Investigation of mechanisms involved in subversion of dendritic cell-function by the parasitic filarial nematode product, ES-62

Russell John Eason

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The Institute for Infection, Immunity & Inflammation,

College of Medical, Veterinary and Life Sciences

University of Glasgow

The Institute of Infection, Immunity & Inflammation,
Glasgow Biomedical Research Centre,
120 University Place,
University of Glasgow,
Glasgow,
G12 8TA.

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Declaration

This work presented in this thesis represents the original work carried out by the author and has not been submitted in any form to another University. Where use has been made of materials provided by others, due acknowledgements have been made.

Russell ‘Rusty’ John Eason

University of Glasgow

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Acknowledgements

Throughout my four years in the University of Glasgow and the Harnett lab, I have made friends and memories that I shall always cherish, wherever the wind takes me. They have not only made the good times great but have supported and carried me through the storms that always assail someone who undertakes something as crazy as a PhD (especially in immunology). First and foremost however, I would like to thank Maggie and Billy Harnett, for their constant support and parental like guidance that has moulded my enthusiasm (or in other words, crazy ideas) to produce something of merit and made the journey an enjoyable and unforgettable experience.

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To a Dimity Ball, although we could not avoid the rocks on horizon the experiences we shared cannot attest to how much the time we spent together meant to me, which were some of the best of my life.

And finally, I would like to leave you with a dialogue that has made me smile not matter what the weather or my mental condition, so please sit back and relax, take a deep breath and close your eyes to picture a brilliant sun beating is rays upon crystal clear waters and swaying palm trees on a beach. Now imagine two men sitting on their surfboards in a gently rolling ocean, Kunu, the wise Hawaiian surf instructor and his student Peter:

Peter: “Thanks for taking me out here it’s the first time I have felt good in three weeks or so…”

Kunu: “No worries, you just need to get back on that board, it’s what you need to do…”

A beautiful wave winks a foamy eyelash as it slides past.

Kunu: “Hey…. Here’s the deal when life gives you lemons you just have to say screw the lemons and make lemonade”

Peter “… Yeah … No… you said it… totally”

Kunu “ You just got to pull yourself up by your wetsuit and get back on your board… hey look man if you got attacked by a shark are you gonna give up surfing?”

Peter “ Probabaly…yeah…”

Kunu “… mmm … well let’s go in. Taco’s are on me”

Now re-place the sun with a constant drizzle, swaying palm trees with highland cows bracing near hurricane winds, three weeks with 3 years, the shark with the fourth chapter of this thesis and taco’s with sausage suppers and you have distilled the essence of my time in Glasgow, which I wouldn’t trade in for a kilo of gold (well ok, maybe two).

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List of Abbreviations

7-AAD, 7-amino-actinomycin D

Ag, Antigen

NH_4Cl, Ammonium chloride

AP-1, Activator protein-1

APC, Antigen presenting cells

APC, Allophycocyanin

aPKC, Atypical PKC

ASM, Acid sphingomyelinase

ATG, Autophagy related genes

BCR, B-cell receptor

BLP, Bacterial lipopeptide

Bm, Bone marrow

Cbl, Casitas b-lineage lymphoma

CD, Cluster of differentiation

CERase, Ceramidases

CFSE, 5,6-carboxy-succinimidyl-fluoresceine-ester

CIA, Collagen induced arthritis

cIAP1, Apoptosis protein 1

C-PAF, Carbamyl platlet activating factor

cPKC, Conventional PKC

COX-2, Cyclooxygenase-2

DAG, Diacylglycerol

DC, Dendritic cells

DFCP1, Double FYVE-containing protein 1

DNA, Deoxyribonucleic acid

dNTP, 2'deoxynucleotide 5'-triphosphate

dsRNA, Double stranded RNA

DTT, 1,4-dithiothreitol

EBI3, Epstein-Barr virus induced molecule 3

EBV, Epstein-Barr virus

EDTA, Ethylene diamine tetra acetic acid

EGTA, Ethylene glycol-bis(β-aminoethyl ether) tetra acetic acid

EGR-1, Early growth response protein 1

ER, Endoplasmic reticulum

ERK, Extracellular-regulated kinase

ES, Excretory- secretory product

ES-62, Excretory-secretory product 62

FACS, Fluorescence activated cell sorting

FAM, 5'-6-carboxyfluorescein

FcR, Fc receptor

FCS, Fetal calf serum

FIP200, Focal adhesion kinase family interacting protein of 200 kDa

FITC, Fluorescein isothiocyanate

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

GATA3, GATA binding protein 3

GM-CSF, Granulocyte/macrophage colony stimulating factor

GPCR, G-protein coupled receptor

gp130, Glycoprotein 130

GRAIL, Gene related to anergy in lymphocytes

HDL, High density lipoportein

HECT, Homologous with E6-associated protein C terminus

HSV, Herpes simplex virus

HPRT, Hypoxanthine phosphoribosyltransferase

HRP, Horse radish peroxidase

IAP, Inhibitors of apoptosis

ICAM-1, Inter-cellular cell adhesion molecule 1

IDO, Indoleamine 2,3-dioxygenase

IFN, Interferon

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

iNOS, Inducible nitric oxide synthase

IP_3 , Inositol triphosphate

IRAK, IL-1 receptor-associated kinase

IRF, Interferon regulatory factors

ITAM, Immuno-receptor tyrosine-based activatory motif

ITCH, Itchy homologue E3 ubiquitin protein ligase

JNK, c-Jun N-terminal kinases

kDa, Kilo Daltons

LAMP2, Lysosome associated membrane glycoprotein-2

LAP, LC3 associated phagocytosis

LC3, Light chain 3

LDS, Lithium Dodecyl Sulfate

LPS, Lipopolysaccharide

LRR, Leucine-rich repeats

LTA, Lymphotoxin alpha

Lys, Lysine (**K**)

3-MA, 3-methyladenine

MALP-2, Macrophage-activating lipopeptide-2

MAP, Mitogen activated protein

MAPK, MAP kinases

MD-2, Myeloid Differentiation factor-2

MHC, Major Histocompatibility Complex

mTOR, Mammalian target of rapamycin

MyD88, Myeloid Differentiation factor-88

NBR1, Neighbour of BRCA1 gene 1

NEDD4, Neural precursor cell expressed, developmentally down-regulated 4

NEMO, NF-κB essential modifier

NF-κB, Nuclear factor-kappa B

NIK, NF-κB-inducing kinase

NK, Natural killer cells

nPKC, Novel PKC

NO, Nitric oxide

NOX2, NADPH oxidase-2

OVA, Ovalbumin

PAF, Platelet activating factor

PAFR, Platelet activating factor receptor

PAMP, Pathogen associated molecular patterns

PB1, Phox and Bem1p-1

PBS, Phosphate buffered saline

PC, Phosphorylcholine

PE, Phycoerythrin

PE, Phosphatidylethanolamine

PEL1, Pellino 1

PFA, Paraformaldehyde

PIP_2, Phosphatidylinositol 4,5-bisphosphate

PI-3-K, Phosphoinositide-3-kinase

PKC, Protein Kinase C

PKB, Protein kinase B (**Akt**)

PKM, Protein kinase M

PLA_2, Phospholipase 2

PLC, Phospholipase C

PLD, Phospholipase D

PMSF, Phenylmethylsulfonylfluoride

PM, Plasma membrane

PRR, Pathogen recognition receptor

PtdIns3P, Phophatidylinositol-3-phosphate

PTK, Protein tyrosine kinase

RANK, Receptor activator of NF-κB

RNA, Ribonucleic acid

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

RHD, Rel-homology domains

RING, Really new interesting gene

RIPA, Radio-Immunoprecipitation Assay

RIP1, Receptor-interacting protein 1

RT-PCR, Reverse transcription polymerase chain reaction

S1P, Sphingosine 1 phosphate

S1PR, Sphongosine 1 phosphate receptor

S1Pase, Sphingosine 1 phosphate phosphatase

SARM, Sterile α and HEAT-Armadillo Motifs

SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser, Serine

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

SKP1, S-phase kinase-associated protein 1

SLAM, Signalling lymphocytic activation molecule

SMase, Sphingomyelinases

SOCS1, Suppressor of cytokine signalling

SPHK, Sphingosine kinase

SQSTM1, Sequestosome 1

SR-A, Scavenger receptor-A

ssRNA, Single stranded RNA

STAT, Signal transducer and activator of transcription

TAB, TAK1 Binding Protein

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

T-bet, T box 21

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

TRAM, TRIF-related adaptor molecule

TRAF, TNF receptor-associated factor

TRAF6, TNF Receptor-Associated Factor-6

T_{reg} , Regulatory T_H cells

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

Tyr, Tyrosine

Ubc13, Ubiquitin carrier protein N

ULK1, UNC-51-like kinase

VMP1, Vacuole membrane protein 1

VSP, Vacuolar sorting protein

WIPI, WD-repeat domain phosphoinositide-interacting

g, gram

mg, milligram

μg, microgram

M, molar

mM, millimolar

μM, micromolar

ml, millilitre

μl, microlitre

min, minute

s, second

h, hour

rpm, revolutions per minute

g, times gravity

V, volts

Summary

The developed world is experiencing a steep increase in diseases based on aberrant autoimmune inflammatory responses and allergic conditions. By contrast, such diseases are uncommon in countries endemic for parasitic helminths, suggesting that parasite-derived immunomodulators may protect against their development. One such molecule, ES-62, which is a phosphorylcholine (PC)-containing glycoprotein originally identified from the filarial nematode *Acanthocheilonema viteae*, has shown therapeutic potential in a range of inflammatory diseases. Although its mechanism of action is not fully understood, ES-62 is known to target dendritic cells (DCs) to subvert T helper cell (T_H) differentiation and hence modulate the subsequent host immune response to an anti-inflammatory phenotype. ES-62 requires Toll-like receptor 4 (TLR4) to mediate its effects, and here, we have investigated the mechanisms underpinning ES-62-mediated subversion of TLR4-pro-inflammatory signalling as exemplified by its canonical ligand, bacterial endotoxin (lipopolysaccharide, LPS) that results in the activation of the transcription factor, nuclear factor-κB (NF-κB) and consequent secretion of proinflammatory cytokines.

This study investigated the mechanism of ES-62-mediated uncoupling of NF-κB activation by its modulation of TLR4 expression and its associated LPS recognising complex TLR4/Myeloid differentiation factor-2 (MD2), as the internalisation, trafficking and degradation of this receptor complex has previously been shown to regulate its responses to different stimuli. ES-62 and LPS were found to differentially modulate the dynamics and expression of the total cellular pool of TLR4, relative to that of the 'active' LPS recognising TLR4/MD2 complex. Specifically, ES-62 acted to antagonise the dynamics of the LPS response, providing a mechanism for its induction of TLR4 hyporesponsiveness. In addition, as preliminary studies demonstrated an interaction between ES-62 and the Platelet activating factor receptor (PAFR), the possibility that ES-62 might signal via TLR4 in concert with one or more co-receptors was investigated but the data did not conclusively support a role for the PAFR as a target of ES-62 action.

The dissection of the effects of ES-62 on TLR4 signalling was further pursued by defining its modulation and/or sequestration of transduction elements associated with the TLR4 mediated activation of NF-κB. Of particular interest were PKCα, PKCε and PKCδ members of the protein kinase C family that directly interact with TLR4 and its immediate signalling adaptors to mediate proinflammatory cytokine (TNF-α, IL-6 and IL-12p70)

production. Several isoforms including PKCα have previously been shown to be targeted in B cells and mast cells for degradation by ES-62. Here, we utilised pharmacological inhibitors of proteosomal and lysosomal protein degradation pathways to demonstrate that whilst LPS induces the turnover of PKCs through the proteosome, ES-62 directs their degradation through the lysosomes. In conjunction with this, ES-62 and LPS were also found to differentially regulate the expression of tumour necrosis factor receptor associated factor-6 (TRAF6) and c-Casitas B lineage lymphoma (c-Cbl), E3 ubiquitn ligases, which act as scaffolds (via Lysine 63 linked polyubiquitination chains) in the activation and stabilisation of NF-κB. However, it was found that ES-62 did not prevent recruitment of these various TLR4 signal transducers upon subsequent recognition of LPS, suggesting that it acts to attenuate hyper-inflammatory responses without leaving an individual immunocompromised to infection. In addition, by mimicking an inflammatory environment with exogenous granulocyte macrophage-colony stimulating factor (GM-CSF), it was found that the actions of ES-62 had more profound effects on the modulation of the aforementioned signal transducers and its range of targets were expanded to include, myeloid differentiation primary responses gene 88 (MyD88), which transmits the early response signals from all the TLR family receptors, except TLR3.

The lysosomal degradation of these cystolic proteins induced by ES-62 indicated a potential role for subversion of TLR-regulated autophagy. This cellular process provides a negative feedback mechanism for TLR4 signalling through its targeting of intracellular proteins for lysosomal degradation. Detailed investigation revealed that ES-62 induced autophagic flux, as defined by an increase in the ratio of light chain-3 type II (LC3-II; a component the characteristic autophagic vesicle, the autophagosome) to LC3–I. Moreover, ES-62 was observed to induce this mechanism to target the lysosomal degradation of p62 (an LC3 binding autophagy adaptor protein), which is strongly upregulated by LPS to transduce NF-κB activation, potentially limiting its positive role in TLR4 signalling. In this context, this study demonstrates a novel interaction between PKCδ and p62 (but not LC3) upon LPS stimulation, suggesting as with other systems that the predominant role of p62 in DCs is as a mediator of the TLR4-NF-κB signalling axis.

These effects on DC activation were associated with the capacity of ES-62 matured DCs to limit T_H1/T_H17 pro-inflammatory responses both relative to and following LPS stimulation. ES-62 was found to have little effect on costimulatory marker expression (cluster of differentiation-40 (CD40), CD80/86 and major histocompatibility complex (MHC) class II), placing a major emphasis of its modulation of TLR signalling on the DC

mediated cytokine milieu and not its direct interactions with T_H cells. In this context, the plastic nature of immunomodulation by ES-62 was reflected by its lack of affect on LPS induced IL-6 and IL-27 production, crucial for initiating T_H1 or T_H17 differentiation required to fight infection. Rather, it attenuated IL-12p70, IL-23 and TNF-α cytokine secretion, which augment adaptive immune responses and drive the hyper-inflammatory potential of the T_H1 and particularly T_H17 phenotype of autoimmune pathology. Subsequently, ES-62 matured DCs displayed a limited potential to induce a T_H17 phenotype, as defined by a reduction in IL-17 and IL-22 secretion coupled to an impaired ability of such DCs to stimulate antigen (Ag) specific naïve T_H cell proliferation, lowering the number of responding cells and their proliferative capacity. This occurred even upon subsequent stimulation with LPS, however a level of plasticity was observed as these effects were partially overcome when DCs were presenting high levels of Ag to naïve T_H cells, indicating the important role of the microenvironment in regulating the induction of different T_H phenotypes.

Collectively these data further advance our understanding of the mechanisms underlying ES-62 mediated manipulation of NF-κB activation and cytokine production. This not only provides valuable information on how helminths can both augment and evade the immune system but demonstrates the therapeutic potential of ES-62 and reveals the underlying mechanisms it employs to attenuate pathogenic inflammation.

1 Introduction

1.1 The innate immune system

In all animals, the innate immune system provides essential protection against invading pathogens. This ancient and evolutionary conserved system is the first line of defence against invading pathogens; thus many of the cells which make up its network are found beneath the skin and other physical barriers of the body to protect us from the environment we inhabit. A key component of the innate system is a collection of germ-line encoded receptors called pathogen recognition receptors (PRRs), which recognise highly conserved molecular structures specific to microbes (Pathogen associated molecular patterns, or PAMPs)[1]. There are many examples of conserved PAMPs: mannans, lipopolysaccharide (LPS), peptidoglycans, lipoteichoic acids, unmethylated deoxynucleic acid (DNA) or CpG motifs, present in a number of pathogens but not on mammalian cells. Upon the recognition of a PAMPs by its relevant PRRs, rapid responses are activated by innate cells such as phagocytosis and chemotaxis, which are coupled to the active secretion of a number of soluble proteins and bioactive molecules. These include proteins such as cytokines (e.g. tumour necrosis factor-α [TNF-α]) and complement as well as lipid mediators (e.g. prostaglandins, leukotrienes and platelet activating factor [PAF]), which go on to regulate the later functional responses of innate cells that contribute to inflammation. Inflammation is an essential component of the innate immune system, as it leads to the recruitment and activation of cells such as dendritic cells (DC), macrophages, mast cells, eosinophils and neutrophils that exhibit key antimicrobial functions.

The best understood subgroup of PRRs is the Toll-like receptor family. The mammalian Toll-like receptors (TLRs) are derived from an evolutionary ancient recognition and signalling system, originally discovered for its part in regulating dorsal polarity in developing embryos of the fruit fly, *Drosophila Melanogaster*. The susceptibility of drosophila strains to fungal infections that carried loss-of-function mutations in Toll suggested TLRs played an important role in host defence[2]. Soon after this discovery the first mammalian homologue of Toll, TLR4 was identified and shown to be essential for the response of mammals to the bacterial endotoxin, LPS[2,3]. The TLR-family of receptors has the ability to recognise pathogens or pathogen-derived products and initiate signalling events leading to the activation of innate host defences and acute inflammatory responses by the induction of anti-microbial genes, inflammatory cytokines

and chemokines[3]. However the scope of TLR function extends beyond the innate immune system as it also has important roles in the activation of adaptive immune responses[4-6].

1.1.1 The Toll-like receptors

The TLRs, members of the Toll/Interleukin-1 receptor superfamily[3], are transmembrane spanning proteins that are comprised of extraceullar leucine-rich repeats, a transmembrane region and an intracellular domain that contains a conserved TIR (Toll/Interleukin-1 receptor) domain, the latter of which is also present on their intracellular adaptor proteins[7]. The 11 human and 13 mouse TLRs identified to date sense a diverse range of PAMP structures covering lipids, proteins, nucleic acids and lipopeptides[8] and upon their recognition TLRs form homo- or heterodimers. For example, TLR2 forms heterodimers with TLR1 or TLR6 to recognise a large variety of lipopeptides found on gram negative bacteria, fungi, parasites and viruses. By contrast, TLR3 homodimers sense double stranded ribonucleic acid (dsRNA) produced during viral replication whilst TLR 7/8 recognise guanosine- or uridine- rich single stranded RNA (ssRNA) and TLR9 unmethylated CpG from viral RNA and DNA respectively[8]. TLR5 and TLR11 recognise protein ligands, for example TLR5 is expressed by intestinal cluster of differentiation-11c^{+} (CD11c^{+}) lamina propia cells where it senses bacterial flagellin[9]. TLR10 may heterodimerise with TLR2, but the ligand it recognises is currently unknown. The current knowledge of TLR proteins, their stimulatory ligands and the dimers they form is summarised in **Figure 1.1**.

The most well characterised TLR, TLR4, in conjunction with accessory molecules Myeloid Differentiation factor-2 (MD-2) and CD14 recognises LPS from gram-negative bacteria[10]. TLR4 dimerisation results in propagation of the intracellular signals that lead to the gene induction of inflammatory cytokines, such as TNF-α, interleukin (IL)-6, IL-1β and IL-12 and up-regulation of co-stimulatory molecule expression, which is required for the induction of pathogen specific adaptive immune responses. The role of TLR4 in innate immunity was first discovered in C3H/HeJ mice, where a missense mutation in TLR4 renders it unable to respond to bacterial endotoxin[11]. From these early studies it was observed that ligand specificity is not the only distinguishing feature of each TLR. In addition to recognising distinct classes of micro-organism individual TLRs differentially trigger different signal transduction pathways. For example receptors such as TLR3 and TLR4 induce the production of pro-inflammatory cytokines and the type I interferons (IFN), whereas others like TLR2 and TLR5 do not induce the latter type I IFN response[12].

These differences in signalling outcome were explained by the discovery of multiple TIR domain-containing adaptor proteins, which link activated receptors to downstream kinases that trigger differential signalling pathways[13]. Thus, different receptors engage distinct adaptor combinations and the particular adaptor(s) used determine which signalling pathway(s) will be activated. These TIR-containing cytoplasmic adaptor proteins include Myeloid Differentiation factor-88 (MyD88), TIR-containing adaptor protein/MyD88 adaptor-like (TIRAP/Mal), TIR-containing adaptor inducing IFN-β/TIR containing adaptor molecule-1 (TRIF/TICAM-1), TRIF-related adaptor molecule (TRAM) and Sterile α and HEAT-Armadillo Motifs (SARM)[7]. The intracellular adaptors utilised by the various TLR's are summarised in **Figure 1.1**.

The association of MyD88 with TLRs leads to the activation of the mitogen activated protein kinases (MAPKs), extracellular-regulated kinase (ERK), c-Jun N-terminal kinases (JNK) and p38, coupled with nuclear factor κB (NF-κB) activation resulting in the expression of inflammatory cytokine genes[14]. The LPS sensor TLR4 mediates NF-κB activation through the adaptors TIRAP-MyD88, while also utilising TRAM and TRIF to induce IRF3 activation that is pivotal for the production of type I IFNs[15]. This latter response was identified from analysis of cells from MyD88 deficient mice that were still able to respond to endotoxin, but with a noticeably delayed activation of NF-κB activation[16,17]. This delayed activation was shown to reflect the later recruitment of TRIF to TLR4 and this pathway was observed to not only activate NF-κB but also IRF3 and the MAPKs. TRAM was then found to selectively participate in the activation of the TRIF-dependent pathways downstream of TLR4, but not TLR3.

1.1.2 The recognition of LPS by TLR4

The release of the cell wall component, LPS occurs in infections with Gram-negative bacteria (e.g. Salmonella). Its recognition by innate cells prepares the immune system to eradicate the bacterium and hence, prevent the further spread of infection. The regulation of this response is a very important process as an amplified or uncontrolled inflammatory response to LPS can lead to septic shock syndrome and death. Structurally, LPS is a glycolipid located in the outer membrane of gram negative bacteria, that is composed of an amphipathic lipid A component and a core of hydrophilic polysaccharides[18]. The lipid A constituent is the main inducer of immunological responses to LPS and is conserved between different bacterial species.

The recognition of LPS is mediated by TLR4 on cells of the immune system such as macrophages, neutrophils and DCs. However, TLR4 alone cannot recognise LPS, it also requires the accessory cell surface proteins, CD14 and MD-2. Specifically, LPS becomes associated with LPS-binding protein (LBP) in the circulation, enabling it to bind to the cell-surface protein, CD14 which presents LPS to the TL4/MD-2 complex[19], where MD-2 initially binds TLR4 intracellularly and is important in targeting TLR4 to the cell surface[20]. The extracellular domains of members of the TLR family consist of leucine-rich repeats (LRRs) with a horseshoe-like shape[20] and upon LPS binding the receptor dimerises, triggering the recruitment of adaptor proteins to the intracellular TIR domains of the TLRs and the initiation of signalling leading to activation of NF-κB. These include MyD88, which interacts with all the TLRs except TLR3 and in the case of TLR2 and -4, TIRAP (Mal), serves as a bridge for the recruitment of MyD88. The endocytosis of the TLR-MyD88 complex is required for the recruitment of further signalling adaptors.

1.1.3 The downstream signalling adaptors and events involved in TLR4 mediated activation of NF-κB

The recognition of LPS by TLR4 results in the activation of the transcription factor NF-κB, which in turn induces the production of pro-inflammatory mediators. The NF-κB family comprises dimeric transcription factors that contain Rel-homology domains (RHDs) that bind to discrete DNA sequences known as κB sites present in promoter and enhancer regions of various genes[21]. The NF-κB family proteins form hetero- and homo-dimers, the most common of which activated by TLR signalling is the RelA-p50 complex. Both these proteins are transcription factors that regulate the expression of distinct but overlapping genes involved in the regulation of innate and adaptive immunity, inflammation, apoptosis, proliferation, stress responses and cancer progression[21]. In the resting cell, NF-κB is sequestered in the cytoplasm in an inactive form through its interaction with inhibitory protein-κB (IκB isoforms α, β, γ and ε) that masks the nuclear localisation signal, preventing nuclear translocation.

There are several key steps in the activation of NF-κB by TLR4 (summarised in **Figure 1.2**), which begins with the cytoplasmic recruitment of the TIR domain-containing adaptor proteins TIRAP (Mal) and MyD88. MyD88 then interacts with the death domains of members of the IRAK (IL-1 receptor-associated kinase) family of protein kinases, which include IRAK1, -2, -4 and –M[22]. In this process it is IRAK4 that is initially activated and consequently, it phosphorylates and activates IRAK1. After this sequential

phosphorylation of IRAK4 and IRAK1, they dissociate from MyD88 and interact with TNF Receptor-Associated Factor-6 (TRAF6), a RING-fingered domain E3 ubiquitin ligase, which together with the Ubc13 (Ubiquitin carrier protein N) and Uev1A promote the Lys63-linked polyubiquitination of target proteins, including TRAF6 itself[23]. The ubiquitinated form of TRAF6 subsequently recruits a protein kinase complex containing TAK1 (transforming growth factor-β-activated kinase-1) and TABs (TAK1 binding proteins)[24]. This complex then activates the IκB kinase (IKK) complex, which consists of IKKα, IKKβ and NF-κB essential modifier (NEMO), the latter being the regulatory component of the complex. This IKK complex phosphorylates specific serine residues on IκB, initiating its rapid polyubiquitination and degradation by the 26S proteosome, releasing NF-κB to translocate into the nucleus and mediate inflammatory responses. This pathway is known as the 'canonical pathway' and is responsible for the TLR mediated induction of inflammatory cytokines such as TNF-α and IL-6[25]. However, the NEMO-TAB-TAK1 complex also initiates the activation of a number of MAPKs (ERK, JNK, p38), coupling TLR4 signalling to other cellular processes. The direct role of TRAF6 in TLR4 signal propagation and DC maturation is evidenced by studies with TRAF6-deficient mice. In response to LPS, DCs from these mice are unable to effectively secrete IL-6 and IL-12 and up-regulate Major Histocompatibility Complex (MHC) class II to induce T cell priming, in response to various microbial stimuli including LPS, both *in vitro* and *in vivo*[26,27].

1.1.3.1 Ubiquitination and its role in mediating TLR4 responses

1.1.3.1.1 What is ubiquitination?

Ubiquitination is an important mechanism for regulating cellular trafficking and the induction of proteosomal and non-proteosomal-dependent mechanisms of signal degradation that result in the regulation of cellular responsiveness and inflammation[28]. Although the addition of ubiquitin (a 76 amino acid protein) was originally associated with targeting misfolded or damaged proteins for proteolysis. It is now known to be important for the formation of scaffolding complexes in signal transduction cascades and the subsequent activation and regulation of proteins[29]. Due to its diverse range of functions the ubiquitination of the target proteins lysine residues is mediated by three different classes of protein: the ubiquitin activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the E3 ubiquitin-protein ligase[30-32]. For ubiquitin to be added to a protein the formation of a thioester bond between the C-terminal carboxyl group of ubiquitin and a catalytically

active cysteine residue of an E1 enzyme in an ATP dependent manner is required. Subsequently ubiquitin is transferred to a cysteine residue within the active centre of an E2 enzyme and finally, ubiquitin is attached to the substrate protein in a process mediated by the E3 ligase[33]. Depending on the class of E3 ligase, this can involve ubiquitin transfer onto the E3 ligase, which occurs with HECT (homologous with E6-associated protein C terminus) domain containing proteins[34], or directly to the target protein from the E2 enzyme, as with RING (really new interesting gene) and U-box domain containing classes[35,36]. This process is summarised in **Figure 1.3**.

As ubiquitin has several lysine (Lys, K) residues in its structure it can itself be a target for ubiquitination, allowing formation of polyubiquitin chains, which differ in linkage type depending on the lysine residue[35,36]. Several different chain types (in terms of linkage and length) exist, which differ not only structurally but functionally[37-40]. However, the best studied are K48 and K63 linked chains, where K48 linked chains are involved in the proteosomal degradation of target proteins[41] and K63 linked chains can act as scaffolds for the formation of signalling complexes[24] as well as having roles in protein trafficking, DNA repair and other proteosome-independent mechanisms[42].

1.1.3.1.2 The importance of ubiquitin and the E3 ligases in TLR4 mediated activation of NF-κB

The ubiquitination of proteins down stream of TLR4 stimulation is required for the activation of NF-κB. For example, the RING-fingered domain E3 ubiquitin ligase, TRAF6, is a key member of the TRAF (TNF receptor-associated factor) family of proteins that participates in both TLR and TNFR signalling[27]. This E3 ligase acts as a key scaffolding protein involved in the propagation of TLR4 signals and is required for both MyD88 dependent and independent activation of NF-κB. TRAF6 is coupled to the TLR4 signaling complex at the plasma membrane (PM) by IRAK-1/2 and -4 after their phosphorylation and activation consequent to their association with MyD88. Subsequently, TRAF6 in conjunction with the E2 ligases, Ubc13 and Uev1a, promotes the Lys63-linked polyubiquitination of target proteins including TRAF6 itself, the cIAP1/2 (cellular inhibitor of apoptosis protein 1/2) proteins and the IKK complex component NEMO[23,24]. The polyubiquitylated cIAP proteins promote the K48-linked polyubiquitination and proteosomal degradation of TRAF3[43], a process which mediates the release of the MyD88-TRAF6 complex into the cytosol[44]. Ubiquitinated NEMO and TRAF6 subsequently recruit a protein kinase complex involving TAK1[24]. The activation of TAK1, subsequently

activates the IKK complex and several MAPKs, culminating in the activation of NF-κB and AP-1, respectively[24]. This involves the IKK mediated phosphorylation of IκB proteins, which are recognised by the F-box protein βTrCP – a subunit of the ubiquitin E3 ligase complex known as $SCF^{\beta TrCP}$, which also contains S-phase kinase-associated protein 1 (SKP1), CUL1 and RBX1. The $SCF^{\beta TrCP}$ E3 complex polyubiquitinates phosphorylated IκB proteins, which are subsequently degraded by the proteosome. This allows NF-κB to enter the nucleus and turn on target gene expression[45]

The activation of the TRAM-TRIF pathway by TLR4 occurs subsequent to MyD88 signalling and acts to maintain the activation of NF-κB and MAPKs as well as to induce the production of the type I IFNs via the activation of IFR3[15]. In detail the activation of TRIF results in its recruitment of receptor-interacting protein 1 (RIP1)[46], which undergoes K63-linked polyubiquitination by an E3 ligase of the pellino family, PELI1[47]. Ubiquitylated RIP1 then recruits TAB2 and NEMO, leading to NF-κB activation. The endocytosed TLR4-TRIF complex also recruits TRAF6 and TRAF3, but not the cIAPs to the endosome. TRAF3 subsequently undergoes K63-linked polyubiquitination, which activates the kinase TBK1 and IKKε, leading to IRF3 phosphorylation and subsequent production of type I IFN. Interestingly, the ability to induce IFNs can be conferred to TLR pathways that normally do not induce IFNs (such as the TLR2 pathway) by forced PM localisation of TRAF3[48]. The processes and the importance of ubiquitination in both MyD88 and TRIF mediated activation of NF-κB is summarised in **Figure 1.4**. Thus it is becoming clear that ubiquitination provides an important mechanism for the regulation of protein interactions, activation of signalling pathways and subcellular localisation of signalling elements in the activation of NF-κB by TLR4 signalling.

1.1.3.2 The Protein Kinase C (PKC) family and its involvement in TLR signalling and NF-κB activation

1.1.3.2.1 The PKC family

The PKC family is comprised of several lipid-sensitive protein serine/threonine kinases, which play central roles in the regulation of intracellular signal transduction and currently have an expanding repertoire of functions in both adaptive and innate immune responses[49]. Originally identified in 1977 by Nishizuka and colleagues[50], the PKC family now comprises of at least ten mammalian isoforms divided into 3 sub-families depending on their activation requirements. The conventional isoforms: PKC-α, -βI, -βII and -γ

require calcium, DAG and phosphatidylserine for activation; the novel isoforms, PKC-δ, -ε, -θ and -η, require diacylglycerol (DAG) and phosphatidylserine but are calcium independent; the atypical isoforms, PKC-ζ, -λ and -ι, require only phosphatidylserine for activation[51]. Thus many of these requirements revolve around the upstream stimulation of phospholipase C (PLC), an enzyme that hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate membrane-bound DAG and inositol triphosphate (IP_3), which results in the mobilisation of intracellular calcium[49]. In this respect, it is important to note that many PKCs can be pharmacologically activated by tumour-promoting phorbol esters (e.g. PMA), which act as DAG substitutes and anchor PKCs in their active conformations to cellular membranes[52].

Despite their expression within a broad range of cells and tissues[49], each isoform shares a similar overall structure consisting of an N-terminal regulatory domain coupled to a highly conserved C-terminal kinase domain (Summarised in **Figure 1.5 A**). In resting cells or in the absence of lipid hydrolysis, PKCs are localised primarily in the cytosol[53]. The regulatory N-terminal portion therefore serves two roles; the first being to engage lipid second messengers/proteins to tether the enzyme to particular cellular locations, including the PM, through two discrete targeting modules, termed C1 and C2[54]. The second is to regulate its kinase activity through a pseudo-substrate domain that binds to the substrate binding cavity in the catalytic site. This maintains the enzyme in an inactive form until binding of lipid second messengers restores catalytic activity by removing this blocking portion[54]. Between the regulatory (N-terminal) and kinase (C-terminal) domains lies a non-conserved 'hinge' region, which is liable for proteolytic cleavage when the PKC is activated, leading to the release of an isolated kinase domain fragment termed 'PKM' (protein kinase M)[55].

1.1.3.2.2 Regulation of PKC activity, localisation and function

Although often being labelled collectively as protein serine/threonine kinases, PKCs are anything but generic in their properties and functions as evidenced by recent studies showing they can be: **1)** controlled through phosphorylation on serine, threonine and tyrosine residues that influence the stability, protease/phosphatase resistance, protein-protein interactions, subcellular targeting and substrate specificity of the enzyme[52]; **2)** cleaved by caspases, generating a catalytically active kinase domain (in some cases, with altered enzymology) and a free regulatory domain fragment that can act both as an inhibitor of the full length enzyme and as a activator of certain signalling responses[52], **3)**

activated by other lipid cofactors (such as ceramide or arachidonic acid) or lipid-independent mechanisms allowing PKC to signal throughout the cell, not just at DAG-containing membranes[52]. The understanding of these key mechanisms is an important step in defining their roles in mediating TLR responses and as such will be outlined.

1.1.3.2.3 The cleavage of certain PKC isoforms in response to different stimuli

The hinge regions of certain PKCs have been identified as targets for caspase-dependent cleavage, protein-protein interactions and tyrosine phosphorylation. PKCδ, -θ, -ε and -ζ undergo caspase-dependent cleavage in response to a range of apoptotic stimuli where cleavage at the hinge region results in the release of a catalytic domain fragment[53], uninhibited by the NH_2-terminal regulatory domain[56]. However, activation loop transphosphorylation is required for this cleaved form to be active in all isoforms, except PKCδ. In this respect, the PKCδ isoform displays differences in the enzymology and roles of the full length and cleaved 40-kDa catalytic domain fragment, the latter of which is phospholipid independent and constitutively active[57,58]. Several studies have found that the relative levels of full length and cleaved (active) PKCδ and -ε can have dramatic effects on cellular function and the phosphorylation of targets depending on the stimulus[56]. The localisation of the PKCδ catalytic domain is also quite unique, as lacking the membrane targeting modules located within the NH_2-terminal regulatory domain, it accumulates in the nucleus through a NLS and this accumulation is sufficient to induce apoptosis in certain cell types[59].

1.1.3.2.4 The phosphorylation of PKCs

The phosphorylation of PKCs is crucial in the regulation of their activity, localisation and stability[53] (summarised in **Figure 1.5B**). Phosphorylation of three key sites located at the C-terminal 'tail', on most of the PKC family members is important for their function; these sites are the activation loop (A-loop), the turn motif (TM) and hydrophobic motif (HM) and have been described as maturational and priming events as the majority of PKCs are phosphorylated at these sites shortly after synthesis within quiescent cells[60,61]. The absence of phosphorylation at one or more of these sites leads to impairment of catalytic activation and enzyme stability. The A-loop is a highly conserved 20-30 residue sequence in the cleft of the kinase domain that is flexible and forms part of the peptide substrate binding surface. A-loop phosphorylation (at a threonine residue) stabilises the active conformation of the enzyme and is generally attributed to the master kinase, PDK-1

(3-phosphoinositide-dependent protein kinase-1)[62]. The TM motif is conserved in all PKC isoforms and upon phosphorylation makes important contacts with other residues to help stabilise its kinase core[63]. Interestingly the down-regulation of phosphorylation at the A-loop, TM and HM sites has been shown to precede the degradation of the PKC isoforms, most commonly through a proteosomal-ubiquitin system[64-66]. This generally occurs upon the metabolism of DAG, which results in the translocation of the cPKCs and nPKCs from the PM back to the cytoplasm. Dephosphorylation of PKCs can also occur at the sites such as the A-loop in response to TNF-α and is a key step in turning off the signalling capacity of these kinases[67]. However despite dephosphorylation serving as an important switch to turn off the activity of these enzymes, it does not necessarily predispose the enzyme to degradation. For example the down-regulation of PKCα, which occurs through the ubiquitin/proteosome pathway does not involve dephosphorylation of the enzyme[64,68].

Recently however, the phosphorylation of these C-terminal regions on nPKCs have been implicated in controlling their association with certain proteins and membranes and not just in maintaining the enzyme in a conformation that is favourable for catalysis[69,70]. The reasoning for this is: **1**) PKCδ can function as a kinase even without A-loop phosphorylation (at Threonine 505 [Thr505] in the case of PKCδ)[71]; **2**) Several phosphorylations of the nPKCs are not constitutive but are dynamically regulated[72]; **3**) Phosphorylations of the nPKCs at the TM and HM can occur by both auto-phosphorylation and transphosphorylation mechanisms (depending on the isoform and context).

Among all the nPKCs, PKCδ appears to be unusual as it retains a modest catalytic ability when not phosphorylated at these sites through its ability to stabilise its activation loop[73,74]. Whether it is phosphorylated (Thr505) or self-stabilised, the A-loop can dictate the substrate specificity of PKCδ. For example a Thr-507A mutation, which lacks A-loop phosphorylation, mimics only some of WT-PKCδ responses in cells, where it can trigger apoptosis but cannot activate NF-κB or AP-1 reporter constructs in Jurkat T cells[74]. The notion that the A-loop phosphorylation regulates PKCδ substrate specificity (rather than absolute activity) goes some way to resolving discrepancies in the literature regarding Thr505 phosphorylation in the control of PKCδ catalytic function[72,75]. PKCδ also has several sites outside the C-terminal tail that are phosphorylated in response to a diverse range of stimuli[53,73], including Tyrosine 311 (Tyr311), which has generated quite a lot of interest due to its role in acting as a docking site for the interaction of PKCδ with several

signalling molecules[52]. Thus current evidence suggests that its intracellular localisation and phosphorylation status “fine tune” the enzymological capacity of PKCδ in cells.

1.1.3.2.5 The localisation of PKCs and its importance in their function

The spatiotemporal localisation of PKCs is very important in mediating their activation and function, which can vary substantially depending on the cell type and the particular stimulus[52]. In this respect the C1 and C2 portions of the NH_2-terminal regulatory domain are important in tethering and directing PKCs to different cellular locales. For example, under certain stimulations, nPKCs (including PKCδ, -ε, -θ, -η) localise through their C1B domain to the golgi complex where PKCδ and -θ can induce apoptosis, while PKCε modulates secretion from the organelle[76,77]. However, PKC localisation is not restricted to cytoplasmic organelles as full length PKCδ can be detected in the nucleus during the initial phase of genotoxic stress in many cell types where its translocation is mediated by a bipartite NLS (6 basic amino acids) in its C-terminus[52]. Interestingly, there is some literature suggesting that PKC activity itself can influence PKC cycling (between the cytosol and PM), translocation and down-regulation in cells[78].

In terms of cPKCs, for stimulations that induce a biphasic DAG response, PKCα and PKCβII are released from the PM and collect in a ‘perinuclear’ site dubbed the “pericentron” (a subset of recycling endosomes) through a PLC-independent mechanism involving Phospholipase D (PLD)[79,80]. Interestingly the lipid, ceramide, can act as an inhibitory regulator of PKCα/PKCβII signalling at perinuclear membranes[81]. This localisation to the pericentron plays an important role in controlling trafficking of continuously recycling membrane signalling proteins such as caveolin-1 (important in the formation of caveolae) and certain cell surface receptors.

Indeed, several PKC isoforms are known to translocate to specialised membrane compartments known as caveolae, which are sphingolipid/cholesterol-enriched detergent-resistant membranes that form flask like invaginations (60-80 nm in diameter) at the PM[82]. Relating to this, the activation of PKCδ is important for the function of acid sphingomyelinase (ASM), an enzyme that catalyses the hydrolysis of sphingomyelin to form ceramide at the PM[83], as this lipid is important in the formation of lipid rafts and the localisation of proteins for internalisation via caveolae. Thus, PKCδ promotes local ceramide accumulation at the PM and drives raft fusion, providing a mechanism for the localisation of signalling proteins to membrane rafts. cPKC and nPKCs can be recovered

both at rest or following agonist activation in the caveolae fraction of several cell types[59], and some caveolin isoforms (caveolin-1 and -3) can physically interact with certain PKC isoforms such as PKCα and PKCγ but not others including PKCε[84]. Indeed the recruitment of PKCε and -δ to the PM is important in driving the clustering of FCγRIIA and caveolin-1 in lipids rafts to facilitate phagocytosis[85,86]. For example, PKCε translocates to the phagosomes and promotes efficient phagocytosis in macrophages where the engagement of IgG opsonised particle by FcγR results in the localised accumulation of signalling molecules through PLC/PLD production of DAG[87].

PKCα, -δ, and -ε have been found to accumulate in the caveolae fraction isolated from cardiomyocytes treated with PMA[88]. Interestingly, in cardiomyocytes this stimulation with PMA not only directs PKCδ to caveolae but subsequently induces its tyrosine phosphorylation (at Tyr311) that can be disrupted by cyclodextrin (a cholesterol-binding agent that undermines caveolae structural integrity)[89]. The phosphorylation at Tyr311 by Src family kinases, acts as a docking sites for several signalling molecules on PKCδ[52] and this has been reported in a variety of cell types to occur in response to non-apoptotic stimuli[53]. In this respect, the Src-dependent Tyr-311 and Thr-505 phosphorylation of PKCδ may regulate its localisation and subsequent substrate specificity[58,90].

1.1.3.2.6 The role of the PKCs in TLR signalling

There is now a growing body of evidence indicating that PKCs play important roles in TLR mediated activation of NF-κB (Summarised in **Figure 1.6**), with the majority of studies showing that alteration of the activity of particular PKCs in cells of the innate immune system, including DC, affected their ability to secrete pro-inflammatory cytokines in response to TLR stimuli. Subsequent to this, the TLR4 agonist, LPS has been shown to activate most PKC isoforms in innate cells such as monocytes, macrophages, DCs and neutrophils[91-95]. In conjunction with this, studies have shown that acute activation of the PKCs with phorbol esters, increases LPS-stimulated cytokine secretion[94,96-98], while pharmacological inhibition of PKCs attenuates cytokine production[96,98,99].

Recent evidence indicates that each of the TLRs can initiate the activation/regulation of different PKCs. In this context, several isoforms including PKCα, -δ and -ε appear to have important roles in the propagation of signals from TLR4, particularly in relation to the secretion of certain cytokines. For example, it has been demonstrated that both PKCε and PKCδ, directly interact with MyD88[49,100] and TIRAP[101],

via their TIR domains, respectively. In this respect, the downregulation of PKCδ leads to a decrease in NF-κB activation and the secretion of inflammatory cytokines, in a range of innate cells[93,102,103]. Consistent with this, as demonstrated in PKCδ deficient RAW264.7 macrophages, PKCδ is important for the TLR4 mediated activation of IKK and subsequently, NF-κB[101]. Interestingly, PKCδ has been suggested to be able to act like an IKK to phosphorylate IκB, which is then subsequently ubiquitinated and degraded by the 26S proteosome allowing nuclear translocation of NF-κB and its binding to κB elements[104,105]. PKCδ is also reported to increase the transactivation potential of NF-κB via an IKK/IκB independent pathway that is speculated to involve the direct phosphorylation of NF-κB by kinases downstream from PKCδ such as p38 or Akt[106-108]. Interestingly studies have shown a reciprocal regulation of PKCδ by NF-κB, by showing that a NF-κB responsive regulatory element in the PKCδ promoter links TNF-α stimulation to increased PKCδ mRNA and protein expression[109].

In relation to the diverse cell types in which PKCδ plays a prominent role, PKCδ$^{-/-}$ mice exhibit autonomous hyper-proliferation of B cells, leading to the development of immune-complex mediated glomerulonephritis and lymphocyte infiltration in many organs, indicating PKCδ as an important negative regulator of B cell proliferation[52]. By contrast, in endothelial cells, PKCδ mediated activation of NF-κB is observed to enhance protein expression of ICAM-1 (inter-cellular cell adhesion molecule 1) and increased neutrophil adhesiveness. There is also a link between PKCδ mediated activation of NF-κB and the induction of certain 'Inhibitors of apoptosis' (IAP) protein family members in human colon cancer cells and increased expression of pro-inflammatory mediators in airway epithelial cells[52]. Interestingly in microglia cells, which express all the TLRs and act as the primary sensors of PAMPs in the CNS[110], LPS activation of several caspases did not induce apoptosis but rather contributed to the downstream signalling of TLR4 in microglia. This was attributed to the role of caspases in PKCδ cleavage and ultimately NF-κB activation. Therefore, inhibition of caspase activity was shown to protect neurons in animal models of Alzheimer's disease and Parkinson's disease[111].

Another novel isoform, PKCε is an important mediator of macrophage function, with evidence indicating a role in LPS signal transduction through its involvement in initiating MAPK signalling[112]. Indeed, although PKCε $^{-/-}$ mice are healthy, they display the major defect of being unable to clear gram positive (TLR2) and negative (TLR4) bacterial

infections, due to the inability of their macrophages to activate IKK and NF-κB resulting in reduced production of TNF-α, Il-1β and nitric oxide (NO)[113]. PKCε appears to fulfil a similar role in DCs[112], as experiments utilising specific peptide inhibitors of translocation based on PKC-isoenzyme-specific pseudosubstrates, showed that PKCε is crucial for LPS-induced IKK and NF-κB activation resulting in TNF-α and IL-12p70 secretion but not the ability to up-regulate co-stimulatory molecules in monocyte derived DC[114]. Recently a direct link between TLRs and PKCε has been shown[100], where stimulation with LPS results in PKCε phosphorylation and association with MyD88. Interestingly, knock-down of MyD88 specifically results in the reduction of PKCε phosphorylation at Ser346. Thus PKCε may be important in integrating different signalling cascades that impact on the establishment of an effective immune response.

However, it is not only the novel isoforms that have a role in the TLR-NF-κB signalling axis as the convential isoform, PKCα has also been implicated in mediating a number of effects in the TLR signalosome. This conventional PKC isoform has been found to be immunoprecipitated with TLR2 from DCs following activation, an association which is ablated in MyD88$^{-/-}$ mice suggesting it is MyD88 that acts as the link between PKCα and potentially TLR2/4[115]. This hypothesis is further reinforced by evidence that the inhibitor Gö6976 (targets PKCα and -β) prevents TLR-stimulated cytokine secretion by neutrophils[91] and macrophages[116-118]. Moreover, 264.7 RAW macrophages expressing a dominant negative (DN) PKCα have reduced LPS-stimulated TNF-α and IL-6 secretion[119,120], suggesting a direct link in its propagation of TLR4 signals. However, these cells also show defects in phagocytosis and phagosome maturation which are non-TLR mediated aspects of innate immunity[121,122]. In human and mouse DC, the role of PKCα has been elucidated by use of its inhibitor Gö6976, DN constructs and PKCα$^{-/-}$ mice[115]. In these studies, PKCα was shown to be crucial for TLR2 mediated activation of MAPK and NF-κB leading to the secretion of TNFα, IL-6 and IL-10.

1.2 Bridging the gap; TLRs are an important component of both the adaptive and innate immune systems

1.2.1 The basics components of the adaptive immune system

Vertebrates have a second line of defence against invading pathogens, called the adaptive immune system which confers Ag-specific responses with memory of infection.

This complex network of cells employs a diverse set of somatically rearranged receptors including T-cell receptors [TCRs] and B-cell receptors [BCR] expressed by T and B lymphocytes that allows recognition of an extremely diverse range of unique Ags. Although T and B cells of the adaptive immune system express receptors of enormous diversity, full activation of these cells depends on the secretion of cytokines and chemokines coupled with the interaction and Ag presentation by the innate immune system. The discovery that innate immune cells sense pathogens through PRRs such as TLRs revolutionised our understanding of the role of the innate immune system in infection and driving adaptive immune responses particularly in relation to T cells[1]. The type of agonist and the PRR it binds is important for promoting the maturation of APCs such as DCs (through the activation of proinflammatory transcription factors such as NF-κB[6]) and their production of specific cytokines, depending on the pathogen encountered, promotes the development of an appropriate effector subset from the pool of naïve $CD4^+$ T cells. In this context, naïve T lymphocytes specifically recognise Ags presented by MHC complexes on antigen presenting cells (APC) through direct interactions via their TCR, resulting in their proliferation (clonal expansion) and differentiation into effector T cells. In a process referred to as 'priming', naïve T cells initially encounter an Ag intiating their differentiation and clonal expansion. The differentiated effector cells gain abilities that allow them to direct other cells of the immune system to locate and remove the recognised Ag, inducing active secretion of various cytokines. The primary T-cell response also generates memory T cells, long-lived cells that give an accelerated response to Ag, providing protection from subsequent challenge by the same pathogen.

In this context, T cells can be divided into two broad groups based on the expression of the transmembrane glycoproteins, CD8 and CD4. $CD8^+$ T cells recognise Ags presented by MHC class I molecules inducing the differentiation of naïve cells into cytotoxic effector T cells that recognise and kill virus infected cells. On the other hand, $CD4^+$ T cells (T helper cells; T_H) have a more flexible repertoire whereby after recognition of pathogenic peptides presented by MHC class II molecules, naïve CD4 T cells can differentiate down distinctive pathways that generate effector subsets such as T_H1, T_H2 and T_H17. For example; the secretion of IL-12p70 promotes the differentiation of T_H1 cells and cellular mediated immunity; IL-4 and IL-13 stimulates the clonal expansion of T_H2 cells which promote humoral immunity; IL-10 induces the development of regulatory T_H cells (T_{reg}) cells, which help to regulate the immune response; while combinations of IL-1, IL-6, TGF-β, IL-18 and IL-23 promote the generation of T_H17 cells, which in addition to combating infection have been shown to promote infammation[123-125]. Another interesting T

cell subset to emerge in recent years are γδ T cells, which secrete various pro-inflammatory cytokines following stimulation with IL-23 in combination with IL-1 or IL-18 (which are also secreted by DCs) with no requirement for TCR engagement[126,127]. Differentiation of effector T_H cells also allows them to provide help to B cells to trigger immunoglobulin (antibody) production.

The humoral (antibody) immune response mediated by B cells allows the specific targeting of pathogens in the extracellular spaces of the body and makes up a significant arm of the adaptive immune response. Interesingly, B cells do not merely produce antibodies, they also actively secrete cytokines and serve as APCs, where through direct interactions (does not require MHC) of the BCR with Ag activated B cells then differentiate into memory B cells and/or antibody-secreting plasma cells. In the context of natural infections, the BCR can bind native proteins, glycoproteins, polysaccharides, virus particles and bacterial cells by recognising certain epitopes on their surfaces. The BCR has two roles in B cell activation; firstly it signals to the interior of the cell when Ag is bound, and secondly, the B cell Ag receptor delivers bound Ag to intracellular sites, where it can be degraded to give peptides that are returned to the B-cell surface bound to MHC class II molecules. These peptide presenting MHC class II complexes are recognised by Ag specific T_H cells that have already differentiated in response to the same Ag. However this T-B cell interaction is bidirectional as effector T cells secrete cytokines that induce B cells to proliferate and differentiate into antibody secreting and memory B cells.

Based on their phenotypical, functional and topographical characteristics, B cells can be divided into innate-like and adaptive immune cells[128]. Follicular B cells have an important role in T cell dependent immune responses and belong to the adaptive arm of the immune system. Through somatic hypermutation in B cell follicles situated in lymphoid tissue they generate a clonally rearranged Ag specific BCR, which upon Ag stimulation develop either into antibody secreting plasma cells or memory B cells (for long-term repsones) both of which rely on T cells to maximise their potential. By contrast, B-1 and marginal zone B cells are considered to be more like innate immune cells, generating rapid but lower affinity antibody responses that are independent of T cell help. The term ‘B-1’ refers to the idea that this population develops earlier during ontogeny where they are predominantly enriched in the peritoneal and pleural cavities but can also be found in the spleen[129]. This is relative to the more conventional B-2 cells, which is a term used to traditionally describe the main population of mature B cells that develop, after birth, from common bone marrow precursors and are located in the bone marrow, spleen and lymph

nodes. B-2 cells therefore include both follicular and marginal zone subsets, which are currently referred to as separate populations because of their distinct phenotypical and functional characteristics.

1.2.2 The expression of TLRs on T and B cells; enhancing adaptive immune responses

It is well known that positive signalling through the TLRs on APCs dictates the maturation state and cytokine milieu that is important in driving the proliferation and differentiation of T cells and the adaptive immune response. For example, the TLR4 ligand LPS has been shown to promote IL-17 production by Ag-specific memory T cells in mice through the induction of IL-1 and IL-23 production by DCs[130]. However, within the last couple of years a number of studies have defined the expression of TLRs on various T cells subsets where it is emerging that TLR signalling can promote cytokine secretion and regulate their function[131]. For example, in relation to effector $CD4^+$ T cells, TLR2 signalling has been demonstrated in T_H1 cells to promote their proliferation and production of IFNγ[132]. In relation to the development of a T_H17 population, stimulation of $CD4^+$ T cells with PAM_3Cys, a specific TLR1/2 complex stimulant, induces their proliferation and production of IL-17, which is enhanced by concurrent treatment with IL-23[133]. It has also been demonstrated that $CD8^+$ effector T cells also express various TLRs[134,135]. In this context stimulation of mouse $CD8^+$ T cells with TLR2 agonists, enhances the proliferation of these cells in response to a CD3-specific antibody by lowering the threshold required for co-stimulatory signals[136]. Recently, IL-17 producing γδ T cells have been demonstrated to express higher levels of TLR1, -2, -4, -6 and -9 than IL-17 secreting $CD4^+$ T cells[133]. In this context, γδ T cells that express TLR1 and -2, secrete IL-17 *in vitro* following stimulation with their appropriate ligands, particularly in the presence of IL-23[126]. Recent evidence suggests that certain subsets of γδ T cells are already differentiated in the periphery and produce IL-17 in response to local IL-23[137], without requiring stimulation with either CD3 or CD28 specific antibodies or stimulation with IL-23 and IL-1 derived from PAMP activated immune cells[127].

The stimulation of TLRs on T cells can also lead to a number of inflammatory and suppressive effects as demonstrated by mouse T_{regs}. These cells are known to express TLR4, -5, -7 and -8[138], which is accompanied by mounting evidence that TLR agonists can modulate the function of these cells, where the persistent activation of the TLRs can overcome the suppressive effects of T_{reg} cells and abrogate tolerance to tumor cells[139]. In

contrast, it has also reported that TLR agonists can directly enhance T_{reg} function as LPS (TLR4 agonist) can induce the proliferation and enhance the suppressive function of T_{reg} cells[138]. The TLR1/2 ligand Pam_3Cys induces the proliferation of mouse T_{reg} cells and although this was associated with loss of the cells suppressive activity, this was temporary as it was restored following removal of the TLR agonist[140].

With respect to B cells, they express both Ag-specific receptors (BCR) and the TLRs[141] and dual BCR and TLR engagement can fine-tune functional B cell responses, directly linking cell-intrinsic innate and adaptive immune programmes. Importantly, the expression of distinct profiles of TLRs and BCR help to specify differentiation and function of key innate like versus adaptive B cell populations. Although B cell development and survival both appear unperturbed in the absence of TLR signals[142], patients with a deficiency in the TLR signalling molecules, MyD88 or IRAK4, possess an altered BCR repertoire with an increased proportion of autoreactive B cells, presumably owing to alterations in B cell selection processes[143]. During T cell-independent immune responses in mice, dual BCR and TLR signalling rapidly induces marginal zone B cell and B-1 cell migration and antibody production[141]. In addition, following the triggering of T cell-dependent immune responses, TLR responsiveness is directly modulated in activated follicular B cells, thereby affecting germinal centre responses[141]. Therefore, individual TLR expression profiles permit various effector B cell populations to manifest specific response profiles based on antibody production, Ag presentation and cytokine secretion following TLR engagement[144,145].

