and IL-5 induction. Positive fractions were analysed by mass spectrometry and peptides detected in the fractions were compared with 2 *F. hepatica* organism databases. FHKTM was identified, the gene synthesised, cloned into pPIC9 expression vectors and amplified in *E. coli*, then transferred and expressed in *P. pastoris* yeast, and the proteins purified. The *P. pastoris* strains used contain a mutation in the histidinol dehydrogenase gene (his4) which prevents them from synthesising histidine. pPIC9 vectors contain the his4 gene [376]. Therefore, yeast cells that have successfully been transformed were selected for their ability to grow on histidine-

deficient medium. Expression from the pPIC9 vector is controlled by a methanol-induced promoter AOX1 so the yeast cells are cultured the methanol containing medium BMMY to express FHKTM. The vector also contains an N-terminal protein secretion signal to ensure the proteins will be secreted into the medium from which they are purified. The *P. pastoris* expression system is commonly used to express recombinant proteins. It is easier and quicker to work with than mammalian cells. As *P. pastoris* is a eukaryote, protein processing, folding, and post-translational modification that is required for correct protein function will occur [377]. It is also a system free from endotoxin which is a major concern when using *E. coli* expression systems [378].

While FHKTM was identified from FHES fractions that had induced eotaxin production, i.p. injection of the expressed and purified protein did not promote eosinophil infiltration or expansion. In the MSU sterile peritonitis model, *F. hepatica* exosomes induced eosinophilia in the peritoneal cavity and suppressed MSU-induced neutrophil infiltration. There was a negative correlation between eosinophil and neutrophil recruitment suggesting an antagonistic relationship between the cell types (this was shown not to be the case). FHKTM did not induce eosinophil recruitment in the MSU sterile peritonitis model and was unable to inhibit neutrophil recruitment.

Eosinophils have been shown to mediate the anti-inflammatory activity of FHES [157]. However, FHKTM did not induce eosinophil recruitment. It is therefore unlikely that the protein can mediate any potential anti-inflammatory activity via eosinophils. Nonetheless, FHKTM consistently enhanced IL-4-induced type-2 macrophage activation *in vitro*. FHKTM enhanced IL-4-induced arginase activity in BMDMs and peritoneal exudate cells. FHKTM also enhanced IL-4--induced expression of Arg1, Mrc1, and IL-1RN in BMDMs. The mechanism of FHKTM enhancement of IL-4 type-2 response induction has not yet been defined. It may be via the induction of IL-6. IL-6 has traditionally been considered a pro-inflammatory cytokine but it is now recognised to have pleiotropic effects and to contribute to immune modulation [150]. It is known to potentiate IL-4-induced M2 macrophage polarisation. In BMDMs, IL-6 induced the expression of the IL-4 receptor (IL-4R), priming the macrophages for IL-4 stimulation and leading to enhanced IL-4-promoted M2 gene expression[151]. IL-6 induced less IL-4R expression in macrophages derived from mice with a myeloid-specific deletion of the IL-6 receptor

(IL6ra$^{\Delta myel}$) and the IL6ra$^{\Delta myel}$ transgenic mice were more susceptible to LPS-induced endotoxemia[151]. These findings suggest that IL-6 has an anti-inflammatory role that is mediated by IL-4 signalling. The present study demonstrated that FHKTM induced IL-6 production by BMDMs and peritoneal exudate cells. Future work is required to determine if IL-6 mediates FHKTM potentiation of IL-4 responses and if it is due to the induction of IL-4R expression.

Although FHKTM enhanced IL-4-induced Arg1 and Mrc1 expression, it suppressed IL-4-mediated Retnla induction in BMDMs. Relm-α production is induced by helminth infection and acts as a negative regulator of the helminth-induced type-2 immune response; helminth infected Retnla$^{-/-}$ mice developed a more robust Th2 response than wild-type mice [379, 380]. Helminth-induced attenuation of autoimmunity was mediated by the induction of a type-2 immune response [157]. As Relm-α negatively regulates the type-2 response, the ability of FHKTM to inhibit Relm-α induction would enhance the type-2 immune response induced and so may be beneficial in the treatment of autoimmunity.

As well as enhancing IL-4-mediated type-2 responses, FHKTM attenuated pro-inflammatory responses, inhibiting LPS-induced IL-1β, IL-12-p70, and TNF production and CD38 and Frp2 gene expression. Other helminth-derived products have been shown to suppress pro-inflammatory responses *in vitro*. FhHDM-1 and *Schistosoma mansoni* soluble egg antigens were demonstrated to inhibit LPS-induced production of pro-inflammatory cytokines by macrophages *in vitro* [243, 325]. The only previous description of FHKTM described the inhibition of LPS cytokine production induction [369] which are confirmed with the findings in the present study. FHKTM induced IL-6 production *in vitro*. IL-6 has been shown to suppress LPS-induced expression of the pro-inflammatory molecules TNF and Nos2 in BMDMs [150]. This highlights the potential importance of IL-6 as a mediator in anti-inflammatory activity of FHKTM.

Studies using an animal model of CNS autoimmunity, EAE, demonstrated that infection of mice with live *F. hepatica* or administration of FHES at the induction of EAE attenuated the clinical severity the disease [157, 205]. The present study demonstrated that when mice were treated therapeutically every second day with FHKTM (starting one day

before induction), disease onset was significantly delayed. With *F. hepatica* infection and FHES administration, delayed EAE onset and reduced disease severity was accompanied by the suppression of MOG-specific Th1 and Th17 responses. It is to be determined whether FHKTM inhibit the induction, migration or function of pathogenic Th1 and Th17 cells in the CNS and peripheral immune organs.

IL-10 is a cytokine with an important role in preventing pathological inflammation including autoimmunity [381]. The induction of IL-10 production in the CNS was sufficient to supress the development of EAE [181] and the transfer of IL-10-producing $CD25^{+}CD4^{+}$ T cells from naïve mice into mice with EAE decreased disease severity [182]. FHKTM promoted the production of IL-10 *in vitro* in innate cells. In FHES-treated mice, protection against EAE was independent of IL-10 and dependent on IL-5 and IL-33 [157]. Unlike FHES, FHKTM did not induce eosinophilia. It is possible that the ability of FHKTM to delay EAE development may be mediated by the induction of IL-10 production rather than the induction of type 2 cytokine production and eosinophil recruitment.

Work in our lab has demonstrated that administration of FHTE 21 and 7 days before EAE induction prevented disease development. This protection is thought to be an antigen-independent mechanism whereby FHTE is training innate cells to be less responsive leading to diminished T cell responses. While immunological memory was thought to be confined to antigen-specific T and B lymphocytes, recently studies have shown that innate cells can be trained or made tolerant in response to stimulation. This has been named "trained immunity" or "innate immune memory" [84, 382]. Prophylactic administration of FHKTM did not inhibit the development of EAE. The results from experiments involving FHKTM in EAE demonstrate that continuous administration of the proteins is required for their protective effects.

Comments for pPIC9:
8023 nucleotides

5′ *AOX1* promoter fragment: bases 1-948
5′ *AOX1* primer site: bases 855-875
α-Factor secretion signal(s): bases 949-1215
α-Factor primer site: bases 1152-1172
Multiple Cloning Site: bases 1192-1241
3′ *AOX1* primer site: bases 1327-1347
3′ *AOX1* transcription
termination (TT): bases 1253-1586
HIS4 ORF: bases 4514-1980
3′ *AOX1* fragment: bases 4870-5626
pBR322 origin: bases 6708-6034
Ampicillin resistance gene: bases 7713-6853

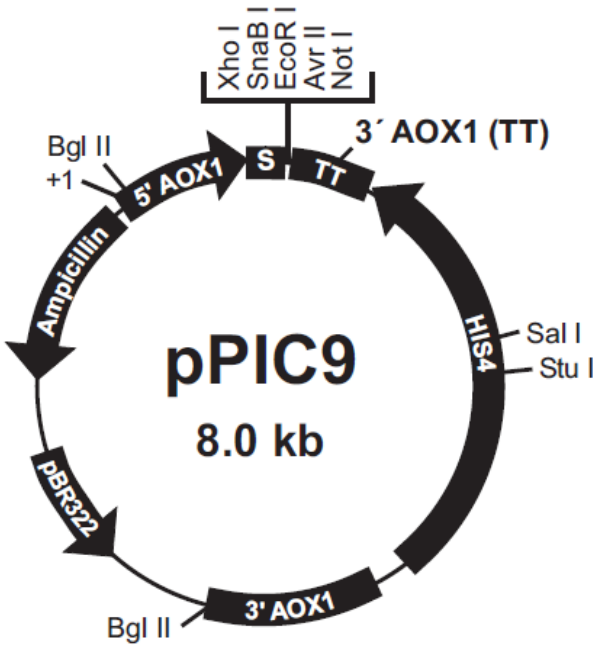


Figure 5.1: Map of pPIC9 expression vector used to express FHKTM in *Pichia pastoris*.

The gene encoding FHKTM was His-tagged at the N-terminus, synthesised, cloned into a pPIC9 expression vector, and transformed into *P. pastoris* yeast. The *P. pastoris* yeast strains have a mutation in the histidinol dehydrogenase gene (his4) that prevents them from synthesising histidine. The pPIC9 expression vector carries the HIS4 gene. Therefore, successfully transplanted yeast cells are selected for their ability to grow on histidine-deficient medium. 5'AOX1 promoter fragment is an ~ 1000 base pair fragment containing the AOX1 promoter. This allows methanol-induced high expression in the yeast. In order to ensure FHKTM is secreted by the yeast cells, the vector contains an α-secretion signal between the Xho I and Snab I cloning sites. Adapted from Invitrogen, Life Technologies 'User Guide - Pichia Expression Kit' (2014).

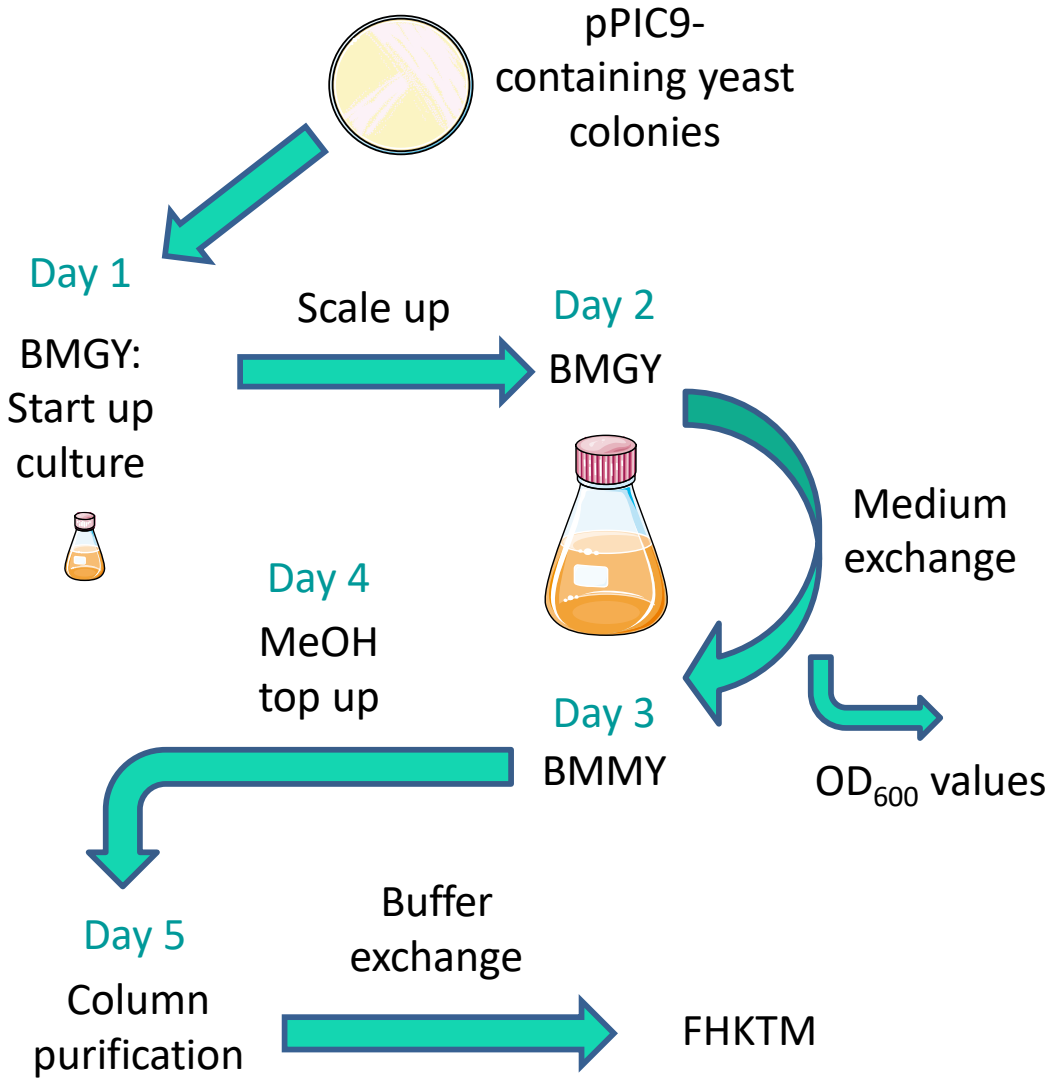


Figure 5.2: Flow chart detailing the protocol for expressing and purifying FHKTM from *P. pastoris* yeast stocks.

