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MODULATION OF T CELL RESPONSES BY MANIPULATING SIGNALLING PATHWAYS IN DENDRITIC CELLS

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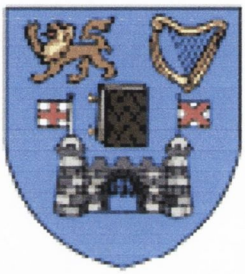

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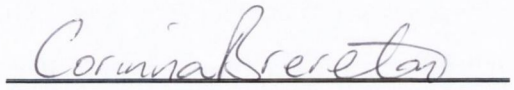

Corinna Brereton

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ABSTRACT

Heat labile enterotoxin from *E. Coli* (LT) is a powerful vaccine adjuvant, capable of enhancing Th1 and Th2 responses to co-administered antigens. The present study demonstrates that LT also promotes the induction of Th17 cells. The ability of LT to promote the induction of IL-17-producing T cells (Th17 cells) was mediated in part by modulation of dendritic cell (DC) activation. LT induced IL-23p19 mRNA expression in DC and enhanced TLR-induced IL-23 secretion. The adjuvanticity and immunomodulatory potential of LT has largely been attributed to the ADP-ribosyltransferase activity associated with the A subunit, and consequent accumulation of intracellular cAMP. However, studies with the B subunit and LT mutants lacking enzyme activity demonstrated that these retained adjuvant properties. The findings of this study demonstrate that LTR72, a partially enzymatically active derivative of LT, also promoted the induction of Th17 cells, as did LTK63, an enzymatically inactive mutant, albeit to a lesser extent. LTR72 and LTK63 induced IL-23p19 mRNA expression and enhanced TLR-induced IL-23 production by DC.

LT and LTK63 suppressed TLR-induced IL-12 and enhanced TLR-induced IL-10 production by DC. While LT can exert these effects rapidly, LTK63 required a pre-treatment of at least 12 h. Therefore, there are roles for enzyme–dependent and enzyme–independent pathways in the immunomodulatory actions of LT. The present study revealed that, despite being devoid of enzyme activity, LTK63 increased the concentration of intracellular cAMP, indicating that LTK63 can mediate cAMP-dependent effects, independently of the enzymatically active A subunit. Protein kinase A (PKA) is a principal downstream target of cAMP, however, the suppression of LPS-induced IL-12 production by LT and LTK63 was PKA-independent.

The MAPK ERK negatively regulated TLR-induced IL-12p70 production, but did not play a role in mediating the suppression of IL-12 secretion by LT. However, inhibition of ERK inhibited the expression of IL-23p19 mRNA induced by LT and LTK63. LT and LTK63 induced phosphorylation of Akt, one of the principal downstream targets of PI3K activation. The suppression of IL-12 and enhancement of IL-10 production by LT and LTK63 was PI3K-dependent, indicating that PI3K signalling plays an important role in mediating the immunomodulatory effects of the LT.

IL-1β and IL-23 are two of the cytokines that mediate the induction and/or expansion of Th17 cells, which are pathogenic in many autoimmune diseases. This study showed that inhibition of ERK suppressed IL-23 and IL-1β production by DC stimulated with TLR or dectin-1 agonists. Incubation of antigen-pulsed TLR-activated DC with an ERK inhibitor suppressed their ability to induce antigen-specific Th17 cells *in vivo*. Treatment with an ERK inhibitor attenuated experimental autoimmune encephalomyelitis (EAE), when administered either at the induction phase of acute EAE or during remission in the remitting-relapsing model. This was associated with significant suppression of autoantigen-specific Th17 responses. The inhibitory effect of the ERK inhibitor on IL-17 responses and EAE was reversed by administration of IL-1β and IL-23. Therefore, ERK MAP kinase plays a critical role in activating the IL-23-IL-17 axis, and represents an important target for therapeutic intervention against autoimmune diseases.

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Brereton, C.F., C.E. Sutton, S. Lalor, E.C. Lavelle and K.H.G. Mills. 2008. Inhibition of ERK MAP kinase suppresses IL-23-driven IL-17 production and attenuates autoimmune disease. (Manuscript under review)

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LIST OF ABBREVIATIONS

ACT	Adenylate cyclase toxin (also known as CyaA)
ADP	Adenosine-diphosphate
Ag	Antigen
AKAP	A-kinase-anchoring-proteins
AP	Activator protein
APC	Antigen presenting cell
aPKC	Atypical PKC
ARF	ADP-ribosylation factors
ASK	Apoptosis signal-regulating kinase
ATF	Activating transcription factor
BCR	B cell receptor
BMDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
bZIP	Basic and leucine zipper
cAMP	Cyclic 3',5'-adenosine monophosphate
CARD	Caspase-recruiting domain
CBP	CREB-binding protein
CD40L	CD40 ligand
cDC	Conventional DC
C/EBP	CCAAT/enhancer-binding protein
CFA	Complete Freund's Adjuvant
CNS	Central nervous system
CpG	Cytosine-phosphate-guanine
CpG-ODN	Cytosine-phosphate-guanine synthetic oligonucleotide motif
cPKC	Classical PKC
CRE	cAMP response element (5'-TGACGTCA-3')
CREB	CRE-binding protein
CREM	cAMP response element modulator
CD	Crohn's disease

CSIF	Cytokine synthesis inhibiting factor
CSF	Cerebrospinal fluid
CT	Cholera toxin
CTB	Cholera toxin B subunit
CTL	Cytotoxic T lymphocyte
CVID	Common variable immunodeficiency
DAG	Diacylglycerol
DC	Dendritic cell
d-cAMP	Dibutyryl-cAMP
DNA	Deoxyribonucleic acid
DNB	DNA-binding domain
ds	double stranded
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveitis
EBI3	Epstein-Barr virus-induced molecule 3
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme linked immunosorbent assay
Epac	cAMP-dependent guanine nucleotide exchange factor
ER	Endoplasmic recticulum
ERK	Extracellular-regulated kinase
ETEC	enterotoxigenic *E.coli*
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FHA	Filamentous hemagglutinin
FITC	Fluorescein isothiocyanate
GITR	Glucocorticoid-induced TNF receptor
G proteins	guanine nucleotide binding proteins
GA	Glatiramer acetate
GM-CSF	Granulocyte/macrophage colony stimulating factor
GPCR	G protein-coupled receptor
GSK-3	Glycogen synthase kinase 3
GTPase	Guanosine triphosphatase

HCV	Hepatitis C virus
HDACs	Histone deacetylases
HRP	Horseradish peroxidise
HSP	Heat shock protein
IBD	Inflammatory bowel disease
ICAM	Intracellular adhesion molecule
ICER	Inducible cAMP early repressor
ICOS	Inducible costimulator
iDC	Immature DC
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of NFκB
i.p.	intraperitoneal
KID	Kinase inducible domain
IKK	IκB kinase
IL	Interleukin
IL-R1	Interleukin receptor 1
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
JAK	Janus-family tyrosine kinase
Jak2	Janus kinase 2
JNK	c-jun NH_2-terminal kinase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LT	Heat-labile enterotoxin
LTA	Lipoteichoic acids
MAL	MyD88-adaptor-like protein
MARCO	Macrophage receptor with collagenous structure
MAPK	Mitogen-activated protein kinase
MAPKAPKs	MAPK-activated protein kinases (also known as MKs)
MBL	Mannan-binding lectin
MBP	Myelin basic protein

MCP-1	Macrophage chemoattractant protein-1
MDP	Muramyl dipeptide
MEK	MAP kinase/ERK kinase
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
MMDC	Monocyte derived dendritic cell
MNK	MAPK-Integrating Kinase
MPL	Monophosphoryl lipid A
MR	Macrophage mannose receptor
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSK	Mitogen- and Stress-Activated Protein Kinase
MS	Multiple sclerosis
MyD88	Myeloid differentiation factor 88
NDV	Newcastle disease virus
NF-AT	Nuclear factor of activation in T cells
NFκB	Nuclear factor κB
ng	nanogram
NK	Natural killer
NLR	NOD-like receptor
NLS	Nuclear localisation sequence
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
nPKC	Novel PKC
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PGIA	Proteoglycan-induced arthritis
pDC	Plasmacytoid DC
PDE	Phosphodiesterases

PDK1	Phosphoinositide-dependent kinase 1
PE	Phycoerythrin
pg	picogram
PGE_2	Prostaglandin E_2
PGRP	Peptidoglycan recognition proteins
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PKA/C	Protein kinase A/C
PMA	Phorbol mysteric acid
PMN	Polymorphonuclear neutrophils
PP	Primary progressive
PP	Peyer's patches
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PT	Pertussis toxin
RANTES	Regulated on activation normal T cell expressed
RIP1	Receptor interacting protein 1
RA	Rheumatoid Arthritis
RPMI	Roswell Park Memorial Institute
RSK	Ribosomal S6 kinase
RR	Relapsing-remitting
RSV	Respiratory syncytial virus
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SARM	Sterile α- and β- armadillo-motif-containing protein
SDS	Sodium dodecylsulphate
SEM	Standard error of the mean
SP	Secondary progressive
SV	Sendai virus
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
s.c.	subcutaneous

TAK-1	TGF-β-activated kinase 1
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th cell	T helper cell
TIR	Toll/IL-1R
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TMEV	Theiler's murine encephalomyelitis virus
TNF	Tumor necrosis factor
Treg	T regulatory cell
TRAF	TNFR-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing interferon-β
UV	Ultra violet
VSV	Vesicular stomatitis virus

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Chapter 1

General Introduction

1 GENERAL INTRODUCTION

1.1 OVERVIEW OF THE IMMUNE SYSTEM

The major role of the immune system is to defend against infectious pathogens. The mammalian immune response relies on a close interplay between its two components, the innate and adaptive systems. The innate immune system responds rapidly to invading pathogens, providing the first line of defence. The adaptive immune response acts at a later phase of infection and is associated with the generation of immunological memory. Adaptive immunity is characterized by specificity and is mediated by T- and B- lymphocytes that proliferate clonally in response to foreign antigens. Since the innate immune response is a more rapid process, it takes centre stage in the recognition of invading pathogens and is responsible for directing the nature of the ensuing adaptive immune response (1).

1.2 INNATE IMMUNE RECOGNITION

The survival of the host relies on its ability to recognize infectious microbes and to induce appropriate defence responses. These protective mechanisms appeared early in the evolution of multicellular organisms and are referred to, collectively, as innate immunity (2). The innate immune system detects the presence and the nature of infecting microbes, provides the first line of host defence, and controls the initiation and the effector class of the adaptive immune response. The effector mechanisms of innate immunity, which include antimicrobial peptides, phagocytes, and the alternative complement pathway, are activated immediately after infection and rapidly control the replication of the infecting pathogen (3, 4). There are two main types of innate defence mechanisms used by the host against pathogens: constitutive and inducible. The constitutive mechanisms include the barrier functions of the body surfaces and mucosal epithelia of the respiratory, gastrointestinal and reproductive tracts. Inducible responses of the innate immune system are triggered upon pathogen recognition by a set of pattern recognition receptors (PRRs) (5).

If pathogens evade constitutive mechanisms, innate cells and soluble protein effectors may be induced. Innate cellular responses are mediated to a large extent by phagocytic cells, such as polymorphonuclear neutrophils (PMN), macrophages and dendritic cells (DC), that either kill the invading microbe or secrete proteins, known as cytokines, which trigger an influx of defensive cells to the site of infection. In addition to their phagocytic activity and ability to destroy pathogens, these cells secrete a variety of inflammatory mediators and cytokines that are important in the regulation of adaptive immune responses. Recognition of pathogens by innate cells is achieved by means of cell-surface and intracellular receptors expressed by the immune cells, including CD14, macrophage mannose receptor, scavenger receptors, integrins, C-reactive protein, Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (1, 6).

1.2.1 Pathogen-Associated Molecular Patterns

Innate immune recognition is based on the detection of conserved molecular structures associated with microbial pathogens, but not with the host (5). These structures were originally called pathogen-associated molecular patterns (PAMPs) and the receptors that recognised these PAMPs were termed PRRs (1, 5). However, PAMPs may not be an appropriate term as they are also expressed by commensal microorganisms and it is specific molecules and not 'patterns' that are actually recognised. The term 'microbial activator' instead of PAMP has been recently proposed (7). However, PAMPs remains the term universally used by researchers.

The best known activators of innate immunity include lipopolysaccharide (LPS) from Gram-negative bacteria, lipoteichoic acids (LTA) from Gram-positive bacteria, cytosine-guanine dinucleotide (CpG) motifs, lipoproteins and double stranded (ds) RNA, produced by viruses during their infection cycle. These structures are only expressed by particular classes of microorganisms allowing the innate immune system to distinguish between self and non-self molecules. PAMPs are often shared by large groups of microorganisms, which allows a limited number of germline-encoded PRRs to recognize a vast variety of potential pathogens (8). As PAMPs often represent 'molecular signatures' of a pathogen class, (e.g. LPS is a signature of Gram-negative pathogens), the immune system induces the effector mechanism that is most efficient against a given

class of pathogen (2). The recognition of microbial 'molecular signatures' by the innate immune system, signals the presence of infection (9, 10).

1.2.2 Pattern Recognition Receptors

The innate immune system detects infection via PRRs that recognise microbial molecular structures and trigger effector responses (1). PRRs can be expressed on the cell surface, in intracellular compartments, or secreted into the blood stream and tissue fluids (1). Several types of protein domains have been found to be involved in pattern recognition, the most prominent being the C-type lectin domain, scavenger receptor cysteine-rich domain and the leucine-rich repeat (LRR) domain (3). The principal functions of PRRs include opsonization, activation of complement, phagocytosis, activation of pro-inflammatory signalling pathways, and induction of apoptosis (11). PRRs are structurally and functionally heterogeneous proteins and can be categorised according to their function (8).

Secreted PRRs can activate complement, opsonize microbial cells to facilitate phagocytosis and function as accessory proteins for pathogen recognition by transmembrane receptors. Examples of secreted PRRs include mannan-binding lectin (MBL) and the peptidoglycan recognition proteins (PGRPs). Phagocytic (or endocytic) PRRs are expressed on the surface of DC, macrophages, and neutrophils. They recognise microbial molecular structures on the surface of pathogens and mediate their uptake. Examples of these receptors include the macrophage mannose receptor (MR), the macrophage receptor with collagenous structure (MARCO), complement receptors, such as CR3 and CR4, and a C-type lectin, dectin-1. Cytoplasmic PPRs can be grouped into 3 classes: the intracellular nucleotide-binding oligomerization domain (NOD) proteins, interferon-inducible proteins and caspase-recruiting domain (CARD) helicases. The TLRs are the major signal-generating family of PRRs and recognize pathogens at either the cell surface or lysosome/endosome membranes (12). Recognition of pathogens by PRRs activates conserved host defence signalling pathways that control the expression of a variety of immune response genes (2).

1.2.3 TOLL-LIKE RECEPTORS

TLRs are the best characterized signal-generating receptors among the PRRs and represent a crucial component of the innate immune system. Toll, the founding member of the TLR family, was initially identified as a gene product essential for the development of embryonic dorsoventral polarity in *Drosophila melanogaster* (13). Subsequently, the role of Toll in innate immunity of adult flies was demonstrated; Toll mutants were unable to mount effective anti-microbial responses and succumbed to overwhelming fungal infection (13). In 1996, the gene encoding this protein, later known as TLR1, was mapped to chromosome 4 in humans (14). The first mammalian TLR was cloned in 1994 by Nomura and co-workers (15). To date, 11 human TLRs and 13 mouse TLRs have been identified, of which TLR1-9 are conserved between the human and the mouse (10).

TLRs are type 1 integral membrane glycoproteins, characterized by an ectodomain composed of LRRs that are responsible for recognition of PAMPs and a cytoplasmic signalling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (16). The TIR domain is a conserved protein-protein interaction module located in the face of each TLR, which is also found in a number of transmembrane and cytoplasmic proteins in animals and plants (17). In TLRs the TIR domain functions in the recruitment of adaptor proteins, of which there are five, MyD88, MyD88-adaptor-like (MAL; also known as TIRAP), TIR-domain-containing adaptor protein inducing IFNβ (TRIF; also known as TICAM1), TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo-motif-containing protein (SARM).

Since their discovery, it has been established that TLRs play important roles in recognizing specific microbial components derived from pathogens, including bacteria, fungi, protozoa and viruses. Activation of TLRs by TLR agonists or pathogens leads to the induction of numerous genes that function in inflammatory and immune responses. These include inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6 and IL-12), chemokines (MIP-1α, MIP-2), MHC and co-stimulatory molecules (1, 8, 18). Activation via mammalian TLRs can also induce molecules, such as inducible nitric oxide synthase and antimicrobial peptides, which can directly destroy microbial pathogens (8). Each TLR

recruits a specific combination of adaptors which ultimately leads to the activation of specific transcription factors, giving appropriate and effective responses to particular pathogens (19).

TLRs can be grouped into families according to the types of ligands they recognize. TLR2, in combination with TLR1 or TLR6 as heterodimers, recognizes lipid based structures, as does TLR4 as a homodimer (20, 21). One of the most studied examples of lipid-based recognition is bacterial LPS, which is recognized by TLR4. TLR4 can also recognize the plant diterpene, paclitaxel, the fusion protein of respiratory syncytial virus (RSV), fibronectin and heat-shock proteins, all of which have different structures (12). Viral and/or bacterial nucleic acids are recognized by TLR3, TLR7, TLR8 and TLR9, which are localized intracellularly. The best characterized of these interactions are the recognition of double stranded RNA (dsRNA) by TLR3 and recognition of unmethylated CpG motifs in DNA by TLR9. TLR5 and TLR11 recognize proteins, flagellin in the case of TLR5 and profilin in the case of TLR11 (only in mice) (19). TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface, while TLRs 3, 7, 8 and 9 are found almost exclusively in intracellular compartments, such as endosomes. Moreover, different TLRs are expressed by distinct cell populations. For example, TLR4 is expressed on the surface membrane of human myeloid DC and monocytes and is essential for the recognition of LPS (22). In contrast, TLR9, which is involved in the recognition of viral and intracellular bacterial DNA, is expressed on plasmacytoid DC (pDC) and B cells (23).

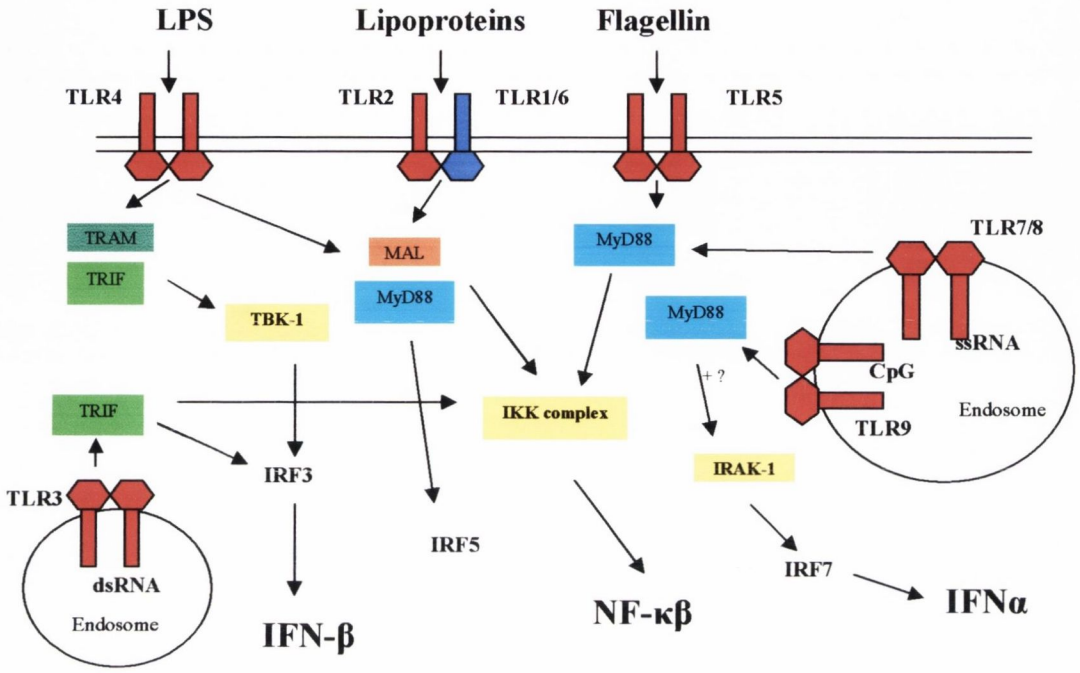

Figure 1.1 Overview of major TLR signalling pathways

All TLRs signal through MyD88, with the exception of TLR3. The TLR2 subfamily (TLR1, TLR2 and TLR6) require MAL to enable MyD88 recruitment. TLR9 causes sustained recruitment of MyD88 to endosomes for interferon-regulatory factor 7 (IRF7) activation to occur via IL-1R-associated kinase (IRAK-1). TLR3 recruits TRIF, which leads to NF-κB and IRF3 activation. TLR4 activates NF-κB via MAL and MyD88, but can also trigger IRF3 activation through TRAM and TRIF. A number of TLRs can also activate IRF5, through a MyD88- and IRAK-1-dependent pathway, which is involved in TNF gene expression.

1.2.3.1 Adaptor proteins in TLR signalling

MyD88

MyD88 was the first adaptor to be described and, with the exception of TLR3, all TLR members recruit it. Members of the IRAK family are recruited immediately downstream of MyD88. IRAK4 appears to be the MyD88-proximal kinase, which in turn recruits and activates IRAK1. A key downstream target for IRAK1 is TNFR-associated factor 6 (TRAF-6), which acts as a ubiquitin ligase (E3). A series of ubiquitinylation reactions occur on TRAF-6 and on the protein kinase TGF-β-activated kinase 1 (TAK-1). TAK-1 phosphorylates the inhibitor of NFκB kinase (IKK) complex and MAP kinase kinase 6 (MKK6), leading to the activation of NFκB and MAP kinases, which results in the induction of genes involved in inflammatory responses (12, 19, 24).

With the exception of TLR3, the MyD88 dependent pathway is the core pathway activated by all TLRs. The first indication of the existence of a MyD88-independent pathway came when it was observed that while MyD88-deficient mice were unable to activate NFκB and MAP kinases, or to upregulate surface marker expression of MHC and costimulatory molecules in response to IL-1 and many TLR ligands (25-27), the TLR4 agonist LPS could still activate NFκB and MAPK (27). MyD88-deficient cells retained their ability to upregulate costimulatory and MHC molecules (28). Furthermore, LPS-dependent activation of IFN regulatory factor (IRF)-3 was preserved in MyD88-deficient cells (28). These studies demonstrated that although MyD88 was essential for signalling events downstream of some TLRs, such as TLR2 and TLR9, MyD88 was clearly dispensable for some TLR4-induced signals, suggesting the existence of a MyD88-independent pathway. This hypothesis was borne out by the discovery of the second TIR domain-containing adaptor, MAL (29), also known as TIRAP protein (TIRAP) (30).

The main role of MyD88 is the activation of NFκB which is a key transcription factor that controls the expression of a number of inflammatory genes. MyD88 is required for the induction of type 1 IFNs by TLR7, TLR8 and TLR9, and has also been shown to be essential for the activation of IRF7 by these TLRs (31). A key function for IRAK1 is the phosphorylation of IRF7 as its activation was totally abolished in IRAK1-deficient cells, whereas NFκB activation was only partially impaired (32). MyD88 also

interacts with IRF1 and IRF5, two transcription factors that are crucial for the induction of inflammatory cytokines.

MAL

The second TIR domain-containing adaptor to be discovered was MAL (29). MAL-deficient mice have impaired responses to TLR2 and TLR4 agonists but have normal responses to other TLRs and IL-1 (33, 34). While MAL-deficient mice respond normally to TLR5, TLR7 and TLR9 ligands, as well as to IL-1 and IL-18, they have impaired responses to ligands for TLR1, TLR2 and TLR6 (33). A dominant negative form of MAL inhibited TLR4-mediated NFκB activation, but did not inhibit activation by IL-1RI or IL-18R (29). Cytokine production by LPS stimulated Mal-deficient macrophages is severely impaired (e.g. TNF-α, IL-6) (33, 34), similar to findings with MyD88-deficient mice (27). For TLR4 signalling the phenotypes in MyD88- and MAL-deficient cells were very similar, in that TLR2- and TLR4- induced TNF-α is abolished and activation of NFκB and p38 MAPK is delayed. Importantly, the induction of IFN-β and activation of IRF3 is normal in both single and double knockouts, further indicating the existence of a MyD88-independent pathway. A role for MAL as a bridging adaptor for MyD88 has been shown. It appears that MyD88 does not directly bind to TLR4, but instead interacts with MAL in association with TLR4. Importantly, MAL has also been shown to function independently of MyD88. MAL has a TRAF6-binding domain and might therefore recruit TRAF6 to the signalling complex. More recently, it was demonstrated that cleavage of MAL by caspase-1 was required for MAL to activate NFκB (35).

TRIF

TIR-related adaptor protein inducing interferon (TRIF) was the third adaptor to be described. TRIF controls the TLR4-induced MyD88-independent pathway, and is the exclusive adaptor used by TLR3 (36). The discovery of TRIF provided an explanation for the ability of TLR3, TLR4, TLR7 and TLR9 ligands, but not TLR2 ligands, to induce

type 1 interferon production. Furthermore, TLR3 and TLR4 have the ability to induce IFN-β and IFN-inducible genes in MyD88 deficient cells. Importantly, LPS-induced NFκB and MAPK activation was completely abolished in MyD88 and TRIF double-deficient cells, showing that TRIF accounts for the MyD88-independent aspect of TLR4 signalling (37). Distinct regions at the N- and C- termini of TRIF activate distinct signalling pathways. The N-teminal region signals through an IKK-like kinase, termed TBK-1 (38), and together with inducible IKK (IKKi), mediates IRF3 phosphorylation, dimerization and nuclear translocation mediating type I IFN induction. The N-terminal region can also activate the NFκB promoter but the C-teminal region has also been shown to signal via a distinct route to NFκB (35).

TRAM

The fourth adaptor to be described was Trif-related adaptor molecule (TRAM). TRAM interacts with TRIF and is specifically involved in TLR4 signalling, and therefore is the most restricted of the adaptors in terms of TLR action (39, 40). LPS-induced cytokine responses are more impaired in TRAM-deficient cells than in TRIF-deficient cells, which suggests that TRAM does not only serve as a bridging adaptor for TRIF. TRAM is required for the induction of TNF, CD86 and a number of IRF3-dependent genes, as well as for the late activation of NFκB (35).

SARM

The last of the adaptors to be assigned a role in TLR signalling was SARM. Unlike the other 4 adaptors, SARM is a negative regulator of NFκB and IRF activation (41). SARM expression was shown to specifically block gene induction and transcription factor activation downstream of TRIF but not of MyD88.

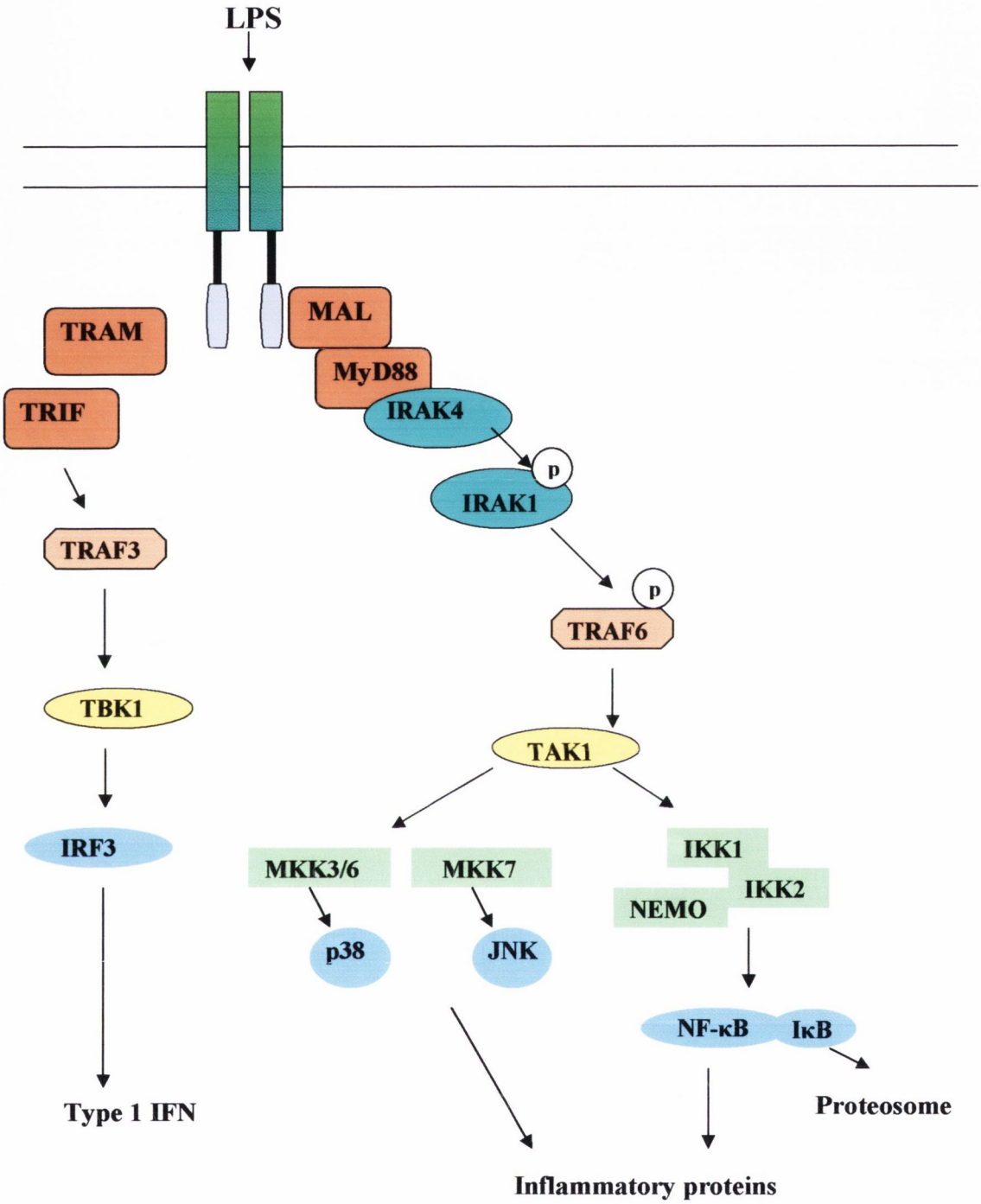

Figure 1.2 LPS-induced TLR4 mediated signalling

1.2.3.2 Regulation of TLR signalling

TLR signalling is governed by a number of factors, including the ligand itself, the localization of the receptor and the sub-cellular localization of its associated adaptors. Recently it has become clear that TLR signal transduction is subject to complex regulation at a number of different levels, including regulation by phosphorylation, targeted degradation, and sequestering of signalling molecules ultimately resulting in the inhibition of transcription (19, 42). Both exogenous and endogenous factors can inhibit TLR signalling, primarily by targeting adaptors. A member of the IL-1R subgroup of TIR proteins, ST2, was shown to inhibit TLR signalling in macrophages by sequestering MyD88 and MAL (43). SIGIRR also belongs to the IL-1 receptor family and has been shown to interact with IRAKs and TRAF6 to block TLR signalling (44). TGF-β blocks TLR signalling by causing ubiquitinylation and proteasomal degradation of MyD88 (45).

The adaptors play a central role in the signalling processes activated by TLRs and as such, these proteins are targets for the inhibition of TLR signalling. Evasion of the immune response is central to the ability of many viruses and other pathogens to persist in the host. Two viruses that use this evasion strategy are vaccinia virus and hepatitis C virus (HCV). Vaccinia virus produces the protein A46R which blocks both IL-1 and TLR4 signalling (46). A46R sequesters MyD88, MAL, TRIF and TRAM and inhibits TLR- or IL-1R-induced NF-κB activation (47). The HCV protein NS3/4A blocks IRF3 activation by cleaving and inactivating TRIF, and thereby limits TLR3-mediated activation of IFN responses (48).

Recently, Sun *et al.* have discovered a role for the tripartite-motif protein TRIM30α in curtailing TLR4-mediated NFκB activation (49). TRIM30α was induced by LPS, and also the TLR9 agonist, CpG, and the TLR3 agonist, polyinosinicpolycytidylic acid, in bone marrow-derived DC. It was demonstrated that TRIM30α bound to TAK1, preventing its recruitment to and activation by TRAF6. Furthermore, *in vivo* transfection of mice with DNA encoding TRIM30α increased the survival of recipient mice after intraperitoneal challenge with LPS. This study established a role for TRIM30α in a negative feedback loop used to control TLR-mediated inflammation and suggests that other TRIM family members may be important regulators of innate immunity (50).

1.2.4 INTRACELLULAR SENSORS OF INFECTION

Pathogens that invade the cytoplasm are recognized by intracellular PRRs. IFN-inducible proteins and CARD helicases are involved in antiviral defense, while NLRs are mainly involved in antibacterial immune responses.

1.2.4.1 CARD Helicases

This family of cytoplasmic senors recognizes viruses that enter the cytoplasm of cells directly or dsRNA synthesized during active viral replication. The CARD-helicases, RIG-1 and MDA5 are thought to detect viruses, such as Newcastle disease virus (NDV) and Sendai virus (SV) (51).

1.2.4.2 NOD-like Receptor Family

NLRs are a family of cytoplasmic PRRs that contain 3 distinct domains: C-terminal LRRs, mediating ligand sensing, a centrally located NACHT domain, mediating self-oligomerization and the activation of NLRs, and an N-terminal domain, mediating protein-protein interactions for initiating downstream signalling. NLRs have been grouped into several subfamilies based on the basis of their effector domains and also on the history of their NACHT domains: NODs, NALPs, IPAF and NAIPs. The NOD and IPAF families contain CARD effector domains, while the NALPs and NAIPs contain pyrin (PYD) effector domains and 3 BIR domains, respectively (52). While the ligands and functions of many of these PRRs are unknown, it appears their major role is to recognize cytoplasmic microbial PAMPs and/or endogenous danger signals, initiating immunological responses.

The best characterised members of the NOD family are NOD1 and NOD2, which have one or two N-terminal CARD effector domains, respectively. The expression of NOD1 is ubiquitious, whereas NOD2 expression is restricted to monocytes/macrophages and epithelial cells. NOD1 recognizes the peptide γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and mainly acts as a sensor for gram-negative bacteria, while NOD2 detects muramyl dipeptide (MDP), found in most bacteria (53). When ligands bind to

NOD1 and NOD2 they oligomerize, resulting in activation of NFκB through the recruitment of RIP2/RICK, a serine-threonine kinase, via their respective CARD domains by homophilic interactions (12). NODs have also been shown to activate members of the MAPK family. Macrophages lacking either NOD1 or NOD2 failed to produce cytokines in response to their corresponding ligands (54, 55). A missense point mutation in the human NOD2 gene is correlated with susceptibility to Crohn's disease, a common inflammatory disease of the intestinal tract (56).

Members of the NALP subfamily, of which there are at least 14, do not activate NFκB or MAPK but are involved in the activation of caspase-1. Several NALPs have been shown to play important roles in the production of pro-inflammtory cytokines, including IL-1β and IL-18. Upon activation NALPs form inflammasomes, which initiates their interaction with caspase-1 via a CARD-CARD interaction. Dostert *et al.* recently demonstrated that asbestos and silica are sensed by the NALP3 inflammasome, whose subsequent activation leads to IL-1β secretion (57).

1.3 DENDRITIC CELLS

DC play a critical role in bridging innate and adaptive immunity (58). DC are professional antigen presenting cells (APCs) and are unique in their ability to stimulate naïve T cells. They are derived from haematopoietic stem cells in the bone marrow and can be separated into subsets, which differ in phenotype, function and location (59). One such division separates DC into blood-derived and tissue-derived DC (60), although the origin of different DC subtypes is not completely understood. Many phenotypic distinctions between human and murine DC may reflect differences in tissue origin and differences between cultured and freshly isolated DC. However, human and mouse DC can be divided into several subtypes on the basis of their surface antigen expression. Three subsets of mature human DC have been described, $CD11c^{hi}$ myeloid, a minor population of $CD34^{+}$ cells and $CD11c^{low}$ pDC (61, 62). Murine DC express the CD11c integrin and can be subdivided on the basis of CD8α expression. There are at least three subsets of murine classical $CD11c^{hi}$ DC, including $CD8\alpha^{+}$, $CD4^{+}$ and double negative DC, as well as a population of $CD11c^{low}$ DC with plasmacytoid morphology. The diverse

DC subsets vary in their expression of many of their surface molecules and therefore a number of distinct populations have been reported in the literature. A population of murine $CD11c^+$ cells also expressing B220 and Gr-1, are equivalent to human plasmacytoid DC (63), whereas murine myeloid DC are $CD11c^+$, $CD8\alpha^-$, $CD11b^+$ (62). $CD8\alpha^-$, $CD8\alpha^+$, and plasmacytoid $B220^+$ DC share a common origin in that they can be generated by both myeloid and lymphoid precursors (64). Recently, it was reported that a splenic precursor population gave rise to all splenic $CD8^+$ and $CD8^-$ conventional DC (cDC) but not plasmacytoid DC or other lineages (65). The existence of distinct subsets of DC raises the question of whether they are functionally specialized to promote distinct immune responses. There is evidence to suggest that distinct DC subsets vary in their microenvironmental localization, lifespans, PRR expression and responsiveness to microbes, indicating that DC may exhibit considerable functional plasticity (62, 66).

DC take up antigen in the tissue and migrate to lymph nodes, where they present antigen to naïve T cells. DC are sentinels for invading pathogens, acting as immunological sensors through the expression of PRRs (66, 67). TLRs are highly expressed on innate immune cells, in particular DC and macrophages. It has been suggested that the functional specialization of different DC subtypes may be in part due to differential distribution of TLRs. For example, in humans, TLR7 and TLR9 are preferentially expressed on pDC. Adaptive immunity to pathogens is often initiated by DC maturation after ligation of TLRs, which therefore are crucial proteins that link innate and adaptive immune responses (4).

DC can also sense pathogens indirectly by detecting inflammatory mediators produced by various cells, including macrophages, natural killer (NK) cells and endothelial cells (68). These inflammation-associated tissue factors include cytokines produced in response to microbes, chemokines, eicosanoids, heat shock proteins (HSPs), and extracecllular matrix components (61). Evidence that HSPs and uric-acid crystals are recognized by DC provides a molecular basis for the 'danger' theory proposed by Matzinger (69). Matzinger argues that tissues send signals to the immune system that determine the appropriate immune response to that tissue. Therefore, the ability of DC to sense pathogens, either directly or indirectly is central to the activation of an appropriate adaptive immune response.

1.3.1 DC MATURATION

DC sense invading pathogens and undergo a process, known as maturation, that allows them to effectively activate naïve T cells, thus inducing an adaptive immune response. DC migrate from the tissue to the lymph nodes and undergo profound changes during maturation. The conventional model of DC maturation, states that DC exist in two functional states, immature and mature (70, 71). According to this model, immature DC can internalize exogenous antigens and process them for MHC class II-mediated presentation, but cannot prime immune responses. Immature DC express low levels of MHC class II and almost no costimulatory molecules. Upon activation, immature DC migrate towards T cells areas in the lymph node, whilst undergoing a maturation process, which shuts off their antigen-capturing and processing capacity. Mature DC express high levels of MHC molecules and the costimulatory molecules CD86, CD80, CD40 and CD83 (62, 72). Expression of these molecules renders the DC capable of T cell priming and therefore they are termed "immunogenic". Therefore, mature DC are primed by pathogens to induce pathogen-specific T cells with signals that determine effector function.

1.3.2 DC LINK THE INNATE AND ADAPTIVE IMMUNE RESPONSES

DC play a central role in shaping the nature of the adaptive immune response. The ability of DC to direct T cell responses depends on several parameters, including the nature of the PRR and the tissue microenvironment. Activation and subsequent maturation of DC, through TLRs, leads to upregulation of surface molecules, and the production of cytokines, which direct different subtypes of T cells (66). Several studies have suggested that distinct subsets of DC differentially modulate T cell responses. It has been proposed that $CD8\alpha^+$ DC may be specialized for promoting Th1 responses and that $CD8\alpha^-$ DC promote Th2 responses (73, 74). However, subsequent studies have shown that the nature of the microbial stimulus and the environmental milieu also play important roles in directing different T cell subtypes (59, 75).

Recent evidence suggests that DC can express MHC, adhesion and costimulatory molecules under non-immunogenic conditions and phenotypically mature DC can induce tolerance. This has led to revisions of the classical model of DC maturation and T cell polarization (72). While the original model proposes that in the absence of costimulation, DC induce tolerance, it has been demonstrated that tolerogenic DC can express substantial levels of costimulatory markers (76, 77). Expression of CD80 and CD86 by APCs is crucial for delivering the costimulatory signals through CD28, however, the same molecules also engage cytotoxic T-lymphocyte antigen 4 (CTLA4), a negative regulator of T cell activation. However, a distinct molecule that delivers signal 2, but is not expressed by tolerogenic DC, has yet to be found. One suggestion is that quantitative differences in the expression of surface markers may determine whether a DC becomes immunogenic or not. One way to explain how some maturation signals might induce the generation of tolerogeneric DC is the view that immature DC become "effector cells" with the potential to instruct distinct T cell fates. However, tamoxifen administration induced DC antigen expression promoting peripheral $CD8^+$ T cell tolerance (78, 79).

Recently Blander and Medzhitov have put forward a model for antigen selection in DC for presentation by MHC class II that is based on the origin of the antigen (80). The report demonstrates that 'immunophagosomes' containing cargo in association with TLR ligands, are required for efficient presentation of antigens, whereas antigens within non-immunophagocytic cargo, such as apoptotic cells, are not presented on MHC class II molecules. Therefore, such differences in presentation of self antigens and microbial antigens, on MHC class II, might explain how DC can induce immunity against invading pathogens but also tolerance to peripheral self antigens.

Ultimately DC are central to shaping the adaptive immune response and the induction of self-tolerance. Through the upregulation of MHC and co-stimulatory molecules and the production of key cytokines and chemokines, DC direct the fate of naïve T cells. The conditions of activation and degree of maturation of DC are critical for the delivery of the three activation signals to the T cells (61).

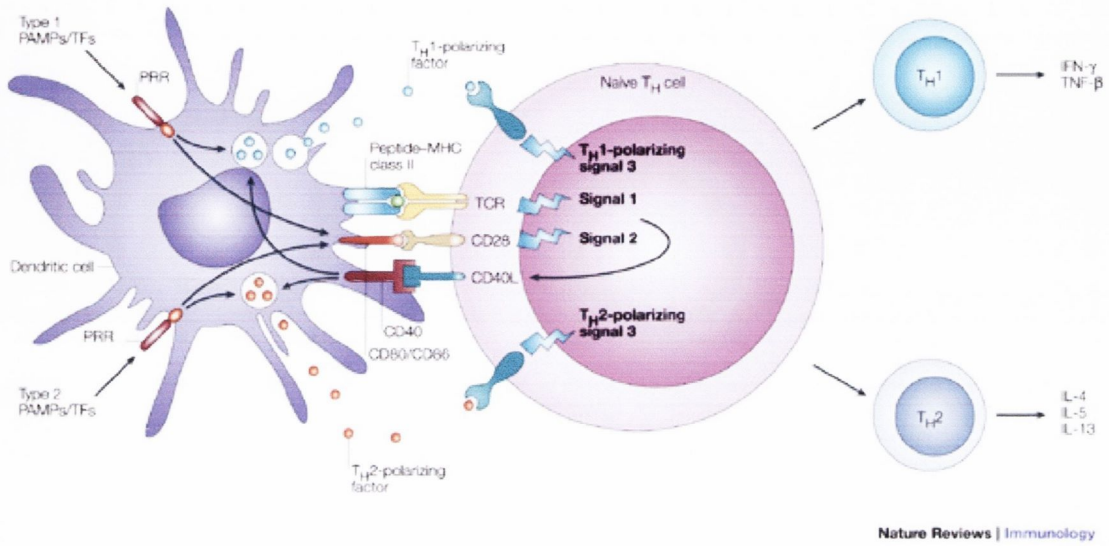

Figure 1.3 DC link the innate and adaptive immune responses.

T cell stimulation and Th cell polarization requires three DC-derived signals. Signal 1 is delivered through the TCR when it engages the appropriate MHC class-II-associated peptides. Signal 2 is the co-stimulatory signal, mainly mediated by triggering of CD28 by CD80 and CD86 that are expressed by DC after ligation of PRRs, such as TLRs. Signal 3 is the polarizing signal that is mediated by various soluble or membrane-bound factors, such as cytokines. The nature of signal 3 depends on the activation of particular PRRs by PAMPs or inflammatory tissue factors. Optimal T cell polarization often requires feedback stimulation by CD40 ligand (CD40L) expressed by T cells after activation by signals 1 and 2. While the diagram shown here (61) illustrates the activation of Th1 and Th2 cells, the other T cell subtypes, Tr1 and Th17 cells, are also activated by APCs.

1.4 THE ADAPTIVE IMMUNE SYSTEM

Microorganisms that overcome or circumvent the non-specific innate immune mechanisms are met by the host's second line of defence, the adaptive or acquired immune system. APCs, including DC and macrophges, initiate the activation of this form of immunity by presenting antigens of the invading pathogen to lymphoid cells capable of responding to a particular epitope of the antigen. The adaptive immune response takes two forms, humoral and cell-mediated immunity, that are usually operated in parallel. B cells respond to antigens by differentiating into antibody-producing plasma cells (humoral immunity), while T cells are responsible for cell-mediated immunity. B cells develop from progenitor cells in the bone marrow while T cells are differentiated in the tymus.

1.4.1 B CELLS

B cells comprise a major component of the adaptive immune system, having the exclusive ability to produce and secrete immunoglobulins (Ig), also known as antibodies, of various forms (isotypes). The antigen-specific membrane receptors of B cells are Ig molecules whose expression occurs through random rearrangement and splicing together of multiple DNA segments that code for the antigen-binding areas of the receptors. Gene rearrangement occurs early in the development of the cells, before exposure to antigen, and leads to the production of a repertoire of over 10^{10} antibody specificities (81). Immature B cells, which have never been exposed to an antigen, express only the IgM isotype but once activated begin to express both IgM and IgD, which renders them mature and ready to respond to antigen. The majority of B cells carry MHC class II antigens, which are important for cognate interactions with T cells.

Activated B cells differentiate into antibody-producing plasma cells or memory cells and are normally restricted to the secondary lymphoid organs and tissues. Antibodies produced by a single plasma cell are of one specificity and immunoglobulin class that have defined roles in the immune system. There are five Ig classes in mammals, known as IgM, IgG, IgD, IgA and IgE. IgG is the most abundant immunoglobulin and constitutes 75% of serum Ig and IgG antibodies can be subdivided further into subclasses in mice (IgG1, IgG2a, IgG2b and IgG3) and in humans (IgG1,

IgG2, IgG3 and IgG4). One of the main ways that antibodies contribute to immunity is by coating pathogens making them easier to phagocytose, a process known as opsonisation. Furthermore, antibodies can activate the complement system which leads to the direct destruction of pathogens. Most plasma cells have a short lifespan while memory B cells tend to be longer-lived and can therefore respond quickly upon second exposure to an antigen.

1.4.2 T CELLS

Lymphocytes, which include NK cells, B cells and T cells, constitute about 20% of all leukocytes present in the adult circulation. The dominant T cell population has T cell antigen receptors (TCRs) that consist of α and β chains, while less than 5% of T cells use the γ and δ TCR chains. Both T cell populations develop in the thymus before entering the periphery. αβ T cells are subdivided into two distinct, non-overlapping populations; cytotoxic $CD8^+$ T cells that kill virus-infected cells and T helper (Th) cells, that express CD4 on their surface. $CD4^+$ T cells recognize their specific antigen in association with MHC class II molecules, whereas $CD8^+$ T cells recognize antigen in association with MHC class I molecules. Thus, the presence of CD4 or CD8 restricts the type of cell with which the T cell can interact.

Like αβ T cells and B cells, γδ T cells are evolutionary conserved and found in all jawed verterbrates, but in contrast to αβ T cells, which reside primarily in secondary lymphoid organs, γδ T cells form the majority of intraepithelial lymphocytes, where they may play a role in protecting the mucosal surfaces of the body. In the mouse, each tissue has its own specific subset of γδ T cells, each displaying a limited TCR diversity (82). Resident γδ T cells are thought to represent a first line of defence against infection and provide certain other tissue-specific functions, including the regulation of wound healing (83). γδ T cells are thought to function largely like their αβ counterparts, however, there is evidence to suggest that γδ T cells do not routinely rely on APC for antigen recognition and they themselves have recently been shown to efficiently process and present peptide antigen to αβ T cells (84).

1.4.3 TH CELL DIFFERENTIATION

The differentiation of naïve $CD4^+$ T cells into distinct subsets of Th cells was first described by Mosmann and colleagues, on the basis of their function and the cytokines they secrete (85). The fate of naïve Th cells is determined by three signals that are provided by pathogen-primed mature DC. Signal 1 is delivered through the TCR when it engages an appropriate peptide-MHC complex. Signal 2 is referred to as 'co-stimulation'; an accessory signal required in addition to signal 1, to induce immunity (72). Signal 2 is often equated to interactions between CD28, CTLA4, ICOS and members of the B7 family, such as CD80 and CD86 (86). In the absence of Signal 2, Th cells become anergic, which can lead to tolerance. The costimulatory molecule ICOS (inducible costimulator) is expressed only on activated T cells and binds to B7-H2 on APCs, this interaction is important for IL-10 production and the polarization of Th2 cells (87). Signal 3 refers to signals, primarily cytokines, delivered from the APC to the T cell, that determine its differentiation into an effector cell (i.e. to a Th1, Th2 or T regulatory (Treg) cell phenotype). The balance of these cytokines and the resulting polarization of T cell response strongly depend on the conditions under which DC are primed for the expression of signal 3 (88).

Protective immunity against certain diseases is dependent on the differential induction of Th1 or Th2 responses. Th1 cells mediate cellular immunity against viruses, bacteria and small parasites (89). In several studies, Th1 responses have been shown to decrease rapidly in the absence of IL-12, resulting in a loss of protective immunity to intracellular pathogens such as *Leishmania* and *Toxoplasma* (90, 91). However, exaggerated Th1 cell differentiation can be detrimental to the host. Th2 cells are induced in response to helminth parasites, but are also induced by allergens and by immunization with soluble or alum-adsorbed antigens (89). A population of T cells with regulatory or suppressive activity, termed regulatory T cells, has been described, which can control Th1/Th2 and inflammatory responses during infection (92).

1.4.3.1 Th1 cells

Th1-polarizing factors, include IL-12 family members; IL-12 and IL-27 (93, 94), IL-18 (95), type 1 interferons (IFNs) (96) and cell-surface expressed intercellular adhesion molecule 1 (ICAM1) (97). Th1 cells secrete IFN-γ and TNF-α, and mediate cell-mediated immune responses, including delayed-type hypersensitivity, macrophage activation and inflammatory responses (98). Th1 cells also promote IgG2a synthesis (99). IL-12 production contributes to inflammatory responses at the site of infection and induces or enhances IFN-γ production by NK cells or effector $CD4^+$ T cells (94). In particular, DC-derived IL-12 potently stimulates IFN-γ production in naïve T cells resulting in the polarization of a strong Th1 response. It was initially thought that Th1 cells mediated autoimmune diseases, including rheumatoid arthritis (RA), insulin-dependent diabetes mellitus and experimental autoimmune encephalomyelitis (EAE) (100). However, it is now known that the IL-12 family member, IL-23, and Th17 cells also play an important role in mediating organ-specific autoimmunity (101).

1.4.3.2 Th2 cells

Th2-polarizing DC-derived factors include monocytic chemotactic protein 1 (MCP1) and OX40 ligand (102). In addition, IL-4 was initially characterized as the dominant cytokine influencing Th2 differentiation, however, IL-10 and transforming-growth factor (TGF-β) have also been shown to play important roles in Th2 and Treg cell polarization (103, 104). In contrast to Th1 cells, Th2 cells produce IL-4, IL-5, and IL-13 and promote humoral immunity, allergic reactions to environmental antigens, and resistance to helmintic infections (105). During Th2 responses, IL-4 and IL-13 induce B cell switching to IgE production. An important prerequisite for the induction of Th2 responses by DC, is that their capacity to produce IL-12 is down-regulated. It has been shown that IL-12 can induce the production of IFN-γ and inhibit IL-4 production in established effector Th2 cell clones (106). It has been suggested that Th2 responses are a 'default', that occurs in the absence of Th1-polarizing factors, in particular IL-12, however, recently it was reported that the differentiation of Th2 cells induced by

helminth-primed DC requires Th2-polarizing factors from these cells. Research into the Th2-polarizing factors is still ongoing and unlike Th1 cells, little is known about the promotion of Th2 cells by DC. However, IL-4 produced by T cells themselves acts in an autocrine manner, amplifying IL-4 production by developing Th2 cells. Recently, the Notch family member Jagged was reported to be responsible for Th2 differentiation. Jagged-mediated Notch activation promoted Th2 responses independent of IL-4 and STAT6 (107). In addition, MCP1 was found to enhance IL-4 production in murine T cells (108) and has been associated with Th2 responses in both infectious and allergic disease models (88). An important role for OX40L-OX40 interactions in Th2 cell development has been demonstrated *in vivo* in a murine model of experimental leishmaniasis (109). Resting murine and human DC do not express OX40L, however, it is rapidly induced upon CD40 ligation (88).

1.4.3.3 T regulatory cells

Treg cells are unique populations of lymphocytes capable of regulating the immune response. Distinct subtypes of $CD4^+$ T cells, discriminated on the basis of phenotype or cytokine secretion, play a major role in maintenance of self tolerance, largely by their ability to suppress responses mediated by other populations of T cells. The existence of suppressor T cells and their role in the maintenance of immune homeostasis was first proposed in the early 1970s (110). Due to the inability to identify specific markers for these cells, their existence was discredited for some time. However, they re-emerged as Treg cells in the mid 1990s. Several subsets of Treg cells with distinct phenotypes and mechanisms of action have been described. These include, naturally occurring $CD4^+CD25^+$ Treg cells, type 1 T reg (Tr1) cells, which secrete high levels of IL-10 but not IL-4 or IFN-γ and Th3 cells, which secrete TGF-β. Treg cells have dramatic ability to suppress both adaptive and innate immune responses (111).

Naturally occurring Treg are generated in the thymus and represent 5-10% of the peripheral $CD4^+$ T cell population. Natural Treg cells constitutively express CD25, however, as this marker is not unique to Treg, other markers have been proposed, including CTLA-4 (112), and glucocorticoid-induced TNF receptor (GITR) (113). The

transcriptional repressor Foxp3 is critical for both the development and function of natural Tregs (114). Both mice and humans deficient in functional Foxp3 protein suffer from severe autoimmune disease (115, 116).

In contrast to naturally occurring $CD4^+CD25^+$ Treg cells, adaptive Treg cells (i.e.Tr1 and Th3 cells), originate from uncommitted peripheral naïve or central memory Th cells upon activation by APCs in a certain context (117). Adaptive Tr1, often referred to as inducible Tr1 cells, secrete high levels of IL-10 and exhibit variable expression of CD25 and other surface markers described for naturally occurring Treg cells (103). The suppressor activity of these Tr cells is cell-cell contact independent and is mediated primarily through the actions of the anti-inflammatory cytokines IL-10 and TGF-β. Tr1 cells were found to inhibit antigen-specific T cell responses and prevent colitis (117). These Tr1 cells produced IL-10, low to moderate levels of IL-5 and TGF-β, but with little or no IL-2, IL-4 or IFN-γ.

Th3 cells were originally identified in mice in experiments on oral tolerance induction to myelin basic protein (MBP). Th3 cells preferentially secreted TGF-β together with varying amounts of IL-4 and IL-10 and suppressed the induction of EAE *in vivo* in a TGF-β-dependent manner. Th3 cells provide help for IgA production and can suppress both Th1 and Th2 cells (118).

The mechanism of suppression mediated by Treg cells remains controversial. Recently, TLRs have been proposed to play a role in modulating Treg cell function, directly or indirectly via TLR-activated DC. The TLR-2 ligand, zymosan, induced DC that produced abundant IL-10, but little or no IL-6 or IL-12p70. Injection of zymosan plus OVA into mice resulted in OVA-specific T cells that secreted high concentrations of IL-10, but no Th1 or Th2 cytokines (119). Pathogen-derived molecules, including filamentous haemagluttinin (FH)A and adenylate cyclase toxin (CyaA) from *B. pertussis*, non-structural protein (NS) 4 from hepatitis C virus and cholera toxin (CT) have been shown to promote the induction of Tr1 cells, through their interaction with DC or other innate cells, such as macrophages (103, 120-122).

1.4.3.4 Th17 cells

A T cell population, distinct from Th1, Th2 and Treg cells, that produces IL-17, but not IFN-γ or IL-4, has recently been described (123, 124). It was demonstrated that the transcriptional factor RORγt directed development of IL-17-secreting T cells (125), whereas T-bet and GATA3, which are involved in the development of Th1 and Th2 cells respectively, inhibited differentiation of Th17 cells (123). It was initially reported that a member of the IL-12 family, IL-23, was the major stimulus for promoting the differentiation of Th17 cells (123, 124, 126). Although T cells are a major source of IL-17 the precise subpopulation(s) of T cells that secrete IL-17 are still unclear. The study by Cua and colleagues demonstrated that it was possible to induce EAE in IL-12p35$^{-/-}$ but not in IL-23p19$^{-/-}$ or IL-12p40$^{-/-}$ mice (127). However, naïve murine T cells do not express IL-23R (128) and do not differentiate into Th17 cells when stimulated with IL-23 *in vitro* (129-131). Indeed, three independent studies then demonstrated that IL-6 and TGF-β could promote differentiation of Th17 cells from naïve CD4$^+$ T cells co-stimulated with anti-CD3 and CD28 or APC *in vitro* (129-131). It was later reported that IL-21 was a key cytokine in the differentiation of Th17 cells and that it may act in an autocrine loop, involving IL-6 and TGF-β (132, 133). At the same time it was also demonstrated that IL-1α or IL-1β could synergize with IL-23 to promote IL-17 production by murine T cells in the presence or absence of TCR engagement, whereas T cells from IL-1 receptor type I-deficient (IL-1RI$^{-/-}$) mice failed to induce IL-17 production in response to IL-23 (134).

Although there is general agreement that a number of cytokines are required for the generation of murine Th17 cells, the crucial differentiation factors for human Th17 cell development are less clear. It has been reported that TGF-β with IL-6, or TGF-β with IL-21 failed to induce the differentiation of naïve human T cells into Th17 cells (135-137). Chen *et al.* showed that IL-23 drove Th17 cell differentiation, while Wilson *et al.* demonstrated that either IL-23 or IL-1 alone stimulated the Th17 phenotype. However, more recently TGF-β has been 're-instated' as a critical factor in inducing both human and mouse Th17 differentiation from naïve CD4$^+$ T cells, along with inflammatory mediators including IL-1, IL-21, IL-6 and IL-23 (138-140).

As well as IL-17A (IL-17) and IL-17F, Th17 cells can also produce IL-21 and IL-22. Furthermore, murine pathogenic Th17 cells were shown to produce TNF-α and IL-6 (101). IL-21 is a member of the IL-2 family of cytokines and has been shown together with TGF-β to amplify Th17 cell differentiation (132, 133, 141). Futhermore, IL-21 and IL-6 can upregulate the expression of IL-23R on Th17 cells (132). IL-22 is one of the IL-10 family of cytokines and is mainly produced by Th cells and NK cells. IL-23 together with IL-6 can directly induce production of IL-22 from naïve human and murine T cells (142). Th17 cells are potent inducers of autoimmunity and elevated levels of IL-17 are present in the target organs of several human autoimmune diseases, including multiple sclerosis (MS), RA and psoriasis (136, 143-145). While much of the focus in recent years has been on the role of IL-17-secreting T cells in promoting organ-specific autoimmunity, there is increasing evidence of a role for IL-17 in mediating immune responses to extraceullular bacteria and fungi (146).

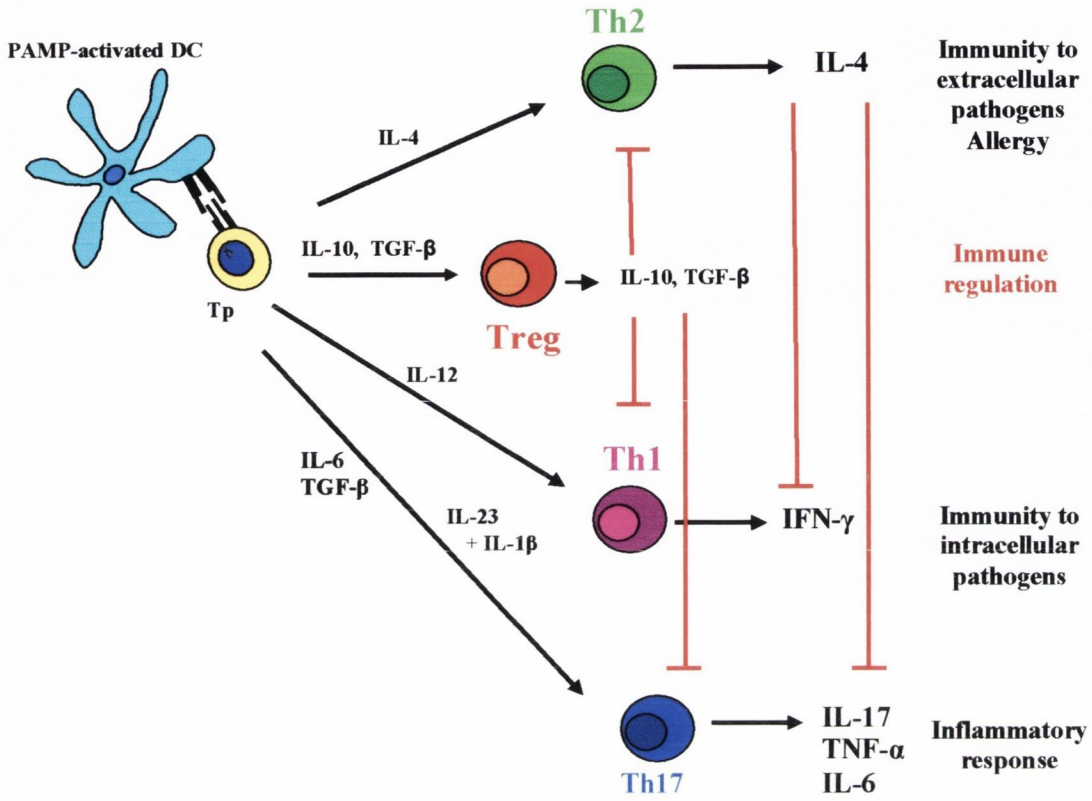

Figure 1.4 T cell polarization

Precursor T lymphocytes can give rise to all four types of T cell described in the text. IL-4 is known to drive the differentiation of Th2 cells, which mediate their effects via the anti-inflammatory cytokines IL-4, IL-5 and IL-13. Th1 cells differentiate in response to IL-12, and produce IFN-γ and IL-2. Th17 cells are thought to be differentiated from naive T cells in the presence of TGF-β and IL-6, while IL-1 and IL-23 are thought to drive IL-17 production by memory T cells. IL-10 is involved in the differentiation of T reg cells, which mediate their effects largely through IL-10 itself and TGF-β.

1.5 IL-12, IL-23 AND IL-10 PRODUCTION BY DC IS AN IMPORTANT DETERMINANT OF TH CELL DIFFERENTIATION

While a number of cytokines play pivotal roles in Th cell polarization, including IFN-γ and IL-4, IL-12 and IL-10 are central to the differentiation of Th1 and Th2/Treg cells, respectively.

IL-12

IL-12 is a heterodimeric cytokine composed of two subunits, p35 and p40 (147), and is secreted by activated APCs, primarily DC, monocytes and macrophages (148). IL-12 induces the proliferation of T cells to a Th1 phenotype. TLR agonists, including LPS and CpG, are potent inducers of IL-12 from DC and macrophages (67, 149, 150). IL-12 can induce the proliferation of NK cells and T cells, resulting in the secretion of IFN-γ. Recent data has shed light on the involvement of IFN-γ in Th1 polarization. It was demonstrated that DC migration from the periphery to draining lymph nodes, provides inflammatory signals that attract NK cells. The NK cells can then provide the source of IFN-γ needed to induce Th1 responses (151).

IL-12 signals through the IL-12 receptor complex, composed of IL-12Rβ1 and IL-12Rβ2 chains. Resting T cells do not express β1 or β2, but expression is upregulated upon TCR ligation. IL-12p40 binds to the IL-12β1 chain, which is associated with Tyk2, whereas IL-12p70 binds to the IL-12β2 chain, which is associated with Jak2. Signalling through the IL-12R complex induces the phosphorylation, dimerization and nuclear translocation of STAT1, 3, 4 and 5, leading to the activation of IL-12-responsive genes (152). Mice deficient in either the IL-12 components (p35 or p40) or in its receptor components (IL-12R -β1 or -β2) have deficient Th1 responses *in vitro* and *in vivo*, confirming that IL-12 is a critical Th1 polarising cytokine (94, 148).

IL-23

In addition to forming bioactive IL-12p70 with p35, the p40 subunit can combine with p19, a subunit distantly related to p35, to form IL-23 (153). IL-23 signals through a heterodimeric receptor complex consisting of IL-12Rβ1 and IL-23R (93). Similar to IL-12, IL-23 is expressed predominantly by activated DC and phagocytic cells and IL-23R is expressed on activated T cells, bone-marrow-derived DC, and activated and inflammatory macrophages. Signalling through the IL-23R results in the activation of Stat3, with limited Stat4 phorphorylation, by association with Janus kinase 2 (Jak2). IL-12 and IL-23 have some overlapping functions, for example IL-23 can induce IFN-γ production from T cells (154). However, a unique function of IL-23 is the induction of IL-17 producing T cells, which have been implicated in promoting autoimmune diseases (93).

Up until the discovery of IL-23, IL-12-dependent Th1 cells were thought to be essential for the induction of autoimmunity. However, Cua and colleagues showed that both IL-12p40- and IL-23p19- deficient mice were resistant to EAE, a murine experimental model of MS, which was once thought to be a classical Th1-mediated disease (127). Around the same time, IL-23 was shown to expand a population of IL-17-producing $CD4^+$ T cells (101, 126) and these observations led to the proposal that IL-23 directly drives Th17 cell differentiation (101, 123, 124). Langrish *et al.* showed that passive transfer of IL-23-driven IL-17-producing cells are highly potent at inducing EAE. Futhermore, IL-23p19$^{-/-}$ mice mounted normal Th1 responses but have a profound defect in Th17 cells (155). Cua and colleagues demonstrated that IL-23 is produced by both resident microglia and inflammatory macrophages during an autoimmune reaction in the central nervous system (CNS).

However, since naïve T cells do not express IL-23R, a major issue with the IL-23-Th17 hypothesis was that IL-23 could not drive the induction of de novo Th17 cells. It was soon after demonstrated that TGF-β in the presence of other inflammatory cytokines, particularly IL-6, were required for the differentiation of Th17 cells (129-131). Recent reports have suggested that the function of IL-23 is to expand differentiated Th17 cells or maintain IL-17 production. This was concluded on the basis that IL-23 increases the frequency of $IL\text{-}17^+$ cells and that IL-23 is required during re-stimulation of TGF-β and IL-6 stimulated cells to maintain the secretion of IL-17 (101, 129). The suggestion that

IL-23 maybe stabilize the Th17 phenotype does not incorporate the fact that IL-23 can promote the expression of IL-22, another pathogenic cytokine associated with Th17 cells (142). Moreover, IL-23R expression is upregulated remarkably early in response to IL-6, suggesting that IL-23 may have a role in the early differentiation of Th17 cells (132).

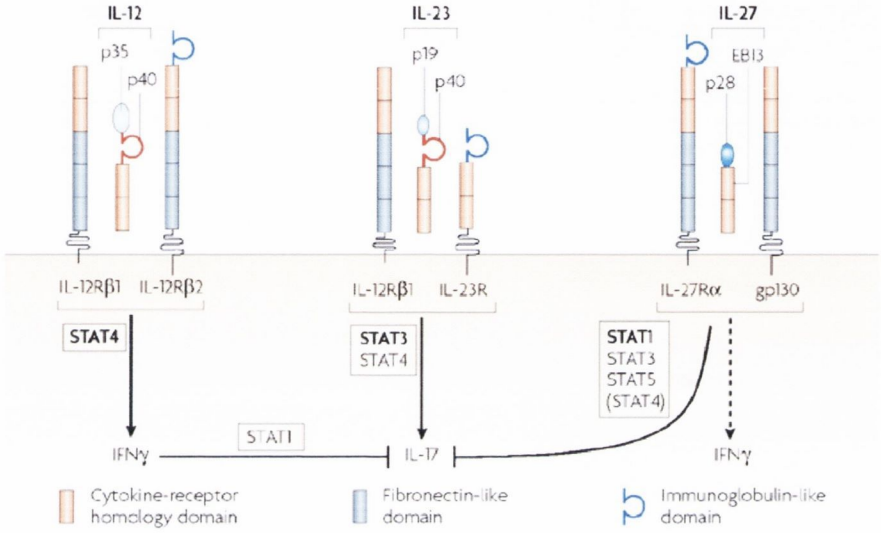

Figure 1.5 The IL-12 cytokine family (Goriely, 2008)

IL-10

IL-10 is a pleiotrophic immunomodulatory cytokine, which exerts potent effects on numerous cell populations, although its primary targets are APCs and lymphocytes. IL-10 was first described in 1989 as a cytokine synthesis inhibiting factor (CSIF) (156). The anti-inflammatory role of IL-10 was demonstrated in IL-10 deficient mice which develop lethal inflammation of the intestine, which correlated with increased IL-12 production (157). Furthermore, IL-10 treatment was shown to be beneficial in EAE, a murine model for MS (158). IL-10 promotes the development of a Th2-biased response, by inhibiting the IFN-γ production by T lymphocytes, particularly via the suppression of IL-12 (159). In addition, the optimal induction of Th2 cells by $CD8^-$ DC was IL-10-dependent (160). IL-10 potently inhibits macrophage activation and reduces pro-

inflammatory cytokine and chemokine production, thus limiting the course of an inflammatory response (159). IL-10 suppresses T cell activation by inhibiting expression of MHC class II, CD80 and CD86 on DC and macrophages (161, 162). Besides these indirect effects on T cells, IL-10 directly suppresses IL-2, IFN-γ, IL-4 and IL-5 production from $CD4^+$ T cells (163).

The mechanism by which IL-10 mediates the suppression of pro-inflammatory cytokine synthesis has not been fully elucidated. It was suggested that IL-10 may exert its anti-inflammatory properties by inhibiting the transcription factor, NFκB. (164). IL-10 was reported to block the nuclear translocation of the p65, but not p50, subunit of NFκB, resulting in the accumulation of inhibitory p50 homodimers in the nucleus and an associated inhibition of pro-inflammatory genes (165, 166). In contrast, other reports proposed that IL-10 has negligible effects on NFκB activity (163, 167). Two main signalling cascades have been implicated in IL-10 signalling: the Jak/STAT pathway and the phosphatidylinositol 3 kinase (PI3K) pathway. STAT3 has been shown to be essential for IL-10-mediated cytokine suppression. Studies using Jak1 and STAT3 knockout mice have shown an absolute requirement for these two molecules in IL-10-mediated cytokine suppression (163, 168). In contrast, research into the role of PI3K signalling in IL-10 activation and IL-10 production has generated contradictory results. In human monocytes, inhibition of IL-10- induced PI3K activation had no effect on IL-10 activity (169). However, pre-treatment of BMDC with IL-10 inhibited LPS-induced PI3K activation (170). Recent data demonstrated that IL-10 acting through STAT3, inhibited gene transcription, independently of posttranscriptional mRNA processing, suggesting that the effects of IL-10 are indirect and require the synthesis of intermediary proteins (167). PI3K can phosphorylate and thereby inactivate glycogen synthase kinase 3 (GSK3), which, when active, inhibits IL-10 production. The inhibition of PI3K using RNA interference or small molecule inhibitors, enhanced TLR-induced IL-10 production (171).

Although IL-10 exerts potent anti-inflammatory effects, more recent data has demonstrated a role for IL-10 in the development of Treg cells. The presence of IL-10 during activation of $CD4^+$ T cells promotes the development of Treg cells (117, 172). T cells from OVA-TCR-transgenic mice, repeatedly stimulated with OVA and IL-10,

differentiated into Tr1 cells *in vitro* (117). Filamentous haemagluttinin from *Bordetella pertussis*, inhibited IL-12 production and augmented IL-10 secretion by immature DC, enabling the DC to direct naïve T cells into a regulatory Tr1 type phenotype (103).

1.6 MULTIPLE SCLEROSIS

MS is an inflammatory demyelinating disease of the CNS that is characterised by inflammatory infiltrates composed of T cells, B cells and macrophages and focal demyelinating plaques within the CNS. MS leads to substantial disability through deficits of sensation and of motor, autonomic, and neurocognitive function. There are three major forms of MS. Relapsing-remitting (RR)-MS is the most frequent (85-90%) and is characterised by clinical exacerbations interspersed by periods of inactivity (173). However, with time, the extent of recovery from attacks often decreases and most RRMS patients later enter into a slow progressive neurodegenerative process, known as secondary progressive (SP)-MS (174). While earlier RRMS is sensitive to immunosuppression, responsiveness to immunotherapy decreases and may even disappear in SPMS. About 10-15% of patients are diagnosed with primary progressive (PP)-MS which is characterized by the absence of acute attacks present and instead involves a steady progression of disease (173). MS continues to be diagnosed on clinical basis, although this is supported by histopathological evaluation and neuroligic examination using magnetic resonance imaging (MRI). The precise etiology of MS remains unknown but populations vary in their susceptibility to MS and there is evidence to suggest that genetic and environmental factors are involved. The age of onset of MS is highly variable but usually peaks between 20-40 years and therefore, it is the most common CNS disorder in young people. MS affects roughly 2.5 million people worldwide and a clear gender difference in RRMS, but not PPMS is evident: females being more susceptible than males (~1.6:1).

The pathological hallmark of MS, whether determined histopathologically or by MRI, is the inflammatory plaque (175). Inflammation is thought to be a major contributor to the formation of these acute demyelinating lesions. It is hypothesized some form of systemic infection may result in the activation of peripheral lymphocytes as well as the up-regulation of adhesion molecules on the endothelium of the brain and spinal

cord. Autoreative T cells, generated by unknown events in early childhood, can become activated and home to the CNS, attach to the endothelium and penetrate the blood brain barrier. Having entered the CNS, autoreative T cells programmed to recognize myelin antigen exist within the cell infiltrate, they may be reactivated by antigenic myelin fragments presented in an MHC-restricted pattern by APCs, such as microglia, astrocytes and macrophages (173). This results in the release pro-inflammatory cytokines, opening up of the blood-brain barrier and an influx of more inflammatory cells recruitment, leakage of antibody and plasma proteins; ultimately triggering a cascade of events resulting in the formation of an acute inflammatory, demyelinating lesion (175). These typically develop in the white matter where they target the myelin sheath and the myelinating cell, the oligodendrocyte. White matter plaques can be found in the optic nerve, brainstem, spinal cord and periventricular regions but it is now apparent that changes in grey matter also play a role in the pathology of the disease (176). While MS predominantly affects the CNS myelin, axonal damage dictates to a large extent the development of neuropathological deficit and axonal loss may be the major determinant of long-term (permanent) disability in MS.

MS has long been considered a $CD4^+$ Th1-mediated autoimmune disease due to the cellular composition of brain and cerebrospinal fluid (CSF)-infiltrating cells and data from the animal model EAE. All individuals, patients with MS and healthy persons, have T cells in their peripheral blood that have potential to react against MBP and other CNS antigens. However, myelin-reactive T cells from patients with MS exhibit a memory or activated phenotype and the ability of these myelin-specific T cells to produce cytokines causes inflammation in the CNS (177). Myelin-reactive T cells from patients with MS produce cytokines more consistent with a Th1-mediated response, whereas myelin-reactive T cells from healthy persons are more likely to produce cytokines with a Th2-type profile (178). In recent years, Th17 cells have been shown to play a crucial role in the pathogensis of autoimmune disease. In support of this, microarray studies of MS lesions from patients demonstrated increased expression of IL-17, suggesting that it may play an important role in the development of inflammatory demyelination (179).

1.6.1 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

EAE is an animal model of inflammatory demyelinating disease used to dissect the mechanisms of the autoimmune inflammatory response and, as such, much of our knowledge on MS stems from experiments in this model. EAE is induced in different strains of mice, rats or non-human primates by subcutaneous (s.c.) immunization with myelin proteins or defined peptides, including MBP and proteolipid protein, usually emulsified in complete Freund's adjuvant. This immunization leads to a disease that shares clinical and neuropathological changes with MS (180).

It had been accepted for many years that EAE was largely a $CD4^+$ Th1-mediated disease. However, MOG-specific $CD8^+$ T cells have been shown to induce EAE, demonstrating a pathogenic role for $CD8^+$ T cells in mediating this disease (181). More recently, it has been demonstrated that an IL-17-producing T cell subset, distinct from Th1 and Th2, is the critical cell type mediating the pathogenesis of EAE (101). Langrish and colleagues showed that both IL-12- and IL-23-expanded PLP-specific Th1 and Th17 cells, respectively, could migrate to the CNS upon adoptive transfer into naïve mice, but that only the IL-23-driven Th17 cells induced disease. While there is still some debate in the literature, it is likely that Th1 and Th17 cells cooperate to induce the development of organ-specific autoimmunity. A recent study by O'Connor *et al.* demonstrated that only Th1 cells can access the noninflamed CNS and as such are required to facilitate the entry of Th17 cells into the CNS during EAE (182).

1.6.2 MS therapies

Several therapies for MS exist and are directed at resolving acute attacks, reducing the number of exacerbations, treating the sequelae of previous attacks, and preventing progression of disability. Administration of glucocorticoids, which act broadly as anti-inflammatory agents, intravenously at high doses is a routine theapy during acute clinical exacerbations of MS. Immunomodulatory therapies approved for RRMS include the type-1 beta interferons and glatiramer acetate. The mechanism of action of type-1 interferons is largely unknown, but they have been found to suppress T cell proliferation, reduce the influx of T cells into the CNS, and induce a more polarized Th2-type response

(183). Glatiramer acetate (GA) is a random polymer of four amino acids (L-glutamic acid, L-lysine, L-alanine, and L-tyrosine) in proportions that resemble the content of MBP. Its mechanism of action is also unclear, but evidence points to induction of tolerance to MBP-specific T cells and it was reported that GA induced Th2-polarized immune responses in MS patients (184). Other promising therapeutic strategies include humanized monoclonal antibodies against VLA-4 (natalizumab), marketed as Tysabri, and mitoxantrone. Natalizumab was shown to block T cell activation and migration across the blood brain barrier (BBB), as well as reduce brain inflammation (185, 186). Treatment of advanced forms of MS is more difficult than RRMS but mitoxantrone and natalizumab have shown positive effects with secondary progressive disease (173).

1.7 ADJUVANTS

Vaccination is a highly effective means of alleviating the impact of infectious diseases. Since the discovery by Edward Jenner in 1796 that cowpox, or vaccinia, could induce protection against human smallpox, vaccines have been developed against a broad range of diseases, including diphtheria, whooping cough, polio, measles, mumps and rubella and have saved countless lives worldwide. However, the failure to produce vaccines against tuberculosis, malaria and HIV, highlights the need to improve our understanding of the immune system, so it can be manipulated effectively to generate protective immune responses. Traditional vaccines, generally based on live attenuated pathogens, killed whole cell organisms or inactivated toxins, have constituted the majority of clinically used vaccines to date, however the emergence of new pathogens, and concerns over the safety of killed and attenuated vaccines have highlighted the requirement for new, safer and more effective vaccines. The use of subunits of these organisms as vaccines is becoming more widespread. Subunit vaccines do not generally contain immunostimulatory components, and so are poorly immunogenic, therefore, strategies to enhance the immunogenicity of vaccines are required (187).

Since the majority of pathogens that infect humans do so via mucosal sites, principally the digestive, respiratory and genitourinary tracts, effective vaccination strategies that induce immunity at these sites is essential. Most currently used vaccines are administered via intramuscular injection, however, this generally stimulates poor

mucosal immune responses. The development of effective mucosal vaccines is dependent on the use of delivery systems that protect the antigen from degradation and enzymatic digestion and enhance uptake at mucosal surfaces. Although oral vaccination has many advantages, including easy administration and distribution, difficulties in inducing immune responses by the oral route has moved research on mucosal vaccines towards the development of nasally delivered vaccines (188).

Enhancing immunogenicity to subunit vaccines involves augmenting the strength of the innate immune response and subsequently influencing the induction of cellular and antibody responses. Adjuvants are substances that enhance immune responses to co-administered antigen. In the late 1920's, it was demonstrated that aluminium based compounds could enhance the immunogenicity of a diphtheria toxoid vaccine. Since then, a considerable number of compounds have been evaluated as adjuvants, however, toxicity and adverse reactions have precluded the use of many of these candidates and therefore aluminium-based mineral salts and MF59 are the only adjuvants approved by the U.S. Food and Drug Administration (FDA) organization. In recent years, research focusing on innate immunity has led to the appreciation of the fact that activation of innate immune responses, via highly conserved PRRs on APCs, is central to protection against infectious diseases. Traditional vaccines, such as pertussis whole cell vaccine and attenuated viral vaccines, contain components, such as TLR agonists, that specifically activate innate immune responses. In contrast, subunit vaccines do not contain these immunostimulatory factors, necessitating the inclusion of adjuvants to enhance their immunogenicity.

There is limited knowledge about the mechanism of action of vaccine adjuvants, however, in recent years it has been established that activation of innate immunity plays a central role in their ability to enhance adaptive immune responses. DC are the principle mediators of these innate responses, and activation through TLRs is integral to their function. Activation of TLRs, by factors such as LPS, leads to the maturation of DC which ultimately results in T cell polarization (Refer to sections 1.4 and 1.5). TLR-activated DC induce both Th1 and Th2 polarizing cytokines (61). For example, the TLR4 agonist, LPS, induced higher concentrations of IL-12p70 from DC when compared with the TLR2 agonist, zymosan. In contrast, DC stimulated with zymosan produced higher concentrations of IL-10, than those stimulated with LPS (119). Emerging evidence

suggests that triggering different TLRs on DC results in distinct patterns of gene expression and cytokine secretion that direct the type of immune response induced (189, 190). These findings suggest that DC and TLRs are important targets in the design of effective vaccine adjuvants.

1.8 HEAT-LABILE ENTEROTOXIN AND CHOLERA TOXIN

Heat-labile enterotoxin (LT) from enterotoxigenic strains of *E.coli* and cholera toxin (CT) from *Vibrio cholerae* are two of the most powerful mucosal adjuvants described to date. LT and CT belong to the AB class of bacterial toxins and the nucleotide sequences of the two toxins are approximately 80% homologous. CT is the causative agent of the severe diarrhoea associated with the sometimes fatal disease Cholera, while LT is responsible for traveller's diarrhoea (191). Both of the enterotoxins generate powerful immune responses at mucosal surfaces and can also stimulate strong responses against co-administered antigens. As a consequence of these properties, both CT and LT have been used as mucosal adjuvants with a wide variety of antigens in animal models and in a number of human clinical trials. Mucosal delivery of CT and LT has been shown to significantly enhance IgG and IgA antibody responses to a broad range of antigens (192-195). In addition, CT and LT have been shown to act as parenteral adjuvants in studies involving immunization via the subcutaneous, intraperitoneal, intravenous, intradermal and transcutaneous routes (196-199). While there has been extensive research into the application of the toxins as adjuvants, their mechanism of action is not fully understood. Derivatives of the toxins, including enzymatically inactive mutants and molecules composed only of purified B subunits are available, which are useful tools for investigation into their mechanism of action.

1.8.1 STRUCTURE OF LT AND CT

LT and CT are composed of two subunits, an enzymatically active A subunit, with adenosine-diphosphate (ADP)-ribosyltransferase activity, which is responsible for the toxicity, and a B subunit that mediates binding of the molecule to its receptor on

eukaryotic cell surfaces (191). The B subunits of LT (LTB) and CT (CTB) are formed by five monomers of 11 kDa each, that are arranged in a cylinder-like structure with a central cavity. The B subunit monomers interact with each other to form a non-covalently linked pentameric ring which is highly stable. The principal receptor of LTB and CTB is GM1-ganglioside [Gal(β1-3)GalNAc(β1-4)(NeuAc(α2-3))Gal(β1-4)Glc(β1-1)ceramide], a glycosphingolipid found ubiquitously on the surface of mammalian cells. LTB and CTB can also bind to GD1b-ganglioside, but with a lower affinity than to GM1. In addition, LTB may bind to other glycosphingolipids (asialo-GM1), to glycoprotein receptors present in the intestine of rabbits and humans, to polyglycosilceramides and to paragloboside (200). The different receptor binding specificities of LT and CT indicates that the two toxins have distinct immunomodulatory activities and modes of action.

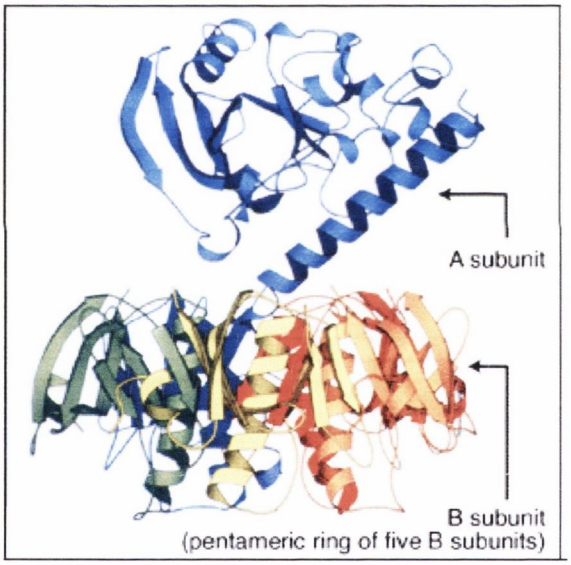

Figure 1.6 Crystal structure of LT

The A subunit (blue), comprising the A1 and A2 fragments, forms a wedge-shaped structure. The C-terminal of the A2 fragment is inserted into the pore formed by the B-subunit monomers which are arranged in a doughnut-shaped pentameric ring.
Williams *et al. Immunol Today 20:95*

The A subunits of LT and CT are composed of a globular structure linked to the B oligomer, the C-terminus of which anchors the A subunit to the B pentamer, by entering into the central cavity of the B subunit. The A subunit is divided into the globular (enzymatically active) A1 domain and an A2 domain, whose primary function is to interact with the B subunit. The two domains are attached via a trypsin-sensitive loop and a disulphide bridge between Cys_{187} and Cys_{199}. The ADP-ribosyltransferase activity is facilitated following proteolytic cleavage of the trypsin-sensitive loop between the two domains and reduction of the disulphide bond. The fate of CT after receptor binding has

been more intensively studied than LT. The holotoxin binds surface receptors and undergoes vesicular transport from the plasma membrane to the Golgi compartment with subsequent separation of the A and B subunits. The A subunit is then transported to the endoplasmic reticulum, while the B subunit remains in the Golgi (201), and subsequently the A subunit is redirected to the plasma membrane. The A1 fragment ADP-ribosylates the stimulatory α subunit of a GTP-binding protein (Gsα), causing permanent activation of adenylate cyclase, resulting in an elevation in intracellular cyclic adenosine monophosphate (cAMP) concentration. Modification of Gsα results in the accumulation of salts and water in the intestinal lumen, producing the watery diarrhoea characteristic of cholera (202). The C-terminus of the A2-fragment also contains a sequence motif associated with retrieval of proteins from the *trans*-Golgi network to the endoplasmic reticulum. It has been suggested that this may be important in the delivery of the A1-fragment to the correct cellular compartment (195). The basal ADP-ribosyltransferase activity of LT and CT is enhanced by interaction with GTP-binding proteins, known as ADP-ribosylation factors (ARFs). In addition to cofactor activity for the bacterial toxin, ARFs play a crucial role in vesicular membrane trafficking, principally between the ER and Golgi, and contribute to the maintenance of organelle integrity and the assembly of coat proteins.

1.8.2 Adjuvant action of LT and CT

Co-administration of LT or CT with antigen via the nasal, oral or other mucosal routes potently enhances antigen-specific mucosal and cellular immune responses. Most studies indicate that CT induces a Th2 biased response to itself and to bystander antigens. These responses are characterized by $CD4^+$ T cell production of IL-4, IL-5, IL-6 and IL-10 (203-205) and IgG1, IgA and IgE antibodies (203, 205), but elevated IFN-γ and IgG2a antibodies have also been reported. CT has also been shown to induce a population of IL-10 producing Treg cells that did not secrete IL-4 and exhibited suppressive effects on Th1 cell proliferation and cytokine production (121). In contrast, mucosal delivery of LT has been shown to induce a more mixed Th1/Th2 type response, with the production of antigen-specific IL-4, IL-5 and IFN-γ and IgG antibodies of the IgG1, IgG2a and IgG2b subclasses and significantly less antigen-specific IgE than CT (193, 206-208).

LT and CT can also potentiate antigen-specific class I restricted CTL responses (209-211). OVA-specific CTL was detected in the spleen and local draining lymph nodes following intranasal or oral immunization of mice with OVA or OVA peptide together with CT or LT. LT can also elicit CTL responses to OVA independently of $CD4^+$ T cell help, suggesting that it may activate host APCs directly or indirectly, such that they are able to prime an OVA-specific $CD8^+$ T cell response (211).

In addition to their proven activity as mucosal adjuvants in animal models, CT and LT have also proven to be strong mucosal adjuvants in humans. Two nasal vaccinations with LT-adjuvanted virosomal influenza vaccine induced antigen-specific immune responses comparable to those induced after a single parenteral vaccination with influenza virus haemagglutinin in adult volunteers (212). Humans are exquisitively sensitive to LT and CT; diarrhoeagenic responses may be triggered at low doses that are non-toxic in mice (191, 213). Therefore, the high toxicity of LT and CT renders them unsuitable for clinical use, and so for this reason it is desirable to separate the toxic effects of these molecules from their adjuvanticity.

1.8.3 Site-directed mutants of LT and CT

To address the significant toxicity associated with the use of LT and CT as adjuvants, attempts have been made to reduce the toxicity of the native toxins without affecting their adjuvanticity, most of which have focused on eliminating or attenuating the enzymatic activity of the A subunit. The majority of efforts have involved the use of site-directed mutagenesis to introduce amino acid substitutions in residues associated with the active site of the toxins that are critical for enzymatic activity. Mutants with amino acid substitutions at the active site, such as LTR72 (Ala72 → Arg) (214, 215) or in the proteolytically sensitive region of the biologically active domain, including LTR192G (Arg192 → Gly), are significantly less toxic than the wild-type toxins. LTR72 has approximately 1% of the ADP-ribosylating activity of the wild-type toxin but has been shown to exhibit comparable or even superior adjuvant activity to LT (216). Although weaker than the wild type and partially active toxins, mutants that are completely devoid of enzyme activity, including LTK63 (Ser63 → Lys), also retain mucosal adjuvant properties (217). These mutants have no enzyme activity and have been shown to be non-

toxic *in vitro* and *in vivo*, however they maintain all other biological properties associated with the wild type toxins, including receptor and ARF binding. The X-ray structure of LTK63 has been determined and the only difference between it and LT is in the active site, where the bulky side chain of the lysine residue fills the catalytic cavity.

The mucosal adjuvanticity of these mutants has been demonstrated using viral, bacterial, and parasitic pathogens or antigens. Mucosal delivery of LTK63 or LTR72 together with antigens from *Bordetella pertussis* (218), pneumococci (219), *Helicobacter pylori* (220), *Shigella flexneri* (221), *Toxoplasma gondii* (222) or a peptide from respiratory syncytial virus (223) conferred protection in the appropriate animal models. LTR72 appears to exert the strongest mucosal adjuvanticity of mutant toxins, with properties similar to those of wild type LT (216, 224-226). LTK63 and LTR72 have also been shown to induce CTL responses to a number of antigens, including peptides (227), OVA (210), HIV gag p55 protein (228) and respiratory syncytial virus (223). LTK63 when delivered transcutaneously with tetanus toxin promoted antibody responses (229) and when delivered via the respiratory tract improved efficacy of the group C meningococcal conjugate vaccine (230). The first study conducted in humans of an intranasal vaccine with LTK63 as an adjuvant demonstrated strong mucosal IgA responses to three influenza virus strains, A/H3N2, a B strain, and a novel avian pandemic H5N3 strain (231).

