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# Regulatory T cell induction and function

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A handwritten signature in blue ink, reading "Kevin Walsh", is written over a horizontal line.

Kevin Walsh

## Abstract

The main objective of this study was to examine novel methods of inducing regulatory T (Treg) cells by modulating dendritic cell (DC) activation. It has previously been reported that TLR agonists stimulate IL-12 production from DC and thereby induce Th1 cells. However, recent studies have suggested that TLR agonists may simultaneously induce IL-10 producing Treg (Tr1) cells. In the present study, this property was explored and exploited in order to develop methods of selectively inducing Tr1 cells, in the development of potential therapies for auto-immune diseases mediated by Th1 and IL-17 producing T (Th17) cells.

This study demonstrated that the cAMP activators dibutyryl cAMP, forskolin and PGE<sub>2</sub> can modulate TLR induced cytokine production by DC. Dibutyryl cAMP enhanced TLR induced IL-23 and IL-10 production and inhibited IL-12 production. Forskolin inhibited TLR stimulated IL-12 and IL-23, while PGE<sub>2</sub> was shown to enhance TLR induced IL-10 and inhibit TLR stimulated IL-12 and IL-23 production by DC. Transfer of DC treated with antigen, dibutyryl cAMP and LPS to naive mice promoted the induction of antigen specific Th17 cells *in vivo*. Co-administration of antigen, LPS and PGE<sub>2</sub> promoted the induction of antigen specific Tr1 cells that inhibited IFN- $\gamma$  but not IL-17 production by autoantigen specific T cells from mice with experimental autoimmune encephalomyelitis (EAE).

Filamentous haemagglutinin (FHA), a virulence factor from *Bordetella pertussis* has previously been shown to play a major role in the evasion of the immune response by the bacteria. This study demonstrated that FHA induced the production of IL-10 by DCs through a p38 dependent mechanism. Furthermore, FHA when used as an adjuvant promoted the development of Tr1 cells specific for a co-administered antigen *in vivo*. These Tr1 cells inhibit Th1 cells and the proliferation of Th17 cells. Tr1 cells generated from mice immunized with antigen and FHA suppressed IL-17 production by T cells but not as potently

as IFN- $\gamma$  production. Prophylactic immunization of mice with myelin oligodendrocyte (MOG) and FHA attenuated the development of EAE which suggested that the protective effect of FHA is mediated by the induction of autoantigen specific Treg cells. MOG specific T cells generated from the mice that were immunized with MOG and FHA produced higher levels of IL-10 and reduced concentrations of IL-17 when compared with MOG specific T cells from control mice with EAE.

*Fasciola hepatica* is a helminth pathogen which has been shown to induce immunosuppression in the infected host. This study showed the infection of mice with *F. hepatica* resulted in an influx of immune cells into the peritoneal cavity that displayed an anti-inflammatory phenotype. Furthermore, it was demonstrated that *F. hepatica* infection suppressed the development of MOG specific Th1 and Th17 responses and attenuated the clinical symptoms of EAE. Investigations into the mechanisms involved ruled out a role for IL-10 but demonstrated that it was dependent on helminth induced TGF- $\beta$ . *In vitro* studies with recombinant TGF- $\beta$  showed that it inhibited LPS induced IL-23, IL-12 and IL-1 production by DCs and also inhibits IFN- $\gamma$  but not IL-17 production by MOG specific T cells from mice with EAE.

This report demonstrates that pathogenic autoimmune responses can be inhibited by immunomodulatory approaches that promote the induction of Tr1 cells. Although Th17 cells are resistant to direct suppression by Tr1 cells, this study demonstrated that recombinant TGF- $\beta$  inhibited IL-12, IL-23 and IL-1 production by DCs suggesting that suppression may be mediated at the level of the antigen presenting cell (APC).

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## **Publications and abstracts**

**Walsh, K.P,\*** Brady, M.T,\* Finlay, C, Boon,L, Mills, K.H.G, Infection with a helminth parasite attenuates autoimmunity through TGF- $\beta$  mediated suppression of Th17 and Th1 responses. (returned after revision to the Journal of Immunology). \*both authors contributed equally to this work

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## List of Abbreviations

<b>AIG</b>	Autoimmune Gastritis
<b>ANOVA</b>	Analysis of Variance
<b>APC</b>	Antigen Presenting Cell
<b>AP-1</b>	Activator Protein 1
<b>APC</b>	Allophycocyanin
<b>ATP</b>	Adenosine Triphosphate
<b>BAL</b>	Bronchoalveolar Lavage
<b>BCG</b>	Bacille Calmette-Guérin
<b>BMDC</b>	Bone marrow-derived DC
<b>BSA</b>	Bovine Serum Albumin
<b>cAMP</b>	Cyclic Adenosine Monophosphate
<b>CD</b>	Cluster of Differentiation
<b>CFA</b>	Complete Freund's Adjuvant
<b>CIA</b>	Collagen induced arthritis
<b>CLR</b>	C-type lectin receptor
<b>CNS</b>	Central Nervous System
<b>Con A</b>	Concanavalin A
<b>COX</b>	Cyclooxygenase
<b>CpG</b>	Cysteine-phosphate-Guanosine motif
<b>cpm</b>	counts per minute
<b>CR3</b>	Complement Receptor 3
<b>CREB</b>	cAMP responsive element binding protein
<b>CT</b>	Cholera Toxin
<b>CTL</b>	Cytotoxic T lymphocyte

<b>CTLA-4</b>	Cytolytic T lymphocyte-associated antigen 4
<b>Cy</b>	Cyanine
<b>CyaA</b>	Adenylate Cyclase Toxin
<b>DAP</b>	Diaminopilimic acid
<b>DC</b>	Dendritic Cell
<b>DNA</b>	Deoxyribonucleic Acid
<b>DMSO</b>	Dimethyl sulphoxide
<b>DUSP</b>	Dual specificity phosphatases
<b>EAE</b>	Experimental Autoimmune Encephalomyelitis
<b>EAU</b>	Experimental Autoimmune Uveitis
<b>EDTA</b>	Ethylenediaminetetraacetic Acid
<b>ELISA</b>	Enzyme Linked Immunosorbent Assay
<b>EP</b>	E prostanoïd
<b>Epac</b>	Exchange protein directly activated by cAMP
<b>ERK</b>	Extracellular signal-regulated protein kinase
<b>ES</b>	Excretory secretory product
<b>FACS</b>	Fluorescence Activated Cell Sorter
<b>FCS</b>	Fetal Calf Serum
<b>FHA</b>	Filamentous Haemagglutinin
<b>FITC</b>	Fluorescein Isothiocyanate
<b>FIM</b>	Fimbriae
<b>Foxp3</b>	Forkhead Box P3
<b>GITR</b>	Glucocorticoid-induced TNF receptor related protein
<b>GM-CSF</b>	Granulocyte Macrophage-Colony Stimulating Factor
<b>GPCR</b>	G protein coupled receptor

<b>HLA</b>	Human Leukocyte antigen
<b>HRP</b>	Horseradish Peroxidase
<b>IBD</b>	Inflammatory Bowel Disease
<b>IFN</b>	Interferon
<b>Ig</b>	Immunoglobulin
<b>IL-</b>	Interleukin
<b>iNOS</b>	inducible Nitric Oxide
<b>i.p</b>	intraperitoneal
<b>IRF</b>	Interferon regulatory factor
<b>JAK</b>	Janus Activated Kinase
<b>JNK</b>	c-Jun NH <sub>2</sub> -terminal kinases
<b>KLH</b>	Keyhole Limpet Haemocyanin
<b>LPS</b>	Lipopolysaccharide
<b>MAPK</b>	Mitogen activated protein kinase
<b>MCP</b>	Monocyte Chemotactic Protein
<b>MHC</b>	Major Histocompatibility Protein
<b>MIP</b>	Macrophage Inflammatory Protein
<b>MKKK</b>	Mitogen activated kinase kinase kinase
<b>MPL</b>	Monophosphoryl Lipid A
<b>MOG</b>	myelin oligodendrocyte glycoprotein
<b>NF-<math>\kappa</math>B</b>	Nuclear Factor- $\kappa$ B
<b>NLR</b>	nucleotide-binding domain and leucine-rich repeat containing molecules
<b>ng</b>	nanogram
<b>NO</b>	Nitric Oxide
<b>NOD</b>	Nucleotide Binding Oligomerization Domain

<b>NK</b>	Natural Killer Cell
<b>OVA</b>	Ovalbumin
<b>OPD</b>	O-phenyldiamine
<b>PAMP</b>	Pathogen-Associated Molecular Pattern
<b>Pam-3CSK4</b>	Palmitoyl-3-cysteine-serine-lysine-4
<b>PBMC</b>	Peripheral Blood Mononuclear Cells
<b>PBS</b>	Phosphate Buffered Saline
<b>PE</b>	Phycoerythrin
<b>PI3K</b>	Phosphoinositide 3-kinase
<b>pg</b>	picogram
<b>PGN</b>	Peptidoglycan
<b>pH</b>	power of hydrogen
<b>PKA</b>	Protein Kinase A
<b>PMA</b>	Phorbol Myristic Acid
<b>PMN</b>	Polymorphonuclear
<b>Poly I:C</b>	Polyinosinic:Polycytidylic Acid
<b>PRN</b>	Pertactin
<b>PRR</b>	Pathogen Recognition Receptor
<b>PLP</b>	Proteolipid peptide
<b>PT</b>	Pertussis Toxin
<b>RAG</b>	Recombination Activating Gene
<b>RANTES</b>	Regulation on Activation Normal T cell Activation
<b>RLR</b>	RIG-I like receptors
<b>RNA</b>	Ribonucleic Acid
<b>RPMI</b>	Roswell Park Memorial Institute

<b>RT</b>	Room temperature
<b>s.c</b>	sub-cutaneously
<b>SD</b>	Standard Deviation
<b>STAT</b>	Signal Transducer and Activation of Transcription
<b>TCR</b>	T cell receptor
<b>TCT</b>	Tracheal Cytotoxin
<b>TEMED</b>	Tetramethylethylenediamine
<b>TGF</b>	Transforming Growth Factor
<b>Th</b>	T helper cell
<b>TLR</b>	Toll-like receptor
<b>TNF</b>	Tumour Necrosis Factor
<b>Treg</b>	Regulatory T cell
<b>Tr1</b>	T regulatory type 1
<b>UV</b>	Ultra Violet
<b>WHO</b>	World Health Organisation
<b>Yop</b>	Yersinia effector protein



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# **Chapter I**

## **Introduction**



# **1.1 General introduction to the immune system**

## **1.1.1 Overview of the immune system**

The Immune System has evolved to protect animals from invading pathogenic organisms and cancer. It functions by distinguishing between the body's own cells and proteins and those of foreign pathogens. On recognition of a foreign organism, the immune system launches an effector response, which involves the recruitment of a variety of cells and molecules, which work to eliminate or neutralize the threat to the homeostasis of the host. Immunity has a non-specific and specific component. The non-specific component involves the innate immune system which provides the first line of defence against infection. Most of its components are present before the onset of disease and recognition is not specific to a particular pathogen. In contrast to this broad reactivity, adaptive immunity is antigen specific. The adaptive immune system is activated to assist in pathogen clearance when the innate immune system has failed to effectively eliminate the threat to the host. Following the initial exposure to the pathogen, an adaptive immune response takes 5-6 days to develop. However, adaptive immunity has the remarkable property of memory. On a repeat exposure to the same antigen, a second recall response occurs more quickly and is stronger than the first response. In most cases a second infection is asymptomatic due to the rapid elimination of the microbe. Immunologists have harnessed the immune systems property of memory through the development of vaccines, which has been one of the fields greatest contributions to human health.

## **1.1.2 Innate immunity**

Physical and anatomical barriers form the first line of host defence against invading microorganisms. The outer layer of the skin consists of several layers of tightly packed dead



cells. Sebaceous glands produce sebum which maintains the pH of the skin between 3 and 5, thereby inhibiting the growth of most microorganisms. The conjunctivae and the alimentary, respiratory and urogenital tracts are lined with mucous membranes, which produce tears, saliva and mucous secretions, which act to wash away potential pathogens. They also contain antibacterial and antiviral substances such as lysozyme, a hydrolytic enzyme which is able to cleave the peptidoglycan layer of bacterial walls. In the lower respiratory tract, the mucous membrane is covered by cilia which through their synchronous movement remove mucous entrapped organisms from the tract. Non-pathogenic organisms also influence innate immunity by colonizing the epithelial surfaces and out competing pathogens for binding sites.

Molecular components of innate immunity include interferons, complement and collectins. Interferons are produced by virally infected cells and induce an anti-viral state in nearby cells. Complement consists of a group of serum proteins that circulate in an inactive state. Mechanisms of both innate and adaptive immunity can convert complement components to their active forms, allowing them to damage the membranes of pathogens, which aids their elimination. Collectins are surfactant proteins, which have been shown to kill certain bacteria by disrupting their lipid membranes.

If the physical barriers of the innate immune system are breached leading to the invasion of a pathogenic organism, an inflammatory response ensues. Inflammation can be initiated by mediators derived from damaged cells, several plasma enzyme systems and leukocytes. One of the major mediators of inflammation released by damaged cells is histamine, which binds to receptors on nearby capillaries and venules causing vasodilation and increased permeability. This facilitates the influx of fluid and cells. Another cellular initiator of inflammation is the macrophage. One of the principal ways this immune cell recognises

pathogens is through pathogen recognition receptors (PRRs) which are discussed in detail below.

Macrophages are derived from monocytes which circulate in the bloodstream for about 8 hr before migrating into the tissues. Some macrophages become fixed, whereas others remain motile. On activation by a variety of stimuli, including PRR ligation, macrophages exhibit greater phagocytic activity, an increased ability to kill ingested microbes, increased secretion of inflammatory mediators and an increased ability to activate T cells. Expression of MHC class II and various co-stimulation molecules, including CD80, CD86 and CD40 are enhanced following activation.

Another group of innate immune cells are the granulocytes/polymorphonuclear leukocytes consisting of neutrophils, eosinophils and basophils. Neutrophils, like most immune cells are produced in the bone marrow and are then released into the peripheral blood, where they circulate for 7-10 hrs before migrating into the tissues. Like macrophages, neutrophils are phagocytic. Granules within neutrophils containing lytic and bactericidal substances, fuse with the phagosomes thereby killing the pathogen. Activated neutrophils and macrophages generate superoxide anions (respiratory bursts), that are extremely toxic to ingested organisms.

Eosinophils are capable of phagocytosis and release granules that are important in parasite clearance. Basophils are unable to phagocytose but function by releasing pharmacologically active substances. Another cellular component of innate immunity are natural killer (NK) cells which are derived from the same cellular progenitor as T and B cells of the adaptive immune system. NK cells function in the recognition and killing of abnormal cancerous or

virally infected cells. Traditionally NK cells have been classified as non antigen specific cells that are an important component of the innate immune system. However recent evidence published by Sun *et al* demonstrates that NK cells bearing the Ly49H receptor specifically recognize a protein produced by some strains of mouse cytomegalovirus (MCMV) (1). Furthermore, following MCMV infection a subset of the virus experienced Ly49H NK cells survive and maintain the characteristics of effector NK cells (1). On transfer to newborn immunodeficient mice, these cells conferred protection against infection suggesting the existence of “memory” NK cells (1).

Dendritic cells (DC) are the most effective antigen presentation cells. It is currently accepted that there are two main DC subsets in humans-CD11c<sup>+</sup> myeloid DC and CD11c<sup>-</sup>CD123<sup>+</sup> plasmacytoid DC (2). Immature conventional (myeloid) DC include langerhan cells, dermal DCs and interstitial DCs and are found in the blood, skin and mucosal tissues (2). They are both phagocytic and macropinocytic. Upon encountering a pathogen, immature DC rapidly mature and migrate to the lymph node, where they present antigens to T cells. Myeloid DCs secrete high levels of IL-12 on activation and express higher levels of TLR4 than plasmacytoid DCs (3). Myeloid cells are therefore more responsive to LPS than plasmacytoid DCs (3). Plasmacytoid DCs are located in blood and secondary lymphoid organs but are recruited to sites of inflammation (2). Plasmacytoid DCs secrete high levels of type I IFNs (4). The cytokines secreted by the antigen presenting cell are determined by innate receptor ligation on the cell surface which in turn determine the phenotype of the T helper cell induced.

### 1.1.3 Adaptive immunity

Adaptive and innate immunity do not function independently of each other. Macrophages and dendritic cells are crucial to non-specific innate immune response, but are intimately involved in activating the adaptive immune response. Lymphocytes, which include T and B cells, are the main cell type within adaptive immune system. The induction of adaptive immune responses initially involves the presentation of an antigen on a MHC molecule to a T cell or the direct recognition of certain antigens by B cells.

T cells originate in the bone marrow and then migrate to the thymus where they mature. T cells that have yet to encounter antigen are known as naïve T cells and continually recirculate between the blood and the lymph systems often residing in the secondary lymphoid tissues such as the lymph nodes. Each T cell expresses a unique T cell receptor (TCR) type that only recognises one specific antigen. The activation of the T cell is dependent on the presentation of the antigen to the T cell while bound to an MHC molecule. There are two major types of MHC molecules. Class I molecules are expressed on nearly all nucleated cells and Class II molecules are solely expressed on antigen presenting cells.  $CD4^+$  T cells recognise antigen presented on MHC class II molecules and  $CD8^+$  T cells recognise antigen presented on MHC class I molecules. TCR-CD3 ligation is the initial signal for activation but a second signal is also required. In the case of TCR-MHC class II/ peptide complex ligation the second signal comes from members of the B7 family. B7.1 (CD80) and B7.2 (CD86) are expressed on the surface of activated APCs. Members of the B7 family must ligate with CD28 on the surface of the T cell. An alternative ligand for the B7 family of molecules is CTLA-4 which following interaction with CD80/CD86 leads to the inhibition of T cell activation and results in T cell anergy. It is however, only expressed on the surface of activated T cells and Treg cells.

Approximately 48 hours after an encounter with its specific antigen, the naïve T cell enlarges into a blast cell and undergoes repeated rounds of division. The result is a population of effector T cells. There are two distinct populations of T cells.  $CD4^+$  T cells are known as T helper cells and recognise antigen presented on MHC class II molecules by professional antigen presenting cells of which there are three types-macrophages, B cells and dendritic cells.

The cytokine environment during antigen presentation determines the phenotype of an activated  $CD4^+$  T helper cell. Secretion of IL-12 by the APC induces Th1 cells. Th1 cells evolved to enhance the clearance of intracellular pathogens and are characterised by the production of IFN- $\gamma$  (5). IFN- $\gamma$ , activates the microbicidal activity of macrophages, enhances expression of MHC class II molecules and upregulates IL-12 production, which in turn induces more  $CD4^+$  T cells to differentiate into Th1 cells. IFN- $\gamma$  also induces B cells to produce opsonising IgG<sub>2a</sub> antibodies that bind to high affinity Fc receptors on the surface of macrophages and interact with the complement system. Ligation of CD40 ligand on the surface of the T helper cell and CD40 on the B cell is in most cases required for the survival of activated B cells. CD40 is also expressed on antigen presenting cells. Binding of CD40 on the APC to CD40L on an activated T cell transmits activating signals to the T cell, as well as activating the APC to upregulate B7 molecule expression, therefore stimulating further T cell proliferation. Three additional  $CD4^+$  T helper cell types- Th2 cells, IL-17 producing T cells (Th17) and regulatory T cells (Treg) are discussed in detail below.

T cells that express CD8 are known as cytotoxic T lymphocytes (CTLs) and are MHC class I restricted. Similarly to  $CD4^+$  T cells,  $CD8^+$  T cells can differentiate upon antigen stimulation into specific CTL subsets. Type 1 CTLs (Tc1 cells) are critical in the recognition and the

elimination of altered host cells such as virus infected cells or tumour cells. They have lytic capability and secrete high levels of IFN- $\gamma$ . Type 2 CTLs (Tc2 cells) produce IL-4, IL-5, IL-10 and TGF- $\beta$  and have been shown to induce tumour regression in mice with pulmonary tumours (6, 7). Furthermore, Tc2 cells can modulate DC function towards the induction of type 2 immune responses (7). IL-17 producing CD8<sup>+</sup> cells are known as Tc17 cells and have been shown to be protective during viral infection (8).

## 1.2 Pathogen Recognition Receptors

### 1.2.1 Toll like receptors

The immune system initially recognises pathogen components directly through various PRRs which are expressed on a variety of innate immune cells including DCs, macrophages, mast cells, neutrophils and endothelial cells. Toll like receptors (TLRs) are the most well known of the PRRs and recognize a vast array of pathogen associated molecules termed pathogen associated molecular patterns (PAMPs)- carbohydrates, lipids, proteins and nucleic acids. To date 13 murine TLRs and 10 human TLRs have been identified and each is specific for differing PAMPs. For example, TLR4 recognises LPS from Gram negative bacteria, TLR3 recognizes dsRNA and TLR2 recognizes various pathogen derived molecules including peptidoglycan, specific components of *Saccharomyces cerevisiae* zymosan and purified LPS from *Porphyromonas gingivalis* (9). TLR5 recognizes flagellin and TLR9 recognizes microbial DNA with hypomethylated CpG motifs. TLRs consist of multiple leucine rich repeats (LRRs) and one Toll IL-1 receptor homology (TIR) domain. The LRRs of TLR1, 2, 4, 5 and 6 are located on the outside of cell and the LRRs of TLR3, 7, 8 and 9 are found within cytosolic membrane compartments such as endosomes and lysosomes (10). TLRs share many of the same signalling components with IL-1 and IL-18 receptors. Ligation of TLRs activates NF- $\kappa$ B and MAP kinase signalling pathways and leads to the production of

pro-inflammatory cytokines including IL-1, TNF- $\alpha$  and IL-6 (11). These signalling pathways are discussed in more detail below

TLR ligation directs cytokine production by DCs and therefore plays a major role in influencing the antigen specific adaptive immune response. Classically TLRs have been perceived as inducers of Th1 responses and LPS, CpG and Poly I:C all drive IL-12p70 production by DCs (9). However, recent studies have shown that certain TLRs such as TLR2 also mediate Th2 responses. Both Pam3Cys and the schistosome derived lysophosphatidylserine (lyso-PS) bind to TLR2 and induce DCs that promote the differentiation of IL-4 and IL-10 producing T cells (12, 13). Interestingly, the filarial nematode ES product ES-62 binds to TLR4 and in contrast to LPS, inhibits the secretion of IL-12p70 thereby driving an anti-inflammatory/Th2 phenotype (14).

### **1.2.2. TLR independent pathogen recognition**

Other innate immune receptors include nucleotide-binding domain and leucine-rich repeat containing molecules (NLRs), RIG-I like receptors (RLRs) and C-type lectin receptors (CLRs).

NLRs are a series of cytosolic receptors that are characterized by the presence of a central NOD domain, a C-terminal LRR and either a caspase recruitment domain (CARD) or a PYRIN domain which are responsible for signalling (10). NOD1 and NOD2 possess a CARD domain and have been shown to recognise iE-DAP and MDP respectively (15). Both molecules are breakdown products of peptidoglycan. NLRs that are members of the NALP family contain PYRIN domains (10). The detection of microbial components by NALP1, NALP2 and NALP3 triggers the formation of large caspase-1 activating complexes termed

inflammasomes which in turn leads to the secretion of IL-1 $\beta$  and IL-18 (10). NALP3 has been shown to recognize LPS, lipoprotein, CpG, bacterial RNA and products of damaged cells such as uric acid crystals (10, 16-18). Agonists have also been identified for two other inflammasome forming NLRs, namely Ipaf and AIM2, which have been shown to recognise flagellin and cytosolic dsRNA respectively (19, 20).

The RLR family of PRRs are essential for the recognition of viruses in the cytoplasm and includes RIG-I and MDA5. RIG-I has been shown to recognise paramyxoviruses while MDA5 is responsible for the recognition of picornaviruses (21). Another family of PRRs consist of C-type lectins (C-TLs) which are calcium dependent carbohydrate binding proteins and include collectins, the mannose receptor family, selectins and DC specific ICAM-3 grabbing non-integrin (DC-SIGN). The C-TL, Dectin 1 has been reported to recognise beta-glucan which is found in fungal cell walls (22, 23). There is also evidence emerging that CT-Ls are involved in the recognition of helminths. Glycans from *Schistosomes mansoni* and *Toxocara canis* have both been shown to interact with DC-SIGN (24, 25). The effect of this ligation on the immune response against the parasites has yet to be established.

## **1.3 Intracellular Signalling**

### **1.3.1 Introduction to MAP Kinases**

The three major groups of MAP kinases include the extracellular signal-regulated protein kinases (ERK), the p38 MAP kinases and the c-Jun NH<sub>2</sub>-terminal kinases (JNK). These kinases have 60-70% sequence homology and differ in the sequence and size of their activation loop (26). In general, the ERK MAP kinases are activated by mitogenic and proliferative stimuli, whereas p38 and JNK MAP kinases are induced by environmental stress stimuli, such as inflammatory cytokines, UV light and osmotic shock (26). Each MAP kinase



subfamily consists of several isoforms, which often have distinct functions (26). Activation occurs by dual phosphorylation at the tripeptide motif, Thr-X-Tyr (27). Where X is glutamate in ERK, it corresponds to glycine in p38 and proline in JNK (27). The ERK MAP kinases are activated by the MAP kinase kinases (MKK), MKK1 and MKK2, the p38 MAP kinases are activated by MKK3, MKK4 and MKK6 and the JNK pathway by MKK4 and MKK7 (27). Further upstream are serine/threonine kinases called MAP kinase kinase kinases (MKKK), which are often activated by G proteins (27). The ERK pathway has been shown to be activated by Ras via the Raf group of MKKK. p38 and JNK MAP kinases can be activated by the Rho family of G proteins, including Rac and Cdc42 (27). It has recently emerged that p38 can be activated by an MKK independent mechanism involving TAB-1 (TGF- $\beta$  activated protein kinase 1 (TAK1) binding protein 1) (28). TAB-1 mediated activation was comparable to that of MKK6 (28). The TLR/IL-1 receptor family has also been shown to induce the MAP kinases p38 and JNK, as well as NF- $\kappa$ B (27).

Activated MAP kinases shuttle into the nucleus and initiate the rapid transcription of immediate-early genes through the phosphorylation of pre-existing transcription factors, in particular Elk1 or ATF-2 (29). MAP kinases also activate many downstream protein kinases including ribosomal S6 kinases (RSKs), mitogen and stress activated kinases (MSKs), MAP kinase interacting kinases (MNKs) and the real MAP kinase-activated protein kinases (MAPKAPs/MKs) (30). ERK MAP kinases activate RSKs. ERK and p38 MAP kinases activate MSKs (31). MNK1 and MNK2 are activated by both ERK and p38 MAP kinases (32). RSKs and MSKs have been shown to be involved in the regulation of gene expression at the transcriptional level by phosphorylating substrates, such as c-Fos and cAMP-responsive element binding protein (CREB)(RSKs) or histone H3(MSKs) (30). MNKs are involved in the regulation of translation by targeting the eukaryotic translation

factor (eIF)4E (30). There are three enzymes in the MAPKAP/MK subfamily-MK2, MK3 and MK5. MK2 has been shown to be essential for LPS induced upregulation of cytokine mRNA and is therefore necessary for the inflammatory response (33). MK3 regulates chromatin remodelling and MK5 is important in development (34, 35).

### **1.3.2 The role of p38 MAPK in the induction of pro-inflammatory cytokines**

The MAPK p38 is extremely important in the induction of a number of cytokines. Several p38 inhibitors have been shown to block the production of IL-1, TNF and IL-12 (36, 37). p38 activates MK2 and MK3 (38). Spleen cells from MK2<sup>-/-</sup> mice have severely decreased production of TNF- $\alpha$  and IFN- $\gamma$  in response to LPS (33). The production of IL-1 $\beta$  and IL-6 are also decreased when compared with cells from wild type mice (33). MK2<sup>-/-</sup> mice are resistant to LPS induced endotoxic shock (33). A potential mechanism for the role of p38 is through regulation at the post transcriptional level. The messenger RNAs for IL-1, MIP-2 and TNF are short lived and contain an AU-rich region. Normally AU binding proteins occupy the AU-rich region which results in the high turnover of the mRNA (26). Phosphorylation of the AU binding protein releases the mRNA which is then available for translation. The importance of AU-rich elements (ARE) found in the 3' UTR of TNF- $\alpha$  mRNA was demonstrated by the development of mice that lack the ARE of TNF- $\alpha$ . Studies performed on macrophages from these mice showed that p38 directly interacts with the ARE of TNF mRNA and permits its translation (39). Zhao *et al* have recently investigated the induction of IL-6 expression by IL-1 and reported that p38 MAP kinase induced by IL-1 is necessary for the stabilization of IL-6 mRNA, via its multiple ARE (40). A similar mechanism may be utilized in the regulation of other cytokines by p38. In macrophages, IL-10 suppresses the production of TNF- $\alpha$  by the inhibition of p38 MAP kinase activation of MK2 mediated ARE activity (41). Lu *et al* have shown that MKK3 activation of p38 is

required for the induction of IL-12 by LPS in both macrophages and DC (37). TNF- $\alpha$  signalling also induces MKK3, which then activates p38 MAPK. TNF- $\alpha$  stimulation of mouse embryonic fibroblasts from MKK3<sup>-/-</sup> mice resulted in a failure to upregulate IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$  mRNA or downregulate IL-1 receptor antagonist (IL-1Ra) mRNA (42). Another potential mechanism behind p38 MAPK induction of pro-inflammatory cytokines is phosphorylation of histone H3, which has been shown to mark and recruit NF- $\kappa$ B to otherwise cryptic promoters resulting in cytokine expression (26).

### **1.3.3 The role of ERK MAPK in the induction of cytokines**

ERK MAP kinase has a role in the regulation of LPS induced TNF- $\alpha$ . Macrophages from mice deficient in the MKKK Tpl2/Cot are defective in TNF- $\alpha$  production (43). The transport of TNF- $\alpha$  mRNA from the nucleus to the cytoplasm was inhibited by an ERK inhibitor, suggesting a role for ERK. The activation of ERK has also been shown to inhibit LPS induced production of IL-12p70 which contrasts with the proposed role of p38 and JNK MAPKs in the cytokines induction (44-46). The TLR2 agonist Pam3cys has been shown to stimulate sustained ERK MAPK activation in human DCs (45). ERK MAPK stabilizes the transcription factor c-fos, which functions as a suppressor of IL-12 (45). The inhibition of c-fos expression using siRNA interference led to an enhancement of IL-12p70 induction by Pam3cys (45). Studies have also been carried out in murine macrophages and show that calcium influxes activate ERK MAPK which then inhibits LPS-induced IL-12p40 transcription by suppressing the synthesis of the transcription factor interferon regulatory factor-1 (IRF-1) (46). IL-23 is composed of the subunit p19 and the p40, which is common to IL-12p70. IL-12p40 inhibition by calcium influx suggests that ERK MAPK may regulate production of IL-23. Goodridge *et al* also showed that inhibition of IL-12 by ES-62 a secretory product of the fimbrial nematode *Acanthocheilonema viteae* was reversed by the

addition of the ERK MAPK inhibitor PD 98059 (46). Interestingly TGF- $\beta$  inhibits p38 MAP kinase mediated pro-inflammatory cytokine production by ERK activation. TGF- $\beta$  induced ERK upregulates MAPK phosphatase 1, which inactivates p38 MAPK (47).

#### **1.3.4 p38 MAPK activation induces IL-10 production**

The evidence discussed above is part of a large body of data that indicates that p38 plays a crucial role in the induction of pro-inflammatory cytokines, whereas ERK MAP kinase is involved in their suppression. p38 inhibitors have been shown to block the production of IL-1, TNF and IL-6 in vitro and in vivo and have also been shown to be efficacious in reducing inflammation in animal models of rheumatoid arthritis (48). There is however substantial evidence that p38 also plays a role in the induction of the anti-inflammatory cytokine IL-10. The infection of human macrophages infected with *Mycobacterium avium* activates MAP kinases through CD14 (49). Using MAP kinase inhibitors, Reiling *et al* showed that ERK activation is responsible for TNF- $\alpha$  production, whereas p38 signalling was responsible for IL-10 production (49). Human monocytes treated with LPS produce IL-1, TNF- $\alpha$  and IL-10. Foey *et al* reported that the induction of IL-10 was IL-1 and TNF- $\alpha$  dependent and that IL-10, IL-1 and TNF- $\alpha$  production was inhibited by the p38 MAP kinase inhibitor SB203580. Inhibition of ERK MAPK partially blocked IL-1 and TNF- $\alpha$ , but had no effect on IL-10 production (50). Ma *et al* showed that in human macrophages, p38 mediates induction of IL-10 by the activation of the transcription factor Sp1 (51). A52R, a vaccinia virus protein has been shown to modulate the host immune response by inhibiting NF- $\kappa$ B activation (52). The viral protein has also been shown to enhance TLR-induced p38 or JNK MAP kinases (52). A52R interacts with TRAF6 to p38 which enhances LPS mediated IL-10 production (52).

### **1.3.5 Role of MAPK in DC maturation**

MAP kinases have been shown to be important in the maturation of DC (44). DC maturation is accompanied by the upregulation of the co-stimulatory molecules CD40, CD80 and CD86 and the adhesion molecules CD54 and CD58 (44). The p38 inhibitor SB203580 has been shown to inhibit LPS or TNF- $\alpha$  induced maturation of DCs, whereas the ERK inhibitors U0126 and PD98059, enhance maturation (44, 53, 54). In summary p38 MAPK induces DC maturation and ERK MAPK inhibits it.

### **1.3.6 Dual specificity phosphatases**

The activity of MAP kinases can be downregulated by dual specificity phosphatases (DUSPs), that regulate MAPK activity through TXY motif dephosphorylation (55). There are ten mammalian DUSPs identified to date. DUSP1, DUSP2, DUSP4 and DUSP5 localize to the nucleus and are induced by growth or stress signals. DUSP6, DUSP7 and DUSP9 are cytoplasmic and preferentially recognize ERK MAPK. DUSP8, DUSP10 and DUSP16 preferentially recognize p38 and JNK MAPK (56). DUSP1 regulates p38 and JNK activity. DUSP1<sup>-/-</sup> mice are more susceptible to LPS induced endotoxic shock with increased IL-6 and TNF- $\alpha$  production (57). DUSP2 (also called phosphatase of activated cells 1 (PAC-1)), is only expressed in leukocytes, where it is high expressed in activated immune cells (58). Cells from PAC-1<sup>-/-</sup> mice had increased activity of JNK MAPK but unexpectedly impairment of ERK and p38 MAPK activity (58). PAC-1<sup>-/-</sup> mice also had decreased immune effector cell function suggesting that PAC-1 is necessary for inflammatory MAPK signalling (58).

### **1.3.7 Pathogen manipulation of MAPK signalling**

Pathogens have evolved mechanisms of manipulating MAP kinase signalling pathways. The induction of p38 MAP kinase by A52R allows the vaccinia virus to modulate the immune

response (52). *Yersinia enterocolitica* has been shown to suppress TNF- $\alpha$  by inhibiting the activity of ERK, p38 and JNK MAP kinases (59). Ibata-Ombetta *et al* reported that *Candida albicans* activates MAPK phosphatase-1 (MKP-1), which down regulates ERK MAPK activation (60). The authors suggest that as the phagocytosis of the yeast by macrophages is ERK dependent, the induction of the phosphatase allows the yeast to escape macrophage killing (60). IL-12, IFN- $\gamma$  and IL-10 are all induced following activation of CD40 on the surface of macrophages (61). Mathur *et al* showed that weak stimulation of CD40 results in ERK MAPK induction and IL-10 production, whereas stronger signals activate p38 MAPK which results in IL-12 production (61). The parasite *Leishmania major* skews CD40 signalling towards ERK MAPK activation which results in IL-10 production and ineffective parasite clearance (61).

### **1.3.8 NF- $\kappa$ B**

NF- $\kappa$ B is an extremely important transcription factor in the regulation of the immune system. It plays a leading role in the innate immune system, where it regulates many cytokines, chemokines, adhesion molecules and inducible enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (62, 63). In particular, NF- $\kappa$ B signalling is involved in the production of IL-1, TNF- $\alpha$ , IL-6, TNF- $\beta$  and IFN- $\gamma$  (62). IL-1 and TNF- $\alpha$  can directly activate the NF- $\kappa$ B pathway, therefore establishing a positive autoregulatory loop that can amplify the immune response (63). NF- $\kappa$ B also regulates MHC and co-stimulatory molecule expression which are crucial for the induction of the adaptive immune response (62). NF- $\kappa$ B is composed of many dimeric complexes of the Rel Protein family- Rel (c-Rel), Rel A (p65), Rel B, NF- $\kappa$ B1(p50 and its precursor p105) and NF- $\kappa$ B2 (p52 and its precursor p100) (11). The most common dimer is p50-p65. In resting cells, NF- $\kappa$ B proteins are predominantly cytoplasmic and are associated with members of the inhibitory I $\kappa$ B family,

such as I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$  and I $\kappa$ B- $\epsilon$  which bind the transcription factor and prevent its DNA-binding activity (11). The release of NF- $\kappa$ B depends on the degradation of I $\kappa$ B, which is initiated by a complex comprising IKK (I $\kappa$ B kinase) $\alpha$ , IKK $\beta$  and a scaffold protein IKK $\gamma$ /NEMO. The complex phosphorylates two key N-terminal serines on I $\kappa$ B. E3<sup>I $\kappa$ B</sup> ubiquitin ligase complex  $\beta$ -TrCP-SCF then polyubiquitinylates I $\kappa$ B and targets it for degradation by the 26S proteasome (64). Activators of the IKK complex include, MKKK1, MKKK3 and TAK-1 (62). There are numerous stimuli further upstream including ligation of the TLR, IL-1/IL-18 receptors, NLRs, the TNF receptor superfamily and B and T cell receptors (62).

These receptors share a conserved Toll/IL-1R (TIR) domain which recruit TIR domain containing adaptor proteins. Four adaptor proteins have been well characterised- MyD88, Mal, TRIF and TRAM and are involved in the induction of NF- $\kappa$ B. With the exception of TLR3, MyD88 is recruited by all of the TLRs. TLR3 recruits TRIF and TLR4 recruits all of the aforementioned TIR adaptor proteins (65). The adaptors recruit and activate members of the IRAK family. MyD88 initially recruits IRAK-4 which associates with Toll interacting protein (Tollip). IRAK-4 in turn recruits IRAK-1. IRAK-1 then disassociates with MyD88 and Tollip, and interacts with TRAF-6. The interaction of IRAK-1 and TRAF-6 leads to the activation of TAK-1 (66). TRAF-6 can activate other MKKKs that stimulate the IKK complex and MKKK3 has been shown to be essential for activation of IKKs by TLR4 or the IL-1R (67). A fifth TIR domain containing adaptor protein SARM has been identified. SARM does not activate NF- $\kappa$ B, but functions as a negative regulator of TRIF mediated TLR signalling (68).

The TNF receptor superfamily can also activate NF- $\kappa$ B. Some members of the receptor family contain death domains, including TNFR1 and Fas which activate NF- $\kappa$ B by the recruitment of TRADD, which associates with TRAF2 and RIP (69). TRAF2 recruits the IKK complex and RIP then activates it via MKKK3 (70). Members of the receptor family that lack a death domain such as TNFR2 and CD40 directly recruit TRAF proteins (69)..

### **1.3.9 Pathogen manipulation of NF- $\kappa$ B signalling**

As with MAP kinase signalling many pathogens manipulate NF- $\kappa$ B signalling pathways to their own advantage. The viral protein A52R has been shown to block the activation of NF- $\kappa$ B by associating with IRAK-2 and TRAF-6 (71). Many effector proteins of *Y. enterocolitica* including YopP, are delivered by a Type III secretion system into the host cell (72). YopP has been shown to bind and inhibit IKK $\beta$  thereby preventing NF- $\kappa$ B activation (72). Other pathogens have been shown to induce NF- $\kappa$ B activation. *Shigella flexneri* triggers sustained activation of NF- $\kappa$ B in the gut, which leads to the disruption of the epithelial layer and easier invasion by the bacteria (73).

### **1.3.10 The interferon regulatory factor family**

The interferon regulatory factor (IRF) family has been shown to play a crucial role in TLR induced cytokine production. MyD88 deficient mice lack the capacity to produce the pro-inflammatory cytokines TNF- $\alpha$ , IL-1, IL-6 and IL-12p40 in response to LPS (74). The TIR adaptor proteins TRIF and TRAM are still functional in the MyD88 deficient mice and they retain the ability to induce IFN-inducible genes such as RANTES and IP-10 and to upregulate co-stimulatory molecules (75). DCs from MyD88 deficient mice mature in response to LPS (75). TLR3 signals through TRIF, while TRAM acts as a bridging adaptor between TRIF and TLR4 (76). TLR3 or TLR4 ligation activates IKK-i and TBK-1 via TRIF which results



in the phosphorylation of IRF-3 (77). IRF-3 primarily activates the IFN- $\beta$  promoter. IFN- $\beta$  is produced and induces the expression of IRF-7 which becomes activated and stimulates IFN- $\alpha/\beta$  promoters, thereby establishing a positive feedback loop (78).

Plasmacytoid DCs produce extremely high levels of type I IFNs and this occurs by TLR7/TLR9 activation of IRF7, which is MyD88 dependent (79). IRF-5 has been shown through the use of IRF-5 deficient mice to be crucial in the induction of TNF- $\alpha$ , IL-6 and IL-12 by TLR3, TLR4, TLR5, TLR7 and TLR9 (80). IRF-5 binding sites are present in the promoters of several pro-inflammatory cytokines (80). It has been proposed that IRF-5 and NF- $\kappa$ B may both function co-operatively (80). IRF-5 has been shown to associate with both MyD88 and TRAF-6 but the exact mechanism of its activation awaits further elucidation (80). Liu *et al* have investigated the roles of IRFs in IL-12p70 production from innate immune cells. Macrophages from IRF8 deficient mice were found to be highly defective in IL-12p70 production and IRF1 and IRF8 were shown to synergistically stimulate the IL-12p35 promoter (81).

### **1.3.11 STAT and Smad pathways**

Other pathways important in cytokine signalling include the Smad and STAT pathways. Binding of interleukins, interferons and haemopoietins to their receptors results in homodimerization and the activation of receptor associated JAK kinases. The JAK kinases then phosphorylate tyrosines on the receptors cytoplasmic domains creating docking sites for signal transducers and activators of transcription (STATs) (82). While docked to the receptor subunits the STATs undergo JAK catalyzed phosphorylation. STATs then disassociate from the receptor, dimerize and translocate to the nucleus where they function as transcription factors. Different STATs are induced by different cytokines. STAT-1 is induced by IFN- $\gamma$ ,

STAT-3 by both IL-10 and IL-6, STAT-4 by IL-12 and STAT-6 by IL-4 (82). STAT signal transduction is negatively regulated by a family of inhibitor proteins known as suppressors of cytokine signalling (SOCS) (83). The Smad family of proteins are induced by TGF- $\beta$  receptors type I and II (82). The type I receptor recognizes and phosphorylates Smad2 and Smad3 which associates with Smad4 to form complexes that participate in DNA-binding and transcription factor recruitment (82). Smad6 and Smad7 are inhibitory and bind to activated receptors and inhibit Smad 2 and Smad3 binding (84).

### **1.3.12 Cyclic adenosine monophosphate (cAMP) signalling.**

Most signalling molecules are too large and too polar to pass across the cell membrane and therefore rely on membrane associated receptors to transfer information to the intracellular domain. Second messengers relay information from the ligand bound receptor by activating protein kinases or transcription factors. cAMP was the original second messenger to be discovered and it is involved in many signal transduction pathways throughout the body. Remarkably most of the effects of cAMP are achieved by the activation of one protein kinase- protein kinase A (PKA). The generation of cAMP is initiated when an extracellular first messenger binds to a seven transmembrane helix G protein coupled receptor (GPCR) that is coupled to a stimulatory G protein  $\alpha$  subunit. The  $\beta$  adrenergic receptor induces cAMP through this pathway and provides a good example. The binding of adrenalin results in a conformational change in the intracellular domains of the receptor which then activates a G protein. The activated  $G\alpha$  subunit then binds to the membrane bound enzyme adenylyate cyclase and stimulates its enzymatic activity. Adenylyate cyclase converts ATP to cAMP.

An increase in the intracellular cAMP concentration to 10 nM results in the activation of PKA. Activated PKA then phosphorylates serine and threonine residues in many target

proteins to alter their activity. Adrenaline induced PKA activates phosphorylase kinase which is an enzyme involved in glycogen breakdown. PKA also phosphorylates cyclic AMP response element binding protein (CREB), which is a transcription factor that binds to specific DNA sequences as a dimer. Recently PKA independent intracellular targets were identified and include cyclic nucleotide-gated channels and two isoforms of exchange protein directly activated by cAMP (Epac) (85). Epac 1 is widely distributed in cells throughout the body whereas Epac 2 has limited tissue distribution (86). cAMP has powerful effects on cellular processes and levels of the second messenger are tightly regulated by a variety of mechanisms. 10 isoforms of adenylate cyclase have been characterized which are differentially expressed in different cell types (87). Additionally, phosphodiesterases are a group of enzymes that degrade cAMP and therefore also regulate cAMP levels (88). Increases in intracellular cAMP concentration have in general been shown to inhibit immune cell function. Much research has therefore investigated the effects of cAMP inducing agents on immune cells in order to better understand intracellular immune cell signalling and to identify potential therapeutic agents for immune mediated diseases.

### **1.3.13 Prostaglandin E<sub>2</sub>**

Prostaglandins are members of the eicosanoid family and are found ubiquitously in cells throughout the body (89). They are synthesized from arachidonic acid via the actions of the COX 1 and 2 enzymes either constitutively or in response to cell stimuli (89). The most abundant prostanoid in the body is prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) which is primarily synthesized by the COX-2 enzyme (89). Its effects are varied and it has been shown to induce both pro-inflammatory and anti-inflammatory responses as well as mediating homeostasis. Inhibition of PGE<sub>2</sub> synthesis has been an important anti-inflammatory therapy for more than 100 years (89). Like the  $\beta$  adrenergic receptor, prostanoid receptors are seven transmembrane helix

GPCRs (90). The cell membrane receptors for PGE<sub>2</sub> are called the EP receptors, of which there are four subtypes, namely EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>4</sub> (91). All of the EP receptor subtypes are expressed on the cell surface and EP<sub>3</sub> and EP<sub>4</sub> are also localized on the nuclear membrane (91). In the mouse, the rank order of affinity of PGE<sub>2</sub> for the EP subtypes is EP<sub>4</sub> > EP<sub>3</sub> > EP<sub>1</sub> > EP<sub>2</sub> (91). Each EP subtype results in the initiation of specific signalling pathways. Ligation of EP<sub>1</sub> activates phospholipase C resulting in the generation of the second messengers inositol triphosphate, diacylglycerol and Ca<sup>2+</sup> (91). EP<sub>4</sub> and EP<sub>2</sub> activate adenylate cyclase resulting in PKA signalling (91). Both these EP subtypes have also been shown to initiate the phosphoinositide-3-kinase (PI3K) signalling pathway (92). The EP<sub>3</sub> receptor can activate G<sub>i</sub> subunits, which inhibit adenylate cyclase activity and G<sub>s</sub> subunits which activate the enzyme (91).

#### **1.3.14 The effect of PGE<sub>2</sub> on the immune cell system**

There have been many studies performed that have established the anti-inflammatory properties of PGE<sub>2</sub> *in vivo*. Yin *et al* have shown the protective effect of the molecule in a mouse model of immune mediated liver injury. The treatment of COX-2<sup>-/-</sup> mice with Concanavalin A (Con A) resulted in increased liver damage when compared with the damage seen in COX1<sup>-/-</sup> mice (93). The PGE<sub>2</sub> analog misoprostol had a protective effect on administration to the Con A treated COX-2<sup>-/-</sup> mice (93). Furthermore, *in vitro* studies demonstrated the ability of misoprostol to inhibit IFN- $\gamma$  production from Con A treated liver derived COX-2<sup>-/-</sup> NK, NKT and T cells (93). Nomi *et al* investigated the effects of specific EP receptor agonists in a murine cardiac transplantation model and found that both EP<sub>2</sub> and EP<sub>4</sub> agonists inhibited acute graft rejection (94). In a mouse model of mammary carcinoma, PGE<sub>2</sub> produced by the tumour was shown to inhibit the production of IL-12 which is a cytokine associated with tumour clearance (95). The administration of a COX-2 inhibitor to

tumour bearing mice resulted in a reduction in tumour metastases in the lung (95). Peritoneal macrophages taken from these mice produced higher levels of IL-12 in response to LPS and IFN- $\gamma$  when compared with cells from mice that had not been treated with the inhibitor (95). The inhibition of IL-12 production by PGE<sub>2</sub> was mediated by the activation of activator protein 1 (AP-1) (95). A previous study by Procopio *et al* reported that the inhibition of LPS induced IL-12 by PGE<sub>2</sub> was IL-10 dependent (96). They also showed that PGE<sub>2</sub> inhibited LPS induced TNF- $\alpha$  production (96).

cAMP analogs that are highly specific for PKA and Epac-1 have been used in an attempt to elucidate the intracellular effects of PGE<sub>2</sub> on alveolar macrophages by Marc Peters Golden and colleagues. They reported that PGE<sub>2</sub> inhibited LPS induced TNF- $\alpha$  and MIP-1 $\alpha$  and augmented LPS induced IL-6 and IL-10 (97). These effects on LPS induced cytokine production were dependent on PKA activation (97). Furthermore PGE<sub>2</sub> inhibited Fc $\gamma$ R mediated phagocytosis and this effect was shown to be dependent on Epac-1 activation (98). They also studied the effects of the PKA and Epac-1 specific agonists on DC cytokine production. Both agonists inhibited LPS induced MIP-1 $\alpha$  and TNF- $\alpha$  and the PKA agonist inhibited LPS induced IL-6 (97).

Investigations have also been carried out on the effect of PGE<sub>2</sub> on the ability of DCs to induce T cell differentiation. LPS stimulation of human monocyte derived DCs developed in the presence of PGE<sub>2</sub> resulted in reduced levels of IL-12 and enhanced IL-10 production (99). Furthermore, DCs developed in the presence of PGE<sub>2</sub> promoted the development of Th2 cells whereas DCs developed in the absence of PGE<sub>2</sub> promoted Th1 cell development (99). The ability of PGE<sub>2</sub> to activate DCs that promote Th2 cells was directly attributed to the inhibition of innate IL-12 production (99).

Two reports have shown that PGE<sub>2</sub> produced by tumours induces Tr1 cells thereby creating a tolerogenic environment (100, 101). Akasaki *et al* showed that human DCs that phagocytose COX-2 overexpressing tumour cells produce more IL-10 and less IL-12p70 and induce a Tr1 response (100). The Tr1 cells produced high levels of IL-10 and TGF- $\beta$  and inhibited the proliferation of autologous lymphocytes by an IL-10 dependent mechanism (100). The ability of the tumour treated DCs to induce Tr1 cells could be replicated by the addition of PGE<sub>2</sub> (100). It has also been reported that LPS activated monocytes produce PGE<sub>2</sub>, which upregulates Foxp3+ expression in CD4<sup>+</sup>CD25<sup>-</sup> cells (102). In contrast to the evidence proposing a suppressive effect of PGE<sub>2</sub> on the immune system, Ganea and colleagues have reported that PGE<sub>2</sub> exacerbates CIA and IBD through the induction of Th17 cells (103, 104). They reported that PGE<sub>2</sub> promotes IL-23 and inhibits IL-12p70 production by DCs stimulated with LPS (104).

### **1.3.15 The effect of forskolin and dibutyryl cAMP on the immune system**

Forskolin is a cAMP activator that was originally derived from the Indian Coleus plant. It binds directly to adenylate cyclase and has therefore been used as a tool to quantify the number of adenylate cyclase molecules within a cell (105). Since forskolin can induce cAMP, it has been studied for its ability to modulate immune cell function. Dahle *et al* have shown that forskolin inhibits TNF- $\alpha$  and IL-10 production by LPS stimulated rat Kupffer cells (106). Furthermore PGE<sub>2</sub> inhibited LPS induced TNF- $\alpha$  but had no effect on LPS induced IL-10 production by Kupffer cells (106). The authors reported that Kupffer cells express high levels of the forskolin insensitive adenylate cyclase 9 and proposed that this may explain the differing effects of alternate cAMP inducing agents (106). Woo *et al* have studied the effects of forskolin on LPS stimulated microglia and reported that it inhibits TNF-

$\alpha$ , IL-6 and iNOS expression, while enhancing IL-10 production (107). The enhancement of LPS induced IL-10 was dependent on PKA signalling (107).

The cell permeable cAMP analog dibutyryl cAMP has also been studied for its effect on immune cell function. *In vivo* administration of dibutyryl cAMP has been shown to protect mice from TNF- $\alpha$ /D-galactosamine induced liver injury (108). Egawa *et al* reported that LPS pretreatment of murine macrophages enhanced their ability to inhibit the intracellular growth of *Legionella pneumophila* (109). The anti-*L. pneumophila* activity of the LPS treated macrophages was inhibited by dibutyryl cAMP (109).

Other studies have examined the role of intracellular signalling pathways involved in dibutyryl cAMP mediated immune suppression. Dibutyryl cAMP treatment of LPS stimulated microglia decreased TNF- $\alpha$  expression and enhanced IL-10 and IL-1 $\beta$  production (110). Dibutyryl cAMP was shown to inhibit p38 activation while enhancing ERK phosphorylation (110). The analog potentiated the activity of AP-1 by enhancing c-fos binding (110). Furthermore, dibutyryl cAMP repressed LPS stimulated NF- $\kappa$ B transcriptional activation (110).

### **1.3.16 The activation of cAMP production by pathogens**

Increasing concentrations of intracellular cAMP suppress the immune system and pathogens have therefore devised systems to induce cAMP as part of their immune evasion strategies. The bacteria *Vibrio cholerae* produces an enterotoxin, cholera toxin (CT), which consists of an A subunit noncovalently linked to a pentamer of cell binding B subunits (111). The B subunits facilitate the intracellular entry of the A subunit. The A subunit activates a G protein  $\alpha$  subunit which stimulates adenylate cyclase to produce cAMP (111). Lavelle *et al*

reported that the stimulation of DCs with CT inhibits LPS induced IL-12, TNF- $\alpha$ , MIP-1 $\alpha$ , MIP-1 $\beta$  and MCP-1 and enhanced LPS stimulated IL-10 production (112). Parenteral immunization of mice with CT and a model antigen induced Tr1 cells, which suppressed Th1 cells (112). Adenylate cyclase toxin (CyaA) is produced by *Bordetella pertussis* and is one of a number of virulence factors that are essential for bacterial colonization (113). CyaA specifically binds to the  $\alpha_M\beta_2$  integrin (CD11b/CD18) on the surface of macrophages and DCs (114). This allows the enzyme to enter the cell cytoplasm (114). CyaA is then activated by calmodulin and begins producing supraphysiological levels of cAMP (114). In common with the studies carried out on CT, CyaA enhanced TLR ligand induced IL-10 and inhibited IL-12 and TNF- $\alpha$  production in macrophages and DCs (115). CyaA also promoted the induction of Th2 and Treg cells (115). The immunomodulatory properties of CyaA were dependent on the toxins ability to generate cAMP (115). An additional study has reported that CyaA inhibits TLR induced IRF-1 and IRF-8 activation, which provides a potential mechanism for the ability of the toxin to inhibit IL-12 production (116).

## **1.4 Th2 Cells**

### **1.4.1 Introduction to Th2 Cells**

Th2 cells are involved in humoral immunity against extracellular pathogens and are characterized by the production of IL-4, IL-5, IL-9 and IL-13. These cytokines promote B cells to produce large amounts of IgM, IgE and non-complement activating IgG isotypes and also activate eosinophils which along with IgE antibodies are important in parasite clearance.. Th2 cells also play a role in the induction of asthma and other allergic diseases.



### **1.4.2 The induction of Th2 cells**

The DC derived cytokines and co-stimulatory molecules that are required for Th1 cell differentiation have been well documented. However, the events that lead to Th2 cell differentiation are somewhat less clear. One theory suggests that in the absence of IL-12p70 production, DCs promote Th2 cell development by default (117). It is clear that IL-4 promotes the differentiation of Th2 cells (5). However DCs do not make this cytokine. A recent study has reported that IL-4 producing basophils support the induction of Th2 cell differentiation (118). Many studies have highlighted the role that cell surface molecules play in the induction of Th2 cells. Amsen *et al* have shown that the Notch ligand families delta and jagged which are expressed on the surface of DCs influence Th1 and Th2 cell differentiation respectively (119). MacDonald *et al* have shown that the interaction between CD40 on the DC and CD154 on the naive T cell is crucial for the development of Th2 cells (120). Another interaction of importance in the induction of Th2 responses is the binding of OX40 and OX40L. Jember *et al* have reported that OX40<sup>-/-</sup> mice have a severely reduced ability to generate a Th2 response and develop significantly reduced inflammation in a model of allergic asthma (121).

### **1.4.3 Th2 cell differentiation**

The differentiation of an effector Th2 cell is dependent on TCR signalling and IL-4 receptor mediated STAT6 activation which in turn leads to the upregulation of GATA3 transcription (122). GATA3 is the master transcription factor of Th2 cell differentiation and enforced expression of GATA3 in Th1 cells has been shown to result in the induction of IL-4 producing Th2 cells (123). GATA3 serves as transcription factor for the Th2 cytokine genes namely IL-4, IL-5 and IL-13 and Yamashita *et al* have shown that the depletion of the GATA3 gene in Th2 cells *in vitro* leads to a decrease in the production of Th2 type cytokines

and an increase in IFN- $\gamma$  suggesting that GATA3 serves as a principal switch in determining Th1 and Th2 responses (124).

#### **1.4.4 The role of Th2 cells during helminth infection**

Helminths are the main examples of Th2 cell inducers in both humans and animals. Secondary infection with the intestinal nematode parasite *Heligmosomoides polygyrus* results in a highly polarized Th2 response which leads to the expulsion of the worm (125). The activation of parasite specific memory CD4<sup>+</sup> Th2 cells initially drives an immune response against the parasite larva which reside in the gut submucosa (126). Neutrophils and alternatively activated macrophages are recruited and enclose the parasite in a granuloma (126). The Th2 cytokines IL-4, IL-13, IL-10 and IL-21 all induce alternative activation of macrophages which are identified by high expression of arginase-1, IL-4R $\alpha$ , CD206 and a lack of iNOS expression (127, 128). IL-4 and IL-13 produced by the memory CD4<sup>+</sup> T cells may also induce effects in the cells of the small intestine. *H. polygyrus* infection induces STAT6 dependent changes in epithelial cell function, increased smooth muscle contractibility and increased mucus production (129). It has been proposed that this weep (increased fluids) and sweep (increased muscle contractibility) approach creates an inhospitable environment for the helminth and results in live parasite expulsion (125).

In contrast to *H. polygyrus* infection, the blood fluke *S. mansoni* initially induces a Th1 response (125). However the immune response is modified to a Th2 phenotype once the helminth produces eggs (125). This transition to a Th2 type response is critical as it suppresses an otherwise pathologically damaging Th1 responses which can lead to the death of the host (125). Indeed wild type mice become chronically infected after 8 weeks whereas

IL-4 deficient mice die due to uncontrolled TNF- $\alpha$  production (130). Interestingly both groups have similar numbers of adult worms and eggs (130).

