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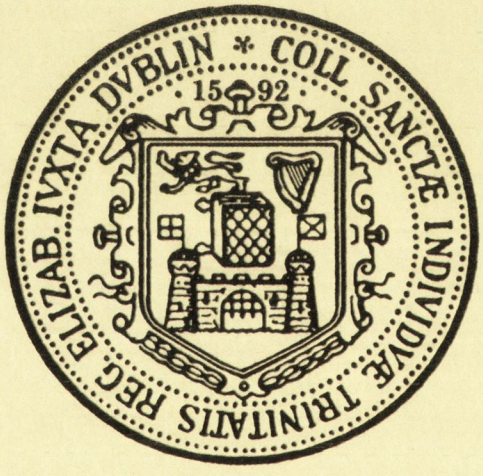
Immune modulation by the helminth parasite *Fasciola hepatica*

2013

Conor Michael Finlay



Trinity College Dublin

A Thesis submitted as completion of the degree of

Doctor of Philosophy

Supervisor: Prof Kingston Mills

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ABSTRACT

The incidence of allergic and autoimmune disease in the developed world has increased dramatically in the past century. This has coincided with a reduction in the incidence of infectious disease. The hygiene hypothesis suggests that reduced exposure to infectious organisms, and in particular parasitic worms (helminths), has led to disordered regulation of the immune system, and hence to an increase in these inflammatory disorders. Infection with parasitic helminths induces polarised Th2 immune responses but this is largely ineffective at clearing the parasite. Indeed, the host remains remarkably tolerant to helminths. There is a growing consensus that the host and parasite work in tandem to mediate this effect, with the helminth benefiting through survival, while the host benefits from not mounting destructive inflammatory reactions. Host immunity to helminths has thus developed, perhaps through co-evolution with helminths, to limit pathology and to balance immune resistance with susceptibility and immune pathology. In recent years, it has been recognised that helminths not only suppress immune responses against themselves, but promote an environment of general immune suppression.There is now convincing epidemiological and experimental evidence to suggest that parasitic helminth infections can prevent the induction of and suppress the severity of autoimmune (Th1/Th17-mediated) and allergic disease (Th2-mediated). This study identifies novel immunomodulatory mechanisms utilised by the helminth parasite *F. hepatica* that mediate the suppression of autoimmunity and allergy. Infection with *Fasciola hepatica* protects mice against experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis, mediated predominantly by Th17 cells. This protection was associated with the recruitment of regulatory T cells and the production of the anti-inflammatory cytokine, TGF-β. The excretory/secretory products (ES) of *F. hepatica* were investigated to determine their immunoregulatory properties. *F. hepatica* ES also attenuated the clinical severity of EAE, in an IL-5 dependent but IL-4 and TGF-β independent manner. Moreover, ES had suppressive activity on dendritic cells. *F. hepatica* ES inhibited LPS-induced production of pro-inflammatory cytokines and the maturation and antigen presenting cell capacity of dendritic cells; thereby preventing the induction of Th1 and Th17 immune responses *in vivo*. Furthermore, ES induced the release of TGF-β from cells of the innate immune system through the induction of reactive-oxygen species. However, ES also promoted the differentiation of Th2 cells and induced eosinophilia *in vivo*. Despite this, ES was capable of suppressing pathogenic Th2 responses in a model of allergic asthma. These findings provide experimental evidence for the validity of the hygiene hypothesis and describe the immunological mechanisms which promote immunosuppression. This study suggests that the specific components of *F. hepatica* ES which mediate these effects may have therapeutic potential in the treatment of allergic and autoimmune disease in humans.

In memory of Mabel Doyle,
my second mother.
Who taught me, amongst other things,
the value of dedication.

PUBLICATIONS

Walsh, K. P., M. T. Brady, **C. M. Finlay**, L. Boon, and K. H. G. Mills. 2009. Infection with a Helminth Parasite Attenuates Autoimmunity through TGF-β-Mediated Suppression of Th17 and Th1 Responses. *J Immunol* 183:1577-1586.

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The Siphonaptera

"The vermin only teaze and pinch
Their foes superior by an inch.
So, naturalists observe, a flea
Has smaller fleas that on him prey;
And these have smaller still to bite 'em,
And so proceed ad infinitum."

Jonathon Swift, 1733

An extract from "On Poetry: a Rhapsody",

Inspired by Leeuenhoek's discovery

that fleas which infest man were

in turn, paratisised

by mites

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Kingston, for his guidance and advice. Since I first saw a lecture from him as an undergrad I have been inspired by his drive, his curiosity and aptitude. I couldn't think of a better place to do a PhD than in his lab. I've met so many great scientists in this lab and as such I have so many people to thank for everything over the last four years. I just hope I don't leave anyone out!
Now, In order of importance!

Viv, running this lab with her would be like waking up in the morning and trying to make tea only to find there was no running water, or electricity, and somebody had let farm animals into the house. I know everyone says it, in their PhD thesis but we really do thank you!

I'd like to thank the few 'teachers' I've had in the lab, while I learnt things from everyone, these were the people I never stopped pestering in the first few years. So thanks to Kevin, Neil, Padraic, Jean, Lisa and Pat, for all the techniques, experimental design, how to look for 'the bigger picture,' and stay interested in science! It's safe to say that these people made me the scientist I am today.

Barry. What a guy!

I want to thank all the post-docs that helped me, in million different ways, throughout my PhD. Thanks to Sarah, Aishling, Padraig, Caroline, Rowan, Jim (especially for the skiing accident) and Sarah J.
And as usual, the PhD students come last (total fear of leaving someone out here). All the reading rooms have been a blast guys!: Paddy (the only other true nerd), Lara (I hope you one day complete your Diet Coke tower of Babel), Karen (for making me feel shrewd with money), Anna (for the science arguments, that I could never win!), Andres (ironically, the person I had most in common with in the whole department), Alicjia, Sharee, Shane, Steven, Corrina Michelle, Anna M, Keith, Nick, Sarah E, Celia and all those Undergrad students and such riff-raff that came and went with seasons.

Thanks Catherine, for all her help in the world of finances; Prof, Joe Cassidy for all the help with histology; Gavin for help with the microscopes; Mary-Pat for keeping the department running and in high spirits!

I want to send out a special thanks to Ania, my partner in crime. We have worked together for four years, and I hope she will agree that we have been the better for it, it's been great to have someone that you can argue your own results with and who comes up with ideas you'd never think you. It has also made me realise that science can be, and should be, a more collaborative exercise.

I have made some great Friends over the past few years, since the great summer of '09 to every Friday night since. It will be great to see you all again! the boyz (see what I did there?), Falcon, Cpt ignorance, Tenner, Johnny boy, The Beast, Bomber, Smurf, Murtag, Sé, The fear Mcglynn, Dr.Mc.D, Donk, Toole, Mac, BOB, Cooney, Welsh, Dales, The Bag, the McGivneys and Feore, The dragon, T, The Shark, In the name of the Casley, Mcateer, Minty, double-Beef, Morrissey and various other shams. I'm not even going to try and list all the Killenard/Port crew because there is too many, but I can't leave out the extended family, especially Liam, Michéal, Mag, Johnny, Eoin and Hatch.

Ciaran and Kevin, who don't have nicknames and who I'm glad I'll get to see more of. I could go with nicknames for the girls, although if any of them actually read this.... I'll just make do with the boring versions, Síle, Coleen, Claire C, Claire Mc, Mary, Emer, Eimear, Eimear, Dee, Banana, Maria, Sinead, Kate, Aimee Caroline, Aine, Emma and more!

To Mam, Dad, Clara, Cáitríona and Fionnuala. Thank you for everything for every kind of support over the past four years and beyond. Thanks especially for putting up with me writing back in Laois. It was great to go home for a while, it made writing up significantly less septic, although it was still pretty awful. Thanks to, and best wishes to the Doyle family, I never would have got off the starting blocks without Mabel.

There must be someone else? Who is it? Eh, eh, eh.

Oh yeah, Cheryl I should thank Cheryl this is how much I should thank her,

Cheryl, thank you!

LIST OF ABBREVIATIONS

AMcam	Acidic mammalian chitinase
APC	Antigen presenting cell
APC	Allophycocyan
Arg	Arginase-1
BCR	B cell receptor
BMDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albmin
CD	Cluster of differentiation
CD3-ζ	CD3-zeta chain
CFA	Complete Freund's adjuvant
Chaffs	Chitinase and Fizz Family Members
CIA	Collagen induced arthritis
CNS	Central nervous system
CpG	Cytosine-phosphate-guanine synthetic oligonucleotide motif
Tc	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
CXCL	CXC chemokine ligand
DC	Dendritic cell
DAMPs	Damage-associated molecular patterns
Derp1	House dust mite allergen
DNA	Deoxyribonucleic acid
dPBS	Dulbecco's PBS
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ES	*F. hepatica* excretory-secretory products
ES-62	*Acanthocheilonema viteae* excretory-secretory product molecule-62
EtOH	Ethanol
FACS	Fluorescence activated cellular sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FIZZ1/Retnla	Found in inflammatory zone 1 / Resistin like alpha
FOXP3	Forkhead winged box transcription factor 3
GATA-3	GATA binding protein 3
GG	Gordon Graham
GMCSF	Granulocyte-macrophage colony-stimulating factor
Gr-1	Granulocyte differentiation antigen 1
^{3}H	Tritiated Thymidine
HBSS	Hanks balanced salt solution
HCV	Hepatitis C virus
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
i.p.	Intraperitoneal
IL	Interleukin
IL-R1	Interleukin receptor 1
iNOS	Inducible nitric oxide synthase
IPEX	Immune dysregulation polyendocrinopathy X-linked syndrome
IRAK	IL-1R-associated kinase
iTreg	Inducible regulatory T cell
KLH	Keyhole limpet hemocyanin
LAP	Latency associated peptide
LFH	Liver fluke homogenate
LNF-III	Lacto-*N*-fucopentaose III
LNnT	Lacto-*N*-neotetraose
LTE_4	Leukotriene E_4
LPS	Lipopolysaccharide
M2	Alternatively activated macrophages
MAL	MyD88-adaptor-like protein
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MBP	Major basic protein
MCP-1	Macrophage chemoattractant protein-1
MDSC	Myeloid-derived suppressor cells.
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metallo-proteases
MR	Macrophage mannose receptor
mRNA	Messenger RNA
MS	Multiple sclerosis
MyD88	Myeloid differentiation factor 88
NES	*N. brasiliensis* excretory-secretory product
NFκB	Nuclear factor κB
ng	Nanogram
NK	Natural killer
NLR	NOD-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOD	Non-obese diabetic
nTreg	Natural regulatory T cell
OVA peptite	$OVA_{(323-339)}$ peptide fragment of chicken ovalbumin protein
PAMPs	Pathogen-associated molecular patterns

PBS	Phosphate-buffered saline
PD-1	Programmed death 1
pg	Pictogram
PEC	Peritoneal exudates cells
PGE_2	Prostaglandin E_2
PMN	Polymorphonuclear neutrophils
PRR	Pattern recognition receptor
PT	Pertussis toxin
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcriptase-polymerase chain reaction
s.c.	Subcutaneous
SEA	*Schistosoma mansoni* egg antigens
SFHEP	*F. hepatica* egg proteins
Siglec	Sialic acid binding Ig-like lectins
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th	T helper cell
TIR	Toll/IL-1R
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
ES-H	ES higher molecular weight fraction (>3 kDa)
ES-L	ES low molecular weight fraction (<3 kDa)
AHR	airway hypersensitivity
AB-PAS	Alican-Blue Periodic Acid-Schiff
BMEOS	Bone marrow-derived dendritic cells
BMM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
H&E	Haematoxylin and eosin
PBMC	Peripheral blood mononuclear cells
TBHP	tert-Butyl hydroperoxide
TE	The total extract of *F. hepatica*
PI	propidium iodide
ION	ionomycin
PMA	Phorbol 12-myristate 13-acetate
ROR	Retinoic Acid Orphan receptor
GMSCF	Granulocyte-macrophage colony stimulating factor
SCF	Stem cell factor
MCP-1	monocyte chemotactic protein-1
OVA	Chicken Ovalbumin
ova	eggs of helminths

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Chapter 1 Introduction

1.1 The immune system

The mammalian immune system protects the host from infection with pathogenic bacteria, viruses, protozoa and parasitic worms. In order to respond to such a wide variety of insults, the immune system has evolved a complex system of receptors, cells and organs, which act in concert to detect and eliminate pathogens. At its foundation is the discrimination between self and non-self. Detection of 'non-self' pathogens activates the inflammatory response; a mobilisation of immune cells and histological changes that culminates in the elimination of the invader. However, inflammation is a destructive process that can cause collateral damage to the tissue. Indeed, inappropriate activation is the underlying cause of many inflammatory diseases, including allergy and autoimmunity. To prevent such outcomes the host has evolved an array of regulatory mechanisms that control inflammatory processes.

The immune response can be broadly classified into two categories, innate and adaptive. The innate immune system provides the first line of defence against pathogens, being immediate in its response and broad in its reactivity. It is activated by conserved molecules shared by many pathogens. The adaptive immune response is essential to the elimination of infections that fail to be controlled by the innate response. While being slower to respond, the adaptive immune system is highly specific and results in the development of 'immunological memory' and immunity to subsequent infection. Upon repeat exposure to the same organism, the second, adaptive immune response is quicker, more pronounced and leads to rapid clearance of the pathogen.

The concept of memory was first recognised in 1796 when Edward Jenner demonstrated that prior exposure to vaccinia virus (the causative agent of the bovine disease, cowpox) induced protection against human smallpox, a discovery that led to the development and widespread use of vaccination, and eventual eradication of smallpox. Jenner achieved this feat without any understanding of the underlying mechanisms, but research conducted in the past 200 years has provided not only an explanation for immunological memory but has revealed the great complexity of the immune system and its importance for human health.

1.2 The innate immune system

Innate defence mechanisms fall into two broad categories: constitutive and inducible immunity. Constitutive mechanisms are the barrier functions of the skin and mucosal membranes that prevent entry of pathogens. However, if these barriers are breached, inducible defence mechanisms play a crucial role in eliminating or controlling infection. Here, the cells of the innate immune system play a crucial role in the detection and destruction of pathogens once they gain entry to the body.

1.2.1 Pattern recognition receptors

The mechanism by which the immune system detected pathogens was unknown until relatively recently. In 1989, Charles Janeway published his theory of pattern recognition, where he proposed that cells of the innate immune system were responsible for the detection of pathogens via the detection of essential conserved molecules (molecular patterns) shared by wide variety of pathogens (1). Research conducted in the last 20 years has validated this theory (2), and it is now accepted that the innate immune system detects pathogens via a set of germline encoded nonclonal receptors, termed pattern recognition receptors (PRRs). PRRs recognize conserved microbial components called pathogen associated molecular patterns (PAMPs) (3).

PAMPs are molecules essential for survival and replication of microbes and include lipopolysaccharide (LPS) from Gram-negative bacteria, lipoteichoic acid from Gram-positive bacteria, cytosine-guanine dinucleotide (CpG) motifs and lipoproteins, also from bacteria and double stranded (ds) RNA, produced by viruses during their infection cycle. PRRs are expressed on the cell surface, intracellularly on endosomal membranes or within the cytoplasm. Recognition of PAMPs by PRRs activates host defence signalling pathways, which control the expression of a variety of immune response genes (3). Downstream effects of this includes the expression of pro-inflammatory cytokine, the enhancement of phagocytosis and other killing mechanisms in phagocytes and the expression of molecules required for antigen presentation. Some PPRs may also recognise endogenous ligands that associated with damage and inflammation such as heat-shock proteins, HMGB1, host DNA and Uric acid crystals. Such ligands are referred to as damage-associated molecular patterns (DAMPS)

Currently identified transmembrane and cytoplasmic PRR belong to four different classes, toll-like receptors (TLR), C-type lectin receptors (CLR), retinoic acid-inducible gene (RIG)-I-like receptors (RLR) and NOD-like receptors (NLR). The TLR family are the most intensively studied PRRs. TLRs are expressed on the cell surface or on the membranes of lysosomes and endosomes (4). Thirteen TLRs have been identified in mammals and are found to be expressed on a wide variety of immune cells (5). Specific TLR are involved in recognition of distinct classes of PAMPs (6). The cytoplasmic signalling domain is known as the Toll/IL-1R (TIR) domain (6). Signalling by TLR utilises a number of TIR containing adaptor proteins, including myeloid differentiation factor 88 (MyD88, used by TLR1, 2, 4, 5, 7, 8, 9), MyD88 adaptor like (MAL), TIR domain containing adaptor protein inducing IFN-β (TRIF, used by TLR3 and TLR4) and TRIF related adaptor like molecule (TRAM) and results in the downstream activation of signalling cascades leading to cellular activation. Each TLR uses different combinations of adaptor proteins , and depending on the identity of the TLR activated, a number of specific signalling pathways can be induced, leading to an appropriate and effective immune response (7).

1.2.2 Cells of the innate immune system

The cells of the immune system reside in tissues of the body or circulate in the blood and in a specialized system of vessels called that comprise the lymphatic system. Innate immune cells originate in the bone marrow where they develop from pluripotent hematopoietic stem cells. They follow two main pathways of differentiation, the lymphoid lineage that gives rise to T and B lymphocytes and natural killer (NK) cells, and the myeloid lineage that gives rise to the mononuclear cells, including macrophages, mast cells, monocytes, dendritic cells (DC) and polymorphonuclear leucocytes (PMN) or granulocytes, which include eosinophils, neutrophils, and basophils.

Macrophages and neutrophils actively kill invading bacteria by phagocytosis, a process by which extracellular bacteria are engulfed and destroyed in internal vesicles. The word phagocytosis comes from the Greek word, phagein, meaning "to devour" was first identified by Ilya Mechnikov in 1882, and its specific mechanisms elucidated by the work of Zanvil Cohen in the 1960s and 1970s (8).

The short-lived neutrophil granulocyte is the most abundant phagocyte; they constitute 50-70% of blood leukocytes but are largely absent from healthy tissue. They are particularly responsive to tissue injury and other inflammatory signals and can enter the site of injury or infection within minutes. Circulating neutrophils respond to early mediators of inflammation such as histamine, prostaglandins and leukotrienes. These mediators act on tissues to induce the localised clinical symptoms associated with inflammation: pain, swelling and vasodilation, which activates and further enhances the recruitment of leukocytes, which enter the tissues from the blood stream by passing through the endothelial wall in a process known as extravasation. Once in the tissue neutrophils play an important antimicrobial role through the phagocytosis of bacteria and the release of toxic metabolites, such as reactive oxygen (ROS) and nitrogen species, proteases and anti-microbial proteins. Neutrophils are also an important source of late mediators, including protein factors, such as cytokines and chemokines, which primarily act on other leukocytes, enhancing chemotaxis and the function of these cells.

The other major granulocytes, the eosinophils, constitute 1-6% of circulating leukocytes and are recruited during parasitic worm infections and during allergic disorders. Eosinophils play a role in controlling infection and mediating inflammation not by phagocytosis but through the release of pre-formed pro-inflammatory molecules, such as histamine and proteins such as eosinophil peroxidase, ribonuclease (RNase), deoxyribonucleases (DNAase), and major basic protein. These mediators are released by a process called degranulation, which follows activation of the eosinophil, and are toxic to both parasite and host tissues. Eosinophils are also a source of cytokines and growth factors that modulate the immune response (9).

Depending on the nature of the infection, mast cells or basophils may migrate to the site of infection. Mast cells are an important source of histamine, and are primarily located in the tissue associated with blood vessels and epithelial surfaces. Basophils are similar to mast cells in that both cell types can be activated by immunoglobulin (Ig) E, but are found in blood. Mast cells are activated during parasitic infections and allergy (9) and play a role in the development of the adaptive immune responses (10). NK cells are innate lymphocytes that play a role in anti-viral and anti-cancer immunity. NK cells kill virally-infected and malignant cells and limit infection and tumour growth prior to activation of the adaptive immune response.

The macrophage is a large, long-lived, tissue-resident immune cell. There are numerous macrophage populations in the body, each associated with a specific organ, and as such they

have highly heterogeneous functions within their specific tissues. In the steady state most macrophages are anti-inflammatory and exert homeostatic functions, such as regulation of metabolism homeostasis, fibrosis, wound repair and removal of dead cells (11).

Through the high expression of PRRs, macrophages also act as tissue resident immune sentinels and respond to both pathogens (PAMPs) and tissue injury (DAMPs) through the production of cytokines that enhance inflammation. Macrophages are highly responsive to cytokines produced by the adaptive immune system. Interferon (IFN)-γ enhances the microbicidal activity of macrophages in a process known as classical activation. Classical activation also enhances the production of inflammatory cytokines including tumour necrosis factor (TNF)-α and interleukin (IL)-1, as well as the toxic metabolite, nitric oxide (NO). This inflammatory state is important for the efficient destruction of bacteria by phagocytosis, but can cause collateral damage to the tissue. In contrast, alternative activation of macrophages is mediated by the action of IL-4 and IL-13. This activation state is anti-inflammatory and plays an important role in wound repair following infection as well as providing a protective role during worm infections (11).

Circulating monocytes give rise to the variety of tissue-resident macrophages and to specialized cells such as dendritic cells (DC). Monocytes originate in the bone marrow from a common myeloid progenitor that is shared with granulocytes. Monocytes constitute 5 to 10% of peripheral blood leukocytes in humans. They circulate in the blood for several days before entering tissues and replenishing the tissue macrophage populations (12). The production of monocytes is enhanced during inflammation when they are required to increase the numbers of macrophages and DC at the site of inflammation (11).

DC are functionally and developmentally similar to macrophages but were identified much later; by Ralf Steinman and Zanvil Cohn in 1973 (13). DC serve as immune sentinels through their high expression of TLRs (14). DC also detect pathogens indirectly by responding to cytokines and other inflammatory factors released by macrophages, NK cells and endothelial cells (15). The two major populations of DC are $CD11c^{hi}$ myeloid and $CD11c^{low}$ plasmacytoid DC (pDC) (16, 17). It has been suggested that the different subsets of DC may play distinct roles in immunity to infection (18), for example human and murine pDC play a vital role in viral immunity by producing large amounts of anti-viral type-1 IFNs (19, 20). DC are the principal cell type that bridge the innate and adaptive immune systems. DC and to a lesser extent macrophages activate the adaptive immune response in a process known as antigen

presentation, thus, these cells are sometimes referred to as antigen presenting cells (APC). DC internalise antigen by phagocytosis or by receptor-mediated endocytosis, and present a fragment of the antigen, bound to a histocompatibility complex (MHC) class II molecule, on the cell surface (14). In order to illustrate the process of antigen presentation, it is first necessary to describe the components of the adaptive immune system.

1.3 The adaptive immune system

The adaptive immune system is unique to jawed vertebrates, and allows the host to 'recognise' and 'remember' specific antigens on pathogens, which leads to a more robust secondary immune response.

The key features of the adaptive immune system are antigen specificity, diversity and memory. The T and B lymphocytes, which together comprise the adaptive immune system, recognise antigens through their antigen receptors, the T cell receptor (TCR) and the B cell receptor (BCR). T and B cells are named after their site of maturation, the Thymus and the Bursa/bone marrow. Each lymphocyte expresses an individual antigen receptor with unique specificity for a particular antigen. This specificity is determined by the amino-acid sequence in the antigen binding region of the antigen receptor. Billions of lymphocytes with unique receptors are created through a complex gene rearrangement of the V-J-D genes that code for the variable region of the BCR and TCR. This gene recombination is under the control of the enzyme coded for by recombination activating gene-1 and -2. Specificity of each lymphocyte clone is determined early in life and is irreversible, each clone gives rise to daughter cells with the same specificity. The mammalian host generates billions of lymphocytes, each with a distinct specificity that allows the host to detect an exhaustive number of unique antigens. Lymphocytes are long-lived but in the resting state are non-proliferative. However, upon encountering cognate antigens, lymphocytes become activated and proliferate readily. After clearance of the infection some lymphocytes take on a memory phenotype and if the same pathogen is re-encountered these lymphocytes are poised to rapidly expand and re-exert their effects. This second, memory response is usually strong enough to clear infection prior to the onset of symptoms. While both T and B cells originate from the bone marrow, T cells are maturated in the thymus in a process known as T cell education.

The induction of adaptive immunity is tightly regulated to prevent unwarranted immune activation but also to prevent the recognition of self-antigens. Innate cells are required for the initiation of the adaptive immune response. The induction of T cell response requires the presentation of antigen bound to MHC molecules expressed on APC. B cells, which can directly recognise antigens through the BCR, nonetheless require activation signals supplied by either activated T cells or innate cells.

1.3.1 B cells and the production of antibody

The antigen-recognition molecules of B cells are the Ig molecules. These proteins are produced by B cells in a vast range of antigen specificities, with each B cell producing immunoglobulin of a single specificity. Membrane-bound Ig on the B-cell surface serves as the BCR. Once a B cell recognises its cognate antigen it differentiates into a plasma cell. The terminally differentiated plasma cells secrete large amounts of the soluble form of the BCR called antibodies. The secretion of antibodies is the main function of B cells and forms the core of humoral immunity. Antibodies bind to specific antigens on pathogens or to their products, including toxins. This has several benefits including the enhancement of phagocytosis (opsonisation), the neutralisation of the effects of toxins and in the prevention of microbes from entering host cells (18).

B cells produce distinct classes of antibodies each with unique effects. There are five Ig classes in mammals, IgM, IgG, IgD, IgA and IgE. Immature B cells express IgM on their surface, but upon activation begin to express IgD. Activated B cells are characterised by secretion of IgG, IgA and IgE subtypes: IgA has been shown to play a role in mucosal immunity, IgE in allergic and parasitic worm infections and IgG subtypes in opsonisation and neutralisation (18). The Ig subclass expression is influenced by multiple factors, including the prevailing cytokine environment. For example, in mice, IL-4 preferentially induces switching to IgG1 and IgE, whereas TGF-ß induces switching to IgG2b and IgA (21, 22). In contrast, IFN-γ induces switching to IgG2a, 2b, and 3 (21).

1.3.2 T cells

T cells develop in the thymus and orchestrate cell mediated immunity, but also function in humoral immunity by providing help for B cell differentiation and survival. Unlike B cells, T

cells do not secrete their antigen recognition receptors. Ligation of the TCR functions to activate the T cell itself. The vast majority of T cells express a dimeric TCR composed of α and β TCR chains. $\alpha\beta^+$ T cells can be subdivided into two categories, $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T cells. $CD8^+$ T cells act to kill virally-infected or tumour cells using an armoury of cytolytic proteins or by inducing apoptosis that limits the spread of infection and tumours. The function of $CD4^+$ T cells is to modulate and orchestrate effector immune responses of other cells (both immune and tissue cells), primarily through the release of cytokines. $CD4^+$ T cells exist in three activation states, naïve (unresponsive cells that have not encountered their cognate antigen), effector (T cells activated following antigen exposure) and memory (a resting state of T cells that have formally encountered antigen, and which rapidly divide upon re-exposure).

The TCR is structurally similar to the Ig molecules that comprise antibodies, and also possess highly variable regions, which account for their specificity. However, unlike the BCR, the TCR does not recognise and bind antigen directly nor does it recognise antigens in their native form; instead it recognises short peptide fragments presented by MHC molecules expressed on the surface of APC. Importantly, each TCR specificity recognises the combination of MHC and peptide, thus T cells are said to be MHC-restricted. The small peptide fragments which bind to MHC (via the peptide binding cleft) can be derived from either intracellular pathogens, such as viruses or bacteria that replicate in the cytoplasm, or from extracellular pathogens that have been internalised by phagocytes from the extracellular fluid. Antigens derived from intracellular pathogens are degraded into short peptide fragments by the cytosolic proteasome and presented on the cell surface by MHC-class I molecules. $CD8^+$ T cells are activated by peptides bound to MHC-class I molecules, this ensures destruction of virally infected cells. Conversely, antigens which are derived from extracellular pathogens activate $CD4^+$T cells. Pathogens or large pathogen-derived molecules are delivered to a phagosome during phagocytosis. The phagosome fuses with another organelle, the lysosome to form the phagolysosome, the resulting drop in pH activates low-pH-dependent proteolytic enzymes, which degrade large proteins to smaller peptides. Peptides obtained in this fashion are presented on MHC-class II molecules. Unlike MHC-class I that is expressed on every cell, the cellular expression of MHC-class II is tightly controlled, being restricted to APC.

Whereas the vast majority of T cells express TCR composed of α and β chains around 5% of circulating T cells express the γ and δ TCR chains. Unlike αβ T cell, γδ T cells function as part of the innate immune response. They are abundant in the gut mucosa and form the majority of the intraepithelial lymphocyte population. γδ T cells are not necessarily MHC-restricted, they respond to a limited diversity of atypical antigens, including non-peptide antigens, phospho-antigens and heat shock proteins. γδ T cells are considered to play a role in the first line of defence against pathogens and are importance sources of T cell associated cytokines early in infection (18).

NKT cells are a population of cells that share properties of both T cells and NK cells. NKT cells recognize the CD1d molecule, an antigen-presenting molecule that binds self- and foreign lipids and glycolipids. They constitute only approximately 0.1% of all peripheral blood T cells (23).

1.3.2.1 T cell education and tolerance

The immune system can discriminate between self and non-self and is tolerant to self-antigens in healthy individuals (24). A breakdown in tolerance to self-antigens can result in the development of autoimmune diseases. The prevalence of autoimmunity is relatively rare since the immune system has developed a number of mechanisms which function to maintain tolerance. Joshua Lederberg in 1959 and Bretescher and Cohen in 1970 provided the first working theories on how this was achieved, suggesting that T cells that recognise self-antigens would be eliminated in the thymus (25).

In the thymus T cells undergo two separate selection processes, collectively known as T cell education. Positive selection or clonal deletion ensures the survival of only those T cells that express a TCR capable of recognising MHC-peptide complexes, thus ensuring MHC-restriction. $CD4^+$ and $CD8^+$ T cells diverge from double positive precursors in this manner and those T cells that recognise MHC class I differentiate into $CD8^+$ T cells, whereas those that recognise MHC-Class II develop into $CD4^+$ T cells.

However, the diversity of TCR generated during T cell development also creates T cells that express TCR that recognise self-antigens. Self-reactive T cells normally undergo negative selection; thymic medulla stromal cells present self-peptides loaded on MHC class I and class II molecules to thymic T cells. T cells which express TCRs with high affinity for MHC-self

peptide complexes undergo apoptosis (26). This is the origin of peripheral naïve $CD4^+$ and $CD8^+$ T cells which recognise only foreign antigens. However, education gives rise to another distinct lineage of T cells, the naturally occurring regulatory T cells (nTreg).

nTreg cells possess a self-reactive TCR, but unlike cells that are deleted by negative selection the affinity of the interaction is low and results in the production of anti-apoptotic molecules (such as GITR), that protect the T cell from apoptosis. IL-2 and co-stimulatory molecule interaction is also required for the differentiation of nTreg cells. Indeed, CD25, the IL-2R alpha chain is highly expressed on nTreg cells (27).

nTreg cells are involved in immune regulation; in the periphery they act to suppress immune responses to self-antigens and thus maintain tolerance. nTreg cells were first identified by Shimon Sakaguchi in 1995 and were defined as $CD4^+$ $CD25^+$ T cells which compose approximately 5-10% of peripheral $CD4^+$ T cells (28). nTreg cells are anergic and do not respond in a proliferative manner to TCR activation (29). Instead, nTreg cells are hyper-proliferative in response to stimulation with IL-2 (30). Mice which are deficient in either IL-2 or the IL-2R α or β chains have fewer nTreg cells and suffer from severe lymphoproliferative autoimmune syndromes, thus demonstrating the importance of IL-2 in nTreg cell function (31). Moreover, depletion of nTreg cells in mice results in the development of lethal autoimmunity, which can be reversed by reintroduction of nTreg cells into depleted mice (28).

The function of nTreg cells is under the control of the master regulatory transcription factor, FOXP3. The expression of FOXP3 in naïve T cells results in T cell anergy and in the development of a regulatory immune phenotype (32). FOXP3 was first described as the gene bearing a mutation which gave rise to scurfy mice. Scurfy mice are characterised by lethal multi-organ infiltration of T cells which is associated with autoimmune reactions (32). A mutation in FOXP3 also causes the human disease immune dysregulation polyendocrinopathy X-linked syndrome (IPEX), which clinically resembles the symptoms in scurfy mice. Both scurfy mice and humans suffering from IPEX are totally lacking nTreg cells. This suggests that FOXP3 is essential for the function of nTreg cells which themselves are indispensable in preventing the development of autoimmunity.

nTreg cells exert their suppressive functions through several mechanisms, some of which require cell-cell contact (33), while other mechanisms of suppression involve the production

of the anti-inflammatory cytokines IL-10 and TGF-β (34). nTreg cells can also suppress the activation of T cells indirectly through the modulation of APC function (35).

1.3.2.2 Antigen presentation

Activation of T cells requires MHC-peptide-TCR interactions, co-stimulation and the presence of polarising cytokines. All three signals are provided by APC including DC, macrophages and B cells. DC are the most effective APC and may be essential for priming of naïve T cells (36). In their immature state, DC express low levels of MHC-class II and costimulatory molecules and are poor activators of naïve T cells. However, in their immature state, DC function as sentinel cells and express high levels of TLRs and other PRRs. Immature DC are very efficient at internalizing and processing antigen (14). DC are activated upon encounter with pathogens. Activated DC migrate to the lymph nodes and undergo a maturation process. Maturation is associated with a reduction in the antigen capturing and processing capacity of DC with a concurrent enhancement in the expression of MHC and the costimulatory molecules, such as CD80, CD86 and CD40 (36, 37). Antigen that was acquired by DC in an immature state, is now presented on MHC-II to cognate T cells in the presence of strong co-stimulatory activity (14). High levels of co-stimulation are required for successful T cell activation; the well characterised co-stimulatory molecules, CD80 and CD86 bind to CD28 on T cells. This ligation reinforces the signal provided by MHC-peptide-TCR interaction (38). Without co-stimulation T cells that are activated through the TCR, undergo apoptosis. This mechanism ensures that DC do not activate T cells in the absence of the correct microbial stimulus and thus, prevents the induction of adaptive immune responses to self and environmental antigens (39).

T cells are initially activated as undifferentiated Th0 cells, but the release of cytokines from activated DC 'educate' T cells to elicit an immune response tailored to the specific pathogen encountered by the DC. As such, DC are central to not only the activation of adaptive immune responses, but also in directing the nature of the adaptive response.

1.3.2.3 Functional phenotypes of $CD4^+$ T cells

$CD4^+$ T cells play a central role in directing and regulating immunity. $CD4^+$ T cells orchestrate immune responses through the production of various cytokines and chemokines, which provide help to B cells and thus promote the production of antibodies, but also

through the activation of the microbicidal activity of macrophages and through the recruitment of inflammatory granulocytes (neutrophils and eosinophils). The cytokines produced by $CD4^+$ T cells also instigate changes in tissue morphology that promote the removal of pathogens and which activate wound repair mechanisms following infection.

The initial description of discrete T helper (Th) cell subsets was provided by Mossmann and Coffmann in 1986. They divided $CD4^+$T cells into two lineages, Th1 and Th2, according to the cytokines that these cells produced. Th1 cells are characterised through their production of IFN-γ while Th2 cells produce IL-4 and IL-13 (40). Mossmann and Coffmann also observed that the differentiation of $CD4^+$ T cells into either subset was dependent on the cytokines present at initial activation. These cells appeared to be reciprocally regulated and had divergent roles in immunity. Early reports suggested that Th1 and Th2 cells were induced by infections with different pathogens, with Th1 cells being induced by intracellular pathogens, whereas Th2 cells were activated during parasitic infection and allergy (21). Another, more recently described subset, the Th17 cells, are thought to be induced in response to extracellular bacterial infections and fungi (34). A fourth subset, induced regulatory T cells (iTreg) act in a suppressive capacity and prevent immune responses directed against self and environmental antigens and in dampening down chronic immune responses that lead to immune pathology.

The discovery of that distinct T cell sub-types expressed distinct transcription factors, (Th1- (T-bet and STAT4), Th2- (GATA-3 and STAT6), Th17 (RORγt and STAT3) and iTreg- (STAT5 and FOXP3)) which control the expression of associated cytokines added to the validity of the Th1/Th2/Th17/iTreg paradigm. Moreover, the individual subsets appear to be cross-reciprocally regulated as lineage commitment antagonises the development of T cells into other subtypes.

1.3.2.3.1 Th1 cells

Th1 cells play a crucial role in immunity to intracellular parasites, including infection with viruses, mycobacterium and protozoa (21). However, until relatively recently Th1 cells were also thought to be responsible for delayed-type hypersensitivity and were thus implicated in the pathogenesis of autoimmune disease (41).

The principal cytokine products of Th1 cells are IFN-γ, IL-2 and tumour necrosis factor (TNF)-α and lymphopoietin. IFN-γ is important in the activation of macrophages, increasing their microbicidal activity through classical activation (42). Classical activation leads to the expression of inducible nitric oxide synthase (iNOS) in macrophages and induces reactive oxygen and nitrogen mediators that kill microbes. IL-2 plays a role in the proliferation of $CD4^+$ T cells and primes $CD8^+$ T cells and NK cells, which kill infected cells (34). TNF-α is a cytokine implicated in destructive inflammatory processes (18).

IL-12 is critical for the development of Th1 cells. IL-12 is primarily produced by activated DC and macrophages (stimulated through PRR with bacterial/viral products) and induces the production of IFN-γ by T cells and NK cells. IFN-γ in turn acts on differentiating T cells to enhance the STAT-4-dependant expression of T-bet, the master transcription factor of Th1 cells (43).

The expression of T-bet is essential for the production of IFN-γ (44). T-bet also enhances the expression of the IL-12Rβ2 on T cells, which enables T cells to directly respond to IL-12 (43). T-bet expression leads to Th1 lineage commitment and the overexpression of T-bet in Th2 cells promotes production of IFN-γ, whilst simultaneously suppressing their capacity to produce IL-4 (43). Cells from mice deficient in T-bet have severe defects in Th1 cell differentiation both *in vitro* and *in vivo* (45).

1.3.2.3.2 Th2 cells

Th2 cells mediate host defence against extracellular parasitic worms and play a key role in promoting wound repair following infection (21). Th2 cells also mediate the pathogenic immune responses that cause asthma and other allergic diseases. Th2 cells produce a range of cytokines including IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25 that have pleiotropic effects central in immunity to parasitic worms (34). IL-4 plays a role in T cell and B cell activation and is the major mediator of IgE class switching in B cells (46). IgE binds to FcεRI on basophils and mast cells and antigen recognition results in their degranulation and secretion of active mediators including histamine and type-2 cytokines, such as IL-4 and IL-13. IL-5 is important in the activation and recruitment of eosinophils and enhances antibody secretion by B cells (47). IL-9 enhances production of mucin during allergic reactions (34). IL-10, produced by Th2 cells, suppresses the proliferation of Th1 cells and inhibits the function of APC (48). The effector cytokine IL-13 is involved in the expulsion of helminths by inducing

changes in the macroscopic nature of mucous membranes, including mucus secretion epithelial/goblet cell hyperplasia. IL-13 also mediates many of the effects of allergy (49). IL-25 (IL-17E) further enhances the production of IL-4, IL-5 and IL-13 through the activation of a newly described innate lymphoid cell called nuocytes (also called natural-helper cells or Innate lymphoid cell-2 cells), which play an important role in type-2 immunity, particularly in the early activation of Th2 cells (50, 51).

IL-4 is essential for Th2 differentiation and thus is both a major effector cytokine and differentiating factor for Th2 cells (52). Studies *in vitro* have shown that IL-4 exerts its effects through the IL-4Rα, which activates the transduction of the transcription factor STAT6 and in turn, controls the expression of GATA3, the Th2 master transcription factor (53). However, the role of IL-4 may be more complicated *in vivo*, as Th2 cells can differentiate in the absence of IL-4, for example, infection with the gastrointestinal helminth parasite *Nippostrongylus brasiliensis* or the administration of the schistsome product Omega-1 induces a robust Th2 response in mice that lack the IL-4Rα and are thus unresponsive to IL-4 (54, 55). The adjuvant alum also induces Th2 responses independent of IL-4 signalling; Th2 responses appear to be induced instead by inflammatory factors released by dying cells (56, 57). Cytokines such as IL-25, thymic stromal lymphopoietin (TSLP) and IL-33 have been implicated in IL-4-independent Th2 differentiation (53). TSLP, primarily synthesized by epithelial cells, acts on DC to generate a type-2 inducing APC phenotype; it has also been implicated as a primary mediator in models of airway hypersensitivity and asthma (58). The cellular source of IL-4 has also been hotly debated, as Th2 responses can exist in the absence of DC. Indeed, DC are poor producers of IL-4 (59). It has been reported that basophils and eosinophils can produce high concentrations of IL-4 (60), and it was shown that basophils can act as both a source of IL-4 and as APC which promote Th2 differentiation (61-63). Other reports contest these findings with the demonstration that mice deficient in DC are devoid of Th2 cells (64).

Parasitic worm products and allergens such as house dust mite and bee venom, can induce Th2 responses. However, the specific mechanisms of how these products promote Th2 differentiation via DC are unclear. Indeed some molecules that induce Th2 cells via DC can act independent of TLR ligation. Proteases, for example, are potent inducers of Th2 cells and are detected via proteases-activated receptors in a poorly understood mechanism (65) The cysteine protease, such as papain can promote the induction of Th2 cells via ROS production

that subsequently activates the release of TSLP from epithelial cells (66). However, TLRs are required for the detection of other proteases. The house dust mite protease, Derp1 induces the activation of TLR-4, which results in the production of TSLP and IL-33 by epithelial cells, which in turn acts on DC to promote the differentiation of Th2 cells (67). FcεR1 and OX40L when expressed on DC, are also associated with the induction of Th2 responses (68, 69).

1.3.2.3.3 Th17 cells

Th17 cells are an inflammatory cell subset which play a crucial role in immunity to extracellular bacteria such as *Klebsiella pneumonia*, and fungi such as *Candida spp.* Th17 cells are central to the pathology of chronic inflammatory and autoimmune diseases (70). Th17 cells are characterised by the production of IL-17A, but also produce IL-17F, IL-21, IL-22 and GMSCF (34). IL-17 promotes inflammation, primarily through recruitment and activation of neutrophils, but can also induce the production of GM-CSF, IL-6 and IL-8 (a major chemotactic factor for neutrophils) and a range of other chemokines by macrophages (34, 71). Furthermore, the IL-17 receptor is ubiquitously expressed on many cell types, enabling IL-17 to exert global pro-inflammatory effects (71). IL-22 enhances the barrier function of epithelial cells and is considered to play a protective role in the gut, where barrier integrity is essential to contain commensal bacteria in the lumen. However, IL-22 also mediates the pathogenic skin hyperplasia in psoriasis (72). IL-21 acts in an autocrine manner on Th17 cells to amplify the early IL-17 response (73). GMCSF primarily stimulates the differentiation of granulocytes in the bone marrow (18).

Until recently the Th1-IL-12 axis was thought to play a central and unique role in organ-specific autoimmune diseases. However, Cua et al demonstrated that IL-23 and not IL-12 was required for the development of experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (74). Further studies demonstrated that IL-23 plays a central role in the development of Th17 cells which were subsequently described as a unique Th subset (75, 76). A firm role for Th17 cells in many T cell-mediated autoimmune diseases is now well established (70).

The Kuchroo and Stockinger groups demonstrated in 2006 that IL-6 and transforming growth factor-beta (TGF-β) play a central role in the differentiation of Th17 cells (77, 78). These cytokines were subsequently shown to induce the STAT3-dependent expression of RORγt, the master transcription factor of Th17 cells (79). RORγt is essential to Th17

function as mice deficient in *rorc* (the gene coding for RORγt) have reduced numbers of Th17 cells and are resistant to EAE (79). However, it was later shown, that only low concentrations of TGF-β were required for the differentiation of Th17 cells and higher concentrations of TGF-β in fact suppressed their development (34). It was subsequently reported that TGF-β was only required indirectly, to inhibit T-bet and GATA-3 expression and thus allow ROR-γt to dominate (80). However, IL-6 does appears to be required for Th17 cell differentiation as mice deficient in IL-6 are resistant to actively induced EAE (81).

Later, findings from this laboratory demonstrated that IL-1β in combination with IL-23 can induce IL-17 production by Th17 cells independent of TGF-β and IL-6 (82). However, the exact role of IL-23 in the development of Th17 cells was still unclear. While IL-23 was shown to be dispensable for the *de novo* differentiation of Th17 cells (77, 83), several reports demonstrated that IL-23 was essential later in the development of Th17 cells to expand, maintain and stabilise committed Th17 cells (34, 77, 83, 84). Importantly, IL-23 is essential for the pathogenic function of Th17 cells in EAE (74).

Gamma-delta (γδ) T cells, a non-classical innate T cell subset, were shown to produce IL-17 in response to IL-1 and IL-23 (85, 86) and are intimately involved in the induction of autoimmune processes. Since the discovery that Th17 and not Th1 cells mediate the pathogenesis of EAE, numerous groups have demonstrated a pathogenic role for IL-17 and Th17 cells in human autoimmune diseases including MS, psoriasis and Crohn's disease (34).

1.3.2.3.4 iTreg cells

Treg cells play a critical role in maintaining self-tolerance and in the prevention of immune pathology (33). Approaches aimed at increasing the frequency of Treg cells numbers have therapeutic potential in the treatment in autoimmune diseases. On the other hand, depletion of Treg cells can enhance immunity against tumours (87). There are two major types of Treg cells, nTreg and inducible Treg cells (iTreg). Unlike nTreg, iTreg cells differentiate in the periphery (outside the thymus) in a similar manner to other Th cell lineages and while nTreg cells are sufficient to control immune responses to self-antigens, iTreg cells are essential in maintaining tolerance to food, environmental and commensal antigens.

High concentrations of TGF-β, in the absence of proinflammatory cytokines (which may favour Th17 cell differentiation) acts as the differentiating factor for iTreg cells. (88, 89). In

2003, Chen et al demonstrated that TGF-β can convert naïve T cells into iTreg cells which express FOXP3 (88). IL-2 is also required for the expression of FOXP3. TGF-β and IL-2 together, enhance the survival and function of iTreg cells (90). TGF-β can also act on DC to enhance the expression of indoleamine 2,3 dioxygenase (IDO) an enzyme that confers suppressive activity on DC (91). These tolerogenic DC express costimulatory molecules at low levels and preferentially induce iTreg cells.

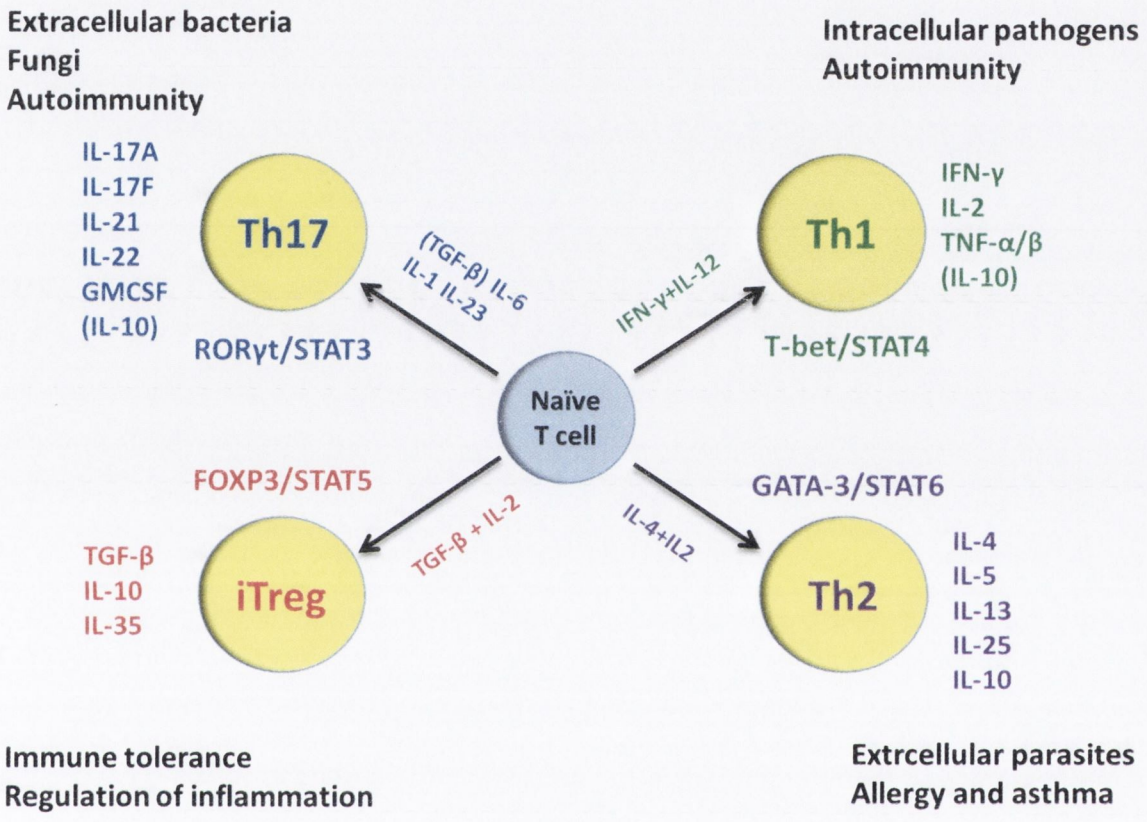


Figure 1.1 Summary of the four CD4 T helper cell Lineages: their functions, their cytokine products, their characteristic transcription factors, and cytokines critical for their differentiation. Figure modified from Zhu and Paul, 2008 (34)

iTreg like nTreg cells can exert their suppressive function by cell-cell contact (33). Cell contact mechanisms can involve the expression of the inhibitory T cell ligand CTLA-4 which binds to CD28 and antagonises CD28-CD80/CD86 co-stimulation (92). However, iTreg cells also mediate suppression via the production of regulatory cytokines.

Regulatory-type-1 (Tr1) cells, a subset of iTreg cells were originally classified by the high production of IL-10. IL-10 exerts a variety of suppressive effects. IL-10 is important in limiting immune mediated pathology during infection. For instance, during infection with *Leishmania* spp, Treg-derived IL-10 protects mice against catastrophic organ damage at the cost of reduced clearance (93). However, IL-10 is not under control of FOXP3 and has recently been shown to be produced by all four Th cell subsets under certain conditions; thus Tr-1 may represent a functional state, rather than a truly differentiated cell type (94). Regardless, iTreg cells produce high amounts of IL-10 and mice deficient in Treg-derived IL-10 develop colitis and lung inflammation early in life suggesting an important role for IL-10 in the regulation of commensal and environmental antigens (95).

iTreg cells that produce TGF-β were initially classified as Th3 cells, although this name is no longer used (96). TGF-β is a pluripotent cytokine, with effects including, immunosuppression, fibrosis and in development (97). TGF-β1 deficient mice die within 3-4 weeks of birth due to a multi-organ inflammatory syndrome, which is characterized by the hyper-activation of T cells and a progressive infiltration of leukocytes into the affected organs (98). TGF-β affects T cell proliferation, differentiation and survival. One of the mechanisms underlying the inhibition of T cell proliferation by TGF-β is the blockade of IL-2 transcription (99). TGF-β inhibits the proliferation of naive T cells, but does not affect activated T cells, which have reduced expression of TGF-βRII (100). Interestingly, IL-10 induces the expression of TGF-βRII, thereby conferring TGF-β responsiveness upon activated T cells (100), suggesting that these cytokines may work in tandem to regulate immune responses.

1.3.2.3.5 Plasticity

Recent work has questioned the concept that $CD4^+$ T cell subsets are terminally differentiated An emerging view is that these subsets may be more plastic that previously appreciated and under certain conditions may convert into a different subsets. Thus, $CD4^+$ subsets may represent functional states of activated cells. One factor contributing to this change in dogma is increasing complexity; since the original description of the Th1/Th2 dichotomy, the paradigm has been amended to include the two more types, Treg cells and Th17 cells. In addition, T follicular helper (Tfh) cells have been recently considered to be a separate lineage. These cells are committed to helping B cells to produce antibody specific for T cell dependent antigens. Tfh also express a unique master transcription factor, Bcl6 (101). However, these cells can share many properties with Th1 and Th2 cells, including the production of

associated cytokines and are considered by many to represent a functional state of Th1/Th2 cells similar to the memory phenotype of T cells.

Similarly, IL-10 was originally considered to be a Th2 cytokine and more recently a Treg cytokine. However, IL-10 is now known to be produced by all T cell subsets (102). Moreover, although reciprocally regulated, IL-17 and IFN-γ can be expressed together in Th17 cells. These cells are potent inducers of inflammation *in vivo*. Th17 cells can also completely loose expression of their signature cytokine and become sole expressers of IFN-γ (102). Th17 cells differentiated *in vitro* lose their expression of IL-17 without constant exposure to TGF-β and IL-6 (103) and may convert to a Th1 or Th2 phenotype in the presence of IFN-γ or IL-4, respectively (103). In addition, IL-17$^+$IFN-γ$^+$ T cells have been reported in the CNS of mice with EAE (104), these cells are highly pathogenic and can induce EAE upon transfer (105). Similarly, Th1 cells have been shown to produce IL-13, a cytokine once thought to be produced exclusively by Th2 cells (101).

This plasticity may be explained by expression of multiple transcription factors/repressors concurrently in one T cell. For instance, FOXP3, the transcriptional repressor originally thought to be expressed exclusively by committed Treg cells is now known to be co-expressed with T-bet, GATA-3 and RORγt in Th1, Th2 and Th17 cells under certain conditions. Under these conditions FOXP3 binds to lineage specific genes including IL-2, NFAT, AP1, IL-4, GM-CSF and TNF-α, which results in a reduction in cytokine expression and induction of regulatory activity (106).

Effector function is thus controlled by gradients of expression of multiple transcription factors. This plasticity may be advantageous to the host as they adapt to shifting conditions during the natural course of infection. The plasticity hypothesis need not replace the theory of CD4$^+$ subsets, which has a strong molecular basis, but rather does not consider that this commitment is absolute, adding another level of complexity that confers on the immune system more options for the maintenance of homeostasis.

1.4 Immune-mediated diseases

The loss of tolerance to self, environmental or commensal antigens leads to the induction of adaptive immune responses that are deleterious to the host. Autoimmune diseases are mediated by adaptive immune responses against self-antigens, which cause organ-specific

tissue damage. Such diseases include type-1 diabetes, MS, rheumatoid arthritis and systemic lupus erythematous. Colitis and Crohn's disease are mediated by a breakdown in the tolerance to commensal bacteria in the gut. Allergic disorders are caused by inappropriate immune responses against innocuous environmental antigens and include allergic asthma, allergic rhinitis and eczema.

1.4.1 Autoimmunity

1.4.1.1 Multiple Sclerosis

MS is an inflammatory demyelinating human disease of central nervous system (CNS) (107). MS is a chronic debilitating disease and is the second leading cause of disability in young adults in the developed world (108). The aetiology of disease is still largely unknown, but there is evidence to suggest that genetic susceptibility, environmental contributions and/or infectious agents may play a role. Individuals with HLA DRB1*1501 are three to four times more susceptible than individuals with a different HLA type at this locus, suggesting a role for APC (108). Low levels of sunlight have also been identified as a contributing factor, with higher incidence of disease at higher latitudes (108). Studies have also shown that MS exacerbations often follow viral infections, suggesting that infections may play a role in disease development and progression (108). Molecular mimicry has been proposed as a mechanism to explain how infections may induce MS; suggesting that self-reactive T cells are activated by pathogen antigens that are homologous to self-antigens (108).

Several studies have indicated that MS is a classic T cell-mediated autoimmune disease, of which the autoantigen(s) is contained within myelin. It is noted that healthy individuals possess anti-myelin specific T cells, but in MS patients these myelin reactive T cells are of an activated phenotype (109, 110). It is thought that MS involves the activation of myelin-specific Th1 and Th17 (111). Both IFN-γ and IL-17 are involved, leading to the recruitment and activation of macrophages and neutrophils (111, 112). These macrophages exhibit increased phagocytic activity and release NO metabolites, matrix metallo-proteases (MMP) and complement, which damage myelin (111). Auto-antibodies raised against myelin are also important in demyelination (113). Typically, the disease course involves periods of remission and exacerbation, which can last for decades prior to progression to a stage of continuous inflammation and neurodegeneration (107).

1.4.1.2 Experimental autoimmune encephalomyelitis

EAE is a widely used animal model of autoimmunity. EAE is a demyelinating CNS disease that shares clinical and pathological features with MS (111) and has been used in the development of therapies for MS, including natalizumab and copaxone (114). EAE is actively induced in susceptible strains of mice and rats by immunisation with one of a number of purified recombinant myelin antigens emulsified in complete Freund's adjuvant (CFA), followed by injection of pertussis toxin (115). EAE is primarily a $CD4^+$ T cell mediated autoimmune disease and initiates with the permeabilization of the blood-brain-barrier, followed by translocation of myelin-specific $CD4^+$ T cells into the CNS. Upon entering the CNS, myelin-specific $CD4^+$ T cells are reactivated by local and infiltrating APC, which present myelin-associated peptides to T cells, resulting in subsequent inflammatory reactions that eventually damage CNS tissue (115).

Myelin-specific Th1 and Th17 cells have both been implicated in the initiation of EAE and in almost all models of the disease, these T cells can be isolated and used to transfer disease to naive animals (116).

EAE can be induced in susceptible mice by adoptive transfer of IFN-γ producing T cells (117), Moreover, T-bet deficient mice are protected from EAE (118). However, the role of IFN-γ in the pathogenesis of this autoimmune disease has been questioned, since mice deficient in IL-12p35, STAT1 or IFN-γR are more susceptible to EAE (74) and animals with disrupted IFN-γ genes are not protected from disease (119). In addition, a number of groups have reported that EAE can be induced in mice lacking the IL-12R (120) and IL-12 (121).

Cua et al demonstrated that IL-23, not IL-12 was essential for the induction of EAE (74). Mice deficient in the IL-23p19 or IL-12p40 subunit and therefore lacking in IL-23 or IL-12 and IL-23 respectively were resistant to EAE, whereas mice lacking in the IL-12p35 subunit alone were susceptible to disease (75, 76). Previous studies investigating the role of IL-12 had utilised animals deficient in IL-12p40, but these were deficient in IL-23 as well as IL-12. It has since been shown that Th17 cells expanded by IL-23 induced EAE following transfer to naïve mice (122). However, a study investigating the kinetics of T cell infiltration during the course of EAE revealed a decrease in the infiltration of Th17 cells during the late stages of disease, which corresponded to an increase in Th1 cells and resolution of EAE, suggesting a possible regulatory role of IFN-γ (104).

Thus, the precise role of Th1 and Th17 cells in EAE has become rather complicated. Indeed, abrogation of genes associated with either Th1 or Th17 cells often provides contradictory results as to which cell is more important; adding complexity is the apparent cross-regulation of these two cell types.. However, another study reported that EAE could be induced by adoptive transfer of Th1 or Th17 cells, but each cell induced disease with distinct clinical outcomes and with different localization within the CNS (123). Transfer of Th1 cells induced classical EAE with only spinal cord involvement, whereas Th17-induced EAE was characterized by atypical disease involving inflammation of the brain (123). Thus it appears that Th1 and Th17 are both involved in complementary pathogenic processes in CNS autoimmunity.

1.4.2 Allergy

Allergy is the hypersensitivity to innocuous environmental antigens (allergens) (124). Common allergies include asthma, hay fever (allergic rhinitis), insect venom and food allergies, such as those to peanuts. It is estimated that 40% of the population of Western Europe are atopic (prone to allergic responses). These conditions range from minor irritants to potentially fatal diseases (124).

The symptoms of allergy are caused by type-1 (or immediate) hypersensitivity reactions to the allergen. Allergen-specific IgE produced by plasma cells binds to FcεR1 which is constitutively expressed on mast cells. The allergen cross-links IgE on the surface of mast cells, inducing degranulation and release of allergic mediators, such as histamine, prostaglandins and leukotrienes. This results in the development of early phase symptoms of disease, including itching, inflammation and the contraction of smooth muscle. Mast cells also releases cytokines and chemokines which attract T cells, eosinophils and basophils, in what is referred to as the late phase response (6 hours or more) and contributes significantly to inflammation (124). Eosinophils release toxic granule proteins such as eosinophil peroxidase and major basic protein which cause significant damage. Activated eosinophils can also enhance the expression of FcεR1, which enables them to respond directly to IgE (125).

Th2 cells predominately mediate allergy. The mechanism by which allergic responses are initiated in atopic individuals is still not clear but both environmental and genetic factors are thought to play a compounding role. As most allergic disorders present clinically at epidermal

and mucosal surfaces, a breakdown of the physical barrier is thought to play a role in pathogenesis. Experimental studies in mice have shown that the route of administration of antigen can influence the immune response as the mucosal membranes seem to be predisposed to a Th2 response; possibly as an adaptation that prevents destructive type-1 and 17 responses at these sites. The mechanism of induction of allergy by some but not other antigens is unclear, but molecular mimicry of antigens present in parasitic worms is a possible explanation e.g. bee venom and peanuts. Other factors include high solubility of allergen (allowing penetration into the mucosa) and protease activity (which disrupts epithelial borders) (124).

1.4.2.1 Asthma

Asthma is a common chronic allergic disease characterised by airflow obstruction and bronchospasm. Symptoms include wheezing, coughing, chest tightness, and shortness of breath. Acute attacks of asthma can be fatal and it is estimated that 250,000 people die each year from the disease worldwide (126). Asthma appears to be more prevalent in urbanised and developed countries, although it is associated with higher mortality rates in less developed regions, owing to the lack of treatment options available (126). Management options involve avoidance of allergens and triggers (tobacco smoke for example), while common therapeutics target bronchoconstriction e.g. albutamol, a short-acting β2-adrenergic receptor agonist used for the relief of bronchospasm, and general immunosuppressive therapies such as inhaled or oral glucocorticoids. Allergens that are implicated in allergic asthma include house dust mite, animal dander and aspergillus mould, but also include any number of inhaled particulate proteins and chemicals. The aetiology of disease is complex and thought to include genetic and environmental factors. Obesity and the male gender are associated, as is air pollution and tobacco use. Chronic exposure to allergens is a mitigating factor but not a direct cause. Genome-wide linkage studies have revealed an atopy linkage region on chromosome 5q31-33 (127). This region contains genes for IL-4, IL-13, CD14 (co receptor along with TLR4 for LPS) and the B_2 adrenergic receptor (ADRB2, regulates smooth muscle contraction). Certain HLA haplotypes have also been associated with progression of disease, underpinning the importance of antigen presentation. Single nucleotide polymorphisms (SNPs) in the genes coding for STAT6, CTLA-4, IL-4Rα, FCεR-1, TLR 4, TGF-β, IL-10, CD14 and NOD2 are also associated with development of disease, suggesting defects in microbial sensing, Th2

biology and immune regulation may play a role. Other genes associated with epithelial cell barrier function and airway remodelling also appear to be important (127).

Asthma attacks are triggered by allergen-induced activation of sub-mucosal mast cells in the lower airway and follow an early/late phase pattern similar to many other atopic diseases. Allergen-induced mast cell degranulation causes the immediate release of histamine and prostaglandins, which act on the airways causing self-resolving bronchoconstriction. However, several hours later mast cells produce cytokine, chemokines and leukotrienes (which must be synthesised by the mast cells before release), resulting in the late phase response, characterised by influx of Th2 cells, eosinophils and basophils (124).

A compounding problem of asthma is this late phase response does not self-resolve. Prolonged inflammation and damage results in the generation of positive feedback mechanisms, whereby influx and activation of Th2 cells continually recruit other cells (124). The airways thus become characteristically hyper-reactive; in this stage, factors other than re-exposure to allergen can trigger acute attacks, which explains why tobacco smoke or infections can exacerbate disease in the absence of allergen. In the long term, the original antigen may become irrelevant as the self-perpetuating atopic inflammation takes over. The constant interplay of inflammatory cells and the release of mediators leads to tissue remodelling of epithelial cells, goblet cells, smooth muscle and results in chronic damage of the airways.

While mast cell degranulation mediates bronchoconstriction, obstruction of the airways is caused by mucus deposition, a severe problem in asthma as this obstructs the already constricted airways. IL-13 acts on the bronchial epithelial layer to induce goblet cell hyperplasia. Goblet cells produce and secrete mucin into the airway lumen, which acts to aggregate and obstruct the airways (128).

Thus, Th2 cells, through the production of IL-4, IL-5 and IL-13 can initiate and maintain the key pathogenic features of asthma. IL-4 is important for original allergic sensitisation and IgE production. IL-5 is crucial for eosinophilia and eosinophil survival and IL-13 mediates many of the tissue–specific features of disease, such as the remodelling of tissue and deposition of mucus (129).

There has been considerable interest regarding the protease activity of allergens in the aetiology of asthma. The faeces of house dust mites contain a papain like cysteine protease, called Derp-1. Cysteine proteases active Th2 cells by inducing cytotoxic damage or by activating protease activated receptors, which are linked with DC maturation (65). Proteases may also reduce the integrity of the mucosal epithelial barrier, allowing allergen to be taken up by sub-epithelial APC (124). The Th2-inducting activity of proteases is supported by the fact that native but not inactivated papain induces IgE responses in mice (130). The presence of low amounts of endotoxin in Derp-1 seems to be important for initial immune activation (67). Low concentrations of LPS are known to preferentially induce Th2 responses (131). Interestingly, Derp-2, a related protease also found within house dust-mite faeces, is structurally similar to MD2 a co-receptor for TLR-4 and thus may act as an adjuvant as well as an allergen (132).

Recent studies have demonstrated the importance of non-canonical type-2 cytokines released by epithelial cells in the generation of Th2 cells in the lung. Proteases such as papain also induce Th2 cells via the production of TSLP and IL-25 by epithelial cells (66). Derp-1 acts on TLR-4 expressed on airway epithelial cells to promote the production of TSLP and IL-33 (67). TSLP was found to be required for AHR in mice (133) TSLP is also thought to condition DC to generate a Th2-inducing phenotype. Downstream, IL-25 and IL-33 acts on nuocytes, which are an innate source of type-2 cytokines, aiding the development of Th2 cells (134). Indeed these cells may contribute to the overall atopic environment as a recent report demonstrated that airway hypersensitivity could be induced in mice that lacked T or B cells. This was mediated via the production of IL-4 and IL-13 by nuocytes (135).

1.5 Parasites

The word parasite is derived from the Greek word, Parasitos which means literally "one who eats at the table of another." Küchenmeister, in 1855 defined parasites in the following manner:

> "Parasites are independent organised beings... in order that they may be enabled to complete their development, growth, or reproduction... take up their abode either constantly or temporarily in or upon a second animal... from which they also derive their nourishment."

Parasitism is a complicated phenomenon that not only includes internal parasite infections but external infestation by lice and fleas and any behaviour where one organism benefits at the expense of another. Parasites increase their fitness by exploiting hosts for resources necessary for their survival, including nutrients, heat, habitat, and transmission. It differs from predation in that the host is usually much larger than the parasite and parasite does not intend to kill the host. Internal parasitism usually refers to infections with macroparasites and infections with viruses and bacteria, while technically parasitical in nature are, in general use, excluded from the term. Protozoa and parasitic worms are macroparasites which infect mammals. One common feature of such infections is that parasites generally have a complicated life cycle that require infections of both definite (mammalian) hosts, and intermediate hosts, which can include arthropods and other invertebrate animals or even other mammals. Diseases of humans caused by protozoal infections include African trypanosomiasis (sleeping sickness) and Chagas' disease caused by infection with flagellate protozoa of the genus *Trypanosoma* or malaria which is caused by infection with the protists of the genus *Plasmodium.*

1.5.1 Helminths

Worms are metazoan invertebrate animals, which belongs to one of several phyla. Parasitic worms are called helminths, in order to differentiate them from free living worms. Helminths include species from two phyla, Nematoda and Platyhelminthes.

Nematoda or roundworms: are elongated and cylindroidal in shape with a basically bilateral symmetrical arrangement. The outer surface consists of a non-nucleated cuticle secreted by the underlying hypodermis. Unlike cnidarians (free-living worms) and platyhelminthes, nematodes have tubular digestive systems with openings at both ends and those that are parasitic in humans are unisexual (136). Those nematodes of interest to human and mouse immunology include, the intestinal nematodes *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis* and *Necator americanus*, *Strongyloides stercoralis,* the filarial nematodes *Litomosoides sigmodontis* and *Brugia malayi* and the whipworms *Trichuris suis* and *Trichuris muris.*

Platyhelminthes or flatworms: These helminths are bilaterally symmetrical and are compressed dorsoventrally to give a flat leaf-like (trematodes) or tape-like shape (cestodes). Unlike nematodes, they lack a body cavity and they have no

specialized circulatory or respiratory organs which restricts them to flattened shapes to enable oxygen and nutrients to pass through their bodies by diffusion. Instead, they are covered in tegument, a biologically active outer layer of multinucleated cells covered with a carbohydrate glycocalyx. The tegument performs all the vital activities such as protection, absorption, excretion and secretion. It has a large surface area for the enhanced absorption of nutrients. The tegument is also the host-parasite interface, protecting the worm from digestive enzymes and immune system of the host. Flatworms are subdivided into two classes:

Cestoda or tapeworms: The adult worms are usually segmented with the body being divided transversely into separate, sexually complete units called proglottids. In cestodes the tegument is further covered with highly specialized microvilli, which project from the outer membrane of the tegument. Since cestodes are devoid of any digestive and excretory systems, the tegument constitutes the principal site of absorption of nutrients and elimination of waste materials. Adult worms of different species live almost exclusively in the intestine and are hermaphroditic. Species of the genera *Taenia* and *Echinococcus* are parasites of mammals. *Taenia solium* infection in humans is widely known to be contracted by consumption of undercooked pork since the pig is the intermediate host.

Trematoda or flukes: These worms are unsegmented and the adult forms have suckers and an incomplete gut for digestion. Thus, trematoda use the tegument for excretion and gas exchange. The tegument contains a number of invaginations or surface pits, and is dispersed with bristle-like projections called spines which are composed of actin filaments. Trematodes are hermaphroditic and reproduce both sexually and asexually in the definite host. Almost all trematodes use molluscs as intermediate hosts. The two best described flukes are the blood flukes of the genus *Schistosoma*, and the liver flukes of the genus *Fasciola.*

1.5.1.1 Heterogony and transmission

Most helminths require more than one host in order to complete their life cycle. A definite host is one in which the adult worm reproduces and an intermediate host, is one which contains one or more types of larval stages. However, there are exceptions, namely hookworms, which exist as independent soil-living worms in their larval stage and do not require an intermediate host. These concepts are called heterogony, and were first conceived by Peter Christian Abildgaard, who in 1790 discovered the larva form of a tapeworm known to infect ducks in the intestines of fish and demonstrated that ducks became infected by

eating these fish. Such observations helped dispel the prevailing view, held since classical times that worms arose from "spontaneous generation" in the host (136).

Helminths enter the definite host via contaminated food or water, soil, insect bites, and even sexual acts. Poorly washed vegetables, eaten raw may contain eggs of nematodes such as *Ascaris*, *Enterobius*, *Trichuris*, and/or cestodes such as *Taenia* and *Echinococcus*. Plants such as watercress can be contaminated with liver fluke metacercariae. Undercooked meats may transmit *Taenia* (pork, beef and venison), *Trichinella* (pork and venison), *Diphyllobothrium* (fish), *Clonorchis* (fish), and *Paragonimus* (crustaceans). *Schistosomes* and nematodes such as hookworms (*Ancylostoma* and *Necator* and *Strongyloides*) infect the host by actively penetrating the skin upon contact with contaminated water or soil.

1.5.1.2 Epidemiology

It is estimated that one quarter of the population is infected with helminth parasites. However, in developing counties this prevalence is much higher (137, 138). Hookworm infection affects between 750-1300 million people worldwide, but this is almost entirely restricted to sub-Saharan Africa, Latin America and south-east Asia (138, 139). There are several reasons for the disparity in the geographic spread of helminthiasis: the endemic regions overlap with the natural geographical range of the intermediate hosts of many helminths. In addition, the greater reliance on the use of untreated water supplies can provide habitats for the intermediate mollusc host of schistsomes and these regions generally lack availability to anti-helminthic drugs. In these regions, infection of individuals with numerous species is common (polyparasitism) as is coinfection with HIV and malaria, which is likely due to the overlapping geographical distribution of these diseases (140, 141).

Helminths cause long lasting infections that inflict harm on their host via competition for nutrients, mechanical/enzymatic damage during tissue translocation and immune pathology. Common symptoms include chronic anaemia, malnutrition and fatigue. However, a major feature of many helminth infection is that they are often totally asymptomatic in otherwise healthy adults (142). In most cases, infection with species of the genera *Schistosomes, Necator* and *Trichuris,* which collectively infect over a billion people, cause mild or benign disease, although the severity of disease is proportional to worm burden. However, other species of helminths are associated with significant morbidity, disability and reduction in life expectancy. For example *Brugia malayi* and *Onchocerca volvulus* are the causative agents of the disabling and

disfiguring diseases, lymphatic filariasis (elephantiasis) and river blindness, respectively. Infection with these parasites results in a chronic inflammatory disease, tissue damage and fibrosis, which may persist even after the worm is expelled (143).

Most helminth species have a propensity to infect children. Children and adolescents harbour greater number of worms and are more vulnerable to infection than adults (143, 144). Moreover, in addition to the aforementioned symptoms observed in adults, infection of children has more insidious effects including growth retardation, diminished physical fitness and even impairment in cognitive development (145). Helminth infections are thus viewed as not only an important individual health problem, but also as one of social and economic development (140). It is estimated that the disability-adjusted life years lost each year as a result if helminth infection is 39 million, a figure higher than that of malaria and almost comparable with tuberculosis (146). Such disease also has an immeasurable effect on societal development through days lost in school and work and diminished fitness of the population.

1.5.1.3 Treatment of helminth infections

Helminth infections are regarded as neglected tropical diseases (138). Deaths are rarely directly caused by helminth infection and as a result the impact of these pathogens on human health has been largely overlooked. Helminth infections also primarily affect poorer people in resource-constrained regions of the world, those people that lack the resources to protect themselves from infection. As disease results largely from poverty, it in turn contributes to further poverty by impairing agricultural and economic productivity (139).

International investment into the problem of helminthiasis is quite poor, especially when compared with diseases typically associated with the developed world (heart disease, diabetes and cancer). This is also reflected in research spending; less than 1% of global research funds are targeted for helminth diseases, with little investment from the pharmaceutical industry, likely due to a perceived lack of return on investment due to helminth infections being restricted to the world's poorest people. These facts go a long way to explaining why there has been little development in drugs or vaccines for the treatment of helminth infections (143). However in the past two decades NGOs and charities have tasked themselves to deal with this problem. Of note are the WHO's Neglected tropical diseases initiative, the Bill and Miranda Gates foundation's Global Health Program and that Mass Drug administration

program (139). The primary aim of these organisations/initiatives is to promote the eradication of tropical diseases including helminth infections.

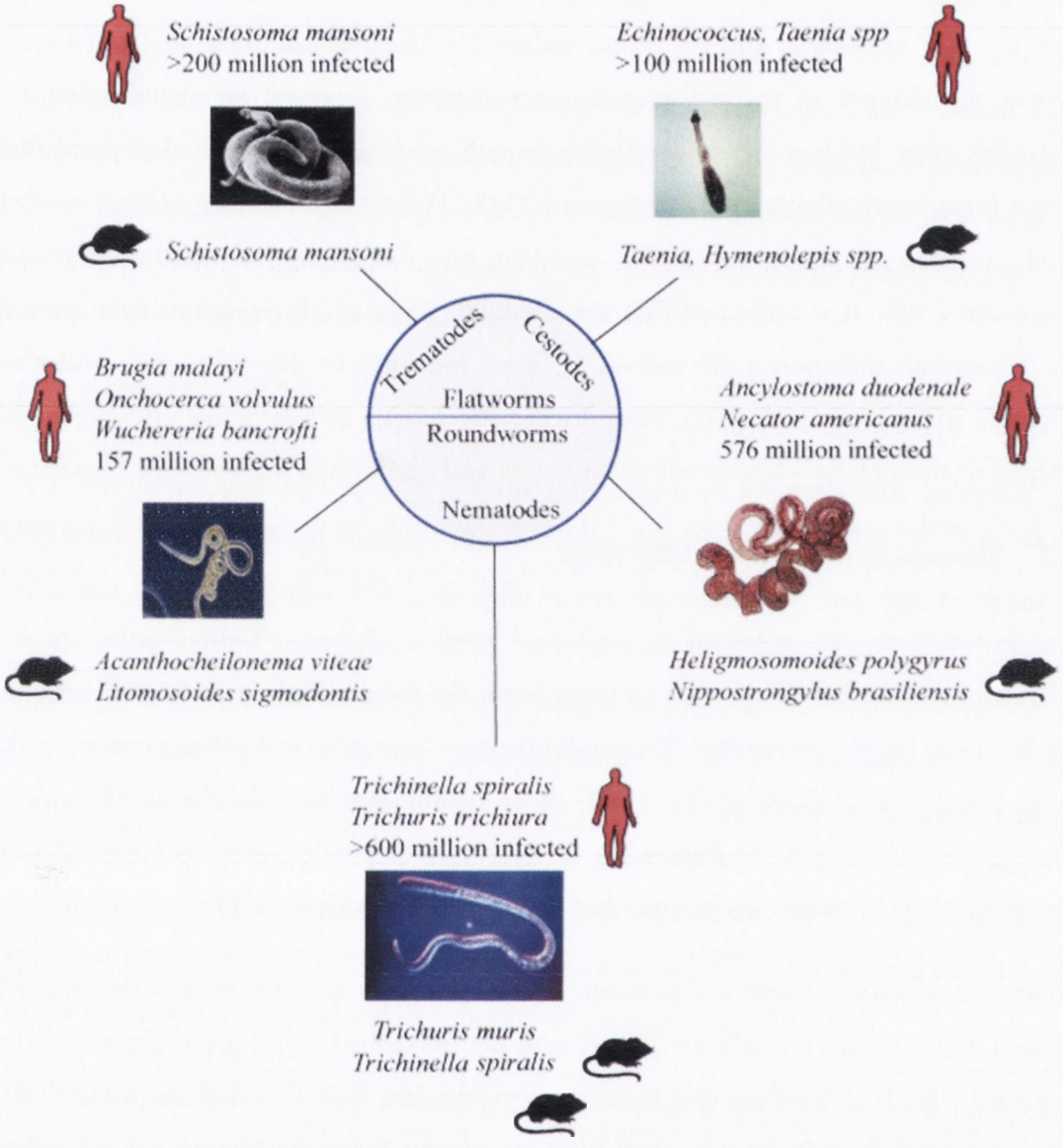


Figure 1.2 Classification of helminths infecting mouse and man. A summary of the major helminth infections of humans and their mouse model counterparts. Estimates of human infection numbers are taken from Hotez et al. (2008) (143). Figure reproduced from Maizels et al (2010) (147).

The elimination of disease may be best achieved through public health initiatives which target the intermediate host or vector (143). Reducing transmission by use of clean water supplies, provision of housing and education regarding the risks of swimming and bathing in open water is of paramount importance, given the prevalence in poor regions of soil transmitted helminths that are transmitted via contaminated water (138).

Systematic use of anti-helminthic drugs on the entire population of endemic areas regardless of whether an individual is infected or not, is another method being used: this has the added benefit of not only treating the affected individuals but also preventing transmission by eradicating helminths from treated regions. Some of these initiatives have made astounding progress. The WHO's global program to eliminate lymphatic filariasis (elephantiasis) for example is on track to eliminate the disease by 2020, through the systematic blanket administration of diethylcarbamazine or ivermectin plus albendazole to large geographic regions, where the causative filarial nematodes are endemic and thus far 900 million people have been treated (148). Eradication of helminthic diseases will require significant investment, with a multi-faceted approach that not only targets populations using drugs but aims to reduce vectors together with societal development that deals with the underlying cause, poverty.

One worrying aspect is the lack of investment in primary research. Advancement in genomic and molecular biology has revolutionised the fight against diseases such as malaria and tuberculosis and while the genomic sequence of some helminths have been completed they have yet to be annotated. Basic research remains especially important given the selection pressure on helminths to evolve resistance to the major anti-helminthic drugs. Helminth vaccines are another approach being developed. While there is, as of yet, no helminth vaccines approved for use in humans, there are several vaccines being used for veterinary cestode infections (149-151).

No doubt the development of such interventions will be aided by research into the host-parasite interactions. Why do some people clear infection while others do not? How can a macroscopic creature evade the host's immune system for years or even decades? The next section will deal with the question: how does the immune system respond to infection with helminth and in turn how do helminths evade immunity?

1.5.1.4 Host-parasite interactions: The Protective Immune response to helminths

> "A Man, whose body abounds with a certain sort of Humour, will produce Worms ... and he who has no Humour proper for the Eggs of Worms, will produce none, and so be free of them."
>
> Nicolas Andry, 1700 foreshadowing the concept of immunity to helminths.

Adult helminths are rarely killed by the immune system. Thus, it is suggested that the immune response limits infection by reducing the viability and reproductive capacity of helminths or to physically expel them from the mucosal membranes in which they characteristically reside (e.g., the intestine, lungs and skin). Protective immunity to helminths is mediated by Th2 responses (152). The importance of the Th2 response in helminth immunity is supported by population genetics which have demonstrated an association between loci that control Th2 responses and their effector cytokines with susceptibility to infection (153). However, it may be more useful to describe the response to helminths as a "type-2 response" rather than a "Th2 response," as this term more accurately describes the multiple cell types (innate and adaptive) mobilised during anti-helminth response, rather than suggesting immunity is mediated by Th2 cells alone. For instance, it was shown that the archetypal Th2 cytokine IL-4, is not required for the expulsion of the gastrointestinal nematode *N. brasiliensis,* however, subsequent studies revealed that IL-13, IL4Rα and STAT6 were all required; suggesting that separate facets of type-2 immunity work in tandem to mediate protective immunity against helminths (154). IL-4, once considered to be the hallmark type-2 effector cytokine is now viewed to be more important for the polarisation of T cells and in class switching (IgE class) of B cells rather than in mediating protective immunity. Indeed, IL-13 and IL-5 appear to be more important for the actual expulsion of helminths. Liang et al showed that IL-13-producing cells in mice infected with *N. brasiliensis* home to the infected tissue, while IL-4 producing cells remain in the LN to provide 'help' to B cells (155).

IL-13 is involved in mediating changes to the physiology of target organs and is particularly associated with the induction of goblet cell hyperplasia, mucus production, epithelial shedding and smooth muscle contraction that can act to physically expel helminths from these membranes in what is referred to as the 'weep and sweep' response. During *T. Muris* infection, IL-13 increases epithelial cell turnover in the gut, which removes parasitiased cells

(156). Similar flushing effects are controlled by the action of IL-13 and IL-25 in response to infection of the gut by *H. polygyrus* and *N. brasiliensis.*

Upon allergen encounter, helminth specific IgE bound to mast cells via FcεR1 induces mast cell degranulation and release of factors including TNF-α and LTE which act on endothelial and epithelial cells to induce vasoconstriction, permeability and the expression of VCAM-1/Eotaxin that promotes the recruitment of eosinophils (9).

IL-5 recruits and activates eosinophils (157). A classic, but simplified understanding is that eosinophils kill helminths. *In vivo,* helminths are too large to be phagocytised and are thought to be killed by eosinophils through the release of preformed or metabolic mediators, such as major basic protein (MBP), or through antibody-dependent cell-mediated cytotoxicity. As a purified protein, MBP has potent *in vitro* toxicity against a number of parasites, including *Schistosomes* spp. (9). However, few studies could replicate this finding *in vivo.* It is hotly debated whether eosinophils kill worms at all. Worms are commonly found *in vivo,* coated with eosinophils, but the chronic nature of helminth infections argues against direct killing mechanisms (152). However, the action of eosinophils, coating the parasite and releasing toxic substances, may have evolved to reduce the viability of the parasite, restricting its growth and fecundity.

Recently, the importance of innate type-2 cytokines has been appreciated; the 'alarmin' cytokines IL-25, IL-33 and TSLP are produced by epithelial cells in response to helminth infections and induce the production of IL-5 and IL-13 by nuocytes (50). In fact, Yatsuda et al recently demonstrated that the lung/intestinal nematode *Strongyloides venezuelenis* was expelled in the absence of T cells and B cells by eosinophils that were recruited by IL-5 produced by nuocytes (158), suggesting that, in this model at least, innate effector cells are sufficient to control infection. However, such innate systems would require constant stimulation and thus cannot fully replace a sustained antigen-specific Th2 response, nor could this allow antigen-specific immunity to develop. Nevertheless, innate lymphoid cells may function to sound the alarm and potentiate a type-2 response in the early stages of primary infection; where there is not sufficient time to allow for the expansion of helminth-specific Th2 cells.

Helminth infections provide the canonical setting for the generation of M2 macrophages. Macrophages form a major constituent of type-2 inflammatory infiltrates and are alternatively

activated via the abundance of IL-4 and IL-13 in the milieu. M2 macrophages perform a variety of discrete effects during helminth infection, including suppressing inflammation, wound repair, fibrosis, and further polarisation of Th2 cells (156, 159).

M2 macrophages are a major constituent alongside eosinophils in type-2 granulomas, which involve the encasement of helminths or helminth eggs. Similar to eosinophils, M2 macrophages secrete anti-helminthic compounds including chitinase, which can degrade the glycan coats of helminths, such as *B. malayi in vitro* (156). M2 macrophages implement other methods of reducing the overall viability of the parasites; RELM-β produced in response to IL-13 interferes with the chemosensory apparatus and inhibits the chemotaxic function of nematodes. M2 macrophages are characteristically defined by the high expression of arg1, which depletes the tissue micro environment of L-arginine, thus starving the helminth of this essential amino acid. Helminths cause considerable damage while migrating through tissues and a tissue repair function has been attributed to M2 macrophages (142, 147). M2 macrophages are thought to induce fibrosis through the expression of profibrotic factors, including fibronectin, matrix metalloproteinases (MMPs), IL-1β and TGF-β (142). Secreted Fizz1 also has proliferative and angiogenic properties and plays a role in wound repair (160). M2 macrophages are essential to repair tissue damage in the lung following infection with *N. brasiliensis* (161). Fibrosis during helminth infection is supported by IL-3 and IL-9 which induce the expression of pro-fibrotic mediators by mast cells which in turn activates fibroblasts (156). Fibrosis is a complicating factor in helminth infections and can result in irreversible organ damage.

Recent studies have identified some of the helminth-derived products that induce Th2 cells. Omega-1, derived from the *S. mansoni* egg antigens (SEA) primes DC to induce Th2 responses *in vivo,* even in the absence of IL-4 (55). Omega-1 also suppresses the development of Th1 responses and has been shown to inhibit DC activation in response to LPS, which further amplifies type-2 responses. *N. brasiliensis* excretory-secretory products also drive Th2-biased responses and have be used as an Th2-inducing adjuvant for unrelated antigens, even in the presence of CFA, a powerful Th1 adjuvant (142, 162). Moreover, ES-62, a molecule derived from the secreted products the filarial helminth *Acanthocheilonema viteae* primes DC to induce Th2 cells (142).

Failure to mount a type-2 response to helminths may result in an adverse outcome. For instance, susceptibility to *T. muris* is linked to the development of a Th1 response rather than

a protective Th2 response (163). Paradoxically however, the induction of 'protective' type-2 responses may be advantageous to both the host and the helminth. The induction of Th1/Th17 responses, which are significantly more inflammatory and destructive than Th2 responses, reduce the viability of helminths directly (through inflammatory killing) but also indirectly by destroying the mucosal membranes they inhabit.

One striking example of an inappropriate, pathological Th1/Th17 response during infection is infection with *S. mansoni,* which is associated with liver damage, splenomegaly, portal hypertension and even death. Immunopathology during *S. mansoni* infection is due to an inflammatory response to *Schistosoma* eggs deposited in the liver. However, the severity of immunopathology differs greatly between individuals and between different strains of mice. Initially, SEA induces a Th1 response in the host, which in most cases converts to a non-damaging Th2 response. Pathologically, this Th2 response presents as small fibrotic type-2 granulomas in the liver which surround the eggs. Failure to develop an effective Th2 response in some individuals and certain strains of mice, results in exacerbated type-1 granulomatous inflammation driven by Th1 and Th17 cells (164). In one study Th1-predisposed CBA mice lacking IL-23p19 and thus Th17 responses, were protected from this immunopathology. In these mice, the expression of IL-5 and IL-10 was increased, whilst IL-17 was suppressed. In addition, the granulomas formed were smaller in size and no longer expressed a Th1 phenotype (composed of neutrophils and M1 macrophages), but instead displayed a type-2 phenotype (composed of eosinophils and M2 macrophages), similar to those induced during a non-damaging Th2 response. Another study showed that M2 macrophages were essential for the development of an appropriate Th2 response to SEA; removal of M2 macrophages (through use of macrophage specific IL-4Ra knockout mouse), resulted in death caused by severe Th1 responses (165). Likewise, infection with *N. brasiliensis* initially induces a Th17 response that damages the lung, which is suppressed by IL-4Rα signalling that subsequently prevents further immunopathology and helps maintain tissue homeostasis (161).

However, what the examples above do not convey is that for both parasites, Th1/Th17 responses may induce eradication of the worm, at the cost of immune pathology (166). Thus, a revision of terminology may be necessary as type-2 responses may not be as 'protective' as it is 'appropriate.' Conversely, inflammatory Th1/Th17 responses may be more 'protective' in the literal sense of eliminating the pathogen but they are certainly more 'inappropriate' due to the damage they cause. Interestingly, the evolutionary origin of the type-2 response is believed

to be an expansion of ancient wound repair mechanisms and there is a view that rather than trying to kill the pathogen, Th2 responses are more concerned with the damage they cause (167). Thus preventing the induction of inflammatory Th1/Th17 responses and favouring tolerance of infection may have originated from an evolutionary cost/benefit analysis – perhaps it's better to live with the worm than to risk utter ruin in an attempt to rid one's self of it.

1.5.1.5 Host-parasite interactions: immune evasion and regulation

Host immunity has developed - perhaps through co-evolution with helminths- to limit pathology and to best balance susceptibility, resistance and immune pathogenesis. The majority of helminth parasites survive for long periods in the host in most cases without compounding immune pathology, this situation reflects a combination of active immunoregulatory mechanisms and immune evasion strategies implemented by the parasite (156, 168).

Successful infection requires that the helminth suppress protective Th2 responses. There is a large body of evidence in the literature to support the hypothesis that helminths employ a variety of mechanisms to suppress the immune response. Infection with helminths causes considerable damage in the host and provides a high antigenic stimulus for the immune system. However, T cells from humans and animals infected with helminths typically display weak antigen-specific responses. For instance, infection with *Onchocerca* spp leads to a weak Th1/Th2 response with concurrent robust production of IL-10 and TGF-β (169). Moreover, infection with *N. brasiliensis* enhances the expression of CTLA-4 on Th2 cells, which inhibit proliferative responses and maintain weak anti-helminth responses (170).

Treg cells play a crucial role in immune regulation that protects the host from immunopathology. Several helminth spp can exploit the function of Treg cells to evade the host immune response. Indeed, *H. polygyrus* has been shown to enhance the frequency of Treg cells in the host during infection to establish long-term persistence (142, 156). Likewise, Taylor et al have shown that infection with the filarial helminth *Litomosoides sigmodontis* induces hyporesponsiveness in mice which can be reversed by depletion of Treg cells *in vivo* (171). Taylor et al later demonstrated that induction of Treg cells is required for the establishment of chronic infection with *L. sigmodontis* (172). Depletion of Treg cells leads to the induction of a protective immune response, which results in expulsion of the parasite (172). Further

investigations revealed that neutralising Treg-derived IL-10 and TGF-β *in vivo* also restored anti-parasite responses, suggesting that Treg-derived cytokines are crucial mediators of immunoregulation by parasites (142).

The role of TGF-β in promoting the expansion of iTreg cells is well established. Thus, induction of TGF-β has been implicated in the helminth-mediated expansion of Treg cells (173-175). *H. polygyrus* enhances the expression of host TGF-β during infection (174, 175), while *Leishmania* spp activate latent-TGF-β using parasite-derived cathepsins, a process which is essential for the survival of the parasite (176). Likewise, *Plasmodia* spp can activate latent TGF-β by activation of endogenous MMPs (177). *H polygyrus* secretes a TGF-β homologue that acts to enhance the expression of FOXP3 in naïve T cells (178). In certain cases, *Onchocerca* spp and *B. malayi* also express distinct molecules homologous to mammalian TGF-β (173). *In vitro* studies have shown that SEA induces the expression of FOXP3 in naive T cells via TGF-β, which confers tolerogenic and Treg cell expanding properties on DC (179-181). These mechanisms are also applicable to human infections, and recent publications demonstrated that patients infected with filarial nematodes or *Schistosomes* had higher frequencies of Treg cells in the peripheral blood (152, 182). It is likely that the host tolerates these interventions as a method of reducing host-pathology; depletion of Treg cells from mice during infection with *T. muris* enhanced the expulsion of worms but at the cost of significant intestinal pathology (183).

Helminth products such as SEA and E62 are well described compounds which modulate DC to selectively induce Th2/Treg cells (184, 185). Some of these products, including E62, inhibit the ability of DC to respond to Th1 associated ligands, such as LPS (186). Helminths can also target macrophages. In a mouse model of *S. mansoni* infection, M2 macrophages are essential for parasite survival in the host (165). M2 macrophages are also required for the survival of *Nippostrongylus* spp, *Toxocara* spp and *Taenia* spp during infection (187). M2 macrophages may utilise the anti-inflammatory properties of arg-1, as depletion in L-arginine from the tissue micro environment not only affects the helminth, but also inhibits T cell function, particularly effector T cells, which have a high metabolic turnover.