Yeast stocks were scraped from MD plates into 25 mL BMGY medium. The following day the culture was scaled up by adding the 25 mL to 400 mL BMGY. On day 3, the BMGY medium was exchanged for 400 mL BMMY medium. Before exchanging the medium, the OD_{600} values of the cultures were calculated to confirm they were approximately 10. The BMMY cultures were incubated for two days adding 2 mL methanol after 24 hours. On day 5 the protein was purified from the cultures by column purification and the buffer exchanged for PBS.

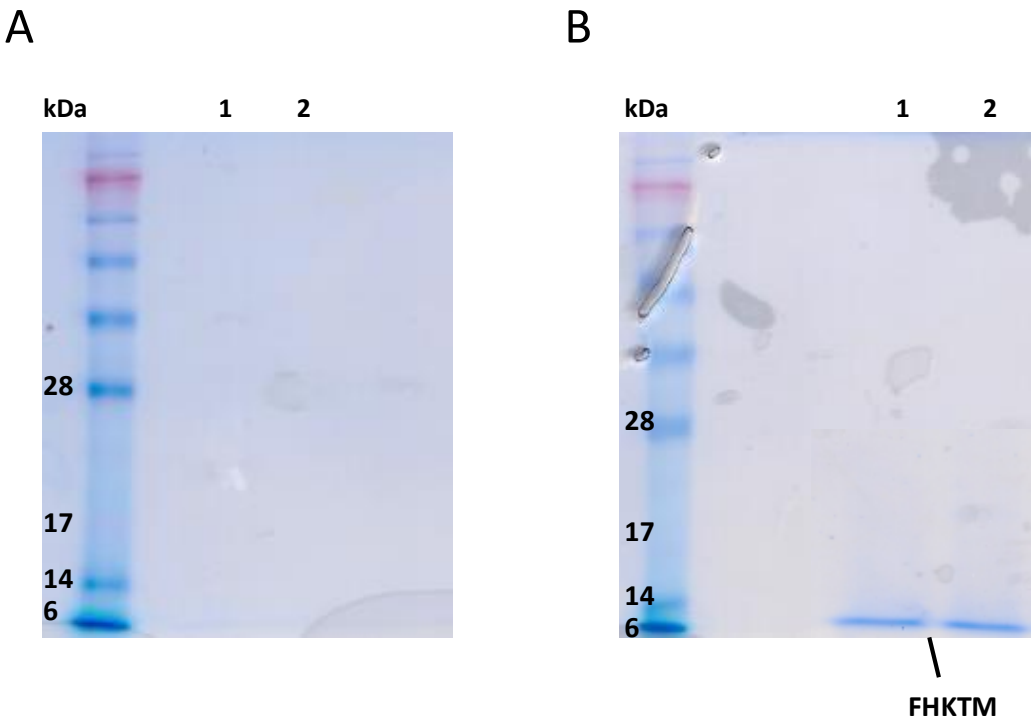


Figure 5.3: Resolving problems with the purification of the recombinant protein FHKTM from *P. pichia* yeast cells.

FHKTM protein samples produced by *P. pichia* yeast cells and purified by NiNTA agarose were separated by SDS polyacrylamide gel electrophoresis to confirm their purification. The gel was stained with Coomassie Brilliant Blue R-250 to visualise the protein (A). Lanes 1-2 contain FHKTM. *P. pichia* yeast cell supernatants before purification were separated by SDS polyacrylamide gel and the gel stained with Coomassie Brilliant Blue R-250 (B). Lanes 1-2 contain the supernatants. The molecular weight of FHKTM is 7kDa.

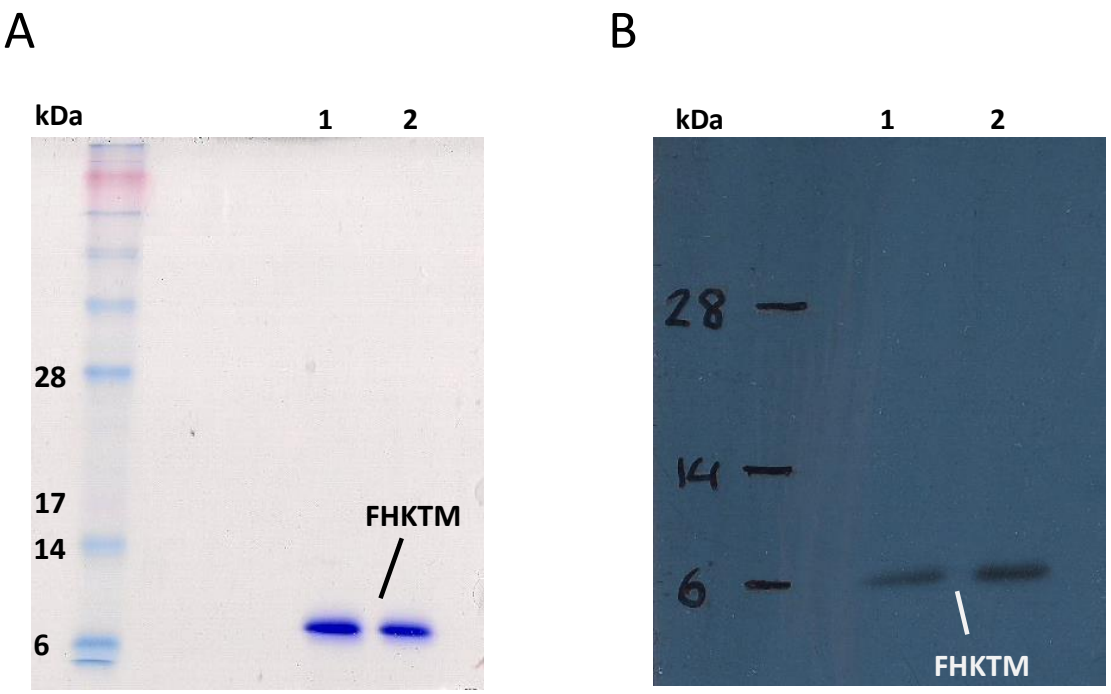


Figure 5.4: Production of the recombinant protein FHKTM identified from FHES.

FHKTM protein samples produced by *P. pichia* yeast cells and purified by NiNTA agarose were separated by SDS polyacrylamide gel electrophoresis. The gel was stained with Coomassie Brilliant Blue R-250 to visualise the protein (A). Lanes 1-2 contain FHKTM. FHKTM was run on a separate gel and transferred to a polyvinylidene fluoride membrane for Western blot analysis using HisProbe-HRP (B). Lanes 1-2 contain FHKTM. The molecular weight of FHKTM is 7kDa.

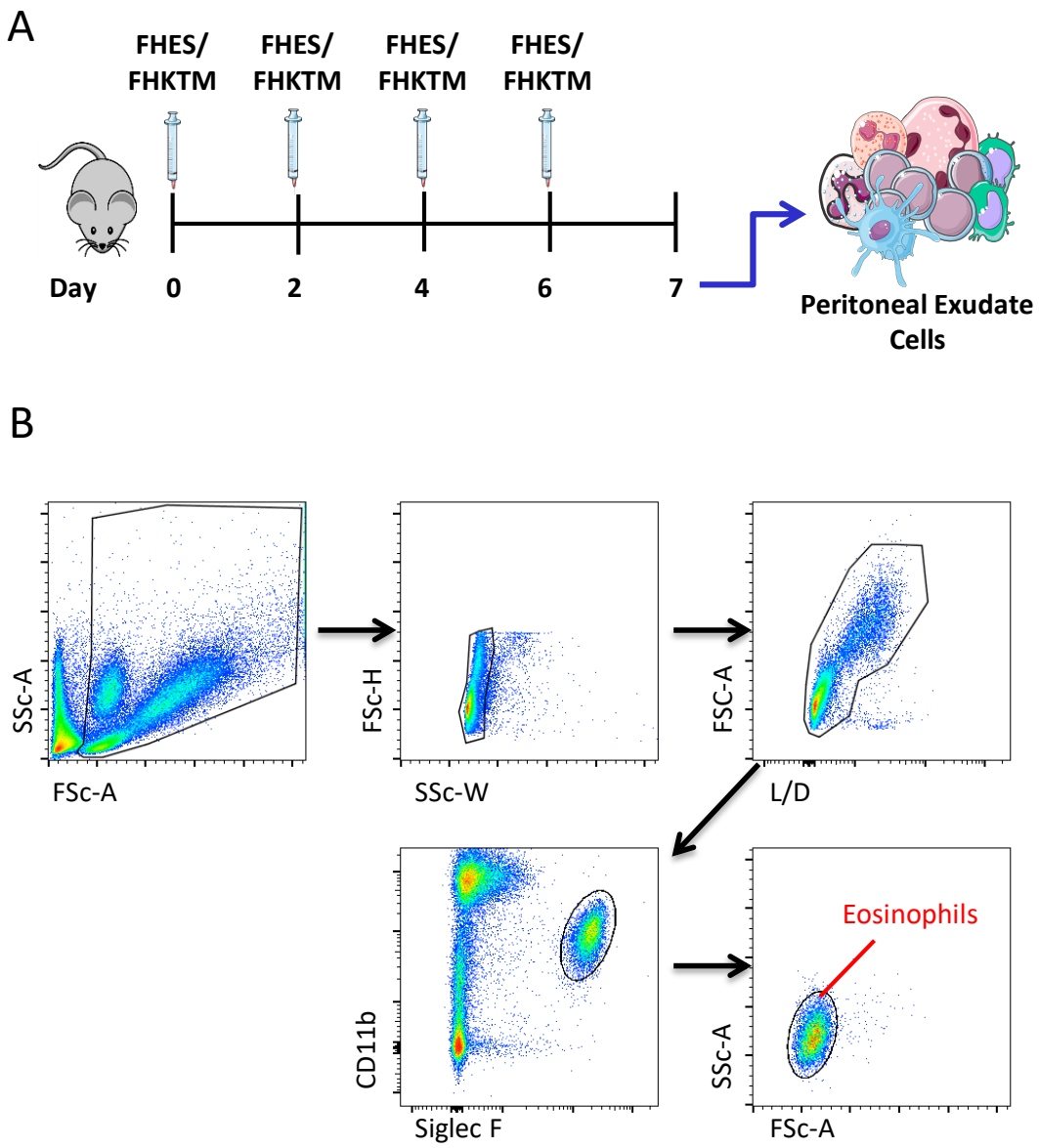


Figure 5.5: Experimental design and flow cytometric gating strategy to examine the *in vivo* activity of FHKTM.

(A) C57BL/6 mice were injected i.p. with FHKTM (2.5 µg/mouse/day), FHES (100 µg/mouse/day), or PBS on days 0, 2, 4, and 6 (n = 5). On day 7, peritoneal exudate cells were isolated for flow cytometry analysis. (B) Gating strategy to identify eosinophils.

