Regulation of cytokines by Tpl-2 in dendritic cells and macrophages

Dorthe Skeel Cook

The National Institute for Medical Research

The Ridgeway

Mill Hill

London

NW7 1AA

Declaration

I Dorthe Skeel Cook, confirm that the work presented in this thesis is my own. Where

information has been derived from other sources, I confirm that this has been in

indicated in this thesis

Abstract

Antigen presenting cells such as macrophages and dendritic cells play an important role between the interface of the innate and adaptive immune responses. One property shared by dendritic cells and macrophages is that upon activation they secrete either pro- or anti-inflammatory cytokines dependent on the pathogen derived product and the T cell derived signal they encounter. The panel of cytokines they produce determines the class of the adaptive immune response.

Tpl-2 was originally described as a proto-oncogene. It was later found to function as a Map-3 kinase leading to phosphorylation of MEK and ERK. Upon stimulation of dendritic cells and macrophages with Toll like receptor ligands (TLR) such as LPS and CpG, Tpl-2 phosphorylates MEK, which in turn phosphorylates ERK, leading to production of cytokines.

In the Tpl-2 knockout mice, it has been shown that TNF production is decreased. We have confirmed this finding and shown that this is both at the transcriptional and post-transcription level. In the complete absence of Tpl-2 we have shown that production of the suppressive cytokine IL-10 is reduced in response to TLR stimulation in macrophages and dendritic cells. On the other hand, when macrophages and dendritic cells are stimulated with LPS or CpG in the absence of Tpl-2, production of IL-12 is increased. IFN-β is also upregulated in absence of Tpl-2. Tpl-2 can regulate IL-12 either directly via IL-10, but also independently of IL-10 via ERK.

IL-10 is an important cytokine in regulating the immune response in order to inhibit immune pathology. We crossed the Tpl-2 knockout with an IL-10 knockout mouse in order to investigate whether Tpl-2 regulates IL-12 and IFN-β in the complete absence of IL-10. These studies are currently in progress. Preliminary results however,

show that the Tpl-2/IL-10 double knockout develop diarrhoea and colitis around 8 weeks of age, leading to minimal weight gain and even weight loss compared to littermates. This is in contrast to the Tpl-2 knockout, which does not develop colitis and the IL-10 knockout, which only develops colitis at 4 months of age. These findings have important implications for treatment of autoimmune diseases with drugs that inhibit ERK, to inhibit TNF mediated pathology.

The aim of this thesis was to investigate to role ERK has in regulating cytokine production in DC and macrophages. We used two different strategies to do this. Firstly we pharmacologically blocked ERK phosphorylation with an inhibitor. Secondly we used genetically modified mice lacking Tpl-2.

Acknowledgements

Abbreviations

APC	Antigen presenting cell
ARE	AU-rich element
CNK	Connector enhancer of KSR
DC	Dendritic cell
EGF	Epidermal growth factor
ERK	Extracellular regulated protein kinase
GPCR	G-protein-coupled receptor
IFN	Interferon
IGF	Insulin-like growth factor
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-23	Interleukin-23
IRF	Interferon regulatory factor
JNK	c-Jun NH2-terminal kinase
KSR	Kinase suppressor of Ras
LBP	LPS binding protein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MEKK1	MEK kinase 1
MHC	Major histocompatibility complex
MP1	MEK-partner 1

MPK	MAP kinase phosphatases
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor- κB
NGF	Nerve growth factor
NK cells	Natural killer cells
NO	Nitric oxide
pDC	Plasmacytoid precursor dendritic cell
PDGF	Platelet-derived growth factor
PRR	Pattern recognition receptor
PTPases	Protein tyrosine phosphatases
SUR-8	Suppressor of RAS-8
TACE	TNF-alpha converting enzyme
TGF-β	Transforming growth factor-β
Th1	T helper 1
TIR	Toll/IL-1 receptor homologous
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
Tpl-2	Tumour progression locus-2
TRADD	Tumour-necrosis-factor-receptor-associated death domain
TRAF	Tumour-necrosis-factor-receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain–containing adapter protein inducing IFN-β

Table of contents

11

14

Chapter 1

Introduction

1.1 Regulation of the immune response

1.1.1   Cytokines in the innate and adaptive immune response.

The immune system can be divided into the innate and the adaptive immune responses. These are characterised by which cell types are activated. The innate responses involve macrophages, dendritic cells (DC), natural killer (NK) cells and neutrophils, and the adaptive responses involve B cells and T cells. The innate immune system is the first line of defence during infections. The adaptive immune response only comes into action at a later stage (Palucka and Banchereau 2002).

The cells of the immune system have very specific roles to play during an immune response. Innate immune cells can recognise pathogens through several surface receptors such as pattern recognition receptors and Toll-like receptors (TLR) (discussed in detail later). These receptors enable the cells to take up the antigen. This process leads to production of cytokines, which will activate more cells including those of the adaptive response. DC are very specialised cells (Palucka and Banchereau 2002; Steinman and Hemmi 2006). They will take up antigen, which they will present to T cells and thereby activate the adaptive immune response (Janeway and Medzhitov 2002).

T cells of the adaptive immune system can be further subdivided into two groups, comprising CD4$^+$ T cells divided into T helper 1 (Th1), T helper 2 (Th2) (O'Garra 1998), CD25$^+$foxp3 regulatory T cells (Treg) (O'Garra and Vieira 2004; Sakaguchi 2005), T helper 17 (Th17) (Veldhoen *et al.* 2006); and CD8$^+$ cytotoxic T

18

cells. The type of T cell that becomes activated depends on the signal received from the DC and on signals from co-stimulatory molecules, and whether the antigen is presented on class I (CD8) or class II (CD4) MHC molecules. Furthermore, DC produce IL-12 upon interaction with a naïve CD4$^+$ T cell, will induce a Th1 response. Under certain conditions and in the absence of IFN-γ, CD4$^+$ T cells will be driven in a Th2 direction, which is dependent on IL-4 (O'Garra 1998). Th17 cells differentiate from CD4$^+$ naïve T cells in response to TGF-β and IL-6 and are expanded by IL-23 (Bettelli *et al.* 2006; Veldhoen *et al.* 2006).

Th1 responses are characterised by production of IFN-γ and are responsible for eradication of intracellular pathogens, but if uncontrolled can cause inflammatory pathologies (O'Garra 1998). Th2 responses are characterised by production of IL-4, IL-5 and IL-13 and are responsible for eradication of nematodes but are also implicated in allergy (Sher and Coffman 1992; O'Garra 1998). Treg (CD25$^+$Foxp3) cells are important in controlling both the innate and adaptive immune system (Sakaguchi 2005). Their suppressive effects can in some cases be mediated by IL-10 and TGF-β, in order to downregulate an inflammatory response (O'Garra and Vieira 2004). It has been shown that Treg can inhibit T cell expansion and function in certain disease models (reviewed in (O'Garra *et al.* 2004). More recently a new T cell subset, Th17 was described. Th17 cells produce the inflammatory cytokine IL-17, which plays a part in autoimmune diseases (Cua *et al.* 2003). The differentiation into Th17 effector cells requires TGF-β production, which paradoxically is normally associated with downregulation of inflammatory immune responses (Veldhoen *et al.* 2006).

The cytokines produced during an immune response are critical in order to achieve complete eradication of the pathogen without causing damage to the host. Two cytokines responsible for downregulating the immune response are IL-10 and TGF-β. IL-10 is produced by a variety of cells including macrophages, DC, T cells and B cells (Moore *et al.* 2001). IL-10 works by inhibiting macrophage and DC function including their production of multiple proinflammatory cytokines such as TNF and IL-12. IL-12 produced by DC can drive a Th1 response, which can be downregulated by various factors such as IL-10 and TGF-β (Moore *et al.* 2001). TGF-β works by inhibiting Th1 and Th2 cell differentiation but also actively drives Th17 differentiation in the context of IL-6. IL-23 then expands committed Th17 effectors (Veldhoen and Stockinger 2006).

IL-10 is a very important cytokine for immune regulation. The effects of IL-10 have been studied in IL-10 deficient mice, which spontaneously develop colitis caused by high levels of TNF and IL-12 and also the Th1 response (Kuhn *et al.* 1993) and the recently described IL-23 (Hue *et al.* 2006; Kullberg *et al.* 2006). However, too much IL-10 can lead to deactivation of macrophages and in an infection study with *Toxoplasma gondii*, it was shown that IL-10 inhibited parasite killing which led to an uncontrolled infection (Gazzinelli *et al.* 1992). However, in the absence of IL-10, infection with *Toxoplasma gondii* led to more effective clearance of the parasite, but the mice died of uncontrolled immunopathology (Gazzinelli *et al.* 1992).

IFN-γ, which is produced by Th1 cells, plays an important part in the immune response to intracellular pathogens (Novelli and Casanova 2004). In the absence of IFN-γ, an individual becomes susceptible to infections with various intracellular microorganisms (Novelli and Casanova 2004). It was recently shown that patients with

defects in IFN-γ receptors were susceptible to infections with mycobacteria (Casanova and Abel 2002).

Regulation of the balance of the immune response is very important, as too much of a Th1 response can cause autoimmune or inflammatory pathology but is required for effective eradication of pathogens. On the other hand, too much of a Th2 response can cause allergy, but is required for eradication of nematodes (Sher and Coffman 1992; O'Garra 1998). Th17 thus far have only been shown to affect the clearance of *Klebsiella pneumoniae* (Happel *et al.* 2005) and if dysregulated cause anti-inflammatory pathologies (Chen *et al.* 2006; Veldhoen and Stockinger 2006). In order to understand this balance, it is important to study the antigen presenting cells particularly macrophages and DC, which are responsible for communication with T cells. The cytokines produced by DC and macrophages may determine the type of immune response elicited to pathogens (Banchereau and Steinman 1998).

1.1.2   *Dendritic cells as initiators of the immune response.*

Dendritic cells (DC) are described as professional antigen presenting cells (APC) and they function at the interface between innate and adaptive immune responses where they take up and process antigen for presentation to T cells (Banchereau and Steinman 1998; Liu *et al.* 2001; Robinson and O'Garra 2002; Shortman and Liu 2002; Kapsenberg 2003; Smits *et al.* 2004).

DC are found in most tissues as immature cells unable to present antigen to T cells. Their role is to capture antigen they encounter. Upon antigen uptake, the DC will

start to undergo maturation. The antigen will be processed in specialised MHC class II-rich compartments, where the antigen is broken down for presentation of peptides on MHC class II molecules on the cell surface to CD4^{+} T cells or MHC class I molecules to CD8^{+} T cells (Banchereau and Steinman 1998). It has been suggested that DC become activated to secrete either pro- or anti-inflammatory cytokines dependent on the pathogen derived product and the T cell derived signal they encounter. Upon activation and maturation, DC start to migrate to the lymph nodes, where processed antigen is presented to T cells through interaction with MHC and costimulatory molecules. DC can drive the immune response in either a T helper 1 (Th1) or Th2 direction depending on a number of factors, including the cytokines produced in their microenvironment (O'Garra 1998).

Different subsets of DC have been identified (Liu *et al.* 2001; Shortman and Liu 2002). These include the myeloid, plasmacytoid and splenic DC. Myeloid DC (CD11c^{+} CD11b^{+}) can be generated directly *in vitro* from murine bone marrow by culturing in medium containing GM-CSF (Inaba *et al.* 1992). Upon appropriate stimulation with LPS or other ligands for pattern recognition receptors expressed on these cells (Medzhitov 2001), myeloid DC produce IL-12, TNF and IL-10 (Boonstra *et al.* 2003). Although mouse DC expressing CD11c^{+}CD11b^{+} can be isolated directly from the spleen, it is still unclear how these or other so-called "steady-state" DC relate to the bone marrow derived myeloid DC (Banchereau and Steinman 1998; Liu *et al.* 2001; Shortman and Liu 2002).

Murine plasmacytoid precursor DC (pDC) (CD11c[dull] B220^{+} GR1^{+}) can be obtained directly from the spleen and lymph nodes (Asselin-Paturel *et al.* 2001). Upon

stimulation with virus, pDC produce large amounts of IFN-α (Asselin-Paturel *et al.* 2001; Bjorck 2001; Nakano *et al.* 2001; Asselin-Paturel *et al.* 2003; Boonstra *et al.* 2003). Plasmacytoid DC (pDC) (CD11c$^+$ CD11b$^-$ B220$^+$) can also be generated from murine bone marrow by culturing with FLT3L instead of GM-CSF (Gilliet *et al.* 2002). FLT3L generated pDC produce large amounts of IL-12, some TNF but no IL-10 in response to stimulation through TLR7 or TLR9 (Boonstra *et al.* 2003).

1.1.3 Macrophages in the immune response.

Macrophages belong to the group of innate immune cells. Their main functions are to detect, ingest by phagocytosis and destroy pathogens. They can also present antigen to T cells (Morrissette *et al.* 1999). Macrophages are highly phagocytic and are capable of ingesting pathogens as well as apoptotic cells. They have several surface receptors involved in innate immune recognition including toll-like receptors (described in detail below), and other pattern recognition receptors (PRR) such as scavenger receptors, which they use to detect pathogens and accelerate them to produce cytokines (Taylor *et al.* 2005).

Activation of macrophages can occur through a number of signals such as ligands for PRR and cytokines. One cytokine signal is IFN-γ produced by NK and T cells, which primes the macrophages for activation. Another cytokine signal is TNF, which is produced by macrophages and DC in response to many pathogens upon PRR and also engagement by T cells (Mosser 2003). Upon activation, macrophages migrate to the site of inflammation, where they start to degrade pathogens. Both resting and

23

activated macrophages have an enhanced ability to phagocytose the pathogen, however, activated macrophages have an enhanced ability to kill and degrade intracellular microorganisms (Mosser 2003).

Macrophages can kill pathogens in many ways. Some of these include phagocytosis, release of antimicrobial peptides and reactive oxygen intermediates, or by activation of the complement system via the alternative pathway (Bogdan *et al.* 2000). One of the main defence mechanisms of macrophages is their ability to produce nitric oxide (NO) in order to kill pahogens. Production of NO is only induced after macrophage activation with IFN-γ produced by Th1 and NK cells. It has been shown that in infection with many intracellular pathogens such as *Leishmania major*, macrophages produce NO to inhibit the replication of the parasite. NO deficient mice, showed non-healing lesions in response to *Leishmania major* infection. (Celada and Nathan 1994; Bogdan *et al.* 2000).

Macrophages are very poor at presenting antigen to T cells, as compared to DC (Morrissette *et al.* 1999). However, they play an important role in innate immunity by killing pathogens. Macrophages can, however, be very damaging to the host if their activation is not controlled due to the production of toxic radicals and inflammatory cytokines. Therefore it is very important that there are mechanisms to downregulate macrophage activation. Such regulation can be provided by other cells or even by the macrophage itself. It has been found that transforming growth factor-β (TGF-β) and IL-10 are important in turning off activated macrophages (Bogdan *et al.* 1991; Fiorentino *et al.* 1991; Fiorentino *et al.* 1991; Gazzinelli *et al.* 1992).

1.1.4 Toll-like receptors: Pattern recognition receptors expressed by DC and macrophages.

Toll like receptors (TLRs) are conserved PRR that recognise patterns on microbes (Martin *et al.* 2003; Takeda *et al.* 2003). TLRs were originally identified in *Drosophila Melanogaster* as an essential receptor for dorso-ventral development in embryos (Hashimoto *et al.* 1988). It was later demonstrated that mutant flies lacking TLRs were highly susceptible to fungal infections (Hoffmann *et al.* 1999). TLRs comprise a family of type I transmembrane receptors characterised by an extracellular leucine-rich repeat domain and an intracellular Toll/IL-1 receptor domain (Medzhitov 2001).

To date, 11 TLR have been identified in mammals. The extracellular domain confers sensitivity for specific ligands. The conserved Toll/IL-1 receptor (TIR) domain couples TLR to intracellular signalling pathways by recruiting cytoplasmic adaptor proteins following ligand stimulation. The specific ligands for each receptor are summarised in Table 1 and have been validated using TLR knockout animals (Hoshino *et al.* 1999; Takeuchi *et al.* 1999; Hemmi *et al.* 2000; Alexopoulou *et al.* 2001; Hayashi *et al.* 2001; Takeuchi *et al.* 2001; Hemmi *et al.* 2002; Takeuchi *et al.* 2002).

Toll-like receptor	Ligand	Origin
TLR1	Triacyl lipopeptides	Bacteria
TLR2	Lipopeptides	Various pathogens
	Peptidoglycan	Gram-positive bacteria
	Zymosan	Fungi
TLR3	Double stranded RNA	Virus
TLR4	**Lipopolysaccharide**	**Gram-negative bacteria**
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides	Mycoplasma
	Zymosan	Fungi
TLR7	Single-stranded RNA	Virus
TLR8	Imidazoquinoline	Synthetic compounds
	Single-stranded RNA	Virus
TLR9	**CpG-containing non-methylated DNA**	**Bacteria** **Virus**
TLR10	Not determined	Not determined
TLR11	Not determined	Uropathogenic bacteria

Table 1. Summary of Toll-like receptors and their major ligands. Modified from (Akira and Takeda 2004). The TLR ligands we are working with are highlighted in bold.

When a TLR specific ligand interacts with its appropriate TLR, a signalling cascade is initiated and several adaptor proteins are recruited to the TIR domain of the TLR. All TLRs signal through MyD88, an adaptor protein, apart from TLR3, which utilises a MyD88 independent pathway through TRIF (discussed in more detail below) (Akira *et al.* 2003). Many of the TLRs also use several other adaptor proteins, and as a result of these signalling cascades, transcription of cytokines is initiated. We are mainly focussed on TLR4 and TLR9 for this thesis, which are described in detail below.

1.1.5 Differential TLR expression by DC and macrophages.

TLRs are expressed in many cells of the immune system. These include; B cells, macrophages and DC. Mouse and human DC subtypes express different TLRs and, therefore, respond to different microbial products (Kadowaki *et al.* 2001; Edwards *et al.* 2002; Boonstra *et al.* 2003). TLR4 (LPS) is expressed by macrophages, mouse myeloid DC, human monocyte-derived DC, and human macrophages but not by pDC (Kadowaki *et al.* 2001; Boonstra *et al.* 2003). TLR9 (CpG) is expressed by both human pDC and human B cells. In the mouse however, pDC, myeloid DC, splenic DC, B cells, and macrophages express TLR9 (Kadowaki *et al.* 2001; Boonstra *et al.* 2003). TLR2 (lipopeptides, peptidoglycans, zymosan) is expressed by macrophages and myeloid DC, whereas TLR7 (single-stranded RNA) is expressed by pDC and some myeloid cells (Diebold *et al.* 2004). The differential expression of TLRs by different immune cells determines whether they respond to particular signals to induce activation including cytokine production.

1.1.6 Adaptors molecules involved in Toll-like receptor signalling.

Upon TLR ligation, signalling cascades are activated through the TIR domain containing adaptors, such as MyD88 and TRIF leading to cytokine production. All TLRs, except TLR3, use MyD88, whereas TLR3 only uses TRIF. TLR4 uses both MyD88 and TRIF and TRAM (Akira and Takeda 2004). Activation through MyD88 or

27

TRIF lead to activation of a cascade of signalling pathways including nuclear factor-κB (NF-κB) and mitogen activated protein (MAP) kinases, which initiate transcription of cytokines and other functional molecules (Akira *et al.* 2003).

1.1.7 Adaptor molecules involved in Toll-like receptor 4 signalling.

TLR4 was first discovered as a receptor for LPS by cloning of the *Lps* gene in the LPS non-responsive C3H/HEJ mouse (Poltorak *et al.* 1998) and this was later confirmed in the TLR4 knockout mouse (Hoshino *et al.* 1999). Recognition of LPS by TLR4 requires several accessory molecules, including LPS-binding protein (LBP), CD14, and MD2 (Medzhitov 2001). LPS is first bound to LBP, which transfers LPS monomers to CD14 (Wright *et al.* 1989). CD14 is a high-affinity LPS receptor that can be expressed on the surface of APCs or secreted into serum. CD14-deficient mice show a defect in responsiveness to LPS (Wright *et al.* 1990; Haziot *et al.* 1996). MD2 is a small protein that lacks a transmembrane region and is expressed at the cell surface together with TLR4 (Shimazu *et al.* 1999). Its precise function is presently unknown.

TLR4 is one of the most complex TLR as it utilises a MyD88 dependent and a MyD88 independent signalling pathway (Fitzgerald *et al.* 2003). TLR4 mainly recognises lipopolysaccharide (LPS) from gram-negative bacteria. When TLR4 is triggered with LPS, MyD88 and TIRAP (TIR domain-containing adaptor protein) are recruited to the Toll/IL-1 receptor (TIR) domain of TLR4. This leads to activation of nuclear factor-κB (NF-κB) and MAP kinases through interaction of IRAK and TRAF6, which leads to transcription of cytokine genes (Li and Verma 2002). The MyD88

independent pathway uses adaptors TRAM (TRIF-related adaptor molecule) and TRIF (TIR domain-containg adaptor inducing interferon β) upon ligation of TLR4, which also trigger NF-κB, MAP kinase activation and transcription of cytokines. Interferon regulatory factor 3 (IRF3) is also activated through TRAM and TRIF, leading to type 1 interferon (IFN) transcription (Figure 1.1.7) (Akira *et al.* 2003; Takeda *et al.* 2003). Recent reports showed that protein kinase PKR works downstream of TRIF leading to induction of apoptosis in response to certain pathogens that activate TLR4 (Hsu *et al.* 2004). PKR also promotes IFN-α production in a TLR-independent manner (Diebold *et al.* 2003).

1.1.8 Adaptor molecules involved in Toll-like receptor 9 signalling.

TLR9 is an intracellular TLR which is located on endocytic vesicles below the plasma membrane (Takeshita *et al.* 2004). TLR9 was first identified as the receptor for CpG using the TLR9 knockout mouse (Hemmi *et al.* 2000). CpG is a non-methylated DNA motif that is expressed by bacteria, fungi and viruses, but not in mammals (Krieg *et al.* 1995). CpG can be divided into 3 distinct subtypes; CpG-A, B and C, each with its own function (Krieg 2002; Guiducci *et al.* 2006). CpG-A are capable of inducing high amounts of IFN-α in pDC (Honda *et al.* 2005) but are poor at inducing differentiation of these cells and induction of costimulators, which lead to B cell activation (Guiducci *et al.* 2006). CpG-B induces expression of costimulators on pDC but lower amounts of IFN-α (Guiducci *et al.* 2006). CpG-C has recently been described as being able to induce

both high levels of IFN-α and costimulators (Guiducci *et al.* 2006). In our lab we use CpG-B.

When a cell is stimulated with CpG both the TLR9 and the CpG is transported to the endosome, where the signalling cascade is initiated (Takeshita *et al.* 2004). The signalling cascade uses adaptor protein MyD88 and leads to activation of NF-κB, MAP kinases and transcription of inflammatory cytokines (Takeda and Akira 2004). MyD88-deficient mice have been shown to be unresponsive to CpG, showing that MyD88 is essential for CpG mediated signalling (Kawai *et al.* 1999). To date, no other adaptors for TLR9 have been identified (Figure 1.1.8).

1.1.9 Production of proinflammatory and anti-inflammatory cytokines by macrophages and DC through TLR stimulation.

Upon stimulation of macrophages and DC through TLRs, cytokine production is induced. This includes the production of proinflammatory cytokines IL-12, TNF and interferons. IL-10 can also be induced in both macrophages and myeloid DC but not plasmacytoid DC upon TLR stimulation (Boonstra *et al.* 2006). It has been suggested that the IL-10 production observed in the macrophages and myeloid DC is preferentially induced by stimulation through TLR2 and dectin-1 (Dillon *et al.* 2006). This report showed that upon stimulation of DC with zymosan, which is recognised by both TLR2 and C-type lectin, production of IL-10 was enhanced and production of proinflammatory cytokines was reduced compared to stimulation with LPS (TLR4). Dillon *et al.* (2006) conclude that the IL-10 production upon TLR2 and Dectin-1 stimulation is regulated by

30

ERK activation. In another report, it was shown that both macrophages and myeloid DC produced IL-12 and IL-10 in response to TLR stimulation through both the MyD88 dependent or the MyD88 independent pathway, whereas plasmacytoid DC did not produce any IL-10 (Boonstra *et al.* 2006). This indicates that IL-10 can be produced by a range of stimuli including TLR ligands and not just through TLR2 and Dectin-1, but its production may be restricted to certain cell types.

1.2 Cytokines produced by the innate and adaptive immune response

1.2.1 Interleukin 10 (IL-10).

IL-10 plays an important role in the control of inflammation by directly inhibiting DC and macrophage function (Bogdan *et al.* 1991; Fiorentino *et al.* 1991; Fiorentino *et al.* 1991; Moore *et al.* 2001). IL-10 is produced by a variety of cell types, including activated macrophages, DC, B cells, and mast cells. IL-10 is also secreted by T cells, in particular by the effector subset of Th2 cells and under some conditions by Th1 and CD8$^+$ T cells, and by some types of regulatory T cells (O'Garra and Robinson 2004; O'Garra and Vieira 2004; O'Garra *et al.* 2004). As discussed earlier, absence of IL-10 leads to spontaneous development of inflammatory bowel disease (IBD) (Kuhn *et al.* 1993), showing the importance of IL-10 in regulating immune responses.

IL-10 has been shown to inhibit production of several proinflammatory cytokines, including IL-1, IL-6, IL-12 and TNF. It also inhibits production of many chemokines implicated in the recruitment of monocytes, DC, neutrophils and T cells.

The inhibition of these cytokines and chemokines show how IL-10 plays an important role in downregulating inflammatory immune responses (Moore *et al.* 2001). Studies carried out in IL-10 deficient mice show increased production of IL-12 and TNF (Yi *et al.* 2002), and in infections with lethal doses of *Listeria monocytogenes* where the IL-10 receptor was blocked with anti-IL-10R monoclonal antibodies, treated mice survived and also had a lower bacterial burden as compared with untreated mice (Silva *et al.* 2001). In another infection with *Toxoplasma gondii* of IL-10 deficient mice, it was found that these mice compared with littermate controls all died. This was not due to uncontrolled infection as parasite numbers were similar in both mice strains, however, an increase in IL-12, TNF and IFN-γ was observed. By depleting CD4 T cells, IFN-γ levels fell and the mice survived (Gazzinelli *et al.* 1996). This again confirms the critical role of IL-10 in regulating inflammatory responses. For this reason the understanding of how IL-10 and proinflammatory cytokines are regulated is of great importance.

It has been shown that in absence of MAP kinase ERK activation in splenocytes and a macrophage cell line, IL-10 production is decreased, whereas, IL-12 production is increased possibly by the action of reducing IL-10. This indicates yet another level of IL-10 regulation of inflammatory cytokines (discussed in detail later).

The molecular mechanisms that regulate expression of the *Il-10* gene are poorly understood. Recent studies have described changes in the chromatin structure at the *Il-10* locus that relate to the control of *Il-10* gene expression in cells that produce this cytokine (Ansel *et al.* 2003; Jones and Flavell 2005; Saraiva *et al.* 2005; Wang *et al.* 2005). Transcription factor NF-κB have been shown to regulate IL-10 induction in macrophages, as mice deficient in IKK developed severe atherosclerosis due to low

levels of IL-10 when compared to wild type mice (Kanters *et al.* 2003). A specific chromatin site has been identified in the IL-10 locus. This site was only found in macrophages but not in T cells (Saraiva *et al.* 2005). This shows that NF-κB plays an important part in regulating IL-10 induction and production in antigen presenting cells. However, a master regulator that initiates expression of the *Il-10* gene, by inducing remodelling of the *Il-10* locus to allow access and function of ubiquitous enhancing or silencing transcription factors, has not yet been identified for macrophages and DC, although GATA3 plays an important role in T cells inducing chromatin remodelling of the *Il-10* locus (Saraiva *et al.* 2005). At the transcriptional level, Sp1 (Brightbill *et al.* 2000), Sp3 (Tone *et al.* 2000), CCAAT/enhancer-binding protein β (Liu *et al.* 2003), IFN regulatory factor-1, and STAT3 (Ziegler-Heitbrock *et al.* 2003) transactivate various constructs of the IL-10 promoter in reporter assays, in both mouse and human cell lines. Smad-4 (Kitani *et al.* 2003) and Jun proteins (Wang *et al.* 2005) have been shown to regulate IL-10 expression in primary Th1 and Th2 cells, respectively. Finally Ets-1 has been suggested to play a role in repressing the production of IL-10 in Th1 cells, as Ets-1-deficient mice show an increase in the production of IL-10 (Grenningloh *et al.* 2005).

1.2.2 Interleukin 12 (IL-12).

IL-12 is an inflammatory cytokine that is produced by DC and macrophages in response to TLR triggering. It induces Th1 differentiation and IFN-γ production (Trinchieri 2003). IL-12 belongs to a family of structurally similar cytokines, IL-12, IL-

23 and IL-27. IL-12 is a heterodimer composed of a 35kD light chain, p35, and a 40kD heavy chain, p40, forming the bioactive IL-12p70 (Kobayashi *et al.* 1989). IL-23 is composed of the p40 chain and a p19 chain. In contrast IL-27 is composed of a p28 chain and EBI3 or p40 (Figure 1.2.2) (Trinchieri 2003).

IL-12p40 can be secreted as a homodimer or monomer, since it is produced in excess over the IL-12p70 and IL-23 heterodimers. IL-12p40 can bind to the IL-12 receptor at lower affinity than IL-12p70 or IL-23, but its function is not yet clear (Trinchieri 2003). IL-12p70 is produced in high amounts by DC upon TLR ligation. This leads to activation of Th1 cells to produce IFN-γ. IL-12 also enhances the phagocytic ability of phagocytes and their production of inflammatory cytokines such as IL-6, TNF and IL-12 (Ma and Trinchieri 2001). IFN-γ enhances the production of IL-12p70 to form a positive feedback loop during inflammatory and Th1 responses (Ma *et al.* 1996). The T cell enhancement of IL-12p70 production also takes place through direct cell-cell interactions, primarily through ligands of the TNF family such as CD40L (Schulz *et al.* 2000).

IL-12p40 can be secreted as a homodimer or monomer, since it is produced in excess over the IL-12p70 and IL-23 heterodimers. IL-12p40 can bind to the IL-12 receptor at lower affinity than IL-12p70 or IL-23, but its function is not yet clear (Trinchieri 2003). IL-12p70 is produced in high amounts by DC upon TLR ligation. This leads to activation of Th1 cells to produce IFN-γ. IL-12 also enhances the phagocytic ability of phagocytes and their production of inflammatory cytokines such as IL-6, TNF and IL-12 (Ma and Trinchieri 2001). IFN-γ enhances the production of IL-12p70 to form a positive feedback loop during inflammatory and Th1 responses (Ma *et al.* 1996). The

T cell enhancement of IL-12p70 production also takes place through direct cell-cell interactions, primarily through ligands of the TNF family such as CD40L (Schulz *et al.* 2000).

IL-12p70 is a very potent cytokine to induce Th1 differentiation which is important for the eradication of pathogens. However, the tight regulation of IL-12p70 is critical as too much IL-12 can lead to uncontrolled Th1 differentiation, which can cause pathology (O'Garra 1998). Studies in IL-10 deficient splenocytes show that IL-12 production is elevated (Yi *et al.* 2002), indicating the importance of IL-10 in downregulating IL-12 production. Consistently, IL-10 has been found to block the transcription of both the p40 and p35 genes (Zhou *et al.* 2004).

The role of IL-23 is important in the establishment and maintenance of organ-specific inflammatory autoimmune diseases (Cua *et al.* 2003; Murphy *et al.* 2003). IL-23 deficient mice show normal immune responses to intracellular pathogens, whereas IL-12p35 deficient mice cannot mount a Th1 immune response but are highly susceptible to autoimmunity (Langrish *et al.* 2005). One report showed that collagen-induced arthritis is blocked in IL-23 knockout mice, whereas IL-12 deficient mice showed enhanced pathology (Murphy *et al.* 2003). Another report shows that the role of IL-12 and IL-23 are different in induced inflammatory bowel disease (IBD). In CD40-induced colitis IL-23 was responsible for induction of inflammation but not wasting disease or enhanced proinflammatory serum cytokine production, whereas IL-12 was responsible for systemic inflammation (Uhlig *et al.* 2006). Taken together this indicates that IL-23 has an important role in autoimmune responses.

1.2.3 Tumour necrosis factor (TNF).

TNF is an inflammatory cytokine, which was first identified about 30 years ago, as a cytokine produced by the activated immune system, which was able to exert cytotoxicity on tumour cell lines and cause tumour necrosis in animal models (Wajant *et al.* 2003). TNF is primarily produced as a type II transmembrane protein arranged in stable homotrimers and the soluble form is released by proteolytic cleavage by the metalloprotease TNF alpha converting enzyme (TACE) (Black *et al.* 1997). TACE belongs to a family of mammalian metalloproteases called adamlysins (ADAMs) (Soond *et al.* 2005). TACE cleaves the membrane bound TNF in order to release the active soluble form (Wajant *et al.* 2003). It has been shown that ERK can phosphorylate TACE at threonine 735 in LPS stimulated macrophages leading to activation and cleavage of TNF, showing that TACE is a physiological substrate for ERK1/2 in LPS stimulated macrophages (Soond *et al.* 2005; Rousseau *et al.* 2008). Rousseau *et al* (2008), further suggested that phosphorylation of TACE at threonine 735 may be a prerequisite for the LPS stimulated cell surface expression and cleavage of pre-TNF. However, they also showed that inhibition of ERK did not affect the transport of pre-TNFα from the Golgi to the plasma membrane and thus did not affect levels of membrane bound TNF. However, levels of soluble TNF were reduced upon inhibition of ERK showing that Tpl-2/ERK1/2 is crucial for processing of pre-TNFα to the secreted form of TNF.

Once TNF has been cleaved it exerts its effects by binding to either a 55kDa TNF receptor (TNFR1) or a 75kDa TNF receptor (TNFR2). The TNFR family proteins are type I transmembrane proteins containing conserved cysteine-rich domains. Binding

of TNF to its receptor results in receptor trimerisation and initiation of signal transduction (Arron *et al.* 2002; Wajant *et al.* 2003). Signal transduction can occur through protein binding to the TNFR intracellular extension, which contains a death domain, but also through a family of adapter proteins called TNFR-associated factor (TRAF) (Vandenabeele *et al.* 1995; Arron *et al.* 2002). The death domain-containing proteins shown to bind to the TNFR include TNFR-associated death domain (TRADD), which can bind the additional signal transducer TRAF (Yuasa *et al.* 1998). TRAF proteins consist of a family of six proteins, which can interact directly or indirectly with members of the TNFR superfamily. TRAF proteins mediate the assembly of cytoplasmic signalling complexes at the TNFR. TRAF can activate the signalling pathway leading to the activation of NF-κB and AP-1 (Arron *et al.* 2002; Beinke and Ley 2004). TRAF proteins can also activate members of the mitogen-activated protein kinase (MAPK) family (Raingeaud *et al.* 1995).

TNF uses TNFR1 and TNFR2 for signal transduction. TNFR1 contains a death-domain and does not bind directly to TRAF proteins, whereas TNFR2 does not have a death-domain, but can interact directly with TRAF proteins (Arron *et al.* 2002). TNFR1 recruits TRADD to its receptor complex. This can either lead to recruitment of FADD, which activates the caspase cascade leading to apoptosis, or TRAF2 can be recruited initiating the activation of NF-κB and AP-1. TNFR2 can bind directly to TRAF2 activating survival pathways (Arron *et al.* 2002).

AU-rich elements (ARE) found in the 3'-untranslated region of transcripts encoding cytokines, growth factors, oncogenes and transcription factors have been shown to regulate mRNA stability and translation (Kontoyiannis *et al.* 1999). Bioactive

TNF has many actions and therefore the biosynthesis is under the control of several factors. It has been indicated that there are additional translational controls in the 3'-untranslated region of *Tnf* mRNA, as experiments using reporter constructs transfected into cell lines show no activity in cells that lack of the 3'-untranslated region of *Tnf* mRNA (Han *et al.* 1990). In another study, it was found that LPS induced JNK plays a role in the translational activation of *tnf* mRNA as a kinase dead mutant of JNK blocked the activation of the translational blockade imposed by the 3'-untranslated region of *Tnf* mRNA (Swantek *et al.* 1997). TNF transcription has also been found to be controlled by several transcription factors including NF-κB (de Winther *et al.* 2005) and NF-AT (Collart *et al.* 1990; Tsytsykova *et al.* 2007).