There is wide variation among the results pertaining to the Th1/Th2 responses induced with the toxins and their derivatives. The outcome of these studies are likely to be influenced by the nature of the toxin, the dose and the route of administration, the strain of mice and the nature and dose of the co-administered antigen (206, 218, 224, 226, 228).

In the LT mutant, LTR192G, an arginine residue at position 192 is replaced by a glycine, which eliminates the susceptibility of the toxin to the cleavage required for activation of the enzymatic activity (232). Mucosal immunization with LTR192G as the adjuvant has been shown to enhance humoral immunity to a range of antigens (207, 233-235). In addition, LTR192G promoted protective immunity against several viral and bacterial infections in murine models (236-238). LTR192G also enhanced IgA responses and Th1 type cytokines (in *H. pylori* negative subjects) following mucosal delivery with

formalin-inactivated and *Helicobacter pylori* whole-cell vaccine in human volunteers (239). Intrarectal immunization of macques with a synthetic-peptide vaccine and LTR192G induced mucosal CTLs specific for simian immunomodulatory virus (240).

Adjuvant studies with derivatives of the toxins have also shown variable T cell responses. The partially enzymatically active LTR72 was shown to induce mixed Th1/Th2 cytokine and mixed IgG1/IgG2a antibody responses *in vivo*, while enzymatically inactive LTK63 promotes a predominant Th2 cytokine response with higher IgG1 than IgG2a antibody production (195, 215, 216). The response to LTK63 was enhanced by increasing the dose (216), or by administering the toxin intranasally (228, 241). In a study investigating the ability of the enterotoxins to boost the immunogenicity and efficacy of live attenuated vaccines directed against shigellosis, Hartman *et al.* found that LTK63 was as effective as native LT, for the mucosal delivery of *E. coli-Shigella flexneri* hybrid vaccine strains to guinea pigs (242). In addition, LTK63 promoted higher systemic antibody responses to intranasally delivered pneumococcal conjugate vaccines than LTR72, when used as a mucosal adjuvant (219).

1.8.4 USE OF ISOLATED A OR B TOXIN SUBUNITS AS MUCOSAL ADJUVANTS

Another approach to avoiding the toxicity associated with the native toxins, is the use of native or recombinant B subunits. These isolated B subunits were the first non-toxic derivatives of CT and LT to be produced. Initial studies using purified native CTB showed that it was a strong mucosal adjuvant, however, it was later found that much of this activity was derived from contamination by the holotoxin (243). Recombinant LTB and CTB have now become available and studies using these molecules have shown that rLTB and rCTB have activity as mucosal adjuvants, but this is generally weaker than that observed with the native toxins (205, 206, 214, 243-246). However, supplementation of rLTB or rCTB with a trace amount of LT or CT has been shown synergistically enhance antibody responses, up to levels observed following immunization with the holotoxins alone (217). The adjuvant activities of LTB have been demonstrated using a number of antigens including herpes simplex virus glycoproteins (246), influenza antigens (247), KLH (248) and DT (249).

Using rCTB as an adjuvant, intranasal immunization with tetanus toxoid induced high levels of tetanus toxoid-specific serum IgG antibodies and protection in mice challenged with lethal doses of tetanus toxin (250). An oral vaccine containing cholera and CTB is widely used in animal models and humans (251, 252). Data on the enhancement of either Th1 or Th2-type responses by recombinant B subunits are varied. Some studies found enhanced IFN-γ and/or elevated IgG2a over IgG1 (199, 205), while others report augmentation of both Th1 and Th2-type responses and IgG1 and IgG2a to the co-administered antigen or to the toxin (253, 254). In contrast, Richards *et al* reported selective enhancement of Th2 cytokines and increased IgG1 over IgG2a with LTB, as well as the generation of antigen-specific CTL responses (246).

The ability of the B subunits to modulate immune responses is dependent on receptor binding. This requirement has been demonstrated using a non-receptor binding mutant of LTB, LTBG33D, containing a Gly33 to Asp substitution, which lacked the adjuvant properties of the B subunit (248, 255, 256). A CTB-mediated GM1 vesicle delivery system has been designed to enhance localization of protein antigens at mucosal cells, due to the ability of the pentameric CTB to bind to GM1 on both epithelial cells and GM1 vesicles. Intranasal administration of a recombinant HIV envelope protein formulated in CTB-associated GM1 lipid vesicles enhanced IgA antibody responses (257, 258). However, LTB monomer expressed in *Streptococcus gordonii*, despite being unable to bind GM1, was highly immunogenic in mice following parenteral and mucosal delivery (259). More recently it was reported that a mutant LTB (H57S), which has a His57 to Ser substitution but retains its GM1 binding ability, failed to act as a mucosal adjuvant, implying that GM1 binding alone is not sufficient to trigger the signalling events responsible for the immunomodulatory properties of LTB (260).

In further attempt to separate the adjuvant activity of LT and CT from toxicity, a completely non-toxic fusion protein was constructed that combines the enzymatic activity of the A subunit of CT A1 with a dimer of an Ig-binding fragment, D, from *Staphylococcus aureus* protein A. This molecule, CTA1-DD, retains the enzymatically active CTA1 moiety, while the CTB subunit and the ability to bind to the GM1-ganglioside receptor were removed (261, 262). CTA1-DD has been shown to be an effective mucosal and parenteral adjuvant, capable of enhancing IgG1 and IgG2a antibodies to co-administered antigens (262, 263).

1.8.5 MECHANISM OF ADJUVANT ACTION OF LT AND CT

The mechanism of adjuvant action of CT and LT has long been controversial but there is now an appreciation that a number of factors may be involved in their immunomodulatory effects. Studies using derivatives of the toxins, that are partially or completely enzymatically inactive and purified A or B subunits, have suggested that the effects of the toxins are derived from independent contributions of the enzymatic activity, the non-toxic AB complex and the B subunit interaction with GM1 (207, 216, 246, 255, 259, 262, 264).

Enhanced uptake at mucosal surfaces is an important mechanism by which adjuvants can enhance the immunogenicity of co-administered antigens. When orally delivered with KLH and dextran, CT, but not CTB, strongly increased the gut permeability for dextran, with a concomitant enhancing effect on the KLH-specific immune response in the lamina propria (265). The enzymatic activity may therefore play a role in gut permeability, facilitating access of luminal antigens to the gut mucosal immune system, and thereby enhancing mucosal immune responses. Furthermore, it was shown that oral delivery of CT in mice increased the number of DC in the follicle-associated epithelium and redirected DC from the Peyer's patches (PP) to B cell follicles and parafollicular T cell zones (266). Sphingolipid- and cholesterol-rich plasma membrane lipid microdomains, termed lipid rafts, function as platforms for both receptor signalling and membrane trafficking. Indeed, membrane lipid rafts selectively enriched in GM1 were selectively targeted to the MHC class II peptide-loading compartment after B cell receptor (BCR) cross-linking (206).

Since APCs, particularly DC, play a vital role in linking innate and adaptive immune responses it is likely that activation of APCs is involved in the ability of LT and CT to exert immunomodulatory effects. CT enhanced CD80 and CD86 expression on B cells (262), murine Flt3L-expanded DC (267) and on BMDC (268, 269). In contrast, other studies have shown that CT enhances CD86, but not CD80, expression by bone marrow-derived macrophages (270) and PP-derived macrophages and B cells (271). LT selectively upregulated the expression of CD80, but not CD86, on bone marrow-derived DC (223) and murine macrophages (272). The partially active LT derivative, LTR72, and the enzymatically inactive, LTK63, also enhanced CD80 on murine macrophages (272).

CT- and LT-treated APC enhanced proliferative responses in T cells stimulated with anti-CD3 monoclonal antibody and these responses were blocked by anti-CD86 monoclonal antibody (270, 271). Furthermore, administration of anti-CD86 monoclonal antibodies has been shown to inhibit KLH-specific antibody responses in mice orally immunized with KLH and CT (270). Therefore, the enhancement of co-stimulatory molecule expression appears to play an important role in the adjuvant effects of the toxins.

This ability to upregulate co-stimulatory molecule expression has been attributed to the enzyme activity of the toxins as well as GM1-binding. Incubation of native or mutant CT with GM1 blocked CD86 expression (273), indicating that binding of the B subunit to APC via GM1 is necessary for enhancement of CD86 expression. However, it was reported that while CT, alone or together with TNF-α and IL-1β, induced DC maturation, CTB did not (268). Bagley *et al.* reported that the ability of CT and LT to induce the maturation of human monocyte-derived DC is cAMP dependent (274). Furthermore, these researchers recently reported that the maturation of DC induced by CT was dependent on prostaglandin E_2 (PGE_2) and nitric oxide (NO) production (275). In addition, rCTB failed to enhance CD86 expression on macrophages (270, 276). cAMP elevating agents, including dibutyryl-cAMP (d-cAMP) and forskolin, have been used, with conflicting results, to examine the role of cAMP in the ability of the toxins to mature APCs. One study reported that forskolin was unable to induce maturation in human DC (268), while another report showed upregulation of CD80 and CD86 on human DC in response to d-cAMP and forskolin (274). The latter group suggested that these conflicting data resulted from the use of different concentrations of forskolin. The study by Cong *et al.* showed that treatment of macrophages with d-cAMP upregulated CD86 expression (270).

CT or LT do not induce the production of cytokines by DC, however, CT induced the production of the chemokine MIP-2 (121). However, both CT and LT have been shown to modulate cytokine production in response to TLR-agonists. CT and LT suppressed IL-12 and TNF-α production by human monocytes and DC, or murine macrophages in response to a variety of stimuli, including LPS and CD40L (268, 272, 277). Furthermore, CT inhibited the expression of the β1 and β2 chains of the IL-12 receptor on activated T cells, leading to a suppression of Th1 cell differentiation and enhancement Th2 responses (277). As well as inhibiting IL-12 and TNF-α production,

CT also inhibited LPS-induced MIP-1α, and MIP-1β production from murine DC, while IL-10, IL-1β and IL-6 production was enhanced. The inhibitory effects of CT were independent of IL-10 (269). In contrast to the wild type toxins, it has been demonstrated that CTB did not affect IL-12p70 production by SAC and IFN-γ-stimulated (277) or CD40L- or LPS-induced (268) human DC. Based on these data, cAMP has been proposed as the dominant factor involved in the modulatory effects of the toxins on cytokine/chemokine production (268).

It has also been established that CT and LT may directly affect T cells. CT inhibited Th1 responses from Peyer's patch $CD4^+$ T cells activated via the TCR-CD3 complex. In contrast, LT enhanced Th1 type responses by $CD4^+$ TCR-activated T cells, but significantly inhibited IL-4 production (276). These studies may explain in part the different T cell subtype responses that have been reported with the two toxins. In addition, oral immunization of IL-$4^{-/-}$ mice with LT, but not CT, has been shown to induce antigen-specific Th2 type responses, including IL-5, IL-6 and IL-10, suggesting a role for IL-4 in the induction of Th2 type responses by CT (271, 276).

The immunomodulatory effects of the toxins and their efficacy as mucosal adjuvants is likely to be dependent on a number of factors, including increased permeability at mucosal surfaces, activation of APCs and direct interactions with T cells, resulting in enhancement of T cell and Ab responses of the Th polarization adaptive immune system.

1.8.6 Safety issues associated with LT, CT and their mutant derivatives

The toxicity problems associated with the use of LT and CT as vaccine adjuvants has been largely attributed to the enzyme activity of the toxins. Early reports showed that CT, even at low doses that are non-toxic in mice, could cause severe diarrhoea in humans (213). However, in recent years the principal concerns have been the neurotoxic effects of nasally administered toxins. Clinical trials with an intranasally administered influenza vaccine formulated with LT showed that the vaccine stimulated local and systemic anti-influenza virus antibody (212, 278). However, subsequently a number of cases of Bell's palsy were reported in vaccine recipients and consequently the vaccine was withdrawn from clinical use (279). In an unrelated study, CT and CTB were reported to bind to

olfactory nerves and epithelium, and undergo retrograde transport to the olfactory bulb following intranasal administration in mice (280). It was also reported that intranasal vaccination with tetanus toxoid together with CT as adjuvant resulted in antigen uptake into the olfactory nerve/epithelium, whereas tetanus toxoid alone did not enter the CNS (280). These studies suggested that uptake into olfactory regions was GM1-dependent. In contrast, it was demonstrated that intranasal administration of CTB or LTB, supplemented with small amounts of holotoxin, did not localize to the brain or cause any histological changes to brain tissue in mice (281). Nevertheless, there are serious safety concerns for the contribution of the GM1-binding ability of the toxins and the role this might play in targeting of vaccines to neuronal tissues. Recent studies using the non-toxic fusion protein, CTA1-DD, demonstrated that in contrast to CT and CTB, CTA1-DD did not bind to or accumulate in the nervous tissues of the olfactory bulb (282). This fusion protein has enzyme activity but the ability to bind GM1-ganglioside has been removed, suggesting that the neurological effects observed with the toxin or their derivatives are GM1-dependent. It has been reported that neurological responses to bacterial toxins are dependent on the route of delivery. It was shown that intranasal delivery of CT induced pro-inflammatory cytokine production in the murine hypothalamus, whereas parenterally delivered CT had little effect (283).

The potential side effects associated with the native and recombinant toxins led to the development and testing of site-directed mutants of CT and LT, that retain their adjuvanticity but with minimal toxicity. LTK63 and LTR72 were found to have no adverse side effects in a number of preclinical trials (200, 284, 285). Intranasal administration produced no histological inflammatory changes in the respiratory tract or olfactory or meningeal tissues in mice. More recently, LTK63 has exhibited an excellent safety profile in clinical trials with a nasally delivered influenza vaccine (231). In addition, clinical trials have been performed with CTB mixed with killed enterotoxigenic *E.coli* (ETEC) or inactivated *Vibrio cholerae* as oral vaccines against diarrhoeal diseases, including cholera (252, 286, 287). One of the main issues in this field is the sensitivity of the method used to assess toxicity. Comparisons between trials are difficult because of the different clinical settings and also because the methods used, for example histology as compared with PCR, to determine toxicity may have different varied sensitivities.

Therefore, future studies with vaccines that include bacterial toxins should be carefully monitored for their potential side effects.

1.9 SIGNALLING IN INNATE IMMUNE RESPONSES

1.9.1 G PROTEIN-COUPLED RECEPTOR SIGNALLING (GPCR)

The prototypical GPCR is comprised of a seven-transmembrane spanning receptor which is coupled to a heterotrimeric-G-protein complex (288). The extracellular components determine receptor ligand specificity, while the G-protein complex mediates downstream signalling events. In the inactivated state, G proteins exist as a heterotrimer consisting of α, β and γ subunits. Upon ligation of a G protein-coupled receptor, the G protein undergoes a change in conformation, whereby GTP replaces GDP followed by dissociation of the GTP-Gα subunit from the G β/γ subunits, and subsequent independent activation of intracellular signalling pathways. Dephosphorylation of the α subunit terminates this activation, favouring reassembly of the trimeric complex (289).

The GTP-Gα family of G proteins fall into four groups, G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$, which are characterized by their ability to alter the activity of adenylate cyclase (AC). The βγ dimer can also activate signalling pathways, including the MAPK pathway, PI3K, and phospholipase C (PLC) (290). CT and LT activate Gsα by ADP-ribosylation, rendering it resistant to GTPase activity. This leads to spontaneous dissociation of the heterotrimeric complex, followed by Gsα-GTP binding to and activating AC, resulting in synthesis of cAMP from ATP.

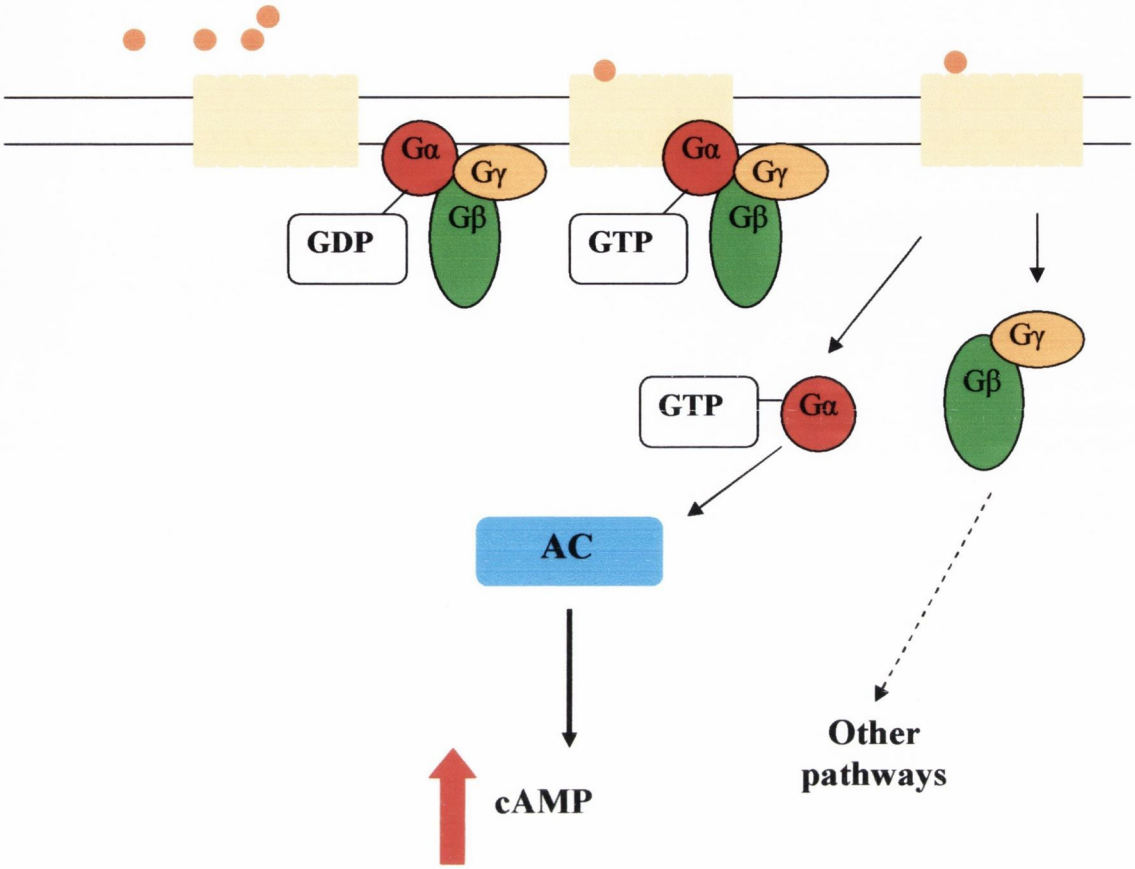

Figure 1.7 Activation of GPCR signalling

Upon receptor activation, the G-protein exchanges GDP for GTP, causing the dissociation of the GTP-bound α and β/γ subunits and triggering diverse signalling cascades. To date, over 20 known G-alpha subunits have been classified into four families, G-α (s), G-α (i/o), G-α (q) and G-α (12), based on structural and functional similarities. Activation of Gαs (as shown here) leads to activation of adenylate cyclase which results in increased intracellular cAMP. The βγ subunits can activate signalling pathways including the phosphoinositide 3-kinase (PI3K) and MAPK pathways.

The increased concentration of cAMP can affect a wide range of cellular processes. The principal cAMP receptor in mammalian cells is the cAMP-dependent protein kinase A (PKA). However, cAMP has been demonstrated to activate cyclic nucleotide gated channels involved in transduction of olfactory and visual signals, and the cAMP-activated guanine exchange factors Epac 1 and Epac 2 (291, 292). cAMP is broken down by cAMP phosphodiesterases (PDE) to 5'-AMP, which does not regulate cellular processes to any great extent. The balance between cAMP synthesis and degradation allows very precise regulation of the events controlled by cAMP.

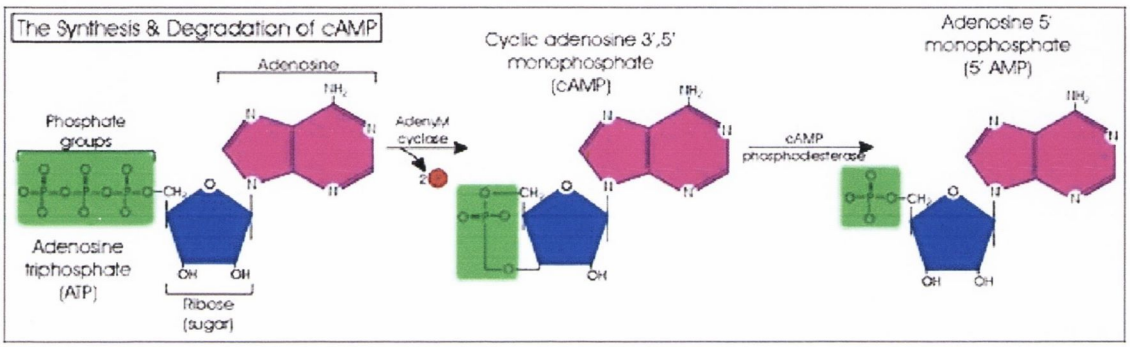

Figure 1.8 Synthesis of cAMP from ATP and its degradation by phosphodiesterases

1.9.1.1 PROTEIN KINASE A

PKA is a primary target of cAMP in a diverse number of tissues and cell types. In its inactive state, PKA has two regulatory subunits (R) and two catalytic subunits (C). In the absence of cAMP the R and C subunits form a R_2C_2 complex that is enzymatically inactive. A pseudocatalytic domain in the regulatory subunits binds to the catalytic domain of the catalytic subunits. Binding of two molecules of cAMP to the regulatory subunits changes the conformation of the pseudocatalytic domains, and results in the dissociation of the now enzymatically active PKA catalytic subunits (292). These free catalytic subunits then phosphorylate specific serine and threonine residues in many targets to alter their activity (292, 293).

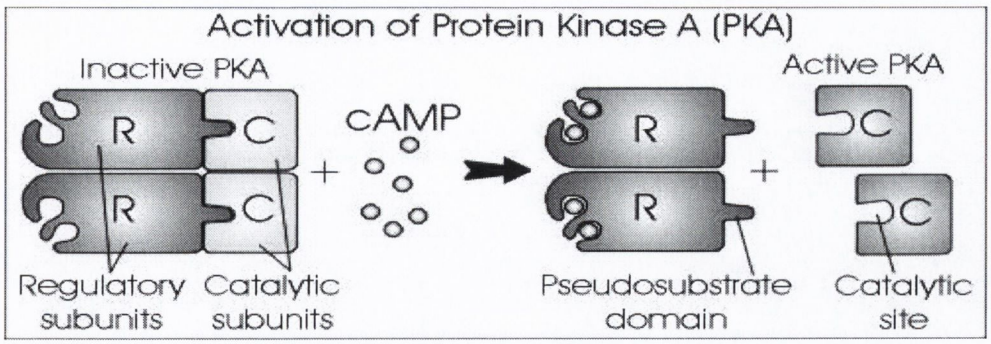

Figure 1.9 Activation of PKA

Binding of two molecules of cAMP to the regulatory subunits of PKA results in a conformational change allowing dissociation of the now enzymatically active PKA catalytic subunits

PKA is a serine/threonine kinase that regulates many cellular processes. Modulation of signal transduction by PKA is a complex and diverse process, and differential isozyme expression, holoenzyme composition and subcellular localization via A-kinase-anchoring-proteins (AKAPs) contribute specificity to the PKA signalling pathway. AKAPs play an important role in regulating cAMP/PKA signalling, by attracting PKA to distinct cellular locations, where these multidomain proteins sequester signalling molecules such as phosphatases, kinases and PDEs (294).

PKA can regulate immune function at a number of levels, although the substrates of PKA appear to be cell-type specific. For example, PKA can modulate transcription factors that are activated upon TCR engagement, including NFκB, AP-1 and CREB/ATF families (295). In addition, PKA-mediated modulation of the MAPK pathway has been demonstrated in a range of cell types. It was reported that PKA mediated ERK activation in HEK-293 cells by the β2-adrenergic receptor via the GTPase, Ras, and the MAPKKK, Raf-1 (296). In contrast, it was demonstrated that β2-adrenergic receptor-mediated activation of ERK was mediated via Rap-1, but not Ras (297). More recent studies identified a cAMP-dependent guanine nucleotide exchange factor (Epac) as the mediator of Gs-coupled receptor-activation of Ras and ERK in HEK-293 cells and N1E-115 neuroblastoma cells (298). Importantly, it has been reported that PKA plays a role in the

impaired T cell-mediated activation of B cells found in a high percentage of patients with common variable immunodeficiency (CVID). The impaired secretion of IL-10 by T cells in CVID patients was suggested to be as a result of cAMP/PKA signalling (299).

1.9.1.2 Signal transduction via cAMP and PKA

cAMP-mediated gene expression is principally mediated by the cAMP element (CRE)-binding protein (CREB) and PKA plays a major role in this process (300). cAMP/PKA signalling pathways have been shown to modulate the activities of important transcription factors, such as CREB, NF-κB, MAPK and CCAAT/enhancer-binding protein (C/EBP), that play central roles in immunity. Besides CRE, other regulatory sequences, such as AP-1, AP-2, Sp1, Pit-1, Y box (inverted CCAAT element) and estrogen response element may also mediate the cAMP-dependent gene activation. Furthermore, transcription of some c-fos genes, which depends on the activity of other binding proteins, e.g. C/EBP, are induced by cAMP. Elevation of cAMP and activation of PKA in both endothelial and monocytic cells inhibited NF-κB activity and transcription (301). The transcriptional coactivator CREB-binding protein (CBP) plays an important role in PKA-mediated inhibition of NFκB transcription. Overexpression of CBP rescued cAMP inhibition of NFκB-mediated transcription, while overexpression of CREB inhibited transcription, suggesting that the phosphorylation of CREB, results in competition between p65 and CREB for limiting amounts of the coactivator CBP (302).

1.9.2 PROTEIN KINASE C

Protein kinase C (PKC) is a family of phospholipid-dependent serine-threonine kinases involved in a wide variety of cellular processes that impact on cell growth and differentiation, cytoskeletal remodelling and gene expression in response to diverse stimuli. The PKC family can be divided into three categories based on their N-terminal regulatory domain and requirements for lipid second messengers (303). Conventional PKCs (cPKCs) comprise the α, β, γ isoforms, which are Ca^{2+} dependent via their C2 domains and are regulated by phosphatidylserine (PS) and diacylglycerol (DAG). The novel PKCs (nPKCs) comprise δ, ε, η, θ, μ isoforms and are Ca^{2+} independent but are regulated by PS and DAG. The atypical PKCs (aPKCs) ζ and λ/ι are $Ca2^{+}$ and DAG independent but PS-dependent (304).

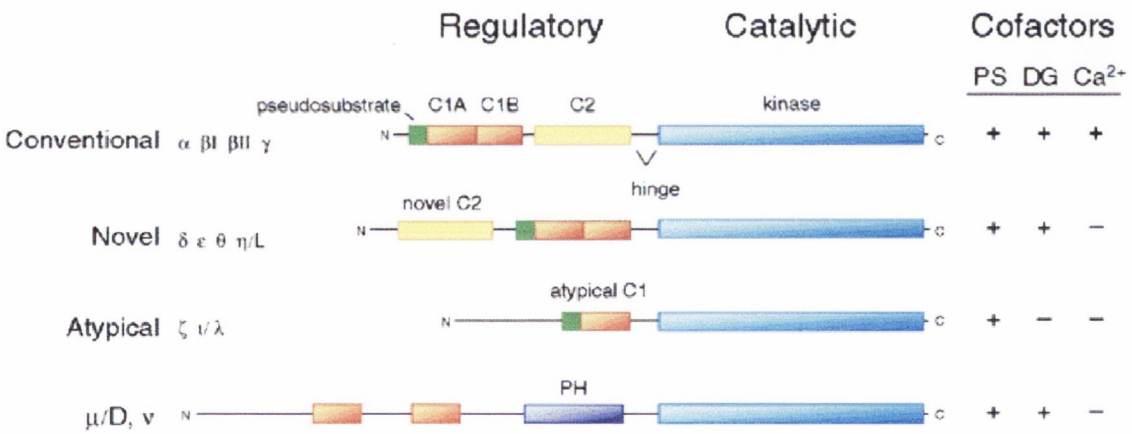

Figure 1.10 Schematic of primary structures of PKC family members showing domain composition and activators

PS, phosphatidylserine; DG, diacylglycerol; Ca^{2+}, calcium. (304)

Members of these three PKC groups contain a pseudo-substrate or autoinhibitory domain that binds to the substrate binding site in the catalytic domain, preventing its activation in the absence of cofactors or activators. The cPKC and nPKCs contain three conserved residues that are essential for catalytic activity, intracellular distribution and stability. These sites are known as the activation loop, the turn-motif, and the C-terminal hydrophobic-motif and are also conserved on many other kinases, termed ABC kinases, such as PKA and PKB. Atypical PKC isoforms can not be phosphorylated in the hydrophobic region, which has a Glu in place of the Ser or Thr residue found in other PKC isoforms. Phosphoinositide-dependent kinase 1 (PDK1) phosphorylates the activation loop of many ABC kinases but the mechanism of phosphorylation at the other sites is unclear. Different PKCs are selectively expressed in particular cell and tissue types and have distinct structural features as well as regulatory mechanisms. This accounts for the diverse biological roles of PKCs.

An essential role for PKCθ in TCR activation and signalling was demonstrated in T cell lines from PKCθ deficient mice, which exhibited reduced TCR-CD28-induced proliferation and IL-2 production. In addition, TCR/CD28-induced AP-1 and NFκB activation was significantly impaired (305, 306). More recently, it was demonstrated that anti-CD3 and anti-CD28 and MHC alloantigen-induced T cell proliferation and IFN-γ production was severely impaired, and antigen-specific IgG2a and IgG2b responses were diminished in PKCα deficient T cells (307). PKCε plays an important role in LPS-mediated signalling in DC and macrophages. LPS and IFN-γ -stimulated PKCε deficient murine macrophages had reduced NO, TNF-α, and IL-1β and were defective in the induction of nitric oxide synthase (NOS)-2. $PKC\varepsilon^{-/-}$ mice displayed a major defect in their capacity to clear both Gram-positive and Gram-negative bacterial infections (308). Furthermore, inhibition of PKCε reduced LPS-induced TNF-α and IL-12 production and IKK and NF-κB activation in monocyte-derived human DC (309).

1.9.3 MAPK SIGNALLING

The mitogen-activated protein kinase (MAPK) cascade is a highly conserved signalling pathway involved in the control of immune responses. There are three major groups of MAPKs in mammalian cells, the extracellular signal-regulated protein kinases (ERK), the p38 MAPKs, and the c-Jun NH_2 – terminal kinases (JNK) (310, 311). A number of recent studies have demonstrated a major role for components of the MAPK cascade in the regulation of innate and adaptive immune responses, including Th cell polarization (189, 190, 312, 313).

1.9.3.1 MAPK SIGNAL TRANSDUCTION

The MAPKs, ERK, JNK and p38, are activated by dual phosphorylation of Thr and Tyr residues in a 'TXY' (where X is Glu, Pro and Gly in ERK, JNK and p38 MAPKs respectively) activation motif by a dual-specificity MAPK kinase (MAPKK or MKK). The MAPKK, in turn, are activated by a serine-threonine kinase, termed MAPKK kinase (MAPKKK or MKKK) (311, 314). Each protein kinase subfamily consists of several isoforms and members, which often have distinct functions. For example, the ERK MAPKs are activated by the MKKs, MKK1 and MKK2; the p38 MAPKs are activated by MKK3, MKK4, and MKK6; and the JNK MAPKs are activated by MKK4 and MKK7. Downstream targets of MAPKs include three structurally related MAPK-activated protein kinases (MAPKAPKs or MKs), MK2, MK3 and MK5, the ribosomal S6 kinase (RSK) subfamily, the mitogen- and stress-activated kinase (MSK) subfamily and the two MAPK-interacting kinases, MNK1 and MNK2, which form the MNK subfamily. Therefore, differential activation of MAPKKK results in signal transduction to diverse cellular targets.

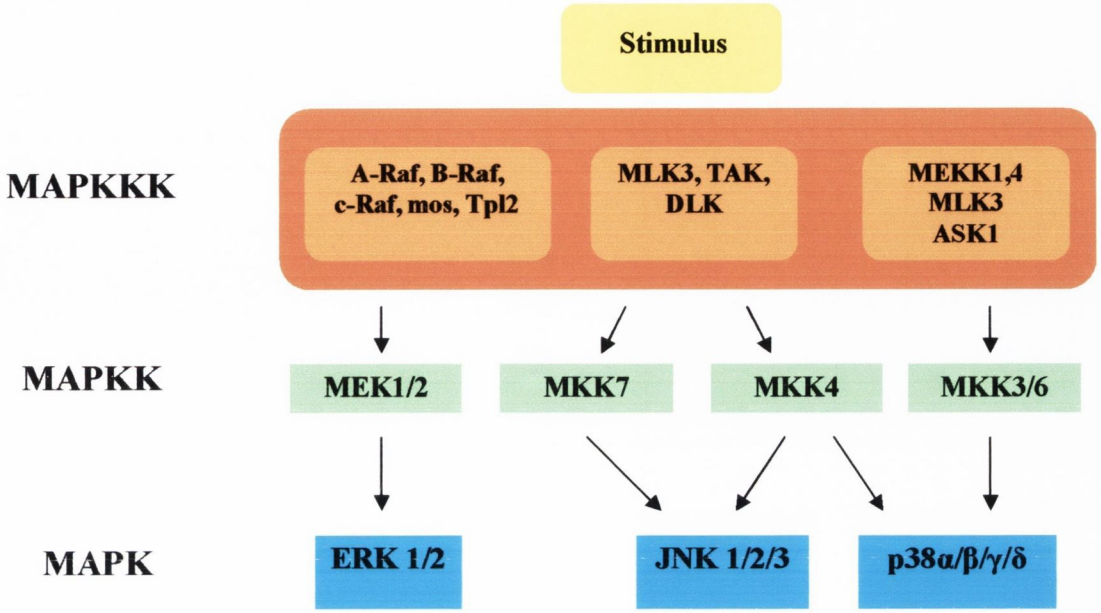

Figure 1.11 MAPK signalling

The MAPK signalling cascade is activated by a wide variety of receptors, including GPCRs, receptor tyrosine kinases (RTKs), integrins, TLRs, stress, inflammatory cytokines and ion channels. The specific components of the cascade vary greatly among different stimuli. MAPK signalling comprises a three-component protein kinase cascade of a serine-threonine protein kinase MAPKKK, which phosphorylates a dual-specificity protein kinase MAPKK that, in turn, phosphorylates and activates a MAPK. The MAPKKKs are activated by interaction with the family of small GTPases and/or other protein kinases, connecting the MAPK module to cell surface receptors or external stimuli.

Depending on the stimulus, receptors transduce their signals via different MAPK subtypes. Activation of specific MKKKs links MAPKs to receptors involved in controlling immune responses. A total of 21 kinases have been shown to function as MAPKKKs, including apoptosis signal-regulating kinase 1 (ASK1), tumour progression locus 2 (TPL-2), and MEK kinases 1-4 (315). ASK1 has been shown to be required for TLR4-mediated, but not TLR2-mediated, activation of p38 in DC (316). Current evidence suggests that specific MAPKKKs are utilized in a cell-type- and stimulus-

specific manner and that MAPKKKs might function cooperatively to optimally activate downstream MAPKKs (315). MAPKKK regulation has been found to be very complex, as demonstrated by the regulation of the most studied mammalian MAPKKK, Raf-1 (317). The prominent mechanisms by which MAPKKK activation are controlled involves regulation by phosphorylation and association with other proteins. In general, the ERKs are activated by mitogenic and proliferative stimuli, whereas the JNK and p38 MAPKs respond to environmental stresses, including ultraviolet light, heat, osmotic shock and inflammatory cytokines (318).

MAPK signalling cascades are central to the polarization of T cell responses. In particular, p38 MAPK has been proposed as an essential factor for IL-12 production from macrophages and DC (319). $MKK3^{-/-}$ macrophages exhibited a defect in LPS-induced IL-12 production at both the protein and mRNA levels, while MKK3 deficiency resulted in a reduction of CD40L-induced IL-12 by bone marrow-derived DC. This group also showed that LPS-induced IL-12 production by macrophages was inhibited by the p38 inhibitors, SB203580 and SB202190. In addition, mice deficient in the p38α substrate, MAPKAP K2, display diminished production of LPS-induced IFN-γ and TNF-α by spleen cells (320). In contrast, it was reported that inhibition of p38 suppressed TLR-induced IL-10 production by DC, and incubation of CpG-pulsed DC with a p38 inhibitor promoted the induction Th1 responses (321). Consistent with this study, Bowie and colleagues also showed that inhibition of p38 with SB203580 suppressed LPS-induced IL-10 production (322). Naïve T cells from $JNK1^{-/-}$ mice preferentially differentiate into a Th2 phenotype on activation with anti-CD3 +/- anti-CD28, and consistent with this, infection of JNK1 deficient mice with *Leishmania* leads to greatly exacerbated disease (323, 324). There are reports in the literature of positive and negative regulation of IL-12 by ERK MAPK. Inhibition of ERK suppressed IL-12p70 production by DC (189, 190) and $ERK1^{-/-}$ mice exhibit Th1 cell polarization and increased susceptibility to EAE (325). In contrast, it was demonstrated that ERK negatively regulated IL-12p40 but not IL-12p70 production by peritoneal macrophages (326).

1.9.4 PI3K SIGNALLING

Phosphoinositide 3-kinases (PI3Ks) are a family of kinases that modulate numerous biological processes, including cell survival, cytoskeletal remodelling and tumourigenesis. PI3Ks are dual-specific lipid and protein kinases that are composed of a catalytic subunit (p110) and a regulatory subunit and can be divided into three classes. Class 1 PI3Ks are heterodimers made up of a catalytic subunit, called p110, and a tightly associated regulatory subunit. Class 1 PI3Ks can be further subdivided into class 1A and class 1B, which signal downstream of tyrosine kinases and GPCRs, respectively. Mammals have three class 1A p100 isoforms, (p110α, p110β and p110δ), which form a complex with the SH2-containing regulatory p85 subunits, of which there are five isoforms (327, 328). Class 1B PI3Ks contains a single member, p110γ, which is complexed to a regulatory protein, p101, and is mainly activated by Gβγ subunits of heterotrimeric G proteins (329). Class 1B PI3K is expressed highly on cells of the immune system (328).

1.9.4.1 PI3K SIGNAL TRANSDUCTION

PI3K activation involves the binding of Src-homology 2 (SH2) domains, of the regulatory subunit, to adaptor proteins with phosphorylated tyrosine residues present in activated cellular receptors on the plasma membrane, including TLRs (329). Activation of class 1 PI3K results in the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) at the cell membrane, which then acts as a second messenger by localizing a subset of signalling proteins with pleckstrin homology (PH) domains to the membrane, where they become activated and initiate downstream signalling events. In addition to being activated by RTKs, the p110 catalytic subunit can also bind directly to the small GTPase Ras, which contributes to PI3K activation. Akt (also known as protein kinase B) is a key physiological mediator of the PI3K pathway. Akt is activated by phosphorylation at Thr 308 by PDK1, and Ser 473 by an as yet unknown kinase (330).

Activated Akt phosphorylates a number of downstream targets, including the constitutively active serine-threonine kinase, GSK3. Two isoforms of GSK3, GSK3α and GSK3β, have been identified with high homology and similar but not identical

biochemical properties (331). Phosphorylation by protein kinases, including Akt, at Ser9 in GSK3β and Ser21 in GSK3α, inactivates GSK3 resulting in its inhibition (332). GSK3 regulates the phosphorylation status of a number of substrates. In its active state, GSK3 increases cell adhesion through the phosphorylation of β-catenin and MUC1. GSK3 phosphorylates several transcription factors, including c-Myc, c-Jun and c-Myb and the translation factor eIF2B (333).

GSK3 can both positively and negatively affect a variety of transcription factors involved in immune responses, including NFκB, AP-1, NF-AT and CREB, that regulate pro- and anti-inflammatory cytokine production. TLR-mediated IL-12 production was increased by PI3K or Akt inhibition, whereas TLR-induced IL-10 production was inhibited in human monocytes (171). Using RNA interference to selectively inhibit CREB expression, it was demonstrated that enhancement of TLR-induced IL-12 and suppression of IL-10 production were dependent on inhibition of GSK3 which results in the subsequent enhancement of CREB. It was proposed that the mechanism through which PI3K negatively regulates IL-12 is via competition between CREB and NFκB for the coactivator CBP. Furthermore, PI3K negatively regulates TLR-induced IL-12 production in DC and in addition, $PI3K^{-/-}$ mice demonstrated enhanced Th1 responses upon *Leishmania major* infection (334).

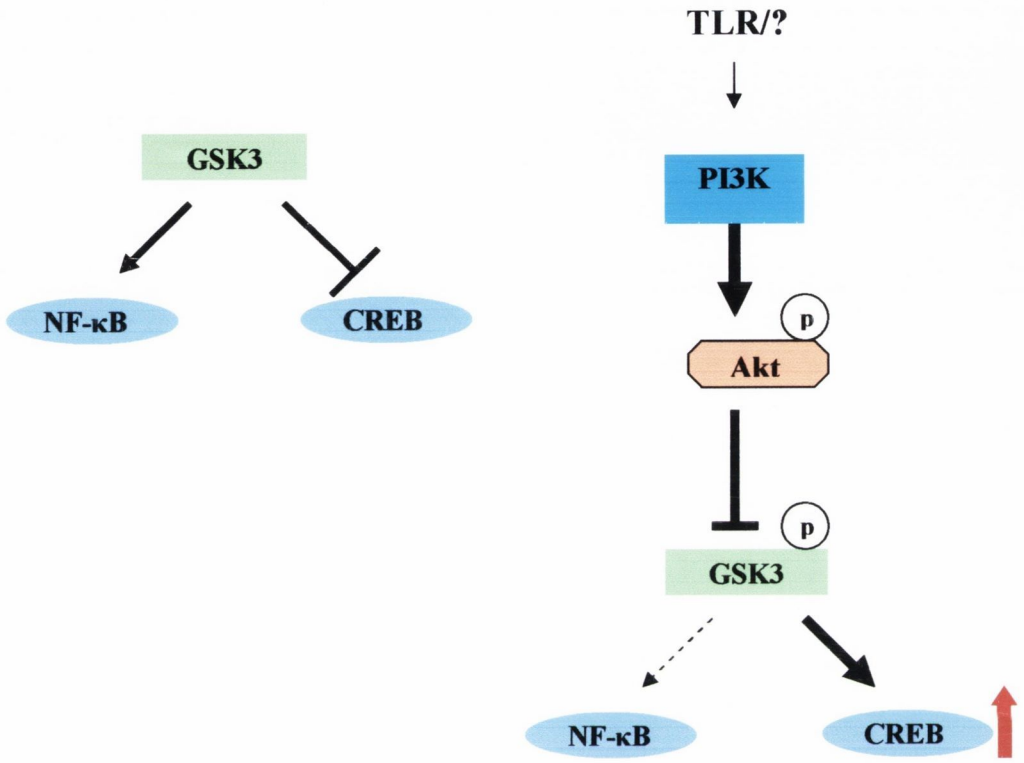

Figure 1.12 PI3K signalling

TLR activation induces multiple pathways that mediate pro-inflammatory responses. For example, TLR4 activation leads to the predominant activation of the NFκB pathway and in this situation there is limited PI3K stimulation. Activation of PI3K results in the phosphorylation of a variety of protein kinases, one of which is Akt. GSK3 is a serine-threonine kinase that is constitutively active in resting cells but can be inactivated through phosphorylation by other kinases, including Akt. GSK3 has been linked to the regulation of many transcription factors, including NFκB, CREB, NF-AT and AP-1, and therefore plays an important role in regulating TLR signalling. (Modified from (335))

1.10 TRANSCRIPTION FACTORS IN IMMUNE RESPONSES

1.10.1 NFκB

The transcription factor NFκB is one of the key mediators of both innate and adaptive immune responses, and also plays a role in stress responses, cell survival and development. The members of the NFκB family include p50, p52, p65 (RelA), RelB and c-Rel. NFκB is maintained in the cytoplasm as inactive homo- or hetero-dimers, the most common being p50-p65, via interactions with a class of inhibitory proteins, called IκBs (336). It was thought that the IκB proteins sequestered NFκB in the cytoplasmic compartment by masking the nuclear localisation sequence (NLS) however, more recently they have been found to interact via multiple ankyrin repeats (337). In response to a variety of stimuli, IκBs are phosphorylated and degraded, allowing the translocation of NFκB to the nucleus, where it carries out its transcriptional activity at specific κB sites within the promoter regions of target genes. NFκB-responsive genes include those encoding inflammatory cytokines and chemokines and adhesion molecules (338). Most of the NFκB family members contain a consensus phosphorylation site (RRXS) localized close to the NLS (339).

NFκB is activated rapidly in response to a wide range of stimuli, including pathogens, stress signals and pro-inflammatory cytokines, such as IL-1 and TNF-α. Activation of NFκB depends on the degradation of IκB, which occurs via IKKs that reside in a large protein complex. Activators of the IKK complex include MAPKKKs, such as MEKK1, MEKK3 and TAK1. Ligands for TLRs, IL-1/IL-18 receptors, the TNF receptor superfamily and B and T cell receptors can all activate this complex. NFκB is one of the pivotal regulators of pro-inflammatory gene expression and as such a major downstream target of TLR signalling. The activation of NOD proteins also leads to activation of the IKK complex via receptor-interacting protein (RIP2). TCR-induced activation of NFκB requires a costimulatory signal from CD28, and involves the activation of PKCθ. Likewise, BCR-mediated activation of NFκB involves activation of PKCβ (337).

Inappropriate or excessive activation of the NFκB pathway can lead to inflammatory and autoimmune diseases, including RA, MS, inflammatory bowel diseases

and asthma. Blocking NFκB by overexpression of IκBα inhibited the inflammatory response and tissue destruction in rheumatoid synovium (340). Regulation of this pathway through IκB and the IKK complex is central to NFκB activation, but regulation of the transactivation of NFκB represents another level of control. p65 has multiple sites at which it can be phosphorylated and as such a number of kinases have been reported to have the ability to regulate it. Mice deficient in GSK3β, TBK1 and PKCζ exhibited normal IκB degradation but impaired activation of NFκB-dependent target genes (341). p65 is phosphorylated on Ser276 by PKA in response to LPS (342) and mice deficient in mitogen- and stress- activated protein kinase 1 and 2, showed impaired Ser276 phosphorylation in TNF-treated fibroblasts (343). p65 is phosphorylated on Ser311 in response to TNF, however, this phosphorylation is lost in PKCζ$^{-/-}$ cells (344). The phosphorylation at these sites is associated with an increase in the transcriptional activity of p65, and is accompanied by enhanced binding to transcriptional cofactors such as CBP/p300. Histone deacetylases (HDACs) play an important role in repressing transactivation and also terminating NFκB activation by increasing the affinity of NF-κB for IκBα (338).

1.10.2 CREB

cAMP stimulates target gene expression via a conserved cAMP-responsive element (CRE), which consists of an eight-base pair palindrome and is typically found within 100 nucleotides of the TATA box (300). CRE-binding protein (CREB) is a 43-kDa phosphoprotein, one of a family of leucine zipper transcription factors, exclusively found in the nucleus, and under regulation by a number of factors, including cAMP and calcium. CREB regulates a diverse array of genes involved in numerous cellular processes, including glucose metabolism and neuronal development, but also in immune responses. Other CREB family members include activating transcription factor-1 (ATF-1) and cAMP response element modulator (CREM). The proteins in this family contain consensus PKA phosphorylation sites and are regulated by cAMP in vivo. CREB binds to the CRE typically as a homodimer, but is transcriptionally inactive until phosphorylated at Ser133 by any one of several kinases including PKA, calcium/calmodulin-dependent protein kinase IV and p38 mitogen-activated protein

kinase. Phosphorylation of Ser133 promotes recruitment of the coactivator CBP or its paralog p300 (345). CBP/p300 mediates gene transcription via its association with the basal transcriptional machinery of the RNA polymerase II holoenzyme complex and its intrinsic histone acetyltransferase activity. However, it has been reported that Ser133 phosphorylation alone is not sufficient to mediate CREB transactivation and that a number of cofactors may be required in addition to CREB phosphorylation for full CREB-dependent transcription. Indeed, the activity of the coactivator CBP has been reported to be regulated by a variety of kinases, including PKA, CaMKIV and ERK, possibly by direct phosphorylation.

It has been suggested that Ser133 phosphorylation specifically affects the transactivation potential of CREB rather than its DNA binding or nuclear targeting activities. The CREB transactivation domain is bipartite, consisting of a glutamine-rich constitutive activator Q2 and a kinase inducible domain (KID), which function synergistically to stimulate target gene expression. The KID comprises a 58 AA region that contains the PKA phosphorylation site and is highly conserved in CREB family members ATF-1 and CREM. Residues in KID appear to perform two critical functions: PKA recognition and association with the transcriptional apparatus. Q2 encodes a constitutive transactivation domain that is critical for PKA-dependent induction of target genes.

1.10.3 CCAAT/ENHANCER-BINDING PROTEIN

CCAAT/enhancer-binding proteins (C/EBP) are transcription factors involved in cAMP-dependent gene expression, for example nuclear factor for IL-6 (NF-IL-6) and IL-1, and belong to the family that includes CREB/ATF. Six members of the family have been characterized (C/EBPα, -β, -δ, -γ, -ε and -ζ) and are distributed in a variety of tissues (346). C/EBP proteins mediate cAMP responsiveness by indirect mechanisms, which include their increased expression and translocation into the nucleus in response to elevated cAMP levels. They possess domains that mediate cAMP-inducible activities that are independent of direct phosphorylation by PKA. A synergistic interaction of CREB and C/EBP transcription factors has been shown to mediate a tissue-specific

response to cAMP. A dominant role was shown for C/EBP in the basal as well as cAMP-induced IL-10 transcription, in two cell lines of myelomonocytic origin (347).

1.10.4 AP-1

Activator protein-1 (AP-1) is a pleiotropic, dimeric transcription factor involved in many cellular functions related to apoptosis, cell differentiation, metabolism, tumour evasion and inflammation (348). A family of proteins that include, Fos, Jun and ATF can homo- and hetero- dimerize to form the active AP-1 complex that modulates gene expression. The diverse functions which AP-1 regulates can be accounted for by different combinations of these proteins. AP-1 activity is induced by stimuli such as growth factors, cytokines, bacterial and viral infections, T cell activators and neurotransmitters. Most of the genes that encode AP-1 components behave as "immediate-early" genes, that is, genes whose transcription is rapidly induced, independently of *de novo* protein synthesis (349). MAPKs are central to the activation of AP-1 in response to many stimuli. For example, the induction of AP-1 by pro-inflammatory cytokines and genotoxic stress is mostly mediated by the JNK and p38 MAPKs. AP-1 is strictly regulated at the posttranscriptional level by phosphorylation. Some of the kinases that are thought to phosphorylate and activate Jun include ERK1/2, JNK, PKC and activated Ras, while GSK3β has been implicated in the phosphorylation and negative regulation of AP-1 activation.

1.10.5 IRFs

IFN-regulatory factors (IRFs) are a family of transcription factors that have extensive homology in the DNA-binding domain (DBD). In humans and mice nine family members have been identified: IRF-1 to -7, IRF8 (IFN consensus sequence-binding protein), IRF9 (IFN-stimulated gene factor 3γ/p48). In addition, IRF10 was identified in chickens (350, 351). The major function of the IRF family of transcription factors is the regulation of immune responses, especially those directed against viruses (337). They are widely involved in signalling events downstream of most PRRs, including TLRs and NLRs.

IRF1

IRF1 is critical for the enhancement of TLR-dependent gene induction by IFN-γ. IRF1 directly interacts with MyD88 but requires activation of TLR9 to efficiently undergo posttranslational modifications and migrate to the nucleus. The induction of genes encoding IFN-β, iNOS and IL-12p35 was impaired in IRF1-deficient cDC and macrophages stimulated with IFN-γ and CpG (352). IRF1 is indispensable for the development of Th1 responses. IRF1-dependent induction of IL-12Rβ1 was essential for IFN-γ-IL-12 signaling, but was dispensable for IL-23-IL-17 signalling (353). $IRF^{-/-}$ mice fail to develop NK cells, which produce IFN-γ to stimulate IL-12 production by macrophages. IRF2 appears to cooperate with IRF1 in the induction of IL-12p40 gene expression (354).

IRF3 and IRF7

Five members of the IRF family (IRF1, IRF3, IRF5, IRF7, and IRF8) have been implicated as positive regulators of type I IFN gene induction. Two RNA helicases, RIG-I and MDA5 have been identified as essential cytosolic receptors for intraceullular viral RNA to induce type I IFN genes in a number of cell types, except pDC. IRF3 and IRF7 have been shown to be necessary for the RIG-I/MDA5-mediated type I IFN gene induction pathway. In response to viral infection, specific tyrosine residues in the C-terminal regulatory regions are phorphorylated, which causes the IRFs to rapidly translocate to the nucleus and activate the transcription of type I IFNs and other cytokines. IRF5-deficient mice are highly susceptible to vesicular stomatitis virus (VSV) (ssRNA) infection demonstrating a role for IRF5 in the RIG-I signalling pathway (355). IRF8 has also been shown to be required for type I IFN induction in virus-stimulated DC (356). TLR3 and TLR4- induced type 1 IFN production is mediated through a TRIF-dependent pathway via IRF3 and IRF7 activation (337). In contrast, TLR7 and TLR9 utilise MyD88 to induce type I IFN expression. TLR9 induces IRF7 via MyD88 and IRAK-1, as confirmed by IRAK-1 deficient mice which displayed complete impairment of IFN-α production in response to both TLR7 and TLR9 activation (32).

IRF5

Similar to IRF7, IRF5 also interacts with MyD88 and TRAF6. TLR9-induced activation of IRF5 causes IRF5 to bind to the promoter regions of a number of target genes, such as *IL12b*, that contain an ISRE. IRF5-deficient splenic DC and macrophages displayed severely impaired TLR-induced IL-6, TNF-α and IL-12 production and consistent with this, $IRF5^{-/-}$ mice showed increased resistance to lethal shock induced by unmethylated DNA and LPS (357). However, recent evidence suggests a cell-type dependency for the role of IRF5 in cytokine gene induction. $IRF5^{-/-}$ bone marrow-derived DC and macrophages showed normal production of IL-6 in response to LPS or PGN but not to TLR3, TLR7 or TLR9 agonists (358).

IRF4 and IRF8

IRF4 and IRF8 are highly homologous immune-cell specific IRFs. Unlike other MyD88-interacting IRFs, IRF4 negatively regulates TLR-signalling. IRF4 mRNA is induced by TLR-activation, and because IRF4 and IRF5 bind to the same region of MyD88, IRF4 can compete with and inhibit the binding of IRF5 to this adaptor. IRF8 plays an essential role in TLR9-MyD88-dependent production of pro-inflammatoy cytokines, including TNF-α and IL-6 by DC. This defect is associated with the inabilitiy of TLR9 to activate NFκB and MAPKs in IRF8-deficient DC (359). IRF8 is required for the expression of the gene encoding IL-12p40 upon stimulation of DC and macrophages with a number of PAMPs and for the induction of type I IFN genes by TLRs and viruses in DC. IRF8-deficient mice are impaired in developing Th1 responses, while IRF4-deficient mice are defective in mounting Th2 responses (354).

HYPOTHESIS AND AIMS

Escherichia coli heat labile enterotoxin (LT) is a potent immunomodulator and vaccine adjuvant. However, the means by which it enhances and directs adaptive immunity is not fully understood. The hypothesis on which this thesis is based is that LT mediates its effects on adaptive immunity by promoting the activation of dendritic cells. The specific aims were to determine the roles of the enzymatically active A subunit and binding B subunit in the ability of LT to modulate DC activation and to dissect the relationship between the type of DC activation induced by the toxin and its constituent subunits and the adaptive immune response induced.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 REAGENTS USED FOR TISSUE CULTURE

2.1.1.1 AMMONIUM CHLORIDE LYSIS SOLUTION

0.87% (w/v) NH_4Cl dissolved in endotoxin free dH_2O filter sterilized. Stored at 4°C.

2.1.1.2 CELL CULTURE MEDIUM

Roswell Park Memorial Institute (RPMI)-1640 medium (BioSera) was supplemented with 8% (v/v) heat inactivated (56°C for 30 min) foetal calf serum (FCS) (Biosera), 100 mM L-Glutamine (Gibco) and 100 μg/ml penicillin/streptomycin (Gibco). Complete RPMI (cRPMI) medium was used to culture all cells *in vitro*.

2.1.2 PRR AGONISTS USED *IN VITRO*

LIGAND	RECEPTOR	SUPPLIER	CONCENTRATION RANGE
LPS	TLR4	Alexis	1-100 ng/ml
CpG	TLR9	Sigma Genosys	0.1-1 μg/ml
Curdlan	Dectin-1	Wako	1-1000 μg/ml

2.1.3 MATERIALS USED IN IMMUNIZATION STUDIES

ADJUVANT	SUPPLIER	CONCENTRATION RANGE
KLH	Calbiochem	2-50 μg/ml (*in vitro*) 20 μg/mouse (*in vivo*)
MOG_{35-55}	GenScript	2-50 μg/ml (*in vitro*) 150 μg/mouse
$PLP_{139-151}$	GenScript	1-25 μg/ml (*in vitro*) 150 μg/mouse

2.1.4 BACTERIAL DERIVED MOLECULES

E. coli heat labile toxin (LT) and the LT derivatives, LTK63 and LTR72, created by site-directed mutaganesis as previously described (Magagnoli *et al.*, 1996), were kindly provided by Chiron Corporation, Siena, Italy. LTB was kindly provided by Prof. Neil Williams (University of Bristol, UK). *Mycobacterium tuberculosis* was sourced from Chondrex Inc., USA.

Preparations contained the following endotoxin concentrations as supplied or as determined by using a PyroGene Recombinant Factor C Assay (Lonza Group Ltd, Basel, Switzerland):

LT: < 0.16 EU/mg
LTK63: 0.048 EU/mg
LTR72: < 0.14 EU/mg
LTB: < 30 EU/mg

2.1.5 LIST OF INHIBITORS USED *IN VITRO* AND *IN VIVO*

INHIBITOR	SPECIFICITY	IC50
Rp-8-Br-cAMPs	PKA	
U0126	MEK1/2	MEK1 = 72 nM; MEK2 = 58 nM
PD98059	MEK	2-7 μM
MEK1/2 inhibitor	MEK1/2	MEK1 = 180 nM; MEK2 = 220nM
SB203580	p38	50 nM
SB202190	p38	50 nM
p38 MAP kinase inhibitor (506126)	p38	35 nM
JNK inhibitor II	JNK 1/2/3	JNK-1 and JNK-2 = 40 nM JNK-3 = 90 nM
Rottlerin	novel PKCs	PKCδ (3-6 μM) PKCθ
BAY 11-7082	NFκB	10 μM
SB-216763	GSK3 (α and β)	34 nM
Wortmannin	PI3K	5 nM
LY294002	PI3K	1.4 μM

The GSK3 inhibitor was sourced from Sigma. Rp-8-Br-cAMPs was sourced from BIOLOG Life Science Institute, Germany. All other inhibitors were sourced from Calbiochem (Merck Biosciences), Germany.

2.1.6 Enzyme linked immunosorbent assay (ELISA) and fluorescence activated cell sorting (FACS) reagents

2.1.6.1 Buffers and solutions for ELISAs

Phosphate-buffered saline (PBS) 20X

320 g Sodium chloride (NaCl, 1.4M)
46 g Sodium hydrogen phosphate (Na_2HPO_4, 0.08M)
8 g Potassium dihydrogen phosphate (KH_2PO_4, 0.01M)
8 g Potassium chloride (KCl, 0.03M)

Dissolved in 2 L of dH_2O and adjusted to pH 7.0.
Diluted to 1X with dH_20 before use.

Wash buffer

PBS 0.05% (w/v) Tween 20
500 ml 20X PBS pH 7.0
5 ml Tween 20
Made up to 10 L with dH_2O.

Reagent diluent

BD Pharmingen kits: PBS
R & D Duosets: 1% Bovine serum albumin (BSA)
eBioscience: 1X reagent diluent (supplied)

Block solution

BD Pharmingen: PBS 10% marvel (except IL-6 : 3% BSA)

R & D Duosets: PBS 1% BSA

eBioscience: 1X reagent diluent (5X diluent supplied)

Phosphate citrate buffer

20.38 g Citric Acid ($C_6H_8O_7$)

73.8 g di-Sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4.12H_20$)

Made up to 2 L with dH_2O, pH 5.0. Stored at 4°C.

Stop solution (1M H_2SO_4)

26.74 ml 18 M H_2SO_4

473.26 ml dH_2O

2.1.6.2 Buffers and solutions for FACS

FACS buffer

2% FCS

0.1% Sodium azide

Made up in 1X PBS. Stored at 4°C.

FACS blocking buffer

50% FCS

50% FACS Buffer

Or anti-CD16/CD32 (Fcγ Block; BD Pharmingen)

TABLE 2.1.1 ORIGIN AND SPECIFITIES OF ANTIBODIES USED IN CYTOKINE ELISAS

ANTIBODY SPECIFICITY	Clone	PURIFIED/CONJUGATE	SUPPLIER
Mouse IL-17	-	Purified	R & D Systems
Mouse IL-17	-	Biotin	R & D Systems
Mouse IL-12p70	-	Purified	R & D Systems
Mouse IL-12p70	-	Biotin	R & D Systems
Mouse IL-10	-	Purified	R & D Systems
Mouse IL-10	-	Biotin	R & D Systems
Mouse TNF-α	-	Purified	R & D Systems
Mouse TNF-α	-	Biotin	R & D Systems
Mouse IL-1β	-	Purified	R & D Systems
Mouse IL-1β	-	Biotin	R & D Systems
Mouse MIP2	-	Purified	R & D Systems
Mouse MIP2	-	Biotin	R & D Systems
Mouse GM-CSF	-	Purified	R & D Systems
Mouse GM-CSF	-	Biotin	R & D Systems
Mouse IL-23p19	-	Purified	eBioscience
Mouse IL-23p19	-	Biotin	eBioscience
Mouse IL-12p40	C17.8	Purified	BD Pharmingen
Mouse IL-12p40/70	C17.8	Biotin	BD Pharmingen
Mouse IFN-γ	R4-6A2	Purified	BD Pharmingen
Mouse IFN-γ	XMG1.2	Biotin	BD Pharmingen
Mouse IL-4	11B11	Purified	BD Pharmingen
Mouse IL-4	BVD6-24G2	Biotin	BD Pharmingen
Mouse IL-5	TRFK5	Purified	BD Pharmingen
Mouse IL-5	TRFK4	Biotin	BD Pharmingen
Mouse IL-6	MP5-20F3	Purified	BD Pharmingen
Mouse IL-6	MP5-32C11	Biotin	BD Pharmingen

TABLE 2.1.2 ORIGIN AND SPECIFITIES OF ANTIBODIES USED IN ANTIBODY ELISAS

ANTIBODY SPECIFICITY	CLONE	PURIFIED/CONJUGATE	SUPPLIER
Mouse IgG1	A85-1	Biotin	Caltag
Mouse IgG2a	R19-15	Biotin	Caltag
Mouse IgG	-	Biotin	Caltag

TABLE 2.1.3 ORIGIN OF RECOMBINANT CYTOKINES USED AS ELISA STANDARDS

CYTOKINE	SOURCE
Murine IL-17	R & D Systems
Murine IL-12p70	R & D Systems
Murine IL-10	R & D Systems
Murine TNF-α	R & D Systems
Murine IL-1β	R & D Systems
Murine MIP2	R & D Systems
Murine GM-CSF	R & D Systems
Murine IL-23p19	eBioscience
Murine IL-12p40	BD Pharmingen
Murine IL-6	BD Pharmingen
Murine IFN-γ	BD Pharmingen
Murine IL-4	BD Pharmingen
Murine IL-5	BD Pharmingen

TABLE 2.1.4 FLUOROCHROME LABELLED ANTIBODIES

SURFACE MARKER	CLONE	FLUORESCENT LABEL	SUPPLIER
CD3ε	145-2C11	APC	BD Pharmingen
	17A2	APC-AlexaFlour 750	eBioscience
γδ	GL3	FITC	BD Pharmingen
CD4	RM4-5	PE-Cy7	eBioscience
CD11c	HL3	APC	BD Pharmingen
	N418	Tri	Caltag
CD40	3/23	PE	BD Pharmingen
CD86	GL1	FITC	BD Pharmingen
CD80	16-10A1	PE	BD Pharmingen
ICAM-1	M17/4	FITC	BD Pharmingen
I-A/I-E (MHC II)	2G9	PE	BD Pharmingen

INTRACELLULAR CYTOKINE	CLONE	FLUORESCENT LABEL	SUPPLIER
IL-17	TC11-18H10	PE	BD Pharmingen
IFNγ	XMG1.2	APC	BD Pharmingen

2.1.7 Buffers and solutions used in the analysis of protein expression by Western blotting

Sample preparation buffer

62.5 mM Tris pH 6.8
2% (w/v) SDS
10% glycerol
0.01% (w/v) bromophenol blue
50 mM DTT (added just prior to cell lysis)

1.5 M Tris-HCl pH 8.8

18.15 g Tris Base
75 ml of dH_20
Made up to 100 ml with dH_2O. Stored at 4°C.
pH to 8.8 with 1 M HCl.

0.5 M Tris-HCl pH 6.8

6 g Tris Base
60 ml of dH_20
Made up to 100 ml with dH_2O. Stored at 4°C.
pH to 6.8 with 1 M HCl.

5X Running buffer

15 g Tris Base
72 g glycine
5 g SDS
Made up to 1 L with dH_20. Stored at 4 °C.

Transfer buffer

0.19 g Tris Base

4.32 g Glycine

60 ml MeOH

0.15 g SDS

Made up to 300 ml with dH_20. Stored at 4 °C

TABLE 2.1.5 COMPONENTS OF STACKING AND RESOLVING GELS

For 4 gels:

SOLUTION COMPONENTS	STACKING GEL	RESOLVING GEL (12%)
dH_20	2.7 ml	6.6 ml
30% (w/v) acrylamide/Bis solution (BIO-RAD)	0.67 ml	8.0 ml
1 M Tris pH 6.8	0.5 ml	
1.5 M Tris pH 8.8		5.0 ml
10% (w/v) ammonium persulphate (APS)	40 µl	200 µl
10% (w/v) SDS	40 µl	200 µl
TEMED	4 µl	8 µl

TABLE 2.1.6 PRIMARY ANTIBODIES FOR WESTERN BLOT ANALYSIS

PROTEIN	1° Ab Dilution	SUPPLIER	2° ANTIBODY
p-ERK (E-4)	1/1000	SC	anti-mouse
c-Fos (H-125)	1/400	SC	anti-rabbit
β-actin	1/1000	S	anti-mouse
c-Jun	1/1000	CS	anti-rabbit
ERK (K-23)	1/1000	SC	anti-rabbit
p-p38	1/1000	CS	anti-rabbit
p-Akt (Thr 308)	1/1000	CS	anti-rabbit
p-GSK3 (Ser 9)	1/1000	CS	anti-rabbit
p-JNK (G-7)	1/1000	SC	anti-mouse

SC = Santa Cruz Biotecnology, INC.

CS = Cell Signalling Technology, INC.

S = Sigma-ALDRICH, INC.

TABLE 2.1.7 SECONDARY ANTIBODIES FOR WESTERN BLOT ANALYSIS

2° ANTIBODY	2° AB DILUTION	SUPPLIER
anti-mouse	1/1000	S
anti-rabbit	1/1000	S

TABLE 2.1.8 REAL TIME RT-PCR PRIMERS

Cytokine	Ref. Seq.	Product Code	Supplier
18S rRNA	X03205.1	4319413E	ABI
IL-1β	NM_008361.3	Mm00434228_m1	ABI
IL-6	NM_031168.1	Mm00446191_m1	ABI
IL-10	NM_010548.1	Mm99999062_m1	ABI
IL-23(p19)	NM_031252.2	Mm01160011_g1	ABI
TNFα	NM_013693.2	Mm00443258_m1	ABI

2.2 METHODS

2.2.1 ANIMALS

Specific pathogen-free BALB/c (H-2^d), C57BL/6 (H-2^d) and SJL/J mice were purchased from Harlan UK Ltd. (Bicester, Oxon, UK), and were maintained according to the regulations and guidelines of the Irish Department of Health and Children. Experiments were performed under license from the Department of Health and with the approval the Trinity College Dublin BioResources Ethics Committee. All mice used were female 6-12 weeks old at the initiation of each of the experiments.

2.2.2 INDUCTION OF EAE

EAE was induced in C57BL/6 or SJL/J mice by subcutaneous (s.c.) injection of 150 μg of myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ (GenScript) emulsified in complete Freund's adjuvant (CFA; Chondrex Inc.) supplemented with 4 mg/ml heat killed H37 Ra *Mycobacterium tuberculosis* (Chondrex Inc). Alternatively, SJL/J mice were immunized s.c. with 150 μg of PLP peptide $_{139-151}$ (GenScript) emulsified in CFA supplemented with 4 mg/ml heat killed H37 Ra *M. tuberculosis*. All mice were injected intraperitoneal (i.p.) with 500 ng pertussis toxin (PT; Kaketsuken, Japan) on days 0 and 2. Animals were monitored daily for signs of clinical disease. Disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis; grade 5 – tetraparalysis/death.

2.2.3 TREATMENT OF MICE WITH A MEK1/2 INHIBITOR

In the MOG-induced EAE model, the MEK1/2 inhibitor U0126 (50 μg/mouse) was administered s.c. to C57BL/6 mice with the MOG and CFA on day 0 and again on days 1 and 2. In the PLP-induced relapsing-remitting EAE model, the MEK1/2 inhibitor U0126 (50 μg/mouse) was administered s.c. to SJL/J mice every 2 d from day 18 post immunization.

2.2.4 DIRECT IMMUNIZATION WITH ADJUVANTS

BALB/c mice were immunized by s.c. injection into the footpad (25 µl/footpad) with PBS, KLH (20 µg/mouse) or KLH with LT, LTK63, LTR72 or LTB (1 µg/mouse). Mice were sacrified after 7 d, spleen and lymph nodes were removed for analysis of T cell responses. Serum samples were also collected for analysis of antibody production.

2.2.5 CELL CULTURE

Cells were cultured in an incubator at 37°C with an atmosphere maintained at 95% humidity and 5% CO_2.

2.2.5.1 CELL COUNTS

Cell counts were performed by diluting cells (usually 1/10 dilution) with trypan blue (Sigma). A 10 µl volume of the cell suspension was then loaded onto a disposable hemocytometer (Hycor Biomedical, UK). The number of viable cells (white) was counted using a light microscope. The number of cells per ml was calculated using the following formula: Number of cells/ml = cell number x 10^4 x dilution factor

2.2.5.2 CULTURING OF THE J558 CELL LINE

The granulocyte-macrophage colony-stimulating factor (GM-CSF) secreting J558 cell line was originally established by cloning the mouse gene for GM-CSF into a mammalian expression vector, which was subsequently transfected into the plasmacytoma line V53-Ag (Karasuyama *et al.*, 1990), and was obtained from Dr. Nathalie Winters (Pausteur Institute, France). The first two passages of the J558 cell line were grown in selection medium (RPMI supplemented with geneticin (G418 sulphate; Gibco BRL)). After the second passage in selection medium, the cells were grown to a 'medium' density, harvested and washed in cRPMI. The cells were then returned to culture for 3-4 days. The supernatant from the cell line was harvested by pelleting the

cells by centrifugation (1200 rpm for 5 min at 4°C). The cells were resuspended in cRPMI medium and cultured for a further 8 passages. Supernatant collected at each passage was pooled and stored at –20°C until GM-CSF was quantified by enzyme linked immunosorbent assay (ELISA) (section 2.2.6.2) and aliquoted into smaller volumes for DC culture (section 2.2.5.3).

2.2.5.3 GENERATION OF MURINE BONE MARROW-DERIVED DENDRITIC CELLS

Bone marrow-derived dendritic cells (BMDC) were generated from BALB/c mice or C57BL/6 mice, using a method similar to that described by Lutz *et al.* (360). Mice were euthanized and their femurs and tibiae removed and dissected from the surrounding muscle tissue. The bone marrow was flushed out with cRPMI medium using a 27 G needle attached to a 20 ml syringe. The cell aggregates were dissociated using a 19 G needle attached to a 20 ml syringe. The cell suspension was pelleted by centrifugation (1200 rpm for 6 min at 4 °C) and cells were resuspended in 2 ml of heated ammonium chloride lysis solution (section 2.1.1.1) for 2 min, to lyse red blood cells. The cells were washed in fresh cRPMI then pelleted by centrifugation (1200 rpm for 6 min at 4 °C) and resuspended in 10 ml of cRPMI medium. Cell viability was assessed by trypan blue method (section 2.2.5.1). Immature BMDC were cultured at 1 x 10^6/ml for BALB/c or 0.4 x 10^6/ml for C57 cells in cRPMI medium supplemented with 20 ng/ml of GM-CSF in the form of supernatant from a GM-CSF expressing J558 cell line. After 3 d incubation, 20 ml of fresh cRPMI containing 20 ng/ml GM-CSF was added to each culture flask.

On day 6, the flasks were gently removed from the incubator and the cell culture supernatant was carefully removed to eliminate contaminating cells (e.g. granulocytes) from the culture. 25 ml of sterile PBS (BioSera), heated to 37°C, was added to each flask and the flasks were gently agitated before the PBS suspension was transferred into 50 ml tubes containing 10 ml of fresh medium. Sterile EDTA (20 ml) (0.02%; Sigma), heated to 37 °C, was added to each culture flask before returning the flask to the incubator for 10 min. Meanwhile, the cells removed in the PBS step were pelleted by centrifugation (1200 rpm for 6 min at 4°C) and resuspended in cRPMI medium. Culture flasks were removed from the incubation and the EDTA solution was pipetted over the layer of remaining

cells, before being added to fresh, warm cRPMI medium and centrifuged at 1200 rpm for 6 min. The cell pellet was resuspended in cRPMI medium and pooled with the pellet obtained in the PBS step. Cells were then recultured at 1 x 10^6/ml for BALB/c or 0.4 x 10^6/ml for C57 cells in cRPMI medium supplemented with 20 ng GM-CSF/ml. After a further 2/3 d of incubation (day 8/9), 20 ml of cRPMI medium, containing 20 ng/ml GM-CSF, was added to each tissue culture flask of cells. After a further 2 d in culture (day 10), the loosely adherent cells were harvested by gentle pipetting of the culture medium. Viability of BMDC was assessed and the cells were cultured at required concentrations (e.g. 1 x 10^6 cells/ml) in tissue culture plates. DC were left overnight in medium containing 10 ng/ml GM-CSF before stimulation with bacterial-derived molecules.

The purity of the BMDC culture was routinely analyzed using flow cytometric analysis. The following antibodies were used to surface stain the cells: DC marker: CD11c; monocyte/macrophage marker: F4/80, CD11b; myeloid differentiation marker: Gr1 (Ly-6G); B cell marker: B220. Typically, the percentage of $CD11c^+$ cells was 70-95 %. A representative plot showing forward scatter and side scatter and a typical $CD11c^+$ population is shown in Appendix Figure 7.1. This method of DC culture results in a very low percentage of contaminating B cells, usually < 1 %. The cell cultures characteristically contained cells that stained positive for F4/80, a macrophage marker, usually 35 – 45 % of the population. Furthermore, ~ 15–25 % of the cells in culture were $Gr1^+$, a protein expressed by the myeloid lineage and often used as a granulocyte and neutrophil marker. Figure 7.2 in the Appendix shows the frequency of these populations in a typical 12 d BMDC culture.

2.2.5.4 Adoptive Transfer of DC

BALB/c BMDC were cultured in medium alone, KLH (20 μg/ul) or KLH (20 μg/ul) with LT, LTK63, LTR72 or LTB (1 μg/ml) for 24 h. Cells were washed twice in PBS and injected s.c. into the footpads of naïve mice (5 x 10^5/mouse). After 7 d popliteal lymph nodes and spleens were removed for assessment of T cell responses.

2.2.5.5 STIMULATION OF ANTIGEN-SPECIFIC CYTOKINE PRODUCTION BY LYMPH NODE AND SPLEEN CELLS *EX VIVO*

Isolated spleen and draining lymph nodes were homogenized and passed through a 70 μm cell strainer (Nunc) to obtain single cell suspensions. Spleen cells were centrifuged at 1200 rpm for 5 min and red blood cells were lysed by resuspending the cell pellet in 1 ml of 0.87% ammonium chloride solution for 2 min. Cells were washed and resuspended in fresh cRPMI-1640 medium. Spleen mononuclear cells (2×10^6) or lymph node cells (1×10^6) were cultured in triplicate wells of 96-well 'U bottomed' microtitre plates at 37°C and 5% CO_2 with various concentrations of antigen, or with the mitogens PMA (25 μg/ml; Sigma) and anti-mouse CD3 (1 μg/ml; Pharmingen, SanDiego, USA) or medium only as positive and negative controls respectively. Supernatants were collected for analysis of cytokine production by ELISA. The frequency of cytokine producing cells was assayed by intracellular staining with anti-cytokine antibodies following exposure to Brefeldin A for final 4-6 h of culture.

2.2.5.6 *IN VITRO* STIMULATION OF BMDC

Precise conditions for stimulation of innate responses by DC *in vitro* are outlined in each figure legend. In brief, studies examining the effect of LT, LTK63 or LTB (1 μg/ml) on cytokine production, BMDC (5×10^5/ml) were cultured in 96-well tissue culture plates (Nunc) at 37°C, and supernatants were removed at various time points. In the investigation of the immunomodulatory properties of LT, LTK63, LTR72 or LTB on PRR-induced cytokine production, BMDC were pre-treated with LT, LTK63, LTR72 or LTB (1 μg/ml), with or without prior incubation in the presence of various inhibitors, before stimulation with PRR agonists. Supernatants were removed after various time points and analyzed for cytokine concentrations by ELISA using pairs of antibodies (Table 2.1.1) as described in the following sections. For analysis of cytokine mRNA expression, BMDC (1×10^6 cells/ml) were cultured with LT, LTK63, LTR72 or PRR agonists for various periods of time. Total RNA was isolated from BMDC by lysing the cells in TRIzol (Invitrogen) reagent and storing the lysates in sterile RNase-free tubes at -

20°C.

For analysis of protein by Western blot, BMDC (1 x 10^6/ml) were cultured in 6-well tissue culture plates (3 ml/well) and supernatants were removed at appropriate time points. Sample preparation buffer was added to the cells (100 µl/ 1 x 10^6 cells). In experiments investigating DC maturation in response to LT, LTK63 or LPS, BMDC (1 x 10^6/ml) were cultured in 24-well plates for 24 h before the cells were gently removed, washed and used for immunofluorescence analysis (Section 2.2.7). Where required, BMDC were pre-treated with inhibitors for 30 min or 1 h prior to incubation with other stimuli.

2.2.6 Analysis of immune responses

2.2.6.1 Measurement of proliferative responses of lymph node and spleen cells

Antigen and mitogen induced T cell proliferation was assessed by monitoring ^{3}H thymidine incorporation into newly synthesized DNA. Briefly, after the removal of supernatants for analysis of cytokine concentration, lymph node or spleen cells were pulsed with 0.5 µCi of [^{3}H]-thymidine/well (Amersham, GE Healthcare Life Sciences, UK) diluted in fresh, warm cRPMI medium. After 6 h incubation at 37°C, cells were harvested onto glass fibre filter mats (Wallac) with an automatic cell harvester. Once completely dry, the filter mats were sealed in plastic sample bags with 5 ml of non-aqueous scintillation fluid (BetaScint, Wallac). [^{3}H]-thymidine incorporation was detected by liquid scintiallation counting using a micro-β-counter (Wallac). Results are expressed as mean counts per minute (CPM) of [^{3}H]-thymidine incorporation for triplicate cultures of spleen cells or lymph node cells.

2.2.6.2 Measurement of cytokine production

IL-12p40, IFN-γ, IL-4 and IL-5 ELISA

IL-12p40, IFN-γ, IL-4 and IL-5 were detected by ELISA using commercially available antibodies (PharMingen) as described in Table 2.1.1. The IL-12p40 antibody recognises IL-12p40 either as a monomer, dimer, or as part of the IL-12p70 or as part of the IL-23 heterodimer. High binding certified ELISA plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of purified rat anti-mouse IL-5, IL-4 and IFN-γ (1 µg/ml) or a purified anti-IL-12p40/p70 antibody at a concentration of 1 µg/ml. After washing (1X PBS/0.05% Tween 20), non-specific binding sites were blocked by adding 200 µl/well of blocking solution (10% (w/v) dried milk (Marvel) in PBS) at room temperature for 2 h. Plates were washed and 50 µl/well of test supernatant (diluted 1/300 in PBS for IL-12p40 or diluted 1/2 in PBS for IL-4, IL-5 and IFN-γ) or a serial dilution of the top working standard recombinant protein for each cytokine, (IL-4 - 2500 pg/ml, IFN-γ - 10 ng/ml, IL-5 – 2500 pg/ml and IL-12p40 – 5 ng/ml; all diluted in PBS), was added to the plates and left to incubate overnight at 4°C. The plates were washed and incubated for 1 h at room temperature with 50 µl/well biotinylated rat anti-mouse IL-12p40, IL-5, IL-4 or IFN-γ detection antibodies (all 1 µg/ml) diluted in PBS. Plates were washed again and incubated with 50 µl/well HRP-conjugated strepavidin (BD Pharmingen, 1:1000 in PBS) for 30 min at room temperature in the dark. Finally, plates were washed and incubated with 50 µl/well of OPD substrate in phosphate citrate buffer (0.4 mg/ml). The enzyme reaction was quenched by the addition of 25 µl/well 1M H_2SO_4. Absorbance was read at 492 nm on a Versamax Tunable Microplate Reader (Molecular Devices). Cytokine concentrations in the test supernatants were determined by reference to a standard curve, prepared using recombinant murine IL-12p40, IL-5, IL-4 or IFN-γ of known concentration.

IL-17, IL-1β, IL-10, IL-12p70 AND TNF-α ELISA

Cytokine concentrations of IL-17, IL-1β, IL-10, IL-12p70 and TNF-α were measured using commercially available ELISA kits (R&D systems) (Table 2.1). High-binding certified 96-well microtiter plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of rat anti-mouse IL-17 (2 µg/ml), IL-1β (4 µg/ml), IL-10 (2 µg/ml), IL-12p70 (4 µg/ml), TNF-α (0.8 µg/ml) capture antibody in PBS. After washing, non-specific binding sites were blocked by addition of 200 µl/well of blocking solution (1% (w/v) BSA in PBS) for 2 h at room temperature. Plates were washed and 50 µl/well of test supernatant or serially diluted top working concentration of each standard, IL-17 (1500 pg/ml), IL-1β (1000 pg/ml), IL-10 (2000 pg/ml), IL-12p70 (1500 pg/ml), TNF-α (2000 pg/ml) diluted in 1% BSA/PBS solution were added and plates were incubated overnight at 4°C. Test supernatants for IL-17, IL-10 and IL-12p70 were added neat to the plates, while those for TNF-α were diluted 1:2 and IL-1β 1:5 in 1% BSA/PBS. Plates were washed and incubated for 2 h at room temperature with 50 µl/well biotinylated goat anti-mouse IL-17 (200 ng/ml), IL-1β (100 ng/ml), IL-10 (400 ng/ml), IL-12p70 (400 ng/ml) or TNF-α (150 ng/ml) detection antibodies diluted in 1% BSA/PBS. Plates were washed and incubated with 50 µl/well HRP-conjugated strepavidin (R&D, 1:200 in 1% BSA/PBS) for 30 min at room temperature in the dark. Plates were washed and 50 µl/well of OPD substrate in phosphate citrate buffer (0.4 mg/ml) was added to each well. The enzyme reaction was quenched by addition of 25 µl/well of 1 M H_2SO_4 and absorbance was read at 490 nm on a Versamax Tunable Microplate Reader (Molecular Devices). Cytokine concentration in test supernatants was determined by reference to the standard curve, prepared using recombinant mouse IL-17, IL-1β, IL-10, IL-12p70 or TNF-α of known concentration.

IL-23p19 ELISA

Cytokine concentrations of IL-23p19 were measured using a commercially available ELISA kit (eBioscience). High binding certified 96-well microtitre plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of purified anti-mouse IL-

23 (2 μg/ml) capture antibody in eBioscience coating buffer. Plates were washed in wash buffer (PBS/0.05% Tween 20) and non-specific binding sites blocked by addition of 200 μl/well of blocking buffer (eBioscience assay diluent) for 2 h at room temperature. Plates were washed thoroughly before the addition of 50 μl per well of sample supernatant (diluted 1/2 in assay diluent) or an eight point two-fold serial dilution from the top working standard recombinant protein for IL-23p19 (4000 pg/ml in eBioscience assay diluent). Plates were incubated overnight at 4°C. Plates were washed five times and incubated for 1 h at room temperature with 50 μl/well biotinylated anti-mouse IL-23p19 detection antibody (2 μg/ml) diluted in eBioscience assay diluent. Plates were washed five times, leaving the plates to soak for at least 2 min between each wash, and incubated with 50 μl/well HRP-conjugated strepavidin (eBioscience, 1:250 in eBioscience assay diluent) for 30 min at room temperature in the dark. Plates were washed seven times, soaking the wells for 2 min between washes, and 50 μl TMB substrate solution (eBioscience) added to each well. The enzyme reaction was quenched by addition of 25 μl stop solution (1M H_2SO_4) per well and absorbance was read at 450 nm on a Versamax Tunable Microplate Reader (Molecular Devices). Cytokine concentration in test supernatants was determined by reference to the standard curve, prepared using recombinant mouse IL-23p19 of known concentration.

GM-CSF ELISA

GM-CSF concentration of the J558 cell line supernatant was measured using a commercially available ELISA kit (R&D systems) (Table 2.1.1). High-binding certified ELISA plates (Greiner Bio-one) were coated overnight at 4°C with 50 μl/well of rat anti-mouse GM-CSF antibody (2 μg/ml) capture antibody in PBS. After washing, non-specific binding sites were blocked by adding 200 μl/well of blocking solution (1% BSA/5% sucrose in PBS) and incubating plates for 2 h at room temperature. Plates were washed and 50 μl/well of test supernatant (neat or at various dilutions in 1% BSA/ PBS) or serially diluted GM-CSF standard (0-2000 pg/ml) in 1% BSA/PBS solution were added and plates were incubated overnight at 4°C. After washing, plates were incubated with 50 μl/well of biotinylated goat anti-mouse biotinylated GM-CSF (50 ng/ml), in 1% BSA/PBS were added for 2 h at room temperature. Plates were washed and GM-CSF

was detected by incubating plates in the dark for 30 min with 50 µl/well of horseradish-peroxidase (HRP) conjugated streptavidin (1:200 in 1% BSA/PBS) followed by washing and adding 50 µl/well of OPD substrate in phosphate citrate buffer (0.4 mg/ml). The colour reaction was allowed to develop for approximately 10 min after which time 25 µl/well of 1 M H_2SO_4 was added to stop the enzymatic reaction. The OD values were determined by measuring absorbance at 492 nm using a microtiter plate reader and cytokine concentrations of test samples were evaluated using a standard curve prepared using recombinant mouse GM-CSF of known concentration.

2.2.6.3 Determination of serum total IgG and IgG subclass titres

Serum samples were prepared from peripheral blood removed from the thoracic cavity of mice after sacrifice. Blood was allowed to clot for 2 h at room temperature and then placed at 4°C for 2-4 h. The samples were centrifuged for 8 min at 12,000 g and the serum was transferred into fresh tubes and stored at -20°C. The levels of antigen specific IgG antibodies in sera of immunized mice were determined by ELISA. KLH (5 µg/ml in PBS) was added to flat-bottomed 96-well microtitre plates and incubated overnight at 4°C. Excess antigen was removed by washing the plates with wash buffer (PBS/0.05% Tween 20) and non-specific binding sites were blocked by incubating the plates with PBS supplemented with 10% (w/v) of dried milk (Marvel) for 2 h. Plates were washed and the sera were serially diluted in PBS to determine the endpoint titres starting at a dilution of 1/50 or 1/100. The plates were incubated with serum samples overnight at 4°C. After washing, plates were incubated for 1 h at room temperature with 50 µl/well of a rat anti-mouse IgG monoclonal antibody (Sigma) (1/1000 dilution in PBS), with specificity for the whole IgG molecule or 50 µl/well of a 1/10,000 dilution of either biotin conjugated IgG2a (0.05 µg/ml) or IgG1 (0.05 µg/ml). Plates were washed again and incubated for 30 min at room temperature with 50 µl/well of HRP-conjugated streptavidin at a 1/1000 dilution in PBS. The plates were then washed and 50 µl of OPD in phosphate citrate buffer (pH 5.0; 0.4 mg/ml) was added to each well. The enzyme reaction was stopped with 25 µl 1M H_2SO_4. The absorbance was measured at 492 nm. Results are expressed as $\log_{10}$ endpoint antibody titres.

2.2.7 FLOW CYTOMETRY – FLUORESCENCE ACTIVATED CELL SORTING

2.2.7.1 SURFACE STAINING

BMDC co-stimulatory molecule expression was analyzed by flow cytometry using a FACS Caliber™ flow cytometer (Becton-Dickinson, San Jose, CA). The FACS was calibrated using the Autocomp software in conjunction with commercially prepared fluorescent beads (Calibrate beads, Becton-Dickinson). Cells were harvested from wells in 1 ml of FACS buffer (2% FCS, 0.1% NaN_3 made up in PBS) and pelleted by centrifugation. Cell pellets were resuspended in 1 ml block buffer and left on ice for 20 min. The cells were then washed twice in FACS buffer and transferred to FACS tubes (Falcon) in 100 µl of FACS buffer. Cells were incubated for 30 min with biotin-conjugated antibodies (PharMingen) (Table 2.1.4) and isotype control antibodies for 20 min in the dark. Cells were washed and resuspended in 100 µl of FACS buffer and immunoflourescence analysis was performed. The results were analysed using CELLQuest™ software using a FACS Caliber™ flow cytometer (Becton-Dickson, San Jose, CA). 50,000 cells were analysed per sample, unless otherwise stated.

In some cases, analysis of samples was performed using a CyAn ADP flow cytometer (DakoCytomation). The flow cytometer was calibrated using the compensation function in the Summit software in conjunction with BD Comp Beads (BD Biosciences). Results were analyzed using Summit (DakoCytomation) or FloJo (Stanford University) software.

2.2.7.2 INTRACELLULAR STAINING

Isolated cells were restimulated at 37°C/5% CO_2 with PMA (10 ng/ml), ionomycin (1 µg/ml), and cytokine secretion blocked with Brefeldin A (5 µg/ml). Cells were then washed, non-specific binding blocked by incubation with 1 µg/ml anti-CD16/CD32 (Fcγ Block; BD Pharmingen) for 20 min and stained for surface markers (Table 2.1.4). Cells were fixed and permebilized with Caltag Fix & Perm Kit, and stained for intracellular cytokines with anti-IFNγ (APC) and anti-IL-17 (PE). Flow cytometric

analysis was performed using a CyAn ADP flow cytometer (DakoCytomation). The flow cytometer was calibrated using the compensation function in the Summit software in conjunction with BD Comp Beads (BD Biosciences). Results were analyzed using Summit (DakoCytomation) or FloJo (Stanford University) software.

2.2.7.3 VIABILITY STAINING

Cell viability was assessed by addition of propidium iodide directly to the samples (final concentration 1-5 μg/ml) immediately prior to acquisition on the FACS machine. Non-viable cells fluoresce in the FL3 channel.

