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The effect of small molecule analogues of the immunomodulatory helminth product ES-62 on dendritic cell responses

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Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:

Date:

Abstract

ES-62, a glycoprotein secreted by the parasitic filarial nematode *Acanthocheilonema viteae,* targets immune system cells including dendritic cells (DCs), to subvert inflammatory responses. The post-translational addition of phosphorylcholine (PC) is responsible for many of the immunomodulatory properties of this molecule and as ES-62 is potentially immunogenic, and therefore unsuitable as a drug, a library of small molecule analogues (SMAs) of ES-62 based on its PC moiety has been synthesised. The aim of this project was to investigate the effects of these SMAs on DCs. From a library of 79 compounds, 6 SMAs (11a, 12b, 11e, 11h, 11i and 11k) were selected as they significantly down-regulated LPS-induced cytokine production *in vitro*. ES-62 requires TLR4 and MyD88 to mediate many of its anti-inflammatory effects. However, cytokine inhibition mediated by SMAs 11a, 12b, 11e and 11i is generally intact in TLR4 knock-out (KO) and MyD88 KO bmDCs. Cytokine inhibition was found to be mediated through inhibition of LPS-induced cytokine gene expression and to be associated with reduction in LPS-induced activation of NF-κBp65, and MAPKs p38 and ERK. The SMAs also suppress LPS-induced up-regulation of CD40 and CD86; priming a DC phenotype that inhibits the production of IFN-γ by naïve T cells *in vitro*. Pre-exposure of DCs to 11a or 11i prior to LPS stimulation *in vitro* primes the cells to suppress the expansion of Ag-specific T cells *in vivo*, and this is associated with significantly inhibited numbers of IL-17A^{+}CD4^{+} cells. Furthermore, mice subjected to collagen-induced arthritis pre-treated with 11a/12b-DCs had significantly reduced disease compared to mice inoculated with untreated DCs, and this was accompanied by a significant inhibition of IL-17^{+} cells in the draining lymph nodes. Thus overall this study has found that SMAs based on the PC moiety of ES-62 can modulate DCs such that they prime reduced T_H17 responses *in vivo*.

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List of Abbreviations

7-AAD, 7-amino-actinomycin D

Ag, Antigen

AHL, N-acetylated-L-homoserine lactones

AID, Activation –induced cytidine deaminase

ANA, autoantibodies against nuclear Ag

APC, Antigen presenting cells

APC, Allophycocyanin

ASC, Apoptosis-associated speck-like protein containing CARD

ASM, acid sphingomyelinase

ATM, Ataxia telangiectasia mutated kinase

ATP, Adenosine triphosphate

BAFF, B cell activating factor

BCR, B-cell receptor

BLP, Bacterial lipopeptide

Bm, Bone marrow

bmDC, Bone marrow-derived dendritic cells

BMMC, bm-derived mast cells

BSA, Bovine serum albumin

CBP, CREB-binding protein

CIA, Collagen induced arthritis

CLR, C-type lectin receptors

CpG, unmethylated 2′-deoxyribo(cytidine-phosphateguanosine)

CSR, Class switching DNA recombination

CTL, Cytotoxic T cells

DAMP, Damage-associated molecular pattern

DC, Dendritic cells

dLN, draining lymph node

DNA, Deoxyribonucleic acid

dsRNA, Double stranded RNA

DUSP, Dual-specificity phosphatases

EAE, Experimental autoimmune encephalomyelitis

EBI3, Epstein-Barr virus-induced gene 3

EBV, Epstein-Barr virus

EDTA, Ethylene diamine tetra acetic acid

ELISA, enzyme-linked immunosorbent assays

ENTPD1, Ectonucleoside triphosphate diphosphohydrolase 1

ER, Endoplasmic reticulum

ERK, Extracellular-regulated kinase

ES, Excretory- secretory product

ES-62, Excretory-secretory product 62

FACE, Fast-activated cell-based ELISA

FACS, Fluorescence activated cell sorting

FAM, 5’-6-carboxyfluorescein

FcR, Fc receptor

FCS, Fetal calf serum

FhTeg, *Fasciola hepatica* tegumental coat

FITC, Fluorescein isothiocyanate

FOXP3, Forkhead box P3

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

GATA3, GATA binding protein 3

GM-CSF, Granulocyte/macrophage colony stimulating factor

gp130, Glycoprotein 130

GPI, glycosylphosphatidylinositol proteins

HEL, Hen egg lysozyme

HES, *Heligomoisoides polygyrus* exrecetory/secretory products

HO-1, Heme oxygenase-1

HRP, Horse radish peroxidase

HSP60, Heat shock protein 60

IFN, Interferon

Ig, Immunoglobulin

Ii, invariant chain

IκB, NF-κB inhibitory proteins

IKK, I-kappa B kinase complex

IL, Interleukin

IL-6R, IL-6 receptor

IL-6Rα, IL-6 receptor-α

IL-10R, IL-10 receptor

IL-12R, IL-12 receptor

IL-27RA, IL-27 receptor

iNOS, Inducible nitric oxide synthase

IP3 , Inositol triphosphate

IRAK, IL-1 receptor-associated kinase

IRF, Interferon regulatory factors

IFN-γ, Interferon-γ

ITAM, Immuno-receptor tyrosine-based activatory motif

JAK, Janus kinase

JNK, c-Jun N-terminal kinases

kDa, Kilo Daltons

KO, Knock-out

LBP, Lipopolysaccharide binding protein

LN, lymph node

LPS, Lipopolysaccharide

LTA, Lymphotoxin alpha

MAP, Mitogen activated protein

MAPK, MAP kinases

MAPK-APK, MAPK-activated protein kinases

MAPKK, MAP kinase kinases

MAPKKK, MAPKK kinases

MARCH1, membrane associated RING-CH

MD-2, Myeloid Differentiation factor-2

MHC, Major Histocompatibility Complex

MKK,

MyD88, Myeloid Differentiation factor-88

NEMO, NF-κB essential modifier

NES, *Nippostrongylus brasiliensis* excretory/secretory products

NFAT, nuclear factor of activated T cells

NF-κB, Nuclear factor-kappa B

NK, Natural killer cells

NLK, Nemo-like kinase

NLR, NOD-like receptor

NOD, nucleotide binding and oligomerization domain

OVA, Ovalbumin

OxPAPC, oxidised phospholipids

PAF, Platelet activating factor

PAFR, Platelet activating factor receptor

PAMP, Pathogen associated molecular patterns

PBS, Phosphate buffered saline

PC, Phosphorylcholine

pDC, plasmacytoid DCs

PDMC, Peritoneal-derived mast cells

PE, Phycoerythrin

PIP2 , Phosphatidylinositol 4,5-bisphosphate

PI-3-K, Phosphoinositide-3-kinase

PKC, Protein Kinase C

PLC, Phospholipase C

PLD, Phospholipase D

PolyI:C, Polyinosinic-polycyidylic acid

PRR, Pathogen recognition receptor

RA, Rheumatoid arthritis

RIG, Retinoic acid-inducible gene

RING, Really interesting new gene

RIP, Receptor-interacting protein

RLR, RIG-I-like recptor

RNA, Ribonucleic acid

ROR-γt, Retinoic acid receptor-related orphan receptor-γt

RT-PCR, Reverse transcription polymerase chain reaction

SARM, Sterile α and HEAT-Armadillo Motifs

SAv-HRP, Streptavidin HRP

SEA, Schistosome soluble egg antigen

SHM, Somatic hypermutation

SHP-2, Src homology domain containing protein tyrosine phosphatase-2

SLE, Systemic lupus erythematosus

SMA, Small molecule analogue

SOCS, Suppressor of cytokine signalling

ssRNA, Single stranded RNA

STAT, Signal transducer and activator of transcription

TAB, TAK1 Binding Protein

TACE, TNF-α converting enzyme

TAK1, Transforming Growth Factor-β-Activated Kinase-1

TANK, TRAF-family-member associated nuclear factor-κB (NF-κB) activator

T-bet, T box 21

TBK, TANK-binding kinase 1

TCR, T-cell receptor

TGF-β, Transforming growth factor β

T helper cells; **T_H**, $CD4^+$ T cells

TIR, Toll/Interleukin-1 receptor

TIRAP/Mal, TIR-containing adaptor protein/MyD88 adaptor-like

TLR, Toll-like receptor

TMB, Tetramethylbenzidine

TNF-α, Tumour necrosis factor-α

TNFR, TNF Receptor

TRAM, TRIF-related adaptor molecule

TRAF, TNF receptor-associated factor

TRAF6, TNF Receptor-Associated Factor-6

Treg , Regulatory T_H cells

TRIF/TICAM-1, TIR-containing adaptor inducing IFN-β/TIR containing adaptor molecule-1

Ubc13, Ubiquitin carrier protein N

WT, Wild type

g, gram

mg, milligram

μ**g**, microgram

μ**M**, micromolar

ml, millilitre

μ**l**, microlitre

rpm, revolutions per minute

g, times gravity

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Chapter 1: Introduction

1.1 Introduction

Helminth infections are still very common throughout the world, despite the availability of effective treatments and preventative measures. It is currently estimated that over one billion people are infected with one or more helminths, with the vast majority of infected people residing in developing countries in Asia, Sub-Saharan Africa and Latin America. There are two major phyla of helminths: the nematodes (or roundworms) and the platyhelminths (flatworms). The nematodes include the intestinal helminths, which cause the most infections worldwide, and the filarial nematodes which cause onchocerciasis and lymphatic filariasis. The flatworms include the flukes such as the schistosomes, and the tapeworms [1]. Helminth infections typically induce a strong T_H2 response with associated modulatory anti-inflammatory responses; the latter enabling the pathogens to survive in the host, sometimes for up to ten years, without causing any overt pathology. The resulting immunological phenotype can have a significant effect on the ability of the host to react to subsequent secondary infections, as helminth infections have been shown to suppress the necessary T_H1 responses required for immunity to diseases such as tuberculosis and malaria. This helminth-induced suppression of immune responses may help to explain the inverse correlation observed between helminthiasis and atopy, asthma, colitis, type 1 diabetes, arthritis and other autoimmune diseases. Certainly, there has been a sudden increase in these diseases in developing countries that appears to coincide with the elimination of childhood infections such as helminthiasis. The 'Hygiene' or 'Old Friends' Hypothesis, suggests that environmental microbes, such as helminths and gut microflora, we have co-evolved with, have developed means to regulate our immune system to promote their own survival. The elimination of these pathogens in a short period of time, evolutionarily speaking, could have led to immunoregulatory defects in our immune systems which make us more susceptible to autoimmune and allergic diseases [2]. The link between parasite infections and rheumatoid arthritis was first suggested by Greenwood in 1968 who noted the lack of arthritis cases in West Africa [3]. It has now been demonstrated through the use of human and murine studies that helminth infections can suppress the immune responses to alleviate autoimmune and allergic diseases such as asthma and rheumatoid arthritis. Proteins secreted by helminths within the host have also been demonstrated to modified immune responses and protect against disease in murine immune systems. Several of these helminth molecules have been well characterised and ES-62 is one of the most studied helminth products. It is a glycoprotein secreted by the rodent filarial nematode

Acanthocheilonema viteae, and has been found to have potent immunomodulatory properties in multiple cells types, and has also been demonstrated to be protective in several immune disease models including collagen-induced arthritis, ovalbumin-induced airway hyper-responsiveness and the MRL/lpr mouse model of lupus.

In this chapter I will review in greater detail the components of both the innate and adaptive immune system, focussing on the mechanisms of pathogen recognition by the innate immune system, specifically dendritic cells and their central role in priming the adaptive immune system and the different T cell phenotypes that can be induced. I will then introduce helminths and go into detail regarding the immune response they promote before exploring the mechanisms they use to generate this response, focussing on excretory-secretory molecules produced by helminths within the host. Specifically this chapter will describe the immunomodulatory effects of the secreted glycoprotein ES-62 on the immune system and introduce the concept of small molecule analogues of ES-62.

1.2 The innate immune system

The immune system has evolved through the selective pressure of infectious pathogens and all multi-cellular organisms have the capacity to trigger some form of defensive mechanism against invading microorganisms. There are two branches of the immune system in vertebrates: the innate and the adaptive immune system. The innate immune system is the first line of defence and is found in all organisms. It does not react with the same specificity as the adaptive immune system however it can respond immediately to pathogen invasion and therefore we rely on our innate immune response in the first few days of infection. After recognition most pathogens are quickly engulfed by phagocytic cells such as macrophages which use a combination of degradative enzymes, reactive oxygen species and antimicrobial peptides to kill the invading microorganisms. In addition they help to promote an inflammatory response and secrete signalling molecules to recruit immune cells such as neutrophils, monocytes and dendritic cells to the site of infection. Neutrophils are short-lived cells that are normally found in the blood and are amongst the first cells to be recruited in large numbers to the site of infection. There are multiple other innate cell types that are essential in the immune response including eosinophils, basophils, mast cells and innate lymphoid cells. Mast cells are found in the mucous membranes and connective tissues and release a number of factors such as histamine and pro-inflammatory cytokines upon activation which are essential for protection against parasites but have

also been implicated in allergic reactions. Basophils and eosinophils are related to neutrophils and, like mast cells, are also essential for immunity to parasites but are also involved in allergy. Dendritic cells are phagocytic cells that are often referred to as the 'sentinels' of the immune system as they patrol the periphery, constantly sampling the environment. They are also recruited to the site of infection by complement proteins and cytokines. Dendritic cells provide the critical link between the immune systems as after recognition and uptake of pathogens in the periphery they migrate to the draining lymph nodes where they present the antigen in the context of MHC II molecules on their surface, along with co-stimulatory molecules, to T cells to prime and direct the activation of the adaptive immune system.

The innate immune system was originally thought to be a crude, non-specific response that merely contained infection until 'real' immunity – the adaptive system - kicked in [4]. However, in 1989 Charles Janeway proposed that the innate system, via germ line-encoded receptors termed Pattern Recognition Receptors (PRRs), could specifically recognise conserved molecular structures on pathogens to initiate an immune response and to discriminate between self and non-self antigen [5]. These conserved molecular structures, known as Pathogen-Associated Molecular Patterns (PAMPs), are generally essential for microbe survival and as such are less subjected to mutations [6]. A classic PAMP example is lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria. It is secreted in minute amounts during infection and as the lipid A structure within it is conserved throughout all species of Gram-negative bacteria recognition of this structure allows the innate immune system to detect all forms of infection by this type of pathogen [7]. These receptors are also able to recognise endogenous proteins released from damaged or stressed cells, collectively called Damage-Associated Molecular Patterns (DAMPs) [8]. It is in this way that a limited number of receptors can recognise a diverse range of pathogens. There are currently four known classes of PRRs: the transmembrane proteins, Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and the cytoplasmic proteins, nucleotide binding and oligomerization domain (NOD) -like receptors (NLRs) and Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) [8]. TLRs recognise a wide variety of pathogens and are discussed in more detail below. The CLRs are carbohydrate-recognising PRRs found on the plasma membrane of phagocytes. They mediate recognition primarily through mannose, fucose and glucan structures allowing them to sense most classes of human pathogen [9]. CLR activation can mediate a diverse range of immune responses including activation of NF-κB and cross-talk with TLR signalling pathways

[10]. The discovery of the two cytoplasmic families of receptors indicated that there was a ‘second line’ of PRRs able to recognise any pathogens that got past the extracellular receptors. RIG-I-like receptors have been shown to recognise viral dsRNA and play a key role in the generation of antiviral responses in various immune system cells [11] [12]. The NOD-like receptors play multiple roles in the inflammatory response. For example, NLR family members Nalp1 and Cryopyrin recruit the adaptor protein ASC to form the NLRP3 inflammasome that regulates caspase-1 [13] [14] while other NLR members can recognise molecules from the synthesis or degradation of bacterial peptidoglycan [15]. Through the recognition of peptidoglycan NLRs can generate a response against these bacterial pathogens in synergy with TLRs. They have also been shown to play a role in host defence against respiratory syncytial viral infection through RNA-induced type I interferon production [16].

1.3 The Toll-like receptors

The Toll-like receptors (TLRs) are the best understood subgroup of PRRs and were the first to be identified. They sense a wide variety of PAMPs and recognition by these receptors induces a downstream signalling cascade that results in the activation of transcription factors such as NF-κB and IRF3, as well as mitogen-activated protein kinases (MAPKs), leading to the production of pro-inflammatory cytokines, type 1 interferons and the up-regulation of co-stimulatory molecules on DCs. TLRs are type 1 integral membrane proteins consisting of three major domains: a leucine-rich extracellular domain that mediates PAMP recognition; a transmembrane domain, and a cytoplasmic tail domain that is essential for signal transduction. To date, 10 and 13 TLRs have been identified in humans and mice respectively, TLR1-9 are conserved between mice and humans but TLR11, 12 and 13 have been lost from the human genome and TLR10 is non-functional in mice due to a retrovirus insertion [17]. TLRs are expressed on multiple immune system cells including antigen presenting cells, B cells, specific T cell subsets and mast cells, as well as on non-immune system cells such as fibroblasts and epithelial cells. Cellular expression of each TLR varies across cell types with haematopoietically-derived cells such as dendritic cells expressing almost a full repertoire [18]. TLRs each respond to different stimuli and their cellular location largely depends on the type of ligand they recognise. TLRs that recognise bacterial components such as lipids, lipoproteins and proteins (TLR 1, 2, 4, 5 and 6) are all expressed on the cell surface while TLR 3, 7, 8 and 9, which largely recognise

viral products, are located intracellularly on endosomal membranes and the endoplasmic reticulum (ER) [18] [19].

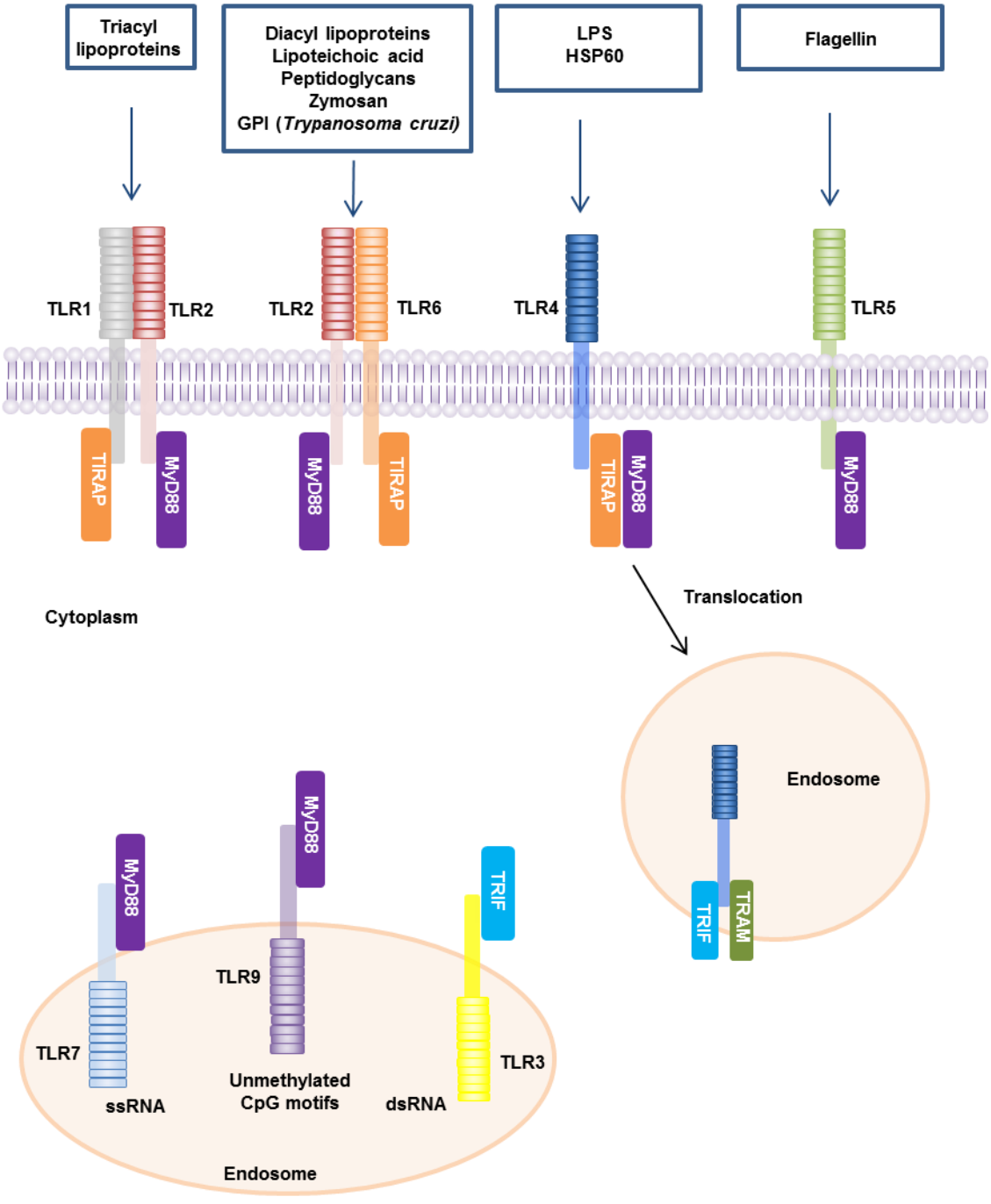
The TLRs present on the cell surface are key receptors for the recognition of bacteria, generally through recognition of conserved membrane components. The most important immune-stimulant from Gram-negative bacteria is LPS and this component is recognised by the first TLR discovered, TLR4. Gram-positive bacterial membranes do not contain LPS, instead the main component that stimulates innate immunity is lipoteichoic acid (LTA). TLR2 plays a key role in recognising Gram-positive bacteria through LTA [20]. The receptor also recognises lipoproteins which are present in both groups of bacteria and interacts with TLR1 and TLR6, which require dimerisation with TLR2 to be functional, to facilitate the discrimination between triacylated and diacylated lipoproteins respectively [21] [22]. TLR2 is also known to interact with non-TLR proteins for the recognition of peptidoglycan, *Trypanosoma cruzi* glycosylphosphatidylinositol-anchored-mucin-like glycoproteins, porins and haemagluttinin proteins and so is important for recognition of bacteria-, virus and parasite-derived proteins [19]. TLR5 is required for recognition of the constant D domain of flagellin, the major component of flagella; the motility apparatus of Gram-negative and Gram-positive bacteria [23]. The intracellular TLRs – TLR3, TLR7, TLR8 and TLR9 - mainly recognise nucleic acids and are important in host defence against viruses. TLR3 was originally found to recognise polyinosinic-polycytidylic acid (PolyI:C), a synthetic double-stranded RNA (dsRNA) [24] and was subsequently discovered to recognise genomic RNA of reoviruses and the dsRNA produced by replication of single-stranded RNA (ssRNA) by viruses [17]. The genes for TLR7 and 8 show high homology. Murine TLR7 and human TLR8 recognise ssRNA from RNA viruses such as influenza A and HIV as well as RNA from bacteria. TLR8 is present in mice but appears to be non-functional [17]. The other intracellular receptor, TLR 9 recognises unmethylated 2′-deoxyribo(cytidine-phosphate guanosine) (CpG) DNA motifs common in bacteria and viruses but rare in mammalian cells [25] [26]. Recognition of foreign nucleic acids by these TLRs triggers potent anti-viral immunity characterised by production of type I interferons and pro-inflammatory cytokines [17]. It has also been shown that TLR7, 8 and 9 can interact with each other [27] further enhancing the range of molecules they can recognise. The location of the TLRs is shown in Figure 1.1.

The first step in TLR identification occurred when Jules Hoffmann's group discovered that the Toll protein in *Drosophila melangaster* played a key role in the insect's defence

against invading microorganisms [28]. A key mechanism of host defence in the fruit fly is the rapid synthesis of peptides in response to infection. There are two types of peptides – antimicrobial peptides such as cecropins, and an antifungal peptide drosomycin – and expression of these has been shown to be independently controlled [29]. The Toll protein was found to be involved in the control of the expression of the drosomycin gene in adult flies and is important for fly survival during fungal infection [28]. Mammalian homologues to Toll had been predicted in PubMed database in 1994 but the first one, termed hToll, was first cloned and studied by Ruslan Medzhitov and Janeway in 1997 [30]. They demonstrated that transfection of human monocytes with a $CD4^{+}$hToll chimeric protein induced the activation of NF-κB-dependent genes. By 1998 five mammalian Toll homologues had been described and were re-named Toll-like receptors, including hToll which had been re-named TLR4 [31].

Figure 1.1: Toll-like Receptors

Schematic diagram of the different murine toll-like receptors with their adaptor proteins, cellular location and primary ligands. Abbreviations: GPI – glycosylphosphatidylinositol-anchored proteins; LPS – lipopolysaccharide and HSP60 – Heat shock protein 60


Triacyl lipoproteins
Diacyl lipoproteins
Lipoteichoic acid
Peptidoglycans
Zymosan
GPI (Trypanosoma cruzi)
LPS
HSP60
Flagellin
TLR1
TLR2
TLR2
TLR6
TLR4
TLR5
TIRAP
MyD88
MyD88
TIRAP
TIRAP
MyD88
MyD88
Cytoplasm
Translocation
Endosome
TRIF
TRAM
MyD88
MyD88
TRIF
TLR9
TLR7
TLR3
ssRNA
Unmethylated
CpG motifs
dsRNA
Endosome

1.3.1 LPS recognition by TLR4

The ligand for TLR4 was identified using genetic studies with the C3H/HeJ mutant mouse strain. These mice have a defective response to LPS as a result of a spontaneous mutation in the *lps* locus (*lps*^{d}) which became fixed in the population in the 1960's. Positional cloning of this mutation identified *lps*^{d} locus as the gene expressing TLR4 [32]. It was then shown that TLR4 knock-out mice were unable to respond to LPS, indicating that TLR4 was required for LPS recognition *in vivo* [20]. LPS is a glycolipid in the outer cell wall of Gram-negative bacteria and is composed of core hydrophilic polysaccharides, an O-Antigen and an amphipathic lipid A component which is the conserved molecular structure and main inducer of immune responses against LPS [7]. TLR4 cannot bind LPS alone, it requires several other proteins - lipopolysaccharide binding protein (LBP), CD14 and myeloid-differentiation factor 2 (MD-2) in order to recognise the PAMP. TLR4 and MD-2 form a heterodimer that recognises the conserved lipid A component of structurally diverse LPS molecules. LBP extracts LPS from the outer bacterial wall and facilitates binding to CD14 which then presents LPS to TLR4/MD-2 on the cell surface [33].

1.3.2 Downstream signalling by TLRs following binding of cognate antigen

Upon binding their cognate ligand TLRs form homo- or hetero-dimers that are essential in the recruitment of adaptor proteins containing the Toll-interleukin 1 Receptor (TIR) homology domain that mediate further downstream signalling pathways. There are five known TIR-containing adaptor proteins – myeloid differentiation primary-response protein 88 (MyD88), MyD88-adaptor-like protein (MAL; also known as TIRAP), TIR domain-containing adaptor protein inducing IFNβ (TRIF; also known as TICAM1), TRIF-related adaptor molecule (TRAM; also known as TICAM2) and sterile-α- and armadillo-motif-containing protein 1 (SARM1). A sixth protein termed B cell adaptor for PI3K (BCAP; also known as PIK3AP1) which contains a domain that is related to the TIR domain has been proposed as a sixth adaptor protein [31]. TLR signalling pathways can be classified as being a MyD88-dependent pathway, which drives the production of pro-inflammatory cytokines or a TRIF-dependent pathway, which induces type 1 interferon production as well as inflammatory cytokines. MyD88, a TIR and death domain containing protein, was the first adaptor protein to be identified and is essential for all TLR signalling pathways excluding TLR3 and activates the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) to induce pro-inflammatory cytokines. TRIF is used by TLR3 and TLR4 to induce alternative

pathways that result in the activation of transcription factors IRF3 and NF-κB for the induction of inflammatory cytokines and type 1 interferons. MAL and TRAM are used as bridging adaptors to TLR4 by MyD88 and TRIF respectively. MAL is also used to recruit MyD88 to TLR2. TLR4 is the only TLR to utilise all four adaptor proteins and so it activates the MyD88- and TRIF-dependent pathways. Both pathways are required for NF-κB activation and robust production of pro-inflammatory cytokines [34].

1.3.3 MyD88-dependent pathway

Upon recruitment to TLR4 by MAL, MyD88 interacts with members of the IL-1R-associated kinase (IRAK) family via homophilic interaction with the death domains. There are four members of the IRAK family – IRAK1, IRAK2, IRAK4 and IRAKM. IRAKM functions to inhibit TLR responses - IRAKM-deficient mice show increased pro-inflammatory cytokine production [35]. IRAK4 is activated initially and has an essential downstream role in NF-κB and MAPK activation [36]. IRAK1 and IRAK2 are activated sequentially and are required for robust activation of NF-κB [35]. IRAK activation then recruits tumour necrosis factor receptor associated factor 6 (TRAF6), an E3 ubiquitin ligase, which contains a highly conserved RING domain that is essential for NF-κB activation. TRAF6 functions in conjunction with the E2 ubiquitin-conjugating enzymes Ubc13 and Uev1A to mediate the assembly of K63-linked polyubiquitin chains on target proteins, including TRAF6 itself [37] [38]. Ubiquitination is the reversible covalent addition of ubiquitin, catalysed by three ubiquitin enzymes – E1, E2 and E3. Initially ubiquitin is activated in an ATP-dependent reaction by ubiquitin-activating enzyme E1. Activated ubiquitin is then transferred to E2, an ubiquitin-conjugating enzyme. E3 ubiquitin ligases then attach ubiquitin to a target protein via an isopeptide bond between the carboxy terminal on ubiquitin and the amino terminal on a lysine residue on the target protein. Ubiquitin itself has 7 lysine residues and so can attach to another ubiquitin to create a polyubiquitin chain, usually on lysine63 or 48 [38]. These K63-linked polyubiquitin chains bind to TGF-beta activated kinase 1 (TAK1), MAP3K7 binding protein 2 (TAB2) and TAB3 via a highly conserved zinc finger domain (NZF). These proteins make up the regulatory components of the TAK1 complex, which activates TAK1 and the NF-κB essential modulator (NEMO), the regulatory component of the IκB complex. Upon activation TAK1 phosphorylates IKKβ at two serine residues (ser32 and ser36) leading to the phosphorylation and subsequent degradation of IκB proteins, which frees NF-κB to translocate to the nucleus. TAK1 also induces phosphorylation of MKKs leading to the activation of JNK and p38 kinase pathways

[38]. Cellular inhibitors of apoptosis 1 and 2 (cIAPs) are RING domain ubiquitin E3 ligases, which are also recruited to MyD88 and bind to TRAF6-catalysed K63 polyubiquitin chains, which promotes K48 polyubiquitin chains and causes the subsequent degradation of TRAF3. This then allows the cytosolic translocation of MyD88, TAK1 and MAPKs, resulting in the activation of MAPKs and pro-inflammatory cytokines [34] [39].

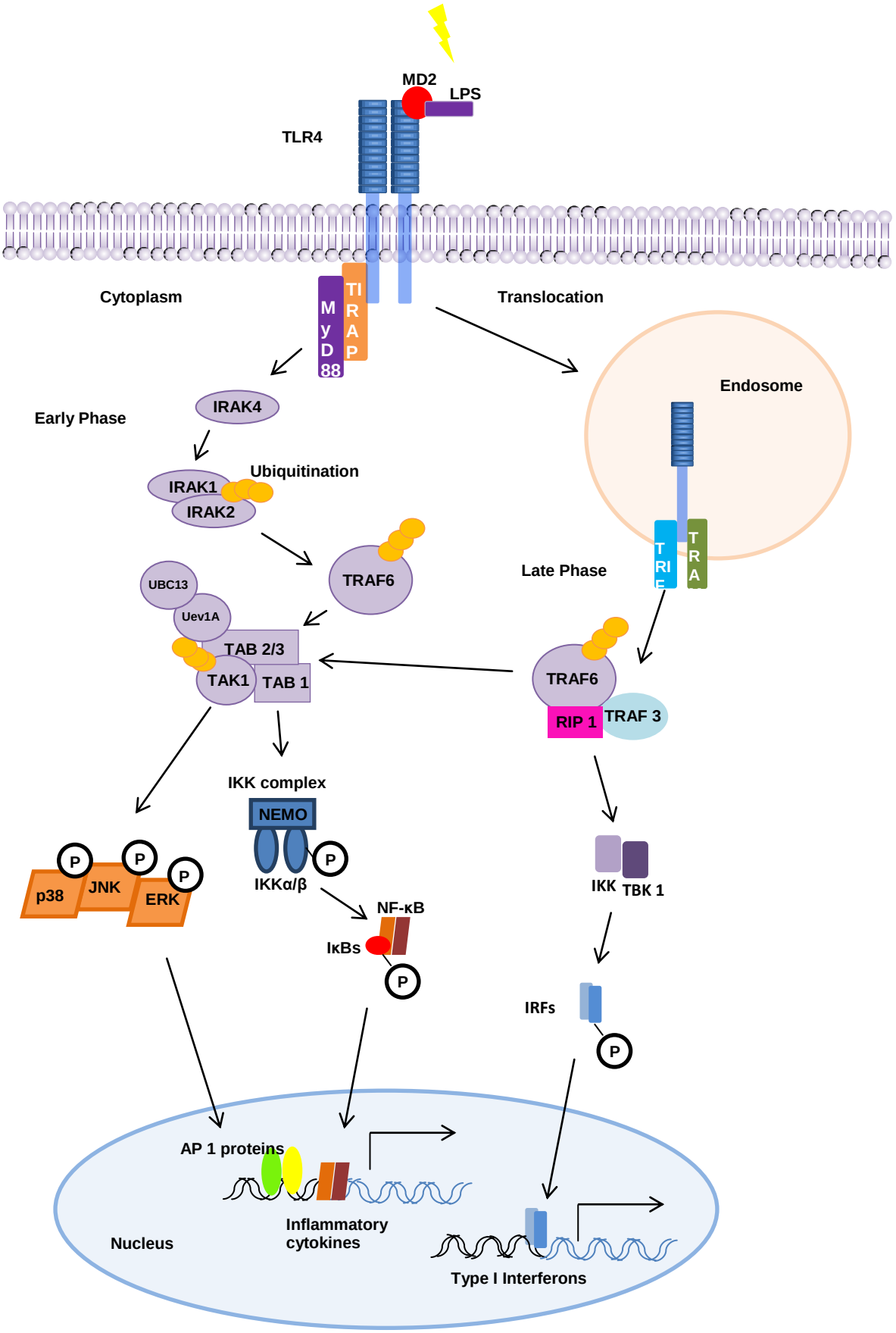
1.3.4 MyD88-independent pathway

The MyD88-independent pathway is unique to TLR4 and TLR3 and leads to the production of interferon (IFN)-β as well as the production of pro-inflammatory cytokines. The adaptor protein TRIF is essential for this pathway. A fourth adaptor protein, TRAM, is required to serve as a bridging adaptor between TLR4 and TRIF, in a similar role to MAL in the MyD88-dependent pathway. Initially, TRIF binds TLR4 via TRAM and recruits the death domain kinases receptors interacting protein (RIP)-1 and RIP3 through its RIP homotypic interaction motif [40] and TRAF 6 to mediate downstream activation of NF-κB in a manner that is likely very similar to the MyD88 pathway [41]. TRIF also activates TBK1 (TRAF-family-member associated nuclear factor-κB (NF-κB) activator (TANK)-binding kinase 1) and IKK-ε leading to the phosphorylation of transcription factor IRF3 (IFN-regulatory factor 3). This results in the dimerization of IRF3 and its association with coactivators CREB-binding protein (CBP) and p300 and the formation of the IRF3 complex which can translocate from the cytoplasm to the nucleus and mediate the expression of IFN-β [42] [43]. Both MyD88-dependent and independent TLR4 signalling pathways are summarised in Figure 1.2.

Figure 1.2: TLR4 Signalling Pathway – MyD88-dependent and MyD88-independent pathways

Binding of its cognate ligand, LPS, to TLR4 initiates downstream signalling that results in the production of inflammatory cytokines and type I interferons. Recognition of LPS causes dimerisation of TLR4 and recruits the adaptor proteins MyD88 and TIRAP. MyD88 then recruits the IRAK proteins which in turn recruit TRAF 6, an E3 ligase which promotes the ubiquitination of several proteins including Ubc13 and Uev1A and itself. UBC13 and Uev1A form an E2 ubiquitination ligase complex. Ubiquitination activates the TAB1/TAB2/3 and TAK1 complex which activates the IKK complex. This causes the phosphorylation of IκB proteins, targeting them for degradation and freeing NF-κB to translocate to the nucleus where it promotes production of pro-inflammatory cytokines. Simultaneously, activated TAK 1 also activates ERK, JNK and p38 MAPKs which activate AP-1 which are also important in cytokine induction. TRIF, via TRAM, mediates MyD88-independent effects through the activation of TRAF3 and RIP1. TRAF3 mediates downstream activation of IRFs which leads to the production of type I interferons while RIP1 is responsible for downstream activation of NF-κB which results in late phase pro-inflammatory cytokine production. Both MyD88 and TRIF activation are required for production of cytokines in response to TLR 4.

Adapted from [31]


MD2
LPS
TLR4
Cytoplasm
Translocation
MyD88
TIRAP
Endosome
Early Phase
IRAK4
Ubiquitination
IRAK1
IRAK2
TRAF6
UBC13
Uev1A
TAB 2/3
TAK1
TAB 1
Late Phase
TRIF
TRA
TRAF6
RIP 1
TRAF 3
IKK complex
NEMO
IKKα/β
P
p38
JNK
ERK
NF-κB
IκBs
IKK
TBK 1
IRFs
AP 1 proteins
Inflammatory cytokines
Nucleus
Type I Interferons

1.4 NF-κB

The NF-κB transcription factor family plays a crucial role in many cellular processes as well as a key role in activating the immune response. Members can be activated by over 200 different stimuli including ligated cell receptors such as TLRs, bacterial and viral products, mitogens, growth factors and environmental stress [44]. NF-κB was originally discovered in B cells by Sen and Baltimore in 1986 [45]. It was identified as a nuclear factor that bound to the κ light chain enhancer in B cells and was activated by LPS [45]. There are five subunits of NF-κB – RelA(p65), c-Rel, RelB, p50/ NF-κB1 and p52/ NF-κB2 – which exist as homo- or heterodimers in resting cells. All five subunits share a highly conserved Rel homology domain that is responsible for DNA binding, nuclear translocation, dimerization and interaction with IκB proteins [44]. Knockout studies have demonstrated that the different subunits play distinct roles in regulating innate and adaptive immunity as well as cell survival and lymphocyte functions [46]. Knockout of p65 is embryonically lethal due to liver degeneration but while deletion of one of the other four subunits does cause immune system deficits, mice develop normally. However, mice that lack more than one subunit have more severe phenotypes, indicating a functional redundancy between subunits. Using DCs from $RelA^{-/-}$, $p50^{-/-}$ or $c\text{-}Rel^{-/-}$ mice, Ouaaz *et al* found no differences in DC generation, maturation or APC ability but in $p50^{-/-}RelA^{-/-}$ double knock-out mice there were very few $CD11c^{+}$ cells detected [47]. Mice with both p50 and c-Rel deleted displayed normal DC generation with no difference in MHC II and co-stimulatory molecule expression – either constitutive or LPS-induced – but these DCs had reduced IL-12p40 expression and IL-12p70 production. These findings demonstrate that NF-κB subunits differentially regulate DC development and maturation [47].

In resting cells, NF-κB is associated with a family of proteins called Inhibitors of NF-κB (IκBα, IκBβ and IκBε) which maintain multiple contacts with NF-κB and this has the effect of blocking the nuclear localization sequence of NF-κB and thus sequestering it in the cytoplasm. Activation of NF-κB proteins requires the sequential phosphorylation, ubiquitination and degradation of IκBα and IκBβ [48]. Phosphorylation is mediated by the IκB kinase (IKK) complex which consists of two kinases – IκB kinase 1 and 2 (IKKα and IKKβ), and a non-catalytic element IKKγ also known as NEMO [49]. In response to stimuli such as pro-inflammatory cytokines the IKK complex is rapidly activated and phosphorylates two crucial serine residues in the N-terminal domain of IκB proteins [50] [51]. The regulatory component NEMO, although devoid of catalytic activity, is essential

for the activation of the canonical NF-κB pathway [49]. NF-κB can be activated via 3 pathways: the canonical pathway, the alternative/non-canonical pathway and the atypical pathway. The canonical pathway is triggered by microbial products and pro-inflammatory cytokines, such as IL-1 and TNF-α, and results in the activation of RelA and c-Rel complexes. The second NF-κB pathway – the non-canonical pathway- has only a few known stimuli including lymphotoxin-β and B-cell activating factor (BAFF) and results in processing of the p100 molecule to generate RelB/p52 complexes [44]. Activation of these pathways requires different IKK subunits. IKKβ regulates activation of the canonical pathway through phosphorylation of the regulatory component NEMO while IKKα appears redundant [52] but is required for the activation of the non-canonical pathway through phosphorylation and processing of p100 and p102 molecules [53]. DNA damage caused by agents such as UV radiation can activate the third, weakest, NF-κB pathway. This pathway is the least studied and to date, understanding of its physiological role is in its infancy. Progress has been made elucidating the molecular mechanisms behind how DNA damage in the nucleus can activate NF-κB sequestered in the cytoplasm however. The IKK regulatory component, NEMO, plays an IKK-independent role, mediated by the C-terminal zinc finger domain, in linking DNA damage to NF-κB activation via the ataxia telangiectasia mutated kinase (ATM) [54].

1.5 MAPKs

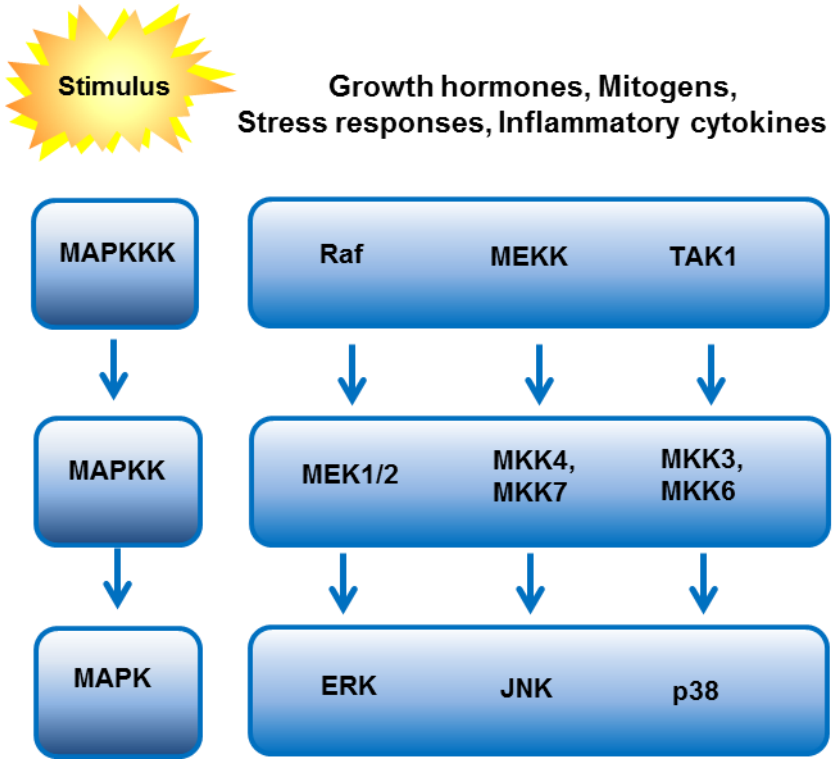
The mitogen-activating protein kinases (MAPKs) are signal transducing enzymes that play a crucial role in connecting extracellular stimuli recognised by surface receptors such as TLRs, to a wide range of regulatory targets within the cell. The signalling pathways are highly conserved and are involved in a diverse number of cellular processes including gene expression, cell proliferation, differentiation, survival and apoptosis [55]. There are currently 14 MAPKs known in mammals and they can be classified as classical or atypical MAPKs. There are four main subgroups of classical MAPKs – extracellular signal-regulated protein kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK) 1/2/3, p38 α, β, γ and δ, and ERK 5. The atypical MAPKs consist of ERK3/4, ERK 7 and nemo-like kinase (NLK) [56]. Each group of classical MAPKs is composed of a cascade of three conserved, sequentially activated kinases: MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK) (Summarised in Figure 1.3). The MAPKs share a common activation motif Thr-X-Tyr in the kinase subdomain VII activation loop and are activated by proline-directed phosphorylation of the threonine

and tyrosine residues within that activation motif by MAPKK. The MAPKKs have, in turn, been activated through phosphorylation of their serine and threonine residues by MAPKKKs, which are activated via small GTP-binding proteins, such as those from the Ras/Rho family, in response to extracellular stimuli. Once activated MAPKs can then activate a diverse range of substrates including members of a family of protein kinases termed MAPK-activated protein kinases (MAPK-APKs). MAPK substrates contain specific MAPK docking domains which mediate strong and selective interaction between the substrate and its cognate MAPK (MAPK activation is reviewed in [57] [58]. The activation of MAPKs is tightly regulated by multiple mechanisms including dephosphorylation by phosphatases, scaffolding of MAPK cascades and compartmentalisation of MAPKs within the cell [59]. As MAPKs can phosphorylate a wide range of substrates in different compartments controlling their intracellular localisation is important in order to elicit the appropriate response. For example, in resting cells ERK1/2 is found in the cytoplasm in association with MEK1 (an ERK-specific MAPKK) due to the NES sequence in the N-terminal region of MEK1. Upon stimulation, ERK 1/2 dissociates from MEK1 and moves into the nucleus [60]. MAPKs can also be deactivated by dephosphorylation of threonine and/or tyrosine in the activation motif by phosphatases. Dual-specificity phosphatases (DUSPs) play a key role in the regulation of MAPK activation but as both threonine and tyrosine must be phosphorylated for activation of MAPKs, serine/threonine phosphatases such as PP2A, and tyrosine phosphatases like PTP-SL, are also sufficient to deactivate MAPKs [61] [62].

ERK MAPK was the first MAPK to be discovered and is characterised by the motif Thr-Glu-Tyr. It is encoded by two genes – ERK1 and ERK 2. ERK MAPK appears to primarily respond to growth factors through Ras/Raf to mediate signals to control cell proliferation and survival [63], but it is also activated in a Raf-independent manner by PAMPS, DAMPS cytokines such as TNF-α. JNK MAPKs are characterised by the activation motif Thr-Pro-Tyr while p38 MAPK contains Thr-Gly-Tyr in its activation motif. These MAPKs are primarily activated in response to pro-inflammatory cytokines, hormones and environmental stress such as radiation and osmotic shock. There are three distinct genes for JNK encoding for Jnk1, Jnk2 and Jnk3 while there are four distinct isoforms of p38 - p38α, p38β, p38γ and p38δ. For both MAPK groups further structural diversity is generated through alternative mRNA splicing. JNK1 and JNK2 are ubiquitously expressed while JNK3 is brain-specific [64].

Figure 1.3: The MAPK Signalling Cascade

Schematic of mitogen-activated protein kinase (MAPK) signalling cascades, with examples of each level of the cascade. Activation of these pathways by stimuli such as inflammatory cytokines or stress responses results in activation of MAPK kinase kinases (MAPKKK) which then phosphorylate and activate MAPK kinases (MAPKK) which can then go on to phosphorylate and activate the MAPKs ERK, JNK and p38 to initiate biological responses.


Stimulus
Growth hormones, Mitogens,
Stress responses, Inflammatory cytokines
MAPKKK
Raf
MEKK
TAK1
MAPKK
MEK1/2
MKK4,
MKK7
MKK3,
MKK6
MAPK
ERK
JNK
p38

1.6 The adaptive immune system

The second branch of the immune system in vertebrates is the adaptive immune response. This generates an antigen (Ag)-specific response along with memory to infection using somatically generated Ag receptors (TCR and BCR) on lymphocytes that arise from random recombination of variable gene segments encoded in the heavy and light chain of immunoglobulin and the TCR loci. Induction of an adaptive response to a pathogen is dependent upon its recognition by PRRs on cells of the innate immune system. Although B and T cells are capable of recognising a diverse range of Ag through their receptors full activation is dependent on the cytokine and chemokine milieu produced by the innate cells along with co-stimulatory molecules present upon the APCs when presenting Ag. This is especially true for T cells. Naïve T lymphocytes recognise specific Ag presented by APCs such as DCs via their TCR. After interaction with their cognate Ag, and appropriate additional co-stimulatory signals, naive T cells become committed to clonal expansion and differentiation into effector cells called T helper cells (T_H) or cytotoxic T cells (CTLs), depending on their expression of transmembrane glycoproteins CD4 and CD8 respectively [65]. CD8+ T cells recognise Ag in the context of MHC I, promoting their differentiation into cytotoxic T cells (CTLs), which locate and destroy virus-infected cells [66]. $CD4^+$ T cells have a much more flexible repertoire in that they can be differentiated into multiple effector subsets depending on the environment they encounter Ag in. These subsets are defined by the set of transcription factors they express; their cytokine profiles and their distinct effector functions and are summarised in Figure 1.4. In 1986 Mosmann & Coffman showed mouse CD4 T cell clones could be divided into two groups they designated T_H1 and T_H2 [67]. In both cases the key driving cytokine is often also one of the major products of the mature T_H cells. Thus, IFN-γ and IL-12 are key cytokines that promote naïve $CD4^+$ T cells towards a T_H1 phenotype, which is characterised by secretion of IFN-γ. The transcription factor T-bet is required for the commitment to the T_H1 lineage. $CD4^+$ T cells from mice lacking T-bet fail to produce IFN-γ and display a 'default' T_H2 phenotype with increased IL-4, IL-5 and IL-13 production [68]. IL-12 also activates STAT4 which in turn induces IFN-γ and so the T_H1 lineage can reinforce its development through this positive feedback loop. T_H1 immunity is important in mediating cellular immunity against intracellular pathogens but an over-aggressive or dysregulated response has been linked to autoimmune diseases. By contrast, IL-4 drives T_H2 polarisation, characterised by IL-4, IL-13 and IL-5 production, which drives humoral immunity against extracellular pathogens such as helminth infections. As with

T_H1 immunity, the T_H2 response can also cause immune pathology, in this case allergic diseases and asthma. The transcription factor GATA3 is essential for the promotion of the T_H2 response, as it drives the production of T_H2 cytokines while at the same time down-regulating T-bet expression and so inhibiting the T_H1 response. It is also essential for the production of IL-5 and IL-13 [69] [70]. Originally it was thought that the T_H1- T_H2 paradigm covered all immune reactions but work with two murine models of autoimmune diseases initially designated T_H1 diseases – experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) – discovered that IL-17-producing $CD4^+$ T cells were important in terms of pathogenesis rather than IFN-γ-producing T cells. It was then discovered that IL-23 played a key role in autoimmunity [71] and was originally thought to promote T_H17 cells from naïve $CD4^+$ T cells [72] [73]. However it was subsequently shown that although IL-23 plays a role in the maintenance of T_H17 cells, IL-6 and TGF-β are essential for the initial priming of this effector subtype [74]. The T_H17 lineage is characterised by IL-17A and IL-17F, as well as IL-22 production, and is important in mediating defence against some bacterial and fungal pathogens at the mucosal barriers but is also implicated in promoting chronic inflammation in a number of autoimmune diseases including EAE, CIA and psoriasis [71]. Both *in vitro* cytokine priming of T_H17 cells and *in vivo* T_H17 mediated inflammatory responses require the expression of RORγt [75]. Interestingly, TGF-β, along with IL-10, can also induce the polarisation of a fourth $CD4^+$ effector phenotype termed T regulatory cells which play a role in regulating the magnitude of immune responses to foreign antigen as well as protecting against autoimmune diseases [76] [77]. Mice lacking T regulatory cells through a mutation in the major driving transcription factor, FOXP3, develop fatal autoimmune pathology early in life, led by $CD4^+$ effector cell responses [78]. Thus, TGF-β can induce the anti-inflammatory T regulatory effector cells or the pro-inflammatory T_H17 effector cells depending on the presence of IL-6.

The humoral branch of the adaptive immune response is mediated by B cells and is primarily responsible for protection against extracellular pathogens through the generation of antibodies. Antibodies mediate protection through a variety of mechanisms including neutralising pathogens and toxins by binding to them and preventing them from entering cells and targeting them for phagocytosis or complement-mediated degradation. Antibodies recognise specific epitopes on a wide range of pathogen-derived molecules including proteins and carbohydrates. Once Ag is bound it can be internalised for processing and presentation in the context of MHC II

and the B cell can present Ag to activated effector $CD4^+$ T_H cells, enhancing T cell activation. This interaction is bidirectional as cytokines secreted by these T_H cells which have previously been activated by interaction with APCs to the same Ag, induce B cell proliferation and some differentiate into antibody-secreting plasma cells while others enter the germinal centre where they become high affinity memory B cells [79].

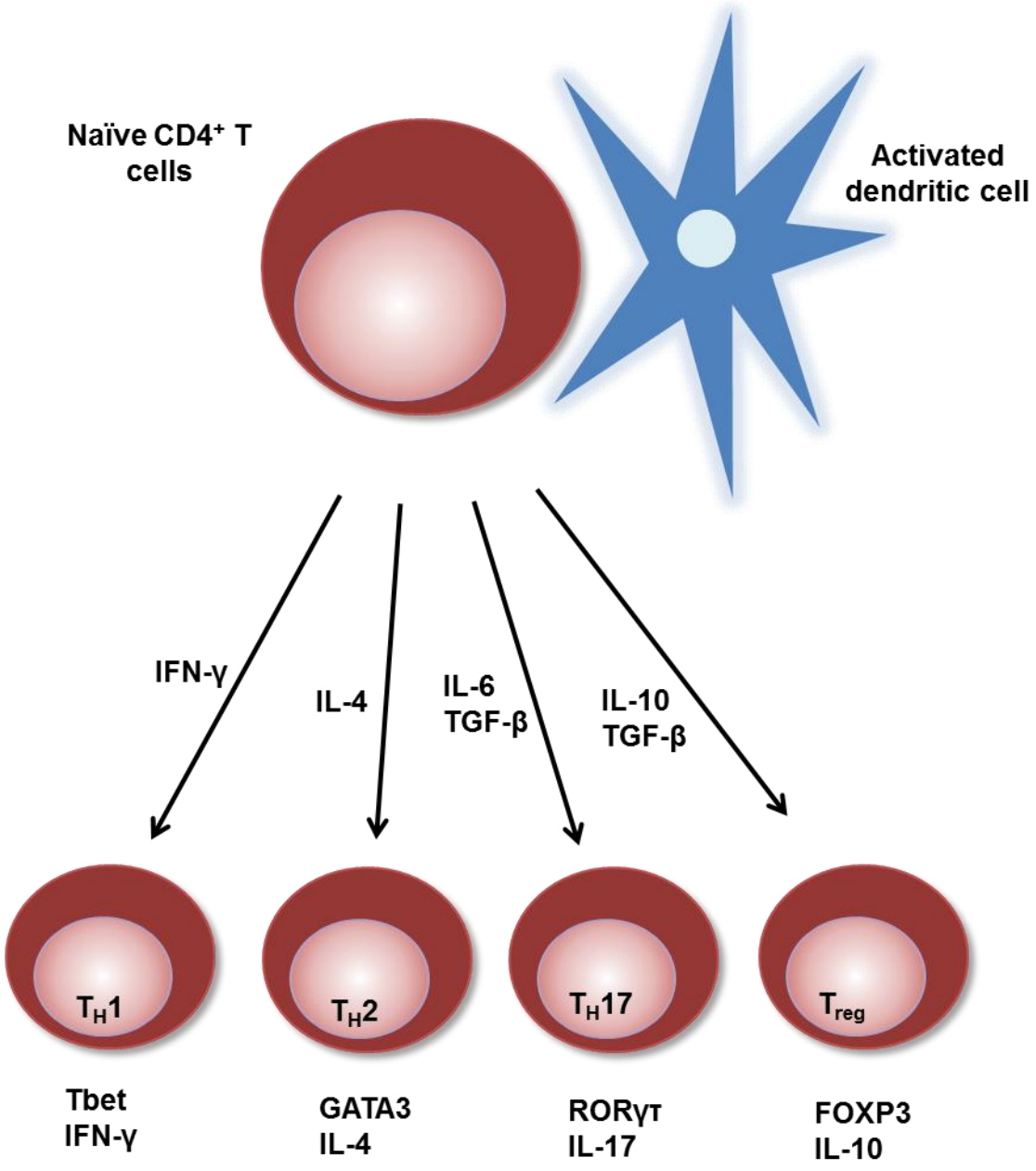
Naive B cells react to Ag by producing large amounts of IgM but following activation B cells are further differentiated by somatic hypermutation (SHM) and class switching DNA recombination (CSR), both of which require activation-induced cytidine deaminase (AID) [80]. B cells can be activated in a T cell-dependent or independent manner. Both CSR and AID are efficiently induced following T cell-dependent B cell activation but it has also been noted that IgG and IgA can also arise early during some viral and bacterial infections suggesting that CSR can in induced in B cells independently of T cells. It was subsequently discovered that BCR signalling can synergise with TLR 1/2, 4, 7 or 9 signalling to induce efficient CSR in marginal zone B cells resulting in low affinity antibody responses that can bridge the gap until high affinity antibodies can be produced by follicular B cells [80]. This difference in antibody responses by different B cell subsets demonstrates that B cell can be divided into innate-like lymphocytes and conventional, adaptive-like lymphocytes. Follicular B cells are conventional lymphocytes as they recognise highly specific epitopes via their BCR and upon Ag recognition they differentiate into high affinity antibody-producing plasma cells or memory cells, and these processes are maximised by T_H cells. Marginal zone B cells, as well as B-1 cells, are known as innate-like lymphocytes and they have a recurrent expression of families of germ-line encoded BCRs with a limited diversity that often recognise self-antigens that arise through stress or tissue damage. These cells have an activated/memory or effector phenotype and are able to react instantly to Ag indicating they have encountered these Ags previously [81]. B-1 cells are predominantly found in the peritoneal and pleural cavities and develop earlier in ontogeny compared to B2 conventional B cells which develop from bone marrow precursors after birth and are found in the bone marrow, spleen and lymph nodes. Marginal zone and follicular B cells are both B2 cells, however due to their distinct functional characteristics they are described as separate populations.

Another key aspect of the adaptive immune response is the generation of immunological memory. This consists of specialised memory B and T cells, and long-lived, neutralising antibody-producing effector B cells (plasma cells). Memory T cells

can be $CD4^+$ or $CD8^+$ T cells which can rapidly re-acquire effector function to either kill infected cells or secrete cytokines to inhibit pathogen replication. $CD4^+$ memory T cells can also provide assistance to the development of B cells and $CD8^+$ T cells via activation of APCs and the secretion of cytokines such as IL-2, IL-4 and IL-5 [65].

Figure 1.4: Thelper (T_H) cell subtypes

Following interaction with activated DCs naïve $CD4^+$ T cells can differentiate into multiple subsets depending on the signals received from the DC and the environment in which they interact with the DCs. Distinct T cell subsets can be distinguished by the cytokines they produce and the expression of 'master' transcription factors. The most well characterised subsets are T_H1, T_H2, T_H17 and Tregulatory (Treg) cells and are shown here along with their differentiating signals, cytokine secretion profiles and master transcription factors. IFN-γ, Interferon-γ; IL, interleukin; GATA3, GATA-binding protein 3; RORγδ, retinoic acid receptor-related orphan receptor-γδ; FOXP3, forkhead box P3; TGF-β, transforming growth factor-β


Naïve CD4+ T cells
Activated dendritic cell
IFN-γ
IL-4
IL-6
TGF-β
IL-10
TGF-β
TH1
TH2
TH17
Treg
Tbet
IFN-γ
GATA3
IL-4
RORγT
IL-17
FOXP3
IL-10

1.7 Dendritic cells: bridging the gap between adaptive and innate immunity

Dendritic cells were originally discovered in 1973 as a novel cell population in mouse spleen, clearly distinguished from macrophages by their unique morphology [82]. DCs play a key role both in the induction of adaptive immunity and maintenance of self-tolerance through their interactions with naive $CD4^+$ T lymphocytes. Due to their unique distribution throughout our body surfaces, often intimately associated with the epithelium, they are the perfect sentinels to constantly sample the environment for foreign antigens [83]. Peripheral DCs exhibit an immature phenotype characterised by high phagocytic capabilities and low expression of MHC II and co-stimulatory molecules CD40, CD80 and CD86 [84]. These DCs are capable of phagocytosing large quantities of antigens (Ag) in a highly regulated manner. Adhesion and phagocytosis of Ag by DCs is dependent on a range of receptors including CD14, scavenger receptor A and Fc receptors [85]. At this point they are poor APCs as they retain most MHC II internally and are unable to load Ag and form peptide-MHC II complexes [86]. In order to efficiently present Ag immature DCs require an inflammatory stimulus to induce their maturation. This causes a significant difference in membrane traffic in the cell along with a decrease in phagocytosis. Phagocytosis is an actin-dependent process controlled by Rho family GTPases. Specifically, DCs control the level of Cdc42 to developmentally regulate phagocytosis as only immature DCs were found to have activated Cdc42 required for phagocytosis [86]. Upon recognition of an inflammatory signal DCs can then load internalised Ag and transport the peptide-MHC II complex to the plasma membrane in non-lysosomal vesicles [87]. In murine bone marrow-derived DCs (bmDCs) these vesicles also contain the co-stimulatory molecule CD86. Once on the cell surface the peptide-MHC II complex and CD86 remained clustered together, perhaps in order to facilitate more efficient T cell activation [87]. DC maturation following recognition and uptake of Ag results in the migration of the cell to the draining lymph nodes (dLN) where it joins a network of DCs in the T cell zone of the LN and interacts with naive $CD4^+$ T lymphocytes. Interaction of DCs with T cells specific for the cognate peptide can result in either activation or tolerance of the T cell. How the immune system decides between dangerous pathogens and harmless foreign or self Ag is still being elucidated but it appears that the maturation state of the presenting DC plays an important role. During the steady state DCs that phagocytose Ag retain their immature phenotype as they enter the LN and any naive T lymphocytes that encounter them either undergo transient activation followed by rapid cell death by apoptosis, become primed towards a Tregulatory cell phenotype or become anergic [84]. On the

other hand, DCs that recognise Ag in the context of inflammation and/or tissue damage undergo partial activation and induce T lymphocyte activation and expansion [84]. Hugues *et al*, demonstrated that interaction between DC and T lymphocytes that results in T cell activation is transient and dynamic between 8-10 hours and 1-2 days post immunisation but is more stable, with interactions lasting at least an hour, at the time point in between [88]. During T cell tolerance, however, DC-T cell interactions remained dynamic at all time points measured [88].

DCs, like all other leukocytes, develop from bone marrow-derived haematopoietic stem cells. However, unlike most leukocytes, we are only just beginning to understand the exact origins and routes of development of DCs. DCs constitute a heterogeneous cell population, with many distinct subsets, each differing in cell surface marker expression, function, anatomical location and origins [89] [89].

1.8 The role of cytokines in mediating immune responses

Cytokines are critical mediators of immune regulation and are key inter-cellular communicators that help to connect the innate and adaptive immune response. Cytokines are small (~5-10KDa) proteins that mediate inter-cellular communication. The first characterised cytokine, IFN-α, was discovered in 1957 and it is now known that cytokines are produced by a broad range of cells, and that they can act on a large number of cell types depending on the situation. Almost every aspect of the immune response is mediated through cytokines. The main signalling pathway activated through cytokine receptors is relatively simple, consisting of the Janus kinases (JAKS) of which there are four, and the signal transducers and activators of transcription (STATs), of which there are seven. Stimulation of this pathway leads to the activation of specific sets of transcription factors for each STAT family member [90]. This pathway can also interact with multiple other signalling pathways activated by heterologous receptors which results in diverse cellular activation [91].

1.9 Important cytokines associated with DC function

1.9.1 Interleukin-6 (IL-6)

IL-6 is a pleiotrophic cytokine from the IL-6 cytokine family that has both pro- and anti-inflammatory members including IL-11, IL-27, IL-31, Leukaemia inhibitory factor, oncostatin and cardiotrophin [92]. IL-6 is a glycosylated 21-28 kDa protein and has the typical 4-helix bundle structure characteristic of all IL-6-type cytokines. The IL-

6sreceptor contains two subunits: the low affinity IL-6 receptor chain (IL-6R), which can exist as a membrane bound protein or in a soluble form [93], and the membrane-bound glycoprotein common to all IL-6-type cytokines, gp130. Initially IL-6 binds to IL-6R which then binds to gp130, causing it to form dimers [94] and leading to the activation of non-covalently associated Janus kinases (JAK1, JAK2, TYK) which become autophosphorylated and subsequently phosphorylate tyrosine residues in the cytoplasmic tail of gp130. This results in the recruitment of Src homology domain containing protein tryrosine phosphatase-2 (SHP-2) and the activation of MAPKs as well as JAK/STAT pathways [95]. IL-6R can also be secreted as a soluble protein (sIL-6R) [93] and IL-6/sIL-6R complexes can also activate cells via gp130, known as trans-signalling and this greatly enhances the repertoire of IL-6 signalling. Generally, pro-inflammatory IL-6 effects are mediated via trans-signalling whereas classic signalling to the membrane-bound IL-6R is most important in anti-inflammatory IL-6 effects [92].

IL-6 is secreted by multiple cell types including B and T lymphocytes, dendritic cells, macrophages, endothelial cells, fibroblasts, mesenchymal cells, glial cells and keratinocytes in response to a variety of stimuli [96] [92]. Originally discovered as a T cell secreted factor that enhanced B cell differentiation to immunoglobulin-producing cells [92]it is now known to play numerous roles in innate and adaptive immunity, liver regeneration [92], lipid metabolism, angiogenesis [97] and bone biology [98].

IL-6 has been shown to be involved in the transition from innate to adaptive response in a number of ways. For example, one of the hallmarks of an initial inflammatory infection is the infiltration of neutrophils for the first 24-48 hours before monocytes become the dominant cell type. Recruitment of these cells is dependent on adhesion molecules and chemokines such as IL-8, for neutrophils, and MCP-1 for monocytes. Neutrophils shed sIL-6R and this combines with IL-6 produced by endothelial cells to then activate these cells to produce MCP-1 but not IL-8, attracting monocytes to the site of inflammation. In addition, IL-6 may trigger neutrophil apoptosis, reducing these numbers at the site, but also phagocytosis of apoptotic neutrophils by macrophages also induces them to produce MCP-1 [99]. IL-6 also plays a key role in the priming and differentiation of naïve $CD4^+$ T cells. The cytokine promotes IL-4-mediated T_H2 differentiation through IL-4-dependent and independent mechanisms. IL-6 was found to increase GATA3 expression in $CD4^+$ T cells which required IL-4 but also increased the expression of NFAT (nuclear factor of activated T cells) independently of IL-4 [100]. Simultaneously, IL-6 was able to inhibit T_H1 differentiation by inhibiting IFN-γ gene

expression and production through activation of SOSC1, which inhibits STAT1 activation required for IFN-γ production [100]. As described above, IL-6 is also essential for the polarisation of naïve $CD4^+$ T cells towards the T_H17 phenotype along with TGF-β and IL-21 and suppresses TGF-β-mediated induction of FOXP3 required the differentiation of all four T_H phenotypes [101].

1.9.2 Tumour necrosis factor-α (TNF-α)

TNF-α was first discovered in 1975 as a secreted factor that could cause necrosis of some tumours, hence the name [102]. It is encoded for by a single gene located in the MHC region on chromosome 6p21.3 in humans and on chromosome 17 in mice [103]. It is initially translated as a 26 kDa type II transmembrane precursor that is then cleaved by the metalloprotease, TNF-α converting enzyme (TACE) to a 17 kDa soluble protein that exists as homotrimers [104]. Both forms appear to be biologically active [105]. Cytokine regulation is complex and occurs at translational, transcriptional and post-transcriptional levels [106] [107].