## 1.5 Th17 cells

### 1.5.1 An introduction to Th17 cells

CD4<sup>+</sup> effector T cells that produce IL-17 have been termed Th17 cells (131). Th17 cells are involved in the immune response to infection but have arguably received more attention for the major role they have in autoimmune inflammation. It was initially believed that IL-23 acted as a differentiation factor for the induction of Th17 cells. IL-23 is a heterodimeric cytokine composed of a unique p19 subunit and a common p40 subunit shared with IL-12 (132). However, evidence has since emerged that TGF- $\beta$  and IL-6 are the cytokines responsible for the differentiation of naïve T lymphocytes into effector Th17 cells and that IL-23 plays a role in the expansion and survival of the cell type (131, 133-137). Th17 cells produce IL-17, IL-17F, IL-6, TNF- $\alpha$ , IL-22, IL-21 and CCL20 (also known as MIP3 $\alpha$ ) (138-142). T helper cell commitment is mediated by lineage specific transcription factors and two transcription factors have been shown to regulate Th17 cell differentiation. ROR $\gamma$ t and ROR $\alpha$  are induced by TGF- $\beta$  and IL-6 and have both been reported to promote Th17 cell differentiation when Th1 and Th2 cell differentiation is inhibited (143, 144). Zhang *et al* have recently identified a key role for the transcription factor Runx1 in the activity of ROR $\gamma$ t (145). Runx1 induces the transcription of ROR $\gamma$ t and binds to and acts with ROR $\gamma$ t during IL-17 transcription (145). Interestingly, Runx1 has also been shown to interact with Foxp3 and this interaction is required for the inhibitory effect of Foxp3 on Th17 cell differentiation (145). Langrish *et al* compared the gene expression profiles of effector Th1 and Th17 cells developed using recombinant IL-12 and IL-23 respectively. 80% homology was seen in the genes expressed. Both cell types expressed high levels of IL-12R $\beta$ 1. Th1 cells expressed

high levels of IL-12R $\beta$ 2, IFN- $\gamma$ , Granzyme F and G, TRAIL, BLYS, TRAILR2 and FASL (138). Many of these genes are involved in host defence and cytotoxicity. Th17 cells expressed high levels of IL-23R, Integrin  $\alpha$ 3, CCL7, CCL17, CCL20, CCL22, CCR1 and GM-CSF (138). Unlike the Th1 cells, expression of IL-12R $\beta$ 2, IFN- $\gamma$  and Granzyme G were all dramatically reduced in Th17 cells (138).

### **1.5.2 IL-17A**

The hallmark cytokine produced by Th17 cells is IL-17 which is associated with many inflammatory diseases, such as rheumatoid arthritis, asthma, systemic lupus erthematosus (SLE) and allograft rejection (146). IL-17 signals through the IL-17 receptor A (IL-17RA). The receptor is distributed ubiquitously in various tissues on mesenchymal cells, such as epithelial, endothelial and fibroblastic cells (147). Its engagement leads to NF- $\kappa$ B and MAP kinase activation (148). Chang *et al* have shown that the adaptor protein ACT1 is essential for IL-17RA mediated NF- $\kappa$ B activation (149). Early studies performed by Fossiex *et al* showed that the stimulation of human fibroblasts with recombinant IL-17 induced IL-6, IL-8, G-CSF and PGE<sub>2</sub> production (150). Park *et al* have since shown that murine embryonic fibroblasts treated with IL-17 upregulate approximately 60 genes which included the chemokines CCL2, CCL7, CCL20, CXCL1 and the matrix metalloproteinases 3 and 13 (151).

### **1.5.3 IL-17F**

When compared with IL-17A, less is known about the function of IL-17F. Like IL-17A, IL-17F signals through the IL-17RA and induces the expression of cytokines and chemokines from murine embryonic fibroblasts (152). The exact function of IL-17F *in vivo* is still being elucidated, but initial findings suggest it has a different role to IL-17A. Yang *et al* developed

mice deficient in IL-17A and IL-17F respectively. Consistent with the role of IL-17A in autoimmune disease, mice deficient in IL-17A had a significant delay in the onset and progression of the model autoimmune disease EAE. Mice deficient in IL-17F only had moderately reduced disease severity when compared with wild type mice (152). Furthermore mice deficient in IL-17F but not IL-17A have defective airway neutrophilia in response to allergen challenge (152). Wantanabe *et al* have identified a role for IL-17F in the neutrophilia that results in psoriasis (153). IL-17F induced higher levels of IL-8 production by epidermal keratinocytes than IL-17A and furthermore, IL-17F was shown to be upregulated in lesional psoriatic skin (153). In an attempt to investigate the role of T cell derived IL-17A and IL-17F in experimental colitis, Leppkes *et al* transferred IL-17A, IL-17F or ROR $\gamma$  deficient T cells into RAG1 null mice. The mice that received the IL-17A or IL-17F deficient cells developed colitis (154). Interestingly, increased levels of IL-17F were observed in the animals that received IL-17A<sup>-/-</sup> T cells and mice that received IL-17F<sup>-/-</sup> cells and an anti-IL-17A antibody developed significantly reduced disease (154). These data suggest that cytokine redundancy exists in this disease model.

#### **1.5.4 IL-22, IL-21 and MIP3 $\alpha$**

IL-22 is a member of the IL-10 family of cytokines and is also produced by effector Th17 cells (155). It functions as a pro-inflammatory cytokine and induces the expression of  $\beta$  defensin 2 and 3 through STAT3 signalling (156). It also mediates IL-23 induced dermal inflammation (139). IL-21 is a member of the common gamma chain cytokine family along with IL-2 (131). It is produced by Th17 cells upon stimulation with IL-6 and has been shown to function in an autocrine loop to amplify the Th17 response and induce its own expression (140, 141, 157). Th17 cells have been shown to both produce MIP3 $\alpha$  and express CCR6 (142). The chemokine MIP3 $\alpha$  binds to CCR6 which is expressed by B cells and subsets of T

cells and DCs (146). This suggests that Th17 cells might regulate their own recruitment. IL-17 also stimulates MIP3 $\alpha$  production from tissues (151). It is therefore possible that CCR6-MIP3 $\alpha$  signalling may have a major role in maintaining Th17 cells at the site of inflammation. Indeed it has been reported that CCR6 deficiency or blockade protects against Th17 cell mediated EAE (146).

### **1.5.5 The role of IL-17 in pathogen clearance**

Th17 cells and their signature cytokine IL-17 play important roles in the immune response to various pathogens. Th17 cells play a role in neutrophil recruitment and granuloma formation (158). IL-17R deficient mice have impaired host defences against microbial infection, due to a reduction in G-CSF and MIP2 production (158). IL-17 has been shown to be a significant factor in effective host defence against the Gram negative bacteria *Klebsiella pneumoniae*. Infected IL-17RA deficient mice showed an enhanced bacterial burden and consequently increased mortality (159). Ye *et al* also showed that intratracheal administration of an adenovirus that over expresses IL-17 led to neutrophil recruitment and survival (160). IL-17 is also required for the clearance of *Citrobacter rodentium*, *Toxoplasma gondii* and *Bacteroides fragilis* (135, 161, 162). In addition, Acosta-Rodriguez *et al* have found *C. albicans* specific Th17 responses in the peripheral blood of human donors (163).

### **1.5.6 Th17 cells in autoimmunity**

Th17 cells have received arguably the greatest attention due to the mounting evidence that this cell type is essential for the establishment of organ-specific inflammation in many autoimmune diseases. Langrish *et al* showed that Th17 cells were highly encephalogenic and the transfer of PLP specific Th17 cells developed through the use of recombinant IL-23 resulted in the development of EAE in recipient mice (138). On transfer to a naïve recipient,

the cells were shown to cross the blood brain barrier and traffic to the CNS (138). The effect of IL-17 on the disease pathogenesis was also investigated using a neutralizing anti-IL-17 antibody (138). Treatment of PLP immunized mice with an anti-IL-17 antibody partially protected against the development of EAE (138). In an attempt to define the mechanism of action of IL-17 in the induction of EAE, Park *et al* showed that an anti-IL-17 antibody considerably delays disease onset by preventing mononuclear cell infiltrate into the white matter of the spinal cord (151). The MOG specific T cells in the antibody treated mice produced comparable levels of IL-17, TNF- $\alpha$  and IFN- $\gamma$  suggesting that the autoreactive T cells are not inhibited by neutralization of IL-17 (151). Anti-IL-17 appears to reduce EAE by inhibiting IL-17 induced chemokines such as CCL2, CCL17 and CXCL1, which in turn reduces the recruitment of autoreactive T cells and macrophages to the CNS (151). Komiyama *et al* have since reported that EAE is attenuated in IL-17<sup>-/-</sup> mice. The mice exhibited delayed onset of disease as well as reduced maximum disease scores (164). IL-17<sup>-/-</sup> mice are also resistant to adjuvant induced rheumatoid arthritis (165). Tzartos *et al* recently reported the presence of IL-17 positive perivascular lymphocytes in brain lesions from patients with active MS, confirming the major role that IL-17 plays in human autoimmune disease (166). Hsu *et al* have shown that IL-17 promotes the formation of germinal centres in an animal model of an SLE-like disease (167). Transgenic mice that overexpress IL-17 in lung epithelial cells develop several pathological symptoms including hypertrophic lung epithelium, alveolar wall thickening, the presence of multi-nucleated macrophages and excess mucus production by epithelial cells (151).

### **1.5.7 The role of Th1 cells in autoimmunity**

In 2005, Langrish *et al* reported that PLP specific Th1 cells developed using recombinant IL-12 traffic to the CNS on transfer to naïve mice. These mice did not however develop the

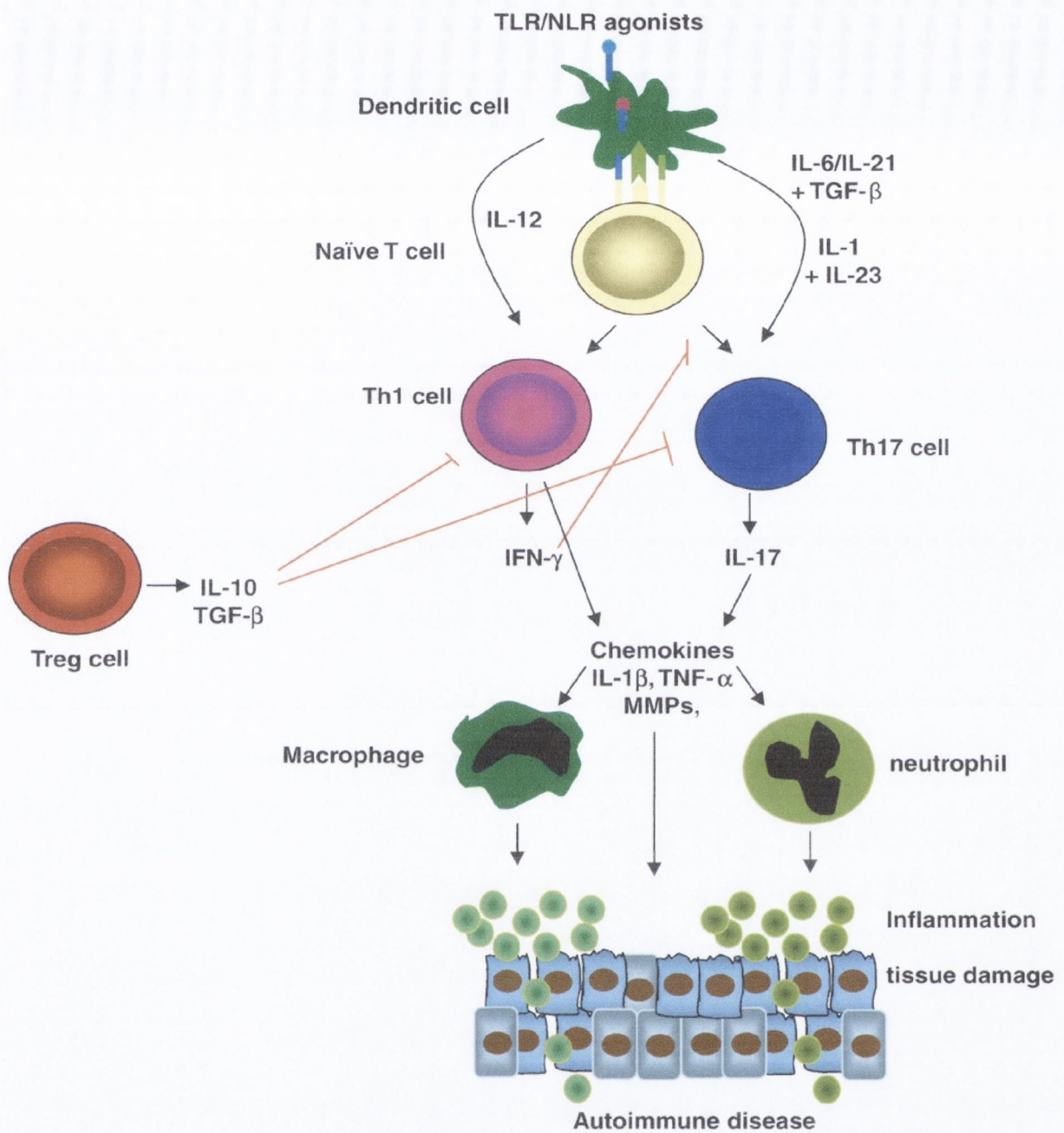
clinical signs of EAE (138). They also showed that the treatment of mice with an anti-IFN- $\gamma$  antibody exacerbated EAE increased mortality (138). This suggested that Th1 cells may play an inhibitory role in the development of EAE. However conflicting evidence has recently emerged suggesting that Th1 cells may be pathogenic in autoimmune disease. Kroenke *et al* showed that either Th1 or Th17 polarized myelin reactive T cells could induce EAE. Ascending paralysis was observed in both models. The cellular infiltrate of the mice that received Th1 cells was rich in macrophages whereas in the mice that received the Th17 cells, neutrophils predominated (168). In an animal model of uveitis (EAU), Luger *et al* have shown that the transfer of an IRBP specific Th1 clone caused severe disease (169). O'Connor *et al* have since reported that in an EAE model, the transfer of antigen specific Th17 cells lacking IFN- $\gamma^+$  cells does not result in disease. In contrast myelin reactive Th1 cells devoid of contaminating IL-17 $^+$  cells are highly pathogenic (170). They conclude that only Th1 cells can access the CNS, and that Th17 cells are recruited once EAE lesions have been established (170). Our current understanding of autoimmune disease is therefore unclear. A growing body evidence suggests that both Th1 and Th17 cells play a role in autoimmune pathogenicity.

### **1.5.8 Th17 cell differentiation**

The cytokines involved in Th17 differentiation and expansion have received considerable attention over the past few years. It was initially thought that IL-23 was the cytokine responsible for the differentiation of Th17 cells from naïve T cells. Langrish *et al* showed that lymph nodal cells taken from mice immunized with PLP could be directed into Th17 cells if cultured for 5 days with antigen in the presence of recombinant IL-23 (138). Nevertheless it was still possible to direct Th17 cell induction using recombinant IL-23 in







**Fig 1.1 Overview of the induction and expansion of pathogenic Th1 and Th17 cells in autoimmune disease.**

Reproduced from Mills KH, European Journal of Immunology 2008 (171)

lymph node cells taken from IL-12p40<sup>-/-</sup> mice. This suggested that the absence of IL-23 at the time of priming has no effect on Th17 induction.

It then emerged that TGF- $\beta$  and IL-6 were the cytokines responsible for the development of Th17 cells (133-135). Veldhoen *et al* initially demonstrated that Th17 cells are induced in the presence of naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> T regs and TLR3, TLR4 or TLR9 stimuli (133). Co-culture of dendritic cells, LPS and naïve CD4<sup>+</sup> cells led to Th1 cell induction but further addition of Treg cells led to Th17 differentiation (133). They then showed that Treg cells can be substituted with TGF- $\beta$  and the TLR agonist with IL-6 (133). The addition of anti-IL-23 had no effect on the induction process whereas the addition of anti-TGF- $\beta$  inhibited Th17 cell development. The authors also reported that Th17 cells do not express the negative regulator of TGF- $\beta$  signalling, Smad 7 (133). A further link was then made by Bettelli *et al* between the induction of Th17 cells and natural Foxp3<sup>+</sup> Treg cells (134). TGF- $\beta$  alone induced natural Tregs, but the addition of IL-6 led to the induction of Th17 cells (134). The natural Tregs were anergic and were shown to inhibit the proliferation of the Th17 cells (134). Bettelli *et al* developed a TGF- $\beta$  transgenic mouse, in which TGF- $\beta$  is under the IL-2 promoter (TgTGF- $\beta$ ). They crossed this mouse with a MOG specific T cell receptor (TCR) transgenic mouse to create a 2D2 X TgTGF- $\beta$  transgenic mouse. *In vitro* activation of T cells from this mouse with MOG resulted in high TGF- $\beta$  production and these cells suppressed EAE on adoptive transfer (134). However the 2D2 X TgTGF- $\beta$  mice developed the most severe EAE when compared with the 2D2 and wild type mice (134). The immunization of the 2D2 X TgTGF- $\beta$  mice with MOG and CFA led to an increase in levels of IL-6 which in the presence of TGF- $\beta$  induced Th17 cells (134). 75% less Foxp3<sup>+</sup> Treg cells infiltrated the CNS in the 2D2 X TgTGF- $\beta$  mice compared with 2D2 transgenic mice, which is consistent with a reciprocal relationship between Th17 and natural Treg cells (134).

Further evidence came from Mangan *et al* who showed that the differentiation of Th17 cells by TGF- $\beta$  and IL-6 was antagonized by IFN- $\gamma$  and IL-4 (135). However, it is still unclear for how long Th17 cells are susceptible to IL-4 and IFN- $\gamma$  mediated suppression (131). Li *et al* observed that Th17 cells are absent in mice that lack TGF- $\beta$  production from CD4+ T cells, thereby identifying the *in vivo* source of TGF- $\beta$  involved in Th17 cell differentiation (172).

Th17 cells have been shown to develop via signalling pathways distinct from those employed by Th1 or Th2 cells and does not involve STAT1, STAT4 or STAT6 (173). STAT3 has been reported to be a crucial signalling component in the development of Th17 cells. Yang *et al* reported that the overexpression of a hyperactive form of STAT3 resulted in Th17 cell differentiation and that the induction of Th17 differentiation by TGF- $\beta$  and IL-6 is defective in STAT-3 deficient cells (174). They also showed that STAT3 deficiency resulted in decreased expression of ROR $\gamma$ t (174). The role of T-bet was less clear. Antigen receptor stimulation has been shown to induce IFN- $\gamma$  independent expression of T-bet and the progression of Th17 cells to a Th1 cytokine secretion pattern (175). Mice that lack T-bet produce greater numbers of Th17 cells (151). However T-bet deficient mice are resistant to EAE suggesting that the transcription factor plays a role in autoimmune disease development (176).

### **1.5.9 IL-23**

The role of IL-23 in Th17 cell induction and function is somewhat unclear. IL-23 has been shown to be necessary in the development of many autoimmune diseases. IL-23p19<sup>-/-</sup> mice are resistant to EAE, CIA and IBD (138, 177, 178). IL-23 is also required for the clearance of certain pathogens such as the bacterial pathogen *C. rodentium* (135). Infection of wild type mice with *C. rodentium* results in transient distal colitis but resolution of the lesions and

clearance of bacteria within 14-21 days (135). In contrast, infected IL-23p19<sup>-/-</sup> mice developed significantly less colonic inflammation but fail to clear the bacteria suggesting that IL-17 plays a role in protective immunity as well as in immune pathology (135). The IL-23 receptor is not expressed on naïve T cells but is upregulated by TCR ligation in the presence of IL-6 (136, 143). IL-23 has been shown to induce IL-17 production from effector and memory T cells. Happel *et al* used a transwell system to show that cell-cell contact between the dendritic cell and a T cell is not necessary for IL-17 production by T cells. Neutralization of IL-23 using an antibody led to a significant reduction in IL-17 production (179). Aggarwal *et al* showed that IL-23 induces IL-17 production from memory T cells (CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>low</sup>) but not from naïve T cells (CD4<sup>+</sup>CD44<sup>low</sup>CD62L<sup>high</sup>) (180). It was later shown that Th17 cells developed using TGF-β and IL-6 required IL-23 during restimulation to maintain IL-17 production (133). McGeachy *et al* have found that the restimulation of activated PLP specific Th17 cells in the presence of TGF-β and IL-6 results in Th17 cells that produce both IL-17 and IL-10. The transfer of these cells into naïve mice did not result in the symptoms of EAE (136). In contrast, myelin specific Th17 cells restimulated in the presence of IL-23 did not produce IL-10 and on transfer induced severe EAE (136). Mice transferred with TGF-β and IL-6 stimulated Th17 cells and IL-23 stimulated Th17 cells remained healthy (136). This suggests that the IL-10 producing Th17 cells may have a regulatory role and that IL-23 is necessary for the development of pathogenic Th17 cells.

#### **1.5.10 The role of IL-1 in Th17 cell function**

IL-1β has also been shown to be important in the development of Th17 cells. It promotes Th17 cell development in the presence of TGF-β and IL-6 (133). Sutton *et al* reported that the induction of antigen specific Th17 cells is abrogated in IL-1R<sup>-/-</sup> and that the incidence and

severity of EAE is significantly lower in these mice (181). Further evidence for the role of IL-1 in the Th17 immune response has come from the development of *Tir8<sup>-/-</sup>* mice. TIR8 is a member of the IL-1 receptor family and acts as a negative regulator of IL-1 and TLR signalling. *C. albicans* infection of the *Tir8<sup>-/-</sup>* mice resulted in increased inflammation and susceptibility to infection that was linked to a Th17 response (182).

#### **1.5.11 TLR signalling in the induction and maintenance of a Th17 response**

TLRs have an important role to play in the induction of Th17 cells. Veldhoen *et al* showed that dendritic cells from *MyD88<sup>-/-</sup>* mice were unable to induce Th17 cells, thereby demonstrating that TLR-MyD88 signalling is necessary for the induction of Th17 cells (133). In addition, TLR stimuli have been shown to induce IL-23 and are therefore also important in the maintenance of an effective Th17 response. Incubation of spleen cell cultures with the TLR agonists LPS, Lipotechoic Acid or Lipopeptide leads to IL-17 production (180). The TLR agonists CpG, LPS and Poly I:C have been shown to enhance expression of IL-12p40, IL-23p19 and IL-12p35 resulting in the release of bioactive IL-12 and IL-23 (183). *Bordetella pertussis* has also been shown to preferentially induce strong IL-23 responses over IL-12 as does peptidoglycan from *Staphylococcus aureus* (183). Happel *et al* reported that TLR4 signalling is required for effective Th17 cell induction against *K. pneumonia* (179). Infected C3H/HeJ mice had reduced Th17 responses and succumbed to the infection with *K. pneumonia* (179). Some pathogen molecules induce Th17 cell induction through TLR independent pathways. Fungal  $\beta$ -glucans bind to C-type Dectin-1 on the surface of DCs and induce IL-23 but little IL-12, thereby preferentially inducing Th17 responses (184).

### **1.5.12 The inhibition of Th17 cells.**

The role of Th17 cells in the pathogenesis of autoimmune disease has meant that there has been considerable interest in understanding and developing approaches for their inhibition. IL-27 has been shown to suppress the development of Th17 cells (185, 186). Fitzgerald *et al* showed that IL-27 induces a population of effector T cells that secreted IFN- $\gamma$  and IL-10 and inhibit IL-17 secretion from activated spleen cells by an IL-10 dependent mechanism (187). It has since been reported that engagement of the type I interferon receptor induces IL-27 via an intracellular translational isoform of Osteopontin termed Opn-i (188). This provides a potential mechanism whereby type I IFNs inhibit autoimmune disease. IL-25 and IL-35 have also been implicated in the inhibition of Th17 responses. IL-25 deficient mice are more susceptible to EAE than wild type mice and have increased levels of IL-23 in the periphery (189). IL-35 has been shown to inhibit Th17 cell differentiation (190).

### **1.5.13 IL-17 production by non Th17 cells**

Th17 cells are not the only cell type to produce IL-17. Both CD8<sup>+</sup> T cells and invariant NKT cells have been shown to be a source of the cytokine (179, 191).  $\gamma\delta$  T cells have also been shown to be a major source of IL-17. Lockhart *et al* demonstrated that  $\gamma\delta$  T cells are the major source of IL-17 in mice infected with *Mycobacterium tuberculosis* (192). There is also evidence emerging that  $\gamma\delta$  T cells have an inherent ability to produce IL-17. Romani *et al* reported that  $\gamma\delta$  T cells from naive animals produce IL-17 following an overnight stimulation with CD3 antibody and LPS. Furthermore naive  $\gamma\delta$  T cells have been shown to produce IL-17 after stimulation with IL-23 alone, an ability which differs from CD4  $\alpha\beta$  Th17 cells.

## 1.6 Treg cells

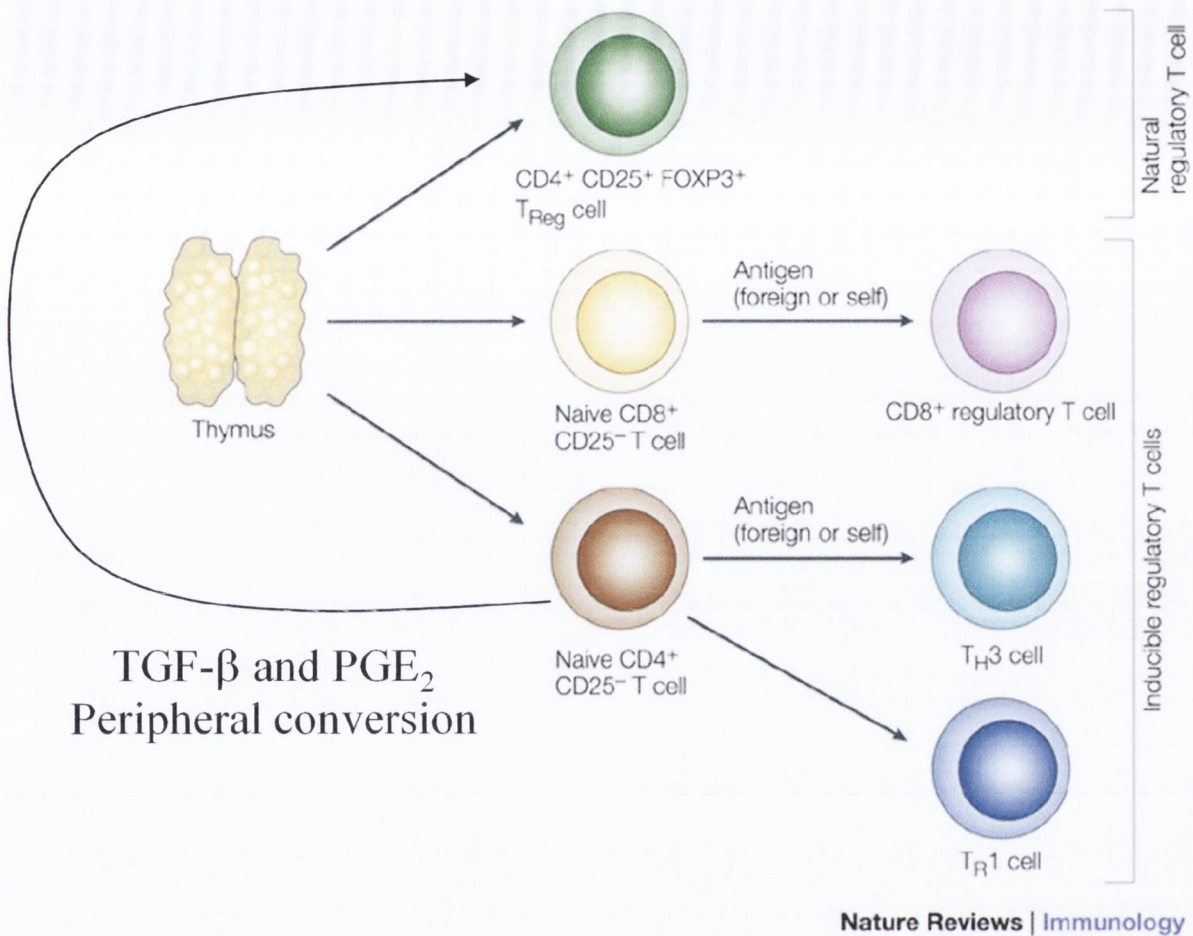
### 1.6.1 An introduction to Treg cells

The existence of Treg cells was first suggested in 1969 when organ specific autoimmunity was found to be induced in specific strains of mice that had been thymectomized 3 days after birth, but did not develop in mice when the thymus was removed at 0 or 7 days (193). It was deduced that self reactive T cells emerge from the thymus immediately after birth, but that there is also a suppressive population of T cells that does not emerge until day 3 of life. It wasn't until 1995 that these cells were characterised as CD4<sup>+</sup>CD25<sup>+</sup> natural Treg cells (194). It is now accepted that there are two main regulatory T cell types categorized as natural and inducible regulatory T cells. Both Treg cell types are capable of suppressing a variety of different immune cells, including naive and memory CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells, B cells, monocytes and DCs (195). CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells express the transcription factor Foxp3, whereas inducible Treg cells are antigen specific, derived from CD4<sup>+</sup>CD25<sup>-</sup> naïve T cells and show variable expression of CD25 on activation (196, 197).

### 1.6.2 Natural Treg cells

Natural Treg cells develop in the thymus and enter peripheral tissues where they suppress self reactive T cells thereby preventing autoimmune diseases (197). These cells account for 5-10% of the peripheral T cells in mice (198). A recent report has showed that Foxp3<sup>+</sup> natural Tregs can also differentiate in the periphery from CD4<sup>+</sup>CD25<sup>-</sup> cells (199). Davidson *et al* have shown that TGF- $\beta$  induces the differentiation of Foxp3<sup>+</sup> Tregs from naive CD4<sup>+</sup> precursors in the presence of IL-2 (200). These TGF- $\beta$  induced Treg cells resembled thymic derived natural Treg cells and on transfer to scurfy mice prevented the fatal lymphoproliferative syndrome that spontaneously develops in these animals (201). CD25 is the  $\alpha$  chain of the IL-2 receptor and natural Treg cells are the only cells to constitutively





**Fig 1.2 Overview of Treg cell differentiation**

**Adapted from Mills 2004, Nature Reviews Immunology (197).**

Natural Treg cells that express CD4, CD25 and the transcription factor Foxp3 develop in the thymus before migrating to the periphery where they constitute 5-10% of the T cell population (197). Natural Treg cells have also been shown to develop in the periphery where both TGF- $\beta$  and prostaglandin E<sub>2</sub> can induce the conversion of CD4<sup>+</sup>CD25<sup>-</sup> cells to CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells (102, 202). Other populations of antigen specific Treg cells are induced in the periphery by regulatory DCs that present antigen and activate naive CD4<sup>+</sup>CD25<sup>-</sup> cells. Tr1 cells and CD8<sup>+</sup> regulatory T cells are characterized by the production of high levels of IL-10 and Th3 cells secrete high levels of TGF- $\beta$  (197).

Th1 effector cells. The CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells did not prevent migration of effector cells into the gastric lymph node or inhibit the expansion of autoreactive T cells (209). Natural Treg cells are also very important in the context of cancer. Treg cells can prevent effective tumour clearance by suppressing effector T cells specific for tumour antigens. Recently, an agonistic antibody against GITR was shown to increase the efficacy of a xenogeneic DNA vaccination, which induces anti-tumour CD8<sup>+</sup> T cell responses (210).

### **1.6.3 Mechanisms of suppression by natural Treg cells**

The mechanisms of suppression of natural Treg cells can be grouped into four basic modes of action, suppression by inhibitory cytokines, suppression by cytolysis, suppression by metabolic disruption and suppression by modulation of DC maturation and function (211). There is conflicting evidence concerning the roles of the anti-inflammatory cytokines IL-10 and TGF- $\beta$ , in suppression by natural Treg cells. Neutralization of IL-10 and TGF- $\beta$ 1 *in vitro* does not abrogate suppression and natural Treg cells isolated from IL-10 deficient and TGF- $\beta$ 1-deficient mice are functionally operative, which suggests that suppression is mediated by cell-cell contact or by other anti-inflammatory cytokines (212-214). In addition, natural Tregs inhibit target T cells that lack the ability to respond to TGF- $\beta$  (TGF- $\beta$ 1RII dominant negative transgenic or Smad3-deficient mice (214). However there is mounting evidence that TGF- $\beta$  and IL-10 may mediate suppressive activity *in vivo*. Asseman *et al* showed that IL-10 production by Tregs is essential for the prevention of colitis in a mouse model of IBD (215). Furthermore, in UV induced carcinogenesis, IL-10 production by Tregs was shown to be important in blocking anti-tumour immunity (216). Rubtsov *et al* developed mice lacking IL-10 in Treg cells. The deletion did not result in spontaneous systemic autoimmunity, but the mice developed colitis at 10 weeks of age and had an increased pathology in a model of airway inflammation (217). It has therefore been suggested that Treg derived IL-10 may be

restricted to the control of inflammatory responses induced by pathogens or allergens (211). TGF- $\beta$  produced by Tregs has been shown to be important in preventing colitis in an IBD model (172). Furthermore in the same autoimmune model, effector cells that are resistant to TGF- $\beta$  mediated suppression are not controlled by Tregs (218). IL-35 is a member of the IL-12 cytokine family and has been shown to be preferentially expressed by Treg cells (219). Collison *et al* reported that ectopic expression of IL-35 in naive cells confers regulatory activity and that recombinant IL-35 suppresses T cell proliferation. The role of the anti-inflammatory cytokines *in vivo* is supported by evidence from two-photon laser-scanning microscopy of the priming of diabetogenic T cells in pancreatic lymph nodes (220). It was found that natural Treg cells compromised the arrest and swarming of autoreactive CD4<sup>+</sup>CD25<sup>-</sup> Th cells. However no stable interactions between the Treg and CD4<sup>+</sup>CD25<sup>-</sup> Th cells were observed during suppression, therefore supporting the role of anti-inflammatory cytokines (220).

Natural Treg cells have also been shown to suppress by granzyme B dependent cytotoxicity. It has been reported that Treg cells from granzyme B deficient mice had reduced suppressive activity *in vitro* (221). In a tumour model, Treg cells have been shown to kill NK cells and CTLs by a granzyme B dependant and perforin dependent mechanism thereby suppressing tumour clearance (222). Treg cells express the ectoenzymes CD39 and CD73, which generate pericellular adenosine. The binding of pericellular adenosine to the adenosine receptor 2A on the surface of effector T cells results in suppression (223). Bopp *et al* have also shown that natural Tregs suppress effector T cells by the transfer of the inhibitory second messenger cyclic AMP (cAMP) across membrane gap junctions (224). Natural Treg cells have also been shown to influence DC maturation. Cederbom *et al* initially reported that Treg cells could downregulate the expression of the co-stimulatory molecules CD80 and

CD86 on DCs thereby affecting their ability to activate effector T cells (225). Natural Treg cells express lymphocyte activation gene (LAG3) which is a homologue of CD4. Binding of LAG3 to MHC class II molecules on DC induces an immunoreceptor tyrosine based activation motif ITAM mediated inhibitory pathway which also suppresses the ability of DCs to activate effector T cells (226).

#### **1.6.4 Tr1 cell differentiation**

Inducible Treg cells are generated when naïve CD4<sup>+</sup>CD25<sup>-</sup> T cells in the periphery encounter antigen presented by a DC with a regulatory activation status (197). Inducible Treg cells that produce IL-10 are called Tr1 cells and like natural Treg cells, can suppress naïve and memory T cell responses *in vitro* and *in vivo* (112, 195, 227, 228). The regulatory DC that induce Tr1 cells produce IL-10 and have an intermediate phenotype with increased MHC class II and CD86 but low levels of CD40 and ICAM-1 (227). Martin *et al* have shown that RelB is responsible for CD40 up regulation in DC and that inhibition of RelB or the use of DC from CD40<sup>-/-</sup> mice leads to the induction of antigen specific Treg cells that secrete IL-10 (229). Wakkach *et al* have demonstrated that *in vitro* culture of bone marrow cells in the presence of IL-10 induces a distinct subset of dendritic cells that are CD11c<sup>low</sup>CD45RB<sup>high</sup>, have plasmacytoid morphology and secrete high levels of IL-10 (230). On incubation with OVA, the DCs induced Tr1 cells *in vitro* and *in vivo* (230). IL-10 inhibits the production of IL-1 $\beta$ , TNF, IL-6 and IL-12 by DCs and macrophages therefore preventing the induction of effector T helper responses (231). IL-10 has also been shown to down regulate the expression of MHC class II, CD80 and CD86, thereby preventing DC maturation (232). A population of tolerogenic DCs, termed DC-10 that are present in peripheral human blood has recently been identified. These cells are CD14<sup>+</sup>CD16<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>CD83<sup>+</sup>HLA-DR<sup>+</sup>CD1a<sup>-</sup>CD1c<sup>-</sup> and display a mature myeloid phenotype (195). They express high levels of immunoglobulin-like

transcript-2 (ILT-2), ILT-3, ILT-4 and human leukocyte antigen (HLA-G) and secrete high levels of IL-10 and low levels of IL-12 (195). DC-10 were found to be more potent inducers of Tr1 cells than immature DCs. The induction of Tr1 cells by DC-10 required autocrine IL-10, which upregulated the expression of ILT-4 and HLA-G on DC-10 and HLA-G on the surface of T cells. The interaction between ILT-4 and HLA-G on the DC-10 and HLA-G on the T cell has been shown to be crucial for the induction of Tr1 cells (195).

### **1.6.5 Th3 cells**

Inducible Treg cells that secrete TGF- $\beta$  are known as Th3 cells. TGF- $\beta$  has been shown to inhibit Th1 cell responses by inhibiting T-bet expression (233). TGF- $\beta$  has also been found to induce IL-10 production from Th1 effector cells, via Smad4 activation of the IL-10 promoter (234). Th3 cells were first identified as T cells with suppressive or anergic activity that could be generated under certain conditions, such as in oral tolerance (235). Chen *et al* showed that the oral administration of myelin basic protein led to the induction of Th3 cells, which suppressed EAE by inducing peripheral tolerance (235).

### **1.6.6 The induction of Tr1 cells**

Antigen exposure by intranasal, intradermal or oral route, appears to selectively induce T cells with a regulatory phenotype and these cells appear to have a role in the maintenance of tolerance to food or inhaled antigens. Sundstedt *et al* developed and characterised myelin basic protein specific Tr1 cells by intranasal administration of antigen (236). The Tr1 cells were anergic, failed to produce IL-2, but responded to antigen by secretion of IL-10 (236). Inducible regulatory T cells can also be induced using immunosuppressive drugs. Barrat *et al* developed Tr1 cells by culturing APCs and antigen with vitamin D3 and dexamethasone (237). The T cells produced substantial concentrations of IL-10 and IL-4 and low

concentrations of IFN- $\gamma$  and IL-5 (237). The addition of neutralizing antibodies to IL-12, IFN- $\gamma$ , and IL-4 at the induction of the culture led to the development of a Tr1 population that secreted IL-10 and low levels of IL-2 but no IL-4, IL-5 or IFN- $\gamma$  (237). They also showed that the combination of vitamin D3 and dexamethasone was capable of inducing Tr1 cell following stimulation of T cells with anti-CD3 and anti-CD28 (237). Interestingly, the induction of the Tr1 cells was shown to be dependent on a cytokine that binds to the IL-10 receptor as differentiation of the Tr1 cells was significantly reduced on the addition of an anti-IL-10R antibody (237). McGuirk *et al* have shown that pathogen specific Tr1 cells are induced during infection with *B. pertussis*. Furthermore, Filamentous Haemagglutinin (FHA) an immunomodulatory molecule derived from *B. pertussis* was shown to induce Tr1 cells specific for a bystander antigen (227). Again the development of Tr1 cells was IL-10 dependent.

There are conflicting reports on the role of IL-4 in the induction of Treg cells. STAT-6 is essential for IL-4 responses in T cells. CD4<sup>+</sup> T cells from STAT6<sup>-/-</sup> mice failed to differentiate into IL-10 secreting T cells (238). The lack of Tr1 cell development in IL-4-defective mice could be reversed with the addition of exogenous IL-4 (238). In contrast Yamamoto *et al* have reported that oral administration of *Escherichia coli* derived heat labile enterotoxin (LT) induces Treg cells in IL-4-defective mice (239). IFN- $\alpha$  has also been implicated in the induction of Tr1 cells. It has been demonstrated that IFN- $\alpha$  and IL-10 induce Tr1 cells from human cord blood and peripheral blood derived naive T cells (240).

It has been suggested that inducible regulatory T cells generally function *in vivo* and *in vitro* in a cytokine dependent manner. However O'Garra and Vieira have argued that suppressor function of Tr1 cells is IL-10 dependent *in vivo* but not *in vitro* (241). This argument is

supported by evidence showing that Tr1 cells developed using immunosuppressive drugs could inhibit EAE *in vivo* in an IL-10 dependent fashion (237). However the inhibition of *in vitro* T cell proliferation by the same cells was shown to be cytokine independent (237).

### **1.6.7 Tr1 cell induction by pathogens**

The ability of Treg cells to inhibit effective adaptive immune responses has been exploited by many pathogens as part of their immune evasion strategies. Infection with *Plasmodium falciparum* has been shown to induce Tr1 cells. The immunodominant CD4+ T cell epitope region Th2R, of the circumsporozoite is highly polymorphic (242). Many of the variants induce T cells that produce IL-10 and IL-4 or IL-10 alone, instead of a Th1 effector immune response (242). There is also evidence that *M. tuberculosis* induces Tr1 cells (243). Most MTB infected patients have normal Th1 cell dominated DTH responses, however 15% of infected patients fail to respond to intradermal injection with purified protein derivative (PPD) (243). The T cells from these hosts proliferate poorly and secrete high levels of IL-10 (243).

Certain pathogen-derived immunomodulatory molecules promote IL-10 and inhibit IL-12 production from DC and macrophages and activate DC into a semi-mature phenotype. Many of these molecules can therefore act as adjuvants for the induction of Tr1 responses *in vivo*. Lavelle *et al* demonstrated that bone-marrow derived DC's treated with Cholera Toxin (CT) have enhanced expression of CD80, CD86 and OX40 (CD134) on DC and secrete MIP-2 (112). CT synergized with low levels of LPS to induce substantial levels of IL-10 from DCs (112). CT was capable of promoting the generation of Tr1 cells against a bystander antigen (112). T cell clones that were developed from mice immunized with antigen and CT were capable of inhibiting IFN- $\gamma$  production by Th1 cells (112).

express this marker in normal disease free animals. Experimental evidence has now confirmed that IL-2 is a key growth and survival factor for natural Treg cells. Setoguchi *et al* have shown that the neutralization of circulating IL-2 with a monoclonal antibody results in the development of autoimmune gastritis in BALB/c mice. The antibody treated mice had reduced numbers of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells in the thymus and the periphery, whereas the numbers of CD4<sup>+</sup>CD25<sup>-</sup> cells were unaffected (203). The source of IL-2 necessary for natural Treg cell survival appears to be other T cells especially activated CD4<sup>+</sup>CD25<sup>low</sup> T cells (203). A problem with using CD25 as a marker for Treg cells is that it is also upregulated on effector T helper cells. Other putative markers for natural Tregs include CD38, CD62L<sup>hi</sup>, CD103, GITR, CD45RB<sup>low</sup>, CTLA-4 and the transcription factor Foxp3 (198, 204). Foxp3 has emerged as the most promising marker with studies showing that transfection of CD4<sup>+</sup>CD25<sup>-</sup> T cells with the transcription factor confers regulatory activity (205). There is also emerging evidence that some CD4<sup>+</sup>CD25<sup>-</sup> cells especially CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>low</sup> cells can express Foxp3 at low levels (206). These cells can suppress autoimmune activity, albeit less effectively than CD4<sup>+</sup>CD25<sup>+</sup> natural Treg cells (207, 208).

From an experimental perspective the intracellular location of Foxp3 limits its use as a tool to purify Treg cells. GITR (glucocorticoid-induced TNF receptor related protein) has recently emerged as potentially the most reliable extracellular marker of natural Treg cells. The vast majority of Foxp3 cells, whether CD25<sup>+</sup> or CD25<sup>-</sup>, have been shown to have constitutively high levels of GITR (208). Transfer of a T cell or thymocyte suspensions depleted of GITR<sup>high</sup> cells into BALB/c nude mice produced a wider spectrum and more severe forms of autoimmune diseases, when compared with the transfer of CD25 depleted cells (208). In a mouse model of autoimmune gastritis, Di Paolo *et al* found that natural Tregs inhibited the development of disease by subverting the differentiation of autoantigen-specific T cells to



Pathogen-specific Tr1 cell responses are not exclusively detrimental to the host. In *B.pertussis* infected mice, TLR4 has been shown to be vital for effective Tr1 induction and bacterial clearance (244). Infected TLR4 defective mice exhibited enhanced inflammatory cytokine production, cellular infiltration and severe pathological changes due to a defect in the induction of IL-10 producing T cells (244). These findings suggest that PRR induced Treg cells prevent excessive collateral damage by an uncontrolled immune response during infection (244).

### **1.6.8 The use of Tr1 cells to attenuate autoimmune disease**

The development of autoimmune diseases involves a breakdown in the mechanisms that control the discrimination between self and non-self antigens. Tolerance to self antigens is primarily maintained by thymic clonal deletion of self reactive T cells. However, self reactive T cells that escape deletion are present in the periphery of all individuals and yet only 5% of the population suffers from autoimmune disease. It has now been shown that natural Tregs and Tr1 cells can suppress the activation of self reactive T cells (245). A breakdown in this regulation leads to the development of autoimmune diseases. Th1 and Th17 cells have both been implicated in the pathology of autoimmune diseases. Regulatory T cells can inhibit Th1 cells and may inhibit Th17 cells, for which the induction of regulatory T cells *in vivo* has therapeutic potential in the treatment of autoimmune disease (112, 227). Expansion of the existing natural Treg cell population could alleviate autoimmune pathology. However this strategy could lead to the suppression of immune responses to pathogens and tumours. Therefore the induction of Treg cells that are specific for pathogenic autoantigens could be a more powerful and safer therapy. Pathogen derived molecules have the potential to induce Treg cells specific to autoantigens. The problem with this approach is that the autoantigen target must be identified for each autoimmune disease.

A number of studies have underlined the potential of inducible Treg cells in the prevention of autoimmune disease in animal models. CD3 $\epsilon$  antibodies have been shown to induce a population of TGF- $\beta$  producing inducible Treg cells that inhibit overt diabetes in nonobese diabetic (NOD) mice and maybe effective in human type I diabetes (246). Massey *et al* showed that antigen specific Tr1 cells induced by the intranasal administration of MOG, inhibited the induction of EAE. IL-10 was shown to be crucial to the protective mechanism as peptide administration in IL-10<sup>-/-</sup> mice did not prevent disease progression (247). Barrat *et al* generated OVA-specific Tr1 cells *in vitro* through the use of Vitamin D3 and Dexamethasone and neutralizing antibodies to Th1 and Th2 cytokines (237). On adoptive transfer these cells were capable of preventing the induction of EAE (237). Protection was only observed when OVA absorbed in Alum was injected intracranially to provide a reservoir of antigen to activate the Treg cells (237). IL-10 was shown to be a key mediator of protection, as the administration of a neutralizing anti-IL-10R mAb at the time of T cell transfer abrogated the protective effect (237).

## **1.7 *Bordetella pertussis***

### **1.7.1 Disease**

*B. pertussis* is the causative agent of whooping cough a highly contagious acute respiratory illness. The pathogen is a small gram negative aerobic coccobacillus that only infects humans (248). The genus *Bordetella* contains 9 species, 4 of which are pathogenic to humans or animals- *B. bronchiseptica*, *B. parapertussis*, *B. holmesii* and *B. pertussis* (113). Unlike *B. pertussis*, *B. bronchiseptica* infects a wide range of animal hosts (249). Remarkably little genetic diversity has been observed between *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. *B. bronchiseptica* has been identified as the evolutionary progenitor, while *B. pertussis* and *B. parapertussis* can be classified as subspecies with human adaptations

(250). *B. pertussis* and *B. parapertussis* evolved independently, with the latter emerging more recently. As a consequence of this, even the most effective *B. pertussis* vaccines confer poor protection against *B. parapertussis* (251).

The first reported epidemic of *B. pertussis* occurred in 1578 and the disease was named “pertussis” meaning violent cough in 1679 (113). The term whooping cough comes from the sound of the desperate inspiration of air between paroxysmal coughing episodes of children infected with the disease (249). However it was not until the twentieth century that the organism was first observed and cultured by Bordet and Gengou in the sputum of a patient with pertussis (113). At the time the disease was a major cause of child mortality so vaccine development began in earnest.

### **1.7.2 Pertussis vaccines**

The first whole cell pertussis vaccine was licensed in 1914 and consisted of killed bacteria (248). By the 1940s and 1950s the widespread use of the whole cell pertussis vaccine had brought epidemic pertussis under control in the United States (113). Incidences of whooping cough had decreased, but the age distribution shifted to younger and older age groups (248). By 1948 the whole cell vaccine was combined with diphtheria and tetanus toxoids (DTwP) (248). The use of acellular vaccines, which consist of highly purified antigens from *B. pertussis* began in Japan where its use led to a dramatic decline in the number of cases of whooping cough (248, 252). The acellular pertussis vaccine is now the pertussis vaccine of choice in most of the developed world. The acellular pertussis vaccine is associated with less frequent and less severe adverse reactions when compared with the whole cell vaccine (248). However the extent of the memory response induced by the acellular vaccine has not been established. Primary antibody response and limited follow-up data suggest a comparable if not improved duration of protection when compared with the whole cell vaccine (253).

Five acellular vaccines are licensed in the US that consist of differing combinations of the following components- pertussis toxin (PT), filamentous haemagglutinin (FHA), pertactin (PRN), Fimbriae (Fim) 2 and 3. Four of the five licensed acellular vaccines contain FHA with concentrations ranging from 5 µg-35 µg (248). 3 component vaccines have been shown to have a higher efficacy than 2 component product and the 5 component vaccine was found to be effective against milder disease, as well as typical pertussis (252). In a study carried out by Watanabe *et al* it was found that 2 component vaccines that consisted of large amounts of detoxified PT and small amounts of FHA were more effective than the common formula seen in many of the currently available acellular vaccines where the FHA dose is high and the PT levels dose is low (254). The reason PT content is usually kept low is that potential adverse reactions by the vaccine may be related to residual activity of PT. A comparison of whole cell vaccine and acellular vaccine revealed that the whole cell vaccine induces lower antibody titres to PT, FHA and PRN than the acellular vaccine (255). Conversely macrophages from mice immunized with the whole cell vaccine secreted higher nitric oxide (NO) concentrations than cultures from acellular vaccine immunized mice (255). The whole cell vaccine protected 90% of mice from intracerebral challenge, whereas the acellular vaccine only protected 40% of immunized mice (255). The whole cell vaccine is the more effective pertussis vaccine and induces a Th1 response characterised by high levels of the cytokines IL-2 and IFN- $\gamma$  and the induction of cell mediated immunity (255). The acellular vaccine induces a Th2 type response with elevated levels of the cytokines IL-4 and IL-5 and higher antibody production (255).

Currently in Europe acellular vaccines are examined for their induction of antibodies and the protection they confer in mice against intranasal challenge (256). It has however been reported that antibodies to antigen doesn't correlate to vaccine efficacy (256). CMI as well as

humoral immunity is required and it has indeed been shown that some acellular vaccines don't even induce T cells against pertussis toxin (256). Much improvement is therefore needed if the acellular pertussis vaccine is to match the efficacy of the whole cell pertussis vaccine without the potential side effects.

### **1.7.3 Virulence factors of *B. pertussis***

Bacterial virulence factors enable pathogens to enter the host, interact with a specific target tissue, evade host defence in order to proliferate, develop localised damage at the site of infection and cause systemic disease by dissemination of itself or their products (257). Infection with *B. pertussis* begins with the introduction of the organism into the respiratory tract in droplets (258). The bacteria attaches to the cilia of epithelial cells of the upper respiratory tract, using the virulence factors- FHA, fimbriae, PT, LPS, TcfA, BrkA, Vag8 and PRN (113, 248, 258). However there is redundancy among the attachment factors. It has been reported that synergy exists between FHA and PT in the binding of the bacterium to the cilia (259). Paralysis of cilia and death of ciliated cells follows the attachment of the bacteria which is thought to be mediated by IL-1 and nitric oxide (NO) (260). This leads to defective mucociliary clearance and is therefore advantageous to the survival of the bacteria in the host. FHA and adenylate cyclase toxin (CyaA) have both been shown to aid the evasion of the immune response by *B. pertussis* (227, 261, 262). There is some evidence to suggest that *B. pertussis* can invade and survive within phagocytes (263). This would provide the organism with another important immune evasion strategy.

The expression of their virulence factors (with the exception of TCT) by *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* is controlled by the Bordetella virulence regulon which is encoded by the bvgAS locus (248). BvgA and BvgS are members of a 2-component signal

transduction system that uses a four step His-Asp-His-Asp phosphotransfer signalling mechanism (113). FhaB the gene that encodes FHA, one of the main virulence factors of *B. pertussis*, and a major focus of this report, is one of the strongest BvgAS activated genes with a high affinity BvgA binding site in its promoter (264).

## **1.8 Filamentous Haemagglutinin (FHA)**

### **1.8.1 The molecular properties of FHA**

FHA is a dominant attachment factor for *B. pertussis* and antibodies against FHA have been shown to prevent *B. pertussis* adherence to human bronchial epithelial cells (265). The mature FHA protein is a hairpin shaped protein with a filamentous structure of 2nm x 40-100nm (266). FHA is a 500Å long secreted protein rich in  $\beta$  structure and contains two regions R1 and R2 of tandem 19-residue repeats (267). The central shaft consists of a  $\beta$ -helix in which the polypeptide chain is coiled to form three long parallel  $\beta$ -sheets (267).

The structural gene of FHA, FhaB has an open reading frame of 9783bps and FHA is initially synthesized as a single 3,590 amino acid (367kDa) precursor called FhaB (268). FHA is one of the most efficiently secreted proteins in Gram negative bacteria. It is secreted by means of a TPS pathway, where TpsA is the secreted protein FhaB and TpsB is an associated specific outer membrane transporter FhaC (269). Initially the N-terminal 71 amino acids signal sequence is removed by proteolysis (270). The amino acids up to position 322 are thought to form the TPS (two partner secretion) domain, which targets FhaB to FhaC in the periplasm (269). FhaB is secreted through the pore in FhaC. It was initially proposed that on the cell surface a specific protease SphB1 cleaved the precursor protein FhaB at the C terminus facilitating the release of the mature protein (271). The 160 kDa C terminal fragment remained associated with the bacteria. Coutte *et al* developed a SphB1 mutant of *B.*

*pertussis* which released virtually no FHA into the extracellular milieu and had large amounts of FHA related polypeptides on its surface (272). Contrary to this report, Mazar *et al* have reported that SphB1 mutants of *B. pertussis* and *B. bronchiseptica* release FHA albeit at reduced levels. They however did acknowledge that SphB1 is required for the production of mature FHA (273). Furthermore, they proposed that the C-terminus as oppose to the N-terminus of FHA is exposed on the cell surface. It was reported that the C-terminus of FHA was required for the adherence of *B. bronchiseptica* to epithelial cells (273).

Two genes regulated by the Bvg have been shown to encode putative protein homologues of FHA (274). FhaL and FhaS seem to be well expressed albeit at much lower levels than FhaB (274). There are many potential explanations for the redundancy. FHA is an extremely important protein and back-up copies may be necessary (274). Alternatively the two additional genes might act as reservoirs for homologous recombination with the master gene to generate antigenic diversity (274). It is also possible that the three proteins may have related yet distinct functions (274).

### **1.8.2 The adjuvanticity of FHA**

The adjuvant properties of FHA have been investigated by intranasal, subcutaneous and oral administration with model antigens (275). FHA was found to enhance IgG titres to co-administered antigens by all routes (275). However the adjuvant activity after oral administration was lower which may be due to proteolytic cleavage (275). Interestingly, no pro-inflammatory cytokines were detected in the lungs after intranasal immunization (275). CD80, TGF- $\beta$  and MHC-Class II mRNA expression was elevated (275). The adjuvanticity of FHA was also observed in mice that had been pre-immunized with the pertussis vaccine, which is important if FHA is to have clinical applications as an adjuvant or

immunomodulator. Poulain-Godefroy *et al* have since reported that the mucosal delivery of FHA alone results in enhanced total IgG and IgA concentrations (276).

### 1.8.3 The role of FHA in *B. pertussis* infection

Mutants of *B. pertussis* defective in either FHA or PT expression colonize mice nearly as effectively as wild type bacteria (272). In mice infected with FHA mutants of *B. pertussis*, the ADP-ribosylating activity of PT results in a strong inflammatory response characterized by pulmonary lesions (272). This allows the FHA mutant to colonize the lungs and therefore obscures the role of FHA in bacterial colonization (272). In mice infected with PT mutants of *B. pertussis*, there is a mild inflammatory response by the host and FHA is required for colonization (272). This redundancy between FHA and PT led Coutte *et al* to develop a double SphB1 and PT mutant, therefore allowing a study into the role of released FHA in the colonization of the lung by *B. pertussis* during infection. The infection of mice with the double mutant demonstrated that FHA is necessary for the normal colonization of the mouse respiratory tract by *B. pertussis* (272). *In vitro* the double mutant showed increased binding to epithelial and macrophage cell lines when compared to wild type *B. pertussis* (272). Menozzi *et al* had previously found that FHA causes the auto-agglutination of *B. pertussis in vitro* (277). It is therefore possible that there may be an exacerbation of this phenotype in the SphB1-deficient mutant. The high adherence of *B. pertussis* to respiratory cells may be counter productive *in vivo*. Coutte *et al* suggest that *in vivo* FHA may initially facilitate binding and then subsequent release, which would facilitate the detachment and dispersal of the bacteria (272).

*B. pertussis* is a strict human pathogen and large infectious doses are therefore required for animal colonization (113). *B. bronchiseptica* animal models have been developed which



require smaller infectious doses (113). Inatsuka *et al* constructed a *B. bronchiseptica* strain which expressed *B. pertussis* FHA (FHA<sub>Bp</sub>) as opposed to the wild type strain that expresses *B. bronchiseptica* FHA (FHA<sub>Bb</sub>) in an attempt to investigate the role of FHA as a primary adhesin and an immunomodulator (278). FHA<sub>Bp</sub> was able to substitute for FHA from *B. bronchiseptica* *in vitro* allowing the mutant bacteria to bind to several epithelial and macrophage cell lines (278). However *in vivo* the FHA<sub>Bp</sub> strain was unable to colonize the lower respiratory tract of rats (278). Further studies were carried out using a mouse infection model. FHA<sub>Bb</sub> allowed the wild type bacteria to colonize the respiratory tract in mice and to modulate the immune response resulting in decreased lung damage and increased bacterial persistence (278). The infection peaked at 4 days post inoculation and then gradually decreased for the following 4 weeks (278). In contrast, mice inoculated with an FHA deficient strain of *B. bronchiseptica* displayed a bimodal response; by day 4 of infection half the mice remained healthy and cleared the infection by day 11 whereas the other half became moribund (278). The authors suggest that the healthy mice developed a robust inflammatory response, whereas the moribund group developed very severe inflammation in the absence of FHA, which resulted in increased bacterial growth, pneumonia and death (278). Infection of mice with the mutant strain expressing *B. pertussis* FHA resulted in the same bimodal response, however on Day 11 more bacteria was recovered from the lungs of these mice compared with the FHA deficient mutants suggesting that the *B. pertussis* FHA had some ability to suppress the immune system (278).

SCID-beige mice that lack T and B cells and some NK cell function were used to examine the effect of FHA on innate immunity (278). The infection of SCID-beige mice with wild type bacteria caused 100% lethality by Day 88 (278). SCID-beige mice infected with the FHA deficient strain remained healthy. The majority of the SCID-beige mice infected with

the FHA<sub>Bp</sub> expressing mutant remained healthy (278). The authors concluded that FHA from *B. pertussis* is capable of subverting the immune response, but not to the same degree as FHA from *B. bronchiseptica* (278). An explanation could be that FHA<sub>Bp</sub> fails to facilitate attachment to the epithelial cells in the lower respiratory tract (278). The authors also suggest that the receptor that *B. pertussis* FHA binds to may not be present in animals and that this could explain why *B. pertussis* is a strict human only pathogen (278).