2.2.8 WESTERN BLOT ANALYSIS

2.2.8.1 PREPARATION OF WHOLE CELLS LYSATES

Cells were plated in 6 well plates at 1 x 10^6 cells/ml, 3 ml per well, and left overnight in an incubator. After appropriate stimulation (as indicated in figure legends) supernatants were removed using a transfer pipette. Cells were lyzed in 100 μl 1x sample buffer (See 2.1.7) using the barrel of a 1 ml syringe the cells were carefully scraped from the wells and the lysates were transferred to 500 μl flip top eppendorph tubes on ice. Samples were boiled (95-99 °C) in a heating block for 5 min and then frozen at -20°C.

2.2.8.2 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The separating/resolving gel was prepared and poured in between two glass plates and the gel was allowed to set. The stacking gel was carefully poured on top of the set separating gel and a comb immediately inserted in between the plates. The set gels were used straight away or left overnight at 4 °C. The plates were placed in the gel rig before the addition of running buffer. 10 μl of sample was loaded into the required lanes, keeping one lane for 5 μl of a molecular weight marker (Precision Plus Protein Standards, Bio-Rad). The gels were run at 120V until the gel front was coming near the end of the glass plate.

2.2.8.3 TRANSFER OF PROTEINS TO NITROCELLULOSE MEMBRANE

The transfer of proteins from the gel to a nitrocellulose membrane was carried out using a wet transfer system. Gels were gently removed from between the glass plates and kept moist using transfer buffer. Gels were then placed on nitrocellulose between layers of moist filter paper. The apparatus for transferring the gel was wiped down with transfer buffer before the 'gel sandwich' was placed on the apparatus, making sure that there were no trapped air bubbles. The gels were transferred at 180 mA for 2 h.

2.2.8.4 DETECTION

Membranes were blocked using blocking buffer (5% (w/v) non-fat dried milk (Marvel) in 1x PBS Tween or 5% (w/v) BSA) for 1 h at room temperature on a rocker. Blots were then rinsed twice in PBS/Tween. Membranes were incubated at 4°C overnight with the appropriate primary Ab according to the manufacturer's specifications (Table 2.1.6). Membranes were washed in PBS-Tween for 2 x 5 min followed by 2 x 15 min. Membranes were incubated with the specified secondary Ab for 1 h at room temperature on a rocker (Table 2.1.7). Membranes were then washed as before in PBS-Tween. Blots were developed by chemiluminesence (LiteAblot, Euroclone) according to the manufacturer's instructions.

2.2.9 REAL TIME QUANTITATIVE RT-PCR

Total ribonucleic acid (RNA) was extracted from BMDC using the TRIzol/chloroform method. Briefly, BMDC were collected in TRIzol (1 ml/10 cm^2) reagent in sterile RNase-free tubes. 200 µl chloroform was added to each tube, vortex mixed, stood at room temperature for 3 min, and then centrifuged at 12,000 rpm for 15 min. The resulting upper aqueous phase was removed to sterile RNase-free tubes, and 500 µl isopropanol added, mixed and stood at room temperature for 15 min, before centrifugation at 12,000 rpm for 15 min. Supernatants were decanted and the pellet washed twice (7,600 rpm for 3 min) in 1 ml 75% ethanol in nuclease-free H_2O. Finally, remaining ethanol was removed with a pipette and the tubes inverted and allowed to air-

dry for 10 min. RNA was resuspended in 80 µl nuclease-free H_2O, incubated on ice for 10 min, heated to 65°C for 10 min and then stored on ice for 20 min. RNA concentration was determined spectrophotometrically using an Eppendorf BioPhotometer 6131 (Eppendorf) by diluting 2 µl RNA in 98 µl nuclease-free H_2O and reading the absorbance at 260 nm and determining the 260/280 nm ratio. 1 µg of each RNA sample was reversed transcribed into cDNA using the QuantiTect Reverse Transcription kit (Qiagen), according to the manufacturer's protocol, and the cDNA reverse transcription product diluted 1:5 with nuclease-free H_2O. Transcripts were quantified by real time quantitative PCR on an ABI 7500 Fast Real Time PCR System with Applied Biosystems predesigned Taqman Gene Expression Assays and reagents according to the manufacturer's instructions. For each sample, mRNA abundance was normalized to the amount of 18S ribosomal RNA (rRNA) and is expressed as fold difference compared to DC incubated in cRPMI only.

2.2.10 Statistics

Statistical analyses were performed using the computer-based mathematical package GraphPad InStat 3 or GraphPad Prism software. Statistical differences in mean cytokine values between experimental groups were determined by ANOVA or student's *t* test as indicated in figure legends. P-values of 0.05 or less were considered significant.

Chapter 3

Adjuvant activity of LT

3.1 INTRODUCTION

A move towards safer subunit vaccines in the past decade has increased the need for potent and safe adjuvants to enhance immunogenicity of purified native or recombinant antigens. A central requirement of an effective adjuvant is the activation of the innate immune system, which directs the induction of the adaptive immune response. *E.coli* heat-labile enterotoxin (LT) is one of the most potent mucosal adjuvants available. Co-administration of antigen with LT by injection via the nasal, oral or other mucosal routes potently enhances antigen-specific IgA and IgG and cellular immune responses (192-195, 203).

LT is composed of two subunits, an enzymatically active A subunit with ADP-ribosyltransferase activity, which is responsible for the toxicity, and a B subunit that mediates binding of the molecule to receptors on eukaryotic cell surfaces (191). The A subunit comprises the globular (enzymatically active) A1 domain and an A2 domain, whose primary function is to interact with the B subunit. The A1 fragment enters the cell cytosol and ADP-ribosylates the stimulatory α subunit of a GTP-binding protein (Gsα), which causes permanent activation of adenylate cyclase, resulting in elevation of intracellular cAMP concentration. The induction of cAMP by LT has been proposed as a dominant factor in the adjuvant effects of the toxin (246, 268). However, reports that site-directed mutants and the isolated B subunit (LTB) retain immunomodulatory properties indicate that other factors are also involved (217, 285, 361, 362).

Although LT has been used clinically, it is generally accepted that non-toxic derivatives may be safer as adjuvants for human vaccines. Derivatives of the toxin with reduced toxicity have been generated, and these site-directed mutants with reduced or no enzymatic activity also provide useful tools to elucidate the mechanism of action of LT (207, 216, 245). Studies in animal models using the partially active LT derivative, LTR72, and the enzymatically inactive derivative, LTK63, have shown that these molecules retain at least some of the adjuvant properties of the wild-type toxin (195, 207, 215, 216, 231, 285, 363). These findings clearly indicate that the immunomodulatory properties associated with LT are not entirely dependent on ADP-ribosyltransferase activity. Most studies indicate mucosal delivery of CT, a highly related AB toxin,

induces a biased Th2 type response to itself and to bystander antigens, characterized by $CD4^+$ T cell production of IL-4, IL-5, IL-6 and IL-10 (203-205) and IgG1, IgA and IgE antibodies (203, 205). In contrast, mucosal delivery of LT has been shown to induce a more mixed Th1/Th2 type response, with the production of antigen-specific IL-4, IL-5 and IFN-γ and IgG antibodies of the IgG1, IgG2a and IgG2b subclasses (193, 206-208). Adjuvant studies with derivatives of the toxins have shown that they can induce mixed T cell responses but these findings are likely to be influenced by a number of factors, particularly the dose and the route of administration. The partially enzymatically active LTR72 was shown to induce Th1 and Th2 responses and IgG1 as well as IgG2a antibody production. An enzymatically inactive mutant, LTK63, promoted a predominant Th2 response, with higher IgG1 than IgG2a antibody production (195, 215, 216).

Innate immunity is central to protection against infectious diseases and determines the nature of the adaptive immune response that is induced. DC are major mediators of innate immune responses and the only cells capable of acting as APCs for naïve T cells (72). Therefore, the activation of innate immune responses, and as a consequence adaptive immune responses, by adjuvants may be mediated in part by their interaction with DC. The differentiation of naïve Th cells is determined by three signals that are provided by DC. Initial activation of T cells is dependent on two signals; signal 1 is provided by the interaction of the antigen-specific TCR with peptide-MHC, and signal 2 by interactions between co-stimulatory molecules, such as CD28 on T cells with CD80 or CD86 on APCs (86). It has been proposed that in the absence of signal 2, Th cells become anergic, which can lead to tolerance (364). Binding of the co-stimulatory ligands CD80 and CD86 to the T cell co-stimulatory receptors CD28 is essential for the activation and regulation of T cell immunity (365). CD80 and CD86 also engage CTLA4, a negative regulator of T cell activation (366). The co-stimulatory molecule ICOS (inducible costimulator) is expressed only on activated T cells and binds to B7-H2 on APCs, this interaction is important for IL-10 production and the polarization of Th2 cells (87). Signal 3 is provided primarily by cytokines, produced by cells of the innate immune system, acting on T cells to determine their differentiation into different effector cells, including Th1, Th2, Th17 or Treg cells. The cytokines produced by TLR-stimulated DC and macrophages are instrumental in shaping antigen-specific T cell responses.

Pathogen-derived molecules have the capacity to activate T cells and direct their differentiation, by providing signals 1, 2 and 3, through DC activation.

CT, LT and their derivatives, have been shown to promote DC maturation alone or in the presence of additional stimuli, such as LPS. CT enhanced CD80 and CD86 expression on B cells (262), murine Flt3L-expanded DC (267), human blood-monocyte DCs (268) and on murine BMDC (269). In contrast, other studies have shown that CT enhances CD86, but not CD80, expression by bone marrow-derived macrophages (270) and by macrophages and B cells obtained from PP (205). LT has been reported to selectively up-regulate the expression of CD80, but not CD86, on BMDC (210) and murine macrophages (272). Furthermore, the partially enzymatically active LT derivative, LTR72, and the enzymatically inactive derivative, LTK63, also enhanced CD86 expression on murine macrophages (272).

The importance of enhancing CD86 expression by the toxins was demonstrated by the finding that anti-CD86 monoclonal antibody blocked the ability of LT- or CT-treated APCs to induce proliferation of anti-CD3–triggered $CD4^+$ T cells (271). Furthermore, administration of an anti-CD86 monoclonal antibody inhibited KLH-specific antibody responses in mice orally immunized with KLH and CT (270). Blocking CD80/CD86 co-stimulation using CTLA4-Hγ1 transgenic (Tg) mice, resulted in severely impaired specific mucosal and serum IgA responses, following oral immunizations using CT as an adjuvant (367). Studies in CD28-deficient mice revealed that these mice had normal gut IgA responses but impaired systemic responses (368). These findings demonstrated that CD28 signalling was an essential component of systemic antibody responses, but that the enhancement of gut IgA responses by CT was independent of signalling through the B7-CD28 pathway. In contrast to its effects on CD80 and CD86 expression, CT inhibits CD40 and ICAM-1 on BMDC (121). This contrasts the effect of LPS, CpG and poly I:C, which enhance the expression of co-stimulatory molecules (369). Moreover, CT enhanced LPS-induced CD80, but inhibited CD40 and ICAM-1 expression (121).

GM1-binding has been shown to be essential for upregulation of co-stimulatory molecule expression by the toxins (273, 370). Incubation of native CT or an enzymatically inactive mutant (CT E112K) with GM1-ganglioside suppressed their ability to upregulate CD80 and CD86 expression on macrophages and B cells (273). However, it was reported that while CT, alone or together with TNF-α and IL-1β, induced

co-stimulatory molecule expression on DC, CTB did not (268), suggesting that the A subunit of the toxin was required to induce DC maturation. Studies with the CTA1-DD, a fusion of the intact CT A1 subunit with a dimmer of an Ig-binding fragment D of *S. aureus* protein A, suggested that potent adjuvant activity could be mediated via a ganglioside-GM1 receptor-independent pathway (371). The ability of CT and LT to upregulate co-stimulatory molecule expression has also been attributed to the enzyme activity of the toxins. Bagley *et al.* reported that toxin-mediated maturation of human monocyte-derived DC was cAMP-dependent (274), while rCTB failed to enhance CD86 expression on macrophages (270, 276). Furthermore, cAMP elevating agents, including d-cAMP and forskolin, have been shown to upregulate CD80 and CD86 on human DC (274) and to enhance CD86 expression on macrophages (270).

In contrast to the ability of the holotoxins to promote DC maturation, CT and LT alone do not stimulate DC to secrete cytokines or chemokines, with the exception of MIP-2, but can modulate the response to other pathogen-derived molecules (121, 268, 269, 277). CT and LT suppress LPS-induced pro-inflammatory cytokine and chemokine production in human monocytes and DC (268, 277), J774 macrophages (272) and murine BMDC (121, 269). It was demonstrated that CT not only inhibited LPS-induced IL-12p40, IL-12p70, TNF-α, MIP-1α, and MIP-1β production from murine DC, but also enhanced IL-10, IL-1β and IL-6 production. The inhibitory effects of CT were independent of IL-10 (269).

While it is well established that LT acts as a strong adjuvant, capable of promoting the induction of Th1 and Th2 responses, the ability of LT to promote the induction of Th17 cells has yet to be examined. Therefore, one of the aims of the present study was to investigate the ability of LT to promote the induction of Th17 cells. There is limited knowledge about the mechanism through which the enterotoxins LT and CT exert their immunomodulatory effects, especially the mechanism of activation of DC. Since much of the published work points to a role for elevation of intracellular cAMP concentration in the adjuvanticity of the toxins, one of the aims of this chapter was to examine the role of the ADP-ribosyltransferase activity in the ability of LT to promote the induction of T cell responses. Furthermore, this study set out to determine the specific role of the enzymatically active A subunit and the B subunit in mediating the modulatory effects of LT on DC cytokine production.

3.2 RESULTS

3.2.1 LT, LTK63 and LTR72 are strong vaccine adjuvants

LT has been shown to act as an adjuvant when delivered with antigen via the subcutaneous, intraperitoneal, intravenous, intradermal and transcutaneous routes (197-199). Mucosal delivery of LT has been shown to induce a mixed Th1/Th2 type response, with the production of antigen-specific IL-4, IL-5 and IFN-γ and IgG antibodies of the IgG1, IgG2a and IgG2b subclasses. However, the ability of LT to promote Th17 cells has not been investigated. This study examined the adjuvant effect of LT delivered by s.c. injection and assessed the role of the enzymatic activity using the site-directed mutants LTK63 and LTR72 and a recombinant subunit, LTB. BALB/c mice were immunized s.c. into the footpad with PBS alone, KLH (20 μg/mouse) or KLH with LT, LTK63, LTR72 or LTB (1 μg/mouse) and the cellular immune responses were assessed in popliteal lymph nodes 7 d later.

Immunization with KLH alone did not induce any cytokine production from lymph node cells following antigen stimulation *ex vivo*. Immunization with antigen and LT or LTR72 induced antigen-specific IL-17 production by lymph node cells (Fig. 3.1). Moreover, LTK63 induced KLH-specific IL-17, but to a much lesser extent than LT or LTR72. This suggests that while LTK63 promoted the induction of IL-17-producing T cells, the enzyme activity of LT and LTR72 plays a major role in the induction of Th17 cells. Lymph node cells from mice immunized with antigen and LTR72 produced more KLH-specific IFN-γ than mice immunized with antigen in the presence of wild-type toxin (Fig. 3.1). LTK63 promoted a comparable KLH-specific IFN-γ response to LT-immunized mice, but less than LTR72. While LT promoted the induction of the most potent KLH-specific IL-10 response, LTK63 and LTR72 also promoted KLH-specific IL-10. Similarly, immunization with antigen and LT promoted the induction of antigen-specific IL-4 and IL-5 production by lymph node cells, which was greater than that induced by immunization with antigen and LTR72 or LTK63. Furthermore, mice immunized with KLH and LT, LTK63 or LTR72 had strong KLH-specific proliferative responses in lymph node cells compared to mice immunized with KLH alone. The

isolated B subunit of LT, LTB, did not promote the induction of KLH-specific IL-17, IFN-γ or IL-4 responses but marginally increased KLH-specific IL-10 and IL-5 compared with that induced by KLH alone (Fig. 3.1).

LT promoted robust KLH-specific IgG1 and IgG2a antibody production (Fig. 3.2). The KLH-specific IgG1 titres in mice immunized with antigen and LTR72 were only slightly less than that induced by LT, but LTR72 promoted greater IgG2a production compared with LT. LTK63 also promoted KLH-specific IgG1 and Ig2a, albeit to a lesser extent than LT or LTR72. LTB also promoted antigen-specific IgG1 and IgG2a, responses but the antibody titres were less than those induced by LT.

Therefore, parenteral immunization with LT promotes mixed Th17, Th1 and Th2 type responses. Comparing the effects of the site-directed mutants of LT at the same concentration may not be reflective of their adjuvanticity. However, the results show that the partially enzymatically active derivative, LTR72, can also induce a strong Th17 and Th1 response, but a less robust Th2 response compared to wild-type toxin. In this study, LTK63 promoted a weak Th17 response but immunization with LTK63 resulted in IFN-γ production by lymph node cells comparable to LT and also promoted the induction of a Th2 response comparable to LTR72.

3.2.2 LT, LTK63 and LTR72 modulate the activation status of DC to promote the induction of antigen-specific T cell responses

The mechanism of adjuvant action of LT, and its highly related family member CT, has long been controversial. Since DC are the most effective APC and the only cell capable of activating naïve T cells, the ability of LT to activate innate immune responses, and as a consequence adaptive immune responses, may be mediated in part by their interaction with DC. Therefore, the next study examined the role of DC in mediating the adjuvant effects of LT and its derivatives. BALB/c BMDC were pulsed with KLH (20 μg/ml) alone or KLH with LT, LTK63, LTR72 or LTB (1 μg/ml) *in vitro* for 24 h and transferred s.c. to naïve BALB/c mice. Antigen-specific T cell responses were assessed 7 d later from lymph node cells stimulated with antigen *ex vivo*.

DC pulsed with antigen in the presence of LT induced antigen-specific IL-17 production when transferred *in vivo* (Fig. 3.3). The induction of KLH-specific IL-17

production *in vivo* by DC that were pulsed with KLH and LTK63 was less than that induced by LT. DC stimulated with KLH and LTR72 promoted the induction of the most potent KLH-specific IL-17 response compared with LT or LTK63. Immunization with DC pulsed with antigen and LT promoted the induction of KLH-specific IFN-γ production by lymph node cells. Injection of DC pulsed with antigen and activated with LTK63 or LTR72 also induced KLH-specific IFN-γ production, but to a lesser extent than LT. DC stimulated with LTK63 promoted a strong KLH-specific IL-10 response, while LT- or LTR72- pulsed DC did not increase IL-10 production above the concentration induced by DC stimulated with KLH alone (Fig. 3.3). There was no KLH-specific IL-4 or IL-5 detected in lymph node cells of mice immunized with DC pulsed with LT or LTR72 (Fig 3.3). However, LTK63 induced KLH-specific IL-4 and to a lesser extent IL-5 production by lymph node cells. LT-, LTK63- and LTR72- pulsed DC also increased proliferation of lymph node cells above the level induced by DC stimulated with KLH alone (Fig. 3.3). DC pulsed with antigen and LTB did not promote KLH-specific T cell cytokine production, but did increase the proliferative response of lymph node cells compared to KLH-pulsed DC (Fig. 3.3).

In conclusion, DC stimulated with antigen and LT or LTR72 promoted Th1 and Th17 biased responses, while DC stimulated with antigen and LTK63 induced a mixed Th1/Th2/Th17 response. Overall, these data demonstrate that the ability of LT, LTK63 and LTR72 to modulate the induction of an antigen-specific T cell response *in vivo* appears to result, at least in part, from their immunomodulatory effects on DC.

3.2.3 LT and LTK63 do not stimulate DC cytokine secretion alone, but can induce IL-1β, IL-6 and IL-10 mRNA expression

Having established that DC play a significant role in mediating the adjuvant effect of LT, the next study investigated the ability of LT to induce DC cytokine and chemokine production *in vitro*. In addition, to determine if the effects of LT are mediated by its ADP-ribosyltransferase activity, the effect of LTK63, a derivative of LT that lacks the enzyme activity associated with the native toxin, was also examined and the responses to both were compared with those induced by the TLR agonist, LPS.

LPS stimulated the production of IL-12p70, IL-12p40, TNF-α, IL-10, IL-1β and MIP-2 by DC (Fig. 3.4). In contrast, neither LT nor LTK63 induced the production of IL-12p70, IL-12p40, IL-10, IL-1 or TNF-α, but LT did induce the production of the chemokine MIP-2, to a level comparable to that induced by LPS (Fig. 3.4). In contrast, LTK63 did not induce MIP-2, suggesting that LT-induced MIP-2 production is dependent on the enzyme activity of the toxin.

In comparison with LPS, LT or LTK63 had little effect on cytokine mRNA expression. Incubation of DC with LT for 2 h increased IL-1β and IL-6 mRNA expression but this effect was transient and was not evident when DC were stimulated with LT for 13 h (Fig. 3.5). LTK63 also induced IL-6 and IL-1β mRNA expression, albeit to a lesser extent than LT, but only when DC were incubated for 13 h or more (Fig. 3.5). However, LPS increased IL-6 mRNA by ~ 1200-fold after 12 h while the maximum fold increase observed in response to LT or LTK63 was ~ 20-fold. Likewise, the induction of IL-1β mRNA expression by LT was much less than that induced by LPS, 10-fold as compared with ~140-fold. LT and LTK63 induced an ~ 20 fold increase in IL-10 expression when DC were incubated for 1 and 12 h respectively (Fig. 3.5). Similar to IL-1β and IL-6 expression, IL-10 mRNA expression after 24 h stimulation had returned to the level expressed in unstimulated DC. In contrast to the strong increase in IL-12p40 mRNA expression by LPS, LT and LTK63 had little effect IL-12p40 expression.

3.2.4 LT and LTK63 upregulate CD80 and CD86 and downregulate CD40 and ICAM-1 expression on the surface of DC

Mature DC express high levels of MHC molecules and co-stimulatory molecules, including CD86, CD80, CD40 and CD83, and expression of these molecules is one of the key signals that enables DC to activate naïve T cells (72). To examine the effects of LT and LTK63 on DC maturation, bone-marrow derived immature DC were cultured for 24 h with LT (1 μg/ml), LTK63 (1 μg/ml) or LPS (10 ng/ml), as a positive control, and analysed for surface expression of co-stimulatory molecules by immunofluorescence analysis with antibodies specific for CD80, CD86, CD40 and ICAM-1. Analysis was carried out on $CD11c^+$ cells (see Appendix 7.3). Stimulation of BMDC with LPS enhanced CD80, CD86, CD40 and ICAM1 expression (Fig. 3.6). Incubation of BMDC

with LT or LTK63 enhanced CD80 and CD86 expression. While the level of enhancement of CD86 by LT and LTK63 was comparable, the enhancement of CD80 expression was more pronounced with LT than with LTK63. In contrast to LPS, LT and LTK63 suppressed CD40 and ICAM-1 expression (Fig. 3.6). The data demonstrate that upregulation of co-stimulatory molecules by the toxin is not dependent on its associated ADP-ribosyltransferase activity and that DC activated with LT or LTK63 have a semi-mature phenotype.

3.2.5 LT and LTK63 do not stimulate DC cytokine production alone but can modulate TLR-ligand induced responses

The previous data demonstrated that LT does not induce cytokine secretion from DC but can upregulate the co-stimulatory molecules that render DC capable of priming naïve T cells. The next study examined the ability of LT and LTK63 to modulate DC cytokine production in response to the TLR4-agonist LPS. BMDC were stimulated with LT or LTK63 in the presence or absence of LPS and cytokine production was examined after 36 h. LT or LTK63 alone did not stimulate the production of IL-12p40, IL-10 or TNF-α by DC following 1 or 12 h incubation, whereas LPS induced the production of IL-12p40, IL-10 and TNF-α (Fig. 3.7). Pre-incubation of BMDC with LT for 1 or 12 h inhibited LPS-induced IL-12p40 production. In contrast to LT, LTK63 required at least a 6 h pre-treatment to inhibit LPS-induced IL-12 production and enhance LPS-induced IL-10 production by DC. LT inhibited IL-12 and TNF-α production by LPS-treated DC following a 1 h or 12 h pre-treatment. LTK63 did not inhibit LPS-induced IL-12p40 when added to cells 1 h prior to LPS, however, significant inhibition of IL-12 was observed following a 12 h pre-incubation (Fig. 3.7). LTK63 marginally decreased LPS-induced TNF-α production after 1 h, but more significant inhibition was observed following a 12 h pre-treatment. LT enhanced LPS-induced IL-10 production when DC were pre-incubated for 1 h and this effect was diminished after a 12 h pre-treatment. There was no enhancement of LPS-induced IL-10 by LTK63 with a 1 h pre-incubation. However, LTK63 enhanced LPS-induced IL-10 when DC were pre-treated for 12 h (Fig. 3.7).

These findings demonstrated that LT and LTK63 increase LPS-induced IL-10 production but inhibit IL-12 production by DC. To investigate if the toxin exerts the same effect on IL-10 and IL-12p40 mRNA expression, BMDC were incubated with LT for 1 h or LTK63 for 12 h prior to stimulation with LPS for 30 min, 1, 12 or 24 h. Total RNA was isolated from DC, purified, reverse transcribed into cDNA, and amplified by real-time PCR for the detection of IL-12p40, IL-10, IL-6 and IL-1β mRNA expression.

Stimulation of DC with LPS alone induced IL-10 mRNA (Fig. 3.8). LT and LTK63 significantly increased the expression of IL-10 mRNA in DC pulsed with LPS for 30 min or 1 h (Fig. 3.8). By 12 h, the level of expression of IL-10 in response to stimulation was diminished and decreased further by 24 h. Moreover, the enhancing effect of the toxins had also declined after 12 h culture in the presence of LPS. The kinetics of IL-12p40 induction in response to LPS were more notably delayed than the induction of IL-10. While there was an approx. 3000 fold increase in p40 expression following 1 h stimulation with LPS, by 12 h there was a fold increase of ~ 12000. LT and LTK63 suppressed LPS-induced IL-12p40 expression in DC cultured with LPS for 1, 12 or 24 h.

LT and LTK63 can enhance LPS-induced IL-1β and IL-6 production in DC, but this effect is observed only when DC are co-incubated with the toxin and LPS or pre-treated with LT for a short period (Lavelle; unpublished observations). Following 30 min stimulation with LPS, DC that had been pre-incubated with LT (1 h) or LTK63 (12 h) had greater IL-β and IL-6 mRNA expression than cells stimulated with LPS alone (Fig. 3.9). The enhancement of LPS-induced IL-6 mRNA, and to a lesser extent IL-1β mRNA, by LT was still evident after 1 h stimulation with LPS. In contrast, pre-treatment of DC with LTK63 suppressed IL-1β and IL-6 mRNA expression in response to LPS stimulation for 1 h. The expression of IL-6 in response to LPS was diminished by 12 h. Furthermore, DC pre-treated with LT or LTK63 had decreased expression of IL-6 as compared to DC stimulated with LPS alone. LPS-induced IL-1β mRNA remained elevated in DC stimulated for 12 h, but by this stage, DC that were pre-incubated with LT or LTK63 before LPS had decreased IL-1β expression (Fig. 3.9). After 24 h stimulation, the expression of IL-1β and IL-6 in response to LPS and the suppression of IL-1 and IL-6 by LT or LTK63 had diminished further. Consistent with observations on cytokine

secretion, these results demonstrate that LT and LTK63 inhibit IL-12 and enhance IL-10, IL-1β and IL-6 mRNA expression in DC.

Overall, these findings established that the inhibition of LPS-induced IL-12 and TNF-α production and enhancement of LPS-induced IL-10 production by LT is not entirely dependent on ADP-ribosyltransferase activity, but the kinetics are different; LTK63 requires a longer pre-treatment than LT to exert its immunomodulatory effects in DC.

3.2.6 LT and LTK63 induce IL-23p19 mRNA expression in DC

IL-23 is an IL-12 family member that has been recently shown to be required for the maintenance and expansion of Th17 cells (129). IL-23 shares a common p40 subunit with IL-12p70 but has a unique p19 subunit. Having established that LT, LTK63 and LTR72 promote the induction on Th17 cells *in vivo*, in part due to their modulatory effects on DC, the next study examined the ability of the toxins to induce p19 expression in DC. BALB/c BMDC were incubated with LT, LTK63 or LTR72 (1 μg/ml) for 12 h. Total RNA was isolated from DC, purified, reverse transcribed into cDNA, and amplified by real-time PCR for the detection of cytokine mRNA expression.

Stimulation of DC with LT for 12 h induced IL-23p19 expression in DC (Fig. 3.10a). Moreover, LTR72 induced IL-23p19 expression, and to a greater extent than wild-type toxin. LTK63 also induced IL-23p19 mRNA expression, albeit to a lesser extent than LT or LTR72. These results indicate that LT can induce IL-23p19 in an enzyme-independent manner. To determine if the kinetics of IL-23p19 induction by LTK63 were slower that wild-type toxin, BMDC were stimulated with LT or LTK63 over a time course from 1 to 36 h. Incubation of DC with LT for 1 h induced ~ 7 fold increase in p19 expression compared to unstimulated DC, however, a dramatic ~ 570 fold increase in p19 was observed following 12 h stimulation with LT (Fig. 3.10b). The level of expression of IL-23p19 was reduced by 25 h but was still ~ 110 fold higher than that expressed by DC incubated with medium alone. Incubation of DC with LTK63 for 12 h induced ~ 6 fold increase in p19 expression (Fig. 3.10b). Similar to LT, LTK63 increased IL-23p19 expression by ~300 fold after 24 h but this was further increased to ~ 450 fold

over the level of IL-23p19 expression in unstimulated DC after 36 h (Fig. 3.10b). These findings were surprising as neither LT nor LTK63 had a strong effect on IL-12p40 expression in DC (Fig. 3.8) and as shown earlier, both inhibit TLR-agonist induced IL-12p70 and IL-12p40 production from DC. The results suggest the possibility that LT promotes Th17 induction by inducing IL-23p19 expression in DC.

The significant finding of these experiments is that LT or LTK63 alone induce a substantial level of IL-23p19 mRNA expression in DC. Furthermore, the data presented earlier demonstrated that LT and LTK63 can also promote IL-1 and IL-6, all of which have been implicated in driving the induction or expansion of Th17 cells.

3.2.7 LT and LTK63 increase LPS-induced IL-23p19 expression in DC

Previous results indicated that LT and LTK63 had opposing effects on IL-12 cytokine family members. Wild-type toxin and LTK63 inhibit the production of IL-12p70 and IL-12p40 by DC, but significantly induced IL-23p19 expression. Experiments were carried out to investigate the effect of LT and LTK63 on LPS-induced IL-23p19 mRNA expression. BMDC were stimulated with LT or LTK63 (1 or 12 h respectively) prior to incubation with LPS for 12 h. Total RNA was isolated from DC, purified, reverse transcribed into cDNA, and amplified by real-time PCR for the detection of IL-23p19 mRNA expression.

Stimulation with LPS for 30 min induced the expression of IL-23p19 in DC (Fig. 3.11). Pre-treatment of DC with LT and LTK63 increased the expression of LPS-induced IL-23p19. Incubation with LPS for 1 h dramatically increased p19 expression (from ~ 500 fold to ~ 75000 fold) compared with DC incubated in medium only. Again, pre-incubation with LT (1 h) or LTK63 (12 h) increased the expression of p19 in DC stimulated with LPS for 1 h. Following 12 h stimulation, the level of p19 expression induced by LPS had further increased. By this time, the enhancement of p19 by LT was suppressed while DC pre-treated with LTK63 still had increased p19 expression compared to DC stimulated with LPS alone (Fig. 3.11). This might be explained by the overall time in culture, as DC were incubated with LT and LPS for a total of 13 h, but with LTK63 and LPS for a total of 24 h. The data indicates that LTK63 can continue to exert its enhancing effect over the 12 h period of stimulation with LPS. It is consistent

with the observation that stimulation of DC with LTK63 alone for up to 36 h induces p19 expression. By 24 h, the induction of IL-23p19 by LPS had begun to diminish. DC stimulated with LT (1 h) prior to LPS for 24 h had marginally enhanced expression of p19. However, DC incubated with LTK63 for 12 h before LPS had diminished p19 expression compared to DC stimulated with LPS alone. As discussed previously, this might be explained by the length of the culture period, a total of 25 h with LT and LPS, while DC stimulated with LTK63 and LPS were in culture for a total of 36 h.

Having established that LT and LTK63 enhance expression of IL-23p19 mRNA in LPS-stimulated DC, the next study investigated their effect on LPS-induced IL-23 protein secretion. BMDC were pre-treated with LT (1 μg/ml) or LTK63 (1 μg/ml) for 1 or 12 h respectively, before stimulation with LPS (100 ng/ml) for 24 h. In contrast to the enhancement of LPS-induced IL-23p19 mRNA expression, both LT and LTK63 significantly inhibited IL-23 production (Fig 3.12). This is consistent with their ability to suppress LPS-induced IL-12p40 and IL-12p70 production.

Therefore, in contrast to the suppressive effect of LT and LTK63 on IL-12p70, IL-12p40 and also IL-23 production, these data clearly show that LT and LTK63 enhance LPS-induced IL-23p19 mRNA expression in DC.

3.2.8 Pre-treatment of DC with LTK63 for up to 1 h increases LPS-induced IL-23 production

While LT and LTK63 induced IL-23p19 mRNA expression, these molecules inhibited LPS-induced IL-23 protein secretion. Analysis of cytokine mRNA expression highlighted that the kinetics of IL-23p19 expression appear to be different to IL-12p40, with the induction of IL-23p19 occuring faster than that of IL-12p40 expression. Therefore, the next study examined the ability of LT to modulate LPS-induced IL-23 production when DC were pre-incubated for less time and over a wider LPS concentration range. BMDC were pre-treated with LT (1 μg/ml) or LTK63 (1 μg/ml) for 30 min, 1 or 12 h before stimulation with LPS (1, 10 or 100 ng/ml) for 24 h.

Stimulation of DC with LPS induced IL-23, IL-12p40, IL-12p70 and IL-10 production. Following a 30 min or 1 h pre-treatment, LT increased LPS-induced IL-23 production, from ~ 1500 pg/ml to ~ 1900 pg/ml, but only when DC were stimulated with 1 ng/ml of LPS (Fig. 3.13). LT suppressed IL-23 production when the concentration of LPS was increased or when the pre-incubation period with LT was lengthened to 12 h. However, LTK63 significantly increased LPS-induced IL-23 production and this was observed when DC were pre-treated for 30 min or 1 h with LTK63 followed by LPS over the range of concentrations (1 – 100 ng/ml) (Fig. 3.13). Nevertheless, similar to LT, pre-treatment of DC with LTK63 for 12 h prior to LPS, completely abolished IL-23 production in response to LPS.

The effects of LT and LTK63 on IL-23 production might be explained by negative regulation of IL-23 by IL-10. DC pulsed with LPS alone induced very low concentrations of IL-10 (Fig. 3.13). Stimulation with LT alone did not induce IL-10 but pre-incubation with LT for 30 min increased IL-10 production by DC pulsed with LPS. While the enhancement of IL-10 remained, the concentration of IL-10 diminished when the pre-treatment time was increased to 12 h. As previously demonstrated, LTK63 increased LPS-induced IL-10 production but only after pre-incubation of DC with LTK63 for 12 h (Fig. 3.13). Inhibition of LPS-induced IL-23 and enhancement of LPS-induced IL-10 by LTK63 appears to occur at the same time, when DC were pre-incubated with LTK63 for 12 h. This suggests that the delayed induction of IL-10 by LTK63 might play an important role in its suppressive effect on IL-23 production. However, since LTK63 alone can induce p19 expression in DC, experiments to determine whether LTK63 can enhance IL-23 in the absence of IL-10 (using DC from IL-$10^{-/-}$ mice) would shed some light on this observation.

Consistent with earlier findings, LT and LTK63 inhibited LPS-induced IL-12p40 production but in addition, also inhibited the production of bioactive IL-12p70 (Fig. 3.13). LT inhibited IL-12p70 after 30 min pre-incubation, while the inhibitory effect was more dramatic after 1 or 12 h. Similarly, the suppressive effect of LT on LPS-induced IL-12p40 production was more pronounced when DC were pre-treated with LT for 1 or 12 h. LTK63 inhibited LPS-induced IL-12p70 and IL-12p40 production but only following a pre-treatment of 12 h prior to stimulation with LPS. Of note here however, is

that LTK63 increased IL-12p40 production by DC stimulated with low dose LPS (1 ng/ml), but only when DC were pre-treated for 30 min or 1 h with LTK63. This effect was abolished when DC were stimulated with LTK63 for 12 h before stimulation with LPS.

These data establish that LTK63 increases IL-23 production but this occurs soon after stimulation with LTK63 and the effect is diminished over time. The findings suggest the possibility that wild-type LT might increase IL-23 production but might exert this effect earlier than its regulation of other TLR-induced cytokine responses. Furthermore, experiments using IL-$10^{-/-}$ DC would shed light on the role of IL-10 in the regulation of IL-23.

3.2.9 LT and LTK63 increase CpG-induced IL-23 production

The previous results indicated that LT might increase IL-23 production but that this effect may occur rapidly after the DC is activated by TLR-stimulation. In addition, the activation status of the DC and as such the cytokine environment appeared to be playing an important role. Since CpG induces lower concentrations of IL-10 than LPS, this study investigated the effect of LT on the ability of the TLR-9 agonist-CpG, to induce IL-23 production. BMDC were pre-incubated with LT, LTK63 or LTR72 (1 μg/ml) for 1 or 12 h before stimulation with LPS (10 or 100 ng/ml) or CpG (1 μg/ml) for 24 h.

Consistent with previous findings, LT, LTK63 and LTR72 inhibited LPS induced IL-12p70 production and the inhibition of IL-12p70 by LTK63 required up to a 12 h pre-treatment (Fig. 3.14). Similarly, LPS-induced IL-12p40 secretion was suppressed when DC were pre-incubated with LT for 1 or 12 h, while the suppressive effect of LTR72 on LPS-induced IL-12p40 was more marked at 12 h. LTK63 inhibited LPS-induced IL-12p40 when DC were pre-treated for 12 h prior to LPS. CpG did not induce IL-12p70 production but DC stimulated with CpG produced a comparable concentration of IL-12p40 to LPS. LT suppressed CpG-induced IL-12p40 when DC were pre-treated for 1 h and IL-12p40 production was completely abolished when DC were pre-incubated with LT for 12 h (Fig. 3.14). LTK63 and LTR72 suppressed CpG-induced IL-12p40

production, but only when DC were pre-treated with the toxins for 12 h prior to stimulation with CpG. However, LTK63 increased IL-12p40 production by CpG-pulsed DC when the cells had been pre-treated with LTK63 for 1 h.

Stimulation of DC with LPS induced a comparable concentration of IL-23 to CpG (Fig. 3.14). LT had no effect on LPS-induced IL-23 when DC were pre-treated for 1 h with the toxin but as before, inhibited IL-23 following a 12 h pre-incubation. However, LT significantly increased CpG-induced IL-23 production when DC were pre-treated with LT for 1 h before incubation with CpG. The enhancement of CpG-induced IL-23 by LT was diminished when DC were pre-incubated for 12 h with LT, but the level of IL-23 still remained above that observed with DC treated with CpG alone. Pre-treatment of DC with LTK63 or LTR72 increased LPS-induced IL-23 production when DC were cultured with the molecules for 1 h before stimulation with LPS but suppressed IL-23 production when cells were pre-treated for 12 h. LTR72 significantly increased CpG-induced IL-23 secretion when DC were pre-incubated with LTR72 for 1 h. Again, this enhancement was suppressed, but not abolished, when DC were pre-treated with LTR72 for 12 h. LTK63 also increased CpG-induced IL-23 production but to a lesser extent than LT or LTR72.

As previously discussed, negative regulation of IL-23 by IL-10 has been reported. In this experiment, CpG did not induce IL-10 production by DC, while LPS induced a low concentration of IL-10 (Fig. 3.14). Consistent with previous results, LT, LTK63 and LTR72 enhanced LPS-induced IL-10 production. When DC were pre-incubated with LT or LTR72 for 1 h the concentration of IL-10 in response to LPS increased substantially. Enhancement of LPS-induced IL-10 by LTK63 was evident when DC were pre-treated for 12 h before LPS stimulation (Fig. 3.14). While LT, LTK63 and LTR72 increased IL-10 production by CpG-pulsed DC, the concentration of IL-10 was markedly lower than that produced by LPS-stimulated DC.

These findings suggest that LT and its derivatives enhance IL-23 production rapidly, but unlike their suppressive effect on IL-12p40 and IL-12p70, their ability to augment IL-23 is weakened the longer the DC are exposed to TLR-stimulation. Together with earlier findings, the data indicate that IL-23p19 is transcribed faster than other inflammatory cytokines in DC, which is a significant observation as the cytokines

produced by activated DC play a crucial role in the polarization of naïve T cells *in vivo*. Therefore, activation of DC mediated by TLR or non-TLR pathways, including signalling through C-type lectins or GPCRs, might promote a Th17 response quickly before the DC is programmed to induce the differentiation of other T cell subtypes.

3.2.10 Co-incubation of DC with LT, LTK63 or LTR72 increases LPS-induced IL-23 production

Previous data showed that pre-incubation with LT or LTK63 for short periods enhanced TLR-induced IL-23 production. Therefore, experiments were designed to determine the effect of co-incubation with the molecules on the ability of LPS to induce IL-23 protein secretion. BMDC were co-incubated or pre-treated with LT, LTK63 or LTR72 (1 μg/ml) for 15 min, 1 or 6 h before stimulation with LPS for 24 h.

DC incubated in medium alone did not produce IL-23, IL-12p40, IL-12 p70, IL-1β or IL-10. Stimulation of DC with LPS induced IL-23, IL-12p40, IL-12p70, IL-1β and IL-10 production (Fig. 3.15). LT increased LPS-induced IL-23 production when DC were co-incubated with LT and LPS. The enhancement of IL-23 was still evident when DC were pre-treated with LT for 1 h, but this effect was abolished after longer pre-treatment periods (Fig. 3.15). LTK63 also increased LPS-induced IL-23 production and this enhancement remained for up to 6 h pre-treatment with LTK63 prior to LPS stimulation. LTR72 increased LPS-induced IL-23 production to a greater extent than either LT or LTK63, but like LT, the ability of LTR72 to enhance IL-23 diminished when the pre-treatment time was extended to 6 h before incubation with LPS.

Consistent with earlier results, stimulation of DC with LT alone did not induce IL-10 but co-incubation or pre-incubation with LT increased IL-10 production from DC pulsed with LPS. When DC were pre-treated with LT for 6 h the enhancement of IL-10 was still evident but the concentration of IL-10 diminished as the pre-treatment time was extended to 6 h. Furthermore, co-incubation or pre-treatment of DC with LTR72 increased LPS-induced IL-10 production. Like LT, the ability of LTR72 to enhance IL-10 diminished over time. LTK63 did not increase IL-10 production, but this was not

surprising as DC were only pre-treated with LTK63 for up to 6 h, and as shown before LTK63 requires up to 12 h pre-incubation to enhance IL-10 production.

In line with previous data, LT, LTR72 and LTK63 inhibited LPS-induced IL-12p70 production (Fig. 3.15). Co-incubation or pre-treatment with LT for 15 min suppressed LPS-induced IL-12p70 production but the inhibitory effect was more dramatic when DC were pre-treated with LT for 1 or 6 h. Similarly, the suppressive effect of LT on LPS-induced IL-12p40 production was more pronounced the longer DC were stimulated with LT prior to LPS. LTK63 slightly inhibited IL-12p70 production but only when DC were pre-treated for 6 h prior to stimulation with LPS. LTK63 did not inhibit IL-12p40 production, but similar to the enhancement of IL-10, LTK63 requires up to 12 h pre-incubation prior to LPS-stimulation in order to exert this effect.

IL-1β, together with IL-23, plays a crucial role in the regulation of Th17 cells (134). Having established that LT can induce IL-23p19 expression and enhance LPS-induced IL-23 production by DC, the effect of LT on IL-1β production was examined. LT alone did not promote the induction of IL-1β, but dramatically increased LPS-induced IL-1β production (Fig. 3.15). The ability of LT to enhance IL-1β was diminished when DC were pre-incubated for longer periods of time. Similarly, LTR72 and LTK63 increased LPS-induced IL-1β production, albeit to a lesser extent that LT.

These findings confirm that LT increases IL-23 production by DC in an enzyme-independent manner but that the timing is critical. The cytokine milieu, particularly the presence of IL-10, may also play a role, and future experiments will investigate the regulation of IL-23 by IL-10. Furthermore, LT enhances LPS-induced IL-1β production, which appears to be only partially mediated by its enzyme activity. Collectively, the data suggest that the induction of Th17 cells by LT may be mediated by its enhancing effects on IL-23 and IL-1β.

3.2.11 LT inhibits LPS-induced IL-12 production by DC even up to 4 days in culture

The ability of LT to promote the induction of IFN-γ producing T cells is inconsistent with the fact that LT inhibits the production of the Th1-polarizing cytokine IL-12p70 by DC. While the enhancement of IL-23 production and IL-23p19 mRNA expression by LT is transient, previous studies indicated that pre-treatment with LT for 12 h suppressed LPS-induced IL-12p40 and IL-12p70. To determine if LT loses its ability to inhibit LPS-induced IL-12 if DC are cultured for a longer duration, BMDC were pre-incubated with LT, LTK63 or LTR72 (1 μg/ml) for 1, 12, 24, 48 or 72 h before stimulation with LPS (100 ng/ml) for a further 24 h.

Consistent with previous results, LT, LTK63 and LTR72 inhibited LPS induced IL-12p70 production, and the inhibition of IL-12p70 by LTK63 was only evident when DC were pre-incubated for 12 h (Fig. 3.16). LT suppressed IL-12p40 production when DC were pre-treated for 1 h, while the suppressive effect of LTR72 on LPS-induced IL-12p40 was more marked when cells were pre-incubated for 12 h. In line with data shown earlier, LTK63 inhibited LPS-induced IL-12p40 but only when DC were pre-incubated for 12 h. The suppressive effect of LT, LTK63 or LTR72 on LPS-induced IL-12p70 and IL-12p40 remained up to 4 d of culture. These data clearly indicate that the suppressive effect of LT on IL-12 is stable and that stimulation of LT-treated DC with LPS even after a long pre-incubation period does alter the ability of DC to produce IL-12.

Stimulation of DC with LPS induced substantial IL-23 production (Fig. 3.16). LT had no effect on LPS-induced IL-23 when DC were pre-treated for 1 h but as before, LT inhibited IL-23 as the pre-incubation period was extended to 12 h or more prior to LPS stimulation. Pre-treatment with LTK63 or LTR72 increased LPS-induced IL-23 production when DC were cultured with LTK63 or LTR72 for 1 h before stimulation with LPS (Fig. 3.16). This effect was diminished when cells were pre-treated for 12 h or longer. Consistent with previous results, LT, LTK63 and LTR72 enhanced LPS-induced IL-10 production (Fig. 3.16).

These findings confirm that the suppressive effect of LT on IL-12 is not transient, and therefore does not provide an explanation for the ability of LT to induce IFN-γ production *in vivo*. A role for IL-23 in the regulation of IFN-γ has previously been described and may account for how LT promotes the induction of Th1 cells.

3.2.12 The enzymatically inactive derivative of LT, LTK63, increases intracellular cAMP

The activation of G-protein coupled receptor signalling enhances the concentration of intracellular cAMP. LT is composed of two subunits, an enzymatically active A subunit, with ADP-ribosyltransferase activity and a B subunit that mediates binding of the molecule to its receptor. The A fragment ADP-ribosylates the stimulatory α subunit of a GTP-binding protein (Gsα), causing permanent activation of adenylate cyclase, resulting in an elevation in cAMP concentration. To assess whether LT or its derivatives, LTK63 and LTR72 increase cAMP, BMDC were stimulated with LT, LTK63 or LTR72 (1 μg/ml) for 1, 12 or 24 h. cAMP is present in some tissue culture media, therefore, RPMI alone was included as a control in the assay. In addition, there is a basal level of cAMP in cells and as such the concentration of cAMP in unstimulated DC was also tested. DC incubated in medium alone expressed ~ 0.5 pmol/ml of cAMP (Fig. 3.17). LT enhanced intracellular cAMP after 1 h, which accumulated further by 12 h and remained elevated up for to 24 h. DC stimulated with LTR72 for 1 h expressed slightly elevated cAMP, but by 12 h, the elevation of cAMP by LTR72 was comparable to that induced by wild-type toxin. After 24 h, the increase in cAMP in response to LTR72 had decreased, but was still above the concentration expressed by DC incubated in medium alone. The enzymatically inactive derivative of LT, LTK63, elevated cAMP when DC were stimulated for 12 h and this had decreased by 24 h. However, while LTK63 increased cAMP, the maximum concentration detected was only twice that observed in DC incubated in medium alone (Fig. 3.17).

These data indicate that the kinetics of cAMP elevation may play an important role in the ability of the molecules to exert their effects. Stimulation of DC with LT for 1 h enhanced the concentration of intracellular cAMP, which was further increased by 12 h.

The results suggest that the significant differences observed in the earlier studies demonstrating the importance of kinetics in the modulatory effects of LT or its derivatives may be largely dependent on the enhancement of cAMP. Moreover, these findings demonstrate that LT can increase intracellular cAMP in an enzyme-independent as well as enzyme-dependent manner. Since LTR72 has less catalytic activity compared with wild-type LT (216), it is surprising that LTR72 can increase cAMP to the same extent as LT. Furthermore, while LTK63 is enzymatically inactive it also enhanced cAMP. Intracellular cAMP becomes elevated in cells following the activation of adenylate cyclase through GPCR signalling. As LTK63 is devoid of ADP-ribosylase activity, and therefore unable to activate Gsα, this data indicates that LTK63 increases cAMP in an indirect manner.

3.2.13 LT induces phosphorylation of CREB and ATF-1 in DC

One of the principal targets of cAMP is PKA which can activate the transcription factor CREB. To assess whether LT induces signalling pathways, which result in activation of CREB, BMDC were stimulated with LT over a time course from 10 min to 48 h. LT induced early activation of CREB as indicated by phosphorylation at Ser 133 by 10 min. This phosphorylation was sustained and was most intense between 1 and 6 h. LT also induced phosphorylation of ATF-1, a CREB family member. Phosphorylation of both proteins diminished after 12 h and was undetectable by 48 h (Fig. 3.18).

3.2.14 The enhancement of LPS-induced IL-10 and inhibition of IL-12 by LT or LTK63 is PKA-independent

Both LT and LTK63 enhanced LPS-induced IL-10 production but inhibited LPS-induced IL-12 production by DC (Fig. 3.7). The induction of cAMP is considered to be the dominant factor in the immunomodulatory effects of CT and LT (246, 268). Elevation of intracellular cAMP concentration has been reported to be responsible for DC maturation in response to CT and LT (274). As PKA is a principal target of cAMP,

experiments were carried out to examine the role of PKA in the immunomodulatory effects of LT and LTK63. BMDC were pre-treated with the selective PKA inhibitor, Rp-8-Br-cAMPs, prior to LT or LTK63, followed by stimulation with LPS. Preliminary experiments were set up to determine the optimum concentration of Rp-8-Br-cAMPs. The results indicated that Rp-8-Br-cAMPs had little effect on cell viability up to 1 mM and this concentration significantly inhibited LPS-induced p-CREB expression, which is commonly used as a readout for activation of signalling events downstream of PKA. As previously demonstrated, LT and LTK63 inhibited LPS-induced IL-12 and TNF-α production, and enhanced LPS-induced IL-10 production. Inhibition of PKA enhanced LPS-induced IL-12p40, IL-12p70 and TNF-α production and inhibited IL-10 production (Fig. 3.19). The inhibition of LPS-induced IL-12p40 and IL-12p70 by LT or LTK63 was not abrogated by the PKA inhibitor. Furthermore, synergy between LT and LPS for IL-10 production was not affected by inhibition of PKA. However, the enhancement of LPS-induced IL-10 production by LTK63 was partially reversed by the PKA inhibitor. Blocking PKA reversed the inhibition of LPS-induced TNF-α production by LT and LTK63 (Fig. 3.19). These data suggest that endogenous PKA may negatively regulate pro-inflammatory cytokine production by DC. In addition, the data demonstrate that PKA is not responsible for the enhancement of LPS-induced IL-10 by LT or for the inhibition of IL-12 by LT or LTK63.

3.2.15 Upregulation of CD80 and CD86 expression by LT or LTK63 is NFκB-independent

The previous findings established that the suppressive effect of LT on IL-12 production and its ability to enhance IL-10 secretion by DC is not mediated by PKA. Furthermore, the up-regulation of CD80 and CD86 by CT, a closely related AB toxin, was PKA-independent (Brereton; unpublished observations). It was reported that CT-induced DC maturation was dependent on binding to GM1-ganglioside and subsequent NFκB translocation (370). To investigate the role of NFκB signalling in the ability of LT and LTK63 to increase CD80 and CD86 expression, and decrease CD40 expression, BMDC were treated with BAY 11-7082, an inhibitor of IκBα phosphorylation which

prevents NFκB activation, before stimulation with LT, LTK63 or LPS for 24 h. Experiments carried out to determine the optimum concentration of BAY 11-7082 revealed that the inhibitor was toxic to DC at a concentration > 10 μM. A dose response with BAY 11-7082 showed that at > 1 μM, the inhibitor suppressed TLR-induced IL-12p40 and IL-12p70 production by DC. In subsequent experiments BAY 11-7082 was used at 4 and 8 μM.

Incubation of DC with the NFκB inhibitor alone had no effect on CD80 expression but decreased CD86 expression (Figs. 3.20 and 3.21). LT, LTK63 and LPS upregulated CD80 and CD86 on the surface of DC. The upregulation of CD80 by LT and LTK63 was unaffected by BAY 11-7082, while the enhancement of CD80 by LPS was slightly reduced with BAY 11-7082 at 15 μM (Fig 3.20). Similarly, blocking NFκB did not reverse the enhancement of CD86 by LT and LTK63 (Fig. 3.20). In contrast, the upregulation of CD86 in response to LPS was completely abolished by the NFκB inhibitor (Fig. 3.21). These results demonstrate that the ability of LT and LTK63 to upregulate CD80 and CD86 expression is NFκB-independent. Furthermore, while LPS-induced CD80 expression is independent of NFκB, the enhancement of LPS-induced CD86 expression is NFκB-dependent.

In contrast to LPS, LT and LTK63 downregulated CD40 expression on DC (Fig. 3.22). Incubation of DC with BAY 11-7082 alone did not affect the expression of CD40 expression. The suppression of CD40 by LT and LTK63 was unaffected by the NFκB inhibitor at a concentration of 3 μM, although a slight increase in CD40 expression was observed at a higher concentration (15 μM). The enhancement of CD40 expression in response to LPS was reversed when DC were pre-treated with the NFκB inhibitor (Fig. 3.22). Thus, enhancement of CD40 by LPS is NFκB-dependent.

3.3 DISCUSSION

The bacterial enterotoxins, CT and LT, are probably the most powerful mucosal adjuvants described to date, yet their clinical use is restricted due to their toxicity (195, 215). The enhancement of intracellular cAMP by the toxins has been proposed as a dominant factor in their adjuvant effects (246, 268). However, the demonstration that derivatives of the toxin with reduced or no enzyme activity and the purified B subunit (LTB) retain immunomodulatory properties indicate that other factors are also involved (217, 285, 361, 362). The partially enzymatically active mutant, LTR72, has been shown to be less toxic than the wild type toxin but exhibited comparable or even superior adjuvant activity to LT (216, 224-226). The enzymatically inactive derivative, LTK63, also retains mucosal adjuvant properties (220, 231, 269, 363), demonstrating that the toxin can exert immunomodulatory effects independent of its ADP-ribosyltransferase activity.

While the adjuvant effects of LT are well documented, the ability of LT to promote the induction of the most recently identified Th cell subset, Th17 cells, has not been examined. The findings presented here show that LT promoted the induction of antigen-specific IL-17 production by lymph node cells from mice immunized with LT and the bystander antigen KLH. The induction of Th17 cells was not completely dependent on its associated enzyme activity as the partially enzymatically active derivative LTR72 also induced a potent Th17 cell response. Moreover, LTK63, which is devoid of enzyme activity, promoted the induction KLH-specific IL-17 production, albeit to a lesser extent that LT or LTR72. Consistent with previous reports, LT and LTR72 also induced a mixed Th1/Th2 response, while LTK63 induced a more Th2- polarized response (193, 195, 207). Furthermore, LT, LTK63 and LTR72 promoted the production of IL-10, which is consistent with the study by Lavelle *et al.* which showed that adoptive transfer of KLH- and CT-treated BMDC into naïve mice induced IL-10-secreting T cells (121).

Significantly this study showed that LT promoted the induction of Th17 cells at least in part, by its modulatory effects on DC. Adoptive transfer of DC pulsed with

antigen and LT into mice resulted in the induction of KLH-specific Th17 cells. Similarly, DC pulsed with antigen in the presence of LTK63 or LTR72 generated Th17 responses. A recent study by Raveney and colleagues demonstrated that intranasal administration of LTB inhibited Th1 but not Th17 responses in established experimental autoimmune uveoretinitis (EAU). They showed that the ratio of IL-17- to IFN-γ- producing cells was higher in mice that were treated with LTB compared to mice immunized with antigen in CFA alone at the peak of disease (372).

A number of reports have shown that the polarization of the immune response is affected not only by the different adjuvant molecules used, but also by the route of immunization (203, 207). In this study, mice were immunized s.c. into the footpad with antigen in the presence of LT or its derivates (1 μg/mouse). Therefore, the type of Th response that was observed may reflect the overall poorer adjuvanticity of these molecules compared with wild-type LT. Since LTK63 is devoid of enzyme activity, 1 μg/mouse may be sub-optimal, whereas LT may exert more potent adjuvant effects at lower doses. A detailed analysis of the immune responses to these molecules over a wide concentration range would be extremely informative for future studies. In line with these observations, the Th responses induced by adoptive transfer of DC stimulated with antigen and LT or LT derivatives appeared to be slightly at variance with those observed after direct immunization. DC pulsed with antigen and LTK63 promoted the strongest antigen-specific IL-10, IL-4 and IL-5 production. While this is consistent with reports demonstrating that mucosal immunization with LTK63 promotes a biased Th2 response, surprisingly, LT- or LTR72- pulsed DC did not enhance KLH-specific IL-10 or IL-4 production. However, DC stimulated with antigen in the presence of LT or LTR72 promoted the induction of KLH-specific IL-17 production to a greater extent than LTK63. This may be as a result of a dominant role for the enzyme activity of LT in the promotion of Th17 or regulation of IL-17 production by other cytokines in the local environment. It has been reported that IL-12, IFN-γ and IL-4 may negatively regulate Th17 cell differentiation (124, 127).

The appreciation in recent years that innate immune responses are central to the induction of adaptive immunity, suggest that activation of innate immunity may be crucial to adjuvanticity. The data presented here clearly showed that activation of DC plays an important role in the ability of LT to exert its immunomodulatory effects. A

number of reports have demonstrated that CT can modulate cytokine production by DC and can induce DC maturation (121, 268, 269). A major aim of this study was to examine the immunomodulatory effects of LT on cytokine production by DC. In addition, an enzymatically inactive derivative of the holotoxin was used to determine if the effects of LT were dependent on ADP-ribosyltransferase activity.

Emerging evidence suggests that signalling through different TLRs on DC can result in distinct patterns of cytokine secretion that differentially regulate adaptive immune responses (119, 189, 373, 374). Furthermore, a screen of macrophage responses to a wide variety of TLR ligands, revealed that co-activation of many receptors resulted in complex cross-talk between signalling pathways that were non-additive. For example, stimulation of RAW cells with either IL-6 or LPS induced IL-10, but a combination induced a dramatic synergistic secretion of IL-10 (375). Moreover, a detailed study by Napolitani *et al.* investigating the synergistic effects of various combinations of TLRs on the induction of specific genes in DC revealed that TRIF-coupled TLRs (TLR3 and TLR4) synergize with endosomal TLRs to increase the production of IL-12p70 (376). Thus, combined activation of receptors on immune cells may provide a more representative picture of the response to an infectious agent. In contrast to CT, which is secreted by *Vibrio cholera* across the outer membrane, LT interacts with LPS on the surface of enterotoxigenic *E.coli.* Thus, LT remains bound to the bacterial surface and is released in association with outer membrane vesicles (377, 378). Since LT is naturally found in a complex with LPS, it is important to determine the responses of DC to a combination of both stimuli.

Activated DC secrete cytokines that are instrumental in the polarization of T cell responses. Previous studies have shown that CT alone did not stimulate the secretion of cytokines or chemokines, with the exception of MIP-2, from DC (121, 268, 269, 277). The present study showed that neither LT nor LTK63 induced production of the pro-inflammatory cytokines IL-12, IL-23, or TNF-α or the anti-inflammatory cytokine, IL-10 from BMDC or macrophages. However, like CT, LT induced the production of the chemokine MIP-2. In contrast, LTK63 did not induce MIP-2, demonstrating that the induction of MIP-2 production was dependent on the enzyme activity of the toxin. This is consistent with a report that identified a role for cAMP in hydrogen peroxide-mediated MIP-2 gene expression (379).

In contrast to the effects on protein secretion, analysis of cytokine mRNA expression revealed that LT and LTK63 induced the expression of IL-1, IL-6 and IL-10 mRNA. Surprisingly, both LT and LTK63 also induced the expression of IL-23p19, another member of the IL-12 family. This finding was significant given that it is now well established that IL-23 plays a crucial role in the induction or expansion of Th17 cells (127, 134). LT also induced early expression of IL-6, IL-10 and IL-1β mRNA, detected 2 h after stimulation. However, while LTK63 induced the expression of IL-1β, IL-10, and to a lesser extent IL-6 mRNA, the induction of these cytokines was more delayed; its effects were more apparent after 13 h of incubation. The expression of IL-6 and IL-1β in response to LT was abolished when DC were incubated for 13 h with LT, suggesting that LT induces rapid induction of the genes controlling these cytokines. It should be noted however, that the induction of cytokine expression by LT or LTK63 was markedly less that induced by LPS.

A number of reports have demonstrated that CT modulates TLR-induced cytokine production (121, 268, 269, 277). CT suppressed LPS-induced IL-12p70 by human monocytes and DC (268, 277), and by murine BMDC (121, 269). CT also inhibited LPS-induced TNF-α, MIP-1α and MIP-1β production (268, 269), and enhanced LPS-induced IL-10 and IL-1β production (269). Braun *et al.* showed that CT suppressed IL-12p70 secretion and IL-12p40 and IL-12p70 mRNA from *Staphylococcus aureus*, Cowan's strain I (SAC) and IFN-γ treated human monocytes, which was independent of IL-10 and TGFβ (277). The present study demonstrated that LT inhibited LPS-induced IL-12 and TNF-α production, and enhanced LPS-induced IL-10 production by DC. In contrast to previous reports, the ADP-ribosyltransferase activity of LT was not essential for the modulation of LPS-induced cytokine production by the toxin. Similar to LT, the enzymatically inactive mutant, LTK63, inhibited LPS-induced IL-12 and TNF-α and enhanced LPS-induced IL-10 production. However, investigation into the immunomodulatory effects of LT and LTK63 revealed that the ability of LTK63 to exert its suppressive effect on IL-12 and TNF-α, and to enhance IL-10 production required that DC were pre-treated with LTK63 for a longer period of time before stimulation with LPS. Wild-type LT modulated cytokine production when co-incubated with LPS or after a short pre-treatment, but LTK63 required a 12 h pre-incubation to inhibit LPS-induced IL-12 and TNF-α, and enhance LPS-induced IL-10 production. Previous studies compared

the effects of LT and LTK63 when co-incubated or pre-treated for short periods with LPS, therefore, this may explain why the ability of LTK63 to modulate cytokine production was not observed.

There are contradictory reports in the literature regarding the involvement of cAMP in mediating the immunomodulatory effects of CT and LT. One study concluded that, since the cAMP elevating agent, forskolin (10 μM), inhibited LPS-induced IL-12 and TNF-α, and enhanced LPS-induced IL-10 production by monocyte-derived DC (MDDC) but did not induce DC maturation, adenylate cyclase-independent pathways must also contribute to the effects of CT (268). In contrast, Bagley *et al.* reported that the effects of CT and LT on MDDC were mediated principally by cAMP. In that study, forskolin (10, 50 and 100 μM) was used at higher doses and other pharmacological agents that increase cAMP, such as dibutyryl-cAMP (d-cAMP), were also found to mimic the effects of CT on DC. PKA is one of the principal mediators of cAMP signalling (300). Inhibition of PKA, using the selective inhibitor Rp-8-Br-cAMPs, prevented the upregulation of CD80 and CD86 by CT (274). A study in murine microglial cells showed that enhancement of LPS-induced IL-10 production by the cAMP analog, d-cAMP, was completely reversed by the PKA inhibitor H-89, while the inhibition of TNF-α was only partially reversed. These researchers suggested that the effects of dbcAMP were mediated through both PKA -dependent and -independent pathways (380). However, a number of studies have reported non-specific effects of H-89, suggesting that this inhibitor should be used with caution for dissecting the specific role of PKA (381, 382). While H-89 interferes with the phosphorylation process of PKA, Rp-cAMPS and its analogues prevent the PKA holoenzyme from dissociating, and thus act one step earlier.

In the present study, inhibition of PKA, with the selective inhibitor, Rp-8-Br-cAMPs, enhanced LPS-induced IL-12p40, IL-12p70 and TNF-α production, indicating that PKA plays an endogenous anti-inflammatory role in DC. This is consistent with a report which demonstrated that elevation of cAMP and activation of PKA in both endothelial and monocytic cells inhibited NFκB activity and transcription (301). Furthermore, overexpression of the coactivator CBP rescued cAMP inhibition of NFκB-mediated transcription, while overexpression of CREB inhibited transcription, suggesting that the phosphorylation of CREB, results in competition between p65 and CREB for

limiting amounts of the coactivator CBP (302). This data suggests that PKA plays a negative role in the induction of LPS-induced pro-inflammatory responses in DC.

In contrast to previous reports which suggested that the inhibitory effects of CT and LT were mediated by PKA, the data presented here indicate that inhibition LPS-induced IL-12 and enhancement of LPS-induced IL-10 production by LT is PKA-independent. Pre-treatment of BMDC with Rp-8-Br-cAMPs, did not reverse the suppression of LPS-induced IL-12p40 or IL-12p70 production by LT or LTK63. Furthermore, inhibition of PKA had no effect on the enhancement of LPS-induced IL-10 production by LT, while increased LPS-induced IL-10 production by LTK63 was partially inhibited. This suggests that the augmentation of LPS-induced IL-10 production by LTK63 is partially PKA-mediated. The inhibition of LPS-induced TNF-α production by LT was reversed by the PKA inhibitor, while blocking PKA partially reversed the inhibition of LPS-induced TNF-α by LTK63. The modulation of TLR-signalling by cAMP via a PKA-independent process was reported in studies with RAW macrophages, where inhibition of PKA with H-89 did not significantly affect the inhibition of LPS-induced TNF-α mRNA by the cAMP analog, 8BrcAMP (375). The results presented here show that the ability of LT to inhibit LPS-induced TNF-α production is PKA-mediated, but the inhibitory effect of LTK63 on TNF-α production is only partially PKA-mediated. While these data contradict the study in microglial cells, where dbcAMP-induced IL-10 was PKA-dependent, it might be explained by differential signalling in different cell types. This indicates that while cAMP analogs increase cAMP, they may also activate other pathways that play a role in mediating their anti-inflammatory effects. In addition, it has been suggested that H-89 can interact with signalling intermediates other than PKA, and therefore behaves unpredictably so cannot be used with confidence in elucidating the effects of PKA in biological responses (383).

The results of this study demonstrate that LT can inhibit LPS-induced IL-12 via an enzyme-independent pathway, as LTK63, which is devoid of ADP-ribosyltransferase activity, was capable of exerting the same effect. Furthermore, the enhancement of LPS-induced IL-10 by LT is not dependent on its enzyme activity, as LTK63 synergized with LPS to promote IL-10. Although LTK63 mimics LT in its ability to inhibit LPS-induced IL-12 and enhance IL-10 production, it does so with delayed kinetics. This suggests that LTK63 triggers intracellular signalling events that require a longer time to activate the

downstream targets responsible for its effects. LT inhibited LPS-induced IL-12 and enhanced LPS-induced IL-10 when DC were treated with LT and LPS simultaneously. In contrast, LTK63 did not modulate LPS-induced cytokine production when DC were treated simultaneously with LPS and LTK63, indicating an essential role for the ADP-ribosyltransferase activity of the toxin in mediating these effects. Therefore, these data suggest that the immunomodulatory effects of the toxin may be mediated by the activation of both enzyme –dependent and –independent pathways.

The partially enzymatically active LT derivative, LTR72, is another useful molecule for dissecting the mechanism through which LT exerts its immunomodulatory effects. The findings in this study demonstrated that similar to LT, co-stimulation of DC with LTR72 and LPS enhanced IL-10 production. The suppressive effect of LTR72 on LPS-induced IL-12p40 was more delayed and required up to 1 h pre-treatment before stimulation with LPS. In contrast, like LT, LTR72 inhibited LPS-induced IL-12p70 production by DC following co-incubation. Similar to LT and LTK63, stimulation of DC with LTR72 alone did not induce cytokine secretion. However, like LT and LTK63, LTR72 induced the expression of IL-23p19 mRNA.

A detailed study of the contribution of kinetics to the ability of LT, LTK63 and LTR72 to modulate DC cytokine production led to a number of important observations. Firstly, the induction of IL-1β and IL-6 cytokine mRNA expression by the molecules did not result in cytokine secretion. Secondly, the expression of different cytokines appeared to be time-dependent, for example, maximal induction of IL-10 mRNA occurred 1 h after stimulation with LPS, while LPS-induced IL-12p40 mRNA expression peaked 12 h after stimulation. It was also found that LT and LTK63 enhanced LPS-induced IL-6 and IL-1β mRNA when DC were stimulated with LPS for up to 1 h, but suppressed the induction of these cytokines when the incubation time was increased to 12 h or longer.

LT and LTK63 exerted opposing effects on IL-12 and IL-23. LT and LTK63 alone induced IL-23p19 expression and enhanced LPS-induced IL-23p19 mRNA expression. The enhancement of IL-23p19 was most dramatic when DC were incubated with LPS for only 30 min. Having established that the molecules enhanced LPS-induced IL-23p19 expression, it was important to determine if they could increase IL-23 secretion. Early experiments revealed that in line with their suppressive effect on IL-12p40 and IL-12p70, LT and LTK63 inhibited IL-23 production. However, in these initial studies DC

were pre-treated with the molecules for at least 1 h before stimulation with LPS. Subsequent experiments, using shorter pre-treatment periods or co-stimulating DC with the molecules and TLR agonist, established that co-incubation of DC with LT, LTK63 or LTR72 increased LPS- and CpG- induced IL-23 production. A significant finding is that the enhancement of IL-23 by LT is abolished when DC are pre-treated with LT for more than 30 min. In contrast, LTK63 retained its ability to enhance LPS-induced IL-23 when DC were pre-incubated for up to 6 h, while the enhancement of IL-23 in response to LTR72 was not evident when DC were pre-treated with LTR72 for 6 h. These data indicate that the effect of wild-type LT on IL-23 reflect differential temporal regulation of the p19 and p40 subunits. While LT can rapidly induce the induction of IL-23p19, its suppressive effect on IL-12p40 mRNA is more delayed and as such the balance is in favour of IL-23 production. If DC are pre-treated for a longer length of time, LT strongly suppresses LPS-induced IL-12p40 and consequently, IL-12p70 and IL-23 secretion. These findings are particularly significant given the role of IL-12 and IL-23 in the induction of Th1 and Th17 cells, and therefore, indicate that the balance of IL-12 and IL-23 can easily be tipped if favour of either cytokine. The consequences of this are highlighted by the essential roles that Th1 and Th17 cells play in fighting infectious disease and regulating autoimmunity.

In light of these observations, the effect of LTK63 on intracellular cAMP was examined in DC. LTK63 increased the concentration of cAMP above the endogenous concentration expressed in DC. While LTK63 did not increase cAMP to the same extent as wild-type toxin or LTR72, it is a significant finding since this molecule does not exhibit ADP-ribosyltransferase activity. These data suggested that the ability of LTK63 to indirectly increase cAMP may be an explanation for the delay in the ability of LTK63 to inhibit LPS-induced IL-12 and enhance LPS-induced IL-10 production, as compared with the wild-type toxin. Furthermore, since LTK63 is devoid of enzyme activity, it was surprising that some of the modulatory effects of LTK63 were regulated by PKA, one of the primary targets of cAMP; specifically the enhancement of IL-10 and inhibition of TNF-α production by LPS-stimulated DC. cAMP is degraded through the action of PDEs and indeed, PDE4 inhibitors can serve as potent anti-inflammatory agents (384). The catalytic subunit of some members of the PDE4 family contains a single consensus site for phosphorylation by ERK, and it has been demonstrated that ERK activation of PDE4

long forms can generate a transient increase in intracellular cAMP (385). cAMP-induced activation of PKA can negate the inhibitory effect of ERK on phosphorylation of PDE4 long isoforms, as demonstrated in smooth muscle cells and COS1 cells, therefore re-programming ERK action on these enzymes (386). Thus, the ability of LTK63 to exert its anti-inflammatory effects may be mediated by its sustained phosphorylation of ERK resulting in the suppression of PDEs and subsequent elevation of cAMP.

The differentiation of naïve T cells by activated DC is determined not only by cytokines, but also by expression of MHC class II and co-stimulatory molecules (270, 273). In agreement with studies on CT (121), the results presented here demonstrate that LT enhanced expression of CD80 and CD86 and downregulated expression of CD40 and ICAM-1 on BMDC. Bagley *et al.* reported that enzymatically inactive derivatives of the enterotoxins failed to induce maturation of human monocyte-derived DC, and demonstrated that inhibition of PKA blocked the ability of CT and LT to upregulate CD80, CD86 and CD83 expression on these cells (274). In contrast, the data shown here demonstrate that LT-induced DC maturation was not solely dependent on its ADP-ribosyltransferase activity, as the enzymatically inactive derivative, LTK63, also upregulated CD80 and CD86 and downregulated ICAM-1 and CD40 expression on the surface of BMDC.

The NFκB pathway has been shown to play an essential role in antigen presentation by DC and in the regulation of co-stimulatory molecule expression (387, 388). In this study, the induction of co-stimulatory molecule expression by LPS was differentially regulated by NFκB signalling. LPS-induced CD86 expression was suppressed by the NFκB inhibitor BAY 11-7082, whereas, the enhancement of CD80 expression by LPS was unaffected. This suggests that LPS-induced CD80 and CD86 expression is regulated by distinct signalling cascades. In contrast, the upregulation of both CD80 and CD86 expression by LT and LTK63 was NFκB-independent. Thus, unlike NFκB-dependent upregulation of CD86 by LPS, the enhancement of CD86 expression by LT is mediated through an alternative signalling pathway. Furthermore, as in the case of LPS, the results indicate that the ability of LT and LTK63 to increase CD80 expression is regulated by an NFκB-independent pathway. A role for NFκB in the regulation of CD40 expression was also investigated in this study. Blocking NFκB inhibited LPS-induced upregulation of CD40 expression, demonstrating that NFκB

activation is required for the induction of CD40 expression on DC in response to LPS. This suggests that inhibition of NFκB may be a possible mechanism through with LT mediates its suppressive effect on CD40 expression.

The ability of LT to alter the surface phenotype of DC, and thereby influence their capacity to direct Th cell polarization, provides further evidence that LT can promote distinct T cell responses. The results clearly show that the ability of LT to upregulate CD80 and CD86 and suppress CD40 and ICAM-1 expression is not dependent on its enzyme activity as LTK63 also exerted these effects. This is consistent with earlier findings that LTB, the receptor binding subunit of LT, upregulated CD80 and CD86 expression on BMDC (Lavelle; unpublished observations). In line with this, LTB (EtxB), but not a non-binding mutant of EtxB, EtxB(G33D), was shown to upregulate MHC Class II and CD25 expression on B cells (389). The authors also showed that LTB binding to the GM1 receptor did not induce detectable lipid raft formation or tyrosine phosphorylation, an event that has been shown to occur during interactions between gangliosides and transmembrane receptor tyrosine kinsases. While it is clear that LT alters the activation status of DC, how the binding of the B subunit to its receptors initiates signalling events remains to be elucidated.

Taken together, these findings establish that LT exerts its potent adjuvant effects by modulating DC cytokine production and co-stimulatory expression. The data clearly show that LT inhibits IL-12, and enhances IL-23 and IL-10 production by a process that is not dependent on its ADP-ribosyltransferase activity. Moreover, LTK63 upregulated CD80 and CD86, indicating that DC maturation in response to LT can occur in the absence of enzyme activity. The current results also reveal that inhibition of LPS-induced IL-12 and enhancement of IL-10 by LT was PKA-independent, while inhibition of PKA partially reversed the enhancement of IL-10 by LTK63 but did not affect the suppression of IL-12 production. The modulation of TLR-signalling by cAMP via a PKA-independent process was reported in studies with RAW macrophages, where inhibition of PKA with H-89 did not significantly affect the inhibition of LPS-induced TNF-α mRNA by the cAMP analog, 8BrcAMP (375). However, the significant finding of this study is that stimulation of DC with LT alone induces the expression of IL-23p19. Moreover, the results demonstrate that LT promotes the induction of Th17 cells, in part mediated by its modulatory effects on DC.

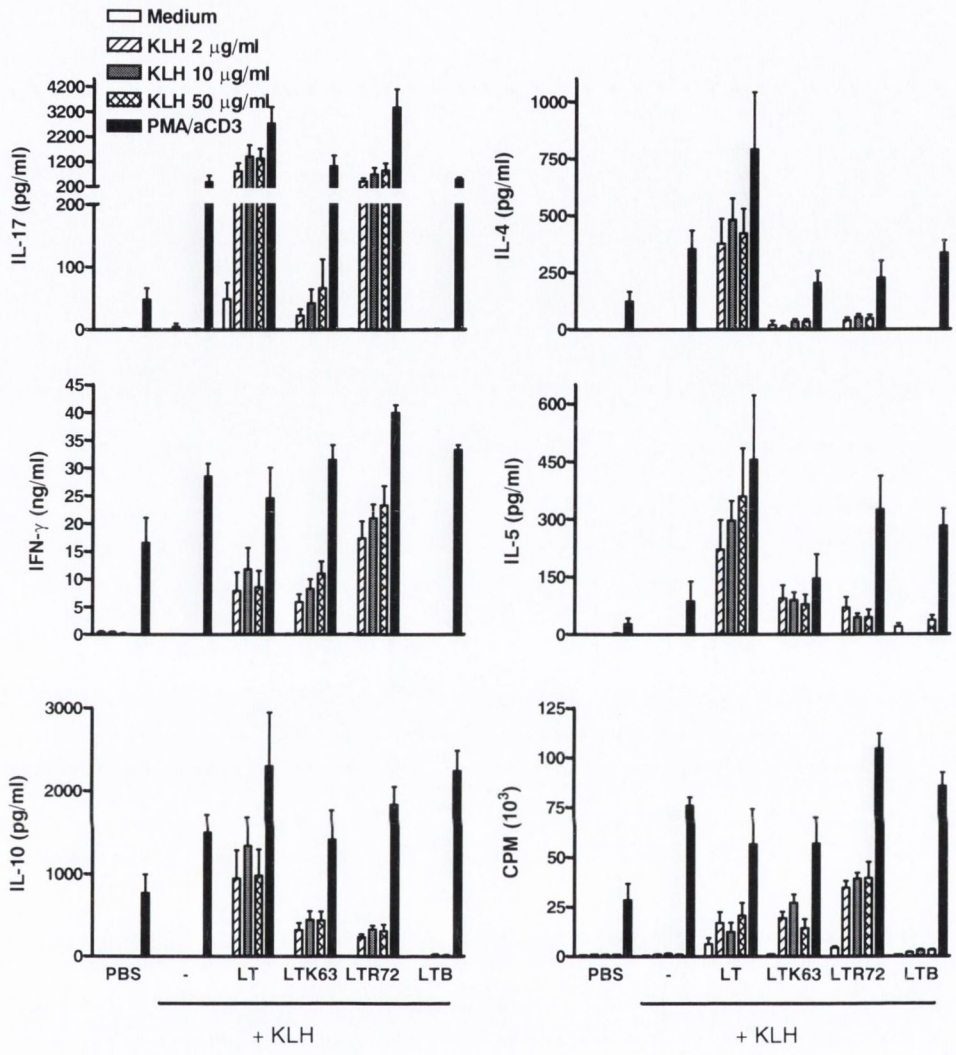

Figure 3.1 LT, LTK63 and LTR72 act as adjuvants to promote the induction of Th17 cells, as well as Th1 and Th2 responses to co-injected antigen. BALB/c mice were injected s.c. with PBS, KLH alone (20 μg/ml) or KLH with LT (1 μg/ml), LTK63 (1 μg/ml), LTR72 (1 μg/ml) or LTB (1 μg/ml). After 7 d lymph node cells from individual mice (1 x 10^6 cells/ml) were re-stimulated *in vitro* with KLH (2-50 μg/ml), medium only or with PMA (25 ng/ml) and anti-CD3 (1 μg/ml). Supernatants were recovered after 72 h and IL-17, IFN-γ, IL-10, IL-4 and IL-5 concentrations quantified by ELISA. The proliferative response (CPM) of Th cells was quantified by [^{3}H]thymidine incorporation. Data are the mean ± SEM of triplicate stimulations of lymph node cells from individual mice (n = 5) and are representative of two independent experiments.

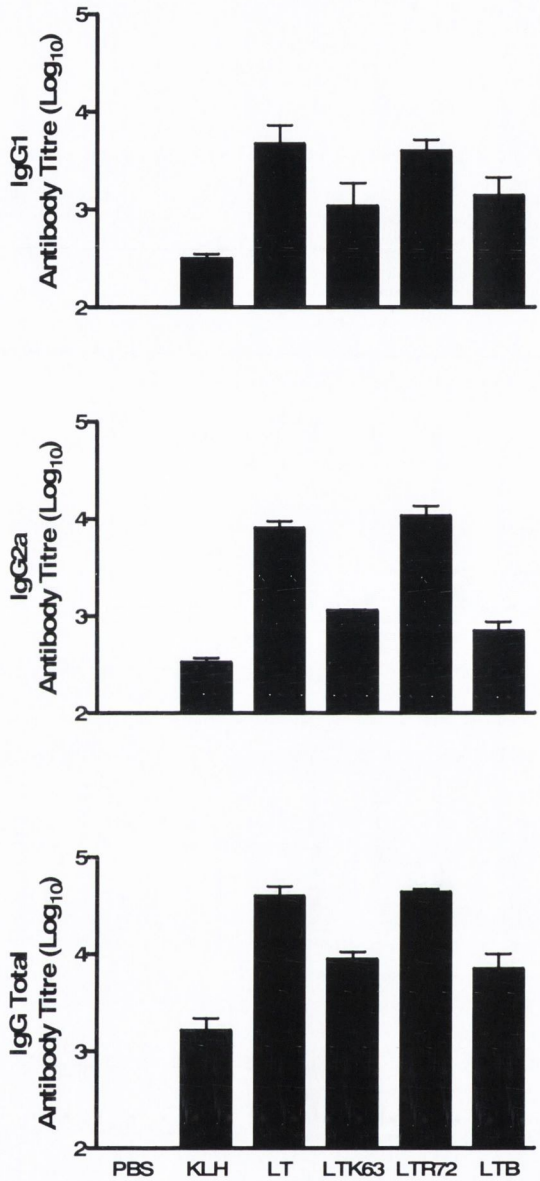

Figure 3.2 Co-administration of antigen with LT, LTK63, LTR72 or LTB enhances antigen-specific antibody production. BALB/c mice were injected s.c. with PBS, KLH alone (20 μg/ml) or KLH with LT (1 μg/ml), LTK63 (1 μg/ml), LTR72 (1 μg/ml) or LTB (1 μg/ml). After 7 d serum samples were collected and analyzed for KLH-specific IgG, IgG1 and IgG2a by ELISA. Data are the mean ± SEM of serum samples from individual mice (n = 5) and are representative of two independent experiments.

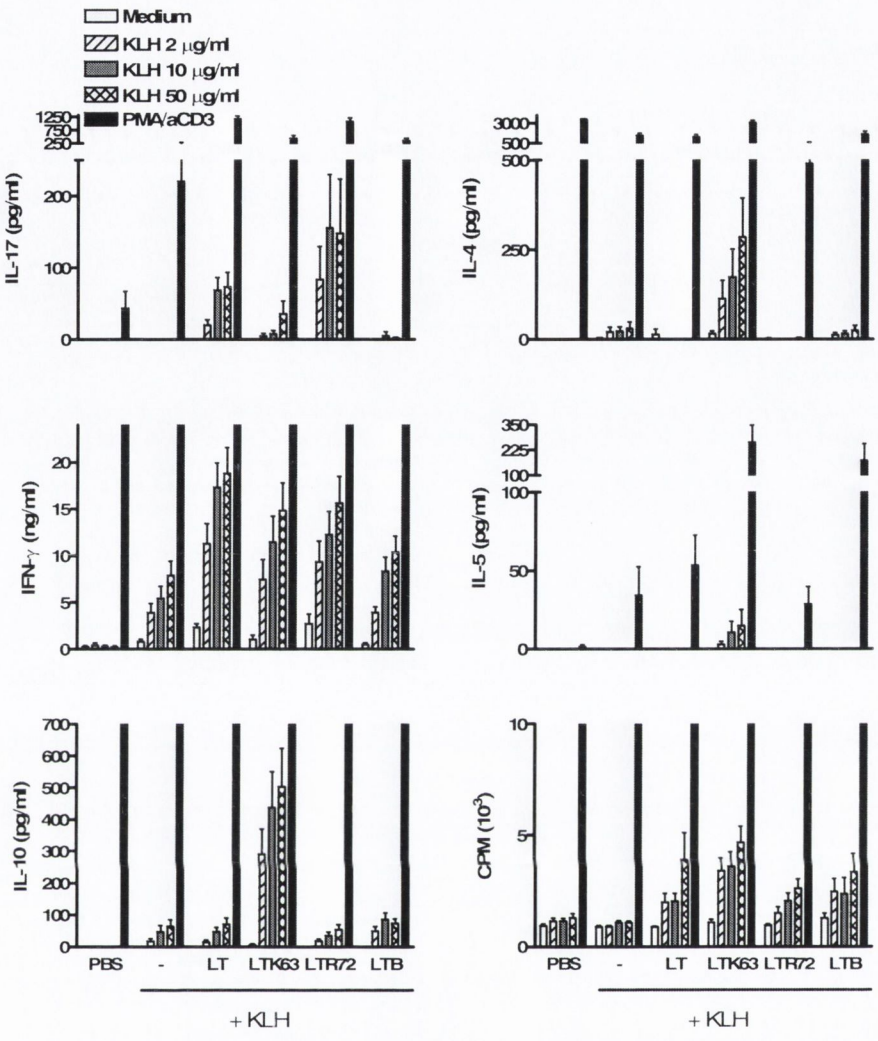

Figure 3.3 Exposure of DC to LT, LTK63, LTR72 or LTB enhances their ability to promote antigen-specific cellular immunity. DC were stimulated for 24 h with medium only, KLH (20 μg/ml) alone or with LT (1 μg/ml), LTK63 (1 μg/ml), LTR72 (1 μg/ml) or LTB (1 μg/ml). Cells (5 x 10^5/mouse) were washed and transferred s.c. to naïve mice. After 7 d lymph node cells from individual mice (1 x 10^6 cells/ml) were re-stimulated *in vitro* with KLH (2-50 μg/ml), medium only or with PMA (25 ng/ml) and anti-CD3 (1 μg/ml). Supernatants were recovered after 72 h and IL-17, IFN-γ, IL-10, IL-4 and IL-5 concentrations quantified by ELISA. The proliferative response (CPM) of Th cells was quantified by [^{3}H]thymidine incorporation. Data are the mean ± SEM of triplicate stimulations of lymph node cells from individual mice (n = 5) and are representative of two independent experiments.

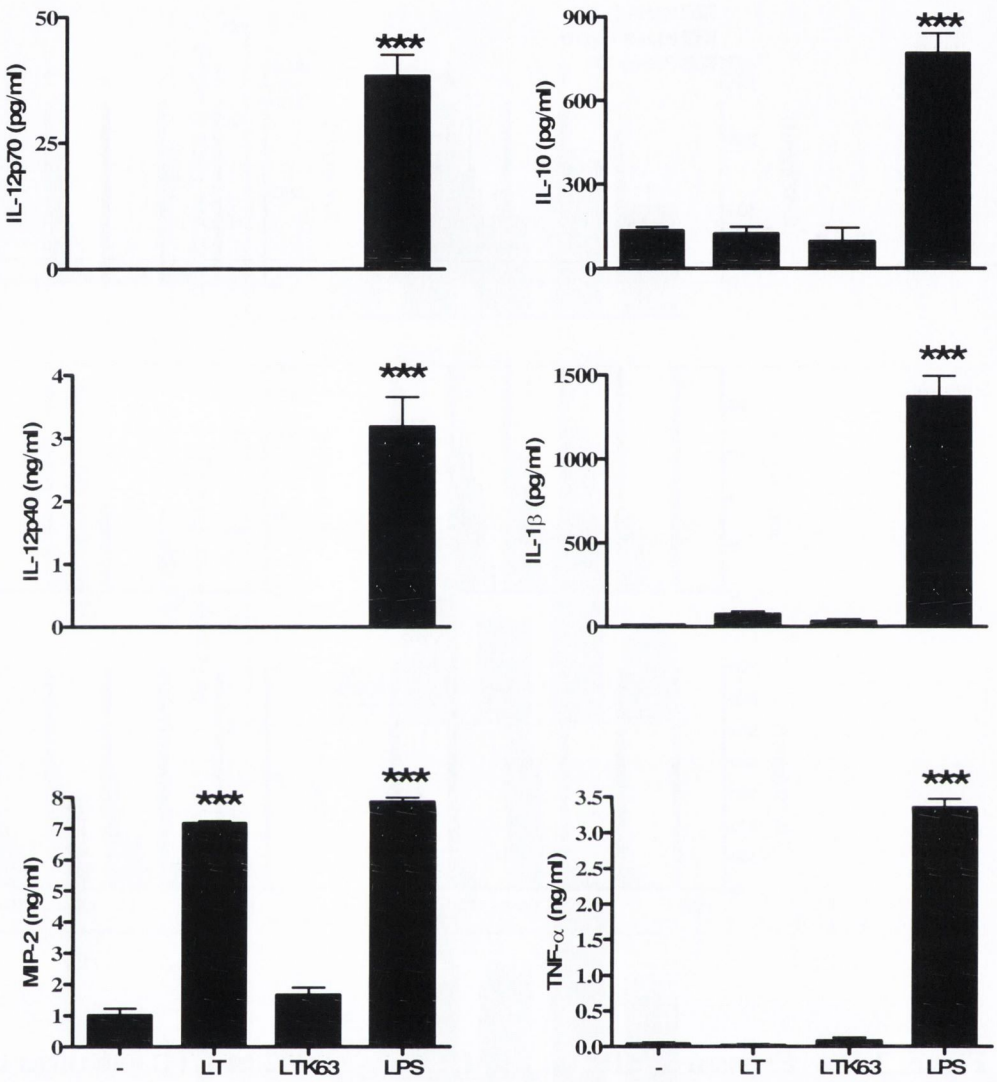

Figure 3.4 Neither LT nor LTK63 induce inflammatory cytokines, but wild type LT induces the production of the chemokine MIP-2 by DC. BMDC (5 x 10^5 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only. Supernatants were collected after 24 h and IL-12p70, IL-12p40, IL-10, IL-1β, TNF-α and MIP-2 concentrations were determined by ELISA. LPS or LT versus medium control: ***, $P < 0.001$. Data are the mean ± SEM of triplicate stimulations and are representative of at least five independent experiments.