1.3 Dendritic cells; the definitive link between the innate and adaptive immune systems

DCs are recognised as the most highly potent APCs that interpret multiple signals through various PRRs, including the TLRs to orchestrate the initiation of the adaptive immune response (summarised in **Figure 1.7**). In this context, many intracellular and extracellular pathogens, such as bacteria and viruses will be recognised by PRRs expressed on DCs, leading to the upregulation of costimulatory molecules and Ag presentation as well as the secretion of a host of cytokines and chemokines, which promote T cell responses and enhance B cell responses[146]. Indeed, in addition to regulating priming or tolerance of T cell responses, differential DC maturation dictates the polarisation of T_H responses and the general phenotype of the immune response to fight distinct types of infection. For example, the production of IL-12 and TNF-α is commonly associated with

gram negative bacterial infection and leads to the subsequent generation of T_H1 responses, characterised by the production of IFNγ, bactericidal activity in macrophages and production of opsonising antibodies of the IgG2a class (in the mouse) by B cells[147]. Indeed, many pathogenic PAMPs mature DC to a phenotype that can induce such T_H1 mediated responses, including bacterial lipopeptide (BLP), LPS and CpG rich nucleic acid fragments activate TL2, TLR4 and TLR9, respectively[148]. Such a DC phenotype is typically characterised by strongly up-regulated expression of MHC class II Ags and co-stimulatory molecules, like ICAM-1, CD40, CD80 and CD86, leading to the expansion of naïve $CD4^+$ T cells and their differentiation to T_H1 $CD4^+$ T cells [147]. This occurs in conjunction with activation of $CD8^+$ T cells and the enhancement of cytotoxic lymphocyte activity, the maturation of B lymphocytes, Ig class-switching and the production of antibody[146].

By contrast, infection by parasitic worms does not induce T_H1 responses; instead helminths typically induce production of the regulatory cytokine IL-10 by DCs, which acts to down-regulate T_H1 responses. DCs also respond to parasitic worms by inducing T_H2 responses, characterised by the production of IL-4, IL-5 and IL-13 by $CD4^+$ T cells. This T_H2-polarising phenotype of DCs is associated with lower levels of MHC and co-stimulatory activity relative to those seen with T_H1-polarising DCs[149]. However, the mechanisms underlying the generation of T_H1 responses (i.e. the PRRs involved, signalling molecules activated and the molecular T_H1 signature) are better understood relative to those associated with the T_H2 responses as little is known about molecules that function as T_H2-inducing PAMPs. Indeed, over the last decade many studies have shown a tremendous versatility in the functions of DC, such that they are able to tailor selective immune responses to individual classes of pathogens, although the key regulatory mechanisms involved have not been defined[146].

Nevertheless, it appears that many of these processes are accompanied by endocytosis of the invading pathogens (or their products) by DCs, and this can be mediated by a variety of receptors such as: CD14, scavenger receptor-A (SR-A) and the Fc receptors: FcγRI1, FcγRI2b and FcγRIII. The receptor-mediated uptake of these agents leads to the activation of Ag processing mechanisms that load microbial peptides, typically onto MHC Class II, for presentation to $CD4^+$ T lymphocytes. However, the $CD8^+$ DC subtype are efficient in shuttling phagocytosed Ags into the MHC Class I pathway for the presentation to $CD8^+$ T lymphocytes[146]. Upon endocytosis and subsequent activation, DCs found residing in peripheral tissues traffic to the draining lymph nodes where they undergo further maturation and present Ag to naïve T_H cells to stimulate development of certain

subsets specific for the cognate peptide (e.g. T_H1, T_H2, T_H17 etc.) depending on the cytokine environment.

Although the engagement of various TLRs results in the production of several key proinflammatory cytokines including the type I interferons, TNF-α, IFN-γ, IL-12 and IL-1[150]. It appears that different DC phenotypes/subsets express unique repertoires of TLRs potentially allowing specialised responses to each class of pathogen at the site of inflammation and dictating the precise adaptive immune response required. For example, plasmacytoid DCs uniquely express TLR9 and TLR7 allowing them to respond to CpG DNA and viral ssRNA[151]. TLR9 ligation results in the high production of IFN-α and IFN-β that promote MHC expression in neighbouring APCs to enhance anti-viral immunity. However, these cells do not express TLR4 resulting in a poor response to LPS and bacterial infections that are primarily dealt with by TLR4 expressing $CD11c^+CD11b^+$ myeloid DCs. Phagocytosis in the presence of TLR signals results in trafficking of the endocytosed material to a specific phagosome maturation pathway that promotes enhanced degradation and the efficient presentation of Ags[152].

1.4 The role of cytokines in mediating immune responses

The interlocking innate and adaptive branches of the vertebrate immune system allow the host to fine tune immune responses to diverse invading pathogens to optimize elimination of the pathogen without damage to host tissues. Cytokines are small glyco-proteins that mediate inter-cellular communication and are important in maintaining this balance. Cells from both the innate and adaptive systems respond as well as produce and actively secrete cytokines, which have crucial functions in the development, differentiation and regulation of immune responses. For example, upon stimulation of TLRs by PAMPs, DCs secrete a specific range of cytokines in response to the type of pathogen encountered which for LPS/TLR4 signalling include IL-12, IFN-α, IL-6 and TNFα[153]. These cytokines can act directly on newly activated $CD4^+$ T-cells and as stated above bias their clonal expansion towards different immune phenotypes. The $CD4^+$ T_H cell is a key player of the immune system 'orchestra' and distinct lineages of these cells selectively produce certain cytokines that direct other cells to combat the specific type of invading pathogen encountered. Within recent years there has been an expansion of the number of lineages beyond the T_H1-T_H2 paradigm established by Mosmann & Coffman in 1986[154], which now includes T_H17 and T_{reg} cells (A summary of these T cell phenotypes and the stimuli required to generate them is provided in **Figure 1.8**).

The nature of the pathogenic stimulus influences the ability of DCs to induce particular T_H cell phenotypes. For example, the T_H1 cell population (associated with cellular immunity) produces the cytokines IFN-γ and lymphotoxin-α and their development is predominantly mediated by IL-12 secreted by DCs, which activates STAT4 (signal transducer and activator of transcription 4) in naïve lymphocytes[155]. Interestingly, the production of T_H1 inducing cytokines (IL-12p70 and potentially IL-27) by DCs is often amplified by positive feedback signals that are provided by the differentiating T cells[156]. These DC cytokines can also activate natural killer (NK) cells, which also produce IFNγ and indirectly promote the same type of immunity[157]. IFNγ is the cytokine that defines T_H1 differentiation and it is known to activate APCs and promote further T_H1 differentiation by up-regulating the signature transcription factor T-bet (T box 21).

By contrast, helminth products usually induce the maturation of DCs that drive the development of T_H2 cells and such differentiation has been associated with the selective expression of members of the jagged family of notch ligands[158-160]. The T_H2 phenotype is associated with driving humoral responses and constraining cell-mediated inflammation alongside the production of IL-4, IL-5 and IL-13. In relation to this, T_H2 cells are often additionally found to be active producers of the immuno-suppressive cytokines IL-10 and transforming growth factor β (TGF-β)[155], which are more generally associated with the T_{reg} cells. Within recent years there has been the identification of an ever growing number of individual T_H cell phenotypes dependant on the cytokines they secrete with one of the most interesting to emerge being T_H17 cells. This T_H subset functions mostly at mucosal surfaces, triggering pro-inflammatory danger signals to enhance neutrophil mobilisation and the expression of antimicrobial factors. However, T_H17 cells are also associated with driving inflammatory pathology in various autoimmune conditions[161]. Interestingly, some T_H17 cells have been shown to secrete IL-10 indicating that they have the capacity to regulate the potent inflammatory responses they induce[162]. Studies of the role of DCs and the cytokines they secrete in the development of a T_H17 population has identified that their differentiation is regulated by the cytokines TGF-β, IL-1, IL-6 and IL-23, which stabilise and expand the cell population through the induction of the transcription factors: STAT3 and retinoic acid receptor-related orphan receptor-γt (ROR-γt)[161]. Interestingly, the DC secreted cytokine, IL-27 specifically inhibits the proliferation of T_H17 cells[163].

1.4.1 Important cytokines associated with DC function

1.4.1.1 Tumour necrosis factor-α (TNF-α)

TNF-α was first discovered due to its ability to cause necrosis of some tumours[164]. The regulation of TNF-α production is complex and stimulus-dependent and occurs at both the transcriptional and post-transcriptional levels. Human TNF-α is encoded by a single gene, which is located on chromosome 6 in the MHC coding region[165]. It is initially expressed as a 26 kDa transmembrane protein, which is subsequently cleaved by a metalloproteinase[166] to release soluble TNF-α molecules that can then form non-covalently linked homotrimers. Mature TNF-α is non-glycosylated and both the membrane bound and soluble forms are known to be biologically active[167].

TNF-α is a pro-inflammatory cytokine, which is principally produced by monocytes, macrophages and DCs in response to a range of pro-inflammatory stimuli[168,169]. Typically exposure to LPS up-regulates its gene and subsequent protein production[170]. The overproduction of TNF-α is well established as contributing to pathogenesis in various autoimmune disorders[171] as well as in septic shock[172]. TNF-α induces the activation of B and T cells[170] and stimulates APCs, such as DCs, to secrete pro-inflammatory cytokines (for example IL-1 and IL-6) and chemokines. Interestingly, the production of IL-6 in response to TNF-α can lead to the promotion of T_H2 differentiation through IL-4 induction[155] and in relation to this, at the same time IL-6 attenuates T_H1 differentiation through its effects on SOCS1 (suppressor of cytokine signalling) signalling promoting the development of T_H17 cells.

TNF-α can influence the function of APCs in a number of ways. It is known to activate immature APCs by augmenting their Ag presenting capabilities and up-regulating co-stimulatory molecule expression on their surface. However, in mature DCs it can also inhibit these functions and may even induce apoptosis and impair Ag presentation[155,173]. Thus, it has emerged that TNF-α is a molecule that can induce a complex array of responses. Reflecting this, it exhibits both pro- and anti-apoptotic functions, for example TNF-α activates NF-κB, a key inflammatory and apoptotic factor that has also been shown to be to inhibit T cell signalling[173].

1.4.1.2 Interleukin-6 (IL-6)

IL-6 is defined by several structural motifs including a 'four helix bundle' and is associated with acute phase responses and the regulation of innate and adaptive immunity[174]. IL-6 was originally discovered in 1986 as a B-cell differentiation factor that promotes the differentiation of activated B-cells into immunoglobulin-producing cells[175]. However since that time the effects of IL-6 have been found to be far reaching indeed, ranging from a key polarising cytokine in immunity, through to playing important roles in the biology of bone[176], lipid metabolism and angiogenesis[177]. Its receptor comprises the IL-6 receptor-α (IL-6Rα) and glycoprotein 130 (gp130) subunits, the latter being a common component of several members of this receptor family. IL-6 is produced by various types of cell, such as T-cells, B-cells, monocytes, DCs, fibroblasts and endothelial cells[177]. However, the IL-6 receptor (IL-6R) is mainly expressed by haemopoietic cells, such as T cells, monocytes, activated B cells and neutrophils[177] and as such the elevated production of IL-6 contributes to the pathogenesis of various autoimmune and inflammatory diseases. The cytoplasmic domain of gp130 contains several motifs for the propagation of intracellular signalling including those for recruiting and activating SHP-2 (Src homology domain containing protein tryrosine phosphatase-2)[177]. Consistent with this, the binding of IL-6 to its receptor induces the homodimeriation of gp130 and association with IL-6Rα, resulting in the activation of the SHP2-mediated ERK/MAPK pathway and the JAK/STAT pathway[178].

Although discovered as a B-cell differentiation factor, IL-6 has also been shown to promote antibody production by inducing $CD4^+$ T cells to secrete more IL-21. Thus, it has been proposed to enhance humoral immunity[179] and supporting this transgenic mice that overexpress IL-6 exhibit hypergammaglobulinaemia and autoantibody production[180]. IL-6 also plays an important role in the proliferation and differentiation of T_H cells, where it significantly enhances the proliferation of T_H cells stimulated with mitogen[181]. Within this context, IL-6 promotes IL-4-mediated T_H2 differentiation and inhibits IL-12 induced T_H1 differentiation[182]. Moreover, *in vitro* studies in mice have shown that co-stimulation with IL-6 and TGF-β is essential for the differentiation of T_H17 cells from naïve T_H cells and this role of IL-6 in T_H17 development has been confirmed in several autoimmune disease models[177].

1.4.1.3 Interleukin-10 (IL-10)

IL-10 plays a central role in preventing inflammatory and autoimmune pathologies by limiting the immune response to pathogens and host tissues[183]. The anti-inflammatory properties of IL-10 are evidenced by, studies in IL-10-deficient mice, which develop inflammatory bowel disease following colonisation of the gut with particular micro-organisms and display other exaggerated inflammatory responses to microbial challenge[184,185]. Although originally thought to be specifically expressed by T_H2 and T_{reg} cells, it is currently understood that IL-10 can be expressed by many cell types and has been reported to be produced by the T_H1, T_H2, T_H17 and T_{reg} $CD4^+$ subsets as well as $CD8^+$ T cells and B cells[186,187]. It is also expressed by cells of the innate immune system, including DCs, macrophages, mast cells, NK cells, eosinophils and neutrophils[188]. This broad expression across immune cells highlights its important role as a feedback regulator of many effects of the immune system and not just those associated with T_H1 cells but also T_H2 cells and allergic responses[189,190]. The anti-inflammatory response of IL-10 is mediated by the IL-10 receptor (IL-10Rα), which is coupled to signalling through STAT3[188]. The production of IL-10 by DCs and macrophages acts in an autocrine fashion on the IL-10Rα expressed by these cells to help attenuate the extent of their responses and in turn the development of T_H1, T_H2 and allergic responses that can be driven by continued activation of these immune cells[188,191]. The production of IL-10 by T_H1, T_H2 and T_H17 cells adds another feed back loop to limit innate effector functions of DCs and macrophages and the subsequent activation of T cells.

Both macrophages and DCs can express IL-10 following activation of specific PRRs, including TLR4 recognition of LPS both *in vitro* [192] and *in vivo*[193,194]. Interestingly, ligation of CD40 on DCs enhances their IL-10 production in response to TLR and dectin 1-stimulation[195], while in macrophages a similar effect is observed upon TLR stimulation and ligation of Fc receptors[196]. Following the stimulation of TLRs (including TLR4) the activation of ERK has been shown to be important in IL-10 production[197,198], as in ERK-deficient cells, TLR-mediated secretion of IL-10 by DCs is decreased[199]. However, other signalling elements have been implicated in the LPS induced production of IL-10, including TRAF3 and p38[200]. The production of IL-10 from T_H1 cells requires they be triggered via the TCR by a high Ag dose in the presence of endogenous IL-12[162], whilst in T_H2 cells, IL-10 secretion is controlled by the IL-4 signalling pathways in conjunction with the transcription factors STAT6 and GATA3 (GATA binding protein 3)[201,202]. By contrast, IL-10 production by T_H17 cells predominantly occurs in a STAT3 and STAT1 dependent

manner[203]. However the production of IL-10 from all of these subsets is dependent on the activation of ERK[162]. Interestingly the recently identified cytokine IL-27, which is produced by DCs has been shown to enhance the expression of IL-10 by T_H1, T_H2 and T_H17 cells[203].

1.4.1.4 Transforming growth factor β-1 (TGF-β1)

TGF-β comprises a well-defined multipotent cytokine family known to regulate several patho-physiological events, with TGF-β of T cell, monocyte/macrophage or granulocyte origin typically being detected at sites of inflammation[204]. Among the three isoforms, TGF-β1 exhibits the broadest spectrum of biological activities, promoting early DC development whilst suppressing immature DC activation and maturation and modulating T_H polarisation[205]. It mediates these effects on DC maturation by inhibiting the up-regulation of the co-stimulatory molecules CD80 and CD86 thereby reducing the efficacy of such DC cells to stimulate T cells[146]. One way TGF-β may achieve these functions is by the down-regulation of key immunomodulatory receptors, for example murine bmDC treated with TGF-β1 show decreased expression of TLR4 and this correlates with their reduced sensitivity to LPS induced maturation[206]. It has also been noted that TGF-β treatment of splenic $CD11c^+$ DCs inhibits their ability to produce IL-6 and IL-12 in response to LPS[207]. In terms of modulation of T_H responses, TGF-β acting alone induces FoxP3 expression leading to T_{reg} induction whereas in the presence of IL-6, it results in the promotion of T_H17 responses[123,124].

1.4.1.5 IL-12p40 and its extended family; IL-12p70, IL-23 and IL-27

IL-12 was identified as a product of Epstein-Barr virus (EBV)-transformed human B-cell lines that can activate NK cells, generate lymphokine-activated killer cells (LAKs) and induce IFN-γ production and T cell proliferation[208]. After further investigation, the biologically active form IL-12p70 was found to be a heterodimeric (IL-12p40p35) pro-inflammatory cytokine that induces the production of IFNγ (from multiple sources) and favours the differentiation of T_H1 cells forming a vital link between innate resistance and adaptive immunity[209]. The major sources of this cytokine are phagocytes and DCs, which produce it in response to parasitic and bacterial infections[210]. The production of this cytokine is able to promote naïve $CD4^+$ T cells to differentiate into mature T_H1 effector cells[211] and is a potent stimulus for T_H1, NK and $CD8^+$ T cells to produce IFN-γ[212-214]. As stated above, this heterodimeric protein is composed of two covalently linked subunits, the

p35 subunit (IL-12p35) and the p40 subunit (IL-12p40), which is structurally related to soluble IL-6 receptor. The IL-12 receptor (IL-12R) is composed of two chains, IL-12Rβ1 and IL-12Rβ2, and coupled to the JAK-STAT pathway of signal transduction[215], with the major effector being STAT4. IL-12R is expressed predominantly on activated T cells and NK cells[215] but has also been identified on other cell types such as DCs[216] and B cells[217]. The activation of T cells through the TCR up-regulates the transcription and expression of both chains of IL-12R and this can be enhanced by the cytokines IFN-γ and TNFα[218]. Large quantities of IL-12 p40 are produced relative to IL-12 p70 and IL-12p40 is secreted both as homodimers and monomers[209]. In mice the production of p40 homodimers is observed *in vivo*[219] and although not biologically active as an agonist, it binds to the IL-12R with an affinity similar to IL-12 p70 and thus competes for binding[220]. On this basis it has been proposed that in mice formation of the IL-12p40 homodimer may act as a natural inhibitor of IL-12. Interestingly the p35 subunit can only be secreted when associated with IL-12 p40 and hence, the low abundancy of its transcripts even in activated inflammatory cells suggest that its expression is the rate limiting step for IL-12 p70 production[221].

The ability of DCs to produce IL-12 during interactions with T cells and to induce T_H1 responses including the subsequent production of IFN-γ were first shown *in vitro*[210]. This was soon followed by *in vivo* data demonstrating $CD8\alpha^+$ DCs being the first cells to secrete IL-12p70 in the spleens of mice exposed to LPS or soluble extracts of *Toxoplasma gondii*[222]. The initial production of IL-12 p70 occurs rapidly and is independent of IFN-γ and signals from T cells[223] with several TLR agonists, including those for TLR2, TLR4, TLR5 and TLR9, strongly inducing IL-12 p70 production in monocytes, macrophages and DCs in a T cell-independent manner[224]. However, IL-12p40 is also produced during the Ag-specific phase of the immune response in a T cell-dependent manner[225] with the production of IFN-γ up-regulating IL-12p70 production in a positive feedback manner to stimulate the further production of IFN-γ by T_H and NK cells. This in turn then acts to enhance IL-12p70 production from macrophages and DCs[226]. This positive feed back loop is required for optimal IL-12p70 production to induce protective responses against certain pathogens such as *Leishmania major* or *Bacillus Calmett-Guerin* [227]. The activation of IRF (interferon regulatory factors) transcription factors by IFN-γ is central to the production of IL-12p35 and p40. Specifically IRF1, -2, -5, -7 and -8 are directly involved in IL-12p35 and p40 production[228], with mice lacking IRF1 and -2 being unable to express IL-12p35 and p40 and IRF8 knock-out mice failing to synthesise IL-12p40[229-231].

Interestingly, T cells can also enhance the production of IL-12 through direct cell-cell interactions, such as the interaction of CD40L on activated T cells with CD40 on DCs and macrophages[232]. IL-12p70 only induces the proliferation of resting peripheral blood T_H and NK cells in conjunction with mitogenic lectins, alloantigens and CD3 specific antibodies[233] but it does have a direct proliferative effect on pre-activated T_H and NK cells[233]. Stimulation through CD28 (by CD86 on DCs) is synergistic with IL-12p70 such that the engagement of both by activated, Ag-presenting DCs results in T_H cell proliferation and IFN-γ production[234]. The activation of NK cells by IL-12p70 also promotes their production of IFN-γ and enhances their cytotoxicity[235]. Although IL-12p70 does not directly affect B-cell proliferation, through its induction of IFN-γ it can induce B cell activation and immunoglobulin switching[217].

Although IL-12p70 is important in host defence, its over expression can cause persistent inflammation in autoimmune disorders such as multiple sclerosis (MS). However, both TGF-β and IL-10 are potent inhibitors of IL-12p70 production[236], by decreasing NF-κB and AP-1 activation[237,238] and the association of the IL-12p40 promoter with RNA polymerase[239]. The inhibition of IL-12p70 production by TGF-β occurs through the ability of the latter cytokine to reduce the stability of IL-12 p40 mRNA[240]. The complexity of these cytokine networks is evidenced by the finding that TNF-α, which has many overlapping functions with IL-12p70 in regulating inflammatory responses and IFN-γ production can suppress production of TGF-β[241].

More recently, there has been a major focus on two other cytokines of the IL-12 family, IL-23 and IL-27. Neither of these cytokines play a role in classical T_H1 associated immunity. Rather, IL-23 has been shown to promote the expansion of naïve T_H cells to the T_H17 phenotype and hence, this cytokine also plays a dominant role in the development of autoimmune inflammation[242]. On the other hand the principal function of IL-27 *in vivo* is to limit the intensity and duration of innate and adaptive immune responses[242], in part by antagonising T_H17 responses[209]. Although all of the IL-12 family cytokines IL-12p40, IL-12p70, IL-23 and IL-27 are produced in response to microbial and host stimuli such as positive signalling through the TLR[243] and IFN receptors[244,245], it is becoming clear that these cytokines play divergent roles in guiding the immune system to eliminate invading pathogens and prevent excessive damage to host tissues.

IL-23 is composed of a p19 (IL-23p19) subunit, identified in 2000 by Kastelein et. al[246], covalently linked to the IL-12p40 subunit and it is now known that the activation of NF-κB through LPS induced TLR4 activation, is essential for the up-regulation of the p19 subunits mRNA and protein expression in murine DCs [247]. Interestingly mRNA for the receptor of IL-23 (IL-23R) is found to be upregulated in activated T and NK cells, regardless of the polarising conditions[242] and consistent with this, the IL-23R is expressed on activated/memory T cells, NK cells, DCs and macrophages but not naïve T cells [248]. Thus, it has been proposed that the relative amounts of IL-12p70 and IL-23 might determine whether T cell production of IL-17 or IFN-γ predominates[242]. Both monocyte and myeloid derived DCs have been shown to produce IL-23 in response to LPS and the polarisation of T_H1 and T_H17 responses has again been correlated to the balance of IL-12p70 and IL-23 secretion[249].

IL-27 comprises the IL-12p40 homolog, Epstein-Barr virus induced molecule 3 (EBI3) and the IL-27p28 subunit. The IL-27 receptor is composed of the two subunits, WSX1 and gp130[242] and exhibits an extremely broad expression, not only being expressed by various cells of the immune system (APCs, mast cells, NK cells, B and T lymphocytes) but also by endothelial cells[250]. Initial studies indicated a role of IL-27 in the generation of T_H1 responses, as IL-27 deficient T cells produced less IFN-γ *in vitro* than wild type cells and hence, it was thought to direct early events leading to the development of T_H1 responses[251]. Consistent with this, IL-27 was shown to induce the early activation of STAT1 and expression of the T-bet transcription factor (whose target genes encode signature T_H1 responses and IFN-γ[242]) and subsequently the expression of the IL-12Rβ2, which is the pre-requisite for mounting a IL-12 dependent T_H1 driven immune response[252]. However, more recent studies indicate that in the presence of strongly polarising stimuli, i.e. parasitic or bacterial infection, the ability of IL-27 to promote T_H1 cell responses is secondary to its role as a suppressor of effector T-cell proliferation and cytokine production[253,254]. In this context it has also been shown to specifically inhibit the proliferation of T_H17 cells[163] and act as a negative regulator of mast cell and macrophage function[253]. Studies using the parasitic nematode *Trichris muris* also indicate a direct regulatory role for IL-27 on T_H2 responses through inhibition of the secretion of the cytokines IL-4, IL-5 and IL-13[255]. IL-27 can regulate the kinetics and intensity of T cell responses but these effects are not restricted to a particular T_H cell subset[256]. In this respect, it can enhance the clonal expansion of $CD4^+$ T cells but this effect is most apparent in the

absence of the dominant T cell growth factor IL-2, which acts as a negative regulator of IL-27 receptor expression[257].

1.5 The role of co-stimulatory molecules in DC function

Co-stimulatory molecules are cell-surface proteins on APCs that interact with counter molecules on naïve T cells to transmit signals in concert with antigenic stimulation that is required for priming or tolerance of T cell responses. Their main function therefore is to transmit the appropriate signals to naïve T cells to promote (e.g. CD40-CD40L, CD80-CD28 and CD86-CD28) or inhibit (e.g. CTLA-4-CD28) T cell survival, expansion and lineage commitment. When DCs arise from myeloid- or lymphoid-derived presursors they exhibit an immature phenotype characterised by a high phagocytic capacity and low expression of the co-stimulatory molecules CD80, CD86 and CD40[258]. However, upon encountering bacterial, viral and parasite PAMPs, which induce DC maturation via TLR signalling and NF-κB activation, cell surface expression of these co-stimulatory molecules is upregulated[259]. During this process DCs will adhere and engulf pathogens via phagocytosis utilising a range of receptors including CD14, scavenger receptor-A (SR-A) and the Fc receptors: FcγRI, FcγRIIb and FcγRIII[146]. Once engulfed, proteolysis of the pathogens generates antigenic microbial peptides, which are loaded onto MHC Class II molecules and presented to $CD4^+$ T_H cells, prolonging DC-T cell interactions in the lymph node[260]. Thus, TCR engagement of the peptide-MHC Class II complex in concert with the particular signals derived from DC co-stimulatory molecule expression and cytokine secretion dictates whether T cell tolerance or priming is established[146].

1.5.1 CD40

Despite the well defined roles of co-stimulatory molecules in DC-T cell interactions the intracellular signalling pathways and regulation of these proteins reveals a complex biology that is as yet not fully defined. One such DC-expressed molecule is CD40, a 48kDa transmembrane glycoprotein surface receptor that is a member of the TNFR superfamily. Its natural ligand is CD40L, also referred to as CD154, a 34-39 kDa type II integral membrane protein. Initially CD40L was found to be expressed on $CD4^+$ cells, suggesting its main function was in the ability of T cells to stimulate B cell proliferation through ligation of CD40. However, recent mRNA and protein expression studies have shown CD40L to be expressed on a variety of DCs (including plasmacytoid and spleenic DCs), monocytes, epithelial cells and even endothelial cells[261,262] and

crosslinking of CD40 has even been shown to stimulate non-haemopoietic cells, such as epithelial cells, to produce GM-CSF[263]. Interestingly, it has been reported that CD40 signalling in DCs induces changes that make them more effective APCs, including the up-regulation of MHC Class II and other co-stimulatory molecules such as CD80 and CD86[264,265] as well as producing high levels of inflammatory cytokines and chemokines[266]. The wide expression of CD40 and CD40L, particularly on DC subtypes, suggests that DCs may interact with one another through this receptor to regulate their functions. Together with the proposed interactions of CD40 with several other proteins, such as C4BP, a complement protein that can signal through CD40 on B cells, these findings indicate this receptor can act as a bridge between innate and adaptive immunity[267]. Focusing on the intracellular effects of CD40, its cytoplasmic tail contains sites for the recruitment of TRAFs and indeed, following CD40 ligation, recruitment of TRAF6 is important in the activation of p38 MAPK and JNK, mediating inflammatory cytokine (IL-12p40 and IL-6[262]) production and up-regulation of the co-stimulatory markers CD80 and CD86[26,268]. TRAF6 also plays an important role in TLR signalling and consistent with the importance of TRAF6 for both TLR and CD40 signalling, DC from TRAF6 $^{-/-}$ mice are unable to up-regulate MHCII and CD86 and produce IL-12p40 or IL-6 in response to either CD40L or LPS[27]. The overlap of CD40 and TLR signalling pathways through TRAF6 is further reinforced by the fact that these signalling cascades converge on the activation of the non-canonical NF-κB pathway as evidenced by the finding that CD40-mediated DC Ag presentation required the activation of NF-κB-inducing kinase (NIK) to activate NF-κB2 (p52/p100)[262].

1.5.2 CD86

CD86 is a co-stimulatory molecule that is mainly expressed on APCs such as DCs and engages with CD28 on T cells, significantly lowering their activation threshold and allowing them to be readily activated[269]. DCs dynamically regulate CD86 expression levels depending on their need to harness or reduce their T cell activating capacity: for example, DCs up-regulate CD86 in response to microbial Ag[270] to facilitate microbe-specific activation of T cells. By contrast, DCs down-regulate CD86 in response to IL-10 and as DC secrete IL-10 in response to LPS, this generally immunosuppressive cytokine acts in an autocrine manner to prevent the excessive expression of CD86[271]. Interestingly, the E3 ubiquitin ligases have been shown to play an important role in regulating CD86 expression and Ag presentation by DC in an IL-10 dependent manner[272]. Ubiquitination is known to mediate endocytosis and the subsequent lysosomal or proteosomal degradation of many

transmembrane proteins[273,274] and it is in this context that the E3 ubiquitin ligase MARCH1 mediates the ubiquitination of CD86 and MHC Class II molecules to regulate their lysosomal degradation.

Interestingly DC-T cell interactions are frequently viewed as monodirectional with T cell activation being dependent on the interaction with DC (or other APCs). However, the engagement of various cell surface molecules on the DC by T cells induces bidirectional signals that affect DC maturation and activation. For example, ligation of CD40 by CD40L expressed on the surface of T cells delivers a potent activation signal for DCs[275]. By contrast, CTLA-4 expressed on the surface of T_{regs} binds and signals through CD80 and CD86, leading to the production of IFN-γ, which acts in an autocrine fashion on DC leading them to secrete the enzyme indoleamine 2,3-dioxygenase (IDO)[146]. When naïve T cells interact with such IDO-producing DC, they undergo activation and then rapid cell death by apoptosis, resulting in Ag-specific tolerance[146]. However, T cell-mediated feedback of a pro-inflammatory signal to DCs via CD28-CD86 interactions[146], induces MAP Kinase (p38) and NF-κB signalling resulting in IFN-γ and IL-6 production[276], which prevents IDO production by IFN-γ.

1.5.3 Major histocompatibility complex class II (MHC Class II)

MHC Class II molecules (encoded by the gene locus *H2* in mice*)* are expressed predominantly by professional APCs, mainly macrophages, DCs and B cells. MHC Class II molecules are heterodimers composed of an α-chain and a β-chain, which are type I integral membrane proteins with short cytoplasmic domains and large extracellular domains[277]. Their main function is to present peptides processed from exogeneous protein Ags to $CD4^+$ T_H cells and hence they are involved in directing the processes of positive and negative selection in the thymus, shaping the repertoire during T cell maturation and lineage commitment[260], as well as triggering TCR-mediated responses of mature T cells in the periphery. MHC Class II molecules acquire antgenic cargo for presentation by intersection of the endocytic and class II biosynthetic pathways[278]. This process requires the efficient proteolysis of both the Ag to be presented and Class II associated invariant chain (Ii), which associates with both the α- and β- dimers to mediate proper folding and trafficking of the complex to the late endosomes by occupying the peptide binding cleft and preventing unwanted peptide binding[278]. TLR signalling transiently increases Ii and MHC Class II synthesis to optimise Ag presentation under conditions of infection[279].

However, MHC Class II molecules can also act as receptors to mediate reverse signal transduction after interaction with T cell receptors or CD4 molecules (mimicked *in vitro* with agonist antibodies). Thus, engagement of MHC Class II can regulate multiple cellular functions in both the APC and stimulated T cells such as cell adhesion, cytokine production and co-stimulatory molecule expression[280]. Signalling via protein kinase C (PKC) isoforms[280,281] has been shown to play roles both in regulating MHC Class II expression and also reverse signalling through these proteins in APCs. For example, various PKC isoforms are activated upon LPS-TLR4 binding and PKCδ has been found to not only play an important role in the coupling of TLR4 to NFκB but also to the upregulation of MHC Class II expression by DCs[281]. In the context of TLR4 signalling, deficiency in MHC Class II expression results in the diminished production of LPS-induced TNF by human peripheral blood monocytes and mouse macrophages[280], resulting in greater resistance to endotoxic shock and the reduced production of pro-inflammatory cytokines in response to lethal doses of LPS[280]. Interestingly, it has also been found that intracellular pools of MHC Class II via endosome-localised CD40 and the kinase Btk, are able to interact with and promote, the activation of both MyD88- and TRIF-dependent pathways indicating a novel role for MHC Class II signalling in the TLR4-NF-κB pathways[280].

1.6 Immunomodulation by Filarial nematodes

1.6.1 The West and the rise of inflammatory disease

The developed world is experiencing a steep increase in the prevalence of a diverse range of diseases based on aberrant inflammatory responses, including autoimmune disorders like rheumatoid arthritis and allergic conditions such as asthma. The immunological phenotype of these diseases reflect both T_H1 (typically characterised by TNFα/IFN-γ based responses associated with bacterial infections and autoimmune disease) and T_H2 (classically associated with IL-4/IL-5 driven responses important in helminth infections and allergic diseases) immune responses[282]. The emergence of this diverse range of disorders is too rapid to support a solely genetic explanation for their increased incidence[283]. Rather, Strachan formulated the 'hygiene hypothesis' to account for this phenomenon[282], which postulates that the rise of allergic diseases is directly linked to inappropriate priming of the immune system, as a result of the decline in infectious diseases associated with the implementation of antibiotics, improved hygiene and wide spread use of vaccinations. This leaves the immune system with insufficient T_H1

stimulation and consequently T_H2 mediated pathologies such as asthma have become more prevalent. Indeed, the geographical distribution of the aforementioned inflammatory conditions virtually excludes areas where T_H2 driving helminth infections are endemic[284]. However, the hygiene hypothesis does not explain why T_H2 type allergies are rising in concert with T_H1 mediated autoimmune diseases (such as arthritis, multiple sclerosis and diabetes) and hence this conundrum suggests that the rise in inflammatory disease cannot merely be accounted for by an imbalance in T_H1-T_H2 priming. This has led to recent proposals that, although helminth infections and atopic diseases are associated with similar immunological phenomena, immunoregulation by the parasites within the host could dampen responses to allergens and T_H2 associated pathologies[285].

1.6.2 Filarial nematodes and their orchestration of a T_H2 immune phenotype

Helminth parasites are the most common infections that affect deprived communities worldwide. With nearly 2 billion individuals infected with a wide range of symptoms, ranging from intestinal problems, general malaise, weakness to anaemia, there are continuing problems with morbidity and even mortality[286-288]. Filarial nematodes represent a group of helminths that can induce particularly severe morbidity with a significant proportion of sufferers presenting with debilitating health problems including severe skin lesions, elephantiasis and several forms of eye damage, which can lead to blindness. Infection with these organisms is long-term, with individual worms surviving for up to 10 years[289]. The ability of these parasites to survive for these lengths of time within a host are dependent on the modulation and suppression of the host immune system[290]. This modulation has been shown to reflect impaired lymphocyte proliferative responses and polarisation of cytokine and antibody responses, generally evidenced by reduced T_H1 associated IFN-γ production and increased IL-4 and anti-inflammatory IL-10 secretion coupled with elevated levels of IgG_4, responses typically associated with a T_H2 phenotype. The switch to IgG4 is also important as this isotype is unable to activate complement or bind with a high affinity to phagocytic cells and hence cannot contribute to the elimination of pathogens. Indeed, the suppression of T_H1 inflammatory mediators by APC populations is emerging as a common theme in helminth infection. For example, studies on *N. brasiliensis* excretory-secretory (NES) products indicate that they also actively mature DCs to induce a T_H2 response by upregulating CD86 and OX40L, switching the cytokine profile to release IL-6[291].

It was from these findings that the hypothesis arose that this phenotype not only prolongs parasite survival but is conducive to host health by limiting the development of autoimmune disorders that arise from aggressive, pro-inflammatory T_H1-like immune responses. There is now a large body of evidence that immunomodulators synthesised and secreted by the nematodes may be responsible for such immune system deviation.

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

For millennia pathogens have been continuously adapting to their complex biological niche, a ubiquitously hostile environment as the host immune system actively works to eliminate the pathogen. This has driven many infectious agents including parasitic worms to evolve immunomodulatory systems to circumvent the normal host immune response to foreign agents and limit the inflammatory response. For example, it is now known that viral genomes encode decoy proteins that resemble host proteins[292], intracellular bacteria such as Salmonella have evolved proteins that mimic intracellular signalling molecules[293] and filarial nematodes secrete molecules that suppress parasite-induced inflammatory responses and local tissue destruction or disruption. However, these evolutionary steps taken by the parasite, have also resulted in "anti-inflammatory" intervention mechanisms that prevent their rejection without leading to immunosuppression of the host[294]. This has led to the concept that through this evolutionary process, the pathogens themselves have provided a complex method of molecular target validation to act as a basis for the development of novel classes of anti-inflammatory agents.

The parasitic helminths actively produce excretory and secretory (ES) molecules to occupy this biological niche as these immunomodulatory agents suppress inflammation and redirect the focus of the hosts immune response. Genomic and expression-based analysis of parasitic helminths of veterinary and medical importance (approximately 20,000 protein coding genes) has yielded large scale repositories of such novel anti-inflammatory mediators[295]. Studies investigating several such molecules are beginning to provide an insight into such "anti-inflammatory" mechanisms involved in the intervention and subversion of host responses. For example, *Schistosoma mansoni, (*a major species of the disease schistosomiasis) secretes a number of defined products including: lysophosphatidylserine[296], lacto-*N*-fucopentaose III (LNFPIII)[297] and double-stranded DNA (dsDNA)[298,299] all of which exhibit immunomodulatory actions. These observations

have been made alongside other parasite products such as phosphatidylserine secreted by *S. Mansoni* and *Ascaris lumbricoides*[300] and ES-62 secreted from the *Acanthocheilonema viteae*[301,302] being reported to suppress pro-inflammatory immune responses through subversion of TLR function.

The TLRs are emerging as prominent targets of many ES products, with direct interaction with the receptor commonly associated with motifs that are specific to the helminths. For example, Schistosome–derived lysophosphatidylserine contains a helminth-specific acyl chain, which interacts with TLR2 to promote the differentiation of DCs that induce IL-10-producing regulatory T cells[296]. Many of these molecules target DCs to bias them to induce T_H2-type immune responses, indicating this to be a conserved evolutionary step that takes advantage of the high expression of TLRs on this APC. Many examples of this can be found throughout the literature as both *S. mansoni*- and *A. lumbricoides*-derived phosphatidylserine-containing extracts have been demonstrated to utilise TLR2 to mature DCs such that they induce T_H2-type immune responses[300]. This is not always restricted to DC modulation as, LNFPII, by inducing alternative activation of macrophages[303,304] (similarly to other helminth products) and modulating DC maturation through its α1,3-linked fucose moiety in a TLR4-dependent manner[297,305] can promote T_H2-type immune responses[297]. In addition, the schistosome egg component dsRNA is able to activate DCs through TLR3[298] and priming of ovalbumin-specific T cells by DCs towards a T_H2 cell phenotype in the presence of these egg Ags is suppressed in a TLR3-dependent manner[299]. Commonly if these modulators are not directly targeting the TLRs, helminth ES products will redirect their associated signalling adaptors and pathways to induce T_H2-type anti-inflammatory responses. For example, this is reflected by the Sm16 lipid bilayer-binding protein released by *S. mansoni* during skin invasion potently inhibiting proinflammatory cytokine production from macrophages mediated by TLR3- and TLR4- but not, TLR2[306]. Alongside this the cysteine proteases secreted by *S. mansoni* and *Fasciola hepatica* can prevent TLR3- and TLR4- mediated induction of nitric oxide, TNF, IL-6 and IL-12 from macrophages, which in turn protects mice from endotoxic shock[307].

The molecular pattern phosphorylcholine (PC) is observed throughout nature but is commonly associated with pathogen products from a diverse range of organisms, including bacteria, fungi, and protozoa, as well as filarial and gastrointestinal nematodes[308]. In this context, the molecule provides a pattern for the hosts immune system to recognise these pathogens (for example via antibodies or C-reactive protein), but can also function to promote pathogen survival and in particular that of parasitic helminths through the

modulation of the host immune response. For example, it has been proposed that *Echinococcus granulosus* utilises the PC-containing component of glycoprotein 5 as a candidate TLR ligand for its promotion of T_H2-type anti-inflammatory responses[309]. Specifically, in this laboratory group extensive research has shown that many of the anti-inflammatory functions of ES-62, a secreted product from *A. viteae* secreted product, ES-62 is dependent on its PC moiety[310]. Through this work both synthetic and natural PC based compounds have shown great potential in mimicking the immunomodulatory activities of ES-62 and other ES products[311,312].

1.6.4 ES-62

ES-62 is a PC-containing glycoprotein (the number of PC-containing glycans is currently unresolved) that was first identified in the rodent filarial nematode *Acanthocheilonema viteae*[313], but has well conserved orthologues in human filarial nematode parasites including *Brugia malayi* and *Onchocerca volvulus*[314,315]. ES-62 is transcribed throughout the helminth lifecycle but its mRNA levels are considerably higher in adult worms than L3 larvae (~5 % adult levels) and microfilariae (<0.2 % adult levels)[315]. This is translated into a 62 kDa protein which includes the parasite-specific post-translational modification of PC attached via an *N*-type glycan, the latter being trimmed to the tri-mannosyl core and then substituted with between one and four *N*-acetylglucosamine residues during oligosasccharide processing[316]. This immunomodulator is produced from cells that underlie the oesophagus at post infective lifecycle stages (L4 larvae and adult worms) of the *A. viteae* parasite and can be detected in the serum of the jird host, *Meriones lybicus*[313,315]. The native form of ES-62 is a tightly bound tetramer formed from dimers[317], with a relatively high sequence homology with members of the M28 peptidase family, where a weak *in vitro* (amino)peptidase activity has been observed against synthetic substrates[283]. Furthermore, divalent cations are known to be critical to the function of aminopeptidases and ES-62 has a putative metal co-ordination motif in its sequence with a strong magnesium ($Mg2^+$) signal being detected in its atomic spectrum[318].

1.6.5 ES-62; a molecular platform for understanding the immune response

ES-62 has been found to have potent immunomodulatory effects on DC/macrophages[291,301,319], B cells[311,320], mast cells[321], and to some extent T cells[322], where the PC moiety is important in these affects. In broad terms, rather than acting in an immunosuppressive manner, ES-62 induces a T_H2/anti-inflammatory phenotype

characterised by reduced levels of IL-12, IFN-γ and pro-inflammatory cytokines as well as the production of IgG1 rather than IgG2a antibodies. These effects and our current understanding of the signalling pathways targeted by ES-62 in a variety of immune cells will be covered in the following sections. A summary of the known interactions and immunomodulatory effects ES-62 exerts on the immune system are presented in **Figure 1.9**.

1.6.5.1 The immunomodulation of APCs; the importance of TLR4 in ES-62 immunomodulation

The PC moiety of the 62 kDa glycoprotein ES-62, is unusual in that it is attached to the protein core via a unique N-type glycan[301] and it appears to be the active moiety in subverting the host immune response, predominantly by targeting DCs and macrophages[291,301,323]. Both of these cell types are important in establishing the T_H1-T_H2 immune phenotypes and the subsequent inflammatory response of the host. Indeed, DCs and macrophages derived from bone marrow progenitors exposed to ES-62 *in vivo* become hyporesponsive to subsequent stimulation with LPS[160,291,319], promoting a DC phenotype that induces a T_H2 biased, yet anti-inflammatory response[160]. However, the modulation of these innate immune cells by ES-62 is not, restricted to driving maturation of APCs that induce T_H2 responses as ES-62 has been shown to prevent the development of both T_H1/T_H17-mediated collagen-induced arthritis[310,324], and T_H2-associated ovalbumin-induced airway hypersensitivity in mice[321,325].

So how does ES-62 achieve the modulation of these APCs? Studies investigating the effects of ES-62 on macrophages and DCs derived from knockout mice showed that its immunomodulatory effects were dependent on the presence of MyD88 and TLR4 but not TLR2[301]. In these studies it was observed that ES-62 subverts the normal pro-inflammatory function of TLR4 as well as utilising the receptor for its internalisation into APCs[326]. The ES-62 dependent suppression of IL-12 and TNF-α secretion in response to LPS stimulation and the internalisation of this immunomodulator were abrogated in cells from TLR4 deficient mice[301] and the low level cytokine responses observed in response to ES-62 are lost in MyD88-deficient cells.

The internalisation and immuno-modulatory effects of ES-62 may depend on the dynamics of pro-inflammatory TLR4 signalling, which is divided into two separate stages; the first being an early MyD88-dependent phase that is triggered by receptor-mediated endocytosis in clathrin coated pits followed by a later endosomal TRIF mediated phase[327].

This presents a potential mechanism for inducing TLR4 hypo-responsiveness and the suppression of TLR4 associated autoantigen presentation in DCs[48,327], as these phases are followed by the degradation of TLR4 along with any associated microbial proteins in late endocytic vesicles[278], resulting in the peptide loading of MHC class II molecules for the presentation of Ag and activation of $CD4^+$ T_H cells[328,329]. The activation-induced trafficking of TLR4 provides a mechanism by which innate and adaptive immune responses are linked and therefore identifies a potential route for ES-62-mediated uncoupling of TLR4 associated NF-κB activation.

Generally activation of TLR4 by LPS induces sustained activation of the NF-κB pathway leading to secretion of IL-6, IL-12 and TNFα from DCs[330]. These pro-inflammatory responses ultimately promote priming of T_H1/T_H17 phenotypes[248]. It has been shown that ES-62, despite inducing the transient activation of this pathway is able to retain the cell in an immature phenotype that is associated with a decrease in the secretion of these pro-inflammatory cytokines[331]. It is hypothesised that ES-62 subverts TLR4 signals by interacting with the receptor to 're-wire' the cell by the degradation and inhibition of signalling elements important in NF-κB activation and cytokine secretion. There are several proposed targets for ES-62 action in DCs, which revolve around proteins that associate or are important in TLR4 mediated pro-inflammatory signalling. Of particular interest are the PKC family, which were first identified as important targets of ES-62 in the immunomodulation of B cells.

1.6.5.2 Subverting the activation and antibody production of B cells; ES-62 uncouples BCR signalling

ES-62 suppresses B cell proliferation following BCR cross-linking. The BCR comprises a clonotypic Ag-binding component (surface immunoglobulin, sIg) and its accessory immuno-receptor tyrosine-based activatory motif (ITAM)-containing signal transducing molecules Igα and Igβ[332]. Ligation of the BCR triggers protein tyrosine kinase (PTK) activity, resulting in tyrosine phosphorylation of the ITAMs and the recruitment of a number of key signal-transducing pathways implicated in cellular activation and proliferation. These include the PLCγ, phosphoinositide-3-kinase (PI3K) and the Ras-ERK MAPK signalling cascades. ES-62 specifically targets key signalling events following BCR ligation to attenuate the activation and proliferation of B cells. It does this by selectively modulating the expression and activity of certain PKC isoforms in resting and BCR stimulated B cells[320,333]. In this context, ES-62 selectively down-regulates the

expression of the α, β, δ, ξ and ι/λ isoforms, predominantly by stimulating proteolytic degradation whilst it up-regulates PKCγ and PKCε in murine splenic B cells[320]. This is coupled with modulation PKC signalling resulting from Ag-receptor ligation of B cells through the disruption of normal activation and nuclear translation patterns of the PKCα and PKCι/λ isoforms. These data were consistent with results which indicated PKCα, -ε and -ι/λ transduced key activation signals involved in the regulation of Ag-driven DNA synthesis and proliferation in B cells[334], such as phosphorylation of nuclear protein lamin B and the induction and activation of NF-κB, Fos, Egr-1 and Myc[335-339].

However ES-62 regulation of BCR signalling also targets the receptors recruitment of additional key proliferative signals, such as the PI3K and Ras/Erk MAP kinase cascades. It does this by activating a low level of signalling through molecules such as ERK and MAPK resulting in the induction of negative feedback signalling such that when subsequent BCR ligation occurs, ERK activity is rapidly uncoupled by activation of the inhibitory phosphatase, PAC-1 preventing the activation of the cell[340].

Although ES-62 attenutates the proliferation of B cells, they still mount an antibody response against the molecule, although the isotype and subclass of antibody produced is very specific. For example BALB/c mice injected with ES-62 mount an antibody response to the molecule resulting in the production of the T_H2-associated isotype IgG1, but not IgG2a, the latter of which is associated with the T_H1 phenotype. Many human studies reveal an indirect association between the presence of circulating filarial nematode products and the levels of parasite specific IgG1, IgG2 and IgG3 whilst the T_H2 associated IgG4 subclass is usually found to be elevated and often to a considerable extent[341]. Interestingly the cytokine IL-4, which is commonly associated with filarial infection and has the potential to elevate the levels of IgG4, is believed to play an important role in determining the isotype and subclass of antibody produced in responses to filarial infection. In this context, IL-4 has been shown to synergise with ES-62 to cause B-lymphocyte proliferation rather than hypo-responsiveness[283]. This IL-4 mediated rescue appears to arise as a consequence of exposure to IL-4 preventing the ES-62 mediated degradation of PKCα, as PKCα is important for the mitogenic activation of B lymphocytes[320]. Thus although ES-62 may render conventional B lymphocytes hyporesponsive and impair their ability to produce antibody *in vivo*, in the presence of IL-4, the cells may be induced to produce antibodies of the IgG4 subclasses. This may be the reason why total and specific subclasses of antibody are increased in filariasis patients.

Consistent with these findings the T_H2 bias observed for the anti-ES-62 response is dependent on IL-4, as IL-4 knockout mice fail to produce IgG1; an IL-10 dependent role for PC in blocking the IgG2a response has also been implicated[342]. Interestingly exposure of B1 cells to ES-62 released from osmotic pumps results in their proliferation and IL-10 production, even in the absence of further stimuli[343].

Similarly, with respect to T cells, ES-62 has been observed to suppress anti-CD3-induced proliferation of Jurkat T cells and promote concanavalin A-induced growth arrest of these cells[344], an affect that can be mimicked by PC[283]. The precise sequence of signalling events responsible for these affects remain to be fully elucidated. However the ES-62 mediated desensitisation of TCR signalling is associated with disruption of TCR coupling to PLD, PKC, PI3K and Ras-ERK MAP kinase signalling. The PC molecule appears to be the active moiety in mediating these affects as culture with PC or PC-BSA has comparable effects as ES-62 on the coupling of the TCR to PTK activation (ZAP-70, Lck and Fyn recruitment) and the Ras-Erk MAP kinase signalling cascades[283,308,344]. These findings are consistent with an earlier report showing that PC-containing molecules of the human filarial nematode, *Brugia Malayi*, inhibit the response of human T cells to mitogens[345].

1.7 Aims of the thesis

The aims of this project were to dissect the mechanisms by which ES62 modulates the maturation and activation of bone marrow derived DCs.

Specifically, the aims of this project were to:

1. Identify how engagement of TLR4 by ES-62 and/or LPS induces differential effects on the maturation of DCs and subsequent induction of effector T cell subsets.

2. Investigate whether ES-62 utilises additional co-receptors in conjunction with TLR4, to mediate its immunomodulatory effects.

3. Dissect the specific effect of ES-62 on the expression of key proteins important in the propagation of TLR4 signals, focusing on mediators required for the activation of NF-κB, a proinflammatory transcription factor.

4. Explore the mechanisms by which ES-62 modulates signal transducer expression to downregulate proinflammatory responses.

These objectives were investigated in murine, bone marrow progenitor derived DCs; a well established system for the study of these APCs and the modulation of the immune responses by ES-62[291,319,326].

Figure 1.1 | Transcription-factor activation through TIR-domain containing adaptors of the TLR family and the various ligands that stimulate these receptors.

Collectively, the TLR family recognise a wide variety of PAMPs, and the specificity for each receptor within this family is often based on particular motifs shared between pathogenic molecules. For example, TLR2 can recognise lipoarabinomannan from mycobacteria, peptidoglycan from Gram-positive bacteria , hemagglutinin protein from measles virus and tGPI-mutin from *Trypanosoma*. Members of the TLR family can also form complexes to further enhance their recognition repertoire as TLR2/6 recognises zymosan from *Saccharomyces cerevisiae* (*S. cerevisiae*), while TLR2/1 and TLR2/6 are able to discriminate between triacyl- and diacyl-lipopeptide lipid structures, respectively. TLR4 recognises gram negative bacterial LPS and envelope proteins from respiratory syncytial virus (RSV) and mouse mammary tumor virus (MMTV), where TLR5 is a little more limited in its spectrum as to date its is only known to recognise bacterial flagellin. In mice, TLR11 recognises components of uropathogenic bacteria and a profilin-like molecule of *Toxoplasma gondii*. The aforementioned receptors generally reside at the plasma membrane (TLR1, -2, -4, -5, -6, -11) while TLR3, -7, -8 and -9, are located in the endosomes, where they are ideally situated for the recognition of their PAMPs, which include nucleic acids synthesised from viruses and bacteria. Specifically, TLR3 recognises dsRNA, a product of the replication of viruses and poly IC, while TLR7 detects another derivative of many viruses, ssRNA. TLR9 recognises CpG DNA motifs present in the genomes of bacteria and viruses, which also includes non-nucleic acids such as hemozoin from *Plasmodium*. The differential engagement of various intracellular adaptors by the TLR receptor complexes (for example; TIRAP-MyD88, TRAM-TRIF) allow the positive regulation of transcription-factor activation. For example, TLR1, 2 and 6 utilise MyD88 and TIRAP as adaptors while TLR5, -7, -9 and -11 utilise MyD88 alone. TLR4 utilises a range of adaptors including MyD88, TIRAP, TRIF and TRAM. TLR3 utilises TRIF as its sole adaptor. The exception to these various adaptors is SARM, which inhibits TRIF-mediated transcription factor activation.

(Adapted from Kawai, T. et al., 2007[14])

Figure 1.2 | **The activation of NF-κB through LPS-TLR4 signalling.**

Pro-inflammatory TLR4 signalling can be divided into two separate stages; the first being an early MyD88-dependent phase that is triggered by receptor-mediated endocytosis in clathrin coated pits, the second a later endosomal TRIF mediated phase. The LPS recognition complex is composed of TLR4/MD2 and CD14, which initiates both MyD88- (requiring TIRAP) and TRIF-dependent (requiring TRAM) intracellular signalling pathways via its TIR domain. The MyD88 pathway stimulates the recruitment of a number of intracellular adaptor proteins including members of the IRAK family and TRAF6, the latter of which utilises K63-linked ubiquitinaton to form a scaffold for the activation of the TAK1 complex. Resulting in the activation of another multiprotein IKK complex, composed of IKKα, IKKβ and its regulatory component NEMO, to catalyse the rapid phosphorylation of IκB and induce its polyubiquitination and proteosomal degradation. This releases the NF-κB complex to translocate into the nucleus, which alongside AP-1 induces the production of the inflammatory cytokines IL-6, IL-12 and TNF-α from DCs. The phosphorylation and translocation of AP-1 is dependent on TAK1 mediated activation of the MAPK pathway. In the later TRIF dependent phase, the recruitment of both TRAF3 and TRAF6 are important for the activation of the kinases TBK1 and IKKi, which mediate the phosphorylation and dimerisation of IRF3, resulting in its nuclear translocation for the regulation of gene transcription. TRIF also interacts with TRAF6 and RIP1 to mediate the activation of NF-κB, where this pathway is required for the induction of the type I IFNs, particularly IFN-β. The dissociation of TRIF from TRAM occurs in the late endosomes and terminates its signalling capacity, while TLR4 is ubiquitinated via K48 linkages for its eventual degradation in the lysosomes.

(Adapted from Jiang, X. et al., 2011[346]; Barton G. M. et al., 2009[13]; Kawai, T. et al., 2007[14]; McGettrick, A. F. et al., 2010[347])

Figure 1.3 | The regulation of ubiquitination and fates of the ubiquitin associated proteins.