#### **1.8.4 The interaction of FHA with eukaryotic proteins**

FHA contains two RGD sites, one of which is surface exposed, hydrophilic and antigenic, and has a role in host cell binding during infection (268). In eukaryotes, an RGD sequence is present in fibronectin and other extracellular matrix proteins which allows their interaction with the integrin receptor superfamily (279). A *B. pertussis* mutant with an internal in frame deletion within the FhaB gene in the region encompassing the RGD sequence, showed very poor binding to rabbit respiratory epithelial cells (268). Addition of purified FHA allowed the mutant to bind (268). Relman *et al* initially suggested that the integrin CD11b/CD18 (CR3) on macrophages is involved in the recognition of the RGD motif on FHA (268). The integrin CR3 normally functions in the recognition of endothelial cells during leukocyte recruitment (280). Ishibashi *et al* amended this finding by showing that the RGD motif within FHA interacts with the monocyte signal transduction leukocyte response integrin and its integrin associated protein (CD61 and CD47 respectively) on human monocytes (281). This interaction initiates a signalling event that up regulates CR3, thereby facilitating FHA mediated host-pathogen binding (281). Antibodies developed against FHA have been shown to crossreact with the serum complement component iC3b (280). This antibody maps to the carboxy terminal of FHA and examination of the FHA sequence identified C3b homology,

within an 11 amino acid sequence from 1408 to 1418 (280). There is therefore a high probability that it is this sequence that represents the CR3 binding site of FHA.

*B. pertussis* has been shown to invade epithelial cells. However a *B. pertussis* strain expressing a mutated form of FHA where the glycine in the RGD site had been replaced by an alanine, has significantly diminished invasiveness (282). A monoclonal antibody against the very late antigen (VLA-5) (an epitope within the  $\alpha 5\beta 1$  integrin heterodimer) completely diminished the ability of *B. pertussis* to invade epithelial cells (282). These results led to the conclusion that FHA promotes bacterial entry into epithelial cells through the interaction of the RGD sequence with VLA-5 on the host cell (282). The upregulation of CR3 by RGD-VLA-5 interaction is mediated by phosphatidylinositol 3-kinase (PI3-kinase) signalling (283). The binding of FHA to the CR3 leads to the phagocytosis of *B. pertussis* by macrophages which allows the bacterium to avoid an oxidative burst (284).

FHA has also been shown to up regulate ICAM-1 on the surface of epithelial cells through the interaction between the RGD motif of FHA and VLA-5 (285). ICAM-1 is a cell surface glycoprotein that has a crucial role in inflammatory cell recruitment (285). The binding of the RGD motif of FHA to VLA-5 leads to I $\kappa$ B degradation (286). The infection of epithelial cells with *B. pertussis* has been shown to initiate DNA binding of specific Rel protein family members. It was demonstrated that p50 and p65 are induced and there is a partial upregulation of c-Rel (286).

Factor X of the coagulation cascade binds CR3 during inflammation initiating procoagulant activity (287). FHA also binds to the CR3 and prevents Factor X binding. The blockade of the CR3 by FHA prevents blood clotting and the transendothelial migration of leukocytes

(287). FHA and the CR3 binding loops of Factor X have amino acid sequence homology (287). The binding region of FHA for CR3 appears to reside at least in part in the sequence ETKEVDG (287). FHA and the peptide sequence FHA II (ETKEVDG) were shown *in vivo* to prevent leukocyte extravasation from the blood to the cerebral spinal fluid in a pneumococcal meningitis mouse model (287).

FHA is also a heparin binding protein (288). Heparins are highly sulphated polysaccharides which are used clinically as an anticoagulant. FHA binds to the epithelial cell surface molecule, heparan sulphate glycosaminoglycan (288). Heparan sulphate glycosaminoglycans commonly associates with core proteins to form proteoglycans which are ubiquitous components of eukaryotic cell surface (288). FHA has also been shown to bind to sulfatides, which are sulphated glycolipids (288). The heparin binding domain of FHA was identified by the use of four recombinant fusion proteins and has been mapped to a region near the N terminus between amino acid 442 and 863 (288).

FHA has the ability to agglutinate erythrocytes (289). Using monoclonal antibodies against various epitopes within the bacterial adhesin, the haemagglutination activity of FHA was located to the N-terminus (290). A recent study mapped the haemagglutination domain to the region of FHA between amino acid 430 and 873 (291).

FHA also interacts with eukaryotic carbohydrates (290). The carbohydrate recognition domain (CRD) was identified through the use of monoclonal antibodies (290). An antibody specific for the region of FHA between amino acid 1141 and 1279 prevented FHA binding to lactosylceramide and prevented the binding of *B. pertussis* to ciliated cells (290). An 18kDa polypeptide designated Fragment A corresponding to the putative CRD, bound to

lactosylceramide and sulfatides in a pattern similar to FHA (290, 292). Anti-fragment A sera inhibited the binding of *B. pertussis* to rabbit ciliated cells (292).

### **1.8.5 The immunomodulatory properties of FHA**

The infection of mice with *B. pertussis* leads to an increase in IFN- $\gamma$  concentrations in the murine bronchoalveolar lavage fluid by day 1. A decrease in IFN- $\gamma$  concentrations to near pre-infection levels is observed by day 3 and remain low until at least day 15 (293). Monocytes infected with *B. pertussis* have a significantly impaired capacity to stimulate T cell proliferation to the exogenous antigen, tetanus toxoid *in vitro* (293). A Bvg<sup>-</sup> strain of *B. pertussis* (BP537) had a slight inhibitory effect on T cell proliferation to tetanus toxoid although the inhibition was not nearly as effective as that seen with monocytes infected with the Bvg<sup>+</sup> strain of *B. pertussis* (293). This suggests that non-Bvg regulated factors play a small role in the inhibition. The infection of monocytes with a strain of *B. pertussis* that has a mutation in the haemolytic domain of CyaA, a mutation in the catalytic domain of CyaA or in the expression of FHA induced in T cell proliferation that was comparable to levels observed when monocytes were infected with the Bvg<sup>-</sup> strain (293). This demonstrated that both FHA and CyaA play a major role in *B. pertussis* mediated inhibition of T cell responses to exogenous antigen (293). BP1098 expresses a mutated form of FHA where the glycine in the RGD site had been replaced by an alanine (293). Infection of monocytes with BP1098, resulted in comparable inhibition of T cell proliferation to monocytes that were infected with wild type *B. pertussis*. This suggests the RGD site of FHA does not have a role in the inhibition of T cell proliferation (293).

Studies by McGuirk *et al* have shown that FHA has anti-inflammatory properties. Incubation of macrophages with FHA induced moderate levels of IL-10 and high levels of IL-6

production (227). FHA also suppressed LPS induced IL-12 in an IL-10 dependent manner in macrophages and bone marrow derived dendritic cells (227, 294). Ligation of CR3 with an anti-CD11c antibody has also been shown to modulate IL-12 activity (294-296). FHA was also found to inhibit pro-inflammatory cytokines *in vivo* using a murine septic shock model (294). Mice pre-injected intra-peritoneally with FHA one hour before the injection of LPS had reduced serum concentrations of IL-12 and IFN- $\gamma$  (294).

A study of the effect of FHA on DC maturation showed that it moderately enhanced surface expression of CD86 and CD40 expression but had no effect on CD80 or CCR5 surface marker expression (227). These semi-mature FHA treated DCs were shown to direct the induction of Treg cells that produced high levels of IL-10 and IL-5 and low levels of IL-4 and IFN- $\gamma$  (227). McGuirk *et al* also succeeded in developing an FHA specific Treg cell clone from the lungs of *B. pertussis* infected mice (227). The Treg cell clone secreted high levels of IL-10 and IL-5 and low IFN- $\gamma$  and no IL-2 and IL-4 and was capable of inhibiting a Th1 cell clone specific for an unrelated antigen, influenza virus haemagglutinin (227). The Treg cell clone inhibited IFN- $\gamma$  and IL-2 production and the proliferation of the Th1 cell clone. The mechanism of suppression was shown to be IL-10 dependent (227).

The FHA specific Treg cells expressed high levels of CCR5 and T1/ST2, which contrasted with the surface marker expression of Th1 and Th2 cells, which expressed CCR5 or T1/ST2 respectively (227). The transfer of the FHA specific Treg cell clone to *B. pertussis* infected mice exacerbated the course of infection with bacteria still remaining in the respiratory tract after 3 weeks (227). In contrast, the transfer of *B. pertussis* specific Th1 cells to *B. pertussis* infected mice resulted in a complete clearance of infection after 3 weeks (227). Mice infected with a mutant of *B. pertussis* that is deficient in FHA expression (BP409) cleared the

infection within 3 weeks whereas mice infected with wild type *B. pertussis* did not clear the infection until 5 weeks post inoculation (227). IFN- $\gamma$  levels were elevated in the lung and surrounding lymph nodes of mice infected with the BP409 mutant (227). In mice infected with wild type *B. pertussis*, significant levels of IL-10 were detected in the bronchoalveolar lavage (BAL) fluid whereas IL-10 was absent in the BAL fluid of mice infected with BP409 (227). These findings support a role for FHA in the induction of IL-10 production from innate immune cells and the induction of Treg cells during *B. pertussis* infection.

The 3 component acellular pertussis vaccine (PTd, FHA and PRN) has been shown to attenuate colitis in the *Gai2*<sup>-/-</sup> mouse model (297). It was found that mice immunized with the acellular pertussis vaccine had increased levels of IL-10 in intestinal tissue (297). The immunization of mice with FHA was shown to inhibit T cell proliferation and stimulate apoptosis of activated Th1 cells but was unable to prevent colitis when administered to the *Gai2*<sup>-/-</sup> mice (297).

Another study demonstrated that FHA effectively attenuated the clinical symptoms of experimental colitis. Colitis was induced by the transfer of naive CD4<sup>+</sup>CD45RB<sup>high</sup> cells into SCID mice. These mice were treated with four subcutaneous injections of FHA every two weeks and had significantly reduced symptoms of colitis compared with mice that had been injected with PBS (298). Injection of FHA stimulated IL-10 and TGF- $\beta$  production in the mesenteric lymph nodes and Peyer's patch within 2-6 hours (298). The protective effect of FHA in attenuating colitis was independent of IL-10 production by T cells (53).

Abramson *et al* have also reported that FHA induces apoptosis in human macrophages and bronchial epithelial cells (299). FHA stimulation led to a dose dependent increase in TNF- $\alpha$  production but an antibody against the cytokine did not drastically reduce levels of apoptosis

(299). This suggests that the induction of apoptosis by FHA is not mediated by TNF- $\alpha$ . Blocking TNFR1 with an antibody reduced apoptosis by 50%, suggesting the receptor may play a role in FHA mediated apoptosis (299).

## **1.9 *Fasciola hepatica***

### **1.9.1 Taxonomy of *F. hepatica***

Parasites can be defined as symbiotic organisms that live at the expense of a host organism. All of the eukaryotic kingdoms of plantae, animalia, fungi and protozoa contain parasitic organisms (300). The parasites of the animal kingdom include a group of worms known as helminths. Parasitic helminths infect more than a billion people worldwide and are a major cause of morbidity and disability (301). Helminths are multicellular organisms and can be divided into three groups- the cestodes, trematodes and nematodes (302). The first two groups are all members of Phylum Platyhelminths, meaning flatworms while the latter group make up Phylum Nematoda. The trematodes are known as the flukes and are nearly all parasites of molluscs and vertebrates. The majority of flukes are monoecious. The trematodes can be further sub divided into the tissue flukes such as *Fasciola hepatica* or liver fluke and the blood flukes such as *S. mansoni*.

### **1.9.2 Life cycle of *F. hepatica***

Fascioliasis is an infection caused by *F. hepatica* and has traditionally been considered to be an important veterinary disease because of the substantial cases of the disease in ruminants particularly cattle and sheep. Liver fluke infection results in productivity losses (eg reduced meat, wool and milk yield) and therefore has economic consequences. *F. hepatica* can also infect humans and in parts of the developing world such as the Bolivian Altiplano, the prevalence of infection can be as high as 67% of the local population (303). *F. hepatica* is



one of the largest parasites to infect humans with an average adult liver fluke measuring 30 mm in length and 13 mm in width (304). The life cycle of the helminth involves the infection of a definitive host which is a vertebrate and an intermediate host, which is a lymnaeid snail that inhabits shallow ponds (304). The vertebrate infection is initiated by the consumption of plant material that harbours encysted metacercariae. The metacercariae excysts in the duodenum and migrates through the intestinal wall. It then crosses the peritoneal cavity before entering the liver. The juvenile liver fluke spends between 8-12 weeks feeding on the parenchymal cells of the liver which causes extensive haemorrhaging and perforation (305). The parasite then moves to the bile duct where it achieves sexual maturity. The hermaphroditic adult liver fluke burrows through the walls of the bile duct and feeds on blood which provides the nutrients necessary for egg production (304). The unembryonated eggs are dispatched into the duodenum and are subsequently passed onto pasture in the faeces. The eggs must be deposited in fresh water in order to complete their development and hatch. Hatching normally occurs within 10-15 days and the ciliated miracidium that are released actively swim in search of the intermediate host (304). The miracidium then undergo morphogenesis sequentially to become sporocysts and then rediae. Each stage results in an increased number of immature flukes. Each redia gives rise to many cercariae which burrow out of the snail, into water and eventually encyst on water plants becoming metacercariae (304).

### **1.9.3 Host immune response against *F. hepatica***

The life cycle of *F. hepatica* involves long periods within a vertebrate organism and the parasite is therefore a target of the immune system. The parasite has evolved an array of immune evasion strategies in order to survive and many studies have begun to elucidate the complex mechanisms. Studies from experimentally infected cattle show that the host mounts

a Th2 dominated response against *F. hepatica* infection, characterized by IL-4 production, eosinophilia and the production of IgG1 antibodies (306-308). Time course studies conducted by Waldvogel *et al* reported that within 10 days the immune response against *F. hepatica* is Th2 biased. PBMCs taken from cattle 10 days postinfection, expressed high amounts of IL-4 and no IFN- $\gamma$  in response to parasite antigen stimulation *in vitro* (309). The host immune response against liver fluke is usually not protective and within 12 weeks the infection becomes chronic. PBMCs taken from chronically infected animals have reduced levels of IL-4 and produce high levels of IL-10 and TGF- $\beta$  in response to parasite antigen (309, 310). Neutralization of IL-10 and TGF- $\beta$  results in increased production of both IL-4 and IFN- $\gamma$  suggesting that the parasite may induce these anti-inflammatory cytokines to evade the immune response (310). 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. Confirming this observation, studies have shown that fasciolosis suppresses immune responses against bacterial infections. 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 (311). In a mouse model of *F. hepatica* infection, Brady *et al* reported that the infection of mice with the parasite suppressed a protective Th1 response against *B. pertussis* which resulted in increased bacterial numbers in the lung (312).

#### **1.9.4 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. Many studies have shown that the ability of *F. hepatica* to modulate and evade the immune response is often achieved by these molecules. The surface of the parasite in the vertebrate host is covered with a 40 nm layer of glycoprotein, known as the glycocalyx.

In juvenile flukes, the glycocalyx is replaced every 3 hours which prevents antibody bound immune effector cells, such as eosinophils, from damaging the parasite (313, 314). The secretome of *F. hepatica* is known as excretory-secretory products (ES) and has many immunomodulatory properties. Serradell *et al* reported that ES induces apoptosis in rat peritoneal eosinophils (315). In a separate study bovine macrophages stimulated with ES produced reduced levels of NO and IFN- $\gamma$  in response to LPS (316). Proteomic analysis of ES has revealed that constituent proteins include glutathione S-transferase, peroxiredoxin and cysteine proteases. Glutathione S-transferase is an oxidant scavenging enzyme and may allow *F. hepatica* to disable the toxic reactive oxygen products of innate immune cells (317). Peroxiredoxin has been shown to convert macrophages to an alternatively activated phenotype (AAM $\Phi$ ) characterized by the production of high levels of IL-10 and PGE<sub>2</sub> and low levels of IL-12 (318). In a subsequent study, *F. hepatica* AAM $\Phi$  enhanced the secretion of IL-4, IL-5 and IL-13 from naive T cells (319). Cysteine proteases are known as cathepsins and *F. hepatica* expresses and secretes cathepsin L and P proteases. Interestingly, the helminth expresses different cathepsins at different stages of its life cycle. On entering the liver, the juvenile fluke downregulates the expression of cathepsin B and upregulates the expression of cathepsin L (320). In the immunologically safe environment of the bile ducts, the adult liver fluke solely expresses cathepsin L proteases, which digest blood for the parasite (320). Cathepsin L proteases have also been shown to modulate the immune response. The incubation of human and ovine T cells with fluke cathepsin L resulted in reduced surface CD4<sup>+</sup> expression, and in a mouse model fluke cathepsin L, suppressed the development of a Th1 response against a pertussis whole cell vaccine (321, 322).

## **1.10 Helminth infection and the hygiene hypothesis**

### **1.10.1 Helminth infection suppresses the immune response against bystander infections**

Many studies have shown that helminth infection can result in immune hyporesponsiveness against co-infected pathogens. As discussed above *F. hepatica* infection attenuates the immune response against *M bovis* and *B. pertussis*. Studies involving other helminths have led to further insights into the mechanisms involved in parasite induced immune suppression. The chronic infection of mice with the gastrointestinal nematode *Heligmosomoides polygyrus* results in an impaired ability to control a *Plasmodium chabaudi* infection (323). Coinfected mice produced significantly lower levels of IFN- $\gamma$  in response to malaria antigen and had reduced levels of plasmodium specific IgG2a (323). Blood plasma taken from *H. polygyrus* infected mice had significantly increased levels of TGF- $\beta$  and the authors of this study suggest that this may provide the mechanism for the suppression of the malaria specific Th1 immune response (323). An epidemiological study conducted in Burkina Faso reported that the incidence of lepromatous leprosy which is caused by *Mycobacterium leprae* was twice as high in areas where the nematode *Onchocerca volvulus* was hyperendemic (324).

### **1.10.2 Helminth infection attenuates allergic inflammation**

Helminths have received considerable interest in the developed world due to their ability to alter the immune responses that mediate allergy and autoimmune diseases. These observations have resulted in a major shift in the hygiene hypothesis, which initially proposed that Th2 mediated allergies could be counteracted by micro-organisms that induce Th1 responses (325). This theory was based on the hypothesis that Th1 cells can inhibit Th2 cell responses (326). It however did not explain how helminth infections, that also induce Th2 responses could attenuate allergy. The revised model proposes that regulatory immune responses induced by helminth infection inhibit allergic inflammation (327). Studies in

animal models of allergy have begun to elucidate the mechanisms. Wilson *et al* reported that mice infected with *H. polygyrus* had reduced inflammatory infiltrates in models of experimentally induced airway allergy. The treatment of the infected mice with antibodies to CD25 reversed the suppression indicating that helminth induced Treg cells are important (328). Another study conducted by Mangan *et al*, showed that *S. mansoni* infection protects mice from an experimental model of fatal systemic anaphylaxis (329). IL-10 and B cells provide the protective mechanism and the transfer of B cells from IL-4 deficient mice that had been modulated by worms *in vitro* conferred resistance to anaphylaxis (329).

### **1.10.3 Helminth infection attenuates autoimmune inflammation**

In a seminal study conducted by Correale and Farez, helminth infected MS patients were monitored for a period of 4.6 years. When compared with uninfected patients, parasite infected MS patients had a significantly reduced number of relapses and lesions in the CNS (330). PBMCs taken from the infected patients produced significantly higher levels of IL-10 and TGF- $\beta$  in response to myelin basic protein when compared with PBMCs from uninfected patients (330). Furthermore the cloning frequencies of Tr1, Th3 and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells were substantially higher in the infected patients (330). This data provides strong evidence that helminth induced Treg cells can protect against MS. Studies in mouse models of autoimmune disease have yielded similar findings. *S. mansoni* infection can protect diabetes and colitis in mouse models (331, 332). Smith *et al* have shown that the attenuation of colitis by the parasite is macrophage dependent (332). The intestinal trematode *Trichinella spiralis* also suppresses colitis in the mouse and is now being used to treat patients with ulcerative colitis and Crohns disease (333, 334). *S. mansoni* infection is also protective in murine EAE where the parasite and disease are not co-localized (335). Sewell *et al* reported

that the helminth did not attenuate EAE in STAT-6 deficient mice indicating that the signalling molecule plays a critical role in parasite mediated protection (335).

## **1.11 TGF- $\beta$**

### **1.11.1 The synthesis of TGF- $\beta$**

In mammals there are three members of the TGF- $\beta$  cytokine family- TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 (336). These cytokines are produced by a wide variety of lymphoid and non lymphoid cells and have pleiotropic functions (337). TGF- $\beta$ 1 is the predominant form produced by immune cells (337). Murine TGF- $\beta$  is synthesized as a 391 amino acid long precursor polypeptide which contains a hydrophobic signal sequence, a pro region and the mature peptide. The polypeptide is processed in the Golgi and the product is a disulfide linked homodimer (25kDa) (338). This active homodimer is non covalently associated with the remainder of the precursor which is known as latency-associated peptide (LAP) to form a 75kDa complex (339). This latent complex may be secreted or can associate with latency-TGF- $\beta$ -binding protein (LTBP), which targets TGF- $\beta$  to the extracellular matrix (340). The active TGF- $\beta$  homodimer must be released from LAP and LTBP in order to bind its receptor. TGF- $\beta$  activators (TA) trigger LAP degradation or conformational change that releases the active cytokine (341). Interestingly, the cells that secrete TA can differ from those that secrete TGF- $\beta$  (341).

### **1.11.2 T cell derived TGF- $\beta$**

One could argue that TGF- $\beta$  exerts the greatest impact on T cells. TGF- $\beta$ 1 deficient mice die within 3-4 weeks of birth of a multi-organ inflammatory syndrome which is characterized by the hyperactivation of T cells and a progressive infiltration of leukocytes into the affected organs (342). This demonstrates the pivotal role of TGF- $\beta$  in the regulation of lymphocyte

activation. TGF- $\beta$  affects T cell proliferation, differentiation and survival. One of the mechanisms behind the inhibition of T cell proliferation by TGF- $\beta$  is the blockade of IL-2 transcription (343). TGF- $\beta$  inhibits the proliferation of naive T cells but does not affect activated T cells which have reduced expression of TGF- $\beta$ RII (344). Interestingly, IL-10 induces TGF- $\beta$ RII expression thereby conferring TGF- $\beta$  responsiveness upon activated T cells (344). CD28 stimulation attenuates TGF- $\beta$  mediated inhibition of proliferation (345). This provides a model to explain how TGF- $\beta$  inhibits aberrant T cell responses against self antigens in the periphery. Under steady state conditions TGF- $\beta$  inhibits the proliferation of naive T cells (336). However this inhibition is reversed by strong co-stimulatory signals such as CD28 ligation which are usually associated with infection (336). In the presence of CD28 stimulation, TGF- $\beta$  actually inhibits T cell apoptosis and promotes T cell expansion (345). TGF- $\beta$  has been shown to be important in the induction of both pro and anti-inflammatory T cell subsets. TGF- $\beta$  and IL-2 induce Foxp3<sup>+</sup> expression in naive T cells and TGF- $\beta$  induced regulatory T cells have been shown to be phenotypically identical to thymic derived Treg cells (200). TGF- $\beta$  and IL-6 are the key differentiation factors for Th17 cells (133). The source of the TGF- $\beta$  required is CD4<sup>+</sup> T cells (172). TGF- $\beta$  also inhibits Th1 and Th2 cell differentiation. It prevents the development of Th1 cells by inhibiting T-bet expression and similarly in Th2 cells TGF- $\beta$  prevents differentiation by inhibiting GATA-3 expression (233, 346). Interestingly fully differentiated Th2 cells are unaffected by TGF- $\beta$  whereas IFN- $\gamma$  expression is inhibited by TGF- $\beta$  in fully differentiated Th1 cells (347).

Models of oral tolerance have helped to establish the existence of a subset of CD4 T helper cells known as Th3 cells which produce TGF- $\beta$  as their effector cytokine (235). Myelin basic protein (MBP) specific Th3 cell clones have been shown to inhibit MBP specific Th1 cells and suppress EAE (235). Th3 cell mediated suppression of EAE was abrogated by an anti

TGF- $\beta$  antibody (235). The role of TGF- $\beta$  in natural regulatory T cell mediated suppression of immune responses is less clear. Natural Treg cells from TGF- $\beta$ 1<sup>-/-</sup> mice have been shown to inhibit the proliferation of effector T cells *in vitro* (214). However *in vivo* studies have been less conclusive. Powrie *et al* reported that an anti-TGF- $\beta$  antibody abrogated the protective effect of a CD4<sup>+</sup>CD45RB<sup>high</sup> T cell transfer in a SCID mouse IBD model (348). In contrast, Mamura *et al* showed that natural Treg cells from TGF- $\beta$ 1<sup>-/-</sup> were protective in a mouse model of autoimmune inflammation induced by the transfer of CD25<sup>-</sup> spleen cells into Rag2<sup>-/-</sup> recipients (349).

### 1.11.3 The effect of TGF- $\beta$ on innate immune cells

The effect of TGF- $\beta$  on the monocyte/macrophage cell lineage appears to depend on the cells stage of development. TGF- $\beta$  recruits monocytes to sites of inflammation by the induction of adhesion molecule expression, which allows the cells to attach to the extracellular matrix (350). TGF- $\beta$  also induces IL-1 and IL-6 production in monocytes, thereby potentiating inflammation (351, 352). However the effect of TGF- $\beta$  on macrophages is predominantly inhibitory. TGF- $\beta$  downregulates Fc $\gamma$ RI and Fc $\gamma$ RII, which affects the phagocytic capability of the macrophage and also inhibits LPS induced TNF- $\alpha$  and IL-1 $\beta$  production (353, 354). Interestingly, TGF- $\beta$  inhibits CD14 expression in LPS stimulated macrophages which attenuates further LPS induced TLR4 signalling (355). TGF- $\beta$  also affects the antigen presentation ability of macrophages through the inhibition of CD40 expression and IL-12p40 production (356). DCs are the most important APCs and studies have shown that TGF- $\beta$  also inhibits their function. Geissmann *et al* have reported that the stimulation of Langerhans cells with TGF- $\beta$  inhibits LPS induced MHC Class II, CD80 and CD86 upregulation and IL-1 and TNF- $\alpha$  induced IL-12p70 production (357).



#### **1.11.4 Cell surface expression of LAP**

Howard Weiner and colleagues have identified cell surface expression of LAP as indicative of suppressor function. LAP is expressed on the surface of *ex vivo* immature human DCs (358). These cells were shown to inhibit Th1 cell differentiation and enhance the differentiation of Foxp3<sup>+</sup> Treg cells by a TGF- $\beta$  dependent mechanism (358). Furthermore LPS stimulation of the immature DCs resulted in a loss of membrane LAP (358). LAP is also expressed on the surface of T cells. CD4<sup>+</sup>CD25<sup>+</sup> T cells that express LAP have been shown to suppress the proliferation of CD4<sup>+</sup>CD25<sup>-</sup>LAP<sup>-</sup> T cells *in vitro*.(359) The suppression was both cell contact and soluble factor dependent (359). The transfer of CD4<sup>+</sup>CD25<sup>+</sup>LAP<sup>+</sup> cells but not CD4<sup>+</sup>CD25<sup>+</sup>LAP<sup>-</sup> cells attenuated the clinical symptoms of disease and the suppressive function of the LAP<sup>+</sup> T cells was found to be TGF- $\beta$  dependent (359).

### **1.12 Multiple sclerosis and experimental autoimmune encephalomyelitis**

#### **1.12.1 Multiple Sclerosis**

Multiple Sclerosis (MS) is an inflammatory demyelinating human disease of CNS white matter that affects more than 2.5 million individuals in Europe and North America (360). MS is a chronic debilitating disease and its socio economic burden in young adults is second only to trauma (361). The disease derives its name from the scleroses which are better known as plaques or lesions that develop in the white matter of the CNS. It is widely accepted that MS involves the activation of myelin specific CD4<sup>+</sup> T helper cells in the periphery (362). These T cells are captured on the vascular endothelium by selectins and integrins and migrate across the endothelium in response to chemotactic signals (362). Matrix Metalloproteinases (MMPs) degrade extracellular proteins and facilitate T cell entry into the basal lamina (362). The T cells are reactivated in the CNS by microglial cells and astrocytes (362). The T cells

secrete cytokines such as IFN- $\gamma$  and IL-17 which activate macrophages and possibly neutrophils (166, 362). The macrophages exhibit increased phagocytic activity and release NO metabolites, proteases and complement that damage myelin (362). Autoantibodies against myelin are also important in demyelination (363). The hypothesis that T cells initiate disease is supported by the cellular composition of CNS and CSF infiltrating cells (364). Furthermore, MMPs, IL-6, TNF- $\alpha$ , IFN- $\gamma$  and IL-17 have all been detected in the inflammatory lesions of MS patients (362).

There are two main disease courses in multiple sclerosis. Approximately 85% of MS cases begin as relapsing remitting disease (RRMS). Discrete attacks involving white matter tracts that cause neurologic impairment are followed by a period of clinical stability (360). Periods of remission are characterized by the re-organization of axonal Na<sup>+</sup> channels and the remyelination of axons, which results in clinical recovery (360). The relapsing remission stage of disease can last for years or even decades. However the majority of RRMS patients eventually enter a secondary progressive stage of disease (SPMS) with continuous neurological inflammation and no periods of remission (360). About 15% of MS patients develop primary progressive disease (PPMS), which is characterized by increasing neurological deficits from the onset of disease (360).

The aetiology of disease is still largely unknown, but evidence suggests 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 (361). Levels of sunlight have also been identified as a causative factor with a north-south disease gradient (361). Studies have also shown that MS attacks often follow viral relapses, suggesting that infections may play a role in disease

development and progression (361). The observance of MS epidemics in previously isolated communities, such as the one on the Faroe Islands supports an association between an infectious agent and disease development (361). Molecular mimicry has been proposed as a mechanism to explain how infections may induce MS. It proposes that self antigen specific T cells are activated by antigens on pathogens that are homologous to self antigens (361).

### **1.12.2 Experimental autoimmune encephalomyelitis**

Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS. The model originated from the vaccination of humans with rabies infected rabbit spinal cord by Louis Pasteur. A few years after vaccination some individuals developed neuroparalytic incidents which were later attributed to the contaminating spinal cord components in the inoculums (365). Current models employed involve the immunization of the animal with whole spinal cord homogenate, myelin proteins, or defined peptides such as myelin oligodendrocyte glycoprotein (MOG), emulsified in CFA. Chronic models of EAE have been developed which include exacerbations and remissions as seen in MS. SJL/J mice immunized with myelin proteolipid protein (PLP) in CFA develop relapsing remitting EAE. In other EAE models, the animal develops an acute form of disease from which they do not recover. An example of this form of disease is the EAE seen in C57BL/6 mice immunized with MOG in CFA. The clinical and neuropathological signs in EAE resemble those seen in MS (362). Myelin specific CD4<sup>+</sup> Th1 and Th17 cells have both been implicated in the initiation of EAE and in almost all models these T cells can be isolated and used to transfer disease to naive animals (170). Antibody responses against myelin proteins can be detected at the site of vesiculating myelin in the brains of mice with EAE which is analogous to observations made in patients with MS (363).

EAE models have been vital for the study of multiple sclerosis and the model has led to the development of widely used MS drugs such as Copaxone which is a synthetic polymer analog of myelin (362). There are however some key differences between EAE and MS and many therapeutics that are effective against EAE, do not attenuate MS and in some cases have actually been shown to exacerbate disease. EAE is induced and therefore cannot mimic a spontaneous disease such as MS (365). It develops over days in most models, whereas MS manifests insidiously over years, by which time antibodies and complement may be more important than in mouse models (365). EAE is also studied using inbred strains of animals which differs from the heterogenous population in which MS develops (365). It has been argued that EAE is more similar to the human disease acute disseminated encephalomyelitis which is an autoimmune disease of the brain that is predominantly induced by viral infection (362). However at present EAE is the most powerful tool for dissecting the molecular mechanisms and immune responses involved in the pathogenesis of MS.

**Hypothesis behind the studies performed in Chapter III: Modulation of the innate and adaptive immune response by cAMP activators.**

**IL-10 produced by DCs during antigen presentation is required for the differentiation of antigen specific Tr1 cells. This CD4<sup>+</sup> T cell type has considerable potential as a therapy for autoimmune disease. TLR agonists have been shown to induce Tr1 cells as well as the Th1 and Th17 cells. cAMP activators have been shown to inhibit TLR agonist induced IL-12 and enhance TLR agonist induced IL-10 production by DCs. It was therefore postulated that the combination of a cAMP activator and a TLR agonist might enhance the ability of a TLR agonist to induce Tr1 cells while inhibiting TLR induced Th1 and Th17 cell differentiation. This approach could therefore provide a novel therapy for autoimmune disease.**

#### **Objectives of the project**

- Determine the optimal concentration of specific TLR agonists for the induction of IL-10 production by DCs**
- Investigate the effects of different cAMP activators on TLR agonist induced cytokine production by DCs**
- Examine if the ability of cAMP activators to modulate TLR induced cytokine production by DCs, altered the T cell subtype induced by the DCs *in vivo***
- Determine the optimal combination of TLR agonist and cAMP activator for the induction of antigen specific IL-10 producing T cells and investigate can these cells suppress autoantigen specific Th1 and Th17 cells.**

## Hypothesis behind the studies performed in Chapter IV: The immunomodulatory properties of Filamentous Haemagglutinin

FHA is a major virulence factor of *B. pertussis* and has been shown to play a role in the evasion of the immune response by the bacteria. It has been previously reported that FHA induces IL-10 and inhibits TLR agonist induced IL-12 production by DCs and macrophages. It was therefore decided to investigate the potential intracellular signalling pathways induced by FHA in DCs. Specific inhibitors of the signalling pathways could then be used to examine their role in FHA induced cytokine production. DCs treated with antigen and FHA have been shown to induce Tr1 cells *in vitro* by an IL-10 dependent mechanism. The next logical step was to investigate the potential of FHA to function as an adjuvant *in vivo* for the induction of Treg cells specific for a co-administered antigen. Previous studies have shown that adjuvants that induce Treg cells can be co-administered with auto-antigens and attenuate the clinical symptoms of model autoimmune diseases. This report shows that mice immunized with MOG and FHA before the induction of EAE, develop attenuated symptoms of the disease. It was therefore postulated that FHA may induce autoantigen specific Treg cells which might be capable of suppressing the pathogenic Th1 and Th17 cells that cause autoimmune disease. Furthermore FHA may inhibit cytokine production by innate cells which is required for the differentiation and survival of Th1 and Th17 cells.

### Objectives of the project

- Investigate potential intracellular signalling pathways induced by FHA in DCs and to determine their involvement in FHA induced cytokine production through the use of specific inhibitors.

- To examine the effect of FHA on DC maturation
- Determine the adjuvant properties of FHA *in vivo*.
- Develop antigen specific Treg cell lines through the use of FHA and investigate the potential of these cells to suppress Th1 and Th17 cell lines.

Hypothesis behind the studies performed in Chapter V: Infection with *F. hepatica* attenuates EAE through TGF- $\beta$  mediated suppression of Th1 and Th17 responses.

Previous studies have shown that parasitic infections can attenuate the immune responses that lead to autoimmune and allergic inflammation. Infection with the parasitic trematode *F. hepatica* has been shown to activate bystander suppression against Th1 responses induced by other pathogens. Consistent with these findings, work carried out in the lab showed that mice infected with *F. hepatica* developed attenuated clinical symptoms of EAE. It was decided to investigate the mechanisms involved in the suppression of pathogenic autoimmune responses by the helminth infection. Studies had reported that the immunoregulatory cytokines IL-10 and TGF- $\beta$  play an important role in the suppression of *F. hepatica* specific Th1 and Th2 responses. I therefore investigated their potential role in the ability of *F. hepatica* to attenuate the clinical symptoms of EAE and to suppress autoantigen specific Th1 and Th17 responses. Having shown that TGF- $\beta$  is required for the attenuation of EAE by the trematode infection, it was decided to look at the effect of the recombinant cytokine on autoantigen specific Th1 and Th17 responses and on the production of pro-inflammatory cytokines by DCs. This would help elucidate how parasite induced TGF- $\beta$  attenuates the clinical symptoms of EAE.

## Objectives of the project

- Further investigate the effect of *F. hepatica* infection on immune cells at the site of infection namely the peritoneal cavity.
- Examine the potential role of IL-10 and TGF- $\beta$  in the attenuation of the symptoms of EAE by *F. hepatica* through the use of IL-10<sup>-/-</sup> mice and a TGF- $\beta$  neutralizing antibody.
- Determine the effect of recombinant TGF- $\beta$  on autoantigen specific Th1 and Th17 cell responses and on TLR agonist induced cytokine production by DCs.



# **Chapter II**

## **Materials and Methods**



## **2.1 Materials**

### **2.1.1 Reagents used in tissue culture and *in vivo***

#### **2.1.1.1 Cell culture medium**

Roswell park memorial institute (RPMI)-1640 medium (Biosera) was supplemented with 8% heat inactivated (56°C for 60min) foetal calf serum (FCS) (Biosera), 100mM L-Glutamine (Gibco), 100 µg/ml penicillin/streptomycin (Gibco).

X-VIVO 15 medium with gentamicin and phenol red (Lonza)

#### **2.1.1.2 Ammonium chloride lysis solution (0.87%)**

4.35g Ammonium Chloride NH<sub>4</sub>Cl

Dissolved in 500 mls ddH<sub>2</sub>O

#### **2.1.1.3 MACS running Buffer**

Dulbeccos PBS supplemented with 1% (v/v) FCS and 0.5 mM EDTA

#### **2.1.1.4 MACS rinsing Buffer**

Dulbeccos PBS supplemented with 0.5 mM EDTA

### 2.1.1.5 TLR ligands

Ligand	TLR	Supplier	Concentration used <i>in vitro</i>	Dose used <i>in vivo</i>
LPS	4	Alexis	0.1 ng/ml- 10 µg/ml	10 µg/mouse
CpG	9	Sigma-Genosys	0.1 ng/ml- 100 µg/ml	25 µg/mouse
Pam3CSK4	1 and 2	Invivogen	0.1 ng/ml- 10 µg/ml	
Poly I:C	3	Sigma	1 ng/ml- 100 µg/ml	
MPL	4	Invivogen	0.1 ng/ml- 100 µg/ml	

The doses of LPS and CpG used *in vivo* were based on optimization experiments carried out by others in the lab.

### 2.1.1.4 cAMP activators

Activator	Supplier	Concentration used <i>in vitro</i>	Dose used <i>in vivo</i>
Prostaglandin E <sub>2</sub>	Calbiochem	1 µM and 10 µM	0.05 mg/kg, 0.5 mg/kg and 5 mg/kg
Forskolin	Calbiochem	10 µM and 100 µM	0.1 mg/kg, 1 mg/kg and 10 mg/kg
Dibutyryl cAMP	Calbiochem	10 µM and 100 µM	0.5 mg/kg, 5 mg/kg and 50 mg/kg

The concentrations/doses of the cAMP activators used *in vitro* and *in vivo* were chosen after a review of the literature (96, 107, 108, 366-368).

### 2.1.1.5 Antigens

Antigen	Supplier	Concentration used <i>in vitro</i>	Dose used <i>in vivo</i>
KLH	Calbiochem	10 µg/ml, 25 µg/ml and 100 µg/ml	10 µg/mouse
MOG <sub>35-55</sub>	Cambridge Biosciences (UK)	10 µg/ml, 25 µg/ml and 100 µg/ml	150 µg/mouse
ES	Obtained from adult liver fluke harvested at a local abattoir	20 µg/ml	

The concentrations/doses of antigens used were based on optimization experiments carried out by others in the lab.

### 2.1.1.6 Bacterially derived molecules

Molecule	Supplier	Concentration used <i>in vitro</i>	Dose used <i>in vivo</i>
FHA	CAMR	0.1 µg/ml- 10 µg/ml	0.2 µg/mouse- 10 µg/mouse
PT	Kaketsuken		500 ng/mouse

### 2.1.1.7 Inhibitors used *in vitro*

Inhibitor	Supplier	Specificity	IC50	Concentration used	Acts on
U0126	Calbiochem	p-ERK	58- 72 nM	5 $\mu$ M	MEK 1/2
SB203580	Calbiochem	p-p38	50- 100 nM	5 $\mu$ M	p38 activating kinase
BAY 11-7082	Calbiochem	NF- $\kappa$ B	10 $\mu$ M	5 $\mu$ M	I $\kappa$ B $\alpha$ phosphorylation

The concentrations of inhibitors used were based on optimization experiments carried out by others in the lab.

### 2.1.1.8 Recombinant Cytokines

Cytokine	Supplier	Concentration Used <i>In vitro</i>
rIL-10	R&D	500 pg/ml
rIL-12	R&D	1 ng/ml
rIL-23	R&D	10 ng/ml
rTGF- $\beta$	R&D	1 pg/ml- 10 ng/ml
rIL-2	R&D	10 U/ml= 5 ng/ml

The concentrations of cytokines used were based on optimization experiments carried out by others in the lab

### 2.1.1.9 Antibodies Used

Antibody	Clone	Supplier	Concentration used <i>in vitro</i>	Dose used <i>in vivo</i>
Anti-IL-12	C17.8	BD Biosciences	10 µg/ml	
Anti-IL-4	BVD4-1D11	BD Biosciences	10 µg/ml	
Anti-IFN- $\gamma$	R4-6A2	BD Biosciences	10 µg/ml	
Anti-IL-10	JES5-16E3	BD Biosciences	10 µg/ml	
Anti-CD3e	145-2C11	BD Biosciences	1 µg/ml	
Anti-TGF- $\beta$	1D11	Bioceros BV	10 µg/ml	100 µg/mouse and 200 µg/mouse
Anti- $\beta$ galactosidase	GL113	Bioceros BV	10 µg/ml	100 µg/unit and 200 µg/mouse

The concentrations of antibodies used *in vitro* were based on optimization experiments carried out by others in the lab. The dose of anti-TGF-beta antibody used *in vivo* was chosen after a review of the literature (369, 370).

## **2.1.2 Reagents used for ELISA and Flow Cytometry**

### **2.1.2.1 Phosphate-buffered saline (PBS) 20X**

320g Sodium chloride (NaCl, 1.4 M)

46g di-Sodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ , 0.08 M)

8g Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ , 0.01 M)

8g Potassium chloride (KCl, 0.03 M)

Dissolved in 2L  $\text{dH}_2\text{O}$ , pH 7.0

### **2.1.2.2 ELISA stopping solution (1 M)**

26.6 mls 18.8M ( $\text{H}_2\text{SO}_4$ )

473.4 mls  $\text{dH}_2\text{O}$

### **2.1.2.3 Phosphate Citrate Buffer**

10.2 g Citric acid anhydrous ( $\text{C}_6\text{H}_8\text{O}_7$ )

36.9 g di-Sodium hydrogen orthophosphate dodecahydrate ( $\text{NaHPO}_4 \cdot 12\text{H}_2\text{O}$ )

Dissolved in 1L of  $\text{dH}_2\text{O}$ , pH 5.0

### **2.1.2.4 FACS Buffer**

PBS (Dulbeccos)

2% FCS

0.1% sodium azide ( $\text{NaN}_3$ )



### 2.1.2.7 ELISA Antibody Concentrations

Cytokine	Coating Antibody Concentration/ Dilution	Top Working Standard	Detection Antibody Concentration/ Dilution
<b>BD ELISA Kits</b>			
IFN- $\gamma$	1 $\mu\text{g/ml}$	10 ng/ml	1 $\mu\text{g/ml}$
IL-4	1 $\mu\text{g/ml}$	2000 pg/ml	1 $\mu\text{g/ml}$
IL-5	1 $\mu\text{g/ml}$	2500 pg/ml	1 $\mu\text{g/ml}$
IL-6	1 $\mu\text{g/ml}$	5000 pg/ml	1 $\mu\text{g/ml}$
IL-12p40	1 $\mu\text{g/ml}$	5000 pg/ml	1 $\mu\text{g/ml}$
IL-13	1 $\mu\text{g/ml}$	4000 pg/ml	1 $\mu\text{g/ml}$
<b>R &amp; D Duosets</b>			
IL-10	1/180	2000 pg/ml	1/180
IL-17	1/180	1000 pg/ml	1/180
IL-1 $\beta$	1/180	1000 pg/ml	1/180
IL-12p70	1/180	1500 pg/ml	1/180
TNF- $\alpha$	1/180	2000 pg/ml	1/180
GM-CSF	1/180	2000 pg/ml	1/180
TGF- $\beta$	1/180	1000 pg/ml	1/180
<b>eBioscience ELISA Kit</b>			
IL-23	1/250	4000 pg/ml	1/250

### 2.1.2.8 Antibodies used in Flow Cytometry

Antibody Specificity	Fluorochrome	Secondary Antibody	Clone	Isotype of Primary Ab	Supplier
<b>Myeloid Lineage markers</b>					
CD11c	PE-Cy5		N418	Hamster IgG	eBioscience
F4/80	PE-Cy5		BM-8	Rat IgG2a	eBioscience
<b>Maturation markers</b>					
CD80	PE		16-10A1	Hamster IgG2κ	BD Pharmingen
CD86	FITC		B7.2	Rat IgG2aκ	BD Pharmingen
CD40	FITC		HM40-3	Hamster IgM	BD Pharmingen
CCR5	PE		HM-CCR5(7A4)	Hamster IgG1	eBioscience
<b>Lymphoid Lineage Markers</b>					
CD4	PE-Cy5		GK1.5	Rat IgG2b	eBioscience
	PE-Cy7		GK1.5	Rat IgG2b	eBioscience
<b>Activation Markers</b>					
CD25	PE-Cy5		PC61.5	Rat IgG1	eBioscience
<b>Cytokines</b>					
IL-10	FITC		JES-1683	Rat IgG <sub>2b</sub>	BD Pharmingen
IL-17	PE		TC11-18H10	Rat IgG1	BD Pharmingen
IFN-γ	APC		XMG1.2	Rat IgG1	eBioscience
LAP	Biotinylated	APC-Cy7			R & D
TGF-β	Biotinylated	PE			R & D

## **2.1.3 Reagents Used for SDS-PAGE and Western Blot Analysis**

### **2.1.3.1 Electrophoresis running buffer**

125 mM Tris base (15g)

0.96 M Glycine (72g)

17 mM Sodium dodecyl sulphate (5g)

Dissolved in 1L dH<sub>2</sub>O (1L)

### **2.1.3.2 Transfer buffer**

25.5 mM Tris-HCl pH 8.3 (2.73g)

0.2 M Glycine (13g)

20% v/v Methanol (180ml)

0.05% w/v SDS (0.45g)

Dissolved in dH<sub>2</sub>O

### **2.1.3.3 1X Sample Buffer**

2.5 mM Tris-HCl pH 6.8

11% Glycerol

2% SDS

0.1% bromophenol blue

dH<sub>2</sub>O

50mM DTT- only added before use.

### 2.1.3.4 Antibodies used in Western Blotting

Antibody Specificity and Clone	Primary Antibody Dilution	Secondary Antibody	Primary Antibody Source
p-ERK E-4	1/750	1/2000 HRP-linked goat anti-mouse IgG (Sigma)	Santa Cruz (CA)
ERK1 (Total) K-23	1/2000	1/1000 HRP-linked goat anti-rabbit IgG (Sigma)	Santa Cruz (CA)
p-p38 (Thr180/Tyr182) 2B10	1/2000	1/2000 HRP-linked goat anti-mouse IgG (Sigma)	Cell Signalling (MA)
p38 (Total) C-20	1/2000	1/1000 HRP-linked goat anti-rabbit IgG (Sigma)	Santa Cruz (CA)
c-Fos H-125	1/200	1/1000 HRP-linked goat anti-rabbit IgG (Sigma)	Santa Cruz (CA)
I $\kappa$ B- $\alpha$	1/100	1/2000 HRP-linked goat anti-mouse IgG (Sigma)	Gift from Luke O' Neill Lab

The concentrations of antibodies used were based on optimization experiments carried out by myself and others in the lab.

## **2.2 Methods**

### **2.2.1 Animals**

Specific pathogen-free BALB/c (H-2<sup>d</sup>), C3H/HeN (H-2<sup>k</sup>), C3H/HeJ (H-2<sup>k</sup>) and C57BL/6 (H-2<sup>k</sup>) mice were purchased from Harlan UK Ltd., Bicester, Olac, UK. IL-10<sup>-/-</sup> mice were obtained from the Jackson Laboratory, USA. All mice were maintained according to the regulations and guidelines of the Irish Department of Health. Experiments were performed under license from the Department of Health and with the approval of the University Ethics Committee. All mice used were female and 6-12 weeks old at the initiation of the experiments.

### **2.2.2 Immunizations and infections**

#### **2.2.2.1 Subcutaneous immunizations into the flank**

C57BL/6 and BALB/c mice were immunized by subcutaneous injection into the flank using a total volume of 200 µl. Mice were injected with PBS, KLH (10 µg per mouse), KLH and FHA (0.2, 1, 5 or 10 µg per mouse), KLH and LPS (10 µg per mouse) or MOG(50 µg per mouse) and FHA(5 µg per mouse). The mice were boosted after 21 days. All experiments included 4 mice per group. Mice were sacrificed 7 days following booster immunization and antigen specific immune responses were assessed.

#### **2.2.2.2 Subcutaneous immunizations into the footpad**

C57BL/6 mice were immunized by subcutaneous injection into the footpad using a total volume of 50µl/mouse (25 µl/foot). Mice were injected with KLH (10 µg), KLH and FHA (5 µg), KLH and CpG (25 µg), KLH and LPS (10 µg), KLH, LPS and dibutyl cAMP (0.5-50

mg/kg), KLH, LPS and forskolin (0.1-10 mg/kg) and KLH, LPS and PGE<sub>2</sub> (0.05-5 mg/kg). In the DC transfer experiment, mice were injected with 5 x 10<sup>5</sup> cells/50 µl/mouse. All experiments included 4 mice per group. Mice were sacrificed 7 days post immunization and antigen specific responses were assessed.

### **2.2.2.3 Intraperitoneal injections**

BALB/c mice were injected intraperitoneally with a total volume of 200µl. The mice were injected with PBS, FHA (10 µg), anti-TGF-β antibody (100 µg and 200 µg) or an isotype control antibody (100 µg and 200 µg).

### **2.2.2.4 Induction and assessment of EAE**

C57BL/6 and IL-10<sup>-/-</sup> mice were immunized s.c. with 150 µg of MOG peptide emulsified in complete Freund's adjuvant (CFA) supplemented with 5mg/ml killed *Mycobacterium tuberculosis* (Chondrex) in 100µl. Mice were injected i.p. with 500ng of PT on day 0 and day 2. The mice were observed daily for clinical signs of disease. Disease severity was recorded as follows: grade 0, normal; grade 1, limp tail; grade 2, wobbly gait; grade 3, hind limb weakness; grade 4, hind limb paralysis. Mice with a disease severity of grade 4 were sacrificed.

### **2.2.2.5 Infection with *Fasciola hepatica***

10 viable metacercariae of *F.hepatica* (Gerald Coles, Department of Veterinary Clinical Sciences, University of Bristol) were selected using a light microscope and entangled in cotton wool. C57BL/6 and IL-10<sup>-/-</sup> mice were then infected by oral inoculation. The dose of metacercariae was chosen after optimization experiments that were carried out by others in the lab.

## **2.2.3 *In vitro* tissue culture**

### **2.2.3.1 Culture of the J558 cell line**

The J558 cell line (provided by Natalie Winter, Institute Pasteur, Paris, France) was selected for cells transfected with the mouse gene for GM-CSF by culturing the cells for two passages in G418 (Gibco) antibiotic (1 mg/ml). Cells were further cultured for passages 3-9 in fresh complete RPMI-1640, the supernatant of which was collected and measured for the presence of GM-CSF using the commercially available ELISA Kit (R&D) (2.2.4.1).

### **2.2.3.2 Generation of murine bone marrow derived dendritic cells.**

Bone marrow derived dendritic cells (BMDC) were generated from BALB/c (H-2<sup>d</sup>), C3H/HeN (H-2<sup>k</sup>), C3H/HeJ (H-2<sup>k</sup>) and C57BL/6 (H-2<sup>k</sup>) mice. Naïve mice were sacrificed by cervical dislocation and their femurs and tibiae were removed and dissected from the surrounding muscle and tissue. The bone marrow was then flushed from both of the bones using a 25 gauge needle attached to a 20 ml syringe containing complete RPMI-1640. A single cell suspension from the flushed bone marrow was obtained using a 19G needle attached to an empty 20 ml syringe. The single cell suspension was centrifuged at 1200 rpm for 5 min and the cells re-suspended in 1 ml of heated 0.87% ammonium chloride for 2 min, to lyse contaminating red blood cells. The cells were washed in complete RPMI-1640 and pelleted by centrifugation at 1200 rpm for 5 min. The washed mononuclear cells were then re-suspended in 2 ml complete RPMI-1640, cell viability and a total cell count was performed using trypan blue as described in (2.2.3.4). The immature BMDC were cultured at  $5 \times 10^5$  cells/ml of complete RPMI with no more than  $25 \times 10^6$  cells in a T175 tissue culture flask (Greiner). 20 ng/ml of granulocyte macrophage-colony stimulating factor (GM-CSF) in the form of supernatant obtained from the J558 cell line was added (2.2.3.1). After culture

for 3 days at 37°C and 5% CO<sub>2</sub>, 20 ml of fresh medium containing 20 ng/ml of GM-CSF was added to each T175 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 removed contaminating cells, such as granulocytes from the culture. 10 mls of heated sterile PBS (Biosera) was then added to each flask and the flask was gently rolled dislodging the loosely adherent cells. The PBS was then collected into sterile 50 ml tubes containing 15 ml of warm complete media. 16 ml of sterile heated EDTA (0.02%: Sigma) was then added to each flask and the flasks incubated for 10 min at 37°C. The PBS suspension was then pelleted by centrifugation at 1200 rpm for 5 min and re-suspended in 1 ml complete RPMI-1640. 15 ml of media was added to the flasks that contained EDTA and the cells were collected after vigorous pipetting. These cells were then centrifuged at 1200 rpm for 5 min and re-suspended in 1 ml of fresh medium. The suspensions from the PBS and EDTA steps were pooled and cell viability and a total cell count was performed using the trypan blue as described in (2.2.3.4). Cells were re-cultured at  $5 \times 10^5$  cells/ml again with no more than  $25 \times 10^6$  cells in a T175 tissue culture flask in complete RPMI-1640 supplemented with GM-CSF as before. After a further 2 days at 37°C, (day 8) 20 ml of fresh medium containing GM-CSF (20 ng/ml) was added to each of the flasks and the cells returned to the incubator. On day 10 the loosely adherent cells were harvested by gently removing the supernatant and 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. The purity of the DCs was in general greater than 90% based on surface staining for CD11c. The protocol for the generation of bone marrow derived DCs was developed and optimized by others in the lab.



### **2.2.3.3 Preparation of BMDCs for transfer *in vivo***

Immature DCs were obtained by the culture method outlined in 2.2.3.3 and cultured overnight in 6 well plates at  $5 \times 10^5$  cells/ml. On day 11, the appropriate cells were pre-treated with KLH (25  $\mu\text{g/ml}$ ) for 1 hr. The cells were then treated with LPS (100 ng/ml), LPS and dibutyryl cAMP (100  $\mu\text{M}$ ), LPS and forskolin (100  $\mu\text{M}$ ), PGE<sub>2</sub> (1  $\mu\text{M}$ ) or medium. After 24 hours the DCs were collected using a cell scraper and the DCs were pelleted by centrifugation at 1200 rpm. The cells were then resuspended in 10 ml of sterile PBS (Biosera) and were centrifuged at 1200 rpm. The resulting pellet was resuspended in 1 ml of PBS and the DCs were counted using the trypan blue method (2.2.3.4). The cells were diluted with PBS to a concentration of  $5 \times 10^5$  cells/50  $\mu\text{l}$  before transfer *in vivo* as described in 2.2.2.2.

### **2.2.3.4 Cell counts**

Cell counts were performed by diluting cells (normally 1:50 or 1:10) in 50% trypan blue (Sigma)/ 50% PBS (Biowest). A 10 $\mu\text{l}$  volume of the cell suspension was then loaded onto a disposable haemocytometer (Hycor Biomedical, UK). Viable cells, which did not stain and appeared light under a light microscope, were then counted.

### **2.2.3.5 Peritoneal lavage.**

Naïve, injected or infected mice were sacrificed by cervical dislocation. The peritoneal cavity was exposed taking care not to puncture peritoneal sack. A 25 gauge needle was inserted and 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. The body of the mouse was then gently shaken to remove the peritoneal exudate into the PBS. The needle was then re-inserted and the maximum amount of the injected PBS was removed, taking care not to

pierce the intestines. The cells were then centrifuged at 1200 rpm for 5 min. The resulting pellet was resuspended in complete RPMI and the cells were counted. (2.2.3.4)

### **2.2.3.6 Detection of murine T cell responses ex vivo**

Immunized mice were sacrificed by cervical dislocation. Spleens and lymph nodes were dissected and removed into ice cold complete RPMI-1640 (Biosera). In the DC transfer experiment, the lymph nodes were removed into ice cold EX-VIVO medium and this medium was used for the remainder of the experiment. Isolated spleen and lymph nodes from both the immunized and naïve mice were passed through sterile 70 µm sterile cell strainers (BD Biosciences) to obtain single cell suspensions. Cells were centrifuged at 1200 rpm for 5 min. Spleen cells were resuspended in 1 ml of heated 0.87% ammonium chloride for 2 min, to lyse contaminating red blood cells. The cells were then washed and centrifuged at 1200 rpm for a further 5 min. Both spleen cells and nodal cells were then re-suspended in complete RPMI-1640 medium (Biosera)/ EX-VIVO medium. Spleen or lymph node cells ( $2 \times 10^6$  cells/ml) were cultured in triplicate wells of 96-well 'flat-bottomed' microtitre plates at 37°C and 5% CO<sub>2</sub> with various concentrations of antigen, or with PMA (1 ng/ml: Sigma) and anti-mouse CD3e (1 µg/ml: BD Biosciences) antibody or with medium alone. Supernatants were removed after 72 hours in order to determine cytokine concentrations by ELISA.

### **2.2.3.7 Generation of KLH specific T cell lines**

BALB/c or C57BL/6 mice were immunised subcutaneously as in (2.2.2.1) and (2.2.2.2) with KLH and FHA, KLH and LPS or KLH, LPS and PGE<sub>2</sub>. After 7 or 28 days the appropriate lymph nodes and/or spleens were harvested and re-stimulated ex-vivo in the presence of KLH (25 µg/ml). In some cases specific cytokines and antibodies were used to selectively expand specific T helper cell subtypes. Th1 lines were developed using inguinal nodal cells and

spleen cells ( $2 \times 10^6$  cells/ml) from mice immunized with KLH and LPS and were incubated with IL-12 (1 ng/ml) at the initiation of the culture. Th17 cell lines were also developed using the inguinal lymph node cells and spleen cells ( $2 \times 10^6$  cells/ml) from mice immunized with KLH and LPS and were incubated with IL-23 (10 ng/ml) at the initiation of the culture. IL-10 producing T cell lines were developed using lymph nodal cells and/or spleen cells ( $2 \times 10^6$  cells/ml) from mice immunized with KLH and FHA or KLH, LPS and PGE<sub>2</sub>. In addition to KLH, some of the Treg cell lines developed from mice immunized with KLH and FHA were initially incubated with IL-10 (500 pg/ml) and in some cases anti-IL-12, anti-IFN- $\gamma$  and anti-IL-4 (all-10  $\mu$ g/ml) were added. These cells were defined as a manipulated Treg cell line. After 4 days, IL-2 (10 U/ml) was added to all T cell cultures. After a further 7 days the T cell cultures ( $1 \times 10^5$  cells/ml) were re-stimulated with irradiated APCs ( $2 \times 10^6$  cells/ml) and KLH (25  $\mu$ g/ml). The T cell lines were maintained at  $1 \times 10^5$  cells/ml with irradiated APC and antigen for 4 days followed by 7 days of culture with IL-2 (10 U/ml). Cell mixing experiments were carried out at the initiation of this cycle when the APCs and antigen was added. The T cell lines were co-cultured at the ratios of 1:3, 1:1 and 3:1. 1 refers to  $2 \times 10^5$  cells/ml. The concentration of APCs was kept at  $2 \times 10^6$  cells/ml and the concentration of KLH was kept at 25 $\mu$ g/ml. T cell line mixing experiments were carried out in triplicate.