TNF-α is a pro-inflammatory cytokine primarily secreted by monocytes, macrophages and dendritic cells in response to infection. It activates B and T cells, macrophages, dendritic cells, endothelial cells, fibroblasts and induces IL-6, IL-1 and chemokine production by dendritic cells [90]. TNF-α signals through two receptors, TNF receptor 1 (TNFR1) and TNFR2, which can exist in both membrane-bound and soluble forms [108]. TNFR1 is almost universally expressed on cells whereas TNFR2 is tightly regulated and is only expressed on cells of the immune system [108]. TNF-α has been shown to activate a complex mix of responses including gene expression through NF-κB activation and caspase-mediated apoptosis. TNFR1 can induce caspase activated apoptosis through recruitment of proteins in its death domain and can also induce p38 and JNK MAPK activation through indirect recruitment of TRAF2. TNFR2 directly binds TRAF2, inducing gene expression and crosstalk with TNFR1. Activation of NF-κB induces the production of anti-apoptotic factors and so TNF-α can induce both pro- and anti-apoptotic responses [108].

TNF-α was the first cytokine to be implicated in the pathogenesis of autoimmune diseases [96] and it is now well established that dysregulation of the cytokine contributes to the pathogenesis of rheumatoid arthritis [109] [110], pulmonary disease and endotoxin shock [111]. Indeed, anti-TNF antibodies have been approved for use as drugs in Rheumatoid arthritis (RA), psoriasis and Crohn's disease. However the exact

mechanisms of action have not been fully elucidated and approximately one third of patients do not respond to these drugs. A suppressive effect of TNF-α on the production of type I IFNs by plasmacytoid DCs (pDCs) has also been demonstrated [96] again demonstrating that TNF-α signalling is very complex and clearly has multiple functions within the immune system as a whole.

1.9.3 Interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine that plays a critical role in suppressing immune responses to prevent inflammation and autoimmunity in the host. Mice that are deficient in IL-10 develop severe enterocolitis in response to the gut microflora [112] and while they can clear infections with Toxoplasmosis this is often accompanied by excessive immune reactions, which can be fatal [113]. IL-10 was first identified in 1989 as Cytokine Synthesis Inhibitory Factor, a factor secreted by T_H2 cells that could inhibit cytokine production by T_H1 cells [114]. Since then it has been reported to be broadly expressed by both adaptive and innate immune cells - B cells, CD8+ T cells, T_H1, T_H2, T_H17 and Treg cells, DCs, macrophages, mast cells, neutrophils, eosinophils and endothelial cells have all been found to produce IL-10. IL-10 signals through the IL-10 receptor (IL-10R), consisting of two subunits, IL-10R1 and IL-10R2, both of which are members of the IFN receptor family [115]. IL-10/IL-10R binding initiates a STAT3-dependent signalling cascade that ultimately results in the suppression of transcription of several target genes, although the full pathway is not yet understood [116]. IL-10 has been shown to down-regulate LPS-induced cytokine production by macrophages and DCs [117] as well as the surface expression of MHC II and co-stimulatory molecules CD80 and CD86, effectively down-regulating their ability to activate and sustain an adaptive T cell response [118]. These cells can also produce IL-10 which acts in an autocrine manner on IL-10R on these cells. T_H cells, which rely on signals from APCs, also produce IL-10 which further down-regulates APC function and so IL-10 acts in a negative feedback loop to limit host immune responses.

Originally IL-10 was thought to be a T_H2-derived cytokine, and then it was mostly associated with T regulatory cells. However it has now been demonstrated that all subsets of T_H cells mentioned (T_H1, T_H2 and T_H17) can produce IL-10 and in each case it seems that the mechanisms which drive TH differentiation induce these cells to produce IL-10. For example, a strong antigen dose and endogenous IL-12 is required for T_H1 cells to secrete IL-10 [119] while the typical T_H2 inducers, IL-4, GATA3 and

STAT6 are required for IL-10 production from T_H2 cells [120]. Similarly, IL-10 is produced by T_H17 cells in a STAT3- and sometimes STAT1-dependent manner [121].

1.9.4 Interleukin-12 (IL-12)

The IL-12 cytokines, IL-12, IL-23, IL-27 and IL-35, belong to the IL-6 superfamily of cytokines but are unique in that they are all heterodimer cytokines which consist of an α chain (p35, p28 or p19), which contains the 4-helices structure common to IL-6 superfamily of cytokines, and a β chain (p40 or Ebi3), which shares homology with class I receptor chains such as the IL-6R [122]. Despite sharing many structural features, receptors and down-stream signalling components, the four cytokines mediate a diverse range of functional effects. In a certain respect they mirror the balance displayed by the immune system as IL-12 and IL-23 are mainly pro-inflammatory cytokines while IL-27 and IL-35 play prominent roles in controlling aberrant immune responses through generation of T regulatory cells. Many of their mechanisms of action on the immune response arise as a result of their influence on the differentiation and function of T cells.

The patriarch of the family, IL-12, was identified in 1989 as a factor (Natural killer cell stimulatory factor) in the supernatants of EBV-transformed B lymphoblastoid cell lines that augmented Natural Killer cell-mediated cytotoxicity as well as inducing IFN-γ production by T cells [123]. IL-12 is a heterodimeric cytokine composed of two disulphide-linked subunits, p35 and p40. Large quantities of p40 are produced in comparison to p35 and bioactive IL-12, and it can be secreted as a monomer or homodimer. The p35 subunit can only be secreted in association with p40 (or Ebi3, see below). The subunits are differentially regulated and it appears that the availability of p35 is the rate-limiting step of IL-12 production and secretion [124] [122]

IL-12 is a pro-inflammatory cytokine primarily produced by macrophages, dendritic cells and monocytes in response to bacterial and parasitic infections [125]. It signals through the IL-12R consisting of two subunits – IL-12Rβ1 and IL-12Rβ2 [126] - and is coupled to the JAK/STAT pathways, primarily via activated STAT4 [127]. The receptor is expressed on T cells, NK cells and DCs [128] [129]. Receptor expression is tightly regulated on T cells – resting, naïve cells have low levels of the IL-12R but upon activation via TCR and co-stimulatory molecule CD28, expression is upregulated and this is enhanced by IL-27, IL-2, TNF-α and IFN-γ [122]. The expression of IL-12Rβ2 is lost from T_H2 cells rendering them insensitive to IL-12 signalling [68]. IL-12 positively

regulates its own induction through the production of IFN-γ by $CD4^+$ T cells [130] and NK cells [123], which, in turn, primes APCs to produce more IL-12, polarising the T cells to a T_H1 phenotype. T cells can also enhance the production of IL-12 through engagement of CD40 via CD40L during cognate APC-T cell interactions. Conversely, IL-12 can also be suppressed by cytokines IL-4, IL-13, and IL-10 [131] [132].

IL-23 shares the same p40 subunit as IL-12, covalently linked via a disulphide bond to the p19 α chain. Similar to IL-12p35, the p19 subunit requires association with p40 to be secreted [133]. It is produced by activated dendritic cells, monocytes and macrophages as well as B and T lymphocytes, NK cells and endothelial cells [133]. IL-23 signals through the IL-23 receptor which consists of the IL-12Rβ1 chain and the novel IL-23R subunit and the receptor is found on APCs, NK cells, B cells and activated T cells [134]. IL-23 plays a key role in mediating T_H17 immune responses and as such has been linked to several autoimmune diseases and chronic inflammation. The findings that IL-23-deficient mice were protected from autoimmune diseases such as EAE [135] [136] caused a shift in the established T_H1 or T_H2 paradigm and led to the identification of T_H17 phenotype [74]. Early reports suggested IL-23 directly drove the differentiation of the T_H17 lineage [72] [73]; however it was then demonstrated that IL-23 could not prime T_H17 cells *in vitro*, likely due to a lack of IL-23R on resting, naïve T cells [137] and IL-6 and TGF-β were shown to be the key T_H17 polarising cytokines [74] along with IL-1β and IL-18. IL-23 has since been shown to maintain the T_H17 phenotype but does so without promoting proliferation or survival of the cells and IL-23 alone does not commit the cells to this lineage as they can be induced to secrete cytokines from T_H1 and T_H2 subsets [138]. STAT3 and RORγt, activated by cytokines such as IL-6, cooperate to activate both IL-23 and IL-23R, initiating a positive feedback loop that up-regulates IL-23 and IL-17, stabilising pathogenic T_H17 phenotype [71]. Specifically, IL-23 drives a pathogenic T_H17 phenotype, likely due to a lack of IL-10 production that is observed when T_H17 cells are stimulated with IL-6 and TGF-β [121].

IL-27 is composed of the p28 subunit and the Epstein-Barr virus-induced gene 3 (EBI3), mainly produced by APCs. It was identified in 2002 in the supernatant of EBV-transformed B cell lines and was found to trigger the clonal expansion of naïve T cells [139]. It signals through the IL-27 receptor which consists of the novel IL-27 receptor alpha chain (IL-27Ra or WSX-1) [140] [141] and the common IL-6 receptor chain gp130. Gp130 is ubiquitously expressed on cells in the host but expression of IL-27RA is mostly restricted to immune cells with activated T cells and NK cells displaying the

highest expression levels. Originally, IL-27 was thought to be, like IL-12, a pro-inflammatory cytokine as it had been reported to induce T-bet expression and IFN-γ in $CD4^+$ T cells while suppressing GATA3 expression [142]. However IL-27 signalling alone could not fully differentiate naïve T cells to the T_H1 phenotype and IL-27RA-/- mice could mount a strong T_H1 response to challenge with infection suggesting this cytokine was not only involved in generating T_H1 responses. Indeed IL-27RA-/- mice went on to develop lethal T cell-mediated inflammatory disease [143] indicating that IL-27 may play a role in suppressing T_H1 responses. Several further studies indicated that IL-27 could suppress T_H1, T_H2 and T_H17 responses as IL-27RA-/- mice developed more severe asthma with an increase in T_H2 cytokines [144] and also developed worse EAE disease [145]. These studies have supported an anti-inflammatory role for IL-27 in T_H1, T_H2 and T_H17 responses.

IL-27 produced from DCs that had been primed by TGF-β-induced T regulatory cells drove the generation of IL-10-producing Tr1 cells which could suppress T cell effector responses in a FOXP3-independent manner [146] through the up-regulation of transcription factor c-Maf, growth factor IL-21 and co-stimulatory molecule ICOS [147]. Up-regulation of c-Maf and IL-21 by IL-27 has also been implicated to have an important role in the differentiation of T follicular helper (Tf_H) cells. In this manner IL-27 also supports germinal centre function and is an important survival factor for Tf_H cells *in vivo* and so IL-27 plays a key role in T cell-dependent antibody responses [148]. Indeed IL-27 could have a pro-inflammatory function in diseases which are dependent on a high affinity antibody response.

The majority of work on IL-27 has focussed on the effect of the cytokine on T cells but it has been reported that IL-27 can also affect DCs in an autocrine manner. Pre-treatment of DCs with IL-27 resulted in the significant inhibition of LPS-induced IL-12, IL-6 and IL-23 and down-regulation of MHC II, CD80 and CD86 with a simultaneous increase in IL-10 and TGF-β resulting in DCs with a reduced ability to stimulate antigen-specific T cell proliferative responses and a decrease in production of IFN-γ and IL-17 by these T cells [149]. Further work revealed that IL-27 acted *in vivo* on conventional DCs to limit the development of encephalitogenic T cells and EAE disease and this was dependent on the up-regulation of ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1) [149]. ENTPD1 catalyses the degradation of extracellular ATP, which is important for the activation of the NLRP3 inflammasome,

and accordingly IL-27 was found to decrease this as evidenced by reduced detection of activated caspase 1 and mature IL-1β as well as decreased production of IL-1β [149].

IL-35 is the latest IL-12 cytokine to be identified and is composed of the p35 and Ebi3 subunits [150] [151]. Its receptors consist of the common IL-6 receptor chain gp130 and the IL-12Rβ2. The biological effects of IL-35 are still being elucidated but it is known to be a potent inhibitory cytokine. Unlike the other IL-12 cytokines which are all secreted mainly by APCs, IL-35, to date, is reported to be secreted by lymphocytes. Treg cells have been shown to secrete IL-35 and upon contact with naïve $CD4^+$ T cells, Tregs substantially upregulate the production of IL-35. Loss of IL-35 results in reduced suppressive effects of Tregs *in vivo* [150]. IL-35 has been shown to inhibit the differentiation of T_H17 cells and to be protective in the CIA murine model [151]. Recently, it has been reported that B cells can secrete IL-35 after activation through TLR4 and CD40 and that these IL-35-producing B cells were found to limit disease severity in EAE but also afford protective immunity to Salmonella infection [152]. As stated above, IL-27 is able to induce the generation of IL-10-producing Treg cells and it has now been reported that IL-35 is able to generate human and mouse $CD4^+FOXP3^-$ Tregs that exclusively secrete IL-35. These cells were able to restore immune homeostasis and prevent autoimmunity in $FOXP3^{-/-}$ mice and also confer protection to EAE disease [153]. These cells appear to be induced by natural Treg cells through IL-35 and can also been generated *in vivo* during *Trichuris muris* infection [153].

1.10 The role of co-stimulatory molecules in DC function

T lymphocyte activation is often viewed as being dependent on TCR engagement with its cognate Ag presented in the context of MHC II by DCs. However, it also requires a second signal provided by co-stimulatory molecules present on the DCs. This actually represents a reciprocal interaction as signals from various T cell receptors induce further activation of the DC, which, in turn, signals the T cell towards full activation. Co-stimulatory molecules CD40, CD80, CD83 and CD86 are up-regulated on the surface of DCs following Ag recognition and uptake and can either promote (CD40-CD40L; CD80/CD86-CD28) or inhibit (CD80/CD86-CTLA-4) T lymphocyte survival, proliferation and differentiation. This immunological synapse between T cells and DC is shown in Figure 1.5.

1.10.1 Major histocompatibility complex class II (MHC II)

MHC Class II molecules, heterodimers composed of an α and β chain, are type I integral membrane proteins with a small cytoplasmic domain and four large extracellular domains [154]. They are expressed primarily on APCs - macrophages, dendritic cells and B cells and their main function is to present processed peptides from exogenous antigens to naive $CD4^+$ T cells to initiate and shape the immune responses as well as involvement in positive and negative selection of T cells in the thymus [155] [156]. Both subunits are synthesised in the ER and assemble with a non-polymorphic protein, the chaperone Invariant chain (Ii), which binds the peptide-binding groove of the MHC II molecule, preventing inappropriate peptide binding. The Ii protein traffics the MHC class II molecule to the endocytic compartment where both the invariant chain and the antigen to be presented are proteolytically degraded by aspartic and cysteine proteases, and the accessory protein H-2/HLA-DM then catalyses the binding of the peptide to the MHC class II molecule, which is then trafficked to the surface [157]. In addition to antigen presentation, MHC II molecules are now known to function as receptors that can induce reverse signal transduction in the APC. MHC II ligation may regulate cell adhesion, cytokine production, co-stimulatory molecule expression and apoptosis. In addition, mice deficient in MHC II have been shown to be more resistant to endotoxin shock by LPS and dendritic cells lacking MHC II have attenuated responses to TLR4, TLR3 and TLR9 stimulation indicating a role for MHC II in activation of TLR signalling responses. Indeed, intracellular MHC II was found to activate the tyrosine kinase Btk, which then associated with MyD88 and TRIF, activating both TLR signalling pathways [158].

1.10.2 CD40

CD40 is a member of the TNF Receptor superfamily and is expressed on a wide variety of cells including B cells, monocytes, endothelial and epithelial cells, and dendritic cells [159]. It is a 45kDa transmembrane glycoprotein [160] and binds to CD154/CD40L, a 34-39kDa type II integral membrane protein. The binding of CD40L to CD40 causes clustering of CD40 in lipid rafts at the immunological synapse between the two interacting cells to transmit intracellular signals [161]. This clustering of CD40 is dependent on reciprocal clustering by CD40L in acid sphingomyelinase (ASM) and ceramide-enriched rafts, a process that is dependent on p53 [161]. Initially CD40L was discovered on $CD4^+$ T cells, suggesting the main function of CD40L is the activation of B cells by T cells via ligation of CD40. Indeed, CD40/CD40L signalling is very important

in the activation and proliferation of B cells by T cell-dependent antigens as well as associated processes such as germinal formation, Ig class switching and the generation of memory and plasma B cells [159]. A lack of CD40L on T lymphocytes leads to hyper IgM syndrome in humans where patients are unable to switch from IgM to IG, IgA or IgE [162]. Recently CD40L mRNA and protein were also discovered in a range of dendritic cell subsets including plasmacytoid and splenic DCs as well as monocytes, activated B cells and endothelial and epithelial cells [163]. Activation of CD40 on DCs by CD40L increases the secretion of pro-inflammatory cytokine IL-12p70, causes the up-regulation of other co-stimulatory molecules such as CD80 and CD86, and increases the ability of the DCs to stimulate T cell proliferation and IFN-γ production [164]. The wide range of expression of CD40 and CD40L by different DC subsets suggests that DCs may use this signalling pathway to interact with each other and regulate their function. C4 binding protein, an inhibitor protein in the complement pathway, is also a ligand for CD40 and this suggests that CD40 can link the adaptive and innate immune systems [163]. Despite the well-defined role of CD40 in DC-T cell and T-B cell activation, the intracellular signalling pathways associated with these events have not yet been fully elucidated. CD40 expression increases on DCs after encounter with a microbial antigen, such as LPS. The surface receptor lacks intrinsic catalytic activity but contains two TRAF binding sites in its cytoplasmic tail and it is known that TRAF 1, 2, 3, 5 and 6 can bind to the receptor and so link CD40 to PI3K, PLC-γ, MAPKs ERK, JNK and p38 and NF-κB pathways [165][163]. TRAF 6 recruitment by CD40 after DC activation results in the non-canonical activation of NF-κB.

1.10.3 CD86 and CD80

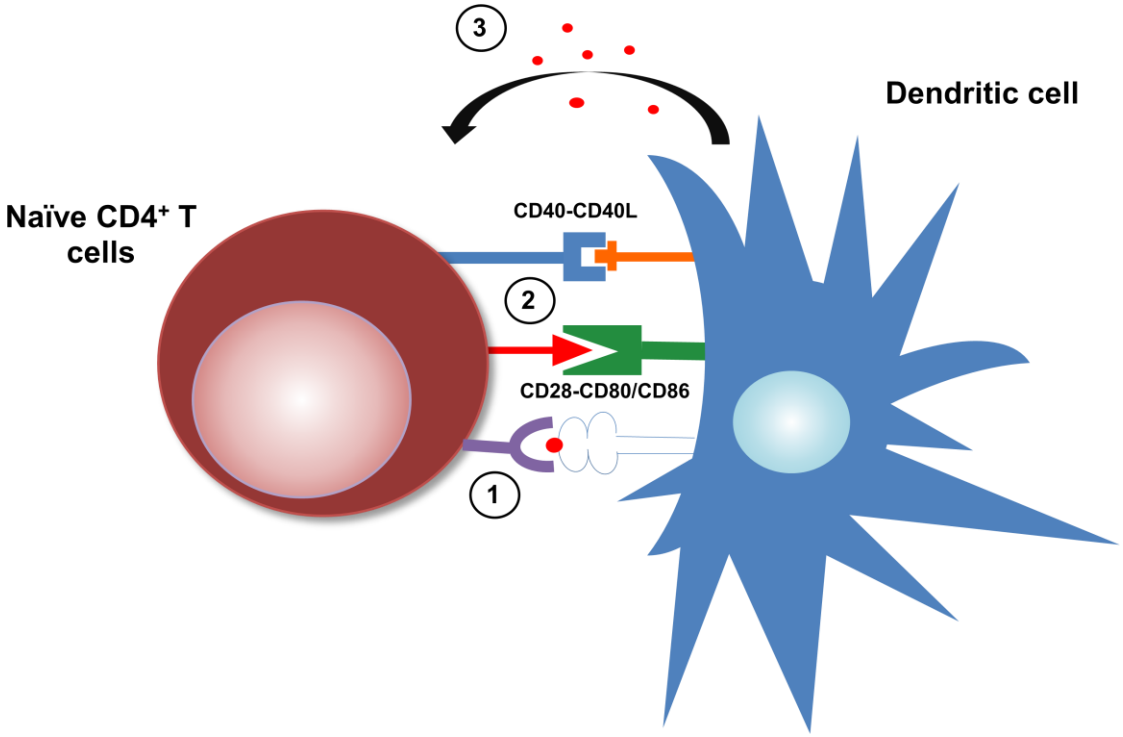
CD86 and CD80 are co-stimulatory molecules from the B7 protein family. They share the same natural ligands – CD28 and CTLA-4 - but have different patterns of expression on different cell types [166]. CD86 is more widely expressed than CD80 and is usually expressed at higher levels. CD86 is an important co-stimulatory molecule on DCs and B cells and is constitutively expressed on DCs and monocytes but expression increases after microbial activation of these cells. CD80, on the other hand, is expressed at very low levels on immature dendritic cells and is up-regulated following DC activation [167]. Both CD80 and CD86 bind to CD28 and CTLA-4 on $CD4^+$ T lymphocytes. Binding to CD28 provides the second stimulatory signal to the T cell, lowering the activation threshold of the cell thereby allowing the naïve T cell to be

readily activated. Binding of CD80 and CD86 to CTLA-4, however, causes inhibition of T cells [168]. It is not yet completely understood how CTLA-4 blocks T cell activation, or how CD80/CD86 binding to each receptor is regulated, however it has been reported that CTLA-4 removes CD80 and CD86 from the surface of APCs via trans-endocytosis to mediate cell-extrinsic inhibitory effect [169]. Data obtained from $CD86^{-/-}$ mice suggest that CD86 may be the stronger activator of T cells as they have a more suppressed T cell phenotype compared to $CD80^{-/-}$ but it is known that CTLA-4 has around 100 fold higher affinity for CD80 and so a lack of CD86 may enhance CD80/CTLA-4 signalling [170]. CD28 is constitutively expressed on $CD4^{+}$ cells whereas CTLA-4 is only up-regulated following T cell activation, except on Tregs where both markers are constitutively expressed [171]. It has been noted that human T cell proliferation responses inhibited by CTLA-4 were only observed when using CD80 as a ligand [172] [173] reported that antibodies against CD80 exacerbated disease in NOD mice, suggesting that CD80 plays a role in immune suppression and tolerance. DCs dynamically regulate CD86 expression depending on their need to activate or suppress the adaptive immune response, with expression increasing after LPS stimulation and decreasing in response to IL-10. DCs produce IL-10 in response to LPS and this then acts in an autocrine manner to suppress CD86 expression and thus prevent prolonged T cell activation [174] [175].

The E3 ubiquitin ligase membrane associated RING-CH (MARCH) 1 [176] has been shown to play a key regulatory role in the expression of MHC II and CD86 in DCs and B cells. MARCH 1 ubiquitinates both MHC II and CD86 in DCs at a critical lysine residue in the cytoplasmic tail, thereby mediating endocytosis and targeting them for lysosomal degradation [177] [178] [167]. DC maturation triggers the up-regulation of another co-stimulatory molecule, CD83. It is a glycosylated, 40-45 kDa member of the Ig superfamily, first described in 1992 that is present on mature human and murine DCs [179]. The exact function of CD83 is still unclear but it is also present on T and B cells and is thought to play a role in selection of $CD4^{+}$ T cells in the thymus [180]. Tze *et al*, however, have recently shown that activation of CD83 by TLR binding causes the transmembrane domain of CD83 to interact with MARCH 1, thereby decreasing its association with MHC II and CD86 [181] and Pinho *et al* have demonstrated that blocking CD83 decreases calcium signalling in T cells, suggesting that CD83 may play a role in controlling activation of the adaptive immune response [182].

Figure 1.5: The immunological synapse

The interaction between antigen in the context of MHC II on dendritic cells with the cognate TCR on naïve $CD4^+$ T cells, referred to as the immunological synapse, is the crucial first step for the induction of an adaptive immune response. In addition co-stimulatory molecules provide a second 'stimulatory' (CD28-CD80 and CD40-CD40L) or inhibitory (CD28-CD86) signal to the T cells. The third signal comes from cytokines secreted by the DCs such as IL-12 and IL-6 which play a key role in priming the type of immune response generated by the T cells.


3
Dendritic cell
Naïve CD4⁺ T cells
CD40-CD40L
2
CD28-CD80/CD86
1

1.11 The rise of inflammatory diseases in developed countries

In recent years there has been a sudden increase in the incidence of autoimmune diseases such as type 1 diabetes and multiple sclerosis, as well as allergic diseases such as asthma and eczema in developed countries. This has not been mirrored in the developing world and there are also differences observed between rural and urban areas, with a higher incidence of disease in urban regions. In 1989, Strachan reported that children from smaller families were more likely to develop allergic rhinitis than children from larger families based on a study of over 17,000 British children born in 1958 [183]. He proposed the 'Hygiene hypothesis' - that a lack of childhood infections could increase the risk of developing allergic rhinitis. The subsequent discovery that T cells could be primed to a T_H1 or a T_H2 phenotype which was accompanied by a reciprocal down-regulation of the other phenotype supplied an immunological framework for this proposal and there have been many further studies to confirm that a lack of intense infection due to increased hygiene, vaccinations and the use of antibiotics may have altered the immune system such that it then reacts inappropriately to harmless antigens [184]. This does not however, explain the concurrent increase in autoimmune diseases associated with T_H1 and T_H17 responses. Despite both inducing a strong T_H2 phenotype the incidences of allergic disease and helminth infection do not overlap, and several epidemiological studies have demonstrated an inverse correlation between helminth infections and autoimmune/allergic diseases. This has led to the hypothesis that helminth infections, through host immunomodulation, can be protective against aberrant immune responses.

1.12 Helminth infection

Helminth infections are a major healthcare problem, particularly in the tropical and sub-tropical areas of Africa, Asia, and Central and Southern America, infecting more than 2 billion people worldwide [185]. In most cases infection is associated with public health problems such as poor living conditions, inadequate sanitation and water supplies, poor personal hygiene and poor health awareness. Helminth infections are rarely fatal but can cause permanent and debilitating morbidity - anaemia, vitamin A deficiency, stunted growth, poor intellectual development, impaired cognitive function, and damage to the liver, intestine, and urinary tract are all associated with chronic worm infection [1]. However, most people infected with helminths show no overt signs of disease. These people generally develop chronic, long-lived helminth infections [186] and this

seems to be due to the parasites' ability to modulate the host immune response to allow it to survive without causing host pathology.

Helminths are a diverse group of organisms, comprising both parasitic and free-living species. Despite varying greatly in biology – for example helminths employ a wide range of infection routes and occupy a diverse set of niches within the host – the mammalian host response to these parasites is remarkably similar. Helminth infections are one of the main inducers of a T_H2 cell response in humans, however it is often associated with regulatory components. This creates a 'modified T_H2 response' that promotes parasite survival and longevity but also seems to benefit the host as it limits pathology [184]. The development of this immune response over time is summarised in Figure 1.6. Filarial nematodes cause some of the most persistent helminth infections, with adults living up to 10 years in some hosts [187] [188]. Infections with filarial nematodes generally result in three broad outcomes which are characterised by specific immune responses. A small proportion of people infected develop an uncontrolled T_H1 cell response characterised by a severe inflammatory response that leads to pathology such as elephantiasis and hydroceole in lymphatic filariasis and sowda in onchocerciasis. Some patients show a balanced T_H1/T_H2 response and are resistant to infection. It is thought that Treg cells in these cases tightly control the number of each type of T cell. The majority of people infected remain largely asymptomatic, despite a high parasite load, and show the modified T_H2 response that is so characteristic of this type of infection. These patients are susceptible to infection and are the main reservoir for continued transmission of disease and can remain infected for many years. The parasites' ability to establish such a long-lived, chronic infection suggests an ability to modify and evade the host immune response. This "immunomodulation" is also beneficial to the host as it limits host pathology generated through excessive pro-inflammatory reactions. The immunological phenotype typically involves a strong T_H2 and low T_H1 cell response, production of T_H2 cytokines such as IL-4 and IL-13, as well as high levels of immunoregulatory cytokine IL-10 and a skewed T_H2 antibody response. Asymptomatic individuals also have a reduced T cell proliferative response to specific parasite antigen, and often to non-specific antigens and mitogens as well, compromising immunity to other diseases or the efficacy of vaccines [189] [190] [184]. The uncoupling of specific IgG4 and IgE antibody responses is an important aspect of the response to helminths. In a normal T_H2 response the level of IgE is very high whereas during the typical response to helminths IgE, is down-regulated and there is a much higher level of IgG4 produced, however IgE is still

detected. IL-4, a T_H2 cytokine, promotes IgE and IgG4 production by naïve B cells and in many experimental models helminth antigens induce IgG4 and IgE. Il-10, TGF-β and other immunoregulatory cytokines are important inducers of IgG4 in humans [191] and during helminth infections it is thought that these cytokines promote IgG4 production. IL-10 also inhibits B cell switching to IgE while stimulating these cells to switch to IgG4 production. IgG4 is the protective, non-cytolytic, blocking subclass of IgG antibody as, unlike IgG1 and IgG3, it cannot fix complement to induce antibody-dependent cell mediated cytotoxicity [191].

1.13 The filarial nematode excretory/secretory products and immunomodulation of the host

Humans have been infected with helminths for thousands of years: clinical symptoms of helminth infections are described in Egyptian medical papyri and in the medical writings of the Greeks, particularly Hippocrates, and Roman and Arabic physicians [192]. In 1910 Marc Armand Ruffer noted the presence of calcified *Schistosoma haematobium* eggs in the kidneys of two mummies dating from 1250 to 1000 BC [193], and, while there are no written records, a statue of the Pharaoh Mentuhotep II from around 2000 BC has swollen limbs suggesting he suffered from lymphatic filariasis [192]. Throughout this period helminths have been constantly evolving in order to survive within their hosts and one of the main mechanisms employed by these pathogens is the release of “excretory/secretory” products that actively dampen the host immune response to the parasite. As some of these products are anti-inflammatory in nature they provide us with a natural avenue of potential new anti-inflammatory drugs and also an insight into how these suppressive pathways operate which also provides new molecular drug targets.

The activation or suppression of the immune system is linked to dendritic cells, which constantly sample their environment and so acquire the signals required to generate an appropriately biased adaptive immune response. It is perhaps unsurprising therefore that many of the excretory/secretory products of helminths target these cells. For example, the excretory-secretory proteins released by *Fasciola hepatica* during its migration through host tissues to the bile duct have been shown to impair the ability of murine DCs to respond to TLR-ligand stimulation and prime a T_H2/Treg phenotype *in vitro* [194]. In addition the tegumental coat of *F. hepatica* (FhTeg), which is shed while in the host to avoid antibody binding, has been shown to suppress dendritic cell responses. DCs treated with FhTeg have decreased production of TLR- and non TLR-

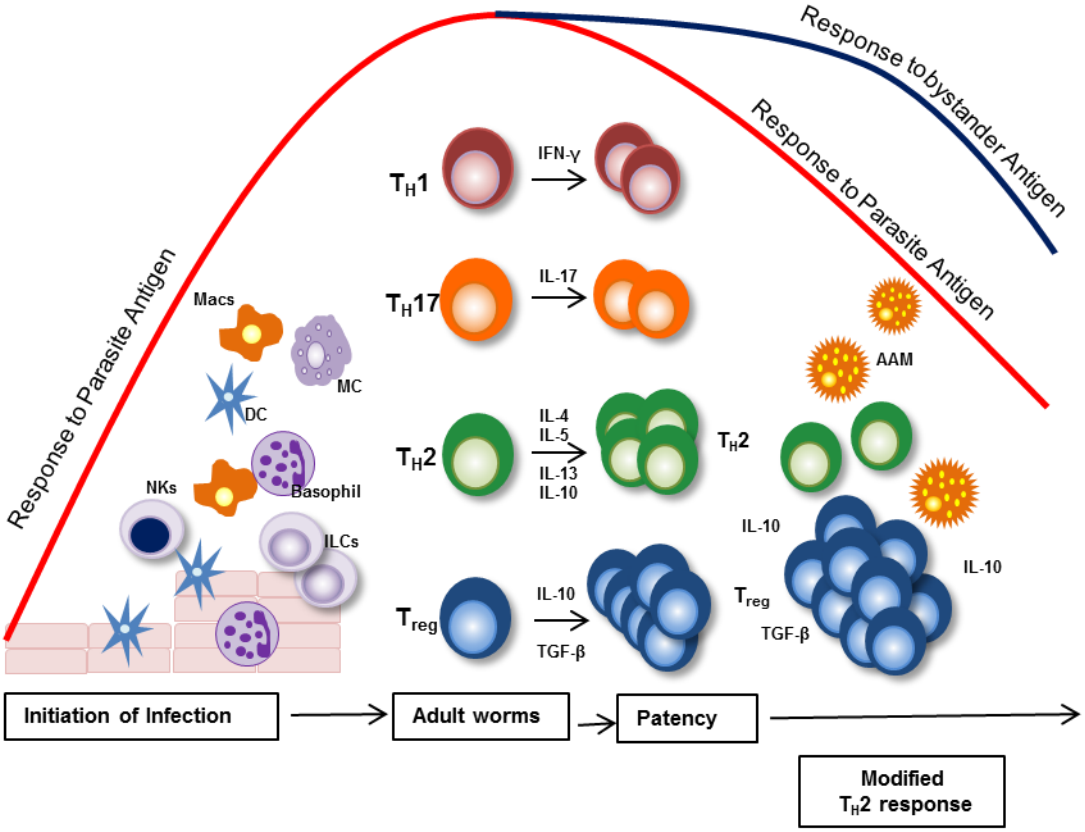
ligand induced IL-12, IL-6, IL-10 and TNF-α and decreased surface expression of CD80, CD86 and CD40 [195]. These DCs attenuate the T_H17 phenotype *in vivo* but do not seem to prime for a T_H2 response [196]. A similar DC phenotype is induced by the excretory/secretory products of the gastrointestinal nematode *Heligomoisoides polygyrus* (HES) and characterised by inhibition of TLR-induced cytokines and decreased surface expression of co-stimulatory molecules which suppress Ag-specific antibody responses *in vivo* [197]. By contrast, ES from *Nippostrongylus brasiliensis* (NES), which induces a strong T_H2 response *in vivo*, actively matures DCs towards the T_H2 phenotype by selectively up-regulating OX4OL and CD86 and inducing IL-6 and IL-12p40, while also blocking LPS-induced IL-12p70 [198].

Helminths and their excretory/secretory products have also been shown to be protective in several animal models of disease (Table 1). EAE is currently the best available animal model for multiple sclerosis and is characterised by the breakdown of myelin by autoantigen-specific T cells in the CNS [199]. Several studies have demonstrated that pre-infection with *Schistosoma mansoni* [200] or injection of *S. japonicum* soluble egg antigen (SEA), a complex extract of soluble molecules from mechanically disrupted eggs, can modulate EAE in mice [201]. Helminth infection has also been demonstrated to prevent the onset of type 1 diabetes in NOD mice. In this case infection with *Litomosoides sigmodontis* completely prevented the onset of disease. This protection was associated with IL-4, which has previously been shown to ameliorate T_H1-driven autoimmune diseases, through a shift to a T_H2 phenotype and an increase in splenic $CD4^+CD25^+FOXP3^+$ T cells [202]. Further work however has suggested that the T_H2 response is not critical and protection is dependent on TGF-β as injection of a neutralising antibody abrogated helminth-mediated protection [202].

Figure 1.6: Immune responses to helminth infections over time.

Initial parasite infection promotes a strong innate response which activates a number of cell types such as mast cells (MC), basophils, dendritic cells (DCs), macrophages (macs), NK cells and innate lymphoid cells (ILs). This early response stimulates TH1, TH2 and TH17 effector cells, along with IgE, which may play a role in allowing establishment of infection. The release of eggs or microfilariae by the parasite (patency) results in a small increase in T_H2 cells and a concomitant decrease in T_H1/T_H17 responses. Long-term, chronic infection promotes high levels of IL-10 and TGF-β which stimulates the expansion of Treg cells, resulting in further IL-10 production which causes antibody switching to IgG4.

Adapted from [185]



0 ----------2 weeks ---------- up to 6 months ----------\\ \\---------- Decades

Table 1.1: Suppression of autoimmune and allergic disease by helminths

The effects of helminth infections on different murine models of disease are summarised, along with any known mechanisms of protection.

Animal Disease Model	Helminth	Proposed mechanism	Reference
Experimental autoimmune encephalomyelitis (EAE)	*Schistosoma .mansoni*	↓MOG-specific IFN-γ, TNF-α, NOD	[203]
	Fasciola hepatica	↓IFN-γ, IL-17, dependent on TGF-β	[204]
	Trichinella spiralis	↓IFN-γ, IL-17 ↑IL-4, IL-10	[205]
Type 1 Diabetes in NOD mice	*S.mansoni*	↓ anti-insulin IgG (inhibition of AB class switching)	[206]
	Litomosoides sigmondontis	↑Treg cells, dependent on TGF-β	[207]
	Heligomosoides polygyrus		[208]
	Fasciola hepatica ES	↑Bregs and M2	[209]
Streptozotocin-Induced Diabetes	*Taenia crassiceps*	↑IL-4, alternatively activated macrophages	[210]
Collagen-induced arthritis (CIA)	*A.viteae* (ES-62)	↓IFN-γ, IL-17, TNF-α, IL-6, ↓anti-collagen IgG	[211]
	S.mansoni	↓IFN-γ, IL-17, TNF-α, IL-6, RANKL ↓anti-collagen IgG	[212]
	Ascaris suum		[213]
TNBS/DNBS-induced colitis	*S.mansoni*	↑IL-4	[214]
	S.mansoni (eggs)	↓IFN-γ ↑IL-4	[215]
	S.japonicum	↓IFN-γ ↑IL-4, IL-5, IL-13, Treg cells	[216]
	Hymenolepsis diminuta	↑IL-4, IL-10	[217]
	T.sprialis	↑IL-4, IL-13 ↓IL-12	[218]
DSS-induced colitis	*S.mansoni*	↑ colon-infiltrating macrophages	[219]
Asthma/Airway sensitisation or inflammation	*A.viteae* (ES-62)	↑IFN-γ, T-bet	[220]
	S.mansoni (male worm)	↑IL-4, IL-13, IL-10 ↓IL-5	[221]
	S.japonicum (egg Ag)	↑Treg cells	[222]
	H. polygyrus	↑Treg cells, Bregs ↓innate lymphoid cell responses*	Summarised in [223]
	Strongyloides stercoralis		[224]

*different mechanisms depending on asthma model

1.14 ES-62

ES-62, a 62kDa glycoprotein, is the major ES protein of the rodent filarial nematode *Acanthocheilonema viteae* and is perhaps the best characterised of all the secreted helminth products. It was discovered by Harnett *et al* in 1989 and contributes to > 90% of the proteins secreted by these worms [225]. An adult female worm, the greatest producers of the protein, can secrete 0.038-0.092 µg/hour [226]. Within the jird, the natural host for *A. viteae*, ES-62 can be detected in the bloodstream 4 hours after release, where its half-life is dependent on whether the animal is infected and the length of infection [225]. There appears to be a clearance window and so the longer the animal is infected the higher the concentration of detectable ES-62, thus an animal that has been infected 14 weeks has a higher level of ES-62 in its blood than a jird which has only been infected for 5-6 weeks. This may be linked to the differences in the size of ES-62-antibody-containing immune complexes over the course of infection [225] [227]. Production of ES-62 is stage-specific – it is only secreted by post-L3 stages – but the mRNA can be detected in all stages [228]. ES-62 contains the unusual post-translational modification of phosphorylcholine (PC) moieties attached via *N*-linked glycans [229]. The presence of PC on ES-62 was first suggested after it was discovered that anti-PC antibodies were responsible for the recognition of ES-62 in serum samples from infected human patients [225]. Initial studies suggested that the PC moieties could be present on carbohydrate-containing molecules. Thus, [^{3}H]-choline labelled ES-62 was subjected to N-glycosidase F (an enzyme known to cleave N-glycans from proteins), which resulted in a complete loss of radioactivity suggesting that the PC molecule is attached via a N-type glycan to the protein backbone [230]. Further inhibitor studies indicated that addition of PC was a post-ER event as treatment of *A. viteae* with Brefeldin A blocks secretion of the protein, and studies with inhibitors of *N*-linked oligosaccharide processing events demonstrated that PC addition is dependent on the generation of an appropriate substrate during oligosaccharide processing. These studies suggested that PC is transferred in the lumen of the medial golgi and the substrate is the 3-linked branch of Man5GlcNAc3 or Man3GlcNAc3 [229]. Fast atom Bombardment mass spectroscopy confirmed that the substrate was Man3GlcNAc3 and that PC is attached to an *N*-glycan which has a trimannosyl core (with and without core fucosylation) and which has one to four *N*-acetlyglucosamine residues. Two other glycans were detected on ES-62, one was fully trimmed to the trimannosyl core and had been sub stoichiometrically fucosylated while the other had a high mannose content [231]. Structural analysis demonstrated that each glycan

contains 1-2 PC residues and sequence analysis of ES-62 suggested the protein may have three N-linked glycosylation binding sites indicating that each ES-62 molecules may have up to 6 PC residues [231] [227].

Homologues of ES-62 have been found in the ES of the human filarial nematodes *Brugia malayi* and *O.voluvolus* [230] and this type of binding of PC to *N*-glycans appears to be conserved among filarial nematodes [231]. PC is a conserved structural component of many organisms and while it plays multiple roles in the different species a common function of PC is the modulation of the host immune response [232]. As ES-62 is present in the blood of *A.viteae* hosts it would have ample opportunity to interact with the cells of the immune response. Indeed, ES-62 has since been demonstrated to be a key immunomodulator during filarial infections. It has been shown to target multiple cells including T and B lymphocytes, antigen presenting cells - macrophages and DCs, and mast cells, to generate an overall biased immune response towards a regulated T_H2/anti-inflammatory phenotype, characterised by an increase in cytokines IL-4, IL-5 and IL-13, a reduction in IL-12, IL-6 and TNF-α, and a skewed antibody response [233] [234][235]. The effects of ES-62 on the cells of the immune system are described in detail below and summarised in Figure 1.7.

1.14.1 ES-62 and lymphocytes

A common characteristic of filarial nematodes is hyporesponsiveness of patients' lymphocyte populations in response to parasite antigen. ES-62 significantly inhibits the anti-Ig-mediated activation of murine splenic B cells *in vitro* [235]. The effects of ES-62 on B cells have been extensively studied and are perhaps the best understood of all of ES-62's immunomodulatory effects. Treatment with ES-62 does not completely block B cell function, rather, B cells are desensitised to B cell Receptor (BCR) signalling and ES-62 achieves this by selectively targeting key downstream signalling pathways. Exposure to ES-62 enhances SHP-1 tyrosine phosphatase recruitment after subsequent BCR ligation. SHP-1 dephosphorylates the Immunoreceptor Tyrosine-based Activation Motif (ITAM) on accessory transducing molecule Igβ in the BCR complex and thus keeps it in the 'resting' state. This blocks recruitment of ShcGrb2Sos complexes to the plasma membrane, which are required for coupling of BCR to RasERK - MAP kinase cascades [236]. Additionally, ES-62 targets negative regulators of BCR signalling; specifically it promotes BCR-driven upregulation of RasGAP to terminate ongoing Ras signalling and association of nuclear MAPkinase dual threonine/tyrosine phosphatase Pac-1 with ERK MAP kinase to terminate any ongoing

ERK signals. ES-62 also reduced the expression levels of PKC isoforms (α, β and δ) via proteolytic degradation [237]. These PKCs have been shown to play a role in regulating B cell proliferation. Interestingly, ES-62-mediated down regulation of these PKC isoforms can be rescued by IL-4 suggesting the specific microenvironment could influence the PKC-dependent effects of ES-62 [237].

ES-62 does not just target B cells; it also suppresses anti-CD3-induced proliferation of Jurkat T cells by desensitising T cell Receptor (TCR) signalling through disrupting coupling of the TCR to downstream Phospholipase D (PLD), PKC, PI-3-K and Ras MAPK cascades [238]. As in B cells, ES-62 does not target the generation of intracellular second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG). Treatment with ES-62 causes a reduction in expression of PKC-α and, to a lesser extent, PKC-ε but in these cells this is in association with downregulation of phosphatidylcholine PLD (PtdCho-PLD) activation [238].

The effect of ES-62 on lymphocytes *in vitro* was corroborated *in vivo* by the use of osmotic pumps, which releases ES-62 into the mouse at a steady rate to mimic natural infection. Conventional (B2) splenic B cells from mice exposed to ES-62 for 14 days were stimulated *ex vivo* and were found to be hyporesponsive to BCR-induced and LPS-induced proliferation, and this was associated with a decrease in activated ERK MAPK [239]. Interestingly, peritoneal B1 cells exposed to ES-62 *in vivo* demonstrated increased spontaneous and anti-IgM- and LPS-driven proliferation, as well as increased levels of spontaneous and anti-IgM-driven IL-10 release [239] demonstrating that ES-62 does not target these two B cell subtypes in the same manner. This differential targeting of B cell subsets was also observed when investigating the use of ES-62 in the MRL/lpr mouse model of lupus. ES-62 was found to increase the number of IL-10-producing B cells while suppressing pathogenic plasmablast-like B cells in the joint [240].

Adoptive transfer experiments where T cells with a transgenic TCR specific for the immunodominant epitope of OVA are injected into recipient BALB/c mice in numbers large enough to trace *in vivo* but not interfere with physiological responses to antigen, demonstrated that ES-62 treatment reduced the proliferative capacity of these T cells in response to *ex vivo* stimulation with OVA [241]. In addition, ES-62 inhibited the clonal expansion of, and total numbers of OVA-specific T cells in the draining lymph nodes (dLN). Analysis of antibody levels in the serum of these mice demonstrated that ES-62 caused an increased OVA-specific IgG1 and a decrease in IgG2a antibody responses.

Despite a strong T_H2 antibody bias response, however, there was not a classical cytokine T_H2 response. Rather, ES-62 caused a reduction in antigen specific IL-2, IL-17, IFN-γ as well as IL-4 and IL-13 production [241], demonstrating an overall suppression of cytokine production rather than a polarised T_H1 or T_H2 response. In order to determine if these effects by ES-62 were as a result of disrupting the B cell – T cell interaction a double transfer system was employed where recipient mice received OVA-specific tg T cells and hen egg lysozyme (HEL)-specific transgenic B cells and were stimulated with conjugated OVA-HEL that, due to its linked epitopes promote B-T cell co-operation [242]. Perhaps surprisingly, transfer of HEL-specific B cells rescued the reduced proliferation effects of ES-62 treatment in T cells. However, these ES-62-treated mice also displayed a more generalised immunosuppressed phenotype with overall lower antibody levels with an inhibition of both IgG1 and IgG2a, and reduced levels of IL-2, IFN-γ, IL-17, IL-4, IL-5 and IL-10 [242]. This study indicates that although ES-62 may be able to modulate B-T cell co-operation it is not the mechanism by which ES-62 mediates its suppressive/polarisation effects on T cells.

1.14.2 ES-62 and antigen presenting cells

The effects of ES-62 on antigen presenting cells – macrophages and dendritic cells, was analysed to investigate if modulation of these cells was responsible for the suppressive effects on T cells and indeed, ES-62 was the first helminth molecule found to have the capacity to polarize the nature of the immune response towards a T_H2 phenotype through DC modulation. Whelan *et al* demonstrated that LPS-matured DCs promote a T_H1 phenotype as evidenced by production of IFN-γ from naïve $CD4^+$ T cells co-cultured with these DCs, while ES-62-matured DCs stimulated T cells to produce IL-4, the characteristic T_H2 cytokine [243]. Further analysis found that stimulation with LPS caused the maturation of DCs as they up-regulated several co-stimulatory molecules including CD40, CD80, CD86 and CD54 but this effect was absent with ES-62-treated DCs. Surprisingly, blocking CD80 and CD86 resulted in inhibition of both IL-4 and IFN-γ, suggesting that they may be more important in T cell proliferation rather than polarisation [243]. As well as priming DCs towards a T_H2 phenotype, ES-62 also modulates the function of macrophages *in vitro* and *in vivo*. Treatment of murine macrophages *in vitro* with ES-62 results in the cells becoming hypo-responsive to stimulation with their classical inducers, LPS and IFN-γ, with a reduced capacity to produce T_H1 cytokines IL-12, IL-6 and TNF-α, but not NO. Through the use of osmotic pumps in mice, which release ES-62 in low, regular doses (0.05 µg/hour) to mimic

natural filarial infection, it was demonstrated that this suppression is also achieved *in vivo*, as macrophages from ES-62-treated mice produced less IL-12 and TNF-α when stimulated *ex vivo* with LPS and IFN-γ compared to control mice [226]. Washing of the cells pre-treated with ES-62 before LPS stimulation resulted in the same effect suggesting that ES-62 induces a state of hyporesponsiveness in the macrophages and affects the cells directly, not as a soluble mediator or through the induction of another inhibitory molecule. Real-time PCR studies showed that the suppression of IL-12 induction by LPS and IFN-γ by ES-62 was achieved by inhibition of both subunits of the bioactive cytokine at the transcriptional level [226]. Interestingly, treatment of macrophages with ES-62 causes initial, low level production of the pro-inflammatory cytokines, IL-12, IL-6 and TNF-α before cells subsequently become refractory to further release of these cytokines when stimulated with LPS and IFN-γ [226]. This transient release of inflammatory cytokines could be the result of abortive signalling that then suppresses production of these cytokines. It is in keeping with the literature as pro-inflammatory cytokines have been found to dominate the host response during the early stages of filarial nematode infection. In response to infective L3 live parasites Babu & Nutman found that PBMCs from unexposed, naïve individuals responded in a predominantly T_H1 manner. Thus, there was an increase in the frequency of T cells expressing IFN-γ, TNF-α, GM-CSF, IL-8 and IL-6 but not IL-4, IL-5, IL-10 or IL-13 [244]. Not only does ES-62 modulate the function of macrophages and DCs in the blood but it appears to affect the bone marrow precursors of these cells as well. Pre-exposure of bone marrow precursors of macrophages and dendritic cells to ES-62 renders the cells hypo-responsive to subsequent LPS stimulation. As above, this appears to be through the inhibition of IL-12 production via a decrease in the mRNA and protein of the p40 and p35 subunits of the cytokine [245].

The unusual post-translational modification of an addition of PC to ES-62 appears to be responsible for many of the immunomodulatory properties of the molecule as the majority of the activities of ES-62 can be mimicked by PC conjugated to OVA or BSA (or even PC alone). Pre-treatment of macrophages and DCs with such molecules results in initial low levels of IL-12 production, subsequent suppression of full activation of macrophages and DCs following LPS stimulation and inhibition of IL-12 induced by BLP and CpG. Importantly, mock-conjugated OVA protein had no effect of macrophages and DCs [233]. As PC is a common PAMP and so a target for immune

cell recognition it was investigated whether ES-62 was recognised through TLRs. Modulation of macrophages and DCs by ES-62 was investigated using TLR4 and TLR2 knockout (KO) mice. Consistent with wild-type mice, low level induction of IL-12 and TNF-α as well as subsequent suppression of the cytokines was observed in macrophages and DCs from TLR2 KO mice indicating that TLR2 was not required for ES-62 modulation of APCs. In TLR4 KO mice, however, both effects were ablated. As LPS is also recognised by TLR4, BLP (TLR2 ligand) and CpG (TLR9 ligand) were used to determine the effects of ES-62 in TLR4 KO mice. ES-62 pre-treatment suppressed induction of IL-12 and TNF-α after stimulation with IFN-γ/ BLP and IFN-γ/CpG in wild type but not TLR4 KO mice, indicating TLR4 is necessary for ES-62 activity. The use of C3H/HeJ mice, which have a Pro712His point mutation in the TIR domain of TLR4 preventing LPS recognition and response, demonstrated ES-62 does not need a fully functioning TLR4 receptor to mediate its effects as IL-12 and TNF-α were both initially produced and then suppressed by macrophages and DCs from these mice in response to IFN-γ/BLP stimulation [246].

Studies have shown that ES-62 does not affect detection of LPS by peritoneal macrophages in mice as the surface expression of TLR-MD-2 was unaffected after treatment of the cells with ES-62. It is likely therefore that ES-62 mediates its effect through modulating intracellular signalling pathways. The signalling adaptor MyD88 is required for downstream signalling following TLR4 activation and the low level induction of IL-12p40 in macrophages and DCs by ES-62 appears to be dependent on this adaptor as it is absent in MyD88 KO cells [246]. It has since been demonstrated that ES-62 down-regulates MyD88 expression in macrophages [247] , mast cells [247], T_H17 cells during CIA [211] and, most recently, in B cells and kidney cells in MRL/Lpr mice [240]. The exact mechanism of action of ES-62 on APCs has not yet been elucidated but it is known that the parasite molecule can modulate the activation of ERK, JNK and p38 MAPKs and NF-κB required for pro-inflammatory cytokine production. ES-62 differentially regulates the two subunits required for synthesis of bioactive IL-12p70, IL-12p40 and IL-12p35, by suppressing the stimulation of p38 and JNK by LPS required for p35 (and IL-6 and TNF-α) production, while also augmenting the LPS-induced, calcium-dependent activation of ERK MAPK, which negatively regulates p40 production [246] [124].

1.14.3 ES-62 and mast cells

Mast cells are bone marrow-derived cells that play an important role in parasitic infections but have also been implicated in pathogenic roles in allergic disease [248]. Upon classical activation, via cross-linking of IgE bound to FcεR1, mast cells degranulate and release allergic mediators such as histamine, prostaglandins and leukotrienes as well as preformed cytokines, heparin and platelet activating factor (PAF). Mast cells can also be activated independently of FcεR1 by PAMPs such as LPS, allergic stimuli or circulating cytokines [249]. ES-62 has been shown to inhibit FcεR1-induced degranulation in human mast cells by forming a complex with TLR4 on the cell surface, resulting in the sequestration of PKCα and so targeting this molecule for proteasome-independent, caveolae/lipid raft-mediated degradation. This blocks the coupling of Phospholipase D-coupled, sphingosine kinase mediated calcium mobilisation required for degranulation [250]. Antisense reduction of TLR4 demonstrated that TLR4, as in macrophages and DCs, is essential for inhibition of mast cell function by ES-62 [250]. It was noted that ES-62 also reduced the expression of other PKC isoforms (PKCs -β, -δ, -ι and –ζ) and recent work using PKC isoform deficient mice has found that ES-62 was still able to inhibit IL-6 and TNF-α release from BMMCs from PKCα KO mice suggesting that degradation of this isoform is not the only mechanism involved in the desensitising effects of ES-62 [251]. This study found that in PKCα-deficient BMMC, PKCε was strongly up-regulated. PKCε was shown to be a negative regulator of IL-6 release and so it could be that by degrading PKCα ES-62 not only disrupts the cytokine signalling pathway and calcium mobilization but could also promote a PKCε-mediated cytokine suppressive pathway [251]. Mast cells constitute a very heterogeneous cell population as they arrive in the tissue before the bone marrow progenitors have fully matured which means the specific microenvironment they encounter in the tissues dictates their final maturation state, creating site-specific mast cells [249]. Recent studies with ES-62 employing several murine mast cell subsets demonstrated that the parasite-derived molecule is able to inhibit PKCα and calcium signalling in bone marrow derived mast cells (BMMC), which represent an immature phenotype, as well as in peritoneal-derived mast cells (PDMC), which are typically represent a mature phenotype [247]. These mast cell subsets have differential functional responses as PDMCs are strong FcεR1-induced degranulators but produce low levels of cytokines and chemokines, while BMMCs are stronger producers of cytokines and can also be activated by molecules such as LPS. Consistent with this, inhibition of BMMC responses is accompanied by down-regulation of PKCδ and MyD88

but this is not seen in PDMCs. ES-62 did not alter TLR4, FcεR1 or ST2 expression on any mast cell subset, suggesting that it targets mast cell functional plasticity rather than phenotypic status [247]. This is in keeping with data obtained in dendritic cells and macrophages where treatment of bone marrow progenitors of these cells with ES-62 programmed them to an anti-inflammatory phenotype [233], demonstrating that ES-62 can target cells at different stages of development.

1.14.4 Treatment of murine models of autoimmune and allergic diseases by ES-62

As ES-62 has such a broad range of anti-inflammatory effects it has been tested as a therapeutic in several animal inflammatory disease models. Indeed, ES-62 was found to be an effective treatment in a number of disease models including collagen-induced arthritis (CIA) and an OVA-induced model of allergic asthma [234]. Intriguingly, ES-62 seems to be capable of reducing pathology in 'T_H17' autoimmune diseases and T_H2 allergic diseases but has less effect on T_H1 diseases as ES-62 treatment could not alleviate T_H1 immunopathology in *Toxoplasma gondii* infection model, nor did it have any effect on *Plasmodium chaubaudi* parasitemia in mice [252] [253].

CIA is a well-established murine model of rheumatoid arthritis (RA). It was originally thought to be primarily mediated through T_H1 responses and so with ES-62's potent ability to suppress aggressive T_H1 responses the prophylactic and therapeutic potential of ES-62 in this model was investigated. ES-62 was found to exert a potent anti-inflammatory effect in CIA in both prophylactic and therapeutic models [254]. This was associated with a significant inhibition of collagen-specific pro-inflammatory cytokines (TNF-α, IL-6 and IFN-γ) as well as reduced levels of collagen-specific IgG2a [254]. More recently it has been found that ES-62 exerts this protective effect through suppression of T_H17 responses by targeting a complex network of inflammatory cells including γδ T cells and DCs [211]. As with so many of the immunomodulatory effects of ES-62 protection against CIA can be mimicked by the use of PC-OVA or PC-BSA [255].

Systemic lupus erythematosus (SLE) is an autoimmune disease that can affect the skin, joints, kidney, brain and other organs. SLE is characterised by high titres of autoantibodies, often against nuclear antigens (ANA). These antibodies can generate immune complex-mediated inflammation in the skin, kidney and joints. Many patients go on to develop joint pain and arthritis and glomerulonephritis, which are leading

contributors to morbidity. ES-62 was found to reduce proteinuria, a marker for kidney damage, and creatinine levels in MRL/lpr mice, which spontaneously develop lupus-like pathology within 4 months but had no effect on glomerular hypercellularity [240]. ES-62-treated mice also had decreased incidence and severity of arthritis and while there was no difference in total IgG1, IgG2a or IgM production, the treated mice had reduced production of pathogenic ANA. The mechanism of action appears to be via ES-62 resetting the balance between effector and regulatory B cells in MRL/lpr mice and indeed transfer of purified splenic B cells from ES-62-treated mice is enough to confer the protection afforded by the helminth product [240].

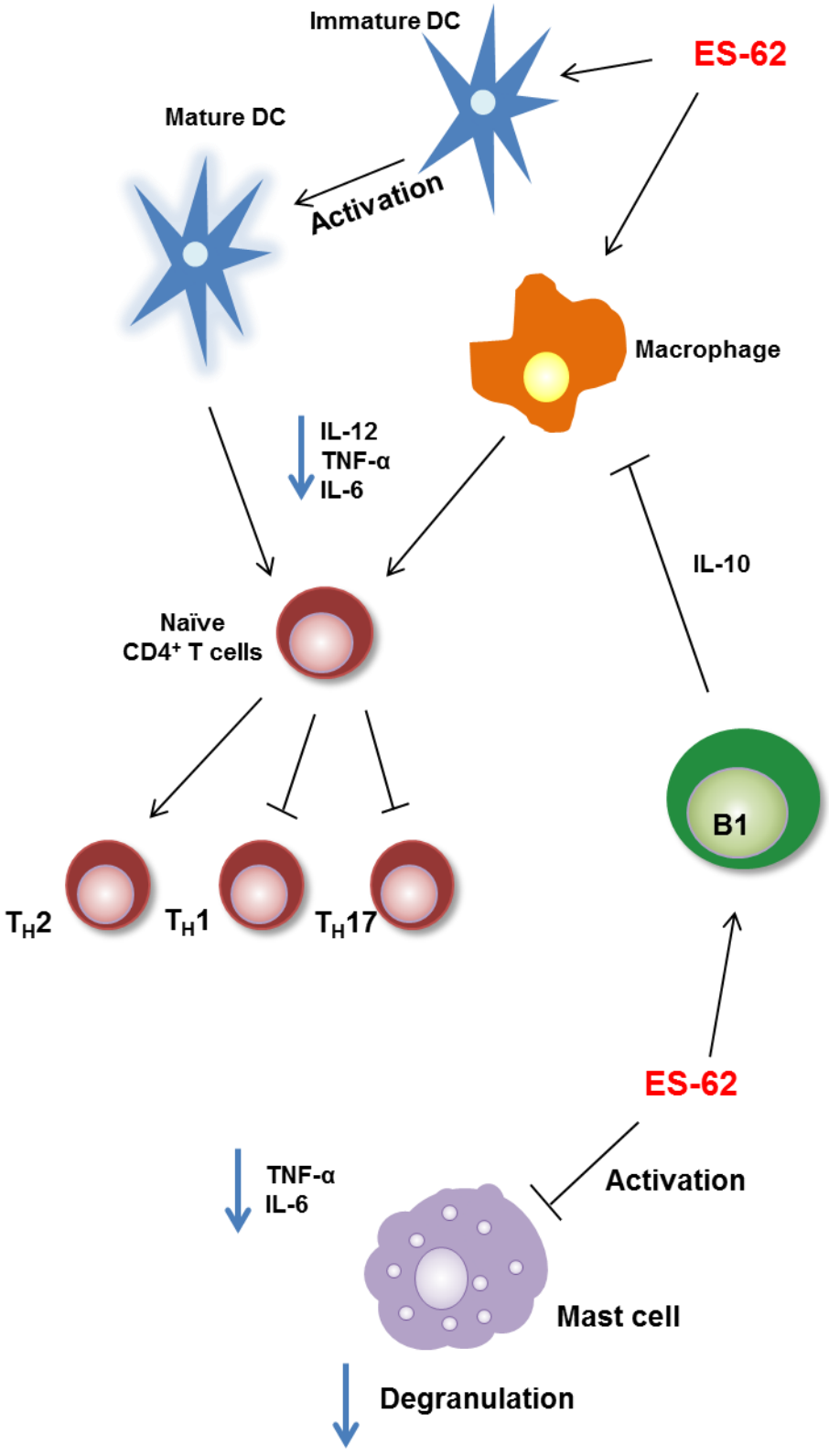
SLE and RA patients have a much higher risk of developing accelerated atherosclerosis, causing a consequent increased risk of cardiovascular disease. Atherosclerosis is the leading cause of cardiovascular disease in the Western world and is caused by lipid deposition and intimal thickening of the aorta and larger arteries such as the coronary artery [256]. The apoE-/- mouse is a well-established model for the study of atherosclerosic lesion formation [257] while the gld.ApoE-/- mouse show accelerated atherosclerosis and is utilised as a model for the study of for the accelerated cardiovascular disease that can occur in some lupus patients [258]. When treated with ES-62 in osmotic pumps for 12 weeks these mice demonstrated reduced atherosclerotic lesion area of nearly 60% compared to PBS-treated mice, with reduced numbers of macrophages and collagen at the lesion site. They also had some evidence of reduced renal disease as measured by decreased proteinuria, as well as decreased levels of ANA indicating that ES-62 is also protective against lupus-associated accelerated atherosclerosis in a mouse model [256].

In addition to being protective against autoimmune diseases ES-62 has been found to display protective effects in mouse models of allergic diseases and asthma. Prophylactic exposure to ES-62 *in vivo* has been found to reduce pathology in the murine OVA-induced model of allergic asthma [250]. This protection is associated with ES-62-induced desensitisation of mast cells, reduced lung infiltration by eosinophils, neutrophils and lymphocytes as well as inhibited local T_H2 cytokine responses [250] [220]. IL-17 has been shown to play a role in promoting T_H2 responses while suppressing T_H1 [259] and ES-62 was found to suppress IL-17-producing γδ, CD4 and CD8 T cells [220]. It appears ES-62 does not increase the regulatory T cell population but rather protects against T_H2-associated airway inflammation by a switch in priming towards a T_H1-like phenotype with enhanced IFN-γ production and cells expressing the

T_H1 signature transcription factor *Tbet,* while suppressing T_H2/T_H17 responses [220]. Consistent with this, ES-62-mediated protection against airway inflammation in terms of pro-inflammatory cell infiltration and lung pathology was blocked with the use of neutralising anti-IFN-γ antibodies [220].

Figure 1.7: The Immunomodulatory effects of ES-62

ES-62 targets multiple cell types to mediate its overall anti-inflammatory effects on the immune system. In APCs (DCs and macrophages) ES-62 down-regulates the pro-inflammatory cytokine responses of these cells resulting in decreased ability to prime T cells towards T_H1 or T_H17 phenotype while promoting T_H2 responses. ES-62 induces IL-10 production by B1 cells, further down-regulating pro-inflammatory responses by APCs. In addition, ES-62 desensitises mast cells to FcεR1 and LPS stimulation with decreased IL-6 and TNF-α production and reduced degranulation responses.


Immature DC
ES-62
Mature DC
Activation
Macrophage
IL-12
TNF-α
IL-6
IL-10
Naïve
CD4+ T cells
B1
TH2
TH1
TH17
ES-62
TNF-α
IL-6
Activation
Mast cell
Degranulation

1.14.5 Small molecules analogues of ES-62

Studies with ES-62 in various animal models have demonstrated that the helminth molecule has therapeutic potential against autoimmune and allergic diseases. However, it is a large and hence potentially immunogenic protein and therefore it is not a suitable drug candidate. As it has been demonstrated that many of the immunomodulatory properties of ES-62 are due to the PC moiety of the molecule a library of small molecule analogues (SMAs) were developed by Drs Abedawn Kalaf and Judith Huggan under the supervision of Professor Colin Suckling in the Department of Pure & Applied Chemistry at the University of Strathclyde. The immunomodulatory potential of these SMAs was initially investigated in macrophages *in vitro* and two sulfones, 11a and 12b, were found to mimic the inhibitory effect of ES-62 on TLR-induced pro-inflammatory cytokines [260] [261].

These SMAs were subsequently found to confer protection against CIA in mice and mirrored ES-62's mechanism of action by downregulating the TLR adaptor protein MyD88 [260] [261]. 11a-mediated protection also closely mimicked ES-62 with suppressed IFN-γ and IL-17 responses [260]; however 12b-mediated protection was also associated with novel immunomodulatory properties not previously seen with ES-62. Thus, treatment with 12b significantly decreased the expression of a number of genes in macrophages that regulate the inflammasome and specifically inhibits IL-1β production *in vitro*. This is also observed *in vivo* as joints from CIA mice treated with 12b had significantly less IL-1β than PBS-treated mice [261].

Therapeutic treatment of 11a and 12b with MRL/lpr mice reduced proteinuria but, like ES-62, had no effect on glomerular hypercellularity. Both SMAs reduced ANA levels and kidney MyD88 expression levels as well as the secretion of IL-6 by kidney cells [262].

The SMA library was also screened against mast cells responses to investigate their potential against allergic responses. The sulfones 11a and 12b were again found to mimic ES-62 actions as pre-treatment with these SMAs inhibited FcεR1- and LPS-mediated pro-inflammatory cytokine production and decreased FcεR1-mediated calcium mobilisation and degranulation in mast cells. Following on from these *in vitro* screening studies 11a and 12b were then tested in the OVA-induced model of airway inflammation. Both SMAs were found to inhibit OVA-induced T_H2-associated airway inflammation and eosinophil infiltration of the lungs in mice [263].

In addition, both SMAs were also found to be protective in the mouse oxazalone-induced contact hypersensitivity model, employed to study human allergic contact dermatitis. Prophylactic treatment with the SMAs 11a and 12b reduced ear swelling following antigen challenge with reduced cellular infiltration and collagen deposition in the ear. Inflammatory cytokines IL-4, IFN-γ, IL-17a and TNF-δ mRNA were all up-regulated upon oxazalone challenge and treatment with 11a and 12b caused a significant decrease in IFN-γ mRNA [264]. These studies indicate that drugs based on the PC moiety of ES-62 are viable drug candidates for allergic diseases.