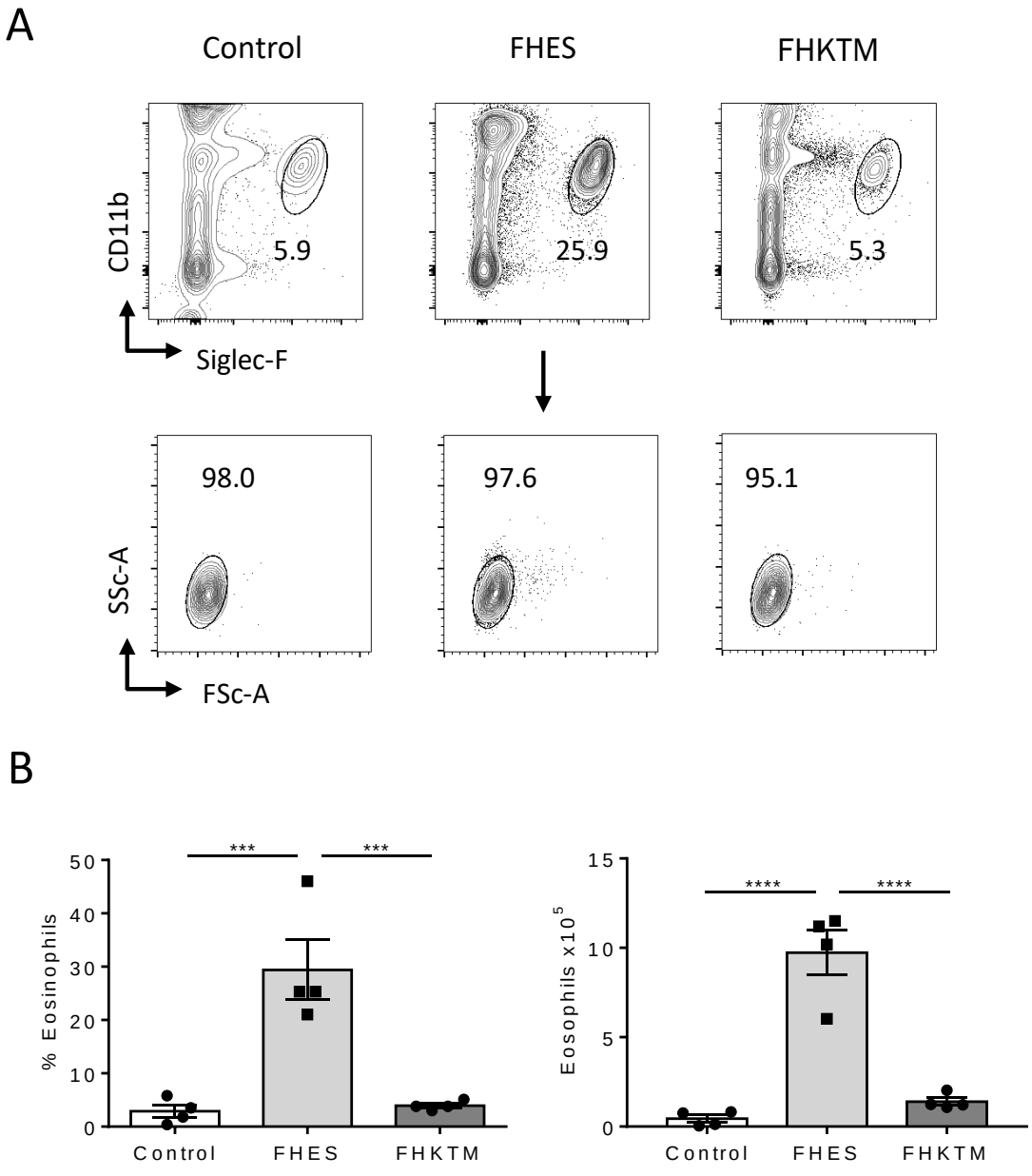


Figure 5.6: I.p. injection of mice with FHKTM does not induce eosinophil recruitment.

C57BL/6 mice injected FHKTM, FHES or PBS (Figure 5.11). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile (A). Data from the mice is presented as means +/- SEM (n=4) (B). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test. *** P<0.001, **** P<0.0001.

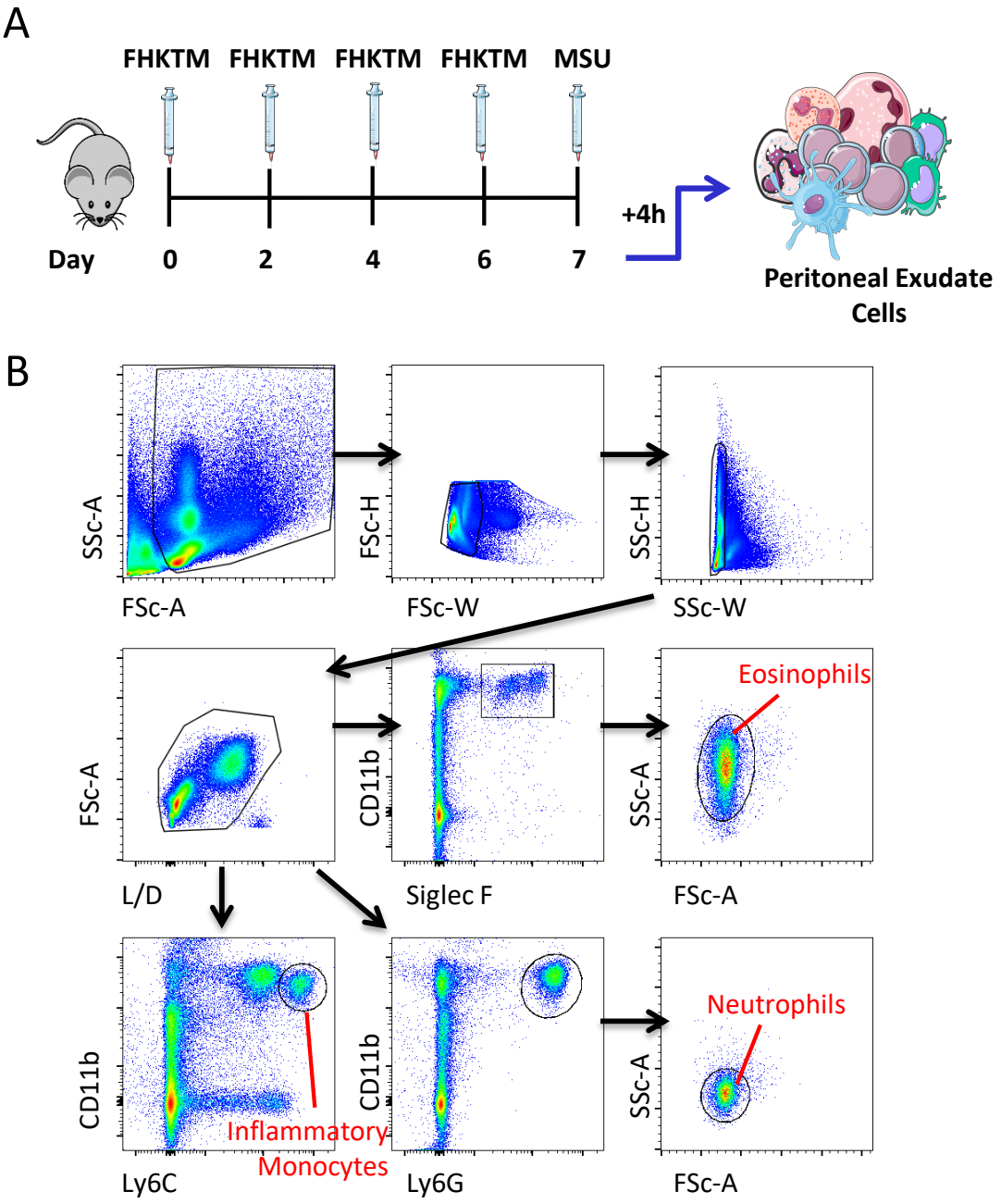


Figure 5.7: Experimental design and flow cytometric gating strategy for assessing the ability of FHKTM to suppress MSU crystal -induced inflammation.

(A) C57BL/6 mice were injected i.p. with FHKTM (2.5 µg/mouse/day) or PBS on days 0, 2, 4, and 6 (n=5). On day 7 the mice were injected with MSU crystals (250 µg/mouse). Naïve mice were also injected with PBS as a control (n=3). Four hours post-MSU crystal /PBS injection, peritoneal exudate cells were isolated for flow cytometry analysis. (B) Gating strategy to identify eosinophils, neutrophils, and inflammatory monocytes.

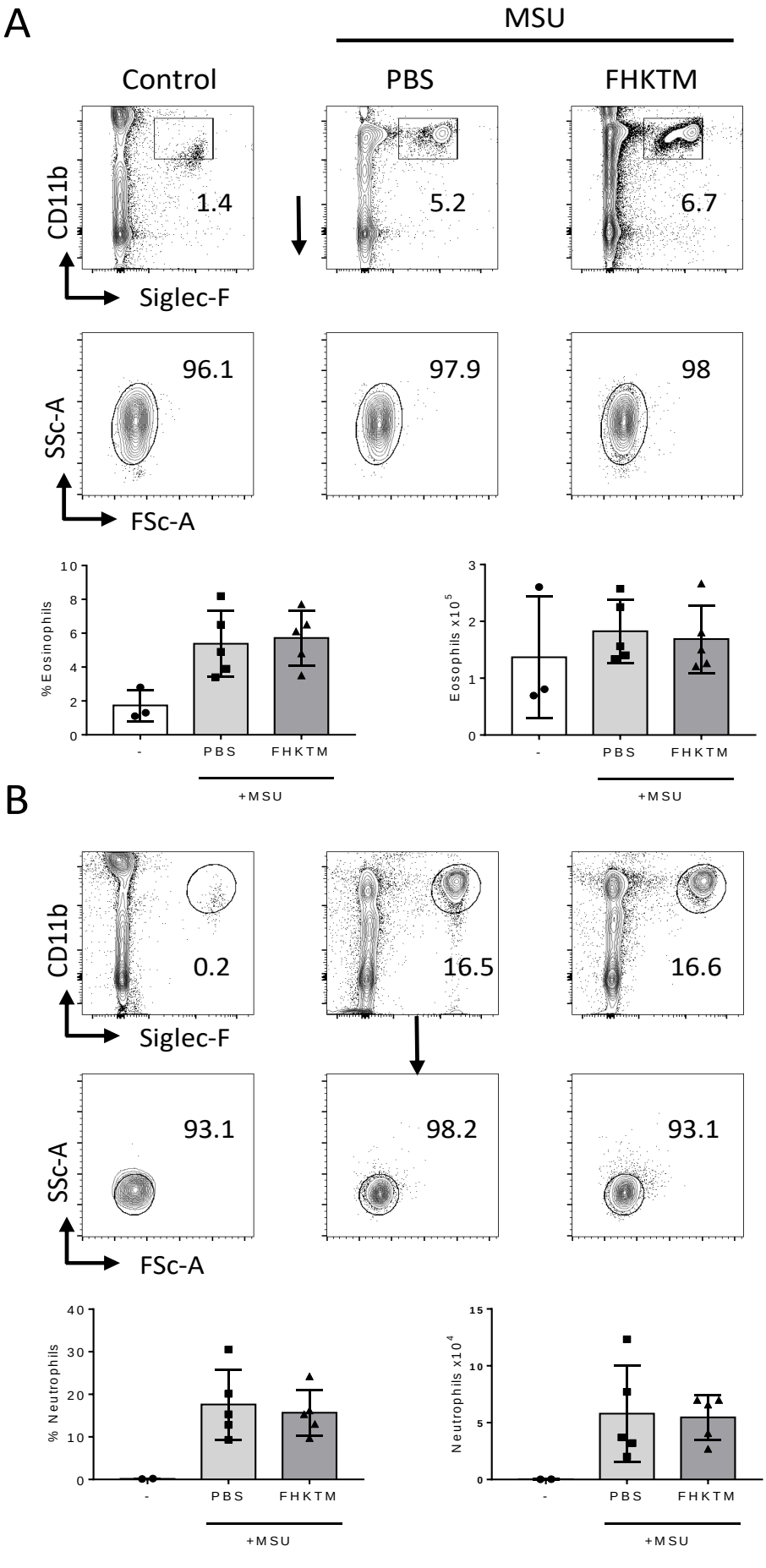

A
MSU
Control
PBS
FHKTM
CD11b
Siglec-F
1.4
5.2
6.7
SSc-A
FSc-A
96.1
97.9
98
% Eosinophils
Eosophils x10^5
-
PBS
FHKTM
+MSU
B
0.2
16.5
16.6
93.1
98.2
93.1
% Neutrophils
Neutrophils x10^4

Figure 5.8: I.p. injection of mice with FHKTM did not induce eosinophil recruitment or suppress MSU crystal -induced neutrophil infiltration in the peritoneal cavity.
C57BL/6 mice were injected with FHKTM or PBS and challenged with MSU crystals (Figure 5.13). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Eosinophils were identified by the expression of the surface marker Siglec-F and confirmed by their high SSc-A/low FSc-A profile (A). Neutrophils were identified by the expression of the surface marker Ly6-G and confirmed by their low SSc-A and FSc-A profile (B). Data from the mice is presented as means +/- SEM (n=3-5). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test.