In a study on TNF ARE it was found that mice were lacking TNF ARE (TNF$^{\Delta ARE}$) had elevated levels of TNF in their sera as compared to the wildtype control (Kontoyiannis *et al.* 1999). Upon LPS administration the level of TNF rose 3-fold as compared to controls. Macrophages from TNF$^{\Delta ARE}$ mice also showed elevated production of TNF in the absence of stimulation. These results were also confirmed at the mRNA level. This group also found that the TNF$^{\Delta ARE}$ mice showed reduced weight gain and increased mortality within 5-12 weeks of age as a consequence of increased TNF in the serum. Kontoyannis *et al.* (1999) conclude that in the absence of stimulation ARE acts as determinants of *Tnf* mRNA instability, but upon LPS stimulation the ARE mediate posttranscriptional enhancement of *Tnf* mRNA accumulation.

Another study showed that mice deficient in Tpl-2/ERK showed normal *Tnf* mRNA induction but TNF protein was dramatically reduced in response to stimulation with LPS, suggesting that ERK regulates TNF at the post-transcriptional level. These

Tpl-2 deficient mice were found to be resistant to LPS induced septic shock, due to defective TNF protein production (Dumitru *et al.* 2000).

However, in the study by Rousseau *et al.* (2008), it was shown that *Tnf* mRNA induction was reduced although not completely inhibited in macrophages from Tpl-2 deficient mice upon LPS stimulation. This reduction in *Tnf* mRNA expression was shown to be time dependent as by 6 hours of stimulation the *Tnf* mRNA levels in the Tpl-2 deficient mouse were the same as compared to the wildtype control. All these studies indicate that TNF regulation is complex as the Tpl-2/ERK pathway is important in controlling the maturation of pre-TNF to TNF, but also plays a role in the production of pre-TNF at the level of transcription/translation.

TNF is produced by many immune cells but high levels are induced in DC and macrophages in response to pathogens. In macrophages the action of TNF has been shown to upregulated pathways such as iNos and ROI to affect the killing of intracellular pathogen (Taylor *et al.* 2005). On the other hand, high concentrations of TNF are critical in the development of septic shock, whereas, prolonged exposure to low concentrations of TNF can cause wasting disease and many inflammatory disorders (Vassalli 1992; Feldmann *et al.* 2005) and therefore it is important that the TNF response is regulated (Wajant *et al.* 2003). Consistent with its strong proinflammatory and immunostimulatory properties, TNF has been shown to be an important mediator of autoimmune diseases such as rheumatoid arthritis and Crohn's disease, which can be effectively treated with anti-TNF antibodies (Choy and Panayi 2001; Feldmann *et al.* 2005). In keeping with its role in the host defense TNF has been shown to play an important role in the defence against many intracellular pathogens such as

Mycobacterium tuberculosis, *Listeria monocytogenes*, and *Leishmania major* in mouse models of disease (Flynn *et al.* 1995; Vieira *et al.* 1996; Mocci *et al.* 1997). Indeed treatment of rheumatoid arthritis or Crohn's disease with anti-TNF antibodies has been shown to lead to reactivation of latent TB (Keane 2005).

1.2.4 Interferons.

IFN is divided into many distinct types. Type I IFN include IFN-α and IFN-β and are produced in response to viral and intracellular infections by DC and macrophages. Type II IFN is composed of IFN-γ, which is structurally unrelated to Type I IFN. IFN-γ is produced mainly by Th1 cells, NK cells and CD8$^+$ T cells. Type III IFN genes are a relatively newly described family and are induced in virally infected cells (Honda *et al.* 2006).

Type I IFN was described nearly 50 years ago, as a soluble factor produced by virally infected cells (reviewed in (Stetson and Medzhitov 2006). It has later been shown that pDC are mainly responsible for the production of high levels of type I IFN, particularly IFN-α, during the early stages of a viral infection (Asselin-Paturel and Trinchieri 2005). The early production of type I IFN can inhibit production of IL-12 (Dalod *et al.* 2002). However, the effects of type I IFN on IL-12 production are complex, as high levels of IFN blocks IL-12 production by decreasing the transcription of the IL-12p40 chain, whereas low levels of IFN are required for transcription of the IL-12p35 chain (Gautier *et al.* 2005).

Type I IFN production is primarily controlled at the transcriptional level where a family of transcription factors, Interferon Regulatory Factors (IRF), play an important role. IRF can be divided into several family members named IRF1 to IRF9 (Mamane *et al.* 1999). IRF3 and IRF7 are highly homologous and are the key players in IFN type I gene expression. IRF7 forms homodimers or heterodimers with IRF3 and each of these dimers act differentially on type I IFN family members. IRF3 activates IFN-β more potently than IFN-α, whereas IRF7 activates both IFN-α and IFN-β (Honda *et al.* 2006).

Two pathways for type IFN I induction have been identified, triggered respectively by cytosolic pattern recognition receptors (RIG-I and MDA5) and TLRs (Stetson and Medzhitov 2006). All TLRs except TLR3 activate MyD88 which can induce type I IFN production in response to TLR ligands, for example, pDC induce high levels of type I IFN through TLR7 and TLR9. MyD88 interacts directly with IRF7 but not IRF3 (Honda *et al.* 2004; Kawai *et al.* 2004). TLR3 and TLR4 use the adaptor TRIF, which interacts directly with IRF3. TLR4 is the only receptor to use both MyD88 and TRIF upon LPS ligation leading to IFN-β production but not IFN-α in macrophages and myeloid DC (Honda *et al.* 2006).

Recently, it was shown that MyD88 recruits TRAF3 and TRAF6 to induce type I IFN (Hacker *et al.* 2006). It was shown that TRAF3 was essential for type I IFN and IL-10 induction, whereas TRAF6 was essential for other proinflammatory cytokine production. It was shown that TRAF3 deficient myeloid cells stimulated with LPS (TLR4) or CpG (TLR9) synthesised high levels of IL-12, but no IL-10 or type I IFN. Poly(I:C) (TLR3) also did not induce type I IFN in TRAF3 deficient cells. TRAF6

deficient cells stimulated with CpG lead to reduced IL-12 and IL-10 but no type I IFN production (Hacker *et al.* 2006).

1.3 Signalling pathways in dendritic cells and macrophages

1.3.1 Nuclear factor-κB transcription factors.

Upon TLR stimulation, several signalling pathways are activated. These include the NF-κB and MAP kinase pathways. Nuclear factor-κB (NF-κB) transcription factors play a critical role in regulating innate and adaptive immune responses (Li and Verma 2002). NF-κB dimers are held in the cytoplasm by NF-κB inhibitory (IκB) proteins. Upon agonist stimulation, IκBs are degraded, leading to NF-κB dimer release, entry to the nucleus and their binding to NF-κB sites in promoters of target genes and modulation of gene expression.

The NF-κB transcription factor family compromises five members, RelA (p65), RelB, c-REL, NF-κB1 (p50) and NF-κB2 (p52) (Li and Verma 2002). Each of these has a conserved Rel-homology domain at the N-terminus, which contains the nuclear localisation signal and DNA binding domains. NFκB1 and NFκB2 are synthesized as large precursors of 105kDa (p105) and 100kDa (p100), respectively. These are partially proteolysed by the proteasome (termed processing), which removes their C-terminal halves, to produce the active NF-κB1 p50 and NF-κB2 p52 subunits (Beinke and Ley 2004). The IκB family includes IκBα, IκBβ, and IκBε (Li and Verma 2002). NF-κB1 precursor, p105, and NF-κB2 precursor, p100, also function as IκBs containing IκB-like

C-terminal halves, which retain REL proteins in the cytoplasm (Belich *et al.* 1999; Pomerantz and Baltimore 2002).

TLR stimulation activates the IKK complex (IKK1, IKK2 and NEMO). This leads to phosphorylation, ubiquitination and proteasome-mediated proteolysis of the IκB family by members of the IκB kinase (IKK) family. NF-κB subunits are released and enter the nucleus to transcribe cytokine genes.

There are three signalling pathways, by which NF-κB is activated. These are the canonical, p105 and the alternative p100 pathways. In the canonical pathway, the NF-κB dimer p65/p50 is held in the cytoplasm by IκBα (Pomerantz and Baltimore 2002). In the p105 pathway, p50 dimers are held by p105. In the alternative pathway, p100 retains RelB in the cytoplasm. (Beinke and Ley 2004). Upon stimulation of these pathways, IKK1 or IKK2 phosphorylate the relevant IκB, p105 or p100, leading to proteolysis by the proteasome and release of active dimers. These pathways are activated in response to different stimuli.

1.3.2 MAP Kinases.

MAP kinases are evolutionarily conserved in eukaryotes and play an important role in cell differentiation, cell movement, cell division, and cell death (Schaeffer and Weber 1999). MAP kinases can be divided into three main groups: the extracellular signal-regulated protein kinases 1/2 (ERK), the p38α/β/γ/δ MAP kinase, and the c-Jun NH$_2$-terminal kinases 1/2/3 (JNK) (Chang and Karin 2001; Symons *et al.* 2006). MAP kinases are activated by phosphorylation on conserved tyrosine and threonine residues in

the activation loop by dual-specificity MAP kinase kinases (MAP2-Ks), which are in turn, phosphorylated on conserved serine and threonine residues by MAP kinase kinase kinases (MAP3-Ks) (Marshall 1994). ERK1/2 MAP kinases are activated by MEK1/2 (MAP2-K). JNK1/2/3 are activated by MKK4 and MKK7. p38 is activated by MKK3 and MKK6. These MAP-2K are themselves activated by several different MAP3-K (Dong *et al.* 2002; Symons *et al.* 2006).

1.4 Extracellular signal-regulated protein kinase (ERK)

1.4.1 ERK activation control by Raf.

Raf is a serine/threonine kinase, which is activated by a series of events including recruitment to the plasma membrane mediated by Ras-GTP, dimerisation of Raf proteins, phosphorylation and association with scaffold complexes such as KSR downstream of antigen and mitogen receptors in lymphocytes (McCubrey *et al.* 2007). Ras is a small GTP-binding protein, which is the common upstream molecule of several signalling pathways including Raf; MEK; ERK pathway, PI3K and AKT (Yoon and Seger 2006; McCubrey *et al.* 2007)

Raf specifically activates MEK but not other MAP2-K. The interaction of MEK with Raf is dependent on a proline-rich sequence only found in MEK and not other MAP2-K. Deletion of this proline-rich sequence has been shown to diminish the ability of Raf to activate MEK (Schaeffer and Weber 1999). Raf can be subdivided into three groups, A-Raf, which preferentially activates MEK1, c-Raf-1, which activates both

MEK1 and MEK2 equally well and B-Raf, which binds to both MEK1 and MEK2 but activates MEK1 better than MEK2 (Schaeffer and Weber 1999).

The Ras/Raf/MEK/ERK pathway (Figure 1.4.1) can be activated by a wide range of stimuli including epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), nerve growth factor (NGF) and phorbol esters. This wide range of stimuli all activate the same MAP kinase pathway but with different end results (Marshall 1995). This leads to the question of how a specific signal induces a specific response when activating a signaling pathway shared by many receptors.

1.4.2 Scaffold proteins.

Scaffold proteins have been shown to interact with components of MAP kinase pathways and found to play an important part in regulating MAP kinase signalling by creating a functional signaling molecule and to maintain the specificity of the signal transduction (Morrison and Davis 2003; Sacks 2006). Several functions have been proposed for scaffold proteins. 1). Binding to the scaffold may be necessary for kinase activation, 2). Scaffold proteins may allow for faster signalling by inhibition of phosphatases, 3). Scaffold proteins may recruit other kinases to the receptor, 4). Scaffold proteins can control protein interaction and 5). Scaffold proteins can control subcellular location (Sacks 2006).

Several scaffold proteins have been shown to play a role in the ERK signalling pathway. These include; KSR (Kinase suppressor of Ras), MP1 (MEK-partner 1), β-

Arrestin-1 and –2, MEKK1 (MEK kinase 1), CNK (connector enhancer of KSR), SUR-8 (suppressor of Ras-8) and IQGAP1 (Morrison and Davis 2003; Sacks 2006). Evidence suggests that KSR binds directly to c-Raf-1, MEK1/2 and ERK1/2. KSR and MEK are constitutively associated, whereas binding to c-Raf-1 and ERK occurs through activation of Ras. In studies using cells from KSR deficient mice, it was found that KSR modulates the intensity and duration of the ERK signal. KSR was found to be required for maximal ERK activation (Kortum *et al.* 2005; Kortum *et al.* 2006).

MP1 associates with MEK1 and ERK1, where it promotes signalling between MEK and ERK. β-Arrestins interacts with Raf, MEK and ERK contributing to G-protein-coupled receptor (GPCR) mediated activation of ERK. MEKK1 acts constitutively with c-Raf-1, MEK1 and ERK2. CNK and SUR-8 interact with Raf kinase and play a role in coupling membrane signalling events to Raf activation. IQGAP1is a widely expressed protein that binds directly to MEK1/2 and ERK1/2 after EGF activation (Morrison and Davis 2003; Sacks 2006).

1.4.3 Control of ERK activation by Tumour progression locus-2 (Tpl-2); (MAP3K8).

Tpl-2 or Cot was originally described as a proto-oncogene in T cell lymphomas (Patriotis *et al.* 1993; Erny *et al.* 1996; Ceci *et al.* 1997). Tpl-2 is a proto-oncogene encoding a serine-threonine kinase and was identified as the target for provirus insertion in MMLV in the last intron leading to the enhanced expression of a carboxy-terminally truncated protein. This leads to activation of Tpl-2 (Ceci *et al.* 1997).

Studies in cell lines have shown that overexpressed Tpl-2 activates the ERK MAPK pathway (Patriotis *et al.* 1994; Salmeron *et al.* 1996; Chiariello *et al.* 2000). *In vitro* experiments have shown that this activity results from Tpl-2 acting as a MAP-3 kinase for MEK1 and -2 (MAP-2 kinase), which phosphorylates ERK (Salmeron *et al.* 1996; Chiariello *et al.* 2000) (Figure 1.4.3). It is now known that Tpl-2 functions as a MAP3K in innate immune cells (Symons *et al.* 2006). A Tpl-2 knockout mouse was generated in order to study the physiological effects of Tpl-2 (Dumitru *et al.* 2000). Tpl-2 deficient macrophages stimulated with LPS were unable to phosphorylate MEK and ERK, but the activation of p38 and JNK MAP kinases were normal. It was shown that the Tpl-2 knockout mouse was resistant to LPS induced endotoxic shock, due to a defect in the TNF mRNA transport (Dumitru *et al.* 2000).

Tpl-2 binds to the C-terminal half of NF-κB1 (p105) (Belich et al. 1999). I unstimulated macrophages Tpl-2 does not exist in a p105-free form, and p105 deficient macrophages suggests that p105 is needed for Tpl-2 stability (Beinke et al. 2003). Tpl-2 is held in its inactive state by binding to p105 and upon stimulation of macrophages, Tpl-2 is released from p105 in order to exert its MAPK properties on ERK. The truncated oncogenic form of Tpl-2 is unable to form a stable complex with p105 and is therefore able to cause persistent phosphorylation of MEK (Cowley et al. 1994; Beinke et al. 2003).

It has been shown that that Tpl-2 is essential for induction of ERK in macrophages (Dumitru *et al.* 2000; Eliopoulos *et al.* 2003) and in DC (Sugimoto *et al.* 2004). In unstimulated cells Tpl-2 forms a ternary complex with NF-κB1 p105 and A20-binding inhibitor of NF-κB 2 (ABIN-2), which are both required for Tpl-2 stability.

Upon TLR stimulation Tpl-2 is released from the complex, which is required for its activation (Beinke *et al.* 2003; Lang *et al.* 2004). It has been shown that Tpl-2 release from NF-κB1 is not sufficient for ERK phosphorylation but phosphorylation of the C-terminal of Tpl-2 is also required before it can induce ERK activation (Robinson *et al.* 2007). ABIN-2 has been shown to be essential for Tpl-2 protein stability and consequently ABIN-2 deficient macrophages stimulated with LPS show reduced ERK activation, due to downregulation of Tpl-2. However, ABIN-2 is not required for Tpl-2 activation (Papoutsopoulou *et al.* 2006).

1.4.4 Importance of ERK regulation.

Regulation of ERK is essential as uncontrolled activation of ERK through either the Ras/Raf or Tpl-2 pathway has been implicated in many cancers (Patriotis *et al.* 1993; Patriotis *et al.* 1994; Ceci *et al.* 1997; McCubrey *et al.* 2007; Tsatsanis *et al.* 2008). Stimulation of the ERK pathway leads to activation of various transcription factors including c-Fos, Ets, Elk1, c-Myc, c-Jun and CREB, as well as regulation of apoptosis by phosphorylation of Bad, Bcl-2 and caspase-9 (Marshall 1994; McCubrey *et al.* 2007). The outcome of signalling through the ERK MAP kinase pathway depends on whether the signal is transient or sustained. In studies using PC12 neuronal cells, it has been found that stimulation with NGF causes differentiation of the PC12 cells due to sustained ERK activation, whereas stimulation with EGF leads to proliferation of the cells due to transient ERK activation (Marshall 1995; Keyse 2000; Murphy and Blenis 2006). The correlation between transient and sustained ERK activation with respect to

alteration of cell behaviour has also been found in fibroblasts, macrophages and T cells (Murphy and Blenis 2006). Sustained ERK activation can be achieved by mutations in components of the Ras/Raf/MEK/ERK pathway leading to carcinogenesis.

One of the targets of the ERK cascade is the Elk1 transcription factor, which belongs to the family of Ets-domain transcription factors. Elk1 directly interacts with ERK at two docking sites present in both ERK and Elk1, directly phosphorylated by Elk1 (Yoon and Seger 2006). Another transcription factor activated by ERK is c-Fos. This is one of the earliest transcriptional events to take place after cell stimulation. c-Fos and c-Jun comprises one form of AP-1, which is an important regulator of early transcriptional processes. The regulation of c-Fos is very important as it controls proliferation, differentiation and oncogenic transformation. (Eferl and Wagner 2003). The phosphorylation of c-Fos requires active ERK and RSK in the nucleus, which is achieved after sustained stimulation of the ERK pathway (Yoon and Seger 2006).

Other substrates of ERK includes the MAP kinase activated protein kinases (MAPKAPs). These include RSK1-4, MNK1/2, MSK1/2, MAPKAPK3 and MAPKAPK5. The RSK family is a key component of the ERK signalling cascade, as it contributes to both cytosolic and nuclear signalling and can dictate a number of ERK-mediated cellular outcomes such as prevention of apoptosis and induction of the cell cycle (Yoon and Seger 2006). The other MAPKAPs activated by ERK are not specifically ERK substrates as they can also be phosphorylated by p38, but are also required for ERK signalling with additional substrates necessary for ERK-mediated responses (Roux and Blenis 2004).

1.4.5 Phosphatases.

Regulation of ERK MAP kinase activity is essential as this kinase controls a range of cellular functions such as proliferation, differentiation and apoptosis. In order for a MAP kinase to become activated, phosphorylation is required on the tyrosine and threonine residues within the activation loop but dephosphorylation of either of the residues is sufficient for inactivation (Keyse 2000; Martin *et al.* 2005). Phosphatases are responsible for this dephosphorylation. They can be divided into three groups; the dual specificity phosphatases, which dephosphorylate both the tyrosine and threonine residues on ERK2 MAP kinase, the tyrosine-specific phosphatases (PTPases), which dephosphorylate the tyrosine residue on ERK2 MAP kinase and the serine/threonine-specific phosphatases, which dephosphorylate either the serine or the threonine residue on ERK2 MAP kinase (Keyse 2000; Saxena and Mustelin 2000; Martin *et al.* 2005; Owens and Keyse 2007). MAP kinase phosphatases (MKP) act to dephosphorylate and inactivated MAP kinase isoforms. Some MKPs are encoded by genes which are transcriptionally upregulated upon MAP kinase activation. Individual MKPs can also differentially regulate MAP kinase isoforms. Overall, MKPs form a negative regulatory network that controls MAP kinase activity (Owens and Keyse 2007).

The dual-specificity phosphatases consists of a family of 10 MKPs, which are structurally similar. They can be subdivided into three groups dependant on their actions. The first group comprises the inducible nuclear phosphatases DUSP1, DUSP2, DUSP4 and DUSP5. The second group contains the cytoplasmic related phosphatases DUSP6, DUSP7 and DUSP9, which preferentially inactivates ERK. The third group

consists of DUSP8, DUSP10 and DUSP16, which show substrate selectivity towards JNK and p38 (Owens and Keyse 2007). In two studies using a DUSP1 deficient mouse, it was found that these mice were highly susceptible to endotoxic shock upon LPS injection as compared to wildtype controls. Elevated levels of TNF, IL-6 and IL-10 were found in the serum from these mice (Chi *et al.* 2006; Hammer *et al.* 2006). Bone marrow derived macrophages from DUSP1 deficient mice also produced increased levels of TNF, IL-6 and TNF upon stimulation with LPS, whereas IL-12p40 levels were normal as compared to wildtype macrophages (Hammer *et al.* 2006).

The protein tyrosine phosphatases were first identified in yeast and some mammalian homologues have since then been identified such as STEP (Striatal enriched phosphatase), PTP-SL (STEP-like phosphatase), HePTP (Haemopoietic phosphatase) and LC-PTP (Leukocyte phosphatase). STEP and PTP-SL are found in neuronal cells where they were found to physically associate with ERK. The lymphoid specific phosphatases HePTP and LC-PTP were found to block antigen-dependent T cell activation and cellular targets of these phophatases were identified as ERK (Keyse 2000).

Activated MAP kinases are also substrates for the cytosolic serine/threonine phosphatases, PP2A and PP2C (Saxena and Mustelin 2000; Martin *et al.* 2005). PP2A has been indicated to play a major role in downregulating the ERK pathway, where it can act on both MEK and ERK (Saxena and Mustelin 2000).

1.4.6 The role of Tpl-2/ERK induction of IL-10 and IL-12.

Studies using the Tpl-2 knockout mouse, show that not only TNF production is reduced upon TLR stimulation of macrophages (Dumitru *et al.* 2000), but also IL-12p40 production is increased due to abrogation of ERK phosphorylation (Sugimoto *et al.* 2004). ERK1 knockout mice have been shown to have reduced IL-10 and increased IL-12p70 production upon TLR stimulation of DC (Agrawal *et al.* 2006). Based on these studies, ERK has been proposed to function as a negative regulator of Th1 immune responses. Consistent with this hypothesis infection of Tpl-2 deficient mice with *Leishmania major* led to a skewed Th1 response (Sugimoto *et al.* 2004), and conversely ERK1 knockout mice were shown to have increased susceptibility to experimental autoimmune encephalomyelitis (Agrawal *et al.* 2006).

Furthermore, ERK phosphorylation has been implicated in the regulation of IL-10 and IL-12 in macrophages although the mechanism is not clear as of yet (Yi *et al.* 2002; Agrawal *et al.* 2003). The role of ERK activation in regulation of these cytokines in DC is controversial, as there have been conflicting reports claiming that ERK does play a role (Agrawal *et al.* 2003) or ERK does not play a role (Hacker *et al.* 1999).

MAP kinases play an important part in regulating the immune system. Activation of the MAP kinases signalling cascade as discussed earlier is required for transcriptional regulation of the inflammatory cytokine TNF. A possible role of ERK activation in macrophages for induction of IL-10 has also been found, where absence of ERK activation lead to increased IL-12 (Yi *et al.* 2002), although it is unclear whether the effects on IL-12 are direct or via action on IL-10.

52

In DC, there are two conflicting studies with respect to ERK activation. One finds that ERK phosphorylation cannot be induced in DC upon TLR stimulation but only in macrophages (Hacker *et al.* 1999). The other shows that ERK activation in DC induces IL-10 and low levels of IL-12 and upon ERK inhibition, the IL-12 levels are increased, while IL-10 was decreased (Agrawal *et al.* 2003). However, it is not known whether the upregulation of IL-12 seen in the absence of ERK activation is the consequence of decreased IL-10 or via direct ERK activation alone. Little is known about the role of MAP kinase activation on the production of type I IFN, although one study suggests that IL-10 and type I IFN are co-ordinately regulated (Hacker *et al.* 2006). For more detail on IL-12 and IL-10, please see section 1.2.

1.5 Aims of the project

We wanted to investigate the role ERK has in regulating cytokine production in DC and macrophages stimulated with TLR ligands LPS and CpG focussing particularly on production of TNF, IL-10, IL-12p40, IL-12p70 and IFN-β, as no systematic and thorough studies have been carried out on these cytokines, which plays a key role in innate and/or adaptive immune responses as discussed earlier. We used two different approaches to study this. One was inhibition of ERK activation with a pharmacological MEK inhibitor that blocks ERK activation downstream of MEK. The second approach was using genetically modified mice lacking Tpl-2. With these approaches we have found that in DC and macrophages IL-12 was regulated negatively through IL-10, but

also through direct ERK activation in the complete absence of IL-10. We also show that

Tpl-2 not only regulates ERK activation but also affects activation of p38.

Chapter 1

Figures

LPS

CD14

TLR4

MD2

TRAM/TIRP

TIRAP/
MAL

TRIF/TICAM

MyD88

PKR

MAPK

NFκB

IRF3

Inflammatory
cytokines

Type 1
IFNs
RANTES
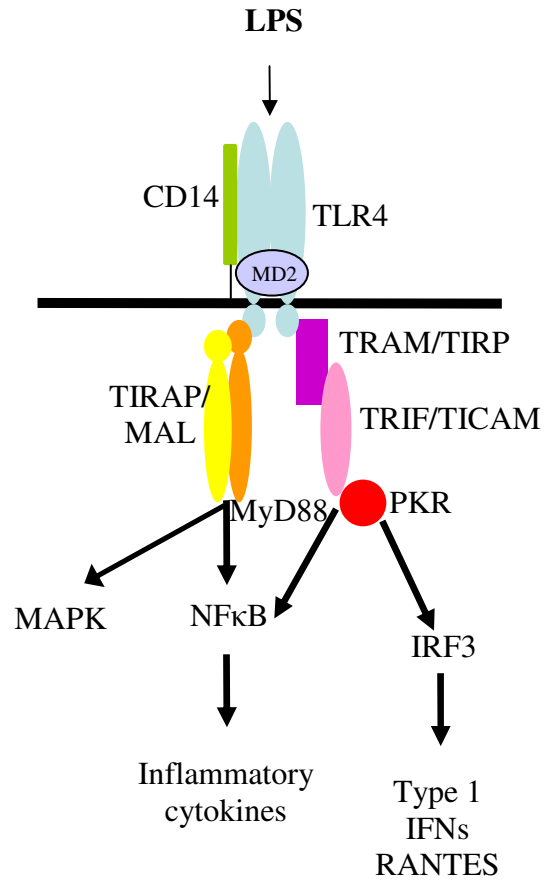
Figure 1.1.7. Toll-like receptor 4 signalling pathway (Akira *et al.* 2003)

CpG

TLR9

MyD88

NFκB

MAPK

Inflammatory
cytokines
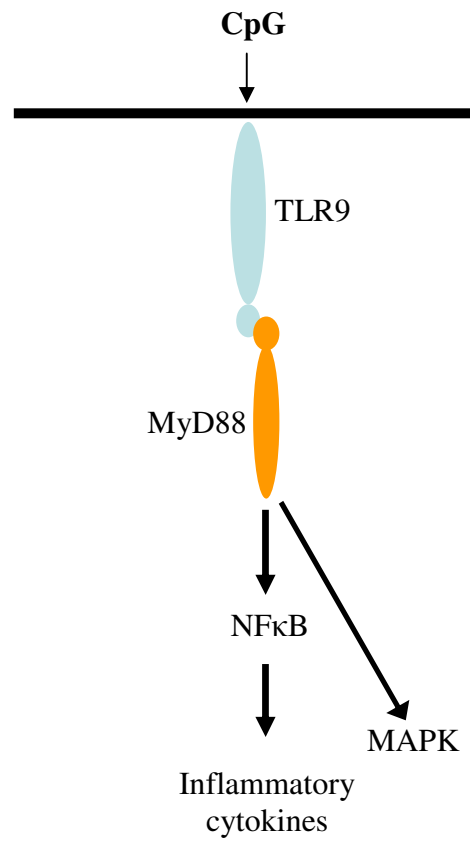
Figure 1.1.8. Toll-like receptor 9 signalling pathway (Akira *et al.* 2003)
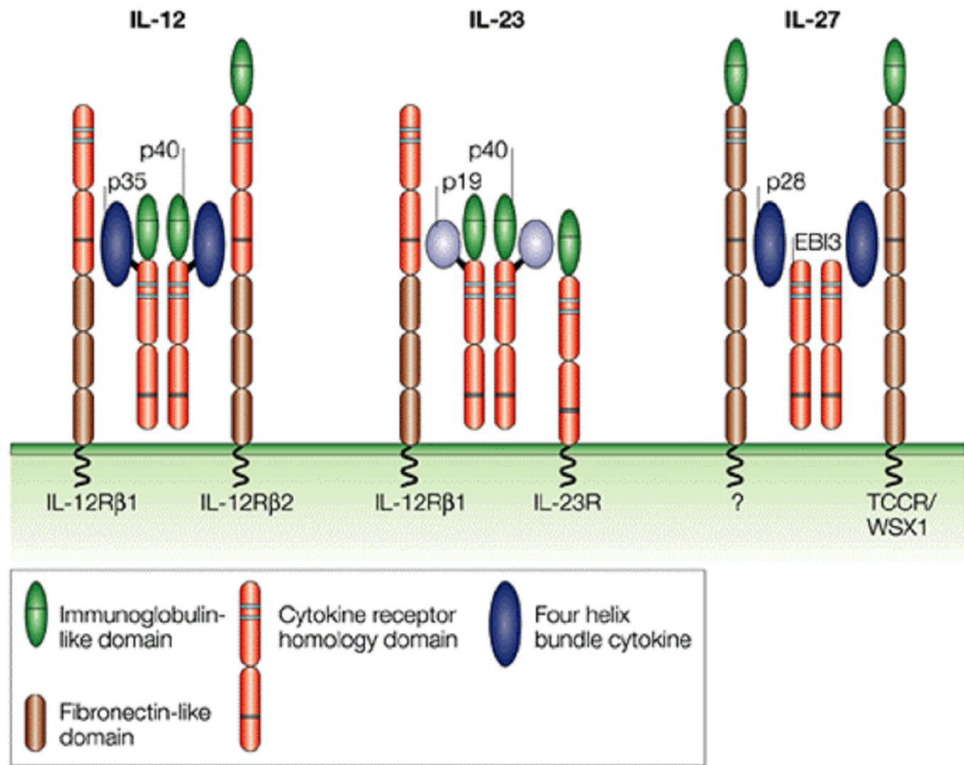
Figure 1.2.2: IL-12 family members from (Trinchieri 2003)

Growth factors
Phorbol esters
Antigens

Ras

Raf

MEK1/2

ERK1/2
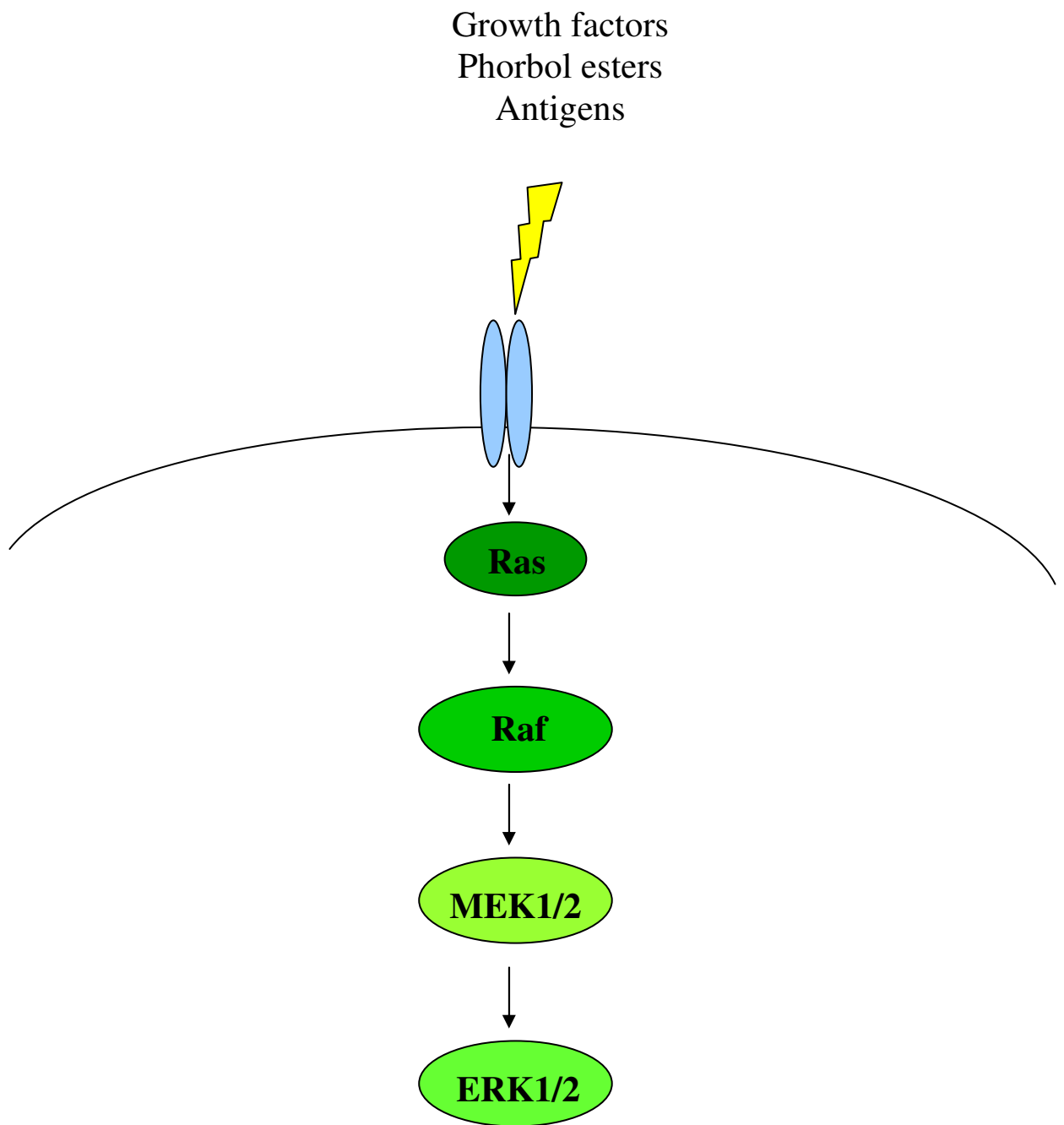
Figure 1.4.1 Activation of the Ras/Raf/MEK/ERK signalling pathway
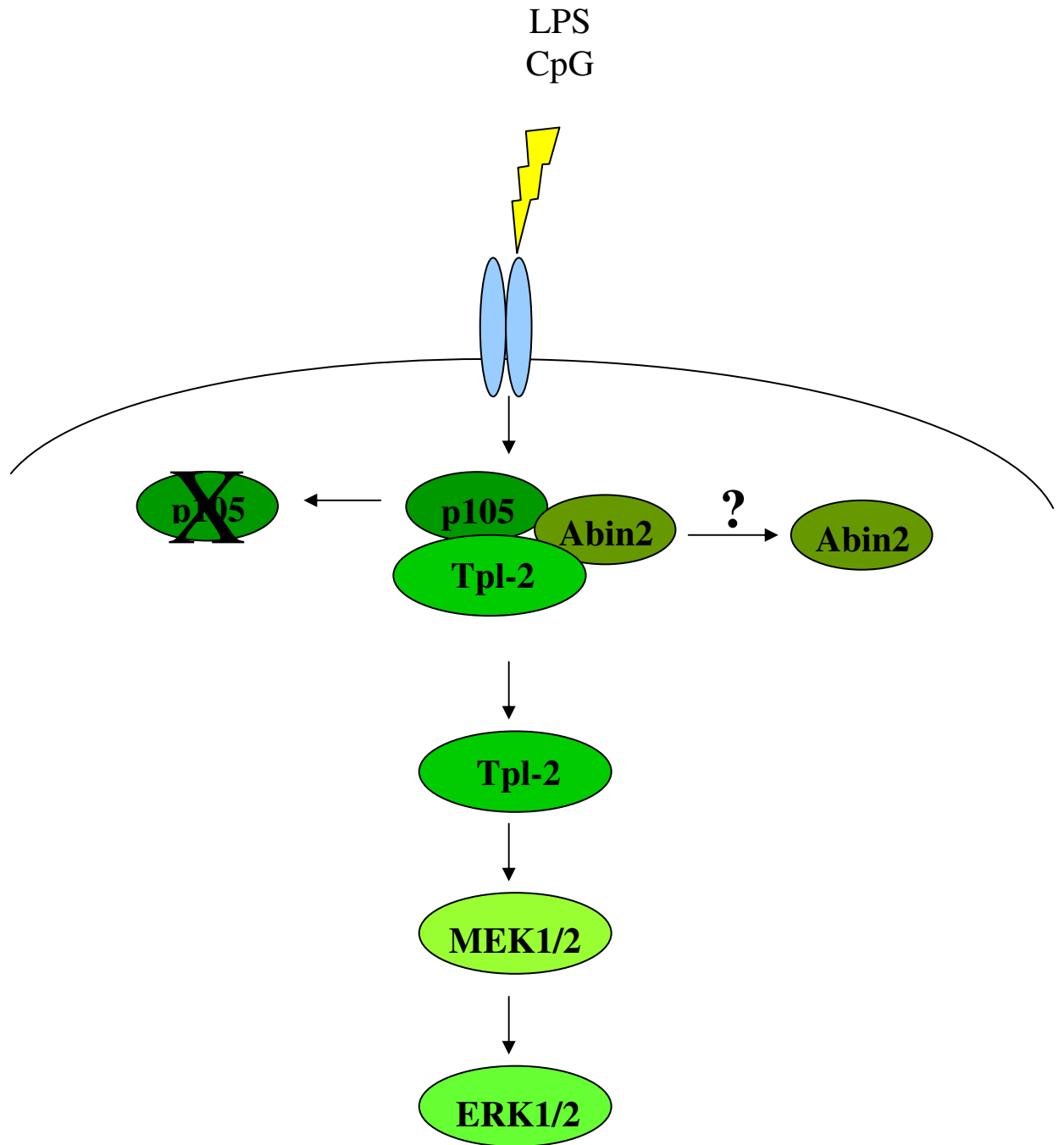
Figure 1.4.3 Activation of the Tpl-2/MEK/ERK signalling pathway

Chapter 2

Material and methods

2.1 Plastics

2.1.1 Tissue culture.

For all types of tissue culture 6-well, 12-well, and 24-well plates (Corning Incorporated, USA), 60mm tissue culture dishes (Nunc, Denmark), 60mm petridishes (Sterillin, UK), 50ml and 15ml Falcon tubes (Corning Incorporated, USA), 70μm cell strainers (BD, USA) were used.

