Regulation of Macrophage Function and its Subversion by Pathogens.

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Abbreviations

AP-1, activator protein-1

BCR, B cell receptor

BSA, bovine serum albumin

C/EBP, CCAAT/enhancer binding protein

cAMP, cyclic adenosine monophosphate

cNOS, constitutive NOS

CNS, central nervous system

CR3, complement receptor 3

CRE, cAMP response element

CREB, CRE binding protein

CSF, colony stimulating factor

dATP, 2'-deoxyadenosine 5'-triphosphate

dCTP, 2'-deoxycytosine 5'-triphosphate

dGTP, 2'-deoxyguanosine 5'-triphosphate

DNA, deoxyribonucleic acid

dNTP, 2'deoxynucleotide 5'-triphosphate

DTT, 1,4-dithiothreitol

dTTP, 2'-deoxythymidine 5'-triphosphate

dUTP, 2'-deoxyuracil 5'triphosphate

EBV, Epstein Barr virus

EDTA, ethylene diamine tetra acetic acid

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

EMSA, electrophoretic mobility shift assay

ELISA, enzyme-linked immunosorbance assay

ER, endoplasmic reticulum

ERK, extracellular regulated kinase

ES, excretory-secretory

FAD, flavin adenine dinucleotide

FAM, 6-carboxy-fluorescin

FCS, foetal calf serum

FMN, flavin mononucleotide

GAS, IFN-γ-activated sequence

GRE, glucocorticoid response element

HIV, human immunodeficiency virus

HPRT, hypoxanthine-guanine phosphoribosyltransferase

HRP, horse radish peroxidase

HSC, haematopoietic stem cell

ICSBP, IFN consensus sequence binding protein

IFN, interferon

Ig, immunoglobulin

IFNGR, IFN-γ receptor

I-κB, inhibitor-κB

IKK, I-κB kinase

IL, interleukin

IL-1R, interleukin-1 receptor

iNOS, inducible NOS

IRAK, IL-1R-associated kinase

IRE, IFN response element

IRF, IFN regulatory factor

ISRE, IFN-stimulated response element

JAK, Janus kinase

JNK, c-Jun N-terminal kinase

LBP, LPS binding protein

LPG, lipophosphoglycan

LPS, lipopolysaccharide

LTA, lipoteichoic acid

MAP, mitogen activated protein

MEK, MAP kinase kinase, or MKK

MFR, mannose-fucose receptor

MHC, major histocompatibility complex

MRE, multi-response element

mRNA, messenger RNA

MKK, MAP kinase kinase, or MEK

MKKK, MKK/MEK kinase

NADPH, β-nicotinamide adenine dinucleotide phosphate

NFAT, nuclear factor of activated T cells

NF-IL6, nuclear factor-IL6

NF-κB, nuclear factor-κB

NIK, NF-κB-inducing kinase

NK, natural killer

NKSF, NK cell stimulating factor, or IL-12

NO, nitric oxide

NOS, NO synthase

OD, optical density

OVA, ovalbumin

PAF, platelet-activating factor

PBS, phosphate-buffered saline

PC, phosphorylcholine

PDGF, platelet-derived growth factor

PG, phosphoglycan

PI-3K, phosphoinositide-3 kinase

PKA/PKC, protein kinase A/C

PLC, phospholipase C

PMSF, phenyl methyl sulphonyl fluoride

PTK, protein tyrosine kinase

RNA, ribonucleic acid

RT-PCR, reverse transcriptase polymerase chain reaction

SAPK, stress activated protein kinase, or JNK

SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

SHP, SH2 domain-containing protein tyrosine phosphatase

sPG, synthetic phosphoglycan

SRE, serum response element

STAT, signal transducer and activator of transcription

TAB, TAK binding protein

TAK, transforming growth factor (TGF)-β activating kinase

TAMRA, 6-carboxy-tetramethyl-rhodamine

TBE, Tris-borate/EDTA electrophoresis buffer

TBS, Tris-buffered saline

TCR, T cell receptor

TE, Tris-EDTA buffer

TLR, Toll-like receptor

TNF, tumour necrosis factor

TRAF-6, TNF-R-associated factor-6

Tris, Tris (hydroxymethyl) methylamine

U, units

UTR, untranslated region

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I lift my eyes up to the hills – where does my help come from?

My help comes from the LORD, the Maker of heaven and earth.

Psalm 121:1-2

Summary

Macrophages are key mediators of innate immunity to infection and are also involved in the initiation of adaptive immune responses. Their functions include phagocytosis of invading microorganisms, killing of pathogens by the generation of toxic mediators such as reactive oxygen intermediates and nitric oxide (NO), and antigen presentation to lymphocytes. Activated macrophages also release various cytokines that can induce or direct lymphocyte activation. For example, the pro-inflammatory cytokines IL-6 and TNF-α promote B and T cell activation, and the heterodimeric cytokine IL-12 drives the differentiation of IFN-γ-producing type 1 T helper cells.

Bacterial endotoxin or lipopolysaccharide (LPS), a potent activator of macrophages, has been used extensively to study macrophage function. Multiple intracellular signalling cascades are triggered following binding of LPS to the recently-identified Toll-like receptor 4 (TLR4), resulting in the induction of macrophage mediators including cytokines and inducible nitric oxide synthase (iNOS), the enzyme that catalyses NO synthesis. Pre-treatment or co-stimulation with interferon (IFN)-γ, which is itself a potent inducer of iNOS, results in modulation of LPS-induced cytokine production, including priming for IL-12 production.

The mitogen-activated protein (MAP) kinases are important intracellular regulators of a diverse range of cellular functions. In this study, the three major MAP kinase subfamilies - extracellular–regulated kinases (ERKs), p38 and c-Jun N-terminal/stress-activated protein kinases (JNK/SAPKs) – are shown to be activated by

stimulation of macrophages with LPS in the presence/absence of IFN-γ. Furthermore, regulatory roles for the ERK and p38 MAP kinases in the induction of IL-12, IL-6, TNF-α and iNOS are demonstrated. p38 MAP kinase activation is required for induction of all of these macrophage mediators, regulating the induction of IL-12 and iNOS at the transcriptional level, and the translation or release of TNF-α. Induction of TNF-α and iNOS, but not IL-12 p35 or IL-6, is also dependent on ERK MAP kinases. In contrast, transcription of the IL-12 p40 gene is negatively regulated by the ERKs to promote the formation of bioactive IL-12 heterodimer without excessive production of antagonistic p40/p40 homodimers.

The modulation of macrophage activation by pathogens was also examined using the immunomodulatory products of two parasites: a surface glycoconjugate of intracellular *Leishmania* parasites and a glycoprotein secreted by an extracellular filarial nematode.

Leishmania sp. parasites, which invade and parasitise macrophages, have been shown to evade NO-mediated killing by suppressing macrophage IL-12 production and thereby preventing the production of IFN-γ by Th1 cells. In this study, the repeating phosphodisaccharide (phosphoglycan, PG) component of the *Leishmania* surface molecule lipophosphoglycan (LPG) is shown to be responsible for the suppression of bioactive IL-12 production and a mechanism for this inhibition is proposed. Following binding of a synthetic version of this portion of LPG (sPG) to macrophages, probably via interaction with either the mannose-fucose receptor (MFR) or complement receptor 3 (CR3), cellular levels of dual-phosphorylated ERK MAP kinases were enhanced, thereby

suppressing the induction of bioactive IL-12 by preventing synthesis of the p40 subunit at the transcriptional level.

The effects on macrophage function of an excretory-secretory product of the filarial nematode *Acanthocheilonema viteae* (ES-62) were also investigated. ES-62 has previously been shown to modulate the activation of B and T lymphocytes and the differentiation of dendritic cells. *In vitro* and *in vivo* exposure of macrophages to ES-62 is shown to inhibit induction of IL-12, IL-6 and TNF-α by LPS. ES-62 targets the synthesis and activation of multiple signalling molecules including tyrosine kinases, MAP kinases and transcription factors such as NF-κB and IRF family members. The roles of MAP kinases in the ES-62-mediated suppression of cytokine induction were also investigated.

The results described provide valuable information about the mechanisms underlying the production of cytokines and NO by activated macrophages, and demonstrate how pathogens target these responses to evade detection and destruction by the host immune system.

Chapter 1 – General Introduction.

1:1 Macrophages and their roles in innate and adaptive immunity.

Invasion of tissues of healthy, genetically-normal mammals with harmful pathogens results in the generation of an immune response to clear the infection. Initially, non-specific mechanisms of innate immunity result in local inflammatory responses, which are mediated by a variety of cell types, including macrophages, dendritic cells, polymorphonuclear leukocytes, mast cells and natural killer cells. These innate immune cells co-operate to contain the infection and to remove pathogens by mechanisms including phagocytosis and destruction by toxic products. Adaptive immunity is established several days after the initial infection and involves the specific recognition of pathogens and their products by B and T lymphocytes. This leads to the generation of cytotoxic lymphocytes and the production of specific antibodies, which mediate killing of infected cells and clearance of the pathogen. The establishment of immune memory is another feature of the adaptive response, protecting the individual from subsequent re-infection with that pathogen.

The long-established theory of self/non-self immune recognition states that an immune response is initiated when cells of the immune system recognise foreign (non-self) antigen (via binding to pattern recognition receptors on cells of the innate immune system and lymphocyte antigen receptors). Self-reactive lymphocytes are removed by deletion to ensure that the host is tolerant to self-antigens. Recently, this long-held view of the immune system has been challenged by the Danger Theory (Matzinger, 1994 and 1998), which is now becoming widely accepted. This new theory suggests that where there is no damage caused to an individual by an infectious organism, there is no need to mount an immune response to it. However, if infection with pathogens results in distress,

damage, destruction or necrotic death, endogenous alarm signals from affected cells are thought to be detected by dendritic cells, which then activate innate and adaptive immunity.

Macrophages play critical roles in immune responses, both as mediators of innate immunity and also in the establishment of adaptive immune responses. They provide a first line of defence against infection due to their phagocytic activity and toxicity to a variety of pathogens, and are thus critical during the early stages of infection. Their principle functions (summarised in Figure 1.1) include detection of pathogens and pathogen products such as LPS, phagocytosis of infectious microorganisms, toxic killing of pathogens by the production of reactive oxygen intermediates and NO release, antigen presentation to T cells and production of pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and TNF-α. As well as detecting and destroying invading organisms, macrophages are responsible for the careful removal of apoptotic and necrotic cells, which might represent sources of danger signals.

Macrophages develop from pluripotent haematopoietic stem cells (HSCs) of the bone marrow along the myeloid lineage. In mammals, HSCs are found in the foetal liver, spleen and bone marrow; after birth and throughout adulthood they are present only in the bone marrow. Myelopoiesis proceeds by the action of the colony stimulating factors (CSFs). HSCs differentiate in the presence of macrophage-CSF (M-CSF or CSF-1), granulocyte/monocyte-CSF (GM-CSF) and IL-3 to become monocytes, which enter the bloodstream. Monocytes migrate into tissues e.g. at sites of infection, in response to chemotactic molecules released by endothelial cells, and there they mature into macrophages (Unanue, 1984). There are two major types of macrophage: those that are

resident in particular tissues (e.g. liver, lung, skin, CNS, gut, kidney, endocrine organs and lymphoid tissue; Gordon, 1986) and those that are recruited to sites of injury or infection. The latter group can be further subdivided into inflammatory and activated macrophages. Inflammatory macrophages are recruited to non-immunological inflammatory sites e.g. following injection with thioglycollate broth; they show increased size and competence for phagocytic activity, chemotaxis and antigen presentation compared with resident macrophages (Adams and Hamilton, 1984). Macrophages can be activated by pathogen products such as lipopolysaccharide (LPS) and cytokines such as IFN-γ and TNF-α. In addition to the properties displayed by inflammatory macrophages, activated macrophages produce toxic oxygen intermediates and secrete regulatory molecules that activate B and T lymphocytes (Beaman and Beaman, 1984).

1:2 Activation of macrophages by LPS.

One of the best characterised and most potent activators of macrophages is lipopolysaccharide (LPS). LPS, or bacterial endotoxin, is a surface glycolipid of Gram negative bacteria. It is composed of a hydrophilic repeating polysaccharide region (O-antigen and core) and a hydrophobic lipid domain, lipid A (see Figure 1.2). The structure of LPS varies between species, especially in the O-antigen region (reviewed by Seydel *et al.,* 2000). The function of LPS is largely attributed to the conserved lipid A portion, since virtually all LPS-induced biological activities are lipid A-dependent (Rietschel *et al.,* 1994) and synthetic lipid A has full endotoxic activity.

1:2:1 Induction of pro-inflammatory cytokines and other macrophage mediators by LPS.

LPS on intact bacteria or shed from the bacterial surface can be recognised by a variety of cells including macrophages and B cells via the lipid A portion of the molecule. Activation of these cells by LPS results in the generation of an array of mediators that direct an immune response to destroy the invading bacteria. During infection, LPS is a potent mediator of local inflammatory responses. The effects of LPS include activation of macrophages to produce pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, IL-12 and tumour necrosis factor (TNF)-α. LPS also induces production of other inflammatory mediators such as prostaglandins, oxygen radicals, hydrogen peroxide, nitric oxide (NO), leukotrienes and platelet-activating factor (PAF). TNF-α and IL-1 stimulate vascular permeability, allowing local accumulation of fluid, immunoglobulins and complement proteins. Furthermore, expression of adhesion molecules by vascular endothelial cells allows recruitment of circulating neutrophils and monocytes to the site of infection. Mediators of innate immunity also direct activation of the adaptive immune response e.g. IL-1 and IL-6 activate lymphocytes; IL-12 is critical for differentiation of type 1 T helper (Th1) cells. Overstimulation with LPS can have toxic systemic effects on the host, such as multiple organ failure, shock and death (Bone, 1991; Glauser *et al.,* 1991).

1:2:2 LPS binding and activation of signalling.

1:2:2:1 CD14 and Toll-like receptors (TLRs).

Unlike the receptors of the adaptive immune system (the TCR and BCR), which are generated with random antigen specificity and then clonally selected, cells of the innate immune system possess pattern recognition receptors with predetermined specificity for a limited number of molecular patterns that are common to a broad range of pathogens. These include lipopolysaccharide (LPS) and lipoteichoic acid (LTA) of Gram negative and Gram positive bacteria respectively, lipoproteins produced by bacteria and parasites, phosphorylcholine (PC) which has been detected in a variety of prokaryotic and eukaryotic pathogens, mycobacterial glycolipids, yeast mannans and viral double-stranded RNAs (reviewed by Medzhitov and Janeway Jr, 1997 and Harnett and Harnett, 1999).

The association of LPS with a variety of serum proteins has been shown to have positive and negative effects on macrophage activation by LPS (Marra *et al.,* 1992; Wright *et al.,* 1992). The best characterised interaction is between LPS and LPS binding protein (LBP), a 60 kDa glycoprotein produced by hepatocytes (Ramadori *et al.,* 1990; Grube *et al.,* 1994). A role for LBP in LPS recognition and activation of macrophages was confirmed by targeted deletion of the LBP gene in mice, which resulted in impaired sensitivity to LPS (Jack *et al.,* 1997). LPS aggregates in solution, but LBP disaggregates LPS molecules and transports them to the macrophage cell membrane (Schromm *et al.,* 1996).

The LPS-LBP complex is able to bind to the monocyte/macrophage specific surface receptor CD14, allowing responses to even low doses of LPS (Wright *et al.*, 1990; Martin *et al.*, 1992). Targeted deletion of the CD14 gene in mice demonstrated the importance of CD14 in LPS responsiveness, since only modest amounts of TNF-α and negligible IL-1β and IL-6 were induced in these mice (Haziot *et al.*, 1996). Furthermore, stable expression of CD14 in Chinese hamster ovary (CHO)-K1 fibroblasts conferred LPS-responsiveness on these cells (Golenbock *et al.*, 1993), and the overexpression of a human CD14 transgene in mice resulted in hypersensitivity to LPS (Ferrero *et al.*, 1993).

CD14 exists in two forms: as a glycosylphosphoinositide (GPI)-anchored membrane glycoprotein, or in serum as a soluble form (sCD14) that lacks the GPI tail (Bazil *et al.*, 1989). sCD14 is involved in the LPS-mediated activation of non-myeloid cells such as endothelial and epithelial cells (Frey *et al.*, 1992; Pugin *et al.*, 1993), but at high concentrations it inhibits LPS action on macrophages (Haziot *et al.*, 1995). GPI-anchored CD14 binds LPS with a stoichiometry of 1:1 (Kitchens and Munford, 1995) and is essential for LPS signalling, but it is not capable of transducing signals since it lacks an intracellular domain. Therefore it was postulated that it interacts with another LPS receptor to achieve activation. The requirement for CD14 was shown to be bypassed by treatment with high doses of LPS (Beaty *et al.*, 1994; Wright *et al.*, 1990), suggesting that CD14 acts to present low doses of LPS to a less abundant receptor.

Recently a family of pattern recognition receptors, the Toll-like receptors (TLRs), has been identified and its members have been implicated in the binding of a range of pathogen molecules including LPS (reviewed by O'Neill and Dinarello, 2000 and Brightbill and Modlin, 2000). The TLRs were identified on the basis of their homology to

Drosophila Toll protein, which, as well as being involved in dorsoventral patterning of *Drosophila* embryos during development, is crucial for anti-fungal responses in these flies, which possess only innate immunity to pathogens. Similarly, another member of the *Drosophila* Toll family, 18-wheeler (18w), is required for production of an anti-bacterial peptide (Eldon *et al.,* 1994; Williams *et al.,* 1997). Toll-like proteins have now been identified in a broad range of organisms, including plants (van der Biezen and Jones, 1998).

The first mammalian Toll homologue was identified by Medzhitov *et al.* (1997). They isolated hToll4 (TLR4) mRNA from dendritic cells, γδ T cells, Th1 and Th2 αβ T cells and B cells, and demonstrated that a dominant active form of the receptor induced cytokine expression when expressed in monocyte cell lines. The murine homologue of this receptor was subsequently implicated as the LPS receptor. This was suggested by the demonstration that LPS-hyporesponsive C3H/HeJ mice have a point mutation in the gene encoding TLR4 that results in the production of inactive receptor (Poltorak *et al.,* 1998). Similarly, C57BL/10ScCr mice, which are also LPS-insensitive, have a null mutation in the TLR4 gene (Qureshi *et al.,* 1999). Subsequently, Chow *et al.* (1999) demonstrated that transient transfection of HEK 293 cells with human TLR4 conferred sensitivity to stimulation with LPS/CD14 complexes on these cells. Furthermore, an LPS antagonist blocked TLR-4-mediated activation of an NF-κB reporter construct in a dose-dependent manner.

To date, ten mammalian TLRs are known (WIPO, 1998); nine have been published (Rock *et al.,* 1998; Chuang and Ulevitch, 2000; Du *et al.,* 2000), although ligands for only TLR2 and 4 have been identified. TLRs are members of the IL-1R/TLR

superfamily which also includes the receptors and accessory proteins for IL-1 and IL-18, and the Th2 cell-specific marker ST2 (reviewed by Wright, 1999 and O'Neill and Dinarello, 2000). In contrast to the intracellular signalling domain, which shows a high degree of conservation between TLR family members, the extracellular portion is considerably more diverse. A critical feature of the extracellular domain is the presence of leucine-rich repeats (LRRs), which are believed to mediate the response to conserved pathogen-associated molecular patterns and thought to play an important role in the specificity of host responses to infection.

TLR family members studied so far also achieve transcriptional regulation of effector genes through identical signal transduction cascades (see Figure 1.3). In *Drosophila*, production of anti-microbial proteins is dependent on the activation of the NF-κB pathway: Rel family members such as Dorsal, Dif and Relish are activated following the degradation of the I-κB homologue Cactus. In the same way, mammalian TLRs have been demonstrated to signal via NF-κB. Following the binding of ligand to a TLR, MyD88, which is itself a member of the IL-1R/TLR superfamily but which lacks an extracellular domain, associates with the intracellular signalling domain of the receptor. MyD88 acts as an adaptor for the recruitment of IL-1R-associated kinases (IRAKs), which activate TNF receptor-associated factor 6 (TRAF-6), resulting in nuclear translocation of NF-κB and activation of mitogen activated protein (MAP) kinases.

1:2:2:2 LPS signalling.

The intracellular signalling pathways triggered by LPS are outlined in Figure 1.4. Signalling molecules previously identified as targets of LPS via CD14 are presumably regulated through TLR4, although this assumption has not yet been fully confirmed.

Increased tyrosine phosphorylation and activation of Src family kinases Lyn, Hck and Fgr occurs within 1 minute of macrophage activation with LPS (Ziegler *et al.,* 1988; Stefanova *et al.,* 1991 and 1993; Beaty *et al.,* 1994). LPS has also been shown to induce tyrosine phosphorylation of Vav (English *et al.,* 1997) as well as p145 and Shc, which are often associated with Syk (Crowley *et al.,* 1996). However, activation of Syk by LPS has not been shown and Syk-deficient cells respond normally to LPS (Crowley *et al.,* 1997). LPS-induced Lyn activation (Stefanova *et al.,* 1993) leads to activation of PI-3-kinase (Herrera-Velit and Reiner, 1996) which in turn activates protein kinase C (PKC)-ζ (Herrera-Velit *et al.,* 1997). However, macrophages from $Lyn^-/Hck^-/Fgr^-$ triple knockout mice have no major defects in LPS-induced stimulation of NO, IL-1, IL-6 and TNF-α secretion, and normal activation of ERK1/2 and JNK MAP kinases and the transcription factor NF-κB (Meng and Lowell, 1997).

LPS is a strong activator of all three subfamilies of mitogen activated protein (MAP) kinases – the extracellular-regulated kinases (ERKs), p38 and the c-Jun N-terminal or stress-activated protein kinases (JNKs or SAPKs) (Weinstein *et al.,* 1992; Han *et al.,* 1994; Liu *et al.,* 1994; Raingeaud *et al.,* 1995; Hambleton *et al.,* 1996). These serine/threonine kinases have important roles in a range of cell functions including protein synthesis, cell cycle control, division and differentiation, and have been suggested

to regulate cytokine and iNOS production (Davis, 1994; Karin, 1994). The MAP kinase kinases (MKKs or MEKs) responsible for dually phosphorylating (Thr-X-Tyr) and thereby activating the MAP kinases in response to stimulation with LPS have also been identified: MEKs 1 and 2 activate ERKs 1 and 2, MEKs 3 and 6 activate p38, and MEKs 4 and 7 activate JNKs 1 and 2 (Geppert *et al.,* 1994; Reimann *et al.,* 1994; Buscher *et al.,* 1995; Sanghera *et al.,* 1996; Swantek *et al.,* 1997; Yao *et al.,* 1997b).

The Ras→Raf→MEK pathway for activation of ERK MAP kinases is well characterised but there are indications that in LPS-stimulated macrophages it is not the main activatory pathway for ERK1/2 (Buscher *et al.,* 1995). Indeed, there is evidence that LPS doesn't even activate the Ras→Raf pathway (Guthridge *et al.,* 1997). PKC has been implicated in the activation of the MEK1/2→ERK1/2 pathway in other systems, but this connection has not been established for LPS.

LPS stimulation induces the activity of a number of transcription factors, including NF-κB, NF-IL6, members of the CREB/ATF1 and AP-1 families, Ets family members (including Ets, Elk, Erg and PU.1) and Egr (Sweet and Hume, 1996; Groupp and Donavon-Peluso, 1996). These may be activated by phosphorylation by specific kinases (e.g. CREB/ATF1, or the AP-1 family member c-Jun) or synthesised *de novo* (e.g. the LPS 'early response genes' Jun-B, c-Fos and Egr-1).

As described above, activation of NF-κB family members by TLR4 occurs via MyD88, IRAKs and TRAF-6. This leads to activation of IKKα/β which phosphorylates I-κB; I-κB is then ubiquitinated and degraded, releasing NF-κB to translocate to the nucleus

(reviewed by Karin, 1999) where it can bind to and regulate the transcription of a variety of LPS-inducible genes including IL-12, IL-6, TNF-α and iNOS.

1:2:3 Priming of macrophages by IFN-γ.

Pre-incubation or co-stimulation of macrophages with IFN-γ has been shown to enhance responses to LPS, including IL-12, IL-6, TNF-α and iNOS production (Ma *et al.,* 1996b; Xie *et al.,* 1993; Martin *et al.,* 1994; Lowenstein *et al.,* 1993; Weisz *et al.,* 1994; Sanceau *et al.,* 1991; Darnell *et al.,* 1994). Priming of different responses is achieved by distinct mechanisms: for example, while priming for iNOS is achieved by direct targeting of pre-existing transcription factors, maximal priming for IL-12 induction may require *de novo* synthesis (Ma *et al.,* 1996b).

The IFN-γ receptor is composed of two distinct subunits, IFNGR1 and IFNGR2 (Stark *et al.,* 1998). IFNGR1 is the major ligand-binding subunit; both IFNGR1 and IFNGR2 have critical signalling functions (reviewed by Bach *et al.,* 1997 and Platanias and Fish, 1999). Binding of IFN-γ to its receptor results in association of the Janus kinases JAK1 and JAK2 with the intracellular signalling portions of the receptor. This activates the JAKs, which phosphorylate IFNGR1, providing a docking site for the cytoplasmic protein signal transducers and activators of transcription 1 (STAT1). The JAKs phosphorylate STAT1, enabling the formation of STAT1 homodimers, which translocate to the nucleus where they bind IFN-γ-activated sequence (GAS) elements on target genes. Other signalling molecules activated by IFN-γ include the Src tyrosine kinase family member Fyn, the tyrosine kinase Pyk-2 which has been linked to ERK-2

activation, the adaptors CrkL and CrkII, the guanine exchange factor Vav, and the tyrosine phosphatases SHP-1 and SHP-2, but their functions in the priming of macrophages for cytokine induction by LPS are not clear.

1:3 Regulation of pro-inflammatory cytokine and NO production.

1:3:1 Interleukin-12 (IL-12) production.

IL-12 was originally identified in the culture supernatants of Epstein-Barr virus (EBV)-transformed human B cell lines due to its ability to activate NK cells to produce IFN-γ, and was initially known as NK cell stimulatory factor (NKSF; Kobayashi *et al.,* 1989; Stern *et al.,* 1990). However, it was subsequently found to be produced mainly by phagocytic cells (monocytes, macrophages and neutrophils) in response to both Gram negative and positive bacteria, bacterial products such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), viruses and intracellular parasites (D'Andrea *et al.,* 1992; Cleveland *et al.,* 1996; Kanangat *et al.,* 1996). Bacterial DNA has also been shown to be a potent inducer of IL-12; this activity is dependent on nonmethylated CpG repeats (Halpern *et al.,* 1996; Ballas *et al.,* 1996). Dendritic cells, polymorphonuclear cells and mast cells also produce IL-12 (Smith *et al.,* 1994; Cassatella *et al.,* 1995; Cella *et al.,* 1996; Heufler *et al.,* 1996; Kang *et al.,* 1996; Koch *et al.,* 1996).

Bioactive IL-12 p70 is a heterodimer comprising two glycosylated polypeptides - a 35 kDa (p35) chain and a 40 kDa (p40) chain - which are linked by disulphide bonds. These subunits are encoded separately, on chromosomes 3 and 5 respectively (Sieburth *et*

al., 1992), and regulation of their expression is distinct. p35 is ubiquitously and constitutively expressed in a variety of cell types, whereas p40 expression is more restricted and highly inducible. It has been hypothesised that the IL-12 heterodimer may have been derived from a primordial cytokine and its receptor since p35 shares structural similarities with IL-6 and granulocyte-CSF (G-CSF; Merberg *et al.,* 1992), while p40 is structurally related to the extracellular portion of the IL-6 and G-CSF receptors (Gearing and Cosman, 1991).

The p40 subunit is produced in large excess over p70 heterodimer (10 – 20 times) and, in addition to forming bioactive IL-12 heterodimers, it can form homodimers ($p40_2$), which are antagonistic to bioactive IL-12 (Ling *et al.,* 1995). p35 has also been found in complex with the product of EBV-induced gene 3 (EBI3; Devergne *et al.,* 1997) although the function of EBI3 and the p35:EBI3 complex are unknown.

The *in vivo* induction of IL-12 can be either T cell-independent or -dependent. Infection with bacteria or intracellular parasites results in rapid IL-12 production by direct stimulation of phagocytes; indeed, T cell-deficient SCID mice produce bioactive IL-12 upon infection (Gazzinelli *et al.,* 1994; Tripp *et al.,* 1994). However, T cell-dependent mechanisms have also been demonstrated. IL-12 production occurred in response to presentation of T cell-dependent antigens such as OVA via triggering of CD40 molecules on antigen presenting cells and was dependent on TCR ligation (DeKruyff *et al.,* 1997; Maruo *et al.,* 1997). CD40-CD40L interaction plays a critical role in bioactive IL-12 production by regulating p40 but not p35 mRNA accumulation (Kato *et al.,* 1996).

IL-12 is a key mediator of innate immunity and is also involved in the establishment of adaptive immune responses (reviewed by Trinchieri, 1995). It directs the differentiation of helper T cells towards a type 1 phenotype, which is characterised by the production of IFN-γ and down-regulation of IL-4. IL-12 also stimulates IFN-γ production by NK cells, and thus establishes a positive feedback loop resulting in enhanced activation of macrophages, including stimulation of NO production. This is especially important for the effective removal of intracellular pathogens such as *Listeria monocytogenes*, *Toxoplasma gondii* and *Leishmania major* (reviewed by Ma *et al.*, 1996a). Animals treated with neutralising doses of monoclonal antibodies against IL-12 p40, or lacking either the IL-12 p40 or p35 gene are highly susceptible to such intracellular pathogens (Biron and Gazzinelli, 1995; Trinchieri and Scott, 1995).

Both subunits must be co-expressed in the same cell to generate bioactive heterodimer and since p35 is constitutively-expressed in a variety of cell types, it was originally assumed that p70 generation was mainly controlled at the level of p40 transcription. However, there is now much evidence of regulation of p35 transcripts (Hayes *et al.*, 1995; Snijders *et al.*, 1996; Aste-Amezaga *et al.*, 1998; Kincy-Cain and Bost, 1997), and it is therefore more likely that p35 is the limiting subunit. Indeed, the formation of antagonistic $p40_2$ may predominate even under optimal conditions (Hayes *et al.*, 1995; Snijders *et al.*, 1996) and it has therefore been suggested that a temporal balance between $p40_2$ and bioactive p70 determines the IL-12 response (Schultze *et al.*, 1999).

Synthesis of both subunits is induced by LPS; IFN-γ, which alone can induce low levels of p35 but not p40, primes monocytes for transcription of p35 and p40 and is

thereby synergistic with LPS for bioactive IL-12 production (Ma *et al.*, 1996b). Regulation of p40 production is thought to be largely at the level of transcription. The IL-12 p40 promoter has been characterised (reviewed by Ma *et al.*, 1997a) and is summarised in Figure 1.5. The human and murine promoters are highly conserved up to approximately 400 bp upstream of the transcription start site.

There is an ets element between –211 and –206 and putative PU.1 and NF-κB elements between –124 and –105. In non-stimulated macrophages, the ets element is occupied by PU.1, but this is displaced following stimulation with IFN-γ or LPS by a transcription factor complex, F1, which binds to the ets element in a complex manner, interacting with flanking sequence in the region –292 to –196 (Ma *et al.*, 1997b). F1 comprises a number of transcription factors, including Ets-2, IRF-1, c-Rel and Glp109, a 109 kDa protein highly induced by IFN-γ or LPS stimulation. Similarly, an F2 complex is induced by IFN-γ to interact with this element in the region –243 to –196, but its identity has not been established. The NF-κB 'half-site' between –116 and –106 has been reported to bind p65/p50 and c-Rel/p50 complexes in response to LPS stimulation (Murphy *et al.*, 1995).

Investigations using knockout mice have indicated that proteins of the IRF family, including IRF-1 and IRF consensus sequence binding protein (ICSBP), play significant roles in IL-12 expression. IRF-/- and ICSBP-/- mice are deficient in IL-12 production, do not efficiently develop Th1 immune responses and are hence highly susceptible to infection with pathogens such as *Listeria monocytogenes*, *Toxoplasma gondii* and *Leishmania major* (Scharton-Kersten *et al.*, 1997; Fehr *et al.*, 1997; Giese *et al.*, 1997; Wu *et al.*, 1999; Taki *et al.*, 1997; Lohoff *et al.*, 1997). Wang *et al.* (2000) recently

demonstrated that ICSBP is an important regulator of IL-12 p40 expression. They proposed that synthesis of ICSBP occurs following priming with IFN-γ (via STAT-1) and stimulation with LPS (via NF-κB), and that ICSBP then binds to the Ets element of the IL-12 p40 promoter and acts synergistically with IRF-1 (which is also induced by IFN-γ via STAT-1) to promote transcription.

Other transcription factor binding sites include an IRF-1 site at –730 to –719 and a binding site for a nuclear factor originally identified as a regulator of IL-6 production (NF-IL6); however, deletion of these regions did not alter the response to IFN-γ and LPS (Ma *et al.,* 1996b).

The IL-12 p35 gene has not been so well characterised but, in contrast to p40 expression, it is thought to require only pre-synthesised activators since its expression is not inhibited by treatment with cycloheximide (Aste-Amezaga *et al.,* 1998). Although p35 is constitutively expressed, it has been suggested that regulation of p35 expression or post-translational modification, which would affect its association with the p40 subunit, may contribute to the regulation of p70 production (Snijders *et al.,* 1996). The murine p35 gene and its cDNA have been cloned and sequenced, and putative promoter elements have been identified including Sp1, AP-1, ISRE, ICSBP, NF-κB, GATA-1 and GAS elements (Schoenhaut *et al.,* 1992; Yoshimoto *et al.,* 1996; Tone *et al.,* 1996). Tone *et al.* (1996) showed that transcription can initiate from either of two 5' exons, resulting in mRNA isoforms with different 5' untranslated regions (UTRs). Subsequently, Babik *et al.* (1999) detected 4 isoforms of p35 mRNA in a murine B cell lymphoma line (A20) and bone marrow-derived dendritic cells. Two of these were predominant in non-stimulated cells and contained an additional upstream ATG in the 5' UTR, which resulted in

abortive translation of p35. Following stimulation with LPS, transcription of the p35 gene was not only upregulated, but the initiation of transcription occurred from alternate positions, resulting in transcripts lacking the additional upstream ATG. These were successfully translated to produce p35 subunit, which could subsequently dimerise with p40 to form bioactive IL-12 p70.

Processing of the IL-12 pre-protein subunits represents a further level of IL-12 regulation. Murphy *et al.* (2000) recently demonstrated that p40 is processed in the normal way for a secretory protein, with cotranslational targeting to the endoplasmic reticulum (ER), removal of the signal peptide and partial glycosylation. In contrast, p35 processing involves two sequential cleavages, which occurred after migration of the intact pre-protein into the ER; the second cleavage is accompanied by complex glycosylation.

Several cytokines have activatory or suppressive effects on the stimulation of IL-12 production by phagocytic cells. While IFN-γ and GM-CSF enhance IL-12 (Cassatella *et al.,* 1995; Kubin *et al.,* 1994; D'Andrea *et al.,* 1993), IL-10, IL-4, IL-13 and TGF-β inhibit IL-12 production by suppressing both p40 and p35 accumulation (Kubin *et al.,* 1994; D'Andrea *et al.,* 1995). However, IL-4 and IL-13 inhibit IL-12 and TNF-α production by monocytes when added simultaneously to the inducing stimulus, but prime for IL-12 production in response to various stimuli when added to the culture at least 18 hours before the stimulus (D'Andrea *et al.,* 1993).

1:3:2 Interleukin-6 (IL-6) production.

IL-6 is a pleiotropic, pro-inflammatory cytokine with roles in the immune response, haematopoiesis and regulation of the acute phase reaction (reviewed by Hirano, 1992). It promotes the growth and proliferation of T cells and has been shown to induce IL-2 receptor expression in a T cell line and thymocytes (Noma *et al.,* 1987; Le *et al.,* 1988). It also synergises with IL-1 for IL-2 and IL-2 receptor alpha chain production (Le *et al.,* 1988; Houssiau *et al.,* 1989), plays critical roles in the generation and function of cytotoxic T lymphocytes (Takai *et al.,* 1988; Renauld *et al.,* 1989; Liu *et al.,* 1990; Smyth *et al.,* 1990), and induces immunoglobulin synthesis by activated B cells (Yoshizaki *et al.,* 1982; Muraguchi *et al.,* 1988; Hilbert *et al.,* 1989; Splawski *et al.,* 1990).

IL-6 is produced by many different cell types including macrophages, fibroblasts, T and B lymphocytes, synovial cells, endothelial cells, glial cells and keratinocytes, in response to a variety of inducers such as IL-1, TNF-α, PDGF, serum and LPS (reviewed by Ray *et al.,* 1989a and Hirano, 1992). T cells, for example, can be induced to produce IL-6 by mitogens or antigenic stimulation; LPS enhances IL-6 production by monocytes and fibroblasts, but glucocorticoids and IL-4 are inhibitory. Various viruses induce IL-6 production in fibroblasts and in the central nervous system, and HIV has been demonstrated to induce IL-6 in monocytes. Disregulation of IL-6 production has also been found to contribute to a variety of diseases including autoimmune diseases and malignancies (reviewed by Hirano, 1992).

IL-6 is a single chain glycoprotein with a molecular mass ranging from 21 to 28 kDa (reviewed by Hirano, 1992). Human and murine IL-6 are 66% and 42% homologous

at the DNA and protein levels respectively; IL-6 is also homologous to G-CSF (Hirano *et al.,* 1986). The human IL-6 gene is 5 kb in length and is located on chromosome 7 (Sehgal *et al.,* 1986); the 7 kb murine gene mapped to chromosome 5 (Mock *et al.,* 1989). Both consist of five exons and four introns (Yasukawa *et al.,* 1987; Tanabe *et al.,* 1988). The promoter region is highly conserved up to approximately 350 bp upstream of the transcription start site (Tanabe *et al.,* 1988), and contains several potential transcriptional control elements, such as glucocorticoid responsive elements (GREs), an AP-1 binding site, a c-fos serum response element (SRE), a cyclic AMP responsive element (CRE), and an NF-κB binding site (Tanabe *et al.,* 1988; Ray *et al.,* 1988 and 1989b; see Figure 1.6).

The CRE element, which mediates cAMP inducibility and binds the phosphoprotein CREB (Montminy *et al.,* 1986; Silver *et al.,* 1987), is located within a 23 bp multi-response element (MRE) which is induced by a variety of stimuli (Ray *et al.,* 1988 and 1989b). A novel 14 bp regulatory element containing an inverted repeat was also identified in this region and the nuclear factor that binds to it was cloned and named NF-IL6 (Isshiki *et al.,* 1990). NF-IL6 is a leucine zipper protein that shares structural homology with CCAAT/enhancer binding protein (C/EBP; Akira *et al.,* 1990). It is induced by stimulation with LPS, IL-1, TNF-α and IL-6 in a variety of tissues (Akira *et al.,* 1992). NF-IL6 has been reported to be activated by phosphorylation of its activation domain, which is mediated by a Ras-dependent MAP kinase cascade (Trautwein *et al.,* 1993; Nakajima *et al.,* 1993). NF-IL6 may form homodimers or heterodimerise with other C/EBP family members (Kinoshita *et al.,* 1992). Furthermore, Fos and Jun have been demonstrated to repress NF-IL6 through association with the leucine zipper region (Hsu *et al.,* 1994).

There have also been numerous reports of regulation of IL-6 production by NF-κB (Ray and Ray, 1995; Zhang *et al.,* 1994; Mori *et al.,* 1994; Akira and Kishimoto, 1997). Sanceau *et al.* (1995) showed that triggering of the human IL-6 gene in monocytes by IFN-γ and TNF-α involves co-operation between the IRF-1, NF-κB and Sp1 transcription factors, and Kannabiran *et al.* (1997) demonstrated that the transcriptional repressor RBP (CBF1) regulates IL-6 gene expression.

Post-translational N- and O-linked glycosylation and phosphorylation of IL-6 pre-protein represent additional levels of regulation (May *et al.,* 1988; Santhanam *et al.,* 1989).

1:3:3 Tumour necrosis factor-α (TNF-α) production.

Tumour necrosis factor (TNF)-α was so named because it was first discovered due to its ability to cause necrosis of some tumours (Carswell *et al.,* 1975). Also known as cachectin, it is a pro-inflammatory cytokine, which is produced principally by monocytes and macrophages (Beutler and Cerami, 1988; Ziegler, 1988; Old, 1987; Vassalli, 1992; MacNaul *et al.,* 1990); exposure to LPS typically results in up to 10,000-fold induction (Beutler *et al.,* 1986). It has also been implicated in the pathogenesis of both acute and chronic inflammatory disease (Beutler and Cerami, 1986; Mannel and Echtenacher, 2000). Like IL-6, TNF-α can induce production of acute phase proteins and activation of B and T cells. It is also capable of stimulating fibroblast proliferation, and activating endothelial and synovial cells. Furthermore, it activates macrophages to produce pro-

inflammatory cytokines: antibodies against TNF-α caused repression of IL-1 and IL-6 production in response to LPS *in vivo* (Fong *et al.,* 1989).

Regulation of TNF-α production is complex and stimulus-dependent, and occurs at transcriptional and post-transcriptional levels. Human TNF-α is encoded by a single gene, which is located on chromosome 6 in the middle of the major histocompatibility complex (Spies *et al.,* 1989). It is initially expressed as a 26 kDa transmembrane protein which is subsequently cleaved by a metalloproteinase (Black *et al.,* 1997; Moss *et al.,* 1997). Soluble TNF-α molecules can then form non-covalently linked homotrimers. Mature TNF-α is non-glycosylated, and both the membrane-bound and soluble forms are biologically active (Perez *et al.,* 1990).

Sequence analysis revealed that the proximal promoter contains multiple transcription factor binding sites (see Figure 1.7), including NF-κB (Collart *et al.,* 1990; Drouet *et al.,* 1991; Shakhov *et al.,* 1990; Trede *et al.,* 1995), NF-IL6 (also known as C/EBPβ; Pope *et al.,* 1994; Natsuka *et al.,* 1992) and c-Jun (Newell *et al.,* 1994), which have been shown by deletion and mutational analysis to contribute to activation of the TNF-α promoter in macrophages (Economou *et al.,* 1989; Pope *et al.,* 1994; Wedel *et al.,* 1996; Yao *et al.,* 1997a; Udalova *et al.,* 1998). NF-κB binding sites have also been identified downstream of the TNF-α gene (Kuprash *et al.,* 1995; Kwon *et al.,* 1996). All of these transcription factors can be activated via ERK and JNK MAP kinase pathways following LPS stimulation (Nakajima *et al.,* 1993; Trautwein *et al.,* 1993; Scherle *et al.,* 1998; Foey *et al.,* 1998).

However, the relative contribution of each transcription factor and the effects of synergy between them appear to depend on the differentiation stage of the macrophage. NF-IL6 and c-Jun interacted to mediate LPS-induced TNF-α production by the monocytic cell line, U937 (Zagariya *et al.,* 1998), but Liu *et al.* (2000) demonstrated that while NF-κB and c-Jun are involved in LPS-mediated TNF-α production in primary human macrophages, NF-IL6 is not. Whilst NF-κB and NF-IL6 have been shown to interact through their Rel and b-Zip domains to synergistically activate IL-6 and IL-12 p40 (Stein *et al.,* 1993; LeClair *et al.,* 1992; Plevy *et al.,* 1997; Matsusaka *et al.,* 1993), regulation of TNF-α expression by NF-κB was independent of c-Jun and NF-IL6 (Liu *et al.,* 2000). NF-κB p65/p50 heterodimers bound to the κB1 and κB3 sites of the human TNF-α promoter, while c-Jun bound to an AP-1 site adjacent to κB3, but these transcription factors operated independently. Down-regulation of TNF-α production in response to a second LPS challenge occurred by competition between NF-κB p50 homodimers and p65-p50 complexes for binding to the κB1 site.

TNF-α expression in LPS-stimulated macrophages has also been shown to be regulated by the ERK MAP kinase substrates Ets and Elk-1, which bind to two nuclear factor of activated T cells (NFAT)-binding sites that are required for induction of TNF-α in lymphocytes (Kramer *et al.,* 1995; Tsai *et al.,* 2000). Egr-1 and Sp1 binding is also induced by LPS, and the coactivators CREB binding protein (CBP) and p300 are required for assembly of the LPS-stimulated TNF-α enhancer complex (Tsai *et al.,* 2000).

Message stability is thought to be a particularly significant step in the regulation of TNF-α production (Beutler *et al.,* 1986; Shaw and Kamen, 1986). For example, AU-rich sequences in the 3'-untranslated region (UTR) of TNF-α mRNA predispose for

degradation by RNases and regulate the efficiency of translation (Caput *et al.,* 1986; Han *et al.,* 1990). In addition, post-translational control mechanisms regulate the proteoloytic cleavage of the membrane-bound TNF-α precursor to release soluble TNF-α from the cell surface (Kriegler *et al.,* 1988).