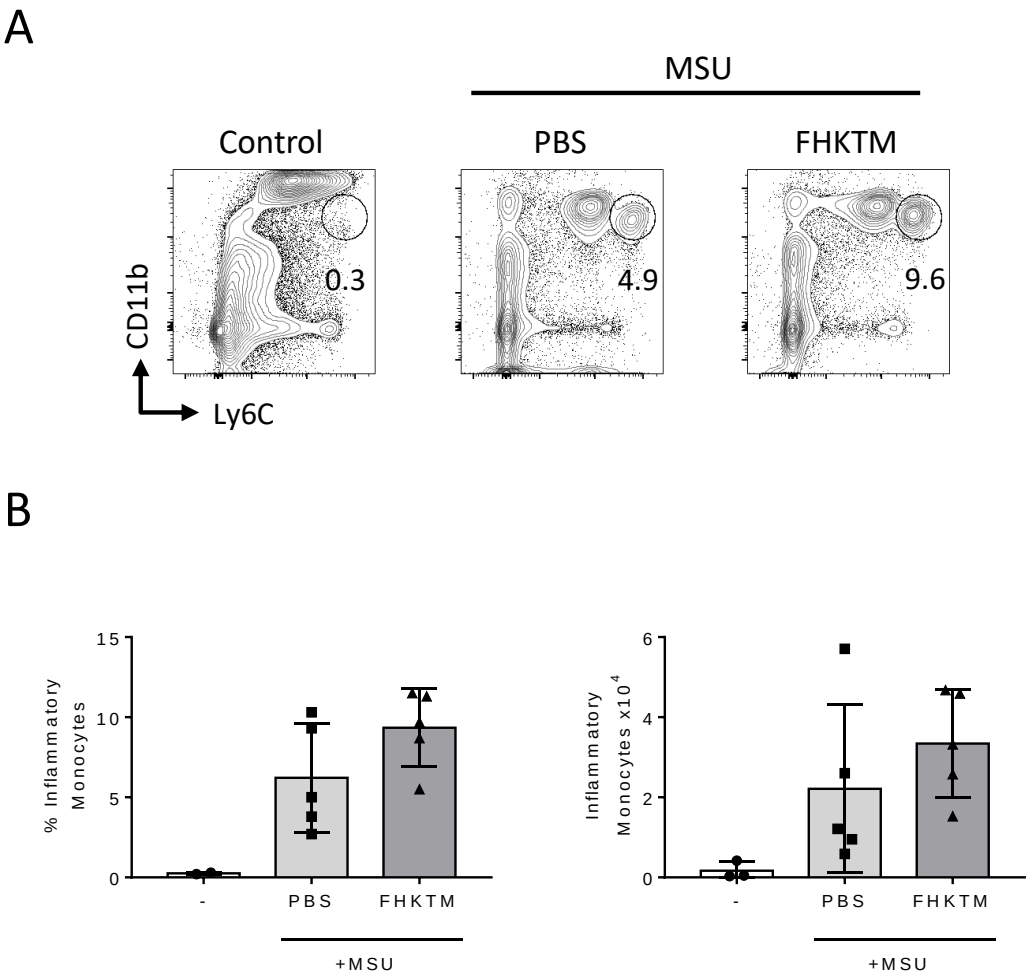


Figure 5.9: I.p. injection of mice with FHKTM did not suppress MSU- crystal induced inflammatory monocyte recruitment in the peritoneal cavity.

C57BL/6 mice were injected with FHKTM or PBS and challenged with MSU crystals (Figure 5.13). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Inflammatory monocytes were identified by the very high expression of the surface marker Ly6C (A). Data from the mice is presented as means +/- SEM (n=3-5) (B). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test.

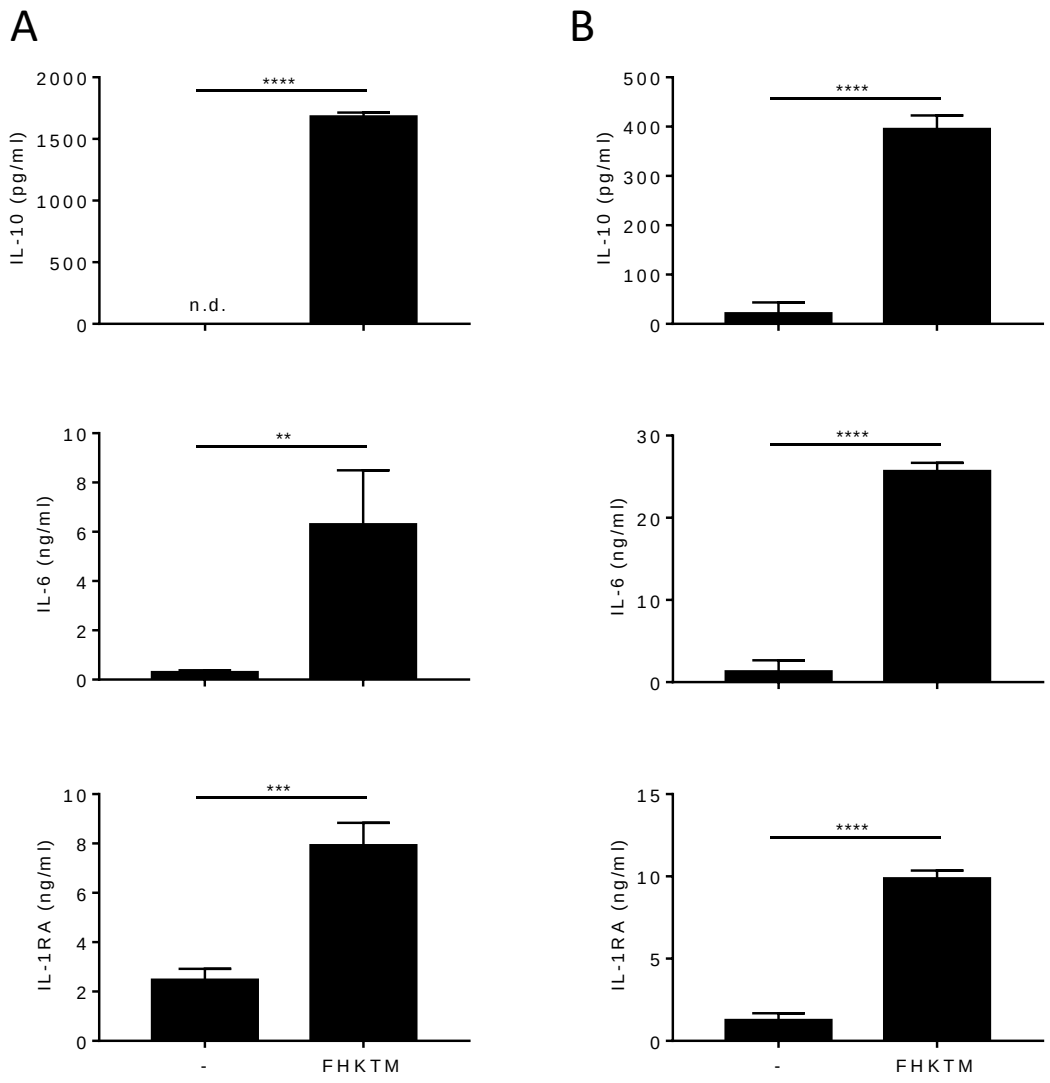


Figure 5.10: FHKTM induces IL-10, IL-1RA and IL-6 production by M-CSF-expanded bone marrow macrophages and peritoneal exudate cells.

M-CSF-expanded bone marrow macrophages (A) and peritoneal exudate cells (B) isolated from C57BL/6 mice were treated with FHKTM (2.5 µg/ml) or medium for 24h. The concentrations of IL-10, IL-1RA, and IL-6 in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by Student's t-test v medium control. ** P<0.01, *** P<0.001, **** P<0.0001.

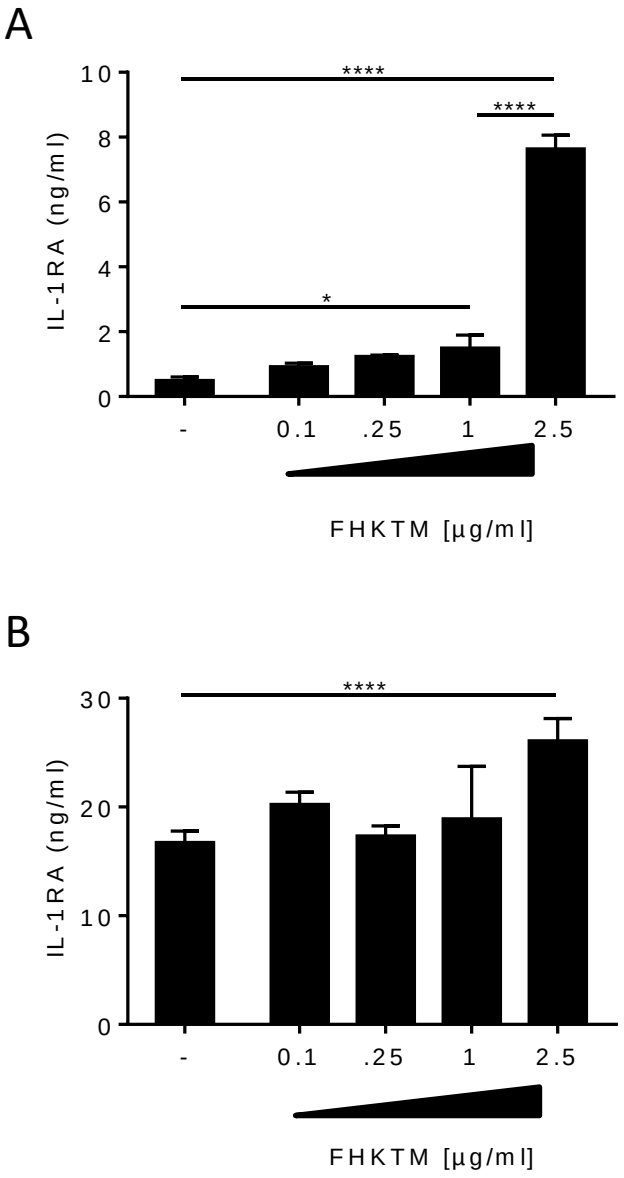


Figure 5.11: Dose response of FHKTM-induced IL-1RA production in M-CSF-expanded bone marrow macrophages and GM-CSF-expanded bone marrow dendritic cells.

M-CSF-expanded bone marrow macrophages (A) and GM-CSF-expanded bone marrow dendritic cells (B) were treated for 24h with FHKTM (0.1, 0.25, 1, and 2.5 µg/ml) or medium control. The concentration of IL-1RA in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test *P<0.05, ****P<0.0001.

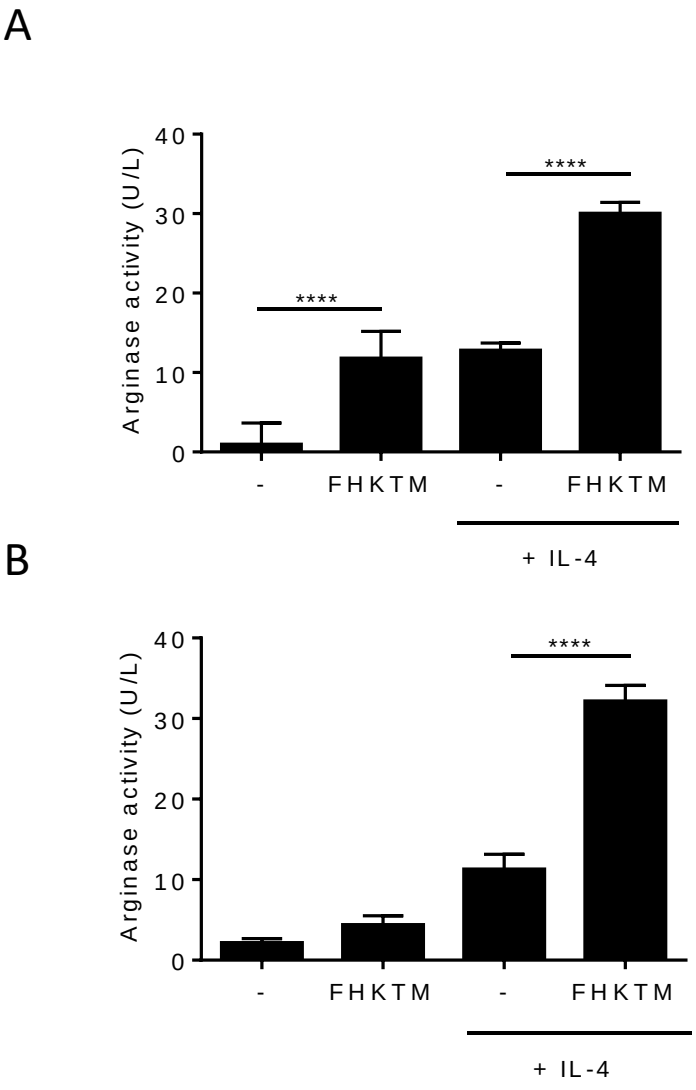


Figure 5.12: FHKTM induces arginase activity in peritoneal exudate cells and enhances IL-4-induced arginase activity in M-CSF-expanded bone marrow macrophages and peritoneal exudate cells.