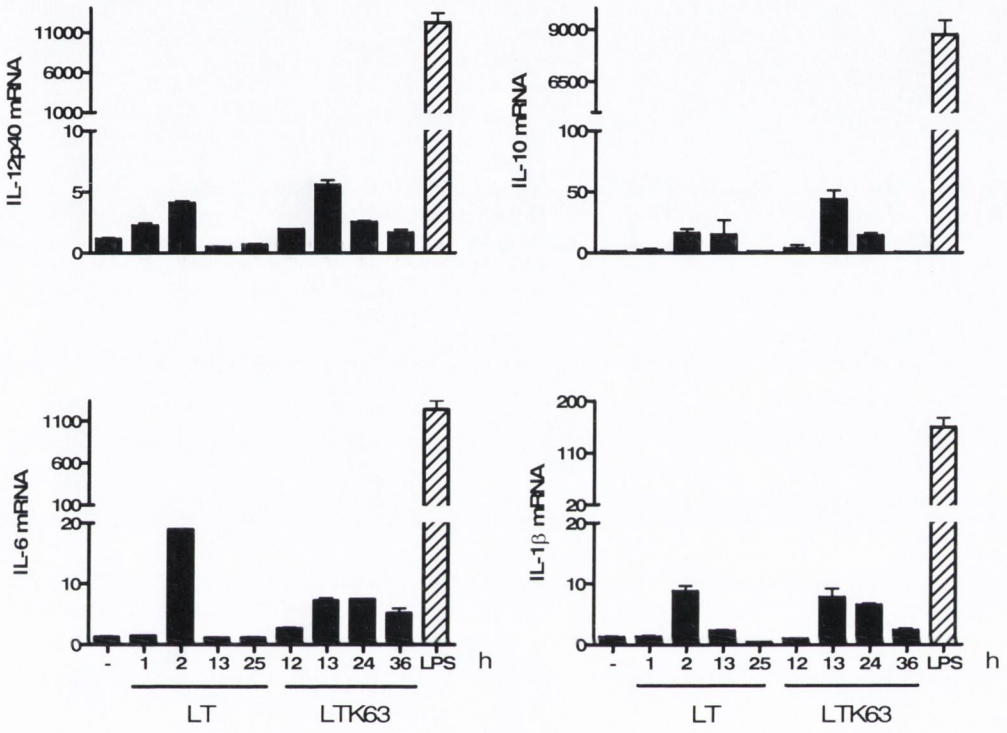

Figure 3.5 LT and LTK63 induce IL-6, IL-1 and IL-10 mRNA expression in DC. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml) or medium only for the indicated times (h). BMDC were stimulated with LPS (100 ng/ml) for 12 h as a positive control. RNA was isolated from BMDC and cytokine expression determined by real time RT-PCR. The data represent the fold change for IL-12p40, IL-6, IL-10 or IL-1β mRNA expression relative to cells incubated in medium only following normalization to the endogenous control, 18S rRNA. Data are the mean ± SEM of triplicate reactions from duplicate cultures and are representative of two independent experiments.

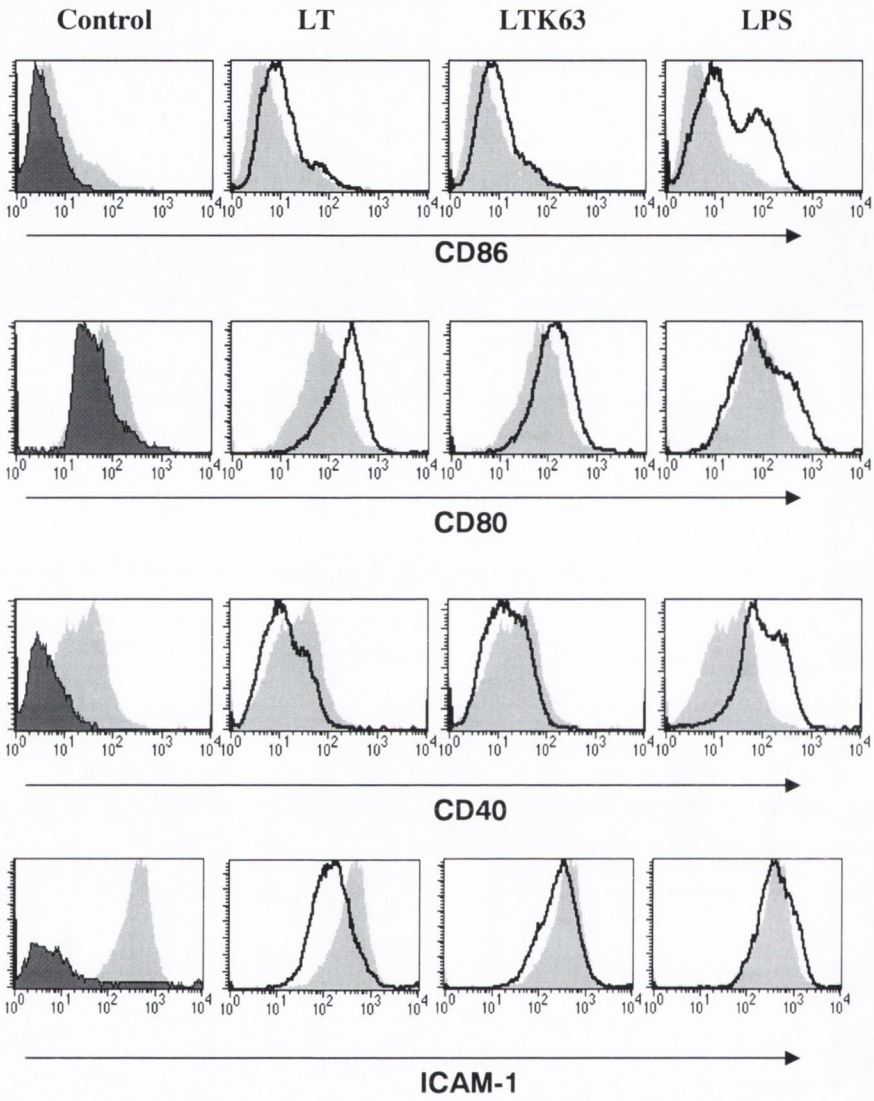

Figure 3.6 ADP-ribosyltransferase activity is not essential for LT-induced upregulation of CD80 and CD86 expression, and inhibition of CD40 and ICAM-1 expression. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only. Cells were harvested after 24 h and stained with antibodies specific for CD80, CD86, CD40 and ICAM-1 or with isotype matched controls. Results from immunofluorescence analysis are shown as histograms and are representative of at least two independent experiments. Filled light grey histograms represent untreated $CD11c^+$ cells (see Appendix 7.3), filled dark grey histograms represent isotype controls and the thick black line represents cells stimulated with LT, LTK63 or LPS.

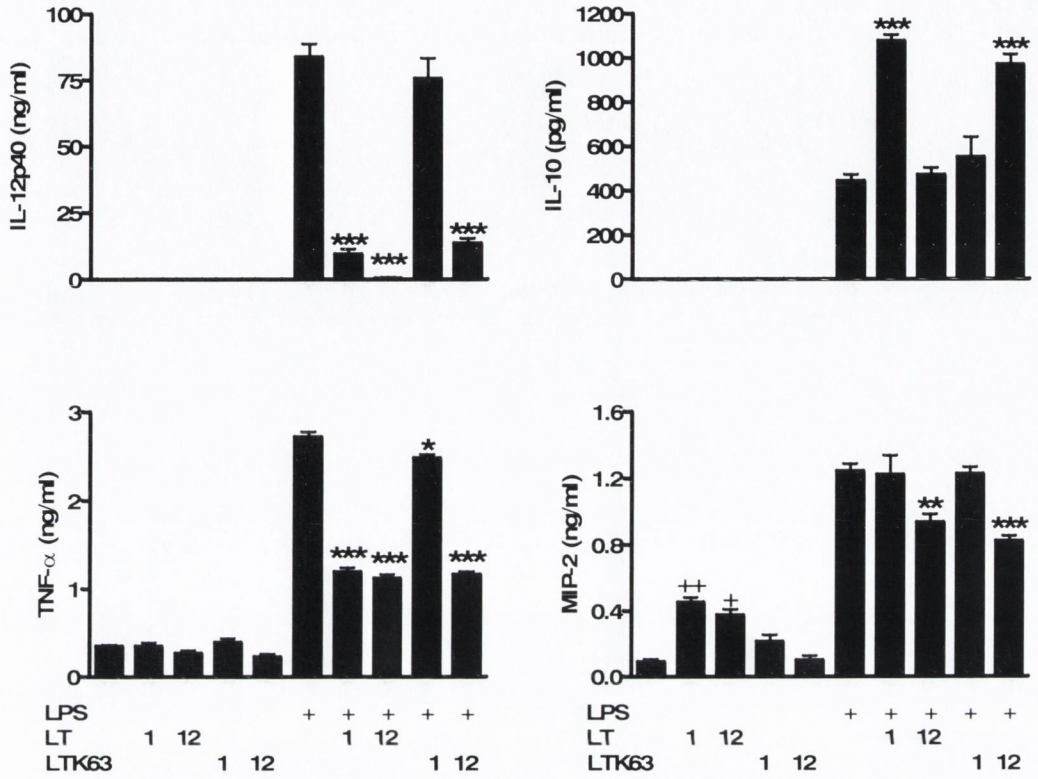

Figure 3.7 LT and LTK63 synergize with LPS for the production of IL-10 but inhibit LPS-induced IL-12 and TNF-α. BMDC (5 x 10^5 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml) or LTK63 (1 μg/ml) for 1 or 12 h prior to stimulation with LPS (5 ng/ml). After 24 h, supernatants were collected for analysis of IL-12p40, IL-10, TNF-α and MIP-2 production by ELISA. LPS versus LPS plus LT or LTK63: *, $P < 0.05$; ** $P < 0.01$; ***, $P < 0.001$. Control versus LT or LTK63: +, $P < 0.05$; ++, $P < 0.01$. Data are the mean ± SEM of triplicate stimulations and are representative of at least three independent experiments.

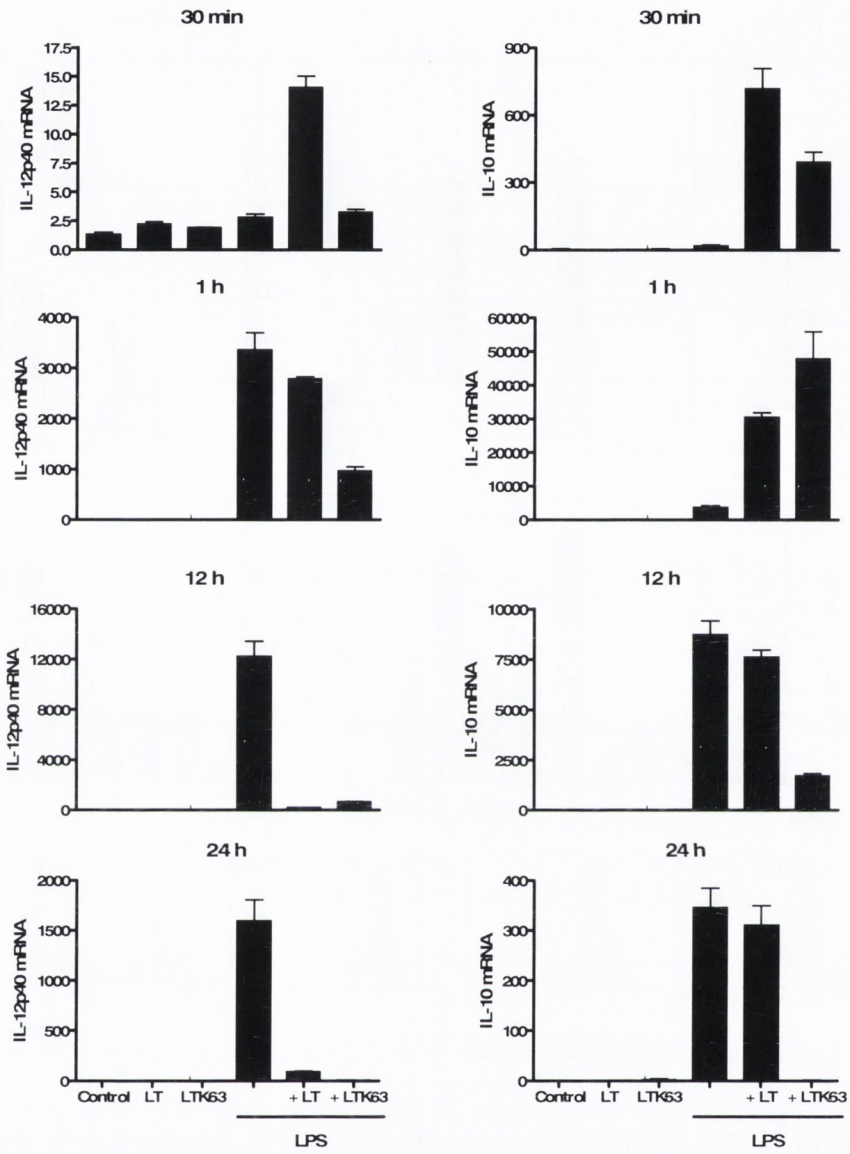

Figure 3.8 LT and LTK63 inhibit LPS-induced IL-12p40 and enhance LPS-induced IL-10 mRNA expression. BMDC (1 x 10^6 cells/ml) from BALB/c mice were pre-incubated with LT (1 μg/ml) or LTK63 (1 μg/ml) for 1 or 12 h respectively or medium only before stimulation with LPS (100 ng/ml) for 30 min, 1, 12 or 24 h. RNA was isolated from BMDC and IL-12p40 and IL-10 expression determined by real time RT-PCR. The data represent the fold change for IL-12p40 or IL-10 mRNA expression relative to cells incubated in medium only, following normalization to the endogenous control, 18S rRNA. Data are the mean ± SEM of triplicate reactions from duplicate stimulations and are representative of two independent experiments.

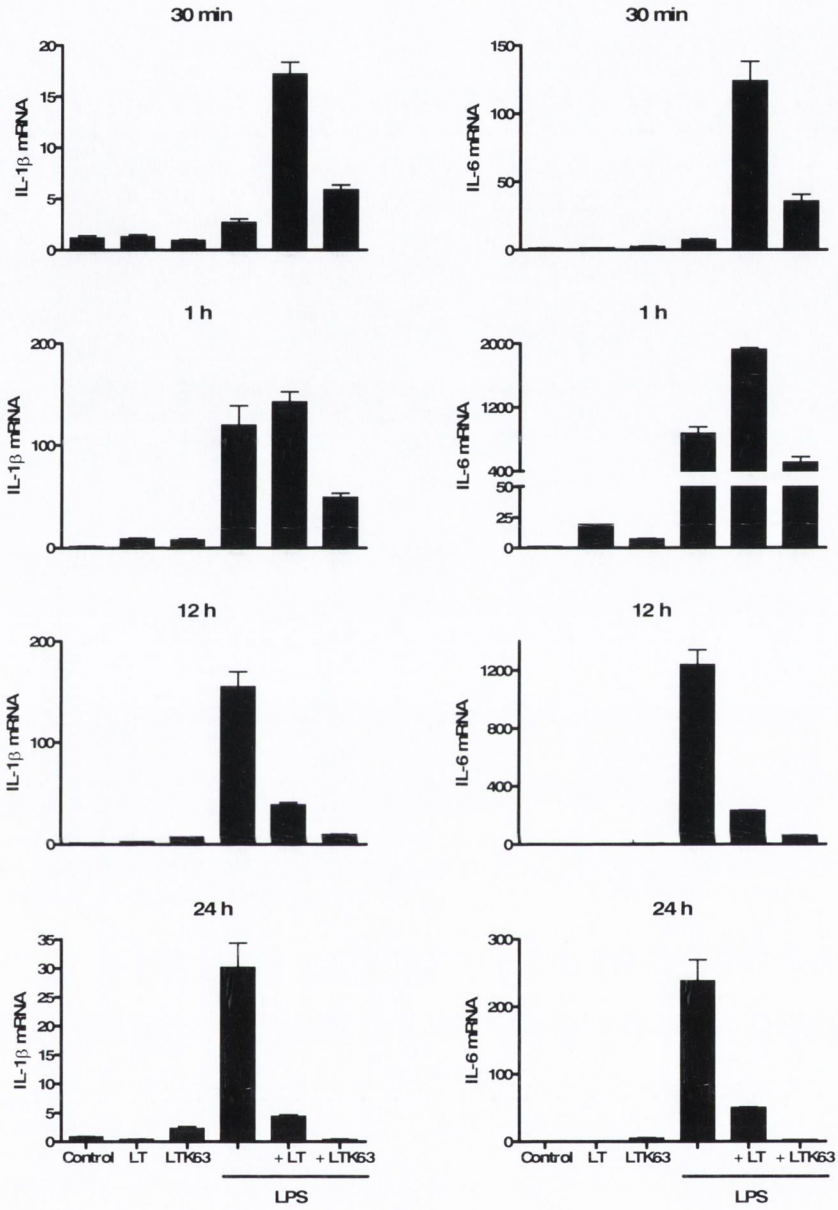

Figure 3.9 LT and LTK63 enhance the early induction of IL-1β and IL-6 mRNA expression in response to LPS. BMDC (1 x 10^6 cells/ml) from BALB/c mice were pre-incubated with LT (1 μg/ml) or LTK63 (1 μg/ml) for 1 or 12 h respectively or medium only before stimulation with LPS (100 ng/ml) for 30 min, 1, 12 or 24 h. RNA was isolated from BMDC and IL-1β and IL-6 expression determined by real time RT-PCR. The data represent the fold change for IL-1β or IL-6 mRNA expression relative to cells incubated in medium only, following normalization to the endogenous control, 18S rRNA. Data are the mean ± SEM of triplicate reactions from duplicate stimulations and are representative of two independent experiments.

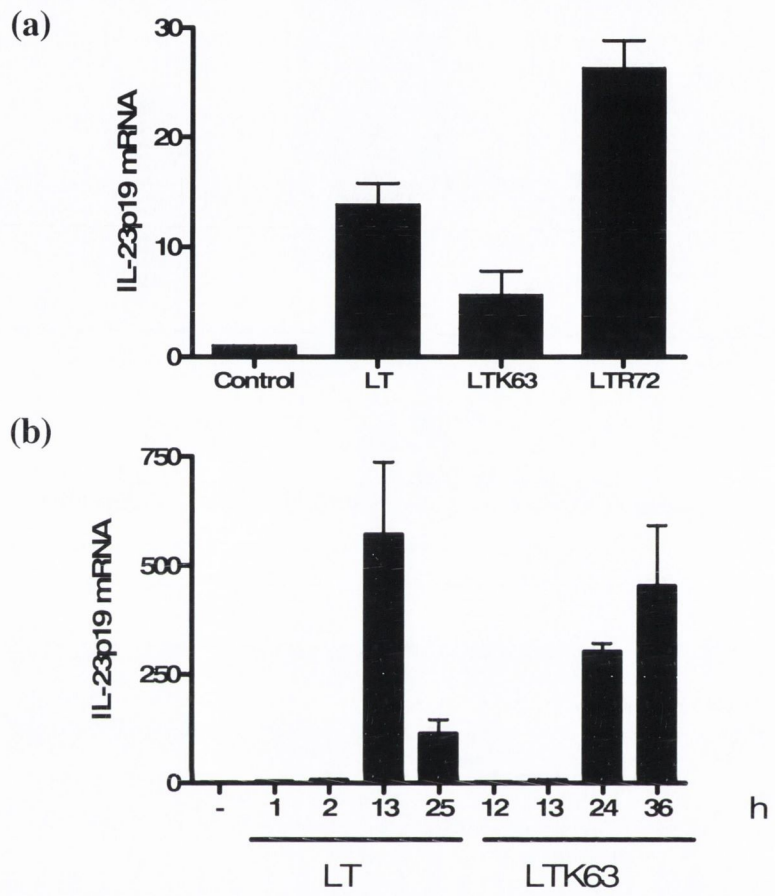

Figure 3.10 LT, LTK63 and LTR72 induce IL-23p19 mRNA expression in DC.
(a) BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LTR72 (1 μg/ml) or medium only for 12 h. **(b)** BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml) or medium only for indicated times (h). RNA was isolated from BMDC and IL-23p19 expression determined by real time RT-PCR. The data represent the fold change for IL-23p19 mRNA expression relative to cells incubated in medium only, following normalization to the endogenous control, 18S rRNA. Data are the mean ± SEM of triplicate reactions from duplicate stimulations.

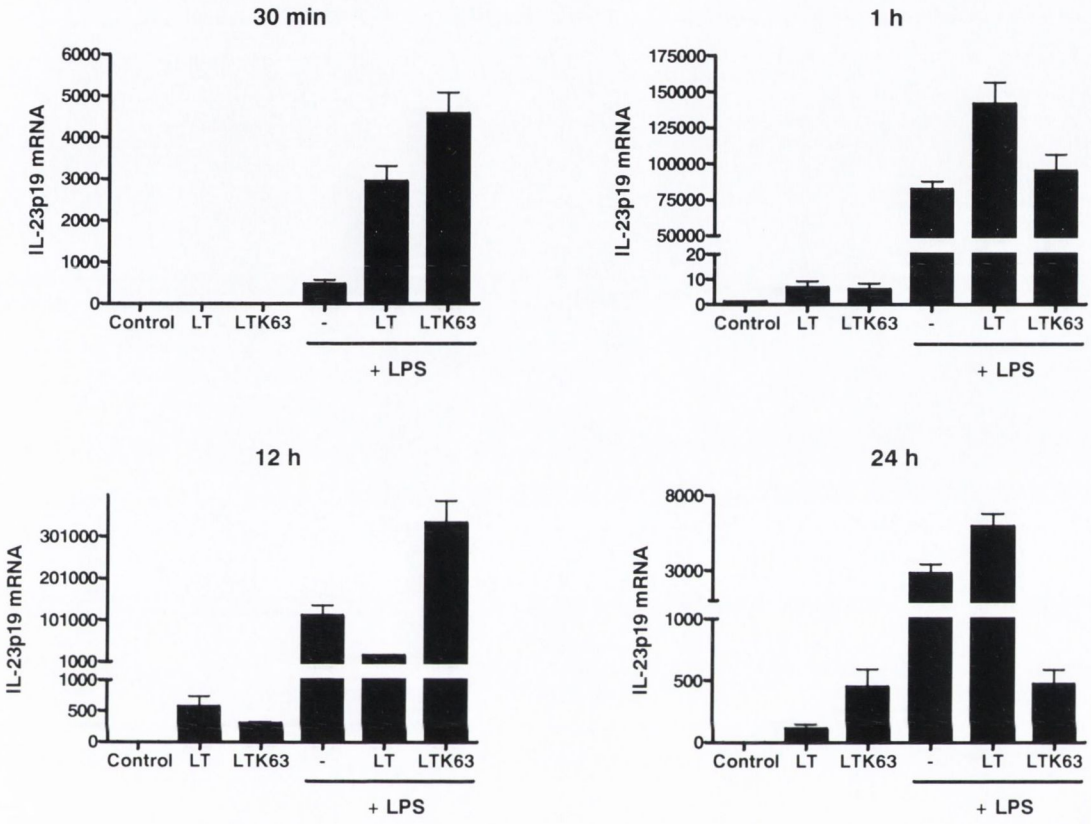

Figure 3.11 LT and LTK63 enhance LPS-induced IL-23p19 mRNA expression. BMDC (1 x 10^6 cells/ml) from BALB/c mice were pre-incubated with LT (1 μg/ml) or LTK63 (1 μg/ml) for 1 or 12 h respectively or medium only before stimulation with LPS (100 ng/ml) for 30 min, 1, 12 or 24 h. RNA was isolated from BMDC and IL-23p19 expression determined by real time RT-PCR. The data represent the fold change for IL-23p19 mRNA expression relative to cells incubated in medium only following normalization to the endogenous control, 18S rRNA. Data are the mean ± SEM of triplicate samples from duplicate stimulations and are representative of two independent experiments.

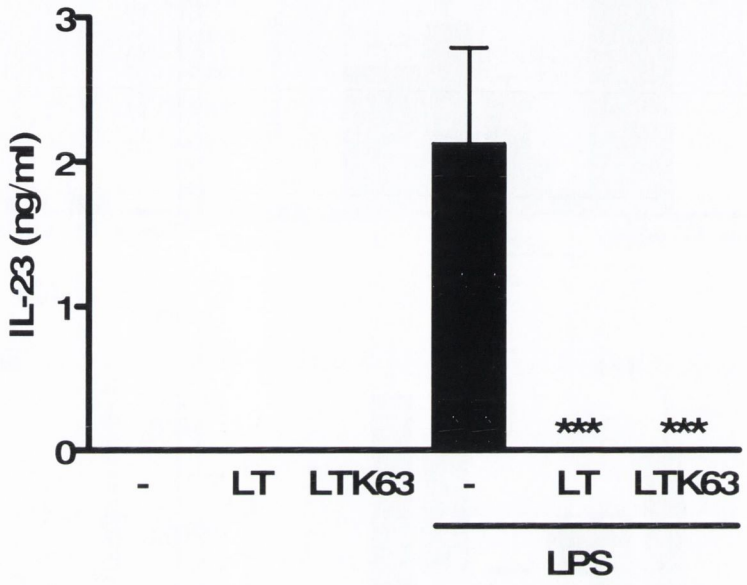

Figure 3.12 LT and LTK63 inhibit LPS-induced IL-23 production by DC. BMDC (5×10^5 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml) for 1 or 12 h respectively before stimulation with LPS (100 ng/ml). After 24 h, supernatants were collected for analysis of IL-23 production by ELISA. LPS versus LPS plus LT or LTK63: ***, $P < 0.001$. Data are the mean ± SEM of triplicate stimulations and are representative of at least two independent experiments.

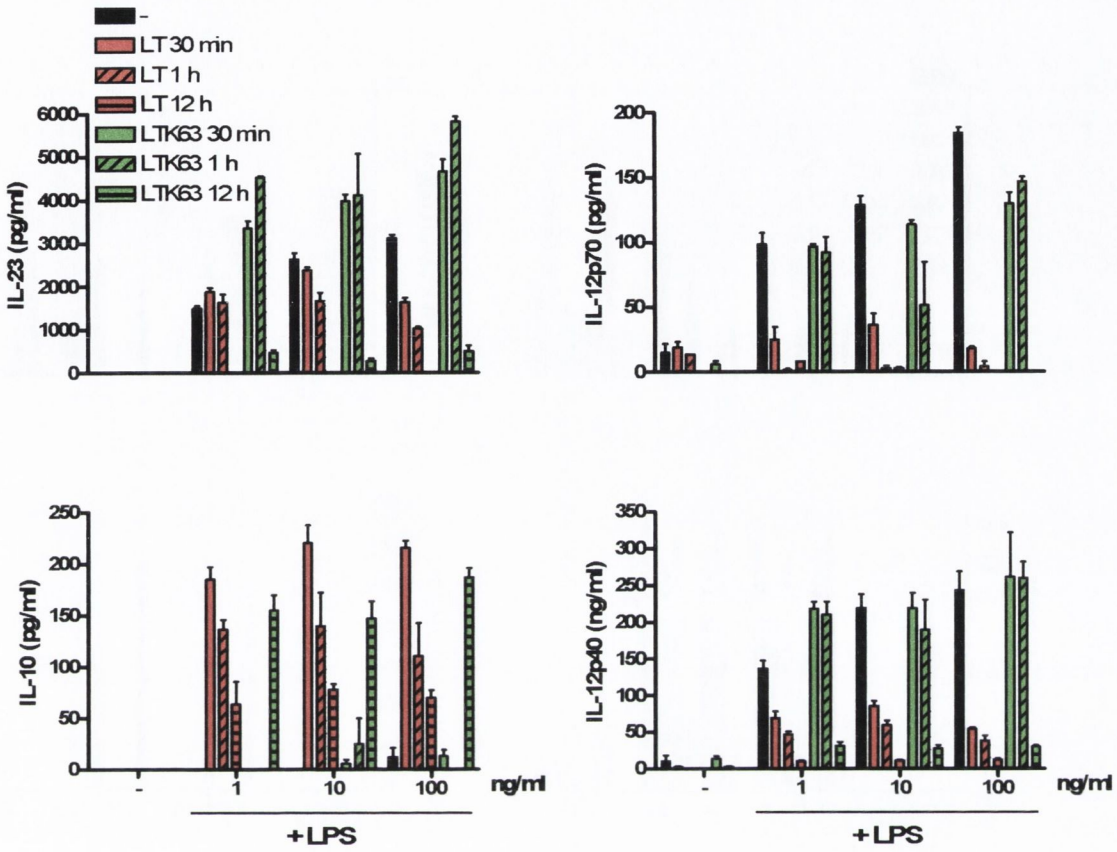

Figure 3.13 Pre-incubation with LTK63 for 30 min - 1 h enhances LPS-induced IL-23 production. BMDC (5 x 10^5 cells/ml) from BALB/c mice were pre-incubated with LT (1 μg/ml) or LTK63 (1 μg/ml) for 30 min, 1 or 12 h prior to stimulation with LPS (1, 10 or 100 ng/ml) for 24 h. Supernatants were collected for analysis of IL-12p40, IL-12p70, IL-23p19, and IL-10 production by ELISA. Data are the mean ± SEM of triplicate stimulations and are representative of two independent experiments.

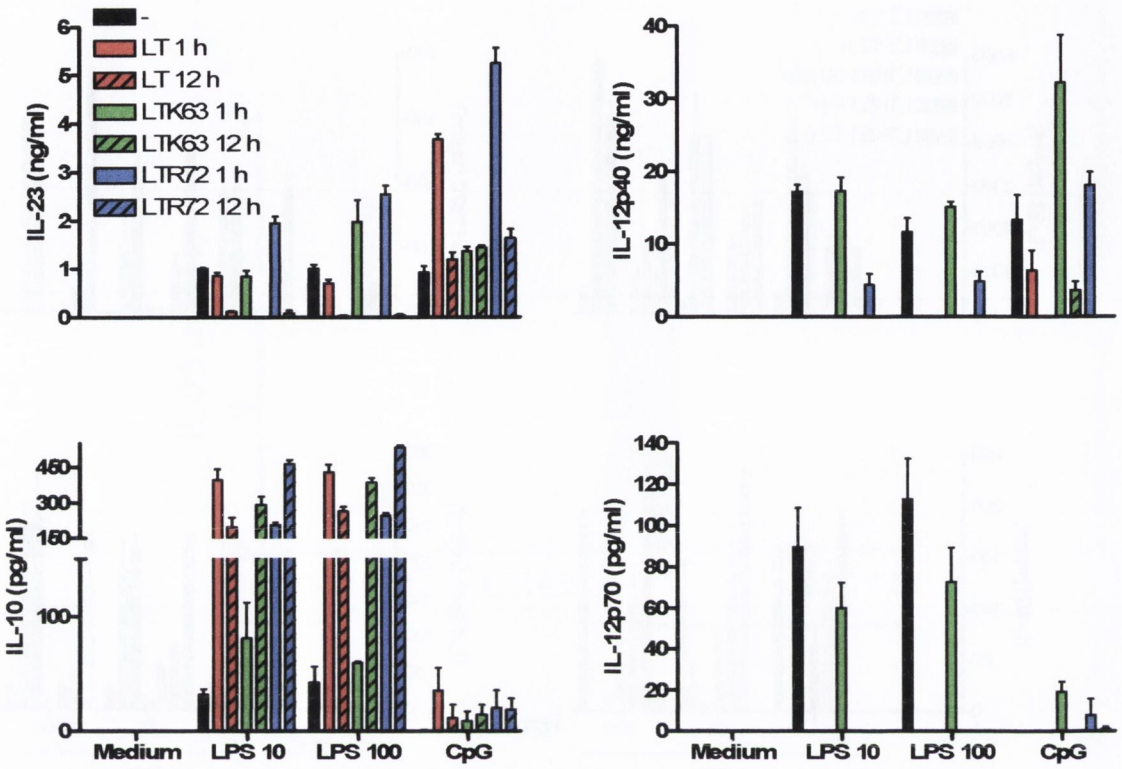

Figure 3.14 LT, LTK63 and LTR72 increase CpG-induced IL-23 but inhibit CpG-induced IL-12 production. BMDC (5 x 10^5 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml) or LTR72 (1 μg/ml) for 1 or 12 h prior to stimulation with LPS (10 or 100 ng/ml) or CpG (1 μg/ml). After 24 h, supernatants were collected for analysis of IL-12p40, IL-10, IL-12p70 and IL-23 production by ELISA. Data are the mean ± SEM of triplicate stimulations and are representative of two independent experiments.

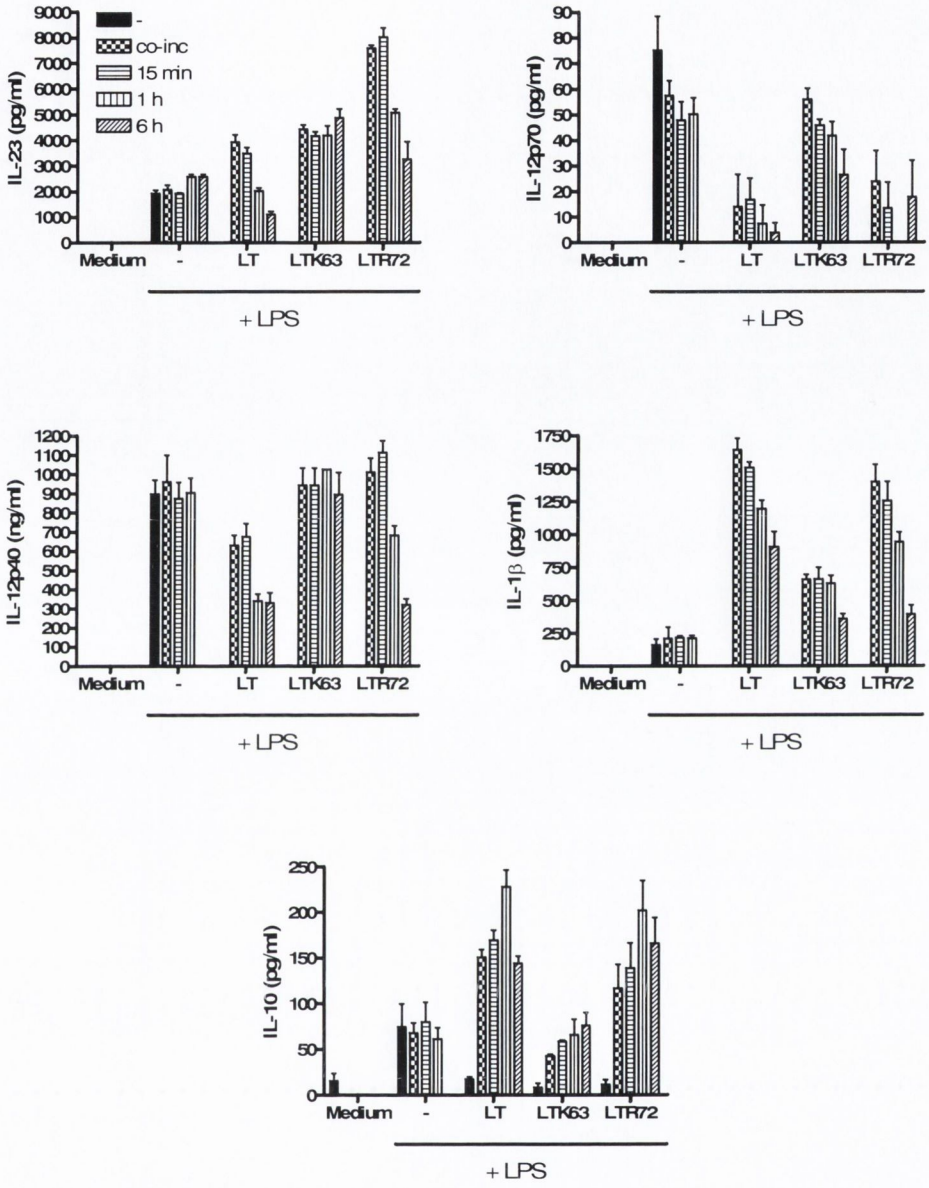

Figure 3.15 Co-incubation of DC with LPS and LT, LTK63 or LTR72 increases LPS-induced IL-23 production. BMDC (5 x 10^5 cells/ml) from BALB/c mice were co-incubated with LT (1 μg/ml), LTK63 (1 μg/ml) or LTR72 (1 μg/ml) or pre-incubated for 15 min, 1 or 6 h prior to stimulation with LPS (100 ng/ml). After 24 h supernatants were collected for analysis of IL-12p40, IL-12p70, IL-23, IL-10 and IL-1β production by ELISA. Data are the mean ± SEM of triplicate stimulations.

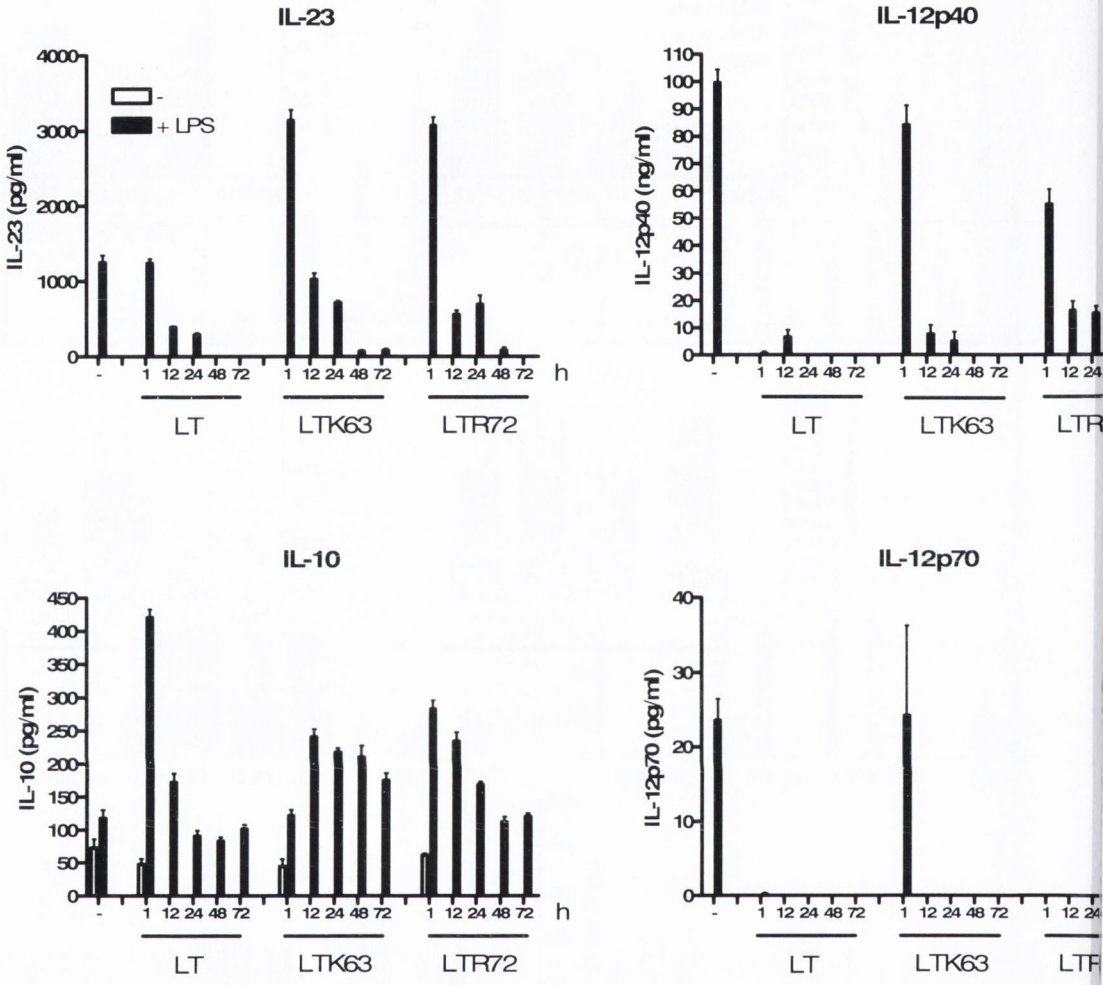

Figure 3.16 LT, LTK63 and LTR72 inhibit LPS-induced IL-12 by DC when cultured with the molecules for up to 4 d. BMDC (5 x 10^5 cells/ml) from BALB/c mice were incubated with LT, LTK63 or LTR72 (1 μg/ml) for 1, 12, 24, 48, 72 h prior to stimulation with LPS (100 ng/ml). After 24 h supernatants were collected for analysis of IL-12p40, IL-10, IL-23 and IL-12p70 production by ELISA. Data are the mean ± SEM of triplicate stimulations.

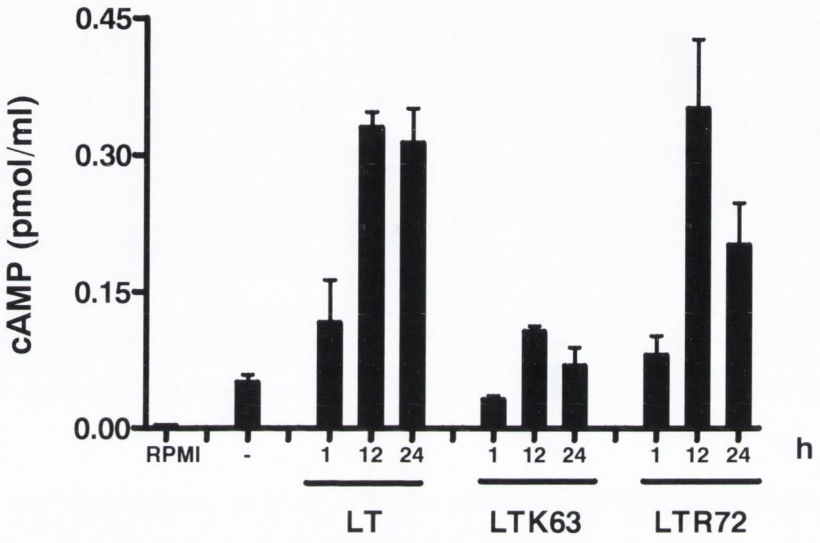

Figure 3.17 The ability of LT to increase intracellular cAMP is not completely dependent on its catalytic activity. BMDC (5 x 10^5 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LTR72 (1 μg/ml) or medium only (Con). Cells were collected after 1, 12 and 24 h and lysates were assayed for cAMP concentration. RPMI medium was also included as a control in the assay. Data are the mean ± SEM of triplicate stimulations assayed in duplicate and are representative of two independent experiments.

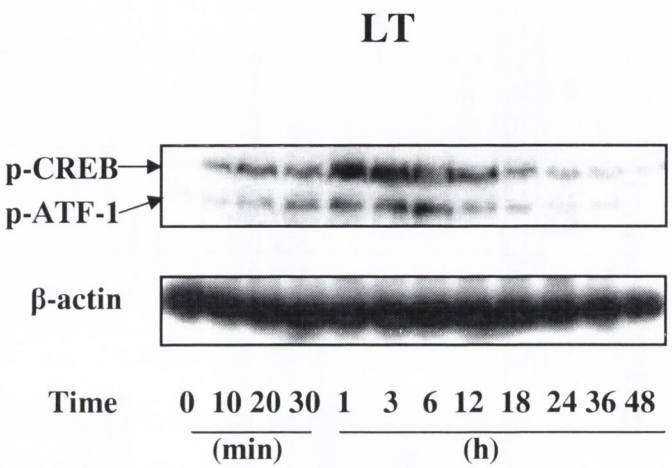

Figure 3.18 LT induces phosphorylation of CREB and ATF-1 in DC. BMDC (1 x 10^6 cells/ml) from BALB/c mice were stimulated with LT (1 μg/ml) for 10 min to 48 h. Cell lysates were probed with specific antibodies for p-CREB and β-actin (as a loading control) by Western blot analysis.

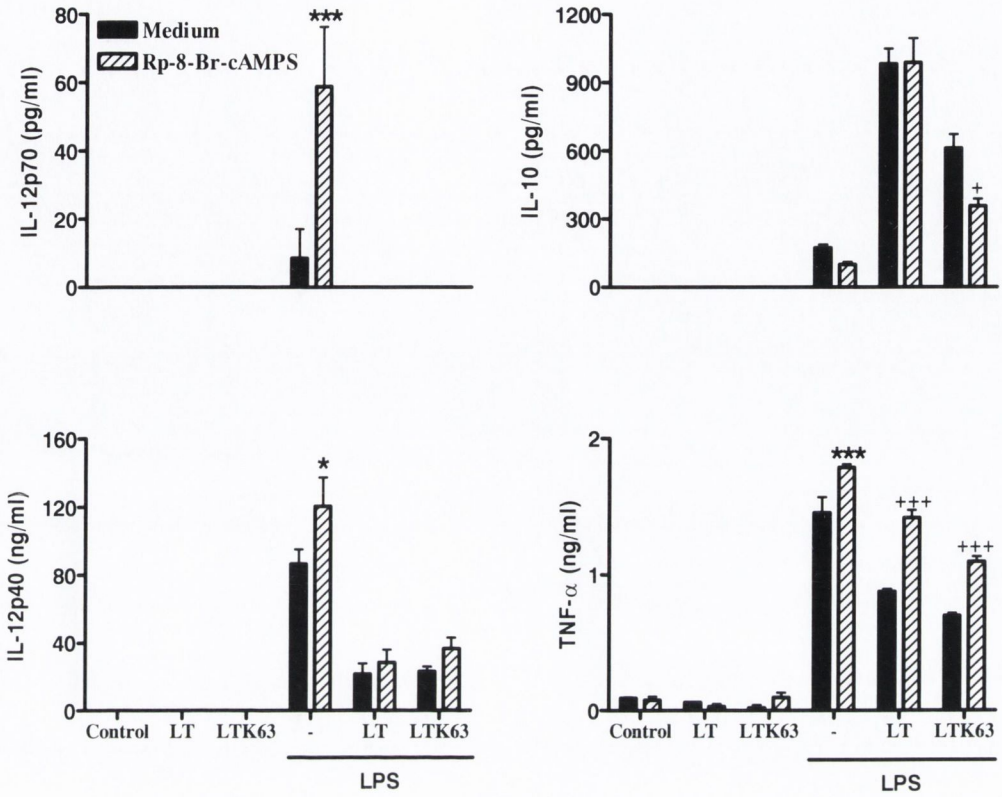

Figure 3.19 Enhancement of IL-10 and inhibition of IL-12 by LT or LTK63 is PKA-independent. BMDC (5 x 10^5 cells/ml) from BALB/c mice were incubated with the selective PKA inhibitor, Rp-8-Br-cAMPs (1 mM), for 6 h prior to a 1 or 12 h pre-treatment with LT (1 μg/ml) or LTK63 (1 μg/ml) respectively, followed by stimulation with LPS (5 ng/ml). After 24 h, supernatants were collected for analysis of IL-12p70, IL-12p40, IL-10 and TNF-α production by ELISA. LPS versus LPS + Rp-8-Br-cAMPs: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. LPS + LT/LTK63 versus LPS + LT/LTK63 + Rp-8-Br-cAMPs: +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$. Data are the mean ± SEM of triplicate stimulations and are representative of two independent experiments.

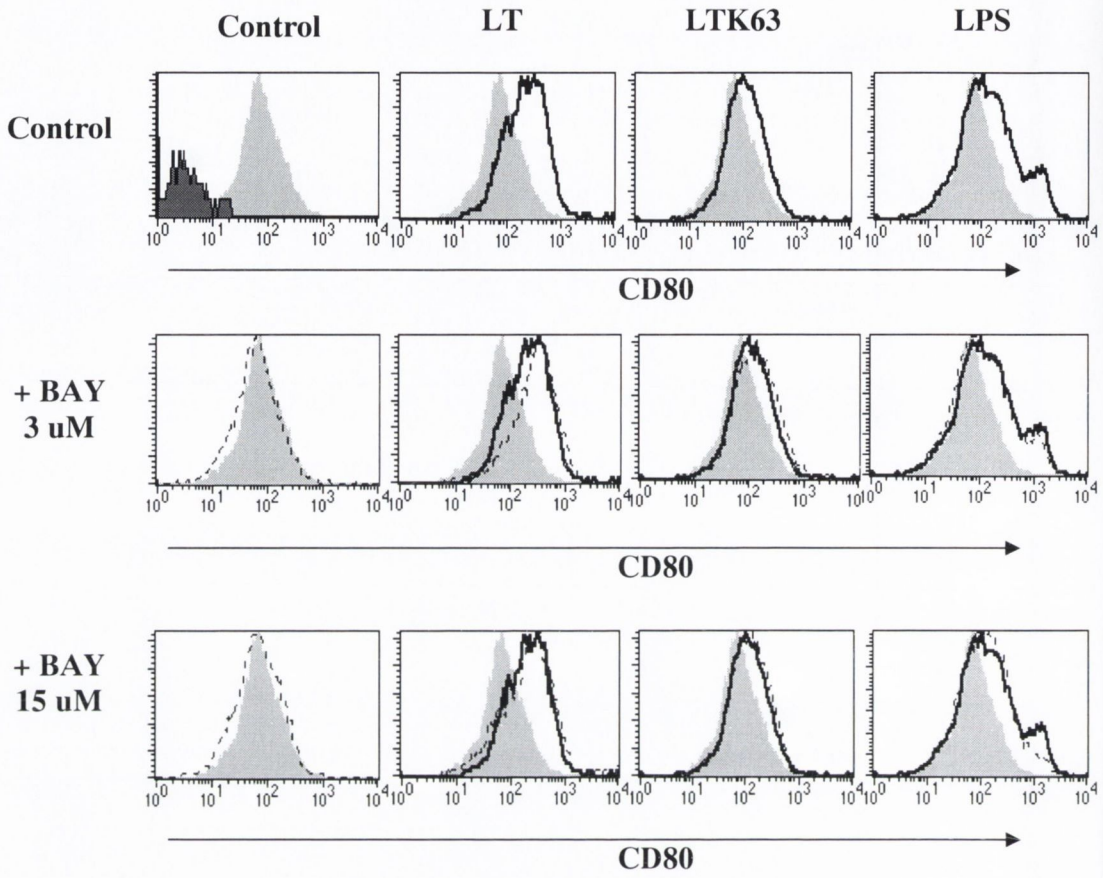

Figure 3.20 Upregulation of CD80 expression by LT, LTK63 and LPS is NFκB-independent. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with BAY 11-7082 (3 or 15 μM). Cells were harvested after 24 h and stained with antibodies specific for CD80 or with the isotype matched control. Results from immunofluorescence analysis are shown as histograms and are representative of two independent experiments. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype controls, the thick black line represents cells stimulated with LT, LTK63, or LPS and the black dashed line represents cells treated with BAY 11-7082.

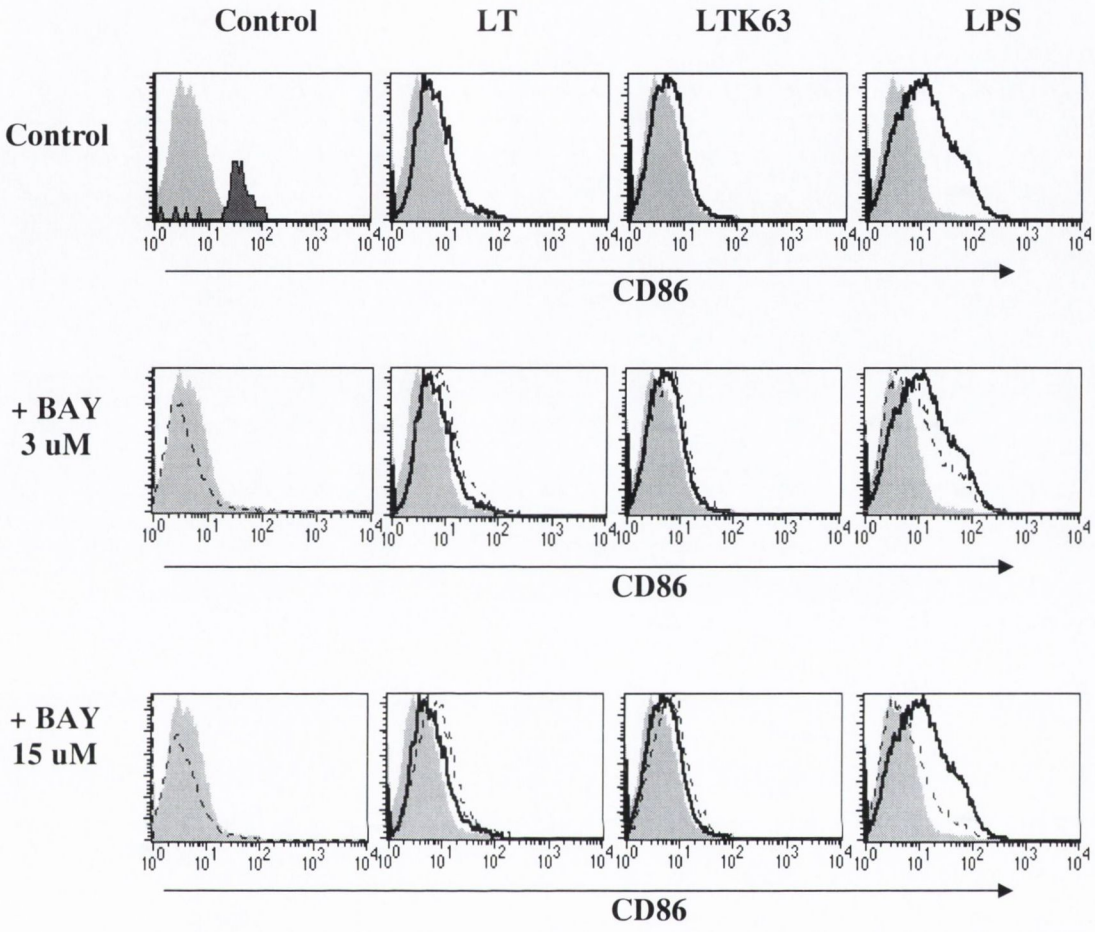

Figure 3.21 Upregulation of CD86 expression by LT and LTK63 is NFκB-independent, while upregulation of CD86 expression by LPS is NFκB-dependent. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with BAY 11-7082 (3 or 15 μM). Cells were harvested after 24 h and stained with antibodies specific for CD86 or with the isotype matched control. Results from immunofluorescence analysis are shown as histograms and are representative of two independent experiments. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents cells stimulated with LT, LTK63 or LPS and the black dashed line represents cells treated with BAY 11-7082.

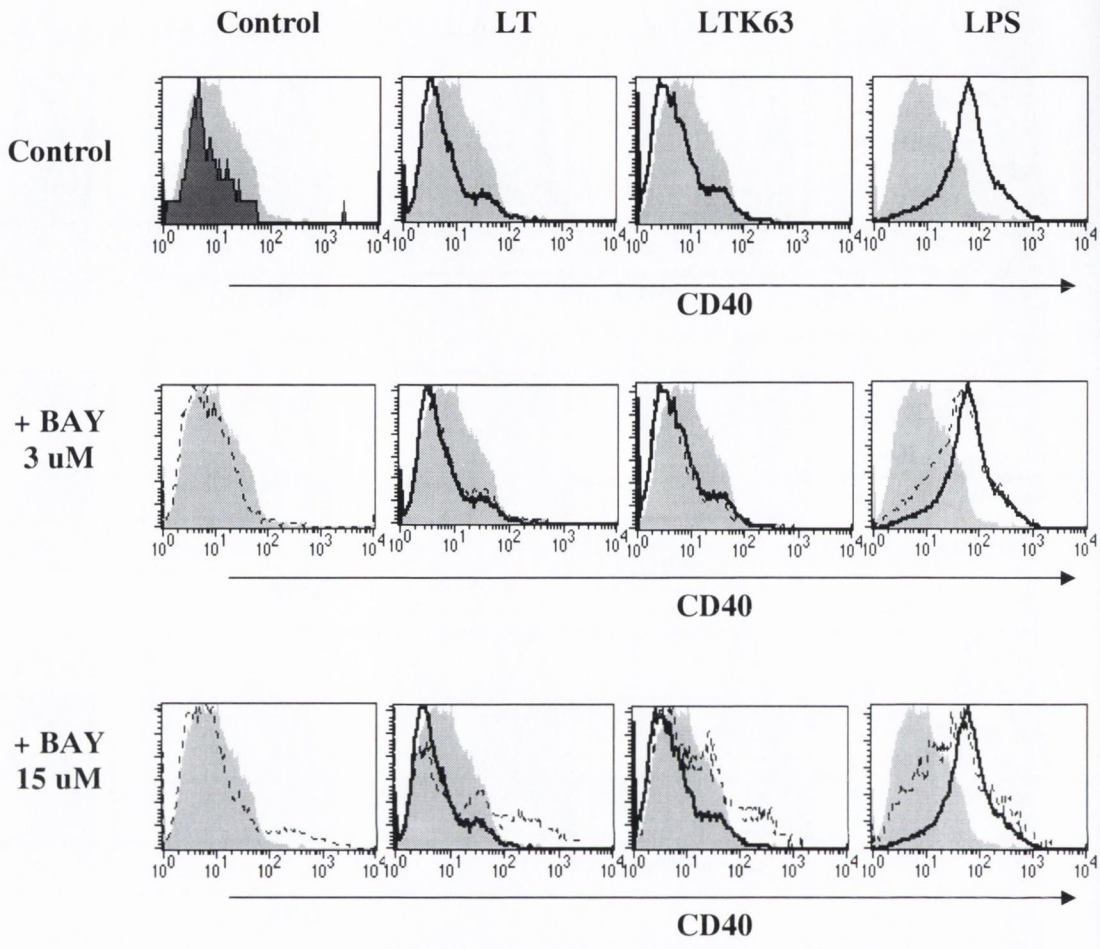

Figure 3.22 Enhancement of CD40 expression by LPS is partially NFκB-dependent. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with BAY 11-7082 (3 or 15 μM). Cells were harvested after 24 h and stained with antibodies specific for CD40 or with the isotype matched control. Results from immunofluorescence analysis are shown as histograms and are representative of two independent experiments. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents cells stimulated with LT, LTK63 or LPS and the black dashed line represents cells treated with BAY 11-7082.

Chapter 4

Mechanism of action of LT

4.1 INTRODUCTION

The innate immune response to pathogens can shape the adaptive immune response, through the stimulation of DC, which act as APC to direct the differentiation of naïve T cells. Activation of DC through PRRs results in their maturation, characterized by upregulation of MHC class II, co-stimulatory molecules including, CD80, CD86, and CD40, but also activation of inflammatory cytokine production, including IL-12 and TNF-α. The maturation and cytokine production by DC enables them to direct the induction of naïve T cells. In the classical model of DC maturation, the immature DC (iDC) internalize exogenous antigens and process them for MHC class II-mediated presentation, but cannot prime T cell responses (70, 364). iDC express the chemokine receptors, CCR5 and CCR6, and low levels of MHC class II and the co-stimulatory molecules, CD80 and CD86. Upon activation, iDCs migrate to lymph nodes, whilst undergoing a maturation process, which shuts off their antigen capturing and processing capacity. After antigen acquisition by the DC, the expression of the chemokine receptors CCR5 and CCR6 is downregulated, while CCR7 is upregulated. This enhances the responsiveness of DC to the CCR7 ligands, CCL19 (MIP-3β) and CCL21 (secondary lymphoid tissue chemokine, SLC) that are produced in the T cell areas of the lymph nodes, and as such CCR7 is a key receptor for the encounter of antigen bearing mature DC and responder T cells (390). Mature DCs express high levels of MHC molecules and the co-stimulatory molecules, CD86, CD80, CD40 and CD83 (62, 72). Expression of these molecules renders the DCs capable of T cell priming and therefore they are termed "immunogenic". Maturation signals can be provided by PAMPs, such as LPS and CpG motifs, CD40L expressed on T cells or IL-1 and TNF-α, secreted by macrophages in response to pathogen-derived molecules.

The intracellular signalling pathways involved in the modulation of co-stimulatory molecule expression by the enterotoxins are poorly understood. The upregulation of co-stimulatory molecules on DC and macrophages by LPS is reported to be NFκB-dependent (391, 392). Using adenoviral transfer of IκBα, it was demonstrated that blocking NFκB function inhibited antigen-presentation by DC (388). The contribution of specific components of the NFκB pathway to the upregulation of co-stimulatory molecule

expression has been examined using knockout mice. A number of studies have shown that TLR4- and TLR3- induced upregulation of CD80, CD86 and CD40 is MyD88-independent. Hoebe *et al.* demonstrated that LPS-TLR4-mediated upregulation of these molecules required TRIF and type 1 IFN signalling. In contrast, TLR3-mediated upregulation of co-stimulatory molecules and MHC Class II was only partly dependent on the TRIF-IFN-β-IFNR-1 axis (387). Kawamura *et al.* reported that CT activated DCs through GM1-binding which resulted in NFκB translocation (370). However, the findings presented in chapter 3 demonstrated that upregulation of CD80 and CD86 in response to LT or LTK63 was NFκB-independent.

Much of the published work points to a role for elevation of intracellular cAMP in the adjuvanticity of the toxins, however, the results shown in chapter 3 established that LT could modulate cytokine production by DC independently of its enzyme activity. The data showed that, similar to wild-type toxin, the enzymatically inactive derivative of LT, LTK63 inhibited LPS-induced IL-12 and enhanced LPS-induced IL-10 production. However, a significant finding was that the modulation of cytokine production by LTK63 was more delayed compared with LT. LTK63 required 12 h to suppress IL-12 production, while LT could exert this effect very rapidly. In contrast to the delayed suppression of IL-12 and enhancement of IL-10 by LTK63, co-incubation or a short pre-treatment with LTK63 or LT augmented TLR-agonist induced IL-23 production by DC. Furthermore, the data established that the suppression of IL-12 by LT or LTK63 was independent of PKA, the major downstream target of cAMP. In addition, the enhancement of IL-10 production by LT was PKA-independent. It is well documented that MAPK signalling plays an essential role in regulating cytokine production in APCs (189, 190, 312). Specifically, it has been reported that ERK MAP kinase negatively regulates IL-12 production and that IL-10 production is p38-dependent (190, 312). The PI3K pathway also plays a role in the regulation of IL-12 production. DC from $PI3K^{-/-}$ mice produced more IL-12 in response to TLR agonists compared with DC from wild-type mice (334).

The aim of this study was to examine the role of MAPK signalling in the ability of LT to modulate the production of pro- and anti- inflammatory cytokines by DC, particularly the enhancement of LPS-induced IL-10 and inhibition of LPS-induced IL-12

secretion. Furthermore, this study aimed to elucidate the intracellular signalling pathways underlying LT-induced DC maturation.

4.2 RESULTS

4.2.1 LT and LTK63 induce phosphorylation of ERK and increase the expression of c-Fos in DC

A number of studies have demonstrated that components of the MAPK pathway play a major role in the regulation of innate and adaptive immune responses, including the modulation of DC and macrophages that promote Th1/Th2 cell differentiation (119, 190, 312). In particular, the MAP kinase ERK and the immediate early gene c-Fos have been shown to have an important role in regulating IL-12 production by DC (189). To determine if LT and LTK63 activate ERK and c-Fos, BMDC were stimulated with LT or LTK63 and antibodies specific for p-ERK and c-Fos were used to detect these proteins by Western blotting. LT and LTK63 induced early activation of both ERK1 and ERK2 (p44/p42) which was sustained for 6 h (Fig. 4.1). Both LT and LTK63 increased the expression of c-Fos although with different kinetics. c-Fos expression was greatest at 3 h and 6 h, following stimulation with LT, and at 12–18 h after stimulation with LTK63 (Fig. 4.1).

4.2.2 Enhancement of LPS-induced IL-10 production by LT or LTK63 is partially ERK dependent, while inhibition of IL-12 is ERK-independent

Having shown that both LT and LTK63 activate phosphorylation of ERK and increase c-Fos expression, experiments were carried out to determine if their modulatory effects on LPS-induced cytokine production were ERK dependent. U0126 is a specific inhibitor of the dual specificity kinases MEK1 and MEK2, with IC50 values of 72 nM and 58 nM for MEK1 and MEK2 respectively (393). This inhibitor is widely used to block the activation of ERK1 and 2. Similar to other reports preliminary experiments revealed that pre-treatment of cells with U0126 at 5 - 10 μM was required for total inhibition of TLR-stimulated phosphorylation of ERK1/2 in DC. BMDC were pre-treated

with the MEK1/2 inhibitor, U0126, prior to incubation with LT or LTK63 followed by stimulation with LPS for 24 h. Inhibition of ERK significantly enhanced LPS-induced IL-12p70 production, but did not reverse the inhibitory effect of LT and LTK63 on LPS-induced IL-12p70 production (Fig. 4.2). In contrast to the enhancing effect of ERK inhibition on bioactive IL-12 production, the MEK1/2 inhibitor attenuated LPS-induced IL-12p40 production. Furthermore, the inhibition of IL-12p40 by LT or LTK63 was more pronounced in the presence of the MEK1/2 inhibitor. LPS-induced IL-10 production was slightly inhibited in cells pre-treated with U0126 and the enhancement of LPS-induced IL-10 by LT or LTK63 was partially inhibited by the MEK1/2 inhibitor. The MEK1/2 inhibitor also reduced LPS-induced TNF-α production and exacerbated the inhibition of LPS-induced TNF-α production by LT or LTK63 (Fig. 4.2). These findings indicate that ERK plays a negative role in IL-12p70 production by DC but is not responsible for the inhibition of LPS-induced IL-12 production by LT or LTK63.

4.2.3 LT-induced c-Fos expression is only partially ERK-dependent, but LTB-induced c-Fos expression is totally ERK-dependent

Although the effects of LT and LTK63 on IL-12 and IL-10 production were not reversed by the MEK1/2 inhibitor, this did not exclude a role for c-Fos. Experiments were therefore carried out to examine the effect of ERK inhibition on LT-induced c-Fos expression. The role of the isolated B subunit of LT, LTB, in the modulatory effects of the toxin was also investigated by examining ERK activation by LTB and its effect on c-Fos induction. LTB has been shown to act as a mucosal adjuvant, but promotes weaker responses than native LT or its site-directed mutants. It has been reported that binding of LT to its receptor alone is not sufficient to activate the signalling pathways responsible for the immunomodulatory properties of LTB (260). Both LT and LTB induced early activation of ERK which was sustained for 6 h. In addition, both LT and LTB increased the expression of c-Fos, although with different kinetics. c-Fos expression was increased between 1.5 h and 6 h following stimulation with LT, while LTB-induced expression of c-Fos was detected at 2.5 h and 6 h (Fig. 4.3). The MEK1/2 inhibitor U0126 completely

inhibited LT and LTB-induced activation of ERK activation. However, inhibition of ERK only partially blocked the enhancement of c-Fos expression in response to LT. In contrast, blocking ERK completely prevented the induction of c-Fos expression by LTB (Fig. 4.3). These findings demonstrate that LTB-induced c-Fos expression is ERK dependent, while c-Fos induction by wild type LT is only partially ERK dependent. This suggests that the wild-type LT can induce c-Fos expression through an alternative, ERK-independent mechanism.

4.2.4 Enhancement of IL-10 production by LPS-treated DC by LT, LTK63 or LTB is partially ERK1-dependent

While both LT and LTK63 activated ERK signalling, the modulatory effects of LT on DC cytokine production appear to be only partially ERK dependent. $ERK1^{-/-}$ BMDC were used to confirm these findings. Consistent with previous experiments, LT, LTK63 and LTB enhanced LPS-induced IL-10 production by DC from C57BL/6 mice (Fig. 4.4). As before, unlike LT, LTK63 required up to 12 h pre-incubation before LPS to significantly augment IL-10 and this was also the case for LTB. The enhancement of LPS-induced IL-10 production by C57BL/6 BMDC after 1 h pre-treatment with LT was diminished in $ERK1^{-/-}$ BMDC but still greater than that observed with DC stimulated with LPS alone. After a 12 h pre-treatment with LT, the enhancement of IL-10 was still observed in BMDC from wild-type mice, but to a lesser extent, and LT also retained its ability to augment LPS-induced IL-10 production by DC from $ERK1^{-/-}$ mice. Pre-incubation with LT for 24 h did not enhance LPS-induced IL-10 production by DC from wild-type mice, however an increase was observed in DC from $ERK1^{-/-}$ mice. Pre-treatment with LTK63 for 12 or 24 h enhanced LPS-induced IL-10 production by C57BL/6 BMDC. The enhancement of LPS-induced IL-10 production by LTK63 following 12 h pre-treatment was diminished but still evident in $ERK1^{-/-}$ BMDC. However, when DC were pre-incubated with LTK63 for 24 h the concentration of IL-10 induced by LTK63 and LPS was comparable between $ERK1^{-/-}$ and wild type BMDC. The augmentation of LPS-induced IL-10 by LTB, when DC were pre-incubated with

LTB for 12 h, was diminished, but still significant, in ERK1$^{-/-}$ BMDC compared with C57BL/6 BMDC (Fig. 4.4). Together with the results shown in Fig. 4.2, these findings demonstrate that inhibition of ERK plays some role in the regulation IL-10 production. However, as LT and its derivatives could synergize with LPS for the production of IL-10 in ERK1-deficient DC, this data indicates that activation of ERK1 is not the principal mechanism by which LT or LTK63 enhance LPS-induced IL-10 production.

4.2.5 The increase in c-Fos expression mediated by LT or LTK63 is only partially ERK1-dependent

Since both LT and LTK63 retained most of their ability to synergize with LPS for IL-10 production in ERK1$^{-/-}$ BMDC, experiments were carried out to determine if LT and LTB-induced c-Fos expression was altered in these cells. BMDC from C57BL/6 or ERK1$^{-/-}$ mice were treated for 6, 12 or 24 h with LT or LTK63. LT enhanced c-Fos expression at 6 h and this expression was only slightly diminished in ERK1$^{-/-}$ cells. LTK63 also enhanced c-Fos expression at 12 h, although not to the same extent as LT and this expression was still observed in ERK1$^{-/-}$ cells (Fig. 4.5). Therefore, although LTK63 induced the phosphorylation of both ERK1 and ERK2, it could synergize with LPS to drive the production of IL-10 in ERK1$^{-/-}$ BMDC. In addition, inhibition of ERK1 and ERK2, using the MEK1/2 inhibitor U0126, only partially inhibited the enhancement of LPS-induced IL-10 by LTK63. These findings suggest that both the enhancement of LPS-induced IL-10 and the induction of c-Fos in response to LTK63 are partially ERK-dependent.

4.2.6 LT and LTK63 induce phosphorylation of p38 in DC

The p38 MAP kinase signal transduction pathway is a highly conserved cascade, important for diverse aspects of the immune response. Studies with inhibitors of p38, as well as mice defective in MKK3 a major upstream kinase of p38, have implicated the p38

signalling pathway in LPS-induced IL-12 and TNF-α production in macrophages (319, 394) and DC (189). p38 also played a critical role in IL-10 production in response to TLR agonists (312). Therefore, experiments were carried out to examine the influence of LT and LTK63 on phosphorylation of p38. Phosphorylation of p38 was examined following stimulation of BMDC with LT or LTK63 for 10 min to 48 h. Both LT and LTK63 induced the activation of p38, as soon as 10 min after stimulation (Fig. 4.6). In contrast to the activation of ERK1/2 by both LT and LTK63 (Fig. 4.1), the activation of p38 gradually decreased after 10 min.

4.2.7 Enhancement of LPS-induced IL-10 production by LT or LTK63 is suppressed by the p38 inhibitor SB203580, while the inhibition of IL-12 is p38-independent

Having demonstrated that both LT and LTK63 induce phosphorylation of p38, a p38 inhibitor was used to examine the role of this kinase in the immunomodulatory effects of LT and LTK63. SB203580 is a widely used highly selective and cell permeable inhibitor of p38 MAPK with IC50 values of 50 and 500 nM for p38/SAPK2a and p38/SAPK2b respectively. Early experiments showed that SB203580 at > 5 μM completely inhibited LPS-induced phosphorylation of p38 in DC. BMDC were pre-treated with SB203580 prior to incubation with LT or LTK63, followed by stimulation with LPS for 24 h. The results in Fig. 4.7 demonstrate that inhibition of p38 enhanced LPS-induced IL-12p70 production, but did not affect IL-12p40 production. In contrast, the inhibition of LPS-induced IL-12p70 production by LT or LTK63 was not reversed by SB203580. However, inhibition of p38 partially reversed the suppression of LPS-induced IL-12p40 by LT or LTK63. LPS-induced IL-10 production was completely inhibited by SB203580. Blocking p38 prevented LT or LTK63 synergizing with LPS for the production of IL-10. Inhibition of p38 enhanced LPS-induced TNF-α production and partially reversed the inhibition of LPS-induced TNF-α production by LT or LTK63 (Fig. 4.7). These results show that p38 signalling is essential for IL-10 production, while p38 differentially regulates the production of the p40 and p35 subunits of IL-12.

These findings suggested that p38 negatively regulates LPS-induced IL-12 production by DC. To determine if this effect was specific for SB203580, experiments were carried out with an alternative commercially available synthetic inhibitor of p38 (Calbiochem). BMDC were pre-treated with SB203580 or with p38 MAP kinase inhibitor prior to stimulation with LT or LTK63 in the presence or absence of LPS. Both of the p38 inhibitors enhanced LPS-induced IL-12p70 and IL-12p40 production (Fig 4.8). Inhibition of LPS-induced IL-12p70 by LT or LTK63 was not reversed by either of the p38 inhibitors. In contrast, inhibition of IL-12p40 by LT or LTK63 was partially reversed by blocking p38. While pre-treatment of DC with either p38 inhibitor decreased IL-10 production in response to LPS in the presence or absence of LT or LTK63; inhibition of IL-10 was more significant with SB203580 (Fig. 4.8). Of note here is the observation that pre-incubation of DC with the Calbiochem p38 inhibitor augmented LPS-induced IL-12p70 to a greater extent than SB203580. Therefore, inhibition of p38 enhanced IL-12 production even when the p38 inhibitor had little effect on IL-10 production. These findings confirm that p38 signalling negatively regulates IL-12 production, but appears to be required for IL-10 secretion by DC. However, they also indicate that SB203580 exerts a potent suppressive effect on IL-10 in DC regardless of the stimulus, which suggests that the ability of LT to enhance IL-10 production may be independent of p38.

Having established that p38 or ERK inhibition alone did not alter the modulatory effect of LT or LTK63 on LPS-induced IL-12 production, experiments were carried out to determine the effect of blocking both p38 and ERK signalling. BMDC were pre-treated with the MEK1/2 inhibitor, U0126, or the p38 inhibitor, SB203580, alone or in combination, prior to stimulation with LT or LTK63 followed by LPS stimulation for 24 h (Fig. 4.9). Blocking both ERK and p38 resulted in enhanced production of IL-12p70 in response to LPS; the increase in IL-12p70 observed with the ERK and p38 inhibitors combined was greater than that observed with U0126 or SB203580 alone. The inhibition of LPS-induced IL-12p70 production by LT was not reversed when DC were pre-incubated with a combination of the ERK and p38 inhibitors. However, the suppression of LPS-induced IL-12p70 by LTK63 was partially reversed, though not significantly, in the presence of both ERK and p38 inhibitors. Inhibition of both ERK and p38 attenuated LPS-induced IL-12p40 production. However, inhibition of both ERK and p38 did not

affect the suppression of LPS-induced IL-12p40 by LT or LTK63. In contrast, inhibition of p38 with SB203580 completely suppressed IL-10 production in response to LPS, or LPS and LT or LTK63. Therefore, the inhibition of LPS-induced IL-12 production by LT or LTK63 is both ERK and p38 independent, while the enhancement of LPS-induced IL-10 production by DC is partially ERK dependent, but p38 appears to play a dominant role. These results suggest that the toxin uses different mechanisms to modulate signalling pathways involved in the promotion of IL-10 and inhibition of IL-12 in DC.

4.2.8 Enhancement of LPS-induced IL-10 production by LT or LTK63 is p38-dependent and partially ERK-dependent, while inhibition of IL-12 is p38- and ERK-independent in a macrophage cell line

The data presented in figures 4.7 and 4.8 indicated that p38 negatively regulates IL-12 production. However, a number of reports have shown that TLR agonist-induced IL-12 is mediated by p38 in macrophages and DC (189, 312, 395). Therefore, experiments were carried out in the J774 macrophage cell line to determine if the effects observed in DC were also seen in macrophages. J774 cells were pre-treated with the MEK1/2 inhibitor U0126 or the p38 inhibitor SB203580, alone or in combination, prior to incubation with LT or LTK63, followed by stimulation with LPS for 24 h. The results in Fig. 4.10 show that inhibition of ERK and p38 enhanced LPS-induced IL-12p40 production. Inhibition of LPS-induced IL-12p40 production by LT or LTK63 was not reversed by the MEK1/2 inhibitor but was partially reversed by the p38 inhibitor alone or in combination with the MEK1/2 inhibitor. In contrast, LPS-induced IL-10 production was inhibited by U0126 or SB203580 alone or in combination. The enhancement of LPS-induced IL-10 by LT or LTK63 was partially inhibited with the ERK or p38 inhibitor and completely inhibited when cells were pre-treated with both inhibitors (Fig. 4.10). These results demonstrate that both ERK and p38 play negative roles in LPS-induced IL-12 and suggest that the inhibition of LPS-induced IL-12 by LT or LTK63 in macrophages is p38 dependent. Furthermore, LPS-induced IL-10 production and its enhancement by LT or LTK63 in macrophages are both ERK and p38-dependent.

4.2.9 Activation of ERK plays an important role in LPS-induced IL-23 and IL-1β production by DC

IL-1β, together with IL-23, plays a crucial role in the regulation of Th17 cells (134). Having established that LT and LTK63 can induce IL-23p19 and IL-1β expression and increase LPS-induced IL-23 and IL-1β production by DC, the effect of inhibiting ERK on the ability of LT and LTK63 to modulate IL-1 and IL-23 was examined. BMDC were pre-treated with U0126 prior to incubation with LT or LTK63 for 15 min, 30 min, 1 or 6 h, followed by stimulation with LPS for 24 h. LPS-induced IL-23 and IL-1β production was decreased when DC were pre-incubated with the MEK1/2 inhibitor (Fig. 4.11). The enhancement of LPS-induced IL-23 production by LT, when DC were pre-treated for 15 min, was abolished in the presence of U0126. Moreover, the enhancement of LPS-induced IL-23 secretion by LTK63, which was evident up to 1 h pre-incubation, was also blocked by inhibition of ERK (Fig. 4.11). Similarly, the enhancement of LPS-induced IL-1β production by LT was diminished when DC were pre-incubated with the MEK1/2 inhibitor.

4.2.10 ERK activation plays a role in the ability of LT and LTK63 to enhance CD80 expression

Much of the published literature points towards an essential role for NFκB in regulating DC maturation, however, a number of other signalling pathways have been reported to be involved in the upregulation of co-stimulatory molecules on APC in response to LPS, including ERK, p38 MAPK, p38SAPK, and Raf1 (396). The previous data demonstrated that the enhancement of LPS-induced IL-10 production by LT and LTK63 was partially ERK-dependent. To investigate the role of MAPK signalling in the modulation of CD80, CD86, CD40 or ICAM-1 expression by LT, BMDC were pre-treated with the MEK1/2 inhibitor, U0126, the p38 inhibitor, SB203580, or the JNK inhibitor II before stimulation with LT (1 μg/ml), LTK63 (1 μg/ml) or LPS (100 ng/ml) for 24 h.

Incubation of DC with any of the MAPK inhibitors alone did not affect the expression of CD80, CD86, CD40 or ICAM-1 on DC (Figs 4.12-4.15). LT- and LTK63-increased the expression of CD80 and this enhancement was partially suppressed in the presence of the MEK1/2 inhibitor (Fig. 4.12). The p38 or JNK inhibitors did not affect the upregulation of CD80 expression by LT or LTK63, indicating that ERK plays a specific role in mediating the enhancement of CD80 by LT. Inhibition of ERK, p38 or JNK had no effect on LPS-induced CD80 expression. Similarly, the enhancement of CD86 by LT, LTK63 or LPS was not affected by blocking ERK, p38 or JNK (Fig. 4.13).

Inhibition of ERK, p38 or JNK had no effect on endogenous CD40 expression. Stimulation of DC with LPS enhanced CD40 expression and there was no effect in the presence of the ERK, p38 or JNK inhibitor (Fig. 4.14). Incubation of DC with SB203580 or JNK inhibitor II alone did not affect ICAM-1 surface expression while blocking ERK slightly decreased ICAM-1 on DC incubated in medium alone. The upregulation of ICAM-1 by LPS and the suppression of ICAM-1 expression by LT or LTK63 were not reversed in the presence of the ERK, p38 or JNK inhibitors (Fig. 4.15).

These findings demonstrate that the upregulation of CD80 by LT is partially ERK-dependent but independent of p38 and JNK activation. However, inhibition of ERK, p38 or JNK had no effect on the enhancement of CD80 by LPS. The data indicate that MAPK signalling does not play a role in the regulation of CD86, CD40 or ICAM-1 expression.

4.2.11 The enhancement of LPS-induced IL-10 and inhibition of LPS-induced IL-12 production by LT or LTK63 is PI3K-dependent

It has recently been established that PI3K signalling negatively regulates IL-12 production by DC (334). One of the principal targets of PI3K is the serine/threonine kinase Akt. Stimulation of certain receptors, such as RTKs, integrins and GPCRs, induces PI3K-mediated signalling cascades leading to activation of Akt, which is associated with increased phosphorylation of Ser-473 and Thr-308 of Akt (397). Phosphorylation of Akt is often used as a downstream readout for activation of PI3K signalling, therefore, the effect of LT and LTK63 on Akt phosphorylation in DC was examined. BMDC were stimulated with LT or LTK63 for 15 min to 8 h in the presence

or absence of the PI3K inhibitor, LY294002. LT and LTK63 induced activation of p-Akt, which was strongest at 1 h and was sustained for at least 8 h (Fig. 4.16). Pre-incubation with LY294002 partially inhibited LT- or LTK63- mediated phosphorylation of Akt (Fig. 4.16).

To assess if PI3K activation by LT and LTK63 is responsible for their immunomodulatory effects, BMDC were pre-treated with the PI3K inhibitor, LY294002, prior to incubation with LT or LTK63, followed by stimulation with LPS. LPS-induced IL-12p40 production was inhibited in the presence of LY294002 (Fig. 4.17). Furthermore, the inhibition of LPS-induced IL-12p40 by LT or LTK63 was unaffected by LY294002. Likewise, LY294002 inhibited LPS-induced IL-12p70 and the inhibition of IL-12p70 by LT or LTK63 was not reversed by this PI3K inhibitor. LPS-induced IL-10 production by DC was completely inhibited by LY294002 and the synergy between LT/LTK63 and LPS for the production of IL-10 was also completely inhibited by this PI3K inhibitor. TNF-α production by LPS-treated DC was unaffected by LY294002 and the inhibition of LPS-induced TNF-α by LT was slightly reversed by LY294002, while LY294002 had no effect on the inhibition of LPS-induced TNF-α production by LTK63. These findings demonstrate that the enhancement of LPS-induced IL-10 production by LT or LTK63 is PI3K-dependent, and indicate that, in contrast to previous reports, inhibition of PI3K with LY294002 inhibited IL-12 production by DC.

The effect of PI3K inhibition on IL-12 production was also examined using an alternative inhibitor, Wortmannin. In contrast to the effect of LY294002, LPS-induced IL-12p40 and IL-12p70 production was enhanced in the presence of the PI3K inhibitor Wortmannin (Fig. 4.18). Furthermore, the inhibition of LPS-induced IL-12p40 and IL-12p70 by LT or LTK63 was completely reversed by inhibition of PI3K. LPS-induced IL-10 production by DC was partially inhibited by Wortmannin, and the enhancement of LPS-induced IL-10 by LT or LTK63 was significantly inhibited by the PI3K inhibitor (Fig. 4.18). These data are consistent with the suppressive effect of PI3K inhibitor LY294002 on IL-10 production. Similar to observations with LY294002, inhibition of PI3K with Wortmannin had little effect on LPS-induced TNF-α production however, the suppression of LPS-induced TNF-α by LT or LTK63 was reversed by Wortmannin (Fig. 4.18).

Overall, these findings demonstrate that the enhancement of LPS-induced IL-10 production by LT or LTK63 is PI3K-dependent. Inhibition of PI3K using two different inhibitors, LY294002 and Wortmannin, had distinct effects on IL-12 production. However, the data obtained using Wortmannin are consistent with reports in $PI3K^{-/-}$ DC that implicate PI3K as a negative regulator of IL-12 secretion.

4.2.12 The PI3K inhibitors LY294002 and Wortmannin differentially regulate phosphorylation of GSK3 in DC

The results shown in Figs 4.17 and 4.18 indicated that the PI3K inhibitors, Wortmannin and LY294002, have different effects on IL-12 production by DC. These results were surprising given that $PI3K^{-/-}$ DC show increased IL-12 production and as such warranted further investigation into the effects of the inhibitors on DC responses. GSK3 is an ubiquitously expressed serine/threonine protein kinase that phosphorylates and inactivates glycogen synthase. The activity of GSK3 can be reduced by phosphorylation at Ser21 of GSK3α and Ser9 of GSK3β. Several kinases have been shown to be capable of mediating this modification, including p90Rsk (also called MAPKAP kinase-1), Akt, certain isoforms of PKC, and PKA (398). Activation of the PI3K pathway can result in the phosphorylation of GSK3 and p-GSK3 is often used as a marker for PI3K induction. Therefore, experiments were carried out to investigate the effects of the two PI3K inhibitors on p-GSK3β expression in DC. BMDC were stimulated with LT or LTK63 for 15 min to 8 h in the presence or absence of LY294002 or Wortmannin and Western blotting was performed on cell lysates to detect p-GSK3β using an antibody specific for p-GSK3β (Ser 9). High constitutive expression of p-GSK3 was observed in DC incubated in medium alone, therefore no clear effect was observed following stimulation with LT or LTK63. This is consistent with previous reports that suggest that serum-containing medium increases p-GSK3β expression (399). However, reduced p-GSK3β expression was observed in LT or LTK63-treated DC, or cells incubated with medium only, in the presence of LY294002 (Fig. 4.19). The inhibition of

p-GSK3β in LT- or LTK63- stimulated DC by Wortmannin was less pronounced than that observed with LY294002 (Fig. 4.19).

These findings suggest that these two PI3K inhibitors may have different mechanisms of action and may exhibit different target specificities and as such may exert more potent inhibitory effects on different signalling molecules in the PI3K cascade. As there are multiple mechanisms that can regulate the inactivation of GSK3 via Ser 9 phosphorylation, inhibition of PI3K alone may not prevent other signalling molecules maintaining the activity of GSK3.

4.2.13 PI3K negatively regulates IL-23 and IL-12 production by DC

Previous results indicated a differential effect of the PI3K inhibitors, Wortmannin and LY294002, on IL-12 production, however, both inhibitors decreased the expression of p-GSK3β and suppressed LPS-induced IL-10 secretion by DC. Therefore, the next study examined the effect of inhibitor concentration and pre-incubation time of LY294002 on LPS-induced IL-12 production by DC as compared with the effects of Wortmannin. BMDC were pre-treated with LY294002 (5 μM) or Wortmannin (0.1 or 1 μM) for 1 or 12 h prior to incubation with LT or LTK63 (1 μg/ml) for 1 or 12 h respectively, before stimulation with LPS (100 ng/ml) for 24 h.

The data in Fig. 4.17 demonstrated that LY294002 suppressed LPS-induced IL-12 secretion. A number of reports in the literature documented the use of LY294002 at a concentration range of 10-50 μM, therefore, in earlier experiments DC were incubated with 20 or 40 μM LY294002. However, a dose response with LY294002 revealed that at < 5 μM LY294002 enhanced LPS-induced IL-12 secretion. The data presented in Fig 4.21 revealed that pre-treatment of DC with LY294002 at 5 μM for 1 h increased LPS-induced IL-12p40 production, which mimics the enhancing effect of Wortmannin. The ability of Wortmannin or LY294002 to increase IL-12p40 production was lost when DC were pre-treated for 12 h prior to LPS stimulation, but IL-12p70 production remained elevated, in the presence of Wortmannin, albeit to a lesser extent. The suppressive effect of LT or LTK63 on LPS-induced IL-12p70 was partially reversed when DC were pre-

treated with Wortmannin (1 μM) for 1 h prior to LT or LTK63 (Fig. 4.20). However, the reversal of suppression was diminished when DC were pre-treated with either inhibitor for 12 h. This is similar to the observation that IL-12p70 production by DC treated with LPS alone was only slightly increased when the cells were cultured with either Wortmannin or LY294002 for 12 h prior to stimulation with LPS. However, the suppression of LPS-induced IL-12p40 production by LT or LTK63 was partially reversed when DC were incubated with either Wortmannin or LY294002 for 1 or 12 h prior to stimulation (Fig. 4.21).

In this experiment, LPS did not increase IL-10 production, and as expected Wortmannin or LY294002 did not induce IL-10 production by DC (Fig. 4.20). Here, pre-treatment with LT for 1 h did not increase IL-10 production by LPS-stimulated DC. Surprisingly, 1 h pre-treatment with Wortmannin, before stimulation with LT and LPS, increased IL-10 production. This suggests that Wortmannin requires longer than an hour to exert its inhibitory effect on IL-10 production. DC that were left to rest for 12 h before stimulation with LT produced IL-10 when stimulated with LPS (Fig 4.20). Pre-treatment with LY294002 or Wortmannin for 12 h suppressed the enhancement of LPS-induced IL-10 production by LT. These data are consistent with previous results that showed that the enhancement of IL-10 production by LT is PI3K-dependent. However, the results indicate that Wortmannin requires longer than 1 h to effectively inhibit PI3K signalling in DC.

The results in Figs 3.14 and 3.15 demonstrated that LT enhanced LPS- and CpG-IL-23 production when DC were co-incubated or pre-incubated for a short time before TLR-stimulation, but inhibited IL-23 secretion when the pre-incubation time was increased to 1 h. Therefore, the effect of inhibiting PI3K on the delayed suppressive effect of LT on IL-23 production was examined in DC. Inhibition of PI3K with Wortmannin or LY294002 increased LPS-induced IL-23 production by DC that were pre-incubated with either inhibitor for 1 h. However, pre-treatment of DC with Wortmannin for 12 h prior to LPS did not enhance IL-23 production, while LY294002 retained its ability to augment IL-23 even after 12 h in culture before further stimulation. Incubation of DC with either PI3K inhibitor for 1 or 12 h reversed the suppressive effect of LT (1 h pre-treatment) or LTK63 (12 h pre-treatment) on LPS-induced IL-23 production. These

data suggest that the length of time in culture and the inhibitor concentration are particularly important when examining the effect of these PI3K inhibitors.

A significant finding of this study is that inhibition of PI3K enhances the production of the IL-12 family member IL-23 by DC. Furthermore, together with data presented earlier, these results demonstrate that PI3K plays a major role in mediating the suppressive effect of LT and LTK63 on LPS-induced IL-12 and their enhancement of LPS-induced IL-10 production.

4.2.14 Upregulation of CD80 expression by LT and LTK63 is mediated by PI3K whereas PI3K play a negative role in CD86 expression on DC

The previous data established that PI3K signalling is central to the ability of LT and LTK63 to modulate LPS-induced cytokine production. The inhibition of LPS-induced IL-12 and the enhancement of LPS-induced IL-10 production by LT and LTK63 are PI3K-dependent. To investigate if PI3K signalling plays a role in the ability of LT to increase CD80 and CD86 and decrease CD40 expression, BMDC were pre-treated with either of two PI3K inhibitors, Wortmannin or LY294002, before stimulation with LT, LK63 or LPS for 24 h.

Wortmannin decreased the expression of CD80 on DC cultured in medium only (Fig. 4.22), suggesting that PI3K is required for the endogenous expression of CD80 on the cell surface. Likewise, LY294002, at a concentration of 40 μM, decreased CD80 expression on DC incubated with medium alone, although not to the same extent as Wortmannin (Fig. 4.23). The upregulation of CD80 expression by LT and LTK63 was unaffected by either Wortmannin at 1 μM or LY294002 at 20 μM, but the enhancement of CD80 expression in response to LT and LTK63 was inhibited by both Wortmannin and LY294002 at higher concentrations. This indicates that upregulation of CD80 by LT and LTK63 is PI3K-dependent. The upregulation of CD80 by LPS was slightly inhibited by 1 μM Wortmannin, but slightly enhanced at 10 μM (Fig. 4.22). The upregulation of CD80 expression by LPS was markedly enhanced by LY294002 at both concentrations (Fig. 4.23). These findings demonstrate that PI3K plays a negative role in LPS-induced

upregulation of CD80 expression on BMDC, while the ability of LT and LTK63 to increase CD80 expression is PI3K-dependent.