Rodriguez et al demonstrated that T cells stimulated in the absence of L-arginine lose their expression of the CD3ζ-chain which is required for TCR-signalling and results in the suppression of T cell proliferation and cytokine production (188, 189). Similarly, SEA can suppress pathological inflammation by promoting the expression of arg1 in M2 macrophages

(190). M2 macrophages from mice infected with *L. sigmodontis* were shown to suppress T cell responses via IL-4 and IL-13 (142). In addition to their characteristic expression of arg1, M2 macrophages express IL-4R, TGF-β and chitinases including FIZZ1 (191, 192). Macrophage-derived IL-10 and TGF-β can inhibit LPS induced pro-inflammatory cytokine release, induce Treg cells and inhibit Th1 responses (193). FIZZ1, is a secreted protein that helps to control inflammation induced by SEA and mice lacking FIZZ1 develop SEA-induced inflammation of the liver. This was associated with enhanced Th2 responses and could be reversed upon treatment with recombinant FIZZ1 (194).

1.6 The hygiene hypothesis

The incidence of autoimmune and allergic diseases in the developed world has increased dramatically since the beginning of the 20th century (195, 196). This has coincided with a dramatic change in lifestyle and healthcare, including the eradication of many infectious diseases, a trend of migration from rural to urban areas and a decrease in the number of children per family (196). Autoimmune and allergic diseases are common in the developed world. In contrast, autoimmune and allergic diseases are less common in rural areas of the developing world, areas where multiple infectious diseases are endemic [Fig 1.3] (166).

The hygiene hypothesis has been put forward to explain this phenomenon and it attributes the rise in autoimmune and atopic diseases to the simultaneous decrease in infectious disease, in particular, helminth infections [Fig 1.4]. The main tenet of this hypothesis is that helminth infection induces a variety of immunoregulatory mechanisms that aid parasite survival by suppression and evasion of the immune system; with the systemic side-effect of suppressing inflammation associated with allergic and autoimmune disorders.

Hay fever, the most common allergic disease is a relatively modern disease, the first description of which dates to the early 19th century; however by the end of that century the disease was commonplace (197). In 1989, David Strachan conducted an epidemiological study which correlated the incidence of hay fever in children with low family size and with an older position within the family. He coined the term "hygiene", postulating that "unhygienic" contact with older siblings lead to unspecified infections in early life that conferred protection from atopy (198).

A seminal study by Correale and Farez published in 2007 examined the effect of helminth infections on the severity of MS in patients in South America. Over a five year period, MS patients who were infected with parasites had significantly lower rates of relapses, reduced disability scores and a reduction in the number of brain lesions compared with uninfected MS patients. T cells from infected MS patients produced less IL-12 and IFN-γ, but more IL-10 and TGF-β in response to myelin basic protein, compared with those from uninfected MS patients. The frequency of Treg cells also increased in patients infected with helminths (199). A recent study reported that patients infected with Schistosomes had lower levels of autoantibody compared with patients free from infection. Moreover, the frequency of autoantibodies increased in infected patients in response to treatment with anti-helminthic drugs (200).

As for allergy, a number of studies have shown that helminth-infected cohorts display lower allergic propensity. In a Brazilian study, a lower frequency of children infected with Schistosomes tested positive for skin-prick tests for house dust mite allergy compared with uninfected children (201). Furthermore, a study in Gabon found that eradication of worms resulted in increased skin reactions to allergens among children (202). A recent meta-analysis of all published data statistically correlated *Trichuris* and *Ascaris* infection with a reduction in atopy (203).

Helminth infections have also been shown to suppress beneficial immune responses including those induced to bacterial infection and vaccination. For instance, in Gabon, children with helminth infections vaccinated against influenza demonstrated poor influenza-specific T cell responses compared with uninfected children (147, 204). Furthermore, *N. brasiliensis* infection induces M2 macrophages that result in compromised resistance to mycobacterium (205).

1.6.1.1 Helminth infections and Allergy

There are some excellent experimental models which describe the effect of helminth infections on allergic diseases. A study by *Wilson* et al reported that helminth infection resulted in the suppression of a mouse model of allergic airway inflammation (206). Infection with the intestinal helminth *H. Polygyrus* reduced the symptoms of OVA- and Derp1-induced experimental allergic asthma (AHR) in mice, including respiratory cell infiltrates, eosinophilia and goblet cell hyperplasia (206). Increased nonspecific IgE was proposed as a mechanism to explain how a Th2-inducing helminth infection could in fact suppress Th2-mediated atopy.

High nonspecific IgE may out-compete allergen-specific IgE bound to mast cells and basophils. In addition, infection was shown to enhance the expression of $CD4^+CD25^+$ $FOXP3^+IL\text{-}10^+TGF\text{-}\beta^+$ T cells that suppressed inflammation upon transfer to uninfected allergen exposed recipients (206). The protective effect was maintained in recipient mice following transfer of T cells from IL-10 deficient mice. The secreted products of *H. polygyrus* were subsequently shown to induce Treg cells *in vitro* that upon transfer could induce protection from AHR (178).

Infection of mice with *L. sigmodontis* has similar effects in protecting against AHR and is associated with a reduction in Th2 cytokines with a corresponding increase in TGF-β and Treg cells (207). In a peanut allergy model, infection with *H. polygyrus* protected mice from IgE-mediated anaphylaxis (208). Specific helminth products of *N. brasiliensis*, *Ascaris* and *S. mansoni* have all been shown to attenuate the symptoms of allergy in experimental models (166).

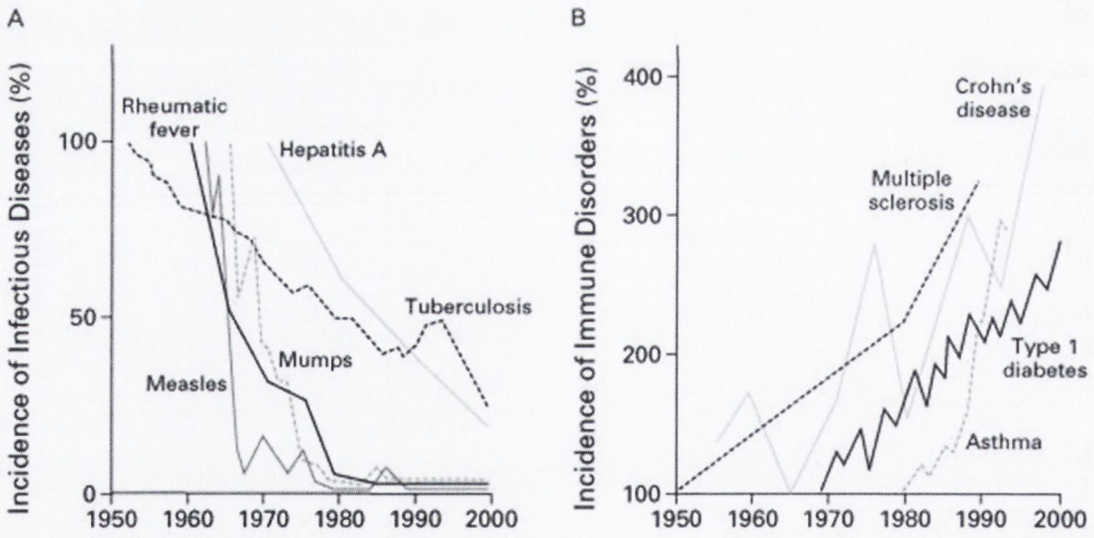


Figure 1.3 Inverse Relation between the Incidence of prototypical Infectious Diseases (Panel A) and the Incidence of Immune Disorders (Panel B) from 1950 to 2000)Figure reproduced from Bach, 2002 (209).

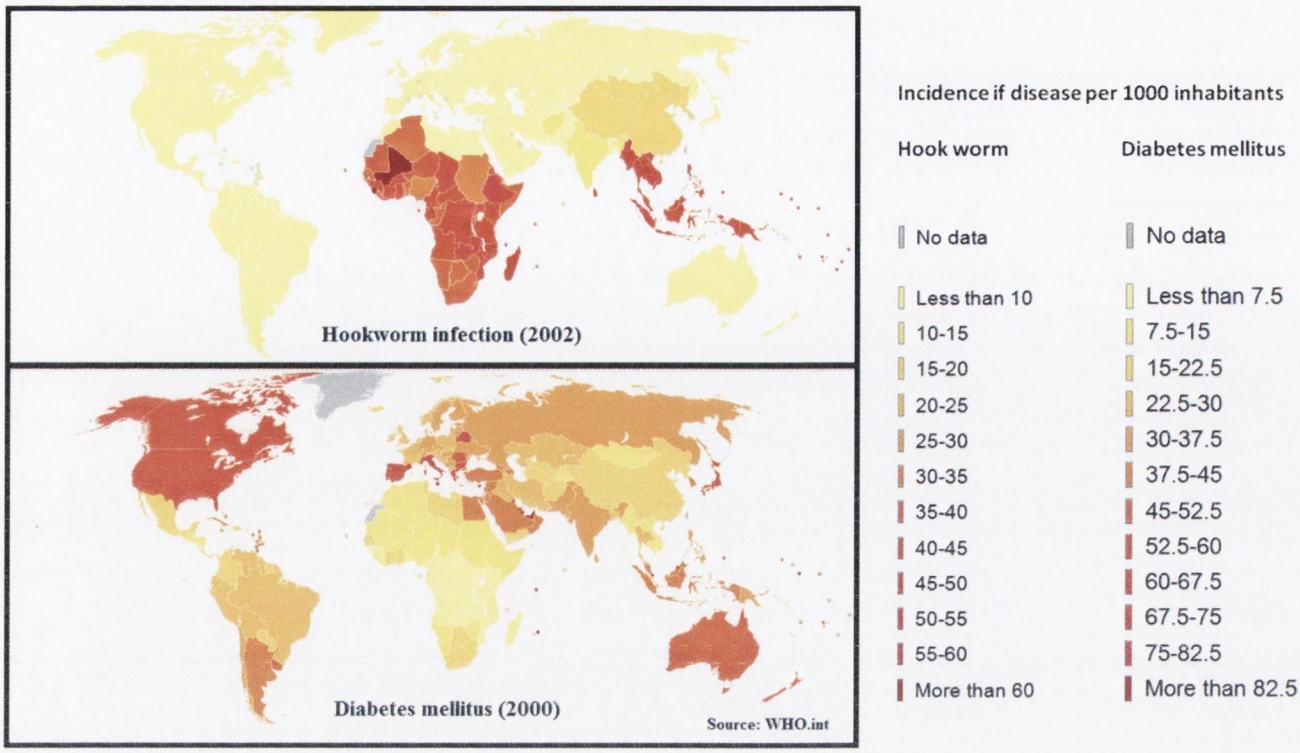


Figure 1.4 Incidence of Hookworm infection and diabetes mellitus worldwide.
Created using data from WHO World health report 2009 (137).

1.6.1.2 Helminth infections and autoimmunity

There is a growing body of experimental evidence to suggest that helminth infections are associated with protection against a number of autoimmune disorders. The first report, came from Pearson and Taylor, in 1975 where they showed that infection with the nematode *Symphacia obvelata* attenuated the severity of collagen-induced arthritis (CIA) in rats (210). Since then, protection from arthritis induced with the helminth *S. mansoni,* was found to be associated with a reduction in pathogenic TNF-α and IL-17 and an enhancement of IL-4 and IL-10 (211).

Several helminths have also been shown to protect mice from diabetes. Saunders et al used the gastrointestinal nematodes *T. spiralis* and *H. polygyrus* to prevent the onset of Th1 mediated type-1 diabetes in NOD mice (212). The authors concluded that the induction of strong Th2 responses suppressed the development of autoimmune Th1 responses (212). However, a

recent study by Hübner et al has shown that protection from diabetes in NOD mice due to infection with *L. sigmodontis* was not mediated by a switch to a type-2 response but via the expansion of TGF-β-induced Treg cells (213).

Likewise, murine infection with *H. polygyrus* was shown to reduce IL-17 production, and suppress the development of experimental colitis in both wild-type and IL-10$^{-/-}$ mice (214). Several studies have shown attenuation of EAE by helminths including *T. spiralis* and *S. mansoni* (215). Infection with *S. mansoni* reduced the severity and delayed the onset of EAE which was associated with a reduction in MOG-specific IFN-γ (216).

In an attempt to bring these concepts into the clinic, the helminths *T. muris* and *N. americanus* which cause largely asymptomatic infections, have been tested in patients with Crohn's disease with promising results (217, 218). *T. muris* infection resulted in an improvement in clinical symptoms of Crohn's in 79% of patients with no reported adverse effects (218).

Gaining regulatory health care approval for the use of helminths to treat human diseases has both ethical and medical concerns. As such, the immunosuppressive products secreted by helminths have generated much interest as potential therapeutics (219). Several helminth-derived molecules have been shown to be beneficial in the treatment of experimental autoimmune animal models. The *S. mansoni* molecule, LNFPIII was shown to prevent the development of psoriatic-like lesions in flaky skin mice as well as to promote transplant tolerance in mice (220, 221). ES-62, derived from *A. viteae* was able to attenuate the symptoms of CIA which was associated with a suppression of anti-collagen Th1 responses and a reduction in synovial cell derived TNF-α (222). Administration of SEA has been shown to reduce the severity of EAE as well as to prevent the development of both trinitrobenzene sulfonicacid-induced colitis and diabetes in the NOD mouse model (223-225). Studies *in vitro* have shown that SEA triggered TGF-β dependent expression of FOXP3 in naive NOD T cells (179).

1.7 *Fasciola hepatica*

The trematode, *Fasciola hepatica* or liver fluke is the causative agent of fasciolosis or liver rot. It has important economic consequences as a major pathogen of domesticated ruminants and is associated with significant reduction in agricultural output by infected animals. However, *F. hepatica* can infect a range of mammals including humans, with an estimated 18 million people

infected worldwide (147). The clinical symptoms of liver fluke infection both in humans and in the livestock include jaundice, anaemia, weight loss, submandibular oedema and diarrhoea. In addition to the definitive mammalian host, freshwater snails belonging to the family of *Lymnaeidae* are needed as intermediate hosts (226).

The adult fluke resides in the biliary tract (bile ducts) of the liver, where it requires only a simple digestive system to feed on bile, blood, lymph and cell debris of the host. In contrast, the reproductive system is highly developed. The fluke is a hermaphrodite but cross fertilization is common and allows for the generation of greater genetic diversity. Adult flukes have a huge reproductive capacity and produce up to 25,000 eggs per day (136, 227). In addition, the fluke reproduces asexually by polyembroyony in the intermediate host, which can greatly increase the number of parasites. The life cycle is described in detail below [Fig 1.6].

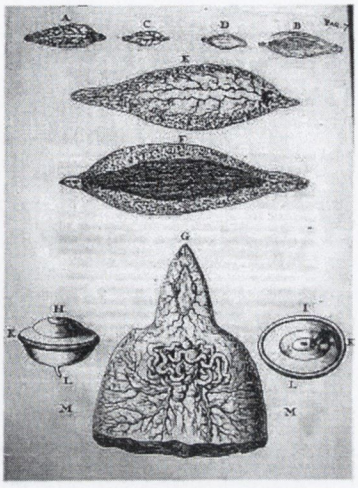


Figure 1.5 Bidloo's 17th century illustration of *F. hepatica*.

Phylogenetic studies place the origin of the genus *Fasciola* in Africa 50 million years ago (228). Since this time *F. hepatica* has diversified into nine spp with different geographical ranges and host preferences. *Fasciola* spp are distantly related to the schistosomes and most likely diverged from these as a result of a switch in the intermediate host. Of the nine known *spp* of Fasciola three can infect humans *F. hepatica, Fasciola gigantica,* and *Fasciola buski,* while all others are associated only with large herbivorous mammals (228). *F. hepatica* is unique in the genus in that it has a global distribution due to its ability to adapt to a variety of aquatic snails as intermediate hosts but also due to the increase in the transport of infected ruminants over the last 200 years (228).

1.7.1 Life cycle

F. hepatica was the first fluke of human importance to be discovered. It is likely that *F. hepatica* has affected man directly or indirectly since the domestication of ruminants. Indeed, fossilised *F. hepatica* eggs were found on the remains of a Neolithic farmer and his cattle in Germany (229). The first written account comes from the Frenchman de Brie, who in 1379 described

liver rot in sheep as part of a treatise on herd management, he quite accurately suggested that sheep should be removed from wet land during the summer months, a practice still in use today (230). In England in 1523, Fitzherbert coined the term flokes (flukes) and gave an accurate clinical description of fasciolosis in infected sheep (231). In 1670, Faber observed that worms were located in the biliary tree of the liver of infected animals (136). Twenty years later the Dutchman Govard Bidloo discovered that the adult flukes were hermaphroditic and produced an abundance of eggs (232). Bidloo correctly postulated on how the worms caused liver rot, suggesting inflammation, blockage of the biliary network and that flukes compete with the host for nutrients. However, Bidloo incorrectly assumed that the eggs produced by *F. hepatica* could cause infection upon ingestion. However, Antony van Leeuwenhoek in correspondence with Bidloo was convinced that animals were infected rather by feeding on contaminated pastures, but alas failed to identify the infectious agent (136).

Working out the correct lifecycle of *F. hepatica* would prove difficult given the apparatus available at the time and the complex heterogeneous life cycle of the fluke, a concept difficult to conceive in the 17th century. While Bidloo and Leeuwenhoek made many correct observations and postulations, it took nearly 200 years before the life cycle of the fluke would be resolved (136).

In 1758, Linnaeus named the worm *Fasciola hepatica* and classified it into the class *Vermis*. At the time it was one of only six parasites described in his *Systema Naturae,* the origin of modern taxonomy (233) He also gave the first detailed accounts of human infection (232, 234).

The Prussian Bojan-Us in 1880, upon dissecting infected snails, discovered that cercariae crept out of sacs (radiae) in the viscera of the snails and concluded that they were probably generated within them. It caused much astonishment that cercariae were not derived from parents resembling themselves but instead came from the morphologically distinct radiae (136). It was unknown at the time that these life forms represented intermediate stages in the development of *F. hepatica* and were indeed classified as unique species. Similarly, surprise greeted the publication by Braun who demonstrated that the eggs of *F. hepatica* hatched into ciliated larvae, the miracidium, thus demonstrating that animals could have distinct stages in its life cycle (136). Since it was also noted at this time that miracidium were motile, it was suggested that they may actively seek out their host.

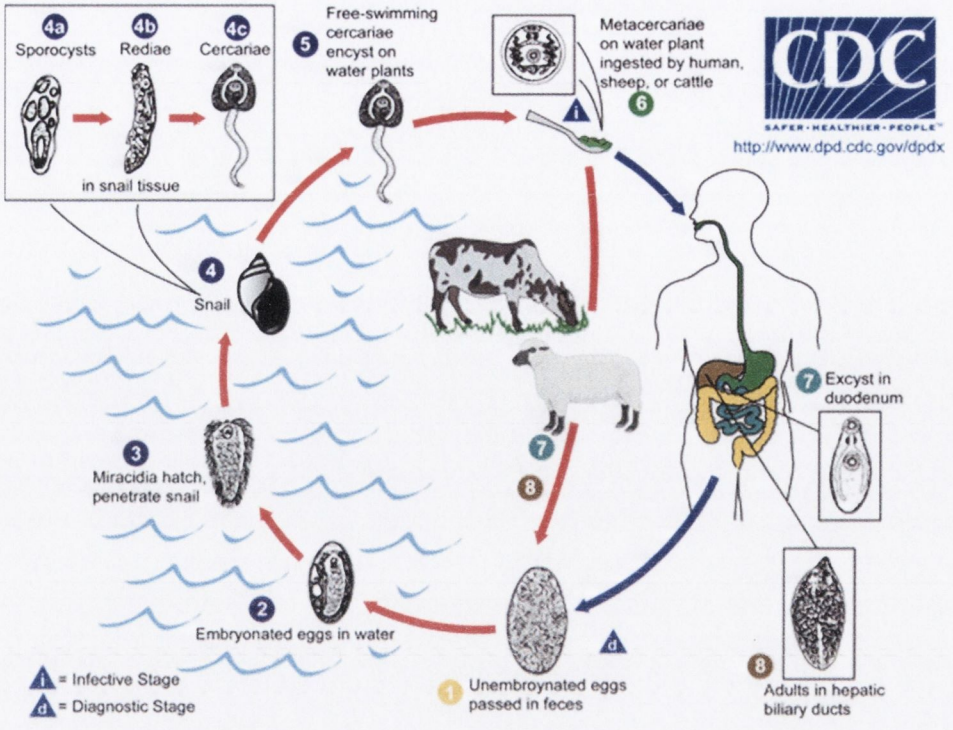


Figure 1.6 The life cycle of *F. hepatica* In addition to the definitive mammalian host, freshwater snails belonging to the family of *Lymnaeidae* are needed as intermediate hosts for *Fasciola hepatica* to complete its life cycle. **1,** Immature eggs are discharged in the biliary ducts and pass from the mammalian host in the faeces. **2,** The eggs become embryonated in water within 2 weeks after exposure to water, sunlight and in temperatures of 20-25 °C. **3,** The embryo differentiates into a miracidium larva **4,** The free swimming miracidium seeks out a suitable snail host whom they invade via the pulmonary aperture. Within the snail, the parasite develops into the second larva stage, the sporocyst or brood sac (**4a**) Over the course of one month the larva multiply mitotically (within the sporocyst) by polyembryony producing several daughter radiae (**4b**). Radiae feed on nutrients provided by the host causing much morbidity to the host. Each radiae multiplies to form several daughter cercariae, which develop within each radiae (**4c**). This asexual expansion can produce 1000 cercariae for every miracidium. **5,** The cercariae leave the radiae and burrow out of the snail. The free swimming cercariae encyst as metacercariae on aquatic vegetation or other surfaces. **6,** Mammals acquire the infection by eating vegetation containing metacercariae. **7,** After ingestion, the metacercariae excyst in the duodenum and migrate through the intestinal wall into the peritoneal cavity. The immature flukes burrow into the surface of the liver, travel to the liver parenchyma and eventually into the biliary ducts, where they develop into adults. **8,** In humans, maturation from metacercariae into adult flukes takes approximately 3 to 4 months. The adult flukes which can measure up to 30 mm by 13 mm, reside, feed and reproduce in the large biliary ducts (235).

The greatest leap forward in the understanding of the life cycle of flukes came from Leuckart and Thomas who in 1882 independently described the development of miracidium through to cercariae in the snail, *Lymnaea truncatula* (236). In a series of epidemiological studies and laboratory experiments, both authors discovered that miracidium hatched from *F. hepatica* eggs, invaded *Lymnaea* snails and there metamorphosed into sporocysts in which, radiae subsequently developed. They showed that that radiae then developed into cercariae. However, the matter of transmission was not settled until 1893 when Lutz showed that *F. hepatica* cercariae upon leaving damaged or dead snails, encysted on plant or other material. He then demonstrated that when these cysts were fed to guinea pigs, adult flukes could be isolated after a month after infection. Thus, the lifecycle was completed (237-239).

However, the route of migration and the processes of maturation of worms in the definitive host were still unclear. It was assumed that the maturing fluke reached the biliary tree via the gall duct in the duodenum. However, Thomas had previously described adult flukes in the peritoneal cavity. In 1914 the Russian, Dimitry Ssinitzin demonstrated the pathway of migration of flukes in the definitive host (240). He showed that after infecting rabbits the metacercariae excysted in the intestine, penetrated the intestinal wall and passed into the peritoneal cavity, where they remained for 2 weeks (240). They then attached themselves to the surface of the liver using the mouth suction cap, penetrated into the liver parenchyma with the aid of hard pointed spines, passed further into the biliary passages, where they matured and produced eggs within two months of infection (240).

Fasciolosis results in economic losses in livestock from mortalities, reduced fertility, abortions, slow growth, reduction of milk and meat production, infected livers and withered carcasses. Knowledge of the life cycle of *F. hepatica* has allowed farmers to implement measures to treat and eradicate infection. Of paramount importance is preventing animals from grazing on wet pastures, as is the destruction of infected animals to prevent transmission. These methods alone may not prove sufficient in preventing diseases as each fluke can produce at least 25,000 eggs a day and a single miracidium has been known to generate over 1,000 cercariae. Although the vast majority of these do not reach their intended hosts, it highlights the potential magnitude of transmission (241). As such, control of transmission requires the use of anti-helminthic drugs. Triclabendazole is the front line treatment for infection in ruminants. It is the only anti-helminthic drug effective against both

the immature and mature fluke. Triclabendazole interferes with microtubule formation in the flukes and reduces the worm's capacity to feed and reproduce effectively by inhibiting the formation of the tegument.

Drug resistance is a growing problem, particularly in Ireland where *F. hepatica* isolates have been found to be triclabendazole resistant. This is perhaps unsurprising given the widespread use of the drug and the high reproductive capacity of the fluke, particularly the capacity of *F. hepatica* to reproduce asexually in the snail and thereby greatly expand any resistant mutant (241, 242). The use of alternative anti-helminthics, including nitroxylin and albendazole is compounded by the lack of efficacy against the destructive immature fluke (243). However, recent reports do suggest that anti-helminthics directed against intestinal nematodes, such as luxabendazole, in combination with triclabendazole are effective against triclabendazole-resistant infection of sheep (244). Praziquantel, a drug commonly used for the treatment of schistosomiasis is licenced for use in humans, although triclabendazole is also effective, but unlicensed (136). Praziquantel increases the concentrations of calcium in the flukes thereby inducing paralysis that leads to their detachment and phagocytosis by leukocytes (143, 245).

Future approaches include the continued development of new anti-helminthic drugs and the development of drugs which target the cathepsins produced *F. hepatica.* Novel approaches include the development of vaccines using parasitic antigens (243, 246). Several vaccine formulations have been tested with moderate success and include the use of fatty acid-binding proteins, glutathione S-transferases, cathepsin L proteases, leucine aminopeptidase, fluke haemoglobin, thioredoxin peroxidase and paramyosin as targets (247). Some formulations have achieved reductions in worm burden of up to 70% along with the retardation of growth of surviving flukes and reductions in egg output and viability. While not as efficacious as pharmaceutical interventions, they may provide a cost effective method of reducing transmission by impeding the production of eggs by remaining flukes (243, 247).

1.7.2 Immunomodulation by *F. hepatica* infection

The life cycle of *F. hepatica* involves long periods within the definitive host. Thus, the parasite is a target of the mammalian immune system. The parasite causes significant damage as it translocates across to the peritoneal cavity and in its immature stage in the liver parenchyma. Thus, the parasite has evolved an array of immune evasion strategies in order to survive in its

host. Studies from experimentally infected cattle have shown that the host mounts a Th2-dominated response against *F. hepatica* characterized by the production of IL-4, IgG1 antibodies and eosinophilia (248-250). Time course studies conducted by Waldvogel et al showed that within 10 days of infection the immune response against *F. hepatica* is skewed towards a Th2 phenotype. Peripheral blood mononuclear cells (PBMC) isolated from cattle 10 days post-infection expressed high levels of IL-4 but no IFN-γ in response to parasitic antigens (251). However, the host immune response usually fails to protect against infection with liver fluke and within 12 weeks the infection becomes chronic.

Chronic infection suppresses the production of IL-4 but enhances IL-10 and TGF-β by bovine PBMC in response to parasite antigen (251, 252). Neutralization of IL-10 and TGF-β results in increased production of both IL-4 and IFN-γ, suggesting that the parasite may induce these anti-inflammatory cytokines to evade the immune response (252). The production of IL-4 by PBMCs from acutely infected animals suggests that the immune response against liver fluke is not restricted to the site of the infection. It has been shown that infection with *F. hepatica* suppresses immune responses against bacterial infections as calves that were co-infected with *Mycobacterium bovis* and *F. hepatica* had reduced Th1 responses to *M. bovis* antigen compared with calves that were not infected with the parasite (253). In a mouse model of *F. hepatica* infection, Brady et al reported that the infection of mice with the fluke suppressed Th1 responses directed against *B. pertussis,* and delayed bacterial clearance from the lung (254). Flynn et al reported that liver fluke antigens could promote the alternative activation of macrophages, which expressed arg1 and had chitinase activity (255). However, in contrast to these findings, Miller et al demonstrated that co-infection of mice with *F. hepatica* and the intracellular parasite *Toxoplasma gondii,* which typically induces strong Th1 response, did not affect the production of NO or IFN-γ *in vivo.* In contrast, infection with *T gondii* suppressed the production of IL-4, IL-5 and the alternative activation of macrophages associated with *F. hepatica* infection. These findings suggest that the induction of Th2 responses to *F. hepatica* is dependent the immunological environment of the host

1.7.3 The excretory/secretory products of *F. hepatica*

The surface of the parasite and the molecules it secretes are in direct contact with the immune cells of the host. Studies have shown that the ability of *F. hepatica* to modulate and evade the immune response is achieved via secreted molecules. The surface of the parasite in the

vertebrate host is covered with tegument, a 40 nm layer of glycoprotein. In juvenile flukes, the glycocalyx is replaced every 3 hours, which prevents bound antibody from activating immune effector cells, including eosinophils which can directly damage the parasite (256, 257). The tegument of *F. hepatica* has been shown to drive alternative activation of macrophages, inhibit the maturation of DC, suppress the production of TLR-agonist induced pro-inflammatory cytokines and suppress serum concentrations of IFN-γ and IL-12, in a model of septic shock (258, 259).

Recently Moxon et al showed that an immune response was elicited by *F. hepatica* egg proteins and that these antigens could induce the production of TGF-β and IL-10 by PBMC isolated from cattle chronically infected with *F. hepatica;* suggesting that embryonated eggs may induce suppressive immune responses (260).

The secretome of *F. hepatica* is known as excretory-secretory products (ES) and has known immunomodulatory properties. Serradell et al reported that ES induces apoptosis of rat peritoneal eosinophils in a caspase/tyrosine kinase dependent manner (261). Flynn et al demonstrated ES suppresses LPS-induced production of NO and IFN-γ by bovine macrophages (262). ES has been shown to inhibit the TLR agonist-induced maturation of DC, including pro-inflammatory cytokine release and expression of MHC-class II, CD40 and CD86. DC matured in the presence of ES promotes the expression of IL-4, IL-5, IL-10 and TGF-β by T cells, moreover these T cells were shown to inhibit the production of IFN-γ during a mixed lymphocyte reaction (263).

Proteomic analysis of ES has revealed that it contains a variety of cysteine proteases as well as glutathione S-transferase and peroxiredoxin. Glutathione S-transferase is an oxidant scavenging enzyme and may enable *F. hepatica* to counter-act the toxic reactive oxygen products of innate immune cells (264). Many products affect the activation and function of macrophages, another antioxidant, a mu class glutathione transferase significantly suppressed the production of NO by rat macrophages and inhibited the proliferation of spleen cells in response to conA or LPS (265). Peroxiredoxin has been shown to promote the alternative activation of macrophages which highly express arg1 and IL-10 (266). It was subsequently shown that these M2 macrophages enhanced the secretion of IL-4, IL-5 and IL-13 by naive T cells (267).

Other major components of ES include the cysteine proteases or cathepsins. *F. hepatica* utilises a variety of cathepsins during infection including, cathepsin B for penetration of the gut, a combination of cathepsins B and L for migration through the liver before ceasing production of cathepsin B and solely producing cathepsin L in the bile ducts (268). Cathepsin L, which is used by the parasite primarily to digest blood in the bile duct, has also been shown to modulate the immune response to the parasite (269). Incubation of human and bovine T cells with cathepsin L reduces the expression of CD4 on $CD4^+$ T cells and in a murine model, cathepsin L suppressed the development of a Th1 response to the *B. pertussis* whole cell vaccine (270, 271). Donnelly et al reported that cathepsin L could protect from LPS-induced septic shock, which was associated with a reduction in serum concentrations of NO, IL-6 and TNF-α. The cysteine protease was shown to degrade TLR3 leading to an impairment in the TRIF-dependent signalling pathway, thereby suppressing Th1 responses induced via this pathway (272).

1.8 Aims

The aim of this study was to investigate the immunomodulatory properties of *F. hepatica* infection and *F. hepatica* ES. Experiments were designed to examine the effect of ES on APC function both *in vitro* and *in vivo* and on the recruitment of cells into the peritoneal cavity. This study also examined the effect of infection and ES on the function of T cells *in vivo*. Finally, this study examined whether infection with *F hepatica* or the administration of ES products of *F. hepatica* could modulate autoimmune and allergic disease in mouse models.

Chapter 2 Materials and Methods

2.1 MATERIALS

2.1.1 AMMONIUM CHLORIDE LYSIS SOLUTION

0.77g Ammonium Chloride (NH_4Cl)

Dissolved in 100 ml ddH_2O

2.1.2 CELL CULTURE MEDIUM

Roswell Park Memorial Institute-1640 medium (RPMI BioSera) or Dulbecco's modified eagle medium (DMEM) was supplemented with 10% heat-inactivated (56°C for 30 min) foetal-calf serum (FCS, South American origin, Biosera), L-glutamine (Gibco, 100 mM) penicillin/streptomycin (Biowest, 100 μg/ml)). RPMI was used as general purpose medium and was supplemented with β-mercaptoethanol (2-ME 50 μM) where indicated for culture of T cells *ex vivo*. X-VIVO 15 medium (Lonza) supplemented with L-glutamine (100 mM) was used where indicated for experiments requiring detection of TGF-β or for DC transfer experiments (Section 2.2.13.7).

2.1.3 CARBONATE BUFFER

4.2 g $NaHCO_3$

1.7 g Na_2CO_3

Dissolved in 500 ml ddH_2O

pH 9.5

2.1.4 EDTA (200 μM)

0.0093 g of EDTA

Dissolved in 50 ml Baxters Water.

2.1.5 ELISA DEVELOPING SOLUTION

1 OPD tablet

Dissolved in 25 ml of phosphate citrate buffer

7 µl of hydrogen peroxide H_2O_2

2.1.6 ELISA WASHING BUFFER

1X PBS

0.2% Tween

2.1.7 ELISA BLOCKING SOLUTION

5 % w/v milk (Marvell)

Dissolved in 1 X PBS

Or

1% w/v Bovine serum albumin (BSA)

Dissolved in 1 X PBS

2.1.8 ELISA STOPPING SOLUTION

1 M H_2SO_4

2.1.9 FACS BUFFER

2% FCS

0.1% Sodium azide

Made up in 1X PBS

2.1.10 MACS BUFFER

2% FCS

5 ml 200 μM EDTA

Made up in 500 ml 1X PBS

2.1.11 PHOSPHATE-BUFFERED SALINE (PBS) 20X

800g Sodium chloride (NaCl, 1.4M)

92g Sodium hydrogen phosphate (Na_2HPO_4, 0.08M)

20g Potassium dihydrogen phosphate (KH_2PO_4, 0.01M)

20g Potassium chloride (KCL, 0.03M)

Dissolved in 5L of dH_20, pH 7

2.1.12 PHOSPHATE CITRATE BUFFER

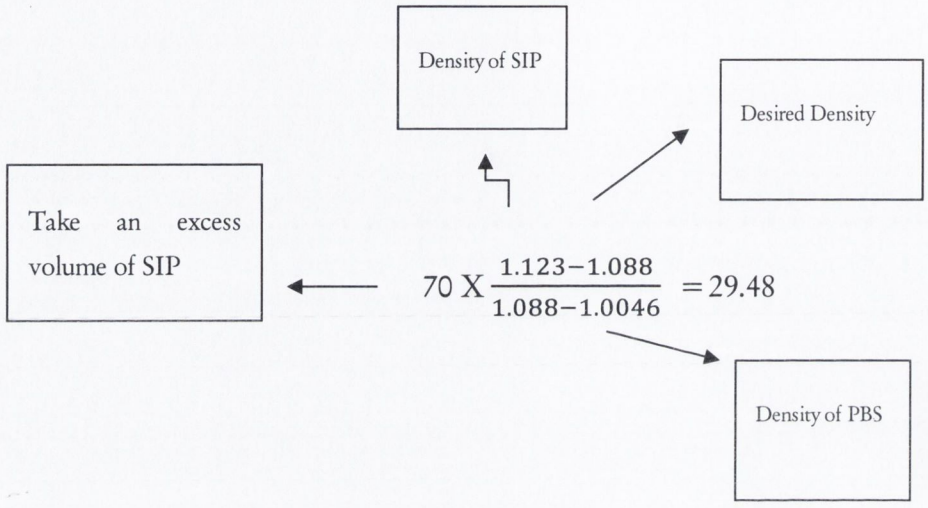
10.19g Anhydrous citric acid

36.69g $Na_2HPO_4.12H_2O$

Made up in 1L of dH_2O, pH 5

2.1.13 STOCK ISOTONIC PERCOLL

Stock isotonic Percoll® (SIP) is made isotonic by diluting 9:1 with 10X PBS (Sigma-Aldrich). The different density gradients are calculated using the equations described in the Percoll manual (Sigma Website). Briefly, for 1.088 g/ml: if you require ~100 ml,



Therefore: 70 ml SIP + 29.38 ml 1X PBS = 9.38 ml of 1.008 g/ml Percoll

Table 2.1 TLR ligands

Ligand	TLR	Supplier	Concentration used *in*	Dose used *in*
LPS	4/2	Alexis	0.1 ng/ml- 1 μg/ml	10 μg/mouse
CpG	9	Sigma-Genosys	0.1 ng/ml- 100 μg/ml	50 μg/mouse
Ultra-pure LPS	4	Sigma-Aldrich	100ng/ml	n/a

Table 2.2 Antigens

Antigen	Supplier	Concentration used	Dose used *in vivo*
KLH	Calbiochem	10 μg/ml, 25 μg/ml	10 μg/mouse
MOG_{35-55}	Cambridge	10 μg/ml, 25 μg/ml	150 μg/mouse
ES	See section 2.2.4	0.125-5%(v/v)	20-45 μl/mouse
$OVA_{(323-339)}$	Genscript	1 μg/ml, 5 μg/ml,	100 μg/mouse
LFH	Homogenised adult	20 μg/ml, 40μg/ml	n/a
OVA (protein)	Sigma-Aldrich	2-50 μg/ml	6% w/v aerosolised

Table 2.3 Bacterially derived molecules

Molecule	Supplier	Concentration used	Dose used *in vivo*
PT	Kaketsuken	n/a	500 ng/mouse

Table 2.4 Mouse Cytokines

Cytokine	Type	Supplier	Concentration used *in*
FLT3-L	recombinant	preprotech	100 ng/ml
GMCSF	J558 Supernatant	In-house	40 ng/ml
IL-1β	recombinant	R&D	10 ng/ml
IL-2	recombinant	R&D	10 U/ml= 5 ng/ml
IL-23	recombinant	R&D	10 ng/ml
IL-5	recombinant	Miltenyi-Biotech	5 ng/ml
MCSF	L929 Supernatant	In-house	20% v/v
SCF	recombinant	Miltenyi-Biotech	100 ng/ml
TGF-β	recombinant	R&D	1 pg/ml- 10 ng/ml

Table 2.5 Small molecule inhibitors biologically active chemicals

Chemical	Biological Target	Supplier	Concentration
BrdU	DNA	BD	100 µg/mouse
Cycloheximide	Protein translation	Sigma	100 ng/ml
E-64	Cathepsins	Sigma	5 µM
N-acetylcysteine	Antioxidant	Sigma	0.2-2 µM
SB-431542	Alk-5 (TGF-βR)	Sigma	10 µM

Table 2.6 Antibodies.

Antibody	Clone	Supplier	Concentration used	Dose used *in*
Anti-IL-12	C17.8	BD Biosciences	10 µg/ml	n/a
Anti-IL-4	BVD4-	BD Biosciences	10 µg/ml	n/a
Anti-IFN-γ	R4-6A2	BD Biosciences	10 µg/ml	n/a
Anti-IL-10	JES5-16E3	BD Biosciences	10 µg/ml	n/a
Anti-CD3e	145-2C11	BD Biosciences	1 µg/ml	n/a
Anti-TGF-β	1D11	Bioceros BV	10 µg/ml	100 µg/mouse
Unspecified IgG_1	n/a	BioXcell	10 µg/ml	100 µg/mouse
Anti-IL-5	TRFK-5	Bioceros BV	n/a	80 µg/mouse

Table 2.7 ELISA Antibody Concentrations

Antigen	Coating Antibody	Top Working	Detection Antibody
IFN-γ	1 µg/ml	10 ng/ml	1 µg/ml
IL-4	1 µg/ml	2000 pg/ml	1 µg/ml
IL-5	1 µg/ml	2500 pg/ml	1 µg/ml
IL-6	1 µg/ml	5000 pg/ml	1 µg/ml
IL-12p40	1 µg/ml	5000 pg/ml	1 µg/ml
IL-2	1 µg/ml	2000 pg/ml	1 µg/ml
IL-17A	1/180	1000 pg/ml	1/180
IL-1β	1/180	1000 pg/ml	1/180
IL-12p70	1/180	1500 pg/ml	1/180

GM-CSF	1/180	2000 pg/ml	1/180
TGF-β	1/180	1000 pg/ml	1/180
IL-23	1/180	4000 pg/ml	1/180
TSLP	1/180	1000 pg/ml	1/180
IL-25	1/180	4000 pg/ml	1/180
IL-13	1/180	4000 pg/ml	1/180
IL-27	1/180	1000 pg/ml	1/180
IL-10	1/180	2000 pg/ml	1/180

Table 2.8 Antibodies used in Flow Cytometry

Antigen	Flurochrome	Clone	Isotype	Supplier
BrdU	APC	B44	Mouse IgG1 κ	BD Pharmingen
CD115	PE	ASF298	Rat IgG2aκ	eBioscience
CD11b	A780	M1/70	Rat IgG2aκ	eBioscience
CD11b	FITC	M1/70	Rat IgG2aκ	eBioscience
CD11c	PE-Cy5	N418	Hamster IgG	eBioscience
CD11c	PE-Cy7	N418	Hamster IgG	eBioscience
CD11c	FITC	N418	Hamster IgG	eBioscience
CD25	PE-Cy5	PC61.5	Rat IgG1	eBioscience
CD3	A780	145-2C11	Hamster IgG	eBioscience
CD3	FITC	145-2C11	Hamster IgG	eBioscience
CD3	V500	17A2	Rat IgG2a	eBioscience

CD4	PE-Cy5	GK1.5	Rat IgG2b	eBioscience
CD4	PE-Cy7	GK1.5	Rat IgG2b	eBioscience
CD4	V450	GK1.5	Rat IgG2a	eBioscience
CD40	FITC	HM40-3	Hamster IgM	BD Pharmingen
CD80	PE	16-10A1	Hamster IgG2κ	BD Pharmingen
CD86	FITC	B7.2	Rat IgG2aκ	BD Pharmingen
D0.11.10 TCR	PE	KJI-26	IgG2a κ	BD Pharmingen
F4/80	PE-Cy5	BM-8	Rat IgG2a	eBioscience
F4/80	PE-Cy7	BM-8	Rat IgG2a	eBioscience
FcεR-1	APC	MAR-1	Rat IgG2a	eBiosciences
FOXP3	PE	FJK-16A	Rat IgG2aκ	eBioscience
FOXP3	APC	FJK-16S	Rat IgG2aκ	eBioscinece
GM-CSF	PE	MP1-22E9	Rat IgG2a	eBioscience
Gr-1	PE	RB68-C5	Rat IgG2bκ	eBioscience
Gr-1	PE-Cy7	RB68-C5	Rat IgG2bκ	eBioscience
I-A/I-E	PE	M5/114.15.2	Rat IgG2aκ	eBioscience
I-A/I-E	Pacific-Blue	M5/114.15.2	Rat IgG2aκ	eBioscience
IFN-γ	APC	XMG1.2	Rat IgG1	eBioscience
IFN-γ	PerCp Cy5.5	XMG1.2	Rat IgG1	eBioscience
IFN-γ	Pe-Cy-7	XMG1.2	Rat IgG1	BD
IFN-γ	PerCp-Cy5.5	XMG1.2	Rat IgG2a	eBioscience
IL-10	FITC	JES-1683	Rat IgG_{2b}	BD Pharmingen

IL-10	APC	JES5-16E3	Rat IgG2bκ	eBioscience
IL-13	PE	eBio13A	Rat IgG2a	eBioscience
IL-17 A	PE	TC11-18H10	Rat IgG1	BD Pharmingen
IL-17 A	A647	TC11-18H10	Rat IgG1	eBioscience
IL-17A	V450	TC11-18H10	Rat IgG2a	eBioscience
IL-4	FITC	BVD6-24G2	Rat IgG κ	eBioscience
IL-4	PE-Cy7	BVD6-24G2	Rat IgG2a	eBioscience
IL-5	APC	TRFK5	Rat IgG2a	BD
LAP	APC	27232	Mouse IgG1	R & D
Ly6C	A488	ER-MP20	Rat IgG2aκ	ABD Serotec
Ly6C	Pe-Cy7	AL-21	Rat IgGM1 κ	eBioscience
Ly6C	PerCP-Cy5.5	AL-21	Rat IgG2a	eBioscience
Ly6G	A647	1A8	Rat IgG2aκ	eBioscience
Ly6G	Pe-Cy7	1A8	Rat IgG2aκ	eBioscience
PD-1	PE	RMP120	Rat IgG2bκ	eBioscience
Siglec-F	PE	E50-2480	Rat IgG2aκ	eBioscience
TGF-β	PE	9016	Mouse IgG1	R & D
TGF-β	PE	1D11	Rat IgG2a	IQ Products

Table 2.9 Real Time RT-PCR primers used in mouse studies

Cytokine	Ref. Seq	Product Code	Supplier
18S rRNA	X03205.1	4319413E	ABI
Arg1	NM_007482.3	Mm00475988_m1	ABI
CFSR	NM_009969.4	Mm01290062_m1	ABI
IFN-γ	NM008337.3	Mm00801778_m1	ABI
IL-10	NM0105481.1	Mm00439616_m1	ABI
IL-17	NM010552.3	Mm00439619_m1	ABI
IL-27 p28	NM145636.1	Mm00461164_m1	ABI
IL-4	NM_021283.2	Mm00445259_m1	ABI
iNOS	NM_010927.3	Mm01309897_m1	ABI
RETNLA	NM_020509.3	Mm00445109_m1	ABI
TGF-β	NM_007482.3	Mm00441724_m1	ABI

2.2 METHODS

2.2.1 Mice

Female specific pathogen-free C57BL/6 and BALB/c mice were purchased from Harlan UK Ltd (Bicester, UK). IL-$10^{-/-}$ and IL-$4^{-/-}$ mice on a C57BL/6 background and DO.11.10 mice on a BALB/c background were bred in-house in the BioResorces Unit, Trinity College Dublin. Mice were 6-12 weeks old at the initiation of each experiment and were sacrificed by cervical dislocation or by asphyxiation with CO_2 except where an intracranial perfusion was required, where mice were chemically anesthetised (section 2.2.15.4). Animals were maintained according to the regulations and guidelines of the European Union and the Irish Department of Health and Children. Experiments were conducted under license from the Department of Health and with the approval from the Trinity College Dublin BioResorces Ethics Committee.

2.2.2 Basic cell culture protocols

2.2.2.1 Preparation of single-cell suspensions

Organs were isolated from freshly killed mice and collected in tubes containing ice-cold PBS RPMI or DMEM. Organs were dissociated through a sterile 40 or 70 μm cell strainer using the plunger of a sterile 1 ml or 5 ml syringe. Cells were washed through with medium and were pelleted by centrifugation at 1200 rpm for 5 min prior to use.

2.2.2.2 Lysis of Red blood cells

Single cell suspensions (section 2.2.2.1) of cultured or freshly isolated cells were centrifuged at 1200 rpm for 5 min and the pellet was resuspended in 1 ml of heated RBC lysis solution (section 2.1.1) for 2 min, to lyse contaminating red blood cells. The cells were washed in 20X volume of RPMI and pelleted by centrifugation at 1200 rpm for 5 min. The washed mononuclear cells were then resuspended in RPMI and counted prior to culture.

2.2.2.3 Cell culture

Single cell suspensions were cultured in various media in an incubator with temperature maintained at 37 °C in an atmosphere containing 5% CO_2.

2.2.2.4 Cell counts

Cell counts were performed by diluting cells (1:1 to 1:20) in trypan blue (Sigma-Aldrich) and loading 10 µl of this mixture onto a disposable plastic haemocytometer (Hycor Biomedical, UK). Viable cells which do not stain with trypan blue appear white under a light microscope, whereas dead cells stain blue. Alternatively, in cases where discrimination of live and dead cells was required, or when cell suspensions contained a large proportion of red blood cells (RBCs), cell counts were performed in ethidium-bromide/acridine-orange (Sigma-Aldrich) at a 1 in 2 dilution and this mixture was loaded onto a neubauer glass haemocytometer (Hawsley). Using this method viable cells fluoresce green when viewed under a UV fluorescent microscope, while dead cells appear orange. The number of cells per ml was calculated using the following formula:

$$\#\text{cells in central square of haemocytometer} \times 10^4 \times \text{dilution factor} = \text{cells/ml}$$

2.2.3 *Fasciola hepatica* infection

Mice were infected with metacercariae of the Cullompton strain (Gerald Coles, Department of Veterinary Clinical Sciences, University of Bristol) or Shrewsbury/South Gloucestershire strains (Paula Martin, Ridgeway Research Ltd, St. Briavels, Gloucestershire) of *F. hepatica*. Ten viable metacercariae, selected using a light microscope, were entangled in cotton wool. C57BL/6 or IL-$10^{-/-}$ mice were orally infected by placing inoculated cotton wool at the back of the mouth using a forceps. Swallowing was induced by giving the animal drinking water. Mice were observed for about 30 s to ensure swallowing was successful and that they did not regurgitate the cotton wool.

2.2.4 Isolation of ES

ES was purified from *F. hepatica* parasites collected from the infected livers of cattle slaughtered at a local abattoir (Kildare Chilling Ltd). Briefly, livers from freshly killed adult cows were identified by an on-duty veterinarian as exhibiting the morphological changes associated with *F. hepatica* infection. The infection state of the livers was determined by an examination of dissected bile ducts for the presence of adult fluke. Live flukes, collected with a sterile forceps, were transferred into PBS containing penicillin/streptomycin (100 μg/ml, Biowest). Flukes were transported to the laboratory and washed 5 times with PBS to remove large debris. Dead flukes were discarded and live flukes were incubated at 5-10 worms/3ml PBS at 37 °C. After 24 hours, flukes were carefully removed using a sterile forceps. ES was aspirated from the culture vessel and filtered through a 0.45 μm filter (Millipore) to remove fluke tissue and eggs. After filtration, ES was centrifuged (30,000 g 30 min) to remove smaller impurities. The supernatant was aliquoted in small volumes, stored at -80 °C, and used within 6 months of purification. Nine separate biological isolations of ES were used in this study. Each isolate was tested for LPS contamination by Limulus amebocyte lysate assay which was consistently <0.1 ng/ml. The relative biological activity of each isolate was determined by ability to induce release of TGF-β and inhibit the LPS-induced production of IL-12p70 by BMDC.

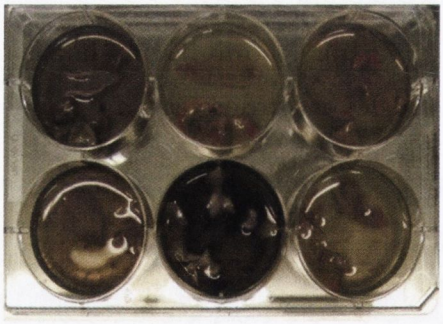


Figure 2.1 Flukes in culture.
Live adult *F. hepatica* were cultured in PBS in 6 well plates at 37 °C. After 24 hours the culture vessel was removed and photographed.

2.2.4.1 Fractionation of ES

Immediately following isolation, ES was fractionated into >3kDa and <3kDa fractions. 4 ml of ES was applied to a sterile 3kDa concentrating filter device (Amicon Ultra-4® , Millipore) and centrifuged at 3000 g for 2 hours. The elute, hereafter referred to as "<3kDa ES", was removed without modification and stored at -80°C. The remaining 50 µl of now concentrated solute remaining in the filter device, hereafter referred to as ">3kDa ES", was replenished to 4ml with PBS, removed from the device, homogenised and stored at -80°C.

2.2.5 Intraperitoneal injections

C57BL/6 mice were injected intraperitoneally (i.p.) with various reagents (Table 2.1, Table 2.2, Table 2.3 and Table 2.6) diluted in PBS to a total volume of 100 µl. I.p. injections are 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 (section 2.2.5.1) (273).

2.2.5.1 Peritoneal lavage

The abdominal skin of mice was cut away to expose the intact peritoneal sack. A 25 gauge needle was inserted through the peritoneum and, taking care not to pierce any internal organs, 6 ml of ice cold sterile PBS (Biosera) was injected into the cavity. An air bubble was then injected as a reference point for re-insertion of the needle and also to prevent the regress of liquid through the injection site. The body of the mouse was gently shaken to aid the removal of the peritoneal exudate into the PBS. The needle was then re-inserted and the maximum possible volume of PBS was removed. The cells were placed on ice before being pelleted by centrifugation at 1200 rpm for 5 min. The resulting pellet, the peritoneal-exudate cells (PEC), was resuspended in RPMI; counted and used in culture, or analysed by flow cytometry.

2.2.6 Subcutaneous injections into the footpad

Mice were injected subcutaneously (s.c.) into the footpad with reagents dissolved or suspended in PBS in a total volume of 25 µl (50µl/mouse). The lymphatics of the foot drain preferentially to the easily isolated popliteal LN (pLN), located behind the knee (274). As

such, footpads injections and subsequent removal of the pLN is used as a model system for the study of the local immune response following injection of substances of interest.

2.2.7 Arginase Activity

PEC or spleen cells were isolated from naïve and *F. hepatica*-infected mice by peritoneal lavage (section 2.2.5.1). $1x10^6$ PEC were treated with 100 µL of passive-lysis buffer (Promega) containing protease inhibitor cocktail (Roche). Arginase activity of the cell lysate was determined using the quantichrom arginase assay kit (Bioassay Systems). Samples were incubated with the substrate L-arginine for 2 hours and arginase activity was quantified by the production of the product, urea. The kit utilises a chromogen that forms a colored complex (A_{max} 430 nm) specifically with urea.

2.2.8 Isolation of peripheral blood mononuclear cells

Blood was collected immediately after the cardiac puncture of killed mice and was mixed 1:1 with 8% sodium citrate in PBS. The mixture was layered over an equal volume of Ficoll (Histopaque, 1071 g/ml, Sigma-Aldrich) followed by centrifugation at 670 g (without the brake) for 20 min at RT. The PBMC layer was recovered, washed twice with sterile PBS and pelleted by centrifugation at 203g for 7 min at RT. PBMC were resuspended in RPMI and counted.

2.2.8.1 Isolation of blood serum

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

2.2.9 Cytospin preparations

Single cell suspensions were prepared from PEC (section 2.2.5.1) or BAL cells (section 2.2.16.3). $20\text{-}50x10^3$ cells in a maximum volume of 100 µl RPMI or PBS were pipetted into cytocentrifuge cuvette with attached glass slide in a metal holder. Cells were centrifuged at

1200 RPM for 3 min in a cytocentrifuge (cytospin 3, Shandon) The glass slides were removed from the metal holders and allowed to air-dry. Dry slides were fixed with methanol and stained with haematoxylin and eosin (H&E, DIFF-QUICK, Thermo-Scientific).

2.2.10 Detection of ROS

Cellular oxidant production was visualised using the dye 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CH2-DCFDA, Molecular Probes, Life Technologies). The compound readily permeates live cells; wherein it is deacetylated by non-specific cellular esterases. This modification prevents regress from the cell and makes the compound oxidation sensitive. In the reduced state the dye is non-fluorescent, however, in the presence of nonspecific ROS (produced during oxidative stress) it is oxidised to produce a fluorescent fluorescein (ex/em 488/529 nm).

A594 human lung carcinoma cells (0.5 x 10^6/ml) were allowed to adhere to sterile glass dishes (35 mm μ-Dish, ibidi). Adherent cells were treated with PBS or ES (5% v/v) for 18 hours or with tert-butyl hydroperoxide (TBHP, 100 μM), an inducer of ROS, for 2 hours. Following treatment, cells were washed with warm phenol red-free DMEM (Sigma-Aldrich) and incubated with warm medium containing CH2-DCFDA (40 μM). After 20 min the cells were washed, repleted with phenol red free DMEM and visualised by confocal microscopy for fluorescence at 535 nm.

2.2.11 Cell purification

2.2.11.1 $CD4^+$ T cell magnetic separation

Freshly isolated spleen or LN cells were suspended in ice-cold MACS buffer 1 x 10^6cells/9 μl. Cells were incubated with anti-CD4 coated magnetic beads (Miltenyi Biotec) at a ratio of 10:1 at 4°C. After 15 min, unbound beads were washed with 10X labelling volume of MACS buffer and pelleted by centrifugation at 300 g for 10 min. Cells were resuspended in 1 ml of MACS buffer and $CD4^+$ T cells were isolated by positive selection on an autoMACS machine (Miltenyi Biotec). Cells were washed with RPMI and counted using the ethidium-

bromide/acridine-orange method (section 2.2.2.4). T cell purity was determined by flow cytometry and was routinely >95%.

2.2.11.2 CD11b$^+$ isolation by MACS

Freshly isolated PEC (section 2.2.5.1) from naïve and *F. hepatica* infected mice were suspended in ice-cold MACS buffer 1 x 10^6cells/9 µl. Cells were incubated with anti-CD11b coated magnetic beads (Miltenyi Biotec) at a ratio of 10:1 at 4°C. After 15 min, unbound beads were washed with 10X labelling volume of MACS buffer and pelleted by centrifugation at 300 g for 10 min. Cells were resuspended in 1 ml of MACS buffer and separated into the labelled and unlabelled fractions using the "possel" program on an autoMACS machine (Miltenyi Biotec). Each fraction was washed with RPMI and counted using the ethidium-bromide/acridine-orange method (section 2.2.2.4). The percentage purity of separated cell populations was determined by flow cytometry.

2.2.11.3 Siglec F$^+$ isolation by FACS

Freshly isolated PEC (section 2.2.5.1) from *F. hepatica* infected or ES-treated mice (section 2.2.5, 2.2.5.1 and 2.2.3) were washed and resuspended in a maximum volume of 1 ml of RPMI. Cells were surface stained with fluorescent antibodies directed against CD11b, Gr-1 and Siglec F (section 2.2.18.3). CD11b^{int} Siglec F$^+$ Gr-1$^{low\text{-}int}$ SSc^{high} cells were purified by FACS with a MoFLo cell sorter (Beckman Coulter). Cellular purity was determined by flow cytometric analysis and was consistently >95%.

2.2.11.4 Isolation of myeloid-derived suppressor cells

A single cell suspension of PEC was surface-stained with anti-CD11b PE and Gr-1 PE-Cy7. CD11b^{int} Gr-1^{hi} cells were purified by FACS with a MoFLo cell sorter (Beckman Coulter).

Alternatively, Ly6G$^+$ and Ly6G$^-$ Gr-1^{dim} cells were purified from PEC by sequential MACS sorting as follows: Gr-1^{high} Ly6G$^+$ cells were indirectly labelled with anti-Ly6G-biotin and anti-biotin microbeads. LY6G$^+$ cells were purified by positive selection on an autoMACS machine using the "posseld2" program. The unlabelled fraction, depleted of Ly6G$^+$ Gr1^{high} cells, was stained with an anti-Gr-1-biotin antibody and sequentially with streptavidin microbeads. Gr-1^{dim} Ly6G$^-$ cells were purified by positive selection on an autoMACS machine using the

"posseld2" program. Cellular purity of separated populations was determined by flow cytometric analysis and was routinely >90%.

2.2.12 Growth factor-induced cell differentiation from bone marrow

2.2.12.1 Generation of murine bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDC) were generated from BALB/c (H-2^d), and C57BL/6 (H-2^d) mice: the femurs and tibiae of freshly killed mice were removed and dissected from the surrounding muscle and tissue. The bone marrow was then flushed using a 25 gauge needle attached to a 20 ml syringe containing RPMI. A single cell suspension was prepared from the bone marrow by aspirating through a 19G needle. RBCs were lysed with a hypotonic ammonium chloride solution (section 2.2.2.2). After washing, cells were pelleted by centrifugation at 1200 rpm for 5 min. The cells were resuspended in 2 ml of RPMI and counted (section 2.2.2.4). Cells were cultured at 0.5 x 10^6 cells/ml (BALB/c) or 1 x 10^6 cells/ml (C57BL/6) in RPMI with no more than 25 x 10^6 cells in a T175 tissue culture flask (Greiner). Granulocyte macrophage-colony stimulating factor (GM-CSF, 20 ng/ml) in the form of supernatant obtained from the J558 cell line was added and cells were cultured for 3 days. At this time, 20 ml of fresh medium containing 20 ng/ml of GM-CSF was added to each flask. On day 6, the flasks were gently removed from the incubator and the supernatant was carefully removed so as not to dislodge the loosely adherent immature BMDC cells (this step removes contaminating cells, such as granulocytes from the culture). 10 ml of heated sterile PBS was then added to each flask and the flask was gently rolled to dislodge loosely adherent cells. The PBS was then collected into sterile 50 ml tubes containing 10 ml of warm complete medium. Sterile heated EDTA (16 ml, 0.02%: Sigma) was then added to each flask and the flasks were incubated for 10 min at 37°C. Medium (15 ml) was added to the flasks that contained EDTA and the cells were collected after vigorous pipetting. The 'PBS' and the 'EDTA' cell suspensions were pooled and pelleted by centrifugation at 1200 rpm for 5 min. The pellet was resuspended and cells counted (Section 2.2.2.4). Cells were re-cultured in RPMI supplemented with GM-CSF as before. On day 8, 15 ml of fresh medium containing GM-CSF (20 ng/ml) warmed to 37°C was added to each of the flasks and the cells returned to the incubator. On day 10 the loosely adherent cells were harvested by removing the supernatant through gentle pipetting. The viability of BMDC was assessed and the cells were

cultured at the required concentration in tissue culture plates. The immature DC were allowed to 'rest' overnight at 37°C with 10 ng/ml of GM-CSF before use.

2.2.12.2 Generation of bone marrow-derived eosinophils

Bone marrow-derived eosinophils (BMEOS) were generated from BALB/c mice. Bone marrow mononuclear cells were prepared as in section 2.2.12.1 and were cultured at 1 x 10^6/ml in RPMI containing mouse-recombinant stem cell factor (SCF, 100 ng/ml) and mouse-recombinant FLT3-L (100ng/ml). After 2 days, 50% of the medium was carefully removed and the remaining medium containing non-adherent cells was aspirated and transferred to a fresh culture vessel. The culture was topped up to 100% of original volume with medium containing SCF and FLT3-L (100ng/ml). On day 4, non-adherent cells were removed and pelleted by centrifugation at 1200 rpm for 5 min. Cells were resuspended and cultured at 1 x 10^6 cells/ml in a new flask containing mouse recombinant IL-5 (10 ng/ml). On day 6 and day 8, 50% of the medium was carefully removed and replaced with fresh medium containing IL-5 (10 ng/ml). On day 10, cells were counted and the percentage Siglec-F^+ $CD11b^+$ eosinophils was determined by flow cytometric analysis.

2.2.12.3 Generation of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMM) were generated from BALB/c mice. Bone marrow mononuclear cells were prepared as in section 2.2.12.1. and cultured in petri dishes at 1 x 10^7/10 ml of DMEM supplemented with supernatant of the macrophage colony-stimulating factor (MCSF)-producing cell line, L929 (20 % v/v). On day 3 and day 5, the culture was replenished with 1 ml of L929 supernatant. On day 6, non-adherent cells were removed by washing with DMEM and adherent cells were repleted with DMEM containing 10% v/v L929 supernatant. Adherent cells were removed on day 7 using a cell scraper, and were transferred to a fresh culture vessel. BMM were allowed to adhere before use.

2.2.13 APC function

2.2.13.1 Cytokine production

BMDC were generated as in section 2.2.12.1 and were cultured at 1 x 10^6 cells/ml in 96-well flat bottom tissues culture plates (CellStar). BMDC were stimulated with a variety of TLR-agonists as indicated in Table 2.1, or with ES as indicated in Table 2.2 or left untreated. After 24 hours, supernatants were recovered and concentrations of cytokines were determined by ELISA (see section 2.2.17.1).

2.2.13.2 Phagocytosis: *in vitro*

BMDC expanded from C57BL/6 mice were stimulated with PBS, ES (5% v/v), LPS (100 ng/ml) or ES + LPS for 2 hours. Without washing, cells were incubated for 30 min with 3.64 x 10^8 YG-fluorescent labelled latex microbeads (1 μm diameter, polysciences Inc.). Cells were then washed and resuspended in 100 μl of FACS buffer. Cells were surface stained with anti-CD11c PE-Cy7 (section 2.2.18.3) and acquired by a flow cytometer. Percentage uptake was determined as the frequency of $CD11c^+$ cells that displayed Y-G fluorescence.

2.2.13.3 Phagocytosis: *in vivo*

C57BL/6 mice were injected s.c. into the footpad with 3.64 x 10^8 0.5μm YG-fluorescent latex beads and LPS (10 μg) with or without ES (10 μl). After 24 hours, the popliteal LN were excised and a single cell suspension of LN cell was surface stained with fluorescent antibodies directed against CD11b and CD11c. Percentage uptake was determined as the frequency of $CD11c^+$ or $CD11b^+$ cells which displayed YG-fluorescence.

2.2.13.4 Antigen processing

BMDC, generated from C57BL/6 mice were allowed to adhere to a glass slide and were stimulated with PBS, ES (5% v/v), LPS (100 ng/ml) or ES + LPS. After 18 hours, cells were incubated with DQ-OVA (5μg/ml, molecular probes, Life Technologies). DQ-OVA is an ovalbumin protein which contains fluorescent BODIPY dye residues, however when the protein remains in its native form, the dye is quenched in hydrophobic pockets. After

phagocytosis the protein is degraded in the phagolysosome and the dye residues are released. Cells were imaged by live confocal microscopy for fluorescence at 515 nm.

For quantitative determination of antigen processing: BMDC grown from C3H/HEJ mice were treated with medium or ES (2% v/v) for 6 hours. Cells were then washed and resuspended in RPMI. Cells were surface stained with a fluorescent anti-CD11c antibody. Immediately prior to acquisition by a flow cytometer, cells were incubated with DQ-OVA (1μg/ml). Fluorescence at 515 nm of $CD11c^{hi}$ cells was tracked for 60 min

2.2.13.5 APC function: *ex vivo*

PEC or purified eosinophils from day 21 *F. hepatica* infected or naive BALB/c mice were used as APC for $CD4^+$ T cells isolated from naive DO.11.10 mice in the presence or absence of OVA peptide (Table 2.2). Supernatants were removed after 72 h and the concentrations of cytokines were determined by ELISA.

Alternatively, $CD11b^+$ cells or eosinophils were purified (section 2.2.11.2 and 2.2.11.3) from PEC cells isolated from naive and ES treated mice (section 2.2.5) and cultured with $CD4^+$ T cells isolated from mice with EAE in the presence or absence of the MOG antigen (Table 2.2).

2.2.13.6 APC function: *in vitro*

BMDC expanded from C57BL/6 (section 2.2.12.1) were cultured at $2x10^5$ cells/ml in RPMI containing 20 ng/ml GMCSF and were 'rested' for 24 hours. BMDC were stimulated with medium, KLH (20 μg/ml), KLH + ES (5% v/v), KLH + LPS (100 ng/ml) or KLH + LPS + ES. After 24 hours, BMDC were co-cultured with $CD4^+$ T cells ($1x10^6$ cells/ml, section 2.2.11.1), purified from the inguinal LN and spleens of C57BL/6 mice that were previously immunized with KLH (20μg) and LPS (10μg), at a ratio of (1:5). After 72 hours the concentrations of cytokines in supernatants were determined by ELISA. Cell proliferation was quantified by [^{3}H] thymidine incorporation (section 2.2.14.4)

2.2.13.7 DC transfer experiments

BMDC, expanded from either BALB/c or C57BL/6 mice (section 2.2.12.1), were cultured at $3x10^6$/well in 6 well plates with medium, antigen alone, antigen+ES, antigen+LPS or antigen+ES+LPS. DC were loaded with antigen (Table 2.2) at time 0. After 2 hours, DC were treated with either PBS or ES (5% v/v). After a further 30 min DC were treated with LPS (100 ng/ml). After 24 hours, BMDC were removed from culture using a cell scraper, washed twice and resuspended in PBS. DCs ($0.5x10^6$/footpad) were transferred into the footpads of naive mice by s.c. injection.

2.2.14 T cell responses

2.2.14.1 Generation of antigen-specific T cells

To determine memory T cell responses to a model antigen, C57BL/6 or DO.11.10 mice were immunized i.p. on day 0 and day 21 with KLH or $OVA_{(323-339)}$ peptide respectively. Mice were injected i.p with antigen (KLH or $OVA_{(323-339)}$, 100 μg, Table 2.2) and CpG (50 μg). On day 28 mice were sacrificed and inguinal LN and spleens were removed. $CD4^+$ T cells were purified from a pooled single cell-suspension of spleen and LN cells (section 2.2.11.1).

2.2.14.2 Irradiated APC

Whole spleens from C57BL/6 or BALB/c mice were placed in 50 ml tubes containing ice-cold RMPI. The tubes were placed into the irradiation chamber of a Nordian Gammcell 3000 Elan irradiator and were irradiated at a dose of 40 Gy. Irradiation prevents cell proliferation while retaining APC function. A single cell suspension was prepared, and the cells were counted prior to use in T cell assays.

2.2.14.3 Detection of T cell cytokine production *ex vivo*

Single-cell suspensions from the spleens, lungs, draining LNs or PECs of naïve or immunized mice and mice with EAE (section 2.2.15.1) or airway hypersensitivity (section 2.2.16) were prepared and cultured at in 2 x 10^6/cells in RPMI. Cells were incubated with increasing concentrations of relevant antigen (Table 2.2), or with anti-mouse CD3e (1 μg/ml: BD Biosciences, Table 2.6) in the presence or absence of increasing concentrations of ES or were

left untreated. The concentration of cytokines in the supernatant after 72-86 hours was determined by ELISA (section 2.2.17.1).

2.2.14.4 Proliferative responses of T cells: radioactive thymidine.

LN, spleen or purified T cells were stimulated as described above (section 2.2.14.3). For the last 18 hours of culture, cells were pulsed with 2.5 μCi/ml of [^{3}H]-thymidine (Amersham Pharmacia Biotech). At the end of the culture, cells were aspirated onto glass fibre filters (Wallac) with an automatic cell harvester. The filters were allowed to dry on a heating block (70 °C) before being sealed in plastic sample bags (Wallac) containing non-aqueous scintillation fluid (5ml, BetaScint, Wallac). [^{3}H]-thymidine incorporation was quantified with a Beta-plate scintillation counter (Wallac). Results are expressed as mean counts per minute (cpm) of [^{3}H]-thymidine incorporation for triplicate cultures of T cells.

2.2.14.5 Fluorescent membrane dye staining

Spleen or purified $CD4^+$ T cells were stained with the fluorescent membrane dyes carboxyfluorescein succinimidyl ester (CFSE, ex/em 492/517 nm Sigma-Aldrich) or CellTraceViolet (CTV, ex/em 400/452 nm, Molecular Probes Life Technologies,). Cell suspensions were washed twice in an excess of PBS to remove protein. Cells were resuspended in warm PBS (10 x 10^6 cells/ml) and stained with either CFSE (1-2.5 μM) or CellTraceViolet (5 μM). After 5 min the reaction was quenched by the addition of FCS at a concentration of 2% v/v. Cells were washed twice prior to use.

2.2.14.6 Proliferative responses of T cells: Membrane dye dilution.

Staining T cells with fluorescent membrane dyes allows for quantification of proliferation by flow cytometric analysis because of membrane dilution effect (and thus fluorescence dilution) of diving cells. Spleen or T cells were stained with a membrane dye (section 2.2.14.5), and then stimulated as in section 2.2.14.3. At 72 hours, post stimulation cells were aspirated from culture and stained for fluorescent antibodies directed against the cellular antigens CD3 and CD4. Stained cells were acquired by a flow cytometer (section 2.2.18) and the percentage-cellular proliferation was determined as the percentage of $CD3^+CD4^+$T cells that had lower, or had lost, CFSE or CTV fluorescence, compared with unstimulated T cells.

2.2.14.7 Proliferative responses of T cells *in vivo*

DO.10.11 mice were immunized on day -28 by i.p. injection with either OVA peptide (100 μg) + CpG (50 μg) ('immunized') or PBS alone ('non-immunized'). On days -1, 0, 1 and 2, mice were injected i.p. with either PBS or ES (40 μl) as indicated in Figure 2.2. On day 0 all mice received OVA peptide (100 μg) by i.p. injection. On days 1, 2 and 3 all mice were injected i.p. with Bromodeoxyuridine (BrdU, 100 μg). BrdU, a synthetic nucleotide, is incorporated into the newly synthesized DNA of replicating cells (during the S-phase of the cell cycle), substituting for thymidine during DNA replication. Mice were sacrificed on day 4 and single-cell suspensions of spleen, IG LN and peritoneal cells were prepared. Cells were surface stained with fluorescent antibodies specific for CD4 and the DO.10.110-transgenic TCR. Finally, cells were permeabilised and treated with DNase (to allow antibodies access to DNA residues) and stained with a fluorescent anti-BrdU antibody. Flow cytometric analysis was used to select antigen-specific T cells by gating on $CD4^+$ OVA Tg TCR^+ cells; while proliferation was quantified as percentage BrdU incorporation in these cells.

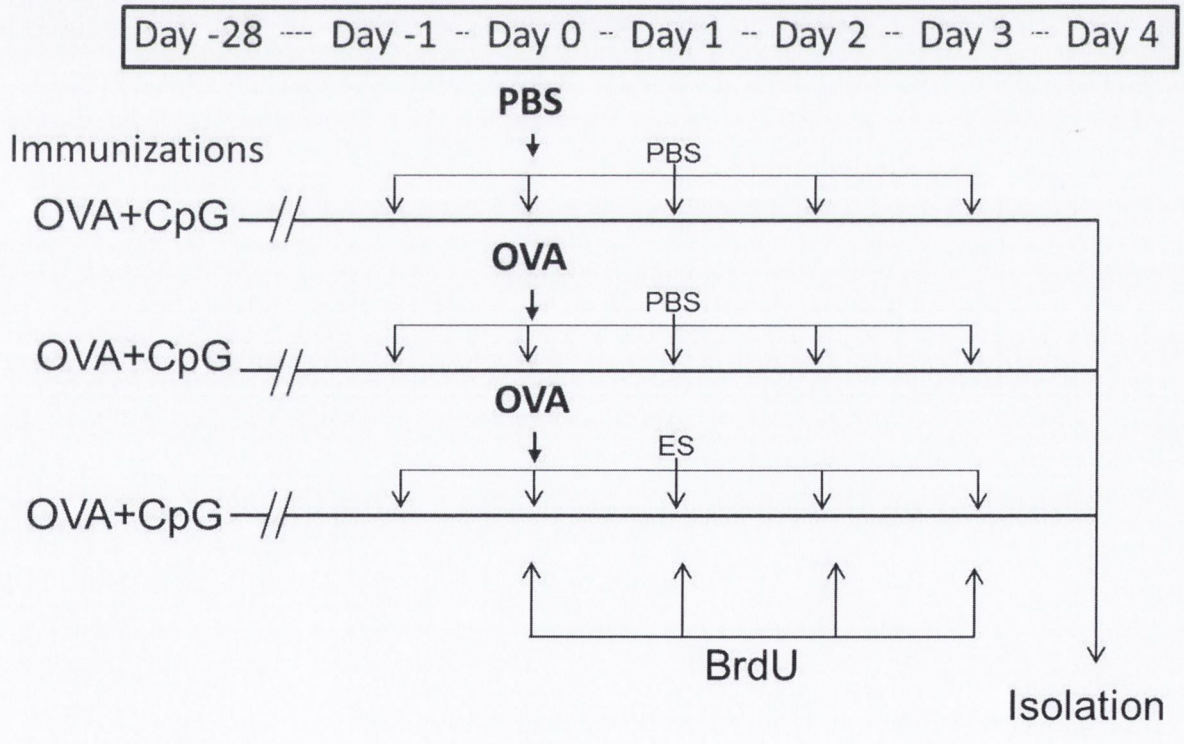


Figure 2.2 Proliferative T cell responses *in vivo*:
Experimental setup

2.2.14.8 Suppressive function of myeloid-derived suppressor cells on T cell responses

Purified $CD4^+$ T cells were activated with αCD3ε/CD28 (Table 2.6) and cocultured with myeloid-derived suppressor isolated from *F. hepatica* infected mice (section 2.2.11.4) at varying ratios (1:1, 1:2, 1:5, 1:10 MDSC:CD4, with the number of T cells remaining constant). After 72 hours, proliferation was measured by thymidine incorporation (section 0), and cytokine concentrations was quantified by ELISA.

2.2.15 EAE

2.2.15.1 Active induction of EAE

EAE was induced in C57BL/6 mice by s.c. injection with myelin oligodendrocyte glycoprotein$_{35-55}$, (MOG, 150 μg, GenScript) emulsified in 200 μl of complete Freund's adjuvant (CFA, Chondrex Inc.) supplemented with 4 mg/ml (400 μg/mouse) of heat killed H37 Ra *M. tuberculosis* (Chondrex Inc). Mice were injected i.p. with 500 ng of pertussis toxin (PT; Kaketsuken, Japan) on days 0 and 2.

2.2.15.2 Passive induction of EAE

A donor group of mice were immunized by s.c. injection of 150 μg/mouse of MOG emulsified in CFA (See section 2.2.15.1) without administration of PT. On day 10, spleens and inguinal, auxiliary, brachial, cervical and mesenteric LN of immunized mice were excised and cultured at $1x10^7$ cells/ml in RPMI in the presence of MOG (100 μg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml). After 72 hours, non-adherent cells were aspirated from the culture vessel, washed and resuspended in RPMI. $CD4^+$T cells ($1x10^7$), purified from the cell suspension (section 2.2.11.1), were resuspended in PBS and i.p. injected into naïve recipient mice.

2.2.15.3 Treatment and clinical evaluation of EAE

EAE was induced in mice by the active or passive method. Mice with EAE were treated by i.p. injection of ES every other day (Table 2.2) and/or with various neutralising antibodies

(Table 2.6). Animals were monitored daily for signs of clinical disease. Disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis and grade 5 – tetraparalysis/death. Animals were sacrificed if they reached a score of 4 or above.

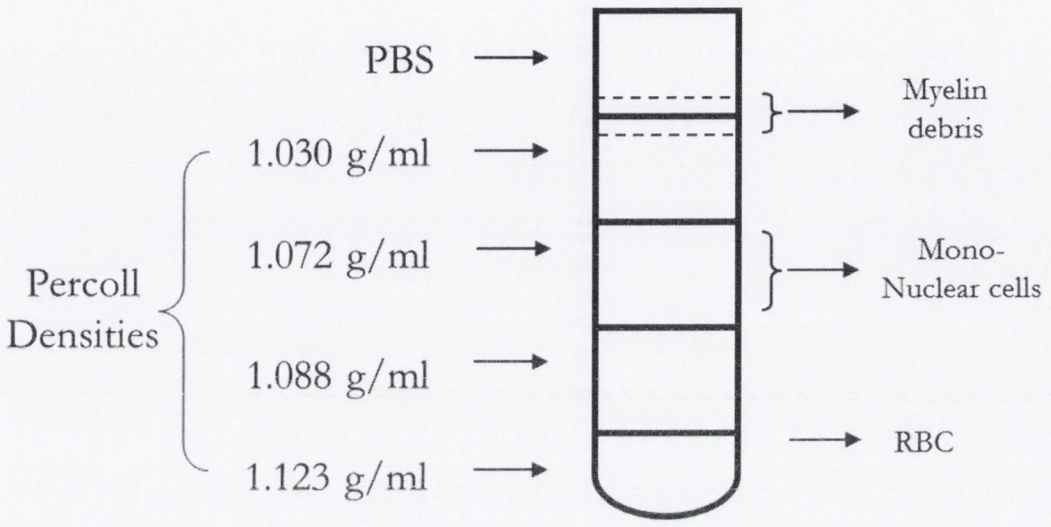


Figure 2.3 Percoll density layers
Percol densities were applied to a 50 ml tube as shown on the left with cells were suspended in the 1.088 g/ml layer. After centirfugation cells layered to the speccific regions shown on the right.

2.2.15.4 Intracranial perfusion of mice and mononuclear cell isolation from the CNS

Mice with EAE were terminally anaesthetised with sodium pentobarbital (Euthethal®, 40 µl). Skin over the chest was dissected to expose the rib cage, which was subsequently excised, to allow access to the heart. The left ventricle was pierced with a 23-guage needle attached to a syringe mounted in a syringe pump. A puncture was made in the right atrium, to allow flow-through, and mice were intracardially perfused with 20 ml of ice-cold PBS. The brains were then excised and placed in ice-cold HBSS containing 3% FCS. Brains were dissociated through a 100 µm cell strainer and cells were pelleted by centrifugation at 170 g for 10 min. Cell were resuspended in HBSS containing collagenase D (1 mg/ml) and DNase (10 µg/ml) and incubated at 37 °C with gentle agitation for 1 hour. Cells were pelleted as before and resuspended in 9 ml of 1.0088 g/ml Percoll (see section 2.1.13 for description of Percoll

densities) in a 50 ml tube. This was under layered with 5ml of 1.122 g/ml Percoll. The 1.088 g/ml Percoll layer was over-layered with 9ml of 1.072 g/ml Percoll; and this with 9 ml of 1.030 g/ml Percoll. Finally, 9 ml of PBS was layered on top. Percoll gradients were centrifuged at 1250 g for 45 min at RT with no ac/deceleration boost. Red blood cells layer to the 1.123:1.088 g/ml interface, myelin debris layers to 1.030 g/ml/PBS interface while mononuclear cells layer into the 1.072 g/ml Percoll layer. Mononuclear cells were removed from this layer and washed twice in HBSS. After washing cell counts were performed using the ethidium bromide/acridine orange method (see section 2.2.2.4).

2.2.16 Airway hypersensitivity

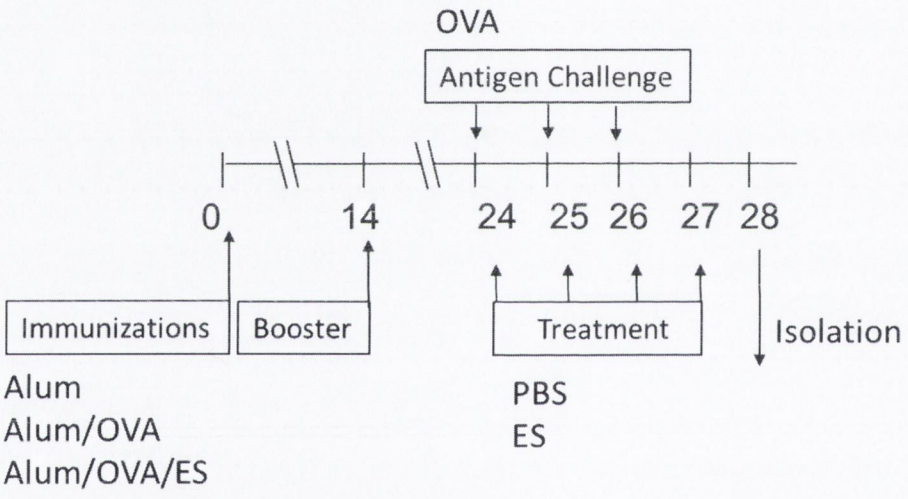


Figure 2.4 Induction of airway hypersensitivity
BALB/c mice were immunized by i.p. administration of either alum; alum with OVA; or alum, OVA and ES on day 0 and day 14. Mice were challenged for 30m with aerosolised OVA on days 24, 25 and 26. Mice were injected i.p. with PBS or ES on days 24, 25 and 26

2.2.16.1 Induction of airway hypersensitivity

BALB/c mice were sensitised by i.p. administration of OVA (50 μg, ovalbumin protein, Sigma-Aldrich) emulsified with alum (1 mg) and boosted 14 days later. On days 24-27, mice were challenged with OVA (6%, 30 minute exposure) aerosolised via a nebuliser. Mice were

killed 48 h after final airway challenge to assess airway inflammation (Figure 2.6). In some experiments mice were injected i.p. with PBS or ES (20 μl) on days 24-27 (Figure 2.4).

2.2.16.2 $CD4^+$ Transfer in airway hypersensitivity model.

Mice were sensitised as in section 2.2.16. On day 21 $CD4^+$T cells were isolated by magnetic separation from the spleens and inguinal, mediastinal, mesenteric and auxiliary LN of mice immunized with alum, OVA and ES. Purified $CD4^+$T ($1x10^7$) cells were i.p. transferred into mice previously immunized with alum and OVA. On days 24-27, mice were airway-challenged with OVA (6%, 30 minute exposure), aerosolised using a nebuliser. Mice were sacrificed 24 h after final airway challenge to assess airway inflammation.

2.2.16.3 Bronchial-alveolar lavage.

Mice were sacrificed by CO_2 asphyxiation to ensure preservation of the trachea. Skin was removed from the rib cage and cervical region and the tissue overlying the trachea was excised. A 23-gauge IV catheter (Angiocath™, BD) was used to pierce the upper trachea and the protruding needle was removed from the catheter after successful insertion. 1ml of ice-cooled PBS was injected into the lungs and aspirated immediately. Lungs were injected/aspirated a total of 3 times. BAL fluid was collected and placed on ice. Differential cell counts were quantified via cytospin or flow cytometric analysis.

2.2.16.4 Lung Histopathology

The left lung lobe of mice was excised and fixed in 10% formalin. Formalin-fixed lungs were embedded in paraffin and 5 μm sections were cut with a microtome. Sections were stained with either haematoxylin and eosin (H&E) or Alican-Blue-Periodic Acid Schiff (AB-PAS). H&E stained sections were analysed for morphological changes to the alveolar spaces, smooth muscle, and for peribroncular inflammatory cell infiltration. AB-PAS stained sections were analysed for luminal mucus, which appears pink and for mucin-positive goblet cells which is indicative of goblet-cell hyperplasia.

2.2.17 Enzyme-linked immunosorbent assay

2.2.17.1 Standard cytokine ELISA

The concentrations of murine IL-12p40, IL-12p70, IL-10, IL-6 and IL-1β, IL-10, IL-1β, IL-17, GM-CSF, IL-12p70, TNF-α, IL-25, IL-27, IL-13, IL-23, TSLP and TGF-β were quantified using pairs of antibodies (Table 2.7). High-binding 96-well microtiter plates (Greiner Bio-one) were coated over night at 4°C with 50µl/well of rat anti-mouse capture antibody in coating buffer according to manufacturer's instructions. After washing in PBS tween (section 2.1.6), non-specific binding sites were blocked by the addition of 150µl/well of

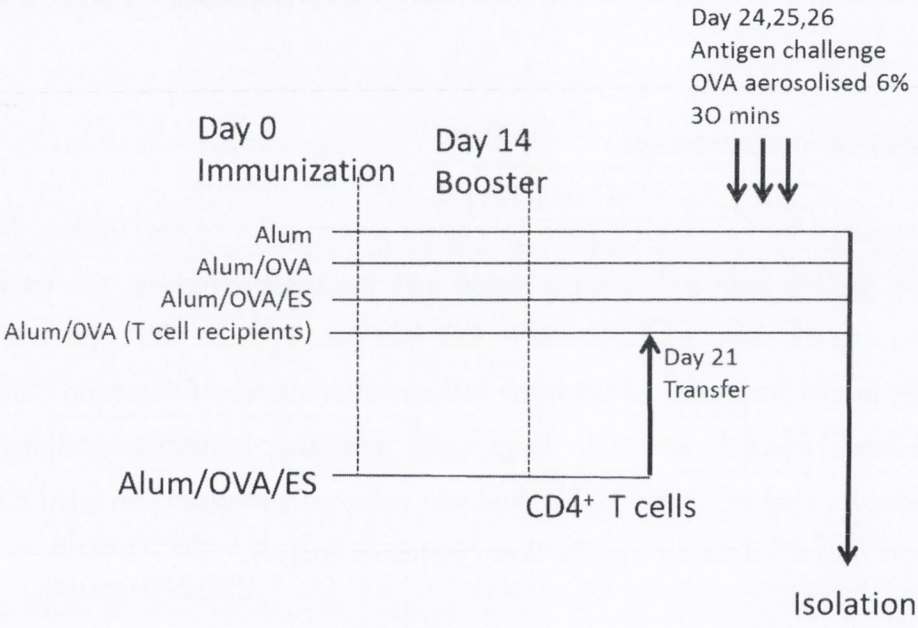


Figure 2.5 CD4$^+$ Transfer into mice with airway hypersensitivity
BALB/C mice were immunized by i.p. administration of either alum; alum with OVA; or alum, ovalbumin and ES on day 0 and day 14. On day 21 "OVA/alum recipient" mice received CD4+ cells isolated from mice which were immunizaed identically to "alum+OVA+ES" mice were challenged for 30m with aerosolised ovalbumin on days 24, 25 and 26.

blocking solution for 2 hours at room temperature. After blocking, plates were washed and 50µl/well of sample supernatant or serially diluted recombinant cytokine (0-1000 pg/ml) in assay diluent were added to plates and incubated overnight at 4°C. Samples to be assayed for the presence of latent TGF-β were heated using a PCR thermocycler to 80°C for 10 min. After washing, 50µl of biotinylated goat anti-mouse detection antibody was added per well and incubated for 2 hours at RT. Plates were washed again and incubated, in the dark for 20 min, with 50µl/well of horseradish-peroxidase (HRP)-conjugated streptavidin (BD Pharmingen 1:1000 in assay diluent) followed by washing and addition of 50µl/well of OPD substrate in phosphate citrate buffer (section 2.1.5, 0.4 mg/ml). ELISA plates were allowed to develop in the dark for 2-30 min after which, the enzyme reaction was stopped by the addition of 25µl/well of 1 M H_2SO_4. The OD values were determined by measuring absorbance at 492 nm using a microtiter plate reader and cytokine concentrations for supernatant samples were calculated using the standard curve prepared with recombinant mouse cytokine.

2.2.17.2 Antibody ELISA.

Antigen coating solution (50 µl/well, section 2.2.17.1) was added onto the wells of medium binding 96 well ELISA plates (Greiner Bio-One) and incubated at 4°C overnight. Plates were washed 4 times with wash buffer. Non-specific antibody interactions were blocked by incubating the plates for 2hrs at RT with 100 µl/well of 10% w/w Skimmed milk (Fluka) in PBS. After blocking, plates were washed 4 times with wash buffer and 50 µl of diluted (1/50) serum or neat BALF sample were added to each well and then incubated at 37°C for 24 hrs. Plates were washed 4 times and 50 µl/well of biotinylated antibody was added at the appropriate concentration (see Table 2.7) to each well and plates were then incubated at 37°C for 1hr. Following this, plates were washed 4 times and then incubated for 20 min at RT in the dark with 50 µl/well of Streptavidin Horseradish Peroxidase solution at 0.7 g/ml (Xtravidin, Sigma). Then, plates were washed 4 times and incubated with 50 µl/well of developing solution (section 2.1.5). The enzymatic reaction was stopped by the addition of 20 µl/well of 1 M H_2SO_4. Finally the absorbance was measured at 492 nm in a microplate reader.

2.2.18 FACS analysis

2.2.18.1 Cell activation prior to cytokine expression analysis

Cell suspensions of interest were added to a sterile FACS tube at 1 x 10^6 cells/100 µl/tube. Cells were activated with PMA (10 ng/ml) and Ionomycin (1 µg/ml) in the presence of Brefeldin A (BFA) (5 µg/ml) in RPMI supplemented with 2-ME (50 µM) and incubated at 37°C and 5% CO_2 for 5 hours. Alternatively, cells were incubated with BFA alone for 12 hours at 37°C. After incubation, cells were washed twice with 3 ml of FACS buffer and pelleted by centrifugation at 1200 rpm for 5 min. The cells were then resuspended in 50 µl of FACS buffer.

2.2.18.2 Discrimination of Live cells

Preparation, handling or stimulation of cells in culture can cause cell death. Dead cells or cell debris can interfere with flow cytometric analysis. Discrimination of live and dead cells was achieved by 2 methods. Firstly, propidium iodide (PI, Sigma-Aldrich), which is a fluorescent DNA dye that only binds dead cells as intact membranes prevent the ingress of the dye into live cells. 1 µl of PI (500 µg/ml) was added to a 100 µl cell suspension immediately prior to acquisition by a flow cytometer (section 2.2.18.5). PI has ex/em maxima of 488/645 nm and can be detected using filter bands for PE-Texas-RED or PE. Secondly: LIVE/DEAD® Fixable Aqua Dead Cell stain (Aqua-Live/dead, Molecular probes, Life technologies) is amine-reactive fluorescent dye that binds dead cells with a 50-fold affinity compared with live cells. Here, cells were incubated with a 1/1000 dilution in PBS of supplied stock of LIVE/DEAD for 15 min prior to or during surface-staining. Aqua-Live/dead has ex/em maxima of 375/512 nm and can be detected on a flow cytometer using a filter band for V500/Am-Cyan.

2.2.18.3 Surface marker staining

Single cell suspensions, prepared and if required, stained with Aqua-Live/Dead (section 2.2.18.2), were added to a FACS tube at <1 x 10^6 cells/100 µl/tube. 1 µl of anti-mouse CD16/CD32 at 2.5 g/ml (Fcγ Block stock solution, ebiosciences) was added to each tube, to prevent non-specific binding of antibodies to cells via the somatic Fc region to cellular Fcγ

receptors. Without washing, the cell suspension was then stained with the appropriate surface FACS antibodies or surface isotype-control (see Table 2.8) for 15 min at RT in the dark. In parallel, single stained compensation controls were prepared using FACs comp beads (BD). Excess staining antibody was removed by washing each tube twice in FACS Buffer, 3 ml/tube, with centrifugation at 1200 rpm for 5 min between each wash.

2.2.18.4 Intracellular-cytokine staining

Cells were stimulated and stained as described in sections 2.2.18.2, 2.2.18.1 and 2.2.18.3. In order to stain for intracellular epitopes, cells were permeabilised with a permeabilization kit (Caltag). Briefly, 100 µl of fixation buffer was added to each tube and incubated for 15 min at RT in the dark. The cells were washed twice in FACS Buffer, 3 ml/tube with centrifugation at 1200 rpm for 5 min between each wash. Cells were resuspended in 100 µl of permeabilisation buffer (saponin) containing the appropriate amount of fluorescent antibodies directed against intracellular molecules (Table 2.8). The cells were incubated for 15 min at RT in the dark. After incubation, cells were washed twice, first with saponin-containing FACS buffer and second with FACS buffer.