Peritoneal exudate cells (A) and M-CSF-expanded bone marrow macrophages (B) isolated from C57BL/6 mice were treated with FHKTM (2.5 µg/ml) or medium +/- IL-4 (10 ng/ml) for 24h. Cells were harvested, and arginase activity was determined using an Arginase Activity Assay Kit from Sigma. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test. **** $P<0.0001$.

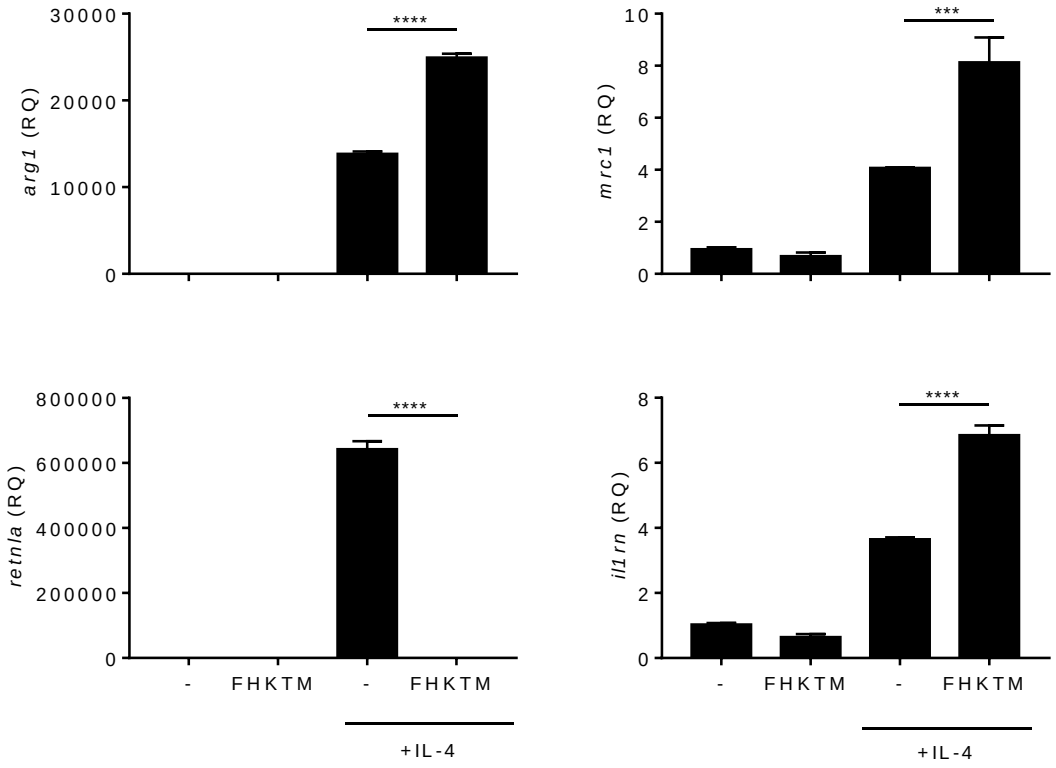


Figure 5.13: FHKTM enhances IL-4-induced Arg1, Mrc1 and IL-1RN gene expression and supresses IL-4-induced Retnla expression in M-CSF-expanded bone marrow macrophages.

M-CSF-expanded bone marrow macrophages generated from C57BL/6 mice were treated for 24h with FHKTM (2.5 µg/ml) or medium +/- IL-4 (10 ng/ml). The expression of Arg1, Mrc1, Retnla, and IL-1RN was determined by rtPCR. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test. *** P<0.001, **** P<0.0001.

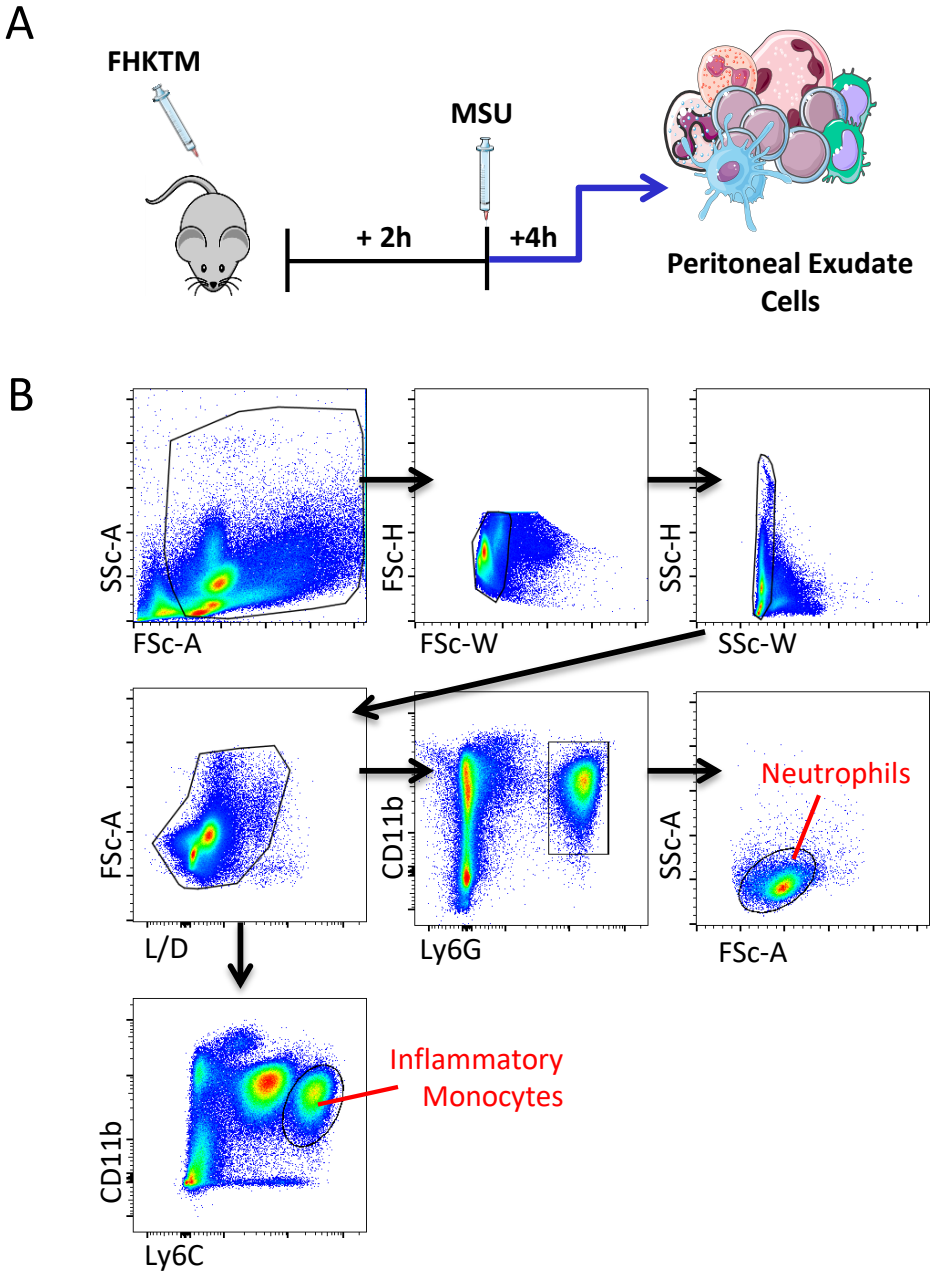
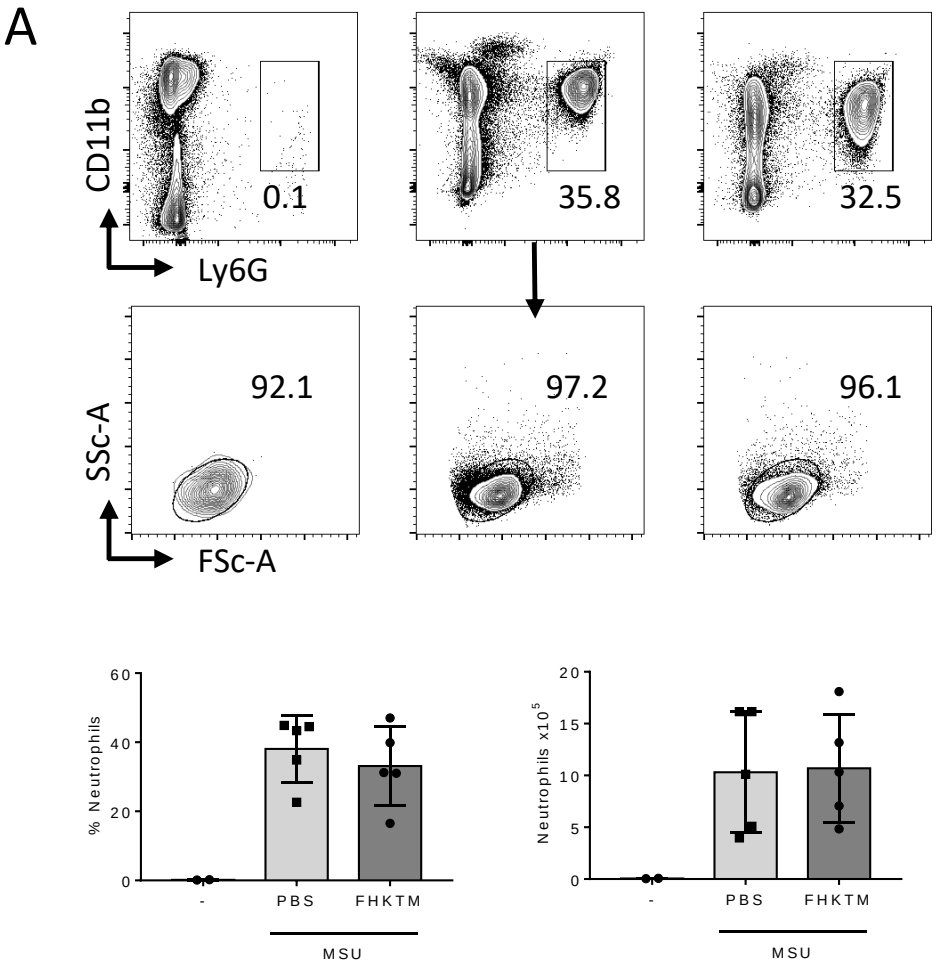


Figure 5.14: Experimental design and flow cytometric gating strategy for assessing the ability of FHKTM to suppress MSU crystal-induced inflammation.

(A) C57BL/6 mice were injected i.p. with FHKTM (2.5 µg/mouse) or PBS (n=5). 2 hours later the mice were injected with MSU crystals (250 µg/mouse). Naïve mice were also injected with PBS as a control (n=2). Four hours post-MSU crystal/PBS injection, peritoneal exudate cells were isolated for flow cytometry analysis. (B) Gating strategy to identify neutrophils and inflammatory monocytes.