Cyclic AMP (cAMP) has also been implicated in the regulation of cytokine synthesis by LPS-activated macrophages (Benbernou *et al.,* 1997; Eigler *et al.,* 1998, Meisel *et al.,* 1996, van der Pouw Kraan *et al.,* 1995). Indeed, prostaglandin E_2 (PGE_2), a potent physiological inducer of cAMP, and derivatives of cAMP have been shown to inhibit IL-12, IL-6 and TNF-α production by LPS-stimulated macrophages (Eigler *et al.,* 1998; Kambayashi *et al.,* 1995a and b; Panina-Bordignon *et al.,* 1997; van der Pouw Kraan *et al.,* 1995). It has been suggested that these inhibitory effects of cAMP are due, at least in part, to release of IL-10; however the inhibition of TNF-α production is probably a direct effect of cAMP, and largely independent of IL-10, although this is controversial (Eigler *et al.,* 1998; Kambayashi *et al.,* 1995a and b; Seldon *et al.,* 1998; Procopio *et al.,* 1999).

1:3:4 Inducible nitric oxide synthase (iNOS) production and nitric oxide (NO) release.

The generation of the toxic mediator nitric oxide (NO) has been shown to be critical in non-specific innate immunity. NO is formed by the action of the NO synthase (NOS) enzyme on the substrate L-arginine. Three isoforms of NOS have been identified:

two are constitutively expressed (cNOS) – ecNOS and ncNOS expressed by vascular endothelium and neuronal cells respectively – and the third is inducible (iNOS). Whilst the cNOS isoforms have limited tissue distribution, multiple cell types have the capacity to express iNOS (Oswald *et al.,* 1994). All three are flavoproteins containing bound flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), and are dependent on NADPH as a cofactor (Hevel *et al.,* 1991; Mayer *et al.,* 1991; Schmidt *et al.,* 1992; Stuehr *et al.,* 1991). While cNOS isoforms are only capable of generating small amounts of NO, iNOS can catalyse the production of high NO levels. iNOS was first identified in murine macrophages; these cells have negligible basal iNOS activity but this can be massively enhanced within a few hours by treatment with IFN-γ and LPS (Nathan and Hibbs, 1991).

NO has been demonstrated to play critical roles in responses to pathogens, acute and chronic inflammation, autoimmunity, and apoptosis. For example, inflammatory responses were reduced by treatment of rats with the inhibitor of NO generation, L-NMMA, but enhanced by treatment with L-arginine (Ialenti *et al.,* 1992 and 1993). Similarly, patients with ulcerative colitis have increased NO levels (Middleton *et al.,* 1993), and inhibition of NOS ameliorated experimentally-induced chronic ileitis (Miller *et al.,* 1993). Rheumatoid arthritis and osteoarthritis patients also have increased NO levels in their plasma and synovial fluid (Farrell *et al.,* 1992). Furthermore, in addition to killing invading microorganisms, NO is cytotoxic to the cells that produce it and neighbouring cells (Moncada, 1992), and has been shown to mediate apoptosis by causing DNA damage (Nguyen *et al.,* 1992; Cui *et al.,* 1994).

The importance of NO in immune responses against pathogens has been most extensively studied in relation to infection with *Leishmania* parasites, which invade and parasitise mammalian macrophages. Liew *et al.* (1990) showed that *L. major* promastigotes are killed when incubated *in vitro* at room temperature in PBS containing NO. They also demonstrated that injection of L-NMMA into lesions of *L. major*-infected CBA mice resulted in a 10^4-fold increase in parasite burden in the lesion. Furthermore, iNOS-deficient mice were highly susceptible to infection and developed visceral disease (Wei *et al.,* 1995). Murine peritoneal macrophages can be induced to produce NO *in vitro* by stimulation with IFN-γ and LPS (Stuehr and Marletta 1985 and 1987; Ding *et al.,* 1988; Drapier *et al.,* 1988); this is inhibited by treatment with L-NMMA (Liew *et al.,* 1990). IFN-γ-treated macrophages are efficient killers of *Leishmania*, and this can be completely abrogated by L-NMMA treatment (Green *et al.,* 1990; Liew *et al.,* 1990).

NO generation is dependent on substrate and enzyme availability; iNOS production is regulated at transcriptional and post-transcriptional/translational levels. The human and murine iNOS promoters have been cloned and the functions of human (reviewed by Morris and Billiar, 1994) and murine (Xie *et al.,* 1993) promoter/enhancer elements have been investigated. The murine iNOS promoter contains at least 24 elements with homology to transcription factor consensus sequences known to be induced by cytokines and microbial products.

Two regions (I and II) of the iNOS gene are required for maximal induction of transcription; both regions are necessary for activation by LPS, while region II is critical for the response to IFN-γ (Lowenstein *et al.,* 1993). Within these regions multiple putative transcription factor binding sites have been identified. Region I contains an

octamer site, an NF-IL6 site and a tumour necrosis factor response element (TNF-RE), while region II has an ISRE and a PU-box/IFN element (PIE); both regions also have NF-κB binding sites.

The ISRE matches the IFN-regulatory factor element (IRF-E) consensus sequence (Martin *et al.,* 1994; Tanaka *et al.,* 1993) which binds IRF-1 (Lorsbach *et al.,* 1993). IRF-1 is induced by IFN-γ (Fujita *et al.,* 1989; Miyamoto *et al.,* 1988) and regulates the transcription of a number of IFN-inducible genes (Reis *et al.,* 1992; Pine, 1992). IRF-1 is essential for the induction of iNOS by IFN-γ since neither NO release nor iNOS mRNA could be detected in macrophages from IRF-1 knock-out mice stimulated with IFN-γ in the absence/presence of LPS (Kamijo *et al.,* 1994; Martin *et al.,* 1994).

Both the IRF and NF-κB binding domains are necessary for full transcriptional activation of the iNOS promoter (Lowenstein *et al.,* 1993). The role of these transcription factors in the induction of iNOS expression by IFN-γ and LPS has previously been investigated in this laboratory (Feng, 1997; see Figure 1.8). IFN-γ activates JAK1 and JAK2, which catalyse the phosphorylation and homodimerisation of STAT1. STAT1 homodimers translocate to the nucleus and induce IRF-1 synthesis; IRF-1 then stimulates iNOS expression. An NF-κB pathway transduces the LPS signal, stimulating iNOS expression directly by binding to the iNOS promoter, and indirectly by stimulating the synthesis of IRF-1 and an IRF-like protein.

iNOS expression is also regulated by post-transcriptional mechanisms such as mRNA stability, translation and post-translational modification. For example, TGF-β suppresses iNOS production by decreasing message stability, efficiency of translation and

protein stability (Vodovotz *et al.,* 1993). At least some of the regulatory effects are cell-type specific; iNOS mRNA induction is attenuated by TGF-β in RAW 264.7 cells, but enhanced in Swiss 3T3 cells (Morris and Billiar, 1994).

NO generation is also dependent on the availability of the substrate L-arginine, which is itself limited by the rate of arginine synthesis and uptake (Nussler *et al.,* 1994; Bogle *et al.,* 1992). Furthermore, NADPH is required for the donation of electrons and tetrahydrobiopterin (BH_4) acts as a cofactor to enhance iNOS activity (Tayeh and Marletta, 1989; Kwon *et al.,* 1989). Tetrahydrobiopterin is synthesised from GTP by the enzymatic activity of GTP-cyclohydrolase I, which is absent or present at only very low levels in unstimulated macrophages but is strongly co-induced with iNOS following stimulation with cytokines and LPS (Morris and Billiar, 1994).

1:4 Aims of the thesis.

Macrophages activated by cytokines such as IFN-γ, and microbial products such as LPS, produce pro-inflammatory cytokines and NO, which protect the host from a variety of infectious organisms, including bacteria, viruses, parasites and fungi. Excessive or inappropriate production of these immune mediators can however lead to pathology such as chronic inflammation, septic shock and autoimmune disease. A thorough understanding of the regulation of these processes is therefore extremely important.

Evasion of the host immune response by infectious organisms is critical to ensure their survival in the hostile environment of the host. Immunosuppression and immunomodulation are common mechanisms employed by a variety of pathogens, which target both innate and adaptive branches of the immune response. For example, a type 1 T helper (Th1) cell response is critical for the successful clearance of infection with *Leishmania* parasites, which invade and parasitise macrophages (reviewed by Mattner *et al.,* 1997a). However, lipophosphoglycan (LPG) molecules released from the surface of these parasites mediate immune evasion by a variety of mechanisms including the suppression of IL-12 production by macrophages (Piedrafita *et al.,* 1999). This results in a bias in the T helper cell response towards a type 2 (Th2) phenotype. In the absence of IFN-γ and the presence of IL-4, the production of toxic oxygen intermediates and NO by macrophages is suppressed, enabling parasite survival.

Immunomodulation and immunosuppression by filarial nematodes can be achieved by the production of excretory-secretory (ES) molecules, which modulate the activity of a variety of immune cell types. For example, ES-62, the major ES product of

Acanthocheilonema viteae, has been demonstrated to modulate the activation of B and T lymphocytes and the differentiation of dendritic cells, and bias the T helper response towards a type 2 (Th2) phenotype (Whelan *et al.*, 2000; Harnett *et al.*, 1999a). The effects of ES-62 on macrophage function have not previously been investigated.

Given the critical roles of macrophages in both innate and adaptive immunity, it is extremely important to understand how pathogens such as these parasites disrupt normal activation of macrophages.

Therefore this project aims to:

1 Dissect the signalling mechanisms underlying the activation of pro-inflammatory cytokine (IL-12, IL-6 and TNF-α) and NO production by LPS-stimulated macrophages, in particular to establish the roles of the MAP kinases in the induction of these macrophage mediators.

2 Determine the signalling mechanisms targeted by a phosphoglycan molecule of *Leishmania* sp. protozoan parasites to suppress IL-12 production by macrophages.

3 Examine the effects of the filarial excretory-secretory glycoprotein ES-62 on cytokine and NO production by macrophages, and investigate the signalling pathways involved.

These objectives will be investigated in the murine macrophage system, which is well established for both the study of cell function and the establishment and clearance of *Leishmania* infection.

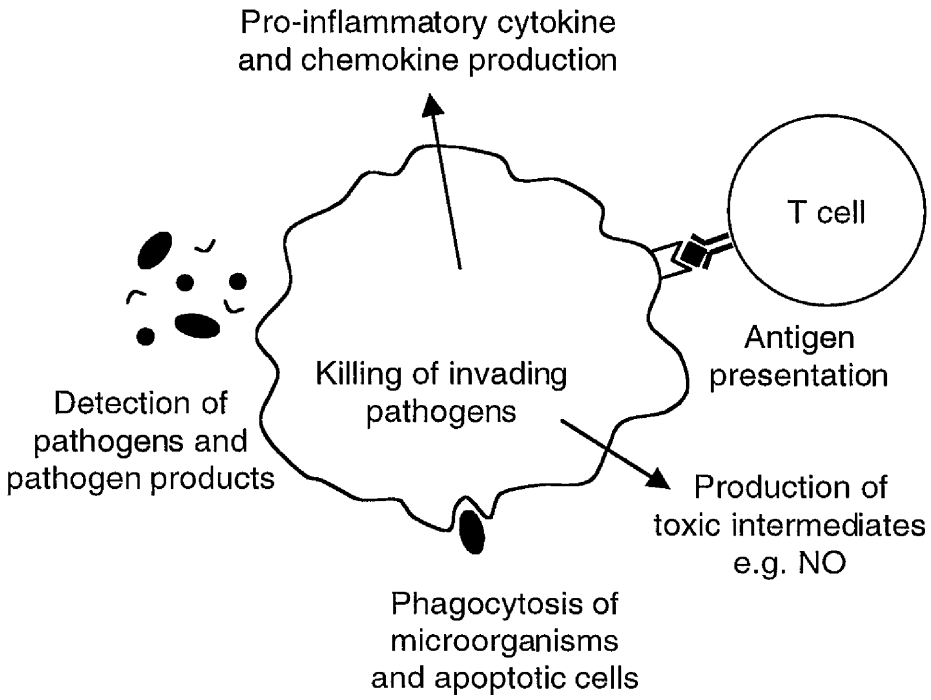


Figure 1.1. Major functions of macrophages.

Macrophages have multiple functions in innate immunity as well as in the induction of adaptive immune responses. They recognise pathogens and pathogen products via receptors including the Toll-like receptors, Fc receptors, complement receptors and scavenger receptors. They can phagocytose apoptotic cells, as well as small microorganisms, which they kill with toxic intermediates such as oxygen radicals and nitric oxide (NO). They digest pathogens and present antigen to T cells via MHC II. Furthermore, they produce pro-inflammatory cytokines (such as IL-1, IL-6, IL-8, IL-12 and TNF-α) and chemokines, which mobilise other cells of the immune system to the site of infection.

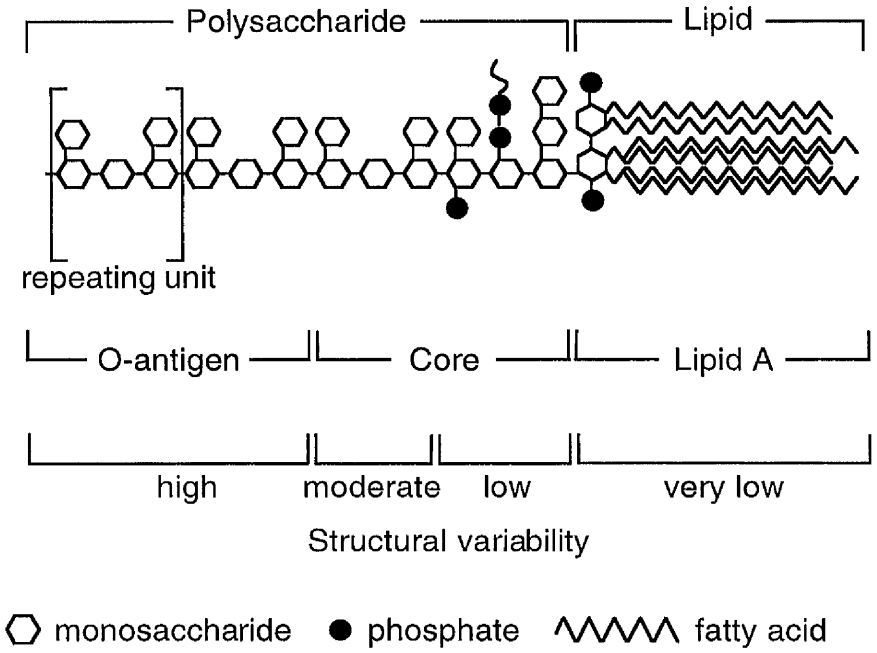


Figure 1.2. Structure of bacterial LPS.

LPS is a complex glycolipid, composed of a hydrophilic repeating polysaccharide (O-antigen and core) and a hydrophobic lipid A domain. The frequency of structural variation between species is indicated; the lipid A portion is conserved while the O-antigen is highly variable.

(Adapted from Seydel *et al.*, 2000)

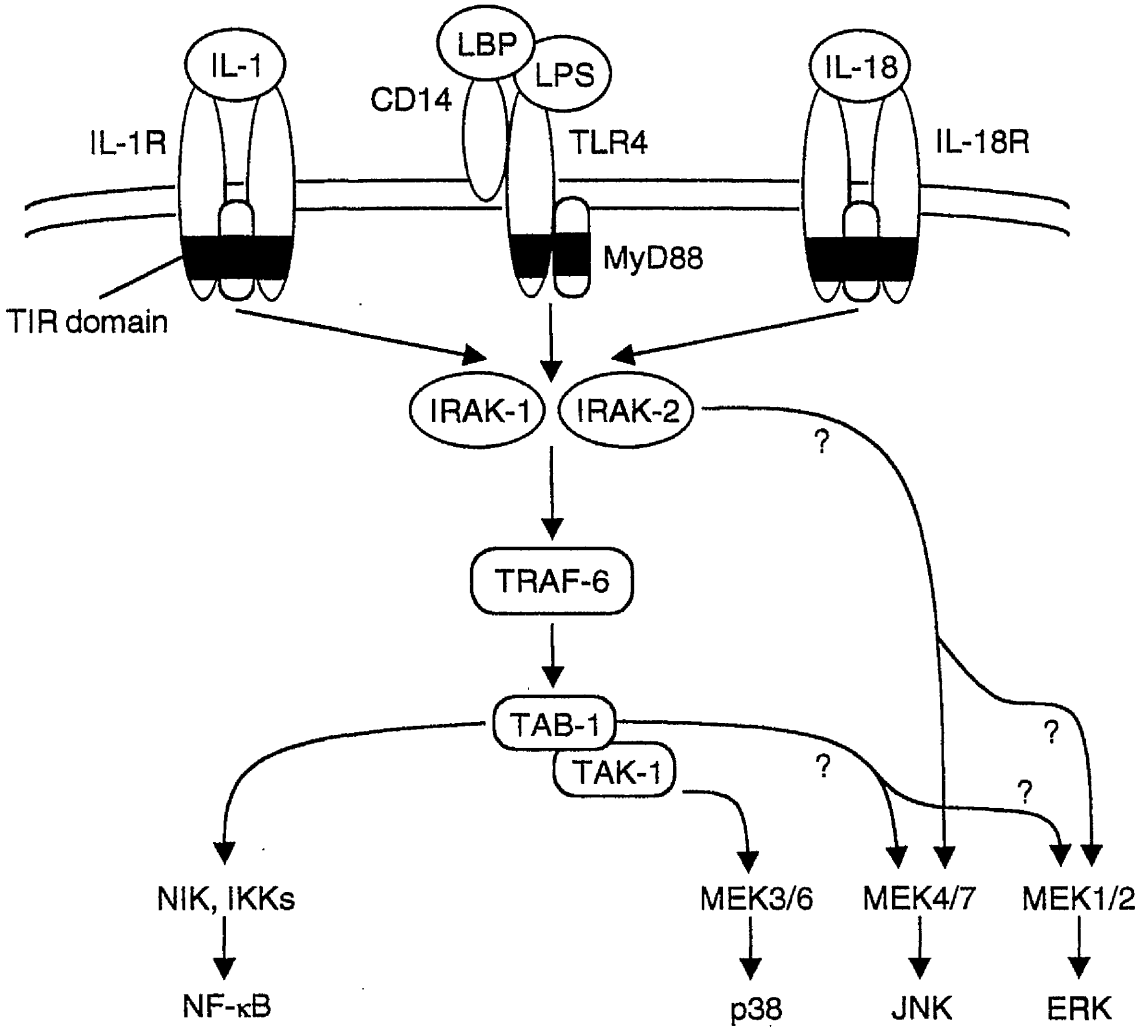


Figure 1.3. Conserved IL-1R/TLR signalling pathways.

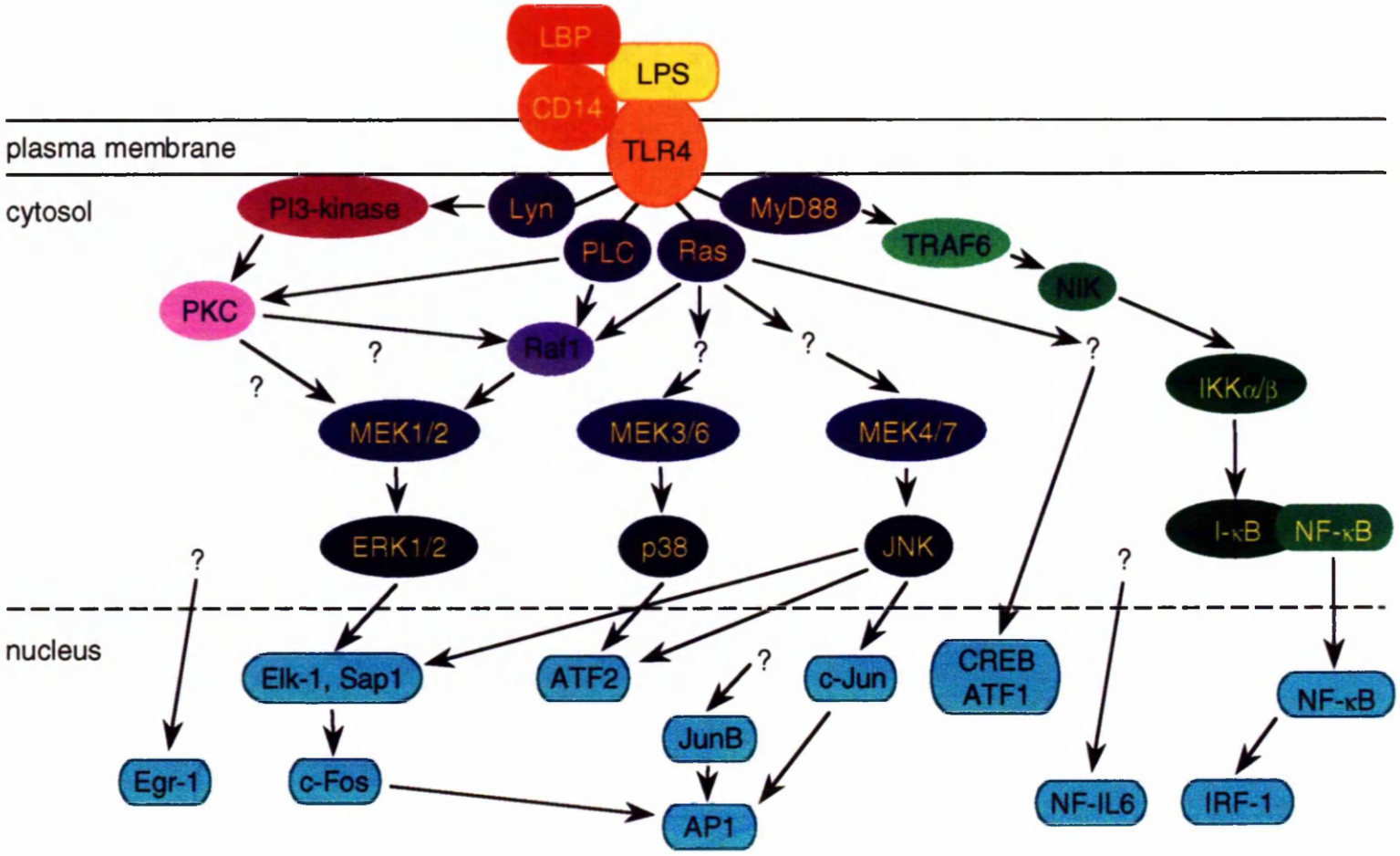
IL-1, IL-18 and LPS trigger similar signalling pathways in target cells. A conserved region in the intracellular portion of their receptors, known as the Toll/IL-1R (TIR) domain, interacts with MyD88, which is also a member of the IL-1R/TLR superfamily. This enables the recruitment of IRAK-1 and IRAK-2, which in turn recruit TRAF-6. Activation of the kinases TAB-1 and TAK-1 leads to activation of NF-κB and MAP kinase pathways.

(Adapted from O'Neill and Dinarello, 2000)

Figure 1.4. LPS signalling pathways.

The LBP-LPS complex binds to the macrophage surface receptors CD14 and TLR4, initiating intracellular signalling via TLR4. Multiple signalling pathways are activated, including NF-κB and MAP kinase cascades, resulting in the activation or *de novo* synthesis of a variety of transcription factors, which regulate the induction of macrophage response genes including those encoding pro-inflammatory cytokines and iNOS.

(Adapted from Dziarski et al., 2000)


LBP
LPS
CD14
TLR4
plasma membrane
cytosol
PI3-kinase
Lyn
MyD88
TRAF6
PLC
Ras
NIK
PKC
Raf1
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IKKα/β
MEK1/2
MEK3/6
MEK4/7
I-κB
NF-κB
ERK1/2
p38
JNK
?
?
nucleus
Elk-1, Sap1
ATF2
?
c-Jun
CREB
ATF1
NF-κB
JunB
Egr-1
c-Fos
AP1
NF-IL6
IRF-1

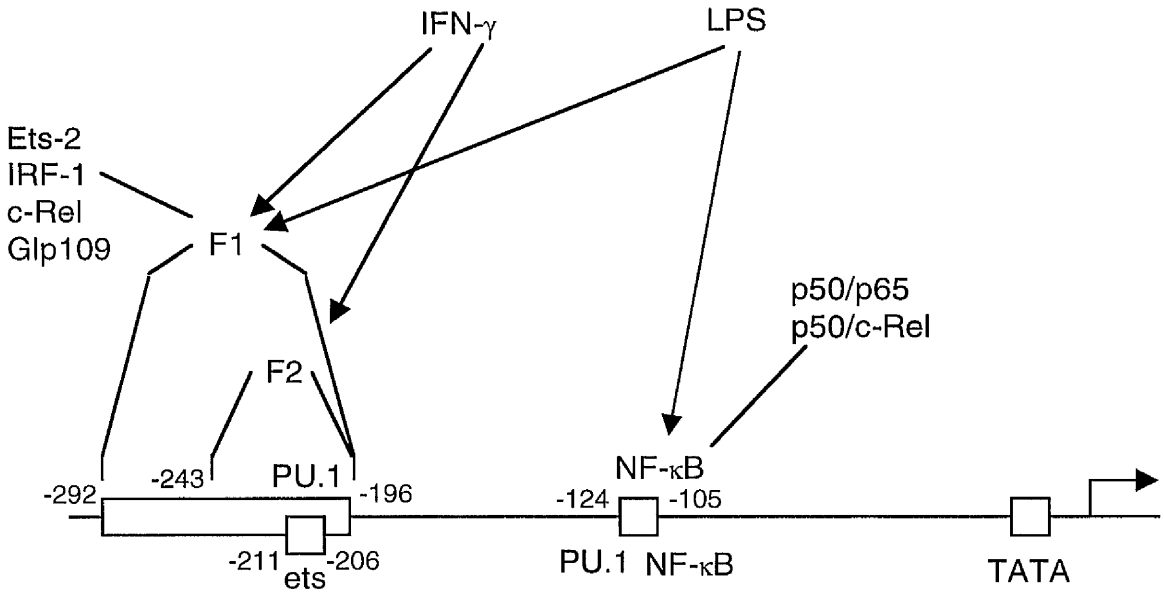


Figure 1.5. The IL-12 p40 promoter.

The human and mouse IL-12 p40 genes are highly conserved up to approximately 400 bp upstream of the transcription start site. In unstimulated cells, PU.1 occupies the ets element, but this is displaced upon stimulation with IFN-γ or LPS by the F1 complex, which binds at this site and interacts with a large flanking region (Ma *et al.*, 1997b). The F1 complex includes Ets-2, IRF-1, c-Rel and Glp109 (a 109 kDa protein, highly inducible by IFN-γ or LPS). The F2 complex, which responds more to IFN-γ than LPS, also binds in this region but its identity has not been established. The NF-κB 'half-site' between -106 and -116 has been reported to bind p50/p65 and p50/c-Rel complexes in response to LPS stimulation (Murphy *et al.*, 1995).

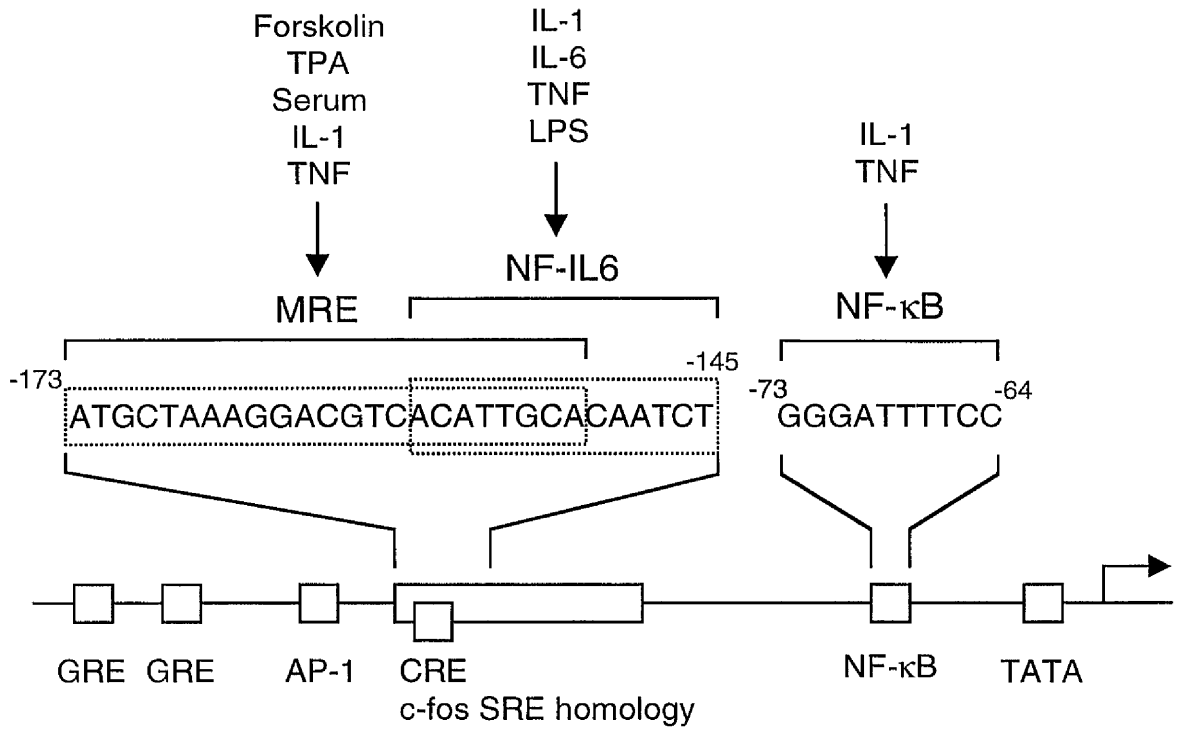


Figure 1.6. The IL-6 promoter.

The human and mouse IL-6 genes are approximately 5 kb (mapped to chromosome 7) and 7 kb (chromosome 5) respectively, and both consist of 5 exons and 4 introns. Highly homologous regions extend approximately 350 bp upstream of the transcription start site.

GRE = glucocorticoid response element, CRE = cyclic AMP response element, SRE = serum response element, MRE = multi-response element

(Adapted from Hirano *et al.*, 1992)

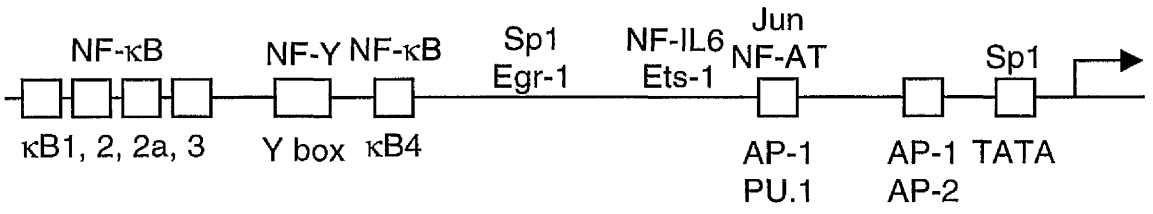


Figure 1.7. The TNF-α promoter.

The 5' promoter region of the TNF-α gene contains numerous transcription factor binding sites, including at least five NF-κB binding sites (-850, -655, -625, -510, -210 bp). An MHC class II-like Y box is located at -255 bp, and there are several AP-1 and AP-2 binding elements. Multiple transcription factors, including Sp1, Egr-1, Ets, NF-AT and NF-IL6, have been shown to bind or interact with other factors in the 5' promoter region to regulate the transcriptional machinery. There are further control elements downstream of the TNF-α gene, including an additional NF-κB binding site.

(Adapted from Jongeneel, 1992)

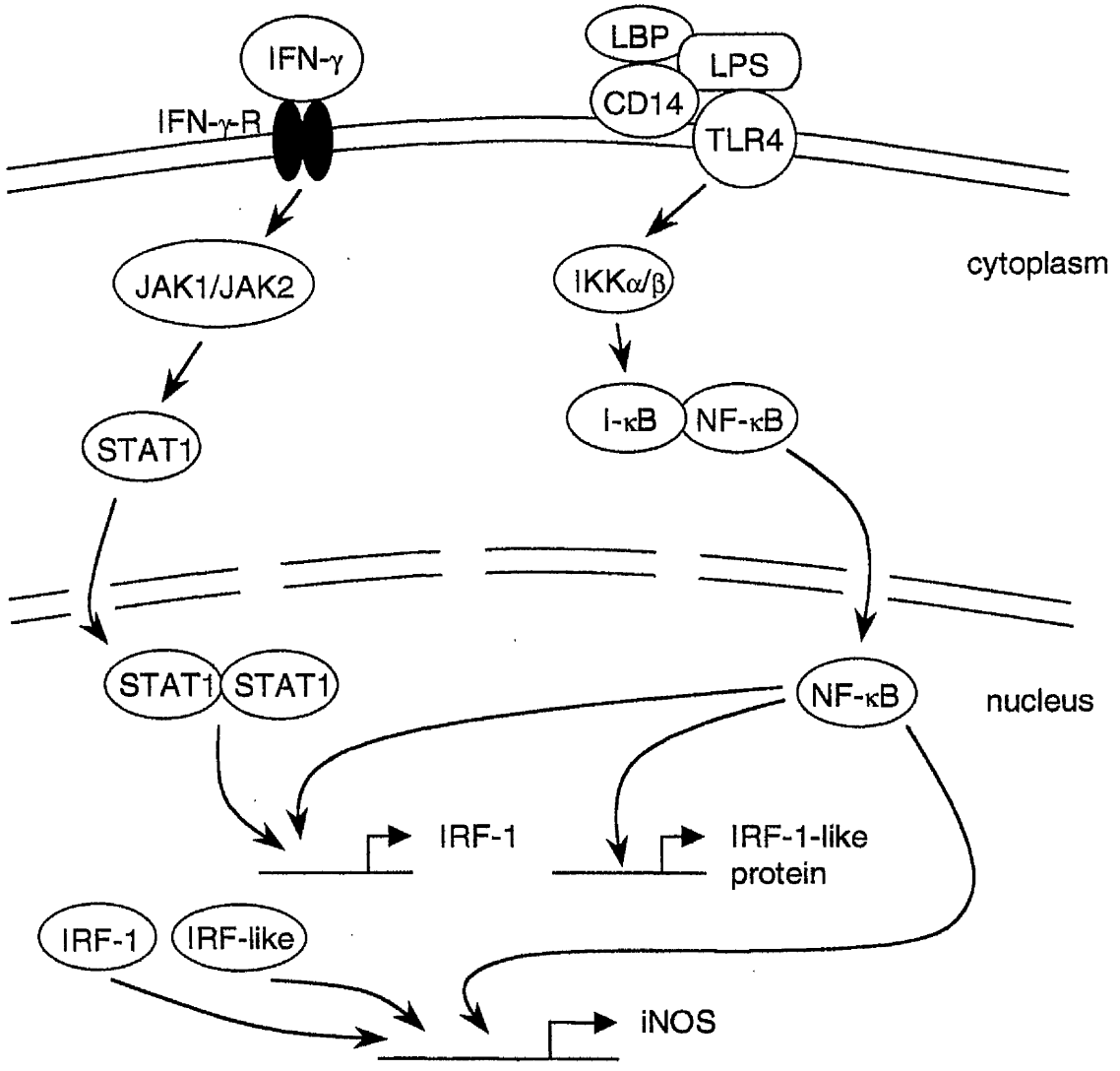


Figure 1.8. Transcriptional regulation of iNOS induction by IFN-γ and LPS.

Stimulation of macrophages with IFN-γ results in IRF-1 synthesis via a JAK-STAT pathway. IRF-1 and an IRF-like protein are also produced following stimulation with LPS via an NF-κB pathway. iNOS promoter activation is achieved by the binding of NF-κB and members of the IRF family of transcription factors.

(Adapted from Feng, 1997)

Chapter 2 – Materials and Methods.

2:1 Materials.

Alexis	PD 098059, SB 203580 (MAP kinase inhibitors)
American Type Culture Collection	J774 murine macrophage cell line
Amersham	[α^{32}P]-dATP
	ECL detection system
	Rainbow protein molecular weight markers
Amicon	Centricon tubes
	Stirred cells with PM10 membranes
Aventis Pasteur	Halothane-RM
Biogenesis Ltd.	RNAzol™ B
BioRad	Bis-acrylamide
	Nitrocellulose membrane
Charles River UK Ltd.	ALZET osmotic mini-pumps
Costar	Eppendorf tubes
	Filter tips
	Plastic scrapers
	Tissue culture flasks and plates
Dynex Technologies	Immulon-4 ELISA plates
	MRX platereader
Fluka	Nonidet P-40 (NP-40)
Genosys	Oligo synthesis
Gibco BRL	dATP, dGTP, dCTP, dTTP
	Dulbecco's Modified Eagle Medium (DMEM)
	Foetal calf serum (FCS)

	Formaldehyde
	HEPES
	High salt buffer (for annealing)
	Klenow (DNA polymerase)
	L-Glutamine
	Penicillin/Streptomycin
	Phosphate buffered saline (PBS)
	Random priming kit
	RNA markers
	RPMI
	S-MEM (Calcium-free DMEM)
Harlan Olac	BALB/c and 129 mice
Hoeffer Scientific Ltd	Electrophoresis system
Kodak	X-ray film cassettes
	X-OMAT AR autoradiography film
KPL	TMB substrate
Life Technologies	SuperScript II (reverse transcriptase)
New England Biolabs	MAP kinase antibodies
PE Biosystems	ABI-prism 7700 SequenceDetector
	Sequence Detection software
	TaqMan reagents
Pharmacia	Nylon membrane
	Poly (dI-dC).poly (dI-dC)
	Superose 6 columns
PharMingen	ELISA antibodies

Pierce	Coomassie protein assay reagent
Promega	RNA markers
	U0126 (MEK inhibitor)
Santa Cruz	NF-κB, I-κB, IRF-1/2, Ets-2 and iNOS antibodies
Sigma	Aprotinin
	DEPC
	Dithiothreitol (DTT)
	ExtrAvidin peroxidase conjugate
	Leupeptin
	Phenyl methyl sulphonyl fluoride (PMSF)
	Salmonella minnesota Lipopolysaccharide (LPS)
	Sodium orthovanadate
	TEMED
Scottish Antibody Production Unit	HRP-conjugated anti-mouse IgG
	HRP-conjugated anti-rabbit IgG
Transduction Laboratories	Anti-phospho-tyrosine RC20
Upstate Biotechnology	Anti-phospho-tyrosine 4G10

All other chemicals used in this study are commercially available from Sigma or BDH.

2:2 Reagents and antibodies.

Reagents were obtained from Sigma (Poole, UK) unless otherwise indicated. IFN-γ was a gift from Dr. G. Adolf (Vienna). LPS (*Salmonella minnesota*) was from Sigma (Poole, UK).

Antibodies were obtained from the following sources: MAP kinase antibodies (p42/p44 ERK; Thr202/Tyr204 phospho-p42/p44 ERK; p38; Thr180/Tyr182 phospho-p38; p46/p54 SAPK/JNK; Thr183/Tyr185 phospho-p46/p54 SAPK/JNK) were from New England Biolabs (Hertfordshire, UK). ELISA antibodies (IL-12 p40 and IL-6 antibody pairs, and IL-12 p70 and TNF-α kits) were from PharMingen (San Diego, USA). NF-κB, I-κB, IRF-1, IRF-2, Ets-2 and iNOS antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA). Horse Radish Peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG antibodies were obtained from the Scottish Antibody Production Unit (Lanarkshire, UK). The anti-phospho-tyrosine antibodies 4G10 and RC20 were from Upstate Biotechnology (Lake Placid, USA) and Transduction Laboratories (San Diego, USA) respectively.

The MAP kinase inhibitors PD 098059 and SB 203580 were obtained from Alexis (Nottingham, UK); U0126 was from Promega (Madison, USA).

2:3 Preparation of synthetic phosphoglycan, sPG.

Synthetic fragments of *Leishmania* lipophosphoglycan (see Figure 4.2) were prepared by Drs. A.V. Nikolaev and A.P. Higson, Department of Chemistry, University of Dundee, UK (Nikolaev *et al.,* 1995).

2:4 Purification of ES-62.

ES-62 was purified from secretions from adult *Acanthocheilonema viteae* worms in culture by Ms. D. Rose (Department of Immunology, University of Strathclyde, UK). Spent culture medium from *A. viteae* maintained in RPMI (Gibco BRL, Paisley, UK) at 37°C, 5% CO_2 was passed through filtration units with 0.22 μM membranes (Sigma, Poole, UK) to remove microfilariae. The medium was concentrated to a volume of 10ml, washed in PBS (pH 7.2), in a stirred cell with a PM10 membrane (Amicon, Upper Mill, UK). A further concentration step was undertaken using Centricon tubes (Amicon) with a 100 kDa membrane to give a final volume of 0.5 ml. This was applied to a 30 x 1 cm Superose 6 column (HR 10/30; Pharmacia Biotech, St. Albans, UK), fitted to an isocratic FPLC system (Pharmacia Biotech), previously equilibrated with PBS (pH 7.2) at room temperature. The column was eluted at a flow rate of 0.5 ml/min and monitored for absorbance at 280 nm. >95% of the protein eluted as a single peak, which was confirmed to be ES-62 by SDS PAGE analysis.

2:5 Macrophages and cell culture.

Macrophages were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (Gibco BRL, Paisley, UK) supplemented with 10% heat-inactivated foetal calf serum (FCS; Gibco BRL), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (complete DMEM). Cells were cultured in 96-well plates ($2x10^5$ cells/well) or in 25 cm^2 flasks ($4x10^6$ cells/flask) and rested overnight following isolation or passaging before use.

Macrophages were obtained as follows: J774 cells. The murine macrophage cell line, J774, was obtained from American Type Culture Collection (Atlanta, USA). Peritoneal macrophages. 6-8 week old mice (BALB/c and 129 strains; Harlan Olac, Bicester, UK) were injected intraperitoneally with 2 ml thioglycollate broth and after three days peritoneal macrophages were removed by peritoneal washing and enriched by plastic adherence for 2 hrs. Total spleen cells and splenic macrophages. Spleens were removed from mice and gently mashed through a small sieve in medium and aspirated to obtain a single cell suspension. Splenic macrophages were selected by plastic adherence for 2 hrs. Bone marrow-derived macrophages. Bone marrow cells were removed from the femurs of BALB/c mice by gently flushing out the marrow plug with a syringe containing medium, and a single cell suspension was created by gentle aspiration. Cells were cultured in DMEM containing 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10 ng/ml CSF-1 for 6 days. Adherent cells were washed twice and rested before use.

Macrophages were stimulated with 100 U/ml IFN-γ and 1 μg/ml (J774 cells) or 100 ng/ml (murine macrophages - from BALB/c mice unless otherwise stated) *Salmonella minnesota* LPS (Sigma, Poole, UK). Culture supernatants were assayed for cytokine production by ELISA or for NO release by Griess reaction after 24 hrs culture unless otherwise stated.

2:6 Griess Reaction.

Nitrite concentrations in culture supernatants were determined by Griess reaction (Ding *et al.*, 1998). Briefly, 50 μl culture supernatant was mixed in a 96-well plate with an equal volume of Griess Reagent (1% sulphanilic acid, 0.1% napthylene diamine dihydrochloride, 5% orthophosphoric acid) at room temperature for 10 mins. The absorbance at 570 nm was measured using an MRX microplate reader (Dynex Technologies, Chantilly, USA). Sodium nitrite was used as a standard to determine nitrite concentrations, which are equivalent to NO production.

2:7 Enzyme-linked immunosorbance assay, ELISA.

ELISA was performed according to the antibody supplier's recommendations. Briefly, Immulon-4 ELISA plates (Dynex Technologies, Chantilly, USA) were coated overnight at 4°C with capture antibody in 0.1 M $NaHCO_3$ buffer (pH 7.4) and then blocked for 2 hrs with 10% calf serum (Gibco BRL, Paisley, UK) in PBS at 37°C. Samples and standards were diluted in culture medium and incubated for 1 hr at 37°C or

overnight at 4°C. Detection antibodies and ExtrAvidin (Sigma) were diluted in blocking buffer and incubated for 1 hr each at 37°C. Plates were washed at least five times between stages. Finally, plates were developed using TMB substrate (KPL, Gaithersberg, USA) and absorbances at 630 nm were determined.

2:8 MTT assay.

Following removal of culture supernatants for cytokine analysis, cell viability was assessed by replacing medium and adding 500 μg/ml MTT reagent (Sigma, Poole, UK). After 3 hrs at 37°C all medium was removed, the precipitate dissolved in isopropanol, and absorbances at 600 nm were determined.

2:9 Osmotic pumps.

ALZET osmotic mini-pumps model 2002 (Charles River UK Ltd., Margate, UK) were used as directed by the manufacturer (see also Theeuwes and Yum, 1976) to mimic the *in vivo* release of ES-62. The following procedures were performed by Dr. M. Deehan (Department of Immunology, University of Glasgow, UK). Pumps were loaded with 200 μl of 10, 20, 40 or 80 μg ES-62 or PBS pH7.4, and left overnight at room temperature submerged in sterile 0.9% saline solution, according to the manufacturer's instructions (see Figure 2.1). 6-8 week old, male BALB/c mice weighing at least 20g were anaesthetised with Halothane-RM (Aventis Pasteur MSD, Maidenhead, UK). The back of the neck was swabbed with disinfectant (0.1% benzalkonium chloride) and the area

shaved. A mid-scapular incision was made and the connective tissue severed to create a pocket to insert the mini-pump. The pump was placed with the flow moderator inserted first. The wound was sutured and the animal observed until consciousness was regained. Animals were sacrificed on day 14 and peritoneal macrophages were removed by peritoneal washing and cultured as above.

2:10 Preparation of total cell protein extracts.