For detection of intra-nuclear epitopes, cells were prepared using the FOXP3 permeabilisation buffers (eBioscience). Cells were surface stained as described in section 2.2.18.3. After washing, cells were incubated in 100 µl of FOXP3 fixation/permeabilisation buffer for 30 m to 18 h. Cells were washed with FOXP3 permeabilisation buffer, and resuspended in 50 µl of permeabilisation buffer containing fluorescent-conjugated antibodies directed against intra-nuclear epitopes. After 15 m, the cells were washed with FACS buffer containing saponin.

2.2.18.5 FACS data acquisition

Stained cell suspensions were acquired using DAKO $CyAN_{ADP}$, BD CANTO-II or BD LSRFortessa flow cytometers according to the manufactures instructions. The flow cytometer was calibrated using single-stained suspensions of compensation beads (BD). Where possible, voltages were set such that all negative and positive populations appeared within a standard plot.

2.2.18.6 FACS data analysis

FACS data analysis was performed using FLoJo (Treestar) software. In order to accurately assess the composition of purified leukocyte subsets, all FACS analysis included the use of discriminatory gates and back-gating strategies: initial gating excluded cell debris and RBC by applying a FSc/SSc gate. Doublets were identified and excluded from analysis using pulse-width. Dead cells were excluded by use of fluorescent dyes as described in section 2.2.18.2. Primary cell gates were based on fluorescent marker expression with the positions of gates

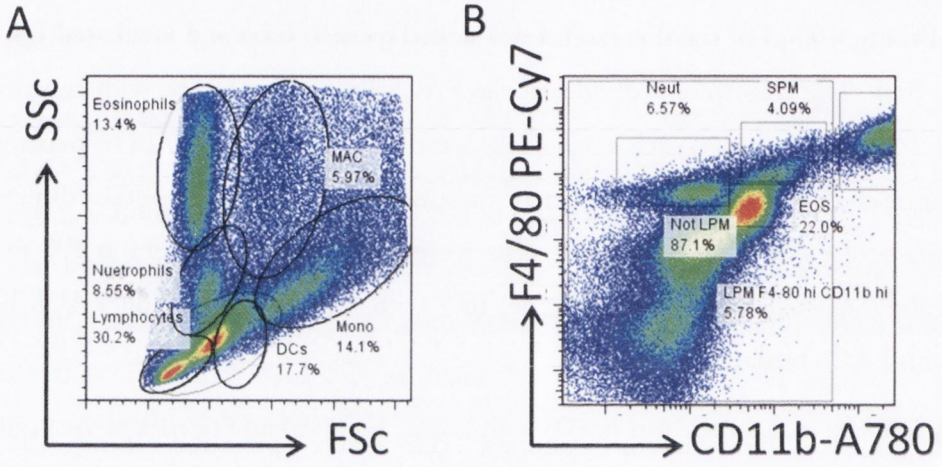


Figure 2.6 Flow cytometric analysis gating strategies.
PEC from naïve mice was analysed by flow cytometery for the expression of F4/80, Cd11b and for size (FSc) and granularity (SSc).A, SSc/FSc secondary analysis gates for common leukocytes. B, Gates enacted to remove autofluorescent macrophages

being determined by isotype controls or fluorescence minus one (FMO) controls. Secondary gates were assigned for each leukocyte type based on their unique FSc/SSc profile (Figure 2.6). Percentage expression and total cell numbers calculated thereof which are described in this study were based on secondary gates as percentage of total live single cells (Figure 2.6). Macrophage autofluorescence is a problem which can account for false positives in flow cytometric analysis. (275) Macrophages are autofluorescent between 475-625nm which precludes the use of FITC and PE as suitable fluorescent markers for the analysis of macrophage populations (275). To prevent autofluorescent macrophages creating false positives macrophages were gated out as depicted in Fig 2.2 B and analysed in isolation.

2.2.18.7 Multi-functional T cell cytokine analysis.

Cell suspensions were stimulated with PMA/ION in the presence of BFA and stained for CD3, CD4, IL-4, IL-5, IL-10, IL-13 IL-17 and IFN-γ as in sections 2.2.18.3 and 2.2.18.4. Cells were acquired and analysed as in sections 2.2.18.5 and 2.2.18.6. Cells staining positive for each parameter were determined by histogram gates and the percentage of cells producing each of the 64 possible combinations of IL-4, IL-5, IL-10, IL-13 IL-17 and IFN-γ was determined by sequential Boolean gates for each parameter. Bar-graph and pie chart representation of results were produced by analysis with SPICE version 5.1, downloaded from National Institute of Allergy and Infectious Diseases Exon Website. Bar charts display the mean response in each of the treatment groups (treatment group colour defined in the bar chart legend). For pie charts, polyfunctional T-cell populations are plotted according to the colours indicated on the bar chart, with the coloured arcs representing the secretion of each individual cytokine as detailed in the pie chard arc legend (276, 277).

2.2.19 Real Time Quantitative RT-PCR

Total ribonucleic acid (RNA) was extracted from PBMC/DC or brain tissue using the TRIzol/chloroform method. Briefly, cells/tissue was collected in TRIzol reagent (1ml/10^6 cells/10cm^2 Life Technologies) in sterile RNase-free tubes. 200 µl of chloroform was added to each tube, vortex mixed, stood at RT for 3 min and then centrifuged at 12,000 g for 15 min at 4° C. The resulting upper aqueous phase was removed to sterile RNase-free tubes, and 500 µl isopropanol added, mixed and stood at RT for 10 min, before centrifugation at 12,000 g for 10 min at 4° C. Supernatants were decanted and the pellet washed in 75% ethanol in nuclease-free water at 7,600g for 5 min at 4° C. Finally, remaining ethanol was removed with a pipette and the tubes were inverted and allowed to air dry for 20 min. RNA was resuspended in 40 µl nuclease-free water. RNA concentration was determined spectrophotometrically using a Nanodrop (Thermo Scientific) according to the manufacturer's instructions. 300 ng (PBMC/ DC studies) or 2 µg (brain tissue) of each RNA sample was reverse-transcribed into cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcriptase kit, according to the manufacturer's protocol, and the cDNA reverse transcriptase product was diluted 1:8 with nuclease free water. Transcripts were quantified by real time quantitative PCR on an ABI 7500 Fast Real Time PCR System with Applied Biosystems predesigned Taqman

Gene Expression Assays and reagents according to the manufacturer's instructions. For each sample, mRNA abundance was normalised to the amount of 18S ribosomal RNA (rRNA) and is expressed as fold difference compared to cells incubated in medium only or as fold difference compared to control mice.

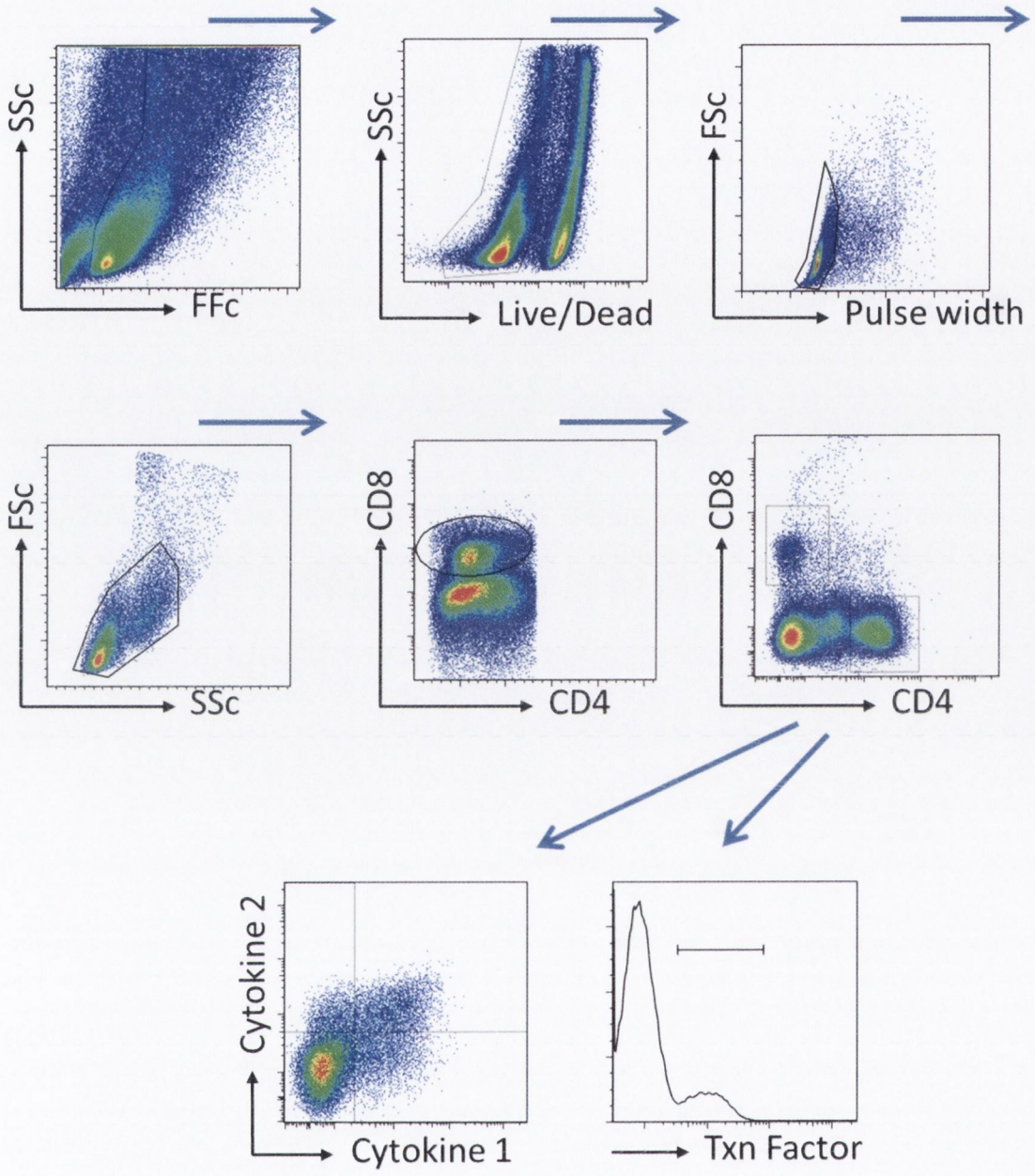


Figure 2.7 Flow cytometery gating strategy for $CD4^+$ T cells

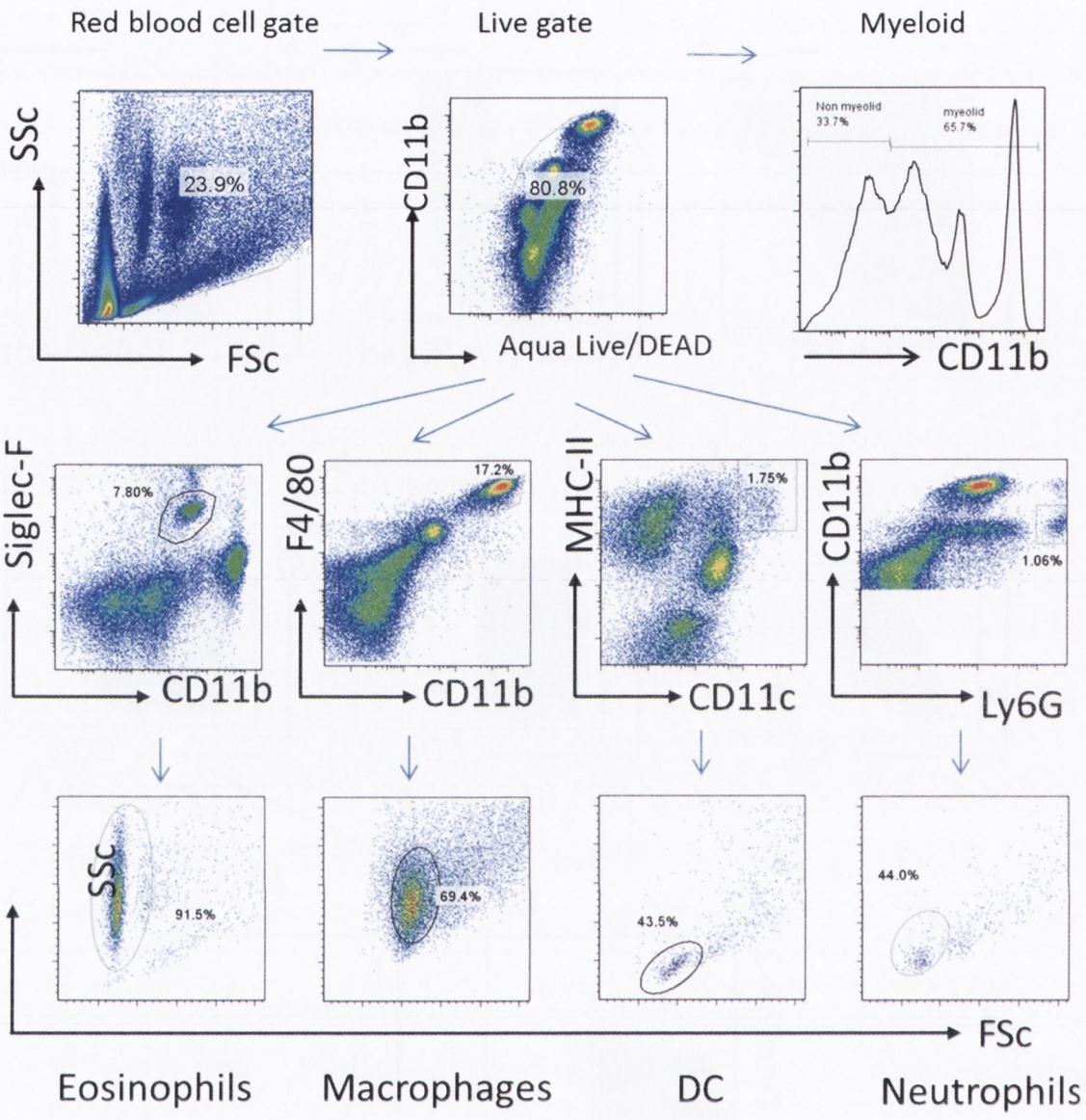


Figure 2.8 Flow cytometery gating strategy for innate leukocytes cells

Chapter 3 Infection with *Fasciola hepatica* induces immunoregulatory cells that attenuates autoimmunity

Chapter 3 Infection with *Fasciola hepatica* induces immunoregulatory cells that attenuates autoimmunity

3.1 Introduction

F. hepatica establishes a chronic infection in its natural ruminant hosts, such infections may last a number of decades, a reflection of the induction of host immunoregulation by the fluke. However, the murine model of *F. hepatica* infection represents a better model in which to investigate immune modulation by *F. hepatica,* because their immune systems are highly characterised, a wider availability of commercial reagents and a variety of well-established murine models of disease. However there is a major caveat in using mice to investigate infection with *F. hepatica:* Da Paula et al reported that infection of BALB/c mice with *F. hepatica* resulted in a mortality rate of 73% by day 35 post infection (278) and while adult flukes are found in infected mice, they fail to reach sexual maturity.

Nevertheless, a number of studies have investigated the immune response to *F. hepatica* in mice. Mice infected with *F. hepatica* mount a response to the fluke that is dependent on the number of metacercariae used to infect the animal. Lower numbers of metacercariae induce a mixed Th1/Th2 response, whereas higher numbers induce strongly polarised Th2 responses (279). Infection with *F. hepatica* has also been associated with induction of host immune suppression (251, 252). In mice, infection with *F. hepatica* inhibits the protective Th1 response to *B. pertussis* (254). However, the effect of *F. hepatica* infection on Th17 or Treg responses is unknown. This is of particular interest due to the growing body of evidence that several helminths spp can suppress the induction of autoimmune responses mediated by Th17 cells.

The major aim of this chapter was to characterise the effect of *F. hepatica* infection on the function/phenotype of regulatory immune cells and to investigate the role of *F. hepatica* in a model of autoimmunity. Specific aims include:

1) **To determine the effect of *F. hepatica* infection on the recruitment of cells to the peritoneal cavity**

The life-cycle of *F. hepatica* requires the parasite to translocate from the duodenum to the liver through the peritoneal cavity (280). This causes significant damage in the host and is associated with the production of inflammatory cytokines/chemokines in the peritoneal cavity (278, 281). Thus, experiments were conducted to determine if infection with *F. hepatica*

induced the recruitment of immune cells to the peritoneal cavity. This study also characterised the composition and cytokine production of immune cells isolated from the peritoneal cavity during infection.

2) **To determine the effect of *F. hepatica* infection on recruitment of suppressive cell populations**

Treg cells play an important role in immune regulation, protecting the host against autoimmunity but also from immunopathology during infection. Helminths spp such as *H. polygyrus* and *L. sigmodontis* have exploited this mechanism for their survival, acquiring the ability to increase the frequency of Treg cells in the host during infection, thus, enabling long-term parasite persistence through the suppression of protective Th2 responses (142, 156, 172).

Myeloid-derived suppressor cells (MDSC) are a heterogeneous group of immature myeloid cells that share many characteristic with M2 macrophages. The role these cells play in helminth infections is unclear, despite the existence of several reports which demonstrate that certain helminths spp, such as *S. mansoni* and *Taenia crassiceps,* recruit cells that resemble MDSC phenotypically and functionally (187, 282, 283). LNF-PIII and LNnT, two molecules from *S. mansoni* recruit MDSC/M2 cells that suppress the activation of T cells (282, 284, 285). Thus, an objective of this study was to determine whether infection affected the recruitment of Treg cells or MDSC to the peritoneal cavity and to characterise the phenotype of recruited cells

3) **To determine whether *F. hepatica* infection can modulate autoimmunity in mice.**

Several helminths have been shown to protect mice from experimental models of autoimmunity (166). Infection with *T. spiralis* and *S. mansoni* have both been shown to suppress the development of EAE (215, 216). However, the role of *F. hepatica* in a model of autoimmunity has not been addressed to date. Therefore, this study investigated whether infection with *F. hepatica* could alter the course of EAE or modulate the induction or function of pathogenic Th1 and Th17 cells, associated with the progression of autoimmune disease.

3.2 Results

3.2.1 Oral infection of mice with *Fasciola hepatica* results in the recruitment of macrophages, DC and T cells to the peritoneal cavity

In this study, mice were infected with *F. hepatica* by oral inoculation with 10 metacercariae; the rate of infection was consistently 80-90% positive, as confirmed by the presence of macroscopic changes in liver morphology, including fibrotic tracts caused by fluke migration and abdominal haemorrhaging in the peritoneal cavity. This is indicative of the damage caused to the host by parasitic worms during infection. The present study terminated infected mice at 21-days post infection as infection causes high mortality by week 4 post-infection (286).

Sibille et al, 2004 described that infection of rats with *F. hepatica* results in the recruitment of cells to the peritoneal cavity (287), however the types of cells recruited and the kinetics of their infiltration have not been well described. In order to assess the effect of *F. hepatica* infection on immune cell recruitment, peritoneal exudate cells (PEC) were isolated by peritoneal lavage from C57BL/6 mice 7, 14 and 21 days post-infection (p.i.) infection with *F. hepatica* and compared with PEC from uninfected controls. While there was no change in number of PEC on day 7 p.i. compared with uninfected controls, on day 14 and 21 there was a significant increase in cell numbers, with a 10 fold increase by day 21 [Fig 3.1 A].

Inspection of H&E stained cytospin preparations revealed an infiltration of polymorphonuclear cells as well as a reduction in the relative number of lymphocytes in PEC from infected mice compared with uninfected mice. In addition, macrophages from infected mice also appeared to have more granular cytoplasm [Fig 3.1 B]. Macrophages and DC play a major role during infections both as effector cells and in their capacity as APC. DC in particular have been shown to be essential for the initiation of protective immunity to certain parasites (288, 289).

Since infection is associated with significant damage as well as recruitment of cells to the peritoneum [Fig 3.1], it is likely that the inflammatory infiltrate would contain DC, macrophages and T cells. To investigate this, PEC of infected and uninfected animals were

analysed by flow cytometry for the expression of CD11c, F4/80 and CD4. The proportion as well total number of macrophages, DC and T cells increased in the peritoneum during the course of infection [Fig 3.2 A]. While the relative numbers of macrophages in the PEC initially declined on day 7, from 6.3% of total cells in uninfected controls to 1.4% in infected mice, the numbers of macrophage increased to 16.4% and 13.6% on days 14 and 21 p.i., respectively [Fig 3.2 B]. The total number of macrophages increased during the later stages of infection, from $0.2x10^6$ in uninfected animals to $0.1x10^6$, $1.8x10^6$ and $2.5x10^6$ on days 7, 14 and 21, respectively. The total number of DC was comparable in the PEC of infected and uninfected mice on day 7 p.i. but increased thereafter from $0.1x10^6$ cells in uninfected mice to $4x10^6$ cells in day 14 infected mice and remained at this level until at least day 21. There was an eventual 10-fold increase in the number of DC and macrophages by day 21 in the peritoneal cavity of infected compared with uninfected mice [Fig 3.2 C, D]. While the percentage of $CD4^+$ T cells peaked at day 7 p.i (14%), and declined thereafter, the total number of T cells increased dramatically from $0.3x10^6$ in uninfected mice to a peak of $4x10^6$ cells on day 14 and a subsequent decline to $2.5x10^6$ by day 21 [Fig 3.2 C, D]. These results demonstrate the influx of both innate and adaptive immune cells into the peritoneum following infection with *F. hepatica,* which is suggestive of an on-going immune response.

3.2.2 *F. hepatica* infection increases the concentration of IL-10, IL-27 and TGF-β in the peritoneal cavity

During the acute stage of infection with *F. hepatica,* the host mounts a Th2 response, characterised by IL-4 production (248-250) (251). Flynn et al, 2008, demonstrated that 10 days after experimental infection of cows, bovine PBMC produce IL-4 but not IFN-γ in response to a mixture of parasite antigens, liver fluke-homogenate (LFH) (252). However during the chronic stage of infection (week 12) PBMCs predominately produce IL-10 and TGF-β in response to LFH (252). Furthermore, it has been reported that C57BL/6 mice mount a mixed Th1/Th2 response during *F. hepatica* infection (279).

This study has demonstrated that *F. hepatica* infection is associated with an inflammatory cell infiltration of the peritoneal cavity, including the recruitment of DC, macrophages and T cells [Fig 3.1-2]. Since infection has been associated with mixed T cell responses, experiments were conducted to determine the effect of infection on the expression of cytokines and

chemokines associated with Th1/Th2/Th7/Treg cells. The concentration of IL-23, IL-10, IFN-γ, monocyte/DC chemokine monocyte chemoattractant protein-1 (MCP-1), IL-27, IL-4, granulocyte-macrophage colony stimulating factor (GMCSF), TGF-β and IL-17 in peritoneal exudate fluid (PEF) of uninfected and *F. hepatica* infected (day 14/21 p.i) mice was quantified by ELISA. On days 14 and 21 p.i. the concentration of the MCP-1 was enhanced in PEF of infected compared with uninfected mice [Fig 3.3]. In contrast, the expression of GM-CSF remained unchanged throughout the course of disease [Fig 3.3]. Neither IFN-γ nor IL-17 were detected at significant levels [Fig 3.3]. The concentration of IL-4 was significantly enhanced on day 14 p.i. but returned to control levels by day 21 [Fig 3.3]. The concentrations of the anti-inflammatory cytokines IL-10 and IL-27 were enhanced on day 21 p.i., but this did not reach statistical significance. However, in contrast, the concentration of the other major anti-inflammatory cytokine, TGF-β, was significantly enhanced on both day 14 and 21 p.i in PEF from infected compared with uninfected mice [Fig 3.3]. These results demonstrate that that *F. hepatica* infection induces the local production of type-2/anti-inflammatory cytokines.

3.2.3 Innate immune cells from *F. hepatica* infected mice express IL-10, TGF-β, and total PEC express genes associated alternative activation of macrophages

This study has shown that infection of mice with *F. hepatica* results in recruitment of macrophages and DC to the peritoneal cavity and enhanced concentrations of IL-10 and TGF-β in the peritoneal fluid [Fig 3.3]. Thus, experiments were designed to determine whether DC or macrophages were a source of IL-10 and TGF-β during *F. hepatica* infection. TGF-β is transcribed as pro-form and becomes biologically active only after the proteolytic cleavage of the molecule into its secreted C-terminal mature region and an N-terminal region, LAP which remains on the cell surface after secretion, thus surface bound TGF-β and LAP are commonly used as markers of TGF-β secretion (290).

The expression of IL-10, TGF-β, latency-associated peptide (LAP) and IFN-γ in macrophages and DC (as defined in Fig 3.2) in the PEC of infected and uninfected mice was quantified by intracellular cytokine staining and analysed by flow cytometry, following a 10-hour incubation with BFA. The expression of IL-10 was enhanced in macrophages and DC from *F. hepatica* infected compared with uninfected mice [Fig 3.4, day 21]. Intracellular expression of TGF-β was enhanced in macrophages and surface expression of LAP was

enhanced by both DC and macrophages from infected compared with uninfected mice [Fig 3.4]. These data suggest that DC and macrophages are a source of anti-inflammatory cytokines produced in the peritoneal cavity during infection with *F. hepatica.*

Macrophages exposed to IL-4 or IL-13 become alternatively activated (291, 292). M2 macrophages, are associated with type-2 immune responses and are strongly-induced during helminth infections, including *F. hepatica* (255, 267, 291, 293). M2 macrophages are anti-inflammatory (156, 159); express Fizz1, a secreted anti-inflammatory molecule (294); CD115, the MCSF receptor expressed on monocytes; TGF-β; IL-4 and IL-13 (187). M2 macrophages enhance expression of arg1, the gene coding for arginase and supress the expression of the inducible form of nitric-oxide synthase (iNOS), the reciprocal state to classically activated macrophages (291). Arginase-mediated depletion of L-arginine suppresses T cell function via amino-acid starvation. (188, 189). In order to determine if infection of mice with *F. hepatica* enhances the expression of genes associated with M2 macrophages, PEC were isolated from the peritoneal cavity of infected C57BL/6 mice on day 21 p.i. and the relative gene expression of Arg1, FIZZ1, CSF-R, IL-4, TGF-β, IL-10, IL-27, IFN-γ and IL-17 was analysed by RT-PCR. The mRNA expression of Arg1 (48,000 fold induction; F/I), FIZZ1 (390 F/I), CSF-R (80 F/I), IL-4 (32 F/I), TGF-β (3.8 F/I), IL-10 (6 F/I), IL-27 (13.5 F/I), IFN-γ (15 F/I) and IL-17 (9 F/I) were all enhanced in the PEC from infected compared with uninfected mice [Fig 3.5]. In contrast, the expression of iNOS mRNA was suppressed in PEC from infected compared with uninfected PEC. The increase in expression of Arg1 suggested a corresponding increase in arginase protein in the cytoplasm and thus arginase activity. To test this, cellular lysate of PEC from infected and uninfected mice was assayed for the ability to metabolise L-arginine to urea. The arginase-specific activity in lysate of 10^6 PEC increased from 1 U in uninfected mice to 39 U in infected mice [Fig 3.5]. Taken together, these results demonstrate that *F. hepatica* infection of mice is associated with the recruitment and alternative activation of macrophages.

3.2.4 Infection induces eosinophilia in the peritoneal cavity which is exacerbated in IL-10$^{-/-}$ mice

This study has shown that infection leads to the increase in the number of immune cells in the peritoneal cavity and morphologically, a large proportion of cells were from the

granulocyte lineage. Eosinophils and neutrophils are the two most common granulocytes. Neutrophils are thought to be the first cell type to respond to a worm infection, and are essential for protective immunity to certain helminths, such as *H. polygyrus* (156) Eosinophilia however remains synonymous with helminth infections and is the most common diagnostic feature of such infections in humans (9). Moreover, the recruitment of eosinophils has been previously described in bovine fasciolosis (295). To investigate the infiltration of eosinophils and neutrophils during *F. hepatica* infection, the expression of Gr-1, F4/80 and Siglec-F, which are associated with neutrophils, macrophages and eosinophils respectively, was determined in PEC of mice infected with *F. hepatica* and compared with PEC of uninfected mice (296). Consistent with previous data [Fig 3.3], there was an increase in percentage of macrophages infiltrating the peritoneal cavity. Furthermore, infection enhanced the percentage of Gr-1^{hi} (neutrophils) and Gr-1^{int} cells (mostly monocytes and macrophages) from 2.1% to 13.8% and from 10% to 22.6% respectively. The percentage of Siglec F^+ eosinophils in the PEC increased from 2.9% of total cells in uninfected mice to 31.8% in infected mice [Fig 3.6].

IL-10 has a role in immune regulation and has been shown to supress eosinophil recruitment during an allergic response (297). Peritoneal macrophages and DC from infected mice expressed more intracellular IL-10 than cells from uninfected mice [Fig 3.4]. To examine the role of IL-10 on the recruitment of innate cells during *F. hepatica* infection, PEC from IL-$10^{-/-}$ mice (on a C57BL/6 background) were analysed by flow cytometry and compared with PEC from WT C57BL/6 mice. The numbers of cells infiltrating the peritoneal cavity was significantly higher in infected IL-$10^{-/-}$ ($3.2x10^8$) compared with WT mice ($1.1x10^7$). The composition of the cellular infiltrate was also different; Siglec-F^+ eosinophils made up 80% of total PEC in infected IL-$10^{-/-}$ mice compared with 33% in WT mice. Although the percentage of macrophages declined in IL-$10^{-/-}$ with infection (due to the proportional increase in eosinophils) the total numbers of macrophages was higher in infected ($19.5x10^6$) compared with uninfected ($2x10^6$) IL-$10^{-/-}$ mice. The percentage of Gr-1^{int} cells in IL-$10^{-/-}$ did not change with infection while Gr-1^{hi} neutrophils increased from 1.7% to 5.3%, a smaller increase than that observed in WT mice [Fig 3.6]. These results suggest that eosinophils play a major role in the immune response to *F. hepatica* and that IL-10 is involved in limiting eosinophil recruitment.

3.2.5 Defective APC function of peritoneal cells from infected mice

This study has shown that infection of mice with *F. hepatica* recruited macrophages and DCs that produced IL-10 and TGF-β into the peritoneal cavity [Fig 3.2-4]. In addition, studies from this laboratory have shown that the expression of CD80, CD40 and MHC-II on DC is reduced during *F. hepatica* infection (298). Taken together, these results indicate that APC in the peritoneal cavity during *F hepatica* infection may be functionally impaired. To test this hypothesis, PEC cells, isolated from both *F. hepatica* infected (day 21 p.i.) and uninfected BALB/c mice, were used as APC for $CD4^+$ T cells purified from DO.10.11 mice (which transgenically express a TCR specific for OVA peptide). OVA specific $CD4^+$T cells produced IL-17, IFN-γ and IL-10 when co-cultured *in vitro* with PEC from uninfected animals [Fig 3.7]. In contrast, OVA-specific IL-17 and IL-10 were completely abrogated and there was a significant reduction in IFN-γ production when PEC from infected animals were used as APC [Fig 3.7]. These results suggest that either APC in the peritoneal cavity of *F. hepatica* infected mice are defective at priming T cells, or that other cells in the PEC from infected mice suppress the function of the APC.

3.2.6 *F. hepatica* infection results in the recruitment of IL-10^+ TGF-β^+ $Foxp3^+$ CD4 T cells to the peritoneal cavity.

DC with relatively low surface expression of co-stimulatory markers preferentially induce the expansion of Treg cells (299). TGF-β is required for the differentiation of iTreg cells, acting on naive T cells to induce the expression of FOXP3 (88). Since the expression of various co-stimulatory molecules by DC is suppressed during *F. hepatica* infection (298), and having shown that macrophages and DCs produced TGF-β during infection, the hypothesis was generated that DC may be capable of expanding Treg cells during *F. hepatica* infection. To test this hypothesis, the expression of FOXP3 by $CD4^+$T cells from PEC of infected (on days 7, 14 and 21 p.i) and control mice was quantified by flow cytometry. The expression of FOXP3 in $CD4^+$T mice was significantly enhanced from 7.5% in uninfected controls to 18.5% on day 21 after infection; FOXP3 expression was unchanged on day 7 or 14 p.i. [Fig 3.8].

The Treg subsets, Tr1 and Th3, have been shown to secrete the anti-inflammatory cytokines IL-10 and TGF-β respectively. In order to determine if these subsets were present during infection with *F. hepatica*, $CD4^+$ T cells from the peritoneal cavity of infected and uninfected mice were analysed by FACS for their intracellular expression of FOXP3, IL-10 and LAP. Moreover, $CD4^+$ T cells were analysed for intracellular expression of IFN-γ and IL-4 as markers of Th1 and Th2 cells, respectively. While the expression of IL-10, TGF-β, IFN-γ or IL-4 by $CD4^+$ was unchanged on days 7 and 14, the expression of IL-10 and LAP was significantly enhanced in $CD4^+$ T cells from PEC by day 21 p.i. with *F. hepatica* [Fig 3.9]. In addition, the majority of $IL\text{-}10^+$ and LAP^+ T cells co-expressed FOXP3 [Fig 3.9]. The expression of IL-10 or LAP was not increased in $FOXP3^-$ T cells [Fig 3.9]. Co-staining analysis revealed that LAP and TGF-β were simultaneously expressed in $FOXP3^+$ T cells; below 1% in uninfected controls compared with to 6.3% and 7.3 respectively on day 21 p.i. These data suggest that *F. hepatica* infection actively recruits a Treg cell which secretes anti-inflammatory cytokines. *F. hepatica* infection recruits $CD11b^{int}$ $Gr\text{-}1^+$ cells to the peritoneal cavity.

MDSCs are a heterogeneous population of immature myeloid cells containing macrophages, granulocytes, immature DC and early myeloid precursors that are associated with infections and malignancies; they are functionally described by the ability to suppress T cell responses (300). MDSCs are thought to be induced by infections that are typically cleared by Th1 or Th17 responses as a strategy to evade elimination by the immune system. They were initially phenotypically characterised by the intermediate expression of CD11b and Gr-1 (300). Gr-1 is the antigen for the antibody RB68-C5, which has been shown to bind two separate molecules, Ly6G and with lower affinity, Ly6C (301). The differential expression of these markers has been used to subdivide MDSCs into two groups, the monocyte-like suppressor cells ($Gr\text{-}1^+$ $Ly6C^+$) and neutrophil-like suppressor cells ($Gr\text{-}1^+$ $Ly6G^+$) (300). MDSCs have been described in helminth infections, including *S, mansoni* and *T. crassiceps* (187, 282-285). In these infections macrophages express M2 markers such as Arg1, MR, Fizz1 and Ym1 but they also express Gr-1. These $CD11b^+$ $Gr\text{-}1^+$ cells are phenotypically and functionally related to MDSC. This suggests that macrophages considered as MDSC, due to the expression of CD11b and Gr-1 could perhaps be reclassified as M2 and *vice versa* (187, 282, 283). The helminth glycan lacto-*N*-fucopentaose III (LNF-III, also called $Lewis^x$ antigen) and the

related glycan structure lacto-*N*-neotetraose (LNnT) derived from *S. mansoni* has been shown to drive the generation of M2/MDSC (282).

This study has already shown that infection of mice with *F. hepatica* leads to the recruitment of Gr-1$^+$ cells [Fig 3.5]. To determine if these cells express markers associated with MDSC, PEC isolated from infected mice (day 21 p.i.) were analysed by flow cytometry for the expression of CD11b, PD-1, TGF-β, Ly6G and Ly6C. Infection with *F. hepatica* induced recruitment of cells into the peritoneal cavity that expressed Gr-1 and had intermediate expression of CD11b [Fig 3.11 D]. In addition, CD11b^{int} Ly6C$^+$ and CD11b^{int} Ly6G$^+$ cells infiltrated the peritoneal cavity and expanded from 3.2% and 0.2%, respectively in uninfected to 18.7% and 16.1% in infected mice [Fig 3.11 A]. PD-1, an inhibitory T cell receptor, has been linked to the suppressive activity of MDSC (302). The expression of PD-1 was enhanced on CD11b^{int} cells, from 20% in uninfected animals to 63% in infected mice [Fig 3.11 B]. The expression of intracellular TGF-β was also enhanced in CD11b^{int} cells from infected mice compared with CD11b^{int} cells from uninfected control mice [Fig 3.11 C]. The expression of IL-10, however, did not increase [Fig 3.11]. Finally, in contrast to WT mice, *F. hepatica* infection did not lead to the recruitment of F4/80$^+$ Gr-1$^+$ in IL-10 $^{-/-}$ mice [Fig 3.12].

3.2.7 CD11b^{int} Gr-1$^+$ MDSC isolated from infected mice suppress in vitro CD4$^+$ T cell proliferation.

Some studies have shown that the suppressive activity of MDSCs is dependent on IFN-γ-induced NO production, while other studies have demonstrated that suppression is rather mediated through arginase-dependent L-arginine depletion (187, 300, 303, 304). Having established that infection with *F. hepatica* is associated with recruitment of cells that were phenotypically identified as MDSC [Fig 3.13], experiments were conducted to determine if these cells could regulate Th cell responses *ex vivo*. CD11b^{int} Gr-1$^+$ cells were purified by FACS from the PEC of C57BL/6 mice infected with *F. hepatica* (day 21 p.i.). These cells were co-cultured with CD4$^+$ T cells purified from the spleens of naïve mice in the presence or absence of plate-bound αCD3 and αCD28. Co-culture with MDSCs suppressed the proliferation of T cells at CD4:MDSC a ratio of 1:1. In addition, MDSC significantly enhanced the concentrations of NO, IL-10 and IL-27 produced in response to αCD3/CD28 stimulation [Fig 3.13] Conversely, MDSC enhanced proliferation of T cells at lower ratios

(1:0.1 CD4:MDSC) [Fig 3.13]. These data confirm that cells that phenotypically resemble MDSCs, recruited during *F. hepatica* infection, can regulate T cell function *in vitro*.

3.2.8 Monocytic and granulocytic MDSC isolated from the peritoneal cavity show differential ability to suppress T cell proliferation.

MDSC have been classified into two groups, monocytic and granulocytic MDSC (305). To investigate whether different subsets of MDSC exert similar suppressive effects, $Ly6G^+$ "granulocytic" and $Ly6G^-$ $Gr\text{-}1^{dim}$ "monocytic" populations were purified, using a commercially available magnetic sorting kit (306), from PEC and non-parenchymal hepatic mononuclear cells of mice infected with *F. hepatica*. While $Ly6G^+$ cells displayed polymorphonuclear morphology, $Ly6G^-$ $Gr\text{-}1^{dim}$ cells had macrophage/monocyte morphology as indicated by inspection of H&E stained cytospin preparations of the purified populations [Fig 3.14 A].

The frequency of MDSC expressing TGF-β, quantified by flow cytometry, was high in both populations; 8% of peritoneal and 31.3% of hepatic $Ly6G^-$ $Gr\text{-}1^{dim}$ cells expressed TGF-β and 3.5% of peritoneal and 3.4% of hepatic $Ly6G^+$ expressed TGF-β [Fig 3.14 B]. To further characterise these cells, the expression of CD11b, Gr-1, Ly6C, Siglec-F, CD115 and LAP on total uninfected and infected PEC as well as on the $Ly6G^+$ and $Ly6G^-$ $Gr\text{-}1^{dim}$ populations purified from the PEC of infected animals was analysed by flow cytometry. The expression of Ly6C was greatest on $Ly6G^-$ $Gr\text{-}1^{dim}$ cell populations, but both purified and total PEC from infected mice also expressed this marker [Fig 3.15]. Up to 15 % of PEC cells from infected animals expressed Siglec-F, indicating a large eosinophilic infiltrate. The proportion of $CD11b^{hi}$ cells was low in both sorted populations of PEC cells indicating that there were few contaminating mature macrophages [Fig 3.15]. CD115, the CSF-1 receptor is known to be expressed by MDSC but also inflammatory monocytes (307). This study has shown that $CD11b^+$ cells from naive mice have very low expression of CSF-R, but $CD11b^+$ cells isolated from the PEC of infected mice highly expressed CD115. $Ly6G^+$ cells also highly expressed CD115. The $Ly6G^-$ $Gr\text{-}1^{dim}$ population had diminished expression of CD115, although this may be explained by the lower total $CD11b^{hi}$ cells found in this population. The expression of LAP was enhanced on $Ly6C^+$, $Gr\text{-}1^+$ and $Siglec\text{-}F^+$ cells isolated from infected mice compared with uninfected control mice, with expression of LAP greatest on eosinophils [Fig 3.15].

Finally, to test the suppressive capacity of these populations, each of the four populations were co-cultured at increasing ratios with $CD4^+$T cells (1:02, 1:0.5 and 1:1 CD4:MDSC) that had been purified from naive mice and activated with αCD3/αCD28. $Ly6G^-$ $Gr\text{-}1^{dim}$ cells from the peritoneal cavity of *F. hepatica* infected mice significantly inhibited the proliferation of T cells at a high (1:1/1:0.5) but not at low ratios of CD4: MDSC (1:0.2). In contrast, $Ly6G^+$ cells from the peritoneal cavity did not suppress, but instead enhanced the proliferation of T cells. Conversely, MDSC populations isolated from the liver behaved in the opposite manner, where the $Ly6G^+$population significantly inhibited T cell proliferation, the $Ly6G^-$ $Gr\text{-}1^{dim}$ population enhanced proliferation [Fig 3.14]. These results suggest that infection of mice with *F. hepatica* is associated with the recruitment of functionally heterogeneous MDSC populations.

3.2.9 Infection delays the onset and clinical severity of EAE

Infection of mice with *F. hepatica* promotes the induction of IL-10/TGF-β-producing $FOXP3^+$ CD4 T cells [Fig 3.8-9]. In mice, helminth infections have been associated with protection against a number of experimental autoimmune disorders (212, 214) and in some cases helminth-induced expansion of Treg cells has been shown to play a role in suppression of Th1 and Th17 cells (213).

To examine the effect of *F. hepatica* infection on a model of autoimmunity, EAE was induced in C57BL/6 mice 1 day after infection with *F. hepatica*. Infection of mice with *F. hepatica* significantly delayed the onset and attenuated the clinical severity of EAE, compared with uninfected mice [Fig 3.16]. EAE is a T cell-mediated disease; myelin-specific Th1 and Th17 cells have both been implicated in the pathogenesis of EAE (116). To determine if infection with *F. hepatica* could modulate the induction of antigen-specific Th1or Th17 cells, spleen cells from uninfected and infected mice with EAE were restimulated *ex vivo* with the auto-antigen MOG. MOG-specific IL-17 and IFN-γ production was significantly reduced in spleen cells from infected mice compared with uninfected mice. There was a corresponding significant increase in MOG-specific IL-10, and MOG-nonspecific TGF-β production [Fig 3.16]. These results demonstrate that infection with *F. hepatica* attenuates autoimmunity and shifts the autoantigen-specific T cell response away from the pathogenic Th1/17 response towards an immunomodulatory phenotype.

3.2.10 Immunoregulation induced by *F. hepatica* is strain-specific.

The present study has demonstrated that infection with *F. hepatica* is associated with the recruitment of innate and adaptive cells with an anti-inflammatory phenotype [Fig 3.1-10]. In addition, infection of mice attenuates the clinical symptoms of autoimmunity, in a mouse model [Fig 3.11].

The metacercariae used in this study were obtained from researchers who maintain the snail colonies required to produce them. The intermediate host, freshwater aquatic snails of the family Lymnaeidae are infected with the eggs of *F. hepatica*, that were collected from the gall-bladder's of infected ruminants or from incubation of adult fluke isolated from these animals (308, 309). In order to ensure reproducibility, specific strains are maintained under laboratory conditions by serial passage in laboratory colonies of *Galba truncatula* (309). For reasons of availability, it was necessary to change the strain of *F. hepatica* during this study. The strain of metacercariae used thus far in this study is known as the Cullompton isolate; it originated from eggs collected from sheep slaughtered at the Cullompton abattoir, Devon in 1998 by Gordon Graham, Imperial College, UK.

Two separate strains, Shrewsbury and South Gloucestershire were purchased from Paula Martin at the Ridgeway Research Institute in Gloucestershire and they were investigated to determine if they displayed similar immunoregulatory effects to the Cullompton strain. As such, EAE was induced in mice one day after infection with either the Shrewsbury or South Gloucestershire stains of *F. hepatica* and compared to uninfected controls. Unlike the Cullompton strain neither of these stains protected mice from EAE [Fig 3.17 A]. In addition, MOG-specific proliferation and IFN-γ/IL-17 production was unchanged compared with uninfected controls [Fig. 3.17 B, C]. While there was no change in MOG specific IL-4, IL-10 or TGF-β production [Fig 3.15] there was a large increase in Liver fluke homogenate (LFH)-specific IL-10 and IL-4. Interestingly there was no increase in the production of TGF-β [Fig 3.17].

Having demonstrated that *F. hepatica* Shrewsbury and South Gloucestershire strains failed to attenuate EAE, experiments were conducted to determine if either could induce the expansion of Treg cells. The expression of FOXP3 by peritoneal $CD4^+$T cells from *F. hepatica* infected mice on days 7, 14 and 21 p.i was quantified by flow cytometry. Unlike the

Cullompton isolate, neither Shrewsbury nor South Gloucestershire strains of *F. hepatica* enhanced the expression of FOXP3 in peritoneal $CD4^+$ T cells compared with uninfected controls. In fact, expression of FOXP3 was significantly reduced in mice infected with these strains compared with uninfected controls (mean of 1.3% and 4.2%, for Shrewsbury and South Gloucestershire, respectively, compared with 7.8% in uninfected mice) [Fig 3.18]. These results demonstrate that different isolates of *F. hepatica* have fundamental differences in their ability to induce immunoregulation in the murine host. These findings provide evidence that Treg cells induced by the infection with the Cullompton strain of *F. hepatica* may play a role in bystander suppression of auto-antigen-specific T cell responses that mediate autoimmunity.

3.3 Discussion

There is a growing body of experimental evidence to suggest that infection of humans with helminth parasites can actively protect the host from autoimmune diseases. The present study found that infection of mice with one strain of *F. hepatica* protected mice from EAE, a model of autoimmunity mediated by autoantigen-specific Th1 and Th17 cells. These findings provide new evidence to support the hygiene hypothesis and suggest that regulatory mechanisms induced by parasite infection can attenuate pathogenic Th17-mediated autoimmune diseases. This study also demonstrated that *F. hepatica* infection recruits cells with immunosuppressive phenotypes and functions. Infection of mice with *F. hepatica* recruited $CD4^+FOXP3^+IL\text{-}10^+LAP/TGF^+$ cells to the peritoneal cavity. It also enhanced the expression of IL-10 and TGF-β in peritoneal macrophages and DC. Furthermore, it blocked the APC function of PEC and recruited a population of $CD11b^+Gr\text{-}1^+$MDSC that suppressed proliferation of $CD4^+$ T cells *in vitro*. This study also found significant differences between distinct isolates of *F. hepatica;* protection against EAE and expansion of Treg cells was restricted to only one of three isolates tested, demonstrating the importance of genetic diversity in helminth immunosuppression.

Previous studies have demonstrated a role for helminths in protection against EAE. Infections with both *H. polygyrus* and *T. spiralis* have been shown to attenuate the course of the disease (215, 310). In contrast, infection with *Strongyloides venezuelensis* did not alter the progression of disease (311). The present study is the first and only report to demonstrate that infection of mice with *F. hepatica* protects mice from EAE, indeed it is the first report to investigate the effect of this parasite on any model of autoimmunity (298).

The present study showed that a significant proportion of mice infected with *F. hepatica* were completely protected from the paralytic symptoms of EAE; although in practice, the level protection varied somewhat, with mice in the same experimental groups, or between different experiments displaying a range of severity of symptoms. This variation may be due to the number of juvenile flukes that succeeded in reaching the liver and thus was dependent on both the viability of the metacercariae and the quality of the experimental inoculation. This hypothesis is consistent with a previous study which showed that the nature of T cell response elicited by infection with *F. hepatica* was dependent on the number of metacercariae

used to inoculate mice (279). O'Neill et al demonstrated that C57BL/6 mice developed a mixed Th1/Th2 response when inoculated with a low dose of metacercariae, while infection with a higher number of metacercariae resulted in a more pronounced Th2 response (279). These data suggest that strong Th2 responses induced during infection with *F. hepatica* may inhibit the differentiation of Th1 and Th17 cells and thus may play a role in the attenuation of EAE. Indeed, other helminth infections that confer protection against autoimmunity in animal models are dependent on the induction of Th2 responses. Infection with the tapeworm *Hymenolepis diminuta* was shown to suppress the development of collagen-induced arthritis in WT mice, but had no effect on disease in mice deficient in the IL-4Rα (312). Indeed, the induction of Th2 responses by *F. hepatica* has already been shown to suppress the development of Th1 responses (204). Brady et al demonstrated that infection with *F. hepatica* suppressed the development of protective Th1 responses to *B. pertussis,* which delayed clearance of the bacteria (204).

During induction of EAE, mice develop MOG-specific Th1 and Th17 responses (116). The present study showed that infection with the Cullompton strain of *F. hepatica* in mice with EAE decreased MOG-specific IFN-γ and IL-17 production by spleen cells, whereas, MOG-specific IL-10 production was enhanced. Infection with *F. hepatica* also enhanced the production of TGF-β, although this was not MOG-specific, and most likely represents parasite-driven TGF-β production, or production of TGF-β by innate cells. Regardless of its cellular source, TGF-β may nonetheless be capable of suppressing MOG-specific responses through bystander suppression. This observation provides a potential mechanism to explain the suppression of autoimmunity by *F. hepatica*, since both IL-17 and IFN-γ play a crucial role in the pathogenesis of EAE. Conversely, the induction of IL-10 and TGF-β in response to MOG, suggests that parasite-induced immunosuppressive cytokines may be involved in attenuation of disease. Barrat et al reported that IL-10 can suppress the clinical symptoms of EAE (313). Moreover, certain helminths have been shown to induce immune suppression via IL-10. Infection with *S. mansoni* or *H. diminuta* protected mice from experimental models of colitis in a manner depended on macrophage-derived IL-10 (314, 315).

However, studies from this laboratory have since shown that while IL-10 played a role in the modulation of *F. hepatica*-specific Th responses, it was dispensable for the suppression of EAE induced by *F. hepatica* infection, as suppression of EAE was maintained during infection

of IL-10$^{-/-}$ mice. (298). Consistent with this finding, it has been reported that suppression of both colitis and airway hypersensitivity by infection with *H. polygyrus* was also independent of IL-10 (206, 214). Furthermore, infection of NOD mice with *L. sigmodontis* confers protection against diabetes independent of the action of IL-10 (213).

The administration of recombinant TGF-β *in vivo* was shown to attenuate the clinical symptoms of EAE, even when administered during active disease (316). Unpublished data from this laboratory show that TGF-β inhibited IFN-γ but not IL-17 production by MOG-specific T cells isolated from mice with EAE, suggesting that memory Th17 cells, like memory Th2 cells, are resistant to TGF-β mediated suppression (K. P. Walsh, unpublished findings). However, another study from this laboratory showed that recombinant TGF-β suppressed TLR-induced IL-23, IL-1 and IL-12 production by DC and since IL-23 and IL-1 are crucial for the development of pathogenic Th17 cells (317, 318), it is possible that helminth-induced TGF-β can inhibit autoantigen-specific Th17 cells by modulating innate cytokines involved in the differentiation of Th17 cells. While IL-10 was not involved in the attenuation of EAE, neutralisation of TGF-β, using an anti-TGF-β antibody administered throughout the course of EAE, completely reversed *F. hepatica*-induced suppression of EAE. Thus, TGF-β plays an essential role in the suppression of EAE by *F. Hepatica*. This is consistent with a report by Hübner et al which demonstrated that *L. sigmodontis* protects mice from collagen induced arthritis in manner dependent on TGF-β (213).

Treg cells play an important role in immune regulation in the host. Helminth infections have been shown to expand Treg cells, most likely as a mechanism designed to enhance survival of the parasite through regulation of anti-parasite immune responses. Indeed, Treg cells have been shown to play a crucial role in mediating *H. polygyrus*-induced suppression of autoimmunity and atopy (147). In the present study, infection of mice with *F. hepatica* led to the recruitment of CD4$^+$FOXP3$^+$ T cells in the peritoneal cavity of infected mice. Further research from this laboratory has shown that the expression of CTLA-4 and FOXP3 was enhanced on parasite-specific T cell clones (298). Moreover, these clones failed to produce IFN-γ in response to LFH, but instead produced large quantities of IL-5 and IL-10 (298). T cells that were isolated from the peritoneal cavity of mice infected with *F. hepatica* suppressed the proliferation and cytokine production of OVA-specific Th1 and Th17 cells (298). These data suggest that helminth infection can suppress parasite-specific, but also T cell responses

to unrelated antigens, through bystander suppression. This finding is consistent with other studies which showed that depletion of Treg cells reversed filarial helminth-induced hyporesponsiveness in mice (172). Furthermore, in a model of *L. sigmodontis* infection, depletion of Treg cells induced a helminth-specific immune response that expelled the larva (172).

The results of the present study demonstrated that infection with *F. hepatica* was associated with the recruitment of Treg cells, which co-expressed TGF-β and FOXP3. In addition, spleen cells isolated from mice with EAE that were infected with *F. hepatica* produced TGF-β. As TGF-β is required for the protection against EAE during infection with *F. hepatica* (298), this suggests a role for Treg-derived TGF-β in protection, or conversely a role for TGF-β in expanding Treg cells. However, preliminary data has shown that transfer of $CD4^+$ T cells from *F. hepatica* infected mice to uninfected mice with EAE failed to protect from disease (K P Walsh, C M Finlay, unpublished observations), suggesting that Treg cells may be dispensable for the suppression of autoimmunity. While there is a large body of evidence to demonstrate that Treg cells mediate immunosuppression during helminth infection, there are also many reports which show that helminth infections mediate immunosuppression independent of the activity of Treg cells. For example, infection with *L. sigmodontis* protects NOD mice from diabetes in a manner dependent on TGF-β, but not dependent on Treg cells, as protection was maintained following depletion of $CD25^+$ cells and in mice deficient in FOXP3 (213, 319). Depletion studies are required to address the role of Treg cells in the attenuation of disease during *F. hepatica* infection. Alternative sources of TGF-β, in particular innate sources, may be important, especially considering infection with *F. hepatica* recruited DC and macrophages that expressed TGF-β to the peritoneal cavity of infected mice.

The present study also showed that infection with *F. hepatica* inhibited the APC function of PEC. PEC isolated from mice infected with *F. hepatica* had a reduced ability to prime Th cells that produced IL-17, IFN-γ and IL-10 compared with PEC isolated from uninfected mice. A study from this laboratory has shown that the expression of MHC class II, CD40, CD80 and CD86 was reduced on DC isolated from mice infected with *F. hepatica* compared with those isolated from uninfected mice (298). However, transfer of T cells from *F. hepatica* infected mice into mice with EAE was unable to confer suppression of disease (K P Walsh, C M Finlay, unpublished findings). Therefore, it is possible that *F. hepatica*-induced suppression of

APC function plays a role in the suppression of T cell responses. The APC capacity of DC matured in the presence of *F. hepatica* or its secreted products warrants further investigation. Most studies on immunosuppression during helminth infection have concentrated on adaptive immune responses and few have considered the role of innate cells. However, Smith et al reported that infection of mice with *S. mansoni* prevents the development of colitis by a mechanism dependent on macrophages; depletion of macrophages was shown to reverse the protective effect in infected mice, while transfer of macrophages from infected mice to uninfected recipient mice with colitis attenuated disease (315).

Alternative activation of macrophages is a characteristic of helminth infections. M2 macrophages have been implicated in immunosuppression observed during helminth infections (147). It has also been shown that arg1 expression by M2 macrophages plays a role in SEA-induced suppression of inflammation (190). It was reported that *F. hepatica* infection leads to the alternative activation of macrophages (255). Infection with *F. hepatica* was shown to induce the mRNA expression of genes associated with alternative activation of macrophages, such as Fizz1, Arg-1, Ym1 (320). Furthermore, another study showed that *F. hepatica* ES could promote the alternative activation of macrophages that expressed IL-10 and had high specific arginase activity (321). The present study showed that infection of mice with *F. hepatica* increased the expression of IL-10 and TGF-β by peritoneal macrophages and DC. In addition, the expression of Arg1 was enhanced in the PEC from infected mice compared with uninfected mice. In contrast, the expression of iNOS was decreased in the PEC from infected mice. Moreover, high antigen-specific arginase activity was detected in lysate of PEC from infected animals when compared with uninfected mice. Taken together, these data suggest the *F hepatica* infection promotes the development of M2 macrophages. M2 have an anti-inflammatory phenotype and can inhibit T cell proliferation through depletion of L-arginine in the cellular micro-environment (188, 189). In addition, M2 macrophages have a strong Th2 bias and can suppress the development of Th1 cells. M2 macrophages generated by the *in vivo* administration of peroxiredoxin derived from ES was able to induce an IL-4 response by activated spleen cells, while inhibiting the production of IFN-γ (267). Together, these findings suggest a role for M2 macrophages in infection-induced suppression of inflammation.

The present study showed that infection of mice with *F. hepatica* led to the recruitment of MDSC into the peritoneal cavity. MDSC, characterised by co-expression of CD11b and Gr-1, are a population of heterogeneous immature myeloid cells (187). There is a limited body of evidence to suggest that helminths promote the recruitment of MDSC. (221, 282, 284, 285). One such study showed that the forced accumulation of MDSC aided in the expulsion of *N brasiliensis* in a manner associated with a more pronounced Th2 response (322). In the present study, infection with *F. hepatica* led to the recruitment of $CD11b^{+}Gr\text{-}1^{+}$ cells into the peritoneal cavity possibly by the high expression of MCP-1 in infected mice. Infection with *F. hepatica* enhanced the expression of the inhibitory T cell receptor PD-1 and TGF-β by $CD11b^{+}Gr\text{-}1^{+}$ cells. Furthermore, $CD11b^{+}Gr\text{-}1^{+}$ cells isolated from infected mice were able to suppress T cell proliferation *in vitro,* suggesting that these cells may play a role in suppression of autoimmunity. The present study has also shown that MDSC are composed of at least two separate populations. $Gr1^{high}$ $Ly6G^{+}$ MDSC were isolated from *F. hepatica* infected livers and included cells with a ring shaped nuclei, possibly representing myeloid cells at their early developmental stages and cells with characteristic neutrophil-like morphology. In contrast, cytospin preparations of isolated $Gr1^{dim}$ $Ly6G^{-}$, included cells with monocytic and macrophage morphology, but also cells with eosinophil-granulocyte like morphology. This study also showed that the $Ly6G^{-}Gr\text{-}1^{dim}$ population isolated from the peritoneal cavity of infected mice significantly inhibited the proliferation of T cells. In contrast, the $Ly6G^{+}$ population was unable to suppress T cell proliferation, but instead enhanced the proliferation of T cells. Conversely, MDSC populations isolated from the liver of infected mice behaved in the opposite manner; the $Ly6G^{+}$ population significantly inhibited T cell proliferation, while the liver $Ly6G^{-}Gr\text{-}1^{dim}$ population enhanced the proliferation of T cells. In the liver, both MDSC populations were found to express extremely high levels of IL-10 and TGF-β and the $Ly6G^{-}Gr\text{-}1^{dim}$ population also expressed CD11c (323). Thus, the present study was unable to ascertain the differences between MDSC populations isolated from the liver and peritoneal cavity of mice infected with *F. hepatica.* It is likely that these differences are a result of the highly heterogeneous nature of MDSC populations as defined by the literature.

The relation between various immature myeloid cells in this model and their ability to contribute to the immunosuppressive environment during infection or following treatment of ES needs to be investigated in more detail. One interesting observation is that MDSC were

not recruited during infection in mice deficient in IL-10. MDSC share many similarities with M2 macrophages, indeed, IL-4 and IL-13 promotes the development of MDSC. IL-10, TGF-β, IL-1β and LPS in combination with IFN-γ also play a role in the differentiation of MDSC. While many studies have shown that monocyte-like MDSC can mediate suppression of T cell responses through arg1 in a similar manner to M2 macrophages, other studies have shown that monocyte-like MDSC differ from M2 in that they are dependent on IFN-γ to produce NO, which mediates the suppression of T cell responses. Thus, monocyte-like MDSC display an array of M1 and M2 macrophage characteristics (187, 300, 303, 304). Studies have described that neutrophil-like MDSC also express M2 macrophage phenotypic markers (284, 285). Therefore, a proportion of Gr-1$^+$Ly6G$^+$ cells that are identified in studies as neutrophils may in fact be neutrophil-like MDSCs (187). In support of this, alternatively activated neutrophils (N2) have been shown to play a role in suppression of immune responses during cancer (324). LNF-PIII and LNnT were found to drive the IL-13-dependent recruitment of F4/80$^+$CD11b$^+$Gr-1$^+$Fizz1$^+$ cells consisting of both neutrophil and macrophage type cells, which were able to suppress mitogen-activated T cells in an IFN-γ/NO dependent manner and could drive the production of IL-10 and IL-13 by T cells (282, 284, 285). It has recently been shown that LNFPIII can prolong heart transplant tolerance in mice and this effect required PD-1L expression by M2 macrophages. (221). Interestingly, granulocytic MDSC recruited during infection of *N. brasiliensis* aided protection from the parasite, as their removal prolonged survival of the helminth

The present study reports important differences between distinct isolates of *F. hepatica*. For reasons of convenience and repeatability most studies investigating the various aspects of *F. hepatica* biology have made use of a limited number of strains. These strains are maintained by serial passage in snail colonies (309). The strain used in this study was the Cullompton isolate which was isolated by Gordon Graham at the Cullompton abattoir, Devon in 1998. However, due to serial passage this strain has undergone genetic transformation. The Cullompton isolate is triploid and does not produce sperm and thus, reproduces via parthenogenesis (309). The change in reproductive strategies suggests that this isolate may differ in many other respects from WT flukes. While this study initially utilised the Cullompton isolate, due to supply issues, other isolates were later used, which enabled a comparison to be made between several strains. Indeed, the two new strains tested (Shrewsbury and South Gloucestershire)

did not protect mice from the clinical symptoms of EAE, in fact, infection with these strains exacerbated disease, in direct contrast to the Cullompton isolate. In addition, MOG specific production of IL-17, IFN-γ, IL-10, IL-4 or TGF-β was not modulated by infection with Shrewsbury or South Gloucestershire strain of *F. hepatica.* Importantly, the production of TGF-β by spleen cells from mice infected with Shrewsbury or South Gloucestershire did not increase in response to parasite antigens *in vitro.* Infection with either the Shrewsbury or the South Gloucestershire strain of *F. hepatica* also failed to enhance the expression of FOXP3 in $CD4^+$T cells from the peritoneal cavity. Thus, Cullompton strain-induced attenuation of EAE was associated with a modulation of autoantigen-specific T cell responses and an increase in frequency of Treg cells *in vivo.* In contrast, the failure to attenuate disease was characterised with an inability of Shrewsbury or South Gloucestershire strains of *F. hepatica* to induce various immunomodulatory effects.

Populations of *F. hepatica* are genetically diverse and studies have shown that the fluke population within a single animal can be diverse. Indeed, fluke populations from farms even quite closely spaced geographically can differ significantly and fluke populations from a single farm can change quite rapidly over a short time-frame (243, 325). The unique reproductive strategies used by *F. hepatica* may account for this genetic diversity. The adult fluke which reproduces both asexually and sexually is very productive and can produce up to 25,000 eggs per day (326, 327). In addition, the fluke reproduces asexually, by polyembryony, in the intermediate snail host. One miracidium can produce over a thousand identical cercariae, this goes a long way to explaining how one fluke population can rapidly replace another. No doubt an effective technique in the presence of the selection pressure provided by the use of anti-helminthic drugs, genetically resistant flukes that are generated by chance from sexual reproduction in the host can expand rapidly by asexual reproduction in the intermediate host (309). A comparative study of the immune response to infection with these strains may prove useful in the identification of the mechanisms, in particular the genes which confer protection against autoimmunity.

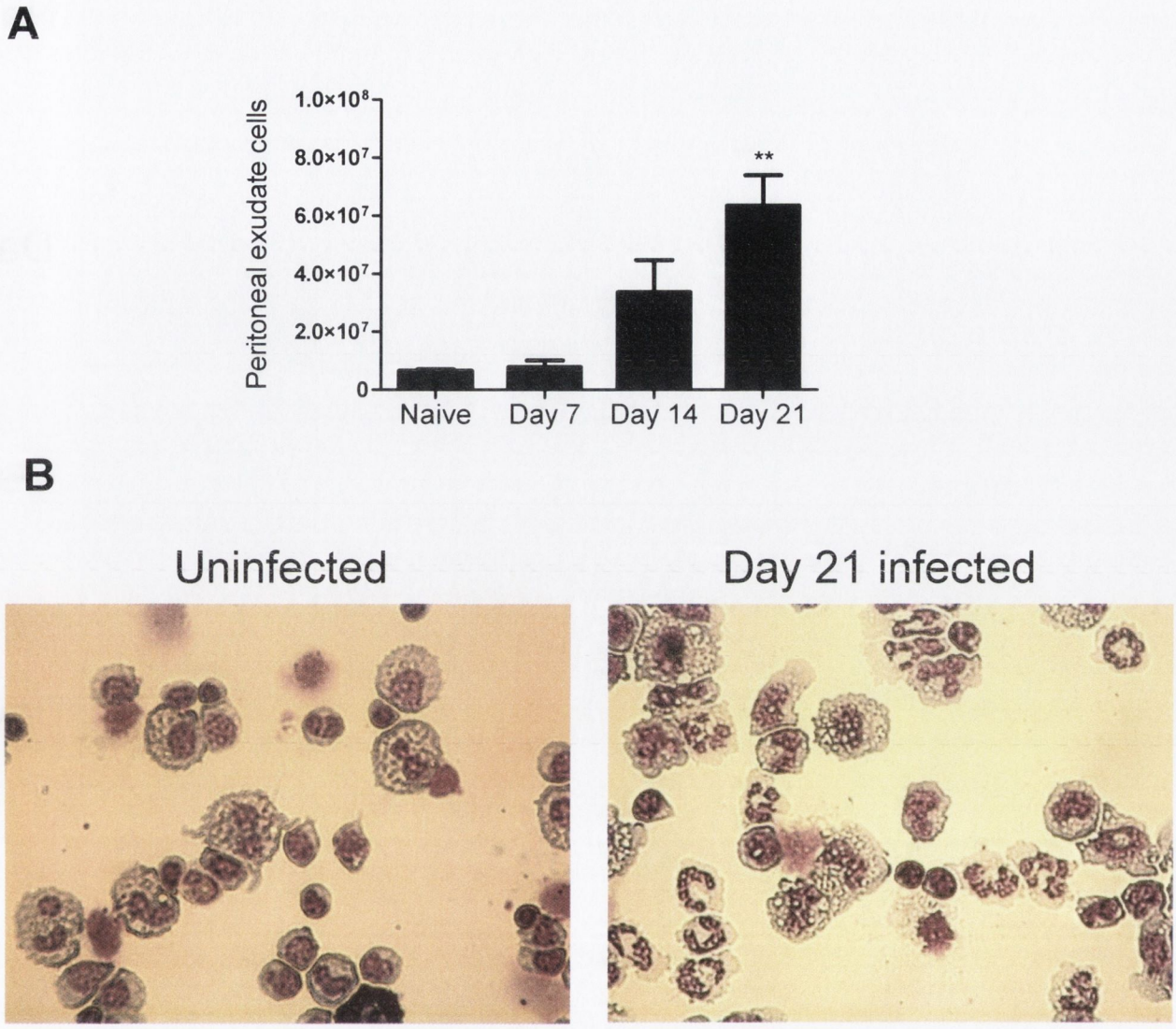


Fig 3.1 *Fasciola hepatica* infection induces an influx of inflammatory cells into the peritoneal cavity of mice. C57BL/6 mice were infected with 10 viable metacercariae of *F. Hepatica (*Gordon Graham strain). On days 7, 14 and 21 days post challenge PEC were isolated by peritoneal lavage. A, viable peritoneal exudate cells were counted by trypan blue staining. Data presented are means +/- SEM of 4 mice per group. Statistical assessment was performed as indicated vs naïve control using Students t-test. ** $P<0.001$, B, Selected photomicrographs (400X) of cytospin preparation of cells fixed and stained with eosin and hematoxylin. ** $P<0.05$ versus uninfected. Data representative of 5 separate experiments.

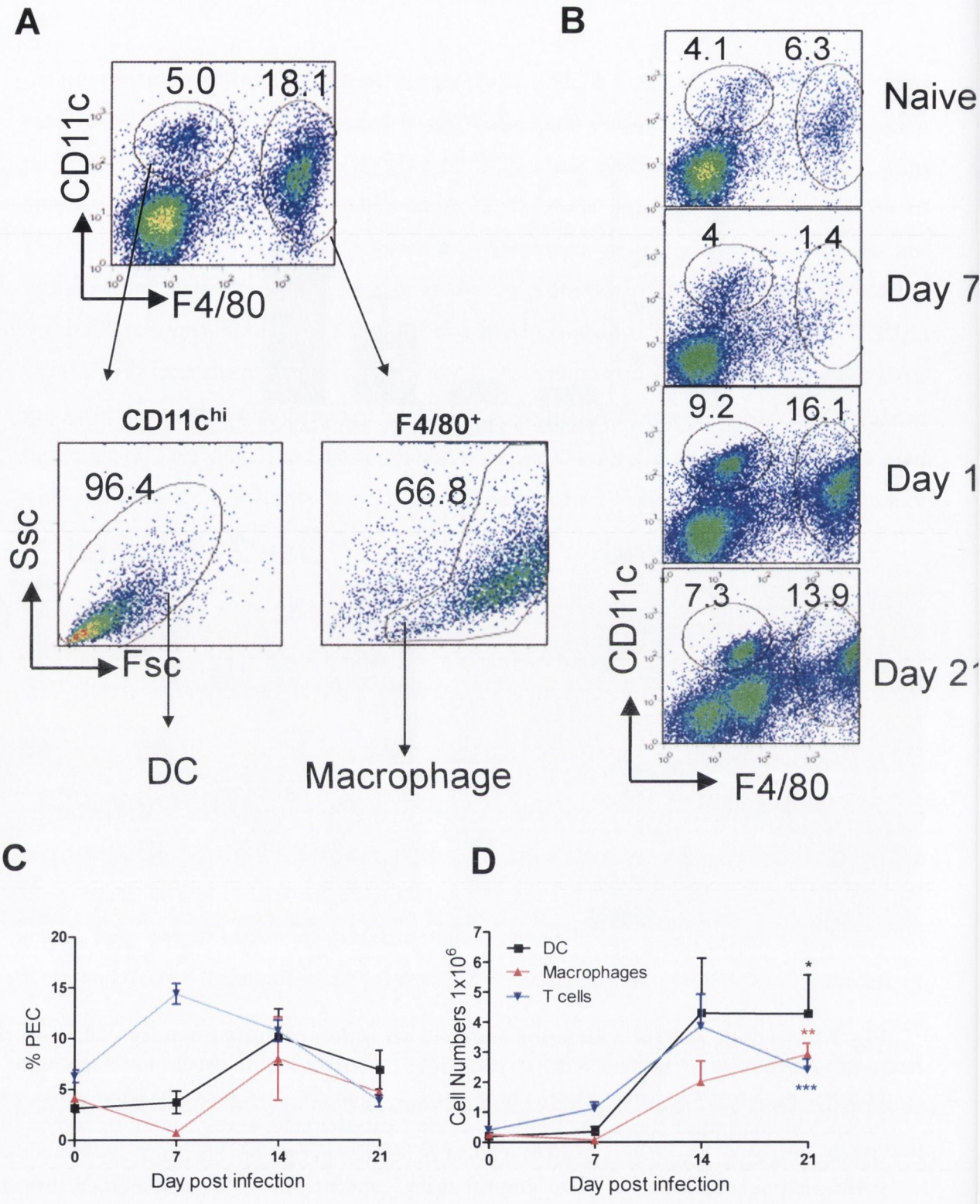


Fig 3.2 *F. hepatica* infection is associated with an influx of DC and macrophage into the peritoneal cavity. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica* Gordon Graham strain. On days 7, 14 and 21 post challenge, PEC were isolated by peritoneal lavage. PEC were surface stained for CD11c, F4/80 and CD4 and analysed by flow cytometrey. A, The gating strategy for DC and macrophages. Displayed flow cytometric plots are gated on live cells. B, Differential gating of DC and macrophage populations during course of infection. C, Relative abundance of DC and macrophage as a percentage of total PEC and D, as total cell numbers. Data presented are means +/- SEM of 4 mice per group. Statistical assessment was performed as indicated vs naïve control using Students t-test. ** $P<0.001$. Data representative of 2 separate experiments.

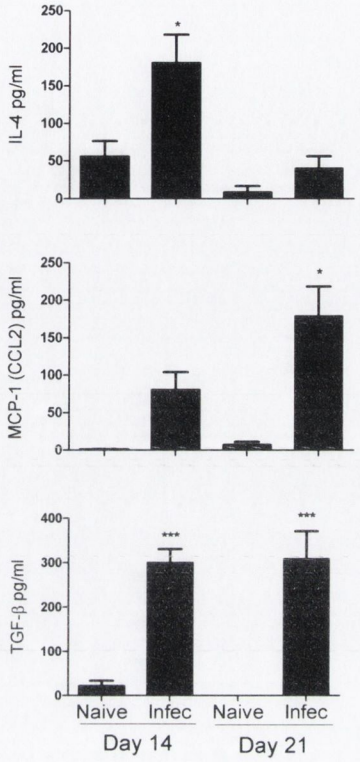


Fig 3.3 *F. hepatica* infection increases the concentrations of TGF-β, IL-27 and IL-10 in the peritoneal fluid. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica* (Cullompton strain) On day 14 and day 21 post infection, peritoneal lavages were preformed on infected and naïve mice as controls. The peritoneal exudate fluid was concentrated 8X using centrifugal protein concentrators (Millipore). The concentration of cytokines was determined by ELISA. Data presented are means +/- SEM of 4 mice per group and ELISA in triplicate. Statistical assessment was performed as indicated vs naïve control using Students t-test. ** $P<0.001$, * $P<0.05$. Data is representative of 2 separate experiments.

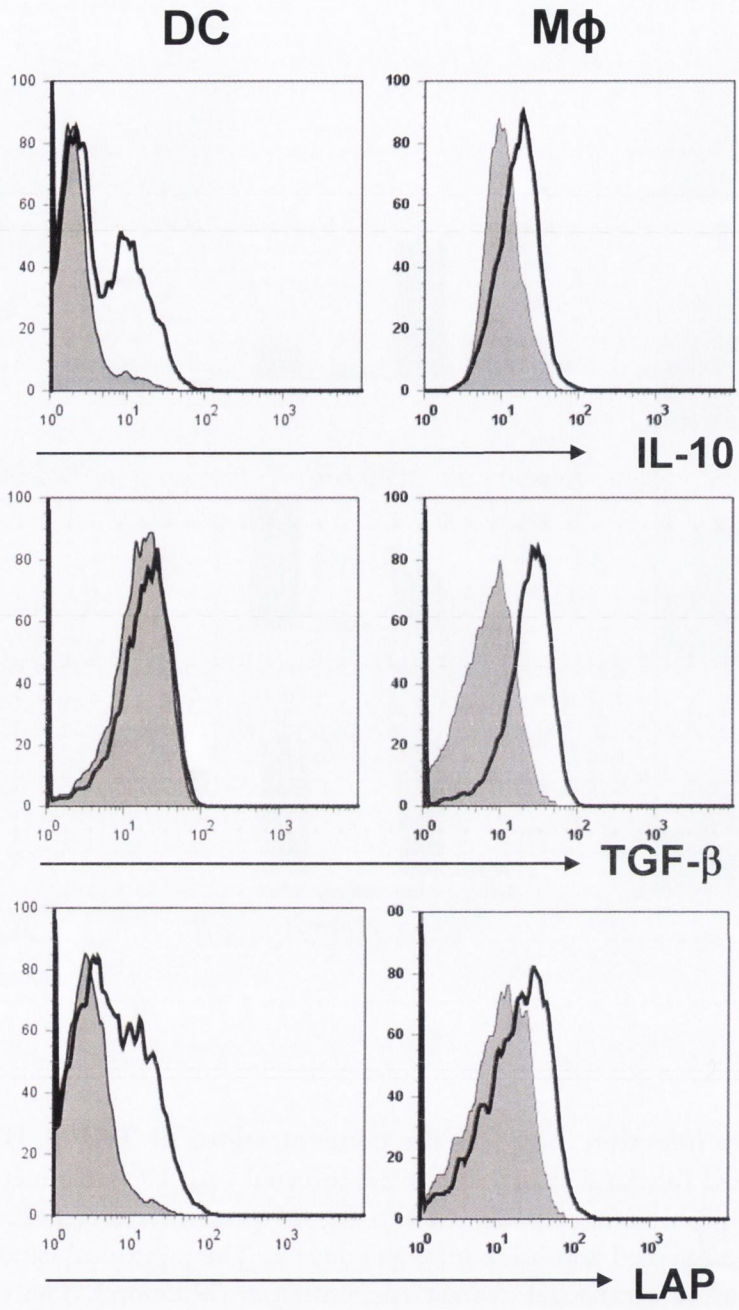


Fig 3.4 *F. hepatica* infection enhances cytokine production by peritoneal macrophages and DC. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica* (Cullompton strain). On days 21 post challenge, PEC were isolated from naïve and *F. hepatica*-infected mice by peritoneal lavage. The cells were treated with Brefeldin A for 10 hours and were surface stained for CD11c and F4/80 and intracellular stained for IL-10, TGF-β and LAP. Results from immunofluorescence analysis are shown for peritoneal $CD11c^+$ and $F4/80^+$ cells from uninfected (grey histogram) and infected (black bar). Data is representative of 3 separate experiments.

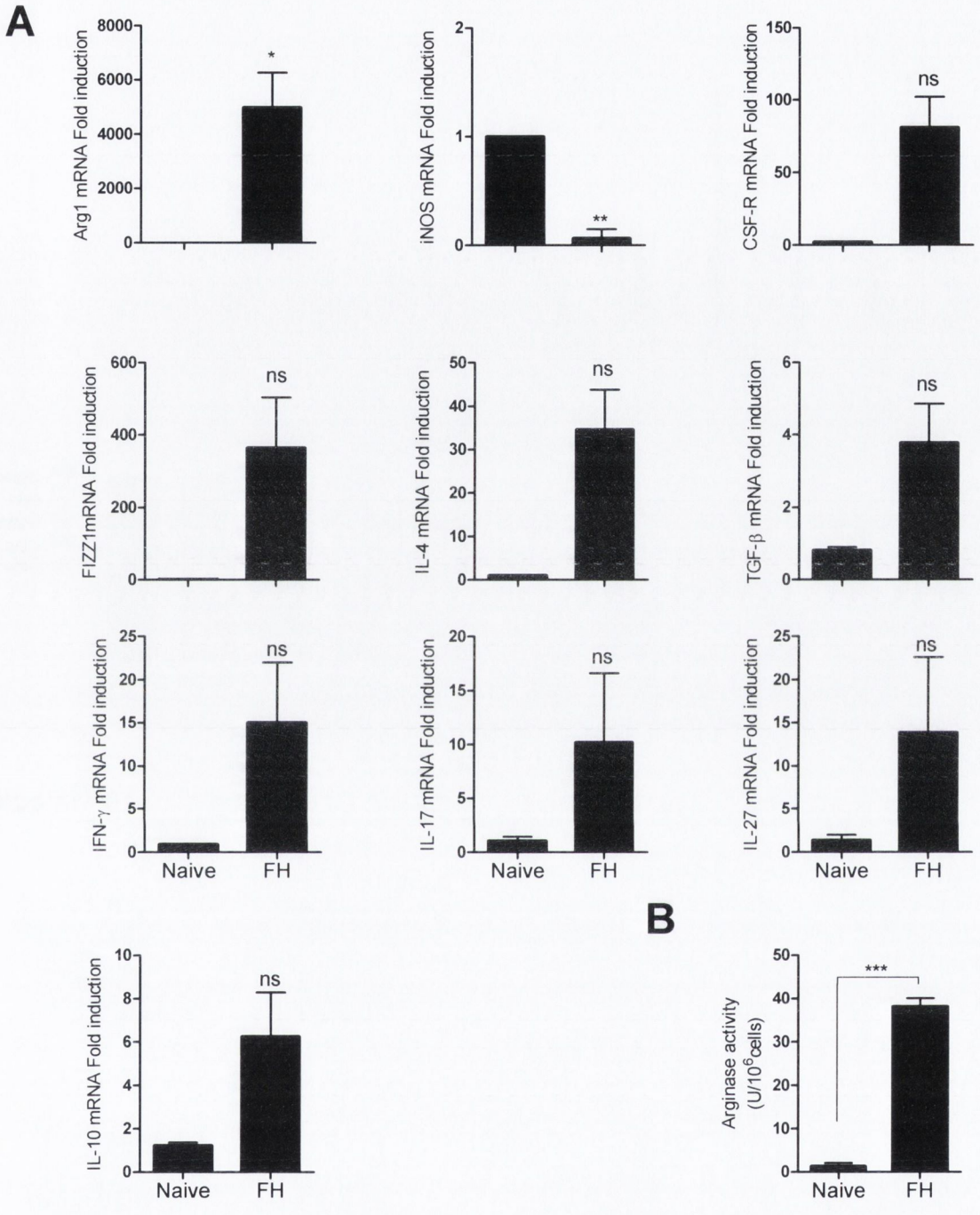


Fig 3.5 *F. hepatica* infection induces the expression of genes associated with alternative activation of macrophages. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica* Shrewsbury strain. After 21 days PEC were isolated from naïve and *F. hepatica*-infected mice by peritoneal lavage. A, The mRNA expression of arg1, nos2, fizz1, il-4, tgf-β, csf-R, il-27, il-10, ifn-γ and il-17 was assessed by RT-PCR. Data presented are means +/- SEM of 4 mice per group and rtPCR in triplicate. B, 10^6 PEC were lysed and the arginase activity of cell lysate was determined using the Quantichrom Arginase Assay Kit (Bioassay Systems). Data presented are means +/- SEM of triplicate assay. Statistical assessment was performed vs uninfected controls using unpaired Students's test. * $p<0.05$, **$p<0.01$ *** $p<0.001$. Data is representative of 2 separate experiments.

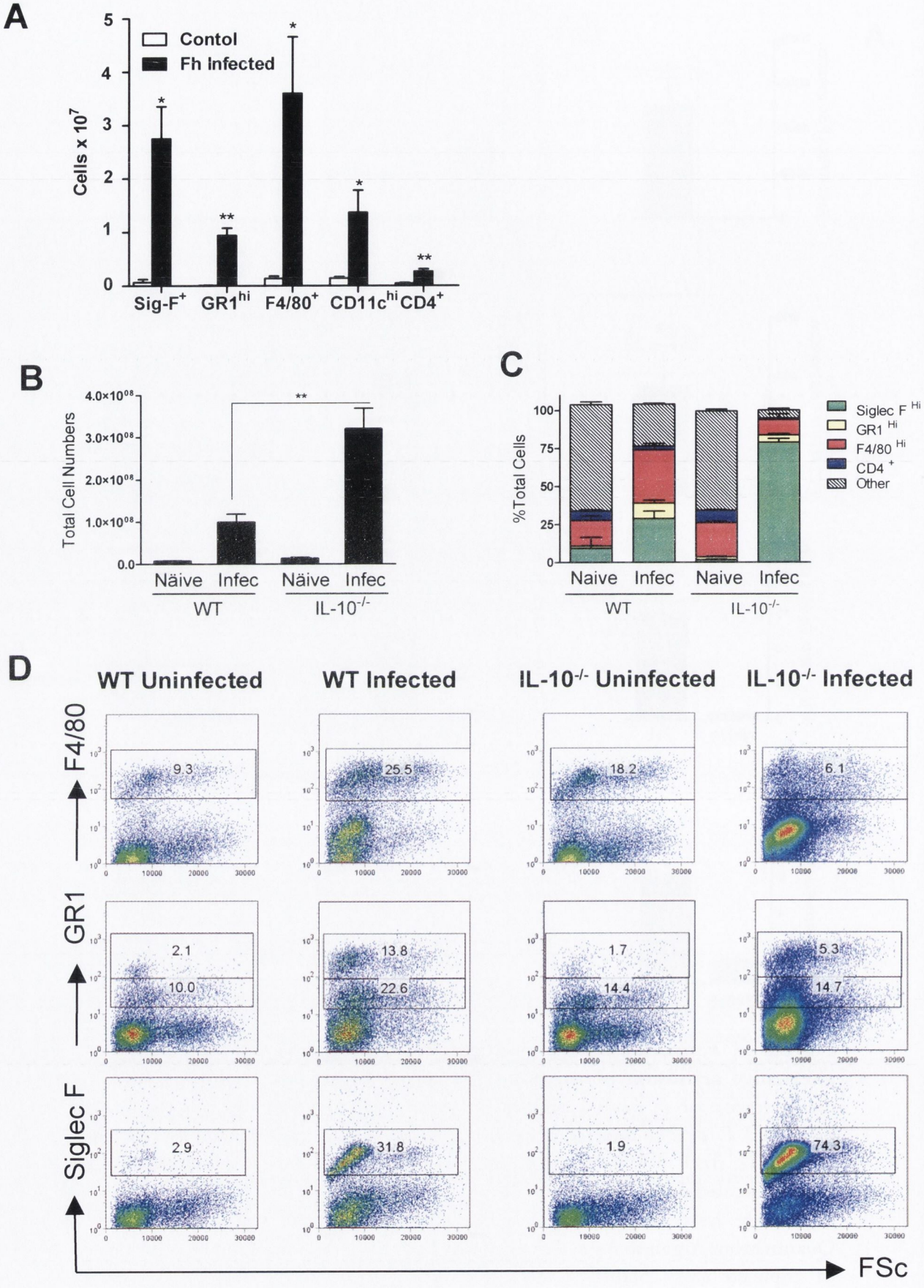

A
Contol
Fh Infected
Cells x 10^7
Sig-F+
GR1hi
F4/80+
CD11chi
CD4+
B
Total Cell Numbers
Näive
Infec
WT
IL-10-/-
C
%Total Cells
Siglec F Hi
GR1 Hi
F4/80 Hi
CD4 +
Other
D
WT Uninfected
WT Infected
IL-10-/- Uninfected
IL-10-/- Infected
F4/80
GR1
Siglec F
FSc

Fig 3.6 *F. hepatica* infection is associated with the recruitment of F4/80$^+$, GR1$^+$ and Siglec F$^+$ cells to the peritoneal cavity of WT and IL-10$^{-/-}$ mice. C57BL/6 and IL-10$^{-/-}$ (on a C57BL/6 background) mice were infected with 10 viable metacercariae of *F. hepatica* (Cullompton strain). On day 21 post challenge PEC were isolated from naïve and *F. hepatica*-infected mice by peritoneal lavage. A, numbers of Siglec-F$^+$, CD11c^{hi}, F4/80$^+$, GR-1^{hi} and CD4$^+$ cells in the peritoneal cavity of uninfected and infected mice B, Total viable PEC in infected and uninfected WT and IL-10$^{-/-}$ mice C, proportion of cells in PEC of infected and uninfected WT and IL-10$^{-/-}$ mice. Data presented are means +/- SEM of 5 mice per group. D, Representative FACS plots of individual mice. Data is representative of 2 separate experiments.

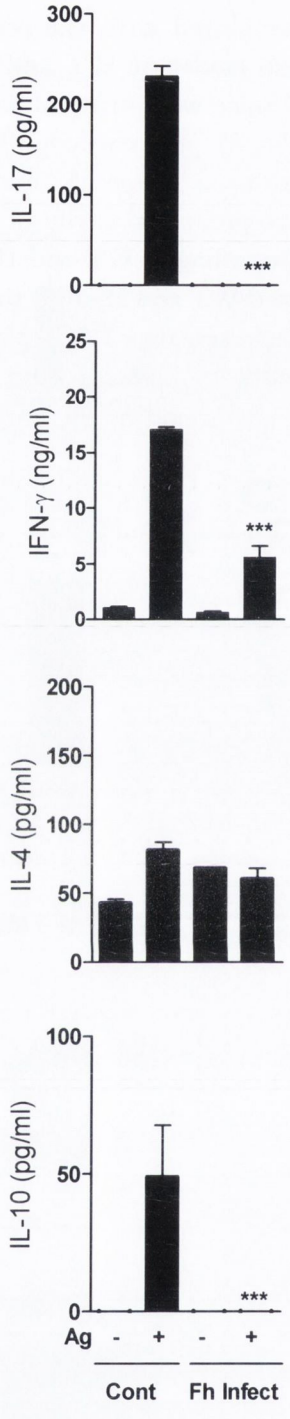


Fig 3.7 Defective APC function of PEC from infected mice. PEC from *F. hepatica* Cullompton strain)-infected or PEC from naive control (Cont) mice were cocultured with purified $CD4^+$ T cells from DO11.10 mice and Ag (OVA, 2 μg/ml) or with medium only. Supernatants were removed after 72 h, and the concentrations of IFN-γ, IL-17, IL-4, and IL-10 were quantified by ELISA, ***$p<0.001$ vs control by unpaired t test. Data presented are means +/- SEM of 5 mice per group. Results are representative of four mice per group and of two experiments.

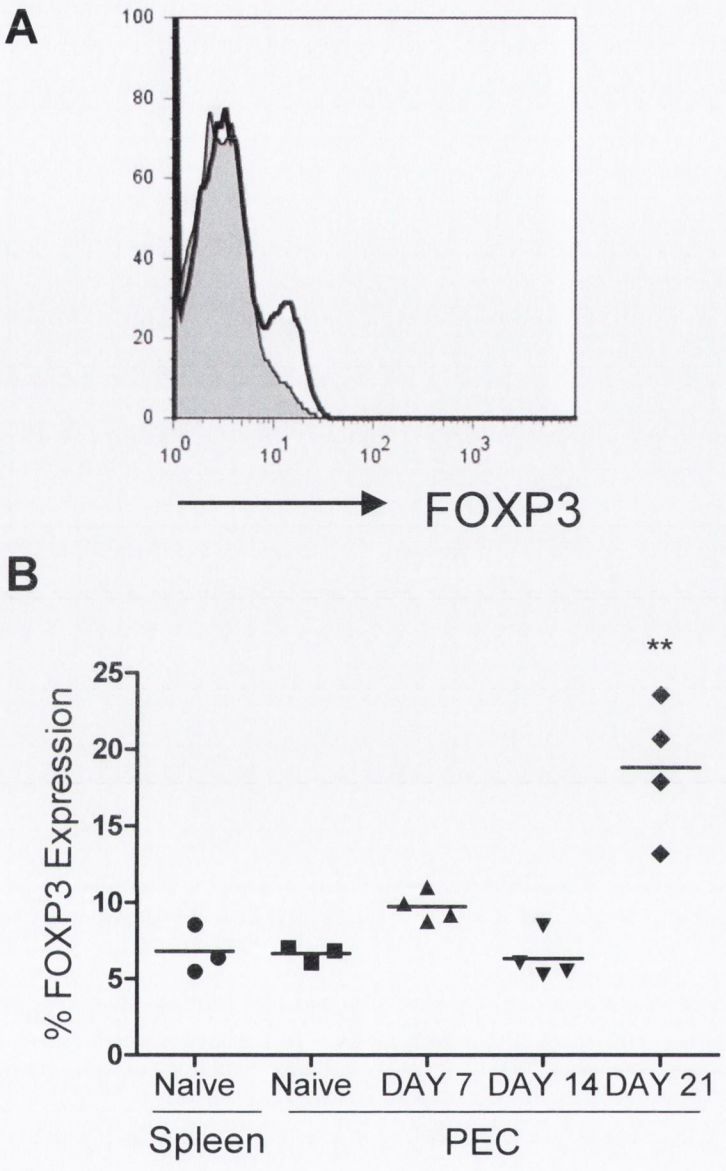


Fig 3.8 *F. hepatica* infection enhances the expression of FOXP3 in peritoneal CD4$^+$ T cells. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica* (Cullompton strain). On days 7, 14 and 21 post challenge, PEC were isolated from naïve and *F. hepatica*-infected mice by peritoneal lavage. The cells were treated with Brefeldin A for 10 hours and then stained for CD3, CD4 and FOXP3. A, representative plot of expression of FOXP3 in uninfected PEC (shaded line) and Day 21 post infection (black line) CD4$^+$cells. B, mean FOXP3 expression in CD4$^+$ cells from uninfected spleen and PEC from naïve and infected mice. ** $P<0.005$ vs naïve PEC. Data presented are means +/- SEM of 5 mice per group. Data is representative of 3 separate experiments.