2.1.2 ELISA.

For ELISA 96-well Maxisorp immuno plates (Nunc, Denmark) were used.

2.1.3 Miscellaneous.

1.5ml tubes (Corning Incorporated, USA) were used for RNA, DNA and protein samples. 0.2ml Thermostrip PCR tubes (ABgene, UK) were used for PCR reactions. 96-well PCR plates (ABgene, UK) were used for real time PCR reactions. 1ml syringes (BD, USA) and 25G needles (BD, USA) were used for homogenising cells for RNA purification.

2.2 Chemicals and buffers

2.2.1 Radio-Immunoprecipitation Assay Lysis buffer (RIPA).

For lysis of cells for Western blot, RIPA lysis buffer consisting of 1% (v/v) NP40 (BHD Laboratory Suppliers, UK), 0.1% (v/v) SDS (Biorad, Germany), 0.5% (w/v),

deoxycholate acid (Sigma, USA), 50mM Tris HCl pH 8 (Sigma, USA), 150mM NaCl (Sigma, USA), 2mM EDTA (Sigma, USA), 2mM Sodium-Pyrophosphate (Sigma, USA), 50mM sodium fluoride (Sigma, USA), 100mM vanidate (Sigma, USA) and 10μl complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Germany) was used.

2.2.2 4x Resolving gel buffer.

The resolving gel buffer was made from 1.5M TRIS (Sigma, USA), 0.4% (v/v) SDS (Biorad, Germany), in distilled H_2O and the pH was adjusted to 8.8 with HCl.

2.2.3 4x Stacking gel buffer.

The stacking gel buffer was made from 0.5M TRIS (Sigma, USA), 0.4% (v/v) SDS (Biorad, Germany), in distilled H_2O and the pH was adjusted to 6.8 with HCl.

2.2.4 SDS-acrylamide SDS-PAGE resolving gel.

For running western blot samples the resolving gel contained 8ml protogel (30% Acrylamide: 0.8% Bis-Acrylamide stock 37.5:1) (National Diagnostics, UK), 7.5ml 4x resolving gel buffer, 15ml distilled H_2O, 100μl of 10% APS (w/v) (Biorad, Germany) and 20μl TEMED (Biorad, Germany).

2.2.5 SDS-acrylamide SDS-PAGE stacking gel.

For running western blot samples the stacking gel contained 1.6ml protogel (National Diagnostics, UK), 3.1ml 4x stacking gel buffer, 7.6ml distilled H_2O, 62.5μl of 10% APS (w/v) (Biorad, Germany) and 12.5μl TEMED (Biorad, Germany).

2.2.6 4x SDS-PAGE loading buffer.

The loading buffer for Western blot samples contained 0.454g TRIS pH6.8 (Sigma, USA), 15μl 20%SDS (Biorad, Germany), 4.8ml Glycerol (Sigma, USA), 3ml 2-mercaptoethanol (Sigma, USA), 0.5g Bromophenol Blue (Sigma, USA) and 30ml distilled H_2O.

2.2.7 10x Running buffer.

The Western blot running buffer was purchased as 10x TRIS/Glycine/SDS running buffer (Biorad, Germany).

2.2.8 10x Transfer buffer.

The Western blot transfer buffer was made from 44.26g CAPS (100mM) (3-[Cyclohexylamino]-1-propanesulfonic acid) (Sigma, USA) in 2L distilled H_2O and the pH was adjusted to 11 with NaOH.

2.2.9 Stripping buffer.

The Western blot stripping buffer contained 3.9ml 2-mercaptoethonol (Sigma, USA), 100ml 10% SDS (Biorad, Germany), 31.25ml 1M Tris pH6.7 (Sigma, USA) in 500ml distilled H_2O.

2.2.10 Western blot wash buffer.

The Western blot wash buffer contained 1xPBS (media kitchen, NIMR, UK) and 0.5% TWEEN 20 (Sigma, USA)

2.2.10 Western blot blocking solution.

The Western blot blocking solution was made from 5% (w/v) dry milk powder (Western Laboratory Supplies, UK) dissolved in western blot wash buffer.

2.2.11 Development substrate.

The Western blot development substrate was purchased and mixed at an equal ratio of LumiGlo substrate A and LumiGlo substrate B (KPL, USA).

2.2.12 ELISA wash buffer.

The ELISA wash buffer was made from 1xPBS (media kitchen, NIMR, UK) and 0.25% TWEEN 20 (Sigma, USA).

2.2.13 ELISA substrates.

ELISA substrate a) was ABTS made from 0.15g ABTS (2,2'-Azino-bis(3-Ethylbenz-Thiazoline-6-sulfonic Acid)) (Sigma, USA), 0.1M citric acid (Fisher Scientific, UK) and the pH was adjusted to 4.35 with NaOH. 2μl peroxide/10ml (Sigma, USA) was added before use.

ELISA substrate b) was purchased as TMB (E-biosciences, UK).

2.2.14 ELISA stop solutions.

Stop solution a) contained 1.25% (w/v) Sodium fluoride dissolved in H_2O (Sigma, USA) for ABTS and stop solution b) contained 2N H_2SO_4 (Signa, USA) for TMB.

2.2.15 Ear punch buffer for digestion of mouse tissue.

Buffer for digestion of mouse tissue contained 50mM TRIS pH 8 (Sigma, USA), 2mM NaCl (Sigma, USA), 10mM EDTA pH 8 (Sigma, USA), 1% (v/v) SDS (Biorad, Germany) and 1mg/ml Proteinase K (Roche Diagnostics, Germany).

2.2.16 DNA precipitation.

For DNA precipitation 1/10 sample volume 3M NaAc pH 5.2 was mixed with 2x sample volume100% Ethanol (Fisher Scientific, UK) and added to the sample.

2.2.17 PCR mix.

PCR reactions contained 2.5μl 10x PCR buffer (ABgene, UK), 2.5 μl 25mM $MgCl_2$ (ABgene, UK), 0.5μl 10mM dNTPs (ABgene, UK), 0.5μl Primer A, 0.5μl Primer B,

0.5μl Primer C, 0.2μl 5U/μl Taq polymerase (ABgene, UK), 15.8μl H$_2$0 and 2μl sample DNA.

2.2.18 2% Agarose gel.

Agarose gel contained 4g Agarose (Biorad, Germany) mixed with 200ml 1x TBE buffer (Media kitchen, NIMR, UK) and 15μl Ethidium bromide (Biorad, Germany).

2.2.19 DNA 5x loading buffer.

The loading buffer for DNA samples contained 0.4% (w/v) Bromophenol blue (Sigma, USA), 0.4% (w/v) Xylene Cyanol (Sigma, USA), 25ml Glycerol (Sigma, USA) made up in 25ml H$_2$0.

2.2.20 Formalin.

Formalin for tissue fixing contained 100ml formaldehyde (Sigma, USA), 8.2g NaCl (Sigma, USA) made up in 900ml H$_2$0.

2.3 Medium

2.3.1 Complete RPMI.

Tissue culture medium used for generation of all cells contained RPMI (Cambrex, Belgium), 5% heat-inactivated foetal calf serum (FCS) (Labtech, USA), 1% L-Glutamine (Cambrex, Belgium), 1% penicillin/streptomycin (Pen/Strep)(Cambrex,

Belgium), 1% HEPES (Cambrex, Belgium), 1% Sodium-pyruvate (Cambrex, Belgium) and 0.1M 2-mercaptoethanol (Sigma, USA)

2.3.2 PBS.

Calcium and magnesium free endotoxin free phosphate buffered saline (PBS) was purchased from Gibco, UK.

2.3.3 Sorting buffer.

For cell sorting 500ml PBS (Gibco, UK), 5ml Pen/Strep (Cambrex, Belgium), 0.2mM EDTA (Cambrex Belgium) and 5ml FCS (Labtech, USA) was mixed.

2.4 Equipment

2.4.1 Western blot.

Equipment used for Western blot included Hoefer SE 400 vertical unit (Amersham, UK) for casting and running gels, Transblot unit (Biorad, Germany) for transferring protein, Power pack E865 (Consort, USA) for all electrical output, Bio-spec-mini spectrophotometer (Shimadzu, UK), PDVF transfer membranes (Millipore, USA), 18x24cm Film cassette (Kodak, UK), 18x24cm X-ograph film (Kodak, UK) and Film developer (Fuji Film).

2.4.2 ELISA.

The ELISA reader was a Multiscan Ex (Labsystems, UK)

2.4.3 PCR and electrophoresis.

Equipment used for PCR included PCR machine GeneAmp PCR system 2700 (Applied Biosciences, USA), Thermo EC Maxicell Primo gel running tank (USA) and UV DNA transilluminator (BioDoc-it system, USA).

2.4.4 Real-time PCR.

ABI Prism 7000 detection system (Applied Biosciences, USA) was used for real-time PCR quantification.

2.4.5 Cell culture.

Equipment used during cell culture included Hera Cell incubator (Heraeus, UK) and a Haemocytometer for cell counting.

2.4.6 Cell sorter.

Equipment used for cell sorting included AutoMACS (Milteney, Germany) and MoFlo Cell sorter (Cytomation, USA).

2.4.7 Computer programs.

All graphs were illustrated using Prism 4 (Graphpad software). Calculations of RT-PCR results were done using Excel (Microsoft). All text was typed using Word (Microsoft). All western blots were scanned into an image processing software (Arcsoft Photostudio) and copied into Powerpoint (Microsoft).

2.5 Reagents and antibodies

2.5.1 Western blot antibodies and reagents.

Phospho-p38 (9215), total-p38 (9212), and total-JNK (9252) were purchased from Cell Signaling, USA and used at 1:1000. Phospho-ERK (44-680G) and phospho-JNK (44-682G) were from Biosource, USA and used at 1:1000. Anti-ERK16 was a gift from Jeremy Tavaré (University of Bristol) and used at 1:10000. Anti-Tpl-2 antibody (sc-720) and anti-IκBα antibody (sc-1643) were purchased from Santa Cruz, USA and used at 1:1000. α-Actin antibody (sc-58670) was from Oncogene, USA and used at 1:10000. HRP-conjugated anti-mouse IgG (1021-05) and anti-rabbit IgG (4020-05) were purchased from Southern Biotechnology, USA and used at 1:10000. 5x Protein dye for Bradford assay was from Biorad, Germany and the Rainbow protein marker was from Amersham, UK.

2.5.2 ELISA.

IL-12p70 and TNF ELISA kits were purchased from e-biosciences, UK. IL-10 and IL-12p40 ELISA antibodies were purchased from Pharmingen, USA. IFN-β ELISA kit was from PBL Biomedical Laboratories, USA.

2.5.3 Antibodies for cell sorting.

Anti-CD11c-PE and Anti-FcR III/II were purchased from BD, USA. CD11c microbeads was from Milterney, Germany.

2.5.4 PCR Primer sequences.

Tpl-2 A: 5' CTT CAG TCA TCT TAA CAC TCA GGC 3'

Tpl-2 B: 5' CTG CTT GGA ACT TGC TGT TCT AGA TG 3'

Tpl-2 C: 5' CTG CAC GAG ACT AGT GAG ACG TGC 3'

IL-10 T1.4: 5' GCC TTC AGT ATA AAA GGG GGA CC 3'

IL-10 T2.2: 5' GTG GGT GCA GTT ATT GTC TTC CCG 3'

Neo 5: 5' CCT GCG TGC AAT CCA TCT TG 3'

All primes were supplied by Eurogentec (UK)

2.5.5 Real-time PCR Primer sequences.

IL-10: 5' TTT GAA TTC CCT GGG TGA GAA 3'

      3' GCT CCA CTG CCT TGC TCT TAT T 5'

IL-12p40: 5' CAA ATT ACT CCG GAC GGT TCA 3'

       3' AGA GAC GCC ATT CCA CAT GCT 5'

IL-12p35: 5' CCT GCA CTG AAG ACA TC 3'

      3' GCT CCC TCT TGT GGA AG 5'

TNF: 5' GCC ACC ACG CTC TTC TGT CT 3'

     3' TGA GGG TCT GGG CCA TAG AAC 5'

IFN-β: 5' GCA CTG GGT GGA AGT AGA CT 3'

      3' AGT GGA GAG CAG TTG AGG ACA 5'

Ubiquitin: 5' TGG CTA TTA ATT ATT CGG TCT GCA T 3'

     3' GCA AGT GGC TAG AGT GCA GAG TAA 5'

All supplied by Eurogentec (UK)

2.5.6 CpG 1668 sequence.

5' TCC ATG ACG TTC CTG ATG CT 3' all bases are on a phosphorothioate backbone (InVitrogen, UK)

2.5.7 Real-time PCR.

Fluorescent DNA binding dye SYBR Green was purchased from Applied Biosciences, USA.

2.5.8 Other reagents.

U0126 MEK inhibitor was purchased from Biomol (USA), Lipopolysaccharide (LPS) was from Alexis, Canada, DMSO (Dimethyl sulfoxide 99.5%) was purchased from Sigma, USA, Granulocyte-macrophage stimulating factor (GM-CSF) was from DNAX, USA, Macrophage stimulating factor (M-CSF) containing L-cell supernatant was made at the large scale lab (NIMR, UK). Liberase Cl was purchased from Roche Diagnostics, Germany and 0.83% Ammonium Chloride was from the Media kitchen (NIMR, UK)

2.6 Mice

2.6.1 C57BL/6 mice.

C57BL/6 mice were bred under pathogen free conditions at the NIMR (UK). All animals were sacrificed according to regulated procedures (Schedule 1). The mice were between 8-12 weeks of age.

2.6.2 IL-10$^{-/-}$ mice.

IL-10$^{-/-}$ mice on the C57BL/6 background bred under pathogen free conditions at the NIMR (UK). The mice have been backcrossed to C57BL/6 for 10 generations.

2.6.3 Tpl-2$^{-/-}$ mice.

Tpl-2$^{-/-}$ mice on the C57BL/6 background were a kind gift from Phillip Tsichlis (Tufts University, USA). As the mice were only backcrossed to the 6th generation, we continued to breed to the mice as heterozygous crossing and were screening all the offspring. The mice were bred in a quarantine unit at NIMR (UK).

2.6.4 Tpl-2$^{-/-}$/IL-10$^{-/-}$ mice.

Tpl-2$^{-/-}$/IL-10$^{-/-}$ mice were generated by intercrossing the *Tpl-2$^{-/-}$* mouse to an *IL-10$^{-/-}$* mouse. The offspring was screened and crossed to generate 4 genotypes: wildtype, *Tpl-2$^{-/-}$, IL-10$^{-/-}$* and *Tpl-2$^{-/-}$/IL-10$^{-/-}$* mice. These mice were bred in a quarantine unit at NIMR (UK).

2.7 Genotyping of mice.

When the mice were weaned a small piece of the ear was collected. The tissue was digested in earpunch buffer at 56°C overnight. The proteinase K was inactivated by boiling for 3 minutes. The DNA was precipited at -20°C overnight. Next day the samples were spun down (13000 rpm for 30min at 4°C) and the supernatant removed. The precipitate was left to dry at room temperature for 10 minutes before being resuspended in water. Amplification of the DNA was carried out by PCR.

The amplification cycles for identification of the Tpl-2 genotype was:

94°C for 7 minutes

94°C for 45 seconds

52°C for 45 seconds   35 cycles

72°C for 45 seconds

72°C for 7 minutes

The amplification cycles for identification of the IL-10 genotype was:

94°C for 7 minutes

94°C for 1 minute

60°C for 1 minute   30 cycles

72°C for 1 minute

72°C for 7 minutes

The DNA was run on an agarose gel containing Ethidium bromide until separation of the DNA was visible under UV light.

2.8 Generation of myeloid dendritic cells.

Myeloid dendritic cells were generated according to the original protocol by Inaba (Inaba *et al.* 1992). In brief, femurs and tibiae from mice were flushed with complete RPMI. Red blood cells were lysed with ammonium-chloride for 2 min. Cells were plated out in 6-well plates at $1x10^6$ cells/ml in medium containing 10ng/ml GM-CSF. On day 2, non-adherent cells were removed, cells gently washed and fresh medium supplemented with GM-CSF added. This was repeated on day 4 omitting the wash step. On day 6, non-adherent cells were collected and replated in tissue culture at $0.5x10^6$ cells/ml with medium containing GM-CSF. On day 7, non-adherent cells (DC) were harvested after 16 hours replating and used as required. The cells generated contained a 70% pure population of myeloid DC ($CD11c^+$) according to Flow cytometry analysis. However, if a greater purity was required, the cells were sorted for $CD11c^+$ using a MoFlo Cell sorter. The cell sorting was carried out by the FACS Lab (NIMR, UK).

2.9 Generation of bone marrow derived macrophages.

Femurs and tibiae were flushed and cells prepared as described in section 2.8. The cells were plated out at $4x10^6$ cells/ml in petridishes in complete RPMI containing 20% L-cell supernatant. On day 4, cells were fed with 10ml complete RPMI containing 20% L-cell

supernatant. On day 7, adherent cells were harvested with icecold PBS. Briefly, the supernatant was removed and replaced with 5ml icecold PBS and incubated at 4°C until the macrophages detached.

2.10 Generation of splenic dendritic cells.

Mouse spleens were collected and injected with 0.4mg/ml liberase Cl. After 30 min incubation at 37°C, the spleens were mashed though 70μm cell strainers and red blood cells were lysed with ammonium chloride. The cells were stained with 1μg/μl CD11c-PE and incubated on CD11c microbeads for 30 min at 4°C, before being purified by AUTOMACS, which separates the labelled cells into $CD11c^+$ and $CD11c^-$ fractions. After purification, the positive fraction was taking to the cell sorter, where it was further sorted for $CD11c^+$ using MoFlo Cell sorter.

2.11 Cell stimulation

2.11.1 Immunoassays.

For cytokine immunoassays, $1x10^6$ cells/ml were plated out in 24-well plates. The cells were stimulated with LPS or CpG at specified concentrations in presence or absence of MEK inhibitor U0126. DMSO was used as vehicle control. The supernatant was collected after 1, 3, 6 and 20 hours and cytokines measured by ELISA.

2.11.2 Real-time quantitative PCR.

For real-time quantitative PCR, the cells were plated out and stimulated as described in section 2.11.1. The cells were lysed and RNA purified using an RNeasy kit after 0, 1, 3 and 6 hours of stimulation.

2.11.3 Western blot.

For Western blot, $1x10^6$ cells/ml were plated out in 12-well plates and rested for 5 hours in complete RPMI containing 1% FCS to decrease MAP kinase phosphorylation background signals. Cells were stimulated with the indicated concentrations LPS or CpG with or without inhibitor U0126. If the U0126 inhibitor was used, the cells were pre-incubated with the inhibitor for 15 minutes prior to stimulation.

2.12 Methods

2.12.1 Western blot.

Cells were lysed in RIPA buffer, and the protein concentration was checked by the Bradford assay. Briefly, 5x dye was added to the samples and the wavelength was measured at 595nm on a spectrophotometer. All protein samples were run on 8% SDS-acrylamide SDS-PAGE gels. The proteins were transferred overnight to a PVDF membrane. The membranes were blocked in blocking solution. The membranes were probed with specific antibodies to the phosphorylated or constitutive form of the protein of intend. Proteins were visualised using the LumiGlo kit. Membrane were stripped at 56°C for 20min before reprobing

2.12.2 Real-time quantitative PCR.

Total RNA was prepared and purified using Qiagen RNeasy minicolumn kit following the manufacturers protocol. To measure the relative amount of cytokine mRNA, amplification of cDNA was monitored with SYBR Green and specific primers in combination with the ABI Prism 7000 detction system. Cytokine mRNA values were normalised to ubiquitin signals using the following formula was used: 1.8*(Ubiquitin value –Cytokine sample value)*100000

2.12.3 ELISA.

ELISA plates were coated with the capture antibody over night at 4°C. The plates were then blocked for 1 hour with FCS. Samples were incubated on plates for 2 hours. Detection antibody was added for 1 hr. Conjugated HRP was added for 1 hour and ELISA was developed using correct substrate. All steps were carried out at room temperature in a humidity chamber unless otherwise stated. Plates were washed 8 times in wash buffer between each step.

2.12.4 Histology.

Colon and caecum was removed from sacrificed mice and fixed in buffered formalin at room temperature over night. The histology procedure was carried by the NIMR histology department. Sections were cut from the colon and caecum and embedded in paraffin blocks. Sections were cut and stained with hematoxylin (H) and eosion (E) and fixed on slides. The sections were then visualised by light microscopy.

Chapter 3

Establishing the experimental system

3.1 Cytokine production in myeloid dendritic cells can be initiated at low doses of LPS and CpG.

Mouse myeloid DC and macrophages express TLR4 and TLR9 and thus respond to their respective ligands, LPS and CpG (Akira and Takeda 2004). However, the optimal dose of these stimuli had not been established and doses of LPS range between 100ng/ml to 10μg/ml, whereas, CpG was used at 1μM in published studies. We stimulated myeloid DC with LPS or CpG at increasing doses in order to find the optimal concentration for induction of the cytokines IL-10, IL-12p40/p70 and TNF (Figure 3.1).

The LPS titration was initiated at 10µg/ml and titrated down to 6pg/ml. The CpG titration was initiated at 1000nM, a concentration used in the literature, and titrated down to 0.5nM. As seen in Figure 3.1, the induction of cytokines started to titrate at doses of LPS doses below 10ng/ml and 60nM of CpG. Thus both LPS and CpG are normally used at saturating concentrations at which subtle modulation of cytokine production might not be detectable. Based on these titration curves, we selected the LPS dose of 10ng/ml and the CpG dose of 500nM for optimal stimulation of the production of cytokines, which was just at the beginning of the plateau (Figure 3.1) of cytokine induction. Dose response curves for induction of cytokines in macrophages were similar as compared to the dose response curves for myeloid DC.

3.2 Kinetics of LPS and CpG for cytokine transcription in myeloid DC and macrophages.

The literature is very limited with information on the kinetics of induction of different cytokines with different TLR ligands in myeloid DC and macrophages, therefore we determined which time point was appropriate for optimal mRNA and cytokine induction after stimulation with the optimal doses of LPS or CpG in both myeloid DC and macrophages. To do this, we set up a time course of stimulation with LPS and CpG and collected total RNA at 0, 1, 3, and 6 hours. *Tnf, Il-10, Il-12p40,* and *Il-12p35* mRNA were measured by real-time quantitative PCR (Figure 3.2).

As shown in Figure 3.2, induction of *Tnf* mRNA peaked early at 3 hours in both myeloid DC and macrophages. *Il-10* mRNA induction had different kinetics in DC and macrophages. Following LPS or CpG stimulation of DC, *Il-10* mRNA induction peaked late at 6 hours, whereas in macrophages IL-10 mRNA peaked at 1 hour. Induction of *Il-12p40* mRNA was also different between DC and macrophages. LPS or CpG stimulation of DC induced maximal *Il-12p40* mRNA at 6 hours, whereas in macrophages *Il-12p40* mRNA peaked earlier at 3 hours. Induction of *Il-12p35* mRNA was only detectable in DC and after both LPS or CpG stimulation peaked between 3 to 6 hours. It was therefore not possible to select a single time point for LPS or CpG stimulation. In future experiments we decided to continue to perform a time course in order to allow for the differences in the kinetics and to ensure that subtle changes in the induction of any of the cytokines could be observed.

3.3 Kinetics of cytokine protein production in myeloid DC and macrophages.

Having investigated the kinetics of mRNA induction, we investigated the kinetics for cytokine protein production. To do this, myeloid DC and macrophages were stimulated with LPS or CpG for 0, 3, 6, 20, and 48 hours and the supernatants were collected and assayed for cytokines by immunoassay (Figure 3.3).

LPS and CpG followed the same kinetics for induction of IL-10 and IL-12p40 production in the myeloid DC, reaching a plateau at 20 hours. TNF, however, reached a plateau at 6 hours where it remained for CpG, but peaked around 20 hours for LPS and then dropped off (Figure 3.3a).

Similarly in macrophages (Figure 3.3b) stimulated with LPS, the IL-10 and IL-12p40 protein levels reached a plateau at 20 hours and TNF at 6 hours and were sustained. The CpG kinetics in macrophages were a bit different from LPS, as both IL-10 and IL-12p40 did not reach a plateau during the time course, but kept on increasing up to 48 hours upon stimulation with LPS. TNF reached a plateau at 6 hours. Taking all the cytokine data into account, we decided that the optimal stimulation times were 20 hours for IL-10 and IL-12 protein induction and 6 hours for TNF protein induction for both myeloid DC and macrophages stimulated with LPS or CpG.

3.4 Kinetics of activation of MAP kinases by LPS and CpG.

Interested in determining the role of ERK in regulating cytokine production as it has been reported that LPS or CpG cannot induce ERK phosphorylation in myeloid DC,

82

although in the same report both LPS and CpG induced ERK phosphorylation in a RAW macrophage cell line and peritoneal macrophages (Hacker *et al.* 1999). However, in another study , it has been shown that LPS can induce phosphorylation of ERK in DC (Dillon *et al.* 2004). We investigated the kinetics for MAP kinase activation in detail in the myeloid DC following stimulation with LPS and CpG (Figure 3.4), using phospho-specific ERK and p38 antibodies as a readout for MAP kinase activation.

Myeloid DC were stimulated with either LPS or CpG for 0, 7.5, 15, 30, 45, and 60 minutes. Lysates were then immunoblotted for phosphorylated and total forms of p-38, ERK and total IκBα. As seen in Figure 3.4, LPS induced phosphorylation of ERK and p38 after only 7.5 minutes. After 45 minutes the signal started to weaken. CpG showed a much slower kinetics of induction of ERK phosphorylation. Only after 30 minutes of stimulation was it possible to detect a signal for ERK and p38 phosphorylation. After 60 minutes however, the signal weakened.

3.5 Stimulation of myeloid DC with LPS and CpG can induce MAP kinase phosphorylation even at low concentrations.

The concentrations of LPS and CpG required to induce MAP kinase phosphorylation, which have been used in the literature range from 1μg/ml to 10μg/ml LPS and on average of 1μM for CpG. We wished to determine the optimal dose of each of these stimuli to ensure the signal induced was on the plateau but at a level at which one could still detect subtle differences in signal strength. Figure 3.5a shows that high doses of both LPS (0.5-100ng/ml) and CpG (50-1000nM) potently activated MAP

kinase phosphorylation as detected by immunoblotting. Figure 3.5b shows that the ERK phosphorylation was observed at 500pg/ml LPS but was not detectable at 50pg/ml and that the CpG induced ERK phosphorylation could still be observed down to a concentration of 50nM below which the signal was lost. Interestingly these findings reflect the cytokine assay results (Figure 3.1), where LPS started to titrate out between 10ng/ml and 1ng/ml and however, CpG titrated out between 60 and 250nM. However, for both LPS and CpG stimulation, IκBα degradation was only induced at higher concentrations of stimuli. For LPS, IκBα degradation can be detected at doses above 500pg/ml and for CpG above 100nM. Taken together with the cytokine titration curves (Figure 3.1), we chose 10ng/ml LPS and 500nM CpG for all future assays to ensure a signal that could reveal any modulation in subsequent experiments.

3.6 Discussion.

Based on the titration experiments in this section, we were able to select the optimal dose of LPS and CpG for stimulation of myeloid DC and macrophages for production of cytokines and activation of ERK and p38 MAP kinases (Figure 3.1 and 3.5). We chose to use 10ng/ml LPS and 500nM CpG, as these doses were sufficient to induce clear phosphorylation of both MAP kinases and where such concentrations were at the beginning of the plateau for induction of cytokine production. Such concentrations were predicted to be at a level that could allow observation of subtle changes in cytokine production resulting from interference with specific MAP kinase signalling pathways.

Kinetic analysis of cytokine mRNA expression by real-time quantitative PCR showed that the peak time point for *Tnf* mRNA was 3 hours and for *Il-10, Il-12p40* and *Il-12p35* mRNA was 6 hours for both DC and macrophages. However, due to differences between macrophages and DC in the rate of induction or *Il-10* and *Il-12p40* mRNA, we concluded that a full time course of stimulation would be necessary for subsequent experiments (Figure 3.2). For kinetic experiments assaying cytokine protein production, 20 hours was chosen as the optimal time to assay IL-10, IL-12p40 and IL-12p70, whereas TNF, which peaked earlier, was assayed at 6 hours (Figure 3.3). The immunoassays measure the accumulation of the cytokine in the supernatant. During the kinetics experiment, we collected the supernatant at the described times without replacing the medium. Upon TLR stimulation the cell signalling cascade will become activated leading to the transcription of cytokines. As seen in Figure 3.2, this can be detected by quantitative PCR as soon as 1 hour of stimulation (peak times are dependent on the cytokine). After cytokine mRNA transcription takes place, translation and eventually production and secretion of the soluble protein can be detected by immunoassay. The soluble cytokines are released into the culture medium, where they can interact with their cytokine receptor on the cells in the medium signalling into the cell and potentially influencing the production of cytokines in the local environment. For example, IL-10 produced by macrophages and DC can affect the production of IL-12 by the same cell population by negatively regulating its production. Therefore, at each time point it is only possible to measure the accumulated cytokines in the medium and not quantify the exact production due to absorption and even degradation of the proteins. For example, IL-2 production by T cells is often difficult to measure accurately since IL-

85

2 is a growth factor for T cells and is readily consumed. To overcome this issue and measure IL-2 protein in the supernatants of activated T cells accurately anti-IL-2 receptor antibodies have been used to block the IL-2 consumption (Meuer *et al.* 1984). Thus far this does not appear to be a major issue in measuring cytokine production in DC and macrophages. To support this in our experiments (Figure 3.3) we show that the cytokines measured increased with time after stimulation all reached a plateau and that this plateau was maintained over time, which suggests that they are not consumed or degraded and therefore blocking the various cytokine receptors does not seem necessary.

Myeloid DC stimulated with LPS resulted in phosphorylation of ERK and p38 after 7.5 minutes, however following stimulation with CpG stimulation phosphorylation of ERK and p38 was observed after 30 minutes (Figure 3.4). We therefore picked 15 minutes for LPS and 30 minutes for CpG for subsequent experiments, as the signals detected were strongest at these time points.