#### **2.2.3.8 Preparation of APCs**

Whole spleens from the appropriate strain of mice were placed in 50 ml tubes (Greiner) containing ice cold complete medium. These 50 ml tubes were placed into the irradiation chamber of a Nordian Gammcell 3000 Elan irradiator and irradiated at a dose of 30 Gy, which prevents subsequent cell proliferation. A single cell suspension was prepared, the red blood cells were lysed and the cells were counted as in (2.2.3.4).

### **2.2.3.9 Generation of MOG specific T cell lines.**

C57BL/6 mice were pre-immunized with MOG<sub>35-55</sub> (50 µg) and FHA (5 µg) or PBS, 21 days and 7 days before EAE was induced (2.2.2.4). The MOG specific T cells lines described in Chapter 3 were developed from mice with EAE. After the clinical symptoms of severe EAE (ie Grade 4) had appeared in the control mice, the mice were sacrificed and the spleens and were harvested. The spleen cells were re-stimulated ex-vivo with MOG (25 µg/ml). IL-2 (10 units/ml) was added after 4 days and the cells were incubated for a further 4 or 7 days. The cell lines ( $1 \times 10^5$  cells/ml) were then re-stimulated with MOG (25 µg/ml) and irradiated APCs ( $2 \times 10^6$  cells/ml). The T cell lines were maintained at  $1 \times 10^5$  cells/ml with irradiated APC and antigen for 4 days followed by 7 days of culture with IL-2 (10 U/ml). Cell mixing experiments were carried out at the initiation of this cycle when the APCs ( $2 \times 10^6$  cells/ml) and MOG (25 µg/ml) is added. The T cell lines were co-cultured at the ratios of 1:3 and 1:1. 1 refers to  $2 \times 10^5$  cells/ml.

### **2.2.3.10 Proliferative responses of T cells *in vitro*.**

KLH specific Th1, Th17 and Treg cell lines were developed as outlined in section 2.2.3.7. Cell mixing experiments were performed where a Treg cell line was co-incubated with a Th1 or Th17 cell line for 72 hours. The cells were then pulsed with 0.5 µCi of [<sup>3</sup>H]-thymidine (Amersham Pharmacia Biotech) in 200 µl of complete RPMI for the final 18 hours of culture. The cells were harvested onto glass fibre filters (Wallac) with an automatic cell harvester. The filters were then dried and sealed in plastic sample bags (Wallac) with 5 ml of non-aqueous scintillation fluid (BetaScint, Wallac). [<sup>3</sup>H]-thymidine incorporation was assessed using a Beta-plate scintillation counter (Wallac). Results are expressed as means counts per minute (cpm) of [<sup>3</sup>H]-thymidine incorporation for triplicate cultures of T cells.

### **2.2.3.11 CD4<sup>+</sup> T cell isolation by MACS**

Freshly isolated peritoneal exudate cells (2.2.3.5) or spleen cells (2.2.3.6) were suspended in ice cold MACS running buffer  $9 \mu\text{l}/1 \times 10^6$  cells. Anti-CD4 coated magnetic beads (Miltenyi Biotec) were added  $1 \mu\text{l}/1 \times 10^6$  cells. The suspension was mixed well and incubated at  $4^\circ\text{C}$  for 15 min. Unbound beads were washed away by adding 10 times the volume of MACS running buffer and centrifuging at 300 g for 10 min. Cells were resuspended in 1 ml of MACS running buffer and separated into the labelled and unlabelled fractions using the “possel” program on an autoMACS machine (Miltenyi Biotec). The resulting cells were washed immediately in cRPMI.

## **2.2.4 Determination of cytokine concentrations by ELISA**

### **2.2.4.1 IL-10, IL-1 $\beta$ , IL-17, GM-CSF, IL-12p70, TNF- $\alpha$ and TGF- $\beta$ ELISA.**

Concentrations of IL-10, IL-1 $\beta$ , IL-17, GM-CSF, IL-12p70, TNF- $\alpha$  and TGF- $\beta$  were measured using commercially available ELISA Kits (R&D Systems). High binding certified 96-well microtitre plates (Greiner Bio-one) were coated overnight at  $4^\circ\text{C}$  with  $50 \mu\text{l}/\text{well}$  of rat anti-mouse IL-10 ( $2 \mu\text{g}/\text{ml}$ ), IL-1 $\beta$  ( $4 \mu\text{g}/\text{ml}$ ), IL-17 ( $2 \mu\text{g}/\text{ml}$ ), GM-CSF ( $2 \mu\text{g}/\text{ml}$ ), IL-12p70 ( $4 \mu\text{g}/\text{ml}$ ), TNF- $\alpha$  ( $0.8 \mu\text{g}/\text{ml}$ ) and TGF- $\beta$  ( $4 \mu\text{g}/\text{ml}$ ) capture antibody in PBS. Plates were then washed in wash buffer (PBS/0.05% Tween 20) and non-specific binding sites were blocked by adding  $200 \mu\text{l}$  of blocking buffer (1% BSA in PBS) for 2hrs at room temperature. After this plates were washed again in wash buffer and  $50 \mu\text{l}/\text{well}$  of test supernatant were added as well as serially diluted standard recombinant proteins for each cytokine, IL-10 standard (0-2000pg/ml), IL-1 $\beta$  standard (0-1000pg/ml), IL-17 standard (0-1000pg/ml), GM-CSF standard (0-2000 pg/ml), IL-12p70 standard (0-1500 pg/ml), TNF- $\alpha$  standard (0-2000 pg/ml) and TGF- $\beta$  (0-1000 pg/ml) all diluted in 1% BSA (Sigma)/PBS. Samples to be

assayed for the presence of latent TGF- $\beta$  were activated by the addition of 10  $\mu$ l 1N HCL to 50  $\mu$ l culture supernatant, followed by the addition of 10  $\mu$ l of 1.2 N NaOH/ 0.5 M HEPES to restore the pH. Plates were then incubated overnight at 4°C. The plates were then washed in wash buffer and the plates were incubated with 50  $\mu$ l/well of biotinylated goat anti-mouse IL-10 (400 ng/ml), IL-1 $\beta$  (100 ng/ml), IL-17 (200 ng/ml), GM-CSF (50 ng/ml), IL-12p70 (400 ng/ml), TNF- $\alpha$  (150 ng/ml) and TGF- $\beta$  (200 ng/ml) diluted in 1% BSA. These plates were incubated for 2 hrs at room temperature, before washing and incubation with 50  $\mu$ l/well of horseradish peroxidase (HRP)-conjugated streptavidin (1:200 in 1% BSA) for 25 min at room temperature in the dark. The plates were then washed one final time. Cytokine concentrations were determined after addition of 50  $\mu$ l/well OPD substrate (o-phenyldiamine dihydrochloride (Sigma) in phosphate citrate buffer (0.4 mg/ml). Once standard curves for each cytokine had developed to the desired intensity, the enzyme reaction was quenched using 25  $\mu$ l/well of the stop solution H<sub>2</sub>SO<sub>4</sub>. The absorbance of each well was read at 492nm using a microtiter plate reader. Cytokine concentrations contained in the test samples were evaluated with reference to the standard curve prepared using recombinant mouse IL-10, IL-1 $\beta$ , IL-17, GM-CSF, IL-12p70, TNF- $\alpha$  and TGF- $\beta$  of known concentration.

#### **2.2.4.2 IL-12p40, IFN- $\gamma$ , IL-4, IL-13, IL-5 and IL-6 ELISA**

Concentrations of IL-12p40, IFN- $\gamma$ , IL-4, IL-13, IL-5 and IL-6 in supernatants were detected by ELISA using commercially available paired antibodies. (BD Pharmingen). High binding certified 96-well microtitre plates (Greiner Bio-one) were coated overnight at 4°C with 50 $\mu$ l/well of purified anti-mouse IL-12p40 (1  $\mu$ g/ml), IFN- $\gamma$  (1  $\mu$ g/ml), IL-4 (1  $\mu$ g/ml), IL-13 (1  $\mu$ g/ml), IL-5 (1  $\mu$ g/ml) and IL-6 (1  $\mu$ g/ml) capture antibody in PBS. After washing with wash buffer (PBS/0.05% Tween 20), non-specific binding sites were blocked with 200  $\mu$ l/well 10% dried milk (Marvel) w/v in PBS, for 2hrs at room temperature. Plates were

washed once again in wash buffer and 50µl of test supernatant (in the case of IL-12p40 the supernatants were diluted 1:300 in PBS) were added as well as serially diluted recombinant proteins for each cytokine, IL-12p40 standard (0-5000 pg/ml), IFN- $\gamma$  (0-10 ng/ml), IL-4 standard (0-2000 pg/ml), IL-13 (0-4000 pg/ml) standard, IL-5 standard (0-2500 pg/ml) and IL-6 standard (0-5000 pg/ml) all diluted in PBS were added. The plates were incubated overnight at 4°C. The plates were then washed again in wash buffer and incubated for 1 hr at room temperature with 50 µl/well of biotinylated rat anti-mouse IL-12p40 (1 µg/ml), IFN- $\gamma$  (1 µg/ml), IL-4 (1 µg/ml), IL-13 (1 µg/ml), IL-5 (1 µg/ml) and IL-6 (1 µg/ml) detection antibody in PBS. The plates were then washed and incubated with HRP-conjugated streptavidin (BD Pharmingen, 1:1000 in PBS) 50 µl/well for 25 min at room temperature in the dark. Plates were washed in wash buffer one final time. Cytokine concentrations were determined after addition of 50 µl/well OPD substrate (o-phenyldiamine dihydrochloride (Sigma) in phosphate citrate buffer (0.4 mg/ml). Once standard curves for each cytokine had developed to the desired intensity, the enzyme reaction was quenched using 25 µl/well of the stop solution H<sub>2</sub>SO<sub>4</sub>. The absorbance of each well was read at 492nm using a microtiter plate reader. Cytokine concentrations contained in the test samples were evaluated with reference to the standard curve prepared using recombinant mouse IL-12p40, IFN- $\gamma$ , IL-4, IL-13, IL-5 and IL-6 of known concentration.

#### **2.2.4.3 IL-23 ELISA**

Cytokine concentrations of IL-23 were measured using a commercially available ELISA Kit (eBioscience). High binding certified 96-well microtitre plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of purified anti-mouse IL-23 (2 µg/ml) capture antibody in eBioscience coating buffer. Plates were then washed in wash buffer (PBS/0.05% Tween 20) and non-specific binding sites were blocked by adding 200 µl of blocking buffer

(eBioscience Assay Diluent) for 2 hrs at room temperature. Plates were washed again in wash buffer and 50 µl/well of test supernatant was added as well as serially diluted recombinant IL-23 (0-4000 pg/ml) diluted in eBioscience assay diluent. Plates were then incubated overnight at 4°C. The plates were then washed in wash buffer and the plates were incubated with 50 µl/well of biotinylated anti-mouse IL-23 (2 µg/ml) diluted in eBioscience assay diluent. The plates were incubated for 1hr at room temperature before washing and incubation with 50 µl/well of horseradish peroxidase (HRP)-conjugated streptavidin (eBioscience 1:250 in eBioscience assay diluent) for 25 min at room temperature in the dark. The plates were then washed one final time. Cytokine concentrations were determined after addition of 50 µl/well TMB substrate (eBioscience). The enzyme reaction was quenched using the stop solution H<sub>2</sub>SO<sub>4</sub>, 25 µl/well once standard curves for the cytokine had developed sufficiently. The absorbance of each well was read at 450 nm using a microtiter plate reader. Cytokine concentrations contained in the test samples were evaluated with reference to the standard curve prepared using recombinant mouse IL-23 of known concentration.

## **2.2.5 FACS analysis**

### **2.2.5.1 Surface marker staining of BMDCs**

The DCs were harvested from wells, post stimulation, in 1 ml FACS Buffer/1 x 10<sup>6</sup> cells. The cells were centrifuged at 1200 rpm for 5 min and re-suspended in 1 ml FACS buffer /1 x 10<sup>6</sup> cells and 1 µg/ml of Fcγ Block (BD Biosciences) was added. The cells were left for 20 min on ice. Cells were then transferred to FACS tubes (Falcon) in a volume of 100-200 µl of FACS Buffer and incubated with the appropriate FACS antibody or isotype control antibodies (2.1.2.8) for 20 min at room temperature 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 mls/tube, with centrifugation at 1200 rpm for 5min between each wash. Immunofluorescence analysis was then performed using a DAKO CyAN<sub>ADP</sub> flow cyometer. The flow cytometers were calibrated using the Autocomp software in conjunction with commercially prepared fluorescent beads-Calibrate beads (BD)

### **2.2.5.2 Surface marker and intracellular cytokine staining**

The cells to be analysed were added to a sterile FACs tube at  $1 \times 10^6$  cells/ml/tube. The T cells were activated using PMA (10 ng/ml) and Ionomycin (1  $\mu$ g/ml) and incubated at 37°C and 5% CO<sub>2</sub>. After 2 hours Brefeldin A (5  $\mu$ g/ml) was also added and the cells were incubated for a further 6 or 10 hours. The peritoneal exudate cells were incubated with Brefeldin A alone for 10 hours at 37°C and 5% CO<sub>2</sub>. This protocol was optimized by myself and others in the lab. The cells were then washed twice with 3 ml of FACs buffer by centrifugation at 1200 rpm for 5 min at room temperature. The cells were then re-suspended in 1 ml FACS buffer /tube and 1  $\mu$ g/ml of Fc $\gamma$  Block was added. The cells were left for 20 min on ice. The appropriate surface FACS antibodies or surface isotype control antibodies were then added (2.1.2.8) for 20 min at room temperature 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. The cells were then prepared for intracellular staining using a cell permeabilization kit (Caltag). 100  $\mu$ l of fixation buffer A was added to each tube and the cells were gently resuspended in it. The cells were then incubated for 15 mins at room temperature in the dark. The cells were washed twice in FACs Buffer, 3mls/tube with centrifugation at 1200 rpm for 5 min between each wash. 100  $\mu$ l of

permeabilization buffer B was then added to each tube along with the intracellular antibodies and the appropriate isotype control antibodies (2.1.2.8). The cells were again incubated for 15 min at room temperature in the dark. The cells were then washed twice in FACS Buffer, 3 ml/tube with centrifugation at 1200 rpm for 5 min between each wash. Immunofluorescence analysis was then performed using either a FACS Caliber flow cytometer (Becton Dickinson, CA) or a DAKO CyAN<sub>ADP</sub> flow cytometer. The flow cytometers were calibrated using the Autocomp software in conjunction with commercially prepared fluorescent beads-Calibrate beads (BD).

### **2.2.5.3 FACS data acquisition**

Compensation settings were adjusted such that the median fluorescence intensity of both positive and negative populations of single stained compensation beads or cells were equal, and verified by visual inspection of experimental samples. Where possible, voltages were set such that all negative populations appeared within a standard plot.

### **2.2.5.4 FACS data analysis**

FACS data analysis was performed using Summit or CELLQuest software. In order to accurately assess composition of purified leukocyte subsets, FACS analysis included all events from the macrophage, lymphocyte and dendritic cell zone. For phenotypical analysis of specific cell types, initial gating was performed on the zone in which they reside, and confirmed by backgating. Position of gates was determined by isotype controls and fluorescence minus one (FMO) controls.

## **2.2.6 Western Blot Analysis**

### **2.2.6.1 Preparation of samples for gel electrophoresis**

BMDCs were seeded at a density of  $1 \times 10^6$  cells/ml, 1 ml per well in 24 well tissue culture plates (Greiner). After the appropriate stimulation, the supernatant was removed and the cells were lysed in 100  $\mu$ l of 1X Sample buffer/well. Samples were stored at  $-20^\circ\text{C}$  and boiled at  $100^\circ\text{C}$  for 5 mins before being run on an SDS PAGE gel.

### **2.2.6.2 SDS Polyacrylamide Gel Electrophoresis**

Samples were resolved on sodium dodecylsulphate (SDS) polyacrylamide gel using a constant current on a 60 mA gel. Samples were first run through a stacking gel (162  $\mu$ l 30% acrylamide/bisacrylamide (BioRad), 312  $\mu$ l 1M Tris-HCl pH 6.8, 12.5  $\mu$ l of 10% ammonium persulphate (APS), 12.5  $\mu$ l 10% SDS, 7.5  $\mu$ l of TEMED and 762  $\mu$ l of  $\text{dH}_2\text{O}$ ) and then resolved using a 12% polyacrylamide gel (2.08 ml 30% acrylamide/bisacrylamide, 1.25 ml of 1.5 M Tris-HCl pH 8.8, 50  $\mu$ l of 10% SDS, 15  $\mu$ l of TEMED and 1.59 ml of  $\text{dH}_2\text{O}$ ). Samples were run with pre-stained protein markers (Precision Plus Standards; Bio-Rad) as molecular weight standards.

### **2.2.6.3 Transfer of proteins to membrane**

The resolved proteins were transferred to nitrocellulose (Sigma) using a semi-dry transfer system. The gel, nitrocellulose paper and filter paper were soaked in transfer buffer. The nitrocellulose paper was placed on top of two pieces of filter paper followed by the gel and then two other sheets of filter paper to make a layered sandwich. Air bubbles were then removed and the sandwich was placed on the anode of the semi-dry blotter, which had been

pre-moistened with transfer buffer. The lid, containing the cathode was also pre-moistened and was firmly placed on top and the transfer was carried out at 125 mA for 90mins.

#### **2.2.6.4 Antibody blotting**

Membranes were blocked to prevent non-specific binding by incubation in blocking buffer (5% (w/v) dried milk (Marvel)) in wash buffer (PBS/0.05% Tween 20) for 1 hr at RT with constant movement. The membrane was rinsed in wash buffer and incubated with the antibody of interest (2.1.3.4) at 4°C overnight with constant movement. The membrane was then washed and incubated with the appropriate secondary horseradish peroxidase (HRP) linked antibody (2.1.3.4) for 1 hr at RT. Again the nitrocellulose was washed with wash buffer. Blots were developed by SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The membranes were placed between two sheets of transparent acetate before been exposed to photographic film for the required time. The film was processed using a Fuji X-ray processor.

#### **2.2.7 Statistical analysis**

Statistical analyses were performed using the computer-based mathematical package InStat 3. Statistical differences in mean cytokine values and EAE clinical scores between experimental groups were determined by student T test if there was only two groups, by one way ANOVA and by two way ANOVA with repeated measures. Where significant differences were found, the Tukey-Kramer multiple comparisons test was used to identify differences between individual groups. P values of less than 0.05 are considered significant.

# **Chapter III**

## **Modulation of the innate and adaptive immune response by cAMP activators**



### 3.1 Introduction

Most innate immune responses begin with the recognition of pathogen derived molecules by PRR, such as TLRs. TLR ligation results in the production of chemokines and cytokines including IL-1, IL-10, IL-6, IL-12p40 and IL-12p70 (371) . The response of antigen presenting cells to a pathogen can be decisive in both the nature and magnitude of the adaptive immune response. TLR induced IL-12 production by DCs, promotes Th1 cell differentiation. Th1 cells enhance the clearance of intracellular pathogens and are characterized by the production of IFN- $\gamma$ . Studies conducted by Veldhoen *et al* have shown that DCs stimulated in the presence of TLR3, TLR4 or TLR9 agonists and natural Treg cells induce Th17 cell differentiation (133). Moreover, the Tregs could be substituted with TGF- $\beta$  and the TLR agonist with IL-6 (133). Th17 cells have a major role in organ specific inflammation in many autoimmune diseases. McGeachy *et al* have recently reported that the presence of IL-23 is necessary for the pathogenesis of Th17 cells which supports previous studies that showed that IL-23p19<sup>-/-</sup> mice are resistant to EAE, CIA and IBD (136, 138, 177). Many TLR agonists such as LPS, CpG and Poly I:C have been shown to induce the production of IL-23 by innate immune cells and may therefore play a role in autoimmune inflammation (183, 372).

There has also been evidence that TLR activation induces the differentiation of Tr1 cells. Den Haan *et al* have reported that LPS induced CD4<sup>+</sup> T cells suppress CD8<sup>+</sup> T cell responses. Immunization of mice with OVA and LPS elicited weak CD8<sup>+</sup> T cell responses in wild type mice, but in mice that lacked CD4<sup>+</sup> T cells the immunization resulted in strong CD8<sup>+</sup> T cell responses (373). The neutralization of IL-10 *in vivo* restored strong CD8<sup>+</sup> T cell responses in wild type mice suggesting that LPS induces IL-10 producing Tr1 cells that inhibit CD8<sup>+</sup> responses (373). Jarnicki *et al* have shown that the inhibition of p38 suppresses the ability of

CpG activated DC to induce IL-10 producing T cells (374). In the same study, the p38 inhibitor SB203580, enhanced the antitumor therapeutic efficacy of DCs that were pulsed with Ag and CpG and also enhanced the protective effect of a pertussis vaccine that was formulated with CpG (374). Furthermore, KLH specific Tr1 cells were generated from mice immunized with KLH and CpG and were shown to suppress Th1 cells in co-culture experiments (374). The importance of TLR induced Treg cells in the development of an appropriate immune response to a pathogen was demonstrated in a mouse model of *B.pertussis* infection. Pathogen clearance was delayed in TLR4 defective mice and this was associated with enhanced inflammatory cytokine production, cellular infiltration and severe pathological changes, due to a defect in the induction of IL-10 producing T cells (244).

Many studies have reported that the activation of the intracellular second messenger cAMP, suppresses immune cell function. The cAMP activator PGE<sub>2</sub> has been shown to inhibit LPS induced IL-12, TNF- $\alpha$  and MIP-1 $\alpha$ , while enhancing IL-10 production in macrophages (96, 97). Similar findings have been generated using DCs (97, 99). Woo *et al* have examined the effects of the cAMP activator forskolin and the cAMP analog dibutyryl cAMP on microglial cells and found that they both inhibited LPS induced TNF- $\alpha$  and enhanced IL-10 production (107, 110). Many pathogens have evolved the ability to enhance intracellular cAMP concentrations to inhibit immune cell function as part of their immune evasion strategies. The enterotoxin CT produced by *V. cholera* activates a G protein  $\alpha$  subunit which activates adenylate cyclase (111). CT has been shown to inhibit LPS induced IL-12, TNF- $\alpha$ , MIP-1 $\alpha$ , MIP-1 $\beta$  and MCP-1 and enhance LPS induced IL-10 production in DCs (112). Similar findings were reported for CyaA, a virulence factor produced by *B. pertussis* (115).



IL-10 producing T cells termed Tr1 cells can be induced *in vitro* by stimulating naive T cells with antigen in the presence of vitamin D3, dexamethasone and neutralizing antibodies against Th1 and Th2 cytokines (237). The transfer of these Tr1 cells was found to prevent the induction of EAE but only when OVA was injected intracranially (237). The induction of the Tr1 cells was dependent on DC derived IL-10 which supports findings made by McGuirk *et al* (227, 237).

cAMP activation enhances TLR agonist induced IL-10 and inhibits IL-12 production in DCs. This observation led us to postulate that the ability of TLR agonists to induce Tr1 cells might be enhanced by the addition of a cAMP activator. This study therefore investigated the effects of the cAMP activators PGE<sub>2</sub> and forskolin and the cAMP analog, dibutyryl cAMP on TLR agonist stimulated DCs and their ability to induce Tr1 cells. Dibutyryl cAMP inhibited TLR agonist induced IL-12 and enhanced IL-23 and IL-10 production in DCs. The transfer of these DCs into naive mice resulted in the differentiation of an antigen specific Th17 cells. PGE<sub>2</sub> inhibited TLR agonist induced IL-12 and IL-23 and enhanced IL-10 production in DCs. Immunization of mice with antigen in the presence of LPS and PGE<sub>2</sub> directed the differentiation of IL-10 producing T cells specific for a co-administered antigen *in vivo*. These T cells inhibited IFN- $\gamma$  production, but not IL-17 production from a MOG specific T cell line developed from mice, with EAE.

## 3.2 Results

### 3.2.1 TLR agonist induced IL-10 and IL-12 production by DCs is dose dependent

It has previously been reported that TLR agonists induce both IL-12 and IL-10 production by DCs (374). The aim of this study was to manipulate TLR induced cytokine production in DCs, through the use of cAMP activators. The first experiments focused on examining the effect of different doses of TLR agonists on the induction of IL-10 and IL-12 by DC. DCs were incubated with increasing doses of LPS, CpG, Poly I:C, Pam3CSK and MPL. MPL is a TLR4 agonist and a derivative of lipid A from *E.coli* LPS, which is being used as an adjuvant in a human papilloma virus vaccine that is nearing clinical approval (371). After 24 hours, supernatants were taken for cytokine analysis. LPS induced the production of IL-10, IL-12p40 and IL-12p70 at all concentrations used (0.1 ng-10,000 ng/ml) [Fig 3.1A]. Increasing doses of LPS from 0.1 ng/ml to 100 ng/ml resulted in an increase in the production of IL-10, IL-12p40 and IL-12p70 [Fig 3.1A]. Doses of LPS higher than 100 ng/ml, resulted in a slight decrease in cytokine production [Fig 3.1A]. This observation and reports that high doses of LPS have been associated with the induction of apoptosis led to the conclusion that 100 ng/ml was the optimal dose of LPS in DCs (375). Low concentrations of CpG (0.1 ng-100 ng/ml) did not induce the production of substantial levels of IL-10, IL-12p40 or IL-12p70 by DCs [Fig 3.1B]. 1 µg/ml of CpG resulted in the highest levels of IL-10, IL-12p70 and IL-12p40 [Fig 3.1B] and was therefore adjudged to be the optimal dose. High doses (10 µg and 100 µg/ml) of Poly I:C and MPL were required for the production of significant levels of IL-10, IL-12p40 and IL-12p70 by DCs [Fig 3.1C and E]. 100 µg/ml of either TLR agonist induced the highest levels of IL-12p40 and IL-12p70 [Fig 3.1C and E]. 10 µg/ml of MPL and 100 µg/ml of Poly I:C resulted in the highest concentration of IL-10 [Fig 3.1C and E]. Pam3CSK did not induce significant IL-10 production by DCs [Fig 3.1D]. Increasing doses (0.1 ng-1000 ng/ml) of Pam3CSK resulted in increasing levels of IL-12p70 [Fig 3.1D]. 10

ng/ml of the TLR agonist resulted in the highest concentration of IL-12p40 produced [Fig 3.1D].

### **3.2.2 The activation of cAMP modulates TLR agonist induced cytokine production**

The activation of cAMP by PGE<sub>2</sub> has been shown to enhance TLR induced IL-10 and inhibit LPS induced IL-12 production by human monocyte derived DCs (99). It was therefore decided to investigate the effects of the cAMP activators, PGE<sub>2</sub>, forskolin and dibutyryl cAMP on TLR induced cytokine production by murine BMDCs. TLR agonists have also been shown to induce IL-23 production from innate immune cells, a cytokine which is essential for the development of many organ specific autoimmune diseases (138, 183). The effect of the cAMP activators on TLR induced IL-23 was therefore examined. DCs were pre-incubated with the cAMP activators for 30 mins before the addition of the LPS, Poly I:C, Pam3CSK, MPL or CpG. Dibutyryl cAMP and PGE<sub>2</sub>, both enhanced LPS, Poly I:C and MPL induced IL-10 production [Fig 3.2 A,B and D]. Pam3CSK did not induce the production of IL-10, however pre-incubation of DC with PGE<sub>2</sub> (1 µM or 10 µM) or dibutyryl cAMP (100 µM) resulted in IL-10 production [Fig 3.2 C]. 100 µM of forskolin enhanced MPL induced IL-10 but the cAMP activator had no significant effect on the concentration levels of IL-10 induced by the other TLR agonists [Fig 3.2]. cAMP activation did not enhance CpG induced IL-10 production and 100 µM of dibutyryl cAMP significantly inhibited the production of this cytokine [Fig 3.2 E]. Consistent with previous studies, PGE<sub>2</sub> and the high dose of dibutyryl cAMP inhibited LPS, Poly I:C, Pam3CSK and MPL induced IL-12p40 and IL-12p70 production [Fig 3.2 A, B, C and D]. Forskolin suppressed LPS and Poly I:C induced IL-12p40 and IL-12p70, and also inhibited Pam3CSK induced IL-12p70 [Fig 3.2 A,B and C]. All of the cAMP activators tested suppressed CpG induced IL-12p70 production, but appeared to have no effect on CpG induced IL-12p40 [Fig 3.2 E]. PGE<sub>2</sub> and

Forskolin inhibited LPS, Poly I:C, Pam3CSK and MPL induced IL-23 [Fig 3.2 A, B ,C and D]. This observation contradicts reports by Sheibanie *et al* that show that PGE<sub>2</sub> enhances LPS induced IL-23 in murine DCs (104). Interestingly, the cAMP analog dibutyryl cAMP does enhance LPS, Poly I:C and MPL induced IL-23 [Fig 3.2 A, B and D]. Analogous to their effect on CpG induced IL-10 and IL-12p40, the cAMP activators did not affect CpG induced IL-23, suggesting that increases in cAMP production do not affect CpG induced TLR signalling [Fig 3.2 E]. DCs treated with PGE<sub>2</sub>, forskolin or dibutyryl cAMP in the absence of TLR agonists did not produce IL-10, IL-12 or IL-23 [Fig 3.2 F].

### **3.2.3 Treatment of LPS stimulated DCs with cAMP activators modulates the phenotype of T cell induced *in vivo*.**

The cytokines produced by DCs during antigen presentation ultimately determine the phenotype of the activated CD4<sup>+</sup> T helper cell. The result in Fig 3.2 showed that cAMP activators modulate TLR agonist induced cytokine production by DCs [Fig 3.2]. It was decided to investigate the phenotype of T cells induced by DC treated with LPS and cAMP activators using an adoptive transfer protocol. DCs were treated with KLH, LPS and a cAMP activator and then injected *in vivo*. After 7 days, the lymph nodes were removed and the recall response of the lymph node cells were tested against KLH *in vitro*. DCs were co-incubated with KLH, LPS and dibutyryl cAMP (100 µM), forskolin (100 µM) or PGE<sub>2</sub> (1 µM). The optimal concentration of the cAMP activators was determined by the data in Fig 3.2. Before the DCs were transferred *in vivo*, the innate cytokine production and the maturation status of the DCs was examined. DCs treated with KLH alone produced very low levels of IL-10 and IL-12p70 and no IL-23 or IL-12p40 which confirmed that the antigen was free of endotoxin contamination [Fig 3.3]. KLH and LPS induced the production of substantial levels of IL-10, IL-23, IL-12p40 and IL-12p70 by the DCs [Fig 3.3]. Dibutyryl

cAMP enhanced LPS induced IL-23 and IL-10 and inhibited IL-12p40 and IL-12p70 production [Fig 3.3]. Forskolin inhibited LPS stimulated IL-23, IL-12p40 and IL-12p70 and had no effect on IL-10 production [Fig 3.3]. PGE<sub>2</sub> enhanced LPS induced IL-10 and inhibited IL-23, IL-12p40 and IL-12p70 production by the DCs [Fig 3.3]. Comparison of the data in Fig 3.2A and Fig 3.3 suggests that the pre incubation and the co-incubation of cAMP activators results in the same modulation of LPS induced cytokine production by DCs.

During antigen presentation, the ligation of CD80 or CD86 on the surface of DCs with CD28 on the surface of a T cell provides an essential secondary signal for the activation of T cells. The binding of CD40 on an APC to CD40L on a T cell, transmits activating signals. The upregulation of CD80, CD86 and CD40 expression on the surface of a DC is therefore indicative of a mature phenotype which is capable of antigen presentation to T cells. The chemokine receptor CCR5 is expressed on the surface of DCs and its downregulation is indicative of DC maturation. The expression of CD80 and CD86 on the DCs to be transferred *in vivo* was initially examined using flow cytometry analysis. Approximately 50% of the DCs treated with medium or KLH alone solely expressed CD80 and approximately 25% of the cells co-expressed CD80 and CD86 [Fig 3.4]. None of DCs expressed CD86 alone [Fig 3.4]. The addition of LPS resulted in an increase in cells that co-expressed CD80 and CD86 to 57% [Fig 3.4]. A higher percentage of DCs (33%) treated with KLH, LPS and dibutyryl cAMP or PGE<sub>2</sub> expressed CD80 alone, when compared with DCs treated with KLH and LPS (16%) [Fig 3.4]. KLH, LPS and forskolin stimulated DCs expressed a slightly higher percentage of cells that solely expressed CD80 (23%) when compared with KLH and LPS stimulated DCs [Fig 3.4]. The expression of CCR5 and CD40 on the DCs was then examined. Approximately 50% of the DCs treated with medium or KLH expressed CD40 and 22% expressed CCR5 [Fig 3.5]. The stimulation of the cells with

KLH and LPS resulted in a dramatic increase in the percentage of DCs that solely expressed CD40 to nearly 70% [Fig 3.5]. The cAMP activators did not effect LPS induced upregulation of CD40 expression, however the treatment of DCs with KLH, LPS and dibutyryl cAMP or PGE<sub>2</sub> reduced expression of CCR5 [Fig 3.5]. We concluded that dibutyryl cAMP, forskolin or PGE<sub>2</sub> did not affect LPS induced DC maturation and in some cases actually enhanced it.

The DCs ( $5 \times 10^5$  cells/ml) were injected s.c. into the footpad. After 7 days, the popliteal lymph nodes were removed and the lymph node cells were cultured with KLH in X-VIVO medium for 3 days. The lymph node cells from the mice that received the KLH and LPS stimulated DCs, produced IFN- $\gamma$ , IL-10 and IL-17 in response to KLH, confirming that LPS promotes the induction of Th1, Th17 and Treg type immune responses [Fig 3.6]. Lymph node cells from mice that received DCs treated with KLH, LPS and dibutyryl cAMP, produced higher levels of KLH specific IL-17 and lower KLH specific IL-10 and IFN- $\gamma$  when compared with the response induced by DCs stimulated with KLH and LPS [Fig 3.6]. KLH, LPS and Forskolin treated DCs induced a strong Th1 response *in vivo*, characterised by high levels of antigen specific IFN- $\gamma$  and no IL-17, IL-4 or IL-10 [Fig 3.6]. Lymph node cells from mice that received DCs incubated with KLH, LPS and PGE<sub>2</sub>, produced increased levels of KLH specific IL-10 and reduced levels of IL-17 and IFN- $\gamma$  compared with the response induced by LPS treated DCs [Fig 3.6]. This study confirms that the manipulation of TLR stimulated cytokine production can ultimately alter the phenotype of T cell induced by a DC. It also identifies PGE<sub>2</sub> as a cAMP activator that may enhance the LPS induced differentiation of Tr1 cells.

### **3.2.4 LPS and PGE<sub>2</sub> (5 mg/kg) promotes the induction of antigen specific T cells that produce high levels of IL-10.**

Having shown that cAMP activators could modulate the T cell response induced by LPS stimulated DCs, it was decided to examine the effect of direct injection of mice s.c. into the footpad with KLH, LPS and three doses of each cAMP activator. After 7 days, the popliteal lymph nodes were removed and the recall response to KLH was analysed *in vitro*. Cells from mice immunized with KLH and LPS, produced high levels of IFN- $\gamma$ , IL-10, IL-17 and moderate levels of IL-5 in response to KLH stimulation *in vitro* [Fig 3.7]. The injection of increasing doses of dibutyryl cAMP (0.5 mg-50 mg/kg) with LPS, resulted in decreasing levels of KLH specific IFN- $\gamma$ , IL-17 and IL-10 when compared to mice that received LPS alone [Fig 3.7]. Lymph node cells from mice that received KLH, LPS and the lowest dose of forskolin (0.1 mg/kg) with LPS, produced reduced levels of KLH specific IFN- $\gamma$ , IL-10 and IL-17, when compared with cells from mice immunized with KLH and LPS [Fig 3.7]. In contrast, the highest dose of forskolin (10 mg/kg) enhanced LPS induced IFN- $\gamma$ , IL-17 and IL-10 [Fig 3.7]. The immunization of mice with KLH, LPS and increasing doses of PGE<sub>2</sub> (0.05 mg- 5 mg/kg), resulted in decreasing levels of KLH specific IFN- $\gamma$ , IL-17 and IL-10 when compared with KLH and LPS immunized mice [Fig 3.7]. None of the cAMP activators had a significant effect on KLH specific IL-5 production induced by immunization with KLH and LPS [Fig 3.7]. These experiments showed that immunization of mice with KLH, LPS and PGE<sub>2</sub> (5 mg/kg) was the optimal method for the induction of IL-10 producing antigen specific T cells *in vivo*. Lymph node cells from these mice, produced high levels of IL-10 (700 pg/ml), low levels of IFN- $\gamma$  (3 ng/ml) and no IL-17 in response to restimulation with KLH *in vitro*[Fig 3.7].

Treg cells are defined by their ability to suppress effector T cell populations. In order to investigate the regulatory potential of antigen specific T cells induced by LPS and PGE<sub>2</sub>, T cell lines were developed from the spleen or lymph node cells from mice with EAE and mice that had been immunized with KLH and LPS and KLH, LPS and PGE<sub>2</sub>. After 15 days of culturing the T cell lines, supernatants were taken and analysed for cytokine production. The MOG specific T cell line developed from mice with EAE produced moderate levels of IFN- $\gamma$ , low levels of IL-17 and IL-5 and no IL-10 [Fig 3.8]. The KLH specific T cell line developed from mice with immunized with KLH and LPS produced low levels of IFN- $\gamma$ , IL-17 and IL-10 and moderate levels of IL-5 [Fig 3.8]. The T cell line developed from the mice immunized with KLH, LPS and PGE<sub>2</sub> produced high levels of IL-10 and IL-5 and low levels of IL-17 and IFN- $\gamma$  [Fig 3.8].

### **3.2.5 IL-10 producing T cells developed from mice immunized with KLH, LPS and PGE<sub>2</sub> inhibit IFN- $\gamma$ production by a MOG specific Th1/Th17 cell line.**

The T cell lines developed from mice immunized with KLH, LPS and PGE<sub>2</sub> produced high levels of IL-10 (~1600 pg/ml) which is the signature cytokine of Tr1 cells and essential for their function. We conducted a mixing experiment at the start of the third round of antigen stimulation. The IL-10 producing T cell line developed from mice that had been immunized with KLH, LPS and PGE<sub>2</sub> was co-incubated with the MOG specific T cell line developed from mice with EAE at the ratios of 1:3 and 1:1. The MOG specific T line produced high levels of IL-17 and IFN- $\gamma$  [Fig 3.8]. The KLH specific T cell line produced very high levels of IL-10, low levels of IL-4 and IFN- $\gamma$  and no IL-17 [Fig 3.8]. The IL-10 producing T cell line inhibited IFN- $\gamma$  production by the MOG specific cell line but had little effect on IL-17 production [Fig 3.8]. This observation suggests that cAMP agonists can enhance the ability of a TLR agonist to induce IL-10 producing T cells while inhibiting the induction of Th1 and



Th17 cells. It also appears that IL-10 producing T cells can suppress IFN- $\gamma$  but not IL-17 production by T cells.

### 3.3 Discussion

The production of IL-12p70 by DCs drives Th1 cell differentiation, while IL-10 production results in the differentiation of Tr1 cells. TLR agonists, such as CpG, induce both IL-12p70 and IL-10 production by DCs (374). CpG treated DCs induce both IFN- $\gamma$  and IL-10 producing T cells (374). This is a vital part of an effective immune response. The Th1 cells direct the clearance of intracellular pathogens and the Tr1 cells ensure that the immune response is limited thus not damaging to the host. The autoimmune disease MS is initiated when myelin specific T cells become activated in the periphery and develop into Th1 and Th17 cells (166, 362). The activated T cells recruit innate immune cells such as macrophages and neutrophils which damage the myelin sheath of neurons, leading to the clinical symptoms of disease (362). There is growing evidence that autoimmune inflammation in MS lacks the suppressive effect of Treg cells (376).

The transfer of Tr1 cells developed *in vitro* has been shown to inhibit the development of EAE in mice, thereby highlighting the therapeutic potential of this cell type (237). TLR agonists induce Tr1 cells and many TLR agonists are in clinical trials for use as adjuvants in humans (371, 373). This study set out to examine the hypothesis that if TLR induced cytokine production by DCs could be manipulated to produce more IL-10 and less IL-12 and IL-23, TLR agonists would only induce Tr1 cells and could therefore be used as a viable therapy in autoimmune disease.

Initial experiments demonstrated that LPS, CpG, Poly I:C and MPL all induced IL-10, IL-12p40 and IL-12p70 production by DCs. Interestingly, increases in IL-10 appeared to correlate with increases in IL-12p40 and IL-12p70. This observation is intuitive as an increase in inflammation requires a similar increase in regulation in order to prevent an

aberrant immune response. Indeed in a mouse model of graft versus host disease (GVHD), disease prevention required an equal number of Tregs to effector T cells (377).

The effects of dibutyryl cAMP, forskolin and PGE<sub>2</sub> on TLR agonist induced cytokine production by DCs were then investigated. This study tested the effects of the cAMP activators on LPS, Poly I:C, Pam3CSK, MPL and CpG treated DCs. With the exception of CpG, consistent trends were observed in the effects of the cAMP activators on TLR induced cytokine production by DCs. Dibutyryl cAMP enhanced TLR agonist induced IL-23 and IL-10 and inhibited TLR agonist induced IL-12p40 and p70. Forskolin inhibited TLR agonist induced IL-12p40, IL-12p70 and IL-23 and had no effect on TLR agonist induced IL-10 production. PGE<sub>2</sub> enhanced TLR induced IL-10 and inhibited TLR agonist induced IL-12p40, IL-12p70 and IL-23. These initial findings identified PGE<sub>2</sub> as potentially the most promising candidate for the enhancement of TLR agonist mediated Tr1 cell induction.

Dibutyryl cAMP, forskolin or PGE<sub>2</sub> had little effect on CpG induced IL-10, IL-23 or IL-12p40 production, which contrasted with the ability of the cAMP activators to modulate LPS, Poly I:C, MPL and Pam3Cys induced cytokine production. CpG binds directly to TLR9 (378). Latz *et al* have reported that TLR9 is initially located in the endoplasmic reticulum (ER) of DC and macrophages (378). Furthermore, it was demonstrated that CpG is internalized into the DC by a clathrin dependent endocytic pathway and is subsequently transported to a tubular lysosomal compartment (378). TLR9 was then shown to translocate from the ER to the CpG lysosomal compartment for ligand binding and signal transduction (378). The intracellular localization of TLR9 may explain why increased cAMP levels failed to effect CpG induced cytokine production. Adenylate cyclases, phosphodiesterases and PKA can also be localized to specific spatial compartments within the cell (379). This

creates subcellular pools of intracellular cAMP and its effector PKA. It is therefore possible that the cAMP induced by dibutyryl cAMP, forskolin or PGE<sub>2</sub> may not be localized to the same cellular microdomain as CpG containing lysosomes, which would explain why the cAMP activators failed to modulate CpG induced IL-12p40, IL-10 and IL-23 production by DCs.

Dibutyryl cAMP, forskolin and PGE<sub>2</sub> all increase the intracellular concentration of cAMP. Dibutyryl cAMP is a cAMP analog, forskolin binds to and directly activates adenylate cyclase and PGE<sub>2</sub> binds to the surface receptors EP<sub>2</sub> and EP<sub>4</sub> which activate adenylate cyclase (91, 105). It is therefore difficult to explain why each cAMP activator has a different effect on TLR agonist induced cytokine production. However a study by Dahle *et al* provides a possible explanation. Forskolin was reported to inhibit TNF- $\alpha$  and IL-10 production by LPS stimulated Kupffer cells (106). In the same study, PGE<sub>2</sub> had no effect on IL-10 and inhibited TNF- $\alpha$  production by LPS stimulated Kupffer cells (106). The authors reported that Kupffer cells express high levels of forskolin insensitive adenylate cyclase 9 which might explain the differing effects of the cAMP activators (106).

Hickey *et al* have reported that CyaA and PGE<sub>2</sub> both inhibit TLR induced IRF-1 and IRF-8 activation, which provides a possible mechanism for the inhibition of IL-12 production by cAMP activators (116). However, more studies will be required to fully elucidate the intracellular pathways involved. The use of cAMP analogs that are highly specific for PKA and Epac would allow the identification of the cAMP induced intracellular signalling pathways that are responsible for the modulation of TLR stimulated cytokine production.

Having shown the effect of cAMP activators on TLR stimulated cytokine production by DCs, a DC transfer experiment was performed. The pre-incubation and co-incubation of cAMP activators resulted in identical effects on TLR agonist induced cytokine production by DCs. This observation, allowed us to perform a direct injection study whereby antigen, LPS and cAMP activator were simultaneously injected *in vivo*. DCs treated with KLH and LPS exhibited a mature phenotype characterised by high surface expression of CD80, CD86 and CD40 and low levels of CCR5 expression. Our aim was to enhance the induction of Tr1 cells by TLR agonists. Traditionally DCs that display an immature phenotype are associated with the differentiation of Tr1 cells. Martin *et al* have shown that DCs from CD40<sup>-/-</sup> mice induce IL-10 producing T cells (229). In this study, we report that the cAMP activators had no effect on LPS induced maturation. However, a recent report by Allan *et al*, has identified a population of mature DCs in human peripheral blood, that display a mature phenotype and are more potent inducers of Tr1 cells than immature DCs (195). This suggests that the inhibition of DC maturation may not be crucial to the induction of Tr1 cells.

The transfer of DCs treated with LPS *in vivo* resulted in antigen specific IFN- $\gamma$ , IL-17 and IL-10 production which is indicative of a mixed Th1, Th17 and Tr1 cell response. DCs treated with LPS and dibutyryl cAMP induced a Th17 response. TGF- $\beta$  and IL-6 are the key differentiation factors for the induction of Th17 cells and IL-23 is required during restimulation for the maintenance of IL-17 production. It was demonstrated that dibutyryl cAMP enhances LPS induced IL-23. It is possible that in the presence of T cell derived TGF- $\beta$ , TLR agonists can always drive Th17 differentiation. The maintenance of this Th17 response may be dependent on IL-23 production. Further studies will be required to confirm this hypothesis. DCs treated with KLH, LPS and PGE<sub>2</sub> induced high levels of antigen specific IL-10, and reduced levels of IFN- $\gamma$  and IL-17 when compared to DCs treated with

LPS. This suggested that the combination of LPS and PGE<sub>2</sub> enhances the induction of IL-10 producing T cells. It also confirms that the use of PGE<sub>2</sub> to manipulate LPS induced cytokine production by DCs, predictably modulates the antigen specific T cell response.

A direct injection study was then performed whereby mice were immunized with KLH, LPS and three doses of each cAMP activator. The optimal method for the induction of antigen specific IL-10 producing T cells was the immunization of mice with KLH, LPS and PGE<sub>2</sub> (5 mg/kg). Using this approach, we developed an IL-10 producing T cell line which suppressed IFN- $\gamma$  production by a MOG specific T cell line developed from mice with EAE. Jarnciki *et al* have previously developed Tr1 cells from mice that were immunized with KLH and CpG. These Tr1 cells were established in the presence of antigen and an anti-IFN- $\gamma$  antibody and were shown to inhibit IFN- $\gamma$  production by a Th1 cell line (374). The IL-10 producing T cells developed in this report, from mice immunized with KLH, LPS and PGE<sub>2</sub> were not manipulated *in vitro* and were more potent suppressors of IFN- $\gamma$  production by T cells. We therefore believe that the use of PGE<sub>2</sub> to modulate LPS induced T cell responses represents an advancement in the development of IL-10 producing T cells, over the use of TLR agonists alone.

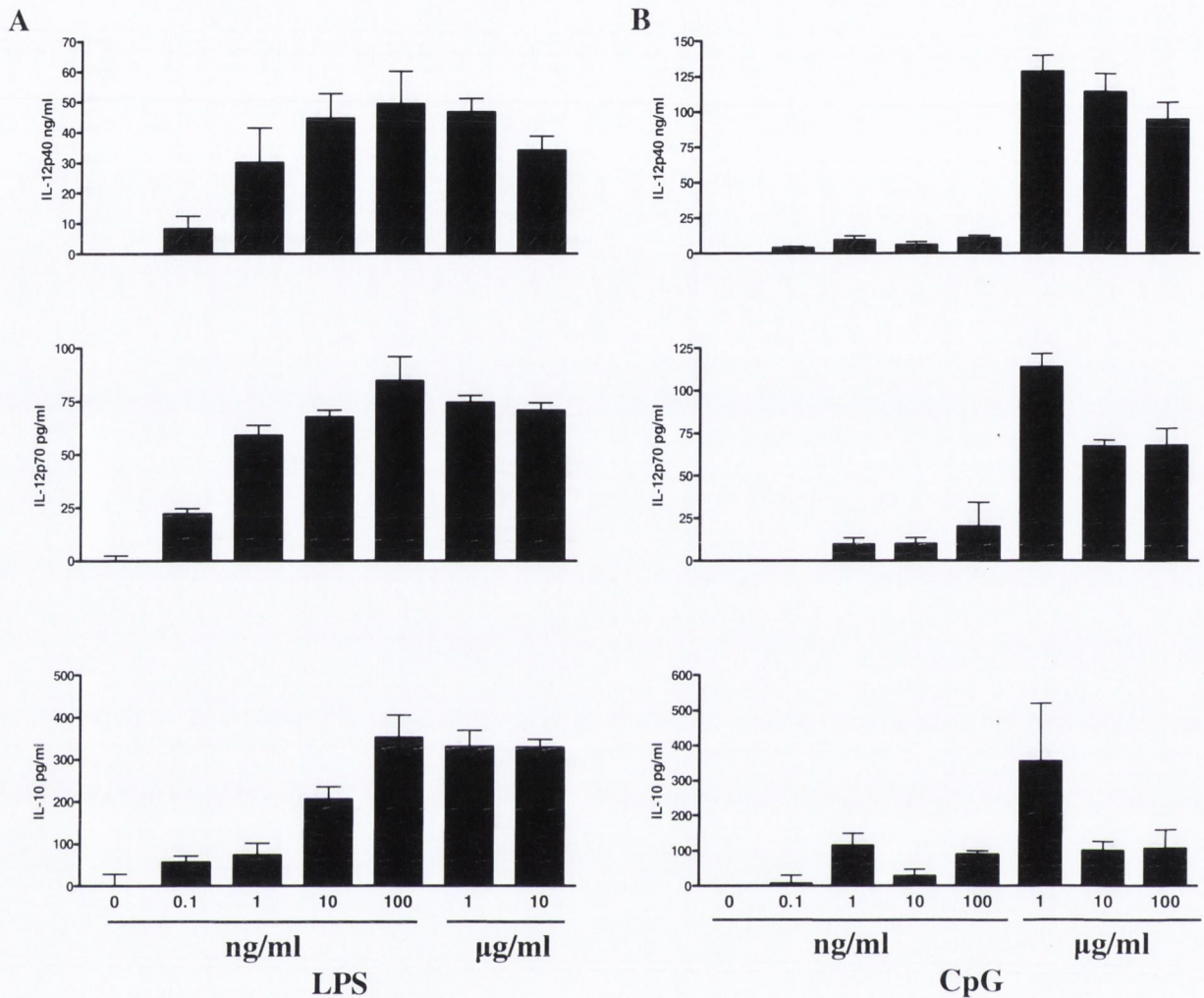
Studies conducted by Langrish *et al*, reported that myelin specific Th17 cells developed by stimulation with antigen in the presence of recombinant IL-23 were highly encephalogenic and induced EAE in recipient mice (138). In contrast, the transfer of myelin specific Th1 cells did not induce EAE (138). This suggested that myelin specific Th17 cells played a key role in directing autoimmune inflammation. However, conflicting evidence has recently emerged that myelin specific Th1 and Th17 can both induce EAE (168). O'Connor *et al* have recently suggested that Th17 cell populations that lack IFN- $\gamma$ <sup>+</sup> cells do not induce EAE

due to their inability to access the CNS (170). They propose a model for the development of EAE, whereby myelin specific Th1 cells initially enter the CNS which results in the formation of EAE lesions (170). Th17 cells are only recruited to the CNS once inflammation has been established (170). These data suggest that the inhibition of both Th1 and Th17 cell responses will be important in the treatment of autoimmune disease. This report showed that IL-10 producing T cells developed from mice immunized with KLH, LPS and PGE<sub>2</sub> inhibit IFN- $\gamma$  but not IL-17 production by a MOG specific pro-inflammatory T cell line.

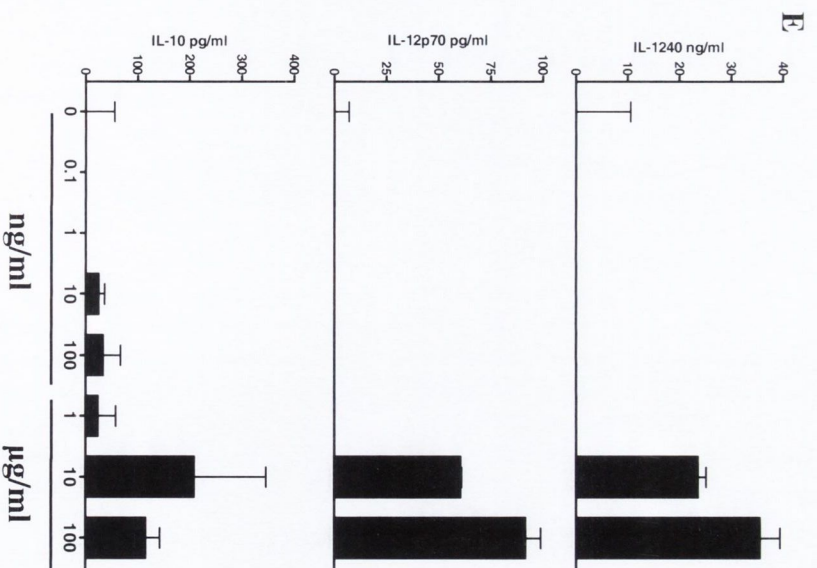
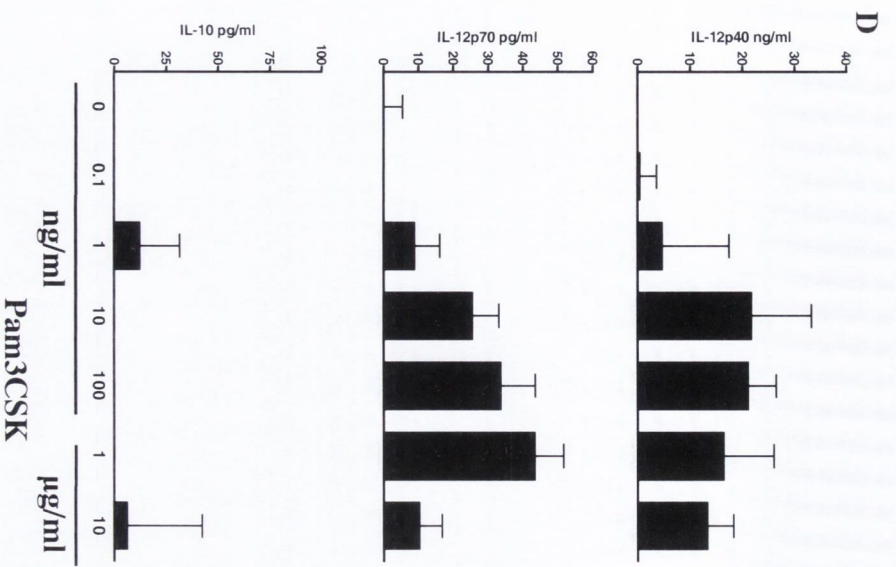
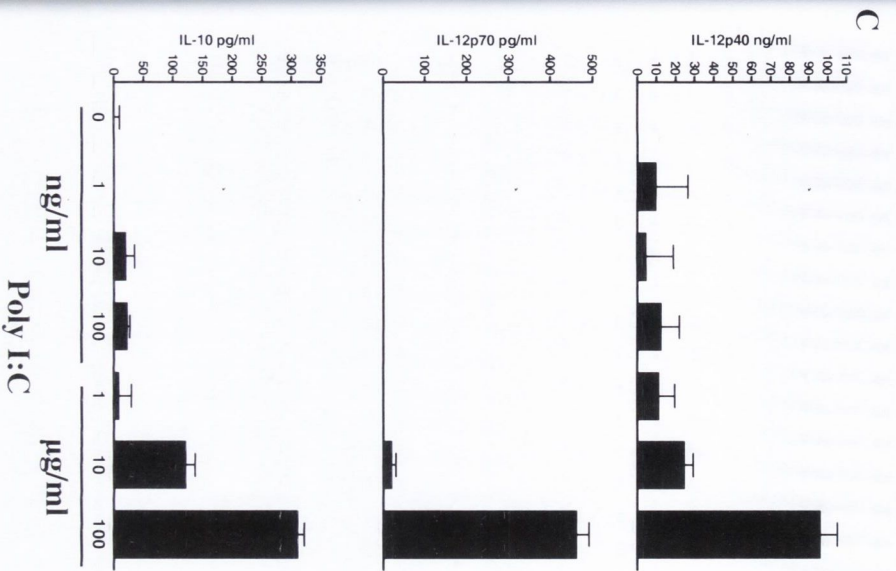
The role of Th17 cells in autoimmune inflammation has resulted in significant research focused on developing approaches for their inhibition. Bettelli *et al* have shown that natural Treg cells suppress the proliferation of Th17 cells (134). However, Allan *et al* have suggested that the analysis of proliferation *in vitro* does not always mirror the situation *in vivo* (195). They propose that a better indicator of Treg function *in vitro*, is their ability to suppress cytokine production (195). There is limited published data showing that Treg cells or even immunosuppressive cytokines inhibit IL-17 production by effector Th17 cells which suggests that the cell type may be resistant to suppression. Kleinschek *et al* have reported that IL-25<sup>-/-</sup> mice are highly susceptible to EAE suggesting that IL-25 plays a role in the inhibition of Th17 cells (189). IL-25 induces IL-13 production, which inhibits the production of IL-23, IL-1 $\beta$  and IL-6 by DCs and results in suppressed Th17 responses (189). This suggests that the natural suppression of Th17 responses *in vivo* may depend on the inhibition of innate cytokines such as IL-23 which promote the expansion of Th17 cells.





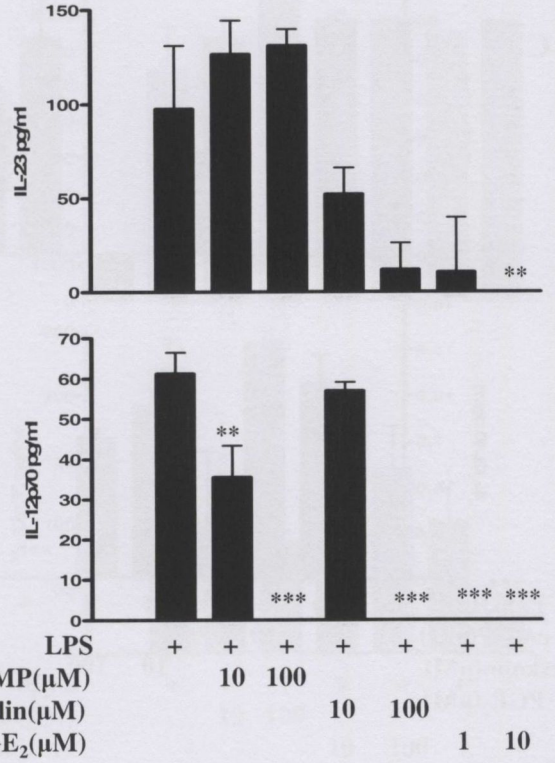
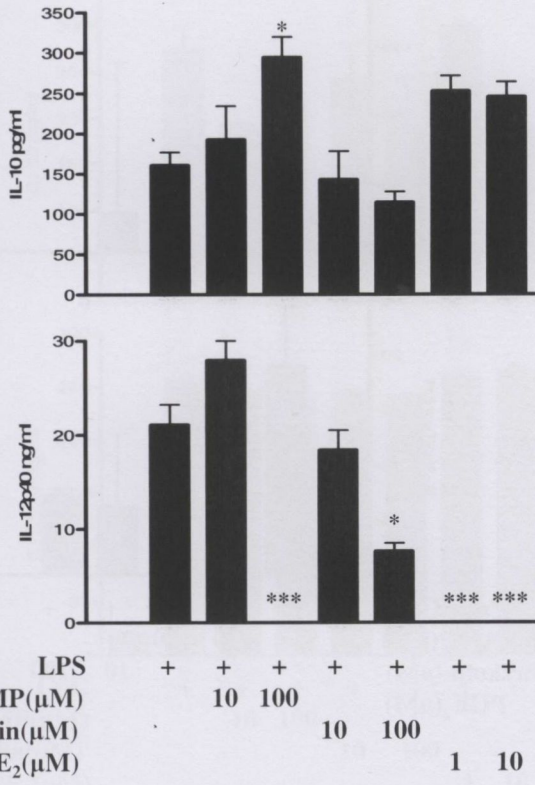


**Fig 3.1 TLR agonists induce IL-10 and IL-12 production from conventional DC.** BMDCs ( $5 \times 10^5$  cells/ml) from C57BL/6 mice were incubated with increasing concentrations of the TLR agonists, (A) LPS (0.1 ng/ml-10  $\mu$ g/ml), (B) CpG (1 ng/ml-100  $\mu$ g/ml), (C) Poly (I:C) (1 ng/ml-100  $\mu$ g/ml), (D) Pam3CSK (0.1 ng/ml-10  $\mu$ g/ml), and (E) MPL (1 ng/ml-100  $\mu$ g/ml). Supernatants were removed after 24 hours and IL-12p40, IL-12p70 and IL-10 were quantified by ELISA. These results are representative of two experiments which were carried out in triplicate.