(**A**) The process of ubiquitnation begins with the utilisation of ATP by the ubiquitin-activating enzyme (E1) to form a high-energy, labile E1-thiol ester intermediate to activate Ubiquitin (Ub), which is then transferred to a ubiquitin-conjugating enzyme (E2). The method of ubiquitin transfer to a substrate is dictated by the type of ubiquitin-protein ligase (E3), a protein important in ensuring the specificity of such associations. The E3 proteins can be defined by whether they contain either a HECT or RING finger domain. (**B**) A HECT domain-containing E3 ligase transfers the activated ubiquitin from the E2 protein onto itself to generate another labile thiol ester intermediate. This is then conveyed to an associated substrate through the formation of a stable isopeptide bond between ubiquitin and an ε-NH_2 group of an internal Lys residue in the substrate. In the generation of a polyubiqutin chain additional ubiquitin moieties are added to the initial moiety in a similar process. (**C**) On the other hand, a RING finger domain-containing E3 ubiquitin ligase directly transfers the activated ubiquitin from E2 to an internal Lys residue onto an associated substrate. (**D**) In the majority of cases, polyubiquitin chains linked through Lys48 of ubiquitin are associated with proteasomal degradation. (**E**) In contrast, linkages through Lys63 of ubiquitin are associated with a raft of proteosomal independent (including lysosomal degradation) functions. However, recently evidence has emerged suggesting a role for this linkage in targeting proteins for proteosomal degradation[348]. The association of ubiquitinated proteins to the proteosome occurs either directly or by shuttling of proteins, where they are degraded to short peptides by the 26S proteosome. (**F**) Subsequently, most of the ubiquitin chain is disassembled by deubiquitinating enzymes (DUBS), where in the minority of cases select substrates are processed rather than completely degraded by the proteosome.

(Adapted from Weissman, A.M. et al., 2011[349])

Figure 1.4 | Ubiquitin-mediated signalling in TLR pathways, particularly focusing on TLR4 signalling and the different types of polyubiquitination involved.

Stimulation of TLR4 leads to the formation of the 'myddosome' at the plasma membrane, which is composed of the adaptor proteins, MyD88 and TIRAP as well as two members of the IRAK family, IRAK1 and IRAK4. The subsequent recruitment and activation of the E3 ligase TRAF6, is required for the synthesis and linkage of K63-linked polyubiquitin chains to cIAP1 and cIAP2 for the ubiquitination and degradation of TRAF3. The degradation of TRAF3 releases TRAF6 associated complexes from TLR4 into the cytosol, where it can interact and recruit additional components through its associated polyubiquitin chains. These include the TAK1 and IKK kinase complexes, which do so through their ubiquitin-binding subunits TAB2/TAB3 and NEMO, respectively. The binding of these complexes to the K63-linked polyubiquitin chains on TRAF6 induces the phosphorylation of IKKβ by TAK1 and results in the actvication of IKK. This induces IKK to phosphorylate IκB proteins leading to their polyubiquitination by $SCF^{\beta TrCP}$ (an E3 ubiquitin ligase complex), targeting IκB for degradation by the proteosome and freeing NF-κB to translocate to the nucleus for the activation of genes involved in immune and inflammatory responses. The endocytosis of TLR4, which occurs after MyD88 associaiton, induces the recruitment of the adaptor proteins TRAM and TRIF. This complex recruits a number of E3 ligases including PEL1, TRAF3 and TRAF6 to mediate the ubiquitination of the signalling proteins such as RIP1 to induce the sustained acticaiton of NF-κB and production of the type I IFNs.

(Adapted from Jiang, X. et al., 2011[346])

Figure 1.5 | Structure and maturation of the various PKC subfamilies.

In panel **A**, the structural characteristics associated with the classification of various PKC isoforms are defined. The catalytic domains of the various PKCs are conserved but this family to be categorised into three subgroups based on their differing regulatory domains. The conventional PKC isoforms (cPKC) are composed of: the autoinhibitory pseudosubstrate motif, two DAG binding C1 doamins (C1a and C1b) and the calcium-binding C2 domain. The novel PKC isoforms (nPKC) can be regulated by DAG but lack a calcum-biniding motif and rather uniquely contain an extended N-terminal domain important for receiving regulatory signals. Finally, the catalytic activity of atypical isoforms (aPKC) is independent of DAG and calcium, where the regulation of this isoform occurs mainly through intracellular localisation, which in itself is regulated by the interaction with regulatory proteins and a nuclear localisation signal/nuclear export signal in their regulatory domains. The various PKC isoforms share a highly conserved C-terminal tail which contains certain motifs termed the A-loop and C terminal associated turn-motif (TM) and hydrophobic motic (HM), all of which are important phosphorylation sites.

In panel **B**, the prevailing model of the activation and localisation through the phosphorylation of the cPKC and nPKC isoforms is presented. After synthesis, the PKCs are phosphorylated at a series of sites (represented by the yellow circles) including the A-loop (mediated by PDK-1), followed by the TM and HM regions (via mTORC1/mTORC2-dependent pathways), which allows the proteins to enter a closed, enzymatically 'competent' state. There is mounting evidence that these phosphorylations occur at the plasma membrane (PM). The pseudosubstrate occupies the substrate-binding site and in this state the enzyme predominantly localises to the cytosol, where it is ready for activation by the second messenger DAG (and in the case of the cPKC isoforms, calcium released by inositol triphosphate production [IP_3]) produced after receptor stimulation. It is tyrosine kinases coupled to the receptors that are responsible for triggering of PLC-mediated hydrolysis of PtdIns(4,5)P_2 to DAG. DAG binds to the C1 domains on the PKC as well as DGK, which phosphorylates DAG to produce phospatidic acid (PA) to terminate DAG signalling. Binding to second messengers is required for the release of the pseudosubstrate motif from the active site and for this to be made accessible for signalling effectors. There is mounting evidence however that stimulation of receptors may directly induce various phosphorylations on a range of PKC isoforms.

(Adapted from Spitaler, M. et al., 2004[54]; Freeley, M. et al., 2011[53]; Parker, P.J. et al., 2003[350])

Figure 1.6 | The known roles of the PKCs in TLR signalling and activation of NF-κB.

The TLRs mediate the activation and regulation of different PKCs, which can then directly interact with various components of the NF-κB activation pathway. The cPKC, PKCα associates with TLR2 and TLR4 following its activation, an association which is reliant on MyD88, where it mediates the activation of MAPK and NF-κB leading to the secretion of TNFα, IL-6 and IL-10. This isoform is also required for the TLR3-mediated IRF-3 binding of CBP and IFN-β gene induction. The nPKCs, PKCε and PKCδ interact with TLR4 through MyD88 and TIRAP, respectively. This recruitment to the TLR4 signalosome is important in the activation of IKK complex and NF-κB, where PKCε has also been shown to be required for the phosphorylation of TRAM. Interestingly, PKCε is important in TLR4 signal transduction through its involvement in initiating MAPK signalling and is known to be directly involved in the LPS induced secretion of TNF-α and IL-12p70 in DCs but not the up-regulation of co-stimulatory molecules. PKCζ is activated upon ligation of TLR2 or TLR4. During signalling by these receptors, active PKCζ binds to TLR2, associates with TRAF6 and is required for the full transcriptional activation of p65.

(Adapted from Loegering, D. J. et al., 2011[351])

Figure 1.7 | **Dendritic cells and pathogens**

(**A**) When immature DCs encounter pathogens and their inflammatory stimuli, such as LPS, maturation ensues resulting in the activation and nuclear translocation of NF-κB and the interferon regulatory factors (IRFs) for the transcription of multiple genes associated with immunity and infalmmation. The costimulatory molecules CD40, CD80 and CD86 as well as proinflammatory cytokines (IL-6, IL-1β, TNF-α, IL-12p70, IL-12/23p40 and IL-23) are produced and support the activation of T cells. (**B**) In the process of activation and maturation, DCs uptake endogenous Ags from the environment and present this on their surface through MHC class II molecules to Naïve T cells. MHC class II α- and β-chains assemble in the endoplasmic reticulum (ER) and form a complex with the invariant chain (Ii). The Ii–MHC class II heterotrimer is transported through the Golgi to the MHC class II compartment (MIIC), either directly and/or via the plasma membrane. Endocytosed pathogens/proteins and Ii are degraded by resident proteases in the MIIC. A portion of the li peptide; the class II-associated Ii peptide (CLIP) fragment remains in the peptide-binding groove of the MHC class II dimer and is exchanged for an antigenic peptide with the help of the dedicated chaperone HLA-DM (known as H2-M in mice). MHC class II molecules are then transported to the plasma membrane to present antigenic peptides to $CD4^+$ T cells. The key ligand pairs required in the formation of the DC-T cell immunological synapse consists of a central interaction of the T cell receptor (TCR) and MHC:peptide complex (**C**). This reaction is stabilised by a scaffolding network consisting of CD80/86 interactions with CD28 and CTLA4 alongside CD40 and its ligand CD40L. There is also a major input from cytokine milieu secreted in response to various pathogens by the DCs for the maturation of various phenotypes from the naïve T_H cell precursors.

Figure 1.8 | T helper (T_H) cell subset generation

Activated APCs such as DCs and macrophages engage the T cell receptor (TCR) on naïve $CD4^+$ T cells through peptide presenting MHC class II molecules and the appropriate costimulatory molecules, triggering the activation of distinct signal transducer and activator of transcription (STAT) pathways and their proliferation. The APCs then guide the phenotypic maturation of these cells through the specific cytokine environments they create (determined by the type of pathogen encountered), dictating the type of immune response mounted by the adaptive immune response. The cytokine IL-27 is involved in the priming of naïve T cells, where high doses of Ag presented by DCs or the secretion of IL-12p70 activate STAT4 and favour the development of T_H1 cells, which produce IFN-γ and mediate cellular immunity. In the case of T_H2 cells, IL-4 and the activation of STAT6 signalling is required for their development, resulting in a cell population that drives humoral immunity and the secretion of high quantities of IL-4, IL-5 and IL-13. The secretion of IL-23 is involved in the induction of T_H17 cells, alongside IL-6, TGF-β and the autocrine production of IL-21 by this developing cell population. The presence of IL-27 inhibits the development of T_H17 cells, which secrete IL-17, IL-21 and IL-22 inducing strong inflammatory responses. The novel cytokine IL-35 has been demonstrated to play a role in the immunosuppressive effects of T regulatory (T_{reg}) cells, alongside IL-10 and TGF-β.

Figure 1.9 | **Immunomodulation by ES-62.**

ES-62 targets multiple cells of the immune system (black arrows [+/induction] and 'bulb' arrows [-/inhibition]) to achieve immunomodulation, broadly biasing the immune response to a T_H2/anti-inflammatory phenotype. It targets DCs and macrophages, which are important regulators of T cell polarisation, by interacting with TLR4 to interfere with the TLR4-NF-κB signalling axis and thus modulate the maturation and secretion of cytokines from these innate cells. This is characterised by the production of low levels of IL-12, IFN-γ, TNF-α and IL-6 by these APCs. In respect to its modulation of B and T cell responses, ES-62 targets the signalling triggered following cross-linking of the B- and T-cell antigen receptors (BCR and TCR), hence disrupting the responses of these cells to specific Ags. This modulation results in the secretion of IL-4 by T_H2 cells and production of the T_H2 associated antibody isotypes IgG1 (mouse) and IgG4 (human) by B cells alongside the production of IL-10 specifcally by B1 cells. With the recent emergence of the T_H17 phenotype it has been hypothesised that ES-62 may suppress inflammation by targeting IL-17 responses as it is implicated in the pathogenesis of diseases resulting from both T_H1 and T_H2 driven inflammation. This targeted suppression of IL-17 would limit both types of inflammation as well as that driven by T_H17 cells but it is as yet unknown whether ES-62 may target APC driven development of T_H17 cells or the direct secretion of cytokines from this cell population. The majority of evidence to date suggests ES-62 acts on T_H cells through DCs, however recent data has suggested it can act directly on T_H17 cells *in vitro*[352].

2 Materials and Methods

2.1 Animals

Male and female, 7-10 week old, BALB/c or C57BL/6 background, Sphingosine 1 and Sphingosine 2 knock-out (KO) mice were used to generate bone marrow derived DCs for this study. For the *in vitro* co-culture 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 (DO.11.10 on a BALB/c background)[353] and I-A^b (OTII on a C57BL/6 background) were used as T cell donors. The tg TCR was detected by flow cytometry using the clonotypic monoclonal antibodies KJ1.26 (DO.11.10) and Vα2 (OTII). All animals were specified pathogen free and were maintained under standard animal house conditions with free access to both water and standard rodent pellets at the University of Glasgow Research Facilities in accordance with local and home office regulations.

2.2 Purification of ES-62

Endotoxin free ES-62 was prepared from adult *Acanthocheilonema viteae* worms in culture by Dr. lamyaa Rayami and Dr. Justyna Rzepecka (SIPBS, University of Strathclyde, UK). Briefly, ES-62 was prepared from 500 ml spent culture medium of adult *A. viteae* (endotoxin-free RPMI 1640 (Invitrogen Life Technologies)) with added endotoxin-free glutamine (2 mM), endotoxin-free penicillin (100 U/ml), and endotoxin-free streptomycin (100 μg/ml)). To remove larval forms (microfilariae) released by the adult female worms, the medium was passed through a 0.22-μm pore size filter (Sigma-Aldrich). It was then transferred to a stirred cell ultrafiltration unit containing a YM10 membrane (Amicon). After reducing the volume of the sample to 5–10 ml and transferring the holding medium to endotoxin-free phosphate buffered saline (PBS), pH 7.2 (Cambrex Bioscience), it was further concentrated to 200–500 μl using Centricon microconcentrators with a 30-kDa cutoff membrane (Amicon). The sample was then applied to a 30 x 1-cm Superose 6 column (HR 10/30; BD Biosciences), fitted to an isocratic FPLC system (BD Biosciences) previously equilibrated with endotoxin-free PBS, pH 7.2, at room temperature. The column was eluted at a flow rate of 0.5 ml/min and monitored for absorbance at 280 nm. More than 95% of the protein eluted as a single peak, and this was purified ES-62. The purity and identity of each batch were confirmed by a combination of SDS-PAGE and Western blotting, the latter using a rabbit antiserum specific for ES-62. Finally the level of endotoxin in the ES-62 samples was confirmed using an Endosafe kit

(Charles River Laboratories, Kent, UK). ES-62 is used at a working concentration that has an endotoxin reading of <0.0015 endotoxin units per μg of ES-62.

2.3 Generation of bone marrow derived dendritic cells

Bone marrow (bm) was isolated from mouse femurs by flushing with cold PBS followed by homogenisation with a 23-gauge needle. This solution was then treated to remove red cells by incubation with 0.168 M NH_4Cl (pH 7.2) on ice for 10 mins. The cells were then filtered through Nitex nylon mesh (Cadisch Precision Meshes, London, UK) to remove dead cells prior to culture. To prepare bmDC, bone marrow cells were cultured in complete medium containing RPMI-1640 (Invitrogen), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 10% Fetal Calf Serum (FCS; Lonza) supplemented with 10 ng/ml recombinant murine Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF; Peprotech) for 6 days in 90 mm petri dishes (Greiner) at 37^{o}C/5% CO_2 ($2x10^6$ cells per dish), with fresh medium supplied on day 4. On day 6, immature bmDC were harvested by gentle scraping and used as a source of DCs, which were approximately 65-85% $CD11c^+$ DC. Where indicated, these bmDCs (day 6) were further purified ($\geq$ 95%) prior to maturation by positive selection using MACS microbead separation (Miltenyi biotec) against the CD11c marker. The cells were prepared in MACs buffer - PBS pH 7.2 supplemented with 0.5% bovine serum albumin (BSA) and 2 mM Ethylene diamine tetra acetic acid (EDTA) and then passed through Nitex nylon mesh before being blocked with Fc receptor blocking agent (anti-CD16/32) and positively selected on CD11c Microbeads using magnetic separation according to the manufacturer's instructions.

2.3.1 ES-62 and modulator treatment of bone marrow derived dendritic cells

Purified bmDCs were stimulated with combinations of 2 μg/ml ES-62 and/or 1 μg/ml LPS (*Salmonella Minnesota* [Sigma-065K4078]) in 6 well plates for the stipulated times. After such times, the culture supernatants were removed for cytokine analysis and the cells were assayed by flow cytometry, western blotting and mRNA transcript analysis as indicated.

A number of pharmacological treatments were utilised to inhibit different cellular processes throughout the cell. These included inhibitors of lysosomal degradation such as ammonium chloride (NH_4Cl; 50μM) or the combination of E-64-D (10 μg/ml) and

Pepstatin A (10 μg/ml), which raises the pH of lytic granules and inhibits the function of lysosomal proteases, respectively. However, it is important to note that ammonium chloride has a number of subsidiary effects as it raises the pH of many intracellular compartments resulting in interference with transcytosis, endocytosis, fusion of membrane vesicles[354-357], and receptor recycling[358-360].

The proteosomal degradation inhibitor lactacystin (10 μM), which inhibits the ATP independent activities of the proteosome, was also utilised in these studies and the viability of bmDCs with this treatment was analysed due to frequent reports in the literature of its cytotoxicty. The fluorochrome, 7-AAD (7-amino-actinomycin D) was utilised to identify cell viability. This compound is commonly utilised for the exclusion of nonviable (unhealthy) cells due to its ability to inerchelate into the DNA cells with compromised membranes (a common feature of dying cells). Immature bmDCs treated with lactacystin over an 18 h time period (**Fig. 2.1**), were observed to induce a substantial level of cell death (41.9 %, of the total cell population) after 18 h when utilised at 10 μM but not 1 μM (12.3 %). Interestingly, this was reduced when the cells were stimulated with LPS (24.4 %; with 10 μM lactacystin). Despite the reduction in cell viability, 10 μM lactacystin was generally utilised as it is commonly cited as being required to completely inhibit proteosomal degradation. However, any potential effects on cell viability were taken into account. All other specific pharmacological treatments are covered in the relevant chapters.

2.4 Purification of DO.11.10 $CD4^+$ $CD62L^+$ T cells

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

Purified naïve $CD4^+CD62L^+$ T cells from DO.11.10.BALB/c or OTII C57BL/6 mice were utilised for *in vitro* culture with bmDC to assess differentiation of T_H phenotype. Single cell suspensions of lymphocytes were prepared and pooled from the spleens, peripheral (axillary, brachial, inguinal, cervical; PLN) and mesenteric lymph nodes from DO.11.10 BALB/c mice and forced through Nitex nylon mesh using a syringe plunger. The suspensions were washed in 10 ml of sterile RPMI 1640 before being centrifuged at 300 x g for 10 mins and the supernatant discarded. These cells were then purified using a $CD4^+CD62L^+$ T cell isolation kit (Miltenyi Biotec) according to the manufacturers instructions. Briefly, lymphocytes were resuspended in MACS buffer (PBS pH 7.2 supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA) and incubated with a biotin-antibody cocktail which contains biotin-conjugated antibodies

against CD8α (Ly-2), CD45 (B220), CD49β (DX5), CD11b (Mac-1), Ter-119, CD25 and TCRγδ. Cells were then incubated with anti-biotin microbeads and after washing, the $CD4^+$ T cell population was obtained by negative selection using MACS separation. The enriched $CD4^+$ T cell population was then incubated with anti-CD62L microbeads and the naïve $CD4^+CD62L^+$ population was obtained using positive MACS selection. The percentage of $KJ1.26^+CD4^+$ DO.11.10 T cells in these preparations was determined by flow cytometric analysis (**Fig. 2.2**).

2.4.2 DNA synthesis assay

To be able to divide, cells have to replicate their DNA. Thus, if cells are cultured in the presence of radiolabelled bases, these become incorporated into their DNA with the levels of signal being proportional to the amount of DNA synthesised. The most common label used to determine DNA synthesis is [^{3}H]-labeled thymidine, which can be detected by scintillation counting. To measure DNA synthesis, triplicate samples of T cells (2.5×10^5), co-cultured with bmDC (2.5×10^4) were incubated (37^{o}C in a 5% [v/v] CO_2 atmosphere at 95% humidity) in T cell culture medium (RPMI-1640, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 10% FCS, 50 μM β-mercaptoethanol [Invitrogen], 1% Non-essential amino acids (Invitrogen) and 1mM Sodium Pyruvate [Sigma] for 72 h, unless indicated otherwise). [^{3}H] thymidine (0.5 μCi/well, Amersham) was added for 16 hours before cell harvesting with an automated cell harvester (Perkin Elmer). Incorporated label was analysed by liquid scintillation analysis (Microbeta Trilux, Perkin Elmer).

2.4.3 5,6-Carboxy-Succinimidyl-Fluoresceine-Ester (CFSE) staining of cell division

CFSE is a cell permeable dye that permanently stains cells following its binding to exposed amine groups on intracellular proteins[361]. During each cell division the cell undergoes thereafter the amount of cellular dye is halved and this can be detected as a shift to lower fluorescence intensity by Fluorescence Acitvated Cell Sorting (FACS) analysis, with each 50% reduction of the fluorescence signal representing one division. Briefly, prior to the analysis, cells (10^5/sample) were washed twice (in ice cold PBS) and then loaded with 5 μM CFSE in PBS for 10 min at 4^{o}C. The cells were then washed with 40 ml ice cold PBS three times, to remove any excess CFSE, after which the cells were resuspended in warm T cell culture medium (**section 2.4.2**). The cells are analysed for CFSE-staining (intensity) by FACS analysis of cells in the FL-1 channel (Becton Dickinson

FACSCalibar^{TM} flow cytometer). The dilution of CFSE in the cells was then analysed using Flowjo software (Tree Star Inc, OR, USA, version 8.8.6) creating gates correlating with the number of cycles of proliferation (generations) the cells have undergone (**Fig. 2.2**).

In conjunction with these analyses the division index, proliferation index and percentage of dividing cells (% divided) was calculated from these samples utilising the proliferation platform program provided by the FlowJo software package. The division index is defined as the average number cell divisions that a cell in the original population (generation 0 or the undividing cell peak) has undergone and includes the cells that remain within the undivided cell peak. The proliferation index is the total number of divisions divided by the number of cells that started dividing. As such, the proliferation index only takes into account the cells that undergo at least one division, thus only responding cells are reflected in the proliferation index. In general, the proliferation index more faithfully reflects the biology of the responding system (dividing cells), while the division index reflects what the entire system is doing. The percentage of dividing cells (% divided) provides a percentage value for the number of cells, which have divided (assuming that no cells died during the culture). These statistics are related in the following way:

Division index = (Proliferation index)(% Divided)

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

BmDC were prepared and purified as described in **section 2.3** The cells were then seeded at 2 x10^6 cells/ml in 6 well plates before being matured (day 6) in the presence or absence of 2 μg/ml ES-62 and/or 1 μg/ml LPS (*S. Minnesota*; Sigma-065K4078) for 24 hours or the indicated time. On day 7 (or day 8 when cells were pretreated with ES-62 for 18 h prior to stimulation with LPS) the bmDC were harvested and pulsed with 0, 10, 30, 100 or 300 nM $OVA_{323-339}$ peptide for 3 h at 37^{o}C, 5% (v/v) CO_2 atmosphere at 95% humidity. Following this the cells were washed 3 times with sterile RPMI 1640 medium and then cultured (2.5x10^4 cells) in T cell culture medium (**section 2.4.2**) with MACS sorted, CFSE stained, purified $CD4^+CD62L^+$ T cells (2.5 x 10^5 cells) from DO11.10/BALB/c or OTII/C57BL/6 mice in a total volume of 1 ml in 24 well plates for 72 h at 37^{o}C and 5% CO_2. A portion of the cells was analysed by FACS for CFSE staining using antibodies raised against the TCR specific for the chicken $OVA_{323-339}$ peptide bound to I-Ad MHC class II molecules (KJ1.26) in the case of the DO11.10/BALB/c mice

derived cells or the Vα2 chain of the TCR for OTII/C57BL/6 mice derived cells. The remaining cells were treated for RNA extraction and mRNA analysis and the supernatants were frozen at -20^{o}C for future cytokine analysis.

2.6 FACS Analysis

2.6.1 Staining for surface protein

Aliquots of cells (10^5-10^6 per sample) in 5 ml polystyrene tubes (Falcon, BD) were washed (all wash steps were followed by centrifugation at 300 *g* for 5 min) with 200 μl cold FACS buffer (PBS and 10% FCS) at 450 *g* for 5 min at 4^{o}C. Cells were re-suspended in 200 μl Fc receptor (FcR) blocking buffer (anti-CD16/32, clone 2.4G2, hybridoma supernatant, 10% mouse serum, 0.1% sodium azide) for 15 min before washing with 1 ml cold FACS buffer. Anti-CD16/32 binds to FcγRII/III whilst the immunoglobulin in mouse serum binds to FcγRI resulting in the blocking of all FcR preventing non-specific binding of antibodies. FcR blocking was followed by the addition of the appropriate fluorochrome-conjugated or biotinylated primary antibodies for 15-30 min in the dark at 4^{o}C. Details of the Ab clones, their specificities and isotype controls used are provided in **Table 2.1**. The cells were then washed with 1 ml cold FACS buffer as before and where appropriate, biotinylated antibodies were detected following incubation with fluorochrome-conjugated streptavidin for 15-30 min in the dark at 4^{o}C. Finally the cells were washed again in FACS buffer, re-suspended in 100-300 μl FACS buffer for analysis using a Becton Dickinson FACSCalibar^{TM} flow cytometer and analysed using Flowjo software (Tree Star Inc, OR, USA, version 8.8.6) (**Fig. 2.3**). Where appropriate the fluorchrome 7-AAD was added to the samples to exclude nonviable cells (for a detailed descripiotn of this compound please refer to **section 2.3.1**) before two to four-colour analysis was performed on a minimum of 30,000 events.

Many of the analyses compare the fluorescence intensity of a stained antigen (Ag) between two samples and as such the histograms y-axis is defined as the percentage of maximum cell numbers (%-Max). In FlowJo software all recorded events are divided into 256 “bins”, which are numerical ranges for the parameter on the x-axis for example the fluorescence intensity of an Ag. Thus the %-Max is derived from the number of cells in each bin divided by the number of cells in the bin that contains the largest number of cells, where each graph is scaled to the percentage of its maximum bin (100%).

2.6.2 Intracellular protein staining

Following labelling of appropriate cell surface phenotypic markers as described in **section 2.6.1**, where indicated the cells were processed for intracellular staining in the dark. Briefly, cells were resuspended in 500 µl Fixation buffer (BioLegend) for 20 min at room temperature, before washing twice in Permeabilisation buffer (BioLegend). After resuspension in 100 µl Permeabilisation buffer the appropriate fluorochrome-conjugated or unconjugated primary antibodies were added for 15-30 min in the dark at room temperature. In the case of the unconjugated antibodies, the cells were washed and an appropriate Fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG was then added for a further 15-30 min in the dark at room temperature. The cells were then washed one final time before resuspension in 100-300 µl FACS buffer for analysis using a Becton Dickinson FACSCalibar^{TM} flow cytometer and analysed using Flowjo software (Tree Star Inc, OR, USA, version 8.8.6) (**Fig. 2.3**). Two or three-colour analysis was performed on a minimum of 30,000 events.

2.7 Enzyme-linked immunosorbance assay, ELISA

Cytokine ELISA analysis was performed using the ELISA kits outlined in **Table 2.2 A** according to the manufacturer's instructions. Briefly IMMULON® 4 HBX flat bottomed 96 well plates (Thermo scientific, UK) were coated overnight at 4^{o}C with the relevant cytokine capture antibody in PBS, pH 7.4 (IL-22, IL-23, IL-12p70, IL-12p40) or 0.1 M sodium bicarbonate buffer, pH 9.5 (IL-17A, IL-6, IFN-γ). The plates were then blocked with 10 % FCS (IL-17A, IL-6, IFN-γ) or 1 % BSA (IL-22, IL-23, IL-12p70, IL-12p40) in PBS for 1 hour at room temperature before incubation with the diluted (in reagent diluent) samples and standards for 2 h at room temperature. Biotinylated, cytokine-specific, detection antibodies were diluted and added to the plates for 1 h at room temperature. Then streptavidin-HRP (Horse Radish Peroxidase) was added for approximately 30 min at room temperature before the plates were developed using tetramethylbenzidine (TMB) substrate (Insight Biotechnology Limited, Middlesex, UK). Plates were washed at least five times between all these stages before the addition of TMB with PBS containing 0.05% Tween-20 (PBS Tween). Samples tested with the BD kits had the detection antibody and streptavidin-HRP added at the same step for 1 h. Plates were left to develop in the dark for 15-30 min and absorbance at 450 nm was determined in an ELISA plate reader (Sunrise microplate reader, Tecan, Switzerland). The cytokine concentration was determined using the

absorbance values obtained for the standards. The limit of detection for all cytokines was no higher than 15 pg/ml.

2.8 Preparation of cellular extracts

2.8.1 Preparation of total cell protein extracts

Stimulated bmDC were treated as indicated ($2x10^6$ cells/treatment) and then reactions terminated by washing with ice cold PBS before the addition of 50 μl modified Radio-Immunoprecipitation Assay (RIPA) buffer (50 mM Tris buffer, pH 7.4 containing 150 mM sodium chloride, 2 % (v/v) NP 40, 0.25 % (w/v) sodium deoxycholate, 1 mM Ethylene glycol-bis [β-aminoethyl ether] tetra acetic acid [EGTA], 0.5 mM phenylmethylsulfonylfluoride [PMSF], 1 in 100 dilutions of Halt™ Protease and Phosphatase Inhibitor Cocktails [Thermo Scientific, IL, USA]). After resuspending the pellet, the cells were solubilised for 30 minutes on ice before centrifugation of the lysates at 16,000 *g* for 15 minutes. The supernatants containing the whole cell lysates were stored at -80°C before being analysed by SDS-PAGE gel electrophoresis and Western Blot analysis.

2.8.2 Preparation of nuclear protein extracts

An Active Motif Nuclear Extract kit was used to produce nuclear and cystolic fractions. The protocol was followed as per the manufacturer's instructions. Briefly, cells (2×10^6) were incubated with the appropriate stimuli at 37°C in a 5% (v/v) CO_2 atmosphere at 95% humidity. Samples were washed with 5 ml of PBS containing phosphatase inhibitors to terminate any cellular reactions and then centrifuged at 400 *g* for 5 min. The pellets were resuspended in 500 μl hypotonic buffer by pipetting and then incubated on ice for 15 min. Following this, 25 μl of detergent (supplied with kit) was added and the samples vortexed on the highest setting for 10 s. Samples were then centrifuged at 14,000 *g* for 30 s and the supernatant removed. This supernatant contained the cystolic fraction and was stored at -80°C for further use. The pellet was then resuspended in 50 μl of complete lysis buffer (containing a 1,4-dithiothreitol (DTT) and protease inhibitor cocktail) and vortexed for 10 s. The samples were then left on ice for 30 min and after a further vortexing centrifuged at 14,000 *g* for 10 min. The resultant supernatant contained the nuclear fraction and was stored at -80°C before being analysed by SDS-PAGE gel electrophoresis and Western blotting analysis.

2.9 Protein Co-Immunoprecipitation

To identify specific protein-protein interactions occurring between certain proteins a Dynabeads® Co-Immunoprecipitation kit (Invitrogen) was used. The kit allows the covalent immobilisation of antibodies onto the dynabeads for the co-immunoprecipitation of proteins and their associated protein complexes directly on the bead surface. This ensures low background binding as the magnetic nature of the beads allows the easy separation of proteins and protein complexes from a lysate and also facilitates their washing, buffer changes and elution. However, it is important to note that none of the antibodies utilised with the kit were stabilised in glycerol as this compound interferes with the coupling process.

Briefly the coupling process first involved the removal of antibody aggregates from the antibody stocks by centrifugation at 16,000 g for 10 min at 4^{o}C. Then 5 μg of antibody was coupled per mg of beads by incubation with the appropriate amounts of coupling buffers '1' (C1) and '2' (C2) for18 h at 37^{o}C on a shaker (170 rpm). The beads were then harvested on the DynaMag^{TM}-2 magnet, the supernatant removed and the beads resuspended to a concentration of 10 mg/ml in storage buffer (SB).

Samples were prepared for co-immunoprecipitation as follows, cells (2 x10^{6}) were treated with the appropriate stimulations at 37^{o}C in a 5% (v/v) CO_2 atmosphere at 95% humidity. The cells were then harvested, washed in ice cold PBS and lysed using 'Extraction buffer A', which consisted of IP buffer (supplied), NaCl (100 mM), protease inhibitors (Thermo Scientific) and phosphatase inhibitors (Thermo Scientific) diluted to their appropriate concentrations in dH_2O. The volume of extraction buffer added to the cells was determined on the basis of the total concentration of the harvested cells in a 9 to 1 ratio, i.e: 50 μg of cells required 450 μl of extraction buffer. During this time, 1.5 mg of antibody coupled Dynabeads were washed in 900 μl extraction buffer and the supernatant discarded, before the cell lysate was applied and the mixture incubated on an orbital rotor at 4^{o}C for 1 hour. The magnet was then used to separate the immune complexes from the depleted supernatant, and the beads washed in extraction buffer by gentle agitation three times. The beads were then washed with the provided last wash buffer (LWB) and incubated on an orbital rotor at room temperature for 5 min before the antibody-protein interaction broken with extraction buffer (EB), by incubating on an orbital rotor for 5 min at room temperature. The supernatants, containing the purified protein complex, generated after the beads were removed from suspension by the magnet were analysed by SDS-

PAGE gel electrophoresis and then Western Blotting. A conformation specific HRP conjugated secondary antibody was utilised to detect the primary Western blotting antibodies to prevent recognition of heavy and light chain. All antibodies utilised in this experimental procedure are listed in **Table 2.3**.

2.10 Enrichment of Ubiquitinated Proteins

The UbiQapture^{TM} –Q kit (Enzo life sciences) facilitates the isolation of both mono- and polyubiquitinated proteins (independent of lysine residue chain linkage) from cell extracts through the use of a high-binding affinity matrix. Briefly, cells (2 x10^6) were treated with the appropriate stimulations at 37^{o}C in a 5% (v/v) CO_2 atmosphere at 95% humidity. The cells were then harvested, washed in ice cold PBS and lysed using modified RIPA buffer (formula in **section 2.8.1**) and 40 μg of protein from each sample diluted with PBS at 4^{o}C to a final volume of 100 μl, in a clean screw-top tube. A control ubiquitinated-protein lysate (8 μl) was also diluted with PBS at 4^{o}C, to a final volume of 100 μl. To prepare the UbiQapture-Q matrix, 40 μl of matrix suspension was washed in 200 μl of PBS and mixed gently by inversion, before pelleting by centrifugation at 5000 g for 10 s at 4^{o}C. The buffer was carefully removed using gel-loading pipette tips and the sample/control solution (100 μl) was added to the washed UbiQapture-Q matrix, before gentle inversion was utilised to thoroughly mix the samples. The ubiquitinated protein conjugates were allowed to bind to the matrix for 18 h at 4^{o}C on an orbital rotor. After this incubation the samples were centrifuged at 5000g for 30 s at 4^{o}C and the supernatant carefully removed and stored at -80^{o}C as the 'Unbound Fraction'. The matrix was then washed twice with 200 μl PBS at 4^{o}C and gently mixed by inversion during each wash. The ubiquitinated proteins were then eluted using NuPAGE LDS (Lithium Dodecyl Sulfate) loading buffer with 5% (v/v) β-mercaptoethanol diluted in LDS in the proportions: 25 μl LDS buffer, 5 μl β-mercaptoethanol and 70 μl PBS followed by quenching of the reaction by heating to 70^{o}C for 10 minutes. These solutions were then pulse centrifuged to collect the protein solutions for analysis by SDS-PAGE gel electrophoresis and then Western blotting.

2.11 SDS-PAGE gel electrophoresis

The protein concentration of total cellular and nuclear protein lysates was assessed by the BCA protein assay (Pierce). Equal protein amounts of samples were resolved using the XCell *SureLock* Mini-cell kit with NuPAGE Novex 'high performance pre-cast Bis-Tris gels and NuPAGE buffers and reagents (all supplied by Invitrogen). The appropriate

volume of 4 x NuPAGE LDS sample buffer and 10 x NuPAGE reducing agent were added prior to heating samples to 70^{o}C for 10 min and samples were resolved using NuPAGE Bis-Tris gels (10%, 12% and 4-12%) with NuPAGE MOPS or MES running buffer (supplemented with NuPAGE antioxidant) at 200 V for 50 min following the manufacturers instructions. Proteins were then transferred onto a nitrocellulose membrane (Amersham) using NuPAGE transfer buffer with 20% (v/v) methanol at 30 V for 2 h.

2.12 Western Blotting

Following transfer, nitrocellulose membranes were washed once in TBS/Tween (0.5 M NaCl and 20 mM Tris pH 7.5 with 0.1% (v/v) Tween-20) and blocked for 1 h in TBS/Tween containing 5% non-fat milk protein. Membranes were then incubated with the appropriate primary detection antibody overnight at 4^{o}C. All antibodies were diluted in TBS/Tween with 5% BSA. Following incubation with primary antibody, nitrocellulose membranes were washed (5 x 5 min) with TBS/Tween and incubated in the appropriate HRP-conjugated secondary antibody (diluted up to 1:5000 in wash buffer containing 5% non-fat milk protein) for 1 h at room temperature. Nitrocellulose membranes were then washed (5 x 5 min) with TBS/Tween and protein bands were visualised using the ECL detection system. Nitrocellulose membranes were incubated in a mixture of equal volumes of ECL solution A (2.5 mM luminol, 0.4 mM p-coumaric acid and 100 mM Tris pH 8.5) and ECL solution B (0.002% hydrogen peroxide and 100 mM Tris pH 8.5) for 1 min before exposing membranes to KODAK X-Ray film. The nitrocellulose films were often stripped either at room temperature for 1 h in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris pH 7) or for 30 min shaking at 60^{o}C. Nitrocellulose membranes were washed thoroughly in TBS/Tween and checked for residual signal before re-starting the Western Blotting protocol. All antibodies and the dilutions utilised in this experimental procedure are listed in **Table 2.3.**

2.13 Preparation of RNA extracts

Following *in vitro* culture, cells were washed twice with cold sterile PBS (Gibco, Paisley, UK.) and transferred to microcentrifuge tubes. RNA was extracted using the RNeasy micro kit (Qiaqen) according to the manufacturer's instructions. Briefly, cells were lysed and homogenised in a high-salt buffer, which also acts to denature native RNases before being applied to a column supporting a silica based membrane which binds high levels of RNA. Ethanol is utilised to promote the selective binding of RNA to the

column and contaminants are efficiently washed away using additional supplied buffers. The purified RNA is then eluted using RNase free water. DNase (Invitrogen) digestion was then performed to remove possible genomic DNA contamination by mixing 0.5-2 μg of RNA with the appropriate concentration of DNase I reaction buffer and DNase I (amplification grade). This solution was then incubated for 15 min at room temperature before enzyme inactivation by the addition of 25 mM EDTA solution and heating the reaction mixture for 10 min at 65^{o}C. The samples were then stored at -80^{o}C, ready for amplification prior to quantitative RT-PCR.

2.14 Real-time PCR by TaqMan®

To transcribe the mRNA samples into cDNA the "High Capacity cDNA Reverse Transcription Kit" (Applied Biosystems) was used as per the manufacturer's instructions. Briefly the DNase digested mRNA (0.5-2 μg) was incubated with random primers, 4mM dNTPs and 50 units MultiScribe^{TM} Reverse Transcriptase diluted in nuclease free water. The samples were transferred to an Eppendorf Mastercycler gradient PCR machine which heated the samples for 10 min at 25^{o}C followed by 120 min at 37^{o}C before a final step for 5 min at 85^{o}C. The cDNA samples were stored at -20^{o}C before Taqman quantitative PCR was performed.

TaqMan real-time RT-PCR was performed according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) in a 7900 HT Sequence Detector (Applied Biosystems). Triplicate PCR amplifications were performed for each sample in a total volume of 10 μl per well containing 1 μl cDNA sample and 9 μl PCR amplification buffer. The amplification buffer consisted of: 900 nM of each of forward and reverse primers, 250 nM 6-FAM™ dye-labelled, TaqMan® MGB probe and 1 x Taqman® Fast Universal PCR master mix (Applied Biosystems). The probe, forward and reverse primers for each gene investigated were pre-mixed when supplied by Applied Biosystems and diluted 1 in 20 with the Universal PCR master mix and nuclease free H_2O to make the amplification buffer. The endogenous controls utilised throughout the analysis are, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT), both of which are pre-designed Applied Biosystems primer/probe kit (GAPDH; Cat # -4352339E, HPRT; Cat # - 4331182). The thermal cycler conditions were as follows: 20 seconds at 95^{o}C, followed by 45 two-temperature cycles (3 seconds at 95^{o}C and 30 seconds at 60^{o}C) with a final step of 60^{o}C for 30 seconds.

Data analysis was completed using RQ Manager Software (Applied Biosystems), which calculates the threshold cycle, Ct, which represents the PCR cycle when an increase in fluorescence above a set threshold can be detected. Samples were normalised by their reference reporter GAPDH, by subtracting the Ct value of GAPDH from the Ct value of the gene of interest; creating the ΔCt. To obtain a value for a fold increase relative to GAPDH the formula $2^{-\Delta Ct}$ was utilised. Multiplication of this value by 100 gives the expression of the gene of interest as a percentage of GAPDH. The positive error is the standard deviation of the difference, $s=\sqrt{(s_1^2+s_2^2)}$, where s_1 and s_2 are the standard deviations of the Cts of GAPDH and the gene of interest. Details of the primer/probes mixes purchased from Applied Biosystems are given in **Table 2.2 B**. The probes for each gene assay consisted of a reporter dye 5'-6-carboxyfluorescein (FAM) and a non-fluorescent quencher coupled to a minor groove binding (MGB) moiety that stabilises the hybridised probe improving allelic discrimination.

2.15 Immunofluorescence staining

For all immunofluorescence studies, bmDC were treated and analysed in lab-tek permanox (plastic) chamber slides (Nunc, Naperville, IL). The following staining protocol was carried out at room temperature, in a darkened chamber and each washing step included 3 x 1 min incubations with PBS, unless otherwise stated. Media was removed from the cells, which were quickly washed three times in PBS at 37^{o}C before their fixation with 4% paraformaldehyde (PFA) for 10 min and permeabilisation with 0.1% triton-x in PBS for 15 min. After washing, the cells were incubated with 0.3 M glycine in PBS to reduce cellular autofluorescence. Blocking of non-specific binding was performed with 1% BSA and 10 % normal goat serum (Invitrogen), or the appropriate species for the secondary antibody, for 30 min. The cells were then incubated with primary antibody (appropriate dilutions are listed in **Table 2.4A**), for either 1 h at room temperature or 4^{o}C overnight; the specificity of binding was validated by staining with the same primary antibody conjugated to its relevant epitope blocking peptide (appropriate dilutions are listed in **Table 2.4B**). After washing, the cells were incubated for 1 h with an appropriate fluorophore labelled anti-Ig antibody (appropriate dilutions are listed in **Table 2.4 A**). Finally, the cells were washed and stained with 4',6-diamidino-2-phenylindole (DAPI) at 300 nM in PBS for 5 min. Following a final wash, the cells were mounted in Pro-long Gold anti-fade mounting medium (Invitrogen). Images were acquired and analysed on a Zeiss LSM510 confocal microscope with scale bars determined by "LSM Image Browser" software (Zeiss).

2.16 Densitometry and statistical analysis

Data presented are representative of the stated number of independent experiments involving the indicated statistical analysis using Graphpad software. Densitometry was performed using the program ImageJ (NIH).

Figure 2.1 | **FACS analysis of the effect of lactacystin on the viability of dendritic cells.**

To establish whether the proteosomal degradation inhibitor, lactacystin affected the viability of DCs during lengthy treatments, Bm cells matured with GM-CSF for 6 days were treated with either 1 μM or 10 μM lactacystin for the indicated times over an 18 h period before being analysed by flow cytometry. The dead/nonviable cell control consisted of cells heated to 60^{o}C for 15 min. In panels **A**-**B**, dot plots of forward scatter (FSC-H) versus side scatter (SSC-H) are presented alongside histograms of 7-AAD staning (to identify live cells) of viable (live; panel **A**) and nonviable (dead; panel **B**) cell controls. The dot plots allowed cells of the correct size and granularity associated with DCs to be gated where the exclusion (the gating for which was defined from the nonviable cell control) of the fluorescent compound 7-AAD marked them as being healthy/viable cells. In panels **C**-**E**, the gating of viable cells on the basis of 7-AAD staining is presented for cells treated with medium or lactacystin (1 μM or 10 μM) for 1 hour before stimulation with medium (panel **C**) or LPS (panel **D**; 1 μg/ml) for the indicated times (hours). The percentage of deceased cells, as defined and gated from the control established in (panel **B**) was plotted for the differentially treated and stimulated cells (panel **E**).

Figure 2.2 | FACS analysis of proliferation of transgenic (Tg) OVA-specific TCR $CD4^+$ T cells from D0-11-10 BALB/c mice

To validate CFSE dilution as a method for the detection of proliferation form BALB/c Ag-specific transgenic TCR ($KJ1.26^+$) T cells, lymph nodes were harvested from these mice and $CD4^+CD62L^+$ cells purified by MACS microbead magnetic separation and stained with CFSE. These were then cultured (2.5×10^5 cells) with LPS-matured bmDCs (2.5×10^4 cells), for 3 days. The proliferative analysis of T cells in response to either no (panels **A, C, E & G**) or 100 nM (panels **B, D, F & H**) $OVA_{323-339}$ peptide are shown. Dot plots of forward scatter versus side scatter allowed gating of a healthy population of lymphocytes on the basis of their size and granularity (panels **A & B**).

Dot plots displaying lymphocytes double stained with anti-CD4-PerCP and a rat IgG isotype control for the anti-clonotypic KJ1.26-biotin-streptavidin-APC (panel **C**) and anti-KJ1.26-biotin-streptavidin-APC and a rat IgG isotype control for anti-CD4 PerCP (panel **D**) show the gates set to allow identification of $CD4^+KJ1.26^+$ T lymphocytes (panels **E & F**). Analysis of the gated $CD4^+KJ1.26^+$ T cells responding to no (panel **E**) and 100 nM (panel **F**) $OVA_{323-339}$ peptide in terms of CFSE fluorescence allows the proliferation of the Ag specific T cells to be clearly identified and assessed by flow cytometry (panels **G & H**) using FLowJo software, with generation 0 (the gate set at the highest fluorescence of CFSE [on the far right of each histogram]) representing undivided cells.

Table 2.1 | Antibodies utilised in FACS studies

Figure 2.3 | FACS analysis of costimulatory molecule and surface/intracellular TLR4 expression

In panels **A** to **C**, bone marrow cells were differentiated in the presence of GM-CSF for 6 days, stained with 7-AAD (to identify live cells) and antibodies against the murine DC marker, CD11c and the markers of interest, before being analysed by flow cytometry. The dot plots of forward scatter (size) versus side scatter (granularity) allowed the gating of a population of cells of the correct size and granularity associated with DCs (panel **A)**. Then the viable (live) cells were gated on their ability to exclude 7-AAD as healthy cells don't acquire and accumulate this fluorochrome (panel **B**). The live cells were subsequently gated on CD11c expression, an integrin highly expressed on DCs, and approximately 70-85 % of the resulting populations were observed to be $CD11c^+$ relative to the appropriate isotype control (panel **B**). Finally the expression of the Ag or marker of interest, which in this case was stained with an APC conjugated antibody, on $CD11c^+$ cells is then defined by plotting a histogram and analysing its fluorescence relative to an appropriate isotype (panel **C**).

In panels **D** to **E**, bone marrow cells were differentiated in the presence of GM-CSF for 6 days, stained with antibodies against CD11c (unless stated otherwise) and any other markers of interest, before being fixed, permeabilised and stained for both the intracellular and surface associated fractions of a protein of interest, which was then analysed by flow cytometry. The dot plots of forward scatter (size) versus side scatter (granularity) allowed the gating of a population of cells of the correct size and granularity associated with fixed and permeabilised DCs (panel **D)**. These cells were subsequently gated on CD11c expression, an integrin highly expressed on DCs, and approximately 70-85 % of the resulting populations were observed to be $CD11c^+$ relative to the appropriate isotype control (panel **E**). Finally the expression of the Ag or marker of interest, which in this case was stained with an APC conjugated antibody, on $CD11c^+$ cells is then defined by plotting a histogram and analysing its fluorescence relative to an appropriate isotype (panel **F**).

Tables 2.2 | ELISA kits and real-time PCR TaqMan® primers and probes utilised

(**A**) ELISA kits used for cytokine quantification

(**B**) Applied biosystems real-time PCR TaqMan® primers and probes

Table 2.3 | Antibodies utilised in co-immunoprecipitation and Western blotting studies

Table 2.4 | **Antibodies utilised in immunofluorescence studies**

(**A**) List of antibodies used for immunofluorescence staining

(**B**) List of the immunogenic peptides for the immunofluorescence staining antibodies

3 The effect of ES-62 on the maturation of dendritic cells

3.1 Introduction

3.1.1 ES-62 and TLR4; old friends

ES-62 is known to directly interact and exert its immuno-modulatory effects on a variety of immune cells including B-lymphocytes[311,320], macrophages, DCs[291,301,319] and mast cells[321]. Specific examples of the effects of ES-62 on cells of the immune system are: the inhibition of BCR-mediated B lymphocyte proliferation[311,320,340], the induction of IL-10 production by B1 cells[343], reduced levels of pro-inflammatory cytokine secretion by DC and macrophages in response to a range of TLR ligands (BLP, LPS, CpG)[301] and the desensitisation of FcεRI-mediated degranulation and pro-inflammatory mediator release by mast cells[321]. ES-62 has been shown to act via the PRR, TLR4, to selectively modulate the activity of a number of signal transduction molecules including MAPKs, PI3Ks, the PLD/SPHK/Ca^{2+} cascade and NF-κB. Thus, rather than acting in a non-specific immunosuppressive manner, ES-62 specifically targets the initiation of a pro-inflammatory immune response to, for example, prevent the induction of T_H1 responses[323]. However ES-62 also has the ability to block T_H2 responses in situations where they are associated with aberrant inflammation[321]. The latter finding has led to a broadening interest in the effects of ES-62 on cytokines such as IL-17. This is due to the current understanding that IL-17 can act as a master regulator of both T_H1 and T_H2-type inflammation, in the context of both pathogen removal and inflammatory disorders[362].

TLR4 is key for the activity of ES-62. As the most widely studied TLR, it is a critical member of the receptor complex responsible for the recognition and response to gram-negative bacterial LPS. It also mediates the responses to a range of stimuli including host derived heat shock proteins (HSPs), extracellular matrix components (the oligosaccharides, hyaluronan, soluble heparin sulphate and fibrinogen) and taxol (a product of the pacific yew, *Taxus brevifolia,* which exhibits potent antitumor activity in humans)[3]. This important role for TLR4 in mediating the effects of ES-62 was identified from studies on genetically modified mice where macrophages and DCs from TLR4-KO (but not TLR2 or TLR6 KO) mice were found to be refractory to the immunomodulatory effects of ES-62[301]. Moreover, in macrophages TLR4 has recently been shown to be crucial for the internalisation of ES-62[326]. Interestingly, presumably through a TLR4 mediated

mechanism, ES-62 appears to utilise MyD88 for the transduction of intracellular signals in macrophages and bmDC as suggested by the use of cells from MyD88-knockout mice[326]. However this interaction with TLR4 is quite unique as macrophages and bmDC from the TLR4-mutant C3H/HeJ mice are unable to transduce LPS signals due to a point mutation in the intracellular TIR domain but can still respond normally to ES-62[301].

3.1.2 ES-62 and dendritic cells, the role of TLR4 and potential co-receptor involvement.

As stated above the anti-inflammatory effects of ES-62 reflect the subversion of TLR4 signalling in DCs and macrophages[160,291,301,363]. Interestingly, evidence of the binding partners of ES-62 from pull-down, Far Western and surface plasmon resonance (BiaCore^{TM}) kinetic studies indicates differential, PC-dependent receptor-binding of ES-62 to monocyte, B- and T-lymphocyte membranes[326]. Moreover, it has been shown that many of the anti-inflammatory effects of ES-62 are dependent on its PC-moiety[325]. Through this work both synthetic and natural PC based compounds have shown great potential in mimicking the immunomodulatory activities of ES-62 and other helminth ES products[311,312]. The structure of PC from which these compounds is based is presented in **Figure 1A**. It has also been found that the function of plasmacytoid DCs, which do not express TLR4 (at least in resting cells), can be modulated by ES-62 (C. Steiger & M. M. Harnett, unpublished data). Collectively, these findings may imply that ES-62 achieves its effects by signalling via TLR4 in concert with other receptors or a co-receptor, suggesting an additional target for its interference of TLR-induced inflammatory responses via an alternative signalling pathway. Consistent with this, preliminary analysis of the intracellular signalling pathways (p38 MAPK, PI3K-Akt and NF-κB) triggered by LPS and ES-62 in macrophages and DCs identified differential signalling, potentially indicating differences in the receptor-coupling mechanisms utilised[291,301,326].

3.1.2.1 Molecular mimicry; ES-62 and platelet activating factor (PAF)

As stated above, ES-62 appears to modulate DC maturation by subverting signalling via the LPS receptor, TLR4[301]. However, unlike LPS, it does not necessarily require TLR4 to be fully functional to mediate its' subversive effects[301]. Intriguingly, it has emerged that the PC-moiety of the inflammatory glycerophospholipid, platelet activating factor (PAF)[295,364], is important for binding to the PAF receptor (PAFR), a G-protein coupled receptor (GPCR) expressed on many cells of the immune system. This led to the hypothesis that the large number of PC-moieties present on the surface of ES-62 could

facilitate its interaction with the PAFR followed by its modulation and internalisation into the cell. This is the case with a number of pathogen species which employ the ability of PC to bind the PAFR for receptor recruitment, entry into the cell and as a mechanism to evade the immune system. For example, *Salmonella pneumoniae* utilises the PC expressed on its surface to bind to the PAFR on host cells and become rapidly internalised[365,366]. In the context of immune cell function, PAF binding induces the modulation of certain B cell activation responses in a similar fashion to the suppression of B cell responses induced by ES-62[367].

Preliminary studies using native ES-62 and PC-free rES-62 on rat brain capillary endothelial cells (RBCEC) and primary neurons from wild type and PAFR knockout mice (PAFR KO) suggested that ES-62 can also bind to the PAF receptor in a PC-dependent manner (E. Tuomamen and W. Harnett, unpublished results). These findings could suggest that ES-62 may achieve its effects by signalling via TLR4 in concert with a co-receptor, such as the PAFR. This is an attractive hypothesis as the PAFR can be recruited as a secondary effector of LPS/TLR4 signalling[322] leading to the exacerbated release of several pro-inflammatory cytokines (TNF-α, IL-6, IL-1) and other mediators including PAF[368,369]. Moreover, Nakamura and colleagues showed that bacterial LPS can also directly activate the PAFR, enhancing the effect of PAF and the local production of TNF-α, resulting in the increased ability of leukocytes and other immune system cells to deal with invading pathogens[295,364,369-372]. In any case, there is some degree of crosstalk between LPS/TLR4 and PAF/PAFR mediated signalling as LPS primed mice showed enhanced sensitivity towards PAF resulting in increased mortality associated with the accumulation of PAF receptor mRNA in peritoneal macrophages[370]. The mechanisms behind this priming effect are gradually being elucidated by *in vitro* studies, with the PAF induced phosphorylation of p38 being greatly potentiated in neutrophils pre-treated with LPS[364]. The subversion of PAF, its receptor and/or its intracellular signalling pathways could therefore allow ES-62 to engineer a favourable phenotype in those immune system cells, which do not express TLR4, such as plasmacytoid DCs.

3.1.2.2 The biology of PAF and its G-protein couple receptor, PAFR

PAF (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (**Fig. 1 B**) is a potent proinflammatory mediator, the production of which is dysregulated in pathological situations where it can acts as a primary and secondary messenger[373]. For example, it appears to play a key role in the pathogenesis of bronchial asthma and anaphylactic

shock[374]. PAF mediates its effects through homotypic and heterotypic cell-to-cell communication, resulting in the activation of platelets, neutrophils, macrophages, lymphocytes and endothelial cells[295,375-377]. All of these cells can produce PAF, further promoting the migration of granulocytes to sites of inflammation and mediating the adhesion of neutrophils to endothelial cells[377]. In addition, PAF is mitotic for fibroblasts and regulates lymphocyte function[378]. PAF is produced both *de novo* and through remodelling pathways, the latter being regulated by extracellular signals[379], with the half-life of PAF being relatively short due to rapid degradation by PAF acetylhydrolases[380]. A model of *Candida albicans* infection reinforced the importance of the PAFR in determining the early cytokine environment, especially IFN-γ and TNF-α, in pathogen removal[381].

The PAFR gene is encoded on human chromosome 1 and generates two distinct mRNA species (transcripts 1 & 2) although only a single 39kDa protein is produced[382], with NF-κB and SP-1 upregulating the expression of transcript 1 in immune system cells[382,383]. As stated above, following the interaction of the PAFR with its ligand, it modulates certain B cell activation responses in a similar fashion to the suppression of B cell responses induced by ES-62[367]. Signalling through the PAFR has also been implicated in mediating the production of IL-8, phagocytosis and CD11/CD18 expression by neutrophils. Moreover, the activation of neutrophils in response to Zymosan particles, a *Saccharomyces cerevisiae* PAMP, appears to require an initial burst of PAF secretion[26]. The PAFR becomes rapidly desensitised upon stimulation with PAF due to phosphorylation-dependent internalisation and consequent ubiquitin-dependent, proteosomal- and lysosomal-mediated degradation. Thus, intracellular PAFR is localised to the endosomes, due to ligand-driven receptor internalisation, where recruitment of cell trafficking and intracellular signalling proteins can occur[384]. Interestingly functional PAFR has also been identified as being expressed on the nuclear envelope of isolated piglet brain microvascular endothelial cells *in vitro* and *in situ*[385]. Thus, the ability of extracellular PAF to activate pro-inflammatory responses including its own intracellular generation, suggests locally produced PAF could be generated in the vicinity of its cognate nuclear receptors to modulate gene transcription[385].