A
CD11b
Ly6G
0.1
35.8
32.5
SSc-A
FSc-A
92.1
97.2
96.1
% Neutrophils
Neutrophils x10^5
-
PBS
FHKTM
MSU

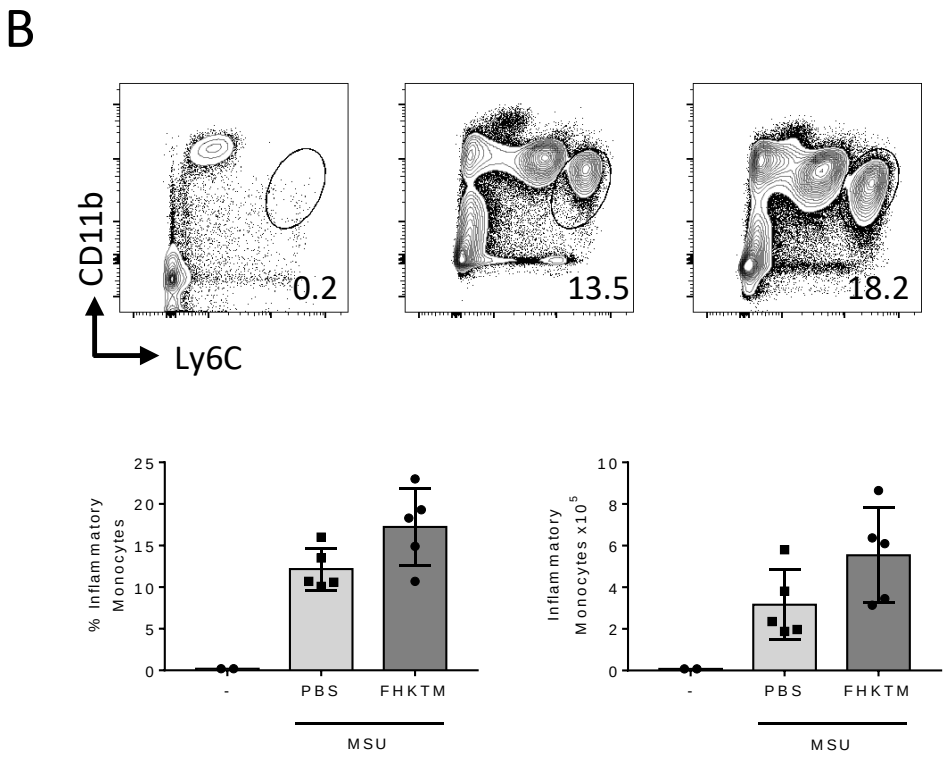
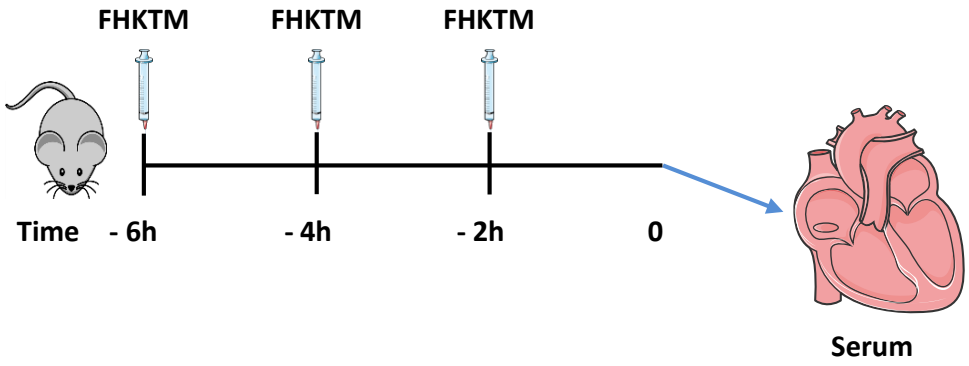

B
CD11b
Ly6C
0.2
13.5
18.2
% Inflammatory Monocytes
Inflammatory Monocytes x10^5
-
PBS
FHKTM
MSU

Figure 5.15: I.p. injection of mice with FHKTM 2 hours prior to MSU crystal challenge did not suppress MSU crystal -induced neutrophil or inflammatory monocyte infiltration in the peritoneal cavity.

C57BL/6 mice were injected with FHKTM or PBS and challenged with MSU crystals (Figure 5.16). Peritoneal exudate cells were isolated and flow cytometry analysis was carried out. Neutrophils were identified by the expression of the surface marker Ly6-G and confirmed by their low SSc-A and FSc-A profile (A). Inflammatory monocytes were identified by the very high expression of the surface marker Ly6C (B). Data from the mice is presented as means +/- SEM (n=2-5). Statistical assessment was performed by one-way ANOVA with Tukey's honestly significant difference post hoc test.

A



B

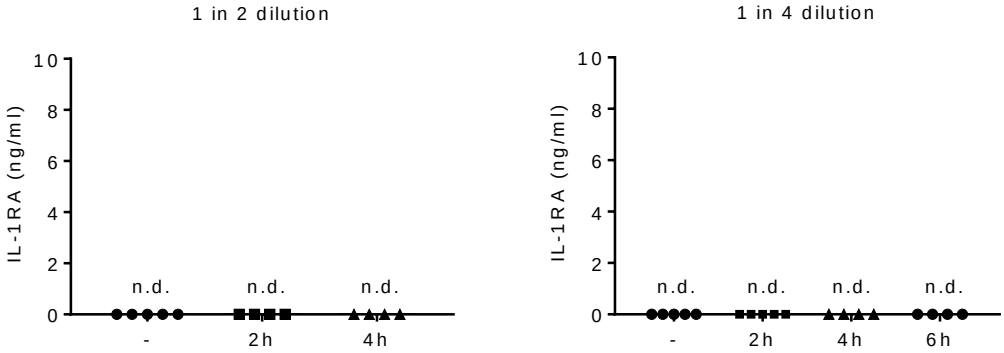


Figure 5.16: FHKTM does not induce an increase in circulating IL-1RA.

C57BL/6 mice were injected with FHKTM *(*at 6 hours, 4 hours, or 2 hours prior to sacrifice) or PBS and serum was collected and diluted as described. The concentration of IL-1RA in the serum were determined by ELISA. Data from the mice is presented as means +/- SEM (n=4-5).

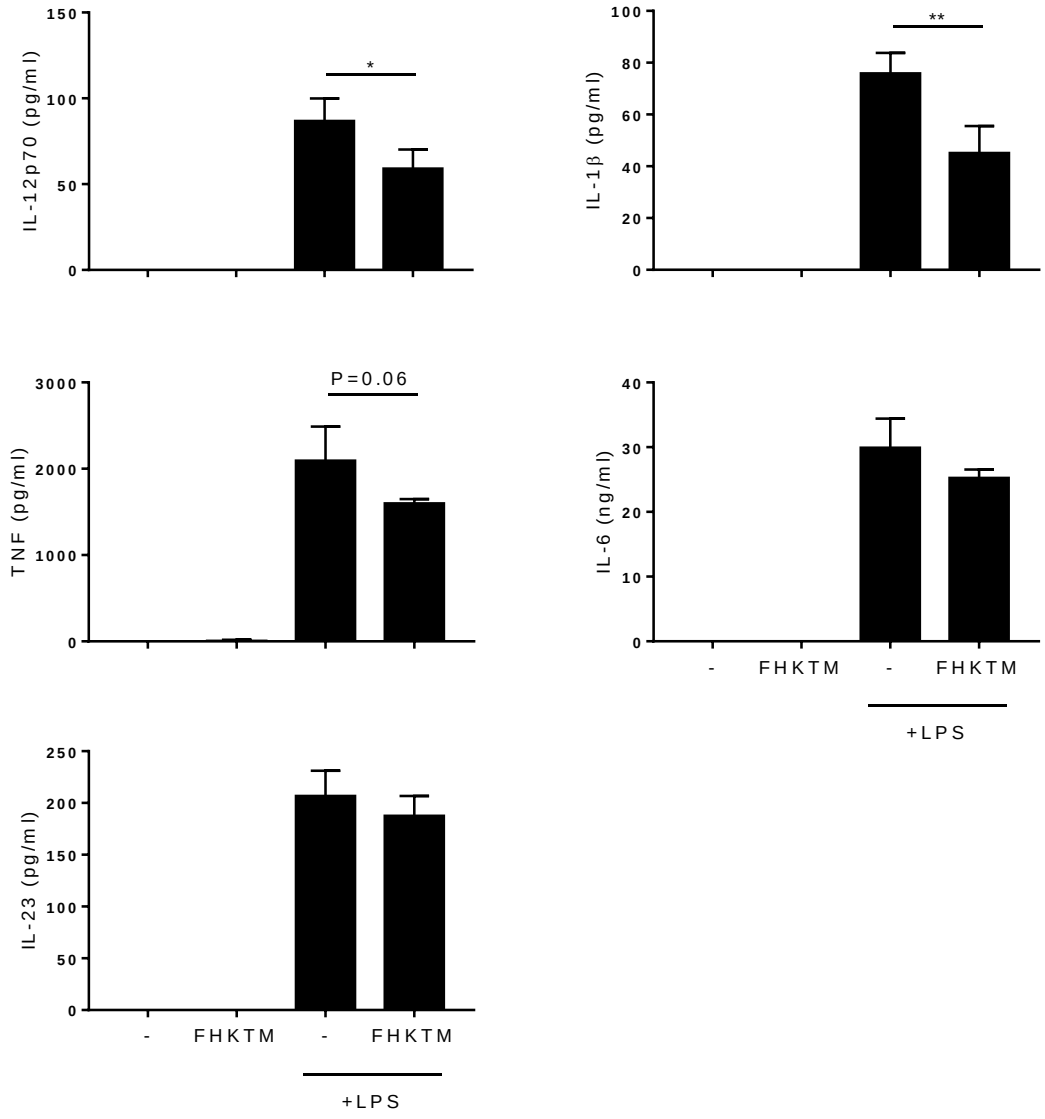


Figure 5.17: FHKTM suppresses LPS-induced pro-inflammatory cytokine production. GM-CSF-expanded bone marrow dendritic cells generated from C57BL/6 mice were treated for 24h with FHKTM (2.5 µg/ml) or medium +/- LPS (100 ng/ml). The concentrations of IL-12p70, IL-1β, TNF-α, IL-6, and IL-23 in the supernatants were determined by ELISA. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test. * $P<0.05$, ** $P<0.01$.

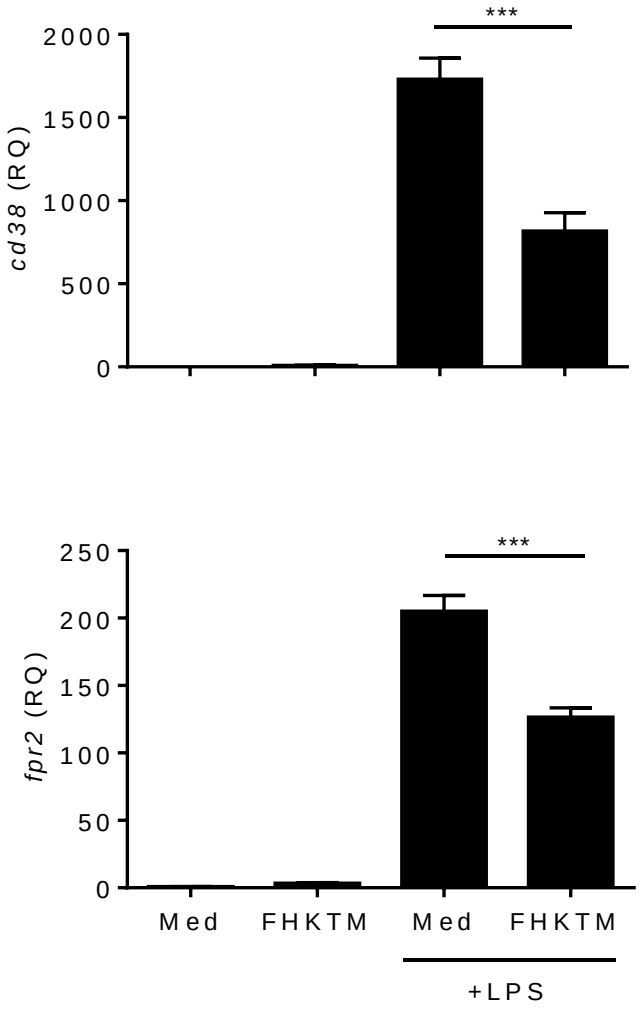


Figure 5.18: FHKTM suppresses LPS-induced CD38 and Frp2 expression.

M-CSF-expanded bone marrow macrophages generated from C57BL/6 mice were treated for 24h with FHKTM (2.5 µg/ml) or medium +/- LPS (100 ng/ml). The expression of CD38 and Frp2 was determined by rtPCR. Data presented are means +/- SD of triplicate assays. Statistical assessment was performed by two-way ANOVA with Tukey's honestly significant difference post hoc test. *** P<0.001.