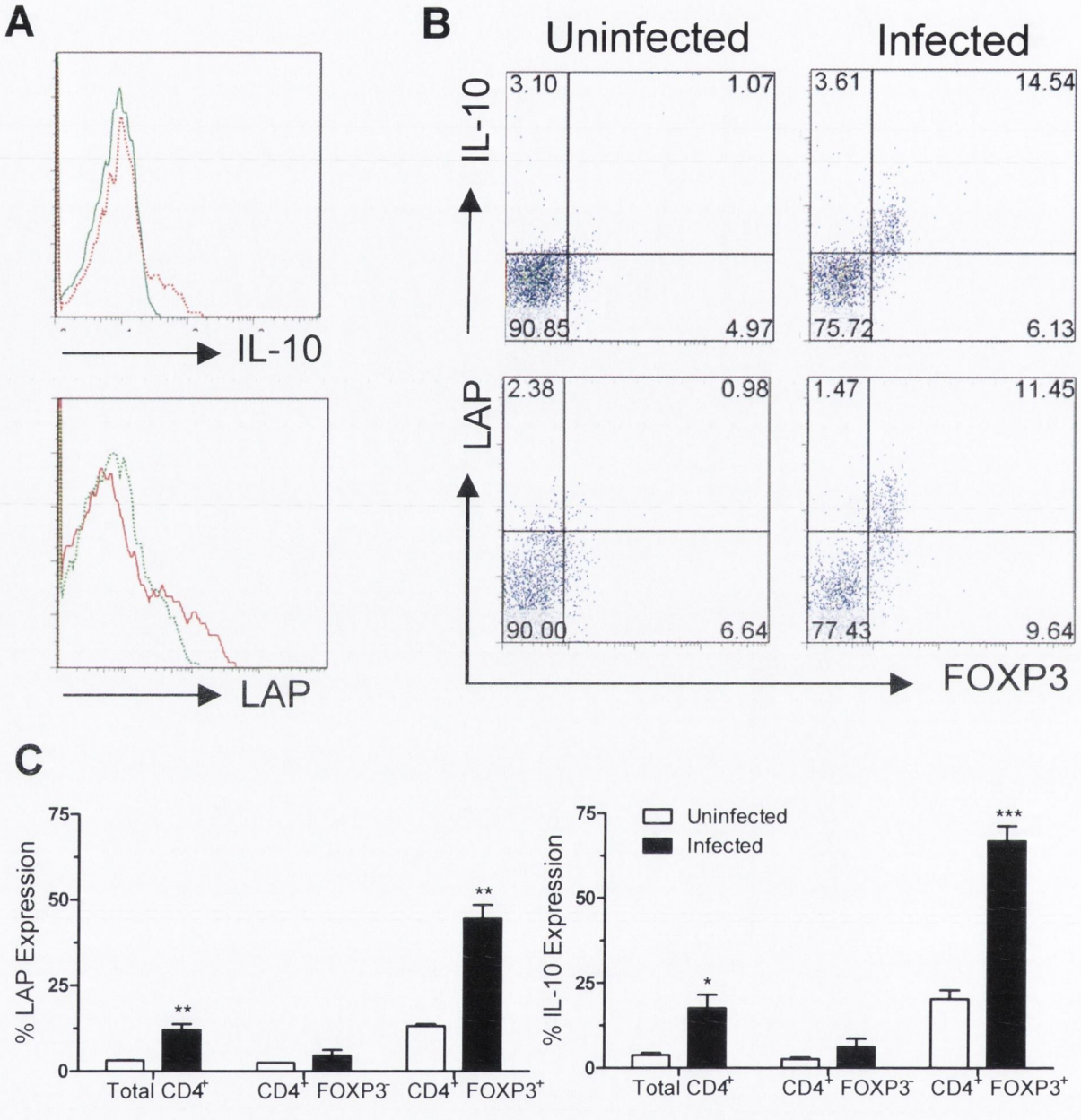


Fig 3.9 *F. hepatica* infection enhances the expression of IL-10 and LAP in peritoneal CD4$^+$ FOXP3$^+$ T cells. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica* (Cullompton strain). On day 21 post challenge PEC were isolated from naïve and *F. hepatica*-infected mice by peritoneal lavage. The cells were treated with Brefeldin A for 10 hours and then surface stained for CD3, CD4, LAP and were intracellularly stained for IL-10. A, Expression of IL-10 and LAP on CD4$^+$ cells from infected animals (red line) compared with uninfected controls (green line). B, representative plot of expression of IL-10 and LAP vs FOXP3 expression gated on CD4$^+$ cells. C, Mean (+/- SEM, of 5 mice per group) expression of IL-10$^+$ and LAP$^+$ from the total CD4$^+$ population compared with the CD4$^+$ FOXP3$^-$ and CD4$^+$ FOXP3$^+$ populations. ** P<0.005.. vs uninfected. Data is representative of 2 separate experiments.

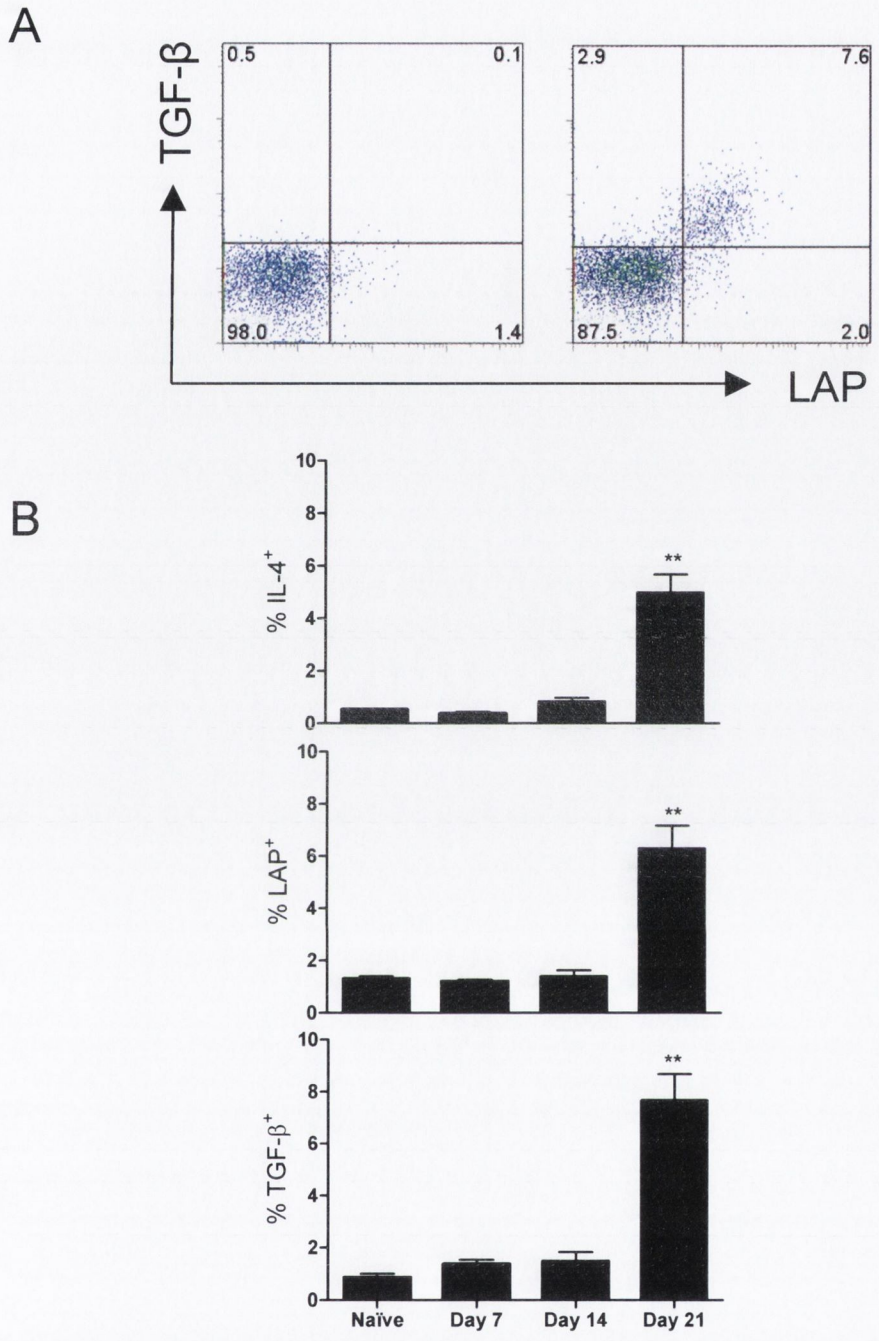


Fig 3.10 *F. hepatica* infection enhances the expression of TGF-β, and IL-4 in peritoneal CD4⁺ T cells. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica.* (Cullompton strain). On days 7, 14 and 21 post challenge, PEC were isolated from naïve and *F. hepatica*-infected mice by peritoneal lavage. The cells were treated with Brefeldin A for 10 hours and then surface stained for CD3, CD4, LAP and intracellularly stained for TGF-β and IL-4. A, representative plots of expression of TGF-β and LAP gated on $CD4^+$ cells. B, mean cytokine expression by $CD4^+$ cells. Data presented are means +/- SEM of 5 mice per group. Statistical assessment was performed using unpaired Student's t test vs naïve controls. * $P<0.01$ vs naïve $CD4^+$ cells. ** $P<0.005$. Data is representative of 2 separate experiments.

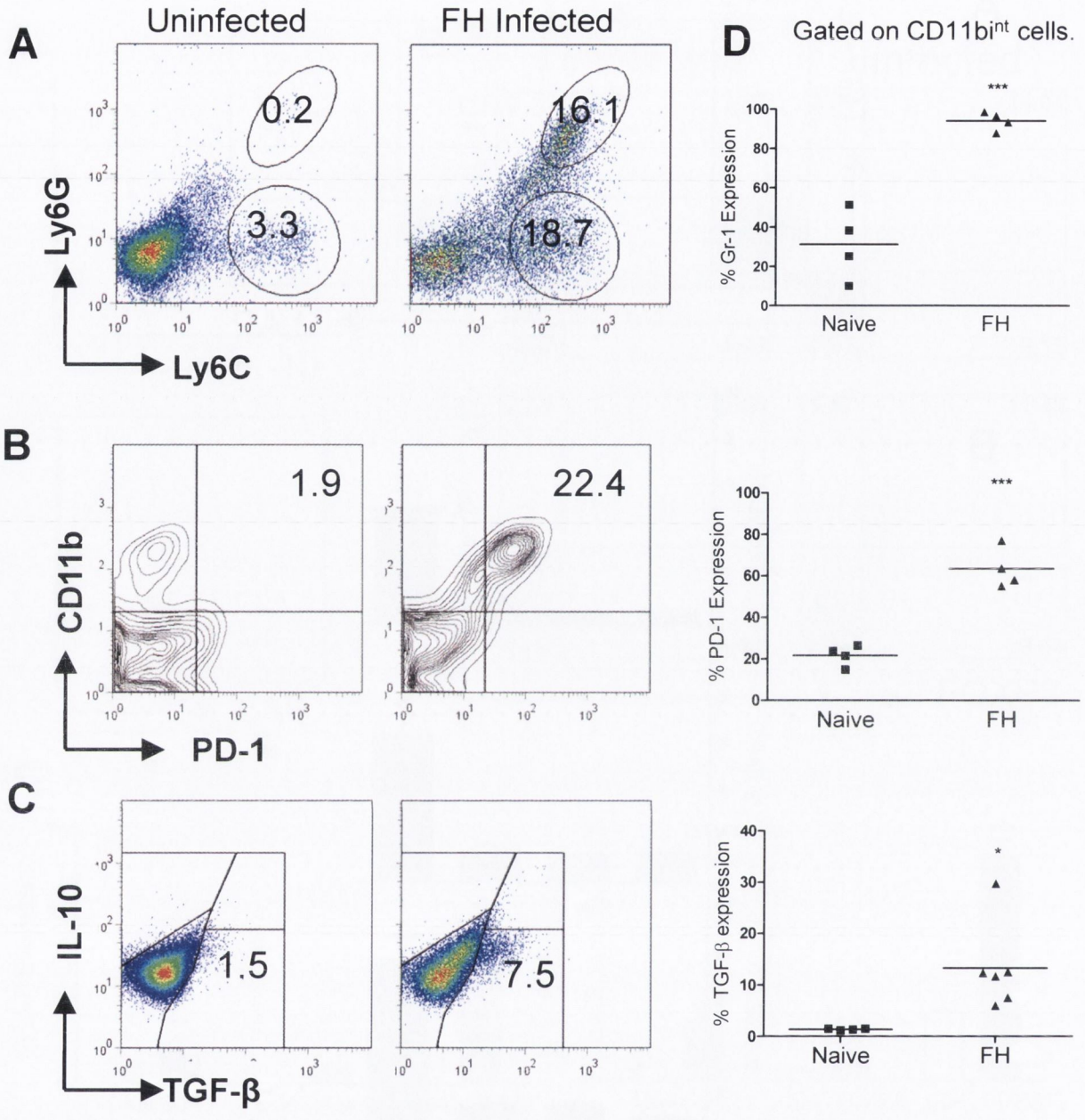


Fig 3.11 ***Fasciola hepatica*** **infection results in the recruitment of myeloid cells into the peritoneal cavity which express higher levels of Ly6G, Ly6C, PD-1 and TGF-β.** C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica* (Cullompton strain). After 21 days PEC cells were isolated by peritoneal lavage from naive and infected animals. PEC were surface stained for CD11b GR-1, Ly6G, Ly6G and PD-1 and intracellularly stained for IL-10 and TGF-β. A-C Representative flow cytometric plots shown are gated on CD11b^{int} cells. Collated data of expression of Gr-1 (Ly6G), PD-1 and TGF-β by CD11b^{int} cells. Statistical assessment was performed using Student's t test vs naïve controls with 4 mice per group. * $P<0.01$ and *** $P<0.001$. Data is representative of 3 separate experiments.

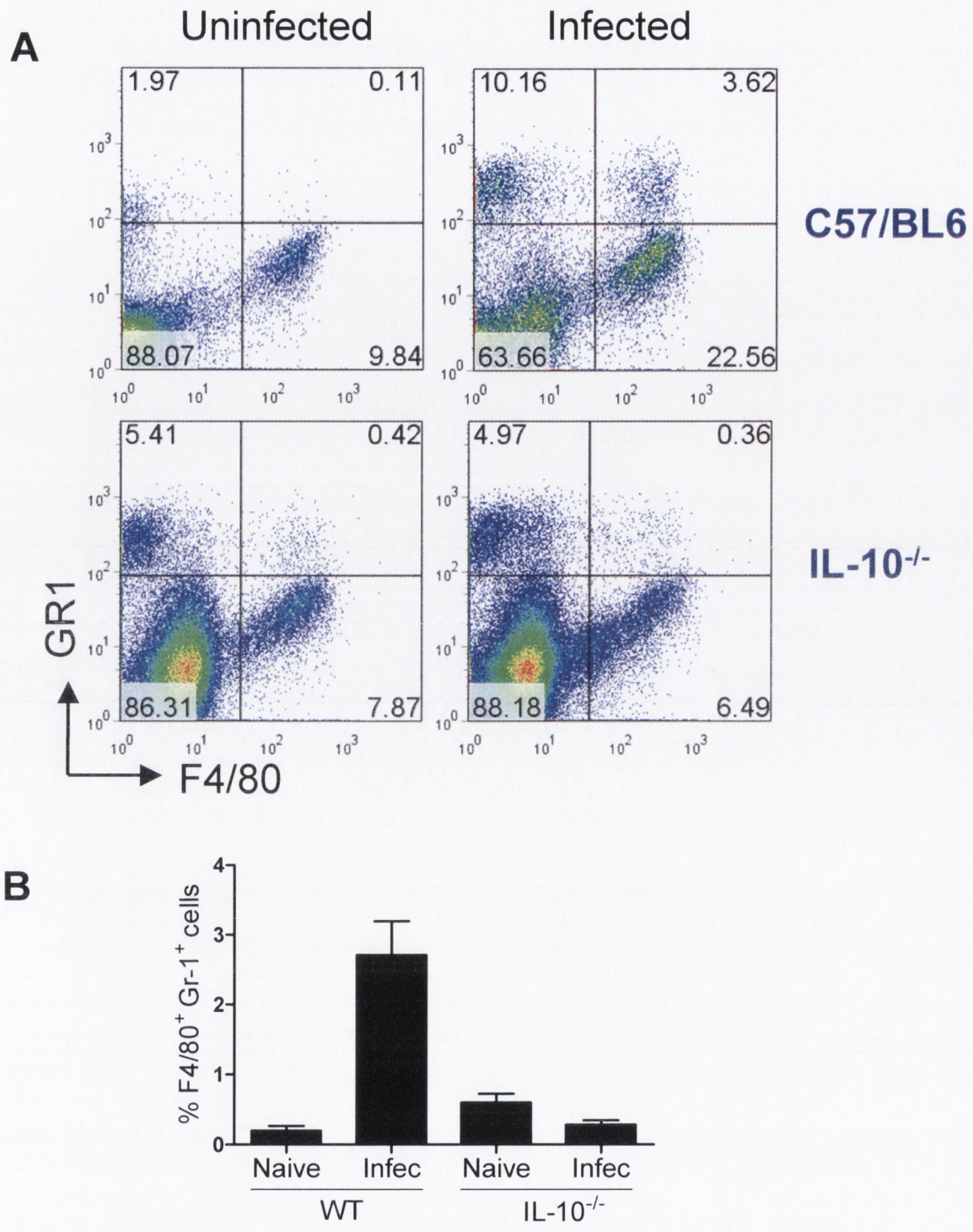


Fig 3.12 *F. hepatica* infection recruits F4/80$^+$ GR1$^+$ cells to the peritoneal cavity of WT but not IL-10$^{-/-}$ mice. C57BL/6 and IL-10$^{-/-}$ (on a C57/BL6 background) mice were infected with 10 viable metacercariae of *F. hepatica* (Cullompton strain). After 21 days PEC were isolated from naïve and *F. hepatica*-infected mice by peritoneal lavage. A, representative flow cytometric plots of F4/80 vs Gr-1, gated on total viable cells. B, The mean percentage infiltration of F4/80$^+$ Gr1$^+$ into infected and uninfected WT and IL-10$^{-/-}$ mice was determined by flow cytometry. Data is representative of 2 separate experiments.

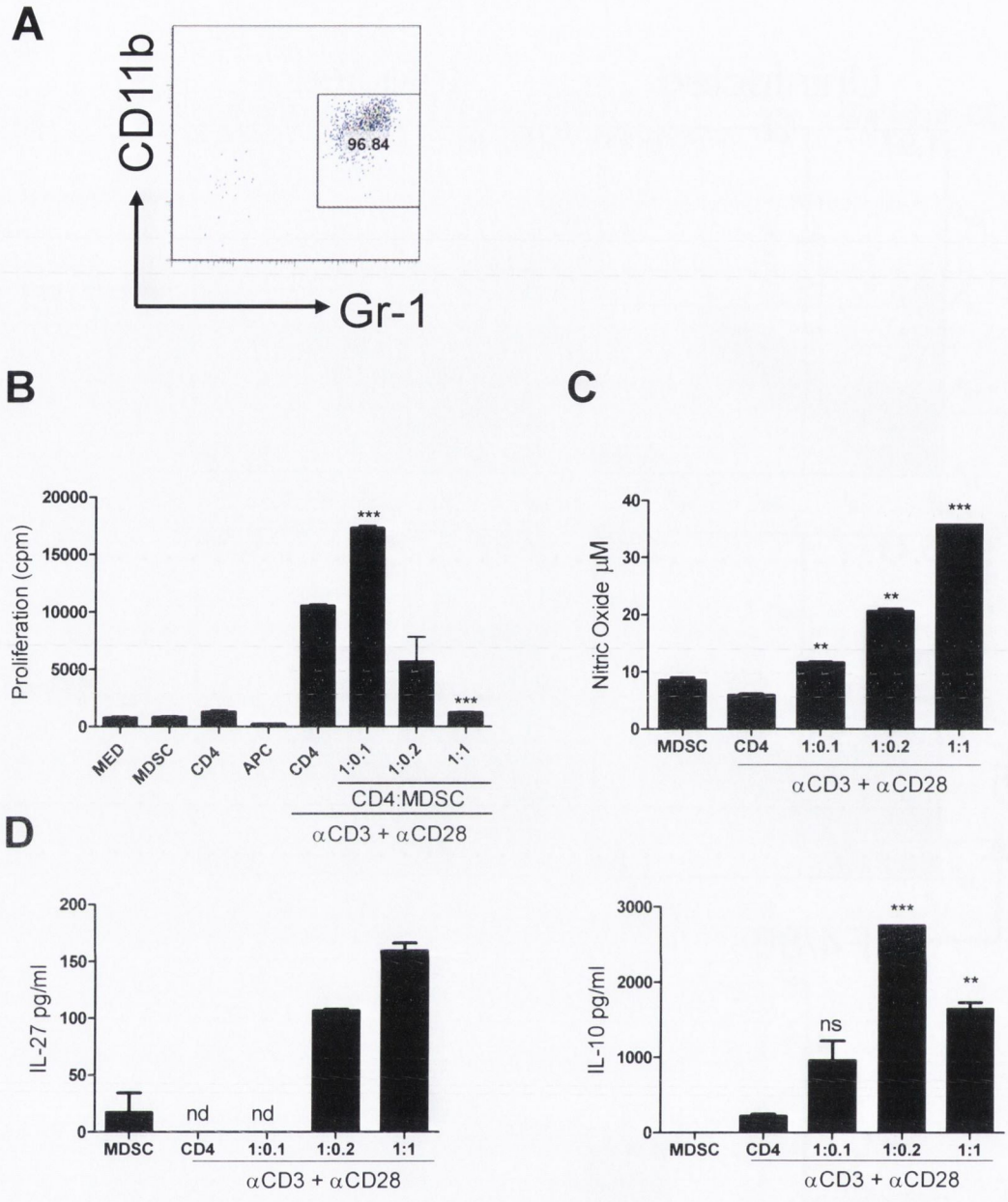


Fig 3.13 CD11b^{int} GR-1^{hi} cells isolated from *F. hepatica* infected mice suppress CD4^{+} T cell proliferation. C57BL/6 mice were infected with 10 viable metacercariae *F. hepatica* Cullompton strain. After 21 days PEC were isolated by peritoneal lavage and CD11b^{int} GR-1^{hi} MDSCs were isolated by flow cytometry. Purified cells were co cultured with CD4^{+} T cells isolated from naive mice and activated with plate bound αCD3 (1µg/ml) and αCD28 (2.5µg/ml) for 84 hours. A, purity of sorted MDSC population. B, Proliferation was measured by [^{3}H] thymidine incorporation during the final 12 hours of culture. Data is representative of 2 separate experiments. C and D, Supernatants were removed and cells were supplemented with fresh media at 72 hours. Concentration of Nitric oxide and cytokines (IL-27 and IL-10) were detected by Griess Assay and ELISA, respectively. ** $P<0.005$ vs naïve PEC. Data presented are means +/- SEM of triplicate assay.

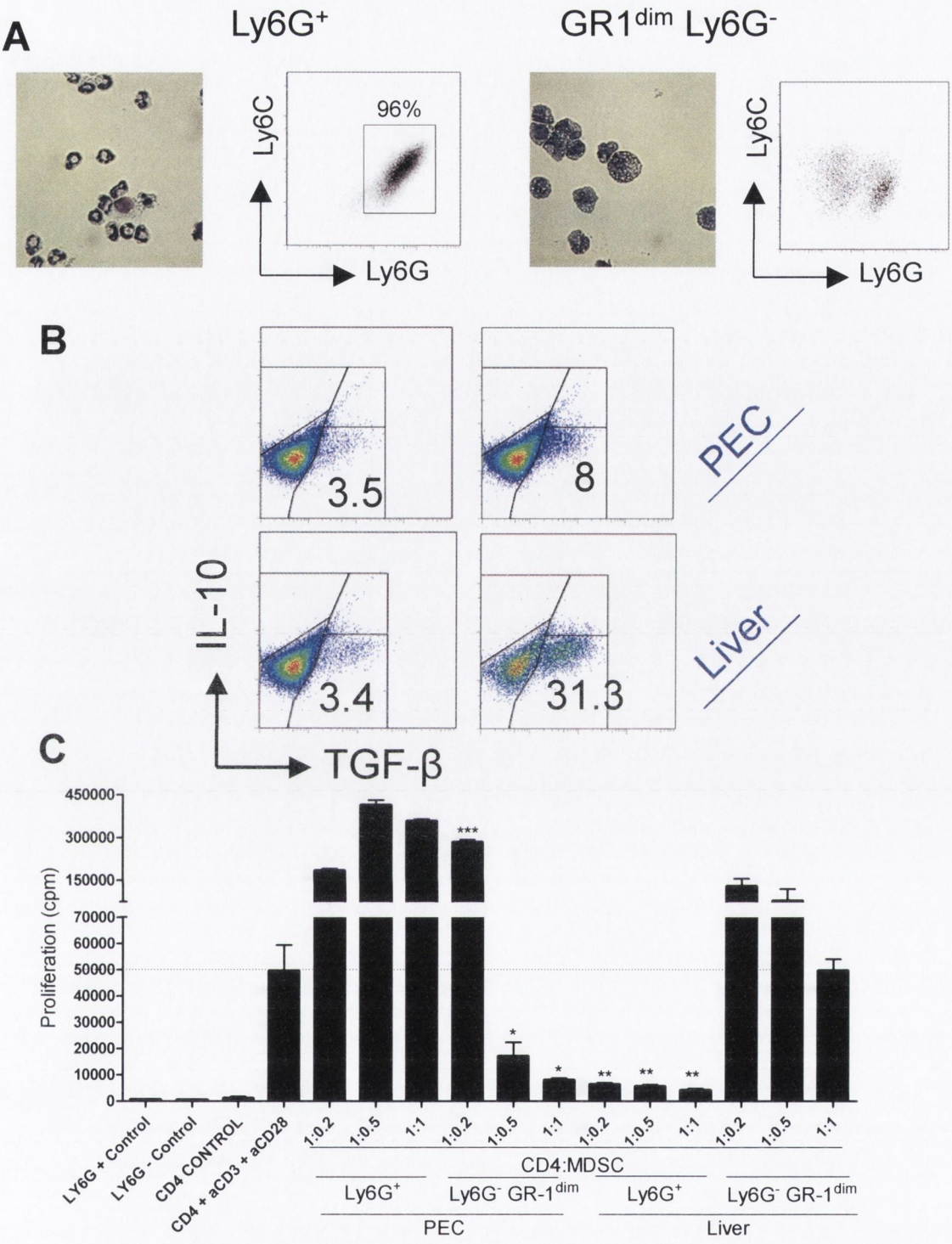
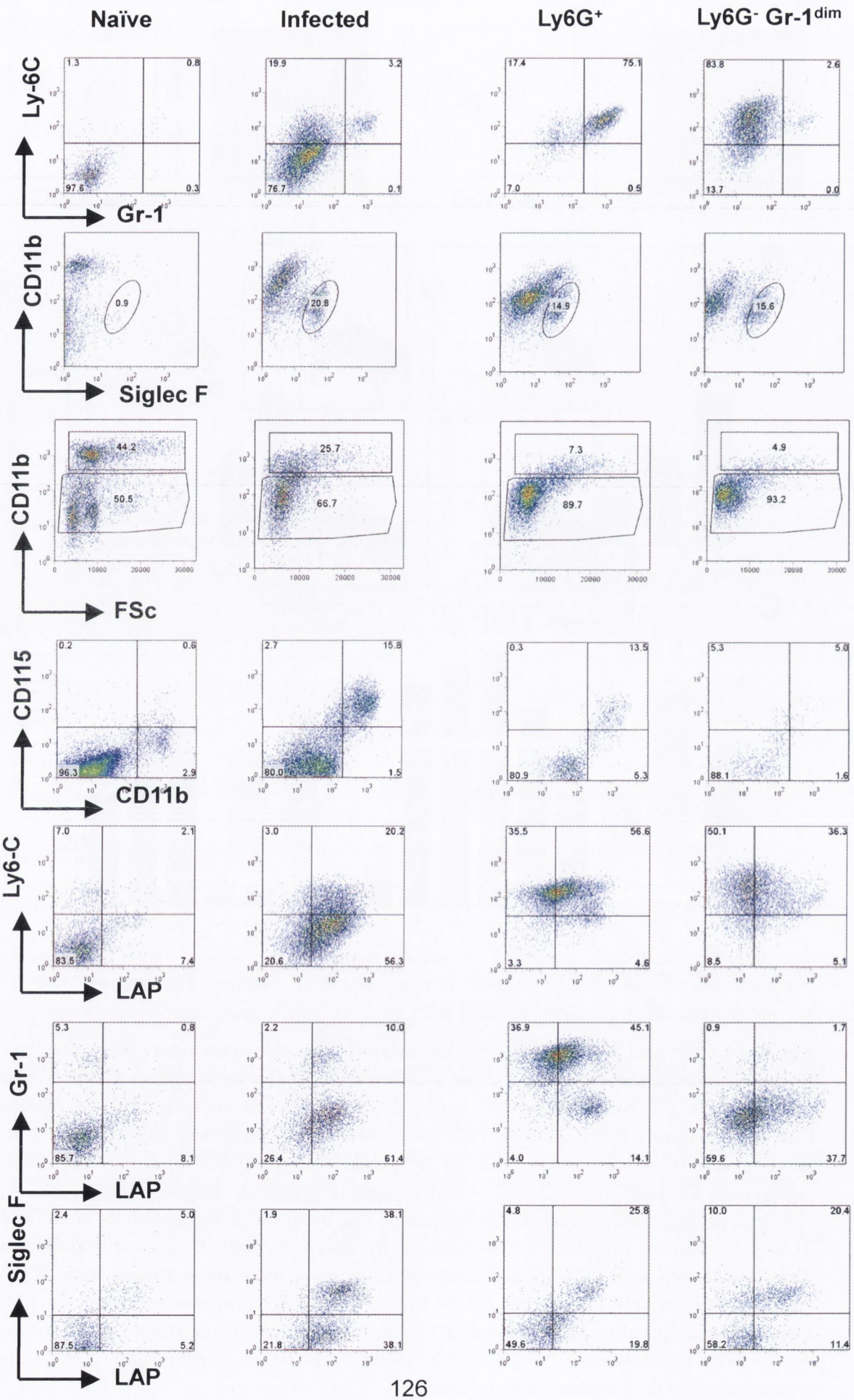


Fig 3.14 Ly6G$^+$ and Ly6G$^-$ GR-1^{dim} cells isolated from the peritoneal cavity and liver from *F. hepatica* infected mice show a differential ability to suppress CD4$^+$ T cell proliferation. C57BL/6 mice were infected with 10 viable metacercariae *F. hepatica* Cullompton strain. After 21 days PEC were isolated by peritoneal lavage while non-parenchymal hepatocytes were isolated by portal vein perfusion and digestion with collagenase D. Ly6G$^+$ and Ly6G$^-$ GR-1^{dim} were isolated by magnetic separation A, Cytospin preparations of sorted populations. B, The intracellular expression of IL-10 and TGF-β by purified populations was assessed by flow cytometry. C, Sorted populations were co cultured with CD4$^+$ T cells isolated from naive mice and activated with αCD3 (1μg/ml) and αCD28 (2.5μg/ml) for 84 hours. C, Proliferation was measured by [^{3}H] thymidine incorporation during the final 12 hours of culture. Data presented are means +/- SEM of triplicate assay. * P<0.01 ** P<0.005 *** P<0.001 vs stimulated CD4$^+$. Data is representative of 2 separate experiments.


Naïve
Infected
Ly6G+
Ly6G- Gr-1dim
Ly-6C
Gr-1
1.3
0.8
97.6
0.3
19.9
3.2
76.7
0.1
17.4
75.1
7.0
0.5
83.8
2.6
13.7
0.0
CD11b
Siglec F
0.9
20.8
14.9
15.6
CD11b
FSc
44.2
50.5
25.7
66.7
7.3
89.7
4.9
93.2
CD115
CD11b
0.2
0.6
96.3
2.9
2.7
15.8
80.0
1.5
0.3
13.5
80.9
5.3
5.3
5.0
88.1
1.6
Ly6-C
LAP
7.0
2.1
83.5
7.4
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20.2
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56.3
35.5
56.6
3.3
4.6
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36.3
8.5
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Gr-1
LAP
5.3
0.8
85.7
8.1
2.2
10.0
26.4
61.4
36.9
45.1
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14.1
0.9
1.7
59.6
37.7
Siglec F
LAP
2.4
5.0
87.5
5.2
1.9
38.1
21.8
38.1
4.8
25.8
49.6
19.8
10.0
20.4
58.2
11.4

Fig 3.15 Phenotypic analysis of myeloid-derived suppressor cells from naive and *Fasciola hepatica* infected mice. C57BL/6 mice were infected with 10 viable metacercariae *F. hepatica* Shrewsbury strain. After 24 hours PEC were isolated by peritoneal lavage. $Ly6G^+$ and $Ly6G^-$ $Gr\text{-}1^{dim}$ cells were isolated by magnetic separation. Naïve PEC, infected PEC and the purified populations, $Ly6G^+$ and $Ly6G^-$ $Gr\text{-}1^{dim}$ were surface stained for CD11b, Siglec-F, Ly6C, Gr-1, CD115 and LAP. Cells were analysed by flow cytometry. Plots representative of 4mice per group Data is representative of 2 separate experiments.

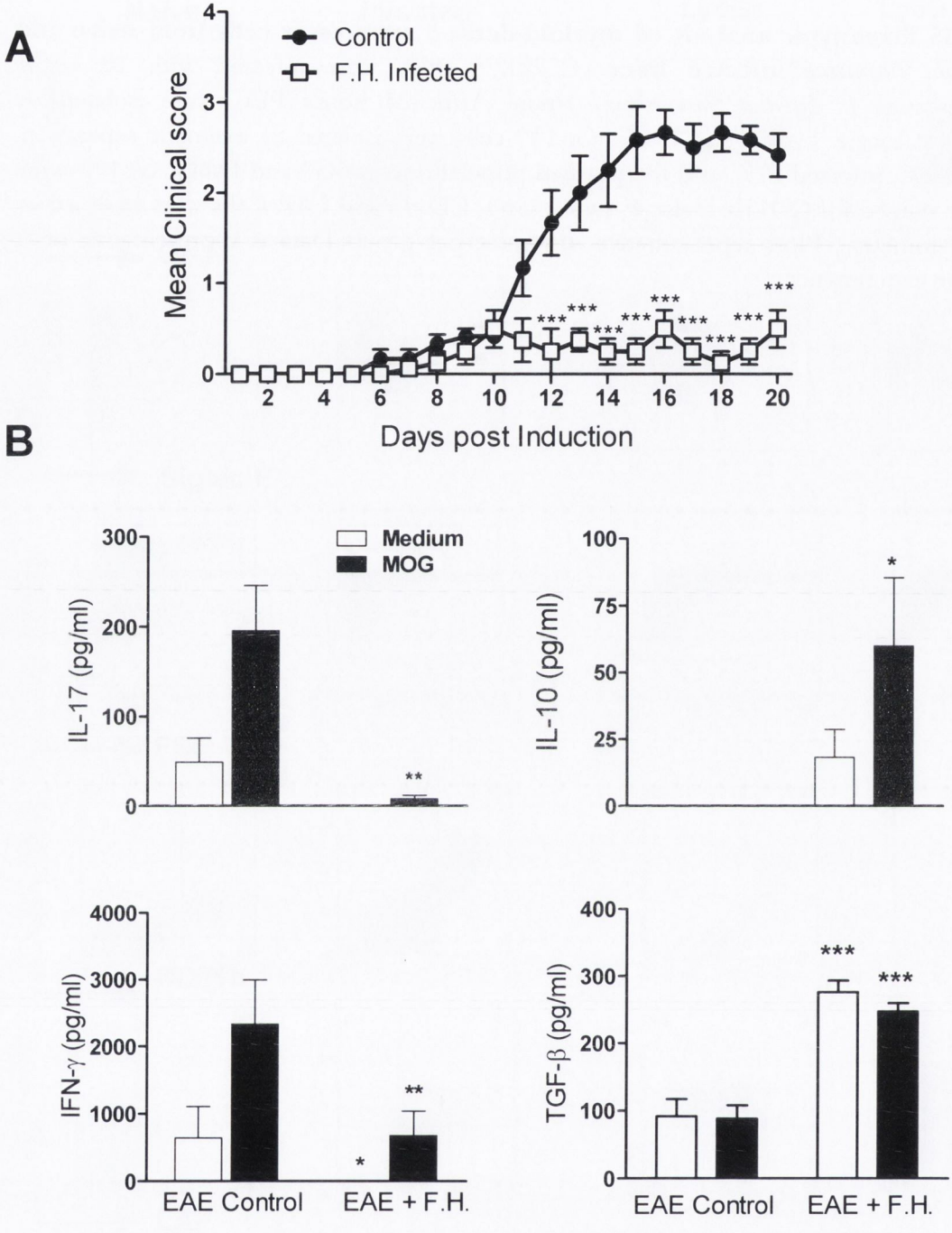


Fig 3.16 *F. hepatica* infection delays the onset and reduces the clinical severity of EAE. On day -1 C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica* (Cullompton strain) (□) or left untreated (●). On day 0 EAE was induced in both infected and uninfected mice by s.c. injection with 100 μg of MOG_{35-55} in CFA, followed by injection i.p. with 500 ng of PT on days 0 and 2. A, Clinical scores were monitored daily. B, Mice were sacrificed on day 20 and splenocytes were isolated and were activated with MOG antigen (100 μg/ml). After 72 hours the concentrations of IL-10, IL-17, IFN-γ and TGF-β were determined by ELISA. Data presented are means +/- SEM of 6 mice per group. Statistical assessment was performed using 2-way ANCVA for EAE and unpaired Students t test vs EAE control for cytokine concentrations. * $P<0.01$ ** $P<0.005$ *** $P<0.001$ vs uninfected. Data is representative of 4 separate experiments.

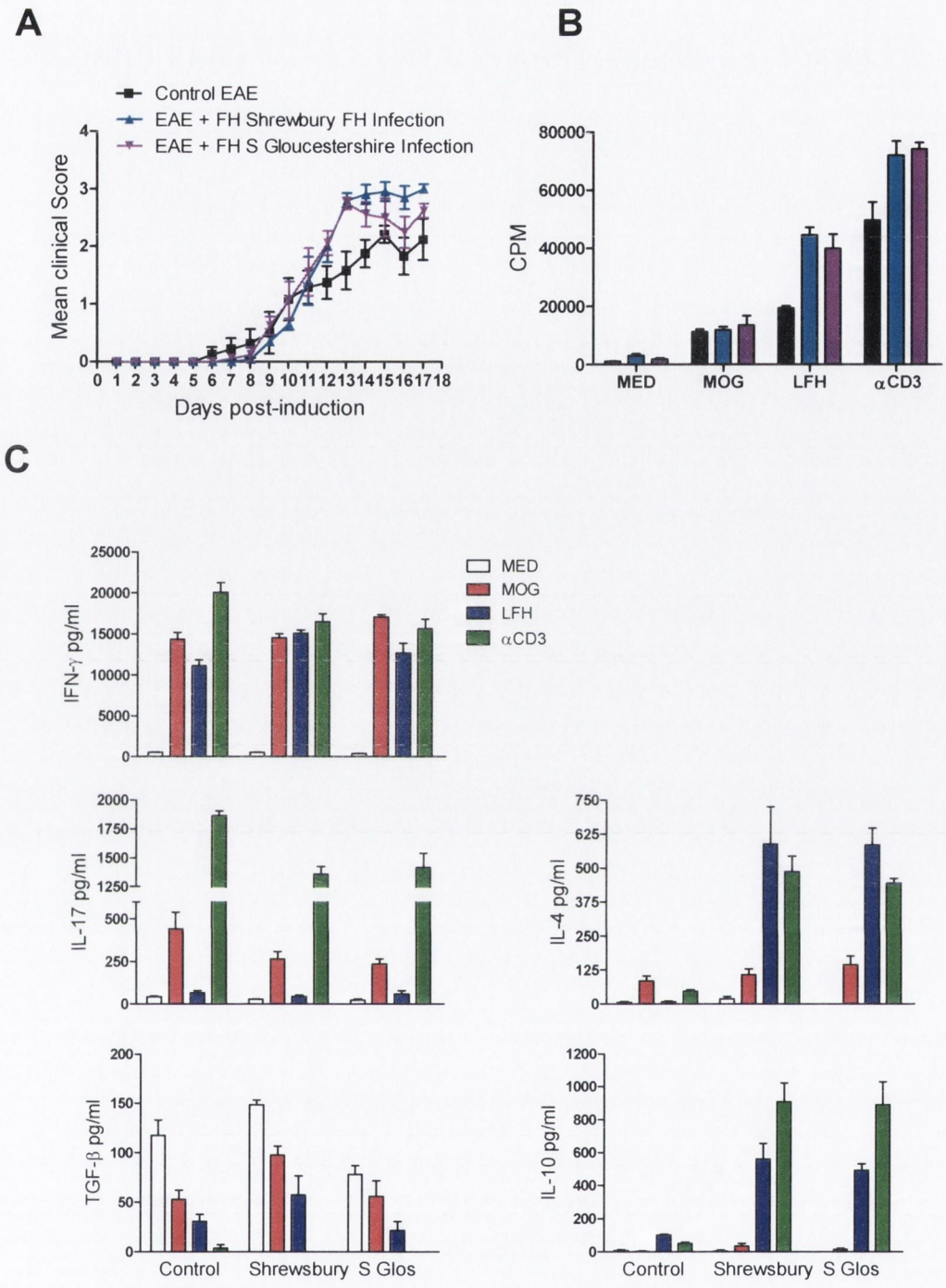


Fig 3.17 Infection with *Fasciola hepatica* Shrewsbury and South Gloucestershire strains fails attenuate EAE. On day -1 C57BL/6 mice were infected with 10 viable metacercariae of *F. Hepatica* Shrewsbury or South Gloucestershire strains. On day 0 EAE was induced in both infected and uninfected mice by s.c. injection with 100 μg of MOG_{35-55} in CFA, followed by i.p. administration with 500 ng of PT on days 0 and 2. A, Clinical scores were monitored daily. Data presented are means +/- SEM of 6 mice per group. On day 18 splenocytes were isolated and were restimulated *ex vivo* with MOG (50μg/ml), Liver-Fluke Homogenate (LFH, 50μg/ml), αCD3 (1μg/ml) or left unstimulated. B, cell proliferation was determined by [^{3}H] thymidine incorporation during the last 18 hours of a 72 hour culture. C, After 72 hours the concentrations of IFN-γ, IL-4, IL-10, IL-17 and TGF-β were quantified by ELISA. Data presented are means +/- SEM of triplicate assays.

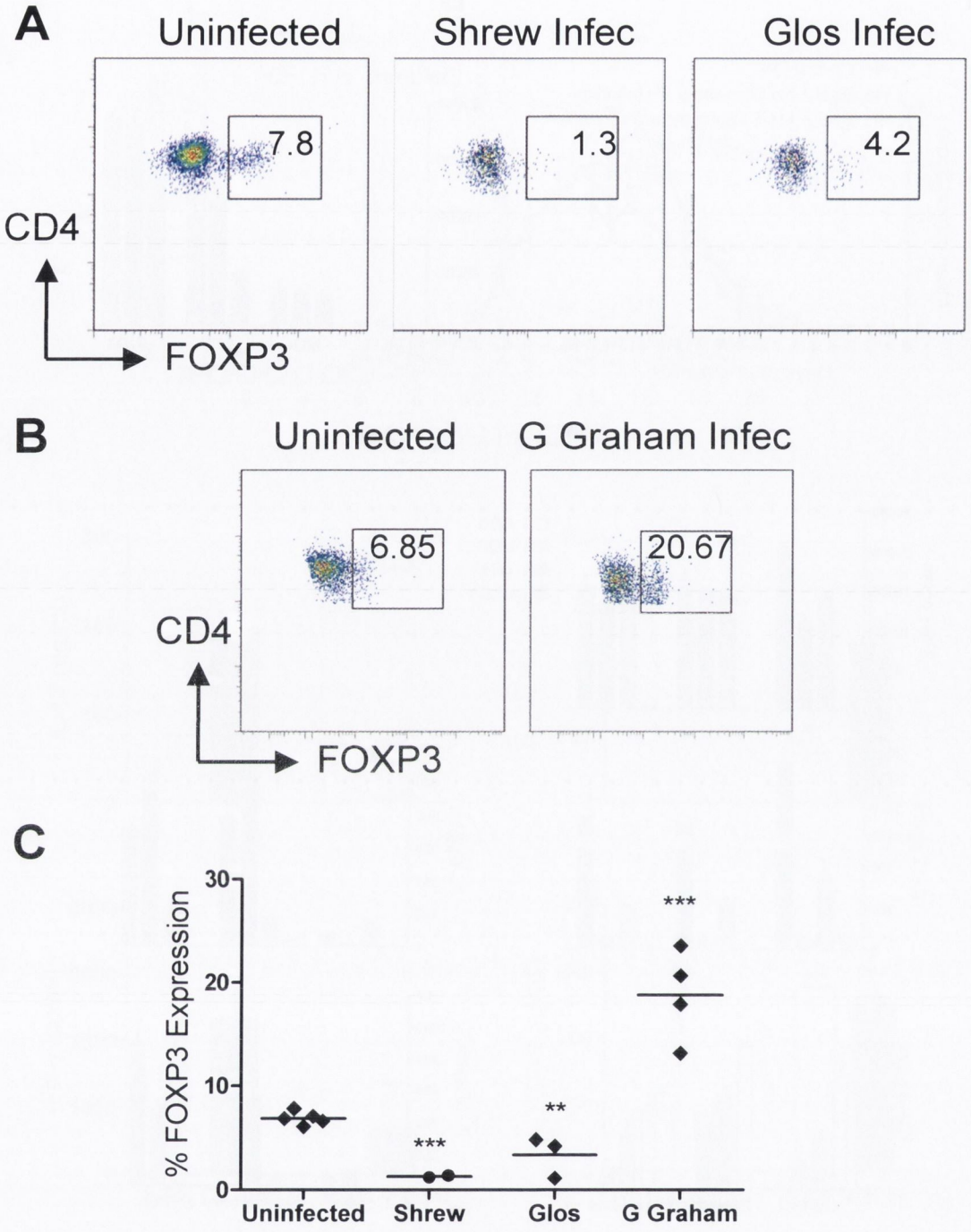


Fig 3.18 *F. hepatica* Shrewsbury and South Gloucestershire strains do not enhance the expression of FOXP3 by peritoneal CD4$^+$ cells. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica* Shrewsbury, South Gloucestershire and Cullompton strain strains. After 21 days PEC were isolated from infected and uninfected animals by peritoneal lavage. PEC were surface stained for CD4$^+$ and intra-nuclear stained for FOXP3. A, The percentage expression of FOXP3 by PEC CD4$^+$ T cells. B, FOXP3 expression of Gordon Graham strain infected mice was adapted from Figure 3.8. C, Data from both experiments were combined. Data presented are means +/- SEM of 4 mice per group. Statistical assessment was performed using unpaired Student's t test vs uninfected. * $P<0.01$ ** $P<0.001$ ** $P<0.005$

Chapter 4 The excretory/secretory products of *F. hepatica* modulate the APC function of DC and eosinophils

A. ES inhibits APC function

B. ES induces release of TGF-β

C. APC function of eosinophils

4.1 Introduction

The results of chapter 3 have demonstrated that infection with the Cullompton strain of *F. hepatica* is associated with immunoregulation and protection against the autoimmune disease, EAE. This was associated with the recruitment of cells with suppressive phenotypes, including MDSCs, immature APC and Treg cells into the peritoneal cavity.

The mouse is not a natural host of *F. hepatica*, as recognised by the significant mortality associated with infection. Thus, immune pathology induced during infection of mice may interfere with specific immunoregulatory mechanisms. Moreover, *F. hepatica* cannot survive outside its mammalian host, which prevents the study of specific interactions *in vitro*. Thus, the secreted products of *F. hepatica* represent a more viable option to study the immunoregulatory properties of the fluke. Indeed, ES from *F. hepatica* has already been shown to possess immunomodulatory properties (261-263). However, many of the effects of ES on innate immune cells remain poorly understood. To address this question, the major aim of this chapter was to investigate the effect of *F. hepatica* ES on innate cells with APC function. Specific aims include:

1) **To determine whether *F. hepatica* ES can modulate the APC function of DC**

Several reports have demonstrated that *F. hepatica* ES can suppress TLR-agonist induced pro-inflammatory cytokine production by DC, while only partially maturing DC (258, 328). However, the effect of ES on the APC function is unclear. Thus, this study investigated the effect of *F. hepatica* ES on cytokine production, maturation and APC function of DC *in vitro* and *in vivo*.

2) **To determine the effect of *F. hepatica* ES on the expression of TGF-β by innate immune cells**

The role of TGF-β in immune suppression is well established. Several species of helminths promote the production of host TGF-β as a method of survival (169, 176). Indeed, other helminth spp, such as *Onchocerca spp and B. malayi* secrete a bioactive homologue of TGF-β (173). PBMC from animals infected with liver fluke produce large amounts of TGF-β (329).

The results from chapter 3 have demonstrated that *F. hepatica* infection enhanced the frequency of TGF-β secreting macrophages, DC and Treg cells in the peritoneal cavity. In addition, *F. hepatica* Cullompton strain infection protected mice from EAE in a TGF-β dependent manner (298). However, the role of ES in promoting TGF-β is unknown. Thus, this study investigated the production of TGF-β by innate immune cells.

3) To determine whether eosinophils recruited by *F. hepatica* infection or administration of ES can function as APC

The results presented in chapter 3 has shown that infection of mice with *F. hepatica* was associated with the recruitment of eosinophils in the peritoneal cavity. There is a limited, but growing body of evidence to suggest that eosinophils express MHC-II and can act as APC (330-333). Thus, this study investigated whether eosinophils recruited during infection with *F. hepatica* or following administration of ES displayed APC function.

4.2 Results

4.2.1 ES inhibits TLR agonist-induced inflammatory cytokine production by BMDC

Polarising cytokines provide the third signal required for T cell activation; directing differentiation of naïve T cells into distinct functional lineages. DC are in immediate proximity to T cells during activation and are the main source of these cytokines. ES products of *F. hepatica* were shown by Falcón et al to block TLR agonist-induced production of IL-12p70, IL-6, TNF-α and IL-10 by BMDC (263). The aim of this study was to examine the effect of *F. hepatica* ES on APC function. BMDC were treated with LPS, ES or a combination of both and the concentrations of IL-12p70, IL-1β IL-6 and IL-23 in the supernatants after a 24 hour culture were quantified by ELISA. ES completely abolished LPS-induced production of IL-12p70 and significantly reduced the concentration of IL-1β, IL-6 and IL-23 [Fig 4.1]. This demonstrates that ES can modulate the production of pro-inflammatory cytokines by DC in response to a TLR-4 agonist.

4.2.2 ES does not inhibit phagocytosis or antigen processing by APC

Phagocytosis and antigen processing are essential processes that precede antigen presentation by APC. As such, inhibition of either process would subsequently inhibit APC function. Dowling *et* al demonstrated that two *F. hepatica* ES-derived enzymes, cathepsin L and the antioxidant sigma-class glutathione transferase could inhibit the LPS-induced phagocytosis of sheep erythrocytes by murine DC *in vitro* (334). Other helminths have been shown to inhibit the antigen processing ability of DC, for example, cysteine protease inhibitors derived from a filarial helminth inhibits asparaginyl endopeptidase, an enzyme required for antigen processing in the MHC Class II pathway (335).

Experiments were conducted to determine if ES could affect phagocytosis. BMDC were incubated with ES, LPS or ES + LPS together with an excess of YG-fluorescent-labelled latex microbeads. After 30 min, cells were washed and analysed by flow cytometry for YG-fluorescence, as a positive indicator of phagocytosis. ES significantly enhanced YG-fluorescence by $CD11c^+$ cells *in vitro* [Fig 4.2], demonstrating that ES promotes phagocytosis. Experiments were next undertaken to assess the effect of ES on phagocytosis *in vivo*. YG-

fluorescent-labelled latex microbeads together with 10 μg of LPS were injected, with or without ES, into the footpads of C57BL/6 mice. After 24 hours the popliteal LN were excised and the percentage YG-fluorescence by $CD11b^+$ and $CD11c^+$ cells was quantified by flow cytometry. Consistent with the findings *in vitro,* administration of ES did not inhibit phagocytosis [Fig 4.2].

Antigen processing by APC involves the degradation of proteins into peptides of suitable length to bind to MHC molecules. Phagosomes, containing newly phagocytised material, merge with lysosomes to form phagolysosomes, in which exogenous protein antigens are cleaved by the resultant low pH and presence of proteolytic enzymes (336). Experiments were conducted to assess the effect of ES on antigen processing. Antigen processing was quantified using DQ-OVA (molecular probes, Life technologies), OVA protein containing BOPIDY dye molecules which are trapped in the hydrophobic interior of the protein, the quenched dye fluoresces only after protein degradation. Thus, fluorescence by a cell can be used as an indicator of successful protein degradation within the phagolysosome. BMDC were treated with ES, LPS or ES+LPS in combination for 18 hours. Cells were then incubated with DQ-OVA and percentage BODIPY fluorescence was measured by live confocal microscopy. Addition of ES did not change the intensity of florescence 40 min post addition of DQ-OVA compared with untreated controls [Fig 4.3]. To better quantify and study the kinetics of antigen processing, BMDC were pre-treated with PBS or ES for 6 hours prior to addition of DQ-OVA. BODIPY fluorescence was measured by flow cytometry over time for 1 hour. Pre-treatment with ES enhanced antigen processing of DQ-OVA [Fig 4.3]. Taken together these results show that ES does not negatively affect phagocytosis or antigen processing by APC.

4.2.3 ES inhibits LPS-induced maturation of DC

This study has shown that ES inhibited LPS-induced production of pro-inflammatory cytokines by DC [Fig 4.1], suggesting that ES affects the activation and function of these cells. A series of experiments were conducted to assess the effect ES had on the activation state and APC capacity of DC following incubation with the model antigen, keyhole limpet hemocyanin (KLH) with or without LPS, as a maturation stimulus.

Activation by TLR ligands results in the maturation of DC which is associated with enhanced expression of MHC-II and co-stimulatory molecules including CD40 and CD80/86. This process is required to effectively prime naïve T cells (38). DC which fail to enhance expression of co-stimulatory molecules are said to be tolerogenic and do not activate T cells. Tolerogenic DC also promote the induction of Treg cells (337).

The activation state of DC was determined by the expression of MHC-II, CD40, CD80 and FcεR1 following exposure to combinations of LPS, KLH and ES. The costimulatory molecules CD80, C86 and CD40 are associated with the activation of Th1 and Th17 cells (338, 339), while $FC\varepsilon R1^{+}$DC have been shown preferentially induce type-2 responses (68).

Compared with untreated controls the treatment of DC with KLH partially enhanced the expression of CD40, CD80 and MHC-II but not FcεR1 [Fig 4.4]. Meanwhile, DC treated with ES in combination with KLH had reduced expression of CD40 and MHC-II, similar expression of CD80 and a slight enhancement of FcεR1 compared with DC treated KLH alone. LPS in combination with KLH greatly enhanced the expression of CD40, CD80 and MHC-II by DC [Fig 4.4]. However, a 30 min pre-treatment with ES inhibited the LPS-induced expression of CD40, CD80 and MHC-II; in contrast, pre-treatment with ES, enhanced the expression of FcεR1 [Fig 4.4]. These results demonstrate that ES reduces the ability of DC to mature in response to activation with a TLR-4 agonist.

4.2.4 ES inhibits the ability of BMDC to drive antigen-specific T cell proliferation *in vitro*

The present study has demonstrated that ES inhibited TLR-induced maturation and pro-inflammatory cytokine release by DC [Fig 4.1 and 4.4], suggesting that ES-treated DC may be ineffective at priming T cells. Furthermore, Falcón et al have reported that ES-treated DC supported the production of IL-4, IL-5 and IL-10 and TGF-β by T cells (263). In addition, ES suppressed IFN-γ production during a mixed lymphocyte reaction (263). In contrast, a follow-up study by Falcón et al demonstrated that pre-treatment of DC with ES prior to the addition of LPS generated a Th1 response that protected against fasciolosis in mice (340). To clarify and expand upon these findings, the APC capacity of ES-treated DC was studied *in vitro* and *in vivo*.

In vitro; BMDC were treated with medium, ES, LPS or ES+LPS in combination, in the presence or absence of the antigen KLH. After 24 hours, DC were co-cultured with KLH-specific $CD4^+$ T cells, which were purified from the spleens and LNs of mice previously immunized with KLH and LPS. In a 5 day culture, KLH+ES-treated DC significantly inhibited the proliferation of T cells compared with KLH controls. KLH+ES+LPS-treated DC also induced significantly less T cell proliferation compared with DC treated with KLH+LPS. In addition, KLH+ES+LPS-treated DC induced significantly less IFN-γ by T cells in response to KLH compared with DC treated with KLH+LPS [Fig 4.5]. However, this co-culture produced significantly more IL-17 and IL-10 [Fig 4.5]. Finally, the concentration of TGF-β was increased in all cultures containing DC treated with ES which was independent of the presence of T cells or KLH [Fig 4.5].

In vivo: functional APC-T cell co-cultures *in vitro* while informative, lack the complexities provided by the tissue micro-environment of the host which shape the generation of T cell responses *in vivo*, therefore experiments were conducted to assess the effect of ES on APC function *in vivo*. DC were treated with combinations of KLH, ES and LPS; after 24 hours, DC were washed and injected into the footpads of C57BL/6 mice. Seven days later the LN cells were excised and cultured with KLH to assess KLH-specific proliferation and cytokine production. Adoptive transfer of DC treated with KLH+ES induced significantly less KLH-specific proliferation of T cells in a 5 day culture compared with DC treated with KLH alone [Fig 4.6]. Similarly, T cells from mice that received KLH+ES+LPS-treated DC proliferated less in response to KLH compared with those that received KLH+LPS treated DC, however this reduction was not statistically significant [Fig 4.7]. T cells from recipients of KLH+ES+LPS-treated DC produced significantly less KLH-specific IL-17 than those that received KLH+LPS-treated DC. Moreover, there was also a non-significant reduction in IFN-γ [Fig 4.6].

To address any strain differences, similar experiments were performed in BALB/c mice using OVA as the antigen. DC were treated for 24 hours with combinations of antigen, ES or LPS before being transferred into BALB/c mice by s.c. injection of the footpad. After 7 days, the popliteal LN cells were excised and were reactivated with OVA *ex vivo*. After a 4 day culture, the LN cells of mice that received DC treated with OVA+ES+LPS produced significantly less IFN-γ and IL-5 compared with those mice that received OVA+LPS-treated DC [Fig 4.7].

There was also a non-significant reduction in the concentrations of KLH-specific IL-4, IL-10, IL-13 and IL-17 [Fig 4.7].

4.2.5 ES decreases the expression of co-stimulatory molecules on macrophages and DC *in vivo* and inhibits their ability to act as APC

This study has shown that treatment of BMDC with ES *in vitro* inhibits the APC capacity of these cells *in vitro* and *in vivo* [Fig 4.5-7]. However, the effect of ES on APC *in vivo* has not been addressed. Specifically, to examine the effect of ES on DC maturation *in vivo,* mice were injected i.p. with ES or PBS daily, for five days. PEC and MLN cells were isolated from these mice and the expression of CD40 and CD80 by $CD11b^+$, $CD11c^+$ and $F4/80^+$ cells was determined by flow cytometry. Administration of ES decreased the expression of CD40 and CD80 all 3 populations in the peritoneal cavity [Fig 4.8]. In contrast, the expression of CD40 and CD80 was not affected on mesenteric LN $F4/80^+$, $CD11b^+$ or $CD11c^+$ cells [Fig 4.8].

Since the high expression of co-stimulatory molecules is required for effective antigen presentation, and given that ES-treated BMDC were less effective as APC, experiments were conducted to compare the APC-capacity of peritoneal myeloid cells isolated from mice treated with ES with those from mice treated with PBS. $CD11b^+$ cells were purified from the peritoneal cavity of mice treated with either PBS or ES, daily for five days. Purified cells from ES-treated mice had lower expression of MHC-II and CD86 compared with PBS-treated controls. Gating on $F4/80^+$ or $CD11c^+$ within the purified $CD11b^+$ fraction revealed that the expression of MHC-II and CD86 was also reduced on these populations [Fig 4.9 A]. Purified peritoneal $CD11b^+$ cells were co-cultured *in vitro* with purified $CD4^+$ T cells isolated from mice with EAE. MOG peptide was added to co-cultures to re-activate MOG-specific T cells and MOG-specific T cell proliferation and cytokine production were determined after a 4 days culture. $CD11b^+$ cells isolated from mice treated with ES were significantly less capable at inducing antigen-specific T cell proliferation compared with those from mice treated with PBS [Fig 4.9 B]. In addition, production of antigen-specific IL-17 and IL-10 were significantly reduced, while IFN-γ was non-significantly reduced [Fig 4.9 B].

Taken together these data demonstrate that ES-treatment of mice suppressed the expression of co-stimulatory molecules on macrophages and DC *in vivo,* and inhibited their ability to activate T cell responses *ex vivo.*

4.2.6 ES induces the release of TGF-β from DC

The present study has shown that infection with *F. hepatica* is associated with the accumulation of macrophages and T cells that expressed TGF-β [Fig 3.4 and 3.9]. A published study from this laboratory reported that infection with *F. hepatica* protects mice from EAE in a TGF-β-depended manner (298). This study also showed that ES blocked the LPS-induced production of pro-inflammatory cytokines by BMDC [Fig 4.1]. Experiments designed to test the APC-capacity of ES-treated DC revealed that ES induced an increase in the concentration of TGF-β, which appeared to be produced solely by BMDC [Fig 4.5]. To investigate this further, BMDC were incubated with increasing concentrations of ES in the presence and absence of LPS. The concentrations of TGF-β and IL-10 in the supernatants after 24 hours was determined by ELISA. While addition of ES had no effect on the production of IL-10, there was a significant and dose-dependent increase in the production of TGF-β with or without LPS [Fig 4.10 A]. LPS alone did not induce the production of TGF-β [Fig 4.10 A]. In contrast, LPS alone induced the release of IL-10, ES only enhanced LPS-induced IL-10 at the highest dose of ES employed (5% v/v) [Fig 4.10 A]. ES also induced the release of TGF-β by BMM [Fig 4.10 B].

To study the kinetics of TGF-β release, BMM and BMDC were incubated with ES (5% v/v) for 0, 2, 4 and 6 hours. After 2 hours, the concentration of TGF-β was significantly increased in the supernatants of both BMM and BMDC but did not increase thereafter and was unchanged between the 2 hour and the 4 or 6 hour time points [Fig 4.10 B].

BMDC are an *ex vivo* generated cell type that phenotypically and developmentally resemble myeloid DC. The other major population of DC *in vivo* is the plasmacytoid DC (pDC). pDC are associated with defence against viruses and specialise in rapid secretion of type-I IFNs in response to foreign nucleic acids (341). Experiments were conducted to determine if pDC responded to ES in a similar manner to mDC. Mice were treated i.p. with ES for 6 days and the recruitment of mDC and pDC into the peritoneum on day 7 was determined by flow

cytometry. There was minor increase in the proportion of both $CD11b^{+}$ $CD11c^{high}$ $B220^{-}$ mDC and $CD11c^{low}$ $B220^{+}$ pDC in the peritoneal cavity of ES-treated mice [Fig 4.11, A]. A population of cells that resemble pDC can be generated from bone marrow using the cytokine FLT3L (341). FLT3L-generated pDC were treated for 24 hours with combinations of LPS, CpG and ES. Similar to BMDC, FLT3L-pDC secreted TGF-β in response to ES [Fig 4.11, B]. CpG alone also induced the TGF-β production in the supernatant. A combination of ES and CpG further enhanced the secretion of TGF-β compared with ES or CpG alone [Fig 4.11, B]. While BMDC failed to produce IFN-β in response to any stimulus, FLT3L-pDC produced IFN-β in response to LPS or CpG. CpG stimulated pDC produced significantly more IFN-β compared with pDC stimulated with LPS [Fig 4.11 B]. Interestingly, while ES blocked LPS-induced IFN-β production it enhanced CpG-induced IFN-β.

Taken together these data demonstrate that ES induces the rapid release of TGF-β by mDC, pDC and macrophages.

4.2.7 ES does not induce the transcription or the translation of TGF-β

ES induced the rapid release of TGF-β from macrophages and DC, with concentrations peaking at 2 hours and remaining unchanged thereafter [Fig 4.10]. Two hours is a relatively short period of time to allow for the signal transduction, mRNA transcription and protein translation required for the release of cytokine. While some cytokines can be transcribed, translated and secreted within 2 hours, most cytokines display slower kinetics. For instance Deforge et al reported that the peak expression of *tnf-a* mRNA and TNF-α protein by human PBMC occurs at 3 hours and 4 hours post stimulation with LPS respectively. Moreover, the expression of IL-6 mRNA peaked 4 hours and IL-6 protein did not reach its maximum until 24 hours post stimulation with LPS (342). Avrå et al reported that human PBMC did not induce *il-6*, *ifn-γ, il-10, il-12* or *tnf-β* mRNA expression until 4 hours post incubation with *Streptococcus pneumonia* and while the expression of *il-1β* and *tnf-a* mRNA was enhanced it was 10-fold less than that at 24 hours (343). RTPCR analysis was performed on BMDC to determine the kinetics of *tgf-β* gene expression following treatment with ES. Surprisingly, Relative to 18s RNA, *tgf-β* mRNA did not increase in BMDC at 2, 4 or 6 hours post-incubation with ES [Fig 4.12].

Having shown that ES does not induce transcription of *tgf-β* mRNA, an experiment was conducted to determine if ES induced the translation of TGF-β protein. BMDC were incubated with ES, LPS, or ES+LPS in the presence or absence of cycloheximide. Cycloheximide, is an inhibitor of protein biosynthesis in eukaryotes; it interferes specifically with protein translation by preventing the movement of tRNA within the ribosome (344). The addition of cycloheximide prevented the release of IL-12p40 into the supernatant following a 24 hour stimulation with LPS to DC [Fig 4.13]. ES in the presence or absence of LPS increased the concentrations of TGF-β in the supernatant after 24 hours. However, unlike IL-12p40, the release of TGF-β was not affected by the presence of cycloheximide [Fig 4.13]. Importantly while cycloheximide did result in cell death, as determined by percentage of PI^+ cells, ES-alone did not [Fig 4.13]. These results demonstrate that ES did not induce the transcription of *tgf-β* mRNA or translation of TGF-β protein, suggesting that ES induced the release of pre-formed TGF-β by an unknown mechanism.

4.2.8 Release of TGF-β is not mediated by pH or cathepsins

This study has shown that ES induces the rapid release of TGF-β from DC which is independent of the transcription or the translation of the gene or protein [Fig 4.10-13]. Physiologically, TGF-β is secreted in a latent form as a complex with latent TGF-β binding protein and latency-associated peptide (LAP) (345). The biologically active form of TGF-β must be cleaved from these other proteins. While the mechanisms behind activation are not fully understood, proteases, integrins, pH changes, and ROS have been all shown to promote the release of active TGF-β (345, 346).

Acidic conditions can denature LAP, treatment of latent-TGF-β with extremes of pH or mild acidic treatment, have been shown to activate TGF-β (347). ES has a pH of 5.5 [Fig 4.14 A]. In culture at the dilutions in RPMI used in this study, ES lowered the pH of the medium, although this could be somewhat offset by supplementation with HEPES. To determine if the pH of ES mediated the production of TGF-β, BMDC were incubated with increasing concentrations of native (pH 5.5) or neutralised ES (pH 7.2) in the presence or absence of LPS. Neutralised ES induced the production of similar concentrations of TGF-β compared with native ES [Fig 4.14 B]. The ability of neutralised ES to inhibit LPS-induced production

of IL-12p70 was comparable with that of native ES [Fig 4.14 B]. These results demonstrate that release of TGF-β by DC following treatment with ES is not mediated by changes in pH.

Helminths, including *F. hepatica* secrete a variety of the cysteine proteases known as the cathepsins which have roles in host entry, tissue migration and immunomodulation (348). Since it has been reported that TGF-β can be activated by proteases (345), experiments were conducted to test if cathepsins, contained within ES can mediate release of TGF-β. BMDC were incubated with increasing concentrations of ES in the presence and absence of cycloheximide and L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64). E-64, originally derived from *Aspergillus japonicas,* is a cathepsin inhibitor with a wide range of specificities (349). The addition of E-64 had no effect on the ES-induced increase in TGF-β in the supernatant [Fig 4.15], suggesting that release of TGF-β by DC following treatment with ES is not mediated by the activity of cathepsins.

4.2.9 ES induces an oxidative stress response which mediates the release of TGF-β from DC

Jedlina et al have recently reported that peritoneal macrophages, neutrophils and eosinophils from rats infected with *F. hepatica* produced H_2O_2 (350). Since ROS has been reported to activate TGF-β, experiments were conducted to determine if ES induces the production of ROS that mediates release of TGF-β. The human lung epithelial cell line A549 has been extensively used to assess the production of ROS (351). A549 cells were incubated with medium alone, ES for 18 hours or with TBHP (ROS inducer) for 2 hours before being incubated with the oxidative-dependent fluorescent dye CH2-DCFDA and imaged by confocal microscopy. As determined by CH2-DCFDA fluorescence, untreated cells were negative for ROS, while all TBHP-treated cells were positive. Pre-treatment with ES induced medial fluorescence of CH2-DCFDA by A549 cells indicating the presence of ROS [Fig 4.16]. Next, DC were tested to determine if they upregulated hemeoxygenase-1 (*HMOX-1*) a gene associated with the oxidative-stress response (352). ES-treated DC significantly enhanced the relative expression of *HMOX-1* mRNA at 2 and 4 hours post addition of ES and non-significantly enhanced the expression at 6 hours.

Having shown that ES induces ROS in epithelial cells, an experiment was conducted to determine if ROS mediated the ES-induced release of TGF-β. BMDC were incubated with increasing concentrations of ES with combinations of LPS and n-acetylcysteine (NAC). NAC, a derivative of cysteine and a precursor in the formation of glutathione in the body is a powerful antioxidant itself. NAC has been shown to reduce ROS *in vitro* and *in vivo* and is commonly used as the standard against which to test the antioxidant properties of other compounds (353, 354). BMDC were incubated with increasing concentrations of ES in combinations with LPS and/or NAC. The presence of NAC significantly reduced ES-induced TGF-β release in the absence of LPS. In the presence of LPS, NAC also significantly reduced the release TGF-β at all concentrations of ES tested, except the highest (5% v/v) [Fig 4.17]. Taken together, these data demonstrate that ES induces the release of TGF-β by BMDC via ROS production.

4.2.10 Administration of ES *in vivo* recruits cells to the peritoneal cavity

Most investigations have studied the effect of ES in the context of cells treated *in vitro* largely ignoring the effects *in vivo*. To determine the effects of ES *in vivo*, ES or PBS was i.p administered to C57BL/6 mice, daily for 5 days. On day 6, the expression of CD11b and F4/80 on PEC and mediastinal LN cells was determined by flow cytometry. Consistent with results obtained during infection with *F. hepatica*, administration of ES significantly increased the numbers of cells isolated from the peritoneal cavity [Fig 4.18]. The number of cells isolated from the mediastinal lymph nodes was also enhanced, although this was not significant [Fig 4.18].

The peritoneal cavity of a naïve mouse contains a high proportion of large peritoneal macrophages (LPM), which are known, upon exposure of the peritoneal cavity to TLR-agonists, to rapidly decrease, being replaced by inflammatory monocytes which subsequently differentiate into small peritoneal macrophages (SPM) (355). Following treatment with ES there was a disappearance of $CD11b^{high}$ $F4/80^{high}$ LPM, decreasing from 5.2% in PBS treated animals to 0.6% in ES treated animals. Meanwhile, the proportion of $CD11b^{int}$ $F4/80^{int}$ SPM increased from 6.3% to 11.4% [Fig 4.18]. In keeping with the displacement of LPM with SPM, an examination of the expression CD11b by histogram plot revealed three peaks of CD11b expression corresponding to $CD11b^{low}$ $CD11b^{int}$ $Cd11b^{high}$, in PEC from both PBS

and ES treated mice. Following treatment with ES however, the MFI of CD11b was reduced on the $CD11b^{hi}$ population [Fig 4.18]. $CD11b^{int}$ $F4/80^{-}$ cells, mostly comprised of eosinophils, increased from and 3.2 % in PBS-treated mice to 11.1% in ES-treated animals [Fig 4.18].

An examination of cytospin preparations did not demonstrate reduced numbers of macrophages in the peritoneal cavity of mice injected with ES compared with mice that received PBS; 30% of cells from both groups of mice had a macrophage morphology [Fig 4.19]. Similar to macrophages isolated form the peritoneum of infected mice, macrophages form ES treated mice had a more granular cytoplasm (white arrow), compared with macrophages from mice treated with PBS. In addition, ES administration resulted in an infiltration of cells with a polymorphonuclear phenotype (black arrow) [Fig 4.19].

To further investigate the turnover of cells following treatment with ES, an experiment was designed to distinguish between resident and recruited cells in the peritoneal cavity. PEC from naïve mice were stained with CFSE and injected into the peritoneal cavity of fresh recipients that were subsequently injected i.p. either PBS or ES for 3 consecutive days. On day 4 PEC were isolated from treated mice and the expression of CD11b, F4/80 and Gr-1 by $CFSE^{+}$ (resident only) and $CFSE^{-}$ (resident and recruited) cells was determined by flow cytometry. Importantly, while the expression of CD11b or F4/80 on resident $CD11b^{high}$ $F4/80^{high}$ LPM cells was not affected by administration of ES, the proportion of LPM in the $CFSE^{+}$ resident cell gate decreased from 30.6% to 11.6%; confirming that LPM are either dying or leaving the peritoneal cavity [Fig 4.20 A, B]. The cells which are recruited into the peritoneal cavity were predominantly composed of $Gr\text{-}1^{hi}$ $CD11b^{int}$ cells. The expression of Gr-1 was not enhanced on resident cells [Fig 4.20 A, C]. The Gr-1 antibody binds two separate molecules, Ly6G and Ly6C, albeit it with lower affinity (301). Thus, the expression of Ly6C and Ly6G by peritoneal cells following treatment of ES was determined by flow cytometry. Administration of ES induced the recruitment of both $Ly6C^{+}G^{-}$ cells (mostly monocytes) and $Ly6G^{+}C^{+}$ cells (a mixture of neutrophils and eosinophils) into the peritoneal cavity [Fig 4.20 D].

4.2.11 ES recruits eosinophils in an IL-5 dependent manner.

Eosinophilia is a clinical feature of *F. hepatica* infection in ruminants (295). The present study has shown that eosinophils are recruited during murine infection with *F. hepatica* [Fig 3.6]. Moreover, experiments have revealed that administration of ES recruited $CD11b^+$ $Gr\text{-}1^+$ granulocytes into the peritoneum which are likely composed of a mixture of neutrophils and eosinophils [Fig 4.20]. To expand upon these findings, experiments were conducted to determine if ES could induce eosinophilia in mice. A single i.p. injection of ES into C57BL/6 was sufficient to raise the concentrations of eotaxin, an eosinophil chemokine in the serum after 2 hours [Fig 4.21, A]. While there was no increase in the frequency of eosinophils in the peritoneal cavity 2 hours or 18 hours after a single dose of ES [Fig 4.21 B], repeated administration of ES every second day for 14 days induced a 4-fold increase in the frequency of eosinophils in the peritoneal cavity compared with a repeated administration of PBS [Fig 4.21 B]. The total number of eosinophils in the peritoneal cavity increased significantly, from a mean of $0.19x10^6$ in PBS-treated mice to $6x10^6$ in mice receiving repeated doses of ES [Fig 4.21 C]. There was also a significant increase in the numbers of eosinophils in the mediastinal LN of mice repeatedly injected with ES, compared with mice receiving PBS; increasing from 6000 in PBS-treated mice to 94,000 in ES-treated mice. Interestingly the number of eosinophils in the bone marrow was also significantly enhanced in mice that received repeated administration of ES [Fig 4.21].

A limited number of reports have shown that eosinophils express CD86 and MHC-II and traffic to the lymph nodes upon activation *in vivo* (331, 332, 356-358). Thus, the expression of CD86 and MHC-II by eosinophils in mice given single or repeated i.p. doses of ES was examined by flow cytometry and compared with mice that received PBS. While the expression of MHC-II or CD86 was unchanged 2 hours after a single dose of ES, by 18 hours post administration of ES, the expression of both molecules was significantly increased from 16% and 12% to 37% and 31% for MHC-II and CD86, respectively [Fig 4.22 A, C]. In contrast, repeated administration of ES significantly reduced the expression of both markers to 2% [Fig 4.22 A, C], although it must be noted that the total number of eosinophils dramatically increased in these mice and the overall numbers of eosinophils which expressed $CD86^+$ and MHC-II was increased in the peritoneal cavity [Fig 4.22 C]

A population of cells from the mediastinal LN of mice treated with PBS expressed Siglec-F and CD11b and thus appeared within the eosinophil gate, however, back-gating strategies revealed that they did not display the FSc/SSc characteristics of eosinophils. Thus, this population was excluded from analysis (they appear in figures as "n/a") [Fig 4.22 C]. However, the LN of mice that received repeated administrations of ES did contain eosinophils (at a mean of $0.95x10^6$). Interestingly a higher proportion of eosinophils from the LN expressed MHC-II and CD86 compared with peritoneal eosinophils from the same mice [4.22 B, C].

IL-5 is the major differentiating factor for eosinophils *in vivo* (359) and αIL-5 neutralising antibodies are in clinical trials for the treatment of diseases where eosinophilia is the primary pathology (360). Milbourne et al demonstrated that culture of murine bone marrow with *F. hepatica* ES could induce the differentiation of eosinophils in a similar manner to IL-5 (361). Thus, experiments were conducted to determine if ES-induced eosinophilia was dependent on IL-5. Mice were injected i.p. with ES or PBS every second day for 6 days with or without an anti-IL-5 neutralising antibody. Administration of ES induced eosinophilia of the blood and tissues. The proportion of eosinophils in the blood increased significantly from a mean of 1% in PBS-treated mice to 5.9% in mice treated with ES. Concurrently, the percentage of eosinophils increased in the peritoneal cavity from a mean of $0.25x10^6$ to $1.5x10^6$ in PBS and ES-treated mice respectively [Fig 4.23]. However, co-administration of anti-IL-5 with ES completely abolished eosinophilia in the blood; the percentage of eosinophils decreased from 5.7% to 0.5% [Fig 4.24]. Similarly, the numbers of eosinophils were significantly reduced from $1.5x10^6$ to $0.3x10^6$ in the peritoneal cavity but in contrast, the proportion remained higher than the proportion of eosinophils in PBS controls (7% in PBS-administered mice to 13.93% in ES+anti-IL-5) [Fig 4.23].

Since neutrophils are thought to be the first cell type to respond to a worm infection (156), their infiltration were also examined. A single dose of ES induced a ten-fold increase in the proportion of $CD11b^+$ $Ly6G^{high}$ neutrophils in the peritoneal cavity 2 hours post administration but this had returned to basal levels after 18 hours and did not increase upon repeated exposure to ES [Fig 4.21 B].

4.2.12 Eosinophils act as APC

The present study has shown that both infection with *F. hepatica* and administration of *F. hepatica* ES induced eosinophilia *in vivo* [Fig 3. 6 and 4.21-23]. Moreover, injection of mice with ES is associated with an accumulation of MHC-II$^+$ CD86$^+$ eosinophils in the draining LN. Eosinophils activated in a variety of contexts, including during overexpression of IL-5, in AHR and following infection with helminths, such as *B. Maylai* or *Strongyloides* spp, have enhanced expression of MHC-II and other costimulatory makers such as CD80/86 and CD40 (331, 332, 356-358).

There is a small body of evidence that attributes an APC function to eosinophils (330-333). Tamura et al reported that eosinophils from IL-5 transgenic mice, treated with GMCSF, could induce proliferation of OVA-specific T cells, in a manner dependent on CD86 (362). Eosinophils isolated from the lungs of mice with AHR, can reactivate memory or prime naïve T cells when transferred to the airways of sensitised and naïve mice, respectively (330, 363). In the context of helminth infections, Padigel et al demonstrated that eosinophils loaded with antigens isolated from *Strongyloides* spp and treated with LPS migrate to the spleen where they prime T cells that produce Th2 cytokines on re-exposure. This effect could not be replicated by transfer of eosinophils from MHC-II$^{-/-}$ mice (331).

A series of experiments were conducted to determine whether eosinophils recruited during infection or following administration of ES could function as APC. In order to generate a reservoir of eosinophils for the use in APC assays, eosinophils were isolated initially from the peritoneal cavity of mice infected with *F. hepatica* as the number of cells recruited during infection was much higher than that following treatment with ES [Fig 3.1 and Fig 4.18].

Siglec F$^+$ cells were purified by FACS (purity 96%) from the peritoneal cavity of C57BL/6 mice infected with *F. hepatica* and cultured at increasing ratios with CD4$^+$ T cells that were purified from the spleens of mice with EAE (this provides a useful source of antigen-specific Th1 and Th17 cells). T cell/eosinophil co-cultures were incubated with increasing concentrations of MOG antigen for 72 hours. Titration of eosinophils significantly enhanced MOG-specific T cell proliferation and production of IFN-γ, IL-17 and IL-4, while the production of IL-13 was non-significantly enhanced [Fig 4.24]. The concentrations of IL-5 and IL-10 were unchanged [Fig 4.24].

In a separate experiment, Siglec F^+ cells were purified by FACS from the peritoneal cavity of BALB/c mice infected with *F. hepatica*. Siglec F^+ eosinophils were cultured for 72 hours with $CD4^+$ T cells which were purified from OVA-immunized DO.10.11 mice, in the presence of increasing concentrations of OVA. Co-culture with eosinophils significantly enhanced antigen-specific proliferation of $CD4^+$ T cells and significantly enhanced OVA-specific production of IFN-γ, IL-17 and IL-4 by T cells. In contrast, antigen-specific IL-5 and TGF-β production was not effected [Fig 3.25].

The use of eosinophils *ex vivo* is hampered by the relatively low numbers of cells obtained from mice following purification. The generation of eosinophils *ex vivo* from bone marrow is thus, a useful method to circumvent this problem (364). Bone marrow-derived eosinophils (BMEOS) were generated from the bone marrow of BALB/c mice using SCF, FLT3L and IL-5 as differentiating factors. After a 10-day culture period, 96% of cells were Siglec-F^+ $CD11b^+$ but did not express MHC-II as determined by flow cytometry. BMEOS were treated *in vitro* with increasing concentrations of ES in the presence or absence of LPS to determine if these cells could produce cytokines and act as APC. Eosinophils produced TGF-β in response to ES alone, however they did not produce IL-6, IL-4 or IL-13. Furthermore, in response to LPS alone BMEOS produced both IL-4 and IL-13 which was further enhanced by the presence of ES [Fig 4.26].

In order to assess the APC function of BMEOS *in vivo* cells were pretreated for 2 hours with OVA prior to the addition of combinations of ES and LPS. After 24 hours the cells were washed and injected s.c. into the footpad of mice. After a further 7 days the popliteal LN of recipient mice were excised and cultured *ex vivo*. A 72 hour culture revealed that LN cells of mice receiving BMEOS from any treatment group failed to produce any cytokines in response to OVA [Fig 4.27].

BMEOS failed to activate T cell responses *in vivo* [Fig 2.27]. However, this does not discount the possibility that BMEOS cannot replicate all the functions of eosinophils generated or activated *in vivo*. In order to determine if ES elicited eosinophils could act as APC, $CD11b^+$ Siglec F^+ SSc^{high} eosinophils and $CD11b^+$ $CD11c^+$ myeloid DC cells were purified by FACS from the peritoneal cavity of mice that had been treated with ES. Purified populations were

cultured at increasing ratios with $CD4^+$T cells which were purified from the spleens of mice with EAE.

A 3 day culture in the presence and of absence of MOG antigen revealed that the titration of ES-elicited eosinophils and DC significantly enhanced MOG-specific production of IFN-γ and IL-17 [Fig 4.28]. These results suggest that eosinophils recruited during *F. hepatica* infection or following administration of ES are capable of reactivating antigen-specific T cells.

4.3 Discussion

The evidence presented in this chapter has made three significant advances with regards to the effects of *F. hepatica* ES on innate immune cells: 1) ES inhibited the ability of DC to induce antigen-specific T cell response, 2) ES induced the production of TGF-β from DC by a novel mechanism and 3) ES recruited eosinophils that had the capacity to act as APC.

4.3.1 ES inhibits APC function

Helminths target DC as a means to suppress protective adaptive immune responses. They specifically target the cytokine production by DC required for polarisation of T cells, the co-stimulatory molecules required for effective priming and also DC antigen capture/processing mechanisms.

This study reports that *F. hepatica* ES suppressed LPS-induced production of IL-12p70, IL-1β, IL-23 and IL-6. This is a significant finding as these cytokine are key to the polarisation of Th1 and Th17 cells and thus inhibiting their production *in vivo* may attenuate Th1 and Th17 responses, such as those that mediate EAE. A number of reports published in the past three years have demonstrated that components produced by *F. hepatica,* including ES, interfere with the ability of DC to produce cytokines (258, 263, 365). Falcón et al demonstrated that *F. hepatica* ES blocked TLR-agonist induced production of IL-12p70, TNF-α, IL-6 and IL-10 (263). This group went on to show a similar function for *F. hepatica* total extract (TE), which is primarily composed of the soluble components of homogenised flukes and is likely to contain at least some ES products (340). TE blocked LPS-induced production of TNF-α, IL-6, IL-12p40 from DC when co-administered at the same time with LPS. However, when DC were pre-treated with TE this effect was reversed and the pro-inflammatory cytokines were enhanced (340). The present study did not observe such an effect, as ES was routinely added 30 minutes prior to stimulation with LPS. In partial agreement with these findings, Hamilton et al investigated the effect of *F. hepatica* tegument on DC and found that the timing of addition did not affect cytokine production (258).

There is evidence to suggest that ES can affect multiple TLR pathways. Hamilton et al demonstrated that *F. hepatica* tegument, also known to contain some ES components,

inhibited zymosan (TLR2 agonist) PGN (TLR2), flagellum (TLR5), poly I:C (TLR3), CpG (TLR9) and LPS (TLR4)-induced cytokine production by DC (258, 328). The molecular target of ES is unknown, but it is likely to be component common to all TLRs to have such broad inhibitory effects. Interestingly, *F. hepatica* tegument inhibited cytokine production by PMA-stimulated DC, which is associated with a reduction on NFKB activation, suggesting that the tegument targets a non-TLR pathway (258).

Other studies confirm the importance of TLRs in ES-mediated suppression. ES-62 from *A. vitae* was shown to degrade TLR4 by autophagy. Moreover, ES-62-treated macrophages became refractory to LPS stimulation (366, 367) (note, this paper was subsequently retracted, however the retraction letter stated that this specific observation remained correct (366, 367)). By a similar mechanism, recombinant cathepsin L from ES was shown to degrade TLR3 in the endosomes of macrophages, which mediated the inhibition of LPS-induced production of IL-6, TNF-α, IL-12 and NO. Cathepsin L was also shown to protect mice from LPS-induced septic shock. These studies suggest that certain components of *F. hepatica* may exert their effects by degrading TLRs expressed by innate cells (272). Preliminary evidence from this laboratory also suggests that ES (but only in combination with LPS) degrades MyD88 in macrophages (C M Finlay, preliminary findings). This is an interesting observation and if proven to be correct may explain how components derived from *F. hepatica* have such broad ranging effects, as MyD88 is essential to both TLR and IL-1 signalling. However, it does not explain why ES inhibits PMA-induced responses (258). In order to better characterise the molecular interactions between ES and MyD88, or indeed other pathways, the specific components of ES with immunosuppressive activity must be purified and identified. It is possible that the preparation of ES used in this study contains novel molecules that have not been described elsewhere, for instance no study has shown that ES in isolation induces the production of TGF-β.

In contrast to these studies, there are also reports which show that components of ES can promote the production of proinflammatory cytokines. Dowling et al demonstrated that *F. hepatica* cathepsin L and sigma class glutathione transferase induced the production of IL-6, IL-12p40 by DC in a TLR-depended manner, although these cells did not produce IL-23 or IL-12p70 (334). Indeed, certain preparations of ES used in the present study were found to promote the production of low amounts of pro-inflammatory cytokines in particular, IL-6, in

the absence of TLR-activation. This may to be due to contamination with LPS, however, in the light of the study by Dowling et al, it is possible that molecules contained within ES induce the production of inflammatory cytokines while other ES molecules block such production.

The present study identified the antigen capture/processing machinery of DC as a possible indirect target for ES, since ES induced the expression of TGF-β. TGF-β has been shown to inhibit the phagocytic activity of macrophages by decreasing their expression of FcγRI and FcγRII. (368, 369). In addition, TGF-β can inhibit the APC capacity of macrophages through the suppression of CD40 expression and production of IL-12p40 (370). There are conflicting reports in the literature concerning the effect of products of *F. hepatica* on phagocytosis. Dowling et al reported that neither cathepsin L nor glutathione transferase derived from *F. hepatica* had any effect on phagocytosis, although they did not investigate this in conjunction with a TLR agonist (334). In contrast, Hamilton et al demonstrated that *F. hepatica* tegument suppressed LPS or zymosan-induced phagocytosis of sheep erythrocytes (258).

The present study demonstrated that ES did not negatively affect antigen acquisition or processing. In fact, ES alone, or together with LPS, moderately enhanced phagocytosis and antigen processing. This is perhaps unsurprising given the wide spectrum of helminth products, and thus potential PAMPS, as well as immunomodulatory molecules contained within ES (371). In contrast to the present study, a recent report demonstrated that *F. hepatica* helminth defence molecule-1, (a protein functionally and structurally related to the mammalian antimicrobial peptides, the cathelicidins) was shown to be internalised by macrophages, where it is cleaved by lysosomal proteases to produce a C-terminal product that prevents the acidification of the endolysosomal compartments (via inhibiting vesicle ATPase) (372). This process impedes antigen processing and subsequent MHC-II presentation (372). A recent finding from this laboratory has identified, using mass spectrometry, *F. hepatica* helminth defence molecule-1 as a major component of ES (A M Stefanska, personal communication). Thus the reason why the preparation of ES used in this study did not inhibit phagocytosis remains unclear. Although it is likely that other components contained within ES offsets the effect of *F. hepatica* helminth defence molecule-1 on phagocytosis.

The present study demonstrated that ES inhibited LPS-induced co-stimulatory molecule expression *in vitro* and upon transfer *in vivo,* these cells were less capable of driving the generation of Th1 and Th17 responses than control DC treated with LPS only. Moreover, ES administered *in vivo* reduced the expression of co-stimulatory molecules on macrophage and DC. Furthermore, these cells could not act as APC for antigen-specific T cells *ex vivo.* This is the first evidence to suggest that the products of *F. hepatica* affect APC function *in vivo.* This observation is in agreement with a report by Falcón et al, who demonstrated that ES inhibited TLR agonist-induced maturation of DC, which is associated with a decrease in the expression of CD40 and CD86 *in vitro* (263). They went on to show that transfer of ES-treated DC promoted Th2 responses in a mixed lymphocyte reaction (263). These observations are in contrast with the present study which did not demonstrate the induction of Th2 responses following adoptive transfer of ES treated DC.

Hamilton et al demonstrated that the tegument of *F. hepatica* protects mice from septic shock, this was associated with a decrease in the expression of costimulatory molecules and an inhibition of antigen-specific T cell proliferation and IFN-γ production (258). Interestingly, this group has recently concluded that the methods used to isolate the tegument fraction may also contain components of ES, as isolates were found to contain cathepsin L, a major component of ES (328). Using recombinant cathepsin L, Dowling et al demonstrated that DC treated with cathepsin L failed to drive Th2 responses (334), suggesting that the tegument alone may confer Th2-inducing properties on DC, while ES does not.

Falcón et al have shown that the TE of *F. hepatica* also inhibits LPS-induced maturation of CD40, but not MHC-II expression (340). Surprisingly, they went on to demonstrate that pre-treatment of DC with TE prior to addition of LPS could, upon transfer of DC to mice, protect recipients from infection with *F. hepatica,* in a manner that was dependent on Th1 not Th2 responses (340). This is not the first study to suggest that Th1 responses are more effective at eliminating helminths than Th2 response, but nonetheless it is a break with dogma to suggest that Th1 responses are protective against *F. hepatica.* It also demonstrates the complexities of assessing the interaction of crude preparations of helminth products with DC, where specific interactions and timing are crucial.

4.3.2 ES induces release of TGF-β

It was concluded in the last chapter that the identification of the innate cellular source of TGF-β would be required for a complete understanding of the mechanism by which *F. hepatica* suppressed autoimmunity. It is reasonable to postulate that the parasite itself is responsible for the production of TGF-β, as other helminths have been shown to do so. The filarial nematode *B. malayi* produces a TGF-β homologue that can bind to the host TGF-β receptor (373), while *H. polygyrus* secretes a TGF-β homologue that acts to enhance the expression of FOXP3 in naïve T cells (178). Even if *F. hepatica* is found to produce a TGF-β homologue, it is unlikely to be the sole source of TGF-β-like activity as this study has already shown that the expression of TGF-β by was enhanced in macrophages and DC during infection.

Flynn et al have shown that PBMC from cattle infected with *F. hepatica* for 4 weeks, produce high levels of parasite-specific IL-10 and TGF-β (329). Neutralization of TGF-β enhanced parasite-specific IL-4 and IFN-γ by PBMC (329). In addition, another study found that *F. hepatica* egg antigens could drive the production of TGF-β by PBMC isolated from chronically infected cattle. These observations are likely restricted to T cell-derived TGF-β, thus this study provides the first convincing evidence that ES induces the production of TGF-β by innate immune cells. Importantly, this was independent of TLR-agonist stimulation. In contrast, a report from Carranza et al that demonstrated that TE of *F. hepatica* promoted the production of TGF-β by DC, only did so in presence of CpG; as TE alone had no effect (374).

It has been shown that TGF-β inhibits the expression of CD14 by macrophages following LPS stimulation, which suppressed subsequent TLR4 signalling (375). Therefore, the autocrine action of TGF-β on DC cannot be ruled out as a mechanism by which *F. hepatica* ES can inhibit the production of pro-inflammatory cytokines. However, preliminary data suggested that neutralisation of TGF-β did not affect ES-induced suppression of IL-12p70 or IL-23 by DC (C M Finlay, preliminary findings); suggesting that these two effects are independent of each other. Furthermore, work conducted in this laboratory has confirmed that induction of TGF-β and inhibition of TGF-β by ES separates to different fractions (A M Stefanska, personal communication).

In the present study, a time-course of TGF-β secretion by DC revealed that the cytokine rose to peak concentrations two hours post-addition of ES. As this is a very short time to allow for the transcription, translation and secretion of a protein, it suggests that ES may induce release of pre-existing TGF-β from DC rather than by *de novo* production. Indeed, this study confirmed that ES does not alter the expression of TGF-β mRNA and that cytokine secretion is maintained in the presence of cycloheximide, a potent inhibitor of protein translation. This represents a novel mechanism of TGF-β production. This hypothesis is dependent on the presence of pre-formed TGF-β, however, attempts to demonstrate the presence of pre-formed TGF-β have failed, most likely due to the poor sensitivity of TGF-β specific antibodies used in western blot analysis.