3.1.2.3 Pathogen and parasite utilisation of the PAFR

The PC component common to PAF and ES-62 is also utilised by a number of different pathogens for receptor recruitment and/or entry into host cells. For example, the

PAFR is also rapidly internalised following binding[366] of *S. pneumoniae* which expresses PC on its surface[365]. Within this context it is becoming increasingly apparent that parasites have evolved to exploit this receptor as a means to evade the immune system. For example, the PAFR has been implicated as playing an important role in the protective immune response of the murine host against infection with the intracellular protozoan parasite *Trypanosoma cruzi*. Thus a number of studies have shown that mice pre-treated with a PAFR antagonist and followed by *T. cruzi* infection have increased blood parasitaemia and enhanced infection-associated lethality[386]. A similar protective role for PAF during infection with the closely related parasite *Leishmania amazonensis* has also been shown by utilising PAFR agonists and antagonists *in vitro* and *in vivo*[387]. Thus, the administration of the agonist carbamyl PAF (C-PAF) to C57BL/6 derived macrophages significantly inhibited parasite infectivity and growth in these cells *in vitro*. By contrast, the antagonist WEB2086 increased the infectivity of the parasite in macrophages *in vitro* while it dramatically increased lesion sizes and parasite loads in regional lymph nodes and spleen *in vivo*. Moreover, the infection of $PAFR^{-/-}$ mice with *L. amazonensis* showed dramatic alterations in cytokine production with a shifting away from a protective T_H1 response[381]. Interestingly, entry of the *Leishmania* parasite into the DC, whilst maintaining an "immature" DC phenotype and promoting anti-inflammatory responses, still allows the DC to respond to other exogenous stimuli[388]. The amastigote form of the parasite is thought to avoid inducing maturation of DCs, not through deactivating host processes but rather via selective interaction with particular surface receptors, as antibody-opsonised amastigotes do not result in DC maturation[389].

The roles for PAF and its receptor during filarial/gastrointestinal nematode infection have been poorly documented. However, *Nippostrongylus brasiliensis*-infected rats have been shown to have elevated systemic levels of PAF during the process of worm elimination[390], a stage at which the parasites secrete an acetylhydrolase that functionally inhibits PAF activity[391,392]. In addition, Negrao-Correa *et al.* (2004) have performed preliminary studies on the role of PAFR in mobilising the immune system in response to the parasitic nematode, *Strongyloides venezuelensis*, which infects the gastrointestinal tract using $PAFR^{-/-}$ mice[393]. These authors found that during the course of infection such $PAFR^{-/-}$ mice showed relatively decreased intestinal inflammation against the worms resulting in their increased survival but decreased fecundity. This was associated with reduced levels of T_H2 cytokines, as well as IL-10 and TNF-α and also suppression of goblet cell hyperplasia in these mice.

Collectively these studies suggest that the involvement of PAF and the PAFR in the mobilisation of immune responses, particularly those of the innate immune system, has driven the evolution of pathogens towards strategies that target them for subversion of the immune response. This has led to the idea that ES-62 could utilise its PC moieties to interact with the PAFR and/or elicit its immunomodulatory effect and gain access into host cells or alternatively, it may desensitise cells to subsequent activation by LPS/PAF signalling via the PAFR[283,308]. These are particularly interesting when taking into consideration the above stated preliminary results suggesting that ES-62 can bind to the PAFR in a PC-dependent manner (E. Tuomamen & W. Harnett, unpublished results).

3.1.2.4 PAFR-mediated signal transduction pathways and the effect of agonists and antagonists

The PAFR is known to be expressed at the plasma membrane of many immune system cells including human neutrophils, $P338D_1$ murine macrophages, human peripheral mononuclear leukocytes, rat brain and liver Kupffer cells[364,394]. Where studies utilising a range of PAFR agonists and antagonists have clarified its role in the activation of MAPKs, PI3Ks, PLA_2, PKC and Src pathways on such cells[322,364] and have shown that PAF/PAF receptor signalling plays important roles in a number of pro-inflammatory responses, including LPS induced shock[395]. Due to the importance of PAF in mediating inflammatory responses a wide range of specific PAFR agonists and antagonists are available for its study. For example, C-PAF (**Fig. 1C**), which is an analogue of PAF that exhibits stereo-specific binding to the PAFR, has been demonstrated to induce the expression of the proinflammatory signal transducers, iNOS and COX-2 while increasing ERK1/2 MAPK phosphorylation and activation of NF-κB in various cells of a model of septic shock[57]. In respect to antagonists, the classical compound utilised is WEB2086 (**Fig. 1D**), derived from the hetrazepine family of compounds; it is a potent and highly specific antagonist. Its binding site on the PAFR has been shown, using human platelets, to be identical to that of PAF[364,394,396]. However, characterisation of this antagonist has revealed that whilst the mechanism of action of PAF can be partially explained through its interaction with receptors on the target cell surface, many of its' effects reflect the blocking of the internalisation of PAF and its subsequent activation of intracellular PAFRs[397]. The potency of this compound has been shown in human breast cancer cells where it was able to inhibit their growth[398] and in various models of inflammation[399]. More recently WEB2086 has been suggested to act, as an inverse agonist, as the compound has a higher affinity for the inactive rather than the active state of the PAFR. This would result in the reduction of

basal activation of effector mechanisms and subsequent receptor internalisation and transport within the cell[400].

By contrast, another potent and commonly utilised PAFR antagonist CV3988 (**Fig. 1E**) (rac-3-(N-n-octadecylcarbamoyloxy)-2-methoxypropyl 2-thiazolioethyl phosphate) acts as a competitive antagonist, due to its structural similarity to PAF. It has been shown to effectively inhibit PAF-induced platelet aggregation and hypotension by preventing the specific binding of [^{3}H]PAF to its receptor on platelets[401]. Studies have also shown that it is able to protect mice from gram-positive septic shock induced by LTA[397,401]. Interestingly computational models of the putative PAFR, suggest that both of the antagonists share common spatial features as well as lipophilic and hydrophilic regions with which they interact within the constraints of the hypothetical receptor.

3.1.3 T_H17 cells; could ES-62 target this inflammatory population?

Within recent years there has been a major focus on the role of the cytokine IL-17 in driving inflammation in response to a number of pathogenic stimuli. The most well characterised source of IL-17 within the body is the T_H17 cell. The differentiation of this $CD4^+$ T cell population is regulated by the transcription factors, STAT3 and ROR-γt as well as the cytokines TGF-β, IL-1, IL-6 and IL-23, which stabilise and expand the cell population[161]. This T_H subset functions mostly at mucosal surfaces, triggering pro-inflammatory danger signals that enhance neutrophil mobilisation and expression of antimicrobial factors. However T_H17 cells are also associated with driving the inflammatory pathology in various autoimmune conditions[161]. The current understanding of the differentiation of T_H17 cells from naïve precursors is that it is a three-step process, as summarised in **Figure 3.2**.) The initiation of development of these cells begins with the antigenic stimulation of naïve precursors in the presence of TGF-β1 and IL-6; **2**) Autocrine secretion of IL-21 then reinforces their differentiation; **3**) IL-23 maintains the T_H17 population and increases its pathogenicity[402]. A number of polymorphisms in the receptor for IL-23 are now known to be associated with a wide range of autoimmune diseases, leading to the concept of an IL-23-IL-17 inflammatory axis[402]. However, a lack of IL-23 attenuates host protection against *Citrobacter rodentium* infection despite the presence of normal T_H17 populations[124], indicating IL-23 can operate in a pro-inflammatory manner independently of IL-17.

There are many forms of IL-17 secreted by immune cells (IL-17A-F), but the IL-17A homodimer is by far the best characterised member of the IL-17 family[161]. IL-17A is often associated with driving autoimmunity, due to its potent strength of signalling relative to the other family members[403]. The pro-inflammatory programme of gene expression induced by IL-17A signalling through its receptor (IL-17RA) is mediated by TRAF6 and NF-κB, making it reminiscent of the innate immunity receptors such as TLR4[161]. In this respect, IL-17A is known to activate components of the canonical NF-κB pathway, p50 and p65[404], and TRAF6$^{-/-}$ mice are defective in IL-17A mediated activation of NF-κB[405]. This suggests that T_H17 cells promote signals that are typical of early inflammatory events and this helps to bridge innate and adaptive immunity.

Although the functions of IL-17B-F remain poorly defined (apart from IL-17E), they are known to stimulate the transcription of an array of pro-inflammatory genes similar to that of IL-17A[406-408]. This is also coupled with recent studies indicating that they can act to regulate the function of the T_H17 cells themselves[409], where there is an intriguing level of cross-talk within this cytokine family to promote inflammation, as ectopic expression of IL-17C in conjunction with production of IL-17B by $CD4^+$ T cells exacerbates CIA[408].

Thus, the potential role of IL-17 as a master regulator of T_H1 and T_H2-type responses[362], and indeed many proinflammatory actions within the body, indicate it is potentially a key target of the anti-inflammatory action of ES-62. Through its known modulation of both T_H1 and T_H2 cell driven inflammation, as well as the inherent plasticity of T cell subsets[410], it is believed that ES-62 may target the maturation of DCs to inhibit their induction of a T_H17 subset in response to inflammatory signals. Indeed, a recent publication by this laboratory group has highlighted the importance of this limitation of IL-17 responses by ES-62 through its attenuation of the development and severity of a mouse model of collagen induced arthritis (CIA)[352]. T_H17 cells are also associated with the production of the cytokine IL-22, an important mediator of immune functions that has been shown to act as both a pro- and anti-inflammatory molecule[155,402], where DCs have also been shown to provide a T cell independent source of IL-22[411]. It has been implicated in the development of inflammatory disorders, as for example, blocking IL-22 production prevents the development of IL-23 driven psoriasis[412,413]. However, there is also evidence that IL-22 can protect in several inflammatory disorders[414,415] and upregulate the secretion of anti-microbial peptides from non-hemopoietic cells to enhance mucosal immunity to extracellular bacterial infections in the gut[402].

3.1.4 Sphingosine kinases (SPHK) and their potential as targets for ES-62 mediated responses

The sphingosine kinases (SPHKs) comprise two isoforms, SPHK1 and SPHK2, which catalyze the phosphorylation of sphingosine to generate sphingosine-1-phosphate (S1P). Sphingosine is a membrane lipid that can be rapidly metabolised following the stimulation of various plasma membrane receptors. For example sphingomyelin (a key molecule in the synthesis of several important bioactive lipids[416]) can be converted by sphingomyelinase to ceramide and subsequently, by ceramidase to sphingosine and ultimately to S1P by SPHK (**Fig. 3.3**). The resting levels of S1P in most cells is extremely low as its synthesis and degradation is tightly regulated by the enzymes in the sphingolipid metabolic pathway[417]. Studies indicate that both SPHK1 and SPHK2 are involved in the homeostasis of S1P levels but loss of either of these isoforms does not ablate the blood to tissue S1P gradient, indicating a level of redundancy between these enzymes[418,419]. Having both cell-intrinsic and cell-extrinsic functions in immunity, S1P is the ligand of a family of five G-protein-coupled receptors (GPCRs), labelled S1PR1-5[420]. The expression of S1PR1 is common to most immune system cells but S1PR2-5 have a more limited distribution, for example, T cells express S1PR1 and -4 while macrophages express S1PR1 and -2[417]. The main function of these receptors appears to be associated with regulation of cell migration, where a low concentration of S1P promotes chemotaxis[421]. In the case of DCs this chemotactic response also appears to be associated with its cellular differentiation and activation, as immature DCs express only S1PR1, which switches to S1PR3 upon maturation accentuating its chemotactic responses to S1P[417].

Many studies of the biology of the SPHKs have focused on their roles in inflammation and in respect to DCs showing that both isoforms are important in modulating cellular responses to LPS and other PAMPs[416]. In this context, the recognition of Gram-negative bacterial lipopeptides and LPS by TLR2 and TLR4 respectively, stimulate the up-regulation of SPHK1[422]. Many of these findings have come from the study of Sepsis, an overwhelming systemic inflammatory response resulting from bacterial infections, where mouse models revealed NF-κB to be a major downstream target of SPHK responses[423,424]. The administration of SPHK1 inhibitors to mice suppress LPS-induced production of inflammatory cytokines, the nuclear translocation of the p65 subunit into the nucleus and the degradation of IRAK-1; of which the latter two elements are both associated with NF-κB activation[425]. The activation of TLR2 and TLR4 also enhances the cytoplasmic production of S1P in phagocytes but not its release, suggesting that it is the

intracellular functions of this molecule that are important in TLR signalling[425]. In this context, PKCδ is understood to be involved in the activation and phosphorylation of SPHK1, leading to its translocation to the membrane, where it can metabolise S1P[426]. This combined with its emerging roles in TLR signalling[104,105], suggests direct links in the involvement of the SPHKs in NF-κB activation as summarised in **Figure 3.4**. However, splenocytes from SPHK1 deficient mice have been found to exhibit enhanced LPS induced IL-12 but not IL-6 and IL-10 secretion that can be compensated for by S1PR1 agonists[427]. This suggests additional cell extrinsic roles of S1P during inflammation[425]. Thus, the exposure of human monocyte derived DC to S1P during maturation with LPS impairs their ability to initiate T_H1 cell responses and promotes T_H2 responses of naïve $CD4^+$ T cells by suppressing IL-12p70 and promoting IL-4 and IL-10 production through an unknown mechanism[428,429].

Collectively, many of the reported studies suggest that S1P appears to mature DCs to a phenotype that favours skewing T cell lineage development towards a T_H2 phenotype while dampening T_H1 responses[425], which is accompanied by an increase in endocytosis of mature DCs through the engagement of SIPR3[430]. These observations reflect similar effects of ES-62 on DCs[160,291,301], suggesting the SPHKs and subsequently S1P may be targets of its modulation of DC maturation and cytokine responses in response to positive TLR signalling. In support of this, recent studies have shown that key molecules, such as PKCα and PLD, involved in the activation of SPHKs are targets for the anti-inflammatory effects of ES-62 in mast cells and T cells[321]. Interestingly, it has recently been reported that S1P, predominantly through S1PR1, can substitute for IL-23 *in vitro* to increase the activity and number of IL-17 secreting $CD4^+$ T cells grown in the presence of IL-1β, IL-6 and TGF-β1[431,432]. This S1P-promoted differentiation of T_H17 cells occurs in parallel with the suppression of T_H1 and T_H2 associated cytokines, and similarly to what is observed with IL-23, can be overcome by the actions of IL-27 and the T_H1 and T_H2 cytokines, IFN-γ or IL-4 respectively. With the recently realised potential of ES-62 in limiting T_H17 associated responses[352], this immunomodulator may attenuate S1P production (through the SPHKs) due to its aforementioned roles in the development and expansion of this inflammatory subset.

3.1.5 The localisation and trafficking of TLR4 and its modulation by ES-62

TLRs can be classified not only by the molecules they recognise, but also by their subcellular localisation, either stably or dynamically. In this case they can be roughly split into two groups: TLR1, -2, -4 , -5 and -6 are generally found on the cell surface[10], and recognise pathogenic surface components such as LPS, while TLR3, -7, -8 and -9 are detected intracellularly, localised within endosomal compartments, where they can recognise nucleic acids derived from the genome of viruses and bacteria[347]. Thus, the localisation and trafficking of the particular TLRs is central to the sensing of their ligands, although the precise nature of the compartments where intracellular TLRs meet internalised ligand remains poorly defined. However, it is also clear that TLRs that are normally present at the cell surface can enter into the endocytic pathway following their activation and these receptors are also rapidly recruited to phagosomes or endosomes containing microbial cargo[329,433]. Over recent years it has become apparent that these trafficking pathways play an important role in regulating the activation of APCs, particularly in response to different stimuli and impact on the outcomes of their signalling pathways[347].

The responsiveness of TLR4 to LPS is regulated through two mechanisms: the levels present on the plasma membrane and its localisation within the cell. The movement between these cellular environments acts to prevent prolonged and excessive activation. Studies have shown that the TLR4-MD2-CD14 complex cycles between the plasma membrane and the golgi until it is engaged by LPS[434,435], where it rapidly accumulates in lipid rafts (cholesterol-enriched membrane microdomains) upon which both TIRAP and MyD88 are recruited[436]. Neither of these adaptor proteins co-localise with TLR4 in resting cells, with MyD88 found in condensed particles throughout the cytosol, with no clear association to cellular organelles such as golgi, ER, endosomes or lysosomes[437,438], which provides a barrier to the unregulated activation of the pathway. By contrast, in resting cells TIRAP utilises its PIP_2 binding domain to localise to PIP_2-rich regions such as lipid rafts, to which both TLR4 and MyD88 migrate upon LPS stimulation[48,438]. The presence of TIRAP within these domains is crucial as it serves to recruit its signalling adaptor partner, MyD88 from its cytoplasmic pool to participate in the transduction of TLR4 signals at the plasma membrane[438]. The aggregation of the activated receptor complex briefly recruits several IRAKs and TRAF6, which then dissociate and activate NF-κB within the cytosol. During this process the cell internalises the TLR4 complex through a process known as

endocytosis, during which TIRAP and MyD88 dissociate from the receptor. This involvement of lipid rafts and endocytosis in the formation and internalisation of the TLR4 complex indicates a potential for ES-62 to exploit this mechanism through its own interactions with TLR4 [301,326].

During the initiation of endocytosis and trafficking to the endolysosomes of this complex, the concentration of PIP_2 decreases resulting in the dissociation of TIRAP from TLR4. This allows the association of TRAM and TRIF, resulting in the activation of the type 1 IFN pathway in the endosomes[329]. In this case TRAM functions as to recruit TRIF, which functions as the signalling transducer[48]. The translocation of dynamin and clathrin to the endocytic vacuole that forms around the TLR4/TRAM/TRIF complex is essential for its internalisation[329,439,440]. Dynamins are GTPases that are involved in the budding of vesicles from the plasma membrane and clathrin is a scaffold protein that forms the clathrin coated pits through which endocytosis occurs[329]. Thus TLR4 does not induce the simultaneous activation of both MyD88- and TRIF- dependent pathways: instead, TLR4 induces the sequential activation of these signalling pathways.

It has been noted that in preventing TLR4 internalisation there is an increase in LPS-induced NF-κB activation[329]. Coupled with evidence that TLR4 is ubiquitinated and that this ubiquitination promotes its degradation, it was suggested that the endocytosis and degradation of TLR4 and its associated complex was important in regulating its activation of NF-κB[347]. In conjunction with this, TLR4 has been shown to be tyrosine phosphorylated upon LPS stimulation, a process important for the targeting of transmembrane proteins for lysosomal degradation[441]. However, such tyrosine phosphorylation appears to be essential for the activation of the TRAM-TRIF pathway and the induction of type-1 IFNs[347]. In this respect, in resting cells TRIF and TRAM are localised in the cytosol and the plasma membrane, respectively[440,442] but they both rapidly (within 30 min) translocate to the endosomes upon stimulation with LPS to interact with the CD14, MD-2 and TLR4 complex (all components are essential for this interaction)[439]. Interestingly, in terms of the induction of the type-1 IFNs, the transport of TLR4 into the endolysosomes and the recruitment of TRAM and TRIF serve to engage this complex with the cytosolic E3 ubiquitin ligase,TRAF3, a crucial protein in the propagation of all IFN signals[13].

Following the trafficking of TLR4 in response to external stimuli, any associated microbial proteins are degraded, promoting the loading of microbial Ags onto MHC class II molecules for presentation to and activation of $CD4^+$ T_H cells[328,329]. Thus, LPS-induced

trafficking of TLR4 through the clathrin-dependent endosomal route provides a mechanism for linking innate and adaptive immune responses and provides a potential target for immunomodulation by ES-62. Consistent with this, in mast cells, ES-62 is known to promote the alternative routing of TLR4 to caveolae lipid raft compartments, resulting in its degradation[321]. This therefore provides a mechanism for inducing TLR4 hyporesponsiveness and also suppressing TLR4-associated (auto)-Ag presentation[328,329]. Evidence for this type of immunomodulation also exists from other helminth derived immunomodulators. For example, Schistosoma Egg Antigen (SEA), interacts through multiple receptors including TLR4and targets its trafficking into a lysosome associated membrane glycoprotein 2 (LAMP2; a common lysosomal and endosomal marker) negative compartment in a clathrin-independent manner[443]. This distinctive compartmentalisation of TLR4, relative to that induced by canonical PAMPs, could also be a critical component of the subversion of TLR4 signalling by ES-62 and other helminth products.

3.1.6 Aim of this study

The current understanding of the effects of ES-62, and indeed many parasitic helminth immuno-modulators on APCs, particularly DCs, is that it matures them to induce a T_H2-like, anti-inflammatory immune response. However ES-62 can act to reduce both T_H1 and T_H2 –type inflammatory responses indicating roles for its effects on other, broader regulators of inflammation. One such target that remains a gap in our understanding of the effects of ES-62 is IL-17, which is emerging as a master regulator of T_H1 and T_H2-type responses[362]. Thus a major aim of this section was to identify whether through modulation of DC maturation, ES-62 attenuates the ability of these APCs to induce the lineage differentiation of T_H17 cells and the secretion of IL-17 (and its associated cytokines) in response to TLR4 recognition of LPS. These studies were performed in both C57BL/6 and BALB/c mice which have generally been considered to be genetically pre-disposed to generate T_H1 and T_H2 inflammatory responses respectively, to identify whether the genetic background of the bmDCs influenced the immunomodulatory effects of ES-62. The expression of the transmembrane glycoproteins CD40, CD80, CD86 and MHC class II on bmDCs would also be defined due to their importance in the interaction and transmission of antigenic (MHC class II) and costimulatory signals (CD40, CD80 and CD86) for driving the activation of naïve T cells. This would clarify whether their limited upregulation induced by ES-62 in response to LPS recognition reported for DCs[160,301] is involved in the potential attenuation of T_H17 development by this immunomodulator.

Through TLR4, LPS is known to stimulate and up-regulate SPHK1[422], and recent studies have shown that the SPHKs and key molecules involved in the activation of these enzymes in mast cells and T cells are targets for modulation by ES-62[321]. It was therefore a further aim to exploit the availability of Sphk1 and -2 $^{-/-}$ mice to explore the role of these enzymes in the propagation of inflammatory signals through TLR4 in DCs. These investigations would focus on their involvement in the propagation of a potential T_H17 phenotype and therefore determining whether there are any similarities with the anti-inflammatory effects of ES-62.

Current evidence suggests that ES-62 may utilise its PC moieties to bind and signal via TLR4 in concert with one or more co-receptors[326]. Consistent with this, preliminary analysis of the intracellular signalling pathways (p38 MAPK, PI3K-Akt and NF-κB) triggered by LPS and ES-62 in macrophages and DCs identified differential signalling, potentially indicating differences in the receptor-coupling mechanisms utilised[291,301,326].

The PAFR and its ligand PAF, a PC containing lipid mediator, play a key role in the coordination of pro-inflammatory responses for pathogen removal and as such is considered a strong candidate as an interacting partner for ES-62. However, within the current literature there is little information relating to the role of the PAFR in DC maturation and LPS responses. Thus as a first step to analysing whether the PAFR is a target for ES-62, the role of PAF signalling in LPS-mediated maturation of bmDCs and their subsequent ability to prime and polarise naïve T cells would be defined using the PAFR agonist, C-PAF and the competitive and non-competitive inhibitors, CV3988 and WEB2086, respectively. This characterisation would provide the platform for the investigation of the potential involvement of the PAFR in ES-62 mediated immunomodulation of DCs by identifying whether such signalling is a target of its downregulation of (hyper)-cytokine production in response to LPS. In respect to TLR4, due to the importance of its localisation and trafficking in regulating its activation status to different stimuli, it was planned to determine the effect of ES-62 on the internalisation, trafficking and degradation both TLR4 and its functional complex TLR4/MD2 throughout bmDCs.

In brief the aims of this study were to define whether:

- ES-62 in its interactions with TLR4, induces bmDC maturation that drives the lineage differentiation of T_H17 cells and the secretion of IL-17 (and its associated cytokines) in a similar fashion to that reported for LPS and/or whether it attenuates the ability of LPS-matured APCs to induce the clonal expansion of functional T_H17 cells

- ES-62 exhibits differential effects on bmDCs derived from C57BL/6 and BALC/c in terms of their ability to induce polarised T_H cell subsets.

- the anti-inflammatory/immunomodulatory effects of ES-62 on bmDC were mimicked in bmDC derived from Sphk1 and -2 $^{-/-}$ mice.

- the PAFR plays a role in the LPS-mediated maturation of bmDCs and their ability to stimulate the priming and polarisation of naïve T cells as well as to establish whether ES-62 utilises this receptor in its downregulation of (hyper)cytokine production in response to LPS.

- ES-62 and LPS differentially modulate the internalisation, localisation and expression of TLR4 and its functional complex TLR4/MD2.

3.2 Results

3.2.1 ES-62- and LPS-induced maturation result in the differential ability of DCs to stimulate T_H cell proliferation and T_H17 polarisation

As the basis to define the effects ES-62-mediated maturation may have on the biology of a DC and its subsequent ability to respond to infection as indicated by the pro-inflammatory stimulus, LPS, the effects of the canonical TLR4 agonist versus ES-62 were defined in terms of the ability of the DCs to prime proliferation and cytokine production by naïve, Ag-specific $CD4^+CD62L^+$ T cells. Firstly, proliferation of naïve OVA-specific tg TCR $CD4^+$ T_H cells from a DO.11.10.BALB/c background in response to ES-62- or LPS-matured bmDC loaded with increasing concentrations of OVA peptide. This was achieved by measurement of DNA synthesis as indicated by [^{3}H] thymidine incorporation into replicating DNA in cells (**Fig. 3.5**). This revealed, as expected, the widely established dose dependent OVA peptide stimulated increase in DNA synthesis induced by LPS-matured bmDC indicative of a DC phenotype capable of T_H cell priming. As previously shown[160], bmDC maturation in response to ES-62 was also capable of priming clonal expansion of T_H cells, albeit to a lesser extent than observed with LPS. This was defined by calculating the half maximal effective concentration (EC_{50}) of agonist (OVA peptide) for each stimulus, whereby ES-62 and LPS matured DC exhibited EC_{50} values of 166 nM and 27.74 nM OVA peptide, respectively. Directly related to this was the attenuated ability of ES-62 matured DCs to induce similar maximal and basal levels of cellular proliferation relative to LPS.

However, as this assay only provides a snapshot of DNA synthesis over a short interval (56-72 hours) of the total culture period (3 days) and doesn't indicate whether only a subset of cells are undergoing proliferation, the cell tracking dye, CFSE was utilised to provide a greater depth of analysis. Thus, the number of cell divisions undergone by Ag-specific T_H cells was established in parallel experiments in which the T_H cells were prestained with CFSE prior to culture with the bmDCs. CFSE-labelled naïve DO.11.10/BALB/c $CD4^+CD62L^+$ T_H cells stimulated with ES-62 and LPS-matured bmDC loaded with a range of Ag concentrations (0, 10 and 100 nM OVA peptide), confirmed that maturation of the DCs with LPS enabled them to induce greater levels of Ag specific T_H cell division relative to those stimulated with ES-62, an effect that is exacerbated at the lower concentrations of Ag (**Fig. 3.6 B-D**). On further analysis, the proliferation index (**section 2.4.3**) reveals that ES-62 maturation only marginally lowers the level of division

from the responding Ag specific T_H cell population relative to LPS (**Fig. 3.6 E**). The division index and %-divided values reveal, however, that although there is little difference in the divisions undergone by the responding cell population between ES-62 and LPS, ES-62 maturation is reducing the number of responding Ag specific T_H cells, particularly at low Ag concentrations (10 nM OVA peptide).

Similarly, experiments performed on bmDCs and OVA-specific tg TCR T_H cells (OTII) derived from C57BL/6 background mice revealed differential priming abilities of DC resulting from LPS and ES-62 maturation, which were again exacerbated at the lower concentrations of Ag (**Fig. 3.7 B-D**). However, whilst both ES-62- and LPS-induced maturation produced DCs capable of priming Ag-specific proliferation, in this case, the ES-62-matured DC were more effective at inducing proliferative responses at lower concentrations of Ag. Consistent with this, at low Ag concentrations (10 and 30 nM OVA peptide), ES-62 matured bmDCs induced a higher proliferation index from the responding Ag specific T_H cell population relative to LPS, although this parameter was reversed at the highest concentration of Ag (300 nM OVA peptide) (**Fig. 3.7 E**). Similarly to BALB/c derived bmDCs, however, the division index and %-divided values indicate ES-62 maturation is reducing the number of responding Ag specific T_H cells at Ag concentrations ≤100 nM OVA peptide relative to LPS, although this appears to be overcome at high dosage of Ag.

The production of several important pro-inflammatory and regulatory cytokines, IL-17A, IL-22, IFN-γ and IL-10 from the BALB/c and C57BL/6 background cells was also measured in response to the OVA peptide stimulus (0-300 nM; **Fig. 3.8**). Whilst maturation of BALB/c bmDCs by LPS and ES-62 induced essentially identical levels of OVA-specific IL-10 production by T_H cells, the ability of bmDC to induce OVA-specific IL-17A production was reduced following maturation with ES-62 relative to that observed with LPS over the entire OVA concentration range. Levels of IFN-γ were also assayed but were below detectable levels (**Fig. 3.8 A** and results not shown), consistent with our earlier studies showing that ES-62-matured bmDC induced less IFN-γ than either immature or LPS-matured bmDC on this background[160]. These data suggested that ES-62-matured bmDCs were less effective than LPS-matured bmDCs at priming T_H17 responses in a BALB/c background but this did not reflect an increased propensity to prime counter-regulatory T_{reg} (IL-10) or T_H1 (IFN-γ) responses. Normalised data from several experiments from BALB/c background derived cells confirmed that ES-62 matured bmDCs, only induce low levels of IL-17A secretion relative to LPS at high Ag loads (**Fig.**

3.8 B). In contrast, the C57BL/6 background ES-62 matured bmDCs, were incapable of inducing secretion of IL-17A from T_H cells in response to OVA challenge (**Fig. 3.8 C**). Indeed, on this genetic background, they resembled immature DC which were also incapable of inducing IL-17A secretion and showed reduced production of IL-22 alongside increased IFN-γ generation relative to that induced by LPS-matured bmDC. As previously shown[160], ES-62-matured bmDC appeared to be even less effective at inducing IFN-γ than immature DC, especially at high Ag concentrations.

3.2.2 The induction of an IL-17A secreting T_H17 phenotype from naïve $CD4^+$ T cell precursors; a reversal of ES-62 hyporesponsiveness by LPS

As ES-62 induced a phenotype of DC that was poor at priming T_H17 responses, the plasticity of this phenotype was investigated and whether the T_H17 priming capabilities of a DC could be restored upon their subsequent exposure to LPS (as a surrogate for infection). As IL-17 constitutes a key component of the host response to infection, it would not be of benefit to the host if such a state of ES-62-induced hyporesponsiveness was irreversible rather than dynamic. Investigation of the plasticity of ES-62-matured bmDC (BALB/c background), in terms of their responses to subsequent exposure to LPS revealed that such cells induce an intermediate level of priming of T_H cell clonal expansion relative to maturation with either ES-62 or LPS alone (**Fig. 3.9 B-D**). The division index revealed that at the higher Ag concentrations (100 and 300 nM OVA peptide) subsequent exposure to LPS could induce ES-62 matured bmDCs to stimulate Ag specific T_H cell division to levels more comparable to that observed with LPS-matured bmDC, particularly in terms of the percentage of cells undergoing division (%-divided; **Table 3.1**).

Moreover, analysis of the ability of such ES-62/LPS-matured bmDC to prime development of a OVA-specific T_H17 phenotype from naïve tg TCR $CD4^+$ DO.11.10.BALB/c T cells, revealed that compared to bmDC matured with LPS alone, those “pre-matured” with ES-62 showed reduced IL-17 and IL-22 cytokine responses (**Fig. 3.10 A**). However the IL-17, but not IL-22, responses were higher than those induced by either ES-62-matured or immature bmDCs showing that exposure to ES-62, whilst dampening subsequent responses, does not leave the host in an immunocompromised state to heterologous infection. Such suppression of cytokine release was reflected at the mRNA transcript level suggesting that pre-exposure to ES-62 was acting to prevent the induction of such pro-inflammatory gene expression (**Fig. 3.10 B**).

To further address the mechanisms by which exposure to ES-62 resulted in IL-17 hyporesponsiveness, the effect of ES-62 on DC-derived cytokines that promote/antagonise T_H17 responses was investigated. Thus bmDCs were analysed for a range of cytokines that are associated with driving different immune phenotypes (**Fig. 3.11**): TNF-α, IL-12p70 and IL-12p40 (associated with T_H1 inflammatory responses) as well as IL-6, IL-23 and IL-27 (important mediators in differentiating, maintaining and antagonising the T_H17 phenotype, respectively). As expected, this revealed that whilst immature or ES-62-matured bmDC produced little of any of these pro-inflammatory cytokines, LPS strongly stimulated their production. Interestingly, therefore, the maturation of bmDCs with ES-62 had a significant impact on the levels of TNF-α, IL-12 p70, IL-12 p40 and IL-23 secreted upon subsequent stimulation with LPS, although ES-62 appeared to have little effect on such LPS-induced IL-6 and IL-27 secretion. These data are consistent with previous studies showing that ES-62 prevents bmDCs from driving T_H1 associated inflammatory responses through its reduction of LPS-stimulated IL-12p70 and TNF-α production, whilst itself inducing small increases in cytokine secretion[301]. Moreover, they suggest that ES-62 selectively modulates only certain molecular mechanisms employed by DCs to stimulate T_H17 cellular differentiation and maintenance.

3.2.3 DC co-stimulatory markers are not a major target of ES-62 mediated immunomodulation

To further explore the mechanisms by which ES-62 may be mediating its effects, flow cytometry was utilised to identify whether the parasite product was also affecting DC-T_H cell interactions by modulating the cell surface expression of DC co-stimulatory molecules. The expression of CD40, CD86 and MHC class II were investigated due to the well established finding that they are upregulated in response to LPS/TLR4 signalling, as well as their importance in mediating DC-mediated priming of $T_H1/17$ phenotypes[444,445]. In this context, CD40 signalling has been reported to be involved in the secretion of IL-6 and IL-12p70 making it a potential target during ES-62's subversion of bmDC maturation. Likewise, the role of CD86 as a counterstructure of CD28, the key costimulatory molecule for T cell priming, makes it a prime target for ES-62 modulation. Expression of the antigen presentation machinery, MHC Class II was also analysed to determine whether ES-62 was modulating the Ag availability/presentation to T cells.

LPS-induced maturation resulted in the time (2, 6 and 18 h)-dependent up-regulation of CD40, while analogous treatment with ES-62 had negligible effects on the

percentage of cells expressing this molecule (**Fig. 3.12 A-B**). It is important to note for this result that the very low numbers of CD40 positive DCs analysed under ES-62 simulation likely skewed the corresponding GMF (Geometric Mean Fluorescence) results observed. Interestingly given the inhibitory effects of ES-62 on T_H17 differentiation, exposure to ES-62 prior to stimulation with endotoxin upregulated CD40 expression relative to that seen with LPS alone, particularly at the later time points of LPS stimulation (6 and 18 h) (**Fig. 3.12 C-D)**. However, similar differential patterns of expression in response to LPS and/or ES-62 were not observed for CD86 and MHC Class II molecules (**Fig. 3.13** & **Fig. 3.14**, respectively). These results confirmed earlier studies that ES-62 induced an "immature" phenotype of DC in respect to CD86 and MHC class II expression that is perhaps consistent with its anti-inflammatory responses.

However, mouse immune responses in the BALB/c background are generally associated with T_H2 responses and hence, as responses to ES-62 in healthy mice are also typically T_H2 in nature (e.g. IgG1 antibody responses) it was considered possible that ES-62 may exhibit more dramatic polarising effects on bmDC from the C57BL/6 background which is usually associated with initiating strong $T_H1/17$ responses[445]. Thus, the effect of ES-62 on costimulatory molecule expression on bmDC derived from C57BL/6 mice was also analysed. Perhaps consistent with the T_H1/T_H17 responses observed in C57BL/6 mice, LPS induced a strong up-regulation of CD40, CD86 and MHC class II expression on the surface of bmDC (**Fig. 3.15**). However, as observed with BALB/c-derived bmDC, ES-62-treated bmDCs maintained an immature phenotype and prior exposure of the bmDCs derived from C57BL/6 mice to ES-62 were able to marginally attenuate the subsequent LPS up-regulation of CD86 and MHC class II upregulation (**Fig. 3.16**). Although this limited effect suggests that the modulation of T_H cell differentiation through DCs by ES-62 most likely reflects its effects on cytokine production.

3.2.4 The role of SPHK1 and -2 in LPS mediated maturation of bmDCs; do they play a part in immunomodulation by ES-62

To investigate how ES-62 suppressed priming of T_H17 cells, SPHK, which has been proposed to play a role in development of such responses, was investigated as a potential target of ES-62 in the attenuation of this pro-inflammatory phenotype. In this respect, it was decided to determine whether LPS-matured bmDC from Sphk1 and -2 deficient mice (C57BL/6 background) showed defects in their ability to prime T_H17 differentiation. LPS-matured bmDC from either $Sphk1^{-/-}$ or $Sphk2^{-/-}$ mice were indeed

found to be deficient in their ability to prime wild type (WT) OVA-specific IL-17 responses of OTII $CD4^+$ T cells. This was reflected by an inability to secrete IL-17A coupled with a slight induction of IFN-γ (albeit very low concentrations) suggesting the potential involvement of a compensating shift in the T_H1/T_H17 counter-regulatory balance (**Fig. 3.17**). By contrast, the division and proliferation indices of LPS matured $Sphk1^{-/-}$, but not $Sphk2^{-/-}$ bmDC, revealed they induced a greater number of OVA-specific clonal naïve OTII T_H cells to respond and proliferate to Ag, relative to WT (**Fig. 3.18**). Moreover, both $Sphk1^{-/-}$ and $Sphk2^{-/-}$ bmDC were able to induce proliferation of a large proportion of OTII T cells in the absence of Ag.

There are conflicting results that exist in the current literature about the exact role that the SPHKs play in the secretion of inflammatory cytokines that regulate the T_H1/T_H17 balance. For example, S1P has been suggested to be anti-inflammatory by reducing IL-12p70 production[446], and suppressing T_H1 responses *in vivo*[447]. However, other studies place S1P and SPHK1 at the centre of coordinating pro-inflammatory responses in rheumatoid arthritis and colitis[448,449]. Thus, as ES-62 has been shown to target SPHK responses, the effects on Sphk1 or -2 deficiency on LPS-mediated maturation of bmDCs in terms of costimulatory molecule expression and the secretion of a panel of cytokines associated with the development of T_H1 and T_H17 cells were determined.

These studies showed, consistent with the earlier reports that S1P suppressed IL-12 production, that bmDCs deficient in either of SPHK1 and -2 in bmDC exhibit significantly increased LPS-stimulated IL-12p40, IL-12p70 and IL-27, but not IL-23 responses, indicating that these enzymes are important in the regulation of IL-12 family subunits in response to LPS-TLR4 signalling (**Fig. 3.19**). This is also reflected by the tendency of resting $Sphk^{-/-}$ cells (in medium) to produce higher levels of these cytokines than WT cells (**Fig. 3.19 B**), and in this context, $Sphk2^{-/-}$ bmDCs also show higher levels of TNF-α secretion. Although the basal secretion of TGF-β1 by bmDCs appeared to be relatively high, SPHK1 deficient cells were significantly ($P<0.05$) impaired in their secretion of this cytokine, both under resting and LPS-stimulated conditions. Although these effects do not reflect the actions of ES-62, they are consistent with the promotion of T_H1 differentiation in $Sphk^{-/-}$ cells.

Analysis of surface expression of DC co-stimulatory molecules revealed that these cytokine responses were accompanied by reduction in basal and LPS-stimulated expression of CD80 and CD86 but not MHC Class II molecules, confirming that the effects on T_H17

polarisation were not simply due to lack of Ag presentation (**Fig. 3.20**). Moreover, although reduced presumably because of the effects on basal levels, LPS-mediated upregulation of costimulatory molecule expression was still observed indicating that loss of either SPHK did not simply maintain the cells in an immature state.

Collectively, therefore the effects of ES-62 were not mimicked by either Sphk1 or Sphk2 deficiency: rather, the cells from the Sphk null mice showed opposite effects on IL-12 from that observed with ES-62. Although, these data suggest therefore that ES-62 is not mediating its effects by selective inhibition of either Sphk1 or -2, at this stage this possibility cannot be completely ruled out due potential redundancy/adaptation effects occurring in the Sphk $^{-/-}$ mice.

3.2.5 The role of the PAFR in LPS induced cytokine secretion by bmDC

The lipid mediator PAF plays an important role in leukocyte mediated phagocytosis of particles, including bacteria, and consequent production of pro-inflammatory cytokines, such as TNF-α and IL-8[369]. The role of PAF and its receptor in the stimulation of cytokine secretion in bmDC (BALB/c background) was therefore investigated using the synthetic analogue of PAF, C-PAF in a number of *in vitro* assays. This PAF analogue cannot be metabolised by the cell and thus provides a potent and persistent signal through the receptor. The competitive and non-competitive inhibitors of the PAFR, CV3988 and WEB2086, respectively, the latter of which is reported to act as an inverse agonist that attenuates receptor signal propagation throughout the cell, were used to probe the role of PAFR signalling in basal and LPS-stimulated DC responses.

To identify whether signalling through the PAFR by PAF played a role in DC maturation and pro-inflammatory responses, bmDC were probed for PAFR expression (**Fig. 3.21**) and cytokine production following stimulation with C-PAF (**Fig. 3.22**). Although the antibody appeared to recognise some additional protein bands, it was the p39 band that represented expression of the PAFR by DCs (**Fig. 3.21**), and as such it appeared as though the expression of this receptor could be downregulated by ES-62. Moreover, in relation to its role in immune responses, increasing concentrations of C-PAF stimulated the secretion of cytokines, of both a pro-inflammatory and modulatory nature relative to unstimulated cells (**Fig. 3.22 A-F**), except for IL-12 p40 and p70 with the noticeable up-

regulation of IL-23 production perhaps indicating a role for the PAFR in fine-tuning T_H17 responses.

As PAF had been proposed to mediate some of its pro-inflammatory signals via nuclear signalling leading to induction of gene expression[364,367], and enhance LPS/TLR4 signalling, the expression of Cbl-b, c-Cbl and TRAF6 mRNA in response to C-PAF was analysed by Taqman real time PCR. This was to address whether PAFR signalling facilitated upregulation of these key mediators of TLR4 responses and the secretion of pro-inflammatory cytokines including IL-12 p70, TNF-α and IL-10[146]. Stimulating bmDCs with C-PAF modulated expression of all these transcripts (**Fig. 3.22 G-I**), where the steady increase of TRAF6 mRNA expression from 0.18 % ± 0.02 % (relative to GAPDH) in resting cells to 0.26 % ± 0.02 % was contrasted by the transient downregulation of c-Cbl and Cbl-b transcripts. Collectively, these results suggested that PAFR signalling modulates key adaptors of LPS responses.

To explore the role of the PAFR in LPS signalling and maturation of bmDC, the effect of the non-competitive inhibitor WEB2086 was tested on LPS-mediated pro-inflammatory responses. The pre-incubation of bmDC with WEB2086 appeared to specifically reduce the secretion of IL-12 family (IL-12p40, p70 and IL-23) cytokines as well as IL-10 but not TNF-α in response to LPS in a concentration dependent manner (**Fig. 3.23, A-E**). Incidentally, these studies revealed that the cytokines analysed responded in a differential concentration dependent manner to LPS: for example, TNFα and IL-12p70 responses reached a plateau between 1-10 ng/ml of endotoxin whilst secretion of IL-12p40, IL-23 and IL-10 were maximal at 1 ng/ml. Moreover, the inhibitory effects of WEB2086 were generally overcome at the highest concentration of LPS (1000 ng/ml) perhaps suggesting that at high concentrations of endotoxin, LPS/TLR4 signalling may circumvent its requirement for PAFR signalling or alternatively, stimulate PAFR up-regulation (expression or signalling) by the cell. Interestingly, in the light of these results the competitive PAFR inhibitor, CV3988 (at 30 μM) was only able to attenuate the secretion of TNF-α at LPS concentrations of 10 ng/ml and above (**Fig. 3.23, F-H**) and had little effect on the inhibition of IL-12p70, although it was able to attenuate the secretion of IL-10 in response to LPS in a similar fashion to WEB2086.

3.2.6 The involvement of the PAFR in shaping the LPS induced maturation of bmDC and its ability to stimulate T cell responses

A potential role for the PAFR in exacerbating LPS-stimulated cytokine production was suggested by the above C-PAF and WEB2086 treatment results. However, the ability of C-PAF to induce pro-inflammatory responses in the absence of endotoxin suggests that this receptor may also play LPS-independent roles in DC maturation. To address whether PAF/PAFR signalling would modulate LPS-induced cytokine production, bmDC were matured with LPS in the presence or absence of C-PAF or alternatively, unstimulated and LPS-treated samples were pretreated with WEB2086 and the release of pro-inflammatory cytokines analysed (**Fig. 3.24**).

Firstly it was observed that pretreating bmDCs with WEB2086 to inhibit the PAFR slightly attenuated the ability of the LPS-stimulated DCs to secrete IL-6 but not TNF-α, IL-23, IL-12p70 or IL-10, relative to those exposed to endotoxin alone (**Fig. 3.24**). However in the case of IL-12 p40, an increase was observed but was not found to be significant. On the other hand although C-PAF generally subtlety enhanced (albeit not significantly) secretion of most of these cytokines in response to LPS (1 μg/ml) stimulation, rather unexpectedly C-PAF co-stimulation with LPS inhibited the secretion of IL-6, although again this was not found to be significant. Collectively these results with PAFR agonists and antagonists had only marginal effects on LPS responses suggesting that, if any, the PAFRs role may be to 'fine-tune' cytokine secretion in response to low (limiting) levels of PAMPs (e.g. LPS) and/or pathogen Ag. Thus, the ability of C-PAF or WEB2086 to modulate the ability of LPS-matured bmDC to prime Ag-specific T_H cell responses and polarise them to a T_H1/T_H17 pro-inflammatory phenotype was also investigated.

Consistent with a role for PAFR in LPS-mediated pro-inflammatory responses, treatment with WEB2086 prior to LPS-induced maturation significantly inhibited the ability of bmDC to prime OVA-specific IFN-γ, IL-21 and IL-22 production (**Fig. 3.25**). However, in the case of IFN-γ and IL-21 this only occurred at lower concentrations of OVA peptide perhaps reflecting the well-established finding that high concentrations of Ag drive T_H1 responses[153]. By contrast, IL-17 production was unaffected and IL-4 responses were stimulated. Collectively, therefore, these results indicate that blocking PAFR signalling in bmDC suppresses priming and polarisation of T_H1 but not T_H17 responses,

results consistent with our finding that WEB2086 inhibits IL-12p70 secretion from bmDC cultures (**Fig. 3.23** & **3.24**). Moreover, this suppression of T_H1 responses does not appear to be a result of the induction of T_{regs} as IL-10 is also reduced and hence rather resembles a change in the T_H1/T_H2 balance. Intriguingly, the lack of effect on IL-17 secretion in response to LPS, suggests only certain T_H17-associated cytokines are affected by antagonising the PAFR in DCs, nevertheless the IL-17 results are somewhat inconsistent with our observed inhibition of IL-23 responses as this is important in the development of such a phenotype (**Fig. 3.23 D**). The finding that inhibiting PAFR signalling attenuated induction of IL-22 secretion are consistent with observations from our laboratory group in several animal models of disease, where we are rarely able to detect IL-17 and IL-22 production by the same cell types, thus PAF-R signalling could be important for the development of a T_H22, but not T_H17 phenotype. Interestingly, the concurrent stimulation with C-PAF and LPS stimulated an increase in IL-17 secretion at 100 nM OVA peptide relative to that observed with LPS-matured bmDCs, coupled with a similar although not as marked affect on IL-22 secretion. Counter intuitively, it also resulted in the Ag-independent release of IL-4 but this was downregulated by increasing concentrations of OVA. Given the inhibitory effects of WEB2086, maturation of bmDCs with C-PAF and LPS had surprisingly little effect on bmDC stimulated T_H cell differentiation towards a T_H1/T_H22 phenotype suggesting that the effects of WEB2086 were perhaps targeting PAF-R signalling resulting from secondary production of PAF acting on intracellular PAFR, whilst C-PAF was stimulating surface PAFR.

Analysis of the effects of blocking DC-PAFR signalling on priming of naïve T cells in terms of clonal expansion (proliferation) revealed suppression of the T_H1-like phenotype did not simply reflect inhibition of priming. If anything, WEB2086-treated bmDCs stimulated the T cells to enter further rounds of cell division, with this effect being particularly pronounced at the lowest concentration of OVA peptide (10 nM) and indeed in the absence of Ag (**Fig. 3.26 B-D**). In this context, the proliferation index indicated that inhibition of DC-PAFR signalling with WEB2086 enhanced the average number of divisions as well as the % of responding Ag specific T_H cells in the population upon LPS maturation (**Fig. 3.26 E**). In contrast, concurrent maturation of bmDCs with both LPS and C-PAF reduced the average number of divisions and the % of responding cells relative to those matured with endotoxin alone, albeit only slightly. These results therefore suggest that PAFR signalling may allow a DC to fine-tune the priming and clonal expansion of naïve T_H cells depending on the inflammatory environment.

Although only marginal effects were observed with WEB2086 treatment of immature DC on DC-T_H cytokine secretion (**Fig. 3.27**), there were some limited effects of the inhibitor on T_H cell proliferation induced by such DCs (**Fig. 3.28 A-C**). Thus, immature DC treated with the antagonist WEB2086, were able to induce a larger number of Ag specific T_H cells to respond and begin proliferating at the higher concentrations of OVA peptide relative to the immature DCs, as observed by the higher division index and %-divided values (**Fig. 3.28 D**). Perhaps surprisingly as shown by the proliferation index, C-PAF treated bmDCs were also able to stimulate an increase in the levels of proliferation above that of immature DCs (control) both in the absence and presence of OVA peptide (300 nM) although in contrast to WEB2086, C-PAF generally decreased the levels of cytokines secreted by T_H cells relative to below those observed with control immature DCs (**Fig. 3.27**).

3.2.7 ES-62 maturation of bmDC and its effect on the ability of C-PAF and WEB2086 to modulate responses to LPS

The data outlined above suggested that blocking of PAF-R signalling partially suppressed the pro-inflammatory responses of bmDC and their subsequent ability to modulate the priming of naïve T_H cells into particular subtypes, most notably T_H1. As this latter effect is similar to that previously reported for ES-62, and preliminary data suggests that ES-62 may bind to the PAFR in neuronal cells (E.T. Tuomamen & W. Harnett, unpublished data), it was interesting to note that Western blotting analysis (**Fig. 3.21**) revealed ES-62 to down-regulate the expression of the PAFR over a 6 h period (albeit that the antibody was not stringently specified for the PAFR).

In light of this, the effect of ES-62 on the responses to LPS-maturation of DCs was studied in conjunction with the stimulation or inhibition of the PAFR. The responses of ES-62-matured bmDC to subsequent exposure to LPS showed some interesting differences when the cells were concomitantly treated with C-PAF (**Fig. 3.29**). Thus, the concurrent treatment of C-PAF with LPS significantly rescued ES-62 mediated down-regulation of IL-12 p40 and TNF-α, while this was also the case for IL-23 it was not found to be significant. These trends were replicated by the pretreatment of bmDCs with WEB2086, but in the cases of IL-12 p40 and TNF-α this rescue was not as marked as that with C-PAF but it did have a greater affect on stabilising LPS induced secretion of IL-23. In contrast, both stimulation (C-PAF) and inhibition (WEB2086 and CV3988) of the PAFR in conjunction with ES-62 maturation resulted in a dramatic reduction in IL-6 by the bmDCs

in response to LPS, which was normally rather refractory to modulation by ES-62. Overall however, although the PAFR antagonists mimicked the actions of ES-62 in some cases (e.g. TNF-α) they exhibited opposite effects in others (e.g. IL-23). Thus, as exogenous PAF could generally overcome the effects of ES-62, these findings might suggest that the parasite product could be limiting the release of PAF consequent to LPS-signalling rather than targeting PAFR signalling per se. Moreover, as we found ES-62 to downregulate total PAFR expression, these results may be indicative of ES-62 downregulating intracellular rather than surface receptor expression. Perhaps consistent with this proposal, analysis of the mRNA expression of c-Cbl and TRAF6 revealed that ES-62, like WEB2086, had no effect on the LPS-mediated upregulation of c-Cbl expression but inhibited the LPS-mediated upregulation of TRAF 6 expression, which was not rescued by exogeneous PAF (**Fig. 3.30**).

3.2.8 Targeting the source of NF-κB activation; the modulation of expression and localisation of TLR4 by ES-62

ES-62-mediated inhibition of LPS-induced cytokine production, but not upregulation of costimulatory molecule expression, suggested that it is targeting the specific dynamics and components of the TLR4-NF-κB axis to enforce its actions. In this context, full TLR4-driven NF-κB activation has been shown to be dependent on both early MyD88-TIRAP lipid raft signalling and a late clathrin-mediated endosomal TRAM-TRIF-coupled phase. This presented the question, does ES-62 modulate the surface and/or total expression of TLR4 and/or its active LPS recognising complex, TLR4/MD2 by subverting their intracellular trafficking and degradation?

In answer to this ES-62 and LPS were found to exhibit differential effects on TLR4 expression and trafficking. In bmDCs derived from C57BL/6 mice, FACs analysis using an antibody that recognised all TLR4 expressed, revealed that LPS but not ES-62 induced a significant increase in both total (extra- and intra-cellular sources of the receptor) and surface expression of this receptor over 18 h (**Fig. 3.31**). However, although not significant, ES-62 appeared to transiently upregulate TLR4 expression at early time points and this was also detected in bmDCs derived from BALB/c mice by Western Blot analysis, which was also reflected in the kinetics of TLR4 mRNA transcript expression (**Fig. 3.32**). Interestingly, LPS induced the downregulation of TLR4 mRNA levels, perhaps suggesting that the observed increase in protein expression in response to this TLR4 ligand was as a result of inhibition of TLR4 degradation. Therefore, in the context of ES-62 maturation

inhibiting the secretion of certain cytokines upon challenge with LPS, it also prevented the LPS-mediated upregulation of TLR4 (**Fig. 3.33**).

By contrast, LPS but not ES-62, induced the strong downregulation of the TLR4/MD2 complex from the surface of bmDCs (**Fig. 3.34)** and both TLR4 ligands slightly reduced the levels of this complex throughout the cell (total). This evidence of LPS–induced internalisation of the TLR4/MD2 complex is consistent with its known endosomal trafficking, which is required for LPS-signalling and promotion of Ag processing and presentation. ES-62 maturation of bmDCs was able to significantly prevent this LPS-mediated removal of the TLR4/MD2 complex from the cell surface, and its subsequent LPS-driven reduction that accompanies its internalisation (**Fig. 3.35**). Interestingly, this LPS-induced internalisation of TLR4/MD2 complexes was more pronounced in bmDC from BALB/c mice but in this case, ES-62 maturation was unable to prevent this process (**Fig. 3.36, B-D**). Thus, in conclusion, the ability of ES-62 to dampen LPS functional responses does not reflect the simple downregulation of TLR4 or its active complex, TLR4/MD2 from the surface of bmDCs.

3.3 Discussion

3.3.1 ES-62 maturation of DCs attenuates their ability to induce T_H17 responses upon subsequent TLR4 recognition of LPS

Collectively, and consistent with previous data[291,301,323], the results described above suggest that ES-62 is altering the signalling patterns, maturation and activation of bmDCs to actively modulate the host immune response. Here, ES-62 matured DCs were observed to fail to induce the production of IL-17A and IL-22 from T_H cells, introducing the concept that ES-62 does not induce a strong T_H17 response in a fashion similar to LPS (**Fig. 3.8**). However, rather surprisingly given the tendency of ES-62 to drive T_H2 responses, when stimulated with low concentrations of Ag, the T_H cells primed by ES-62-matured DC, appeared to secrete relatively high concentrations of IFN-γ, suggesting that the particular microenvironment may regulate the induction of different T_H phenotypes depending on the levels of Ag presented to naïve T_H cells. For example, it has been demonstrated that IFN-γ inhibits the development of T_H17 cells both *in vitro* and *in vivo*, through down-regulation of the IL-23 receptor (IL-23R)[450]. Thus, the low Ag induced secretion of IFN-γ by ES-62, coupled with its reduction of IL-23 secretion from the DCs in response to endotoxin (**Fig. 3.11**), may reflect ES-62 induced changes in the environmental cytokine milieu of the cells, which may result in the prevention of naïve T_H cells that receive only a relative low Ag stimulus from developing into a T_H17/pro-inflammatory phenotype. In this context, ES-62 also impaired the ability of DCs to stimulate Ag specific T_H cell proliferation, lowering both the number of responding cells and the divisions undergone (**Fig. 3.5 - 3.7**), again reflecting the ability of ES-62 to dampen but not ablate the host's ability to initiate adaptive immune responses.