Aims of the thesis

The aims of this thesis were to investigate the effects of the ES-62 SMAs on the activation and maturation of bone marrow derived dendritic cells.

Specifically the aims were to:

1. Identify SMAs with immunomodulatory potential through preliminary *in vitro* screening of the library against LPS-induced cytokine responses
2. Investigate the effects of selected SMAs on the activation and maturation phenotype of bone marrow DCs
3. Investigate the mechanisms by which these SMAs modulate signal transduction pathways to downregulate pro-inflammatory cytokines
4. Explore the potential of the selected SMAs to prime DCs to modulate immune responses *in vivo*.

Chapter 2. Materials and Methods

2.1 Animals

Male 6-8 week old BALB/c and C57/BL6 wild type (WT), TLR4 knockout (KO) and MyD88 KO mice were used to generate bone-marrow derived dendritic cells (bmDCs) for this study. BALB/c mice were bred at the University of Strathclyde for all experiments except the adoptive transfer experiment (Section 2.11) where the mice were purchased from Charles River Laboratories (Tranent, Scotland). Wild type C57/BL6 (WT), TLR4 KO and MyD88 KO mice were maintained at Trinity College, Dublin and were a kind gift from Professor Padraic Fallon. For the *in vitro* co-culture experiments and *in vivo* adoptive transfer experiments, mice homozygous for the transgenic TCR which is specific for the chicken Ovalbumin (OVA) peptide$_{323-339}$ in the context of I-A^{d} (D0.11.10 on a BALB/c background) were used as T cell donors. Unless indicated otherwise, all mice were specified pathogen-free and maintained at the University of Strathclyde Biological services Unit in accordance with Home Office UK Licence PL 60/4300. Collagen-induced arthritis (CIA) was induced in male DBA/1 mice (10 weeks; Harlan Olac; Bicester, UK) and these mice were also used to generate bmDCs for use in the CIA study. DBA/1 animals were maintained in the Biological Services Unit of the University of Glasgow in accordance to the Home Office UK Licence PPL 60/26532 and PIL 70/26532 and the respective Ethics Review Boards of the University of Glasgow.

2.2 Preparation of bone-marrow derived dendritic cells (bmDCs)

BmDCs were prepared from bone marrow cells obtained from the femurs and tibias of 6-8 week old BALB/c and C57/BL6 wild type, TLR4 KO and MyD88 KO mice as described previously [265]. Bones were briefly cleaned in 70% ethanol to remove flesh and connective tissue. Both ends of each bone were cut to reveal the marrow, and were flushed with complete RPMI 1460. RPMI complete contained Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza, Slough, UK) with 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin (all Sigma-Aldrich, Poole, UK), 50 µM 2-βMercaptoethanol (Life Technologies, Paisley, UK), 5ml HEPES (Sigma-Aldrich) and 10% Heat-inactivated foetal calf serum (FCS) (Lonza)) using a 25 gauge needle. The resulting cell suspension was mixed using a 21 gauge needle to obtain a homogenous cell solution and any shards of bones and debris were removed by passing the cell suspension through a sterile Falcon™ cell strainer (Thermo Scientific**,** Loughborough, UK). The cell suspension was then centrifuged at 400 xg for 5 minutes, re-suspended

in fresh RPMI complete medium supplemented with 10 ng/ml GM-CSF (PeproTech, London, UK), and counted. Cell concentration was then adjusted to 2 x 10^6 cells/ml and cells were then seeded in sterile bacteriological petri dishes – 2 x 10^6 cells in 10 ml RPMI complete medium supplemented with 10 ng/ml GM-CSF. Cells were maintained in the petri dishes at 37°C in 5% CO_2 in an incubator with 10 ml fresh RPMI complete supplemented with 20 ng/ml GM-CSF added on day 3. On day 6, 10 ml of medium was removed from each petri dish and replaced with fresh RPMI complete supplemented with 20 ng/ml GM-CSF. On day 8 loosely adherent cells were harvested by washing the petri dishes twice with cold, sterile PBS (Lonza). Collected cells were centrifuged at 400 xg for five minutes, the supernatant was discarded and cells were re-suspended in fresh RPMI complete. Cells were then stained with Trypan Blue (Sigma-Aldrich) to ascertain cell viability and counted. Cell identity was confirmed by flow cytometry as described in Section 2.5.1. On average bmDCs were 80% $CD11c^+MHC\ II^+$ and were always at least 65% $CD11c^+$ for use in the initial cytokine screening assays. For all other experiments cells were at least 70% $CD11c^+MHC\ II^+$.

2.3 Cytokine stimulation assay

BmDCs were grown from BALB/c and C57/BL6 WT, TLR4 KO and MyD88 KO mice as described (Section 2.2) and were then plated in triplicate at 2 x 10^5 cells/well in RPMI complete medium in Corning ®Costar® 96 well ultra-low binding plates (Sigma-Aldrich) and rested overnight. The plates were then centrifuged at 400 xg and 50 µl of medium was removed from each well. 50 µl of SMAs (5 µg/ml) in RPMI complete medium or RPMI complete medium alone were added and cells were incubated for 18 hours before stimulation with PAMPS: LPS (100 ng/ml or 1 µg/ml; *Escherichia coli* 055:B5, Sigma-Aldrich); BLP (100 ng/ml; Pam_3CSK_4, InvivoGen, Toulouse, France): CpG (0.1 µM; Sources Biosciences, Nottingham, UK) and PolyI:C (10 µg/ml and 100 µg/ml; Sigma-Aldrich) in 100 µl fresh RPMI complete medium for 24 hours. Plates were then centrifuged and supernatants were removed and stored at -20°C for future analysis.

2.4 Enzyme-Linked ImmunoSorbent Assay (ELISA)

Interleukin-6 (IL-6), IL-12p40, IL-10, TNF-α (all BD Pharmingin, Oxford, UK), IL-12p70, IFN-γ, IL-4 (all eBioscience, Hatfield, UK), IL-17 and IL-1β (both R&D Systems, Abingdon, UK) expression was measured using enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions. The cytokines were detected using biotinylated monoclonal antibodies, streptavidin horseradish peroxidase (SAv-

HRP) and TMB substrate. High binding 96 well ELISA plates (Greiner BioOne) were used for all assays. Plates were coated overnight at 4°C with 50 µl capture antibody diluted according to each cytokine's experimental protocol in coating buffer. For IL-6 and TNF-α, the coating buffer was 0.1 M sodium carbonate, pH 9.5: for IL-10 and IL-12p40, the coating buffer was 0.2 M sodium phosphate, pH 6.5: for IL-12p70, IL-4 and IFN-γ, the coating buffer was provided in the ELISA kit and for IL-17 and IL-1β the coating buffer was PBS (pH 7.2-7.4). Plates were washed three times in wash buffer (PBS with 0.05 % Tween 20) and dried by blotting. The plates were blocked with 200 µl assay diluent (PBS with 10% FCS) and incubated at room temperature for 1 hour. After washing as before, 50 µl samples were added to the plates either neat or diluted with assay diluent. Standard cytokine samples that had been serially diluted were added, 50 µl, per well, to generate a standard curve and incubated at either room temperature for two hours or at 4°C overnight. Plates were then washed five times in wash buffer and dried before addition of the detection antibody, 50 µl per well. Detection buffer was diluted in assay diluent at the concentration recommended by the manufacturer for all cytokines. For IL-6, IL-12p40 and IL-10 the enzyme reagent, streptavidin-horseradish peroxidise conjugate, was diluted in the detection antibody and the plates were incubated for an hour. For all other cytokines, plates were incubated with detection antibody alone for an hour before washing and addition of the enzyme-streptavidin conjugate, diluted according to the manufacturer in assay diluent. After seven washes, with a 30-60 seconds soak for each wash, TMB substrate solution was added to the plates. All reactions were stopped using 2 NH_2SO_4. Plates were read at 450 nm on an Epoch microplate spectrophotometer (BioTek) and the data analysed using Gen5 and Prism 5.

2.5 Fluorescence activated cell sorting (FACS) analysis

2.5.1 Staining for surface proteins

Aliquots of cells (0.25-1 x10^6 per sample) in 5 ml polystyrene tubes (Falcon, BD) were washed in FACS buffer (PBS containing 0.5% Bovine Serum Albumin [BSA; Sigma-Aldrich] and 2 mM EDTA) by centrifugation at 400 xg for 5 minutes. Cells were incubated with anti-mouse CD16/CD32 for five minutes at 4°C. By binding to CD16/CD32 (FcγRII/III) this antiserum blocks non-specific binding of immunoglobulin to Fc receptor. Cells were then incubated with the appropriate fluorochrome-conjugated or biotinylated primary antibodies for 15-30 minutes at 4°C in the dark. Details of the antibodies used are provided in Table 2.1. The cells were then washed with 1 ml FACS

buffer and, where necessary, incubated with fluorochrome-conjugated streptavidin for 15-30 minutes in the dark at 4°C to detect biotinylated antibodies. Cells were then washed as before with FACS buffer and re-suspended in 200 µl FACS buffer before being analysed on a FACSCanto immunocytometry system (BD Pharmingin). Where appropriate, the fluorochrome 7-amino-actinomycin D (7-AAD) (BD Biosciences) was added for 10 minutes before analysis. This compound intercalates in double-stranded DNA in cells with disrupted membranes (a common feature of dying cells) and so is used to investigate cell viability. Prior to running all samples the forward scatter (FSC) and side scatter (SSC) voltages were adjusted in order to gate the cells for analysis. Unstained cells and single-stained cells for each fluorophore present in the experiment were used to set compensation values. Data were analysed using FlowJo software (Tree Star Inc, OR, USA, version 7.6.1).

2.5.1.1 Investigation into the expression of TLR4/MD2 and of co-stimulatory molecules CD40, CD80, CD86 and MHC II on $CD11c^+$ bmDCs

Loosely adherent cells were harvested on day 8 and examined for the expression of CD11c and MHC II. BmDC identity was confirmed by flow cytometry and cells were plated at 2 x 10^6 cells in 2 ml in 6-well plates and rested overnight. BmDCs were pre-treated with SMAs 11a, 12b, 11e, 11i or 19o (5 µg/ml) for 18 hours before stimulation with LPS (100 ng/ml) for 24 hours. Cells were harvested from the plates using cold PBS and rubber syringes. BmDCs were centrifuged at 400 xg for five minutes and re-suspended in 400 µl FACS buffer. Each sample was then examined in triplicate and the remaining cells in each sample were pooled to provide a mixed population for the FACS controls. Cells were next stained as described in Section 2.5.1 and analysed on a FACSCanto system. Data were analysed using FlowJo software. Cells were first gated depending on size and granularity and doublet cells were removed. The positive gates for each antibody were set using fluorescent minus one controls and cells were gated on CD11c expression and the expression of TLR4/MD2, CD40, CD80, CD86 or MHC II on $CD11c^+$ cells was established.

2.5.2 Staining for intracellular proteins

Intracellular cytokine analysis was undertaken on T cells from the draining popliteal lymph node from BALB/c recipients that had received $CD4^+CD62L^+$ D011.10 T cells and OVA-pulsed DCs, and from the dLNs of mice subjected to collagen-induced arthritis pre-treated with SMA-treated or control bmDCs. The lymph nodes were

collected and forced through a Nitrex filter cup with a syringe plunger and the cells were washed in FACS buffer and counted. 1 x 10^6 cells were then used for surface protein analysis as described in Section 2.5.1 to investigate T cell activation status and 1 x 10^6 cells were used to investigate the expression of intracellular cytokines in lymphocytes. Cells were incubated at 37°C with 50 ng/ml PMA and 500 ng/ml Ionomycin (both Sigma-Aldrich) for five hours with Brefeldin A (eBiosciences) added for the last four hours. FACS buffer was then added and the cells were centrifuged at 400 xg for five minutes and the supernatant discarded. Cells were washed a further two times with FACS buffer before being re-suspended in 100 µl FACS buffer and examined for surface expression of cell markers as described above. Cells were next re-suspended in 100 µl ICT Fixation buffer (eBiosciences) and incubated at room temperature for 20 minutes. They were then washed in 1x Permeabilization buffer (eBiosciences), re-suspended in 100 µl of 1x Permeabilization buffer and incubated with IFN-γ, IL-17, IL-4, IL-10, IL-22 or FOXP3 antibodies for 15-30mins in the dark at 4 °C. Details of antibodies used are provided in Table 2.1. The cells were then washed with 1 ml Permeabilization buffer and, where necessary, incubated with fluorochrome-conjugated streptavidin for 15-30 minutes in the dark at 4°C to detect biotinylated antibodies. Cells were next washed as before with Permeabilization buffer and then with FACS buffer and were re-suspended in 200 µl FACS buffer before being analysed on FACSCanto immunocytometry system (BD Pharmingin).

Table 2.1: Antibodies employed in Flow Cytometry

Specificity	Clone	Conjugate	Manufacturer
CD11c	N418	Pe/Cy7	BioLegend
MHC II	M5/114.15.2	PE	BioLegend
MHC II	AMS-32.1	APC	eBioscience
MHC II	AMS-32.1	Biotin	eBioscience
CD11b	M1/70	FITC	BioLegend
CD40	K10	Biotin	eBioscience
CD80	16-10A1	FITC	BioLegend
CD86	GL-1	PerCP	BioLegend
TLR4/MD2	MT5510	PE	BD Biosciences
CD4	RM4-5	PerCP	BD Biosciences
D0.11.10 TCR	KJ1-26	APC	eBioscience
IL-17A	eBio17B7	Biotin	eBioscience
IFN-γ	XMG1.2	Pe/Cy7	BioLegend
IL-4	11B11	PE	BD Biosciences
IL-10	JES5-16E3	PE	eBioscience
IL-22	Poly5164	PE	BioLegend
FOXP3	FJK-16s	APC	eBioscience
CD62L	MEL-14	APC	eBioscience
CD62L	MEL-14	PE	BD Biosciences
CD69	H1.2F3	Pe/Cy7	BioLegend
Isotype Controls			
FITC	Rat IgG2a		eBioscience
PE	Rat IgG2a		BD Biosciences
Pe/Cy7	Hamster IgG		BioLegend
APC	IgG1		eBioscience
PerCP	IgG2a		BioLegend

2.6 Preparation of RNA extracts

BmDCs were grown as described and were plated at 2 x 10^6 cells in 2 ml RPMI complete medium in 6-well plates, and rested overnight. BmDCs were either pre-treated with SMAs 11a, 12b, 11e, 11i, 11h, 11k or 19o at 5 µg/ml for 18 hours and then stimulated with LPS (100 ng/ml) for 4 hours or treated simultaneously with SMAs and LPS for 4 hours. In addition, DCs were stimulated with SMAs alone for 4 or 18 hours. RNA was extracted from these cells using the RNeasy Plus Mini kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions. Briefly, cells were washed with PBS and centrifuged at 400 xg for 5 minutes. Supernatant was discarded and the pellet was re-suspended in 350 µl RLT lysis buffer (a denaturing guanidine-isothiocyanate–containing buffer) containing 1% β-mercaptoethanol and vortexed to lyse cells. The lysate was added to a gDNA Eliminator spin column to remove genomic DNA. The column was spun for 30 seconds at ≥8000 xg and 70% ethanol was added to the flow-through. The sample was then applied to an RNeasy spin column where total RNA binds to the silica based membrane, and contaminants were washed away using additional supplied wash buffers. The RNeasy spin column was then transferred to a fresh collection tube and the RNA was eluted in RNase-free water.

2.7 Real-time PCR by TaqMan®

The High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technology) was used according to the manufacturer's instructions to transcribe the mRNA samples to cDNA. Briefly, 1 µg/ml mRNA was incubated with random primers, 4 mM dNTPs and 50 units/µl MultiScribe™ Reverse Transcriptase diluted in nuclease free water. The samples were transferred to the Veriti™ 96 well plate thermal cycler (Applied Biosystems).

TaqMan® real-time PCR was performed according to manufacturer's instructions (Applied Biosystems). Duplicate PCR amplifications were performed using the StepOne Plus™ real-time PCR system (Applied Biosystems). For each sample the total volume was 15 µl per well consisting of 5 µl cDNA (30 ng) and 10 µl amplification buffer. The amplification buffer contained gene-specific forward and reverse primers (TaqMan® gene expression assays, Applied Biosystems) diluted in 1x TaqMan® Fast Universal PCR Master Mix and nuclease-free H_2O. The primers are supplied as a pair of unlabelled primers with the reporter dye 6-carboxyfluorescien (FAM™) at the 5' end

and a minor groove binder (MGB) and non fluorescent quencher (NFQ) at the 3' end. The endogenous control was glyceraldehyde 3-phosphate dehydrogenase (GAPDH), also supplied by Applied Biosystems. Details of all primers used are provided in Table 2.2. The results were analysed using StepOne software, which calculates the comparative threshold, Ct, for each gene, which is the value where the PCR cycle crosses the set threshold. The lower the Ct value, the more mRNA is present in the sample. Samples were first normalised to the housekeeping gene, GAPDH, to calculate the ΔCt value, and then to unstimulated DCs to calculate the ΔΔCt value. Results are then presented as a fold change (RQ) compared to unstimulated DCs (value of 1), which is calculated with the formula $RQ = 2^{-\Delta\Delta Ct}$.

Table 2.2: TaqMan® Primers for real-time PCR

Gene	Assay Number (Applied Biosystems)
GAPDH	Mm99999915_g1
IL-6	Mm00446190_m1
TNF	Mm00443259_g1
IL-12p35	Mm00434165_m1
IL-12p40	Mm00434174_m1

2.8 Nuclear localisation of NF-κB

BmDCs were grown as described and were plated at 2 x 10^6 cells in 2 ml RPMI complete medium in 6-well plates, and rested overnight. BmDCs were pre-treated with SMAs 11a, 12b, 11e, 11i or 19o (5 µg/ml) for 18 hours then stimulated with LPS (100 ng/ml) for 30 minutes. Cells were then washed with cold PBS and scraped from the plates on ice using rubber syringes, and centrifuged at 13,000 xg for one minute. The supernatant was removed and the pellet re-suspended in a low salt buffer containing 0.5 mM phenylmethanesulfonyl fluoride solution (PMSF, Sigma-Aldrich) and HALT™ protease inhibitor cocktail (Thermo Scientific) and incubated on ice for 15 minutes. 25 µl of detergent (10% v/v Triton X, Sigma Aldrich) was then added, the samples were vortexed briefly and centrifuged for one minute at 13,000 xg. The supernatant containing the cytoplasmic fraction was stored at -20°C for further analysis and the pellet was then re-suspended in a high salt buffer containing 0.5 mM PMSF and HALT™ protease inhibitor cocktail, vortexed briefly and incubated on a shaker at 4°C for 15 minutes before being sonicated on ice for 2 x 30 second intervals. The samples were then centrifuged at 13,000 xg for 15 minutes, the supernatants removed and maintained at -20°C for further analysis.

The amount of protein in each sample was determined using the Pierce® BCA Protein assay (Thermo Scientific). Briefly, protein standards were prepared by diluting the supplied albumin (BSA) according to the manufacturer's instructions and added in duplicate to a 96-well plate. The samples were diluted in the high salt buffer from the preparation of the nuclear extracts, and 10 µl were also added to the plate and 200 µl of working reagent were added to unknown samples and standards and the plates were incubated at 37°C for half an hour and read at 562 nm on a microplate reader. 2 ng/ml of protein was used for each sample and the expression of NF-κBp65 in the nuclear extract fraction was analysed using the Actif Motif TransAM NF-κBp65 Transcription Factor Assay kit according to the manufacturer's instructions. Samples were diluted in complete lysis buffer and added in duplicate to wells to which an oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3') has been immobilized along with complete binding buffer. The active form of NF-κB will specifically bind to this oligonucleotide. The provided Jurkat cell nuclear extract is added in duplicate as a positive control. Blank control wells contained complete binding buffer and complete lysis buffer. The plate was sealed and incubated for an hour at room temperature with mild agitation. The plate was next washed by adding 200 µl of

provided wash buffer, which was removed by flicking and the plate blotted on paper towels. The provided anti-NF-κB antibody (diluted in Antibody Binding buffer) was added to each well; the plate was covered and incubated at room temperature for an hour. The plate was washed as before and an anti-IgG HRP-conjugated antibody was added and the plate incubated for another hour at room temperature. The plates were next washed as before and Developing Solution was added. The plates were then incubated in the dark until the colour representative of the enzyme-substrate reaction developed, at which time the reaction was terminated with provided Stop Solution and the absorbance read on a microplate reader at 450 nm. The absorbance of the blank control wells was subtracted from the sample wells.

2.9 Fast Activated Cell-based ELISA (FACE)

96-well plates were coated with 10 µg/ml Poly-l-Lysine (Sigma-Aldrich) for 30 minutes at 37°C before being washed 3 times with sterile PBS and allowed to dry. BmDCs were grown as described (Section 2.2) and were plated at 1 x 10^5 cells/well. Cells were rested overnight before being treated in triplicate for 18 hours with SMAs 11a, 12b, 11e, 11i or 19o (5 µg/ml). BmDCs were then stimulated with LPS (100 ng/ml) for 10 minutes. The supernatants were discarded and bmDCs were fixed on the plates using 4% (w/v) paraformaldehyde (Santa Cruz Biotechnology, Texas, USA) for 20 minutes at room temperature or at 4°C until required. The plates were then washed twice with 200 µl wash buffer (0.1% Triton X in PBS) and 100 µl Quenching buffer (wash buffer containing 1% H_2O_2) was added for 20 minutes to inactivate the cells' endogenous peroxidase activity. The wells were then washed as before and 100 µl blocking solution (Wash buffer with 5% Milk (Marvel) was added to each well. Plates were then incubated at room temperature for one hour on a rocking platform before being washed as before. The primary antibody was then diluted in antibody diluent (5% BSA in wash buffer) and 40 µl were added to corresponding wells. Details of all antibodies used are provided in Table 2.3. Antibody diluent was added to three wells to act as blank controls. Plates were incubated overnight at 4°C on a rocking platform. Plates were next washed three times as before and the secondary antibody was added (diluted in antibody diluent). The plates were incubated with secondary antibody for one hour at room temperature and then washed three times in wash buffer and twice in PBS before the TMB substrate was added. Plates were read at 450 nm on an Epoch microplate spectrophotometer. The cell number in each well was validated using crystal violet staining: plates were washed twice in wash buffer and then H_2O and 10% crystal violet

solution was added for 30 minutes. Plates were next washed five times with H_2O and then incubated for one hour at room temperature on a rocking platform with 1% SDS solution. The plates were then read at 595 nm.

2.10 *In vitro* bmDC and T cell co-cultures

BALB/c bmDCs were grown as described in Section 2.2. 2.5 x 10^4 bmDCs were plated in 24-well plates and rested overnight. Cells were then treated with SMAs 11a, 12b, 11e or 11i (5 µg/ml) for 18 hours before stimulation with LPS (100 ng/ml) for 24 hours. BmDCs were then washed 3 times with PBS before being loaded with 10 µM, 100 µM or 300 µM OVA peptide$_{323-339}$ for 3 hours. Cells were then washed five times with cold RPMI complete medium and cultured with 2 x 10^6 $CD4^+CD62L^+$ T cells and incubated at 37°C with 5% CO_2 for 72 hours. The cells were centrifuged at 400 xg for five minutes and the supernatants were stored at -20^{o}C for future cytokine analysis by ELISA (as described in Section 2.4).

2.10.1 Isolation of $CD4^+CD62L^+$ T cells

Naïve T cells were isolated from the peripheral (inguinal, cervical, popliteal and auxillary) and mesenteric lymph nodes (LN) of donor D011.10 mice (containing OVA specific $CD4^+$ T cells) using the MACs separation kit (Miltenyi, BioTek) according to the manufacturer's instructions. Briefly, LN cells were re-suspended in 400 µl MACS buffer (PBS containing 0.5% BSA and 2 mM EDTA, pH 7.2) and incubated with a cocktail of lineage specific biotinylated antibodies against CD8a, CD11b, CD11c, CD19, CD45R, CD49b, CD105, MHC class II, Ter-119, CD25 and TCRγδ for 15 minutes at 4°C. The cells were then incubated with anti-biotin microbeads for 15 minutes and then run through an LS column in the magnetic field of a MACS separator to deplete non T cells, as well as regulatory T cells and γδ T cells. Cells were then washed and incubated with anti-CD62L microbeads for 10 minutes before being washed with MACS buffer and then run through an LS MACs column. $CD4^+CD62L^+$ T cell identity and percentage was confirmed by flow cytometry. 2 x 10^5 naïve $CD4^+$ T cells were then re-suspended in T cell medium (RPMI complete with sodium pyruvate, 5 ml MEM nonessential amino acids (both Sigma-Aldrich), 5 ml HEPES and 50 µM β-mercaptoethanol).

2.11 Preparation of cell suspensions for adoptive transfer

$CD4^+CD62L^+$ naïve T cells were isolated from D0.11.10 mice as described in section 2.10.1. Transfer of these transgenic T cells, which are specific for the OVA$_{323-339}$

peptide in the context of 1-A^d MHC Class II molecules, produces a population that can be detected by flow cytometry using an anti-clonotypic antibody but is also small enough that it reacts in a physiological manner following antigen challenge *in vivo* [266].

2.11.1 Transfer of D0.11.10 T cells

The percentage of KJ1.26$^+$CD4$^+$CD62L$^+$ D0.11.10 T cells in these preparations was determined by flow cytometry as described in Section 2.5.1. Cell suspensions containing 1 x 10^6 transgenic T cells in 200 µl PBS were injected intravenously (i.v) through the tail vein of male BALB/c mice.

2.12 Preparation of bmDCs for adoptive transfer

In order to use bmDCs for adoptive transfer, cells were grown as described in Section 2.2. Cells were plated at 2 x 10^6 in 2 ml in 6-well plates and were then treated with SMAs (5 µg/ml) and pulsed with 10 µg/ml of $OVA_{323-339}$ peptide or 10 µg/ml Bovine Type II collagen (CII) (MD Biosciences, Zurich, Switzerland) for 18 hours before stimulation with LPS (100 ng/ml) for 24 hours. Cells were scraped with cold PBS, washed three times with PBS (each wash is followed by centrifugation for 5 mins at 400 xg). For adoptive transfer experiments bmDCs were re-suspended at 5 x 10^6 cells/ml and 2.5 x 10^5 bmDCs were then injected subcutaneously (injection volume 50µl) into the right footpad of male BALB/c mice. BALB/c mice were given non-OVA stimulated DCs, DCs that had been treated with SMA 11a or 11i and pulsed with OVA or with DCs that had been treated with SMAs 11a or 11i and pulsed with OVA and then stimulated with LPS. For the arthritis study bmDCs were re-suspended at 1.25 x 10^6 cells/ml in PBS and injected into the peritoneum (injection volume 200 µl) of DBA/1 mice. BmDCs were either loaded with Bovine Type II collagen (CII) alone (18 hour) or treated with SMA 11a/12b (5 µg/ml) in combination and loaded with CII (18 hours).

2.13 Collagen-induced arthritis (CIA)

10 week old male DBA/1 mice were used for the collagen-induced arthritis model. Arthritis was induced by intradermal immunization with CII emulsified with complete Freud's adjuvant (MD Biosciences) on day 0 (injection volume 100 µl) and with CII in PBS intraperitoneally on day 21 (injection volume 200 µl) and scored for development of arthritis as previously described [254].

2.14 *Ex vivo* analysis of draining lymph node cells

Draining lymph node cells (dLN) (10^6/ml) were incubated with 50 ng/ml PMA and 500 ng/ml Ionomycin for 1 hour before addition of 10 µg/ml Brefeldin A (Sigma-Aldrich, UK) for a further 4 hours at 37°C. Live cells were discriminated by the LIVE/DEAD (eBiosience) and phenotypic markers were labelled as described in section 2.5.1 before cells were fixed, permeabilised and stained for intracellular cytokines as described in section 2.5.2. Cells were gated with appropriate isotype and fluorescence minus one (FMO) controls.

2.15 Detection of serum antibodies from CIA mice

The levels of anti-CII IgG1 and IgG2a titres in the serum of individual CIA mice were detected by ELISA. ELISA plates were coated overnight with 20 µg/ml CII diluted in PBS at 4°C. The plates were washed three times with wash buffer (PBS + 0.05% Tween) then blocked with 4% BSA for an hour at 37°C. The plates were washed as before and serum samples were serially diluted on the plates to reach a final dilution of 1:218700, and incubated at 37°C for one hour, washed as before and then incubated with anti-IgG1 or anti-IgG2a Horseradish peroxidase anti-mouse conjugated antibodies diluted in PBS containing 25% (v/v) FCS for an hour at 37°C and then developed with TMB substrate, stopped with H_2SO_4 and the absorbance read at 450 nm on an Epoch microplate spectrophotometer (BioTek). The endpoint dilution, the point at which no further antibody was detected, was graphed and analysed using Prism 5.

Table 2.3: Antibodies employed in FACE analysis

Primary Antibodies				
Specificity	**Host**	**Clone**	**Dilution**	**Manufacturer**
MyD88	Rabbit	ab2068	1/1000	Abcam
p44/42 MAPK (ERK1/2)	Rabbit	9102	1/250	Cell Signalling Technology
P-p44/42 MAPK (p-ERK1/2)	Rabbit	9101	1/250	Cell Signalling Technology
p38 MAPK	Rabbit	9252	1/250	Cell Signalling Technology
P-p38 MAPK	Rabbit	9251	1/250	Cell Signalling Technology
SAPK/JNK	Rabbit	9212	1/250	Cell Signalling Technology
P-SAPK/JNK	Rabbit	9211	1/250	Cell Signalling Technology
Secondary Antibody				
Anti-Rabbit HRP	Goat	7074	1/1000	Cell Signalling Technology

Chapter 3. Identification of ES-62 Small Molecule Analogues that modulate dendritic cell activities

3.1 Design of ES-62 Small molecule Analogues (SMAs) based around its phosphorylcholine (PC) moiety

ES-62 is a potent immunomodulator secreted by the rodent filarial nematode *Ancanthocheilonema viteae* that is able to directly influence several immune system cell types including B lymphocytes, mast cells and the antigen presenting cells (APCs), macrophages and dendritic cells (DCs). ES-62 renders B lymphocytes hypo-responsive to crosslinking of the B cell receptor (BCR) and inhibits Fcε-R1-induced activation of mast cells (reviewed in [267]). Treatment of macrophages and DCs with ES-62 inhibits pro-inflammatory cytokine production following PAMP stimulation in a TLR4- and MyD88-dependent manner [246]. The unusual post-translational glycosylation and subsequent esterification by phosphorylcholine (PC) of ES-62 appears to be responsible for many of its key anti-inflammatory properties as pre-treatment of macrophages and DCs with PC conjugated to ovalbumin (PC-OVA) or even PC alone results in initial low levels of IL-12 production, subsequent suppression of full LPS-mediated activation of macrophages and DCs and inhibition of IL-12 induced by BLP and CpG. In keeping with ES-62 function, these effects are ablated in TLR4 KO mice. PC is a conserved structural component of many pathogenic organisms including certain Gram-negative and Gram-positive bacteria as well as fungal and protozoan parasites such as *Leishmania major* and *Trypanosoma cruzi* (reviewed in [232]). It has also been identified on the trematode *Schistosoma mansoni* and gastrointestinal and filarial nematodes [268]. Its function in many of these organisms is unknown and it appears to have both advantages and disadvantages to the host. In Gram-positive bacteria such as *Streptococcus pneumoniae*, PC is important for normal growth and cell division but it is also a major target for an antibody response by the infected host. In most nematodes PC appears to be present internally, in the gut and the uterus, and so does not appear as a target for the immune system. In filarial nematodes however, PC is found on the cuticle of infective larval stages of *A. viteae* and *Litomosoides sigmondontis* and is a major component of their excretory/secretory molecules [268]. PC can be attached to the cell surface of pathogens, as with extracellular bacteria that colonize the respiratory tract; be used to modify proteins and glycoconjugates, or be secreted on modified proteins such as ES-62 [225] [232]. *Legionella pneumophillia*, a Gram-negative, intracellular bacterium that invades host macrophages, uses its effector protein AnkX to phosphorylcholinate host Rab GTPases to direct transport and assembly of the vacuole it needs to survive within the host cytoplasm [269]. PC has also been linked to increased cell adhesion by both

bacterial and parasitic pathogens, facilitating host cell invasion and thus evasion of the extracellular immune system clearance responses [232]. For example, Erythrocyte membrane protein 1 (EMP 1) of the *var* gene family and heat shock protein (HSP 70), both of *Plasmodium falciparum* have been found to be PC modified proteins. These two proteins are major virulence factors and play a vital role in erythrocyte cell adhesion and invasion (reviewed by [270]). PC is also part of the recognition domain of platelet-activating factor (PAF), which binds PAF receptor (PAFR) on epithelial cells. Bacterial species such as *S. pneumonia* and *P. aeruginosa* employ PC in an example of molecular mimicry, allowing them to bind to the host PAFR to assist in cell adhesion and invasion [232]. Oxidised phospholipids (OxPAPC) containing PC have also been shown to inhibit LPS-induced, NF-κB-mediated upregulation of inflammatory genes by blocking the PAMP's interaction with TLR4 accessory proteins, LPS binding protein (LBP) and CD14. Administration of OxPAPC was previously found to prevent inflammation and rescue LPS-injected mice from lethal endotoxin shock [271], and has now also been shown to be protective against lung damage caused by heat inactivated *Staphylococcus aureus* (HKSA) by both co- and post-treatment regimens [272]. However how much of the immunomodulatory action of these molecules is solely due to PC remains to be investigated.

Choline itself is an essential nutrient in eukaryotes. In humans it is required for synthesis of several molecules such as PAF and the cell membrane lipids phosphatidylcholine and sphingomyelin. As a result choline is readily available to microbes during infection and therefore they are able to scavenge it for use as a nutrient source, an osmoprotectant and as a tool for facilitating host immune system evasion [232]. The choline derivative cytidine-diphosphocholine (CDP-choline) has been shown to have an anti-inflammatory effect in arthritis and allergen-induced inflammatory mouse models [273], [274]. A recent study by Mehta *et al*, found that a daily choline supplement in addition to regular drug therapy suppressed oxidative stress in asthma patients. After 6 months patients with choline supplement had a lower blood eosinophil count and reduced levels of IL-5, IL-4 and TNF-α compared to control patients who received drug therapy alone [274]. In addition, a methionine/choline-deficient diet is used to induce non-alcoholic steatohepatitis (NASH) in mice [275] and this seems to be due to an increase in IL-1β production and mRNA of components of the inflammasome complex including AIM2, NLRP3, ASC and pro-caspase-1 [276].

Taken together these findings reported in the literature suggest that choline and its derivative PC can play a key role in modulation of the host immune response and supports the observation that many of the immunomodulatory effects of ES-62 appear to be due to its PC moieties. Previous work in the Harnett lab has also shown that PC esters of short peptides (unpublished) and lipids [277] can replicate some of the effects of ES-62 on DC cytokine responses [27]. However, due to the lability of lipid compounds, these are unlikely drug candidates and so a library of drug-like, low molecular weight, Small Molecule Analogues (SMAs) based around the PC moiety of ES-62 were synthesised by Drs Abedawn Kalaf and Judith Huggan under the supervision of Professor Colin Suckling in the Department of Pure & Applied Chemistry at the University of Strathclyde. The general structure of the SMAs is shown in Figure 3.1 and is based on one of the short PC ester peptides containing PC-tyrosine where the labile phosphate ester has been removed and replaced with a sulfone, sulfonamide, phosphonate or carboxamide group (blue shaded area in Figure 3.1; structures are shown in Figure 3.2) attached to the aromatic benzyl group. Small substituents ("x") of differing electronic and steric properties were also added to the benzyl ring (green shaded area of Figure 3.1). On the right-hand side of the SMAs (pink shaded area), dimethylamine, morpholine or pyrrolidine were added. Addition of the benzyl group makes the SMAs lipophilic and is thought to facilitate cell entry through the phospholipid bilayer of the cell membrane.

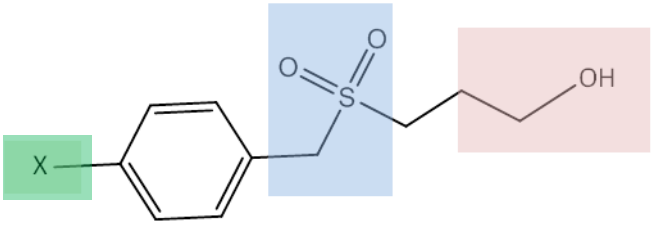


Figure 3.1: Basic SMA Structure

Sulfones have been widely used in medicinal chemistry as potential therapeutics against a range of infections and diseases including malaria, trypanosomiasis and Parkinson's disease. Quorum sensing is a common mechanism used by bacteria to regulate gene expression depending on population density. It is important for virulence in several species of bacteria and thus represents an attractive target for novel antibiotics: pathogenesis can be attenuated without targeting genes, which are essential to bacteria survival and so the selective pressure which can lead to antibiotic

resistance can be avoided. Kapadnis *et al* designed sulfones which mimic the transition state structure for ring hydrolysis of N-acylated-L-homoserine lactones (AHLs), small molecules used by Gram-negative bacteria for quorum sensing, which are able to induce antibodies that will catalyse AHL hydrolysis, inhibiting quorum sensing [278]. Vinyl sulfones are also potent cysteine protease inhibitors in parasitic diseases such as malaria and Chagas disease [279]. In *P. falciparum,* papain-like cysteine proteases falcipain-2 and falcipain-3 are important for host haemoglobin proteolysis. Peptidyl vinyl sulfone has been shown to be a potent inhibitor of falcipain and oral administration of this compound delayed malaria onset and cured 40% of mice [280]. K777, a dipeptide vinyl sulfone, rescued mice from lethal *T. cruzi* infection due to inhibition of the cysteine protease Cruzain [281]. It has also been shown to protect beagles from cardiac damage due to *T. cruzi* infection, to be tolerable in mice and dogs, and was approved for phase I trials in humans by the FDA in 2007 [281]. Vinyl sulfones have also been demonstrated to be protective in mouse models of Parkinson's disease [282]. A chalcone derivative was found to activate Nrf2, a transcription factor that plays an important cellular role in combating oxidative stress [283]. Chalcone contains a unique α,β-unsaturated ketone structure that appears to be responsible for many of its biological functions. Seo Yeon Woo *et al* investigated the effect of addition of vinyl sulfoxide or vinyl sulfone to the α,β-unsaturated ketone and found addition of vinyl sulfone was most potent at inducing heme oxygenase-1 (HO-1), an important antioxidant enzyme [282]. They then designed a series of vinyl sulfone derivatives and tested their propensity to induce HO-1. The ability of the most effective compound, 12g, to activate Nrf2 was then investigated. 12g was found to increase the level of Nrf2 in the nucleus as well as the mRNA and protein levels of antioxidant enzymes HO-1, NAD(P)H quinone oxidoreductase 1 (NQO1) and glutamate-cysteine ligase (GCL). Further, it was shown that 12g protects DAergic neurons *in vitro* and also in the MPTP-induced *in vivo* model of Parkinson's Disease [282].

On the right-hand side of the SMAs, dimethylamine, morpholine or pyrrolidine have been used to enhance diversity (structures shown in Figure 3.2). These secondary amines are used as ingredients in several compounds with anti-inflammatory and/or anti-cancer properties. For instance, investigation of a library of novel dual dithiocarbamates as anti-cancer drugs found that secondary amine substituents dimethylamine and morpholine were the most favoured compounds compared to others tested [284].

Dimethlyamine is also a precursor to several industrial and pharmaceutical compounds including diphenhydramine, a commonly used, first generation antihistamine [285]. In the USA and Canada the over the counter anti-allergy Benadryl contains diphenhydramine and it is also used as an antitussive. Dicpinigaitis *et al* found that it could inhibit cough reflex sensitivity in patients with acute viral upper respiratory infection (common cold) [285]. Myristamidopropyl dimethylamine is an antimicrobial agent in contact lens cleaning solutions and has been shown to be an effective cysticidal compound against *Acanthamoeb*a spp. [286] demonstrating antifungal and antimicrobial activity in relation to keratitis caused by these parasites [287]. Juvenile female *L. sigmondontis* secrete a 160KDa glycoprotein (Juv-p120) that is highly modified by a dimethylamine derivative dimethylethanoamine (DMAE) [288] [289]. Radiolabelling experiments by Houston *et al* suggested that choline might be the precursor for the DMAE that is required for Juv-p120 [290]. The exact function of Juv-p120 is still unknown but it is thought it may play a role in host evasion by the *L. sigmondontis* microfilarae (MF) [291]. It is thought that dimethlyamine will protonate in solution to generate a similar compound to choline in terms of charge.

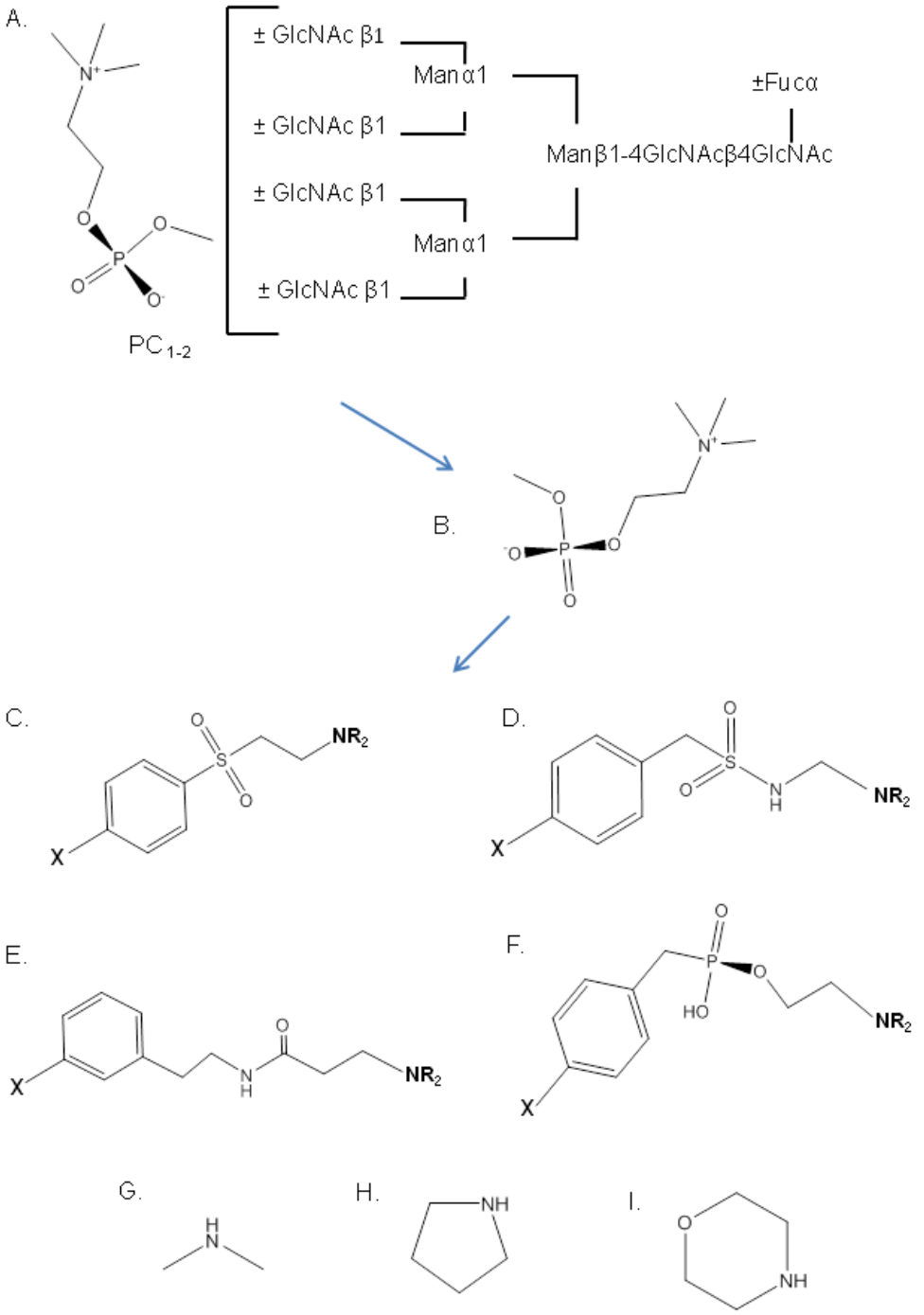
Pyrrolidine is a precursor to licensed drugs Procyclidine, an anticholinergic, and Bepridil, a calcium channel blocker used to treat angina (http://www.drugs.com/mtm/bepridil.html). Procyclidine is an FDA-approved drug used to treat parkinsonism (http://www.drugs.com/cdi/procyclidine.html) but has also been found to demonstrate amoebicidal effects against *Balamuthia mandrillas* and *Acanthamoeba castellanii* [292] [293].

Morpholine is an aliphatic compound that is used commercially in the pharmaceutical, Gefitinib(N-(3-chloro-4-fluoroohenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine). Gefitinib is a small molecule first-generation, reversible inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) that is used to treat non-small cell lung cancer [294]. Morpholine is also a component of a new analogue (GS12021) of 4-O-methylhonokiol, a biologically active agent from *Magnolia officinalis*, which has anti-cancer and anti-inflammatory activities. This analogue significantly inhibited LPS-induced inflammation and phosphorylation of NF-κB p65 in macrophages through activation of AMP-activated protein kinase (AMPK) [295].

Varying the length of the methylene chain between the substituted amine group/choline derivative and sulfone, sulphonamide or carboxamide group generated further compounds for the library. The SMAs are chemically basic in nature and are generally soluble in water and salt solution, which readily enables their use in *in vitro* and *in vivo* experiments. To investigate the potential therapeutic use of these SMAs they were first employed in several assays employing bone marrow derived dendritic cells (bmDCs).

Figure 3.2: Design of SMAs based on the phosphorylcholine moiety of ES-62.

The SMAs were designed based on the phosphorylcholine (PC) moiety (B) of the ES-62 PC-*N*-glycan (A) The basic structures of each of sulfones (C), sulfonamides (D), carboxamides (E) and phosphonates (F) are shown. Aromatic substituents are shown as X, which could be Br, Me, NO_2, NH_2, F or H. The NR_2 groups were secondary amides MeNH (G), pyrrolidine (H) or morpholine (I). GlcNAc = N-acetylglucosamine, Man = mannose and Fuc = fucose, ES-62 structure adapted from Harnett *et al*, 2003.


A.
± GlcNAc β1
Manα1
± GlcNAc β1
± GlcNAc β1
Manα1
± GlcNAc β1
±Fucα
Manβ1-4GlcNAcβ4GlcNAc
PC 1-2
B.
C.
D.
E.
F.
G.
H.
I.

3.2 The effect of Small Molecule Analogues (SMAs) of ES-62 on the LPS-induced cytokine responses of bmDCs

Initial screening of the SMAs (115 in total) was undertaken *in vitro* to investigate the effects they had on the secretion of pro-inflammatory cytokines by macrophages [260]. Macrophages were pre-treated with SMA for 18 hours before stimulation with TLR ligands – LPS (TLR4), BLP (TLR2) or CpG (TLR9) - for 24 hours. The levels of IL-12p40 and IL-6 in the supernatant were measured by ELISA. Several of the SMAs were able to mimic the effects of ES-62 on PAMP-induced macrophage cytokine production but, surprisingly, others showed selectivity in terms of which cytokine they affected, and which PAMP-mediated signalling they could modulate. There were also some SMAs that caused an increase in pro-inflammatory cytokines [260], a result not previously observed with ES-62. ES-62 has been found to inhibit mast cell activation through suppression of calcium signalling [250] and so a number of the SMAs (65) were examined for their ability to affect FcεR1-mediated calcium mobilization. Only a small number of SMAs were found to be able to mimic ES-62 in this capacity. These SMAs were then tested to determine if they could also inhibit mast cell degranulation and production of pro-inflammatory cytokines IL-6 and TNF-α following IgE cross-linking [263]. From these screens two sulfone-containing SMAs, 11a and 12b, were found to be the most effective at inhibiting cytokine responses by macrophages and mast cells and subsequently these SMAs have been found to be as effective as ES-62 in protecting against arthritis and asthma in mouse models, providing proof of concept that these compounds can be active against inflammatory diseases [260], [261], [263].

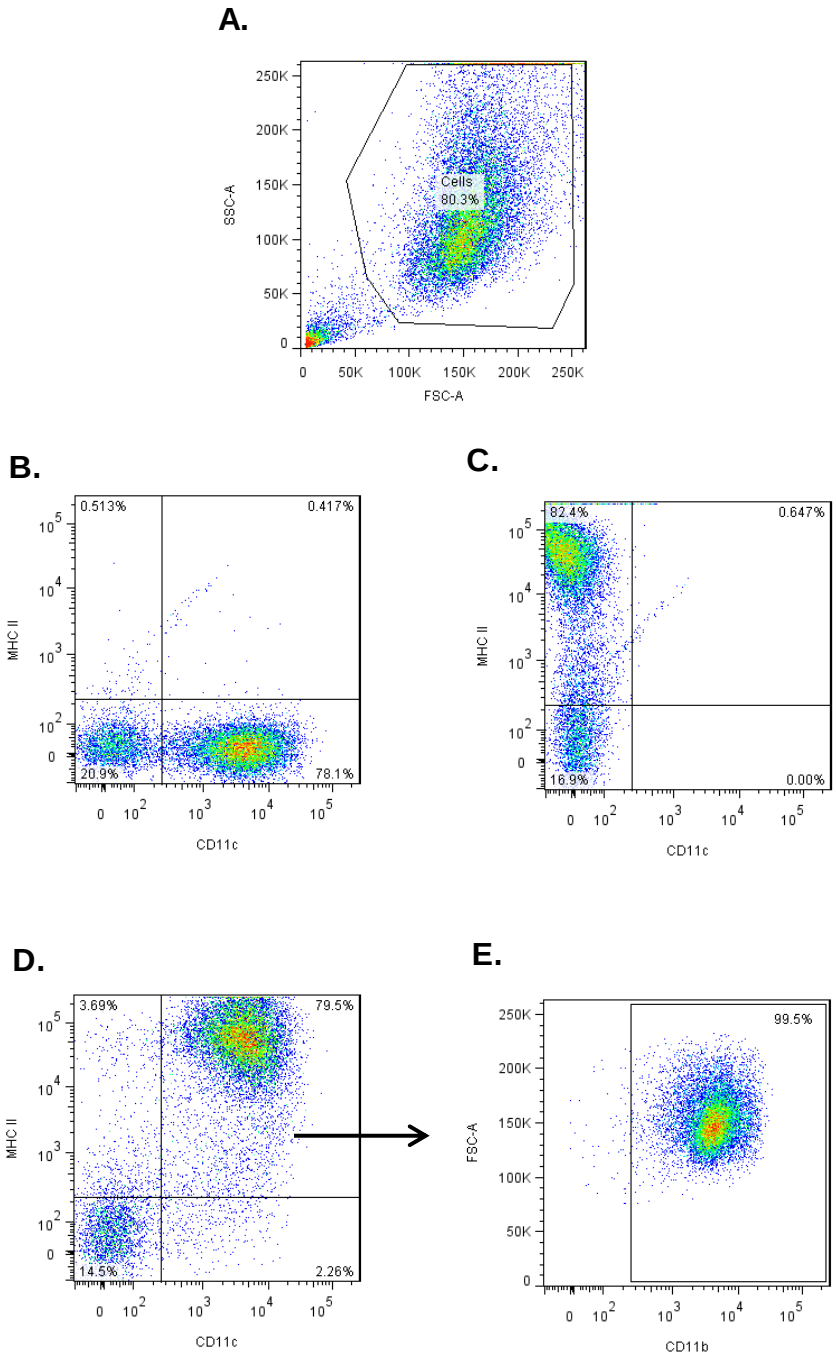
For the initial screening of SMAs with bmDCs it was decided to emulate the screening that had already been undertaken in macrophages and mast cells and thus SMAs (79) were tested for their ability to alter the production of cytokines IL-6, IL-12 and TNF-α following PAMP stimulation. LPS was used as the source of activation as it is the PAMP whose activity has been most studied with respect to the immunomodulatory properties of ES-62. Stimulation via LPS initiates TLR4-dependent downstream signalling pathways that result in the production of pro-inflammatory cytokines and type-1 interferons (Figure 1.2). Upon binding LPS, TLR4 dimerises in order to bind to the adaptor protein MyD88. MyD88 then recruits IRAK proteins, which in turn recruit TRAF6, an E3 ligase, which promotes the ubiquitination of several proteins including itself. TRAF6 then recruits TAK1, which activates the IKK complex. This causes the

phosphorylation of IκBs, which targets them for degradation and thus frees NF-κB to translocate to the nucleus where it promotes production of pro-inflammatory cytokines [17]. LPS-stimulated DCs produce a classical inflammatory cytokine response with high mRNA and protein levels of IL-12p40 and TNF-α and polarise $CD4^+$ T cells towards a T_H1 phenotype. ES-62 was the first molecule to be identified that could prime DCs to polarise the T cell response towards a T_H2 phenotype [243]. BmDCs pre-treated with ES-62 show an altered LPS-induced cytokine response compared to untreated bmDCs in that they secrete less IL-6, TNF-α and IL-12 [243], [245]. The aim of this first component of the work was thus to determine whether any members of the SMA library possessed the same inhibitory properties as ES-62 on bmDCs.

BmDCs were grown from the femurs and tibias of 6-8 week old BALB/c mice in RPMI complete medium supplemented with 10 ng/ml recombinant GM-CSF for 8 days, with fresh medium and growth factor added on days 3 and 6. Cells were harvested on day 8 and assessed by flow cytometry for expression of CD11c and major histocompatibility complex class II (MHC II) before use in the cytokine assays. The gating strategy employed for the phenotyping of the cells by flow cytometry is shown in Figure 3.3. On average bmDCs were 80% $CD11c^+$MHC II^+ and were always at least 65% $CD11c^+$ for use in the initial cytokine screening assays. For all other experiments cells were at least 70% $CD11c^+$MHC II^+. In addition, expression of CD11b on the bmDCs has been analysed and $CD11c^+$ cells were also found to be 98% $CD11b^+$ (Figure 3.3), indicating they are similar to myeloid-like DCs. The cells typically express low levels of co-stimulatory molecules CD80, CD86 and CD40 (results not shown). As with the initial macrophage screen, bmDCs were pre-treated with 5 μg/ml SMA for 18 hours and then stimulated with LPS for 24 hours before cytokine (IL-6, IL-12p40 and TNF-α) production was evaluated by ELISA. The effect of exposure to SMAs in the absence of LPS was also determined. SMAs (total 79; for structures, see Appendix 1) were tested in triplicate assays with 3 biological replicates per assay. SMAs were generally examined in groups of 6 per assay and the percentage inhibition or induction of cytokine compared to the LPS control in each experiment is shown in individual figures and data is summarised in Table 3.1.

Figure 3.3: The phenotype of bmDCs

BmDCs were obtained by culturing bone marrow cells in RPMI complete medium with GM-CSF for 8 days. Their differentiation was verified by flow cytometry before use in experimental assays. Cells were phenotyped with antibodies specific for CD11c and MHC II and cells were first gated depending on size and granularity (A) and the gates were set using fluorescence minus one controls (B) and (C) for MHC II and CD11c respectively. Typically, bmDCs were approximately 80% $CD11c^+$ MHC II^+ (D) with CD11b typically being expressed by >98% of these $CD11c^+$ MHC II^+ bmDCs (E).


A.
Cells
80.3%
SSC-A
FSC-A
B.
0.513%
0.417%
20.9%
78.1%
MHC II
CD11c
C.
82.4%
0.647%
16.9%
0.00%
MHC II
CD11c
D.
3.69%
79.5%
14.5%
2.26%
MHC II
CD11c
E.
99.5%
FSC-A
CD11b

3.2.1 IL-6

There was very little secretion of IL-6 by bmDCs maintained in culture and treatment with SMAs alone had no effect on cytokine release (results not shown). As expected, IL-6 cytokine production significantly increased in response to LPS and it was found that a number of SMAs reduced this. Thus, SMAs 11g, 18b, 19z, 19aa, 21d, 21k, 21n, 21o, 23g, 24a, 24b, 24c, 62, 63, 75, 88 and 97 all significantly inhibited IL-6 secretion in 1/3 experiments; SMAs 11h, 11j, 11k, 12b, 23c, 24e and 72 in 2/3 experiments; and SMAs 11a, 11e and 11i in all 3 experiments performed (Table 3.1). Figure 3.4 shows representative data illustrating the effect of SMAs 11a, 12b, 11e, 11h, 11i and 11k on IL-6 cytokine production.

3.2.2 TNF-α

As with IL-6, there were very low levels of spontaneous TNF-α produced, and treatment with the SMAs alone did not change cytokine release compared to the RPMI control (results not shown). SMAs 11a, 11n, 18b, 19b, 21j, 23g, 24d, 62, 63, 64, 66, 67, 70, 72, 75, 80, 81, 82, 83, 84, 88 and 97 significantly reduced TNF-α production in 1/3 experiments (Table 3.1) following LPS stimulation. SMAs 11g, 11h, 11k, 12b, 19e, 19z and 19aa reduced TNF-α following LPS stimulation in 2/3 experiments and, as with IL-6, SMAs 11e and 11i significantly inhibited TNF-α in all 3 experiments performed (Table 3.1). Figure 3.5 shows representative plots of the effect of SMAs 11a, 12b, 11e, 11h, 11i and 11k on TNF-α cytokine production.

3.2.3 IL-12

Bioactive IL-12p70 is composed of two disulphide-linked subunits – IL-12p40 and IL-12p35, which are encoded for by different genes on separate chromosomes [296]. Consistent with the macrophage SMA screen only IL-12p40 production was investigated initially. BmDCs spontaneously produced high levels of IL-12p40 and in many cases stimulation with LPS did not cause a significant increase (27 of 49 experiments did not demonstrate a significant difference between LPS and RPMI controls) suggesting that bmDCs manufacture and secrete this molecule regardless of activation state. Certainly, as there was little to no spontaneous release of other pro-inflammatory cytokines (IL-6 and TNF-α) it can be concluded that the cells are not already activated. Treatment with the SMAs alone did cause some changes in levels of IL-12p40 but the only two to induce consistent statistical differences were 11a and 12b. Both SMAs caused significant increases in IL-12p40 compared to the RPMI control in

2/3 experiments. Treatment with 11a prior to stimulation with LPS also resulted in a significant up-regulation of IL-12p40 production in 2/3 experiments but 12b only significantly increased p40 compared to LPS in 1/3 experiments (Figure 3.6A and B). Of interest, SMAs 11e, 11h, 11i and 11k, had no consistent significant effects on IL-12p40 production although 11h, 11i and 11k did significantly decrease p40 production compared to LPS in 1/3 experiments (Figure 3.6C), as did 11f, 11g, 19z and 19aa (Table 3.1).

IL-12p40 is produced in excess of bioactive cytokine and, unlike IL-12p35, which is only secreted as part of IL-12p70, IL-12p40 can be secreted alone and exists in monomeric or homodimeric form [297]. Monomeric IL-12p40 (Mo(p40)2) is a potent IL-12 antagonist as it blocks the binding of IL-12p70 to its receptor, IL-12R, by strongly interacting with the beta 1 subunit of the molecule [298]. Furthermore, Snijders *et al* [296] demonstrated that in monocytes the level of p35 expression determines the level of bioactive IL-12p70 expression and Goodridge *et al* demonstrated that p40 and p35 subunits are differentially regulated in macrophages [124]. Taken together, it seems that the increase in p40 by 11a and 12b could have various effects within the cell including relating to production of IL-12p70 and so the levels of this bioactive cytokine were investigated in supernatants from bmDCs following pre-treatment with 11a or 12b and stimulation with LPS. SMAs 11e, 11h, 11i, 11k and 19o were also tested in this assay and results are shown in Figure 3.6D. As anticipated, LPS induced secretion of IL-12p70 and SMAs 12b, 11e and 11i strongly inhibited production of the cytokine in 3/3 experiments while 11a, 11h and 11k inhibited it in 2/3 experiments. Interestingly, SMA 19o, which has no effect on IL-6, TNF-α or IL-12p40 secretion (Figure 3.7 and Table 3.1) also significantly inhibited IL-12p70 in 3/3 experiments (Figure 3.6D).

The differing results with IL-12 again demonstrate the selectivity of the immunomodulatory action of the SMAs that was reported with respect to the macrophage *in vitro* data [260].

Figure 3.4: The Effect of ES-62 SMAs on IL-6 production

BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at $2x10^5$ cells/well, rested overnight and then treated with SMAs (5 µg/ml) 11a, 12b or 11e (A) and 11h, 11i or 11k (B) for 18 hours before stimulation with LPS (100 ng/ml) for 24 hours. The levels of IL-6 in the supernatant were then determined by ELISA. The detection limit of IL-6 was ≥ 15 pg/ml according to the manufacturer's instructions. Data are representative of 3/3 independent experiments for 11a, 11e and 11i and 2/3 for 11h, 12b and 11k. Results are expressed as mean (of triplicate determinations) ± SD and analysed using one-way ANOVA with Bonferroni post-test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

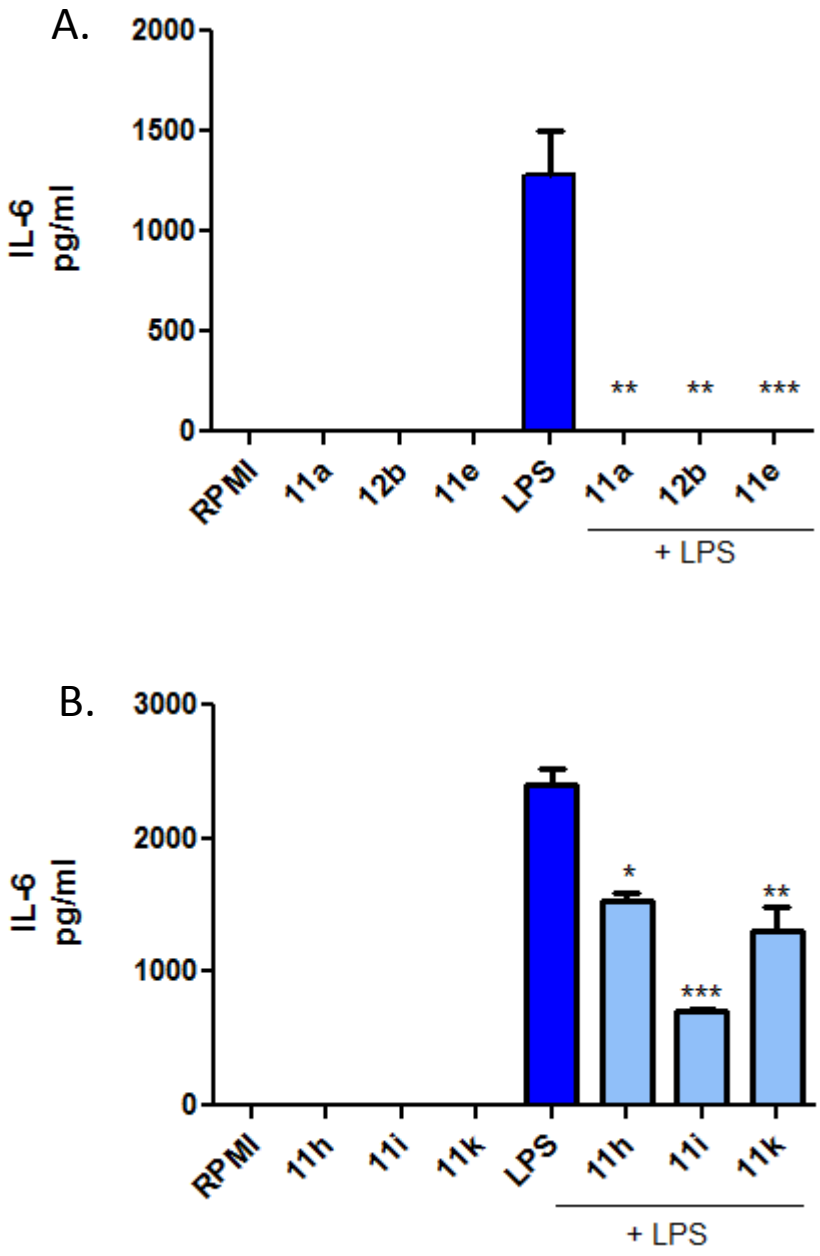
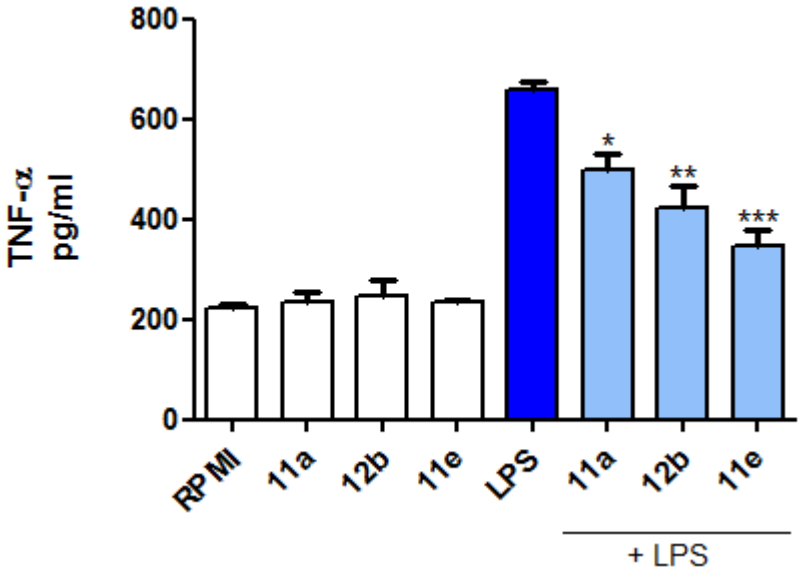

A.
IL-6
pg/ml
2000
1500
1000
500
0
**
**
***
RPMI
11a
12b
11e
LPS
11a
12b
11e
+ LPS
B.
IL-6
pg/ml
3000
2000
1000
0
*
***
**
RPMI
11h
11i
11k
LPS
11h
11i
11k
+ LPS

Figure 3.5: The Effect of ES-62 SMAs on TNF-α production

BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at $2x10^5$ cells/well, rested overnight and then treated with SMAs (5 μg/ml) 11a, 12b or 11e (A) and, 11h, 11i or 11k (B) for 18 hours before stimulation with LPS (100 ng/ml) for 24 hours. The levels of TNF-α in the supernatant were then determined by ELISA. The detection limit of TNF-α was ≥ 15 pg/ml according to the manufacturer's instructions. Data are representative of 3/3 experiments for 11e and 11i and 2/3 for 11h, 11k and 12b. For 11a data are representative of 3 experiments in which inhibition reached significance in 1/3 experiments. Results are expressed as mean (of triplicate determinations) ± SD and analysed using one-way ANOVA with Bonferroni post-test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

A.


800
600
400
200
0
TNF-α
pg/ml
*
**
***
RPMI
11a
12b
11e
LPS
11a
12b
11e
+ LPS

B.