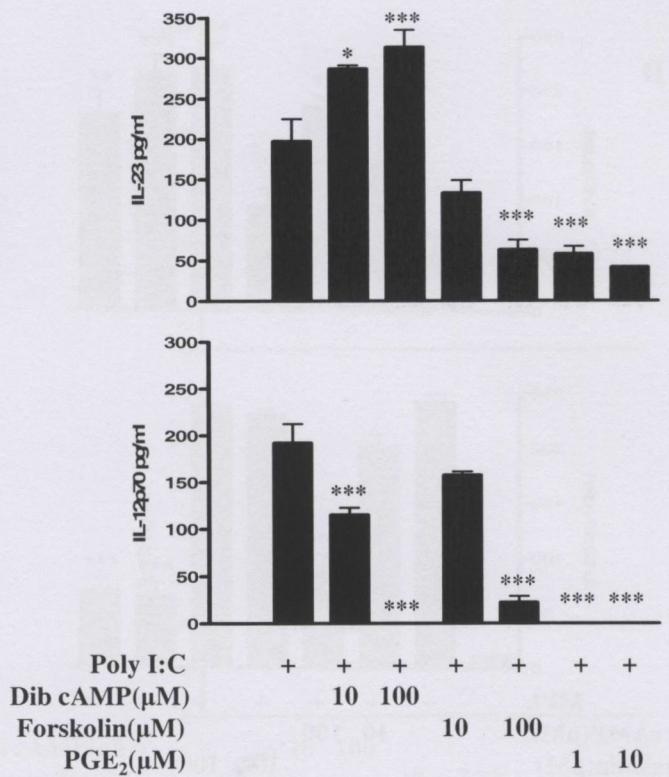
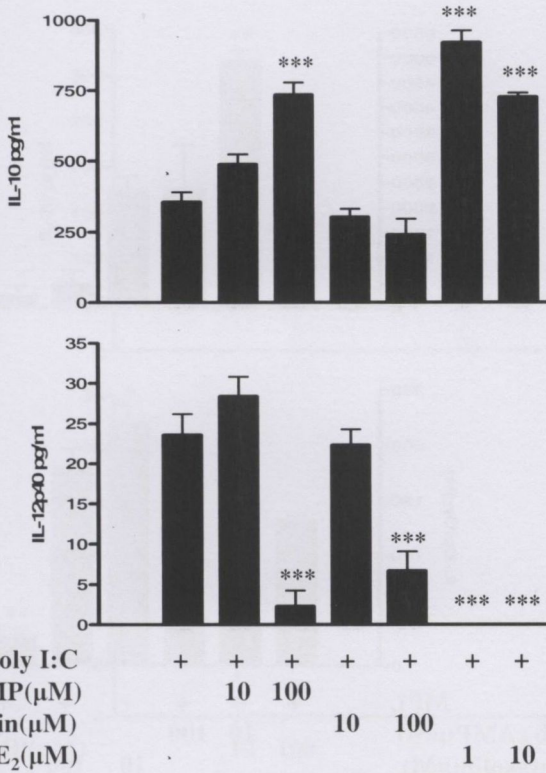


**Fig 3.2 The pre-incubation of DCs with cAMP activators enhances TLR agonist induced IL-10 production and inhibits TLR induced IL-12 production.** BMDCs ( $5 \times 10^5$  cells/ml) from C57BL/6 mice were pre-incubated with the cAMP activators forskolin (10 and 100  $\mu$ M), prostaglandin E<sub>2</sub> (1 and 10  $\mu$ M) or the cAMP analog dibutyryl cAMP (10 and 100  $\mu$ M). After 30 mins (A) LPS (100 ng/ml), (B) Poly (I:C) (100  $\mu$ g/ml), (C) Pam3CSK (100 ng/ml), (D) MPL (100  $\mu$ g/ml), (E) CpG (1  $\mu$ g/ml) or (F) medium alone were added. In the case of Fig (F) no TLR agonist was added. After 24 hours supernatants were removed and IL-12p40, IL-12p70, IL-23 and IL-10 were quantified by ELISA. \*\*\*P<0.001, \*\*P<0.01, \*P<0.05 versus DCs stimulated with a TLR agonist alone. These results are representative of three experiments which were carried out in triplicate.

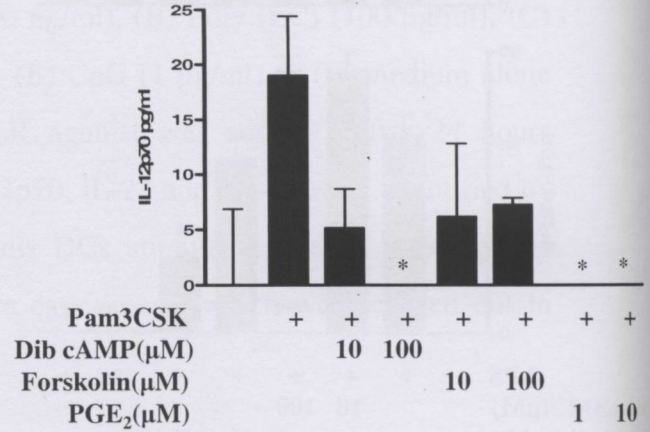
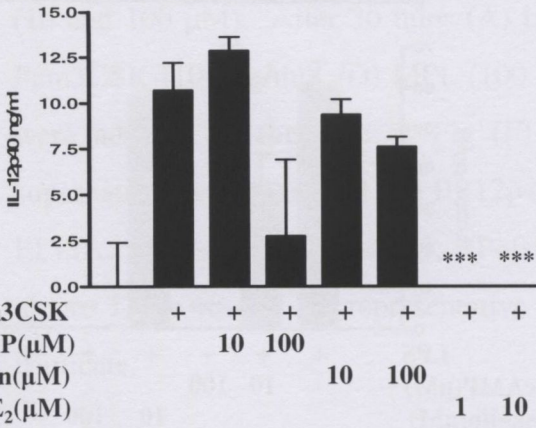
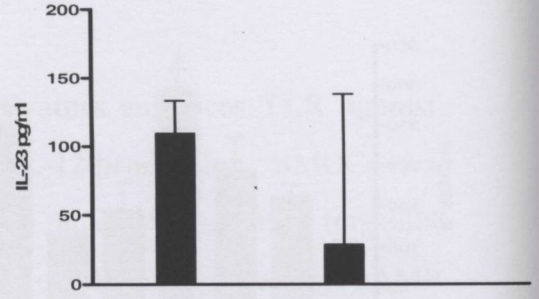
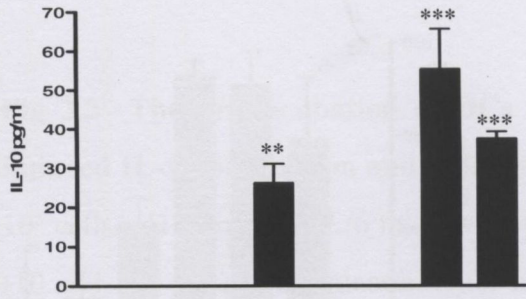
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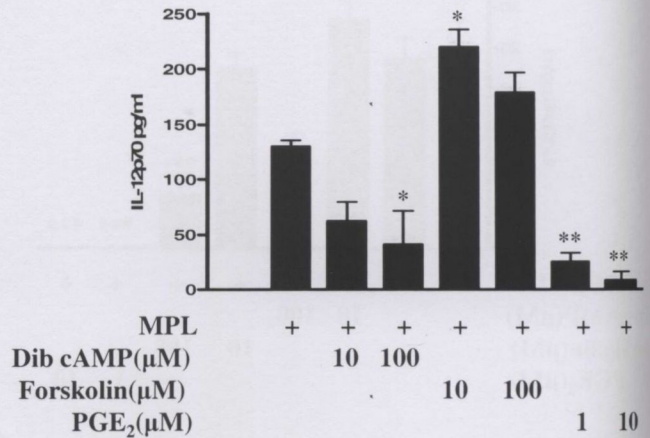
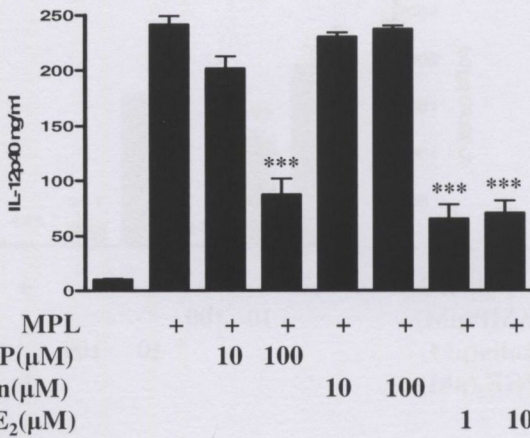
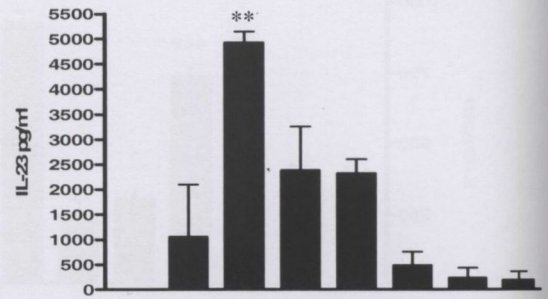
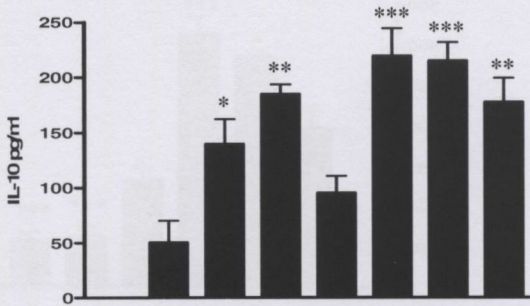
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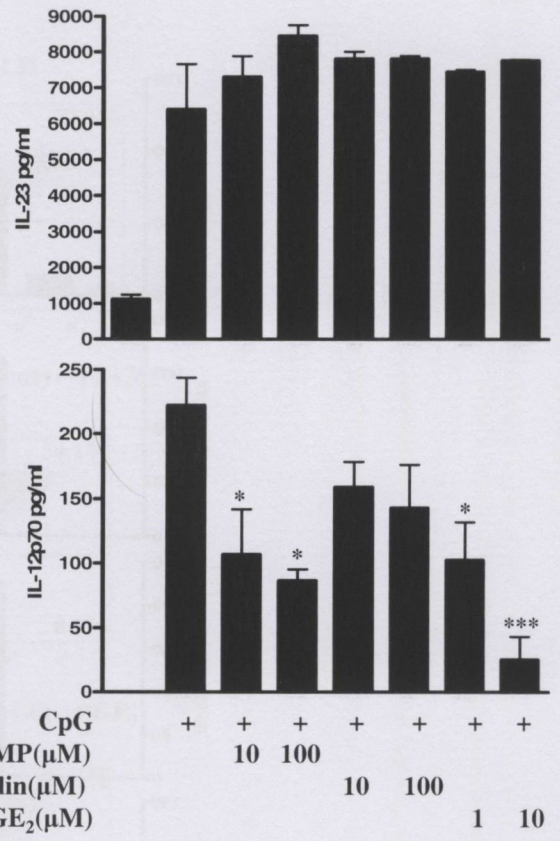
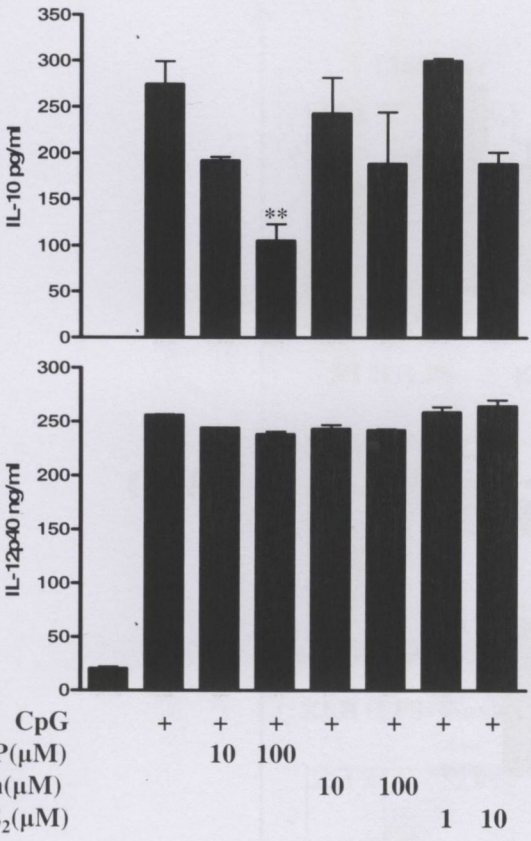
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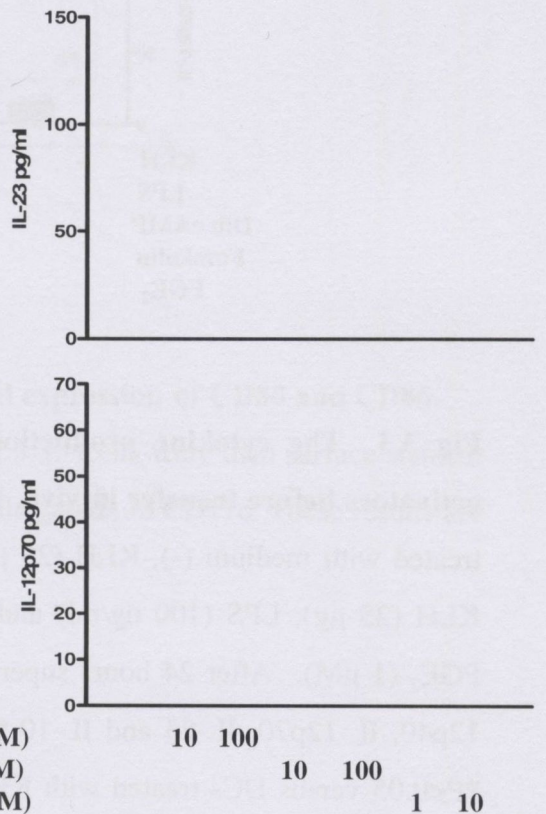
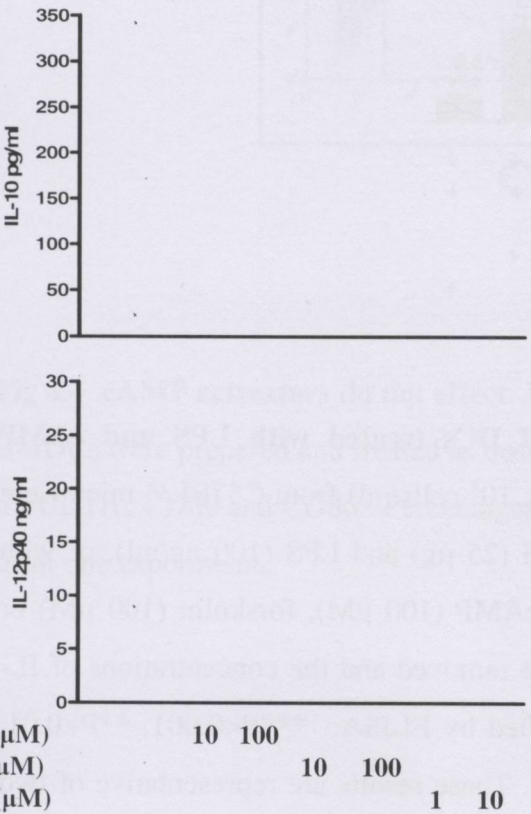
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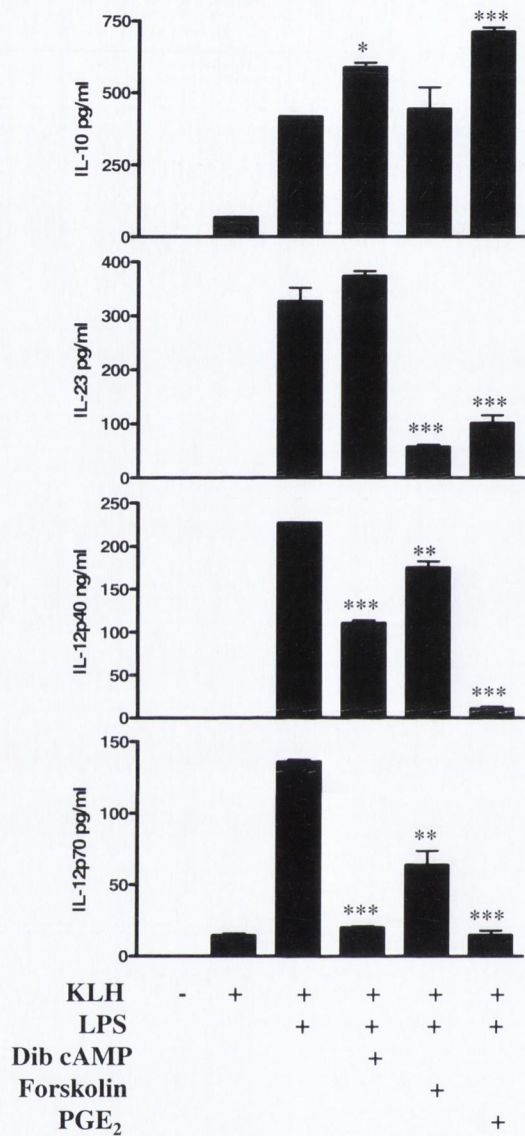


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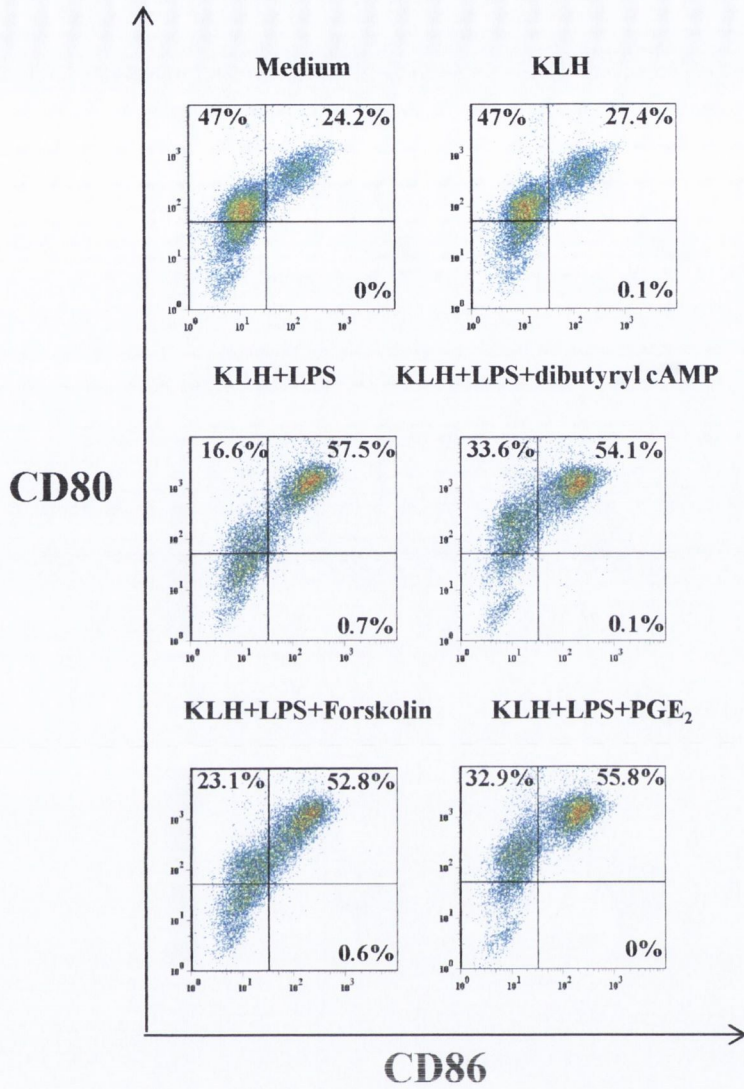


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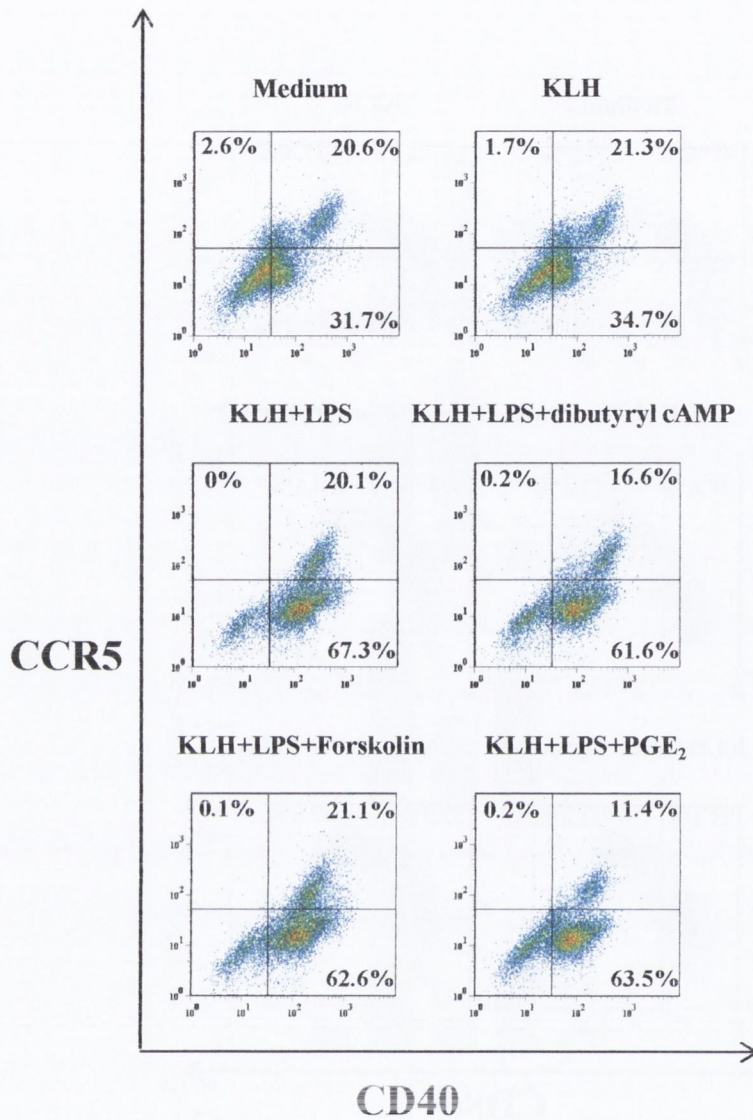
**Fig 3.3 The cytokine production profile of DCs treated with LPS and cAMP activators before transfer in vivo.** BMDCs ( $5 \times 10^5$  cells/ml) from C57BL/6 mice were treated with medium (-), KLH (25  $\mu$ g/ml), KLH (25  $\mu$ g) and LPS (100 ng/ml), or with KLH (25  $\mu$ g), LPS (100 ng/ml) and dibutyryl cAMP (100  $\mu$ M), forskolin (100  $\mu$ M) or PGE<sub>2</sub> (1  $\mu$ M). After 24 hours supernatants were removed and the concentrations of IL-12p40, IL-12p70, IL-23 and IL-10 were quantified by ELISA. \*\*\*P<0.001, \*\*P<0.01, \*P<0.05 versus DCs treated with KLH and LPS. These results are representative of two experiments which were carried out in triplicate.



**Fig 3.4 cAMP activators do not effect LPS induced expression of CD80 and CD86.**

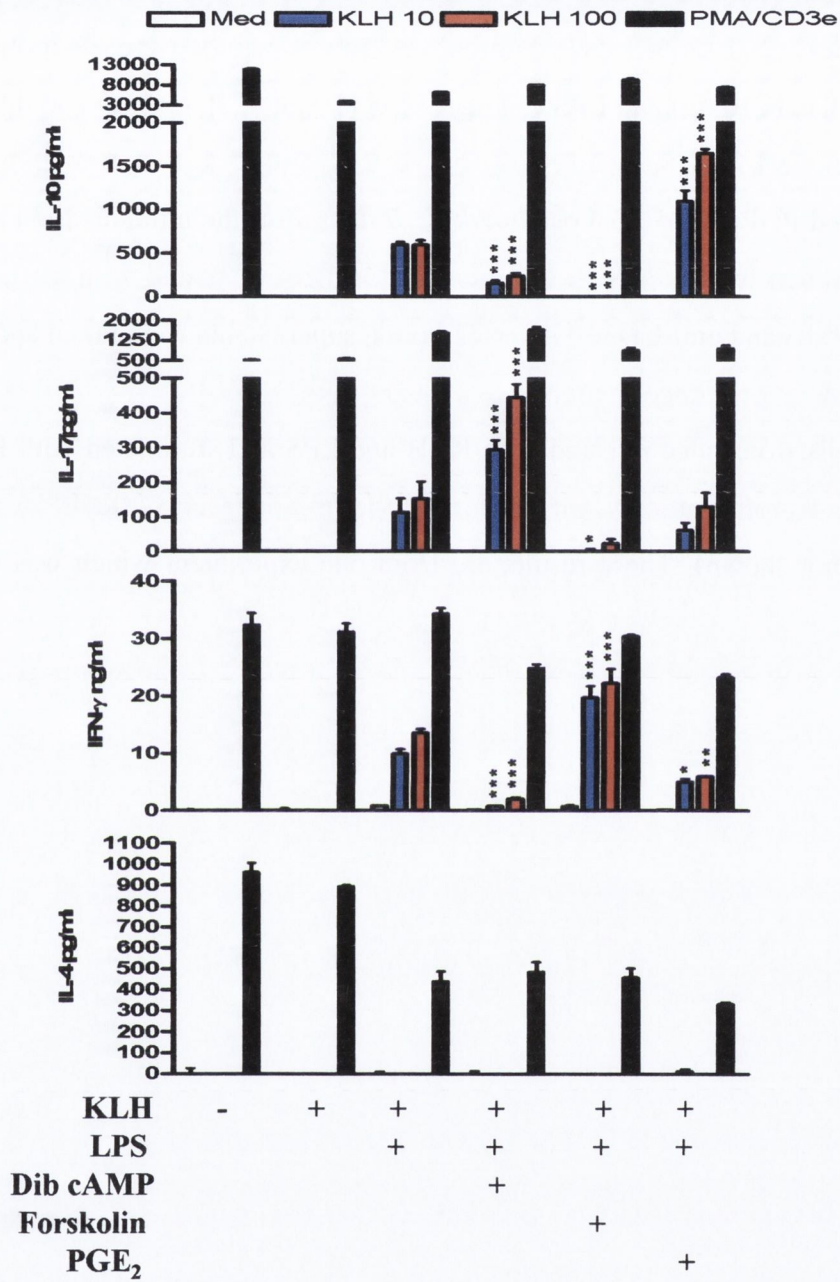
BMDCs were prepared and treated as described in Fig 3.3. Cells were then surface stained for CD11c, CD80 and CD86. Percentages refer to cells gated on CD11c. These results are from one experiment.



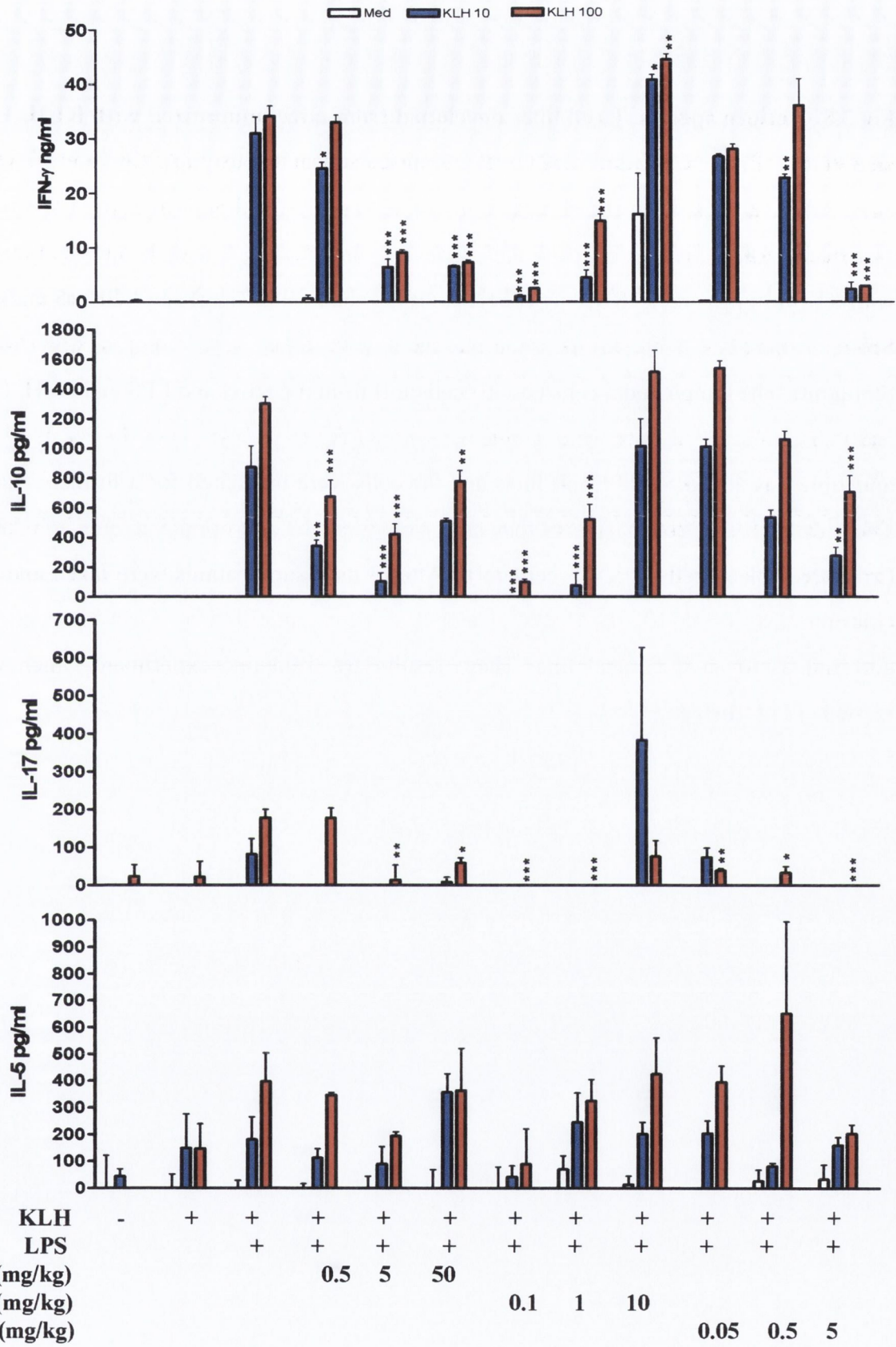


**Fig 3.5 Effect of LPS induced CD40 and CCR5 expression.** BMDCs were prepared and treated as described in Fig 3.3. Cells were then surface stained for CD11c, CD40 and CCR5. Percentages refer to cells gated on CD11c. These results are from one experiment.

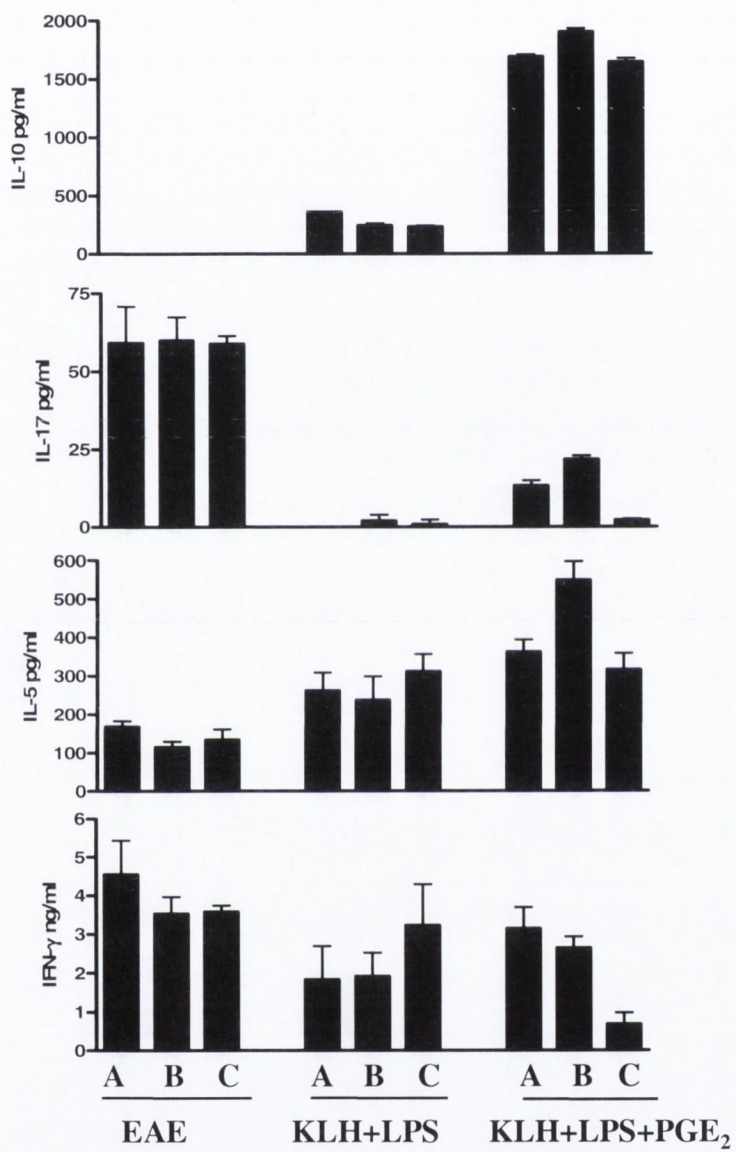
**Fig 3.6 Treatment of TLR agonist activated DC with cAMP activators modulates their ability to promote T cell response *in vivo*.** BMDCs ( $5 \times 10^5$  cells/ml) from C57BL/6 mice were treated with medium (DC) alone (-), KLH (25  $\mu\text{g/ml}$ ), KLH and LPS (100 ng/ml) or with KLH and LPS and Forskolin (100  $\mu\text{M}$ ), Prostaglandin  $\text{E}_2$  (1  $\mu\text{M}$ ) or dibutyryl cAMP (100  $\mu\text{M}$ ). After 24 hrs the cells were washed twice and injected subcutaneously into the footpad ( $5 \times 10^5$  cells/mouse). There were four mice for each DC treatment group. After 7 days the mice were sacrificed, the popliteal lymph nodes were harvested and re-stimulated with *ex vivo* medium, KLH (10 or 100  $\mu\text{g/ml}$ ) or PMA and anti-CD3. After 72 hours supernatants were removed and IL-10, IL-17, IFN- $\gamma$  and IL-4 concentrations were quantified by ELISA. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$  versus cells from mice injected with DCs treated with KLH and LPS and stimulated with KLH. These results are from one experiment which was carried out in triplicate.



**Fig 3.7 The coadministration of LPS and 5mg/kg of PGE<sub>2</sub> induces a Treg cell phenotype *in vivo*.** Groups of four C57BL/6 mice were immunised subcutaneously into the footpad with PBS (-), KLH (10 µg), KLH (10 µg) and LPS (10 µg), KLH (10 µg), LPS(10 µg) and dibutyryl cAMP (0.5, 5 or 50 mg/kg), KLH (10 µg), LPS(10 µg) and forskolin (0.1, 1 or 10 mg/kg) or KLH (10 µg), LPS(10 µg) and PGE<sub>2</sub> (0.05 , 0.5 or 5 mg/kg). 7 days after the immunization the mice were sacrificed, the popliteal lymph nodes were harvested and re-stimulated with medium, KLH (10 or 100 µg/ml) or PMA and anti-CD3e. After 72 hours, supernatants were taken and IL-10, IFN-γ , IL-17, IL-4 and IL-5 concentrations were quantified by ELISA. \*\*\*P<0.001, \*\*P<0.01, \*P<0.05 versus cells from mice injected with KLH and LPS and stimulated with KLH. Lymph nodal cells from all groups immunized produced cytokine in response to PMA and anti-CD3 stimulation (data not shown). These results are from one experiment which was carried out in triplicate.

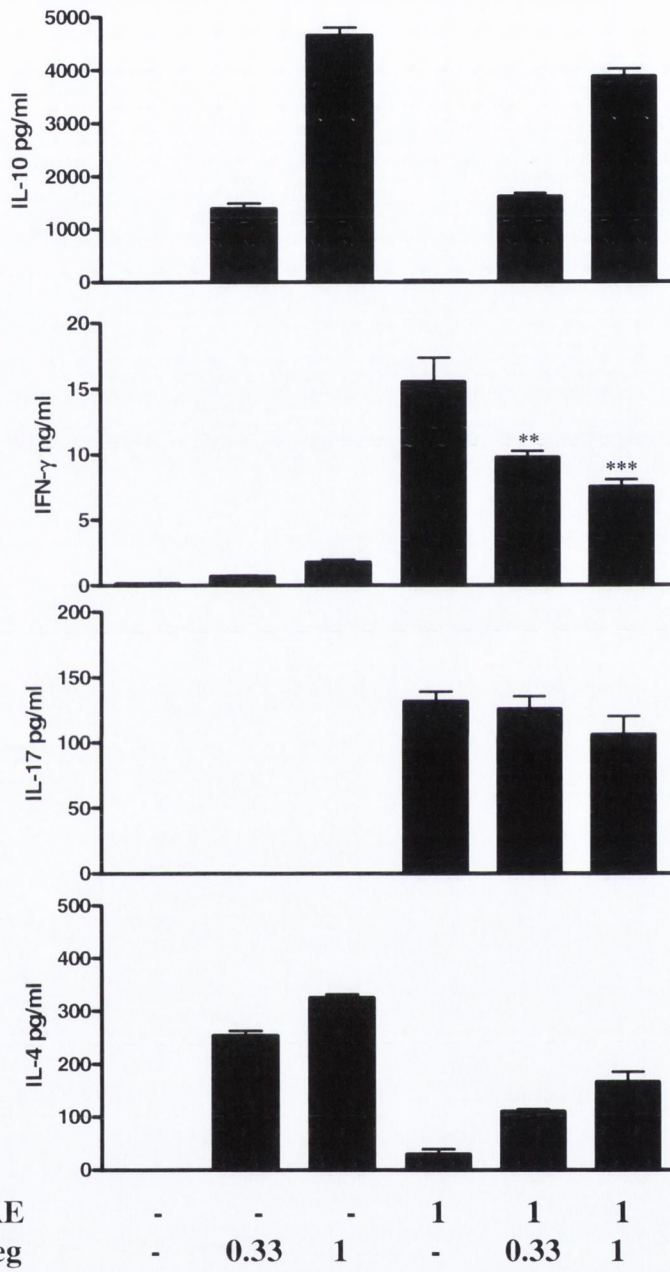


**Fig 3.8 Antigen specific T cell lines developed from mice immunized with KLH, LPS and PGE<sub>2</sub>.** EAE was induced in C57BL/6 mice with MOG (150 µg), CFA and PT (500 ng). After 12 days when the clinical signs of severe EAE had appeared, the mice were sacrificed. Alternatively C57BL/6 mice were immunized subcutaneously into the footpad with KLH (10 µg) and LPS (10 µg) or KLH (10 µg) and LPS(10 µg) and PGE<sub>2</sub> (5 mg/kg). Spleen cells (2 x 10<sup>6</sup>cells/ml) from the mice with EAE were cultured with MOG (25µg/ml). The lymph nodal cells (2 x 10<sup>6</sup>cells/ml) from the KLH and LPS and KLH, LPS and PGE<sub>2</sub> immunized mice were stimulated with KLH (25 µg/ml). After 4 days IL-2 (10 units/ml) was added to all T cell lines and the cells were incubated for a further 7 days. The T cells (1 x 10<sup>5</sup>cells/ml) were then stimulated with the appropriate antigen and APCs (irradiated spleen cells, 2 x 10<sup>6</sup> cells/ml). After 3 days supernatants were taken and the concentrations of IL-10, IL-17, IFN-γ and IL-5 were quantified by ELISA. Each bar corresponds to an individual line. These results are from one experiment which was carried out in triplicate.



**Fig 3.9 An IL-10 producing T cell line developed from mice immunized with KLH, LPS and PGE<sub>2</sub> inhibits cytokine production from Th1/Th17 T cells.** An IL-10 producing KLH specific T cell line was established from mice that had been immunized with KLH, LPS and PGE<sub>2</sub> and a MOG specific T cell line was established from mice with EAE as described in Fig 3.8. At the initiation of the third round of antigen stimulation, the T cell line was co-cultured with the MOG specific T cell line ( $2 \times 10^5$  cells/ml) at the ratios- 1:3 and 1:1 with KLH (25  $\mu$ g/ml), MOG (25  $\mu$ g) and APCs (irradiated spleen cells,  $2 \times 10^6$  cells/ml). After 72 hours, supernatants were removed and the concentrations of IFN- $\gamma$ , IL-10, IL-4 and IL-17 were quantified by ELISA. \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$  versus MOG specific T cell line incubated with APCs and antigen. These results are from one experiment which was carried out in triplicate.





# **Chapter IV**

## **The immunomodulatory properties of Filamentous Haemagglutinin**



## 4.1 Introduction

*B. pertussis* is the causative agent of whooping cough, which is presently one of the ten most common causes of death from infectious disease (257). The World Health Organization (WHO) reported that worldwide, there are 48.5 million cases of whooping cough per year, resulting in 295,000 deaths from the disease (380). The majority of these cases occur in the developing world among unvaccinated individuals where the incubation period of the bacteria can last for up to 6 weeks and the basic reproductive ratio of whooping cough is 15 (249, 259). This makes whooping cough one of the most contagious directly transmitted human infections (249). The success of *B. pertussis* as a pathogen of humans can be largely attributed to the virulence factors of the bacteria which include FHA, fimbriae, PT, LPS, TcfA, BrkA, Vag8, CyaA and PRN (113). These factors allow the bacteria to enter the host, evade the immune response and to induce the paroxysmal cough which provides efficient transmission of the pathogen (113). *B. pertussis* is transmitted in aerosolized droplets and initially attaches to the cilia on the epithelial cells of the upper respiratory tract (113, 259). Two of the virulence factors FHA and PT synergise in allowing *B. pertussis* to bind to the cilia (259). Attachment results in the death of the ciliated cells and defective mucociliary clearance, allowing the bacteria to colonize the lungs (260). An effective Th1 immune response against *B. pertussis* has been shown to clear the bacteria from the lung (381). However the acute phase of infection is associated with hyporesponsiveness of T cells in the lung which facilitates bacterial persistence (382). Studies have shown that the virulence factors CyaA and FHA have major roles in the suppression of the immune response by *B. pertussis* (293).

The expression of virulence factors (with the exception of TCT) by *B. pertussis* are controlled by the Bordetella virulence regulon (Bvg) (248). Boschwitz *et al* reported that monocytes

infected with wild type *B. pertussis* had a significantly impaired ability to stimulate T cell proliferation to the exogenous antigen, tetanus toxoid (293). However the infection of monocytes with a Bvg<sup>-</sup> strain of *B. pertussis* resulted in enhanced T cell proliferation when compared with the infection of the monocytes with the Bvg<sup>+</sup> wild type strain (293). This suggested that Bvg regulated virulence factors might have a role in the suppression of the immune response by the bacteria. The authors consequentially investigated the role of CyaA and FHA in *B. pertussis* mediated immune suppression, through the use of mutants of *B. pertussis*. One mutant strain produced a mutated form of CyaA that lacked catalytic activity and another mutant strain was deficient in the expression of FHA. The infection of monocytes with either mutant resulted in T cell proliferation that was comparable to levels observed when monocytes were infected with the Bvg<sup>-</sup> strain (293). This suggested that both CyaA and FHA play a major role in the inhibition of T cell responses by *B. pertussis*.

Further studies have since confirmed the abilities of CyaA and FHA to modulate the immune response. CyaA enhances TLR induced IL-10 and inhibits IL-12, TNF- $\alpha$  and CCL3 production by macrophages and DCs (115). Furthermore CyaA induces IgG1 Abs, Th2 and Treg cells against a co-administered antigen *in vivo* (115). Similar findings were reported with FHA which induced IL-10 and suppressed LPS induced IL-12 in macrophages and DCs (227, 294). In a murine septic shock model, mice treated with FHA had reduced serum concentrations of IL-12 and IFN- $\gamma$  (294). McGuirk *et al* also generated FHA specific Tr1 cell clones from the lungs of mice infected with *B. pertussis* (227). This Tr1 cell clone inhibited a Th1 cell clone, by an IL-10 dependent mechanism (227). The transfer of the FHA specific Tr1 cell clones to *B. pertussis* infected mice resulted in delayed bacterial clearance and a reduction in *B. pertussis* specific IFN- $\gamma$  production (227). This provided strong evidence that the virulence factor FHA plays a key role in the induction of T cell hyporesponsiveness

during the acute stage of *B. pertussis* infection. Interestingly, FHA was also shown to induce the differentiation of Treg cells specific for a model antigen *in vitro* (227). DCs that had been incubated with OVA and FHA directed the induction of OVA specific Tr1 from naive T cells obtained from DO11.10 OVA TCR Tg mice (227). These Tr1 cells produced high levels of IL-10, low levels of IL-4 and moderate levels of IFN- $\gamma$  (227).

Immunomodulatory molecules that induce Tr1 cells against model antigens have strong therapeutic potential. Barrat *et al* have generated OVA specific Tr1 cells *in vitro* through the use of Vitamin D3, dexamethasone and neutralizing antibodies to Th1 and Th2 cytokines (237). On adoptive transfer, these cells were capable of preventing the induction of EAE when OVA absorbed in alum was injected intracranially to act as an antigen reservoir (237). Although this study demonstrates the ability of Tr1 cells to attenuate the symptoms of autoimmune diseases, its therapeutic potential is somewhat limited. Autoimmune diseases such as MS and EAE are initiated by the activation of auto-antigen specific T helper cells in the periphery (170, 362). Current evidence suggests that both Th1 and Th17 cells play a role in the pathogenesis of EAE (170). Kroenke *et al* have reported that myelin specific Th1 or Th17 cells could induce EAE on transfer (168). Conflictingly, O' Connor *et al* maintain that myelin specific Th17 cells that lack IFN- $\gamma$  producing cells do not induce disease (170). They propose a model whereby Th1 cells can access the CNS, and that Th17 cells are recruited once EAE lesions are established (170). In MS, there is an increase in IL-17<sup>+</sup> T cells in active lesions when compared with inactive areas (166). Th1 responses can also be detected in the lesions of MS patients and in clinical trials the systemic administration of IFN- $\gamma$  worsened disease (362, 383). Th1 cells are induced by antigen presenting cells that produce IL-12p70 whereas TGF- $\beta$  and IL-6 are required for the differentiation of Th17 cells and IL-23 is necessary for the expansion and survival of the cell type (133, 168).

A study conducted by Braat *et al* has shown that FHA effectively attenuates the clinical symptoms of colitis in mice. The mice were injected i.p. with FHA which induced IL-10 and TGF- $\beta$  production in the mesenteric lymph nodes and Peyers patches (298). The protective effect of FHA was independent of IL-10 production by T cells (298), meaning the mechanism of FHA mediated attenuation of disease awaits elucidation.

We investigated the therapeutic potential of FHA in the EAE model. We report that the pre-immunization of mice with MOG and FHA on days -21 and -7 before the induction of EAE, attenuated the clinical symptoms of disease. The pre-immunization with KLH and FHA did not affect disease progression. This suggests that pre treatment with MOG and FHA induces MOG specific Treg cells that attenuate EAE. We show that FHA is capable of acting as an adjuvant for the induction of Tr1 cells that are specific for a co-administered antigen, KLH. These Tr1 cells suppressed KLH specific Th1 cells and the proliferation of KLH specific Th17 cells thereby providing a potential mechanism for the ability of FHA to attenuate autoimmune disease. Studies examining the effect of FHA on DCs, revealed that FHA induces the activation of ERK, p38 and c-fos and stimulates I $\kappa$ B degradation, and that the induction of IL-10 by FHA is both p38 and TLR4 dependent. FHA was also shown to inhibit TLR agonist induced IL-12 and IL-23 production.

## 4.2 Results

### 4.2.1 FHA induces IL-6 and IL-10 production from DC and PEC.

McGuirk *et al* have previously reported that FHA induces moderate levels of IL-10 and high levels of IL-6 from J774 cells (294). This study examined the ability of FHA to modulate cytokine production from DCs. BMDCs from BALB/c mice were incubated with FHA for 24 hours and supernatants were removed for cytokine analysis. FHA induced high levels of IL-6 and IL-10 and low levels of IL-1 $\beta$ , but undetectable IL-12p70 [Fig 4.1]. This contrasts with the cytokine induction profile of the TLR agonists LPS and CpG which both induce significant levels of IL-12p70 as well as IL-6 and IL-10 (133). The ability of FHA to induce cytokines from peritoneal exudate cells (PEC) *in vivo* was also examined. BALB/c mice were injected intra-peritoneally with PBS or FHA. 4 hrs later a peritoneal lavage was performed and the PEC were removed and cultured for 24 hours. When compared with the PBS injected control mice, the PEC from FHA injected mice produced high levels of IL-6 and low levels of IL-10, IL-1 and TNF- $\alpha$ . [Fig 4.2]

### 4.2.2 FHA induces p-ERK, p-p38, c-fos and I $\kappa$ B degradation in BMDCs.

Having demonstrated that FHA can induce IL-6 and IL-10 production in DCs *in vitro* and peritoneal exudate cells *in vivo*. [Fig 4.1, Fig 4.2], it was decided to investigate potential intracellular signalling events involved in FHA mediated cytokine production. The FHA used contained 17 pg/ $\mu$ g of LPS. It was therefore decided to use BMDCs from C3H/HEJ mice to rule out any contribution of contaminating LPS to observed signalling events. The BMDCs were incubated with FHA for 5, 10, 20, 30 min, 1, 3, 6, 9 or 12 hrs. Cells were then lysed and western blotting was performed using antibodies specific for the relevant signalling molecules. FHA induced phosphorylation of ERK which was clearly evident from 20 min-1 hr and was then slightly evident at 9 hrs [Fig 4.3]. p-p38 was induced over a similar



timeframe from 10min-3hrs with the strongest induction from 20 min- 1 hr [Fig 4.3]. c-fos induction was observed from 20min-3hrs and again at 12hrs [Fig 4.3]. I $\kappa$ B degradation occurred from 20 min-1 hr with total restoration of I $\kappa$ B evident at 6 hr [Fig 4.3].

#### **4.2.3 FHA mediated IL-10 production is both p38 and TLR4 dependent in BMDCs.**

Having already shown that FHA induces IL-10 and IL-6 production and the activation of ERK, p38, c-fos and NF- $\kappa$ B in DCs, inhibitors of ERK (U0126), p38 (SB203580) and NF- $\kappa$ B (BAY 11-7082) were used to investigate the role of the induced signalling molecules in FHA mediated cytokine production. DCs from both C3H/HeJ and C3H/HeN mice were incubated with FHA for 6 hours in the presence or absence of the inhibitors. The ERK and NF- $\kappa$ B inhibitors had no significant effect on FHA induced IL-1, IL-6 and IL-10 production [Fig 4.4]. IL-10 production was only evident in the DCs from the C3H/HeN mice [Fig 4.4], suggesting that TLR4 is essential for FHA mediated IL-10 induction. The SB203580 inhibitor completely ablated this IL-10 production demonstrating that the p38 MAP kinase signalling pathway mediates IL-10 production. Inhibition of p38 also led to an increase in IL-1 production [Fig 3.4]. The fact that IL-1 production increases as FHA induced IL-10 production was ablated suggests that although IL-1 is induced by FHA, the cytokine may be inhibited by FHA induced IL-10.

#### **4.2.4 FHA induces IL-10 production by peritoneal exudate cells from C3H/HeJ mice.**

The data in Fig 4.4 shows that DCs from C3H/HeJ mice are unable to produce IL-10 when incubated with FHA. It was therefore decided to investigate the effect of FHA on PEC from C3H/HeJ mice *in vitro*. PEC from both C3H/HeJ and C3H/HeN mice were incubated with increasing concentrations of FHA and supernatants were removed after 6hrs for cytokine analysis. IL-10 production was evident in both the C3H/HeJ and C3H/HeN derived cells at

all three concentrations [Fig 4.5] which contrasts with the effects of FHA on BMDCs. The concentrations of IL-10 produced by peritoneal exudate cells from C3H/HeJ mice were slightly lower than those produced by the peritoneal exudate cells from C3H/HeN cells [Fig 4.5], suggesting that the low levels of LPS contamination may enhance FHA induced IL-10 production. The data also shows that FHA induced IL-10 production was at a maximum when the PEC were incubated with 0.4 µg/ml of FHA with little or no difference in production of the cytokine between the lowest and the highest concentration [Fig 4.5]. In order to investigate the concentrations of FHA required for IL-10 induction in DCs, BMDCs from C57BL/6 mice were stimulated with increasing concentrations (0-10,000 ng/ml) of FHA for 24 hours and IL-10 production was quantified by ELISA [Fig 4.6A]. High levels of IL-10 were induced by 10 µg/ml of FHA, but IL-10 was undetectable on stimulation with lower concentrations of FHA [Fig 4.6A]. This suggests that the concentration required to induce IL-10 production by FHA differs greatly between immune cell types.

#### **4.2.5 FHA inhibits CpG induced IL-12p70 and IL-12p40 in BMDCs from both C3H/HeJ and C3H/HeN mice.**

McGuirk *et al* found that FHA suppresses LPS induced IL-12 in macrophages and dendritic cells.(227, 294) This inhibition was shown to be IL-10 dependent (227, 294). In order to rule out the possibility that the inhibition was a result of tolerance to contaminating LPS that may have been present in FHA it was decided to investigate whether FHA could inhibit IL-12p40 and IL-12p70 production in BMDCs from C3H/HeJ mice. C3H/HeJ and C3H/HeN BMDCs were incubated with FHA (0.1 µg/ml, 1 µg/ml and 10 µg/ml), CpG or FHA and CpG. CpG induced IL-12p40 and IL-12p70 production by DCs from both C3H/HeJ and C3H/HeN mice [Fig 4.7]. The highest concentration of FHA (10 µg/ml) inhibited CpG induced IL-12p40 and IL-12p70 production by DC from C3H/HeJ and C3H/HeN mice.[Fig

4.7] The two lower concentrations of FHA (0.1 µg/ml and 1 µg/ml) enhanced CpG induced IL-12p70 and IL-23 [Fig 4.7]. This indicates that at lower concentrations FHA synergises with CpG in the induction of the pro-inflammatory cytokines IL-12p40 and IL-12p70.

#### **4.2.6 FHA inhibits LPS mediated IL-23 production.**

The TLR agonist LPS induces IL-23, a cytokine that plays a crucial role in the survival and expansion of the Th17 cell type (183). Th17 cells developed using TGF-β and IL-6 required IL-23 during restimulation to maintain IL-17 production (133). Th17 cells play a role in the immune pathology of organ specific autoimmune and chronic inflammatory diseases such as EAE and colitis (138, 384, 385). FHA attenuates the clinical symptoms of colitis in a mouse model of disease (298). It was therefore decided to examine the effect of FHA on the production of IL-23 and whether this was a potential mechanism for the protective effect of FHA. BMDCs from C57BL/6 mice were pre-incubated for one hour with increasing concentrations (0-10,000 ng/ml) of FHA and then incubated with LPS (100 ng/ml) for 24 hours. LPS induced IL-23 decreased with the increasing concentrations of FHA [Fig 4.6]. However significant inhibition of LPS induced IL-23 was only detected with the highest dose of FHA (10 µg/ml) [Fig 4.6].

#### **4.2.7 FHA induces maturation of C3H/HeJ Dendritic Cells.**

The incubation of FHA with BMDCs leads to the induction of intracellular signalling events and resulting cytokine production. Therefore it was decided to examine the effects of FHA on co-stimulatory molecule expression on DCs. It has been previously reported that FHA induces a semi-mature phenotype with enhanced levels of CD86, moderately enhanced CD40 and no effect on CD80 or CCR5 expression (227). DCs from C3H/HeJ were used to rule out any effects of contaminating LPS. The BMDCs were incubated with medium, FHA or CpG.

After 24hrs, the cells were labelled with antibodies for CD40, CD80, CD86 and CCR5 and flow cytometry was performed. CD80, CD86 and CD40 were enhanced by both FHA and CpG [Fig 3.8], whereas expression of CCR5 was reduced by both FHA and CpG. [Fig 3.8] This indicates that FHA induces the maturation of DCs.

#### **4.2.8 FHA induces Treg cells specific for a co-administered antigen *in vivo*.**

This study established that FHA can modulate dendritic cell function, inducing IL-10, IL-6 and IL-1 production and maturation of DC. It was decided to investigate the influence of FHA treated DC on T cell induction. It had been demonstrated *in vitro* that the treatment of DCs with FHA enhanced their ability to prime T cells that produced high levels of IL-10 and IL-5 and low levels of IL-4 and IFN- $\gamma$  (227). It was therefore decided to investigate whether FHA could function as an adjuvant for the induction of Tr1 cells *in vivo*. C57BL/6 mice were immunized subcutaneously with PBS, KLH or KLH and FHA on days 0 and 21. After 28 days, the inguinal nodes were removed and restimulated with KLH *in vitro*. It was found that immunization of mice with KLH and FHA promoted the induction of antigen specific T cells that produced very high levels of IL-10 in response to *in vitro* restimulation with KLH [Fig 4.9]. Cells from mice immunized with KLH and FHA did not produce IL-17, IL-13 or IFN- $\gamma$  on restimulation *ex vivo* with KLH. The data indicates that FHA selectively promotes the induction of an antigen specific Treg cell population.

#### **4.2.9 A high dose of FHA is required for the induction of Treg cells *in vivo*.**

Having established the adjuvant activity of FHA, an experiment was performed to investigate the optimum dose of FHA for the induction of Treg cells *in vivo*. C57BL/6 mice were injected s.c with PBS, KLH or KLH and three different doses of FHA (0.2, 1 and 5  $\mu$ g). After 7 days the spleens were removed and the KLH specific response was assayed. It was

found that the spleen cells from mice injected with KLH and 5  $\mu$ g of FHA produced significant concentrations of IL-10 and low levels of IFN- $\gamma$  [Fig 4.10]. KLH specific T cells from mice immunized with lower doses of FHA and KLH, produced low concentrations of IFN- $\gamma$  and no IL-10 [Fig 4.10]. There was no significant differences in the levels of antigen specific IFN- $\gamma$ , between the different doses of FHA [Fig 4.10]. KLH specific IL-17 was not detected at significant levels for any of the FHA doses used [Fig 4.10].