Interestingly, this dampening effect was demonstrated by the marked reduction in the ability of ES-62 matured DCs to induce the development of a T_H17 phenotype upon stimulation with LPS. This plasticity of immunomodulation is essential to prevent immunocompromising the host with respect to subsequent infection and was demonstrated by these effects being (partially) overcome at the highest concentration of Ag (300nM, OVA peptide), through the secretion of significant amounts of IL-17A but not IL-22 (**Fig. 3.10**). This was accompanied by an increased proliferation of T_H cells, comparable to that observed with endotoxin alone (**Fig. 3.9**). In this respect, the ES-62-mediated attenuation of the LPS induced secretion of IL-23 from DCs (**Fig. 3.11**) may limit the terminal differentiation of the T_H17 cells, as IL-23 is required for cells differentiating down this

route to lose the ability to secrete IL-10 and upregulate IL-22 expression[123]. These results indicate that the modulation of DCs by ES-62 is not, therefore, restricted to driving maturation of DCs that solely induce T_H2 responses[160], but rather allows some flexibility in their biology such that it can respond to other inflammatory stimuli whilst preventing hyperinflamation. Consistent with this, it has been reported that ES-62 can prevent the development of T_H1/T_H17-mediated CIA[310,324], and recent results suggest ES-62 limits the inflammatory potential of T_H17 cells by suppressing their development as well as their capacity to secrete the inflammatory cytokine IL-17A[352].

In the present study, ES-62 alters the cytokine profile secreted by DCs in response to LPS, reducing the levels of IL-12p70, IL-23 and the IL-12/23p40 subunit, shared by both these cytokines, with little effect on IL-6 (**Fig. 3.11**). These data indicate a direct intervention of the cytokine milieu by ES-62 to potentially limit the development of both T_H1 and T_H17 phenotypes of inflammation. The lack of effect on IL-6 secretion in response to LPS, a cytokine that is crucial for the development of a T_H17 phenotype could suggest that ES-62 targets the terminal differentiation of the pro-inflammatory and potentially pathogenic T_H17 cell, but not the initiation of cellular differentiation down this route[444]. Interestingly, therefore, ES-62 pre-treatment did not affect IL-27 production to suppress effector T-cell proliferation and cytokine production[253,254], in relation to the T_H17 phenotype[163]. Indeed, as this cytokine is able to initiate activation of STAT1 and T-bet, the signature T_H1 transcription factors[242], this lack of effect on IL-27 could account for the low level and potentially counter-regulatory T_H1 response that was induced instead. In this context, the reduction of IL-12p70 secretion may act to counterbalance the subsequent IL-27-mediated induction of the expression of the IL-12Rβ2, the pre-requisite for mounting a IL-12 dependent T_H1 driven immune response[252] as it would attenuate this response, limiting the polarisation of a T_H1 phenotype.

Typically exposure to LPS up-regulates the gene and subsequent protein production of TNF-α[170], but ES-62 limits its production as documented previously at both the cytokine and mRNA level in macropahges and DCs[291,363]. TNF-α is known to induce the activation of B and T cells[170], influencing the function of APCs by augmenting their Ag presenting capabilities and up-regulating co-stimulatory molecule expression on the surface of immature but not mature DCs and it is also involved in the activation of NF-κB[155]. Thus ES-62 may maintain a limited production to allow protective immune activation whilst preventing pathological inflammation. By contrast, ES-62 had little effect on the expression of CD40, CD86 and MHC Class II or their upregulation by LPS. The

fact that treatment with ES-62 does not affect the ability of bmDCs to interact with T_H cells (and vice versa), by modulating such costimulatory receptor expression, places a major emphasis on the cytokine milieu induced by its maturation and the subsequent impact on the modulation of T_H responses.

3.3.1.1 Both SPHK1 and -2 potentially have a role in modulation of LPS maturation of bmDCs.

These results posed the question; how does maturation with ES-62 affect the mechanisms utilised by DCs to impact upon the subsequent priming and polarisation of the T_H cells? Recently, the PLD/SPHK axis has emerged as an important target for ES-62[321], as it inhibits FcεRI-mediated degranulation and proinflammatory cytokine secretion in mast cells by inhibiting PLA/SPHK activity in a TLR4-dependent manner. Based on these results the availability of $Sphk^{-/-}$ (KO) mice provided the perfect opportunity to investigate the stated question and whether their absence mimicked the effects of ES-62. However, although the data from SPHK1 and -2 KO DCs suggested similarities they indicated that the effects of this immunomodulator on DC function cannot be solely accounted for by the inhibition of the SPHKs.

For example, LPS-matured DCs from either SPHK KOs, were unable to induce the production of IL-17A from naïve T_H cells upon stimulation with high concentrations of Ag (300nM OVA peptide), in a similar fashion to ES-62 matured bmDCs. These data were also consistent with the production of S1P being implicated in the promotion of T_H17, via the modulation of T_H polarising functions of DCs when exposed to bacterial products[429]. In this context, as with ES-62, SPHK1 and -2 KO cells show a slight induction of IFN-γ under LPS stimulation, which in relation to the down-regulation of IL-17A suggests the SPHKs are important in counter-regulating T_H1-T_H17 responses (**Fig. 3.17**) and perhaps reflects the significant increase in IL-27 production, dually promoting IFN-γ-T_H1 responses whilst suppressing that of T_H17[163]. By contrast, and unlike the situation observed with ES-62, the secretion of IL-23 remains unaffected and a significant increase in both IL-12p70 and the IL-12/23 p40 subunit are observed in the presence and absence of LPS stimulation (**Fig. 3.19**). Directly related to this, splenocytes from SPHK1 deficient mice are also found to exhibit enhanced secretion of IL-12p70 in response to endotoxin[427]. Despite the IL-6 secretion from these cells was not determined, the observed reduction of TGF-β1 may relate to its involvement in the modulation of T_H responses, as TGF-β in conjunction with IL-6, promotes T_H17 responses[123,124]. Thus the reduction in TGF-β1 may reflect a role

of these kinases in the modulation of the T_H1/T_H17 balance in response to LPS-TLR4 signalling.

The above cytokine responses were accompanied by a decrease in the expression of CD80 and CD86 but not MHC class II. Findings consistent with previous reports that pharmacological inhibition or introduction of a DN SPHK1 can result in the reduction of these costimulatory molecules on DCs[423]. Interestingly, therefore, in this study the absence of SPHK1 and -2 was accompanied by a significant increase in LPS-stimulated upregulation of these costimulatory molecules relative to immature cells, suggesting that the absence of the SPHKs does not retain the bmDCs in an immature state, and may reflect aredundancy of functions between SPHK1 and -2.

3.3.1.2 The role of the PAFR in mediating maturation of DCs

Stimulation of the PAFR, coupled with its actions as a secondary transducer of LPS responses[322], results in the exacerbated release of several pro-inflammatory cytokines (TNF-α, IL-6, IL-1) and other mediators including PAF itself[368,369] occurring through activation of MAPK, PI3K, PLA_2, PKC and Src pathways in various cells of the immune system[322,364]. Thus it was not surprising to observe the secretion of several proinflammatory and modulatory cytokines upon the stimulation of the PAFR receptor with the agonist C-PAF on DCs (**Fig. 3.22 A-F**), particularly as this compound has previously been shown to induce the expression of iNOS and COX-2 as well as ERK1/2 MAPK phosphorylation and activation of NF-κB[57]. However, whilst C-PAF induced substantial increases in IL-23 and TNF-α, surprisingly the levels of IL-6 and IL-12/23p40 remained unchanged, suggesting this agonist-receptor interaction is more selective in determining cytokine environments than LPS.

To investigate the effect of C-PAF on the expression of several mediators of LPS-TLR4 responses, the mRNA transcript expression of several E3 ubiquitin ligases, TRAF6, c-Cbl and Cbl-B were assessed (**Fig. 3.22 G-I**). TRAF6 acts as a key scaffolding protein involved in the propagation of TLR4 signals and is required for both MyD88 dependent and independent activation of NF-κB [23,24]. Thus it was interesting to observe that stimulation of the PAFR with increasing concentrations of C-PAF resulted in the gradual increase in the mRNA transcript expression of this key intracellular mediator. Thus, PAFR signalling may promote the ability of DCs to respond to prolonged stimulation with LPS by the upregulation of TRAF6. Where DCs from TRAF6 deficient mice demonstrate

maturation defects in response to LPS, which result in a similar reduction of IL-12 secretion (and notably also IL-6)[26,27]. This reasoning is reinforced by the observation that inhibition of signalling through the PAFR by WEB2086 (between 30-100 μM) attenuates the secretion of IL-12p70, IL-23 and their shared subunit IL-12/23p40, which are all associated with positive TLR4 signalling requiring functional TRAF6 (**Fig. 3.23**). Collectively, these results suggest that the regulation of TRAF6 by PAFR signalling may not have dramatic effects on the secretion of certain cytokines but may play a role in the ability of DCs to regulate certain immune phenotypes. This could perhaps be achieved through the modulation of additional E3 ligases such as c-Cbl, which has been implicated in the regulation of DC activation and maturation[451,452], via the stabilisation of the p50 subunit of NF-κB and its precursor p105[453]. Consistent with this, deficiency of c-Cbl has been shown to lead to higher levels of interleukin (IL)-12 p70 and IL-6 secretion[454], whilst the related E3-ligase, Cbl-B, is directly involved in regulating acute lung inflammatory responses to LPS, by controlling the association between TLR4 and MyD88[455]. Thus, early in the inflammatory response (mimicked by low levels of C-PAF), PAFR signalling may prime the DC to respond to inflammatory stimuli, such as LPS and induce subsequent inflammatory responses by downregulating the ability of these cells to express both c-Cbl and Cbl-B. However at high concentrations, which would reflect induction of a strong inflammatory stimulus, these could be upregulated to limit the inflammatory responses mediated by DCs and stabilise the latent NF-κB complex coupled with disrupting TLR4-MyD88 interactions. Such a mechanism could explain the observed upregulation of c-Cbl and reduction in IL-6 secretion upon the simultaneous stimulation of the PAFR on DCs with LPS and C-PAF (**Fig. 3.24**; **Fig. 3.30**), as c-Cbl expression has recently been directly linked to the regulation of IL-6 secretion[454].

By analysing the concentration dependent effect of LPS signalling, it was clear that although IL-12p70 and IL-23 share the common IL-12/23 p40 subunit (maximal secretion at 1 ng/ml endotoxin), the generation of the IL-12p70-specific p35 subunit is a rate limiting step in the secretion of IL-12p70 (maximal secretion at 10 ng/ml endotoxin). Based on these observations, our preliminary data suggests that inhibition of PAFR signalling (by WEB2086 and CV3988) attenuated the secretion of a range of cytokines analysed (**Fig. 3.23**), although significant levels of inhibition were not achieved. Therefore the secondary role PAF plays in LPS responses may be dictated by the availability and expression of the PAFR, where its expression is positively upregulated by LPS stimulated activation of NF-κB and SP-1[382,383]. In this context, it may also reflect, the role of intracellular PAF-PAFR

interactions, coupled with the potential of LPS to directly bind and influence PAFR signalling, enhancing the effects of PAF and therefore immune cells to deal with invading pathogens[295,364,369-372].

Perhaps consistent with the decrease of IL-6 and TNF-α secretion (**Fig. 3.24**), pretreatment with WEB2086 limited the subsequent ability of DC to stimulate IL-22, IL-10, IFN-γ and IL-21 production, although in most cases (except that of IL-22) these effects could be overcome by high concentrations of $OVA_{323-339}$ peptide loaded onto the APCs (**Fig. 3.25**).These results suggest that positive signalling through the PAFR on DCs by either autocrinely produced PAF or by the direct interaction of LPS is important for the induction of IL-6 secretion and the development of a T_H17 or related phenotype[444]. The selective reduction of IL-22, relative to IL-17, may reflect involvement of PAFR signalling in modulating IL-23 secretion, as despite WEB2086 treatment LPS stimulation induces the production of relatively high levels of IL-17[444]. In this context, the PAFR may impact on the development of a T_H22 population by DCs as within this lab group IL-17 and IL-22 co-production in models of inflammation is rarely observed (M. Pineda & M. M. Harnett, unpublished data). Recently, it has become clear that TNF-α and IL-6 are both important in the differentiation of a T_H22 phenotype[456]. Thus whilst a dramatic reduction in IL-6 secretion is observed upon inhibition of PAFR signalling in DCs, there are little or no effects on TNF-α, perhaps explaining why these DCs stimulate a reduction but not ablation of IL-22 secretion (**Fig. 3.24 - 3.28**), which can drive diseases such as psoriasis[412,413], or protect against several inflammatory disorders[414,415] of a T_H17 phenotype.

The inhibition of PAFR signalling resulted in an increase in the ability of these cells to stimulate T_H cell proliferation in response to immature or LPS-matured DCs, enhancing both the proliferation and division index (including the percentage of dividing cells) above that of endotoxin treated DCs (**Fig. 3.26** & **3.28**). This was consistent with preliminary data showing that DC from PAFR KO mice could induce Ag-independent proliferation of T_H cells (R. Eason & M. M. Harnett, unpublished data). In this respect, it may also reflect the potential of WEB2086 to act as an inverse agonist, as the compound has a higher affinity for the inactive rather than active state of the PAFR, resulting in the reduction of basal activation of effector mechanisms and subsequent receptor internalisation and transport within the cell[400].

3.3.1.3 The potential role of PAFR in mediating the immunomodulatory effects of ES-62

Several of these inhibitory effects on the PAFR demonstrated similarities to observations made when DCs were treated with ES-62; for example inhibition of PAFR signalling resulted in a reduction of IL-23 (**Fig. 3.23**) and TNF-α (**Fig. 3.29**) from the DCs although, unlike the situation with ES-62, inhibition of the PAFR could also result in a reduction in the secretion of IL-6 (**Fig. 3.24**). Similarly, although the reduction in T_H-IL-22 secretion was reminiscent of that observed with ES-62-treated DC, this was not accompanied by a reduction in IL-17 production (**Fig. 3.25**). Interestingly, therefore, utilising C-PAF with LPS reverses ES-62 induced reduction of IL-12/23p40, TNF-α and IL-23 secretion (**Fig. 3.29**). This suggests that engagement and subsequent internalisation of the PAFR by C-PAF attenuates the ES-62 mediated modulation of the secretion of several cytokines in response to LPS. Thus ES-62 may potentially hijack this receptor to engage DCs via its PC moiety and utilise it in its internalisation and subsequent cytokine modulation of the cells in a similar fashion to PAF binding. This results in receptor internalisation and trafficking via the endosomes, where recruitment of cell trafficking and intracellular signalling proteins can occur even to the nuclear envelope[384,385]. Thus by removing or engaging the signalling capacity of this receptor, which in turn affects its internalisation and localisation within the cells, cytokine secretion can be disrupted.

3.3.2 ES-62 and TLR4

The internalisation and immunomodulatory effects of ES-62 on APCs[301,326] appear to be dependent on the presence of TLR4. Thus, the dynamics of pro-inflammatory TLR4 signalling, which can broadly be divided into two separate stages involving an early MyD88-dependent phase, triggered by receptor-mediated endocytosis in clathrin coated pits followed by a later endosomal TRIF mediated phase[327] may provide a potential mechanism for inducing TLR4 hypo-responsiveness and the suppression of TLR4 associated autoantigen presentation[48,327]. In DCs these phases are followed by the degradation of TLR4 along with any associated microbial proteins in late endocytic vesicles[278]. This results in the peptide loading of MHC class II molecules for the presentation of Ag and activation of $CD4^+$ T_H cells[328,329]. Hence, the LPS induced activation and trafficking of TLR4 through the clathrin-dependent endosomal route provides a mechanism for linking innate and adaptive immune responses. Identifying a potential route for ES-62-mediated uncoupling of TLR4 associated NF-κB activation.

One task of this study was to question whether ES-62 not only requires TLR4 for its internalisation and immunomodulatory effects but whether it directly modulates the expression and localisation of TLR4 and its associated complex partners. Interestingly, there appeared to be a disparity between the dynamics and expression of the total cellular pool of TLR4 relative to that of the TLR4/MD2 complex in response to LPS. Thus, LPS stimulated the upregulation of the surface expression of the total TLR4 pool while internalising and downregulating the "active" TLR4/MD2 complex (**Fig. 3.31** & **Fig. 3.34)**. The observed up-regulation of TLR4 on the surface of the DCs may reflect utilisation of this pool for the recycling of TLR4/MD2 complexes. However, as the TLR4/MD2 complex is not replenished on the cell surface within the time frame examined, a negative feedback mechanism may uncouple the ability of TLR4 to associate with MD2 and prevent subsequent LPS signalling and activation of NF-κB, whilst maintaining TLR4 on the cell surface to mediate other potential cellular functions. Interestingly, the expression of TLR4 mRNA shows a decrease in response to LPS stimulation suggesting that the observed increase in the total cellular pool of TLR4 likely reflects a decrease in its degradation but not that of the TLR4/MD2 complex (**Fig. 3.33**). This may also suggest that the increase in TLR4 may result from the recruitment of TLR4 from the pre-existing pool of TLR4/MD2 or the specific degradation of MD2.

In relarion to these observations, ES-62 does not induce internalisation and degradation of TLR4-TLR4/MD2 expression but acts to antagonise the effects of LPS (**Fig. 3.34** & **Fig. 3.36**). Collectively, these antagonistic effects by ES-62 on total and MD2-associated pools of TLR4 by ES-62 may provide a mechanism for inducing TLR4 hyporesponsiveness and the suppression of TLR4-associated (auto)antigen presentation[328,329]. Evidence for this type of immunomodulation exists from other helminth derived immunomodulators. For example, SEA utilises TLR4 (and severlal other receptors) to traffic into a LAMP2 negative compartment in a clathrin-independent manner[443]. Thus, the differential compartmentalisation of TLR4 may be a critical component of the subversion of TLR4 signalling by ES-62 and other helminth products. However, despite earlier studies indicating that the presence of TLR4 is essential for the internalisation of ES-62 by APCs[301,326], neither TLR4 or TLR4/MD2 complexes showed any noticeable down-regulation in response to ES-62, perhaps suggesting that **1**) the internalisation of ES-62 is not accompanied by TLR4 which is being utilised simply as an anchor, or **2**) the internalisation of ES-62 reflects its dissociation from TLR4 in early endosomes and the recycling of TLR4 back to the cell surface and/or perhaps replenishment from TLR4/MD2 localised at the golgi[434,435]. In any case, these data

indicated that in DC, ES-62 was not initiating the internalisation of the receptor complex and the subsequent activation of the TRIF/TRAM signalling cascade perhaps going some way to explaining ES-62's targeting of the TLR4-NF-κB axis. These findings are summarised in **Figure 3.37**.

Figure 3.1 | **Chemical structure of PAF, C-PAF, WEB2086 and CV3988**

(**A**) The structure of phosphorylcholine ($C_5H_{15}NO_4P^+$). (**B**) The natural ligand of the PAFR, PAF (1-O-alkyl-2-acetyl-*sn*-glycero-3-phoshocholine; $C_{26}H_{54}NO_7P$) is a potent phospholipid activator and mediator of many leukocyte functions including platelet aggregation. (**C**) The chemical structure of C-PAF ($C_{26}H_{55}N_2O_7P$), an analogue of PAF with stereo-specific binding to both high and low affinity PAFR, which the cell is unable to metabolise. Stick diagrams of the non-competitive and competitive antagonists of the PAFR, WEB2086 (**D**) and CV3988 (**E**), respectively. Both antagonists have a plethora of effects, but specifically, WEB2086 ($C_{22}H_{22}ClN_5O_2S$) has been shown *in vitro*, to decrease human platelet and neutrophil aggregation, while *in vivo* it has displayed anti-allergic and anti-inflammatory activity in various models. In this context, CV3988 ($C_{28}H_{53}N_2O_7PS$) inhibits PAF-induced human platelet aggregation, while *in vivo* it can attenuate PAF-induced hypotension, contraction of isolated rat colon, acute pancreatitis and the lethality of systemically administered endotoxin in rats.

Figure 3.2 | The differentiation of T_H17 cells.

The differentiation of the T_H17 subset is dependent on the concentration of the growth factor TGF-β in the surrounding environment, which in conjunction with IL-6 activates the transcription factors STAT1 and T-bet on the activated naïve $CD4^+$ T cells. After which, the cells are induced to upregulate the expression of the IL-23R and the production of IL-23 by mature DCs, this alongside IL-1β signalling through the IL-1 receptor, guides the development of cells to a T_H17 phenotype. This is reinforced by the autocrine secretion of IL-21 by these cells. The final steps in the differentiation process are defined by the transcription factors, RORγt and STAT3. T_H17 cells secrete a specific cytokine profile including: IL-17A, IL-17F, IL-22, IL-21, IL-9, IL-26 and CCL20. However, there is now a high degree of flexibility recognised in the T_H17 subset, such that they can switch patterns of cytokine production dependent on the surrounding cytokine milieu. These varied T_H17 derived cytokines are responsible for a number of mediator functions in host defence (although often pro-inflammatory) as well as being linked to several autoimmune diseases.

(Adapted from Olsen, N.J. et al., 2011[444])

Figure 3.3 | The synthesis of S1P.

Stimulation of various tyrosine-kinase, G-protein-coupled, cytokine and immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors activates sphingomyelinases (SMase), which cleave sphingomyelin to yield ceramide, ceramidases (CERase) then cleave ceramide to form sphingosine. Finally sphingosine-1-phosphate (S1P) is synthesised by phosphorylation of sphingosine in a reaction catalysed by sphingosine kinase 1 (SPHK1) at the plasma membrane (**A**). This reaction is also catalysed by SPHK2 at the endoplasmic reticulum (ER), mitochondria and nucleus (**B**). In response to a number of varying stimuli including cytokine recognition, S1P synthesised at the plasma membrane is released by specific transporters to regulate a number of immune functions, by binding to the extracellular portion of specific S1P receptors (S1PRs) to initiate signalling pathways.The production of S1P at the endoplasmic reticulum, mitochondria and nucleus is tightly regulated as its synthesis leads to the activation of numerous intracellular targets. This control comes through the irreversible degradation by S1P lyase or dephosphorylation to sphingosine by S1Pase (S1P phosphatase).

(Adapted from Spiegel et al., 2011[425])

Figure 3.4 | The actions of S1P and the SPHKs in TLR4 signalling.

The recognition of LPS by TLR4 promotes recruitment of MyD88 via TIRAP allowing the formation of a complex, which includes TRAF6 and members of the IRAK family. The recruitment of TRAF6 is important in the activation of the TAK1 complex via its self ubiquitination, which triggers the IKK complex composed of IKKα, IKKβ and the regulatory component NEMO to induce the phosphorylation, polyubiquitination and degradation of IκB via the 26S proteosome, allowing NF-κB to translocate into the nucleus. Concurrently TAK1 also triggers the MAPK pathway, resulting in AP-1 activation (through phosphorylation) and the subsequent production of proinflammatory cytokines. In this context, Sphingosine-1-phosphate (S1P) generation by sphingosine kinase 1 (SPHK1) enhances the autoubiquitinylation of TRAF6. The importance of SPHK1 in this process has been demonstrated via its inhibition, which suppresses the nuclear translocation of the NF-κB subunit, p65/RelA into the nucleus and the degradation of IRAK-1. The dashed arrows indicates the potential involvement of the MAPKs in regulating SPHK1 activity and the subsequent production of S1P, as well as the recently elucidated role of PKCδ in the phosphorylation and activation of SPHK1, leading to its translocation to the membrane.

(Adapted from Spiegel et al., 2011[425])

Figure 3.5 | DNA synthesis induced by BALB/c-derived bmDC, matured by LPS or ES-62, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then stimulated with either LPS (1 μg/ml) or ES-62 (2 μg/ml) for 18 h on day 6. On day 7, the bmDCs were pulsed with $OVA_{323-339}$ peptide at the indicated concentrations for 3 h. The bmDC ($2.5x10^4$ cells/well) were then co-cultured with OVA-specific transgenic (tg) TCR $CD4^+CD62L^+$ T cells (DO.11.10.BALB/c; $2.5x10^5$ cells/well) for 56 hours before DNA synthesis was assessed by pulsing the cells with 0.5 μCi/well [^{3}H] thymdine for the last 16 hours of culture (total of 72 hours), followed by measurement of incorporated label using a scintillation counter. Data are presented as mean counts per minute (cpm) ± SD of triplicate values alongside the maximal, basal and EC_{50} values calculated for each stimulus; the data are from a single experiment.

Figure 3.6 | T cell proliferation induced by BALB/c-derived bmDC, matured by LPS or ES-62, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then stimulated with either LPS (1 μg/ml) or ES-62 (2 μg/ml) for 18 h on day 6. On day 7, the bmDCs were pulsed with $OVA_{323-339}$ peptide at the indicated concentrations for 3 h. The bmDC ($2.5x10^4$ cells/well) were then co-cultured with OVA-specific transgenic (tg) TCR $CD4^+CD62L^+$ T cells (DO.11.10.BALB/c; $2.5x10^5$ cells/well) stained with CFSE (5 μM) for 72 hours before being analysed by flow cytometry (FL1 channel).

In panel **A**, the gating of healthy cells of the correct size and granularity associated with T lymphocytes, were defined by their expression of KJ1.26 (Ag specific TCR). In panel **B**, data are shown as histograms of CFSE fluorescence versus %-Max (**section 2.6.1**) of the $KJ1.26^+$ T cells, for the differentially treated bmDCs (concentration of OVA peptide-top right of plot). The number of cell divisions is indicated by the intensity of CFSE fluorescence relative to the non-proliferating cell peak, with the gating for each generation of cells marked on each plot (Generation 0; peak with highest CFSE fluorescence). In panel **C**, data are shown as histograms of CFSE fluorescence versus the number of $KJ1.26^+$ T cells, for the differentially treated bmDCs. In panel, **D**, the percentage of $KJ1.26^+$ cells in the population undergoing a particular number of divisions beginning at 'Generation 0' is defined relative to the total number of $KJ1.26^+$ cells in the analysis (Concentration of OVA peptide is stated above the graph). In panel **E**, the division index, proliferation index and % divided values are provided for each treatment and subsequent OVA peptide stimulus. The data in panels **B-E** is of a single experiment representative of 2.

Figure 3.7 | T cell proliferation induced by C57BL/6-derived bmDC, matured by LPS or ES-62, *in vitro*.

BmDC were derived from bone marrow cells obtained from C57BL/6 mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then stimulated with either LPS (1 μg/ml) or ES-62 (2 μg/ml) for 18 h on day 6. On day 7, the bmDCs were pulsed with $OVA_{323-339}$ peptide at the indicated concentrations for 3 h. The bmDC ($2.5x10^4$ cells/well) were then co-cultured with OVA-specific transgenic (tg) TCR $CD4^+CD62L^+$ T cells (OT2 C57BL/6; $2.5x10^5$ cells/well) stained with CFSE (5 μM) for 72 hours before being analysed by flow cytometry (FL1 channel).

In panel **A**, the gating of healthy cells of the correct size and granularity associated with T lymphocytes, were defined by their expression of the Vα2 chain of the TCR. In panel **B**, data are shown as histograms of CFSE fluorescence versus the %-Max (**section 2.6.1**) of the $V\alpha2^+$ cells (concentration of OVA peptide-top right of plot); the number of cell divisions is indicated by the levels of CFSE fluorescence relative to the non-proliferating cell peak, with the gating for each generation of cells marked on each plot (Generation 0; peak with highest CFSE fluorescence). In panel **C**, data are shown as histograms of CFSE fluorescence versus the number of $V\alpha2^+$ T cells, for the differentially treated bmDCs. In panel **D**, the percentage of $V\alpha2^+$ cells in the population undergoing a particular number of divisions beginning at 'Generation 0' is defined relative to the total number of $V\alpha2^+$ cells in the analysis. In panel **E**, the division index, proliferation index and % divided values are provided for each treatment and subsequent OVA peptide stimulus. The data in panels **B-E** is of a single experiment representative of 2.

Figure 3.8 | The differential effects of LPS- and ES-62-induced maturation of bmDC on T_H polarisation and cytokine production, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c and C57BL/6 mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$ /well) were then stimulated with either LPS (1 μg/ml) or ES-62 (2 μg/ml) for 18 h on day 6. On day 7, the bmDCs were pulsed with $OVA_{323-339}$ peptide at the indicated concentrations for 3 h before co-culture with D0.11.10 tg or OTII tg TCR $CD4^+CD62L^+$ T cells ($2.5x10^5$ cells/well) for the BALB/c and C57BL/6 derived bmDCs, respectively for 72 h. In panel **A**, the secretion of IL-17A and IL-10 in the culture supernatants from BALB/c background cells was measured by ELISA. Data are presented as the mean values (pg/ml) of triplicate estimates ± SD from a single experiment. In panel **B**, the secretion of IL-17A induced by either LPS (1 μg/ml) or ES-62 (2 μg/ml) matured bmDCs at increasing concentrations of OVA $peptide_{323-339}$ was normalised to LPS matured bmDCs pulsed with 300nM peptide (100 %). Data were acquired from three independent experiments and presented as the normalised values of the mean levels (pg/ml) of triplicate estimates from the individual experiments ± SEM, n=3. In panel **C**, IL-17A, IL-22 and IFN-γ from the culture supernatants of C57BL/6 background cells were measured by ELISA. Data are presented as the mean values (pg/ml) of triplicate estimates ± SD from a single experiment.

Figure 3.9 | The effect of ES-62 on LPS-stimulated bmDCs induction of T cell proliferation, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then pre-treated with medium or ES-62 (2 μg/ml) for 18 h on day 6 and subsequently with medium or LPS (1 μg/ml) for 18 h on day 7. On day 8, the bmDCs were pulsed with $OVA_{323-339}$ peptide at the indicated concentrations for 3 h. The bmDC ($2.5x10^4$ cells/well) were then co-cultured with OVA-specific transgenic (tg) TCR $CD4^+CD62L^+$ T cells (DO.11.10.BALB/c; $2.5x10^5$ cells/well) stained with CFSE (5 μM) for 72 hours before being analysed by flow cytometry (FL1 channel).

In panel **A**, the gating of healthy cells of the correct size and granularity associated with T lymphocytes, were defined by their expression of CD4 and KJ1.26 (Ag specific TCR). In panel **B**, data are shown as histograms of CFSE fluorescence versus the %-Max (**section 2.6.1**) of $CD4^+KJ1.26^+$ T cells, for the differentially treated bmDCs (concentration of OVA peptide-top right of plot). The number of cell divisions is indicated by the levels of CFSE fluorescence relative to the non-proliferating cell peak with the gating for each generation of cells marked on each plot (Generation 0; peak with highest CFSE fluorescence). In panel **C**, data are shown as histograms of CFSE fluorescence versus the number of $CD4^+KJ1.26^+$ T cells, for the differentially treated bmDCs. In panel, **D**, the percentage of $CD4^+KJ1.26^+$ cells in the population undergoing a particular number of divisions beginning at 'Generation 0' is defined relative to the total number of $CD4^+KJ1.26^+$ cells in the analysis (Concentration of OVA peptide-top right of graph). The data in panels **B-D** is of a single experiment representative of 2.

Table 3.1 | Analysis of proliferative parameters of T_H cells – primed by bmDCs treated with ES-62 and/or LPS

Figure 3.10 | The effect of ES-62 on the subsequent LPS maturation of bmDC in terms of polarisation of T_H cells, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then pre-treated with media or ES-62 (2 μg/ml) for 18 h on day 6 and subsequently with ± LPS (1 μg/ml) for 18 h on day 7. On day 8, the bmDCs were pulsed with $OVA_{323-339}$ peptide at the indicated concentrations for 3 h before co-culture with D0.11.10 tg $CD4^+CD62L^+$ T cells ($2.5x10^5$ cells/well) for 72 h. In panel **A**, the secretion of IL-17A and IL-22 in the culture supernatants was measured by ELISA. Data are presented as the mean values (pg/ml) of triplicate estimates ± SD from a single experiment. In panel **B**, the production of IL-17A and IL-22 mRNA transcript from these cells was measured by Taqman real-time PCR and defined as a percentage relative to GAPDH mRNA expression. The results are presented as the mean levels of triplicate estimates ± SD from a single experiment. The statistical analysis of the ELISA results was performed by a Student t-test where *$p<0.05$, ** $P<0.01$ and ***$p<0.001$, relative to the immature (untreated) cells at the relevant OVA peptide concentration.

Figure 3.11 | The effect of ES-62 maturation on the subsequent LPS-stimulated cytokine responses of BALB/c bmDCs.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These bmDC ($2x10^6$/well in a total of 2 ml) were then pretreated with medium or ES-62 (2 μg/ml) for 18 h followed by maturation with medium or LPS (1 μg/ml) for a further 18 h. The culture supernatants were analysed by ELISA for the cytokines, TNF-α; IL-6; bioactive IL-12 p70; IL-12/23 p40; IL-23 or IL-10. Data were acquired from four independent experiments and presented as the mean of the means of triplicate estimates (pg/ml) from the individual experiments ± SEM, n=4. The statistical analysis performed is by one way-ANOVA, utilising a Tukey post test, where *p<0.05, ** P<0.01 and ***p<0.001.

Figure 3.12 | CD40 expression of BALB/c-derived bmDC: modulation by ES-62, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then pre-treated with medium or ES-62 (2 μg/ml) for 18 h followed by stimulation with either medium, ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times over an 18 h period. The control (untreated) cells were incubated under the same conditions for the length of the experiment (36 h). $CD11c^+$, $7\text{-}AAD^-$ gated-cells were analysed by flow cytometry for the CD40 costimulatory marker utilising a FITC conjugated antibody (FL1 channel). In panel **A**, data for bmDCs treated with ES-62 and LPS over 18 h are presented as histograms of fluorescent intensity of CD40 staining versus the %-Max (**section 2.6.1**). In panel **B**, the percentage of positively stained cells induced by both treatments was calculated using the relevant isotype control antibody and is indicated by the marker on the histograms; this is presented alongside the geometric mean fluorescence (GMF) of the CD40 positive cells. In panel **C**, data for bmDCs pre-treated with media or ES-62 before subsequent stimulation with LPS for the indicated times are presented as histograms of fluorescent intensity of CD40 staining versus the %-Max. In panel **D**, the percentage of positively stained cells induced by the differential treatments was calculated using the relevant isotype control antibody and is indicated by the marker on the histograms; this is presented alongside the geometric mean fluorescence (GMF) of the CD40 positive cells. The data presented are from a single experiment.

Figure 3.13 | CD86 expression of BALB/c-derived bmDC: modulation by ES-62, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then pre-treated with medium or ES-62 (2 μg/ml) for 18 h followed by stimulation with either medium, ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times over an 18 h period. The control (untreated) cells were incubated under the same conditions for the length of the experiment (36 h). $CD11c^+$, $7\text{-}AAD^-$ gated-cells were analysed by flow cytometry for the CD86 costimulatory marker utilising a FITC conjugated antibody (FL1 channel). In panel **A**, data for bmDCs treated with ES-62 and LPS over 18 h are presented as histograms of fluorescent intensity of CD86 staining versus the %-Max (**section 2.6.1**) cell numbers. In panel **B**, the percentage of positively stained cells induced by both treatments was calculated using the relevant isotype control antibody and is indicated by the marker on the histograms; this is presented alongside the geometric mean fluorescence (GMF) of the CD86 positive cells. In panel **C**, data for bmDCs pre-treated with media or ES-62 before subsequent stimulation with LPS for the indicated times are presented as histograms of fluorescent intensity of CD86 staining versus the %-Max cell numbers. In panel **D**, the percentage of positively stained cells induced by the differential treatments was calculated using the relevant isotype control antibody and is indicated by the marker on the histograms; this is presented alongside the geometric mean fluorescence (GMF) of the CD86 positive cells. The data presented are from a single experiment.

Figure 3.14 | MHC Class II expression of BALB/c-derived bmDC: modulation by ES-62, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then pre-treated with medium or ES-62 (2 μg/ml) for 18 h followed by stimulation with either medium, ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times over an 18 h period. The control (untreated) cells were incubated under the same conditions for the length of the experiment (36 h). $CD11c^+$, $7\text{-}AAD^-$ gated-cells were analysed by flow cytometry for MHC Class II expression utilising a FITC conjugated antibody (FL1 channel). In panel **A**, data for bmDCs treated with ES-62 and LPS over 18 h are presented as histograms of fluorescent intensity of MHC Class II staining versus the %-Max (**section 2.6.1**). In panel **B**, the percentage of positively stained cells induced by both treatments was calculated using the relevant isotype control antibody and is indicated by the marker on the histograms; this is presented alongside the geometric mean fluorescence (GMF) of the MHC Class II positive cells. In panel **C**, data for bmDCs pre-treated with media or ES-62 before subsequent stimulation with LPS for the indicated times are presented as histograms of fluorescent intensity of MHC Class II staining versus the %-Max. In panel **D**, the percentage of positively stained cells induced by both treatments was calculated using the relevant isotype control antibody and is indicated by the marker on the histograms; this is presented alongside the geometric mean fluorescence (GMF) of the MHC Class II positive cells. The data presented are from a single experiment.

Figure 3.15 | LPS induction of costimulatory molecules in C57BL/6-derived bmDC, *in vitro*.

BmDC were derived from bone marrow cells obtained from C57BL/6 mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then stimulated with either medium or LPS (1 μg/ml) for 18 h. $CD11c^+$, $7\text{-}AAD^-$ gated-cells were analysed by flow cytometry for the expression of MHC Class II and CD86 utilising FITC conjugated antibodies (FL1 channel), while CD40 expression was defined utilising a biotinylated antibody detected by a streptavidin-APC conjugate (FL4 channel). In panel **A**, data for bmDCs treated with medium or LPS for 18 h are presented as histograms of fluorescent intensity of CD40, CD86 and MHC class II staining versus the %-Max (**section 2.6.1**). In panel **B**, the percentage of positively stained cells induced by both treatments was calculated using the relevant isotype control antibody and is indicated by the markers on the histograms; this is presented alongside the geometric mean fluorescence (GMF) of the positive cells. The data presented are from a single experiment.

Figure 3.16 | The effect of ES-62 and/or LPS maturation on CD86 and MHC Class II expression in C57BL/6-derived bmDC, *in vitro*.

BmDC were derived from bone marrow cells obtained from C57BL/6 mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then pre-treated with medium or ES-62 (2 μg/ml) for 18 h followed by stimulation with either medium, ES-62 (2 μg/ml) or LPS (1 μg/ml) for a further 18 h. $CD11c^+$, $7\text{-}AAD^-$ gated-cells were analysed by flow cytometry for CD86 and MHC Class II expression utilising a FITC conjugated antibody (FL1 channel) and bitotinylated antibody detected by a streptavidin-PerCP conjugate (FL3 channel), respectively. In panel **A**, data for the differentially treated bmDCs are presented as histograms of fluorescent intensity of Ag staining versus the %-Max (**section 2.6.1**). In panel **B**, the percentage of positively stained CD86 and MHC Class II cells induced by both treatments was calculated using the relevant isotype control antibody and is indicated by the marker on the histograms. In panel **C**, the geometric mean fluorescence (GMF) of the CD86 and MHC Class II positive cells is presented. The data presented are from a single experiment.

Figure 3.17 | The importance of SPHK1 and SPHK2 in LPS-induced maturation of bmDC on T_H polarisation and cytokine production, *in vitro*.

BmDC were derived from bone marrow cells obtained from Sphk1$^{-/-}$ and Sphk2$^{-/-}$ and wild type (WT) C57BL/6 background mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were treated with medium or LPS (1 μg/ml) for 18 h on day 6. On day 7, the bmDCs were pulsed with $OVA_{323-339}$ peptide at the indicated concentrations for 3 h before being co-cultured with OTII tg $CD4^+CD62L^+$ T cells ($2.5x10^5$ cells/well) for 72 h. In panel **A** and **B**, the secretion of IL-17A (panel **A**) and IFN-γ (panel **B**) in the culture supernatants of both the media and LPS stimulated cells was measured by ELISA. Data are acquired from two independent experiments and presented as the means of the replicates from the individual experiments ± SD, n=6.

Figure 3.18 | The roles of SPHK1 and SPHK2 in T cell proliferation induced by bmDC, matured by LPS, *in vitro*.

BmDC were derived from bone marrow cells obtained from Sphk1$^{-/-}$, Sphk2$^{-/-}$ and wild type (WT) C57BL/6 mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then stimulated with either medium or LPS (1 μg/ml) for 18 h on day 6. On day 7, the bmDCs were pulsed with $OVA_{323-339}$ peptide at the indicated concentration for 3 h. The bmDC ($2.5x10^4$ cells/well) were then co-cultured with OVA-specific transgenic (tg) TCR CD4$^+$CD62L$^+$ T cells (OT2 C57BL/6; $2.5x10^5$ cells/well) stained with CFSE (5 μM) for 72 hours before being analysed by flow cytometry (FL1 channel).

In panel **A**, the gating of healthy cells of the correct size and granularity associated with T lymphocytes, were defined by their expression of the CD4 and the Vα2 chain of TCR. In panels **B**-**C**, data are shown as histograms of CFSE fluorescence versus the %-Max (**section 2.6.1**) of CD4$^+$Vα2$^+$ T cells for medium (panel **B**) and LPS (panel **C**) treated bmDCs (concentration of OVA peptide above the plot). The number of cell divisions is indicated by the levels of CFSE fluorescence relative to the non-proliferating cell peak with the gating for each generation of cells marked on each histogram (Generation 0; peak with highest CFSE fluorescence). In panels **D**-**E**, data are shown as histograms of CFSE fluorescence versus the number of CD4$^+$Vα2$^+$ T cells, for the medium (panel **D**) and LPS (panel **E**) treated bmDCs. In panel **F**, the percentage of CD4$^+$KJ1.26$^+$ cells in the population undergoing a particular number of divisions beginning at 'Generation 0' is defined relative to the total number of CD4$^+$Vα2$^+$ cells in the analysis for the LPS stimulated samples as no discernable proliferation was induced by the medium treated bmDCs (Concentration of OVA peptide-top left of graph). In panel **G**, the division index, proliferation index and % divided values are provided for the LPS treated bmDCs for each phenotype and OVA peptide stimulus. The data in panels **B-G** is of a single experiment representative of 2.

Figure 3.19 | The role of SPHK1 and SPHK2 in LPS stimulated cytokine responses by bmDC, *in vitro*.

BmDC were derived from bone marrow cells obtained from Sphk1$^{-/-}$, Sphk2$^{-/-}$ and wild type (WT) C57BL/6 background mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 ml) were treated with medium or LPS (1 μg/ml) for 18 h. The culture supernatants were analysed by ELISA for the cytokines, TNF-α; bioactive IL-12 p70; IL-12/23 p40; IL-23, IL-27 and TGF-β1. In panel **A**, the expression of these cytokines in response to medium and LPS maturation are presented, while panel **B**, displays the cytokine production of only the medium treated (resting) cells of the various phenotypes analysed. Data were acquired from two independent experiments and presented as the means of the replicates from the individual experiments ± SD, n=6. The statistical analysis performed is by one way-ANOVA, utilising a Tukey post test where *$p<0.05$, ** $P<0.01$ and ***$p<0.001$.

Figure 3.20 | The function of SPHK1 and SPHK2 in LPS stimulated bmDC co-stimulatory molecule expression.

BmDC were derived from bone marrow cells obtained from Sphk1$^{-/-}$, Sphk2$^{-/-}$ and wild type (WT) C57BL/6 mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then stimulated with either medium or LPS (1 μg/ml) for 18 h on day 6. On day 7, CD11c$^+$, 7-AAD$^-$ gated-cells were analysed by flow cytometry for the costimulatory markers CD80, MHC Class II and CD86. A FITC conjugated antibody (FL1 channel) was utilised for each marker. In panel **A**, data for bmDCs treated with medium or LPS over 18 h are presented as histograms of fluorescent intensity of CD80 staining versus the %-Max (**section 2.6.1**). In panel **B**, the percentage of positively stained cells induced by both treatments was calculated using the relevant isotype control antibody and is indicated by the marker on the histogram. This is presented alongside the geometric mean fluorescence (GMF) of the CD80 positive cells. Panels **C-D**, and **E-F**, presents the identical analysis of MHC Class II and CD86 expression, respectively. Data presented are from a single experiment.

Figure 3.21 | **Modulation of PAFR protein expression by ES-62.**

BmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) over a period of 1, 3 and 6 h. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (6 h). In panel **A**, total protein extracts were purified from the samples and assayed by Western blotting for PAFR expression at 39 kDa. In panel **B**, the densitometric analysis of the protein was normalised against the relevant β-actin loading control. The values were then adjusted and expressed as a fold change relative to the control (unstimulated DC, normalised to 1) cells.

Figure 3.22 | The effect of the PAF analogue, C-PAF, on the secretion of a panel of pro-inflammatory and immuno-modulatory cytokines.

BmDC ($2x10^6$/well in a total of 2 mls) were stimulated with the indicated concentrations of C-PAF (0.01, 0.1 and 1 nM) for 18 h. In panels **A-F**, the culture supernatants were analysed by ELISA for the cytokines, TNF-α (panel **A**); bioactive IL-12 p70 (panel **B**); IL-23 (panel **C**) IL-12/23 p40 (panel **D**) or IL-10 (panel **F**). The ELISA Data are representative of two separate experiments and presented as the mean of triplicate values ± SD of a single experiment. In panels **G**-**H**, the production of Cbl-b (panel **G**), c-Cbl (panel **H**) and TRAF6 (panel **I**) mRNA transcript from these cells was measured by Taqman real-time PCR and defined as a percentage relative to HPRT (Cbl-b and c-Cbl) and GAPDH (TRAF6) mRNA expression. The results are presented as the mean levels of triplicate estimates from the individual experiments ± SD.

Figure 3.23 | The effects of the PAFR inhibitors, WEB2086 and CV3988, on LPS-induced cytokine secretion from bmDC.

BmDC ($2x10^6$/well in a total of 2 mls) were pre-treated with medium, WEB2086 (panels **A-E**; 10 μM, 30 μM and 100 μM) or CV3988 (panels **F-H**; 10 μM and 30 μM) for 30 minutes prior to stimulation with LPS (0.01, 0.1, 1 and 10 μg/ml) for a further 18 h. The culture supernatants were analysed by ELISA for the cytokines, TNF-α; bioactive IL-12 p70; IL-12/23 p40; IL-23 or IL-10. The Data are representative of two separate experiments and are presented as the mean of triplicate values (pg/ml) ± SD from a single experiment.

Figure 3.24 | The effect of C-PAF and WEB2086 on the secretion of a panel of pro-inflammatory and immuno-modulatory cytokines in response to LPS.

BmDC ($2x10^6$/well in a total of 2 mls) were pre-treated with medium or 30 μM WEB2086 for 30 min before stimulation with 1 μg/ml LPS. In addition bmDC were stimulated with LPS in combination with 1 nM C-PAF for 18 h, and the culture supernatants were analysed by ELISA for the cytokines, TNF-α; bioactive IL-12 p70; IL-12/23 p40; IL-23; IL-6 or IL-10. The data are pooled from three independent experiments and presented as the mean values of the means of triplicate estimates (pg/ml) from the individual experiments ± SEM, n=3. The statistical analysis performed is by unpaired Students t-test where *$p<0.05$, ** $P<0.01$ and ***$p<0.001$.

Figure 3.25 | The effect of C-PAF and WEB2086 on LPS matured bmDC priming of antigen-specific T cell cytokine production, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. On day 6 these cells ($2x10^6$/well in a total of 2 mls) were pre-treated with medium or 30 μM WEB2086 for 30 min before stimulation with 1 μg/ml LPS or alternatively the bmDC were treated with LPS in combination with 1 nM C-PAF for 18 h. On day 7, the bmDCs were washed and pulsed with $OVA_{323-339}$ peptide at the indicated concentrations for 3 h before co-culture with D0.11.10 tg TCR $CD4^+CD62L^+$ T cells ($2.5x10^5$ cells/well) for 72 h. The culture supernatants were analysed by ELISA for the cytokines, IL-17A; IL-22; IFN-γ; IL-4; IL-21 and IL-10. Data were acquired from two independent experiments and presented as the means of the replicates from the individual experiments ± SD, n=6. The statistical analysis performed is by two way-ANOVA, utilising a Bonferroni post test where *$p<0.05$, ** $P<0.01$ and ***$p<0.001$. The significance of each result represented is relative to the 'LPS stimulated' sample at the relevant OVA peptide concentration.

Figure 3.26 | The effect of C-PAF and WEB2086 on LPS matured bmDC induced antigen-specific T cell clonal expansion, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. On day 6, these cells ($2x10^6$/well in a total of 2mls) were pre-treated with medium or 30 μM WEB2086 for 30 min before stimulation with 1 μg/ml LPS or alternatively the bmDC were matured with LPS in combination with 1 nM C-PAF for 18 h. On day 7, the bmDCs were washed and pulsed with $OVA_{323-339}$ peptide at the indicated concentrations for 3 h, the bmDC ($2.5x10^4$ cells/well) were then co-cultured with OVA-specific transgenic (tg) TCR $CD4^+CD62L^+$ T cells (DO.11.10.BALB/c; $2.5x10^5$ cells/well) stained with CFSE (5 μM) for 72 h before being analysed by flow cytometry (FL1 channel).

In panel **A**, the gating of healthy cells of the correct size and granularity associated with T lymphocytes, were defined by their expression of CD4 and KJ1.26 (Ag specific TCR). In panel **B**, data are shown as histograms of CFSE fluorescence versus the %-Max (**section 2.6.1**) of $CD4^+KJ1.26^+$ T cells, for the differentially treated bmDCs (concentration of OVA peptide-top left of plot). The number of cell divisions is indicated by the levels of CFSE fluorescence relative to the non-proliferating cell peak with the gating for each generation of cells marked on each plot (Generation 0; peak with highest CFSE fluorescence). In panel **C**, data are shown as histograms of CFSE fluorescence versus the number of $CD4^+KJ1.26^+$ T cells, for the differentially treated bmDCs. In panel, **D**, the percentage of $CD4^+KJ1.26^+$ cells in the population undergoing a particular number of divisions beginning at 'Generation 0' is defined relative to the total number of $CD4^+KJ1.26^+$ cells in the analysis (Concentration of OVA peptide-top right of graph). In panel **E**, the division index, proliferation index and %-divided values are provided for each treatment and subsequent OVA peptide stimulus. The data in panels **B-E** is of a single experiment representative of 2.

Figure 3.27 | The effect of C-PAF and WEB2086 on antigen-specific T cell cytokine production by bmDC *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. On day 6 these cells ($2x10^6$/well in a total of 2 mls) were treated with medium, WEB2086 (30 μM) or C-PAF (1 nM) for 18 h. On day 7, the bmDCs were washed and pulsed with $OVA_{323-339}$ peptide at the indicated concentrations for 3 h before co-culture with D0.11.10 tg TCR $CD4^+CD62L^+$ T cells ($2.5x10^5$ cells/well) for 72 h. The culture supernatants were analysed by ELISA for the cytokines, IL-17; IL-22; IFN-γ; IL-4; IL-21 and IL-10. Data were acquired from two independent experiments and presented as the means of the replicates from the individual experiments ± SD, n=6. The statistical analysis performed is by two way-ANOVA, utilising a Bonferroni post test where *$p<0.05$, ** $P<0.01$ and ***$p<0.001$. The significance of each result represented is relative to the 'Immature' sample at the relevant OVA peptide concentration.

Figure 3.28 | The effect of C-PAF and WEB2086 on antigen-specific T cell clonal expansion by bmDC, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. On day 6, these cells ($2x10^6$/well in a total of 2mls) were treated with medium, WEB2086 (30 μM), C-PAF (1 nM) for 18 h. On day 7, the bmDCs were washed and pulsed with $OVA_{323-339}$ peptide at the indicated concentrations for 3 h, the bmDC ($2.5x10^4$ cells/well) were then co-cultured with OVA-specific transgenic (tg) TCR $CD4^+CD62L^+$ T cells (DO.11.10.BALB/c; $2.5x10^5$ cells/well) stained with CFSE (5 μM) for 72 h before being analysed by flow cytometry (FL1 channel).

In panel **A**, $CD4^+KJ1.26^+$ T_H cells were analysed and the data are shown as histograms of CFSE fluorescence versus the %-Max (**section 2.6.1**) of $CD4^+KJ1.26^+$ T cells, for the differentially treated bmDCs (concentration of OVA peptide-top left of plot). The number of cell divisions is indicated by the levels of CFSE fluorescence relative to the non-proliferating cell peak with the gating for each generation of cells marked on each plot (Generation 0; peak with highest CFSE fluorescence). In panel **B**, data are shown as histograms of CFSE fluorescence versus the number of $CD4^+KJ1.26^+$ T cells, for the differentially treated bmDCs. In panel **C**, the percentage of $CD4^+KJ1.26^+$ cells in the population undergoing a particular number of divisions beginning at 'Generation 0' is defined relative to the total number of $CD4^+KJ1.26^+$ cells in the analysis (Concentration of OVA peptide-top right of graph). In panel **D**, the division index, proliferation index and %-divided values are provided for each treatment and subsequent OVA peptide stimulus. The data in panels **A**-**D** is of a single experiment representative of 2.

Figure 3.29 | ES-62 modulation of cytokine secretion by bmDCs treated with WEB2086 and C-PAF.

BmDC ($2x10^6$/well in a total of 2 mls) were pre-treated with medium, WEB2086 (30 μM) or CV3988 (30 μM) for 30 min before maturing the cells with medium or ES-62 for a further 18 h. After which, each group of cells were stimulated with 1 μg/ml LPS in combination with medium or 1 nM C-PAF for 18 h. The culture supernatants were analysed by ELISA for the cytokines, TNF-α; bioactive IL-12 p70; IL-12/23 p40; IL-23; IL-6 and IL-10. The data are pooled from three independent experiments and presented as the mean values (pg/ml) of the means of triplicate estimates from the individual experiments ± SEM, n=3. The statistical analysis performed is by two way-ANOVA, utilising a Bonferroni post test, where; *$p<0.05$, ** $P<0.01$ and ***$p<0.001$. The significance of each result represented is relative to the relevant 'LPS stimulated' sample.

Figure 3.30 | ES-62 modulation of c-Cbl and TRAF6 mRNA production following treatment of bmDCs with PAFR agonists and antagonists.

BmDC ($2x10^6$/well in a total of 2 mls) were pre-treated with medium, WEB2086 (30 μM) or CV3988 (30 μM) for 30 min before maturing the cells with medium or ES-62 for a further 18 h. After which, each group of cells were stimulated with 1 μg/ml LPS in combination with medium or 1 nM C-PAF for 18 h. The production of c-Cbl and TRAF6, mRNA transcript from these cells was measured by Taqman real-time PCR and defined as a percentage relative to HPRT (c-Cbl) and GAPDH (TRAF6) mRNA expression. The data are presented as the mean levels of triplicate estimates from a single experiment ± SD, which is representative of two.

Figure 3.31 | The cell surface and total TLR4 expression of C57BL/6-derived bmDC, following maturation with LPS or ES-62, *in vitro*.

BmDC were derived from bone marrow cells obtained from C57BL/6 mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then stimulated with LPS (1 μg/ml) or ES-62 (2 μg/ml) at the times indicated over an 18 h period. The untreated (control) cells were incubated under the same conditions for the length of the experiment (18 h). In panel **A**, $CD11c^+7\text{-}AAD^-$ gated-cells were analysed by flow cytometry for TLR4 utilising an APC conjugated antibody (FL4 channel). The data for differentially stimulated bmDCs are presented as histograms of fluorescent intensity of TLR4 staining versus the %-Max (**section 2.6.1**). The percentage of positively stained cells induced by both treatments was calculated using the relevant isotype control antibody, indicated by the marker on the histograms. In panel **B**, the percentage of TLR4 positive cells was normalised and expressed relative to untreated (control) cells, which are expressed as 100%. The data was acquired from three independent experiments and presented as their mean normalised values ± SEM, n=3. The statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$), expressed relative to the untreated (control) cells. In panel **C**, the Geometric Mean Fluorescence (GMF) of the TLR4 positive cells determined in panel **B** were normalised to the untreated cells (control; 100 %) and presented as the mean of these normalised values ± SEM, n=3.

In panel **D**, permeablised $CD11c^+$ gated-cells were analysed by flow cytometry for TLR4 expression, with the data generated and presented as in panel **A**. In panel **E**, the percentage of TLR4 positive cells was normalised and expressed relative to untreated (control) cells, which are expressed as 100 %. Data was acquired from ES-62 and LPS stimulated cells from one and three independent experiments, respectively. The data are presented as the mean normalised values ± SEM where for LPS, n=3 and ES-62, n=1. In panel **F**, the Geometric Mean Fluorescence (GMF) of the TLR4 positive cells determined in panel **E** were normalised to the untreated cells (control; 100 %) and presented as the mean of these normalised values ± SEM, where for LPS n=3 and ES-62, n=1. The statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test (where $\alpha = p<0.05$), relative to the untreated (control) cells (100 %).