Cathepsins which are present in ES were identified as a possible candidate to mediate release of TGF-β from DC. Indeed, it was reported that *Leishmania* spp activate TGF-β production via cathepsins, which cleave membrane bound TGF-β, thereby increasing local concentration of active TGF-β. This mechanism is essential for the survival of the parasite in the host (176). However, the present study excluded cathepsins as a mechanism for ES-induced TGF-β release, via the use of cathepsin inhibitors. Matrix metalloproteinases have been shown to activate latent TGF-β during *plasmodia* infection and has been shown to protect the host from immune pathology (177). However experiments conducted in this laboratory did not find a role for MMPs in activating the release of TGF-β from DC after incubation with ES (A Stefanska, personal communication).

While mRNA for TGF-β in DC was not enhanced, ES did induce the expression of mRNA for genes involved in the oxidative stress response. Administration of ES to lung epithelial cells resulted in the generation of oxygen radicals. Furthermore, addition of the antioxidant NAC abrogated ES-induced release of TGF-β by DC following treatment with ES, suggesting that ROS induces TGF-β release by DC. This conclusion is consistent with precious reports which have described that ROS is a potent mediator of TGF-β activation. It has been suggested that ROS invokes a conformational change in the LAP-TGF-β interaction, thus freeing the active component (376). ROS produced following radiation exposure were shown to rapidly activate TGF-β release *in vivo* (376). Furthermore, TCR stimulation was shown to induce latent TGF-β production, which was subsequently activated by the release of ROS from activated T cells. This effect enhanced the expression of FOXP3 in T cells and was

reversed by the administration of an antioxidant (377). Interestingly, TGF-β itself was shown to promote the expression of ROS, through activation of endogenous NADPH oxidase. One effect of TGF-β activity, the cytoskeleton rearrangement of endothelial and epithelial cells, was shown to be dependent on ROS production downstream of TGF-β signalling and cytoskeleton rearrangement was abrogated in the presence of an antioxidant (378). Therefore, it is possible that an ROS-TGF-β positive feedback loop operates in innate immune cells following the addition of ES.

Interestingly, *F. hepatica* secretes large amounts of known antioxidants several of which were shown to have immunomodulatory capacity, including the mu and sigma classes of glutathione transferases and peroxiredoxin. The high expression of these molecules may act to offset the oxidative damage caused by release of oxygen and nitrogen radicals from the fluke itself or indeed eosinophil and macrophages as they attempt to kill the fluke. Thus, it is possible that the activation of TGF-β via ROS may be a host adaptation to deal with damage caused by parasite or eosinophil derived-ROS, as TGF-β is a well characterised promoter of wound repair and fibrosis. Fluke infection of the liver coincides with severe calcification and fibrotic transformation of the bile ducts, the location in which the flukes reside (379). It is possible that TGF-β plays a role in mediating this fibrotic response. Thus, TGF-β production during *F. hepatica* infection may be an underlying cause of pathology in addition to promoting immune tolerance to the fluke.

It should also be noted that αV integrins activates latent TGF-β. αV integrins induce a conformational change in LAP-TGF-β, which releases active TGF-β. The expression of αV integrins on DC is indispensable for the *de novo* generation of Treg cells following administration of TGF-β (380). Further investigation is required to determine if the expression of αV integrins by DC has any effect on the production of TGF-β.

The production of TGF-β by DC acts to expand Treg cells and create a tolergenic environment. Indeed, Carranza et al demonstrated that the TE of *F. hepatica* (in combination with CpG) promoted the generation of Treg cells in a TGF-β-dependent manner (374). In contrast, preliminary data from this laboratory suggested that this is not the case for ES, as ES failed to expand pre-existing Treg cells or the generation of *de novo* Treg cells *in vitro* (A M Stefanska, personal communication).

The compounds in ES which promote the production of TGF-β remain to be defined. Preliminary data suggest that the molecules which promote TGF-β production by DC may differ from those which inhibit the production of pro-inflammatory cytokines (A M Stefanska, personal communication). Also the identification of the cellular receptors that are responsible for signalling following treatment with ES, and the investigation of intracellular signalling networks which precede the secretion of TGF-β will be indispensable in elucidating the mechanism by which the parasite can modulate the host immune system.

4.3.3 APC function of eosinophils

This study found that infection with *F. hepatica* or administration of *F. hepatica* ES is associated with the recruitment of eosinophils in to the peritoneal cavity. This was an expected observation given that that helminth infections are synonymous with eosinophilia and that *F. hepatica*-specific T cell clones produce large quantities of IL-5 in response to parasite antigens (298). ES is known to induce eosinophilia, although the role of eosinophils in host defence to *F. hepatica* is largely unknown. However one study demonstrated that protection from infection with *F. hepatica* was associated with increase of IgE and the infiltration of eosinophils into the gut (381). Moreover, during secondary infection non-encysted juvenile fluked were killed by eosinophils via antibody mediated cell cytotoxicity as the fluke become coated with Ig1 and IgG2a (381).

Milbourne et al showed that culture of murine bone marrow with ES could induce the differentiation of eosinophils in a similar manner to IL-5 (361). However, it is more likely that ES promoted the production of IL-5 by the bone marrow cells, which in turn induces eosinophil differentiation. While it is produced by both haematopoietic and non-haematopoietic cells (as IL-5 is still produced in various lineage deficient and RAG deficient mice) it is primarily associated with Th2 cells, and more recently, nuocytes. IL-5 activates mouse B cells and eosinophils, while in humans it is more restricted to eosinophils function (382). Eosinophils highly express CD125, the IL-5Rα chain (382) and mice deficient in the IL-5Rα have reduced basal levels of eosinophils and display delayed clearance of the nematode *Angiostrongylus cantonensis* (383). Moreover, IL-5 transgenic mice have continuous eosinophilia (384).

This present study has shown that the recruitment of eosinophils following administration of ES was dependent on IL-5. However, while administration of a neutralising antibody to IL-5 completely abrogated eosinophilia in the circulation, it only partially reduced the recruitment of eosinophils to the tissue. Indeed, other factors may play a role in the recruitment of these cells. IL-5 transgenic animals have high numbers of eosinophils in the circulation, but normal numbers of eosinophils in the tissue (385). The eosinophil chemokine, eotaxin is required for baseline tissue eosinophilia and this may explain why neutralisation of IL-5 does not deplete eosinophils from the tissue (386).

The previous chapter demonstrated that infection with *F. hepatica* is associated with the recruitment of eosinophils to the peritoneal cavity of infected mice (323). Interestingly, this effect was significantly enhanced in mice deficient in IL-10. The effect of IL-10 on eosinophil development or function has not been widely investigated, but has been shown to be involved in the regulation of eotaxin. Moreover, administration of recombinant IL-10 to mice reduced eosinophilia associated with inflammation in AHR (297).

ES can modulate the function of eosinophils. Goose et al demonstrated that ES prevents the adhesion of eosinophils to the adult fluke (387). ES was also shown to induce apoptosis in eosinophils in the infected livers of rats (388). The present study provides novel insight into the effect of ES on eosinophils; administration of ES to mice was associated with the accumulation of MHC-II$^+$ CD86$^+$ eosinophils in the draining LN. In partial support to these findings, others have shown that eosinophils express MHC-II. Duez et al demonstrated that eosinophils in the LN of mice with AHR have higher expression of MHC-II (389). The airways of humans with hay fever contain eosinophils that have high expression of MHC-II, whereas eosinophils in the peripheral blood of these individuals do not, suggesting that MHC-II is expressed on activated eosinophils (390). Interestingly, the present study showed that MHC-II$^+$ eosinophils trafficked to the draining LN, much like APC. Moreover, other reports have shown that eosinophils can take up and process soluble antigens (333). Eosinophils have long been considered as end-stage effector cells, acting through the release of lipid mediators and granule-derived cationic proteins. However, there is a limited body of evidence to supports the hypothesis that eosinophils can act as APC. Eosinophils have been shown to act as APC during AHR where they express MHC-II, CD40 and CD80/CD86.

Moreover, the transfer of purified antigen-loaded eosinophils induced airway hypersensitivity in naive pre-disposed mice (391).

The present study has also demonstrated that eosinophils can act as APC. Eosinophils were isolated first from mice infected with *F. hepatica* as this model provided a large reservoir of eosinophils. Purified eosinophils enhanced antigen-specific proliferation and production of IL-17, IL-4 and IFN-γ by OVA-specific T cells suggesting that the eosinophils can drive a range of T cell responses and are not limited to Th2 cells (which might be inferred from their role in helminth infection). Interestingly, eosinophils were also shown to act as APC for MOG-specific T cells purified from mice with EAE. These eosinophils induced the production of IL-17 and IFN-γ as well as IL-4, a cytokine not typically associated with EAE. A direct comparison between DC and eosinophils is thus required to determine if eosinophils can preferentially induce Th2 responses. Eosinophils, purified from mice treated with ES, could also promote IFN-γ and IL-17 production from MOG-specific T cells, confirming that ES also acts on eosinophils to promote APC activity.

Unfortunately, a comparison of the APC capacity of eosinophils from ES-treated or *F. hepatica*-infected and naïve mice was not possible due to the low numbers of eosinophils that can be recovered from untreated mice. An attempt was made to generate eosinophils from the bone marrow (BMEOS) of naïve mice using SCF, FLT3-L and IL-5. These cells highly expressed Siglec-F, but did not express MHC-II. Interestingly, ES promoted the production of TGF-β by eosinophils, suggesting that this effect is not limited to DC. ES also induced the production of low amounts of IL-4 and IL-13 by eosinophils, which was enhanced in the presence of LPS. This suggests that these cells, if they could act as APC would also act as a source of Th2 polarising cytokines. However, adoptive transfer of BMEOS to mice failed to induce an antigen-specific T cell response. There may be several explanations for this, 1) eosinophils may be unable to prime naïve T cells, 2) BMEOS may not fully replicate somatic eosinophils, or 3) expression of MHC-II by BMEOS prior to transfer may be required. Lucey et al have shown that the expression of MHC-II by eosinophils is dependent on the presence of GMCSF (392). Future studies will be required to determine whether the inclusion of GMCSF in transfer experiments effects the expression MHC-II.

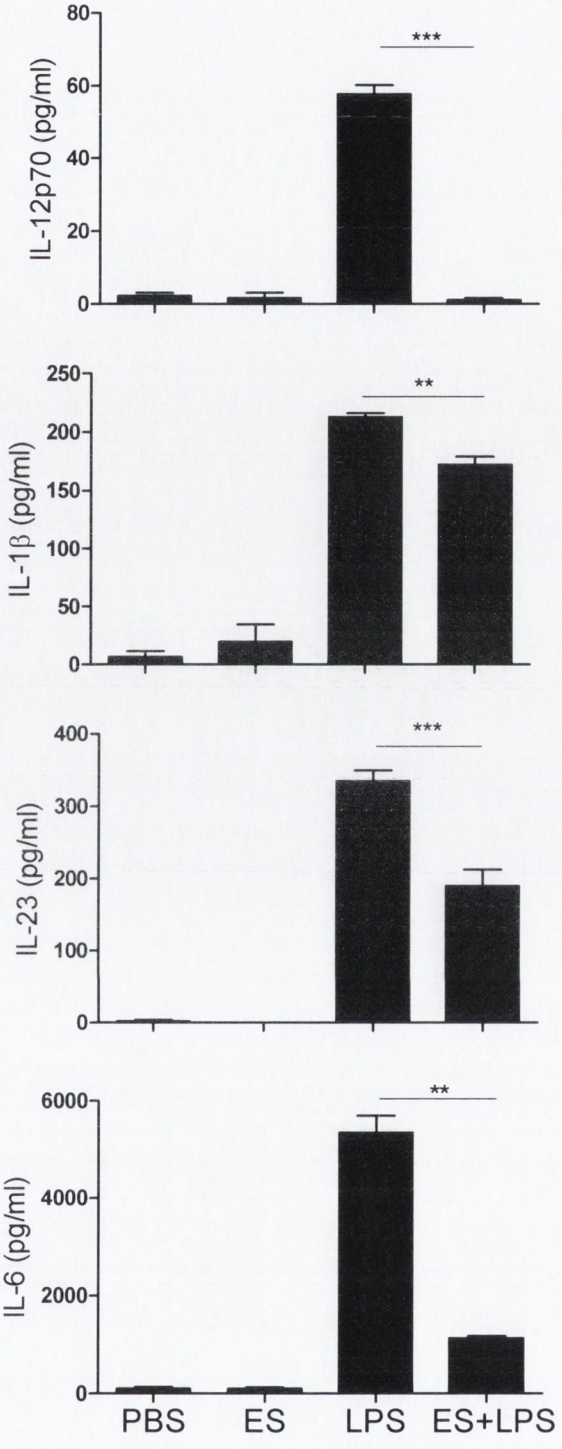


Fig 4.1 *F. hepatica* ES inhibits LPS-induced production of IL-12p70, IL-1β, IL-6 and IL-23 by BMDC BMDC ($1x10^6$/ml) expanded from C57BL/6 mice were treated with either PBS, ES (5% v/v), LPS (100 ng/ml) or ES + LPS (DC were pre-treated with ES for 30 min prior to addition of LPS.) After 24 hours, the concentrations of IL-1β, IL-6, IL-12p70, andIL-23 in the supernatant hours were determined by ELISA. Data presented are means +/- SEM of triplicate assays. Results are representative of 3 independent experiments. Statistical assessment was performed as indicated vs LPS control using Students t-test. ** $P<0.001$, * $P<0.05$

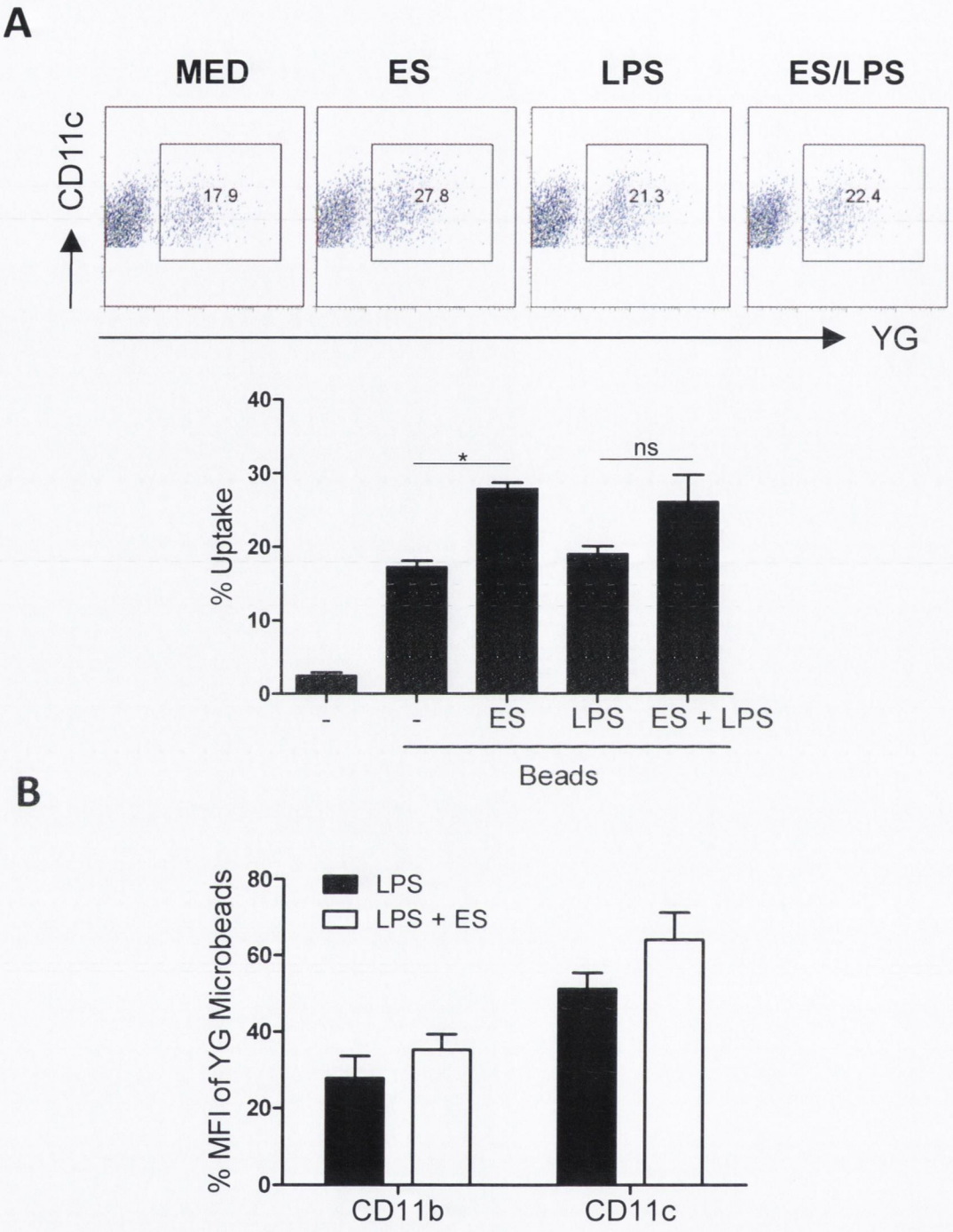


Fig 4.2 ES does not inhibit phagocytosis by DC both *in vivo* and *in vitro*. A, BMDC expanded from C57BL/6 mice were stimulated with either PBS, ES (5% v/v), LPS (100 ng/ml) or ES + LPS. After 2 hours BMDC were incubated with $3.64x10^8$ (1μm diameter) YG-labelled fluorescent latex microbeads. After 30 minutes cells were washed and surface stained for CD11c. A, $CD11c^+$ were analysed by flow cytometry for YG fluorescence. Data presented are means +/- SEM of triplicate assays. Statistical assessment was performed by unpaired Student t-test as indicated. *$p<0.05$. Results are representative of 2 separate experiments. B, mice were injected into the footpad with LPS (10 μg) and $3.64x10^8$ 0.5μm YG fluorescent microbeads with or without ES (10 μl). After 24 hours mice were sacrificed and popliteal lymph node cells were surface stained for CD11b, CD11c and analysed by FACS. Results are representative of 2 separate experiments.

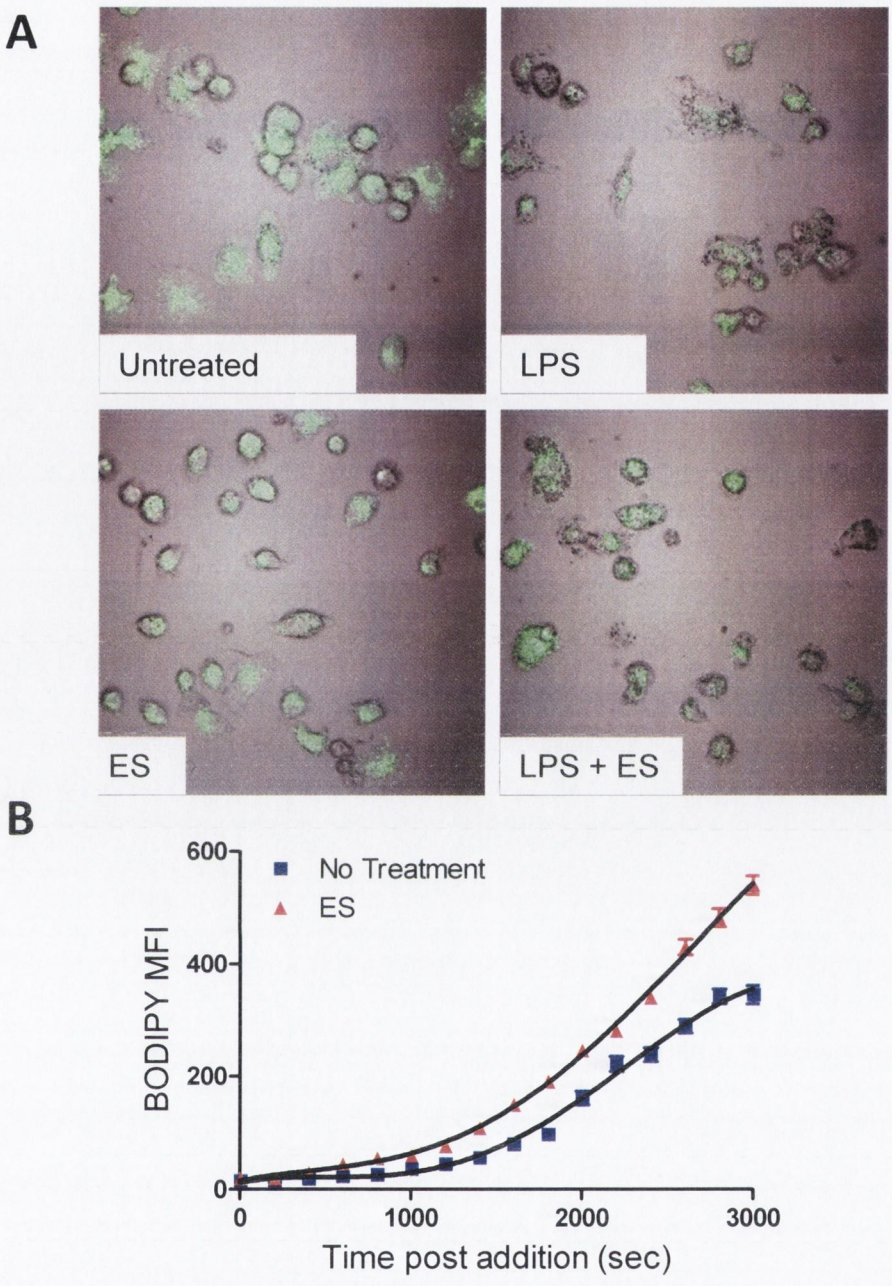


Fig 4.3 ES does not inhibit antigen processing by DC *in vitro*. A, BMDC expanded from C57BL/6 mice were allowed to adhere to a glass confocal slide and treated with either PBS, ES (5% v/v), LPS (100 ng/ml) or ES + LPS. After 18 hours cells were incubated with the BODIPY containing DQ-OVA (5 μg/ml, molecular probes) which fluoresces only after protein degradation. Confocal images were generated from live cells 37°C after 20 minutes treatment with DQ-OVA. B, BMDC grown from HEJ mice were treated with medium or ES (2% v/v) for 6 hours. Cells were washed and stained for CD11c. At time 0 DQ-OVA (1 μg/ml) was added to the cells. BODIPY Fluorescence of $CD11c^{hi}$ cells was measured over time by flow cytometery. Plotted are the linear values of BODIPY MFI. Results are representative of 2 separate experiments.

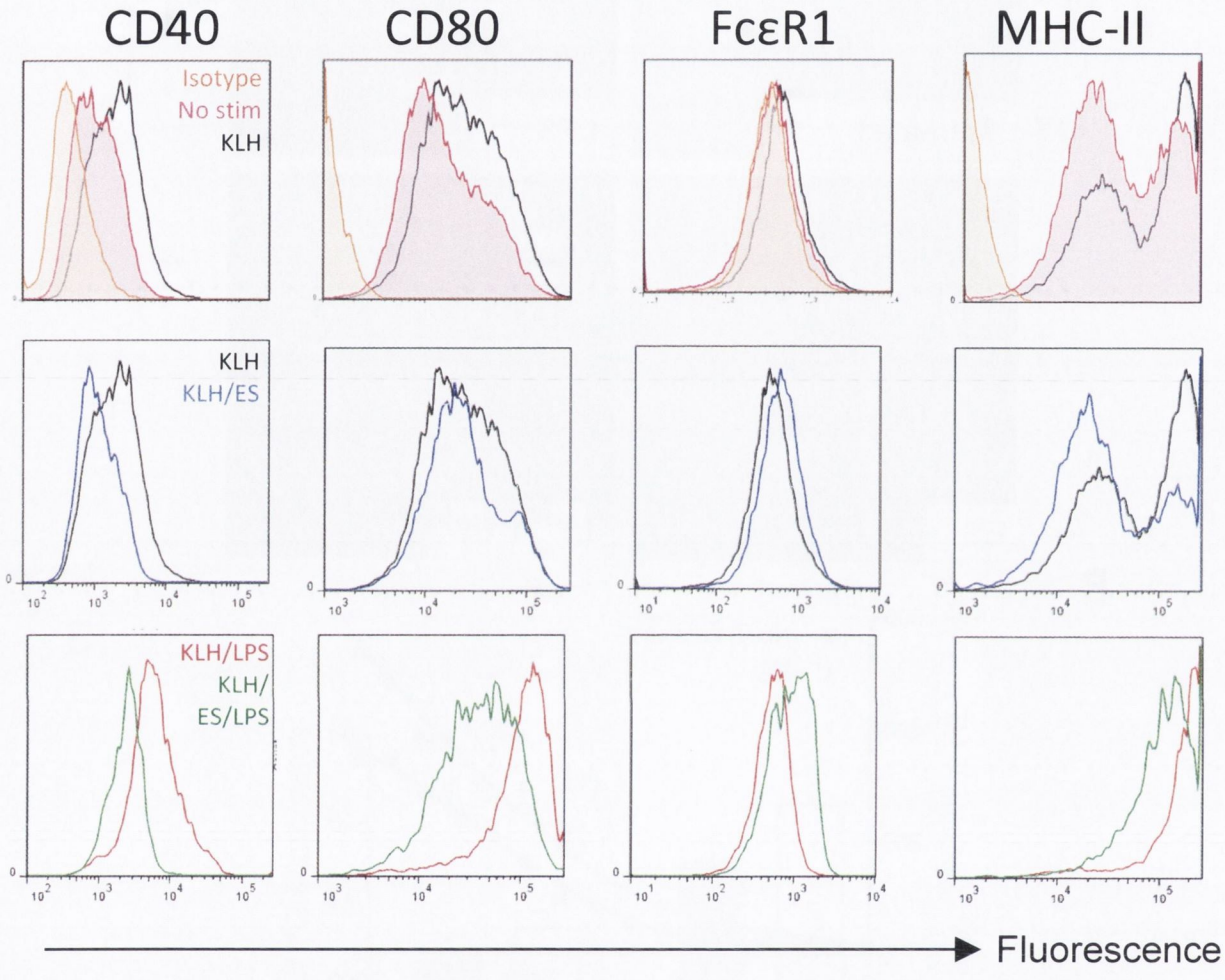


Fig 4.4 *F. hepatica* ES suppresses LPS-induced expression of CD40, CD80 and MHC-II but enhances the expression of FcεR-I on BMDC. BMDC ($1x10^6$/ml) expanded from C57BL/6 mice were treated with either PBS, KLH (50 μg/ml), KLH + ES (5%v/v), KLH + LPS (100ng/ml) or KLH + ES + LPS. KLH or PBS was added at time 0, ES at time 2 hours and LPS at time 2.5 hours. After 24 hours cells were isolated and the expression of CD40, CD80, MHC-II and FcεR-I was determined by flow cytometery. Displayed histograms are gated on $CD11b^+$ $CD11c^+$ cells.. Results are representative of 3 independent experiments. Statistical assessment was performed vs LPS control using Students T test. ** $P<0.001$, * $P<0.05$

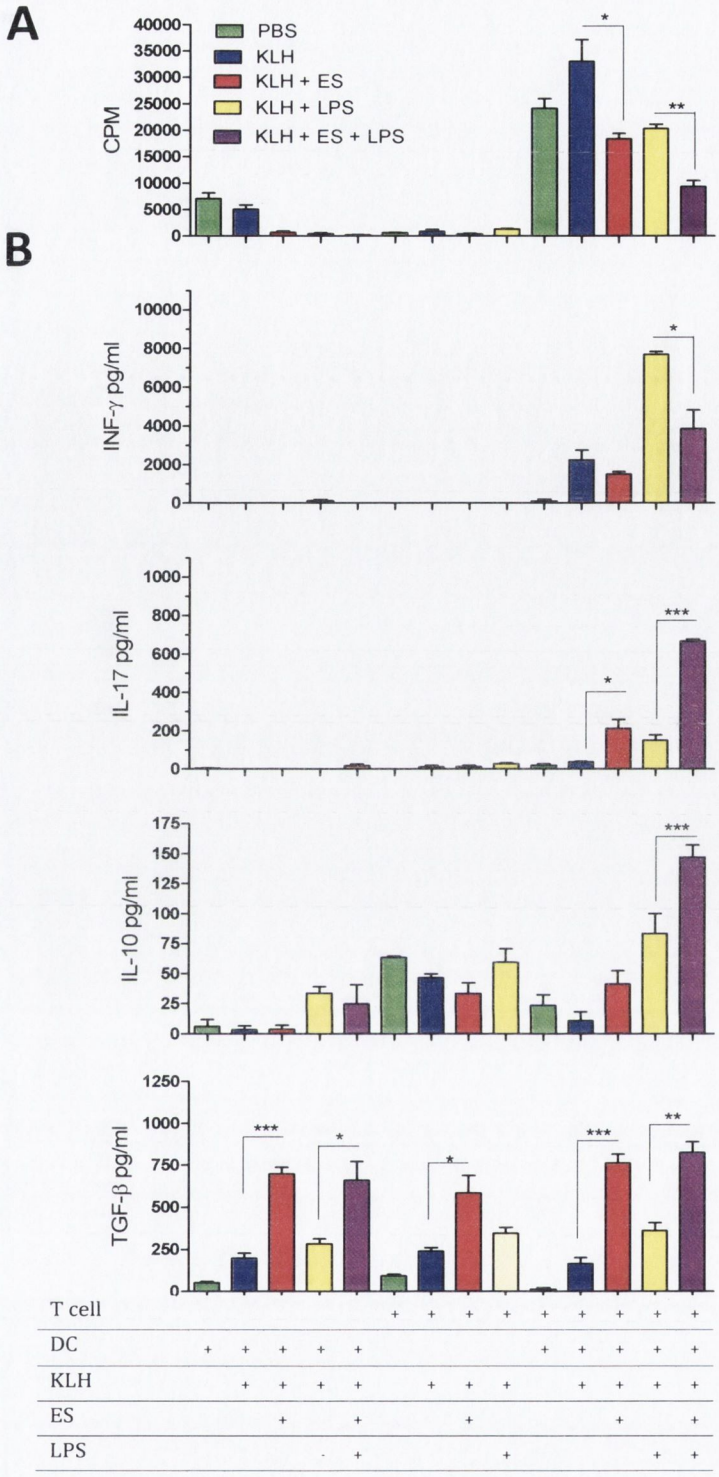


Fig 4.5 *F. hepatica* ES inhibits the ability of DC to promote antigen-specific T cell proliferation and production of IFN-γ *in vitro,* but enhances IL-17, IL-10. BMDC ($2x10^5$ cells/ml) expanded from C57BL/6 mice were incubated with either medium, KLH (20 μg/ml), KLH + ES (5% v/v), KLH + LPS (100ng/ml) or KLH + LPS + ES). After 24 hours, BMDC were co-cultured with $CD4^+$ T cells that were isolated from the inguinal LN and spleens of C57/BL6 mice that were immunized with i.p. injections of KLH (20μg) with LPS (10μg) 14 days previously. A, [^{3}H] thymidine was added for the last 12 hours of a 84 hour culture and cellular proliferation was quantified by CPM. B, The concentrations of IFN-γ, IL-17, IL-10 and TGF-β in the supernatant at 72 hours were quantified by ELISA. Data presented are means +/- SEM of triplicate assays. Statistical assessment was performed as indicated using paired Students T test * $P<0.01$ ** $P<0.005$ *** $P<0.001$. Results are representative of 2 separate experiments.

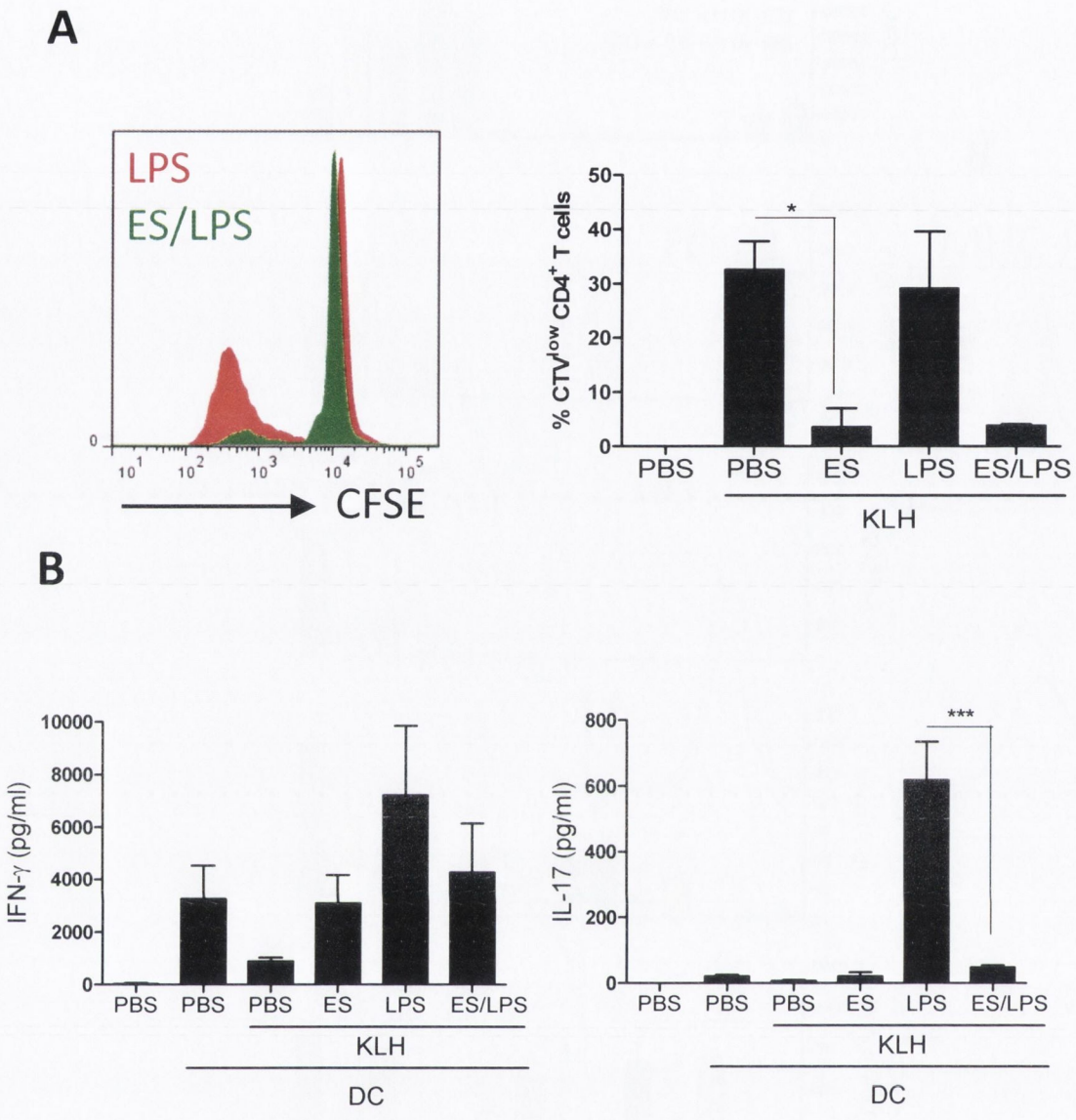


Fig 4.6 ES inhibits the ability of LPS-activated DC to prime antigen specific Th17 cells in C57BL/6 mice. BMDC ($1x10^6$/ml) expanded from C57BL/6 mice were treated with either PBS, KLH (50 μg/ml), KLH + ES (5% v/v), KLH + LPS (100 ng/ml) or KLH + ES + LPS. KLH or PBS was added at time 0, ES at time 2 hours and LPS at time 2.5 hours. After 24 hours BMDC were removed from culture and transferred into the footpads of naive C57BL/6 mice by s.c. injection with 3 mice per group. After 7 days the popliteal LN of recipient mice were excised; stained with CFSE (1 μM) and restimulated *ex vivo* with KLH (100 μg/ml) for 86 hours. A, proliferation of $CD4^+$ T cells was determined by degree of CFSE dilution as measured by flow cytometery. B, The concentrations of IFN-γ and IL-17 in the supernatant after 86 hours was determined by ELISA. Data presented are means +/- SEM of triplicate assays. Statistical assessment was performed as indicated using Students t-test *$p<0.05$ ***$p<0.0001$. Results are representative of 2 separate experiments.

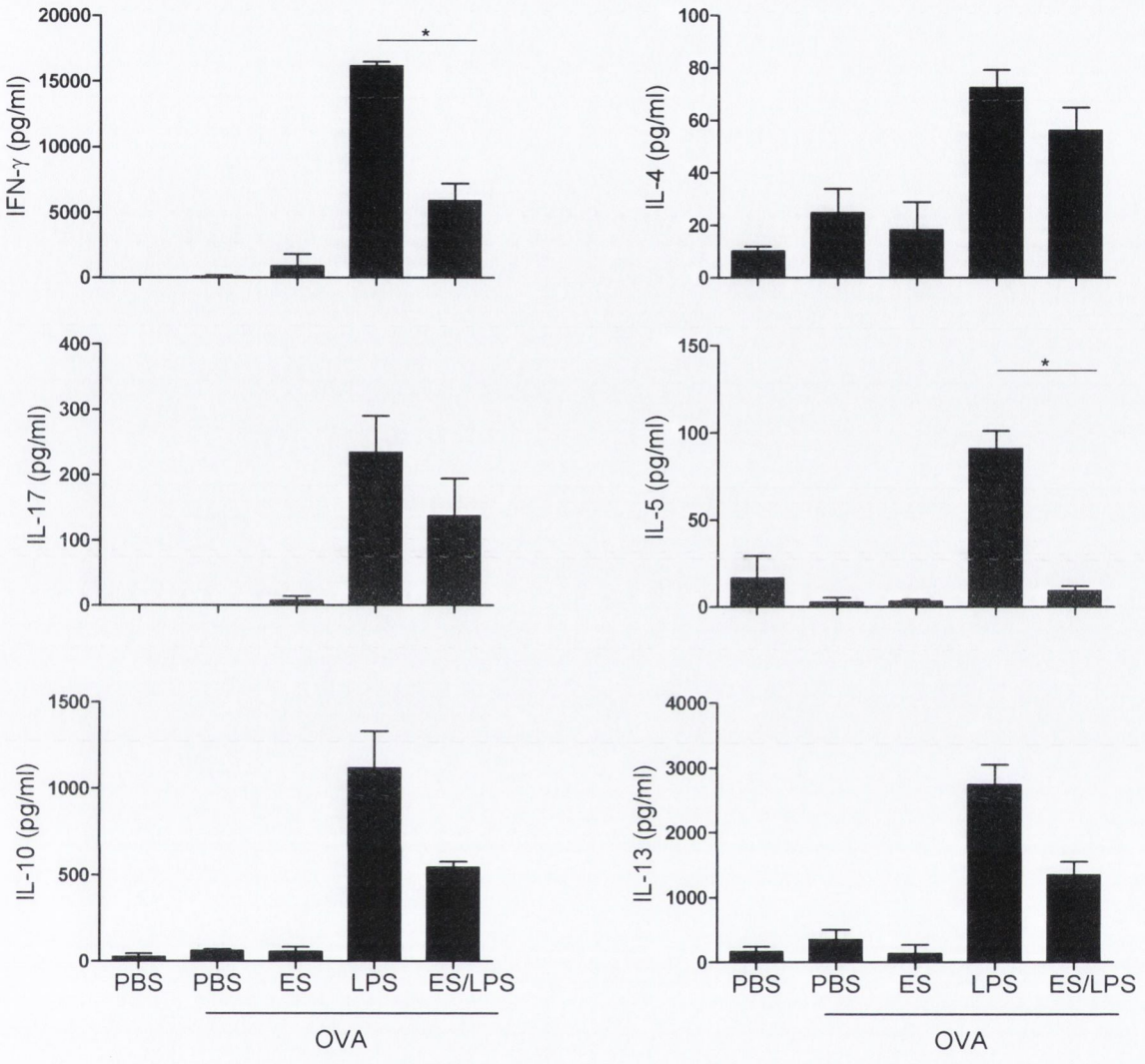
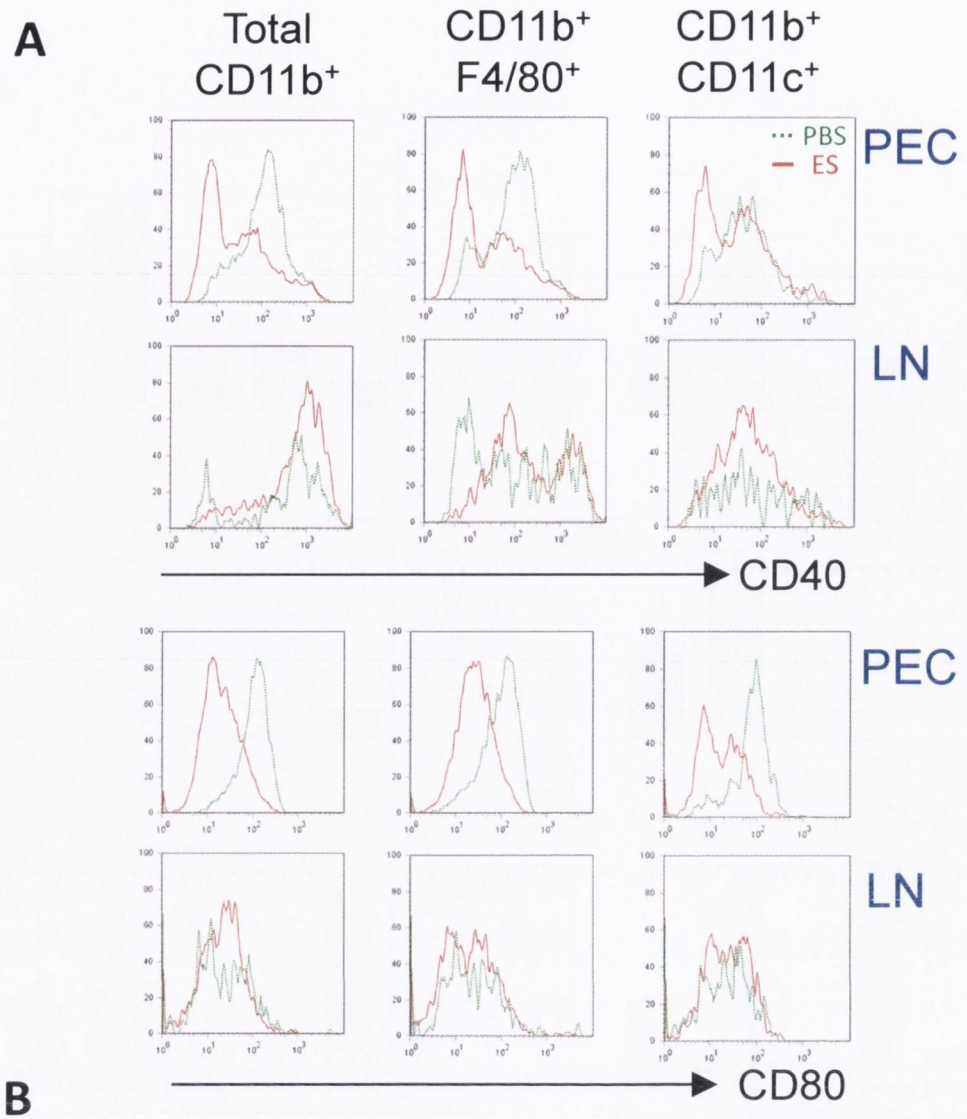


Fig 4.7 *F. hepatica* ES inhibits the ability of LPS-activated DC to prime antigen specific Th1 and Th2 responses in BALB/c mice. BMDC ($1x10^6$/ml) expanded from BALB/c mice were incubated with either PBS, OVA (50μg/ml), OVA + ES (5% v/v), OVA + LPS (100 ng/ml) or OVA + ES + LPS. OVA or PBS was added at time 0, ES at time 2 hours and LPS at time 2.5 hours. After 24 hours BMDC ($1x10^6$) were removed from culture and transferred into the footpads of naive C57BL/6 ($0.5x10^6$/hind footpad) mice by s.c. injection. 7 days after adoptive transfer the popliteal LN of recipient mice were excised and restimulated *ex vivo* with OVA (100 μg/ml) for 86 hours. The concentrations of IFN-γ, IL-17, IL-4, IL5, IL-10 and IL-13 in the supernatant after 86 hours was determined by ELISA. Data presented are means +/- SEM of triplicate assays. Statistical assessment was performed as indicated using Students T test *$p<0.05$ ***$p<0.001$. Results are representative of 2 separate experiments.


A
Total CD11b+
CD11b+ F4/80+
CD11b+ CD11c+
PBS
ES
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CD40
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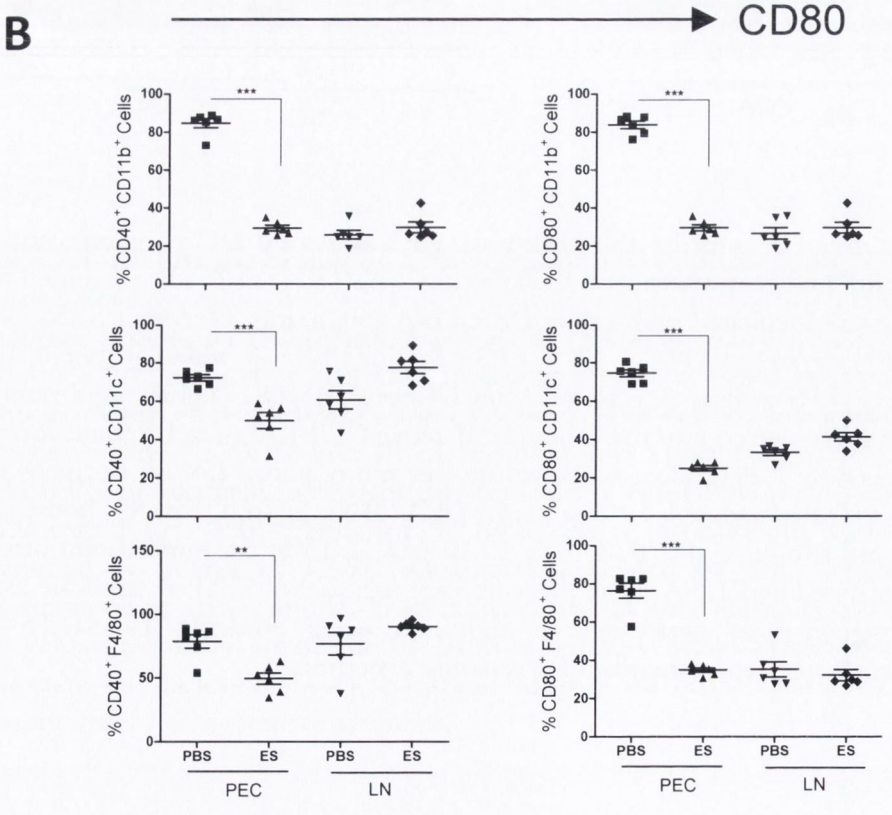
B
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PBS
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PBS
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Fig 4.8 Intraperitoneal administration of *F. hepatica* ES suppresses the expression of CD40 and CD80 by macrophages and DC in the peritoneal cavity. C57BL/6 mice were injected i.p with either ES (200 μg) or PBS daily for 5 consecutive days. On day 6 the mesenteric lymph nodes were excised and peritoneal lavages were performed on sacrificed mice. The expression of CD40 and CD80 by $CD11b^+$, $CD11b^+$ $F4/80^+$ and $CD11b^+$ $CD11c^+$ cells from the mLN and PEC was analysed by flow cytometry. A, Representative flow cytometric histograms of CD40 and CD80 expression are shown with PBS group (green line) and ES group (red line) present. B, The percentage expression of CD40 and CD80 from all mice are shown as replicates. Data presented are means +/- SEM of 6 mice per group. Statistical assessment was performed using unpaired students T test vs PBS group. ** $P<0.01$ *** $P<0.001$. Results are representative of 2 separate experiments.

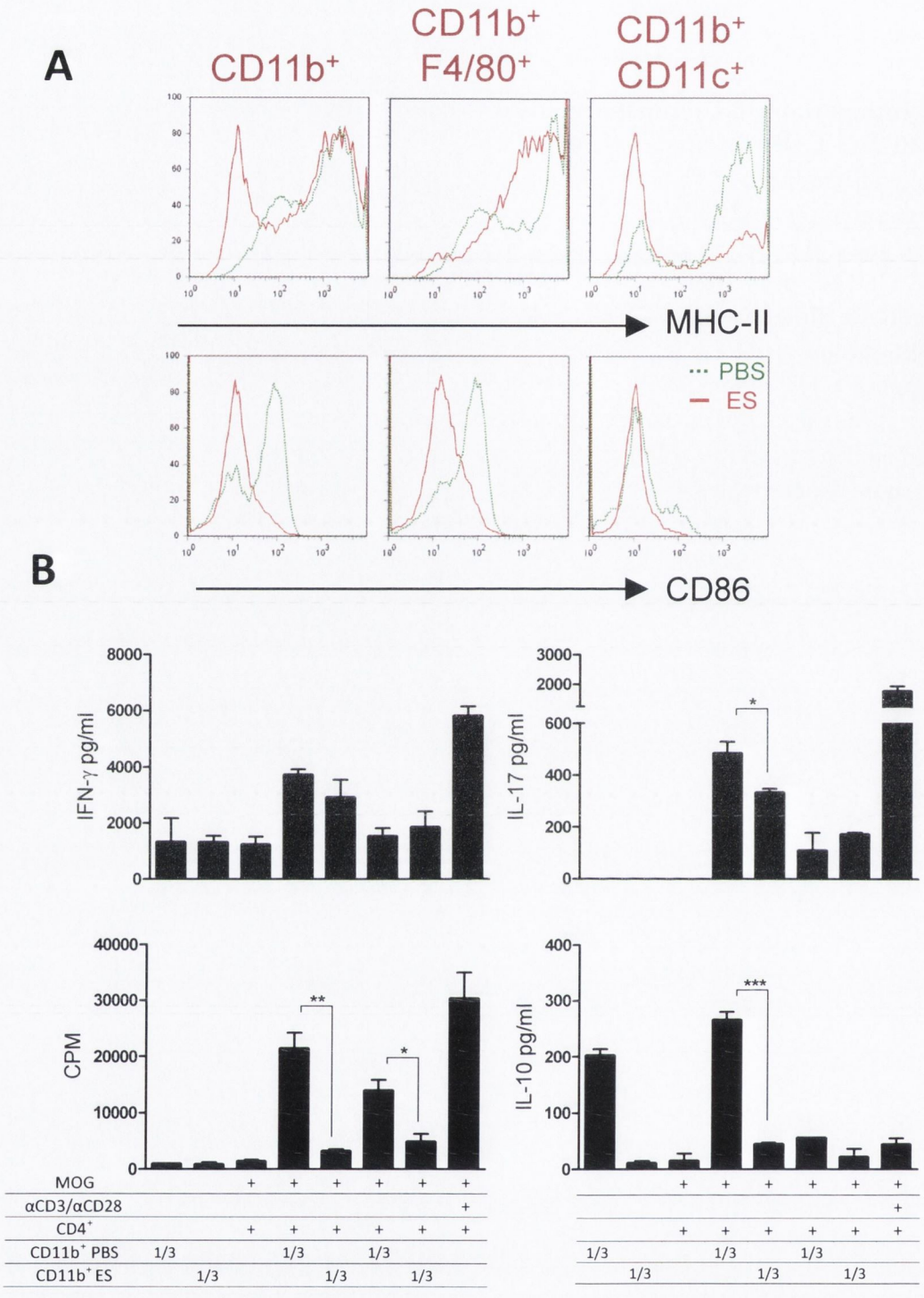


Fig 4.9 CD11b$^+$ cells isolated from mice treated with *F. hepatica* ES have reduced APC function. 10 C57BL/6 mice were injected i.p with either ES (200 μg) or PBS daily for 5 consecutive days. On day 6 PEC were isolated from treated mice by peritoneal lavage, pooled and CD11b$^+$ cells purified by magnetic separation. (A) The expression of MHC-II and CD86 on sorted CD11b$^+$ cells was analysed by flow cytometry. (B) Purified CD11b$^+$ cells were co-cultured with purified CD4$^+$ cells that were isolated from spleens of mice with EAE (day 18 p.i.) in the indicated conditions. Cells were activated with MOG (50μg/ml) or αCD3 (1μg/ml) plus αCD28 (5μg/ml.) as indicated. After 72 h the concentrations of IL-10, IFN-γ and IL-17 in the supernatants was analysed by ELISA. For the last 18 hours of culture [^{3}H] thymidine was added to determine cellular proliferation. Data presented are means +/- SEM of triplicate assays. Statistical assessment was performed as indicated using unpaired Student's t tests. * P<0.01 ** P<0.005 *** P<0.001.

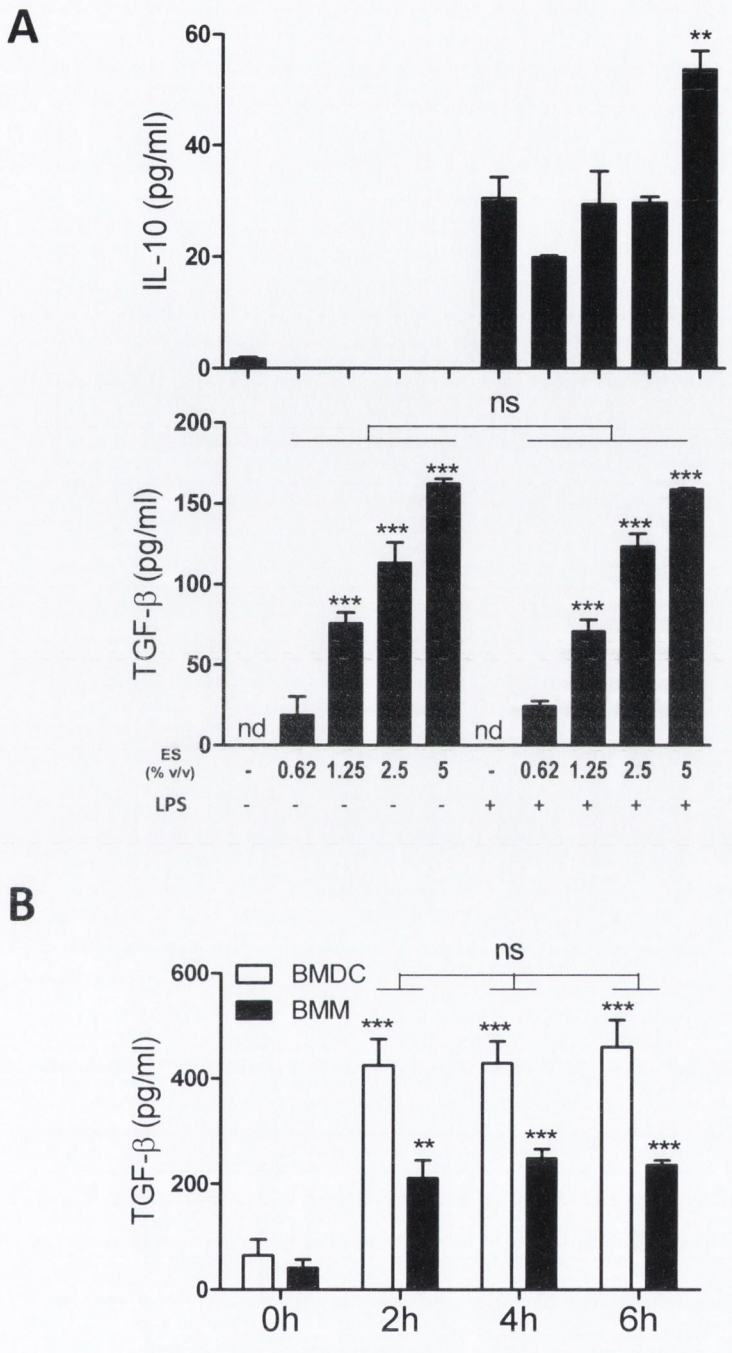


Fig 4.10 *F. hepatica* ES promotes TGF-β production by DC and macrophages and enhances LPS induced IL-10 from DC. A, BMDCs ($1x10^6$cells/ml) from C57BL/6 mice were incubated with increasing concentrations of ES as indicated (0.625-10% v/v). After 24 hours the concentrations of IL-10 and TGF-β in supernatants were determined by ELISA. B, BMDCs and BMM (1×10^6cells/ml) from C57BL/6 mice were incubated with ES (5% v/v) at time 0. Supernatants were removed at time 0, 2, 4 and 6h post incubation and the concentration of TGF-β was determined by ELISA. Results are means (+/- SD) of triplicate assays. Data presented are means +/- SEM of triplicate assays. Statistical assessment in A, was performed by one way ANOVA with Dunnett's post-test vs ES 0% control; and in B, was performed by unpaired Student t-test Vs time=0 *$p<0.05$ **$p<0.01$ ***$p<0.001$. Results are representative of at least 8 independent experiments.

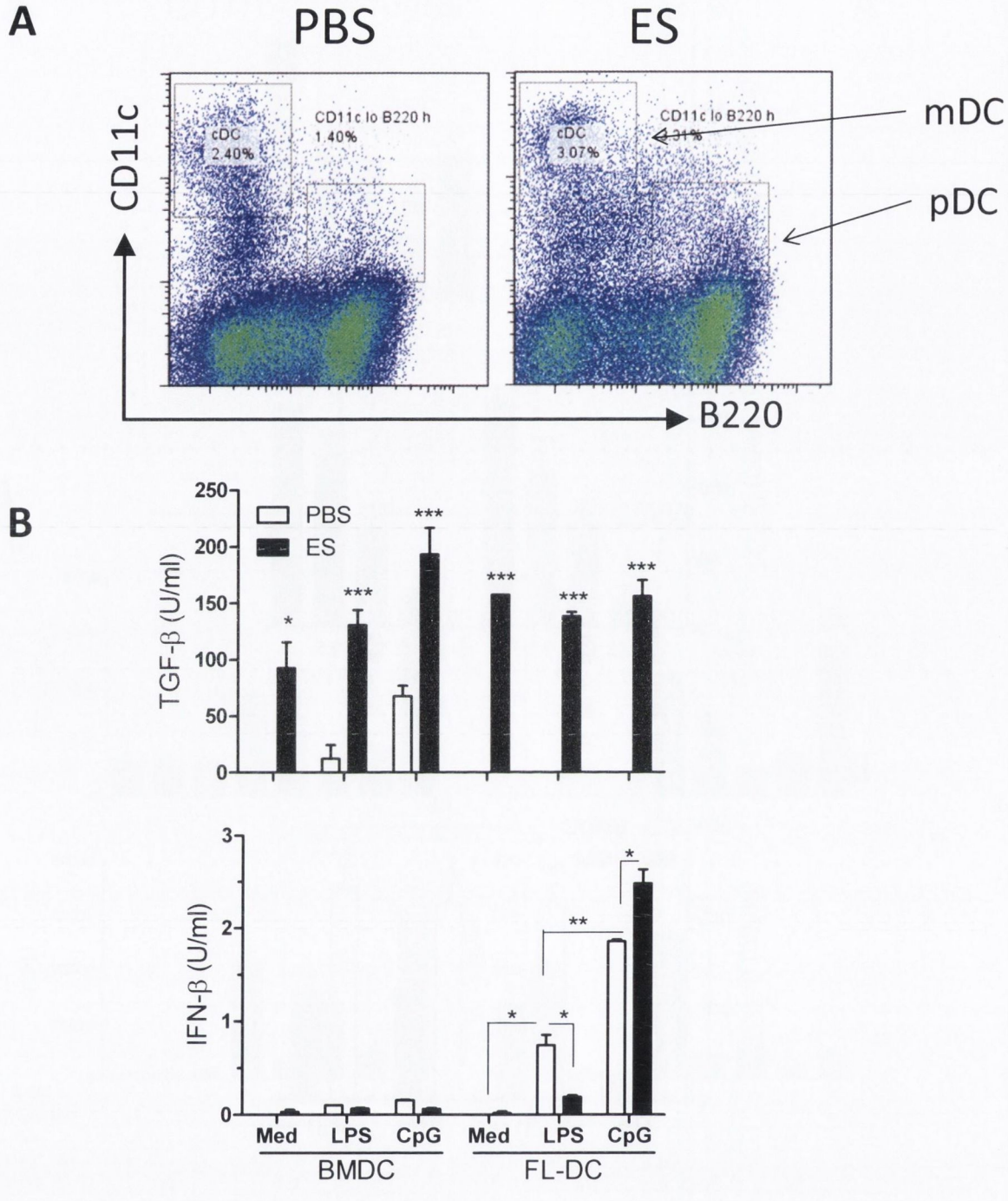


Fig 4.11 *F. hepatica* ES recruits but does not induce release of IFN-β from plasmacytoid DC . A, C57BL/6 mice were treated with ES (40μl) or PBS every second day for 6 days. On day 7 PEC from were isolated and the presence of $CD11b^{+}$ $CD11c^{high}$ $B220^{neg}$ myeloid DC and $CD11c^{low}$ $B220^{+}$ pDC were quantified by flow cytometry. B, pDC and BMDC, expanded from the bone marrow of C57BL/6 mice, were treated with medium, LPS (100ng/ml) or CpG (5μg/ml) in the presence or absence of ES (5% v/v) as indicated. After 24 hours the concentrations of TGF-β and IFN-β in the supernatant was quantified by ELISA. Data presented are means +/- SEM of triplicate assays. Statistical assessment was performed as indicated one way ANOVA with Bonferroni's post-test. *** $P<0.001$, **$p<0.01$* $P<0.05$. Results are representative of 2 separate experiments.

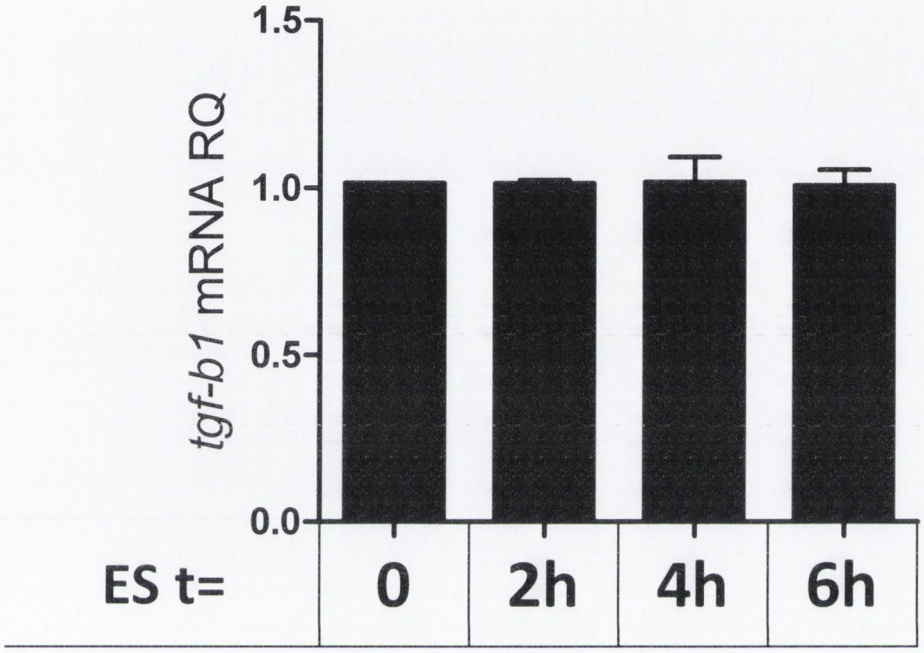


Fig 4.12 ES does not induce the expression of TGF-β mRNA in BMDC. BMDCs ($1x10^6$cells/ml) from C57BL/6 mice were incubated with ES (10% v/v). At 0h, 2h, 4h and 6h post incubation cells were lysed and TGF-β mRNA expression was evaluated by real-time RT-PCR normalized to 18S rRNA. Data are shown as fold induction over DC cultured in medium only. Results are means (+/- SD) of triplicate assays. Statistical assessment was performed by unpaired Student t-test. Vs time=0 *$p<0.05$. Results are representative of 2 separate experiments.

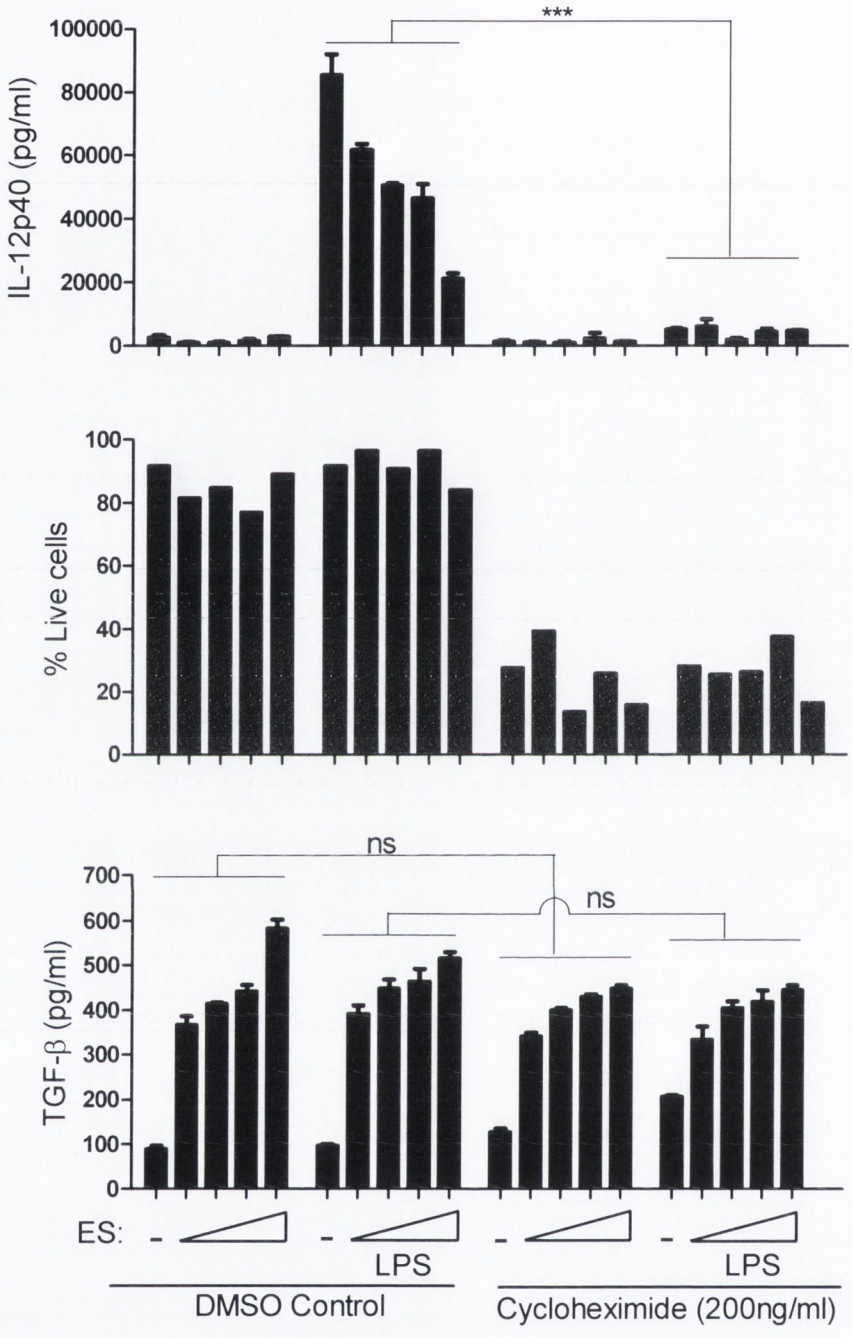


Fig 4.13 Treatment of DC with ES does not induce TGF-β protein translation. A, BMDCs ($1x10^6$ cells/ml) from C57BL/6 mice were incubated with increasing concentrations of ES as indicated (0.625-10% v/v), in the presence or absence of LPS (100 ng/ml) with or without cycloheximide (200 g/ml). After 24 hours supernatants were recovered and the concentration of TGF-β was determined by ELISA. Results are means (+/- SD) of triplicate assays. Statistical assessment was performed by two way ANOVA together with Bonferroni's post test as indicated vs all concentrations of ES incubated with LPS ***$p<0.001$. Results are representative of 2 separate experiments.

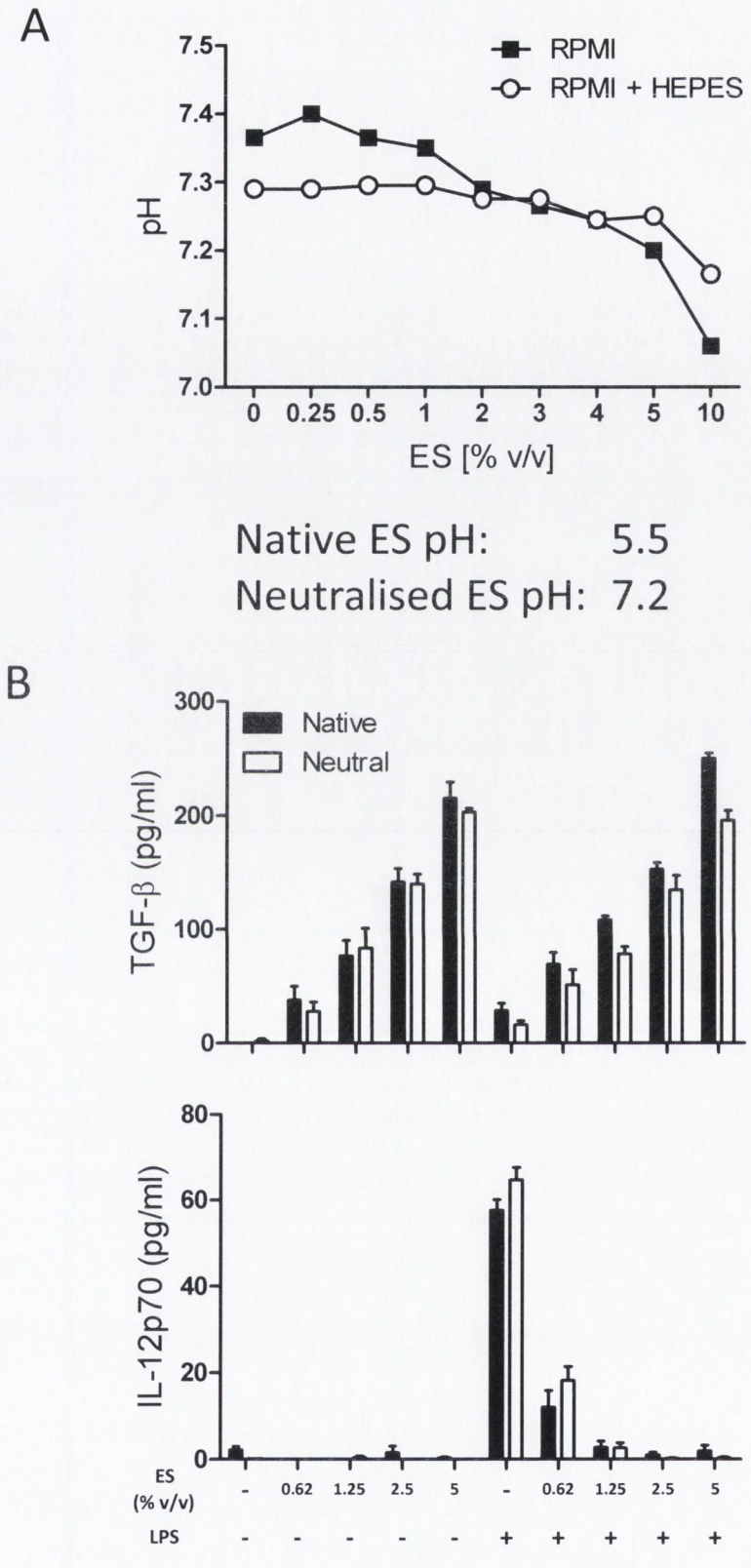


Fig 4.14 Acidity of ES does not effect release of TGF-β. A, BMDCs ($1x10^6$cells/ml) from C57BL/6 mice were incubated with increasing concentrations of either unmodified or pH neutralised ES as indicated (0.625-10% v/v), in the presence or absence of LPS (100 ng/ml). After 24 hours concentrations of TGF-β and IL-12p70 in the supernatant were determined by ELISA. Results are means (+/- SD) of triplicate assays.

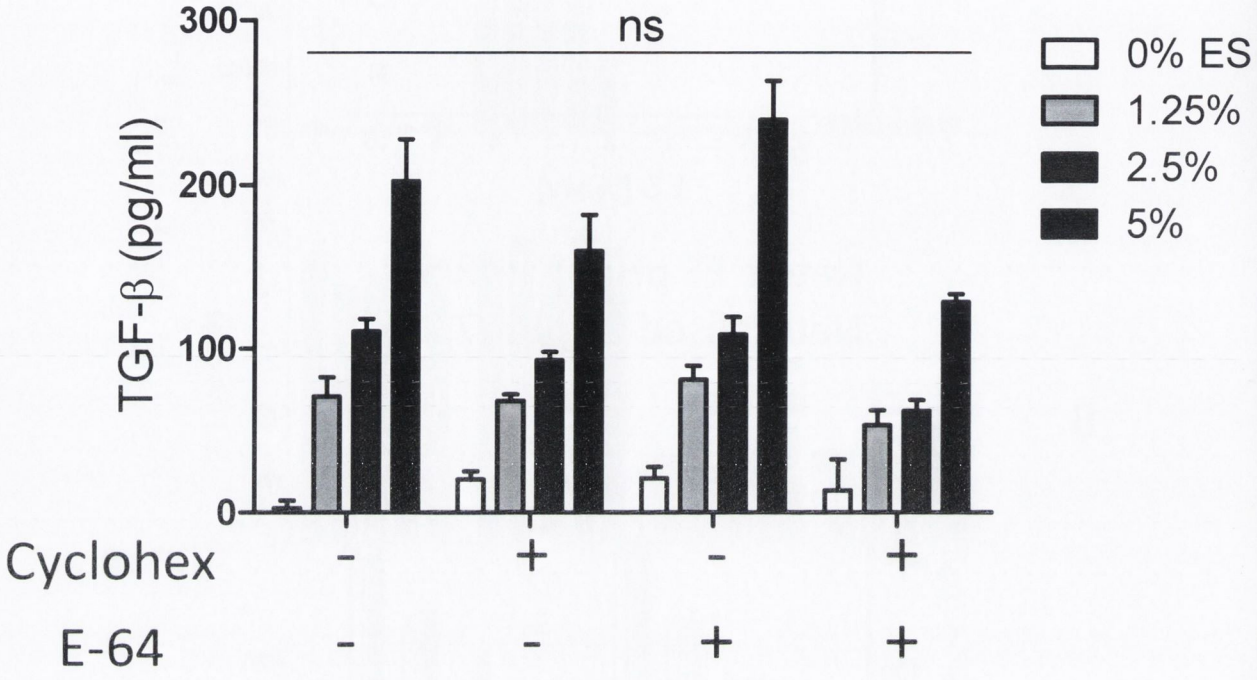


Fig 4.15 ES induced release of TGF-β is not mediated by cathepsins. A, BMDCs ($1x10^6$cells/ml) from C57BL/6 mice were incubated with increasing concentrations of ES as indicated (1.25-5% v/v), in the presence or absence of cycloheximide (clyclohex, 200 ng/ml) and E-64 (5 μM, a competitive inhibitor of chathepsins). After 24 hours supernatants were recovered and the concentration of TGF-β was determined by ELISA. Results are means (+/- SD) of triplicate assays. Statistical assessment was performed by one way ANOVA together with Bonferroni's post-test. Results are representative of 2 separate experiments.



Fig 4.16 ES induces ROS in epithelial cells A, A549 human lung epithelial cells ($1x10^6$ /ml) were pre-incubated with PBS, ES (5% v/v) or *tert*-Butyl hydroperoxide (TBHP, an inducer of ROS) as indicated. Cells were washed and incubated with carboxy-H_2DCFDA (40 μM) for 20 min. Cells were washed and live imaged immediately by confocal microscopy. Selected photomicrographs are taken 10 minutes post-incubation. Results are representative of 2 separate experiments. B, BMDCs ($1x10^6$cells/ml) from C57BL/6 mice were incubated with ES (10% v/v). At 0, 2, 4 and 6 hours post incubation cells were lysed and HO-1 mRNA expression was evaluated by real-time RT-PCR normalized to 18S rRNA. Data are shown as fold induction over DC cultured in medium only. Results are means (+/- SD) of triplicate assays. Statistical assessment was performed by unpaired Student t-test. Vs time=0 *$p<0.05$. Results are representative of 2 separate experiments.

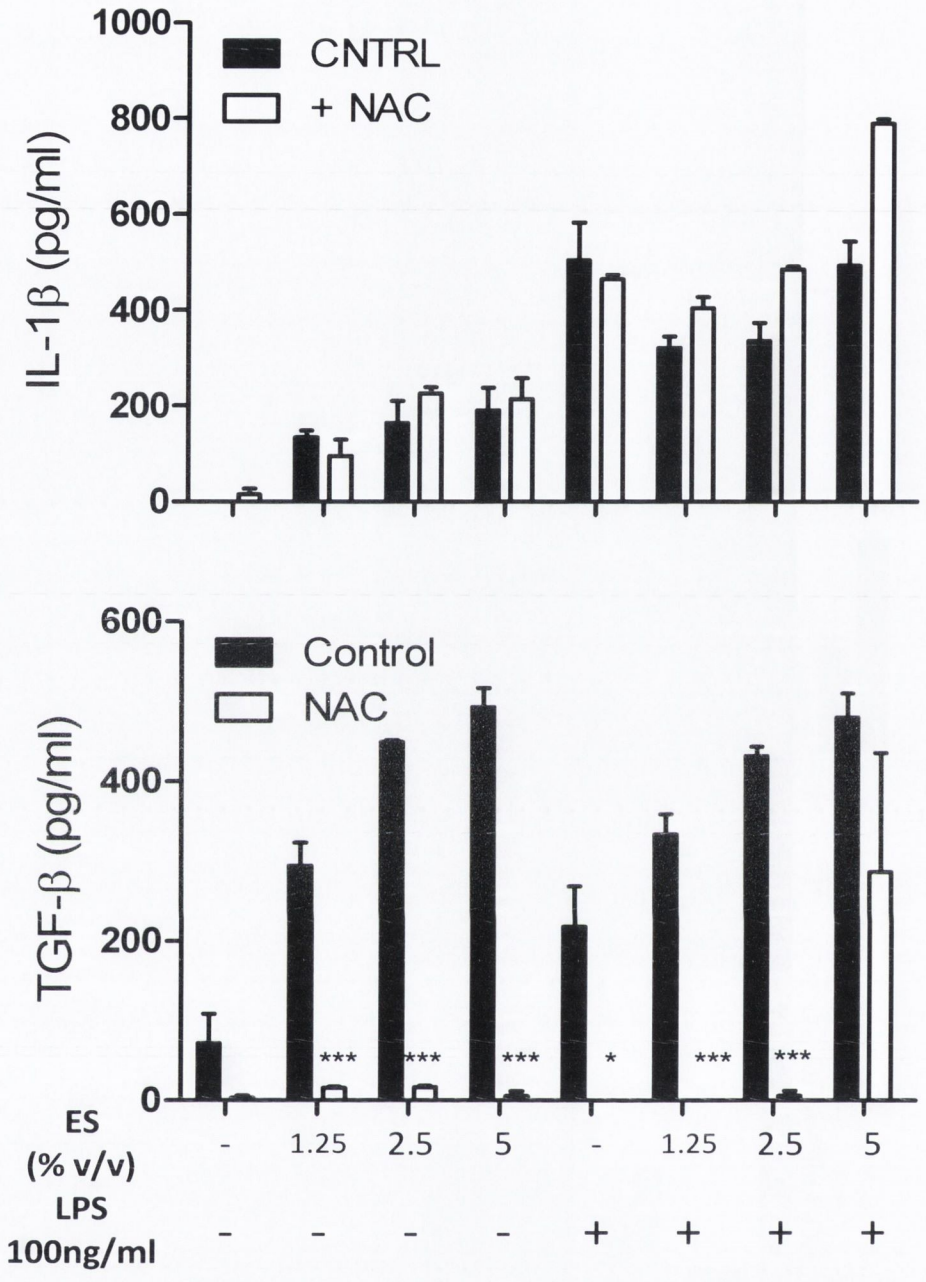


Fig 4.17 The antioxidant N-acetlycystiene inhibits ES-induced release of TFG-β. BMDCs (1 x 10^6cells/ml) from C57BL/6 mice were incubated with increasing concentrations of ES as indicated (0.625-10% v/v), in the presence or absence of LPS (100ng/ml) with or without NAC (2 mM) as indicated. After 24 hours the concentration of TGF-β in supernatant was determined by ELISA. Results are the means (+/- SD) of triplicate assays. Statistical assessment was performed by paired Student t-test as indicated vs corresponding concentration of ES without NAC *$p<0.05$ ***$p<0.0001$. Results are representative of 2 separate experiments.

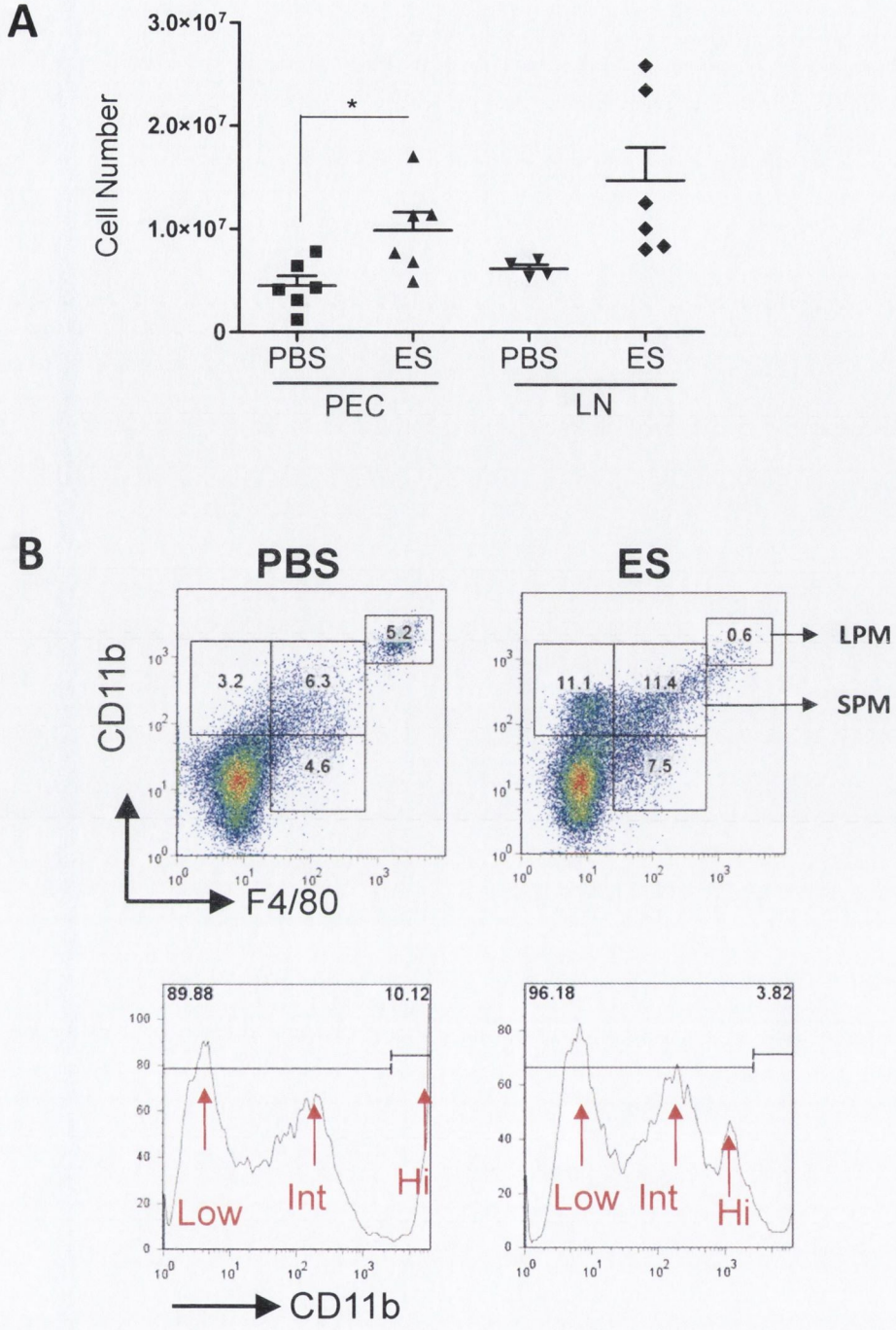


Fig 4.18 ES promotes infiltration of cells including small peritoneal macrophages into the peritoneal cavity while reducing the number of LPM C57BL/6 mice were i.p. injected with PBS or ES (20 μl) daily for 5 consecutive days. On day 6, PEC were isolated from mice by peritoneal lavage. A, Total viable PEC. B, PEC were analysed for expression of CD11b and F4/80 by flow cytometry. Data presented are means +/- SEM of six mice per group. Results are representative of 6 mice and 5 separate experiments. Statistical assessment was performed using unpaired Student's t test vs PBS treated mice.

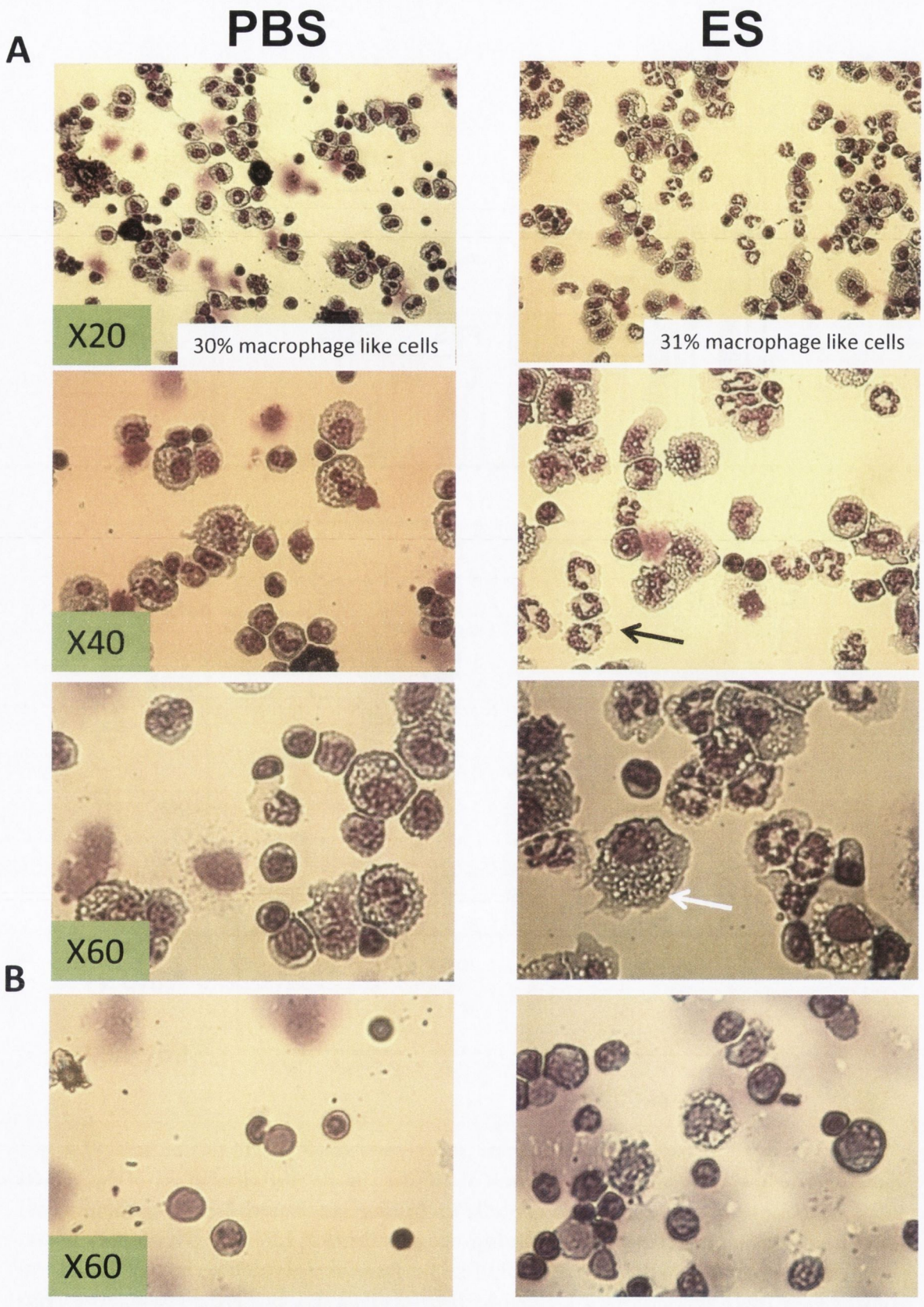
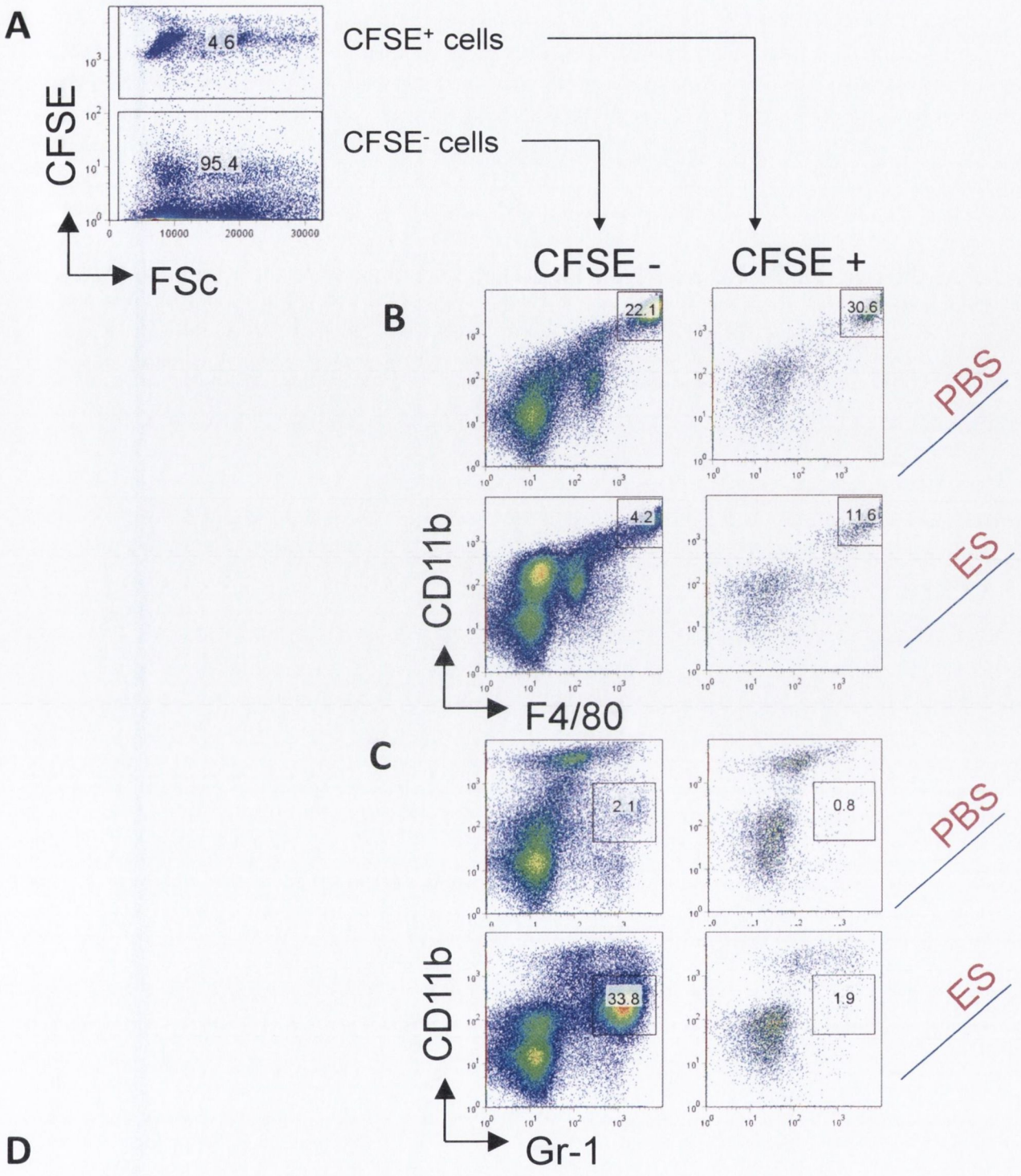


Fig 4.19 Intra-peritoneal administration of ES promotes the infiltration of granulocytes into the peritoneal cavity. C57BL/6 mice were i.p. injected with PBS or ES (20 μl) daily for five consecutive days. On day 6 PEC cells were isolated by peritoneal lavage. Cytospin preparations were fixed (methanol) and stained with H&E. A, Photomicrographs of cytospins preparations of PEC. B, cells from the mediastinal LN. Black arrow indicates a granulocyte. White arrow indicates a macrophage. Results are representative of 3 separate experiments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CFSE
FSc
4.6
95.4
CFSE+ cells
CFSE- cells
CFSE -
CFSE +
B
22.1
30.6
4.2
11.6
PBS
ES
CD11b
F4/80
C
2.1
0.8
33.8
1.9
PBS
ES
CD11b
Gr-1
D
Ly6G
Ly6C
0.7
3.6
80.4
15.2
0.6
36.9
36.9
25.6

Fig 4.20 Intra-peritoneal administration of ES reduces the relative number of macrophages in the peritoneal cavity and promotes infiltration of Gr-1$^+$ cells. PEC were isolated from naive mice by peritoneal lavage. PEC were stained with CFSE (2.5μM) and reinjected into the peritoneal cavity of fresh naïve mice. Recipient mice received either PBS or ES (200 μg) daily for 3 consecutive days. Peritoneal lavage was performed on day 4. PEC were surface stained for CD11b, F4/80 and GR-1. A, FACS analysis was performed on both CFSE$^+$ (Resident only) and CFSE$^-$ (cells which may have been recruited in). B, CD11b and F4/80 expression of both resident and recruited PEC from in PBS and ES treated mice. C, CD11b and Gr-1 expression of both resident and recruited PEC from in PBS and ES treated mice. D, ES (20 μl) or PBS was i.p. administered daily for 5 days. On day 6 PEC were isolated by peritoneal lavage. Expression of CD11b, GR-1, Ly6G and Ly6G on PEC was analysed by flow cytometery.