In a previous study, it was suggested that neither LPS or CpG could induce ERK phosphorylation in bone marrow derived myeloid DC although these stimuli induced ERK phosphorylation in a macrophage cell line and in peritoneal macrophages (Hacker *et al.* 1999). Our data does not support the findings of Hacker *et al*. (1999). We found that LPS and CpG were potent in inducing MAP kinase phosphorylation in macrophages and myeloid DC, although the kinetics of activation were different between LPS and CpG. This could be due to the fact that CpG has to be internalised first before signalling can be initiated (Krieg 2002). A reason for these differences between our results and those of Hacker *et al.* (1999) could be due to high the background in their immunoblot which could mask subtle changes in ERK phosphorylation. To overcome issues with

high background, we rest our cells for 5 hours in medium containing low serum, before the cells are stimulated, which allows observation of the lower levels of phosphorylation of MAP kinases observed in primary cells opposed to cell lines. Consistent with our findings, studies using human monocyte derived DC or murine splenic DC found that LPS induced ERK phosphorylation in both these DC populations (Agrawal *et al.* 2003; Dillon *et al.* 2004)

Chapter 3

Figures

Myeloid DC

LPS
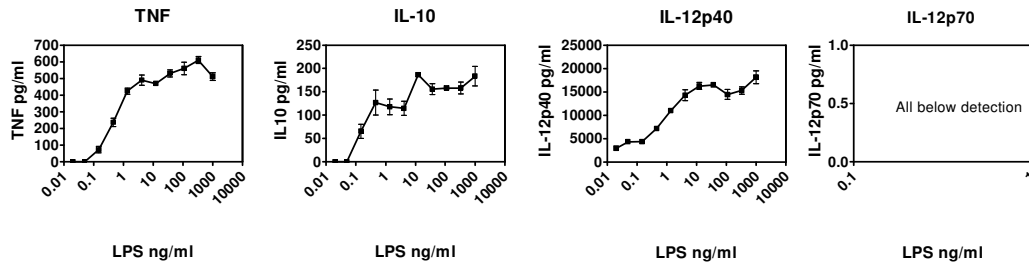
CpG
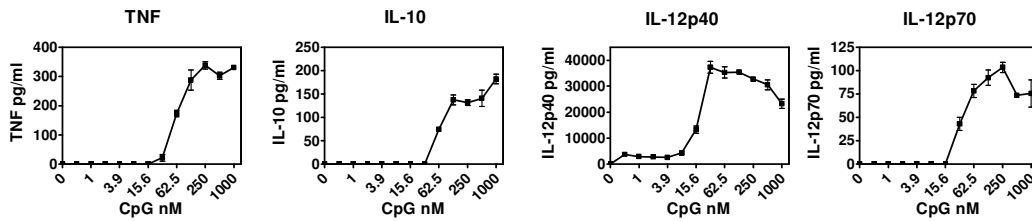
Figure 3.1. Titration of LPS and CpG for cytokine induction in myeloid DC. LPS was titrated from 10000ng/ml to 6pg/ml and CpG from 1000nM to 0.5nM. Cytokine production was measured by ELISA after 20 hours stimulation. The detection limits were: TNF 50pg/ml, IL-10 75pg/ml, IL-12p40 50pg/ml, and IL-12p70 50pg/ml. Triplicate points were tested for each experiment. Results are representative of three similar experiments.
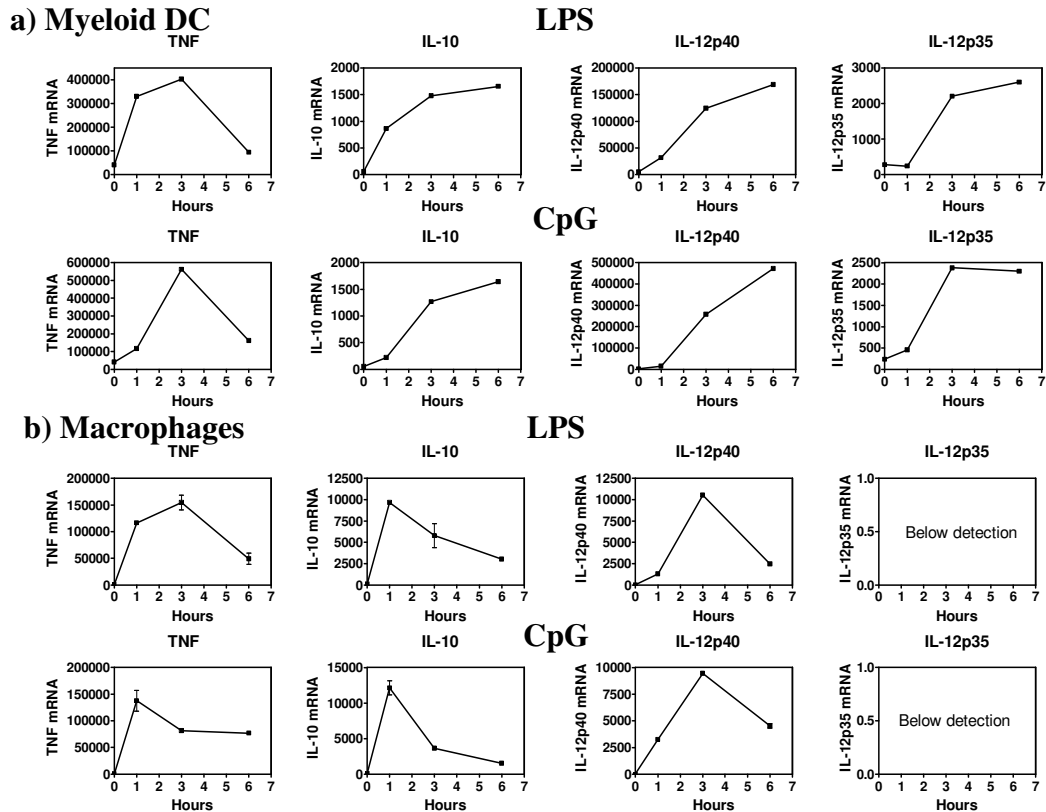
Figure 3.2: Kinetics of induction of cytokine mRNA in myeloid DC and macrophages. a) Myeloid DC or b) macrophages were stimulated with LPS or CpG for the specified times before RNA was purified. Cytokine transcription was checked by real-time quantitative PCR. Triplicate points were tested for each experiment. Results are representative of three similar experiments.

a) Myeloid DC   **LPS**

CpG

b) Macrophages   **LPS**

CpG
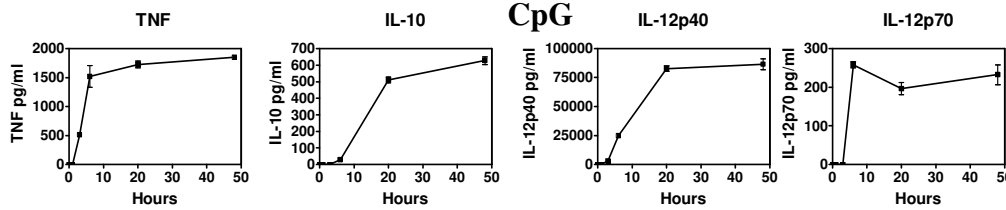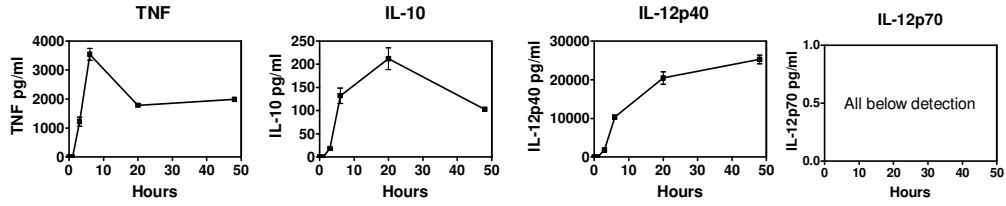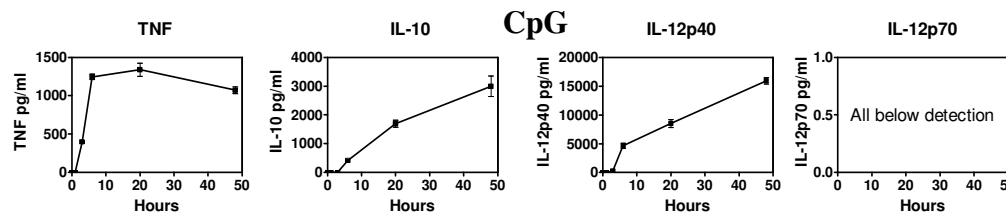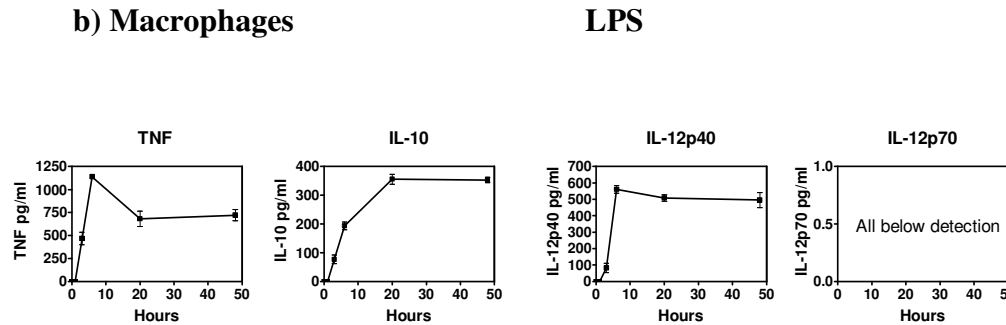
Figure 3.3: Kinetics of LPS and CpG for cytokine protein induction in myeloid DC and macrophages. a) Myeloid DC and b) macrophages were stimulated between 0 and 48 hours LPS or CpG. Supernatants were collected at the indicated times. Triplicate points were tested for each experiment. Results are representative of three similar experiments.
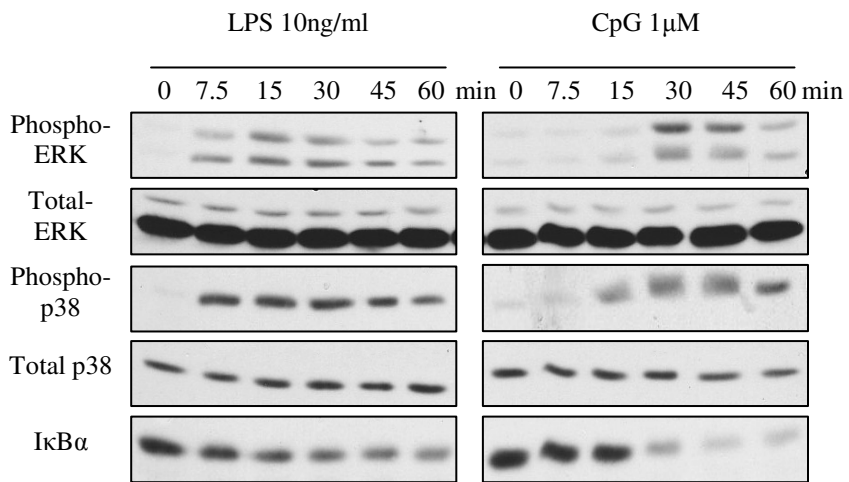
Figure 3.4: LPS activates ERK and p38 with more rapid kinetics than CpG in myeloid DC. Myeloid DC were stimulated with LPS or CpG for the specified time and lysates were immunoblotted for phosphorylated and total form of ERK and p38. LPS and CpG were run on the same gel. Representative of 3 experiments.

a) **High doses of stimuli**

LPS ng/ml   CpG nM

| | 0 | 0.5 | 6 | 100 | 1000 | | 0 | 10 | 50 | 200 | 1000 |

Phospho-ERK

Total ERK

Phospho-p38

Total p38

IκBα

b) **Low doses of stimuli**

LPS pg/ml   CpG nM

| | 0 | 5 | 50 | 500 | 5000 | | 0 | 5 | 10 | 50 | 100 |

Phospho-ERK

Total ERK

Phospho-p38

Total p38

IkBa
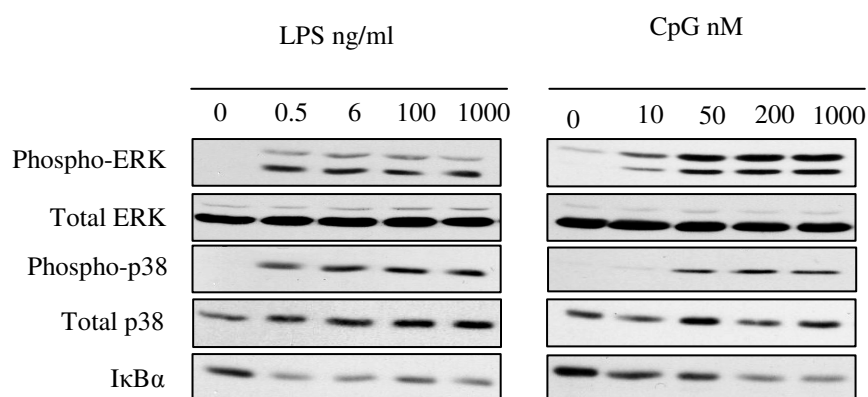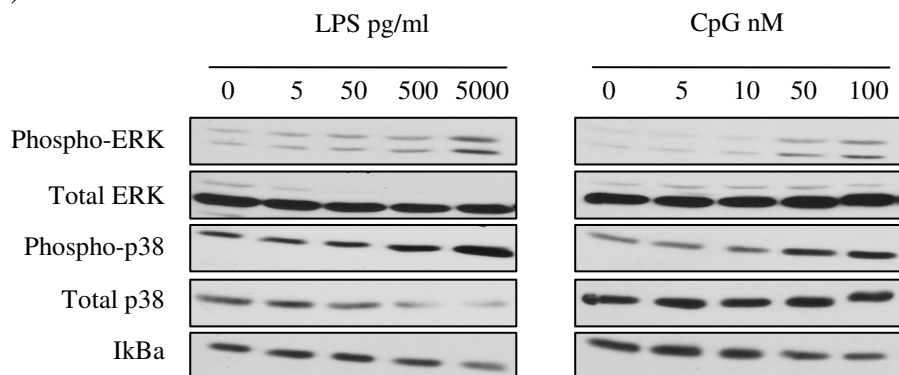
Figure 3.5. Dose response for ERK phosphorylation by LPS or CpG in myeloid DC. Myeloid DC were stimulated with LPS for 15min and CpG for 30min and lysates immunoblotted for the indicated kinases. LPS and CpG were run on separate gels. Exposure times for each antibody was approximately the same between LPS and CpG. Representative of 3 experiments.

Chapter 4

Establishment of specificity of different methods to inhibit

downstream ERK phosphorylation

4.1 Background.

We used pharmacological and genetic methodologies to investigate the role of ERK in regulating TLR induction of cytokine production. First, we used a MEK inhibitor U0126, which has been reported to selectively block ERK phosphorylation (Favata *et al.* 1998). Secondly we used cells from a Tpl-2 deficient mouse strain, which has been shown to be deficient in ERK activation but not in the activation of other MAP kinase after LPS stimulation of macrophages (Dumitru *et al.* 2000).

4.2 Specificity of MEK inhibitor U0126.

As the focus of this project was to determine the effects of ERK phosphorylation on cytokine production, we first determined whether the MEK inhibitor U0126 could block ERK phosphorylation without affecting the activation of other MAP kinases. Myeloid DC were stimulated with different doses of LPS and CpG in the presence or absence of U0126. We initially tested the inhibitor at 10μM against titrations of LPS and CpG, since this dose has been broadly used in the literature (Figure 4.2).

In Figure 4.2, it can be seen that the U0126 inhibitor effectively inhibited ERK phosphorylation at both high and low doses of stimuli in myeloid DC. However, phosphorylation of p38 was also affected at this dose of the U0126 MEK inhibitor, which was very clear for stimulations with CpG although less so with LPS.

4.3 The inhibitory effect of 10μM U0126 on LPS and CpG induced p38 phosphorylation

in macrophages and myeloid DC may be due to non-specific effects of the inhibitor

.

Due to U0126 affecting both the p38 and ERK pathways when used at 10μM, we titrated the inhibitor on myeloid DC and macrophages stimulated with 10ng/ml LPS or 500nM CpG to determine, whether lower doses of U0126 inhibited ERK phosphorylation specifically (Figure 4.3). The titration was started at 5μM and taken down to 0.3μM. U0126 completely inhibited stimulus-induced phosphorylation of ERK when used at doses as low as 0.3-0.6μM. However, doses below 5μM of U0126 did not appear to show any inhibition of the induction of p38 or JNK in either myeloid DC and macrophages stimulated with LPS or CpG.

4.4 Cell surface phenotype of immune cells in the Tpl-2 knock-out mouse.

The Tpl-2 knock-out mouse was generated by disruption of the *Tpl-2* gene in embryonic stem cells by homologous recombination leading to deletion of a portion of the Tpl-2 catalytic domain including the ATP binding site and activation loop (Dumitru *et al.* 2000). The Tpl-2 knock-out mice were kept and bred in a quarantine unit. Some of the Tpl-2 deficient mice were kept up until 7 months of age, during which they did not display any physical signs of illness when compared to littermate controls. They also bred equally well as compared to littermate controls.

To ensure that the development of the immune system was normal, spleen and lymph nodes were collected from three Tpl-2 deficient, three heterozygous and

compared to wild type control mice by flow cytometric analysis. The cells were stained with markers for DC (CD11b, CD11c, 120G8), macrophages (CD11b, F4/80), T cells (CD4$^+$, CD8$^+$) and CD44 (memory marker), CD69 (T cell activation marker) and CD4$^+$ CD25 (T reg marker on the T cells), B cells (CD19) and analysed by flow cytometry. The staining and analysis was carried out by another lab member, Rute Marques. The FACS analysis showed that the percentage and total numbers of DC and macrophages were the same in all the phenotypes. The ratio of T cells to DC, T cells to B cells, and CD4$^+$ to CD8$^+$ in the Tpl-2 deficient mouse were the same and did not differ from the wild type controls. Numbers and percentages of CD44, CD69 and CD25 positive cells were also the same in the *Tpl-2$^{-/-}$* versus the wildtype control mice. In summary there is no difference between the wild type, heterozygous and Tpl-2 deficient mice in terms of cell numbers and cell percentages of DC, macrophages, T cells or B cells.

Expression of TLR4 and TLR9 levels was not checked. This was due to the difficulty in finding an appropriate antibody at the time to quantify the levels of TLR expression. TLR4 is a surface receptor, whereas, TLR9 is an intracellular receptor (Krieg 2002; Yasuda *et al.* 2006). It is possible to determine levels of mRNA expression of TLR4 and TLR9, however, since mRNA may not reflect the protein levels post transcriptionally versus post translationally we did not use this strategy. We however, found that the activation of MAP kinase JNK activated by LPS or CpG was the same in the Tpl-2 deficient macrophages and myeloid DC as compared to the wildtype controls (Figure 4.5), supporting that the level of TLR expression on macrophages and DC may not be altered and thus the same level in the Tpl-2 knock-out mouse as compared to the wildtype controls.

4.5 ERK phosphorylation is blocked and p38 phosphorylation is reduced in the absence of Tpl-2.

It has been shown that LPS does not activate ERK in Tpl-2 deficient macrophages, but phosphorylation of JNK and p38 were unaffected (Dumitru *et al.* 2000). However, another report suggested that CpG could still induce phosphorylation of ERK in Tpl-2 deficient macrophages (Sugimoto *et al.* 2004), suggesting that TLR9 does not utilise Tpl-2 to activate ERK. Our results confirmed that LPS does not activate ERK in Tpl-2 deficient macrophages. However, we also found that CpG activation of ERK was blocked in Tpl-2 deficient macrophages (Figure 4.5). We also showed that neither LPS nor CpG in the absence of Tpl-2 could activate ERK phosphorylation in myeloid DC (Figure 4.5). Our findings are in contrast to those of Sugimoto *et al.* (2004).

We observed decreased induction of p38 phosphorylation in Tpl-2 knockout cells following TLR stimulation of both macrophages and myeloid DC. In macrophages this inhibitory effect on p38 phosphorylation was most pronounced after CpG stimulation and less prominent after LPS stimulation. However, p38 phosphorylation following both LPS and CpG stimulation in myeloid DC was clearly reduced in the absence of Tpl-2. Little or no effect was seen on the induction of phosphorylation of JNK by LPS or CpG in either Tpl-2 deficient macrophages or myeloid DC.

4.6 Discussion.

We found that the commonly used concentration of U0126 inhibitor at 10μM affected not just ERK phosphorylation but also p38 phosphorylation (Figure 4.2) in myeloid DC, which we thought might be the result of non-specific effects of the inhibitor. Therefore, we titrated the U0126 inhibitor on both macrophages and myeloid DC and found that doses between 5 and 0.6μM acted specifically to inhibit ERK phosphorylation without affecting other MAP kinase pathways (Figure 4.3). Based upon the titration experiments on both MAP kinase activation and also cytokine induction, we chose to use the inhibitor at a dose of 2.5μM for our future studies, which did not affect p38 or JNK phosphorylation and thus appeared to specifically inhibit ERK phosphorylation.

We have also shown that Tpl-2 can affect p38 phosphorylation, which is more pronounced in macrophages stimulated with CpG than with LPS. Indeed effects on p38 phosphorylation in the Tpl-2 deficient macrophages stimulated with LPS are only slight (Figure 4.5), which may explain why they were not noticed in the study by Dumitru *et al.* (2000). However, the requirement for Tpl-2 for maximal p38 phosphorylation was marked in myeloid DC stimulated with either LPS or CpG. An explanation for why p38 phosohorylation is not affected in the Dumitru *et al.* (2000) study could be that they use peritoneal macrophages stimulated with 1μg/ml LPS. Since they use high concentrations of LPS and we have shown the effects on p38 phosphorylation is slight it is possible that it is for these reasons they have failed to observe any effect on p38 phosphorylation in the Tpl-2 deficient cells.

In a previous study, it was found that Tpl-2 deficiency only impaired ERK phosphorylation in macrophages stimulated with LPS but not CpG (Sugimoto *et al.* 2004). This is sharp contrast to our findings (Figure 4.5), where we show that ERK phosphorylation is blocked after both LPS and CpG stimulation in Tpl-2 deficient macrophages and myeloid DC. One possible explanation for this discrepancy could be that the Tpl-2 deficient mice we used were generated in a different lab, although both mice show lack of the Tpl-2 kinase. Another explanation could be that the CpG used in the Sugimoto *et al.* (2004) study may be contaminated with a TLR independent ligand, which is responsible for the residual ERK phosphorylation. We test all CpG stocks for purity on TLR9 and MyD88 deficient mice and also confirm that no effects are observed in the TLR4 deficient mice to rule out endotoxin contamination to avoid this concern and can therefore vouch for the purity and specificity of CpG in our system.

We have shown that Tpl-2 may activate p38 as well as its target ERK upon stimulation of macrophages and particularly myeloid DC with LPS and CpG. We also saw this effect on p38 phosphorylation in myeloid DC stimulated with LPS or CpG in the presence of MEK inhibitor U0126 used at doses above 5μM. An explanation for the differences observed between the specificity on the inhibition ERK phosphorylation using U0126 or deletion of Tpl-2 could be that lack of Tpl-2 completely inhibits the activation of MEK, which inhibits the activation of ERK. In cells treated with U0126, both Tpl-2 and MEK are activated, but phosphorylation of ERK by MEK is blocked. Therefore, the effects of the abrogation Tpl-2 pathway may be more dramatic since Tpl-2, MEK and ERK phosphorylation are both inhibited and in some cases p38 phosphorylation is affected.

Chapter 4

Figures
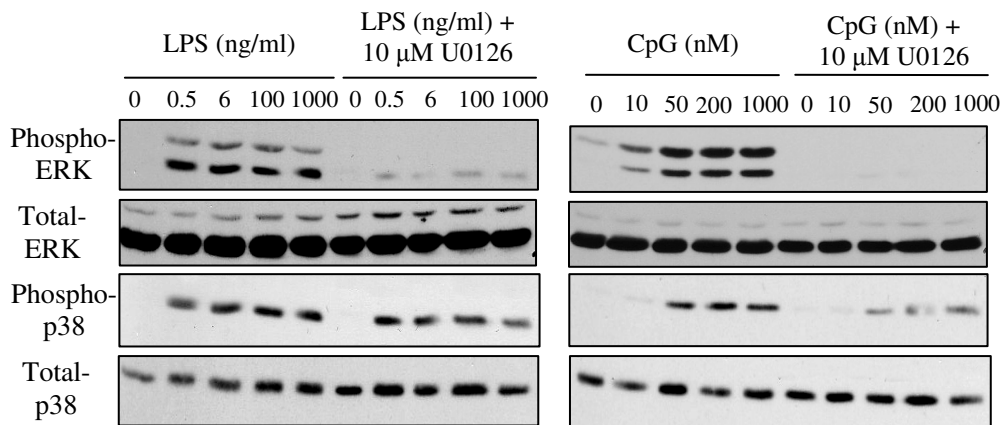
Figure 4.2: 10μM MEK inhibitor U0126 inhibits LPS and CpG induced ERK and p38 phosphorylation in myeloid DC. Myeloid DC were stimulated with LPS and CpG at the given concentrations in the presence or absence of 10μM U0126. Lysates were immunoblotted for the indicated antigens. LPS and CpG were run on separate gels. Exposure times are approximately the same for both LPS and CpG. Representative of 3 experiments.

a) Myeloid DC
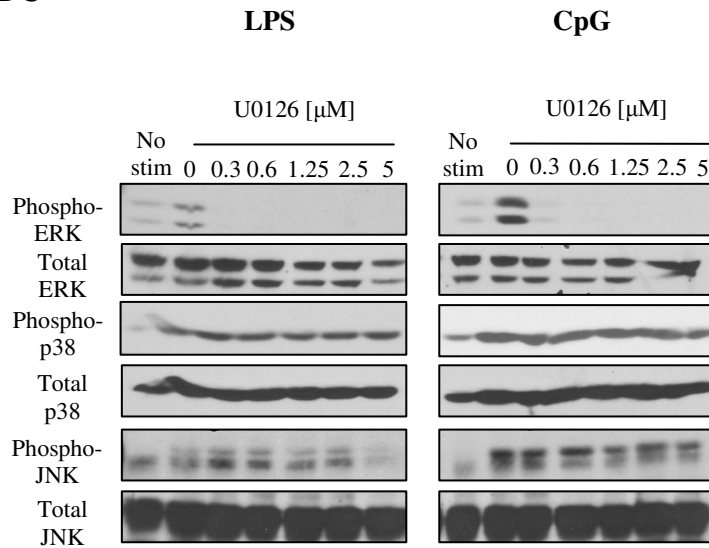
b) Macrophages
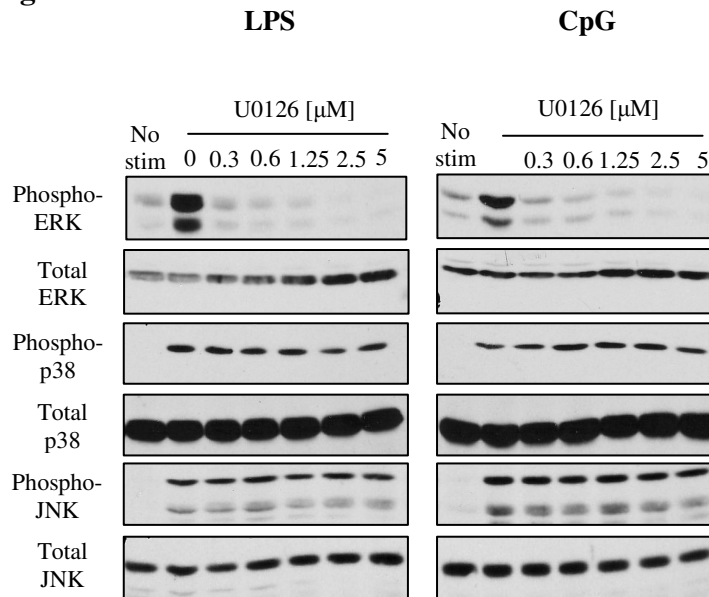
Figure 4.3: Low doses of U0126 inhibit LPS and CpG induced ERK but not p38 phosphorylation in myeloid DC and macrophages. a). Myeloid DC and b). Macrophages were stimulated with LPS (10ng/ml) and CpG (500nM) in presence of varying doses of U0126. Lysates were immunoblotted for phosphorylated and total forms of ERK, p38 and JNK. U0126 titrations have been done 3 times with similar results. LPS and CpG were run on the same gel. Representative of 3 eexperiments.

a) Macrophages LPS CpG

b) Myeloid DC LPS CpG
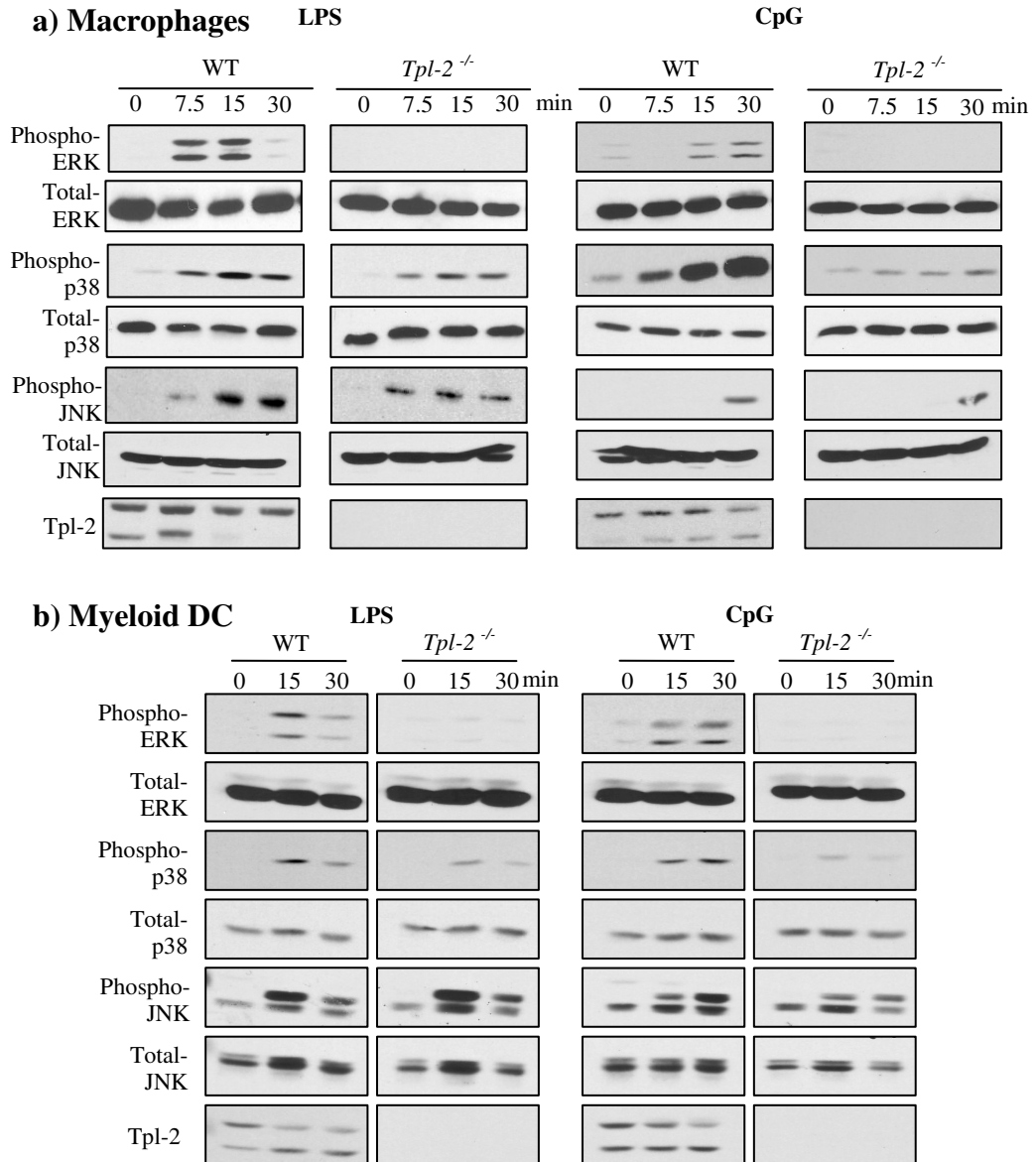
Figure 4.5: Lack of Tpl-2 abrogates TLR-induced ERK phosphorylation, but also impairs p38 phosphorylation. a) Macrophages and b) myeloid DC from *Tpl-2*^{-/-} and littermate controls were stimulated with LPS or CpG and lysates were immunoblotted for MAP kinases. Blots have repeated done 3 individual times with similar results. LPS and CpG were run on separate gels. Exposure times are approximately the same for both LPS and CpG. Representative of 3 experiments.

Chapter 5

Role of ERK phosphorylation in regulating cytokine

production by TLR stimulated macrophages and DC

5.0 Two approaches to determine the effect of ERK on cytokine production.

The main focus of this project was to study the regulation of cytokine production by the ERK MAP kinase pathway in macrophages and myeloid DC. We used the two approaches described in chapter 4 to determine the effect of ERK activation on production of TNF, IL-10, IL-12, and IFN-β.

5.1 Effect of the MEK inhibitor U0126 or Tpl-2 on TNF induction

5.1.1 Addition of MEK inhibitor U0126 inhibits TNF mRNA and protein production in macrophages and myeloid DC in response to LPS and CpG.

It has previously been shown that blocking ERK phosphorylation pharmacologically with U0126 decreases TNF cytokine production in macrophages and human monocytes (Scherle *et al.* 1998; Mancuso *et al.* 2002). We found that by inhibiting ERK activation in myeloid DC and macrophages upon stimulation with LPS or CpG using U0126, *Tnf* mRNA and protein were decreased (Figure 5.1.1). In both the myeloid DC and macrophages stimulated with LPS or CpG, the early *Tnf* mRNA was reduced by about 2-3 fold after treatment with U0126 (Figure 5.1.1a and 5.1.1c). However, by 6 hours stimulation little difference was observed in *Tnf* mRNA upon LPS stimulation in the presence of U0126 versus controls. Upon stimulation with CpG in the presence of U0126, a 2-3 fold inhibition was observed.

The reduction in *Tnf* mRNA resulted in reduced TNF protein production (Figure 5.1.1b and Figure 5.1.1d), in response to LPS or CpG, which was reduced in a dose-dependent manner by U0126 treatment. Again TNF production upon U0126 addition was only partially inhibited by about 3 fold upon stimulation of myeloid DC or macrophages upon stimulation with LPS or CpG.

5.1.2 Induction of both Tnf mRNA and TNF protein is reduced in Tpl-2 knockout macrophages or myeloid DC.

It has previously been reported that TNF induction in the Tpl-2 knockout macrophages is impaired due to post-transcriptional regulation (Dumitru *et al.* 2000). We further investigated the effects of *Tpl-2* deletion on TNF production by stimulating macrophages from 5 individual *Tpl-2* knockout mice and 5 individual littermate controls with LPS or CpG (Figure 5.1.2a). We lysed the cells at 0, 3, and 6 hours, extracted and purified the RNA and quantified the *Tnf* mRNA by real-time quantitative PCR. As seen in Figure 5.1.2a, the *Tnf* mRNA was reduced at 3 hours but returned to the same level as the littermate controls by 6 hours. The early reduction in *Tnf* mRNA is in contrast to the data by Dumitru *et al.* (2000), where the induction of *Tnf* mRNA levels by LPS were reported to be unaffected by the Tpl-2 deficiency (Dumitru *et al.* 2000).

Supernatant was collected from the same cells used for the preparation of mRNA and an immunoassay was performed to determine the effects of deletion of *Tpl-2* on TNF protein production (Figure 5.1.2b). Induction of TNF protein was completely inhibited in the Tpl-2 knockout macrophages in response to LPS or CpG. Our data

suggest that TNF may be regulated at both the transcriptional and post-transcriptional level by Tpl-2.

5.1.3 TLR induction of Tnf mRNA and TNF protein is impaired in Tpl-2 deficient myeloid DC.

We next investigated whether TNF production was regulated by Tpl-2 in myeloid DC. We stimulated purified myeloid DC generated from bone marrow from *Tpl-2* knockout mice and littermate controls with LPS or CpG (Figure 5.1.3). Induction of *Tnf* mRNA was reduced in the *Tpl-2* knockout DC stimulated with LPS or CpG as compared to littermate control cells (Figure 5.1.3a), similar to the effects seen in the macrophages. Likewise induction of TNF protein by LPS or CpG was reduced in *Tpl-2* deficient DC, however, production of TNF protein in the DC in response to LPS or CpG was not inhibited to the same degree as in the macrophages (Figure 5.1.3b).

5.1.4 Splenic DC from the Tpl-2 knockout have reduced TNF production.

As both the myeloid DC and macrophage have been cultured *in vitro* for 7 days before being used in any stimulation assays, we wanted to verify that the Tpl-2 was also required for optimal TNF induction by *ex vivo* DC. DC were purified from *Tpl-2* knockout and littermate control spleens by positive selection for CD11c$^+$ expression by flow cytometry. These cells were then stimulated with CpG for 20 hours and the supernatants collected and TNF protein measured by immunoassay. We chose CpG as

splenic DC have been shown to be more responsive to CpG than LPS stimulation (Boonstra *et al.* 2003). In the splenic DC from *Tpl-2* knockouts, TNF protein production was again reduced as compared to control DC, although not completely inhibited in the absence of *Tpl-2* (Figure 5.1.4). The level of inhibition was very similar to *in vitro* generated myeloid DC from the *Tpl-2* deficient mice (Figure 5.1.3b) and also similar to bone marrow derived myeloid DC treated with the U0126 inhibitor (Figure 5.1.1b).