Figure 3.32 | **Protein and mRNA transcript expression of TLR4 in BALB/c-derived bmDC, matured by LPS or ES-62, *in vitro*.**

In panel **A**, BmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) over a period of 1, 3 and 6 h. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (6 h). Total protein extracts were purified from the samples and assayed by Western blotting for PAFR expression at 39 kDa. This is presented alongside the densitometric analysis of the protein, which was normalised against the relevant β-actin loading control then adjusted and expressed as a fold change relative to the control (unstimulated DC, normalised to 1) cells.

In panel **B**, the total RNA was purified from CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The production of TLR4 mRNA transcript from these cells was measured by Taqman real-time PCR and defined as a percentage relative to GAPDH mRNA expression. The data are presented as the mean levels of triplicate estimates from a single experiment ± SD.

Figure 3.33 | The cell surface and total TLR4 expression of C57BL/6-derived bmDC, matured by LPS and/or ES-62, *in vitro*.

BmDC were derived from bone marrow cells obtained from C57BL/6 mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then pre-treated with medium or ES-62 (2 μg/ml) for 18 h followed by stimulation with either medium, ES-62 (2 μg/ml) or LPS (1 μg/ml) for a further 18 h. The untreated (control) cells were incubated under the same conditions for the length of the experiment (36 h).

In panel **A**, unpermeabilised $CD11c^+7\text{-}AAD^-$ (surface) and permeabilised $CD11c^+$ (total) gated-cells were analysed by flow cytometry for TLR4 utilising an APC conjugated antibody (FL4 channel). The data for the differentially stimulated bmDCs are presented as histograms of the fluorescence intensity of TLR4 staining versus the %-Max (**section 2.6.1**). The percentage of positively stained cells induced by the differential treatments was calculated using the relevant isotype control antibody, indicated by the marker on the histograms. In panel **B**, the percentage of TLR4 positive cells was normalised and expressed relative to the untreated (control) cells, which are expressed as 100 %. The data was acquired from three independent experiments and presented as the mean normalised values ± SEM, n=3. The statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$). In panel **C**, the geometric mean fluorescence (GMF) of TLR4 expression throughout (surface and intracellular) TLR4 positive cells is presented from a single representative experiment.

Figure 3.34 | The cell surface and total TLR4/MD2 expression of C57BL/6-derived bmDC, matured by LPS or ES-62, *in vitro*.

BmDC were derived from bone marrow cells obtained from C57BL/6 mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then stimulated with LPS (1 μg/ml) or ES-62 (2 μg/ml) at the times indicated over an 18 h period. The untreated (control) cells were incubated under the same conditions for the length of the experiment (18 h). In panel **A**, $CD11c^+7\text{-}AAD^-$ gated-cells were analysed by flow cytometry for the TLR4 complex, TLR4/MD2, utilising a APC conjugated antibody (FL4 channel). The data for differentially stimulated bmDCs are presented as histograms of fluorescent intensity of TLR4/MD2 staining versus the %-Max (**section 2.6.1**). The percentage of positively stained cells induced by both treatments was calculated using the relevant isotype control antibody, indicated by the marker on the histograms. In panel **B**, the percentage of TLR4/MD2 positive cells was normalised and expressed relative to the untreated (control) cells, which are expressed as 100 %. The data was acquired from three independent experiments and presented as the mean of normalised values ± SEM, n=3. The statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$), expressed relative to the untreated cells (control; 100 %). In panel **C**, the Geometric Mean Fluorescence (GMF) of the TLR4/MD2 positive cells determined in panel **B** were normalised to untreated cells (control; 100 %) and presented as the mean of these values ± SEM, n=3.

In panel **D**, permeablised $CD11c^+$ gated-cells were analysed by flow cytometry for TLR4/MD2 expression, with the data generated and presented as in panel **A**. In panel **E**, the percentage of TLR4/MD2 positive cells was normalised and expressed relative to the untreated (control) cells, which are expressed as 100 %. In panel **F**, the Geometric Mean Fluorescence (GMF) of the TLR4/MD2 positive cells determined in panel **E** is presented. The data in panels **D**-**F** are from a single representative experiment.

Figure 3.35 | The cell surface and total TLR4/MD2 expression of C57BL/6-derived bmDC, matured by LPS and/or ES-62, *in vitro*.

BmDC were derived from bone marrow cells obtained from C57BL/6 mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then pre-treated with medium or ES-62 (2 μg/ml) for 18 h followed by stimulation with either medium, ES-62 (2 μg/ml) or LPS (1 μg/ml) for a further 18 h. The untreated (control) cells were incubated under the same conditions for the length of the experiment (36 h).

In panel **A**, unpermeabilised $CD11c^+7\text{-}AAD^-$ (surface) and permeablised $CD11c^+$ (total) gated-cells were analysed by flow cytometry for TLR4/MD2 utilising an APC conjugated antibody (FL4 channel). The data for differentially stimulated bmDCs are presented as histograms of the fluorescence intensity of TLR4/MD2 staining versus the %-Max (**section 2.6.1**). The percentage of positively stained cells induced by the differential treatments was calculated using the relevant isotype control antibody, indicated by the markers on the histograms. In panel **B**, the percentage of TLR4/MD2 positive cells was normalised and expressed relative to the untreated (control) cells, which are expressed as 100 %. The data was acquired from three independent experiments and presented as the mean normalised values ± SEM, n=3. The statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$). In panel **C**, the geometric mean fluorescence (GMF) of TLR4/MD2 expression throughout (surface and intracellular) the TLR4/MD2 positive cells is presented from a single representative experiment.

Figure 3.36 | TLR4/MD2 cell surface expression of BALB/c-derived bmDC, matured by LPS and/or ES-62, *in vitro*.

BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These cells ($2x10^6$/well in a total of 2 mls) were then pre-treated with medium or ES-62 (2 μg/ml) for 18 h followed by stimulation with either medium, ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times over an 18 h period. The untreated (control) cells were incubated under the same conditions for the length of the experiment (36 h). $CD11c^+$, $7\text{-}AAD^-$ gated-cells were analysed by flow cytometry for the TLR4 complex, TLR4/MD2 utilising an APC conjugated antibody (FL4 channel).

In panel **A**, data for bmDCs treated with ES-62 and LPS for the indicated times are presented as histograms of fluorescent intensity of TLR4/MD2 staining versus the percentage of maximum cell numbers. In panel **B**, the percentage of positively stained cells induced by both treatments was calculated using the relevant isotype control antibody and is indicated by the marker on the histograms; this is presented alongside the geometric mean fluorescence (GMF) of the TLR4/MD2 positive cells. In panel **C**, data for bmDCs pre-treated with medium or ES-62 before subsequent stimulation with LPS for the indicated times are presented as histograms of the fluorescence intensity of TLR4/MD2 staining versus the %-Max. In panel **D**, the percentage of positively stained cells induced by both treatments was calculated using the relevant isotype control antibody and is indicated by the marker on the histograms; this is presented alongside the geometric mean fluorescence (GMF) of the TLR4/ND2 positive cells. Collectively, the data presented are from a single experiment.

Figure 3.37 | The differential effects of ES-62 on the expression and localisation of TLR4 and the LPS signalling complex TLR4/MD2 in bmDCs

Panel **A**, stimulation of bmDC with LPS progressively increases the pool of TLR4 on the surface whilst decreasing that of the TLR4/MD2 fraction, some of which is known to be targeted for degradation in the lysosomes[347]. The effects of ES-62 are not as striking but there does appear to be a transient up-regulation of total (surface and intracellular) TLR4 expression between 1-6 h (of both protein [as determined by flow cytometry and Western blotting studies] and mRNA transcript), which may suggest a cycling of the receptor between the plasma membrane and endosomes. In contrast, ES-62 appears to maintain a steady-state surface expression of the TLR4/MD2 complex while inducing a slight reduction of the complex throughout the cell, indicating a potential link with its possible lysosomal degradation.

Panel **B**, on the other hand when ES-62 was utilised to mature DCs they could not efficiently internalise TLR4/MD2 in response to LPS, and increased levels were observed throughout the cell. A similar case occurs with TLR4 as ES-62 prevents the up-regulation of TLR4 on the surface in response to LPS, yet also shows higher total levels of TLR4. Within this context the data also suggests that ES-62 maybe utilising TLR4 alone or via the formation of other novel TLR4 signalling complexes (i.e. not associated with MD2) but this is unconfirmed.

4 Immunomodulation of the dendritic cell by ES-62; its effects on the activation of NF-κB by TLR4

4.1 Introduction

4.1.1 The subversion of NF-κB activation by ES-62 requires TLR4

The TLR family of PRRs are important for pathogen recognition by the innate immune system and the most widely studied of these, TLR4, is a critical member of a complex responsible for driving responses to bacterial LPS. This receptor initiates intracellular signalling via TIR domains through the association of adaptor proteins such as MyD88, leading to the activation of the transcription factor NF-κB [21] and the secretion of IL-6, IL-12/23p40, IL-12p70 and TNF-α from DCs[330]. It has been demonstrated that ES-62, despite inducing transient activation of this pathway, is able to retain the DCs in an 'immature' state, which is associated with a decrease in their ability to secrete several proinflammatory cytokines in response to LPS[301,323]. The requirement of both TLR4 and MyD88 in mediating the immunomodulatory effects of ES-62, have been demonstrated in macrophages and DCs derived from TLR4$^{-/-}$ and MyD88$^{-/-}$ mice[301]. Further to this, TLR4 but not MyD88 is crucial for the internalisation of ES-62 into APCs [326], however, the low level cytokine responses induced by ES-62 in MyD88-deficent cells are lost[301].

In recent years the recognition of LPS by TLR4 has been shown to activate most PKC isoforms in several innate cells such as macrophages and DCs, inducing the secretion of a number of pro-inflammatory cytokines (as summarised in **Figure 1.6**)[91-95]. This is of particular interest as the subversion of TLR4 signals by ES-62 in APCs, modulates signalling elements important in NF-κB activation and cytokine secretion. For example, both PKCε and PKCδ are known to directly interact with MyD88[49,100] and TIRAP[101], respectively. These associations induce PKCδ to mediate the activation of p38 and Akt[106-108], which coupled with the phosphorylation and subsequent degradation of IκB through IKK are important in NF-κB signalling[101]. PKCε acts within a similar context but is known to be crucial for LPS stimulated TNF-α and IL-12p70 secretion in DCs[112,114]. Similarly PKCα is also important in LPS-stimulated TNF-α and IL-6 secretion[119,120], where it has been immunoprecipitated with TLR2 in DCs upon its activation. In this context, this association is ablated in MyD88$^{-/-}$ mice, suggesting that MyD88 acts as the link between PKCα and TLR2 with a potential for association to TLR4[115].

In relation to this, the immunomodulation of B cells by ES-62 was observed to involve the selective regulation (expression and activity) of certain PKC isoforms in resting and BCR stimulated B cells[320,333]. This involved the induced proteolytic degradation of the α, β, δ, ζ and ι/λ isoforms, coupled with the up-regulation of PKCγ and PKCε in murine splenic B cells[320]. In these studies, modulation of PKCα was found to be crucial due to its role in proliferation, where it disrupted its normal activation and nuclear translation patterns, contributing to ES-62-mediated inhibition of BCR-stimulated proliferation. In particular, the modulation of PKCα, -δ and -ι/λ, were observed to be important due to their roles in the transduction of key activation signals for the phosphorylation of nuclear protein lamin B and the induction and activation of NF-κB, Fos, Egr-1 and Myc[335-339].

Subsequent to these studies, it has been found that ES-62 causes a similar degradation of PKCα in T cells and mast cells. In mast cells, the degradation of PKCα provides the molecular mechanism by which ES-62 mediates their desensitisation[321], as it is necessary for PLD-coupled, SPHK-mediated, calcium mobilisation. This release of calcium is important for FcεRI-mediated NF-κB activation, mast cell degranulation and pro-inflammatory mediator release. The characterisation of this immunomodulation revealed that the complexing of ES-62 and TLR4 at the plasma membrane resulted in the sequestration of PKCα, which was followed by caveolae/lipid raft-dependent internalisation and non-proteosomal degradation at the peri-nuclear region of the cell[321]. Sequestration of PKCα does not take place when ES-62 is substituted by LPS, highlighting the differences between the two molecules with respect to the consequences of their interaction with TLR4. It has also been found that several other isoforms (PKCδ, -β and -ι) are degraded in response to ES-62, (lymphocytes and mast cells), which is most likely involved in limiting the activation of NF-κB but this has not been confirmed at present. Based on these observations, it has been hypothesised that the PKCs are an important target for modulation in DCs by ES-62. This is due to their emerging importance in NF-κB activation and the induction of proinflammatory cytokine secretion by TLR4 signalling.

4.1.2 Ubiquitination, an important cellular process involved in TLR4 mediated activation of NF-κB and potential mechanism for the effects of ES-62

The ubiquitination of proteins downstream of TLR4 stimulation is required for the activation of NF-κB. For example, the RING-fingered domain E3 ubiquitin ligase,

TRAF6[27], acts as a key scaffolding protein involved in the propagation of TLR4 signals and is required for both MyD88 dependent and independent activation of NF-κB (as described in **section 1.1.3.1.2** and **Figure 1.4**). Briefly, TRAF6 in conjunction with the E2 ligases, Ubc13 and Uev1a, promotes the K63-linked polyubiquitination of target proteins including TRAF6 itself and the IKK complex component NEMO[23,24]. Unlike K48-linked polyubiquitination, which normally targets proteins for degradation by the proteasome, K63-linked polyubiquitin chains act as scaffolds to assemble protein kinase complexes and mediate their activation through proteasome-independent mechanisms[24]. For example, K63 linked polyubiquitination plays an important role in the activation of TAK1 by TRAF6, which subsequently activates IKK and the MAPKs[44], culminating in the activation of NF-κB and AP-1, respectively[24]. Specifically, DCs from TRAF6 deficient mice demonstrate maturation defects in response to LPS, both *in vitro* and *in vivo*[26,27], being unable to effectively secrete both IL-6 and IL-12 and up-regulate numerous costimulatory molecules, limiting their ability to induce T cell priming.

Similarly, recent evidence has indicated that the E3-ligase c-Cbl, may play several roles in the TLR-NF-κB signalling axis and hence has been implicated in the regulation of several innate immune responses, including activation of DCs[451,452]. c-Cbl is a RING-domain containing E3 ubiquitin ligase and is part of the cbl (casita b lineage lymphoma) family of mammalian proteins. It is most commonly associated with its role as a negative regulator of TCR signalling, whereby blocking its RING domain leads to heightened responses[457]. However, c-Cbl has been shown to stabilise the p50 subunit of NF-κB and its precursor p105 in DCs, whilst a deficiency of c-Cbl leads to higher levels of IL-12p70 and IL-6[454]. This finding may reflect that p105 can also act as a IκB protein through its carboxy-terminal ankyrin repeats[453]. Interestingly the closely related E3 ligase, Cbl-b has also been shown to be involved in regulating acute lung inflammatory response to LPS, by controlling the association between TLR4 and MyD88[455].

Because of the important roles of the E3 ubiquitin ligases in innate immune cell function and the TLR-NF-κB signalling axis it is not surprising there is evidence that products secreted by filarial nematodes target this class of proteins. For example, the human filarial nematode, *Brugia malayi*, which releases PC-containing proteins[331], up-regulates expression of the E3 Ubiquitin ligases: CBL, ITCH (itchy homologue E3 ubiquitin protein ligase) and NEDD4 (neural precursor cell expressed, developmentally down-regulated 4) *in vivo*[458]. Moreover, T_H2 hypo-responsiveness during chronic

schistosomiasis in mice is associated with the expression of GRAIL (gene related to anergy in lymphocytes) where it is thought to have a role in the consequent induction of T cell anergy[28]. ES-62 may therefore utilise this regulatory process and modulate key proteins involved in the ubiquitination process to induce its anti-inflammatory effects, potentially via the degradation of TLR4 coupled signalling mediators involved in the activation of NF-κB.

4.1.3 Autophagy and the cell: a potential avenue for immunomodulation by ES-62?

Autophagy, derived from the Greek word meaning to 'eat oneself', is an evolutionary conserved pathway in eukaryotic cells for the transport of cytosolic components for degradation by the lysosomes that is engaged in response to various stimuli including starvation, heat shock and oxidative stress. There are three separate methods of autophagy termed microautophagy, macroautophagy and chaperone-mediated autophagy[459]. Microautophagy involves the direct budding of vesicles with cytosolic proteins into the lysosomal lumen, macroautophagy assembles a double membrane vesicle around the cargo (termed an 'autophagosome'), which is then targeted for fusion with lysosomes and chaperone-mediated autophagy imports signal peptide containing proteins via LAMP-2A into the lysosomes[460].

The eukaryotic cell utilises two main degradation systems to recycle macromolecules: the proteosome and the lysosome and in both systems hydrolytic activity is compartmentalised from the surrounding cytoplasm[460]. Next to the proteosome, which is normally associated with the degradation of malfolded and short lived proteins, the autophagic-lysosomal pathway is the only known disposal mechanism of intracellular organelles and protein aggregates too large for the proteosome in eukaryotic cells[460]. Due to the unique ability of autophagy to degrade cellular components of various sizes, it is now understood to be utilised to dispose of microorganisms, such as viruses, bacteria and protozoa that invade the cell[461,462]. In conjunction with this, the autophagy machinery interfaces with most cellular stress-response pathways, including those involved in controlling immune responses and inflammation[463]. This interface entails the direct interaction and cross-talk of autophagy associated proteins and immune signalling molecules as both systems exert direct affects on one another[464]. This complex relationship may underlie why defects in autophagy (through autophagy gene mutation and/or

microbial antagonism) lead to the pathogenesis of many infectious diseases and inflammatory disorders.

Autophagy is an important mechanism of cellular homeostasis and has also been shown to act as an effector for TLR-mediated innate and adaptive immune responses[465]. In respect to the functions of ES-62, this process has become of particular interest due to its ability in providing a negative feedback mechanism for dampening down LPS/TLR4-mediated pro-inflammatory responses. Thus the mechanisms and immunological functions of autophagy will be outlined, particularly in relation to the mechanisms which ES-62 may exploit.

4.1.3.1 The mechanisms of autophagy

Over the last decade there has been an intense interest in the involvement of macroautophagy, which from this point forward will be referred to as 'autophagy', in immune mechanisms and pathogen removal. This is because the same autophagic machinery used to selectively degrade cytosolic components is utilised in the destruction of microorganisms by redirecting their surrounding phagosome to the lysosomal pathway[466]. In this respect, autophagosomes have now been shown to fuse with endosomes, multivesicular bodies and MHC class II loading compartments[467]. The understanding of the membrane dynamics of autophagosome formation (**Fig. 4.1 A**) has progressed rapidly since the discovery of the ATG (autophagy-related) genes in yeast[468]. The basic dynamics involved in the formation of the double membrane autophagosome begins with the creation of an isolation membrane (or phagophore), which engulfs a portion of the cytoplasm (usually 0.5-1 μm in diameter). The outer membrane of the autophagosome then fuses with a lysosome to become an autolysosome leading to the enzymatic degradation of its contents. Interestingly, recent studies indicate that the endoplasmic reticulum (ER) is crucial for autophagosome formation as electron tomography has demonstrated a direct connection with ER cisternae and autophagosomal membranes[469,470].

The basic molecular mechanisms required for the formation of the isolation membrane and their development into autophagsomes are based on studies of cellular nutrient starvation, through the inhibition of the mammalian target of rapamycin (mTOR). The process is initiated by the translocation of the ULK1 complex (an mTOR substrate), consisting of ULK1/2 (UNC-51-like kinase), ATG13, FIP200 (focal adhesion kinase family interacting protein of 200 kDa) and ATG101 from the cytosol to certain domains of

the ER or closely attached structures[471,472]. The ER-associated multispanning membrane protein, VMP1 (vacuole membrane protein 1) is important at both early and late stages of autophagosome formation due to its interactions with beclin-1[471,473,474]. Specifically, the translocation of the ULK1 complex induces the recruitment of a class III PI3K complex, which includes vacuolar sorting protein 34 (VSP34), VSP15, beclin-1 and ATG14[471,475] to the ER for the localised production of phophatidylinositol-3-phosphate (PtdIns3P). This phospholipid mediates the recruitment of other diffusely distributed effectors such as DFCP1[476] (double FYVE-containing protein 1) and WIPI (WD-repeat domain phosphoinositide-interacting) family proteins, to drive the formation of the ER associated omegasome, a precursor to the isolation membrane[477]. The major isoform of WIPI in many cell types, WIPI2, has been identified as the potential trigger for the development of the omegasome into an isolation membrane[478].

The elongation of the isolation membrane mediated by the ATG12-ATG5-ATG16L1 complex requires the E1-like enzyme ATG7, which activates LC3, a homolog of yeast ATG8 with at least three isoforms, LC3A, –B and –C. ATG7 couples phosphatidylethanolamine (PE) via the E2-like enzyme ATG3 to LC3, converting the soluble LC3-I form to the vesicle associated LC3-II form [468,479]. The shift in LC3 localisation upon the induction of autophagy has been clearly visualised under the microscope, from a diffuse cytosolic distribution to a distinct punctuate pattern associated with the formation of autophagosomes[480]. LC3 has an important role in membrane fusion completing the formation of the autophagosome but also acts as a tether to attach the autophagic cargo to the growing isolation membrane through specific adaptor proteins. For example, p62/SQSTM1 (sequestosome 1), binds polyubiquitinated protein aggregates targeting them for degradation in the autophagolysosomes through its association with LC3-II in the developing autophagosome[460]. In addition to the ER, other membranes sources have been implicated in autophagosome formation including both plasma and nuclear membranes, for which the function of ATG9 maybe essential[481-483]. This multispanning membrane protein is commonly associated with multiple autophagosome precursors throughout the cell and is continual trafficked between the trans-golgi network and endosomes[484]. The details of the complex interplay of proteins involved in mediating autophagic flux is outlined in **Figure 4.1 B**.

4.1.3.2 Selective autophagy and immunity

The selective ability of autophagy allows it to be utilised for the removal of intracellular bacteria, parasites and viruses by phagocytic cells, including DCs and macrophages in a process termed 'xenophagy'[485]. Although the dynamics of this process remain unknown it is capable of forming large vacuoles to accommodate the varying sizes of pathogen. For example, the diameter of group A *Streptococcus*-containing autophagosome-like vacuoles (GcAV) can be as large as 10 μm, markedly bigger than autophagosomes induced in metabolic turnover (0.5-1.5 μm)[486]. These vacuoles are generated by the RAB7-dependent fusion of smaller isolation membranes and indicate a high level of flexibility in the formation of these structures[487]. It is now emerging that autophagy proteins are also important for microbial recognition, where they are recruited to phagosomes to induce phagolysosomal maturation. For example, the self ligand and cell surface receptor SLAM (signalling lymphocytic activation molecule) functions as a microbial sensor that recruits a beclin 1-class III PI3K complex to phagosomes containing Gram-negative bacteria, facilitating phagolysosomal fusion and activation of the antibacterial NOX2 complex[488]. However, autophagy proteins also have autophagosomal independent functions in pathogen removal, as IFN-γ stimulation can induce the ATG5 dependent destruction of *Toxoplasma gondii* vacuoles in infected macrophages[489-491].

The mechanisms utilised by cells to target intracellular bacteria to autophagolysosomal compartments are similar to those utilised for the selective autophagy of endogenous cargo. These include interactions with molecular tags (such as polyubiquitin), adaptor proteins such as p62 or NBR1 (neighbour of BRCA1 gene 1; which recognise these tags and contain an LC3-interacting region (LIR) characterised by a WXXL or WXXI motif) and LC3[485]. In particular, adaptor proteins are important for the selective targeting of designated cargo (endogenous and pathogenic) to newly forming LC3-positive isolation membranes. For example, a mechanism involving ubiquitin and p62 is required for the transfer of intracellular bacteria such as *Salmonella typhimurium*, *Shigella flesneri* and *Listeria monocytogenes* to the autophagosomes[492].

As autophagy is a constitutive process, it has an important role in the continuous input of both endogenous and pathogenic Ags for presentation by MHC complexes on APCs[279]. It is for these reasons that autophagy has been observed to have roles in the enhancement of Ag donor cell cross-presentation to $CD8^+$ T cells[493], DC cross-presentation of phagocytosed Ags to $CD4^+$ T cells[494], and MHC class I presentation of intracellular Ags

to $CD8^+$ T cells[481]. Interestingly, a role for the autophagic delivery of endogenously synthesised Ags has been demonstrated to be important for the MHC class II presentation of certain viral Ags, *in vitro*[493]. It has been proposed that in the process of the generation of peptides for presentation to $CD4^+$ T cells, autophagy-protein-dependent processes allow this machinery access to the interior of the phagosome (much like with autophagosomes). In this respect, the specific deletion of *Atg5* in the DCs of mice infected with HSV (herpes simplex virus) and *Listeria* results in defects in the priming of $CD4^+$ T-cell responses, where the mice succumb more rapidly to lethal disease after intravaginal HSV infection[494]. These DCs specifically showed defects in phagosome-to-lysosome fusion and the cross presentation of MHC class II molecules of phagocytosed Ags containing TLR ligands, with no affect on the activity and cross-presentation of peptides by MHC class I molecules.

4.1.3.3 TLR and cytokine induced autophagy

So how do immune system cells modulate autophagy? Various cytokines and receptor signalling pathways have been implicated in modulating this mechanism, including those of the TLRs, CD40L, TNF-R, TRAIL, TNF-α and IFNs[460,495]. There is now mounting evidence, that TLR2, -3, -4, -7/8 and -9 accelerate the process of phagocytosis and up-regulate the autophagic machinery, as indicated by the increased prevalence of LC3 positive organelles upon positive TLR signalling[496,497]. In this context, both TLR4 and -7/8 mediate the strongest induction of autophagosome formation[460]. In conjunction with this, signalling through TLRs leads to the activation of several PI3Ks implicated in regulating autophagy, which help in modulating the inflammatory response[495]. TLR-dependent autophagy generally requires several hours to occur (spanning 2 to 24 h), suggesting that a TLR mediated transcription program might be involved in the accumulation of mulitlamellar structures in the cytoplasm, with the objective of surveying the cell interior for infectious agents that may have escaped into the cytosol[495,496]. Interestingly, as the PKCs are known to be important in TLR functions, a conserved role for PKCδ in the regulation of gram-negative bacterium degradation by autophagy in mammalian cells has been demonstrated in a DAG and Ubiquitin-dependent manner[498].

In response to TLR ligand stimulation, LC3 can be directly recruited to the surface of the phagosome in a process termed LC3 associated phagocytosis (LAP). This process promotes the rapid maturation of the phagosome as it does not require the formation of the double membrane phagophore as with conventional autophagosomes[495]. In conjunction with this, several reports implicate TLR ligand signalling in the recruitment of both LC3

and beclin-1 to conventional phagosomes, followed by the activation of several PI3Ks, which accelerates their maturation and destructive capacity[279]. Collectively, it is becoming clear that LAP reduces opportunities for microorganisms to subvert their degradation and the subsequent pathogen associated peptide generation for presentation by MHC complexes on APCs. In this context, TLR induced autophagy also enhances peptide presentation on MHC class II molecules, directing the fusion of phagosomes that contain TLR-ligand-enveloped particles with lysosomes[496,497]. For example, induction of autophagy in Macrophages and DCs with LPS or rapamycin enhanced presentation of mycobacterial Ag on MHC class II molecules[499].

The induction of such responses by TLRs occurs through the actions of their direct signalling adaptors MyD88 and TRIF. Both of these adaptors associate with beclin-1, a key autophagy-associated protein, which is upregulated upon TLR activation[279]. However, there is also an important role for the downstream effectors of TLR4-TRIF induced signalling pathways, as the pharmacological inhibition of p38[497] or knockdown of RIP-1 completely blocks conventional LPS induced autophagy. Consistent with this, LPS induces the up-regulation of several autophagy genes, LC3, Atg12 and Atg5[495]. However, p38 inhibitors only have a moderate effect on the recruitment of LC3 to phagosomes, indicating that p38 has a limited role in TLR-dependent LAP[496].

Several cytokines have been shown to regulate autophagy including the type I and –II IFNs, TNF-α and the T_H2 cytokines IL-4 and IL-13. The type I IFNs tend to induce autophagy for the clearance of viruses such as HSV1, while type II has been shown to directly up-regulate autophagosome formation for clearance of mycobacterium[460]. Interestingly, the ability of TNF-α to stimulate autophagy only seems to occur in cells with low levels of NF-κB activation[500]. In general, the T_H1 polarising pro-inflammatory cytokines tend to promote autophagy, while the T_H2 cytokines such as IL-4 and IL-13 appear to block its induction[501]. The rationale behind such a paradigm is that as T_H2 cytokines stimulate humoral based immunity there is no requirement of autophagy to remove intracellular pathogens. Interestingly, it has been hypothesised that the cytoplasmic receptors which recognise PAMPs provide docking sites for the formation of the isolation membrane, as suggested by studies with *Franciscella tularensis*. Although this gram-negative bacterium escapes the phagosome, it is still eventually found in autophagosomes and it is thought its engagement with NALP3 (Nacht domain-, leucine-rich repeat-, and PYD-containing protein 3), a member of the NOD-like receptor (NLR) family is required for this engulfment by autophagosomal vesicles[502].

4.1.3.4 The link between autophagy and NF-κB signalling; p62

The binding of p62 to a cleaved and post-translationally modified form of LC3 termed, LC3 type II (LC3-II), an important component of the autophagosome membrane, allows it to deliver ubiquitinated proteins into the autophagosome for degradation[503]. Interestingly p62 (also known as the 'sequestrome' or SQSTM1) is not only important in the propagation of autophagy but is also an adaptor/scaffold protein crucial for selective signal transduction, preventing non-specific access of enzymes to irrelevant substrates. p62 has a number of domains that allow it to participate in a diverse range of cellular processes including inflammation, neurogenesis, osteoclastogenesis and T cell differentiation. These include: a PB1 (Phox and Bem1p-1) domain, enabling its association to the homotypic region in atypical PKCs, a ZZ zinc finger that binds to the TNF-α signalling adaptor RIP1, a TBS sequence for binding to TRAF6 and finally a UBA (ubiquitin associated) domain allowing it to bind to mono- and poly-ubiquitin chains[504].

It is these multiple binding domains that are important for the proposed role of p62 in NF-κB activation as well as its trafficking of proteins into autophagosomes. For example, it has been shown that TLR induced activation of p62 and its subsequent interactions with TRAF6 help to coordinate orderly regulation of ubiquitin mediated processes in activated macrophages, ensuring cytokine secretion associated with NF-κB activation[505]. The importance of this p62-TRAF6 interaction for the modulation of NF-κB activation has also been shown in a number of different systems including RANK (receptor activator of NF-κB) triggered osteoclasts[506]. Thus, it is becoming clear that p62 acts as a scaffold to regulate K63 poly ubiquitination via its interaction with TRAF6 and subsequently NF-κB activation[507]. In this respect, p62 and TRAF6 have been co-localised to discrete punctuate structures upon stimulation of p62 signalling pathways[508]. Several of the identified affects of p62 have been summarised in **Figure 4.2**.

4.1.4 Aims of this study

The immunomodulation by ES-62, most notably through its degradation (via both proteosomal and lysosomal routes) of several PKC isoforms is an important aspect of its affect on a variety of immune cell types[301,320,321,333]. Thus, based on these observations a major aim of this section was to define the role of these multi-functional kinases in ES-62 modulation of DC responses, particularly in relation to its affects on proinflammatory cytokine secretion. Due to the previously defined induction of PKC degradation by ES-62

through both protesomal and lysosomal routes, another objective of this study was to identify if a similar mechanism was employed in DCs. The investigation also incorporated the characterisation of the effect of LPS on such signals and their potential subversion by ES-62, to provide a comparison between how the expression of these proteins varies upon TLR4 recognition of both pro-inflammatory and anti-inflammatory ligands.

There has been a wealth of information in the recent literature defining the roles of ubiquitination and the mediators of this process, as the E3 ubiquitin ligases, in the downstream transduction of TLR4 coupled activation of NF-κB. Of particular interest to the immunomodulatory affects of ES-62 are the E3 ligases TRAF6 and c-Cbl, both of which are important in regulating the activation of NF-κB[24,451,452] and subsequently the secretion of IL-12 p70 (and IL-6) from DCs[26,27,454]. In this context, the regulation of IL-12p70 (and its subunit IL-12/23p40) secretion is an important aspect of ES-62 immunomodulation of macropahges and DCs in response to inflammatory stimuli (LPS). Thus, this study also set out to identify ES-62's affect on TRAF6 and c-Cbl expression, which was coupled with an investigation of whether ubiquitination has a role in such responses.

Autophagy is a cellular process that targets intracellular proteins for lysosomal degradation, which is commonly utilised in TLR-mediated innate and adaptive immune responses[461,465]. In this context, recent evidence suggests it could provide a negative feedback mechanism for dampening down of LPS/TLR4-mediated pro-inflammatory responses[465]. It was therefore hypothesised that ES-62 could induce/subvert autophagic flux for the trafficking and degradation of TLR4 and its associated signalling adaptor proteins in the lysosomes. This proposal was investigated in this study relative to the effects of proinflammatory TLR4 signalling (LPS).

In brief, due to the emerging roles for the E3 ubiquitin ligases, PKCs and autophagy proteins in the TLR4 signalosome the aims of this study were to define whether:

- ES-62 and LPS differentially modulated the expression of E3 ubiquitin ligases, PKCs and autophagy proteins implicated as components in the TLR4 signalsome in DC

- ES-62 and LPS differentially trafficked TLR4 signalsome components for proteosomal and lysosomal degradation

- ES-62 subverted TLR4-coupled autophagy-mediated negative feedback mechanisms to induce an anti-inflammatory phenotype

- ES-62-mediated degradation of TLR4 signalsome components correlated with suppression of DC pro-inflammatory responses

4.2 Results

4.2.1 Activation of NF-κB and cytokine secretion, the differential effects of LPS & ES-62

As a first step to investigate the molecular changes initiated by ES-62 and LPS on the activation of NF-κB, their effects on two common isoforms of the inhibitory component (IκB) of the complex were analysed. It was observed that LPS induced the early degradation of both IκB-α and -β within 2 h of stimulation (indicating NF-κB activation), after which their levels returned to basal by 18 h (**Fig. 4.3**). By contrast, ES-62 had little or no effect on either IκB-α and -β expression over an 18 h time-course, consistent with its noted lack of induction of cytokine responses (**Fig. 3.11**). This result was found to be representative of the effect of ES-62 on the expression of both isoforms of IκB, from the collated data of several experiments (**Fig. 4.4 A-B**). To address how LPS was inducing the degradation of IκB and whether ES-62 was stabilising its expression, inhibitors of vesicular transport (ammonium chloride[NH_4Cl]), lysosomal degradation (E-64-D/Pepstatin A and ammonium chloride) and proteosomal degradation (lactacystin), were utilised to determine whether blocking of such signals could convert ES-62-TLR4 signalling to an LPS-TLR4 phenotype or vice versa (**Fig. 4.3 & Fig. 4.4 C-D**).

The results indicated that in blocking lysosomal degradation and interfering with vesicular transport (ammonium chloride), IκB-α accumulated upon LPS stimulation, although this was reduced to basal levels after 18 h (**Fig. 4.3**). This perhaps suggests that under these conditions the cell switches to the proteosome to degrade this protein. On the other hand, treatment with E-64-D/Pepstatin A did not alter the early (2 h) transient degradation of either IκB-α or IκB-β but it did prevent their protracted downregulation (≥ 6 h). Under these conditions the suggested lysosomal component of IκB-α and IκB-β degradation could be due to the sustained TRIF-mediated NF-κB activation phase[13]. Here, treatment of the cells with lactacystin did not yield conclusive results due to the toxicity of this compound (as demonstrated in **Fig. 2.1)** as cells treated post 18 h displayed marked variation of protein expression, as defined from the loading control (results not shown). Thus, although this could not be confirmed by the use of lactacystin, it was assumed that the early degradation of IκB induced by LPS (within 2 h), was occurring through the proteosome as this is commonly reported in the literature[330]. The disparity between the effects of ammonium chloride and E-64-D/Pepstatin A suggest the former's subsidiary modulation of vesiclular transport[354-357], in particular the internalisation of cell surface

proteins[358-360], may have a direct effect on the internalisation and function of TLR4 upon LPS recognition. Consistent with the finding that ES-62 did not markedly alter IκB expression, inhibition of lysosomal degradation (E-64-D/Pepstatin A) did little to alter this result (**Fig. 4.3**). However, ammonium chloride did change the dynamics of ES-62 mediated regulation of IκB-α, inducing the gradual accumulation of the protein, which could be attributed to the multiple effects of this agent on cellular homeostasis.

To further define whether ES-62 had any regulatory effects on the expression of IκB-α upon LPS stimulation, combinations of these TLR4 agonists were utilised in conjunction with E-64-D/Pepstatin A, ammonium chloride and lactacystin (**Fig. 4.4** and **Fig. 4.5**). In **Figure 4.5 A-B**, the immature DCs (unstimulated control) yielded a lower expression of IκB-α than would be expected from unstimulated cells as observed in other samples (**Figs. 4.3-4.4**). As such, due to the negligible effects of ES-62 (18 h) on IκB-α expression identified over several experiments, the ES-62 treated sample (Media 18 h + ES-62 18 h) was utilised as the basal control from which the expression of the others were adjusted to. This analysis revealed ES-62 did not inhibit the downregulation of IκB-α in response to LPS, a result unaltered by inhibiting lysosomal degradation, despite previous studies suggesting that this immunoodulator acts to suppress the early (< 18 h) LPS mediated downregulaiton of IκB[291,319] (**Fig. 4.3**).

In this context, ES-62 may act to target hyper rather than normal inflammatory responses. In the above experiments, GM-CSF, the cytokine responsible for the differentiation of DCs, was removed during their maturation with LPS and/or ES-62. This cytokine, in addition to being a key factor in the generation of immature DC from progenitor cells is strongly elevated in pro-inflammatory conditions[509]. Therefore these experiments were repeated with GM-CSF maintained in the media to mimic a pro-inflammatory environment. This approach revealed an interesting pattern of IκB-α and -β expression upon stimulation of bmDCs with both ES-62 and/or LPS (**Fig. 4.6**). For example, ES-62 treatment induced a cyclical expression of IκB-α and -β. Specifically, for IκB-α, the prior maturation of DCs with ES-62 consistently lowered its expression in response to LPS, the greatest of these effects occurring after a 3 h stimulus. By contrast, under the same conditions ES-62 up-regulated the expression of IκB-β.

4.2.2 ES-62 modulation of key proteins in the propagation of TLR4 pro-inflammatory signals; MyD88 and TIRAP

Having established that the TLR4 ligands, LPS and ES-62 were differentially modulating IκB expression, the next step was to analyse their effect on the direct signalling transducers TIRAP and MyD88, which are important for the early activation of NF-κB and several MAPKs (ERK, JNK and p38). Given that previous studies with ES-62 and MyD88$^{-/-}$ mice indicated this adaptor was required for the ES-62 induced low level production of cytokines by macrophages[301,331], it was hypothesised that MyD88 and TIRAP were potential targets of this immunomodulator in DCs.

Western blot analysis of MyD88 expression indicated ES-62 transiently (within 6 h) upregulated this adaptor, albeit only slightly (**Fig. 4.7 A-B**), where over several experiments this was also found to be the case after an 18 h stimulation as well (**Fig. 4.7 C-D**). By contrast, after an initial downregulation (2 h) LPS also stimulated the reproducible upregulation of MyD88 after 18 h (**Fig.4.7 A-D**), a finding supported by flow cytometry of cells stained intracellularly with the same polyclonal antibody from the Western blotting study (**Fig. 4.8 B-C**). Taqman RT-qPCR revealed that MyD88 mRNA expression peaked at 6 h to 3.957 ± 0.641 % above the endogenous GAPDH control in response to LPS (**Fig. 4.8 D**), which precedes the increase in MyD88 protein at 18 h. Consistent with its minor induction of MyD88 protein expression (between 6-18 h), ES-62 had little or no effect on upregulating mRNA transcript. In this respect, ES-62 was observed to transiently increase TIRAP (MAL) expression in a similar fashion to MyD88 (**Fig. 4.9 A-B**). However, neither TLR4 ligand significantly modulated TIRAP expression after a prolonged stimulation of 18 h (**Fig. 4.9 C-D**).

In relation to the results above, when the expression levels of MyD88 and TIRAP were investigated following the pretreatment of the bmDCs with E-64-D/Pepsatain A, ES-62 but not LPS stimulation induced a considerable level of lysosomal turnover of these proteins (**Fig. 4.10**). Indeed, blocking of lysosomal degradation converted the levels of MyD88 expression in not only ES-62-treated but unstimulated DCs to levels approaching that observed in LPS-matured cells (**Fig. 4.10 A-B**). By contrast, this treatment slightly reduced MyD88 upregulation by LPS, with a similar pattern observed for TIRAP, although the inhibitor-induced reduction in LPS-associated expression was more pronounced (**Fig. 4.10 C-D**). Collectively, these results suggest that ES-62 (or immature DCs) may actively suppress MyD88 levels to prevent aberrant (hyper)inflammatory responses and that LPS

traffics some undefined negative regulator of MyD88 and TIRAP through a lysosomal route to maintain such affects. Alternatively, as a high turnover of the protein occurs through the lysosomes, by pharmacologically shutting off this route of degradation, upon a strong LPS signal, the cells may shunt these functions to the proteosome.

Due to the potential of ES-62 modulating its cellular effects dependeing on the inflammatory environment it was decided to analyse MyD88 expression with the addition of GM-CSF to the stimulation media. Interestingly, these preliminary results suggested that when GM-CSF was present (**Fig.4.7 E-F**), ES-62 induced the down-regulation of MyD88 expression whilst LPS maintained its upregulation of this adaptor. These differential effects are consistent with previous studies in this laboratory showing that ES-62 downregulates MyD88 expression in a variety of cells including: T cells undergoing IL-1-driven T_H17 polarisation[352], Bm derived macrophages from BALB/c and MRL/Lpr mice and human monocytes (unpublished data).

4.2.3 Roles for the E3 Ubiquitin ligases, TRAF6 and c-Cbl, in disruption of TLR4-NF-κB based inflammation by ES-62

The differential effects of LPS and ES-62 on MyD88/TIRAP expression suggested that ES-62 was indeed targeting components of the TLR4-NF-κB pathway to imprint its anti-inflammatory effects on DCs. Therefore, the effects of both TLR4 ligands were analysed on the E3 Ubiquitin ligases, TRAF6 and c-Cbl, which are involved in both MyD88-dependent and independent transduction of TLR4 signals. It was hypothesised that based on ES-62-mediated reduction of IL-12p70, IL-23 and TNF-α secretion upon LPS challenge (**Fig. 3.11**), this immunomodulator would down-regulate or otherwise impair the function of TRAF6. In the case of c-Cbl it was hypothesised that it may up-regulate this E3 ubiquitin ligase to stabilise the NF-κB inhibitory complex, preventing effective secretion of pro-inflammatory cytokines.

Both ES-62 and LPS exhibited differential effects on TRAF6 expression with LPS transiently downregulating this protein, which was then steadily replenished to levels above basal (unstimulated cells), while in contrast, following the early upregulation of TRAF6 (2 h), ES-62 then induced its marked reduction (18 h; **Fig. 4.11**). To dissect the mechanisms underlying such differential responses, the effects of these TLR4 ligands was again addressed using the inhibitors E-64-D/Pepstatin A, ammonium chloride and lactacystin. Although E-64-D/Pepstatin A was unable to prevent the degradation of TRAF6

in response to LPS at the 2 h time point (indeed it accentuated it), ammonium chloride had little effect on LPS-induced modulation, acting only to prevent its upregulation after an 18 h treatment (**Fig. 4.11 A-B**). By contrast, lactacystin clearly prevented the reduction of TRAF6 levels in response to LPS, suggesting that endotoxin directs its rapid degradation through the proteosome. Therefore, in support of the hypothesis of differential regulation by the TLR4 ligands, both E-64-D/Pepstatin A and ammonium chloride were able to prevent the oscillating pattern of TRAF6 expression observed in response to ES-62 (**Fig. 4.11 A** & **C)**. In respect to E-64-D/Pepstatin A, a steady time-dependent increase in the expression of this E3 ubiquitin ligase was observed, treatment with ammonium chloride resulted in a similar accumulation although not as marked. In addition, although delaying the effects of ES-62, lactacystin did not ultimately prevent the degradation of TRAF6. Collectively, these data suggest that lysosomal degradation plays a role in ES-62-, but not LPS-mediated down-regulation of TRAF6, which appears to be predominantly occuring via the proteosome. The dynamics of TRAF6 protein expression in response to LPS and ES-62 are consistent with their modulation of its mRNA expression. Thus, ES-62 induced an early increase in the mRNA transcript, within 6 h of stimulation, followed by a return to basal levels by 18 h (**Fig. 4.11 D**). While in contrast, LPS gradually increased TRAF6 mRNA expression progressively during the course of treatment, not reaching maximal production till 18 h of stimulation.

Upon further investigation of the protracted effects of these TLR4 agonists on TRAF6 epxression and the role of the lysosomes in their regulation, both immature and ES-62- but not LPS-treated DCs diplayed a low level of lysosomal turnover of this E3 ligase (**Fig. 4.12**). Indeed, the inhibition of lysosomal degradation (E-64-D/Pepstatin A) converted the ES-62 response to resemble that of LPS (albeit not as strong). Although, when the length of treatment was extended to 36 h the expression of TRAF6 returned to just above basal levels. Rather unexpectedly, prior maturation with ES-62 only marginally suppressed the upregulation of TRAF6 in response to LPS, with E-64-D/Pepstatin A having little effect.

The collated data from several experiments demonstrated that overall, TRAF6 was significantly ($p < 0.05$) and reproducibly downregulated by ES-62 (0.61 ± 0.12, n=6) within an 18 h stimulation relative to its strong up-regulation by LPS (1.99 ± 0.21, n=6) (**Fig. 4.13 A-B**). These downregulatory effects of ES-62 were ablated upon pre-treatment of the cells with either E-64-D/Pepstatin A (1.62 ± 0.31 n=3) or ammonium chloride (1.381 ± 0.41, n=3), with the former inducing a significantly ($p < 0.05$) greater increase.

Moreover, pretreatment with ES-62 repeatedly decreased the LPS induced upregulation of TRAF6 (1.42 ± 0.37, n=6). In addition, ammonium chloride exacerbated TRAF6 expression upon LPS stimulation of ES-62 matured cells while E-64-D/Pepstatin A was unable to replicate this latter effect (**Fig. 4.13 A** & **C**). Of note, neither inhibitor had any significant effects on LPS induced up-regulation of TRAF6 expression. The mimicking of an inflammatory environment with GM-CSF exacerbated ES-62's downregulation of TRAF6 expression throughout an 18 h time course (**Fig. 4.14 A**-**B**). This effect was maintained (exacerbated even) when the cells were subsequently challenged with endotoxin (**Fig. 4.14 C**-**D**). Interestingly, under the same consitions IκB-β was also up-regulated by ES-62 maturation, which was unaffected by subsequent treatment with LPS (**Fig. 4.14 C** & **E**).

These results demonstrated that ES-62 could suppress aberrant hyper-inflammation through the modulation of TRAF6 expression and similarly, both TLR4 ligands induced diametric effects on c-Cbl expression. LPS stimulated the progressive downregulation of c-Cbl with time (within 18 h), whereas ES-62 dynamically upregulated this E3 ligase (**Fig. 4.15 A**-**D**), a reproducible pattern over several experiments (**Fig.4.15 D**). These changes in protein expression were partially mirrored at the mRNA level, with an increase that plateaued within 6 h of ES-62 treatment (**Fig. 4.15 E**), whilst LPS induced an initial transient increase in mRNA, which did not drop below basal levels. This suggested that the decrease in c-Cbl protein reflected degradation rather than suppression of its production. Consistent with this, pretreatment of the bmDCs with E-64-D/Pepstatin A, ammonium chloride and lactacystin rescued LPS induced downregulation of c-Cbl but it was only the latter compound that led to its accumulation within the cells (**Fig. 4.15 A**-**B**). Rather surprisingly, all of the inhibitors blocked the upregulatory effects of ES-62, perhaps indicating some complex time-dependent interplay between endosomal/proteosomal degradation of TLR4-NF-κB regulatory elements (**Fig. 4.15 A** & **B**).

The differential effects on c-Cbl expression exhibited by these anti-(ES-62) and pro-(LPS)inflammatory TLR4 ligands were reproducible with clear up-regulation and substantial down-regulation of c-Cbl by ES-62 (1.88 ±1.01, n=4) and LPS (0.53 ± 0.24, n=4), respectively (**Fig. 4.16**). Moreover, inhibiting lysosomal degradation with E-64-D/Pepstatin A not only blocked ES-62-mediated upregulation of c-Cbl (0.76 ± 0.14, n=2) but also dramatically reduced the down-regulation observed with LPS (1.048 ± 0.08, n=2). Of interest, pretreatment of the bmDCs with ES-62 prevented the LPS induced down-

regulation of c-Cbl, with its expression (0.86 ± 0.08, n=2) remaining above that induced by LPS (0.53 ± 0.24, n=4). Thus, it was very interesting to find that in the presence of GM-CSF, ES-62 profoundly down-regulated c-Cbl (**Fig. 4.17 A-B**) and the subsequent exposure of such ES-62 matured cells to LPS reversed this downregulation after both a 1 h and 6 h stimulation (**Fig. 4.17 C-D**). Interestingly, LPS in conjunction with GM-CSF stimulated a gradual increase in the levels of c-Cbl over 6 h. Collectively, these results indicate that the effect of immuno-modulatory molecules, such as ES-62 and LPS on the signalling components of the TLRs vary depending on the inflammatory environment of the cell.

4.2.4 The emerging role of PKCs in the TLR4-NF-κB signalling axis and the role of ES-62 in its subversion

The TLR4 agonist, LPS has been shown to activate most PKC isoforms in innate cells such as monocytes, macrophages, DCs and neutrophils[91-95]. In conjunction with this, studies have shown that acute activation of the PKCs with phorbol esters, increases LPS-stimulated cytokine secretion[94,96-98], while their pharmacological inhibition attenuates their production[96,98,99]. In this context, several isoforms, including PKCα, PKCδ and PKCε have important roles in TLR4 induced cytokine secretion, through their direct involvement in the TLR4-NF-κB activation axis in cells of the innate immune system, including DCs. The role of PKCs in mediating many important innate cell functions and the regulation of TLR4 responses, particularly in relation to their involvement in pro-inflammatory cytokine secretion was therefore the basis for our investigation of the targeted subversion of DC function by ES-62. Firstly, the effect of ES-62 stimulation on the expression of a wide range of PKC isoforms was defined (**Fig. 4.18-4.22**), whereby the immunomodulator had little affect on the expression of PKCθ, -ι and -β (**Fig. 4.18**) but was observed to markedly regulate the expression of PKCα (**Fig. 4.19**), -ε (**Fig. 4.20**) and -δ (**Fig. 4.22**).

4.2.4.1 PKCα

With respect to PKCα, treatment with both ES-62 (2.17 ± 0.86, n=2) and LPS (1.28 ± 0.01, n=2) progressively and consistently up-regulated its expression over 18 h (**Fig. 4.19 A-D**). Moreover, when the phosphorylation of PKCα at threonine 638 (Thr638; an auto-phosphorylation site located within the C-terminal 'tail') was analysed over an 18 h time course (**Fig. 4.19 E-F**), it appeared that ES-62 may be transiently (< 6h) activating PKCα, a result consistent with our previous studies in B cells[320,333]. However, ES-62-mediated

upregulation of PKCα was not observed in the pro-inflammatory environment mimicked by the presence of GM-CSF, as levels appear to oscillate around basal levels defined by immature (unstimulated) cells within a 6 h stimulatory window (**Fig. 4.19 G-H**). As PKCα is known to play a role in IL-6 secretion, this result provides an explanation for the lack of ES-62-mediated suppression of this cytokines produciton upon subsequent LPS stimulation (**Fig. 3.11**). This result may go some way to explaining how DCs can maintain their ability to allow immune responses to be initiated to LPS and invading gram negative bacteria, when exposed to parasite immunomodulatory products.

4.2.4.2 PKCε

By contrast, it was hypothesised that the reduction of IL-12p70 secretion by ES-62 in response to LPS could be linked to the downregulation of PKCε and indeed, it was found that ES-62 led to the progressive (**Fig. 4.20**), and reproducible (after an 18 h stimulus; **Fig. 4.21**; 0.56 ± 0.14, n=3) reduction of this isoform. These effects were rescued by pre-treating the bmDCs with the lysosomal inhibitors ammonium chloride and E-64-D/Pepstatin A, the former inducing the cyclical up-regulation of PKCε (peaking 2 h after treatment; **Fig. 4.20 B** & **D**). Interestingly, the rescue of PKCε expression by E-64-D/Pepstain A above basal levels after an 18 h stimulus with ES-62 was found to be a significant and consistent phenomenon (1.45 ± 0.19 n=3; **Fig. 4.21 B**) but the proteosomal inhibitor lactacystin, had no effect on such degradation (**Fig. 4.20 B** & **D**). On the other hand, LPS treatment induced the reproducible upregulation of PKCε expression in response to LPS after an 18 h stimulus (1.49 ± 0.21, n=3; **Fig. 4.21 B**), which remained unaltered by prior treatment with E-64-D/Pepstatin A and ammonium chloride. The differential trafficking/signalling of TLR4 and PKCε relative to ES-62 was apparent when, ammonium chloride pretreatment induced an inverse transient effect after 2 h LPS treatment (**Fig. 4.20 C-D**). Interestingly, pretreatment with lactacystin strongly upregulated PKCε, indicating that in response to LPS, the cell may activate negative feedback mechanisms to prevent its excessive accumulation and the hyper-secretion of pro-inflammatory cytokines.

With respect to the induction of PKCε mRNA transcript production, ES-62 appears to uncouple (to some extent) transcription and translation of PKCε, as the high levels of mRNA at 6 h are not translated into similar levels of protein (**Fig. 4.20, E**). This suggests that in an attempt to maintain homeostatsis upon ES-62 downregulation of PKCε, bmDCs

upregulate mRNA transcript (within 6 h) but its turnover through the lysosomes may negate these compensatory effects. Interestingly, LPS induced the up-regulation of both PKCε protein and mRNA transcript expression throughout an 18 h treatment. The prior maturation of bmDCs with ES-62 had a slight inhibitory affect on the LPS induced upregulation of PKCε (1.20 ± 0.30, n=3), which was rescued (albeit not significantly) by pretreatment with E64-D/Pepstatin A (1.57 ± 0.07, n=3). These data reinforce the concept that ES-62 maturation of DCs does not markedly inhibit the cell from responding to normal inflammatory stimuli.

4.2.4.3 PKCδ

The results for PKCε, based on the current literature, may provide a direct link between ES-62's modulation of cytokine secretion in DCs and its down-regulation of components in the TLR4-NF-κB signalling axis. However, in respect to direct NF-κB activation, PKCδ has been shown to play a crucial role for the TLR4 mediated activation of IKK[101], with recent evidence showing it can phosphorylate IκB (which masks the NLS of NF-κB) allowing the nuclear translocation of NF-κB[104,105]. Thus, the effect of PKCδ expression and the potential avenues of its degradation by ES-62 in its attenuation of proinflammatory cytokine responses were investigated. Over several experiments, ES-62 was observed to transiently induce a slight but non-significant up-regulation of PKCδ expression through 2 h (1.09 ± 0.21, n=3), which was significantly down-regulated after 6 h (0.53 ± 0.07, n=3) and maintained at these lower levels over an 18 h stimulation (0.48 ± 0.05, n=6) (**Fig. 4.22 A** & **E**). The effects of LPS were more subtle, with a slight, but non-significant downregulation observed within 2 h of stimulation (0.80 ± 0.07, n=3), that slowly recovered to basal levels over an 18 h treatment (0.98 ± 0.12, n=3) (**Fig. 4.22 B** & **E)**. In a similar fashion to observations made with PKCε, ES-62 appeared to uncouple the translation of rising levels of PKCδ mRNA transcript 2 h post stimulation (**Fig. 4.22, F**). In relation to LPS, the increasing levels of PKCδ transcript may reflect the recovery of protein expression over the 18 h stimulation. Again, the downregulatory effects of ES-62 could be rescued by the pre-treatment of the cells with the lysosomal degradation inhibitors E-64-D/Pepstatin A and ammonium chloride (**Fig. 4.22 A** & **D**) but it is important to note that, unlike PKCε, treatment with E-64-D/Pepstatin A induced the accumulation of the protein, perhaps reflecting the steady increase of mRNA transcript observed over time. However, similarly to that observed for PKCε, ammonium chloride treatment led to the cyclical up-regulation of PKCδ.