#### **4.2.10 A comparison of the effect of CpG and FHA on T cell subtype induction *in vivo*.**

The experiments described in Fig 4.9 and Fig 4.10 suggest that FHA induces T cells that produce high levels of IL-10, low levels of IFN- $\gamma$  and little or no Th2 or Th17 cytokines. In order to clarify that CD4<sup>+</sup> cells produced IL-10, intracellular cytokine staining was performed on cells taken from mice immunized with KLH and FHA. Cells from mice that had been immunized with KLH and CpG were included as controls. C57BL/6 mice were injected s.c. into the footpad with KLH and FHA or KLH and CpG. The popliteal lymph nodes were removed after 7 days and the lymph node cells were stimulated with antigen. After 6 days the cells were re-stimulated with PMA and ionomycin and intracellular flow cytometry was performed for the cytokines IL-10, IFN- $\gamma$  and IL-17 combined with surface staining for CD4. The results shown in Fig 4.11 are from cells gated on CD4 and demonstrate that FHA induces a higher percentage of IL-10 producing T cells than CpG. In contrast, CpG induced a higher percentage of IFN- $\gamma$  producing CD4<sup>+</sup> T cells and higher percentage of IL-17 producing T cells [Fig 4.11]. Immunization with KLH and FHA or CpG induced a substantial population of T cells that produced both IL-10 and IFN- $\gamma$ , whereas IFN- $\gamma$  and IL-17 were produced by distinct T cell subpopulations [Fig 4.11].

#### **4.2.11 FHA induces Treg cells that inhibit Th1 cells.**

Lavelle *et al* have previously shown that like FHA, CT promotes the generation of Treg cells against a bystander antigen and that these Treg cells suppressed IFN- $\gamma$  production by Th1 cells (112). It was therefore decided to investigate whether the Treg cells induced by FHA were capable of inhibiting Th1 cells. FHA has also been shown to attenuate colitis, a disease in which IL-17 plays a major role in pathogenesis (384, 385). We therefore decided to examine the potential suppressive effect of the Treg cells induced by FHA on Th17 cells. KLH specific Th1 and Th17 cell lines were developed from mice that had been immunized with KLH and LPS. Lymph node and spleen cells taken from the immunized mice were restimulated with KLH and IL-12 or IL-23 respectively. KLH specific Treg cell lines were developed from mice that had been immunized with KLH and FHA. Lymph node and spleen cells taken from these mice were restimulated with KLH, IL-10 and anti-IL-12, anti-IFN- $\gamma$  and anti-IL-4. 15 days into the development of the T cell lines, the supernatants were taken and analyzed for cytokine analysis. The Th1 cell line produced IFN- $\gamma$  and low levels of IL-10 and IL-4 [Fig 4.12]. The Th17 cell line produced large amounts of IL-17 and IL-10 [Fig 4.12]. The Treg cell line produced high levels of IL-10 but little or no IL-4, IFN- $\gamma$  and IL-17 [Fig 4.12]. On the initiation of the third round of antigen stimulation a suppression experiment was performed, where the FHA induced Treg cells were incubated at three different ratios 1:3, 1:1, 3:1 with the Th1 cell line. Unfortunately the Th17 cell line had ceased to proliferate or produce IL-17 and was therefore excluded from the experiment. At the two higher ratios of Treg to Th1 (1:1 and 3:1), the IL-10 producing cell line generated by immunization with antigen in the presence of FHA inhibited IFN- $\gamma$  and IL-4 production by the Th1 cell line and also suppressed the proliferation of the Th1 cells [Fig 4.13]. IL-10 production by the Treg cell line was reduced on co-incubation with the Th1 cell line [Fig

4.13]. There appears to be a correlation between the production of IL-10 and the inhibition of the Th1 cell line, which suggests that inhibition may be IL-10 dependent [Fig 4.13].

#### **4.2.12 Treg cells generated from mice immunized with FHA inhibit the proliferation and partially suppress IL-17 production by Th17 cells.**

KLH specific Th1 and Th17 cell lines were developed from mice that had been immunized with KLH and LPS. Lymph node and spleen cells taken from the immunized mice were restimulated with KLH and IL-12 or IL-23 respectively. A separate Th17 line (Th17-10) was also developed which included an anti-IL-10 antibody at the initiation of the culture. 4 days after the first round of antigen stimulation supernatants were removed for cytokine analysis. The Th1 cell line produced high concentrations of IFN- $\gamma$  and IL-10 [Fig 4.14]. The Th17 cell line produced high concentrations of IL-17 and IL-10 [Fig 4.14]. The Th17-10 cell line produced high concentrations of IL-17 and increased concentrations of IFN- $\gamma$  and IL-4 when compared with the Th17 cell line incubated in the absence of the anti-IL-10 antibody [Fig 4.14]. This suggests that IL-10 may have a role in the inhibition of Th1 and Th2 cytokine production by Th17 cells.

Lymph node and spleen cells from the KLH and FHA injected mice were used to develop two Treg cell lines. The first Treg cell line [Treg(A)] was developed by the restimulation of the cells with KLH only. The second [Treg(B)] was developed by an initial stimulation with KLH in the presence of IL-10 and neutralizing antibodies against IL-12, IFN- $\gamma$  and IL-4. There was little difference in the cytokine profiles of the two Treg cell lines. The Treg (B) line produced no IL-4 and slightly higher levels of IL-10 than the Treg (A) cell line [Fig 4.14]. The Treg (A) produced low levels of IL-4 and slightly higher levels of IFN- $\gamma$  when compared with the Treg (B) cells [Fig 4.14]. At the initiation of the second round of antigen

stimulation a mixing experiment was performed. The Th1 cell line ceased to proliferate and was therefore excluded from the experiment. The two Th17 cell lines were cultured with the two Treg cell lines at the ratios 1:3, 1:1 and 3:1 in the presence or absence of IL-23 and the proliferation was assayed after 3 days. The absence of IL-23 did not inhibit the proliferation of the Th17 cell line [Fig 4.15], suggesting that the cytokine is not required in this regard. Both Treg lines strongly inhibited the proliferation of the Th17 cell lines [Fig 4.15]. The inhibition of the Th17 cell lines by the Treg (B) cell line was highly significant with complete inhibition of proliferation of the Th17 cell lines evident even at the lowest ratio Treg:Th17 (1:3) [Fig 4.15]. The inhibition by the Treg (A) cell line, while still dramatic was less pronounced [Fig 4.15].

A second series of Th17 and Treg cell lines were developed from mice that had been immunized and boosted with KLH and LPS or KLH and FHA. At the initiation of the second round of antigen stimulation a mixing experiment was performed. Following co-incubation of the Treg cells with the Th17 cells at two different ratios 1:1 and 3:1 it was clear that the Treg cells inhibit IL-17 cytokine production by the Th17 cells [Fig 4.16]. The Th17 cells also produced large quantities of IFN- $\gamma$ , as well as IL-17 and this IFN- $\gamma$  was also inhibited by the Treg cell line [Fig 4.16]. Indeed the inhibition of IFN- $\gamma$  was more pronounced than the inhibition of IL-17 [Fig 4.16].

#### **4.2.13 Prophylactic immunization of mice with MOG and FHA attenuates the clinical symptoms of EAE.**

IL-10 producing Tr1 cells have been shown to attenuate the clinical signs of EAE, once they are activated by antigen at the site of inflammation (237). It was therefore decided to investigate the potential of FHA to promote auto antigen specific Tr1 cells that prevent the



development of EAE. In EAE, the autoantigen MOG is used to induce the encephalogenic T cells that result in the clinical symptoms of disease. Mice were immunized with MOG and FHA, KLH and FHA, MOG alone or PBS on days -21 and -7 before the induction of EAE. On day 0, EAE was induced and the clinical scores were examined daily. The mice that had been immunized with MOG, KLH and FHA or PBS all developed severe EAE [Fig 4.17]. Mice that had been immunized with MOG and FHA developed a mild form of EAE [Fig 4.17]. These mice had an average clinical score of less than 1 which corresponds to a limp tail [Fig 4.17]. This suggests that FHA induces MOG specific Tr1 cells that attenuate the development of EAE.

**4.2.14 A MOG specific T cell line developed from EAE mice that had been pre-immunized with MOG and FHA produced more antigen specific IL-10 and less IL-17 when compared to a T cell line developed from EAE mice that had been pre-immunized with PBS.**

Immunization with FHA and MOG attenuates the development of EAE and KLH specific Treg cells generated from mice immunized with KLH and FHA have been shown to inhibit Th17 cells. In order to further study the ability of FHA induced Treg cells to attenuate EAE, T cell lines were established from mice with EAE that had been pre-immunized with MOG and FHA or PBS. The T cell line developed from the mice that were pre-immunized with PBS produced moderate levels of IL-17, high levels of IFN- $\gamma$  and low levels of IL-10 [Fig 4.18]. The T cell line developed from mice that were pre-immunized with MOG and FHA produced high levels of IL-10 and IFN- $\gamma$  [Fig 4.18]. Levels of IL-17 were significantly reduced when compared with the PBS cell line. This supports the hypothesis that FHA attenuates EAE by the induction of MOG-specific Treg cells, which inhibit pathogenic MOG-specific Th17 cells.

### 4.3 Discussion

TLR agonists, such as LPS and CpG, induce the production of IL-12 from DCs, which leads to the differentiation of IFN- $\gamma$  producing Th1 cells. This report demonstrates that FHA does not induce IL-12 production by DCs but inhibits CpG induced IL-12p40 and IL-12p70 and stimulates the production of IL-1, IL-6 and IL-10 by DCs. C3H/HeJ (TLR4 defective) mice were used to rule out the role of contaminating LPS in the ability of FHA to induce cytokine production. FHA induced identical amounts of IL-6 and IL-1 production by DCs from both C3H/HeJ and C3H/HeN mice. However, FHA induced IL-10 production was only evident in DCs from C3H/HeN mice. The LPS contamination of the FHA preparation used was 17 pg/ $\mu$ g, which is equivalent to 85 pg/ml of LPS at the concentration of FHA employed. It has previously been shown that this quantity of LPS alone is not sufficient to induce IL-10 production from DCs (112). It is therefore possible that like other pathogen derived immunomodulators, such as CT and CyaA, FHA synergises with LPS to induce an increase in IL-10 production (112, 261).

TLR ligation leads to IL-10 production from innate immune cells and this has been shown to promote the induction of Treg cells which serve as a mechanism for controlling immune responses that may be detrimental to the host. Higgins *et al* showed that C3H/HeJ mice infected with *B. pertussis*, had significantly reduced *B. pertussis* specific Treg cells, exhibited more severe pathological changes and had a prolonged course of infection when compared with wild type mice (244). Pathogens have evolved mechanisms to subvert immune responses and FHA may promote persistence of *B. pertussis* by enhancing TLR agonist induced IL-10.

Another potential explanation for the role of TLR4 in FHA induced IL-10 is that FHA may interact directly with TLR4. The binding of FHA to TLR4 may be necessary to stimulate signalling for IL-10 production. FHA is a very large molecule-220kDa and has already been shown to bind to many eukaryotic proteins and receptors including CD11b/CD18 and CD47/CD61 (268, 287, 288, 290, 386). The immunomodulator ES-62, which is secreted by the filarial nematode *Acanthocheilonema viteae* has been shown to modulate innate immune cell function by a TLR4 dependent pathway (14). This suggests that an interaction between FHA and TLR4 is plausible.

This report shows that FHA stimulates the activation of ERK, p38 and NF- $\kappa$ B in DC. It was decided to use inhibitors of the signalling molecules to establish their possible role in FHA mediated cytokine production. Inhibitors of ERK and NF- $\kappa$ B had no effect on IL-1, IL-6 or IL-10 production from DCs by C3H/HeJ or C3H/HeN mice. The p38 inhibitor did not have an effect on IL-6 production. However, the treatment of DCs from C3H/HeN mice with the p38 inhibitor completely abrogated FHA induced IL-10 production. This finding suggests that the induction of IL-10 by FHA is both TLR4 and p38 dependent. Interestingly, the inhibition of p38 activation enhanced FHA induced IL-1 production by DCs from C3H/HeN mice. This suggests that FHA may inhibit the production of the pro-inflammatory cytokine IL-1 by the induction of IL-10 production. This supports the hypothesis that FHA redirects TLR signalling towards the induction of anti-inflammatory cytokines via activation of p38. The activation of DC with FHA in the presence of the p38 inhibitor leads to a more classical TLR induced cytokine profile. An alternative explanation for the increase in IL-1 production seen on treatment of the DC with the p38 inhibitor, is that FHA may inhibit IL-1 production through p38 signalling and as a consequence lead to an increase in IL-10 production.

Inhibition of p38 signalling could therefore lead to the induction of IL-1 and a corresponding inhibition of IL-10.

Several studies have reported that p38 MAP kinase is involved in the production of pro-inflammatory cytokines such as IL-1, IL-6, TNF- $\alpha$  and IL-12, whereas the activation of ERK MAP kinase suppresses the production of the pro-inflammatory cytokines IL-12 and IL-23 (33, 37, 46). There is however growing evidence that p38 can be responsible for the induction of the anti-inflammatory cytokine IL-10. It has been reported that in human macrophages the transcription factor Sp1 is responsible for p38 mediated induction of IL-10 (51). Further work will be necessary to elucidate the mechanisms downstream of p38 that are responsible for FHA induced IL-10.

It has been reported that FHA activates NF- $\kappa$ B in epithelial cells (286). The binding of the RGD motif within FHA to the VLA-5 integrin on epithelial cells, results in I $\kappa$ B degradation and the upregulation of ICAM-1. Ishibashi *et al* showed that FHA upregulated p50, p65 and c-Rel expression in epithelial cells (286). ICAM-1 is also expressed on dendritic cells where it plays a role in cell-cell interaction

This study also examined the effect of FHA on PEC from C3H/HeJ and C3H/HeN mice. FHA was found to induce IL-10 production by PEC from both C3H/HeJ and C3H/HeN mice. However, the concentrations of IL-10 induced by FHA were slightly less in the PEC from the C3H/HeJ mice than those seen in PEC from C3H/HeN mice. This difference in FHA induced IL-10 between the peritoneal exudate cells from C3H/HeJ and C3H/HeN mice could be attributed to contaminating LPS. Nevertheless, FHA still induced significant IL-10 by PEC from C3H/HeJ mice. PEC are composed of a variety of different cell types that include

macrophages, DCs and T and B cells. Although macrophages are the major cell type and the likely source of the IL-10, further experiments will need to be performed using sorted cell types and an intracellular stain for IL-10.

The identification of the receptor(s) involved in the modulation of innate immune cells by FHA is still unclear. FHA has been shown to bind to a number of receptors, including CD47/CD61, CD11b/CD18, heparan sulphate glycosaminoglycan and lactosylceramide (281, 288, 290). The regions of FHA involved in receptor interactions were elucidated by the use of monoclonal antibodies against various epitopes within the FHA molecule and by the use of recombinant fusion proteins corresponding to fragments of the FHA molecule. Similar techniques could be utilized to establish the region of FHA involved in immunomodulation. The interaction of the RGD motif within FHA and VLA-5 within CD47/CD61 has received considerable attention. A study by Boschwitz et al suggests that the RGD motif is not involved in the modulation of the immune response by FHA. They showed that the inhibition of T cell proliferation, by monocytes that had been infected with wild type *B. pertussis* is comparable with a mutant of *B. pertussis* which expressed a mutant FHA protein where the glycine in the RGD site has been replaced with an alanine (293).

McGuirk *et al* previously showed that FHA mediated suppression of IL-12p40 and IL-12p70 was IL-10 dependent (227, 294). It was therefore decided to investigate the effect of FHA on IL-12 production by DCs from C3H/HeJ mice. The highest concentration of FHA examined (10 µg/ml) inhibited CpG induced IL-12p40 and IL-12p70 from BMDCs from C3H/HeJ and C3H/HeN mice. This suggests that the induction of IL-10 by FHA may not be necessary for the inhibition of IL-12. Indeed, the ability of CyaA to inhibit LPS induced IL-12 production in DCs has been shown to be independent of IL-10 production (116). At lower

concentrations of FHA (0.1 µg/ml and 1 µg/ml), it synergised with CpG to produce higher levels of IL-12p70 than was observed with CpG alone. This suggests that *in vitro*, a high concentration of FHA is required to inhibit IL-12p70 production. Mathur *et al* have reported that CD40 can initiate counteracting immune responses depending on the strength of the signal in macrophages (concentration of a CD40 antibody) (61). A weak CD40 signal resulted in ERK dependent IL-10 production whereas a stronger signal induced p38 dependent IL-12 production (61). A similar mechanism may explain the induction of IL-12 with low doses of FHA and the inhibition of IL-12 at higher doses of FHA.

There are many potential mechanisms for the inhibition of IL-12 by FHA. A fundamental problem with their elucidation is that many of the signalling pathways used by TLR agonists in the induction of IL-12 may also be utilized by FHA. The use of inhibitors is therefore difficult to interpret. Goodridge *et al* have reported a role for ERK MAP kinase in the suppression of LPS induced IL-12p40 in macrophages. A calcium influx activates ERK which in turn suppresses the synthesis of IRF-1 (46). IRF-1 is involved in the transcription of IL-12p40. Sutterwala *et al* have shown that ligation of complement receptors leads to an extracellular calcium influx which inhibits LPS induced IL-12 production (296). Luo *et al* reported that the natural ligand for CR3 iC3b, induces low levels of IL-10 and inhibits IL-12p70 in human monocyte derived DCs (387). The authors also showed that the inhibition of IL-12p70 by iC3b was ERK dependent (387). The ability of FHA to bind to the CR3 suggests that the bacterial molecule may mimic the receptors natural ligand iC3b. Preliminary studies however indicate that this may not be correct as FHA does not induce an extracellular calcium influx (294). Further studies will be necessary to fully exclude a role for FHA-CR3 binding and ERK activation in the inhibition of TLR agonist induced IL-12p40 and IL-12p70 by FHA.

FHA induced c-fos expression in DCs, a signalling molecule with a confirmed role in the suppression of IL-12. The TLR2 agonist Pam3Cys inhibits IL-12 production by the activation of ERK MAP kinase which results in the stabilization of c-fos (45). The Th2 stimulus schistosome egg antigen (SEA) inhibits IL-12 by the same mechanism (45). Interestingly the inhibition of c-fos expression using siRNA interference led to IL-12p70 production from Pam3Cys and SEA treated DCs (45). The present study shows that FHA induced both p-ERK and c-fos. However the induction of the more stable, phosphorylated c-fos is more significant in the inhibition of IL-12 (45). A study must therefore be conducted into the ability of FHA to induce phosphorylated c-fos.

IL-23 is a cytokine that plays a crucial role in the survival and pathogenicity of Th17 cells (133, 136), which play a crucial role in the development of organ specific autoimmune diseases such as colitis, a disease which FHA has been shown to attenuate (298, 385). It was therefore decided to examine the effect of FHA on the production of IL-23 and whether this was a potential mechanism for the attenuation of colitis. Increasing concentrations of FHA led to the inhibition of LPS induced IL-23. There did not appear to be a correlation between IL-10 production and IL-23 inhibition. IL-23 production was reduced on incubation with 100 ng/ml of FHA, a concentration that failed to induce any IL-10. This suggests that the inhibition of IL-23 may be independent of IL-10 production.

McGuirk *et al* have previously shown that FHA treated DCs induce Treg cells *in vitro* (227). The authors also reported that FHA treated DC have a semi-mature phenotype, characterised by enhanced levels of CD86 and moderately enhanced CD40. It was reported that FHA had no effect on CD80 or CCR5. To rule out the role of contaminating LPS, an experiment was

carried out using cells from C3H/HeJ mice to examine the role of FHA on surface marker expression in DCs. The treatment of DCs from C3H/HeJ mice with FHA enhanced the expression of CD80, CD86, CD40 and decreased the expression of CCR5. The cells treated with FHA had a higher state of maturation than DCs treated with CpG. Initial reports suggested that immature DCs optimally induce Tr1 cells. However a recent study conducted by Roncarolo and colleagues identify a population of DCs in human blood that display a mature myeloid phenotype and are more potent inducers of Tr1 cells than immature DCs (195).

FHA specific Treg cells have been cloned from the lungs of *B. pertussis* infected mice (227). FHA can also modulate DCs to induce Treg cells *in vitro* (227). It was therefore decided to investigate the potential of FHA to act as an adjuvant for the model antigen KLH *in vivo*. Lymph node cells from mice immunized with KLH and FHA produced very high levels of IL-10, low levels of IFN- $\gamma$  and no IL-17 or IL-13 in response to *ex vivo* restimulation with KLH. This indicated that as an adjuvant FHA directs a regulatory immune response against a co-administered antigen. Significantly FHA did not promote the induction of IL-17 producing T cells.

Intracellular staining confirmed that the immunization of mice with antigen in the presence of FHA promotes the induction of CD4<sup>+</sup> T cells that produced IL-10, IL-10 and IFN- $\gamma$  and IFN- $\gamma$  only. The frequency of IL-10 producing CD4<sup>+</sup> T cells from mice immunized with antigen and FHA was higher than that seen in mice immunized with KLH and CpG. The reverse was the case for IFN- $\gamma$  production with a higher frequency of CD4<sup>+</sup> T cells in mice immunized with antigen in the presence of CpG than in mice that had been immunized with antigen and FHA.



It has been shown that DCs stimulated with CpG in the presence of CD4<sup>+</sup>CD25<sup>+</sup> natural regulatory T cells promotes the induction of Th17 cells (133). A higher percentage of Th17 cells were detected in the lymph node cells from mice immunized with KLH and CpG than cells from mice immunized with KLH and FHA. Consistent with the findings of Veldhoen *et al*, CD4<sup>+</sup> T cells from mice immunized with antigen and CpG form distinct populations of IFN- $\gamma$  or IL-17 producing T cells, with very few cells producing both cytokines (133).

This study has demonstrated that FHA can act as adjuvant in the induction of a population of T cells that have a Treg cell phenotype. T cell lines developed from mice immunized with FHA produced high levels of IL-10, low levels of IL-4 and no IL-17 or IFN- $\gamma$ . In contrast, Th1 cell lines generated from mice immunized with KLH and LPS produced high levels of IFN- $\gamma$  and the Th17 cell line developed from the same mice produced high levels of IL-17. Interestingly Th17 cell lines also produced high levels of IL-10. This is consistent with a report by Vanden Eijnden *et al* which showed that IL-23 enhanced the secretion of IL-17 and IL-10 from activated human CD4<sup>+</sup> and CD8<sup>+</sup> T cells (388). However it contradicts a recent study by McGeachy *et al*, who reported that Th17 cells restimulated in the presence of TGF- $\beta$  and IL-6 produce IL-17 and IL-10 and that Th17 cells that are restimulated in the presence of IL-23 only produced IL-17 and no IL-10. Addition of an anti-IL-10 to Th17 cell lines led to an increase in IL-4 and IFN- $\gamma$  production. This suggests that the induction of IL-10 by IL-23 may be a mechanism to inhibit the development of Th1 and Th2 cells, thereby allowing the further induction and proliferation of Th17 cells. Th1 and Th2 cells have been shown to inhibit the induction of Th17 cells and there are conflicting reports suggesting that IL-4 and IFN- $\gamma$  may inhibit Th17 cells (151, 173).

Treg cells developed from mice that were immunized with KLH and FHA were capable of inhibiting IFN- $\gamma$  production and the proliferation of Th1 cells. This finding demonstrated that FHA has the ability to induce Treg cells that are specific for a co-administered antigen and capable of suppressing pro-inflammatory effector T cell responses *in vitro*. The levels of IL-10 produced by the Treg cells were reduced following co culture with Th1 cells. This may reflect consumption of IL-10 following the addition of the Th1 cells and suggests that inhibition of Th1 response may be IL-10 dependent.

Treg cell lines developed from mice immunized with KLH and FHA were shown to inhibit the proliferation and partially suppress IL-17 production by Th17 cells. The Th17 cell line also produced very high levels of IFN- $\gamma$  and this was also inhibited following co-incubation with the Treg cells. Moreover, the inhibition of IFN- $\gamma$  was far more pronounced than the inhibition of IL-17 production, suggesting that Treg cells induced under the influence of FHA may be more effective at the inhibition of Th1 cells. The production of IFN- $\gamma$  by Th17 cell types has been addressed in a study by Mathur *et al* who demonstrated that initial antigen stimulation in the presence of IL-23 leads to IL-17 production by T cells. However, a second antigen stimulation in the presence of IL-23 resulted in the upregulation of the transcription factor T-bet and the secretion of IFN- $\gamma$ , thus the Th17 cells had progressed towards a Th1 cell phenotype. If Th17 cells are re-cultured in IL-23 without antigen, the cells continued to produce IL-17 and IFN- $\gamma$  production was not enhanced. The inhibition of Th17 cell proliferation by IL-10 producing Treg cells developed from mice immunized with KLH and FHA, complements the findings by Bettelli *et al*, who reported that Foxp3<sup>+</sup> T cells inhibit proliferation of Th17 cells *in vitro* (134).

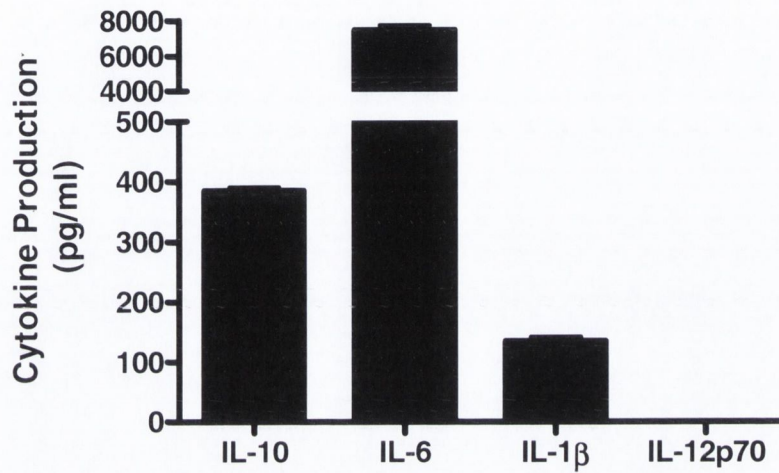
The therapeutic potential of the immunomodulator FHA against autoimmunity was demonstrated through finding that the prophylactic immunization of mice with MOG and FHA attenuated the development of EAE. Significantly, FHA was only protective when co-administered with the auto-antigen MOG. This suggests that FHA promotes the induction of MOG specific Treg cells that attenuate inflammation in the CNS, leading to reduced clinical symptoms of EAE.

An examination of T cell responses in treated and untreated mice with EAE revealed that T cells lines developed from the MOG and FHA pre-immunized mice produced very high levels of IL-10 and lower levels of IL-17 when compared with the cells from the untreated mice. The reduction in IL-17 production by T cells from the mice immunized with MOG and FHA compared with controls was not very pronounced and was similar to the reduction in IL-17 production seen when Th17 cells were co-incubated with Treg cells *in vitro*. The IL-17 levels in both cases are reduced but not completely abrogated. This suggests that there may be other mechanisms by which Treg cells induced by FHA prevent the onset of autoimmune disease. Mononuclear cells such as neutrophils and macrophages have been identified as crucial mediators of the inflammation in the CNS during EAE (151). There is evidence that Treg cells can suppress the recruitment and activation of innate immune cells (389). The Treg cells induced by FHA may therefore inhibit the mononuclear cells that infiltrate into the CNS and lead to the pathology of EAE.

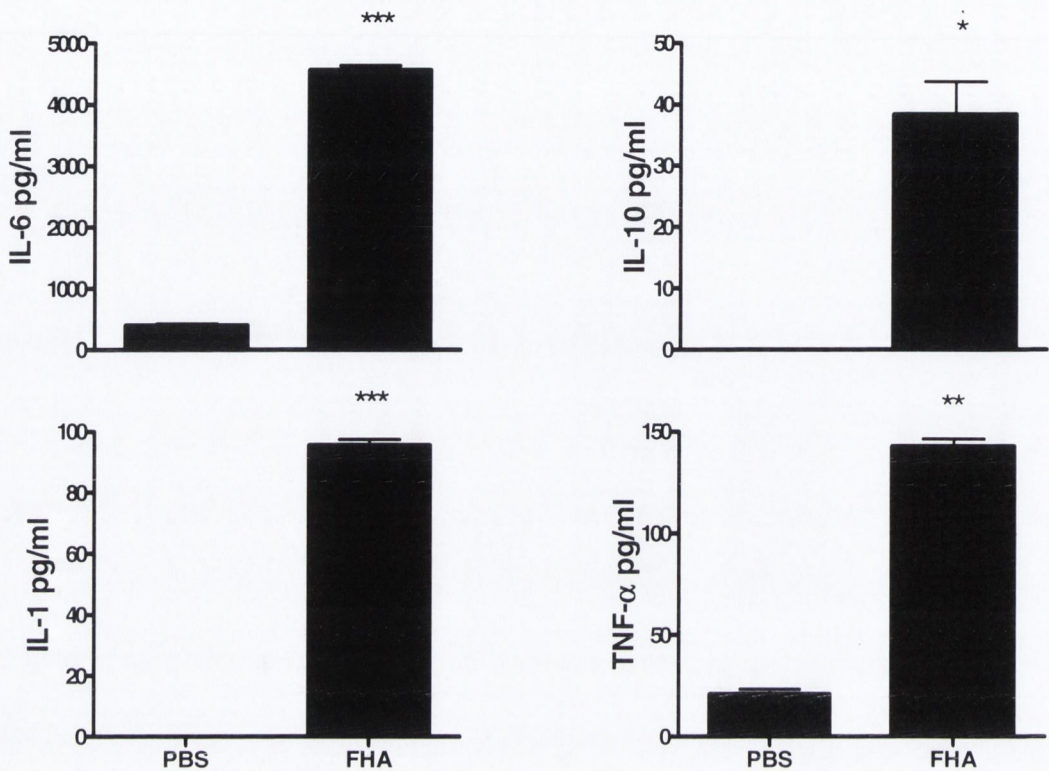
There have been a large number of studies in the last few years proposing that autoimmune diseases such as EAE are primarily driven by Th17 cells. Autoimmune diseases were originally considered to be Th1 diseases. Indeed, some reports even suggested that IFN- $\gamma$  inhibits the development of EAE (138). However, Kroenke *et al* have recently demonstrated

that myelin specific Th1 or Th17 cells can induce EAE (168). The disease induced by the T cell subtypes differs in the CNS chemokine expression patterns and the composition of infiltrating leukocytes (168). However the clinical signs of EAE induced by the Th1 or Th17 were indistinguishable (168). This study has important implications as it suggests that the inhibition of both Th1 and Th17 cells may be viable therapeutic approaches for the attenuation of autoimmune disease. Furthermore, the ability of FHA to potently suppress Th1 cells may partially explain its ability to attenuate EAE.

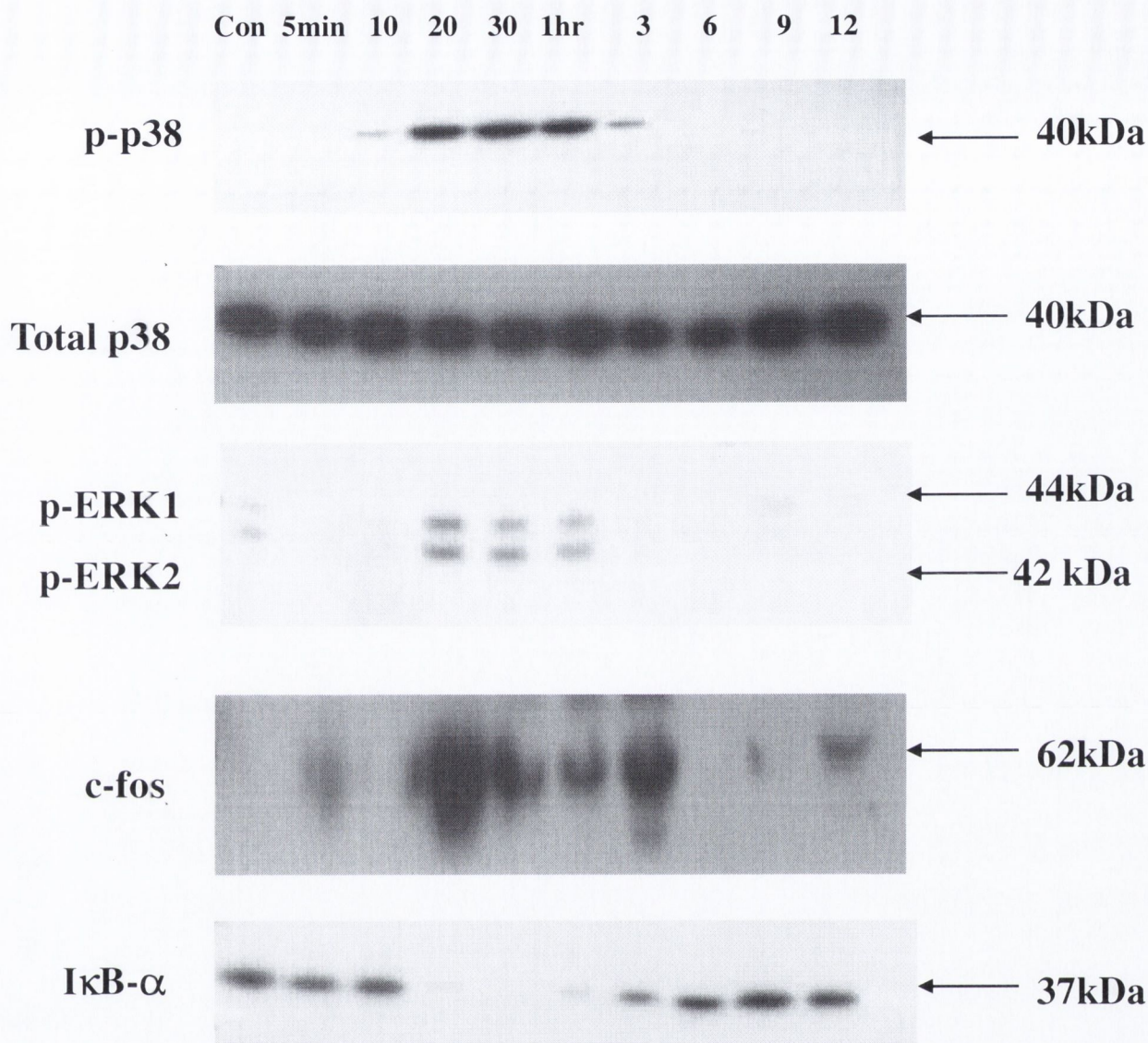
A more recent study by O'Connor *et al* proposed that myelin specific Th17 cell populations that lack IFN- $\gamma$ <sup>+</sup> cells do not induce EAE, whereas Th1 cell populations devoid of IL-17<sup>+</sup> cells are highly pathogenic (170). They also showed that only Th1 cells can access the CNS (170). They propose that once Th1 cells establish the inflammatory lesion, Th17 cells are recruited to the CNS (170). If confirmed, this finding has major consequences for the development of therapies for autoimmune disease. Immunomodulators administered prophylactically may inhibit Th1 cells, but not Th17 cells. Th17 cells would therefore, not gain access to the CNS and consequentially would not cause the inflammation that results in disease. However the therapeutic potential of such an immunomodulator would be limited as in clinical MS the blood brain has already been breached and Th17 cells play a major role in inflammation (166). It is therefore important that the protective effect of FHA is investigated in the relapsing-remitting model of EAE.



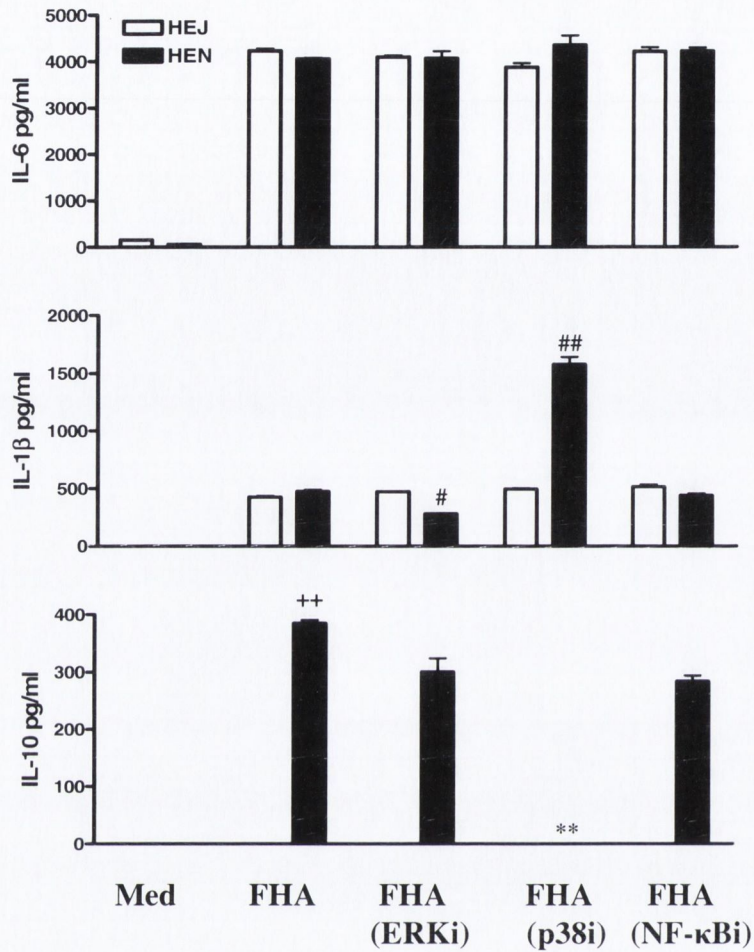
**Fig 4.1** FHA induces IL-10, IL-6 and IL-1 $\beta$  but no IL-12p70 production from bone marrow derived DCs (BMDCs). BMDCs ( $1 \times 10^6$  cells/ml) from BALB/c mice were incubated with FHA (10  $\mu$ g/ml). After 24 hours, supernatants were removed and the concentrations of IL-10, IL-6, IL-1 $\beta$  and IL-12p70 were quantified by ELISA. These results are representative of two experiments which were carried out in triplicate.



**Fig 4.2 In vivo administration of FHA induces IL-6, IL-10, IL-1 $\beta$  and TNF- $\alpha$  production by peritoneal exudate cells.** BALB/c mice were injected intraperitoneally with FHA (10  $\mu$ g) or PBS. Four hours later peritoneal lavage was performed and the peritoneal exudate cells ( $1 \times 10^6$  cells/ml) were cultured in medium. After 24 hours supernatants were removed and IL-6, IL-10, IL-1 $\beta$  and TNF- $\alpha$  concentrations were quantified by ELISA. \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$  versus peritoneal exudate cells from mice injected with PBS. These results are from one experiment. Each bar on the graphs represents three assay wells.

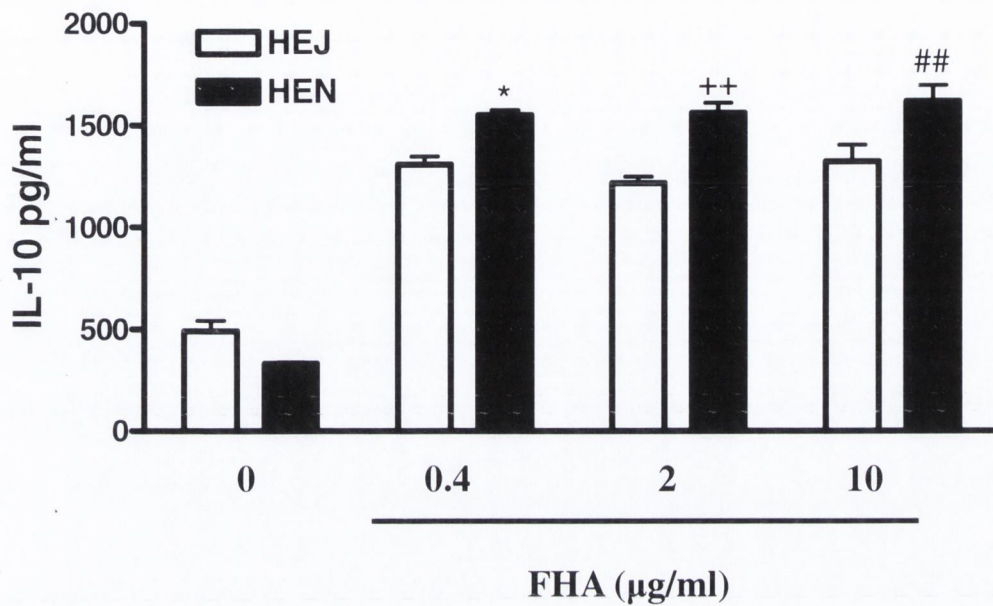


**Fig 4.3 FHA induces p-ERK, p-p38, c-fos and IκB degradation in BMDCs independent of TLR4.** BMDCs ( $1 \times 10^6$  cells/ml) from C3H/HeJ mice were incubated with FHA (5  $\mu$ g/ml) for 5, 10, 20 or 30 min, 1, 3, 6, 9 or 12 hrs. The incubation was terminated by the lysis of the cells with 1X SDS Sample Buffer. Western Blotting was performed on the cell lysates using antibodies specific for p-ERK, ERK, p-p38, p38, c-fos and IκB- $\alpha$ . These results are representative of two experiments.

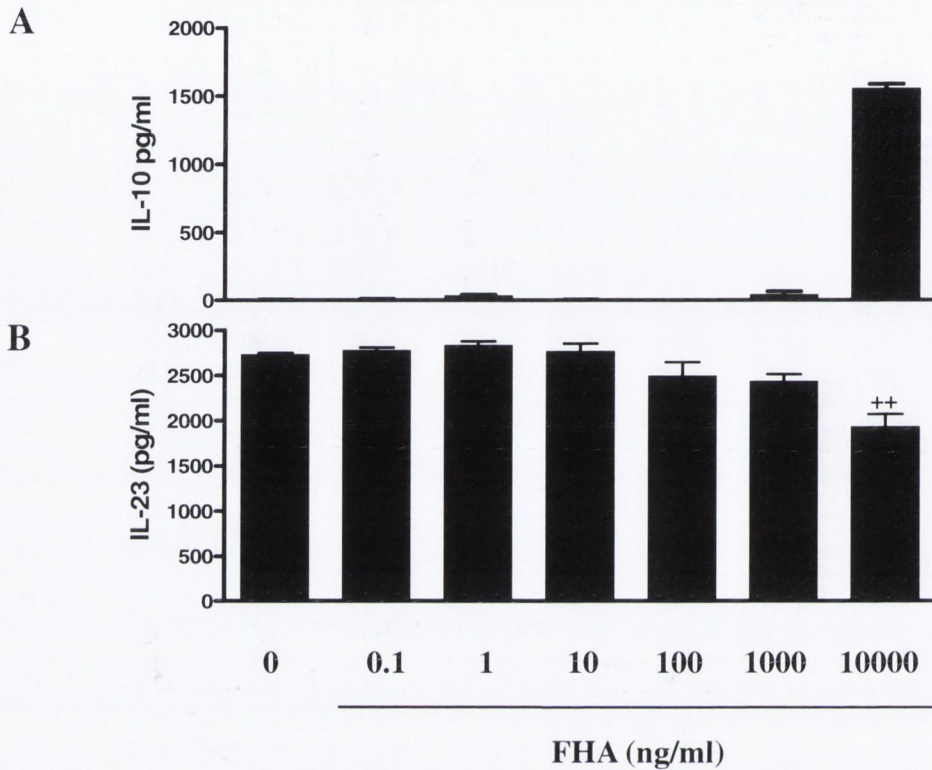


**Fig 4.4 FHA induced IL-6 and IL-1 $\beta$  production is TLR4 independent, but IL-10 production in DCs is both p38 and TLR4 dependent.** BMDCs ( $1 \times 10^6$  cells/ml) from C3H/HeJ and C3H/HeN mice were pre-incubated for 90 mins with the ERK inhibitor U0126 ( $5 \mu\text{M}$ ), the p38 inhibitor SB203580 ( $5 \mu\text{M}$ ) and the NF- $\kappa\text{B}$  inhibitor BAY 11-7082 ( $5 \mu\text{M}$ ). The cells were then incubated with FHA ( $5 \mu\text{g/ml}$ ) for 6 hrs. Supernatants were removed and IL-10, IL-1 $\beta$  and IL-6 concentrations were quantified by ELISA. ##  $P < 0.01$ , #  $P < 0.05$  versus DCs from C3H/HeN mice treated with FHA. ++  $P < 0.01$  versus DCs from C3H/HeJ mice treated with FHA. \*\*  $P < 0.01$  versus DCs from C3H/HeJ mice treated with FHA. These results are from one experiment which was carried out in triplicate.

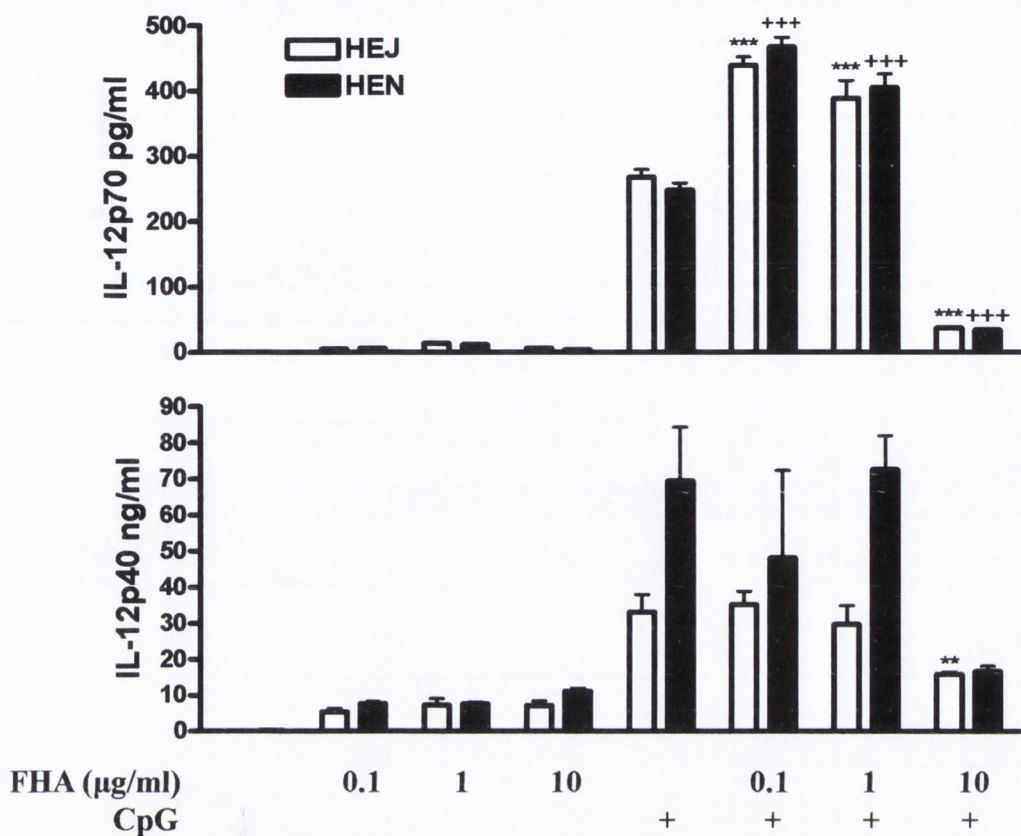




**Fig 4.5** FHA induces IL-10 production by peritoneal exudate cells from C3H/HeJ mice. Peritoneal exudate cells were obtained from C3H/HeJ and C3H/HeN mice by peritoneal lavage. The cells ( $1 \times 10^6$  cells/ml) were incubated with increasing concentrations of FHA (0.4 µg/ml-10 µg/ml). Supernatants were removed after 24 hours and the concentration of IL-10 was quantified by ELISA. \*  $P < 0.05$  versus cells from C3H/HeJ mice treated with FHA (0.4 µg/ml). \*\*  $P < 0.01$  versus cells from C3H/HeJ mice treated with FHA (2 µg/ml). ##  $P < 0.01$  versus cells from C3H/HeJ mice treated with FHA (10 µg/ml). These results are from one experiment which was carried out in triplicate



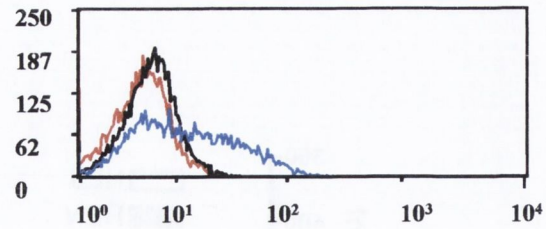
**Fig 4.6 Effect of FHA on LPS induced IL-23 production from DCs.** **A)** BMDCs ( $1 \times 10^6$  cells/ml) from C57BL/6 mice were incubated with increasing concentrations of FHA (0-10,000 ng/ml). Supernatants were removed and the concentration of IL-10 quantified by ELISA. \*\*\*  $P < 0.001$  versus DCs treated with medium alone. **B)** BMDCs ( $1 \times 10^6$  cells/ml) from C57BL/6 mice were also pre-incubated with FHA (0-10,000 ng/ml) for 1hr and LPS (100 ng/ml) was added. Supernatants were removed after 24hrs and the concentration of IL-23 was quantified by ELISA. ++  $P < 0.01$  versus DCs treated with LPS alone. These results are representative of two experiments which were carried out in triplicate.



**Fig 4.7** FHA inhibits CpG induced IL-12p70 and IL-12p40 in DCs from C3H/HeJ and C3H/HeN mice. BMDCs ( $1 \times 10^6$  cells/ml) from C3H/HeJ and C3H/HeN mice were incubated/co-incubated with FHA (0.1, 1 and 10  $\mu$ g/ml), CpG (10  $\mu$ g/ml), FHA and CpG or medium. Supernatants were removed after 24 hours and IL-12p40 and IL-12p70 concentrations were quantified by ELISA. \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$  versus DCs from C3H/HeJ mice treated with CpG alone. +++  $P < 0.001$  versus DCs from C3H/HeN mice treated with CpG alone. These results are from one experiment which was carried out in triplicate.

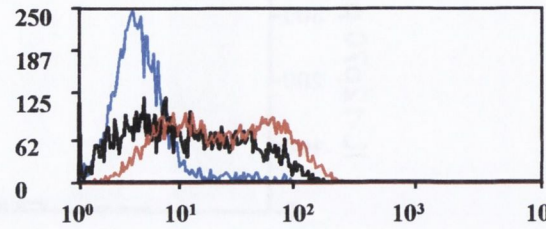
### CCR5 Log PE

Control — (MPF) = 57.05  
CpG — (MPF) = 107.99  
FHA — (MPF) = 161.30



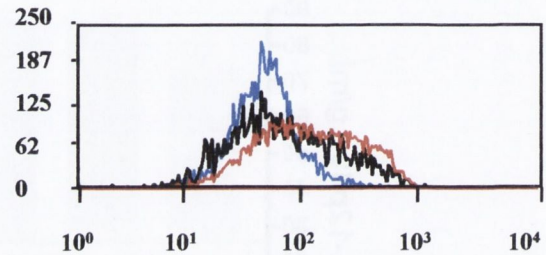
### CD86 Log PE

Control — (MPF) = 15.94  
CpG — (MPF) = 4.48  
FHA — (MPF) = 5.63



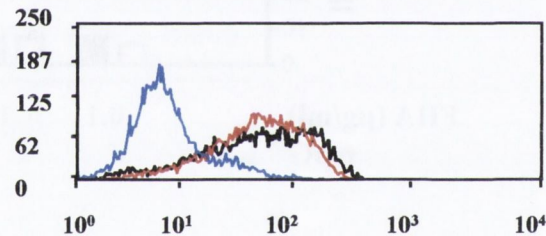
### CD80 Log FITC

Control — (MPF) = 5.45  
CpG — (MPF) = 15.82  
FHA — (MPF) = 28.85

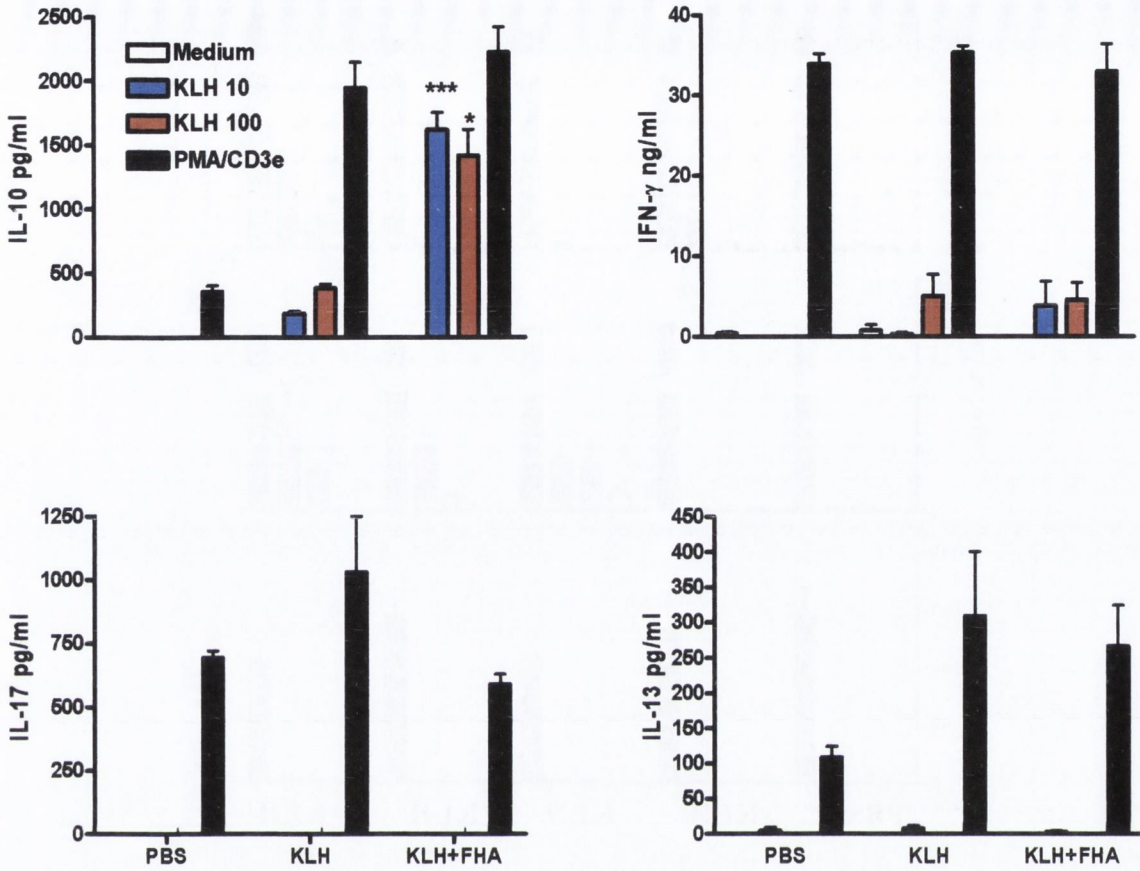


### CD40 Log FITC

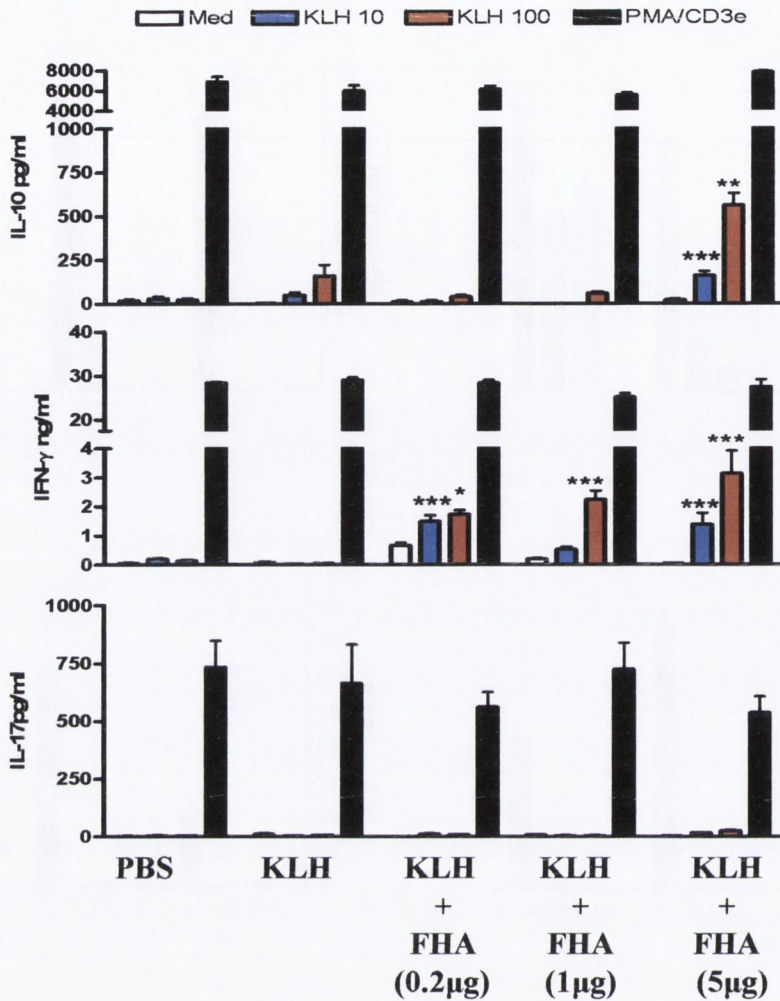
Control — (MPF) = 9.01  
CpG — (MPF) = 47.92  
FHA — (MPF) = 56.73



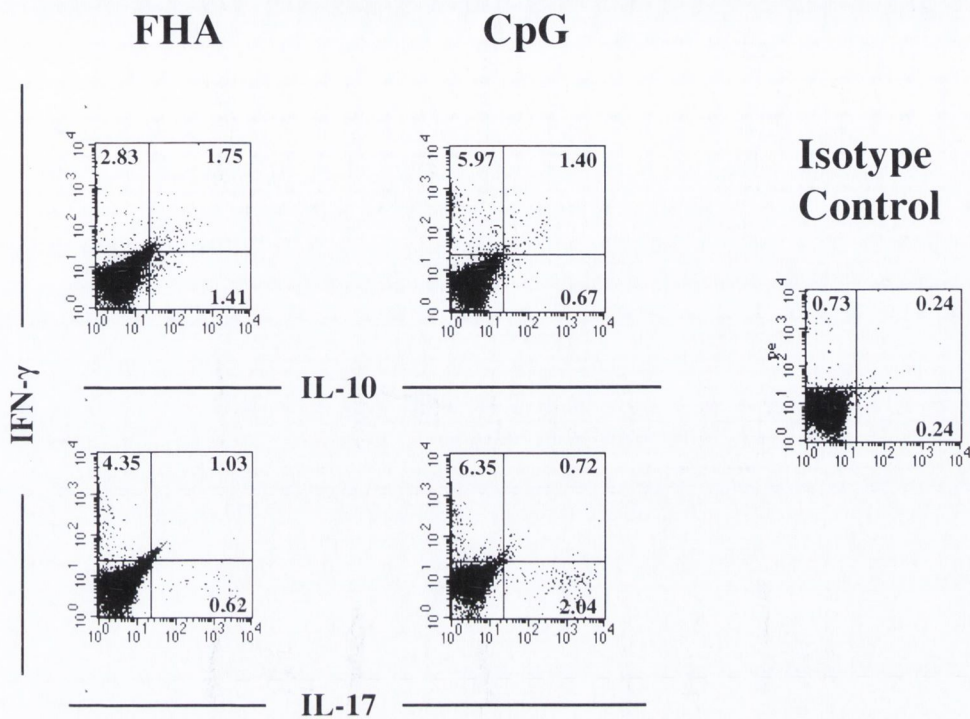
**Fig 4.8** FHA induces the maturation of DC from C3H/HeJ mice. BMDCs ( $1 \times 10^6$  cells/ml) from C3H/HeJ mice were incubated for 24 hours with FHA ( $5 \mu\text{g/ml}$ ), CpG ( $10 \mu\text{g/ml}$ ) or medium alone. The cells were washed and surface stained for CD80, CD86, CD40 and CCR5. Results from flow cytometric immunofluorescence analysis are shown. These results are representative of two experiments.



**Fig 4.9** FHA acts as an adjuvant for the selective induction of Treg cells. Groups of four C57BL/6 mice were immunised subcutaneously into the flank with PBS, KLH (10  $\mu$ g) or KLH (10  $\mu$ g) and FHA (10  $\mu$ g) and were boosted 21 days later. 7 days after the boost the mice were sacrificed, the inguinal nodes were harvested and re-stimulated with KLH (10 or 100  $\mu$ g/ml), PMA and anti-CD3e or medium. After 72 hrs the supernatants were removed and the concentrations of IL-10, IFN- $\gamma$ , IL-13 and IL-17 were quantified by ELISA. \*\*\*  $P < 0.001$ , \* $P < 0.05$  versus cells taken from mice immunized with KLH and restimulated with KLH. These results are representative of two experiments which were carried out in triplicate.