5.1.6 Discussion.

The MEK inhibitor U0126 reduced but did not completely turn off induction of *Tnf* mRNA and TNF protein after stimulation with LPS or CpG in either macrophages or myeloid DC (Figure 5.1.1) indicating that ERK activation was not absolutely essential for TNF production. The effect of U0126 was much more pronounced on induction of the TNF protein than on *Tnf* mRNA. However, TNF protein induction in *Tpl-2* deficient macrophages was completely inhibited upon TLR stimulation (Figure 5.1.2b), whereas the effects on *Tnf* mRNA were transient, a reduction was only observed at 3 hours of stimulation and lost by 6 hours in *Tpl-2* deficient mice as compared to wildtype controls (Figure 5.1.2a). Both myeloid and splenic DC from *Tpl-2* deficient mice produced low levels of TNF protein upon TLR stimulations as compared to the wildtype cells although effects were not as pronounced as in macrophages where TNF protein was completely abrogated in *Tpl-2*^{-/-} mice (Figure 5.1.3 and 5.1.4). The more pronounced effect of U0126/*Tpl-2* deficiency on TNF protein levels versus mRNA levels may be explained by previous studies by Dumitru *et al.* (2000), who showed that the Tpl-2/ERK pathway

regulated TNF production at a post-transcriptional level as they found that LPS stimulated macrophages from *Tpl-2* deficient mice produced no TNF protein but *Tnf* mRNA was not affected as compared to the wildtype macrophages. In a previous report by Dumitru *et al.* (2000), it was found that *Tnf* mRNA was upregulated normally in *Tpl-2* deficient macrophages in response to LPS, which led them to conclude that Tpl-2 and its downstream phosphorylation of ERK regulated TNF post-transcriptionally. In contrast to this study, we have found that induction of *Tnf* mRNA and protein in response to LPS or CpG is impaired in *Tpl-2* deficient macrophages and myeloid DC at early time points. However, in our study at 6 hours the *Tnf* mRNA was the same in both the *Tpl-2*$^{-/-}$ and the littermate control mouse cells (Figure 5.1.2). In the study by Dumitru *et al.* (2000), the levels of *Tnf* mRNA may have been at too high a level to quantitate accurately, since the cells were stimulated with a very high concentration of LPS (1µg/ml) and analysed by Northern blot using only one time point. Furthermore our studies point out small effects at 3 hours after stimulation, which are lost by 6 hours. Therefore subtle changes in *Tnf* mRNA expression in the *Tpl-2* deficient macrophages may have been missed in the Dumitru *et al.* (2000) study. Our data suggest that to a small extent, TNF induction was regulated by Tpl-2/ERK both at the level of mRNA induction but support a role for post-transcriptional regulation in both macrophages and myeloid DC by *Tpl-2*, since the effects of Tpl-2/ERK abrogation on the protein was more pronounced than those on the mRNA (Figure 5.1.2 and 5.1.3). Both these cell types responded in a similar way upon stimulation with LPS and CpG, where we detected a decrease in *Tnf* mRNA in *Tpl-2* deficient macrophages at 3 hours, which returned to normal after 6 hours. We also detected a decrease in TNF protein. We also

110

verified the effects on TNF protein in purified splenic DC. Again we saw a reduction in TNF in the *Tpl-2^{-/-}* cells compared to the littermate controls (Figure 5.1.4). Thus the data obtained from *ex vivo* generated DC are very similar to the data obtained from *in vitro* generated DC and macrophages.

We show here however that *Tnf* mRNA expression was clearly reduced when ERK activation was blocked by U0126 or *Tpl-2* deficiency. These data indicate that the Tpl-2/ERK pathway regulates TNF production at two levels; mRNA induction and post-transcriptionally although the effects on *Tnf* mRNA are small and transient. We found also however that the dramatic effects on TNF protein of blocking ERK signalling were in keeping with recent studies (Rousseau *et al.* 2008). It has been shown that production of TNF is dependent TACE, which cleaves pre-TNF into soluble TNF (Black *et al.* 1997). Recently Rousseau *et al.* (2008) have reported that ERK activation was required to phosphorylate TACE in LPS stimulated macrophages for TNF protein production. This could in part explain the failure to induce TNF protein in *Tpl-2* deficient macrophages as these do not activate ERK upon TLR stimulation. TACE has recently been identified as a substrate of ERK (Rousseau *et al.* 2008), required for TNF production. Our data are in keeping with showing that ERK activation is important for TNF protein production, to a greater extend than *Tnf* mRNA induction (Figure 5.1.2). We observed that whereas in *Tpl-2* deficient cells show complete abrogation of TNF protein in response to LPS or CpG, ERK activation is completely abrogated and p38 activation is decreased (Figure 4.5), whereas cells treated with U0126 show complete block in ERK activation without affecting p38 (Figure 4.3). The difference in the effects with U0126 and *Tpl-2* deficiency on TNF protein induction indicates that other

pathways than ERK may play a role. Indeed we show in Figure 4.5 that p38 phosphorylation is decreased in $Tpl-2^{-/-}$ macrophages and DC. Tpl-2 has been reported to function as a MAP3 kinase directly upstream of MEK and ERK in macrophages and it has been reported that deletion of *Tpl-2* does not affect activation of any other MAP kinase pathways (Dumitru *et al.* 2000; Sugimoto *et al.* 2004). However, in overexpression experiments, Tpl-2 can induce activation of ERK, JNK and p38 (Patriotis *et al.* 1994; Salmeron *et al.* 1996). We have shown that at the doses used, U0126 does not affect the phosphorylation of p38, but we found that the lack of *Tpl-2* not only impaired TLR-induced ERK phosphorylation but also reduced p38 phosphorylation, albeit to a lesser extent in both macrophages and DC (Figure 4.5). In *Tpl-2* deficient macrophages, the reduction of p38 phosphorylation was more pronounced upon stimulation with CpG than with LPS. In *Tpl-2* deficient myeloid DC, however, the reduction of p38 activation was similar with LPS or CpG stimulations. One of the reasons for the differences seen in p38 phosphorylation levels could be that LPS stimulation induces a much stronger p38 phosphorylation signal than is observed with CpG and thus effects may be more difficult to observe. The reasons for the differences in signal strength are not known. One possibility is that CpG has to be internalised before it stimulates the cell in contrast to LPS, which stimulates directly at the plasma membrane. Alternatively, the fact that LPS utilises both the MyD88/TIRAP and the TRIF/TRAM pathways, whereas CpG only utilises the MyD88 pathway may be important (Akira and Takeda 2004). The differences observed in TNF protein production between the macrophages and DC could be due to the fact that DC produce more TNF than macrophages or that DC use an additional pathway to also induce TNF.

The overall conclusion is that Tpl-2 is a MAP3 kinase that is required for ERK phosphorylation, but also plays a minor role in p38 phosphorylation, possibly by functioning as a MAP3 kinase for MKK3/MKK6. This could explain why TNF was more affected in the *Tpl-2* knockout than with the U0126 inhibitor.

In summary we show that ERK/Tpl-2 regulates transcription of TNF protein in keeping with Dumitru *et al.* (2000) but not posttranscriptionally. The U0126 inhibitor completely abrogates ERK activation but not TNF protein, whereas abrogation of *Tpl-2* lead to complete inhibition of TNF protein. We show that abrogation of *Tpl-2* not only inhibits ERK phosphorylation but also affects p38 phosphorylation suggesting that there may be another pathway other than ERK, which plays a role in induction of TNF protein.

5.2 Regulation of IL-12, IFN-β and IL-10 by Tpl-2 and ERK

5.2.1 Background.

It has been shown that the MEK inhibitor U0126 can downregulate IL-10 protein production and enhance the production of IL-12p40 in macrophages stimulated with LPS or CpG (Hacker *et al.* 1999; Yi *et al.* 2002). Conflicting studies on the role of ERK activation in the regulation of IL-12 in myeloid DC have been published (Hacker *et al.* 1999; Agrawal *et al.* 2003). Hacker *et al.* (1999) found that stimulation with LPS or CpG of murine myeloid DC in the presence of a MEK inhibitor did not lead to increased IL-12 production as compared to controls. On the other hand, Agrawal *et al.* (2003) reported that inhibition of ERK activation led to increased IL-12p70 in human DC. Splenic DC from *erk1* deficient mice also produced increased levels of IL-12p70 upon LPS stimulation as compared to wildtype mice (Dillon *et al.* 2004). Since these studies conflicted, it therefore remained unclear whether ERK played a role in regulating IL-12 production in myeloid DC.

It is known that IL-10 production negatively regulates IL-12 production by macrophages and DC in response to many stimuli (Moore *et al.* 2001). Whether the reported upregulation of IL-12 production caused by ERK inhibition in macrophages is a direct effect on the IL-12 gene or due to the reduced levels of induced IL-10 remains to be clarified. Yi *et al.* (2002) have shown that in splenocytes from IL-10 deficient mice pharmacological inhibition of ERK phosphorylation did not further enhance IL-12p40 and IL-12p70 production suggesting that the increased IL-12 was due to decreased IL-10

caused by the MEK inhibitor. However, the splenocytes consist of a mixture of cells, which may mask effects on individual populations, such as macrophages and DC.

In another study, Hacker *et al.* (2006) suggested that IL-10 and type I IFN are co-regulated. ERK has been shown to positively controls IL-10 production in macrophages. Thus we set out to determine the effects of the Tpl-2 pathway on macrophages and DC to resolve the conflicting reports and secondly to investigate whether ERK activity similarly regulated IFN-β production.

5.2.2 MEK inhibitor U0126 inhibits IL-10 but enhances IL-12p40, and IFN-β production in macrophages.

Consistent with earlier studies by Yi *et al.* (2002), macrophages stimulated with LPS or CpG in the presence of U0126 showed decreased *Il-10* mRNA induction and decreased IL-10 protein as compared to control cells (Figure 5.2.2). In contrast, *Il-12p40* mRNA induction and IL-12p40 protein levels were increased. *Il-12p35* mRNA induction or IL-12p70 protein could not be detected in the macrophages in response to LPS or CpG, even in the presence of U0126. Interestingly, levels of *Ifn-β* mRNA and IFN-β protein were also increased in macrophages stimulated with LPS or CpG in the presence of U0126 relative to vehicle control treated cells.

5.2.3 IL-10 is decreased and IL-12p40, IL-12p70 and IFN-β are increased in myeloid DC treated with U0126 upon stimulation with LPS or CpG.

To resolve conflicting results of ERK phosphorylation on the regulation of IL-12 in DC in the literature, we wanted to clarify in our own studies a potential role for ERK activation in the regulation of IL-10 or IL-12 production in this cell type.

Figure 5.2.3 shows that *Il-10* mRNA and IL-10 protein were decreased upon LPS or CpG stimulation in the presence of U0126. In contrast, *Il-12p40* mRNA and IL-12p40 protein levels were increased upon LPS or CpG stimulation in the presence of U0126. The increase in IL-12p40 protein upon U0126 treatment in DC stimulated with LPS was small, although a greater effect was observed at the mRNA level. *Il-12p35* mRNA was increased whereas IL-12p70 protein was not detectable following LPS stimulation in the presence of U0126. *Il-12p35* mRNA and IL-12p70 protein were both increased in the presence of U0126 after stimulation with CpG. *Ifn-β* mRNA and IFN-β protein were also increased upon LPS and CpG stimulation in the presence of U0126. U0126 therefore had similar effects on IL-10, IL-12 and IFN-β production in macrophages and myeloid DC.

5.2.4 The induction of IL-10 is decreased and IL-12 and IFN-β is increased in Tpl-2 deficient macrophages.

To date there has been only been one report on the effect of *Tpl-2* deficiency in macrophages on IL-10 and IL-12p40 production (Sugimoto *et al.* 2004). It was found

that IL-10 protein induction was increased in response to LPS and CpG in the *Tpl-2* deficient macrophages as compared to littermate controls. This result is surprising since our data show that blockade of ERK activation with U0126 decreases IL-10 induction after LPS and CpG stimulation of macrophages. Sugimoto *et al.* (2004) also found that IL-12p40 production was increased in *Tpl-2* deficient macrophages upon CpG stimulation but not altered upon LPS stimulation in the *Tpl-2* deficient macrophages relative to wildtype cells (Sugimoto *et al.* 2004). Sugimoto *et al.* (2004) also showed that CpG induced ERK phosphorylation was found to be unaffected in the *Tpl-2* deficient macrophages, in contrast to LPS stimulation where ERK phosphorylation was completely abrogated. This is in sharp contrast to our studies where ERK activation was blocked in *Tpl-2* deficient macrophages after LPS or CpG stimulation. Furthermore, our U0126 data show that blockade of ERK activation increases IL-12 production after both LPS and CpG stimulation. In this section we investigated further the role of *Tpl-2* in regulating IL-10 and IL-12 production by macrophages stimulated with LPS or CpG. We stimulated *Tpl-2* deficient and littermate control macrophages with LPS and CpG and examined cytokine mRNA induction by real-time quantitative PCR (Figure 5.2.4a and c) and cytokine production by immunoassay (Figure 5.2.4b and d). We found that IL-10 was always decreased at both the mRNA and protein level in *Tpl-2* deficient macrophages stimulated with LPS or CpG compared to littermate control cells. In contrast, *Il-12p40* and *Il-12p35* mRNA as well as IL-12p40 and IL-12p70 protein were increased in the *Tpl-2* deficient macrophages in response to LPS and CpG stimulation as compared to the littermate control macrophages. *Ifn-β* mRNA and IFN-β protein levels were also elevated in the *Tpl-2* deficient macrophages compared to the littermate

117

controls after stimulation with LPS or CpG. These data are consistent with but more pronounced than our findings with the U0126 inhibitor.

5.2.5 IL-10 is decreased and IL-12 and IFN-β is increased in Tpl-2 deficient myeloid DC.

Sugimoto *et al.* (2004) have reported that IL-12p40 was increased but that IL-10 production was also increased in response to LPS and CpG in *Tpl-2* deficient macrophages as compared to littermate control macrophages. However, in *Tpl-2* deficient myeloid DC they only reported an increase in IL-12p40 but did not mention IL-10.

To investigate the effects on Tpl-2 on the production of IL-10, IL-12 and IFN-β, we stimulated *in vitro* generated bone marrow-derived myeloid DC, which were purified by flow cytometry on the basis of CD11c^{+} from *Tpl-2* deficient mice and littermate controls with LPS and CpG and examined cytokine mRNA induction by real-time quantitative PCR (Figure 5.2.5a and c) and cytokine protein production by immunoassay (Figure 5.2.5b and d). The effects of *Tpl-2* deficiency in the myeloid DC were similar to those found in the macrophages. *Il-10* mRNA and IL-10 protein levels were significanty decreased, whereas *Il-12p40* mRNA and IL-12p40 protein levels and *Il-12p35* mRNA and IL-12p70 protein levels were increased upon LPS or CpG stimulation of *Tpl-2* deficient myeloid DC as compared to the littermate controls. Furthermore, *Ifn-β* mRNA and IFN-β protein levels were also increased in the *Tpl-2* deficient myeloid DC

compared to the littermate control cells. Effects on IL-12 protein were more pronounced than on mRNA expression.

5.2.6 Splenic DC from Tpl-2 deficient mice produced increased levels of IL-12 and IFN-β upon stimulation with CpG.

As all the data so far were obtained using macrophages or myeloid DC, which have been cultured *in vitro* for 7 days before stimulation, we wanted to confirm their physiological relevance using *ex vivo* cells that had not been cultured. To do this, we purified splenic DC by flow cytometry from *Tpl-2* deficient mice and littermate controls and stimulated them with CpG (TLR9) for 20 hours after which supernatants were harvested (Figure 5.2.6). Splenic DC only express low levels of TLR4 and therefore respond poorly to LPS stimulation (Boonstra *et al.* 2003). In *Tpl-2* deficient cells, IL-12p40, IL-12p70, and IFN-β protein were increased upon stimulation as compared to splenic DC from littermate controls. However, there was no detectable IL-10 in either the littermate control or knockout splenic DC. The upregulation of IL-12 and IFN-β in the absence of *Tpl-2* were similar to the findings we had with bone marrow derived macrophages and myeloid DC generated *in vitro* from the *Tpl-2* deficient mice.

5.2.7 IL-12 production is regulated through an ERK dependent and independent pathway via IL-10.

We have shown that in both macrophages and myeloid DC from *Tpl-2* deficient mice or in wildtype macrophages and myeloid DC treated with U0126, the inflammatory cytokines IL-12p40 and IFN-β were increased, whereas the regulatory cytokine IL-10 was decreased upon LPS or CpG stimulation. In wildtype macrophages stimulated with LPS or CpG in the presence or absence of U0126, IL-12p70 could not be detected but in wildtype DC stimulated with CpG, IL-12p70 induction could be observed. However, *Tpl-2* deficient macrophages produced IL-12p70 in response to LPS and CpG. Thus Tpl-2 negatively regulated the induction of IL-12p70. The observed increased levels of IL-12 and IFN-β following blockade of the Tpl-2/ERK pathway could result indirectly from the reduced levels of IL-10. Alternatively, the Tpl-2/ERK pathway may directly regulate the induction of IL-12 and IFN-β.

We investigated whether the increase in IL-12p40 and IL-12p70 we detected in the *Tpl-2* deficient cells and in the littermate control cells treated with U0126 was a direct negative effect of the ERK signalling pathway on IL-12 induction or rather the indirect consequence of IL-10 downregulation. To address this question, macrophages from IL-10 deficient mice and littermate controls were stimulated with LPS and CpG in the presence or absence of U0126 (Figure 5.2.7). In the absence of U0126, we observed that both IL-12p40 and IL-12p70 were increased in the IL-10 deficient macrophages as compared to littermate control cells as previously documented (Moore *et al.* 2001).

However, upon addition of U0126 to the IL-10 deficient macrophages stimulated with LPS or CpG, IL-12p40 and IL-12p70 production were further enhanced.

We conclude that the Tpl-2/ERK pathway does downregulate IL-12 in part by inducing the production of IL-10. However, the dramatic increase in IL-12 levels in LPS or CpG stimulated IL-10 deficient macrophages after U0126 treatment, indicates that IL-12 induction was also negatively regulated directly by ERK activation independently of IL-10. This has not previously been reported and is in contrast to data by Yi *et al.* (2002), who showed no effect on IL-12 in IL-10 deficient whole spleen cells treated with U0126.

5.2.8 IL-12 is regulated by both ERK and IL-10 as shown by the Tpl-2/IL-10 double knockout.

To corroborate these pharmacological data, we generated mice that were deficient in both *Tpl-2* and IL-10. We stimulated macrophages from these mice with LPS and CpG and compared them to IL-10 deficient littermate control cells (Figure 5.2.8). We found that in the absence of both *Tpl-2* and IL-10, IL-12p40 and IL-12p70 were dramatically increased as compared to the IL-10 deficient cells. The data are consistent with the effects of the U0126 inhibitor (Figure 5.2.7). We therefore conclude that IL-12 production is negatively regulated directly via ERK signalling independently of IL-10 in addition to the effect mediated via IL-10.

We also found that TNF protein levels were reduced in the Tpl-2/IL-10 deficient macrophages as compared to the IL-10 deficient macrophages upon stimulation with

121

LPS or CpG. This suggests that TNF is upregulated through ERK but also that ERK-induced IL-10 has a negative effect on TNF production.

5.2.9 Discussion.

We have used two approaches to examine the role of ERK activation on the production of IL-10, IL-12 and IFN-β by macrophages and DC stimulated with LPS and CpG. First, we used the MEK inhibitor U0126 to block ERK phosphorylation. U0126 completely inhibited phosphorylation and activation of ERK, whilst not affecting phosphorylation of p38 and JNK at the doses used in this study (Figure 4.1). Second, we used cells from *Tpl-2* deficient mice. LPS and CpG induction of ERK phosphorylation was abrogated in *Tpl-2* deficient macrophages, consistent with published data (Dumitru *et al.* 2000). However, we have now found that phosphorylation of p38 was also reduced, albeit to a lesser extent, in both *Tpl-2* deficient macrophages and myeloid DC upon stimulation with LPS or CpG (Figure 4.3). Thus, the effects of *Tpl-2* deficiency on cytokine production may result from impaired activation of both ERK and p38 after TLR stimulation.

We have shown macrophages stimulated with LPS or CpG in the presence of U0126 displayed decreased induction of IL-10 mRNA and protein (Figure 5.2.1). These data are consistent with Yi *et al.* (2002), who showed that the RAW 264.1 macrophage cell line had decreased IL-10 production in the presence of U0126 upon stimulation with LPS or CpG. We also found that *Tpl-2* deficient macrophages had decreased IL-10 upon stimulation with LPS and CpG as compared to littermate control cells (Figure 4.2.3).

This finding is in contrast data from Sugimoto *et al.* (2004), who showed increased levels of IL-10 protein upon macrophage stimulation with CpG resulting from *Tpl-2* deficiency. One possible explanation for the differences observed between our data and those of Sugimoto *et al.* (2004), could be that the *Tpl-2* deficient mice we used were generated in different labs, although both mice show complete lack of the *Tpl-2* kinase. Another explanation could be that the CpG used in the Sugimoto study was contaminated with a non-TLR ligand that activates ERK independently of Tpl-2. We test all our CpG stock on TLR4, TLR9 and MyD88 deficient mice and have ensured that there is no contamination present.

In another study supporting our findings, it was found that activation of ERK was abrogated in NF-κB1 deficient macrophages, which have markedly reduced Tpl-2 protein, and this led to reduced levels of IL-10 protein upon stimulation with both LPS and CpG. Reconstitution with RAF:ER restored ERK phosphorylation in the NF-κB1 deficient macrophages and induction of IL-10 protein (Banerjee *et al.* 2006). Together with our data, these results strongly indicate that ERK activation positively regulates IL-10 production.

We have shown that both IL-10 mRNA and protein levels are reduced in myeloid DC stimulated with LPS and CpG in the presence of U0126 (Figure 5.2.2). This is in agreement with Agrawal *et al.* (2003), who showed that human DC stimulated with LPS in the presence of the MEK inhibitor U0126 had decreased levels of IL-10 protein. In addition, we confirmed this positive role for ERK activation by demonstrating that LPS and CpG induction of IL-10 is also reduced in *Tpl-2* deficient myeloid DC (Figure 5.2.4).

We have shown that macrophages stimulated with LPS or CpG in the presence of U0126 have increased IL-12p40 at both the mRNA and protein levels (Figure 5.2.1). This is consistent with data from Yi *et al.* (2002), who found that both IL-12p40 and IL-12p70 protein levels are increased in RAW macrophages in the presence of U0126 upon stimulation with either LPS or CpG. We extended these findings using *Tpl-2* deficient mice, where we found that *Il-12p40* mRNA and IL-12p40 protein, as well as *Il-12p35* mRNA and IL-12p70 protein levels were increased upon stimulation with LPS or CpG in *Tpl-2* deficient macrophages as compared to wildtype controls (Figure 5.2.3). This finding agrees with data from Sugimoto *et al.* (2004), who found IL-12p40 protein production is increased in macrophages stimulated with LPS or CpG in the absence of *Tpl-2*. We could not detect any IL-12p70 protein in the presence of U0126 in wildtype macrophages, which could be due to the sensitivity of our assay. In *Tpl-2* deficient macrophages we consistently detected an increase in IL-12p70 levels upon LPS or CpG stimulation. The difference in the effect of *Tpl-2* deficiency compared to U0126 treatment with respect to IL-12p70 induction may suggest a function for Tpl-2 additional to the effect on ERK regulation. One possibility could be via p38 activation since we show a decrease in p38 phosphorylation in *Tpl-2* deficient macrophages and DC (Figure 4.3), although p38 has been suggested to be required for IL-12 induction (Tanaka *et al.* 2002). Thus it is possible that another downstream signal of Tpl-2 independent ERK activation negatively regulates IL-12.

In myeloid DC stimulated with LPS or CpG, we found IL-12p40 and IL-12p70 production was increased at both the mRNA and the protein level by U0126 treatment (Figure 5.2.2). Consistent with our data, Agrawal *et al.* (2003), found that human

monocyte derived DC stimulated with LPS in the presence of U0126 produced increased IL-12p70 protein. However, Hacker *et al.* (1999) found that myeloid DC stimulated with CpG in the presence of a MEK inhibitor did not lead to increased levels of IL-12 in keeping with the lack of observed ERK phosphorylation in the cells used in their study. However, as discussed earlier Hacker *et al.* (1999) did show that IL-12 is increased in macrophages following MEK inhibition. A possible explanation for the discrepancy between our data to those of Hacker *et al.* (1999) could be that the stimulation time points used in the Hacker *et al.* (1999) study are only 3 and 7 hours for IL-12 protein induction whereas we have shown that IL-12 protein was induced only after 20 hours stimulation with CpG (Figure 3.3). Also the lack of ERK phosphorylation reported by Hacker *et al.* (1999) in DC in response to TLR ligation maybe due to lack of sensitivity in their assays compounded by high background levels of ERK phosphorylation in DC in the absence of stimuli.

We confirmed the inhibitory effect of ERK activation reported by Agrawal *et al.* (2003) on the induction of IL-12p40 and IL-12p70 in myeloid DC stimulated with LPS or CpG from *Tpl-2* deficient mice (Figure 5.2.4). These data are in keeping with the results by Sugimoto *et al.* (2004), who showed that *Tpl-2* deficient myeloid DC stimulated with LPS or CpG had increased IL-12p40 levels as compared to littermate control DC.

Using macrophages from IL-10 deficient mice, we have shown that in the absence of IL-10, IL-12 levels are enhanced following LPS or CpG stimulation (Figure 5.2.8 and 5.2.7). Additionally, pharmacological blockade of ERK phosphorylation increased IL-12 levels in the IL-10 deficient cells. Therefore the negative effects of ERK

activation on IL-12 production are mediated both indirectly via IL-10 production and directly via effects on IL-12 transcription by as yet unknown downstream molecules. This is in contrast to data obtained by Yi *et al.* (2002), who showed that splenocytes from IL-10 deficient mice stimulated with CpG in the presence of U0126 did not show enhanced IL-12p40 and IL-12p70 protein levels. An explanation for this discrepancy could be that we used macrophages, whereas Yi *et al.* (2002) used whole spleen cell suspensions, which have a mixed cell population and therefore masked the effects on macrophages and DC. Furthermore of note was that Yi *et al.* (2002) detected IL-10 in splenocytes from *Il-10$^{-/-}$* indicating that the mice could have been mistyped.

A possible mechanism of ERK regulation of IL-12 transcription has been suggested to be dependent on c-Fos induction (Agrawal *et al.* 2003; Dillon *et al.* 2004). Agrawal *et al*. (2003) show that stimulation of human monocyte-derived DC stimulated with LPS (TLR4), flagellin (TLR5), Pam3cys (TLR2) or schistosome egg antigen (SEA) (Th2 stimulus) induced enhanced levels of c-Fos, however, Pam3cys and SEA induced greater levels of c-Fos when compared to DC stimulated with LPS or flagellin. Inhibition of c-Fos expression by si-RNA showed that all the stimuli used produced enhanced levels of IL-12p70. In the absence of c-Fos, even a Th2 stimuli such as SEA produced high levels of IL-12p70 and thus skewing the response towards a Th1 profile. Agrawal *et al*. (2003) concludes that c-Fos is important in regulating IL-12p70 production. Dillon *et al*. (2004), showed that wildtype murine splenic DC stimulated LPS or Pam3cys produce low levels of IL-12p70 and high level of IL-10, whereas *c-fos$^{-/-}$* DC produce enhanced levels of IL-12p70 and reduced levels of IL-10. This again indicates that c-Fos is important in the regulation of IL-12, but c-Fos. It is as yet

unknown whether c-Fos has a direct effect to regulate IL-12 production or does so by induction of IL-10.

It has been shown that sustained ERK activation results in phosphorylation and stabilisation of c-Fos (Murphy *et al.* 2002). The *tpl-2*^{-/-} mouse cannot activate ERK and upon stimulation with LPS or CpG induces enhanced levels of IL-12 and reduced levels of IL-10 as compared to the wildtype. Figure 5.2.9 shows a proposed potential mechanism of regulation of IL-10, IL-12 and IFN-β by ERK. By summarising the data from Agrawal *et al.* (2003), Dillon *et at.* (2004), Banerjee *et al.* (2006) and our own results, three possible pathways of IL-12 and IFN-β regulation by ERK can be identified (Figure 5.2.9). 1). ERK activation negatively regulates IL-12 and IFN-β directly through c-Fos. 2). ERK activation negatively regulates IL-12 and IFN-β indirectly through transcription factor c-Fos via IL-10. 3). ERK activation indirectly regulate IL-12 and IFN-β through another transcription factor via IL-10. 4). ERK activation negatively regulates IL-12 and IFN-β directly through another transcription factor.

Finally, we have shown that IFN-β is upregulated at both the mRNA and the protein levels in macrophages and myeloid DC stimulated with LPS or CpG in the presence of MEK inhibitor U0126 or in the absence of *Tpl-2* (Figures 5.2.1, 5.2.2, 5.2.3 and 5.2.4). We do not know whether this upregulation of IFN-β production is caused by the reduced levels of IL-10 seen in the absence of ERK activation. Recently, it was suggested that IL-10 and type I IFN are co-ordinately regulated (Hacker *et al.* 2006). This group showed that macrophages from TRAF3 deficient mice showed defective upregulation of both IL-10 and type I IFN production, whereas levels of IL-12 and IL-6 were increased in TRAF3 deficient bone marrow derived macrophages stimulated with

127

TLR ligands. TRAF3 is an important adaptor protein in TLR signalling, which is recruited to MyD88 (Hacker *et al.* 2006). From these data the group concluded that IL-10 and type I IFN are co-ordinately regulated and that the other proinflammatory cytokines were enhanced due to reduced levels of IL-10 in the TRAF3 deficient macrophages. However, in another study it was shown that IL-10 production induced by LPS was reduced in macrophages lacking either MyD88 or TRIF (Boonstra *et al.* 2006), however, IFN-β induction by LPS was unaffected in MyD88 deficient macrophages but reduced in TRIF deficient macrophages (Boonstra *et al.* 2006). This already suggested that IFN-β and IL-10 may be independently regulated since IFN-β production in macrophages stimulated with LPS is dependent on TRIF and not MyD88, whereas IL-10 is dependent on both. Consistent with the independent regulation of IL-10 and IFN-β, our data indicate that the Tpl-2/MEK/ERK pathway positively regulates IL-10 production but negatively controls type I IFN production since IFN-β is increased by the addition of U0126 to wildtype cells or in *Tpl-2* deficient macrophages and DC as compared to littermate control cells. However, it is unclear whether the elevated IFN-β levels are the consequence of reduced levels of IL-10 or a direct effect of ERK activation on IFN-β induction. Regardless, although upstream adaptors and signalling molecules such as MyD88, TRIF and TRAF3 may co-ordinately regulate IL-10 and IFN-β there appears to be independent downstream signal in response to ERK phosphorylation, which may lead to differential regulation of IL-10 and IL-12 and IFN-β in macrophages and DC.

In conclusion, we have shown that in the absence of Tpl-2/ERK activation, IL-10 production is decreased and IL-12 and IFN-β production are increased in both

128

macrophages and myeloid DC. Our data show for the first time that IL-12 and IFN-β are negatively regulated by IL-10 as previously reported but we also show that the IL-12 and IFN-β are negatively regulated by Tpl-2/ERK activation in the complete absence of IL-10 in myeloid DC and macrophages.

Chapter 5

Figures

CpG

Figure panels:

- **TNF** (LPS / LPS + U0126) — TNF mRNA vs Hours
- **TNF** (CpG / CpG + U0126) — TNF mRNA vs Hours
- **b)mRNA M0** TNF — TNF mRNA vs Hours
- **c) Protein DC** TNF — TNF pg/ml vs U0126 uM
- **d) Protein M0** TNF — TNF pg/ml vs U0126 uM
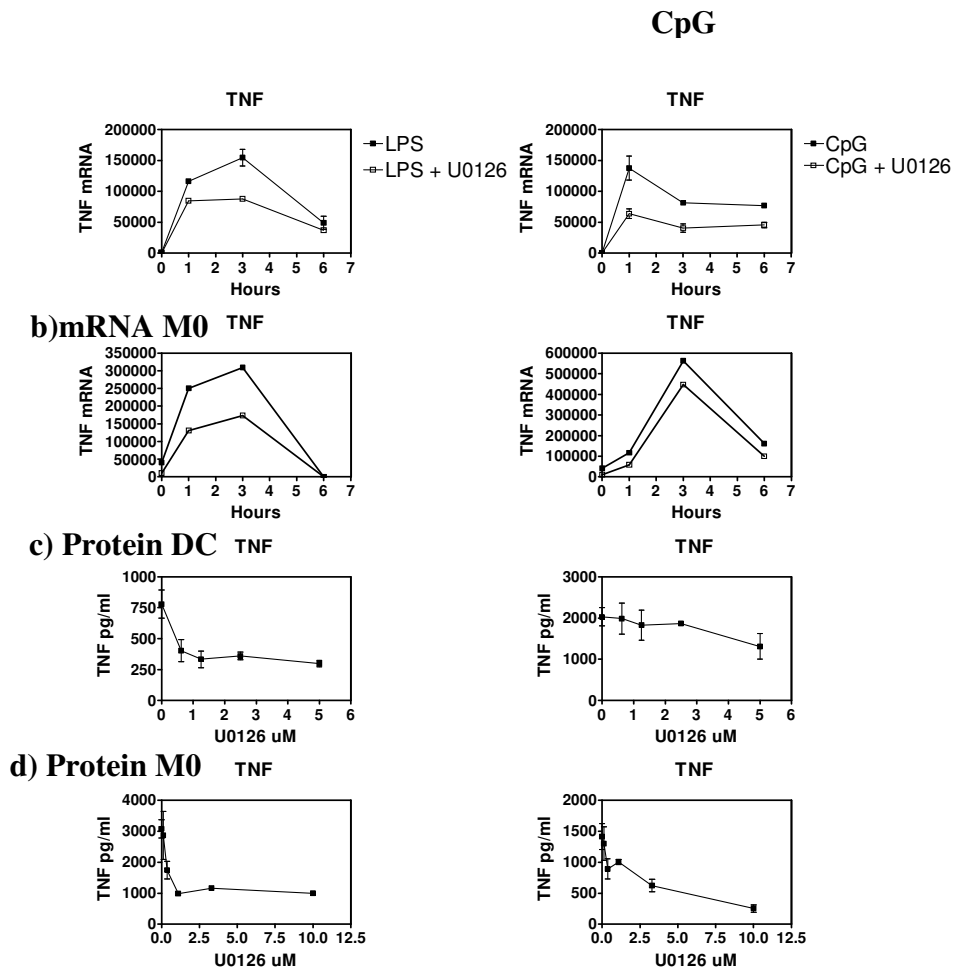
Figure 5.1.1: U0126 inhibits LPS and CpG induction of TNF mRNA and protein in macrophages and myeloid DC. a and b) Myeloid DC and macrophages were stimulated with LPS or CpG and mRNA levels determined real-time quantitative PCR. c and d) Supernatants were collected at 6 hours for quantitation of TNF protein by immunoassay.

a) mRNA

LPS / CpG plots with axes TNF mRNA, WT/KO at 0 hours, 3 hours, 6 hours.

b) Protein

Protein plots with axes TNF pg/ml, WT/KO at 0 hr, 3 hr, 6 hr, 20 hr.
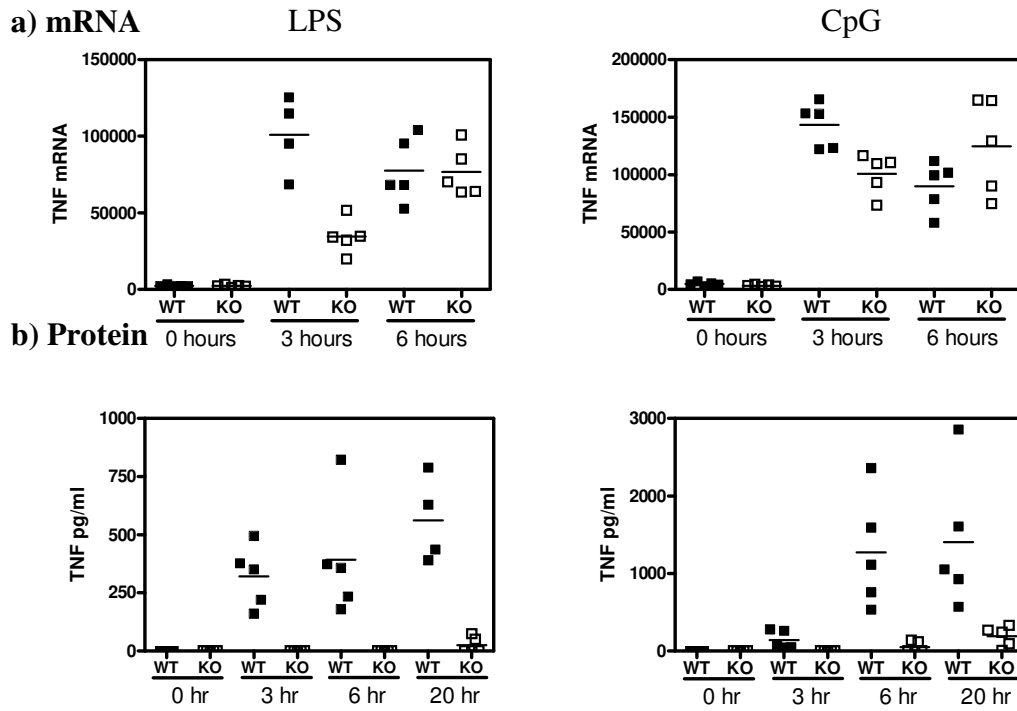
Figure 5.1.2:TLR induction of both TNF mRNA and protein is reduced in the *Tpl-2-/-* macrophages. Macrophages from *Tpl-2-/-* (open squares) and littermate controls (closed squares) were stimulated with LPS or CpG for the given times. a) Cells were lysed for RNA and real-time quantitative PCR preformed. b) Supernatants were collected for immunoassay.