Stimulated cells ($4x10^6$/25 cm^2 flask) were washed twice with ice-cold TBS (25 mM Tris.Cl, pH 7.4, containing 150 mM NaCl, and 100 mM sodium orthovanadate), and harvested with a plastic scraper. The cells were lysed in lysis buffer (25 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM dithiothreitol (DTT), 50 μg/ml each of leupeptin, aprotinin, and phenyl methyl sulphonyl fluoride (PMSF)) by incubation on ice for 30 mins. Lysates were then centrifuged at 13,000 xg at 4°C for 10 mins and the supernatants were transferred to fresh tubes and stored at -70°C until required. Protein concentrations of the lysates were determined using Coomassie Protein Assay Reagent (Pierce, Rockford, USA).

2:11 Western blot analysis.

Cell lysates were resolved by SDS-PAGE (Laemmli, 1970) before transfer to nitrocellulose using a transblot system (Hoeffer Scientific SE600, San Francisco, USA).

Nitrocellulose filters were then incubated with wash buffer 1 (0.01 M Tris.Cl buffer, pH 7.5, containing 0.1 M NaCl and 0.1% Tween-20) containing 2% bovine serum albumin (BSA; Sigma, Poole, UK) for at least 1 hr to block non-specific protein binding. Primary antibodies were diluted in wash buffer 1 containing 1% BSA, and applied to the filter for 1 hr at room temperature or overnight at 4°C. Blots were washed twice in wash buffer 1, twice in wash buffer 2 (0.01 M Tris.Cl buffer, pH 7.5, containing 0.5 M NaCl and 0.1% Tween-20) and finally twice in wash buffer 1 again; each wash was for 10 mins. Following washing, the blots were incubated with the appropriate HRP-conjugated secondary antibody (diluted up to 1:5000 in wash buffer 1 containing 1% BSA) for 1 hr at room temperature. After washing again as before, immuno-reactive bands were visualised by the Enhanced Chemiluminescence (ECL) system (Amersham, Little Chalfont, UK) and exposed to X-OMAT film (Kodak, New York, USA).

2:12 Preparation of nuclear protein extracts.

Treated cells ($4x10^6$/25 cm^2 flask) were washed twice with ice-cold TBS and then resuspended in 400 µl ice-cold buffer A (10 mM HEPES buffer, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 100 µM sodium orthovanadate). Cells were allowed to swell on ice for 15 mins, after which 25 µl of a 10% solution of Nonidet P-40 (NP-40; Fluka, Poole, UK) was added and the cells were vortexed vigorously for 15 secs. The homogenate was centrifuged at 13,000 xg for 30 secs and the resulting nuclear pellet was resuspended in 100 µl ice-cold buffer B (20 mM HEPES buffer, pH 7.9, containing 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 100 µM sodium orthovanadate) and rocked vigorously at 4°C for

15 mins. The nuclear extract was centrifuged for 5 mins at 13,000 xg at 4°C, and the supernatant collected and frozen in aliquots at -70°C until use.

2:13 EMSA/Band shift assay.

Synthetic oligonucleotide probes were designed by Dr. X.-Q. Wei (Department of Immunology, University of Glasgow, UK). After dissolving in distilled water, annealing was carried out in high salt buffer (H buffer; Gibco BRL, Paisley, UK) by boiling at 100°C for 10 mins followed by slow cooling for several hours. Double stranded oligonucleotides were labelled with [α-^{32}P]-dATP (Amersham, Little Chalfont, UK) using a random priming kit containing dCTP, dGTP, dTTP, H buffer and Klenow DNA polymerase (Gibco BRL) and incubating at 37°C for 2 hrs. Probes were purified on an 8% non-denaturing polyacrylamide gel using TBE as running buffer. The gels were then exposed to X-OMAT film (Kodak, New York, USA) to detect labelled probe and gel pieces were incubated in TE buffer overnight at 37°C.

Binding reactions were initiated by pre-incubation of 10 μg nuclear protein extract with 200 ng double-stranded poly (dI-dC).(dI-dC) (Pharmacia Biotech, St. Albans, UK) in reaction buffer (20 mM HEPES buffer, pH 7.9, containing 40 mM KCl, 1 mM MgCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, 10% glycerol and 0.1% NP-40) in a volume of 30 μl for 20 mins on ice. For antibody super-shift assays, 1 μl antibody was added during this pre-incubation period. The appropriate radiolabelled synthetic oligonucleotide DNA probe (0.5 ng) was then added, and the mixture incubated on ice for a further 20 mins. The resultant DNA-protein complexes were resolved by non-denaturing 6% PAGE. The

gels were fixed (in 20% methanol, 10% acetic acid, 10% glycerol) for 20 min and then dried and visualised by autoradiography. The sequences of the oligonucleotide probes used in this work are provided in Table 2.1.

2:14 Preparation of RNA extracts.

Stimulated macrophages ($4x10^6$/25 cm^2 flask) were washed twice with ice-cold PBS before the addition of 1 ml RNAzol™ B (Biogenesis, Poole, UK). After incubation on ice for a few minutes the contents were removed to an Eppendorf tube. 200 µl chloroform was added and samples were incubated for a further 10 mins on ice. Following centrifugation (13,000 xg for 15 mins) the upper phase was removed and an equal volume of isopropanol was added. RNA was precipitated by incubation on ice for 30 mins and separated by centrifugation (13,000 xg for 20 mins). The RNA pellet was washed with ethanol, dried, dissolved in DEPC water and heated to 68°C for 2 mins before use. The concentration of RNA in samples was estimated by UV spectroscopy.

2:15 Northern blot analysis.

IL-12 p40 and β-actin cDNA probes were provided by Dr. X.-Q. Wei (Department of Immunology, University of Glasgow, UK). These cDNA probes were labelled with [α-^{32}P]-dATP using a commercial random priming kit (Gibco BRL, Paisley, UK) according to the manufacturer's instructions, and purified on a NICK column (Amersham, Little Chalfont, UK) using TE buffer. The labelled cDNA was then denatured by heating for 10 mins at 96°C and chilled on ice.

Total macrophage RNA (10 μg in 3 μl DEPC-treated water) was denatured in 7 μl sample buffer (71% formamide, 7% 20x MOPS buffer (400mM MOPS pH 7.0, 122 mM NaAc, 20 mM EDTA) and 8% formaldehyde) for 2 mins at 68°C. Samples were chilled on ice and mixed with 16% nucleic acid gel loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol).

Electrophoresis was carried out through a 1.5% agarose gel containing 5% 20x MOPS buffer and 2.8% formaldehyde. Samples and RNA markers (Promega, Madison, USA) were loaded and gels were run in 1x MOPS buffer at a constant voltage for 3-5 hrs. Following electrophoresis, agarose-formaldehyde gels were rinsed twice in distilled water and the RNA was transferred on to a nylon membrane by capillary blotting (Southern, 1975) overnight at room temperature. The blot was rinsed in 2x SSC (0.3 M NaCl, 42 mM sodium citrate, pH 7.2), air dried and oven baked at 120°C for 20 mins and UV-linked.

The nylon membrane was incubated in pre-hybridisation buffer containing 7% SDS, 40 mM NaH_2PO_4, 1 mM EDTA and 100 mg/ml ssDNA for 3 hrs at 65°C. The labelled probe was then added and incubated for 18 hrs in a hybridisation oven. The filter was then washed sequentially, twice in 2x SSC and 0.1% SDS for 30 mins and twice in 0.2x SSC and 0.1% SDS for 60 mins at 65°C. The membrane was dried between filter paper for 10 mins at room temperature and exposed to X-ray film (Kodak, New York, USA) for 1-3 days.

2:16 TaqMan real-time RT-PCR.

TaqMan real-time RT-PCR (Gibson *et al.* 1996) was performed according to the manufacturer's instructions (PE Biosystems, Foster City, USA) and as described by Overbergh *et al.* (1999). The principle of this method is outlined in Figure 2.2.

RNA (1-5 μg) was reverse transcribed using 100 U Superscript II RT (Life Technologies, Gaithersberg, USA) at 42°C for 50 mins in the presence of 50 mM Tris-HCl (pH8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 0.5 mM dNTPs and 5 μM $Oligo(dT)_{16}$ (Gibco BRL, Paisley, UK).

Primers and fluorogenic probes (see Table 2.2) were designed using the PrimerExpress™ v1.0 program and purchased from PE Biosystems. The fluorogenic probes contained a reporter dye (FAM) covalently attached at the 5' end and a quencher dye (TAMRA) covalently attached at the 3' end, and were HPLC purified. Extension from the 3' end was blocked by attachment of a 3' phosphate group.

PCR reactions were performed in the ABI-prism 7700 Sequence Detector, which contains a Gene-Amp PCR system 9600 (PE Biosystems). PCR amplifications were performed in a total volume of 25 μl, containing 0.5 μl cDNA sample, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 10 mM EDTA, 200 μM dATP, dCTP, dGTP and 400 μM dUTP, 5 mM $MgCl_2$, 300 nM each primer (see Table 2.2), 0.625 U AmpliTaqGold™ and 0.25 U AmpErase Uracil N-Glycosylase (PE Biosystems). Each reaction also contained 200 nM detection probe (see Table 2.2). Each PCR amplification was performed in triplicate

wells, using the following conditions: 2 mins at 50°C and 10 mins at 94°C, followed by a total of 40 or 45 two-temperature cycles (15 secs at 94°C and 1 min at 60°C).

Data analysis was performed using the Sequence Detection software (PE Biosystems) which calculates the threshold cycle, Ct (see Figure 2.3). This represents the PCR cycle at which an increase in fluorescence above a threshold can first be detected. Samples were normalised by their reference reporter (HPRT) by subtracting the Ct value of HPRT from the Ct value of the gene of interest; this value is ΔCt. To obtain a value for fold increase relative to HPRT the formula $2^{-\Delta Ct}$ was used (samples have been checked to ensure 100% efficiency). Multiplication of this value by 100 gives expression of the gene of interest as a percentage of HPRT. The positive error is the standard deviation of the difference, $s = \sqrt{(s_1^2 + s_2^2)}$, where s_1 and s_2 are the standard deviations of the Cts of HPRT and the gene of interest.

2:17 Densitometry and statistical analysis.

Data presented are representative of at least two independent experiments. Cytokine and NO measurements are displayed as means and standard deviations of triplicate culture unless otherwise indicated. Densitometry was performed using the GelPro computer program. Where presented, statistical significance was determined by T-test.

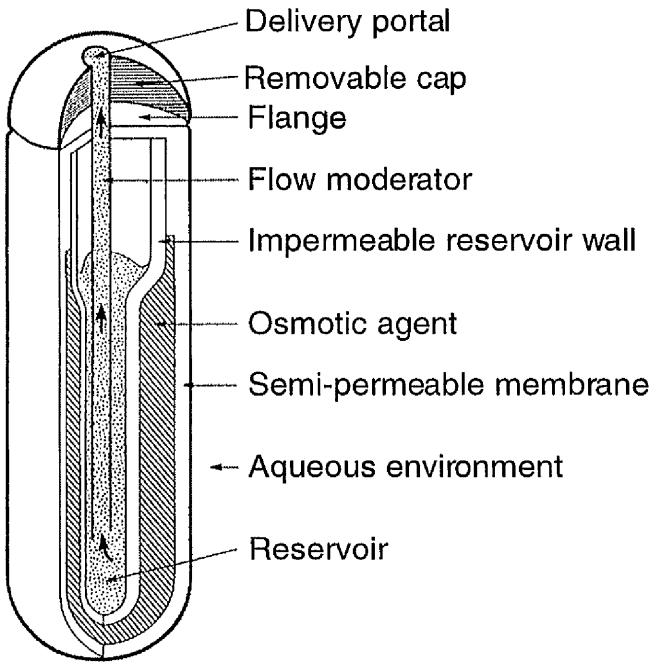


Figure 2.1. ALZET osmotic mini-pumps.

ALZET osmotic mini-pumps are composed of three concentric layers - the drug reservoir, the osmotic sleeve, and the rate-controlling, semi-permeable membrane. The drug reservoir is impermeable, blocking the exchange of material between the reservoir and the surrounding osmotic sleeve, which contains a high concentration sodium chloride solution. The difference in osmotic pressure between the osmotic sleeve and the implantation site drives delivery of the test solution, which is loaded into the drug reservoir before use. Water enters the sleeve along the osmotic gradient, compresses the flexible reservoir, and displaces the test solution through the flow moderator at a constant rate. The mass delivery rate of the test substance can be varied by adjusting the concentration of the test solution loaded into the reservoir.

Table 2.1 - EMSA Oligonucleotide Probes.

NF-κB from iNOS promoter	5' CCC AAC TGG GGA CTC TCC CTT TGG 3'
NF-κB from IL-12 p40 promoter	5' CAA AAC ATT CTG GGG GAA TTT TAA GA 3'
IRF-E from iNOS promoter	5' CAC TGT CAA TAT TTC ACT TTC ATA AT 3'
Fragment of F1 site from IL-12 p40 promoter	5' CAG AGA TAC TAA TTT CTG TTT ACA TCA TGC CTA AGG T 3'

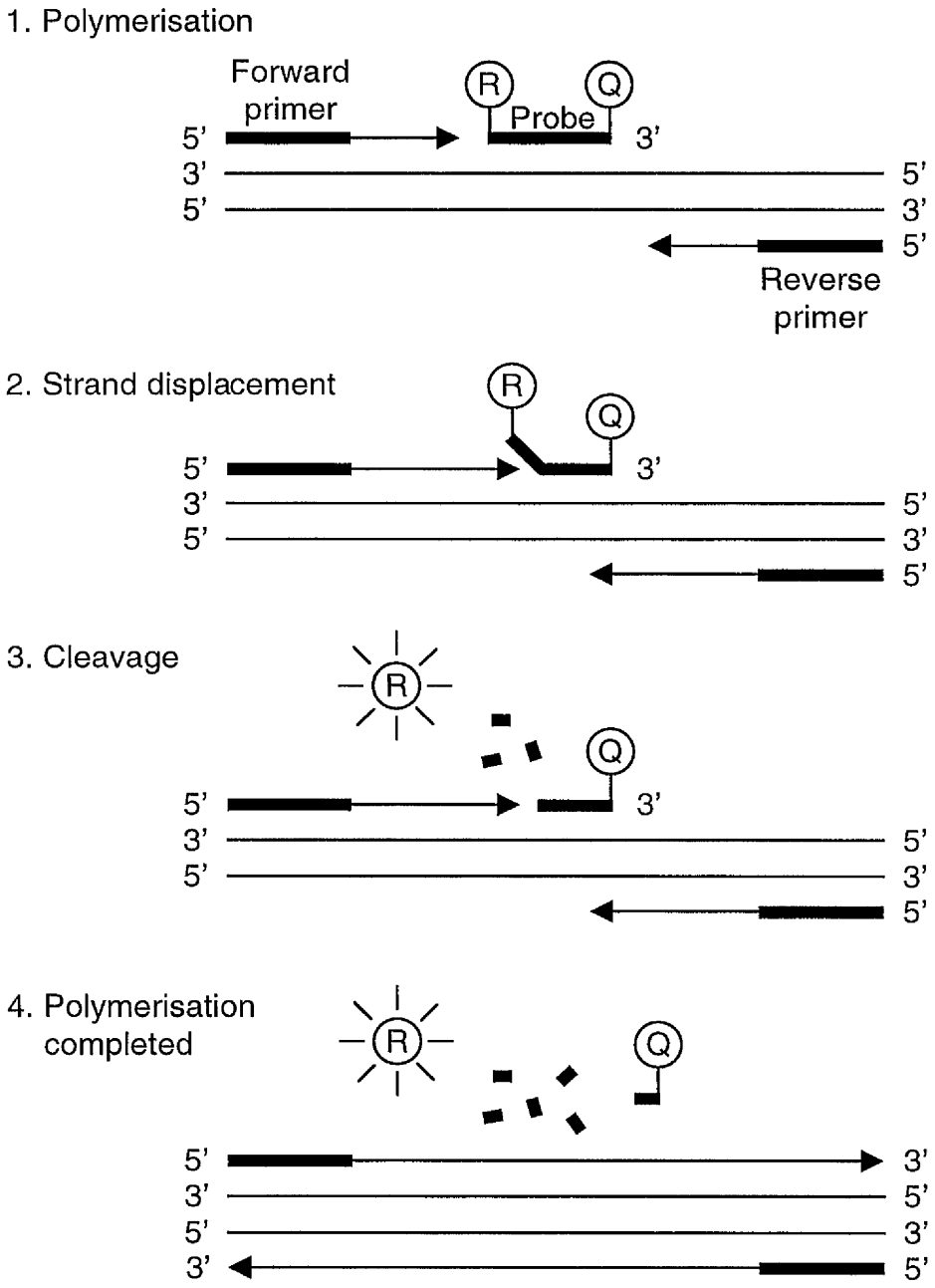


Figure 2.2. TaqMan real-time RT-PCR.

The TaqMan probe consists of an oligonucleotide with a 5' reporter dye (R) and a 3' quencher (Q). When the probe is intact, the proximity of the quencher to the reporter results in suppression of reporter fluorescence. As PCR proceeds, the polymerase enzyme cleaves the probe with its 5'-3'nuclease activity. The reporter and quencher become separated, resulting in increased reporter fluorescence. The 3' end of the probe is blocked to prevent extension of the probe during PCR.

Table 2.2 - TaqMan Primers and Probes.

Primers: F = forward, R = reverse

Probes: 5' FAM (6-carboxy-fluorescein) labelled, 3' TAMRA (6-carboxy-tetramethyl rhodamine; quencher) labelled

Murine IL-12 p40	F Primer: 5' GGA ATT TGG TCC ACT GAA ATT TTA AA 3' R Primer: 5' CAC GTG AAC CGT CCG GAG TA 3' Probe: 5' FAM – AAC AAG ACT TTC CTG AAG TGT GAA GCA CCA AAT - TAMRA 3'
Murine IL-12 p35	F Primer: 5' AAG ACA TCA CAC GGG ACC AAA 3' R Primer: 5' CAG GCA ACT CTC GTT CTT GTG TA 3' Probe: 5' FAM – CAG CAC ATT GAA GAC CTG TTT ACC ACT GGA - TAMRA 3'
Murine TNF-α	Purchased from PE-Applied Biosystems UK (Warrington, UK)
Murine TLR2	F Primer: 5' AAG ATG CGC TTC CTG AAT TTG 3' R Primer: 5' TCC AGC GTC TGA GGA ATG C 3' Probe: 5' FAM – CGT TTT TAC CAC CCG GAT CCC TGT ACT G – TAMRA 3'
Murine TLR4	F Primer: 5' AGG AAG TTT CTC TGG ACT AAC AAG TTT AGA 3' R Primer: 5' AAA TTG TGA GCC ACA TTG AGT TTC 3'

	Probe: 5' FAM – GCC AAT TTT GTC TCC ACA GCC ACC A - TAMRA 3'
Murine HPRT	F Primer: 5' GCA GTA CAG CCC CAA AAT GG 3' R Primer: 5' AAC AAA GTC TGG CCT GTA TCC AA 3' Probe: 5' FAM – TAA GGT TGC AAG CTT GCT GGT GAA AAG GA – TAMRA 3'

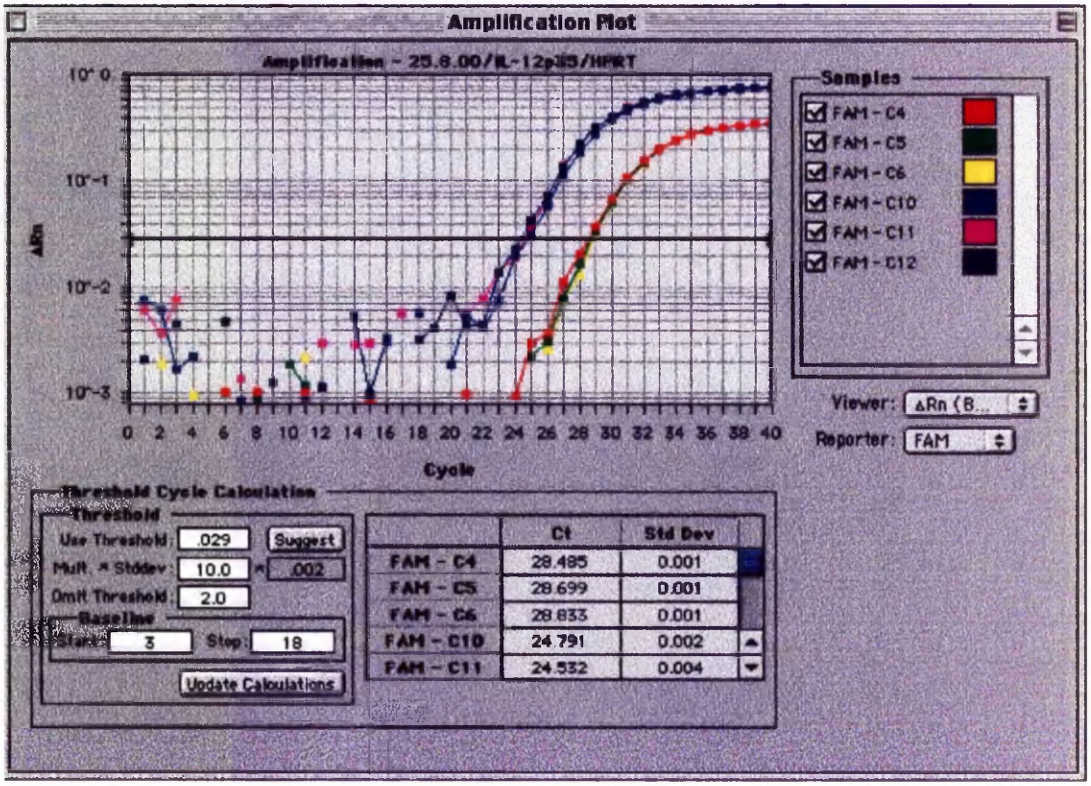


Figure 2.3. Typical TaqMan Amplification Plots.

The amplification plot shows how the fluorescence emission (normalised Reporter, Rn) varies with the PCR cycle number. Initially, the fluorescent signal is below the detection limit of the Sequence Detector. In the second stage, the signal can be detected as it continues to increase in direct proportion to the amount of specific amplified product. As PCR continues, the ratio of polymerase enzyme to PCR product decreases and hence the amount of PCR product ceases to increase exponentially, until eventually the Rn reaches a plateau. The Ct (threshold cycle) is determined during the exponential phase (threshold indicated by black line). Two triplicate samples are shown (C4-C6 and C10-C12).

Chapter 3 – Activation of macrophages by interferon-γ (IFN-γ) and lipopolysaccharide (LPS).

3:1 Introduction.

3:1:1 Mitogen-activated protein (MAP) kinases.

Mitogen-activated protein (MAP) kinases are key signalling molecules involved in the regulation of a diverse range of cellular functions including protein synthesis, cell cycle control, division and differentiation, and cell motility (Davis, 1994; Karin, 1994; Madhani and Fink, 1998). In mammals there are three major subfamilies of MAP kinases – the extracellular regulated kinases (ERKs), p38, and the c-Jun N-terminal kinases (JNKs) which are also known as stress activated protein kinases (SAPKs). The signalling cascades leading to activation of these three MAP kinase subfamilies are summarised in Figure 3.1.

MAP kinases are characterised by the presence of a Threonine-X-Tyrosine motif in the activation loop of the protein. Each MAP kinase is activated by dual phosphorylation of the threonine and tyrosine residues by a MAP kinase kinase (MAPKK, MKK or MEK), which is itself activated by dual serine phosphorylation by a MAP kinase kinase kinase (MAPKKK). For example, the classical MAP kinase pathway is the ERK cascade. Binding of growth factors to their cell surface receptors results in recruitment of tyrosine kinases and adaptor molecules to the intracellular signalling domain of the receptor. Recruitment of the Grb2 and Sos proteins results in activation of the small GTPase Ras, which catalyses the activation of the MAPKKK Raf; Raf then phosphorylates MEK1/2, which activates ERK1/2 MAP kinases. MAP kinases

phosphorylate their downstream targets on serine and threonine residues; these include the transcription factors Elk-1, ATF-2 and c-Jun.

All three subfamilies of MAP kinases (ERKs, p38 and JNKs) have been shown to be activated following stimulation of macrophages with LPS (Weinstein *et al.,* 1992; Han *et al.,* 1994; Liu *et al.,* 1994; Raingeaud *et al.,* 1995; Hambleton *et al.,* 1996), and the roles of some of these in pro-inflammatory cytokine and NO production have been demonstrated (Geppert *et al.,* 1994; Swantek *et al.,* 1997; Bhat *et al.,* 1998; Lu *et al.,* 1999; Zhu *et al.,* 2000).

Selective inhibitors of MAP kinase activation are useful pharmacological tools for dissecting the roles of these signalling molecules. PD 098059 is a selective and potent inhibitor of the ERK MAP kinase cascade, which mediates its effects by binding to and preventing the activation of the ERK-specific MAP kinase kinase, MEK-1 (Alessi *et al.,* 1995; Dudley *et al.,* 1995). It has no effect on any of the components of the JNK or p38 MAP kinase cascades (Pang *et al.,* 1995). U0126 also targets MEK-1, but it blocks MEK-1 activity rather than its activation, and is therefore slightly more effective since it also removes any basal MEK-1 activity present in cells prior to stimulation (Favata *et al.,* 1998; DeSilva *et al.,* 1998). Similarly, the compound SB 203580 has been reported to be a selective and potent inhibitor of p38 MAP kinase activity, which does not affect either ERK or JNK MAP kinases (Cuenda *et al.,* 1995). Unfortunately, there is currently no known selective inhibitor of JNK MAP kinases.

3:1:2 Aim of this study.

The aim of this study was to investigate the involvement of ERK and p38 MAP kinases in the regulation of IL-12, IL-6, TNF-α and NO production. This was achieved by stimulating murine macrophages (J774 cell line, peritoneal macrophages, splenic macrophages and bone marrow-derived macrophages) with LPS in the presence/absence of IFN-γ. MAP kinase activation was assayed by Western Blotting and by employing specific inhibitors of the MAP kinase isoforms to investigate their roles in cytokine and NO production. Cytokine production was measured by ELISA, Northern Blotting and TaqMan real-time PCR; NO release was assayed by Griess Reaction. Furthermore, putative downstream targets were also examined by Western Blotting and EMSA (Band Shift assay) to provide clues to the mechanisms underlying MAP kinase-mediated regulation of cytokine and NO production.

3:2 Results.

3:2:1 Cytokine and NO production by macrophages.

To verify the effects of stimulation, macrophages were cultured with IFN-γ and/or LPS for 24 hours. Culture supernatants were then harvested and assayed for the production of IL-12, IL-6, TNF-α by ELISA, and NO release by Griess Reaction (see Materials and Methods).

3:2:1:1 IL-12 production.

An ELISA antibody pair was used for the detection of IL-12 p40 subunit, which will in fact detect a number of p40-containing species including p40 monomer, bioactive p70 heterodimer and p40/p40 homodimers. Production of the p40 subunit of IL-12 was induced upon stimulation of J774 macrophages with LPS (Figure 3.2A) in a dose-dependent manner (Figure 3.2B). Co-stimulation with IFN-γ reduced LPS-induced IL-12 production (Figure 3.2). In contrast, IFN-γ was synergistic with LPS for the induction of IL-12 p40 from thioglycollate-elicited peritoneal macrophages from BALB/c and 129 mice, which produced only low levels of IL-12 p40 in response to LPS alone (Figure 3.3). IFN-γ treatment alone did not induce IL-12 production from either J774 cells (Figure 3.2A) or murine peritoneal macrophages (Figure 3.3). IL-12 p40 was detected in culture supernatants by ELISA from 8 hours after stimulation with IFN-γ + LPS and was maximal by 12 hours (Figure 3.4).

Macrophages derived from BALB/c bone marrow by culture in the presence of CSF-1 for 6 days also produced IL-12 p40 in response to stimulation with LPS or IFN-γ + LPS (Figure 3.5). Removal of CSF-1 by washing and replacement with normal full medium 2 hours prior to stimulation abolished the response to LPS stimulation and reduced IFN-γ + LPS-induced IL-12 p40 production (Figure 3.5), indicating that these cells need CSF-1 to enable them to respond optimally to activatory signals.

IL-12 p40 production following treatment of spleen cells from BALB/c mice with LPS or IFN-γ + LPS was also measured (Figure 3.6A). IFN-γ had no significant effect on LPS-induced p40 production. This IL-12 can be attributed to splenic macrophages since removal of non-adherent cells by washing and replacement with fresh medium led to similar levels of IL-12 p40 stimulation (Figure 3.6B). The contribution of splenic B cells, which can also produce IL-12 (reviewed by Ma *et al.,* 1997a), could therefore be considered negligible.

To confirm whether stimulation induces production of bioactive IL-12 heterodimer, not simply p40 monomer, IL-12 p70 levels in culture supernatants were measured by ELISA. Peritoneal macrophages produced IL-12 p70 in response to stimulation with IFN-γ + LPS (Figure 3.7); p70 levels were typically 10- to 20-fold lower than p40 levels, consistent with previous reports.

To investigate at what level regulation of IL-12 production occurs, IL-12 p40 and p35 mRNA were detected by TaqMan real-time PCR. IL-12 p40 mRNA expression was induced by IFN-γ + LPS stimulation; it was detectable by 4 hours after stimulation, and maximal by 10 hours, after which levels slowly decreased (Figure 3.8A). Similar

expression kinetics were found by Northern Blotting (not shown). Transcripts for the 'constitutively'-expressed p35 subunit of IL-12 were also enhanced by IFN-γ + LPS treatment (Figure 3.8B); p35 mRNA was detectable by 4 hours but peaked earlier than p40, at 8 hours, before declining to near basal levels by 16 hours. Therefore, the regulation of both p40 and p35 is important for generation of bioactive IL-12. Interestingly, the induction of p40 mRNA was much greater than that of the p35 subunit (~10-fold); this may explain why the p40 subunit is produced in vast excess over bioactive p70.

3:2:1:2 IL-6 production.

J774 cells and peritoneal macrophages from BALB/c mice produced IL-6 in response to LPS stimulation; although IFN-γ alone did not induce IL-6, it was synergistic with LPS for IL-6 production by peritoneal macrophages (Figure 3.9A and B). In contrast, bone marrow-derived macrophages (cultured and stimulated in the presence of CSF-1) produced IL-6 following LPS stimulation, but this was reduced by co-stimulation with IFN-γ (Figure 3.9C).

3:2:1:3 TNF-α production.

TNF-α release by murine peritoneal macrophages was induced by stimulation with LPS alone; IFN-γ, both on its own and in combination with LPS, had little effect on TNF-α production (Figure 3.10A). Bone marrow-derived macrophages behaved in a

similar manner to peritoneal macrophages when stimulated in the presence of CSF-1 (Figure 3.10B). In the absence of CSF-1 they responded poorly to LPS (Figure 3.10B). However, co-stimulation with IFN-γ resulted in similar levels of TNF-α induction to stimulation in the presence of CSF-1, indicating that IFN-γ may be providing the additional signals that these cells need to respond to LPS (Figure 3.10B). In a timecourse experiment, TNF-α was detected in peritoneal macrophage culture supernatant at 4 hours after stimulation and production was maximal by 12 hours (Figure 3.11).

To verify whether the induction of TNF-α occurs at the level of transcription, TNF-α mRNA was detected by TaqMan real-time PCR. Stimulation with IFN-γ and LPS resulted in a rapid and sustained increase in TNF-α mRNA (Figure 3.12). Induction was maximal by 2 hours after stimulation and mRNA levels remained elevated until at least 10 hours, before starting to fall by 16 hours after stimulation. Therefore, induction of TNF-α production does occur at the level of transcription.

3:2:1:4 NO production.

NO is produced by the catalytic action of iNOS on the substrate L-arginine. It is highly reactive and is therefore rapidly converted to nitrite (NO_2^-) in solution; this can be assayed by Griess Reaction. Analysis of culture supernatants in this way showed that J774 cells produced NO in response to stimulation with IFN-γ; this was enhanced by stimulation with LPS, which alone induced very low levels of NO release (Figure 3.13A). Similar results were obtained with murine peritoneal macrophages from BALB/c and 129 strains (Figure 3.13B and C). NO was detectable in peritoneal macrophage supernatants

from between 8 and 12 hours post-stimulation and maximal by about 16 hours (Figure 3.14). Total spleen cells from both mouse strains produced NO in response to stimulation with IFN-γ + LPS (Figure 3.15A), but NO production by splenic macrophages was not detected. IFN-γ + LPS also induced NO release from BALB/c bone marrow-derived macrophages (Figure 3.15B).

3:2:1:5 Cell viability of treated macrophages.

The viability of J774 cells and murine peritoneal macrophages was assessed by MTT assay to determine whether treatment with IFN-γ and/or LPS is toxic to the cells and might account for some of the differences in cytokine production. Macrophage viability was largely unaltered by stimulation with IFN-γ or LPS (Figure 3.16A and B), although occasionally a small decrease in viability was observed following stimulation with both IFN-γ and LPS. A decrease in the viability of bone marrow-derived macrophages following stimulation in the presence of CSF-1 was also seen (Figure 3.16C). Culture of bone marrow-derived macrophages in the absence of CSF-1 resulted in a dramatic decrease in the viability of non-stimulated macrophages, but this was partially rescued by stimulation with either IFN-γ or LPS, or both (Figure 3.16C). However, in general the observed differences in cytokine production were not a consequence of alteration of macrophage viability.

3:2:2 Roles of MAP kinases in cytokine and NO production by macrophages in response to stimulation with IFN-γ and LPS.

Although there are some reports of regulation of cytokines and iNOS by MAP kinases (Bhat *et al.,* 1998; Badger *et al.,* 1998), the role of the different MAP kinase subfamilies in mediating activation of macrophages by LPS is still unclear. Therefore, the activation of MAP kinases by stimulation of macrophages with LPS in the absence/presence of IFN-γ was examined, and the importance of the MAP kinases for induction of IL-12, IL-6, TNF-α and NO was dissected.

3:2:2:1 Activation of MAP kinases by stimulation with IFN-γ and/or LPS.

The LPS-mediated activation of ERK, p38 and JNK MAP kinases in J774 cells and murine peritoneal macrophages was investigated by Western Blotting, using specific antibodies against their dual-phosphorylated (bioactive) forms. LPS strongly stimulated a rapid (within 1 min) and transient increase in the levels of activation of both ERK1 (3.7 fold) and ERK2 (3.2 fold), which peaked at 10 minutes but remained elevated for at least 60 minutes above the low basal levels of ERK activation observed in unstimulated J774 cells (Figure 3.17A). IFN-γ co-treatment enhanced LPS-stimulation of ERK activation in these cells (not shown). Although there was no basal ERK MAP kinase activity in murine peritoneal macrophages, LPS strongly stimulated both ERK1 and ERK2 within 10 minutes (Figure 3.18A). In contrast, IFN-γ only weakly stimulated ERK activity and slightly suppressed LPS-stimulated ERK phosphorylation. It is interesting to note that in J774 cells, there was roughly equivalent activation of both ERK isoforms (Figure 3.17A),

whereas in peritoneal macrophages there was more phospho-ERK1 than phospho-ERK2 (Figure 3.18A). In both cell types there was more total ERK2 than ERK1 protein (Figures 3.17A and 3.18A).

Little or no activation of p38 MAP kinase was detected in unstimulated J774 cells (Figure 3.17B). LPS strongly activated p38, which was maximal at 10 minutes and sustained for up to 60 minutes. Likewise, p38 was activated following stimulation of murine peritoneal macrophages with either LPS or IFN-γ + LPS for 10 minutes (Figure 3.18B). Similarly, although SAPK/JNK MAP kinases were found not to be significantly activated in either unstimulated J774 cells (Figure 3.17C) or peritoneal macrophages (Figure 3.18C), LPS, following a lag period of up to 10 minutes, strongly stimulated both p46 and p54 JNK MAP kinases in J774 cells (Figures 3.17C). Activation of both isoforms was maximal at 30 minutes and declined towards basal levels within 60 minutes. Moreover, p46 and p54 JNK were both stimulated in murine peritoneal macrophages by either LPS or IFN-γ + LPS within 10 minutes; IFN-γ was synergistic with LPS for p54 JNK activation (Figure 3.18C). These results clearly show that LPS stimulates all three MAP kinase subfamilies in macrophages, but with differential activation kinetics.

3:2:2:2 Effects of selective inhibitors of the ERK and p38 MAP kinase signalling cassettes on LPS-stimulated MAP kinase activity.

The ERK and p38 inhibitors (PD 098059 and SB 203580 respectively) were used to investigate whether the induction of IL-12, IL-6, TNF-α and NO by LPS or IFN-γ + LPS is

mediated or regulated by these MAP kinases. First, the effects of these reagents on the dual phosphorylation of their target and heterologous MAP kinases were examined in J774 cells and peritoneal macrophages from BALB/c mice, to verify their specificity. In J774 cells, as expected, PD 098059 (20 μM) profoundly inhibited both the basal and stimulated ERK MAP kinase activation, but had no effect on phosphorylation of p38 or SAPK/JNK (Figure 3.20). Indeed, higher concentrations of PD 098059 completely abrogated ERK MAP kinase activation (not shown). Treatment with PD 098059 also dramatically reduced basal phospho-ERK and suppressed IFN-γ + LPS-induced ERK activation in peritoneal macrophages (Figure 3.21). Conversely, SB 203580 inhibited p38, but not ERK or JNK phosphorylation in J774 cells (Figure 3.20). Pre-incubation of cells with SB 203580 led to an increase in both basal and stimulated ERK (particularly ERK1) activation in J774 cells (Figure 3.20) and peritoneal macrophages (Figure 3.21), suggesting that p38 may act, at least in part, to exert inhibitory effects on ERK signalling in J774 cells. This finding is consistent with the proposed regulatory cross-talk postulated to occur amongst the different MAP kinase signalling cassettes (Zhang *et al.,* 1997; Zhu *et al.,* 2000; Singh *et al.,* 1999).

3:2:2:3 Effects of inhibition of MAP kinases on cytokine and NO production by macrophages.

To investigate whether ERK and p38 MAP kinases are involved in LPS-induced cytokine and NO production by macrophages, J774 cells and murine peritoneal macrophages were pre-treated with either PD 098059 or SB 203580 for 1 hour prior to stimulation, and cytokine and NO levels in 24 hour culture supernatants were measured

as before. Cell viability, as determined by MTT assay, was not altered by treatment with PD 098059 or SB 203580 at the concentrations shown (Figure 3.22).

3:2:2:3:1 Regulation of IL-12 production by ERK and p38 MAP kinases.

Pre-treatment of J774 cells with PD 098059 enhanced LPS-induced IL-12 p40 production in a dose-dependent manner (Figure 3.23A), indicating that ERK MAP kinases negatively regulate p40 production in these cells. 20 μM PD 098059 had maximal effect, so the inhibitor was used at this concentration in subsequent experiments. PD 098059 pre-treatment had the same effect on IFN-γ + LPS-induced IL-12 p40 production by murine peritoneal macrophages (Figure 3.23B). Similarly, p40 production was enhanced by pre-incubation with U0126, which was more potent than PD 098059 (Figure 3.23C).

To determine whether this regulation by ERK MAP kinases occurs at the level of transcription, IL-12 p40 transcript was detected by Northern Blot analysis of total RNA from J774 cells stimulated with LPS following pre-incubation for 1 hour with PD 098059. IL-12 p40 mRNA levels were enhanced by PD 098059 treatment (Figure 3.24), indicating that ERK MAP kinases negatively regulate the transcription of the p40 subunit or message stability. Similar results were obtained by TaqMan real-time PCR analysis of RNA from IFN-γ + LPS-stimulated peritoneal macrophages (Figure 3.25).

Levels of p70 heterodimer in culture supernatants were also measured to determine whether the enhancement of p40 by inhibition of ERK MAP kinases results in

increased production of bioactive IL-12. Interestingly, p70 induction was not enhanced by pre-treatment with PD 098059 or U0126 (Figure 3.26), indicating that enhancement of p40 production is not sufficient to increase production of bioactive heterodimer. This suggests that these MAP kinases might be required for either p35 production or assembly of the two subunits to form bioactive heterodimer, or simply that the p35 subunit is limiting.

To investigate these possibilities, p35 mRNA levels were assayed by TaqMan to determine whether ERK MAP kinases regulate p35 production. PD 098059 pre-treatment did not alter IFN-γ + LPS-stimulated p35 induction (Figure 3.27), indicating that ERK MAP kinases are neither positive nor negative regulators of IL-12 p35 induction. It would therefore appear that the availability of the p35 subunit limits the formation of bioactive IL-12.

The role of p38 MAP kinases was similarly investigated by pre-treating cells with SB 203580. This resulted in decreased production of IL-12 p40 by LPS-stimulated J774 cells and IFN-γ + LPS-stimulated murine peritoneal macrophages (Figure 3.28). Inhibition was dose-dependent and a concentration of 5 μM SB 203580 was chosen for subsequent experiments. IL-12 p70 levels were also suppressed by SB 203580 pre-treatment (Figure 3.29); the extent of p70 inhibition was comparable to that of p40 (82% and 86% inhibition of p40 and p70 respectively).

Northern Blot analysis of total RNA from J774 cells stimulated with LPS following pre-incubation for 1 hour with SB 203580 revealed that regulation of IL-12 p40 induction by p38 MAP kinases occurs at the level of transcription or message stability, since treatment

with SB 203580 resulted in delayed and overall reduced IL-12 p40 mRNA production (Figure 3.30). Similar results were obtained by TaqMan analysis of RNA from IFN-γ + LPS-stimulated peritoneal macrophages pre-treated with SB 203580 (Figure 3.31). IL-12 p35 message was also measured by TaqMan analysis; inhibition of p38 activity by pre-treatment with SB 203580 also suppressed p35 mRNA levels (Figure 3.32).

Taken together, these results suggest that p38 MAP kinases are required for transcription of both the IL-12 p40 and p35 genes, while ERK MAP kinases negatively regulate p40 but not p35 transcription. The modulation of transcripts by both inhibitors correlated with their effects on p40 protein production, indicating that the ERK and p38 MAP kinases exert most or all of their regulatory influence at the transcriptional level.

J774 cells and murine peritoneal macrophages were treated with PD 098059 or U0126 as well as SB 203580 to investigate whether the inhibitors cancel one another out and hence whether the ERK and p38 MAP kinases act antagonistically on the same signalling pathway. However, neither PD 098059 nor U0126 treatment restored IL-12 p40 inhibition by SB 203580 (Figure 3.33). In fact, U0126 treatment further decreased IL-12 p40 beyond the level of SB 203580 reduction (Figure 3.33B). This suggests that ERK MAP kinases can only negatively regulate IL-12 p40 production in the presence of a p38 signal.

3:2:2:3:2 Regulation of IL-6 production by p38, but not ERK, MAP kinases.

Induction of IL-6 in J774 cells and murine peritoneal macrophages was not altered when ERK MAP kinase activation was suppressed by pre-treatment with PD 098059 (Figure 3.34). In contrast, SB 203580 pre-treatment resulted in inhibition of IL-6 production (Figure 3.35), indicating that while ERK MAP kinases do not regulate IL-6 production in LPS- or IFN-γ + LPS-stimulated macrophages, p38 does mediate induction of IL-6.

3:2:2:3:3 Regulation of TNF-α production by ERK and p38 MAP kinases.

Treatment of J774 cells with either PD 098059 or SB 203580 prior to LPS stimulation resulted in dose-dependent suppression of TNF-α production (Figure 3.36A and 3.37A), demonstrating that both MAP kinase subfamilies are required to transduce LPS signals for TNF-α production. Similar results were obtained in murine peritoneal macrophages stimulated with IFN-γ and LPS (Figure 3.36B and 3.37B).

TaqMan analysis was performed to determine whether this regulation of TNF-α production occurs at the level of transcription or message stability. Pre-treatment with PD 098059 did not alter TNF-α mRNA levels at timepoints up to and including 8 hours after stimulation with IFN-γ and LPS (Figure 3.38A), indicating that ERK MAP kinases regulate a later stage of synthesis of this cytokine. At 18 hours, TNF-α mRNA was slightly enhanced in the PD 098059-treated cells. SB 203580 pre-treatment resulted in a reduction in TNF-α transcript levels at intermediate timepoints (6 and 8 hours; Figure

3.38B), suggesting that p38 MAP kinase regulates TNF-α production, at least in part, at the level of transcription or message stability.

3:2:2:3:4 Regulation of NO production by ERK and p38 MAP kinases.

Similarly, both ERK and p38 MAP kinases appear to be required for induction of NO release, since treatment with either PD 098059 or SB 203580 resulted in dose-dependent reduction of NO induction in J774 cells and murine peritoneal macrophages (Figure 3.39). Treatment with PD 098059 (up to 50 μM) only partially inhibited NO generation in J774 cells (Figure 3.39A), but NO production was completely abolished by incubation with 10 μM SB 203580 (Figure 3.39B). These results were confirmed by Western Blot analysis of iNOS expression which showed that whilst PD 098059 (20 μM) only partially blocked the induction of iNOS protein expression (41%), SB 203580 (5 μM) almost completely abrogated LPS-induction of iNOS expression (Figure 3.40). Taken together, these results suggest that whilst both p38 and ERK MAP kinases appear to play positive roles in the LPS-mediated induction of iNOS, p38 activation is more critical, and appears to be necessary and sufficient for effector induction.

3:2:3 Roles of MAP kinases in activation of transcription factor synthesis and binding.

Having clearly demonstrated roles for ERK and p38 MAP kinases in LPS-induced cytokine and NO production, the next step was to attempt to identify their downstream

targets to provide further clues to their mechanisms of action. To analyse the transcription factors targeted by ERK and p38 MAP kinases, cellular protein levels were measured by Western Blotting of total cell lysates, and binding of nuclear proteins to promoter sequences was assayed by EMSA (Band Shift).