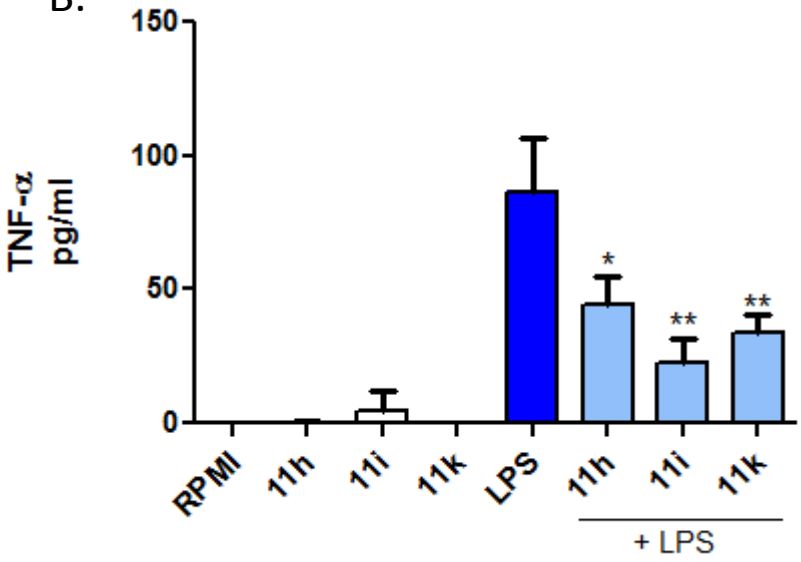

150
100
50
0
TNF-α
pg/ml
*
**
**
RPMI
11h
11i
11k
LPS
11h
11i
11k
+ LPS

Figure 3.6: The Effect of ES-62 SMAs on IL-12 production

BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at $2x10^5$ cells/well, rested overnight and then treated with SMAs (5 μg/ml) for 18 hours before stimulation with LPS (100 ng/ml for measuring IL-12p40 and 1 μg/ml, for IL-12p70) for 24 hours. The levels of IL-12 in the supernatant were then determined by ELISA. The detection limit of both IL-12p40 and IL-12p70 was ≥ 15 pg/ml according to the manufacturer's instructions. Results from a single experiment are expressed as mean (of triplicate determinations) ± SD and analysed using one-way ANOVA with Bonferroni post-test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ for SMA treatment compared to RPMI only and * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ for SMA pre-treatment and LPS stimulation compared to LPS activation only.

The levels of IL-12p40 following treatment with (A) 11a, 11h, 11i and 11k, (B) 12b and (C) 11e are shown. Data are representative of 3/3 experiments for 11e, 11h, 11i and 11k and 2/3 for 11a and 12b compared to RPMI and 3/3 experiments for SMA 11e and 2/3 experiments for 11a, 12b, 11i, 11h and 11k compared to LPS control.

In panel D the effect of 11a, 12b, 11e, 11h, 11i, 11k or 19o on IL-12p70 is shown. Data are representative of 3/3 experiments for 12b, 11e, 11i and 19o and 2/3 experiments for 11a, 11h and 11k

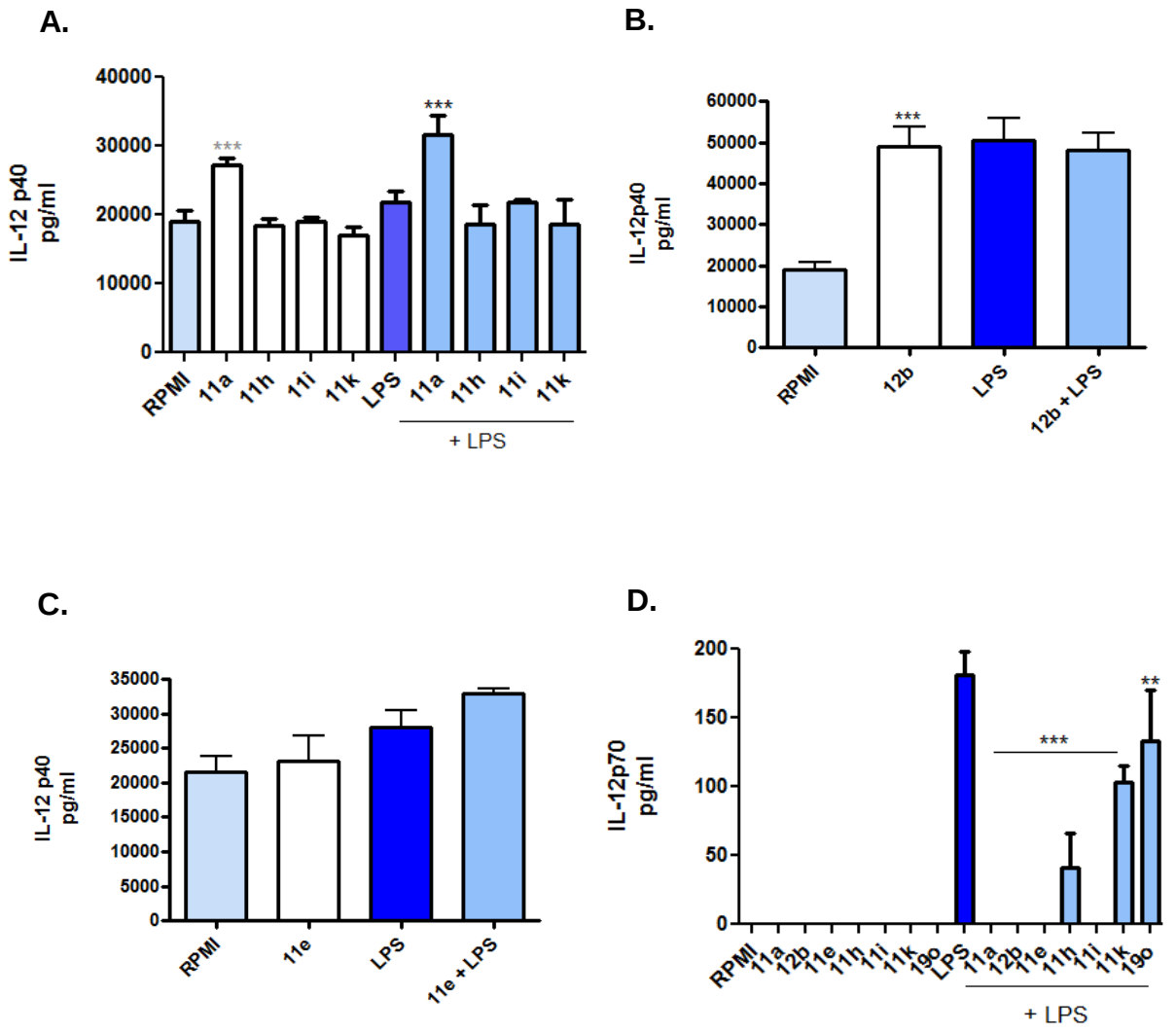

A.
IL-12 p40 pg/ml
40000
30000
20000
10000
0
***
***
RPMI
11a
11h
11i
11k
LPS
11a
11h
11i
11k
+ LPS
B.
IL-12p40 pg/ml
60000
50000
40000
30000
20000
10000
0
***
RPMI
12b
LPS
12b + LPS
C.
IL-12 p40 pg/ml
35000
30000
25000
20000
15000
10000
5000
0
RPMI
11e
LPS
11e + LPS
D.
IL-12p70 pg/ml
200
150
100
50
0
***
**
RPMI
11a
12b
11e
11h
11i
11k
19o
LPS
11a
12b
11e
11h
11i
11k
19o
+ LPS

Table 3.1: The immunomodulatory effect of SMAs on cytokine production by bmDCs exposed to LPS

The percentage of IL-6, TNF-α, and IL-12p40 produced as a result of pre-treatment by each of the selected SMAs compared to the LPS control for each of 3 experiments (except 19o which was only investigated in 2) is shown. Only statistically significant values are shown. ND (not detected) is used in cases where SMA pre-treated resulted in cytokine levels below the detection levels of the ELISA kit (≥ 15 pg/ml) and X signifies no results for that experiment.

SMA	IL-6			TNF-α			IL-12p40		
	1	2	3	1	2	3	1	2	3
11a	ND	32	68		76		187	163	
11e	ND	18	ND	38	35	50			
11f							41		
11g		67		36	61		40		
11h	64		61	51	68		45		
11i	ND	ND	30	49	34	48	56		
11j		40	53						
11k		60	54	39	60		54		
11l									
11n				55					
12b	ND	24		31		95		139	
18a									
18b			78			63			
19a			x			x			x
19b						74			
19c									
19d									
19e				32		73			
19f									
19g									
19i									
19k									
19l									
19p									
19q									
19r					149	65			
19s									
19t									
19o									
19u									
19v									

SMA	IL-6			TNF-α			IL-12p40		
	1	2	3	1	2	3	1	2	3
19w									
19z	57			73		62	68		
19aa	41			70		44	67		
21a									
21c									
21d			65						
21f			161						
21g			152						
21h									
21i									
21j					43				
21k		68							
21l									
21n		71							
21o			57						
21p									
21q									
23a									
23b									
23c	78		71						
23g			39			38			
24a	75								
24b			66						
24c	50								
24d				52					
24e	42		65						
25a									
25b									
25c									
25d						152			
62	52					56			
63		42			52				
64					68				
66					50				
67					63				
70					70				
72	50	47			44				
75	51				82				
80					77				
81					68				
82					84				

SMA	IL-6			TNF-α			IL-12p40		
	1	2	3	1	2	3	1	2	3
83					71				
84					76				
85									
88		64			78		57		
97		35			53				
98									
100									

3.3 Selection of SMAs for further analysis

From the initial cytokine screen 7 SMAs were selected for further analysis of their effects on bmDCs. These SMAs were 11a, 12b, 11e, 11h, 11i, 11k and 19o. Table 3.2 shows a summary of the effect of each SMA on cytokine release by bmDCs. 11a and 12b are already well characterised *in vitro* in macrophages and mast cells and in *in vivo* models of arthritis and asthma [260], [261], [263], [264] and both molecules significantly inhibited LPS-induced IL-6 and IL-12p70 in 2/3 experiments demonstrating they are also capable of modulating DCs. SMA 12b was also able to significantly inhibit TNF-α demonstrating some selectivity between the two sulfones. SMAs 11e and 11i both demonstrated consistent inhibitory effects on IL-6, TNF-α and IL-12p70 production in all experiments demonstrating clear anti-inflammatory activity with respect to bmDC cytokine responses. In addition, both of these SMAs consistently down-regulated IL-6 and TNF-α production by mast cells following activation via cross-linking of IgE [263]. Although with respect to macrophages SMA 11i showed no effect on IL-12p40 or IL-6 after LPS stimulation it did inhibit these cytokines following BLP activation demonstrating that it can act on all three cell types. Interestingly, SMA 11e actually increased pro-inflammatory cytokines IL-12p40 and IL-6 in macrophages demonstrating variation in effects among cell types [260]. SMAs 11g, 11j, 19z, 19aa, 23c, 24e and 72 were also considered for further analysis based on their effects on IL-6 and TNF-α. However, 11j solely acted on LPS-induced IL-6 in bmDCs and macrophages and up-regulated BLP-mediated IL-6 and IL-12 in macrophages [260]. While this selective difference in action on different PAMPS is of interest, in this present study SMAs that closely mimic ES-62 action were selected. 23c and 24e solely acted on IL-6 in bmDCs and had no effect on LPS-induced cytokines in macrophages, although 24e was able to inhibit IL-12p40 after BLP stimulation in the latter cell type [260]. SMAs 19z, 19aa and 72 were not investigated in macrophages and did not have significant effects in mast cells [263] and so were not selected. SMA 11k and 11h inhibited IL-6 and TNF-α in bmDCs in 2/3 experiments, while 11g significantly inhibited TNF-α in 2/3 and IL-6 in 1/3 experiments and so all three SMAs were considered for further work. All three also affected IL-6 in mast cells but only SMAs 11h and 11k inhibited TNF-α secretion in this cell type (Coates and Harnett, unpublished) and so these two were selected for further work in bmDCs. SMA 19o was the last SMA to be selected. It was chosen as a “negative” SMA control as it had no effect on IL-6, TNF-α or IL-12p40 during the cytokine screen (Figure 3.7). It has also been shown to have no

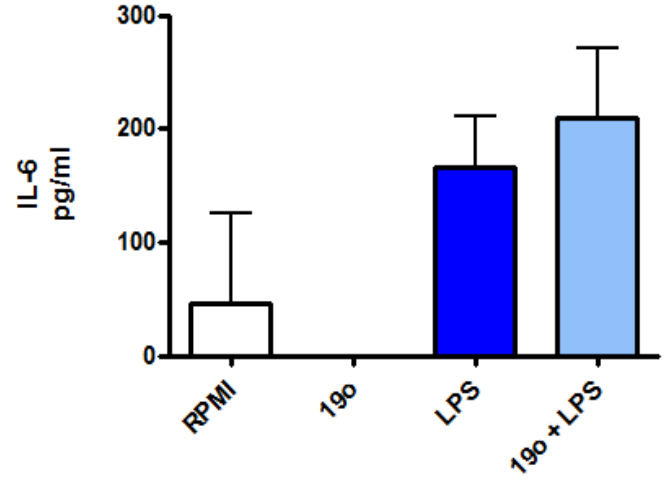
effect on the cytokine profile in macrophages [260]. It does, however, inhibit IL-12p70 production following LPS stimulation, and while it may not be as wide-ranging in effects as the other SMAs it is as consistent as 12b at such inhibition (Figure 3.6).

As the ES-62 SMAs had demonstrated considerable ability to inhibit LPS-induced cytokine responses of bmDCs it was considered prudent to check whether the SMAs exhibited any toxicity in these assays to ensure the cytokine inhibition was due to SMA activity and not toxicity. BmDCs were treated either with SMA only for 18 hours or for 18 hours followed by LPS (100 ng/ml) for 24 hours, and viability was assessed with the cell viability indicator 7-actinomycin D (7-AAD) by flow cytometry. Consistent with previous results in macrophages and mast cells [260], [263] none of the SMAs had any significant effect on the cell viability of bmDCs compared to RPMI or LPS alone (data not shown) and so these SMAs were selected for use in further experiments to investigate their mechanisms of actions on bmDCs.

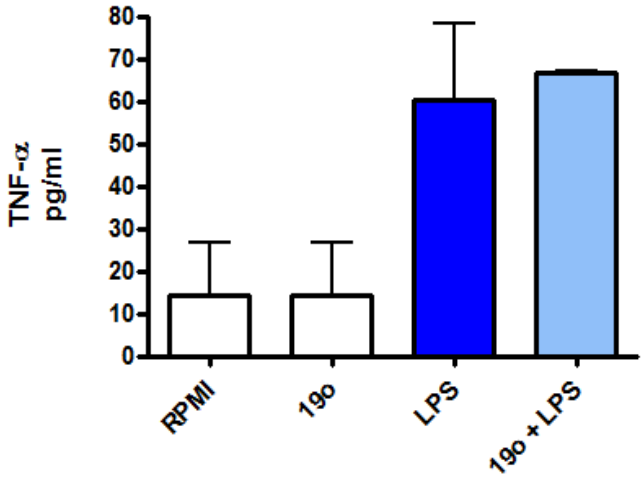
Figure 3.7: The Effect of SMA 19o on LPS-induced cytokine production in bmDCs

BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at $2x10^5$ cells/well, rested overnight and then treated with SMA 19o (5 µg/ml) for 18 hours before stimulation with LPS (100 ng/ml) for 24 hours. The level of (A) IL-6 (B) TNF-α (C) IL-12p40 in the supernatant was determined by ELISA. The cytokine detection limit was ≥ 15 pg/ml according to the manufacturer's instructions. Results are expressed as mean (of triplicate determinations) ± SD and were analysed using an unpaired t-test. Data are from one experiment representative of two

A.


IL-6
pg/ml
300
200
100
0
RPMI
19o
LPS
19o + LPS

B.


TNF-α
pg/ml
80
70
60
50
40
30
20
10
0
RPMI
19o
LPS
19o + LPS

C.

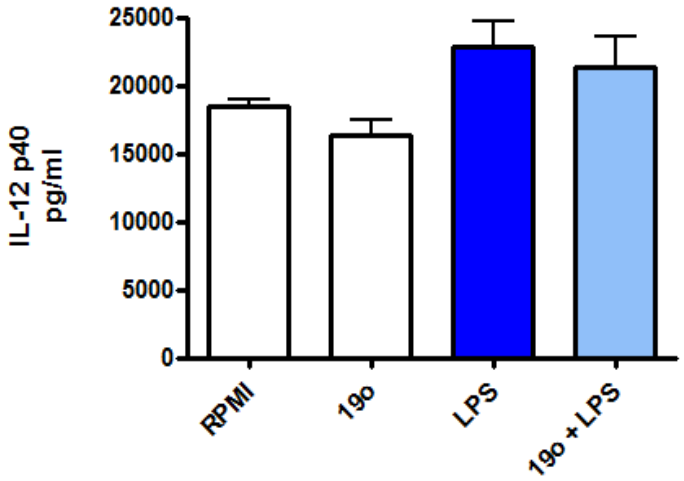

IL-12 p40
pg/ml
25000
20000
15000
10000
5000
0
RPMI
19o
LPS
19o + LPS

Table 3.2: Summary of the effects of selected SMA on bmDC cytokine secretion

The percentage of cytokine produced following pre-treatment by each of the selected SMAs compared to the LPS control for each experiment is shown for cytokines IL-6, TNF-α, IL-12p40 and IL-12p70. Only percentages from experiments where the SMAs induced a significant difference are shown where red $p \leq 0.001$; purple $p \leq 0.01$ and blue $p \leq 0.05$. X signifies no results for that experiment and ND (not detected) is used in cases where SMA pre-treatment resulted in cytokine levels below the detection levels of the ELISA kit (≥ 15 pg/ml).

SMA	IL-6			TNF-α			IL-12p40			IL-12p70		
	1	2	3	1	2	3	1	2	3	1	2	3
11a	ND	32	68		76		187	163		ND	ND	
12b	ND	24		31		95		139		ND	ND	69
11e	ND	18	ND	38	35	50				ND	ND	19
11h	64		61	51	68		45			74	72	
11i	ND	ND	30	49	34	48	56			ND	ND	28
11k		60	54	39	60		54			23	68	
19o			x			x			x	37	57	61

3.4 The effect of treatment with ES-62 SMAs on the subsequent BLP- and CpG-induced cytokine response of bmDCs

As previously described there are multiple TLRs present on cells that can respond to different PAMPs allowing the immune system to recognise a wide range of pathogens. TLR2 is a cell surface receptor that recognises peptidoglycan and lipoteichoic acid (LTA) from Gram-positive bacteria, lipoproteins from various pathogens, zymosan from fungi and glycoinositolphospholipids from *T. cruzi* (reviewed by [41]). Dimerisation of TLR2 with TLR1 or TLR6 adds further specificity to PAMP recognition [299]. TLR9 is found within the cell and recognises genomic DNA rich in unmethylated CpG. Both receptors signal through the MyD88-dependent pathway previously described to produce pro-inflammatory cytokines through activation of transcription factor NF-κB, however, TLR2 is the only TLR, along with TLR4, to utilize the TIR domain containing adaptor protein (TIRAP) to mediate signalling [299].

Both of these receptors, along with TLR3 and TLR4, are thought to play an important role in the pathogenesis of rheumatoid arthritis (RA) [300]. The expression of TLRs in the synovium of RA patients is increased compared to healthy controls with DCs expressing them most strongly, followed by macrophages and synovial fibroblasts [301]. Recently, it has been found that TLRs can also respond to endogenous danger signals or damage-associated molecular patterns (DAMPs) that are important in initiating inflammation in the absence of infection [302] and it is likely that in the case of RA it is DAMPs rather than exogenous PAMPs that the TLRs are reacting to. Indeed, Nic AN Ultaigh *et al* found that blockade of TLR2 with the antibody OPN310, in the absence of a TLR2 agonist, significantly inhibited the spontaneous production of cytokines confirming that TLR2 could be an effective therapeutic target for RA and that endogenous TLR ligands are expressed in RA synovial tissues [303]. TLRs have become attractive therapeutic targets for many conditions and infections and there are a number of molecules currently in preclinical and clinical trials for use as anti-cancer drugs as well as therapeutics for autoimmune diseases such as RA, psoriasis, colitis and systemic lupus erythematosus (SLE). An example of this is the anti-malarial drug, hydroxychloroquine, a TLR9 antagonist that is now being used for the treatment of RA and SLE [304], and IMO-3100, a TLR7/9 antagonist is being developed by Idera as a potential drug against RA, psoriasis and SLE [305].

Given the ability of 11a and 12b to ameliorate disease in the murine model of arthritis, CIA, and the role of these TLRs in inflammatory diseases, it was decided to investigate whether the 7 chosen SMAs were able to modulate the production of IL-6, TNF-α and IL-12 in response to bacterial lipopeptide (BLP), a TLR1/2 agonist and unmethylated cytosine-phosphate-guanine (CpG), a TLR9 ligand. Al-Riyami *et al* investigated the effects of the SMAs on macrophage cytokine secretion in response to activation via these TLRs and observed that the SMAs exhibited selective inhibition with respect to the use of different PAMPs and hence it was of interest to determine if they exhibit the same selectivity when added to DCs [260]. As with the initial screen, bmDCs were pre-treated with SMAs (5μg/ml) for 18 hours, stimulated with BLP (100ng/ml) or CpG (0.1μM) for 24 hours and cytokine production measured by ELISA.

The SMAs were tested in three independent experiments and the level of cytokine compared to PAMP for each SMA in each experiment is expressed as a percentage in Tables 3.3 and 3.4, which follow presentation of the data (Figures. 3.8 and 3.9).

3.4.1 The effect of the ES-62 SMAs on BLP-induced cytokine production by bmDCs

The effects of the selected ES-62 SMAs on BLP-induced IL-6 production were investigated in bmDCs. As with LPS, SMAs 11a, 12b, 11e, 11h and 11i inhibited the BLP-induced IL-6 response (this was significant in 3/3 experiments for 12b, 11e and 11i and 2/3 experiments for 11a and 11h) while 19o had no effect. Interestingly, SMA 11k did not appear to have any consistent effect on BLP-induced IL-6 as it only inhibited production of this cytokine in 1/3 experiments (Figure 3.8A).

SMAs 12b and 11e significantly inhibited BLP-induced TNF-α in all three experiments but none of the other SMAs had any consistent significant effects on the production of this cytokine by bmDCs after activation by the TLR2 ligand (Figure 3.8B).

As with the LPS experiments the production of both IL-12p40 and IL-12p70 was determined following BLP stimulation. Overall the SMAs had no consistent effects on IL-12p40 following BLP activation (Table 3.3). In contrast, 11a, 12b, 11e, 11h and 11i significantly inhibited IL-12p70 in 2/3 experiments (Figure 3.8C) and 11k and 19o significantly reduced cytokine production in 1/3 experiments after BLP stimulation (Table 3.3).

3.4.2 The effect of the ES-62 SMAs on CpG-induced cytokine production by bmDCs

Pre-treatment with 12b, 11e or 11i significantly inhibited CpG-induced IL-6 secretion in all three experiments but these SMAs were the only ones to consistently do so (Figure 3.9A). SMA 11a and 11h were able to significantly inhibit IL-6 production in 1/3 experiments while 11k and 19o had no effect on IL-6 (Figure 3.9A).

By comparison, 11a, 12b, 11e, 11h and 11i significantly inhibited TNF-α in all three experiments performed after CpG stimulation. SMA 11k only inhibited TNF-α in 1/3 experiments and, consistent with IL-6 data, SMA 19o had no effects on TNF-α in any experiments (Figure 3.9B).

Similar to the BLP data none of the SMAs had any consistent effects on IL-12p40 following CpG activation (Table 3.3) but CpG-induced IL-12p70 production was significantly down-regulated by all of the SMAs except 19o (Figure 3.9C).

Figure 3.8: The effect of ES-62 SMAs on BLP-induced cytokine production

BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at $2x10^5$ cells/well, rested overnight and then pre-treated with SMAs (5 μg/ml) for 18 hours before stimulation with BLP (100 ng/ml) for 24 hours and the cytokine levels determined by ELISA. The cytokine detection limit was ≥ 15 pg/ml according to the manufacturer's instructions. Results are expressed as mean (of triplicate determinations) ± SD and was analysed using one-way ANOVA with Bonferroni post-test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Panel A shows the effects of the SMAS on IL-6 production. Data are representative of 3/3 experiments for 12b, 11e, 11i and 19o and 2/3 for 11a, 11h and 11k.

Panel B shows the effects of the SMAs on TNF-α production. Data are representative of 3/3 experiments for 12b, 11e, 11k and 19o and 2/3 for 11a, 11h and 11i

Panel C shows the effects of the SMAs on IL-12p70 production. Data are representative of 2/3 experiments.

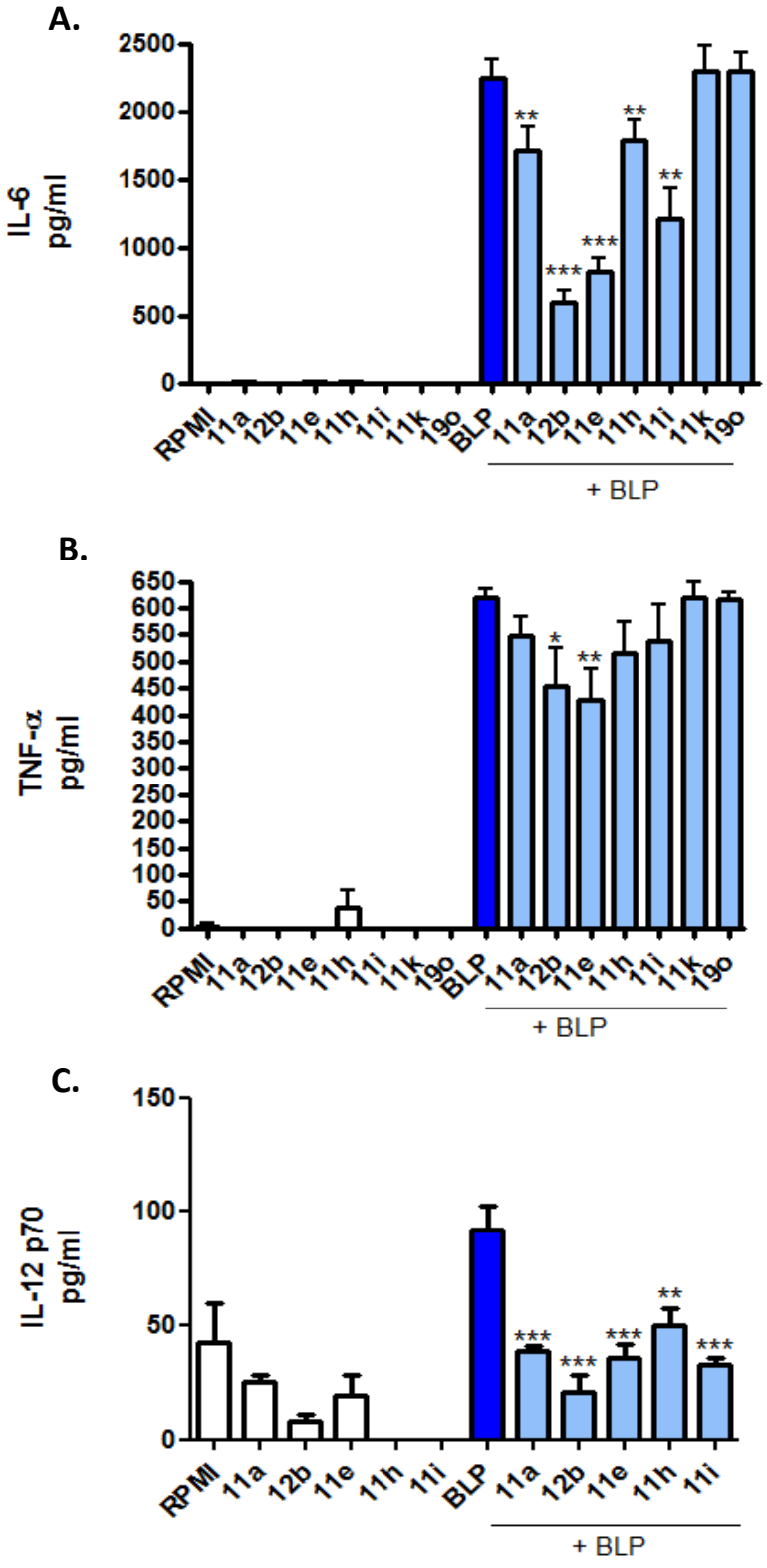

A.
IL-6 pg/ml
2500
2000
1500
1000
500
0
RPMI
11a
12b
11e
11h
11i
11k
19o
BLP
+ BLP
B.
TNF-α pg/ml
650
600
550
500
450
400
350
300
250
200
150
100
50
0
C.
IL-12 p70 pg/ml
150
100
50
0

Figure 3.9: The effect of SMAs on CpG-induced cytokine production

BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at $2x10^5$ cells/well, rested overnight and then pre-treated with SMAs (5µg/ml) for 18 hours before stimulation with CpG (0.1 µM) for 24 hours and the cytokine levels determined by ELISA. The cytokine detection limit was ≥ 15 pg/ml according to the manufacturer's instructions. Results are expressed as mean (of triplicate determinations) ± SD and was analysed using one-way ANOVA with Bonferroni post-test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Panel A shows the effects of SMAs 12b, 11e and 11i on IL-6 production. Data are representative of 3/3 experiments

Panel B shows the effects of SMAs 11a, 11h, 11k and 19o on IL-6 production. Data are representative of 3/3 experiments for 11k and 19o and 2/3 for 11a and 11h

Panel C shows the effects of the SMAs on TNF-α production. Data are representative of 3/3 experiments for 11a, 12b, 11e, 11i, 11h and 19o and 2/3 for 11k

Panel D shows the effects of the SMAs on IL-12p70 production. Data are representative of 3/3 experiments.

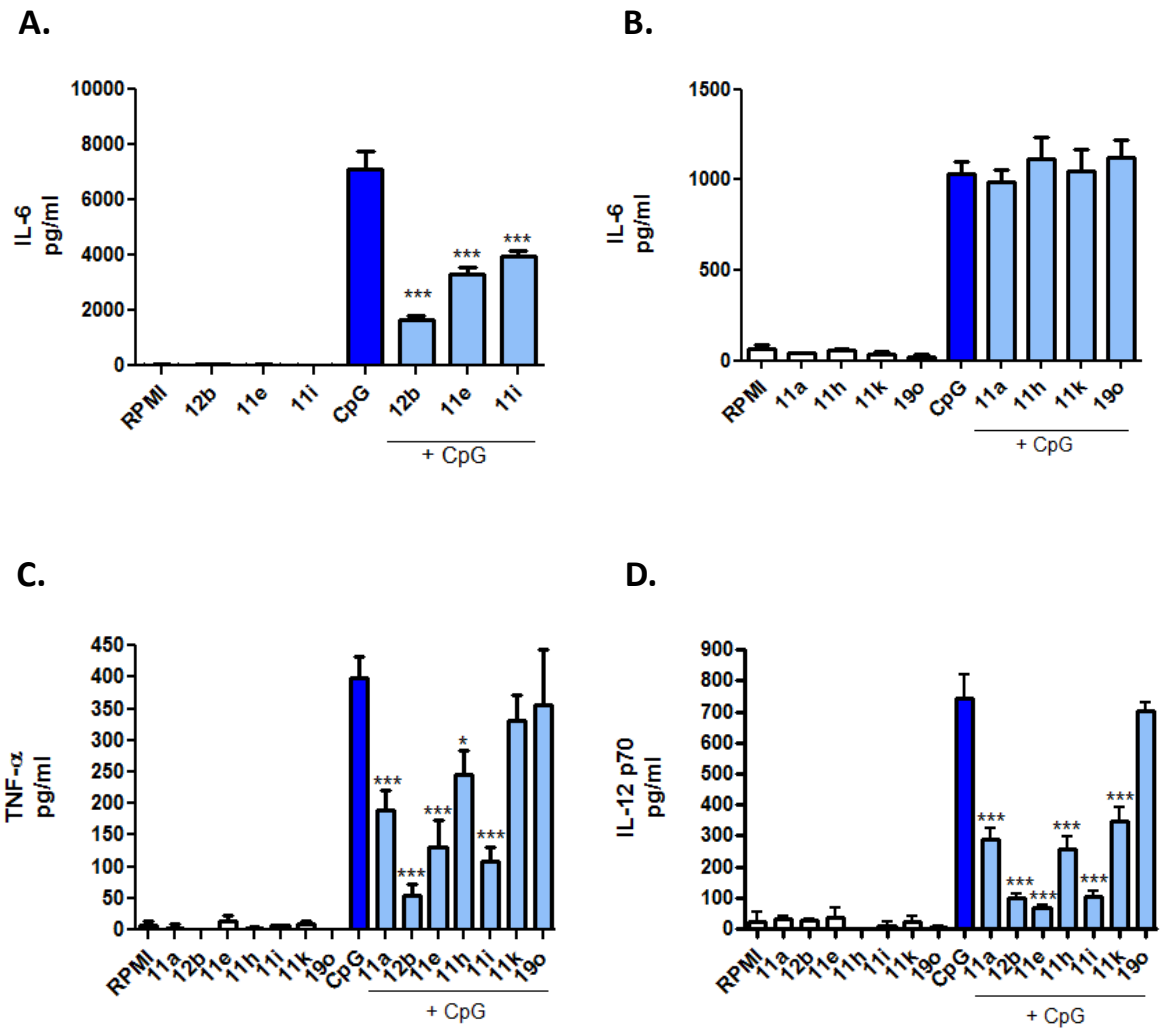

A.
IL-6 pg/ml
10000
8000
6000
4000
2000
0
***
***
***
RPMI
12b
11e
11i
CpG
12b
11e
11i
+ CpG
B.
IL-6 pg/ml
1500
1000
500
0
RPMI
11a
11h
11k
19o
CpG
11a
11h
11k
19o
+ CpG
C.
TNF-α pg/ml
450
400
350
300
250
200
150
100
50
0
RPMI
11a
12b
11e
11h
11i
11k
19o
CpG
11a
12b
11e
11h
11i
11k
19o
+ CpG
D.
IL-12 p70 pg/ml
900
800
700
600
500
400
300
200
100
0
RPMI
11a
12b
11e
11h
11i
11k
19o
CpG
11a
12b
11e
11h
11i
11k
19o
+ CpG

Table 3.3: The effect of ES-62 SMAs on bmDC cytokine production after BLP stimulation

The percentage of cytokine produced compared to the BLP control following SMA pre-treatment is shown for cytokines IL-6, TNF-α, IL-12p40 and IL-12p70. Only percentages from experiments where the SMAs induced a significant difference are shown where red $p \leq 0.001$; purple $p \leq 0.01$ and blue $p \leq 0.05$. X signifies no results for that replicate and ND (not detected) is used in cases where SMA pre-treatment resulted in cytokine levels below the detection levels of the ELISA kit (≥ 15 pg/ml).

SMA	IL-6			TNF			IL-12p40			IL-12p70		
	1	2	3	1	2	3	1	2	3	1	2	3
11a	69	76			23			162		42	28	
12b	76	26	55	20	ND	73		172		23	ND	
11e	19	36	45	41	1	69				39	57	
11h	80	54		77						54	21	
11i	47	54	59		27					36	20	
11k	73									59		
19o										61		

Table 3.4: The effect of ES-62 SMAs on bmDC cytokine production after CpG stimulation

The percentage of cytokine produced compared to the CpG control following SMA pre-treatment is shown for cytokines IL-6, TNF-α, IL-12p40 and IL-12p70. Only percentages from experiments where the SMAs induced a significant difference are shown where red $p \leq 0.001$; purple $p \leq 0.01$ and blue $p \leq 0.05$. X signifies no results for that replicate and ND (not detected) is used in cases where SMA pre-treated resulted in cytokine levels below the detection levels of the ELISA kit (≥ 15 pg/ml).

SMA	IL-6			TNF			IL-12p40			IL-12p70		
	1	2	3	1	2	3	1	2	3	1	2	3
11a		56		47	14	24	157			39	57	54
12b	49	23	75	14	1	3		171	72	13	ND	12
11e	48	47	80	33	ND	13			52	9	28	13
11h		73		62	25	65			42	35	77	49
11i	79	56	88	27	24	29			57	14	35	12
11k					88				70	47	70	68
19o									81			

3.5 The effects of SMA treatment on the surface expression of co-stimulatory molecules on bmDCs

ES-62-matured DCs do not show signs of classical maturation, as they do not up-regulate surface expression of co-stimulatory molecules such as MHC II, CD40, CD80 and CD86. Pre-exposure of DCs to ES-62 prior to LPS stimulation generally had no effect on the up-regulation of these molecules induced by the bacterial product, however exposure of bone marrow progenitors to ES-62 *in vivo* through the use of osmotic pumps to mimic natural infection resulted in development of bmDCs which had decreased MHC II, CD40 and CD80 expression compared to cells derived from unexposed mice and the DCs also had a slight reduction in LPS-induced up-regulation of MHC II and CD80 [245]. Interestingly, treatment with ES-62 *in vivo* caused a slight increase in the expression of CD86 after LPS stimulation *ex vivo* compared to PBS-treated DCs [245]. These effects were generally found to be dependent on the PC moiety of ES-62 as DCs developed from mice that had been treated with PC-free ES-62 were not refractory to subsequent LPS-mediated up-regulation of co-stimulatory molecules (Marshall, Harnett and Harnett, unpublished). Furthermore, PC-OVA was able to mimic the results obtained with ES-62 [246].

As it has been shown that the SMAs can affect the maturation of bmDCs as shown by reduced cytokine production following PAMP activation, it was next investigated whether they could also affect the up-regulation of co-stimulatory molecules CD40, CD80 and CD86 to further inhibit bmDC maturation. SMAs 11h and 11k were initially chosen for further investigation after the LPS cytokine screen as they inhibited not only LPS-induced IL-6 and TNF-α in bmDCs (Figure 3.4 and 3.5 respectively) but also these responses in mast cells (Coates and Harnett, unpublished). However, in subsequent experiments utilising BLP and CpG (Figure 3.8 and 3.9), overall they did not demonstrate consistent effects on cytokine secretion compared to the other SMAs. As a result the effects of these SMAs were not investigated in the current experiment.

BmDCs were incubated for 18 hours with SMA 11a, 12b, 11e, 11i or 19o (5 μg/ml) and then incubated with medium alone or containing LPS (100 ng/ml) for a further 24 hours. Cells were then harvested and the expression of CD80, CD86, CD40 and MHC II on $CD11c^+$ cells analysed by flow cytometry.

3.5.1 CD86

The surface expression of CD86 on $CD11c^+$ cells and also the percentage of $CD11c^+$ $CD86^+$ cells was analysed with respect to bmDCs that had been either treated with SMA for 18 hours or treated with SMA and then stimulated with LPS. Treatment with the SMAs alone had no effect on the mean fluorescent intensity (MFI) of CD86 on $CD11c^+$ cells (data not shown). Out of 3 experiments, stimulation with LPS only caused a significant increase in the MFI for CD86 in 1 experiment and in this experiment SMA 12b-, 11e- or 11i-pre-treatment significantly inhibited the increase (data not shown).

Treatment with SMA only had no effect on the percentage of $CD11c^+CD86^+$ cells (Figure 3.10A). LPS stimulation significantly increased the percentage of $CD11c^+$ $CD86^+$ cells in all experiments and pre-treatment with SMAs 12b or 11e significantly inhibited this response in all experiments while 11a- and 11i-pre-treatment inhibited it in 2/3 experiments (Figure 3.10A). SMA 19o significantly reduced the number of LPS-induced $CD11c^+CD86^+$ cells in 1/3 experiments, but had no effect in the other experiments.

3.5.2 CD80

As with CD86, the surface expression of CD80 on $CD11c^+$ cells and also the percentage of $CD11c^+$ $CD80^+$ cells was analysed with bmDCs that had been treated with SMA alone for 18 hours and subsequently incubated with medium alone or containing LPS. Incubation with SMA 11e alone for 18 hours significantly inhibited the MFI of CD80 in 2/3 experiments and SMAs 11a, 12b and 11i inhibited it in 1/3 experiments (results not shown). Treatment with 19o had no effect on CD80 MFI. As with the majority of experiments for CD86 there was no significant increase in CD80 MFI after LPS stimulation (data not shown). In contrast to CD86, incubation with SMAs 11a, 11e or 11i alone significantly inhibited the number of $CD11c^+CD80^+$ cells in all experiments (Figure 3.10B). 12b significantly decreased the number of $CD11c^+CD80^+$ cells and 19o significantly increased the percentage of $CD11c^+CD80^+$ cells in 1 experiment but both had no effect in the other two experiments (Figure 3.10B).

Stimulation of bmDCs with LPS did not result in any consistent changes in the percentage of $CD11c^+$ cells that express CD80 compared to the RPMI control, however SMA 11a, 11e and 11i pre-treatment did cause a reduction in the number of $CD11c^+CD80^+$ cells compared with the LPS control (data not shown). These results combined with the observation that exposure to the SMAs alone can reduce the

number of $CD11c^+CD80^+$ cells suggests that SMAs 11a, 11e and 11i are capable of modulating the number of $CD80^+$ cells.

3.5.3 CD40

Neither treatment with SMAs or stimulation with LPS caused any consistent changes in the MFI of CD40 on bmDCs (data not shown). Treatment with SMAs alone also had no effect on the percentage of $CD11c^+CD40^+$ cells but activation of bmDCs with LPS for 24 hours significantly increased the percentage of $CD11c^+CD40^+$ cells in 2/3 experiments. Pre-treatment with SMA 11a, 12b or 11e significantly reduced the percentage of $CD11c^+CD40^+$ cells in both experiments and 11i significantly lowered the number of these cells in 1/2 experiments (Figure 3.10C) suggesting that the SMAs can also target the expression of CD40 on bmDCs following PAMP activation. SMA 19o had no significant effects on the number of $CD11c^+CD40^+$ bmDCs.

3.5.4 MHC II

As with the CD86 and CD40 neither treatment with SMAs or stimulation of bmDCs with LPS caused any significant consistent changes in the MFI of MHC II on bmDCs (data not shown). Similarly, in 2/3 experiments LPS did not cause any significant changes in the percentages of $CD11c^+$ cells that express MHC II. However, in both of these experiments over 95% of $CD11c^+$ cells were MHC II^+ in RPMI control samples. In the third experiment LPS stimulation caused a decrease in the percentage of MHC II^+ cells and SMA 11e further reduced this percentage. The percentage of MHC II^+ cells in the medium alone group in this experiment was 45.63 ± 0.74 (mean ± SD) and it could be that this difference in MHC II expression between this experiment and the other two could help explain the differences observed with LPS stimulation. The SMAs had no effect on the percentage of MHC II^+ CD11c cells either alone or after LPS stimulation (Figure 3.10D).

Figure 3.10: The effect of ES-62 SMAs on the surface expression of co-stimulatory CD86, CD80, CD40 and MHC II on bmDCs

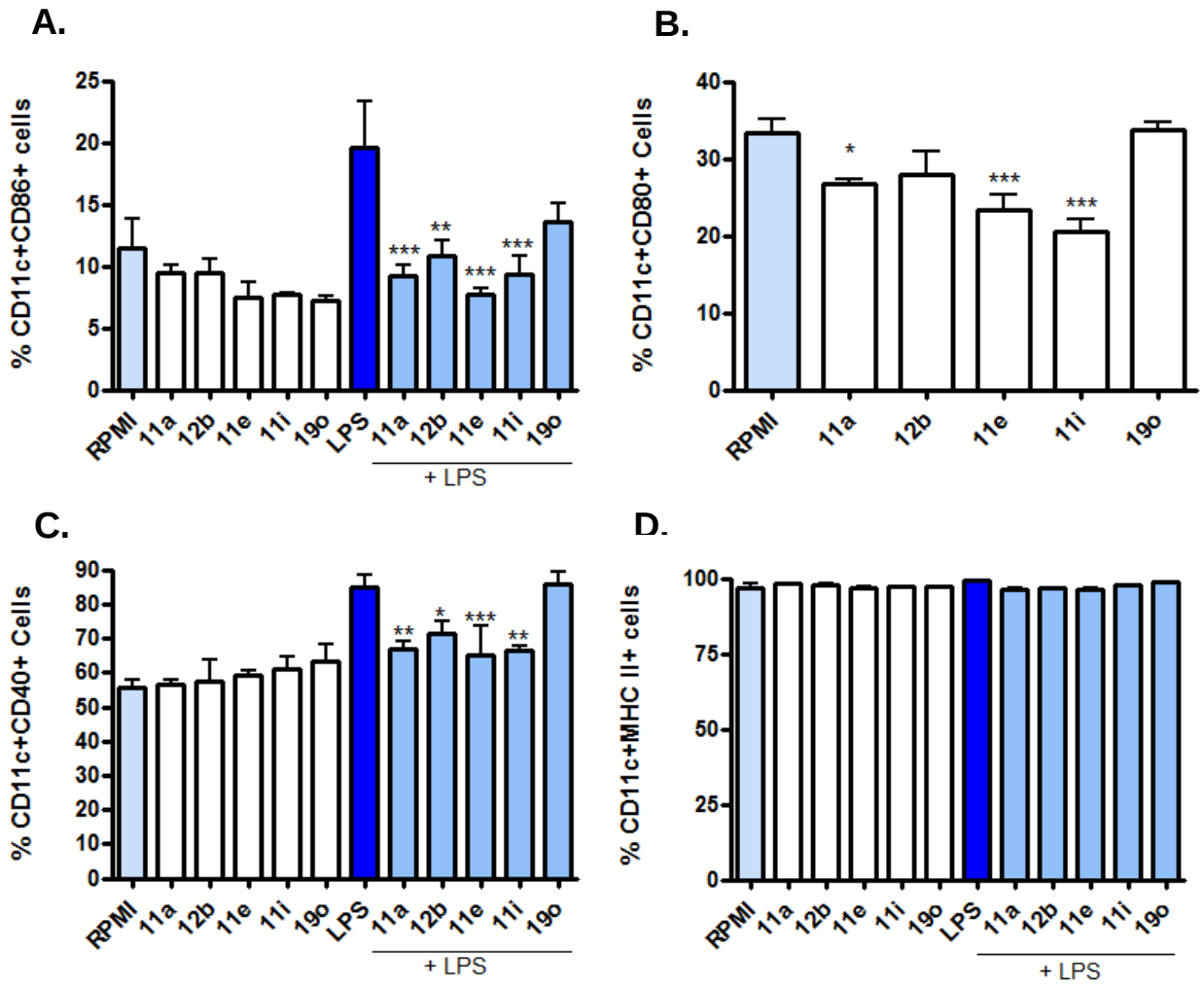
BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity confirmed by flow cytometry. Cells were plated at $2x10^6$ cells/2 ml medium in 6-well plates, rested overnight and pre-treated with SMAs (5 μg/ml) 11a, 12b, 11e, 11i or 19o for 18 hours before incubation with either medium alone or containing LPS (100 ng/ml) for 24 hours. Cells were harvested, stained with anti-mouse CD16/CD32 to block the Fc interactions with IgG to reduce background staining for five minutes before being examined for expression of CD11c (Pe/Cy7 antibody), CD86 (PerCP antibody), CD80 (APC antibody), CD40 (FITC antibody) and MHC II (APC/Cy7 antibody) with analysis in triplicate. Cells were first gated depending on size and granularity and doublet cells were removed. The positive gates for each antibody were set using fluorescent minus one controls and cells were gated on CD11c expression. Data are expressed as mean ± SD and were analysed using one-way ANOVA with Bonferroni post-test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

In panel A the percentage of $CD86^+$ cells after SMA treatment alone or with LPS stimulation is shown from one experiment representative of 3 for 12b, 11e and 2 (of 3) for 11a, 11i and 19o.

As described in the text LPS stimulation had no consistent effect on CD80 and thus panel B shows the effect of SMA treatment on $CD80^+$ cells compared to RPMI alone. The data are representative of 3/3 experiments for 11a, 11e and 11i and 2/3 for 12b and 19o

In panel C the percentage of $CD40^+$ cells after SMA treatment and/or LPS stimulation is shown. These data are representative of 3 experiments for SMA treatment only. As mentioned in the text LPS stimulation only resulted in a significant increase in $CD40^+$ cells in 2 experiments therefore data are representative for these two experiments with respect to 11a, 12b, 11e and 19o and one of two for 11i.

Panel D shows the percentage of MHC II^+ cells following SMA treatment only or with LPS stimulation. This shows a single experiment representative of 2 independent experiments (as described in text, the third experiment demonstrated a lower overall expression of MHC II).


A.
% CD11c+CD86+ cells
25
20
15
10
5
0
***
**
***
***
RPMI
11a
12b
11e
11i
19o
LPS
11a
12b
11e
11i
19o
+ LPS
B.
% CD11c+CD80+ Cells
40
30
20
10
0
*
***
***
RPMI
11a
12b
11e
11i
19o
C.
% CD11c+CD40+ Cells
90
80
70
60
50
40
30
20
10
0
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*
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RPMI
11a
12b
11e
11i
19o
LPS
11a
12b
11e
11i
19o
+ LPS
D.
% CD11c+MHC II+ cells
100
75
50
25
0
RPMI
11a
12b
11e
11i
19o
LPS
11a
12b
11e
11i
19o
+ LPS

Chapter 4. Investigation of the mechanism of action of ES-62 Small Molecule Analogues on dendritic cell responses

4.1 Introduction

Small molecule analogues (SMAs) based on the active PC moiety of the helminth glycoprotein ES-62 have been demonstrated to closely mimic the actions of ES-62 on macrophages and mast cells *in vitro*, and also in *in vivo* models of disease [260]–[264]. Investigation into their effects on cytokine production by DCs has found that the same SMAs, 11a and 12b, inhibit TLR PAMP-induced cytokine responses and also the up-regulation of co-stimulatory molecules CD86 and CD40 in response to LPS (Figures 3.4-3.6; 3.8-3.10). In addition, several other SMAs, 11e, 11i, 11h and 11k, from a screen of 79 were also found to inhibit cytokine responses by DCs and of those, 11e and 11i were also found to inhibit the LPS-induced up-regulation of co-stimulatory molecule expression. In addition, none of the SMAs on their own induced cytokine production or up-regulation of co-stimulatory molecule expression. These results indicate that the SMAs, like many helminth molecules, do not induce classical DC activation and can act to suppress subsequent inflammatory responses. In terms of cytokine modulation these SMAs mimic the action of ES-62 and as they are small, drug-like molecules they have greater therapeutic potential. It is therefore important to understand their mechanisms of action to allow further investigation into their potential as future drugs. Identifying the molecules targeted by the SMAs may also identify additional drug targets through better understanding of inflammatory processes and hence in this study the possible mechanisms of actions of the SMAs on bmDCs were investigated.

Since the SMAs differentially target IL-12p40 and IL-12p70 production, the effects of the SMAs on the mRNA of the two subunits, p35 and p40, of IL-12p70 were investigated. In addition the levels of IL-6 and TNF-α mRNA were determined as cytokine production can be regulated at multiple stages.

ES-62 has been demonstrated to require the TLR4 receptor and also to target the TLR signalling adaptor MyD88 to mediate many of its anti-inflammatory effects. Thus, in macrophages and DCs grown from TLR4 or MyD88 deficient mice the low level induction of pro-inflammatory cytokines induced by ES-62 was absent, as was the ability of the nematode product to suppress CpG- and BLP-induced cytokines, indicating that both the receptor and adaptor are required for activity [246]. It is currently unknown if the SMAs require TLR4, or indeed any receptor, to mediate their effects on cells and the effect of the SMAs was therefore investigated in bmDCs from

TLR4 knock out (KO) mice. Moreover, previously SMAs 11a and 12b have been found to down-regulate MyD88 in macrophages [260], [261] and so the effects of the SMAs were also investigated in MyD88 KO bmDCs.

The exact mechanism of cytokine inhibition by ES-62 in DCs has not been fully elucidated but the molecule has been shown to modulate the activation of the three major families of MAPK, ERK, JNK and p38, and also to interfere with the activation of NF-κB. ES-62 has been found to inhibit activation of p38 and JNK, which are required for IL-6, IL-12 and TNF-α production while inhibition of ERK MAPK through the use of an MEK1 inhibitor rescued the suppression of IL-12 by ES-62 [306]. Based on these results the effects of four of the most potent SMAs (11a, 12b, 11e and 11i) on the activation of ERK, JNK and p38 MAPK and also NF-κBp65 in DCs was investigated.

In brief, the aims of this study were:

- To investigate if the SMAs target cytokine gene expression and whether the different effects of the SMAs on the production of IL-12p40 and IL-12p70 could be explained in part through their effects on the mRNA of the IL-12 subunits, p35 and p40.

- To determine if the SMAs require the receptor TLR4 and/or the signalling adaptor MyD88 to mediate their inhibitory effects on DC cytokine production

- To investigate if the SMAs mediate their inhibitory effects through the altered activation of MAPKs ERK, JNK and p38

- To investigate whether the SMAs can also target the transcription factor NF-κB to mediate suppression of cytokines

4.2 The effect of pre-treatment with ES-62 SMAs on the subsequent LPS-stimulated cytokine mRNA levels of BALB/c strain mouse bmDCs

Cytokine production is tightly regulated as dysregulation of cytokine induced signalling can lead to severe inflammatory disease; for example, overexpression of IL-6 has been shown to play a prominent role in the development and pathogenesis of rheumatoid arthritis [307]. Regulation of cytokines occurs at several different stages, including chromatin remodelling, transcriptional and post-transcriptional control, signal transduction control through proteins such as SOCS and also through cross-talk between cytokines [308]. It has been demonstrated that SMAs 11a, 12b, 11e, 11h, 11i and 11k can inhibit the PAMP-induced cytokine responses of bmDCs, however they show varying effects on cytokines in response to different PAMPs (Chapter 3). To investigate the mechanisms used by these SMAs to suppress the cytokine responses the ability of the SMAs to alter the cytokine gene expression of bmDCs was assessed. As with the experiments measuring cytokine protein levels, bmDCs were pre-treated with SMA (5 µg/ml) for 18 hours prior to stimulation with LPS (100 ng/ml). In these experiments however LPS stimulation was for four hours only as the effect of PAMP activation on mRNA levels can be detected more quickly than on protein levels, and the mRNA levels of IL-6, TNF-α, and IL-12 were determined by qPCR. The effect of simultaneous SMA and LPS treatment was also investigated in that bmDCs were stimulated with SMA (5 µg/ml) and LPS (100 ng/ml) in combination for 4 hours and again cytokine mRNA levels were analysed. The effect of treatment with each SMA alone on bmDC gene expression after 4 and 18 hours was also determined. For each type of analysis, three independent experiments were generally performed. The results were normalised first against the housekeeping gene GAPDH and then to the RPMI control.

4.2.1 IL-6

Analysis of IL-6 mRNA levels demonstrated that the SMAs show selective effects on gene expression when employed either alone (Figure 4.1 A and C), with co-exposure with LPS (Figure 4.1B) or with exposure before LPS stimulation (Figure 4.1D). Treatment with SMA 11a alone for 4 hours (Figure 4.1A) significantly reduced the level of IL-6 mRNA compared to the RPMI control but 18 hour SMA incubation significantly increased IL-6 mRNA levels (Fig. 4.1C). However pre-treatment for 18 hours significantly inhibited LPS-induced production of IL-6 mRNA while treatment with 11a for 4 hours in combination with LPS had no effect on the levels of IL-6 mRNA (Figure

4.1; Tables 4.1 and 4.2). SMA 12b caused a significant increase in the basal level of IL-6 gene expression at 4 hours but significantly inhibited the LPS IL-6 response both in combination with LPS and by pre-treatment with the SMA (Figure 4.1; Tables 4.1 and 4.2). SMAs 11e and 11i had no effect on the basal levels of IL-6 mRNA but pre-treatment with these SMAs significantly inhibited the LPS response (Figure 4.1). These results indicate that after 18 hour pre-treatment, SMAs 11a, 12b, 11e and 11i impact on LPS-mediated IL-6 expression at the transcriptional level. In contrast to the cytokine data SMAs 11h and 11k had no effect on the levels of IL-6 mRNA either in combination with LPS or by pre-treatment before LPS stimulation, however 11h did significantly lower basal IL-6 mRNA compared to RPMI at both 4 and 18 hours (Figure 4.1). SMA 19o, consistent with the cytokine production data, had no effect on the levels of IL-6 mRNA (Figure 4.1). The percentages of IL-6 mRNA in bmDCs that had been incubated in combination with LPS for 4 hours or pre-treated with SMAs for 18 hours before LPS stimulation compared to LPS only bmDCs are shown in Tables 4.1 and 4.2 respectively.

4.2.2 TNF-α

The levels of TNF-α mRNA following SMA treatment for 4 and 18 hours were analysed by RT-PCR. SMAs 11a, 12b, 11h and 11k significantly reduced the basal level of TNF-α compared to RPMI after 4 hours (Figure 4.2A) and SMAs 11e, 11i and 11h significantly lowered TNF-α levels after 18 hours compared to RPMI (Figure 4.2B). Interestingly, and in keeping with IL-6 data, 12b significantly increased TNF-α level after 18 hours incubation (Figure 4.2C). Despite the changes at 4 hours none of the SMAs had any effect on bmDCs when added in combination with LPS (Figure 4.2B). Pre-treatment with SMAs 11a, 12b, 11e and 11i for 18 hours significantly inhibited the LPS-induced TNF-α (Figure 4.2D), consistent with effects on the TNF-α protein production. Similar to IL-6 mRNA levels, pre-treatment with SMAs 11h, 11k and 19o also had no effect on the subsequent LPS increase (Figure 4.2D) The percentages of TNF-α mRNA in bmDCs that had been incubated in combination with LPS for 4 hours and pre-treated with SMAs for 18 hours before LPS stimulation compared to LPS only bmDCs are shown in Tables 4.1 and 4.2 respectively.

4.2.3 IL-12

Pre-treatment of bmDCs with SMAs 11a or 12b results in down-regulation of IL-12p70 production but up-regulation of IL-12p40 following LPS stimulation (Figure 3.6). SMAs 11e, 11h, 11i and 11k administered prior to LPS or CpG stimulation cause suppression

of IL-12p70 production but have no effect on IL-12p40. IL-12, as mentioned previously, is a heterodimer composed of two subunits, p40 and p35, that are located on different chromosomes. Although the cell must co-express both subunits in order to produce bioactive IL-12 [309] production of the two subunits is independently regulated. Manufacture of p40 is predominantly regulated at the level of gene induction while regulation of p35 expression occurs both transcriptionally and translationally [124] and this distinction may explain why the SMAs 11a and 12b target bioactive IL-12 and IL-12p40 differently. Both IL-12p35 and IL-12p40 mRNA levels were analysed and the SMAs displayed differential effects on the two subunits of bioactive IL-12p70. Consistent with cytokine production (Figure 3.6), 11a and 12b significantly increased the basal levels of IL-12p40 mRNA compared to RPMI after 4 (Figure 4.3A) and 18 hours (Figure 4.3C) confirming that these SMAs induce IL-12p40 production by bmDCs. In combination with LPS for 4 hours however, neither 11a nor 12b had any effect on IL-12p40 (Figure 4.3B), nor did pre-treatment with these SMAs affect the following LPS-induced subunit increase (Figure 4.3D). In contrast both SMAs had no effect on the basal levels of IL-12p35 at either time point (Figures 4.4A and C) but pre-treatment with these SMAs significantly inhibited the LPS-induced increase in IL-12p35 (Figure 4.4D) demonstrating selective targeting of the two subunits of bioactive IL-12. SMA 12b in combination with LPS also had reduced levels of IL-12p35 compared to LPS alone (Figure 4.4B). SMAs 11e and 11i significantly inhibit LPS-induced IL-12p70 production but have no effect on IL-12p40 levels (Figure 3.6) suggesting they may target this cytokine in a different manner compared to 11a and 12b. Consistent with this, pre-treatment with 11e and 11i significantly inhibited both LPS-induced IL-12p40 (Figure 4.3D) and IL-12p35 (Figure 4.4D) mRNA levels although they had no effect in combination with LPS after 4 hours (Figures 4.3B and 4.4B). Neither SMA had any effect on the basal levels of either subunit (Figure 4.3 and 4.4). Similar to IL-6 and TNF-α mRNA data, SMA 11h and 11k significantly reduced the basal levels of p40 at 4 and 18 hours but had no effect on the LPS-induced mRNA levels of either subunit (Figure 4.3 and 4.4). Despite inhibiting LPS-induced IL-12p70 production (Figure 3.6) SMA 19o had no effect on the mRNA levels of either subunit either alone or with LPS (Figures 4.3 and 4.4). The percentages of IL-12p40 and IL-12p35 mRNA in bmDCs that had been incubated in combination with LPS for 4 hours and pre-treated with SMAs for 18 hours before LPS stimulation compared to LPS only bmDCs are shown in Tables 4.1 and 4.2 respectively.

Figure 4.1: The effect of ES-62 SMAs on IL-6 mRNA levels in bmDCs

BmDCs were grown in RPMI complete medium with 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at $2x10^6$ cells in 2ml/well and rested for 24 hours before incubation with SMA alone for 4 hours (A), in combination with LPS (100 ng/ml) for 4 hours (B), incubated with SMA alone for 18 hours (C) or pre-treated with SMA (5 µg/ml) for 18 hours and then stimulated with LPS (100 ng/ml) for 4 hours (D). The cells were then harvested, the RNA extracted and mRNA levels of IL-6 analysed by qPCR. The level of IL-6 was normalized to GAPDH and then expressed as a fold change with respect to the relevant RPMI control. RPMI and SMA only were compared using Kruskal Wallis with Dunns post-test or Wilcoxin signed rank test. Due to high LPS variation between experiments the percentage of IL-6 mRNA in each experiment compared to LPS was calculated and compared using a one sample t test. Results are expressed as the mean ± SEM. For statistical analysis, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

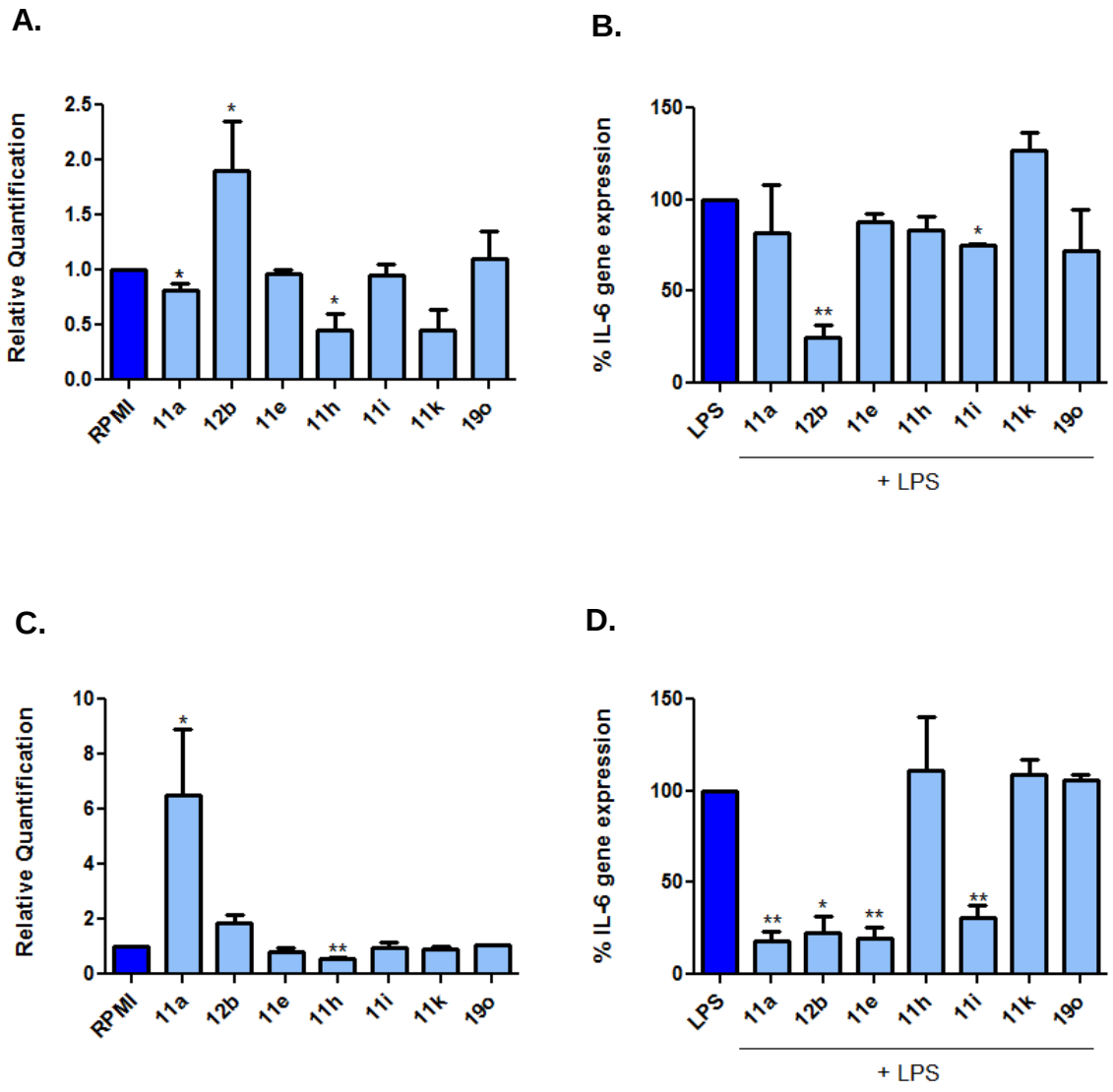
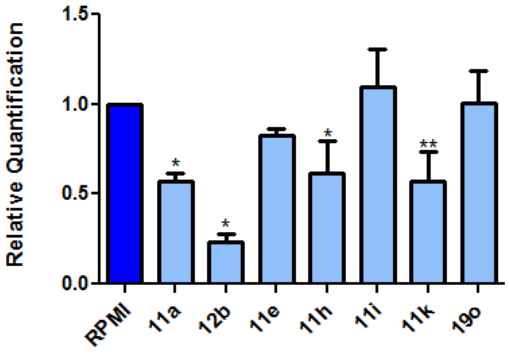

A.
Relative Quantification
2.5
2.0
1.5
1.0
0.5
0.0
RPMI
11a
12b
11e
11h
11i
11k
19o
B.
% IL-6 gene expression
150
100
50
0
LPS
11a
12b
11e
11h
11i
11k
19o
+ LPS
C.
Relative Quantification
10
8
6
4
2
0
RPMI
11a
12b
11e
11h
11i
11k
19o
D.
% IL-6 gene expression
150
100
50
0
LPS
11a
12b
11e
11h
11i
11k
19o
+ LPS

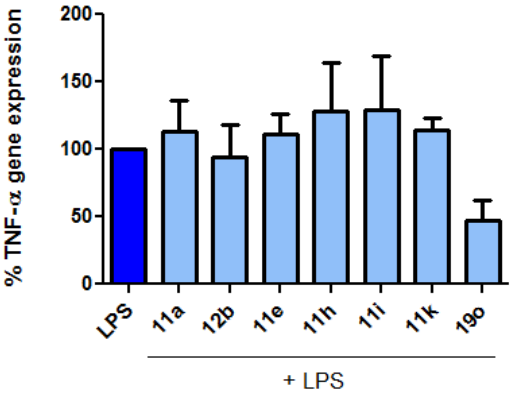
Figure 4.2: The effect of ES-62 SMAs on TNF-α mRNA levels in bmDCs

BmDCs were grown in RPMI complete medium with 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at $2x10^6$ cells in 2ml/well and rested for 24 hours before incubation with SMA alone for 4 hours (A), in combination with LPS (100 ng/ml) for 4 hours (B), incubated with SMA alone for 18 hours (C) or pre-treated with SMA (5 μg/ml) for 18 hours and then stimulated with LPS (100 ng/ml) for 4 hours (D). The cells were then harvested, the RNA extracted and mRNA levels of TNF-α analysed by qPCR. The level of TNF-α was normalized to GAPDH and then expressed as a fold change with respect to the relevant RPMI control. RPMI and SMA only were compared using Kruskal Wallis with Dunns post-test or Wilcoxin signed rank test. Due to high LPS variation between experiments the percentage of TNF-α mRNA in each experiment compared to LPS was calculated and compared using a one sample t test. Results are expressed as the mean ± SEM. For statistical analysis * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

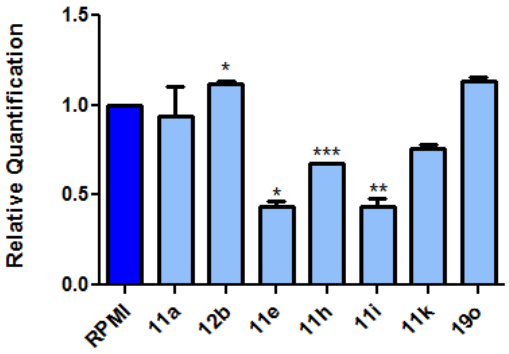
A.