a) mRNA

LPS

TNF

CpG

TNF

b) Protein

TNF

TNF
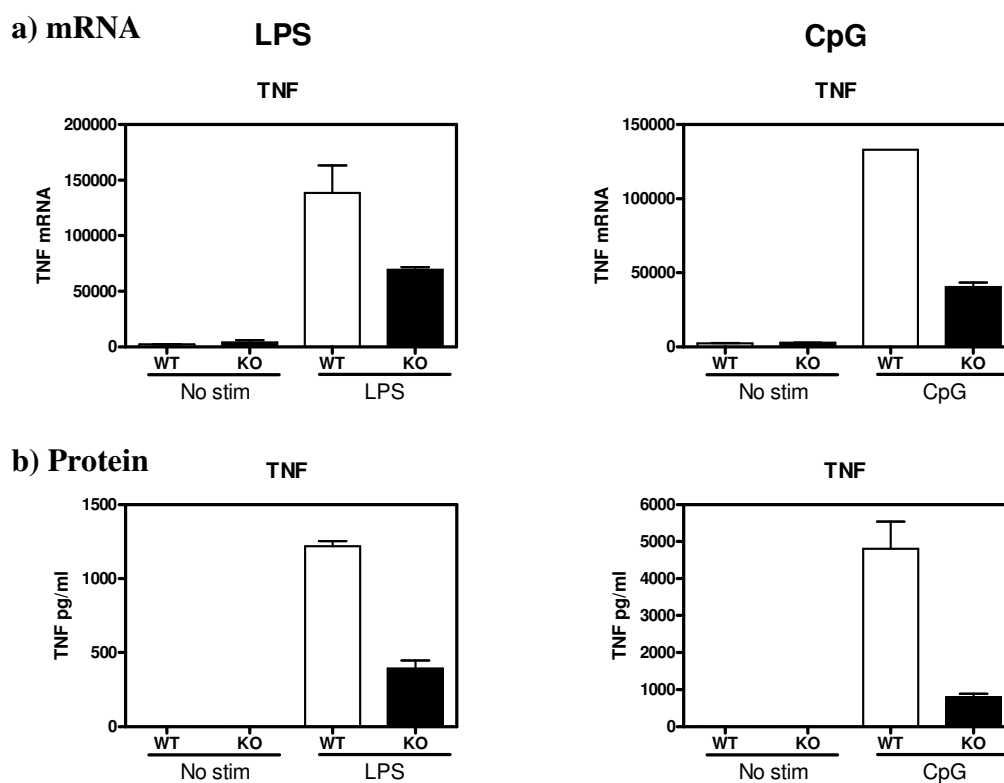
Figure 5.1.3: TNF production is reduced in Tpl-2 deficient myeloid DC. Sorted purified GM-DC were stimulated with LPS or CpG from *Tpl-2$^{-/-}$* (closed bars) or littermate controls (open bars). a) Cells were lysed for RNA after 3 hours and *Tnf* mRNA was quantified by Real-time quantitative PCR. b) Supernatants were collected after 6 hours stimulation for immunoassay. Each bar represents triplicates. This has been repeated 3 times with similar results.
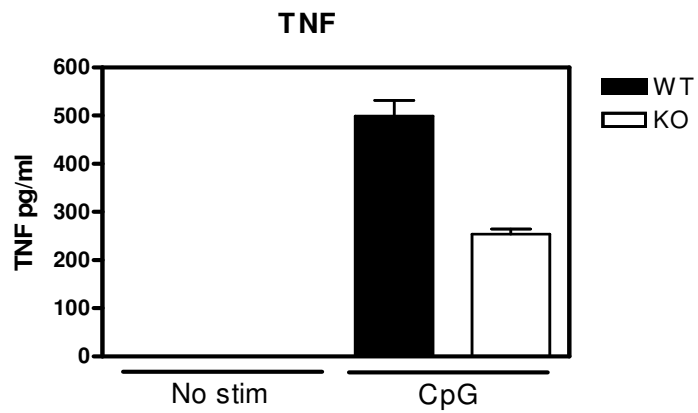
Figure 5.1.4: CpG induced TNF production is reduced in Tpl-2 deficient splenic DC. Splenic DC were purified and stimulated with CpG for 20 hours before supernatant was collected for TNF immunoassay. Each bar represents triplicates. Similar results have been obtained on 3 separate occasions.

a) mRNA   LPS

IL-10

IL-12p40

IL-12p35

IFN-beta

All below detection (IL-12p35)

☐ LPS
■ LPS +U0126

b) Protein

IL-10

IL-12p40

IL-12p70

IFN-beta

All below detection (IL-12p70)

☐ LPS
■ LPS + U0126

c) mRNA   CpG

IL-10

IL-12p40

IL-12p35

IFN-beta

All below detection (IL-12p35)

☐ CpG
■ CpG +U0126

d) Protein

IL-10

IL-12p40

IL-12p35

IFN-beta

All below detection (IL-12p35)

☐ CpG
■ CpG + U0126
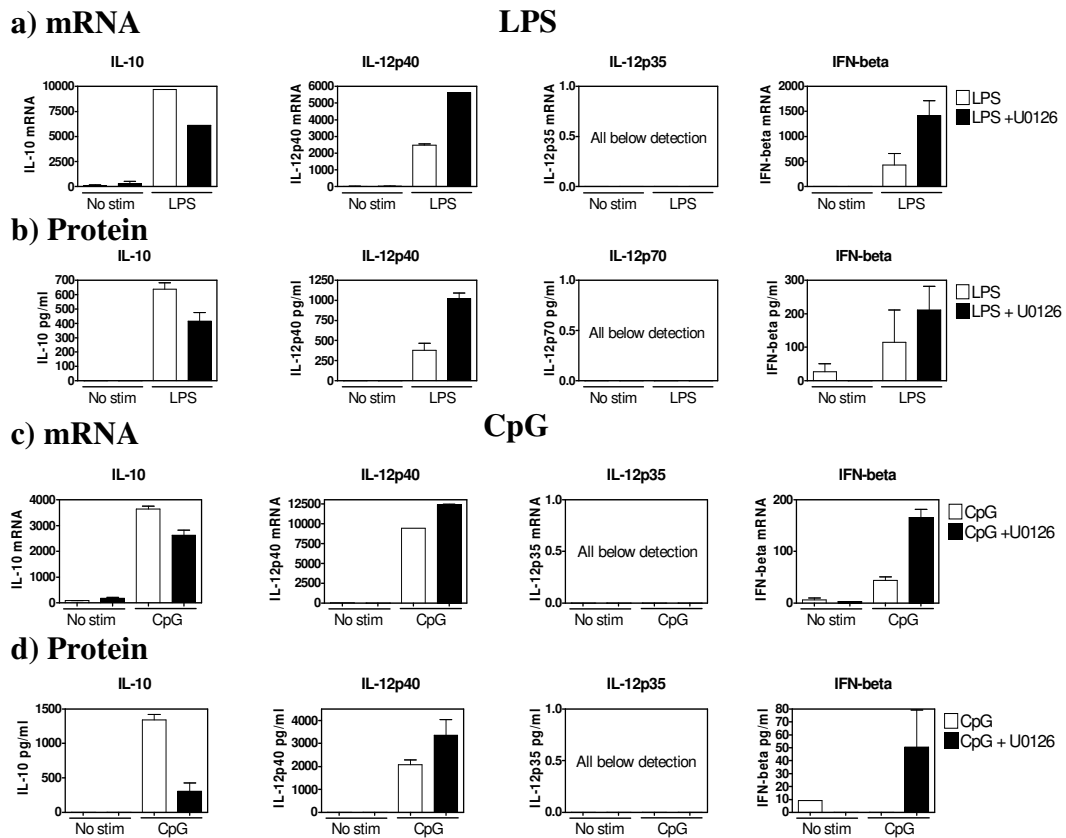
Figure 5.2.2: U0126 treatment decreases IL-10 but increases IL-12p40 and IFN-β in macrophages. a, b) Macrophages stimulated with LPS or, c, d) CpG. mRNA induction (a, c) was analysed after 6 hours stimulation by real-time quantitative PCR and supernatants (b, d) were harvested after 20 hours and to measure cytokine protein by immunoassay.

135

LPS

a) mRNA

b) Protein

c) mRNA   **CpG**

d) Protein
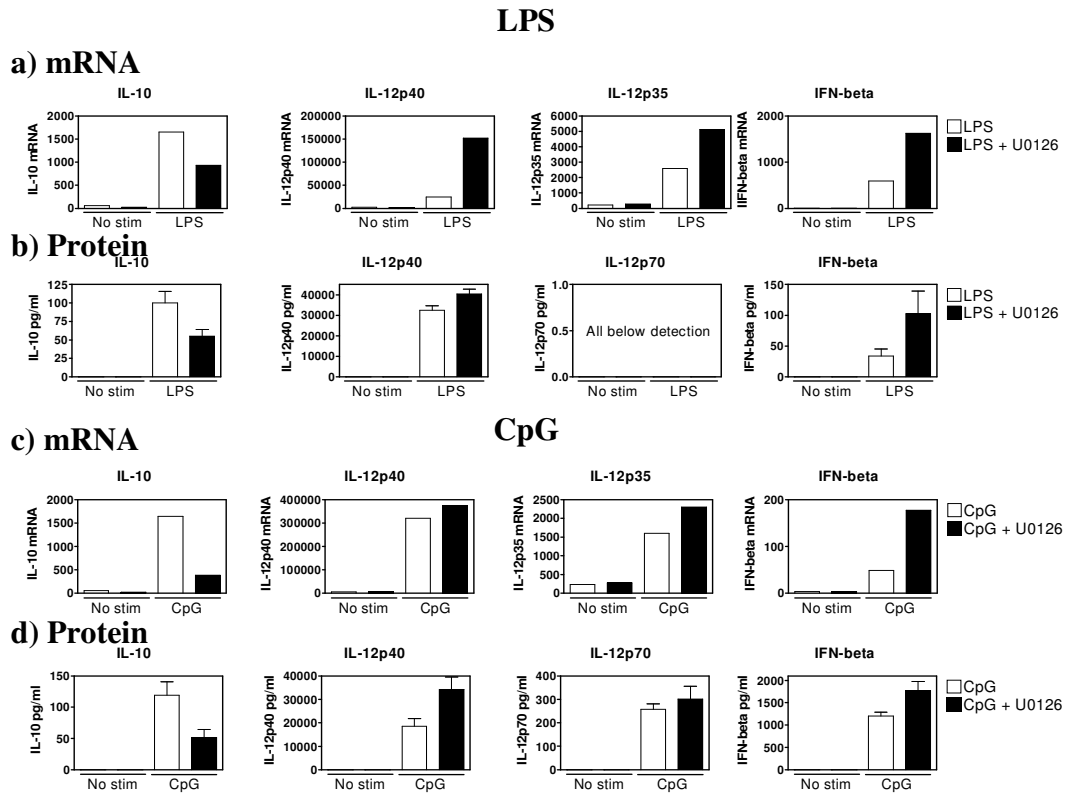
Figure 5.2.3: U0126 treatment decreases IL-10 but increases IL-12 and IFN-β in myeloid DC stimulated with LPS or CpG. a and b) myeloid DC stimulated with LPS or, c and d) CpG. mRNA (a and c) was harvested after 6 hours and quantified by real-time PCR and supernatants (b and d) were harvested after 20 hours and protein levels were quantified by immunoassay. Experiment has been repeated 3 times with similar results.

a) mRNA   **LPS**

b) Protein

CpG

c) mRNA

d) Protein
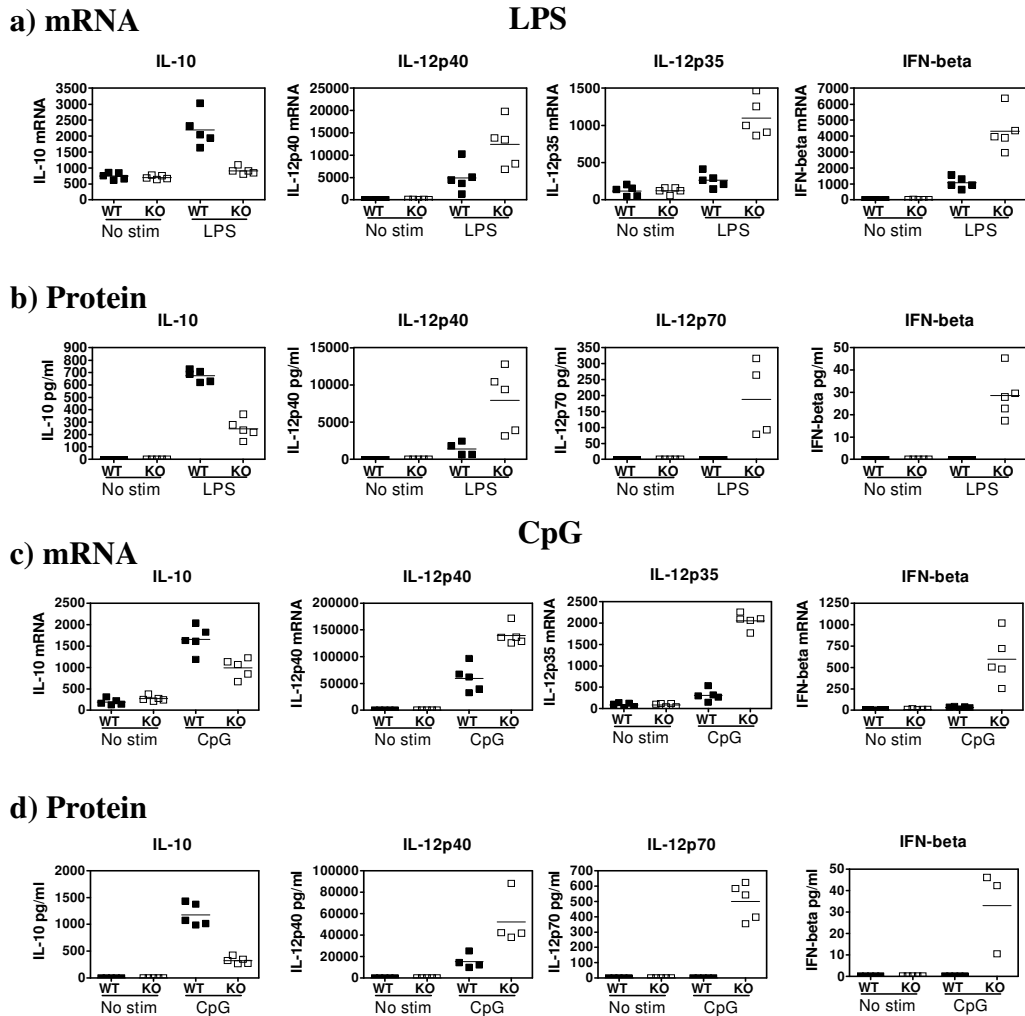
Figure 5.2.4: Lack of Tpl-2 leads to decreased IL-10 but increased IL-12 and IFN-β production in macrophages stimulated with LPS and CpG. a and b) Macrophages from *Tpl-2*^{-/-} and littermate controls were stimulated with LPS or, c and d) CpG. mRNA (a and c) was harvested after 6 hours and cytokine induction was checked by real-time quantitative PCR and supernatants (b and d) were harvested after 20 hours and cytokine protein levels were measured by immunoassay.
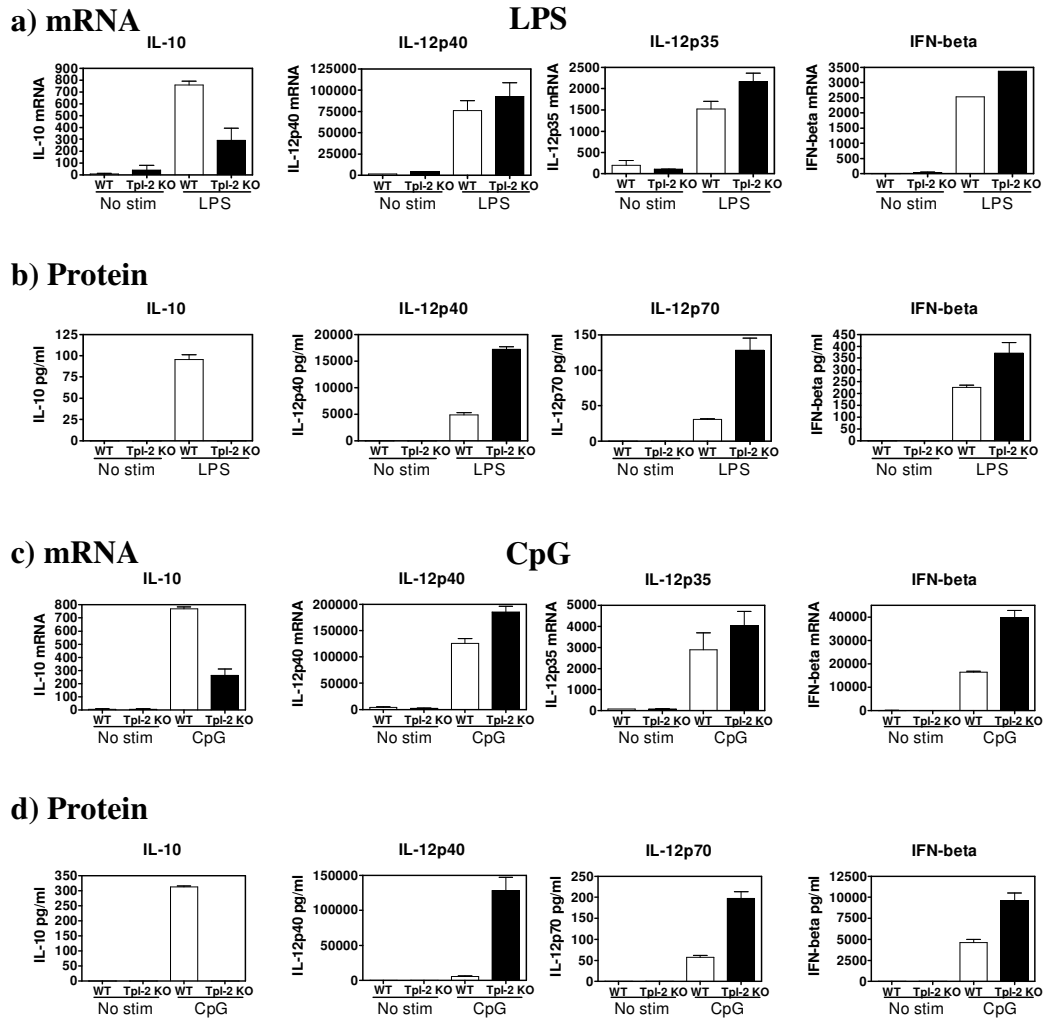
Figure 5.2.5: Lack of Tpl-2 impaired IL-10 induction but increased IL-12 and IFN-β production in myeloid DC stimulated with LPS or CpG. a and b) Myeloid DC from Tpl-2 knockouts and littermate controls were stimulated with LPS or, c and d) CpG. mRNA (a and c) was harvested after 6 hours and cytokine induction was checked by real-time quantitative PCR and supernatants (b and d) were harvested after 20 hours and cytokine protein levels were measured by immunoassay.

IL-10

IFN-beta

IL-12p40

IL-12p70
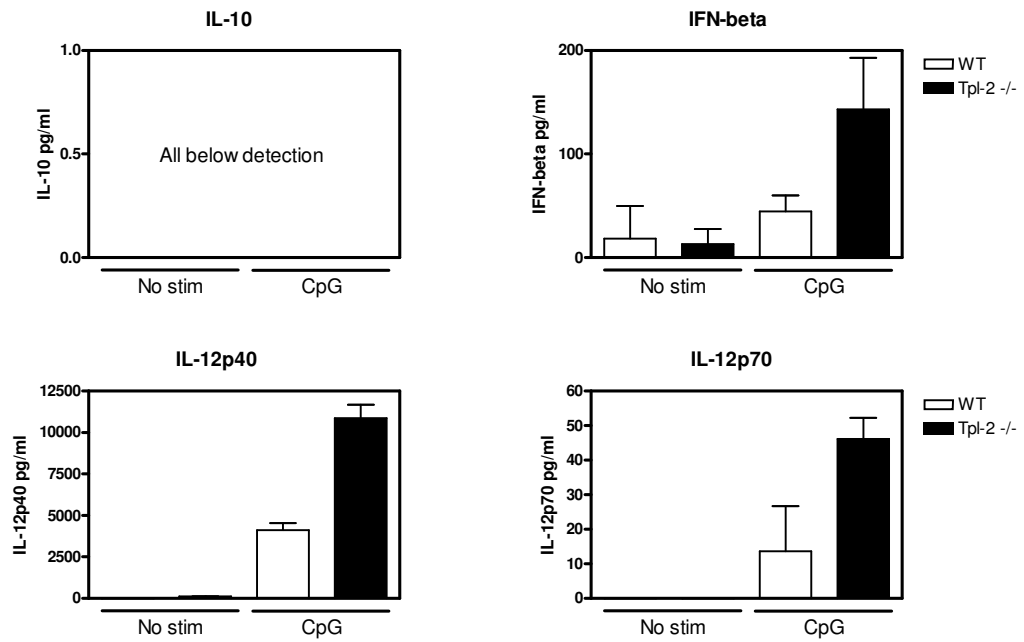
Figure 5.2.6: Lack of Tpl-2 increases IL-12 and IFN-β production in splenic DC. Purified splenic DC from Tpl-2 knockouts and littermate controls were stimulated with CpG for 20 hours and protein levels tested by immunoassay

a) **LPS**

IL-12p40

[Bar chart: y-axis "IL-12p40 pg/ml" from 0 to 75000 (25000 intervals). X-axis groups: WT (DMSO 80.5, U0126 127.5), IL-10 KO (DMSO, U0126). IL-10 KO U0126 bar reaches ~65000.]

IL-12p70

[Bar chart: y-axis "IL-12p70 pg/ml" from 0 to 900 (100 intervals). X-axis groups: WT (DMSO, U0126) labeled "Below detection", IL-10 KO (DMSO ~100, U0126 ~790).]

b) **CpG**

IL-12p40

[Bar chart: y-axis "IL-12p40 pg/ml" from 0 to 250000 (50000 intervals). X-axis groups: WT (DMSO 242.5, U0126 904.2), IL-10 KO (DMSO ~85000, U0126 ~220000).]

IL-12p70

[Bar chart: y-axis "IL-12p70 pg/ml" from 0 to 1500 (500 intervals). X-axis groups: WT (DMSO, U0126) labeled "Below detection", IL-10 KO (DMSO ~100, U0126 ~1200).]

Figure 5.2.7: IL-10 knockout macrophages show increased IL-12 after U0126 treatment. Macrophages from WT or IL-10 knockouts were stimulated with a) LPS or b) CpG in presence or absence of U0126 for 20 hours and protein levels checked by immunoassay. DMSO was used as vehicle control. Repeated 3 times.

a) Macrophages **LPS**
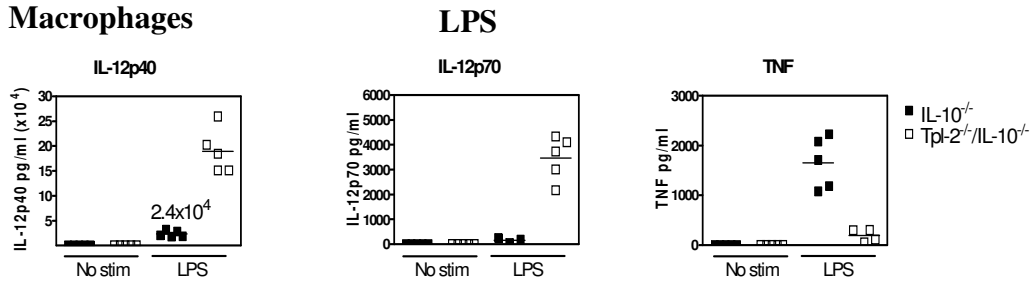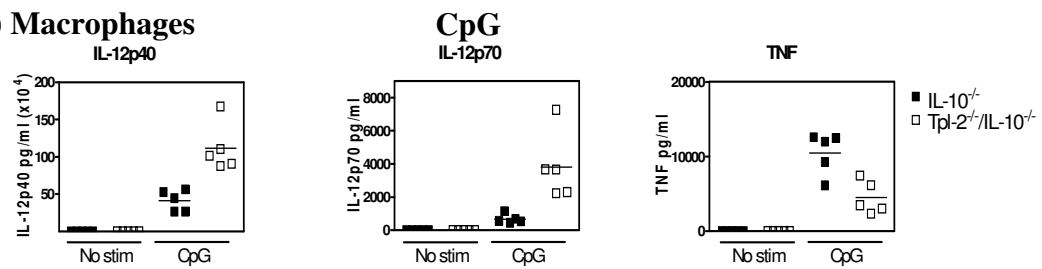
b) Macrophages **CpG**

Figure 5.2.8: Lack of IL-10 and Tpl-2 further increases IL-12, but decreases TNF. Macrophages from 5 individual *IL-10$^{-/-}$* or 5 individual *Tpl-2$^{-/-}$/IL-10$^{-/-}$* mice were stimulated with a) LPS or b) CpG and protein levels measured by immunoassay

LPS/CpG

3

ERK

Other
transcription
factor

3

C-Fos

1

2

IL-10

2

IL-12

IFN-β

4
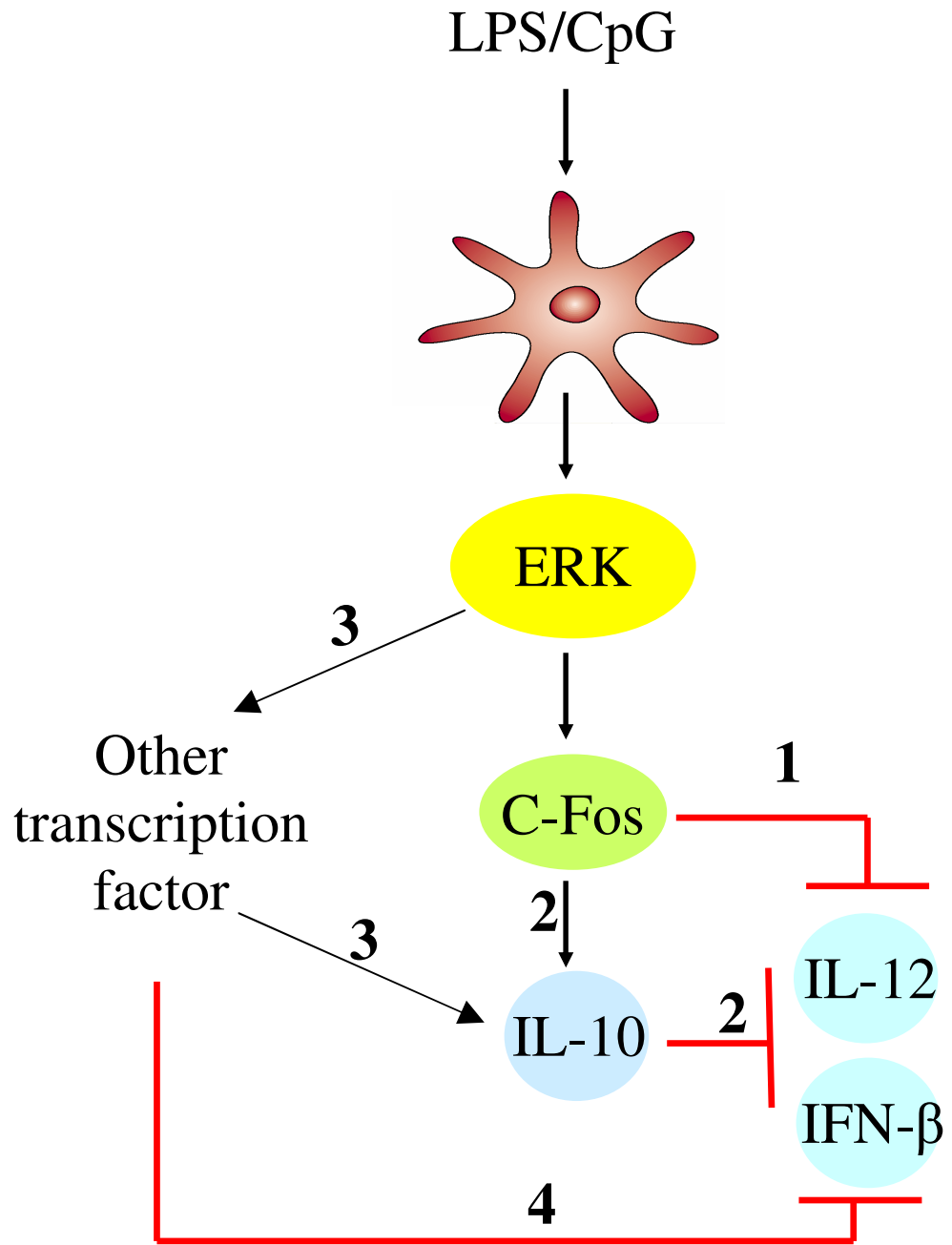
Figure 5.2.9 Proposed mechanisms of ERK regulation by IL-12 and IFN-β.

Chapter 6

Tpl-2/IL-10 double knockout phenotype

6.1 Tpl-2$^{-/-}$ x Il-10$^{-/-}$ mice show weight loss.

We generated *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$* (Tpl-2/IL-10 knockout) mice to investigate a potential mechanism for Tpl-2 in down regulation of IL-12 and IFN-β in the complete absence of IL-10. It became clear that by 8 weeks of age the double knockouts developed diarrhoea, suggesting the development of colitis. It is well established that IL-10 deficient mice can develop colitis, when kept in a non-pathogen free environment (Kuhn *et al.* 1993; Davidson *et al.* 1996). However, when on a C57BL/6 background these mice are usually between 4-6 months old before they develop diarrhoea (Davidson *et al.* 1996) and our results. Tpl-2 deficient mice however never develop colitis. Since our findings demonstrate that the *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$* mice show early onset of colitis compared to the *Il-10$^{-/-}$* mice, implying that the absence of Tpl-2 accelerates disease onset caused by IL-10 deficiency.

We generated mice from 4 genotypes (wildtype, Tpl-2 knockout, IL-10 knockout, and Tpl-2/IL-10 knockout) and the females were mixed in the cages to ensure that they experienced the same environment (Garrett *et al.* 2007). Mice were then weighed weekly from 4 weeks of age until 33 weeks of age as a general indicator of health of the mice (Figure 6.1). It became clear that already at 7 weeks, the *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$* mice were smaller than the single knockouts and the littermate controls, a trend that continued over time. At 13 weeks, the IL-10 deficient mice were also smaller that the wildtype or Tpl-2 deficient mice, but not as small as the *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$* mice.

To begin with there was no noticeable weight loss in the *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$* mice, but only failure to put on weight compared to the other genotypes in the cage. However,

as the mice got older, weight loss was noted, and was sometimes so severe that the mouse had to be culled. This only ever occurred in the *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$* mice within the timeframe of these experiments. The failure to put on weight coincided with the onset of diarrhoea. We never recorded any significant weight loss, but only slower growth as compared to the littermate controls in the IL-10 knockout over the 33 weeks they were monitored.

6.2 Colons from the Tpl-2$^{-/-}$ x Il-10$^{-/-}$ mice are shorter but heavier than those from littermates.

As the *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$* mice either became too ill, and thus had to be sacrificed according to our project license, or as in the case of the single knockout or wildtype reached 33 weeks of age, the intestines were removed and fixed in formalin for further investigation. We noticed a difference in gut size between the different genotypes. Figure 6.2.1 shows a representative photo of the colons from wildtype, Tpl-2 knockout, IL-10 knockout, and the *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$* mice. It is very clear that the *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$* mice have a shorter but thicker colon compared to the wildtype and Tpl-2 deficient mice. The IL-10 deficient mouse colon was slightly thicker and shorter, but not to the same extent as the *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$* mice coincident with the accelerated weight loss in the *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$* mice.

We also recorded the length and weight of the colons when we took them out to quantify these differences. Figure 6.2.2 shows the length and weight of guts measured from the colon to the caecum from Tpl-2 knockout, IL-10 knockout and *Tpl-2$^{-/-}$* x *Il-10$^{-/-}$*

mice aged 33 weeks. The average length and weight for both the Tpl-2 and IL-10 knockouts were around 97mm and 1.2g, whereas for the $Tpl\text{-}2^{-/-}$ x $Il\text{-}10^{-/-}$ mice knockouts the length and weight was 75mm and 1.6g respectively. However, in order to verify the phenotype, larger numbers are required, particularly since is not an absolute phenotype as the $Il\text{-}10^{-/-}$ mice also develop colitis, but rather an accelerated phenotype.

6.3 Intestinal pathology.

We carried out histological analysis on different sections of the colon and caecum to investigate whether the differences in the length and weight of the colon from the $Tpl\text{-}2^{-/-}$ x $Il\text{-}10^{-/-}$ mice were due to colitis. Figure 6.3 shows all 4 genotypes at 20 weeks of age. The morphology of the wildtype and the Tpl-2 knockout colons looked similar with no infiltrates. Colons from the IL-10 knockout mice did not look very different to the wildtype, but there was a slight level of infiltrate in the tissue. However, the $Tpl\text{-}2^{-/-}$ x $Il\text{-}10^{-/-}$ mice showed extensive infiltration of inflammatory cells and the gut structure was completely disrupted, indicating severe colitis.

Taking all the preliminary data together, our results indicate that the $Tpl\text{-}2^{-/-}$ x $Il\text{-}10^{-/-}$ mice develop colitis at an earlier stage than the IL-10 deficient mice. On the other hand there is no evidence that the Tpl-2 deficient mice ever develop colitis.

6.4 Discussion.

We generated *Tpl-2^{-/-} x Il-10^{-/-}* mice by crossing the *Tpl-2^{-/-}* mouse to the *Il-10^{-/-}* mouse. We did this in order to investigate the potential regulation of IL-12 and IFN-β by Tpl-2 in the complete absence of IL-10. Whilst waiting to get enough mice for an experiment, we observed that by 6-8 weeks of age, the *Tpl-2^{-/-} x Il-10^{-/-}* mice developed diarrhoea, whereas the other genotypes (wildtype, *Tpl-2^{-/-}* or *Il-10^{-/-}* mice) at that stage displayed no sign of illness. We show that this phenotype resulted in accelerated weight loss in the *Tpl-2^{-/-} x Il-10^{-/-}* mice. Colons of these mice showed a severe inflammatory infiltrate, which was absent in the control, *Tpl-2^{-/-}* and *Il-10^{-/-}* mice at that age housed in the same cages. This has recently been shown to be important, since *T-bet^{-/-} x RAG2^{-/-}* mice develop spontaneous colitis, which can be transferred to littermate controls when they are housed in the same cage (Garrett *et al.* 2007).

6.5 Potential mechanism.

6.5.1 The role of cytokines in development of colitis.

Colitis has been reported to develop in IL-10 deficient mice (Kuhn *et al.* 1993), although it is dependent on colonisation of the gut flora, it can also be induced by the transfer of CD25⁻ or CD45RB^{high} CD4⁺ T cells into RAG deficient mice (Izcue *et al.* 2006). Colitis has been shown to be dependent on TNF, IFN-γ, IL-12 and IL-23 (Kuhn *et al.* 1993; Hue *et al.* 2006; Kullberg *et al.* 2006). The ability of anti-IL-12p40 antibodies (which recognise both IL-12 and IL-23) and IL-12p40 deficient mice to

protect from colitis was more recently shown to be attributed to blocking of IL-23 (Kullberg *et al.* 2001; Kullberg *et al.* 2006). These different systems have been used as models of ulcerative colitis in humans. In keeping with findings in mouse models, anti-TNF antibodies have also been shown to reduce symptoms of Crohn's disease (Hanauer *et al.* 2006).

IL-10 is important in suppressing the production of proinflammatory cytokines TNF and IFN-γ and also Th1 cell differentiation (Fiorentino *et al.* 1991; Fiorentino *et al.* 1991; Rennick *et al.* 1995). IL-12 also has a role in the development of colitis (Leach *et al.* 1996; Asseman *et al.* 1999). IL-12 was originally thought to be responsible for the gut pathology seen in the IL-10 knockout mouse (Kuhn *et al.* 1993).