3:2:3:1 Effects of MAP kinase inhibitors on NF-κB nuclear translocation and DNA binding.

The transcription factor NF-κB is involved in the induction of the IL-12 p40, IL-6, TNF-α and iNOS genes. In unstimulated cells, NF-κB is held in the cytoplasm by I-κB; LPS stimulation results in activation of I-κB kinases (IKKs) which phosphorylate I-κB, labelling it for ubiquitination and degradation, thereby liberating NF-κB which translocates to the nucleus (reviewed by Karin, 1999). I-κB degradation was not prevented by treatment with either PD 098059 or SB 203580 (Figure 3.41), indicating that neither ERK nor p38 MAP kinases achieve modulation of transcription by regulating the entry of NF-κB into the nucleus.

The translocation of NF-κB to the nucleus of J774 cells and binding to promoter sequences following stimulation with LPS was investigated by EMSA to determine whether ERK and p38 MAP kinases mediate their effects on cytokine and NO synthesis by modulating NF-κB activity. Cells were pre-treated with PD 098059 or SB 203580 for 1 hour prior to LPS stimulation for a further hour. Nuclear protein extracts were incubated for 30 minutes with a ^{32}P-labelled oligonucleotide probe representing an NF-κB binding site from either the iNOS or the IL-12 p40 promoter, prior to separation on a

polyacrylamide gel and visualisation by exposure to X-ray film (see Materials and Methods).

LPS stimulated binding of NF-κB to both the iNOS and IL-12 p40 promoter sequences, with identical patterns of binding complexes (Figure 3.42). Two binding complexes were detected in both resting and LPS-stimulated cells. The lower molecular weight complex (b) was predominant in resting cells and was not dramatically altered upon LPS stimulation. In contrast, a higher molecular weight complex (a) bound at only low levels in unstimulated cells, but binding was strongly induced by LPS stimulation. Supershift assay using specific antibodies against different NF-κB isoforms was performed to identify the components of these complexes. p65, p50 and c-Rel isoforms were present in the higher molecular weight complex (a), which most likely represents p65-p50 and c-Rel-p50 heterodimers. The lower molecular weight complex comprised p50-p50 homodimers. Binding patterns were not altered by pre-treatment of cells with 20 μM PD 098059 or 5 μM SB 203580, suggesting that neither ERK nor p38 MAP kinases regulate NF-κB translocation to the nucleus and binding to promoter sequences. Similar patterns of NF-κB binding were observed using nuclear protein extracts from IFN-γ + LPS-stimulated murine peritoneal macrophages (not shown).

3:2:3:2 Effects of MAP kinase inhibitors on binding to the IRF-E promoter sequence.

The iNOS promoter contains a binding site, known as the interferon regulatory factor (IRF) element (IRF-E), for members of the IRF family of transcription factors.

IRF-1 has also been identified as a member of the large F1 binding complex that regulates IL-12 p40 promoter activity. Therefore the MAP kinase inhibitors PD 098059 and SB 203580 were used to investigate whether ERK or p38 MAP kinases target synthesis of IRF proteins and their binding to promoter sequences.

There was no detectable IRF-1 in unstimulated peritoneal macrophages, but induction of IRF-1 synthesis occurred within 1 hour of stimulation with IFN-γ + LPS and was maximal by 4 hours (Figure 3.43A upper panel). In contrast, another IRF family member, IRF-2, which has been shown to be a negative regulator via interaction with IRF elements (Harada *et al.,* 1989; Yamamoto *et al.,* 1994), was expressed constitutively in resting cells and was not altered by stimulation (Figure 3.43A lower panel). Pre-treatment with PD 098059 for 1 hour prior to, or addition of PD 098059 1 hour after, IFN-γ + LPS stimulation enhanced IRF-1 synthesis by 4 hours post-stimulation; similar treatment with SB 203580 did not alter IFN-γ + LPS-induced IRF-1 synthesis (Figure 3.43B). These results indicate that ERK but not p38, MAP kinases negatively regulate IRF-1 synthesis; neither MAP kinase subfamily regulates IRF-2 production (not shown).

The binding activity of J774 cell nuclear lysates to a ^{32}P-labelled oligonucleotide probe containing the IRF-E from the iNOS promoter was measured by EMSA. Two complexes (a and b) bound at low levels in samples from resting cells and stimulation with LPS for 2 hours enhanced this binding activity (Figure 3.44A). By 3 hours post-stimulation this activity had diminished but was still above basal levels. This indicates that iNOS expression is induced by the transient binding of IRF family members to the IRF-E. Perhaps surprisingly, treatment with PD 098059 alone for 1 hour enhanced IRF-E binding and while PD 098059 pre-treatment did not alter LPS-induced binding at 2 hours,

it did result in sustained induction of binding at 3 hours after LPS stimulation. In contrast, although SB 203580 alone enhanced binding of complex a, pre-incubation with SB 203580 resulted in an overall suppression of complex b binding and promoted complex a binding at 3 hours. Thus, it appears that binding to the iNOS IRF-E is negatively regulated by ERK MAP kinases and modulated by p38 MAP kinases.

3:2:3:3 Effects of MAP kinase inhibitors on binding of transcription factors to the IL-12 p40 promoter.

The F1 complex of transcription factors, which includes IRF-1, Ets-2, c-Rel and Glp109, is an important inducer of IL-12 p40 transcription (Ma *et al.,* 1997b). It binds to an Ets consensus site in a complex manner, interacting with flanking sequences in a region of approximately 96 bp. A ^{32}P-labelled oligonucleotide probe representing a portion of this F1 binding region was used to investigate the binding activity of nuclear extracts from LPS-stimulated J774 cells. Two binding complexes were present in unstimulated cells; 2 hours LPS treatment enhanced this activity and resulted in the induction of a third complex (Figure 3.44B). By 3 hours post-stimulation, binding activity had returned to basal levels. Treatment with neither PD 098059 nor SB 203580 altered this pattern of F1 binding activity, indicating that neither MAP kinase subfamily achieves its regulation of IL-12 p40 transcription by altering binding to the F1 promoter site. Moreover, Western Blotting showed that treatment with neither PD 098059 nor SB 203580 blocked LPS-induced translocation of Ets-2 to the nucleus in J774 cells (Figure 3.45).

3:3 Discussion.

The recent identification of TLR4 as the LPS receptor has clarified the understanding of macrophage signal transduction pathways triggered by LPS stimulation. LPS molecules form aggregates in solution, which must be disrupted for recognition of these bacterial products by macrophages. This is achieved by the serum protein LBP which associates with LPS, enabling it to interact with CD14 on the macrophage surface. CD14 is unable to transduce signals across the plasma membrane so the LPS-LBP-CD14 complex must interact with TLR4. Ligation of the TLR4 receptor results in association and activation of tyrosine kinases and tyrosine phosphatases which regulate activation of multiple signalling pathways including NF-κB and MAP kinase cascades (see Figures 1.3 and 1.4). Multiple transcription factors are influenced, either by modifications such as phosphorylation or by induction of *de novo* synthesis. Ultimately this results in induction of genes encoding pro-inflammatory cytokines such as IL-12, IL-6 and TNF-α, and other innate immune mediators such as iNOS, which catalyses NO production.

In this study, consistent with many previous reports, stimulation of murine macrophages from a variety of sources (J774 macrophage cell line, thioglycollate-elicited peritoneal macrophages, splenic macrophages and bone marrow-derived macrophages) with LPS resulted in induction of IL-12 (both subunits), IL-6 and TNF-α (Figures 3.2 - 3.12). IFN-γ alone was a poor inducer of these cytokines, but it had various effects on the induction of cytokine production by LPS. For example, while it was synergistic with LPS for IL-12 p40 production by peritoneal and bone marrow-derived macrophages (Figures 3.3 - 3.5), it was inhibitory for J774 cells (Figure 3.2) and did not alter p40 production by splenic macrophages (Figure 3.6). Similarly, it enhanced IL-6 production by peritoneal

macrophages but had the opposite effect on bone marrow-derived macrophages (Figure 3.9). These effects may represent the differential abilities of macrophages sourced from distinct locations to process IFN-γ signals, or reflect differential macrophage maturation. Indeed, the priming effect of IFN-γ on LPS-induced IL-12 production has been shown in some cells to be dependent on IFN-γ pre-incubation (Ma *et al.,* 1996b). Furthermore, the effects of IFN-γ on bone marrow-derived cells were also dependent on the absence/presence of CSF-1 in the culture medium (Figures 3.5 and 3.10). In the absence of CSF-1, bone marrow-derived macrophages could not respond to the LPS signal to produce IL-12 and TNF-α, but this ability was restored by IFN-γ. However, IFN-γ was not synergistic with LPS for IL-12 production in these cells in the ~~absence~~ presence of CSF-1. Therefore, the effects of IFN-γ appear to be dependent on multiple factors such as the tissue microenvironment of the macrophages and the maturation status of the macrophage.

Low levels of iNOS expression and NO production were also induced by LPS stimulation (Figures 3.13 - 3.15). However, the effect of treatment with IFN-γ alone was much more significant, and the combination of IFN-γ and LPS produced the strongest induction of NO synthesis. Overall, while LPS stimulation was most critical for induction of cytokine synthesis, IFN-γ induced NO more strongly. The combination of IFN-γ and LPS was most effective for the stimulation of cytokine and NO production by peritoneal macrophages.

MAP kinases have previously been reported to be important downstream mediators of LPS signals (Weinstein *et al.,* 1992; Hambleton *et al.,* 1995 and 1996; Han

et al., 1994; Zhu *et al.,* 2000). Consistent with these reports, the three major subfamilies of MAP kinases (ERKs, p38 and JNKs) were shown in this study to be activated upon treatment of macrophages with LPS or IFN-γ + LPS (Figures 3.17 and 3.18).

Interestingly, in J774 cells, LPS stimulation resulted in roughly equal phosphorylation of both ERK isoforms, whereas in peritoneal macrophages stimulated with LPS in the absence or presence of IFN-γ, the ERK1 isoform was more strongly activated. However, ERK2 was expressed at much higher levels than ERK1 in J774 cells, whereas both isoforms were present at similar levels in peritoneal macrophages. This suggests that LPS stimulation preferentially targets the activation of ERK1, although ERK2 activation is also induced. Since treatment with the inhibitor of the ERK MAP kinase pathway, PD 098059, suppressed the dual phosphorylation of both ERK isoforms (Figures 3.20 and 3.21), this inhibitor cannot be used to dissect the individual functional significance of the two ERK isoforms.

The roles of ERK and p38 MAP kinases in the induction of IL-12, IL-6, TNF-α and NO by LPS and IFN-γ + LPS were investigated using the inhibitors PD 098059 and SB 203580 (Figures 3.23 – 3.40). Synthesis of all of these macrophage mediators was shown to be induced in a p38-dependent manner; ERK MAP kinases were also required for the induction of TNF-α and iNOS, but were negative regulators of IL-12 p40 production (summarised in Figure 3.46).

It has been reported that the anti-inflammatory cytokine IL-10 is produced by LPS-stimulated monocytes in a p38-dependent manner (Foey *et al.,* 1998) and it is therefore possible that the suppression of cytokine and iNOS production observed

following SB 203580 treatment could be due to the induction of IL-10. However, IL-10 release was not detected under any of the conditions tested (not shown), suggesting that the ERK and p38 MAP kinases do not exert any of their regulatory effects on IL-12, IL-6, TNF-α or NO via generation/suppression of IL-10.

Induction of IL-12 p40 transcription by LPS was shown to be mediated by p38 and negatively regulated by ERK MAP kinases (Figures 3.23 – 3.25 and 3.28 – 3.31). The requirement for a p38 signal supports the recent demonstration that mice deficient in MEK3, the kinase upstream of p38, are defective in IL-12 production (Lu *et al.*, 1999). Furthermore, in the absence of p38 MAP kinase activation, inhibition of ERK phosphorylation did not enhance IL-12 p40 production (Figure 3.33), suggesting that the suppressive action of the ERKs is dependent on a positive p38 signal. ERK MAP kinases could target either a regulator of p38 activity or a p38 substrate. However, since treatment with PD 098059 suppressed ERK activation but did not alter p38 (Figure 3.20), this eliminates a target upstream of p38. Therefore the ERKs must negatively regulate a target downstream of p38 to limit p40 monomer production. This may serve to prevent excessive formation of inhibitory p40 homodimers and thus maintain an appropriate balance between p70 heterodimers and p40 homodimers. This suggests that the balance of p38 and ERK MAP kinase signals is critical for the regulation of IL-12 p40 production.

The synergy of IFN-γ with LPS for IL-12 p40 production by peritoneal macrophages (Figures 3.3 and 3.4) can also be explained in terms of negative regulation by ERK MAP kinases, since IFN-γ stimulation reduced LPS-stimulated ERK activation in these cells (Figure 3.18). In contrast, ERK activation in J774 cells was slightly

enhanced by IFN-γ co-stimulation (not shown); hence there was no synergy between IFN-γ and LPS in these cells, rather a slight reduction in IL-12 p40 production (Figure 3.2).

p35 transcription was also p38-dependent but, unlike p40, it was not regulated by ERK MAP kinases (Figures 3.27 and 3.32). Thus, LPS stimulation of macrophages results in the activation of p38 MAP kinase, which induces transcription of both the p40 and the p35 IL-12 genes, resulting in the production of bioactive IL-12. Simultaneous activation of ERK MAP kinases prevents excessive production of the p40 subunit and the formation of antagonistic p40 homodimers.

IL-6 was regulated in the same way as the p35 subunit of IL-12 i.e. induction of IL-6 by LPS was p38-dependent but ERK-independent (Figures 3.34 and 3.35). This is interesting since these two molecules are homologous and IL-12 p35 has been suggested to have been derived from an IL-6-like primordial cytokine (Merberg *et al.,* 1992). p38 MAP kinase has recently been suggested to stabilise IL-6 mRNA (Winzen *et al.,* 1999); this mechanism may also contribute to the p38-dependent regulation of IL-12 p35.

Induction of the TNF-α and iNOS genes was dependent on the activation of both ERK and p38 MAP kinases (Figures 3.36, 3.37 and 3.40). This is consistent with similar reports in the literature (Geppert *et al.,* 1994; Bhat *et al.,* 1998; Larsen *et al.,* 1998; Badger *et al.,* 1998; Zhu *et al.,* 2000), although the production of iNOS and NO does not appear to be dependent on these MAP kinases in some cells (DaSilva *et al.,* 1997; Guan *et al.,* 1997). In this study however, the dependence of iNOS transcription on both ERK and p38 MAP kinase signals in macrophages was underlined by the inhibition of NO production following treatment with PD 098059 or SB 203580 (Figure 3.39). Treatment

with PD 098059 did not alter TNF-α message levels (Figure 3.38), indicating that ERK MAP kinases do not regulate TNF-α transcription or message stability, but must act at a later stage of synthesis such as translation or release from the cell. p38 MAP kinase does appear to partially regulate TNF-α at the message level, but is also likely to act at later stages of TNF-α generation.

The different macrophage effectors exhibited different sensitivities to the inhibitors. For example, treatment of peritoneal macrophages with 5 μM SB 203580 resulted in significant inhibition of IL-6 and NO, but reduced IL-12 p40 levels (in the same samples) even more dramatically and effectively abolished TNF-α production. This indicates that while p38 MAP kinase is required for optimal induction of all of these cytokines and iNOS by IFN-γ + LPS, it would appear to be absolutely necessary for TNF-α production in these cells. Similarly, p38 MAP kinase signals seem to be critical for IL-6 production by J774 cells, but perhaps less important for IL-12 p40 induction.

It has been suggested that ERK activation is negatively regulated by p38 (Singh *et al.,* 1999). The enhanced phosphorylation of ERK that accompanied inhibition of p38 by SB 203580 (Figures 3.20 and 3.21) seems to support this hypothesis. However, Hall-Jackson *et al.* (1999) recently demonstrated that SB 203580 can activate Raf *in vivo* in a p38-independent manner. Thus, it is possible that the enhanced ERK activation occurred as a result of SB 203580 treatment rather than as a consequence of removal of a suppressive signal. Although p38 is unquestionably required for induction of IL-12 p35, IL-6, TNF-α and iNOS, since enhanced ERK activation could not explain the effects of SB 203580 on the induction of these proteins, there is therefore a possibility that the reduction of IL-12 p40 production by treatment with SB 203580 could be due, at least in

part, to increased negative regulation by ERK MAP kinases. However, mice deficient in p38 MAP kinase kinase 3 (MKK3/MEK3) were defective in IL-12 p40 production by macrophages and dendritic cells (Lu *et al.,* 1999), supporting the proposed requirement of p38 MAP kinase for IL-12 p40 production.

The relevance of JNK activation for the induction of cytokine and NO production by LPS could not be demonstrated by such inhibitor studies because a specific inhibitor of this MAP kinase subfamily is not currently available. Alternative techniques could be employed to investigate this, such as stimulation of JNK MAP kinase-deficient macrophages obtained either by transient transfection with dominant negative JNK or antisense oligonucleotides, or from knock-out animals. Indeed, Constant *et al.* (2000) recently showed that IFN-γ + LPS-stimulated peritoneal macrophages from JNK1-deficient mice produce more IL-12 and NO than control mice, while Swantek *et al.* (1997) demonstrated that a kinase-defective JNK mutant blocked the translation of TNF-α.

The effects of inhibition of ERK and p38 MAP kinases on various transcription factors that regulate cytokine and iNOS expression were investigated in an attempt to identify the downstream targets of the ERK and p38 pathways. Treatment with neither PD 098059 nor SB 203580 altered the degradation of I-κBβ or the binding of NF-κB to iNOS or IL-12 p40 promoter sequences (Figures 3.41 and 3.42), suggesting that regulation of LPS-induced cytokine and NO production by these MAP kinase subfamilies occurs independently of the NF-κB pathway.

In contrast, EMSA analysis indicated that regulation of iNOS transcription by p38 may be achieved by members of the IRF family of transcription factors. SB 203580 treatment disrupted binding to the IRF-element (IRF-E) from the iNOS promoter (Figure 3.44A). The identity of the IRF family members involved remains to be established. IRF-1 synthesis was not altered by SB 203580 treatment (Figure 3.43B), indicating that p38 could act further downstream e.g. to phosphorylate IRF-1, or on other IRF family members such as IRF-2, ICSBP, ICSAT or novel IRF proteins.

IRF family members could also play important roles in the regulation of IL-12 p40. PD 098059 treatment enhanced IFN-γ + LPS-induced IRF-1 synthesis in peritoneal macrophages (Figure 3.43B), suggesting a mechanism for the negative regulation of IL-12 p40 expression via suppression of IRF-1 synthesis by ERK MAP kinases. Neither inhibitor altered binding to the F1 site from the IL-12 p40 promoter (Figure 3.44B), indicating that neither ERK nor p38 MAP kinases regulate the assembly of the F1 complex. Therefore it is unlikely that ERK and p38 target IRF-1 within this complex, but there are other IRF-binding sites present on the p40 promoter that represent potential targets. However the oligonucleotide used for F1 binding represents only a portion of this large site so further investigation is required to confirm this. Nuclear translocation of Ets, another component of the F1 complex, was not altered by treatment with PD 098059 or SB 203580 (Figure 3.45), suggesting that Ets does not transduce p38 or ERK signals.

3:4 Summary of this study.

In summary therefore, ERK and p38 MAP kinases play critical roles in the induction of pro-inflammatory cytokines and iNOS by LPS-stimulated macrophages. While p38 MAP kinase transduces positive signals, ERKs can either positively or negatively regulate effector induction. These MAP kinases are likely to exert their regulatory effects at multiple levels including transcription, translation and post-translational modification.

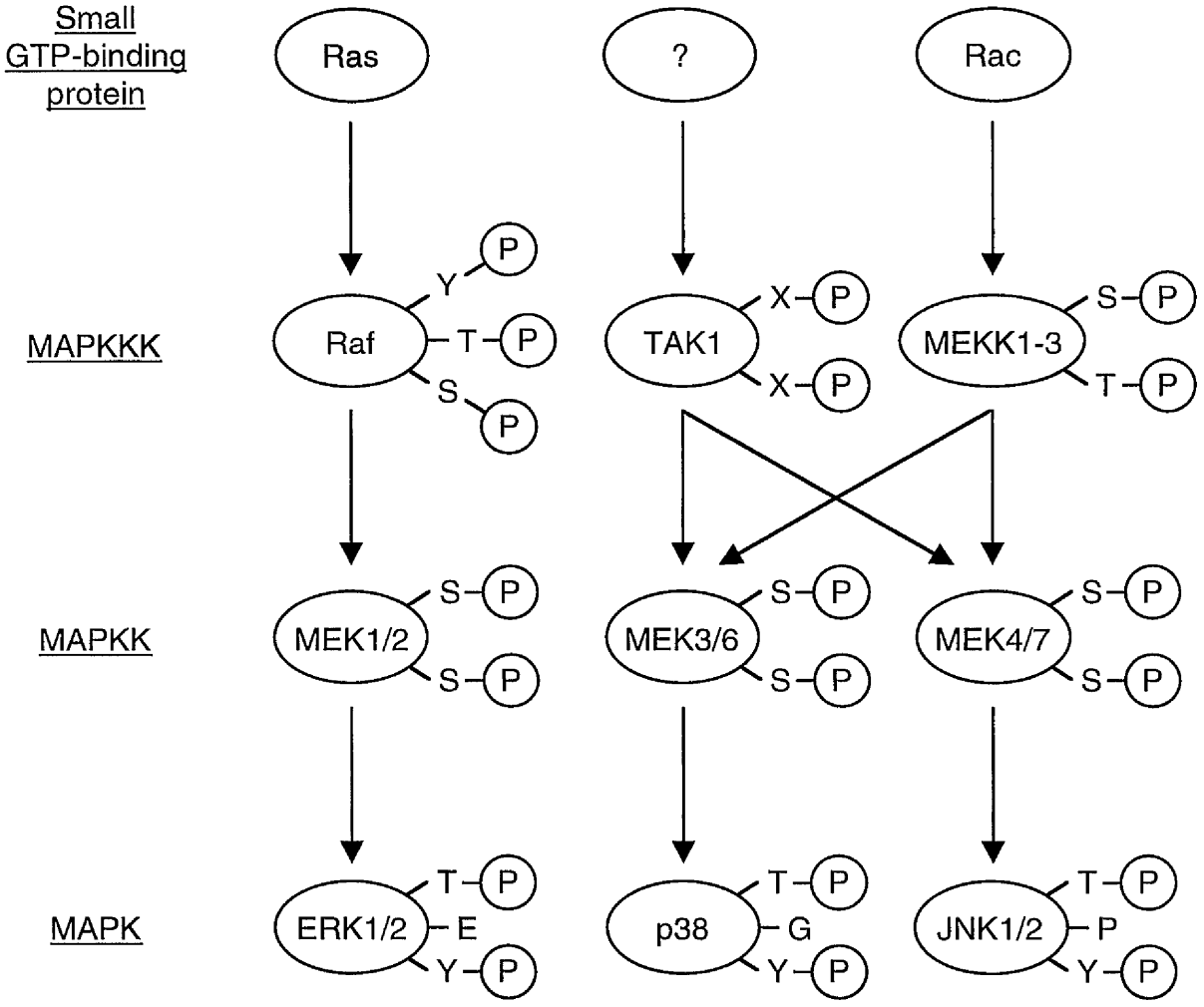


Figure 3.1. MAP kinase cascades.

Binding of ligands to cell surface receptors results in recruitment of tyrosine kinases and adaptor molecules to the intracellular signalling portion of the receptor, leading to activation of small GTP-binding proteins such as Ras and Rac. These catalyse the activation of three MAP kinase cascades - the ERK, p38 and JNK MAP kinase pathways. MAP kinase kinases (MAPKKKs) activate MAP kinase kinases (MAPKKs or MEKs), which in turn activate MAP kinases by dual phosphorylation of a threonine and a tyrosine in the TXY motif in the activation loop. The MAP kinases act by phosphorylating target proteins on serine and threonine residues.

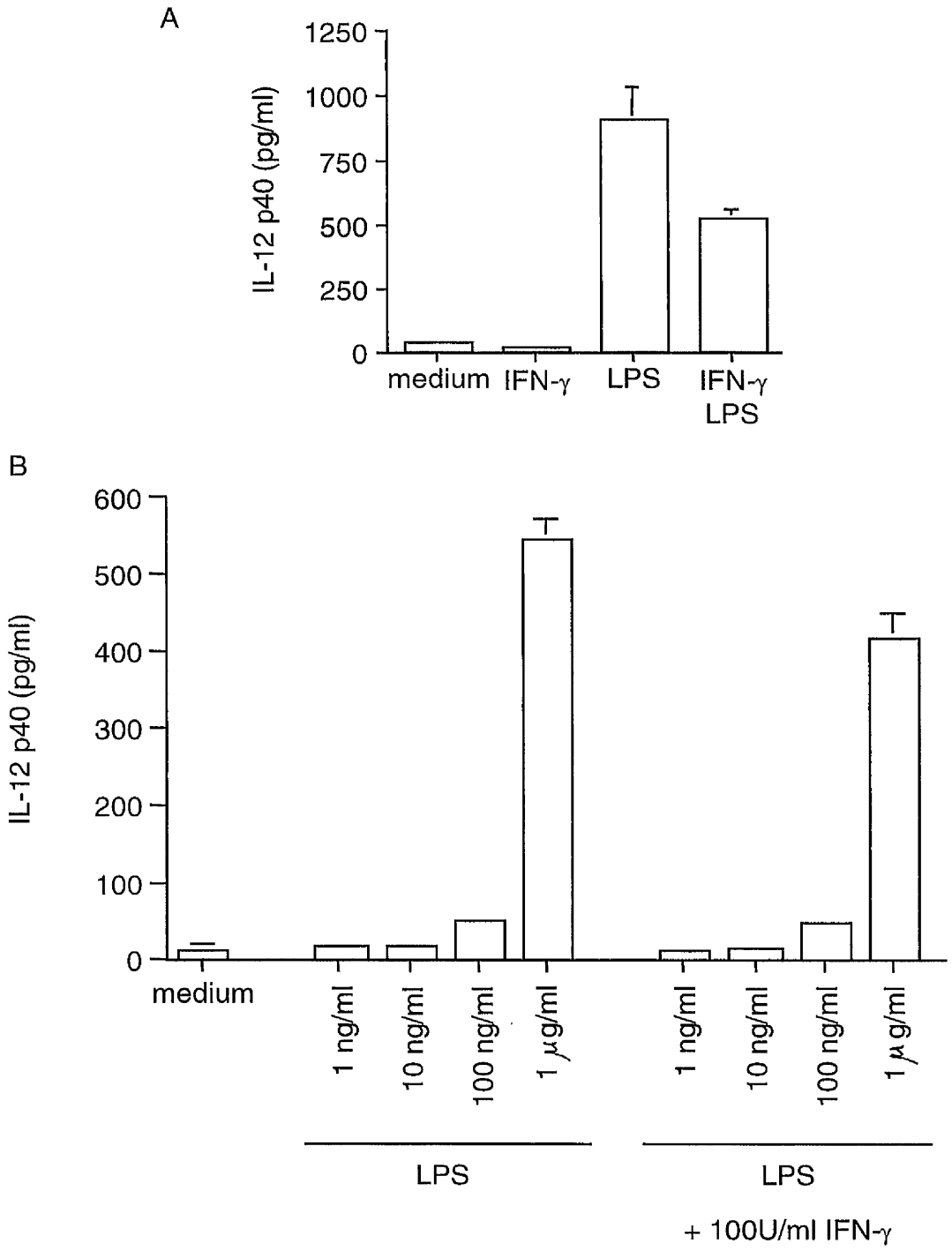


Figure 3.2. IL-12 p40 production by the J774 murine macrophage cell line.

J774 cells ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or 1 μg/ml LPS (A), or treated with various concentrations of LPS in the absence/presence of 100 U/ml IFN-γ (B).

IL-12 p40 (monomer, active p70 heterodimer, p40/p40 homodimer) in culture supernatants was measured by ELISA.

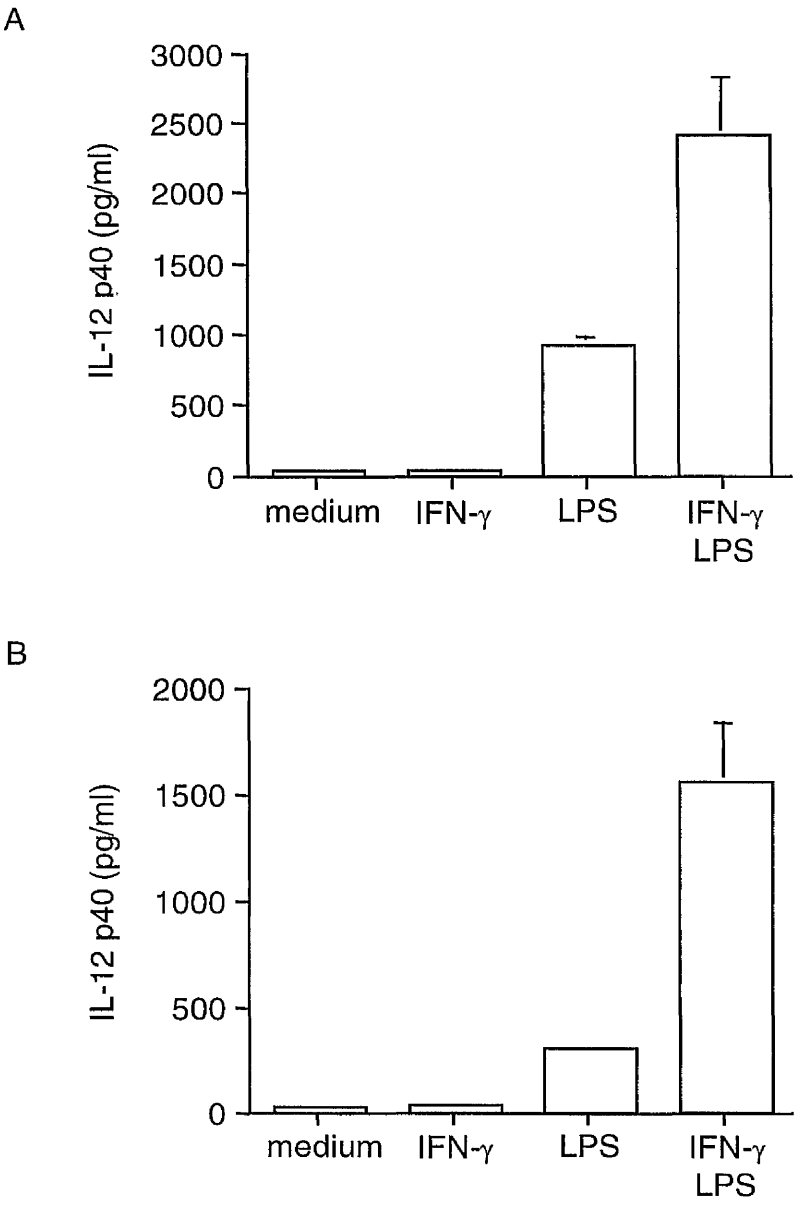


Figure 3.3. IL-12 p40 production by murine peritoneal macrophages.

Thioglycollate-elicited peritoneal macrophages from BALB/c (A) and 129 (B) mice ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS for 24 hours. IL-12 p40 (monomer, active p70 heterodimer, p40/p40 homodimer) in culture supernatants was measured by ELISA.

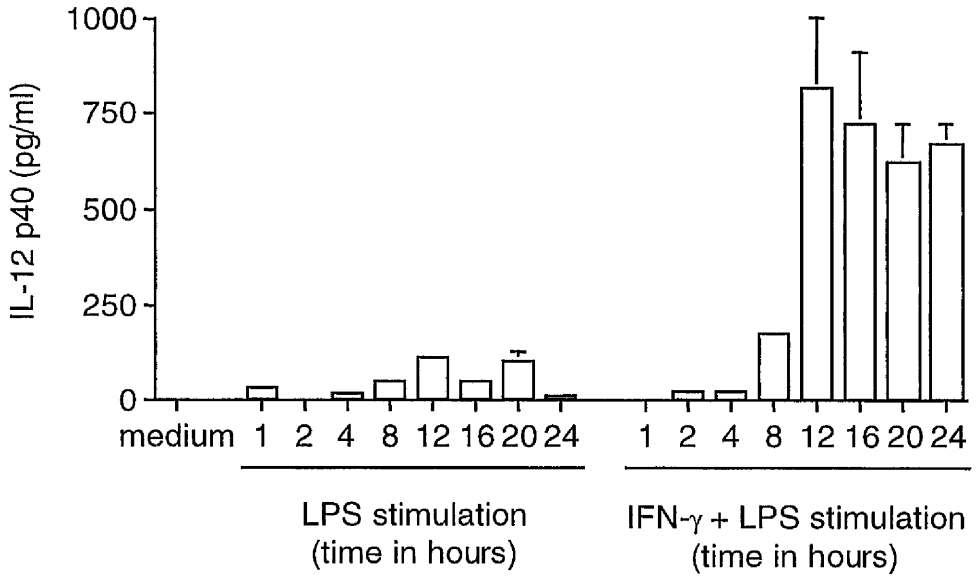


Figure 3.4. Timecourse of IL-12 p40 production by peritoneal macrophages.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($2x10^5$/well in a 96-well plate) were cultured for the times indicated with 100 ng/ml LPS in the absence/presence of 100 U/ml IFN-γ. IL-12 p40 in culture supernatants was measured by ELISA.

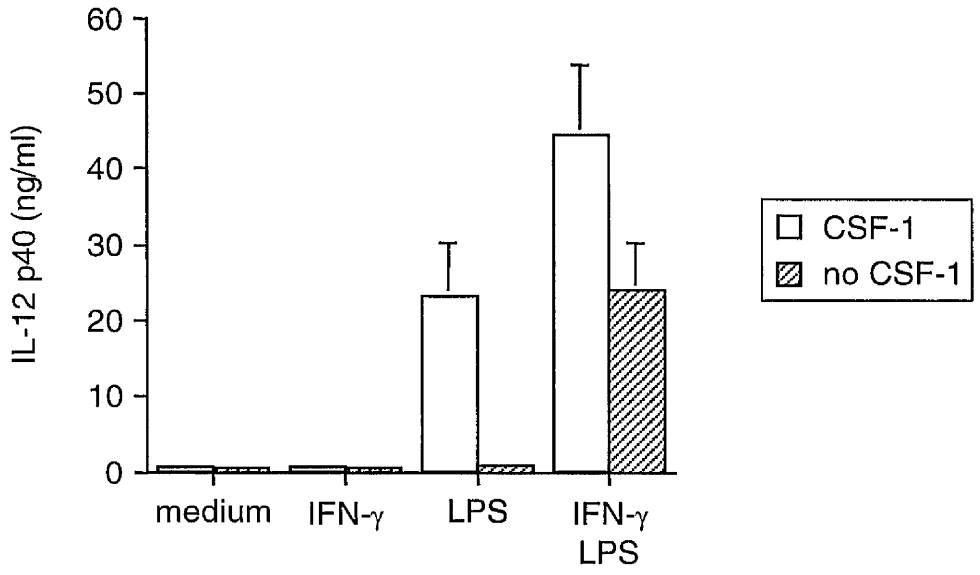


Figure 3.5. IL-12 p40 production by bone marrow-derived macrophages.

Bone marrow-derived macrophages were obtained from bone marrow cells by culture in medium containing CSF-1 for 6 days. For stimulation in the absence of CSF-1, medium was removed and replaced with medium lacking CSF-1 two hours prior to stimulation. Macrophages ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS in the presence/absence of CSF-1; IL-12 p40 in 24 hour culture supernatants was measured by ELISA.

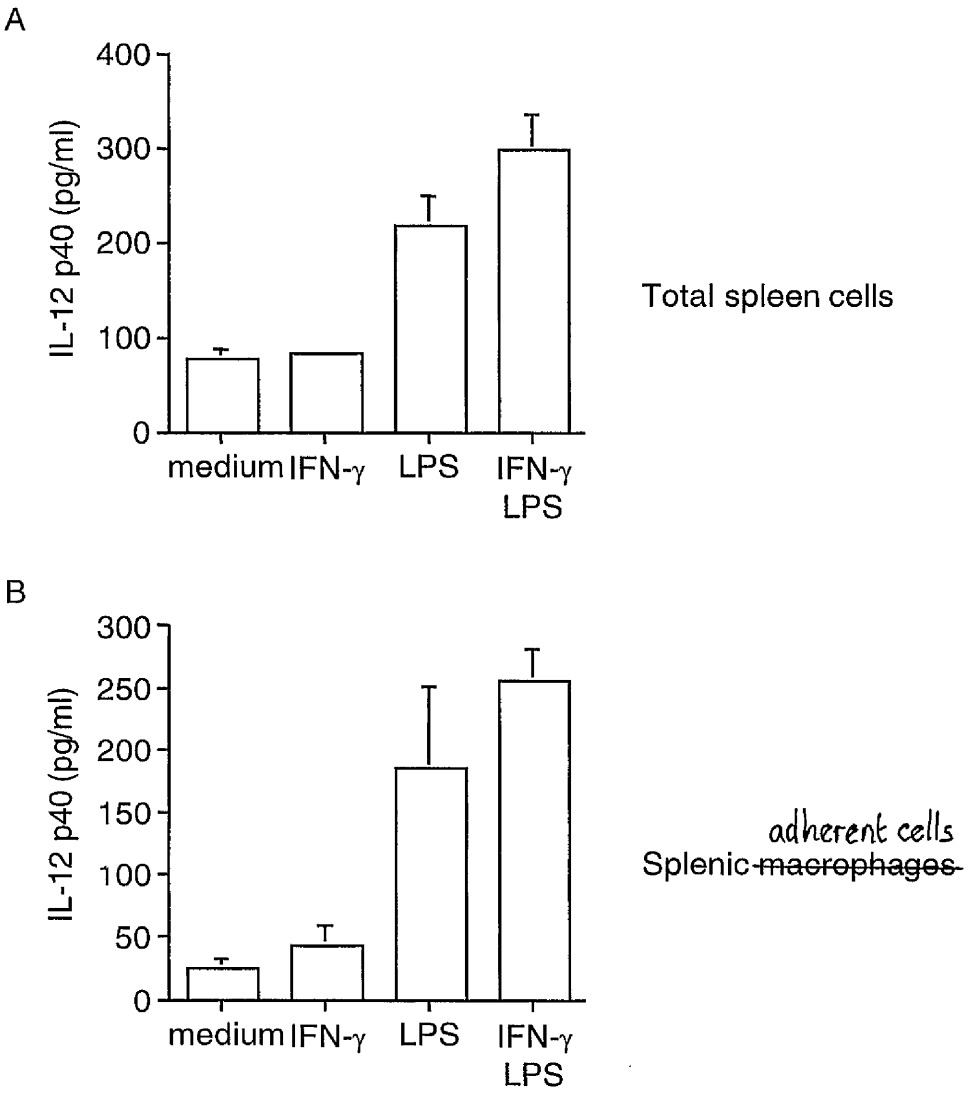


Figure 3.6. IL-12 p40 production by splenic cells.

Spleens were removed from BALB/c mice and cells were prepared as indicated below and stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS.

A. Total spleen cells were plated ($2x10^5$/well in a 96-well plate) and stimulated.

B. In the same experiment, total spleen cells were plated ($2x10^5$/well in a 96-well plate) and splenic macrophages were allowed to adhere for 2 hours. Wells were washed to remove non-adherent cells and medium was replaced prior to stimulation.

IL-12 p40 in 24 hour culture supernatants was measured by ELISA.

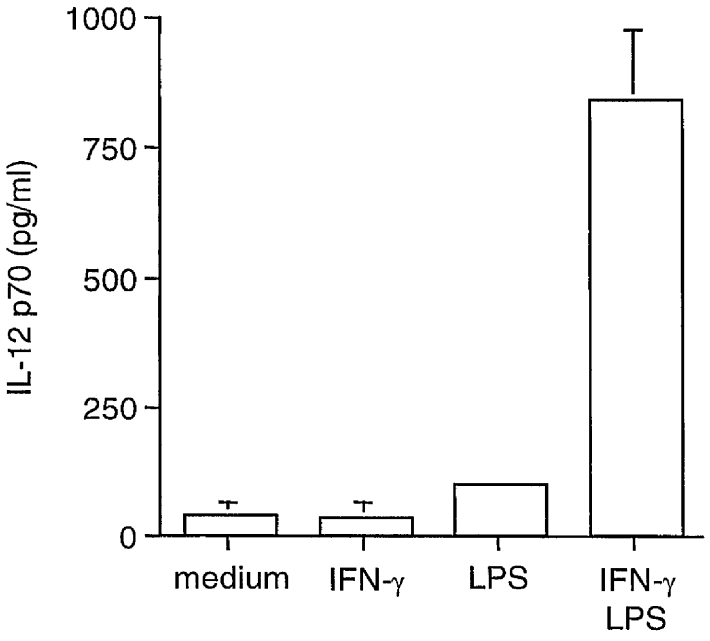


Figure 3.7. IL-12 p70 production by murine peritoneal macrophages.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS for 24 hours. Bioactive IL-12 p70 heterodimer in culture supernatants was measured by ELISA.

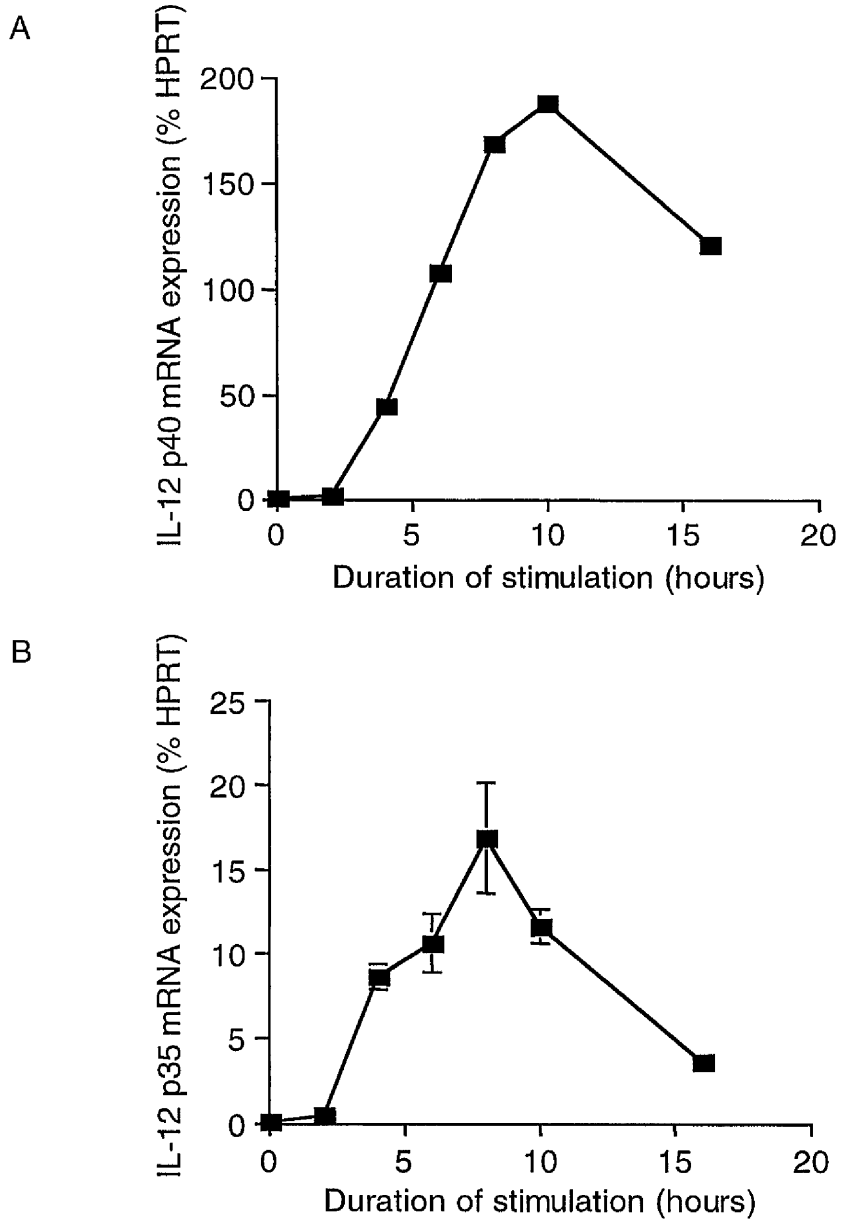


Figure 3.8. Induction of IL-12 p40 and p35 mRNA.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($4x10^6$/25 cm^2 flask) were cultured for the times indicated with 100 U/ml IFN-γ and 100 ng/ml LPS. RNA was purified and IL-12 p40 and p35 message levels were detected by TaqMan real-time PCR. IL-12 p40 and p35 transcript levels are expressed relative to HPRT mRNA.