Relative Quantification
1.5
1.0
0.5
0.0
*
*
*
**
RPMI
11a
12b
11e
11h
11i
11k
19o

B.


% TNF-α gene expression
200
150
100
50
0
LPS
11a
12b
11e
11h
11i
11k
19o
+ LPS

C.


Relative Quantification
1.5
1.0
0.5
0.0
*
*
***
**
RPMI
11a
12b
11e
11h
11i
11k
19o

D.

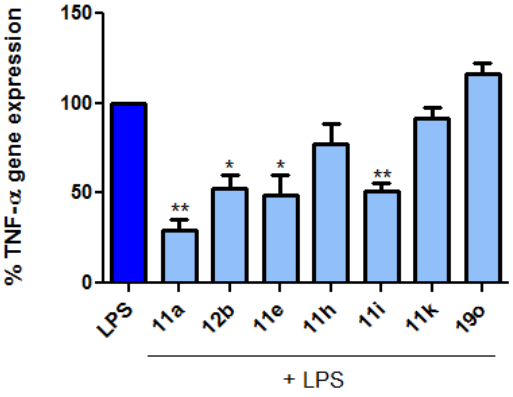
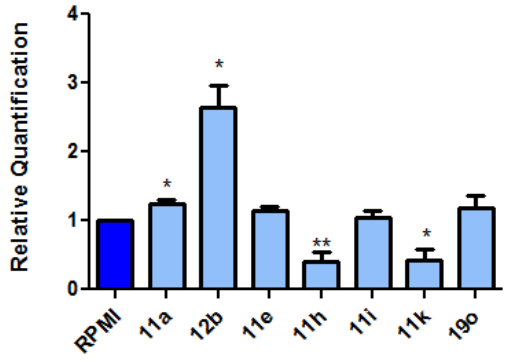

% TNF-α gene expression
150
100
50
0
**
*
*
**
LPS
11a
12b
11e
11h
11i
11k
19o
+ LPS

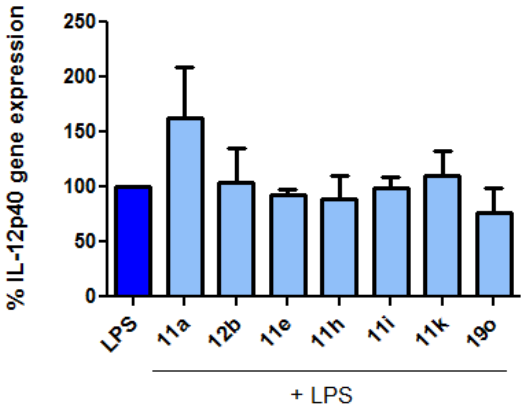
Figure 4.3: The effect of ES-62 SMAs on IL-12p40 mRNA levels in bmDCs

BmDCs were grown in RPMI complete medium with 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at $2x10^6$ cells in 2ml/well and rested for 24 hours before incubation with SMA alone for 4 hours (A), in combination with LPS (100 ng/ml) for 4 hours (B), incubated with SMA alone for 18 hours (C), pre-treated with SMA (5 µg/ml) for 18 hours and then stimulated with LPS (100 ng/ml) for 4 hours (D). The cells were then harvested, the RNA extracted and mRNA levels of p40 analysed by qPCR. The level of p40 was normalized to GAPDH and then expressed as a fold change with respect to the relevant RPMI control. RPMI and SMA only were compared using Kruskal Wallis with Dunns post-test, or Wilcoxin signed rank test. Due to high LPS variation between experiments the percentage of IL-12p40 mRNA in each experiment compared to LPS was calculated and compared using a one sample t test. Results are expressed as the mean ± SEM. For statistical analysis * $p < 0.05$; ** $p < 0.01$

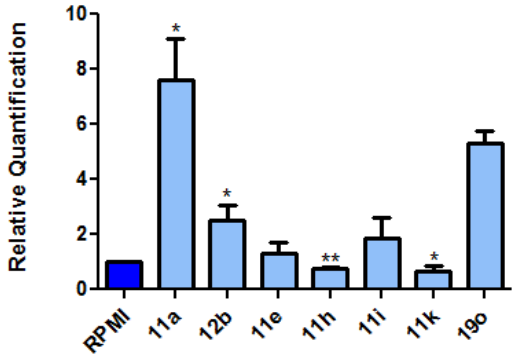
A.


Relative Quantification
4
3
2
1
0
*
*
**
*
RPMI
11a
12b
11e
11h
11i
11k
19o

B.


% IL-12p40 gene expression
250
200
150
100
50
0
LPS
11a
12b
11e
11h
11i
11k
19o
+ LPS

C.


Relative Quantification
10
8
6
4
2
0
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*
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*
RPMI
11a
12b
11e
11h
11i
11k
19o

D.

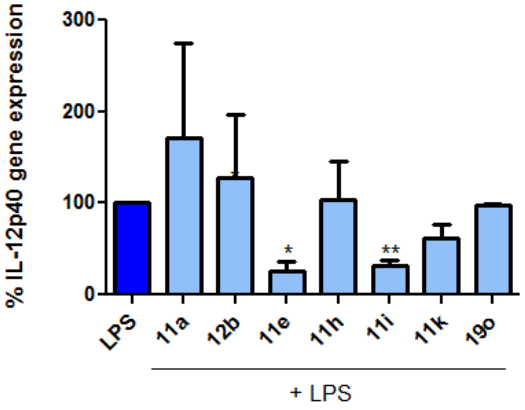
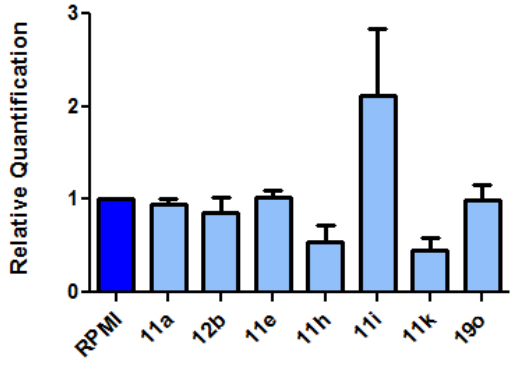

% IL-12p40 gene expression
300
200
100
0
*
**
LPS
11a
12b
11e
11h
11i
11k
19o
+ LPS

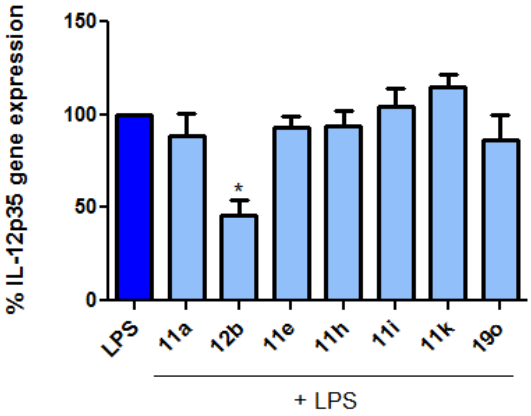
Figure 4.4: The effect of ES-62 SMAs on IL-12p35 mRNA levels in bmDCs

BmDCs were grown in RPMI complete medium with 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at $2x10^6$ cells in 2ml/well and rested for 24 hours before incubation with SMA alone for 4 hours (A), in combination with LPS (100 ng/ml) for 4 hours (B), incubated with SMA alone for 18 hours (C), pre-treated with SMA (5 µg/ml) for 18 hours and then stimulated with LPS (100 ng/ml) for 4 hours (D). The cells were then harvested, the RNA extracted and mRNA levels of p35 analysed by qPCR. The level of p35 was normalized to GAPDH and then expressed as a fold change with respect to the relevant RPMI control. RPMI and SMA only were compared using Kruskal Wallis with Dunns post-test, or Wilcoxin signed rank test. Due to high LPS variation between experiments the percentage of IL-12p35 mRNA in each experiment compared to LPS was calculated and compared using a one sample t test. Results are expressed as the mean ± SEM. For statistical analysis * $p < 0.05$; ** $p < 0.01$

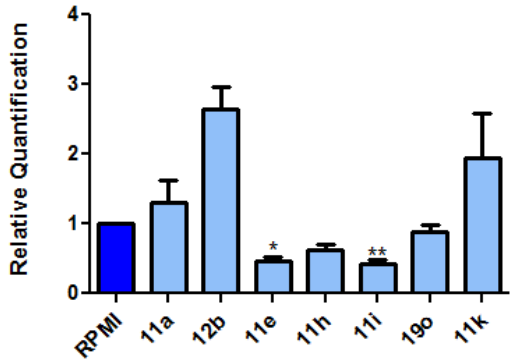
A.


Relative Quantification
3
2
1
0
RPMI
11a
12b
11e
11h
11i
11k
19o

B.


% IL-12p35 gene expression
150
100
50
0
*
LPS
11a
12b
11e
11h
11i
11k
19o
+ LPS

C.


Relative Quantification
4
3
2
1
0
*
**
RPMI
11a
12b
11e
11h
11i
19o
11k

D.

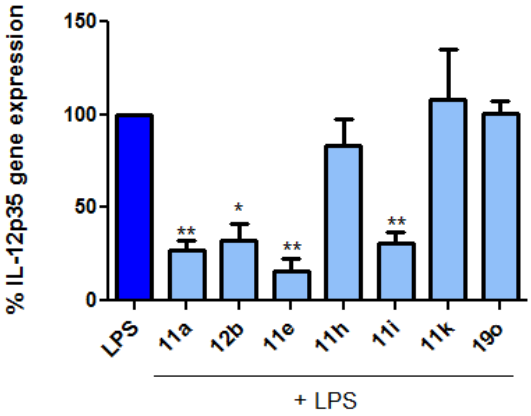

% IL-12p35 gene expression
150
100
50
0
**
*
**
**
LPS
11a
12b
11e
11h
11i
11k
19o
+ LPS

Table 4.1: The effect of ES-62 SMAs on cytokine mRNA levels in bmDCs

The percentage of IL-6, TNF-α, IL-12p35 and IL-12p40 gene expression in bmDCs after incubation with SMA and LPS in combination for 4 hours compared to LPS control for each experiment is shown, along with the mean ± SEM for each SMA. SMAs were compared with LPS using one sample t test. For statistical analysis: in purple $p \leq 0.01$ and blue $p \leq 0.05$. X signifies no results for that experiment.

SMA	IL-6					TNF				
	1	2	3	Mean	± SEM	1	2	3	Mean	± SEM
11a	39	130	77	82	26	69	147	123	113	23
12b	39	18	18	25	7	48	105	129	94	24
11e	83	93	x	88	5	126	97	x	112	14
11h	84	96	70	83	8	199	99	85	128	36
11i	74	76	x	75	1	169	89	x	129	40
11k	144	125	111	127	9	132	103	108	115	9
19o	28	95	95	72	22	77	29	36	47	15

SMA	IL-12p40					IL-12p35				
	1	2	3	Mean	± SEM	1	2	3	Mean	± SEM
11a	92	249	148	163	46	77	100	x	89	11
12b	166	75	71	104	31	62	34	41	46	8
11e	97	89	x	93	4	99	88	x	93	5
11h	49	122	94	89	21	90	110	82	94	8
11i	109	89	x	99	10	114	95	x	104	10
11k	72	107	151	110	23	127	113	105	115	7
19o	34	107	90	77	22	60	100	99	86	13

Table 4.2: The effect of ES-62 SMAs on cytokine mRNA levels in bmDCs

The percentage of IL-6, TNF-α, IL-12p35 and IL-12p40 gene expression in bmDCs after pre-treatment with SMA for 18 hour before LPS stimulation compared to LPS control for each experiment is shown, along with the mean ± SEM for each SMA. SMAs were compared with LPS using one sample t test. For statistical analysis: in purple $p \leq 0.01$ and blue $p \leq 0.05$. X signifies no results for that experiment.

SMA	IL-6					TNF				
	1	2	3	Mean	± SEM	1	2	3	Mean	± SEM
11a	25	20	8	18	5	39	20	30	30	5
12b	12	40	16	23	9	38	65	54	52	8
11e	18	30	10	20	6	37	71	37	49	11
11h	169	89	75	111	29	56	93	84	78	11
11i	31	42	20	31	7	49	60	46	51	4
11k	97	107	124	109	8	83	87	104	91	6
19o	109	102	x	106	3	111	122	x	117	6

SMA	IL-12p40					IL-12p35				
	1	2	3	Mean	± SEM	1	2	3	Mean	± SEM
11a	375	41	98	172	103	30	17	34	27	5
12b	255	22	107	128	68	36	15	46	32	9
11e	42	29	6	26	10	14	29	6	16	7
11h	187	77	48	104	42	111	75	65	84	14
11i	44	26	26	32	6	24	43	26	31	6
11k	69	82	34	62	14	77	87	162	109	27
19o	98	99	x	98	0	95	107	x	101	6

4.3 The effect of pre-treatment with ES-62 SMAs on cytokine release by bmDCs derived from TLR4 knock-out C57-BL/6 mice

ES-62 is recognised by the host immune system via the PRR TLR4 and ES-62 requires this receptor in order to mediate its inflammatory effects [246]. Modulation of macrophages and DCs by ES-62 was investigated using TLR4 and TLR2 knockout (KO) mice. Consistent with wild-type (WT) mice, ES-62-mediated low level induction of IL-12 and TNF-α as well as subsequent suppression of these cytokines in response to PAMPs was observed in macrophages and DCs from TLR2 KO mice indicating that TLR2 was not required for ES-62 modulation of APCs. In TLR4 KO mice, however, both effects were ablated. As LPS is also recognised by TLR4, BLP and CpG were used to determine the effects of ES-62 in TLR4 KO mice. ES-62 pre-treatment suppressed induction of IL-12 and TNF-α after stimulation with IFN-γ/BLP and IFN-γ/CpG in macrophages from wild type but not TLR4 KO mice, indicating that TLR4 is necessary for ES-62-activity. The use of C3H/HeJ mice, which have a Pro712His point mutation in the TIR domain of TLR4 preventing LPS recognition and response, demonstrated that ES-62 does not need a fully functioning TLR4 receptor to mediate its effects as IL-12 and TNF-α were both initially produced and then suppressed by macrophages and DCs in response to IFN-γ/BLP [246]. It is currently not known whether the ES-62 SMAs require a receptor to exert their effects as their structure dictates that many may enter cells by passive diffusion. Nevertheless, in order to investigate whether they, like their parent molecule ES-62, employ TLR4 to exert their effects on cytokines, bmDCs were grown from C57BL/6 TLR4 KO mice and corresponding WT mice that were a gift from Professor Padraic Fallon of Trinity College, Dublin. The bmDCs were grown as previously described and pre-treated with the 7 selected SMAs (5 µg/ml) for 18 hours before stimulation with BLP (100 ng/ml) or CpG (0.1 µM) for 24 hours. The levels of IL-6, TNF-α, IL-12p40 and IL-12p70 were measured by ELISA as before. For WT and TLR4 KO three individual experiments were performed, however some of the WT samples in one of the WT experiments became contaminated during the work and so could not be used meaning there are 3 independent experiments for TLR4 KOs for all SMAs and 3 independent WT experiments for 11a and 12b but only 2 experiments for SMAs 11e, 11h, 11i, 11k and 19o.

4.3.1 IL-6

As with previous experiments there was very little spontaneous IL-6 production by the bmDCs and none of the SMAs alone caused induction of cytokine in WT or KO bmDCs (results not shown).

SMA 12b significantly inhibited IL-6 production by WT bmDCs following 24 hours of stimulation with BLP in all experiments (n=3) and 11a inhibited BLP-induced IL-6 in 2/3 experiments (Figure 4.5A). SMAs 11e and 11i inhibited these responses in all experiments (n = 2; Figure 4.5A) and 11h inhibited BLP-induced IL-6 in 1/2 experiments. In bmDCs from TLR4 KO mice, SMAs 12b, 11e and 11i significantly reduced IL-6 secretion following BLP activation (3/3 for 11e and 2/3 for 12b and 11i) indicating these SMAs do not require TLR4 to modulate TLR2 signalling (Figure 4.5B). Interestingly, SMA 11a only significantly inhibited BLP-induced IL-6 in TLR4 KO bmDCs in 1/3 and SMA 11h had no effect on the levels of IL-6 produced (Figure 4.5C) suggesting that they may require TLR4 in some capacity to modulate BLP responses in bmDCs.

SMAs 12b, 11a, 11e and 11i significantly inhibited IL-6 production by WT bmDCs following 24 hours of stimulation with CpG in all experiments (n=3 for 11a and 12b and n=2 for 11e, 11h and 11i; Figure 4.5D) and 11h significantly inhibited this response in 1/2 experiments. In bmDCs from TLR4 KO mice, SMAs 11a, 12b, 11e, 11i and 11h significantly reduced IL-6 secretion following CpG activation (2/3 experiments for 11a and 3/3 for the other SMAs) indicating that they do not require TLR4 to inhibit TLR9 signalling (Figure 4.5E).

SMA 11k and 19o also generally behaved in a similar negative manner in WT and KO bmDcs following BLP and CpG stimulation (Figure 4.5A, C, D and F), only causing a significant increase in IL-6 in 1/2 experiments in WT but not TLR4 KO bmDCs following BLP stimulation (Table 4.3). SMA 19o also caused an increase in IL-6 following CpG stimulation in 1/2 experiments in WT and both 19o and 11k significantly decreased IL-6 in TLR4 KO cells in 1/3 experiments (Table 4.4).

Overall, there was no difference in 12b, 11e, and 11i action on IL-6 production following BLP and CpG stimulation between WT and KO bmDCs indicating these SMAs do not require TLR4 to exert their effect on this cytokine. SMAs 11a and to some degree 11h, however, showed differential effects between WT and KO cells; both SMAs inhibited BLP- and CpG- IL-6 in WT bmDCs but were only able to down-regulate CpG-induced

IL-6 in TLR4 KO bmDcs indicating they may require TLR4 for their inhibitory activity to alternative PAMPs.

4.2.2. TNF-α

TNF-α production was also measured by ELISA following BLP and CpG stimulation in WT and TLR4 KO bmDCs. There was no spontaneous TNF-α production, and none of the SMAs employed alone induced release of the cytokine (results not shown).

SMAs 11a, 12b, 11e and 11i significantly inhibited TNF-α in all experiments following BLP stimulation in WT bmDCs, and also inhibited TNF-α after BLP stimulation in 2/3 experiments with TLR4 KO bmDCs (Figure 4.6A and B). 11h only significantly inhibited TNF-α in 1/2 and 1/3 in WT and TLR4 KO bmDCs respectively (Figure 4.6A and C) while SMA 19o significantly increased TNF-α in 1/2 experiments employing BLP in WT mice and had no significant effects on bmDCs from TLR4 KO mice. SMA 11k, similarly, had no effect in WT or TLR4 KO bmDCs (Figure 4.6A and C).

SMAs 11a, 12b, 11e, 11h and 11i significantly inhibited TNF-α production following CpG treatment in all experiments in both WT and TLR4 KO bmDCs suggesting that, as with IL-6, these SMAs do not require TLR4 for inhibition of secretion of this cytokine following TLR9 activation (Figure 4.6D and E). Similar to IL-6 data, SMA 11k had no effects in WT or TLR4 KO bmDCs following CpG stimulation and 19o significantly up-regulated IL-6 in 1/2 WT experiments but had no effect in TLR4 KO bmDCs (Figure 4.6D and E).

4.3.2 IL-12

The expression of IL-12p70 was analysed following SMA pre-treatment and BLP or CpG stimulation in bmDCs from WT and TLR4 KO mice. However, IL-12p70 levels were below the detection levels of the kit (15 pg/ml) and so could not be determined in any experiments after BLP stimulation for WT or TLR4 KO bmDCs. Nevertheless, IL-12p70 could be detected in 1/2 experiments with 11e, 11h, 11i, 11k and 19o and 2/3 experiments with 11a and 12b after CpG stimulation of WT bmDCS. SMAs 11a, 12b, 11e, 11h and 11i significantly inhibited cytokine production in all experiments it was detected (Figure 4.7A). 11k had no effect on IL-12p70 in WT bmDCs but 19o caused a significant increase in this cytokine, which has not been seen previously in the study, but this could be a consequence of differing mouse strains. In the TLR4 KO bmDCs, CpG-induced IL-12p70 was detected in 2/3 experiments and 11a, 12b, 11e, 11h and

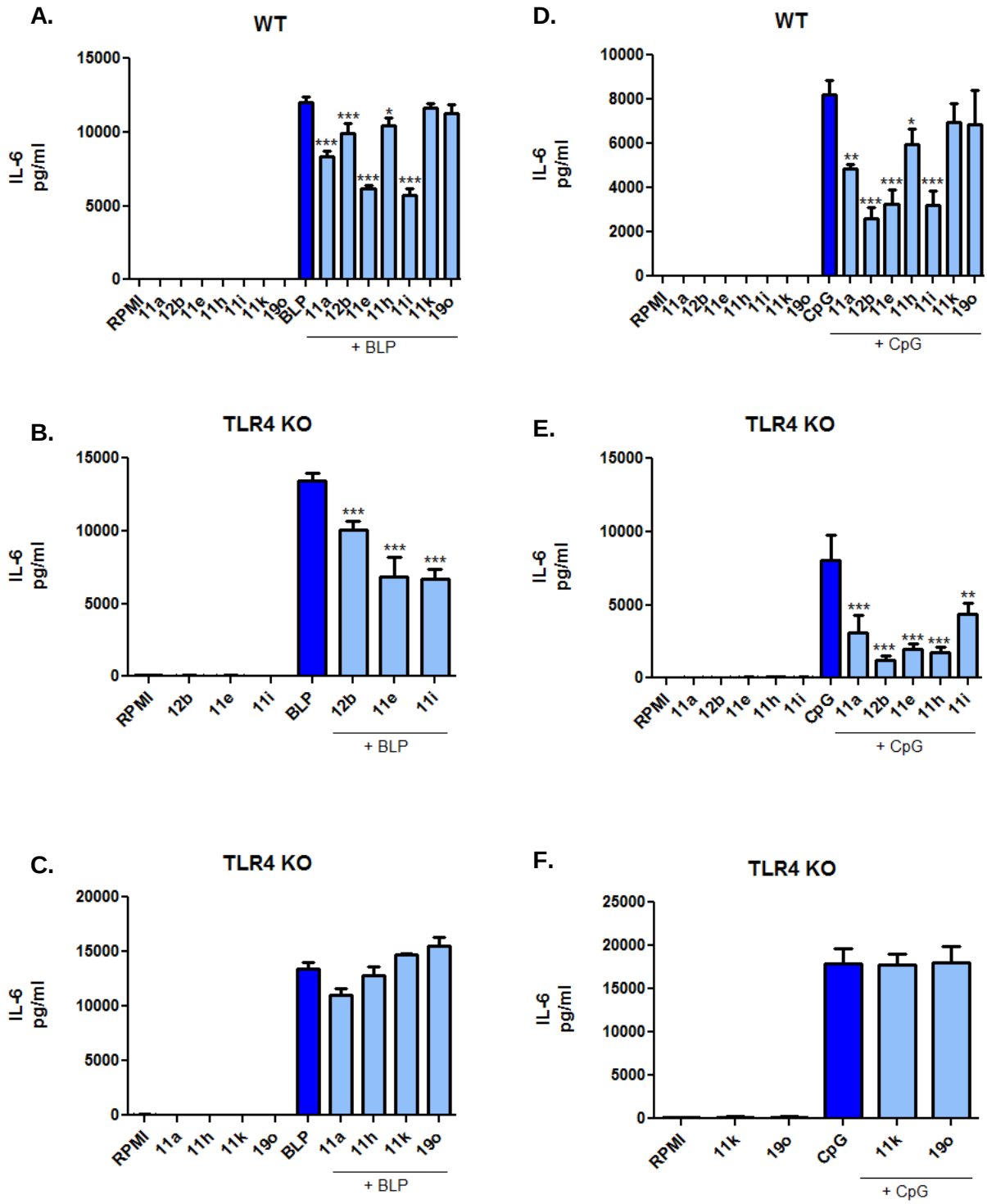
11i significantly inhibited this response in both experiments (Figure 4.7B). SMA 11k perhaps surprisingly, also significantly reduced CpG-induced IL-12p70 in both experiments (Figure 4.7B and Table 4.5). SMA 19o had a variable effect on IL-12p70 production by TLR4 KO cells after CpG exposure – in one experiment it increased levels of IL-12p70 although this was not statistically significant but in another experiment it significantly decreased them (Figure 4.7B and Table 4.5).

Figure 4.5: The effect of ES-62 SMAs on IL-6 production following BLP or CpG mediated-activation of bmDCs from WT and TLR4 KO mice

BmDCs were grown from TLR4 KO and matched WT C57-BL/6 mouse bone marrow for 8 days in the presence of GM-CSF and then plated at $2x10^5$ cells/well and pre-treated with 11a, 12b, 11e, 11h, 11i, 11k or 19o for 18 hours before stimulation with BLP (100 ng/ml) or CpG (0.1 μM) for 24 hours and the levels of IL-6 determined by ELISA. The cytokine detection limit was ≥ 15 pg/ml according to the manufacturer's instructions. Results are expressed as mean (of triplicate determinations) ± SD and data were analysed using one-way ANOVA with Bonferroni post-test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

The effect of SMAs on IL-6 production in WT bmDCs (A) and TLR4 KO bmDCs (B and C) following BLP stimulation are shown. Data are representative of three independent experiments in WT bmDCs for 12b; 2/3 for 11a; 2/2 for 11e and 11i and 1/2 for 11h, 11k and 19o. For TLR4 KO bmDCs data are representative of 3/3 experiments for 11e, 11h, 11k, 19o and 2/3 for 12b, 11a and 11i

The effects of the SMAs on IL-6 production in WT (D) and TLR4 KO bmDCs (E and F) following CpG stimulation are shown. Data are representative of all experiments in WT bmDCs for 11a and 12b (3/3), 11e, 11i and 11k (2/2) and 1/2 for 11h and 19o. For TLR4 KO bmDCs, data are representative of 3/3 experiments for 12b, 11e, 11h and 11i and 2/3 for 11a, 11k and 19o.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WT
IL-6 pg/ml
15000
10000
5000
0
RPMI
11a
12b
11e
11h
11i
11k
19o
BLP
+ BLP
D.
WT
10000
8000
6000
4000
2000
CpG
+ CpG
B.
TLR4 KO
E.
TLR4 KO
C.
TLR4 KO
20000
F.
TLR4 KO
25000

Figure 4.6: The effect of ES-62 SMAs on TNF-α production following BLP or CpG mediated-activation of bmDCs from WT and TLR4 KO mice

BmDCs were grown from TLR4 KO and matched WT C57-BL/6 mouse bone marrow for 8 days in the presence of GM-CSF then plated at $2x10^5$ cells/well and pre-treated with 11a, 12b, 86, 90, 91, 93 or 15 for 18 hours before stimulation with (A) BLP (100 ng/ml) or (B) CpG (0.1 μM) for 24 hours and the levels of TNF-α determined by ELISA. The cytokine detection limit was ≥ 15 pg/ml according to the manufacturer's instructions. Results are expressed as mean (of triplicate determinations) ± SD and data were analysed using one-way ANOVA with Bonferroni post-test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

The effect of SMAs on TNF-α secretion in WT bmDCs (A) and TLR4 KO bmDCs (B and C) following BLP stimulation is shown. Data are representative of 3/3 experiments for 11a and 12b, 2/2 for 11e, 11i and 11k and 1/2 for 11h and 19o for WT bmDCs and 3/3 for 11k and 19o and 2/3 for 11a, 12b, 11e, 11h and 11i for TLR4 KO bmDCs.

Panel B shows the effects of the SMAs on TNF-α secretion in WT and TLR4 KO bmDCs following CpG stimulation. Data are representative of 3/3 independent experiments for 11a and 12b , 2/2 for 11e, 11h and 11i and 1/2 for 11k and 19o in WT bmDCs. For TLR4 KO bmDCs data are representative of 3/3 independent experiments for all SMAs.

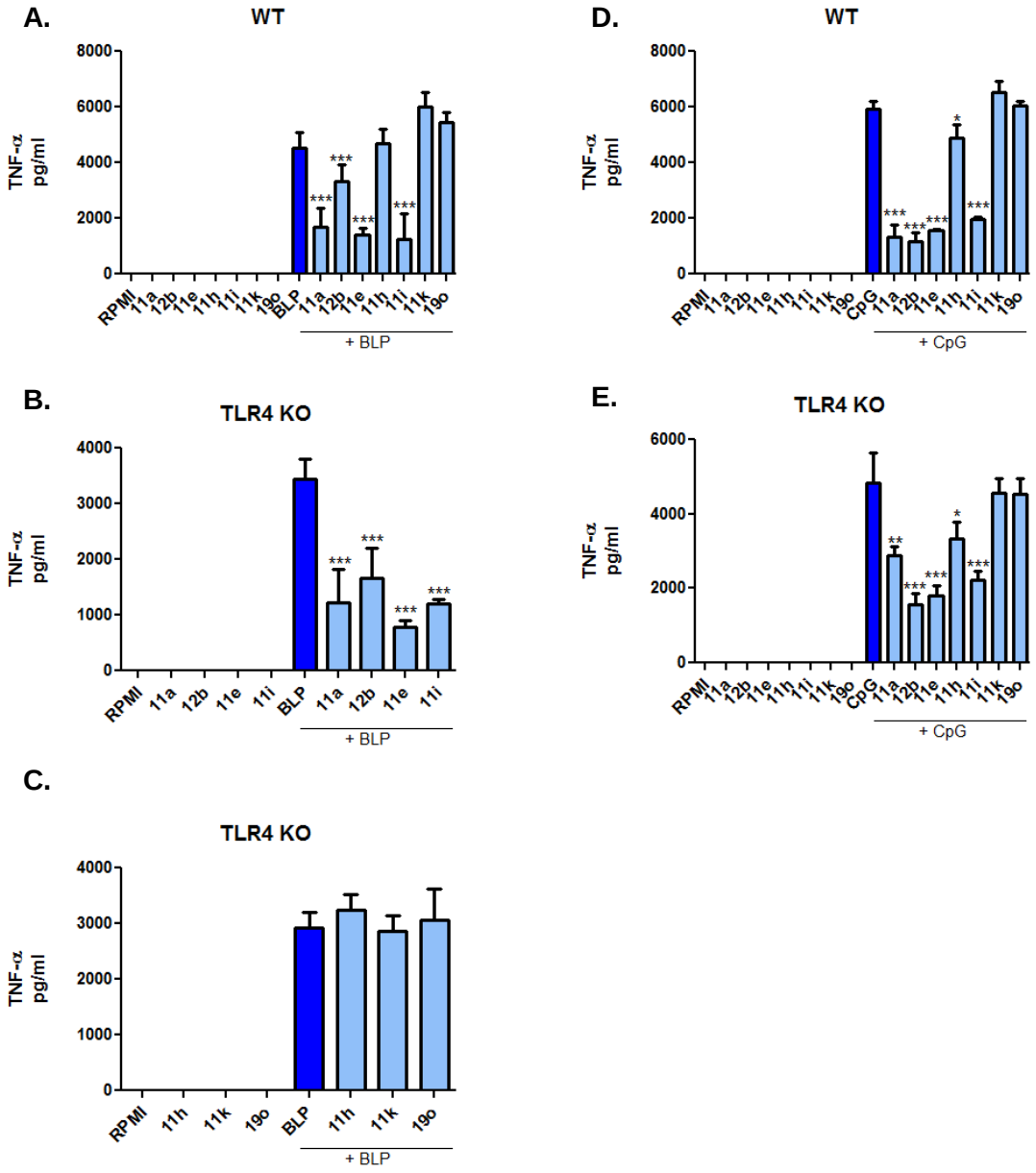

A.
WT
TNF-α pg/ml
8000
6000
4000
2000
0
RPMI
11a
12b
11e
11h
11i
11k
19o
BLP
+ BLP
B.
TLR4 KO
4000
3000
2000
1000
C.
D.
CpG
+ CpG
E.

Figure 4.7: The effect of ES-62 SMAs on IL-12p70 production following BLP or CpG mediated-activation of bmDCs from WT and TLR4 KO mice

BmDCs were grown from TLR4 KO and matched WT C57-BL/6 mouse bone marrow for 8 days in the presence of GM-CSF then plated at $2x10^5$ cells/well and pre-treated with 11a, 12b, 11e, 11i, 11h, 11k or 19o for 18 hours before stimulation with CpG (0.1 μM) for 24 hours and the levels of IL-12p70 determined by ELISA. The cytokine detection limit was ≥ 15 pg/ml according to the manufacturer's instructions. Results are expressed as mean (of triplicate determinations) ± SD and data were analysed using one-way ANOVA with Bonferroni post-test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Panel A shows the effect of SMAs on IL-12p70 secretion in WT bmDCs following CpG stimulation. Data are from the single experiment where IL-12p70 was detected for 11e, 11h, 11i, 11k and 19o and 2/2 for 11a and 12b

Panel B shows the effects of the SMAs on IL-12p70 secretion in TLR4 KO bmDCs following CpG stimulation. Data are representative of 2/2 experiments for all SMAs except 19o where data are representative of 1/2 experiments

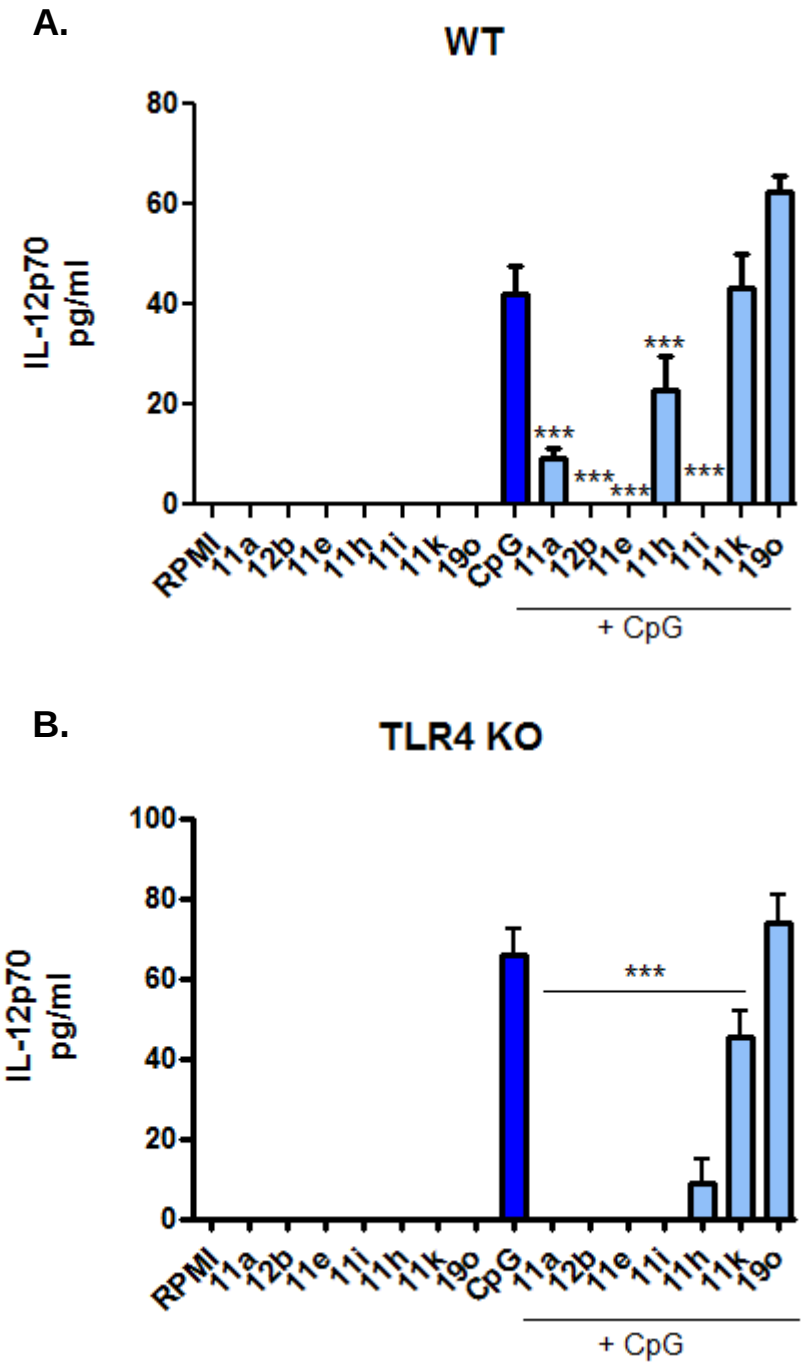

A.
WT
IL-12p70 pg/ml
80
60
40
20
0
***
***
***
***
***
RPMI
11a
12b
11e
11h
11i
11k
19o
CpG
11a
12b
11e
11h
11i
11k
19o
+ CpG
B.
TLR4 KO
IL-12p70 pg/ml
100
80
60
40
20
0
***
RPMI
11a
12b
11e
11i
11h
11k
19o
CpG
11a
12b
11e
11i
11h
11k
19o
+ CpG

Table 4.3: The effect of ES-62 SMAs on the production of IL-6 following BLP- or CpG-mediated activation of bmDCs derived from WT and TLR4 KO C57-BL/6 mice

The percentage of cytokine produced compared to the BLP or CpG control following SMA pre-treatment is shown for IL-6. Only percentages from experiments where the SMAs induced a significant difference are shown where red $p \leq 0.001$ and purple $p \leq 0.01$. X signifies no results for that experiment and ND (not detected) is used in cases where SMA pre-treated resulted in cytokine levels below the detection levels of the ELISA kit (≥ 15 pg/ml).

SMA	BLP						CpG					
	WT			TLR4 KO			WT			TLR4 KO		
	1	2	3	1	2	3	1	2	3	1	2	3
11a	69	40		82			59	27	59		38	36
12b	83	24	61	75	64		32	3	22	54	16	49
11e	51	14	x	51	42	76	39	7	x	64	24	59
11h	87		x				72		x	76	55	79
11i	47	28	x	50	55		39	11	x	65	22	59
11k		125	x						x		69	
19o		134	x					185	x		81	

Table 4.4: The effect of ES-62 SMAs on the production of TNF-α following BLP- or CpG-mediated activation of bmDCs derived from WT and TLR4 KO C57-BL/6 mice

The percentage of cytokine produced compared to the BLP or CpG control following SMA pre-treatment is shown for TNF-α. Only percentages from experiments where the SMAs induced a significant difference are shown where red $p \leq 0.001$; purple $p \leq 0.01$ and blue $p \leq 0.05$. X signifies no results for that replicate and ND (not detected) is used in cases where SMA pre-treated resulted in cytokine levels below the detection levels of the ELISA kit (≥ 15 pg/ml).

SMA	BLP						CpG					
	WT			TLR4 KO			WT			TLR4 KO		
	1	2	3	1	2	3	1	2	3	1	2	3
11a	37	ND	53		36	61	22	ND	12	60	8	65
12b	73	ND	63		48	77	19	ND	12	33	2	44
11e	31	ND	x	23	23		26	ND	x	37	4	47
11h		67	x		76		83	39	x	69	48	79
11i	28	ND	x	23	35		33	ND	x	46	5	52
11k			x					155	x			
19o		141	x					249	x			

Table 4.5: The effect of ES-62 SMAs on the production of IL-12p70 following CpG-mediated activation of bmDCs derived from WT and TLR4 KO C57-BL/6 mice

The percentage of cytokine produced compared to the BLP control following SMA pre-treatment is shown for cytokine IL-12p70 in the two experiments where IL-12p70 was detected. Only percentages from experiments where the SMAs induced a significant difference are shown where red $p \leq 0.001$. X signifies no results for that replicate and ND (not detected) is used in cases where SMA pre-treated resulted in cytokine level below the detection levels of the ELISA kit (≥ 15 pg/ml).

SMA	IL-12p70			
	WT		TLR4 KO	
	1	2	1	2
11a	22	35	ND	ND
12b	ND	8	ND	ND
11e	ND	x	ND	ND
11h	ND	x	ND	ND
11i	54	x	13	ND
11k		x	69	15
19o	148	x		31

4.4 The Effect of ES-62 SMAs on the PAMP-induced cytokine responses of bmDCs from MyD88 knock-out C57BL/6 mice

Downstream TLR signalling after binding of cognate ligand occurs by recruiting a TIR domain-containing adaptor protein. MyD88 is a master TIR adaptor protein used by all TLRs, except TLR3, to initiate the pathway that results in activation of NF-κB and the production of pro-inflammatory cytokines [19]. As many of the immunomodulatory effects of ES-62 are a result of the post-translational addition of PC, a common PAMP, it was theorised that ES-62 could be recognised by one of the TLRs and so the effects of the helminth molecule were examined in cells deficient for MyD88 [246]. Although ES-62 inhibits pro-inflammatory cytokine production after LPS stimulation, treatment of macrophages and DCs with ES-62 alone causes transient, low levels of these cytokines to be released [226], [233]. It was found that in bmDCs grown from MyD88 deficient mice this low level induction of cytokines by ES-62 was abolished, indicating that ES-62 signalled in a MyD88-dependent manner [310]. Since then it has been revealed that ES-62 down-regulates MyD88 expression in macrophages [261], mast cells [247], T_H17 cells during CIA [211] and in B cells and kidney cells in MRL/Lpr mice [311]. It has been demonstrated that SMA 11a is also protective in murine models of arthritis and that it shares ES-62's mechanism of action by down-regulating MyD88 in macrophages in mice with CIA [260].

As it has been found that the SMAs selected from the original screen do not require TLR4 to mediate their inhibitory effects on BLP- and CpG-induced cytokines (Figures 4.5, 4.6 and 4.7) it was next investigated whether they, like ES-62, required MyD88 for inhibition of cytokine production by bmDCs. TLR4 signalling resulting from exposure to LPS utilises the MyD88-dependent and independent pathways to produce cytokines and so bmDCs grown from MyD88 KO and their matching WT mice were pre-treated with SMAs for 18 hours and stimulated with LPS for 24 hours and the levels of cytokines analysed by ELISA. As both pathways are required for strong activation of NF-κB the levels of cytokines produced by the MyD88 KO bmDCs following LPS activation are low but for all cytokines except IL-12 there is a significant upregulation of cytokine after stimulation (Figure 4.8).

4.4.1 IL-6

IL-6 production was analysed in WT and MyD88 KO bmDCs following stimulation with LPS (100 ng/ml) for 24 hours (Figure 4.8A and Table 4.6). In all experiments (n = 3) pre-treatment with 11a, 12b, 11e and 11i significantly inhibited IL-6 production after LPS stimulation in WT bmDCs. SMAs 11h, 11k and 19o significantly inhibited IL-6 in one experiment and caused a slight reduction in IL-6 in the other two. In the MyD88 KO bmDCs 11a, 12b, 11e, 11h, 11i and 11k inhibited IL-6 release after activation with LPS in all experiments and this was significant in 2/3 for 11a, 12b, 11e and 11i and 1/3 for 11h and 11k. SMA 19o significantly inhibited IL-6 secretion in one WT experiment but had no other significant effects on IL-6 production in WT or MyD88 KO bmDCs (Figure 4.8A). Overall, these results suggest that the SMAs, unlike ES-62 [310], can also target the MyD88-independent signalling pathway to inhibit IL-6 production in bmDCs.

4.4.2 TNF-α

The effects of the SMAs on LPS-induced TNF-α secretion were investigated in WT and MyD88 KO bmDCs (Table 4.6). Analysis of TNF-α levels after LPS stimulation of WT and MyD88 KO bmDCs showed that although 11a and 12b inhibited TNF-α production in WT experiments (2/3) they had no significant effects on TNF-α production in MyD88 KO experiments (Figure 4.8C). SMA 11i also significantly inhibited LPS-induced TNF-α in WT bmDCs (2/3 experiments) but only significantly reduced TNF-α in 1/3 MyD88 KO experiments suggesting that it too may require MyD88 for inhibition of this cytokine. SMAs 11e and 11h, surprisingly, only significantly inhibited LPS-induced TNF-α in 1/3 experiments in WT, and had a similar effect in MyD88 KO samples. SMAs 11k and 19o also decreased TNF-α in 1/3 experiments in WT cells but 11k significantly increased TNF-α in one KO experiment and 19o had no effect in the KO samples. These results make it difficult to ascertain whether 11e, 11h, 11k and 19o require MyD88 (Figure 4.8C).

4.4.3 IL-12

Levels of IL-12p70 released from bmDCs grown from WT and MyD88 KO mice after SMA pre-treatment for 18 hours and LPS stimulation for 24 hours were determined by ELISA (Table 4.6). Bioactive IL-12p70 was below the limit of detection for all but one WT experiment and all MyD88 KO experiments when investigating effects of SMAs 11e, 11h, 11i, 11k and 19o and so it could not be determined whether these SMAs require MyD88 for IL-12p70 inhibition. However, in an experiment undertaken with 11a

and 12b LPS-induced IL-12p70 was detected in WT and KO samples and was significantly inhibited in both by the two SMAs (Figure 4.8B). As alluded to earlier, it can been seen in the experiment that the amount of IL-12p70 produced in the KO sample is very low and that there is no significant difference between RPMI only and LPS stimulation suggesting that the cells produce a basal level of IL-12p70 of about 100 pg/ml and that the SMAs can target this production and that they do not require MyD88 to do this (Figure 4.8B).

Figure 4.8: The effect of ES-62 SMAs on LPS-induced IL-6, IL-12p70 and TNF-α secretion from bmDCs derived from WT and MyD88 KO mice

BmDCs were grown from MyD88 KO and age and sex matched WT C57-BL/6 mouse bone marrow for 8 days in the presence of GM-CSF and then plated at $2x10^5$ cells/well and pre-treated with 5 μg/ml 11a, 12b, 11e, 11h, 11i, 11k or 19o for 18 hours before stimulation with LPS (100 ng/ml) for 24 hours and the levels of IL-6, IL-12p70 and IL-12p70 determined by ELISA. The cytokine detection limit was ≥ 15 pg/ml according to the manufacturer's instructions. Results are expressed as mean (of triplicate determinations) ± SD and data were analysed using one-way ANOVA with Bonferroni post-test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Panel A shows the effect of SMAs on IL-6 production in WT and MyD88 KO bmDC. Data are representative of 3/3 experiments for SMAs 11a, 12b, 11e and 11i and 2/3 for SMAs 11h, 11k and 19o for WT bmDCs and 3/3 for 19o and 2/3 for the other SMAs in MyD88 KO bmDCs.

Panel B shows the effects of the SMAs on TNF-α production in WT and MyD88 KO bmDCs following LPS stimulation. Data are representative of 2/3 experiments for all SMAs for WT and 3/3 experiments for 11a, 12b and 19o and 2/3 for 11e, 11i, 11h and 11k treatment of MyD88 KO bmDCs.

Panel C shows the effects of SMAs 11a and 12b on IL-12p70 production in WT and MyD88 KO bmDCs following LPS stimulation. Data are from a single experiment.

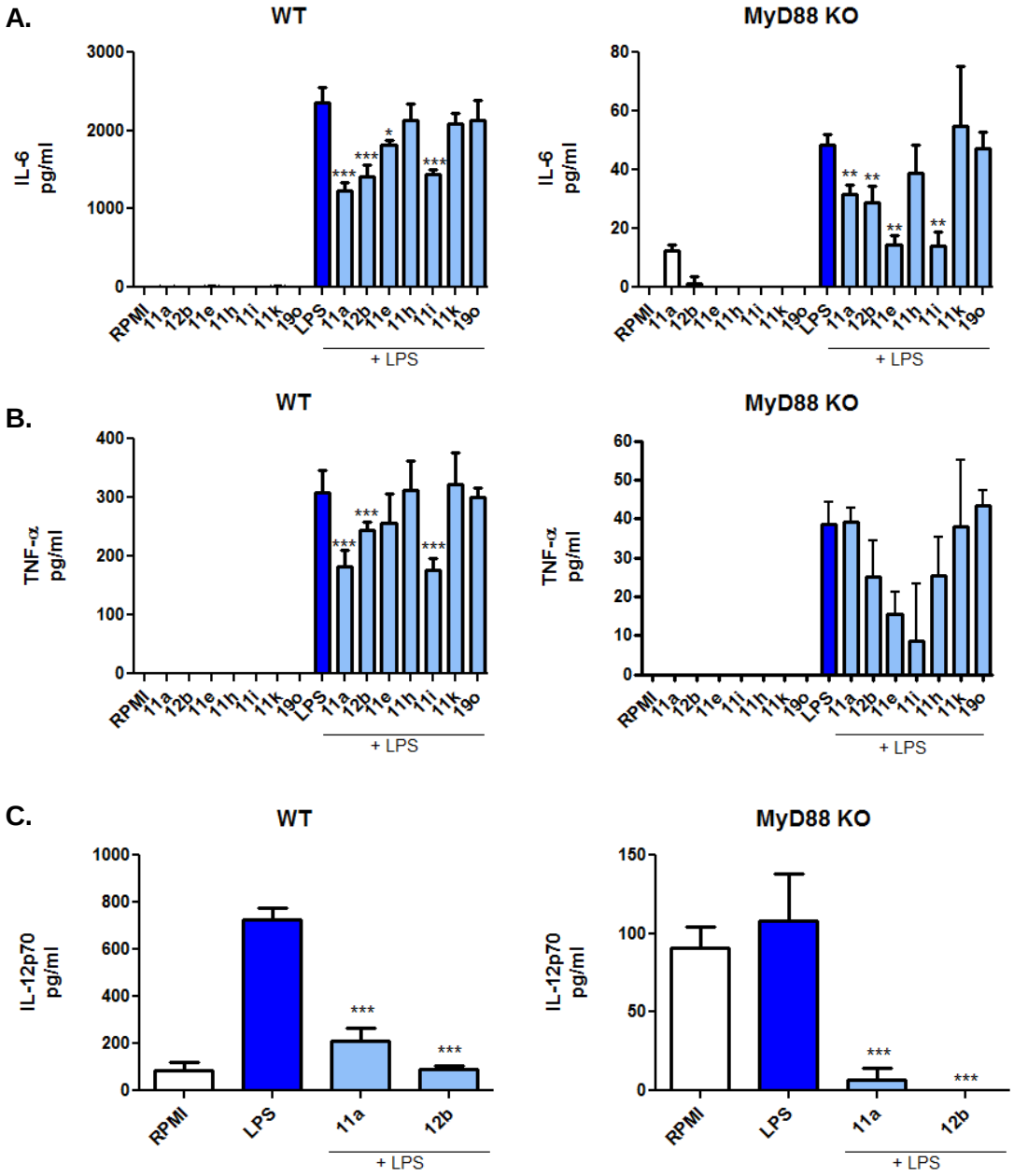

A.
WT
MyD88 KO
IL-6 pg/ml
3000
2000
1000
0
80
60
40
20
RPMI
11a
12b
11e
11h
11i
11k
19o
LPS
+ LPS
B.
TNF-α pg/ml
400
300
200
100
60
50
40
30
C.
IL-12p70 pg/ml
1000
800
600
400
150
100
50
***
**
*

Table 4.6: The effect of ES-62 SMAs on the production of IL-6 and TNF-α following LPS-mediated activation of bmDCs derived from WT and MyD88 KO C57-BL/6 mice

The percentage of cytokine produced compared to the BLP control following SMA pre-treatment is shown for cytokines IL-6, TNF-α and IL-12p70. As described in the text IL-12p70 was only detected on experiment with 11a and 12b. Only percentages from experiments where the SMAs induced a significant difference are shown where red $p \leq 0.001$; purple $p \leq 0.01$ and blue $p \leq 0.05$. X signifies no results for that replicate and ND (not detected) is used in cases where SMA pre-treated resulted in cytokine levels below the detection levels of the ELISA kit (≥ 15 pg/ml).

SMA	IL-6						TNF-α						IL-12p70	
	WT			MyD88 KO			WT			MyD88 KO			WT	KO
	1	2	3	1	2	3	1	2	3	1	2	3	1	2
11a	43	17	53	65		39	59	ND					22.61	ND
12b	52	25	60	59		38	79	6					36.98	ND
11e	77	20	77	30		18		ND				76	x	x
11h		57				34		40				77	x	x
11i	60	22	61	29		17	57	2				69	x	x
11k		67				42		53			153		x	x
19o		69						63					x	x

4.5 The effect of ES-62 SMAs on the expression of MyD88 in bmDCs

One of the key mechanisms of action of ES-62 is the down-regulation of MyD88 expression; this has been demonstrated in multiple cell types including macrophages [261] and mast cells [247]. Interestingly, in DCs ES-62 inhibits the expression of MyD88 but only in the presence of GM-CSF suggesting that ES-62 can sense inflammatory signals in its environment and act to limit on-going inflammation [312]. As SMAs 11a and 12b have also been shown to downregulate MyD88 expression in macrophages [260], [261] and SMA-mediated inhibition of IL-6 and IL-12p70 is intact in MyD88-deficient DCs (Figure 4.8) it was next investigated whether the SMAs modulated the expression of MyD88 in bmDCs.

As SMAs 11h and 11k did not demonstrate consistent effects on bmDC cytokine production in the TLR4 KO and MyD88 KO studies and had no effect on the LPS-induced increase in pro-inflammatory cytokine mRNA they were not selected for further investigation into the mechanisms of SMA action on bmDCs.

BmDCs were treated with either SMA 11a, 12b, 11e, 11i or 19o (5 μg/ml) alone for 1, 4 or 18 hours or with SMAs for these times before stimulation with LPS (100 ng/ml) for 30 mins in the presence or absence of GM-CSF, and the expression of MyD88 was determined by Fast Activated Cell-based ELISA (FACE) assay.

As with ES-62 the SMAs had no effect on the expression levels of MyD88 with or without LPS stimulation at any of the time points in the absence of GM-CSF (Figure 4.9A and C). Perhaps surprisingly however the SMAs also had no significant effects on the expression of MyD88 at any time point in the presence of GM-CSF either alone or with LPS stimulation (Figure 4.9B and D).

Figure 4.9: The effect of ES-62 SMAs on the expression of MyD88

BmDCs were grown in RPMI complete medium containing 10ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated in triplicate at 1 x 10^5 cells/well in 96-well plates that had previously been coated with Poly-l-Lysine, rested overnight and stimulated with SMAs 11a, 12b, 11e, 11i or 19o (5 µg/ml) for 1, 4 or 18 hours before incubation in RPMI only or RPMI containing LPS (100 ng/ml) for 30 minutes. The medium was then discarded and the levels of MyD88 were determined using Fast-activated cell-based ELISA (FACE). Cells number in each well was validated using crystal violet staining (not shown). Data are expressed as mean ± SD of triplicate determinations and were analysed using one way ANOVA with Bonferroni post-test. Data are from a single experiment, representative of three independent experiments.

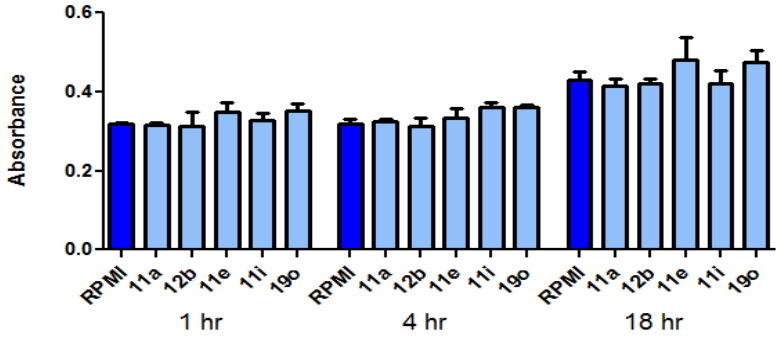
Panel A shows the effects of the SMAs on MyD88 expression levels in the absence of GM-CSF

Panel B shows the effects of the SMAs on MyD88 expression levels in the presence of GM-CSF

Panel C shows the effects of SMA pre-treatment and LPS stimulation on MyD88 expression levels in the absence of GM-CSF

Panel D shows the effect of SMA pre-treatment before LPS stimulation on MyD88 expression levels in the presence of GM-CSF

A.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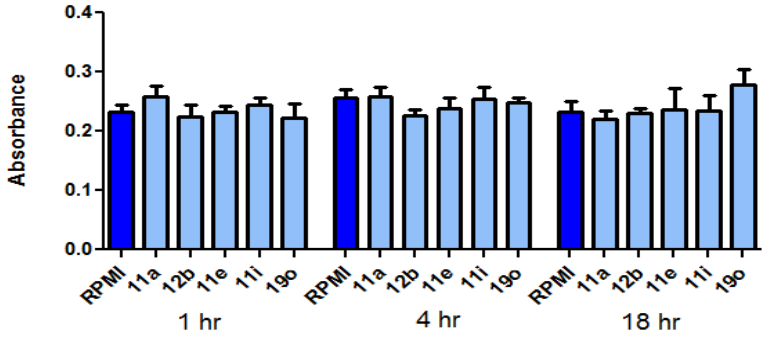
12b
11e
11i
19o
1 hr
4 hr
18 hr

B.


Absorbance
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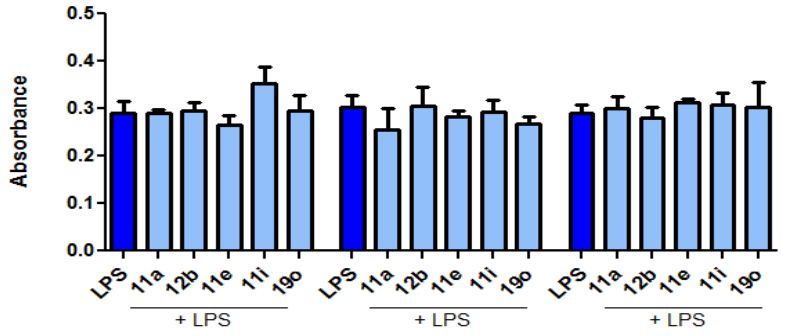
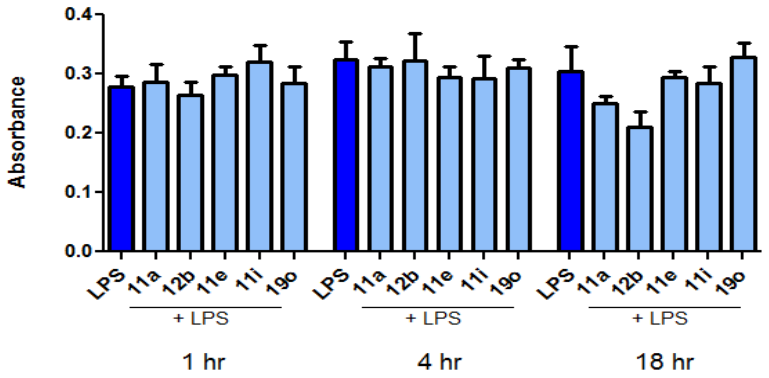
0.2
0.1
0.0
RPMI
11a
12b
11e
11i
19o
1 hr
4 hr
18 hr

C.


Absorbance
0.5
0.4
0.3
0.2
0.1
0.0
LPS
11a
12b
11e
11i
19o
+ LPS
1 hr
4 hr
18 hr

D.


Absorbance
0.4
0.3
0.2
0.1
0.0
LPS
11a
12b
11e
11i
19o
+ LPS
1 hr
4 hr
18 hr

4.6 The effect of ES-62 SMAs on the activation of NF-κB transcription factor

TLR signalling strongly induces the activation of mitogen-activated protein kinases (MAPKs) and the NF-κB transcription factor family of proteins. NF-κB proteins exist as dimeric transcription factors that regulate a diverse range of biological processes including innate and adaptive immune response, inflammation and stress responses. As described in chapter 1 there are five NF-κB subunits – RelA (p65), RelB, c-Rel, p50 and p52 and they can be differentially activated via one of three pathways to mediate the appropriate immune response. TLR signalling activates the canonical pathway which results in the activation of NF-κB/Rel proteins through an IKK complex and leads to the production of pro-inflammatory cytokines such as IL-6, TNF-α and IL-12 as well as anti-viral IFN-β [44]. Many of the stimuli that promote DC maturation also induce activation of NF-κB indicating that these proteins play a key role in this process. The use of the serine protease inhibitor TPCK, which inhibits Rel/NF-κB proteins translocating to the nucleus by blocking IκBα degradation, demonstrated that NF-κB is required for DC maturation as it blocked LPS-induced up-regulation of MHC II and costimulatory molecules in a dose dependent manner [313]. Furthermore, gene silencing of RelB in DCs resulted in immature DCs despite activation via CD40L [314] (Li *et al*, 2007). Most of the signalling pathways that activate NF-κB converge upon the IKK inhibitory complex [46]. Blocking the interaction of the regulatory protein NEMO with IKKβ via a peptide that binds the NEMO-binding domain (NBD) on IKK blocks the activation of NF-κB [315]. NF-κB inhibition by this peptide retains human monocytye-derived DCs in an immature state. This is characterised by reduced LPS-induced up-regulation of HLA-DR, CD83 and CD86 expression, inhibition of CD40L-induced IL-6, IL-12 and TNF-α production and a reduced ability to drive $CD4^+$ T cell proliferation [315]. These studies demonstrate that the activation of NF-κB plays a crucial role in the maturation and activation of DCs and so it is a viable target for the ES-62 SMAs to mediate their immunomodulatory effects. Indeed, previous work in our lab has demonstrated that phosphorylation of NF-κB protein p65 (RelA) induced via stimulation with LPS, BLP or CpG is inhibited by SMAs 11a and 12b in macrophages [260], [261]. It was therefore decided to investigate the effects of the ES-62 SMAs 11a, 12b, 11e, 11i and 19o on the levels of NF-κB protein p65 in the nucleus of bmDCs as an indication of NF-κB activation.

Treatment with SMAs 11a, 11e, 11i or 19o for 18 hours had no effect on the levels of p65 detected in the nucleus of bmDCs compared to unstimulated bmDCs, however

SMA 12b caused a slight reduction in two experiments undertaken (Table 4.7). BmDCs were also pre-treated with the SMAs for 18 hours and then stimulated with LPS for 30 minutes and the level of p65 in the nucleus was determined by ELISA. Pre-treatment with SMAs 11a, 12b or 11e inhibited the LPS-induced increase of p65 in the nucleus in two experiments undertaken (Figure 4.10). These results suggest that these SMAs are capable of modulating the activation of NF-κB p65 protein and so provide a potential mechanism by which they exert their inhibitory effects on pro-inflammatory cytokines IL-6, TNF-α and IL-12p70. SMA 19o, perhaps surprisingly due to its "negative control" status, also decreased the LPS-induced p65 level. SMA 11i was the only SMA to cause inconsistent effects on the p65 levels: in the first experiment pre-treatment with this SMA caused an increase in LPS-induced p65 but there was a decrease in the second experiment (Figure 4.10) and so these results fail to unequivocally indicate whether SMA 11i modulates NF-κB p65 protein to mediate its effects on cytokine release.

Figure 4.10: The effect of ES-62 SMAs on NF-κB p65 activation

BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at $2x10^6$ cells in 2ml in 6 well plates, rested overnight and then treated with SMAs 11a, 12b, 11e, 11i or 19o (5 μg/ml) for 18 hours. The cells were then either harvested at this point or stimulated with LPS (100 ng/ml) for 30 minutes before being harvested. The proteins in the nucleus of the cells were then extracted and the level of NF-κB p65 protein in each sample was determined by TransAM ELISA.

Panel A shows the effects of the SMAs on the expression levels of p65 in the nucleus of bmDCs. Data are representative of 2/2 experiments for SMAs 11a, 12b, 11e and 19o and 1/2 experiments for 11i with inconsistencies described in the text.

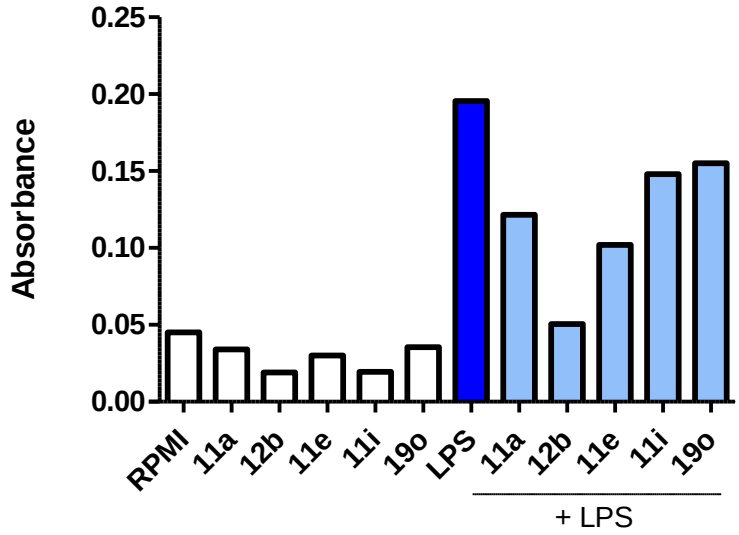

Absorbance
0.25
0.20
0.15
0.10
0.05
0.00
RPMI
11a
12b
11e
11i
19o
LPS
11a
12b
11e
11i
19o
+ LPS

Table 4.7: The effect of ES-62 SMAs on NF-κB p65 activation

The mean (of duplicate determinations) percentage reduction of NF-κBp65 in the nucleus of bmDCs after treatment with SMA compared to RPMI control or pre-treatment with SMA and LPS stimulation compared to LPS alone is shown for each experiment along with the mean of the two experiments. The basal activity level (RPMI) in each experiment was reduced from the LPS and SMA + LPS samples before calculation of each percentage.

SMA	NF-κBp65			SMA + LPS	NF-κBp65		
	1	2	Mean		1	2	Mean
11a	9	24	17	11a	15	49	32
12b	47	58	52	12b	57	96	77
11e	12	33	22	11e	100	62	81
11i	23	57	40	11i	-22	32	5
19o	28	21	25	19o	23	27	25

4.7 The effect of the ES-62 SMAs on the activation of MAPKs

Mitogen-activated protein kinases (MAPK) are evolutionarily conserved signal transduction molecules that regulate multiple cellular processes including proliferation, metabolism, differentiation and apoptosis. There are three main families of classic MAPKs found in mammalian species: the extracellular signal-regulated protein kinases (ERK), the p38 MAP kinases and the c-Jun NH_2-terminal kinases (JNK). MAPKs are activated via a three-tiered kinase phosphorylation cascade. All MAPKs contain a core Thr-X-Tyr activation motif within the activation loop that is essential for their activation. They are activated by dual phosphorylation of Thr and Tyr on the activation loop by MAPK kinases, which are activated by phosphorylation of their Ser/Thr residues by MAPK kinase kinases (reviewed in [57]). This has been extensively studied and reviewed and is discussed in more detail in chapter 1. MAPKs also play a key role in regulating innate and adaptive immune responses [64]. Several studies using chemical inhibitors of p38 or genetic disruption of one of its specific activators, MKK3, have demonstrated the role of p38 MAPK in innate immune responses. Macrophages and dendritic cells from $MKK3^{-/-}$ mice produce less IL-12 in response to LPS or ligation of CD40L compared to WT cells, and naïve $CD4^+$ cells from these mice have an impaired ability to produce IFN-γ suggesting that the p38 MAPK signalling pathway is involved in the production of both IL-12 and IFN-γ [316]. Interestingly in this study the lack of MKK3 did not alter the production of IL-6 and TNF-α or their mRNA levels in macrophages [316] suggesting pro-inflammatory cytokines are differentially regulated by p38 MAPK. Studies using the p38 MAPK inhibitor SD203580 in human monocyte-derived dendritic cells (MoDCs) demonstrated that p38 positively regulates the phenotypic maturation of MoDCs as SD203580 inhibited LPS- and CD40L-induced up-regulation of co-stimulatory molecules CD40, CD80, CD83, CD86 and HLA-DR in these cells [317]–[320]. Treatment with this inhibitor also blocked each of LPS-, flagellin-, TNF-α-, $NiCl_2$- and CD40L-stimulated IL-12p40 production [316], [318]–[321]. Inhibition of JNK1, JNK2 and JNK3 by the chemical inhibitor SP600125 also resulted in a slight inhibition of LPS-induced co-stimulatory molecule upregulation suggesting JNK MAPK works with p38 MAPK to positively regulate DC phenotypic maturation [319]. These studies also revealed that JNK MAPKs play a role in the LPS-induced production of TNF-α and IL-12p70 but not IL-6 and IL-12p40. By comparison, the use of a MEK1 (a specific MAPK kinase of ERK MAPK) inhibitor to block the ERK signalling pathway did not affect the up-regulation of co-stimulatory molecules and in some cases caused a slight increase in these surface molecules suggesting the ERK MAPK

pathway could negatively regulate expression of these molecules [320], [322]. Pro-inflammatory cytokines also appear to be differentially regulated by ERK1/2 as inhibition of the pathway with the chemical inhibitor PB98059 causes an increase in IL-12p40 protein and mRNA [124], [322] but decreases $NiCl_2$-induced TNF-α [318].