In contrast to the effect on CD80 expression, the upregulation of CD86 expression by LT or LTK63 was further enhanced by pre-treatment with either Wortmannin or LY294002 (Figs. 4.24 and 4.25). The level of CD86 expression on DC cultured in medium alone was unaffected by Wortmannin at 1 μM or LY294002 at 20 μM, although a slight increase was observed at higher concentrations of either inhibitor (Figs. 4.24 and 4.25). Inhibition of PI3K signalling with either Wortmannin or LY294002 also increased LPS-induced CD86 expression (Figs 4.24 and 4.25). Therefore, PI3K signalling negatively regulates CD86 expression in response to LT, LTK63 and LPS.

4.2.15 PI3K plays a role in the enhancement of LPS-induced CD40 expression

In contrast to the upregulation of CD40 expression by LPS, LT selectively decreases the expression of CD40 on DC, and this inhibition is independent of the enzyme activity of the toxin (Fig. 3.6). To examine the role of PI3K signalling in the suppression of CD40 by LT or LTK63, BMDC were pre-treated with either Wortmannin or LY294002 prior to incubation with LT, LTK63 or LPS for 24 h.

Inhibition of PI3K with Wortmannin (0.1 μM) or LY294002 (20 μM) alone had little effect on CD40 expression on DC. LY294002 at 40 μM enhanced CD40 expression on DC incubated with medium only. In contrast, Wortmannin at 1 μM decreased CD40 expression on DC (Figs. 4.26 and 4.27). Wortmannin or LY294002 had a minimal effect on the inhibition of CD40 expression by LT and LTK63 (Figs. 4.26 and 4.27). Unlike LT and LTK63, LPS upregulates CD40 expression. Pre-incubation of BMDC with Wortmannin had no effect on the enhancement of CD40 expression by LPS (Fig. 4.26). In contrast, pre-treatment with LY294002 decreased LPS-induced upregulation of CD40 expression (Fig. 4.27). These results indicate that PI3K signalling plays a limited role the enhancement of CD40 expression by LPS.

4.2.16 GSK3 suppresses IL-10 production by DC

GSK3 is a key regulator of several transcription factors including NFκB, AP-1, NF-AT and CREB, and therefore controls the expression of numerous genes involved in innate immune responses (398). GSK3 signalling has been shown to activate the transcription factor NFκB and inhibit the transcription factor CREB (171). SB-216763 is a selective inhibitor of GSK3α and GSK3β and has been shown to have no affect on the activity of other kinases including Akt and PDK1. To examine the effect of inhibiting GSK3 on the immunomodulatory effects of LT and LTK63, BMDC were pre-treated with the GSK3 inhibitor, SB-216763, prior to incubation with LT or LTK63, followed by LPS stimulation for 24 h. The results in Fig. 4.28 show that LPS-induced IL-12p40 and IL-12p70 production were decreased in the presence of the GSK3 inhibitor. Inhibition of GSK3 had no effect on the suppression of LPS-induced IL-12p40 or IL-12p70 production by LT or LTK63. However, LPS-induced IL-10 production from BMDC was enhanced in the presence of the GSK3 inhibitor and IL-10 production was increased in BMDC stimulated with LT or LTK63 and LPS. TNF-α production by LPS-treated BMDC and the inhibition of LPS-induced TNF-α by LT was unaffected by the GSK-3 inhibitor. However, the inhibition of LPS-induced TNF-α production by LTK63 was partially reversed by the GSK3 inhibitor (Fig. 4.28). These findings suggest that PI3K-mediated inactivation of GSK-3 enhances IL-10 production by LPS-treated DC.

4.2.17 Upregulation of CD80 expression by LT and LTK63 is further enhanced by GSK3 inhibition

Having established that inhibition of GSK-3 using the inhibitor, SB-216763, suppressed LPS-induced IL-12 but enhanced LPS-induced IL-10 production, experiments were carried out to investigate the role of blocking GSK3 on the surface expression of CD80 and CD86 expression. BMDC were pre-treated with the GSK3 inhibitor (10 or 20 μM) before stimulation with LT (1 μg/ml), LTK63 (1 μg/ml) or LPS (100 ng/ml) for 24 h.

Incubation of DC with the GSK3 inhibitor alone had little affect on CD80 expression. The enhancement of CD80 expression on LT-, LTK63- or LPS-stimulated

DC was further increased in the presence of the GSK3 inhibitor (Fig. 4.29). This is consistent with the observation that blocking PI3K, which allows GSK3 to remain active, decreased CD80 expression in DC stimulated with LT or LTK63. DC incubated with the GSK3 inhibitor alone expressed increased CD86 on their surface. Inhibition of GSK3 also enhanced the upregulation of CD86 expression induced by LT, LTK63 or LPS (Fig. 4.30). This result was surprising as PI3K inhibition increased the expression of CD86 on DC stimulated with LT, LTK63 or LPS. This might be explained by the enhancing effect of the inhibitor on the endogenous expression of CD86 suggesting that GSK3 does not play a specific role in mediating LT-, LTK63- or LPS-induced upregulation of CD86.

The enhancement of LT- and LTK63-induced CD80 expression by the GSK3 inhibitor also suggests that upregulation of CD80 may be mediated through PI3K activation and subsequent inhibition of GSK-3 signalling.

4.2.18 Inhibition of GSK3 enhances endogenous DC CD40 and ICAM-1 expression

In contrast to the upregulation of CD40 expression by LPS, LT selectively decreases the expression of CD40 on DC, and this inhibition is independent of the enzyme activity of the toxin (Fig. 4.6). To examine the effect of blocking GSK3 on the suppression of CD40 by LT or LTK63, BMDC were pre-treated with the GSK3 inhibitor prior to incubation with LT, LTK63 or LPS, as a positive control, for 24 h.

Inhibition of GSK3 increased CD40 expression on DC incubated in medium alone (Fig 4.31). DC pre-treated with LT or LTK63 also expressed increased levels of CD40 in the presence of the GSK3 inhibitor. However, this increase is possibly as a result of the effect of GSK3 inhibition on the endogenous expression of CD40. Unlike LT and LTK63, LPS upregulates CD40 expression. Pre-incubation of BMDC with the GSK3 inhibitor at 10 μM had no effect on the enhancement of CD40 expression by LPS. Increasing the concentration of the inhibitor to 20 μM resulted in slight suppression of LPS-induced CD40 (Fig. 4.31).

Incubation of BMDC with the GSK3 inhibitor alone increased ICAM-1 expression, suggesting that PI3K negatively regulates ICAM-1 expression (Fig. 4.31). DC stimulated with LPS expressed increased ICAM-1 while LT and LTK63 inhibited ICAM-1 expression. However, the expression of ICAM-1 by LPS-stimulated DC was

inhibited when DC were pre-treated with the GSK-3 inhibitor. The inhibition of ICAM-1 by LT was unaffected by GSK3 inhibition while the suppression of ICAM-1 by LTK63 was reversed in the presence of the GSK3 inhibitor. However, this effect might be due to the effect of the inhibitor on endogenous ICAM-1 expression. These findings demonstrate that PI3K negatively regulate CD40 and ICAM-1 expression.

4.2.19 LT and LTK63 phosphorylate JNK

It has been reported that JNK and PI3K both play a role in the suppression of IL-12 production upon GiPCR triggering (400). Having established that PI3K is essential for the inhibition of LPS-induced IL-12 production by LT and LTK63, experiments were undertaken to investigate the role of JNK activation downstream of PI3K, in the immunomodulatory effects of LT and LTK63. BMDC were stimulated with LT or LTK63 for 15 min to 8 h in the presence or absence of the PI3K inhibitor, LY294002. The results in Fig. 4.33 show that LT- and LTK63- induced activation of p-Akt peaked at 1 h and was sustained for at least 8 h. The PI3K inhibitor LY294002 partially inhibited LT or LTK63-induced p-Akt, however, p-Akt was still detectable 1 h after stimulation with LTK63 and 1, 2 and 8 h after stimulation with LT in the presence of the inhibitor. Phosphorylation of JNK was detected rapidly after stimulation and was sustained in cells treated with LT or LTK63 (Fig. 4.33). Inhibition of PI3K had little effect on LT- or LTK63- induced p-JNK activation (Fig. 4.33). These results indicate that LT or LTK63 phosphorylate JNK but this activation is not mediated by the PI3K pathway.

4.2.20 LT increases c-Fos and c-Jun expression in BMDC

Activation of JNK results in phosphorylation of c-Jun, which can dimerize with c-Fos and activate the transcription factor AP-1. AP-1 refers to a family of transcription factors composed of homo- or hetero- dimer protein complexes, primarily consisting of members of the Jun, Fos, or ATF families of proteins that are encoded by immediate early genes. AP-1 regulates a diverse array of genes and is itself strictly regulated by

phosphorylation by a number of kinases including JNK, ERK, PKC and activated Ras, among others (398). In contrast, GSK3β has been implicated in the phosphorylation and negative regulation of AP-1 activation and has been shown to be a transcriptional inhibitor of the IL-12p40 gene (401, 402). Having established that LT increases c-Fos expression, c-Jun activation was investigated by stimulating BMDC with LT for 10 min to 48 h. LT increased the expression of c-Fos with dramatic expression observed at 3 and 6 h (Fig 4.34). LT increased the expression of c-Jun with the highest level of expression at 3 h (Fig. 4.34). These data demonstrate that LT can activate c-Fos and c-Jun two of the key components of AP-1 which has shown to have a suppressive effect on IL-12 production.

4.2.21 LT- and LT63- induced IL-23p19 expression is ERK-dependent and negatively regulated by PI3K

The data presented in Fig. 3.10 showed that LT and LTK63 induce the expression of IL-23p19 in DC. Furthermore, LT and LTK63 enhanced LPS- and CpG-induced IL-23 production (Fig. 3.14). Having established that PI3K plays an important role in mediating the suppressive effect of LT on IL-12 production and the enhancement of IL-10 production, in which the MAPK ERK was also shown to play a part, a role for these signalling molecules in the induction of p19 expression by LT and LTK63 was investigated. BMDC were pre-incubated with the MEK1/2 inhibitor, U0126 (5 μM) or either of the PI3K inhibitors, Wortmannin (1 μM) or LY294002 (5 μM) for 1 h prior to stimulation with LT or LTK63 for 12 h. Total RNA was isolated from DC, purified, reverse transcribed into cDNA, and amplified by real-time PCR for the detection of IL-23p19 mRNA expression.

LT augmented p19 mRNA expression ~ 30 fold over the level of p19 mRNA expressed in DC incubated with medium alone (Fig. 4.35). Inhibition of ERK suppressed the induction of p19 by LT. Similarly, while LTK63 only induced a 10-fold increase in p19 mRNA expression, this was completely inhibited when ERK was blocked. In contrast, inhibition of PI3K dramatically increased LT-induced p19 mRNA expression.

Inhibition of PI3K also enhanced LTK63-induced p19 mRNA expression, although not to the same extent as LT-induced p19 mRNA.

This data establishes an important role for MAPK ERK in the induction of p19 expression by LT. Furthermore, it reveals PI3K as a negative regulator of IL-23p19 expression in DC, which is consistent with the negative regulation of IL-12p40, IL-12p70 and IL-23 production through activation of this signalling pathway.

4.2.22 PKC signalling is required for the upregulation of CD80 and CD86 in response to LT, LTK63 and LPS

The data presented in Figs 4.12 and 4.23 demonstrated a role for ERK and PI3K signalling in mediating the upregulation of CD80, but not CD86, in response to LT. It has been reported that inhibition of PKC blocked LTB-mediated upregulation of CD25 expression on B cells (389). It is well established that particular isoforms of PKC regulate components of the NFκB pathway. Having established that the ability of LT and LTK63 to upregulate CD80 and CD86 is NFκB-independent, experiments were carried out to investigate the role of PKC in toxin-mediated DC maturation.

Preliminary investigations into the role of PKC in DC maturation revealed that that blocking PKC with rottlerin, a novel PKC isoform specific inhibitor, prevented the upregulation of CD80 by CT or LPS. In the present study, BMDC were pre-treated with rottlerin prior to stimulation with LT, LTK63 or LPS. Rottlerin marginally reduced the expression of CD86 on DC incubated in medium alone, but substantially reduced the expression of CD80 (Figs. 4.36 and 4.37). The upregulation of CD80 expression by LT, LTK63 and LPS was completely inhibited by rottlerin (Fig. 4.36). Similarly, the enhancement of CD86 expression on DC by LT and LTK63 was inhibited by pre-treatment with rottlerin. Partial inhibition of CD86 expression was observed at 0.01 mM rottlerin, while complete inhibition was observed at 0.1 mM (Fig. 4.37). Furthermore, LPS-induced upregulation of CD86 and CD80 expression was completely inhibited by rottlerin. These results demonstrate that PKC signalling, specifically that mediated by novel PKC isoforms, plays an essential role in LT and LPS -induced upregulation of the co-stimulatory molecules CD80 and CD86 on BMDC. As shown in Fig. 3.21, the enhancement of CD86 expression by LPS is also NFκB-dependent, as such there may be

crosstalk between components of the NFκB pathway and PKC in the upregulation of CD86 in response to TLR4 activation. In contrast, the upregulation of CD86 expression in response to LT or LTK63 was NFκB-independent but PKC-dependent, indicating that activation of PKC by LT could be targeting a downstream effector, other than NFκB, for the induction of CD86 expression.

4.2.23 Inhibition of nPKCs suppresses the enhancement of CD40 expression by LPS

Having established that nPKCs play a major role in the regulation of CD80 and CD86 expression on BMDC, experiments were carried out to investigate the role of PKC signalling in the modulation of CD40 expression by LT, LTK63 and LPS. BMDC were pre-treated with rottlerin before stimulation with LT, LTK63 or LPS. At 0.01 mM, rottlerin decreased CD40 expression on DC treated with the inhibitor only (Fig. 4.38), while at 0.1 mM, rottlerin enhanced CD40 expression.

Inhibition of nPKCs with rottlerin at 0.01 mM had a minimal affect on the suppression of CD40 expression by LT and LTK63, when compared with cells treated with the inhibitor alone (Fig. 4.38). Inhibition of PKC with rottlerin at 0.1 mM increased CD40 expression on DC stimulated with LT or LTK63. However, the level of enhancement was comparable to that observed on DC treated with rottlerin alone, therefore the suppression of CD40 by LT or LTK63 appears to be attenuated when cells were pre-treated with rottlerin at this concentration. The enhancement of CD40 expression by LPS was inhibited by rottlerin, demonstrating that nPKCs are required for the upregulation of CD40 expression by LPS. As shown in Fig. 3.22 the enhancement of CD40 expression by LPS is NFκB-dependent, indicating that PKC signalling may be involved in the activation of NFκB leading to the induction of CD40 expression. These results indicate that PKC signalling is required for endogenous CD40 expression on DC and demonstrate that the enhancement of CD40 expression by LPS is nPKC-dependent.

4.3 DISCUSSION

Maturation of DC into competent APCs capable of presenting antigen and eliciting the differentiation of naïve T cells is dependent upon their activation in response to microbial-derived molecules or endogenous danger signals. DC are activated by signalling through PRRs, which results in the upregulation of MHC class II and co-stimulatory molecules, and the production of cytokines that direct the induction of different Th cell subtypes (58). TLRs are one class of PRR and it is well established that stimulation of the TLR family on DC leads to the upregulation of co-stimulatory molecules, including CD80, CD86 and CD40 (403). Activation of TLR signalling also induces the production of various cytokines that control Th cell differentiation (66). Members of the TLR family have been shown to induce IL-12 production by DC and promote the induction of Th1 responses (94). However, research in the past number of years has highlighted that signalling downstream of different TLRs has distinct effects on IL-12 production and in addition, that non-TLR signalling can also induce cytokine production by DC (376). Furthermore, the recent discovery of IL-23 and IL-27, two new members of the IL-12 family, has shed light on the complexity of factors directing Th cell differentiation and prompted a reassessment of the relative contribution of these cytokines in the regulation of Th1 responses, but also of Th17 cells, a newly identified Th cell population (126).

IL-12 stimulates the development of IFN-γ-producing $CD4^+$ Th1 cells, which are key to controlling the immune response against intracellular pathogens. Until recently, IL-12-dependent IFN-γ production was thought to be the mediator of autoimmune diseases, such as IBD, diabetes, MS and CIA (404, 405). As such, there is a fine balance between promoting resistance to pathogens and contributing to organ-specific autoimmunity and therefore, tight regulation of IL-12 production is essential. A number of factors have been shown to inhibit IL-12 production, including IL-10, TGF-β, activation of CD47 by thrombospondin, complement components C3b and iC3b acting via CD46 and CR3, and activation of Fc receptors by immune complexes (94). Furthermore, IL-12 suppression can be achieved by the engagement of GPCRs (406). The inhibitory effect of many of these factors has been attributed IL-10 and the induction

of cAMP. However, a number of reports on GiPCRs have shown that agonists that activate this pathway, including C5a and A_3R, can suppress IL-12 in an IL-10-independent manner (407, 408).

It was initially reported that cAMP mediated the suppression of IL-12 production by PGE_2, histamine receptor 2 and adenosine receptor A2a (409, 410). Furthermore, CT, an activator of Gαs and cAMP, inhibited IL-12p70 production by murine DC (121) and human monocytes (277). The data presented in chapter 3 showed that like LT, LTK63, a site-directed, enzymatically inactive derivative of LT, suppressed IL-12p70 and IL-12p40 production by DC. These findings demonstrated that LT could inhibit IL-12 independently of its enzyme activity. However, the results also established that LTK63 can increase the concentration of intracellular cAMP in DC, suggesting that LTK63 can elevate cAMP in the absence of Gαs-mediated activation of AC. Until the present study there was limited knowledge on the mechanism of suppression of IL-12 by the enterotoxins LT and CT. The studies by Lavelle *et al.* demonstrated that inhibition of LPS-induced IL-12 production by CT was not mediated by IL-10, and Braun and colleagues reported that the suppression of IL-12p70 production by human monocytes stimulated with *S. aureus*, LPS or CD40L with IFN-γ was not dependent on IL-10, TGF-β or PGE_2 (121, 277). The findings in chapter 3 also showed that inhibition of LPS-induced IL-12 by LT was not reversed by blocking PKA, one of the principle mediators of cAMP activity.

MAPK signalling has been shown to play a major role in the regulation of innate and adaptive immune responses, including Th cell differentiation (189, 190). Different TLR agonists induce distinct Th responses in part via modulation of the ERK MAPK pathway in DC. TLR4 and TLR5 activation triggered IL-12p70 production by DC, however, IL-12 was not induced by the TLR2 agonist, Pam3cys (189). In addition, it was recently demonstrated that the TLR2 and dectin-1 agonist, zymosan, induced high concentrations of IL-10, but little or no IL-12 or IL-6 in human and murine DC (119). Since LT modulates TLR-activated DC and consequently influences Th cell polarization, it was important to determine if the toxin could activate MAPK signalling in DC. The findings demonstrated that both LT and LTK63 induced sustained activation of ERK in DC. It was previously shown that Pam-3-cys-mediated ERK activation enhanced the expression of the immediate early gene product c-Fos. DC from c-$fos^{-/-}$ mice secreted

much higher concentrations of IL-12p70, and lower concentrations of IL-10 than wild-type DC, following stimulation with *E.coli* LPS or Pam3cys (190), suggesting that c-Fos expression in DC plays a fundamental role in regulating Th1 and Th2 responses. Furthermore, macrophages from c-fos$^{-/-}$ mice produced significantly more IL-12p70 when compared with macrophages from wild type mice and transfection of RAW264.7 cells with a dominant-negative mutant of AP-1, a transcription factor comprised of Fos and Jun proteins, dramatically enhanced IL-12p40 production (402, 411). Consistent with this, the present study showed that both LT and LTK63 enhanced c-Fos expression. In line with the delayed effect of LTK63 in modulating cytokine production, LTK63 enhanced c-Fos expression after 12 h while LT increased its expression after 3 h.

Having established that LT and LTK63 activated ERK, this study examined the role of ERK in the immunomodulatory effects of the toxin. Surprisingly, the inhibition of LPS-induced IL-12p40 and IL-12p70 secretion by LT and LTK63 was ERK-independent, and the enhancement of LPS-induced IL-10 production by LT and LTK63 was only partially ERK-dependent. This conclusion is based on experiments with inhibitors and from studies in ERK1$^{-/-}$ DC. Inhibition of ERK also affected cytokine production by DC stimulated with LPS. Consistent with previous reports, inhibition of ERK enhanced LPS-induced IL-12p70 production (189, 190, 325). However, it is significant that in some experiments incubation of DC with U0126 had no effect on LPS-induced IL-12p70 production. This may not be surprising given that the concentration of IL-12p70 secreted by DC in response to LPS alone is usually low but also suggests that ERK may not be the dominant regulator of LPS-induced IL-12p70 secretion. Dillon *et al.* showed that IL-12p70 concentrations were significantly elevated by both LPS and Pam-3-cys in ERK1$^{-/-}$ DC when compared with wild-type mice. Furthermore, a concomitant decrease in IL-10 production was observed in DC from ERK1$^{-/-}$ mice (119). The authors suggested that ERK1 negatively regulates IL-12 production through the secretion of IL-10. The current study found that in contrast to the enhancement of LPS-induced IL-12p70, pre-treatment of DC with the MEK1/2 inhibitor U0126 did not significantly affect LPS-induced IL-12p40 production. Differential regulation of the IL-12 p40 and p70 subunits via ERK has been reported in murine peritoneal macrophages, whereby ERK negatively regulated LPS-induced IL-12p40 secretion, but did not alter IL-12p70 production (326). Studies on the role of LPS-induced calcium influx led to the discovery that calcium signalling

negatively regulated both p40 and p35 subunits. Unlike ERK, calcium influx inhibited I-κB degradation, thereby blocking NFκB activation, an essential component of IL-12 transcription. In addition, the current study also demonstrates that the suppression of LPS-induced IL-12p40 by LT or LTK63 was not reversed by blocking ERK in J774 macrophage cells, confirming that ERK activation is not the mechanism by which LT exerts its anti-inflammatory effects. A role for ERK in TNF-α production has been reported and in agreement with this, the present study found that inhibition of ERK decreased LPS-induced TNF-α production.

Although it was established that ERK was not responsible for the inhibition of IL-12 by LT, a role for c-Fos in the suppression of LPS-induced IL-12 could not be ruled out. Therefore, experiments were carried out to examine the effect of ERK inhibition on LT-induced c-Fos expression. In the absence of ERK, the ability of LT to enhance c-Fos, was reduced, but still detectable, demonstrating that c-Fos induction by LT is only partially ERK-dependent. LTB, the purified B subunit of the holotoxin with no enzyme activity, was used to investigate the contribution of the B subunit to the induction of ERK and c-Fos expression by LT. Like, LT and LTK63, LTB phosphorylated ERK and enhanced c-Fos in DC. However, in contrast to LT, ERK inhibition abolished the induction of c-Fos by LTB, demonstrating that LTB-induced c-Fos expression is ERK-dependent. These results suggest that activation c-Fos by LT is mediated by ERK-dependent signalling induced by the B subunit, as well as signal transduction mediated by the A subunit of the toxin in an ERK-independent manner. Furthermore, the data indicate that receptor binding by LTB triggers the activation of signalling pathways that are central to modulating innate immune responses. This is consistent with results demonstrating that LTB, but not a non-receptor binding mutant protein (EtxB(G33D)), induced ERK activation in B cells, which provides further evidence that the B subunit can activate intracellular signalling pathways in innate cells by binding to receptors on the cell surface (389).

It has been reported that p38 MAPK is a positive regulator of IL-12 production (311). A genetic deficiency in MKK3, which is one of the two specific MAPK kinases responsible for p38 phosphorylation, is associated with defective IL-12 production by macrophages and DC, and reduced LPS-induced macrophage IL-12p40 mRNA expression (319). In addition, the p38 inhibitor, SB203580, was found to suppress IL-

12p70 production by DC stimulated with LPS and IFN-γ (334). The present study revealed that both LT and LTK63 induced phosphorylation of p38 in DC. However, the inhibition of LPS-induced IL-12 by LT or LTK63 was not reversed by blocking p38. Inhibition of p38 significantly enhanced LPS-induced IL-12p70, but had little effect on LPS-induced IL-12p40 production. This is consistent with a report which demonstrated that inhibition of p38 using SB203580, enhanced LPS-induced IL-12 production from peritoneal macrophages, through a mechanism that was suggested to be partly through inhibition of IL-10 (412). Blocking p38 with SB203580 inhibited LPS-induced IL-10 production and the enhancement of LPS-induced IL-10 by LT or LTK63. The p38 inhibitor SB203580 completely suppressed IL-10 production by DC suggesting that this may mediate the enhancement of IL-12 production. Similar to the findings with DC, inhibition of p38 with SB203580 suppressed LPS-induced IL-10 production and the enhancement of LPS-induced IL-10 production by LT and LTK63 in J774 macrophages. Moreover, consistent with the findings in DC, inhibition of p38 with SB203580 enhanced LPS-induced IL-12p40. Blocking p38 reversed the inhibition of LPS-induced IL-12p40 by LTK63 in macrophages, and partially reversed the inhibition of LPS-induced IL-12p40 by LT. This suggests that p38 has a more significant role in the negative regulation of IL-12 in macrophages than in DC, and demonstrates that like ERK, p38 plays an endogenous anti-inflammatory role in these APCs. These results also demonstrate that the inhibition of LPS-induced IL-12 by LT and LTK63 is p38-independent.

Consistent with the results on ERK or p38 alone, inhibition of both ERK and p38 together did not reverse the suppression of LPS-induced IL-12 by LT in DC. However, the inhibition of LPS-induced IL-12p70 by LTK63 was partially reversed when cells were pre-treated with both SB203580 and U0126. Similarly, in J774 macrophages, blocking p38 and ERK partially reversed the inhibition of LPS-induced IL-12p40 by LTK63. In contrast to the observations with DC, the suppression of LPS-induced IL-12p40 by LT was reversed by inhibiting both ERK and p38 in the macrophage cell line. These findings confirm that the inhibition of LPS-induced IL-12p70 by LT is both ERK- and p38-independent in DC, but suggests that these MAPK may play a minor role in the suppression of LPS-induced IL-12 by LT and LTK63 in macrophages.

The observation that p38 played a negative role in IL-12p70 production in DC was at variance with a number of reports in the literature, and as such prompted further

investigation into the role of p38 in LPS-induced cytokine responses. Inhibition of p38 with SB203580 enhanced LPS-induced IL-12p40 and inhibited LPS-induced IL-10 production in DC and J774 macrophages. Experiments using an alternative, commercially available synthetic p38 inhibitor from Calbiochem revealed that both inhibitors of p38 increased LPS-induced IL-12p70 production by DC. Significantly, the Calbiochem p38 inhibitor was less effective than SB203580 at inhibiting LPS-induced IL-10 production, but enhanced LPS-induced IL-12p70 production to a greater extent. This suggests that in DC, p38 may inhibit IL-12 production independently of its suppressive effect on IL-10. It also indicates that SB203580 is a potent suppressor of IL-10, indicating that it may be targeting other signalling components required for the induction of IL-10 as well as p38. Indeed, Argast *et al.* reported that pyridinyl imidazole inhibitors of p38, including SB203580, inhibit the activity of RIP2 (413), a serine threonine kinase that has been shown to activate NFκB, JNK and ERK1/2 (414, 415). However, a more recent study showed that in human DC, RIP2 acts downstream of Raf and upstream of p38. In contrast to the inhibition of Raf1, SB203580 did not interfere with phosphorylation of p38 or IκBα, so the authors concluded that RIP2 kinase activity was not sensitive to SB203580 (416). In contrast to other reports, the authors reported that SB203580 inhibited p38 activity but not phosphorylation of p38 (395). Furthermore, while knockdown of RIP2 using siRNA in DC decreased LPS-induced p38 phosphorylation, it was not completely abolished. In addition, LPS-induced p-ERK and pIκBα were also reduced, indicating that RIP2 can activate kinases other than p38, which may be important in mediating its effects (416).

PI3K play an important role in the initial activation of innate immune responses by negatively regulating TLR signalling (417). $PI3K^{-/-}$ mice on a BALB/c background, have enhanced Th1 responses and are resistant to *Leishmania major* infection, unlike wild-type mice. Furthermore, DC from PI3K-deficient mice produced more IL-12 than wild-type DC, pointing towards a crucial negative regulatory role for PI3K during induction of Th1 responses, by suppressing IL-12 production by DC (334). The PI3K pathway has also been implicated in the regulation of MAPK signalling (418). The suppression of LPS and IFN-γ-induced IL-12p70 by GiPCR ligands was mediated by PI3K and JNK activation in human monocytes (400). Importantly, in B cells, LTB-induced ERK activation was PI3K-dependent (389).

The present study showed that LT and LTK63 phosphorylated Akt in DC and this was markedly reduced by the PI3K inhibitor LY294002. Preliminary experiments investigating the role of PI3K in the immunomodulatory effects of LT and LTK63 on LPS-induced cytokine production were carried out using the PI3K inhibitor LY294002, as Wortmannin is reported to be less stable in aqueous solution. In contrast to previous reports, inhibition of PI3K using LY294002 (20 or 40 μM) suppressed LPS-induced IL-12p40 and IL-12p70 production by DC (171). While the study by Martin *et al.* examined LPS-induced IL-12 production in human monocytes, earlier reports using $PI3K^{-/-}$ BMDC or the PI3K inhibitor Wortmannin also showed enhanced TLR-induced IL-12p70 production in the absence of PI3K (334, 400). Therefore, the experiments were repeated using Wortmannin. Inhibition of PI3K with Wortmannin enhanced LPS-induced IL-12p70 and IL-12p40 production by DC, which is in agreement with previous studies (334, 400). Differential effects of these two PI3K inhibitors have previously been reported. LY294002 potently increased IL-1-induced NFκB DNA binding, while Wortmannin only had a marginal effect (419).

Since these two PI3K inhibitors had different effects on LPS-induced IL-12 production, experiments were carried out to examine their effects on the expression of p-GSK3, a downstream target of PI3K-Akt signalling. While both inhibitors reduced p-GSK3 expression in DC, the suppression of p-GSK3β was more marked with LY294002, again demonstrating that these inhibitors may have differential effects. The results revealed that while LY294002 and Wortmannin inhibited p-GSK3β, a readout for PI3K signalling, they had opposing effects on IL-12. Contrary to the different effects of the PI3K inhibitors on LPS-induced IL-12, both Wortmannin and LY294002 inhibited LPS-induced IL-10 production, demonstrating an essential role for PI3K activation in the induction of IL-10 in DC.

Further investigation into the effects of LY294002 on IL-12 over a concentration range from 1-50 μM revealed that the use of this inhibitor at 20 μM may have been too high resulting in the suppression of IL-12, although LY294002 had no effect on LPS-induced TNF-α production, suggesting that the inhibitor was not having a global inhibitory effect on cytokine production. An experiment that directly compared the effect of LY294002, at a lower concentration (5 μM), and Wortmannin on LPS-induced IL-12 production demonstrated that like Wortmannin, pre-treatment of DC with LY294002 (5

μM) enhanced LPS-induced IL-12p40 and IL-12p70 secretion. A recent study examining the effect of PI3K inhibition on NFκB activity in monocytes revealed that LY294002 potently increased IL-1-induced NFκB DNA binding, but that Wortmannin only marginally enhanced NFκB DNA binding. The authors suggested that non-specific effects of Wortmannin may negatively affect NFκB DNA binding activity (419). In addition, it was reported that LY294002 blocks the phosphorylation of eIF4G, a scaffold protein for eIF4E and eIF4A involved in the assembly of the eIF4F complex, which mediates the initiation of translation. It appears therefore that these two PI3K inhibitors may be have different mechanisms of action and may also exhibit cell-type and stimulus specificity. Together with these reports, this study highlights that using small molecular weight inhibitors to elucidate signalling pathways requires careful preliminary investigations into the optimum concentration for specific targets and the pre-treatment time required to exert their inhibitory effects.

The present study revealed the suppression of LPS-induced IL-12p70 and IL-12p40 production by LT and LTK63 was completely reversed by blocking PI3K with Wortmannin. Significantly, Wortmannin had a greater attenuating effect on the suppression of LPS-induced IL-12 production by LTK63 compared with that observed with LT. These data suggest that LT is a more potent inhibitor of IL-12 production than its enzymatically inactive derivative LTK63. Similar to Wortmannin, inhibition of PI3K using LY294002 at 5 μM partially reversed the suppression of LPS-induced IL-12p70 and IL-12p40 production by LT or LTK63, but in contrast to Wortmannin this effect was only observed when DC were pre-treated with LY294002 for 12 h. The suppression of IL-12p40 by LT or LTK63 was partially reversed with LY294002. Therefore, the present study has demonstrated that the ability of LT or LTK63 to inhibit IL-12 production is largely PI3K-dependent.

Blocking PI3K with either Wortmannin or LY294002 inhibited the synergy between LT/LTK63 and LPS for IL-10 production. Significantly, LY294002 inhibited IL-10 production from LT- or LTK63- stimulated DC, in the presence or absence of LPS, to a greater extent than Wortmannin. Another significant observation is that LY294002 consistently inhibited IL-10 production to a greater extent than Wortmannin, and the effect was evident when DC were pre-treated with LY294002 at 5, 20 or 40 μM. This is surprising given that IL-10 has been shown to negatively regulate IL-12, it might be

expected that LY294002 would exert a more potent enhancing effect on IL-12 production (312). However, this is consistent with the observation that LY294002 inhibited p-GSK3β expression to a greater extent than Wortmannin. GSK3 has been linked to the regulation of a number of transcription factors, including NFκB, AP-1, NF-AT and CREB. GSK3 is inactivated through phosphorylation by protein kinases, including Akt, which prevents its ability to activate CREB, an important regulator of IL-10 (335). Therefore, the more marked inhibition of IL-10 by LY294002 may be due to its ability to act as a more effective inhibitor of GSK3β, promoting CREB-mediated induction of IL-10. Indeed, investigation into the role of GSK3 in the immunomodulatory effects of LT and LTK63 suggests that PI3K-mediated inhibition of GSK3 may mediate the modulatory effects of the toxin. The present study revealed that inhibition of GSK3 with SB216763 inhibited LPS-induced IL-12p70 and IL-12p40 production and had no effect on the suppression of IL-12 by LT or LTK63. Furthermore, inhibition of GSK3 augmented LPS-induced IL-10 secretion and enhanced the synergy between LT/LTK63 and LPS in the production of IL-10. These results are consistent with the demonstration that inhibition of GSK3 suppressed LPS-induced IL-12p40 and enhanced LPS-induced IL-10 production by human monocytes (171). These findings clearly show that PI3K plays an important role in the enhancement of LPS-induced IL-10 production by LT and suggest that LT may mediate this effect by suppressing the activity of GSK3.

In contrast to the role of PI3K in suppression of IL-12, blocking PI3K with Wortmannin or LY294002 had no affect on LPS-induced TNF-α secretion by DC. However, partial reversal of the inhibition of LPS-induced TNF-α production by LT or LTK63 was observed when DC were pre-treated with Wortmannin. Blocking PI3K with LY294002 partially reversed the suppression of TNF-α by LT, however, LY294002 had no affect on the suppression of TNF-α by LTK63. This suggests that LPS-induced TNF-α production is PI3K-independent, while the suppression of LPS-induced TNF-α by LT or LTK63 is partly PI3K-mediated.

A role for the MAPK JNK in regulating IL-12 production has been described. Activation of JNK down-regulated LPS-induced IL-12p40 mRNA transcription in human macrophages (420) and JNK activation was necessary for the inhibition of IL-12 following activation of GiPCR (400). The present study revealed that both LT and LTK63 induced sustained phosphorylation of JNK in DC. Inhibition of PI3K using

LY294002 had no effect on LT-induced p-JNK, while the induction of p-JNK by LTK63 was partially suppressed. These results are in agreement with the study that showed that inhibition of PI3K with Wortmannin had no effect on the phosphorylation of JNK by the GiPCR ligand C5a (400).

The results presented in chapter 3 revealed that LT alone can induce IL-23p19 expression and enhance LPS-induced IL-23 production. However, the enhancement of IL-23 production was only evident when DC were co-incubated with LT and LPS, and in fact LT inhibited IL-23 production if DC were pre-treated with LT for longer than 30 min before stimulation with LPS. Having established that the suppression of IL-12 and enhancement of IL-10 production by LT is largely mediated by activation of the PI3K pathway, experiments were designed to examine the effect of blocking PI3K on the delayed inhibition of IL-23 secretion by LT. Firstly, both LY294002 and Wortmannin augmented LPS-induced IL-23 production, indicating that similar to IL-12p40 and IL-12p70, PI3Ks negatively regulate IL-23 in DC. A significant finding of the present study is that the delayed inhibition of LPS-induced IL-23 secretion by LT or LTK63 was completely reversed by blocking PI3K with LY294002 or Wortmannin. The effect of LY294002 was more marked when DC were pre-treated with the inhibitor for 12 h before incubation with LT or LTK63, which is consistent with its enhancing effects on IL-12p40 and IL-12p70. Consistent with a recent study by Kelsall and colleagues, the GiPCR ligand C5a inhibited LPS and IFN-γ induced IL-12p19 expression in human monocytes and blocking PI3K with Wortmannin reversed this suppression (400). In that study monocytes were pre-treated with C5a for 1 h prior to stimulation with LPS and IFN-γ, which is consistent with the suppressive effect observed with LT when DC were pre-treated for more than 30 min.

The findings presented here have shown that LT suppresses IL-12p70 and IL-12p40 and enhances IL-10 production by activating signalling downstream of PI3K. PI3K are stimulated through direct interaction with the active G protein βγ subunit, but can also be activated indirectly by α subunit-induced tyrosine kinase activity (328). This is significant given that the present study established that like LT, LTK63, which is devoid of enzyme activity, can inhibit IL-12 production and enhance IL-10 and these effects were shown to be mediated by PI3K activation. While it still remains unclear how binding of the B subunit of LT to its receptors triggers the activation of signalling events,

data presented here and reports in the literature show that LTB itself can initate signalling pathways including MAPK and PI3K in DC and B cells. Together these findings clearly demonstrate that LT can modulate DC responses independently of its associated enzyme activity. The results in chapter 3 showed that stimulation of DC with LT induced the expression of IL-23p19 in DC, and this effect was not dependent on its associated enzyme activity. Furthermore, the results revealed that inhibition of PI3K with LY294002 or Wortmannin enhanced LT-induced IL-23p19 mRNA expression, providing further evidence that PI3K negatively regulates IL-23 in DC. A study by Harnett and colleagues suggested that the negative regulation of IL-12 by PI3K is mediated in an ERK-, IRF1- and NFκB- independent manner (326). A significant finding of the present study is that inhibition of ERK suppressed the induction of IL-23p19 mRNA expression in response to LT. This result was surprising given that ERK has been shown to negatively regulate IL-12p70 production by DC.

c-Fos and c-Jun are two important regulators of the transcription factor AP-1, a transcriptional inhibitor of the IL-12p40 gene (401). Having established that LT and LTK63 enhance c-Fos expression, experiments were carried out to determine if the toxin could activate other components of AP-1. LT enhanced c-Jun expression, and in line with the delayed induction of c-Fos, the enhancement of c-Jun was also delayed. These data indicate that AP-1 may play crucial role in the ability of LT to inhibit LPS-induced IL-12 production. In contrast to the role of ERK and other kinases, including JNK, PKC and Ras, in increasing AP-1 activity, GSK3β has been implicated in the phosphorylation and negative regulation of AP-1 activation. A study by Boyle *et al.* (421) showed that GSK3β phosphorylated c-Jun *in vitro* at three sites and decreased its DNA binding activity. Furthermore, this group demonstrated that activation of PKC decreased phosphorylation of sites of AP-1 that are negatively regulated by GSK3β, resulting in an increase in AP-1 activity (421).

Having established that the PI3K activity is necessary for the negative regulation of IL-12 by LT, experiments were designed to investigate the role of PI3K in the ability of the toxin to upregulate CD80 and CD86 expression. Blocking PI3K with LY294002 or Wortmannin enhanced LPS-induced CD80 expression, indicating that PI3K negatively regulates CD80 expression in LPS-stimulated DC. However, inhibition of PI3K with LY294002 or Wortmannin suppressed LT- and LTK63- mediated upregulation of CD80

expression. In contrast to CD80 expression, blocking PI3K further enhanced the upregulation of CD86 expression induced by LT, LTK63 and LPS. These findings demonstrate that PI3K negatively regulates CD86 expression on DC, but plays a positive role in the ability of LT and LTK63 to induce CD80 expression. The data presented in chapter 3 showed that LPS-induced CD86 expression was NFκB-dependent. Therefore, the suppression of LPS-induced CD86 expression may be through PI3K-mediated inhibition of NFκB signalling. To further elucidate the role of PI3K signalling, the effect of blocking GSK3 on the ability of LT to upregulate CD80 expression was examined. The results showed that inhibition of GSK3 enhanced the upregulation of CD80 expression by LT and LTK63. This is consistent with the data which demonstrated that blocking PI3K, which allows GSK3 to remain active, suppressed LT- and LTK63-induced upregulation of CD80 expression.

A number of other signalling pathways, including ERK, p38 MAPK, p38SAPK, Trif and Raf1, have been reported to be involved in the upregulation of co-stimulatory molecules on APCs in response to LPS (387, 396). Blocking p38 with SB203580 inhibited LPS-induced upregulation of CD80, CD83 and CD86 expression on MoDC. However, the data presented here showed that inhibition of p38 with SB203580 had no effect on LPS-induced CD80 or CD86 expression. Targets of p38SAPK include the transcription factors ATF-2 and Elk-1, and kinases such as MAPKAP kinase 2 and 3, which activate the transcription factors ATF-1 and CREB. LPS was shown to activate ATF-2 and CREB in a p38SAPK-dependent manner and a binding site for ATF and CREB was found in the promoter sequence of CD86 (396). This suggests that modulation of CREB and ATF activities at the transcriptional level may be a possible mechanism for LPS-induced upregulation of co-stimulatory molecules (396). Upregulation of co-stimulatory molecules by LPS is reported to occur via the TLR4-Trif-IFNβ-IFN-R1 signalling axis in peritoneal macrophages (387). Furthermore, CD40/CD40L-induced CD86 expression in APCs has been reported to be regulated by NFκB, CREB and AP-1 (422). The data presented in chapter 3 demonstrate that the upregulation of CD80 and CD86 by LT and LTK63 was NFκB-independent. The results shown here establish that LT- and LTK63- induced upregulation of CD80 was PI3K-dependent, but that PI3K negatively regulated the ability of LT to induce CD86.

While a role for MAPK signalling in mediating the suppressive effect of LT on LPS-induced IL-12 production had been ruled out, it was important to examine the effect of blocking MAPK activation on the ability of LT to enhance CD80 and CD86 expression. Inhibition of p38 or JNK had no effect on LT-, LTK63- or LPS- induced upregulation of CD80 or CD86 expression. However, a significant finding of this study is that ERK plays a role in LT-induced CD80 expression. Blocking ERK suppressed the upregulation of CD80 in response to LT or LTK63 but not LPS, indicating that ERK has a specific role in mediating the increase in CD80 expression on DC in response to LT.

The data presented in chapter 3 demonstrated that the upregulation of CD80 and CD86 in response to LT was NFκB-independent. However, PKC has been reported to regulate components of the NFκB pathway, particularly in lymphocytes (423). Preliminary experiments investigating the role of PKC, using inhibitors for specific isoforms of PKC, in CT- or LPS- induced CD80 and CD86 expression on DC, revealed that novel isoforms of PKC play an important role in toxin-mediated DC maturation. While pre-treatment of DC with the broad spectrum PKC inhibitor, bisindolylmaleimide (BIM), marginally decreased CT-induced CD80 expression, rottlerin, an inhibitor of novel PKC isoforms, dramatically suppressed the enhancement of CD80 and CD86 expression by CT. In light of this, similar studies were carried out with LT and LTK63, and the present study demonstrates that upregulation of CD80 and CD86 expression by both LT and LTK63 was inhibited by rottlerin. In addition, rottlerin blocked LPS-induced upregulation of CD80 and CD86 expression. These results demonstrate the nPKC isoforms are essential for the regulation of CD80 and CD86 expression on DC. The enhancement of CD40 expression by LPS was inhibited when DC were pre-treated with rottlerin, indicating that nPKCs also play a role in the upregulation of CD40 expression by LPS.

These studies have established that LPS-induced DC maturation is mediated by a number of signalling pathways, and that co-stimulatory molecules that are members of the same family can be differentially regulated. Upregulation of CD86 expression was NFκB-, and nPKC- dependent, which suggests there may be crosstalk between these pathways in regulating the expression of this co-stimulatory molecule. In contrast, LPS-induced CD80 expression was NFκB-independent, but negatively regulated by PI3K. However, like CD86 expression, LPS-induced CD80 is also nPKC-dependent, indicating

that nPKCs play a dominant role, possibly via interaction with other signalling molecules, in the induction of CD80 expression on DC. Furthermore, LPS-induced CD40 expression was NFκB- and nPKC- dependent. The significant finding of the present study is that ERK, PI3K and nPKCs appear to play a role in the upregulation of CD80 expression by LT. In contrast, while inhibition of PKC suppressed LT-induced upregulation of CD86 expression, the ability of the toxin to exert this effect was independent of ERK and negatively regulated by PI3K. Therefore, it appears that many signalling pathways may contribute to DC maturation and furthermore, different stimuli activate distinct pathways involved in the regulation of co-stimulatory molecule expression.

The results of this study demonstrate that PI3K plays a crucial role in the immunomodulatory effects of LT on DC (see proposed model Fig. 4.39 below). The ability of the toxin to suppress IL-12 and enhance IL-10 is dependent on PI3K activation. Furthermore, the PI3K pathway is involved in LT-induced upregulation of CD80 expression on DC. Another significant finding is that LT can induce IL-23p19 expression and when co-incubated with a TLR agonist can enhance IL-23 secretion. The present study has also established that PI3K negatively regulates IL-23 production by DC.

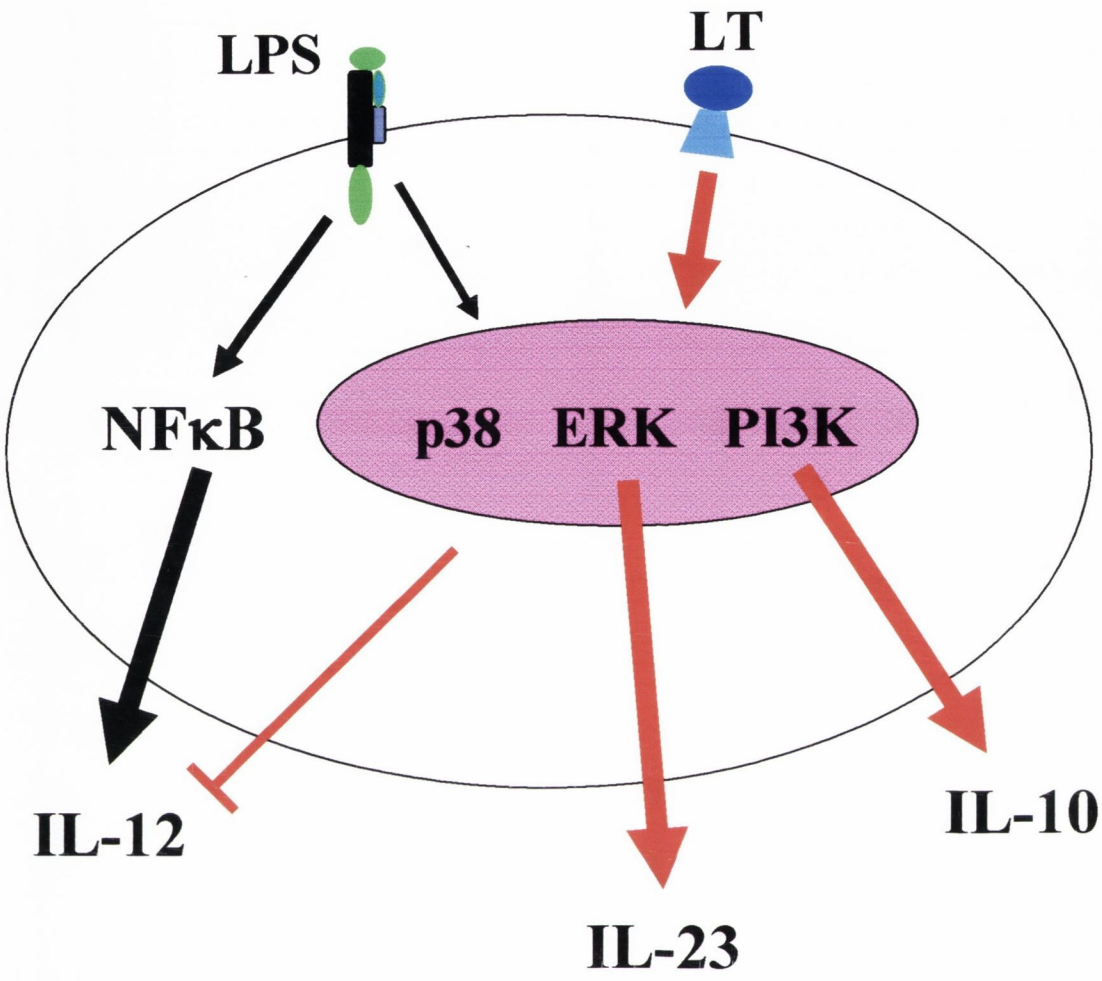

Figure 4.39 Modulation of LPS-induced cytokine production by LT

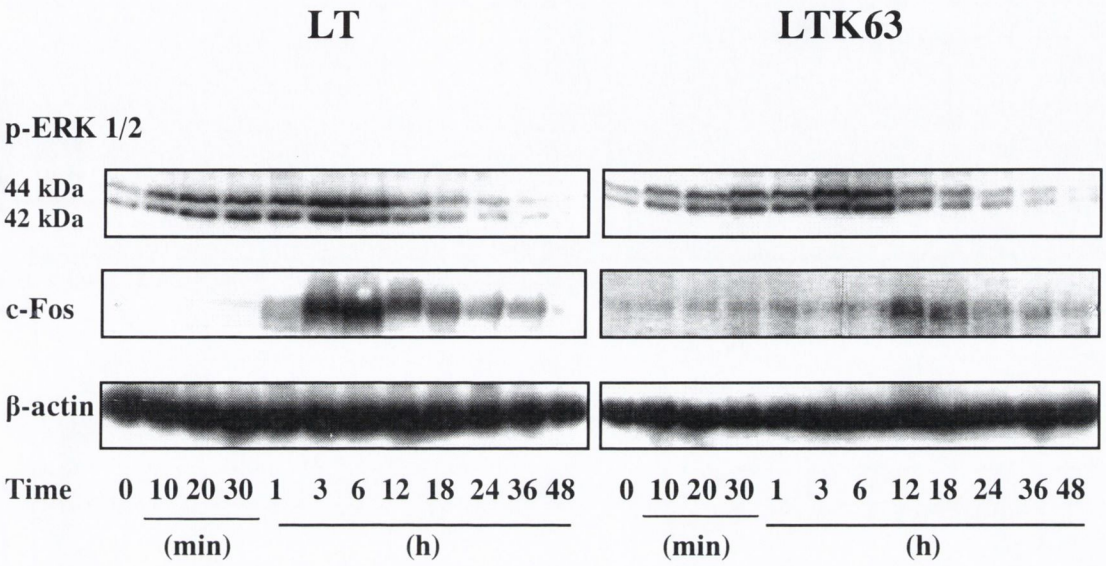

Figure 4.1 LT and LTK63 induce phosphorylation of ERK and increase the expression of c-Fos in DC. BMDC (1 x 10^6 cells/ml) from BALB/c mice were stimulated with LT (1 μg/ml) or LTK63 (1 μg/ml) for 10 min to 48 h. Cell lysates were probed for p-ERK, c-Fos and β-actin (as a loading control) by Western blot analysis. Results are representative of two independent experiments.

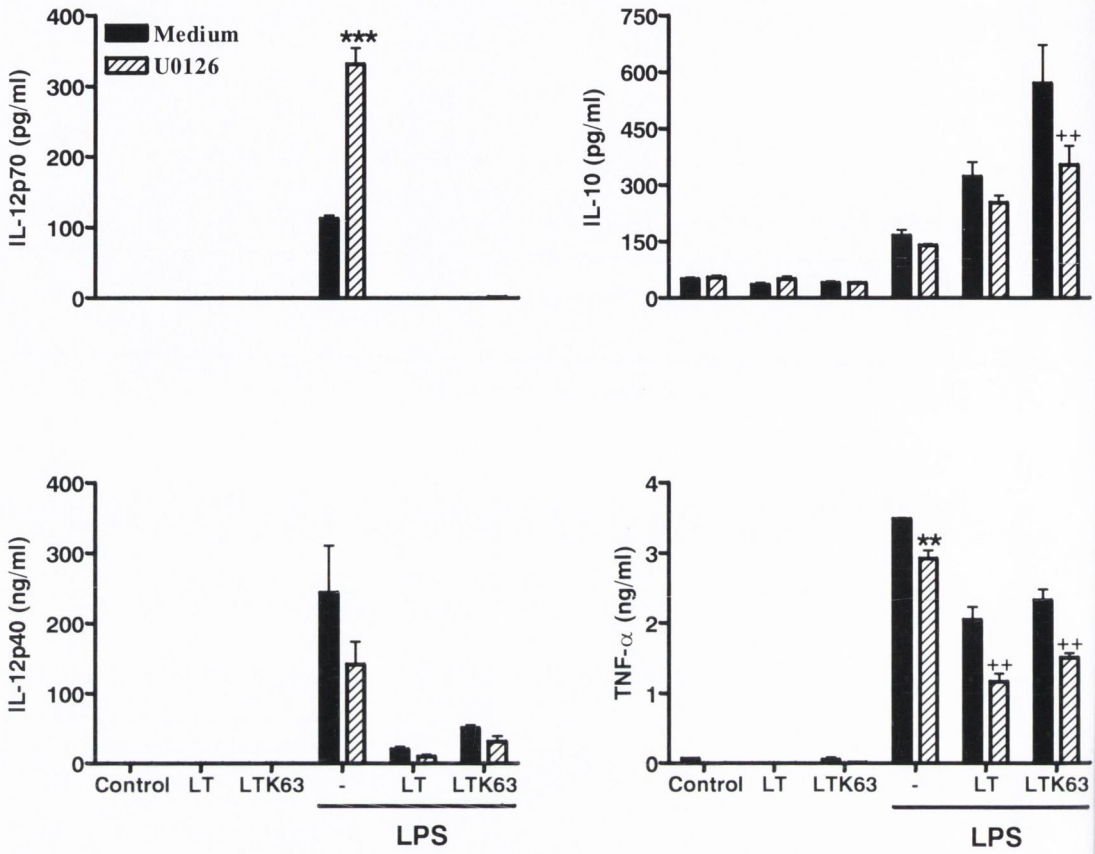

Figure 4.2 Enhancement of LPS-induced IL-10 production by LT and LTK63 is partially ERK-dependent, while inhibition of LPS-induced IL-12 is ERK-independent. BMDC (5 x 10^5 cells/ml) from BALB/c mice were pre-treated with the MEK1/2 inhibitor, U0126 (5 μM), for 30 min, prior to LT (1 μg/ml) or LTK63 (1 μg/ml) for 1 or 12 h respectively, followed by stimulation with LPS (10 ng/ml). After 24 h, supernatants were collected for analysis of IL-12p70, IL-12p40, IL-10 and TNF-α production by ELISA. LPS versus LPS + U0126: **, $P < 0.01$; ***, $P < 0.001$. LPS + LT/LTK63 versus LPS + LT/LTK63 + U0126: ++, $P < 0.01$. Data are the mean ± SEM of triplicate stimulations and are representative of two independent experiments.

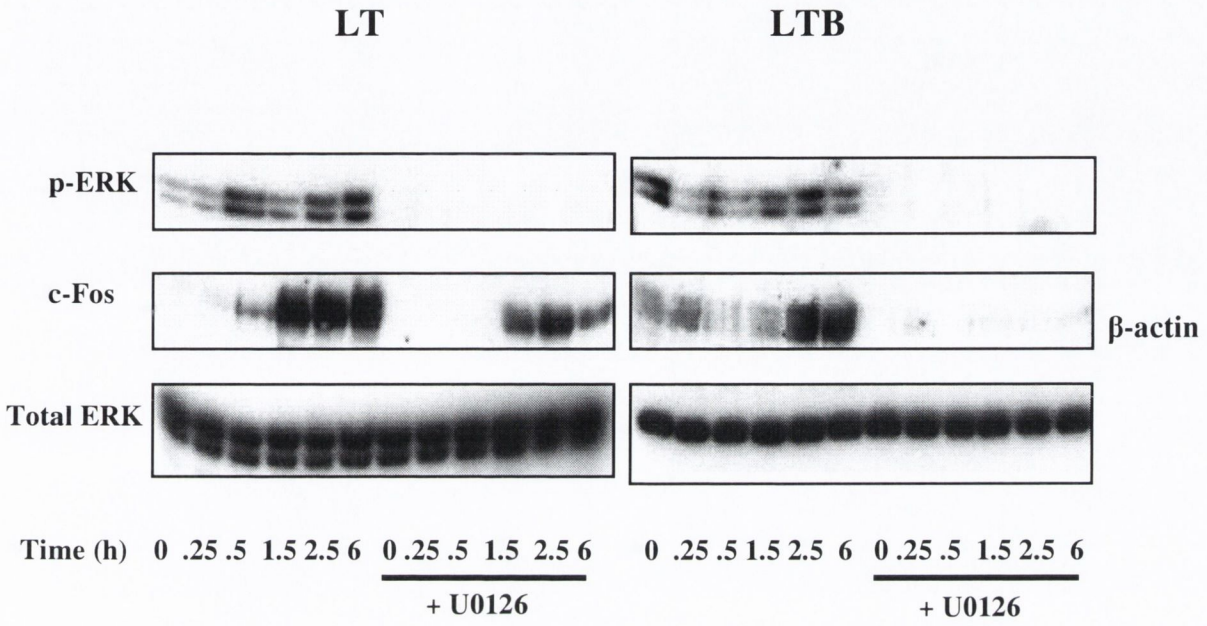

Figure 4.3 Enhancement of c-Fos expression by LT is partially ERK-dependent, while LTB-induced c-Fos is completely ERK-dependent. BMDC (1 x 10^6 cells/ml) from BALB/c mice were pre-treated with U0126 (5 μM) or medium only for 30 min, followed by stimulation with LT (1 μg/ml) or LTB (1 μg/ml) for 15 min to 6 h. Cell lysates were probed for p-ERK, c-Fos and total ERK or β-actin (as a loading control) by Western blot analysis.

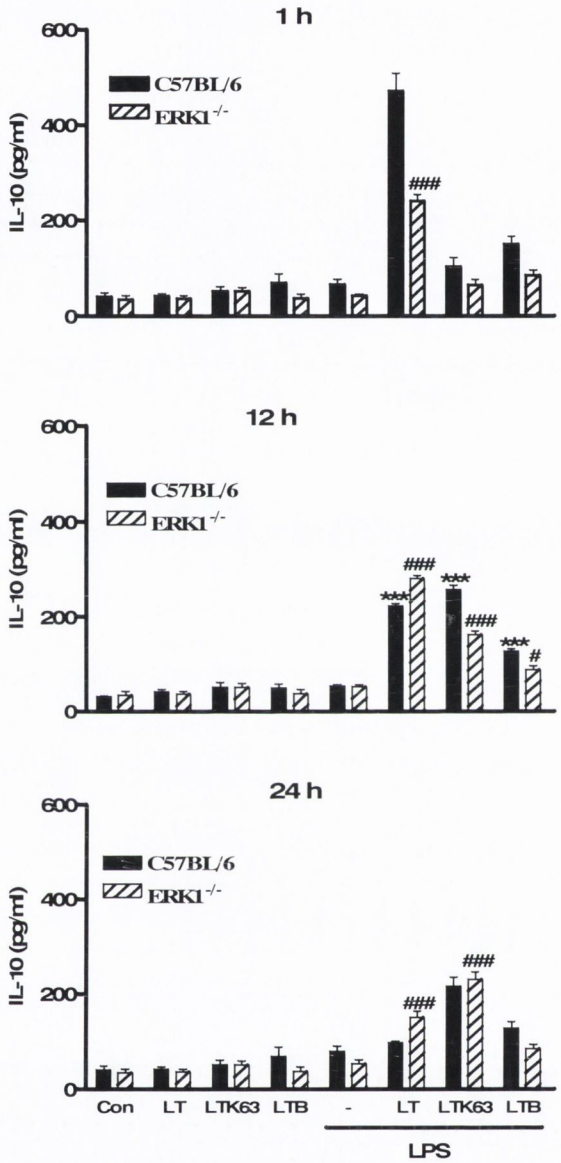

Figure 4.4 Enhancement of LPS-induced IL-10 production by LT, LTK63 or LTB is partially ERK1-dependent. BMDC (5 x 10^5 cells/ml) from C57BL/6 or ERK1$^{-/-}$ mice were pre-treated for 1, 12 or 24 h with LT (1 μg/ml), LTK63 (1 μg/ml) or LTB (1 μg/ml) prior to addition of LPS (5 ng/ml). After 24 h, supernatants were collected for analysis of IL-10 production by ELISA. C57 LPS versus LPS + LT/LTK63/LTB: *, $P < 0.05$; ***, $P < 0.001$. ERK1$^{-/-}$ LPS versus ERK1$^{-/-}$ LPS + LT/LTK63/LTB: #, $P < 0.05$; ###, $P < 0.001$. Data are the mean ± SEM of triplicate stimulations.

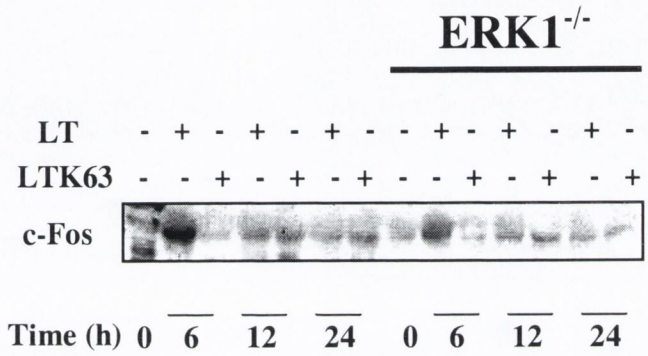

Figure 4.5 LT can enhance the expression of c-Fos in ERK-1-deficient DC. BMDC (1 x 10^6 cells/ml) from C57BL/6 or ERK1$^{-/-}$ mice were treated for 6, 12 or 24 h with LT (1 μg/ml) or LTK63 (1 μg/ml). Cell lysates were probed for c-Fos expression by Western blot analysis.

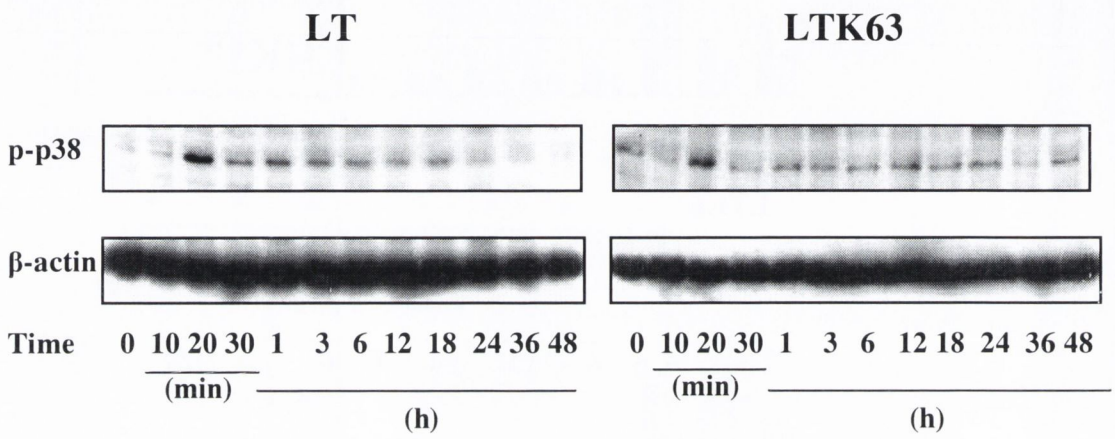

Figure 4.6 LT and LTK63 induce phosphorylation of p38 in DC. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml) or LTK63 (1 μg/ml) for 10 min to 48 h. Cell lysates were probed for p-38 and β-actin (as a loading control) by Western blot analysis.

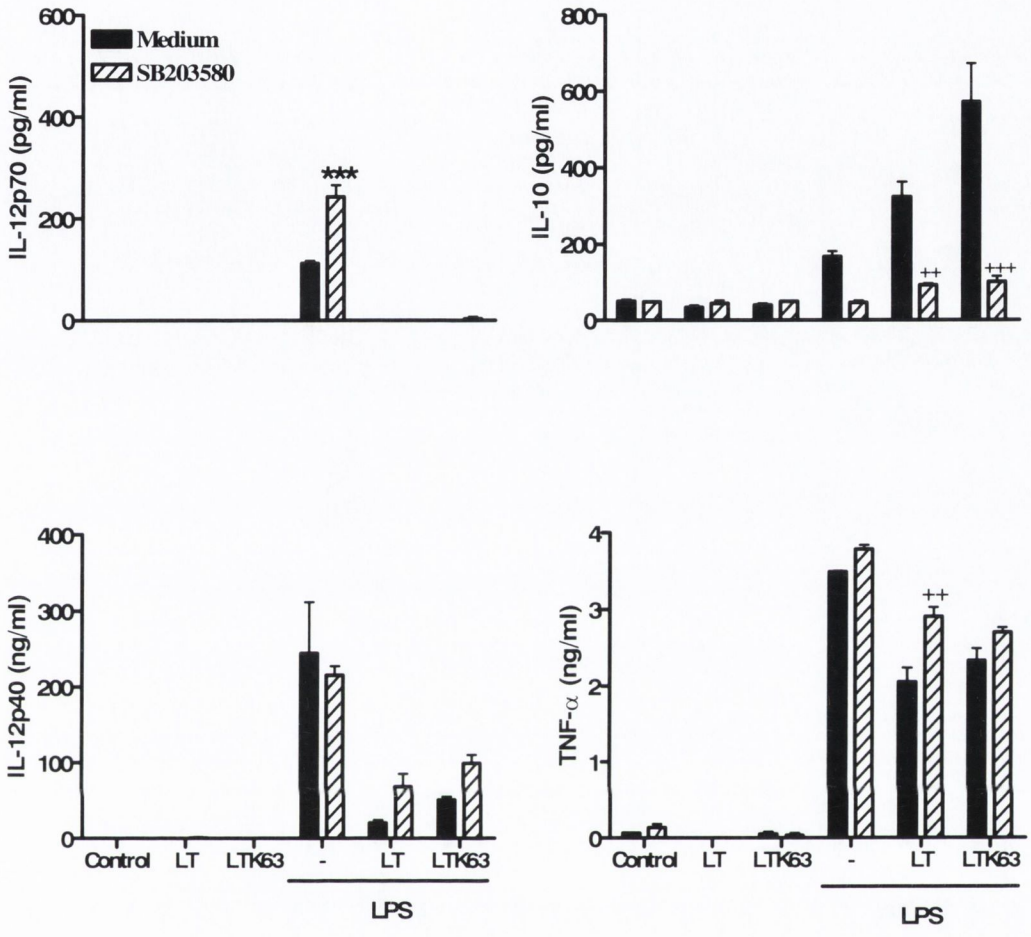

Figure 4.7 Enhancement of LPS-induced IL-10 production by LT or LTK63 is p38-dependent, while inhibition of LPS-induced IL-12 by LT or LTK63 is p38-independent. BMDC (5 x 10^5 cells/ml) from BALB/c mice were pre-treated with the p38 inhibitor, SB203580 (5 μM), for 30 min prior to 1 h or 12 h treatment with LT (1 μg/ml) or LTK63 (1 μg/ml) respectively, followed by stimulation with LPS (10 ng/ml). After 24 h, supernatants were collected for analysis of IL-12p70, IL-12p40, IL-10 and TNF-α production by ELISA. LPS versus LPS + SB203580: ***, $P < 0.001$. LPS + LT/LTK63 versus LPS + LT/LTK63 + SB203580: ++, $P < 0.01$; +++, $P < 0.001$. Data are the mean ± SEM of triplicate stimulations and are representative of two independent experiments.

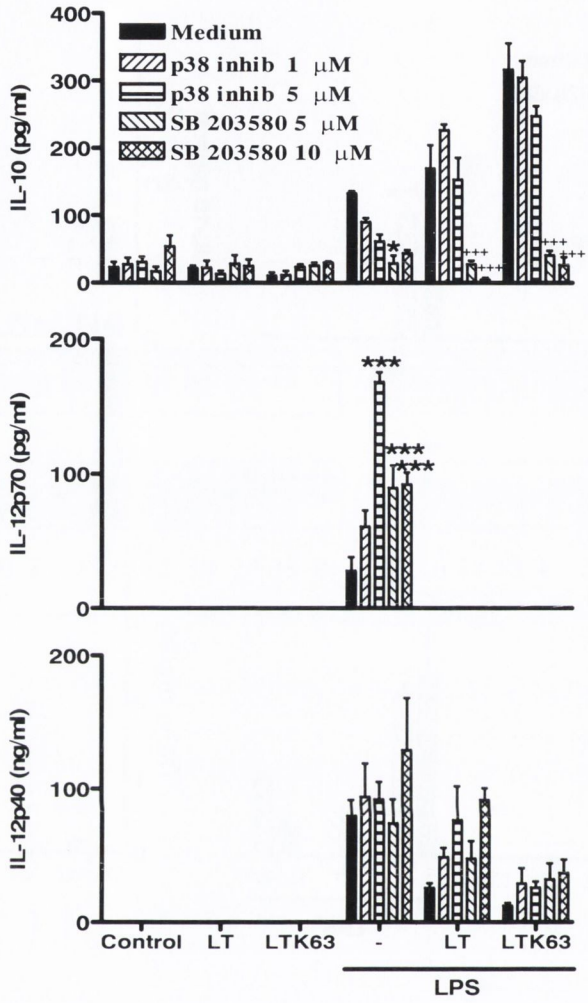

Figure 4.8 Two different synthetic p38 inhibitors enhance LPS-induced IL-12 but inhibition of p38 with SB203580 specifically suppresses LPS-induced IL-10 production. BMDC (5 x 10^5 cells/ml) from BALB/c mice were pre-treated with the p38 inhibitor, SB203580 (5 or 10 μM), or with the Calbiochem p38 inhibitor, (1 or 5 μM), for 30 min prior to 1 h or 12 h pre-treatment with LT (1 μg/ml) or LTK63 (1 μg/ml) respectively, followed by addition of LPS (10 ng/ml). After 24 h, supernatants were collected for analysis of IL-12p70, IL-12p40 and IL-10 by ELISA. LPS versus LPS + p38 inhibitor/SB203580: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. LPS + LT/LTK63 versus LPS + LT/LTK63 + p38 inhibitor/SB203580: +, $P < 0.05$; +++, $P < 0.001$. Data are the mean ± SEM of triplicate stimulations.

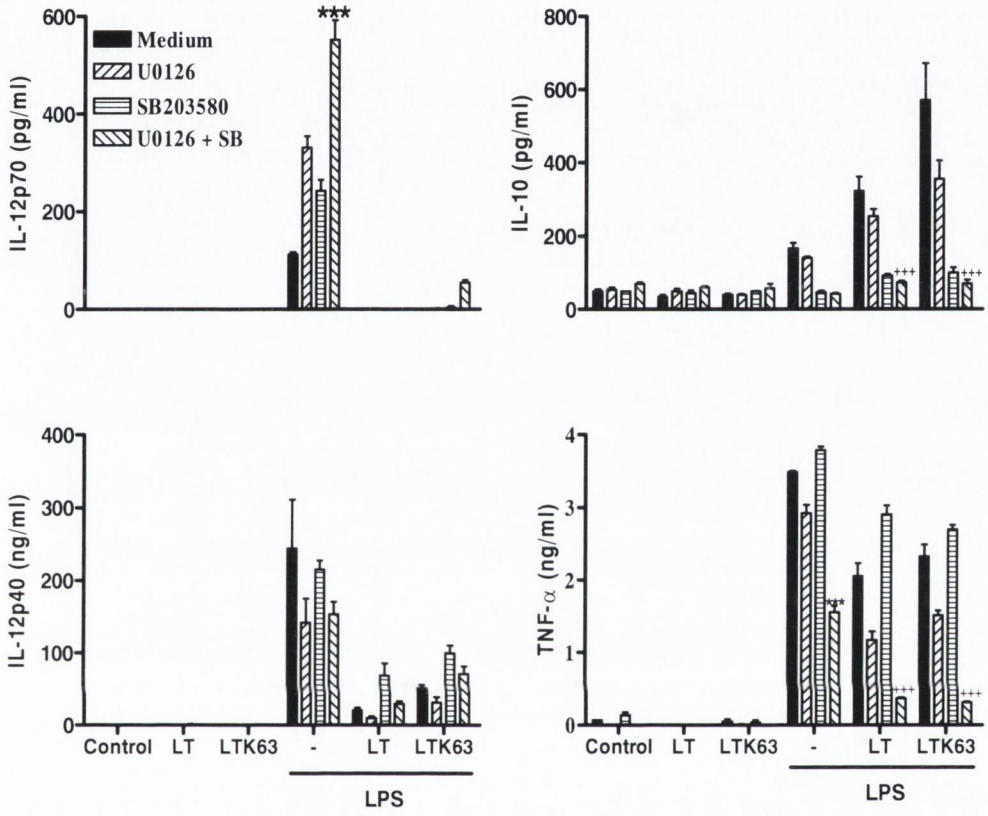

Figure 4.9 Enhancement of LPS-induced IL-10 production by LT or LTK63 by DC is p38-dependent, while inhibition of LPS-induced IL-12 by LT or LTK63 is p38- and ERK- independent. BMDC (5 x 10^5 cells/ml) from BALB/c mice were pre-treated with the ERK inhibitor, U0126 (5 μM), and the p38 inhibitor, SB203580 (5 μM), alone or in combination, for 30 min prior to 1 h or 12 h treatment with LT (1 μg/ml) or LTK63 (1 μg/ml) respectively, followed by stimulation with LPS (10 ng/ml). After 24 h, supernatants were collected for analysis of IL-12p70, IL-12p40, IL-10 and TNF-α production by ELISA. LPS versus LPS + U0126 + SB203580: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. LPS + LT/LTK63 versus LPS + LT/LTK63 + U0126 + SB203580: +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$. Data are the mean ± SEM of triplicate stimulations.

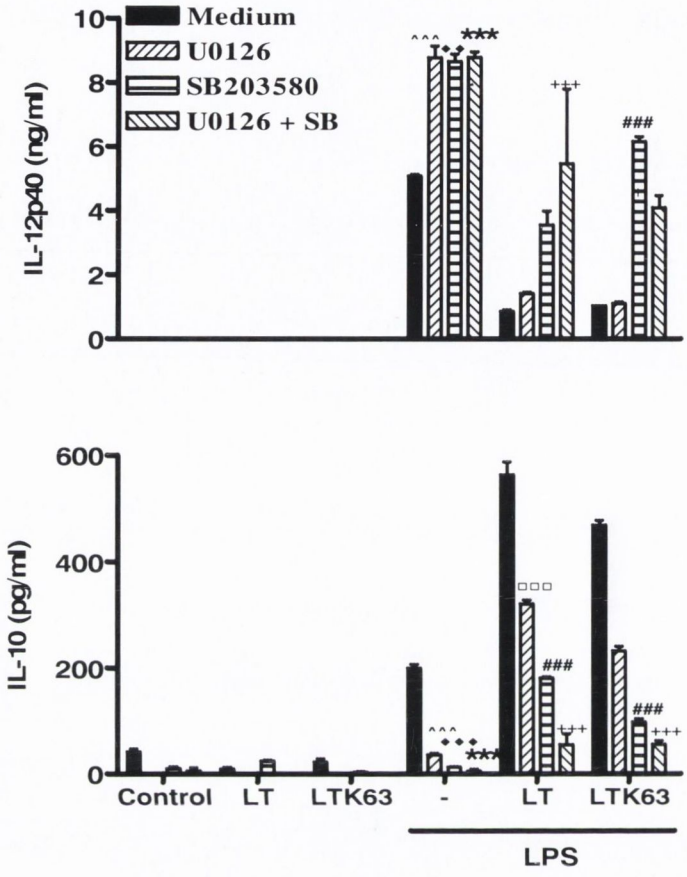

Figure 4.10 Enhancement of LPS-induced IL-10 production by LT or LTK63 is p38-dependent and partially ERK-dependent, while inhibition of LPS-induced IL-12 by LT or LTK63 is p38-dependent and ERK-independent in J774 macrophages. J774 cells (5 x 10^5 cells/ml) were pre-treated with the ERK inhibitor, U0126 (5 μM), or the p38 inhibitor, SB203580 (5 μM), alone or in combination, for 30 min prior to 24 h treatment with LT (1 μg/ml) or LTK63 (1 μg/ml), before stimulation with LPS (10 ng/ml). After 24 h, supernatants were collected for analysis of IL-12p40 and IL-10 production by ELISA. LPS versus LPS + U0126: ^^^, $P < 0.001$. LPS versus LPS + SB203580: ♦♦, $P < 0.05$; ♦♦♦, $P < 0.001$. LPS versus LPS + U0126 + SB203580: ***, $P < 0.001$. LPS + LT/LTK63 versus LPS + LT/LTK63 + U0126: □□□, $P < 0.001$. LPS + LT/LTK63 versus LPS + LT/LTK63 + U0126 + SB203580: +, $P < 0.05$; +++, $P < 0.001$. LPS + LT/LTK63 versus LPS + LT/LTK63 + SB203580: ###, $P < 0.001$. Data are mean ± SEM of triplicate stimulations.

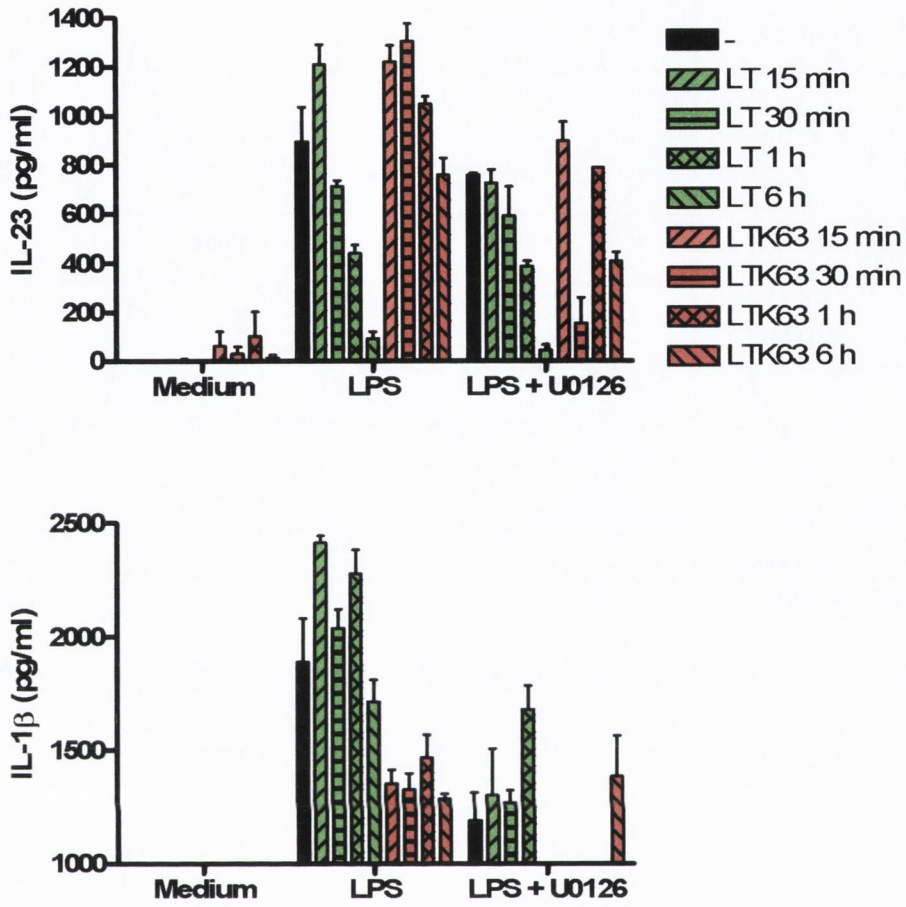

Figure 4.11 ERK activation is required for LPS-induced IL-1β and IL-23 production and the enhancement of IL-23 by LT or LTK63. BMDC (5 x 10^5 cells/ml) from BALB/c mice were pre-treated with the ERK inhibitor, U0126 (5 μM), for 30 min prior to incubation with LT (1 μg/ml) or LTK63 (1 μg/ml) for 15 min, 30 min, 1 or 6 h, followed by stimulation with LPS (100 ng/ml). After 24 h, supernatants were collected for analysis of IL-23 and IL-1β production by ELISA. Data are mean ± SEM of triplicate stimulations.

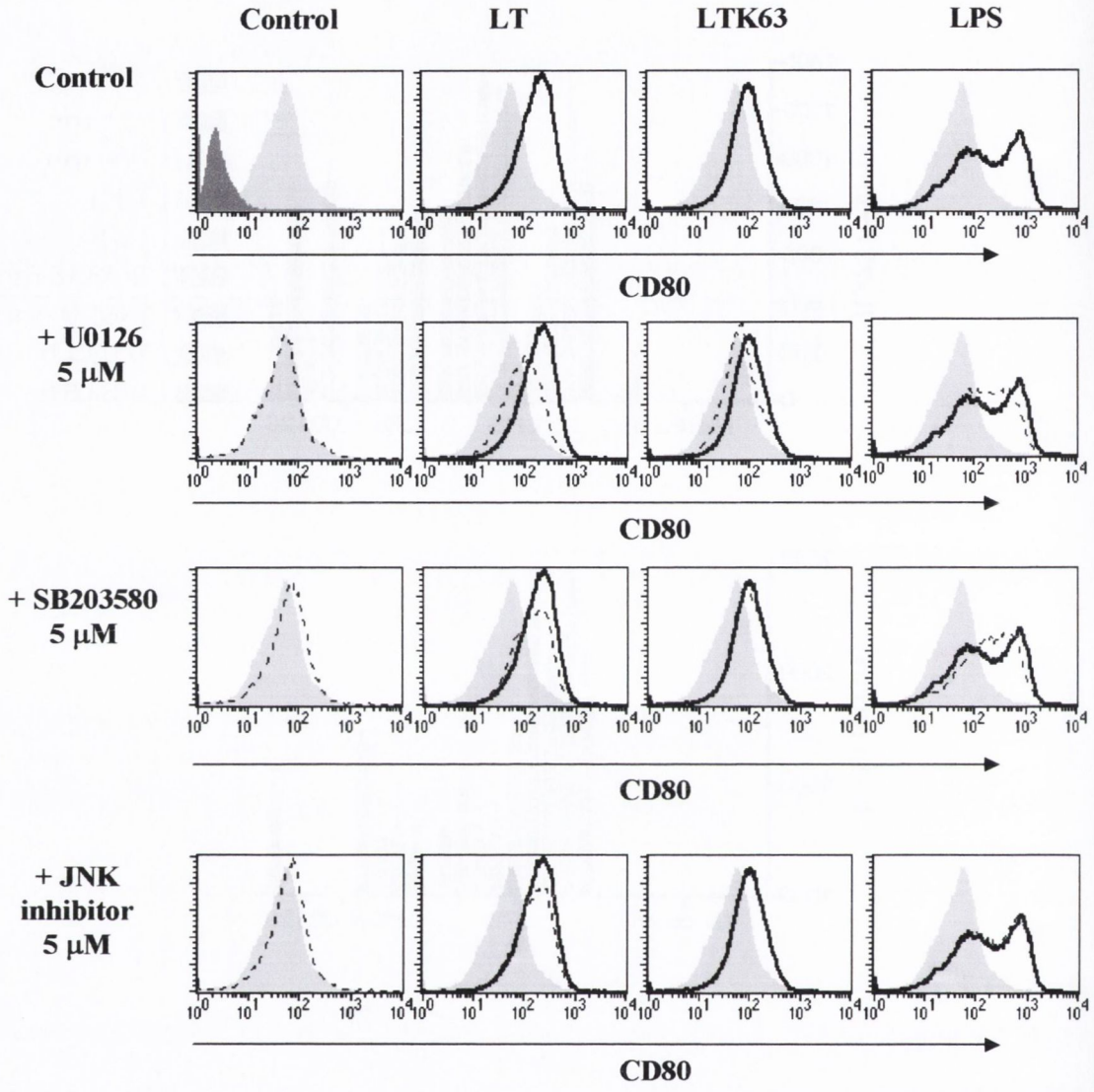

Figure 4.12 Upregulation of CD80 expression by LPS is MAPK-independent but inhibition of ERK partially suppresses the enhancement of CD80 by LT or LTK63. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with U0126, SB203580 or JNK inhibitor II (5 μM). Cells were harvested after 24 h and stained with antibodies specific for CD80, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with U0126, SB203580 or JNK inhibitor II.

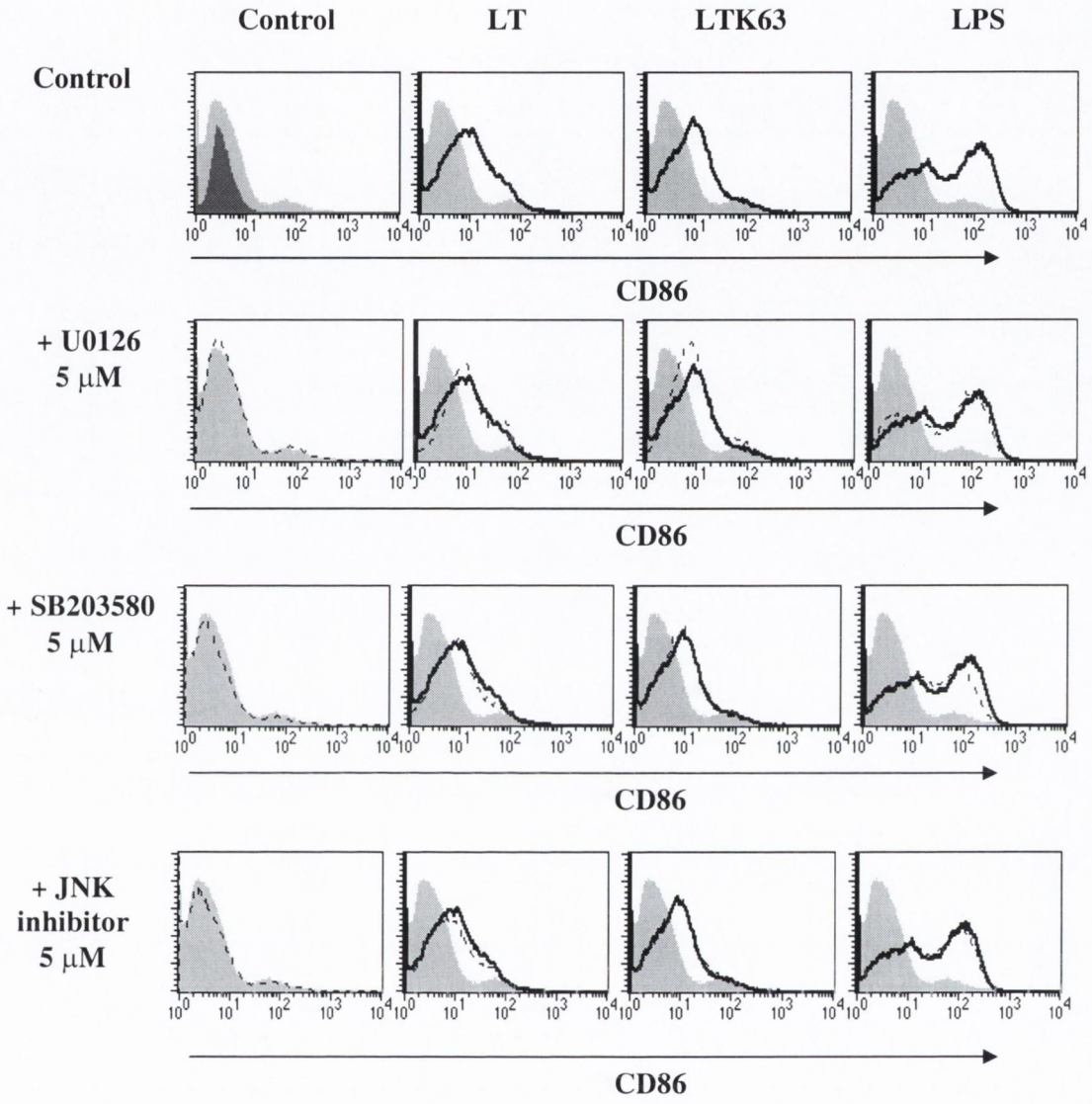

Figure 4.13 Inhibition of MAPK has no effect on the enhancement of CD86 expression by LT, LTK63 or LPS. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with U0126, SB203580 or JNK inhibitor II (5 μM). Cells were harvested after 24 h and stained with antibodies specific for CD86, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with U0126, SB203580 or JNK inhibitor II.

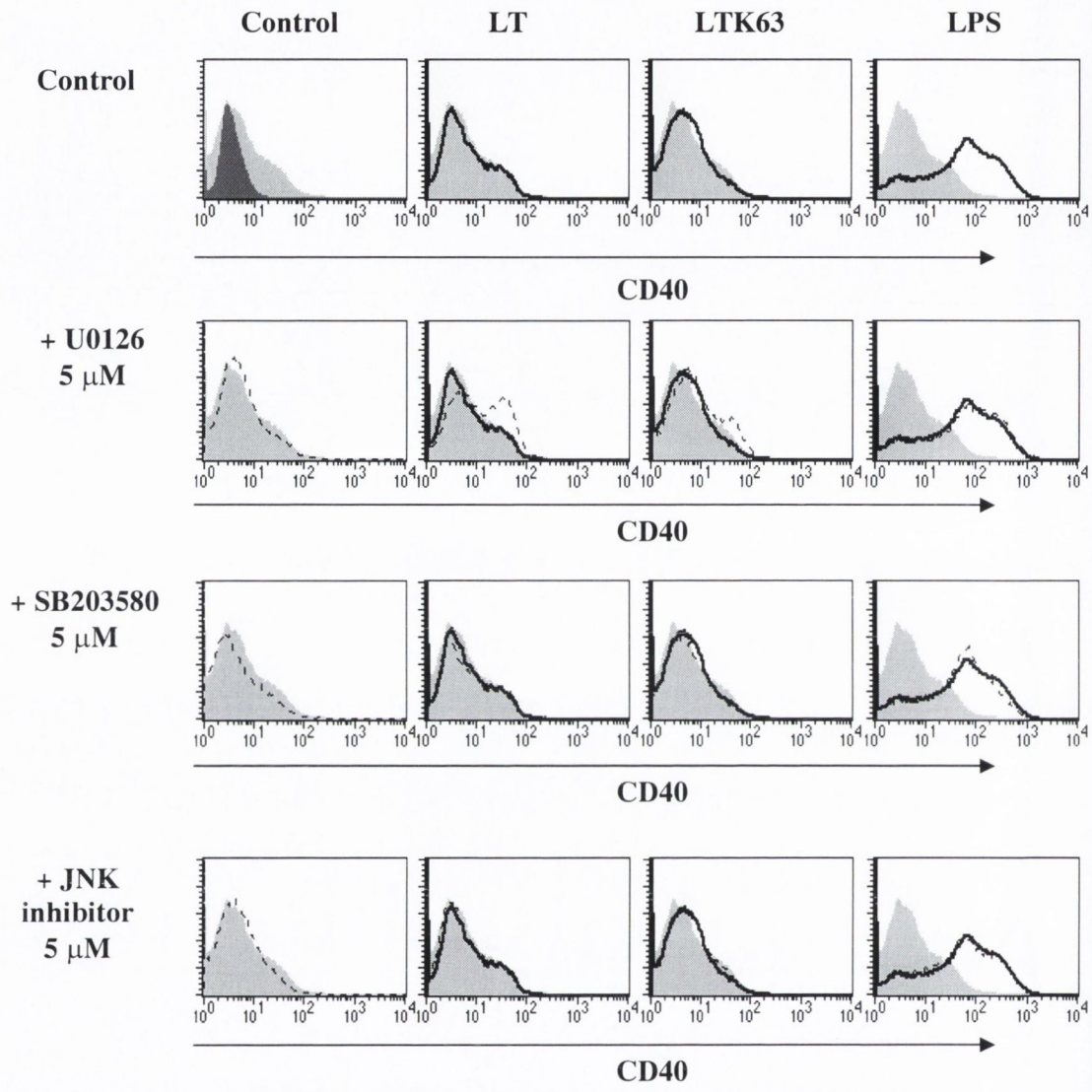

Figure 4.14 Upregulation of CD40 expression by LPS is MAPK-independent. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with U0126, SB203580 or JNK inhibitor II (5 μM). Cells were harvested after 24 h and stained with antibodies specific for CD40, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with U0126, SB203580 or JNK inhibitor II.