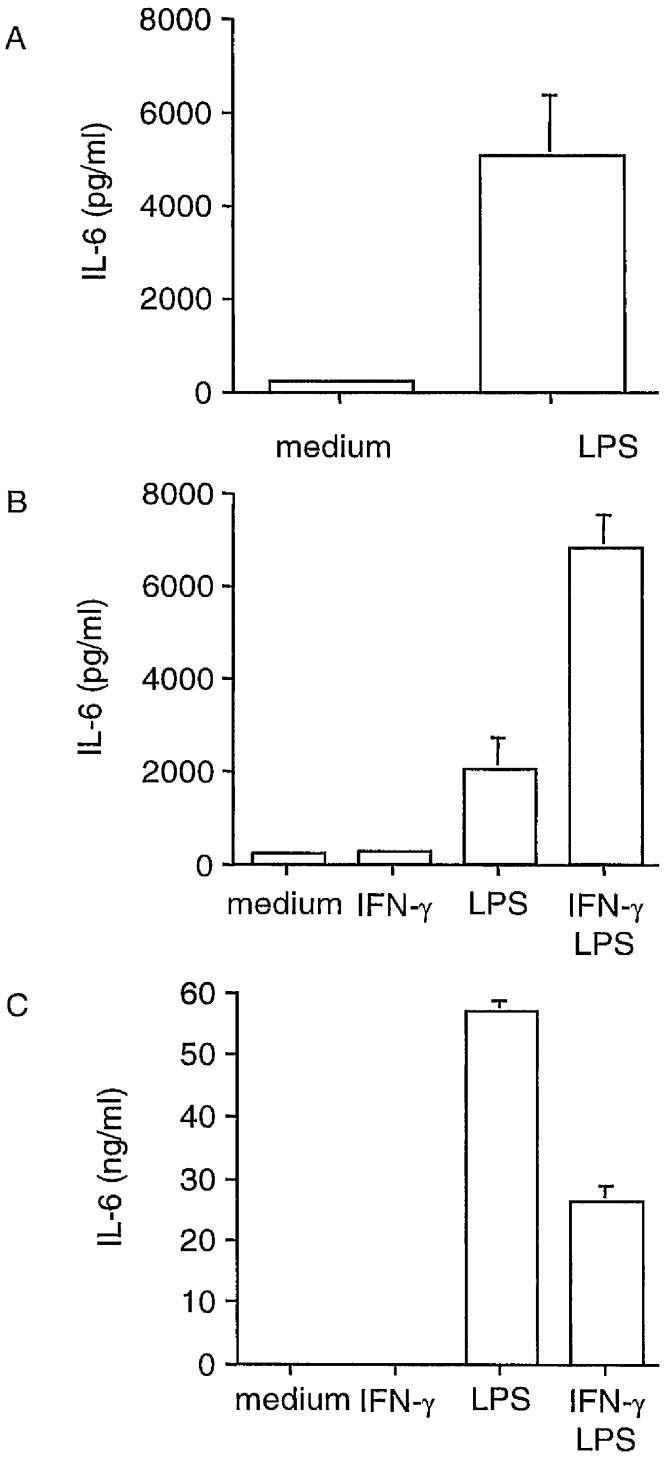


Figure 3.9. IL-6 production by J774 cells, murine peritoneal macrophages and bone marrow-derived macrophages.

J774 cells (A), peritoneal macrophages from BALB/c mice (B) and bone marrow-derived macrophages (obtained by culture of bone marrow cells with CSF-1 for 6 days; C) from BALB/c mice ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or LPS (J774 cells, 1 μg/ml; murine macrophages, 100 ng/ml) for 24 hours; IL-6 in culture supernatants was measured by ELISA.

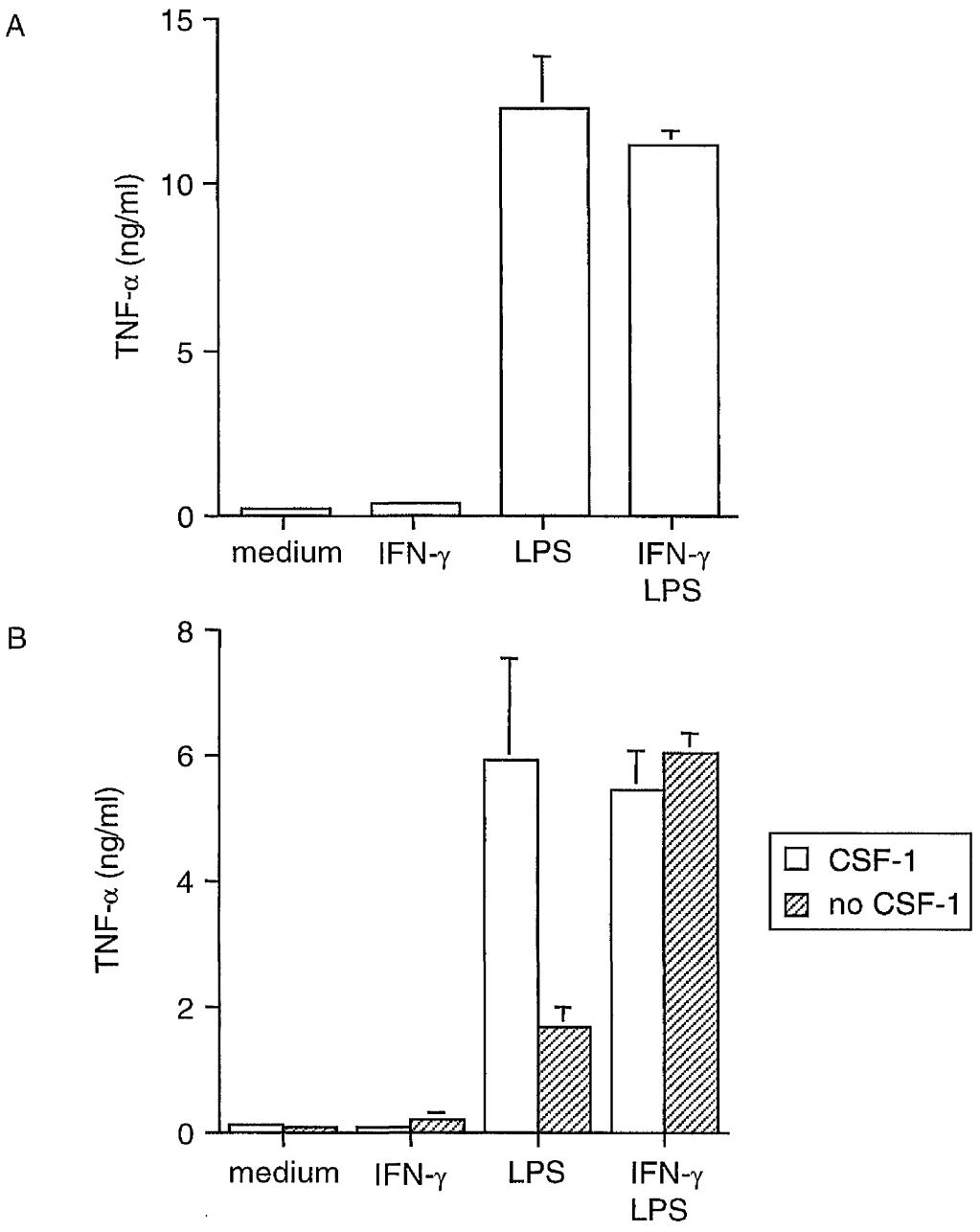


Figure 3.10. TNF-α production by peritoneal macrophages and bone marrow-derived macrophages.

Thioglycollate-elicited peritoneal macrophages (A) and bone marrow-derived macrophages (obtained by culture of bone marrow cells with CSF-1 for 6 days; B) from BALB/c mice ($2x10^5$/well in a 96-well plate) were cultured with 100 U/ml IFN-γ and/or 100 ng/ml LPS. For stimulation of bone marrow-derived macrophages in the absence of CSF-1, medium was removed and replaced with fresh medium lacking CSF-1 two hours prior to stimulation. TNF-α in 24 hour culture supernatants was measured by ELISA.

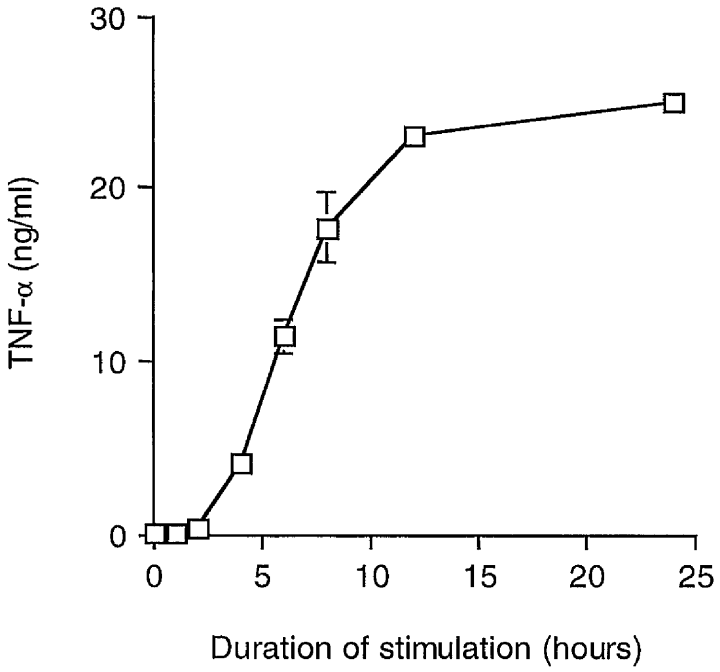


Figure 3.11. Timecourse of TNF-α production by peritoneal macrophages.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($2x10^5$/well in a 96-well plate) were cultured for the times indicated with 100 U/ml IFN-γ and 100 ng/ml LPS. TNF-α in culture supernatants was measured by ELISA.

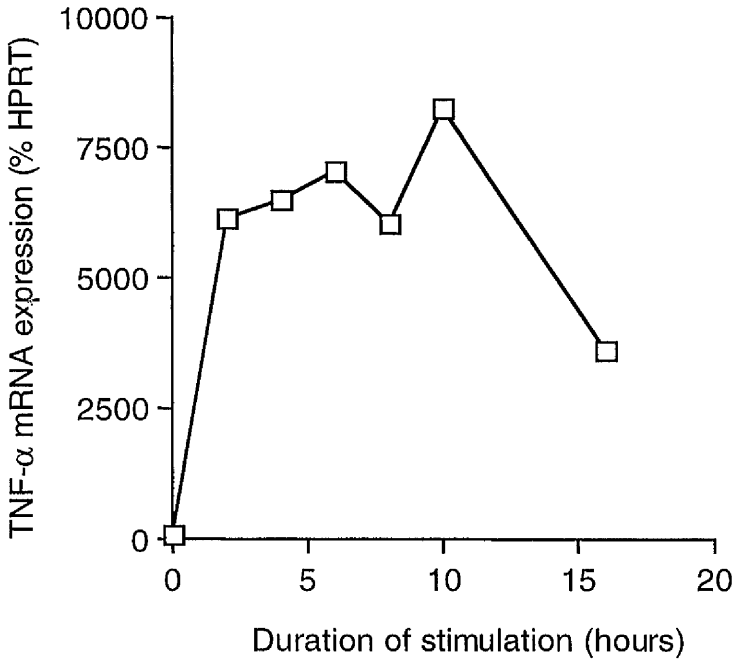


Figure 3.12. Induction of TNF-α mRNA.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($4x10^6$/25 cm^2 flask) were cultured for the times indicated with 100U/ml IFN-γ and 100 ng/ml LPS. RNA was purified and TNF-α message was detected by TaqMan real-time PCR. Transcript levels are expressed relative to HPRT mRNA.

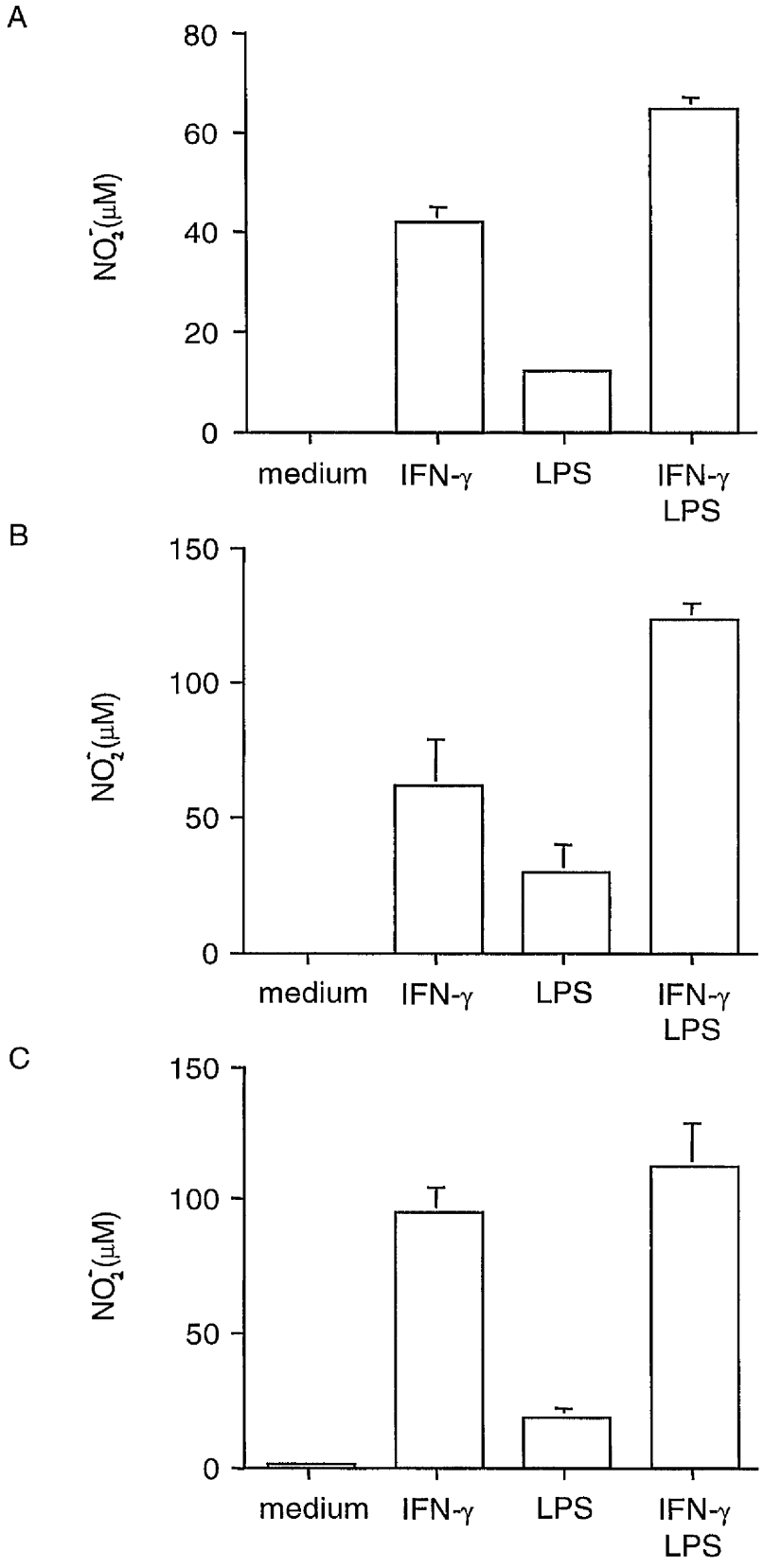


Figure 3.13. NO production by J774 cells and murine peritoneal macrophages.

J774 cells (A) and peritoneal macrophages from BALB/c (B) and 129 (C) mice ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or LPS (J774 cells, 1 μg/ml; murine macrophages, 100 ng/ml) for 24 hours. NO release was assayed by Griess Reaction.

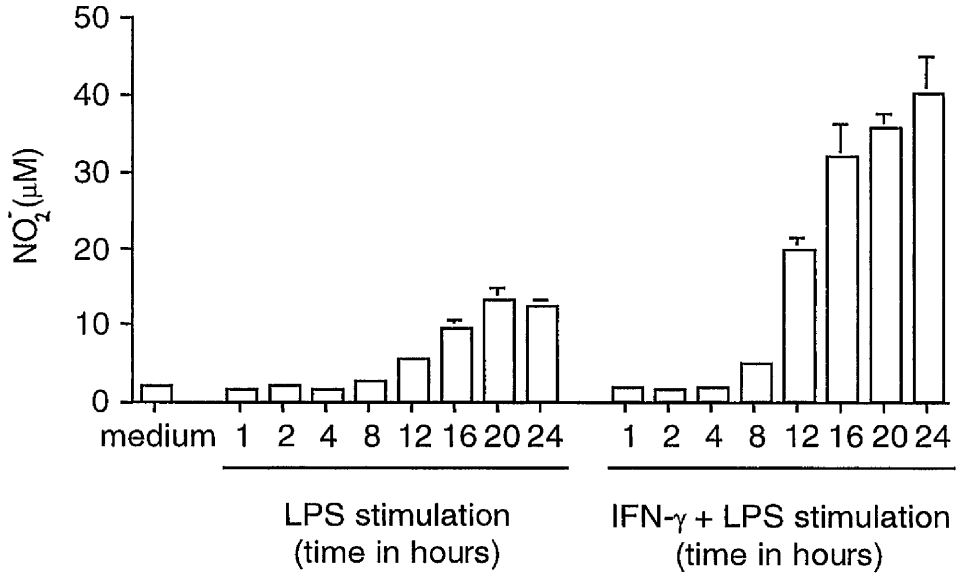


Figure 3.14. Timecourse of NO production by peritoneal macrophages.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($2x10^5$/well in a 96-well plate) were cultured for the times indicated with 100 ng/ml LPS in the absence/presence of 100 U/ml IFN-γ. NO release was assayed by Griess Reaction.

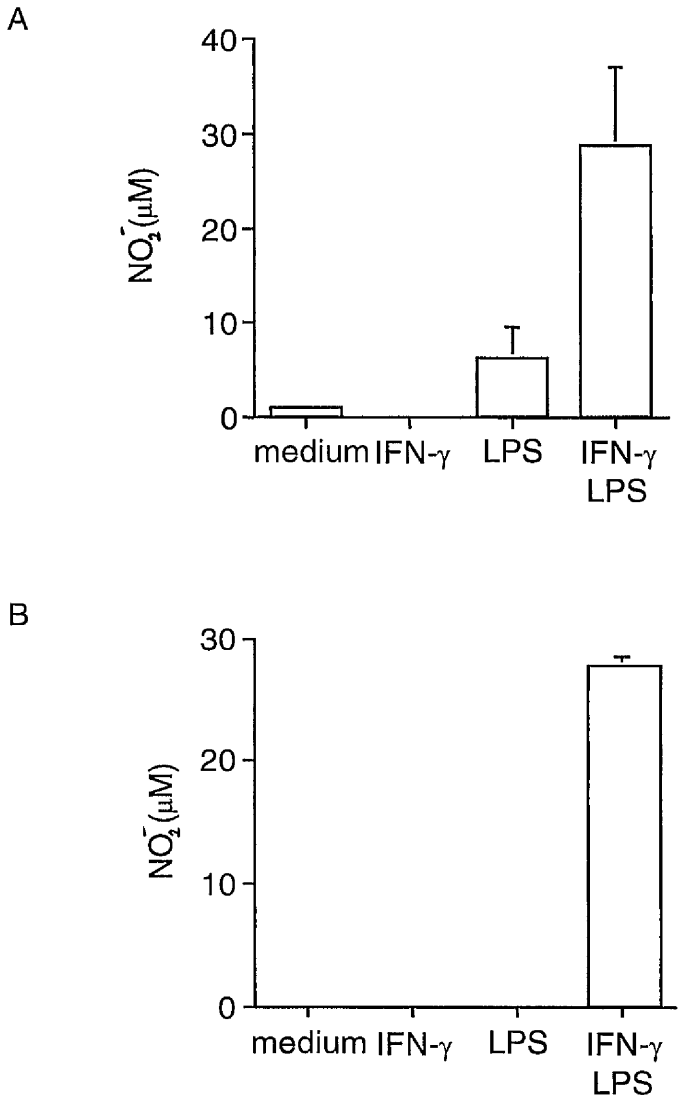


Figure 3.15. NO production by splenic cells and bone marrow-derived macrophages.

Total spleen cells (A) and bone marrow-derived macrophages (B) from BALB/c mice ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS for 24 hours. NO release was assayed by Griess Reaction.

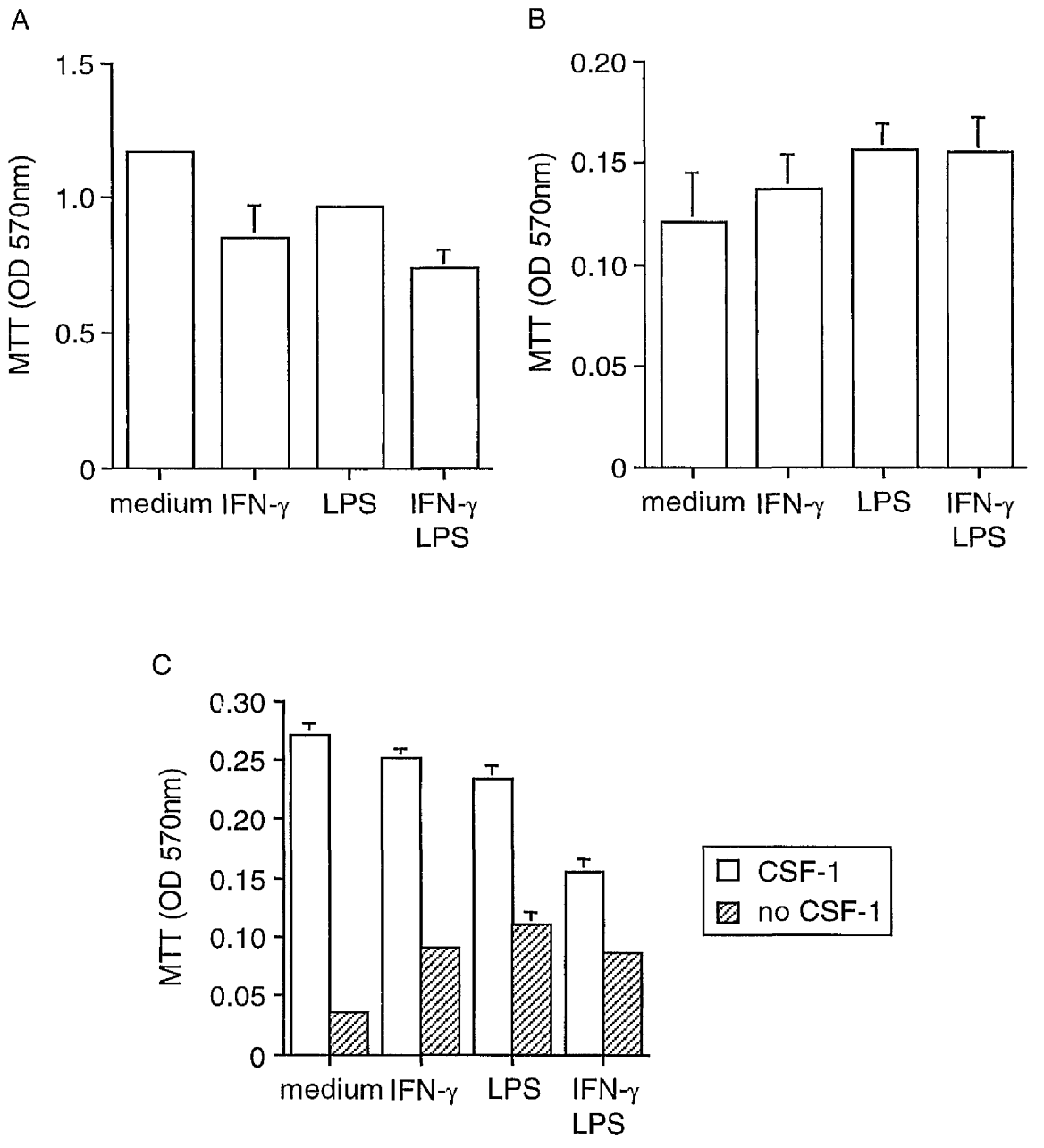


Figure 3.16. Viability of macrophages following stimulation with IFN-γ and/or LPS.

J774 cells (A), and peritoneal macrophages (B) and bone marrow-derived macrophages (in the presence/absence of CSF-1; C) from BALB/c mice ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or LPS (J774 cells, 1 μg/ml; murine macrophages, 100 ng/ml) for 24 hours. Cell viability following stimulation was assessed by MTT assay.

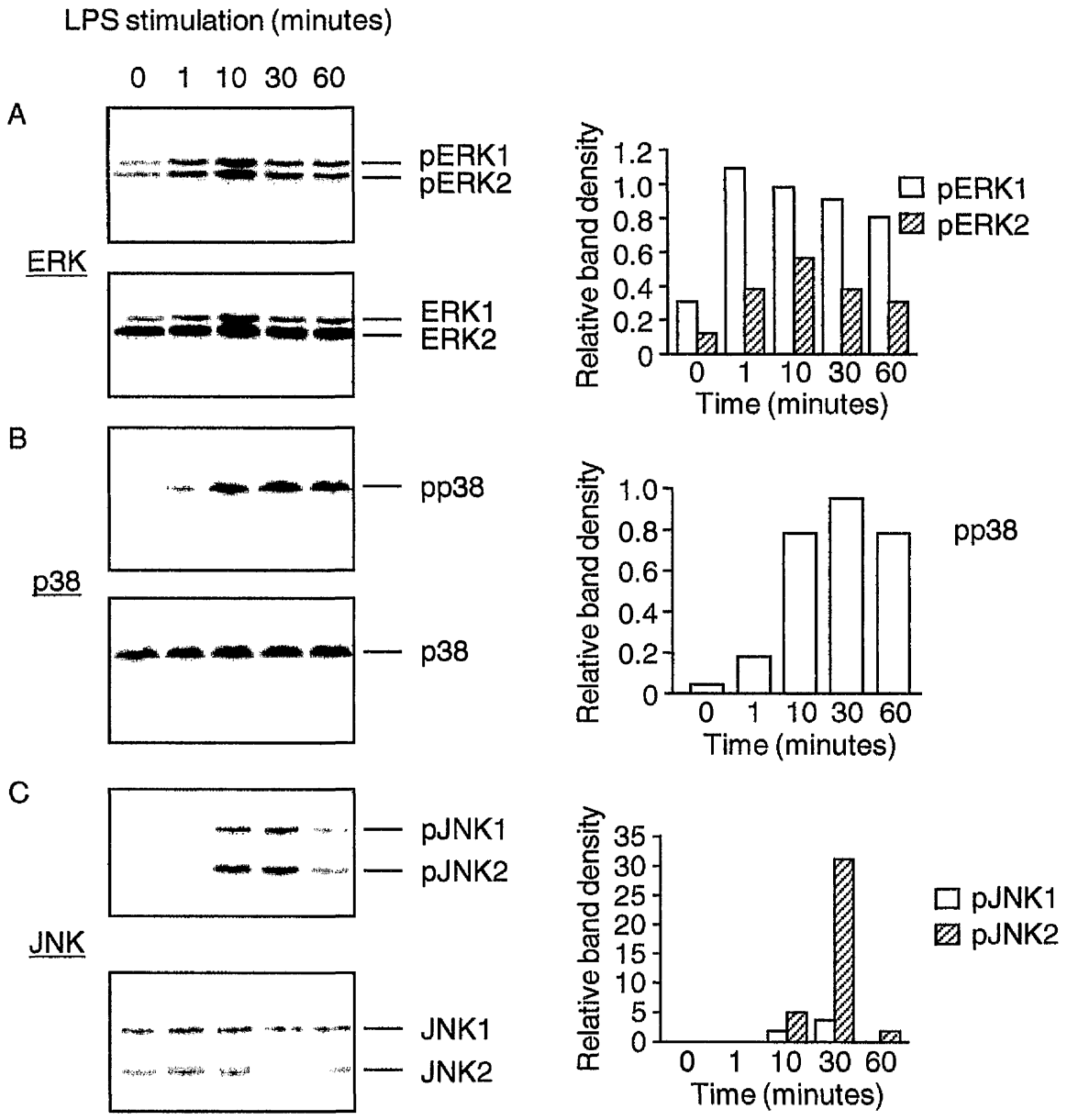


Figure 3.17. Activation of MAP kinases in J774 cells.

J774 cells ($4x10^6$/25 cm^2 flask) were stimulated with 1 μg/ml LPS for the times indicated. Total protein lysates were prepared and equal amounts of protein were separated by SDS PAGE. Gels were blotted on to nitrocellulose and probed with antibodies specific for the active (dual-phosphorylated) isoforms of ERK (pERK1/2; A), p38 (pp38; B) and JNK (pJNK1/2; C) MAP kinases (upper panel of each pair). Blots were stripped and reprobed with antibodies that detect total amounts of each MAP kinase as a protein loading control (lower panel of each pair). Densitometric values show phospho-MAP kinase:MAP kinase protein ratios.

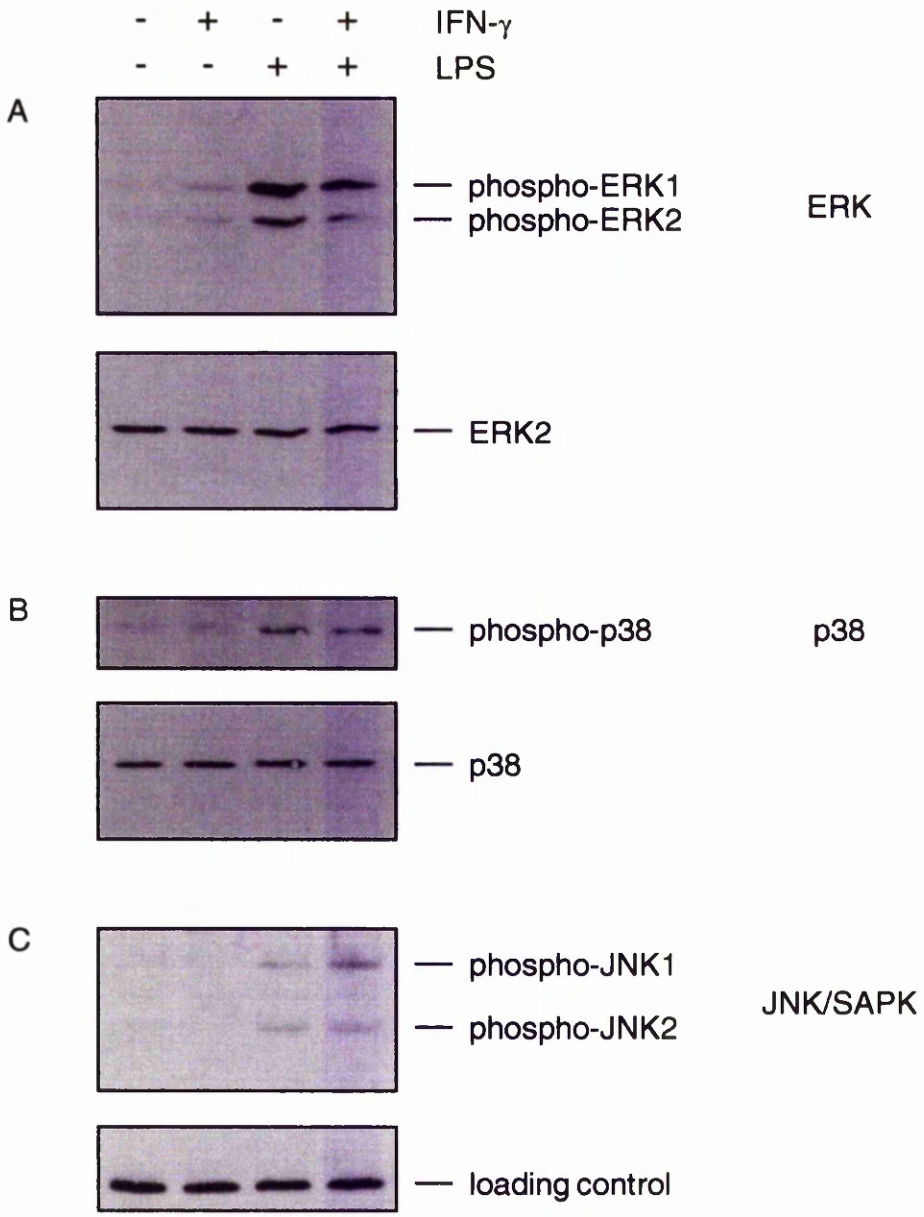


Figure 3.18. Activation of MAP kinases in peritoneal macrophages.

Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS for 10 minutes. Total protein lysates were prepared and equal amounts of protein were separated by SDS PAGE. Gels were blotted on to nitrocellulose and probed with antibodies specific for the active (dual-phosphorylated) isoforms of ERK (A), p38 (B) and JNK (C) MAP kinases (upper panel of each pair). Blots were stripped and reprobed with antibodies that detect total amounts of each MAP kinase as a protein loading control (lower panel of each pair).

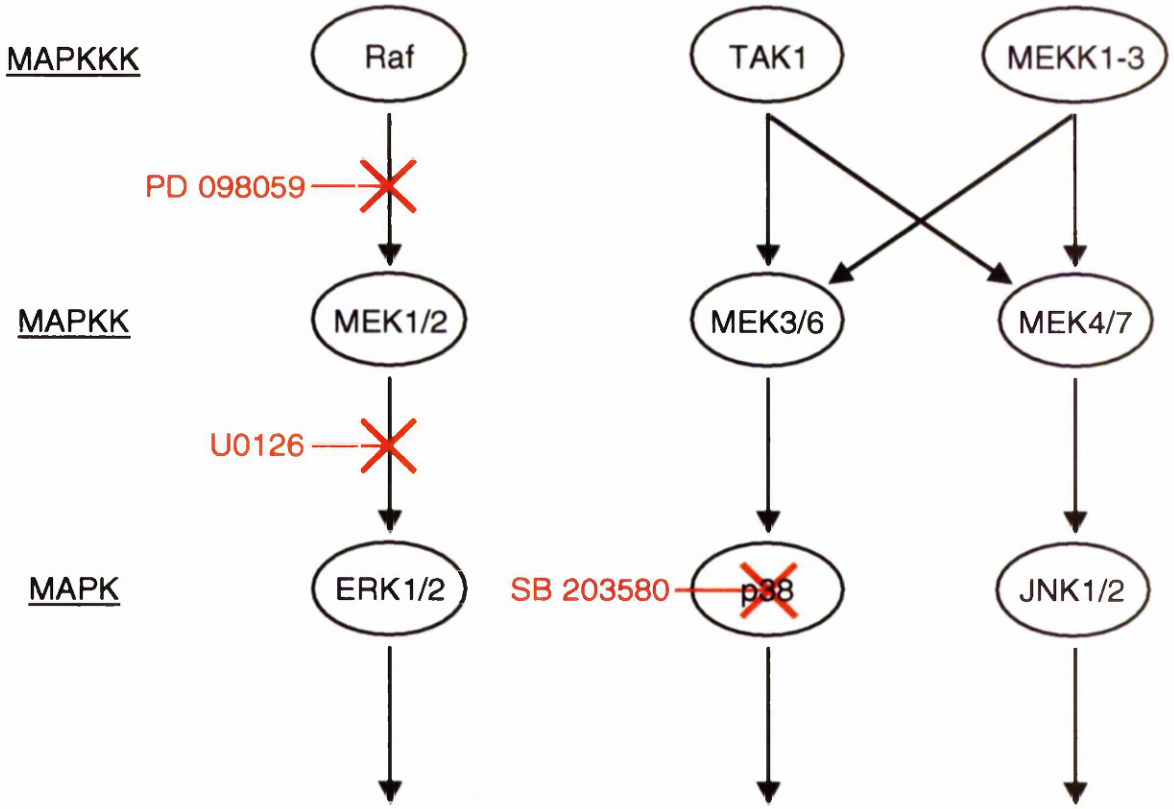


Figure 3.19. Inhibitors of MAP kinase cascades.

MAP kinases (MAPKs) are activated by dual phosphorylation on a tyrosine and a threonine residue by MAP kinase kinases (MAPKKs or MEKs). These are activated by phosphorylation by MAP kinase kinase kinases (MAPKKKs) e.g. Raf activates MEK1/2 which activate ERK1/2. The targets of specific inhibitors of MAP kinase pathways are also indicated. ERK activation can be prevented by treatment with PD 098059, which targets the activation of MEK1/2, or U0126, which inhibits MEK1/2 activity. p38 activity is inhibited by treatment with SB 203580.

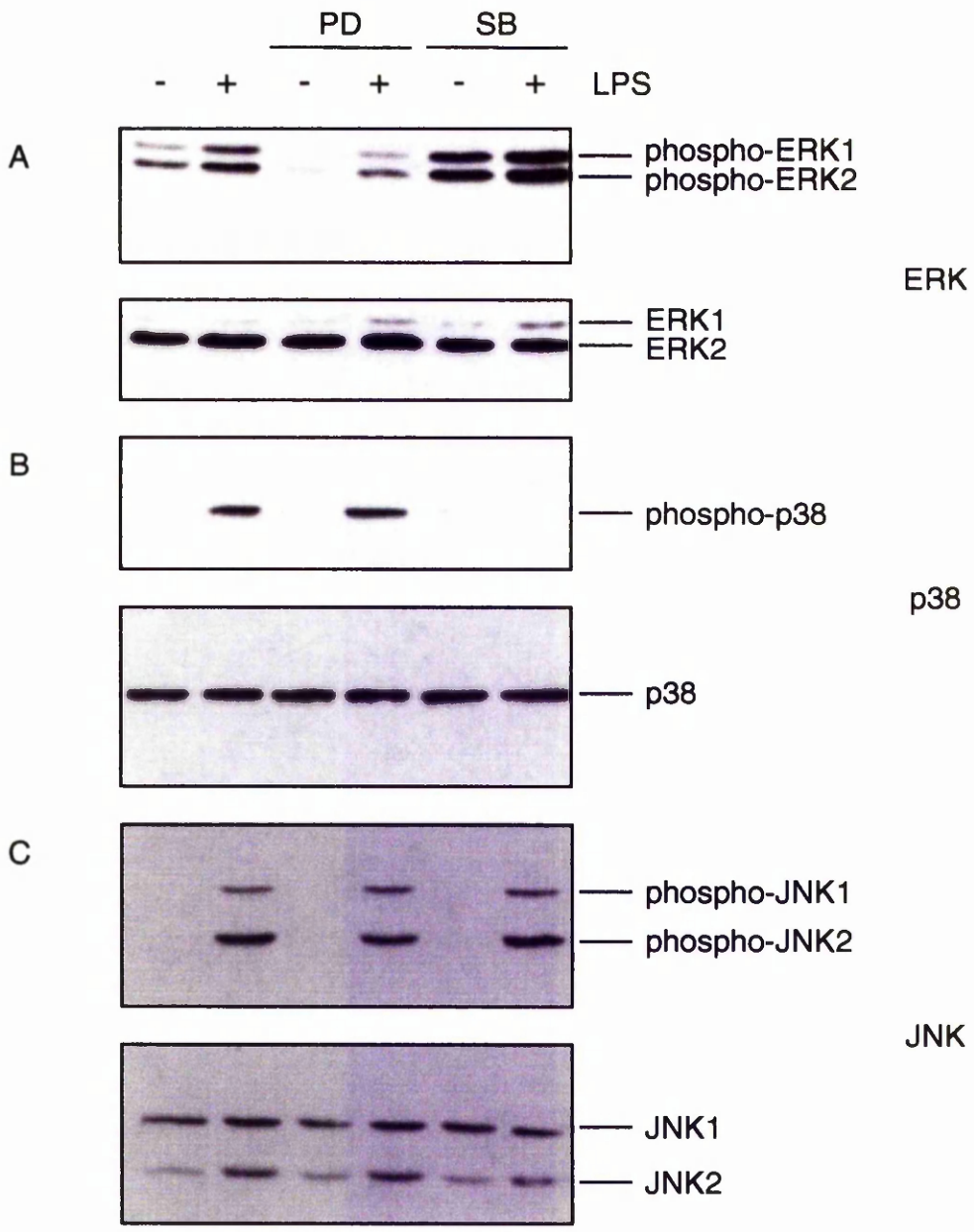


Figure 3.20. Effects of the MEK and p38 MAP kinase inhibitors PD 098059 and SB 203580 on ERK, p38 and JNK MAP kinase activation in J774 cells.

J774 cells ($4x10^6$/25 cm^2 flask) were pre-treated with either the inhibitor of the ERK MAP kinase pathway, PD 098059 (20μM; PD), or the p38 inhibitor, SB 203580 (5μM; SB) for 1 hour prior to stimulation with 1 μg/ml LPS for 10 minutes. Total protein lysates were purified and assayed by Western Blotting using anti-phospho-ERK (A), -p38 (B) and -JNK (C) antibodies (upper panels). The blots were then stripped and reprobed with anti-ERK (A), -p38 (B) and -JNK (C) antibodies as a loading control (lower panels).

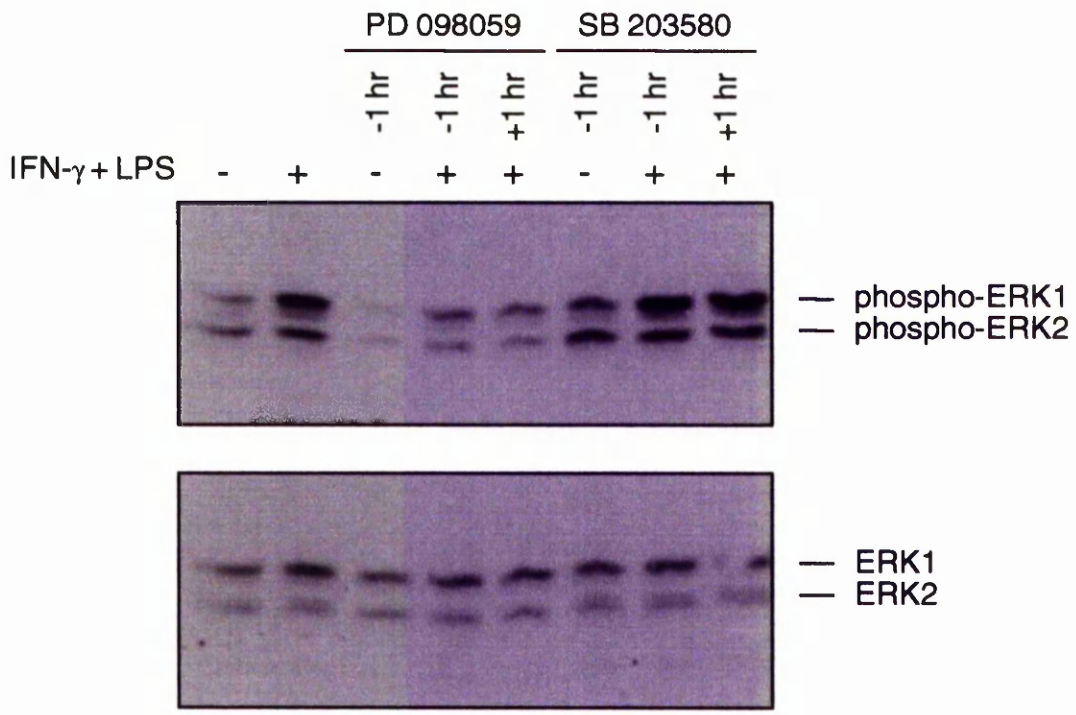


Figure 3.21. Effects of the MEK and p38 MAP kinase inhibitors PD 098059 and SB 203580 on ERK MAP kinase activation in peritoneal macrophages.

Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were stimulated for 4 hours with 100 U/ml IFN-γ and 100 ng/ml LPS, in the absence/presence of PD 098059 (PD; 20 μM) or SB 203580 (SB; 5 μM) which was added at the times indicated relative to IFN-γ + LPS stimulation. Total protein lysates were purified and assayed by Western Blotting using anti-phospho-ERK antibody (upper panel). The blot was then stripped and reprobed with anti-ERK antibody as a loading control (lower panel).

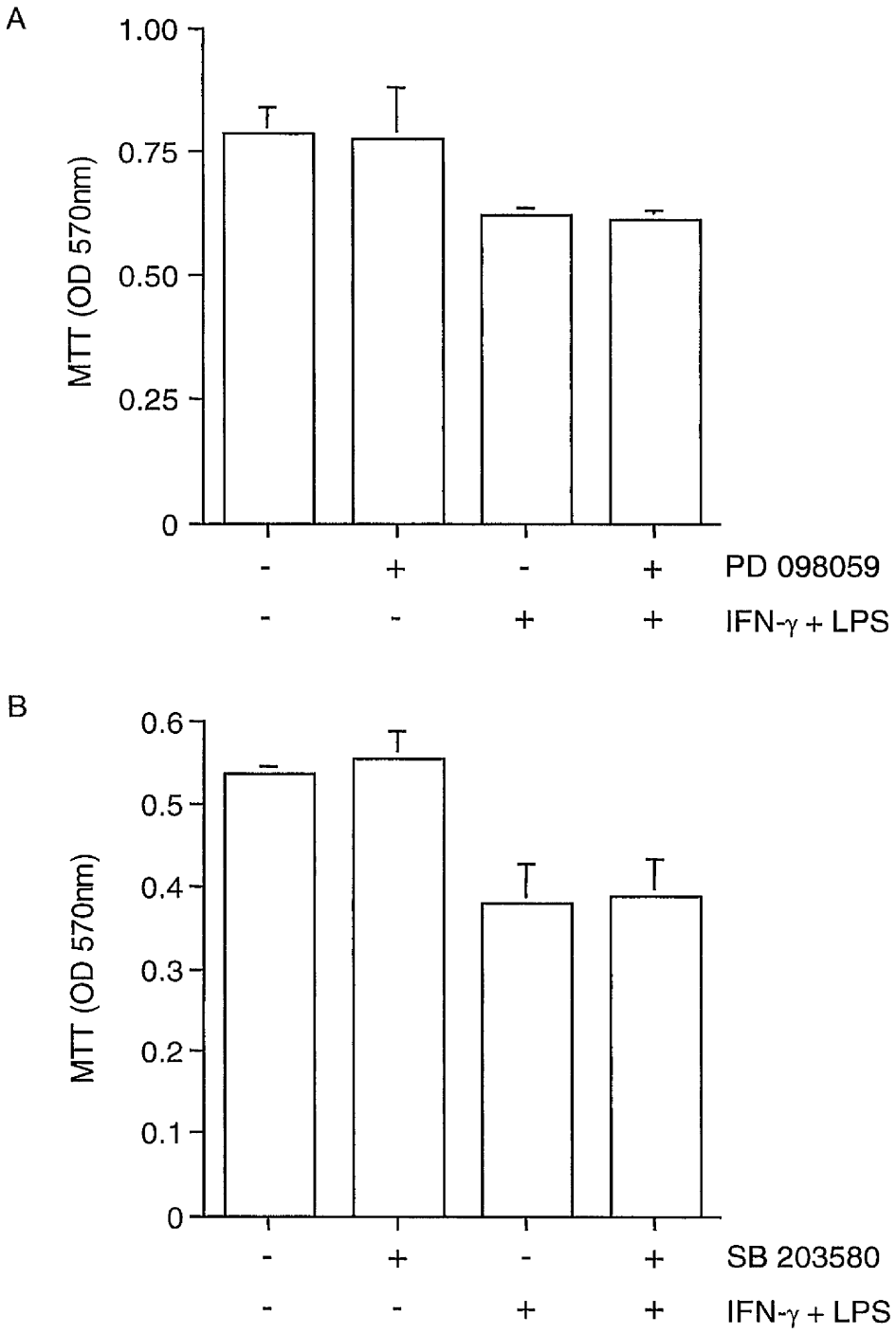


Figure 3.22. Effect of inhibition of ERK and p38 MAP kinases on cell viability of peritoneal macrophages.