The pronounced downregulatory effect of ES-62 on PKCδ expression (0.48 ± 0.05, n=6), by the lysosomal inhibitors, E-64-D/Pepstatin A (1.30 ± 0.05, n=3) and ammonium chloride (1.82 ± 0.54, n=2) were consistent and reproducible (**Fig. 4.23**). In relation to this, when ES-62 matured cells were stimulated with LPS, although not significant, PKCδ expression tended to be reduced below that observed with endotoxin alone and rather unexpectedly, treatment with E-64-D/Pepstatin A had little effect on this trait. Again LPS stimulation had little affect on the long-term expression of PKCδ, with neither lysosomal degradation inhibitor able to change this outcome but lactacystin considerably up-regulated the levels of PKCδ, while not preventing its degradation indued by ES-62 (**Fig. 4.22 B-C**). In this context, the lysosomal inhibitors did not drastically alter the LPS response, although there appeared to be an inverse transient effect at 2 h with ammonium chloride treatment. This suggested that differential trafficking of TLR4 and subsequently PKCδ signalling occurs between ES-62 and LPS. In a similar fashion to PKCε the strong up-regulation of PKCδ in response to the inhibition of proteosomal degradation indicated that in response to LPS, the cell may activate negative feedback mechanisms to prevent its excessive accumulation and the hyper-secretion of pro-inflammatory cytokines.

4.2.4.3.1 The modulation of PKCδ localisation and cleavage by ES-62

The spatiotemporal localisation of PKCδ is very important in regulating its function and activation as it is not restricted to the cytoplasm and is often detected in the nucleus of many cell types, where its translocation is mediated by a bipartite NLS (6 basic amino acids) in its C-terminus[52]. Interstingly, PKCδ is often cleaved upon various cellular stimuli where both the enzymology and roles of the full length and cleaved catalytic domain fragment, which is phospholipid independent and constitutively active, differ considerably[57,58]. Thus, to investigate whether ES-62 induces the cleavage and redistribution of these two different formats of PKCδ from the cytoplasm to the nucleus in bmDCs, nuclear and cytoplasmic preparations of cells stimulated with ES-62 over 18 h were analysed by Western blotting (**Fig. 4.24**). After the normalisation of each result to the relevant control, the stimulation of bmDCs with ES-62, was observed to induce the transient increase in the nucleus of both full length (78 kDa) and cleaved (38 kDa) forms of PKCδ (**Fig. 4.24 A-C**). This was accompanied by a compensatory decrease in the levels of PKCδ in the cytoplasm. This experiment also provided the opportunity to investigate the internalisation and localisation of ES-62 in bmDCs as it is known to be internalised and trafficked to the nucleus of macrophages and B cells (H.S. Goodridge & M. M. Harnett,

unpublished data). After several experiments confirming that ES-62 was internalised by bmDCs after 18 h of stimulation (for example; **Fig. 4.24 D**), the accumulation of the protein in the cytoplasm but not the nucleus was confirmed (**Fig. 4.24 A**). Though lacking in similar subcellular fractionation data for LPS, a clear movement of PKCδ from the cytoplasm into the nucleus upon LPS treatment (18 h) was visualised utilising confocal microscopy in bmDCs (**Fig. 4.25**), where its diffuse expression in resting cells was redistributed to the nucleus (though a cytoplasmic component is still evident).

The data from several ES-62 and LPS stimulations over an 18 h time course revealed both, but particularly LPS, induced the dynamic regulation of PKCδ cleavage in the cell (**Fig. 4.26 A-B**). Endotoxin progressively down-regulated its expression throughout the time course, with the lowest levels observed 6 hours after stimulation (0.048 ± 0.018, n=3). On the other hand, ES-62 transiently increased its expression (peaking at 2 h; 1.22 ± 0.04, n=3) before its downregulation, with the percentages of cleaved PKCδ relative to the total amounts (cleaved and full length protein) in the cell revealing it induced a small but steady increase in the relative amounts of cleaved PKCδ (**Fig. 4.26 B**). However, this is not the case upon LPS stimulation where the large decrease in the levels of cleaved PKCδ is not reciprocated with the full length protein.

Interestingly, the results from the addition of the proteosomal (lactacystin) and lysosomal inhibitors (E-64-D/Pepstatin A and ammonium chloride), revealed differential effects, which were dependent on the nature of the TLR4 stimulus. Inhibition with either E-64-D/Pepstatin A or ammonium chloride did not prevent the long-term degradation of cleaved PKCδ by ES-62, however they did prolong (from 2 to 6 h) and enhance its transient up-regulation (**Fig. 4.26 A** & **C**). The relative percentages of cleaved to total cellular PKCδ, reveal both lysosomal inhibitors induce the transient accumulation of the cleaved form (though ammonium chloride sustains this until 6 h, unlike E-64-D/Pepstatin A), which is followed by a sharp decrease after stimulation for 18 h. Thus, in the absence of lysosomal degradation, ES-62 induces a reduction of cleaved to total cellular PKCδ after 18 h, although this may simply reflect the inhibitors preventing the 78 kDa form of PKCδ from being down-regulated. On the other hand, lactacystin accentuates the degradation of cleaved PKCδ and as revealed by analysis of their relative proportions, there is some correlation between the transient degradation and recovery of both the cleaved and full-length protein (**Fig. 4.22**). By contrast, LPS stimulation induces the marked reduction of cleaved PKCδ (**Fig. 4.26 A** & **D**), while of the lysosomal degradation inhibitors, only E-

64-D/Pepstatin A prevented a small fraction of the LPS induced down-regulation of the cleaved protein. However pre-treating the cells with lactacystin reversed the LPS induced effect while mediating a dramatic transient increase of cleaved protein relative to the total amounts of PKCδ within the cell. Although the cleaved form is a very minor component by 18 h, it suggests that this transient protection may reflect prevention of proteosomal degradation of full length PKCδ while that of the cleaved PKCδ is being processed via a non-proteosomal route. Hence, LPS differentially modulates these distinct forms of PKCδ.

The long-term effects of ES-62 and LPS on the expression of cleaved PKCδ (18 h) were reproducible over several experiments (**Fig. 4.27**) and the maturation of the bmDCs with ES-62 did not modulate its down-regulation in response to LPS. In this context, it was clear that ES-62 was consistently down-regulating both the whole and cleaved forms of PKCδ, as the percentage of the cleaved relative to the total amounts in the cell remained constant (**Fig. 4.27 C**). The collected data for the effects of the proteosomal (lactacytsin) and lysosomal/endosomal trafficking (ammonium chloride) inhibitors confirmed that inhibition of the proteosome rescued down-regulation of cleaved PKCδ and induced its accumulation in response to LPS (**Fig. 4.27 D-F**).

4.2.4.3.2 Phosphorylation of PKCδ

The phosphorylation of PKCδ at various sites is very important in influencing its localisation, stability, protease/phosphatase resistance, protein-protein interactions and substrate specificity[52,72]. This isoform is unusual among the PKC family, however, as it can function as a kinase even without phosphorylation of its A-loop (at Threonine 505 [Thr505]), which is crucial for the other isoforms[71]. However, phosphorylation at the A-loop region is associated with inducing its cleavage and helps to regulate PKCδ substrate specificity, rather than absolute activity of both the cleaved and full length protein. PKCδ can also be phosphorylated at several other conserved C-terminal sites, namely the transmembrane (TM) and hydrophobic motifs (HM) through both auto-phosphorylation and transphosphorylation mechanisms (depending on the isoform and context). The auto-phosphorylation site, Ser643 in the TM is important for maintaining certain catalytic functions of the protein[510,511]. PKCδ also has several sites outside the C-terminal tail that are phosphorylated to a range of diverse stimuli and are important in the protein's function[53,73]. For example, phosphorylation of the Tyr311/Tyr332 residues in the hinge region, which can be mediated via Src tyrosine kinase, provides docking sites for several signalling molecules on PKCδ[52].

Based on these findings the effect of LPS and ES-62 on the state of PKCδ phosphorylation in bmDCs was investigated (**Fig. 4.28 – 4.30**). Both TLR4 agonists induced the phosphorylation of the A-loop on the full length protein (78 kDa), which was consistent with its degradation, while ES-62 specifically stimulated a higher transient increase through 2 to 6 h (**Fig. 4.28 A-B**). Upon longer exposures, LPS was observed to regulate the phosphorylation of the A-loop on both cleaved (38 kDa) and whole (78 kDa) PKCδ, with LPS inducing a transient down-regulation of A-loop phosphorylation over 6 h of stimulation, suggesting that the low levels of cleaved PKCδ remaining at 18 h must be heavily phosphorylated at this site, perhaps indicating it could be associated in its targeting for degradation. It should be borne in mind, however, that these results could reflect either the direct phosphorylation of cleaved PKCδ or the full length protein (78 kDa), which is subsequently cleaved. The relative levels of phosphorylated PKCδ to the total protein in the cell also suggest that ES-62 may utilise this mechanism to downregulate levels of full length PKCδ in the cell (**Fig. 4.28 A** & **C**). The up-regulation of this type of phosphorylation of PKCδ over several experiments by both TLR4 agonists even after 18 h of stimulation was found to be significant for LPS (2.79 ± 0.92, n=4) and consistently reproducible for ES-62 (1.765 ± 0.23, n=3) (**Fig. 4.28 D-E**).

Subsequently, the phosphorylation of PKCδ at a further two sites were investigated, Ser643 and Tyr311, as these are important in maintaining catalytic stability and directing protein-protein interactions, respectively. These studies were performed in conjunction with E-64-D/Pepstatin A, to identify whether lysosomal degradation is associated with these phosphorylated forms of PKCδ. Treatment with ES-62 was found to result in Ser643 phosphorylation above that of resting cells after 36 h of stimulation, while for LPS, this was observed at 18 h, but not 36 h (**Fig. 4.29 A** and **C**). Maturation of the bmDCs with ES-62 had negligible effects upon subsequent LPS induced up-regulation of Ser643 phosphorylation. In this context, pre-treatment of the cells with E-64-D/pepstatin A changed the dynamics of these effects. For example, blocking of lysosomal degradation induced transient (18 but not 36 h) accumulation of ES-62 stimulated Ser643 phosphorylated PKCδ. Thus it appeared to convert ES-62 to a more LPS-like phenotype although this treatment acted to suppress the LPS response, perhaps by increasing the kinetics as ES-62 induced an increase in the levels of Ser643 phosphorylated PKCδ relative to the total amount in the cell and this effect is similarly abolished after 36 h of stimulation by E-64-D/Pepstatin A (**Fig. 4.29 D**).

Under non-stimulatory conditions the dynamics of the phosphorylation of PKCδ on Tyr311 suggest a high turnover through the lysosomes, as E-64-D/Pepstatin induced a strong accumulation of this form of PKCδ in resting cells (**Fig. 4.30 A** & **C**). Upon stimulation with either ES-62 or LPS a transient increase in the phosphorylation of this site occurs over the 36 h period, although this is less pronounced in response to ES-62. The prior maturation of the cells with ES-62 does not inhibit the ability of LPS to induce the Tyr311 phosphorylation of PKCδ. These effects are also observed when the phosphorylation of Tyr311 is expressed relative to the amounts of PKCδ observed in the cell (**Fig. 4.30 A** & **D**). By blocking lysosomal degradation with E-64-D/Pepstatin A, an accumulation is observed of this phosphorylated form of PKCδ in response to ES-62 relative to the immuno-modulator alone (**Fig. 4.30 C**), although this effect is particularly prominent on the ES-62 matured cells subsequently exposed to LPS. By contrast, inhibition of lysosomal degradation inhibition actually reduced LPS induced phosphorylation of Tyr311. Thus, it appears as though lysosomal degradation is associated with ES-62's modulation of this form of PKCδ phosphorylation. In this context, ES-62/LPS stimulated levels of Tyr311 phosphorylated PKCδ decrease relative to the total amounts in the cell upon inhibition of lysosomal degradation (**Fig. 4.30 D**). However, when ES-62 matured cells are stimulated with LPS there is a dramatic increase in PKCδ phosphorylation on Tyr311 relative to total amounts observed in the cell, perhaps suggesting that this phosphorylation may be associated with lysosomal targeting.

4.2.4.4 The effects of specifically inhibiting lysosomal degradation on DC cytokine production in response to ES-62 and/or LPS

Collectively ES-62 was observed to target the lysosomal degradation of important mediators in the activation of NF-κB by TLR4 signalling. Thus, to determine whether ES-62 was suppressing DC cytokine responses by inducing lysosomal degradation of key regulatory elements, the effect of pretreating bmDCs with the lysosomal protease inhibitors E-64-D/Pepstatin A and ammonium chloride prior to ES-62 and/or LPS-stimulation on the secretion of various cytokines was tested (**Fig. 4.31** & **4.32**).

Exposure to E-64-D/Pepstatin A did indeed significantly block ES-62 mediated suppression of the key pro-inflammatory cytokine, Il-12p70 (**Fig. 4.31**). Rather surprisingly, however, this effect was specific to IL-12p70 as the downregulation of IL-23 and TNF-α responses remained unaffected. Moreover, the endotoxin induced secretion of IL-23 and IL-27 was significantly inhibited by E-64-D/Pepstatin A, but in respect to IL-27

this was prevented by the prior maturation of DCs with ES-62. However, pretreatment of the cells with ammonium chloride resulted in very little change in the secretion of LPS induced cytokines and did not disrupt the ability of ES-62 to down-regulate such responses (**Fig. 4.32**).

4.2.5 ES-62 and autophagy; a step towards TLR4 regulation

To explore the role of autophagy in ES-62 mediated lysosomal degradation of key signalling adaptors, the expression of LC3 was quantified as an indicator of the levels of this mechanism engaged in the cells differentially treated with ES-62 and/or LPS (**Fig. 4.33**). LC3 exists in two forms; the soluble LC3 type I (LC3-I) form, which upon conjugation to phosphatidylethanolamine (PE) by the E2-like enzyme ATG3 is transformed into the LC3-II form, which is important in the elongation of isolation membranes and autophagosome formation[468,479]. This lipidated 'LC3-II' migrates with a lower apparent molecular weight of 17 kDa in SDS gel electrophoresis than LC3-I (19 kDa)[480], allowing a quantitative measure of the levels of autophagic flux. The extended treatment of bmDCs with LPS resulted in a noticeable increase and slight decrease of LC3-I and LC3-II expression, respectively (**Fig. 4.33 B** & **E**). When the levels of LC3-II were expressed as a ratio relative to LC3-I as a measure of autophagic flux, endotoxin stimulation was observed reduce the expression of LC3-II relative to LC3-I below basal levels (unstimulated cells; **Fig. 4.33 C** & **F**). On the other hand, ES-62 induced a marginal increase in the expression of LC3-II and the LC3-II/LC3-I ratio (**Fig. 4.33 B-C** & **E-F**). Maturation of DCs with ES-62 also had little effect on the relative changes in LC3-I and LC3-II expression noted upon stimulation with endotoxin. To identify whether LC3 was being turned over and hence, responses masked by autophagic flux induced by the various stimuli, inhibitors of lysosomal trafficking and degradation were utilised including the combination of E-64-D and Pepstatin A to inhibit lysosomal proteases and ammonium chloride. The pretreatment of bmDCs with either E-64-D/Pepstatin A and ammonium chloride resulted in an increase in LC3-II levels in response to ES-62 and/or LPS suggesting that both TLR4 agonists might be inducing autophagy and autolysosomal degradation. This accumulation of LC3-II relative to LC3-I induced by ES-62 was clear when utilising the specific lysosomal protease inhibitor E-64-D/Pepstatin A, an effect that was maintained over a 36 h period (**Fig. 4.33 C**). This was less evident for LPS stimulation as the inhibitor increased both LC3-I and LC3-II levels with the resultant ratio of LC3-II/LC3-I still being less than that for unstimulated cells.

Based on these results another marker of autophagic flux, p62 was investigated to identify whether ES-62 could be utilising this important autophagy adaptor/cargo recognition protein in its immunomodulation of bmDCs (**Fig. 4.34**). TLR signalling in macrophages appears to induce the interaction of p62 with TRAF6 to coordinate orderly regulation of ubiquitin mediated processes to promote cytokine secretion associated with NF-κB activation[505]. In bmDC, two detectable forms of p62, which were approximated to be 47 kDa (p47) and 60 kDa (p60) in size, were identified utilising a polyclonal Ab obtained from NOVUS biologicals (NBP1-42821; **Fig 4.34 A**). Both of these bands could be specifically blocked by pretreating the Ab with its immunising peptide. Of these two protein sizes, ES-62 downregulated the expression of the larger p60 band relative to unstimulated (control) bmDC. Quantitative analysis of ES-62-mediated downregulation of the p60 protein over several experiments revealed it halved its expression relative to unstimulated cells (0.52 ± 0.05 n=5), while having little effect on the p47 protein (1.07 ± 0.23 n=4) (**Fig. 4.34 C**). By contrast, LPS stimulation resulted in strong upregulation of both forms, although this was greater for the p60 (2.59 ± 0.94 n=5) than the p47 protein (2.19 ± 0.92 n=4). Pretreament of the cells with both E-64-D/Pepstatin A and ammonium chloride resulted in the significant upregulation of the p60 form of p62 upon stimulation with ES-62, whereas only a slight increase was observed with the p47 protein, suggesting that ES-62-mediated utilisation of p62 was being masked to some extent by autophagic flux (**Fig. 4.34 B**). However, upon LPS stimulation inhibition of lysosomal degradation resulted only in a slight increase in the expression of both protein sizes suggesting that the strong upregulation by LPS did not reflect autophagy. Moreover, the maturation of bmDCs with ES-62 partially limited their ability to upregulate the expression of p62, particularly the p47 form in response to LPS (**Fig. 4.35 A-C**). Inhibition of lysosomal degradation with E-64-D/Pepstain A increased the expression of both forms of p62; perhaps suggesting it was mediating this downregulation via autophagy (**Fig. 4.35 D**). This proposal was further supported by the finding that an increase in the expression of p60 and to a lesser extent p47 upon stimulation with ES-62, which was achieved through pretreatment of the bmDCs with a pharmacological inhibitor of autophagy, 3-methyladenine (3-MA; **Fig. 4.36**)[512].

Based on the apparent induction of autophagic flux, the ubiquitination of p62 and several other ES-62 modulated TLR4-signalling adaptors (including TRAF6, c-Cbl and PKCδ) was analysed via the selective isolation of ubiquitinated protein conjugates (from cell lysates) utilising a Ubiqapture-Q kit (Enzo life sciences) and analysed by Western blotting (**Fig. 4.37**). The ubiquitination of these proteins was of particular interest due to

the known ability of p62 to directly associate with ubiquitinated (mono and poly) proteins via its UBA domain as well as LC3-II to deliver these proteins into the autophagosome for degradation[503,504]. The downregulation of p62 expression, which was rescued by the autophagy inhibitor, 3-MA, upon ES-62 stimulation suggested this immunomodulator could be inducing their ubiquitination to target their lysosomal degradation via the autohagosomes. The use of the KW0150 antibody, which recognises all ubiquitin conjugates (mono-, multi-, poly-modified and lysine linkage independent) indicated that DCs stimulated with either ES-62 or LPS over an 18 h time frame did not demonstrate any dramatic changes to global ubiquitin expression (**Fig. 4.37 A**). However, upon precipitation of the ubiquitin conjugates from these cells it became clear ES-62 induced the time specific association of TRAF6, PKCδ, c-Cbl and p62 to ubiquitin complexes and potentially their direct conjugation (**Fig. 4.37 B**). In this context, this association was markedly increased after 2 h of stimulation, with the dramatic reduction of ubiquitin associated TRAF6 occuring post 6 h. In contrast, LPS induced the progressive association to ubiquitin complexes of all these proteins except TRAF6, which was quite surprising as the K63 associated (self)polyubiquitination of this E3 ligase is crucial for the propagation of positive signals through TLR4 for NF-κB activation[28]. Interestingly, the levels of ubiquintin associated p62 dramatically increased over the 18 h endotoxin stimulation. This phenomenon is quite intriguing as accumulation of p62 is usually associated with a block in autophagy[480] but for this unique multi-functional scaffold protein, it could also be associated with its continued interactions with TRAF6 upon positive TLR signalling, which is known to maintain NF-κB activation and proinflammatory cytokine secretion[505].

Based on these results the regulation of p62 expression was defined at several points over an 18 h period upon ES-62 and LPS stimulation (**Fig. 4.38**). These experiments were performed in parallel with cells pretreated with inhibitors of proteosomal degradation (lactacystin) and the lysosomal degradation (E-64-D/Pepstatin A and ammonium chloride), to potentially delineate between the role of p62 in autophagy or TLR signalling. In both studies, Western blotting analysis of p62 expression yielded only the p60 form upon stimulation with ES-62 and LPS (in the absence of inhibitors), thus all quantitation cited relates to protein of this size. Treatment with LPS stimulated a clear and sustained increase in p62 expression (**Fig. 4.38 A-B**), which was only enhanced by pretreating the cells with lactacystin, suggesting that p62 degradation in response to LPS is primarily by the proteosome. Rather than for autophagy, the lactacystin data suggest that p62 may predominantly signal to activate NF-κB during the 6-20 h stage of LPS-maturation of

bmDC, a proposal consistent with the known time course of NF-κB-dependent pro-inflammatory cytokine production and release. Perhaps consistent with this, the ammonium chloride-induced reduction of p62 expression may relate to inhibition of LPS-mediated TLR4 activation associated with trafficking of this complex from the surface of the cell and the limiting propagation of signals required for p62 upregulation and NF-κB activation. These data provided further support for the proposal that in terms of TLR4/LPS signalling the role of p62 could be predominantly linked to its functions within the activation of the NF-κB pathway in bmDCs.

In contrast to endotoxin, ES-62 induces the profound down-regulation of p62 expression relative to unstimulated cells over an 18 h time course (**Fig. 4.38 C-D**). Also, as described above, the down-regulation of p62 can be prevented by pre-treating the bmDCs with E-64-D/Pepstatin A or ammonium chloride suggesting that the down-regulation of p62 by ES-62 occurs at least in part via lysosomal degradation. However, pretreating bmDCs with lactacystin also rescued ES-62 induced downregulation of p62, particularly at early time points (< 6h) suggesting a proteosomal mechanism to its degradation, which would be consistent with ES-62 preventing p62-mediated NF-κB activation[507,508,513,514].

Nevertheless, prolonged stimulation by LPS is accompanied by subcellular relocalisation of p62 from a rather disperse yet punctate pattern to that of large foci, some of which are perinuclear whilst others were cytoplasmic (**Fig. 4.39**). Whilst staining of LC3 also exhibited redistribution of LC3 from a dull diffuse pattern of localisation (**Fig. 4.40**) to bright punctuate centres, a hallmark of autophagy[480], this appeared to be predominantly perinuclear and quite different from that of p62 (**Fig. 4.39 C**). By contrast, upon examination of TRAF 6 expression, the bulk of the staining remained disperse but the cytoplasm also contained bright foci reminiscent of those associated with p62 (**Fig. 4.41**), suggesting that there could be interaction between these two proteins in DCs perhaps in mediating TLR4-NF-κB responses[505], as LPS also induces the upregulation of TRAF6 expression (**Fig. 4.13**).

Thus, to further explore whether p62 preferentially signalled for autophagy or NF-κB at various time points of LPS stimulation, p62-containing immune complexes were probed for the expression of p62-associated proteins focusing on LC3, TRAF6 and PKCδ (**Fig. 4.42**). The former was chosen to identify whether p62 was associating with components of autophagic vesicles whilst PKCδ and TRAF6 were investigated for their

potential involvement in TLR4 induced activation of NF-κB. In addition, there was also the potential for PKCδ to directly interact with p62, as the latter has been shown to express domains that allow its direct interaction with various PKC isoforms[504]. Consistent with the proposal that p62 predominantly signals for LPS-mediated activation of NF-κB rather than the initiation of autophagy, PKCδ was found to be present in p62-expressing immune complexes, an association that increased following stimulation with LPS. By contrast, neither TRAF6 nor LC3 expression could be detected in p62-containing immune complexes following an 18 h stimulation of bmDCs with LPS, despite there being expression of LC3-I and -II (only in the unstimulated cells again suggesting its downregulation by LPS) in the depleted lysates. Further analysis revealed that TRAF 6 did not appear to associate with p62 at earlier time points whereas association of p62 with PKCδ increased in a time-dependent manner, showing a substantial increase at 18 h when LPS stimulates strong upregulation of p62 (**Fig. 4.42 B**).

Due to the similarities of the immunomodulatory functions of ES-62 and the proposed role of autophagy as a negative feedback mechanism for dampening down LPS/TLR4-mediated pro-inflammatory responses. The pharmacological inhibitor of autophagy, 3-methyladenine (3-MA)[512], was utilised to identify whether it could disrupt the ES-62-mediated down-regulation of bmDC-derived cytokines associated with driving pro-inflammatory responses (**Fig. 4.43**). As a secondary objective, the effect of this inhibitor was investigated on LPS induced cytokine secretion as although it has been well catalogued that T_H1 and T_H2 polarising cytokines tend to promote and inhibit autophagy, respectively[501], little has been reported on its involvement in the direct secretion of such cytokines, particularly in relation to DCs. It was clear that pretreatment of DCs with 3-MA resulted in a significant reduction in LPS stimulated IL-6, IL-12p70 and TNF-α secretion. In contrast, it induced an increase in the production of IL-23 suggesting a selective role for autophagy in mediating negative feedback inhibition of LPS/TLR4 pro-inflammatory signalling. A similat pattern was replicated with ES-62 matured bmDCs stimulated with endotoxin, albeit the secretion of the cytokines (apart from IL-6) was at a lower level due to the immunomodulatory effects of ES-62. However, 3-MA did not reverse any of the downregulatory effects of ES-62, perhaps suggesting that these were not mediated by autophagy. Although it should be borne in mind that, as a PI3K inhibitor, the effects of 3-MA could reflect dampening of PI3K- dependent signalling pathways, a known target of ES-62 in a variety of cells of the immune system[301,515].

4.3 Disscussion

4.3.1 The inflammatory environment of a DC may dictate the regulation of TLR mediated activation of NF-κB by ES-62

It was clear that LPS induced the early activation of NF-κB with the transient (within 2 h) degradation of both IκB-α and IκB-β (**Fig. 4.3**), which was unaffected by treatment of the cells with the lysosomal protease inhibitor combination, E-64-D/Pepstatin A. However, this treatment did induce a marked increase in both isoforms 6 h post endotoxin stimulation. These results suggested that lysosomal degradation has an important role in the prolonged (post 4-6 h) endosomal TRAM-TRIF mediated phase of LPS recognition by TLR4[327], which maintains the activation of NF-κB[15]. In conjunction with this, the degradation of TLR4 in the late endosomes and lysosomes is an important negative feedback mechanism in regulating TLR4 signalling[328,329]. Thus, by inhibiting lysosomal degradation and the cells ability to regulate TLR4 signalling they may respond by upregulating IκB expression to sequester and limit the activation of NF-κB[330], which could account for the increased expression of both isoforms. However, it could also reflect that in maintaining NF-κB within its activated state the TRAM-TRIF pathway may potentially induce the continual degradation of IκB within the lysosomes. Directly related to these affects on IκB, endotoxin induced the secretion of a range of proinflammatory cytokines (IL-12p70, TNF-α, IL-27 and IL-23) from the bmDCs, all of which were significantly reduced by E-64-D/Pepstatin A treatment (**Fig. 4.31**). This suggested that of the two proposed scenarios, bmDCs upregulate IκB production to sequester and limit the activation of NF-κB, as observed by the attenuated production of the various proinflammatory cytokines.

In contrast, ES-62 induced a slight oscillating pattern of expression for both IκB-α and -β (**Fig. 4.3 C**). This indicated that despite the requirement of TLR4 for the immunomodulation of DCs by ES-62[301], this association does not stimulate the activation (post 2 h) of NF-κB. Several results suggested that ES-62 may progressively upregulate (18 h) expression of IκB-β but not IκB-α **(Fig. 4.3** & **Fig. 4.4 A-B**). As IκB-β is commonly associated in the delayed activation (through its degradation) of NF-κB[516], its upregulation could provide a potential mechanism for attenuating protracted endotoxin responses, however this affect was limited (1.28 ± 0.22, n=3) indicating it is unlikely to be a predominant method of regulation. In relation to this result, the costimulation of GM-

CSF alongside ES-62 maturation, induced the time sensitive upregulation of IκB-β (3 h) in response to stimulation with LPS (**Fig. 4.6**).

GM-CSF is best known as a regulator of the functions of granulocyte and macrophage derived populations acting throughout their development[509] and is frequently employed to differentiate DCs from bone marrow progenitors and peripheral blood[517]. Much of the literature suggests it is involved in mediating the inflammatory responses of these cells (and other more diverse populations), as it is not only able to augment the function of monocytes, macrophages and DCs[518] but prime mice to secrete a range of proinflammatory cytokines in response to the administration of LPS[519]. Thus, in the presence of a potentially more pro-inflammatory environment induced by GM-CSF, ES-62 may take a more proactive role in modulating the maturation of bmDCs through the expression of proteins such as IκB-β[520].

The most compelling evidence for this was the rapid downregulation of MyD88 expression induced by ES-62 (**Fig. 4.7 E-F**), an effect that was reversed upon removal of GM-CSF from the stimulatory culture medium (**Fig. 4.7 C-D** & **Fig. 4.8**). MyD88 associates with all the TLRs (except TLR3) to induce the activation of NF-κB and the expression of a range of proinflammatory cytokine genes[14]. Thus, its specific downregulation in a proinflammatory environment would be a crucial step for ES-62 to uncouple the inflammatory capacity of DCs, which is in keeping with some of its *in vivo* functions in both T_H1/T_H17[310,324] and T_H2[321,325] associated pro-inflammatory responses. In the absence of GM-CSF, MyD88 upregulation could reflect the differential modulation of the components involved in TLR signalling (for example PKCε and PKCδ), indicating the potential environment specific immunomodulatory capacity of ES-62 on DCs. The upregulation of MyD88 may even reflect the maturation of a DC primed to induce a T_H2 biased, yet anti-inflammatory response by ES-62, which is associated with this parasite product[160].

It was clear from these results that ES-62 is targeting several signalling adaptors involved in the propagation of TLR responses (specifically TLR4) in a proinflammatory environment, which is in keeping with its observed abilities to attenuate associated signalling responses[291,301,323,521,522]. Building upon this, the presence of GM-CSF in the stimulation medium induced ES-62 to mediate the degradation of the E3 ligase, c-Cbl (**Fig.4.17 A-B**), which in the cytokines absence was upregulated by ES-62 at both the mRNA transcript and protein levels (**Fig. 4.15**). Collectively, these data may indicate that

in the absence of GM-CSF, ES-62 encourages the upregulation of c-Cbl to limit the potential activation of NF-κB as it is involved in mediating the regulatory capacity of p50 and its precursor p105 (an NF-κB subunit)[453,454]. However, in a proinflammatory environment brought about by the presence of GM-CSF, ES-62 may target its degradation to limit other potential roles it may have in mediating positive TLR signalling.

In contrast, the downregulation of TRAF6 by ES-62 was GM-CSF independent (**Fig. 4.13**) but inclusion of the cytokine did lead to the complete ablation of its expression by ES-62 (**Fig. 4.14 A-B**). In this context, the presence of GM-CSF was also observed to further limit the expression of TRAF6 in response to endotoxin from ES-62 matured DCs (**Fig. 4.14 C-D**). This targeting of TRAF6 expression suggests that ES-62 acts to attenuate its pivotal role in mediating proinflammatory signalling through TLR4, important for the secretion of IL-6 and IL-12p70 and up-regulation of MHC class II in DCs[26,27]. However, IL-6 responses are maintained within ES-62 matured DCs (**Fig. 3.11**). Based upon these observations the GM-CSF independent upregulation of PKCα expression by ES-62 (**Fig. 4.19**) could maintain IL-6 secretion in response to LPS as studies have demonstrated its importance in inducing its secretion from macrophages upon positive TLR4 signalling[116-120]. Contrary to these observations however, a study in peritoneal macrophages conclusively demonstrating that ES-62 renders these cells hyporesponsive to LPS in terms of limited IL-6 secretion both *in vitro* and *in vivo*[363]. This is coupled with observations in the CIA model, where GM-CSF is important in disease progression[523], where ES-62 was able to mediate the downregulation of IL-6 secretion[324,352,524]. Collectively, these results suggest the complex environment specific functions of ES-62.

4.3.2 The differential regulation of PKC expression by ES-62 and LPS

ES-62 was observed to target the degradation of several important TLR4 signalling transducers via the lysosomes (in the absence of GM-CSF) within an 18 h stimulation. Two prominent examples were PKCε (**Fig. 4.20**, **Fig. 4.21**) and PKCδ (**Fig. 4.22, Fig. 4.23**), which are important for the activation of NF-κB through their direct interaction with MyD88 (PKCε)[49,100] and TIRAP (PKCδ)[101]. By contrast, LPS stimulation induced the upregulation of PKCε (exacerbated by blocking proteosomal degradation), which likely reflects it role in the secretion of TNF-α and IL-12p70[114], while only having marginal affects on PKCδ expression. The treatment of DC with E-64-D/Pepstatin A before stimulation with ES-62 rescued both PKCε and PKCδ from degradation (**Fig. 4.21**, **Fig.**

4.23) and restored IL-12p70 secretion (**Fig. 4.31**), converting an ES-62-TLR4 signalling phenotype to that of LPS-TLR4.

These results are particularly interesting as the PKCs are very important in the induction of NF-κB activation by the TLR family of receptors (particularly TLR4) but more specifically the stimulation of proinflammatory cytokine secretion. In this context, PKCδ has been shown to act as an IKK to phosphorylate and target IκB for degradation[104,105], inducing the production of numerous pro-inflammatory cytokines including TNF-α upon the recognition of LPS by TLR4[93,102,103], an effect ES-62 maturation is able to attenuate (**Fig. 3.11**). In DCs, PKCε fulfils a similar role in mediating LPS-induced IKK and NF-κB activation[112] but recently has been directly linked to the secretion of both TNF-α and IL-12p70 in monocyte derived DC[114]. Thus, the degradation of these adaptors by ES-62 likely reflects its attenuation of proinflammatory cytokine responses. However, the parasite product does not ablate the secretion of such cytokines and indeed does not prevent the upregulation of both PKCδ and PKCε in response to LPS (**Fig. 4.21, Fig. 4.23**). In conjunction with these results the modulation of LPS responses by ES-62 left IL-6 secretion intact, whereby even treatment of E-64-D/Pepstatin A had little effect (**Fig. 4.31**). The maintenance of IL-6 responses may relate to the upregulation (**Fig. 4.19 A-C**) and transient phosphorylation (Thr638; **Fig. 4.19 D-E**) of PKCα expression by ES-62 as this isoform is important for the IL-6 secretion in macrophages upon recognition of LPS by TLR4[116-120].

Following on from these results and focusing on the specifics of the degradation of these nPKCs in the lysosomes, studies in mast cells have demonstrated that ES-62 can subvert TLR4 signalling by mediating its sequestration by a caveolae-lipid raft dependent mechanism to target PKCα for lysosomal degradation[321]. Interestingly, the conventional and novel PKCs localise to the caveolae fraction of several cell types[59] to drive the formation of ceramide at the PM, which is crucial in the formation of lipid rafts and caveolae[83]. Thus, ES-62 could induce the transient activation and translocation of PKCδ and PKCε (**Fig. 4.20**, **Fig 4.22**), particularly through the phosphorylation of the former (**Fig. 4.28** – **Fig. 4.30**), to drive caveolae formation and provide a mechanism for their (and potentially other associated adaptors) trafficking to the lysosomes for degradation[82].

As mentioned above the phosphorylation of the A-loop (Thr505; **Fig. 4.28**) and hinge region (Tyr311; **Fig. 4.30**) by LPS may have an important role in mediating the

activation of NF-κB and its interaction with p62, which helps to bridge autophagy and TLR4 signalling[505,507,508] as demonstrated in **Figure 4.42**. In this context, it also provides a potential mechanism for the coupling of these proteins for ES-62 induced lysosomal degradation. These predictions are based on evidence that both phosphorylation at the A-loop and Tyr311 sites of PKCδ are important in regulating the specificity (and consequence) of its interactions with other signalling molecules[52,72,73]. In this context, the induction of phosphorylation at these sites by ES-62 may localise PKCδ (and its associated proteins) to the caveolae, mediating their sequestration from important functional sites or degradation in the lysosomes.

Another intriguing facet of the effect of ES-62 was the transient translocation of both intact (78 kDa) and cleaved (38 kDa) forms of PKCδ into the nucleus of cell (**Fig. 4.24**). Endotoxin induced a similar response (**Fig. 4.25**) but the confocal microscopy analysis did not allow the determination of the proportions of each form of PKCδ. However, the Western blotting studies revealed LPS to downregulate the expression of cleaved PKCδ (over 18 h) through the proteosome (**Fig. 4.26**), while ES-62 modulated its levels in a similar fashion to the full-length protein (**Fig. 4.22**). The enzymology of full length PKCδ and the cleaved (40-kDa) catalytic domain fragment differ considerably as the latter is phospholipid independent, where the relative amounts of both forms can have dramatic effects on cellular function[56-58]. Thus, the LPS induced reduction (via the proteosome; **Fig. 4.26 D**) of the relative levels of cleaved PKCδ to the total (cleaved and full-length) amounts in the cell (**Fig. 4.26 B**), may be important in regulating the activation of NF-κB as the relative amounts in microglia directly contributes to the activation of NF-κB through TLR signalling[110,111]. In contrast, ES-62 mediated only a marginal increase in the relative levels of the cleaved form (**Fig. 4.26 B**) indicating it is not directing the activation of NF-κB. However, the transient translocation of cleaved and full-length PKCδ into the nucleus suggest it induces other differential functions relating to the immunomodulation of DCs. Inhibiting lysosomal degradation extended and accentuated ES-62's regulation of cleaved PKCδ (**Fig. 4.26 C**) suggesting a turnover of the protein, reinforcing the idea that this form of PKCδ plays some part in mediating the functions of ES-62. The induction of the nuclear translocation of PKCδ by ES-62 occurred alongside uncoupling of mRNA transcript production of both PKCε and PKCδ giving rise to the upregulation of their protein levels (**Fig. 4.20 E, Fig. 4.22 F**) despite ES-62 not being directly observed in the nucleus (**Fig. 4.24 A**). This result is quite intriguing as the internalisation of ES-62 into macrophages and B cells results in its accumulation within the

nucleus (H.S. Goodridge & M. M. Harnett, unpublished data), suggesting that in DCs it utilises a different mechanism to uncouple (hyper)inflammatory responses.

Collectively, these results reflect a deep complexity in the function of ES-62 in DCs where it acts to inhibit the maximal output/hyper-inflammatory responses to pathogenic stimuli through their secretion of proinflammatory cytokines, while maintaining their competency in initiating adaptive immune responses (albeit modulated), to what could potentially be a fatal infection in the host if left unchecked. Several of its targets indicate ES-62 acts to limit proinflammatory cytokine secretion through modulation of NF-κB activation, however it does not drastically alter other important aspects of the inflammatory biology of DCs, including co-stimulatory molecule expression[114].

4.3.3 The role of ubiquitination (and its mediators) in the immunomodulation of DCs by ES-62

The mechanism of ubiquitination and the proteins that mediate this process have an important role in shaping the cellular response to inflammatory stimuli. For example, the E3 ubiquitin ligase, TRAF6 is crucial in the propagation of signals from TLR4 and the subsequent activation of NF-κB upon its recognition of LPS through its modulation of the ubiquitination of several TLR signalling adaptors[24,44]. Thus, due to the anti-inflammatory potential of ES-62, it came as little surprise that it induced the lysosomal degradation of TRAF6 (**Fig. 4.11** - **Fig. 4.13**), where a caveolae-lipid raft-dependent mechanism could be involved in its shuttling to the lysosomes[321]. By contrast, endotoxin induced an oscillating pattern of TRAF6 expression resulting in its protracted upregulation (post 6 h), exacerbated by inhibiting proteosomal but not lysosomal degradation (**Fig. 4.11**). This result suggests the cell utilises the proteosome to rapidly degrade and limit the propagation of TAK1 and IKK complex activation by TRAF6 and subsequently that of NF-κB[44]. These affects on TRAF6 were coupled to the increased expression of another E3 ligase by ES-62 that has several emerging roles in TLR4 signalling, c-Cbl (**Fig. 4.15**) and could reflect its involvement in maintaining the integrity of the inactive NF-κB complex[451,452,454]. Interestingly, endotoxin induced its degradation, which maybe involved in the activation, translocation and induction of proinflammatory cytokine secretion of NF-κB[454]. The degradation of c-Cbl is rescued by inhibition of lysosomal and proteosomal degradation but the latter induces the accumulation of the protein, indicating DCs utilise the proteosome as the predominant regulatory mechanism of c-Cbl expression (**Fig. 4.15**). The regulation of these E3 ligases were reflected (to an extent) in the expression of mRNA

transcript produced by the cells (**Fig. 4.11 D, Fig 4.15 E**) and suggest that ES-62 specifically targets their expression (at both the protein and mRNA transcript level) to differentially mature the DC and alter its ability to stimulate hyper-inflammatory responses (such as cytokine production).

The ubiquitination of proteins is important in their trafficking within the cell and the establishment of scaffolding complexes in the propagation of intracellulat signalling cascades[28,29]. In this respect, the differential signalling induced by ES-62, which results in the lysosomal degradation of TRAF6, p62, PKCδ and PKCε was thought to involve the ubiquitination of these signalling adaptors. Thus, the transient association of ubiquitin conjugates to these proteins (≥ 2 h) upon the stimulation of bmDCs with ES-62 suggested that this maybe the required impetus for the degradation of these proteins in the lysosome (**Fig. 4.37**). The proteosome has little involvement in mediating the downregulatory effects of ES-62, indicating this association is unlikely to involve the K48 associated linkage of ubiquitin, which targets proteins for degradation by this complex[41]. In light of this it is more likely to reflect the association of Lys-63-linked polyubiquitin chains, which are associated with directing proteins for non-proteosomal functions and potentially lysosomal degradation[42]. In this respect, through adaptor proteins such as p62, which can directly bind to ubiquitin through a specialised UBA domain, the association of ubiquitin conjugates can allow (and associated complexes) the transport of proteins into autophagosomes, via the direct binding of LC3-II on the inside of forming isolation membranes by p62, facilitating lysosomal degradation[460]. Thus, the ES-62 promoted induction of: the association of ubiquitin conjugates to several modulated proteins (TRAF6, p62 and PKCδ), autophagic flux as defined by increased LC3-II expression (particularly upon pretreatment wit E-64-D/Pepstatin A; **Fig. 4.33**) and downregulation of p62 (**Fig. 4.34**), may reflect its selective targeting of these proteins for lysosomal degradation via autophagosomes/autolysosomes.

By contrast, LPS induced the progressive association of PKCδ, c-Cbl and p62 (which is quite prominent) to ubiquitin conjugates but not TRAF6 (**Fig. 4.37**). The absence of TRAF6 association to ubiquitin conjugates was quite surprising as in canonical activation of NF-κB, (self)polyubiquitination via a K63 linked ubiquitin chain is required for its interaction with the TAK1 complex[24]. Thus, this affect may have been overlooked in this analysis (for example it may occur < 2 h) or the dynamics of this pathway may differ slightly in DCs. However, endotoxin clearly induced the ubiquitination of p62 suggesting this process may have a role in mediating the induction of certain proinflammatory

responses by TLR4 in DCs. In this context, there is growing evidence that p62 acts as a scaffold to regulate K63 polyubiquitination via its interaction with TRAF6 of proteins in a number of complexes associated with the activation of NF-κB[507], with both proteins often observed to co-localise to discrete punctuate structures upon stimulation of p62 signalling pathways[508]. Thus in DCs, the ubiquitination of p62 but not TRAF6 may be important in the protracted (< 2 h) or maintenance of NF-κB activation and proinflammatory cytokine production.

4.3.4 The role of autophagy in LPS and ES-62 stimulated cytokine production

In the investigation into the involvement of autophagy upon the stimulation of bmDCs with ES-62 and/or LPS, it became clear that both stimuli exhibited differential effects on the expression of several important proteins associated with mediating autophagic flux. Consistent with this, when the cells were pretreated with 3-MA, a well known inhibitor of autophagy, through its persistent and transient inhibition of Class I and III PI3K activity, respectively, there was a general down regulation of LPS induced cytokine secretion from the DCs, apart from IL-23 (**Fig. 4.43**). However, as autophagy has previously been proposed to downregulate LPS-induced inflammatory responses, this finding was somewhat surprising, particularly since the TLRs are known to activate Class I PI3Ks via MyD88[525], and we and others have previously shown that activation of Class I PI3Ks is associated with the downregulation of TNF-α, IL-12p70 and IL-12/23p40 secretion via blocking mRNA transcript expression of the p35 and p40 subunit in macrophages and DCs[526,527]. As such, DCs derived from mice which lack the Class I PI3K regulatory subunit p85, ablating Class I PI3K activity, show increased secretion of IL-12p70 and the shared IL-12/23 p40 subunit, which is attributed to an enhanced activation of p38 MAPK, a positive regulator of IL-12 production[526]. This could perhaps suggest that the effect on IL-23 may simply reflect inhibition of PI3K signalling although it has been noted by other groups that treatment of bmDCs with 3-MA increases the production of IL-23 in response to stimulation with LPS but the exact mechanism for this affect has not been defined[528]. However, the clear reduction in LPS induced secretion of TNF-α and IL-6, when the cells are pretreated with 3-MA, has previously been reported in the literature at both the protein and transcriptional level[529] and could potentially reflect proposals that both of these cytokines stimulate autophagy. Moreover, the caveat that because of the generic effects of 3-MA on the inhibition of the PI3Ks these effects cannot be confirmed until further studies are performed[480,530], is perhaps not necessary as no increase in TNF-α and

Il-12p70 (associated with a block of PI3K activity) was observed. That these effects are mirrored by the ES-62 matured DCs, with 3-MA further able to attenuate the LPS induced secretion of cytokines could suggest that either ES-62 does not act to block autophagy as effectively as 3-MA or that it is exerting these inhibitory effects in an autophagy-independent manner: in any case, these data do not identify a clear role for autophagy in mediating modulation of LPS responses by ES-62, although like 3-MA, we have previously shown ES-62 to inhibit PI3K activity[301,331], and presumably via these inhibitory actions, Akt[319]. Interestingly, therefore, in contrast to 3-MA, ES-62 suppressed IL-23 production and co-treatment with 3-MA did not significantly reverse this effect.

4.3.5 The complex interplay between TLR4 signalling and autophagic flux initiated by ES-62 and LPS

These changes in cytokine expression were accompanied by effects on the expression of the autophagy-associated proteins, p62 and LC3 in DCs. Treatment with LPS resulted in the dramatic upregulation of p62 (**Fig. 4.38**), in a similar fashion to that observed in macrophages stimulated with LPS in conjunction with IFN-γ[505]. Perhaps surprisingly therefore, given that p62 is reported to accumulate upon blocking of autophagy, the pretreatment of cells with 3-MA resulted in the marked reduction of p62 (60 kDa) although this could be a contributing factor to the reduction of cytokine secretion observed for IL-6, TNF-α and IL-12p70 (**Fig. 4.43**). Consistent with this, a study on human keratinocytes showed that siRNA knockdown of p62 resulted in the reduced expression of the mRNA transcript and protein of TNF-α and IL-6 as well as NF-κB promoter activity upon stimulation of TLR2/6 (by MALP-2) and TLR4 (by LPS)[531]. This is coupled with data indicating that p62 expression is crucial for the TLR-dependent activation of IKKα/β and degradation of IκB-α in this cell type. Collectively, this study showed that, rather than transducing autophagy, the cystolic accumulation of p62 may trigger TLR4-induced inflammatory responses in keratinocytes through modulation of IKK-NF-κB signalling. Thus, the strong accumulation of p62 in response to LPS stimulation observed in DCs (**Fig. 4.38**) could reflect a similar signalling pathway and the initial LPS-mediated suppression of autophagy in resting cells. Moreover, treatment of the cells with lactacystin (but not lysosomal inhibitors) enhanced LPS induced expression of p62 (**Fig. 4.38**), consistent with the proteosome being the most logical route for the cell to regulate this protein if it is participating in mediating LPS-TLR-NF-κB signals.

Nevertheless, collected data from several experiments showed endotoxin inducing an increase in the levels of LC3-I (1.446 ± 0.189, n=3) and LC3-II (1.124 ± 0.44, n=3) after an 18 h stimulation of LPS (relative to unstimulated cells). This effect displayed a marked increase upon treatment with E-64-D/pepsatain A for both LC3-I (10.09 ± 8.086, n=2) and LC3-II (7.00 ± 5.076, n=2) and was accompanied by an increase in the ratio of LC3-II to LC3-I (**Fig. 4.33 C** & **F**). However, the effect of E-64-D/Pepstatin A treatment on the expression of both LC3-I and LC3-II was accompanied by a decrease in the secretion of a host of cytokines including IL-12p70, IL-23, TNF-α and IL-27 (**Fig 4.31**). These latter results are therefore counter to the idea that the high levels of p62 observed at 18 h were associated with the induction of autophagy to limit responses, as under these conditions it would be expected that the lysosomal inhibitors would increase the levels of LC3-II and promote cytokine production. Interestingly the effects of ammonium chloride were relatively marginal relative to E-64-D/Pepstatin A, suggesting that the subsidiary effects associated with this compound were a factor in its modulation of LC3 expression.

As an atypical PKC (aPKC) scaffold protein, p62 plays an important role in the activation of NF-κB in response to a number of stimuli as it allows the assembly of an aPKC/p62/TRAF6 multiprotein complex[532], whereby, the interaction of p62 with TRAF6 has been shown to be particularly important in the activation of NF-κB by TNF-α or IL-1[533]. To determine whether the substantial accumulation of p62 noted here played a similar role, p62-containing immune complexes were precipitated upon LPS stimulation of the DCs (**Fig. 4.42**). These results showed that p62 formed a complex containing PKCδ but, quite unexpectedly, it did not contain TRAF6 or alternatively, in relation to its potential role in autophagy, LC3. Collectively with all the above data, this association with PKCδ suggested p62 could have a potential role in the activation of NF-κB after the recognition of LPS by TLR4 in DCs.

However, confocal microscopy revealed that LC3, p62 and TRAF6 were upregulated and relocalised to punctate foci upon 18 h stimulation with LPS, where the formation of punctate centres by LC3 (and partially p62) is associated with the induction of autophagy[480]. Although p62 and TRAF6 were observed in foci (**Fig. 4.39** & **4.41**), in general they appeared quite different to those of LC3 (**Fig. 4.40**). Thus, despite the co-immunoprecipitation study indicating a lack of association between TRAF6 and p62, their similar punctuate staining might suggest an interaction (**Fig. 4.39**) and thus, in this regard, it should be noted that a similar pattern did not occur when PKCδ was visualised after LPS

stimulation (**Fig. 4.25**). In relation to these observations, studies have shown that p62 and TRAF6 co-localise to discrete punctuate structures upon stimulation of p62 signalling pathways[508].

The maturation of DCs with ES-62 prior to LPS stimulation subtly altered the expression of p62 (**Fig. 4.35**). Stimulation with endotoxin significantly increased the expression of p62 relative to ES-62 but the power of this significance was reduced upon prior maturation with this immunomodulator before LPS stimulation. This suggested a potential mechanism for the actions of ES-62, as coupled with the downregulation of TRAF6 (**Fig. 4.11 – 4.13**) and the protection of TRAF and p62 expression by lysosomal inhibitors, this could suggest that ES-62 limits LPS responses via autolysosomal degradation. The ES-62 mediated downregulation of p62 occurred quite rapidly upon stimulation of DCs (within 2 h) and was rescued by both lysosomal and proteosomal degradation inhibitors (**Fig. 4.38**). Interestingly treatment with lactacystin resulted in an early increase above both lysosomal degradation inhibitors, suggesting an early proteosomal component to this degradation perhaps to mediate rapid inhibition of NF-κB, which switches to a more lysosomal route with time to allow downregulation of a cargo of signalling elements such as TRAF6, PKCδ, –ε and MyD88 required for TLR4 responses. This would fit with the literature as p62 is a multi-module adaptor/scaffold protein crucial for selective signal transduction that is commonly associated with its ability to take ubiquitinated proteins into forming isolation membranes for degradation in the autophagosomes[503,504]. Consistent with this, the pretreatment of the cells with 3-MA resulted in a dramatic increase in levels of p62 in DCs, further supporting the proposal that there may be an autophagic component to ES-62-mediated downregulation of p62 (**Fig. 4.36**). The rescue of p62 by the lysosomal inhibitors and 3-MA is coupled by the observation that ES-62 induces the interaction of this protein with ubiquitin conjugates (**Fig. 4.37**), suggesting ES-62 may target the degradation of this protein by ubiquitin associated autophagy in which TRAF6 may have a role[503]. In relation to this, in TLR induced activated macrophages the interactions of p62, help to coordinate orderly regulation of K63 polyubiquitination mediated processes ensuring cytokine secretion associated with NF-κB activation[506,507]. Thus the downregulation of this protein by ES-62 could relate to it diminishing the potential of TLR4 induced p62-TRAF6 interactions and NF-κB activation of cytokine secretion. Indeed the upregulation of p62 by LPS is reduced by prior ES-62 maturation (**Fig. 4.35**).

Although ES-62 did not appear to dramatically regulate LC3 expression, inducing a slight increase in LC3-II expression with little effect on LC3-I, treatment with E-64-D/Pepstatin A revealed dynamic lysosomal regulation of their expression indicative of autophagic flux (**Fig. 4.33**). This effect became more apparent when the a clear increase in LC3-II expression was observed relative to LC3-I (**Fig 4.33 C**). Thus, combining results from three experiments, whilst ES-62 appeared to have little effect on their expression after 18 h, with LC3-I (1.076 ± 0.049, n=3) and LC3-II (1.151± 0.045, n=3), treatment of the cells with E-64-D/Pepstatin A resulted in levels of LC3-I (0.06922 ± 0.06408, n=2) and LC3-II (1.157 ± 0.490, n=2), indicating a substantial increase in the ratio of LC3-II to LC3-I masked by autophagic flux and degradation in the autophagolysosomes. By contrast, ammonium chloride had little effect resulting in LC3-I (0.9572 ± 0.2731, n=2) and LC3-II (0.9570 ± 0.468, n=2). Thus collectively, the data indicate that ES-62 is utilising autophagy to mediate down-regulation of certain intracellular adaptors predominantly via the lysosomes, with some potential transient involvement of the proteosome.

Figure 4.1 | **The processes and regulation of autophagy.**

Panel **A**, The main cellular events that occur during macroautophagy begin with the formation of an isolation membrane (induction), followed by the sequestration of cargo that is to be degraded into these elongating vesicles. Once a fully enclosed autophagosome is formed, fusion with lysosomes leads to their maturation into autolysosomes and results in the degradation of the sequestered cargo. Panel **B,** There are multiple reported membrane sources for autophagosome formation, which include both plasma and nuclear membranes but to date the predominant source is the endoplasmic reticulum (ER). In respect to these other membrane sources (non-ER) the multispanning membrane protein that is essential for autophagy, ATG9, is trafficked between the trans-golgi network and endosomes where it maybe important in the formation of autophagosome precursors. Upon the induction of autophagy, the ULK1 complex (ULK1-ATG13-FIP200-ATG101) translocates to the ER where it transiently associates with VMP1 activating an ER-localised autophagy-specific class III PI3K complex. This induces the local (on the ER membrane) production of PtdIns(3)P, which recruits both DFCP1 and WIPIs to the ER, where they induce the formation of the isolation membrane. The ATG12-ATG5-ATG16L1 complex is then recruited to the outer membrane of the forming autophagosome where it helps to drive the elongation process. This process utilises a ubiquitin-like conjugation system where phosphatidylethanolamine (PE) is conjugated to LC3/Atg8 (LC3 is one of the mammalian homologues of Atg3) by the sequential action of the protease Atg4, the E1-like enzyme Atg7 and the E2-like enzyme Atg3. Lipid conjugation leads to the conversion of the soluble LC3 (named LC3-I) to the autophagic-vesicles-associated (LC3-II/LC3-PE). The PE conjugated LC3 is present on both the outer and inner membrane of the isolation membrane, where it can act as a tether to attach the autophagic cargo to the isolation membrane through specific adaptor proteins. LC3 is also important in the completion of the autophagosomes.

(Adapted from Levine, B. et al., 2011[534])

Figure 4.2 | The initiation of autophagy by TLR4 signalling and the interactions between components of this pathway and p62.