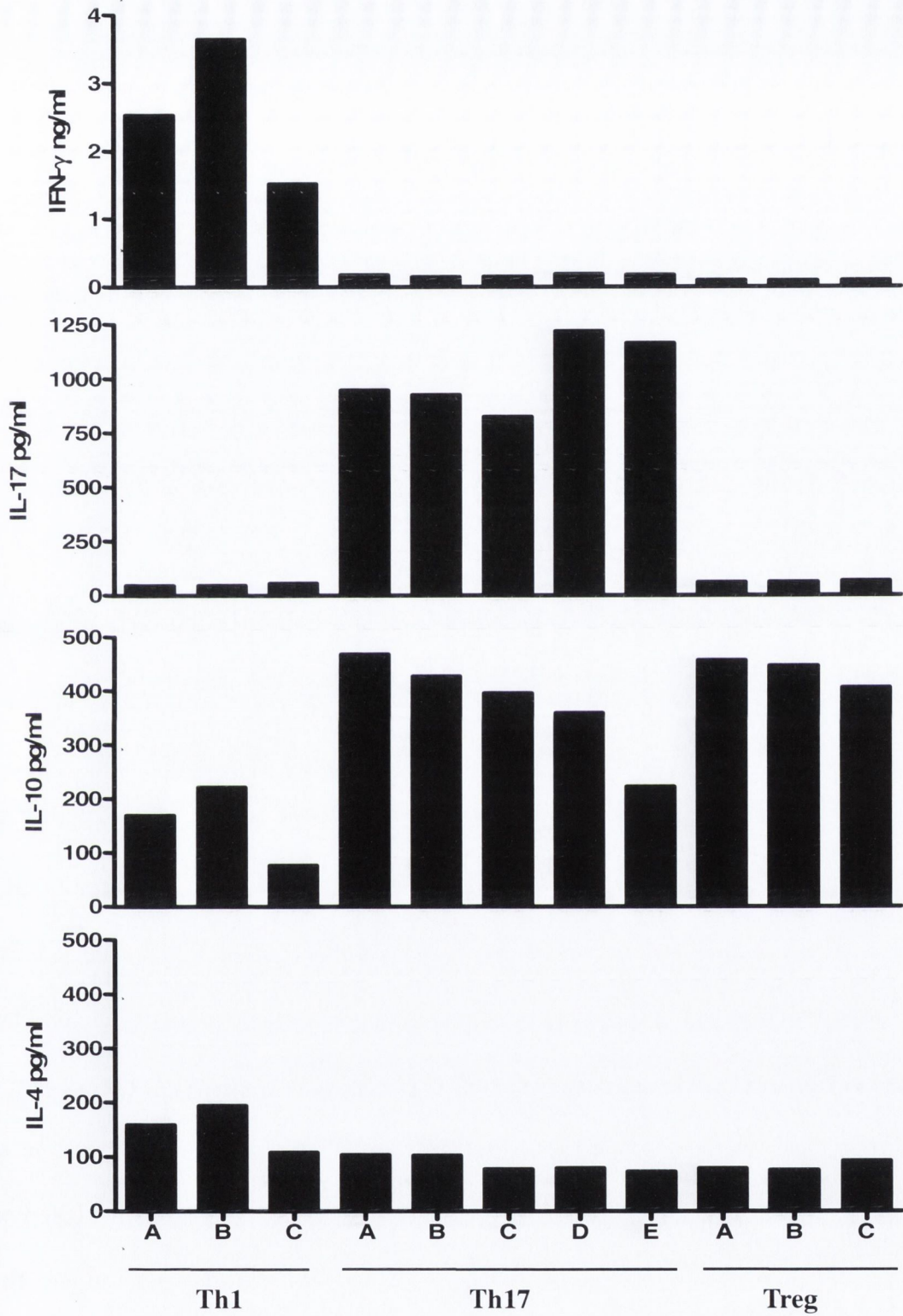
**Fig 4.10 A high dose of FHA is optimal for the induction of a Treg cell phenotype in vivo.** Groups of four C57BL/6 mice were immunised subcutaneously into the flank with PBS, KLH (10 μg), KLH (10 μg) and FHA (0.2 μg, 1 μg or 10 μg) and were boosted 21 days later. 7 days after the boost the mice were sacrificed, the spleens were harvested and re-stimulated with media, KLH (10 or 100 μg/ml) or PMA/CD3. After 72 hours IL-10, IFN-γ and IL-17 concentrations in the supernatants were quantified by ELISA. \*\*\* P<0.001, \*\* P<0.01, \* P<0.05 versus cells from mice immunized with KLH and restimulated with KLH. These results are from one experiment which was carried out in triplicate.

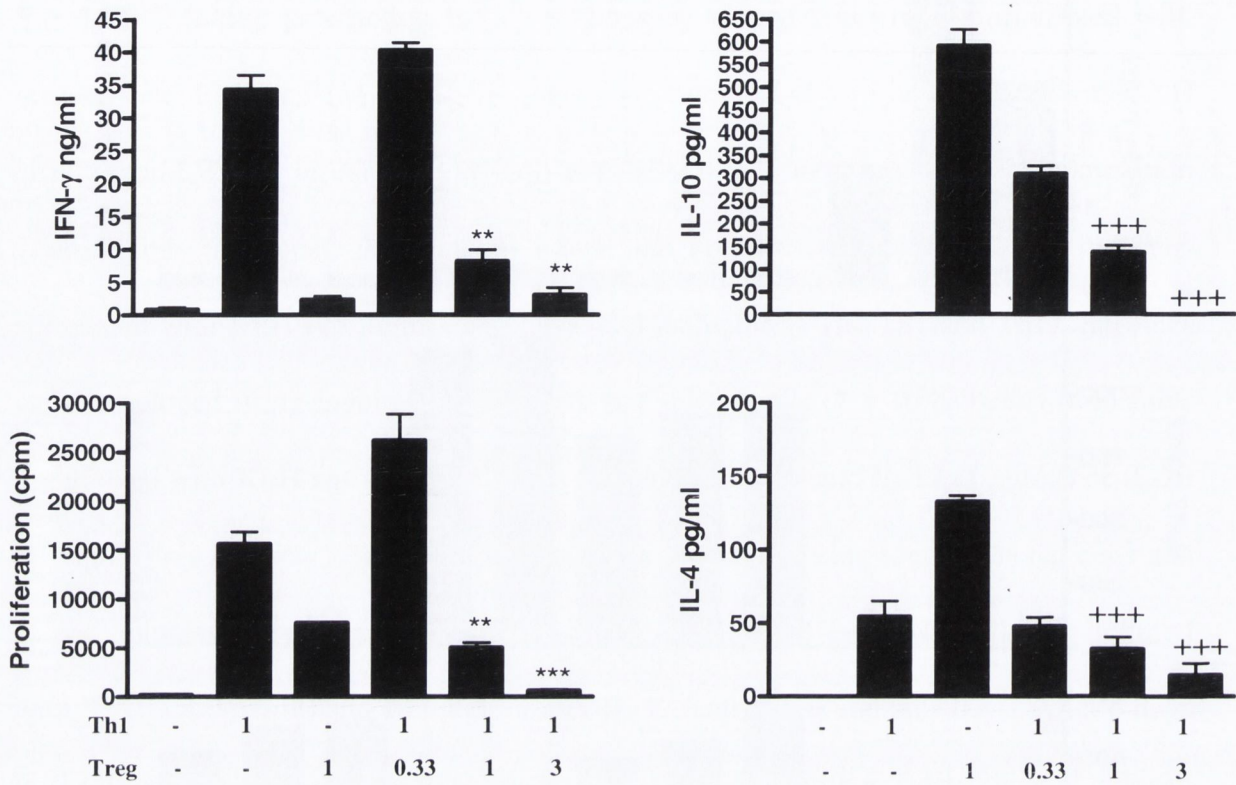


**Fig 4.11 Comparison of the effect of CpG and FHA on the induction of T cell subtypes *in vivo*.** Groups of 4 C57BL/6 mice were immunised s.c. into the footpad with KLH (10  $\mu$ g) and FHA (5  $\mu$ g) or KLH (10  $\mu$ g) and CpG (25  $\mu$ g). 7 days after immunization the popliteal lymph nodes were harvested and re-stimulated with KLH (100  $\mu$ g/ml) for 72 hrs and then incubated with IL-2 (10 units/ml) for a further 3 days. The cells were restimulated with PMA and ionomycin for 6hrs. The cells were washed and surface stained for CD4. The cells were then stained intracellularly staining with antibodies specific for IL-10, IFN- $\gamma$  and IL-17. Results from flow cytometric immunofluorescence analysis are shown. These results are from one experiment.

**Fig 4.12 Cytokine production by T cell lines developed from mice immunized with antigen and LPS or FHA.** BALB/c mice were immunized s.c into the flank with KLH (10 µg) and LPS (10 µg) or KLH (10 µg) and FHA(10 µg) and were boosted 21 days later. 7 days after the boost, the inguinal nodes and spleens were removed and the cells stimulated with KLH (25 µg/ml), cytokines and antibodies. The Th1 and Th17 cell lines were developed using inguinal nodal cells and spleen cells ( $2 \times 10^6$ cells/ml) from mice immunized with KLH and LPS that were cultured with KLH and IL-12 (1 ng/ml) or IL-23 (10 ng/ml) respectively. The Treg cell lines was developed using inguinal nodal cells and spleen cells ( $2 \times 10^6$ cells/ml) from mice immunized with KLH and FHA and were cultured with IL-10 (500 pg/ml) and 10 µg/ml of anti-IL-12, anti-IFN- $\gamma$  and anti-IL-4. After 4 days IL-2 (10 units/ml) was added to the cell cultures. After a further 7 days the cell cultures were re-stimulated ( $2 \times 10^5$ cells/ml), with irradiated APCs ( $2 \times 10^6$ cells/ml) and KLH (25 µg/ml). In the case of the Th17 cell line, IL-23 (10 ng/ml) was also added. 4 days later, supernatants were removed and IL-10, IL-17, IFN- $\gamma$  and IL-4 concentrations were quantified by ELISA. Each bar corresponds to an individual T cell line. These results are from one experiment.

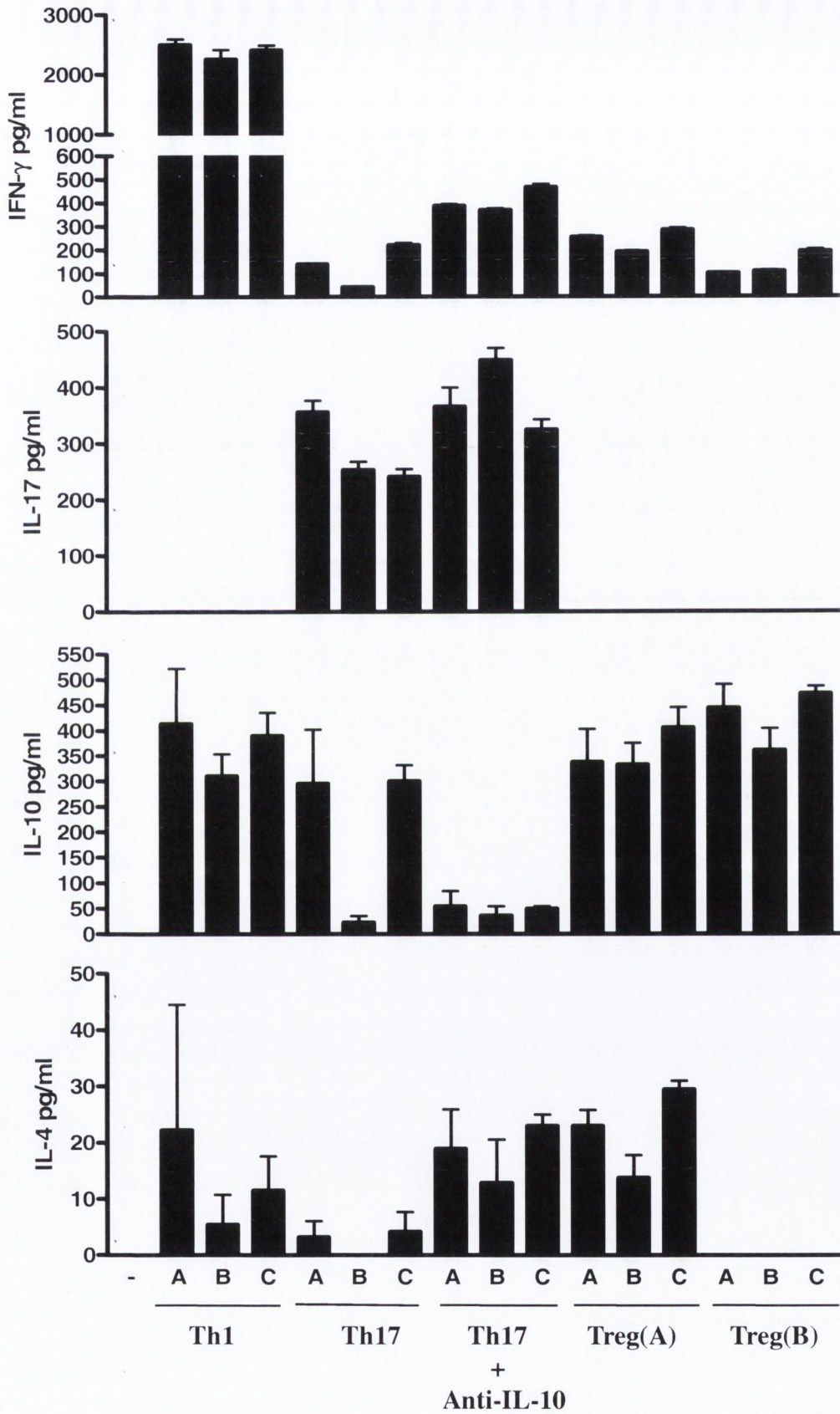


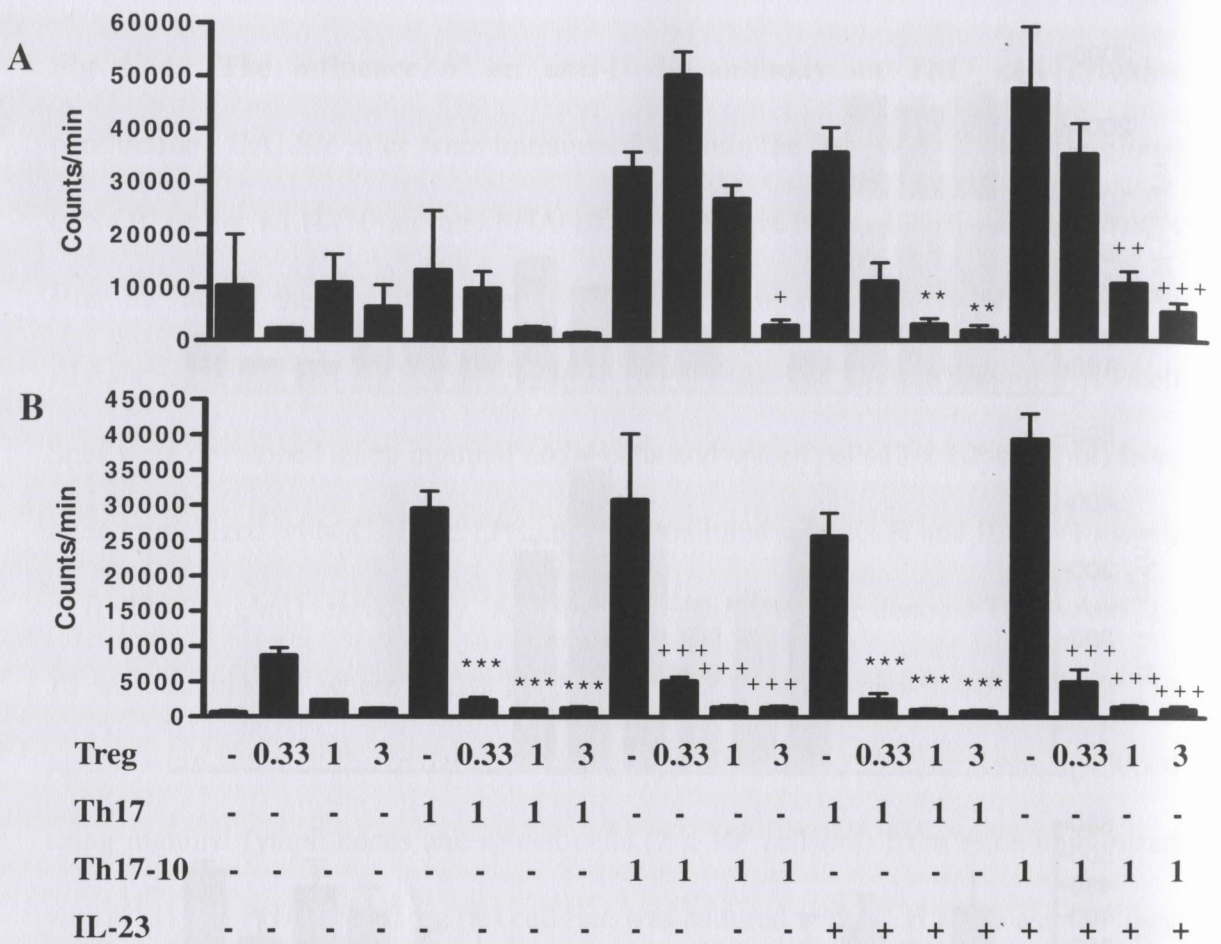




**Fig 4.13** FHA induced Treg cells inhibit IFN- $\gamma$  production and proliferation of Th1 cells. Th1 and Treg cell lines were established as shown in Fig 4.3. At the initiation of the third round of antigen stimulation with KLH (25  $\mu$ g/ml) and APCs (irradiated spleen cells,  $2 \times 10^6$  cells/ml), the Treg cell line ( $2 \times 10^5$  cells/ml) was co-incubated with the Th1 cell line ( $2 \times 10^5$  cells/ml) in a 3:1, 1:1 and 1:3. After 3 days, supernatants were removed and the concentrations of IFN- $\gamma$ , IL-10 and IL-4 were quantified by ELISA. Proliferation was measured by [ $^3$ H] incorporation. \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$  versus Th1 cells incubated with APCs and antigen. +++  $P < 0.001$  versus Treg cells incubated with APCs and antigen. These results are from one experiment which was carried out in triplicate. Each bar on the graphs represents three experimental conditions.

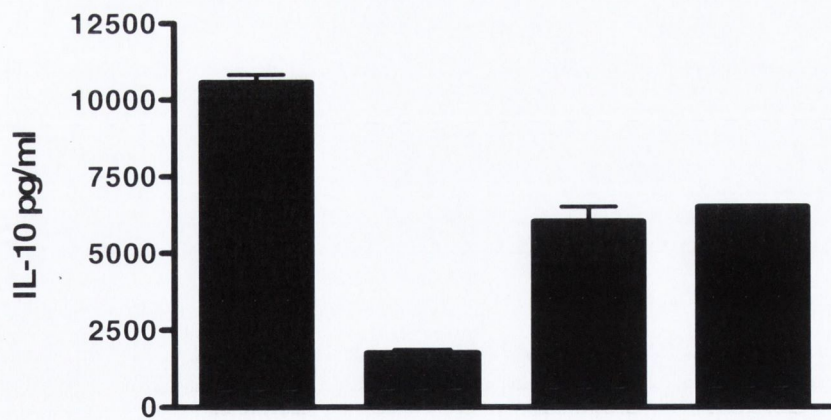
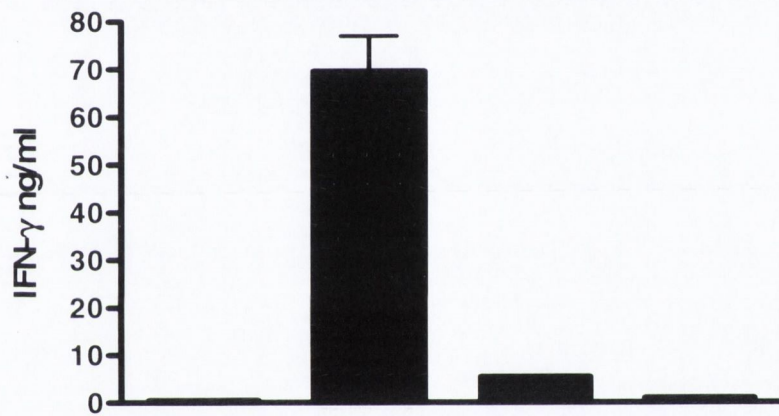
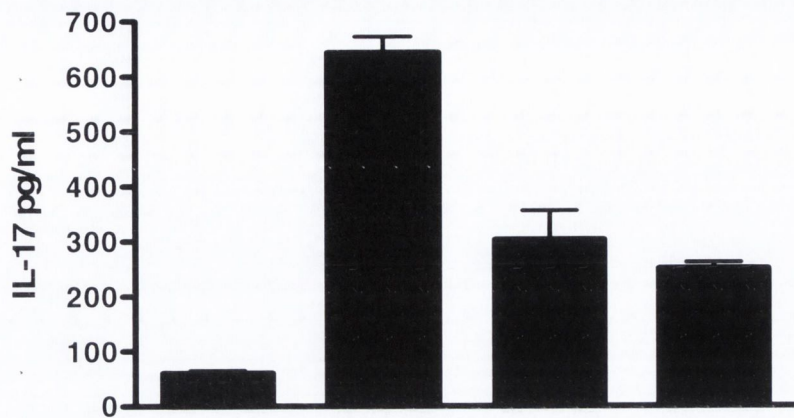
**Fig 4.14 The influence of an anti-IL-10 antibody on Th17 cell cytokine production.** BALB/c mice were immunized s.c. into the flank with KLH (10 µg) and LPS (10 µg) or KLH (10 µg) and FHA(10 µg) and were boosted 21 days later. 7 days after the boost, the inguinal nodes and spleens were removed and the cells were stimulated with KLH (25µg/ml), cytokines and antibodies. The Th1 and Th17 cell lines were developed using inguinal nodal cells and spleen cells ( $2 \times 10^6$  cells/ml) from mice immunized with KLH and LPS and were cultured with KLH and IL-12 (1 ng/ml) or IL-23 (10 ng/ml) respectively. An alternative Th17 cell line denoted Th17+Anti-IL-10 was established where KLH, IL-23 (10 ng/ml) and an anti-IL-10 antibody (10 µg/ml) were added at the initiation of the culture. The Treg cell lines were developed using inguinal lymph nodes and spleen cells ( $2 \times 10^6$  cells/ml) from mice immunized with KLH and FHA. The Treg (A) cell line was cultured with KLH alone and the Treg (B) cell line was cultured with KLH, IL-10 (500 pg/ml) and 10 µg/ml of anti-IL-12, anti-IFN- $\gamma$  and anti-IL-4 antibodies. After 4 days, supernatants were removed and IL-10, IL-17, IFN- $\gamma$  and IL-4 concentrations were quantified by ELISA. Each bar corresponds to an individual T cell line which was assayed in triplicate. These results are from one experiment.



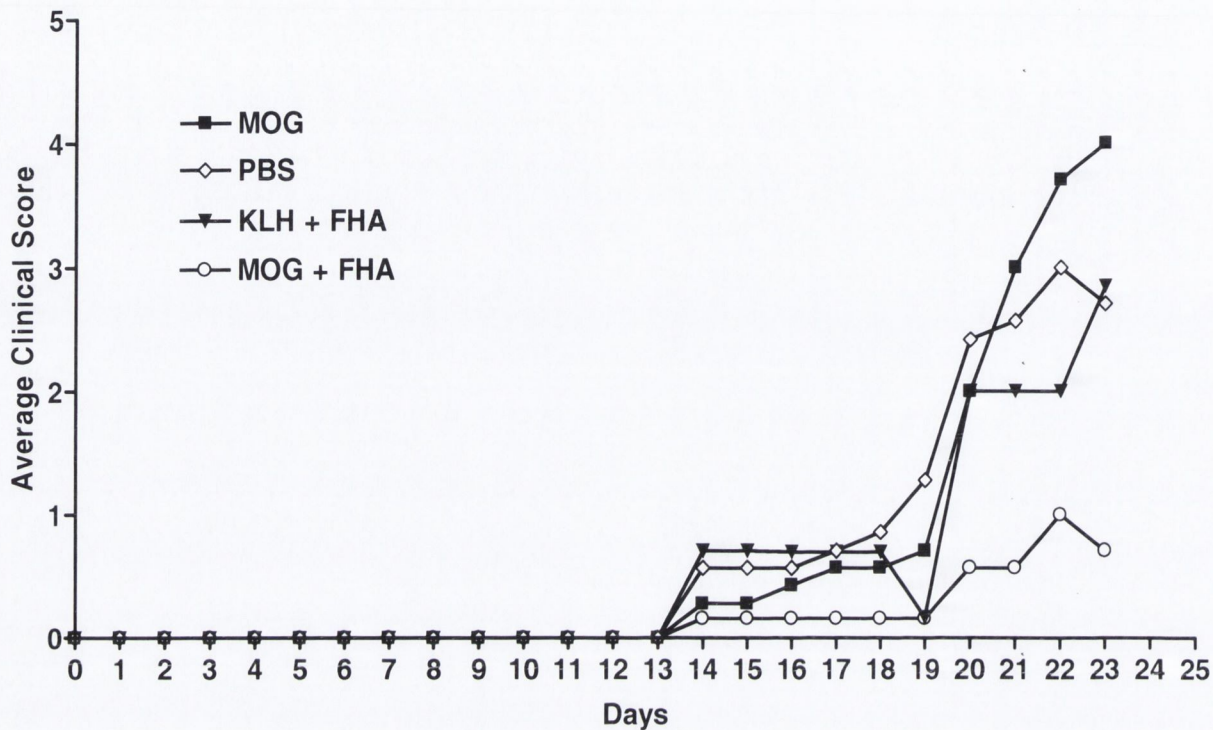


**Fig 4.15 Treg cells developed from mice immunized with antigen in the presence of FHA inhibit the proliferation of Th17 cells.** KLH specific Th17 and Treg cell lines were established as described in Fig 4.6 At the initiation of the second round of stimulation with KLH (25µg/ml) and irradiated spleen cells ( $2 \times 10^6$ cells/ml), the Treg cell lines were co-cultured with the Th17 cell lines ( $2 \times 10^5$ cells/ml) at ratios of 3:1, 1:1 and 1:3. The Th17 cell line established in the presence of the anti-IL-10 antibody is denoted as Th17-10. After 3 days proliferation was measured by [ $^3$ H] incorporation. A) Co-culture of the Treg (A) cell line with the Th17 cell lines. B) Co-culture of the Treg (B) cell line with the Th17 cell lines. IL-23 was added to certain cultures as indicated. \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$  versus Th17 cells incubated with APCs and antigen. +++  $P < 0.001$ , ++  $P < 0.01$ , +  $P < 0.05$  versus Th17-10 cells incubated with APCs and antigen. These results are from one experiment which was carried out in triplicate.

**Fig 4.16 Treg cells generated from mice immunized with FHA can inhibit cytokine production from a mixed Th1/Th17 cell line.** C57BL/6 mice were immunized s.c into the flank with KLH (10  $\mu$ g) and LPS (10  $\mu$ g) or KLH (10  $\mu$ g) and FHA(10  $\mu$ g) and boosted 21 days later. 7 days after the boost the mice were sacrificed and the inguinal nodes and spleens were removed. A mixed Th1/Th17 cell line was developed using inguinal node cells and spleen cells ( $2 \times 10^6$  cells/ml) from mice immunized with KLH and LPS and was cultured with KLH and IL-23 (10 ng/ml). The Treg cell line was developed using inguinal lymph node cells and spleen cells ( $2 \times 10^6$  cells/ml) from mice immunized with KLH and FHA and was cultured with KLH and IL-10 (500 pg/ml). At the initiation of the second round of antigen stimulation the Treg cell line was co-cultured with the Th17 cell line ( $2 \times 10^5$  cells/ml) at the ratios- 1:1 and 1:3 with KLH (25  $\mu$ g/ml) and APCs (irradiated spleen cells,  $2 \times 10^6$  cells/ml). After 3 days supernatants were removed and the concentrations of IFN- $\gamma$ , IL-10 and IL-17 were quantified by ELISA. These results are from one experiment which was carried out in triplicate.



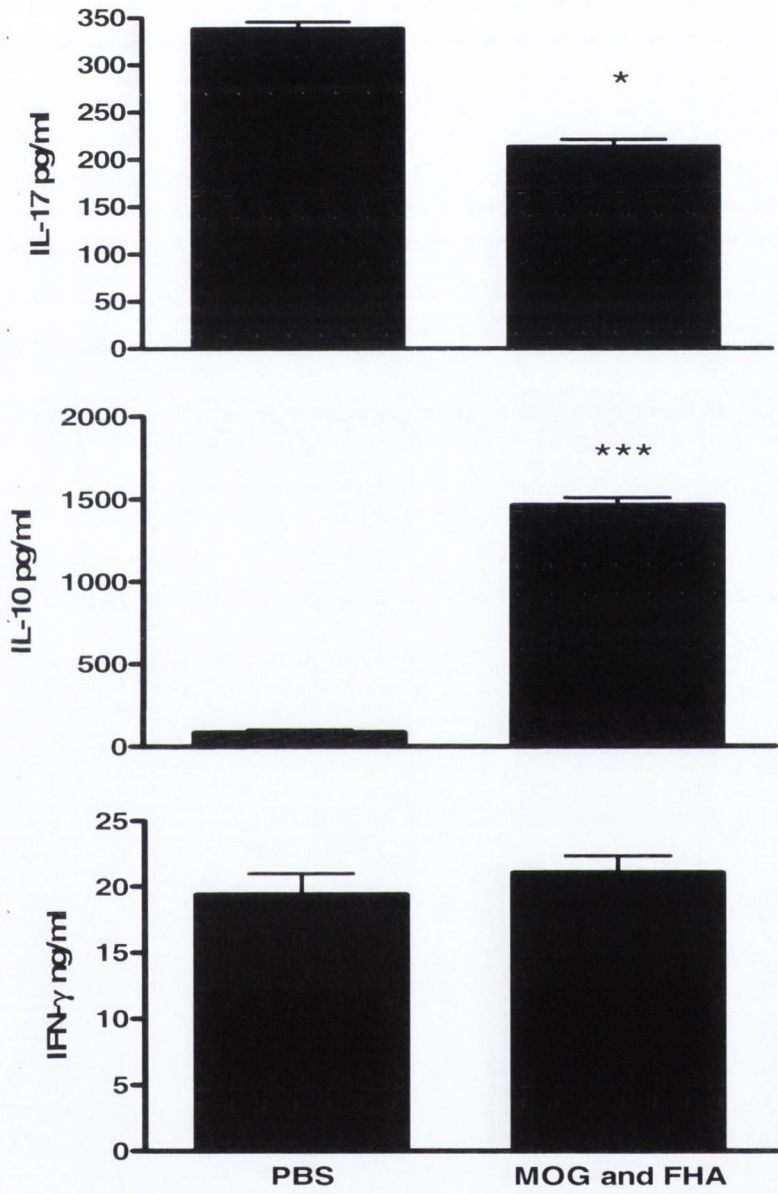
Th17	-	1	1	1
Treg	1	-	1	3



**Fig 4.17 Prophylactic immunization with MOG and FHA attenuates the clinical symptoms of EAE.** Groups of 6 C57BL/6 mice were immunized twice (on Day -21 and Day -7) s.c. into the flank with MOG<sub>35-55</sub> (50 µg) and FHA (5 µg), KLH (10 µg) and FHA, MOG alone or PBS. On day 0, EAE was induced with MOG (150 µg) in CFA, followed by injection i.p. With PT (500 ng) on days 0 and 2. Clinical scores were examined daily and the mice were sacrificed on day 23. This experiment was conducted by Dr. Brian Keogh and the graph shown is included in this thesis with his permission. The results are from one experiment.



**Fig 4.18 T cells from mice immunized with MOG and FHA prior to the induction of EAE produced more IL-10 and less IL-17 when compared with a T cells developed from untreated mice with EAE.** Groups of 6 C57BL/6 mice were immunized twice (on Day -21 and Day -7) s.c. into the flank with MOG (50 µg) and FHA (5 µg) or PBS. On day 0, EAE was induced with MOG, CFA and PT. After the clinical signs of severe EAE had appeared in the group of mice immunized with PBS, 14 days after the induction of EAE, the mice were sacrificed and the spleens were harvested. The spleen cells ( $2 \times 10^6$  cells/ml) from the mice immunized with PBS and the mice immunized with MOG and FHA were stimulated with MOG. After 4 days IL-2 (10 units/ml) was added and the cells were incubated for a further 4 days. The T cells ( $1 \times 10^5$  cells/ml) were then stimulated with MOG (25 µg/ml) and APCs (irradiated spleen cells,  $2 \times 10^6$  cells/ml). After 3 days supernatants were taken and the concentrations of IL-10, IL-17 and IFN- $\gamma$  were quantified by ELISA. \*\*\*  $P < 0.001$ , \*  $P < 0.05$  versus cells taken from mice suffering from severe EAE. These results are from one experiment which was carried out in triplicate.



# Chapter V

**Infection with  
*F. hepatica* attenuates  
EAE through TGF- $\beta$   
mediated suppression  
of Th1 and Th17  
responses**



## 5.1 Introduction

The parasitic trematode *F.hepatica* infects a wide variety of vertebrates. It is commonly associated with the infection of ruminants such as sheep and cattle. However, in parts of the developing world, it can also infect humans (303). The life cycle involves the infection of a vertebrate host and an intermediate host, which is a lymnaeid snail (304). The ingestion of encysted metacercariae initiates the infection of vertebrates. The metacercariae excyst in the duodenum of the host and penetrate the intestinal wall (304). The juvenile fluke then migrate across the peritoneal cavity to the liver (305). The juvenile helminths then spend 8-12 weeks feeding on the parenchyma cells before finally progressing to the bile duct where they reach sexual maturity (305).

The infected host initially mounts a Th2 response against the parasite, which is characterised by IL-4 production, eosinophilia and the production of IgG1 antibodies (306-308). After 10 days of liver fluke infection in cattle, bovine PBMCs produce IL-4 but not IFN- $\gamma$  in response to the parasite antigen ES (309). However, this Th2 response is ineffective at clearing the parasite, and within 12 weeks, a chronic infection develops. PBMCs from chronically infected cattle produce IL-10 and TGF- $\beta$  in response to liver fluke homogenate (LFH) (310). Interestingly the neutralization of TGF- $\beta$  enhanced parasite-specific IL-4 and IFN- $\gamma$  and non-specific IFN- $\gamma$  production by the PBMCs (310). This suggests that both IL-10 and TGF- $\beta$  play a key role in suppressing Th1 and Th2 immune responses against the parasite in cattle.

*F. hepatica* infection can activate bystander suppression of Th1 immune responses induced against other pathogens or following immunization. Cattle that are co-infected with *F. hepatica* and *M. bovis* exhibit reduced Th1 responses against the bacteria (311). Mice that were co-infected *F. hepatica* and *B.pertussis* had an increased bacterial burden in the lung

(312). Spleen cells from co-infected mice displayed a dramatically reduced IFN- $\gamma$  production against *B. pertussis* antigens, when compared with spleen cells from mice that had been infected with *B. pertussis* alone (312). *F. hepatica* infection also suppressed the development of a Th1 response induced with a whole cell pertussis vaccine (312). Interestingly, the inhibition of *B. pertussis* specific Th1 responses was reversed in *F. hepatica* infected IL-4<sup>-/-</sup> mice (312). This suggests that IL-4 plays a role in the suppression of Th1 responses by *F. hepatica*.

Studies have shown that parasite infection can also attenuate autoimmune and allergic immune responses in humans and in animal models of disease. A study conducted over a five year period in Argentina, reported that parasite infected patients with multiple sclerosis (MS) had a significantly reduced number of exacerbations and enlarging MS lesions compared with uninfected MS patients (330). PBMCs from the parasite infected patients produced significantly more IL-10 and TGF- $\beta$  and significantly less IL-12 and IFN- $\gamma$  in response to myelin basic protein when compared to PBMCs from non-infected patients (330). Myelin basic protein-specific T cell clones developed from parasite infected MS patients, produced high levels of IL-10 and TGF- $\beta$  and no IL-2 or IL-4 (330). Furthermore, the cloning frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells was higher in infected than in uninfected MS subjects (330). This suggests that regulatory T cells induced by a parasitic infection attenuate the course of disease in MS patients. A number of studies in animal models of autoimmune disease have confirmed that helminth infection reduces the severity of disease. *S. mansoni*, *T. spiralis* and *H. polygyrus* infections all prevent the onset of diabetes in NOD mice (331, 390). The infection of mice with *S. mansoni* has also been shown to prevent EAE and colitis (332, 335). Smith *et al* have reported that schistosomes infection results in a marked infiltration of F4/80<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>-</sup> macrophages into the colon lamina propria,

which protect the mice from DSS induced colitis (332). There are however, few other studies that demonstrate the mechanisms involved in the attenuation of autoimmune disease by helminth infection.

There has been more progress in studies elucidating the mechanisms of helminth infection mediated attenuation of allergic diseases in animal models. Wilson *et al* have reported that allergic airway inflammation is suppressed by *H. polygyrus* induced Treg cells. *H. polygyrus* infection was associated with significantly reduced levels of IL-5 and eotaxin in the bronchoalveolar lavage fluid of allergen sensitized mice (328). These two key cytokines are responsible for the mobilization and extravasation of the eosinophils involved in allergic inflammation (328). The administration of an anti-CD25 antibody to parasite infected OVA sensitized mice resulted in the restoration of airway infiltration (328). Furthermore, CD4<sup>+</sup>CD25<sup>+</sup> T cells from the mesenteric lymph nodes (MLN) of *H. polygyrus* infected mice transferred protection to uninfected mice (328). The authors concluded that CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells induced by helminth infection were associated with the suppression of allergic inflammation.

There are however some other points to note from this important study. CD4<sup>-</sup> cells from the MLN of infected mice also transferred protection to uninfected allergen sensitized animals (328). Furthermore, CD4<sup>+</sup> and CD4<sup>-</sup> cells from the MLN of IL-10<sup>-/-</sup> infected mice were also able to transfer protection to uninfected mice (328). These data suggest that CD4<sup>-</sup> cells also play a role in the inhibition of inflammation and that IL-10 production is not essential for the parasite induced suppressive effect of either CD4<sup>+</sup> or CD4<sup>-</sup> cells. Mangan *et al* have reported that the infection of mice with *S. mansoni* protects mice from systemic fatal anaphylaxis. *In vivo* depletion studies identified a critical role for IL-10 and B cells in the protection of

helminth infected mice (329). The transfer of worm modulated IL-10 producing B cells from IL-4<sup>-/-</sup> mice to naive mice conferred resistance to anaphylaxis, thereby providing a complete mechanism behind parasite induced suppression of an allergic immune response (329).

The hygiene hypothesis initially proposed that Th2 mediated allergies could be counteracted by infections that induce Th1 responses. However it did not explain how helminth infections that induce Th2 responses could also attenuate the Th2 mediated inflammation that results in allergy. The revised model proposes that helminth induced regulatory mechanisms suppress both autoimmune and allergic inflammation. Since the inception of the hygiene hypothesis, a new T helper subtype has emerged. Th17 cells produce IL-17, IL-6 and TNF- $\alpha$  and play a crucial role in organ specific autoimmune inflammation. In the mouse EAE model, myelin specific Th17 cells track to the CNS and recruit neutrophils which results in inflammatory lesions (168). The presence of IL-23 is essential for the pathogenesis of Th17 cells and IL-23<sup>-/-</sup> mice do not develop EAE (136, 178).

This study shows that mice infected with *F. hepatica* attenuated the development of EAE when compared to uninfected mice. An *in vivo* depletion study, identified TGF- $\beta$  as a key factor in the suppression of the clinical symptoms of EAE by helminth infection. Furthermore, MOG specific Th1 and Th17 responses were substantially reduced by the parasite infection. *In vitro* studies with recombinant TGF- $\beta$  showed that it inhibited MOG specific IFN- $\gamma$  but not IL-17 production by T cells. TGF- $\beta$  also inhibited TLR induced IL-23, a cytokine that is crucial for the survival of Th17 cells. This suggests that the induction of the regulatory cytokine TGF- $\beta$  by *F. hepatica* ultimately inhibits autoantigen specific Th1 and Th17 responses, thereby attenuating autoimmune disease. This observation provides an important addition to the hygiene hypothesis, whereby regulatory immune responses induced



by helminth infection can inhibit Th17 cell responses involved in directing autoimmune inflammation

## 5.2 Results

### 5.2.1 *F. hepatica* infection is associated with an influx of immune cells into the peritoneal cavity

After ingestion, *F. hepatica* metacercariae excyst in the duodenum. The juvenile liver fluke then burrow through the intestinal wall before migrating across the peritoneal cavity to the liver. This study examined the effect of *F. hepatica* infection on the infiltration and activation of cells into the peritoneal cavity after 21 days. Mice were infected with 10 viable metacercariae, and sacrificed after 21 days when a peritoneal lavage was performed. There was a large influx of cells into the peritoneal cavity in the infected mice when compared with naive mice [Fig 5.1]. Flow cytometric analysis conducted on the PEC from naive and *F. hepatica* infected mice, revealed there was an increase in the percentage of DCs and macrophages and a decrease in the percentage of CD4<sup>+</sup> cells [Fig 5.2]. Coupled with the data showing an overall increase in cells in the peritoneal cavity [Fig 5.1], this FACS data indicates that the infection of mice with liver fluke leads to an influx of DCs, macrophages and CD4<sup>+</sup> cells into the peritoneal cavity.

### 5.2.2 PEC from *F. hepatica* infected mice exhibit an anti-inflammatory phenotype

Intracellular cytokine staining was performed on the PEC from naive and parasite infected mice. The cells were not activated with PMA, but were incubated overnight in the presence of Brefeldin A before staining. The results showed an increase in IL-10 expression by both DCs and macrophages and an increase in IFN- $\gamma$  and TGF- $\beta$  by macrophages when compared with cells from uninfected control mice [Fig 5.3 and 5.4]. The production of TGF- $\beta$  by the macrophages was particularly dramatic with 67% of macrophages from infected mice producing the cytokine when compared with 7% of macrophages in naive mice [Fig 5.5]. The effect of *F. hepatica* infection on LAP expression by PEC was also investigated. LAP is

non covalently associated with the TGF- $\beta$  homodimer on the cell surface which renders the cytokine inactive. TGF- $\beta$  activators trigger LAP degradation or conformational change that releases the cytokine. Immature human DCs that express LAP have been shown to inhibit Th1 cell differentiation and enhance the induction of Foxp3<sup>+</sup> Treg cells (358). The data in Fig 5.4 and 5.5 show that *F. hepatica* infection enhances LAP expression on DCs and macrophages in the peritoneal cavity [Fig 5.4 and 5.5]. LAP expression on T cells has also been shown to indicate regulatory function. The transfer of CD4<sup>+</sup>CD25<sup>+</sup>LAP<sup>+</sup> T cells potently suppress the clinical symptoms of EAE by a TGF- $\beta$  dependent mechanism (359). The current study revealed a distinct population of CD4<sup>+</sup>CD25<sup>+</sup>LAP<sup>+</sup> cells in the PEC from *F. hepatica* infected mice that was absent in naive controls [Fig 5.6]. There was a relatively unchanged yet high percentage of Th3 cells (CD4<sup>+</sup>CD25<sup>-</sup> T cells that produce TGF- $\beta$ ) in the peritoneal cavity of both naive and infected mice and an increase in the percentage of T cells expressing CD25<sup>+</sup> and TGF- $\beta$  was seen in the PEC from infected mice [Fig 5.6]. In conclusion, *F. hepatica* infection induces the influx of immune cells to the peritoneal cavity that predominantly display an anti-inflammatory phenotype.

### **5.2.3 IL-10 producing T cells from the peritoneal cavity of *F. hepatica* infected mice do not suppress MOG specific pro-inflammatory T cells from the spleens of mice with EAE.**

Studies previously conducted in the lab had demonstrated that the infection of mice with *F. hepatica* attenuated the development of EAE. In order to establish possible mechanisms for this attenuation, we investigated if the IL-10 producing T cells from the peritoneal cavity of liver fluke infected mice could suppress IFN- $\gamma$  and IL-17 production by MOG-specific T cells from the spleens of mice with EAE. The T cells from the peritoneal cavity of liver fluke infected mice were cultured with and without the *F. hepatica* antigen ES. The T cells

cultured without antigen produced high levels of IL-5, low levels of IL-10 and no IFN- $\gamma$  or IL-17 [Fig 5.7]. The ES stimulated T cells produced significantly more IL-10 and IL-5 [Fig 5.7]. The levels of antigen specific IL-10 produced was very high, suggesting that the helminth may induce a Tr1 cell population that is specific for parasite antigen [Fig 5.7]. The T cells from the mice with EAE produced high levels of MOG specific IFN- $\gamma$  and IL-17 [Fig 5.7]. The co-culture of the MOG specific T cells with the IL-10 producing T cells in the presence and absence of parasite antigen did not result in significant suppression of MOG specific IFN- $\gamma$  or IL-17 [Fig 5.7]. The levels of IFN- $\gamma$  produced by the MOG specific T cells were slightly suppressed by the Treg cells [Fig 5.7]. This inhibition was not dependent on the activation of the Treg cells with ES [Fig 5.7]. The co-culture of the Treg cells with the MOG specific T cells resulted in an increase in MOG specific IL-17 production [Fig 5.7]. These data suggest that the ability of *F. hepatica* infection to attenuate the clinical symptoms of EAE may not be T cell dependent.

#### **5.2.4 *F. hepatica* infection attenuates the clinical symptoms of EAE in IL-10<sup>-/-</sup> mice**

IL-10 production has previously been shown to be an important mediator in the suppression of EAE (237, 391). Therefore the role of IL-10 in *F. hepatica* induced attenuation of EAE was examined using IL-10<sup>-/-</sup> mice. C57BL/6 and IL-10<sup>-/-</sup> mice were infected with 10 *F. hepatica* metacercariae on day -1. On day 0, EAE was induced and the mice were observed daily for the clinical symptoms of EAE. The first clinical signs of EAE were observed on day 7 in the IL-10<sup>-/-</sup> mice and on day 9 in the wild type C57BL/6 mice, which is consistent with the anti-inflammatory role of IL-10 *in vivo* [Fig 5.8]. The uninfected and infected wild type mice exhibited an identical progression in the severity of EAE until day 12 [Fig 5.8]. From this timepoint, the *F. hepatica* infected mice had substantially reduced clinical symptoms of EAE, confirming previous studies conducted in the lab [Fig 5.8]. The

uninfected and infected IL-10<sup>-/-</sup> mice also displayed an identical progression of disease until day 11 [Fig 5.8]. From this timepoint, IL-10<sup>-/-</sup> mice that were infected with *F. hepatica* had attenuated clinical symptoms of EAE [Fig 5.8]. The experiment was terminated on day 20 as the mice begin to die from the liver fluke infection thereafter. The spleens cells of the mice were cultured with MOG *in vitro* for 3 days. Supernatants were then taken for cytokine analysis. The results revealed a reduction in the production of MOG specific IL-17 and IFN- $\gamma$  by the spleen cells from helminth infected C57BL/6 and IL-10<sup>-/-</sup> when compared with the uninfected wild type and IL-10<sup>-/-</sup> mice with EAE [Fig 5.9]. These findings suggest that IL-10 does not play a role in the attenuation of EAE or in the suppression of myelin-specific Th1 and Th17 cell responses by *F. hepatica* infection.

### **5.2.5 Recombinant TGF- $\beta$ inhibits TLR agonist induced IL-23, IL-1 $\beta$ and IL-12p40 production by DCs.**

Having shown that *F. hepatica* infection induced IL-10 and TGF- $\beta$  production by macrophages, DC and T cells and that IL-10 did not mediate suppression of EAE, the role of TGF- $\beta$  was investigated.

Rowan *et al* have previously shown that recombinant TGF- $\beta$  inhibits LPS and IFN- $\gamma$  induced IL-12 and IL-23 production by human monocytes (392). Therefore I examined the effect of TGF- $\beta$  on cytokine production by murine DCs. Increasing concentrations of TGF- $\beta$  inhibited LPS induced IL-23, IL-12p40 and IL-1 $\beta$ , and CpG induced IL-23 and IL-12p40 production by murine DCs [Fig 5.10]. TGF- $\beta$  did not affect CpG induced IL-1 $\beta$  [Fig 5.10]. This study confirmed that the production of pro-inflammatory cytokines by both human and mouse innate immune cells is suppressed by recombinant TGF- $\beta$ .

This assay was also used to test the functionality of a neutralizing anti-TGF- $\beta$  antibody. We treated DCs with a TLR agonist, increasing concentrations of recombinant TGF- $\beta$  and either an anti-TGF- $\beta$  antibody or an isotype control antibody. The anti-TGF- $\beta$  antibody significantly reversed the ability of TGF- $\beta$  to suppress both LPS and CpG induced IL-23 production by the DCs [Fig 5.11]. The isotype control antibody had no effect on the activity of TGF- $\beta$ . We could therefore proceed to an *in vivo* depletion experiment [Fig 5.11].

### **5.2.6 The neutralization of TGF- $\beta$ *in vivo* reverses the protective effect of *F. hepatica* infection**

In order to investigate the role of TGF- $\beta$  in the attenuation of EAE by *F. hepatica* infection, mice were infected with liver fluke on day -1, EAE was induced on day 0 and the mice were injected i.p with the neutralizing TGF- $\beta$  antibody every second day (starting on day 0). Three other groups of mice were included as controls. One group were infected with *F. hepatica*, prior to the induction of EAE and received an isotype control antibody. Another group was immunized for EAE and received an anti-TGF- $\beta$  antibody and a third group was immunized for EAE and received the isotype control antibody. The mice that were immunized with MOG and CFA and received either the anti-TGF- $\beta$  antibody or the isotype control antibody developed severe EAE [Fig 5.12]. Notably, there was no significant difference in the disease scores between the uninfected mice that received the anti-TGF- $\beta$  antibody and the mice that received the isotype control antibody, which suggests that the neutralization of TGF- $\beta$  *in vivo* does not exacerbate EAE [Fig 5.12]. The mice that were infected with *F. hepatica* and received the isotype control antibody did not develop severe EAE, which was consistent with previous studies [Fig 5.12]. The infected mice that received the TGF- $\beta$  antibody developed very severe EAE and were all sacrificed by day 14 for humane reasons [Fig 5.12].

The inguinal lymph nodes cells from all mice were cultured in the presence of MOG for 3 days. Supernatants were then taken for cytokine analysis. The lymph node cells from the mice that were infected with *F. hepatica* prior to the induction of EAE and received the TGF- $\beta$  produced very high levels of MOG specific IL-17 and IFN- $\gamma$  [Fig 5.13]. In contrast, significantly less MOG specific IL-17 and IFN- $\gamma$  was produced by the cells from the other experimental groups [Fig 5.13]. The cells from the mice that were infected and received the control antibody produced the lowest levels of MOG specific IL-17 [Fig 5.13]. The mice in both groups that had been infected with *F. hepatica* produced significant levels of non antigen specific IL-10 and IL-4 [Fig 5.13]. Lymph node cells from the infected mice that received the control antibody produced substantial levels of non antigen specific TGF- $\beta$ , whereas cells from the infected mice that received the anti-TGF- $\beta$  antibody only produced TGF- $\beta$  in response to stimulation with CD3e and PMA [Fig 5.13].

Intracellular cytokine staining and FACs analysis was performed on spleen cells from the infected and uninfected mice with EAE that received the control antibody. The CD4<sup>+</sup> cells from the infected mice had a lower frequency of IL-17 producing cells and had enhanced expression of LAP when compared with the CD4<sup>+</sup> cells from the uninfected mice [Fig 5.14]. It can be concluded from this study, that TGF- $\beta$  has a critical role in the suppressive effect of *F. hepatica* infection on EAE.

### **5.2.7 Recombinant TGF- $\beta$ inhibits the production of IFN- $\gamma$ but not IL-17 from MOG specific T cells**

In an attempt to understand the potential mechanisms involved in the attenuation of EAE by parasite induced TGF- $\beta$ , recombinant TGF- $\beta$  was assessed for its ability to suppress MOG specific T cells from mice with EAE. Increasing doses of TGF- $\beta$  resulted in the suppression

of MOG specific IFN- $\gamma$  production by T cells from mice with EAE [Fig 5.15]. However, TGF- $\beta$  did not inhibit MOG-specific IL-17 production and interestingly, at one of the doses tested (100 pg/ml), TGF- $\beta$  actually significantly enhanced IL-17 production by the T cells [Fig 5.15]. These findings suggest that TGF- $\beta$  inhibits MOG specific Th1 responses but not Th17 responses.



### 5.3 Discussion

This study reports that the infection of mice with *F. hepatica* attenuates the clinical symptoms of EAE by a TGF- $\beta$  dependent mechanism. Rowan *et al* have previously reported that hepatitis C virus induced TGF- $\beta$  suppresses virus specific Th17 responses in infected humans (392). However, this is the first report to show that TGF- $\beta$  induced by a helminth infection protects mice from the development of autoimmunity. The study demonstrates that *F. hepatica* infection inhibits cytokine production by autoantigen specific Th1 and Th17 cells. This finding provides new evidence to support the hygiene hypothesis and suggests that regulatory mechanisms induced by parasite infection can attenuate Th17 immune responses, which have a major role in the development of organ specific autoimmunity.

The results of this study demonstrated that mice immunized with MOG and CFA and injected with an anti-TGF- $\beta$  antibody or an isotype control antibody developed severe EAE. Mice that were infected with *F. hepatica* and received the control antibody did not develop severe EAE, which allowed us to conclude that helminth infection attenuates EAE. Although the average clinical score of the infected mice was consistently below 2, there was a large variance in the severity of EAE among the individual mice in the group. This suggests that, despite infection with 10 viable metacercariae, levels of *F. hepatica* infection may have varied between individual mice, thereby resulting in differing intensities in the helminth induced regulatory responses.

Mice that were infected with *F. hepatica* and received the anti-TGF- $\beta$  antibody developed very severe EAE and had to be sacrificed by day 14. This suggested that TGF- $\beta$  has a critical role in the attenuation of EAE by the helminth. It also demonstrated that administration of an anti-TGF- $\beta$  antibody to parasite infected mice with EAE enhanced the severity of EAE,

when compared to uninfected mice that received the TGF- $\beta$  antibody. Flynn *et al* have shown that PBMCs from cattle infected with liver fluke for 4 weeks, produce high levels of parasite specific IL-10 and TGF- $\beta$  (310). The neutralization of TGF- $\beta$  enhanced parasite specific IL-4 and IFN- $\gamma$  and non specific IFN- $\gamma$  production by the PBMCs (310). This suggests that helminth infection induces Th3 cells that suppress parasite specific Th1 and Th2 responses. This may also explain why parasite infected mice that are treated with an anti-TGF- $\beta$  antibody develop a more severe form of EAE than uninfected control animals. The neutralization of TGF- $\beta$  in helminth infected mice may have resulted in an increase in specific and non specific Th1 responses against the parasite which could have synergised with the autoantigen Th1 response to exacerbate autoimmune inflammation and increase the severity of EAE.

In order to understand the complete mechanism behind the protective effect of *F. hepatica* infection in mice with EAE, the exact cellular source of TGF- $\beta$  will need to be identified. Li *et al* have developed mice that lack TGF- $\beta$  production by CD4<sup>+</sup> T cells. These mice develop an early lethal multifocal inflammatory disorder similar to that seen in TGF- $\beta$  deficient mice (172). This is an obstacle in studying the role of helminth induced TGF- $\beta$  in attenuating the clinical symptoms of EAE. *In vivo* depletion studies such as those used by Smith *et al*, could be utilized to identify key helminth induced TGF- $\beta$  producing cell populations (332). There is also the possibility that the TGF- $\beta$  involved, may be produced by the helminth and not the host. The filarial nematode *Brugia malayi* has been shown to produce a TGF- $\beta$  homolog which binds to host TGF- $\beta$  receptors (393). However the present study shows that *F. hepatica* infection induces TGF- $\beta$  expression by macrophages and T cells suggesting that one or more of these cells could confer protection.

Studies of *F. hepatica* infected cattle and rats have examined the immune response against the helminth over a time course. PBMCs taken from cattle 10 days post infection, produce high levels of parasite specific IL-4 (309). This Th2 response predominates at the acute stage of infection. Within 12 weeks the cattle become chronically infected with the helminth. PBMCs taken from these animals produce high levels of the regulatory cytokines IL-10 and TGF- $\beta$  and reduced levels of IL-4 in response to parasite antigen (310). Studies from *F. hepatica* infected rats report a similar immune response over a shorter time course. Rat liver cells taken on day 7, express high levels of IL-4 and IL-5 mRNA, which are much reduced by day 14 of infection (394). In contrast, the levels of IL-10 and TGF- $\beta$  mRNA in the liver cells is highest on day 14 of infection (394). A time course will need to be conducted in infected mice in order to fully understand the helminth induced immune responses that are required for the attenuation of autoimmune disease. There are indications from the study that the production of regulatory cytokines signifying a chronic infection may begin around day 11 of infection. Before day 11, there was no difference in the clinical scores of EAE between the parasite infected and uninfected mice. After 11 days the infected mice had substantially reduced clinical symptoms of EAE. The study also revealed that low levels of IL-4 and high levels of IL-10 were produced by spleen cells cultured in medium alone [Fig 5.13]. This is compatible with a modified Th2 response, characteristic of a chronic helminth infection.

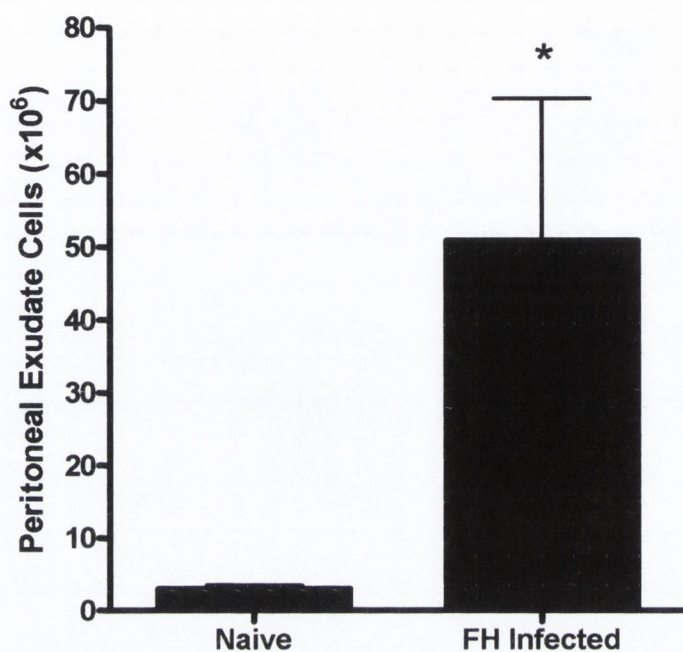
This study showed that *F. hepatica* infection of mice resulted in an influx of immune cells, including DC, macrophages and CD4<sup>+</sup> T cells into the peritoneum. The infiltrating DCs and macrophages produced IL-10 and expressed LAP. A very high percentage of the macrophages in the peritoneal cavity of infected mice produced TGF- $\beta$ . This indicates that the juvenile liver fluke that migrate across the peritoneal cavity produce immunomodulatory molecules that promote an anti-inflammatory response in immune cells. Cysteine proteases

known as cathepsins are produced by *F. hepatica* and cathepsin L has been shown to suppress Th1 immune responses (322). Interestingly, the juvenile liver fluke expresses high levels of cathepsin B, whereas the adult fluke downregulates cathepsin B expression and secretes high levels of cathepsin L (395). This suggests that the cells of the peritoneal cavity interact with cathepsin B and it would be interesting to investigate the immunomodulatory properties of this molecule.