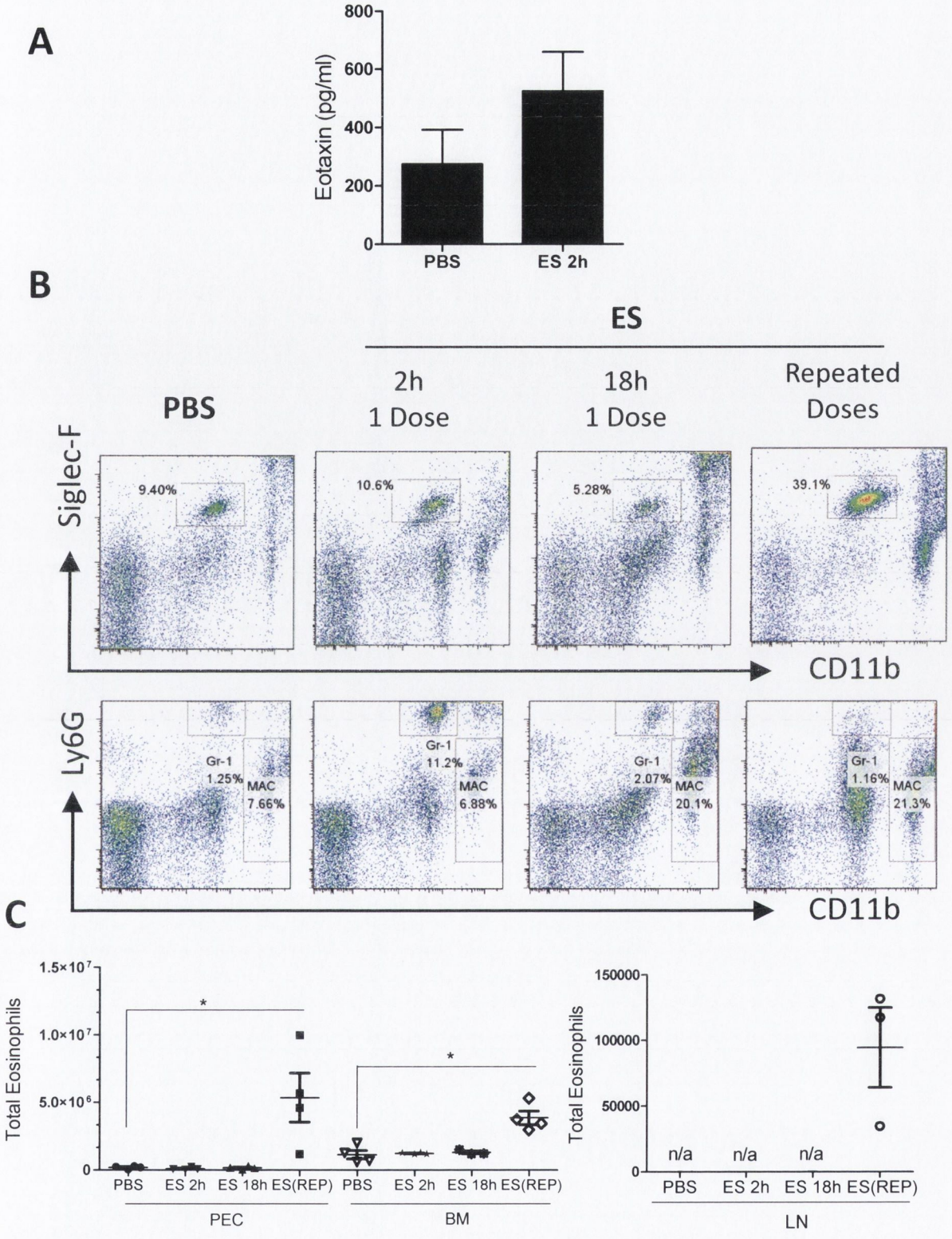


Fig 4.21 Repeated administration of ES promotes recruitment of eosinophils. A, C57BL/6 mice were injected i.p. with ES (20 μl). After 2 hours, Blood serum was extracted and the concentration of eotaxin was quantified by ELISA. B, C57BL/6 mice were i.p. injected with PBS or with ES (20 μl) every 2 days for 14 days. Additionally, groups of mice received a single administration of ES (20 μl) either 18 hours or 2 hours prior to isolation. PEC were isolated by peritoneal lavage and analysed by flow cytometery for the presence of Siglec-F^+ $CD11b^{int}$ eosinophils and $CD11b^+$ $Ly6G^+$ neutrophils. C, total numbers of Eosinophils in the peritoneum bone marrow and medistinal LN. Data presented are means +/- SEM of four mice per group. Results are representative of 3 separate experiments

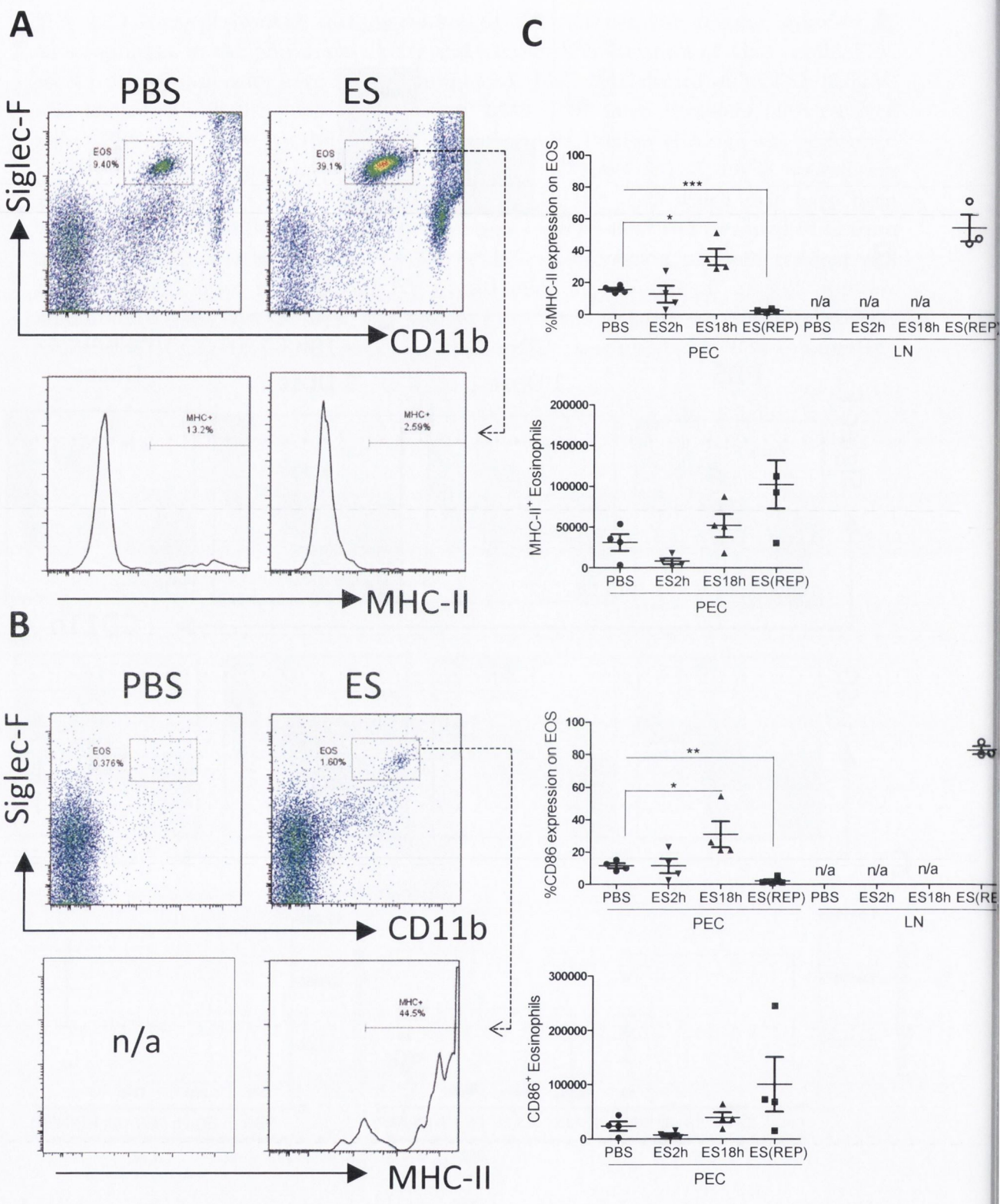


Fig 4.22 ES administration promotes recruitment of eosinophils in the draining LN that express MHC-II and CD86. C57BL/6 mice were i.p. injected with PBS or with ES (20 μl) every 2 days for 14 days. Additionally, groups of mice received a single administration of ES (20 μl) either 18 hours or 2 hours prior to isolation of PEC and mediastinal LN. A, PEC and B, medistinal LN were analysed by flow cytometry for presence of eosinophils and the percentage expression of MHC on eosinophils. C, Mean expression of MHC-II and CD86 on peritoneal and mediastinal eosinophils. Data presented are means +/- SEM of four mice per group. Results are representative of 2 separate experiments

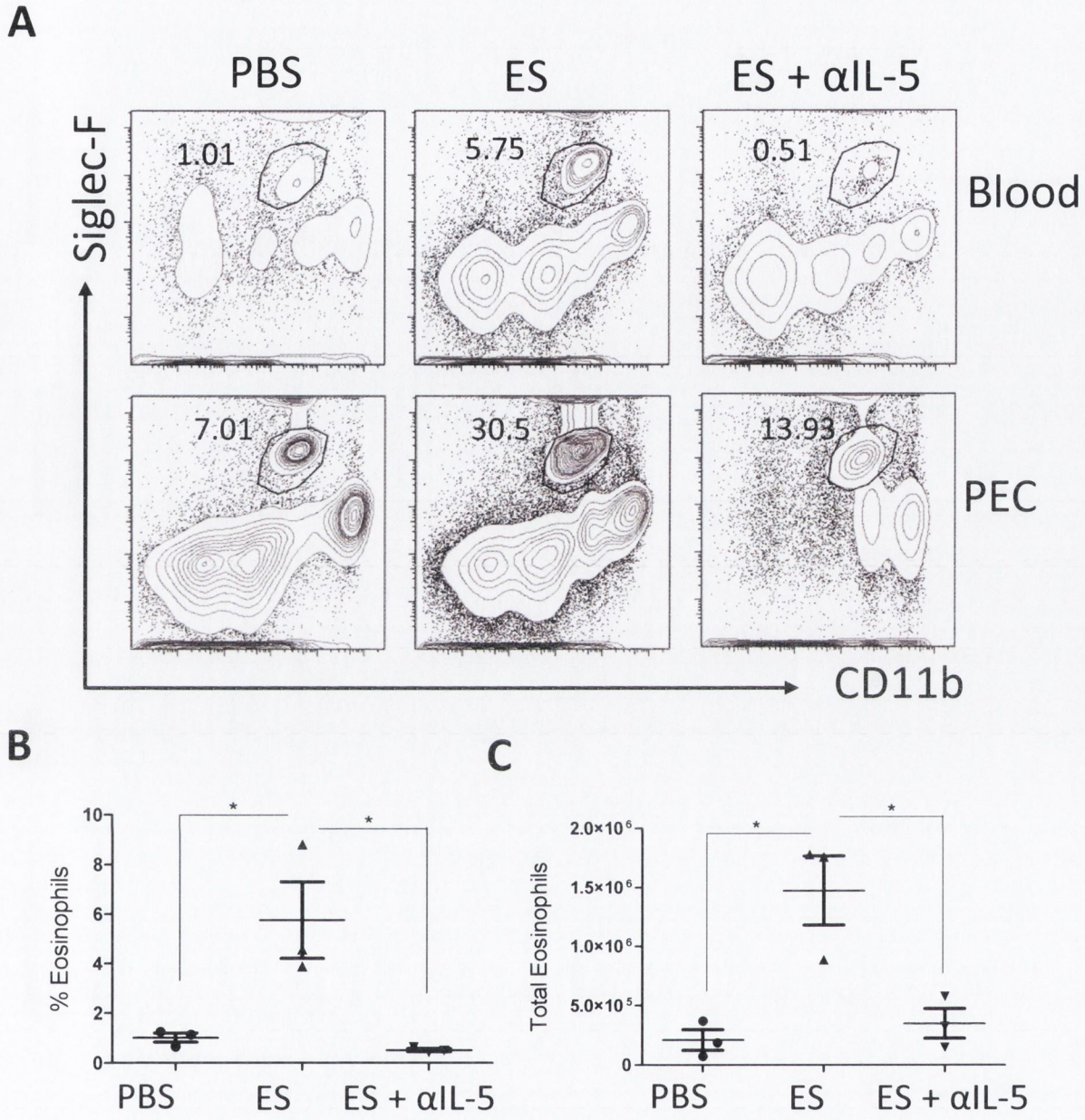


Fig 4.23 ES administration induces eosinophilia in a IL-5 dependent manner. A, PBS, ES (40 μl) or ES + αIL-5 (TRFK-5, 80 μg) was administered i.p. to C57BL/6 mice every 2 days for 6 days . On day 7 blood and PEC were analysed by flow cytometry for presence of eosinophils. A, representative flow cytometric plots demonstrating staining for $CD11b^{int}$ Siglec-F^{+} SSc^{high} Eosinophils. Mean numbers of eosinophils in B, the blood and C, peritoneal cavity. Data presented are means +/- SEM of 3 mice per group. Results are representative of 2 experiments

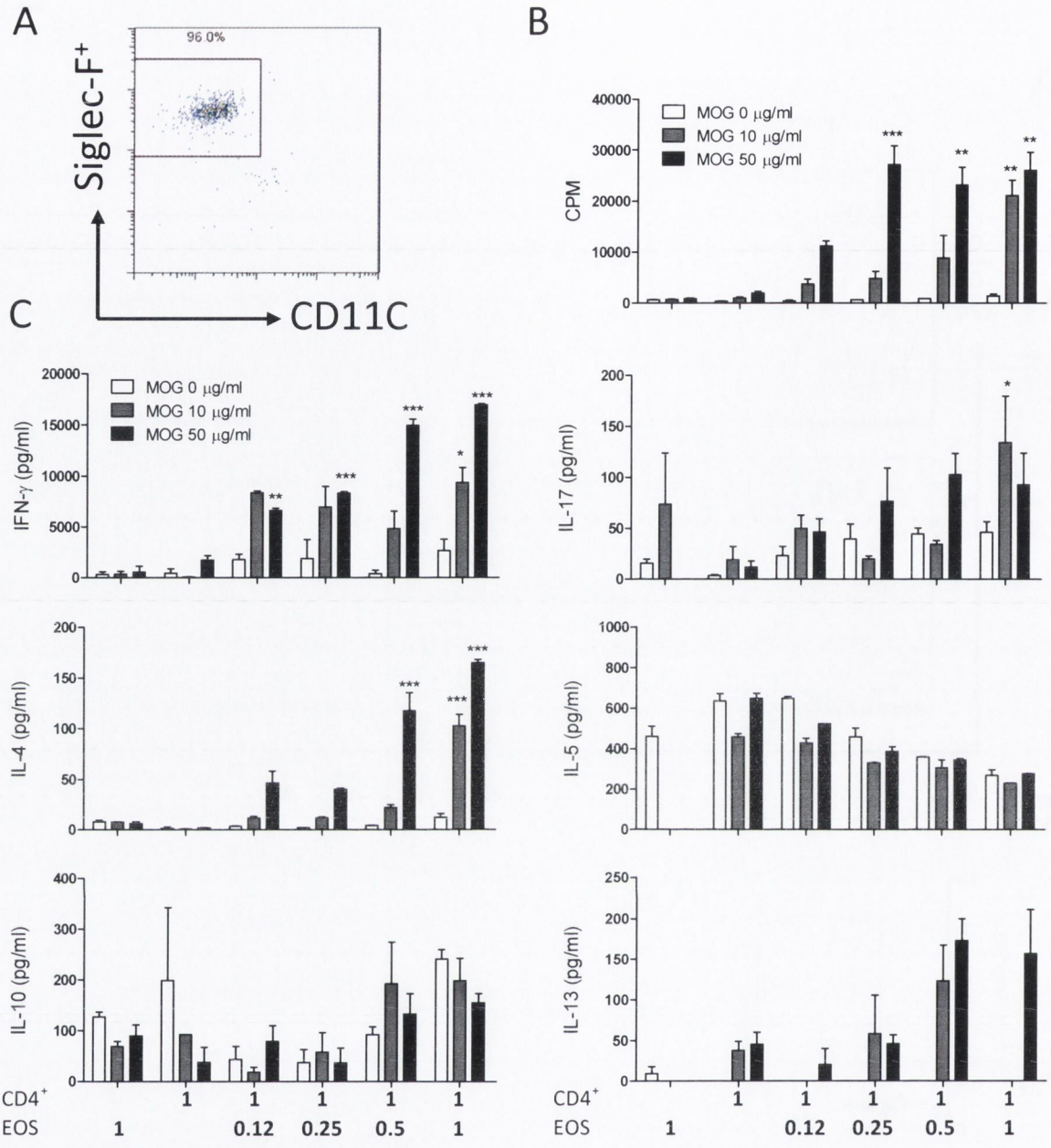


Fig 4.24 Eosinophils isolated from *F. hepatica* infected mice can act as APC for antigen-specific CD4$^+$ T cells. C57BL/6 mice were infected with 10 viable *F. hepatica* metacercariae. After 21 days PEC were isolated by peritoneal lavage and Siglec-F$^+$ eosinophils cells were purified by FACS. A, FACS plot of sorted eosinophils with percentage purity. CD4$^+$ T cells were purified from mice with EAE (day 18) and cultured ($1x10^6$ cells/ml) in the presence of increasing concentrations of MOG peptide (0, 10 and 50µg/ml). Siglec-F$^+$ eosinophils were co-cultured with T cells at the ratios of 1:1, 1:0.5, 1:0.25 and 1:0.125. B, Cellular proliferation was determined by [^{3}H] thymidine incorporation during the last 18 hours of the 72 hour culture. C, The concentrations of IFN-γ, IL-4, IL-5, IL-10, IL-13 and IL-17 in the supernatant after 72 hours were quantified by ELISA. Data presented are means +/- SEM of triplicate assays. Statistical assessment was performed using one way ANOVA with bonferroni's post test vs CD4$^+$ alone. * $P<0.01$ ** $P<0.001$ ** $P<0.005$. Results are representative of 2 separate experiments.

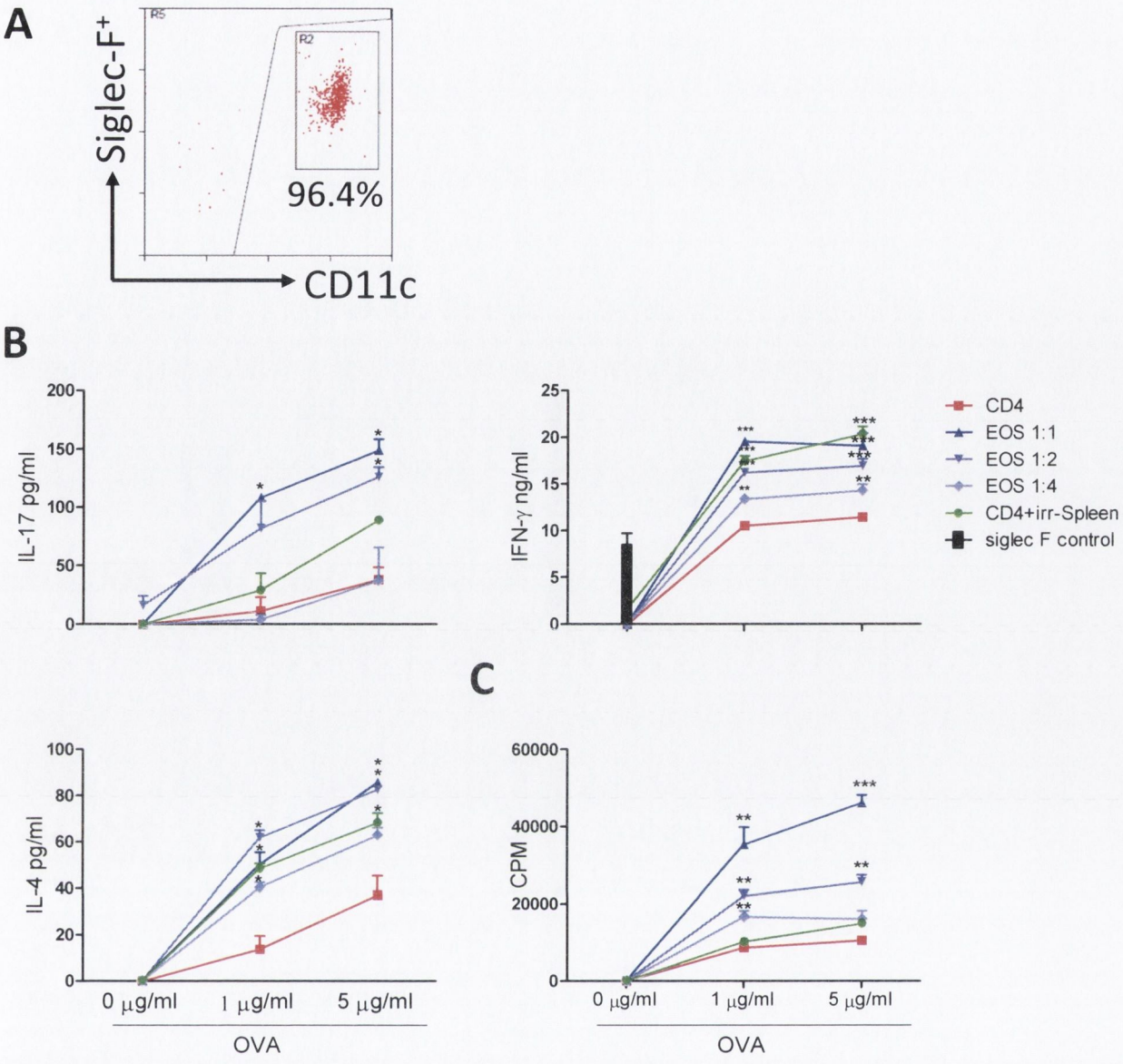


Fig 4.25 Eosinophils isolated from *F. Hepatica* infected mice can act as APC for OVA-specific CD4⁺ T cells. Balb/c mice were infected with 10 viable *Fasciola Hepatica* Shrewsbury strain metacercariae. After 21 days PEC were isolated by peritoneal lavage and Siglec-F⁺ eosinophils cells were purified by FACS A, FACS plot of sorted eosinophils with percentage purity. The siglec-F⁺ population was co-cultured at the indicated ratios with purified CD4⁺ T cells isolated from D0.10.11 mice that had been immunised with OVA peptide (100μg) and CpG (50μg) 14 and 7 days previously. Co-cultures were incubated with increasing concentrations of OVA peptide (0,1 and 5μg/ml). C, After 72 hours the concentrations of IFN-γ, IL-4, IL-5, IL-17 and TGF-β were quantified by ELISA. B, Cellular proliferation was determined by [^{3}H] thymidine incorporation during the last 18 hours of the 72 hour culture. Data presented are means +/- SEM of triplicate assays. Statistical assessment was performed using unpaired Student's t test vs CD4⁺ alone. * P<0.01 ** P<0.001 ** P<0.005

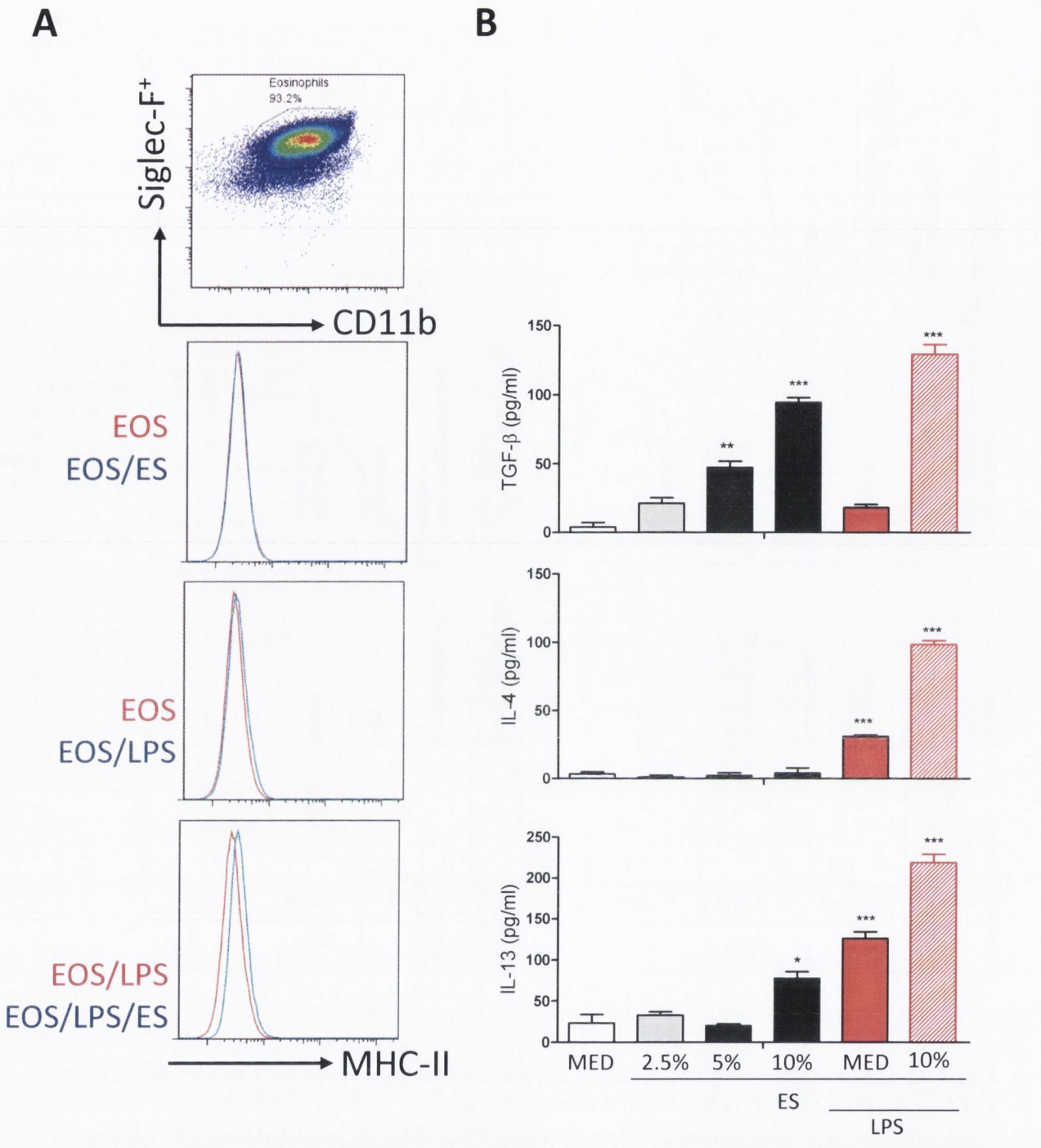


Fig 4.26 ES induces the release TGF-β, and promotes LPS-induced production of IL-4 and IL-13 from bone marrow derived eosinophils but does not enhance expression of MHC-II *in vitro*. BMEOS were expanded from BALB/c mice and incubated with increasing concentrations of ES (0, 2.5, 5, 10% v/v), LPS (100ng/ml) and LPS + ES (10% v/v). A, After 24 hours the expression of MHC-II on eosinophils was analysed by flow cytometry. B, The concentrations of TGF-β, IL-6, IL-4 and IL-13 in the supernatant after 24 hours was quantified by ELISA. Data presented are means +/- SEM of triplicate assays. Statistical analysis was performed using one-way ANOVA with Dunnett's post test vs MED alone.

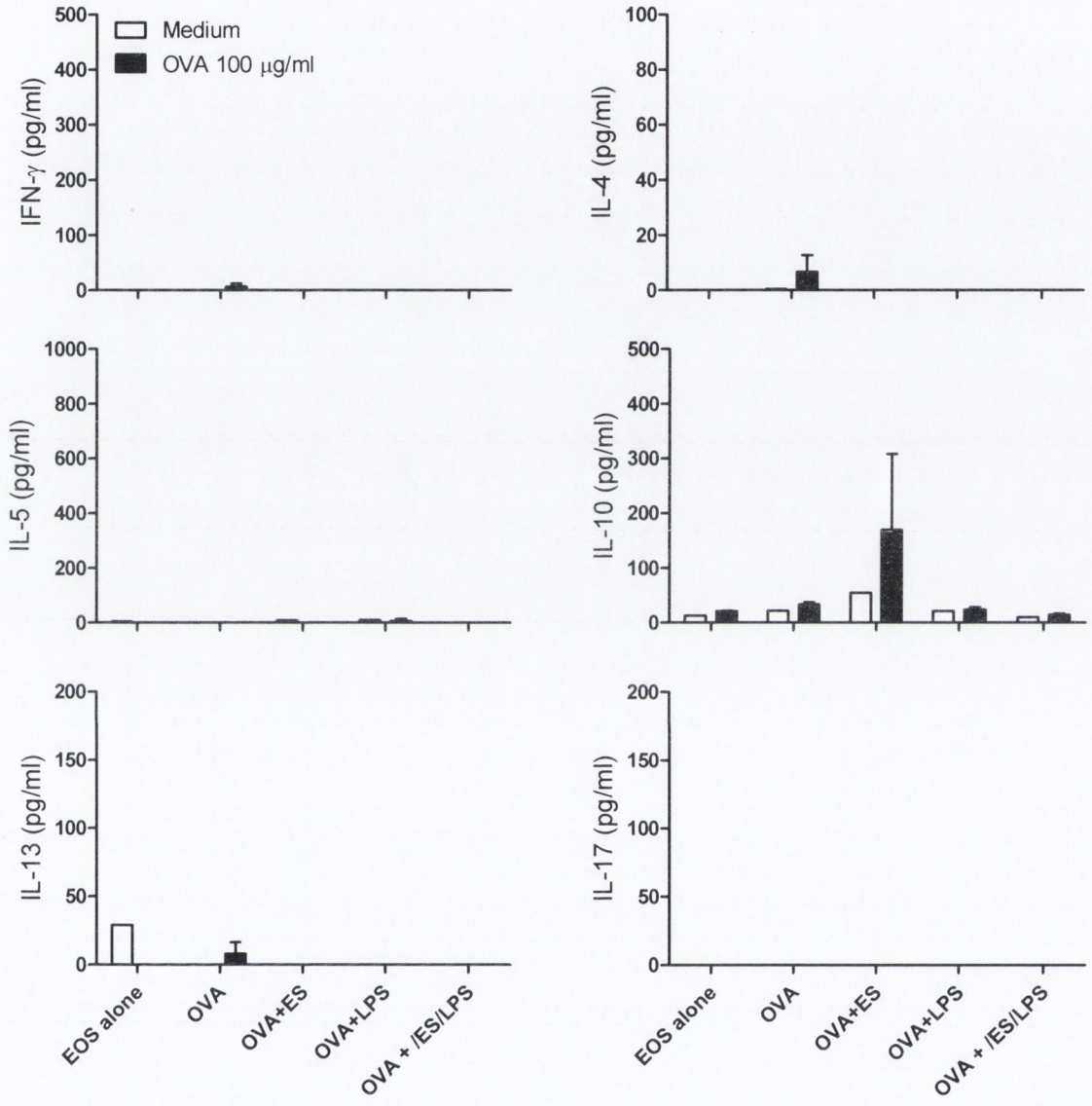


Fig 4.27 ES-treated bone marrow derived eosinophils cannot prime T cell responses *in vivo.* BMEOS were expanded from BALB/c mice and loaded with OVA (50µg/ml). After 2 hours cells BMEOS cells were incubated with either ES (10% v/v), LPS (100ng/ml) and LPS + ES (10% v/v) or left untreated as indicated. After 24 hours $0.5x10^6$ cells were s.c. injected into the footpads of fresh mice. After 7 days the popliteal LN of recipient mice were exised and restimulated with OVA (100µg/ml). The concentration of IFN-γ, IL-4, IL-5, IL-10, IL-13 and IL-17 after a 72 hour culture was determined by ELISA. Data presented are means +/- SEM of triplicate assays.

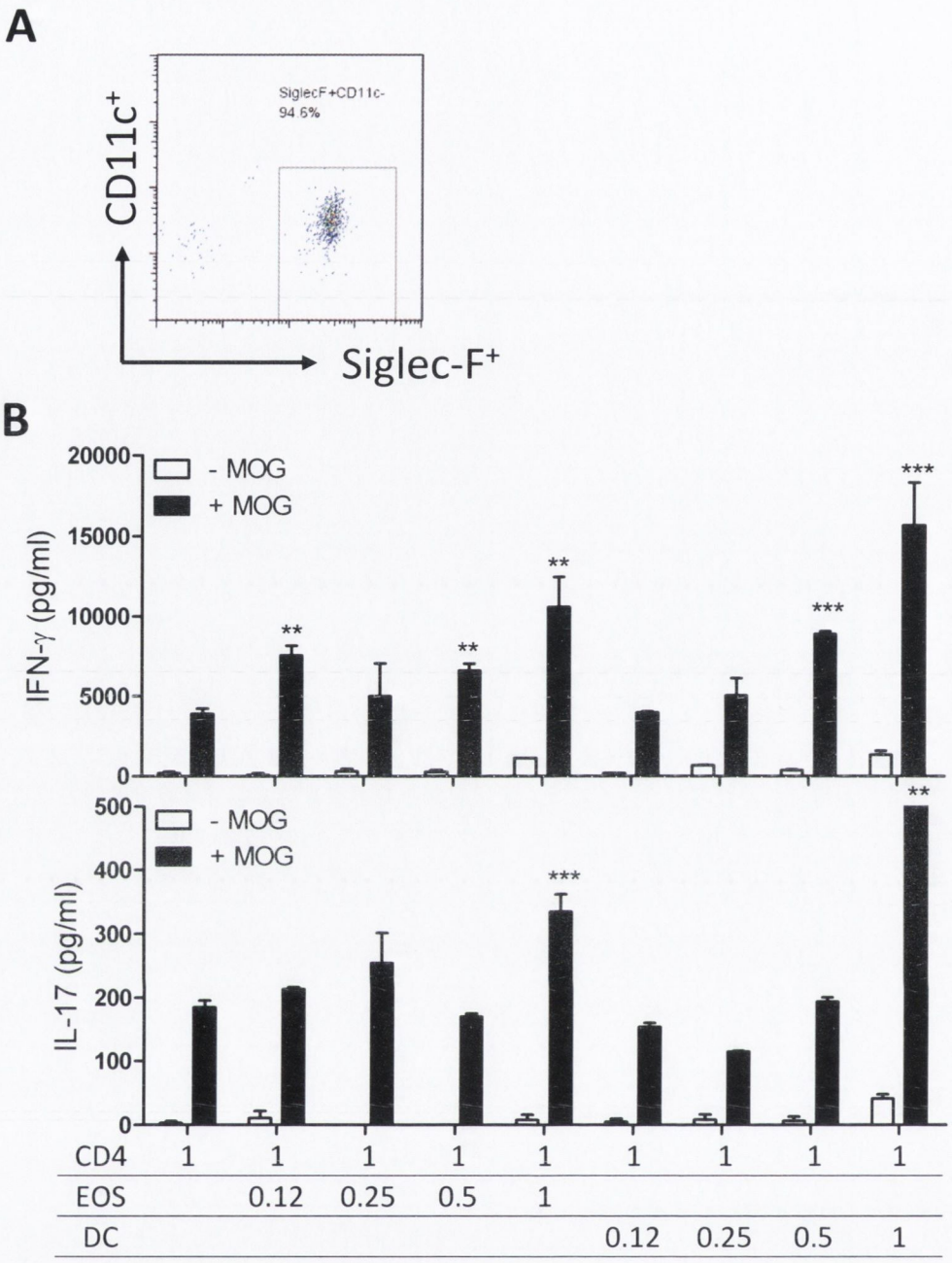


Fig 4.28 ES-elicited eosinophils can reactive antigen specific CD4$^+$ T cells. ES was injected i.p. to C57BL/6 mice every 2 days for 14 days. On day 15 PEC were isolated by peritoneal lavage and Siglec-F$^+$ CD11b^{int} eosinophils were purified by FACS. A, FACS plot of sorted eosinophils with percentage purity. Purified populations were incubated with PBS control or MOG antigen and were co cultured with CD4$^+$ T cells purified by magnetic separation from the spleens and LN of mice with EAE (day 18). B, The concentration of IFN-γ and IL-17 in the supernatant after 72 hours was determined by ELISA. Data presented are means +/- SEM of triplicate assays. Data is representative of 2 separate experiments.

Chapter 5 The excretory/secretory products of *F. hepatica* suppress T cell responses and attenuate autoimmunity

5.1 Introduction

The results presented in chapter 3 have shown that infection of mice with *F. hepatica* attenuated the clinical severity of EAE. This effect was later found to be dependent on the production of TGF-β *in vivo* (298). Helminth therapy is a novel intervention that is currently being investigated as a potential therapeutic against autoimmune diseases (178). However, the helminths of choice (intestinal nematode *T. spiralis* and the hookworm *N. americanus)*, are associated with mild or asymptomatic infections. In contrast, *F. hepatica,* is associated with severe liver damage, wasting disease, fever, and abdominal pain (393), and as such is neither a safe nor viable option for the treatment of autoimmunity. Thus, ES or the components thereof are of greater therapeutic interest than infection with the whole parasite, as these may duplicate the immunomodulatory properties of infection, whilst avoiding the immunopathology associated with disease.

In the previous chapter the secreted components of *F. hepatica,* ES were shown to have several immunoregulatory properties, most notably the inhibition of the APC capacity of DC and induction of TGF-β from these cells. However, the role of ES in the modulation of T cell function and autoimmunity has not been addressed. Thus, the aim of this chapter was to investigate the effect of ES on T cells and on a T cell-mediated model of autoimmunity, EAE. Specific aims include:

1) **To determine whether ES can affect the function of T cells**

The previous two chapters have shown that infection with *F. hepatica* or administration of its secreted products can modulate the APC function of innate cells *in vitro* and *in vivo.* However, the effect of ES on T cell function has not been addressed. Infection with *F. hepatica* is reported to induce a strongly polarised type-2 response *in vivo,* but the nature of the response to ES *in vivo* has not been undetermined. Infection with *F. hepatica* induced the recruitment of TGF-β-producing Treg cells to the peritoneal cavity of mice and it is possible that these cells mediate protection from EAE, as neutralisation of TGF-β reversed protection (298). This study addressed these questions, and investigated the role of ES on T cell function, Treg cell recruitment and induction of Th2 cells.

2) To determine whether *F. hepatica* ES can modulate the course of EAE

The secreted products of other helminths have been shown to be efficacious in the treatment of experimental models of autoimmunity (179, 222). SEA from *S. mansoni* protects mice from EAE which is associated with a decrease in MOG-specific IFN-γ and a concurrent increase in IL-4, TGF-β and IL-10. In that model protection was dependent on the induction of Th2 cells as STAT6 deficient mice were not protected following administration of SEA (223-225). The results of chapter 3 has already shown that infection with *F. hepatica* protected mice from EAE, thus, the objective of the current chapter was to determine if the secreted products of the fluke have a similar protective effect.

5.2 Results

5.2.1 ES inhibits T cell responses in vitro

Having demonstrated that *F. hepatica* ES negatively affected APC function, experiments were conducted to determine if ES could directly affect the activity of T cells *in vitro*. Spleen cells, isolated from naïve C57BL/6 mice, were stained with the membrane dye CTV and activated with soluble αCD3ε in the presence of increasing concentrations of ES (0, 0.625, 1.25, 2.5, 5 and 10% v/v). The proliferation of $CD4^+$ and $CD8^+$ T cells was determined after 4 days as the percentage of these cells which had lowered CTV fluorescence compared with unstimulated controls. Titration of ES inhibited αCD3ε-induced proliferation of $CD4^+$ and $CD8^+$ T cells in a dose dependent manner [Fig 5.1 A]. Indeed, ES completely blocked proliferation at concentrations above 2.5 % v/v. Furthermore, ES reduced αCD3ε-induced production of IFN-γ, IL-17 and IL-4 [Fig 5.1 B]. In contrast, ES enhanced the concentrations of TGF-β in the culture, in a dose dependent manner, albeit independent of the presence of αCD3ε [Fig 5.1 B]. Importantly, titration of ES did not induce death of $CD4^+$ T cells as determined by flow cytometry analysis of Aqua LIVE/DEAD fluorescence on stained cells [Fig 5.1 C].

CTLA-4 and PD-1 are receptors expressed by T cells which inhibit T cell activation upon ligation, in a manner antagonistic to the co-stimulation provided by ligation of the TCR with CD28. CTLA-4 and PD-1 inhibit T cell activation by distinct methods (394, 395). CTLA-4 on T cells is highly upregulated by engagement with CD28 and antagonizes early T cell activation by inhibiting IL-2 production, cell cycle progression and TCR signalling (395, 396). PD-1 ligation inhibits the TCR-induced signals which stop movement of T cells, therefore, allowing for generation of stable DC-T cell interactions (395). Both receptors have roles in preventing immune pathology and autoimmunity (397). To examine the effect of ES on the expression of CTLA-4 or PD-1, spleen cell cultures were incubated with ES in the presence of aCD3 and the expression of CTLA-4 and PD-1 by $CD4^+$ T cells was determined by flow cytometry. Compared with unstimulated controls, ES increased the expression of CTLA-4 from 18% to 65% [Fig 5.2]. However, αCD3ε alone increased the expression of CTLA-4 to 83%, and titration of ES into αCD3ε stimulated cells actually suppressed this effect [Fig 5.2]. Similar to

CTLA-4, ES alone increased the expression of PD-1 by $CD4^+$ T cells from 9% to 60% [Fig 5.2]. However, unlike CTLA-4 expression of PD-1 by T cells stimulated with αCD3ε increased only from 9% to 40% and this was increased further by ES in a dose dependant manner [Fig 5.2]. Furthermore, gating on $CD44^{high}$ $CD62L^{low}$ effector memory (EM) cells revealed that these cells dramatically enhanced expression of PD-1 in response to ES with or without the addition of αCD3ε, indeed, 100% of EM $CD4^+$T cells expressed PD-1 when ES was used at above 5% v/v [Fig 5.2].

5.2.2 ES suppresses antigen-specific $CD4^+$ T cell proliferation *in vivo*.

In order to examine the effect of ES on T cell proliferation *in vivo*, OVA-specific TCR-transgenic DO.10.11 mice previously immunized with OVA peptide and LPS were injected with OVA or PBS (as a vehicle) and were treated with either PBS or ES for 4 days [Figure 2.2]. On days 0-3, all mice received bromodeoxyuridine (BrdU), a synthetic nucleotide that is incorporated into the newly synthesized DNA of replicating cells (398). On day 4, mice were sacrificed, and spleen, inguinal LN and PEC were isolated and surface stained for CD4 and with an antibody specific for the $OVA_{(323-339)}$ transgenic TCR. The results of flow cytometric analysis revealed that, BALB/c control mice lacked $CD4^+$ T cells which expressed the $OVA_{(323-339)}$ transgenic TCR, however, most of the $CD4^+$cells from DO.10.11 mice possessed the transgenic TCR [Fig 5.3]. OVA-specific T cells were gated as those $CD4^+$ T cells that expressed the transgenic TCR and their proliferation of was assessed by immunofluorescence of an antibody directed against BrdU.

Administration of OVA-peptide to mice on day 0 enhanced antigen-specific proliferation of OVA-TCR-Tg $CD4^+$ T cells from the spleen LN and peritoneal cavity on day 4 [Fig 5.3]. However treatment with ES inhibited this proliferation of OVA-specific T cells [Fig 5.3]. Furthermore, spleen, LN and peritoneal cells were reactivated with OVA *ex vivo* for 72 hours and the proliferation of these cells was quantified by ^{3}H-thymidine incorporation in the final 12 hours. Cells isolated from the spleen, inguinal LN but not from the peritoneal cavity of mice treated with ES proliferated less compared with cells isolated from PBS-treated mice [Fig 5.3]. These results demonstrate that ES can suppress antigen-specific T cell proliferation to an unrelated antigen *in vivo*.

5.2.3 TGF-β can inhibit Th1, Th2 and Th17 responses.

TGF-β has been shown to inhibit the proliferation of naive T cells. The effect of ES on activated T cells which have reduced expression of TGF-βRII, is less pronounced (100). Recent reports have shown that low concentrations of TGF-β in association with IL-6, promote the differentiation of human Th17 cells (399, 400). However, it has also been reported that at high concentrations, TGF-β suppresses Th17 differentiation and enhances the expression of Foxp3 in T cells (89). TGF-β has also been shown to suppress the development of EAE (401). Indeed, a publication from this laboratory has shown that TGF-β plays an essential role in the suppression of EAE during infection with *F. hepatica* (298, 402). The present study found that TGF-β was the only cytokine produced by BMDC in response to ES alone [Fig 4.10]. In order to examine the effect of TGF-β on Th cell activation, purified naïve $CD4^+$ T cells were activated with αCD3ε in the presence of increasing concentrations of TGF-β. Irradiated spleen cells from naive mice were used as APC, as CD28 stimulation has been shown to attenuate TGF-β-mediated inhibition of T cell proliferation (403). Increasing concentrations of recombinant TGF-β (1, 10, 100, 1000, 10000 pg/ml) were added to the culture and the concentrations of IL-17, IFN-γ and IL-4 in supernatants were quantified after 72 hours [Fig 5.4]. Addition of recombinant TGF-β significantly suppressed αCD3ε induced IL-4 production even at very low concentrations (1 pg/ml) [Fig 5.4]. IFN-γ production was also suppressed at all concentrations tested. Consistent with previous reports, recombinant TGF-β enhanced αCD3ε-induced IL-17 production at lower concentrations (10 pg/ml) but significantly suppressed the production of IL-17 at higher concentrations (>1000pg/ml) [Fig 5.4]. These findings demonstrate that TGF-β inhibits the production of cytokines associated with Th1, Th2 and Th17 cells.

5.2.4 Inhibition of proliferation is not mediated by TGF-β, cathepsins, IDO, or PD-1

Having shown that recombinant TGF-β inhibited the production of IL-4, IL-17 and IFN-γ by αCD3ε-stimulated $CD4^+$ T cells [Fig 5.4], and that ES induced the release of TGF-β from DC [Fig 4.10], experiments were conducted to determine if ES suppressed T cell proliferation via TGF-β. Spleen cells were activated with soluble αCD3ε in the presence of increasing concentrations of ES (0, 0.1325, 0.625, 1.25, 2.5, 5 and 10%) with or without either a neutralising αTGF-β antibody or with the TGF-βR signalling inhibitor SB-431542. Addition

of αTGF-β or SB-431542 had no effect on the ability of ES to inhibit the proliferation of $CD4^+$ T cells as determined by CFSE dilution in a 72 hour culture [Fig 5.5].

ES enhanced the expression of the inhibitory T cell receptor PD-1 by $CD4^+$ T cells [Fig 5.2]. The role of PD-1 in the ES-induced inhibition of proliferation was tested using blocking antibodies directed against PD-1, and one of its ligands, PDL-2. Neutralisation of PD-1 signalling did not reverse the inhibitory effect of ES on the proliferation of T cells [Fig 5.5].

Two other possible mechanisms were investigated, cathepsins and IDO. ES contains several cathepsins which have been reported to have immunomodulatory activity (272). IDO is an enzyme that is expressed by DC in response to TGF-β and is intrinsically involved in the generation of tolerance and Treg cells (404). However, ES-induced inhibition of T cell proliferation was not affected by the presence of the IDO inhibitor, 1-mythel a cathepsin inhibitor E-64 [Fig 5.5].

5.2.5 ES enhances TGF-β but not FOXP3 expression in CD4+ T cells in vivo

At high concentrations, TGF-β enhances the expression of Foxp3 in $CD4^+$ T cells (88, 89). Infection of mice with *F. hepatica* expanded a population of $FOXP3^+TGF\text{-}\beta^+CD4^+$ T cells in the peritoneal cavity [Fig 3.10]. As ES induced the release of TGF-β from DC [Fig 4.10], experiments were conducted to determine if ES could drive Treg cell differentiation *in vivo*. Mice were injected i.p. with ES every other day for 14 days. On day 15, intracellular expression of TGF-β and FOXP3 in splenic, inguinal LN, mediastinal LN and peritoneal $CD4^+$ T cells was determined by flow cytometry. ES treatment significantly enhanced the expression of TGF-β on $CD4^+$ T cells isolated from the spleens and inguinal LN compared with those isolated from mice which were treated with PBS. Moreover, the expression of TGF-β was enhanced, albeit not significantly, in mediastinal LN $CD4^+$ and splenic $CD8^+$ T cells following treatment with ES [Fig 5.6]. However, ES administration did not enhance the expression of FOXP3 by inguinal LN, mediastinal LN or peritoneal $CD4^+$ T cells compared with those cells from PBS treated mice [Fig 5.6]. These results suggest that while administration of ES enhances TGF-β production by T cells *in vivo*, it does not expand Treg cells.

5.2.6 ES induces Th2 responses *in vivo*

Canonically, helminths drive a type-2 immune response driven by innate and adaptive leukocyte-derived IL-4, IL-5 and IL-13 (152). Protective immunity to the majority of helminths species requires a concerted Th2 response as protection is lost in the absence of IL-4R signalling (152, 405). Several studies have shown that *F. hepatica* infected hosts mount anti-parasite Th2 responses (248-251). Flynn et al, demonstrated that 10 days after experimental infection of cows, bovine PBMC produce IL-4 but not IFN-γ in response to parasite antigens (252). Thus, experiments were conducted to determine if ES could drive a Th2 response *in vivo* to the unrelated antigen, OVA. ES was not used as an antigen in recall experiments since it exerts a variety of suppressive effect on T cells *in vitro* [Fig 5.1]. Mice were immunized by i.p. injection on day 0 and day 21 with OVA alone as a control, OVA+ES and OVA+heat-inactivated ES. Heat inactivated ES was included as a control to determine if any effects of ES were mediated by proteins in their native form. After 7 days, PEC were isolated from all mice and were stimulated with PMA and ION in the presence of BFA. The expression of IL-4, IL-5, IL-13 and IL-10 in peritoneal $CD4^+$ T cells was determined by flow cytometry and the results were analysed using SPICE software. SPICE analysis allows for the graphical presentation of T cells which produce several cytokines in various combinations (polyfunctional T cells) (276, 277). The analysis revealed that $CD4^+$ T cells from OVA control mice expressed relatively little IL-4, IL-5, IL-10 or IL-13 [Fig 5.7]. $CD4^+$ T cells from mice immunised with OVA+ES displayed a more activated Th2 phenotype and expressed higher IL-4, IL-5, IL-10 and IL-13 than mice immunised with OVA alone. Interestingly, there was strong co-expression of IL-4 with IL-5 and IL-13, while IL-5 expression correlated well with IL-13 expression [Fig 5.7]. The expression of Th2 cytokines by $CD4^+$ T cells isolated from mice treated with OVA+heat-inactivated ES was enhanced compared with those treated with OVA only but was lower than that of mice immunised with OVA+ES. Specifically, while the expression of IL-4 was relatively high, the expression IL-5 was very low [Fig 5.7]. IL-10 was not expressed to a high degree by $CD4^+$ T cells from any of the treatment groups.

In addition, the mediastinal LN were excised from all groups of mice, the cells were pooled and reactivated with OVA. After 3 days, there was a significant increase in the production of OVA-specific IL-4 and IL-13 by $CD4^+$ cells isolated from mice treated with OVA+ES compared with OVA control LN cells; the expression of IL-5 while enhanced, did not reach

statistical significance [Fig 5.7]. In contrast LN cells from OVA+heat-inactivated ES did not produce any OVA-specific IL-4, IL-5 or IL-13 [Fig 5.7]. These results demonstrate that ES can drive a Th2 response *in vivo* which is partially suppressed by heat-inactivation of ES.

5.2.7 ES treatment delays onset and reduces the clinical severity of EAE

Since infection with *F. hepatica* significantly attenuated the clinical symptoms of EAE, and having established that ES shares many immunomodulatory properties of *F. hepatica,* experiments were conducted to examine the effect of administration of ES on the clinical outcome of EAE. C57BL/6 mice were injected i.p. with ES or PBS beginning on day -1 and every second day thereafter. EAE was induced by s.c. injection of MOG emulsified in CFA on day 0. Treatment with ES delayed the onset and significantly attenuated the clinical severity of EAE compared with PBS-treated control mice [Fig 5.8]. Results were consistent across separate biological isolates of ES and are displayed as the mean clinical scores from 7 experiments [Fig 5.8].

The spleens of mice with EAE treated with ES or PBS were excised on day 15 post-induction and were reactivated *in vitro* with increasing concentrations of MOG. After a 72 hour culture, MOG-specific IFN-γ and IL-17 production was significantly suppressed in spleen cells isolated from mice treated with ES compared with PBS controls [Fig 5.9].

There are two commonly used protocols for the induction of EAE in C57BL/6 mice: active and passive. Active EAE (above) is induced in mice through the generation of anti-MOG responses by immunization with MOG and CFA. Passive EAE is induced by transfer of encephalitogenic T cells. These cells are generated by culture of spleen and LN cells, isolated from mice 10 days after immunisation with CFA and MOG *ex vivo,* with MOG, IL-1β and IL-23. EAE is commonly induced by active immunisation with MOG emulsified in CFA. However EAE can also be induced by adoptive transfer of MOG-specific T cells from immunized mice which have been expanded *in vitro* with MOG, IL-1β and IL-23. This provides a useful model to examine the effect of ES on pathogenic T cells *in vivo.*

Experiments were conducted to determine if ES could protect mice from EAE induced by the adoptive transfer method. EAE was induced on day 0 by transfer of $10x10^6$

encephalitogenic $CD4^+$ T cells to recipient mice. Starting on day -1 mice were treated by i.p. injection with ES every second day. Clinical scores were monitored for 18 days. Apart from a slight exacerbation of disease at the early stage, ES had no effect on the clinical scores of passively induced EAE [Fig 5.10]. These data demonstrate that administration of ES can attenuate the clinical symptoms EAE induced by the active, but not the passive method and suggests that ES may not directly suppress pathogenic T cells *in vivo*.

5.2.8 The component(s) of ES that most potently protect from EAE are above 3kDa

2D electrophoresis, used together with mass-sepectophtometery, has revealed that ES contains a large number of distinct compounds (406). Experiments were conducted to determine if the active component within ES which protects from EAE, was a small molecule or a protein. Thus, ES was fractionated into the ES >3kDa fraction (ES-H) which contains proteins and macromolecules, and the ES <3kDa (ES-L) fraction which consists of small molecules and peptides. Beginning on day -1 and every 2 days thereafter, C578BL/6 mice were injected i.p. with either PBS, total ES, ES-H or ES-L. On day 0, EAE was induced in all groups by s.c. injection with CFA and MOG. All groups which received formulations of ES had a significantly attenuated course of disease [Fig 5.11 A]. The mean clinical score of mice which received either total ES or ES-H did not rise above 0.25 compared with PBS controls which had a score of 3.25 on day 12 [Fig 5.11 A]. Whilst ES-L initially protected mice from EAE, it was less protective than ES-H; with a mean clinical score of 1.5 at the termination of the experiment [Fig 5.11 A].

EAE is mediated by pathogenic MOG-specific Th1 and Th17 cells that infiltrate the brain and spinal cord (407). Mononuclear cells were isolated by density centrifugation from the pooled perfused brains of all mice in order to determine the number of infiltrating T cells. Mononuclear cells were analysed by flow cytometry for the expression of CD4, CD8, IFN-γ, IL-17, GM-CSF, IL-4 and IL-13 following stimulation with PMA + ION in the presence of BFA. As expected, PBS-treated mice had the greatest infiltration of $CD4^+$ T cells into the brain (936,936 $CD4^+$ T cells) [Fig 5.12 A]; there was a 10 fold reduction in CNS infiltration in mice which were treated with Total ES (8,720 cells), ES-H treated mice had 30,160 cells while those treated with ES-L had the highest number among the ES treated groups at 207,043 cells [Fig 5.12 A].

Analysis of the frequency of cytokine secretion by T cells in the CNS revealed that $CD4^+$ T cells in the brain of mice treated with PBS displayed robust production of IFN-γ (62.1% of total $CD4^+$ T cells), IL-17 (20.9%) and GM-CSF (13.66%) [Fig 5.12 B]. $CD4^+$ T cells from the Total ES treated mice had comparatively reduced expression of IFN-γ (23.4%) and IL-17 (13.1%), although the reduction in IL-17 was mainly due to a 3-fold decrease in proportion of IL-17^+ IFN-γ^+ double positives and not IL-17^+ single producers which were unchanged [Fig 5.12 B]. Similarly, $CD4^+$ T cells from ES-H treated mice had reduced IFN-γ (36%) and IL-17 (8.31%) and in this case the proportion of both IL-17^+ single (5.56%) and IL-17^+ IFN-γ^+ double producers (2.78%) was reduced. Neither treatment group had any effect on the proportion of total GM-CSF^+ $CD4^+$ T cells, although GM-CSF^+ IFN-γ^+ double producers were reduced in both Total ES and ES-H treated groups [Fig 5.12 B]. In contrast, and consistent with disease scores, $CD4^+$ T cells from mice treated with ES-L did not display as large a reduction in IFN-γ (47%), IL-17 (17.3%) and GM-CSF (10.39%) secreting cells [Fig 5.12 B].

Interestingly, Total ES or ES-H but not ES-L treatment enhanced the expression of IL-4 and IL-13 in $CD4^+$ T cells. The expression of IL-4 was increased from 0.7% in PBS controls to 4.91% and 8.28% in total and ES-H, respectively. Meanwhile, the expression of IL-13 was increased from 1.4% to 17.1% and 19.21%, respectively.

$CD8^+$ T cells also produced IL-17 and IFN-γ. In the brains of mice treated with PBS 47% of $CD8^+$ T cells produced IFN-γ and interestingly 16.9% produced IL-17. Moreover, 8.2% of $CD8^+$ T cells produced both IL-17 and IFN-γ. $CD8^+$ T cells from Total ES treated mice expressed less IL-17 (34%) and IFN-γ (8.79%) and had less double producers (2.2%) while ES-H treated mice had a similar reduction in IL-17 (37.9%) and IFN-γ (6.9%) and double producers (3.45%). Similar to results obtained for $CD4^+$ T cells, ES-L treatment did not reduce the expression of IL-17(37.9%), IFN-γ (11%) or IL-17 IFN-γ coproduction (6.37%) by $CD8^+$ T cells in CNS.

Until now mice with EAE were treated by prophylactic administration of ES beginning one day before disease induction. For greater clinical relevance, an experiment was conducted to determine if ES could act therapeutically. EAE was induced in CD57BL/6 mice on day 0. On day 5 and every second day thereafter, mice were injected i.p. with PBS, total ES, ES-H or

ES-L. Similar to results obtained with prophylactic treatment, all mice receiving injections of any type of ES were somewhat protected, but in contrast, no group was completely protected from EAE [Fig 5.11 B]. Treatment with ES-H was the most effective; the clinical score of mice in this mice did not exceed 0.6 compared with a peak of 3.25 in the PBS treated mice [Fig 5.11 B]. Both total and ES-L had a similar course of disease and mean clinical scores reached a peak of just under 2.25 upon termination of the experiment [Fig 5.11 B].

Taken together these data demonstrate that the higher molecular weight fraction of ES protects mice from EAE when administered either prophylactically or therapeutically, and that this protection is associated with a reduction in the number of IL-17 and IFN-γ producing T cells in the CNS.

5.2.9 Protection from EAE is not mediated via TGF-β or IL-4

Infection with *F. hepatica* protects mice from EAE in a TGF-β dependent manner (298). Both infection and administration of ES enhanced the expression TGF-β by innate and adaptive cells [Fig 3.4, 3.10, 4.10 and 5.6]. However, unlike infection [Fig 3.8] administration of ES did not enhance the expression of FOXP3 *in vivo* [Fig 5.6]. Experiments were conducted to determine if ES-induced protection from EAE was mediated via TGF-β.

Starting on day-1 and every 2 days thereafter, mice were injected with PBS or ES along with either 100 μg of either anti-TGF-β or control IgG_{2A}. EAE was induced in all mice on day 0 by s.c. injection with MOG emulsified in CFA. Addition of anti-TGF-β did not reverse the protective effect of ES on EAE [Fig 5.13]; suggesting that protection is not mediated by TGF-β.

This study has shown that mice immunised with an antigen in the presence of ES developed a Th2 response to that antigen [Fig 5.7]. In addition, $CD4^+$ T cells in the brains of mice with EAE that were treated with ES displayed higher expression of IL-4 and IL-13 [Fig 5.12]. These results suggest a shift in MOG-specific responses away from a Th1/Th17 and towards Th2 phenotype. Crucially, Jäger et al reported that EAE could be induced by transfer of activated MOG-specific Th1, Th17 and Th9 but not Th2 cells (408). An experiment was conducted to determine if ES protected from EAE through Th2 cells.

EAE was induced in IL-4$^{-/-}$ mice on a C57BL/6 background. Protection from EAE by ES was maintained in IL-4$^{-/-}$ mice [Fig 5.14]. This suggests that the protection from EAE by ES is not mediated via IL-4

5.2.10 ES induces eosinophilia in mice with EAE

Although IL-4 was not found to have a role in ES-induced protection against EAE [Fig 5.14], this does not discount a role for IL-5. IL-5 is the major differentiating factor for eosinophils *in vivo* (359) and administration of ES to mice induced systemic eosinophilia in an IL-5-dependent manner [Fig 4.21 and 4.23]. Therefore, experiments were conducted to investigate whether ES induced eosinophilia in mice with EAE. EAE was induced in C57BL/6 mice by s.c. injection of MOG emulsified in CFA on day 0. Mice were injected i.p. with ES or PBS beginning on day -1 and every second day thereafter. On day 15, mice were sacrificed and the frequency of eosinophils, mast cells, basophils and neutrophils in the blood was analysed by flow cytometry. Compared with PBS-treated mice, ES treated mice had a significant increase in the proportion of CD11b^{int} Siglec-F^{+} SSc^{high} eosinophils in the blood [Fig 5.15,A, C]. In addition, there was an increase in the proportion of autofluorescence^{-} CD3^{-} CD19^{-} Siglec-F^{-} Ly6G^{-} Ly6C FcεRI^{+} C-Kit^{+} mast cells in the blood of ES-treated mice compared with PBS-treated controls [Fig 5.15 B, C], although, this was not significant. There was no change in the proportion of CD11b^{int} Ly6G^{high} neutrophils of autofluorescence^{-} CD3^{-} CD19^{-} Siglec-F^{-} Ly6G^{-} Ly6C FcεRI^{+} C-Kit^{-} basophils [Fig 5.15 C]. There was an increase in the numbers of eosinophils infiltrating the brains of mice with EAE that were treated with ES compared with PBS-treated mice with EAE [Fig 5.15 D, E]. Interestingly, the numbers of eosinophils in the brains of mice treated with ES was at parity with the number of CD4^{+} T cells [Fig 5.12 A].

5.2.11 IL-5 mediates protection from EAE

The presence of eosinophilia in ES-treated mice with EAE suggests a role for IL-5. For this reason, experiments were conducted to determine if IL-5 mediated the ES-induced protection against EAE. Mice were treated prophylactically with either ES or PBS starting on day -1 and every 2 days thereafter with or without anti-IL-5 (TRFK-5, 80 μg). As expected, mice treated with ES in the absence of anti-IL-5 were protected from EAE [Fig 5.16 A]. However,

protection was reversed upon administration of anti-IL-5 [Fig 5.16 A], indicating that ES protects against EAE via IL-5.

Having shown that IL-5 mediates ES-induced protection from EAE when given by prophylactic administration, the role of IL-5 in protection from EAE by therapeutic administration of ES was investigated. EAE was induced on day 0 by 4 groups of mice by s.c. injection with MOG emulsified in CFA. Beginning on day 7 and every second day thereafter mice were injected with PBS or ES in the presence of anti-IL-5 or control IgG_{2A}. ES protected mice from EAE in those mice receiving the control antibody [Fig 5.16 B]. However, neutralisation of IL-5 reversed the protective effect of ES on EAE [Fig 5.16 B]. These results demonstrate that ES protects mice against from EAE in an IL-15 dependent manner.

ES protected mice from EAE in an IL-5 dependent manner [Fig 5.16]. ES also induced eosinophilia in mice with EAE [Fig 5.15]. In addition, eosinophilia in mice treated with ES alone was mediated by IL-5. ES-elicited eosinophils were even shown to express MHC-II and CD86 and could reactivate MOG-specific T cells *ex vivo* [Fig 4.22 and 4.28]. The results suggest that protection from EAE may be mediated by the downstream effect of IL-5, namely the recruitment of eosinophils. Experiments were conducted to determine if eosinophils recruited by administration of ES could affect the course of EAE. Eosinophils were purified by FACS from the PEC of mice treated with ES. $1x10^6$ eosinophils were injected into C57BL/6 mice by s.c. injection on day 0 [Fig 5.17 A] or i.p. injection on day 5 [Fig 5.17 B]. EAE was induced in these mice and in separate PBS administered controls by s.c. injection of MOG emulsified in CFA. The administration of eosinophils to by on day 0 or day 5 did not affect the outcome of disease compared with PBS injected control mice [Fig 5.17].

5.3 Discussion

5.3.1 *F. hepatica* ES attenuates EAE in an IL-5 dependent manner

The major finding of this chapter is that administration of *F. hepatica* ES protected mice against autoimmunity in the EAE model. This finding demonstrates that the protective effects of *F. hepatica* can be duplicated with its secreted products. This protective effect was associated with a reduction of the numbers of Th1 and Th17 cells in the brains of affected mice.

These observations are in agreement with previous studies that have shown that the products of other helminths have inhibitory activity against autoimmune diseases in mouse models. SEA from *S. mansoni* protects pre-disposed NOD mice from diabetes (225), while the soluble products of *T. suis* and *T. spiralis* were recently shown to attenuate the clinical severity of EAE (409). These findings add significant weight to the hygiene hypothesis, and may help to identify components that hold therapeutic potential for the treatment of autoimmune diseases. Importantly, the present study showed that *F. hepatica* ES protected mice from EAE when administered after the onset of symptoms. In contrast, the eggs from *Schistosoma japonicum* or SEA protected mice from EAE, only when administered prior to the onset of clinical symptoms (410). Likewise, Sewell et al demonstrated that while administration of *S. mansoni* eggs protected mice from EAE, the efficacy was severely diminished when treatment was delayed until the onset of symptoms (223). These differences highlight the potential application of ES as a therapeutic, as therapeutic interventions in humans would only be administered after disease onset. The present study also reported that the protective component of ES is greater than 3 kDa, suggesting that it may be a protein or peptide and not a small molecule.

Further evidence of the therapeutic potential of the products of *F. hepatica* was provided by a report from Carranza et al, who demonstrated that the TE of *F. hepatica* protected mice from CIA, a model for human rheumatoid arthritis (374). In agreement with the present study, attenuation of disease was associated with a reduction in auto-antigen-specific IL-17 and IFN-γ with a concurrent increase in IL-10 and TGF-β production. However, that study differs from the present one in a number of ways, the most important being the preparation of TE

compared with ES. Although precise details were not included in the paper by Carranza and colleagues the (374), previous publications from this group indicated that TE is a soluble somatic extract of homogenised adult flukes. Thus, it is likely to contain tegumental and somatic antigens as well as components from ES, prior to their secretion (411). The route of administration of TE also differed from that used for ES; ES was administered by i.p injection, whereas TE was indirectly administered via DC, as a DC vaccine. DC were loaded with collagen-II and treated with TE and CpG *in vitro* for 18 hours, before transfer, prior to the onset of symptoms of CIA (374). The protective effect of this DC vaccine was found to be dependent on the generation of $CD4^+$ $CD25^+$ Treg cells, as depletion of $CD25^+$ cells abrogated the protective effect. Moreover, transfer of $CD4^+$ $CD25^+$ cells from mice that received TE-treated DC conferred protection to mice with CIA (374). Importantly, the neutralisation of TGF-β *in vitro* during TE and CpG treatment, prior to adoptive transfer, blocked the expansion of Treg cells (374).

TGF-β is a common mediator of helminth-induced suppression of autoimmunity, through its ability to expand Treg cells. Administration of SEA was shown to reduce the clinical severity of EAE and prevented the development of experimental models of colitis and diabetes (223-225). Studies have shown that SEA enhanced the expression of FOXP3 in naive NOD-specific T cells in a TGF-β dependent manner (179). Similarly, infection with *L. sigmodontis* protected mice from diabetes in a Treg/TGF-β dependent manner (213, 319). Moreover; Grainger et al identified that the secreted products of *H. polygyrus* and *Teladorsagia circumcincta* contained molecules that ligated the mammalian TGF-βRII which induced the expression of FOXP3 in naïve T cells (178). Since *F. hepatica* ES induced the release of TGF-β from DC, it is possible that administration of ES would create an environment suitable for the peripheral conversion of Treg cells. However, the results of the present study demonstrated that administration of ES to mice did not expand $FOXP3^+$ Treg cells *in vivo*. In contrast, infection with *F. hepatica* was associated with the expansion of Treg cells. This is at variance with the report from Carranza et al who demonstrated that transfer of DC treated with CpG and the TE of *F. hepatica* enhanced the expression of FOXP3 by T cells in the draining LN (374). Therefore, it is possible that the component of *F. hepatica* that enhanced Treg cells is a somatic or tegumental antigen, rather than a secreted one.

While ES does not expand Treg cells, it enhanced the expression of TGF-β by T cells. Thus, it was initially thought that TGF-β was required for ES-mediated suppression of EAE. However, using neutralising antibody to TGF-β, this study demonstrated that protection against EAE was not dependent on TGF-β.

The role of helminths in the induction of Treg cells has been extensively investigated over the past decade. However, this ignores that cross-regulation of Th1 by Th2 cells, rather than induction of regulatory mechanisms was originally proposed as the mechanism by which helminths suppressed autoimmunity (196). A major shift in this theory was required after it was shown that Th2-inducing helminth infections could suppress Th2-mediated allergic responses (206), and further required when it was discovered that T17 cells were a major mediator of pathology in autoimmune disease, rather than Th1 cells, as the evidence for Th2 cross-regulation of Th17 cells is less convincing that that of Th1 cells (166). It is now thought that helminth-induced suppression of autoimmunity can be classified into two categories, those that inhibit host-antigen-specific Th1 and Th17 responses, via Treg cell induction and those that switch Th1 and Th17 responses towards a non-pathogenic Th2 response. There is now a significant body of experimental evidence that supports the view that these mechanisms can work separately or in concert to exert immunosuppressive effects.

One study which supported a role for Th2-mediated-regulation demonstrated that the gastrointestinal nematodes *T. spiralis* and *H. polygyrus* prevented the onset of Th1-mediated type-1 diabetes in NOD mice by inducing strong Th2 responses (212). Likewise, La Flamme et al demonstrated that protection of mice from EAE following infection with *S. mansoni* was associated with a reduction in the frequency of T cells which infiltrated the brain. Moreover, there was a reduction in MOG-specific IFN-γ with a concurrent increase in systemic IL-4 and IL-5 production (216). Furthermore, Zheng et al showed that when SEA was administered to mice with EAE, MOG-specific responses switched from the production of IFN-γ towards production of IL-4 (410).

In agreement with these studies, administration of *F. hepatica* ES to mice with EAE was associated with a reduction in the migration of $CD4^+$ T cells into the brain. Moreover, the cells that infiltrated the brain had reduced expression of IFN-γ and IL-17, with a concurrent enhancement of IL-4 and IL-13, suggestive of a shift in T cell responses away from

Th1/Th17 phenotype towards that of a Th2. Moreover, there was an accumulation of eosinophils in the brains of mice that were treated with ES, suggesting that IL-5-producting Th2 cells are present in the brain of mice with EAE, following treatment with ES. This is an important observation as Jager et al demonstrated that EAE is induced in naïve recipient mice by transfer of highly polarised MOG-specific Th1, Th9 and Th17, but not Th2 cells (408). IFN-γ and IL-17 produced by Th1 and Th17 cells, respectively and GMCSF, produced from both, activate macrophages, microglia and neutrophils that damage the myelin (73, 412). In contrast, IL-4, IL-5 and IL-13 produced by Th2 cells must be less effective at activating the destructive capacity of these cells.

The results of the present study found that administration of ES with an unrelated antigen can promote a Th2 response to that antigen, as determined by the production of IL-4, IL-5 and IL-13 by T cells *ex vivo*. This is in agreement with previous studies which have shown that infection of mice with *F. hepatica* is associated with the induction of Th2 responses (279). However, the present study showed that DC treated with ES did not promote Th2 responses *in vivo*, suggesting that ES must act in conjunction with other cell types *in vivo* to promote the differentiation of Th2 cells. Thus, the effect of ES on epithelial cells and innate lymphocytes warrants further investigation. Furthermore, the Th2 promoting capacity of ES was partially abrogated by denaturation, suggesting that the component responsible for promoting Th2 responses is a protein. The products of other helminths have been shown to promote potent Th2 responses *in vivo*. Omega-1, a glycoprotein isolated from SEA, was shown to promote the induction of Th2 cells *in vivo*, even in the absence of IL-4R signalling (55), suggesting that factors, other than IL-4 are important for differentiation of Th2 cells. Interestingly, it did not promote Th2 responses *in vitro,* suggesting that like ES, Omega-1 requires factors other than DC to promote Th2 cell differentiation (55).

Previous studies performed in this laboratory have demonstrated that Th2 cells can mediate at least some of the suppressive effects of *F. hepatica*. Brady et al demonstrated that infection with *F. hepatica* suppressed a protective Th1 response to *B. pertussis*. This effect was associated with a switch in *B. pertussis*–specific responses from a Th1 to a Th2 type (254). Although Th17 and Treg responses were not investigated in that study, this observation suggests that administration of ES may attenuate EAE via a switch in MOG-specific Th1/Th17 responses towards a Th2 response. However, the present study demonstrated that attenuation of EAE

by ES was independent of IL-4, suggesting that Th2 responses or at least IL-4 from Th2 cells is not required. This conclusion is at variance with a report showing that the rat tapeworm, *H. diminuta*, attenuated CIA in an IL-4Rα dependent manner (312). In addition, it has been reported that the protective effect of the eggs from *S. mansoni* on the pathogenesis of EAE was dependent on IL-4Rα and STAT6 (223).

Several changes are induced at the chromatin structure of the Th2 cytokines (IL-4/IL-5/IL-13) during Th2 differentiation from naïve T cells. Therefore, these cytokines are usually expressed together in effector Th2 cells (413). IL-4 and the IL-4Rα have been extensively studied as mediators of helminth-induced suppression of autoimmunity. However, the effect of the other major Th2 cytokine, IL-5, has been largely overlooked, despite that fact that its expression is enhanced during attenuation of models of autoimmune disease. Protection of mice from EAE with the eggs from *S. mansoni* was associated with an enhancement of IL-4, IL-13 and IL-5, with the latter enhanced to the greatest extent (223).

The systemic eosinophilia, associated with treatment of ES, is highly indicative of IL-5 activity, and indeed, this study provides the first evidence that helminth-induced attenuation of autoimmunity was mediated by IL-5. Administration of neutralising antibody to IL-5 to mice reversed the protective effect of ES on EAE, when ES was given either prior to disease induction or at onset of clinical symptoms. The source of IL-5 is likely to be Th2 cells as the expression of IL-5 in Th2 cells is STAT6 dependent and is thus provided by IL-4Rα signalling following ligation with IL-4 and IL-13 (413). Therefore, while ES protected IL-4-deficient mice from EAE, in order to fully eliminate the role of Th2 cells, these experiment need to be repeated in IL-4Rα or STAT6-deficient mice. This would also address the question whether IL-5, involved in suppression of EAE, is produced by Th2 cells. However, the source of IL-5 may not necessarily be T cells, since IL-5 is still produced in the lung and gut of RAG-deficient mice, which lack functional T and B cells (414). Potential T cell-independent sources of IL-5 include eosinophils, mast cells and nuocytes or natural-helper cells. Nuocytes rapidly produce IL-5 in response to IL-25 and TSLP (135). However, any cell that expresses GATA-3, such as endothelial cells, may be a potential source of IL-5 as ectopic expression of GATA3 is sufficient to drive the expression of IL-5, but not IL-4 (413).

Unlike IL-4 or IL-13, IL-5 signalling is not mediated via IL-4Rα and STAT6, instead IL-5 signalling is mediated via the IL-5Rα (and a β-chain shared with IL-3 and GMCSF) and STAT1/5. This may explain the divergent roles of IL-5 compared with IL-4 and IL-13 (both of which signal through the IL-4Rα). IL-5 activates mouse B cells and eosinophils *in vivo*, while in the humans, activity is more restricted to eosinophil function (382). In mice, IL-5 promotes the survival of B cells and their differentiation into plasma cells. Moreover, IL-5 promotes IgA and IgG1 production by plasma cells. Thus, the role of antibody in ES-mediated protection from EAE cannot be discounted. In mice, both B cells and eosinophils constitutively express the IL-5Rα. Mice deficient in the IL-5Rα have reduced basal levels of eosinophils and B cells and display delayed clearance of the nematode *Angiostrongylus cantonensis* (383).

The results of the present study suggest that helminth-induced IL-5 may mediate protection against EAE through its action on eosinophils. IL-5 is the archetypal 'eosinophil' cytokine, indeed one of the original names for IL-5 was eosinophil colony growth factor (382). It promotes the differentiation, survival and activation of eosinophils (382). The present study has demonstrated that eosinophilia is associated with *F. hepatica* ES-mediated protection against EAE, indeed eosinophils infiltrated the brains of protected mice. The present study has also shown that ES or infection with *F. hepatica* enhances the APC function of eosinophils. In addition, eosinophils are known to produce IL-5 (382). Thus, the interaction of T cells with eosinophils needs to be properly investigated. However the present study also demonstrated that transfer of eosinophils from mice treated with ES did no modulate disease in mice with EAE However, this intervention may be limited by the low numbers of cells transferred or it is possible that the eosinophils need to be loaded with antigen.

A full understanding of the role of eosinophils in the *F. hepatica* ES-mediated protection against EAE will require the use of mice lacking eosinophils. Commonly used interventions such as the use of anti-Siglec-F or CCR3 (eotaxin receptor) deficient mice have number of problems, thus the use of mice such as the Δdbl-GATA mouse, that totally lacks eosinophils through deletion of the required transcription factor GATA-1, may be a better model as they are on a C57BL/6 background and were previously shown to be susceptible to EAE.

5.3.2 Inhibition of T cell proliferation

In the previous chapter, *F. hepatica* ES was shown to inhibit the ability of APC to promote Th1 and Th17 responses. However, it was not established if ES can interact directly with T cells or if suppression of T cell responses was mediated via suppression of DC maturation and cytokine production. Direct inhibition of anti-parasite T cell responses is advantageous to helminths as indirect suppressive activity on DC alone would have little effect on already activated effector T cells. Indeed, the intestinal nematode *N. brasiliensis* directly suppress T cell responses by inducing the expression of CTLA-4 on Th2 cells, which inhibits proliferative responses and prolongs persistence in the host (176).

The present study provides evidence that *F. hepatica* ES inhibits T cell proliferation *in vitro* and *in vivo*. These findings are in agreement with those of Prowse et al who demonstrated that *F. hepatica* ES suppressed the proliferation of sheep lymphocytes in response to conA (270). Moreover, they identified cathepsins as the active components, as the cathepsin inhibitor E-64, reversed proliferation, and recombinant cathepsin L also possessed inhibitory activity (270). However, the present study found that that inhibitor of cathepsins did not reverse suppression of T cell responses, suggesting that other immunomodulatory molecules from *F. hepatica* ES can also mediate suppression.

It is possible that *F. hepatica* ES inhibits T cell responses by inducing the expression of the inhibitory T cell receptors CTLA-4 and PD-1. These molecules are essential for the regulation of cellular immune responses and mice deficient in CTLA-4 or PD-1 develop spontaneous autoimmune diseases (397). While ES had no effect on the expression of CTLA-4, it did enhance the expression of PD-1. However, neutralising PD-1 had no effect on ES-mediated suppression of proliferation.

The current study has demonstrated that recombinant TGF-β can prevent the induction of Th1, Th2 and Th17 responses from naive T cells. This is consistent with previous findings which have shown that recombinant TGF-β inhibits the differentiation of naive Th1 and Th2 cells and suppresses memory Th1 cells, but not Th2 cells (415). However, the use of neutralising antibody to TGF-β or a receptor signalling inhibitor revealed that TGF-β did not mediate ES-induced suppression of T cell proliferation. Thus, the method by which ES inhibits T cell proliferation is still unclear. Preliminary data generated in this laboratory

suggest that the suppressive component is below 3 kDa and therefore likely to be a small molecule. Furthermore, the suppressive effect of ES was reversed by on an antioxidant (A M Stefanska, personal communication). These data suggest that the production of TGF-β and the inhibition of T cell proliferation are mediated by the same mechanism, but the components which mediate these effects are distinct from those that protect from EAE and induce Th2 responses *in vivo*. Consistent with this hypothesis, a report by Cervi et al showed that the suppressive effect of ES on conA but not LPS, induced proliferation of spleen cells was reversed by antioxidants, specifically, catalase which neutralised peroxides and superoxide dismutase which removes the superoxide anion (416). This evidence further support the hypothesis that a small molecule component contained within ES induces ROS production and thus suppression of T cell proliferation and TGF-β release.

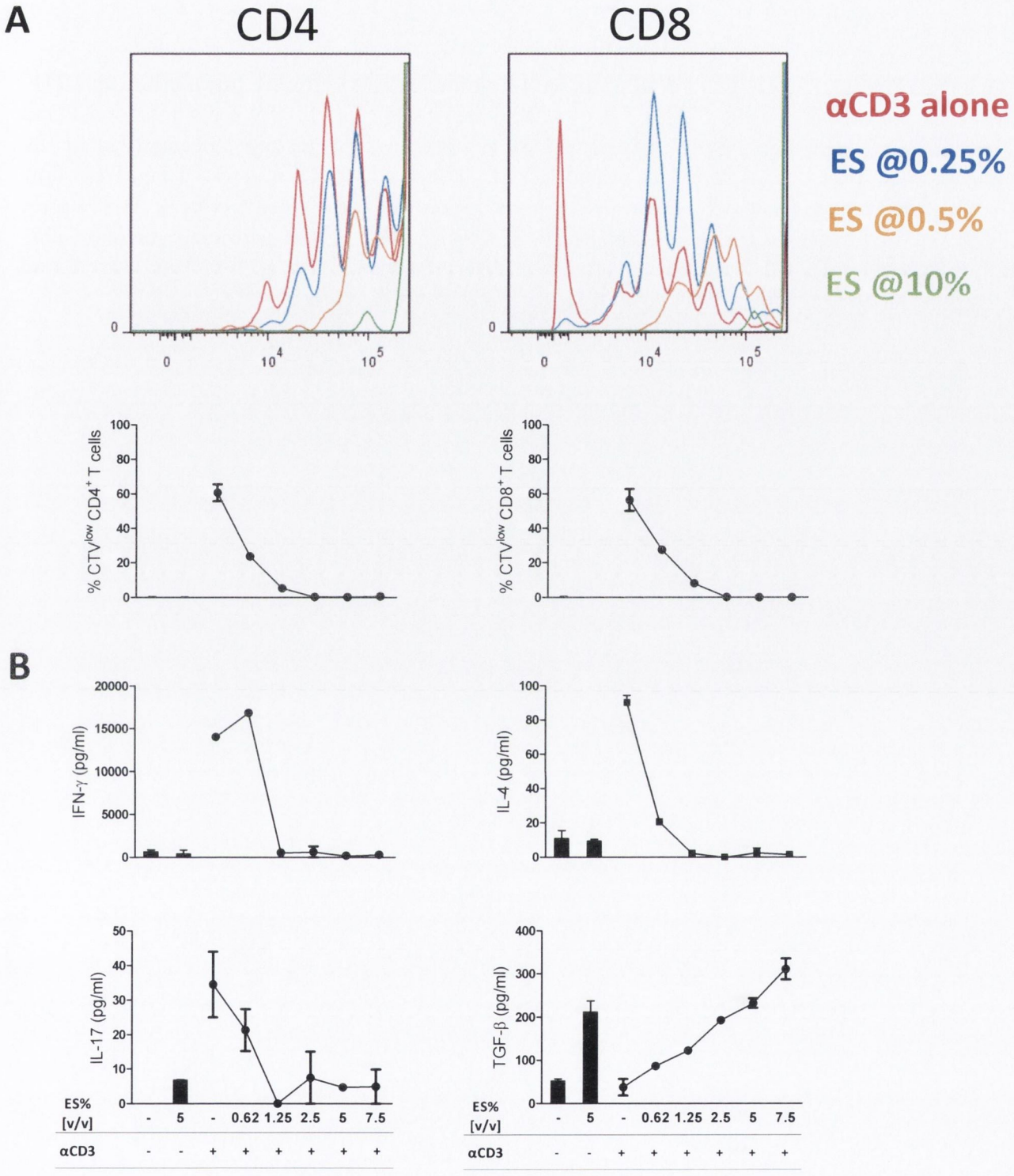

A
CD4
CD8
αCD3 alone
ES @0.25%
ES @0.5%
ES @10%
% CTV^low CD4+ T cells
% CTV^low CD8+ T cells
B
IFN-γ (pg/ml)
IL-4 (pg/ml)
IL-17 (pg/ml)
TGF-β (pg/ml)
ES% [v/v]
αCD3
C
% Live CD4+ cells
ES

Fig 5.1 *F. hepatica* ES inhibits proliferation of and cytokine production by $CD4^+$ and $CD8^+$ T cells. Spleen cells isolated from C57BL/6 mice were stained with CTV and stimulated with anti-CD3ε (1 μg/ml) in the presence of increasing concentrations of ES. A, After 72 hours proliferation of $CD4^+$ and $CD8^+$ T cells was assayed by flow cytometry. Gates for proliferated cells are set on CellTraceViolet^{low} cells. B, At 72 hours, cytokines in supernatants were quantified by ELISA. C, The percentage of live $CD4^+$ cells after 72 hour stimulation was determined by staining for Aqua Live/Dead (invitrogen). Results are representative of 6 separate experiments.

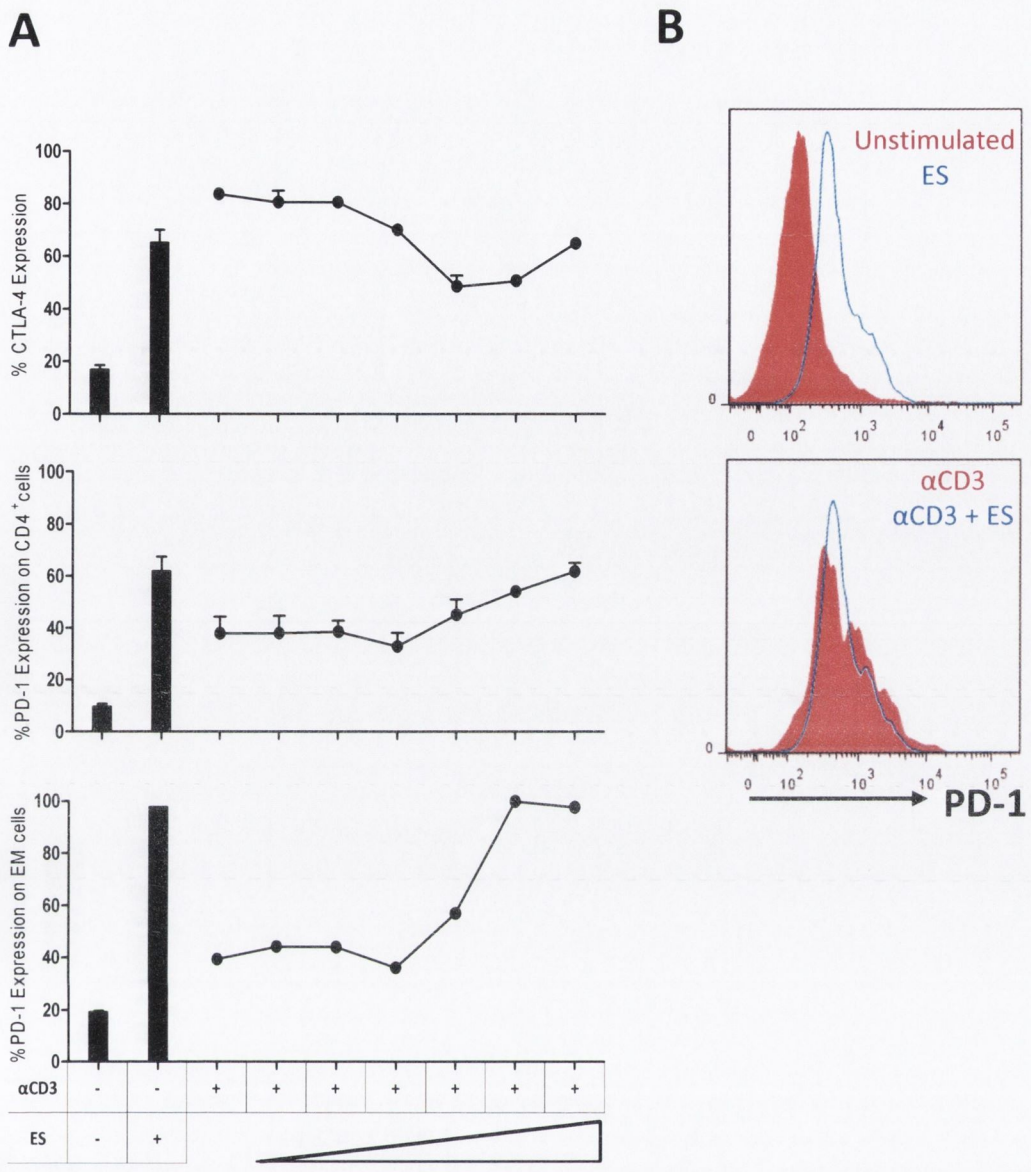


Fig 5.2 *F. hepatica* ES enhances expression of PD-1 on activated CD4⁺ T cells. Spleen cells isolated from C57BL/6 mice were stimulated with anti-CD3ε (1 μg/ml) with increasing concentrations of ES (0.1325%-10% v/v). ES was used at 10% v/v in the absence of anti-CD3ε A, Expression of CTLA-4 and PD-1 on total CD4⁺ and effector-memory (EM, $CD44^{low}$ $CD62L^{high}$) CD4⁺ T cells was determined by flow cytometry. B, representative plots of expression of PD-1 on total CD4+ T cells. n=2. Data presented are means +/- SEM. Data presented are representative of 2 separate experiments.

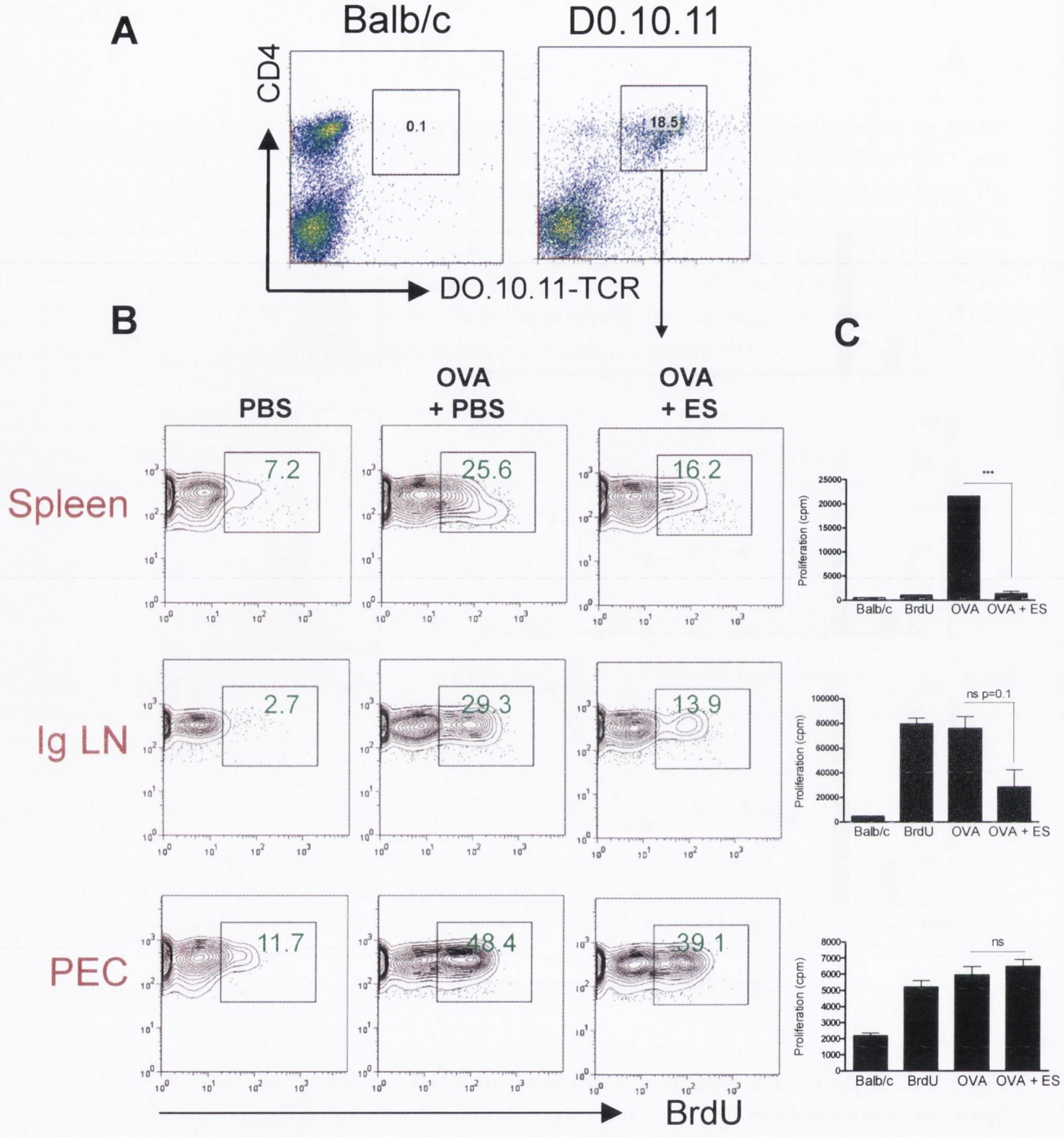


Fig 5.3 *F. hepatica* ES suppresses antigen specific CD4⁺ T cell proliferation *in vivo*. DO.10.11 mice were immunized with OVA peptide (100 μg) and CpG (50 μg) on day -28. On Day 0 mice were injected i.p. with PBS or OVA (100 μg). On day -1, 0, 1 and 2 immunized mice were treated as indicated with i.p. injection of PBS or ES (40 μl). On day 1, 2 and 3 mice received Bromodeoxyuridine (BrdU, 100 μg) by i.p. injection. On day 4, the spleens, inguinal LN and peritoneal cells were isolated from all treated groups and from control group of naïve BALB/c mice. Cells were surface stained for CD4 and with an antibody specific for the OVA transgenic TCR. Cells were permeabilised and treated with DNase and an antibody directed against BrdU. A, Antigen-specific T cells were gated as $CD3^+ CD4^+$ OVA-tg TCR^+. B, Proliferation of antigen specific T cells was quantified by percentage staining for BrdU. Plots are representative of 3 mice per group C, Cells were restimulated *ex-vivo* with antigen (6.25 μg/ml) for 72 hours with [^{3}H] thymidine added for the last 18 hours in order to quantify proliferation. n=2. Data presented are means +/- SEM of triplicate assays of pooled organs from each group. Statistical assessment was performed as indicated by unpaired Student t-test. ***$p<0.001$.