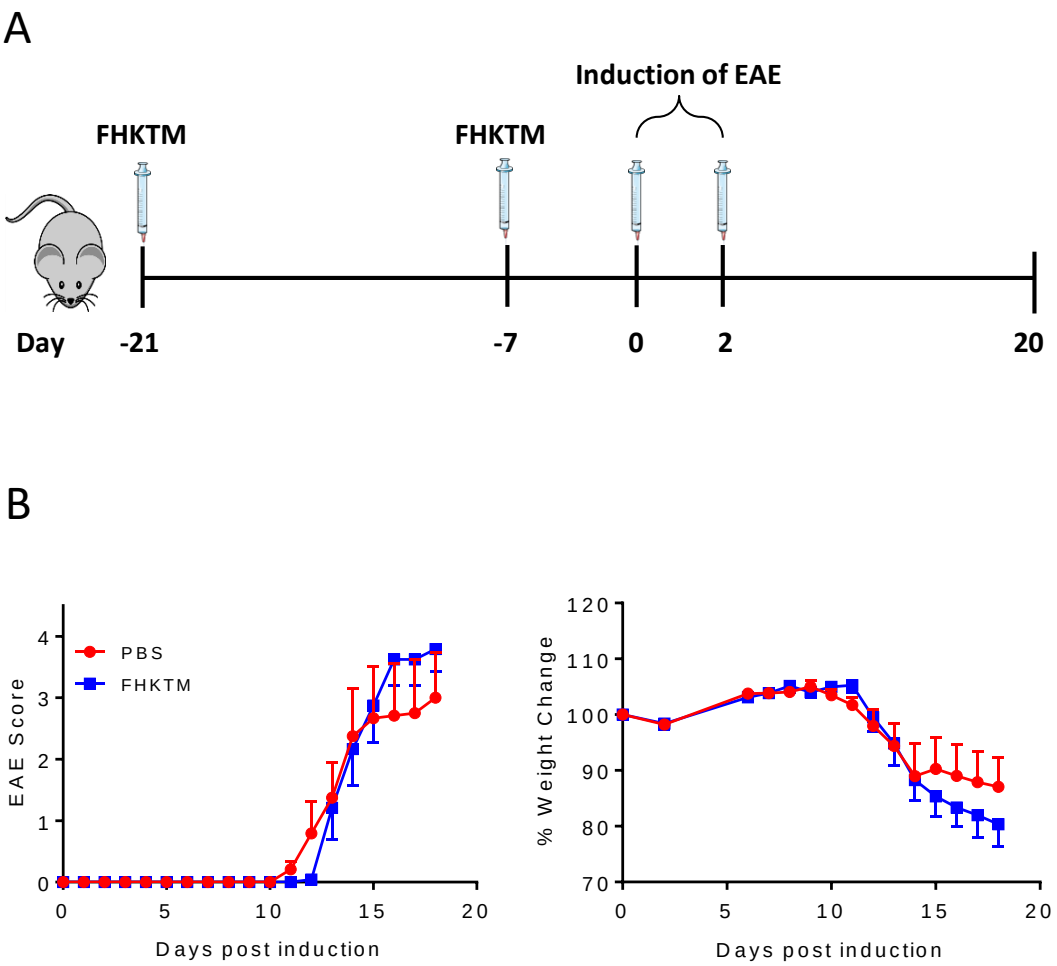


Figure 5.19: Prophylactic administration FHKTM did not affect the course of EAE disease.

(A) C57BL/6 mice were injected i.p. with PBS (vehicle control) or FHKTM (2.5 µg/mouse/day) on days -21 and -7 (n=6). EAE was subsequently induced by injection of MOG/CFA and PT (200 ng/mouse) on day 0 and injection of PT (200 ng/mouse) on day 2. (B) Disease progression was monitored by measuring body weight and clinical score daily. Data presented are means +/- SEM (n=6). Statistical assessment was performed by repeated measures ANOVA with Sidak's post hoc test.

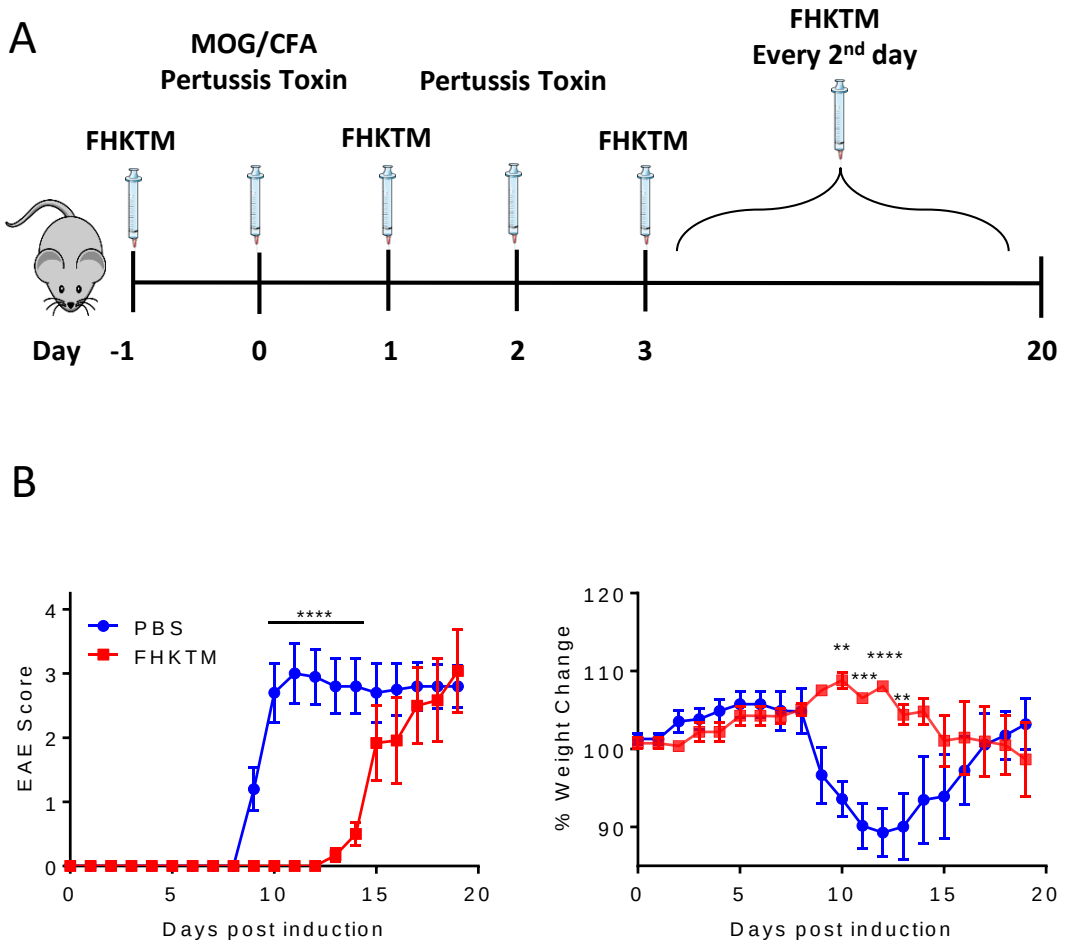


Figure 5.20: Therapeutic administration of FHKTM delayed EAE disease onset.

(A) C57BL/6 mice were injected i.p. with PBS (vehicle control) or FHKTM (2.5 µg/mouse/day) on days -1, and every subsequent second day until the end of the experiment (n=6). EAE was induced by injection of MOG/CFA and PT (200 ng/mouse) on day 0 and injection of PT (200 ng/mouse) on day 2. (B) Disease progression was monitored by measuring body weight and clinical score daily. Data presented are means +/- SEM (n=6). Statistical assessment was performed by repeated measures ANOVA with Sidak's post hoc test. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Chapter 6

General Discussion

6.1 General Discussion

The current study has demonstrated that *F. hepatica* exosomes and the *F. hepatica*-produced protein FHKTM induced anti-inflammatory responses and protected mice from pathological inflammation. The most significant and novel finding was that *F. hepatica* products induced the production of the anti-inflammatory cytokine IL-1RA and that *in vivo* administration of the products attenuated pathological inflammation and suppressed autoimmune disease in the CNS.

The results of this project demonstrate that both eosinophils and M2 macrophages can mediate anti-inflammatory activity of helminth-derived products. *F. hepatica* exosomes-induced eosinophils produced IL-1RA and eosinophil induction by *F. hepatica* exosomes correlated negatively with neutrophils in the MSU-induced peritonitis, a model of sterile inflammation mediated by IL-1β. This suggested an inhibitory relationship between eosinophils and neutrophils. However, suppression of eosinophil mobilisation with anti-IL-5 did not reverse the inhibition of neutrophil infiltration into the peritoneal cavity by *F. hepatica* exosomes (or IL-33). Therefore, eosinophils do not appear to be required for inhibition of MSU crystal-induced inflammation by *F. hepatica* exosomes or IL-33. Data from this project suggests that M2 macrophages are the key cell type that mediate the suppression of MSU crystal-induced inflammation; depletion of macrophages from the peritoneal cavity attenuated IL-33-mediated suppression of neutrophil infiltration. Administration of anti-IL-5 did not inhibit M2 macrophage activation. In mice treated with IL-33 and anti-IL-5, M2 macrophage activation was greater than in mice injected with IL-33 alone. This suggests that eosinophils inhibit M2 macrophage activation. This is an interesting and unexpected result. It has previously been demonstrated that eosinophils induced and sustained M2 macrophage activation rather than inhibiting it [360, 361]. The enhanced expression of the M2 markers may simply be due to the larger percentage of macrophages present in the peritoneal cavity in the absence of any eosinophils or it may be due to the two cell types competing for the same, limited resource. The precise nature of the relationship between eosinophils and M2 macrophages has yet to be defined.

Treatment of mice with *F. hepatica* exosomes every second day delayed the onset of EAE but only had a modest effect on the severity of disease. Pre-treatment of mice with two single doses of *F. hepatica* exosome 21 and 7 days before induction of EAE induced a more pronounced attenuating effect on the course of disease. Recent reports have demonstrated that the innate immune system can be trained to have memory and that immunological memory or training is not just a feature of T and B cells of the adaptive immune system. Innate immune memory is a conserved process through evolution that confers non-specific protection following an initial exposure to a pathogen. Wendeln et al (2018) observed that innate training is possible in microglia (CNS-resident macrophages), peripheral immune stimulation promoted innate immune training in the brain that lasted for at least 6 months [383]. In an inflammatory mouse model of Alzheimer's disease, the induction of memory in innate cells peripherally was shown to enhance immunopathology or to attenuate it depending whether innate training or tolerance was induced [84]. Training promotes the generation of an enhanced immune response following re-stimulation while tolerance primes cells to produce less pro-inflammatory cytokines to subsequent activation [85]. Our lab has recently shown that the anti-inflammatory, rather than the pro-inflammatory, response of innate immune cells can be trained by *F. hepatica* products (FHTE) and that this can protect against autoimmunity (Quinn et al, In review). The present project demonstrated that *F. hepatica* exosomes also induced anti-inflammatory innate training.

Empirical evidence from epidemiological studies and animal models suggests that helminth infections, especially early in life, may protect against autoimmunity (and allergy) through the induction of regulatory immune responses. The use of live infection with helminth parasites as therapies is impractical for a variety of reasons. Helminth migration across mucosal barriers causes tissue damage even if they are non-pathogenic to humans [201]. Therefore, limiting the number of worms administered is important to reduce tissue damage as much as possible. However, the resulting dose of helminths may subsequently be inadequate to generate a therapeutic effect. In any potential clinical trials of autoimmune diseases, it is vital that participants are blinded. This can be difficult to ensure with the use of live helminths as treatments. Therefore, our approach needs to be refined and components of the helminth secretome need to be identified

that mediate the beneficial immunomodulation induced following live infection. In order to address the objective of finding *F. hepatica*-derived products with immunomodulatory properties, this project isolated and characterised *F. hepatica* exosomes. As *F. hepatica* exosomes are a mixture of proteins, nucleic acids and other components, further refinement is possible. A safer, more effective alternative to live infection or the use of the exosomes as therapies would be to identify a specific immune-modulatory molecule produced by *F. hepatica.* This approach would allow the precise immunomodulatory mechanisms of such molecules to be characterised, and their properties modified to increase therapeutic efficacy and stability and reduce potential issue relating to immunogenicity and toxicity. In addition, individual molecules can easily be manufactured on an industrial scale to therapeutic standard [243, 363].

FHTKM is a *F. hepatica*-derived protein with amino acid sequence homology to the Kunitz-type family of protease inhibitors, proteins which can regulate inflammation, tissue damage, and tissue repair by inhibiting proteases [365]. FHTKM is present in *F. hepatica* exosome-like extracellular vesicles [290]. In our lab, we identified FHTKM from FHES (from which *F. hepatica* exosomes are isolated). The protein was initially identified by the mass spectroscopy analysis of a fraction of FHES that mimicked the activity of whole FHES; the fraction induced eotaxin production in the serum, which is an indirect measure of eosinophil induction [167]. However, while FHKTM was identified in an FHES fraction that induced eotaxin production, injection of the protein did not promote eosinophil infiltration or expansion. Even though eosinophil induction was used as the first step in the process to identify individual *F. hepatica* proteins, FHKTM was shown to have other immunomodulatory properties and *in vivo* treatment of mice with FHKTM showed that it could delay the development of EAE. It is possible that attenuation of EAE by FHKTM may be mediated by IL-10. FHKTM induced IL-10 production by macrophages and peritoneal exudate cells. D'Elia and Else (2009) demonstrated that helminth-derived products induced IL-10 production by macrophages and DCs [384]. *Trichuris muris* is a natural helminth of mice and is utilised as a preclinical model for *Trichuris trichiura*, a helminth which infects more than half a billion people in the world [385]. Excretory/secretory (E/S) products isolated from *Trichuris muris* induced IL-10 production in BMDMs and BMDCs. The authors postulated that the induction of IL-10

production by helminth ES products aids parasite survival and persistence by inhibiting the induction of adaptive helper T cell responses. IL-10 can suppress the pathological activity of T cells specific for autoantigens and so is critical in the prevention of autoimmunity. Spontaneous colitis developed in mice with a selected deletion of IL-10 in Treg cells [126]. In EAE, IL-10 knockout mice were susceptible to development of more severe disease [180]. It has been demonstrated that the development of EAE can be suppressed by promoting IL-10 production in the CNS or by the transfer of IL-10-producing Treg cells decreased disease severity [181, 182].