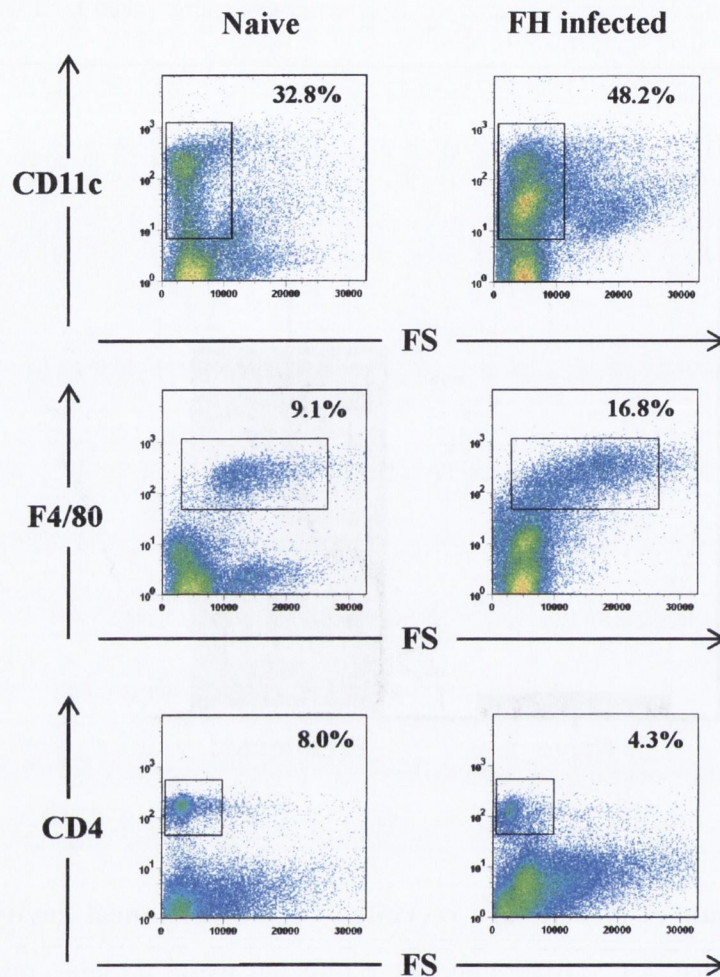
This study also demonstrates the recruitment or activation of a distinct population of CD4<sup>+</sup>CD25<sup>+</sup>LAP<sup>+</sup> cells in the PEC from mice infected with *F. hepatica*. Chen *et al* have demonstrated that the transfer of CD4<sup>+</sup>CD25<sup>+</sup>LAP<sup>+</sup> ( $1 \times 10^5$  cells) attenuates EAE by a TGF- $\beta$  dependent mechanism. The average number of CD4<sup>+</sup>CD25<sup>+</sup>LAP<sup>+</sup> cells in the peritoneum of an infected mouse is  $1.86 \times 10^5$  cells. It is therefore possible that the induction of CD4<sup>+</sup>CD25<sup>+</sup>LAP<sup>+</sup> cells may provide the mechanism for the protective effect of liver fluke infection in EAE.

IL-10 producing T cells from the peritoneal cavity of mice infected with *F. hepatica* did not suppress IFN- $\gamma$  or IL-17 production from MOG specific T cells from mice with EAE. This suggests that the CD4<sup>+</sup> T cells induced by helminth infection do not directly inhibit pro-inflammatory effector T cells. In this experiment, the MOG-specific T cells were initially added to the culture of APCs. The helminth induced Treg cells were then subsequently added 20 mins later. There have been reports that Treg cells can downregulate the expression of CD80 and CD86 on DCs, thereby affecting their ability to activate effector T cells (225). The experiment will need to be repeated where the Treg cells are incubated with the APC to examine if the helminth induced Treg cells indirectly inhibit effector T cells by suppressing APC function.

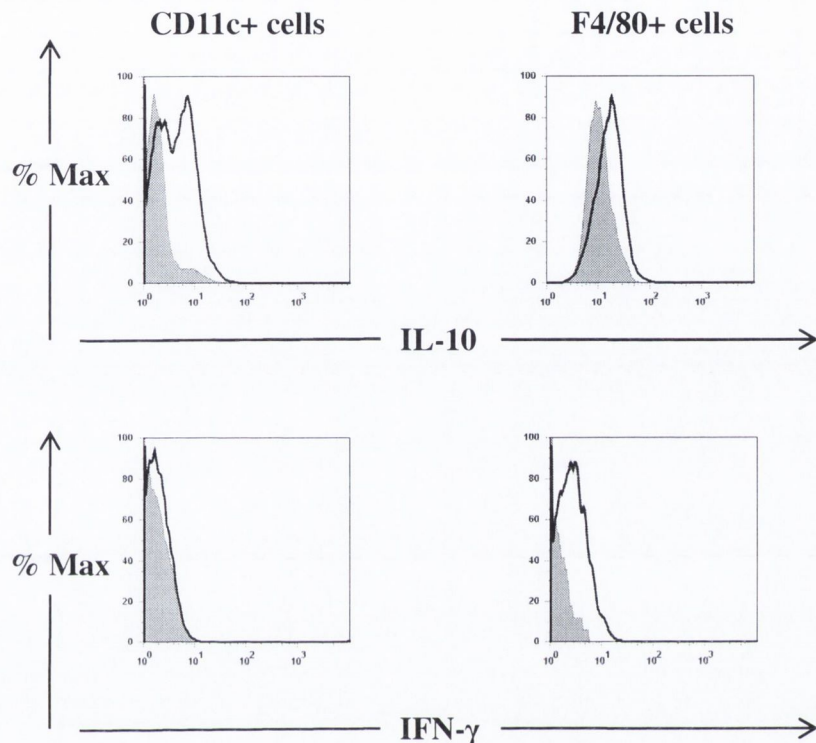
In order to investigate how helminth induced TGF- $\beta$  attenuates EAE, MOG specific T cells from mice with EAE were incubated with increasing concentrations of recombinant TGF- $\beta$ . Increasing doses of TGF- $\beta$ , inhibited IFN- $\gamma$  but not IL-17 production by the T cells. This findings confirms previous reports that TGF- $\beta$  inhibits effector Th1 cells and suggests that like Th2 cells, effector Th17 cells are resistant to TGF- $\beta$  mediated inhibition (347). However, this study found that recombinant TGF- $\beta$  inhibits TLR induced IL-23 and IL-1 $\beta$  production by DCs. Both cytokines have been shown to be crucial for the expansion and survival of pathogenic Th17 cells (133, 136, 181). The findings suggest that helminth induced TGF- $\beta$  inhibits IL-23 and IL-1 $\beta$  production from innate immune cells, thereby suppressing myelin specific Th17 cells that cause inflammation in EAE.



**Fig 5.1** Large numbers of cells are recruited to the peritoneal cavity of *Fasciola hepatica* infected mice. 4 C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica*. Three weeks after challenge, PEC were isolated from naïve and *F. hepatica*-infected mice by peritoneal lavage. Viable peritoneal exudate cells were counted by trypan blue staining. \* P<0.05 versus cell numbers from naïve C57BL/6 mice. This results is representative of two experiments which were carried out in quadruplicate.

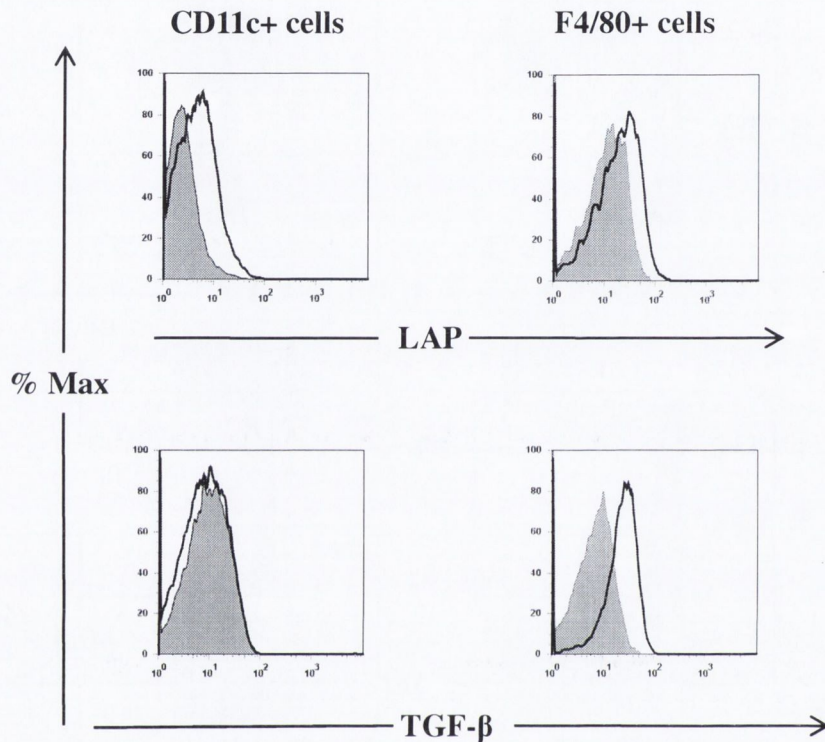


**Fig 5.2** *F. hepatica* infection of mice is associated with an influx of DC and macrophage into the peritoneal cavity. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica*. Three weeks after challenge, PEC were isolated from naïve and *F. hepatica*-infected mice by peritoneal lavage. The cells were treated with Brefeldin A for 10 hours and then surface stained for CD11c, F4/80 and CD4. Percentages refer to live PEC. These results are representative of two experiments.

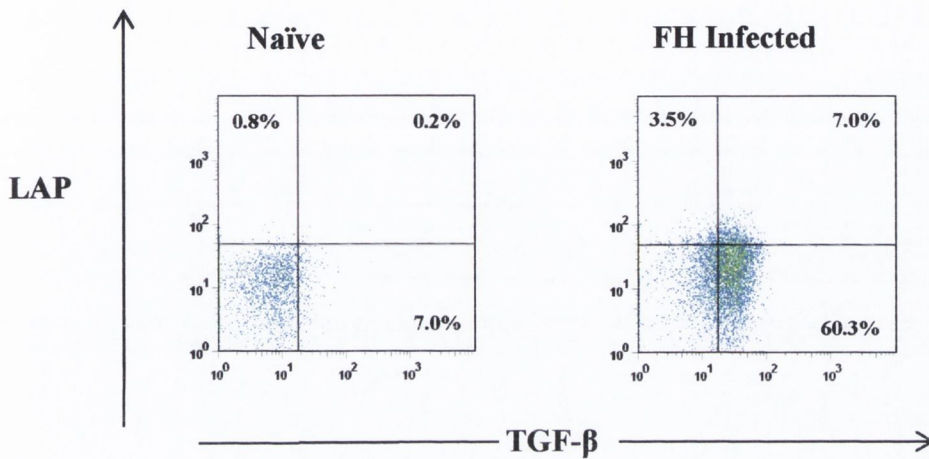


**Fig 5.3** *F. hepatica* infection results in an increase in IFN- $\gamma$  production from peritoneal macrophages and an increase in IL-10 production by both peritoneal DCs and macrophages. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica*. Three weeks after challenge, PEC were 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 CD11c and F4/80 and for intracellular IL-10 and IFN- $\gamma$ . Results from immunofluorescence analysis are shown for PEC from *F. hepatica* infected mice (black line) compared with PEC from naïve mice (grey histogram). These results are from one experiment.

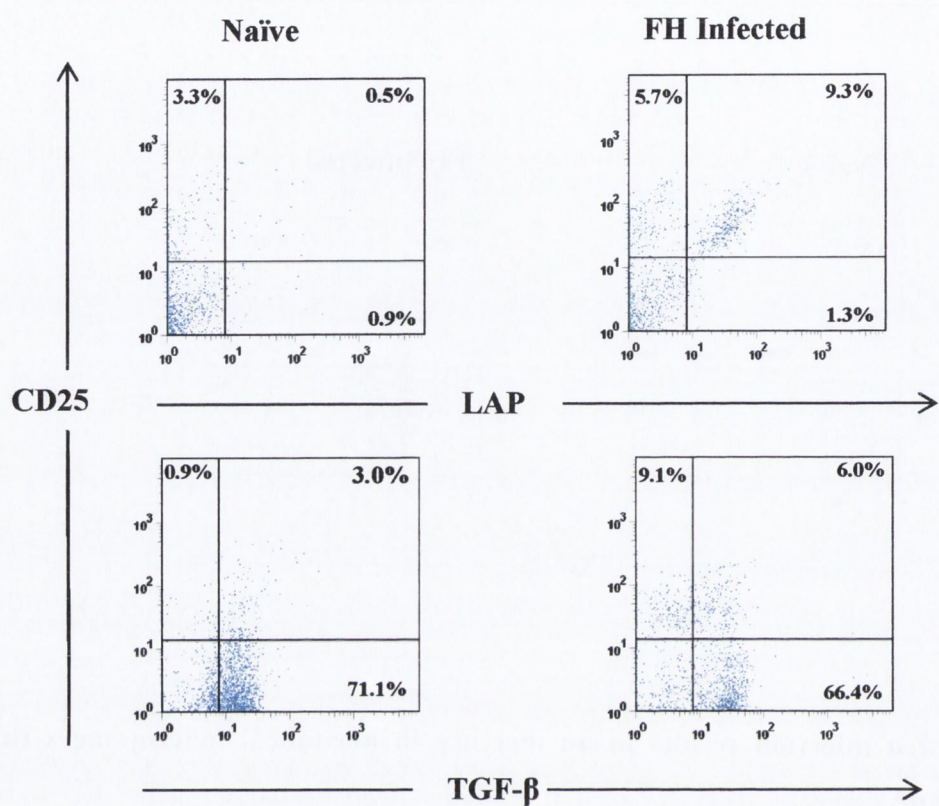




**Fig 5.4** *F. hepatica* infection results in an increase in TGF- $\beta$  production from peritoneal macrophages and an increase in LAP expression on both peritoneal DCs and macrophages. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica*. Three weeks after challenge, PEC were 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 CD11c, F4/80 and LAP and for intracellular TGF- $\beta$ . Results from immunofluorescence analysis are shown for PEC from *F. hepatica* infected mice (black line) compared with PEC from naïve mice (grey histogram). These results are from one experiment.

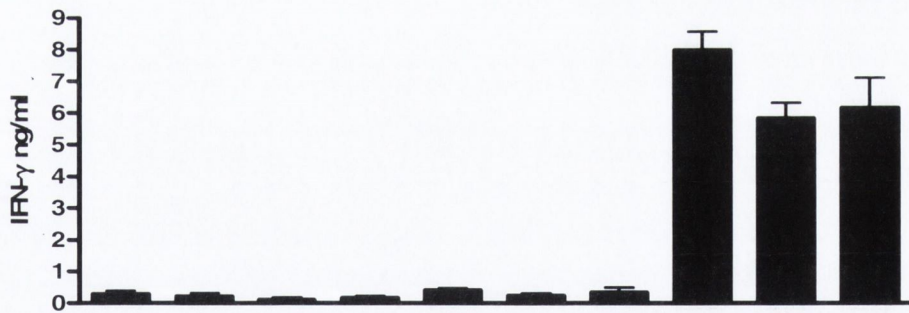
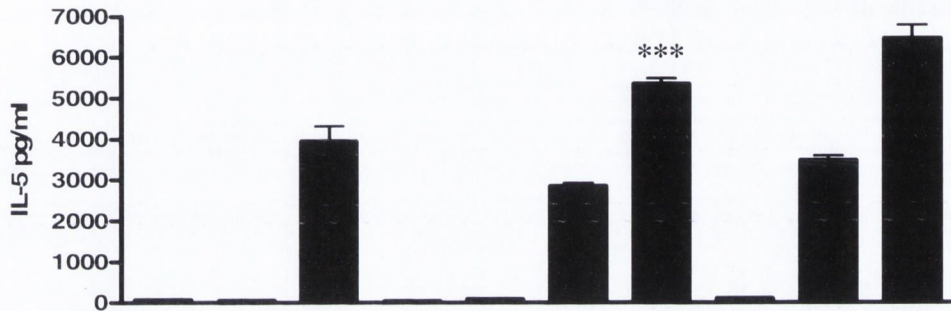
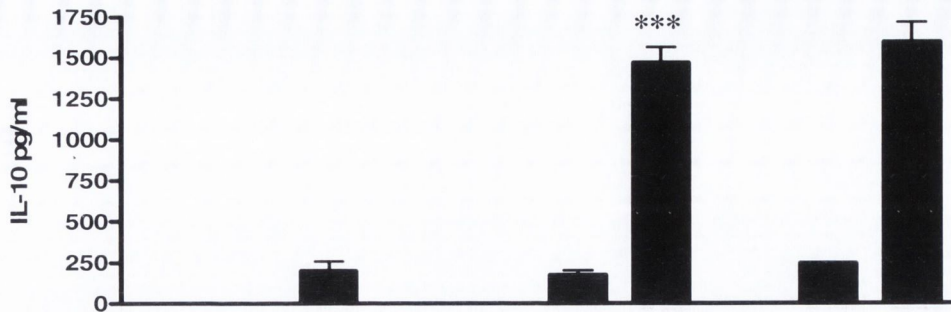


**Fig 5.5** *F. hepatica* infection results in an increase in peritoneal macrophages that express LAP and secrete TGF-β. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica*. Three weeks after challenge, PEC were isolated from *F. hepatica*-infected and naïve control mice by peritoneal lavage. The cells were treated with Brefeldin A for 10 hours and then surface stained for F4/80 and LAP and for intracellular TGF-β. Percentages refer to live peritoneal macrophages. These results are from one experiment.

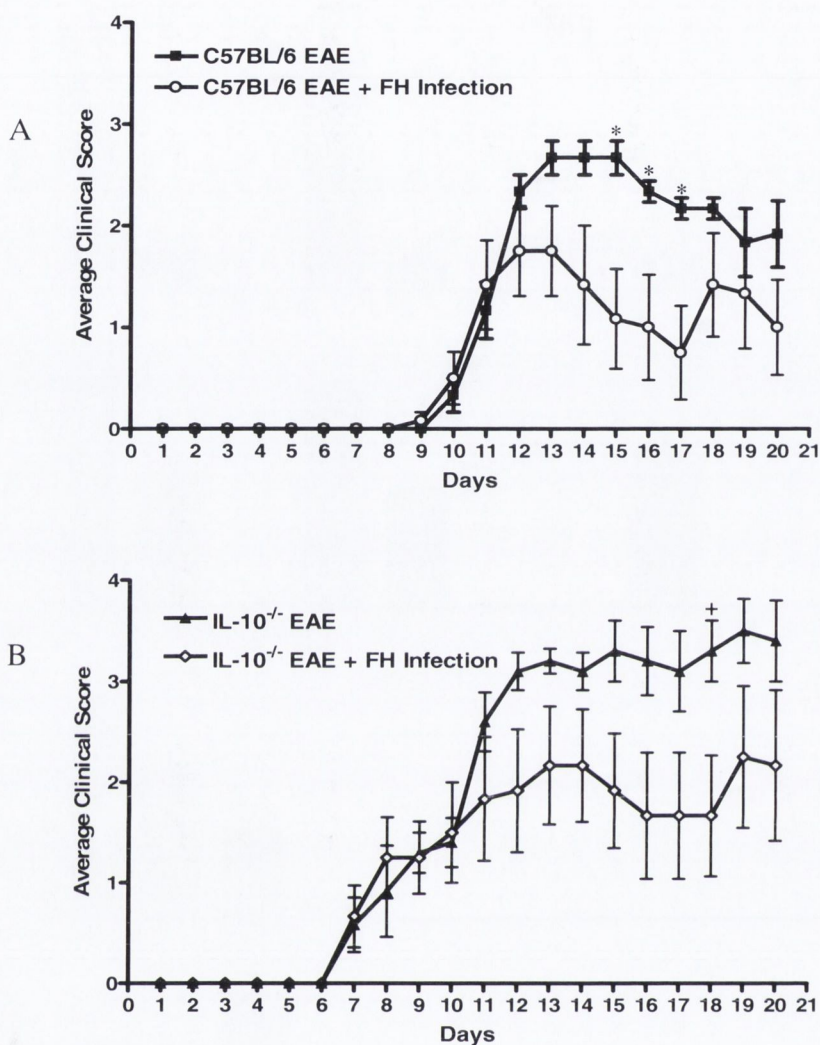


**Fig 5.6** An enhancement of the population of CD4<sup>+</sup>CD25<sup>+</sup>LAP<sup>+</sup> cells occurs in the peritoneal cavity of mice infected with *F. hepatica*. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica*. Three weeks after challenge, PEC were isolated from naïve and *F. hepatica*-infected mice by peritoneal lavage. The cells were treated with brefeldin A for 10 hours and then surface stained for CD4, CD25 and LAP and for intracellular TGF-β. Percentages refer to live CD4<sup>+</sup> cells. These results are from one experiment.

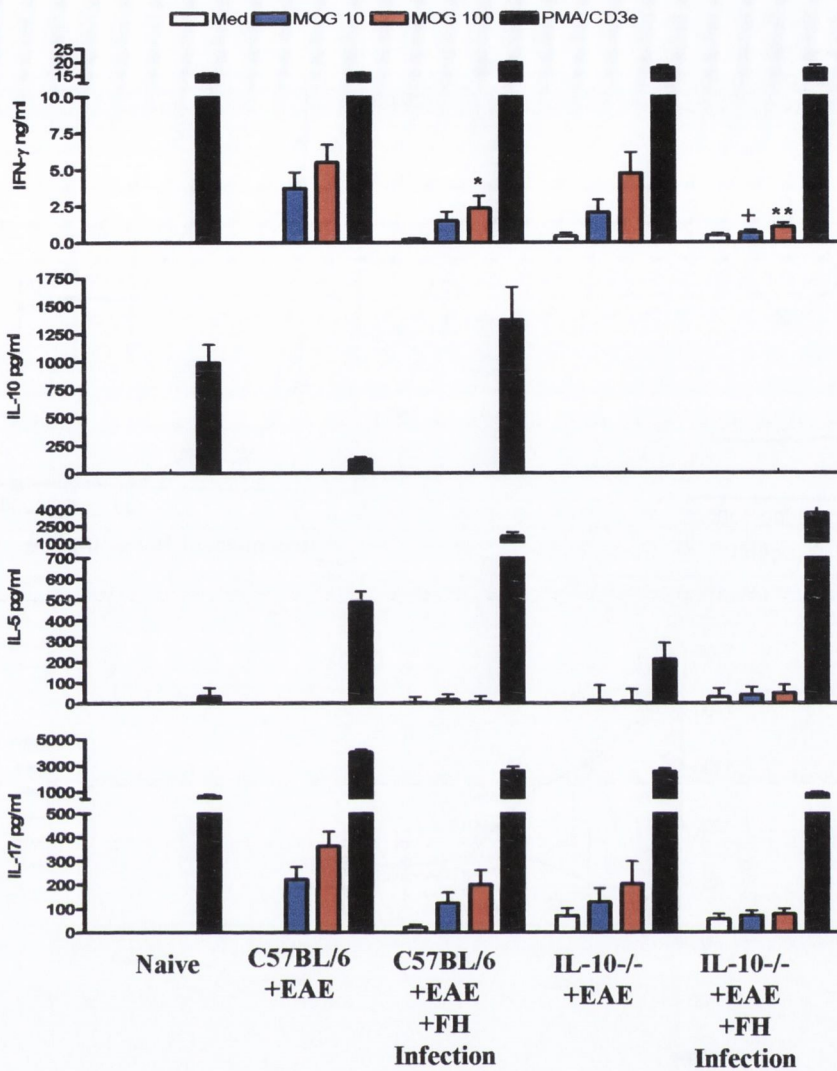
**Fig 5.7 IL-10 producing antigen specific CD4<sup>+</sup> T cells from the peritoneal cavity of *Fasciola hepatica* infected mice do not suppress IL-17 and IFN- $\gamma$  production from CD4<sup>+</sup> T cells from the spleen of mice with EAE.** CD4<sup>+</sup> T cells ( $1.5 \times 10^6$  cells/ml) from the peritoneal cavity of *F. hepatica* infected mice were cultured with APCs (irradiated spleen cells,  $2 \times 10^6$  cells/ml) and with and without ES (20  $\mu$ g/ml). CD4<sup>+</sup> T cells ( $5 \times 10^5$  cells/ml) from the spleens of mice with EAE were cultured with APC and MOG (25  $\mu$ g/ml). After 72 hrs supernatants were taken and the concentrations of IFN- $\gamma$ , IL-10, IL-5 and IL-17 were quantified by ELISA. \*\*\*  $P < 0.001$  versus CD4 T cells from *F. hepatica* infected mice that were cultured without ES. These results are from one experiment which was carried out in triplicate.



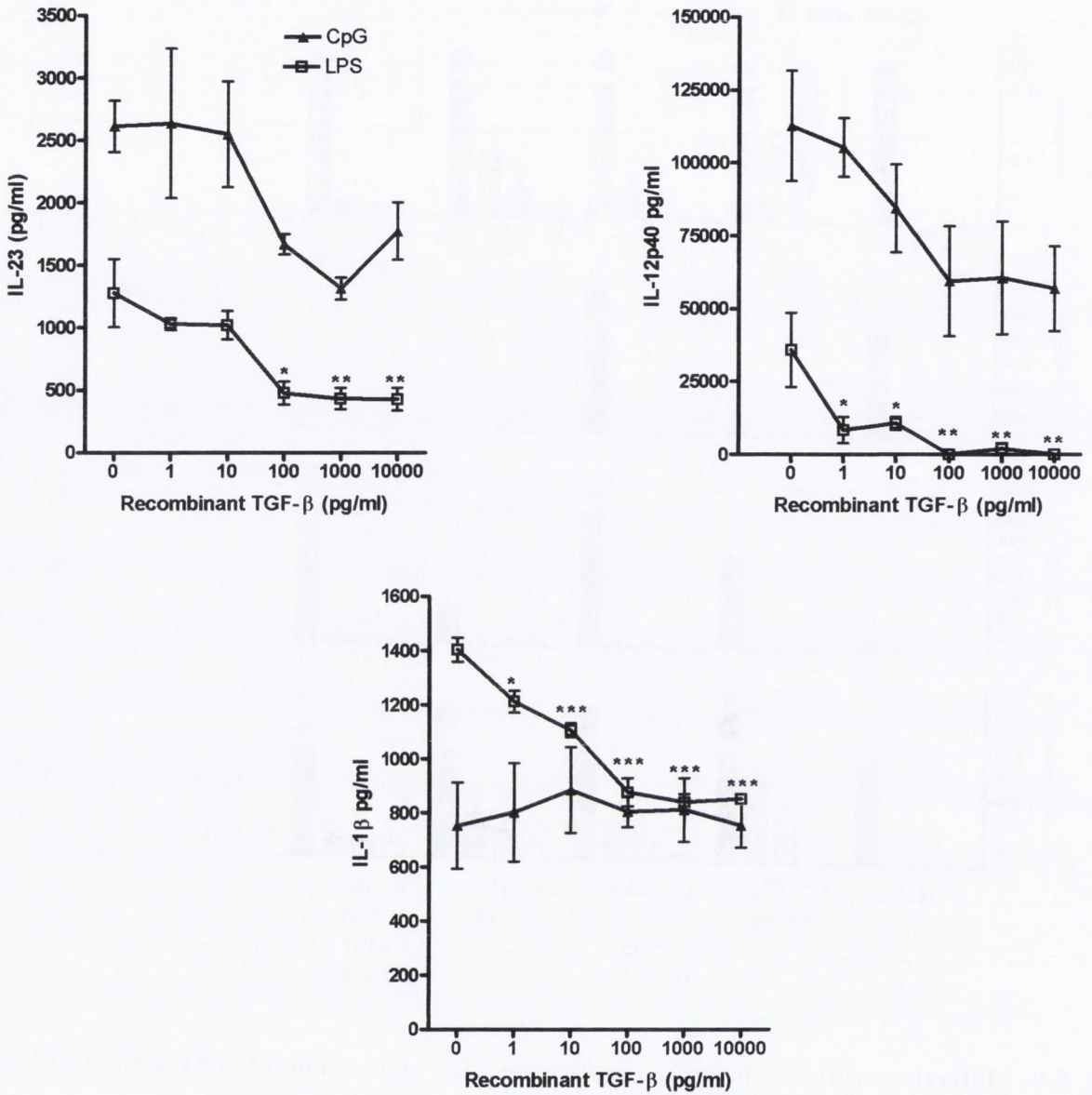
APC	+	+	+	+	+	+	+	+	+	+
ES		+		+			+			+
MOG		+	+					+	+	+
CD4+ (FH)			+			+	+		+	+
CD4+ (EAE)				+	+			+	+	+



**Fig 5.8 Infection with *F. hepatica* ameliorates the clinical symptoms of EAE in C57BL/6 and IL-10<sup>-/-</sup> mice.** Groups of 6 C57BL/6 (A) and IL-10<sup>-/-</sup> (B) mice were infected with 10 viable metacercariae of *F. hepatica* on Day -1 before induction of EAE. On Day 0 all mice were immunized s.c. with 150  $\mu$ g of MOG<sub>35-55</sub> in CFA, followed by injection i.p. with 500 ng of PT on days 0 and 2. Clinical scores were examined daily and mice sacrificed on Day 20. Figures show average clinical EAE scores. \*P < 0.05 versus C57BL/6 mice that were infected with *Fasciola hepatica* and immunized with MOG in CFA and PT. +P < 0.05 versus IL-10<sup>-/-</sup> mice that were infected with *Fasciola hepatica* and immunized with MOG in CFA and PT. These results are from one experiment.

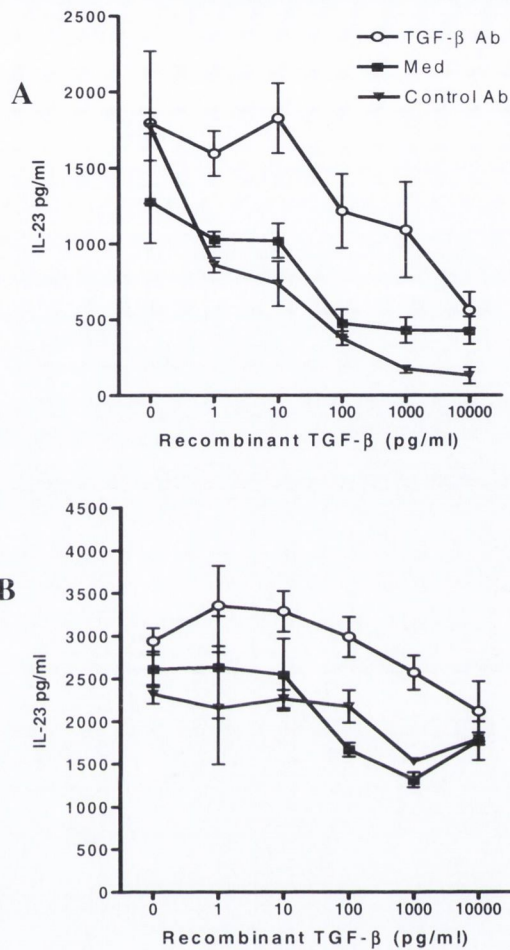


**Fig 5.9 Infection with *F. hepatica* suppresses the generation of Th1 and Th17 responses after the induction of EAE.** The mice in the experiment shown in Fig 5.8 were sacrificed on Day 20 or when their clinical score reached 4. Spleens were removed and the cells were stimulated with MOG peptide (10 or 100  $\mu\text{g/ml}$ ), medium only or PMA and anti-CD3. After 72 hrs supernatants were taken and the concentrations of IFN- $\gamma$ , IL-10, IL-5 and IL-17 were quantified by ELISA. \*\*  $P < 0.01$ , \*  $P < 0.05$  versus cells from uninfected EAE mice stimulated with MOG 100  $\mu\text{g/ml}$ , +  $P < 0.05$  versus cells from uninfected EAE mice stimulated with MOG 10  $\mu\text{g/ml}$ . These results are from one experiment which was carried out in triplicate.

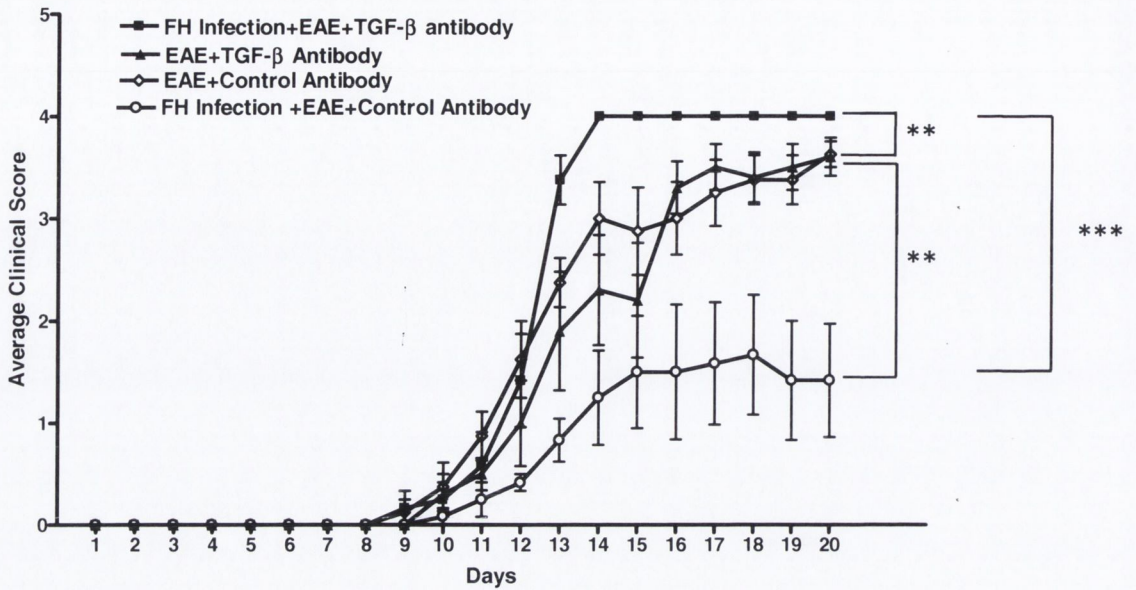


**Fig 5.10 Recombinant TGF-β inhibits TLR agonist induced IL-23 and IL-12p40.** BMDCs ( $5 \times 10^6$  cells/ml) were incubated with increasing concentrations of recombinant TGF-β (1 pg/ml-10 ng/ml) and either LPS (100 ng/ml) or CpG (1 μg/ml). After 24 hours, supernatants were removed and IL-23, IL-12p40 and IL-1β were quantified by ELISA. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$  versus DCs treated with LPS only. These results are from one experiment which was carried out in triplicate.



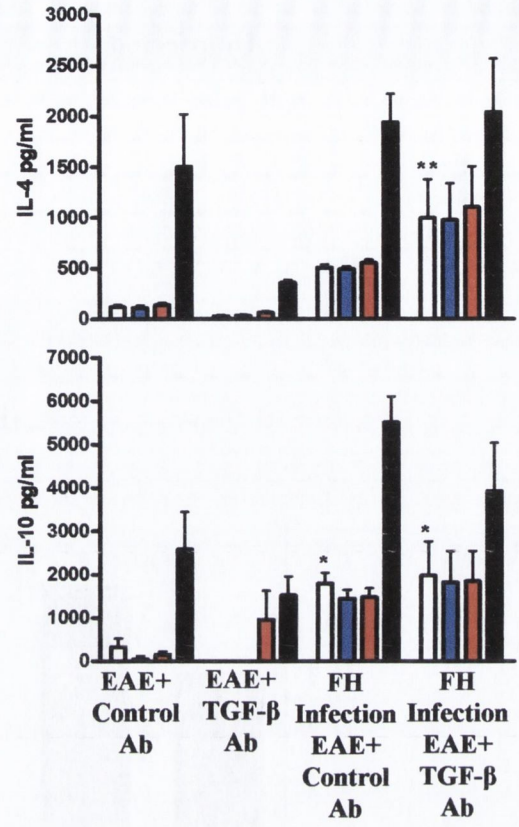
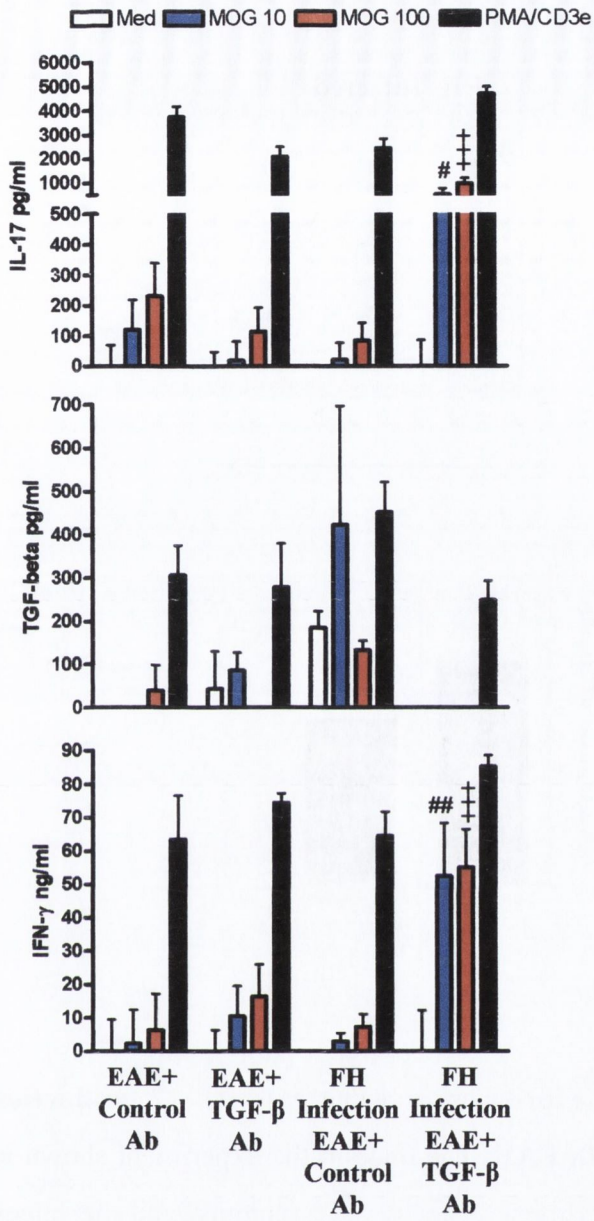


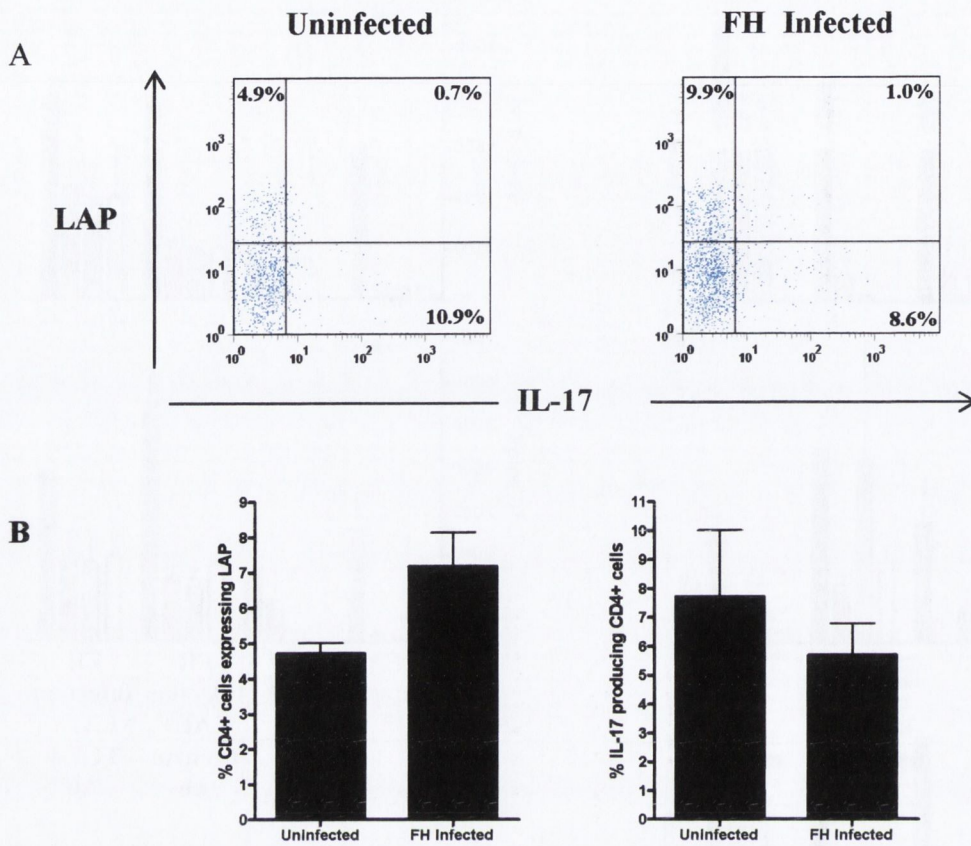
**Fig 5.11 A TGF- $\beta$  antibody inhibits the ability of recombinant TGF- $\beta$  to suppress TLR induced IL-23.** BMDCs ( $5 \times 10^6$  cells/ml) were incubated with either (A) LPS (100 ng/ml) or (B) CpG (1  $\mu$ g/ml), increasing concentrations of recombinant TGF- $\beta$  (1pg/ml-10 ng/ml) and medium, an anti-TGF- $\beta$  antibody (10  $\mu$ g/ml) or an isotype control antibody (10 $\mu$ g/ml). Supernatants were removed after 24 hours and IL-23 was quantified by ELISA. In both graphs A and B a statistical comparison by two way ANOVA with repeated measures was performed.  $P < 0.001$  on comparing cells treated with a TLR agonist, recombinant TGF- $\beta$  and an anti-TGF- $\beta$  antibody and cells treated with recombinant TGF- $\beta$  and a TLR agonist or cells treated with recombinant TGF- $\beta$ , a TLR agonist and an isotype control. These results are from one experiment which was carried out in triplicate.



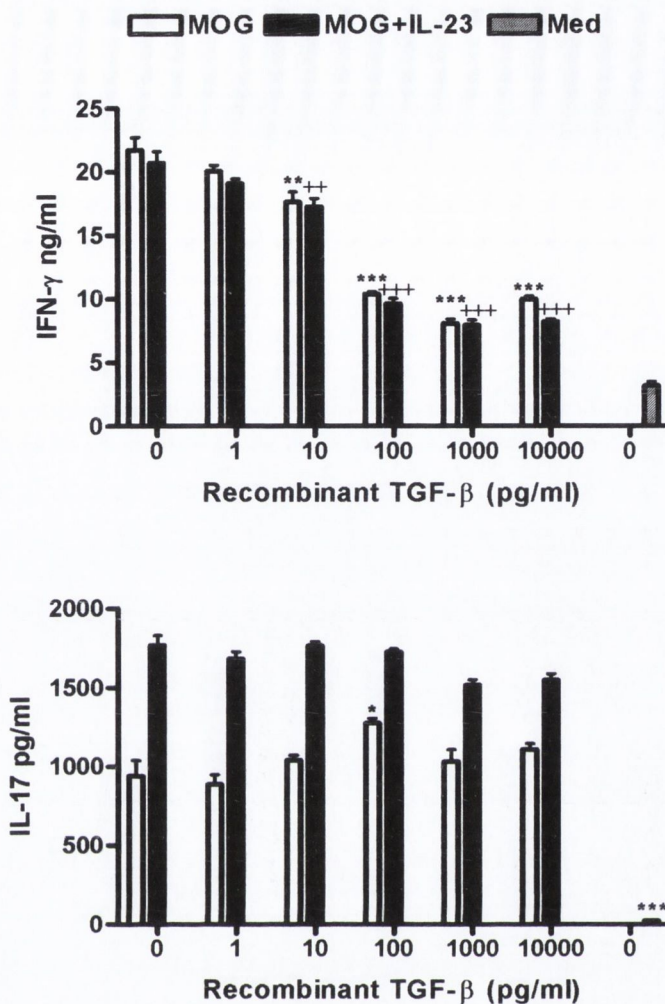
**Fig 5.12 Neutralization of TGF- $\beta$  *in vivo* reverses the protective effect of *F. hepatica* infection on EAE.** Groups of 6 C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica* on Day -1 before induction of EAE. All mice were immunized s.c. with 150  $\mu$ g of MOG<sub>35-55</sub> in CFA on day 0, followed by injection i.p. with 500 ng of PT on days 0 and 2. Mice received i.p. either an isotype control antibody (100  $\mu$ g/mouse) or an anti-TGF- $\beta$  antibody (100  $\mu$ g/mouse) on Days 0, 2, 4, 6, 8, 10, 12, 14, 16, 18. Clinical scores were examined daily and the mice were sacrificed on Day 20. Figures shows average clinical EAE scores. A two way ANOVA with repeated measures was performed between the treatment groups.  $P < 0.001$  on comparing mice that were infected with *Fasciola hepatica* and received an anti-TGF- $\beta$  antibody and mice that were infected and received an isotype control antibody.  $P < 0.01$  on comparing mice that received an isotype control antibody and mice that were infected with *Fasciola hepatica* and received an isotype control antibody.  $P < 0.01$  on comparing mice that received an anti-TGF- $\beta$  antibody and mice that were infected with *Fasciola hepatica* and received an isotype control antibody.  $P < 0.01$  on comparing mice that received an anti-TGF- $\beta$  antibody and mice that were infected with *Fasciola hepatica* and received an anti-TGF- $\beta$  antibody. These results are from one experiment.

**Fig 5.13 Neutralization of TGF- $\beta$  *in vivo* reverses the suppressive effect of *F. hepatica* infection on MOG specific IFN- $\gamma$  and IL-17 production in lymph node cells.** The mice in the experiment shown in Fig 5.12 were sacrificed on Day 20 of the experiment or when their clinical score reached 4. Inguinal nodes were removed and the cells were stimulated with MOG peptide (10 or 100  $\mu\text{g/ml}$ ), medium only or PMA and anti-CD3. After 72 hrs supernatants were taken and the concentrations of IFN- $\gamma$ , IL-10, IL-4, TGF-beta and IL-17 were quantified by ELISA. \*\*P<0.01, \*P<0.05 versus cells from mice with EAE that received an isotype control antibody and incubated with medium. ##P<0.01, #P<0.05 versus cells from EAE mice that were infected with *F. hepatica* and received a control antibody and were stimulated with MOG (10  $\mu\text{g/ml}$ ). +++P<0.001 versus cells from EAE mice that were infected with *F. hepatica* and received a control antibody and were stimulated with MOG (100  $\mu\text{g/ml}$ ). These results are from one experiment which was carried out in triplicate.





**Fig 5.14 *F. hepatica* infection increases LAP expression and decreases IL-17 production from CD4+ cells from the spleens of mice with EAE.** The mice in the experiment shown in Fig 5.12 were sacrificed on Day 20 of the experiment. Spleens were removed and the spleen cells were stimulated with PMA (10 ng/ml), Ionomycin (1  $\mu$ g/ml) and Brefeldin A (5  $\mu$ g/ml) for 10 hours. The cells were then washed and surface stained for CD4 and LAP and for intracellular IL-17. The cells referred to as uninfected are from mice in the EAE and control antibody group and the cells referred to as FH Infected are from mice in the FH infection and EAE and control antibody group. Fig A contains representative flow cytometry dot plots and Fig B is the graphed averages of all mice in the groups.



**Fig 5.15 Recombinant TGF- $\beta$  inhibits the production of IFN- $\gamma$  but not IL-17 from MOG specific T cells from the spleens of mice with EAE.** EAE was induced as described in Fig 5.8. After the clinical signs of severe EAE (Grade 3.5-4) had appeared the mice were sacrificed and the spleens harvested. The spleen cells ( $2 \times 10^6$  cells/ml) were stimulated with medium, MOG (25 $\mu$ g/ml) or MOG and IL-23 (10ng/ml). Recombinant TGF- $\beta$  was added to specific cultures in increasing concentrations (1-10,000 pg/ml). After 72 hours, supernatants were taken and analysed for IL-17 and IFN- $\gamma$  by ELISA. \*\*\*P<0.001, \*\*P<0.01, \*P<0.05 versus cells incubated with MOG. +++ P<0.001, ++P<0.01 versus cells incubated with MOG and IL-23. These results are representative of two experiments which were carried out in triplicate.

# **Chapter VI**

## **General Discussion**





## 6.1 General Discussion

Innate immune responses are activated through PRR following binding of specific microbial components that are widely expressed by bacteria, viruses, protozoa and fungi. One of the best studied families of PRRs are the TLR. TLR 1,2,4,5,6 and 10 are all expressed on the cell surface and migrate to phagosomes after activation (371). In contrast, TLR3, 7, 8 and 9 are expressed in intracellular compartments in many cell types (371). There are three categories of TLRs ligands. Lipids and lipopeptides bind to TLR2/TLR1, TLR2/TLR6 and TLR4 (371). Proteins ligate with TLR5 while nucleic acids bind to TLR3, 7, 8 and 9 (371). TLR ligation activates intracellular signalling pathways which results in cell differentiation, proliferation or apoptosis and the production of cytokines such as IL-1, IL-6, IL-10, TNF- $\alpha$  and IL-12 (11).

Antigen presentation cells, especially DC form the interface between the innate and adaptive immune responses. The cytokines produced by DC and other cells of the innate immune system play a critical role in determining the subtype of CD4<sup>+</sup> T helper cell induced. The classical dogma proposes that TLR agonist stimulated DCs produce IL-12 which promotes the differentiation of Th1 cells. However, evidence has emerged that TLR agonists also induce the differentiation of Th17 and Tr1 cells. Veldhoen et al have reported that in the presence of TGF- $\beta$  producing Treg cells, DCs stimulated with Poly I:C, LPS or CpG induce Th17 cells (133). It was then established that TLR induced IL-6 and T cell derived TGF- $\beta$  are required for the induction of Th17 cells (133). There have also been studies showing that TLR agonist stimulation of DCs leads to differentiation of Tr1 cells. De Haan et al have demonstrated that the LPS induces IL-10 producing Tr1 cells that inhibit CD8<sup>+</sup> cells (373). Other studies have shown that inhibition of TLR agonist induced IL-10 by DCs suppresses the induction of Tr1 cells. This approach enhanced the induction of Th1 cells are thereby, the

efficacy of the pertussis vaccine and antitumour DC therapy (374, 396). It has previously been reported that the production of IL-10 by DCs is required for the induction of Tr1 cells (227, 237). This present study demonstrated that TLR induced IL-10 production by DCs is enhanced by the cAMP activator PGE<sub>2</sub>. This approach enhanced the induction of IL-10 producing T cell differentiation while inhibiting the induction of Th1 and Th17 cells. This study also shows that the pathogen derived molecule FHA also promotes IL-10 but inhibits TLR induced IL-12 production by DCs and acts as adjuvant for the induction of Tr1 cells specific for a co-administered antigen *in vivo*. FHA was unable to induce IL-10 production by DCs from TLR defective C3H/HeJ mice. There are two possible explanations for this observation. First, FHA like other pathogen derived molecules such as ES-62, may bind to TLR4 to mediate its effect (14). Second, FHA synergises with the low levels of contaminating LPS to induce IL-10 production by DCs and thereby promotes the induction of Tr1 cells. If the latter is proved correct, it would suggest that FHA and PGE<sub>2</sub> induce IL-10 producing T cells by similar mechanisms.

There has been mounting evidence demonstrating a role for autoantigen specific Th1 and Th17 cells in the inflammation that lead to organ specific autoimmune diseases, such as EAE. This study examined the ability of the IL-10 producing T cells generated using FHA or co-administered LPS and PGE<sub>2</sub> as an adjuvant, to inhibit Th1 and Th17 responses. The IL-10 producing T cells potently suppressed IFN- $\gamma$  production by Th1 cells *in vitro*. Kroenke *et al* have reported that IFN- $\gamma$  producing Th1 cells can induce EAE that is characterised by macrophage infiltration and NOS2 upregulation (168). In the same disease model O'Connor *et al* have since reported that Th1 cells initially access the CNS and that Th17 cells are recruited once the lesions are established (170). These reports suggest that Th1 cells direct considerable amounts of the pathology observed in EAE and are vital for the initiation of

disease. Methods to generate Tr1 cells that suppress pathogenic Th1 cells may therefore assist in the development of future therapeutics against autoimmune disease.

This study also investigated the potential of IL-10 producing T cells to inhibit Th17 cells which have been shown to direct the recruitment of neutrophils to CNS lesions in EAE (168). Antigen specific Tr1 cells generated *in vivo* using FHA as the adjuvant, potently inhibited Th17 cell proliferation and reduced IL-17 production by the cells. However the inhibition of IL-17 production by T cells was not as substantial as the suppression of antigen specific IFN- $\gamma$  production. Furthermore, the IL-10 producing T cells generated *in vivo* by immunization with antigen in the presence of LPS and PGE<sub>2</sub> did not inhibit IL-17 production by T cells. The resistance of Th17 cells to suppression by Treg cells was recently confirmed in an autoimmune gastritis (AIG) model where disease is induced by gastric parietal cell antigen specific Th1, Th17 or Th2 cells (397). The transfer of polyclonal Treg cells significantly reduced the mean gastritis score in Th1 and Th2 cell mediated AIG, but not in Th17 cell mediated disease (397).

Despite the resistance of Th17 cells to suppression by IL-10 producing T cells, this report demonstrates that MOG specific Treg cells generated by using FHA as an adjuvant, attenuate the development of EAE. This observation suggests that other mechanisms are involved in the inhibition of autoimmune inflammation by the Treg cells. Maloy *et al* have shown that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells inhibit both T cell mediated and T cell independent intestinal inflammation by an IL-10 and TGF- $\beta$  dependent mechanism (389). The FHA induced MOG specific Tregs may therefore inhibit the mononuclear cells such as macrophages and neutrophils that are crucial mediators of the inflammation in EAE (151, 168). Alternatively,

the Treg cells may inhibit the production of inflammatory cytokines that promote the induction of Th1 and Th17 cells.

A number of studies have shown that there is a defect in Treg cell function in patients with MS. Viglietta *et al* have reported that CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells from the peripheral blood of MS patients have a significant decrease in effector function when compared with cells from healthy donors (398). Other studies have identified a dysregulation in Tr1 cell induction in patients with MS. Astier *et al* have shown that CD46 activated Tr1 cells from MS patients produced little or no IL-10 (399). These findings have generated considerable interest in Treg cell based therapies for MS. Roncarolo and colleagues have already demonstrated the clinical potential of Tr1 cells. They report that the infusion of donor derived IL-10 anergized T cells prevented graft versus host disease (GVHD) after haematopoietic stem cell transplantation (195). Glatiramer acetate, a drug that is widely used to treat MS has been reported to induce the generation of Foxp3<sup>+</sup> T reg cells in humans (400). Collectively these findings suggest that methods for Treg cell induction, such as those defined in this report, may be used in future therapies for autoimmune disease and in the prevention of transplant rejection.

The founding principle of the hygiene hypothesis was that Th2 cell mediated allergies could be counteracted by microbial pathogens that induce Th1 cells. Recent studies reporting that helminth infections that induce Th2 cells are associated with a reduction in the inflammation in allergic disease, has resulted in a major shift in this theory. The current revised hygiene hypothesis proposes that helminth induced regulatory mechanisms are associated with the inhibition of Th2 mediated allergic disease. The immunological mechanisms involved are beginning to be elucidated. Wilson *et al* have reported that allergic airway inflammation is

attenuated in mice infected with *H. polygyrus* and identified CD4<sup>+</sup>CD25<sup>+</sup> cells as the most active population involved in the suppression of inflammation (328). IL-10 and B cells have been shown to play a critical role in the protective effect of *S. mansoni* infection in systemic fatal anaphylaxis (329). Other studies have demonstrated that helminth infection can attenuate the inflammation which results in autoimmune disease. Infection with *S. mansoni*, *T. spiralis* and *H. polygyrus* have been shown to prevent the development of diabetes in NOD mice (331, 390). *S. mansoni* infection has also been shown to prevent the development of colitis in a mouse model, by a macrophage dependent mechanism (332). The present study demonstrated that mice infected with *F. hepatica* significantly attenuated the development of EAE. Infected mice exhibited reduced MOG specific IFN- $\gamma$  and IL-17 production when compared with uninfected mice with EAE. It was also shown that the attenuation of EAE by *F. hepatica* infection was independent of IL-10 production and but was mediated by a TGF- $\beta$  dependent mechanism. This is therefore the first report defining a crucial role for a helminth induced cytokine in the suppression of an autoimmune disease and it also demonstrates that helminth induced regulatory mechanisms inhibit autoantigen specific Th17 responses.

The administration of recombinant TGF- $\beta$  *in vivo* has previously been shown to attenuate the clinical symptoms of EAE, even when given during ongoing disease (401). The present study demonstrated that TGF- $\beta$  inhibits IFN- $\gamma$  but not IL-17 production by MOG specific T cells *in vitro* suggesting that analogous to what was observed with Tr1 cells, Th17 cells are resistant to TGF- $\beta$  mediated suppression. Conversely, recombinant TGF- $\beta$  did suppress TLR induced IL-23, IL-1 and IL-12 production by DCs. IL-23 and IL-1 are crucial for the development of pathogenic Th17 cells (136, 181). It is therefore possible that helminth induced TGF- $\beta$  inhibits the innate cytokines that promote the induction and expansion of autoantigen specific Th17 cells.

The helminth *T. suis* is currently been used to treat patients with Crohns disease (334). Although *F. hepatica* does infect humans, it could never be used therapeutically as infection is associated with fever, liver damage and abdominal pain (402). However, the excretory-secretory product of *F. hepatica* may have clinical potential. Studies have shown that the transfer of MOG specific Treg cells generated in mice immunized with MOG and ES attenuate the clinical symptoms of EAE (Miriam Brady, unpublished observations).

This study also demonstrated that Th17 cells can be generated in the absence of TGF- $\beta$ . TGF- $\beta$  and IL-6 have been identified as crucial differentiation factors in Th17 cell development from naive T cells (133). Korn *et al* have previously shown that IL-21 cooperates with TGF- $\beta$  to induce Th17 cells in IL-6<sup>-/-</sup> mice (140). This study showed that EAE could be induced in mice treated with anti-TGF- $\beta$ , suggesting that TGF- $\beta$  may not be required for the generation of pathogenic Th17 cells *in vivo*.

This study has also identified novel mechanisms utilized by pathogens to evade the immune response. FHA is one of the most highly expressed proteins by *B. pertussis* and has previously been shown to induce IL-10 production by DCs and macrophages (227, 264, 294). FHA specific Tr1 cells have also been generated from the lungs of mice infected with *B. pertussis* (227). The present study demonstrated that FHA can function as an adjuvant for the induction of Tr1 cells specific for a bystander protein. It is therefore possible that during *B. pertussis* infection, FHA may also induce Tr1 cells that are specific for other pertussis antigens which would assist the bacteria in suppressing anti-pathogen immune responses. This study also showed that PGE<sub>2</sub> enhanced TLR induced IL-10 production by DCs and the induction of Tr1 cells. Many parasites including *S. mansoni* and *B. malayi* synthesize and release PGE<sub>2</sub> (403, 404). It is plausible that helminth derived PGE<sub>2</sub> may in combination with

endogenous TLR ligands induce innate IL-10 production and therefore promote the induction of helminth specific Tr1 cells.

*F. hepatica* is a very successful parasite and a recent report has suggested that 65% of cows culled in Ireland are infected with liver fluke (405). The present study focused on *F. hepatica* infected mice, but some of the findings may assist in the development of successful treatments for infected humans and livestock. The infection of mice with *F. hepatica* resulted in an influx of immune cells into the peritoneal cavity that had a regulatory phenotype and specifically expressed anti-inflammatory cytokines. Spleen cells from mice with *F. hepatica* produced high levels of IL-10 and low levels of IL-4 which suggests the induction of a modified Th2 response associated with chronic helminth infection. Studies in humans infected with *S. mansoni* and *B. malayi* have demonstrated that individuals that develop modified Th2 responses are the main reservoir for onward transmission of infection (300). The ability of *F. hepatica* to induce a modified Th2 response may help the helminth to avoid being eliminated and facilitates persistence of infection. This study also demonstrated that helminth stimulated TGF- $\beta$  production plays a major role in bystander regulatory mechanisms induced by the parasite. It is therefore possible that TGF- $\beta$  neutralization *in vivo* could result in an appropriate immune response against *F. hepatica* and clearance of the helminth by the host.

# **Chapter VII**

## **References**





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