6.5.2 The role of TNF in colitis.

As discussed above TNF have been shown to an important mediator of colitis. Kontoyiannis *et al*. (1999) show that $Tnf^{\Delta ARE}$ deficient mice, which have a defect in posttranslational regulation of *Tnf* mRNA, develop colitis. This group showed that this overproduction of TNF in the intestine can lead to overproduction of other proinflammatory cytokines such as IL-12 and IFN-γ, which they suggested may cause the tissue damage observed in colitis although they did not look at IL-23 or IL-17 (Kontoyiannis *et al.* 2002).

Based on our *in vitro* studies with macrophages from the $Tpl\text{-}2^{-/-}$ x $Il\text{-}10^{-/-}$ mice (Figure 5.2.9) we show that these mice produce lower levels of TNF as compared to the

Il-10^{-/-} mice. We therefore did not expect that the *Tpl-2^{-/-} x Il-10^{-/-}* mice would develop accelerated colitis.

6.5.3 The role of IL-12 in colitis.

We show earlier that the *Tpl-2^{-/-} x Il-10^{-/-}* deficient *in vitro* macrophages have higher IL-12p40 and IL-12p70 levels compared to the *Il-10^{-/-}* mouse (Figure 5.2.9). As yet however, we have not determined the serum or gut levels of these cytokines in the *Tpl-2^{-/-} x Il-10^{-/-}* mice as compared to the *Tpl-2^{-/-}* or the *Il-10^{-/-}* mice. The colitis we observe may be mediated by increased levels of IL-12, which induces Th1 cell differentiation leading to pathology (Becker *et al.* 2006; Kullberg *et al.* 2006). However, recently another IL-12 family member, IL-23 was identified (Trinchieri *et al.* 2003). IL-23 is composed of the p40 subunit from IL-12 and a p19 subunit. IL-23 has been found to be important for the expansion of precommited Th17 cells (Bettelli *et al.* 2006; Veldhoen *et al.* 2006). It is now known that both IL-12 and IL-23 play an important part in the immune pathology developing in colitis (Becker *et al.* 2006; Uhlig *et al.* 2006; Yen *et al.* 2006). It has been shown that IL-23 is responsible for local intestinal inflammation, whereas IL-12 was responsible for the systemic inflammation (Uhlig *et al.* 2006). Uhlig *et al.* (2006) have shown that IL-12 is important in the development of wasting disease and serum cytokine production seen in colitis, whereas, IL-23 is important for the mucosal immunopathology observed. IL-23 is now thought to be the main inducer of chronic intestinal inflammation via the induction of both IFN-γ and IL-17 production (Hue *et al.* 2006; Kullberg *et al.* 2006). These cytokines have been shown

149

to play an important part in the development of spontaneous colitis in various mouse models (Kontoyiannis *et al.* 1999; Kontoyiannis *et al.* 2002; Uhlig *et al.* 2006).

Although we have no data regarding IL-23 production by macrophages or DC it is possible that elevated levels of IL-23 may also play a role in the *Tpl-2^{-/-} x Il-10^{-/-}* mice resulting in colitis. As the data shown here are only preliminary, more studies will have to be carried out in order to determine the mechanisms leading to the early pathology observed in the *Tpl-2^{-/-} x Il-10^{-/-}* mice. This would include increasing the numbers of mice to verify the difference in the early development of colitis in the *Tpl-2^{-/-}* x *Il-10^{-/-}* as compared to the *Il-10^{-/-}* mice in the same cages to ensure no difference in the gut flora.

We know from our studies with the *Tpl-2* mice that they do not develop colitis although *in vitro* generated macrophages have enhanced IL-12 levels but reduced TNF (Figure 5.2.4) (Dumitru *et al.*, 2000 and our data) but as yet we have no data on IL-23. Thus it is presently unknown whether singularly Tpl-2 and ERK may play a role in the regulation of the immune response and the development of colitis development in the presence of particular gut flora. A recent study in the *Nf-κb1^{-/-}* mouse shows that after challenge with *Helicobacter hepaticus* increased IL-12p40 is observed. This coincides with deficient ERK expression in those mice, suggesting that ERK may play an important role in protecting mice from Helicobacter induced colitis through regulation of IL-12p40 but this is not observed in "spontaneous" colitis resulting from colonisation by gut microflora (Tomczak *et al.* 2006). Of course though regulation of IL-12p40 any effects may be attributable to IL-12 or IL-23, although IL-23 has been attributed with the major role in colitis induction, Kullberg *et al.* (2006) suggest that IFN-γ (hence IL-12) may also still play some role.

150

We do not know as of yet if the colitis observed in the $Tpl-2^{-/-}$ x $Il-10^{-/-}$ mice is dependent on Helicobacter, as since their arrival the mice have been kept in the quarantine unit for studies. Thus a number of questions rising from our study need to be addressed such as: What is the mechanism whereby the $Tpl-2^{-/-}$ x $Il-10^{-/-}$ mice develop accelerated colitis as compared to the $Il-10^{-/-}$ mice, which develop the disease later or as compared to the $Tpl-2^{-/-}$ mice, which never develop the disease? $Tpl-2^{-/-}$ mice show no pathology although these mice have reduced but not absent production of IL-10 by macrophages and DC, but this has not been confirmed *in vivo* in the gut, which may be sufficient to protect them from colitis.

As the phenotype of the $Tpl-2^{-/-}$ x $Il-10^{-/-}$ mice only became obvious towards the end of the thesis, there are still some key experiments outstanding. Originally we bred these mice to study the role of Tpl-2 in regulating IL-12 and IFN-β in the complete absence of IL-10, however, the $Tpl-2^{-/-}$ x $Il-10^{-/-}$ mice developed diarrhoea at around 6 weeks of age. Breeding a sufficient number of mice for RNA and cytokine experiments was not possibly in the time left for completion of this thesis. At this stage, we do not have a mechanism for the accelerated gut pathology and experiments will have to be repeated with increased numbers of mice as explained earlier.

We know that cultured bone marrow-derived macrophages have increased IL-12, but decreased TNF in response to LPS and CpG stimulation (Figure 5.2.10), however, we as yet have not determined the serum cytokines and the local cytokine production in the gut. Based on the factors, which have been implicated in the development of colitis and wasting disease measurements of IL-12p40, IL-12p70, IL-23 and TNF should be performed. RNA could from cultured colonic explants also be measured for cytokine

151

expression as this would help us to get a more complete picture of the cytokine profile of the $Tpl\text{-}2^{-/-}$ x $Il\text{-}10^{-/-}$ mice.

Finally if we find elevated levels of IL-12 and/or IL-23 in the colon or serum, we would like to treat the $Tpl\text{-}2^{-/-}$ x $Il\text{-}10^{-/-}$ mice with anti-IL-12p40 and anti-IL-23p19 antibodies in order to determine the cause of the colitis. As this anti-IL-12p40 blocks both IL-12p70 and IL-23, it would not be enough to determine whether the onset of colitis is mediated by IL-12 or IL-23. Therefore, treatment with IL-23p19 monoclonal antibodies are required to answer this question.

6.5.4 The role of regulatory T cells in colitis development.

$CD4^+CD25^+$ T-regs have been found to be important in maintaining the intestinal homeostasis (Hue *et al.* 2006). It has been shown that transfer of $CD4^+CD45^{high}$ T cells into RAG deficient mice can lead to the development of colitis associated with the expansion and accumulation of T cells and DC in the intestine (Hue *et al.* 2006). However, cotransfer of $CD4^+CD45^{high}$ and T-regs into immunodeficient mice can prevent the development of colitis. Moreover, T cell-induced colitis can be cured by transfer of T-regs (Izcue *et al.* 2006).

We would like to carry out some flow cytometry staining of the bone marrow, lymph nodes, spleen and gut to determine whether there are reduced cell numbers of $CD4^+CD25^+foxP3^+$ regulatory T cells. This would have to be compared to the wildtype, Tpl-2 and IL-10 deficient mice in order to identify whether there are any differences in the cell populations, which could possibly explain the early onset of colitis. Therefore it

is a possibility that the *Tpl-2*$^{-/-}$ x *Il-10*$^{-/-}$ mice may lack T-regs leading to the early onset

of colitis.

Chapter 6

Figures
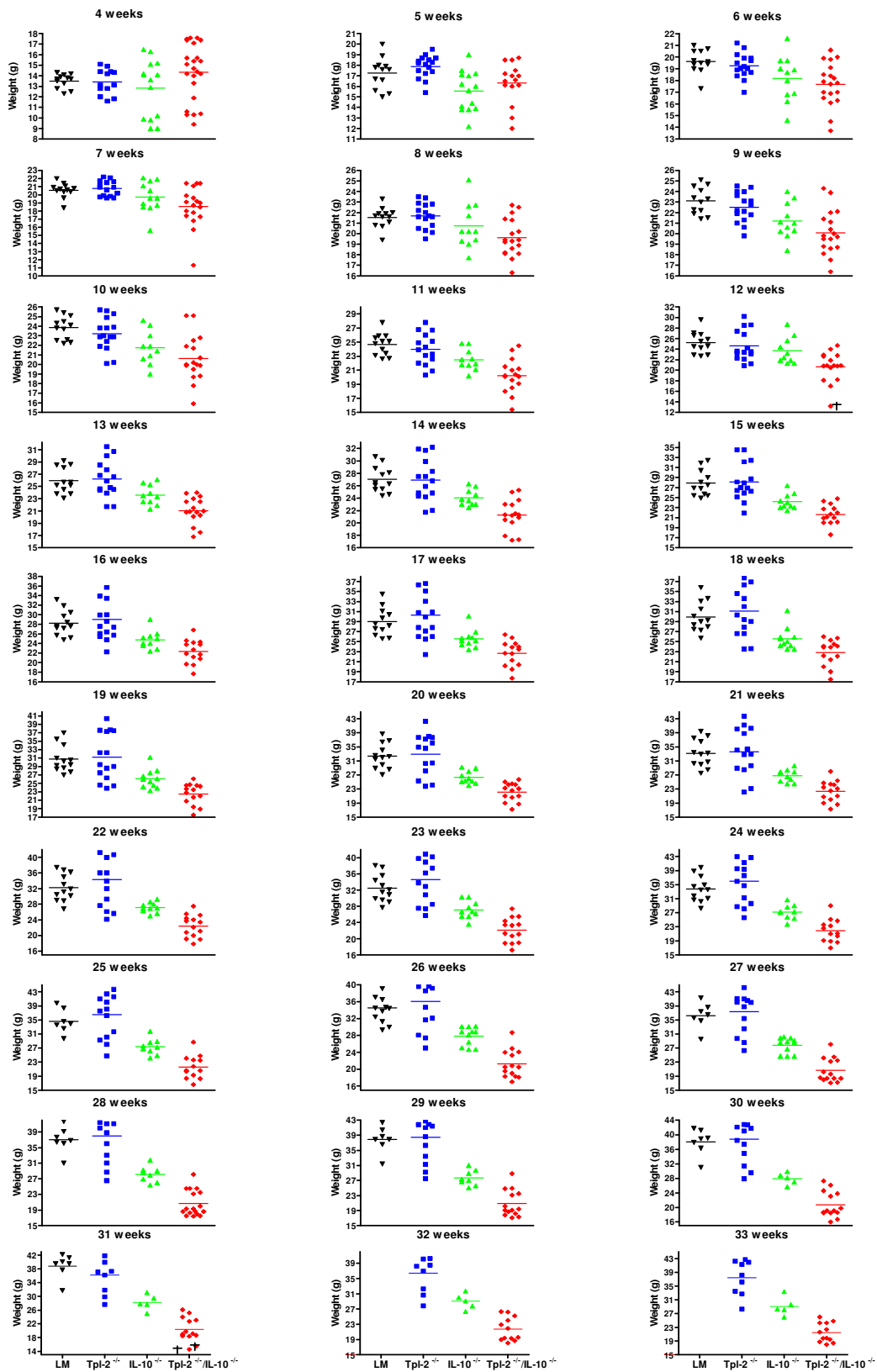
Figure 6.1: Weight chart of mice. Mice were weighed every week to monitor weight loss. LM is the littermate wildtype control. Note scales between graphs are different.

155

WT Tpl-2 -/- IL-10 -/- Tpl-2-/- IL-10 -/-

Figure 6.2.1: Colons from Tpl-2/IL-10 deficient mice are significantly smaller and thicker than littermates. Colons from the 4 phenotypes were removed at 33 weeks of age and photographed to show differences in size.

Gut length

Gut weight
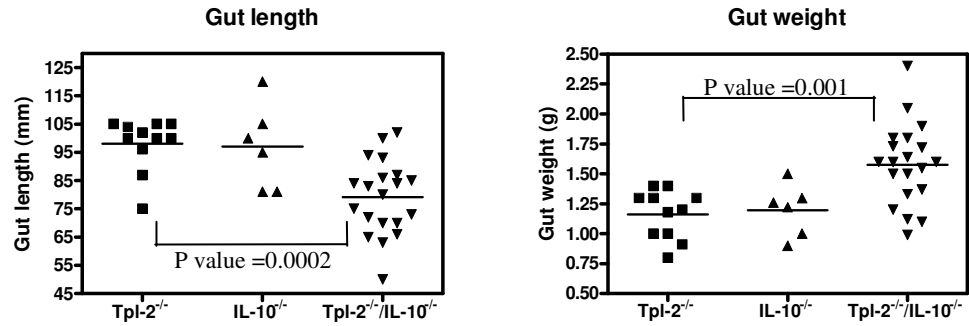
Figure 6.2.2: Colons from Tpl-2/IL-10 deficient mice are shorter and heavier than *Tpl-2^{-/-}* or *IL-10^{-/-}*. Before colons were fixed in formalin they were measured and weighed to show size differences.

157

WT | Tpl-2 -/-
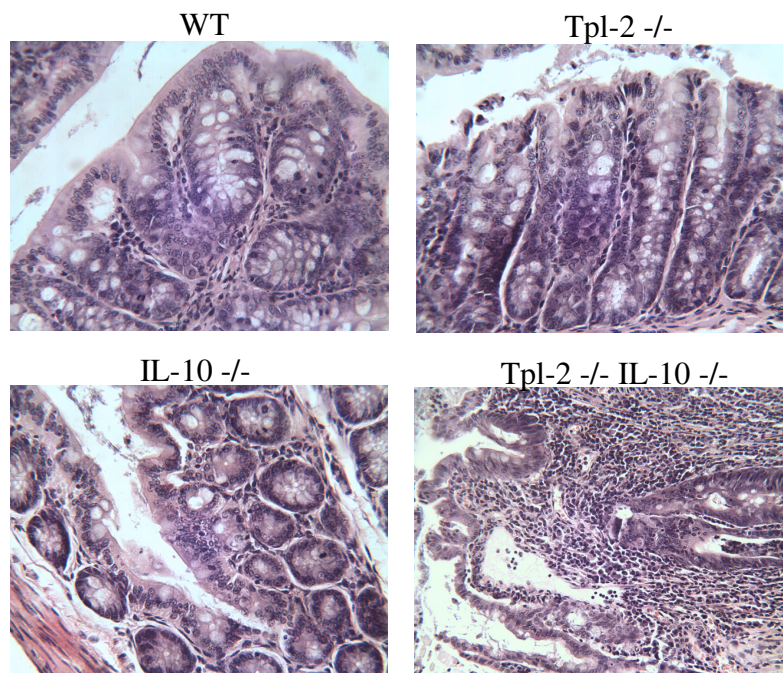
IL-10 -/- | Tpl-2 -/- IL-10 -/-

Figure 6.3: Tpl-2/IL-10 deficient mice show severe colitis. Intestinal samples were fixed in formalin and stained with H and E. Magnification 400 times.

Chapter 7

Future perspectives

7.1 Outstanding questions from work in this Ph.D. thesis

7.1.1 Summary of literature regarding questions at the beginning of our study.

How Tpl-2/ERK phosphorylation in DC as well as macrophages affects cytokine production.

The aim of this thesis was to investigate the role of ERK in regulating cytokine production in DC and macrophages. We used two different strategies to do this. Firstly we pharmacologically blocked ERK phosphorylation with an inhibitor. Secondly we used genetically modified mice lacking Tpl-2, which is a MAP kinase that specifically activates the ERK signalling pathway.

There are conflicting reports whether it is possible to induce ERK phosphorylation in TLR stimulated DC and therefore whether phosphorylation of ERK regulates cytokine production in DC. Hacker *et al.* (1999) showed that CpG could not induce ERK phosphorylation in myeloid DC but could in macrophages. In contrast to this report, Agrawal *et al.* (2003) and Dillon *et al.* (2004) have both shown that ERK phosphorylation could be induced in human or splenic DC following LPS stimulation and led to regulation of IL-12. However, it was unclear in this study whether negative regulation of IL-12 by ERK activation was through the induction of IL-10 or by an IL-10-independent mechanism.

In macrophages it had been shown that negative regulation of IL-12 by ERK phosphorylation in IL-10 deficient splenocytes was not observed (Yi *et al.* 2002), although the results were difficult to interpret because of a mixed population of cells.

We set out to clarify the reported differences by systematically examining direct negative effects of ERK phosphorylation on IL-12 induction in the absence of IL-10, a known regulator of IL-12 using pure populations of macrophages and myeloid DC stimulated with LPS or CpG.

7.1.2 Summary of data presented in this thesis which answers some of the questions in the literature.

CpG as well as LPS leads to ERK phosphorylation in macrophages and DC.

In preliminary experiments in this thesis we first identified the optimal concentrations of LPS and CpG for activation of MAP kinases and induction of cytokines in macrophages and myeloid DC. We also identified the optimal time points for activation of MAP kinases and induction of cytokines. We conclusively show that both LPS and CpG were able to induce phosphorylation of ERK in macrophages and myeloid DC, although Hacker *et al.* (1999) had reported that CpG could not induce ERK phosphorylation in DC, which could potentially be due to the high background in their assay.

Tpl-2 activation results in ERK as well as p38 phosphorylation.

It had been suggested that Tpl-2 exclusively regulates ERK phosphorylation and does not affect other MAP kinases (Dumitru *et al.* 2000). We have now shown that Tpl-

2 not only regulates ERK phosphorylation but also affects p38 phosphorylation. In Tpl-2 deficient LPS or CpG stimulated macrophages and DC, we show ERK phosphorylation is completely blocked, however we also show that p38 phosphorylation is reduced, albeit to a much lesser extent.

TNF is regulated by Tpl-2/ERK to a small extend at the mRNA level.

We have confirmed that TNF induction in macrophages and myeloid DC is regulated by the Tpl-2/ERK pathway and have shown that TNF is regulated both at the induction of mRNA and protein level. This is in contrast to data by Dumitru *et al.* (2000), who showed that TNF was only regulated post-transcriptionally. The effects of Tpl-2/ERK on TNF protein induction can now be explained by studies by Rousseau *et al.* (2008), who show that ERK is required for the phosphorylation of TACE in order to induce TNF protein.

ERK activation negatively regulates IL-12 production in the absence of IL-10.

We have also confirmed studies that IL-10 negatively regulates IL-12 production (reviewed in Moore *et al.* 2001), but shown for the first time that IL-12 production is also regulated negatively by the activation of the ERK MAP kinase pathway in the complete absence of IL-10, which clarifies previous findings of Pulendran *et al.* (2003), which did not establish whether ERK activation in DC was via IL-10 only or also via an IL-10 independent pathway. Our data show that both are the case.

ERK activation leads to negative regulation of IFN-β.

Hacker *et al.* (2006) have suggested that IL-10 and type I IFN are co-ordinately regulated by TRAF3. However, we show that in the absence of ERK activation (U0126 or Tpl-2 deficient mice) that IFN-β production was enhanced in the absence of ERK activation by U0126 or in Tpl-2 deficient cells. As yet we do not know whether the increased levels of IFN-β observed are due to the lack of ERK signalling or solely due to decreased levels of IL-10 when ERK phosphorylation is abrogated, but our data suggest that IL-10 and IFN-β are not co-ordinately regulated..

Tpl-2$^{-/-}$ x Il-10$^{-/-}$ **mice develop accelerated colitis as compared to the** *Il-10$^{-/-}$* **mice.**

Finally we found that in the absence of both IL-10 and Tpl-2, mice developed colitis within 6-8 weeks of age. The onset of colitis was accelerated compared to IL-10 deficient mice, which on this genetic background and in our animal facility develop colitis at 4-6 months of age (Davidson *et al.* 1996).

7.2 Outstanding questions resulting from this Ph.D. thesis work

7.2.1 Tpl-2 possibly affects other signalling pathways than the ERK MAP kinase pathway.

We show that stimulation of macrophages or myeloid DC with LPS or CpG in the presence of the U0126 MEK inhibitor show decreased TNF induction at both the mRNA and protein level as compared to vehicle control. U0126 completely blocked ERK phosphorylation in macrophages and DC but however did not completely block TNF production. Stimulated macrophages and DC from Tpl-2 deficient mice, which also have blocked ERK activation also show a decrease in the TNF level but this inhibition is more pronounced than seen with the U0126 inhibitor. This shows that Tpl-2 regulates TNF production via ERK and potentially another pathway. One possibility is via p38 activation as we have shown that Tpl-2 affects phosphorylation of p38. We found that in the absence of Tpl-2, p38 phosphorylation was impaired and this could possibly explain the differences observed between the U0126 MEK inhibitor and the Tpl-2 deficient cells. No differences in JNK phosphorylation were observed in the Tpl-2 deficient mice. The effect of Tpl-2 deficiency on p38 phosphorylation was clearer in macrophages stimulated with CpG than with LPS stimulation. In myeloid DC stimulated with LPS or CpG the effect on p38 phosphorylation was more pronounced than observed in macrophages. These data suggest that subtle differences in MAP kinase activation may result from activation of through different TLR ligands and/or intrinsic differences in MAP kinase activation in macrophages and DC. Complete blockade of ERK activation

164

by U0126 with no effects on p38 phosphorylation implies that the p38 defect in the Tpl-2 deficient mice was not due to inhibition of ERK activation. Thus, Tpl-2 may directly regulate MKK3/6 or an as yet unidentified signalling pathway.

Consistent with hypothesis that Tpl-2 may activate additional signalling pathways other than ERK, IL-12p70 was not detected in macrophages stimulated with LPS or CpG in the presence of the U0126 MEK inhibitor, but was detected in Tpl-2 deficient macrophages after LPS or CpG stimulation. Since we show that ERK phosphorylation is completely abrogated in the presence of the MEK inhibitor U0126 and that the addition of U0126 to Tpl-2 deficient macrophages or DC showed little or no additional effect, these data suggest that Tpl-2 may function via another signalling pathway to affect IL-12p70. This could be through p38 activation, however Tanaka *et al.* (2002) have previously shown that p38 phosphorylation is required for IL-12p70 induction. This implies that Tpl-2 negatively regulates IL-12 induction via ERK and potentially another pathway. This difference in cytokine levels between cells deficient in Tpl-2 or pharmacological inhibition of ERK phosphorylation indicates that there is potentially another signalling pathway controlled by Tpl-2, independent of ERK activation. Although there is still the possibility that there is still a residual level of ERK activation in the presence of the inhibitor, which is absent in Tpl-2 deficient cells.

7.2.2 Signalling in macrophages and DC suggest that they have different levels of ERK activation, which may account for different cytokine profiles.

Stimulation of macrophages with LPS and CpG lead to strong phosphorylation of ERK. In myeloid DC, we also detect ERK phosphorylation, which is in contrast to data by Hacker *et al.* (1999), although the signal strength appears weaker than that observed in the macrophages, although as yet we have not systematically addressed this. We have also detected differences in signal strength for p38 phosphorylation. This could possibly indicate that macrophages may show higher levels of ERK and p38 activation. This could determine different cytokine profiles observed in macrophages and myeloid DC upon stimulation with LPS and CPG. For example, we cannot detect IL-12p70 in the macrophages stimulated with LPS or CpG in the presence of U0126, (although IL-12p40 was detectable) but can in the myeloid DC after U0126 treatment. As IL-12 is negatively regulated by ERK, it could be that the lower levels of ERK activation in the myeloid DC could account for this difference.

7.2.3 Other potential mechanisms for TNF induction in DC than by Tpl-2 and ERK signalling.

Our data indicates that there are differences in the extent of inhibition of *Tnf* mRNA and TNF protein in macrophages and myeloid DC from the Tpl-2 deficient mice after LPS or CpG stimulation. Tpl-2 deficient macrophages fail to induce TNF protein upon stimulation with LPS or CpG. On the other hand, myeloid DC from the Tpl-2

166

deficient mice still produce significant TNF protein upon LPS or CpG stimulation, although the TNF protein is much decreased as compared to macrophages from the littermate controls.

One possible explanation could be that myeloid DC produce higher amounts of TNF protein compared to the macrophages and therefore, the immunoassay used to measure TNF levels can detect TNF in the Tpl-2 deficient myeloid DC but not in the macrophages as they may produce TNF at a level lower that the sensitivity of the assay. Another possibility is that TNF can be induced by an additional mechanism to Tpl-2/ERK signalling in myeloid DC, but not in macrophages.

The differences observed could also be due to residual ERK activity in the Tpl-2 deficient DC, although we do not believe this is likely as ERK phosphorylation appears to be completely blocked in the Tpl-2 deficient DC and also addition of U0126 MEK inhibitor does not decrease TNF production any further in Tpl-2 deficient DC.

7.2.4 IFN-β regulation through IL-10 or ERK activation.

We have shown that in the absence of ERK activity both by pharmacological inhibition of ERK phosphorylation with U0126 and in the Tpl-2 deficient mice, type I IFN, IFN-β is increased upon stimulation of macrophages and DC with LPS or CpG. At this stage we do not know the mechanism for this increased level of IFN-β. Firstly this elevation could result from the decreased levels of IL-10 observed in the absence of ERK phosphorylation or it could be regulated by the ERK signalling pathway independently of IL-10. This hypothesis will need further testing using the IL-10

167

deficient mice in the presence or absence of U0126 or the Tpl-2/IL-10 deficient mice. There has been a recent report which suggested that type I IFN and IL-10 were co-ordinately regulated by TRAF3 (Hacker *et al.* 2006). However, our data suggests that IL-10 and IFN-β regulation can be distinctly regulated via the Tpl-2/ERK signalling pathway as in the absence of ERK activation we detect reduced IL-10 but increased IFN-β induction both at the mRNA and protein level.

To investigate the potential role of IL-10 in regulating IFN-β, in future experiments IL-10 deficient macrophages and myeloid DC will be stimulated in the presence of the MEK inhibitor U0126 and the IFN-β levels will be investigated at both the mRNA and protein level. If IFN-β is increased in the IL-10 deficient mice, but no further increase is detected upon inhibition of ERK activation, this would indicate that IFN-β is negatively regulated only by IL-10 and not directly by ERK activation of transcription factors directly regulating IFN-β via an as yet unidentified transcription factor in the absence of IL-10. If a further increase is detected upon inhibition of ERK activation in the complete absence of IL-10, this would indicate that IFN-β is also negatively regulated by the ERK pathway as seen with IL-12. This will also need to be confirmed using the Tpl-2/IL-10 deficient mice. If the Tpl-2/ERK pathway negatively regulates IFN-β in the complete absence of IL-10, it will be of interest to determine which downstream transcription factors are responsible for this negative regulation of IFN-β expression.

7.2.5 Mechanism for induction of colitis in the Tpl-2/IL-10 deficient mice.

We observed that the Tpl-2/IL-10 deficient mice developed diarrhoea at the age of 6-8 weeks as compared to the IL-10 deficient mice, which develops colitis at 4-6 month of age, indicating that the Tpl-2 deficiency accelerates the onset of colitis. We found that the onset of diarrhoea coincided with a reduction in weight gain and eventually weight loss. Preliminary histology showed that the Tpl-2/IL-10 deficient mice suffered from premature colitis. As of yet we do not know that exact cause of why these mice show accelerated onset of disease as compared to the IL-10 deficient mice.

We would like to carry out flow cytometry staining of the bone marrow, spleen, gut and lymph nodes, in order to determine whether the Tpl-2/IL-10 deficient mice have the same number of $CD4^+CD25^+foxP3^+$ cells as compared to the wildtype, Tpl-2 deficient and IL-10 deficient mice. Potentially a lack of these regulatory T cells could partly explain the accelerated onset of colitis seen in the Tpl-2/IL-10 deficient mice. T-regs have been found to be important in maintaining the intestinal homeostasis (Schmidt-Supprian *et al.* 2004). It has been shown that transfer of $CD4^+CD45^{high}$ T cells into RAG deficient mice can lead to the development of colitis associated with the expansion and accumulation of T cells and DC in the intestine, however this does not occur if the mice are kept under germ-free conditions (Hue *et al.* 2006). However, cotransfer of $CD4^+CD45^{high}$ and T-regs into immunodeficient mice can prevent the development of colitis. Moreover, T cell-induced colitis can be cured by transfer of T-regs (Izcue *et al.* 2006).

Initially we did not expect these mice to become ill, as from studies with macrophages we found that they have lower levels of TNF, which is one of the mediators of colitis.

However, we expected them to have higher IL-12 levels possibly in the serum and the gut, which was also suggested earlier to be a mediator of colitis. Data using bone marrow derived macrophages from the Tpl-2/IL-10 deficient mice show that compared to the IL-10 deficient mice, they have lower TNF levels but higher IL-12p40 and IL-12p70 levels but as yet we have not studied the effects on IL-23, but we would like to test this, as recent reports have suggested that IL-23 is required for the chronic intestinal pathology observed in colitis (Hue *et al.* 2006; Kullberg *et al.* 2006).

We would like to confirm elevated levels of IL-12 in the serum and gut of the mice and we have collected a large number of serum samples ready for processing. We hope to be able to identify the cause of the accelerated disease from these samples by identifying which cytokines are elevated in the Tpl-2/IL-10 double knockout as compared to the Tpl-2 and IL-10 deficient mice and littermate controls. Examination of the intestinal cytokines produced in would be in order to pinpoint which cytokines are elevated at the early onset of the disease. Samples would have to be collected at various time points throughout the course of the disease to allow a thorough analysis as compared to the IL-10 deficient mice, which develop the disease much later.

If we find as predicted elevated levels of IL-12 or IL-23 in the serum, we would like to treat the Tpl-2/IL-10 deficient mice with monoclonal antibodies to IL-12p40 to investigate whether this would be enough to block the onset of disease. However as this antibody blocks both IL-12p70 and IL-23, it would not be enough to determine whether

the onset of colitis is mediated by IL-12 or IL-23. Therefore, treatment with either antibodies to IL-12p70 (p35) or IL-23 (p19) is required to answer this question.

As the mice have been kept in a quarantine unit and not a pathogen free environment, we would like to examine screen the mice for helicobacter infections in order to determine if this is the cause of the accelerated onset of colitis and see if they still develop colitis when they have been rederived into a clean facility. However, it has been shown that IL-10 deficient mice on the C75BL/6 background develop colitis upon helicobacter infection at 14 weeks (Kullberg *et al.* 1998; Kullberg *et al.* 2001). The IL-10 deficient mice in our unit start showing failure to gain weight around 13 weeks of age, but no other clinical signs of colitis, as confirmed by histology. This result could potentially rule out a helicobacter infection.

Finally, we have also collected the colons from the mice, which will have to be processed and scored in order to show the severity of the disease as compared to the IL-10 deficient mice but this would require larger numbers of mice, particularly since this is not an absolute difference in phenotype but rather accelerated colitis in the Tpl-2/IL-10 deficient mice as compared to the IL-10 deficient mice. This is the scope of future studies that will require time in order to collect enough samples for processing.

References

Agrawal, A., S. Dillon, T. L. Denning and B. Pulendran (2006). "ERK1-/- mice exhibit Th1 cell polarization and increased susceptibility to experimental autoimmune encephalomyelitis." J Immunol **176**(10): 5788-96.

Agrawal, S., A. Agrawal, B. Doughty, A. Gerwitz, J. Blenis, T. Van Dyke and B. Pulendran (2003). "Cutting Edge: different toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos." J Immunol **171**(10): 4984-9.

Akira, S. and K. Takeda (2004). "Toll-like receptor signalling." Nat Rev Immunol **4**(7): 499-511.

Akira, S., M. Yamamoto and K. Takeda (2003). "Role of adapters in Toll-like receptor signalling." Biochem Soc Trans **31**(Pt 3): 637-42.

Alexopoulou, L., A. C. Holt, R. Medzhitov and R. A. Flavell (2001). "Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3." Nature **413**(6857): 732-8.

Ansel, K. M., D. U. Lee and A. Rao (2003). "An epigenetic view of helper T cell differentiation." Nat Immunol **4**(7): 616-23.

Arron, J. R., M. C. Walsh and Y. Choi (2002). "TRAF-mediated TNFR-family signaling." Curr Protoc Immunol **Chapter 11**: Unit 11 9D.

Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Briere and G. Trinchieri (2001). "Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology." Nat Immunol **2**(12): 1144-50.

Asselin-Paturel, C., G. Brizard, J. J. Pin, F. Briere and G. Trinchieri (2003). "Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody." J Immunol **171**(12): 6466-77.

Asselin-Paturel, C. and G. Trinchieri (2005). "Production of type I interferons: plasmacytoid dendritic cells and beyond." J Exp Med **202**(4): 461-5.

Asseman, C., S. Mauze, M. W. Leach, R. L. Coffman and F. Powrie (1999). "An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation." J Exp Med **190**(7): 995-1004.

Banchereau, J. and R. M. Steinman (1998). "Dendritic cells and the control of immunity." Nature **392**(6673): 245-52.

Banerjee, A., R. Gugasyan, M. McMahon and S. Gerondakis (2006). "Diverse Toll-like receptors utilize Tpl2 to activate extracellular signal-regulated kinase (ERK) in hemopoietic cells." Proc Natl Acad Sci U S A **103**(9): 3274-9.

Becker, C., H. Dornhoff, C. Neufert, M. C. Fantini, S. Wirtz, S. Huebner, A. Nikolaev, H. A. Lehr, A. J. Murphy, D. M. Valenzuela, G. D. Yancopoulos, P. R. Galle, M. Karow and M. F. Neurath (2006). "Cutting edge: IL-23 cross-regulates IL-12 production in T cell-dependent experimental colitis." J Immunol **177**(5): 2760-4.

Beinke, S., J. Deka, V. Lang, M. P. Belich, P. A. Walker, S. Howell, S. J. Smerdon, S. J. Gamblin and S. C. Ley (2003). "NF-kappaB1 p105 negatively regulates TPL-2 MEK kinase activity." Mol Cell Biol **23**(14): 4739-52.

Beinke, S. and S. C. Ley (2004). "Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology." Biochem J **382**(Pt 2): 393-409.

Beinke, S. and S. C. Ley (2004). "Regulation and functions of NF-kB2 p100 and NF-kB1 p105." in press.

Belich, M. P., A. Salmeron, L. H. Johnston and S. C. Ley (1999). "TPL-2 kinase regulates the proteolysis of the NF-kappaB-inhibitory protein NF-kappaB1 p105." Nature **397**(6717): 363-8.

Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner and V. K. Kuchroo (2006). "Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells." Nature **441**(7090): 235-8.

Bjorck, P. (2001). "Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice." Blood **98**(13): 3520-6.

Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K. A. Schooley, M. Gerhart, R. Davis, J. N. Fitzner, R. S. Johnson, R. J. Paxton, C. J. March and D. P. Cerretti (1997). "A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells." Nature **385**(6618): 729-33.

Bogdan, C., M. Rollinghoff and A. Diefenbach (2000). "The role of nitric oxide in innate immunity." Immunol Rev **173**: 17-26.

Bogdan, C., Y. Vodovotz and C. Nathan (1991). "Macrophage deactivation by interleukin 10." J Exp Med **174**(6): 1549-55.

Boonstra, A., C. Asselin-Paturel, M. Gilliet, C. Crain, G. Trinchieri, Y. J. Liu and A. O'Garra (2003). "Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation." J Exp Med **197**(1): 101-9.

Boonstra, A., R. Rajsbaum, M. Holman, R. Marques, C. Asselin-Paturel, J. P. Pereira, E. E. Bates, S. Akira, P. Vieira, Y. J. Liu, G. Trinchieri and A. O'Garra (2006). "Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals." J Immunol **177**(11): 7551-8.