The viability of peritoneal macrophages (originally plated at $2x10^5$/well in a 96-well plate) following pre-treatment with 20 μM PD 098059 or 5 μM SB 203580 for 1 hour prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS was assessed by MTT assay.

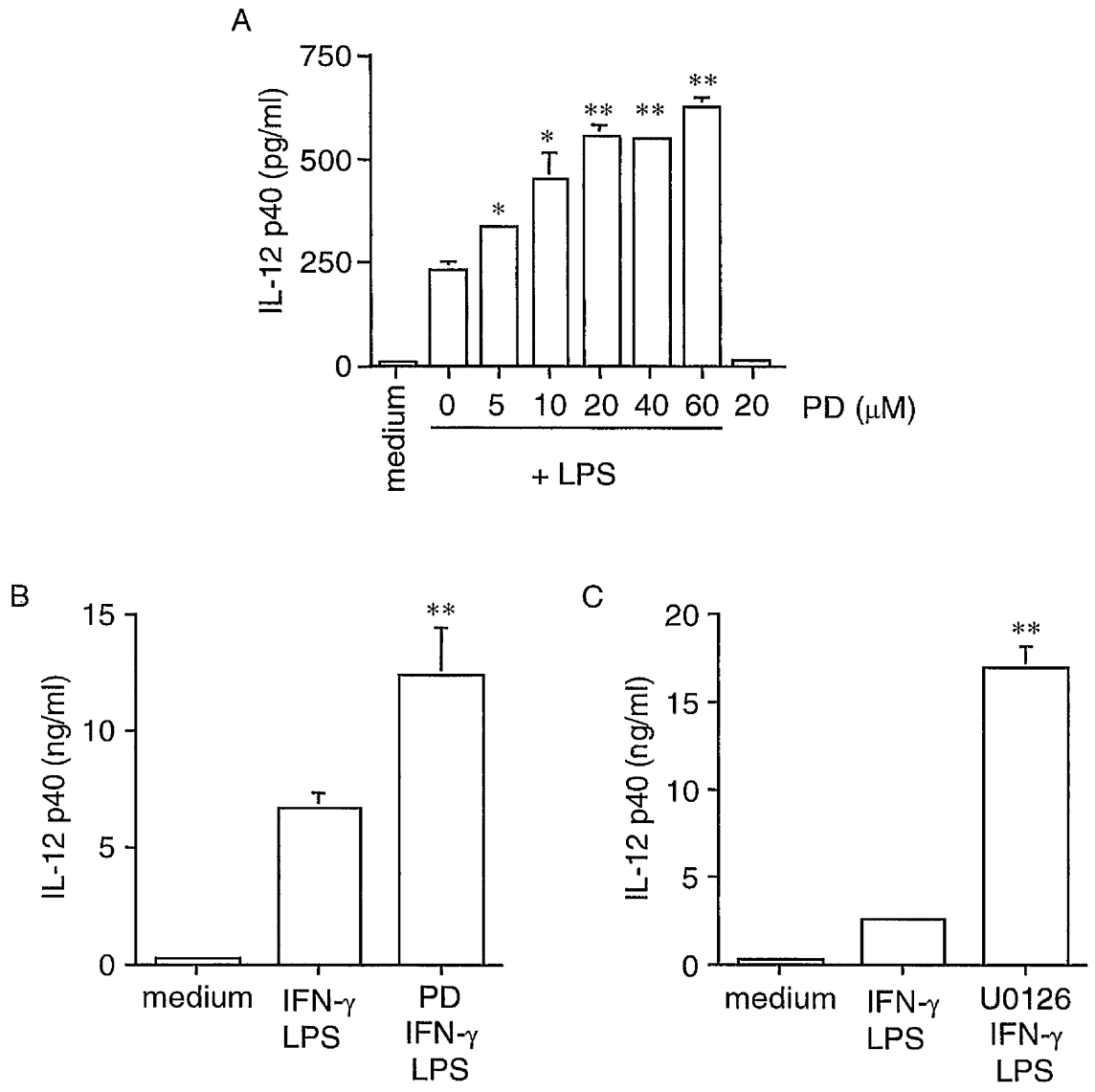


Figure 3.23. Effect of inhibition of ERK MAP kinases on IL-12 p40 production by macrophages.

J774 cells (A) or murine peritoneal macrophages (B and C) ($2x10^5$/well in a 96-well plate) were pre-treated for 1 hour with the inhibitors of the ERK MAP kinase pathway, PD 098059 (PD; 20 μM or as indicated) or U0126 (1 μM), prior to stimulation with 1 μg/ml LPS (A) or 100 U/ml IFN-γ and 100 ng/ml LPS (B and C). IL-12 p40 in 24 hour culture supernatants was measured by ELISA. * $p < 0.05$, ** $p < 0.01$

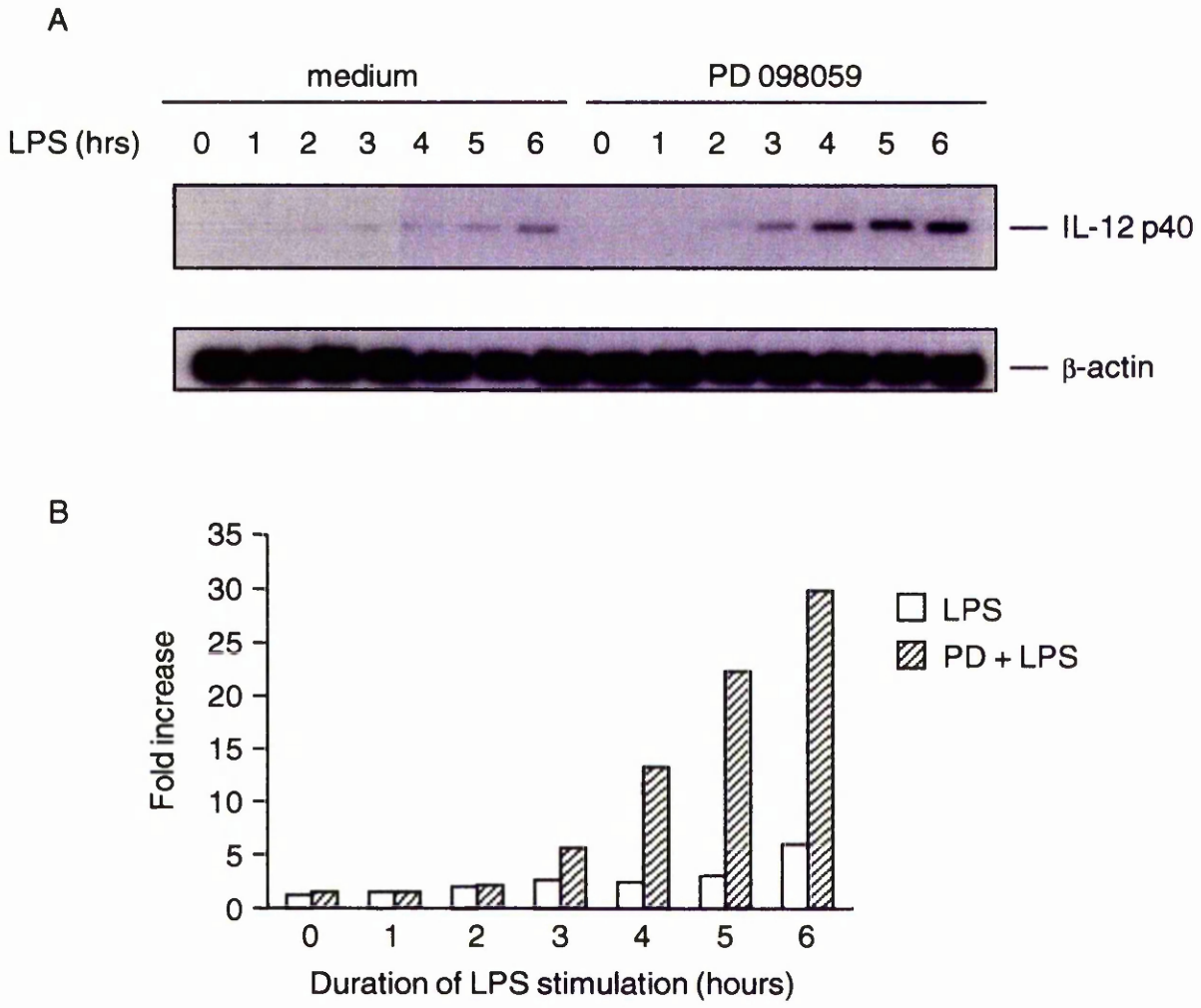


Figure 3.24. Effect of inhibition of ERK MAP kinase activation on IL-12 p40 mRNA induction.

J774 cells ($4x10^6$/25 cm^2 flask) were pre-treated with 20 μM PD 098059 (PD) for 1 hour prior to stimulation with 1 μg/ml LPS for the times indicated. Northern Blot analysis (A) was performed using purified total RNA hybridised with a cloned murine IL-12 p40 cDNA probe. After stripping, a cloned murine β-actin cDNA probe was used as a loading control. Densitometric values (B) are IL-12 p40:β-actin ratios derived from the data, expressed relative to basal levels.

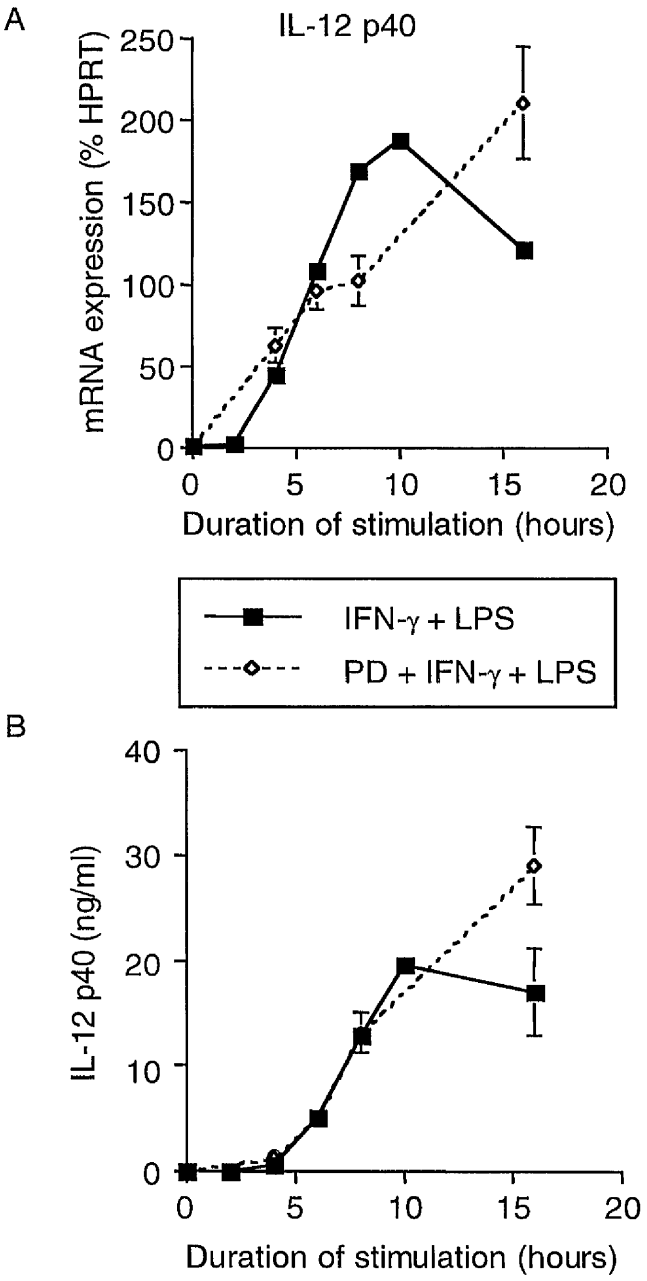


Figure 3.25. Modulation of IL-12 p40 mRNA and protein production by inhibition of ERK MAP kinase activation.

Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were pre-treated with 20 μM PD 098059 for 1 hour prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated. Total RNA was purified and TaqMan real-time PCR analysis was performed to measure IL-12 p40 transcript levels, which are expressed relative to HPRT mRNA (A). IL-12 p40 protein in culture supernatants was measured by ELISA (B).

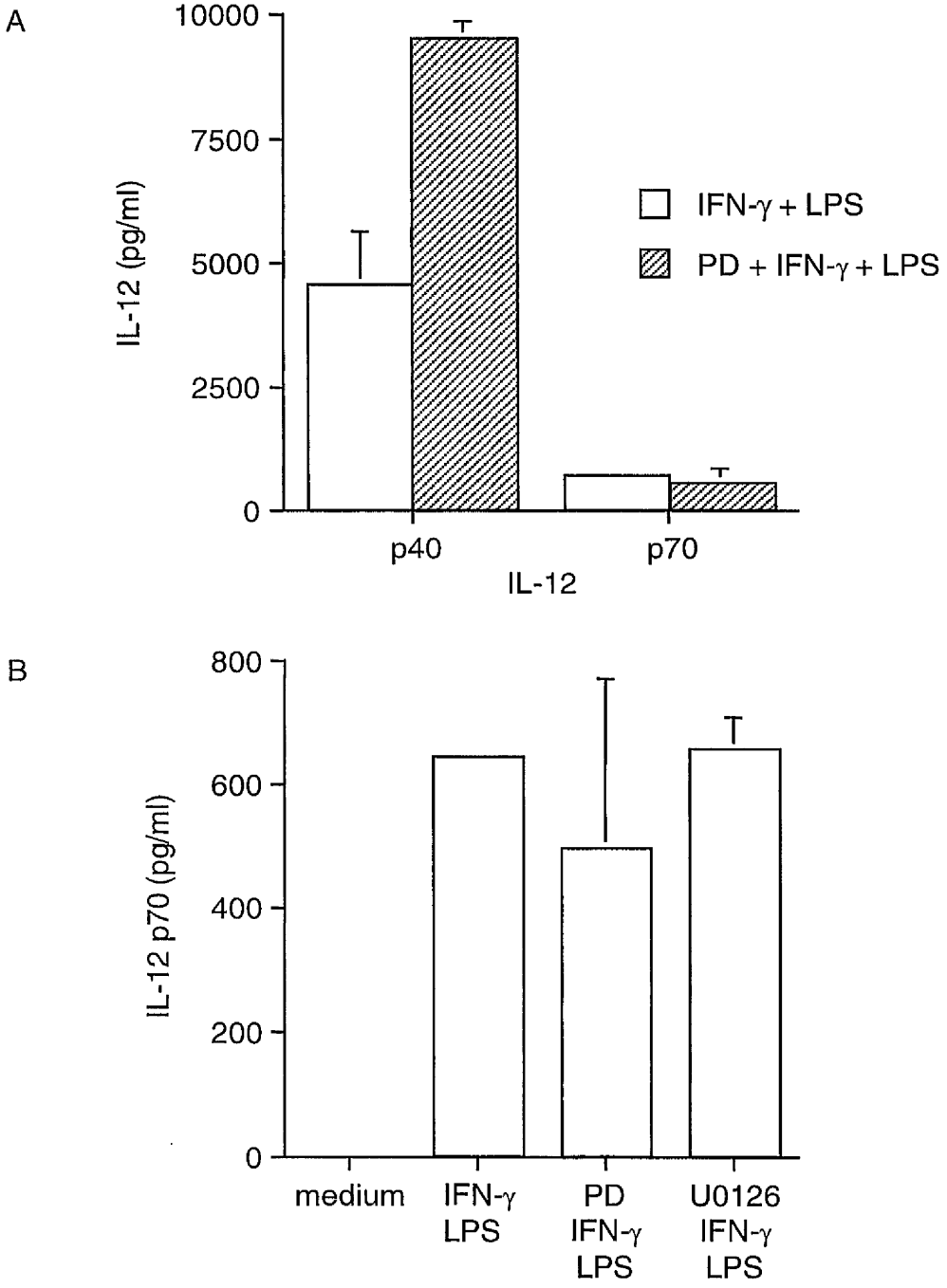


Figure 3.26. Effect of inhibition of ERK MAP kinases on IL-12 p70 production by peritoneal macrophages.

BALB/c peritoneal macrophages ($2x10^5$/well in a 96-well plate) were pre-treated for 1 hour with the inhibitors of the ERK MAP kinase pathway, PD 098059 (PD; 20 μM) or U0126 (1 μM), prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS. IL-12 p40 and bioactive p70 in 24 hour culture supernatants were measured by ELISA.

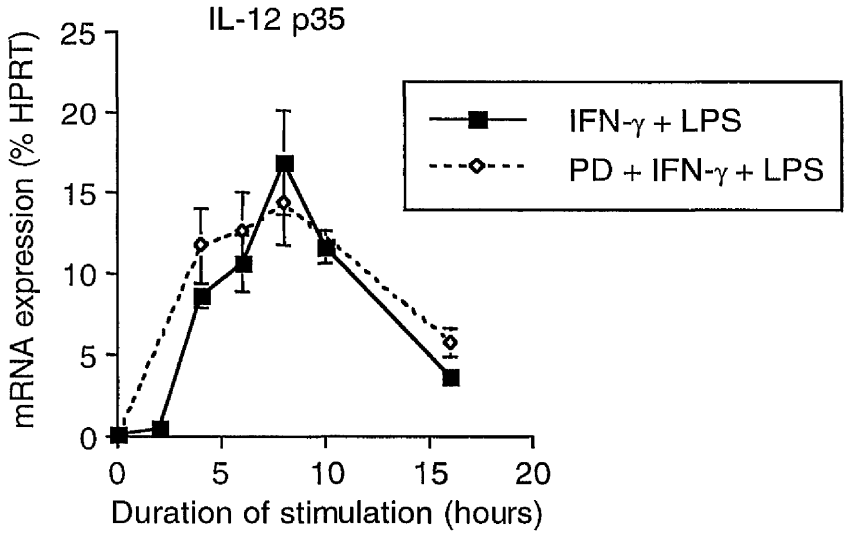


Figure 3.27. Effect of inhibition of ERK MAP kinase activation on IL-12 p35 mRNA induction.

Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were pre-treated with 20 μM PD 098059 for 1 hour prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated. Total RNA was purified and TaqMan real-time PCR analysis was performed to measure IL-12 p35 transcript levels, which are expressed relative to HPRT mRNA.

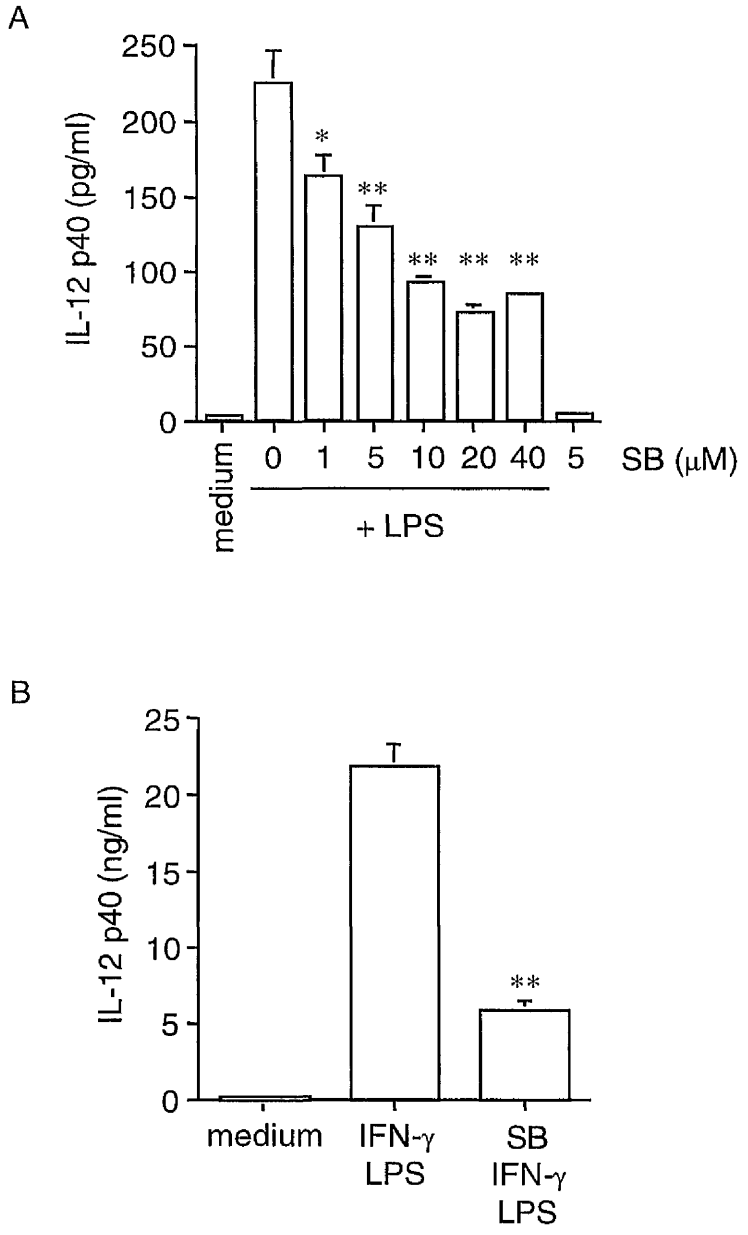


Figure 3.28. Effect of inhibition of p38 MAP kinase on IL-12 p40 production by macrophages.

J774 cells (A) or murine peritoneal macrophages (B) ($2x10^5$/well in a 96-well plate) were pre-treated for 1 hour with the inhibitor of the p38 MAP kinase pathway, SB 203580 (SB; 5 μM or as indicated), prior to stimulation with 1 μg/ml LPS (A) or 100 U/ml IFN-γ and 100 ng/ml LPS (B). IL-12 p40 in 24 hour culture supernatants was measured by ELISA. * $p < 0.05$, ** $p < 0.01$

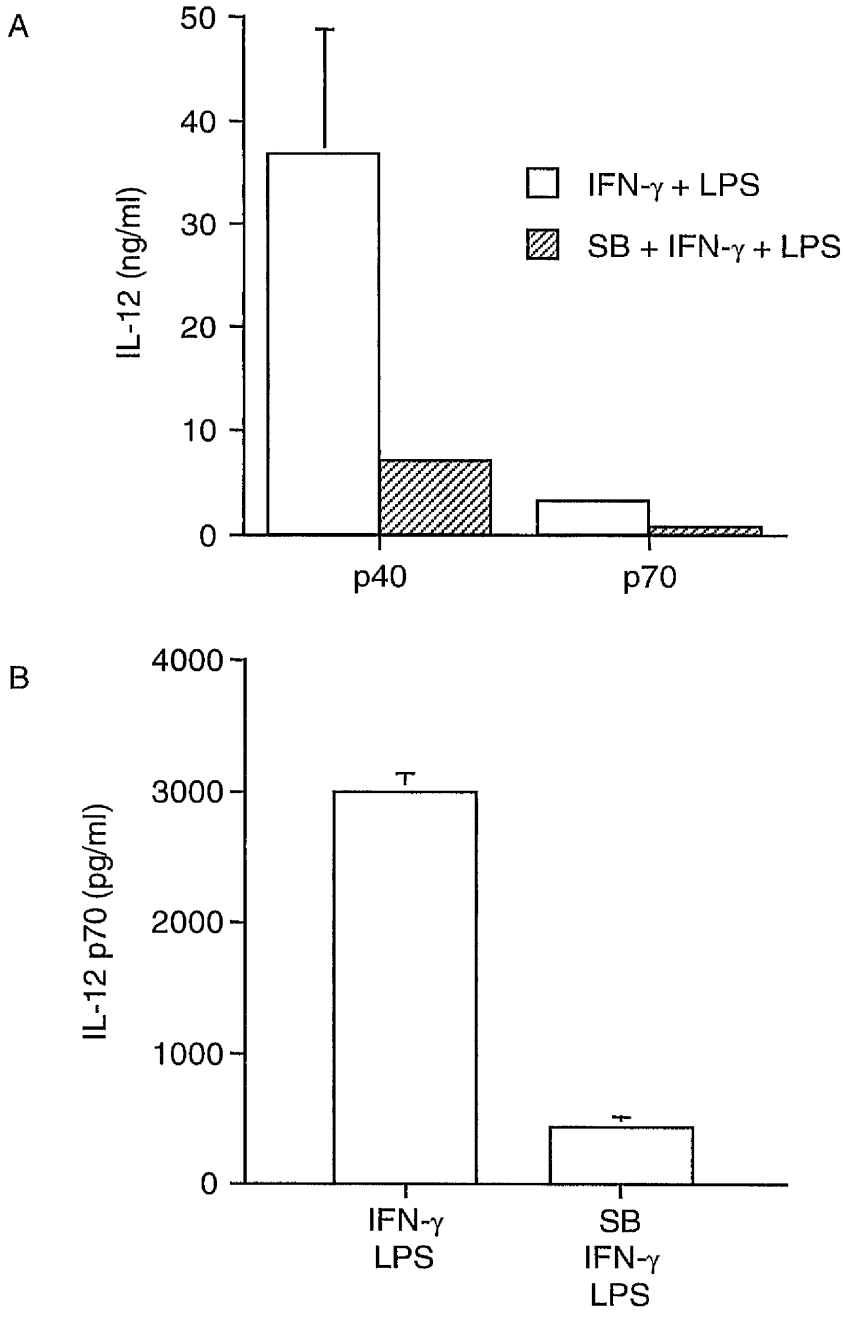


Figure 3.29. Effect of inhibition of p38 MAP kinase on IL-12 p70 production by peritoneal macrophages.

BALB/c peritoneal macrophages ($2x10^5$/well in a 96-well plate) were pre-treated for 1 hour with the inhibitor of the p38 MAP kinase pathway, SB 203580 (SB; 5 μM), prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS. IL-12 p40 and bioactive p70 in 24 hour culture supernatants were measured by ELISA (A); the effect of SB on IL-12 p70 is also shown enlarged (B).

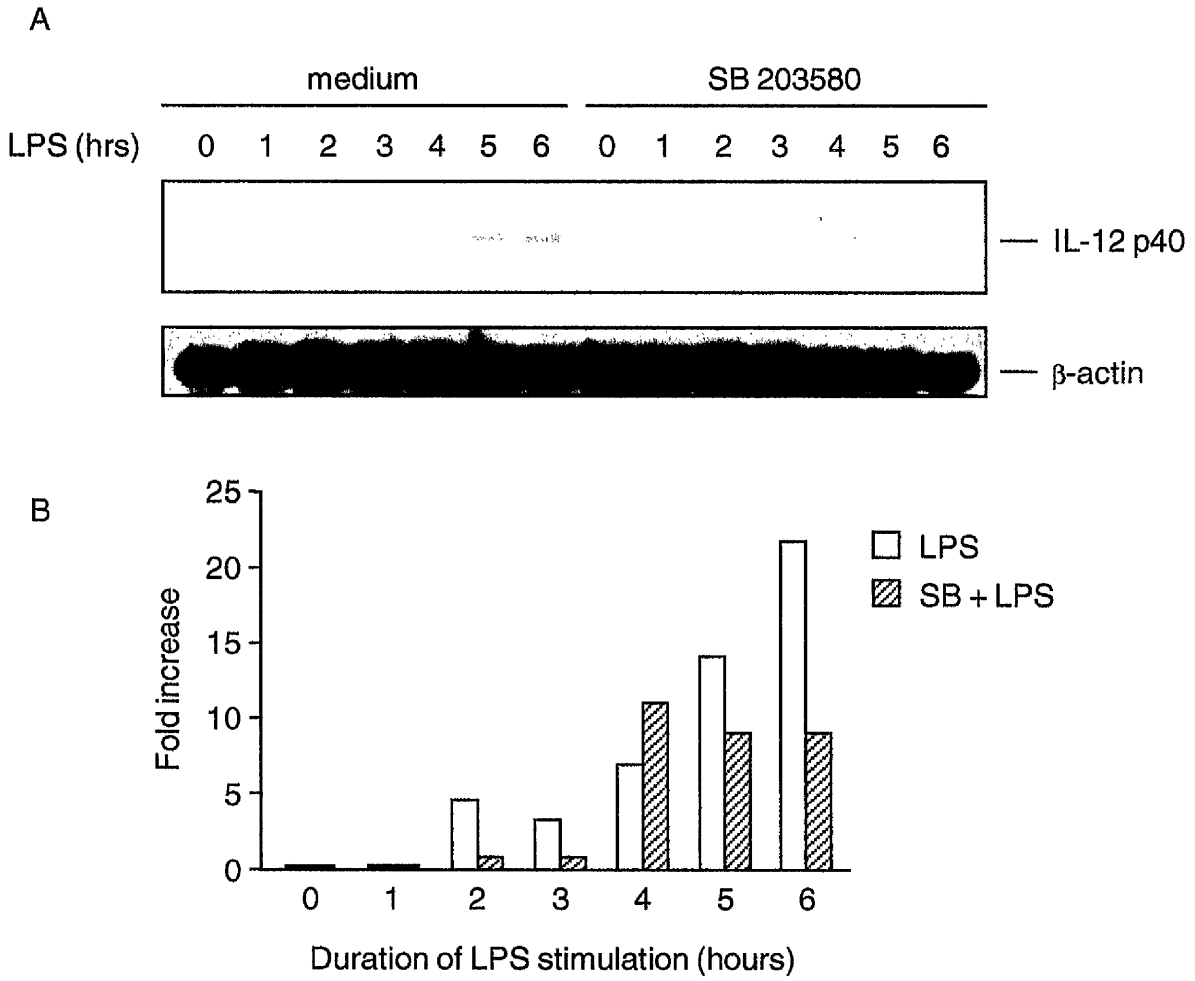


Figure 3.30. Effect of inhibition of p38 MAP kinase activity on IL-12 p40 mRNA induction.

J774 cells ($4x10^6$/25 cm^2 flask) were pre-treated with 5 μM SB 203580 (SB) for 1 hour prior to stimulation with 1 μg/ml LPS for the times indicated. Northern Blot analysis (A) was performed using purified total RNA hybridised with a cloned murine IL-12 p40 cDNA probe. After stripping, a cloned murine β-actin cDNA probe was used as a loading control. Densitometric values (B) are IL-12 p40:β-actin ratios derived from the data, expressed relative to basal levels.

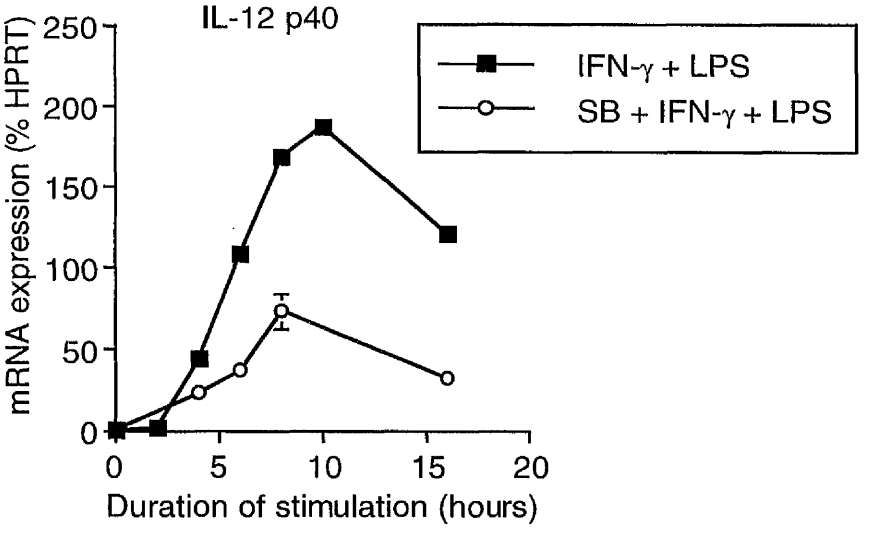


Figure 3.31. Modulation of IL-12 p40 mRNA production by inhibition of p38 MAP kinase activity.

Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were pre-treated with SB 203580 (SB; 5 μM) for 1 hour prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated. Total RNA was purified and TaqMan real-time PCR analysis was performed to measure IL-12 p40 transcript levels, which are expressed relative to HPRT mRNA.

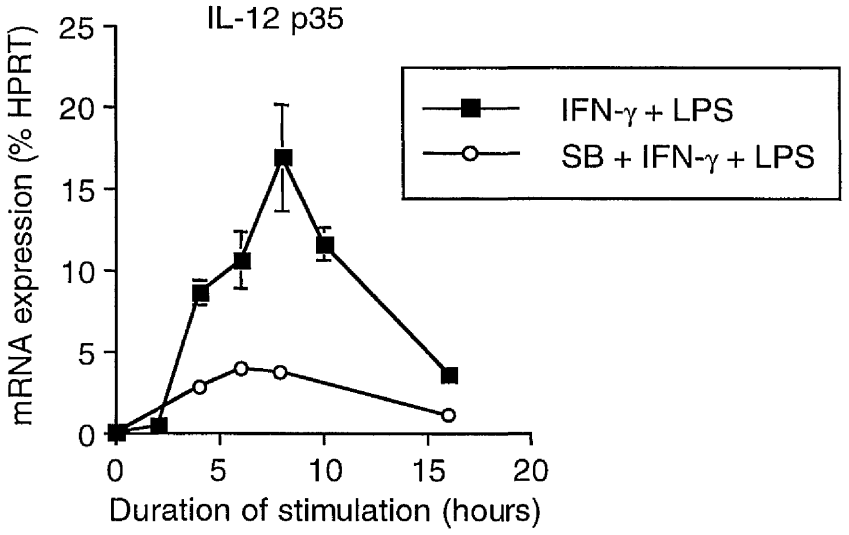


Figure 3.32. Effect of inhibition of p38 MAP kinase activity on IL-12 p35 mRNA induction.

Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were pre-treated with SB 203580 (SB; 5 μM) for 1 hour prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated. Total RNA was purified and TaqMan real-time PCR analysis was performed to measure IL-12 p35 transcript levels, which are expressed relative to HPRT mRNA.

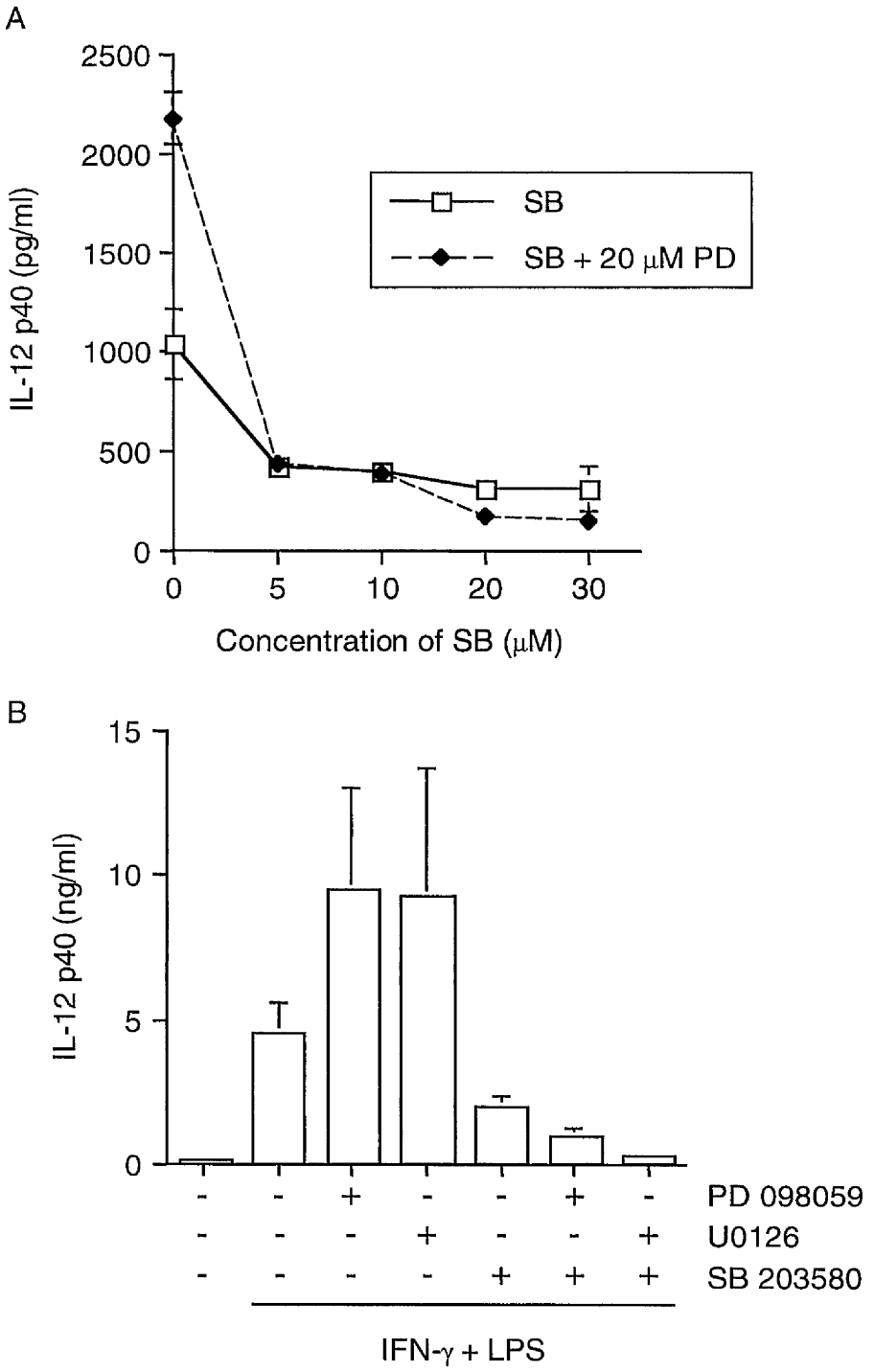


Figure 3.33. Effect of inhibition of both ERK and p38 MAP kinases on IL-12 production.

J774 cells (A) and murine peritoneal macrophages (B) ($2x10^5$/well in a 96-well plate) were pre-treated with the ERK inhibitor PD 098059 (PD; 20 μM) or U0126 (1 μM) in the presence/absence of the p38 inhibitor SB 203580 (SB; 5 μM or as indicated) for 1 hour prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS. IL-12 p40 in 24 hour culture supernatants was measured by ELISA.

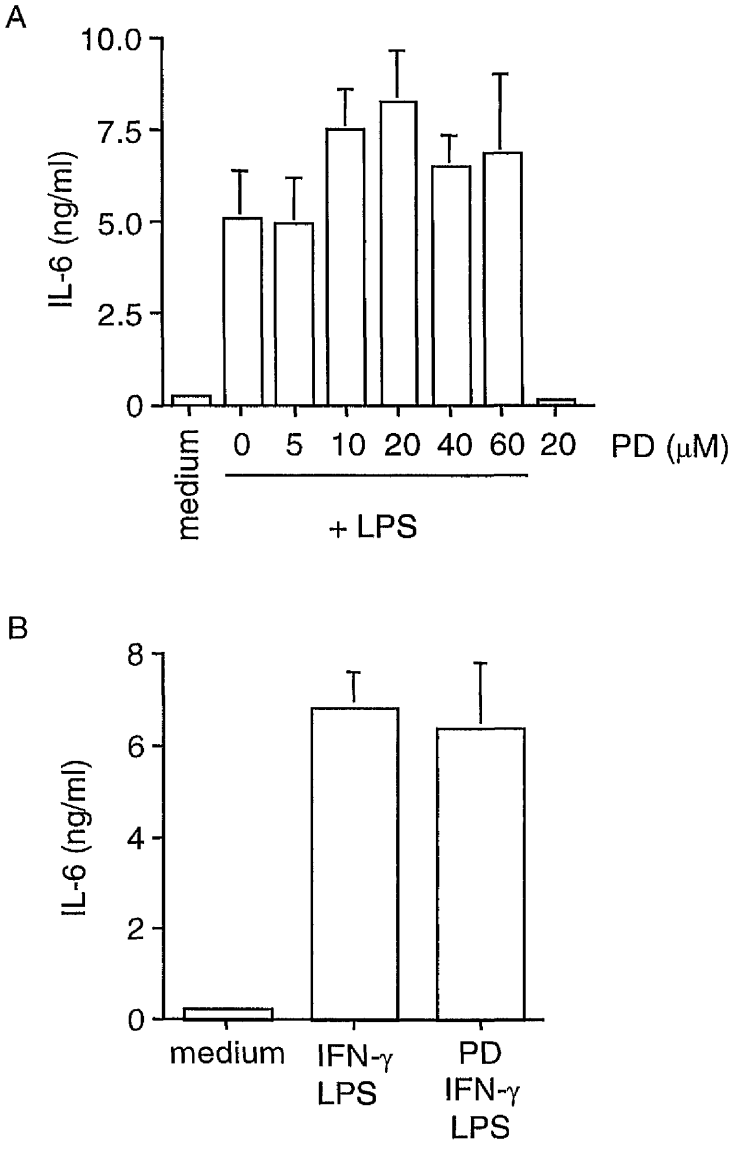


Figure 3.34. Effect of inhibition of ERK MAP kinases on IL-6 production.

J774 cells (A) and murine peritoneal macrophages (B) ($2x10^5$/well in a 96-well plate) were pre-treated for 1 hour with the inhibitor of the ERK MAP kinase pathway, PD 098059 (PD; 20 μM or as indicated), prior to stimulation with 1 μg/ml LPS (A) or 100 U/ml IFN-γ and 100 ng/ml LPS (B). IL-6 in 24 hour culture supernatants was measured by ELISA.

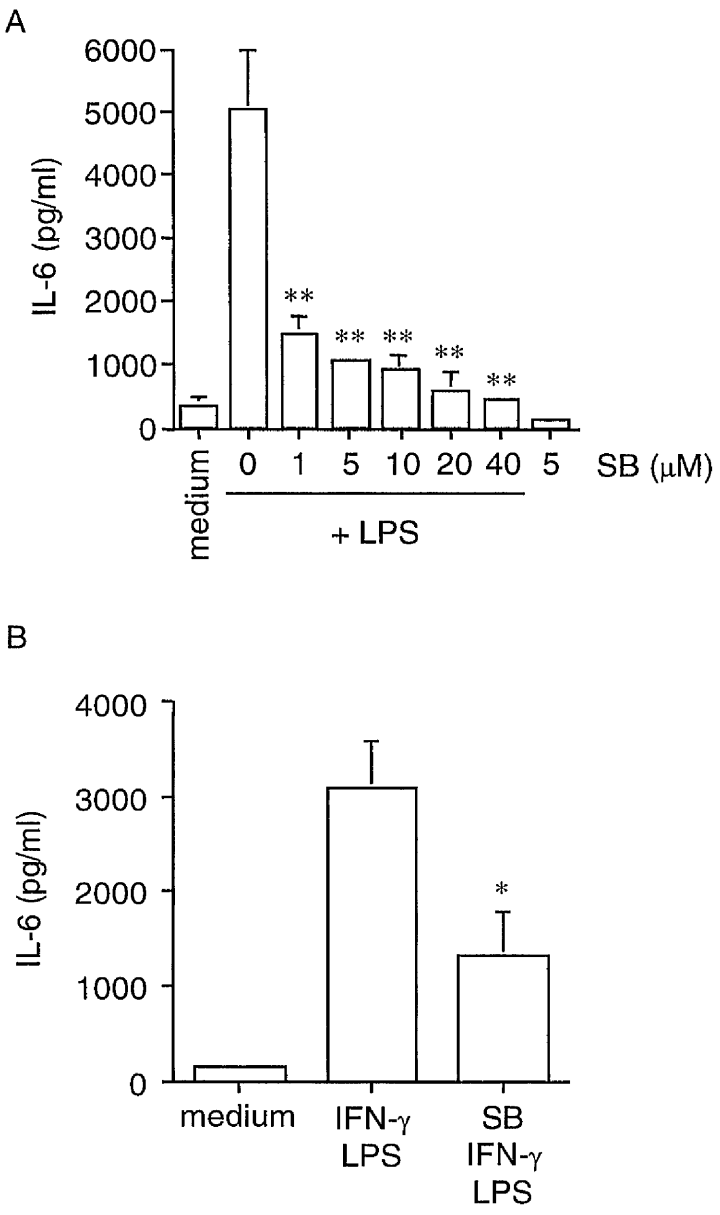


Figure 3.35. Effect of inhibition of p38 MAP kinase on IL-6 production.