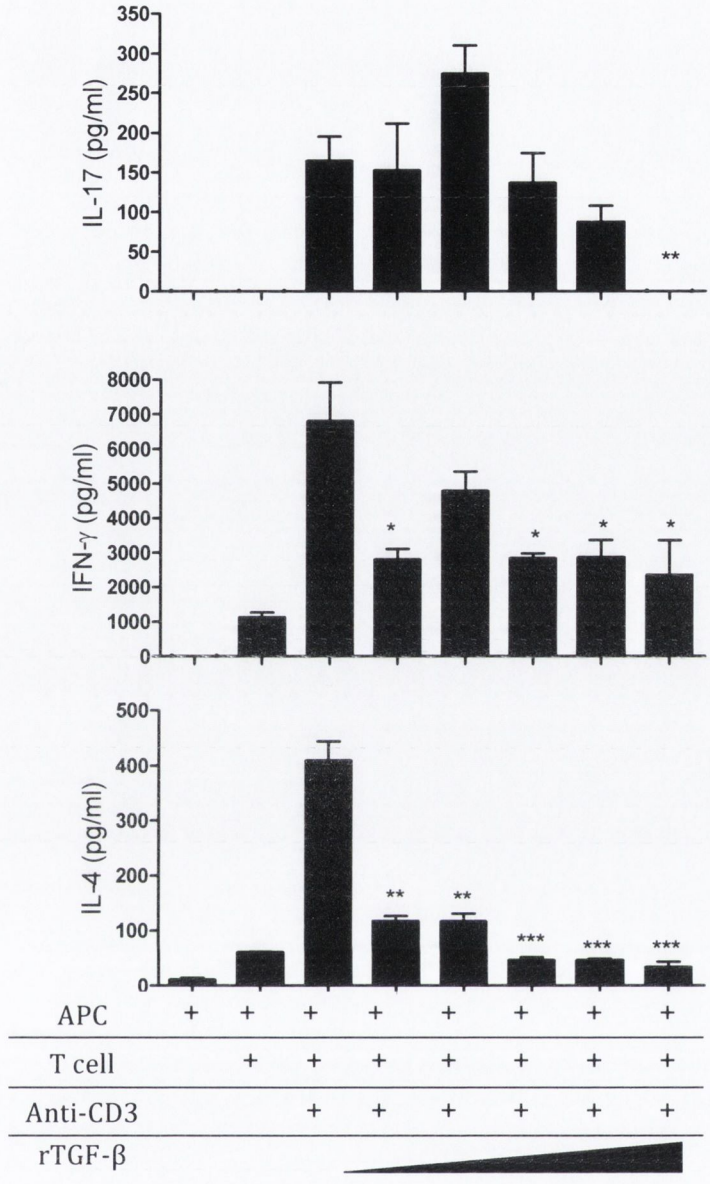


Fig 5.4 Recombinant TGF-β inhibits the production of T cell-derived cytokines in response to anti-CD3 antibody in the presence of APC. $CD3^+$ cells were isolated by MACS separation from naive mice and were cultured in X-vivo medium at $1x10^6$ cells/ml in the presence of an anti-CD3ε antibody (1 μg/ml) and $4x10^6$ cells/ml irradiated splenic APC. Increasing concentrations of recombinant TGF-β were added (0, 1, 10, 100, 1000, 10000 pg/ml). Cells were cultured for 72 hours. Supernatants were removed and concentrations of INF-γ, IL-17 and IL-4 were detected by ELISA. * $P<0.01$ ** $P<0.005$ *** $P<0.001$ vs (0 pg/ml) TGF-β. Data presented are means +/- SEM of triplicate assays Data presented are representative of 2 separate experiments. Statistical assessment was performed by unpaired Student t-test vs anti-CD3ε T cells in the absence TGF-β. *$p<0.05$, **$p<0.01$ ***$p<0.001$

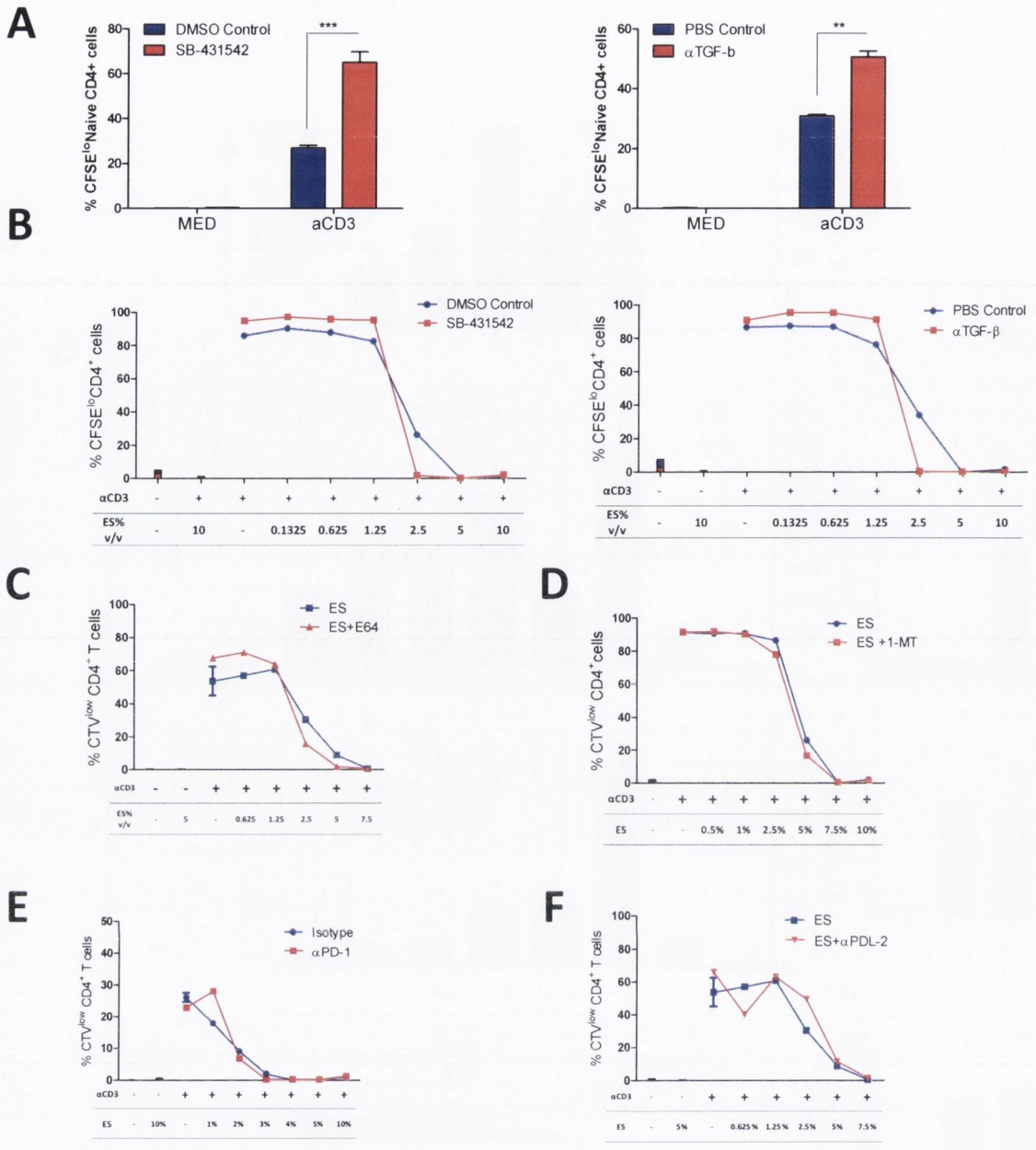


Fig 5.5 Inhibition of proliferation of T cells by *F. hepatica* ES is independent of cathepsins, PD-1, PDL2, TGF-β or IDO. Spleen cells isolated from C57BL/6 mice were stained with CellTraceViolet or CFSE and stimulated with αCD3ε (1μg/ml) with increasing concentrations of ES in the presence (red line) or absence (blue line) of various inhibitors or blocking antibodies as indicated. After 72 hours proliferation of total $CD4^+$ and $CD8^+$ T cells was determined by flow cytometery. Divided cells were gated as CTV^{low} or $CFSE^{low}$ cells. A, Positive controls for TGF-β receptor signalling inhibitor, SB-431542 (10 μM) and a neutralising antibody directed against TGF-β (clone D-11, 100 μg/ml). Proliferation of $CD44^{low}$ $CD62L^{high}$ naïve $CD4^+$ T cells in response to anti-CD3ε in the presence and absence of SB-431542 or anti-TGF-β Statistical assessment was performed by unpaired Student t-test vs anti-CD3ε T cells in the absence TGF-β. $^{**}p<0.01$ $^{***}p<0.001$. B, SB-431542 (10 μM). C, anti TGF-β (clone D-11,). D, the irreversible cathepsin inhibitor E-64 (5 μM). E, 1-methyl-tryptophan (1-MT) the competitive inhibitor of IDO. F, a blocking antibody against PD-1 (clone 112). G, a blocking antibody against PD-L2 (10 μg/ml). Data presented are means +/- SEM of triplicate assays. Data presented are representative of 2 separate experiments.

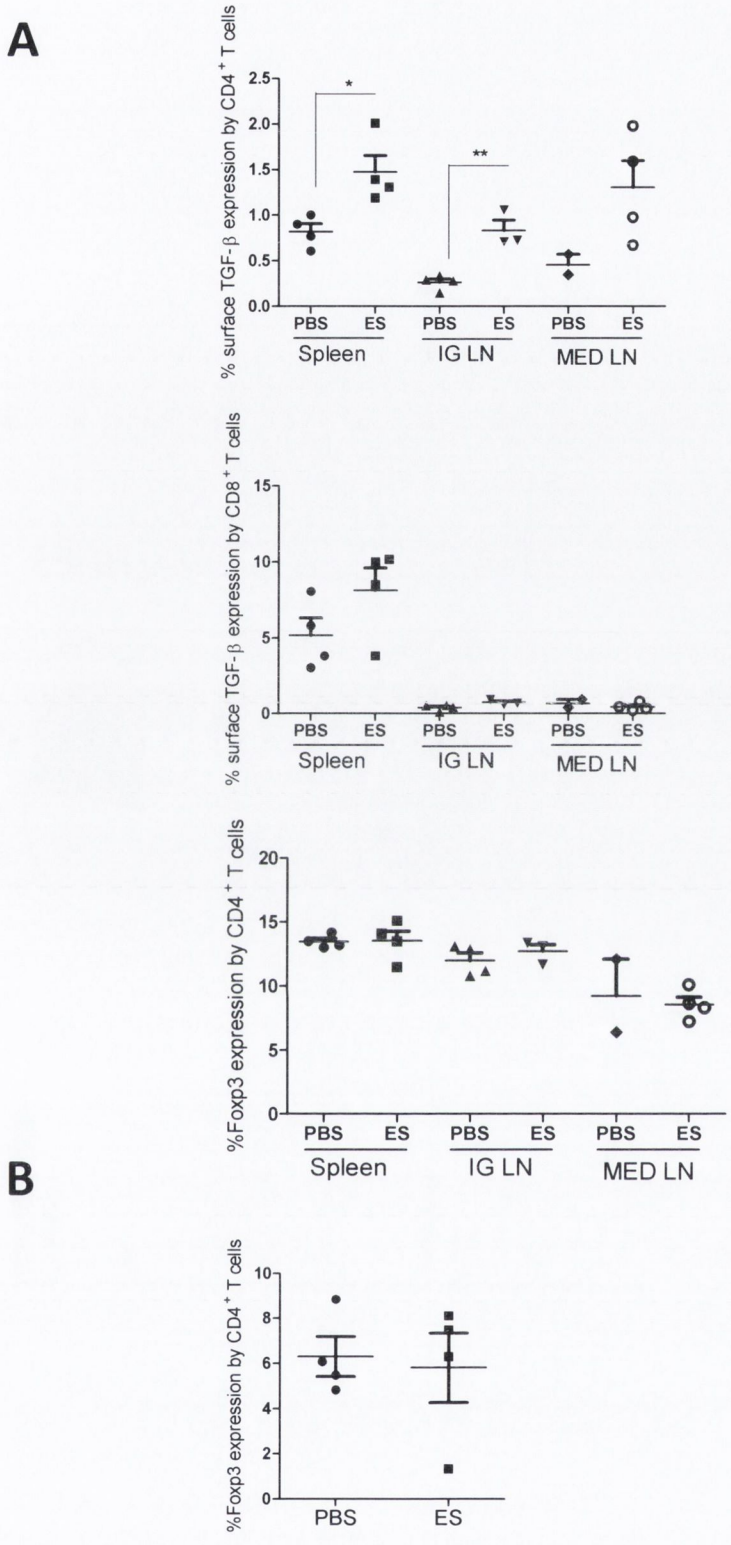


Fig 5.6 ES enhances expression of TGF-β on T cells *in vivo* but ES does not enhance expression of Foxp3. A, C57BL/6 mice were injected i.p. with ES every 2 days for 13 days. On Day 14 the spleens, inguinal LN and mediastinal LNs were excised. T cells isolated from each organ was analysed by flow cytometery for the expression of TGF-β and Foxp3. Statistical assessment was performed as indicated using an unpaired Student's T test * P< 0.05, ** P<0.001 B, Peritoneal $CD4^+$ T cells were analysed for expression of Foxp3. Data presented are means +/- SEM of 4 mice per group. Data presented are representative of 2 separate experiments. Statistical assessment was performed as indicated by unpaired Student t-test. *p<0.05 **p<0.01.

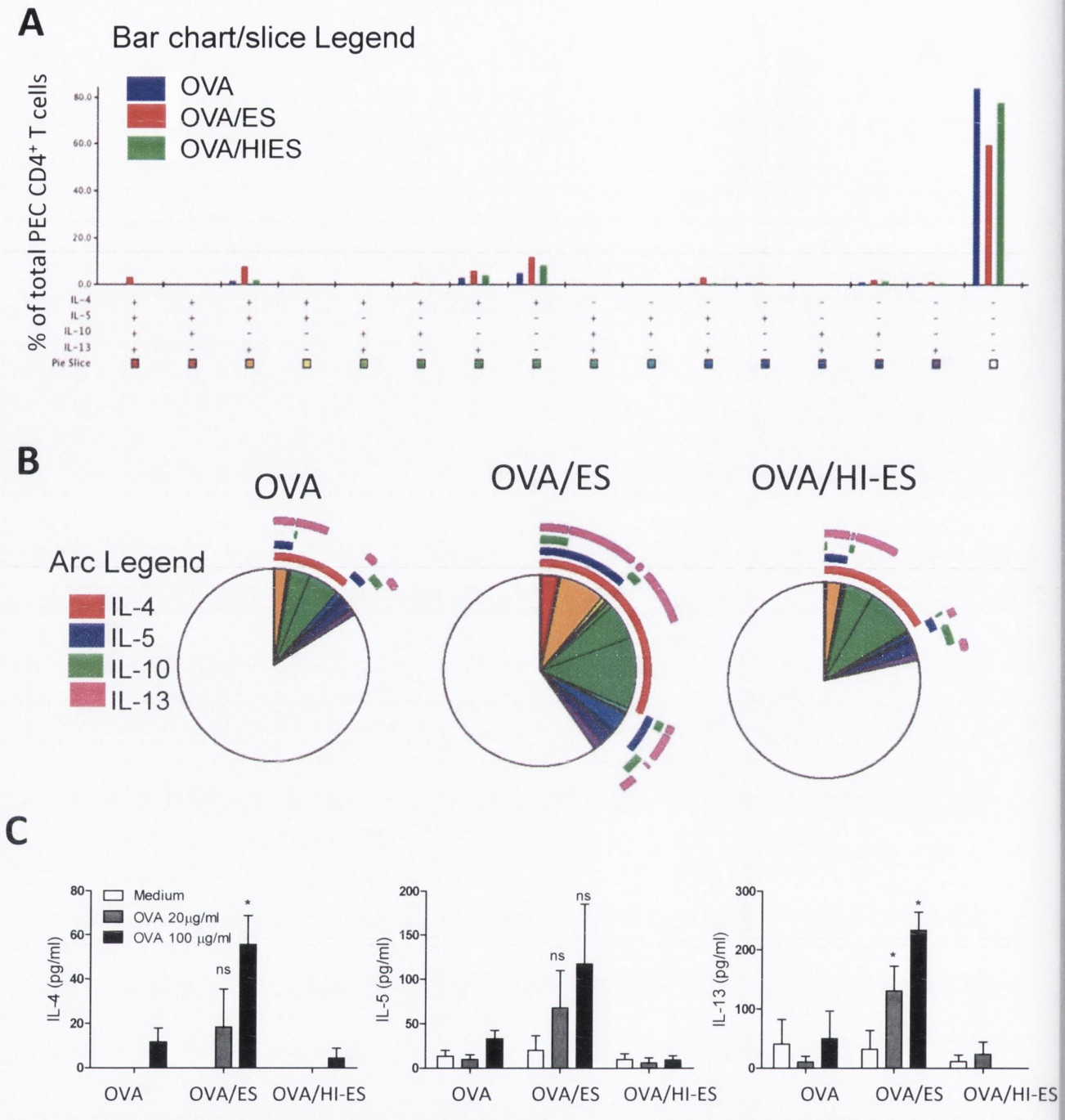


Fig 5.7 Native but not heat-inactivated ES promote Th2 responses. BALB/c mice were immunized twice (day 0 and 21) with either OVA (50 µg) + PBS, OVA+ ES (40 µl) or OVA+HI-ES (40 µl). On day 28 PEC cells were restimulated *ex vivo* with PMA and ionomycin in the presence of BFA for 5 hours. The relative expression of IL-4, IL-5, IL-10 and IL-13 by $CD3^+$ $CD4^+$ T cells was determined by flow cytometry and was analysed using the program SPICE to graphically represent all possible combinations of expression as A, a bar chart and B, pie charts; with pie chart arcs correspond to cytokine expression as indicated by arc colour, overlapping arcs depict cells expressing multiple cytokines. C, On day 28 mediastinal lymph nodes were isolated simulated *ex vivo* with increasing combinations of OVA (0, 20 and 100 µg/ml). The concentrations of IL-4, IL-5 and IL-13 in the supernatants after 72 hours were determined by ELISA. Data presented are means +/- SEM of 4 mice per group. Statistical assessment was performed using an unpaired Student T test vs the OVA group *P<0.05 **P<0.001. Data presented are representative of 2 separate experiments.

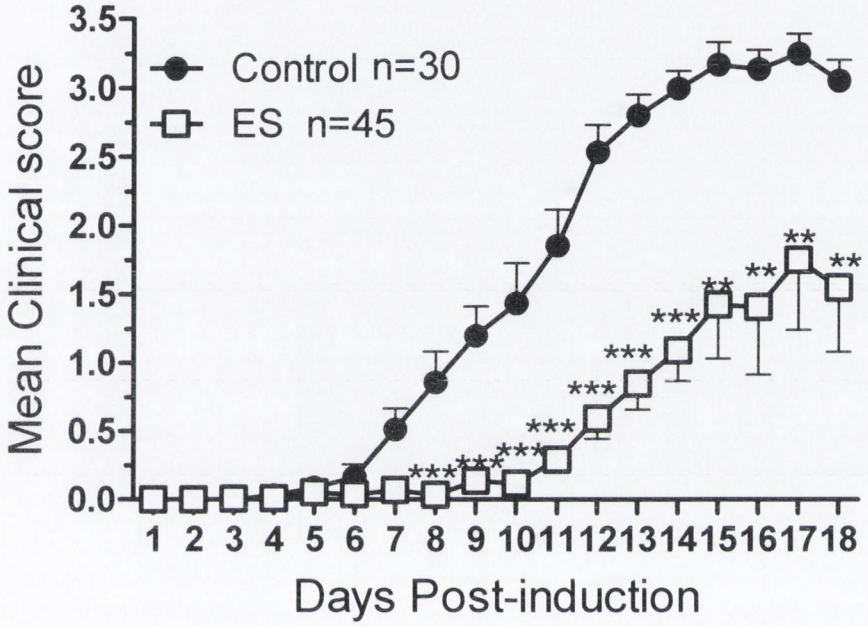


Fig 5.8 *In vivo* administration of ES delays the onset and attenuates the clinical severity of EAE. On day 0, EAE was induced in C57BL/6 mice by s.c. injection with 100 μg of MOG_{35-55} in CFA, followed by i.p. injection with 500 ng of PT on days 0 and 2. From day -1, and for every 2 days thereafter, mice were i.p. injected with either PBS (●) or ES (□, 20-45μl). Clinical scores were examined daily and mice were sacrificed by day 18. Results are mean clinical scores from 7 separate experiments using the same protocol but with different preparations of ES. Control n=30, ES n=45. Data presented are means +/- SEM. Statistical assessment was performed using two-way ANOVA together with Bonferroni's post test $^{**}p<0.001$ $^{***}p<0.0001$.

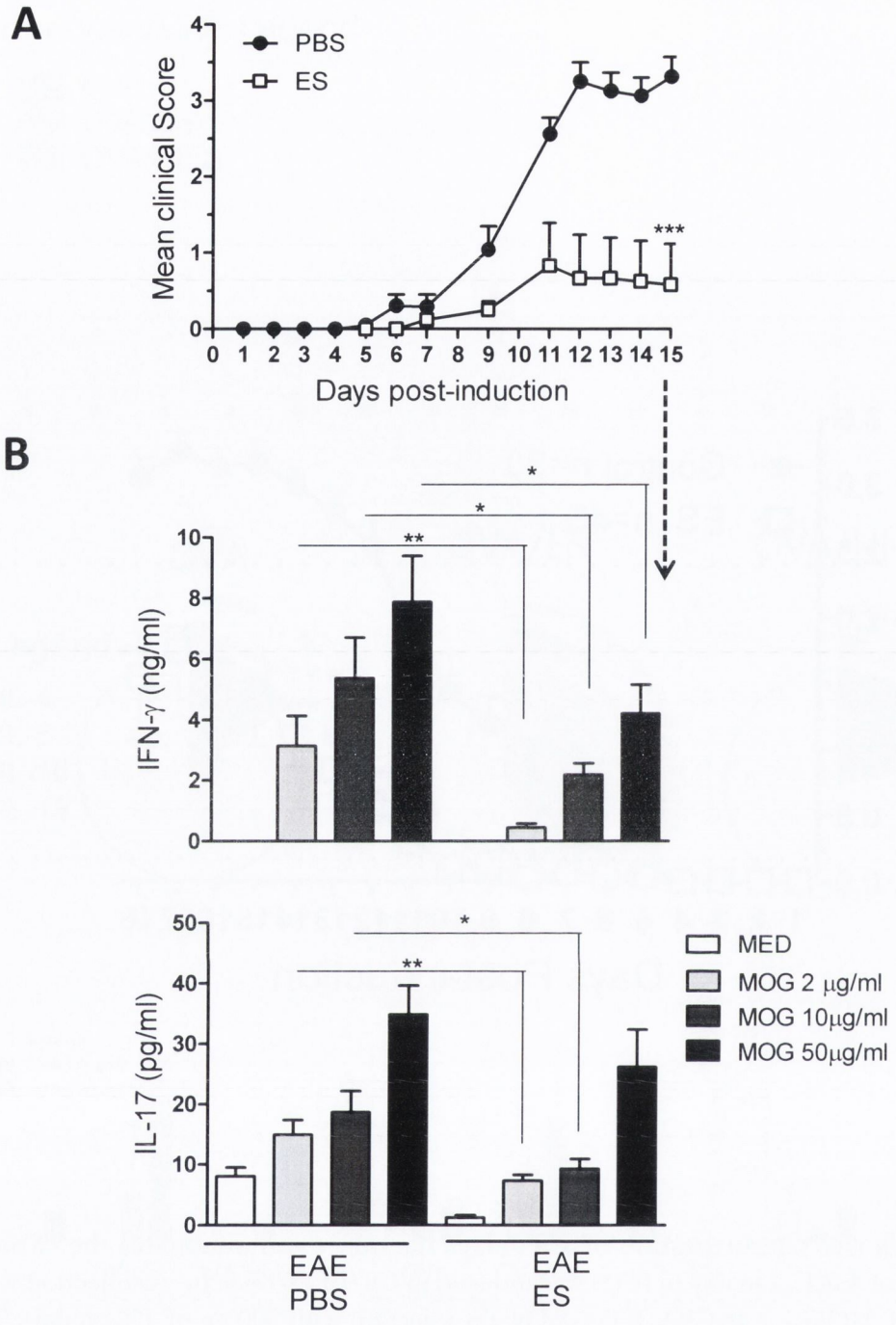


Fig 5.9 The attenuated effect of *F. hepatica* ES on EAE is associated with a reduction in MOG-specific IL-17 and IFN-γ. On day 0, EAE was induced in C57BL/6 mice by s.c. injection with 100 μg of MOG_{35-55} in CFA, followed by i.p. injection with 500 ng of PT on days 0 and 2. From day -1, and for every 2 days thereafter, mice were injected i.p. with either PBS (●) or ES (□, 20-45μl). A, Clinical scores were examined daily. Data presented are means +/- SEM or 6 mice per group. Statistical assessment was performed using two-way ANOVA **p<0.001 ***p<0.0001. B, On day 15 mice were sacrificed and spleen cells were harvested and restimulated ex-vivo with MOG antigen (2, 10 and 50 μg/ml). After 72 hours the concentrations of IFN-γ and IL-17 in the supernatant were determined by ELISA. Data presented are means +/- SEM of triplicate assay Statistical assessment was performed by unpaired Students t-test vs corresponding concentration of MOG *p<0.05 **p<0.001 ***p<0.0001.

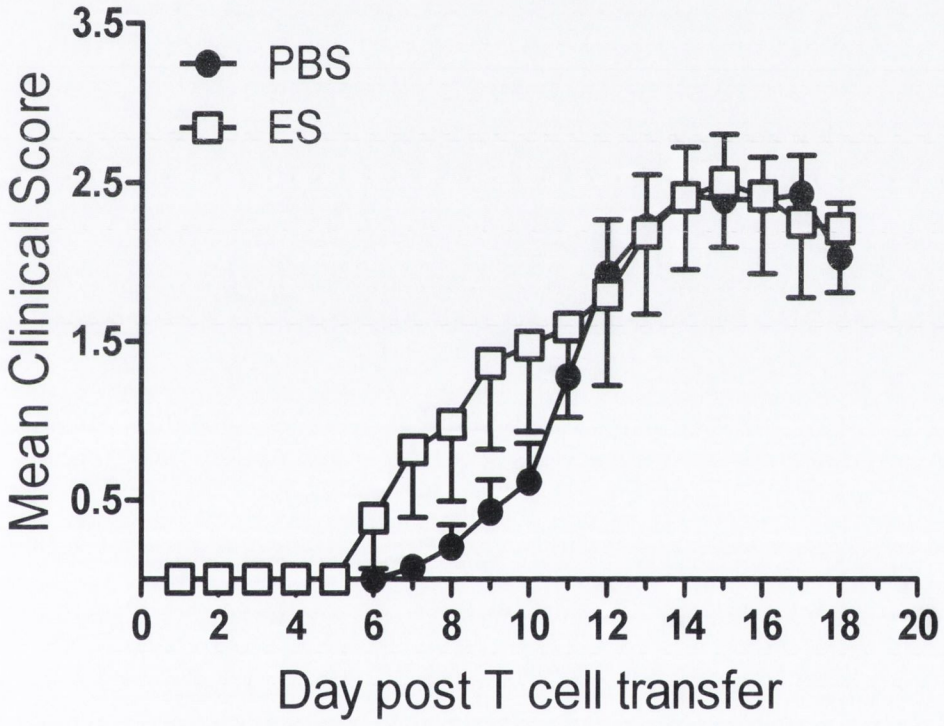


Fig 5.10 ES does not protect mice from passively-induced EAE. On day 0, EAE was induced by transfer of $10x10^6$ encephalitogenic $CD4^+$ T cells isolated from CFA/MOG immunized mice and expanded *in vitro* with MOG, IL-1β and IL-23. Starting on day -1 and every 2 days thereafter mice were Mice were injected i.p. with PBS (●) or ES (40 µl, □) Clinical scores were examined daily and mice were sacrificed at peak of disease. Displayed results are mean clinical scores of 2 separate experiments. Data presented are means +/- SEM of 12 mice per group

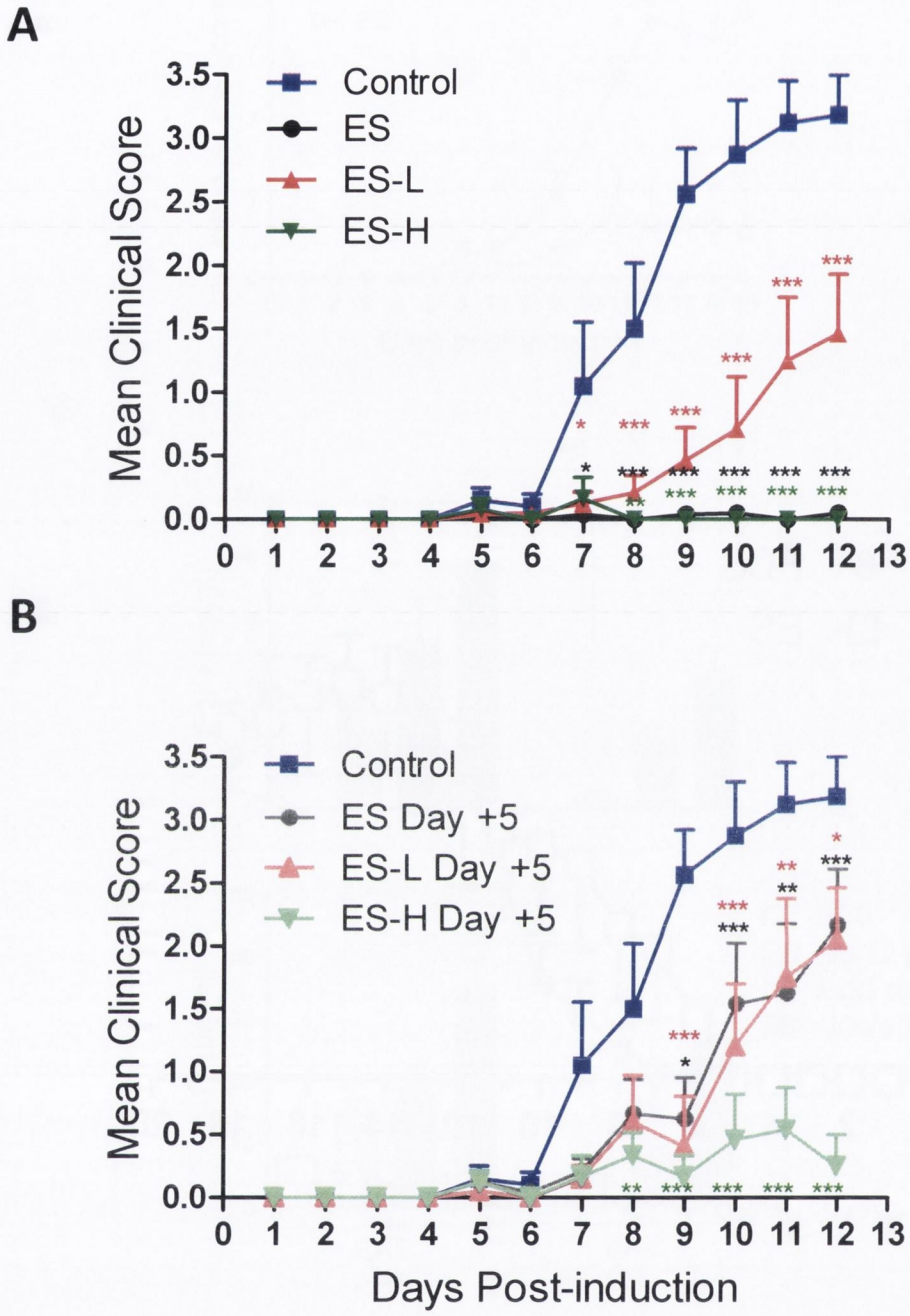


Fig 5.11 Both therapeutic and prophylactic treatment with ES delays onset and attenuates the severity of EAE. On day 0, EAE was induced in C57BL/6 mice by s.c. injection with 100 μg of MOG_{35-55} in CFA, followed by i.p. injection with 500 ng of PT on days 0 and 2. Mice were injected i.p. on day -1 (A) or day + 5 (B) and every 2 days thereafter with PBS (■), Total ES (●), ES-L (▲) or ES-H (▼) (40 μl) as indicated. Clinical scores were examined daily and mice were sacrificed on day 12. Data presented are means +/- SEM of 6 mice per group Statistical assessment was performed using two-way ANOVA together with Bonferroni's post text *$p<0.05$ **$p<0.001$ ***$p<0.0001$. Results are representative of 3 separate experiments.

A

	PBS	ES Total	ES-H	ES-L
$CD4^+$	936,936	8,720	30,160	207,043
EOS	2,462	8,689	199	0

B

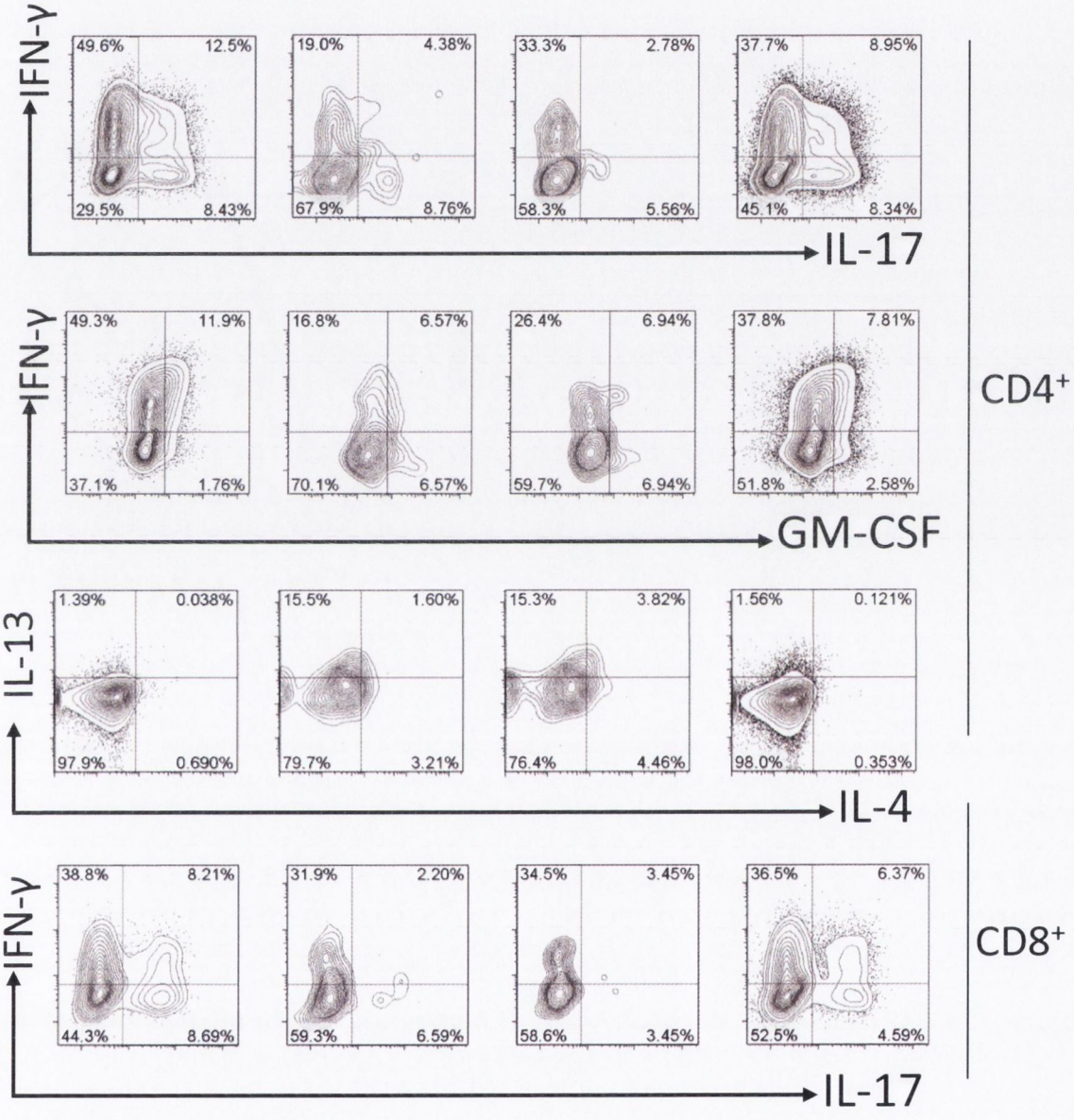


Fig 5.12 Therapeutic and prophylactic treatment with ES delays suppresses the Th1 and Th17 cells that mediate EAE. On day 0, EAE was induced in C57BL/6 mice by s.c. injection with 100 μg of MOG_{35-55} in CFA, followed by i.p. injection with 500 ng of PT on days 0 and 2. Mice were injected i.p. on day -1 or day + 5 and every 2 days thereafter with PBS, Total ES, ES-L or ES-H. A, mice were sacrificed and perfused on day 12 and numbers of $CD3^+$ $CD4^+$ T cells and Siglec-F^+ $CD11b^{int}$ Ssc^{high} eosinophils were determined by flow cytometry of percoll isolated brain mononuclear cells. B, IFN-γ, IL-17, IL-13 GM-CSF and IL-13 cytokine expression by PMA/ION/BFA stimulated brain $CD4^+$ $CD3^+$ and $CD8^+$ $CD3^+$ was determined by intracellular cytokine staining. Results for all cytokines except GMCSF are representative of 4 separate experiments

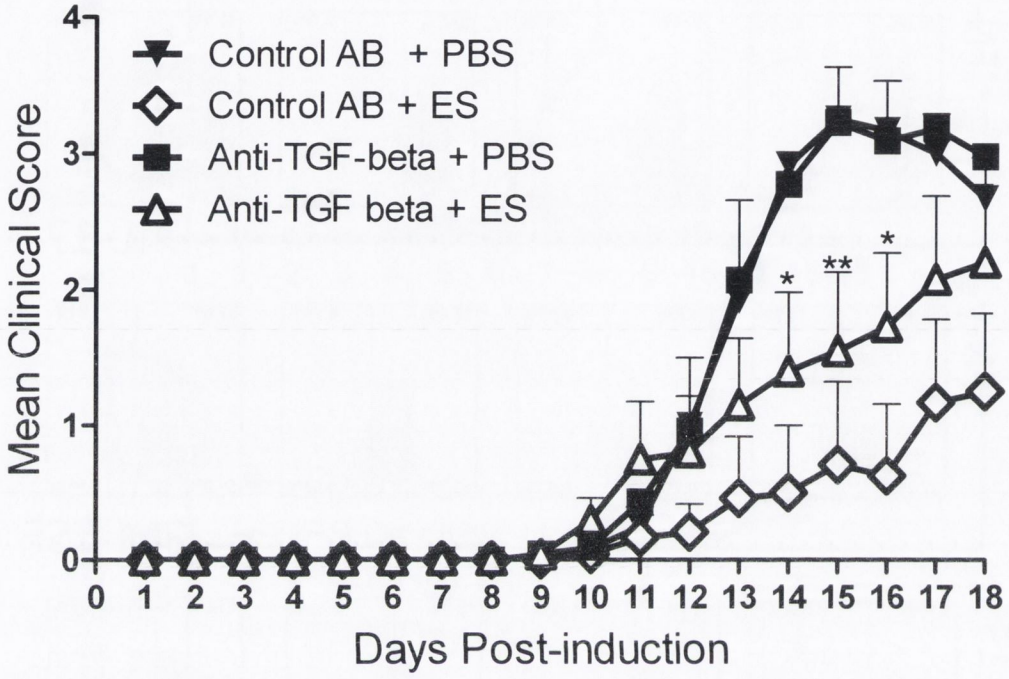


Fig 5.13 Protection against EAE by *F. hepatica* ES is not mediated via TGF-β. On day 0, EAE was induced in C57BL/6 mice by s.c. injection with 100 μg of MOG_{35-55} in CFA, followed by injection i.p. with 500 ng of PT on days 0 and 2. Starting on day -1, and for every 2 days thereafter, mice were i.p. injected with 100 μg of anti-TGF-β (clone, 1D11) or control IgG_{2A} antibody with or without either PBS • or ES (□, 20μl) and Clinical scores were examined daily and mice sacrificed on day 18. Results are representative of two separate experiments. Data presented are means +/- SEM of 10 mice per group Statistical assessment was performed using Two-way ANOVA together with Bonferroni's post text vs Anti-TGF-beta + PBS group *$p<0.05$ **$p<0.001$.

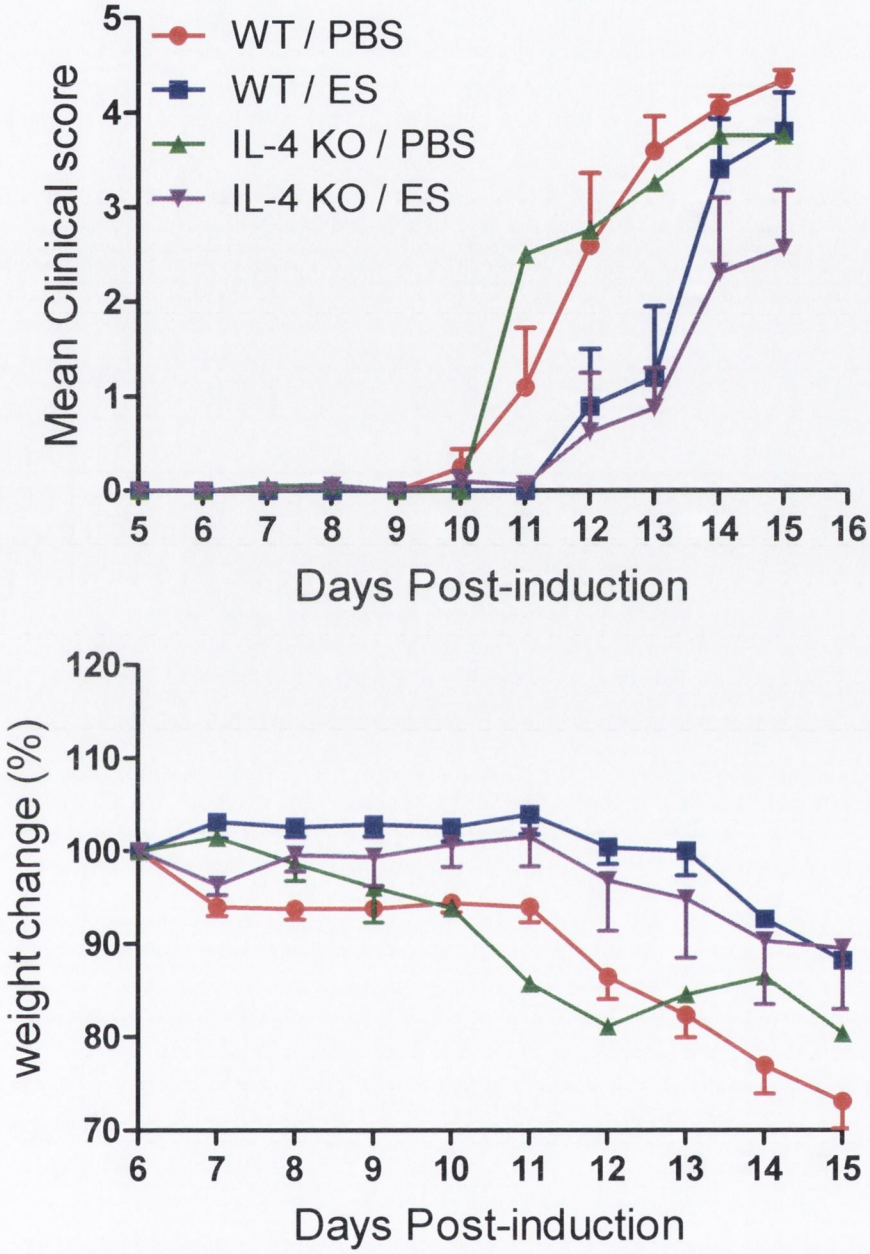


Fig 5.14 ES induced protection from EAE is not mediated by IL-4. EAE was induced in C57BL/6 mice by s.c. injection with 100 μg of MOG_{35-55} in CFA, followed by i.p. injection with 500 ng of PT on days 0 and 2. From on day -1 and for every 2 days thereafter, mice were injected i.p. with either PBS (●), ES (40 μl, ■), anti-IL-4 (100 μg, clone: TRFK-4, ▲) or anti-IL-4 + ES (▼). Clinical scores and weights were examined daily and mice sacrificed on day 15. Data presented are means +/- SEM of 5 mice per group

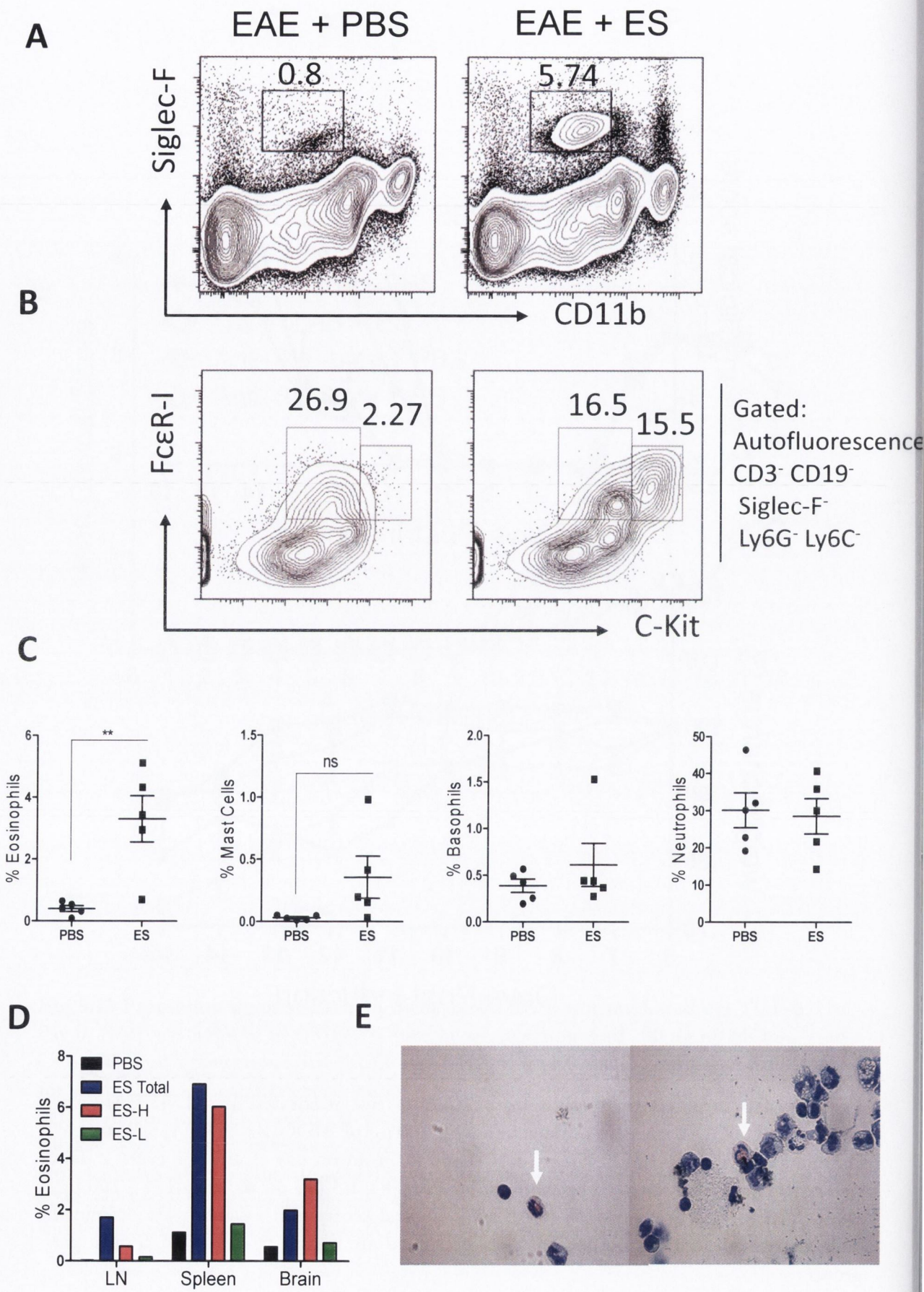

A
EAE + PBS
EAE + ES
0.8
5.74
Siglec-F
CD11b
B
26.9
2.27
16.5
15.5
FcεR-I
C-Kit
Gated:
Autofluorescence
CD3- CD19-
Siglec-F-
Ly6G- Ly6C-
C
% Eosinophils
% Mast Cells
% Basophils
% Neutrophils
PBS
ES
**
ns
D
% Eosinophils
PBS
ES Total
ES-H
ES-L
LN
Spleen
Brain
E

Fig 5.15 ES administration induces systemic eosinophilia in mice with EAE. EAE was induced in C57BL/6 mice by s.c. injection with 100 μg of MOG_{35-55} in CFA, followed by i.p. injection with 500 ng of PT on days 0 and 2. Starting on day -1, and for every 2 days thereafter, mice were injected i.p with PBS or ES (45 μl i.p.). A, on day 15 blood was collected from both groups and tested for the presence of eosinophils, mast cells, basophils and neutrophils was determined by flow cytometery. A, representative flow cyometric plots demonstrating gates for $CD11b^+$ $Siglec\text{-}F^+$ eosinophils. B, representative flow cytometric plots gated on $autofluorescence^-$ $CD3^-$ $CD19^-$ $Siglec\text{-}F^-$ $Ly6G^-$ $Ly6C^-$ depicting gates for $Fc\varepsilon R\text{-}I^+$ $C\text{-}Kit^-$ Basophils and $Fc\varepsilon R\text{-}I^+$ $C\text{-}Kit^+$ mast cells. C, Percentage of eosinophils, mast cells, basophils and neutrophils in blood of treated mice. Data presented are means +/- SEM of 5 mice per group. Results from A-C are representative of 3 separate experiments D, Percentage of eosinophils in the brains of perfused mice determined by flow cytometery. E, Photomicrographs of cytospin preparations of mononuclear cells isolated from perfused brains of ES treated mice with EAE mice; white arrow illustrate infiltrating eosinophils. Results from D-E are representative of 2 separate experiments.

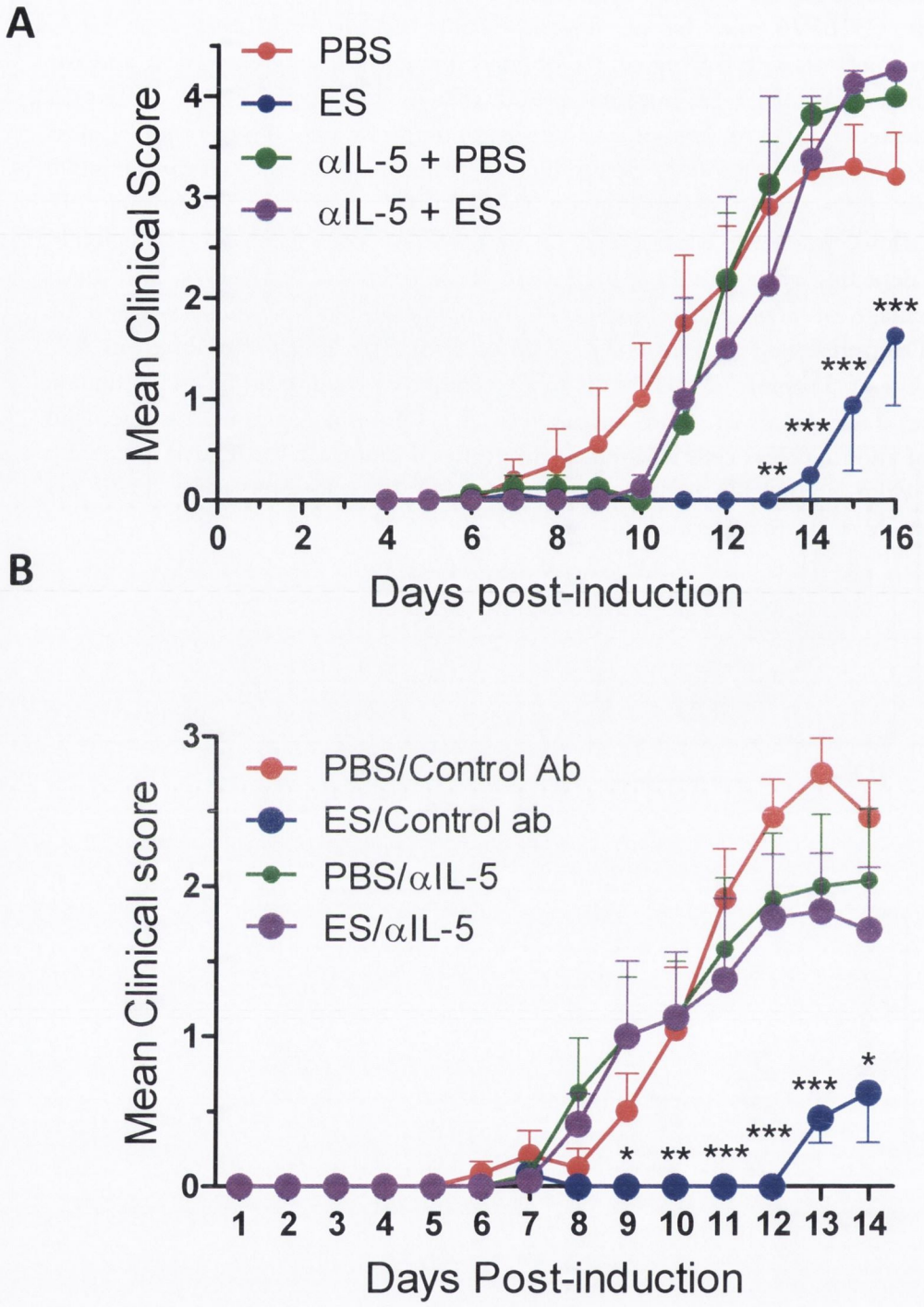
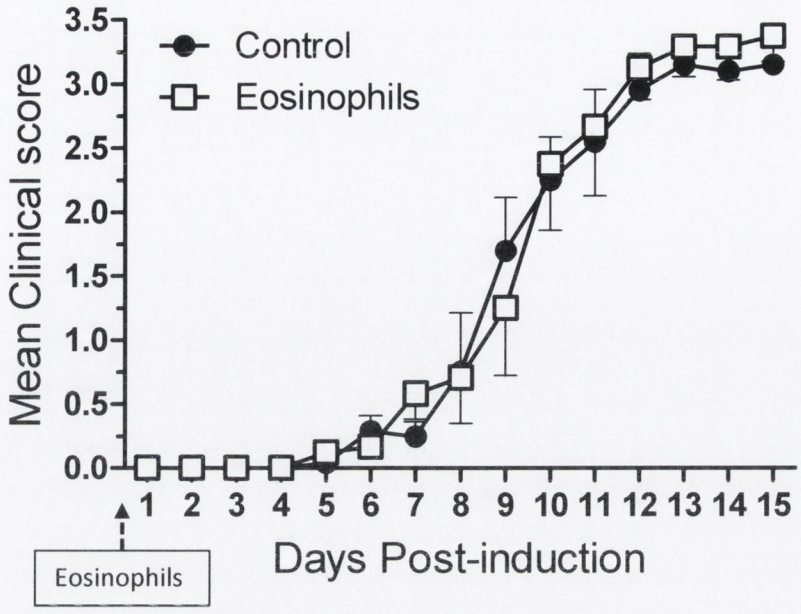


Fig 5.16 The protective effect of *F. hepatica* ES is mediated by IL-5. On day 0, EAE was induced in C57BL/6 mice by s.c. injection with 100 μg of MOG_{35-55} in CFA, followed by i.p. injection with 500 ng of PT on days 0 and 2. From on A, day -1 or B, Day +5 and for every 2 days thereafter, mice were i.p. administered with either control IgG_{2A} (80 μg, ●), ES (40 μl) + control IgG_{2A} (■), anti-IL-5 (80 μg, clone: TRFK-5, ▲) or anti-IL-5+ ES (▼). Clinical scores were examined daily and mice sacrificed at peak of clinical score. Data presented are means +/- SEM of 6 mice per group. Results from A-C are representative of 3 separate experiments Statistical assessment was performed vs ES/Control Ab using Two-way ANOVA together with Bonferroni's post text **$p<0.001$ ***$p<0.0001$. Results from are representative of 3 separate experiments.

A



B

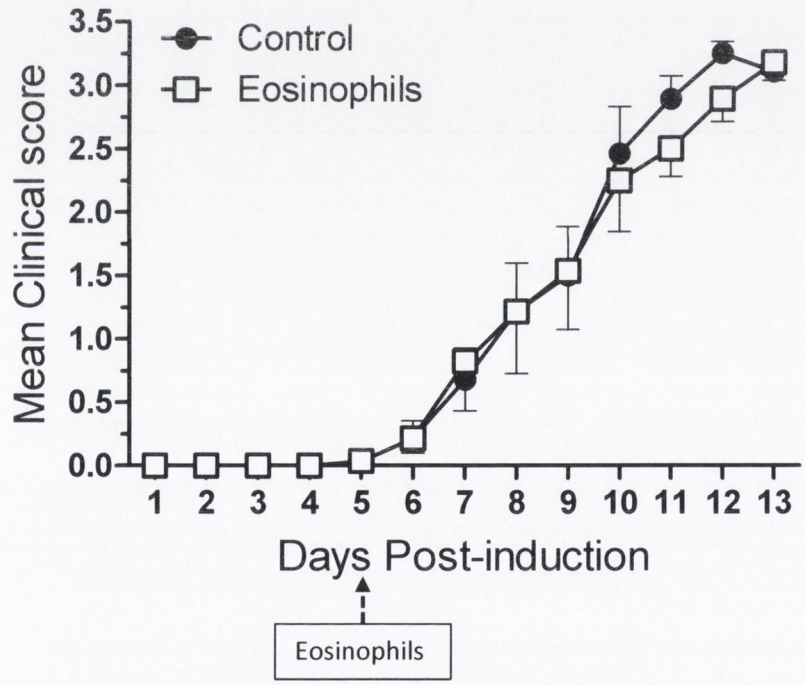


Fig 5.17 ES-elicited eosinophils do not transfer protection against EAE. Siglec-F^{+} CD11b^{+} Ssc^{high} CD11c^{-} eosinophils were isolated by FACS from the peritoneal cavity of C57BL/6 mice which were previously administered ES (40μl) every 2 days for 7 days. On day 0, EAE was induced in fresh C57BL/6 mice by s.c. injection with 100 μg of MOG_{35-55} in CFA, followed by i.p. injection with 500 ng of PT on days 0 and 2. At A, time 0 or B, day 7 post immunization with CFA and MOG mice were A, s.c. or B, i.p. administered either PBS (●) or $1x10^{6}$ eosinophils (□). Clinical scores were examined daily and mice were sacrificed at peak of disease. Data presented are means +/- SEM of 6 mice per group.

Chapter 6 The excretory/secretory products of *F. hepatica* inhibit the induction of T cells responses that mediate allergy

6.1 Introduction

This study has already shown that administration of the secreted products of *F. hepatica* protected mice from EAE, a disease mediated by Th1 and Th17 cells. ES promoted the induction of a Th2 response *in vivo,* and protection from EAE was dependent on the production of IL-5.

Helminth infections and helminth derived products have also been shown to protect from allergy. This might appear paradoxical, as helminths themselves induce powerful Th2 responses *in vivo*; however, helminths also exert powerful immunoregulatory mechanisms to suppress the generation of protective Th2 response. It has also been argued that the host exerts its own regulatory mechanisms to prevent immune pathology associated with a chronic helminth infection. A seminal study by Wilson et al, demonstrated that infection of mice with the Th2/Treg cell–inducing gastrointestinal nematode *H. polygyrus* protected against the development of AHR. In addition suppression was reversed upon treatment with antibodies specific for CD25, demonstrating a role for Treg cells (206).

The majority of animal models of asthma are initiated by one or more peripheral injections of a protein allergen (most commonly ovalbumin) with concurrent administration of the Th2-skewing adjuvant alum. This sensitization phase is followed a few weeks later by the direct administration of allergen to the lung by aerosolisation. This protocol results in the development of AHR which is associated with eosinophilic inflammation in the lung, production of serum anti-allergen IgE and pulmonary Th2 cytokines, including IL-4, IL-5 and IL-13 (129). This model has proved very effective in the study of the pathogenesis of asthma.

The aim of this chapter was to determine the effect of ES on the mouse model of allergic asthma, AHR. Specific aims include:

1) **Determine if *F. hepatica* ES suppress or promote the induction of AHR. Specific aims include:**

Previous studies have shown that the secreted products of *H. polygyrus*, and *N. brasiliensis* and the total antigen of *Ascaris sunum* ameliorate the induction of AHR (165, 424, 425). The effect

of *F. hepatica* ES on the induction of AHR has not been investigated. The previous chapter has shown that ES inhibits the APC function of DC but also promotes the induction of Th2 response *in vivo*. Thus experiments were conducted to determine whether ES inhibited or potentiated allergic responses.

2) **Determine if ES modulates the induction of Th2 cells that mediate allergy via the induction of immune regulatory mechanisms.**

A number of studies have demonstrated that the efficacy of the administration of helminth products to mice with AHR is dependent on the timing of administration (166, 417, 418). Thus, experiments were conducted to determine if administration of ES at challenge or sensitisation differentially altered the severity of AHR. Results from chapter 3 demonstrated that infection of mice with *F. hepatica* protected mice from EAE in a manner associated with Treg cells. However, the previous chapter has shown that ES protected mice from EAE in a manner independent of Treg cells but rather dependent on IL-5. Thus this chapter investigated if ES promoted the expansion of Treg cells in AHR

6.2 Results

6.2.1 Administration of ES at sensitisation inhibits the induction of airway hypersensitivity

Experiments were conducted to determine if ES could affect the induction of AHR. On day 0, BALB/c mice were injected i.p. with alum, alum+OVA or alum+OVA+ES on day 0 and 14. All mice were challenged with aerosolised OVA on days 24, 25, 26 (Figure 2.4). BALs were conducted on sacrificed mice on day 28. BAL cells were counted and cytospin preparations were prepared and stained with H&E for discrimination of macrophages, eosinophils and lymphocytes.

OVA challenge dramatically increased the numbers of cells isolated from the alveolar spaces of mice that were sensitized with alum+OVA compared with those sensitised with alum only, from 30,000 to $1.2x10^6$ cells, respectively [Fig 6.1 B]. In contrast, addition of ES at sensitisation to alum+OVA significantly reduced the numbers of cells isolated via BAL upon challenge with OVA, to a mean of 90,000 cells [Fig 6.1 B]. Cytospin preparations revealed that a large proportion of the infiltrating cells in the alum+Ova sensitised mice were eosinophils and lymphocytes [Fig 6.1 A].

The numbers of Siglec-F^+ $CD11b^+$ $CD11c^-$ SSc^{high} eosinophils and $CD3^+$ or $CD19^+$ lymphocytes in the BAL was quantified by flow cytometry. Both SSc and the expression of CD11c were used to distinguish between eosinophils and $CD11c^+$ Siglec-F^+ $CD11b^-$ SSc^{int} alveolar macrophages. Consistent with results obtained from the examination of cytospin preparations, there was a large increase in the numbers of eosinophils and lymphocytes in the BAL from mice sensitised with alum+OVA, compared with BAL from mice sensitized with alum alone. However, addition of ES to alum+OVA at sensitisation significantly reduced the infiltration of eosinophils into the BAL to the numbers observed following sensitisation with alum alone ($39x10^3$ compared with $796x10^3$) [Fig 6.1, A]. The number of lymphocytes was also reduced significantly from $140x10^3$ to $22x10^3$ in mice sensitized with alum+OVA in the presence of ES [Fig 6.1, C].

IgE and IgG1 are antibody isotypes associated with type-2 responses. Indeed, allergen-specific IgE and IgG1 can passively induce AHR upon transfer to mice challenged with an allergen (419). Allergen specific IgE and IgG1 are enhanced in AHR induced by systemic immunization with alum and allergen (206, 419, 420). However, AHR is maintained following neutralisation of IgE (419, 421) or in B or mast cell deficient mice (419).

Both total and OVA-specific IgG1 and IgE were measured in the BAL fluid and serum of challenged mice on day 28. Total and OVA-specific IgG1 was enhanced in the serum from mice sensitized with alum+OVA with or without ES, compared with mice sensitized with alum only. However, ES given at sensitisation did not alter the serum concentrations of IgG1 between mice sensitised with alum+OVA. Similarly, while total and OVA-specific IgE were enhanced in serum from mice sensitized with alum+OVA or alum+OVA+ES, compared with mice sensitised with alum alone, there was no difference between these groups [Fig 6.2 A].

Total and OVA-specific IgG1 was enhanced in BAL fluid from mice sensitized with alum+OVA compared with BAL from alum controls [Fig 6.2 B]. OVA-specific IgE was also enhanced in BAL from mice sensitized with alum+OVA but total IgE was unchanged compared with BAL from mice sensitized with alum alone [Fig 6.2 B]. Interestingly, the addition of ES at sensitisation with alum+OVA significantly enhanced total and OVA-specific IgE in the BAL fluid compared with BAL from mice sensitized with alum+OVA [Fig 6.2 B]. Similarly, total IgG1 was greatest with addition of ES at sensitisation with alum+OVA compared with that from mice sensitized with alum+OVA only, however this was not significant. The BAL fluid from mice sensitised with both alum+OVA or alum+OVA in the presence of ES had similar amounts OVA-specific IgG1 [Fig 6.2 B].

These data demonstrate that ES inhibits the ability of alum to sensitise mice with an allergen, and subsequently blocks the infiltration of cells into the lung following allergen challenge, however, it does not inhibit type-2 antibody production but in fact enhances IgE production in the lung serum.

6.2.2 Administration of ES at challenge does not affect disease

Co-administered of ES with alum and OVA prevented the infiltration of eosinophils and lymphocytes upon challenge with OVA [Fig 6.1]. Experiments were conducted to determine if treatment with ES at the time of challenge could affect the severity of AHR in animals sensitised with alum and OVA. BALB/c mice were injected i.p on day 0 and 14 with alum alone, alum+OVA or alum+OVA in the presence of ES. Mice sensitised with alum+OVA were either treated with PBS or ES by i.p. injection on days 24-27. On days 24-26, mice were challenged with aerosolised OVA. BALs were performed on sensitised mice on day 28 (Figure 2.4). Consistent with previous results, administration of ES at sensitisation prevented the accumulation of eosinophils in the BAL fluid and lungs of challenged mice [Fig 6.3]. However, ES treatment at challenge did not affect the infiltration of eosinophils into the lungs or the frequency or total numbers of eosinophils in the BAL of mice sensitized with alum+OVA [Fig 6.3].

Alum/OVA sensitised mice had increased numbers of Siglec-F^+ $CD11c^+$ $F4/80^+$ $CD11b^+$ alveolar macrophages, $CD11b^+$ $Gr\text{-}1^{high}$ neutrophils, $CD11b^+$ $F4/80^+CD11c^-$ interstitial macrophages and $CD3^+/CD19^+$ lymphocytes compared with mice sensitised to alum [Fig 6.4 A, B]. Administration of ES at sensitisation, but not challenge, suppressed the increase of alveolar macrophages, neutrophils and lymphocytes, but did not affect the numbers of interstitial macrophages [Fig 6.4 B]. Treatment with ES at challenge did not affect the total numbers of these cells in mice sensitized with alum+OVA [Fig 6.4 B].

These results demonstrate that administration of ES at sensitisation, but not challenge, reduces lung inflammation.

6.2.3 ES inhibits allergic lung pathology associated with airway hypersensitivity

The infiltration of cells into the alveolar spaces is a measure of inflammation in the lung, but it ignores the modifications to the airways which affect vital functions of the animals. Experiments were conducted to investigate the effect of ES on AHR-associated lung pathologies when administrated at sensitisation or during challenge with the model allergen, OVA. Histological sections were prepared from the lungs of OVA challenged mice sensitised

with alum, aluma+OVA or alum+OVA with ES given at sensitisation or challenge. H&E staining was used to examine cellular infiltrates and changes in the bronchial-epithelium or smooth muscle. Alican blue-periodic acid schiff (AB-PAS) was used to identify mucus-producing goblet cells in the epithelial border. H&E staining revealed dense peribronchiolar and luminal inflammatory infiltrate of lymphocytes, and mononuclear and polymorphonuclear cells with epithelial shedding and extended columnal cells in lungs of mice sensitised with alum+OVA following challenge with OVA compared with lungs of mice sensitised with alum only [Fig 6.5 A B]. Consistent with a reduction in BAL cells, the administration of ES at sensitisation reduced the severity of cell infiltration/inflammation in the lung of mice sensitised with alum+OVA [Fig 6.5 C]. In contrast, administration of ES at time of challenge did not affect OVA-induced lung inflammation, in mice sensitised with alum+OVA [Fig 6.5 D].

Furthermore, OVA challenge of mice sensitised with alum+OVA resulted in an accumulation of mucin-containing goblet cells that line the connecting airways, together with a build-up of free mucus in the airway lumen, which was not observed in the lungs of mice sensitised with alum alone [Fig 6.6 A B]. Treatment with ES at sensitisation reduced the proportion of goblet cells in the epithelial border and presence of luminal mucus in mice sensitised with alum+OVA [Fig 6.6 B C]. In contrast, ES treatment at challenge did not suppress the build-up of mucin in airways of mice that were sensitized with alum+OVA [Fig 6.6 B D].

These results demonstrate that ES attenuates a range of allergic airway inflammatory pathologies when administered during sensitisation, but not during challenge with the model allergen OVA.

6.2.4 ES inhibits Th2 responses that mediate airways hypersensitivity

Co-administration of ES during sensitisation prevented a range of pathologies associated with AHR, including the eosinophil/lymphocyte dominated immune cell infiltration of the lungs and goblet cell hyperplasia, but did not reduce production of allergen-specific IgE and IgG1, antibody subclasses associated with type-2 responses.

As AHR is a Th2 cell mediated disease (422), experiments were conducted to determine if co-administration of ES during sensitisation inhibited the induction of Th2 cells in the lungs of challenged mice. Cells isolated from the lungs of mice post OVA-challenge were incubated with increasing concentrations of OVA and after 4 days the concentrations of IL-4 and IL-13 were determined by ELSIA. The production of IL-4 and IL-13 was greatly enhanced in the lungs of mice sensitised with alum+OVA compared with lungs of mice sensitised with alum only [Fig 6.7]. Co-administration of ES at sensitisation significantly reduced the production of IL-4 and IL-13 to concentrations comparable with those in lungs of mice sensitised with alum only Fig 6.7]. In contrast, administration of ES at the time of challenge did not affect OVA-specific IL-4 or IL-13 [Fig 6.7]. These results suggest that ES, administered at sensitisation with alum, prevents the induction of allergen-specific Th2 cells.

6.2.5 Transfer of T cells from protected mice does not confer protection in recipients

ES co-administered at sensitisation with alum and OVA prevented AHR and the induction of Th2 cells that mediate disease [Fig 6.1-6.7]. Wilson et al reported that *H. polygyrus* protects the infected host from AHR and that transfer of $CD4^+$ T cells from mice infected with *H. polygyrus* protects mice against AHR (206). Experiments were conducted to determine if transfer of $CD4^+$T cells from mice immunised with alum+OVA in the presence of ES could confer protection from AHR in recipients. BALB/c mice were sensitized with alum, alum+OVA or alum+OVA in the presence of ES on day 0 and day 14. On day 21, $CD4^+$ cells, purified from the spleens and LN of immunized mice with alum+OVA in the presence of ES, were adoptively transferred by i.p. injection to recipient mice that had been immunized with alum+OVA 21 days earlier. All mice were challenged with OVA by aerosolisation on days 24, 25 and 26. On day 28, BALs were conducted on sacrificed mice.

Consistent with previous experiments, administration of ES at sensitisation reduced the infiltration of eosinophils in the lungs of challenged mice immunised with alum+OVA, as determined by both flow cytometry and analysis of H&E stained BAL cells [Fig 6.8-9].

Transfer of $CD4^+$ T cells from mice immunised with alum+OVA+ES to mice immunised with alum+OVA had no effect on the accumulation of eosinophils or lymphocytes [Fig 6.8-9]. There was a small but non-significant reduction in the total cell count and thus the number

of eosinophils, in mice which received $CD4^+$ T cells compared with mice that did not receive T cell, but this was not-significant [Fig 6.9].

Wilson et al also demonstrated that *H polygyrus* infection increased the expression of FOXP3 by mesenteric LN $CD4^+$ $CD25^+$ T cells and neutralisation of CD25 expressing cells blocked the suppression of AHR, and the transfer of $CD4^+$ $CD25^+$ T cells was more effective at protecting mice from AHR than the transfer of $CD4^+CD25^-$ T cells (206).

To determine if the treatment of alum+OVA immunized mice with ES or by the transfer of $CD4^+$ T cells from mice immunised with alum+OVA+ES could increase the proportion of Treg cells the expression of FOXP3 in lung and cervical LN $CD4^+$ T cells was determined by flow cytometry. The expression of FOXP3 was not altered by either treatment; 18.5% of $CD4^+$ T cells from alum sensitised mice were $FOXP3^+$ while the expression of FOXP3 in $CD4^+$ T cells from mice sensitised with alum+OVA, alum+OVA+ES and alum+OVA/$CD4^+$ recipients was 17.5%, 19.5% and 17.7%, respectively [Fig 6.10]. The overall expression of FOXP3 was higher in cervical LN $CD4^+$ T cells, 29.1%, 24.5%, 23.9% and 30.2% for alum, alum+OVA, alum+OVA in the presence of ES and alum+OVA+$CD4^+$ transfer, respectivelyexpressed FOXP3 although this did not correlate with protection from AHR.

The expression of IL-4 and IL-13 by lung and cervical LN $CD4^+$ T cells were also analysed by flow cytometry. $CD4^+$ T cells from mice sensitised with alum+OVA expressed much more IL-4 and IL-13 compared with $CD4^+$ T cells from mice sensitised with alum. In addition, a higher proportion of $CD4^+$ T cells co-expressed IL-4 and IL-13 than either cytokine alone [Fig 6.11 A]. Consistent with previous results, the expression of IL-4 and IL-13 was reduced in $CD4^+$ T cells from lungs of mice that were immunized with alum+OVA in the presence of ES compared with $CD4^+$ T cells from mice sensitised with alum+OVA only [Fig 6.11 A]. There was a 24 and 30-fold decrease in the absolute numbers of $IL\text{-}4^+$ and $IL\text{-}13^+$ T cells, respectively in lungs of mice treated with ES [Fig 6.11 B]. Furthermore, while ES slightly suppressed the expression of IL-4 and IL-13 in $CD4^+$ T cells from lungs from mice that had received $CD4^+$ T cells, it suppressed the total number of $IL\text{-}4^+CD4^+$ and $IL\text{-}13^+CD4_+$ T cells by 38% and 39%, respectively [Fig 6.11 B]. There was negligible production of IL-4 and IL-13 by cervical LN $CD4^+$ T cells in any treatment group [Fig 6.11 A]

In order to examine antigen-specific T cell responses in the lungs, total lung cells were incubated *ex vivo* with increasing concentrations of OVA. The concentrations of IL-4, IL-5 and IL-13 in the supernatant at 72 hours were determined by ELISA. Consistent with previous data, the lung cells from mice sensitised with alum+OVA in the presence of ES failed to produce any OVA-specific IL-4 and IL-13 in comparison with robust production by the lungs from mice sensitised with alum+OVA only [Fig 6.12]. Consistent with the reduction in the numbers of eosinophils, lung cells from mice sensitised with alum+OVA in the presence of ES produced less IL-5 compared with cells from mice sensitised with alum+OVA [Fig 6.12]. Lung cells from mice that received $CD4^+$ T cells as a treatment produced significantly less OVA-specific IL-5 compared with mice which were sensitised to alum+OVA without transfer of T cells. However, there were non-significant reductions in IL-4 and IL-13 by lung cells from these animals [Fig 6.12].

6.2.6 The protective component of ES is above 3kDa

This study has shown that the higher molecular fraction of ES, ES-H, was the protective fraction of ES against EAE [Fig 5.11]. Administration of this fraction reduced the accumulation of Th1 and Th17 cells in the brain of mice with EAE [Fig 5.12]. Furthermore, ES-mediated protection from EAE was associated with the development of a type 2 response and was dependent on IL-5 [Fig 5.15-6]. Experiments were conducted to determine which fraction of ES was protective in AHR, a model dominated by Th2 responses. BALB/c mice were sensitized with alum, alum+OVA, and alum+OVA in the presence of ES-L or ES-H on day 0 and day 14. All mice were challenged with aerosolised OVA on days 24, 25 and 26. BALs were conducted on sacrificed mice on day 28.

Mice sensitised with alum+OVA in the presence of ES-H had significantly reduced numbers of total BAL cells [Fig 6.13 A]. Furthermore, BAL eosinophils, T cells and inflammatory macrophages were all significantly reduced in following OVA challenge, compared with mice immunized with alum+OVA-sensitised alone [Fig 6.13 A]. Moreover, administration of ES-H non-significantly suppressed the numbers of alveolar macrophages [Fig 6.13 A]. Indeed co-administration of ES-H completely blocked the infiltration of eosinophils, T cells and inflammatory macrophages and their numbers were comparable to numbers in the BAL of mice sensitised with alum only [Fig 6.13]. In contrast, co-administration of ES-L to mice

sensitised with alum+OVA had no effect on the infiltration of eosinophils, T cells or macrophages upon OVA challenge [Fig 6.13]. There was no change in the numbers of neutrophils in any of the treatment groups [Fig 6.13].

The intracellular expression of IL-4, IL-5, IL-13, IL-17 and IFN-γ by lung $CD4^+$ T cells restimulated *ex vivo* with PMA and ION for 5 hours was determined by flow cytometry and the results were analysed using SPICE software. Lung $CD4^+$ T cells from mice sensitised with alum+OVA produced more IL-4, IL-5 and IL-13 and similar amounts of IFN-γ compared with lung $CD4^+$ T cells from mice sensitised with alum alone [Fig 6.14]. SPICE analysis revealed that T cells from these mice produced less IL-4 than IL-5 or IL-13. IL-4 was co-expressed with IL-13 but not with IL-5. There was very strong co-expression of IL-5 and IL-13 [Fig 6.14]. Unexpectedly there was an increase in the expression of IL-17 in $CD4^+$ T cells from mice sensitised with alum+OVA which was not co-expressed with any other cytokine examined [Fig 6.14]. $CD4^+$ T cells from mice sensitised with alum+OVA in the presence of ES-L had a very similar cytokine profile to those from mice sensitised with alum+OVA [Fig 6.14]. Lung $CD4^+$ T cells from mice sensitised with alum+OVA in the presence of ES-H had reduced expression of IL-4, Il-5 and IL-13 but not IFN-γ or IL-17 compared with those from mice sensitised with alum+OVA alone. The expression of IL-4 in these cells was moderately reduced whilst there was a greater reduction on the expression of IL-5 and IL-13. In contrast, the expression of IFN-γ was enhanced slightly in $CD4^+$ T cells from mice sensitised with alum+OVA in the presence of ES-L compared with those from mice sensitised with alum+OVA without treatment [Fig 6.14].

In order to further examine the antigen-specific T cell response lung cells were reactivated *ex vivo* with increasing concentrations of OVA. Lung cells from mice that were sensitised with alum+OVA in the presence of ES-H produced significantly less OVA-specific IL-4, IL-5 and IL-13 compared with those from mice sensitised with alum+OVA alone or in the presence of ES-L [Fig 6.15]. Interestingly, the administration of ES-H at sensitisation enhanced the production of OVA-specific IFN-γ by lung cell of mice sensitised with alum+OVA [Fig 6.15]. Unexpectedly, OVA-specific IL-17 was produced by lung cells from mice sensitised with alum only [Fig 6.15]. These data demonstrate that ES suppresses the induction of Th2 cells that mediate allergy.

6.2.7 ES does not affect the inflammasome activity

Induction of Th2 responses induced by alum differs from those induced by helminths as it does not require IL-4 or IL-13 signalling through the IL-4Rα chain (56). IL-1β was shown to be important from the induction of AHR in response to alum and OVA as disease is attenuated in IL-1α/β$^{-/-}$ mice but aggravated in IL-1R antagonist$^{-/-}$ mice (423). IL-1β is produced as an inactive pro-form that is cleaved by caspase-1 (424). Caspase-1 is produced in response to inflammasome activation. NLRP3, an integral inflammasome component that is activated in innate cells in response to alum (425), has been implicated in the induction of Th2 responses by alum. Several reports have claimed that NLRP3 is required for the adjuvant effect of alum (57, 426). However others have shown that inflammasome activation is not required and that alum-induced Th2 responses are induced by alternative mechanisms (427). Recent studies have demonstrated that alum induces the production of uric acid crystals which can induce AHR independent of inflammasome activity with a role for PGE_2 in the induction of Th2 responses (428, 429).

Experiments were conducted to determine if ES could affect alum-mediated inflammasome activation or PGE_2 production. BMM were primed with LPS in the presence or absence of ES for 3 hours prior to the addition of alum or monosodium urate crystals (MSU). The concentrations of IL-1β in the supernatant at 2 and 24 post-addition of alum+MSU were determined by ELISA. In addition, the concentration of PGE_2 at 24 hours was determined by competitive ELISA. ES reduced the production of IL-1β by alum or MSU-treated LPS primed BMM at 2 hours post addition of alum or MSU, however, this was not significant [Fig 6.16 A]. Furthermore, this effect was transient and by 24 hours ES had no effect on alum or MSU induced activation of LPS primed BMM, as measured by IL-1β production [Fig 6.16 B]. In addition, ES had no effect on alum or MSU induced production of PGE_2 by LPS-primed BMM [Fig 6.16 C]. These data demonstrate that the suppressive capacity of ES on alum-induced Th2 responses is not mediated via inflammasome activated production of IL-1β or PGE_2.

6.3 Discussion

There is strong epidemiological evidence to suggest that atopy and allergy negatively correlates with helminth infections and this has formed the basis of the hygiene hypothesis. This is now supported by experimental studies in mouse models. For example, infection with *N. brasiliensis, S. mansoni, S. japonicum, L. sigmodontis* and *H. polygyrus* have all been shown to suppress the development of AHR, a mouse model of allergic disease (206, 207, 417, 430). However, the efficacy of the secreted products of helminth in regulating lung inflammatory *in vivo* is less convincing. The major finding of this chapter is that administration of *F. hepatica* ES blocked the development of AHR. Administration of ES at sensitisation, but not at aiiergen challenge, blocked the induction of IL-4/IL-5/IL-13 producing Th2 cells that mediated the influx of eosinophils in the airways of challenged mice. These findings are in agreement with others that have shown that the secreted products of *H. polygyrus,* and *N. brasiliensis* and the total antigen of *Ascaris sunum* ameliorate the induction of AHR through various mechanisms (166, 418, 431).

Since the paradoxical discovery that Th2-inducing helminths, or their products, suppress the induction of the Th2 responses that mediate allergy, several mechanisms have been proposed to explain this phenomenon. A revised interpretation of the hygiene hypothesis has proposed that regulatory immune responses induced by helminth infection inhibit allergic inflammation (432). Thus, while helminths ameliorate autoimmune processes using a combination of regulatory responses and via induction of Th2 cells which antagonise Th1 and Th17 responses, their effects on allergy are more dependent on regulatory mechanisms, particularly induction of Treg cells.

Infection with *L sigmodontis* protects mice from AHR through a mechanism involving the expansion of Treg cells (207). This was associated with enhanced production of TGF-β by T cells and protection from AHR was partially reversed by neutralisation of TGF-β. However, in the broader context, TGF-β may not be protective in AHR, as administration of recombinant TGF-β at sensitisation has no effect on disease outcome (166). In addition, TGF-β has been contra-indicated in the treatment asthma as it contributes to pathology, specifically airway remodelling and fibrosis (433).

The present study did not find a role for Treg cells in ES-mediated suppression of AHR. The numbers of $FOXP3^+$ Treg cells in the lungs or lung draining LN were not enhanced following airway challenge of mice treated with ES at sensitisation to allergen. Nevertheless, it was initially thought that a suppressive $FOXP3^-$ T cell might still mediate suppression. However, transfer of $CD4^+$ T cells from protected mice to mice immunised with alum and OVA before airway challenge had no effect on disease outcome, suggesting that Treg cells did not play a role in suppression. These findings contrast with those of Wilson *et al* who reported that Treg cells were expanded in mice infected with *H. polygyrus* and that protection against AHR was transferred from infected allergen-naïve mice to uninfected allergen-sensitised mice via transfer of Treg cells (206).

However, the study by Wilson et al differs greatly from the present one in that suppression of AHR was mediated by an active on-going helminth infection rather than helminth products given at sensitisation. The role of Treg cells in the present study is further compounded by the observation that administration of ES to naïve mice does expand Treg cells. However, infection of mice with *F. hepatica* expands $FOXP3^+$ Treg cells in the peritoneal cavity. Therefore, it may be possible that transfer of Treg cells from mice infected with *F. hepatica* would transfer protection. In a similar manner, McSorley et al have shown that unlike the whole parasite, HES from *H. polygyrus,* which ameliorated AHR when given at sensitisation, did not expand Treg cells (166, 434). In the absence of Treg cell induction, the effect of ES may be restricted to the initial sensitisation phase. Indeed, this study has convincingly demonstrated a suppressive role for ES on the APC function of DC. Moreover, this study demonstrates that ES did not confer Th2 promoting activity to DC.

The present study also found that administration of ES systemically, at allergen challenge had no effect on AHR, further emphasising the initial sensitisation as the time-point when ES inhibits Th2 cell induction. HES, the secreted products of *H. polygyrus* were found to ameliorate the induction of AHR when given at sensitisation, in a similar fashion to *F. hepatica* ES (166). Administration of HES at challenge was sufficient to reduce IL-5 production and the expression of genes associated with M2 macrophages, but in agreement with the present study, it did not affect the infiltration of T cells into the airways nor the cytokine production by these T cells, suggesting that HES has very limited effect when given at challenge. Indeed, this lack of efficacy after the antigen sensitisation phase seems to be a common feature of

many helminths; the secreted products of *N. brasiliensis* inhibited the induction of AHR when given at sensitisation but not when given 7 days later (418). Moreover, infection with *N. brasiliensis* prior to (but not after) allergen sensitisation blocked eosinophil recruitment to the lungs (417).

ES is a mixture of many proteins and small molecules and therefore it has a range of antigens and immunomodulatory molecules. It is possible that ES mediates its suppressive effects on the induction of allergy by simple antigen competition. However, it has been demonstrated that administration of an excess of antigens (using BSA) in the emulsion of alum and OVA during sensitisation had no effect on the induction of AHR, suggesting that high antigenic pressure alone is not a suppressive mechanism in this model (166). However, helminth secretions differ from the relatively inert BSA as they contain immunomodulatory molecules and PAMPS. Interestingly NES, the secreted products of *N. brasiliensis,* while being able to block alum-induced AHR to OVA, simultaneously induce allergic responses to its own components when airway challenged with NES (418). Therefore, it may be possible that ES itself will drive allergic sensitisation to its own antigens, if mice are challenged with ES by administration to the airways.

One possible mechanism that would explain the efficacy of ES may be the degradation of allergen by cathepsins contained within ES to sizes too small for antigen presentation. However, evidence from studies with *Ascaris* spp body fluid have suggested that helminths do not interfere with antigen presentation. *Ascaris* spp body fluid did not affect the number or T cells activated by allergen, but rather limited the proliferative capacity of these activated T cells (435).

The interaction of innate cells during sensitisation may be of importance. The present study has shown that infection with *F. hepatica* was associated with the alternative activation of macrophages. Moreover, an antioxidant derived from ES has been shown to induce alternative activation (320). M2 macrophages may inhibit Th2 cells that mediate allergy through their expression of Arg1 (436). Furthermore, the filarial cystaitn AV17 from *A. vitae* also inhibited allergic pathology when administered at time of allergen sensitisation or prior to challenge in a macrophage (and IL-10) dependent manner (437). Ironically, eosinophils, the major mediator of allergic inflammation may play a role in suppressing the generation of Th2

cells. The present study has shown that eosinophils, derived from *F. hepatica*-infected or ES-administered mice can act as APC. It is therefore possible that eosinophils attracted by ES modulate the generation of alum-induced Th2 responses to OVA. Depletion of eosinophils, by the neutralisation of IL-5, prior to sensitisation would determine any immunomodulatory activity of eosinophils in this model.

Administration of ES at sensitisation was sufficient to reduce inflammation of the airways as confirmed by a reduction in eosinophils and lymphocytes in the BAL of challenged mice, but also by the reduction in histological transformations associated with allergy, particularly mucus production and bronchial epithelial cell hyperplasia. However, in contrast to these findings, ES failed to affect the production of IgE and IgG1, antibodies associated with Th2 responses. Indeed, administration of ES enhanced production of allergen specific IgE; suggesting that type-2 responses are not completely ablated by administration of ES. It also raises the possibility that ES does not completely block AHR, thus, it would be prudent to examine airway resistance using metacholine and a plethysmograph. Interestingly, ES-treated animals have greatly elevated polyclonal (total) IgE titres compared with untreated animals after airway challenge. This underscores a misconception about helminth induced protection from allergy. Protection often appears to be independent of antibody production, including IgE, the archetypal "allergic mediator". Helminth infections or administration of helminth products can stimulate a type 2 antibody response (IgG1 and IgE) to both the parasite and to bystander antigens, including allergen. Elevated polyclonal IgE is symptomatic of most helminth infections and has been proposed as a mechanism of protection from allergy, with parasite-specific IgE out-competing allergen-specific IgE for binding sites on mast cell IgE receptors (420).

In some cases, the inhibition of AHR by helminths or their products is associated with a reduction in antibody production. *N. brasiliensis* NES, when administered at sensitisation, inhibits the production of allergen-specific IgG1 and IgE (418). However, other helminths do not appear to affect allergen-specific antibody. Wilson et al demonstrated that while infection with *H. polygyrus* is sufficient to protect mice from AHR, it does not decrease the production of allergen-specific IgG1 and total IgE. In fact, in agreement with the present study, the expression of these two antibody isotypes were enhanced in infected mice (206). In that study, transfer of Treg cells from infected mice conferred protection to recipients,

independent of a change in antibody production; the authors concluded that increased polyclonal antibody, while it might have a diluting effect on allergen-induced mast cell activation, did not mediate protection.

While antibodies might not have any effect on disease outcome, B cells have been implicated in several studies as mediators of suppression in allergy. Mangan et al showed that *S. mansoni* infection protects mice from an experimental model of fatal systemic anaphylaxis (438). In that model, IL-10 and B cells conferred protection; moreover, transfer of B cells that had been modulated by worms *in vitro* conferred resistance to anaphylaxis (438). Furthermore, a study by Wilson et al demonstrated that transfer of $CD23^{high}$ B cells from allergen-naïve mice infected with *H. polygyrus* also transferred protection from AHR in recipients in a similar manner to Treg cell. This suggests that these B cells act in an antigen-unspecific manner (through bystander suppression) to regulate various types of T cell responses and not via the production of antibodies (310). The role of B cells in mediating *F. Hepatica* ES-induced protection from allergy has not examined and warrants investigation.

The previous chapter has shown that ES suppressed the development of EAE and that the components of ES which conferred protection were over 3 kDa. In addition, ES induced Th2 responses to an unrelated antigen *in vivo*, this effect was heat labile suggesting that the Th2-inducing/protective component was also contained within this higher weight fraction (ES-H). The lower molecular weight fraction was shown to induce the production of TGF-β from innate cells and suppressed the proliferation of T cells *in vitro* (A M Stefanska, personal communication). Thus, it was initially postulated that the lower molecular weight fraction of ES would contain the protective component in AHR. Indeed, NES from *N. brasiliensis* inhibited the induction of AHR when administered at sensitisation, which was unaffected by treatment with heat or proteinase K, suggesting that the protective component is not proteinaceous (418). In contrast, the present study demonstrated that the protective component of ES was contained within the higher weight fraction, suggesting that it is the same molecule which is protective in EAE.