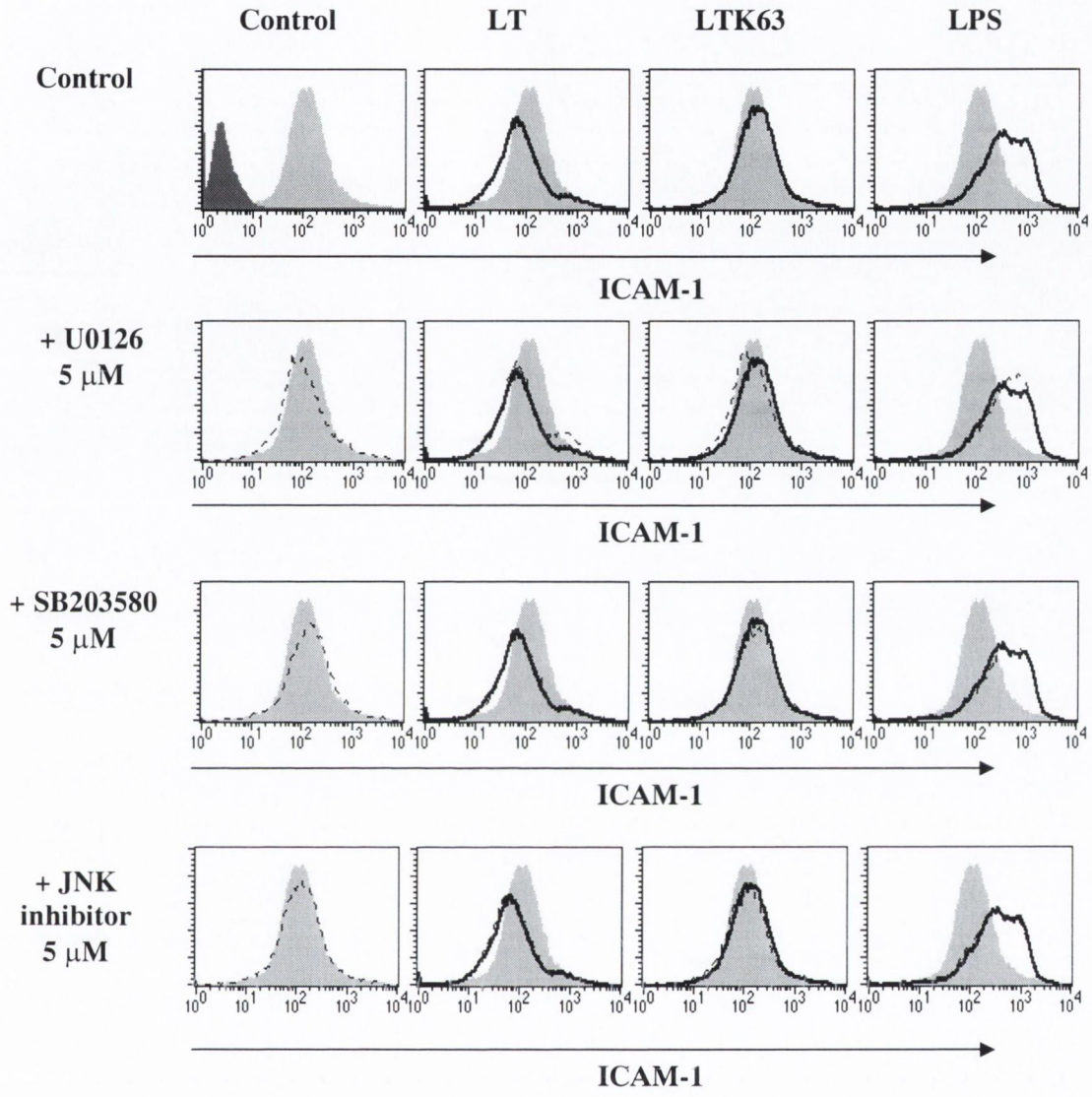

Figure 4.15 Enhancement of ICAM-1 by LPS is MAPK-independent. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with U0126, SB203580 or JNK inhibitor II (5 μM). Cells were harvested after 24 h and stained with antibodies specific for ICAM-1, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with U0126, SB203580 or JNK inhibitor II.

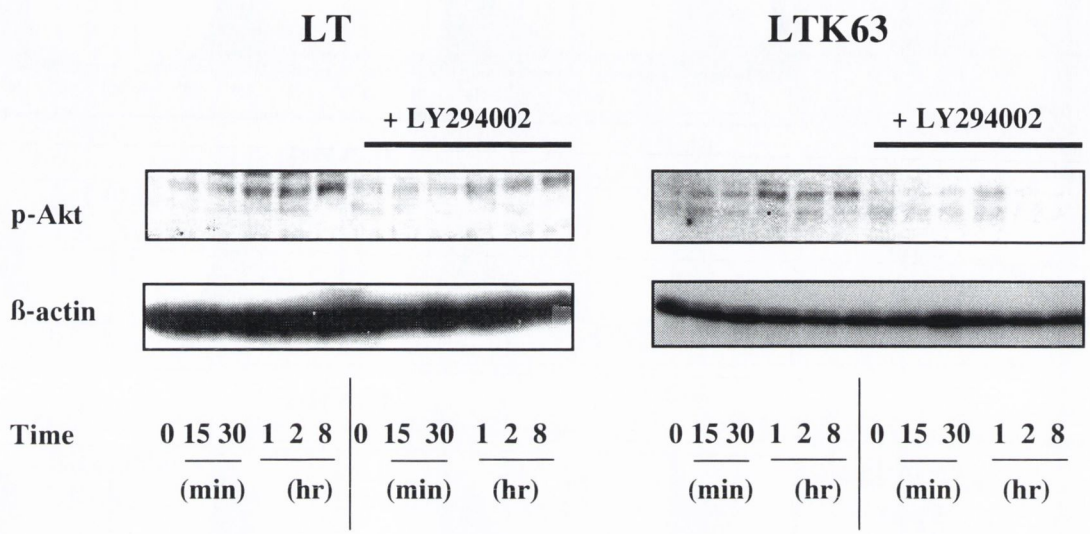

Figure 4.16 The phosphorylation of Akt by LT and LTK63 in DC is partially PI3K-dependent. BMDC (1 x 10^6 cells/ml) from BALB/c mice were pre-treated with LY294002 (20 μM) or medium only for 30 min, followed by stimulation with LT (1 μg/ml) or LTK63 (1 μg/ml) for 5 min, 30 min, 1 h, 2 h or 8 h. Cell lysates were probed for p-Akt, and β-actin (as a loading control) by Western blot analysis.

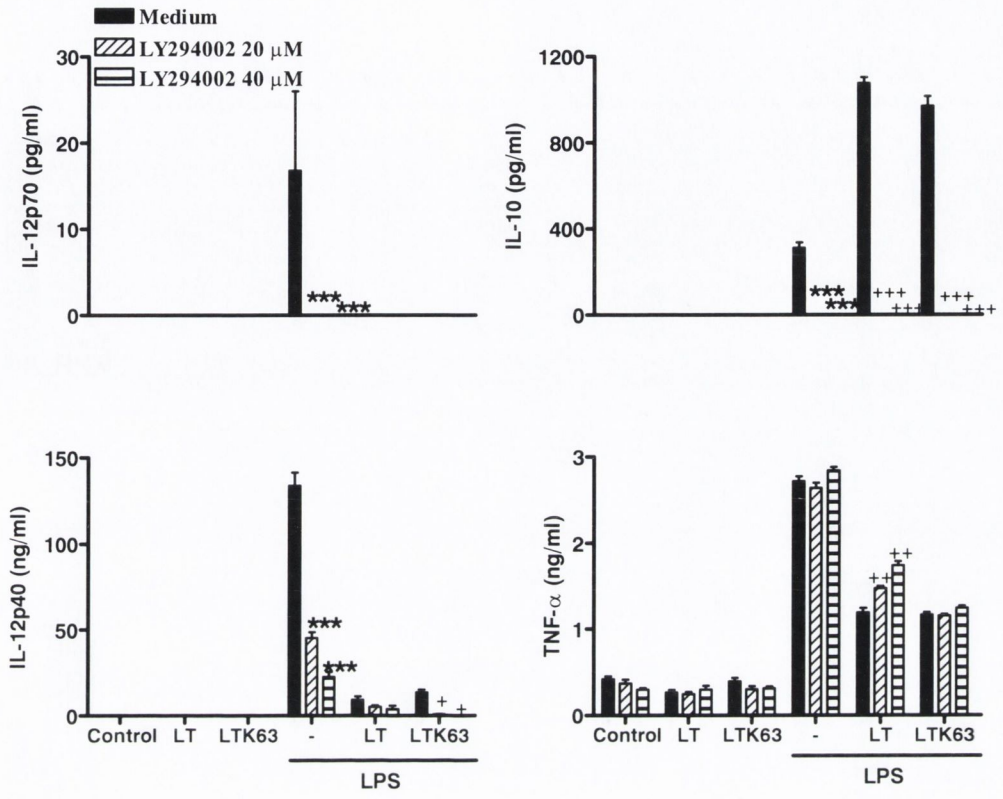

Figure 4.17 The enhancement of LPS-induced IL-10 by LT or LTK63 is suppressed by the PI3K inhibitor LY294002. BMDC (5 x 10^5 cells/ml) from BALB/c mice were pre-treated with the PI3K inhibitor, LY294002 (20 or 40 μM), for 30 min prior to 1 h or 12 h treatment with LT (1 μg/ml) or LTK63 (1 μg/ml) respectively, followed by stimulation with LPS (5 ng/ml). After 24 h, supernatants were collected for analysis of IL-12p70, IL-12p40, IL-10 and TNF-α production by ELISA. LPS versus LPS + LY294002: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. LPS + LT/LTK63 versus LPS + LT/LTK63 + LY294002: +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$. Data are mean ± SEM of triplicate stimulations and are representative of two independent experiments.

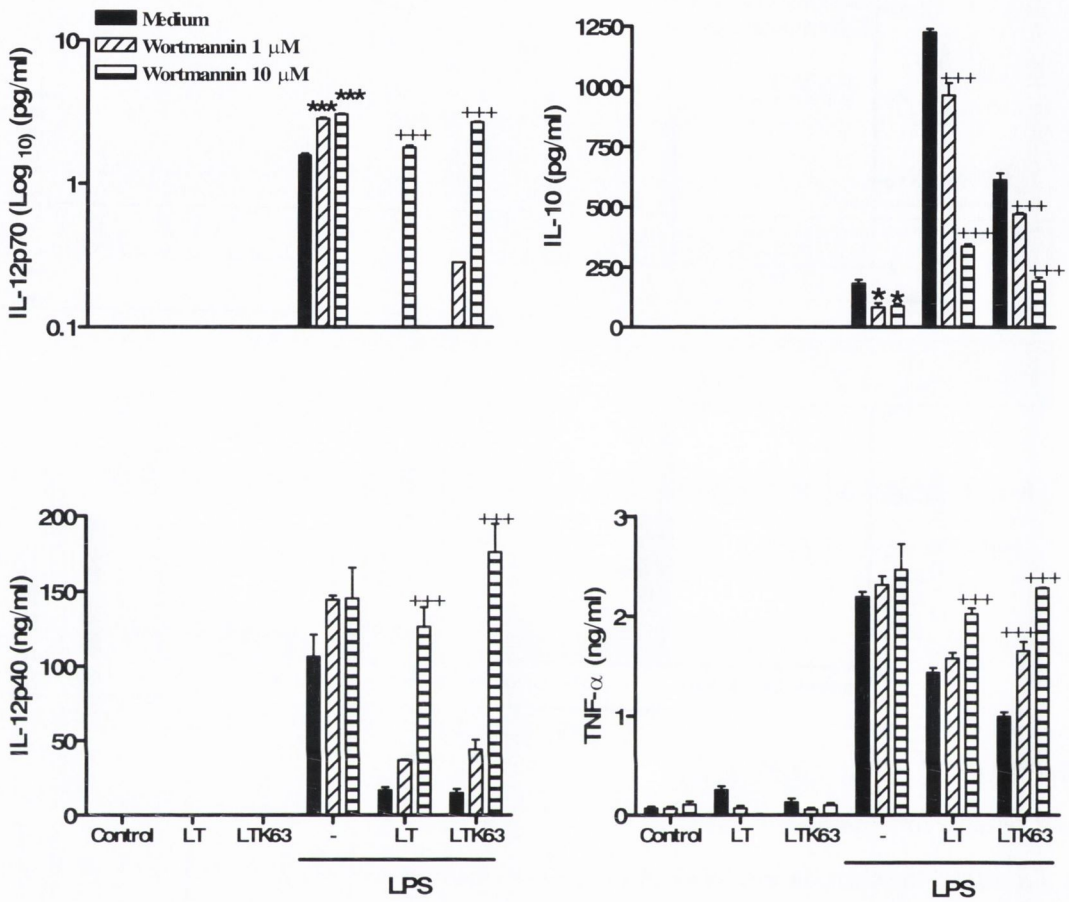

Figure 4.18 The enhancement of LPS-induced IL-10 and inhibition of LPS-induced IL-12 production by LT or LTK63 is reversed by the PI3K inhibitor Wortmannin. BMDC (5 x 10^5 cells/ml) from BALB/c mice were pre-treated with the PI3K inhibitor, Wortmannin (1 or 10 μM), for 30 min, prior to treatment with LT (1 μg/ml) or LTK63 (1 μg/ml) for 1 or 12 h respectively, followed by stimulation with LPS (5 ng/ml). After 24 h, supernatants were collected for analysis of IL-12p70, IL-12p40 and IL-10 by ELISA. LPS versus LPS + Wortmannin: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. LPS + LT/LTK63 versus LPS + LT/LTK63 + Wortmannin: +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$. Data are mean ± SEM of triplicate stimulations and are representative of two independent experiments.

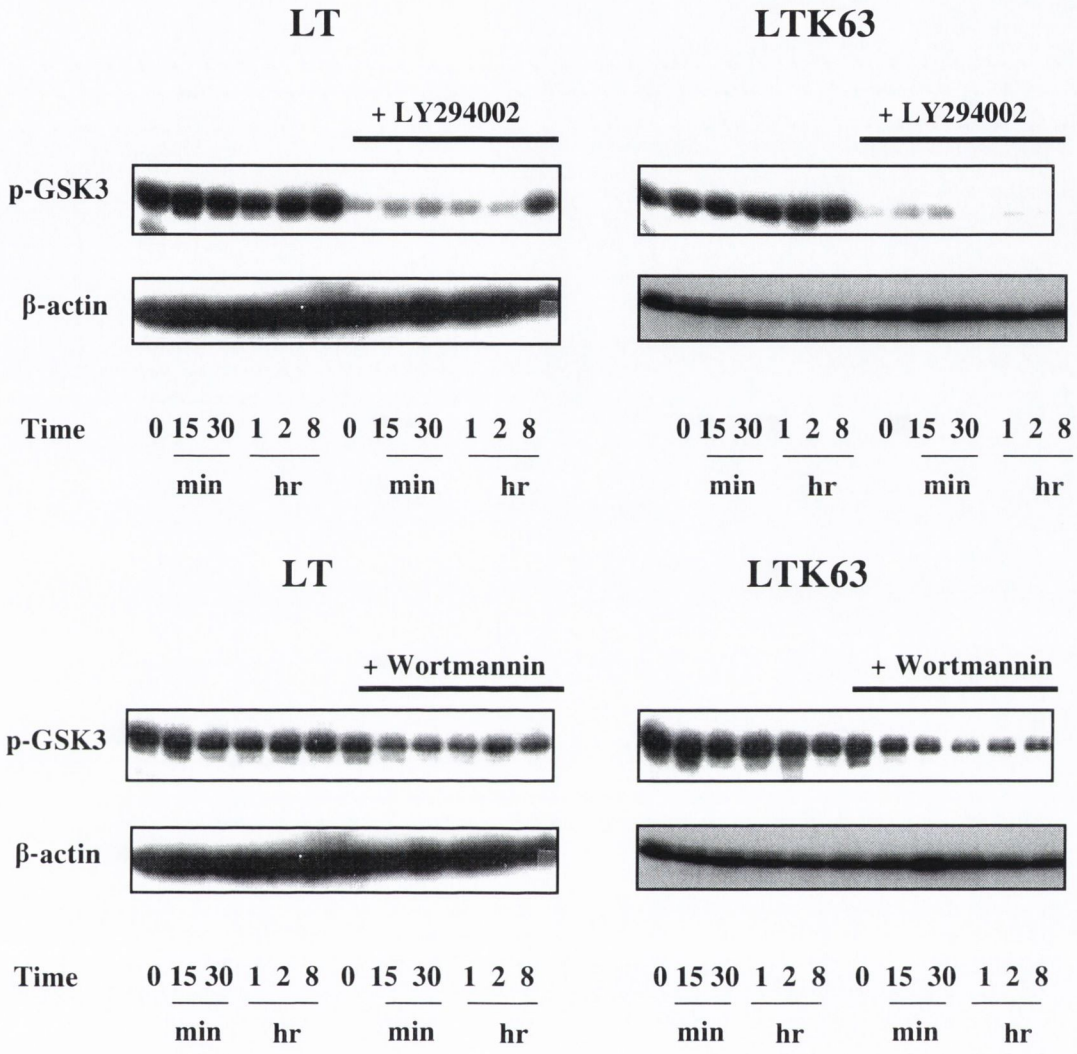

Figure 4.19 The PI3K inhibitors LY294002 and Wortmannin differentially regulate phosphorylation of GSK-3 in DC. BMDC (1 x 10^6 cells/ml) from BALB/c mice were pre-treated with LY294002 (20 μM), Wortmannin (10 μM) or medium only for 30 min, followed by stimulation with LT (1 μg/ml) or LTK63 (1 μg/ml) for 15 min, 30 min, 1 h, 2 h or 8 h. Cell lysates were probed for p-GSK3β and β-actin (as a loading control) by Western blot analysis.

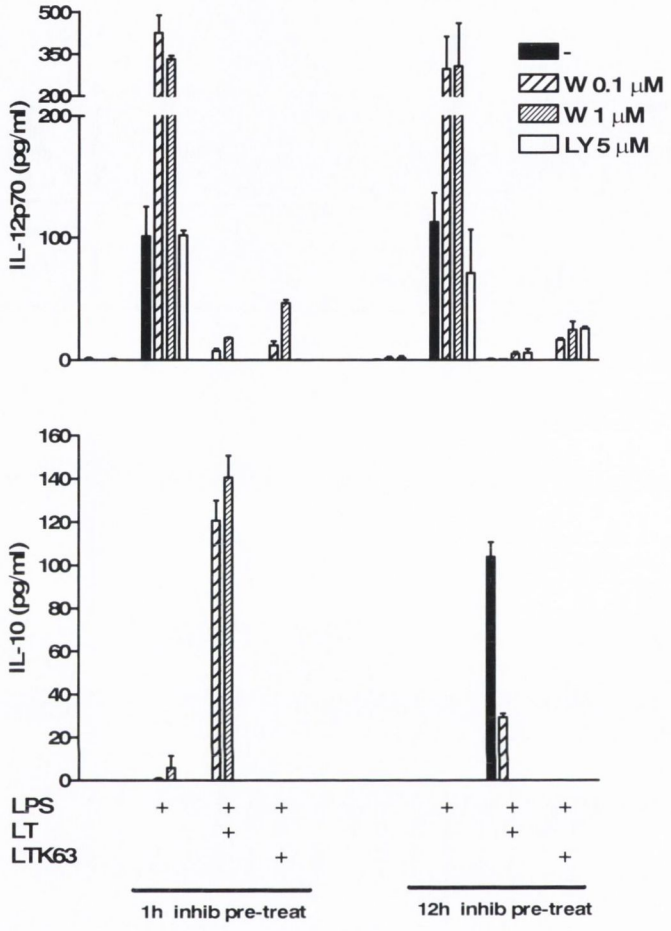

Figure 4.20 Inhibition of LPS-induced IL-12 and enhancement of LPS-induced IL-10 by LT or LTK63 is PI3K-dependent. BMDC (5 x 10^5 cells/ml) from BALB/c mice were pre-treated with the PI3K inhibitor, LY294002 (5 μM) or Wortmannin (0.1 or 1 μM) for 30 min prior to 1 h or 12 h treatment with LT (1 μg/ml) or LTK63 (1 μg/ml) respectively, followed by stimulation with LPS (5 ng/ml). After 24 h, supernatants were collected for analysis of IL-12p70 and IL-10 production by ELISA. Data are mean ± SEM of triplicate stimulations.

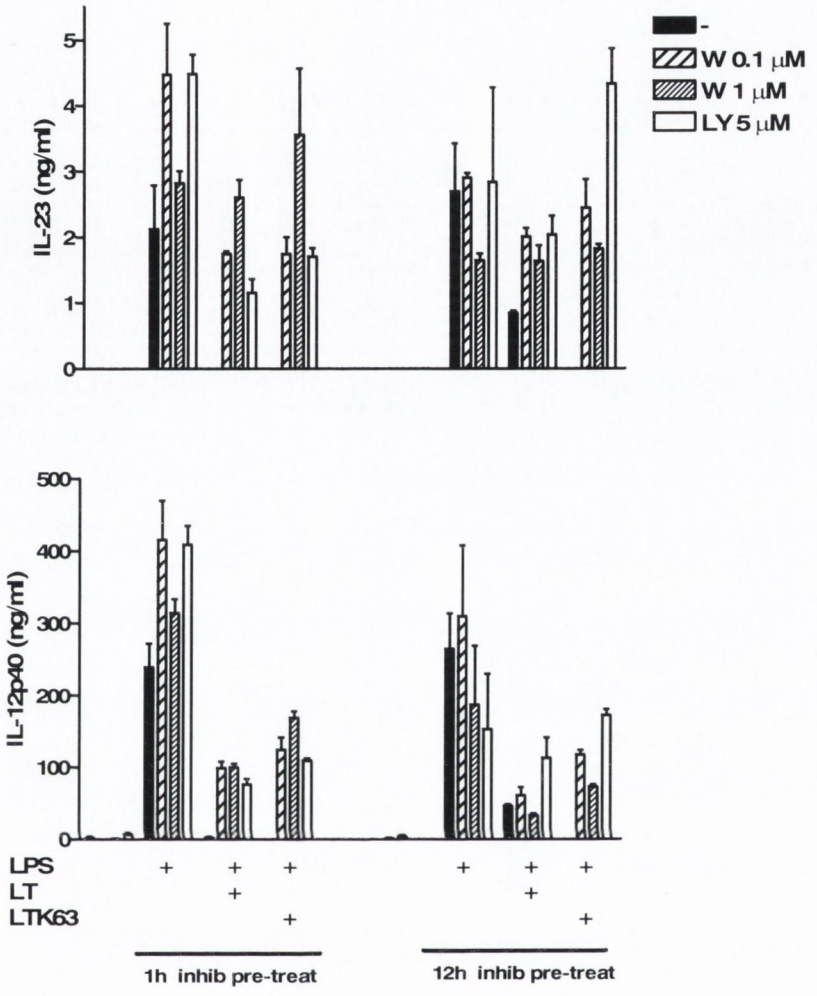

Figure 4.21 The suppression of LPS-induced IL-12 and IL-23 production by pre-incubation of DC with LT or LTK63, for 1 or 12 h respectively, is reversed by inhibition of PI3K. BMDC (5 x 10^5 cells/ml) from BALB/c mice were pre-treated with the PI3K inhibitor, LY294002 (5 μM) or Wortmannin (0.1 or 1 μM) for 30 min prior to 1 h or 12 h treatment with LT (1 μg/ml) or LTK63 (1 μg/ml) respectively, followed by stimulation with LPS (5 ng/ml). After 24 h, supernatants were collected for analysis of IL-23 and IL-12p40 production by ELISA. Data are mean ± SEM of triplicate stimulations.

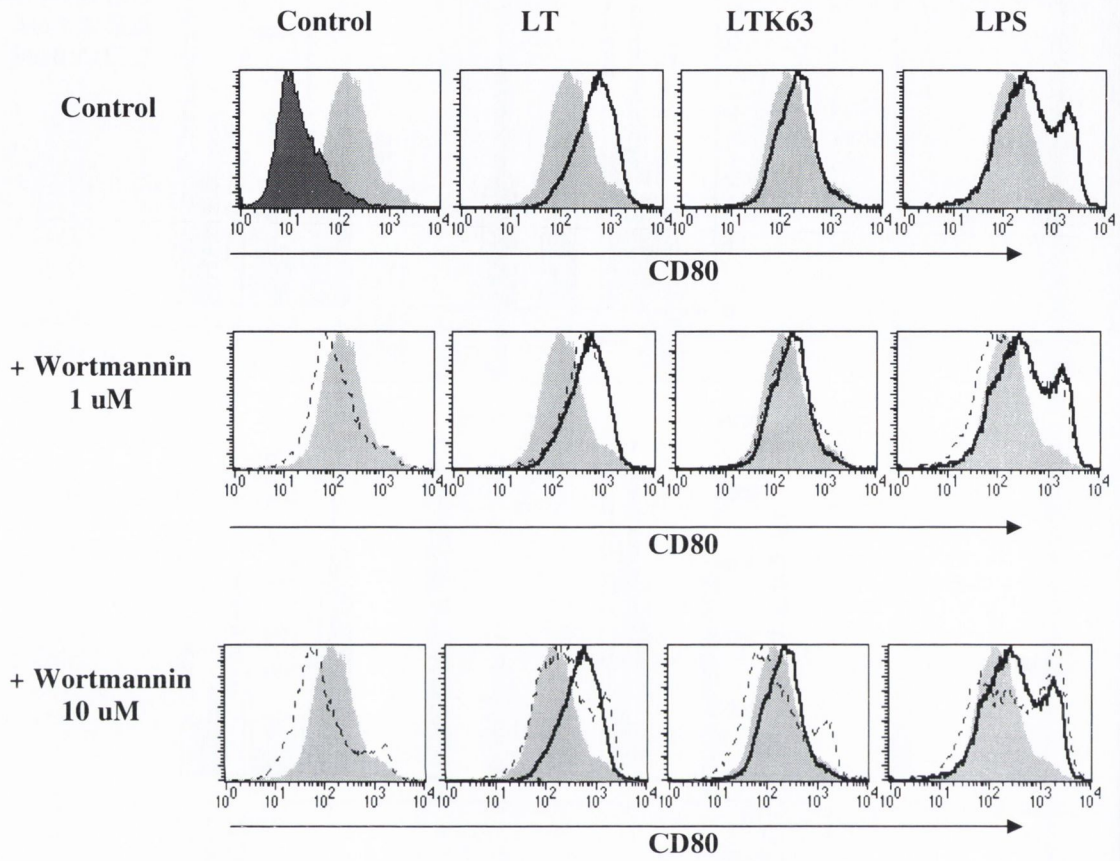

Figure 4.22 Inhibition of PI3K with Wortmannin blocks the expression of CD80 in response to LT and LTK63 but not LPS. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with Wortmannin (1 or 10 μM). Cells were harvested after 24 h and stained with antibodies specific for CD80, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with Wortmannin.

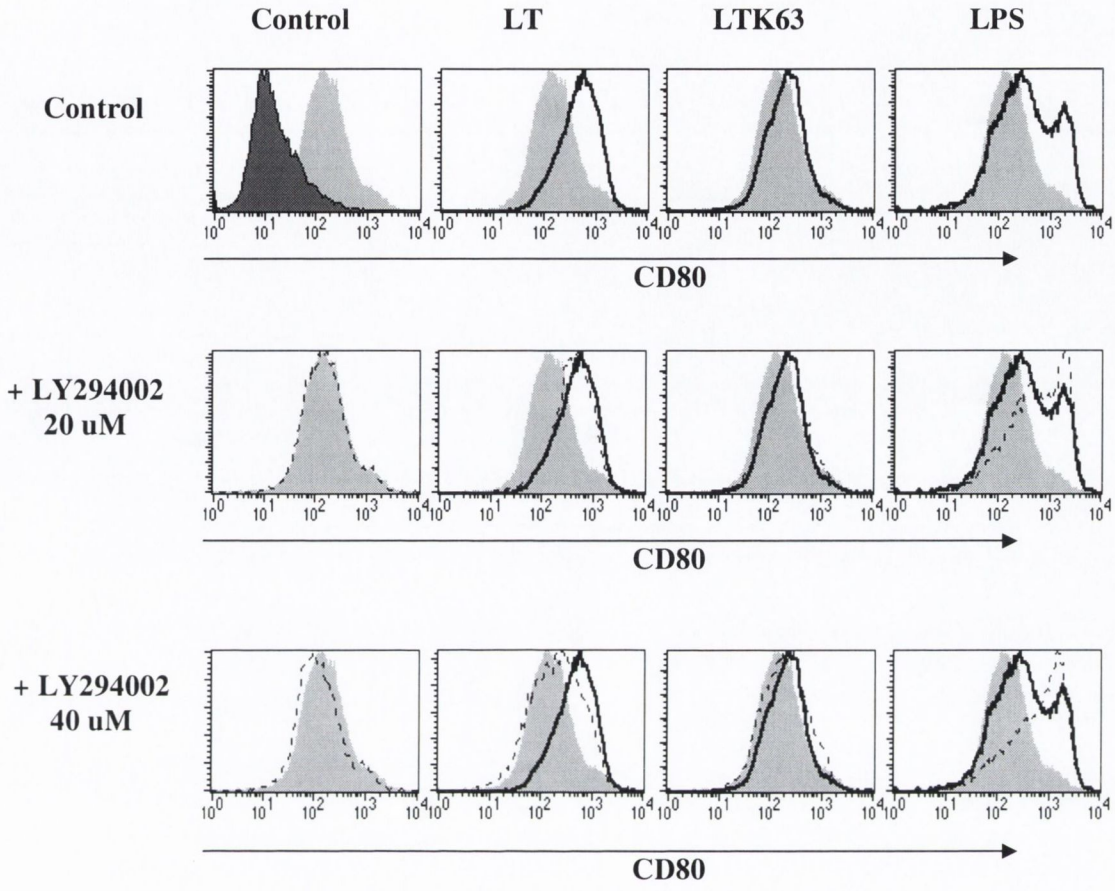

Figure 4.23 Inhibition of PI3K with LY294002 blocks LT- and LTK63-mediated, but not LPS-mediated, CD80 expression. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with LY294002 (20 or 40 μM). Cells were harvested after 24 h and stained with antibodies specific for CD80, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with LY294002.

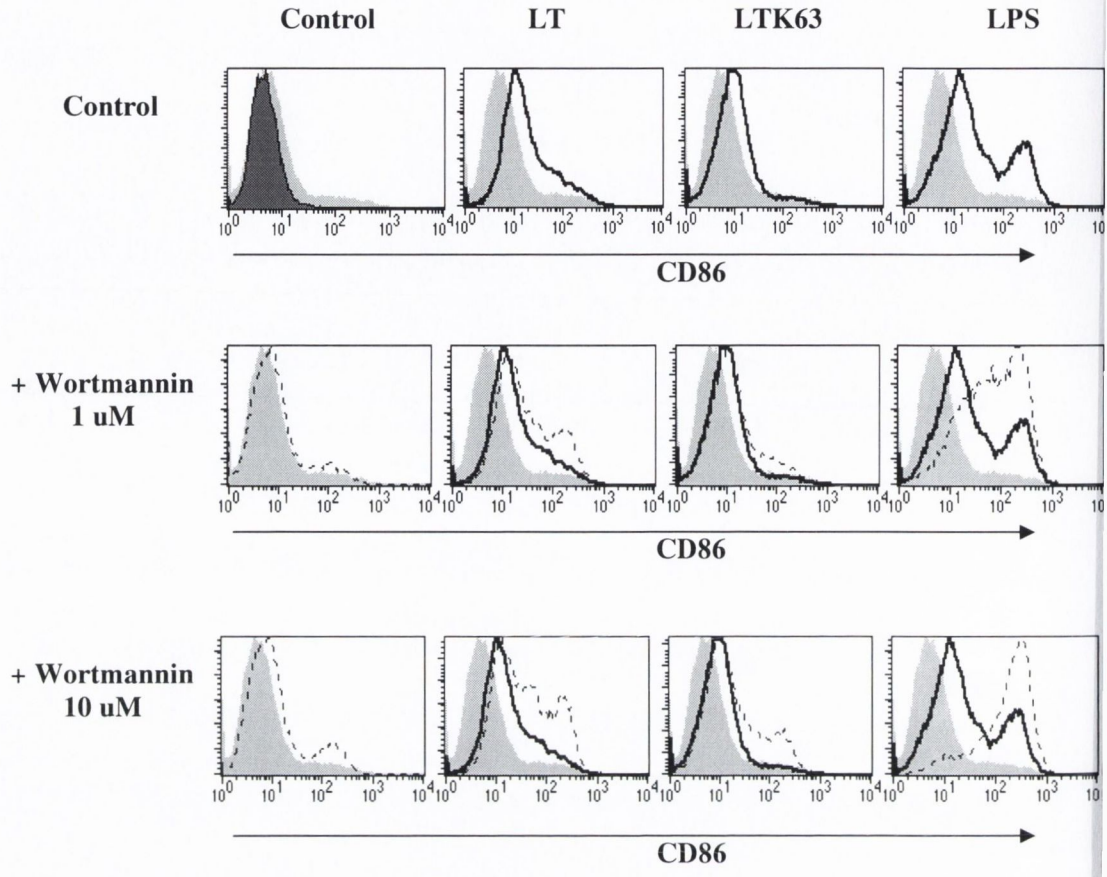

Figure 4.24 Upregulation of CD86 expression by LT, LTK63 and LPS is enhanced by inhibiting PI3K with Wortmannin. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with Wortmannin (1 or 10 μM). Cells were harvested after 24 h and stained with antibodies specific for CD86, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represent isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with Wortmannin.

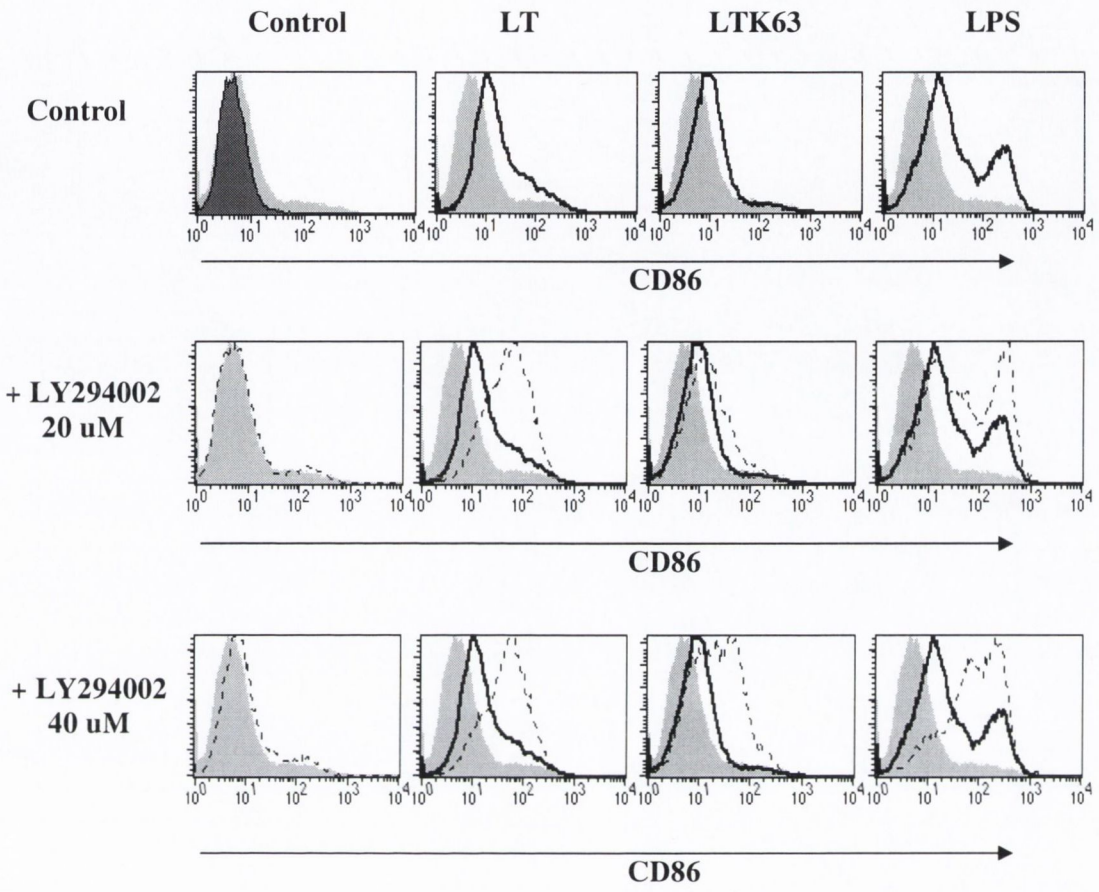

Figure 4.25 Upregulation of CD86 expression by LT, LTK63 and LPS is enhanced by PI3K inhibition with LY294002. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with LY294002 (20 or 40 μM). Cells were harvested after 24 h and stained with antibodies specific for CD86, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with LY294002.

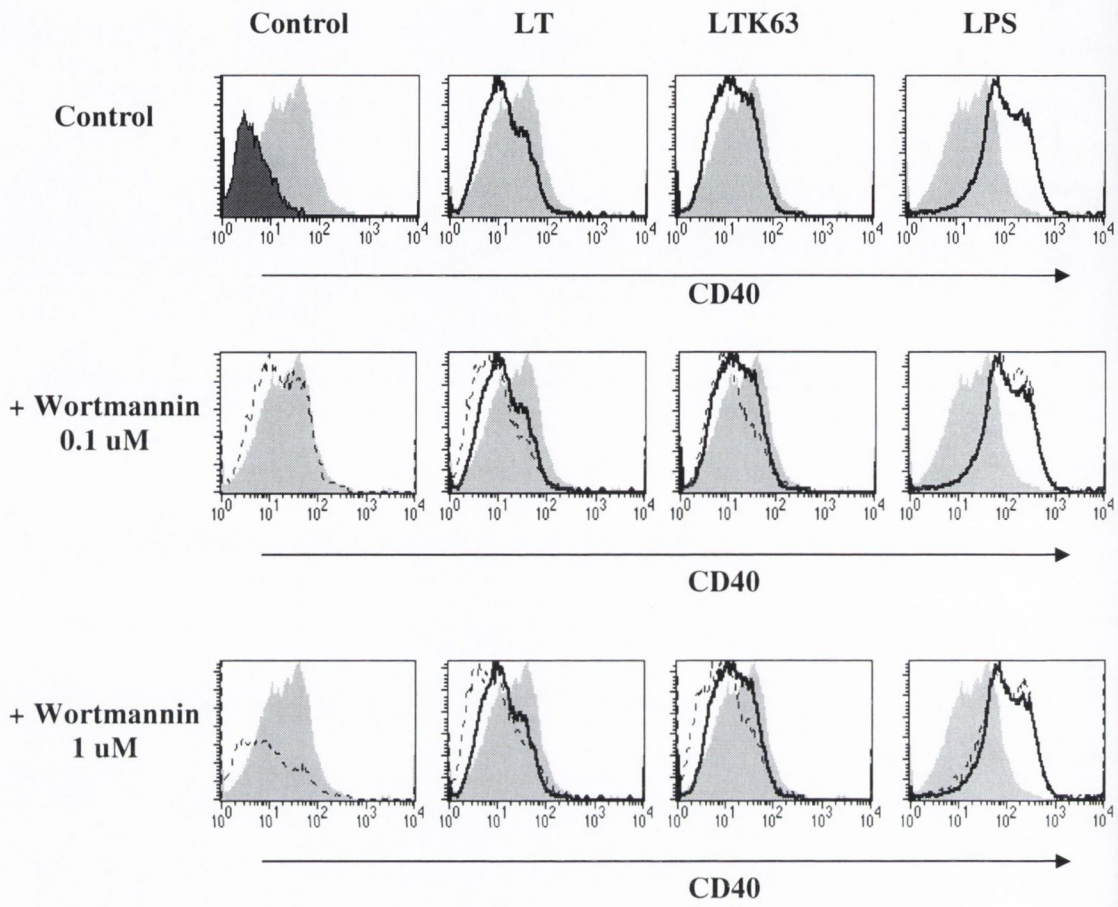

Figure 4.26 Upregulation of CD40 expression by LPS is unaffected by PI3K inhibition with Wortmannin. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with Wortmannin (1 or 10 μM). Cells were harvested after 24 h and stained with antibodies specific for CD40, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with Wortmannin.

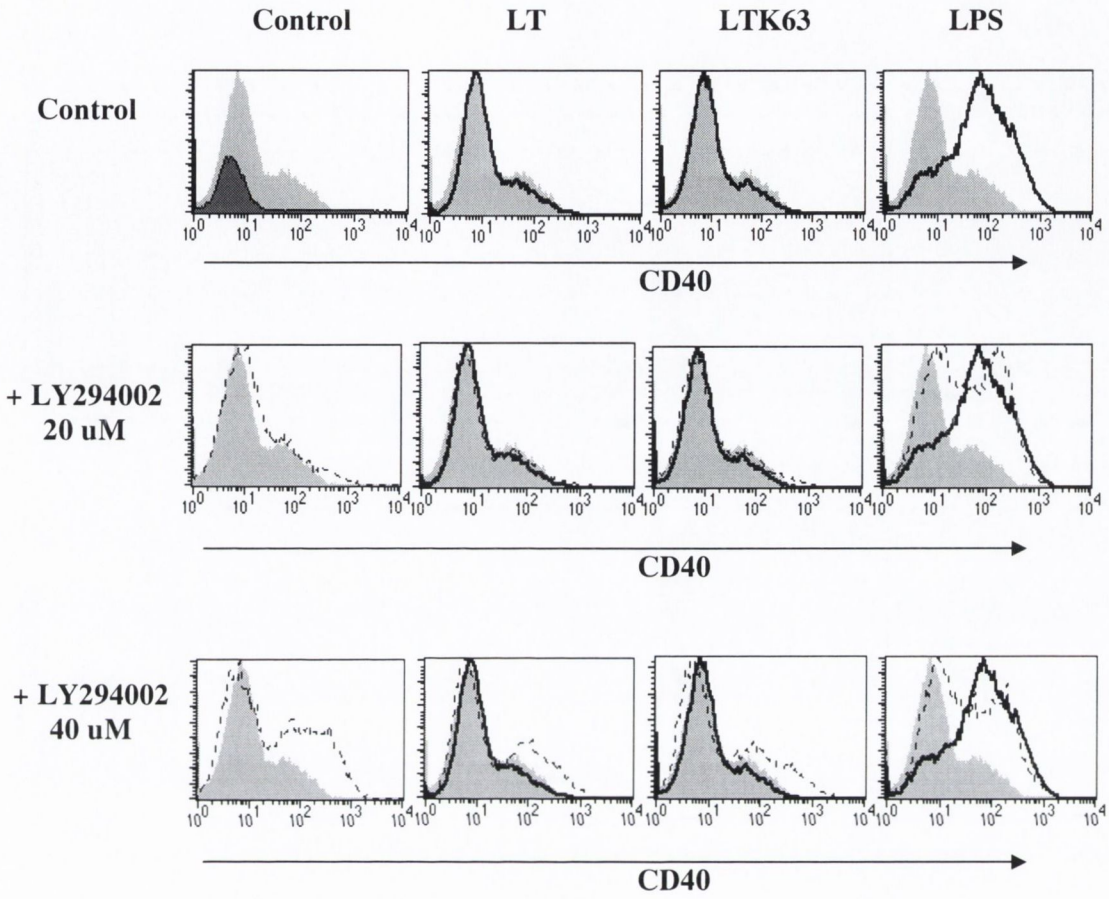

Figure 4.27 Upregulation of CD40 expression by LPS is reduced by PI3K inhibition with LY294002. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with LY294002 (20 or 40 μM). Cells were harvested after 24 h and stained with antibodies specific for CD40, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histograms represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with LY294002.

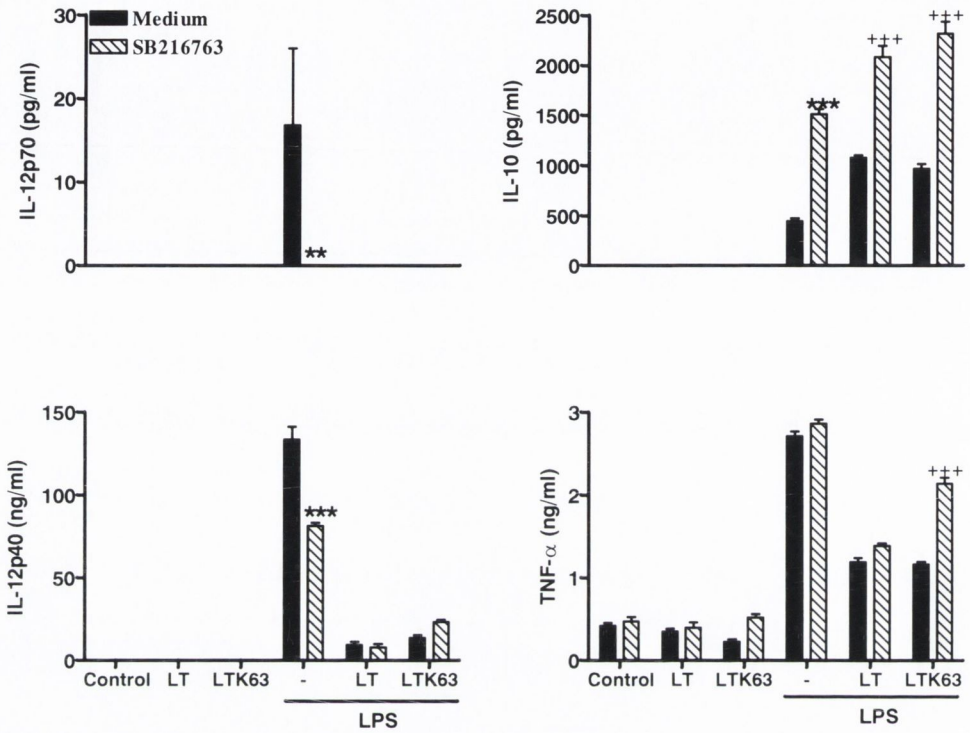

Figure 4.28 Inhibition of GSK3 enhances LPS-induced IL-10 production by DC. BMDC (5 x 10^5 cells/ml) from BALB/c mice were pre-treated with the GSK3 inhibitor, SB-216763 (10 μM), for 30 min prior to 1 h or 12 h treatment with LT (1 μg/ml) or LTK63 (1 μg/ml) respectively, followed by stimulation with LPS (5 ng/ml). After 24 h, supernatants were collected for analysis of IL-12p70, IL-12p40, IL-10 and TNF-α production by ELISA. LPS versus LPS + SB216763: **, $P < 0.01$; ***, $P < 0.001$. LPS + LT/LTK63 versus LPS + LT/LTK63 + SB216763: +++, $P < 0.001$. Data are mean ± SEM of triplicate stimulations.

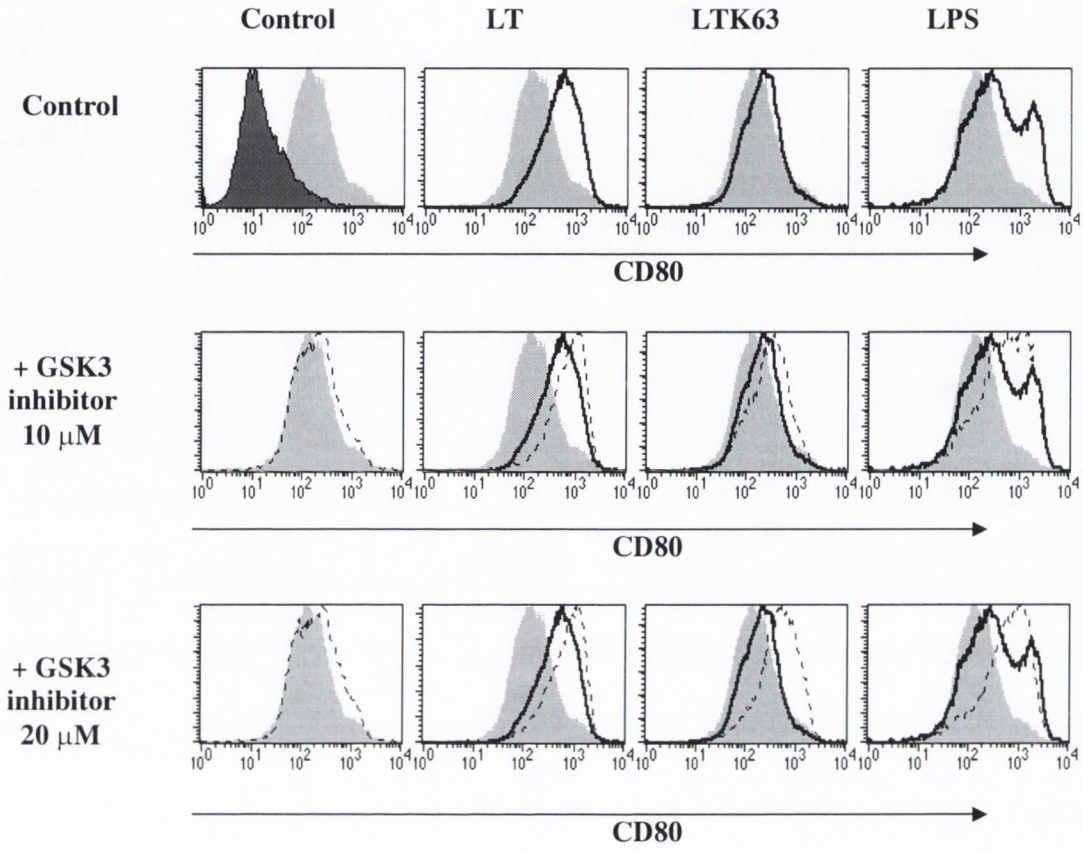

Figure 4.29 Inhibition of GSK3 enhances LT, LTK63 and LPS-induced upregulation of CD80 expression. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with the GSK3 inhibitor, SB-216763 (10 or 20 μM). Cells were harvested after 24 h and stained with antibodies specific for CD80, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with SB-216763.

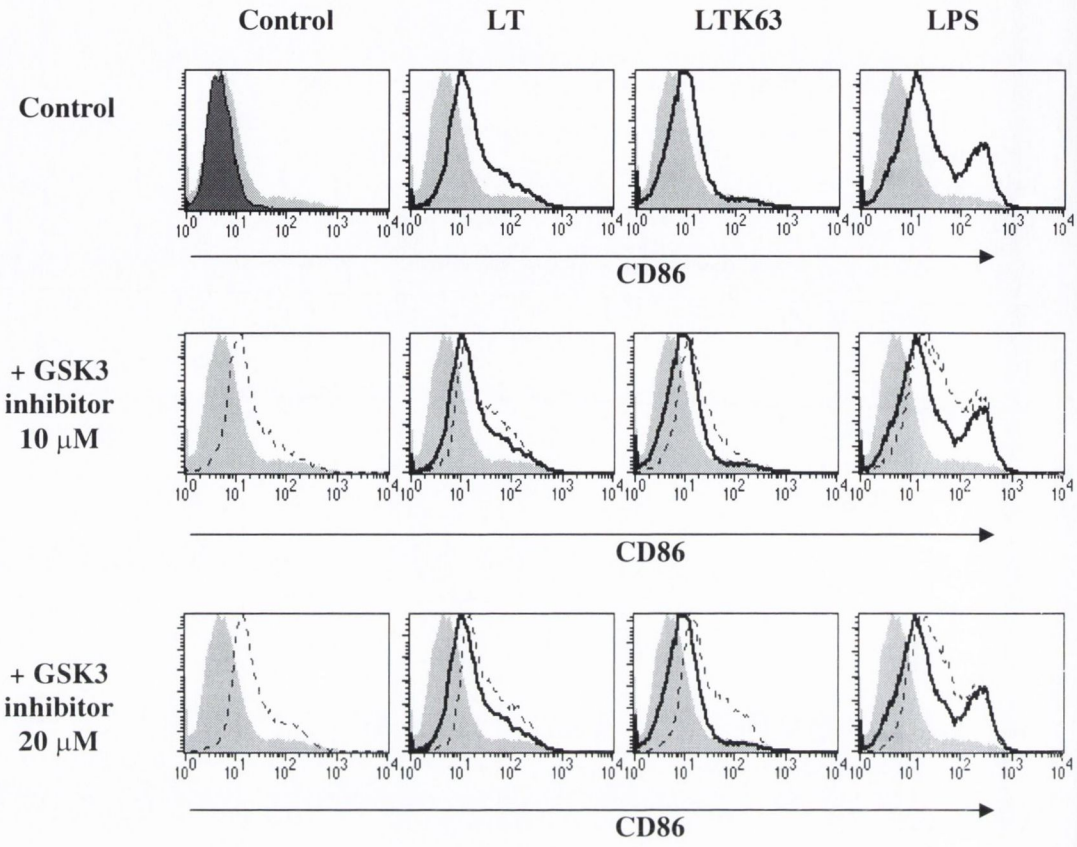

Figure 4.30 Inhibition of GSK3 enhances the endogenous expression of CD86 on DC and increases LT, LTK63 and LPS-induced upregulation of CD86 expression. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with the GSK3 inhibitor, SB-216763 (10 or 20 μM). Cells were harvested after 24 h and stained with antibodies specific for CD86, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with SB-216763.

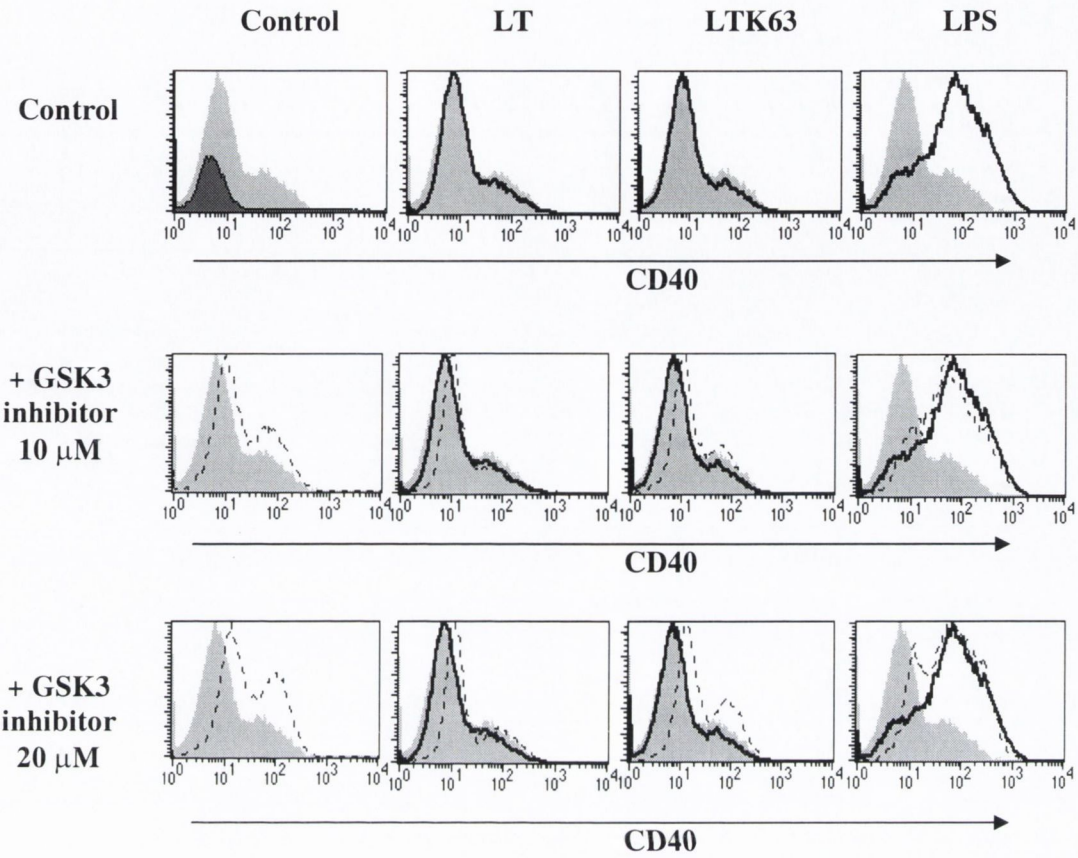

Figure 4.31 Inhibition of GSK3 increases the endogenous expression of CD40 on DC. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with the GSK3 inhibitor, SB-216763 (10 or 20 μM). Cells were harvested after 24 h and stained with antibodies specific for CD40, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with SB-216763.

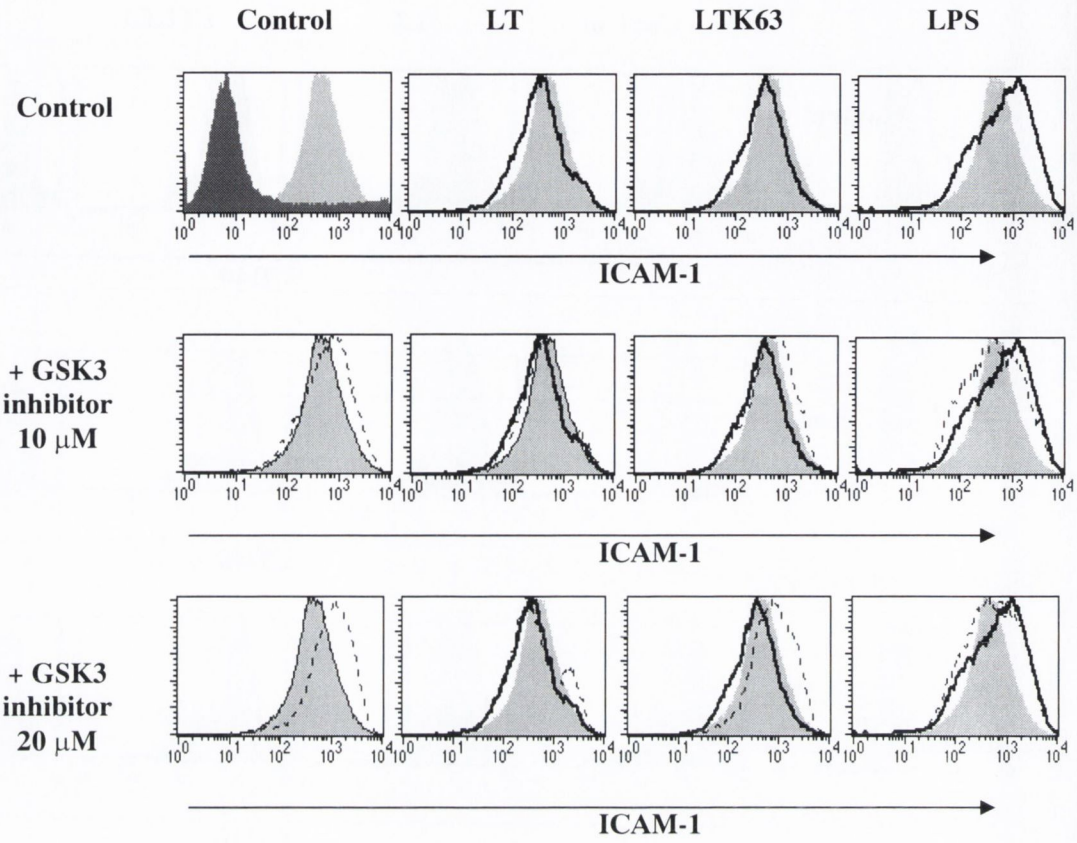

Figure 4.32 Inhibition of GSK3 suppresses the enhancement of ICAM-1 by LPS but increases the endogenous expression of ICAM-1 on DC. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with the GSK3 inhibitor, SB-216763 (10 or 20 μM). Cells were harvested after 24 h and stained with antibodies specific for ICAM-1, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with SB-216763.

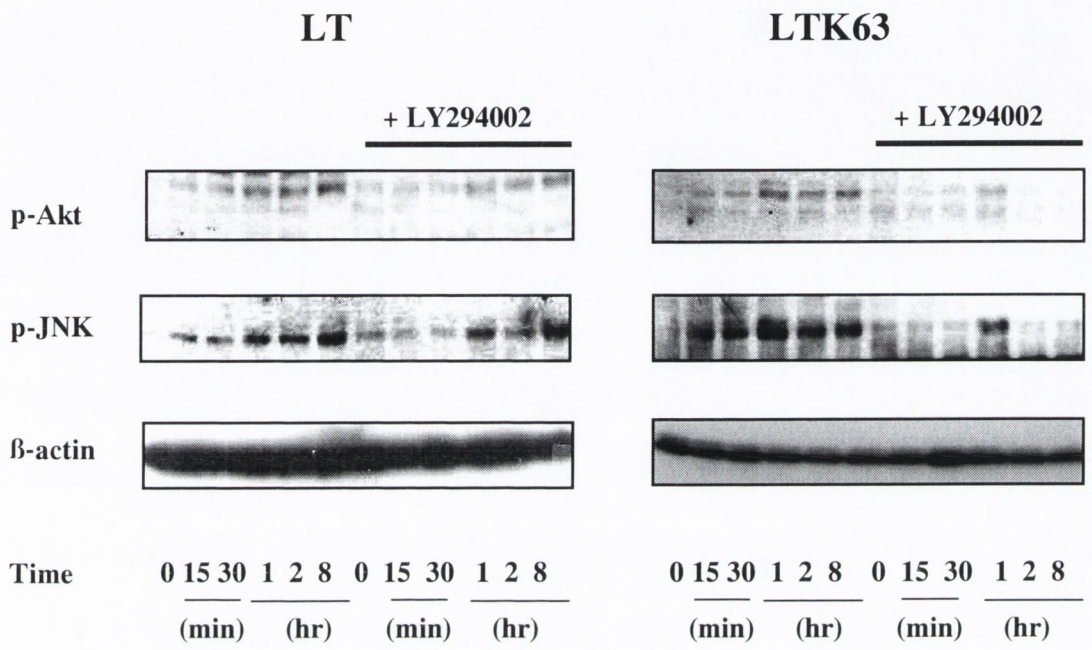

Figure 4.33 LT and LTK63 phosphorylate Akt and JNK in DC and p-JNK activation is only partially PI3K-dependent. BMDC (1 x 10^6 cells/ml) from BALB/c mice were pre-treated with LY294002 (20 μM) or medium only for 30 min, followed by stimulation with LT (1 μg/ml) or LTK63 (1 μg/ml) for 15 min, 30 min, 1 h, 2 h or 8 h. Cell lysates were probed with specific antibodies for p-Akt, p-JNK and β-actin (as a loading control) by Western blot analysis.

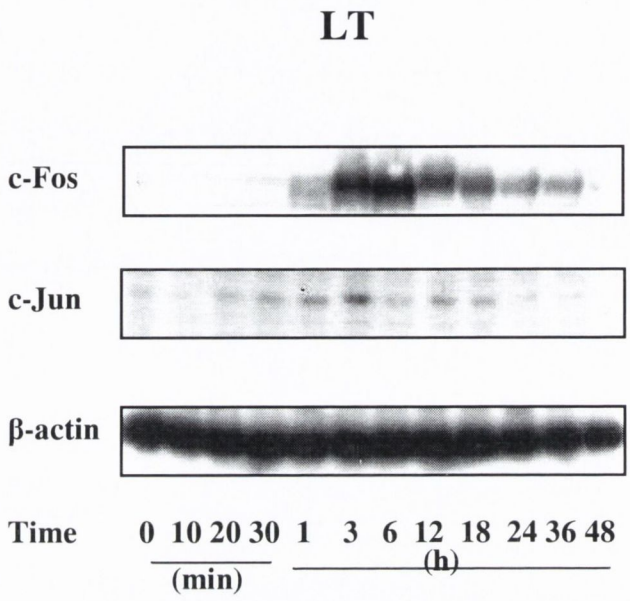

Figure 4.34 LT increases c-Fos and c-Jun expression in DC. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml) for 10 min to 48 h. Cell lysates were probed with specific antibodies for c-Fos, c-Jun and β-actin (as a loading control) by Western blot analysis.

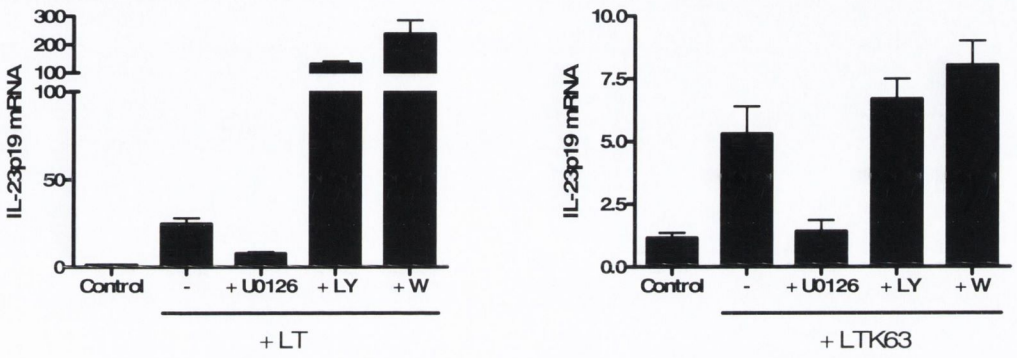

Figure 4.35 Induction of IL-23p19 by LT or LTK63 is ERK-dependent and negatively regulated by PI3K in DC. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml) or medium only, with or without U0126 (5 μM), Wortmannin (1 μM) or LY294002 (5 μM). RNA was isolated from BMDC after 12 h and IL-23p19 expression determined by real time RT-PCR. The data represent the fold change for IL-23p19 mRNA expression relative to cells incubated in medium only following normalization to the endogenous control, 18S rRNA. Data are mean ± SEM of triplicate reactions from duplicate stimulations and are representative of two independent experiments.

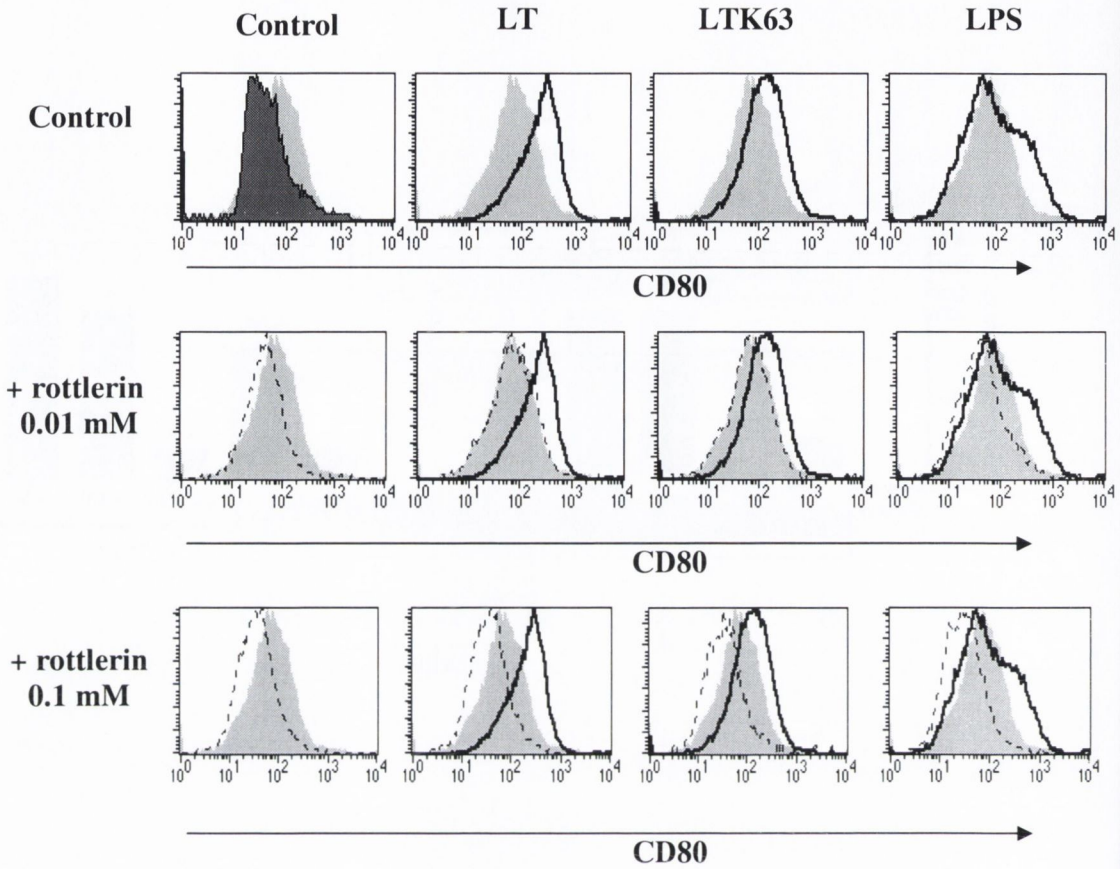

Figure 4.36 The novel PKC inhibitor, rottlerin, inhibits the upregulation of CD80 expression by LT, LTK63 and LPS. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with rottlerin (0.01 or 0.1 mM). Cells were harvested after 24 h and stained with antibodies specific for CD80, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents cells stimulated with LT, LTK63 or LPS and the black dashed line represents cells treated with rottlerin.

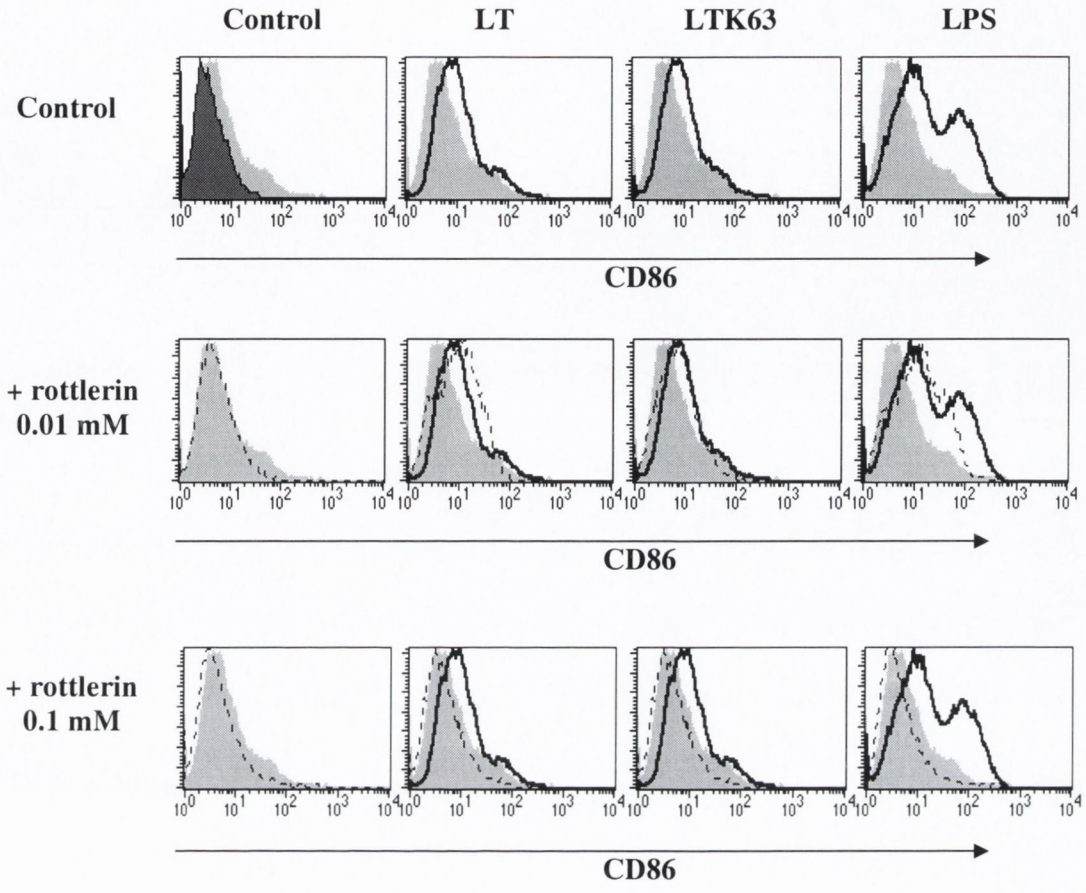

Figure 4.37 Inhibition of novel PKC isoforms with rottlerin suppresses the enhancement of CD86 expression by LT, LTK63 and LPS. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with rottlerin (0.01 or 0.1 mM). Cells were harvested after 24 h and stained with antibodies specific for CD86, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents cells stimulated with LT, LTK63 or LPS and the black dashed line represents cells treated with rottlerin.

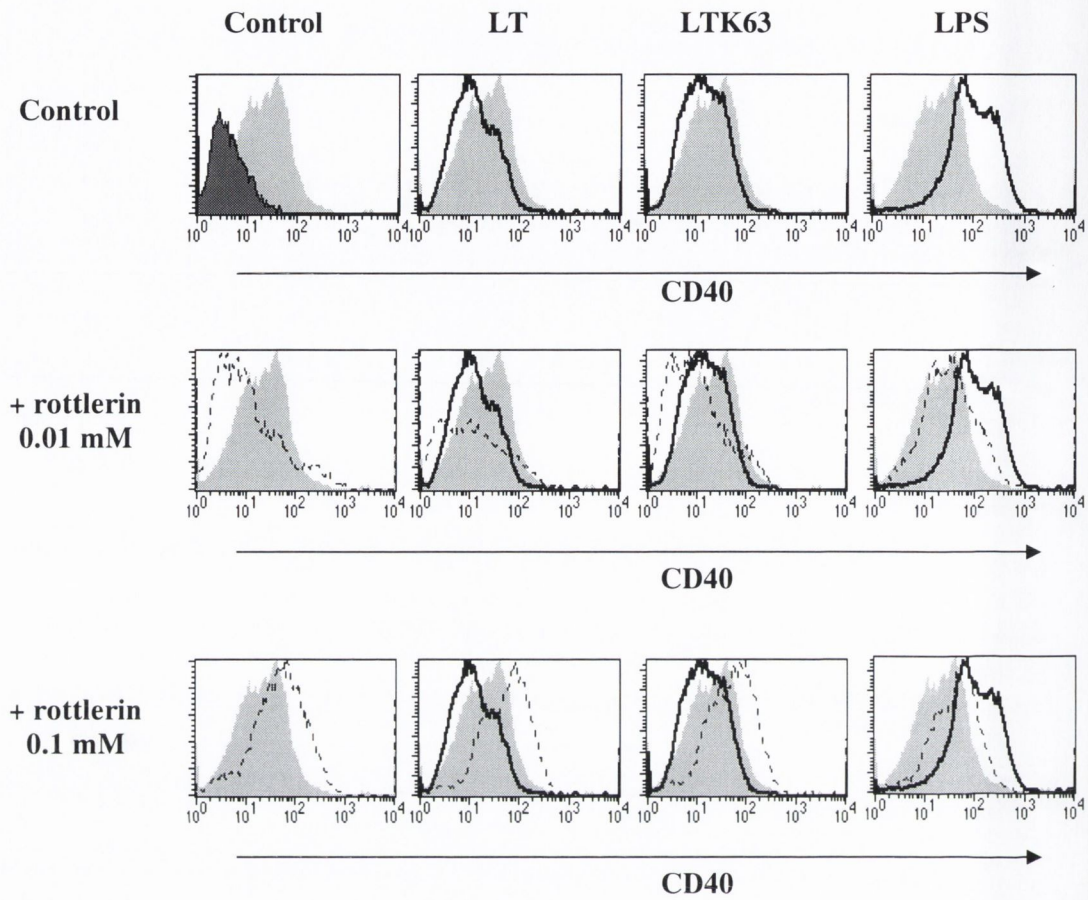

Figure 4.38 Upregulation of CD40 expression by LPS is partially inhibited by rottlerin. BMDC (1 x 10^6 cells/ml) from BALB/c mice were incubated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (10 ng/ml) or medium only, with or without a 30 min pre-treatment with rottlerin (0.01 or 0.1 mM). Cells were harvested after 24 h and stained with antibodies specific for CD40, CD11c or isotype matched controls. Results from immunofluorescence analysis are shown as histograms. Filled light grey histograms represent untreated $CD11c^+$ cells, the filled dark grey histogram represents isotype control, the thick black line represents stimulated cells and the black dashed line represents cells treated with rottlerin.

Chapter 5

Effect of inhibiting ERK on innate cytokine production and T-cell mediated autoimmunity

5.1 INTRODUCTION

Autoimmune conditions, such as MS, RA and the experimental animal models, EAE and CIA, were once considered to be classical Th1-mediated diseases. However, in recent years IL-23-driven Th17 cells have been shown to be pathogenic in many chronic inflammatory and autoimmune diseases (101). Evidence to support a role for Th17 cells in autoimmunity came from reports showing that IL-12(p35)-deficient and IFN-γ-deficient mice developed normal or exaggerated autoimmune inflammation, whereas IL-23(p19)-deficient and IL-17-deficient mice were resistant to the induction of autoimmunity (127, 424, 425). The discovery of the IL-12 family member, IL-23, led to a reassessment of the role of IL-12 in autoimmunity (127).

Th17 cells are a Th cell subset, distinct from Th1 and Th2 cells, that produce IL-17, but not IFN-γ or IL-4 (101, 126). Soon after the discovery of IL-23, a number of reports were published demonstrating that Th17 cells could be induced *in vitro* from naïve mouse CD4 T cells by stimulation through their TCR in the presence of TGF-β and IL-6 (129-131). Although TGF-β and IL-6 are required for the differentiation of Th17 cells, IL-1 and IL-23 are necessary for their survival and full effector function. Studies have shown that expansion of memory Th17 cells is promoted by IL-1 and IL-23. T cells from IL-1 receptor type I-deficient (IL-1RI$^{-/-}$) mice failed to induce IL-17 production in response to IL-23 (101, 134). IL-23R is not expressed on naïve T cells, but once Th17 cells have been differentiated they express high levels of IL-23R (132, 136). Th17 cells also express substantial amounts of IL-1R1 and IL-18Ra, which is consistent with the observation that IL-1R1-deficient mice are resistant to EAE, and this is associated with reduced IL-17 production (134).

Over the last number of years the importance of IL-23 in the pathogenesis of organ-specific inflammation has been demonstrated in a number of animal models including EAE, CIA, and colitis (127, 155, 426). Furthermore, in humans, increased IL-23 expression has been associated with RA, MS, and psoriasis (143, 427, 428). Moreover, polymorphisms in the IL-23R have been linked to susceptibility to two forms of inflammatory bowel disease (IBD): Crohn's disease (CD) and ulcerative colitis (UC) (429). The effects of IL-23 have mainly been linked to Th17 cell responses, indicating

that the IL-23-IL-17 pathway plays a pivotal role in autoimmune inflammation. However, while Th17 cells were shown to be more potent at inducing disease, the relative roles of Th1 and Th17 cells in autoimmune diseases is still a matter of debate (182, 430).

Activated DC are capable of producing key Th17-cell-promoting cytokines including IL-23, IL-1 and IL-6. However, a number of pathogen-derived stimuli, including LPS and CpG, that promote IL-23 also induce IL-12 production by DC and therefore promote Th1 responses. *M. tuberculosis*, a key component of CFA, the widely used adjuvant for murine immunizations, effectively induces Th17 cells, which is associated with its capacity to induce IL-6 and IL-23 as well as TGF-β (129). Veldhoen *et al* showed that LPS-stimulated DC induced the differentiation of naïve T cells into Th17 cells *in vitro*, but required the addition of exogenous TGF-β. IL-23 is produced by DCs and macrophages, soon after exposure to microbial products or endogenous danger signals (431). Subsequently, tissue resident T cells, including αβ, γδ, and NKT cells, produce IL-17 which triggers the production of cytokines and chemokines by epithelial, endothelial and a subset of monocytes, leading to the rapid recruitment of neutrophils (146). It has also been demonstrated that IL-23 is produced by both resident microglia and inflammatory macrophages in the CNS of mice with EAE, but that only macrophages can respond to IL-23, by production of IL-1β and TNF-α (127).

There remains a significant unmet clinical need for new therapies against autoimmune and chronic inflammatory diseases, as existing regimes are only effective in some patients, and crucially leave the host at significantly higher risk of infection, such as with *M. tuberculosis*. Indeed, antibodies, antagonists or receptor antagonists to IL-1 and TNF-α are already in use for a range of inflammatory diseases, including RA (432). It is well recognized that anti-IL-12p40 treatment is highly efficacious in a number of experimental autoimmune disease models, including EAE and CIA (405, 433). Moreover, recent data indicated that anti-IL-12p40 therapy may be effective for the treatment of human autoimmune diseases such as psoriasis and Crohn's disease (434). Clinical trials involving an IL-12p40-specific human monoclonal antibody in Crohn's disease patients showed a reduction of clinical disease and decreased cytokine production by mononuclear cells from the lamina propria (435). However, it was not clear if the therapeutic effects of blocking IL-12p40 resulted from neutralization of IL-12 or IL-23.

Antibodies to IL-23 are in clinical development and pre-clinical studies have already shown efficacy in animal models of autoimmunity (436, 437).

The NFκB and IRF families of transcription factors, as well as MAPK activation, play a critical role in the regulation of the cytokines responsible for the differentiation of naïve T cells (19, 438). Evidence is emerging that DC activated by specific pathogen-derived molecules can selectively promote the induction of distinct T cell subtypes. DC activated through PRRs such as TLRs or NLRs can produce IL-12p40, IL-12p70 and IL-23 following exposure to PAMPs. Different TLR ligands associate with and signal through specific adaptor molecules, which results in the activation of distinct transcription factors (35). In particular, there are differences in the transcriptional control of genes that encode the different polypeptide chains of the IL-12 family members downstream of TLR activation. TLR-stimulation induces expression of the IL-23p19 and IL-12p40 chains which comprise IL-23, but the induction of the p35 gene and subsequent IL-12p70 production is more restricted (438). $IRF1^{-/-}$ macrophages and DC are defective in the induction of IL-12p35, therefore, IRF1 is indispensable for the development of Th1 responses (352). TLR-mediated activation of the genes that encode p40, p35 and p19 is largely mediated by the NFκB family, in particular c-Rel, (439-441). Several IRFs participate in the synthesis of IL-12p70 and IL-23, but importantly, activation of specific IRFs results in the induction of the transcription of particular genes. It has been suggested that IRF5 regulates IL-23 and IL-12p70 production, whereas IRF1, IRF3 and IRF7 are uniquely involved in p35 gene activation and, thereby, IL-12p70 synthesis (351, 354, 438).

Data presented in chapter 4 demonstrated that ERK had opposing effects on IL-12 and IL-23 production by DC. It is well established that, in addition to NFκB activation, MAPK signalling plays an important role in Th cell activation and differentiation. There are reports of positive and negative regulation of IL-12 family members by MAPKs. Analysis of the functional role of p38 is mainly based on results obtained in the p38 kinase MKK3-deficient mice (319). These data demonstrated that p38 MAPK is an essential factor for IL-12 production by macrophages and DC. In contrast, it has been reported that ERK negatively regulates IL-12 production (189, 190). There is more limited knowledge on the signalling pathways involved in the regulation of the most recently discovered member of the IL-12 family, IL-23. A recent report showed that

murine DCs deficient in c-Rel, a member of the NFκB family, were severely compromised in their ability to transcribe the *p19* gene (439).

Having established in chapter 4 that LT-induced IL-23p19 expression was mediated by the MAPK ERK, one of the main aims of this study was to investigate the role of MEK1/2 signalling in the regulation of PRR-induced IL-23 production by DC. In addition, experiments were designed to examine if ERK differentially regulated IL-12p40 and IL-12p70 secretion. Since IL-23 has been shown to play a critical role in the induction IL-17 producing T cells, this study investigated the effect of inhibiting ERK in DC on their subsequent ability to promote Th17 responses. As the IL-23-IL-17 axis is important in a number of autoimmune diseases, experiments were also designed to examine the effect of inhibition of ERK on the clinical course of EAE.

5.2 RESULTS

5.2.1 The MEK1/2 inhibitor, U0126, decreases endogenous and LPS-induced phosphorylated ERK in DC

In studies designed to examine the role of MAPK/ERK in the regulation of LT induced IL-23p19 expression in DC, it was observed that ERK differentially regulated TLR-induced IL-12 family members. While it was clear that the MEK1/2 inhibitor modulated cytokine responses in DC, it was important to confirm that U0126 inhibited ERK activation. In chapter 4, it was shown that DC express endogenous p-ERK which can be further activated by stimulation of DC with the adjuvants LT or LTK63. To verify that the MEK1/2 inhibitor suppresses ERK phosphorylation, the level of expression of p-ERK was examined in DC that were pre-treated with the MEK1/2 inhibitor. BMDC were incubated with increasing concentrations of U0126 (0.2 – 5 μM) followed by stimulation with LPS, and p-ERK and total ERK were detected using specific antibodies. The expression of endogenous p-ERK in DC was suppressed by U0126. In addition, LPS increased the expression of p-ERK and this was inhibited when DC were incubated with the MEK1/2 inhibitor (Fig. 5.1).

5.2.2 LPS-induced IL-23 and IL-1β production by DC is ERK-dependent

Recent research has identified an important role for IL-23 in the maintenance and expansion of Th17 cells (101). IL-6, along with TGF-β, has been shown to be essential for the differentiation of naïve T cells into Th17 cells (129-131). Furthermore, IL-1 has been shown to synergize with IL-23 to promote IL-17 production by memory T cells and γδ T cells (Sutton, unpublished observations). The findings presented in chapter 4 demonstrated that inhibition of ERK enhanced IL-12p70 production in DC (Fig. 5.2). Therefore, experiments were carried out to examine the role of ERK in the regulation of other IL-12 family members. BMDC were pre-treated with the MEK1/2 inhibitor, U0126

(1 or 5 μM), prior to stimulation with LPS (100 ng/ml) for 24 h. The results revealed that inhibition of MEK1/2 significantly inhibited LPS-induced IL-23 production in DC (Fig. 5.2). In this experiment, inhibition of ERK had no effect on LPS-induced IL-12p70 production, while blocking ERK attenuated LPS-induced IL-12p40 production. These findings indicate that MAPK/ERK signalling differentially regulates IL-12 family members in DC.

To determine if ERK is involved in the regulation of other Th17-promoting cytokines, experiments were performed to investigate the effect of blocking ERK on IL-1β and IL-6 production by DC. Inhibition of ERK significantly suppressed LPS-induced IL-1β production and also inhibited IL-6 production (Fig. 5.2). These data demonstrate that inhibition of ERK suppresses IL-23, IL-1 and IL-6, key cytokines involved in Th17 cell polarization.

Consistent with the results shown in chapter 4, U0126 partially inhibited LPS-induced IL-10 production. A reciprocal relationship between IL-10 secretion and IL-12 production has been reported in the literature (94). If IL-10 suppresses IL-23 production then inhibition of ERK might also be expected to enhance IL-23 production. However, these data showed that inhibition of ERK suppressed LPS-induced IL-23 production. Taken together, the results establish that ERK is an important regulator of the three cytokines IL-23, IL-1β and IL-6, all of which are central to the induction and maintenance of Th17 cells.

5.2.3 Two different synthetic MEK1/2 inhibitors exert similar suppressive effects to U0126 on PAMP-induced IL-23 and IL-1β production

While the results in Fig. 5.2 demonstrated that blocking ERK activation with U0126 inhibited LPS-induced IL-23 production, it was important to verify that this effect was not just specific for U0126. Therefore, experiments were carried out using two alternative MEK1/2 inhibitors, PD98059 and the Calbiochem MEK1/2 inhibitor. BMDC were pre-treated with U0126, PD98059 or the Calbiochem MEK1/2 inhibitor at

increasing concentrations (0.1, 1, 5 and 10 μM) prior to stimulation with LPS (100 ng/ml) or the dectin-1 agonist, curdlan (100 μg/ml).

Consistent with the suppression of IL-23 and IL-1β production by LPS-pulsed DC that were incubated with the MEK inhibitors U0126 or PD98059, the MEK1/2 inhibitor (Calbiochem) also suppressed LPS- and curdlan- induced IL-23 and IL-1β production by DC (Fig. 5.3). This data confirmed that ERK plays a pivotal role in the induction of IL-23 and IL-1β in DC, and also showed that inhibition of ERK suppresses IL-23 and IL-1β induced by a dectin-1 agonist.

5.2.4 LPS induces transient IL-23p19, IL-12p40, IL-1β and IL-10 mRNA expression in DC

Formation of biologically active IL-23 requires the synthesis of the IL-12 p19 and p40 subunits within the same cell (153). Unlike the IL-12 p35 subunit, which is extensively posttranslationally modified by N-linked glycosylation, the p19 subunit has no N-glycosylation sites and does not require such modifications. Consequently, it is possible that upon activation, DC expressing p19 and p40 subunits of IL-12 produce IL-23 earlier than bioactive IL-12p70. It was therefore important to determine the kinetics of LPS-induced cytokine mRNA expression in DC before investigating the effect of blocking ERK on IL-23 and IL-1β mRNA expression. At 30 min, 1, 12 and 24 h following stimulation with LPS total RNA was isolated from DC, purified, reverse transcribed into cDNA, and amplified by real-time PCR for the detection of IL-12p40, IL-23p19, IL-1β and IL-10 mRNA expression.

Enhanced expression of IL-23p19, IL-12p40, IL-1β and IL-10 mRNA was observed 1 h after stimulation with LPS. Maximal induction was observed for all tested cytokines when DC were stimulated with LPS for 12 h (Fig. 5.4). By 24 h, LPS-induced expression of IL-23p19, IL-12p40, IL-1β and IL-10 had decreased dramatically. These data indicate that LPS-stimulation induces transient expression of the transcripts required for the translation and secretion of these cytokines.

5.2.5 LPS-induced IL-23 and IL-1β mRNA expression is ERK-dependent

Having established that ERK plays a major role in IL-23 and IL-1β production by DC, experiments were carried out to determine if this was reflected at the level of IL-23 and IL-1β mRNA expression. BMDC were pre-treated with the MEK1/2 inhibitor, U0126 (5 μM), prior to stimulation with LPS (100 ng/ml) for 12 h. The induction of IL-23p19 mRNA by LPS was significantly inhibited in cells treated with U0126 (Fig. 5.5). In contrast, LPS-induced IL-12p35 mRNA was unaffected by blocking ERK. Inhibition of ERK suppressed LPS-induced IL-12p40 expression. These findings are consistent with the results in Fig. 5.2 showing that inhibition of ERK suppressed IL-23 secretion by DC.

While the requirements for the differentiation of Th1 and Th2 cells are well established, studies on the regulation of Th17 cells have only begun in the last number of years, and consequently there is more limited knowledge on the regulation of this T cell population. However, along with IL-23, IL-6 and TGF-β, a crucial role has been demonstrated for IL-1β in the induction of Th17 cells (134). Therefore, it was important to determine if ERK played a role in the regulation of IL-1β mRNA expression in DC. Similar to observations on IL-1β protein secretion by DC, inhibition of ERK significantly decreased LPS-induced IL-1β mRNA expression (Fig. 5.5). These findings suggest a potential role for ERK in regulating Th17 cells by promoting innate IL-23 and IL-1β production.

IL-27 is another member of the IL-12 family of cytokines, and was first characterized as a pro-inflammatory cytokine capable of regulating Th1 cells. It was shown that proteoglycan-induced arthritis (PGIA) was delayed and severity reduced in IL-27R$^{-/-}$ mice and this was associated with decreased IFN-γ production (442). However, subsequent studies demonstrated that IL-27R-deficient mice displayed exacerbated inflammatory responses to a variety of challenges. These mice were hypersusceptible to EAE and had enhanced Th17 responses (443). Therefore, this study examined the role of ERK in regulating IL-27. IL-27 is a heterodimeric cytokine composed of EBI3 and a p28 subunit. The results showed that LPS-induced IL-27p28 mRNA was suppressed in DC pre-treated with the MEK1/2 inhibitor (Fig.5.5).