J774 cells (A) and murine peritoneal macrophages (B) ($2x10^5$/well in a 96-well plate) were pre-treated for 1 hour with the inhibitor of the p38 MAP kinase pathway, SB 203580 (SB; 5 μM or as indicated), prior to stimulation with 1 μg/ml LPS (A) or 100 U/ml IFN-γ and 100 ng/ml LPS (B). IL-6 in 24 hour culture supernatants was measured by ELISA. * $p < 0.05$, ** $p < 0.01$

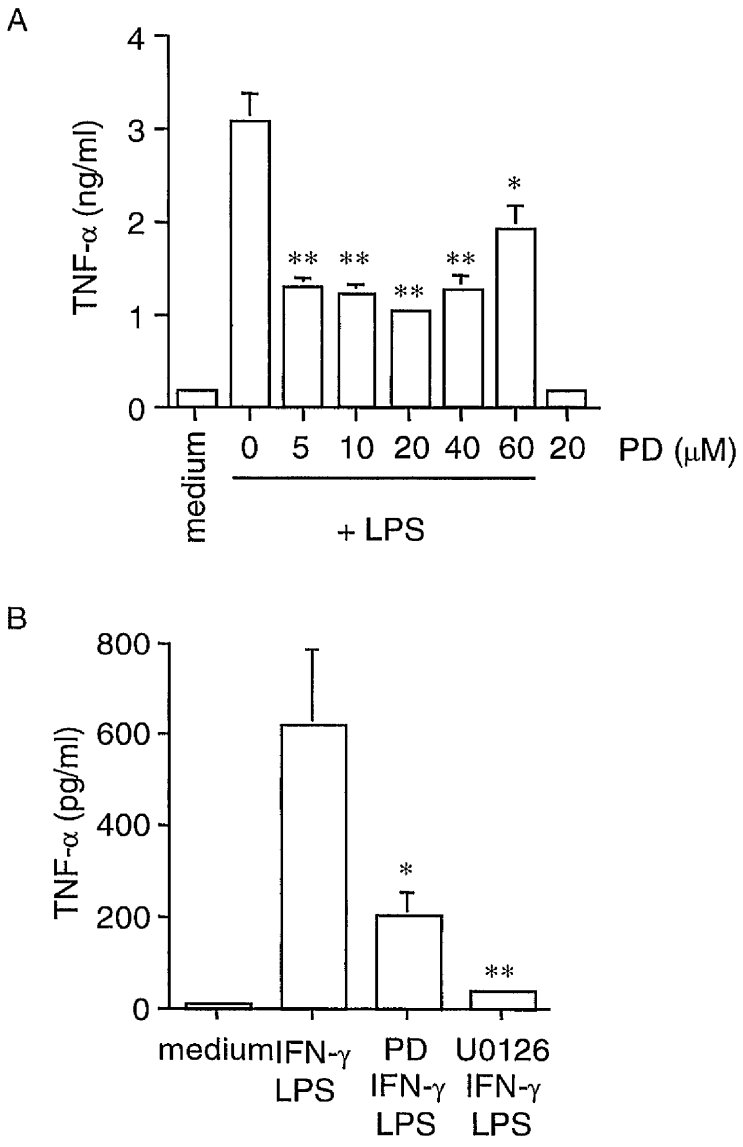


Figure 3.36. Effect of inhibition of ERK MAP kinases on TNF-α production.

J774 cells (A) and murine peritoneal macrophages (B) ($2x10^5$/well in a 96-well plate) were pre-treated for 1 hour with the inhibitors of the ERK MAP kinase pathway, PD 098059 (PD; 20 μM or as indicated) or U0126 (1 μM), prior to stimulation with 1 μg/ml LPS (A) or 100 U/ml IFN-γ and 100 ng/ml LPS. TNF-α in 24 hour culture supernatants was measured by ELISA. * $p < 0.05$, ** $p < 0.01$

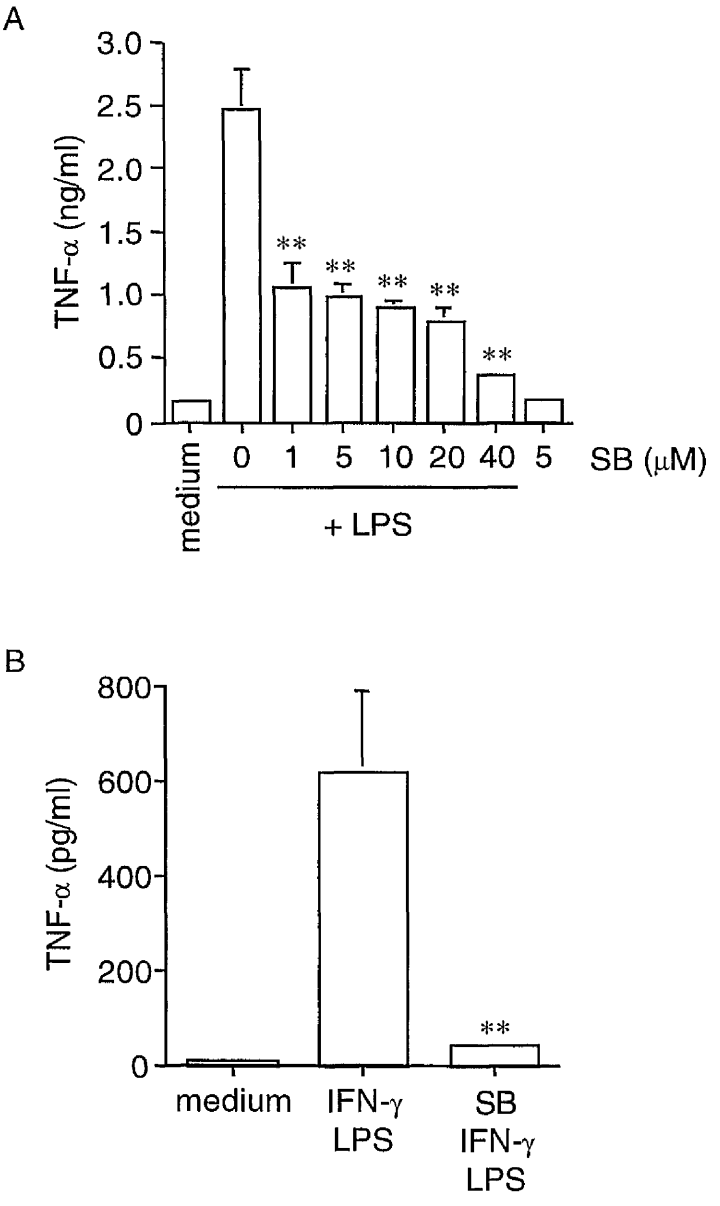


Figure 3.37. Effect of inhibition of p38 MAP kinase on TNF-α production.

J774 cells (A) and murine peritoneal macrophages (B) ($2x10^5$/well in a 96-well plate) were pre-treated for 1 hour with the inhibitor of the p38 MAP kinase pathway, SB 203580 (SB; 5 μM or as indicated), prior to stimulation with 1 μg/ml LPS (A) or 100 U/ml IFN-γ and 100 ng/ml LPS (B). TNF-α in 24 hour culture supernatants was measured by ELISA. ** $p < 0.01$

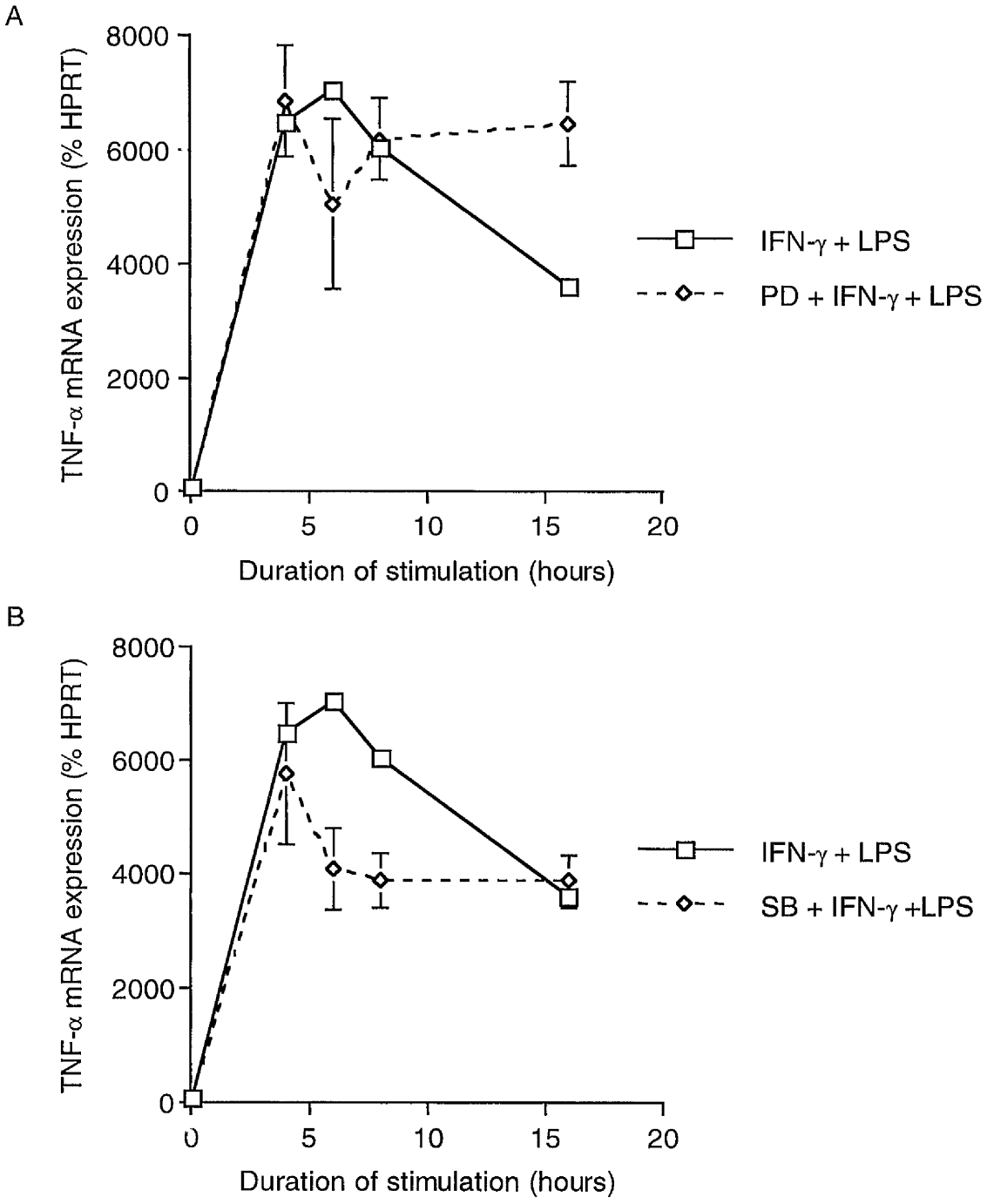


Figure 3.38. Effects of inhibition of ERK and p38 MAP kinases on TNF-α mRNA induction.

Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were pre-treated with 20 μM PD 098059 (PD) or 5 μM SB 203580 (SB) for 1 hour prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated. Total RNA was purified and TaqMan real-time PCR analysis was performed to measure TNF-α transcript levels, which are expressed relative to HPRT mRNA.

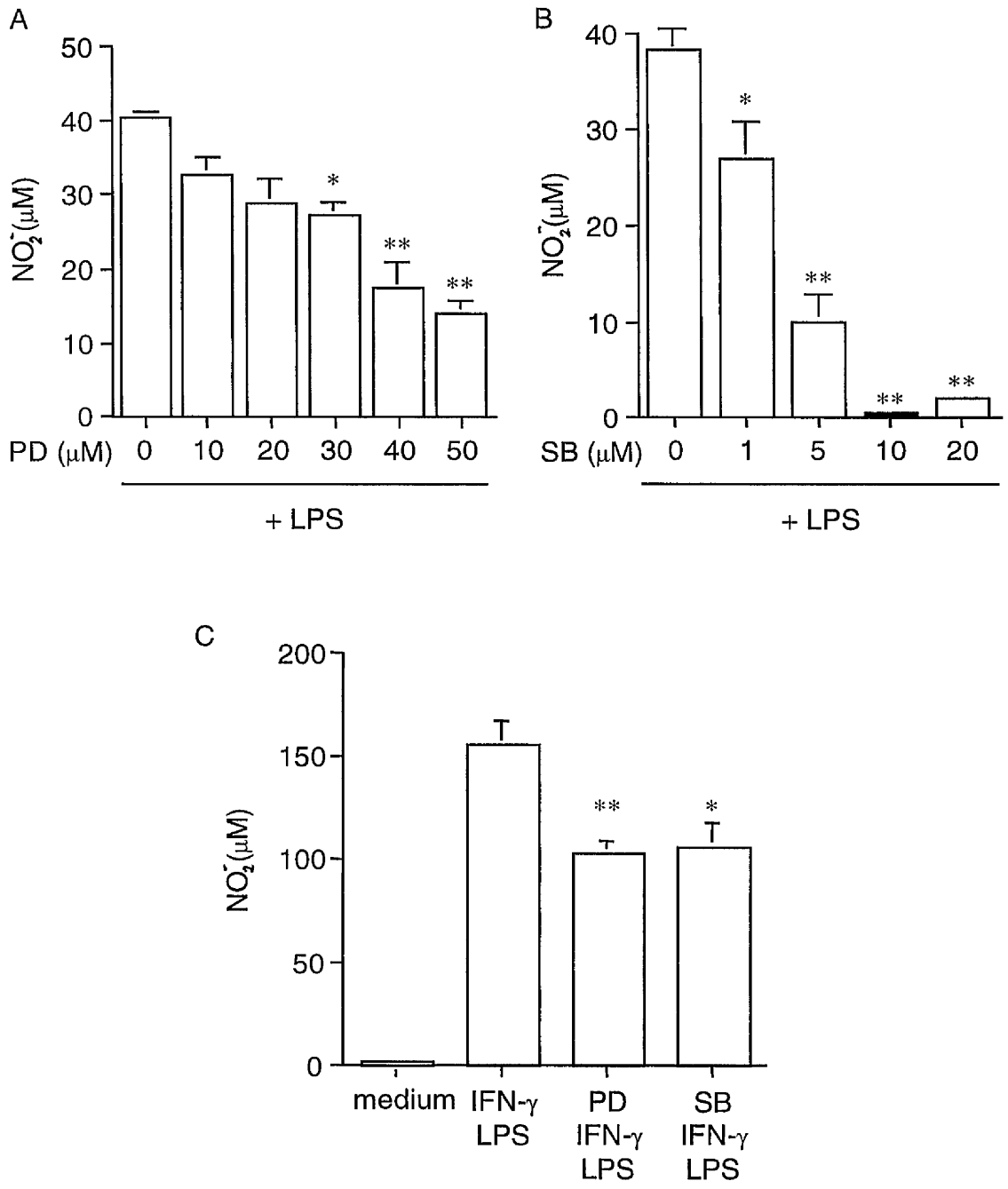


Figure 3.39. Effects of inhibition of ERK and p38 MAP kinases on NO release.

J774 cells (A and B) and murine peritoneal macrophages (C) ($2x10^5$/well in a 96-well plate) were pre-treated for 1 hour with the inhibitor of the ERK MAP kinase pathway, PD 098059 (PD; 20 μM or as indicated), or the p38 inhibitor, SB 203580 (SB; 5 μM or as indicated), prior to stimulation with 1 μg/ml LPS (A and B) or 100 U/ml IFN-γ and 100 ng/ml LPS (C). NO in 24 hour culture supernatants was assayed by Griess Reaction.

* $p < 0.05$, ** $p < 0.01$

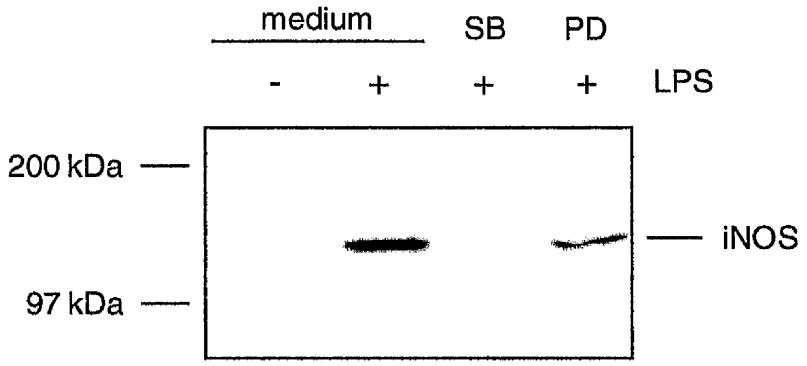


Figure 3.40. Effects of inhibition of ERK and p38 MAP kinases on iNOS induction.

J774 cells ($4x10^6$/25 cm^2 flask) were pre-treated for 1 hour with the inhibitor of the ERK MAP kinase pathway, PD 098059 (20 μM; PD), or the p38 inhibitor, SB 203580 (5 μM; SB), prior to stimulation with 1 μg/ml LPS for 16 hours. Total protein lysates were purified and assayed for iNOS by Western Blotting using an anti-iNOS antibody.

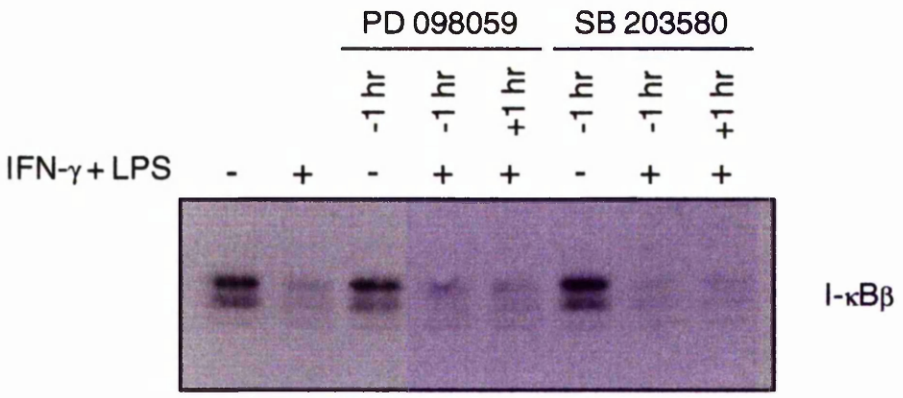


Figure 3.41. Effects of inhibition of ERK and p38 MAP kinases on I-κB degradation following stimulation with IFN-γ + LPS.

Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were stimulated for 4 hours with 100 U/ml IFN-γ and 100 ng/ml LPS, in the absence/presence of PD 098059 (PD; 20 μM) or SB 203580 (SB; 5 μM) which was added at the times indicated relative to IFN-γ + LPS stimulation. Total protein lysates were purified and assayed by Western Blotting using an anti-I-κBβ antibody.

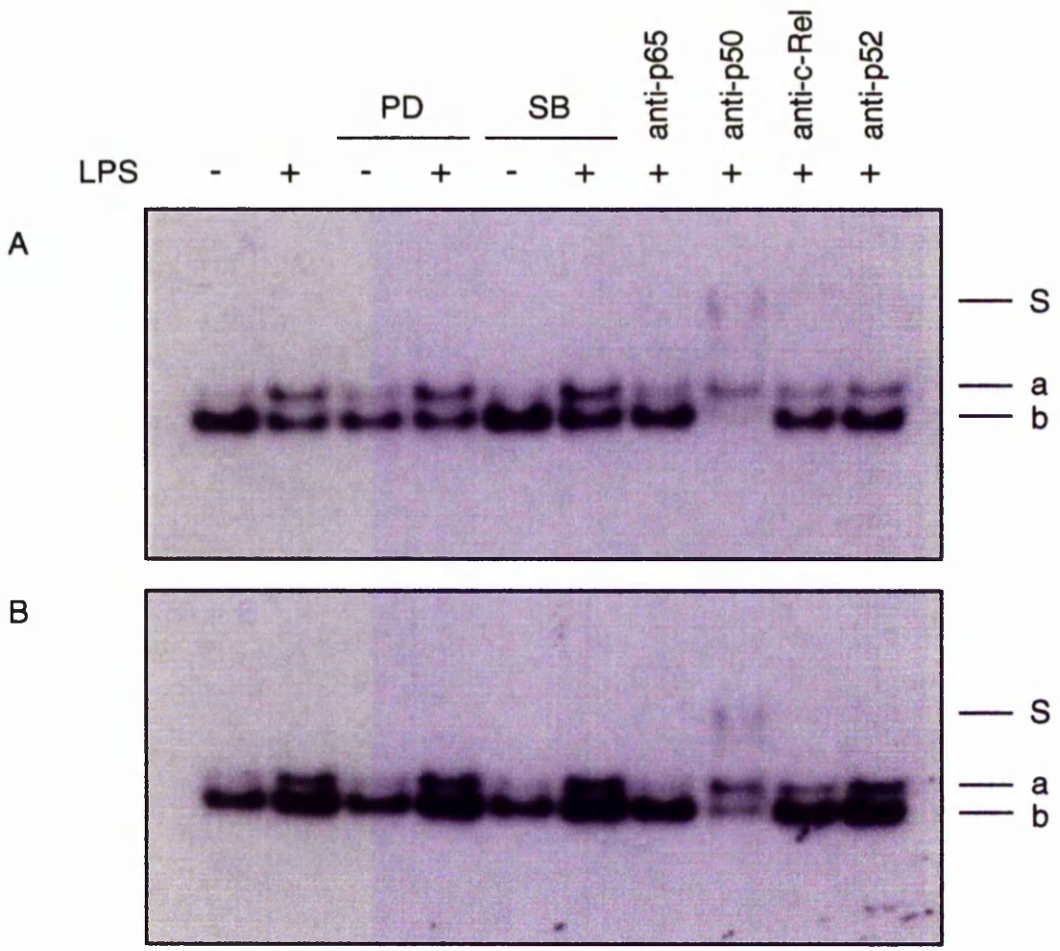


Figure 3.42. Effects of inhibition of ERK and p38 MAP kinases on NF-κB binding to iNOS and IL-12 p40 promoter sequences.

J774 cells ($4x10^6$/25 cm^2 flask) were pre-treated with either PD 098059 (PD; 20 μM) or SB 203580 (SB; 5 μM) for 1 hour prior to stimulation with 1 μg/ml LPS for 1 hour. Nuclear protein was purified and analysed by EMSA for NF-κB binding to ^{32}P-labelled oligonucleotide probes containing NF-κB binding sites from iNOS (A) and IL-12 p40 (B) promoters. Supershift was performed by pre-incubating nuclear protein samples with antibodies against NF-κB isoforms for 30 minutes prior to addition of the probe. 'a' and 'b' indicate the NF-κB binding complexes; 'S' indicates the position of the supershifted band.

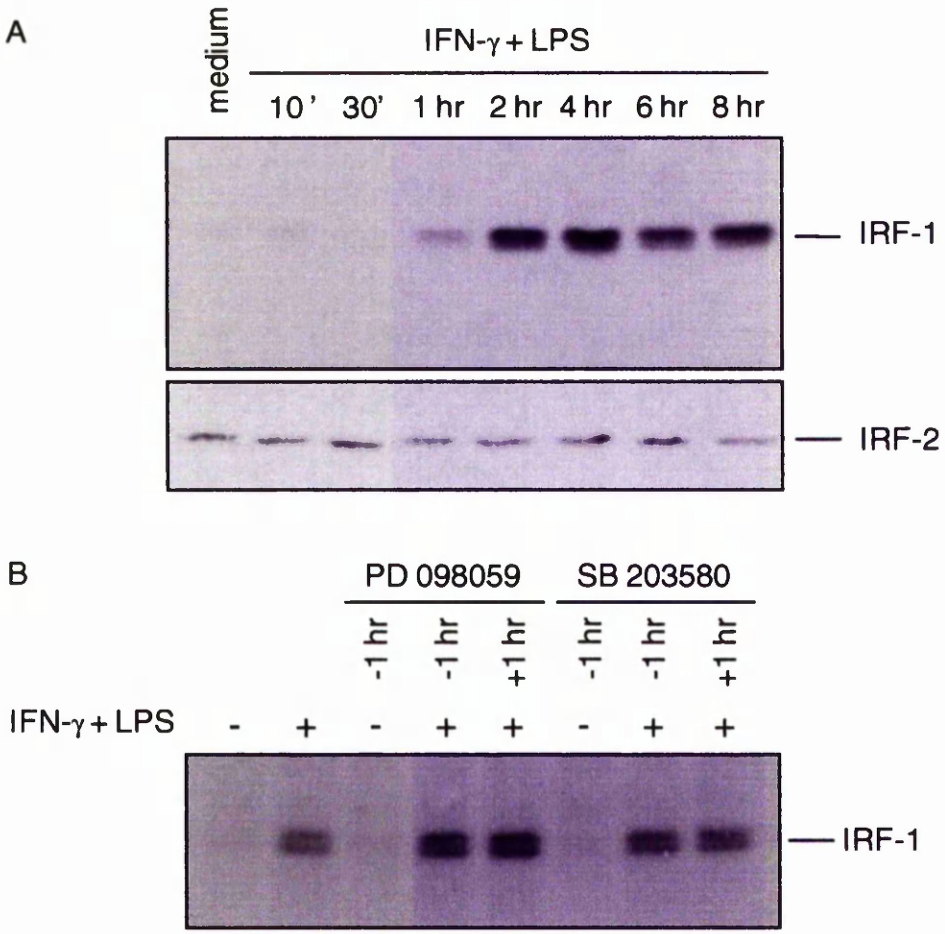


Figure 3.43. Effects of inhibition of ERK and p38 MAP kinases on IRF-1 synthesis following stimulation with IFN-γ + LPS.

A. Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were stimulated with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated.

B. Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were stimulated for 4 hours with 100 U/ml IFN-γ and 100 ng/ml LPS, in the absence/presence of PD 098059 (20 μM) or SB 203580 (5 μM) which was added at the times indicated relative to IFN-γ + LPS stimulation.

Total protein lysates were purified and analysed by Western Blotting using anti-IRF-1 or anti-IRF-2 antibody.

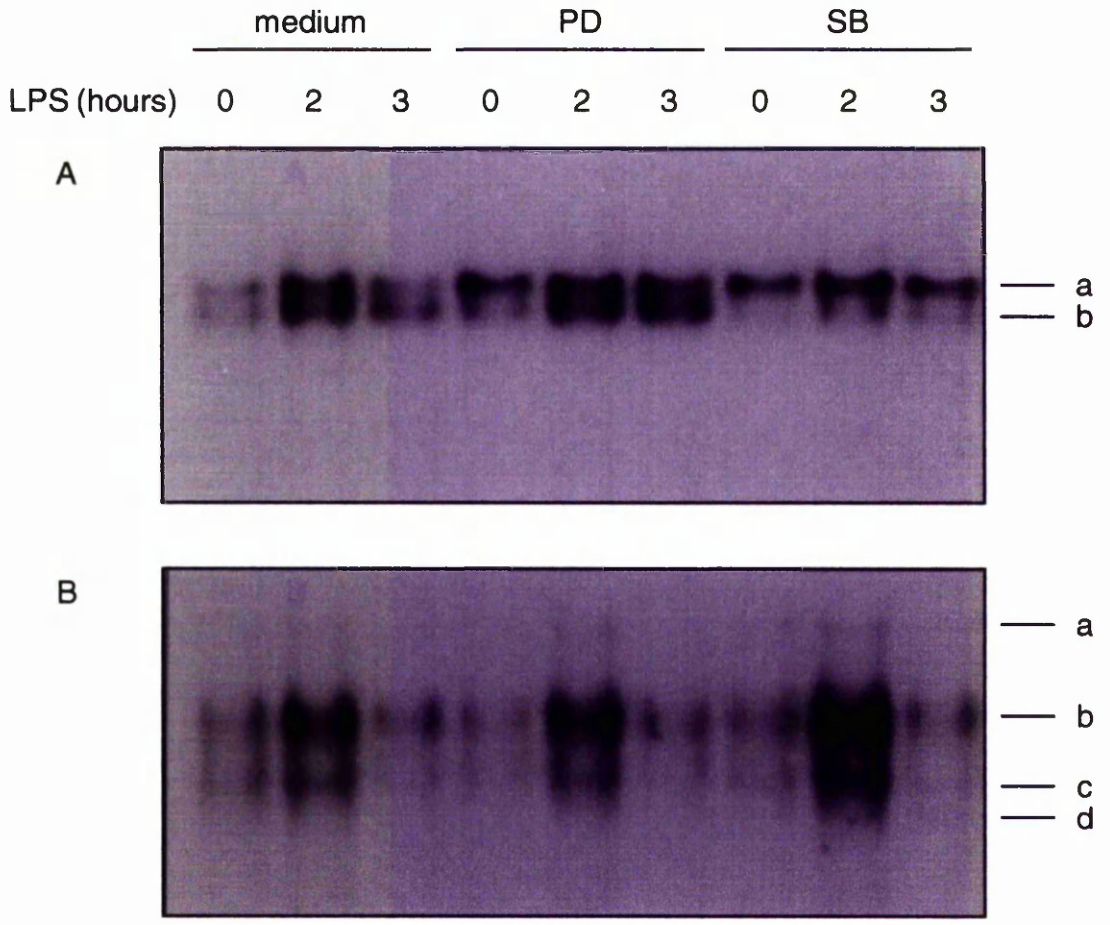


Figure 3.44. Effects of inhibition of ERK and p38 MAP kinases on transcription factor binding to the iNOS IRF-E binding site and a portion of the IL-12 p40 promoter.

J774 cells ($4x10^6$/25 cm^2 flask) were pre-treated with either PD 098059 (PD; 20 μM) or SB 203580 (SB; 5 μM) for 1 hour prior to stimulation with 1 μg/ml LPS for the times indicated. Nuclear protein was purified and analysed by EMSA for binding to ^{32}P-labelled oligonucleotide probes containing the IRF-E binding site from the iNOS promoter (A) and a portion of the IL-12 p40 promoter that is involved in binding of the F1 complex (B). 'a', 'b', 'c' and 'd' indicate binding complexes.

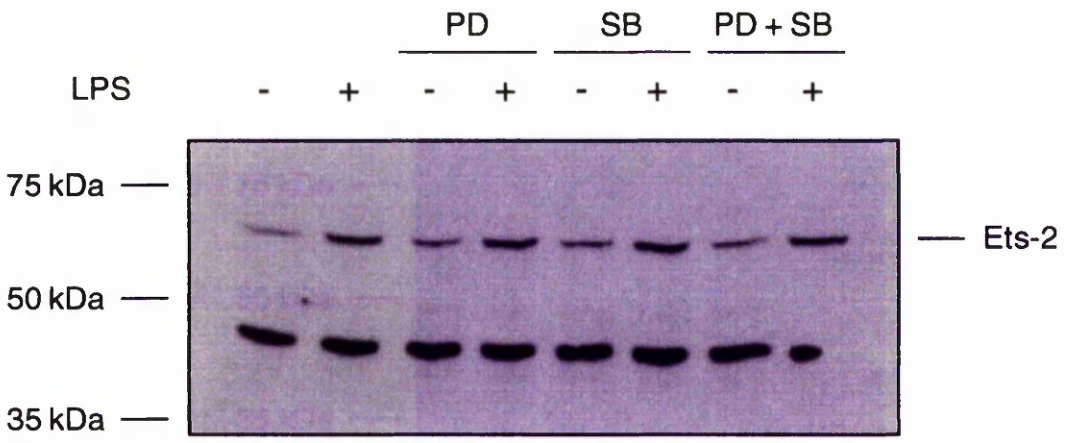


Figure 3.45. Effects of inhibition of ERK and p38 MAP kinases on LPS-induced Ets-2 production.

J774 cells ($4x10^6$/25 cm^2 flask) were pre-treated with either PD 098059 (PD; 20 μM) or SB 203580 (SB; 5 μM) for 1 hour prior to stimulation with 1 μg/ml LPS for 1 hour. Nuclear protein was purified and analysed by Western Blotting using an anti-Ets1/2 antibody.

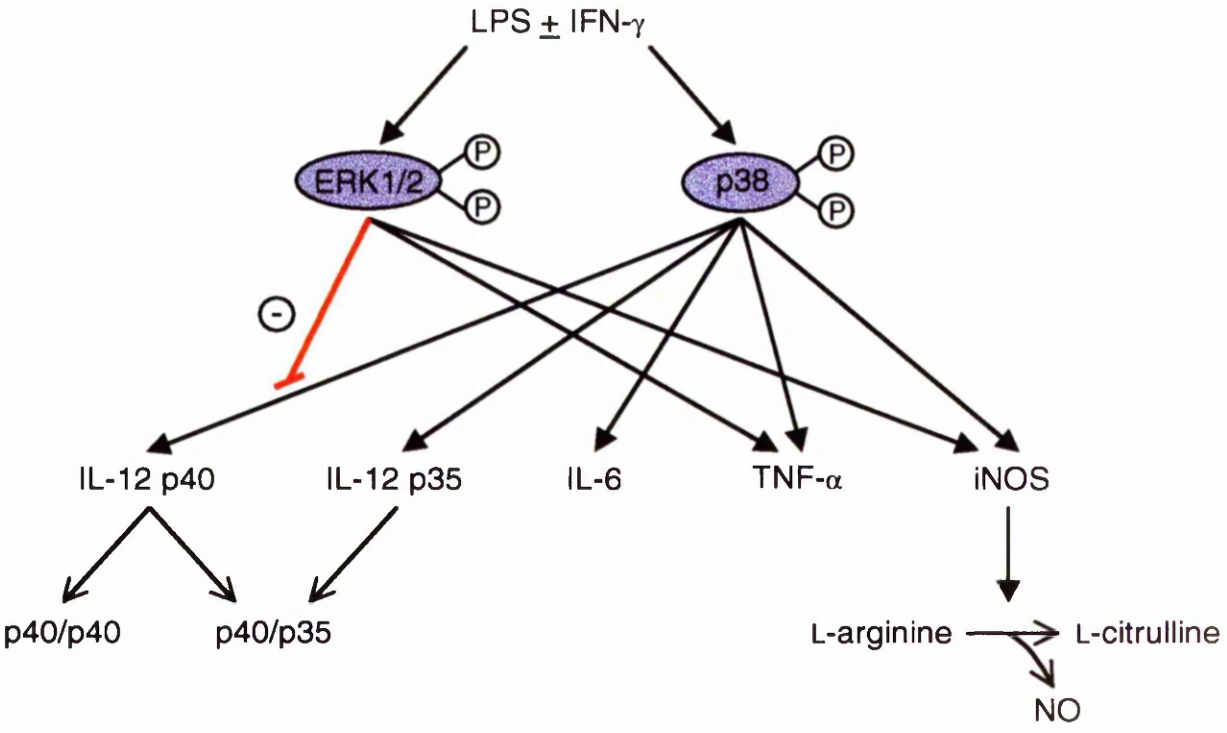


Figure 3.46. Roles of ERK and p38 MAP kinases in the induction of cytokine and iNOS production by LPS.

Stimulation of macrophages with LPS in the presence/absence of IFN-γ results in activation of ERK and p38 MAP kinases by dual phosphorylation. p38 MAP kinase activity is required for synthesis of both subunits of IL-12, as well as IL-6, TNF-α and iNOS. Induction of TNF-α and iNOS is also ERK MAP kinase-dependent. IL-6 and IL-12 p35 are not regulated by ERK MAP kinases. IL-12 p40 production is negatively regulated by ERK MAP kinases, which appear to suppress the p38 signal. IL-12 p40 can heterodimerise with the p35 subunit to form bioactive IL-12, or form inhibitory homodimers. iNOS catalyses the production of NO from L-arginine.

Chapter 4 – Inhibition of IL-12 production by *Leishmania* lipophosphoglycan (LPG).

4:1 Introduction.

4:1:1 *Leishmania* parasites - infection and disease.

It is estimated that 12 million people worldwide are infected with parasites of the genus *Leishmania*, and a further 350 million are at risk (WHO 2000b). Infection occurs mainly in the Tropics since the survival of the parasite is limited by the geographical distribution of its vector (the sandfly) and its susceptibility to cold climates.

Infection of humans with *Leishmania* parasites results in a spectrum of diseases known collectively as leishmaniasis (reviewed by Grimaldi *et al.,* 1989). Visceral leishmaniasis, or kala azar, is the most severe form of the disease and is fatal if left untreated. It is characterised by fever, weight loss, swelling of the spleen and liver, and anaemia. Mucocutaneous leishmaniasis, or espundia, produces lesions that can lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat cavities. Cutaneous leishmaniasis, the most common form, is characterised by large numbers of skin ulcers on the exposed parts of the body such as face, arms and legs, and can cause serious disability and permanent scarring. ~~Disease~~ Visceral leishmaniasis is particularly prevalent and lethal among immunosuppressed patients, and *Leishmania*/HIV co-infection is increasingly common (WHO 2000b).

Parasites are transmitted between mammalian hosts (humans, domestic and wild animals) by sandflies (*Phlebotomus* and *Lutzomyia* spp.) when these insects take a bloodmeal.

4:1:2 The lifecycle of *Leishmania* parasites.

The digenetic lifecycle of *Leishmania* consists of alternating phases in the digestive tract of the sand fly vector and in macrophages of the mammalian host (reviewed by Turco and Descoteaux, 1992; see Figure 4.1). Sandflies acquire *Leishmania* parasites from vertebrate hosts in the amastigote form by ingestion in a bloodmeal. ~~Metacyclogenesis,~~ The process of development from dividing non-infectious amastigotes to resting, flagellated, infectious promastigotes takes place as parasites move through the midgut to the anterior end of the gut and into the mouthparts. When the sandfly feeds again, promastigotes are injected into another mammalian host where they invade and parasitise macrophages. This is achieved by direct association of surface molecules with macrophage receptors, or indirectly via association with molecules present in serum, such as C3, which then bind their receptors on the macrophage (reviewed by Bogdan and Röllinghoff, 1998). Internalisation occurs via formation of a phagosome, which fuses with secondary lysosomes to form a phagolysosome. Inside the phagolysosome, the parasites transform to obligate intracellular amastigotes and multiply, before being released and ingested by the sandfly vector to continue the cycle.

4:1:3 Evasion of the host immune response.

At all stages of the lifecycle parasites are exposed to attack from the vector or the host (reviewed by Turco and Descoteaux, 1992). To survive in the sandfly they must protect against the lytic effects of the insect's gut enzymes. In the mammalian host they

must combat the effects of normal serum in the bloodstream, and degradative enzymes and toxic oxygen products in the macrophage phagolysosome.

Effective immunity against *Leishmania* infection is dependent on the induction of a parasite-specific Th1 cell response (Scott *et al.,* 1988; Boom *et al.,* 1990; Heinzel *et al.,* 1991; Reiner and Locksley, 1995). In the absence of significant IL-4 production, IFN-γ produced by Th1 cells and NK cells stimulates the iNOS-catalysed production of NO by macrophages, resulting in killing of *Leishmania* parasites (Liew *et al.,* 1990; Wei *et al.,* 1995; Stenger *et al.,* 1996). The genetic predisposition of naturally-occurring mouse strains to mount a Th response with a type 1 (Th1) or type 2 (Th2) bias is believed to underlie the resistance versus susceptibility to *Leishmania* infection respectively. C3H and C57BL/6 mice, which naturally resolve their lesions, exhibit a dominant Th1 profile, while BALB/c mice, which develop progressive disease, show a dominant Th2 phenotype (Scott, 1991; Heinzel *et al.,* 1989).

Leishmania parasites employ a number of mechanisms to aid their survival and promote establishment and maintenance of infection, including the suppression of IL-12 production (reviewed by Mattner *et al.,* 1997a). IL-12 is critical for the establishment of Th1 responses and the induction of IFN-γ production, and thus the synthesis of iNOS. It is therefore a key determinant in the outcome of *Leishmania* infections. Indeed, IL-12-deficient, like iNOS-deficient, mice have been shown to be susceptible to *Leishmania* infection (Satoskar *et al.,* 2000; Mattner *et al.,* 1996 and 1997b; Wei *et al.,* 1995). Furthermore, treatment of susceptible BALB/c mice with recombinant IL-12 resulted in a curative response to *L. major* infection due to IFN-γ production and inhibition of Th2 cytokines (Heinzel *et al.,* 1993; Sypek *et al.,* 1993). In contrast, neutralisation of either

IL-12 or IFN-γ led to an adverse outcome in normally resistant mice (Sypek *et al.,* 1993; Belosevic *et al.,* 1989).

Since macrophages are the major producers of IL-12 and it is these cells that *Leishmania* invades, the inhibition of macrophage IL-12 production is thought to be an important parasite survival strategy. In the absence of IFN-γ, NO production by macrophages is significantly reduced and therefore the parasites survive and are able to multiply. Indeed, Reiner *et al.* (1994) and Carrera *et al.* (1996) showed that *Leishmania* promastigotes evade IL-12 induction by macrophages from both resistant C57BL/6 and susceptible BALB/c mice.

4:1:4 *Leishmania* lipophosphoglycan and its role in immune evasion.

Multiple virulence determinants produced by *Leishmania* parasites have been identified with a capacity to modulate the immune response (reviewed by Bogdan and Röllinghoff, 1998). These include molecules expressed on the parasite surface such as lipophosphoglycan (LPG), glycoinositol phospholipids (GIPLs) and the metalloproteinase gp63, as well as secreted factors such as proteophosphoglycans (PPGs) which are secreted by amastigotes into the parasitophorous vesicles of the host cell.

Leishmania parasites are covered with a thick glycocalyx, which consists predominantly of a single molecule called lipophosphoglycan (LPG); as many as 10^6 copies of LPG may be present on a single promastigote (Orlandi and Turco, 1987). LPG is a multifunctional molecule with roles at various stages of the *Leishmania* lifecycle,

both in the sandfly vector and in the mammalian host (reviewed by Turco and Descoteaux, 1992). In the sandfly it is involved in the binding of parasites to the midgut and, subsequently, detachment of the parasite to allow transmission. Metacyclogenesis includes structural modification of LPG, allowing parasites to move through the gut towards the mouthparts; LPG also protects against hydrolytic enzymes in sandfly gut. In the vertebrate host, it has been implicated in the resistance of the parasite to complement-mediated lysis, its binding to and uptake by macrophages, modulation of macrophage signal transduction pathways leading to suppression of effector molecule production and resistance to oxidative attack.

The importance of LPG in the successful establishment and maintenance of infection was suggested by the demonstration that mice vaccinated with LPG displayed a reduced parasite burden upon challenge infection with *L. major*, and had increased numbers of *L. major*-reactive T cells compared with non-vaccinated mice (Handman and Mitchell, 1985). Further evidence was provided from studies in which several LPG-deficient strains of *Leishmania* were shown to be unable to survive in host macrophages; for example, an avirulent clone of *L. major* that lacked LPG was phagocytosed by macrophages and killed within 18 hours (Handman *et al.,* 1986). The ability of LPG to confer resistance to killing was confirmed by passive transfer of purified LPG from a virulent strain into avirulent promastigotes, which were then able to survive in macrophages (McNeely and Turco, 1990).

The structure of LPG is shown in Figure 4.2. It comprises a monoalkyl lipid that anchors LPG to the plasma membrane, a hexasaccharide core and a phosphoglycan region with a neutral cap. The functions of LPG have been investigated and variously

attributed to the different LPG portions. The effects of LPG on macrophage function are summarised in Figure 4.3. LPG is a potent inhibitor of many protein kinase C (PKC)-mediated events which are deleterious to *Leishmania* promastigotes (reviewed by Turco and Descoteaux, 1992). PKC is a multifunctional serine-threonine kinase, which is composed of an ATP-binding catalytic domain and a regulatory domain that contains sites involved in calcium, diacylglycerol (DAG) and phospholipid binding (reviewed by Nishizuka, 1986). Upon binding to membranes, a conformational change occurs and the catalytic and regulatory domains of PKC dissociate. A study by Giorgione *et al.* (1996) indicated that LPG bound on the outside of the plasma membrane inhibits PKCα activation by causing it to bind to the inner membrane without the induction of a conformational change.

LPG inhibits the oxidative burst via its suppression of PKC; monoalkyl lipid fragments exhibit the most potent inhibitory activity, although phosphoglycan was also demonstrated to inhibit purified PKC activity (McNeely and Turco, 1987 and 1990; McNeely *et al.,* 1989). Furthermore, the phosphoglycan portion is a highly effective scavenger of hydroxyl radicals and superoxide ions, thereby limiting the consequences of oxidative attack (Chan *et al.,* 1989). LPG also inhibits the PKC-mediated induction of the immediate early gene *c-fos*, thus preventing the formation of Fos-Jun complexes, which are important for the transcriptional regulation of a number of macrophage effector genes (Descoteaux *et al.,* 1991). PKC-dependent mechanisms mediate the activation of macrophages by TNF-α and IFN-γ (Fan *et al.,* 1988; Hamilton *et al.,* 1985; Schutze *et al.,* 1990) and these have therefore also been implicated as LPG targets. Furthermore, LPG causes the down-regulation of TNF receptors (Descoteaux *et al.,* 1991). In addition, LPG is thought to inhibit chemotaxis via a PKC-dependent mechanism, thereby preventing

accumulation of blood monocytes at sites of infection (Frankenburg *et al.,* 1990; Laskin *et al.,* 1987).