It has been shown that ES-62 targets MAPKS to differentially regulate production of IL-12p40 and IL-12p35. It achieves this by suppressing the activation of p38 and JNK by LPS required for p35 (and IL-6 and TNF-α) production, while also augmenting the LPS-induced, calcium-dependent activation of ERK MAPK which negatively regulates p40 production in macrophages [124], [310]. To date, however, the effect of ES-62 on MAPKs in dendritic cells has not been established. As MAPKs play such an important role in regulating cytokine responses in dendritic cells and ES-62 has been shown to be able to affect these kinases in macrophages it was next investigated whether the ES-62 SMAs were able to modulate the activity of bmDC ERK, p38 and JNK MAPKs in order to exert their immunomodulatory effects on cytokine production and co-stimulatory expression. BmDCs were plated at 1×10^5 cells/well in triplicate in 96 well plates that had previously been coated with Poly-l-lysine, rested overnight and treated with SMAs 11a, 12b, 11e, 11i or 19o (5 µg/ml) for 18 hours before stimulation with LPS (100 ng/ml) for 10 minutes. The medium was then discarded and the expression levels of MAPKs ERK 1/2 and phospho-ERK 1/2; p38 and phospho-p38, and SAPK/JNK and phospho-SAPK-/JNK were determined by Fast Activated Cell-based ELISA (FACE). The expression levels of each phosphorylated MAPK were normalised to the total non-activated form.

4.7.1 The effect of ES-62 SMAs on ERK MAPK

Treatment with the SMAs 11a, 12b, 11e, 11i or 19o for 18 hours had no significant effects on the expression levels of dual phosphorylated ERK1/2 (p-ERK1/2) over three experiments undertaken compared to unexposed cells (Figure 4.11) although some of the SMAs did cause slight, non-significant reductions in p-ERK1/2 levels (Table 4.8). Upon stimulation with LPS for 10 minutes there was a significant increase in activated ERK1/2 in all experiments. Pre-treatment with SMAs 12b, 11e or 11i caused at least an average 45% decrease over the three experiments in the level of activated ERK following LPS stimulation; however this was only significant for 12b, 11e and 11i in one experiment (Figure 4.11A and B, and Table 4.8). Consistent with this trend, SMA 11a caused an average 40% reduction in ERK activation over the three experiments but this was not significant in any experiments. SMA 19o had no effect on the expression levels of activated ERK1/2 (Figure 4.11B). Table 4.8 shows the percentage reduction of levels of activated ERK in SMA + LPS treated samples compared to LPS only treated samples over the three independent experiments. These results raise the possibility that SMAs 12b, 11e and 11i may target the ERK MAPK signalling pathway to mediate their effects.

4.7.2 The effect of ES-62 SMAs on p38 MAPKs

Similar to ERK1/2 MAPK, SMA treatment alone had no significant effects on the levels of activated p38 MAPK. SMAs 11a and 11e did cause a slight reduction in activated p38 levels compared to unstimulated cells in 2/3 experiments (Table 4.9). SMA 11i caused a 30% reduction in 2 experiments but confusingly, a 50% increase in p-p38 levels in the third experiment. The inhibition percentages for each SMA are shown in Table 4.9. LPS stimulation caused a significant increase in activated p38 expression in all experiments. Pre-treatment with SMAs 11a, 12b, 11e or 11i caused at least 50% average reduction in this LPS-induced p38 activation over the three experiments and this was significant for SMA 12b in 2/3 experiments and SMAs 11a, 11e and 11i in 1/3 experiments (Figure 4.12A+B). SMA 19o also caused a slight reduction in p-p38 expression following LPS stimulation in all experiments but this was not significant (Figure 4.12B). These results suggest that SMAs 11a, 12b, 11e and 11i could also target the activation of p38 MAPK in order to mediate their immunomodulatory effects on bmDCs.

4.7.3 The effect of ES-62 SMAs on JNK MAPKs

There were no significant effects on activated JNK expression levels in bmDCs after treatment with SMAs 11a, 12b, 11e, 11i or 19o for 18 hours (Figure 4.13A). There was only a significant increase in activated JNK expression after LPS stimulation in one of three experiments and in this experiment SMA 11e pre-treatment significantly reduced this increase. SMAs 11a, 12b and 11i also caused a slight reduction in P-JNK expression, although this was not significant (Figure 4.13A). The percentage inhibition of the SMAs compared to the RPMI control is shown in Table 4.10. As there was only a significant increase in activation of p-JNK in 1/3 experiments the percentage inhibition for SMA+LPS is not shown. As a result of the lack of LPS-induced JNK activation it is not possible to draw any concrete conclusions on the effect of the SMAs on the JNK MAPK cascade.

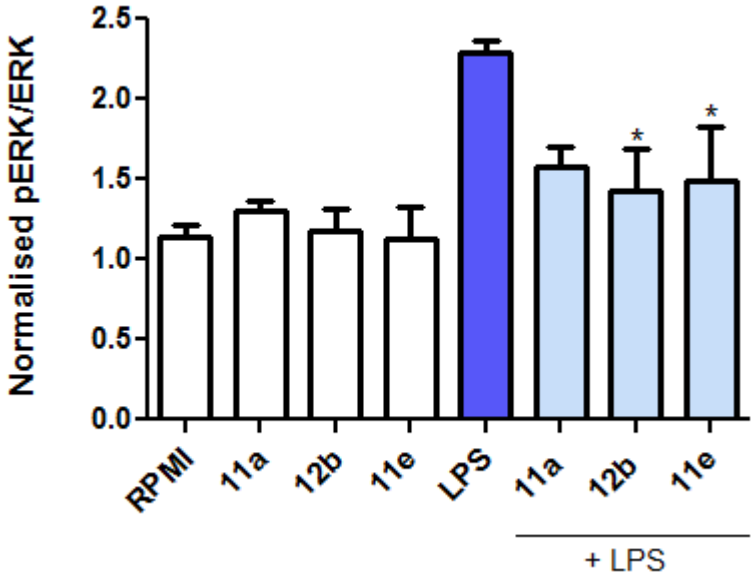
Figure 4.11: The effect of ES-62 SMAs on the activation of ERK1/2 MAPK

BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated in triplicate at 1 x 10^5 cells/well in 96-well plates that had previously been coated with Poly-l-Lysine, rested overnight and stimulated with SMAs 11a, 12b, 11e, 11i or 19o (5 µg/ml) for 18 hours before stimulation with RPMI only or RPMI containing LPS (100 ng/ml) for 10 minutes. The medium was then discarded and the expression levels of total ERK1/2 and dual phosphorylated ERK1/2 determined using Fast Activated Cell-based ELISA (FACE). Phosphorylated ERK1/2 absorbance was normalised to the corresponding total ERK1/2 absorbance and the data expressed as mean (of triplicate determinations) ± SD. Data were analysed using one way ANOVA with Bonferroni post-test where * $p < 0.05$.

Panel A shows the effects of SMAs 11a, 12b and 11e on the levels of activated ERK1/2 MAPK. For SMA only analysis, data are representative of 3/3 experiments for all SMAs. In experiments where bmDCs were stimulated with LPS after SMA pre-treatment, data are representative of 3 experiments for 11a, and for 12b and 11e, data are representative of 3 experiments in which inhibition reached significance in 1/3 experiments.

Panel B shows the effects of SMAs 11i and 19o on the levels of activated ERK 1/2 MAPK. For SMA only analysis, data are representative of 3/3 experiments for all SMAs. In experiments where bmDCs were stimulated with LPS after SMA pre-treatment, data are representative of 3 experiments for SMA 19o and 3 experiments in which inhibition reached significance in 1/3 experiments for SMA 11i.

A.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2.0
1.5
1.0
0.5
0.0
*
*
RPMI
11a
12b
11e
LPS
11a
12b
11e
+ LPS

B.

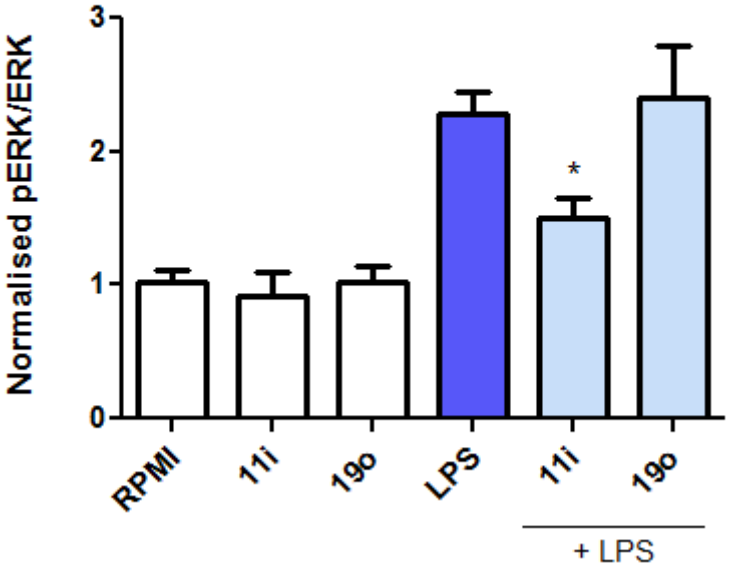

Normalised pERK/ERK
3
2
1
0
*
RPMI
11i
19o
LPS
11i
19o
+ LPS

Table 4.8: The effect of ES-62 SMAs on the activation of ERK1/2 MAPK

The percentage reduction in levels of activated ERK in SMA treated-bmDCs compared to RPMI (SMA only) or LPS (SMA + LPS) control bmDCs for each experiment is shown, along with the Mean ± SEM over the three experiments. The basal activity level (RPMI) of each experiment was reduced from the LPS and SMA+LPS samples before calculation of each percentage. Statistically significant results are depicted in colour: blue $p < 0.05$.

SMA only	pERK normalised to ERK					SMA + LPS	pERK normalised to ERK				
	1	2	3	Mean	± SEM		1	2	3	Mean	± SEM
11a	3	-20	3	-4	8	11a	37	59	24	40	10
12b	1	-7	16	3	10	12b	62	71	47	60	7
11e	6	3	22	10	6	11e	45	66	51	54	7
11i	-4	-7	15	1	11	11i	64	41	29	45	10
19o	-12	-11	3	-7	7	19o	-9	14	-14	-3	9

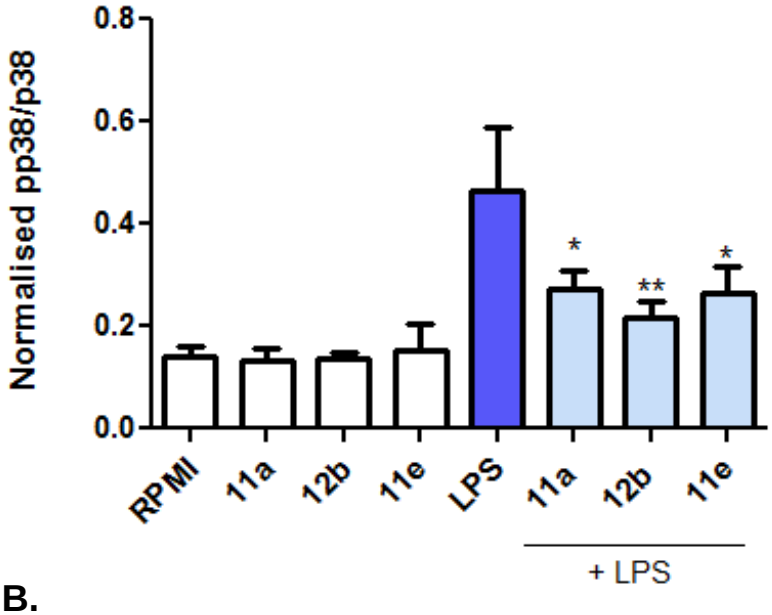
Figure 4.12: The effect of ES-62 SMAs on the activation of p38 MAPK

BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated in triplicate at 1 x 10^5 cells/well in 96-well plates that had previously been coated with Poly-l-Lysine, rested overnight and stimulated with SMAs 11a, 12b, 11e, 11i or 19o (5 µg/ml) for 18 hours before incubation with RPMI only or RPMI containing LPS (100 ng/ml) for 10 minutes. The medium was then discarded and the expression levels of total and phosphorylated p38 were determined using Fast Activated Cell-based ELISA (FACE). Phosphorylated p38 absorbance was normalised to the corresponding total p38 absorbance and the data expressed as mean (of triplicate determinations) ± SD. Data were analysed using one way ANOVA with Bonferroni post-test where * $p < 0.05$, ** $p < 0.01$

Panel A shows the effects of SMAs 11a, 12b and 11e on the levels of activated p38 MAPK. For SMA only analysis, data are representative of 3/3 experiments for all SMAs. In experiments where bmDCs were stimulated with LPS after SMA pre-treatment, data are representative of 2/3 experiments for SMA 12b and 3 experiments in which inhibition reached significance in 1/3 experiments for SMAs 11a and 11e.

Panel B shows the effects of SMAs 11i and 19o on the levels of activated p38 MAPK. For SMA only danalysis, data are representative of 3/3 experiments for all SMAs. In experiments where bmDCs were stimulated with LPS after SMA pre-treatment, data are representative of 3 experiments for SMA 19o and 3 experiments in which inhibition reached significance in 1/3 experiments for SMA 11i.

A.


Normalised pp38/p38
0.8
0.6
0.4
0.2
0.0
*
**
*
RPMI
11a
12b
11e
LPS
11a
12b
11e
+ LPS

B.

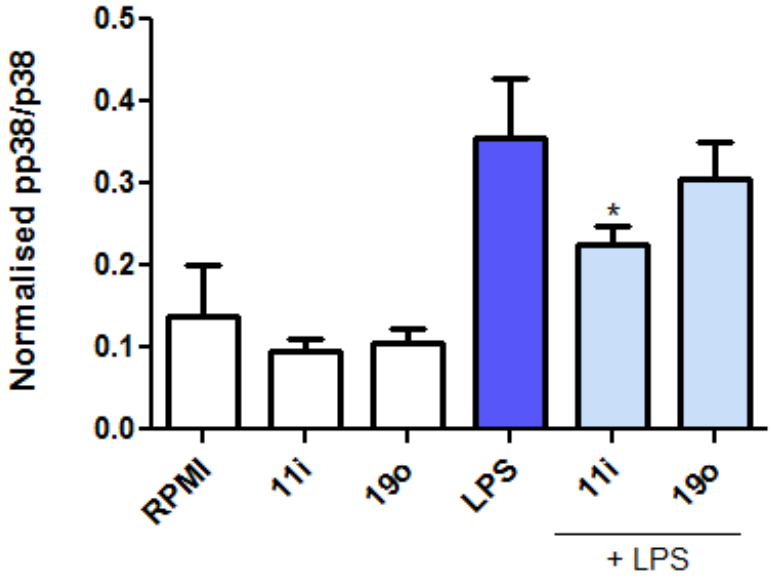

Normalised pp38/p38
0.5
0.4
0.3
0.2
0.1
0.0
*
RPMI
11i
19o
LPS
11i
19o
+ LPS

Table 4.9: The effect of ES-62 SMAs on the activation of p38 MAPK

The percentage reduction in levels of activated p38 in SMA treated-bmDCs compared to RPMI (SMA only) or LPS (SMA + LPS) control bmDCs for each experiment is shown, along with the Mean ± SEM over the three experiments. The basal activity level (RPMI) of each experiment was reduced from the LPS and SMA+LPS samples before calculation of each percentage. Statistically significant results are depicted in colour: blue $p < 0.05$; purple $p < 0.01$

SMA only	pp38 normalised to p38					SMA + LPS	pp38 normalised to p38				
	1	2	3	Mean	± SEM		1	2	3	Mean	± SEM
11a	23	27	4	18	12	11a	57	54	58	57	1
12b	-46	34	3	-3	40	12b	76	79	76	77	1
11e	28	27	-7	16	20	11e	59	57	61	59	1
11i	31	32	-51	4	48	11i	60	38	52	50	7
19o	25	12	-30	2	29	19o	23	32	28	28	3

Figure 4.13: The effect of ES-62 SMAs on the activation of JNK MAPK

BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated in triplicate at 1 x 10^5 cells/well in 96-well plates that had previously been coated with Poly-l-Lysine, rested overnight and stimulated with SMAs 11a, 12b, 11e, 11i or 19o (5 µg/ml) for 18 hours before incubation with RPMI only or RPMI containing LPS (100 ng/ml) for 10 minutes. The medium was then discarded and the expression levels of total and phosphorylated JNK determined using Fast Activated Cell-based ELISA (FACE). Phosphorylated JNK absorbance was normalised to the corresponding total JNK absorbance and the data expressed as mean (of triplicate determinations) ± SD. Data were analysed using one way ANOVA with Bonferroni post-test where * $p < 0.05$

The effects of SMAs 11a, 12b, 11e, 11i and 19o on the levels of activated JNK MAPK are shown. Data are representative of 3/3 experiments for SMA only and a single experiment for SMA+LPS.

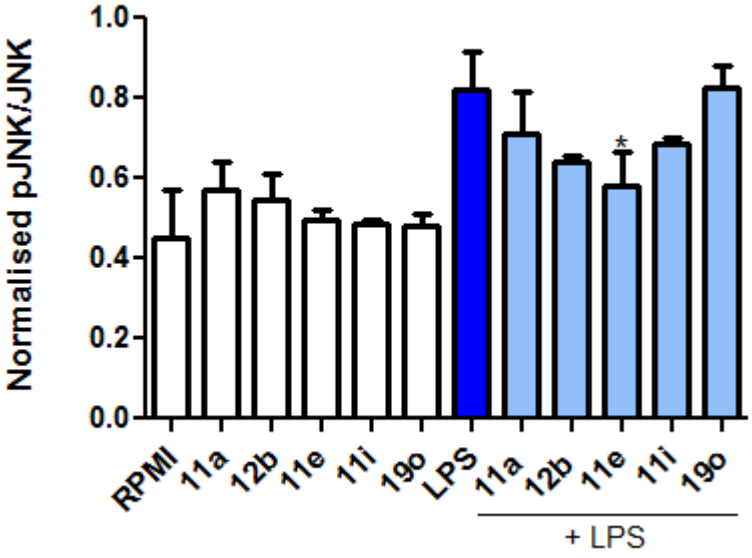

Normalised pJNK/JNK
1.0
0.8
0.6
0.4
0.2
0.0
*
RPMI
11a
12b
11e
11i
19o
LPS
11a
12b
11e
11i
19o
+ LPS

Table 4.10: The effect of ES-62 SMAs on the activation of JNK MAPK

The percentage reduction in levels of activated JNK in SMA treated-bmDCs compared to RPMI (SMA only) control bmDCs for each experiment is shown, along with the Mean ± SEM over the three experiments.

SMA only	pJNK normalised to JNK				
	1	2	3	Mean	± SEM
11a	-2	-8	14	2	7
12b	-4	-10	18	1	23
11e	2	7	15	8	11
11i	5	0	6	4	27
19o	8	19	8	11	16

Chapter 5. Investigation of the effects of ES-62 SMA-primed dendritic cells *in vivo*

Introduction

One of the quintessential features of chronic helminth infection is the induction of lymphocyte hyporesponsiveness, both to parasite-specific antigens and also to heterologous antigens (reviewed by [323]). This was first observed in the 1970s by Ottesen *et al*, who noted that lymphatic filariasis patients had a tendency not to respond to helminth antigen in immunoassays [324]. It appears that filarial parasites can utilise a wide range of mechanisms to induce lymphocyte hyporesponsiveness including induction of T regulatory cells, up-regulation of CTLA-4 on $CD4^+$ effector T cells [325], [326] and modulation of APCs [327]. In 1978 Weiss demonstrated that serum from filarial nematode-infected animals could induce lymphocyte hyporesponsiveness suggesting that the parasite secretes products during infection that can alter the immune response of the host [328]. ES-62 was discovered to reduce the proliferative response of lymphocytes, as splenic B cells and lymph node-derived mononuclear cells from mice exposed to the worm product via osmotic pumps to mimic natural infection displayed a reduced ability to respond *ex vivo* to BCR-driven stimulation [329]. Adoptive transfer experiments where T cells with a transgenic TCR specific for an immunodominant epitope of OVA are injected into recipient BALB/c mice in numbers large enough to trace *in vivo* but not interfere with physiological responses to antigen, demonstrated that ES-62 treatment *in vivo* reduced the proliferative capacity of these T cells to respond to *ex vivo* stimulation with OVA [241].

It has been demonstrated that helminth antigens, particularly helminth ES products, are capable of modulating DC maturation and function such that they generally promote a T_H2 or Treg response and can render the APC less able to respond to other infectious stimuli such as *Mycobacterium tuberculosis* or *Plasmodium falciparum* [185]. For example, DCs exposed to schistosome soluble egg antigen (SEA) retain an immature phenotype characterised by a lack of up-regulation of co-stimulatory molecules CD40, CD80 and CD86 but drive a T_H2 phenotype when injected *in vivo* [330].

ES-62 was the first helminth molecule to be found to prime DCs towards a T_H2 phenotype [243] when it was shown that cells matured with ES-62 promote IL-4 production by T cells and inhibit their IFN-γ response. The ES-62 SMAs also fail to induce classical activation of DCs as they do not stimulate them to produce pro-inflammatory cytokines IL-12p70, IL-6 or TNF-α (Figure 3.4, 3.5 and 3.6), and they do not promote the up-regulation of co-stimulatory molecules CD40, CD80 and CD86 (Figure 3.10). In addition. pre-treatment with the SMAs renders DCs refractory to

subsequent LPS-induced cytokine production and co-stimulatory molecule up-regulation and this is possibly mediated through suppressed activation of MAPKs and NF-κB. These results suggest that SMA-modulated DCs should be capable of impacting on T cell responses. This study will first investigate whether SMA-modulated DCs can alter T cell responses *in vitro* before utilising an adoptive transfer model to measure Ag-specific T cell responses in mice treated with SMA-modulated DCs.

In addition, modulation of DCs has been found to be therapeutic in several murine models of disease such as arthritis and colitis [331] [332] and is also a new line of therapy being developed for cancer treatment [333]. This study will also therefore determine the effect of SMA-modulated DCs on the progression and development of collagen-induced arthritis CIA in mice.

The specific aims of this study were thus:

- To investigate whether the SMAs can modulate the ability of LPS-stimulated DCs to activate T cell responses *in vitro*

- To investigate whether modulation of DCs *in vitro* with SMAs with or without LPS can influence the Ag-specific T cell response *in vivo*

- To investigate whether DCs treated with SMAs *in vitro* can alter the outcome of CIA disease *in vivo*

5.1 The effect of ES-62 SMAs on the ability of bmDCs to drive T_H1 polarisation

As the SMAs have been shown to inhibit the production of PAMP-induced cytokines and also the expression of co-stimulatory molecules it was next investigated whether they could alter the ability of bmDCs to stimulate cytokine production by naïve, Ag-specific $CD4^+CD62L^+$ T cells. BmDCs were incubated overnight with SMAs 11a, 12b, 11e or 11i before further maturation with medium or medium containing LPS and were then loaded with increasing concentration of ovalbumin (OVA) peptide for 3 hours before being co-cultured with naïve $CD4^+CD62L^+$ T cells expressing a TCR that is specific for the immunodominant epitope (323-339) of OVA for 72 hours. The supernatants were then collected and analysed for the presence of IFN-γ, IL-17 and IL-4. Overall the level of cytokine produced was very low and IL-17 and IL-4 were not detected in any experiments (results not shown).

The effects of SMAs 11a and 12b on the ability of bmDCs to prime T cell cytokine responses was investigated in three independent experiments. BmDCs were loaded with OVA peptide at medium concentrations of 10 μM, 100 μM and 300 μM. IFN-γ was not detected following OVA 10 μM in any experiments and was only detected in two when bmDCs were stimulated with OVA 100 μM. IFN-γ was detected in all three experiments at the highest peptide concentration although in the absence of LPS IFN-γ production was very low. Pre-exposure of bmDCs to SMA 11a significantly inhibited the LPS-induced IFN-γ response in all 3 experiments with an average inhibition percentage of 88.67% ± 19.62 (Mean ± SD). SMA 12b significantly inhibited the response in 2/3 experiments with an average inhibition percentage of 56.51% ± 42.27 over the three experiments (Figure 5.1A). These results suggest that both of these SMAs are able to modulate the ability of bmDCs to prime T_H1 cytokine responses from naïve $CD4^+CD62L^+$ T cells.

The effects of SMAs 11e and 11i on the ability of bmDCs to prime T cell cytokine responses was also investigated in three independent experiments. As in experiments with 11a and 12b, bmDCs were loaded with OVA peptide at 10 μM, 100 μM and 300 μM and again IFN-γ was not detected following OVA 10 μM in any experiments. At higher peptide concentrations IFN-γ was inconsistently detected and there was only a significant increase following LPS stimulation in 1/3 experiments. In this experiment pre-exposure to SMAs 11e and 11i significantly inhibited this response (Figure 5.1B). Due to the lack of significant LPS-induced increased IFN-γ from T cells primed by bmDCs in the other two experiments further repeat assays should be undertaken but

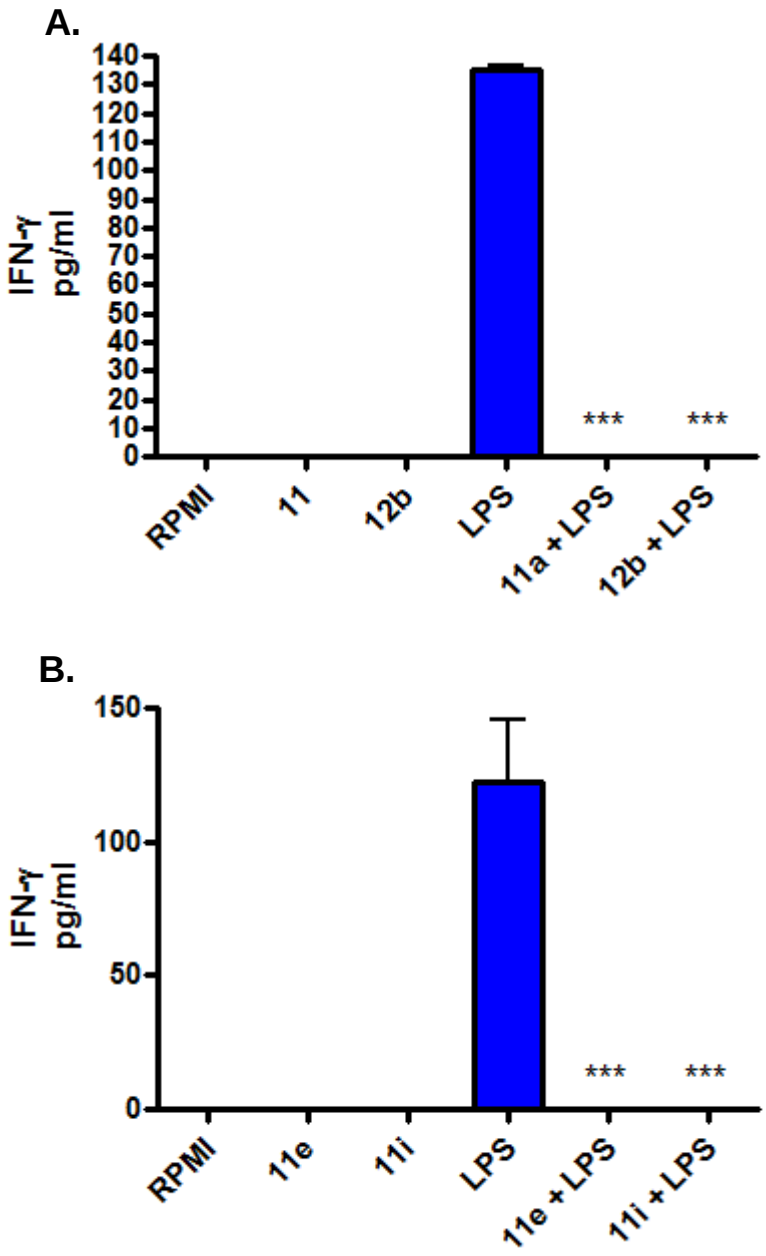
on average over the three experiments 11e caused a 54.43% ± 39.47 and 11i caused a 58.37% ± 37.81 decrease in cytokine production.

Figure 5.1: The effect of ES-62 SMAs on the ability of bmDCs to prime naive T cells to produce IFN-γ

BmDCs were grown in RPMI complete medium containing 10 ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. Cells were plated at 2.5 $x10^4$ cells in 24-well plates, rested overnight and then treated with SMAs (5 µg/ml) 11a, 12b, 11e or 11i for 18 hours before incubation with either medium or medium containing LPS (100 ng/ml) for 24 hours. Cells were then washed and primed with OVA peptide at 300 µM for 3 hours. The OVA peptide was then washed off and 2.5 x 10^5 naive, Ag-specific $CD4^+CD62L^+$ T cells obtained from D011.10 mice were added to the plate. BmDCs and T cells were incubated together for 72 hours and the level of IFN-γ in the supernatant measured by ELISA. The detection limit was ≥ 15 pg/ml according to the manufacturer's instructions. The data are expressed as mean (of triplicate determinations) ± SD and was analysed using one-way ANOVA with Bonferroni post-test where *** $p< 0.001$.

Panel A shows the effect of pre-exposure of bmDCs to SMAs 11a and 12b on the subsequent LPS-induced IFN-γ production by T cells. Data are from a single experiment representative of three independent experiments for 11a and are representative of 2/3 experiments for 12b.

Panel B shows the effects of pre-exposure of bmDCs to SMAs 11e or 11i on the subsequent LPS-induced IFN-γ production by T cells and the data are from a single experiment.


A.
IFN-γ pg/ml
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
***
***
RPMI
11
12b
LPS
11a + LPS
12b + LPS
B.
IFN-γ pg/ml
150
100
50
0
***
***
RPMI
11e
11i
LPS
11e + LPS
11i + LPS

5.2 Modulation of the immune response *in vivo* by ES-62 SMA-exposed DCs

It was demonstrated that DCs exposed to SMAs prior to LPS stimulation have lower expression of co-stimulatory molecules (Figure 3.10) and a reduced ability to prime T cells to produce IFN-γ (Figure 5.1). It was therefore decided to investigate the ability of DCs exposed *in vitro* to SMAs or SMAs + LPS to prime subsequent T cell responses *in vivo*. In order to investigate this, the adoptive transfer model used by Marshal *et al* [241] when investigating ES-62 was employed to determine the ability of SMA-matured bmDCs pulsed with OVA to prime T cell responses in draining lymph nodes, following the regimen shown in Figure 5.2. Recipient BALB/c mice were inoculated with $1x10^6$ OVA-specific TCR tg naive $CD4^+$ T cells from donor D011.10 BALB/c mice and 24 hours later they were inoculated subcutaneously with DCs that had been loaded with OVA peptide and incubated *in vitro* with RPMI, LPS, SMA11a, SMA11i, SMA11a+LPS or SMA11i+LPS. DCs that had not been loaded with OVA were used as a control. The draining popliteal lymph node was collected on 2, 3, 5 and 10 days post immunisation and the T cell responses were examined by flow cytometry with the gating strategy employed shown in Figure 5.3.

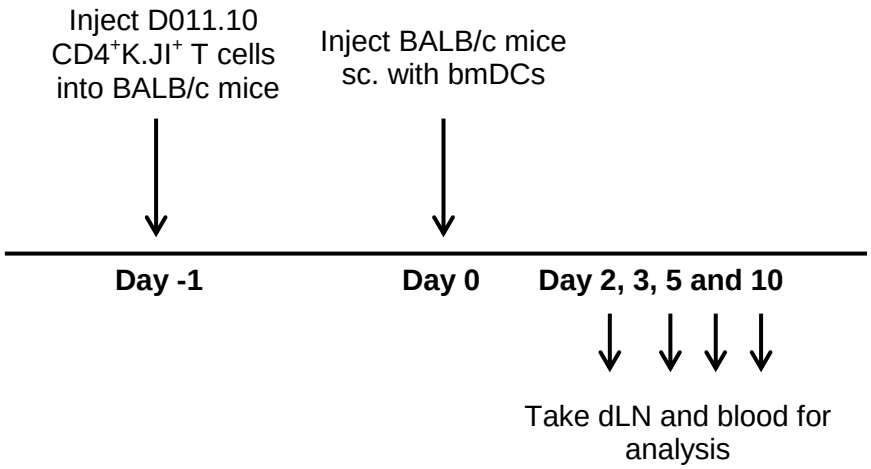


Figure 5.2: Experimental plan for immunising adoptively transferred recipients with in vitro SMA-stimulated DC

SMAs 11a, 12b, 11e and 11i have all been shown to mediate potent inhibitory effects on bmDCs *in vitro* and all four are good candidates for *in vivo* investigation. However, in order to minimise mouse numbers in this initial *in vivo* experiment only SMA 11a and 11i were used. SMA 11a was chosen as it has already been demonstrated to be active in a number of *in vivo* models [260]–[264] and pre-exposure

of bmDCs to this SMA consistently inhibited $CD4^+$ T cell IFN-γ responses *in vitro*. SMA 12b has also been shown to be active in multiple *in vivo* models [260]–[262]; however it was decided to examine the *in vivo* effects of one of the somewhat novel SMAs, which had been discovered to be "active' in the initial LPS screen. Towards this, SMA 11e has previously been tested prophylactically in the OVA-induced asthma model and was found not to display any inhibitory effects (Coates & Harnett, unpublished) and thus SMA 11i, which has not previously been investigated *in vivo*, was selected for this study.

5.2.1 Effect of ES-62 SMA maturation on the ability of DCs to expand antigen specific $CD4^+$ T cells

Efficient antigen presentation by DCs is required for the expansion of Ag-specific T cells and indeed inoculation of mice with OVA peptide-loaded DCs induced high levels of Ag-specific T cell expansion compared to control OVA peptide-free DCs at days 3 and 5 post immunisation, with peak expansion occurring on day 5 (Figure 5.5). Stimulation of (OVA peptide loaded) DCs with LPS slightly enhanced this response but this was not statistically significant. OVA-loaded DCs stimulated with SMAs 11a or 11i ± LPS also induced clonal expansion of AG-specific T cells (Figure 5.5). Pre-exposure of DCs to 11a did not significantly alter the percentage or number of $CD4^+KJ1.26^+$ T cells in the dLN compared to OVA-DCs nor did pre-treatment of DCs with 11a before LPS stimulation affect T cell expansion compared to LPS-stimulated DCs (Figure 5.6 and 5.7). SMA 11i appeared to have greater impact on the ability of DCs to promote T cell expansion. 11i-DCs also showed peak T cell expansion on day 5 but mice inoculated with these DCs had significantly reduced number and percentage of $CD4^+KJ1.26^+$ T cells compared to mice immunised with OVA-DCs on days 3 and 5 (Figure 5.6 and 5.7). Stimulation of 11i-DCs with LPS caused a slight increase in T cell expansion but interestingly this peaked at day 3 rather than day 5 (Figure 5.5). T cell expansion in mice inoculated with 11i-LPS-DCs was significantly lower with respect to percentage and cell number than LPS-DC immunised mice on day 5 but, in contrast to OVA+LPS treated DCs, mice treated with OVA+11i+LPS still had a significantly increased percentage of $CD4^+KJ1.26^+$ T cells in the dLNs on day 10 compared to mice inoculated with control (RPMI) DCs (Figure 5.6 and 5.7).

5.2.2 Expression of early activation markers by Ag-specific $CD4^+$ T cells activated by ES-62 SMA DCs *in vivo*

To investigate whether the reduced T cell expansion was in part due to reduced T cell activation the expression of CD62L, primarily expressed on naive T cells, and CD69, an activation marker, on $CD4^+KJ1.26^+$ T cells was investigated on single cells from the popliteal dLN of mice immunised with the differentially activated bmDCs. The cells were gated on as described in Figures 5.3 and 5.4. Overall, there were no significant changes observed amongst groups in CD62L expression on day 2; however T cells from mice inoculated with LPS-DCs did have a slightly reduced expression level of CD62L compared to RPMI-DCs and 11i+LPS-DCs expressed lower levels of CD62L compared to 11i-DC immunised mice (results not shown). On days 3 and 5, the peak days of expansion, T cells from all mice inoculated with OVA-loaded DCs had significantly lower expression of CD62L compared to RPMI DCs and slightly (non-significant) lower % of $CD62L^+CD4^+$ T cells despite the increase in T cells (Figure 5.8 and results not shown). There were no significant changes in CD69 expression levels on T cells from mice immunised with differentially activated DCs on any of the days investigated, however at the peak of expansion (day 5), OVA-DC and OVA+LPS-DC mice had significantly higher numbers of CD69+ Ag-specific T cells compared to RPMI DC mice. Mice inoculated with 11i-DCs had significantly less CD69+ T cells compared to OVA-DCs and treatment of DCs with either SMA prior to LPS also significantly lowered the number of $CD69^+CD4^+$ cells in the dLN of these mice compared to mice inoculated with OVA+ LPS-activated DCs (Figure 5.9).

5.2.3 Effects of ES-62 SMA matured DCs on the cytokine expression of Ag-specific $CD4^+$ T cells

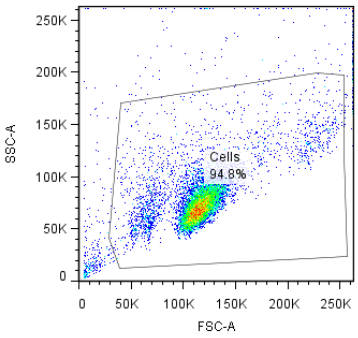
Co-culture of SMA-treated DCs and SMA+LPS-treated DCs with naive $CD4^+$ T cells *in vitro* inhibits the ability of the T cells to produce IFN-γ (Figure 5.1). In order to examine if SMA-DCs and SMA+LPS-DCs have a similar ability to alter the phenotype of $CD4^+$ T cells *in vivo,* dLN cells were stimulated *ex vivo* with PMA/Ionomycin for 1 hour then Brefeldin A for 4 hours, and the expression of IFN-γ, IL-17A and IL-4 on $CD4^+KJ1.26^+$ T cells was analysed by flow cytometry. The cells were gated on as described in Figures 5.3 and 5.4. There was a significant increase in the number of IFN-γ^+ (Figure 5.10A) and IL-$17A^+$ (Fig. 5.10B) T cells in mice inoculated with OVA-loaded DCs and OVA+LPS-DCs on day 5 compared to control RPMI DCs. There was no difference in

the number of IFN-γ+ or IL-17A+ T cells in mice with 11a-DCs but 11i-DC inoculated mice had significantly lower levels of both IFN-γ+ and IL-17A+ T cells in their dLNs compared to OVA-DC inoculated mice. In addition, 11a+LPS-DC and 11i+LPS-DC inoculated mice also had significantly reduced levels of IFN-γ+ and IL-17A+ T cells compared to LPS-DCs (Figure 5.10) suggesting that overall the two SMAs may be able to attenuate T_H1/T_H17 responses *in vivo*. There were no changes in IL-4^+ $CD4^+$ T cells amongst mice immunised with alternatively matured DCs but this is likely due to the very small number of IL-4+ cells detected in the system (results not shown).

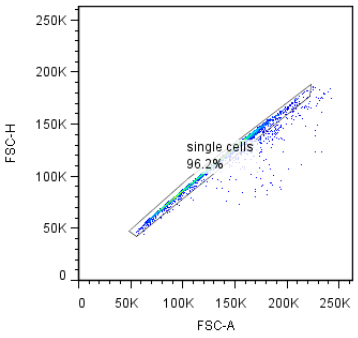
Figure 5.3: Gating strategy for the analysis of T cell responses following immunisation with SMA-stimulated DC.

Cells were initially gated based on size (forward scatter; FSC) and granularity (side scatter; SSC) (A) and doublets were excluded by comparing FSC-Height and FSC-Area (B). Isotype controls (IgG2a APC and IgG2a PerCP) (C) and Fluorescent minus one (FMO) (D) controls were used to determine the expression of $CD4^+$ (PerCP) and $KJ1.26^+$ (APC) (E) cells.

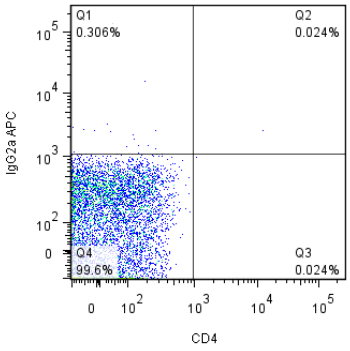
A.

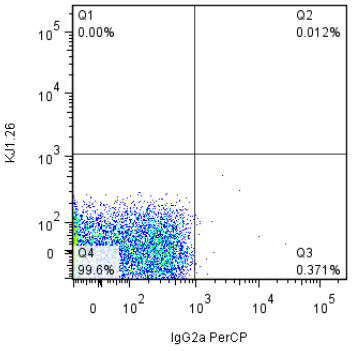

SSC-A
FSC-A
Cells
94.8%

B.

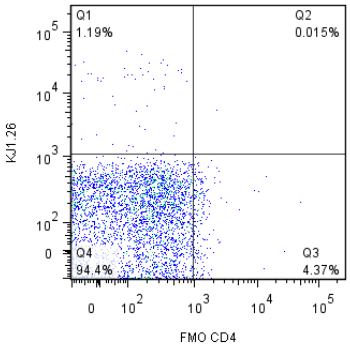

FSC-H
FSC-A
single cells
96.2%

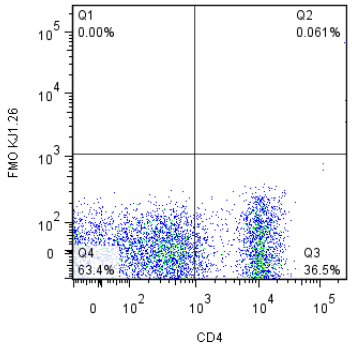
C.


IgG2a APC
CD4
Q1
0.306%
Q2
0.024%
Q4
99.6%
Q3
0.024%


KJ1.26
IgG2a PerCP
Q1
0.00%
Q2
0.012%
Q4
99.6%
Q3
0.371%

D.


KJ1.26
FMO CD4
Q1
1.19%
Q2
0.015%
Q4
94.4%
Q3
4.37%


FMO KJ1.26
CD4
Q1
0.00%
Q2
0.061%
Q4
63.4%
Q3
36.5%

E.

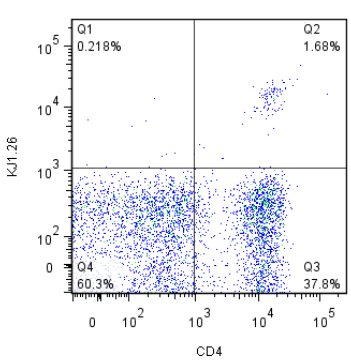
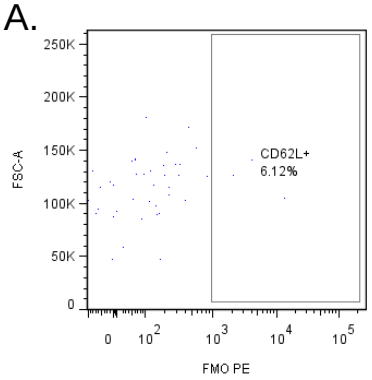
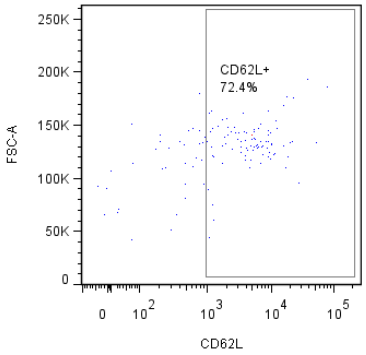

KJ1.26
CD4
Q1
0.218%
Q2
1.68%
Q4
60.3%
Q3
37.8%

Figure 5.4: Gating strategy for the analysis of T cell responses following immunisation with SMA-stimulated DC

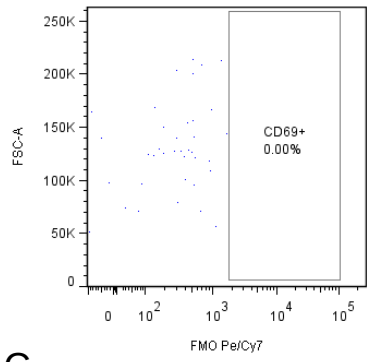
Cells were stained for expression of CD4, KJ1.26, CD62L and CD69. Cells were initially gated as described in Figure 5.3 and the expression of CD62L (PE) (A) or CD69 (Pe/Cy7) (B) by $CD4^+KJ1.26^+$ cells was determined using FMO controls. Lymphocytes from dLNs were also stimulated for 5 hours with PMA and Ionomycin with Brefeldin A added after 1 hour. Cells were then stained for CD4 and KJ1.26 expression before permeabilisation and staining for IFN-γ (Pe/Cy7) and IL-17A (APC/Cy7). Cells were gated as described in Figure 5.3 and the intracellular expression of IFN-γ (C) and IL-17A (D) by $CD4^+KJ1.26^+$ cells was determined using FMO controls.

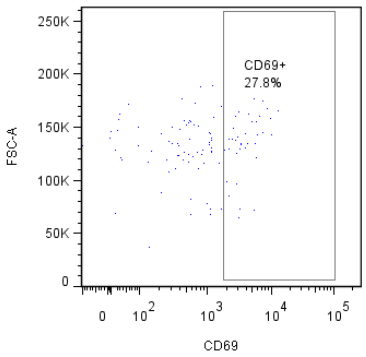
A.


FSC-A
250K
200K
150K
100K
50K
0
CD62L+
6.12%
0
10^2
10^3
10^4
10^5
FMO PE

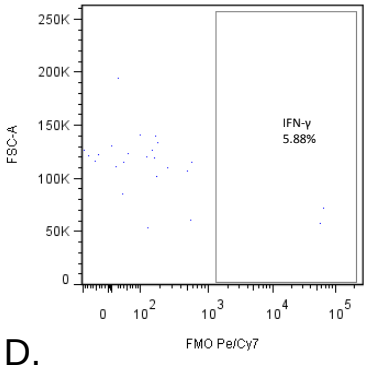

FSC-A
250K
200K
150K
100K
50K
0
CD62L+
72.4%
0
10^2
10^3
10^4
10^5
CD62L

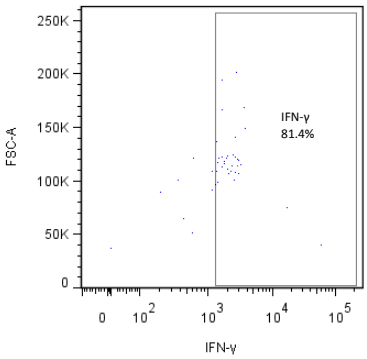
B.


FSC-A
250K
200K
150K
100K
50K
0
CD69+
0.00%
0
10^2
10^3
10^4
10^5
FMO Pe/Cy7

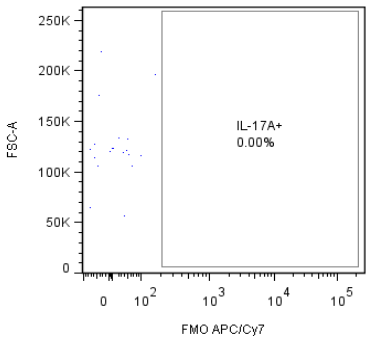

FSC-A
250K
200K
150K
100K
50K
0
CD69+
27.8%
0
10^2
10^3
10^4
10^5
CD69

C.


FSC-A
250K
200K
150K
100K
50K
0
IFN-γ
5.88%
0
10^2
10^3
10^4
10^5
FMO Pe/Cy7


FSC-A
250K
200K
150K
100K
50K
0
IFN-γ
81.4%
0
10^2
10^3
10^4
10^5
IFN-γ

D.


FSC-A
250K
200K
150K
100K
50K
0
IL-17A+
0.00%
0
10^2
10^3
10^4
10^5
FMO APC/Cy7

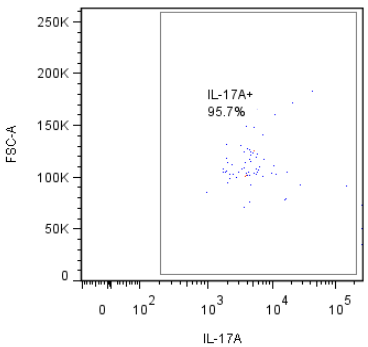

FSC-A
250K
200K
150K
100K
50K
0
IL-17A+
95.7%
0
10^2
10^3
10^4
10^5
IL-17A

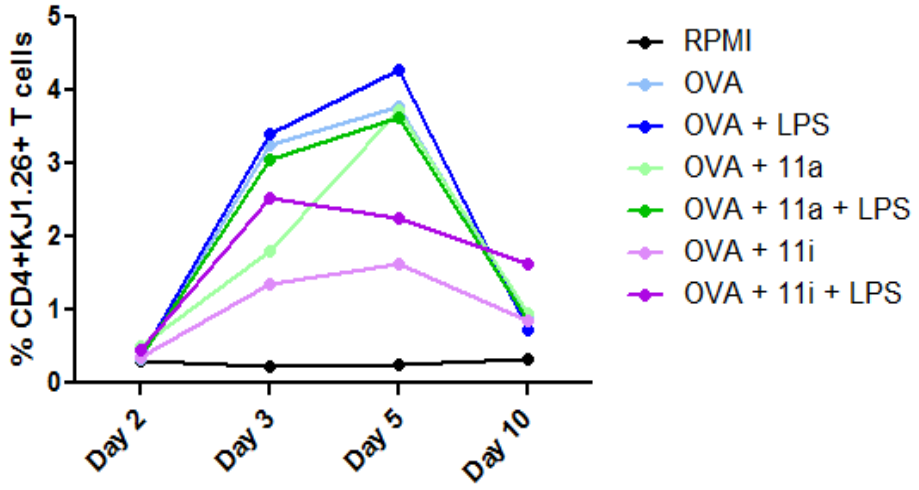
Figure 5.5: Effect of *in vitro* SMA stimulation on the ability of DCs to promote clonal expansion of Ag-specific $CD4^+$ T cells *in vivo*

BmDCs from BALB/c mice were grown in RPMI complete medium containing 10ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. These bmDC were pulsed with OVA peptide and incubated with either SMA 11a or 11i in RPMI or with RPMI alone for 18 hours and then with RPMI or RPMI+LPS for a further 24 hours. BmDCs that were not pulsed with OVA (RPMI DCs) were used as a control. BmDCs were then washed and BALB/c mice that had received $1x10^6$ naïve $CD4^+KJ1.26^+$ T cells from donor DO11.10 mice 24 hours previously were inoculated with $2.5x10^5$ bmDCs. The percentage of $CD4^+KJ1.26^+$ T cells in the draining popliteal lymph nodes of adoptively transferred mice was analysed by flow cytometry. Each time point represents the mean ± SEM for 3 mice per day.

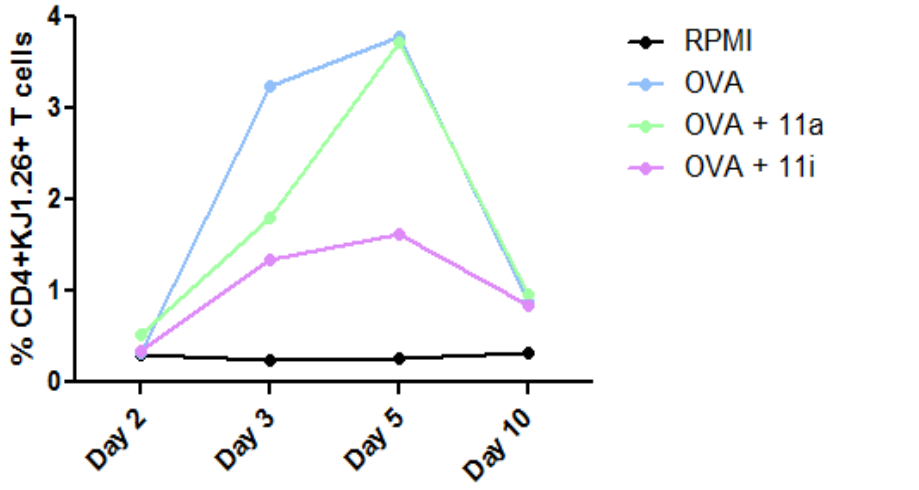
Panel A shows the T cell expansion in mice inoculated with differentially activated DCs.

For clarity panel B shows the T cell expansion in mice inoculated with SMA-activated DCs (along with RPMI and OVA control DC inoculated mice) and panel C shows the T cell expansion in mice inoculated with SMA+LPS activated DCs (along with RPMI and OVA+LPS control DC inoculated mice).

A.


% CD4+KJ1.26+ T cells
0
1
2
3
4
5
Day 2
Day 3
Day 5
Day 10
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS

B.


% CD4+KJ1.26+ T cells
0
1
2
3
4
Day 2
Day 3
Day 5
Day 10
RPMI
OVA
OVA + 11a
OVA + 11i

C.

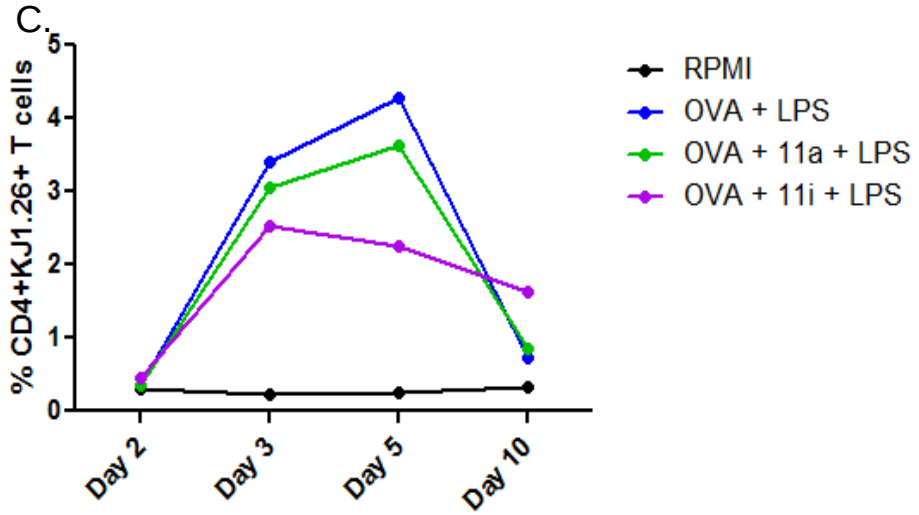

% CD4+KJ1.26+ T cells
0
1
2
3
4
5
Day 2
Day 3
Day 5
Day 10
RPMI
OVA + LPS
OVA + 11a + LPS
OVA + 11i + LPS

Figure 5.6: Effect of *in vitro* SMA- and/or LPS-exposure on the ability of DCs to promote clonal expansion of Ag-specific $CD4^+$ T cells *in vivo*

BmDCs from BALB/c mice were grown in RPMI complete medium containing 10ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. These bmDC were pulsed with OVA peptide and incubated with either SMAs 11a or 11i in RPMI or with RPMI alone for 18 hours and then RPMI or RPMI+LPS for a further 24 hours. BmDCs that were not pulsed with OVA (RPMI DCs) were used as a control. BmDCs were then washed and BALB/c mice that had received $1x10^6$ naïve $CD4^+KJ1.26^+$ T cells from donor DO11.10 mice 24 hours previously were inoculated with $2.5x10^5$ bmDCs. The percentage of $CD4^+KJ1.26^+$ T cells in the draining popliteal lymph nodes of adoptively transferred mice on day 2 (A), 3 (B), 5 (C) and 10 (D) was analysed by flow cytometry. Results are expressed as mean ± SEM for 3 mice per group on each day. Data were analysed using one way ANOVA with Bonferroni post-test where * $p < 0.05$ ** $p < 0.01$ compared to RPMI DCs * $p < 0.05$ ** $p < 0.01$ compared to OVA DCs and * $p < 0.05$ compared to OVA + LPS DCs.

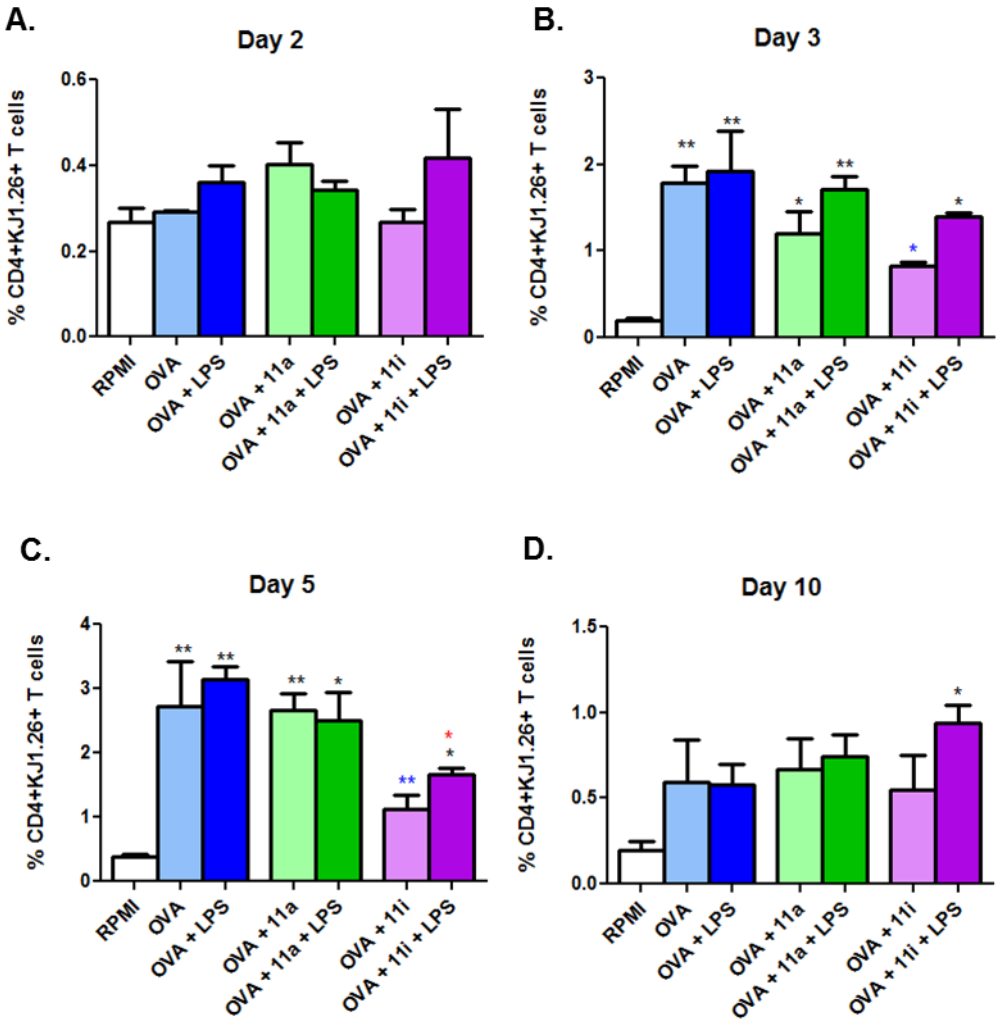

A.
Day 2
% CD4+KJ1.26+ T cells
0.6
0.4
0.2
0.0
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS
B.
Day 3
% CD4+KJ1.26+ T cells
3
2
1
0
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS
C.
Day 5
% CD4+KJ1.26+ T cells
4
3
2
1
0
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS
D.
Day 10
% CD4+KJ1.26+ T cells
1.5
1.0
0.5
0.0
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS

Figure 5.7: Analysis of the number of cells in the dLN from mice immunised with in vitro SMA- and/or LPS-treated

BmDCs from BALB/c mice were grown in RPMI complete medium containing 10ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. These bmDC were pulsed with OVA peptide and incubated with either SMA 11a or 11i in RPMI or with RPMI alone for 18 hours and then RPMI or RPMI+LPS for a further 24 hours. BmDCs that were not pulsed with OVA (RPMI DCs) were used as a control. BmDCs were then washed and BALB/c mice that had received $1x10^6$ naïve $CD4^+KJ1.26^+$ T cells from donor DO11.10 mice 24 hours previously, were inoculated with $2.5x10^5$ bmDCs. The number of $CD4^+KJ1.26^+$ T cells in the draining popliteal lymph nodes of adoptively transferred mice on day 2 (A), 3 (B), 5 (C) and 10 (D) was analysed by flow cytometry. Results are expressed as mean ± SEM for 3 mice per group on each day. Data was analysed using one way ANOVA with Bonferroni post-test where * $p < 0.05$ ** $p < 0.01$ compared to RPMI DCs; * $p < 0.05$ compared to OVA DCs and * $p < 0.05$ compared to OVA + LPS DCs.

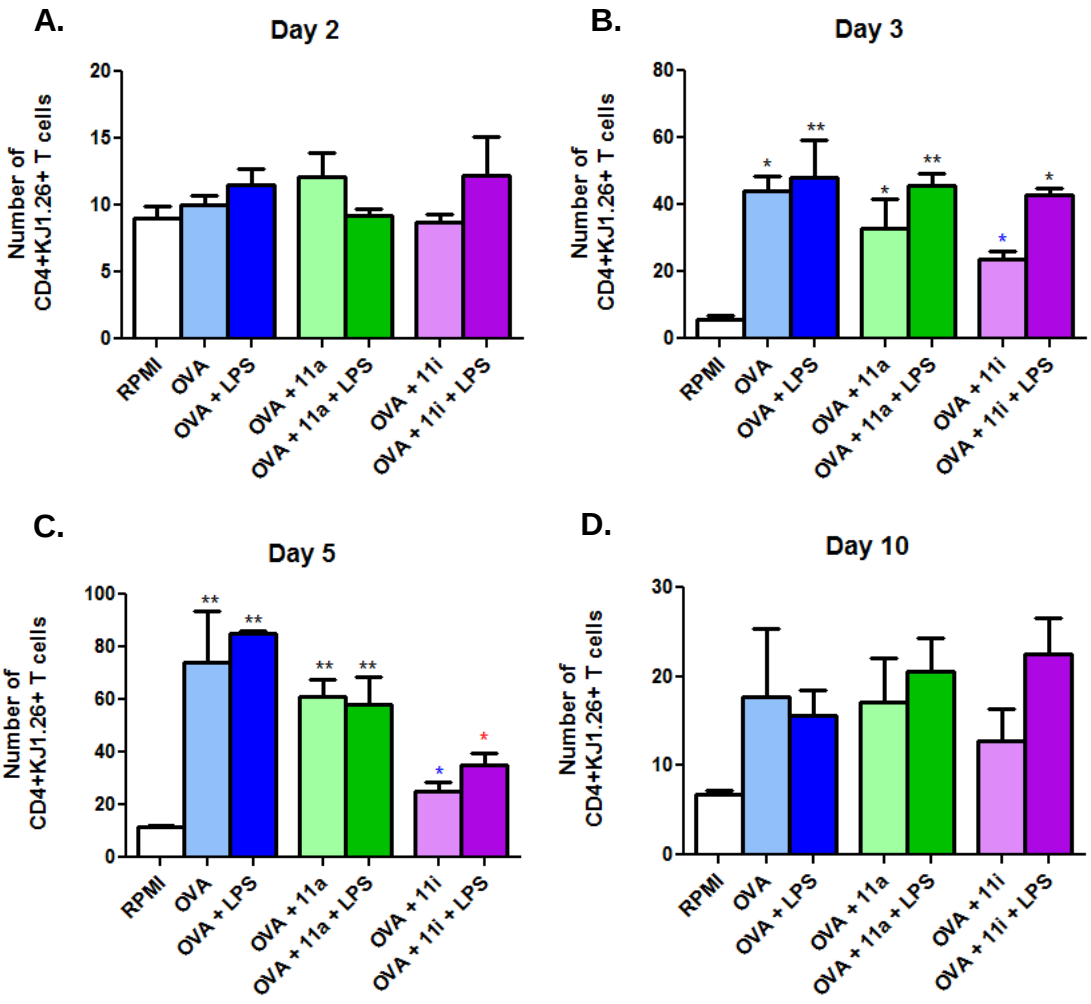

A.
Day 2
Number of CD4+KJ1.26+ T cells
20
15
10
5
0
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS
B.
Day 3
Number of CD4+KJ1.26+ T cells
80
60
40
20
0
C.
Day 5
Number of CD4+KJ1.26+ T cells
100
80
60
40
20
0
D.
Day 10
Number of CD4+KJ1.26+ T cells
30
20
10
0

Figure 5.8: The effects of *in vitro* SMA treatment on the ability of DCs to modulation expression of CD62L on T cells *in vivo*

BmDCs from BALB/c mice were grown in RPMI complete medium containing 10ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. These bmDCs were pulsed with OVA peptide and incubated with either SMA 11a or 11i in RPMI or with RPMI alone for 18 hours and then RPMI or RPMI+LPS for a further 24 hours. BmDCs that were not pulsed with OVA (RPMI DCs) were used as a control. BmDCs were then washed and BALB/c mice that had received $1x10^6$ naïve $CD4^+KJ1.26^+$ T cells from donor DO11.10 mice 24 hours previously were inoculated with $2.5x10^5$ bmDCs. The expression of CD62L on $CD4^+KJ1.26^+$ T cells from the draining popliteal lymph nodes of these mice was analysed by flow cytometry. Results are expressed as mean ± SEM for 3 mice per group on each day. Data were analysed using one way ANOVA with Bonferroni post-test where *** $p < 0.001$ compared to RPMI.

Panel A shows the percentage of $CD62L^+CD4^+KJ1.26^+$ T cells on day 5 in the draining popliteal lymph nodes of adoptively transferred mice

Panel B shows the overall expression levels of CD62L on $CD4^+KJ1.26^+$ T cells.