5.2.6 CpG-induced IL-23 production is ERK-dependent, while ERK negatively regulates CpG-induced IL-12p70 and IL-12p40 production

To determine if the suppressive effect of the MEK1/2 inhibitor on IL-23 production by DC was specific only for TLR4 or dectin-1 agonists, experiments were carried out using the TLR-9 agonist CpG. BMDC were pre-treated with the MEK1/2 inhibitor, U0126 (5 μM), prior to stimulation with CpG for 24 h. Inhibition of ERK significantly suppressed CpG-induced IL-23 production (Fig. 5.6). In contrast, blocking ERK increased CpG-induced IL-12p70 and IL-12p40 production.

Similar to observations with LPS, CpG-induced IL-10 production was inhibited when DC were incubated in the presence of U0126 (Fig. 5.6). Unlike LPS-induced TNF-α production, inhibition of ERK had little effect on CpG-induced TNF-α (Fig. 5.6). In contrast to LPS and other PAMPs, such as curdlan, CpG is a poor inducer of IL-1β in DC. However, blocking ERK did not suppress CpG-induced IL-1β production. These data confirm that ERK is required for IL-23 production by DC, but negatively regulates bioactive IL-12p70 production.

Having established that inhibition of ERK suppresses IL-23 protein secretion by DC stimulated with a TLR- or dectin-1 agonist, and LPS-induced IL-23p19 mRNA expression, the next study examined if ERK was required for the induction of IL-23p19 mRNA by CpG or curdlan. BMDC were pre-treated with U0126 prior to stimulation with LPS, CpG or curdlan for 12 h. Like LPS, the TLR9 agonist, CpG, and the dectin-1 agonist, curdlan, induced IL-23p19 expression in DC (Fig. 5.7). Similar to the effect on LPS-induced IL-23p19 expression, inhibition of ERK decreased CpG- and curdlan-induced IL-23p19 mRNA (Fig. 5.7).

5.2.7 ERK negatively regulates CpG-induced IL-12p40 and IL-12p35 mRNA

Having shown that inhibition of ERK suppressed IL-23p19 expression in CpG- and LPS-stimulated DC, it was important to determine if the expression IL-12p40 and IL-12p35 induced in DC in response to TLR-agonists are also mediated through ERK

activation. BMDC were pre-incubated with U0126 (5 μM) prior to stimulation with LPS (100 ng/ml) or CpG (1 μg/ml) for 12 h. LPS-induced IL-12p35 was marginally enhanced when DC were pre-incubated with the MEK1/2 inhibitor (Fig. 5.8). Previous experiments demonstrated that blocking ERK in LPS-stimulated DC has little effect on IL-12 protein secretion or mRNA expression. CpG-induced IL-12p35 mRNA was enhanced in DC that were pre-treated with U0126 (Fig. 5.8). Moreover, inhibition of ERK augmented CpG-induced IL-12p40 expression (Fig. 5.8). In contrast and in line with earlier data, blocking ERK inhibited LPS-induced IL-12p40 expression. Consistent with the observation that inhibition of ERK suppressed LPS-induced IL-1β mRNA expression, CpG-induced IL-1β mRNA expression was inhibited by U0126 (Fig. 5.8). These data demonstrate that ERK negatively regulates CpG-induced IL-12p40 and IL-12p35 expression, and while LPS-induced IL-12p40 requires ERK, it has little or no effect on the expression of LPS-induced IL-12p70.

5.2.8 *M. tuberculosis*- induced IL-1β and IL-23 production by DC is mediated by ERK activation

IL-1β and IL-23 are crucial for the induction or expansion of Th17 cells, which play a critical pathogenic role in EAE, a mouse model of MS. In this model and other autoimmune models, mice are immunized with self peptides combined with CFA. CFA contains heat killed *M. tuberculosis*, which includes a range of TLR and NLR agonists. Therefore, studies were carried out to examine the effect of blocking ERK on cytokine production by DC stimulated with *M. tuberculosis*.

BMDC were pulsed with heat-killed *M. tuberculosis* (MTB) (2, 10 or 50 μg/ml), LPS (100 ng/ml) or with medium only in the presence or absence of the MEK1/2 inhibitor, U0126 (5 μM). Supernatants were recovered after 24 h for analysis of IL-12p40, IL-23 and IL-1β concentrations. Similar to the observations with individual TLR agonists, inhibition of ERK significantly suppressed IL-23 and IL-1β production by DC in response to MTB (Fig. 5.9). Blocking ERK suppressed IL-12p40 production in response to 10 μg/ml MTB but, did not affect IL-12p40 induced with 50 μg/ml MTB.

These findings provide further evidence of the importance of MAPK/ERK signalling for DC IL-23 and IL-1β production.

5.2.9 The induction of IL-23 and IL-1β by ERK is not reversed by blocking IL-10

Previous data demonstrated that LPS-induced IL-10 production by DC is partly mediated by ERK activation (Fig.s 4.6 and 5.2). IL-10 has been shown to be a negative regulator of IL-12 production (94, 444). This suggests that the suppression of IL-10 by MEK inhibitors would enhance IL-23 production by DC. BMDC were incubated with a neutralizing anti-IL-10 antibody before treatment with U0126 (5 μM) and LPS (100 ng/ml). LPS-induced IL-23 and IL-12p70 production were enhanced in the presence of a neutralizing anti-IL-10 antibody (Fig. 5.10). Inhibition of ERK with U0126 inhibited LPS-induced IL-23 production. Addition of anti-IL-10 did not reverse the suppressive effect of the MEK1/2 inhibitor on LPS-induced IL-23.

TLR-induced IL-10 production was completed abolished by pre-treatment of DC with the p38 inhibitor SB203580 (Fig. 5.10). Inhibition of p38 also suppressed IL-23 production by LPS-stimulated DC (Fig. 5.10). In contrast to the effect on IL-23, inhibition of p38 enhanced IL-12p70 production by LPS-pulsed DC. The enhancement of IL-12p70 by the p38 inhibitor SB203580 was further augmented in the presence of the anti-IL-10 antibody, further emphasising the inhibitory effect of IL-10 on IL-12 production (Fig. 5.10).

Consistent with the results shown in Fig.s 5.2 and 5.3, inhibition of ERK suppressed LPS-induced IL-1β production, and this was not affected by pre-incubation of DC with anti-IL-10 antibody. In contrast, inhibition of p38 with SB203580 enhanced IL-1β production by LPS-stimulated DC (Fig. 5.10). The enhancement of LPS-induced IL-1β production was not affected when DC were pre-incubated with anti-IL-10 antibody prior to SB203580 and LPS. These data indicate that the ability of the p38 inhibitor SB203580 to increase IL-1β production is not mediated by the suppression of IL-10.

5.2.10 PI3K negatively regulates LPS-induced IL-23p19 mRNA expression

The data presented in chapter 4 demonstrated that PI3K negatively regulate LPS-induced IL-12 production by DC and macrophages. Furthermore, it was reported that $PI3K^{-/-}$ mice had enhanced Th1 responses following infection with *Leishmania major* (334). However, there is very little known about the regulation of the *p19* subunit, or the secretion IL-23. A recent study demonstrated that *p19* gene expression is absolutely dependent on c-Rel and its two κB binding sites, and that other transcription factors, including other members of the NFκB family, are unable to compensate for c-Rel (439). The mouse c-Rel transactivation domain contains an ERK consensus site which has been shown to be phosphorylated *in vitro* by recombinant ERK1 (445). Having established that LPS-induced IL-23p19 expression was inhibited by blocking ERK, an experiment was designed to examine the role of PI3K in the regulation of IL-23p19 mRNA expression.

In contrast to the effect of inhibiting ERK, blocking PI3K activation using either Wortmannin or LY294002 as inhibitors, enhanced LPS-induced IL-23p19 mRNA expression (Fig. 5.11). This is consistent with the findings shown in chapter 4, where PI3K inhibition augmented LPS-induced IL-23 secretion by DC (Fig. 4.21). Furthermore, inhibition of PI3K increased LPS-induced IL-12p40 and IL-12p70 production by DC. These data confirm that PI3K negatively regulates a number of members of the IL-12 family in DC and are consistent with a report that showed enhanced bioactive IL-12 production by DC from $PI3K^{-/-}$ mice (334).

Following the demonstration that PI3K activation negatively regulated IL-23p19 mRNA in response to LPS, an experiment was designed to examine the role of this signalling pathway in regulating LPS-induced IL-12p40 and IL-12p35 mRNA expression. BMDC were pre-incubated with LY294002 (5 μM) prior to stimulation with LPS for 12 h. As with LPS-induced IL-23p19 expression, inhibition of PI3K enhanced LPS-induced p40 and p35 expression (Fig. 5.12). In contrast to the suppressive effect of the MEK1/2 inhibitor, blocking PI3K had no effect on LPS-induced IL-1β mRNA expression.

5.2.11 PI3K negatively regulates CpG-induced IL-23p19 mRNA expression

To determine if PI3K activation plays a role in the regulation of CpG or curdlan-induced IL-23p19 expression, BMDC were pre-treated with U0126 or LY294002 prior to stimulation with LPS, CpG or curdlan for 12 h. Inhibition of PI3K, with LY294002, augmented CpG-induced IL-23p19 expression (Fig. 5.12), which is consistent with the enhancement of LPS-induced p19 expression in DC pre-incubated with the PI3K inhibitor. In contrast, blocking PI3K with LY294002 suppressed curdlan-induced IL-23p19 expression, suggesting an alternative role for PI3K in dectin-1-induced IL-23p19 mRNA (Fig. 5.13).

Having established that PI3K activation negatively regulated expression of IL-23p19 mRNA in response to CpG, an experiment was designed to examine the role of this signalling pathway in regulating CpG-induced IL-12p40 and IL-12p35. BMDC were pre-incubated with LY294002 (5 μM) prior to stimulation with CpG (1 μg/ml) for 12 h. In contrast to the enhancement of LPS-induced IL-12p40 and IL-12p35 expression by the PI3K inhibitor, CpG-induced p40 and p35 expression were unaffected in DC pre-incubated with LY294002 (Fig. 5.12). This is consistent with other reports that have documented suppressive effects of some inhibitors on CpG-induced cellular responses. Compounds that block endosomal maturation, such as chloroquine or bafilomycin A, prevent endocytosis of the CpG ODN–TLR9 complex (446) and a similar effect has been suggested for some inhibitors. Unlike other TLRs, endocytosis of the TLR9-ligand complex is required for signal transduction downstream of TLR9. These results demonstrating negative regulation of CpG-induced IL-23 by PI3K prompted an investigation into the effect of LY294002 on LPS and CpG induced IL-1β mRNA expression in DC. Inhibition of PI3K had no effect on LPS-induced IL-1β mRNA but suppressed IL-1β expression in DC stimulated with CpG (Fig. 5.12).

The data presented in Fig. 5.10 indicated that inhibition of p38 suppressed LPS-induced IL-23 production. Therefore, to investigate whether blocking p38 would effect the induction of IL-23p19 mRNA, BMDC were pre-treated with the p38 inhibitor SB203580 prior to stimulation with LPS, CpG or curdlan for 12 h. The data generated on the effects of inhibiting p38 in DC are difficult to interpret due to the critical role that p38 plays in the stabilization of mRNA for a number of cytokines. The endogenous

expression of a number of the cytokines tested, as well as the house-keeping gene (r18S) used to normalize data, was altered in DC treated with the p38 inhibitor. Likewise, pre-incubation of DC with the p38 inhibitor, SB203580, decreased the expression of IL-23p19 mRNA in DC stimulated with LPS, CpG or curdlan (Fig. 5.13). These findings suggest a possible role for p38 in the regulation of IL-23p19 expression. To investigate if the effect of p38 inhibition on cytokine mRNA expression was specific only for SB203580, experiments were carried out with the alternative p38 inhibitor SB212190. This p38 inhibitor also suppressed LPS-induced IL-23p19 mRNA but similar to SB203580, SB212190 altered the expression of 18S mRNA in DC.

5.2.12 Inhibition of MEK1/2 signalling in DC selectively suppresses their ability to induce Th1 and Th17 cells *in vivo*

Given that IL-1 and IL-23 production by innate immune cells promotes IL-17-producing T cells, experiments were carried out to examine the effect of inhibiting ERK on the ability of TLR-activated DC to promote T cell IL-17 production. DC were pulsed for 24 h with the antigen KLH (20 μg/ml) alone or with LPS (100 ng/ml) in the presence or absence of U0126 (5 μM) *in vitro*, washed and injected s.c. into naïve mice. Popliteal lymph nodes and spleens were removed 7 d later and tested for antigen-specific cytokine production.

Lymph node cells from mice injected with LPS-stimulated DC produced antigen-specific IL-17 and IFN-γ (Fig. 5.14). The induction of antigen-specific IL-17 and IFN-γ was significantly suppressed when the DC were pulsed with antigen and LPS in the presence of the MEK1/2 inhibitor, U0126. Intracellular staining of lymph node cells confirmed that KLH-specific IL-17 and IFN-γ production by $CD3^+$ cells was decreased in mice immunized with DC pulsed with KLH and LPS in the presence of U0126 compared with mice injected with DC stimulated with KLH and LPS alone (Fig. 5.16). Similarly, mice injected with DC pulsed with KLH and the TLR-9 agonist, CpG, in the presence of U0126 had lower KLH-specific IL-17 production compared with mice injected with DC pulsed with KLH and CpG alone. In addition, adoptive transfer of DC pulsed with antigen and LPS induced the proliferation of lymph node cells and this was

suppressed when DC were co-incubated with the MEK1/2 inhibitor (Fig. 5.15). These findings suggest that ERK activation in DC plays a major role in their ability to promote the induction of Th17 and Th1 cells *in vivo*.

In order to demonstrate that the inhibition of DC-induced Th17 cells by the MEK1/2 inhibitor was not a non-specific effect on DC function or viability, an experiment was carried out to examine the effect of U0126 (1 μM or 5 μM) on Th2 responses induced with KLH without LPS. Consistent with data in Fig. 5.14, addition of U0126 (at either concentration) to DC pulsed with KLH and LPS suppressed the induction of IL-17 and IFN-γ production. However, IL-10 and IL-13 production induced by DC pulsed with KLH without LPS was not suppressed by U0126 (Fig. 5.17). These findings suggest that the MEK1/2 inhibitor does not cause non-specific suppression of DC activation and that ERK mediates the induction of Th1 and Th17 cells.

5.2.13 Inhibition of MEK1/2 does not affect MHC Class II or CD40 expression on on the surface of DC

DC maturation involves up-regulation of MHC Class II and co-stimulatory molecule expression, which is required for effective antigen presentation by DC and their subsequent activation of naïve T cells. In order to examine if the MEK1/2 inhibitor affected surface marker expression on DC, BMDC were pre-treated with U0126 (1 or 5 μM) prior to stimulation with LPS (100 ng/ml). After 24 h, surface expression of MHC Class II and CD40 was examined by flow cytometry. LPS enhanced surface expression of MHC Class II and CD40 on DC (Fig. 5.18). However, U0126 had no effect on the endogenous or LPS-induced expression of these surface markers. To assess the possibility that U0126 may cause cell death, DC were stained with propidium iodide. The results show that incubation of DC with U0126 at 1 or 5 μM does not affect cell viability (Fig. 5.18).

5.2.14 Inhibition of MEK1/2 suppresses autoantigen-specific Th17 and Th1 cells and attenuates acute EAE

It has been reported that IL-23 and Th17 cells have a pathogenic role in many autoimmune diseases, including EAE (101, 127). Data presented here has shown a major role for ERK in the regulation of IL-23. Furthermore, inhibition of ERK can attenuate arthritis in mice (447). Therefore, experiments were carried to examine the effect of blocking ERK or p38 activation on the clinical course of EAE.

Mice were immunized with MOG in the presence of CFA alone, or CFA with U0126 or SB203580 (50 μg/mouse) using the standard protocol. The control group, immunized with MOG and CFA only, developed severe clinical symptoms of disease. The incidence and severity of EAE was marginally increased in mice treated with the p38 inhibitor, SB203580, at the initiation of disease (day 0) (Fig. 5.19 (b)). Although inhibition of p38 using SB203580 suppresses IL-23 production it also suppresses IL-10 production. Therefore, this data is consistent with the report that demonstrated that IL-10-deficient mice were more susceptible to disease and developed more severe clinical symptoms of EAE when compared with wild-type mice (448).

In contrast, a single administration of U0126, on the same day as antigen immunization, had no significant effect on the clinical symptoms of EAE up to 17 d post immunization (Fig. 5.19 (a)). However, these preliminary data did not rule out a role for ERK in the regulation of EAE and prompted further experiments to investigate the effect of treatment with the MEK1/2 inhibitor before and during the onset of disease.

In the next experiment, EAE was induced in one group of mice by immunization with MOG in CFA, and a second group of mice were immunized with MOG/CFA with U0126 (50 μg/mouse) mixed in the emulsion and then injected s.c. with U0126 in solution on days 1 and 2. The control group of mice that were not treated with U0126 were given vehicle (PBS with DMSO) only. The incidence and severity of EAE were significantly reduced in mice treated 3 times with U0126 during the induction phase of disease (Fig. 5.20). All control mice treated with vehicle only developed severe EAE. In contrast, only 6/8 mice in the U0126 group showed symptoms of EAE and the maximum clinical grade of these mice was 3.

In order to determine if inhibition of ERK was attenuating EAE by inhibiting the induction of Th17 and Th1 cells, lymph node cells were re-stimulated *ex vivo* with MOG and IL-17 and IFN-γ production was examined. MOG-specific IL-17 production was significantly reduced in lymph node and spleen cells of mice treated with the MEK1/2 inhibitor (Fig. 5.21). MOG-specific IFN-γ production was also significantly reduced in the lymph node cells, and to a lesser extent in the spleen cells, of mice injected with U0126.

Intracellular cytokine staining of lymph node cells from control and treated mice 22 d post induction of EAE demonstrated that IL-17 was produced by $CD3^+$ T cells (Fig. 5.22). There was a reduced frequency of IL-17 and IFN-γ producing $CD3^+$ T cells in mice treated with U0126 (Fig. 5.22). In addition, staining for CD4, CD8 and γδ TCR together with intracellular IL-17 and IFN-γ was used to examine the effect of ERK inhibition on IL-17 production by specific T cell subtypes. The production of IL-17 and IFN-γ was significantly inhibited in $\gamma\delta^+$ T cells from lymph node cells from mice treated with the MEK1/2 inhibitor, when compared with lymph node cells from control (untreated) mice with EAE (Fig. 5.23). The percentage of $CD4^+$ cells expressing IL-17 and IFN-γ was low, however, there was a reduced frequency of IL-17 and IFN-γ producing $CD4^+$ T cells in lymph nodes from mice treated with the MEK1/2 inhibitor compared with lymph node cells from control mice.

To address whether inhibiting ERK was affecting differentiation of MOG-specific IL-17 and IFN-γ producing cells, the experiment described above was repeated except mice were sacrificed 5 d post induction of EAE and lymph node cells were re-stimulated *ex vivo* and examined for IL-17 and IFN-γ production. MOG-specific IL-17 and IFN-γ production was reduced in the lymph nodes of mice treated with the MEK1/2 inhibitor (Fig. 5.24). There was no IL-17 or IFN-γ production detected from spleen cells isolated from control mice or mice treated with U0126, re-stimulated with MOG *ex vivo* 5 d after induction of EAE (data not shown). These findings suggest that ERK plays a role in the induction of autoantigen-specific IL-17 and IFN-γ producing T cells.

5.2.15 Delayed treatment with a MEK1/2 inhibitor has limited therapeutic efficacy against MOG-induced EAE

Having established that inhibition of ERK during and immediately after immunization inhibits MOG-specific IL-17 and IFN-γ production, and attenuates the clinical symptoms of EAE, it was important to determine if U0126 would suppress symptoms of EAE when administered after MOG-specific Th1 and Th17 cells were detectable in the lymph nodes (day 5; data shown above). EAE was induced with MOG/CFA using the standard protocol. One group of mice were treated s.c. with the MEK1/2 inhibitor, U0126 (50 μg/mouse) every second day from day 5 to day 18. A control group of mice were injected with vehicle (PBS and DMSO) on the same days. While the mice treated with U0126 developed symptoms of EAE, their clinical disease was less severe than the control EAE mice (Fig. 5.25). There was a small but significant difference in disease severity on days 11, 12 and 13 following the induction of EAE.

These data indicate that while therapeutic intervention with U0126 5 d after the induction of disease attenuated EAE, there was a more significant effect when the MEK1/2 inhibitor was injected into mice with the MOG/CFA emulsion at the induction of EAE. The data suggest that ERK blockade is less effective in inhibiting the proliferation of already committed Th17 cells *in vivo*. This suggests that inhibition of ERK suppresses the innate cytokines that are critical for the induction of the pathogenic T cells that cause the disease.

5.2.16 Administration of the MEK1/2 inhibitor prevents relapse of disease in the relapsing and remitting model of EAE

The course of disease in the PLP-induced relapsing and remitting model of EAE more closely resembles that in patients with multiple sclerosis. Therefore, the MEK1/2 inhibitor was examined for its therapeutic effect against PLP-induced EAE in SJL/J mice. EAE was induced by s.c immunization with PLP and CFA with PT given i.p. on days 0 and 2. Administration of U0126 after the acute phase of disease, when mice were at the onset of relapse (day 18), resulted in almost complete suppression of clinical symptoms. In contrast, control mice treated with vehicle (PBS and DMSO) alone relapsed and

continued to display severe symptoms of EAE (Fig. 5.26). The protective effect of the MEK1/2 inhibitor was associated with significant suppression of PLP-specific IL-17 production by lymph node cells (Fig. 5.27). There was no IFN-γ production detected from lymph node cells of mice treated with U0126 or untreated control mice with EAE.

These data show that ERK blockade is an effective therapy for the treatment of EAE in mice, and demonstrate that the ERK pathway has an important role in mediating IL-23-induced Th17 cells that lead to autoimmunity. The ability of ERK to differentially regulate IL-12 family members involved in the polarization of T cells, by specifically inhibiting the IL-17-polarizing cytokine IL-23, underlines its potential as a drug target for autoimmune disorders.

5.2.17 Inhibition of EAE by ERK blockade is mediated by the suppression of IL-23 and IL-1β

To establish if the MEK1/2 inhibitor was exerting its attenuating effect on EAE and IL-17 producing T cells by suppressing IL-1β and IL-23, with a resulting decrease in IL-17 production, mice were treated with the MEK1/2 inhibitor with or without recombinant IL-1β and IL-23. These cytokines were mixed with the MOG/CFA emulsion containing U0126 (50 μg/mouse) for immunization by the standard protocol. Mice were injected i.p. with U0126 with or without rIL-23 and rIL-1β on days 1 and 2. Control EAE mice were injected with vehicle (DMSO in PBS) only on these days. In addition, another group of mice were immunized with MOG/CFA supplemented with rIL-23 and rIL-1β, and were injected s.c. with rIL-1 and rIL-23 on days 1 and 2.

Consistent with previous data (Fig. 5.20), there was significant inhibition of clinical symptoms of EAE in mice treated with U0126 (Fig. 5.28). While mice that were injected with IL-1 and IL-23 (without the MEK1/2 inhibitor) developed disease, the symptoms were no more severe than observed in the control EAE mice. Co-administration of IL-1 and IL-23 to mice treated with the MEK1/2 inhibitor reversed the attenuating effect of U0126 on clinical symptoms of EAE. This correlated with a reversal of the suppression of MOG-specific IL-17 production by lymph node cells from these mice. Treatment with IL-1 and IL-23 also reversed the suppression of IFN-γ production by the MEK1/2 inhibitor (Fig. 5.29). These data show that the effect of the MEK1/2

inhibitor on the induction of MOG-specific Th1 and Th17 cells was mediated by suppression of IL-1 and IL-23 production.

5.3 DISCUSSION

Multiple cytokines, including IL-1, IL-6, IL-21, TNF-α, IL-23 and TGF-β, can promote the differentiation or expansion of IL-17-secreting T cells (449). However, our understanding of the signalling that drives the IL-17 pathway is still evolving, and in particular there are significant differences between studies with human and murine T cells. The current opinion is that IL-1β and IL-6 drive IL-17 production from central memory $CD4^+$ T cells, whereas TGF-β and a number of other inflammatory cytokines promote the differentiation of naïve T cells into Th17 cells (450, 451).

The differentiation of naïve T cells into effector, pathogenic or regulatory T cells is primarily controlled by the activation status of the APCs and the local cytokine milieu. IL-23, which is produced by activated DC, is essential for the maintenance of Th17 cells and expands previously differentiated Th17 cells (126, 129). While naïve T cells do not express IL-23R, once IL-23R is expressed, IL-23 can bind to this receptor and induce a rapid IL-17 response (132). Depending on the stimulus, activated DC can also produce IL-12p40 and IL-12p70, key cytokines required for the polarization of IFN-γ-producing T cells. Until recently it was assumed that Th1 cells mediated autoimmunity, however, the discovery of IL-23 and its role in the pathogenesis of autoimmune disease has led to a review of the role of Th1 cells in mediating the inflammatory processes involved in these diseases.

This study aimed to examine the role of the MAPK ERK in regulating IL-23 production by DC. Innate sensing of microbial stimuli occurs through the expression of PRRs, including TLRs and NLRs, on DCs and other cells, which results in the activation of the genes that encode inflammatory cytokines (452). TLR-mediated activation of DC is largely dependent on MyD88, an adaptor required by almost all TLRs, that plays a fundamental role in the activation of NFκB (12). However, the IRF and MAPK signalling pathways also regulate cytokine production by DC and Th cell differentiation (311, 438). The present study showed that stimulation of DC with the TLR4 agonist LPS, TLR9 agonist CpG, or the dectin-1-agonist curdlan induced IL-23p19 expression and IL-23 secretion by DC. This is consistent with a number of reports that have demonstrated IL-23 production by DC or macrophages in response to TLR- and non TLR signals (453).

The significant finding of the present study was that inhibition of ERK suppressed TLR4-, TLR9- or dectin-1- agonist induced IL-23 production by DC. These data were surprising since ERK negatively regulated IL-12 production. Pre-incubation of DC with the MEK1/2 inhibitor U0126 prior to stimulation with CpG enhanced IL-12p40 and IL-12p70 secretion but inhibited the production of IL-23. Similarly, LPS-induced IL-23 production was suppressed in the presence of the MEK1/2 inhibitor, while there was little effect on IL-12p70. In contrast to the enhancement of CpG-induced IL-12p40, blocking ERK inhibited LPS-induced IL-12p40 production. Consistent with the effect on IL-23 secretion, the results demonstrated that blocking ERK also suppressed TLR-agonist induced IL-23p19 mRNA expression in DC. In contrast, inhibition of ERK enhanced CpG-induced IL-12p35 expression and CpG-induced IL-12p40 expression. However, U0126 suppressed LPS-induced IL-12p40 mRNA expression, suggesting that IL-12p40 production in response to LPS and CpG is differentially regulated.

Both the NFκB and IRF signalling pathways have been shown to play important roles in regulating the expression of IL-12 and IL-23 (438). IRF1, IRF3 and IRF7 are uniquely involved in p35 activation and subsequent secretion of IL-12p70, while IRF5 contributes to the expression of both p40 and p19. Furthermore, IRF8 is required for p40 expression in response to a number of PAMPs in macrophages and DC, and along with IRF1, IRF8 is a critical factor involved in the enhancement of TLR-induced p40 and p35 expression by IFN-γ (354). A recent study demonstrated that $IRF8^{-/-}$ DC failed to produce TNF-α and IL-6 in response to CpG but not LPS, and furthermore, the induction NFκB and MAPKs in response to TLR9 stimulation was also abolished in $IRF8^{-/-}$ DC (359). The β-glucan agonist curdlan induces IL-23 production by DC without inducing IL-12p70 (453). Consistent with this report, curdlan induced IL-23 and IL-12p40, but no IL-12p70, production by DC. Similar to the observations with CpG and LPS, the induction of IL-23p19 expression and subsequent IL-23 secretion in response to curdlan were suppressed by inhibition of ERK.

These findings are in line with a recent surge of literature highlighting that activation of DCs via individual PRRs will result in distinct cytokine profiles. It is now well established that stimulation of individual PRRs is not sufficient to induce optimal production of IL-12p70 by DC but requires exposure to additional signals, such as IFN-γ, CD40L or association with other TLRs (e.g. TLR7/8 with TLR3 or TLR4 ligands) (376,

454, 455). In contrast and consistent with other reports, on murine and human DC, the findings here demonstrate that individual PRRs can induce IL-23 production (456, 457). Thus, the relative expression of IL-12 and IL-23 appears to be strictly dependent on the PRRs that are activated by different microbial stimuli. Activation of TLR-independent signalling in DC has also been to shown induce IL-23 production. Engagement of C-type lectins has been shown to favour IL-23 production. The β-glucan receptor/dectin-1 agonist induced IL-23p19 expression and IL-23 secretion by murine DC without IL-12p70 production (453). More recently, Gerosa *et al.* reported similar findings in human monocyte-derived DC (457). Therefore, activation of particular PRRs results in the preferential induction of IL-12 or IL-23 by DC, thereby allowing the DC to polarize different T cell responses.

Intact microorganisms contain ligands for multiple TLR and non-TLR PRRs, which adds another layer of complexity. Simultaneous engagement of distinct PRRs has been shown to influence the magnitude and type of cytokine profile induced in response to stimulation. Studies investigating the effect of combinations of TLR ligands have shown synergistic effects which are particularly dramatic for the production of the biologically active heterodimer IL-12p70 (376). Stimulation of human MoDC with LPS and the TLR7/8-agonist R848 synergized for IL-12p70 production. Moreover, incubation of murine BMDC with LPS in combination with R848 or CpG resulted in enhanced IL-12p70 secretion compared with BMDC pulsed with the individual ligands. In addition, stimulation of human DC with LPS in combination with R848 increased IL-12p40, IL-12p35 and IL-23p19 mRNA (376). The TLR2 ligand zymosan is also recognized by dectin-1, and this co-activation induces a different cytokine profile to that induced by TLR2 alone (458).

Unlike IL-12p40 and IL-12p70, the regulation of IL-23 is less well defined. A recent report identified c-Rel, a member of the NFκB Rel proteins, as an important regulator of p40, p35 and p19 in response to TLR-activation (439). Moreover, c-Rel-deficient mice are resistant to the induction of EAE (459). While this group reported a defect in Th1 responses, c-Rel deficiency did not affect T-bet expression. This might suggest that the resistance of c-Rel-deficient mice to EAE might be as a result of the suppression of Th17 cell induction, owing to a defect in IL-23. The findings here demonstrated that the induction of IL-23 in response to PRR-stimulation was suppressed

by inhibition of MEK1/2 signalling. In contrast, inhibition of ERK had little or no effect on LPS-induced IL-12p70 secretion. However, CpG-induced IL-12p70 production was enhanced in the presence of the MEK1/2 inhibitor. While much of the literature suggests that ERK is required for IL-12 production some reports have shown that ERK negatively regulates IL-12 production. Hawlisch *et al.* showed that inhibition of MEK1/2 induced a dose-dependent increase in LPS-induced IL-12p70 production by macrophages (460). Similarly, blocking ERK with the MEK1/2 inhibitors U0126 and PD98059 enhanced IL-12 production by CpG-activated DC and macrophages (446). Furthermore, Goodridge *et al.* reported that ERK negatively regulated p40, but not 35, expression in response to LPS in peritoneal macrophages (326). This group also suggested that ERK suppresses IL-12p40 by targeting IRF1, the transcription factor essential for MyD88-dependent IL-12p70 production (352).

The essential role that ERK plays in the regulation of IL-23 is highlighted by the fact that inhibition of ERK also suppressed the induction of IL-23 in response to the non-TLR agonist curdlan. Curdlan has recently been shown to induce rapid Syk-dependent phosphorylation of ERK, p38 and JNK in DC (453). Signalling via dectin-1 induces responses that can be dependent or independent of the tyrosine kinase Syk. Curdlan-induced IL-23p19 expression and IL-23 secretion was found to be MyD88-independent but Syk-dependent. This suggests that signalling downstream of Syk-ERK might induce activation of transcription factors required for the induction of IL-23p19 in response to curdlan. However, the induction of IL-23 by DC in response to the TLR-9 agonist CpG was MyD88-dependent but Syk-independent (453). This suggests that PRR-induced IL-23 production is regulated via MyD88- dependent and independent pathways. Activation of IRF8 has also been implicated in CpG- but not LPS- mediated activation of NFκB and MAPKs, and IL-6 and TNF-α production by DC (359). Recent analysis of the murine p19 promoter revealed that in addition to NFκB, Sma- and Mad-related protein (SMAD)-3 and activating transcription factor (ATF)-2 are transcription factors essential for IL-23p19 expression (441).

A number of recent studies have highlighted the importance of the CARD-containing protein, CARD9, in mediating downstream signalling events induced by both TLRs and ITAMs. Engagement of ITAM receptors leads to activation of the tyrosine kinase Syk, which is required for signalling downstream of the atypical ITAM, dectin-1.

CARD9 associates with Bcl-10 which results in NFκB activation and subsequent cytokine production in response to dectin-1 stimulation (461). CARD9 has also been shown to be essential for activation of MAPK, but not NFκB, in response to peptidoglycan, a NOD2 agonist (462). Saito and colleagues have recently extended these findings and suggested that CARD9 is a crucial intermediate in the signalling of all TLRs (463). Specifically, they demonstrated that all myeloid receptors that initiate tyrosine kinase signals via ITAMs or an ITAM-like cytoplamic motif (dectin-1) need CARD9, MALT1 and Bcl-10 to activate NFκB. They also showed that CARD9 is required for TLR-mediated activation of MAPK, but dispensable for TLR-induced activation of NFκB. MAPK activation was dependent on the formation of a complex between CARD9 and the serine-threonine kinase RIP2 (463). Consistent with these findings, Hsu *et al.* demonstrated that RIP2 was required for CARD9-dependent MAPK activation downstream of NOD2 (462). However, while these reports agreed that CARD9 plays a critical role in TLR- and NOD2- mediated MAPK activation and tyrosine kinase-mediated NFκB activation, it is not yet clear how CARD9 activates these pathways. Furthermore, there are discrepancies in the extent to which CARD9 is involved in signalling events downstream of TLRs. As discussed above, curdlan-induced IL-12p40 production was independent of MyD88 but Syk- and CARD9- dependent, while CpG-induced IL-12p40 production was completely MyD88-dependent but Syk- and CARD9- independent. CARD9 was critical for the development of Th17 responses in response to fungal infection with *Candida albicans* (453). These findings suggest that CARD9 may be involved in the regulation of IL-23. One possible mechanism might be that PRRs activate CARD9 signalling to induce MAPK activation and subsequent induction of IL-23. A recent report identified a MAP3K, MEKK4, as a negative regulator of NFκB signalling. MEKK4 binds to RIP2 to sequester RIP2 from the NOD2 signaling pathway but exposure to the NOD2 agonist, MDP, results in the dissociation of MEKK4:RIP allowing activation of NOD2-induced NFκB. However, overexpression of MEKK did not affect the activation of JNK by RIP2, indicating that MEKK4 inhibits NFκB but at the same time allows the activation of MAPK (464). The observation that the activation of p38, but not NFκB, in response to the NOD2 agonist MDP, was inhibited by knockdown of MEKK4 is in line with the findings in CARD9$^{-/-}$ DC (453, 464). Therefore, it will be important that future studies

determine whether MEKK4 and CARD9 interact and coordinate signalling downstream of PRRs.

The data from the present study indicated that inhibition of ERK had a greater suppressive effect on LPS- and curdlan- induced IL-23p19 mRNA expression compared with that induced by CpG. A possible explanation of this may be explained by a recent publication on the regulation of IL-1 by IL-23. Harris et al. reported that wheat gliadin- as well as β-glucan- induced IL-23 production by human monocytes was IL-1 dependent. They demonstrated that stimulation of monocytes with rIL-1β alone induced IL-23 production and that IL-23 and IL-1 production in response to β-glucan were suppressed by incubation with IL-1ra (465). Results presented here showed that while CpG induced IL-1β mRNA expression, the secretion of IL-1β was markedly less than that induced by LPS or curdlan. Since inhibition of ERK also significantly suppressed LPS- and curdlan-induced IL-1β production by DC, this may contribute to the suppression of IL-23 production in response to these agonists. Inhibition of MEK1/2 also suppressed *M. tuberculosis*-induced IL-1β and IL-23 production. It has been reported that DC recognize *M. tuberculosis* through a number of PRRs, including TLR2, TLR9, NOD2 and TLR4 (466). Stimulation of NOD2 and TLR2 was shown to induce robust IL-23 production by DC, comparable to that induced by *M. tuberculosis*, but little IL-12 production.

IL-10 is a negative regulator of inflammatory responses in DC and macrophages. The mechanism through which IL-10 antagonizes the genes responsible for inflammatory cytokine production remains controversial. STAT3 has been shown as the key player, as IL-10-mediated inhibition of inflammatory cytokines is abolished in $STAT3^{-/-}$ macrophages (168). Moreover, ERK has been shown to be involved in TLR2- and dectin-1- agonist induced IL-10 production (119). The data presented here show that inhibition of ERK had a minimal effect on TLR-induced IL-10 production. Given that IL-10 has been reported to negatively regulate IL-12 production, it might have been expected that the suppression of IL-10 by inhibition of ERK would enhance IL-23 production. Neutralization of IL-10 enhanced LPS-induced IL-12p70 and IL-23 production. Inhibition of ERK suppressed LPS-induced IL-23, but not IL-12p70, production and this suppression was still evident in the presence of the anti-IL-10 antibody. This finding clearly demonstrates that IL-10 does not mediate the suppression of IL-23 by the MEK1/2 inhibitor. In line with a reciprocal relationship between IL-23

and IL-10, Weiner et al. demonstrated that inhibition of IL-23 by antisence oligonucleotides in human monocytes DC was associated with increased IL-10 production (467). Furthermore, mice deficient in IL-10 develop severe chronic colitis, which is associated with increased substance P, a tachykinin that enhances pathways of inflammation, and IL-23, which are produced at the site of intestinal inflammation (468, 469). In addition splenic $CD4^+$ cells from *IL-10*–deficient mice had increased IL-17 expression when compared with WT mice (470).

Inhibition of p38 with the inhibitor SB203580 abolished LPS-induced IL-10 production. Blocking p38 enhanced LPS-induced IL-12p70 and IL-12p40 production by DC, demonstrating that p38 negatively regulates IL-12. In contrast, inhibition of p38 suppressed LPS-induced IL-23 production. As discussed above, data generated with the p38 inhibitor SB203580 can be difficult to interpret. Indeed, the use of inhibitors to target specific signalling molecules can be problematic due to the effect of the inhibitor on non-specific targets. It has been reported that RIP2 is a target of the pyridinyl imidazole inhibitors of p38. Argast *et al.* demonstrated that pyridinyl imidazole inhibitors suppressed RIP2 activity by competing for the ATP-binding site in RIP2, similar to their effect on p38 (413). These experiments were performed in a hepatocyte cell line or HEK293 cells, but it was suggested that since a number of TLR agonists activate both RIP2 and p38 the contributions of these two proteins to TLR-induced cytokine responses need to revisited. More recently, however, another group has shown that inhibition of p38 with SB203580 in human DC did not affect RIP2 kinase activity (416). There are a number of reports in the literature that indicate that p38 is critical for cytokine mRNA expression but the mechanism through which it exerts this effect is still unclear. The results shown here demonstrated that inhibition of p38 suppressed LPS-, CpG- and curdlan- induced IL-23 mRNA expression. Furthermore, blocking p38 with SB203580 inhibited IL-23 production in response to LPS. The suppression of IL-23 by SB203580 was not altered in the presence of an anti-IL-10 antibody which suggests that the suppression of IL-23 by inhibition of p38 is not mediated by IL-10. While inhibition of p38 suppressed LPS-induced IL-23 secretion, IL-1β production in response to LPS was enhanced in the presence of SB203580. Since IL-1 and IL-23 play a role in the induction of Th17 cells, the enhancement of IL-1β might contribute to an increase in IL-17

production and as such exacerbate autoimmunity. In line with this hypothesis, inhibition of p38 at the induction of EAE resulted in more severe clinical symptoms of disease. This is consistent with the study that showed that IL-10-deficient mice were more susceptible and developed a more severe EAE compared to wild-type mice (448).

The findings presented here showed that inhibition of MEK1/2 in DC pulsed with antigen and LPS significantly suppressed their ability to promote Th17 responses. Furthermore, blocking ERK in DC inhibited the induction of antigen-specific IFN-γ production. There is still some debate about the contribution of Th1 and/or Th17 cells in the pathogenesis of autoimmune diseases. However, these data suggested that ERK activation in DC plays a major role in IL-23 and IL-1 production and in the promotion of Th17 cells *in vivo*. Similar to these findings, dectin-1-stimulated DC promoted Th17 cell differentiation, while CpG-stimulated DC induced a more Th1 biased response (453). Stimulation of DC with curdlan and CpG led to the production of a number of cytokines including IL-6, TNF-α, IL-23, however, CpG also induced a strong IL-12 response. This group elegantly showed that effect of these agonists was mediated by the APCs and not the T cells by demonstrating that the induction of Th17 or Th1 cells were abrogated by the use of DC lacking Syk or MyD88, respectively. In addition, the differentiation of Th17 cells induced by curdlan was shown to be dependent on IL-23 and TNF-α, while the addition of IL-12p70 prevented their differentiation (453).

It has previously been reported that MyD88-mediated activation of NFκB, IRF-1 and IRF-8 is required for IL-12p35 and IL-12p40 gene expression and in driving Th1 responses. However, these signalling pathways did not mediate IL-23-driven Th17 responses. Kano *et al.* demonstrated that expression of IL-12Rβ1 mRNA, which encodes a subunit shared by the IL-12 and IL-23 receptors, was impaired in IRF1-deficient $CD4^+$ T cells, but the expression of IL-23R mRNA was the same as that in wild-type $CD4^+$ T cells. They also showed that IL-12Rβ1 induction by IRF1 was not required for IL-23-dependent IL-17 production. The percentage of IL-17-producing cells in IRF-deficient $CD4^+$ T cell populations after culture with IL-23 was higher than that observed in wild-type $CD4^+$ T cells (353). Taniguchi and colleagues suggest that "distinct thresholds" of IL-12Rβ1 expression determine whether an IL-12 or IL-23 bias response is induced. Differentiation of Th1 cells requires a high level of expression of IL-12Rβ1, which could

possibly be amplified by IFNγ-production in a positive feedback loop. If IL-6 and TGF-β are predominant the balance might shift towards Th17 differentiation. However, in the absence of IRF1, reduced IL-12Rβ1 expression favoured IL-23 production and the induction of a Th17 response.

Evidence for an essential role of IL-17 in T cell autoimmunity is compelling. The first reports of IL-23 driving the induction of an IL-17 secreting $CD4^+$ T cell population came from studies by Langrish *et al.*, who reported that IL-23 promoted the development of a pro-inflammatory T cell population, characterized by the production of IL-17, TNF-a and IL-6 (101). Furthermore, it has been shown that IL-23, IL-1 and Th17 cells have a pathogenic role in many experimental autoimmune diseases, including EAE (101, 127, 134), collagen-induced arthritis (471) and experimental autoimmune uveitis (EAU) (430). The data presented here show that the incidence and severity of acute MOG-induced EAE were significantly reduced in mice treated with the MEK1/2 inhibitor U0126 at the induction phase of disease. This correlated with a significant reduction in antigen-specific IL-17 and IFN-γ production by lymph node cells from these mice. Similarly, inhibition of ERK has been shown to attenuate arthritis in mice, however, the effect of IL-17 was not examined and the mechanism of suppression was unclear (447). Interestingly, Thiel *et al.* also showed that inhibition of MEK suppressed IL-1α-induced stromelysin production and proteoglycan loss from the articular cartilage in the rabbit IL-1α-induced knee arthritis model.

A significant finding of this study was that the suppressive effect of ERK on IL-17 production was mediated by the inhibition of IL-23 and IL-1. The frequency of IL-17 producing T cells in the lymph node cells from mice immunized with MOG and CFA was reduced by administration of the MEK1/2 inhibitor and this suppression was reversed by co-administration of IL-23. Furthermore, the suppressive effect of the MEK1/2 inhibitor on MOG-specific IL-17 production by lymph node cells from mice immunized for EAE was partially reversed by co-administration of IL-23 and completely reversed by co-administration of IL-1β and IL-23. These data clearly show that inhibition of innate IL-23 and IL-1 attenuated the development of EAE and supports previous reports that these cytokines play a critical role in the induction and expansion of Th17 cells.

The findings reported here are in line with the studies which demonstrated that IL-1RI and IL-23 defective mice are resistant to the development of experimental EAE (127, 134), and also that IL-23p19$^{-/-}$ mice produce less IL-17 and were more resistant to EAU (430). Furthermore, Chen *et al.* established that anti-IL-23 therapy reduced IL-17 production and inhibited inflammatory responses that lead to CNS inflammation in EAE (127). Administration of the MEK1/2 inhibitor at the induction phase of disease suppressed acute MOG-induced EAE but when mice were treated with the MEK1/2 inhibitor 5 days after immunization with MOG/CFA, there was only a slight reduction in clinical symptoms. These data suggest that the MEK1/2 inhibitor mediates its suppressive effects by inhibiting the induction or expansion of Th17 cells and is less efficacious in blocking an already differentiated Th17 population. Similar to the data presented here, Cua and colleagues showed that anti-IL-23p19 treatment given on the day of disease onset did not ameliorate acute disease, but inhibited disease relapses. They demonstrated that anti-IL-23p19 blocked epitope spreading to additional encephalitogenic epitopes in the CNS and suggested that anti-IL-23 therapy for ongoing EAE functioned in the brain (127).

Significantly, the present study showed that inhibition of ERK prevented disease relapse in relapsing-remitting EAE, a model that more closely resembles the course of disease in MS patients. Administration of U0126 after the acute phase of disease resulted in significant attenuation of clinical symptoms compared with mice treated with PBS alone. This was associated with significant suppression of PLP-specific IL-17 production. The report by Cua *et al.* showed that administration of anti-IL-23p19 antibody during recovery from acute EAE significantly blocked the induction of pro-inflammatory gene expression in the spinal cord, including the expression of IL-17, IFN-γ, IL-1β, TNF-α and IL-6 (127). Together with the data presented here, these findings suggest that targeting IL-23, and IL-1, can prevent disease relapse in mice that already have active CNS lesions.

A recent study reported that blocking IL-6 with an anti-IL-6 receptor monoclonal antibody inhibited the induction of antigen-specific Th17 and Th1 cells and inhibited the development of EAE (472). Similarly, the data presented here showed that blocking MEK1/2 at the initiation of EAE suppressed MOG-specific IL-17 and IFN-γ production by lymph node cells. Co-administration of IL-23 with the MEK1/2 inhibitor partially

reversed the suppression of MOG-specific IL-17 and IFN-γ, but this was completely reversed by co-administering IL-1β with IL-23. Serada *et al.* also examined the effect of delayed treatment with the anti-IL-6R antibody, but found no significant difference in the clinical scores between treated and control groups (472). Using a passive transfer model, they showed that IL-6 blockade did not prevent the infiltration of activated lymphocytes into the CNS, indicating that the anti-IL-6R antibody mediates its effect by suppressing the induction of antigen-specific Th17 cells in the peripheral lymphoid tissue, rather than via the inhibition of infiltration of activated lymphocytes into the spinal cord (472). Consistent with findings of Cua and colleagues, Serada *et al.* also suggested that specific targeting of cytokines at the relapsing phase of disease might interfere with antigenic spreading of epitopes by naïve T cells. A recent report demonstrated that local APC activation of naïve T cells in the CNS is required for encephalitogenic epitope spreading, and this was shown to be required for chronic disease progression in two different T cell-mediated CNS demyelinating models of multiple sclerosis, relapsing EAE and Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) (473).

Although the focus of most studies on IL-17 production has been on $CD4^+$ αβ T cells, γδ T cells have also been shown to be an important source of IL-17 in murine infectious disease models such as *M. tuberculosis* (474) and *E. coli* (475) and the autoimmune disease model, CIA (476). A recent study by Chien and colleagues has highlighted the important differences in the requirements for the development of effector αβ and γδ T cells. In contrast to αβ T cells, γδ T cells do not require antigen processing and presentation to produce cytokines, including IL-17, in response to TCR engagement (477). Therefore, γδ T cells are ideally suited to provide IL-17 early in an inflammatory response. Consistent with this, the data presented here showed that a significant IL-17 producing γδ T cell population was detected in lymph node cells isolated from mice immunized with MOG and CFA. Importantly, inhibition of ERK suppressed the frequency of IL-17-producing γδ T cells in the lymph node. Chien *et al.* also noted that different subsets of γδ T cells in different lymphoid organs yield distinct cytokine profiles. Specifically, the majority of lymph node γδ T cells produced IL-17, whereas a large population of splenic γδ T cells produced IFN-γ (477).

It is significant that naïve γδ T cells have been shown to produce IL-17 in response to IL-23, which is required for the induction or expansion of Th17 cells. γδ T

cells were shown to be the primary source of IL-17 in *M. bovis*-infected lungs and IL-17 expression was found to be IL-23-dependent (478). Moreover, a study by Shibata *et al.* showed that γδ T cells in the peritoneal cavity of naïve mice produced IL-17 in response to IL-23 (475). Together with the report by Chien *et al.*, these data indicate that γδ T cells are already differentiated in the periphery and are poised to produce IL-17 in a rapid manner and therefore are unique in their ability to respond to pathogen-derived molecules as well as other danger signals. In line with these observations, a study by Lees *et al.* showed that host T cells are the main producers of IL-17 within the CNS during adoptively transferred EAE, and furthermore that this population largely consisted of γδ TCR^+ cells. Moreover, they demonstrated that a high percentage of these γδ TCR^+ cells produced IL-17 upon restimulation (479). It is significant that earlier studies reported significant amelioration of EAE in γδ-deficient mice (480, 481). It is possible that the defect in EAE induction associated with γδ T cell deficiency may be due to reduced IL-17 production.

It is well established that therapeutic targeting of inflammatory cytokines is highly efficacious for the treatment of autoimmune disease. Since the success of anti-TNF therapies in a number of inflammatory diseases (482, 483), there has been a surge of research to develop means to specifically block particular targets known to be responsible for inflammation in particular diseases. It has previously been reported that $ERK1^{-/-}$ mice have increased susceptibility to EAE (325). While these findings appear to be at variance with the data presented in this study they are not directly comparable. There are two isoforms of ERK, ERK1 and ERK2, and U0126, and other ERK inhibitors including PD98059, selectively inhibit MEK, the MAPKK upstream of ERK. ERK2 deletion is fatal in mice, and as such the contribution of ERK1/2 to the induction of EAE has only been examined in $ERK1^{-/-}$ mice. Similar to the findings of Agrawal *et al.*, another group demonstrated that ERK1-deficient mice were more susceptible to EAE. While Agrawal and colleagues showed that immunization of $ERK1^{-/-}$ mice with MOG/CFA resulted in enhanced IFN-γ and IL-5 production, Nekrasova *et al.* reported no difference in T cell cytokine production (484). Furthermore, knockout mice lack ERK1 through the course of their development and it has previously been reported that inhibition of ERK enhanced IL-17 production by T cells *in vitro* (134). In the present study the MEK1/2 inhibitor was only administered during or after the induction of EAE. Since blocking ERK inhibited

IL-1 and IL-23 production by DC, this indicates that the MEK1/2 inhibitor mediated its suppressive effect by inhibiting innate IL-1 and IL-23 production, and therefore the induction of the pathogenic T cells that cause the disease.

This study has shown that inhibition of MEK1/2 suppressed the clinical signs of acute EAE as well as relapse of disease in relapsing-remitting EAE. The results demonstrated that ERK signalling in DC plays a critical role in activating Th17 cells by mediating the production of IL-1β and IL-23. While there are a number of effective therapies for autoimmune disease that target specific cytokines, such as TNF-α, for the treatment of RA, there are patients who do not respond to treatment, and also the therapy is often not sufficient for sustained clinical remission. Taken together the findings presented here suggest that blocking IL-1 and IL-23 by targeting the ERK signalling pathway in innate immune cells is an effective means of suppressing IL-17 production and may be a promising approach in the design of new immunotherapeutic drugs against Th17-mediated autoimmune and chronic inflammatory diseases.

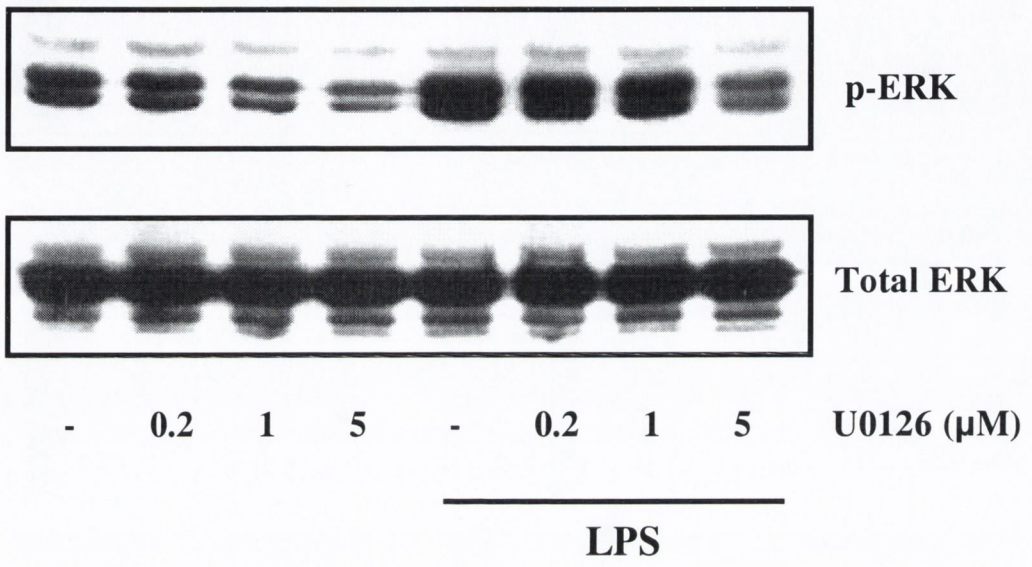

Figure 5.1 DC express endogenous phosphorylated ERK which is enhanced by stimulation with LPS and reduced by incubation with an inhibitor of MEK 1/2. BMDC (1 x 10^6 cells/ml) from C57BL/6 mice were pre-treated with increasing concentrations of the MEK1/2 inhibitor, U0126, or medium only for 30 min, followed by stimulation with LPS (100 ng/ml) or with medium alone for 10 min. Cell lysates were probed for p-ERK and total ERK by Western blot analysis.

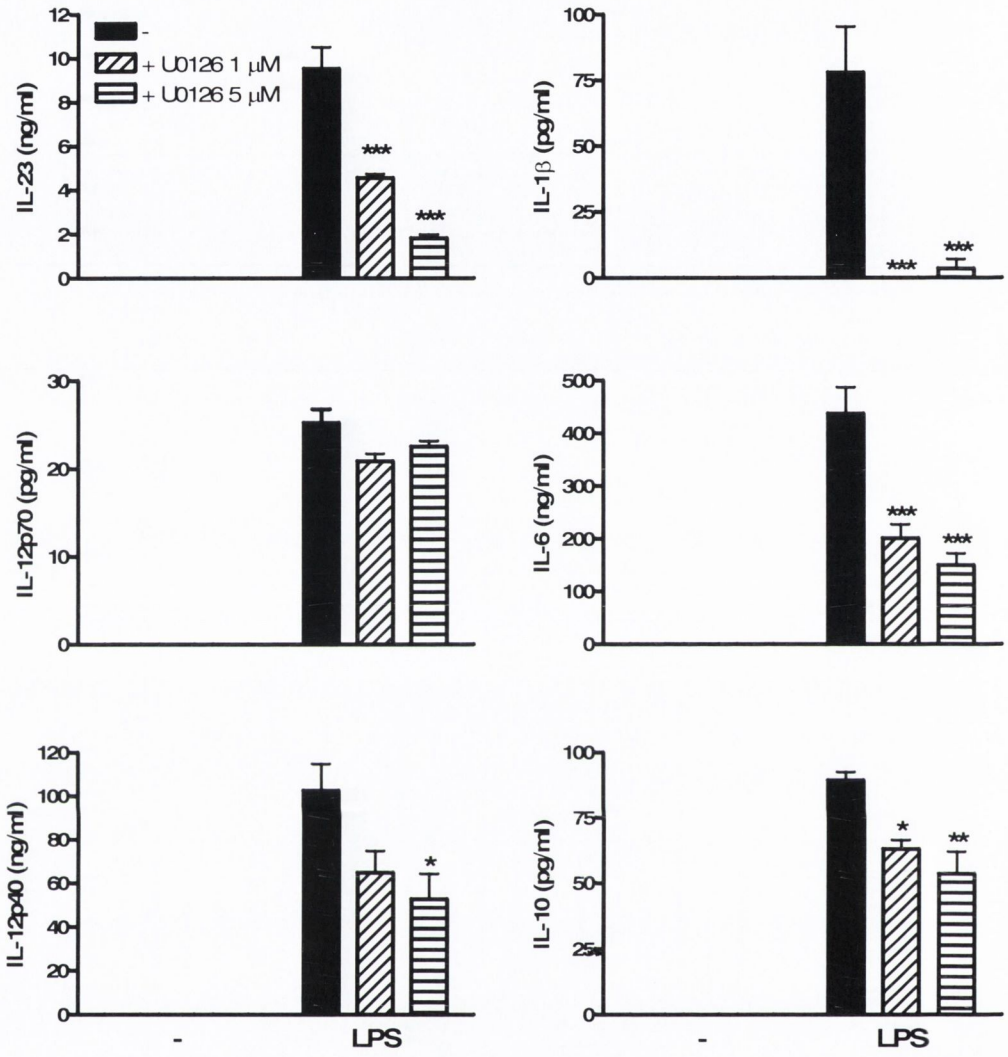

Figure 5.2 LPS-induced IL-23 and IL-1β production by DC is ERK-dependent. BMDC (1 x 10^6 cells/ml) were stimulated with LPS (100 ng/ml) with or without the MEK 1/2 inhibitor, U0126 (1 or 5 μM). Supernatants were collected after 24 h and IL-12p70, IL-12p40, IL-23, IL-6, IL-10 and IL-1β concentrations quantified by ELISA. LPS versus LPS + U0126: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are mean ± SEM of triplicate samples from duplicate stimulations and are representative of at least three experiments.

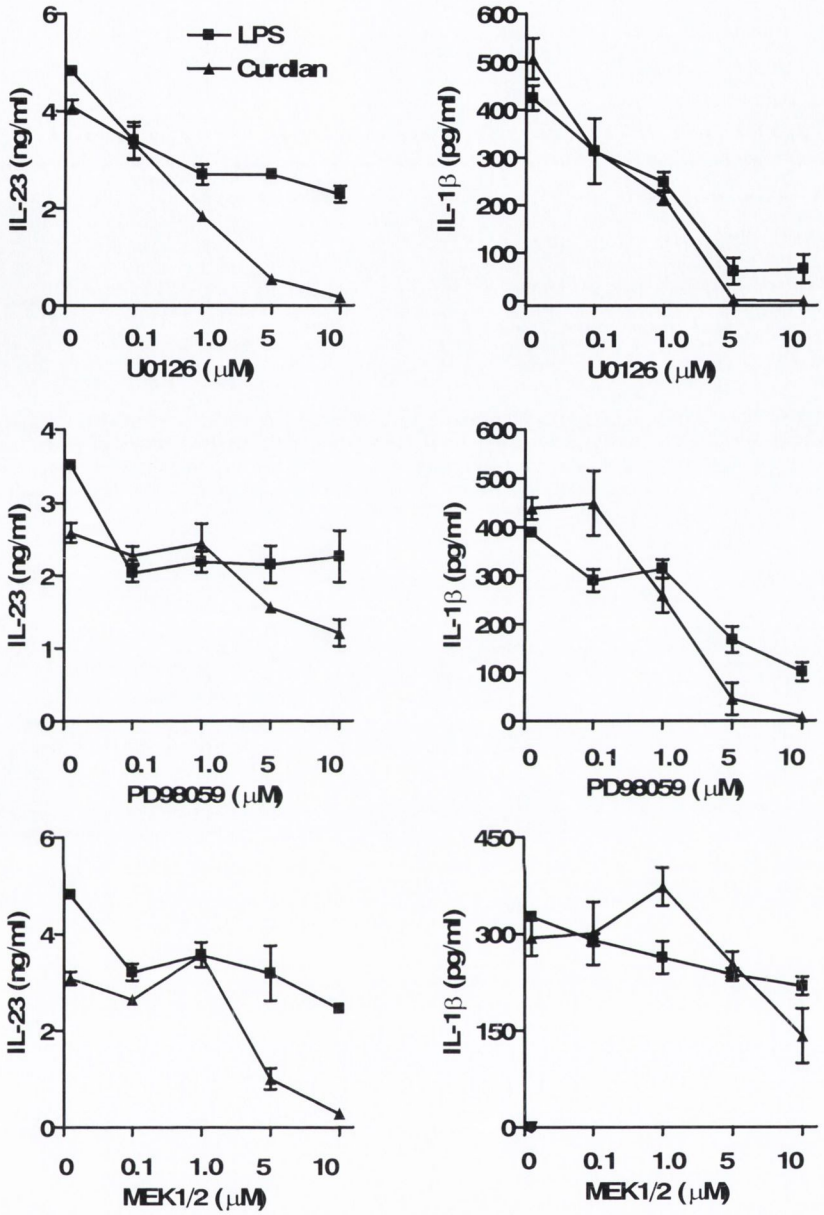

Figure 5.3 Three different MEK1/2 inhibitors suppress LPS- or curdlan- induced IL-23 and IL-1β production. BMDC (5 x 10^5 cells/ml) were stimulated with LPS (100 ng/ml) or curdlan (100 μg/ml) in the presence or absence of U0126, PD98059 or the Calbiochem MEK1/2 inhibitor (0.1, 1, 5 or 10 μM). Supernatants were collected after 24 h and IL-23 and IL-1β concentrations quantified by ELISA. Data are mean ± SEM of triplicate stimulations and are representative of two experiments.

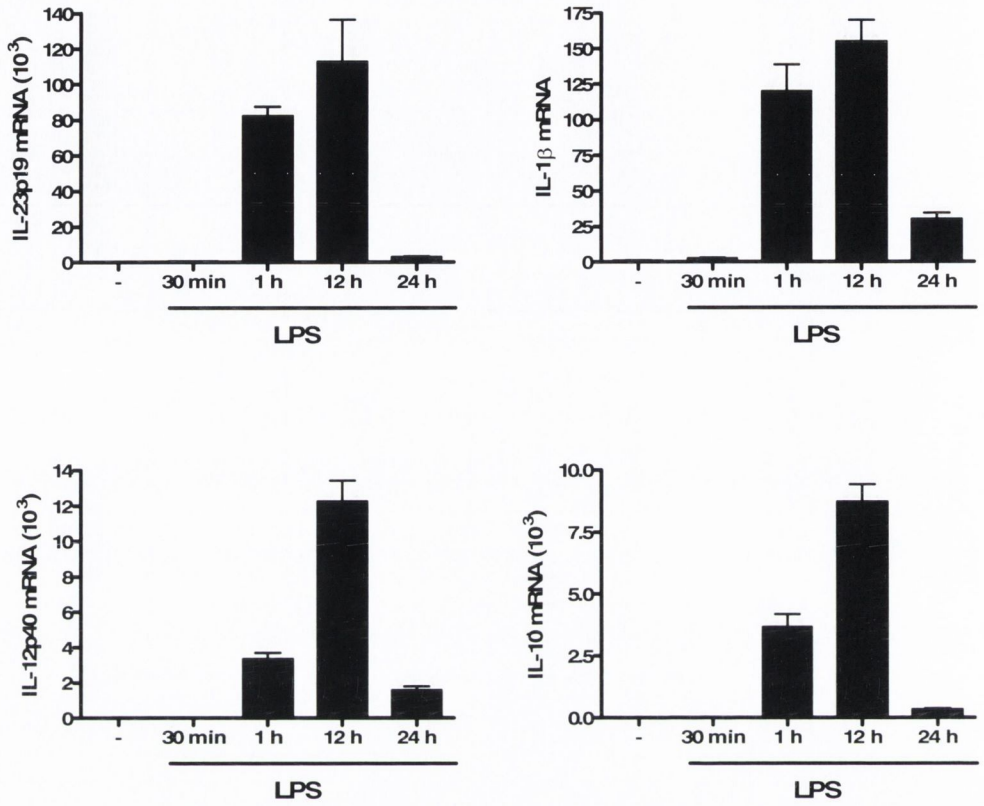

Figure 5.4 LPS induces IL-12p40, IL-23p19, IL-10 and IL-1β mRNA expression in DC. BMDC (1 x 10^6 cells/ml) were incubated with medium or LPS (100 ng/ml) for 30 min, 1 h, 12 h or 24 h. IL-12p40, IL-23p19, IL-10 and IL-1β mRNA expression was evaluated by real-time RT-PCR normalized to 18S rRNA. Data are shown as fold induction over DC cultured in medium only. Data are mean ± SEM of triplicate reactions from each sample and are representative of at least two independent experiments.

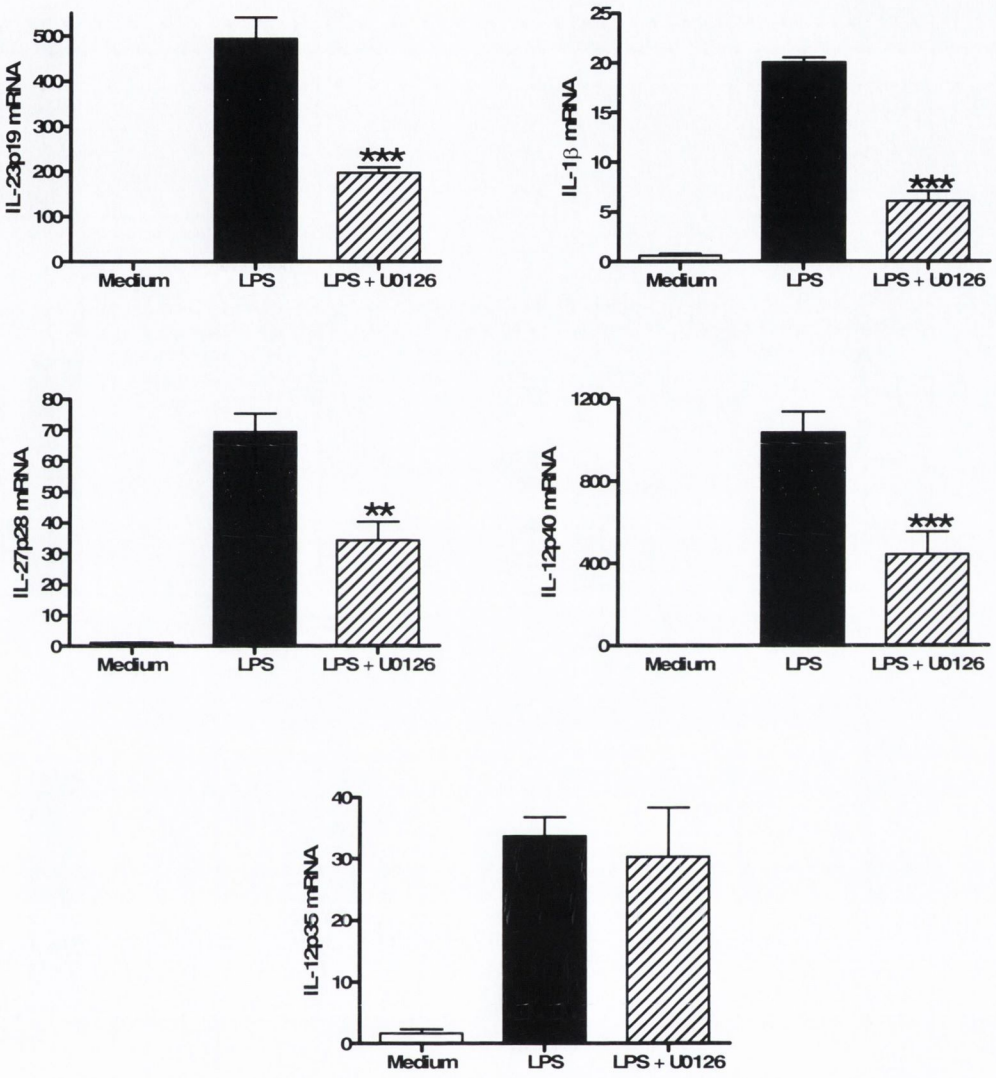

Figure 5.5 Inhibition of ERK suppresses LPS-induced IL-23p19, IL-12p40, IL-27p28 and IL-1β, but not IL-12p35 mRNA expression in DC. BMDC (1 x 10^6 cells/ml) were pre-treated with the MEK1/2 inhibitor U0126 (5 μM) for 1 h prior to stimulation with LPS (100 ng/ml) or medium alone for 12 h. IL-12p40, IL-12p35, IL-23p19, IL-27p28 and IL-1β mRNA expression was evaluated by real-time RT-PCR normalized to 18S rRNA. Data are shown as fold induction over DC cultured in medium only. LPS versus LPS + U0126: ** $P < 0.01$; *** $P < 0.001$. Data are mean ± SEM of triplicate reactions from duplicate stimulations and are representative of at least two independent experiments.

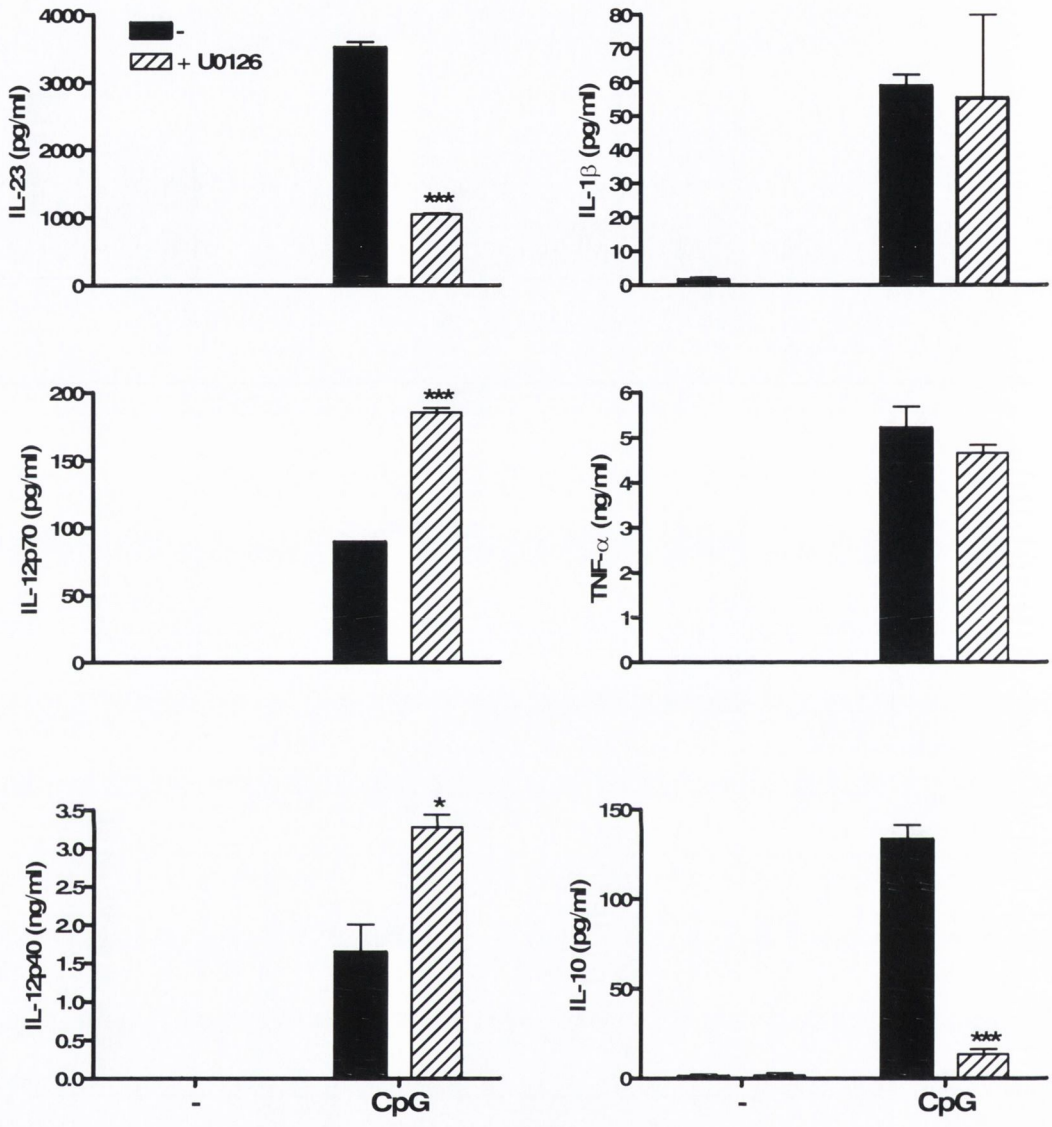

Figure 5.6 ERK is required for CpG-induced IL-23, but negatively regulates IL-12p70 and IL-12p40 production. BMDC (1 x 10^6 cells/ml) were stimulated with CpG (1 μg/ml) with or without the MEK1/2 inhibitor, U0126 (5 μM). Supernatants were collected after 24 h and IL-12p70, IL-12p40, IL-23, TNF-α, IL-10 and IL-1β concentrations quantified by ELISA. CpG versus CpG + U0126: *, $P < 0.05$; ***, $P < 0.001$. Data are mean ± SEM of triplicate samples from duplicate stimulations and are representative of two independent experiments.

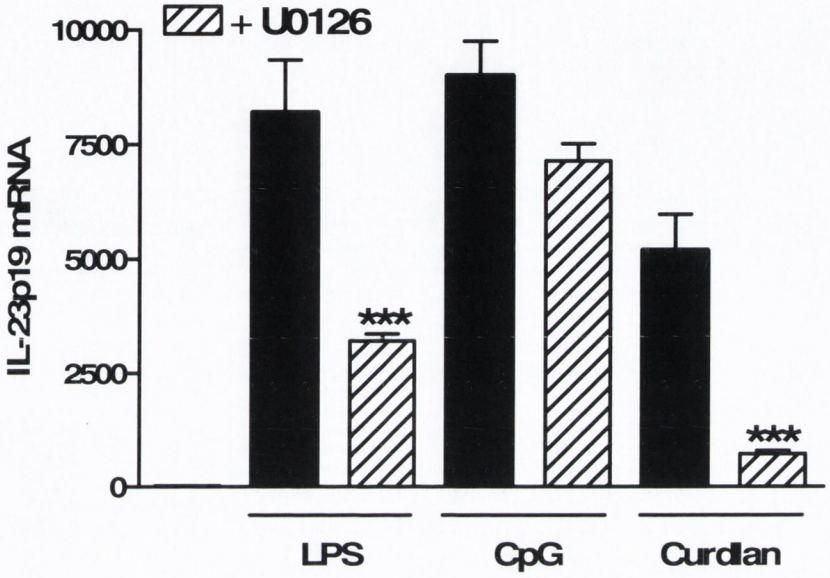

Figure 5.7 Inhibition of ERK suppresses LPS-, CpG- and curdlan- induced IL-23p19 mRNA expression in DC. BMDC (1 x 10^6 cells/ml) were pre-treated with the MEK1/2 inhibitor U0126 (5 μM) for 1 h prior to stimulation with LPS (100 ng/ml), CpG (1 μg/ml), curdlan (100 μg/ml) or medium alone for 12 h. IL-23p19 mRNA expression was evaluated by real-time RT-PCR normalized to 18S rRNA. Data are shown as fold induction over DC cultured in medium only. LPS/curdlan versus LPS/curdlan + U0126: *** $P < 0.001$. Data are mean ± SEM of triplicate reactions from duplicate stimulations and are representative of two independent experiments.

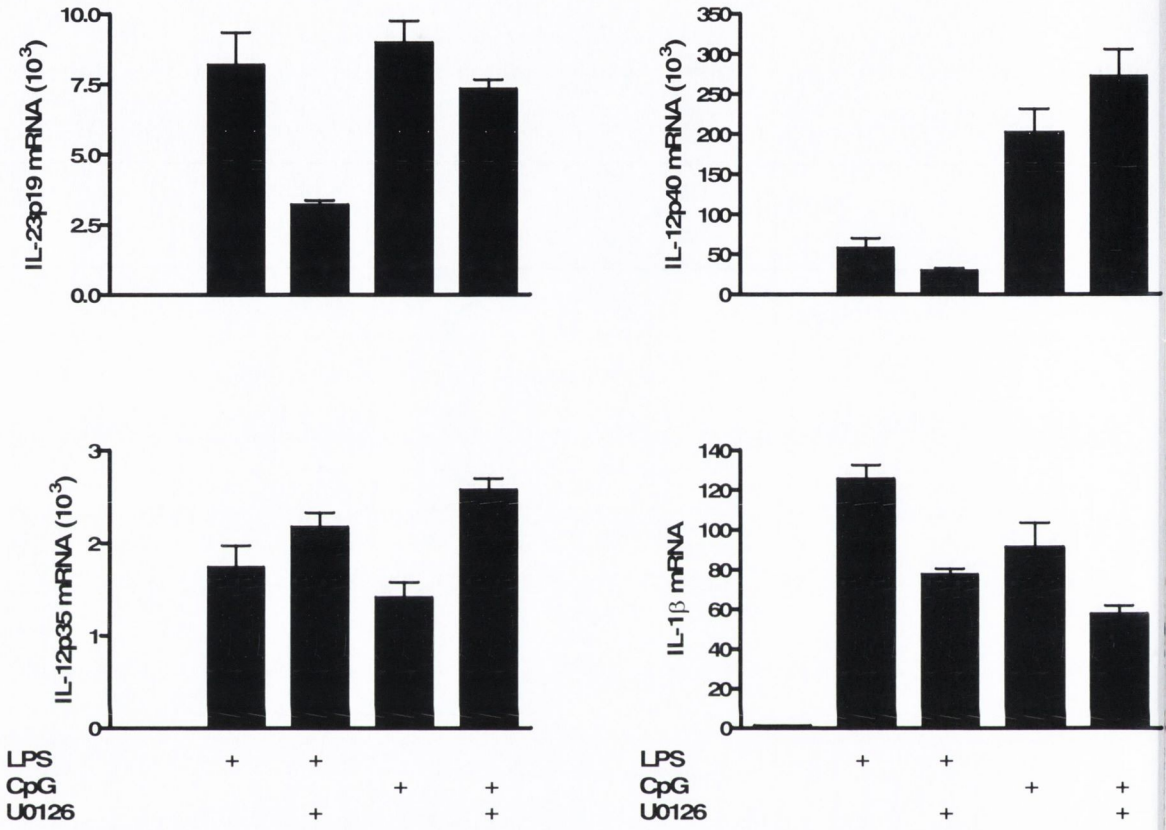

Figure 5.8 Inhibition of ERK enhances LPS- and CpG- induced IL-12p35 and CpG-induced IL-12p40 mRNA expression, but suppresses LPS-induced IL-12p40 mRNA expression in DC. BMDC (1 x 10^6 cells/ml) were pre-treated with the MEK1/2 inhibitor, U0126 (5 μM), for 1 h prior to stimulation with LPS (100 ng/ml), CpG (1 μg/ml) or medium alone for 12 h. IL-23p19 mRNA expression was evaluated by real-time RT-PCR normalized to 18S rRNA. Data are shown as fold induction over DC cultured in medium only. Data are mean ± SEM of triplicate reactions from duplicate samples.

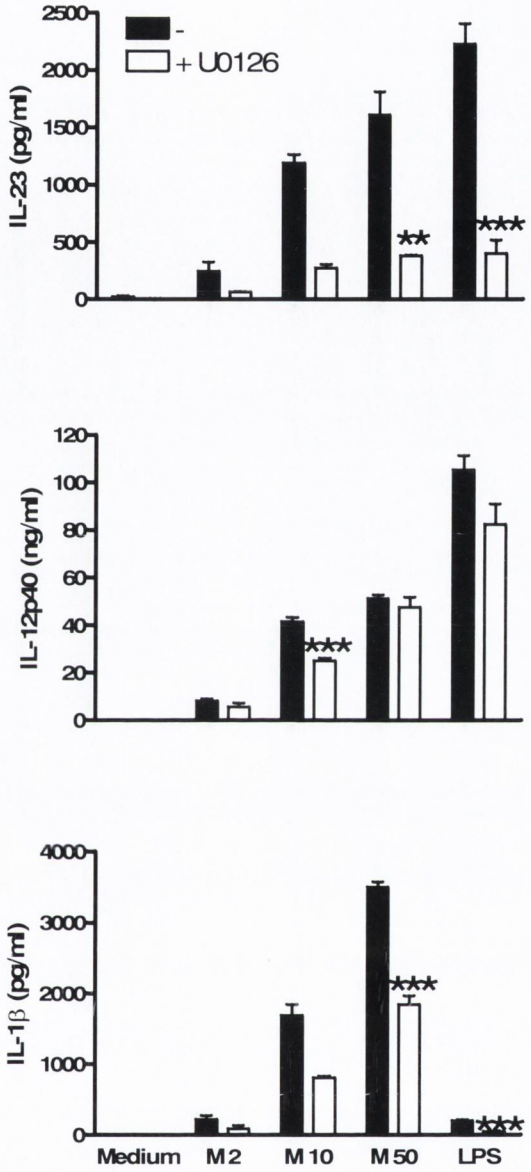

Figure 5.9 ***M. tuberculosis*- induced IL-1β and IL-23 production by DC is mediated by ERK activation.** BMDC (5 x 10^5 cells/ml) were stimulated with heat-killed *M. tuberculosis* (M) (2, 10 or 50 μg/ml), LPS (100 ng/ml) or with medium alone in the presence or absence of the MEK1/2 inhibitor, U0126 (5 μM). Supernatants were collected after 24 h and IL-12p40, IL-23 and IL-1β concentrations quantified by ELISA. M/LPS versus M/LPS + U0126: ** $P < 0.01$; *** $P < 0.001$. Data are mean ± SEM of triplicate stimulations.

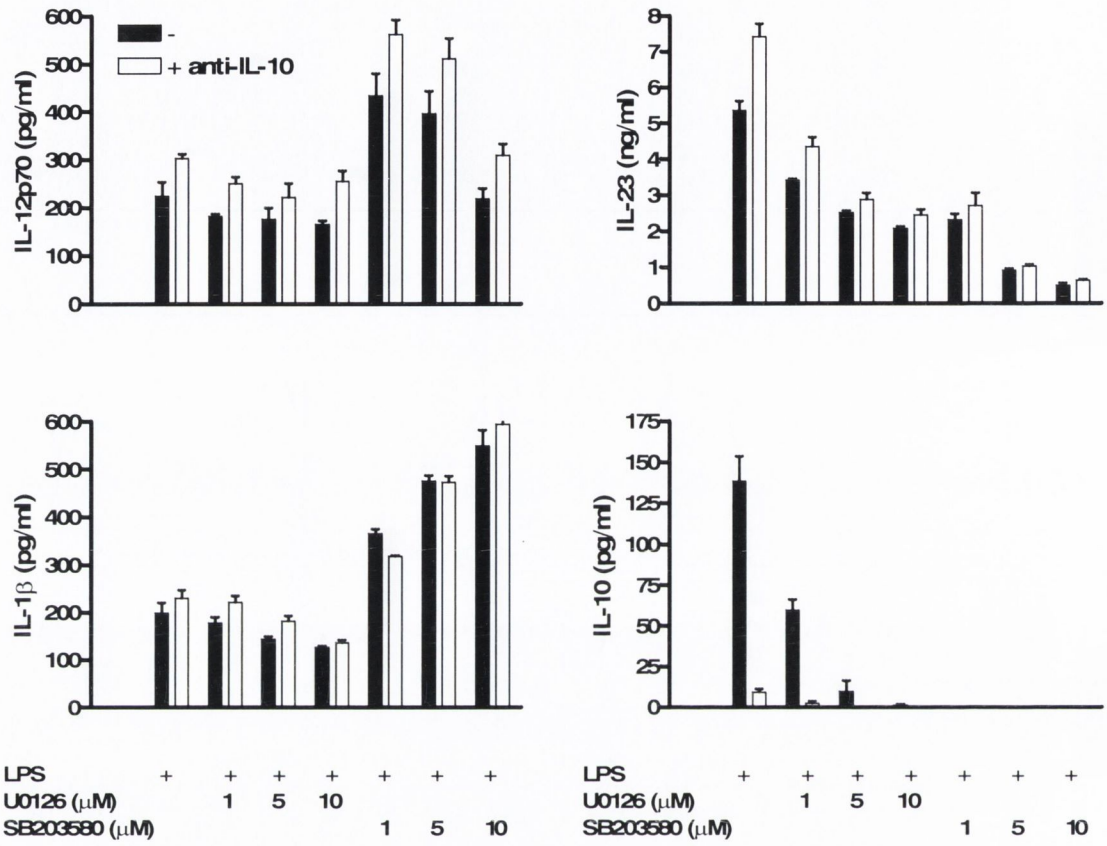

Figure 5.10 The suppressive effect of the MEK1/2 inhibitor U0126 on LPS-induced IL-23 is not mediated by IL-10. BMDC (5 x 10^5 cells/ml) were pre-incubated with a neutralizing anti-IL-10 antibody (10 ng/ml) for 2 h, prior to stimulation with LPS (100 ng/ml) with or without the MEK1/2 inhibitor U0126 or the p38 inhibitor SB203580 (1, 5 or 10 μM). Supernatants were collected after 24 h and IL-12p70, IL-23, IL-10 and IL-1β concentrations quantified by ELISA. Data are mean ± SEM of triplicate stimulations.

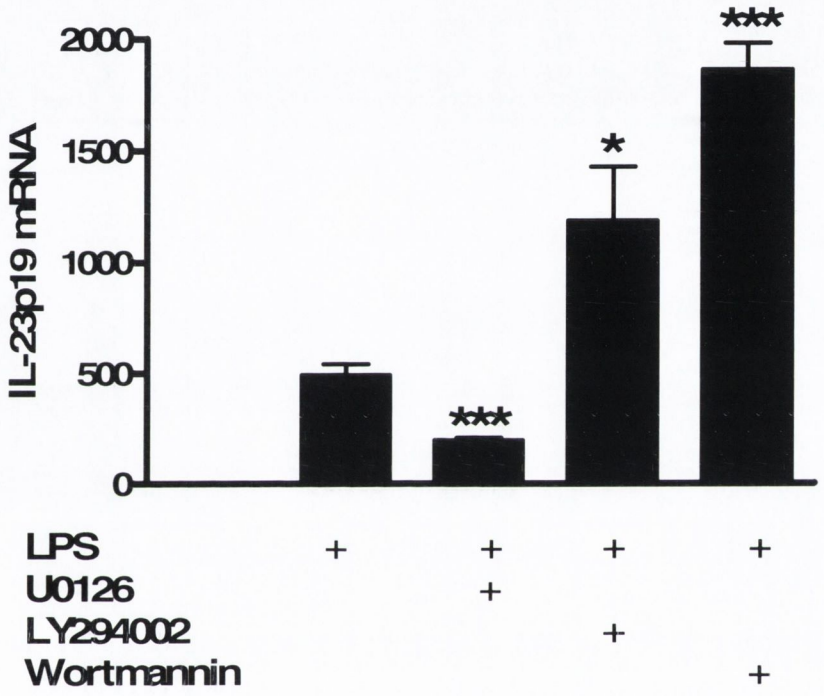

Figure 5.11 LPS-induced IL-23p19 mRNA is suppressed by inhibition of ERK but enhanced by blocking PI3K. BMDC (1 x 10^6 cells/ml) were pre-treated with the MEK1/2 inhibitor U0126 (5 μM), or the PI3K inhibitors, LY294002 (5 μM) or Wortmannin (1 μM), for 1 h prior to stimulation with LPS (100 ng/ml) or medium alone for 12 h. IL-23p19 mRNA expression was evaluated by real-time RT-PCR normalized to 18S rRNA. Data are shown as fold induction over DC cultured in medium only. LPS versus LPS + inhibitor: *, $P < 0.05$; *** $P < 0.001$. Data are mean ± SEM of triplicate reactions from duplicate stimulations.

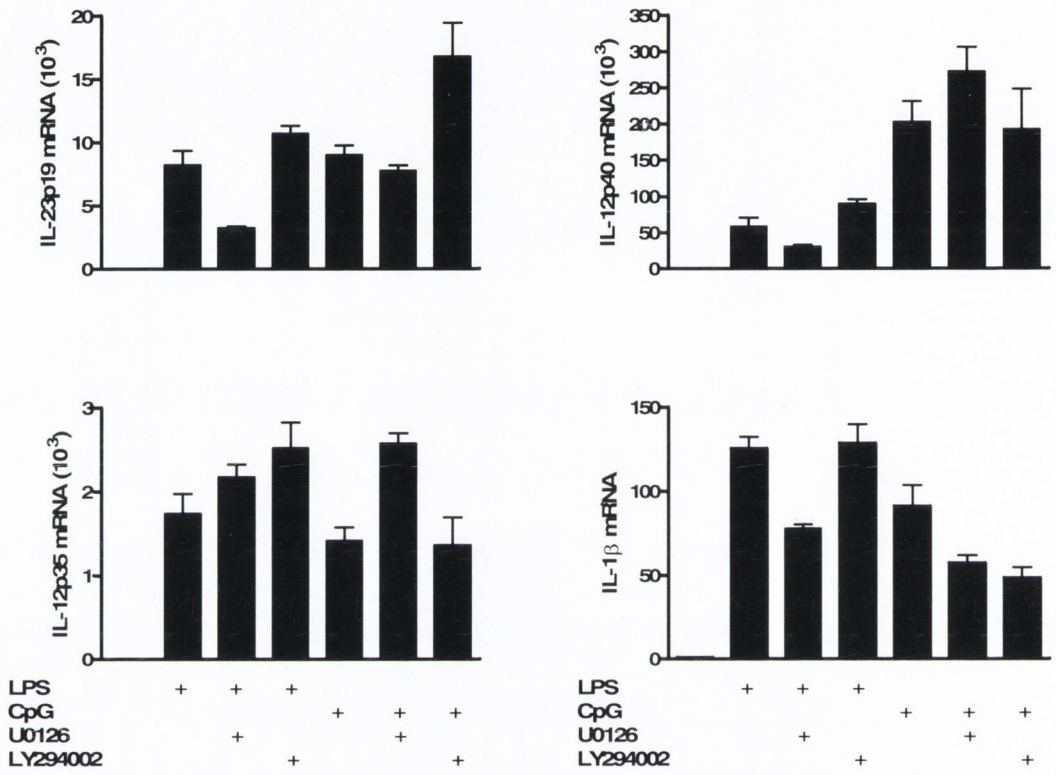

Figure 5.12 Inhibition of PI3K enhances LPS- and CpG- induced IL-23p19 mRNA expression in DC. BMDC (1 x 10^6 cells/ml) were pre-treated with the MEK1/2 inhibitor U0126 (5 μM) or the PI3K inhibitor LY294002 (5 μM) for 1 h prior to stimulation with LPS (100 ng/ml) CpG (1 μg/ml) or medium alone for 12 h. IL-23p19 mRNA expression was evaluated by real-time RT-PCR normalized to 18S rRNA. Data are shown as fold induction over DC cultured in medium only. Data are mean ± SEM of triplicate reactions from duplicate stimulations and are representative of two independent experiments.