Signalling through the TLRs leads to the induction of autophagy through a mechanism dependent on both TRIF and p38 (possibly through activation of members of the PI3K family) but not MyD88. Specifically, TLR4 signalling is known to accelerate phagocytosis and autophagosome formation while up-regulating expression of the autophagic machinery. In this context, there is a conserved role for PKCδ in the regulation of gram-negative bacterium degradation by autophagy in mammalian cells in a DAG and Ubiquitin-dependent manner. The autophagy adaptor/cargo recognition protein, p62 was identified for its role in mediating the transfer of organisms (viruses, bacteria and parasites), organelles and proteins into forming isolation membranes through ubiquitin-dependent and independent mechanisms for lysosomal degradation. This specific targeting to the autophagosome is made possible by p62's LIR (LC3 interacting region)/LRS (LC3 recognition sequence) domain which can recognise and bind LC3 in the isolation membrane but it also has a number of other roles in the cell. Prominent among these is that p62 interacts with the E3 ubiquitin ligase, TRAF6, upon IFN-γ/TLR stimulation, causing its autoubiquitination and the subsequent activation of NF-κB, leading to the induction of pro-inflammatory cytokines. Specifically, following p62 dimerisation through the PB1 (Phox and Bem1p) domain, dimerised TRAF6 binds to p62 at its TRAF6-binding (TB) site allowing transfer of the K63 polyubiquitin chains generated from TRAF6 to specific substrates. In this context, p62 coordinates the K63 polyubiquitination of the TRAF6 specific substrates such as RIP, TRAF6, ERK and caspase 8, allowing p62 to mediate control of apoptosis, NF-κB activation and stress responses. Interestingly, K63 tagged substrates can interact with the UBA (Ubiquitin associated) domain of p62, allowing it to reside at the intersection between two degradation pathways. As the binding of polyubiquitin chains associated to a substrate protein by the UBA domain of p62, allows it to traffic it to the proteosome or the autophagsomes (though this tends to be for larger structures) for degradation.

Figure 4.3 | The effect of inhibiting multiple avenues of protein degradation and sorting on IκB-α and -β expression upon ES-62 or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml), ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for IκB-α and -β expression. In panel **B**, The densitometric analysis of the LPS stimulated samples for both IκB-α and IκB-β were normalised against the relevant β-actin loading control. These were expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1) from a single experiment except for the LPS stimulated samples featuring no chemical treatment (-), where data was acquired from three independent experiments and presented as the mean estimations ± SEM where n=3. In panel **C**, The densitometric analysis of the ES-62 stimulated samples for both IκB-α and IκB-β were normalised against the relevant β-actin loading control and expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1), from a single experiment. E/P, E-64-D/Pepstatin A; NH_4Cl, ammonium chloride.

Figure 4.4 | Effect of inhibiting multiple avenues of protein degradation and sorting on the protracted expression of IκB-α and -β upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for IκB-α and IκB-β expression as indicated. In panel **B**, the densitometric analysis of IκB-α and IκB-β expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line). Data are presented for five (IκB-α) and three (IκB-β) independent experiments as the mean estimations ± SEM where n=5 and n=3.

In panel **C**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for IκB-α expression. In panel **D**, the densitometric analysis of IκB-α expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells in the absence of any chemical treatment (Unstimulated DCs, normalised to 1 and represented by the dashed line). NH_4Cl, ammonium chloride.

Figure 4.5 | Effect of inhibiting lysosomal degradation on the protracted expression of IκB-α and -β upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for IκB-α expression. In panel **B**, the densitometric analysis of IκB-α expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the DCs stimulated with ES-62 for 18 h in the absence of any chemical treatment (media [18] + ES-62 [18], normalised to 1 and represented by the dashed line). E/P, E-64-D/Pepstatin A.

In panel **C**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for IκB-α expression. In panel **D**, the densitometric analysis of IκB-α expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to unstimulated DCs in the absence of any chemical treatment (Media 36 h, normalised to 1 and represented by the dashed line). E/P, E-64-D/Pepstatin A; NH_4Cl, ammonium chloride.

Figure 4.6 | Effect of GM-CSF on modulation of IκB-α and -β expression by ES-62 and/or LPS.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with either ES-62 (2 μg/ml) and/or LPS (1 μg/ml) for the indicated times with GM-CSF maintained in the culture. The key above the blots states the duration of each treatment and the order each stimulant was added, from the top to the bottom . The control (unstimulated) cells were incubated under the same conditions for the relevant length of each set of treatments (i.e. 19, 21 and 24 h respectively). Total protein extracts were purified from the samples and analysed by Western blotting for IκB-α and -β expression. In panel **B** and **C**, the densitometric analysis of IκB-α and IκB-β was normalised against the relevant β-actin loading control and expressed as a fold change relative to the appropriate control (unstimulated DC, normalised to 1) cells for the LPS (**B**) and ES-62 (**C**) treated samples.

Figure 4.7 | The effect of GM-CSF on the modulation of MyD88 expression by ES-62 and LPS.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for MyD88 expression. In panel **B**, the densitometric analysis of MyD88 was normalised against the relevant β-actin loading control and expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1).

In panel **C**, bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for 18 h. Total protein extracts were purified from the samples and analysed by Western blotting for MyD88 expression. In panel **D**, the densitometric analysis of MyD88 expression was normalised against the relevant β-actin loading control and then expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1). Data are acquired from four independent experiments and presented as the mean estimations ± SEM where n=4.

In panel **E**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) for the indicated times with GM-CSF maintained in the culture. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for MyD88 expression. In panel **F**, the densitometric analysis of MyD88 was normalised against the relevant β-actin loading control and expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1). The densitometric analysis of DCs treated with ES-62 in the absence of GM-CSF is included for reference.

Figure 4.8 | Effect of ES-62 and LPS stimulation on the expression of MyD88 protein and mRNA transcript.

BmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with either LPS (1 μg/ml) or ES-62 (2 μg/ml) for the indicated times (hours). These cells were then fixed and permeabilised before being stained with a rabbit polyclonal Ab against MyD88 followed by FITC labelled anti-mouse IgG (FL1 channel). The MyD88 Ab conjugated with its immunising peptide (blocking peptide) was utilised as a specificity control on unstimulated cells. In panel **A**, the gating of healthy cells of the correct size (FSC-H) and granularity (SSC-H) associated with DCs, were defined by their expression of MHC Class II utilising a PE conjugated Ab (FL2-H channel). In panel **B**, data for such gated differentially treated bmDCs are presented as histograms of fluorescent intensity of Ag staining versus the %-Max (**section 2.6.1**). In panel **C**, the percentage of positively stained cells (for MyD88) induced by both treatments was calculated relative to the specificity control as indicated by the markers on the histograms. Data are representative of two experiments.

In panel **D**, the total RNA was purified from CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The production of MyD88 mRNA transcript from these cells was measured by Taqman real-time PCR and defined as a percentage relative to GAPDH mRNA expression. The data are presented as the mean levels of triplicate estimates from a single experiment ± SD.

Figure 4.9 | The effect of ES-62 and LPS on TIRAP expression.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for TIRAP (MAL) expression. In panel **B**, the densitometric analysis of TIRAP expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1).

In panel **C**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for 18 h. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for TIRAP (MAL) expression. In panel **D**, the densitometric analysis of TIRAP expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line). Data are acquired from three independent experiments and presented as the mean estimations ± SEM where n=3.

Figure 4.10 | Effect of inhibiting lysosomal degradation on the expression of MyD88 and TIRAP upon protracted ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for MyD88 expression. In panel **B**, the densitometric analysis of MyD88 expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to unstimulated DCs in the absence of any chemical treatment (Media 36 h, normalised to 1).

In panel **C**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for TIRAP expression. In panel **D**, the densitometric analysis of TIRAP expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to unstimulated DCs in the absence of any chemical treatment (Media 36 h, normalised to 1). E/P, E-64-D/Pepstatin A.

Figure 4.11 | Effect of inhibiting multiple avenues of protein degradation on TRAF6 expression upon ES-62 and LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with lactacystin (10 μM), E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml), ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for TRAF6 expression. In panels **B** & **C**, the densitometric analysis of TRAF6 expression for the LPS (panel **B**) and ES-62 (panel **C**) stimulated samples, including those treated with the various proteosomal and lysosomal inhibitors, were normalised against the relevant β-actin loading control and expressed as a fold change relative to the appropriate control cells (unstimulated DC, normalised to 1). E/P, E-64-D/Pepstatin A; NH_4Cl, ammonium chloride.

In panel **D**, the total RNA was purified from CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The production of TRAF6 mRNA transcript from these cells was measured by Taqman real-time PCR and defined as a percentage relative to GAPDH mRNA expression. The data are presented as the mean levels of triplicate estimates from a single experiment ± SD.

Figure 4.12 | Effect of inhibiting lysosomal degradation on the protracted expression of TRAF6 upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for TRAF6 expression. In panel **B**, the densitometric analysis of TRAF6 expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to unstimulated DCs in the absence of any chemical treatment (Media 36 h, normalised to 1 and represented by the dashed line). E/P, E-64-D/Pepstatin A.

Figure 4.13 | Effect of inhibiting lysosomal degradation on the expression of TRAF6 upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml), ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for TRAF6 expression. In panel **B**, the densitometric analysis of TRAF6 expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line). Data are acquired from four independent experiments and presented as the mean estimations ± SEM where n=4. The statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$). E/P, E-64-D/Pepstatin A; NH_4Cl, ammonium chloride.

Figure 4.14 | The effect of GM-CSF on the modulation of TRAF6 expression by ES-62 and/or LPS.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) for the indicated times (hours), with GM-CSF maintained in the culture. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for TRAF6 expression. In panel **B**, the densitometric analysis of TRAF6 was normalised against the relevant β-actin loading control and these values were adjusted and expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1).

In panel **C**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) and/or LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the relevant length of the experiment (22 h). Total protein extracts were purified from the samples and analysed by Western blotting for TRAF6 and IκB-β expression. In panels **D** and **E**, the densitometric analysis of TRAF6 and IκB-β was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1) for both TRAF6 and IκB-β, respectively.

Figure 4.15 | Effect of inhibiting multiple avenues of protein degradation on c-Cbl expression upon ES-62 and LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with lactacystin (10 μM), E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml), ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for c-Cbl expression. In panels **B** and **C**, the densitometric analysis of c-Cbl expression for the LPS (panel **B**) and ES-62 (panel **C**) stimulated samples, including those treated with the various proteosomal and lysosomal inhibitors, were normalised against the relevant β-actin loading control and expressed as a fold change relative to the appropriate control cells (unstimulated DC, normalised to 1). In panel **D**, the normalised and adjusted data of c-Cbl expression upon ES-62 and LPS stimulation from three independent experiments are presented as the mean estimation ± SEM, where n=3. E/P, E-64-D/Pepstatin A; NH_4Cl, ammonium chloride.

In panel **E**, the total RNA was purified from CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The production of c-Cbl mRNA transcript from these cells was measured by Taqman real-time PCR and defined as a percentage relative to GAPDH mRNA expression. The data are presented as the mean levels of triplicate estimates from a single experiment ± SD.

Figure 4.16 | Effect of inhibiting lysosomal degradation on the expression of c-Cbl upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for c-Cbl expression. In panel **B**, the densitometric analysis of c-Cbl expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented as a dashed line). Data are acquired from three independent experiments and is presented as the mean estimations ± SEM where n=3. E/P, E-64-D/Pepstatin A.

Figure 4.17 | The effect of GM-CSF on the modulation of c-Cbl expression by ES-62 and/or LPS.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) ± GMCSF in the culture medium as indicated for the stated times (hours). The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for c-Cbl expression. In panel **B**, the densitometric analysis of c-Cbl was normalised against the relevant β-actin loading control and these values were adjusted and expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1). The densitometric analysis of DCs treated with ES-62 in the absence of GM-CSF is included for reference.

In panel **C**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) and/or LPS (1 μg/ml) for the indicated times (hours) with GM-CSF maintained in the culture, where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the relevant length of each set of treatments (19 and 24 h). Total protein extracts were purified from the samples and analysed by Western blotting for c-Cbl expression. In panel **D**, the densitometric analysis of c-Cbl was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line).

Figure 4.18 | The effect of ES-62 and LPS maturation on the expression of several PKC isoforms.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCθ expression.

In panel **B**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCι and -β expression.

Figure 4.19 | The effect of ES-62 and LPS on PKCα expression, phosphorylation and the influence of GM-CSF.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCα expression. In panel **B**, the densitometric analysis of PKCα expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1). In panel **C**, of CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) or LPS (1 μg/ml) for 18 h. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCα expression. In panel **D**, the densitometric analysis of PKCα expression was normalised against the relevant β-actin loading control and expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line). Data are acquired from two independent experiments and presented as the mean estimations ± SEM where n=2.

In panel **E**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) for the indicated times alongside unstimulated cells (Control). Total protein extracts were purified from the samples and analysed by Western blotting for expression of phosphorylated PKCα (Thr 638). In panel **F**, the densitometric analysis of phospho-PKCα Thr638 expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1).

In panel **G**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) for the indicated times with GM-CSF maintained in culture. Total protein extracts were purified from the samples and analysed by Western blotting for PKCα expression. In panel **F**, the densitometric analysis of PKCα expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1). The densitometric analysis of bmDCs treated with ES-62 in the absence of GM-CSF is included for reference

Figure 4.20 | Effect of inhibiting multiple avenues of protein degradation on PKCε expression upon ES-62 and LPS stimulation.

In panels **A** and **B**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with lactacystin (10 μM), E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml), ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with either ES-62 (2 μg/ml; panel **A**) or LPS (1 μg/ml; panel **B**) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCε expression. In panels **C** & **D**, The densitometric analysis of PKCε expression for the LPS (panel **B**) and ES-62 (panel **D**) stimulated samples, including those treated with various proteosomal and lysosomal inhibitors, were normalised against the relevant β-actin loading control and expressed as a fold change relative to the appropriate control cells (unstimulated DC, normalised to 1). E/P, E-64-D/Pepstatin A; NH_4Cl, ammonium chloride.

In panel **E**, the total RNA was purified from CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The production of PKCε mRNA transcript from these cells was measured by Taqman real-time PCR and defined as a percentage relative to GAPDH mRNA expression. The data are presented as the mean levels of triplicate estimates from a single experiment ± SD.

Figure 4.21 | Effect of inhibiting lysosomal degradation on the protracted expression of PKCε upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCε expression. In panel **B**, the densitometric analysis of PKCε expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line). Data are acquired from three independent experiments and is presented as the mean estimations ± SEM where n=3. The statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$). E/P, E-64-D/Pepstatin A.

Figure 4.22 | **Effect of inhibiting multiple avenues of protein degradation on PKCδ expression upon ES-62 and LPS stimulation.**

In panels **A** and **B**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with lactacystin (10 μM), E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml), ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with either ES-62 (2 μg/ml; panel **A**) or LPS (1 μg/ml; panel **B**) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCδ expression. In panels **C** & **D**, the densitometric analysis of the LPS (panel **C**) and ES-62 (panel **D**) stimulated samples, including those treated with various proteosomal and lysosomal inhibitors, were normalised against the relevant β-actin loading control and expressed as a fold change relative to the appropriate control cells (unstimulated DC, normalised to 1). In panel **E**, the densitometric analysis of ES-62 and LPS stimulated samples from three independent experiments were normalised against the relevant β-actin loading control and expressed as a fold change relative to the appropriate control cells (unstimulated DC, normalised to 1) and presented as the mean estimations ± SEM where n=3. The statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$), relative to the control (unstimulated) sample. E/P, E-64-D/Pepstatin A; NH_4Cl, ammonium chloride.

In panel **F**, the total RNA was purified from CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The production of PKCδ mRNA transcript from these cells was measured by Taqman real-time PCR and defined as a percentage relative to GAPDH mRNA expression. The data are presented as the mean levels of triplicate estimates from a single experiment ± SD.

Figure 4.23 | Effect of inhibiting lysosomal degradation on the protracted expression of PKCδ upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml), ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCδ expression. In panel **B**, the densitometric analysis of PKCδ expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line). Data are acquired from three independent experiments and is presented as the mean estimations ± SEM where n=3. The statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$). E/P, E-64-D/Pepstatin A; NH_4Cl, ammonium chloride.

Figure 4.24 | **Sub-cellular expression and localisation of PKCδ in response to ES-62.**

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Cytoplasmic and nuclear fractions were prepared from the samples utilising the Nuclear Extract kit from Active motif following the manufacturer's instructions and analysed by Western blotting for the expression of PKCδ, ES-62, GAPDH and VDAC-1. In panels **C** and **D**, The densitometric analysis of both the full length (panel **B**) and cleaved (panel **C**) forms of PKCδ were normalised against their relevant cellular localisation loading control (cytoplasm – GAPDH; nuclear – HDAC-1) and expressed as a fold change relative to the appropriate control cells (unstimulated DC, normalised to 1).

In panel **D**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). After thoroughly washing the cells with PBS, total protein extracts were purified from the samples and analysed by Western blotting for ES-62 expression.

Figure 4.25 | **Visualisation of PKCδ in response to LPS.**

CD11c expressing bmDCs ($3.5x10^4$/chamber in a total of 300 μl) were stimulated with LPS (1 μg/ml) or medium for 18 h. After washing, fixing and permeabilising the cells, PKCδ was visualised using a polyclonal Ab against the protein followed by a FITC-labelled anti-rabbit IgG. The nuclei were stained for with DAPI and the cells visualised by confocal microscopy in 0.8 μm sections. In panel **A**, the PKCδ Ab conjugated with its immunising peptide (blocking peptide) was utilised as a specificity control. Both FITC and DAPI staining is presented (left) alongside both separately (DAPI, middle; FITC, right). In panel **B**, a representative picture in the same format is provided for immature bmDCs and in panel **C**, three representative examples are presented of LPS treated cells.

Figure 4.26 | Effect of inhibiting multiple avenues of protein degradation on PKCδ cleavage upon ES-62 and LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with lactacystin (10 μM), E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml), ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCδ expression, whereby cleaved PKCδ shows a distinctive molecular weight band of 38 kDa. The cleaved form of PKCδ was visible after extensive exposure and the 78 kDa band is shown for reference. In panel **B**, the densitometric analysis of ES-62 and LPS stimulated samples from three independent experiments were normalised against the relevant β-actin loading control and expressed as a fold change relative to the appropriate control cells (unstimulated DC, normalised to 1) and are presented as the mean estimations ± SEM where n=3. In panels **C** and **D**, The densitometric analysis of the ES-62 (panel **C**) and LPS (panel **D**) stimulated samples were normalised against the relevant β-actin loading control and expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1). To the right of panels **B**, **C** and **D**, the relative amounts of the cleaved form of the protein are expressed as a percentage of the total amounts of PKCδ in the cell (cleaved and whole). Statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$), relative to the control (unstimulated) sample. E/P, E-64-D/Pepstatin A; NH_4Cl, ammonium chloride.

Figure 4.27 | Effect of inhibiting lysosomal and proteosomal degradation on the cleavage of PKCδ upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCδ expression. The cleaved form of PKCδ (38 kDa) was visible after extensive exposure, with the 78 kDa band shown for reference. In panel **B**, the densitometric analysis of cleaved PKCδ expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1). Data are acquired from three independent experiments and is presented as the mean estimations ± SEM where n=3 and the statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$), relative to the control (unstimulated sample). In panel **C**, the relative amount of the cleaved form of the protein is expressed as a percentage of the total amounts of PKCδ in the cell (cleaved and full length protein).

In panel **D**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with medium, lactacystin (10 μM) or ammonium chloride (50 μM) as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCδ expression. The cleaved form of PKCδ (38 kDa) was visible after extensive exposure, with the 78 kDa band shown for reference. In panel **E**, the densitometric analysis of cleaved PKCδ expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line). Data are acquired from two independent experiments and is presented as the mean estimations ± SEM where n=2. In panel **F**, the relative amount of the cleaved form of the protein is expressed as a percentage of the total amounts of PKCδ in the cell (cleaved and full length protein). NH_4Cl, ammonium chloride.

Figure 4.28 | The effect of ES-62 and LPS on the phosphorylation of PKCδ at Threonine 505 (Thr505).

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for phosphorylation of PKCδ on Thr505 of both the cleaved full length proteins. In panel **B**, the densitometric analysis of Thr505 phosphorylation of full length and cleaved PKCδ was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1). In panel **C**, PKCδ-Thr505 phosphorylation is represented as a percentage relative to the total amount of PKCδ observed in the cell under ES-62 treatment.

In panel **D**, two batches of CD11c expressing bmDCs, A and B ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for phosphorylation of Thr505 on PKCδ. In panel **E**, the densitometric analysis of Thr505 phosphorylation on PKCδ from three experiments was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented as a dashed line) and presented as the mean estimation ± SEM where n=3. The statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$).

Figure 4.29 | Effect of inhibiting lysosomal degradation on the phosphorylation of PKCδ on Serine 643 (Ser643) upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCδ and its phosphorylation at Ser643. In panels **B** and **C**, the densitometric analysis of PKCδ (panel **B**) and Ser643 phosphorylation (panel **C**) was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to unstimulated DCs in the absence of any chemical treatment (Media 36 h, normalised to 1 and represented by the dashed line). In panel **D**, PKCδ Ser643 phosphorylation is expressed as a percentage relative to the total amount of PKCδ observed in the cell under the varying treatments. E/P, E-64-D/Pepstatin A.

Figure 4.30 | Effect of inhibiting lysosomal degradation on the phosphorylation of PKCδ on Tyrosine 311(Tyr311) upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for PKCδ and its phosphorylation at Tyr311. In panels **B** and **C**, the densitometric analysis of PKCδ (panel **B**) and Tyr311 phosphorylation (panel **C**) was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to unstimulated DCs in the absence of any chemical treatment (Media 36 h, normalised to 1 and represented by the dashed line). In panel **D**, PKCδ Tyr311 phosphorylation is expressed as a percentage relative to the total amount of PKCδ observed in the cell under the varying treatments. E/P, E-64-D/Pepstatin A.

Figure 4.31 | Effect of E-64-D/Pepstatin A on the production of cytokines important in differentiating T helper (T_H) cell phenotypes upon ES-62 and/or LPS stimulation.

In panel **A**, bmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These bmDC ($2x10^6$/well in a total of 2 ml) were then pretreated with medium or ES-62 (2 μg/ml) for 18 h followed by maturation with medium or LPS (1 μg/ml) for a further 18 h. In parallel cultures, bmDCs were treated with a combination of E-64-D (10 μg/ml) and Pepstatin A (10 μg/ml) (**E/P**) for 1 h to block lysosomal degradation before being stimulated with identical combinations of ES-62 and LPS as described above. The culture supernatants were then analysed for a range of cytokines including TNF-α, IL-12/23 p40, IL-12 p70, IL-23, IL-27 and IL-6, associated with the development of particular T_H phenotypes. Data are acquired from four independent experiments and presented as the means of mean values of triplicate estimations ± SEM where n=4. The statistical analysis is by one way-ANOVA, utilising a Tukey post test where P* <0.05, **p<0.01 and ***p<0.001. In panel **B**, control (resting cells) and ES-62 stimulated cytokine secretion results are shown on an expanded scale.

Figure 4.32 | **Effect of ammonium chloride on the production of cytokines important in differentiating T helper (T_H) cell phenotypes upon ES-62 and/or LPS stimulation.**

In panel **A**, bmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These bmDC ($2x10^6$/well in a total of 2 ml) were then matured with ES-62 (2 μg/ml) and/or LPS (1 μg/ml) and in parallel cultures identical stimulations were added to bmDCs pre-treated with ammonium chloride (**NH_4Cl**; 50 μM) for 1 hour to block lysosomal degradation. Data is acquired from one experiment and presented as the mean value of triplicate estimations ± SD where n=3. In panel **B**, control (resting cells) and ES-62 stimulated cytokine secretion results are shown on an expanded scale.

Figure 4.33 | Effect of inhibiting lysosomal degradation on the expression of LC3 upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and/or LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for LC3 expression. In panel **B**, the densitometric analysis of LC3 type I (LC3-I) and LC3 type II (LC3-II) expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to unstimulated DCs in the absence of any chemical treatment (Media 36 h, normalised to 1 and represented by the dashed line). In panel **C**, the expression of LC3-II was calculated as a ratio of LC3-I for the various treamtents. E/P, E-64-D/Pepstatin A.

In panel **D**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and/or LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for LC3 expression. In panel **E**, the densitometric analysis of LC3 type I (LC3-I) and LC3 type II (LC3-II) expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to unstimulated DCs in the absence of any chemical treatment (Media 36 h, normalised to 1 and represented by the dashed line). In panel **F**, the expression of LC3-II was calculated as a ratio of LC3-I for the various treamtents. NH_4Cl, ammonium chloride.

Figure 4.34 | Effect of inhibiting lysosomal degradation and endocytic processing on p62 expression upon ES-62 or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with either medium or ES-62 (2 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were then purified from the samples and analysed by Western blotting for the expression of p62 utilising an antibody against p62 (α-p62) and the same antibody conjugated with its immunising peptide (α-p62 + BP), as a specificity control.

In panel **B**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml), ammonium chloride (50 μM) or medium as indicated. Following these treatments for 1 h, the cells were stimulated with either ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for p62 expression. In panel **C**, the densitometric analysis of the 47 kDa and 60 kDa bands of p62 for each treatment were normalised against the relevant β-actin loading control and expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line). Data from four experiments is presented for the indicated stimulations pretreated with/without, E-64-D/Pepstatin A and ammonium chloride as the normalised and adjusted values ± SEM, where n=4. The statistical analysis performed is a Kruskal-Wallis test utilising a Dunn's Multiple Comparisons post test (α = $p<0.05$). E/P, E-64-D/Pepstatin A; NH_4Cl, ammonium chloride.

Figure 4.35 | Effect of inhibiting lysosomal degradation on the protracted expression of p62 upon ES-62 and/or LPS stimulation.

In panels **A** and **B,** CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with ammonium chloride (50 μM; panel **A**), E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml; panel **B**) or medium as indicated for 1 h. The cells were then stimulated with combinations of medium, ES-62 (2 μg/ml) and LPS (1 μg/ml) for the indicated times (hours), where the order of stimulation follows from the top to the bottom. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (36 h). Total protein extracts were purified from the samples and analysed by Western blotting for p62 expression. In panel **C**, the densitometric analysis of p62 expression was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line). Data are acquired from four independent experiments and is presented as the mean estimations ± SEM where n=4. In panel **D**, the densitometric analysis of p62 expression including the samples treated with ammonium chloride and E-64-D/Pepstatin A was normalised against the relevant β-actin loading control and the values expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line). Data are acquired from three independent experiments and is presented as the mean estimations ± SEM where n=3. The statistical analysis performed is a Kruskal-Wallis test, utilising a Dunn's Multiple Comparison Post Test ($\alpha = p<0.05$).

Figure 4.36 | Effect of inhibiting autophagy on p62 expression upon ES-62 and/or LPS stimulation.

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with either medium or 3-Methyladenine (2 mM; 3-MA) for 1 h before the cells were stimulated with either ES-62 (2 μg/ml) and/or LPS (1 μg/ml) for the times indicated. Total protein extracts were then purified from the samples and analysed by Western blotting for p62 expression.

In panel **B**, the densitometric analysis of the 47 kDa and 60 kDa bands of p62 were normalised against the relevant β-actin loading control and expressed as a fold change relative to the control cells (unstimulated DC, normalised to 1 and represented by the dashed line).

Figure 4.37 | Ubiquitination and association of important proteins in the propagation of TLR4 signalling upon stimulation with LPS or ES-62.

CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with ES-62 (2 μg/ml) or LPS (1 μg/ml) for the indicated times. In order to isolate all ubiquitin conjugated proteins (mono-, multi-, poly-modified and lysine linkage independent) from the cells each sample was carefully lysed and treated with the UbiQapture-Q matrix (Enzo life sciences) as per the manufacturer's instructions. In panel **A**, the expression of ubiquitin (KW0150; mono-, multi-, poly-modified and lysine linkage independent) was analysed directly from the cell lysates by Western blotting. In panel **B**, The matrix precipitates were analysed by Western blotting for ubiquitin conjugate (KW0150), p62, TRAF6, PKCδ and c-Cbl expression.

Figure 4.38 | Effect of inhibiting multiple avenues of protein degradation on p62 expression upon ES-62 and LPS stimulation.

In panels **A** and **C**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were treated with lactacystin (10 μM), E-64-D (10 μg/ml) plus Pepstatin A (10 μg/ml), ammonium chloride (50 μM) or medium as indicated for 1 h. The cells were then stimulated with either LPS (1 μg/ml; panel **A**) or ES-62 (2 μg/ml; panel **C**) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). Total protein extracts were purified from the samples and analysed by Western blotting for p62 expression. In panel **B**, The densitometric analysis of the LPS stimulated samples were normalised against the relevant β-actin loading control and expressed as a fold change relative to the appropriate control cells (unstimulated DC, normalised to 1). In panel **D**, The densitometric analysis of the ES-62 stimulated samples were normalised against the relevant β-actin loading control and expressed as a fold change relative to the appropriate control cells (unstimulated DC, normalised to 1) cells.

Figure 4.39 | **Visualisation of p62 in response to LPS.**

CD11c expressing bmDCs ($3.5x10^4$/chamber in a total of 300 μl) were stimulated with LPS (1 μg/ml) or medium for 18 h. After washing, fixing and permeabilising the cells, p62 was visualised using a polyclonal Ab against the protein followed by a FITC-labelled anti-rabbit IgG. The nuclei were stained for with DAPI and the cells visualised by confocal microscopy in 0.8 μm sections. In panel **A**, the p62 Ab conjugated with its immunising peptide (blocking peptide) was utilised as a specificity control on LPS treated cells. Both FITC and DAPI staining is presented (left) alongside both separately (FITC, middle; DAPI, right). In panel **B**, a representative picture in the same format is provided for immature bmDCs and in panel **C**, two representative examples of LPS treated cells.

Figure 4.40 | **Visualisation of LC3 in response to LPS.**

CD11c expressing bmDCs ($3.5x10^4$/chamber in a total of 300 μl) were stimulated with LPS (1 μg/ml) or medium for 18 h. After washing, fixing and permeabilising the cells, LC3 was visualised using a polyclonal Ab against the protein followed by a FITC-labelled anti-rabbit IgG. The nuclei were stained for with DAPI and the cells visualised by confocal microscopy in 0.8 μm sections. In panel **A**, the LC3 Ab conjugated with its immunising peptide (blocking peptide) was utilised as a specificity control on LPS treated cells. Both FITC and DAPI staining is presented (left) alongside both separately (FITC, middle; DAPI, right). In panel **B**, a representative picture in the same format is provided for immature bmDCs and in panel **C**, two representative examples of LPS treated cells.

Figure 4.41 | **Visualisation of TRAF6 in response to LPS.**

CD11c expressing bmDCs ($3.5x10^4$/chamber in a total of 300 μl) were stimulated with LPS (1 μg/ml) or medium for 18 h. After washing, fixing and permeabilising the cells, TRAF6 was visualised using a polyclonal Ab against the protein followed by a FITC-labelled anti-rabbit IgG. The nuclei were stained for with DAPI and the cells visualised by confocal microscopy in 0.8 μm sections. In panel **A**, the TRAF6 Ab conjugated with its immunising peptide (blocking peptide) was utilised as a specificity control on LPS treated cells. Both FITC and DAPI staining is presented (left) alongside both separately (FITC, middle; DAPI, right). In panel **B**, a representative picture in the same format is provided for immature bmDCs and in panel **C**, two representative examples of LPS treated cells.

Figure 4.42 | **Protein-protein associations formed by p62 upon stimulation with LPS**

In panel **A**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with medium or LPS (1 μg/ml) for 18 h. In order to isolate p62 and its associated proteins each sample was carefully lysed and treated with anti-p62 associated Dynabeads (Invitrogen) as per the manufacturer's instructions. The subsequent cell lysates and Dynabead precipitates were analysed by Western blotting for p62, TRAF6, PKCδ and LC3 expression. In panel **B**, CD11c expressing bmDCs ($2x10^6$/well in a total of 2 ml) were stimulated with medium or LPS (1 μg/ml) for the indicated times. The control (unstimulated) cells were incubated under the same conditions for the length of the experiment (18 h). P62 and its associated proteins were isolated from these samples by anti-p62 associated Dynabeads (Invitrogen) as per the manufacturer's instructions and analysed by Western blotting for p62, TRAF6 and PKCδ expression. A bead only control (no protein lysate) is also included.

Figure 4.43 | Effect of inhibiting the initiation of autophagy on the production of cytokines important in differentiating T helper (T_H) cell phenotypes upon ES-62 and/or LPS stimulation.

In panel **A**, BmDC were derived from bone marrow cells obtained from BALB/c mice by culture *in vitro* in the presence of GM-CSF for 6 days. These bmDC ($2x10^6$/well in a total of 2 ml) were then pretreated with medium or ES-62 (2 μg/ml) for 18 h followed by maturation with medium or LPS (1 μg/ml) for a further 18 h. In parallel cultures, bmDCs were treated with 3-Methyladenine (**3-MA;** used at 2 mM) for 1 h to block autophagy before being stimulated with identical combinations of ES-62 and LPS as described above. The culture supernatants were then analysed for a range of cytokines including TNF-α, IL-12 p70, IL-23 and IL-6, associated with the development of particular T_H phenotypes. Data are acquired from two independent experiments and presented as the means of the triplicate estimations ± SEM where n=2 and the statistical analysis is by one way-ANOVA, utilising a Tukey post test where p*<0.05, **p<0.01 and ***p<0.001. In panel **B**, control (resting cells) and ES-62 stimulated cytokine secretion results are shown on an expanded scale.

5 General Discussion

There is now a wealth of literature proposing that despite both helminth infections and atopic diseases sharing many immunological traits, immunoregulation by the parasites within the host can dampen responses to allergens and T_H2 associated pathologies[285]. It is known that DCs, which are crucial in the interpretation of multiple pathogenic stimuli and the development of tailored immune responses, are a target of the immunomodulatory affects of ES-62 and the results within this thesis demonstrate a number of avenues by which this molecule prevents hyper-inflammatory responses induced by LPS. Although associated with driving the development of T_H2 biased responses, ES-62 can act to attenuate the inflammation associated with various pathological phenotypes *in vivo* that are instigated by aberrant T_H1 and/or T_H17 (CIA[324,352]) as well as T_H2 (OVA induced airway hypersensitivity[321]) phenotypes. The findings in Chapter 3 directly relate to the role of IL-17A proinflammatory responses[444] and the ability of ES-62 to attenuate the ability of DCs to induce the development of T_H17 cells. This is coupled in view of recent evidence from this laboratory that ES-62 suppresses pathogenesis of CIA driven by aberrant T_H17 function by targeting the IL-17-producing cellular network at multiple sites[352]. Importantly for the ability to fight infection a level of plasticity was demonstrated in such suppression of IL-17 responses as these effects could be partially overcome when ES-62 matured DCs presented high concentrations of Ag. This presumably reflects how the microenvironment and pathogenic burden (represented by the increasing concentration of Ag) may overcome the effects of ES-62 on DCs allowing the induction of a limited proinflammatory response to prevent immunocompromising the host with respect to subsequent infection.

The reduction of IL-17A secretion induced by the DCs predominantly stems from alterations to the LPS induced pro-inflammatory cytokine environment, as ES-62 had little effect on costimulatory molecule expression. Consistent with this, secretion of IL-23 was reduced, where this cytokine is important for maintaining a T_H17 cell population and enhancing its pathogenicity[123,444]. The targeting of this cytokine may also reflect ES-62 blunting the potent proinflammatory actions of IL-23, which occurs in conjunction with its induction of the development of T_H17-type immune response[123,209,242,249]. By contrast, the maintenance of IL-6 secretion may reflect the preservation of the differentiation of a T_H17 cell population that is partially functional and allows restricted development of proinflammatory responses. However, it should be noted that contrary to these observations, in the inflammatory emvironment mediated in the CIA model, where the cytokine GM-CSF is particularly important in the progression of the disease[523] and it

commonly associated with inflammatory environments[518,519], ES-62 mediates the downregulation of IL-6[324,352,524]. In relation to this *ex vivo* lymph node cultures from mice whose Ag specific T cells bear a tg TCR specific for $OVA_{323-339}$ deomstrated that upon immnuisation, prior exposure to ES-62 *in vivo* (where GM-CSF would also be present) modulated the responses to heterologous Ag by inhibition of Ag-specific clonal expansion and the secretion of a number of cytokines including IL-6[323]. Therefore the cytokine responses observed upon stimulation of the DCs with combinations of ES-62 and LPS, which were preformed in the absence of GM-CSF, could suggest that some of the effects of this immunomodulator maybe dictated by the inflammatory environment of the DC. Thus, to expand upon these concepts the effects of ES-62 in the presence of GM-CSF should be defined, which can be coupled with an investigation on its affect on TGF-β secretion, as in conjunction with IL-6 it is important in promoting the development of T_H17 cells[123,124].

However, immunomodulation of DCs by ES-62 was not limited to reducing their ability to induce T_H17 differentiation in terms of IL-17A secretion but was accompanied by the ablation of IL-22 production (mRNA and protein) from T_H cells. Previous studies by this laboratory group have identified that the production of IL-17 and IL-22 are rarely observed from the same cells (M. Pineda & M.M. Harnett, unpublished observations). Although a dedicated T_H22 subset has only been recently defined in humans and an equivalent in mice is yet to be discovered[535], both IL-6 and TNF-α have been highlighted to be important for the development of this cell population[536]. IL-22 is quite unusual as it is produced by immune cells but acts only on non-hematopoietic stromal cells and displays both pathogenic[412,413] and protective[414,415,537] properties in a number of inflammatory disorders. Thus the ES-62 induced reduction of TNF-α whilst maintaining IL-6 secretion could be crucial to the reduced secretion of IL-22 either from as yet undefined cell population, or from the many reported to do so including T_H1, T_H17 and γδ T cells[535]. The targeting of the secretion of IL-22 by ES-62 is as yet unclear but it could relate to its importance in inducing the secretion of anti-microbial peptides from non-hematopoietic stromal cells[402,411]. In any case the targeting of the ability of DCs to induce the development of T_H17 cells justifies the excitement associated with the potential of ES-62 to provide a platform for the generation of persistent and effective anti-infalmmatory therapeutics.

The results described demonstrate that ES-62 is able to develop flexibility within DCs to prime the development of specific immune phenotypes while preventing the hyper-

inflammatory associated with such responses and these findings are summarised in **Figure 5.1**.

5.1 Modulation of aspects of LPS signalling upon the canonical activation of TLR4

Following the discovery of the TLR family of receptors over two decades ago, the role of these proteins in innate immunity continues to expand. This is in part due to the increasing diversity of intracellular adaptors they engage to mediate signalling cascades[15], their dynamic movement throughout resting and stimulated cells[13] and the association of a diverse array of accessory molecules to mediate different cellular responses[538]. In this respect, the TLR4/MD2 complex is rapidly internalised via lipid rafts upon engagement with its canonical ligand, LPS, recruiting both TIRAP and MyD88[435,436] for the activation of NF-κB, an important transcription factor for the maturation of DCs to secrete a range of inflammatory cytokines[14,330]. The activation of NF-κB by proteosomal degradation of its inhibitory subunit IκB is key for its induction of proinflammatory responses in DCs[25]. However, results in Chapter 4 demonstrate that inhibition of lysosomal proteases (with E-64-D/Pepstatin A) induce an accumulation of both IκB-α and -β, which is coupled with the significant attenuation of LPS mediated IL-23 and IL-27 secretion, both T_H17 associated cytokines. These data are consistent with lysosomal degradation being important in maintaining the activation of NF-κB during the late-endosomal TRAM-TRIF mediated phase of LPS-TLR4 signalling[327], which in turn is important in the development of T_H17 responses by DCs.

The results presented in Chapter 3 indicate that although this dynamic for TLR4/MD2 holds true within bmDCs, it is remarkably different to that of the total cellular pool of TLR4, defined by its upregulation both intracellularly and on the surface of the cell. This increase in the total cellular pool of TLR4 may reflect the ability of this receptor to mediate further functions via interactions with alternative adaptors. For example, the recently identified RP105 (CD180), a TLR like molecule that lacks a functional TIR domain, is able to interact with TLR4 to limit LPS driven cytokine secretion in DCs, suggesting that the association of this or other such molecules may provide further signals to control DC maturation and induction of immune responses[539].

The dynamic expression of TLR signalling adaptors induced by LPS extends to several members of the E3 ubiquitin ligase family, namely TRAF6 and c-Cbl with both

TRAF6 and c-Cbl being important in TLR4 induced MyD88 (in)-dependent modulation of NF-κB activation[24,44,454]. Consistent with this the data in Chapter 4 indicates that prolonged proteosomal degradation of c-Cbl and the parabolic regulation of TRAF6 is important in the maturation of DCs to endotoxin. The observed upregulation after 18 h of TRAF6 (protein and mRNA) suggests that the intial downregulation may reflect a negative feed back mechanism to LPS, limiting pathogenic TLR4 signalling. By contrast, the proteosmal degradation of c-Cbl induced by LPS most likely reflects its proposed role in stabilising NF-κB subunits as its deficiency leads to higher levels of IL-12p70 and IL-6[454], two direct affects of LPS stimulation of DCs.

This dynamic regulation of NF-κB activation was accompanied by the altered expression, cleavage and phosphorylation of several PKC isoforms upon LPS maturation of DCs. These lipid-sensitive protein serine/threonine kinases are increasingly emerging as important mediators in TLR mediated NF-κB activation, particularly in relation to the induction of cytokine secretion. The results in Chapter 4 support this paradigm as LPS mediated the upregulation of PKCε and PKCα (as well was the phosphorylation of the latter), and directly attributed to these kinases are the release of TNF-α, IL-12p70 and IL-6 from myeloid derived populations[115,119,120]. For example, PKCδ can promote inflammatory cytokine secretion by activation of NF-κB[93,102,103] through IKK dependent[101,104,105,540] and independent[106-108] (through direct phosphorylation of various MAPKs and Akt) pathways.

Although PKCδ expression was not found to be strikingly altered by LPS, the effect on the phosphorylation, localisation and cleavage of the protein suggest a dynamic role in TLR4 signalling. In this context, LPS stimulation induced the phosphorylation of the A-loop, which is important in stabilising the active conformation of the enzyme and defining its substrate specificity to allow the activation of NF-κB and AP-1[62,74]. Thus A-loop phosphorylation of PKCδ appears to be involved in mediating NF-κB activation as defined by upregulation of proinflammatory cytokine and costimulatory marker expression by DCs. Interestingly, this phosphorylation was also observed on the cleaved form of the protein, where the relative levels of cleaved (the isolated kinase domain fragment) to full length PKC can dramatically alter the specificity and cellular function of these kinases[56,58] and often leads to the accumulation of this protein fragment in the nucleus[59]. Thus the observation that LPS induced the proteosomal degradation of the cleaved form suggests that this unregulated kinase fragment is not beneficial upon a strong inflammatory stimuli. As observed by confocal microscopy this modulation of PKCδ (cleaved and whole)

coincides with its distinct nuclear localisation, presumably to mediate the phosphorylation of transcription factors or another as yet undefined role. The scaffolding protein p62 has been reported to interact with aPKCs[506] and TRAF6[504,505,507] to mediate NF-κB activation and the results presented here expand these associations to include PKCδ but perhaps surprisingly not TRAF6. Although the role of these interactions is not clear, the emerging importance of p62 and PKCδ in inflammatory signalling suggests that at least in DCs their interaction is important in their maturation in response to LPS. However, somewhat in disagreement with these results confocal microscopy revealed this same stimulus induced the punctate localisation of p62 and TRAF6, which was not replicated by PKCδ, consistent with studies demonstrating p62 and TRAF6 colocalise to discrete punctuate centres in response to inflammatory stimuli[508].

In relation to the role of TRAF6 the polyubiquitination of this E3 ligase is required for its interaction with the TAK1 complex[24] allowing the activation of NF-κB, however, upon TLR4 stimulation TRAF6 was not observed to associate to ubiquitin complexes but this affect could have been overlooked during this analysis (occurring < 2 h). The progressive ubiquitination of p62, which is required for the interaction with TRAF6 to mediate NF-κB activation[507], could suggest a varying dynamic of this pathway within the maturation process of DCs and could complement the gradual downregulation and assumed regulated signalling of TLR4/MD2 from the cell surface. These results coincided with the dramatic upregulation of p62, in a similar fashion to that observed in macrophages stimulated with LPS and IFNγ, where p62 was also observed to interact with TRAF6 to mediate the activation of NF-κB[505]. Consistent with this a study of human keratinocytes showed that siRNA knockdown of p62 resulted in the reduced expression (mRNA transcript and protein) of TNF-α and IL-6 as well as NF-κB promoter activity upon TLR2/6 (MALP-2) and TLR4 (LPS) activation due to its proposed role in the activation of the IKK complex[531]. Alongside these studies, the results presented here indicate that the LPS induced upregulation and association to ubiquitin complexes of p62 is involved in the activation of NF-κB and the maintenance of such responses for the maturation of DCs. However, as noted in the keratinocyte study, LPS stimulation also induced autophagic flux, where p62 acts as an adaptor protein binding to polyubiquitinated targets to mediate their degradation in autolysosomes through its association with LC3-II in developing isolation membranes[460].

Stimulation of the TLRs, and in particular TLR4, is associated with the acceleration of phagocytosis and the induction of the autophagic machinery in APCs, as defined by the induction of LC3 positive autophagosome formation[460,495-497]. In conjunction with the observations of p62 upregulation, LPS also mediated upregulation of LC3-II, which is the PE conjugated form of LC3 associated with autophagic membranes, indicating autophagic flux was occurring. However, upon blocking lysosomal degradation p62 was not dramatically increased suggesting that although signalling through TLR4 does indeed induce the turnover of the autophagic machinery in DCs, this is not directly related to the functions of p62 as the data indicate it is more likely involved in the maintenance of proinflammatory NF-κB signalling and cytokine responses.

Interestingly, pretreating bmDCs with 3-MA (a well known inhibitor of autophagy) downregulated LPS induced secretion of TNF-α, IL-6 and IL-12p70. This result was somewhat unexpected as induction of autophagy is generally associated with downregulating LPS induced inflammatory responses and the activation of Class I PI3Ks[525]. However, a step associated with initiation of the autophagic machinery has been demonstrated to be involved in the downregulation of TNF-α, IL-12p70 and p40 through the blocking of p35 and p40 mRNA transcript in DCs[526,527]. In this respect DCs from mice which lack Class I PI3K activity show increased IL-12p70 and p40 secretion through enhanced p38 activity[526]. Moreover, the reduction of IL-6 and TNF-α secretion upon 3-MA pretreatment has been previously reported in the literature and could reflect proposals that both of these cytokines feed back into the stimulation of autophagy[528,529]. These findings are summarised in **Figure 5.2**.

5.2 ES-62 modulation of TLR4 function

The TLRs are emerging as targets of many ES products, resulting in the biasing of DCs towards the induction of T_H2-type immune responses. For example, both *Schistosoma mansoni* and *Ascaris lumbricoides* ES products have been demonstrated to utilise TLR2, and potentially TLR4, on DCs to induce a T_H2-type immune response[300]. In regards to ES-62, a requirement of TLR4 (though not its direct recruitment of MyD88) has been defined for the internalisation for ES-62 and its subversion of the proinflammatory functions of this receptor[301,326], which in a DC result in its bias towards promoting T_H2-type responses[160]. The results in Chapter 3, expand upon the current understanding of this dynamic by defining how ES-62 affects the internalisation of TLR4 and its LPS recognising complex TLR4/MD2, a key step in propagating proinflammatory signals and the activation of NF-

κB[347]. The multiple avenues of analysis revealed that although the direct effects of ES-62 on the expression (at both the mRNA and protein levels) of the receptor are relatively subtle, it antagonises the internalisation of TLR4/MD2 and upregulation of TLR4 induced by LPS. Interestingly, ES-62 is observed to promote the alternative routing of TLR4 to caveolae lipid raft compartments for sequestration and degradation in mast cells[321]. Related to this, the results presented demonstrate that ES-62 induces a similar sequestration of the total pools of TLR4 and the TLR4/MD2 complex within the cell but in the DCs this occurs specifically in response to inflammatory stimuli, providing a mechanism for the induction of TLR4 hyporesponsiveness. In a parallel mechanism the schistosome modulator, SEA, which can also function through TLR4, is trafficked into a LAMP2 (an endosome/lysosome marker) negative compartment in a clathrin dependent manner[443]. Such trafficking indicates the potential for a similar differential compartmentalisation of TLR4, relative to that induced by classical PAMPs, for the subversion of TLR4 signalling by ES-62 and (potentially) other helminth products.

Results in chapter 4 demonstrate that the modulation of DC function by ES-62 is not limited to the dysregulation of the receptors involved in the recognition of PAMPs[301,331,515,541]. As described in **section 5.1,** PKCs are directly responsible for the propagation of a number of inflammatory responses including the recognition of LPS by TLR4. In murine splenic B cells ES-62 induced the proteolytic degradation of PKCα, -β and -δ resulting in the inhibition of the function of a host of transcription factors including NF-κB[320,333,335]. A more recent study has identified the non-proteosomal degradation of PKCα following its association to an ES-62-TLR4 complex via a caveolae-lipid raft dependent mechanism in mast cells[321]. Thus it is perhaps no surprise that as with B cells and mast cells, the activities of ES-62 in APCs are dependent on the regulation of several PKC isoforms, as demonstrated by its induction of the lysosomal degradation of PKCε and -δ coupled with the upregulation of PKCα. Accompanying the lysosomal degradation of PKCδ and PKCε, ES-62 uncoupled the transcription and translation of these signalling enzymes. The targeting of these isoforms can be linked to their role in LPS induced activation of NF-κB (particularly PKCδ[93,101,102]) and in the case of PKCε the secretion of TNF-α and IL-12p70 in monocyte derived DC[100,114], as ES-62 attenuated the ability of LPS stimulated DCs to secrete such cytokines. However, there is a complexity to this function as modulation by ES-62 is observed to be plastic, with the subsequent stimulation of LPS attenuating the downregulation of these proteins.

In relation to this the novel PKCs, including PKCδ localise to the caveolae in several cell types to drive the formation of ceramide and lipid rafts at the PM and indeed several isoforms have been shown to physically associate with markers (caveolin-1/-3) in developing caveolae[59,83,84,542]. Thus, the initial transient upregulation of PKCδ and PKCε observed in response to ES-62, may be an important step in the driving of caveolae formation and provide a mechanism for their (and potentially other associated adaptors or TLR4) trafficking to the lysosomes for degradation[82]. The maintenance of PKCα expression (and potentially activation) by ES-62 is important for maintaining IL-6 secretion, as it is known to be important for the maximal production of this cytokine in macrophages and DC[119,120].

In conjunction with the results (Chapter 4) indicating that TRAF6 is upregulated while c-Cbl is degraded in response to LPS, ES-62 was observed to induce the lysosomal degradation of TRAF6 coupled to the increased expression c-Cbl. Such inverse functions by canonical (LPS) and novel (ES-62) TLR4 ligands could reflect the diametric roles of these two E3 ubiquitin ligases in TLR4 signaling. For example, DCs from TRAF6 deficient mice present maturation defects in response to LPS resulting in attenuated production of IL-12p70 and IL-6 secretion[26-28]. By contrast, c-Cbl is involved in stabilising the inactive NF-κB complex for the prevention of excessive secretion of IL-12p70 and IL-6[454]. Thus the combined effects of ES-62 on these E3 ubiquitin ligases suggests that it is maturing a DC phenotype that is unable to induce hyper-inflammatory responses to LPS.

The lysosomal targeting of ES-62 immunomodulation indicated a role for autophagy, which as outlined previously, is an emerging effector of TLR-mediated innate and adaptive immune responses. The induction of autophagic flux by ES-62, defined by the increase in the relative expression of LC3-II to LC3-I was accompanied by the degradation of p62, amplified by the pharmacological inhibition of the lysosomal proteases and/or autophagy. This approach indicated that ES-62 was inducing the induction of autophagy to mediate the selective lysosomal degradation of important adaptors in the TLR4 signalling pathway, such as p62, TRAF6 and PKCδ/ε. In relation to this, ES-62 induced the short-term increase in the association of TRAF6, PKCδ, c-Cbl and p62 to ubiquitin complexes, which could suggest that this maybe the required impetus for the degradation of these proteins in the lysosomes. In this respect, through adaptor proteins such as p62, ubiquitination maybe required for the targeting of these proteins to the autophagosomes for their selective degradation via the autolysosmes, which is rescued upon inhibition of lysosomal proteases

(E-64-D/Pepstatin A)[460]. In this case ES-62, via autophagy may uncouple the signalling capacity of p62 (in relation to TLR signalling and NF-κB activaiton) as ES-62 maturation of the DCs downregulated the expression of this protein and attenuated its upregulation in response to endotoxin.

The immunomodulatory effects of ES-62 in bmDCs were altered in the presence of GM-CSF, a cytokine that is commonly produced in response to inflammatory stimuli such as LPS *in vivo*, where its acts to augment the functions of monocytes, macrophages and DCs, priming for the secretion a range of proinflammatory cytokines in response to further inflammatory stimuli[509]. Here, when experimentally mimicking such a 'proinflammitory' environment by addition of extrinsic GM-CSF, ES-62 dramatically downregulates MyD88, TRAF6 and c-Cbl (although not PKCα). Of particular interest was the downregulation of MyD88, a crucial mediator of proinflammatory responses via TLRs (except TLR3) and the activation of NF-κB[14,543], in the presence of GM-CSF as in its absence ES-62 intially appeared to have little effect. However, on closer inspection in the absence of GM-CSF, use of lysosomal inhibitors revealed that ES-62 acted to maintain basal levels MyD88 by directing its lysosomal degradation. These results indicate the downregulation of not only MyD88 but also TRAF6 and c-Cbl (for which the involvement in inflammatory TLR4 signalling is well documented) is a crucial step in the uncoupling of the inflammatory capacity of DCs by ES-62. This would be in keeping with some of the *in vivo* functions of ES-62 in both $T_H1/17$[310,324] and T_H2[302,321] associated proinflammatory responses. These findings are summarised in **Figure 5.3**.

Figure 5.1 | The effect of ES-62 on LPS induced DC responses

Through the interaction with TLR4 on the surface of immature DCs, ES-62 and LPS induce the differential maturation and trafficking of this receptor. Specifically ES-62 induces the degradation of certain intracellular adaptors and the differential trafficking of TLR4 to potentially attenuate the activation of NF-κB and the IRFs leading to reduced production of IL-12/23 p40, IL12p70, TNF-α and IL-23. In this context, ES-62 matured DCs were observed to induce fewer T_H cells into proliferation upon stimulation with LPS, (compared to DCs matured with LPS alone). However, this effect was Ag dose dependent as proliferative responses induced by DCs presenting high concentrations of OVA peptide weren't inhibited, indicating a ceratin level of plasticity to prevent the host being immunocomprimsed to subsequent pathogenic stimuli. In conjunction with this, evidence in this thesis clearly indicates that ES-62 maturation of DCs is able to limit IL-17 and IL-22 production from differentiating T_H cells upon stimulation with LPS, suggesting that it acts to attenuate the clonal expansion of the proinflammatory T_H17 subset.

Figure 5.2 | The intracellular responses of DCs upon stimulation with LPS

The recognition of LPS by TLR4/MD2 induces the internalisation of the complex alongside the upregulation of both surface and intracellular pools of TLR4. The activation of NF-κB by LPS-TLR4/MD2 signalling occurs relatively rapidly through the proteosomal and degradation of IκB-α and -β. This relates to the early phase of TLR4 signalling, however after prolonged stimulation a distinct lysosomal component is observed, which is accompanied by a change in the expression of TRAF6, PKCε and PKCα coupled with the proteosomal degradation of c-Cbl. These signalling adaptors are involved in the propagation of TLR4 signals and induction of proinflammatory cytokine production. The expression of p62 is progressively increased and is accompanied by a novel interaction with PKCδ but not LC3 (a classical marker of autophagic flux). The phosphorylation and relative levels of cleaved to full-length PKCδ are also altered indicating it may be involved in maintaining the prolonged activation of NF-κB. As repeated elsewhere, the stimulation of TLR4 with LPS induced autophagic flux, as defined by increased levels of LC3-II. However, in contrast to the proposal that autophagy provides a negative feedback mechanism for TLR4 signalling, inhibition of this cellular process results in the attenuated ability of DCs to secrete proinflammatory cytokines.

Figure 5.3 | Immunomodulation of DCs by ES-62

Panel **A**, upon interaction with TLR4, ES-62 induces a slight increase in the surface expression of this receptor, whilst slightly decreasing that of TLR4/MD2. The maturation of DCs with ES-62 partially inhibited LPS-mediated internalisation of the TLR4/MD2 complex and prevented the upregulation of TLR4 to the cell surface. The ES-62 driven upregulation of c-Cbl may relate to the stabilisation of the inactive NF-κB complex and hence prevent its activation and propagation of hyper-inflammatory responses. Moreover, ES-62 specifcally targets the lysosomal degradation of PKCδ, TRAF6 and PKCε, which are all associated with the production of various proinflammatory cytokines. This is accompanied by the induction of autophagic flux (identified upon the accumulation of LC3-II upon inhibition of lysosomal degradation), a process that was accompanied with a reduction of p62 expression, presumably due to its role in LPS-induced NF-κB activation. This could be facilitated by ubiquitination, as p62 can bind to ubiquintinated proteins and deposit them into autophagosomes for degradation. ES-62 also induces the transient translocation of full length and cleaved PKCδ into the nucleus of these cells suggesting that this plays some role in the alternative maturation of bmDCs by ES-62. Panel **B**, under proinflammatory conditions created by the addition of GM-CSF to the stimulatory culture) ES-62 is observed to degrade MyD88, TRAF6 and c-Cbl while maintaining PKCα expression. This indicates that under proinflammatory conditions ES-62 acts to prevent proinflammatory signalling through degradation of key TLR4 signalling adaptors for the activation of NF-κB.

6 References

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