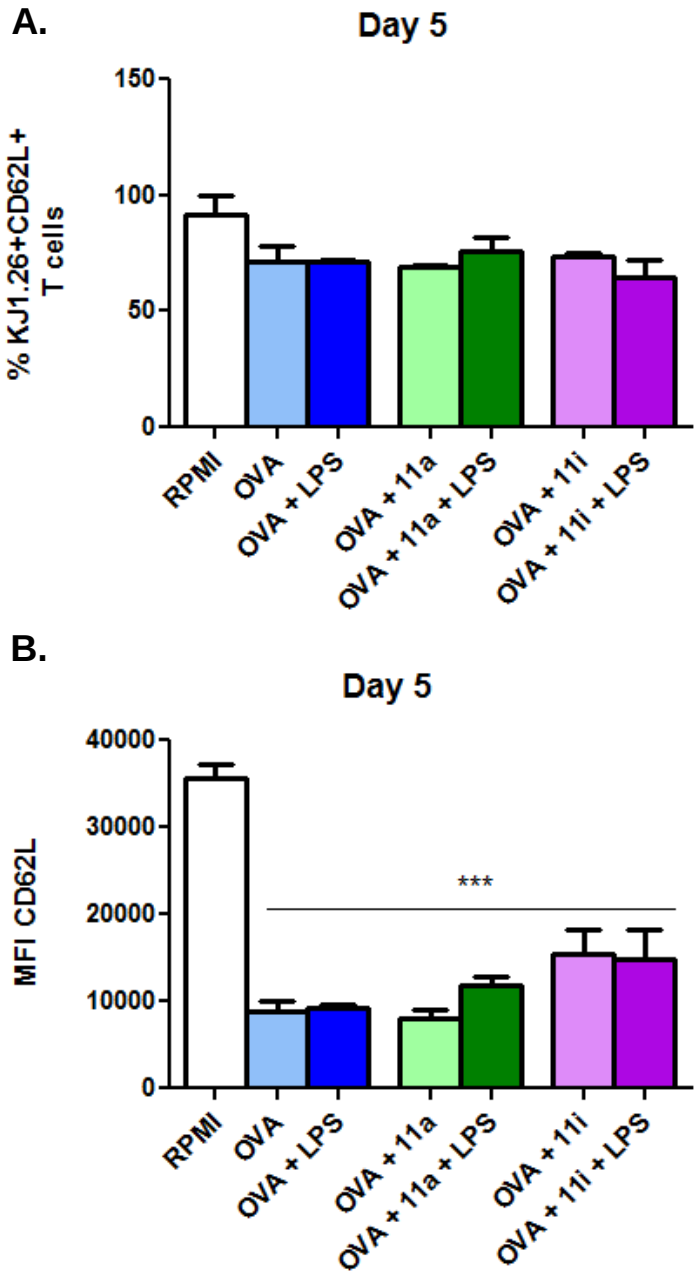

A.
Day 5
% KJ1.26+CD62L+ T cells
150
100
50
0
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS
B.
Day 5
MFI CD62L
40000
30000
20000
10000
0
***
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS

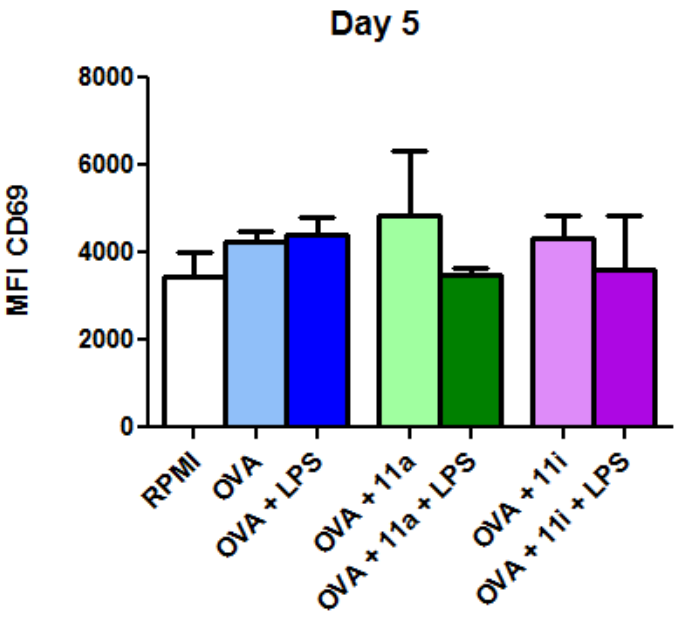
Figure 5.9: The effects of *in vitro* SMA treatment on the ability of DCs to modulate the expression of CD69 on T cells *in vivo*

BmDCs from BALB/c mice were grown in RPMI complete medium containing 10ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. These bmDC were pulsed with OVA peptide and incubated with either SMA 11a or 11i in RPMI or with RPMI alone for 18 hours and then RPMI or RPMI+LPS for a further 24 hours. BmDCs that were not pulsed with OVA (RPMI DCs) were used as a control. BmDCs were then washed and BALB/c mice that had received $1x10^6$ naïve $CD4^+KJ1.26^+$ T cells from donor DO11.10 mice 24 hours previously were inoculated with $2.5x10^5$ bmDCs. The expression of CD69 on $CD4^+KJ1.26^+$ T cells from the draining popliteal lymph nodes of these mice was analysed by flow cytometry. Results are expressed as mean ± SEM for 3 mice per group on each day. Data were analysed using one way ANOVA with Bonferroni post-test where ** $p < 0.01$ compared to RPMI DCs; * $p < 0.05$ compared to OVA DCs; * $p < 0.05$ ** $p < 0.01$ compared to OVA + LPS DCs.

Panel A shows the expression of CD69 on $CD4^+KJ1.26^+$ T cells in the draining popliteal lymph nodes of adoptively transferred mice on day 5

The numbers of $CD69^+CD4^+KJ1.26^+$ T cells from these mice is shown in panel B.

A.


Day 5
MFI CD69
8000
6000
4000
2000
0
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS

B.

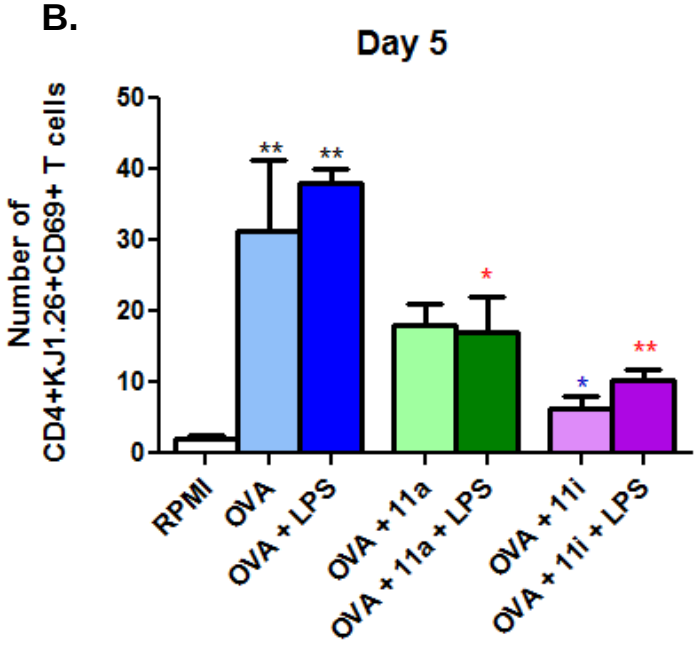
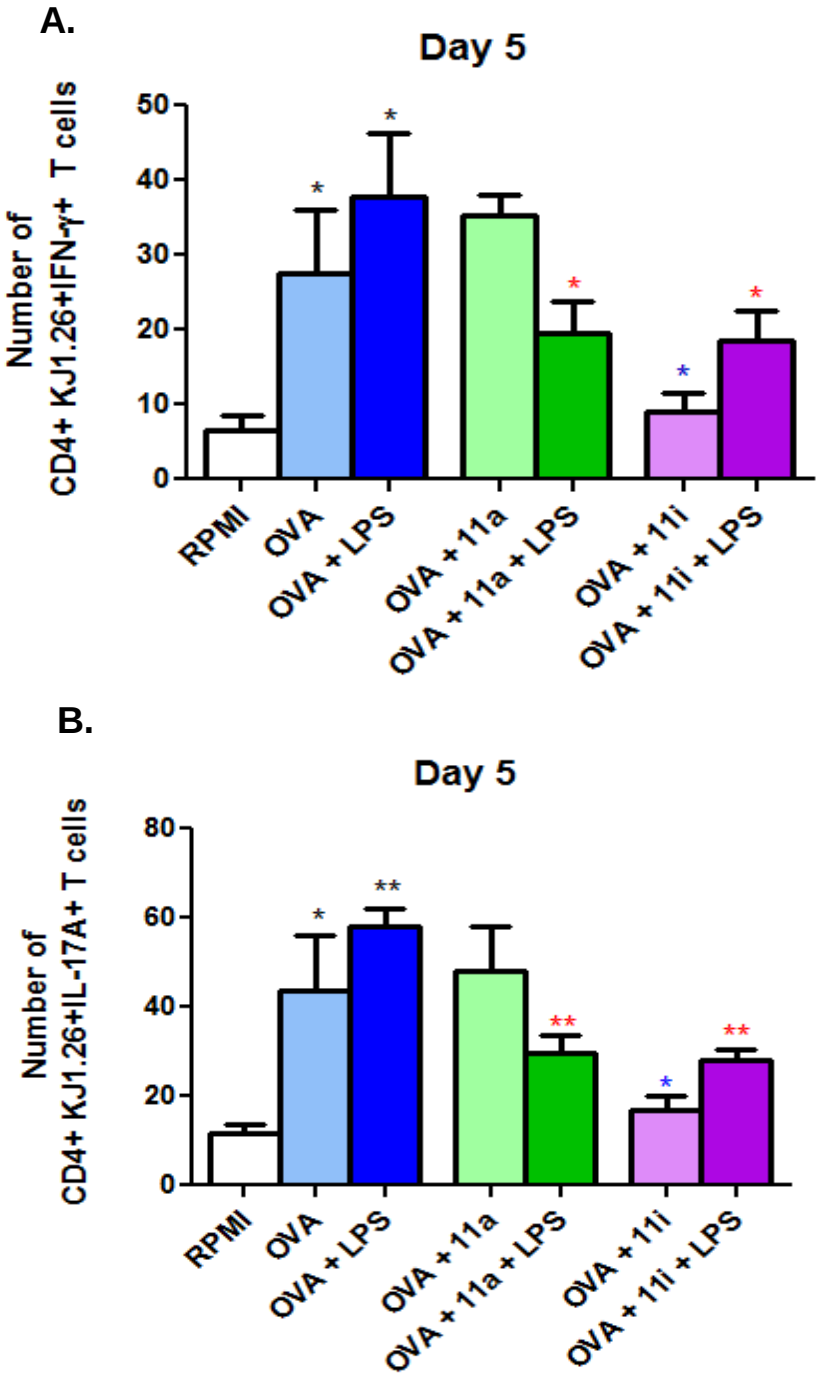

Day 5
Number of CD4+KJ1.26+CD69+ T cells
50
40
30
20
10
0
**
**
*
*
**
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS

Figure 5.10: Effects of immunisation with SMA- and/or LPS-stimulated DC on cytokine expression by OVA-specific $CD4^+$ T cells

BmDCs from BALB/c mice were grown in RPMI complete medium containing 10ng/ml rGM-CSF for 8 days and cell identity was confirmed by flow cytometry. These bmDC were pulsed with OVA peptide and incubated with either SMA 11a or 11i in RPMI or with RPMI alone for 18 hours and then RPMI or RPMI+LPS for a further 24 hours. BmDCs that were not pulsed with OVA (RPMI DCs) were used as a control. BmDCs were then washed and BALB/c mice that had received $1x10^6$ naïve $CD4^+KJ1.26^+$ T cells from donor DO11.10 mice 24 hours previously were inoculated with $2.5x10^5$ bmDCs. The number of (A) IFN-$\gamma^+CD4^+KJ1.26^+$ and (B) IL-$17A^+CD4^+KJ1.26^+$ T cells in the draining popliteal lymph nodes of adoptively transferred mice was analysed by flow cytometry on day 5. Results are expressed as mean ± SEM for 3 mice per group on each day. Data were analysed using one way ANOVA with Bonferroni post-test where * $p < 0.05$ **, $p < 0.01$ compared to RPMI DCs; * $p < 0.05$ compared to OVA DCs; * $p < 0.05$ **, $p < 0.01$ compared to OVA + LPS DCs.


A.
Day 5
Number of CD4+ KJ1.26+IFN-γ+ T cells
50
40
30
20
10
0
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS
B.
Day 5
Number of CD4+ KJ1.26+IL-17A+ T cells
80
60
40
20
0
RPMI
OVA
OVA + LPS
OVA + 11a
OVA + 11a + LPS
OVA + 11i
OVA + 11i + LPS

5.3 The effect of ES-62 SMA-treated DCs on collagen-induced arthritis (CIA)

Helminths and humans have co-evolved over millennia, yet in the West we have mostly eradicated these pathogens in the past fifty years due to increased hygiene. Chronic helminth infections typically induce a T_H2 immune response with immune regulatory components and can 'dampen' the immune system, promoting worm survival within the host. The hygiene hypothesis postulates that the lack of childhood diseases, such as helminth infections, increases the susceptibility to autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis (RA), and allergic diseases [334]. RA is a chronic inflammatory disease prevalent in Western countries. Although the exact aetiology has not been elucidated it is known to be driven by dysregulated TLR responses with pro-inflammatory cytokines such as TNF-α playing a key role in disease pathogenesis [335]. The potent anti-inflammatory effect of ES-62 mediated through modulation of TLR4 signalling prompted an investigation into the protective effects of ES-62 in the murine model of arthritis, collagen-induced arthritis (CIA). This is the most widely studied model of RA as it shares several pathological features with the human condition including synovial hyperplasia, cartilage degradation and mononuclear cell infiltration [336]. ES-62 was found to protect mice from CIA in both prophylactic and therapeutic models [254]. Protection was associated with decreased CII-specific IFN-γ, TNF-α and IL-6 from draining lymph node (dLN) cells stimulated *ex vivo* and an increase in IL-10 [254]. Further study demonstrated that the active PC moiety of ES-62 was responsible for the immunomodulatory actions in this model as PC-OVA but not recombinant ES-62 (rES-62), which lacked PC, reduced CIA disease incidence and severity [255]. The mechanism of action of ES-62/PC-mediated protection is starting to be elucidated. ES-62 has been found to mediate its anti-inflammatory effects by targeting a network of inflammatory cells to down-regulate IL-17 responses. ES-62-treated mice have decreased levels of IL-17 in the bloodstream and joints, and this is associated with decreases in two of the major IL-17 producing cells – T_H17 and γδ T cells. There are also reduced mRNA levels of the IL-17 master transcription factor RORγτ in dLN cells from ES-62-treated compared to PBS-treated CIA mice [211]. Further investigation revealed that bmDCs treated with ES-62 produced less IL-6 and IL-23 and had a reduced ability to generate OVA-specific T_H17 cells *in vitro* and in addition, mature, ES-62-treated DCs were found to be responsible for the modulation of the γδ T cells *in vivo* [211]. ES-62 has also been found to manipulate the IL-17-associated cytokine IL-22 to mediate its effects in CIA [337] via desensitisation of synovial fibroblast IL-17 production in the joint [338]. Of note, IL-22 was found to be

pathogenic in the early phase of disease but after disease onset it acts to dampen pro-inflammatory responses and indeed, neutralisation of IL-22 in late disease onset prevented ES-62-mediated protection [337]. ES-62 has also been shown to increase the number of IL-10-producing B cells infiltrating the joint, which can further suppress inflammatory IL-17 responses [311].

The sulfones 11a and 12b are the most extensively studied ES-62 SMAs and both have been found to be protective in prophylactic and therapeutic models of CIA [260], [261]. SMA 11a appears to closely mimic ES-62's mechanism of action as it acts by down-regulating MyD88 expression and protection in CIA is associated with reduced IFN-γ and IL-17 responses by dLN cells *ex vivo* [260]. SMA 12b mediated protection however was not obviously associated with decreased IL-17 responses in the CIA model but rather, with reduced IL-1β responses as a consequence of activation of transcription factor Nrf2 [261].

As well as their pivotal role in the induction of an immune response, DCs play a central role in tolerance in the thymus and in the periphery. Animals that are deficient in DCs develop fatal autoimmunity [339]. DCs acquire self-antigen from cells undergoing apoptosis, rendering them tolerogenic, and migrate to the lymph nodes where they induce regulatory T cells, T cell death or anergy [340]. There have been numerous studies that have demonstrated ways to manipulate subsets of DCs *in vitro* to become tolerogenic which, when administered *in vivo*, reduce autoimmunity [341] [342]. The exact role of DCs in RA is still not understood but there is evidence suggesting they play a role in maintaining and progressing disease through the presentation of autoantigens and the production of pro-inflammatory cytokines [340]. However, various studies have now demonstrated that DCs modulated *in vitro* can ameliorate CIA disease in mice. For example, TNF-matured DCs but not LPS-matured DCs delayed disease onset and resulted in reduced disease severity [343]. It has also been demonstrated that helminth-matured DCs can influence autoimmune disease progression. Thus, DCs matured with *Fasciola hepatica* total extract and CpG reduced disease incidence and severity in CIA-mice [331]. Similarly, DCs matured with excretory-secretory products from the larva stages of *Trichinella spiralis* reduced EAE-associated disease in rats [344], and, most recently, DCs matured *in vitro* with antigen (Ag) from *Hymenolepis diminuta* have been shown to be protective against the development of experimental colitis in mice [332]. These studies indicate that the activation status of DCs can play a central role in the outcome of autoimmune

diseases. As mentioned earlier, ES-62 and the SMAs 11a and 12b have all demonstrated robust ability to inhibit CIA disease in mice and this is partially mediated through the modulation of DC responses *in vivo* [211]. In addition SMAs 11a and 12b have been shown to modulate *in vitro* bmDC cytokine responses (chapter 3) and to inhibit the ability of bmDCs to prime IFN-γ-producing T cells (Figure 5.1 and 5.10) as well as inhibit NF-κBp65 activation (Figure 4.15). It was therefore investigated whether transfer of SMA-treated DCs into CIA-mice could influence disease outcome and progression.

For this pilot study it was decided to investigate the effects of immature DCs ("RPMI DCs"), and DCs treated with SMAs 11a and 12b ("SMA DCs") on CIA in mice. SMAs 11a and 12b were both chosen as their protective effect in CIA has already been established and as they have differing mechanisms of action in CIA as well as slightly differing *in vitro* effects it was thought employing them in combination could maximise their therapeutic potential. CIA was induced in 10 week old DBA/1male mice by immunization with CII emulsified with Complete Freud's adjuvant (CFA) on day 0 and challenged on day 21 with CII in PBS. BmDCs were pulsed with CII and treated with both SMAs 11a and 12b (total concentration 5 μg/ml) or RPMI only for 18 hours before being harvested, washed and injected into CIA mice on days -2, 0 and 21, following the regime described in Figure 5.11 This regime was chosen to mimic the prophylactic model of ES-62 [254].

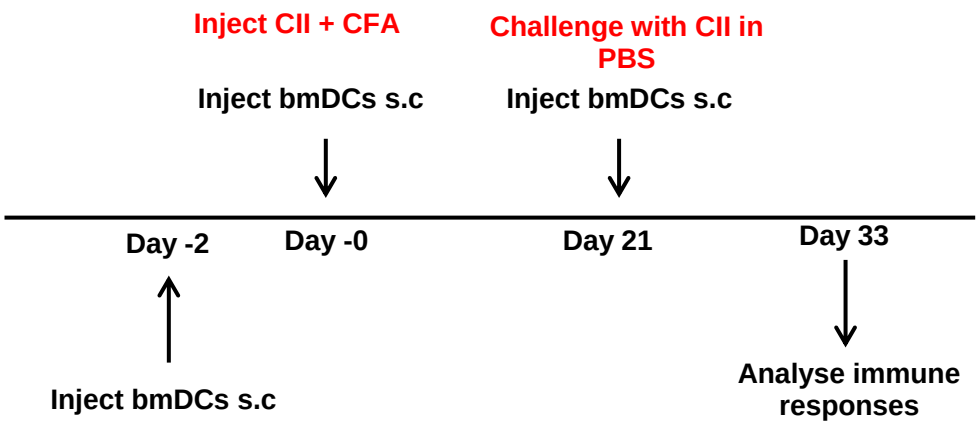


Figure 5.11: Timeline of adoptive transfer of bmDCs in CIA

5.3.1 The effect of administration of SMA-treated DCs on CIA in mice

Administration of *in vitro* SMA-matured bmDCs but not immature bmDCs (RPMI DCs) was found to ameliorate CIA disease as evidenced by reduced arthritic score (Figure 5.12A). Administration of the SMA-matured DCs also reduced hind paw width although this did not reach statistical significance when compared to the PBS-control (Figure 5.12B). Nevertheless the value was significantly less than that obtained with administration of RPMI DCs.

To begin to investigate the mechanisms underlying this modulation of disease, the levels of CII-specific antibodies were determined by ELISA. CII-specific antibodies are crucial to the induction of CIA [345]. While all disease groups had significantly higher antibody levels compared to naïve mice, SMA-treated DCs did not modulate the CII-specific antibody responses as the levels of CII-specific IgG1 and IgG2a were unchanged between PBS-, RPMI DC- and SMA DC- immunised mice (Figure 5.13). This suggests that protection is not mediated through modulation of humoral immunity.

To investigate if protection was mediated through modulation of cellular immunity the dLNs were recovered on day 33 and isolated cells were stimulated with PMA/Ionomycin for 1 hour followed by incubation with Brefeldin A for 4 hours and the expression of IL-17 and IFN-γ in $CD4^+$ cells analysed by flow cytometry, with the gating strategies described in Figure 5.14. SMA DC-treated mice had a significantly reduced proportion of $IL\text{-}17^+CD4^+$ cells in the dLN compared to RPMI DC- and PBS-treated mice and a significantly reduced number of these cells compared to RPMI DC-treated mice (Figure 5.15A-C). In addition, mice treated with SMA-DCs had a significantly

reduced number and proportion of $CD4^-IL\text{-}17A^+$ cells compared to mice treated with RPMI-DCs or PBS. These mice also had a significantly lowered proportion of these cells compared to naïve mice (Figure 5.15D-F). PBS-treated mice had significantly higher expression levels of $CD4^+IFN\text{-}\gamma^+$ cells as well as slightly increased number and proportion of IFN-γ^+ T cells compared to naïve mice (Figure 5.16A-C). RPMI DC and SMA DC-treated mice had lower numbers of IFN-γ^+ T cells but did not alter the increased expression levels of IFN-γ (Figure 5.16B + C). There was also a significant increase in the number of $CD4^-IFN\text{-}\gamma^+$ cells in the dLNs of PBS-treated mice compared to naïve mice but treatment with either RPMI DCs or SMA DCs also had no effect on the levels of these cells (Figure 5.16D-F). ES-62 and 11a mediate protection in CIA through the down-regulation of pro-inflammatory cytokines and specifically inhibition of IL-17 by targeting a complex network of cells. These results suggest that SMA DCs may also mediate protection against arthritis by modulating the immune response away from a T_H17 phenotype.

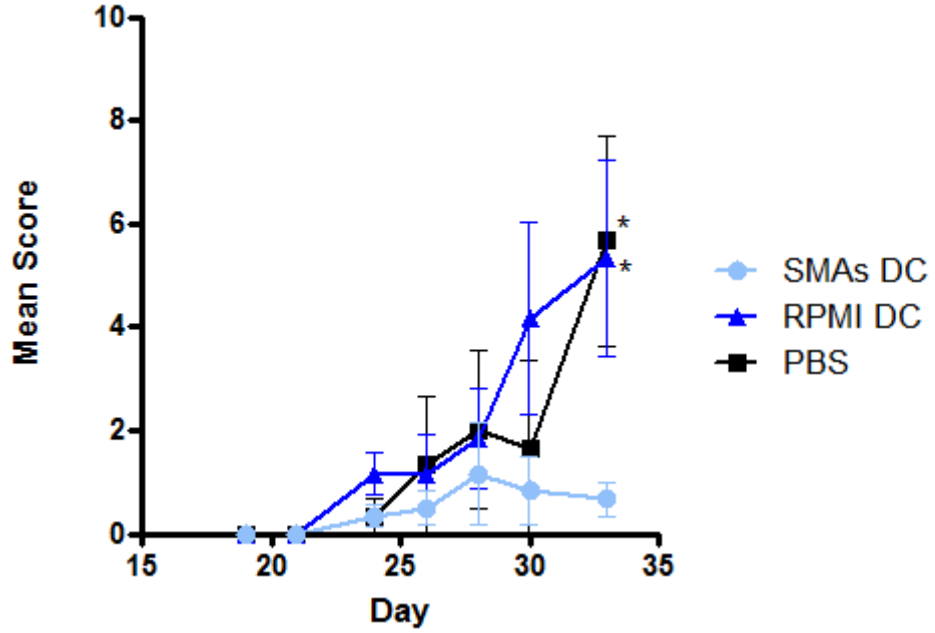
Previous studies, which have demonstrated protective effects of helminth-matured DCs, have reported that protection is mediated through the generation of Treg cells and/or the production of IL-10 [331], [332], [344]. It was therefore investigated whether SMA-matured DCs also mediated protection through these mechanisms. DLNs were recovered on day 33 and cells were stimulated with PMA/Ionomycin for 1 hour followed by exposure to Brefeldin A for 4 hours and the expression of CD4, FOXP3 and IL-10 was analysed by flow cytometry. The gating strategy is shown in Figure 5.17. Consistent with studies with ES-62 [220], [241] there was no difference in the number or percentage of $FOXP3^+CD4^+$ Tregs cells in the dLN of treated mice relative to the other groups, nor was there any difference in the expression of IL-10 by these cells. There was also no difference in the levels of $IL\text{-}10^+CD4^+$ cells, representative of Tr1 regulatory T cells (Figure 5.18). IL-10-producing B cells have been proposed to suppress pathogenic responses in CIA and are found in lower numbers in CIA mice [311]. While ES-62 does not seem to promote modulation through the generation of Treg cells it has recently been found that the nematode product restores IL-10-producing B cell levels in CIA-mice back to those present in naïve mice [311]. As SMA DC-treatment did not induce the generation of $IL\text{-}10^+FOXP3^+CD4^+$ or $IL\text{-}10^+CD4^+$ T cells, it was next investigated whether it could increase the numbers of IL-10^+ B cells. Unfortunately however, for unknown reasons, it was not possible to detect $CD19^+$ cells in these studies and so the levels of $IL\text{-}10^+CD4^-$ cells were analysed instead to provide an insight into whether SMA DCs targeted any non-$CD4^+$ IL-10 producing cell

population. There were no significant differences in the number or proportion of IL-$10^{+}CD4^{-}$ cells amongst groups of mice treated with PBS, RPMI DCs or SMA DCs, indicating that protection is not mediated through induction of IL-10 (Figure 5.19).

Figure 5.12: SMA-treated DCs protect against CIA

DBA/1 male mice were inoculated with bmDCs matured with SMAs 11a and 12b (SMA DCs), immature DCs (RPMI DCs) or PBS on days -2, 0 and 21. Arthritis was induced by injection of collagen on days 0 and 21. Disease is shown by mean arthritic score (A) and (B) hind paw width (PBS, n = 3; RPMI DCs and SMA DCs, n = 6). Results are expressed as mean ± SEM and analysed using an unpaired t test where *$p < 0.05$ ** $p < 0.01$; SMA DC-treated mice compared to RPMI DCs- and PBS-treated mice for mean score and compared to RPMI DCs for paw width.

A.


Mean Score
10
8
6
4
2
0
15
20
25
30
35
Day
*
*
SMAs DC
RPMI DC
PBS

B.

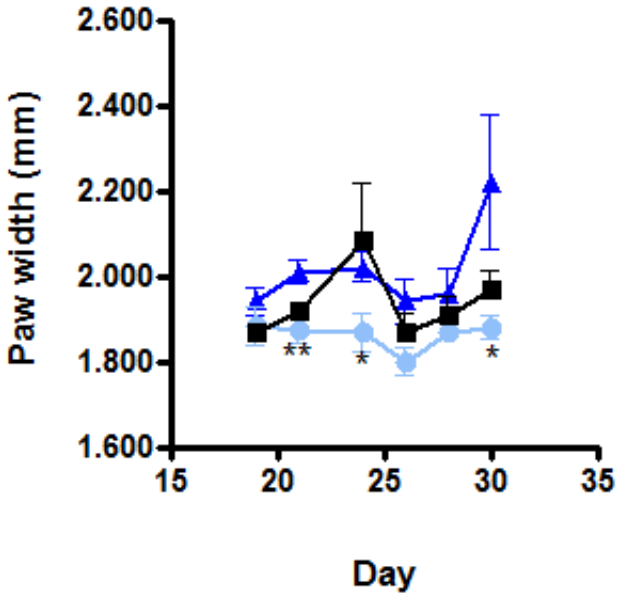
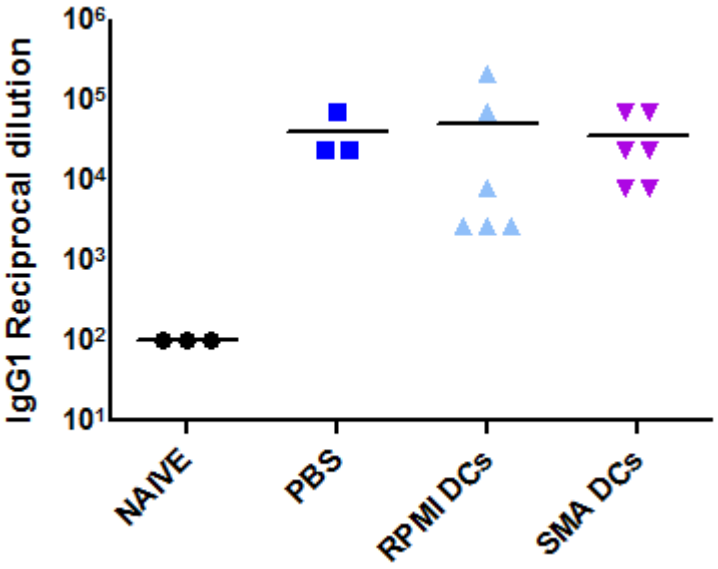

Paw width (mm)
2.600
2.400
2.200
2.000
1.800
1.600
15
20
25
30
35
Day
**
*
*

Figure 5.13: The effect of SMAs on CII-specific antibody response

CII-specific IgG1 (A) and IgG2a (B) levels in serum samples from naïve (no CII treatment; n=3), PBS- (n=3), RPMI DC- (n=6) and SMA DC- (n=6) treated mice were determined by ELISA. Serum was serially diluted and the endpoint dilution, the point at which no further antibody was detected, was plotted. Results are expressed as mean (of duplicate determinations) for each mouse in the group.

A.


IgG1 Reciprocal dilution
10^6
10^5
10^4
10^3
10^2
10^1
NAIVE
PBS
RPMI DCs
SMA DCs

B.

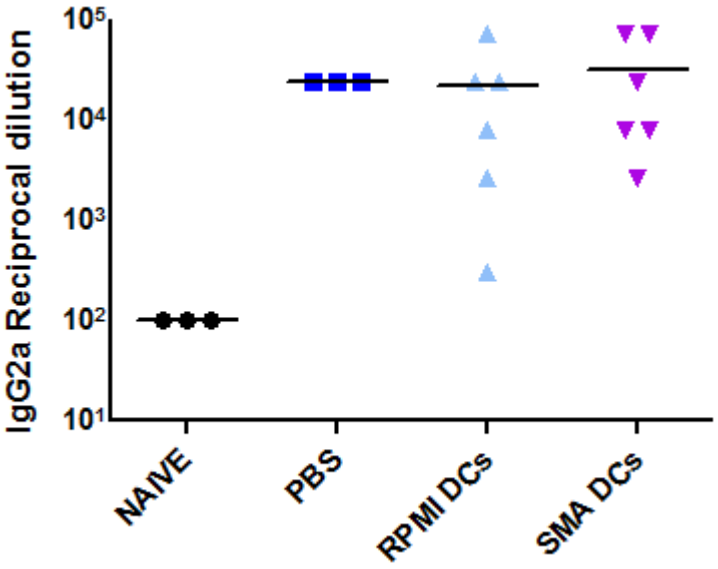

IgG2a Reciprocal dilution
10^5
10^4
10^3
10^2
10^1
NAIVE
PBS
RPMI DCs
SMA DCs

Figure 5.14: Gating strategy for the analysis of T cell subsets

Cells were stimulated with PMA and Ionomycin for an hour before the addition of Brefeldin A for a further 4 hours. Cells were then stained for expression of CD4 (FITC), IL-17A (PerCP) and IFN-γ (APC). Initially cells were gated based on size (FSC) and granularity (SSC), doublets were excluded by comparing FSC height and FSC area and the Fixable Viability Dye eFluor® (APC/Cy7) used to exclude dead cells (A). Relevant isotype and fluorescent minus one (FMO) controls were (B) were used to determine the expression of CD4 on lymphocytes (C). Likewise relevant isotype and FMO controls (D and E) were used to determine the expression of IL-17A by $CD4^+$ (F) and $CD4^-$ (G) T cells, and the expression of IFN-γ by $CD4^+$ (H) and $CD4^-$ (I) T cells.

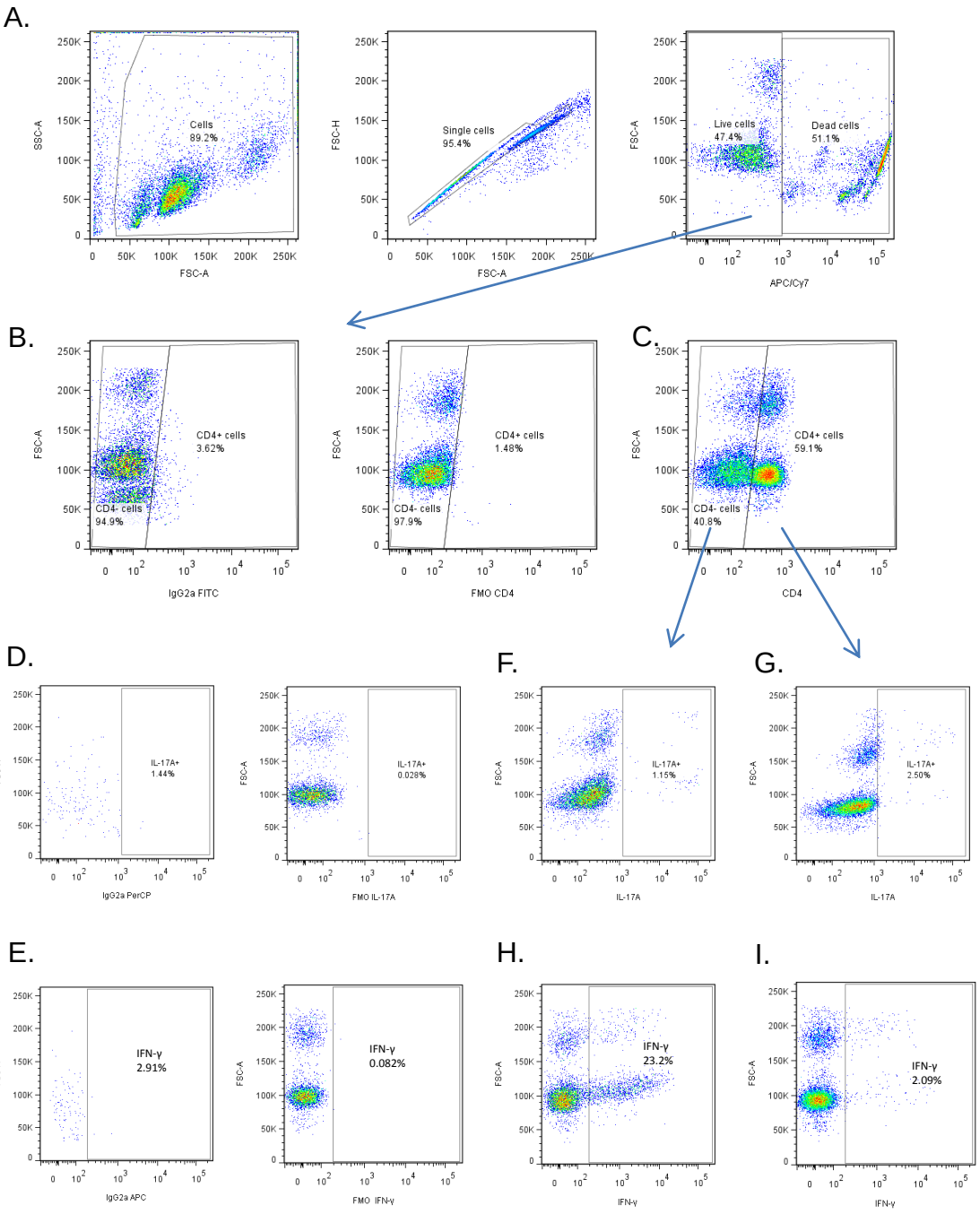

A.
SSC-A
FSC-A
Cells
89.2%
FSC-H
FSC-A
Single cells
95.4%
FSC-A
APC/Cy7
Live cells
47.4%
Dead cells
51.1%
B.
FSC-A
IgG2a FITC
CD4+ cells
3.62%
CD4- cells
94.9%
FSC-A
FMO CD4
CD4+ cells
1.48%
CD4- cells
97.9%
C.
FSC-A
CD4
CD4+ cells
59.1%
CD4- cells
40.8%
D.
FSC-A
IgG2a PerCP
IL-17A+
1.44%
FSC-A
FMO IL-17A
IL-17A+
0.028%
F.
FSC-A
IL-17A
IL-17A+
1.15%
G.
FSC-A
IL-17A
IL-17A+
2.50%
E.
FSC-A
IgG2a APC
IFN-γ
2.91%
FSC-A
FMO IFN-γ
IFN-γ
0.082%
H.
FSC-A
IFN-γ
IFN-γ
23.2%
I.
FSC-A
IFN-γ
IFN-γ
2.09%

Figure 5.15: The effects of SMA DC treatment on IL-17 responses in CIA

DBA/1 mice were immunised with CII on days 0 and 21 and were treated with PBS, RPMI DCs or SMA DCs on days -2, 0 and 21. On day 33 the dLNs of all four paws were collected and cells were stimulated with PMA/Ionomycin for 1 hour prior to treatment with Brefeldin A for 4 hours. The expression of IL-17 on $CD4^+$ cells was analysed by flow cytometry. The percentage (A) and number (B) of $IL\text{-}17^+CD4^+$ cells are shown. The overall expression level (MFI) of IL-17 on $CD4^+$ cells is shown in C. The percentage (D) and number (E) of $IL\text{-}17^+CD4^-$ cells was also determined and the overall expression level of IL-17 on $CD4^-$ cells is shown in F. Results from individual mice in each group (naïve n=3, PBS n=3, RPMI DC n=6 and SMA DC n=6) are shown. Data are compared using one way ANOVA with Bonferroni post-test where $*p < 0.05$ $**p < 0.01$

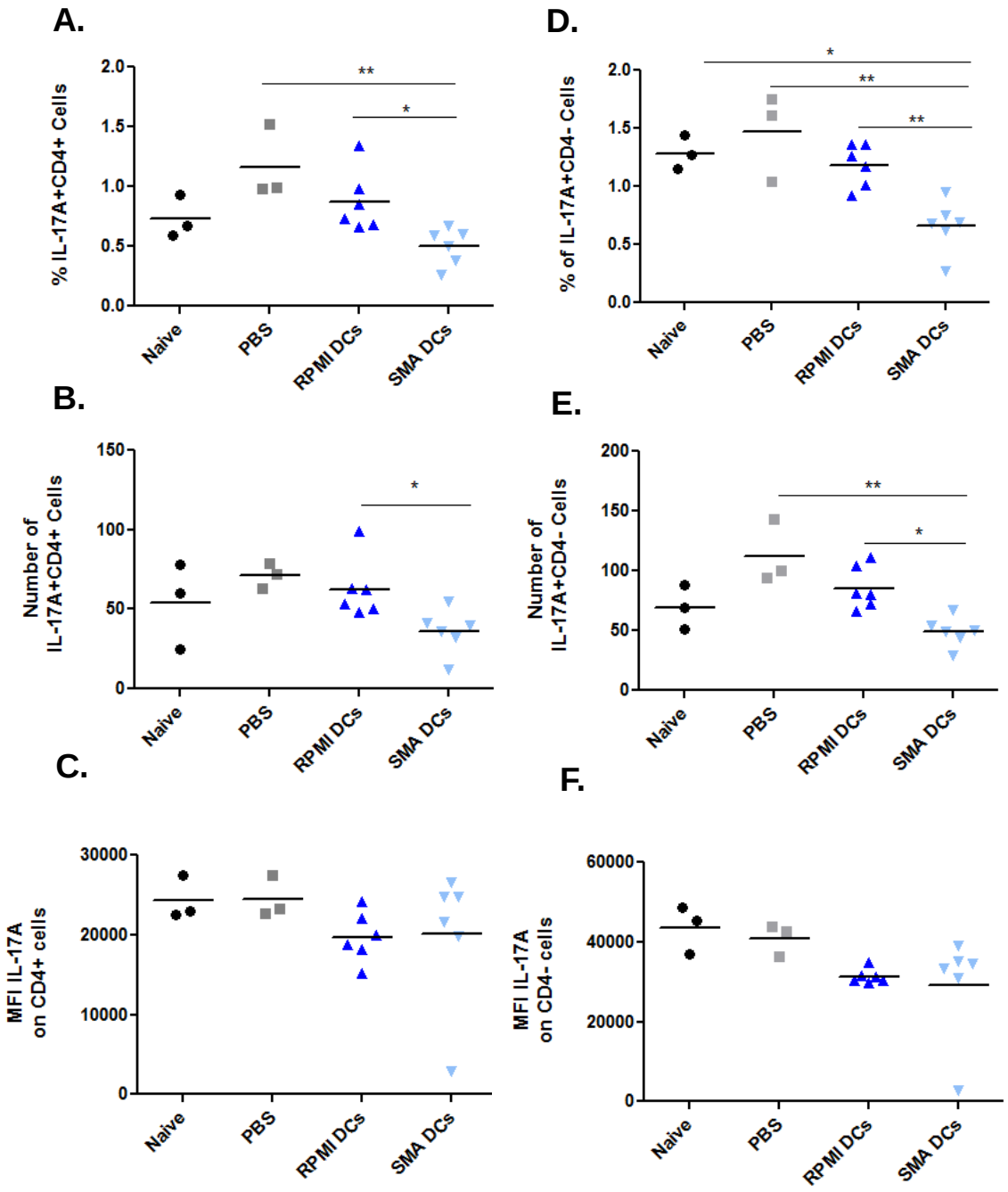

A.
% IL-17A+CD4+ Cells
2.0
1.5
1.0
0.5
0.0
**
*
Naive
PBS
RPMI DCs
SMA DCs
D.
% of IL-17A+CD4- Cells
*
**
**
B.
Number of IL-17A+CD4+ Cells
150
100
50
0
*
E.
Number of IL-17A+CD4- Cells
200
150
100
50
0
**
*
C.
MFI IL-17A on CD4+ cells
30000
20000
10000
0
F.
MFI IL-17A on CD4- cells
60000
40000
20000
0

Figure 5.16: The effects of SMA-DC treatment on IFN-γ responses in CIA

DBA/1 mice were immunised with CII on days 0 and 21 and were treated with PBS, RPMI DCs or SMA DCs on days -2, 0 and 21. On day 33 the draining lymph nodes of all four paws were collected and cells were stimulated with PMA/Ionomycin for 1 hour prior to treatment with Brefeldin A for 4 hours. The expression of IFN-γ on $CD4^+$ cells was analysed by flow cytometry. The percentage (A) and number (B) of IFN-γ^+CD4^+ cells are shown. The overall expression level of IFN-γ on $CD4^+$ cells is shown in C. The percentage (D) and number (E) of IFN-γ^+CD4^- cells was also determined and the overall expression level of IFN-γ^+ on $CD4^-$ cells is shown in F. Results from individual mice in each group (naïve n=3, PBS n=3, RPMI DC n=6 and SMA DC n=6) are shown. Data are compared using One way ANOVA where $^{**}p < 0.01$.

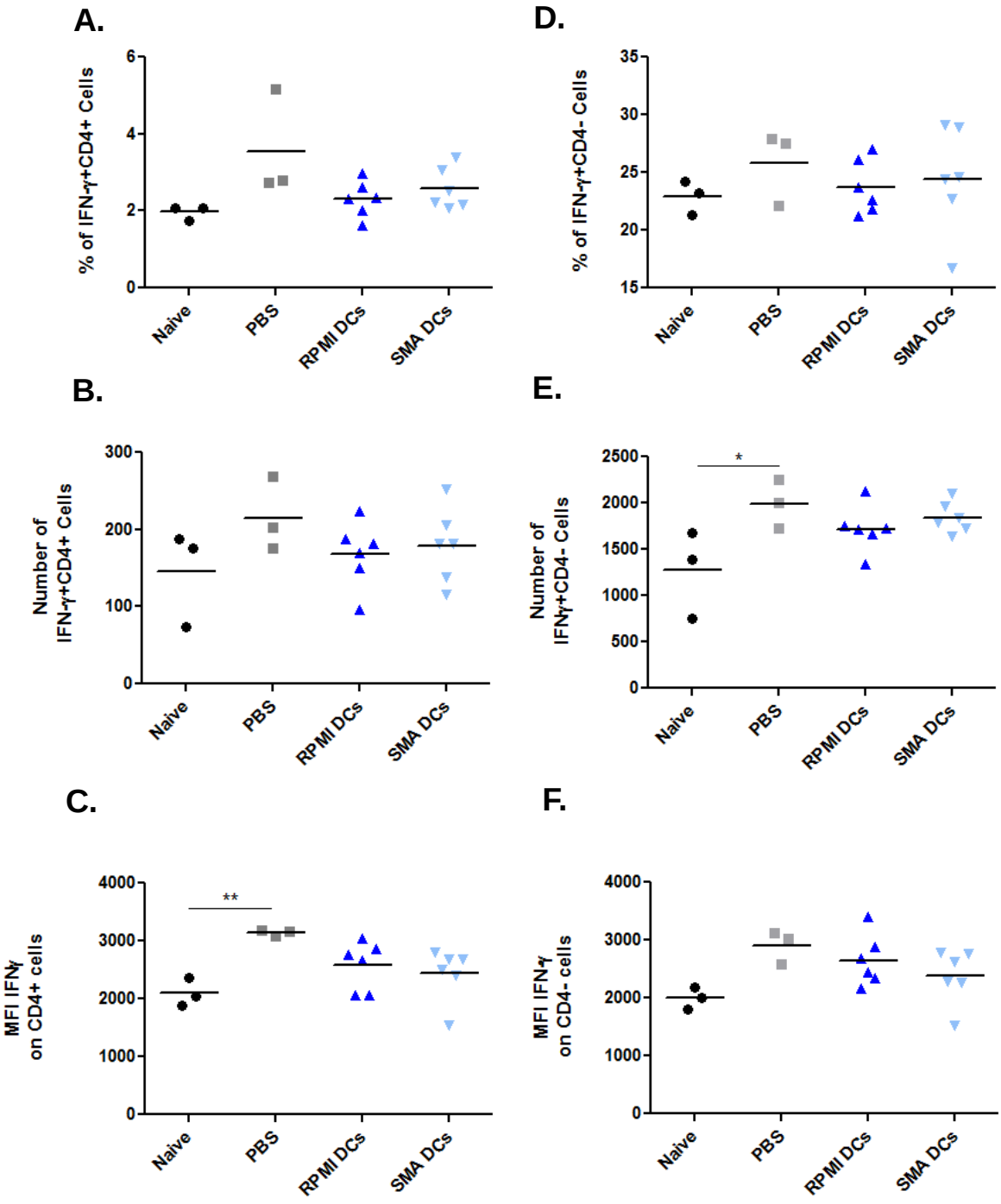

A.
% of IFN-γ+CD4+ Cells
0
2
4
6
Naive
PBS
RPMI DCs
SMA DCs
D.
% of IFN-γ+CD4- Cells
15
20
25
30
35
Naive
PBS
RPMI DCs
SMA DCs
B.
Number of IFN-γ+CD4+ Cells
0
100
200
300
Naive
PBS
RPMI DCs
SMA DCs
E.
Number of IFNγ+CD4- Cells
*
0
500
1000
1500
2000
2500
Naive
PBS
RPMI DCs
SMA DCs
C.
MFI IFNγ on CD4+ cells
**
0
1000
2000
3000
4000
Naive
PBS
RPMI DCs
SMA DCs
F.
MFI IFN-γ on CD4- cells
0
1000
2000
3000
4000
Naive
PBS
RPMI DCs
SMA DCs

Figure 5.17: Gating strategy for the analysis of T cell subsets

Cells were stimulated with PMA and Ionomycin for an hour before the addition of Brefeldin A for a further 4 hours. Cells were then stained for expression of CD4 (PerCP), FOXP3 (APC) and IL-10 (PE). Initially cells were gated based on size (FSC) and granularity (SSC); doublets were excluded by comparing FSC height and FSC area and the Fixable Viability Dye eFluor® (APC/Cy7) used to exclude dead cells (A). Relevant isotype and fluorescent minus one (FMO) controls (B) were used to determine the expression of CD4 on lymphocytes (C). Likewise, relevant isotype and FMO controls (D & F) were used to determine the expression of FOXP3 by $CD4^+$ cells (E) and also IL-10 by these $CD4^+FOXP3^+$ cells (G) These controls were also used to determine the expression of $CD4^+IL\text{-}10^+$ cells (H). The expression of IL-10 by $CD4^-$ cells was also determined (I).

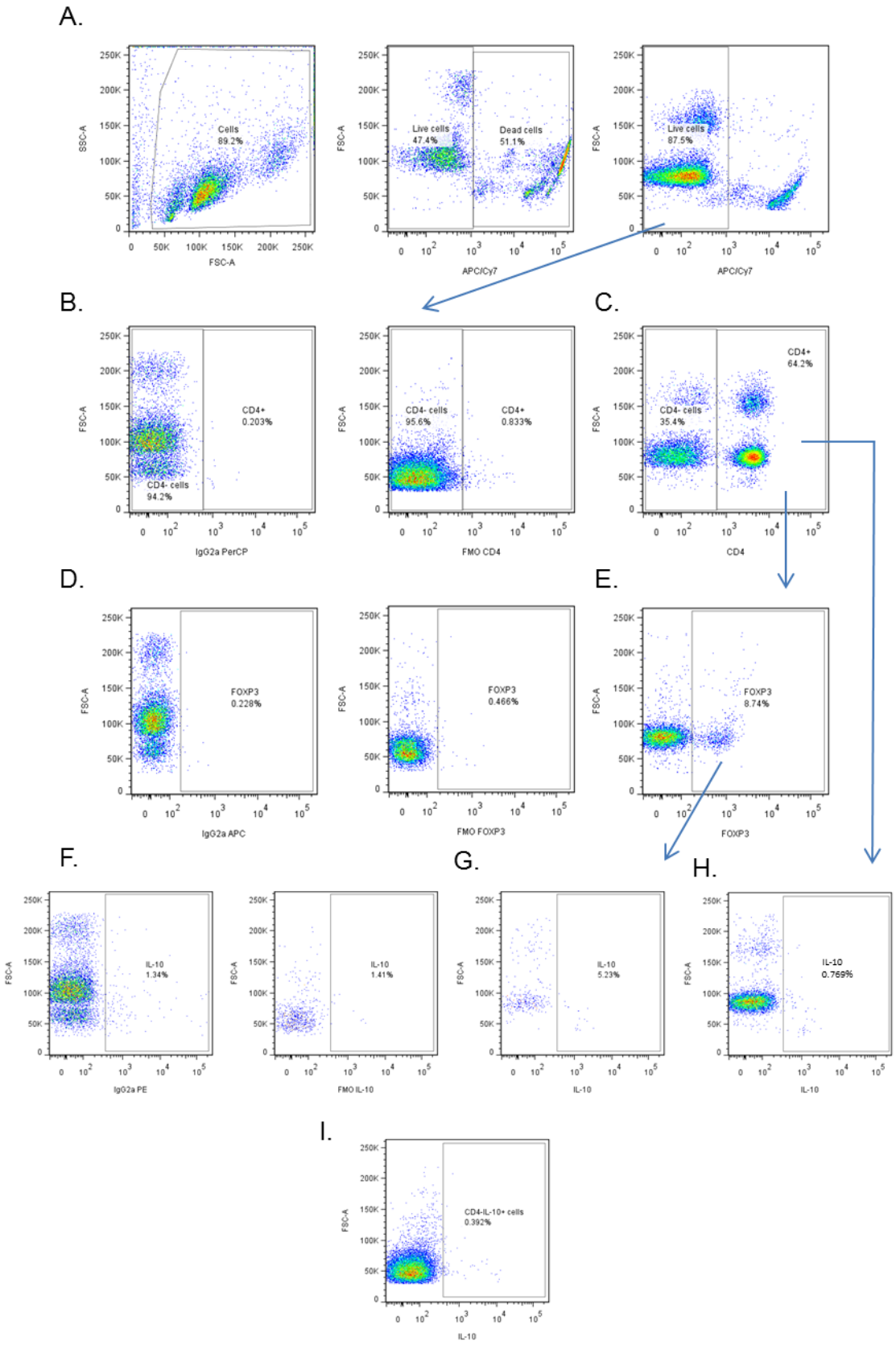

A.
Cells
89.2%
Live cells
47.4%
Dead cells
51.1%
Live cells
87.5%
B.
C.
CD4+
0.203%
CD4- cells
94.2%
CD4- cells
95.6%
CD4+
0.833%
CD4+
64.2%
CD4- cells
35.4%
D.
E.
FOXP3
0.228%
FOXP3
0.466%
FOXP3
8.74%
F.
G.
H.
IL-10
1.34%
IL-10
1.41%
IL-10
5.23%
IL-10
0.769%
I.
CD4-IL-10+ cells
0.392%

Figure 5.18: The effects of SMA-DC treatment on the regulatory T cell response in CIA

DBA/1 mice were immunised with CII on days 0 and 21 and were treated with PBS, RPMI DCs or SMA DCs on days -2, 0 and 21. On day 33 the dLNs of all four paws were collected and cells stimulated with PMA/Ionomycin for 1 hour prior to treatment with Brefeldin A for 4 hours. The expression of CD4, FOXP3 and IL-10 was analysed by flow cytometry and the percentage of $FOXP3^{+}CD4^{+}$ cells (A), $IL\text{-}10^{+}CD4^{+}$ cells (B) and $IL\text{-}10^{+}FOXP3^{+}CD4^{+}$ cells (C); the number of $FOXP3^{+}CD4^{+}$ (D), $IL\text{-}10^{+}CD4^{+}$ cells (E) and $IL\text{-}10^{+}FOXP3^{+}CD4^{+}$ (F), and the MFI of FOXP3 expression on $CD4^{+}$ cells (G), of IL-10 on $CD4^{+}$ cells (H) and of IL-10 on $FOXP3^{+}CD4^{+}$ cells (I) was determined. Results from individual mice in each group (naïve n=3, PBS n=3, RPMI DC n=6 and SMA DC n=6) are shown.

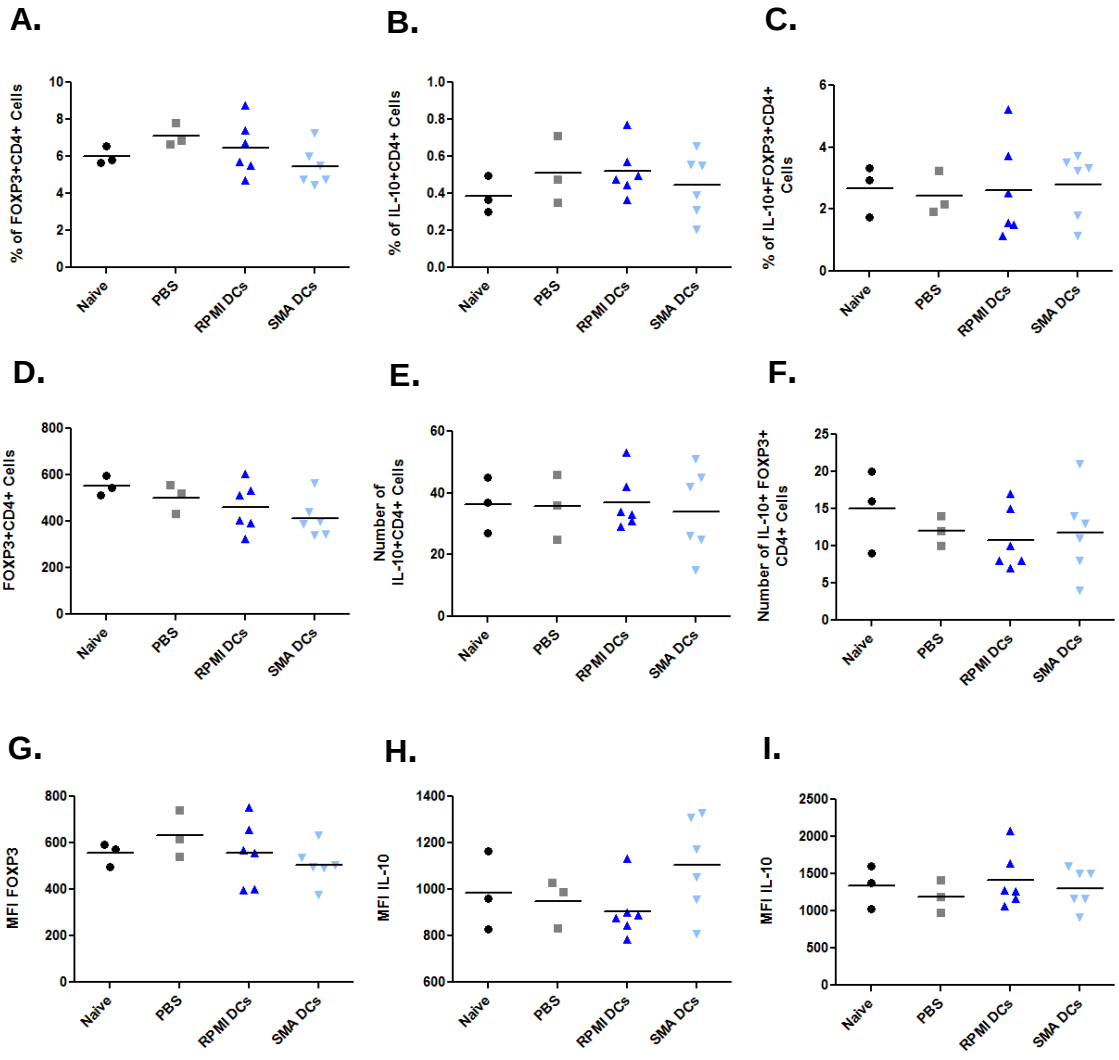
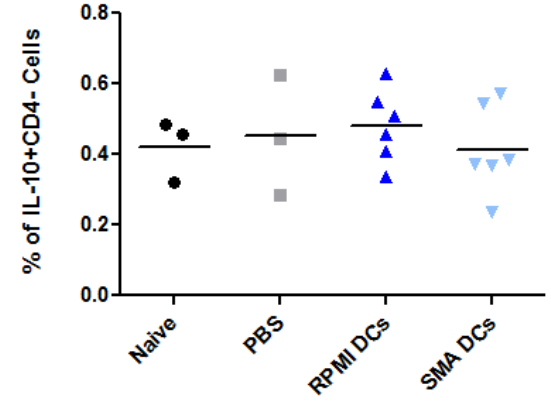

A.
% of FOXP3+CD4+ Cells
10
8
6
4
2
0
Naive
PBS
RPMI DCs
SMA DCs
B.
% of IL-10+CD4+ Cells
1.0
0.8
0.6
0.4
0.2
0.0
Naive
PBS
RPMI DCs
SMA DCs
C.
% of IL-10+FOXP3+CD4+ Cells
6
4
2
0
Naive
PBS
RPMI DCs
SMA DCs
D.
Number of FOXP3+CD4+ Cells
800
600
400
200
0
Naive
PBS
RPMI DCs
SMA DCs
E.
Number of IL-10+CD4+ Cells
60
40
20
0
Naive
PBS
RPMI DCs
SMA DCs
F.
Number of IL-10+ FOXP3+ CD4+ Cells
25
20
15
10
5
0
Naive
PBS
RPMI DCs
SMA DCs
G.
MFI FOXP3
800
600
400
200
0
Naive
PBS
RPMI DCs
SMA DCs
H.
MFI IL-10
1400
1200
1000
800
600
Naive
PBS
RPMI DCs
SMA DCs
I.
MFI IL-10
2500
2000
1500
1000
500
0
Naive
PBS
RPMI DCs
SMA DCs

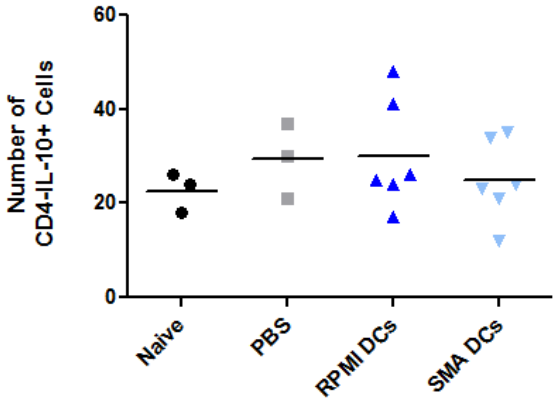
Figure 5.19: The effect of SMA-DC treatment on the $CD4^{-}IL\text{-}10^{+}$ response in CIA

DBA/1 mice were immunised with CII on days 0 and 21 and were treated with PBS, RPMI DCs or SMA DCs on days -2, 0 and 21. On day 33 the dLNs of all four paws were collected and cells stimulated with PMA/Ionomycin for 1 hour prior to treatment with Brefeldin A for 4 hours. The expression of CD4 and IL-10 was analysed by flow cytometry and the percentage of $IL\text{-}10^{+}CD4^{-}$ cells (A); the number of $IL\text{-}10^{+}CD4^{-}$ (B) and the MFI of IL-10 on $CD4^{-}$ cells (C) was determined. Results from individual mice in each group (naïve n=3, PBS n=3, RPMI DC n=6 and SMA DC n=6) are shown

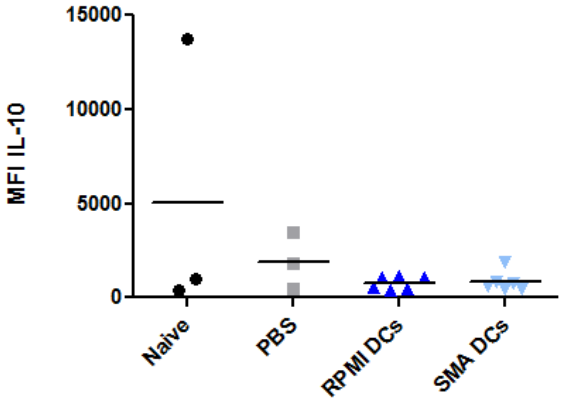
A.


% of IL-10+CD4- Cells
0.8
0.6
0.4
0.2
0.0
Naive
PBS
RPMI DCs
SMA DCs

B.


Number of CD4-IL-10+ Cells
60
40
20
0
Naive
PBS
RPMI DCs
SMA DCs

C.


MFI IL-10
15000
10000
5000
0
Naive
PBS
RPMI DCs
SMA DCs

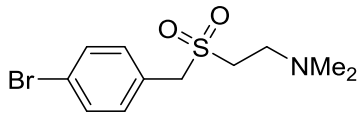
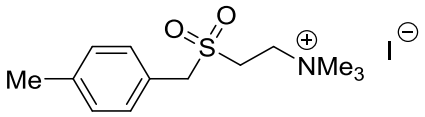
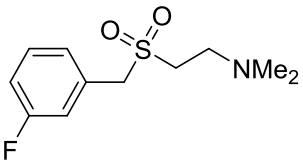
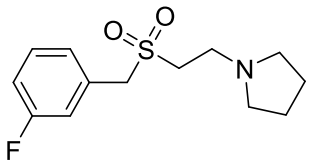
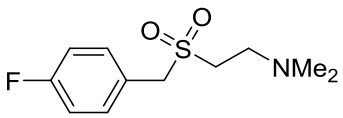
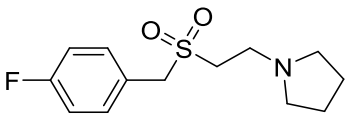
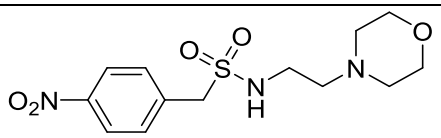
Chapter 6. Discussion

6.1 The effect of ES-62 SMAs on the cytokine profile of bmDCs

ES-62 is a potent immunomodulator secreted by the filarial nematode *A.viteae*, and has been demonstrated to be protective in several murine models of disease including rheumatoid arthritis and asthma (reviewed in [338]). However, it is a large and potentially immunogenic protein that, as yet, cannot be synthesised artificially, making it an unsuitable drug candidate. The immunomodulatory functions of this parasite product have been demonstrated to be dependent on the unusual post-translational addition of PC and therefore we have developed a library of small, drug-like molecules based on the PC moiety that could potentially mimic ES-62 actions [260]. Two of these SMAs (11a and 12b) have demonstrated ability to modulate macrophage and mast cell cytokine responses *in vitro* and are also protective in certain murine arthritis and asthma models [260], [261], [263]. It has now been shown that 11a and 12b can also inhibit PAMP-induced cytokine responses of bmDCs. In order to investigate whether any of the other SMAs had any effect on bmDCs the library was screened using an LPS-induced cytokine assay. SMAs 11a, 12b, 11e, 11h, 11i and 11k were all found to inhibit LPS-induced pro-inflammatory cytokine release and were therefore selected for further analysis. SMA 19o, which generally has little effect on macrophages or DCs was selected for use as a 'negative' control SMA.

SMAs 11e, 11h, 11i and 11k are sulfones, as are 11a and 12b, so it is perhaps not surprising that they can all exhibit similar effects on cytokine release. All contain a two-carbon methylene chain between the sulfone group and choline derivative but vary slightly in their aromatic substituents and secondary amide/choline derivative. 11a and 12b contain bromine and a methyl group respectively on their aromatic ring while 11e, 11h, 11i and 11k all contain fluorine in different positions around the ring (11e and 11h, 3-F and 11i and 11k, 4-F). The position of fluorine does not appear to have any significant effect on immunomodulatory activities but 11e and 11i, which are more consistent and more potent inhibitors of IL-6 and TNF-α, both contain the secondary amide NMe_2, while 11h and 11k contain pyrrolidine. 11a and 12b, which are the best characterised SMAs in all cell types, contain Me_2N and Me_3N^+ groups respectively and it appears that this category of secondary amide is more effective than pyrrolidine at causing cytokine inhibition in bmDCs. SMA 19o is a sulphonamide with NO_2 as its aromatic substituent and morpholine its secondary amide and so it is structurally quite different from the 6 inhibitory SMAs. The structure of the 7 selected SMAs is shown in Table 6.1, with the structures of all of the other SMAs shown in Appendix 1.

Table 6.1: The structures of the 7 selected SMAs along with their molecular weight (MW) and chemical formula

SMA	Structure	Formula/MW
11a		$C_{11}H_{16}BrNO_2S$ 306.22
12b		$C_{13}H_{22}INO_2S$ 383.29
11e		$C_{11}H_{16}FNO_2S$ 245.31
11h		$C_{13}H_{18}FNO_2S$ 271.4
11i		$C_{11}H_{16}FNO_2S$ 245.31
11k		$C_{13}H_{18}FNO_2S$ 271.4
19o		$C_{13}H_{19}N_3O_5S$ 329.37

SMAs 12b, 11e and 11i were also found to inhibit the BLP- and CpG-induced production of IL-6, IL-12 and TNF-α but the other SMAs under analysis demonstrated more selectivity in inhibiting responses to the different TLR ligands. SMAs 11a and 11h had no effect on the production of TNF-α following stimulation with BLP or IL-6 following exposure to CpG but caused inhibition of cytokine secretion in all other cases. SMA 11k had no effect on BLP- induced cytokine responses but significantly inhibited CpG-induced TNF-α and IL-12 production. Consistent with LPS data, 19o had no effect on any of the cytokines induced by BLP or CpG. Interestingly, neither 11a or 12b increased the production of IL-12p40, indicating that this effect could be an LPS-specific response These results suggest that, similar to data observed in the primary screen in macrophages [260], some of the SMAs, unlike ES-62, selectively target different TLR cytokine responses.