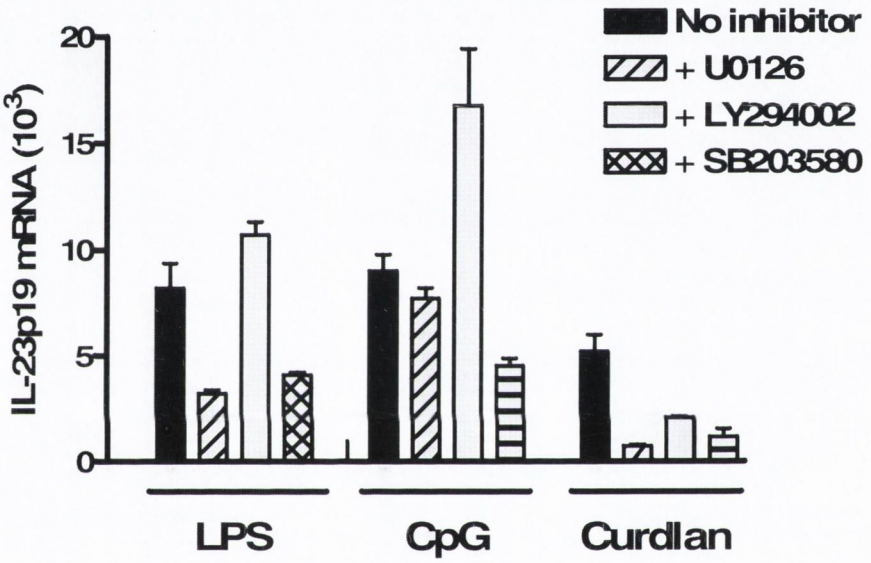

Figure 5.13 Inhibition of PI3K enhances LPS- and CpG- induced IL-23p19 mRNA expression in DC. BMDC (1 x 10^6 cells/ml) were pre-treated with the MEK1/2 inhibitor U0126 (5 μM), the p38 inhibitor SB203580 (5 μM) or the PI3K inhibitor LY294002 (5 μM) for 1 h prior to stimulation with LPS (100 ng/ml), CpG (1 μg/ml), curdlan (100 μg/ml) or medium alone for 12 h. IL-23p19 mRNA expression was evaluated by real-time RT-PCR normalized to 18S rRNA. Data are shown as fold induction over DC cultured in medium only. Data are mean ± SEM of triplicate reactions from duplicate stimulations.

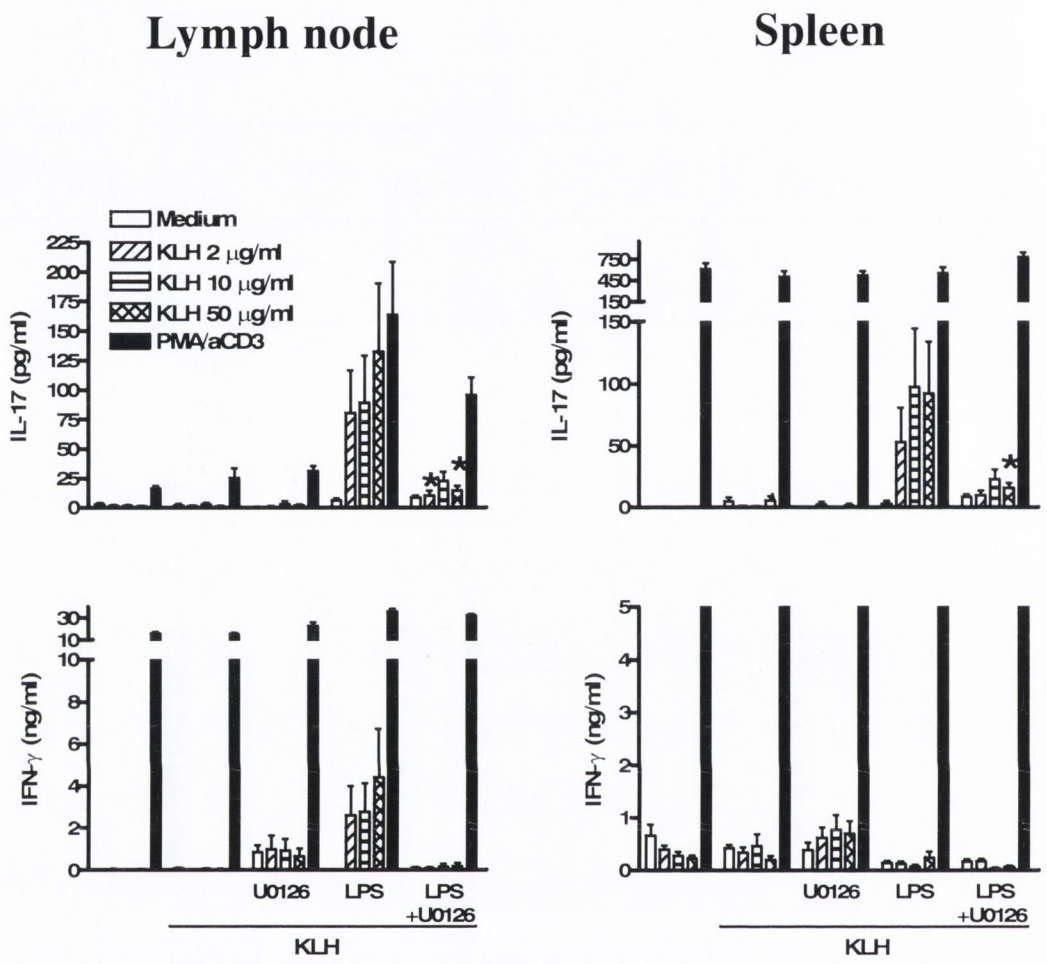

Figure 5.14 Inhibition of ERK signalling in DC suppresses their ability to induce Th1 and Th17 cells. BMDC were stimulated for 24 h with medium only, KLH (20 μg/ml) alone or KLH and LPS (100 ng/ml) with or without U0126 (1 μM). Cells were washed and transferred s.c. to naïve mice (n=5). After 7 d lymph node cells (1 x 10^6 cells/ml) or spleen cells (2 x 10^6 cell/ml) from individual mice were re-stimulated *in vitro* with KLH (2-50 μg/ml), medium only or with PMA (25 ng/ml) and anti-CD3 (1 μg/ml). Supernatants were recovered after 72 h and IL-17 and IFN-γ concentrations quantified by ELISA. KLH + LPS versus KLH + LPS + U0126: * $P < 0.05$. Data are mean ± SEM of triplicate stimulations and are representative of two independent experiments.

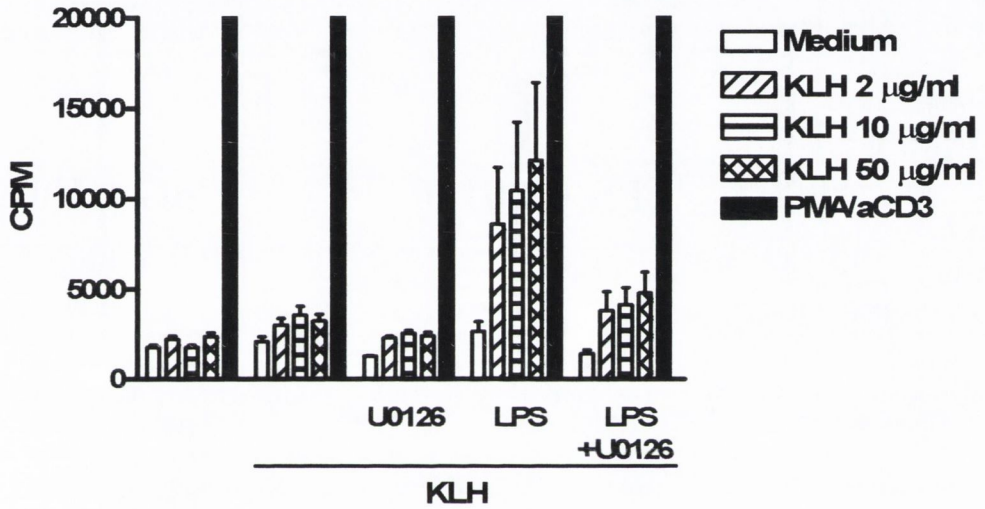

Figure 5.15 Inhibition of ERK signalling in DC suppresses their ability to induce antigen-specific proliferative responses by lymph node cells. BMDC were stimulated for 24 h with medium only, KLH (20 μg/ml) alone or KLH and LPS (100 ng/ml) with or without U0126 (1 μM). Cells were washed and transferred s.c. to naïve mice (n=5). After 7 d lymph node cells (1 x 10^6 cells/ml) from individual mice were re-stimulated *in vitro* with KLH (2-50 μg/ml), medium only or with PMA (25 ng/ml) and anti-CD3 (1 μg/ml). Supernatants were recovered after 72 h and the proliferative response of lymph node cells was quantified by [^{3}H] incorporation over the last 16 h of the 4 d stimulation period. Data are mean ± SEM of triplicate stimulations and are representative of two independent experiments.

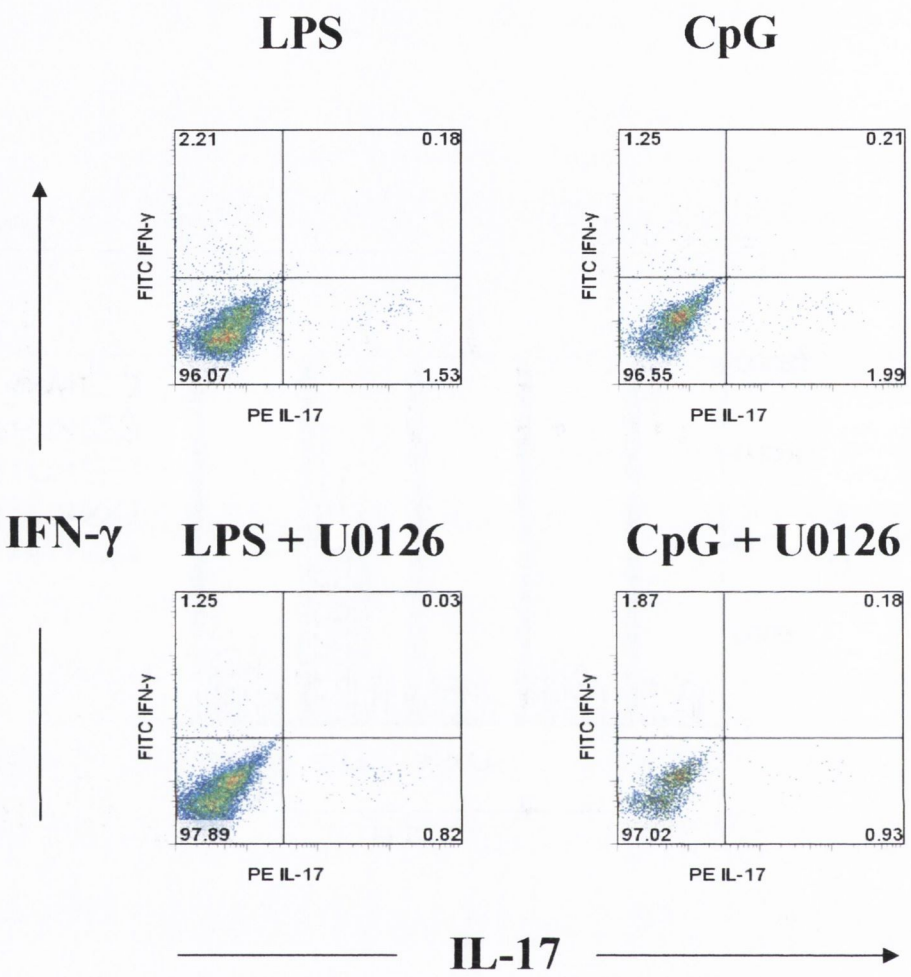

Figure 5.16 Inhibition of ERK signalling in DC suppresses their ability to induce Th17 cells *in vivo*. BMDC were stimulated for 24 h with medium only, KLH (20 μg/ml) alone or KLH and LPS (100 ng/ml) or CpG (1 μg/ml) with or without U0126 (1 μM). Cells were washed and transferred s.c. to naïve mice. After 7 d lymph node cells (1 x 10^6 cells/ml) were from mice (n=5) were pooled and re-stimulated *in vitro* with KLH (20 μg/ml) for 3 d then pulsed with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 18 h, in the presence of Brefeldin A (5 μg/ml) for the last 16 h. Representative flow cytometry demonstrating $CD3^+$ cells that were stained intracellularly for IL-17 and IFN-γ.

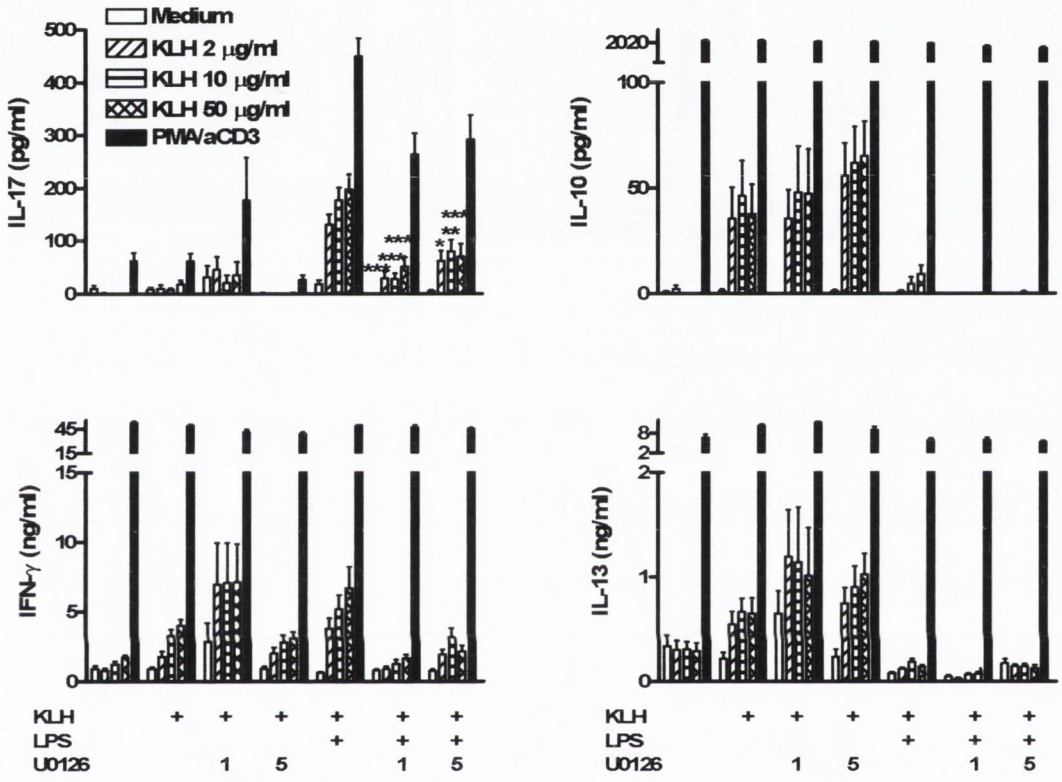

Figure 5.17 Inhibition of ERK signalling in DC suppresses their ability to induce Th1 and Th17 cells *in vivo*, but does not affect KLH-specific IL-10 or IL-13 production. BMDC were stimulated for 24 h with medium only, KLH (20 µg/ml) alone or KLH and LPS (100 ng/ml) with or without U0126 (1 or 5 µM). Cells were washed and transferred s.c. to naïve mice (n=5). After 7 d lymph node cells (1 x 10^6 cells/ml) from individual mice were re-stimulated *in vitro* with KLH (2-50 µg/ml), medium only or with PMA (25 ng/ml) and anti-CD3 (1 µg/ml). Supernatants were recovered after 72 h and IL-17, IFN-γ, IL-10 and IL-13 concentrations quantified by ELISA. KLH + LPS versus KLH + LPS + U0126: * $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are mean ± SEM of triplicate stimulations and are representative of two independent experiments.

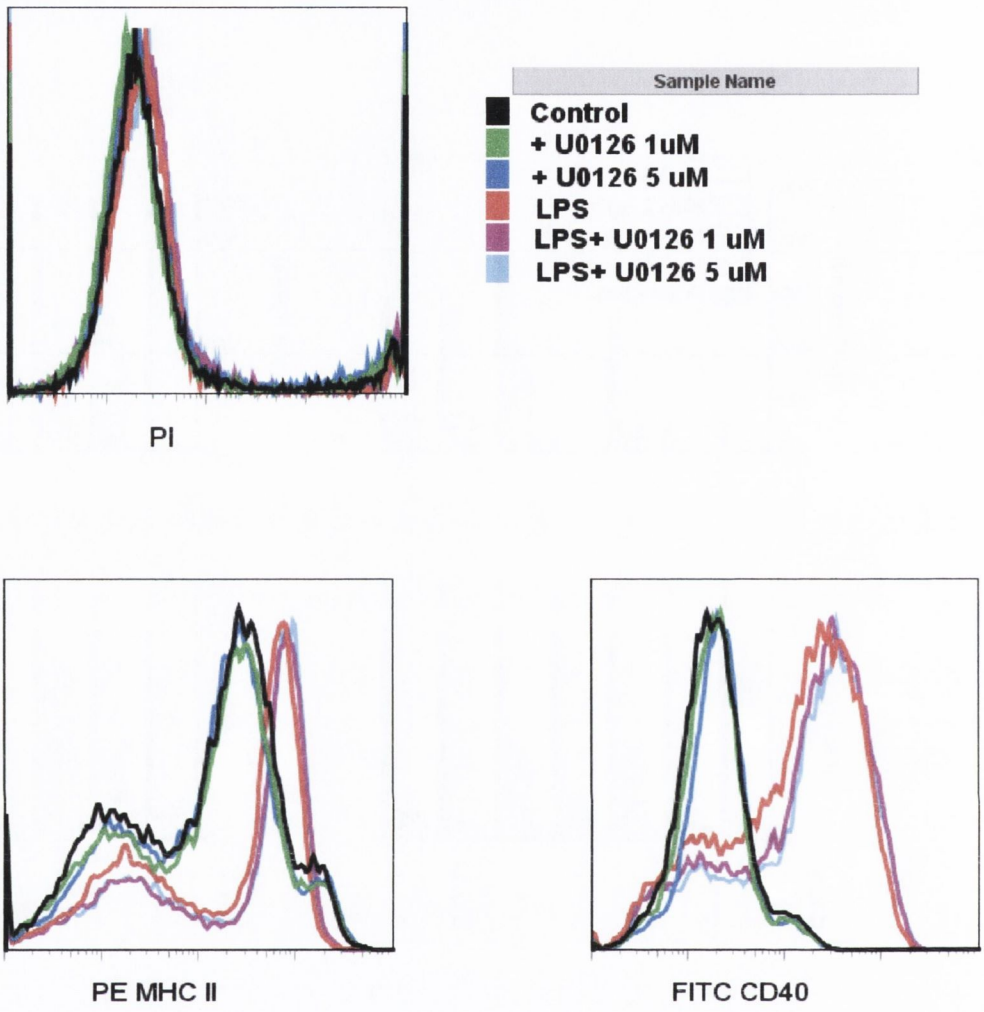

Figure 5.18 Inhibition of ERK does not reduce MHC class II or CD40 expression on DC or reduce cell viability. BMDC (1 x 10^6 cells/ml) were stimulated with LPS (100 ng/ml) or medium alone with or without the MEK1/2 inhibitor, U0126 (1 or 5 μM). Cells were harvested after 24 h and stained with antibodies specific for CD11c, MHC class II and CD40. Propidium iodide was added to the cells immediately before acquisition on the FACS machine to assess cell viability. Representative histograms of $CD11c^+$ cells. Data are representative of two independent experiments.

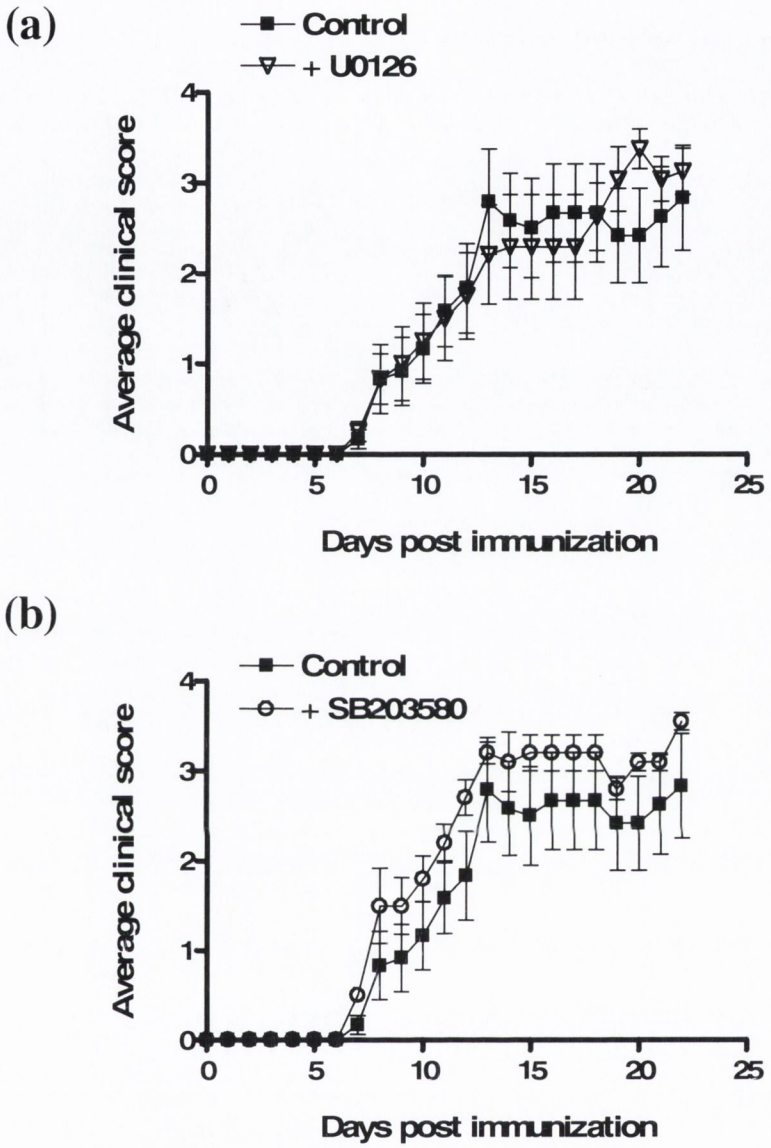

Figure 5.19 Inhibition of p38 exacerbates EAE. EAE was induced in C57BL/6 mice by s.c. injection of 150 μg of MOG_{35-55} emulsified in CFA, supplemented with 4 mg/ml H37 Ra *M. tuberculosis*. All mice were injected i.p. with 500 ng PT on days 0 and 2. Groups of mice were treated with the MEK1/2 or p38 inhibitor by mixing U0126 or SB203580 (50 μg/mouse) with the MOG/CFA emulsion on day 0. Animals were monitored daily for signs of clinical disease. Disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis; grade 5 – tetraparalysis/death. Data are mean score ± SEM for 6 mice per group.

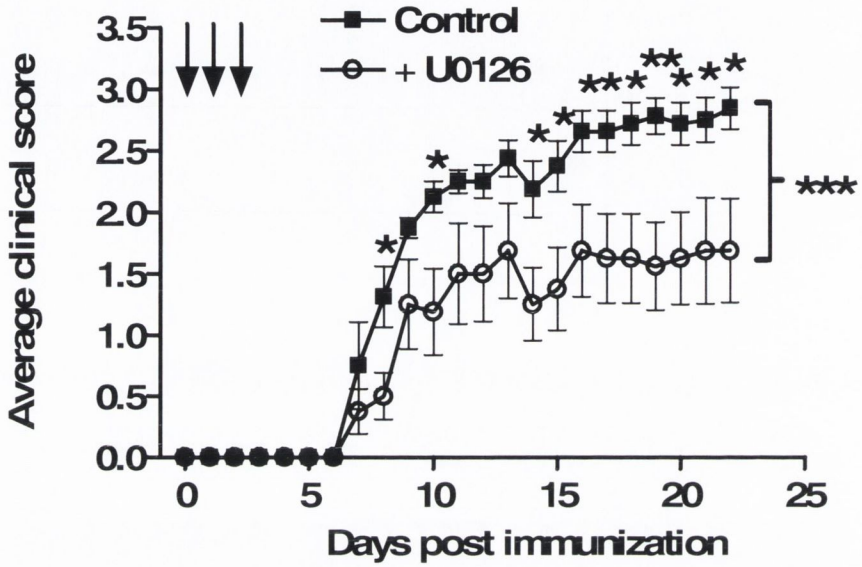

Figure 5.20 Inhibition of ERK suppresses the development of acute MOG-induced EAE. EAE was induced in C57BL/6 mice as described in Fig. 4.19. The group of mice that were treated with the MEK1/2 inhibitor were immunized with MOG/CFA containing U0126 (50 μg/mouse) in the emulsion on day 0 and injected s.c. with U0126 on days 1 and 2. Animals were monitored daily for signs of clinical disease. Disease severity was graded as described in Fig. 5.19. Data are mean score ± SEM for 8 mice per group and are representative of two independent experiments. EAE + U0126 versus untreated EAE on particular days: * $P < 0.05$; ** $P < 0.01$ by unpaired *t*-test. EAE + U0126 versus untreated EAE comparing overall interaction: *** $P < 0.001$ by two-way ANOVA.

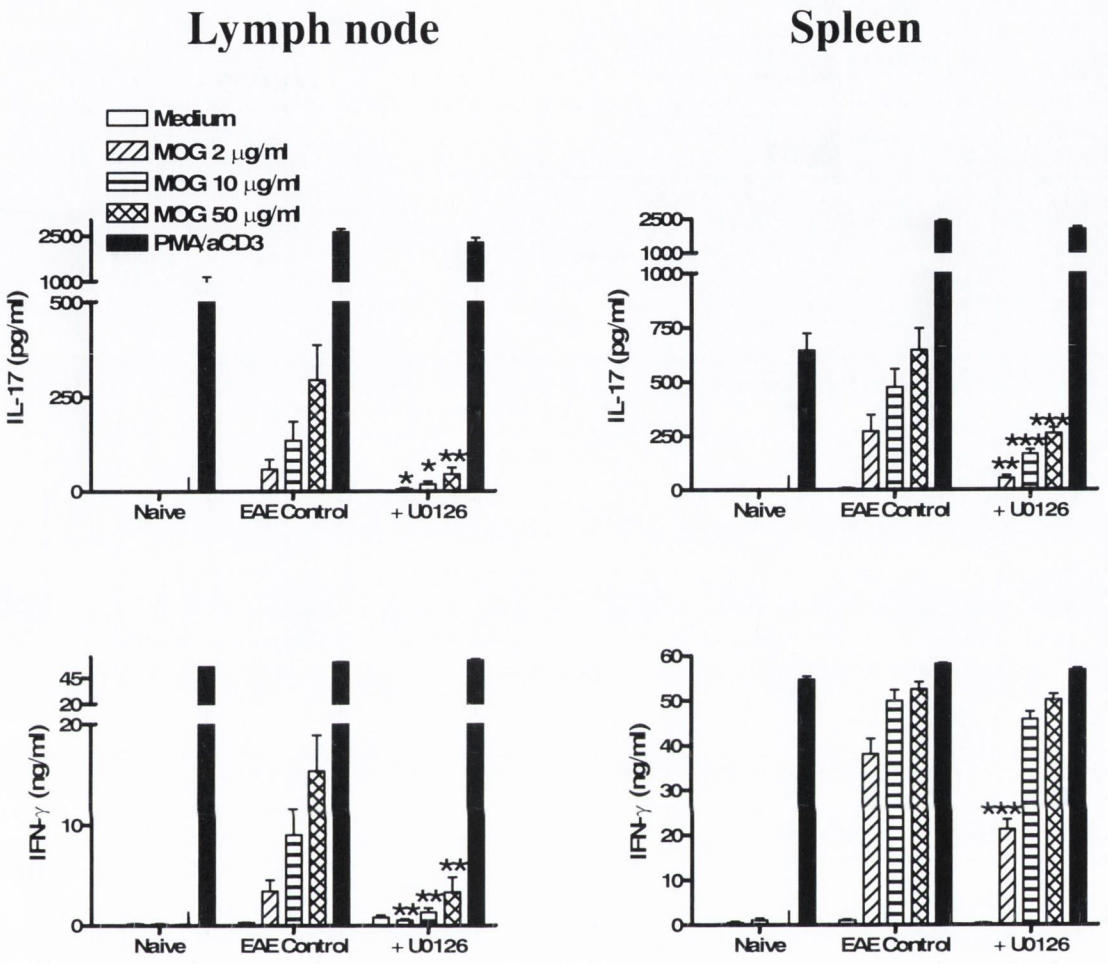

Figure 5.21 Inhibition of ERK suppresses the induction of MOG-specific Th1 and Th17 cells in lymph node and spleen tissue. EAE was induced in C57BL/6 mice as described in Fig. 4.19. The group of mice that were treated with the MEK1/2 inhibitor were immunized with MOG/CFA containing U0126 (50 μg/mouse) in the emulsion on day 0 and injected s.c with U0126 on days 1 and 2. Inguinal lymph node or spleen cells from individual mice (n= 8/group) were recovered 22 d post immunization and re-stimulated *in vitro* with MOG_{35-55} (2-50 μg/ml) or medium only. Supernatants were recovered 72 h later and IL-17 and IFN-γ concentrations quantified by ELISA. EAE Control versus EAE + U0126: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are mean ± SEM for triplicate stimulations of lymph node cells from individual mice (n = 8/group).

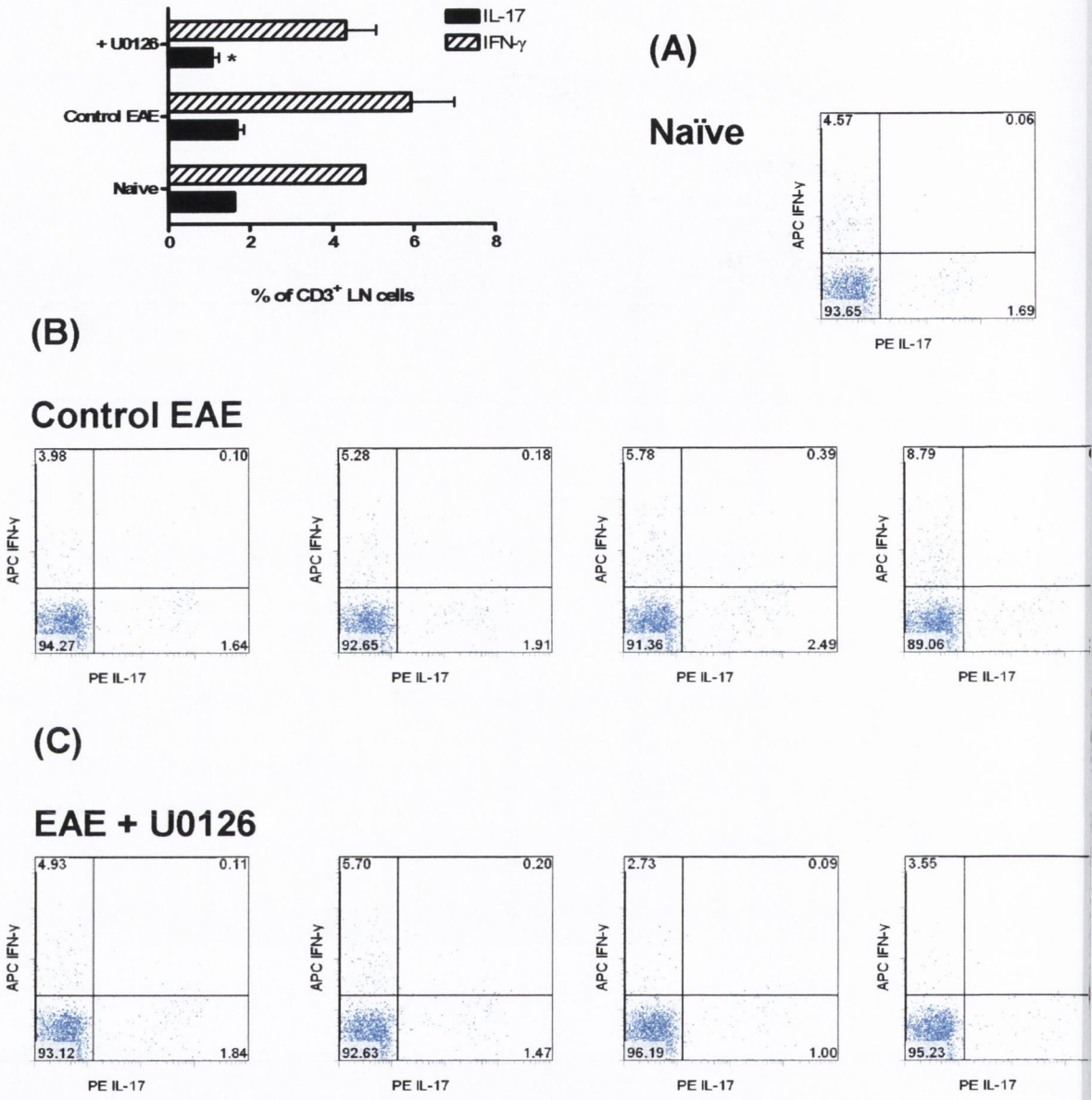

Figure 5.22 Inhibition of ERK suppresses IL-17 and IFN-γ production by $CD3^+$ lymph node cells. EAE was induced in C57BL/6 mice as described in Fig. 4.19. The group of mice that were treated with the MEK1/2 inhibitor were immunized with MOG/CFA containing U0126 (50 μg/mouse) in the emulsion on day 0 and injected s.c. with U0126 on days 1 and 2. Inguinal lymph node cells were recovered after 22 d and re-stimulated *in vitro* with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 16 h the presence of Brefeldin A (5 ng/ml) for the last 4 h. Representative flow cytometry demonstrating $CD3^+$ cells, from (A) naïve mouse, (B) 4 control EAE mice and (C) 4 mice treated with U0126 at the induction of EAE, that were stained intracellularly for IL-17 and IFN-γ.

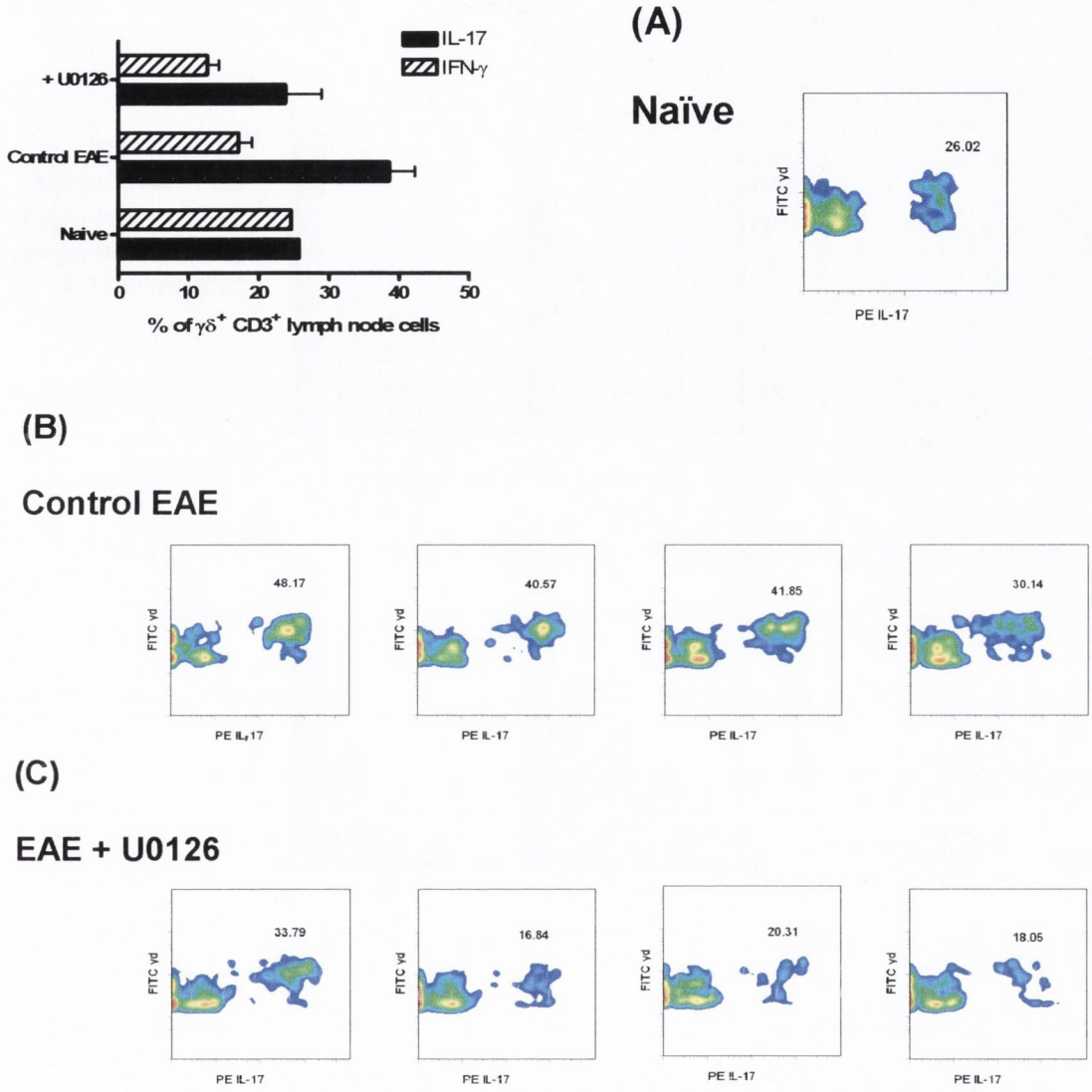

Figure 5.23 Inhibition of ERK suppresses IL-17 production by γδ T cells. EAE was induced in C57BL/6 mice as described in Fig. 4.19. The group of mice that were treated with the MEK1/2 inhibitor were immunized with MOG/CFA containing U0126 (50 μg/mouse) in the emulsion on day 0 and injected s.c with U0126 on days 1 and 2. Inguinal lymph node cells were recovered after 22 d and re-stimulated *in vitro* with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 16 h the presence of Brefeldin A (5 ng/ml) for the last 4 h. Representative flow cytometry demonstrating $CD3^{+}\gamma\delta^{+}$ cells from (A) naïve mouse, (B) 4 control EAE mice and (C) 4 mice treated with U0126 at the induction of EAE, that were stained intracellularly for IL-17 and IFN-γ.

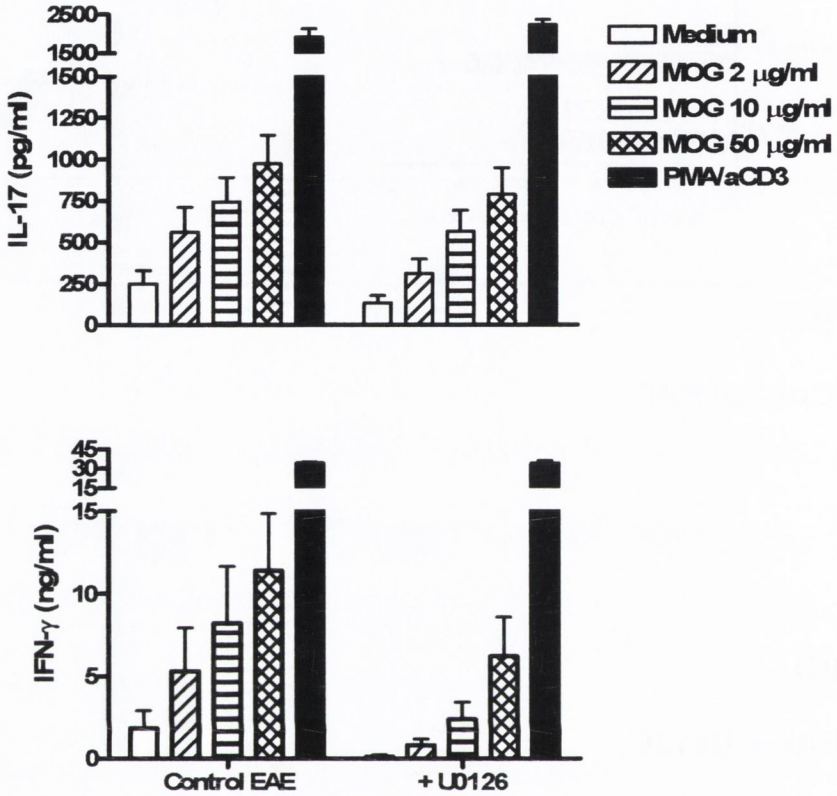

Figure 5.24 Inhibition of ERK suppresses the early induction of MOG-specific Th1 and Th17 cells in the inguinal lymph nodes. EAE was induced in C57BL/6 mice as described in Fig. 4.19. The group of mice that were treated with the MEK1/2 inhibitor were immunized with MOG/CFA containing U0126 (50 μg/mouse) in the emulsion on day 0 and injected s.c with U0126 on days 1 and 2. Inguinal lymph node cells were recovered 5 d post immunization and re-stimulated *in vitro* with MOG_{35-55} (2-50 μg/ml) or medium only. Supernatants were recovered 72 h later and IL-17 and IFN-γ concentrations quantified by ELISA. Data are representative of triplicate stimulations of lymph node cells from individual mice (n = 5/group).

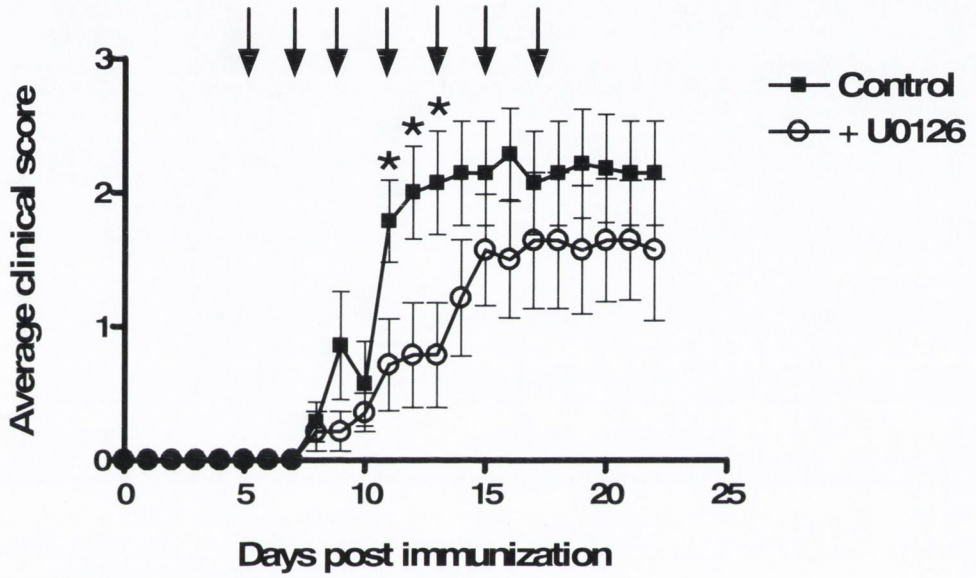

Figure 5.25 The MEK1/2 inhibitor is not as effective at attenuating EAE when administered 5 d after the induction of disease. EAE was induced in C57BL/6 mice as described in Fig. 5.19. Mice were injected s.c. with vehicle (PBS and DMSO) or U0126 (50 μg/mouse) every second day from day 5 to day 18. Animals were monitored daily for signs of clinical disease. Disease severity was graded as described in Fig. 5.19. EAE + U0126 versus EAE control: * $P < 0.05$; ** $P < 0.01$ by unpaired *t*-test. Data are mean score ± SEM (n = 7/group).

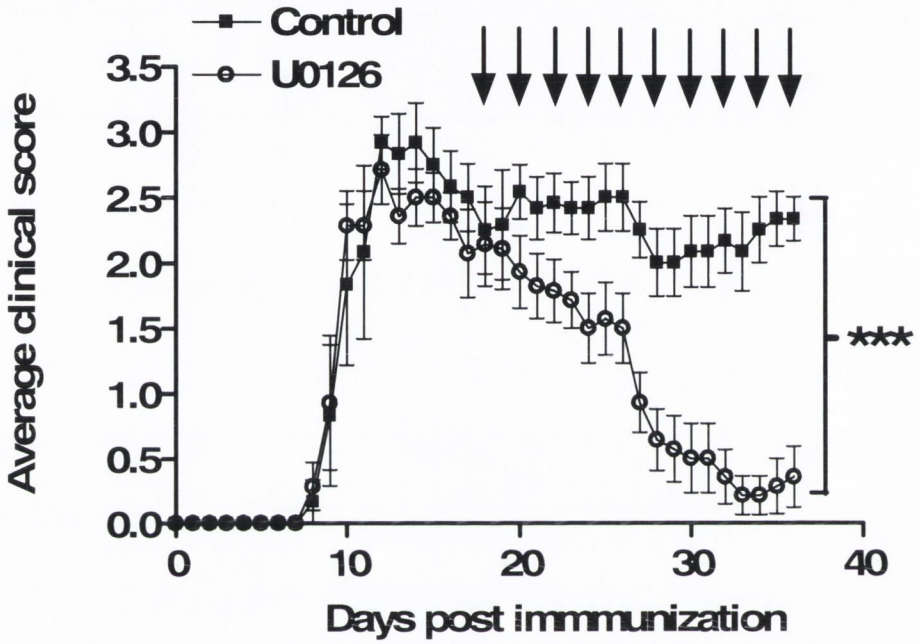

Figure 5.26 Therapeutic intervention with a MEK1/2 inhibitor suppresses relapse during the remission phase of EAE. EAE was induced in SJL/J mice by immunization with PLP in CFA. Mice were injected i.p. with 500 ng PT on days 0 and 2. Mice were separated into 2 groups with equal average clinical scores. Mice were treated with vehicle (PBS and DMSO) or U0126 (50 μg/mouse) every two days from day 18 until day 36. Arrows indicate treatment days. Animals were monitored daily for signs of clinical disease. Disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis; grade 5 – tetraparalysis/death. EAE + U0126 versus EAE control: *** $P < 0.05$ comparing curves by ANOVA. Data are mean score ± SEM (n = 9/group).

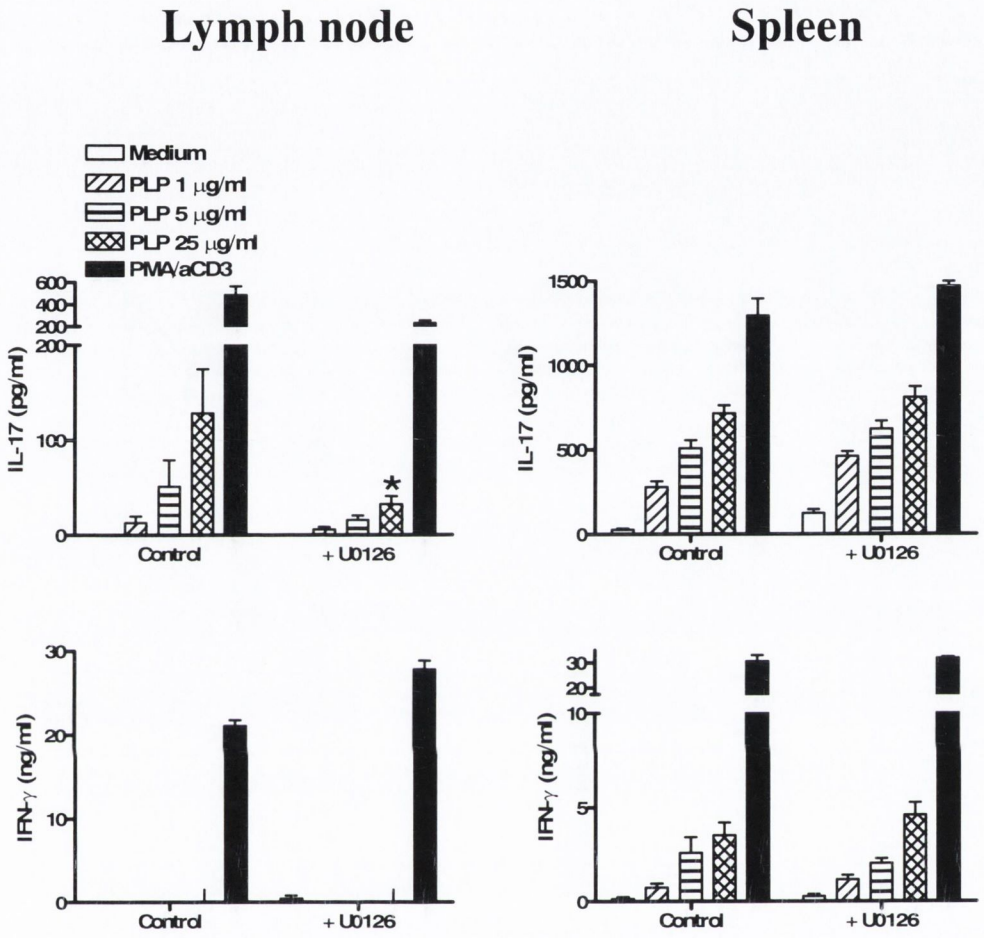

Figure 5.27 Therapeutic intervention with a MEK1/2 inhibitor suppressed PLP-specific Th1 and Th17 cells in lymph and spleen tissue. EAE was induced in SJL/J mice as described in Fig. 5.26. Mice were treated with vehicle (PBS and DMSO) or U0126 (50 μg/mouse) every second day from day 18 until day 36. Inguinal lymph node (1 x 10^6 cells/ml) and spleen cells (2 x 10^6 cells/ml) were recovered 38 d post immunization and re-stimulated *in vitro* with PLP (1-25 μg/ml) or medium only. Supernatants were collected 72 h later and IL-17 and IFN-γ concentrations quantified by ELISA. EAE control versus EAE + U0126: * $P < 0.05$. Data are mean ± SEM for triplicate stimulations of lymph node or spleen cells from individual mice (n = 7/group).

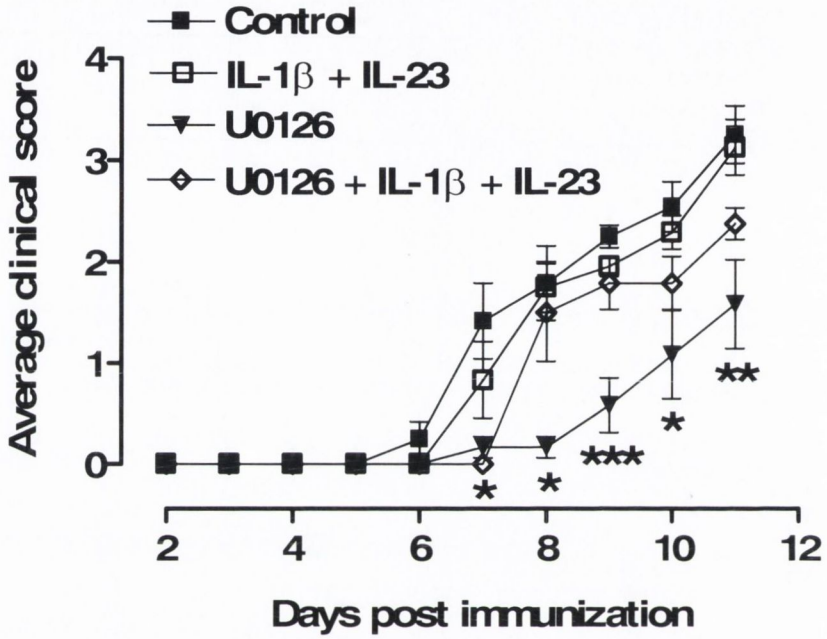

Figure 5.28 The suppression of acute MOG-induced EAE by inhibition of ERK is mediated by the suppression of IL-1 and IL-23. EAE was induced in C57BL/6 mice as described in Fig. 5.19. Treated mice were immunized with MOG/CFA containing U0126 (50 μg/mouse) with or without rIL-1 and rIL-23 (100 ng/ml) in the emulsion on day 0 and injected s.c. with U0126 +/- rIL-1 and rIL-23 on days 1 and 2. Animals were monitored daily for signs of clinical disease. Disease severity was graded as described in Fig. 5.19. EAE + U0126 versus EAE control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by ANOVA. Data are mean score ± SEM (n = 4/group).

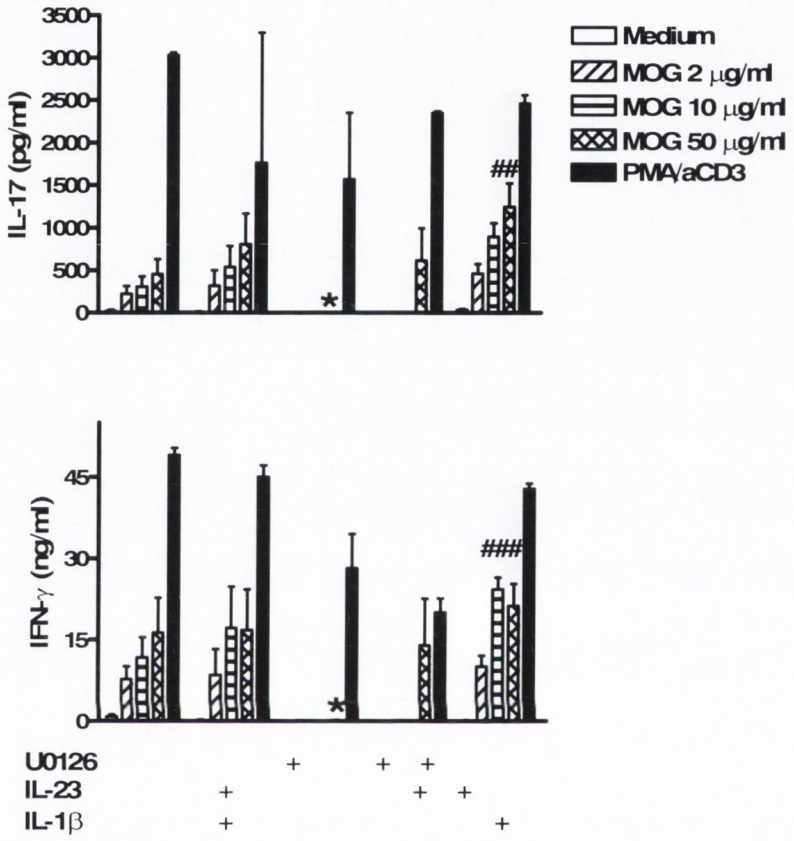

Figure 5.29 The suppression of MOG-specific IL-17 and IFN-γ production by inhibition of ERK is reversed in the presence of IL-23 and IL-1. EAE was induced in C57BL/6 mice as described in Fig. 5.19. Treated mice were immunized with MOG/CFA containing U0126 (50 μg/mouse) with or without rIL-1 and rIL-23 (100 ng/ml) in the emulsion on day 0 and injected s.c with U0126 +/- rIL-1 and rIL-23 on days 1 and 2. Mice were sacrificed 4 d post immunization and inguinal lymph node cells were re-stimulated *in vitro* with MOG_{35-55} (2-50 μg/ml) or medium only. Supernatants were recovered 72 h later and IL-17 and IFN-γ concentrations quantified by ELISA. EAE control versus EAE + U0126 (MOG 50 μg/ml): * $P < 0.05$. EAE + U0126 versus EAE + U0126 + IL-23 + IL-1β: ## $P < 0.01$; ### $P < 0.001$ by unpaired *t* test. Data are mean ± SEM for triplicate stimulations of lymph node cells from 2 individual mice and are representative of two independent experiments.

Chapter 6

General Discussion

6.1 General Discussion

The development of effective and safe vaccines against infectious diseases including, malaria and HIV remains one of the major challenges for immunologists. The trend in vaccination has moved towards the use of subunit vaccines, composed of highly purified antigens, rather than killed vaccines which are reactogenic, or vaccines based on attenuated viruses or bacteria which can revert to virulence. Successful vaccines contain adjuvants that activate the innate immune system and elicit antigen-specific T cell and antibody responses. Over the past number of years adjuvant research has largely been focussed on TLR ligands, given the essential role that TLRs play in the activation of innate immunity and subsequent development of adaptive immune responses (11, 12). However, recent studies have identified that conventional adjuvants such as alum or incomplete or complete Freund's adjuvant elicit efficient adaptive immune responses to antigen in the absence of TLR signalling (485, 486). These findings pointed to the existence of other pathways capable of inducing innate and adaptive immunity, and indeed, newly characterised intracellular innate receptors, such as NLRs and RLRs have been identified as possible candidates (452).

The mechanism of action of most vaccine adjuvants remains unclear. The present study investigated the means by which the enterotoxin LT from enterotoxigenic strains of *E. coli,* one of the most powerful mucosal adjuvants described to date, mediates its adjuvant activity. The use of LT in humans is hampered by toxicity, largely attributed to its enzyme activity, however, site-directed derivatives of LT with reduced or no enzyme activity have been made and these may be more suitable than wild-type LT for clinical use (216, 253). In addition, they provide useful tools to examine the role of the ADP-ribosyltransferase activity and the binding domain of LT in mediating its adjuvant effects.

Effective adjuvants stimulate the innate immune system and enhance adaptive immune responses to associated or bystander antigen. CT and LT have been extensively used as mucosal adjuvants with a wide variety of antigens in animal models. Co-administration of LT or CT with antigen via the nasal, oral or other mucosal routes potently enhances antigen-specific mucosal and cellular immune responses. Mucosal delivery of LT has been shown to induce a mixed Th1/Th2 type response, with the

production of antigen-specific IL-4, IL-5 and IFN-γ and IgG antibodies of the IgG1, IgG2a and IgG2b subclasses (193, 206-208). This study demonstrated that LT also promotes the induction of antigen-specific IL-17 production. Furthermore, immunization with antigen and LTR72, the partially enzymatically active derivative of LT, generated antigen-specific Th17 cells. The enzymatically inactive derivative LTK63 also promoted antigen-specific IL-17 production, but to a lesser extent than LT or LTR72. Adoptive transfer studies revealed that DC stimulated with antigen and LT, LTR72 or LTK63 induced Th responses similar to those induced by direct injection. In addition, adoptive transfer studies showed that DC pulsed with antigen and LT, LTK63 or LTR72 promoted antigen-specific IL-17 production. This suggests that LT exerts its immunomodulatory effects by acting on DC, which induce antigen-specific cytokine production by different Th cell subsets. These findings also indicate that the enzyme activity is not essential for the adjuvant activity of LT.

Due to their unique ability to activate naïve T cells and regulate Th cell polarization, DC represent the most potent APCs of the innate immune system (58). Several adjuvants including LPS and CpG have the capacity to activate DC and trigger the production of cytokines, including the Th1-polarizing cytokine IL-12p70. In addition to forming bioactive IL-12p70 with p35, the IL-12 p40 subunit can combine with p19, a subunit distantly related to p35, to form IL-23 (153). Despite p19 being a homologue of p35 and dimerizing with IL-12p40, IL-23 and IL-12 have been shown to have distinct functions in the development of $CD4^+$ T cell subsets (127, 154). Cua and colleagues showed that both IL-12p40- and IL-23p19- deficient mice were resistant to EAE, a murine experimental model of MS, which was thought to be a Th1-mediated disease (127). Early studies suggested that IL-23 drove the differentiation of Th17 cells (101, 123, 124). However, later evidence led to the proposal that the function of IL-23 is to expand differentiated Th17 cells or maintain IL-17 production (101, 129). A more recent report has shown that the the IL-23R is essential for the terminal differentiation of Th17 cells (487).

IL-23 is expressed predominantly by activated DC and IL-23R is expressed on activated T cells, BMDC, and activated and inflammatory macrophages (431). The present study revealed that LT induced IL-23p19 expression in DC. This was surprising

since LT, in the absence of co-stimulation with TLR agonists, did not induce the production of inflammatory cytokines, including IL-12p70, IL-12p40, IL-10 and TNF-α, which is consistent with studies on CT (121). Other cAMP-activators, including PGE_2 and the P2 receptors for ATP, have been shown to enhance IL-23 production by human DC (488). However, the data presented here demonstrate that LTK63, which is devoid of enzyme activity, also induced IL-23p19 expression, indicating that the activation of Gsα may not be mediating this effect.

Consistent with a report on CT (121), the present study showed that LT suppressed LPS-induced IL-12 production, but synergized with LPS in the production of IL-10. The enhancement of intracellular cAMP by the toxins has been proposed as a dominant factor in mediating their immunomodulatory effects (268, 274). However, the results shown here demonstrate that LTK63, an enzymatically inactive derivative of LT, suppressed IL-12 and enhanced IL-10 production by LPS- or CpG- stimulated DC. Consistent with this, inhibition of PKA, a principal downstream target of cAMP, had no effect on the suppression of IL-12 or the enhancement of IL-10 production by LT or LTK63. A recent study by Boirivant *et al.* supports these findings by demonstrating that CTB, the isolated B subunit of CT, suppressed LPS-induced upregulation of CD40 expression and IL-12 production by human MMDC (489). Moreover, similar to a report by Lavelle *et al.* on CT, DC stimulated with CTB and LPS promoted the induction of Treg cells *in vitro*, and these cells showed reduced ability to proliferate, reduced IFN-γ production, and increased IL-10 production compared to T cells treated with DC stimulated with LPS alone (121, 489).

The ability of LTK63 to suppress IL-12 and enhance IL-10 production required pre-treatment of DC with LTK63 for up to 12 h prior to stimulation with LPS, while co-incubation of wild-type toxin with LPS resulted in the same effect. Similarly, stimulation of DC with LT for 1 h induced the expression of c-Fos, a component of the AP-1 transcription factor. LTK63 also induced c-Fos expression but this was not evident unless DC were stimulated with LTK63 for at least 12 h. AP-1 has been shown to be a negative regulator of IL-12p40 (401, 402). Moreover, it was reported that PGE_2-mediated inhibition of IL-12 production required the activation of AP-1 (402). Despite being devoid of enzyme activity, LTK63 increased the expression of cAMP in DC, albeit to a lesser extent than wild-type toxin or LTR72. This may provide some explanation for the

delayed ability of LTK63 to modulate DC maturation and cytokine production compared with LT.

In contrast to the suppressive effect of LT on LPS-induced IL-12p40 and IL-12p70 production, co-incubation of LT with LPS enhanced IL-23 secretion. However, this effect was abolished when DC were pre-treated with LT for longer than 30 min. LTK63 also enhanced LPS-induced IL-23 production, but unlike its suppression of IL-12, co-incubation of DC with LTK63 and LPS augmented IL-23 secretion. Significantly, the enhancement of LPS-induced IL-23 production was still evident when DC were pre-treated with LTK63 for up to 6 h. The findings indicate that LT enhances TLR ligand-induced IL-23 secretion upon first encountering the TLR-ligand, but subsequently suppresses IL-23 production. This might be explained by the fact that unlike IL-12p35, IL-23p19 has no N-glycosylation sites and does not require extensive posttranslational modifications (490). The p40 subunit can be secreted as a homodimer or as a monomer and is expressed in 10- to a 1000-fold excess over p35 (94). Therefore, LT may be capable of inducing IL-23p19 rapidly, due to the excess of p40 in DC, while its suppressive effect on IL-12p70 may be slightly more delayed. This may suggest that the initial inflammatory response to a pathogen is dominated by IL-23 but once the pathogen-induced signal is processed the IL-12-IFNγ axis may become more prominent.

PI3K negatively regulates IL-12 production by DC (171, 334). Martin and colleagues demonstrated that TLR-dependent activation of PI3K suppressed GSK3β, which resulted in the enhancement of IL-10 and inhibition of IL-12 production by DC. In addition, the authors suggested that inhibition of GSK3β allows the transcription factor CREB to effectively compete for the coactivator CBP that NFκB requires for its function, therefore CREB-dependent IL-10 production is favoured over NFκB-mediated IL-12 production (171). Moreover, $PI3K^{-/-}$ DC produce higher concentrations of IL-12 compared with wild-type DC (334). Consistent with studies by Martin *et al.* and Fukao *et al.*, the data presented here showed that inhibition of PI3K enhanced LPS-induced IL-12p70 and IL-12p40 production as well as IL-12p35 and IL-12p40 mRNA expression. In addition, blocking PI3K also enhanced LPS-induced IL-23 secretion and IL-23p19 mRNA expression.

PI3K activation can also occur upon ligand binding to GPCRs (328). The data presented here showed that LT and LTK63 induced the phosphorylation of Akt, a kinase

that is induced by PI3K activation. A study by Kelsall *et al.* showed that the inhibition of IL-12p70 production by the GiPCR C5a was PI3K-dependent (400). Consistent with these observations, the present study demonstrates that inhibition of PI3K reversed the suppressive effect of LT or LTK63 on LPS-induced IL-12p70 and IL-12p40 secretion. Moreover, blocking PI3K suppressed LT- or LTK63- induced the upregulation of CD80 expression on DC. These data have established that PI3K signalling plays an important role in the ability of the toxin to modulate DC activation. GPCR-induced activation of PI3K can be as a result of direct interaction between the G protein βγ subunit or by α subunit-induced tyrosine kinase activity (328). Whether LT mediates the suppression of IL-12 by activating PI3K via Gsα or βγ warrants further investigation.

IL-12 has also been shown to be regulated by MAPK signalling. In particular, a genetic deficiency in MKK3, which is one of the two specific MAPK kinases responsible for p38 phosphorylation, is associated with defective IL-12 production by murine macrophages and DC (319). In addition, the p38 inhibitor, SB203580, suppressed IL-12p70 production by LPS and IFN-γ stimulated BMDC (334). There are conflicting reports on the regulation of IL-12 by ERK. A number of studies have shown that inhibition of ERK enhanced LPS-induced IL-12p70 (189, 190, 325). Moreover, IL-12p70 concentrations were significantly elevated in LPS or Pam-3-Cys activated DC from $ERK1^{-/-}$ mice compared with DC from wild-type mice (325). A report by Goodridge *et al.* showed that ERK negatively regulated IL-12p40 but not IL-12p70 production. Consistent with these reports, the present study established that ERK played some role in the negative regulation of TLR-induced IL-12 production, but demonstrated that ERK activation did not mediate the suppression of IL-12 by LT. In contrast, LT-induced IL-23p19 expression was inhibited by U0126, an inhibitor of MEK1/2 the MAPK kinase upstream of ERK. These data suggest that the two IL-23 subunits are differentially regulated by ERK MAPK. Recent analyses of the IL-23 promoter have revealed, in both macrophages and DC, that p19 expression is dependent on binding of c-Rel and Rel A NFκB to the proximal p19 promoter (439, 440). A study by Petro and colleagues demonstrated that in addition to NFκB, SMAD-3, ATF-2, and in some cases IRF3, are transcription factors required for IL-23p19 expression (441). Interestingly, ATF-2 is activated by phosphorylation through the MAPK pathways and ERK has been shown to be important for Theiler's murine encephalomyelitis virus (TMEV)-induced p19

promoter activity in RAW264.7 cells (491). Together with the findings presented in this study, these observations suggest that IL-23 transcription is tightly regulated and further analysis of how ERK contributes to the activation of these transcription factors may shed light on the balance between IL-12 and IL-23 production.

There is compelling evidence that IL-23 and IL-17 play essential roles in T cell-mediated autoimmunity. In addition, IL-23 and IL-1 which promote the induction or expansion of Th17 cells are implicated in many experimental autoimmune disease models, including EAE (101, 127, 134), collagen-induced arthritis (471) and EAU (430). Moreover, it is now clear that IL-23 and IL-17 are associated with a number of human autoimmune disorders. IL-17 has been detected in blood, brain lesions and CSF of patients with MS, as well as in the synovial fluid of patients with RA (144, 179). IL-23R has been identified as an inflammatory-bowel-disease associated gene and further genetic studies have shown that an IL-23R polymorphism is linked to psoriasis, ankylosing spondylitis and susceptibility to MS (450). Having established that LT-induced IL-23p19 expression was ERK-dependent, this study investigated the role of ERK in mediating LPS- and CpG- induced cytokine production in DC. ERK negatively regulated CpG-induced IL-12p70 and IL-12p40 secretion, but in contrast, ERK was required for CpG-induced IL-23 production. Similarly, LPS-induced IL-23 production was ERK-dependent, but inhibition of ERK had little effect on IL-12p70 secretion or IL-12p35 mRNA expression. This is consistent with a recent report that inhibition of ERK suppressed the induction of IL-23p19 expression in response to TMEV in RAW264.7 cells (441).

Recent studies have established that non-TLR ligands can promote the induction of Th17 cells *in vitro* and *in vivo* (492). *M. tuberculosis* a key ingredient of CFA, the widely used adjuvant for induction of autoimmune disease in mice, was long thought to induce Th1 responses, but it is now established that it is also a potent inducer of Th17 cell responses. Veldhoen and colleagues showed that the ability of *M. tuberculosis* to induce the differentiation of naïve T cells into Th17 cells was associated with its strong capacity to induce IL-23 and IL-6, as well as TGF-β, whereas LPS required exogenous TGF-β (492). Curdlan, a fungal β-glucan and a component of zymosan, induced IL-23 production by DC but also induced TNF-α and IL-6 (453). In contrast to the TLR9 agonist CpG, curdlan-stimulated DC did not produce substantial amounts of IL-12 and

induced a more polarized Th17 response than CpG. Cytokine production by DC in response to curdlan was independent of MyD88 but Syk- and CARD9- dependent. Significantly, curdlan-induced IL-23 was Syk-dependent but MyD88-independent. The results of the present study revealed that blocking ERK inhibited curdlan-induced IL-23 production and IL-23 mRNA expression, indicating that ERK plays a crucial role in TLR- and non-TLR- induced IL-23 production.

IL-1RI$^{-/-}$ mice are resistant to EAE and this is correlated with a failure to induce autoantigen specific IL-17 production (134). The present study revealed that as well as regulating IL-23 secretion, inhibition of ERK suppressed LPS-, curdlan- and *M. tuberculosis*- induced IL-1β production. The best characterized evidence for interplay between the TLR- and NLR- signalling pathways is the interaction between these two pathways for stimulating IL-1β production and secretion. IL-1R and IL-18R signal via MyD88 and studies using MyD88$^{-/-}$ mice have shown that this adaptor is essential for LPS-induced IL-1β, IL-12, IL-6 and TNF-α, but not IL-18 secretion (493). It has now been established that effective production of mature IL-1β and IL-18 depends on the activation of caspase-containing inflammasomes (494, 495). MyD88$^{-/-}$ mice are resistant to EAE and this was associated with a failure to express IL-17 (496). It is significant that PGN and zymosan are within a minority of microbial components that can be substituted for mycobacteria for the induction of EAE. Components of PGN signal through NOD1 and NOD2 and activate NFκB through RIP2. The NLRs, NOD1 and NOD2, in synergy with TLRs can induce pro-IL-1β and pro-IL-18 (52). Together these studies demonstrated that signalling through the IL-1 receptor is crucial for the induction of Th17 cells and suggest that IL-1 plays a critical role in autoimmunity.

The present study found that the incidence and severity of EAE were significantly reduced in mice treated with the MEK1/2 inhibitor during the induction phase of disease. This is consistent with two independent studies showing that inhibition of MEK or ERK attentuated CIA (447, 497). In contrast, it has previously been reported that ERK1$^{-/-}$ mice exhibit increased susceptibility to EAE (325, 484). While these two studies appear to be at variance with the findings presented here, they are not directly comparable. The MEK1/2 inhibitor U0126 inhibits the two isoforms of ERK by acting on the MAPK kinase MEK upstream of ERK1/2, while ERK1$^{-/-}$ mice are only deficient in the ERK1 isoform, and as such a role for ERK2 can not be excluded. Furthermore, in this study, the

MEK1/2 inhibitor was only administered during or after the induction of EAE, whereas the knockout mice lacked ERK1 through the course of their development. It has also been shown that inhibition of ERK enhanced IL-1- and IL-23- mediated IL-17 production by T cells (134). The present investigation revealed that the attenuation of EAE by inhibition of ERK was associated with significantly reduced autoantigen-specific IL-17 production. It is significant that administration of the MEK1/2 inhibitor to mice 5 d after the induction of EAE had a minimal suppressive effect on clinical symptoms of disease, indicating that once Th17 cells are induced, inhibition of innate IL-1 and IL-23 could not modulate the course of disease.

The relapsing-remitting model of EAE more closely resembles the course of disease in MS patients. The findings shown here demonstrate that administration of the MEK1/2 inhibitor after the acute phase of disease resulted in significant attenuation of clinical symptoms. This was associated with suppression of autoantigen-specific IL-17 production. These results demonstrate that inhibition of ERK attenuates the development of acute EAE when administered during the induction phase, and also prevents relapse in the relapsing-remitting model of EAE. The results also demonstrate that ERK positively regulates IL-23 and IL-1β production by DC, and therefore plays a critical role in the polarization of Th17 cells. Co-administration of IL-1β and IL-23 with U0126 at the induction phase of acute MOG-induced EAE reversed the attenuating effect of the MEK1/2 inhibitor and the suppression of autoantigen-specific IL-17 production. This is consistent previous reports which suggested that IL-23 and IL-1 play a critical role in the induction or expansion of Th17 cells. Studies in EAE and EAU models have demonstrated that IL-23p19$^{-/-}$ mice produce less IL-17 and are resistant to induction of autoimmunity (127, 430). Furthermore, treatment with anti-IL-23 reduced IL-17 production and attenuated EAU, when administered immediately before and after induction of disease, but not at the effector stage of the disease (430). Interestingly, a recent report showed that inhibition of c-Fos, a transcription factor downstream of the MEK-ERK cascade, using a small molecular weight inhibitor, prevented and resolved type II CIA, and demonstrated that the primary action of the molecule was the inhibition of MMPs and IL-1β production (498).

Recent evidence suggests that γδ T cells have an inherent ability to produce IL-17 and are often the major source of this cytokine in infections and autoimmune disease

(499). Depletion of a specific γδ T cell subset, Vγ4/Vγ4+, reduced the incidence and severity of disease in the CIA model (476). Furthermore, naïve γδ T cells have been shown to produce IL-17 in response to IL-23 alone (474, 475). Significantly, the present study demonstrated that the frequency of IL-17 producing γδ T cells was reduced in the lymph nodes of mice that were treated with the MEK1/2 inhibitor at the induction of EAE. These findings suggest that inhibition of ERK suppresses IL-23 and IL-1 production by DC, and therefore prevents the early induction of the pathogenic IL-17-producing T cells, a high proportion of which are γδ T cells. It is significant that the induction of EAE was delayed and symptoms were less severe in γδ T cell-deficient mice (477, 481).

In addition to the participation of IL-17 in the induction of organ-specific autoimmune diseases, there is increasing evidence of a role for IL-17 in protection against extracellular pathogens and fungi. In acute inflammation, the major function of IL-17 appears to be to promote the production of chemokines and pro-inflammatory cytokines and consequent activation and recruitment of neutrophils and monocytes (500, 501). IL-17-producing cells induced by vaccination have been shown to have a role in protective cellular immunity to *B. pertussis* and *M. tuberculosis* (502, 503). Vaccination with *M. tuberculosis* with antigens in a mixed adjuvant induced IL-17-producing $CD4^+$ T cells, which promoted chemokine production and the recruitment of Th1 cells after bacterial challenge (503).

The generation of protective immunity by vaccination is dependent on the ability of adjuvants to boost the adaptive immune responses to protein antigens. Recent studies have suggested that a major function of an adjuvant is to boost innate immunity through activation of DC. The present study has demonstrated LT can promote antigen-specific IL-17 production and established that LT can induce IL-23p19 expression and enhance IL-1β secretion, which are essential for the induction of expansion of Th17 cells. These observations suggest that the ability of LT to act as an adjuvant for the induction of protective immunity to bacteria or other pathogens may in part reflect its ability to promote Th17 responses, which are known to recruit neutrophils to the site of infection.

It has been shown that administration of LTB, the isolated B subunit of LT, via a mucosal route prevented the onset of autoimmune arthritis and autoimmune diabetes in mice (504, 505). A more recent study demonstrated that LTB inhibited Th1, but not

Th17, responses in EAU (372). Raveney *et al.* showed that intranasal administration of LTB immediately before induction of EAU prevented the initiation of disease, however, treatment after induction of EAU did not abrogate disease progression, but appeared to enhance Th17 responses (372). Moreover, the authors showed that addition of LTB to a coculture of $CD4^+$ T cells and macrophages inhibited IFN-γ, but enhanced IL-10 production. Despite the ability of LTB to reduce IFN-γ responses there was no effect on infiltration or structural damage in established EAU, where Th17 responses were shown to predominate.

Since Th17 cells have the capacity to protect the host against invading pathogens but at the same time have the potential to induce immunopathology, it is important that these cells are tightly controlled. Currently, the most successful immunotherapies for autoimmune diseases are antibodies or receptor antagonists to IL-1 and TNF-α. TNF-α neutralization therapies are highly effective in patients with rheumatoid arthritis, Crohn's disease, and psoriasis, but there is evidence of increased *M. tuberculosis* infection in patients treated with infliximab (506, 507). While a number of immunotherapies for autoimmune diseases are aimed at blocking specific cytokines this may prevent the immune system mounting an effective response to infection. One of the significant findings of this study is that inhibition of ERK prevented the onset of EAE and relapse of ongoing disease. It is clear that the balance between IL-12 family members can be selectively tipped by activating particular PRRs on DC or targeting specific components of intracellular signalling pathways. Exploiting the capacity of DC to orchestrate the development of particular Th cell responses has considerable potential in the development of vaccines and immunotherapies against infectious diseases. Furthermore, strategic targeting of specific signalling molecules rather than cytokines may be more effective for the treatment of autoimmune and chronic inflammatory disorders.

Chapter 7

Appendix

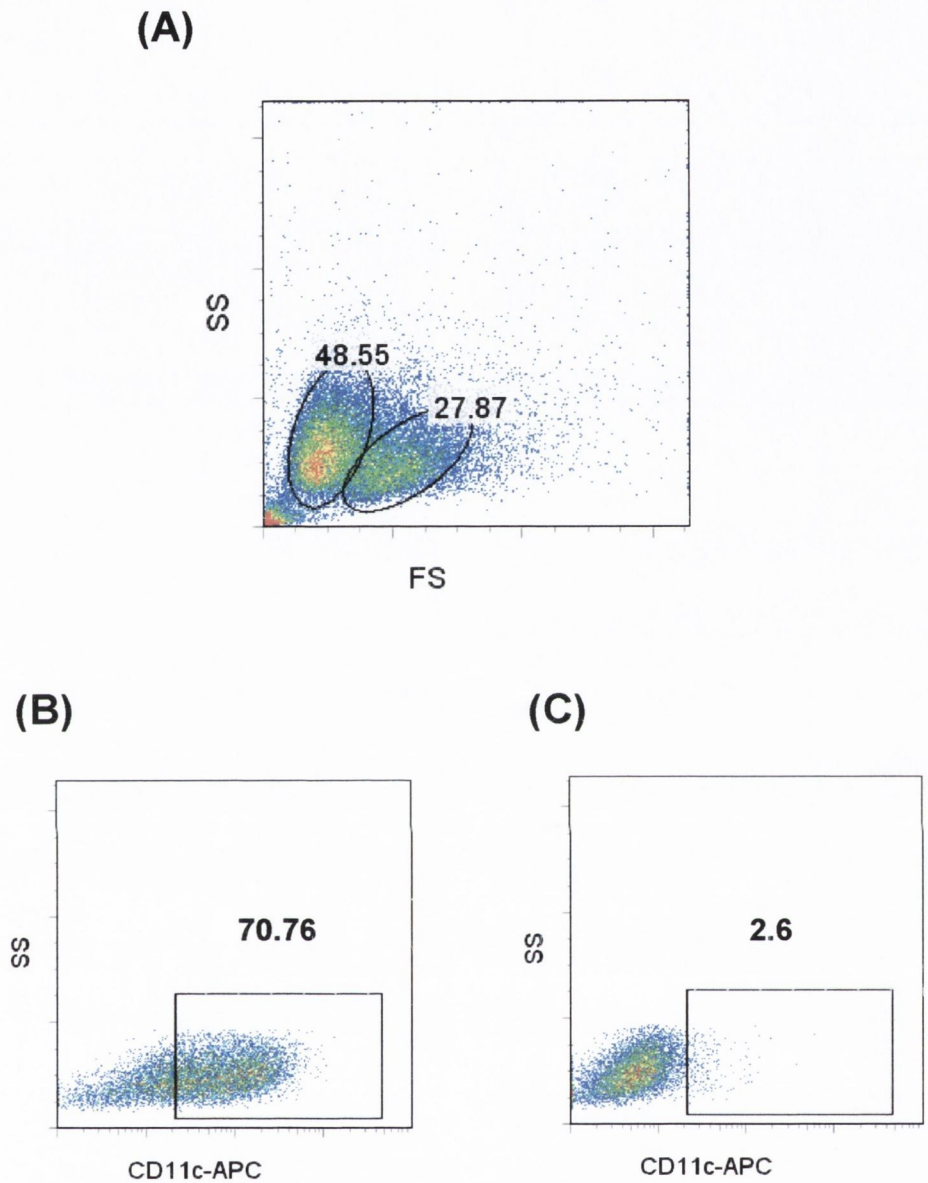

Figure 7.1 Flow cytometric analysis of BMDC after 12 d culture. **(A)** Forward scatter (FS) and side scatter (SS) analysis of unstained BMDC (gate G1 = 27.9 %). **(B)** BMDC (gated on G1) stained with an antibody for CD11c. **(C)** Unstained BMDC (gated on G1). Results were analyzed using FloJo (Stanford University) software and numbers represent the percentage of cells as indicated above.

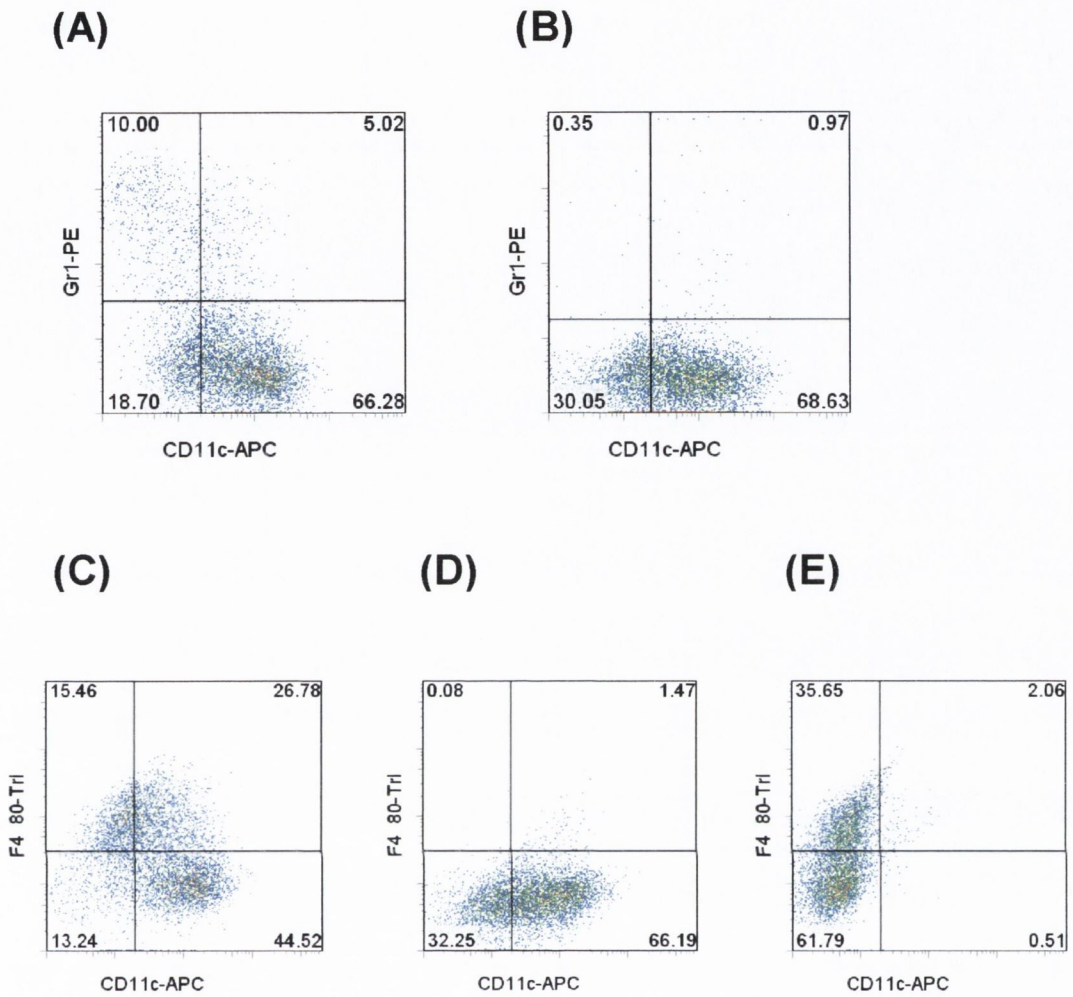

Figure 7.2 Flow cytometric analysis of BMDC. BMDC gated on G1 (see Fig. 7.1), stained with antibodies for CD11c and Gr1 **(A)** or with CD11c only **(B).** BMDC (gated on G1) stained with antibodies for CD11c and F4/80 **(C),** CD11c only **(D)** or F4/80 only **(E).** Results were analyzed using FloJo (Stanford University) software and numbers represent the percentage of cells as indicated above.

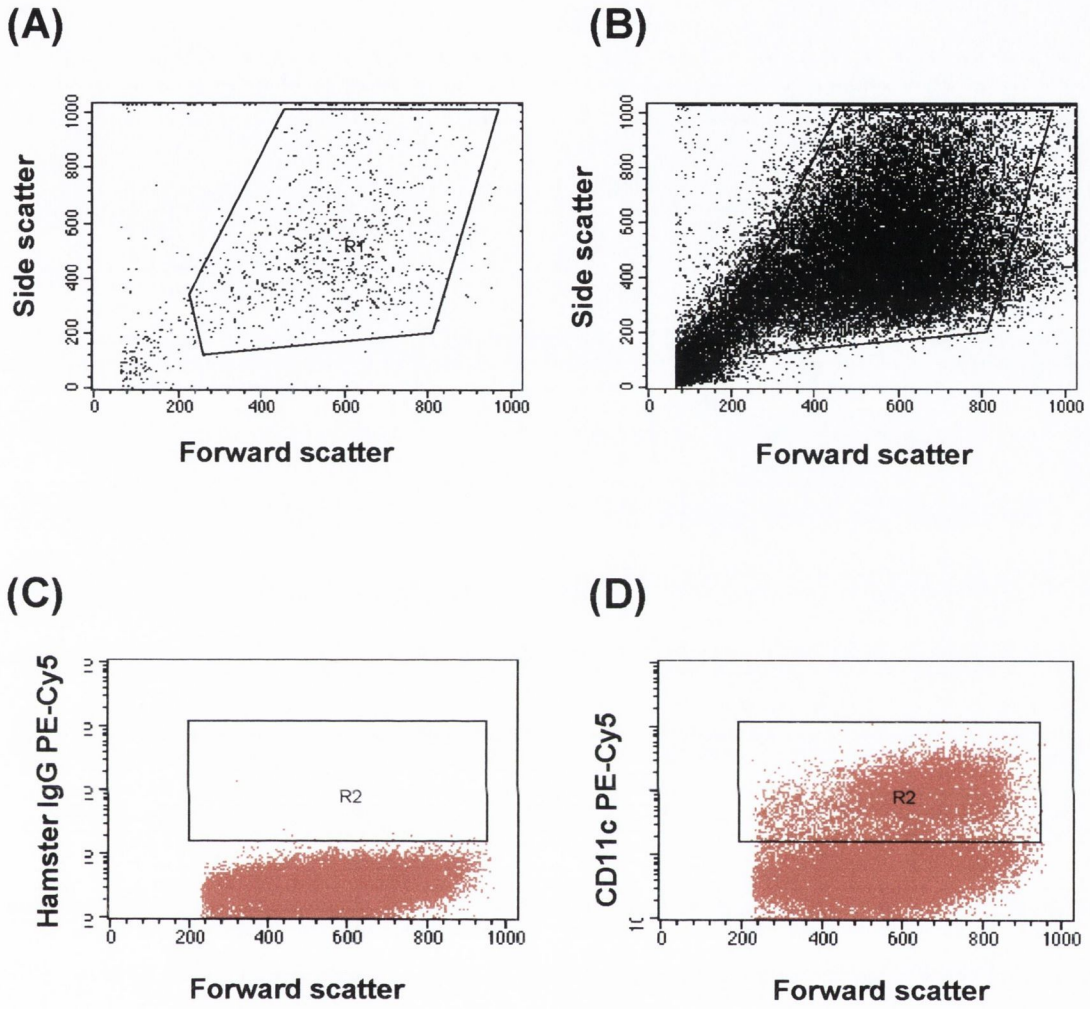

Figure 7.3 Flow cytometric analysis of BMDC. **(A)** FS and SS analysis of unstained BMDC indicating gate R1. **(B)** Larger population of unstained BMDC indicating gate R1. **(C)** BMDC (gated on R1) stained with an antibody for Hamster IgG PE-Cy5, the isotype control for CD11c. **(D)** BMDC (gated on R1) stained with an antibody for CD11c. Results were analyzed using CELLQuest™ software.

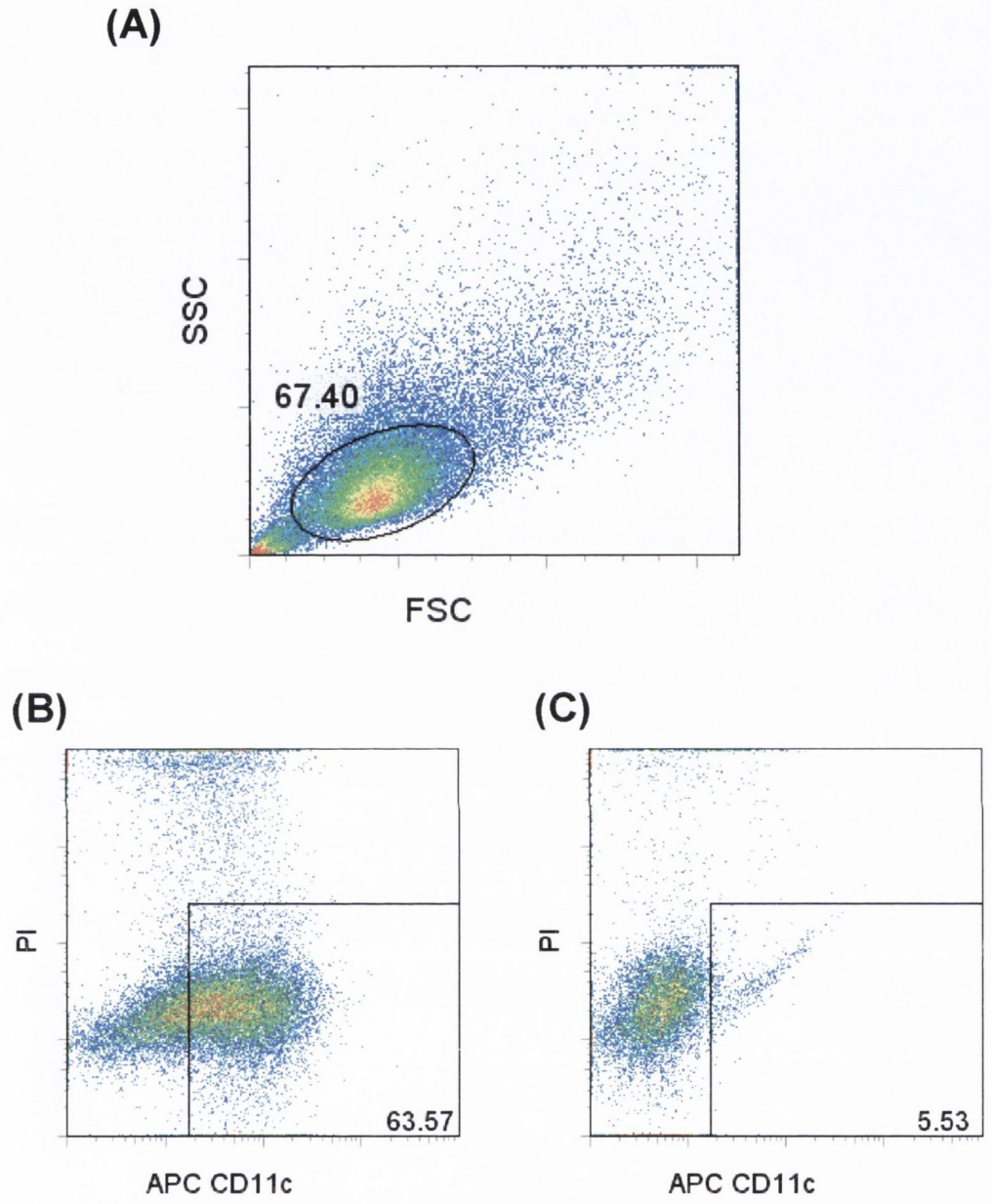

Figure 7.4 Flow cytometric analysis of BMDC. **(A)** Forward scatter (FSC) and side scatter (SSC) analysis of unstained BMDC. **(B)** BMDC (gated on G1 (67.4%)) were stained with an antibody for CD11c and also with propidium iodide, which was added to the cells immediately before acquisition on the FACS machine to assess cell viability. **(C)** BMDC (gated on G1) stained with PI only. Results were analyzed using FloJo (Stanford University) software.

Chapter 8

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