It is interesting to compare the modulatory effects of *F. hepatica* exosomes and FHKTM on EAE. While both are contained in FHES (and FHKTM is a constituent of *F. hepatica* exosomes), they have contrasting activities: *F. hepatica* exosome-pre-treatment is protective while therapeutic administration of FHKTM delays disease onset (Figure 6.1). *F. hepatica* exosomes promote eosinophil recruitment and alternate M2 macrophage activation. The induction of these type 2 immune cells may mediate the protective effect of exosome pre-treatment on EAE, including via the production of IL-1RA, which inhibits the activity of the EAE disease-mediating cytokine IL-1β. Indeed, innate immune cells from mice treated with *F. hepatica* exosomes produce IL-1RA and inhibit pro-inflammatory cytokine production by MOG-specific cells, which mediate pathology in EAE. Unlike *F. hepatica* exosomes, FHKTM pre-treatment does not protect against EAE disease. However, therapeutic administration of FHKTM delays disease development. This may be mediated by the induction of anti-inflammatory cytokines IL-10 and IL-1RA production as well as by M2 macrophage activation. As the aetiology of MS is currently unknown [191], most patients only present in the clinic after symptoms have appeared. Therefore, based on our knowledge of the disease, FHKTM would currently make the better potential therapeutic against MS as it can protect after the disease has been induced.

While FHTKM did not promote eosinophil infiltration or expansion, it did polarise macrophages to an M2 state. Helminth infection promotes eosinophilia and the activation of M2 macrophages as part of the induction of a type 2 immune response [201]. In this project it was demonstrated that *F. hepatica* exosomes (and recombinant IL-33) induced the expansion of eosinophils and M2 macrophages while FHKTM induced

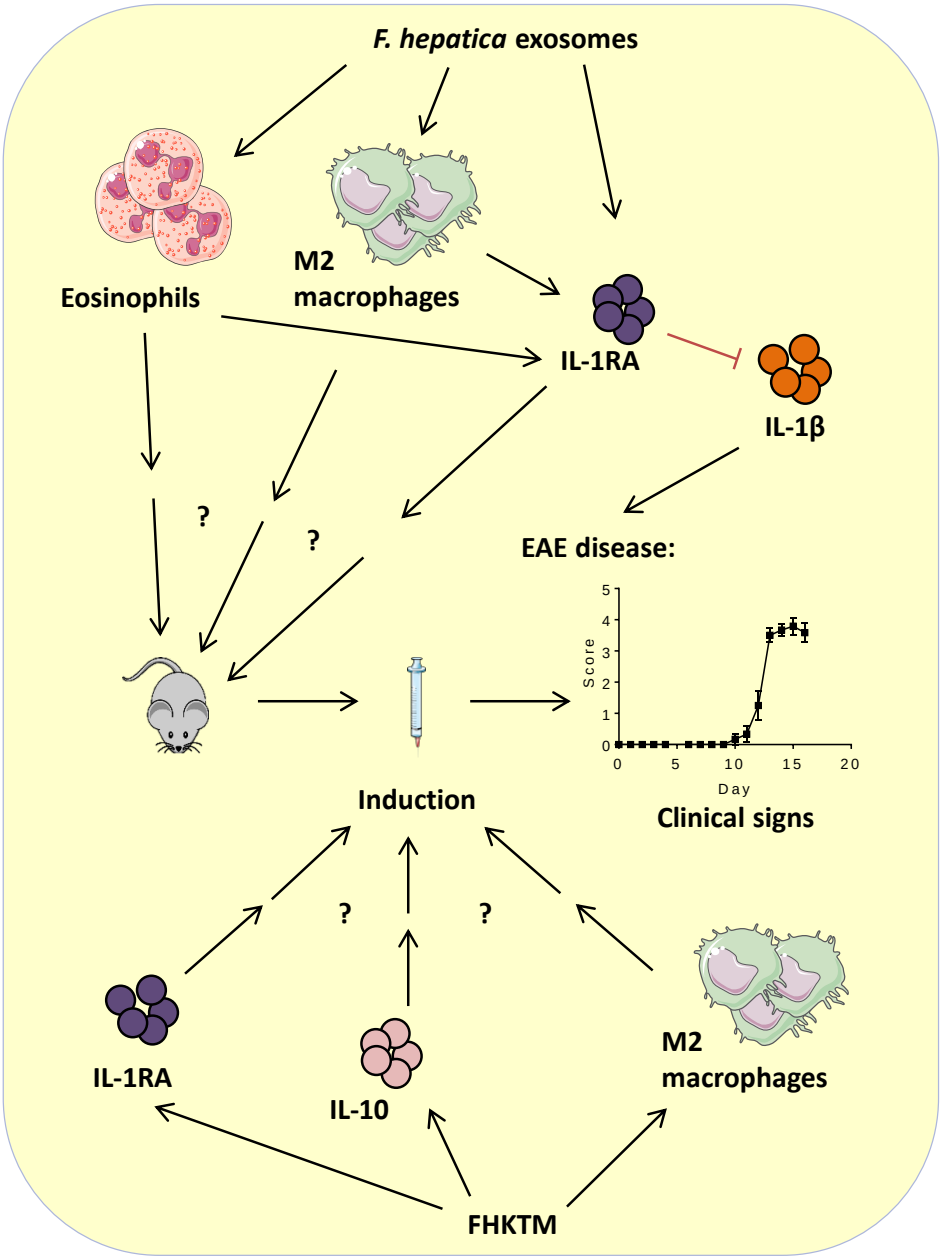


Figure 6.1: The potential mechanisms of inhibition of EAE by *F. hepatica* exosomes and FHKTM.

The induction of eosinophil recruitment and alternate M2 macrophage activation may mediate the protective effect of pre-treatment with *F. hepatica* exosomes on EAE, including via the production of IL-1RA, which inhibits the activity of the EAE disease-mediating cytokine IL-1β. Unlike *F. hepatica* exosomes, FHKTM pre-treatment does not protect against EAE disease. However, therapeutic administration of FHKTM delays disease development, possibly mediated by the induction of IL-10 and IL-1RA production and/or M2 macrophage activation.

M2 macrophage activation. In autoimmune diseases, in particular EAE, both eosinophils and M2 macrophages have been shown to attenuate pathological inflammation. Our lab has demonstrated that FHES-induced protection against EAE requires eosinophils [157]. In mice with EAE, FHES administration induced an increase in the frequency of eosinophils in the blood, LNs, spleen, and CNS. Inhibition of eosinophil mobilisation using anti-IL-5 supressed FHES-induced attenuation of EAE and the transfer of FHES-induced eosinophils into mice with EAE reduced disease severity. Shifting macrophages from a classical (M1) to an alternate (M2) activation state was protective in EAE and IL-33-induced M2 macrophages attenuated disease when injected in mice with EAE [374, 386]. In humans, macrophages in active MS lesions principally express markers of classical M1 macrophage activation including CD40, CD86, CD64 and CD32 [387]. The factors which induce either M1 and M2 macrophage activation reciprocally inhibit each other. Therefore, an increase in the number of M1 macrophages will mean that there will be less M2 macrophages present.

A number of potential new therapies developed against autoimmune diseases target the cytokines that mediate disease, including IL-1β, IL-17, and IL-23. Th17 cells, pathogenic in many autoimmune diseases, are induced by IL-1β and IL-23 (along with IL-6 and TGF-β), which induce RORγT expression, the master regulator of Th17 cells [122]. Th17 cells produce IL-17A, IL-17F, IL-17F, as well as IL-21 and IL-22. Initially it was thought that Th1 cells mediated autoimmunity, but it is now known that Th17 cells are the predominant cell type responsible for mediating pathology. Inhibition of IL-23 and therefore Th17 cell attenuated inflammation unlike suppression of IL-12 and thus Th1 cells, which did not [388]. IL-17 expression is elevated in patients with different autoimmune diseases including MS. IL-1β and IL-23 also promote the differentiation of γδ T cells, important mediators of pathology early in autoimmune diseases [128]. Therefore, targeting the IL-1β/IL-23/IL-17 axis in autoimmunity is potentially of great therapeutic benefit. Treatments against autoimmune diseases have generally be more successful at targeting IL-17 or IL-23. There has been less success in targeting IL-1β to date. In psoriasis and ankylosing spondylitis, two autoimmune diseases, the anti-IL-17A monoclonal antibody secukinumab demonstrated efficacy, reducing inflammation and clinical symptoms [389, 390]. In a phase II clinical trial involving relapse-remitting MS

patients, secukinumab reduced MS legion formation [391]. Focus has since switched from secukinumab to the new anti-IL-17A human monoclonal antibody CJM112 as it has better potential than secukinumab because it has a much higher affinity for IL-17A [392]. In psoriasis, targeting IL-23 with ustekinumab also improved disease outcome in moderate-to-severe disease but the beneficial effect was not as strong as that seen with secukinumab [393, 394]. Clinical trial of MS patients with ustekinumab have been less promising than MS trials with secukinumab; reduced lesion formation was not demonstrated for ustekinumab in patients with relapsing remitting MS [395]. This may be explained by the fact that ustekinumab inhibits IL-12 as well as IL-23. IL-12 induced Th1 cell induction and Th1 cells produce IFN-γ. Our lab has demonstrated a protective role for IFN-γ during the effector phase of EAE [333]. The loss of such protection by inhibiting IL-12-induced Th1 cell differentiation may account for the lack of a therapeutic benefit shown with ustekinumab in MS patients. Nonetheless, targeting IL-17 and/or IL-23 for the treatment of MS appears to be a promising avenue. The evidence supporting the benefits of supressing IL-1 in autoimmunity is less clear. In the treatment of moderate-to-severe rheumatoid arthritis (RA), anakinra (the generic name for IL-1RA) is administered to patients who have not responded to initial disease-modifying anti-rheumatic drugs (DMARD) [143]. Anakinra was relatively safe and had a modest, beneficial effect in RA patients. IL-1RA can cross the blood-brain-barrier (BBB) [144]. Therefore, it could have the potential to suppress CNS autoimmunity. Administration of IL-1RA reduced clinical signs of EAE in rats and IL-1RA gene therapy delayed EAE onset and reduced disease severity [145, 146]. MS patients with endogenously high IL-1β and low IL-1RA production are more susceptible to develop relapses [147]. However, the beneficial effects of Anakinra in other human autoimmune diseases, apart from RA, is less convincing. An interesting finding of this study is that *F. hepatica* products induce IL-1RA production and inhibit the development of autoimmunity in mice. Understanding how IL-1RA may mediate this inhibition could help to discover novel targets in the effort to develop new drugs against autoimmune diseases.

This study has identified, isolated, and characterised *F. hepatica* exosomes and FHKTM, an individual protein secreted by *F. hepatica*. There are still issues with the use of these isolated *F. hepatica* products as potential therapies. *F. hepatica* exosomes contain a

mixture of molecules: proteins, lipids, nucleic acids, and metabolites included [290]. Many of the components of the exosomes may induce immune responses of the host but it is still not clear which individual components are responsible for the immunomodulatory effects of *F. hepatica* exosomes that are beneficial in inhibiting autoimmunity. For example, *F. hepatica* exosome induction of IL-1RA production is heat labile and suggested to be protein-mediated. Supporting this claim, results have demonstrated that at least one protein component of the exosomes, FHKTM, is able to promote IL-1RA production. By comparison, FHKTM did not induce eosinophil recruitment and it was shown that eosinophil induction is induced by a heat stable material in *F. hepatica* exosomes. Treatment of the exosomes with glycosidases, nucleases, and lipases could investigate further the nature of the component(s) responsible for eosinophil recruitment. Non-protein mediators of eosinophilia induction previously described in the literature include lipid mediators [396], the micro RNA miR-126 [397], and the small signalling molecule nitric oxide [398]. For individual recombinant proteins like FHKTM, their use as immunotherapies can be complicated by the fact that the administration of recombinant therapeutic proteins can lead to the production of antibodies that neutralise their function [399, 400]. Therefore, the production of antibodies specific for FHKTM could limit the potential for the recombinant protein to be a successful therapy in the clinic. Issues with recombinant protein therapies can also arise to do with the stability and delivery [401, 402].

Regardless of such issues, studying *F. hepatica* derived-products allows us to better understand how helminth infection can attenuate autoimmunity and to focus on the important cells and molecules responsible for this disease modification. Thus, even if the products themselves may ultimately be unsuitable for progression to the clinic, they may aid in the identification of novel, specific targets for which more suitable therapies can be developed and optimised for the treatment of autoimmune diseases.

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