The present study has also shown that vaccination of mice with ES and OVA, (without alum) was sufficient to drive a Th2 response *in vivo*. This observation is difficult to reconcile in view of the fact that ES blocks alum-induced Th2 differentiation. This is not explained by the fact

that helminths protect from EAE via induction of Treg cells, as ES does not induce the expansion of Treg cells in naïve mice. Moreover, ES-mediated protection from AHR is not associated with the induction of Treg cell responses and transfer of T cells does not confer protection.

The apparent contradiction that ES drives Th2 cells (and not Treg) while also inhibiting alum induced Th2 responses (and thus AHR) may be explained by the fact that Th2 responses induced by ES or alum, are mediated by different mechanisms. Helminths primarily induce Th2 responses via the production of IL-4 or IL-13, which act on T cells to induce STAT6-dependent expression of GATA3. The enhancement of Th2 responses by alum is quite different. Few if any reports have investigated the specific interaction of alum and helminth products. This is a major caveat considering that most reports on the effect of helminths on AHR have shown that infection or administration of helminth products only affect AHR at sensitisation and thus, the effect being tested is not specifically allergic responses, but rather the alum-mediated induction of Th2 cells that drive allergy.

Despite being one of the most widely used adjuvants in vaccine preparations, the mechanisms of action of alum as an adjuvant is still unclear. Charles Janeway went as far as calling this the immunologist's "dirty little secret". (1, 427). Historically alum was considered to act as an antigen depot, absorbing and sequestering antigen at injection site and allowing it to be taken up as particles suitable for phagocytosis (18). Alum induces type-2 responses, characterised by eosinophilia and production of IL-4, IgE and IgG1, but sensitisation is independent of IL-4 or IL-13 signalling (56). Indeed, IL-1 was later found to be more important for allergen-specific Th2 cell activation and the development of airway hypersensitivity response (415). This suggested a role for the inflammasome. It was later reported that alum could activate the NLRP3 inflammasome (57, 425, 426, 439). Uptake of alum was shown to inflict cell damage via liposomal rupture and production of ROS (439). Cell death associated with alum was subsequently shown to release uric acid, which acted as the ligand for the NLRP3 inflammasome (57).

However, these results are controversial and it is still not clear whether NLRP3 plays a role in alum-induced adjuvanticity and induction of type 2 immunity *in vivo*. Whereas some studies found that NLRP3, ASC, and caspase-1 were required for alum-induced adjuvanticity (440),

others failed to find a role for NLRP3 in the adjuvant activity of alum (441), suggesting the IL-1 produced via inflammasome activation is dispensable for the generation of Th2 cells. Indeed two recent reports have supported a NLRP3-independent role for alum-induced adjuvanticity via induction of cell damage, with alum promoting the deposition of uric acid crystals that act on DC to release PGE_2. This PGE_2 was indispensable for the generation of Th2 cells in response to alum (428, 442).

The present study demonstrates that at least *in vitro*, ES does not block alum or MSU mediated production of IL-1β or PGE_2, suggesting an alternative mechanism is at play. Preliminary experiments suggested that that ES does not inhibit alum-mediated responses via its ability to degrade uric acid crystals with uricase. Thus, the most likely candidate remains the effect of ES on APC; while ES does not modulate the molecular response to alum, perhaps it inhibits alum induced APC maturation and function.

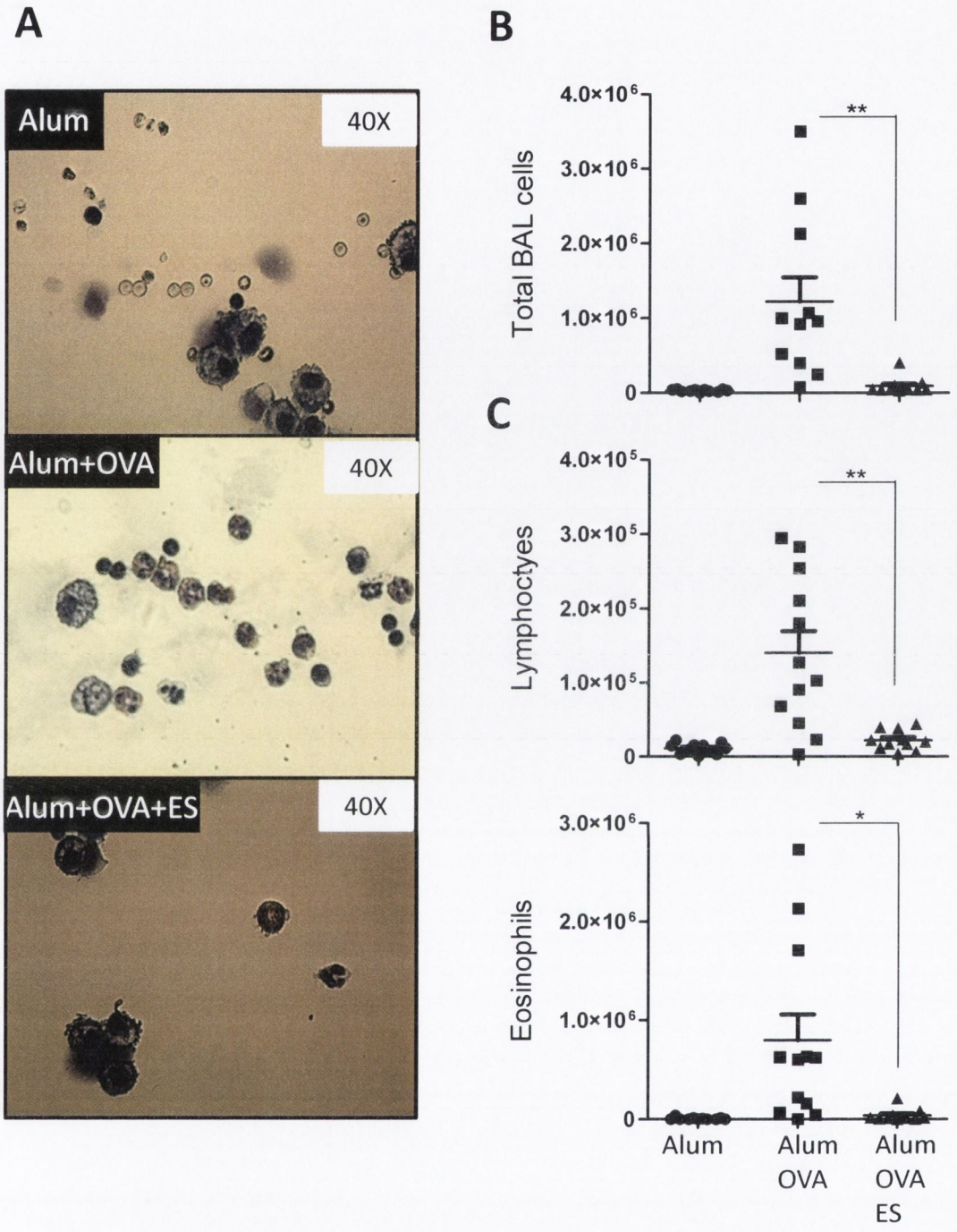


Fig 6.1 Co-administration of ES at sensitisation prevents allergic inflammatory infiltration of cells into the alveolar spaces of the lung. BALB/c mice were sensitized with either alum, alum+OVA or alum+OVA+ES and intra-tracheally challenged with OVA as in Fig. 2.4. On day 28 BALs were conducted on sacrificed mice and cytospin were prepared from isolated bronchial/alveolar cells. A, Photomicrographs of cytospin preparations of BAL; representative of at least 3 separate experiments with 4 mice per group. B, Combined cell count isolated from BAL of mice from 3 experiments with 4 mice per group. C, Combined lymphocytic and eosinophil cell counts from 3 separate experiments determined from flow cytometric staining. n=13. Data presented are means +/- SEM. Statistical assessment was performed by unpaired Student t-test. *$p<0.05$ **$p<0.01$.

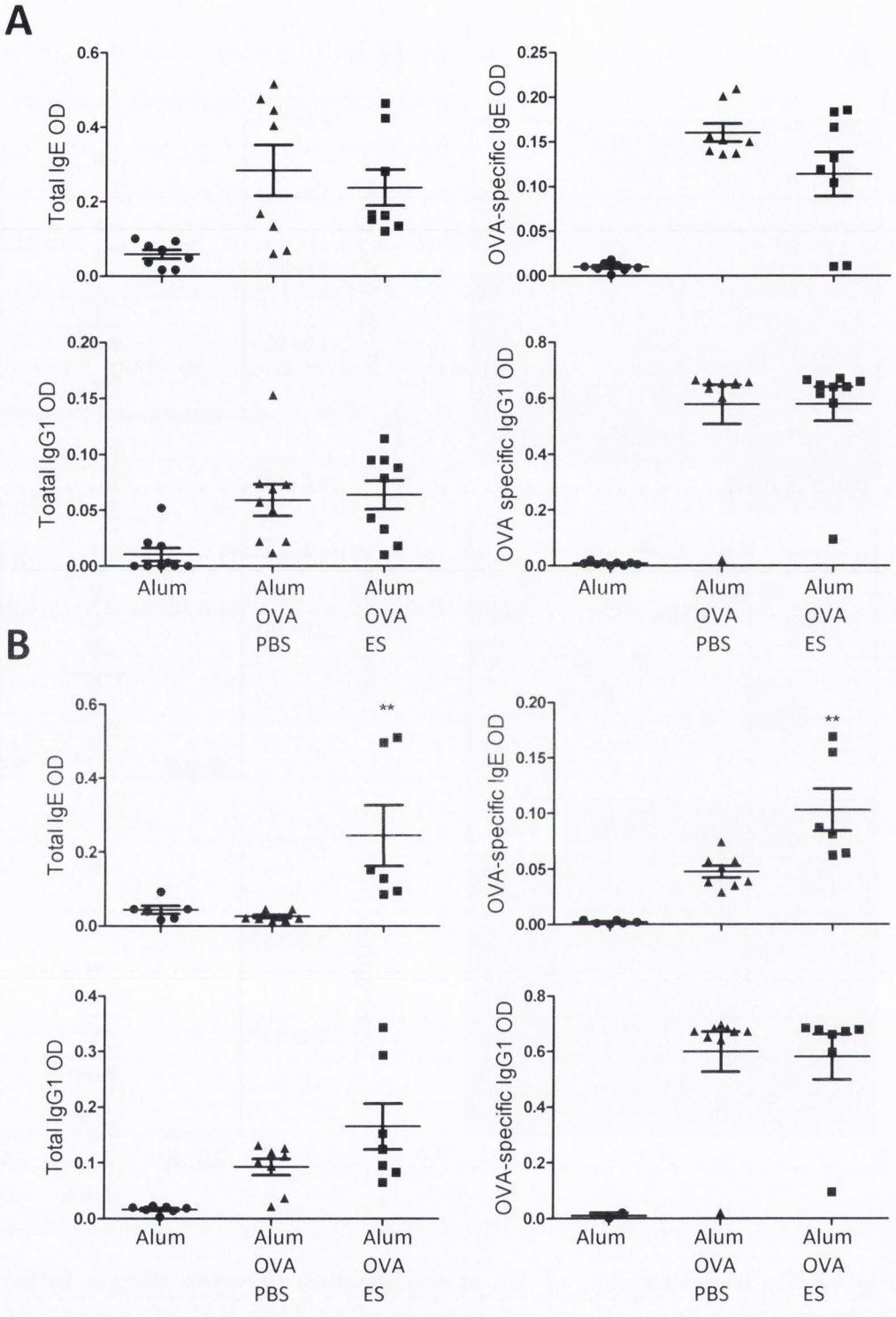


Fig 6.2 Co-administration of ES at sensitisation does not affect IgE/IgG$_1$ antibody production. BALB/c mice were sensitized with either alum, alum+OVA or alum+OVA+ES and intra-tracheally challenged with OVA as in Fig. 2.4. On day 28 the presence total and OVA-specific IgE and IgG$_1$ in a 1/50 dilution of blood serum (A) or neat BAL fluid (B) and was determined by ELISA. Results the combined results of 2 experiments. n=8. Data presented are means +/- SEM. Statistical assessment was performed by an unpaired Student t-test vs mice sensitised with alum alone. **p<0.01.

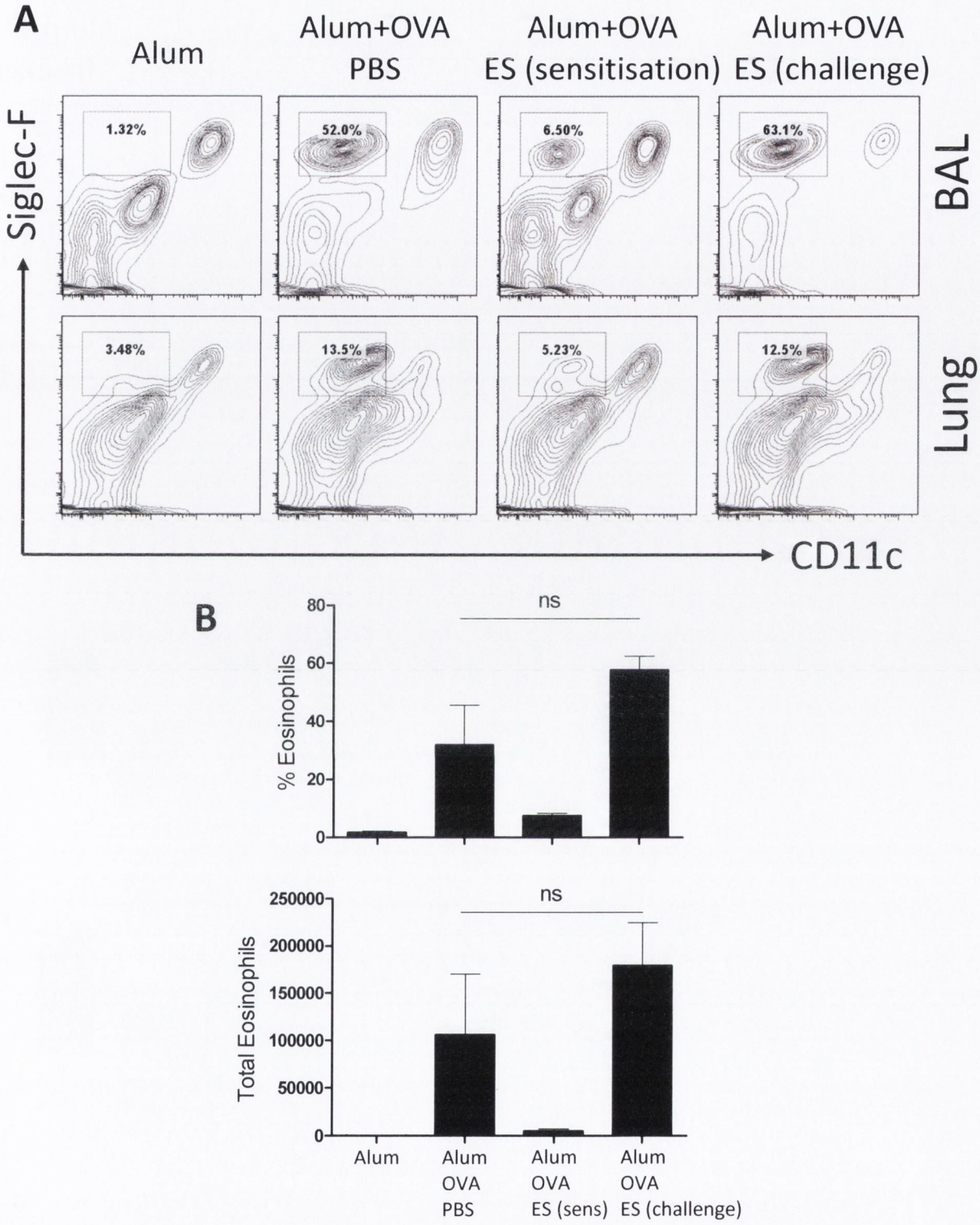


Fig 6.3 Co-administration of ES at sensitisation reduces eosinophilic infiltration into the lungs of challenged mice but ES treatment at challenge fails to prevent disease. BALB/C mice were immunized and challenged as described in Fig 2.4. On days 24-27 mice were treated with a daily i.p. administration of either PBS or ES. On day 28 BALs were conducted on sacrificed mice and proportion and number infiltrating eosinophils were determined by flow cytometry staining of live siglec-F^+ $CD11c^-$ $CD11b^+$ cells (A), and differential cell counts (B). n=4. Data presented are means +/- SEM. Statistical assessment was performed by an unpaired Student t-test vs mice sensitised with alum alone.

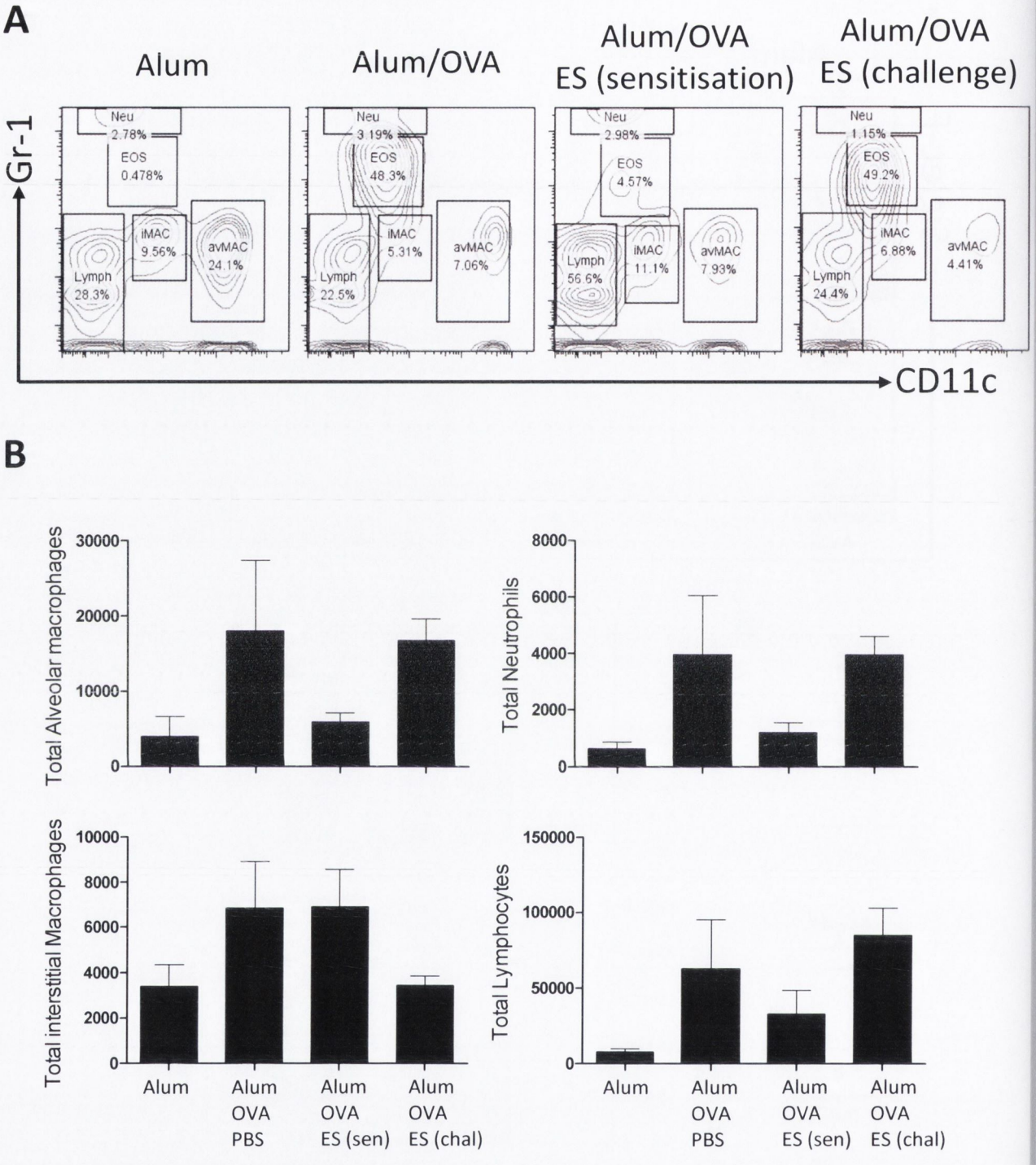


Fig 6.4 Co-administration of ES at sensitisation reduces the frequency of neutrophils, lymphocytes and alveolar macrophages in the BAL of challenged mice. BALB/C mice were immunized and challenged as described in Fig 2.4. On days 24-27 mice were treated with a daily i.p. administration of either PBS or ES. A, On day 28 the percentage and total number of $CD11b^{+}$ $Ly6G^{+}$ neutrophils, $CD3^{+}$ or $CD19^{+}$ SSC^{low} Fsc^{low} Lymphocytes and $CD11b^{high}CD11c^{-}$ $F4/80^{+}$ interstisial/inflammatory and Siglec F^{+} $CD11b^{low}CD11c^{+}$ alveloar macrophages in BAL of challenged mice was determined by multi-colour flow cytometery. A, representative flow cytometery plots with all 4 populations gated on a plot of Gr-1 vs CD11c. B, Absolute cell numbers. n=4. Data presented are means +/- SEM.

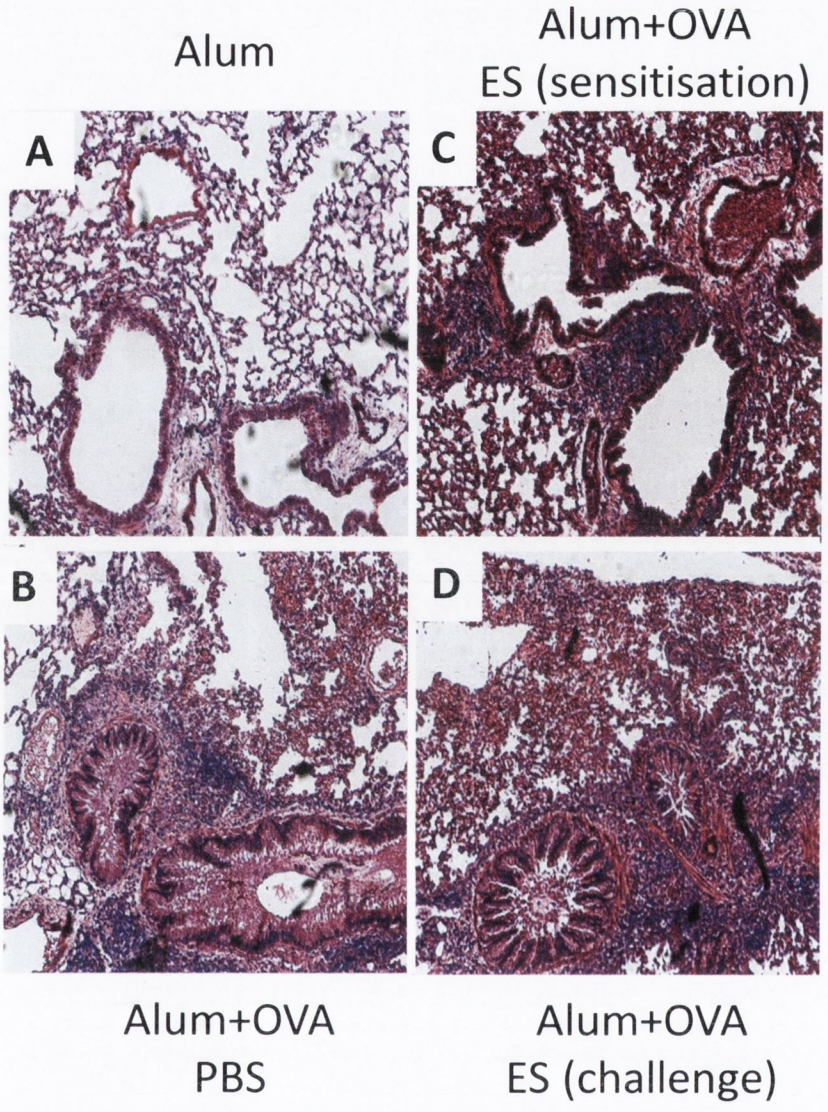


Fig 6.5 A-D. Co-administration of ES at sensitisation but not at challenge inhibits airway inflammation and constriction following challenge. On day 0 and day 14 BALB/c mice were sensitized with either alum (A), alum+OVA or alum+OVA+ES. (C) and intra-tracheally challenged with OVA on days 24-26. On days 24-27 mice that were sensitised to alum+OVA were treated with a daily i.p. administration of either PBS (B) or ES (D). Representative photomicrographs of formalin-fixed, paraffin embedded lung sections stained with haematoxylin and eosin (H&E). Note significantly less luminal and periairway inflammatory cell infiltration in C where mice were given ES at sensitisation. Photomicrographs are representative of 2 separate experiments. Magnification factor x10.

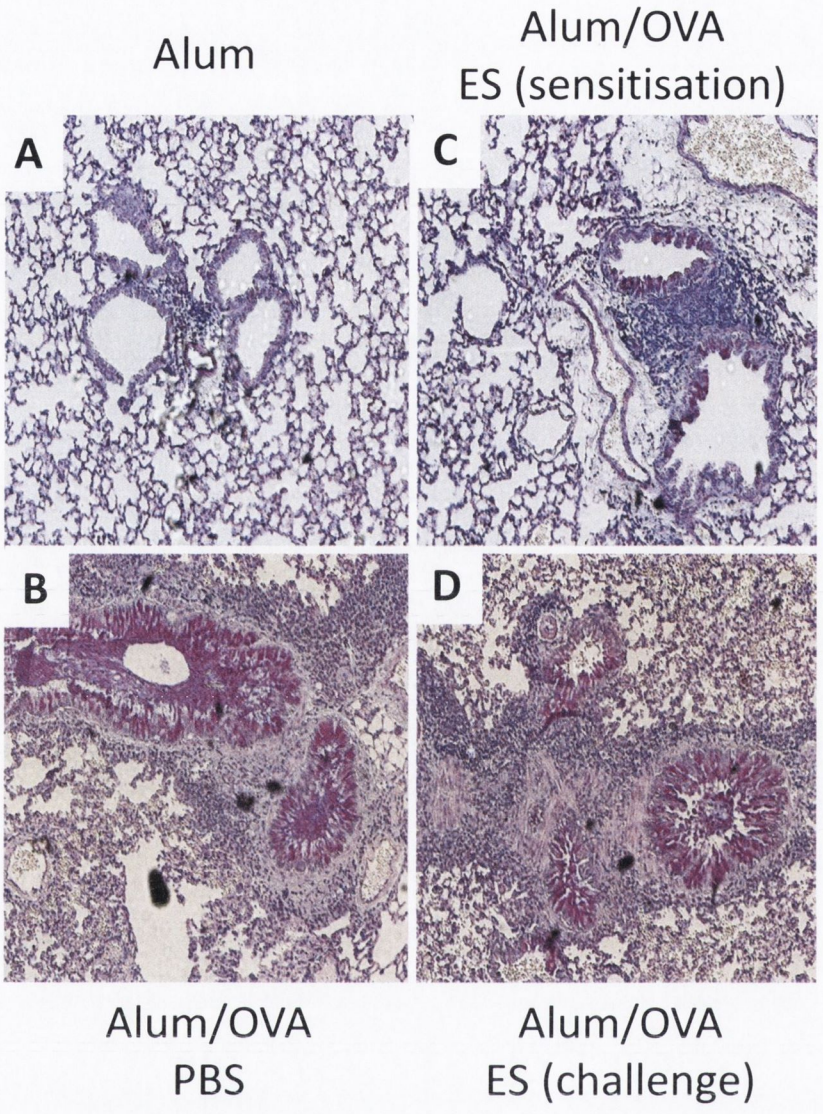


Fig 6.6 A-D. Co-administration of ES at sensitisation but not at challenge inhibits mucus secretion and goblet cell hyperplasia following airway challenge. On day 0 and day 14 BALB/c mice were sensitized with either alum (A), alum+OVA or alum+OVA+ES. (C) and intra-tracheally challenged with OVA on days 24-26. On days 24-27 mice that were sensitised to alum+OVA were treated with a daily i.p. administration of either PBS (B) or ES (D). Representative photomicrographs of formalin-fixed, paraffin embedded lung sections stained with Alican Blue-PAS (mucin and mucin-postivite goblet cells stain pink). Note significantly less luminal mucus and the absence of goblet cell hyperplasia in C where mice were given ES at sensitisation. Photomicrographs are representative of 2 separate experiments. Magnification factor x10.

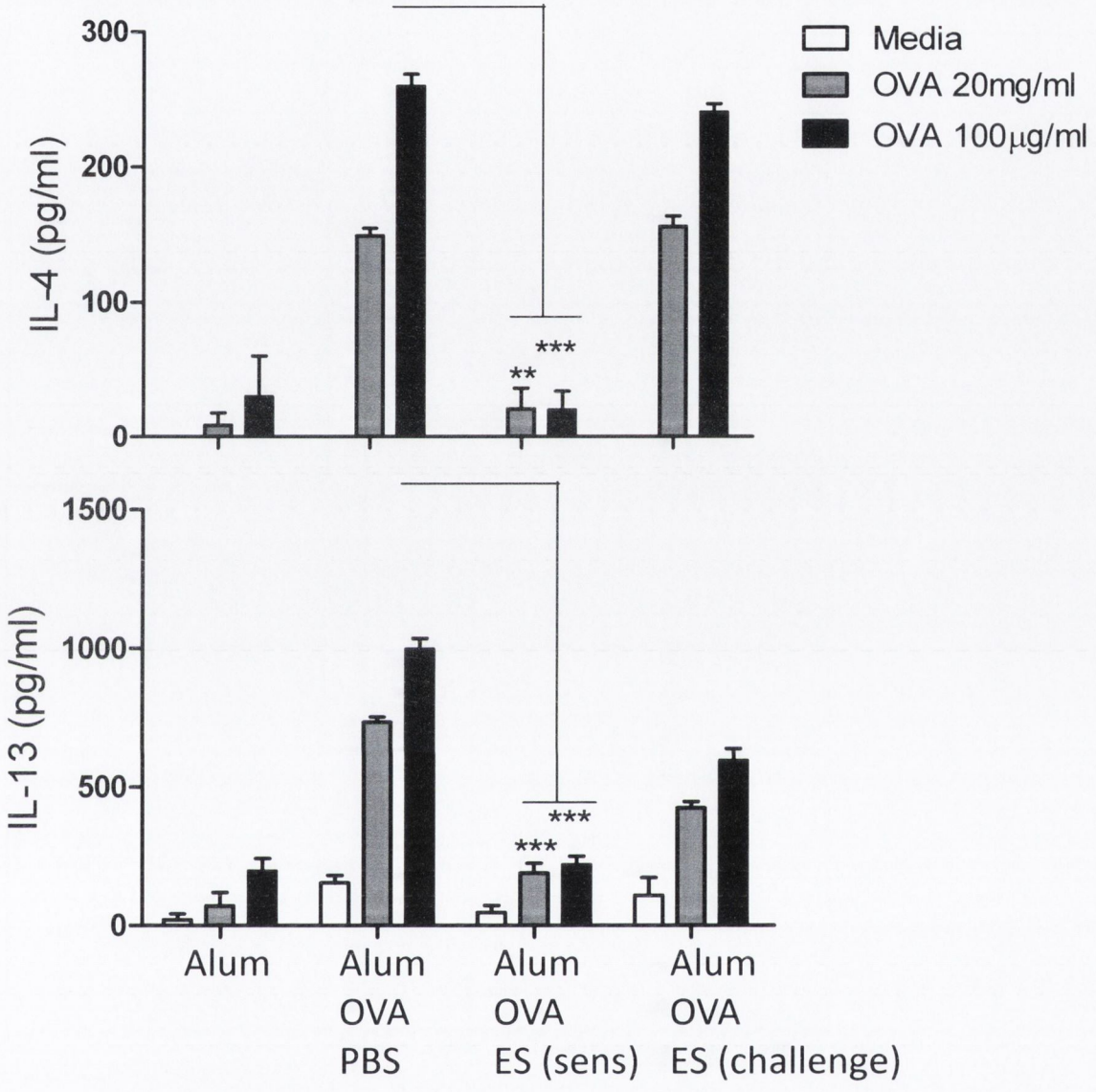


Fig 6.7 Co-administration of ES at sensitisation prevents the accumulation of OVA-specific Th2 cells in the lung. BALB/C mice were immunized and challenged as described in Fig 2.4. On days 24-27 mice were treated with a daily i.p. administration of either PBS or ES. On day 28 lungs of challenged mice were excised and homogenised. Single cell suspensions of homogenised lungs were restimulated *in vitro* with increasing concentrations of OVA. The concentrations of IL-4 and IL-13 in the supernatant after 72h were determined by ELISA. Results are representative of 3 separate experiments. Data presented are means +/- SEM of triplicate assays of lungs pooled from each group. Statistical assessment was performed by unpaired Student t-test vs corresponding concentration of OVA in "Alum/OVA" group. *$p<0.05$ **$p<0.01$.

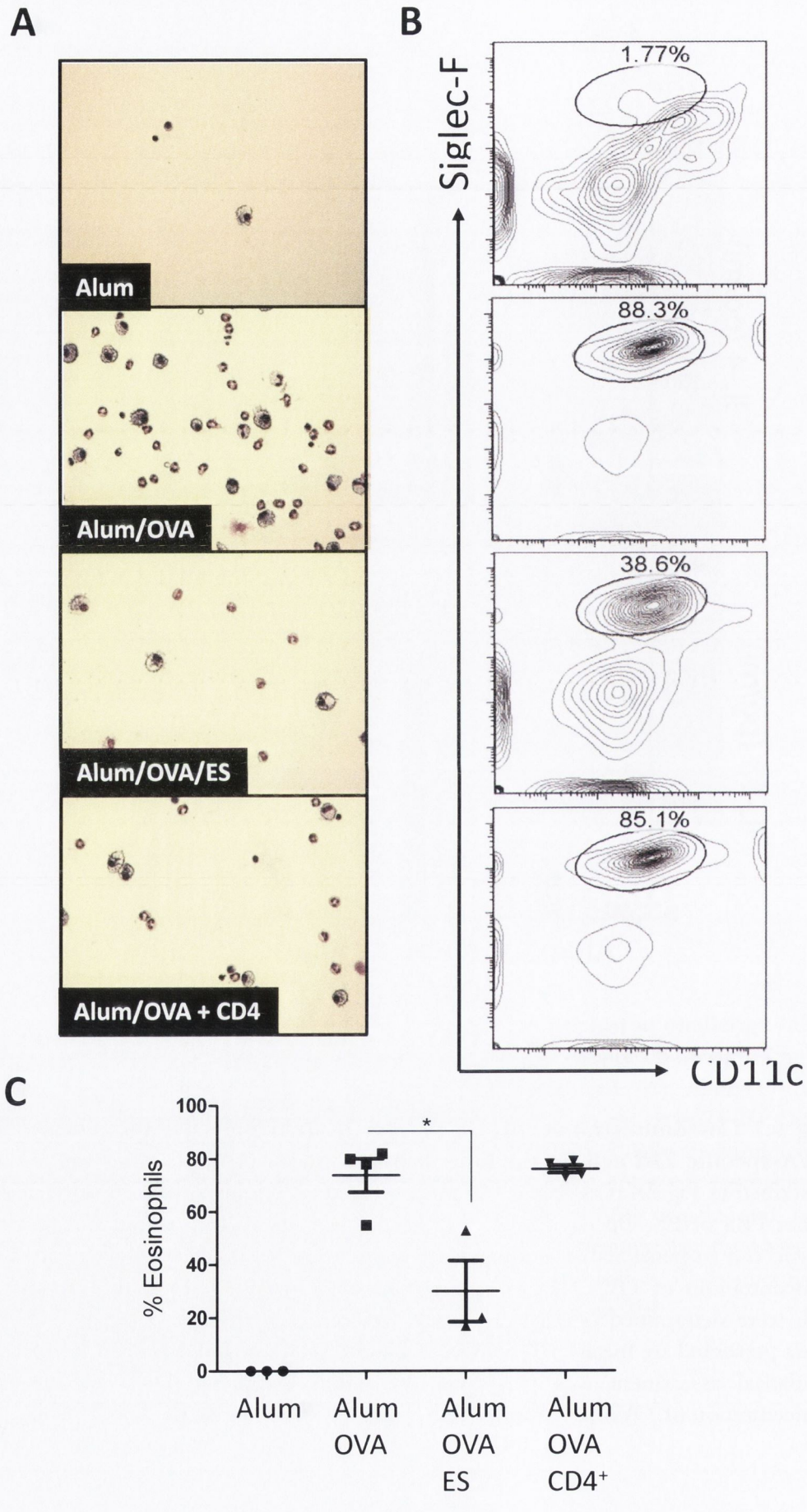

A
Alum
Alum/OVA
Alum/OVA/ES
Alum/OVA + CD4
B
Siglec-F
1.77%
88.3%
38.6%
85.1%
CD11c
C
% Eosinophils
100
80
60
40
20
0
*
Alum
Alum OVA
Alum OVA ES
Alum OVA CD4+

Fig 6.8 Transfer of $CD4^+$ T cells from protected mice fails to prevent eosinophilia in airway hypersensitivity. BALB/C mice were immunized and challenged as described in Fig 2.5. On day 21 $CD4^+$ cells purified from mice immunized with alum+OVA+ES were adoptively transferred by i.p. injection to recipient mice immunized with Alum+OVA. All mice were challenged for 30 min with aerosolised OVA on days 24, 25 and 26. On day 28 BALs were conducted on sacrificed mice and A, H&E stained cytospins were prepared from isolated cells. Displayed photomicrographs are representative of 4 mice per group. The percentage of total counts of eosinophils was determined by multi-colour flow cytometry analysis was performed to determine eosinophilic infiltrate. B, Representative plots show eosinophil gates as siglec F^+ $CD11c^-$ $CD11b^+$ cells. C, percentage eosinophils of total BAL cells with 4 mice per group. Data presented are means +/- SEM Statistical assessment was performed by unpaired Student t-test. *$p<0.05$ **$p<0.01$.

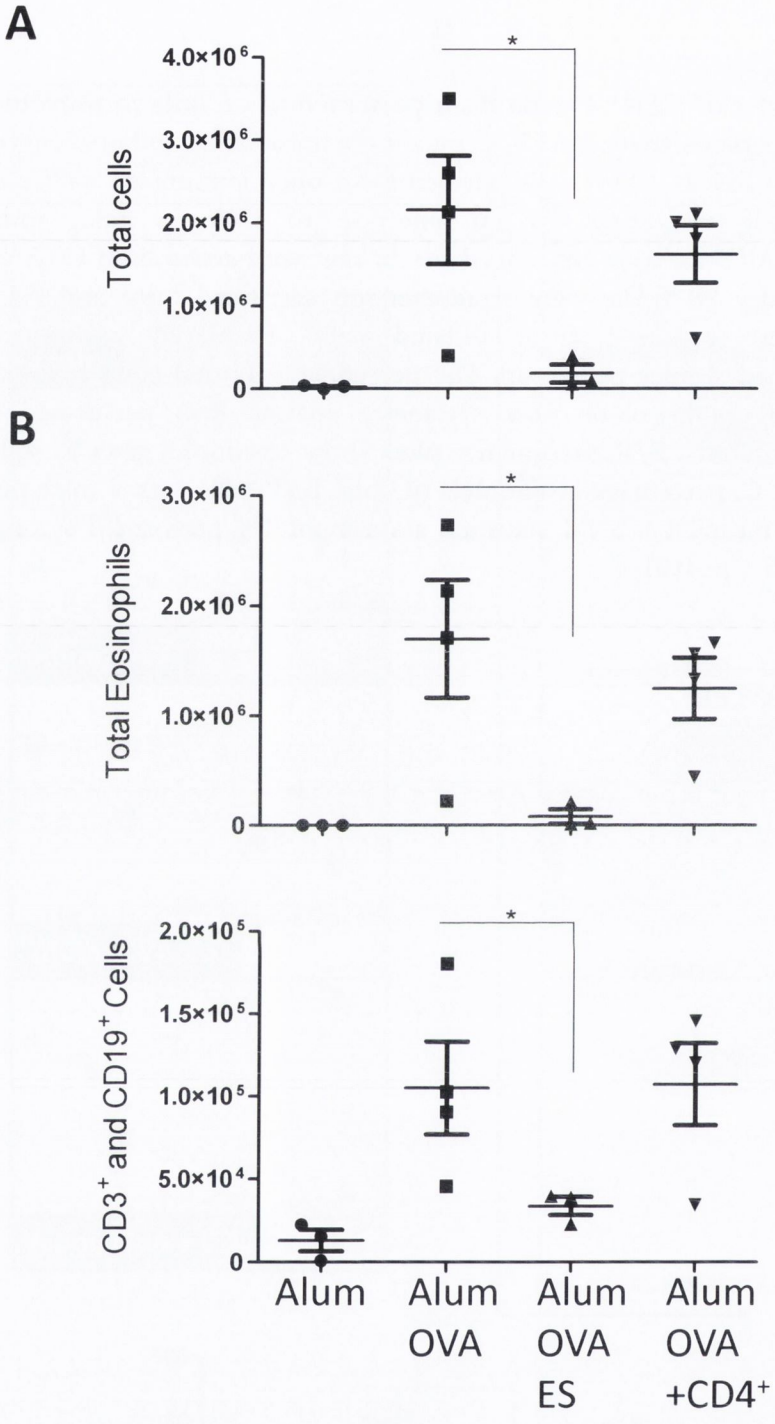


Fig 6.9 Transfer of $CD4^+$ T cells from protected mice fails to prevent infiltration of lymphocytes in airway hypersensitivity. BALB/C mice were immunized and challenged as described in Fig 2.5. On day 21 $CD4^+$ cells purified from mice immunized with alum+OVA+ES were adoptively transferred by i.p. injection to recipient mice immunized with Alum+OVA. All mice were challenged for 30 min with aerosolised OVA on days 24, 25 and 26. A, Total counts of isolated BAL cells. B, Total counts of Singlec-F^+ $CD11c^-$ $CD11b^+$ eosinophils and $CD3^+/CD19^+$ lymphocytes was determined by multi-colour flow cytometry with 4 mice per group. Data presented are means +/- SEM Statistical assessment was performed by unpaired Student t-test. *$p<0.05$ Results for alum, alum+OVA and alum+OVA+ES are representative of 3 separate experiments.

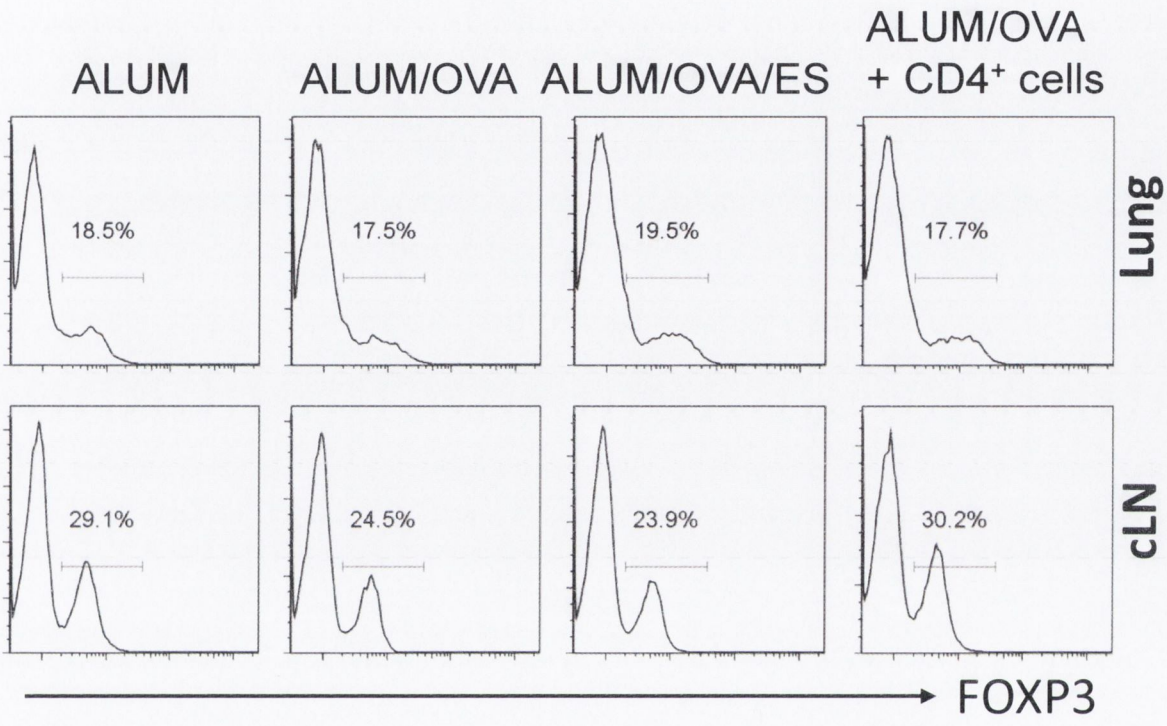
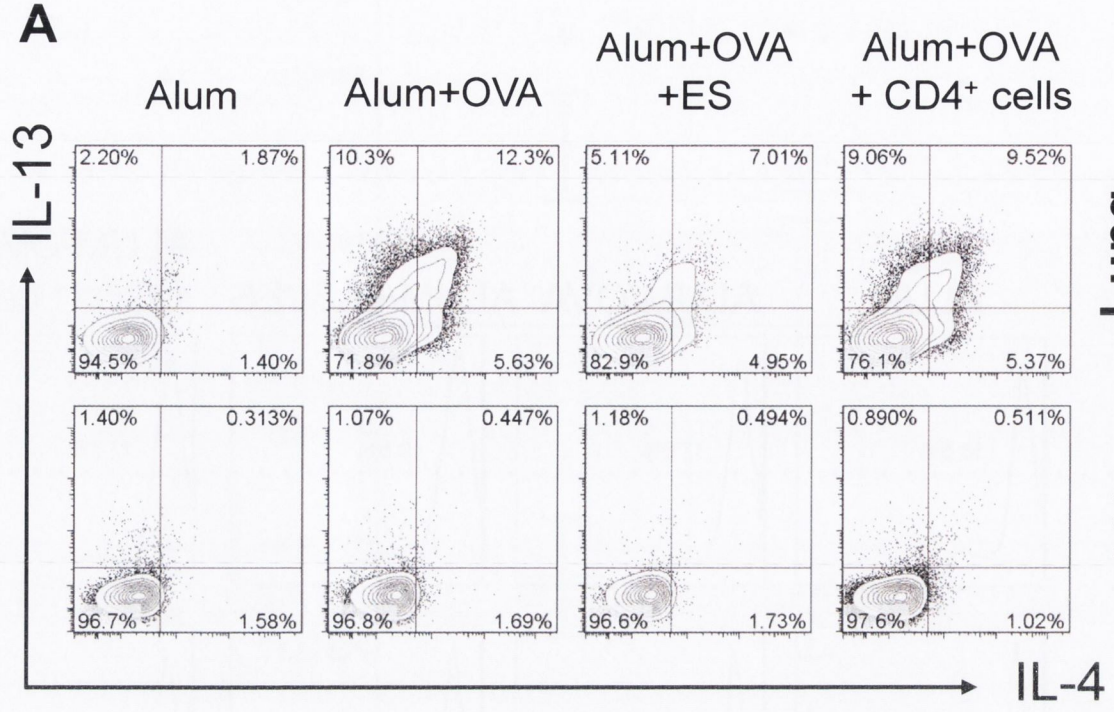


Fig 6.10 Co-administration of ES at allergen sensitisation or transfer of $CD4^+$ T cells at challenge does not affect the frequency of $FOXP3^+$ $CD4^+$ T cells in the lungs and draining LN of challenged mice BALB/C mice were immunized and challenged as described in Fig 2.5. On day 21 $CD4^+$ cells purified from mice immunized with alum+OVA+ES were adoptively transferred by i.p. injection to recipient mice immunized with Alum+OVA. All mice were challenged for 30 min with aerosolised OVA on days 24, 25 and 26. On day 28 lungs and cervical (cLN) lymph nodes were harvested and pooled. The expression of FOXP3 in $CD4^+$ from lung and cLN was determined by flow cytometry.

A



B

	Alum	Alum+ OVA	Alum+ OVA+ES	+ CD4+ T cells
$CD4^+$	1,293	13,7054	8820	10,2781
$IL\text{-}4^+ CD4^+$	42	24,573	1054	15,304
$IL\text{-}13^+ CD4^+$	52	30,974	1068	19,096

Fig 6.11 Co-administration of ES at sensitisation reduces accumulation of Th2 cells in the lungs of challenged mice. BALB/C mice were immunized and challenged as described in Fig 2.5. On day 21 $CD4^+$ cells purified from mice immunized with alum+OVA+ES were adoptively transferred by i.p. injection to recipient mice immunized with Alum+OVA. All mice were challenged for 30 min with aerosolised OVA on days 24, 25 and 26. On day 28 lungs and cervical (cLN) lymph nodes were harvested and digested. Single cell suspensions of pooled homogenised organs were stimulated with PMA and ION in the presence of BFA. A, the expression of IL-4 and IL-13 in $CD3^+$ $CD4^+$ T cells was determined by intracellular flow cytometry. Results for alum, alum+OVA and alum+OVA+ES are representative of 3 separate experiments. B, total counts of $CD3^+$ $CD4^+$; $CD3^+$ $CD4^+$ IL-4 and $CD3^+$ $CD4^+$ $IL\text{-}13^+$ cells.

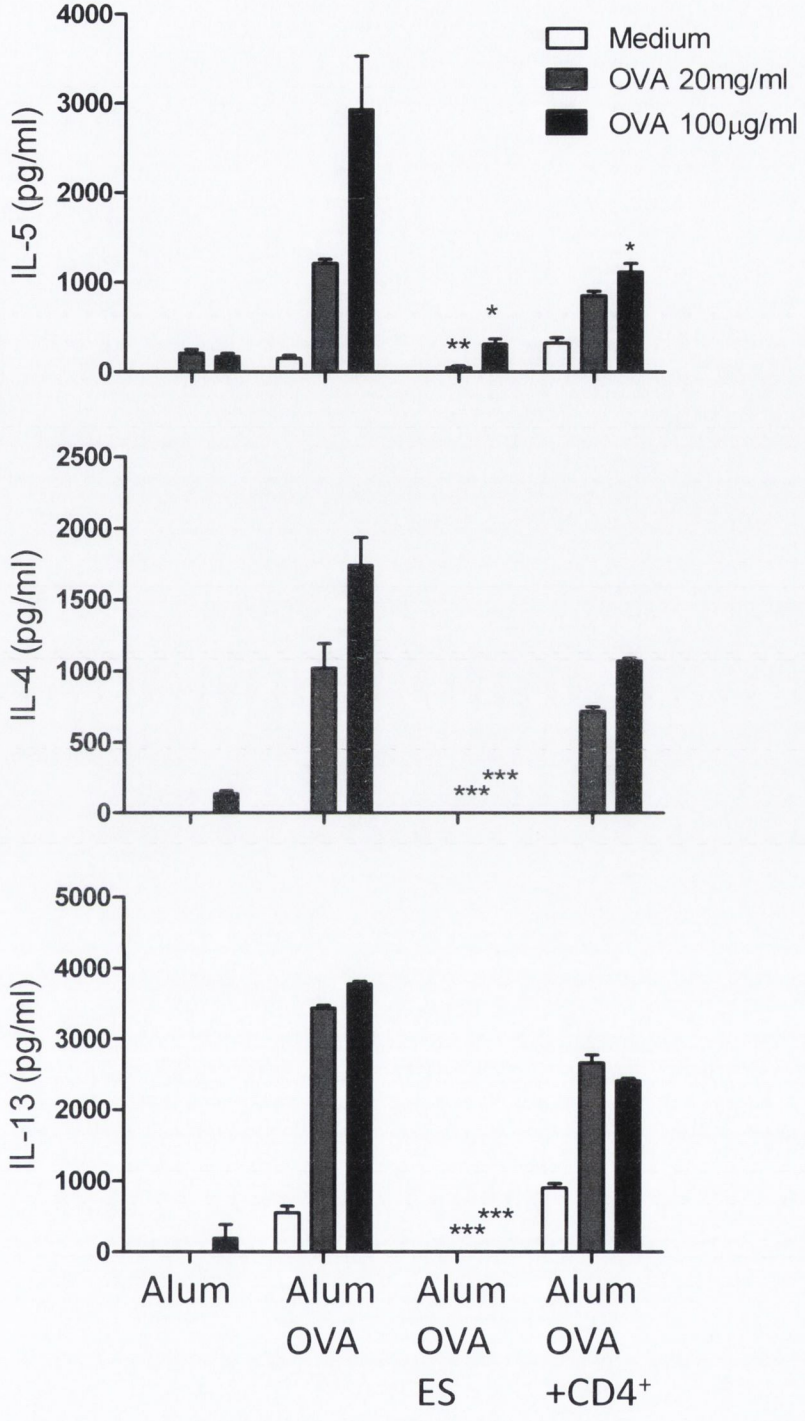


Fig 6.12 Co-administration of ES at sensitisation reduces accumulation of Th2 cells in the lungs of challenged mice BALB/C mice were immunized and challenged as described in Fig 2.5. On day 21 $CD4^+$ cells purified from mice immunized with alum+OVA+ES were adoptively transferred by i.p. injection to recipient mice immunized with Alum+OVA. All mice were challenged for 30 min with aerosolised OVA on days 24, 25 and 26. On day 28 lungs of challenged mice were harvested, pooled and restimulated *in vitro* with increasing concentrations of OVA or left unstimulated. The concentration of IL-4 and IL-13 was tested after 72h by ELISA. Data presented are means +/- SEM. Results for alum, alum+OVA and alum+OVA+ES are representative of 3 separate experiments. Statistical assessment was performed by unpaired Student t-test vs corresponding concentration of OVA in "Alum/OVA" group. *$p<0.05$, **$p<0.01$, ***$p<0.001$

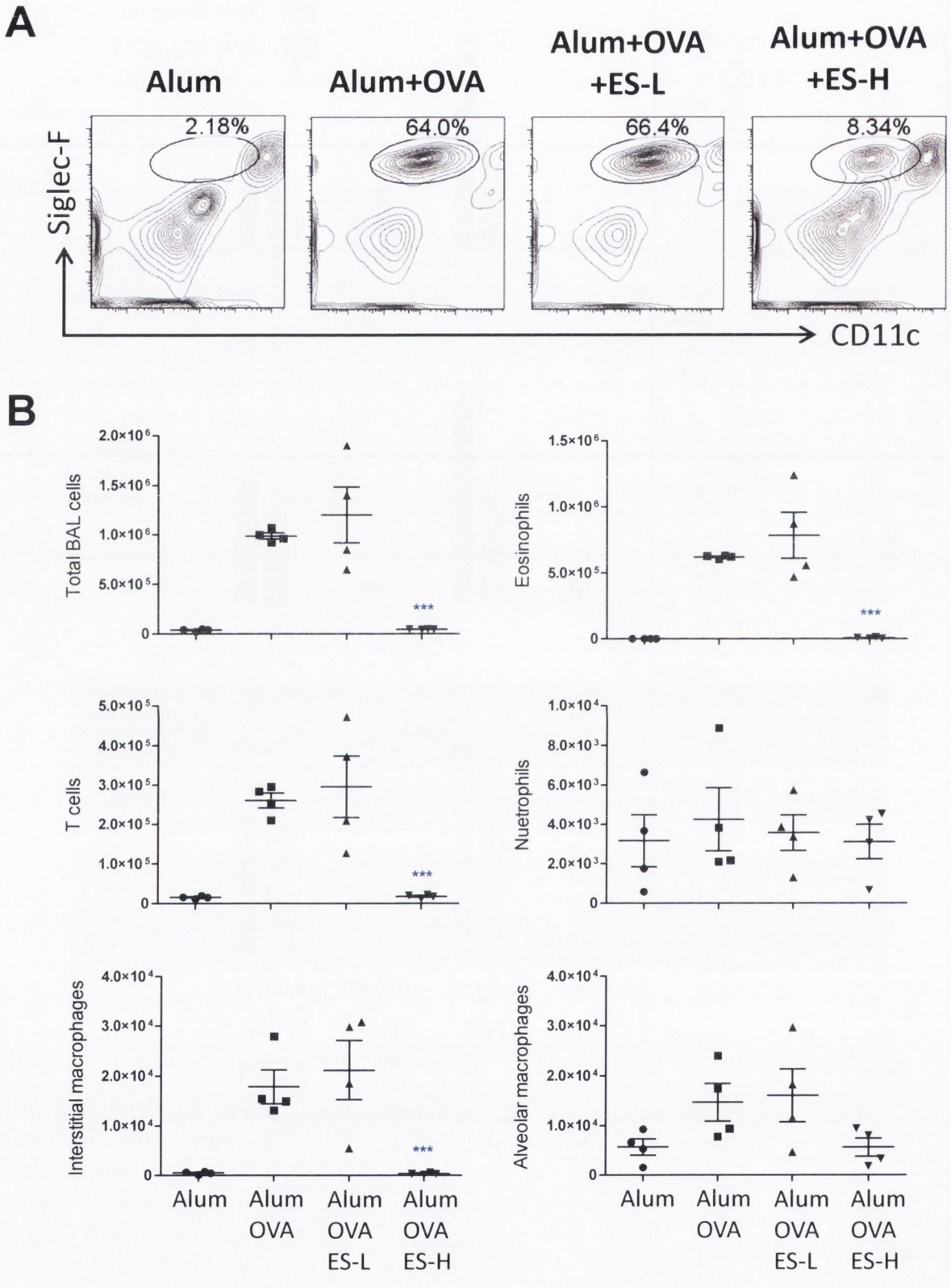

A
Alum
Alum+OVA
Alum+OVA +ES-L
Alum+OVA +ES-H
2.18%
64.0%
66.4%
8.34%
Siglec-F
CD11c
B
Total BAL cells
Eosinophils
T cells
Nuetrophils
Interstitial macrophages
Alveolar macrophages
***
Alum
Alum OVA
Alum OVA ES-L
Alum OVA ES-H

Fig 6.13 Protective component(s) is contained within the large molecular weight fraction of ES. BALB/c mice were immunized as in Fig 2.4 using either ES-L or ES-H. Mice were challenged for 30 min with aerosolised ovalbumin on days 24, 25 and 26. On day 28 BALs were conducted on challenged mice. Eosinophilic infiltrate was quantified by flow cytometery. A, representative plots of siglec F^+ $CD11c^-$ $CD11b^+$ eosinophils. B, Counts of total BAL cells; siglec F^+ $CD11c^-$ $CD11b^+$ Eosinophils, $CD3^+$ T cells, $Ly6G^+$ $CD11b^+$ nuetrophils, $CD11b^+$ $F4/80^+$ $CD11c^-$ interstitial/inflammatory macrophages and $CD11b^{lo}$ CD11c+ Siglec-F^+ $F4/80^+$ aveolar macrophages. Data presented are means +/- SEM. n=4. Results are representative of 2 separate experiments. Statistical assessment was performed by unpaired Student t-test vs "Alum+OVA" mice. ***$p<0.001$

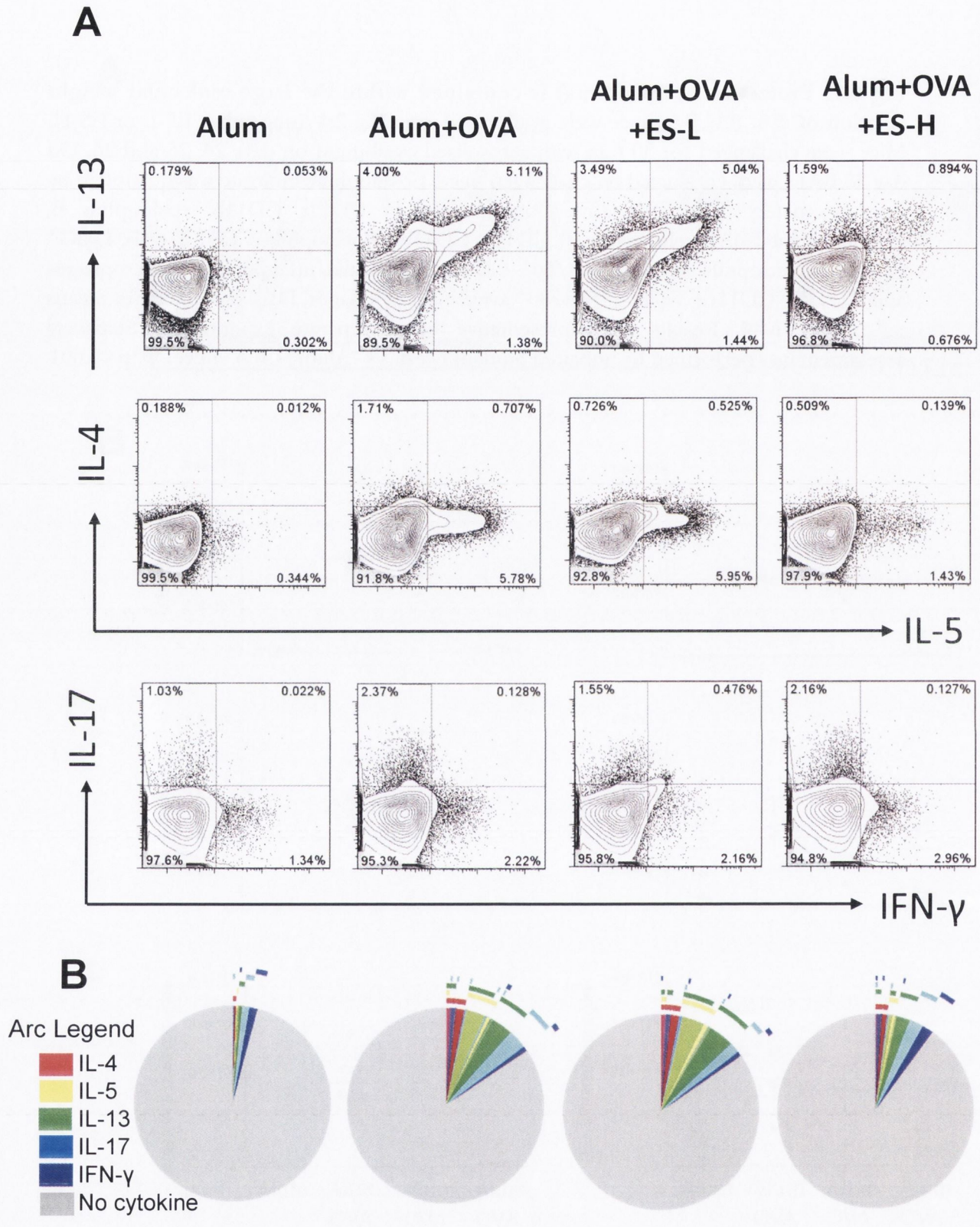

A
Alum
Alum+OVA
Alum+OVA +ES-L
Alum+OVA +ES-H
IL-13
IL-4
IL-5
IL-17
IFN-γ
0.179%
0.053%
99.5%
0.302%
4.00%
5.11%
89.5%
1.38%
3.49%
5.04%
90.0%
1.44%
1.59%
0.894%
96.8%
0.676%
0.188%
0.012%
99.5%
0.344%
1.71%
0.707%
91.8%
5.78%
0.726%
0.525%
92.8%
5.95%
0.509%
0.139%
97.9%
1.43%
1.03%
0.022%
97.6%
1.34%
2.37%
0.128%
95.3%
2.22%
1.55%
0.476%
95.8%
2.16%
2.16%
0.127%
94.8%
2.96%
B
Arc Legend
IL-4
IL-5
IL-13
IL-17
IFN-γ
No cytokine

Fig 6.14 The administration of the large molecular weight fraction of ES at sensitisation inhibits differentiation for polyfunctional Th2 cells. BALB/c mice were immunized as in Fig 2.4 using either ES-L or ES-H. Mice were challenged for 30 min with aerosolised ovalbumin on days 24, 25 and 26. On day 28 whole lungs were harvested and stimulated with PMA and ION in the presence of BFA. $CD4^+$ T cells were analysed by flow cytometery for intracellular experession of IL-4 IL-5, IL-13 IL-17 and IFN-γ. A, representative flow cytometric plots of expression of IL-4, IL-5, IL-13, IL-17 and IFN-γ gated on live $CD3^+$ $CD4^+$ T cells. B, SPICE analysis of cytokine expression. Polyfunctional T-cell populations are plotted according to the overlapping pie arcs; the different coloured arcs representing the percentage of cells secreting each of the individual 5 cytokines.

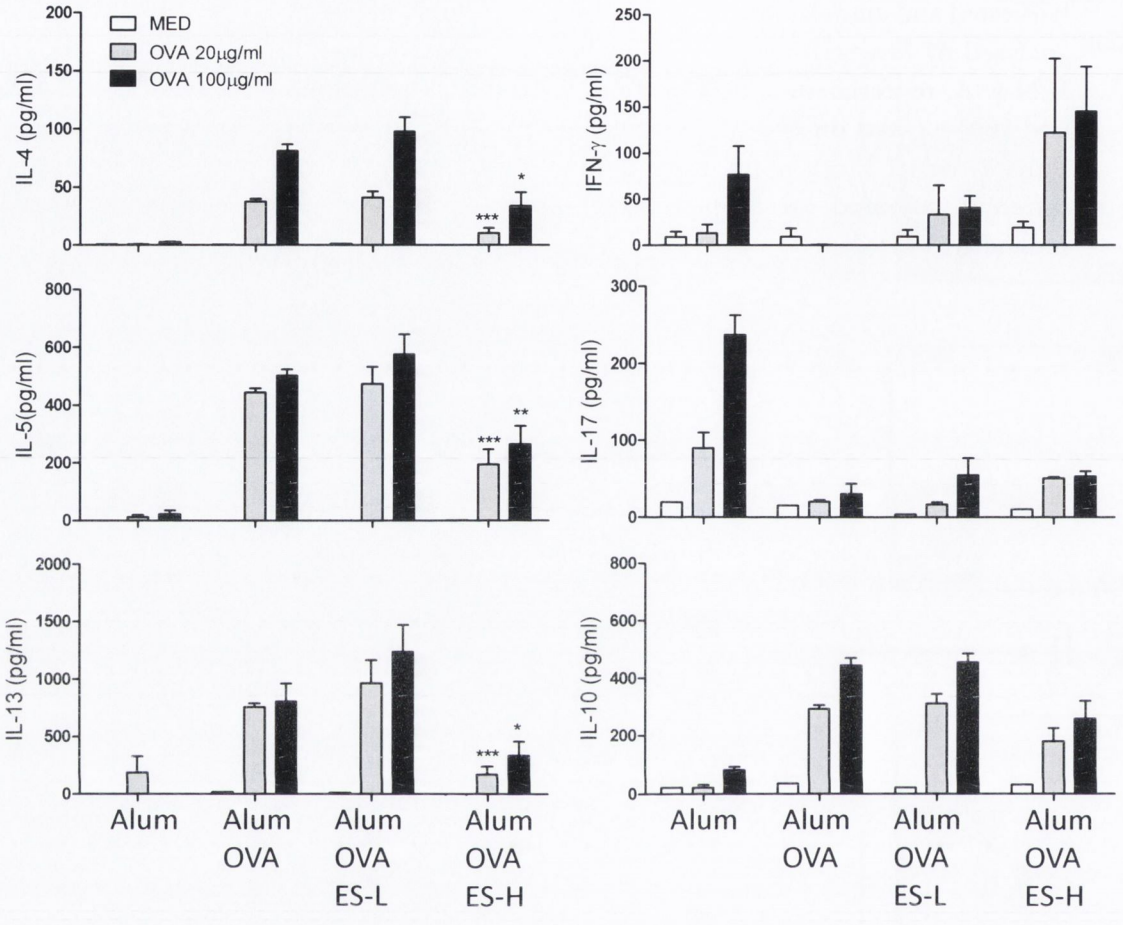


Fig 6.15 The administration of the large molecular weight fraction of ES suppresses the differentiation of Th2 cells in the lungs of challenged mice. BALB/c mice were immunized as in Fig 2.4 using either ES-L or ES-H. Mice were challenged for 30 min with aerosolised ovalbumin on days 24, 25 and 26. On day 28 lungs of challenged mice were harvested, pooled and restimulated *in vitro* with increasing concentrations of OVA or left unstimulated. The concentration of IL-4, IL-5, IL-13, IFN-γ and IL-17 was tested after 72h by ELISA. Data presented are means +/- SEM of triplicate assays. Results are representative of 2 separate experiments. Statistical assessment was performed by unpaired Student t-test vs corresponding concentration of OVA in "Alum/OVA" group. *$p<0.05$ ***$p<0.001$.

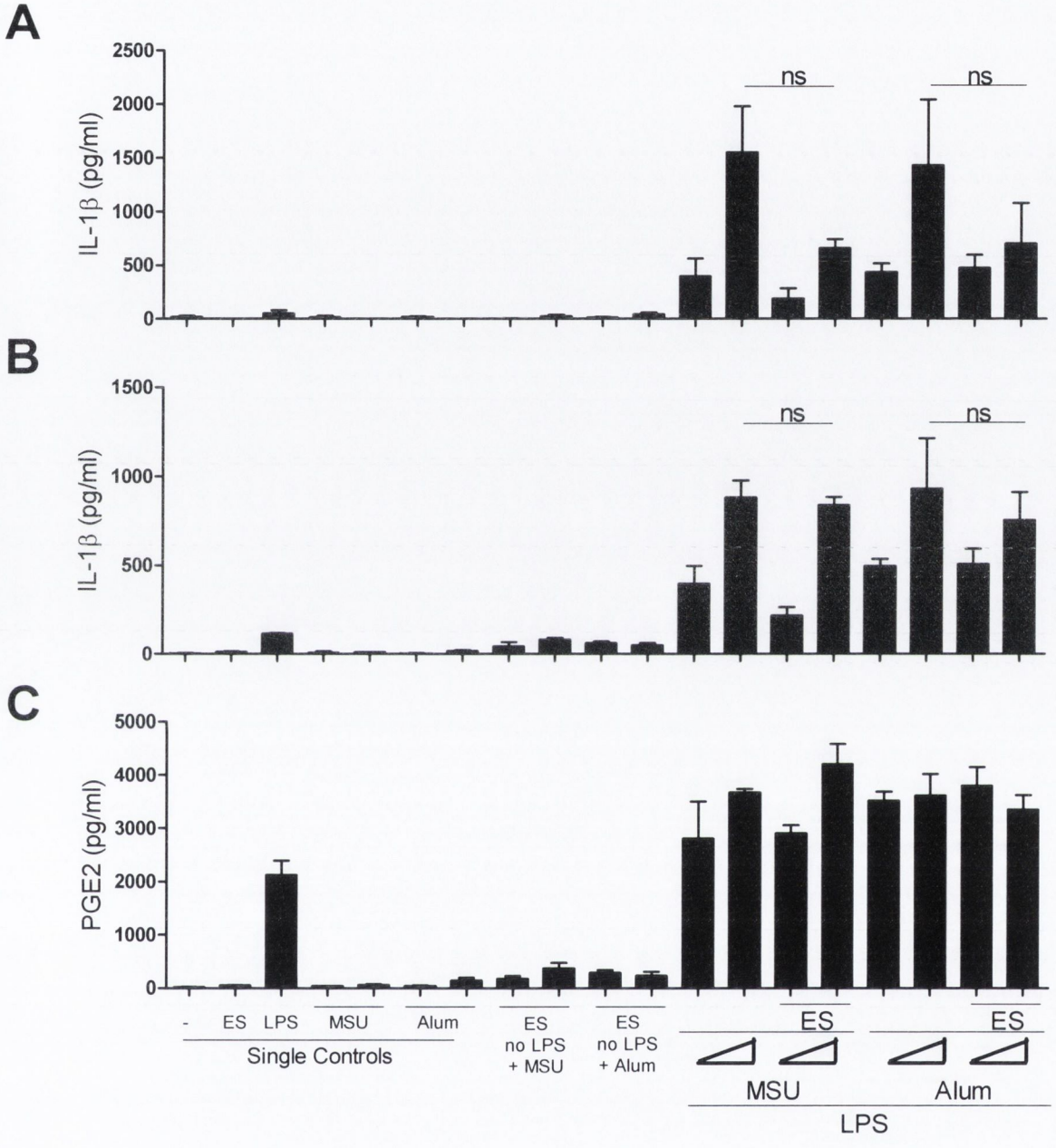


Fig 6.16. ES treatment does not block alum or MSU induced inflammsome activation or PGE_2 production. BMM were primed with LPS (100 ng/ml) for 2 h and ES or PBS for 1 h prior to stimulation with either Alum (200, 400 μg/ml) or MSU crystals (40, 80 μg/ml). A, After 2h 50μl of supernatant was removed and the concentration of IL-1β was quantified by ELISA. B, The concentration of IL-1β at 24 hours. C, the concentration of PGE_2 in the supernatant at 24 hours was determined by competitive ELISA. Data presented are means +/- SEM of triplicate assays. Results are representative of 3 separate experiments.

Chapter 7 General discussion

7.1 General Discussion

This study has shown that infection with the trematode parasite *F. hepatica* attenuated inflammation in murine models of autoimmunity and allergy. The results showed that protection in the autoimmune model, EAE, involved a TGF-β-dependent mechanism (298). Infection of mice with *F. hepatica* was associated with the recruitment of MDSC, immature APC and Treg cells. This study went on to show that the secreted products of *F. hepatica, ES* inhibited the ability of DC to drive Th1 and Th17 responses. Furthermore, ES enhanced the production of TGF-β from innate cells by a novel mechanism independent of protein translation but dependent on the production of ROS. Both infection with *F. hepatica* or the administration of its secreted products recruited eosinophils and enhanced their function as APC. ES was also shown to block the proliferation of T cells, independent of APC function. The administration of ES to mice with EAE suppressed disease in an IL-4 and TGF-β independent and IL-5 dependent manner. Finally, ES blocked the induction of the Th2 cells that mediate AHR, by an unknown mechanism.

7.2 The hygiene hypothesis and helminth therapy

The hygiene hypothesis supports a role for helminths in suppressing the pathogenesis of allergic and autoimmune disease. The findings of the present study add further evidence to support the validity of this hypothesis. The results showed that that infection with *F. hepatica* and/or administration of its secreted products inhibits the induction of autoimmunity (EAE) and allergy (AHR). This is achieved through specific interactions with T cells and APC. *F. hepatica,* is now one of five helminths, the others being *H. polygyrus, L. sigmodontis, A. vitae and S. mansoni,* that have the ability to inhibit both allergy and autoimmunity in murine models.

The epidemiological and experimental evidence to support the hygiene hypothesis has advanced to a point where helminths are being investigated as potential therapeutics for the treatment of inflammatory diseases. This has culminated in several clinical trials involving treatment with live helminth parasites (166). These trials represent the ultimate test for the hygiene hypothesis under the scrutiny of evidence based medicine for the first time. Ten years ago, the first human clinical trial involved administration of *T. suis* eggs (ova) to patients with

inflammatory bowel disease (IBD) (443). Subsequently, infection with *N. americanus* and *Trichuris trichiura* has been tested with success in clinical trials with IBD patients. To date, however, the greatest efficacy has resulted from the use of *T. suis* ova, which significantly improved clinical symptoms in IBD patients with three quarters of treated patients in one trial, entering remission (444).

In support of Carreole and Farez's original description that natural infection of individuals with helminths reduced the progression of MS (199), trials have shown that infection with *T. trichiura, Hymenolepis nana* and *Ascaris lumbricoides T. suis* were clinically effective in MS patients and reduced disease exacerbations and the formation of CNS lesions (445, 446). Some of these treatments were associated with an expansion of Treg cells and enhanced production of TGF-β and IL-10 (445). However, other successful treatment of MS patients with helminths were not associated with induction of TGF-β, IL-10 or Treg cells, but instead clinical suppression was conferred via unknown mechanisms (446).

The ethics of using helminths as a therapy for human diseases raises some interesting questions. The Hippocratic Oath prevents medical practitioners from doing harm and helminths, even those that cause asymptomatic infections, will cause at least some damage in the host as they translocate across mucosal barriers. However, given that nearly all pharmaceuticals approved for use in humans have associated side-effects, could the potential damage from helminth infection simply be considered an "acceptable side-effect"? Surgical intervention is used for the benefit of human health and yet it is intrinsically destructive, causing at least some damage to tissues with a risk of infection. Perhaps helminth therapy can be considered as an invasive, but beneficial intervention, on a par, ethically with invasive surgery? However, will regulatory authorities approve the use of live metazoans as safe treatment? After all it is a treatment that may be difficult to regulate with many unknown variables. However, as its principles it does not differ much from the now popular use of probiotics, i.e. living organisms that are administered to the host which modulate the health of the host, albeit with using smaller and potentially less damaging microorganisms. The fact that the clinical trials themselves have gained ethical approval by regulatory agencies is a positive indicator for future acceptance.

It is likely that the approach of intentionally infecting oneself with parasitic worms may not be acceptable to most individuals. The fact that patients are willing to break with such taboos, and infect themselves with potentially harmful helminth puts in perspective the medical need; the autoimmune and allergic disease that these therapies are designed to treat are chronic, disabling and in many cases, lethal diseases. The reduction in the quality of life these diseases inflict on the patient underscores the need for radical medical interventions. Indeed, those impatient with the slow pace of clinical trials, and convinced of the efficacy of parasitic infection, have sought out alternative sources of helminths from unregulated sources (David Pritchard, personal communication). However, larger scale controlled clinical trials are required before helminths are approved for general use in humans.

Aside from the ethical concerns, there are many practical considerations which may reduce the efficacy of helminth-based therapy. The original understanding of the hygiene hypothesis was that infections early in life provided protection against allergy and possibly other immune-mediated diseases (198). Thus, it is possible that exposure of the human immune system to helminths early in life is required to confer suppressive activity. In addition, experiments in animal models, (including this study) suggest that helminths have the greatest effect on immune activation when present at the time of antigen sensitisation. Thus, the beneficial effect of helminth therapy may be limited to the induction phase of allergy or autoimmune disease. Furthermore, in an attempt to limit the side effects of therapy, under the guidelines of regulatory authorities, many trials have used what may be suboptimal doses of helminths, resulting in lack of efficacy. This explanation was put forward to explain the failure of a trial with *N. americanus* in IBD patients (447). It is also quite difficult, or impossible, to conduct these trials using proper placebo controls. This is due to the obvious symptoms of early infection which will reveal to patients whether they are in placebo or treatment groups (166, 447).

7.3 *F. hepatica* ES

The use of specific helminth-derived proteins and small molecules with immunoregulatory activity represent a more desirable treatment approach compared with infection (448). Soluble helminth-derived products can be synthesised, mass produced and controlled to meet

regulatory guidelines. Helminths, being eukaryotic metazoans, have large genomes and are capable of post-translational modifications that add complex modifications to proteins and may confer further immunomodulatory activity. Helminths have much larger genomes than that of *Caenorhabditis elegans*, a free living worm considered to resemble a common ancestor of parasitic worms (449). Indeed, helminths are thought to possess in the range of 10,000 genes, a figure approaching half that of humans (450). Much of this 'excess' genomic size is thought to be devoted to the host-parasite interactions, and much of this to immunoregulation. These genomes are a veritable untapped source of immunomodulatory molecules with potential clinical efficacy against human immune-mediated diseases. However, despite the extensive body of research demonstrating the efficacy of helminth products in the treatment of animal models of disease (166), no helminth products have yet been administered to humans in clinical trials.

The present study demonstrated that *F. hepatica* ES protected mice from EAE and AHR. These findings suggest that products of *F. hepatica* have potential as therapeutics for the treatment of autoimmunity and allergy. Unlike the hookworms and whipworms already trialled in human disease, *F. hepatica* causes significant damage in the host and as such, would never be considered as a potential treatment. Thus, specific molecules contained within the parasite represent the only chance to develop therapeutics from *F. hepatica*. ES, which consists of soluble products that interact with host immune system are thus the more likely to be mediators of suppression than with somatic extracts, which physiologically do not interact with the host. For this reason, *F. hepatica* ES rather than TE may prove to be a greater source of active components (411).

The identification of the specific components within ES is important, as it will enable systematic evaluation and partition of the many activities of whole ES into specific properties that can be attributed to individual molecules. Indeed, the Dalton group have comprehensively evaluated the immunomodulatory activity of one component of ES, cathepsin L (227, 451, 452). The specific proteins of other helminths such as ES-62 (from *A. viteae*) have been shown to have efficacy in the treatment of animal models of autoimmunity (448), and therefore has potential for use against human diseases.

A recent study may have implications for the delivery of ES products to immune cells. Marcilla et al demonstrated that rather than being conventionally secreted, many components within ES are released from the fluke trapped within small vesicles called exomes, these subsequently fuse with the plasma membranes of host cells (453). While that study did not investigate the actual immune implications of this discovery it is tempting to speculate that this represents a novel in-built delivery mechanism for parasite-derived immunomodulatory molecules (453).

7.4 Helminthiasis

Nearly one-third of the global population is infected with helminth parasites and infection is associated with significant morbidity, disability and disfigurement (139). Furthermore, helminths and in particular, *F. hepatica,* cause significant reduction in agricultural output through infection of cattle and sheep. The eradication or control of helminth infections remains a difficult task and is compounded due to a lack of effective vaccines, limited efficacy of current anti-helminth drugs, emerging drug resistance, and rapid reinfection in environments where transmission cannot be prevented (139). Moreover, the complexity of the immunoregulation exerted by helminths further complicates the control of helminthiasis. New intervention strategies, based on an understanding of the immunological basis of helminth persistence are urgently needed.

The findings of this study have demonstrated that it may be possible to identify novel helminth-derived molecules that could be used as therapeutics for the treatments of human autoimmune diseases; but because successful infection of *F. hepatica* is so critically dependent on immune regulation, these findings have also advanced the understanding of immunity against *F. hepatica.* The specific interactions of *F. hepatica* with the immune system, especially the immunosuppressive pathways, might present new targets for therapy which will interfere with immune regulation and thus aid in the expulsion of the fluke. In parallel, proteomic and genomic profiling of the parasite will further assist in the identification of such targets. In time, these investigations concerning the immunology of the host-parasite interactions of *F. hepatica* may prove beneficial in the control of infection as the findings of Leuckart, Thomas

and Lutz on the life cycle of *F. hepatica* (236-239), which promoted changes in farming practises that reduced transmission between livestock.

7.5 Roles for Treg cells, TGF-β and ROS

The present study has shown that Treg cells do not appear to be required for protection against EAE or AHR. Nevertheless, expansion of Treg cells represents a powerful immunoregulatory mechanism. An interesting observation from this study that warrants further discussion is the finding that infection with *F. hepatica* drives the expansion of Treg cells, while administration of ES does not. However, Carranza et al showed that the TE of *F. hepatica* expands Treg cells (374). This suggests that somatic, rather than secreted antigens may be responsible for the expansion of Treg cells. However, another possibility is that ES needs additional "co-stimulation" to induce Treg cells. Carranza et al showed that Treg cells could be induced *in vivo* by transfer of Dc treated with TE and CpG. Thus it is possible that *F. hepatica* ES lacked a vital stimulus for effective expansion of Treg cells. However, since TLR agonists such as CpG are unlikely to be present during treatment with ES, the results from the present study are more likely to reflect the situation during a helminth infection

The role of TGF-β in the induction of immunosuppression in this study has not been fully resolved. While TGF-β was shown to mediate protection from EAE during infection with *F. hepatica* (298), it did not mediate protection induced by treatment with ES. Furthermore, ES promoted the release of TGF-β by innate cells in a mechanism dependent on ROS. However, ES did not promote the expansion of Treg cells, tentatively suggesting that this TGF-β is functionally inactive. Indeed, preliminarily evidence from this laboratory suggest that ES-induced TGF-β does not activate SMAD-dependent TGF-βR signalling (A M Stefanska, personal communication). Thus, further investigation into the biological role of TGF-β in *F. hepatica* infection and immune regulation is merited.

The production of ROS by neutrophils and eosinophils is a major host immune defence mechanism against helminths (448). Fasciolosis is accompanied with the generation of ROS, which leads to initiation of lipid peroxidation of host tissues that result in damage to the host (454-456). Interestingly, ROS production was shown to be higher upon secondary infection with liver fluke and its magnitude correlated with protection from infection in rats (455, 456).

Infection with *F. hepatica* is accompanied by changes in antioxidative activity in the liver. This is manifested by a decrease in the activity of the main antioxidant enzymes, such as Cu, Zn-superoxide-dismutase (SOD), and by a reduction in concentration of non-enzymatic antioxidants (GSH, vitamins C, A, and E) (457). The present study has shown that ES induced oxidative damage in cultured epithelial cells. In addition, ES-induced ROS was shown to be linked with the production of TGF-β by innate immune cells. Most helminths express high levels of antioxidants including SODs, catalases, glutathione and thioredoxin peroxidases to protect themselves from oxidative damage (455). Proteomic analysis of *F. hepatica* have identified several antioxidants enzymes as prominent components of ES (458). At least one of these antioxidants, thioredoxin peroxidise, has been shown to have immunoregulatory activity, and simulates the alternative activation of macrophages (459). Future work on the immunobiology of *F. hepatica* should consider ROS, and the antioxidants that counteract them, as possible mediators of parasite immune regulation.

7.6 IL-5, eosinophils and protection from autoimmunity

One of the major findings of this study was that *F. hepatica* ES protected mice from EAE in an IL-5 dependent manner. This finding represents the first evidence that helminth induced-IL-5 mediates suppressive effects on autoimmunity. A recent report from Tran et al demonstrated that administration of recombinant IL-5 ameliorated EAE, even when given at onset of symptoms, in a similar manner to ES-mediated suppression (460). The authors convincingly demonstrated that production of IL-4 in response to CFA acted on myelin-specific nTreg cells to enhance their expression of the IL-5Rα. IL-5, also induced in response to CFA, subsequently acted on nTreg, (which were now responsive to the cytokine), to enhance the suppressive activity of these cells. Interestingly, Giang et al showed that IL-4 was required only at induction of EAE. Therefore, IL-4 may still have a role in ES-mediated suppression, although the experiment with the IL-4-defective mice suggest otherwise. However, this mechanism was wholly depended on Treg cells as their depletion reversed the protective effect (460). In contrast, the present study did not observe an expansion of Treg following ES treatment, suggesting that the mechanism may be distinct from the one presented by Giang et al (460). Nevertheless, the precise role of Treg will require depletion

studies with CD25 antibodies or Treg cell defective mice. If Treg are not the target of IL-5, it is likely that the suppression is mediated by eosinophils.

The present study has provided several interesting observations concerning the role of eosinophils in helminth infection and their possible role in immune suppression induced by the parasites. Protection of mice from EAE following treatment with ES was associated with IL-5-dependent recruitment of eosinophils. Moreover, eosinophils infiltrated the brains of protected mice treated with ES. The detection of eosinophils in the brain is indicative of Th2 cell activity in the brain, suggesting a shift in MOG-specific responses from Th17 and Th1 towards Th2 cells. Although the number of eosinophils in the brains of these mice was low, they were at parity with the numbers of $CD4^+$ T cells. This is a potentially important observation since the present study has also demonstrated that eosinophils, activated during infection with *F. hepatica* or following administration of ES displayed APC activity. Since the modulation of DC function by helminth products has been shown to "re-educate" pathogenic T cells towards a suppressive or anergic phenotype (340, 374), it is tempting to speculate that eosinophils may perform a similar function, inhibiting T cell functions or at least polarising responses towards a Th2 phenotype. Indeed, eosinophils have already been shown to promote Th2 responses *in vivo*; Padigel et al demonstrated that transfer of eosinophils, purified from IL-5-transgenic mice, loaded with a helminth antigen to naïve recipient mice induced production of Th2 cytokines upon restimulation with that antigen and this effect was dependent on MHC-II. Interestingly, in that model eosinophils preferentially induced Th2 cells while DC or macrophages isolated from the same animals did not promote such Th2 responses (331).

It is not clear why parasites would induce Th2 responses via eosinophil activation, but it is possible that this is a mechanism to stabilise the anti-inflammatory Th2 responses at the site of helminth infections and ensure they do not deviate towards destructive Th1 or Th17 responses. Moreover, eosinophils, once viewed as end stage effector cells are now known to have a range of immunomodulatory abilities. They produce an array of cytokines, including TGF-β, IL-6, IL-4 and interestingly IL-5. Many of these cytokines are stored preformed in vesicles for immediate release upon activation (461). Indeed, eosinophils may be a dominate source of IL-4 during helminth and allergic sensitisation, an especially important role given that DC do not produce IL-4 themselves (461). Furthermore, eosinophil-derived neurotoxin,

a major component of eosinophil granules, can act on DC to promote Th2 responses in a TLR2-dependent manner (462). In an even broader context, eosinophils have recently been shown to play a role in glucose homeostasis by providing IL-4 "help" to M2 macrophages that control adipose tissue homeostasis and prevent diabetes (463).

However, the role of eosinophils in parasite-induced immunosuppression cannot be fully investigated without the use of mice totally deficient in eosinophil, such as the ΔdblGATA mouse. In contrast, IL-5-transgenic mice may prove useful as a source of eosinophils for transfer studies.

7.7 Allergy; connecting the dots

It is somewhat difficult to reconcile the fact that ES can protect in models of Th1/Th17 mediated disease (EAE) and Th2-mediated disease (AHR), especially in the absence of Treg cell induction. The possible role of IL-5 in ES-mediated suppression of EAE is not compatible with the suppression of allergic responses. ES-H, the shared protective component also induces Th2 responses, and thus possibly allergic sensitisation to its own components.

It is unlikely that IL-5 would have a suppressive role in allergy, since IL-5 is considered to be a major mediator of allergic responses, through its role in promoting of eosinophilia. Indeed, anti-IL-5 antibodies have been assessed in clinical trials, albeit without success, in the treatment of asthma (464). Moreover, AHR cannot be induced in IL-5-deficient mice (465). It is still unclear how helminth products in ES prevent the induction of AHR and future studies will need to include considerations of alum-mediated adjuvanticity and the effect of ES on the innate cell response to alum. It is tempting to speculate that that the immune system can distinguish between signals from helminths (ES) and the signals from allergens (OVA), when both stimuli are present simultaneously at the same site and this may result in the development of a response against the pathogen but not the allergen. This may represent an extension of self-tolerance and form the basis of helminth induced suppression of allergy explaining why individuals with helminth infections display lower atopic responses (466).

To conclude, this study provides further insight into the immunoregulatory networks induced by helminth infection, which underlie the ability of these organisms to attenuate autoimmunity and allergy. These findings may also aid the development of helminth-derived therapeutics for the treatment of human disease.

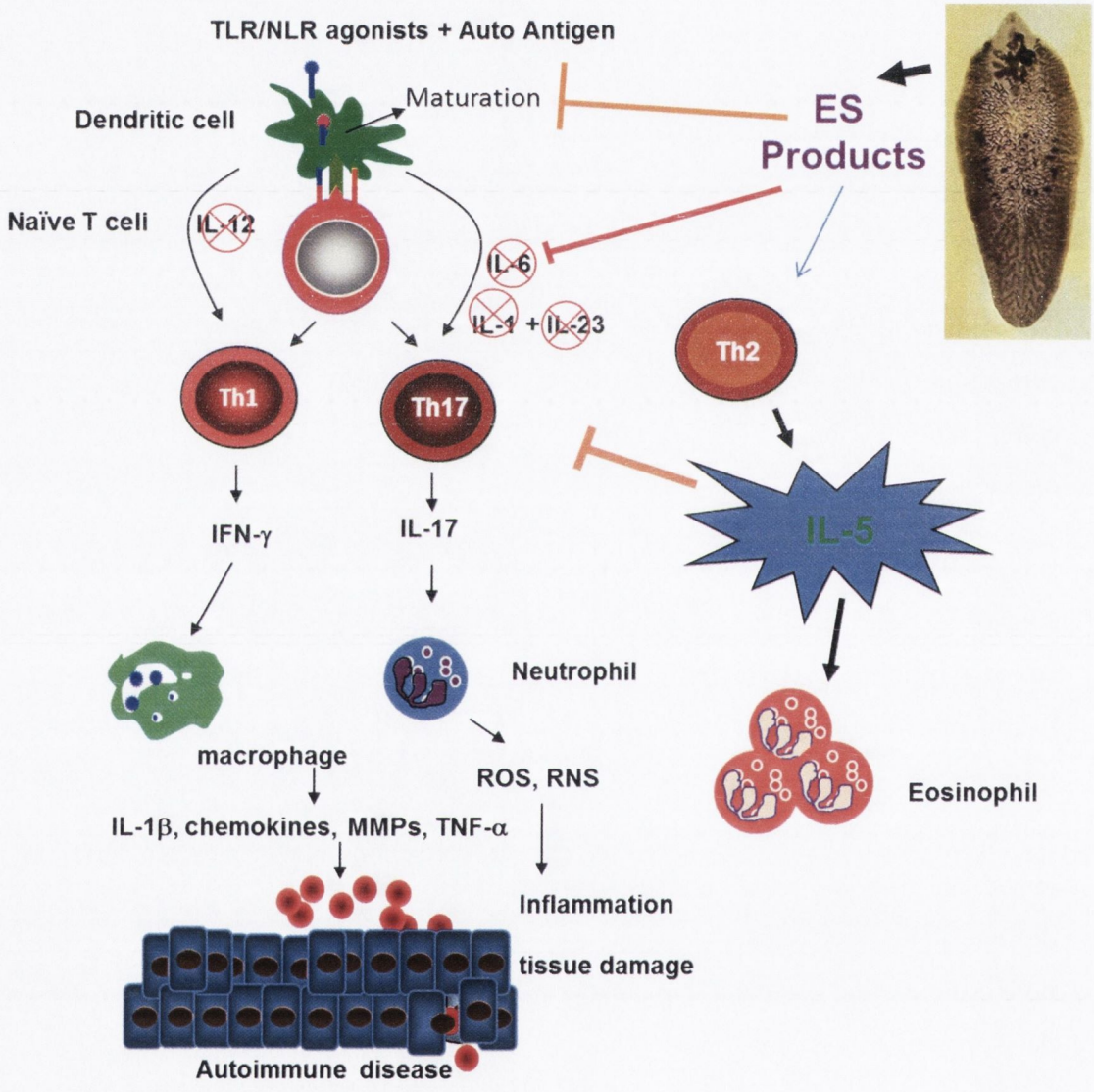


Figure 7.1 Proposed mechanism of attenuation of autoimmunity by *F. hepatica* ES

Chapter 8 References

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