The observation that incubation with 11a or 12b, but none of the other SMAs, significantly increased the production of IL-12p40 by bmDCs (Figure 3.6) was unexpected and further demonstrated selectivity of the SMAs. IL-12p40 is the subunit of both bioactive IL-12 and the T_H17-associated cytokine IL-23 and for both cytokines, it is produced in excess over the second subunits, p35 and p19 respectively [297]. Although a cell must co-express both subunits in order to synthesise bioactive IL-12 [309] production of the two subunits is independently regulated. Manufacture of p40 is predominantly regulated at the level of gene induction while regulation of p35 expression occurs both transcriptionally and translationally [124]. The level of available p35 is thought to be the rate-limiting step in the production of IL-12 and so inhibiting the level of this subunit would have a significant effect on the ultimate production of the bioactive cytokine. IL-12p40 can exist as homodimers (IL-12p80), which can act to inhibit IL-12 signalling by competing for binding to IL-12R1 [346](Gillesson *et al*, 1995) and this could explain why 11a and 12b significantly enhance levels of this subunit. Consistent with protein data 11a and 12b, but none of the other SMAs significantly enhanced the level of p40 mRNA compared to that observed in untreated control samples after 4 or 18 hours incubation. Conversely, pre-exposure to 11a or 12b before LPS stimulation had no effect on p40 mRNA but significantly inhibited the LPS-induced increase in p35 mRNA suggesting 11a and 12b target the p35 subunit to inhibit IL-12p70 production. In contrast, 11e and 11i significantly inhibited the LPS-induced p40 and p35 increases but SMAs 11h, 11k and 19o had no effect on the levels of either subunit (Figure 4.6-4.8) indicating that the latter group may be utilising a different mechanism to mediate inhibition of IL-12p70.

Analysis of the mRNA levels of IL-6 and TNF-α after pre-exposure to SMAs 11a, 12b, 11e, 11h, 11i, 11k or 19o and subsequent LPS stimulation to mimic cytokine experiments revealed that 11a, 12b, 11e and 11i significantly inhibited LPS-induced IL-6 and TNF-α mRNA production suggesting that regulation of these cytokines occurs at the level of gene expression. Interestingly, treatment with 11h alone reduced the basal levels of IL-6, TNF-α, p40 and p35 but the SMA had no impact on the LPS-induced expression of these genes.

Taken together these results show that, similar to ES-62 and other helminth products (Hamilton *et al*, 2009; Massacand *et al*, 2009; McSorley *et al*, 2013 and Harnett *et al*, 2014;) the SMAs do not induce the production of cytokines by bmDCs and pre-exposure renders the cells refractory to full activation by TLR PAMPs.

Although the 6 selected SMAs on the whole demonstrate considerable ability to dampen PAMP-induced cytokine responses there is a lack of consistency within the *in vitro* system and such inconsistency was also noted in SMA screens in mast cells (Coates and Harnett, unpublished results). Because of this inconsistency it would seem difficult to predict which SMAs to select for *in vivo* work. However, despite this inconsistency the two 'original' SMAs (11a and 12b) selected from the macrophage screen (where only a single experiment rather than three assays was undertaken) have both proven to be very consistent and potent in a range of *in vivo* models of inflammation (collagen-induced arthritis; MRL/lpr mouse model of lupus; OVA model of airway hypersensitivity [260]–[264]) indicating that this somewhat crude method of screening is effective. That is not to say that it is always correct: SMA 11e was selected from the mast cell screen and is also efficient at modulating DC responses but had no effect in an experiment employing the OVA-induced asthma model (Coates and Harnett, unpublished). Whether it can modulate DCs *in vivo* would be interesting to investigate. The reason for this lack of consistency is unknown however it is not thought to be generally a result of the SMAs having a tendency to dissemble in solution, although 12b is thought to be converted into a vinyl sulfone, which may explain some of its ability to interact with Nrf2 [261]. One of the reasons for the lack of consistency in data may stem from the fact that primary cells were used for the studies and while the same protocol and recombinant growth factor were used throughout, conditions in each culture may be slightly different as reflected, for example, in the variability in the level of cytokine produced in response to PAMPs amongst experiments. In addition, ES-62 appears to work by targeting multiple cell types and its

surrounding environment has been shown to influence its effects *in vivo*. For example, in DCs ES-62 only modulates the expression of MyD88 in the presence of GM-CSF *in vitro* [312]. As 11a, 11e, 11i and 12b have been demonstrated to be active on a number of cell types [260], [261], [263] it seems likely that they target multiple cells *in vivo* and, like ES-62, their surrounding environment may be important. These signals would not be present in *in vitro* primary cultures and so unknown factors contributing to the DCs requirement for them (differences in basal "activation" amongst different DC populations?) may account for some of the observed inconsistencies.

6.2 The ES-62 SMAs do not require the receptor TLR4 or the signalling adaptor MyD88 to mediate their inhibitory effects on bmDC cytokine production

ES-62 is recognised by the immune system via TLR4 and this receptor is required for ES-62-mediated modulation of cytokine responses in APCs. Interestingly, ES-62 does not require a fully functional TLR4 as these responses are intact in C3H/HeJ mice, which have a Pro712His point mutation in the TIR domain of TLR4, which prevents conventional LPS signalling: this indicates that ES-62 uses TLR 4 in an atypical manner [246]. In addition ES-62 requires the signalling adaptor MyD88, and directly targets this molecule in multiple cells types. The ES-62 SMAs are based around the molecule's PC moiety and 11a and 12b have been shown to modulate MyD88 activity in macrophages [260], [261]. It was therefore investigated whether these SMAs also required TLR4 and MyD88 to mediate their immunomodulatory effects on cytokine gene expression and production.

SMA-mediated inhibition of BLP- and CpG-induced cytokine responses was, on the whole, intact in bmDCs from TLR4 KO mice indicating that the SMAs, unlike ES-62, do not require this receptor to modulate DC cytokine responses. To further investigate whether the SMAs interact with TLR4, the effect of incubation with SMAs, with and without LPS stimulation, on the surface expression of TLR4/MD2 was analysed. However, no consistent effect on the expression of TLR4 was observed and so it was not possible to draw any concrete conclusions about the effects of the SMAs on the surface expression of the PRR (data not shown). TLR4 is known to be continually internalised, degraded and new TLR4 recycled to the cell surface [347] and this may play a part in the difficultly in measuring expression at a given time. TLR4 is required for the internalisation of ES-62 by macrophages but not B cells [348] and ES-62 has also been shown to be active in Jurkat T cells and plasmacytoid DCs which do not express TLR4 ([348]; Steiger and Harnett, unpublished). In addition, ES-62 has been

shown to interact with both the soluble form of the mannose receptor and the PAF receptor (PAFR), although a biological significance has yet to be found for these interactions ([233] and unpublished results). Binding studies have also indicated that ES-62 interacts with a ~135 kDa and ~ 82kDa protein in lymphocytes but only a ~ 82 kDa protein in monocytes. These proteins are yet to be identified but such studies clearly show that ES-62 could utilise a number of receptors in addition to, or independent of, TLR4 to mediate its effects [348]. It is possible that the SMAs may interact with one or more of these, as yet unidentified proteins. More recent work focussing on the PAFR suggests that ES-62 may utilise this receptor on bmDCs for its internalisation and to promote at least in part cytokine modulation via its PC moiety (Eason and Harnett, M, unpublished). As the SMAs are designed around PC it is also possible that they could also interact with the PAFR and this could be an interesting avenue for further research. Moreover, ES-62 is internalised in a complex with TLR4 in mast cells resulting in degradation of PKCα [250], revealing that the parasite product ultimately mediates effects within the cell as well as through receptor modulation. Many of the SMAs have been designed to be accessible to cells through passive diffusion so it is possible they can enter directly and may not require a receptor at all. As the SMAs have been shown to be active in the absence of TLR4 and they also inhibit TLR2 and TLR9 responses it would additionally be interesting to investigate the effects of the SMAs on non-TLR cytokine responses. FhTeg, which appears to have a similar effect on DCs as the SMAs, was demonstrated to inhibit cytokine responses induced by both TLR and non TLR PAMPs [195].

As the SMAs did not appear to require TLR4 it was next investigated whether they required the adaptor MyD88 to mediate cytokine inhibition. ES-62 requires MyD88 for many of its anti-inflammatory effects and indeed directly targets this adaptor in multiple cells to modulate cell signalling [211], [247], [261]. SMAs 11a and 12b have been shown to down-regulate expression of this adaptor in macrophages [260], [261] and so it was perhaps surprising to find that the SMA-mediated inhibitory effect on LPS-induced IL-6 and IL-12 was intact in MyD88 KO bmDCs. MyD88, did, however, appear to be required for inhibition of LPS-induced TNF-α production. It should be noted however that generation of a strong cytokine response through LPS stimulation requires activation of both the MyD88-dependent and independent pathways and therefore the cytokine response to LPS in MyD88 KO bmDCs was much lower than in WT bmDCs. Indeed, in the case of IL-12p70 there was no significant difference between cells maintained in RPMI and exposed to LPS.

Perhaps in keeping with the results obtained when measuring cytokine levels in MyD88 KO bmDCs, none of the SMAs were found to modulate the MyD88 levels compared to RPMI control bmDCs after 1, 4 or 18 hours. Previous experiments with ES-62 in DCs, however, demonstrated that MyD88 was only down-regulated by the nematode product in the presence of the inflammatory mediator, GM-CSF [338]. This suggests that ES-62 'senses' the surrounding environment and acts to inhibit aberrant inflammation. This has also been noted with its effect on IL-22 expression in CIA – in initial stages of disease where IL-22 is pathogenic, ES-62 works to reduce the level of cytokine but after disease onset IL-22 works to dampen joint inflammation and ES-62-mice have an increase in this cytokine at this point [337]. In the case of the SMAs however, no effect on MyD88 expression was observed in the presence of GM-CSF, with or without LPS stimulation. To corroborate these findings the ability of the SMAs to inhibit cytokine production induced by stimulation of the MyD88-independent pathway through activation of TLR3 by PolyI:C was investigated. However, with the exception of IL-12p40, stimulation of bmDCs with PolyI:C caused very low or undetectable levels of cytokines. IL-12p70 was not detected in any experiments and stimulation of bmDCs with PolyI:C only resulted in a significant increase in IL-6 and TNF-α production in 1/3 experiments. Interestingly, in this experiment all of the SMAs were able to significantly inhibit these PolyI:C-induced cytokine responses (data not shown). Consistent with results observed following LPS stimulation (Figure 3.6), 11a and 12b both significantly increased the amount of IL-12p40 produced after PolyI:C stimulation. None of the other SMAs had any effects on IL-12p40 production following PolyI:C stimulation (data not shown). It is not possible to draw any concrete conclusions from these data due to lack of reproducible cytokine production following stimulation with PolyI:C but the results are suggestive of SMAs 11a, 12b, 11e and 11i being able to modulate the MyD88-independent signalling pathway. Further work would be required to confirm this hypothesis. TLR3 stimulation results in the production of type 1 interferons as well as pro-inflammatory cytokines and so analysis of the production levels of the former after SMA and PolyI:C treatment may yield more consistent results. In any case, taken together these results indicate that the SMAs can act independently of MyD88 to mediate their inhibitory effects on bmDC cytokine production and as reported earlier do not appear to have any effect on myD88 levels within the cells. The latter result is in contrast to results in macrophages where both 11a and 12b were found to down-regulate MyD88 expression [260], [261].

To attempt to consolidate the KO cytokine results the effects of SMAs 11a and 12b on the mRNA levels of IL-6, TNF-α and IL-12 were investigated in WT, TLR4 KO and MyD88 KO bmDCs (results not shown). As before, bmDCs were stimulated with SMAs alone for 4 or 18 hours, or in combination with LPS for 4 hours or cells were pre-treated with the SMAs overnight and then stimulated with LPS. Unfortunately, there was not enough RNA recovered from two of the three WT experiments that incorporated 4 hours of SMA exposure and so it is not possible to reliably compare the 4 hour results. Only 11a and 12b were investigated as there was a limited number of KO and matched WT bmDCs available on completion of the protein experiments and as these SMAs were, at the time, the best characterised in investigating bmDC responses, they were chosen. Consistent with experiments measuring cytokine protein, 11a or 12b incubation alone for 18 hours had the same effect in WT and TLR4 KO bmDCs, confirming the SMAs do not require this receptor to mediate their effects. In MyD88 KO bmDCs, contrary to protein data, the significant increase in IL-6 gene expression observed with incubation of SMAs alone in WT cells was absent, as was the significant inhibition of LPS-induced IL-6 gene expression. The levels of IL-6 mRNA stimulated by LPS were very low compared to WT cells however and this may be the reason that the slight reduction attributable to the SMA was not statistically significant (data not shown). SMAs 11a and 12b were shown to be independent of TLR4 but dependent on MyD88 for inhibition of PAMP-induced TNF-α production (Figure 4.6 and 4.7) and to an extent this is mirrored in the KO mRNA experiments. Treatment of WT bmDCS with 11a alone for 18 hours caused a significant decrease in TNF-α mRNA which was not observed in either KO bmDCs while incubation with 12b had no effect on the basal TNF-α level in WT bmDCs, but significantly inhibited TNF-α expression in both KO bmDCs. Consistent with mRNA experiments in BALB/c bmDCs, pre-treatment with 11a significantly inhibited LPS-induced TNF-α gene expression in WT cells but surprisingly 12b had no effect. In MyD88 KO bmDCs both SMAs caused a slight reduction in TNF-α mRNA levels but this was not significant (data not shown). Both SMAs caused a significant increase in basal p40 gene expression in WT, which was not observed in either KO and, interestingly, in the WT bmDCs, which are C57-BL/6 background, both SMAs significantly inhibited the LPS-induced IL-12p40 expression, which again highlights the differences between mouse strains. As with IL-6 and TNF-α there were no significant changes in p40 mRNA in MyD88 KO bmDCs following LPS stimulation. SMA 11a however did inhibit LPS-induced IL-12p35 production in both WT and MyD88 KO bmDCs and 12b caused a reduction in both (only significant in WT) (data not

shown) which could account for the inhibition of IL-12 production observed in MyD88 bmDCs previously (Figure 4.8). Overall, it is difficult to draw any concrete conclusions from the KO mRNA experiments as the SMAs do not appear to always have the same effect on cytokine gene expression in WT and KO bmDCs but they are still having an effect in KO bmDCs suggesting that they can act without TLR4 and MyD88. It may be useful to repeat these experiments with more replicates to improve consistency between experiments and also to investigate the effects of the other two potent SMAs – 11e and 11i in these experiments.

6.3 The effect of the SMAs on the activation of bmDCs

The activation of transcription factor NF-κB through ligation of TLR4 by LPS is crucial for the production of pro-inflammatory cytokines in APCs. Pro-inflammatory cytokine production through TLR4 requires early and late phase signal transduction through signalling adaptors MyD88 and TRIF respectively. Activation of NF-κB depends upon the degradation of IκB-α and -β and in DCs LPS was demonstrated to induce the early degradation of both proteins. In contrast, ES-62 appeared to up-regulate the expression of IκK-β (Eason & Harnett MM, unpublished), degradation of which is required for delayed NF-κB activation, and therefore this up-regulation may account for the attenuation of protracted endotoxin responses by ES-62. Interestingly, ES-62 pre-exposure did not have any effect on subsequent LPS degradation of these proteins. Addition of GM-CSF, a key inflammatory cytokine, altered the expression profile induced by ES-62 on IκB-α and -β with the nematode product inducing the up-regulation of IκB-β in response to LPS (Eason & Harnett MM, unpublished). These results indicate that ES-62 can modulate the activation of NF-κB in DCs; although the differences are slight suggesting this is not a dominant form of regulation. They also serve as further evidence that the inflammatory environment of ES-62 plays a role in determining its immunomodulatory effects, especially in the case of DCs. In mast cells ES-62 suppresses the FcεR1-mediated activation of NF-κB subunits p50 and p65 [250] confirming NF-κB is an ES-62 target. Inhibition of NF-κB activation in APCs is also a mechanism used by other parasites to modulate immune responses including *F. hepatica*, *Brugia malayi* and *Toxoplasma gondii* [195] [349], [350]. Pre-exposure to SMAs 11a, 12b or 11e for 18 hours inhibited the LPS-increased levels of p65 in the nucleus of bmDCs, consistent with their ability to attenuate LPS-induced cytokine responses (Figure 4.15). SMA 11i had no consistent effect on NF-κB activation which is

perhaps unexpected considering it causes significant inhibition of cytokine responses but SMA 19o, which has no consistent inhibitory effect on PAMP-induced cytokine production, except for LPS-induced IL-12, caused a slight reduction in the levels of p65 detected in the nucleus after LPS stimulation. This could suggest that while inhibiting NF-κBp65 activation could contribute to the down-regulation of PAMP-induced cytokine produciton by 11a, 11e and 12b, the transcription factor is unlikely to be the most important target. It has not yet been investigated whether the SMAs target other members of the NF-κB family, such as p50, or other transcription factors such as IRF5, which are also activated by stimulation with LPS.

ES-62 differentially activates MAPKs to mediate its anti-inflammatory effects on cytokine production in macrophages. For example, ES-62 induces ERK MAPK activation to negatively regulate p40 production while suppressing the p38 activation required for manufacture of p35, IL-6 and TNF-α [124]. Consistent with this, SMAs 12b, 11e and 11i significantly inhibited the LPS-induced activation of p38 in bmDCs, which corresponds with their ability to inhibit IL-12, IL-6 and TNF-α production as well as manufacture of p35, IL-6 and TNF-α mRNA. 11a also caused a reduction in p38 activation but this was slight, so while this effect could contribute to the overall inhibitory effects of 11a it is not likely to be the dominant form of regulation. In contrast to ES-62, however, these SMAs also suppressed LPS-induced ERK activation as evidence by reduced levels of pERK in bmDCs. ERK activation has been linked to T_H2 responses and has been shown to be preferentially phosphorylated by helminth products, such as LNFPIII, a helminth glycan found in soluble schistosome egg (SEA), to help drive the T_H2 response conducive to their survival [351]. The results therefore suggest that the SMAs could target both T_H1 and T_H2 responses through modulation of MAPK activation.

ES-62 has also been demonstrated to target the E3 ligases TRAF6 and c-Cbl, molecules which play key roles in signal transduction down-stream of MyD88, demonstrating that the parasite protein is capable of modulating multiple signalling targets to suppress inflammatory signalling via TLR4. Treatment with ES-62 decreases the levels of TRAF6 detected in DCs and this is enhanced in the presence of GM-CSF, indicating it to be a key target of ES-62 during inflammation (Eason & Harnett MM, unpublished). As TRAF6 is a crucial player in both MyD88-dependent and independent pathways of TLR4 activation and it appears the SMAs are capable of targeting MyD88-independent responses, it would be interesting to investigate the effect of the SMAs on

TRAF6 expression. In addition, the Protein Kinase C (PKC) family of signal transducers represent another key target of ES-62 in down-regulating the cytokine responses of cells. In mast cells, ES-62 causes the sequestration and degradation of PKCα thereby inhibiting cytokine production [250] and may also induce expression of PKC-ε, a negative regulator of cytokine production [251]. ES-62 has also been shown to degrade PKCs in dendritic cells (Eason & Harnett MM, unpublished) and it would therefore be interesting to investigate whether these enzymes are also targeted by the SMAs.

6.4 The effect of the SMAs on the phenotypic maturation of bmDCs and their subsequent ability to prime T cell responses

DCs initiate the immune response to pathogens through the presentation of antigen in the context of MHC II. This, along with co-stimulation through CD80, CD86 and CD40, and the cytokines they secrete provides the necessary signals to activate naïve T cells and so bridge the innate and adaptive immune systems. The phenotype and activation status of the presenting DC can alter the outcome of T cell activation, priming towards different T cell phenotypes such as T_H1, T_H17 or T_H2. Helminth infections usually promote a strong T_H2 response and many helminth antigens have been demonstrated to modulate DCs to help drive this response. ES-62 was the first helminth product to be reported to drive DCs towards a T_H2 phenotype, characterised by a reduced ability to produce pro-inflammatory cytokines IL-12p40 and TNF-α, and lower surface expression of co-stimulatory molecules compared to LPS-matured DCs [243]. Consistent with these studies the treatment of bmDCs with SMAs did not result in up-regulation of co-stimulation molecules CD80, CD86, CD40 or MHC II compared to unstimulated bmDCs. In fact, SMAs 11a, 12b, 11i and 11e significantly reduced the percentage of $CD80^+$ cells. Pre-treatment of bmDCs with SMAs 11a, 12b and 11e also inhibited the LPS-induced up-regulation of CD86 and CD40. In addition, SMA 11i inhibited LPS-induced CD86 expression (Figure 3.10). Overall there were no differences in MHC II expression or percentage of cells expressing MHC II between SMA-treated bmDCs and untreated cells. Nor were there any differences in MHC II observed when cells, SMA-treated or otherwise, were stimulated with LPS. These results indicate that the SMAs suppress the maturation phenotype of bmDCs but do not affect their peptide load/antigen presentation ability, and support previous data (Figure 3.4-3.6; 3.10) indicating that treatment with SMAs alone does not induce classical maturation of bmDCs. Consistent with previous studies with ES-62, pre-treatment of bmDCs with SMAs 11a, 12b, 11e or 11i before LPS stimulation resulted in a

significantly decreased ability to prime naïve T cells to produce IFN-γ (Figure 5.1). Treatment of bmDCs *in vitro* with SMA 11a or 11i alone or before LPS stimulation was also found following inoculation to block Ag-specific T cell expansion *in vivo*, associated with reduced T cell activation and suppressed numbers of IFN-γ^+ CD4 T cells and IL-17^+ CD4 T cells (Figures 5.5-5.9).

The precise mechanisms by which SMAs drive bmDCs to modulate T cell expansion *in vivo*, and how pre-exposure to these SMAs attenuates the ability of LPS-stimulated DCs to produce the T_H1 cytokine, IFNγ, *in vitro* are still being investigated. LPS is a classic DC activator, inducing a strong T_H1 phenotype, and consistent with this, bmDCs in this study stimulated with LPS up-regulated co-stimulatory molecules CD40 and CD86, produced high levels of pro-inflammatory cytokines IL-12, IL-6 and TNF-α, and subsequently induced a strong T_H1 response. Attenuation of IFN-γ production by T cells activated by bmDCs exposed to SMAs before LPS stimulation likely reflects the reduced pro-inflammatory cytokine production and decreased surface expression of certain co-stimulatory molecules by these bmDCs. It would be interesting therefore to investigate whether the SMAs target any of the molecules of the T_H1 polarisation signalling pathway such as the transcription factors STAT1, STAT4 or T-bet.

As discussed, the expression of co-stimulatory molecules CD40, CD80 and CD86 is important in T cell proliferation and polarisation. CD80 and CD86 signal through the same receptors on T cells – CTLA4 or CD28 which can either suppress or activate T cells respectively. ES-62-matured DCs suppress T_H1 responses while driving a T_H2 phenotype *in vivo* even though reducing both CD80 and CD86 expression suggesting that these molecules are not crucial to the subsequent T cell polarisation. Indeed, Whelan *et al* demonstrated through the use of neutralising antibodies against CD80 and CD86 that these molecules are required for both IL-4 and IFN-γ production indicating they are more significant for T cell proliferation rather than polarisation. Both CD80 and CD86 have been found to be important in strengthening the interaction between APCs and T cells as blocking these molecules resulted in reduced T cell activation as evidenced by lower production of IL-2 [352]. Consistent with this, both SMA 11a and 11i significantly inhibit LPS-induced CD86 and CD80 expression on bmDCs and cells treated with these SMAs either alone or before being stimulated with LPS, significantly reduce T cell expansion *in vivo.*

Activation of bmDCs by LPS resulted in significant up-regulation of CD40 on bmDCs. The expression of CD40 was differentially affected by the SMAs – pre-exposure to 11a,

12b or 11e significantly inhibited this up-regulation but SMAs 11i and 19o had no effect on this molecule. CD40 is expressed on a wide variety of cells such as DCs, B cells, macrophages and non-haematopoietic cells and CD40-CD40L interactions regulate many aspects of the immune response including DC and B cell activation, DC-T cell interactions and germinal centre formation [159]. Interaction with CD40L on T cells by CD40 on DCs promotes the production of IL-12 by the latter cell-type [353], further promoting the development of a T_H1 phenotype. In contrast, helminth–matured DCs typically show reduced expression of CD40 compared to that induced by stimuli such as LPS. For example, DCs matured with SEA antigen had limited expression of CD40; however, this does not impair their ability to induce an activated T cell response *in vivo*. Indeed by lowering CD40 expressing SEA likely reduces IL-12 production and so helps to drive the T_H2 phenotype [330]. Thus, the reduction of T_H1 responses by SMAs 11a, 12b and 11e could be a result of decreased CD40 expression. CD40 also induces the up-regulation of co-stimulatory molecules CD80 and CD86 and therefore by lowering the expression of CD40, SMAs 11a, 12b and 11e could also be contributing to the corresponding decrease in LPS-induced up-regulation of CD86 on these cells. Interestingly, $CD40^{-/-}$ mice were unable to mount a T_H2 response to *S. mansoni* [354] so perhaps the suppression of CD40 by the SMAs could be targeting T_H2 as well as T_H1 responses. SMA 11i, which modulates the expression of CD80 and CD86, and also inhibits IFN-γ production by $CD4^+$ T cells does not attenuate the LPS-induced CD40 expression on bmDCs. NF-κB plays a role in the activation of many processes in DCs including the up-regulation of CD40 [163] and the suppression of CD40 by 11a, 12b and 11e could be mediated through the down-regulation of NF-κBp65 and therefore this lack of CD40 suppression by 11i could be explained by its inability to modulate NF-κBp65 activation (Figure 4.10). Of relevance, BmDCs grown in the presence of an inhibitor of NF-κB have reduced CD40 but still express CD86 and MHC II [355].

Pre-exposure of bmDCs to SMAs 11a or 11i before stimulation with LPS *in vitro* significantly inhibits the number of Ag-specific $IL\text{-}17^+CD4^+$ T cells following transfer of the DCs *in vivo*. The exact mechanisms underlying this suppression are unknown, however, pre-exposure of bmDCs to these SMAs before LPS stimulation results in significant decrease in TNF-α and IL-6, key cytokines required for the differentiation of T_H17 cells [74]. In addition, SMA 11i–treated bmDCs have significantly reduced IL-12p40 mRNA upon LPS stimulation and p40 constitutes one of the subunits of IL-23, a cytokine that is important for the maintenance of T_H17 cells *in vivo*. While IL-23 was not

detected in these experiments, it has previously been demonstrated by our laboratory that bmDCs treated with ES-62 [211] and SMA 11a [260] show a reduced capacity to produce IL-23 upon LPS stimulation. Given these results it is likely that bmDC pre-exposure to SMA 11a or 11i modulates the subsequent LPS response towards a cytokine profile that does not support T_H17 development. Suppression of T_H17 responses by helminth-modulated DCs has only previously been reported by Dowling *et al* who demonstrated that two molecules from *F. hepatica* ES partially activate DCs, which then attenuate Ag-specific T_H17 responses *in vivo* [196]. Interestingly neither of these molecules induced DCs to drive T_H2 responses *in vivo*, which is also consistent with our data with SMA-modulated DCs. None of the SMAs, unlike ES-62, appeared to promote the production of IL-4 either *in vitro* or *in vivo* suggesting that while they can suppress T_H1/T_H17 responses they may not actually drive the 'opposing' T_H2 phenotype. This is in contrast to the effect on DCs achieved by the majority of helminth products. For example, DCs primed with SEA drive the production of IL-4 by T cells *in vitro* [330], and NES-modulated DCs, when transferred into naïve recipient mice, can prime these mice for T_H2 responsiveness [198]. However, the SMAs 11a, 12b and 11e have been shown to suppress ERK MAPK activation and the surface expression of CD40 during inflammation induced by LPS, both of which have been reported to play roles in the induction of a T_H2 phenotype and therefore it seems possible that the SMAs drive a suppressive rather than polarising response in DCs.

6.5 The effect of SMA-modulated bmDCs on CIA

The use of *in vitro* helminth-modulated DCs to treat or modulate *in vivo* disease has been demonstrated in several murine models of disease indicating that DCs can play a central role in the pathogenesis of autoimmune diseases such as arthritis, EAE and colitis [331], [332], [344]. SMAs 11a and 12b have both been shown to reduce CIA severity in mice and also to have potent immunomodulatory effects on bmDCs *in vitro* and so it was investigated whether bmDCs matured with a combination of both SMAs could modulate CIA disease in mice. Arthritis was induced in DBA/1 mice and mice were treated with PBS, immature DCs (RPMI DCs) or DCs treated with 11a and 12b for 18hours (SMA DCs). Mice treated with SMA DCs were found to have significantly lower disease compared to PBS- and RPMI DC- treated mice as evidenced by reduced arthritic score.

IL-17 is an important pro-inflammatory cytokine expressed in the synovium and synovial fluid of RA patients [356]. It is also involved in the initiation and progression of

murine experimental arthritis and IL-17-deficient mice show reduced incidence and severity of disease [357]. SMA DC-mediated protection was associated with significantly lower numbers of IL-17$^+$CD4$^+$ cells and IL-17$^+$CD4$^-$ cells in the draining lymph nodes compared to PBS- and RPMI DC-treated mice. It would be interesting to investigate the specific CD4$^-$IL-17$^+$ cells targeted by SMA-DCs but these data indicate that SMA DCs could target T_H17 responses to ameliorate disease and are consistent with previously published work within our lab group that both ES-62 and 11a target a complex network of IL-17 responses to mediate their anti-inflammatory effects in CIA [211]. IL-17 was also decreased in the joints of mice treated with *F. hepatica* total extract and matured with CpG (FTegDCs), and dLN from these mice showed reduced levels of IFN-γ and IL-17 when stimulated *ex vivo* with CII [331], indicating that suppression of IL-17 responses may be a common mechanism by which helminths can ameliorate CIA. IL-22 is a T_H17 associated cytokine [358] that appears to have a dual pro- and anti-inflammatory role in asthma and several autoimmune diseases including arthritis [337], [359]. Previous work by our laboratory group has shown that IL-22 is important during initial establishment of disease in CIA but at later stages it acts to promote restoration of joint inflammation by suppressing IL-17 responses [337]. ES-62 enhances IL-22 expression after onset of disease and ES-62-mediated protection can be blocked by neutralising anti-IL-22 antibodies [337]. Based on these results it was therefore investigated whether SMA DCs affected IL-22 expression during CIA in the dLNs. Surprisingly, however, IL-22 was not detected in any of the lymph nodes from any of the mice in this study (data not shown). CIA pathology is not solely mediated through T_H17 responses. The T_H1 cytokine IFN-γ is also elevated during CIA and ES-62 has previously been shown to reduce IFN-γ. Consistent with this, PBS-treated control mice had increased levels of IFN-γ and while not significant, SMA DC-treatment lowered the numbers of IFN-γ$^+$CD4$^+$ cells in the dLNs of CIA mice. It was noted however that this effect was also observed with RPMI DCs raising questions as to whether SMA treatment *per se* is actually having any effect in this context. PBS-treated mice also had significantly increased percentages of CD4$^-$IFN-γ$^+$ cells but neither SMA DCs nor RPMI DCs could affect this response.

The induction of IL-10-producing T regulatory cells is a key mechanism utilised by several helminths in order to prolong their survival within the host [360]. Previous studies, which have demonstrated that helminth-matured DCs can modulate inflammatory disease, have reported an increase in immunomodulatory components such as IL-10 or generation of Treg cells. For example, adoptive transfer of DCs

matured with *Hymenolepis diminuta* antigen confers protection against the development of experimental colitis and this is dependent on the production of IL-10 by adaptive immune cells [332]. Also, protection through transfer of FTegDCs was associated with an increase of $FOXP3^+CD25^+CD4^+$ Treg cells, which were able to confer protection when transferred into recipient mice [331]. It was therefore investigated if SMA 11a- and 12b- matured bmDCs also mediated their protective effect through the generation of T regulatory cells. The level of $FOXP3^+CD4^+$ cells also expressing IL-10 was determined in the dLNs of CIA-mice treated with PBS, RPMI DCs or SMA DCs. In contrast to other models of helminth modulation, there were no differences in the numbers of $IL\text{-}10^+FOXP3^+CD4^+$ cells in the dLNs of any of the mice indicating this is not a mechanism employed by SMA-treated DCs to down-regulate CIA. This is consistent with previous findings with ES-62 and the SMAs, as they have not been found to promote T regulatory responses but rather to act on effector cells to suppress inflammatory responses.

ES-62 has been shown to modulate B cells during CIA by blocking follicular B cell activation and therefore their differentiation into germinal centre B cells as well as inducing IL-10-producing B cells in the joints [311]. It was therefore investigated if SMA DCs could affect levels of $IL\text{-}10^+$ B cells in the draining lymph nodes. Unfortunately however, no $CD19^+$ cells were detected in this experiment, likely due to an antibody problem during staining as $CD19^+$ cells were detected using a different antibody for staining controls. The expression of IL-10 on $CD4^-$ cells was then analysed to investigate if treatment with SMA-modulated DCs had any effect on $IL\text{-}10^+$ populations. As with $IL\text{-}10^+FOXP3^+CD4^+$ populations there were no differences in the numbers or proportion of $IL\text{-}10^+CD4^-$ cells in the dLN of CIA mice treated with PBS, RPMI DCs or SMA DCs suggesting that SMA DCs do not induce IL-10.

Protection was also not associated with a difference in antibody responses between SMA DC-treated mice and PBS or RPMI DC-treated mice. ES-62 has previously been shown to skew the humoral immune response from an IgG2a-dominant to IgG1-dominant anti-CII response [254]. However, PC-BSA, which has been shown to mimic ES-62 protection in CIA and provided the basis for the design of the SMAs, did not have any effect on antibody responses while recombinant ES-62, which lacks PC, did affect the antibody levels suggesting this effect may be a function of the protein backbone of ES-62 [255].

This study has only been conducted once, but it provides positive indication that modulation of bmDCs with SMAs *in vitro* can alter CIA outcome *in vivo*. SMAs 11a and 12b, despite having similar effects on bmDCs *in vitro*, display discrete mechanisms of action in CIA with 11a closely mimicking ES-62 by targeting IL-17 responses whilst 12b primarily acts through IL-17-independent mechanisms including modulation of IL-1β. With this in mind it would also be prudent to analyse the expression of IL-1β in SMA DC-treated mice as well as the T_H17 responses. If SMA DCs are confirmed to be able to ameliorate CIA *in vivo* it would then be interesting to investigate the effects of single SMA treatment on bmDCs *in vitro* on CIA disease outcome utilising a similar immunisation schedule. SMA 11i was also found to be effective at suppressing the expansion of Ag-specific T cells *in vivo* compared to immature DCs and so it would also be interesting to investigate if this SMA could modulate CIA disease. Furthermore, these data highlight the importance of DCs in the initiation of CIA disease and also provide insight into the mechanism of SMA-mediated protection against CIA, particularly with respect to 11a as protection via SMA-DCs closely mimics the mechanisms by which administration of this SMA protects mice from CIA. Moreover, in addition to adding to our knowledge of the mechanism of SMA-mediated protection, these data pinpoint DCs as a potential therapeutic target in RA. This is useful as the drugs available against RA are still inadequate, for example, while cytokine blockers such as infliximab targeting TNF-α have had some success the number of patients achieving remission remains low [338]. Thus, new therapeutics are required, and the SMAs, due to their anti-inflammatory properties, allied to ease and low cost of production are attractive drug candidates. It is therefore important to understand how they function and the cells they target and this study provide some answers to these questions. Indeed, even if the SMAs are not ultimately developed into RA drugs, understanding the pathways targeted by these molecules may define novel therapeutic targets.

6.6 Conclusions

Parasitic helminths can survive within their hosts for years due to their ability to modulate the immune response to avoid clearance and prevent host damage due to aberrant inflammatory responses. This has led to the use of helminths are potential therapeutics in diseases associated with excessive inflammation such as rheumatoid arthritis. Despite a growing body of evidence both in animal models of such diseases and preliminary human studies suggesting that helminths are protective against allergic and autoimmune diseases as well as continuing identification of defined helminth derived immunomodulatory molecules and elucidation of mechanism of action, no anti-inflammatory drugs have been developed. We have recently described the design of a library of SMAs based on the active PC moiety of the helminth protein ES-62 [260]. Two of these SMAs have been demonstrated to be active in macrophages *in vitro* and in our arthritis and lupus murine models of disease [260]–[264]. In this thesis the immunomodulatory actions in bmDCs of these two SMAs and several others screened from the library is described. Overall pre-exposure of bmDCs to SMAs did not induce cell maturation and rendered cells refractory to subsequent PAMP activation. Figure 6.1 and Table 6.1 summarise the effects of these selected SMAs on bmDcs.

SMA 11a

SMA 11a was one of the first SMAs identified from a preliminary screen investigating the effect of SMAs on macrophage TLR-induced cytokine responses and has since been demonstrated to be protective in several murine disease models. In bmDCs, incubation with 11a for 18 hours induces the gene expression and subsequent production of IL-12p40 protein: it also increases IL-6 mRNA but it does not induce production of IL-6, TNF-α or bioactive IL-12. Pre-exposure to this SMA renders DCs refractory to LPS, BLP and CpG stimulation, although it does demonstrate some selectivity in the terms of the PAMPS and/or cytokines affected. Pre-exposure to 11a reduced LPS-induced CD40 and CD86 surface expression, as well as suppressing LPS-induced activation of p38 and ERK MAPKs and also NF-κBp65. This suppressive phenotype induced by 11a caused a corresponding decrease in IFN-γ production by T cells *in vitro* and decreased Ag-specific T cell expansion *in vivo* with a significant inhibition of IL-$17^{+}CD4^{+}$ cells. Injection of DCs treated with 11a and 12b in combination ameliorates CIA in mice and this is also associated with significantly reduced IL-17 responses.

SMA 12b

SMA 12b was the second SMA selected from the initial screen in macrophages and has also been demonstrated to be protective in the same disease models as 11a, although in CIA it surprisingly demonstrates somewhat different mechanisms of protection compared to 11a and ES-62. In bmDCs, 12b acts in a similar manner to 11a, by increasing the production of p40 protein, and the mRNA of IL-6, p40 and TNF-α compared to untreated cells, but ultimately inhibiting PAMP-activated cytokine production. In addition, 12b exposure inhibits LPS-induced up-regulation of CD40 and CD86 and activation of p38, ERK and NF-κB and this DC phenotype also significantly inhibited the production of IFN-γ by T cells.

SMA 11e

SMA 11e displayed an almost identical suppressive phenotype to 12b. The only difference was in relation to IL-12p40 expression: 11e did not induce up-regulation of IL-p40 gene expression or protein production and pre-exposure to this SMA significantly inhibited LPs-induced p40 as well as p35 gene expression. As with 12b, 11e significantly inhibits cytokine production following LPS, BLP or CpG stimulation and mediates this inhibition through suppression of p38, ERK and NF-κB activation in terms of LPS stimulation. Pre-exposure to 11e also significantly reduces LPS-induced surface expression of CD40 and CD86 and this potentially helps 11e to significantly inhibit LPS-induced IFN-γ production by T cells cultured with 11e-modulated DCs *in vitro*.

SMA 11i

SMA 11i is structurally very similar to 11e and consistent with this it exhibits the same effects on PAMP induced cytokine gene expression and production. It also decreases the surface expression of CD80 and inhibits the up-regulation of CD86 following LPS exposure. However, unlike 11a, 12b and 11e, it does not affect the expression of CD40 on LPS-stimulated DCs, nor does it limit LPS-induced activation of NF-κBp65. 11i treatment does target MAPKs, however, as pre-exposure to the SMA inhibits the LPS-induced p38 and ERK MAPK activation and this may be sufficient to inhibit cytokine responses or 11i may target additional, as yet unidentified, signalling molecules. As with the other SMAs, co-culture of 11i + LPS matured DCs with naïve T cells significantly inhibits LPS-induced IFN-γ *in vitro*. In addition, 11i-exposed DCs modulate

T cell responses *in vivo*, as evidenced by suppression of Ag-specific T cells and reduced numbers of IL-17^{+}CD4^{+} cells.

SMA 19o

SMA 19o was originally selected as a 'negative' control SMA as it demonstrated no effect on cytokine production in macrophages or DCs. Further work demonstrated that pre-exposure to 19o could inhibit LPS-induced IL-12 production however. Consistent with lack of cytokine inhibition, 19o pre-exposure did not modulate LPS activated MAPKs or NF-κB, nor did it inhibit LPS-induced up-regulation of co-stimulatory molecules.

SMAs 11h and 11k

SMAs 11h and 11k were initially selected due to their inhibitory effect on LPS-induced cytokine production in bmDCs and also in mast cells (Coates & Harnett, unpublished). SMA 11h displayed an identical 'phenotype' to 11a in terms of inhibition of BLP- and CpG-induced cytokine responses but 11k only inhibited BLP-induced IL-12. Neither SMA had any effect on the LPS-induced increase in IL-6, TNF-α, IL-12p35 or IL-12p40 gene expression, potentially explaining their less potent ability to inhibit cytokines compared to the other SMAs. Due to these inconsistent and weak inhibitory responses these SMAs were not selected for further analysis.

Figure 6.1: A summary of the immunomodulatory effects of the selected SMAs on DC responses

Recognition of LPS via TLR4 triggers a downstream signalling pathway that results in the activation of MAPKs and NF-κB and the production of pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α. It also results in the up-regulation of co-stimulatory molecules CD40 and CD86 and promotes the polarisation of T_H1 and T_H17 cells. Pre-treatment of DCs with the SMAs inhibit several of these processes and suppresses T_H17 responses *in vivo*.

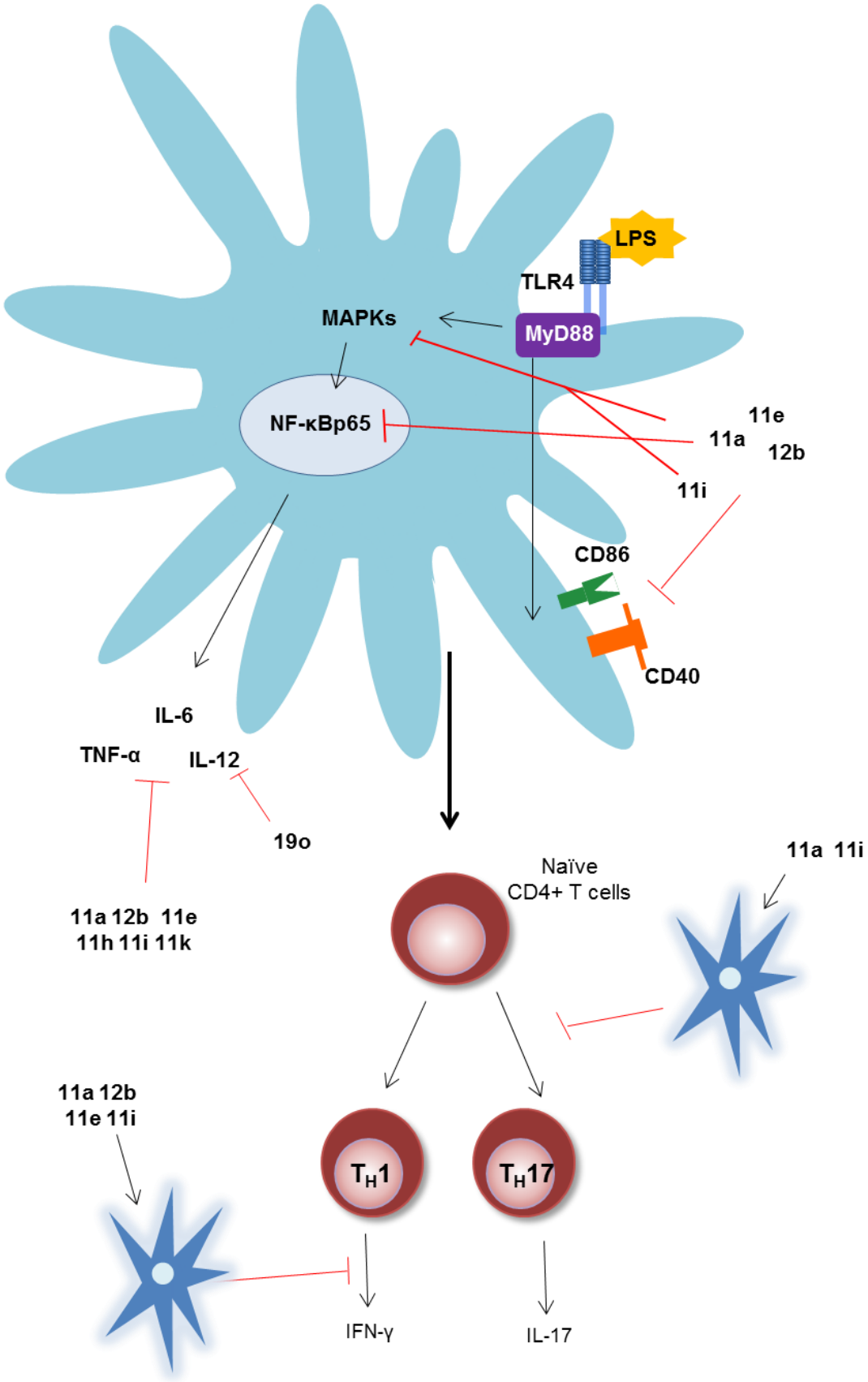

LPS
TLR4
MyD88
MAPKs
NF-κBp65
11e
11a
12b
11i
CD86
CD40
IL-6
TNF-α
IL-12
19o
11a 12b 11e
11h 11i 11k
Naïve
CD4+ T cells
11a 11i
11a 12b
11e 11i
T_H1
T_H17
IFN-γ
IL-17

Table 6.2: A summary of the immunomodulatory effects of the selected SMAs on DC responses

The effects of the SMAs on the cytokine production of DCs was investigated following LPS (TLR4), BLP (TLR2) or CpG (TLR9) activation and is summarised here. In addition the effect of the SMAs on the basal expression of CD80 and LPS-induced up-regulation of CD40 and CD80 and on the LPS-mediated activation of MAPKs and NF-κBp65 is summarised, along with the ability of SMA-treated DCs to prime IFN-γ production by T cells *in vitro*. ↓ represents a significant decrease in the DC response and NS means not selected for that experiment.

	Cytokine									**Co-stimulation**			**Signalling**				**Ability to prime T_H1 responses**
PAMP	**TLR4**			**TLR2**			**TLR9**			**TLR4**				**TLR4**			
SMA	**IL-6**	**IL-12**	**TNF-α**	**IL-6**	**IL-12**	**TNF-α**	**IL-6**	**IL-12**	**TNF-α**	**CD40**	**CD86**	**CD80**	**P-JNK**	**P-ERK**	**P-p38**	**NF-κB p65**	**IFN-γ**
11a	↓	↓	↓	↓	↓			↓	↓	↓	↓	↓		↓	↓	↓	↓
12b	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	-		↓	↓	↓	↓
11e	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓		↓	↓	↓	↓
11h	↓	↓	↓	↓	↓			↓	↓	NS	NS	NS	NS	NS	NS	NS	NS
11i	↓	↓	↓	↓	↓	↓	↓	↓	↓		↓	↓		↓	↓		↓
11k	↓	↓	↓					↓		NS	NS	NS	NS	NS	NS	NS	NS
19o		↓															NS

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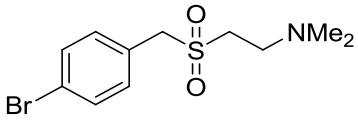
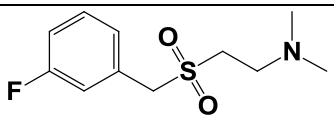
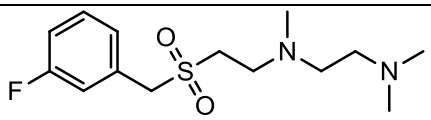
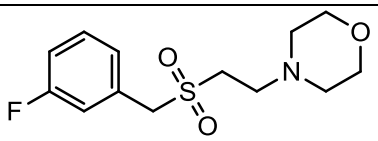
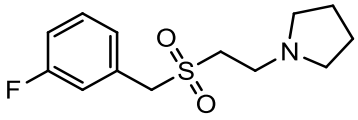
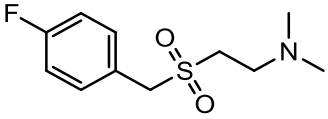
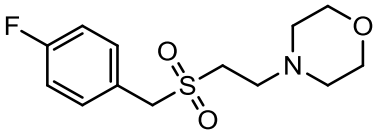
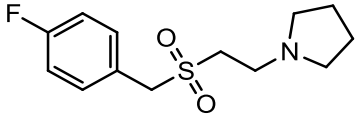
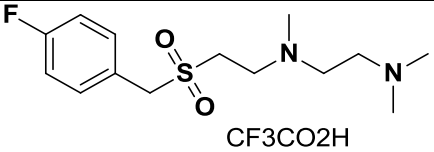
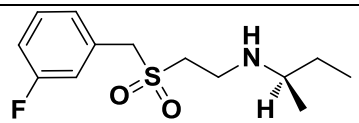
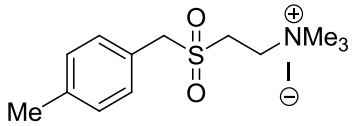
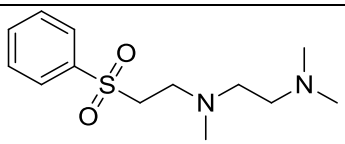
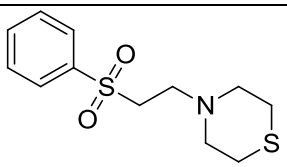
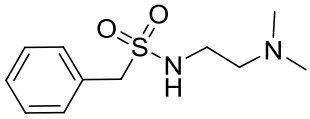
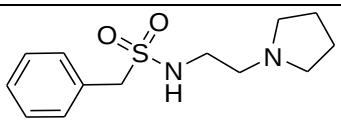
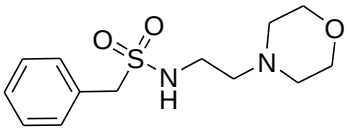
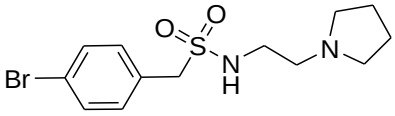
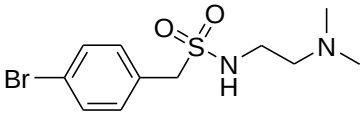
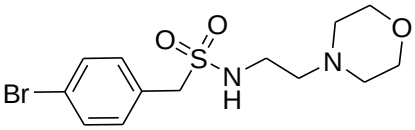
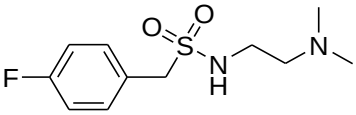
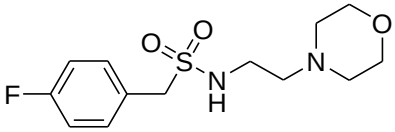
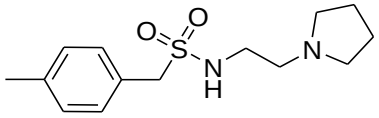
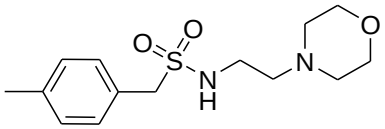
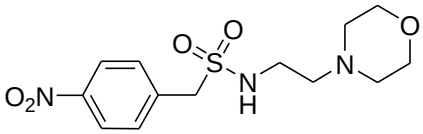
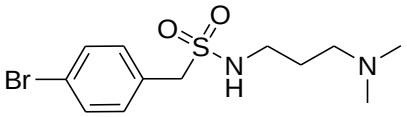
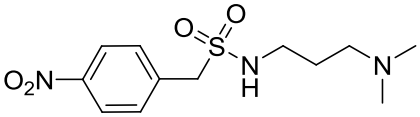
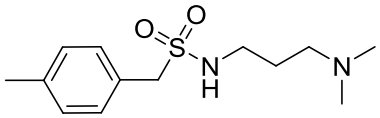
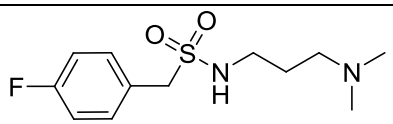
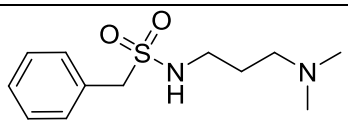
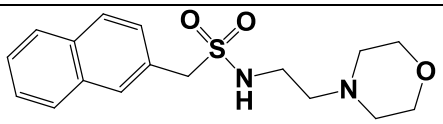
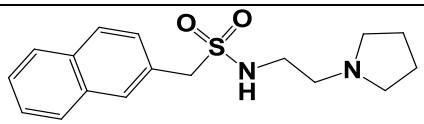
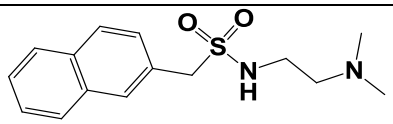
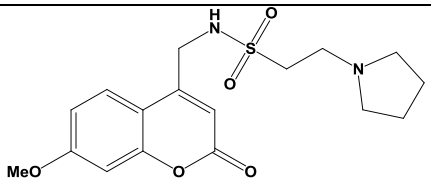
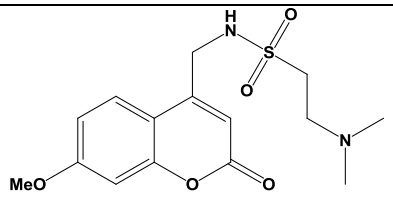
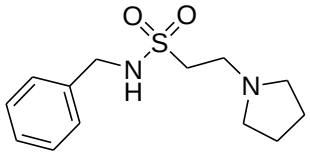
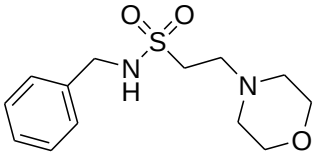
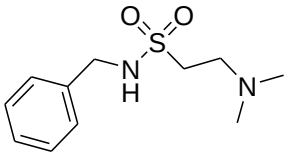
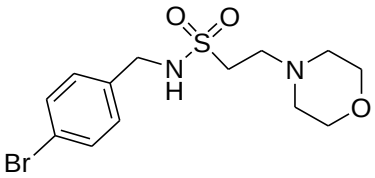
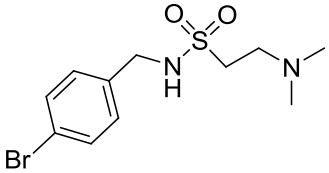
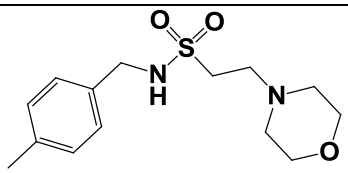
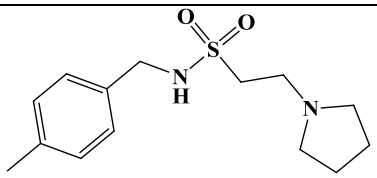
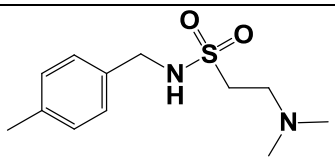
Appendix

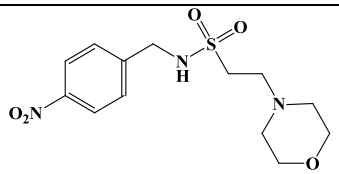
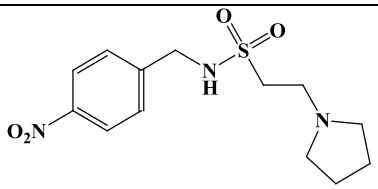
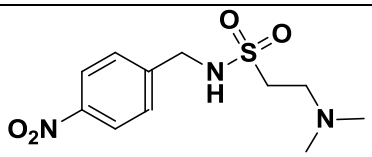
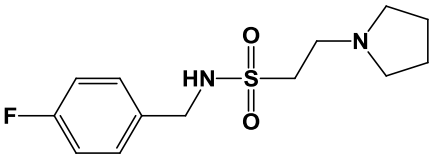
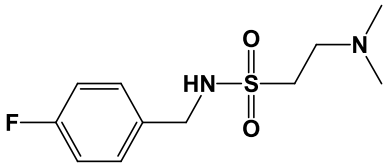
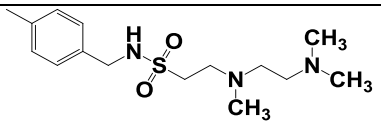
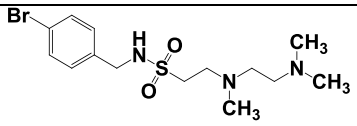
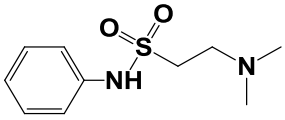
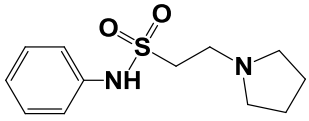
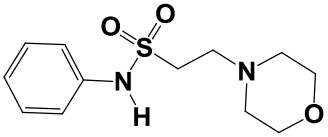
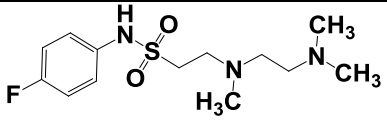
Appendix 1: The structures of the SMAs along with their chemical formula and molecular weight (MW)

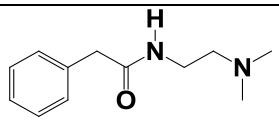
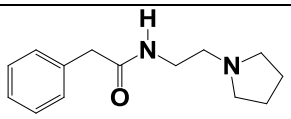
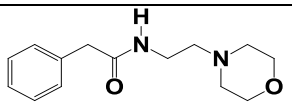
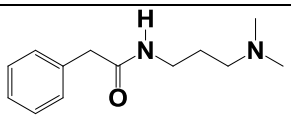
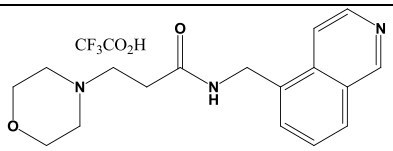
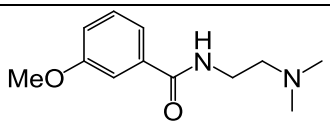
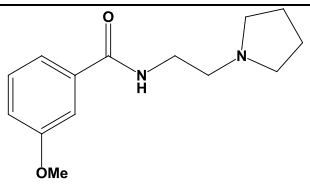
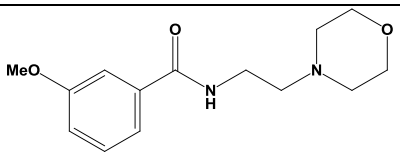
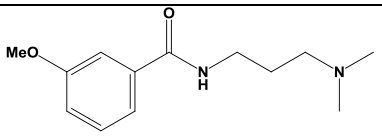
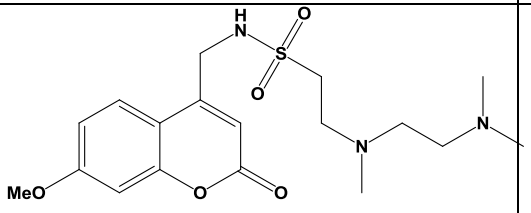
SMA	Structures	Formula (MW)
11a		$C_{11}H_{16}BrNO_2S$ 305
11e		$C_{11}H_{16}FNO_2S$ 245.3
11f		$C_{14}H_{23}FN_2O2S$ 302.4
11g		$C_{13}H_{18}FNO_3S$ 287.4
11h		$C_{13}H_{18}FNO_2S$ 271.4
11i		$C_{11}H_{16}FNO_2S$ 245.3
11j		$C_{13}H_{18}FNO_3S$ 287.4
11k		$C_{13}H_{18}FNO_2S$ 271.4
11l	 CF3CO2H	C16H24F4N2O4S 416.4314

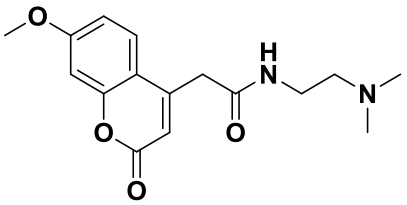
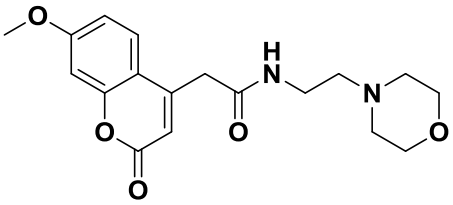
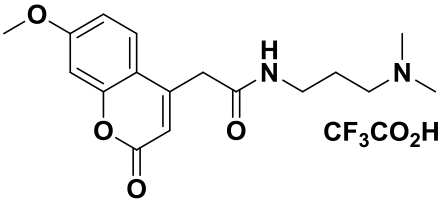
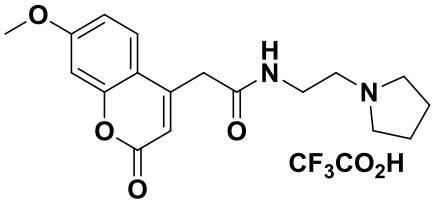
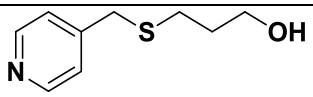
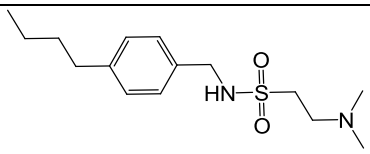
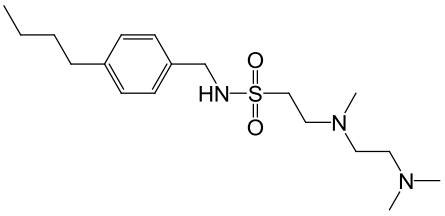
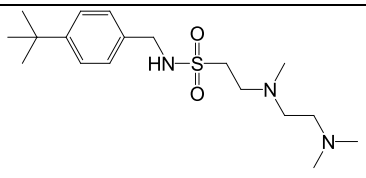
11n		C13H20FNO2S 273.3668
12b		$C_{13}H_{22}INO_2S$ 383
18a		C13H22N2O2S 270.3910
18b		C12H17NO2S2 271.3989
19a		$C_{11}H_{18}N_2O_2S$ 242.3
19b		$C_{13}H_{20}N_2O_2S$ 268.4
19c		$C_{13}H_{20}N_2O_3S$ 284.4
19d		$C_{13}H_{19}BrN_2O_2S$ 347.3
19e		$C_{11}H_{17}BrN_2O_2S$ 321.2
19f		$C_{13}H_{19}BrN_2O_3S$ 363.3
19g		$C_{11}H_{17}FN_2O_2S$ 260.3
19i		$C_{13}H_{19}FN_2O_3S$ 302.4

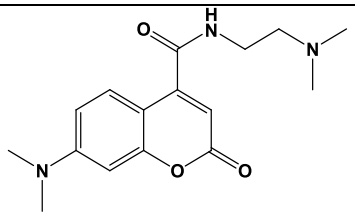
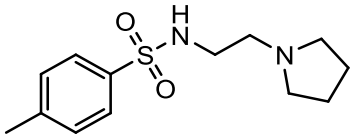
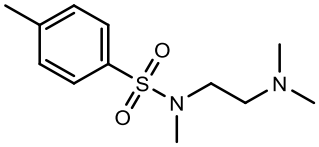
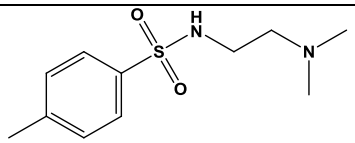
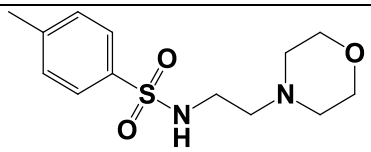
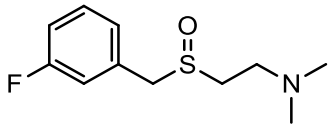
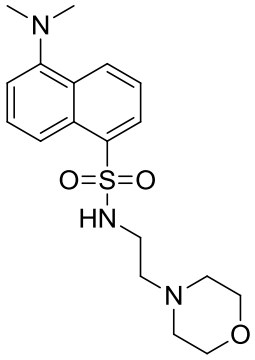
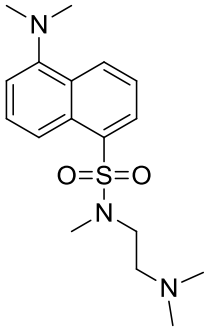
19k		$C_{14}H_{22}N_2O_2S$ 282.4
19l		$C_{14}H_{22}N_2O_3S$ 298.4
19o		$C_{13}H_{19}N_3O_5S$ 329.4
19p		$C_{12}H_{19}BrN_2O_2S$ 335.3
19q		$C_{12}H_{19}N_3O_4S$ 301.4
19r		$C_{13}H_{22}N_2O_2S$ 270.4
19s		$C_{12}H_{19}FN_2O_2S$ 274.4
19t		$C_{12}H_{20}N_2O_2S$ 256.4
19u		$C_{17}H_{22}N_2O_3S$ 334.4
19v		$C_{17}H_{22}N_2O_2S$ 318.1
19w		$C_{15}H_{20}N_2O_2S$ 292.4
19z		$C_{17}H_{22}N_2O_5S$ 366.4

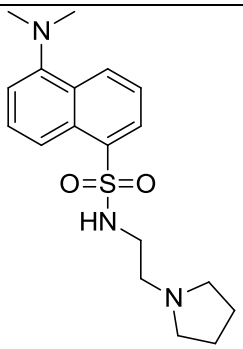
19aa		$C_{18}H_{27}N_3O_5S$ 397.5
21a		$C_{13}H_{20}N_2O_2S$ 268.4
21b		$C_{13}H_{20}N_2O_3S$ 284.4
21c		$C_{11}H_{18}N_2O_2S$ 242.3
21d		$C_{13}H_{19}BrN_2O_3S$ 363.3
21f		$C_{11}H_{17}BrN_2O_2S$ 321.2
21g		$C_{14}H_{12}N_2O_3S$ 298.4
21h		$C_{14}H_{22}N_2O_2S$ 282.4
21i		$C_{12}H_{20}N_2O_2S$ 256.4

21j		$C_{13}H_{19}N_3O_5S$ 329.4
21k		$C_{13}H_{19}N_3O_4S$ 313.4
21l		$C_{11}H_{17}N_3O_4S$ 387.4
21n		$C_{13}H_{19}FN_2O_2S$ 286.4
21o		$C_{11}H_{17}FN_2O_2S$ 260.3
21p		$C_{15}H_{27}N_3O_2S$ 313.5
21q		$C_{14}H_{24}BrN_3O_2S$ 378.3
23a		AIK-21/82
23b		$C_{12}H_{18}N_2O_2S$ 254.4
23c		$C_{12}H_{18}N_2O_3S$ 270.4
23g		$C_{13}H_{22}FN_3O_2S$ 303.4

24a		$C_{12}H_{18}N_2O$ 206.3
24b		$C_{14}H_{20}N_2O$ 232.3
24c		$C_{14}H_{20}N_2O_2$ 248.3
24d		$C_{13}H_{20}N_2O$ 220.3
24e		$C_{19}H_{22}F_3N_3O_4$ 413.2
25a		$C_{12}H_{18}N_2O_2$ 222.3
25b		$C_{14}H_{20}N_2O_2$ 248.3
25c		$C_{14}H_{20}N_2O_3$ 264.3
25d		$C_{13}H_{20}N_2O_2$ 236.3
62		$C_{18}H_{27}N_3O_5S$ 397.5

63		$C_{16}H_20N_2O_4$ 304.35
64		$C_{18}H_{22}N_2O_5$ 346.38
66		$C_{19}H_{23}F_3N_2O_6$ 432.40
67		$C_{20}H_{23}F_3N_2O_6$ 444.41
70		$C_9H_{13}NOS$ 183.27
72		$C_{15}H_{26}N_2O_2S$ 298.4
75		$C_{18}H_{33}N_3O_2S$ 355.6
80		$C_{18}H_{33}N_3O_2S$ 355.5

81		$C_{16}H_{21}N_3O_3$ 303.4
82		$C_{13}H_{20}N_2O_2S$ 268.3751
83		$C_{12}H_{20}N_2O_2S$ 256.4
84		$C_{11}H_{18}N_2O_2S$ 242.3
85		$C_{13}H_{20}N_2O_3S$ 284.4
88		$C_{11}H_{16}FNOS$ 229.3
97		$C_{18}H_{25}N_3O_3S$ 363.5
98		$C_{17}H_{25}N_3O_2S$ 335.5

100		$C_{18}H_{25}N_3O_2S$ 347.5