Brightbill, H. D., S. E. Plevy, R. L. Modlin and S. T. Smale (2000). "A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promoter in macrophages." J Immunol **164**(4): 1940-51.

Casanova, J. L. and L. Abel (2002). "Genetic dissection of immunity to mycobacteria: the human model." Annu Rev Immunol **20**: 581-620.

Ceci, J. D., C. P. Patriotis, C. Tsatsanis, A. M. Makris, R. Kovatch, D. A. Swing, N. A. Jenkins, P. N. Tsichlis and N. G. Copeland (1997). "Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation." Genes Dev **11**(6): 688-700.

Celada, A. and C. Nathan (1994). "Macrophage activation revisited." Immunol Today **15**(3): 100-2.

Chang, L. and M. Karin (2001). "Mammalian MAP kinase signalling cascades." Nature **410**(6824): 37-40.

Chen, Y., C. L. Langrish, B. McKenzie, B. Joyce-Shaikh, J. S. Stumhofer, T. McClanahan, W. Blumenschein, T. Churakovsa, J. Low, L. Presta, C. A. Hunter, R. A. Kastelein and D. J. Cua (2006). "Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis." J Clin Invest **116**(5): 1317-26.

Chi, H., S. P. Barry, R. J. Roth, J. J. Wu, E. A. Jones, A. M. Bennett and R. A. Flavell (2006). "Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses." Proc Natl Acad Sci U S A **103**(7): 2274-9.

Chiariello, M., M. J. Marinissen and J. S. Gutkind (2000). "Multiple mitogen-activated protein kinase signaling pathways connect the cot oncoprotein to the c-jun promoter and to cellular transformation." Mol Cell Biol **20**(5): 1747-58.

Choy, E. H. and G. S. Panayi (2001). "Cytokine pathways and joint inflammation in rheumatoid arthritis." N Engl J Med **344**(12): 907-16.

Collart, M. A., P. Baeuerle and P. Vassalli (1990). "Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B." Mol Cell Biol **10**(4): 1498-506.

Cowley, S., H. Paterson, P. Kemp and C. J. Marshall (1994). "Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells." Cell **77**(6): 841-52.

Cua, D. J., J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S. A. Lira, D. Gorman, R. A. Kastelein and J. D. Sedgwick (2003). "Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain." Nature **421**(6924): 744-8.

Dalod, M., T. P. Salazar-Mather, L. Malmgaard, C. Lewis, C. Asselin-Paturel, F. Briere, G. Trinchieri and C. A. Biron (2002). "Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo." J Exp Med **195**(4): 517-28.

Davidson, N. J., M. W. Leach, M. M. Fort, L. Thompson-Snipes, R. Kuhn, W. Muller, D. J. Berg and D. M. Rennick (1996). "T helper cell 1-type CD4+ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice." J Exp Med **184**(1): 241-51.

de Winther, M. P., E. Kanters, G. Kraal and M. H. Hofker (2005). "Nuclear factor kappaB signaling in atherogenesis." Arterioscler Thromb Vasc Biol **25**(5): 904-14.

Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira and E. S. C. Reis (2004). "Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA." Science **303**(5663): 1529-31.

Diebold, S. S., M. Montoya, H. Unger, L. Alexopoulou, P. Roy, L. E. Haswell, A. Al-Shamkhani, R. Flavell, P. Borrow and C. Reis e Sousa (2003). "Viral infection switches non-plasmacytoid dendritic cells into high interferon producers." Nature **424**(6946): 324-8.

Dillon, S., A. Agrawal, T. Van Dyke, G. Landreth, L. McCauley, A. Koh, C. Maliszewski, S. Akira and B. Pulendran (2004). "A Toll-like receptor 2 ligand

stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells." J Immunol **172**(8): 4733-43.

Dillon, S., S. Agrawal, K. Banerjee, J. Letterio, T. L. Denning, K. Oswald-Richter, D. J. Kasprowicz, K. Kellar, J. Pare, T. van Dyke, S. Ziegler, D. Unutmaz and B. Pulendran (2006). "Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance." J Clin Invest **116**(4): 916-28.

Dong, C., R. J. Davis and R. A. Flavell (2002). "MAP kinases in the immune response." Annu Rev Immunol **20**: 55-72.

Dumitru, C. D., J. D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J. H. Lin, C. Patriotis, N. A. Jenkins, N. G. Copeland, G. Kollias and P. N. Tsichlis (2000). "TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway." Cell **103**(7): 1071-83.

Edwards, A. D., S. P. Manickasingham, R. Sporri, S. S. Diebold, O. Schulz, A. Sher, T. Kaisho, S. Akira and E. S. C. Reis (2002). "Microbial recognition via Toll-like receptor-dependent and -independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering." J Immunol **169**(7): 3652-60.

Eferl, R. and E. F. Wagner (2003). "AP-1: a double-edged sword in tumorigenesis." Nat Rev Cancer **3**(11): 859-68.

Eliopoulos, A. G., C. C. Wang, C. D. Dumitru and P. N. Tsichlis (2003). "Tpl2 transduces CD40 and TNF signals that activate ERK and regulates IgE induction by CD40." Embo J **22**(15): 3855-3864.

Erny, K. M., J. Peli, J. F. Lambert, V. Muller and H. Diggelmann (1996). "Involvement of the Tpl-2/cot oncogene in MMTV tumorigenesis." Oncogene **13**(9): 2015-20.

Favata, M. F., K. Y. Horiuchi, E. J. Manos, A. J. Daulerio, D. A. Stradley, W. S. Feeser, D. E. Van Dyk, W. J. Pitts, R. A. Earl, F. Hobbs, R. A. Copeland, R. L. Magolda, P. A. Scherle and J. M. Trzaskos (1998). "Identification of a novel inhibitor of mitogen-activated protein kinase kinase." J Biol Chem **273**(29): 18623-32.

Feldmann, M., F. M. Brennan, B. M. Foxwell, P. C. Taylor, R. O. Williams and R. N. Maini (2005). "Anti-TNF therapy: where have we got to in 2005?" J Autoimmun **25 Suppl**: 26-8.

Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard and A. O'Garra (1991). "IL-10 inhibits cytokine production by activated macrophages." J Immunol **147**(11): 3815-22.

Fiorentino, D. F., A. Zlotnik, P. Vieira, T. R. Mosmann, M. Howard, K. W. Moore and A. O'Garra (1991). "IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells." J Immunol **146**(10): 3444-51.

Fitzgerald, K. A., D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha and D. T. Golenbock (2003). "LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF." J Exp Med **198**(7): 1043-55.

Flynn, J. L., M. M. Goldstein, J. Chan, K. J. Triebold, K. Pfeffer, C. J. Lowenstein, R. Schreiber, T. W. Mak and B. R. Bloom (1995). "Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice." Immunity **2**(6): 561-72.

Garrett, W. S., G. M. Lord, S. Punit, G. Lugo-Villarino, S. K. Mazmanian, S. Ito, J. N. Glickman and L. H. Glimcher (2007). "Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system." Cell **131**(1): 33-45.

Gautier, G., M. Humbert, F. Deauvieau, M. Scuiller, J. Hiscott, E. E. Bates, G. Trinchieri, C. Caux and P. Garrone (2005). "A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells." J Exp Med **201**(9): 1435-46.

Gazzinelli, R. T., I. P. Oswald, S. L. James and A. Sher (1992). "IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages." J Immunol **148**(6): 1792-6.

Gazzinelli, R. T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri and A. Sher (1996). "In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha." J Immunol **157**(2): 798-805.

Gilliet, M., A. Boonstra, C. Paturel, S. Antonenko, X. L. Xu, G. Trinchieri, A. O'Garra and Y. J. Liu (2002). "The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor." J Exp Med **195**(7): 953-8.

Grenningloh, R., B. Y. Kang and I. C. Ho (2005). "Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses." J Exp Med **201**(4): 615-26.

Guiducci, C., G. Ott, J. H. Chan, E. Damon, C. Calacsan, T. Matray, K. D. Lee, R. L. Coffman and F. J. Barrat (2006). "Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation." J Exp Med **203**(8): 1999-2008.

Hacker, H., H. Mischak, G. Hacker, S. Eser, N. Prenzel, A. Ullrich and H. Wagner (1999). "Cell type-specific activation of mitogen-activated protein kinases by CpG-DNA controls interleukin-12 release from antigen-presenting cells." Embo J **18**(24): 6973-82.

Hacker, H., V. Redecke, B. Blagoev, I. Kratchmarova, L. C. Hsu, G. G. Wang, M. P. Kamps, E. Raz, H. Wagner, G. Hacker, M. Mann and M. Karin (2006). "Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6." Nature **439**(7073): 204-7.

Hammer, M., J. Mages, H. Dietrich, A. Servatius, N. Howells, A. C. Cato and R. Lang (2006). "Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock." J Exp Med **203**(1): 15-20.

Han, J., T. Brown and B. Beutler (1990). "Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level." J Exp Med **171**(2): 465-75.

Hanauer, S. B., W. J. Sandborn, P. Rutgeerts, R. N. Fedorak, M. Lukas, D. MacIntosh, R. Panaccione, D. Wolf and P. Pollack (2006). "Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: the CLASSIC-I trial." Gastroenterology **130**(2): 323-33; quiz 591.

Happel, K. I., P. J. Dubin, M. Zheng, N. Ghilardi, C. Lockhart, L. J. Quinton, A. R. Odden, J. E. Shellito, G. J. Bagby, S. Nelson and J. K. Kolls (2005). "Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae." J Exp Med **202**(6): 761-9.

Hashimoto, C., K. L. Hudson and K. V. Anderson (1988). "The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein." Cell **52**(2): 269-79.

Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill and A. Aderem (2001). "The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5." Nature **410**(6832): 1099-103.

Haziot, A., E. Ferrero, F. Kontgen, N. Hijiya, S. Yamamoto, J. Silver, C. L. Stewart and S. M. Goyert (1996). "Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice." Immunity **4**(4): 407-14.

Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda and S. Akira (2002). "Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway." Nat Immunol **3**(2): 196-200.

Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda and S. Akira (2000). "A Toll-like receptor recognizes bacterial DNA." Nature **408**(6813): 740-5.

Hoffmann, J. A., F. C. Kafatos, C. A. Janeway and R. A. Ezekowitz (1999). "Phylogenetic perspectives in innate immunity." Science **284**(5418): 1313-8.

Honda, K., A. Takaoka and T. Taniguchi (2006). "Type I interferon [correction of inteferon] gene induction by the interferon regulatory factor family of transcription factors." Immunity **25**(3): 349-60.

Honda, K., H. Yanai, T. Mizutani, H. Negishi, N. Shimada, N. Suzuki, Y. Ohba, A. Takaoka, W. C. Yeh and T. Taniguchi (2004). "Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling." Proc Natl Acad Sci U S A **101**(43): 15416-21.

Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida and T. Taniguchi (2005). "IRF-7 is the master regulator of type-I interferon-dependent immune responses." Nature **434**(7034): 772-7.

Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda and S. Akira (1999). "Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product." J Immunol **162**(7): 3749-52.

Hsu, L. C., J. M. Park, K. Zhang, J. L. Luo, S. Maeda, R. J. Kaufman, L. Eckmann, D. G. Guiney and M. Karin (2004). "The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4." Nature **428**(6980): 341-5.

Hue, S., P. Ahern, S. Buonocore, M. C. Kullberg, D. J. Cua, B. S. McKenzie, F. Powrie and K. J. Maloy (2006). "Interleukin-23 drives innate and T cell-mediated intestinal inflammation." J Exp Med **203**(11): 2473-83.

Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu and R. M. Steinman (1992). "Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor." J Exp Med **176**(6): 1693-702.

Izcue, A., J. L. Coombes and F. Powrie (2006). "Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation." Immunol Rev **212**: 256-71.

Janeway, C. A., Jr. and R. Medzhitov (2002). "Innate immune recognition." Annu Rev Immunol **20**: 197-216.

Jones, E. A. and R. A. Flavell (2005). "Distal enhancer elements transcribe intergenic RNA in the IL-10 family gene cluster." J Immunol **175**(11): 7437-46.

Kadowaki, N., S. Ho, S. Antonenko, R. W. Malefyt, R. A. Kastelein, F. Bazan and Y. J. Liu (2001). "Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens." J Exp Med **194**(6): 863-9.

Kanters, E., M. Pasparakis, M. J. Gijbels, M. N. Vergouwe, I. Partouns-Hendriks, R. J. Fijneman, B. E. Clausen, I. Forster, M. M. Kockx, K. Rajewsky, G. Kraal, M. H. Hofker and M. P. de Winther (2003). "Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice." J Clin Invest **112**(8): 1176-85.

Kapsenberg, M. L. (2003). "Dendritic-cell control of pathogen-driven T-cell polarization." Nat Rev Immunol **3**(12): 984-93.

Kawai, T., O. Adachi, T. Ogawa, K. Takeda and S. Akira (1999). "Unresponsiveness of MyD88-deficient mice to endotoxin." Immunity **11**(1): 115-22.

Kawai, T., S. Sato, K. J. Ishii, C. Coban, H. Hemmi, M. Yamamoto, K. Terai, M. Matsuda, J. Inoue, S. Uematsu, O. Takeuchi and S. Akira (2004). "Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6." Nat Immunol **5**(10): 1061-8.

Keane, J. (2005). "TNF-blocking agents and tuberculosis: new drugs illuminate an old topic." Rheumatology (Oxford) **44**(6): 714-20.

Keyse, S. M. (2000). "Protein phosphatases and the regulation of mitogen-activated protein kinase signalling." Curr Opin Cell Biol **12**(2): 186-92.

Kitani, A., I. Fuss, K. Nakamura, F. Kumaki, T. Usui and W. Strober (2003). "Transforming growth factor (TGF)-beta1-producing regulatory T cells induce Smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF-beta1-mediated fibrosis." J Exp Med **198**(8): 1179-88.

Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia and G. Trinchieri (1989). "Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes." J Exp Med **170**(3): 827-45.

Kontoyiannis, D., G. Boulougouris, M. Manoloukos, M. Armaka, M. Apostolaki, T. Pizarro, A. Kotlyarov, I. Forster, R. Flavell, M. Gaestel, P. Tsichlis, F. Cominelli and G. Kollias (2002). "Genetic dissection of the cellular pathways and signaling mechanisms in modeled tumor necrosis factor-induced Crohn's-like inflammatory bowel disease." J Exp Med **196**(12): 1563-74.

Kontoyiannis, D., M. Pasparakis, T. T. Pizarro, F. Cominelli and G. Kollias (1999). "Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies." Immunity **10**(3): 387-98.

Kortum, R. L., D. L. Costanzo, J. Haferbier, S. J. Schreiner, G. L. Razidlo, M. H. Wu, D. J. Volle, T. Mori, H. Sakaue, N. V. Chaika, O. V. Chaika and R. E. Lewis (2005). "The molecular scaffold kinase suppressor of Ras 1 (KSR1) regulates adipogenesis." Mol Cell Biol **25**(17): 7592-604.

Kortum, R. L., H. J. Johnson, D. L. Costanzo, D. J. Volle, G. L. Razidlo, A. M. Fusello, A. S. Shaw and R. E. Lewis (2006). "The molecular scaffold kinase suppressor of Ras 1 is a modifier of RasV12-induced and replicative senescence." Mol Cell Biol **26**(6): 2202-14.

Krieg, A. M. (2002). "CpG motifs in bacterial DNA and their immune effects." Annu Rev Immunol **20**: 709-60.

Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky and D. M. Klinman (1995). "CpG motifs in bacterial DNA trigger direct B-cell activation." Nature **374**(6522): 546-9.

Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky and W. Muller (1993). "Interleukin-10-deficient mice develop chronic enterocolitis." Cell **75**(2): 263-74.

Kullberg, M. C., D. Jankovic, C. G. Feng, S. Hue, P. L. Gorelick, B. S. McKenzie, D. J. Cua, F. Powrie, A. W. Cheever, K. J. Maloy and A. Sher (2006). "IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis." J Exp Med **203**(11): 2485-94.

Kullberg, M. C., A. G. Rothfuchs, D. Jankovic, P. Caspar, T. A. Wynn, P. L. Gorelick, A. W. Cheever and A. Sher (2001). "Helicobacter hepaticus-induced colitis in

interleukin-10-deficient mice: cytokine requirements for the induction and maintenance of intestinal inflammation." <u>Infect Immun</u> **69**(7): 4232-41.

Kullberg, M. C., J. M. Ward, P. L. Gorelick, P. Caspar, S. Hieny, A. Cheever, D. Jankovic and A. Sher (1998). "Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism." <u>Infect Immun</u> **66**(11): 5157-66.

Lang, V., A. Symons, S. J. Watton, J. Janzen, Y. Soneji, S. Beinke, S. Howell and S. C. Ley (2004). "ABIN-2 forms a ternary complex with TPL-2 and NF-kappa B1 p105 and is essential for TPL-2 protein stability." <u>Mol Cell Biol</u> **24**(12): 5235-48.

Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein and D. J. Cua (2005). "IL-23 drives a pathogenic T cell population that induces autoimmune inflammation." <u>J Exp Med</u> **201**(2): 233-40.

Leach, M. W., A. G. Bean, S. Mauze, R. L. Coffman and F. Powrie (1996). "Inflammatory bowel disease in C.B-17 scid mice reconstituted with the CD45RBhigh subset of CD4+ T cells." <u>Am J Pathol</u> **148**(5): 1503-15.

Li, Q. and I. M. Verma (2002). "NF-kappaB regulation in the immune system." <u>Nat Rev Immunol</u> **2**(10): 725-34.

Liu, Y. J., H. Kanzler, V. Soumelis and M. Gilliet (2001). "Dendritic cell lineage, plasticity and cross-regulation." <u>Nat Immunol</u> **2**(7): 585-9.

Liu, Y. W., H. P. Tseng, L. C. Chen, B. K. Chen and W. C. Chang (2003). "Functional cooperation of simian virus 40 promoter factor 1 and CCAAT/enhancer-binding protein beta and delta in lipopolysaccharide-induced gene activation of IL-10 in mouse macrophages." <u>J Immunol</u> **171**(2): 821-8.

Ma, X., J. M. Chow, G. Gri, G. Carra, F. Gerosa, S. F. Wolf, R. Dzialo and G. Trinchieri (1996). "The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells." <u>J Exp Med</u> **183**(1): 147-57.

Ma, X. and G. Trinchieri (2001). "Regulation of interleukin-12 production in antigen-presenting cells." <u>Adv Immunol</u> **79**: 55-92.

Mamane, Y., C. Heylbroeck, P. Genin, M. Algarte, M. J. Servant, C. LePage, C. DeLuca, H. Kwon, R. Lin and J. Hiscott (1999). "Interferon regulatory factors: the next generation." <u>Gene</u> **237**(1): 1-14.

Mancuso, G., A. Midiri, C. Beninati, G. Piraino, A. Valenti, G. Nicocia, D. Teti, J. Cook and G. Teti (2002). "Mitogen-activated protein kinases and NF-kappa B are involved in TNF-alpha responses to group B streptococci." J Immunol **169**(3): 1401-9.

Marshall, C. J. (1994). "MAP kinase kinase kinase, MAP kinase kinase and MAP kinase." Curr Opin Genet Dev **4**(1): 82-9.

Marshall, C. J. (1995). "Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation." Cell **80**(2): 179-85.

Martin, E., B. O'Sullivan, P. Low and R. Thomas (2003). "Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10." Immunity **18**(1): 155-67.

Martin, H., M. Flandez, C. Nombela and M. Molina (2005). "Protein phosphatases in MAPK signalling: we keep learning from yeast." Mol Microbiol **58**(1): 6-16.

McCubrey, J. A., L. S. Steelman, W. H. Chappell, S. L. Abrams, E. W. Wong, F. Chang, B. Lehmann, D. M. Terrian, M. Milella, A. Tafuri, F. Stivala, M. Libra, J. Basecke, C. Evangelisti, A. M. Martelli and R. A. Franklin (2007). "Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance." Biochim Biophys Acta **1773**(8): 1263-84.

Medzhitov, R. (2001). "Toll-like receptors and innate immunity." Nat Rev Immunol **1**(2): 135-45.

Meuer, S. C., R. E. Hussey, D. A. Cantrell, J. C. Hodgdon, S. F. Schlossman, K. A. Smith and E. L. Reinherz (1984). "Triggering of the T3-Ti antigen-receptor complex results in clonal T-cell proliferation through an interleukin 2-dependent autocrine pathway." Proc Natl Acad Sci U S A **81**(5): 1509-13.

Mocci, S., S. A. Dalrymple, R. Nishinakamura and R. Murray (1997). "The cytokine stew and innate resistance to L. monocytogenes." Immunol Rev **158**: 107-14.

Moore, K. W., R. de Waal Malefyt, R. L. Coffman and A. O'Garra (2001). "Interleukin-10 and the interleukin-10 receptor." Annu Rev Immunol **19**: 683-765.

Morrison, D. K. and R. J. Davis (2003). "Regulation of MAP kinase signaling modules by scaffold proteins in mammals." Annu Rev Cell Dev Biol **19**: 91-118.

Morrissette, N., E. Gold and A. Aderem (1999). "The macrophage--a cell for all seasons." Trends Cell Biol **9**(5): 199-201.

Mosser, D. M. (2003). "The many faces of macrophage activation." J Leukoc Biol **73**(2): 209-12.

Murphy, C. A., C. L. Langrish, Y. Chen, W. Blumenschein, T. McClanahan, R. A. Kastelein, J. D. Sedgwick and D. J. Cua (2003). "Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation." J Exp Med **198**(12): 1951-7.

Murphy, L. O. and J. Blenis (2006). "MAPK signal specificity: the right place at the right time." Trends Biochem Sci **31**(5): 268-75.

Murphy, L. O., S. Smith, R. H. Chen, D. C. Fingar and J. Blenis (2002). "Molecular interpretation of ERK signal duration by immediate early gene products." Nat Cell Biol **4**(8): 556-64.

Nakano, H., M. Yanagita and M. D. Gunn (2001). "CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells." J Exp Med **194**(8): 1171-8.

Novelli, F. and J. L. Casanova (2004). "The role of IL-12, IL-23 and IFN-gamma in immunity to viruses." Cytokine Growth Factor Rev **15**(5): 367-77.

O'Garra, A. (1998). "Cytokines induce the development of functionally heterogeneous T helper cell subsets." Immunity **8**(3): 275-83.

O'Garra, A. and D. Robinson (2004). "Development and function of T helper 1 cells." Adv Immunol **83**: 133-62.

O'Garra, A. and P. Vieira (2004). "Regulatory T cells and mechanisms of immune system control." Nat Med **10**(8): 801-5.

O'Garra, A., P. L. Vieira, P. Vieira and A. E. Goldfeld (2004). "IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage." J Clin Invest **114**(10): 1372-8.

Owens, D. M. and S. M. Keyse (2007). "Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases." Oncogene **26**(22): 3203-13.

Palucka, K. and J. Banchereau (2002). "How dendritic cells and microbes interact to elicit or subvert protective immune responses." Curr Opin Immunol **14**(4): 420-31.

Papoutsopoulou, S., A. Symons, T. Tharmalingham, M. P. Belich, F. Kaiser, D. Kioussis, A. O'Garra, V. Tybulewicz and S. C. Ley (2006). "ABIN-2 is required for optimal activation of Erk MAP kinase in innate immune responses." Nat Immunol **7**(6): 606-15.

Patriotis, C., A. Makris, S. E. Bear and P. N. Tsichlis (1993). "Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation." Proc Natl Acad Sci U S A **90**(6): 2251-5.

Patriotis, C., A. Makris, J. Chernoff and P. N. Tsichlis (1994). "Tpl-2 acts in concert with Ras and Raf-1 to activate mitogen-activated protein kinase." Proc Natl Acad Sci U S A **91**(21): 9755-9.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton and B. Beutler (1998). "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene." Science **282**(5396): 2085-8.

Pomerantz, J. L. and D. Baltimore (2002). "Two pathways to NF-kappaB." Mol Cell **10**(4): 693-5.

Raingeaud, J., S. Gupta, J. S. Rogers, M. Dickens, J. Han, R. J. Ulevitch and R. J. Davis (1995). "Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine." J Biol Chem **270**(13): 7420-6.

Rennick, D., N. Davidson and D. Berg (1995). "Interleukin-10 gene knock-out mice: a model of chronic inflammation." Clin Immunol Immunopathol **76**(3 Pt 2): S174-8.

Robinson, D. S. and A. O'Garra (2002). "Further checkpoints in Th1 development." Immunity **16**(6): 755-8.

Robinson, M. J., S. Beinke, A. Kouroumalis, P. N. Tsichlis and S. C. Ley (2007). "Phosphorylation of TPL-2 on serine 400 is essential for lipopolysaccharide activation of extracellular signal-regulated kinase in macrophages." Mol Cell Biol **27**(21): 7355-64.

Rousseau, S., M. Papoutsopoulou, A. Symons, D. Cook, J. M. Lucocq, A. R. Prescott, A. O'Garra, S. C. Ley and P. Cohen (2008). "TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF alpha in LPS-stimulated macrophages." J Cell Sci **121**(Pt 2): 149-54.

Roux, P. P. and J. Blenis (2004). "ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions." Microbiol Mol Biol Rev **68**(2): 320-44.

Sacks, D. B. (2006). "The role of scaffold proteins in MEK/ERK signalling." Biochem Soc Trans **34**(Pt 5): 833-6.

Sakaguchi, S. (2005). "Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self." Nat Immunol **6**(4): 345-52.

Salmeron, A., T. B. Ahmad, G. W. Carlile, D. Pappin, R. P. Narsimhan and S. C. Ley (1996). "Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase." Embo J **15**(4): 817-26.

Saraiva, M., J. R. Christensen, A. V. Tsytsykova, A. E. Goldfeld, S. C. Ley, D. Kioussis and A. O'Garra (2005). "Identification of a macrophage-specific chromatin signature in the IL-10 locus." J Immunol **175**(2): 1041-6.

Saxena, M. and T. Mustelin (2000). "Extracellular signals and scores of phosphatases: all roads lead to MAP kinase." Semin Immunol **12**(4): 387-96.

Schaeffer, H. J. and M. J. Weber (1999). "Mitogen-activated protein kinases: specific messages from ubiquitous messengers." Mol Cell Biol **19**(4): 2435-44.

Scherle, P. A., E. A. Jones, M. F. Favata, A. J. Daulerio, M. B. Covington, S. A. Nurnberg, R. L. Magolda and J. M. Trzaskos (1998). "Inhibition of MAP kinase kinase prevents cytokine and prostaglandin E2 production in lipopolysaccharide-stimulated monocytes." J Immunol **161**(10): 5681-6.

Schmidt-Supprian, M., J. Tian, E. P. Grant, M. Pasparakis, R. Maehr, H. Ovaa, H. L. Ploegh, A. J. Coyle and K. Rajewsky (2004). "Differential dependence of CD4+CD25+ regulatory and natural killer-like T cells on signals leading to NF-kappaB activation." Proc Natl Acad Sci U S A **101**(13): 4566-71.

Schulz, O., A. D. Edwards, M. Schito, J. Aliberti, S. Manickasingham, A. Sher and C. Reis e Sousa (2000). "CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal." Immunity **13**(4): 453-62.

Sher, A. and R. L. Coffman (1992). "Regulation of immunity to parasites by T cells and T cell-derived cytokines." Annu Rev Immunol **10**: 385-409.

Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake and M. Kimoto (1999). "MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4." J Exp Med **189**(11): 1777-82.

Shortman, K. and Y. J. Liu (2002). "Mouse and human dendritic cell subtypes." Nat Rev Immunol **2**(3): 151-61.

Silva, R. A., T. F. Pais and R. Appelberg (2001). "Blocking the receptor for IL-10 improves antimycobacterial chemotherapy and vaccination." J Immunol **167**(3): 1535-41.

Smits, H. H., A. J. Van Beelen, C. Hessle, R. Westland, E. De Jong, E. Soeteman, A. Wold, E. A. Wierenga and M. L. Kapsenberg (2004). "Commensal Gram-negative bacteria prime human dendritic cells for enhanced IL-23 and IL-27 expression and enhanced Th1 development." Eur J Immunol **34**(5): 1371-80.

Soond, S. M., B. Everson, D. W. Riches and G. Murphy (2005). "ERK-mediated phosphorylation of Thr735 in TNFalpha-converting enzyme and its potential role in TACE protein trafficking." J Cell Sci **118**(Pt 11): 2371-80.

Steinman, R. M. and H. Hemmi (2006). "Dendritic cells: translating innate to adaptive immunity." Curr Top Microbiol Immunol **311**: 17-58.

Stetson, D. B. and R. Medzhitov (2006). "Type I interferons in host defense." Immunity **25**(3): 373-81.

Sugimoto, K., M. Ohata, J. Miyoshi, H. Ishizaki, N. Tsuboi, A. Masuda, Y. Yoshikai, M. Takamoto, K. Sugane, S. Matsuo, Y. Shimada and T. Matsuguchi (2004). "A serine/threonine kinase, Cot/Tpl2, modulates bacterial DNA-induced IL-12 production and Th cell differentiation." J Clin Invest **114**(6): 857-66.

Swantek, J. L., M. H. Cobb and T. D. Geppert (1997). "Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK." Mol Cell Biol **17**(11): 6274-82.

Symons, A., S. Beinke and S. C. Ley (2006). "MAP kinase kinase kinases and innate immunity." Trends Immunol **27**(1): 40-8.

Takeda, K. and S. Akira (2004). "TLR signaling pathways." Semin Immunol **16**(1): 3-9.

Takeda, K., T. Kaisho and S. Akira (2003). "Toll-like receptors." Annu Rev Immunol
21: 335-76.

Takeshita, F., I. Gursel, K. J. Ishii, K. Suzuki, M. Gursel and D. M. Klinman (2004).
"Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like
receptor 9." Semin Immunol **16**(1): 17-22.

Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda and S.
Akira (1999). "Differential roles of TLR2 and TLR4 in recognition of gram-negative
and gram-positive bacterial cell wall components." Immunity **11**(4): 443-51.

Takeuchi, O., T. Kawai, P. F. Muhlradt, M. Morr, J. D. Radolf, A. Zychlinsky, K.
Takeda and S. Akira (2001). "Discrimination of bacterial lipoproteins by Toll-like
receptor 6." Int Immunol **13**(7): 933-40.

Takeuchi, O., S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R. L. Modlin and
S. Akira (2002). "Cutting edge: role of Toll-like receptor 1 in mediating immune
response to microbial lipoproteins." J Immunol **169**(1): 10-4.

Tanaka, N., M. Kamanaka, H. Enslen, C. Dong, M. Wysk, R. J. Davis and R. A. Flavell
(2002). "Differential involvement of p38 mitogen-activated protein kinase kinases
MKK3 and MKK6 in T-cell apoptosis." EMBO Rep **3**(8): 785-91.

Taylor, P. R., L. Martinez-Pomares, M. Stacey, H. H. Lin, G. D. Brown and S. Gordon
(2005). "Macrophage receptors and immune recognition." Annu Rev Immunol **23**: 901-
44.

Tomczak, M. F., S. E. Erdman, A. Davidson, Y. Y. Wang, P. R. Nambiar, A. B. Rogers,
B. Rickman, D. Luchetti, J. G. Fox and B. H. Horwitz (2006). "Inhibition of
Helicobacter hepaticus-Induced Colitis by IL-10 Requires the p50/p105 Subunit of NF-
{kappa}B." J Immunol **177**(10): 7332-7339.

Tone, M., M. J. Powell, Y. Tone, S. A. Thompson and H. Waldmann (2000). "IL-10
gene expression is controlled by the transcription factors Sp1 and Sp3." J Immunol
165(1): 286-91.

Trinchieri, G. (2003). "Interleukin-12 and the regulation of innate resistance and
adaptive immunity." Nat Rev Immunol **3**(2): 133-46.

Trinchieri, G., S. Pflanz and R. A. Kastelein (2003). "The IL-12 family of heterodimeric
cytokines: new players in the regulation of T cell responses." Immunity **19**(5): 641-4.

Tsatsanis, C., K. Vaporidi, V. Zacharioudaki, A. Androulidaki, Y. Sykulev, A. N. Margioris and P. N. Tsichlis (2008). "Tpl2 and ERK transduce antiproliferative T cell receptor signals and inhibit transformation of chronically stimulated T cells." Proc Natl Acad Sci U S A **105**(8): 2987-92.

Tsytsykova, A. V., R. Rajsbaum, J. V. Falvo, F. Ligeiro, S. R. Neely and A. E. Goldfeld (2007). "Activation-dependent intrachromosomal interactions formed by the TNF gene promoter and two distal enhancers." Proc Natl Acad Sci U S A **104**(43): 16850-5.

Uhlig, H. H., B. S. McKenzie, S. Hue, C. Thompson, B. Joyce-Shaikh, R. Stepankova, N. Robinson, S. Buonocore, H. Tlaskalova-Hogenova, D. J. Cua and F. Powrie (2006). "Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology." Immunity **25**(2): 309-18.

Vandenabeele, P., W. Declercq, R. Beyaert and W. Fiers (1995). "Two tumour necrosis factor receptors: structure and function." Trends Cell Biol **5**(10): 392-9.

Vassalli, P. (1992). "The pathophysiology of tumor necrosis factors." Annu Rev Immunol **10**: 411-52.

Veldhoen, M., R. J. Hocking, R. A. Flavell and B. Stockinger (2006). "Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease." Nat Immunol **7**(11): 1151-6.

Veldhoen, M. and B. Stockinger (2006). "TGFbeta1, a "Jack of all trades": the link with pro-inflammatory IL-17-producing T cells." Trends Immunol **27**(8): 358-61.

Vieira, L. Q., M. Goldschmidt, M. Nashleanas, K. Pfeffer, T. Mak and P. Scott (1996). "Mice lacking the TNF receptor p55 fail to resolve lesions caused by infection with Leishmania major, but control parasite replication." J Immunol **157**(2): 827-35.

Wajant, H., K. Pfizenmaier and P. Scheurich (2003). "Tumor necrosis factor signaling." Cell Death Differ **10**(1): 45-65.

Wang, Z. Y., H. Sato, S. Kusam, S. Sehra, L. M. Toney and A. L. Dent (2005). "Regulation of IL-10 gene expression in Th2 cells by Jun proteins." J Immunol **174**(4): 2098-105.

Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch and J. C. Mathison (1990). "CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein." Science **249**(4975): 1431-3.

Wright, S. D., P. S. Tobias, R. J. Ulevitch and R. A. Ramos (1989). "Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages." <u>J Exp Med</u> **170**(4): 1231-41.

Yasuda, K., H. Wagner and Y. Takakura (2006). "Role of immunostimulatory DNA and TLR9 in gene therapy." <u>Crit Rev Ther Drug Carrier Syst</u> **23**(2): 89-110.

Yen, D., J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. McKenzie, M. A. Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, E. Murphy, M. Sathe, D. J. Cua, R. A. Kastelein and D. Rennick (2006). "IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6." <u>J Clin Invest</u> **116**(5): 1310-6.

Yi, A. K., J. G. Yoon, S. J. Yeo, S. C. Hong, B. K. English and A. M. Krieg (2002). "Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response." <u>J Immunol</u> **168**(9): 4711-20.

Yoon, S. and R. Seger (2006). "The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions." <u>Growth Factors</u> **24**(1): 21-44.

Yuasa, T., S. Ohno, J. H. Kehrl and J. M. Kyriakis (1998). "Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH2-terminal kinase (JNK) and p38. Germinal center kinase couples TRAF2 to mitogen-activated protein kinase/ERK kinase kinase 1 and SAPK while receptor interacting protein associates with a mitogen-activated protein kinase kinase kinase upstream of MKK6 and p38." <u>J Biol Chem</u> **273**(35): 22681-92.

Zhou, L., A. A. Nazarian and S. T. Smale (2004). "Interleukin-10 inhibits interleukin-12 p40 gene transcription by targeting a late event in the activation pathway." <u>Mol Cell Biol</u> **24**(6): 2385-96.

Ziegler-Heitbrock, L., M. Lotzerich, A. Schaefer, T. Werner, M. Frankenberger and E. Benkhart (2003). "IFN-alpha induces the human IL-10 gene by recruiting both IFN regulatory factor 1 and Stat3." <u>J Immunol</u> **171**(1): 285-90.