LPG also activates HIV-1 replication in monocytes and T cells (Bernier *et al.,* 1995 and 1998) and has been demonstrated to target protein tyrosine kinase- and protein kinase A-dependent pathways and NF-κB binding (Bernier *et al.,* 1998) to achieve up-regulation of HIV-1 long terminal repeat (LTR)-driven transcription.

The critical role of macrophages in the activation of T helper cells is also impaired by *Leishmania* infection. The expression of major histocompatibility complex (MHC) class II molecules and the production of IL-1 are both defective in *L. donovani*-infected macrophages, and LPG has been implicated in these deficiencies (Reiner, 1987; Reiner *et al.,* 1987; Frankenburg *et al.,* 1990).

Proudfoot *et al.* (1996) showed that glycoinositol phospholipids (GIPLs) and LPG are both capable of inhibiting NO synthesis following activation of macrophages with IFN-γ. GIPLs are thought to act via suppression of production of iNOS, the enzyme that catalyses NO synthesis; although LPG may also act in this way, it is believed to achieve NO suppression *in vivo* predominantly via inhibition of IL-12 and hence IFN-γ production.

Reiner *et al.* (1994) demonstrated that non-infective amastigotes induce IL-12 production by bone marrow-derived macrophages, whereas infective promastigotes do not. Furthermore, Sartori *et al.* (1997) showed that both procyclic and metacyclic promastigotes were capable of inhibiting IL-12 production induced by *Staphylococcus*

aureus. The composition of glycans on the surface of the parasites has been linked to this ability to modulate IL-12 production. GIPLs are present on both amastigotes and promastigotes whereas LPG is present predominantly on the promastigote stage of the parasite (Mengeling *et al.,* 1997). Consistent with this theory, IL-12 production is induced by glycoconjugates of GIPLs, but suppressed by LPG glycoconjugates (Piedrafita *et al.,* 1999).

In a previous investigation in this laboratory using the J774 murine macrophage cell line, Piedrafita *et al.* (1999) demonstrated that a synthetic version of the PG portion of LPG (sPG), which consists of an average of 10 repeats of the Galactose-Mannose-phosphate subunit (see Figure 4.2), is able to mimic LPG-mediated suppression of IL-12 p40 production. Thus, the phosphoglycan portion, and not the glycophosphoinositol lipid anchor, is critical for this activity. sPG suppressed IL-12 p40 at the transcriptional level, but did not inhibit IL-6 or TNF-α production by these macrophages.

4:1:5 Aim of this study.

The aim of this investigation was to build on the discoveries described in the previous chapter to determine the signalling mechanisms underlying the sPG-mediated suppression of IL-12. The effects of sPG on the activation of MAP kinases and their regulation of IL-12 p40 production were examined.

4:2 Results.

4:2:1 Effects of sPG on cytokine and NO production by macrophages.

First, the effects of sPG on the production of cytokines and NO by LPS-stimulated J774 cells and IFN-γ + LPS-stimulated murine peritoneal macrophages were analysed. Unless otherwise indicated, cells were stimulated in the absence/presence of sPG for 24 hours, and cytokines and NO in culture supernatants were measured by ELISA and Griess reaction respectively. A shorter PG fragment, called sPG3, which has an average of approximately 4.5 Gal-Man-phosphate repeats and lacks the terminal phosphate group (see Figure 4.2), was also tested.

4:2:1:1 Effect of sPG on IL-12 production.

sPG treatment inhibited LPS-induced IL-12 p40 production in a dose dependent manner (Figure 4.4A). A dose of 50 μM resulted in 57% inhibition and was selected for further experiments. Interestingly, the truncated version of sPG, sPG3, also inhibited IL-12 production, and with a similar potency (Figure 4.4B); 50 μM sPG3 inhibited IL-12 p40 production by 57%. This indicates that a high degree of polymerisation of the Gal-Man repeats is not necessary for inhibition of IL-12, and also that the terminal phosphate group is not critical.

The time of addition of sPG relative to LPS stimulation was important (Figure 4.5). Addition 1 hour before or 10 minutes after LPS was as effective as simultaneous treatment. However, sPG added 30 minutes after LPS resulted in less significant inhibition of IL-12, and by 1 hour after LPS stimulation sPG was totally ineffective.

sPG also inhibited the production of IL-12 p40 induced by treatment of murine peritoneal macrophages with IFN-γ + LPS. Of particular interest, this suppression was independent of the strain of mouse used; sPG inhibited IL-12 p40 production by macrophages from both the *Leishmania*-susceptible BALB/c and the resistant 129 strains to a similar extent (Figure 4.6).

The biological relevance of these data was confirmed by the demonstration that sPG treatment also reduced levels of IL-12 p70 heterodimer in culture supernatants (Figure 4.7). Thus, the PG portion of LPG reduces bioactive IL-12 production by inhibiting production of the p40 subunit.

4:2:1:2 Effects of sPG on macrophage viability and TNF-α and NO production.

Analysis of macrophage viability by MTT assay confirmed that sPG does not inhibit IL-12 production by simply killing cells (Figure 4.8). TNF-α and NO levels in culture supernatants were also measured to assess whether sPG acts specifically to inhibit IL-12 production, or generally, for example to inhibit transcription. sPG did not inhibit the production of TNF-α or NO by J774 cells or murine peritoneal macrophages (Figure 4.9). These data are consistent with previous experiments using sPG, which showed that

it had no effect on IL-6 or TNF-α production (Piedrafita *et al.,* 1999). Therefore, sPG specifically targets the production of IL-12 by macrophages.

4:2:2 Effects of sPG on MAP kinase activity.

The signalling mechanisms underlying the sPG-mediated suppression of LPS-induced IL-12 p40 production are unknown, although a previous study (Piedrafita *et al.,* 1999) suggested that suppression of p40 transcription is not due to reduced NF-κB availability. The requirement of p38 MAP kinase activity for transcription of the IL-12 p40 gene, and the negative regulation of this signal by ERK MAP kinases was demonstrated in the previous chapter. Hence, these MAP kinases represent putative mediators of IL-12 suppression by sPG. Therefore, the effects of sPG on MAP kinase activation and the modulation of IL-12 production by treatment with PD 098059 were investigated.

First, MAP kinase activation in J774 cells and peritoneal macrophages following treatment with sPG and stimulation with LPS or IFN-γ + LPS was assessed by Western Blotting using antibodies specific for the active dual-phosphorylated forms of the ERKs, p38 and SAPK/JNK.

4:2:2:1 Modulation of ERK MAP kinase activation.

Treatment with sPG alone enhanced the activation of both ERK MAP kinase isoforms in J774 cells (Figure 4.10). Increased ERK2 activation was detectable by 30 seconds and ERK1 was enhanced by 1 minute after treatment. Activation of both ERK isoforms was maximal by 5 minutes and remained enhanced throughout a 30 minute timecourse. sPG also enhanced the LPS-induced activation of ERKs (Figure 4.11). As before, enhanced ERK phosphorylation was detectable by 30 seconds after stimulation. Activation of ERK1 was maximal by 5 minutes and ERK2 peaked at about 1 minute after stimulation. Thereafter, phosphorylation of both ERK isoforms declined towards basal levels by 10 minutes.

Similar results were obtained in murine peritoneal macrophages (Figures 4.12 and 4.13). Stimulation for 10 minutes with IFN-γ + LPS in the presence of sPG resulted in the enhanced phosphorylation of both ERK isoforms (Figure 4.12). Another experiment showed sustained enhancement of phosphorylation of both ERK isoforms throughout an 8 hour timecourse of stimulation (Figure 4.13).

4:2:2:2 Modulation of p38 and JNK MAP kinase activation.

Enhanced dual phosphorylation of p38 MAP kinase was detected in J774 cells treated with sPG alone or sPG + LPS for 20 minutes; sPG3 also enhanced p38 production (Figure 4.14). Similar results were observed following stimulation of murine peritoneal macrophages for 10 minutes with IFN-γ + LPS in the absence/presence of sPG (Figure

4.15A). In contrast, dual phosphorylation of JNK MAP kinases at this timepoint was not altered by sPG treatment (Figure 4.15B).

4:2:2:3 Effects of inhibition of ERK MAP kinase activation on sPG-mediated suppression of IL-12.

Since ERK MAP kinases transduce negative signals for the regulation of IL-12 p40 production, the enhancement of ERK activation by sPG could provide a mechanism for the sPG-mediated suppression of IL-12 production. To test this hypothesis the MEK inhibitor PD 098059 was used to investigate whether sPG could still inhibit IL-12 when ERK activation was blocked. As predicted, sPG was unable to suppress IL-12 p40 production following the pre-incubation of J774 cells or murine peritoneal macrophages with 20 μM PD 098059 for 1 hour prior to stimulation (Figure 4.16 and 4.17). However, neither PD 098059 nor U0126 pre-treatment rescued sPG-mediated suppression of bioactive IL-12 p70 production (Figure 4.18).

Taken together, these data demonstrate that sPG, and thus LPG, achieves its inhibition of IL-12 production by enhancing ERK MAP kinase-mediated suppression of IL-12 p40 production.

4:3 Discussion.

Many of the mechanisms employed by *Leishmania* sp. parasites to evade detection and killing by the host immune system are mediated by the major component of the parasite glycocalyx, LPG. In this study, a synthetic version of a portion of *Leishmania* LPG was used to investigate how this molecule suppresses the induction of IL-12, a key cytokine in the clearance of *Leishmania* infection.

Concurrent with a previous report from this laboratory (Piedrafita *et al.*, 1999), the data presented here demonstrate that the phosphoglycan (PG) portion of LPG is capable of suppressing IL-12 p40 production and that this results in lower levels of bioactive IL-12 p70 production (Figures 4.4 – 4.7). Interestingly, the results of this study and the previous report (Figure 4.9 and Piedrafita *et al.*, 1999) demonstrate that sPG does not mimic all the functional properties of LPG; while it was capable of suppressing IL-12 production, sPG did not alter NO induction. This provides further evidence that different structural regions of LPG make distinct contributions to the activity of this multifunctional molecule.

Many of the functions of LPG have been attributed to the PG portion. Indeed, delipidated PG of naturally-extracted LPG exacerbated infection in both resistant and susceptible mouse strains (Mitchell and Handman, 1986). The hexasaccharide core of LPG was not required for inhibition of IL-12, neither was the lipid anchor. Similarly, inhibition of macrophage microbicidal activity and IL-1 production is achieved by the PG portion of LPG, not the lipid anchor (Frankenburg *et al.*, 1990; Giorgione *et al.*, 1996). Preliminary data from this laboratory also showed that vaccination with sPG exacerbated

infection in mice challenged with *L. major* or *L. mexicana* (D. Piedrafita, personal communication). In contrast, inhibition of iNOS by GIPLs is dependent on the lipid anchor (Proudfoot *et al.,* 1995).

A regulatory role for LPG in the induction of iNOS, which results in the inhibition of NO production has been demonstrated, and this activity has been located to the phosphoglycan region (Proudfoot *et al.,* 1996; Liew *et al.,* 1997). sPG, however, did not alter NO production (Figure 4.9), indicating that these Gal-Man-phosphate repeats are not sufficient for this regulation of iNOS. A higher degree of polymerisation is present in LPG molecules and the PG is modified by the attachment of side chains, which vary according to species. It is therefore likely that these additional elements are responsible for modulation of iNOS. Although it appears that sPG is incapable of disrupting iNOS directly, inhibition of IL-12 by sPG would be expected to result in suppression of NO production by macrophages *in vivo* due to reduced IFN-γ production by T and NK cells.

The observation that sPG similarly inhibited IL-12 production by macrophages from both resistant (129) and susceptible (BALB/c) mouse strains (Figure 4.6) is consistent with demonstrations of IL-12 suppression by *L. major* promastigotes in BALB/c versus C3H and C57BL/6 strains (Carrera *et al.,* 1996). Therefore susceptibility to *Leishmania* infection is not due to a defect in IL-12 production or sensitivity to inhibition of IL-12. Rather, it has been proposed that susceptibility to infection may represent a loss of responsiveness of T cells to IL-12. This is suggested by the finding that T cells from BALB/c mice down-regulate IL-12R more rapidly than T cells from resistant mice (Guler *et al.,* 1996).

LPG has been shown to associate with macrophages by both complement-dependent and -independent interactions. It interacts directly via binding to mannose-fucose receptors, MFRs, and complement receptor 3, CR3 (reviewed by Bogdan and Röllinghoff, 1998). It also interacts indirectly via association with serum proteins such as complement factor 3 (C3) and C-reactive protein (CRP) which then bind their own receptors on the macrophage surface. Parasites subsequently enter macrophages by phagocytosis. It is tempting to speculate that sPG would interact with the MFR via its galactose-mannose subunits; indeed galactose and fucose (6-deoxy-galactose) are structurally very similar. This might also explain why such a small number of repeats are sufficient for binding and achieving IL-12 inhibition.

Alternatively, there is evidence in the literature to back up the suggestion that sPG inhibits IL-12 through interaction with CR3. Marth and Kelsall (1997) demonstrated that the induction of IL-12 by a variety of stimuli in human monocytes was specifically down-regulated following exposure to antibodies against CR3 as well as natural CR3 ligands iC3b and *Histoplasma capsulatum.* Thus sPG/LPG could suppress LPS/IFN-γ-induced IL-12 production via binding to and signalling through CR3. Indeed, this receptor has been shown to associate with CD14 in neutrophils following LPS stimulation, suggesting that it may be involved in regulation of LPS signals (Zarewych *et al.,* 1996). The recently identified TLRs, which recognise a variety of pathogen molecules, also represent candidate sPG/LPG receptors.

Sequential development of promastigotes from the non-infective (procyclic) form to the highly infective (metacyclic) form is accompanied by modification of LPG, in particular an increase in the number of phosphodisaccharide repeats (McConville *et al.,*

1992). While the metacyclic form inhibits IL-12 production from PBMCs; procyclic LPG induces high levels of IL-12 production from PBMCs (Sartori *et al.,* 1997). It has been suggested that the ability of promastigotes to inhibit IL-12 production by macrophages could depend, at least in part, on the presence of longer phosphodisaccharide repeats of PG. Consistent with this, Piedrafita *et al.* (1999) showed that a range of synthetic fragments of PG (L1-L5) containing up to three Gal-Man repeats were not effective inhibitors of IL-12. In this study however, a truncated version of sPG (sPG3), which had an average of 4.5 repeats, was capable of inhibiting IL-12 production (Figure 3.4B). This indicates that the binding of sPG and inhibition of IL-12 are dependent on neither a high degree of polymerisation of Gal-Man-phosphate repeats, nor a terminal phosphate group. The apparent differences between these studies could be explained by the presence of $(CH_2)_8CH{=}CH_2$ side chains on the L1-L5 molecules, which may have interfered with the binding of the Gal-Man subunit of such short molecules, thus preventing the inhibition of IL-12.

The inhibition of IL-12 by sPG also explains the apparent anomaly of the R2D2 mutant of *L. ~~major~~ donovani* which is deficient in LPG but still able to inhibit IL-12 release by murine macrophages (Carrera *et al.,* 1996). R2D2 parasites have incomplete LPG assembly but are still able to synthesise repeating phosphodisaccharide (Tolson *et al.,* 1989).

It has been suggested that induction of IL-10 production by LPS and/or LPG could account for suppression of IL-12 production. However, no IL-10 was detected in culture supernatants of J774 cells or peritoneal macrophages stimulated with any combination of sPG, IFN-γ and LPS (not shown). This is consistent with a previous

report, which showed that *L. major* promastigotes were capable of reducing IL-12 p40 production by macrophages from IL-10 knock out mice (Carrera *et al.,* 1996). Thus, IL-10 is not likely to be responsible for the down-regulation of IL-12 synthesis in *L. major*-infected macrophages or the inhibition of IL-12 production by macrophages exposed to sPG.

The results described in this chapter present a mechanism for the suppression of IL-12 p40 production by *Leishmania* LPG, resulting in lower levels of bioactive IL-12 p70 release. sPG treatment, both on its own and in combination with macrophage stimulatory signals, enhanced the activation of both the ERK1 and ERK 2 isoforms (Figures 4.10 – 4.13). Furthermore inhibition of ERK MAP kinase activation by pre-treatment with the MEK1 inhibitors PD 098059 and U0126 rescued macrophages from sPG-mediated IL-12 p40 suppression (Figures 4.16 and 4.17). Taken together, these results suggest that sPG achieves inhibition of IL-12 p70 production by enhancing the negative regulation of p40 transcription by ERK MAP kinases.

Interestingly, pre-treatment with neither PD 098059 nor U0126 ~~pre-treatment~~ rescued bioactive IL-12 production (Figure 4.18), indicating that increased production of the p40 subunit was not enough to rescue p70 production. Since ERK MAP kinases do not appear to regulate p35 production (see Figure 3.27), this suggests that sPG may also target production of the p35 subunit in an ERK-independent manner, or it may influence the assembly or release of bioactive cytokine. Nevertheless, these results demonstrate a mechanism for the suppression of IL-12 p40 production by sPG, resulting in lower levels of bioactive IL-12 p70 production.

The enhancement of p38 activation (Figures 4.14 and 4.15) may at first sight be a little confusing since p38 transduces positive signals for IL-12 production. However, sPG achieved suppression of IL-12 despite enhanced p38 activation. This is consistent with the hypothesis that p38 activation is required for negative regulation by ERKs, which act to disrupt p38 signals.

The upstream mechanisms of ERK activation and the downstream targets of this MAP kinase responsible for regulating IL-12 p40 production remain to be identified. It is possible that sPG alters the binding of different isoforms of NF-κB in a MAP kinase-independent manner, and that this leads for example to the preferential binding of inhibitory p50 homodimers. However, results presented in the previous chapter demonstrated that NF-κB does not transduce p38 or ERK MAP kinase signals. Consistent with this, a previous report suggested that sPG does not inhibit IL-12 by down-regulating the nuclear translocation of NF-κB and its binding to promoter sequences (Piedrafita *et al.*, 1999). Similarly, binding of the F1 binding complex, another potential target of sPG, did not appear to be altered by inhibition of ERK or p38 MAP kinases (Figure 3.43B). However, IRF synthesis was enhanced by inhibition of ERK activation, as was binding of IRF family members to the iNOS promoter sequence, suggesting that ERK MAP kinases mediate their downstream functions by modulating transcriptional regulation by IRF proteins. Hence, this family of transcription factors, which are important regulators of IL-12 production, represent the most likely downstream targets of ERK MAP kinases activated by sPG.

4:4 Summary of this study.

In summary therefore, this investigation has enabled the development of a model for the suppression of IL-12 production by *Leishmania* LPG (Figure 4.19). LPG binds to macrophages via its disaccharide repeats and transduces signals leading to the enhanced activation of ERK MAP kinases. ERKs negatively regulate LPS-induced IL-12 p40 transcription by disrupting p38 MAP kinase signalling. Production of the p35 subunit of IL-12 may also be suppressed by LPG in an ERK-independent manner. The inhibition of p40 and p35 subunits leads to a reduction in the formation and release of bioactive IL-12.

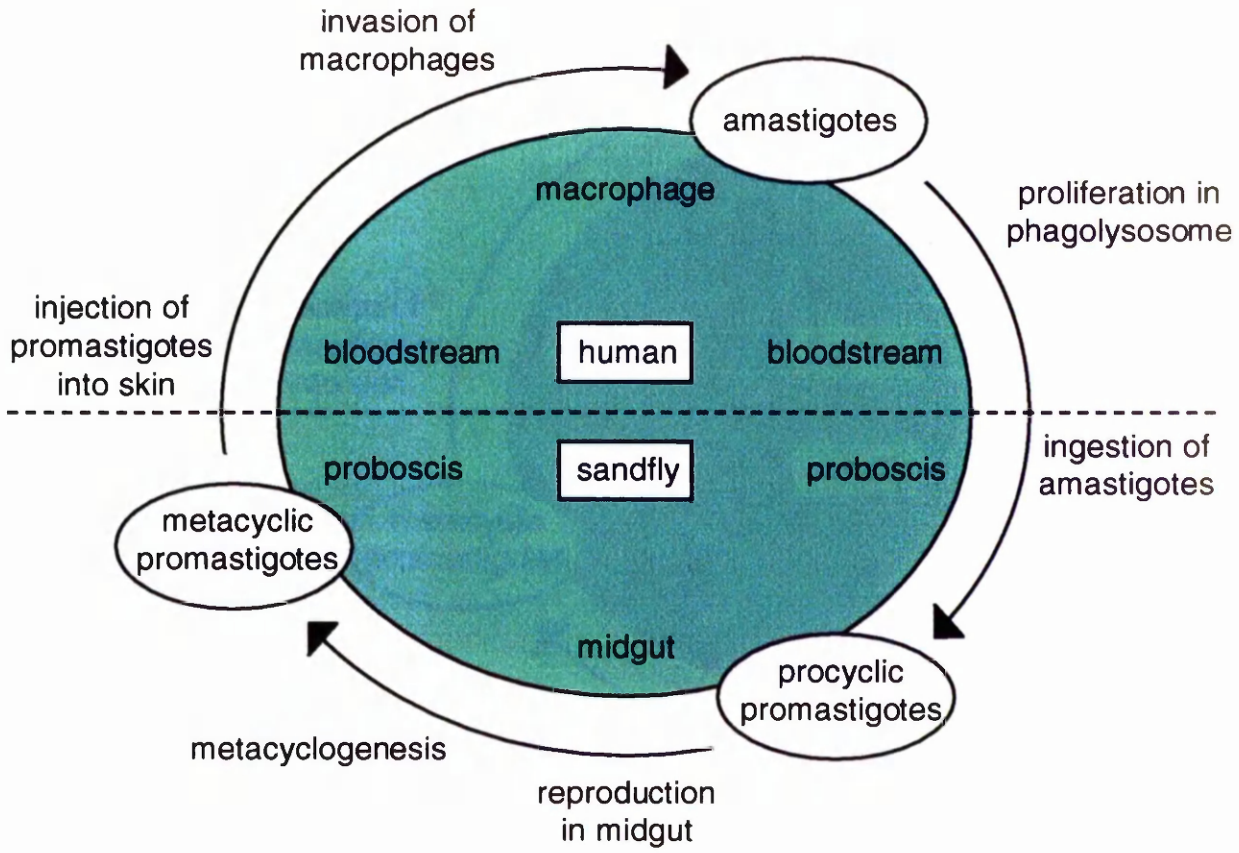
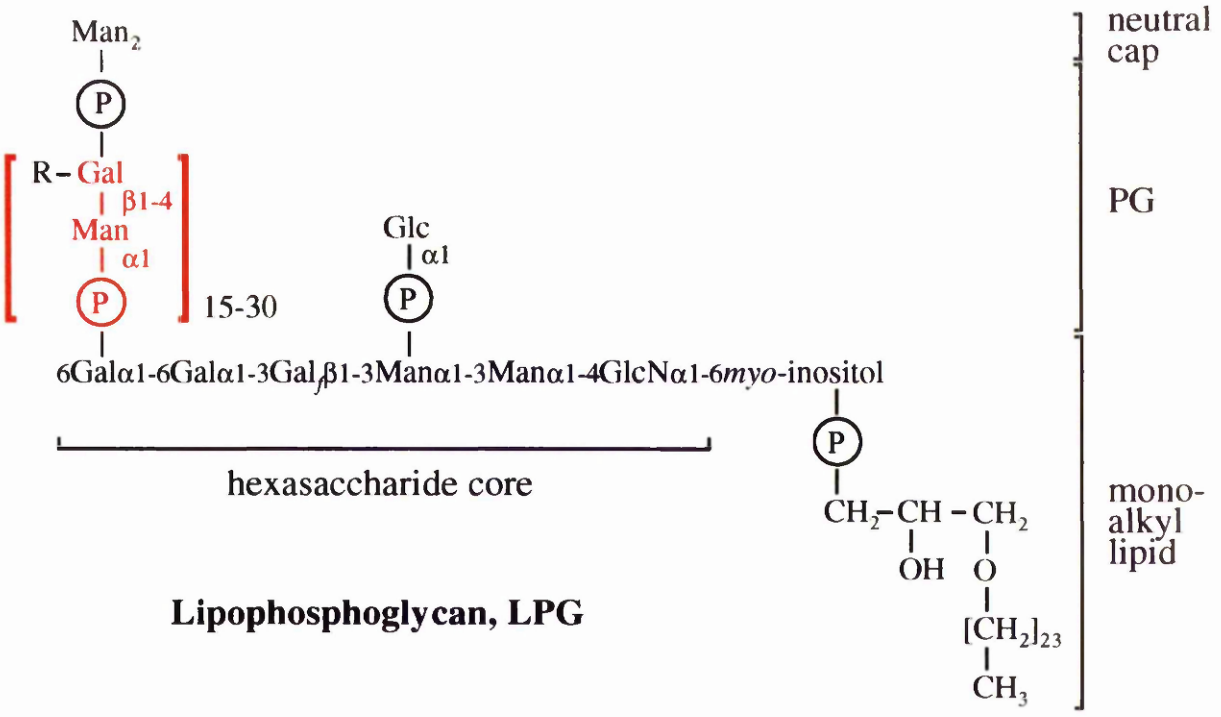


Figure 4.1. *Leishmania* lifecycle.

Typical lifecycle of *Leishmania* sp. parasites. Extracellular flagellated promastigotes reproduce in the midgut of *Phlebotomus* and *Lutzomyia* sandflies. In a process called metacyclogenesis they cease dividing, detach from epithelial cells and migrate to the insect mouthparts. When the sandfly feeds, metacyclic promastigotes are injected into the skin of the mammalian host (e.g. human) and enter the bloodstream. Parasites invade macrophages and differentiate into non-flagellated amastigotes in the phagolysosome. In this hostile environment, amastigotes are able to proliferate and are released by lysis of the host cell to infect other macrophages. Infected macrophages are ingested by feeding sandflies and parasites transform to the promastigote stage again to continue the cycle.



sPG	H-[(6)Gal(β1-4)Man(α1)-$PO_3H.NH_3$]$_{10}$*-OH.NH_3 * = approximate average degree of polymerisation
sPG3	H-[(6)Gal(β1-4)Man(α1)-$PO_3H.NH_3$]$_{3.5}$*- (6)Gal(β1-4)Man-OH.NH_3 * = approximate average degree of polymerisation

Figure 4.2. Chemical structures of *Leishmania* LPG, sPG and sPG3.

LPG comprises a monoalkyl lipid, a hexasaccharide core, a phosphoglycan (PG) region and a neutral cap (adapted from Mengeling *et al.*, 1997). The R side chain and the oligosaccharide cap structure vary according to species (most prevalent *L. donovani* cap shown). The synthetic version, sPG represents the PG (highlighted red) region and is a polymer of galactose-mannose-phosphate repeated an average of 10 times (Nikolaev *et al.*, 1995). Similarly, sPG3 is a shorter PG fragment which additionally lacks the terminal phosphate group.

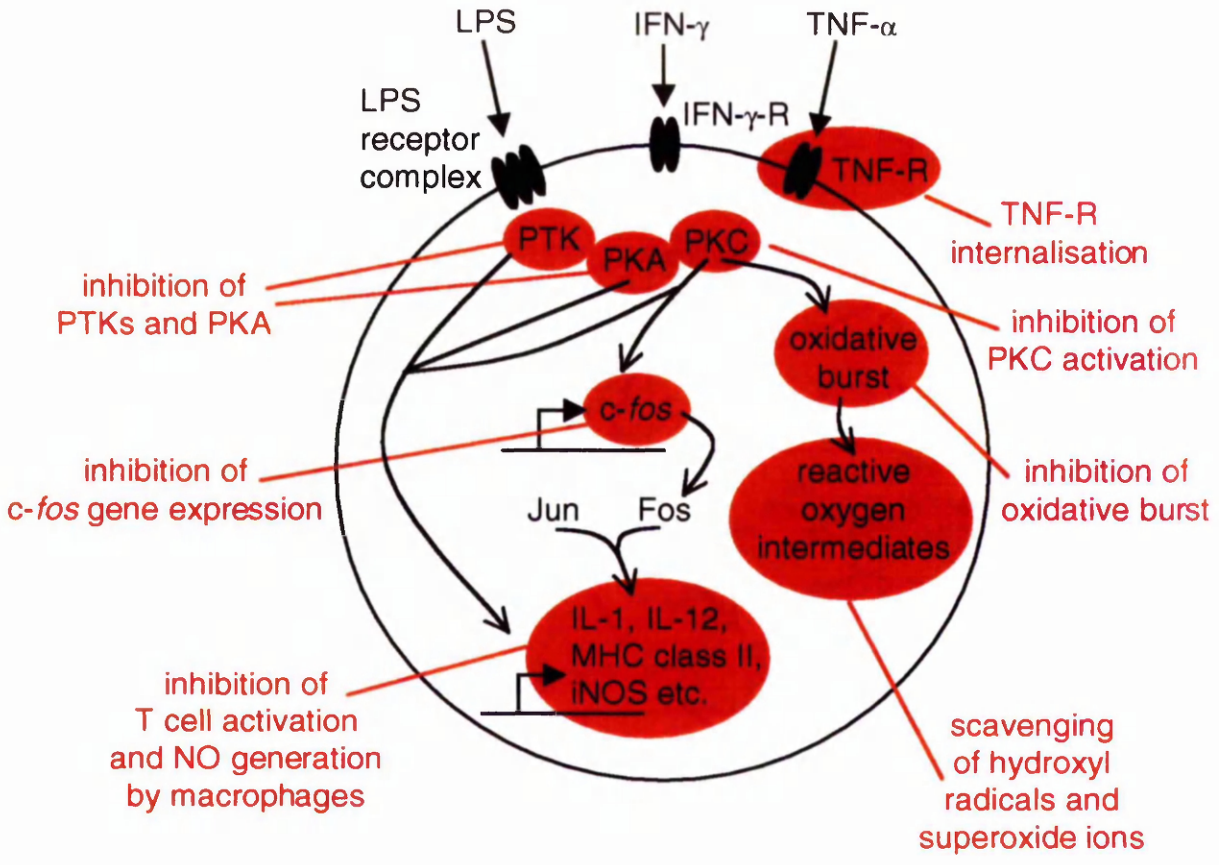


Figure 4.3. Suppression of macrophage function by *Leishmania* LPG.

Leishmania parasites inhibit activation of macrophage responses to evade detection and suppress the host's immune attack against them. The parasite surface molecule lipophosphoglycan (LPG) has been shown to interfere with many macrophage signalling pathways to suppress gene expression and limit the effects of other macrophage responses such as the oxidative burst (reviewed by Turco and Descoteaux, 1992).

PTK = protein tyrosine kinases; PKA = protein kinase A; PKC = protein kinase C.

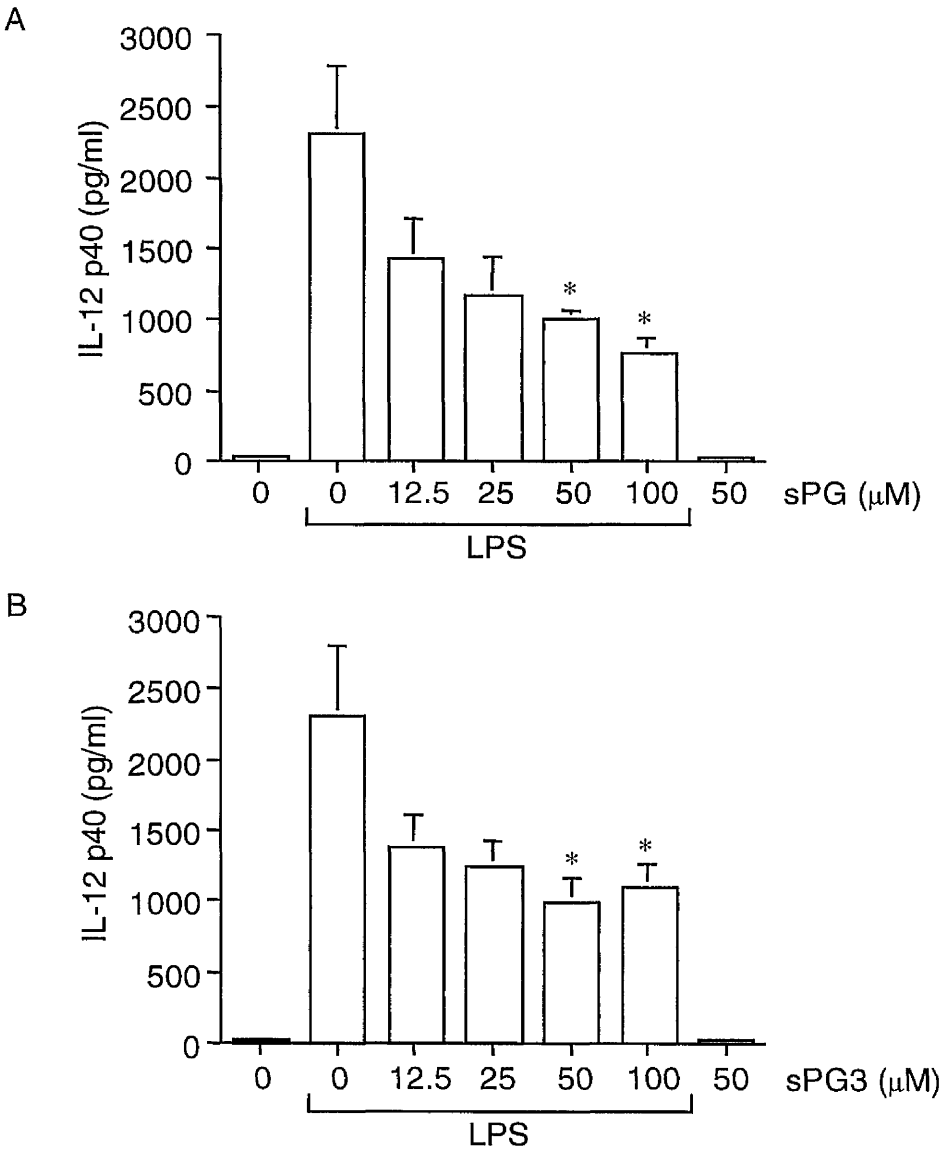


Figure 4.4. Effects of sPG and sPG3 on IL-12 p40 production by J774 cells.

J774 cells ($2x10^5$/well in a 96-well plate) were stimulated with 1 μg/ml LPS in the presence of different concentrations of sPG, or the shorter fragment sPG3, for 24 hours and IL-12 p40 (monomer, active p70 heterodimer, p40/p40 homodimer) in culture supernatants was measured by ELISA. * $p < 0.05$

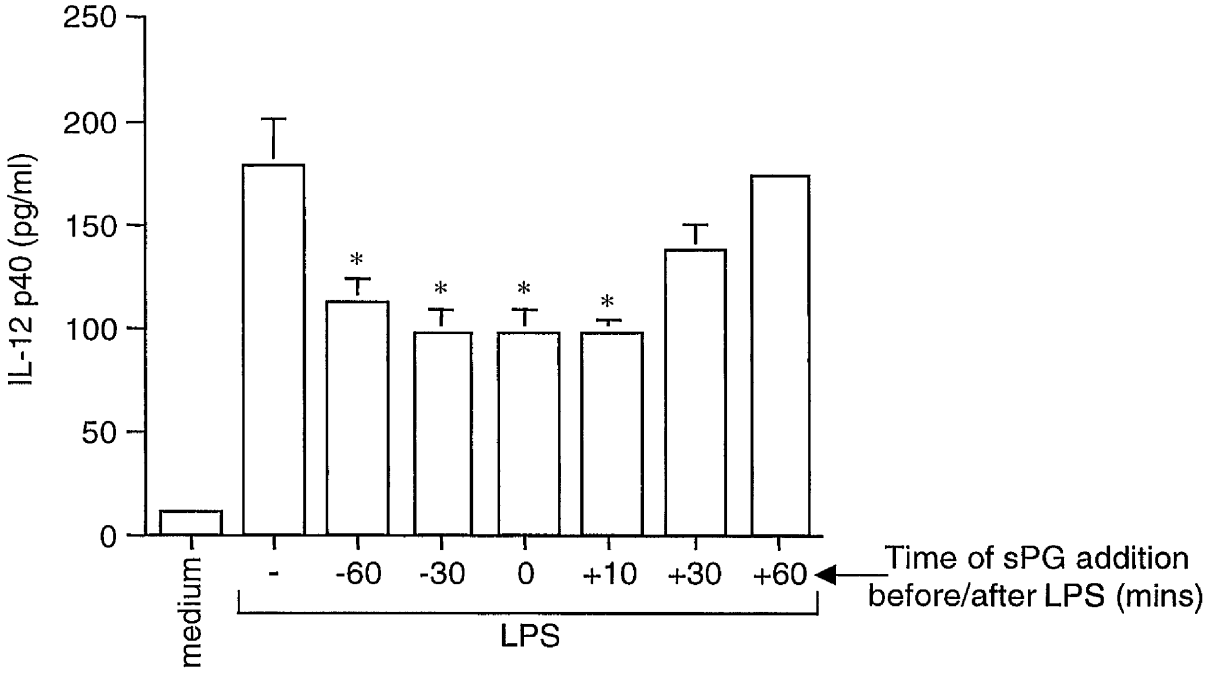


Figure 4.5. Time-dependent inhibition of LPS-induced IL-12 p40 production by sPG.

J774 cells ($2x10^5$/well in a 96-well plate) were stimulated with 1 μg/ml LPS in the presence of 50 μM sPG for 24 hours. sPG was added at the times indicated relative to LPS stimulation, and IL-12 p40 (monomer, active p70 heterodimer, p40/p40 homodimer) in culture supernatants was measured by ELISA. * $p < 0.05$

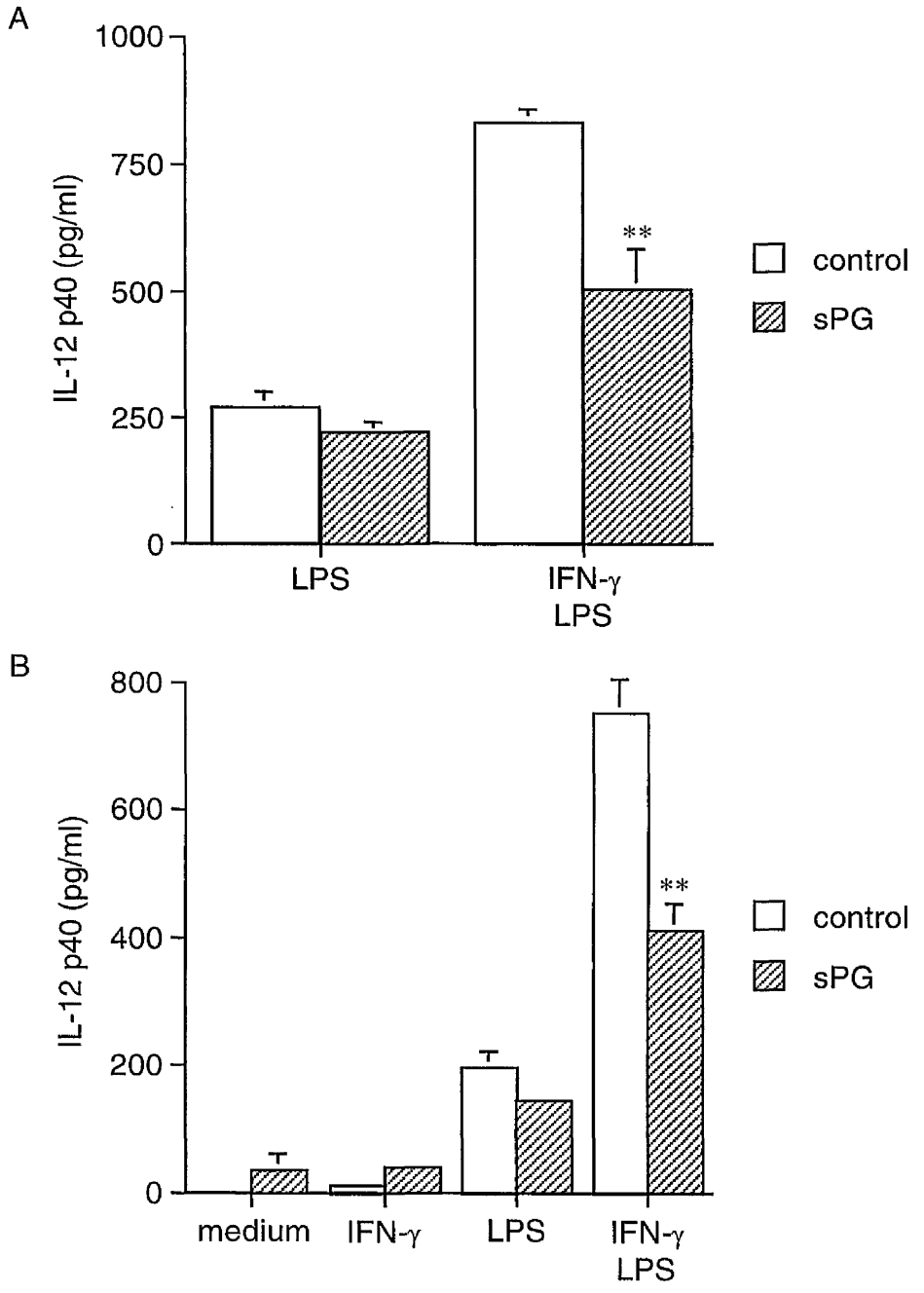


Figure 4.6. Effect of sPG on IL-12 p40 production by peritoneal macrophages.

Thioglycollate-elicited peritoneal macrophages from *Leishmania*-susceptible BALB/c (A) and -resistant 129 (B) mice ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS in the presence/absence of 50 μM sPG. IL-12 p40 (monomer, active p70 heterodimer, p40/p40 homodimer) in 24 hour culture supernatants was assayed by ELISA. ** $p < 0.01$

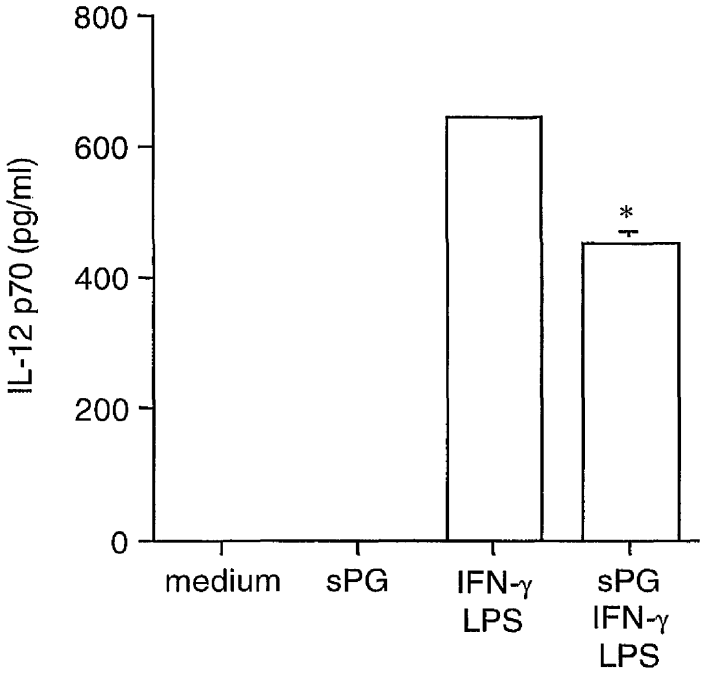


Figure 4.7. Effect of sPG on IL-12 p70 production by peritoneal macrophages.

Thioglycollate-elicited peritoneal macrophages from *Leishmania*-susceptible BALB/c mice ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS in the presence/absence of 50 μM sPG. Bioactive IL-12 p70 in 24 hour culture supernatants was assayed by ELISA. * $p < 0.05$

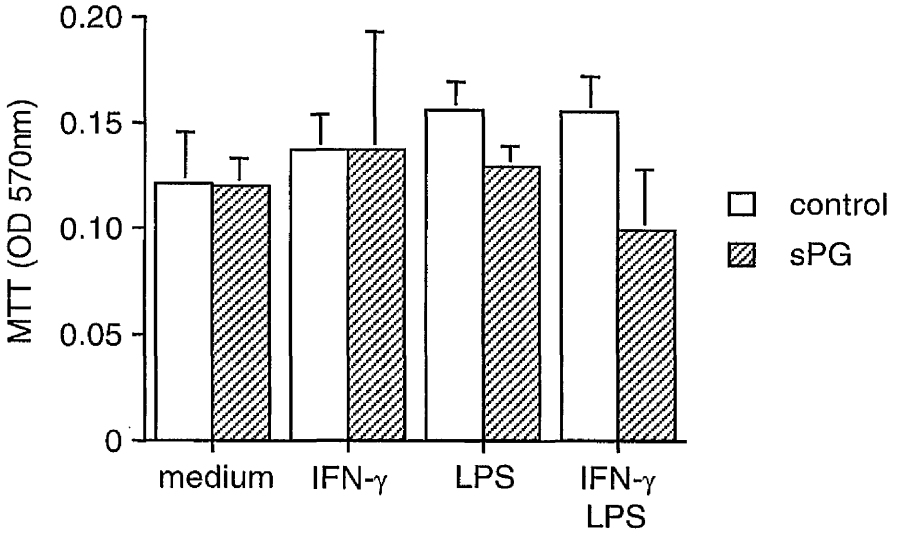


Figure 4.8. Effects of sPG treatment on cell viability.

Thioglycollate-elicited peritoneal macrophages ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS in the presence/absence of 50 μM sPG. The viability of macrophages following treatment was assessed by MTT assay.

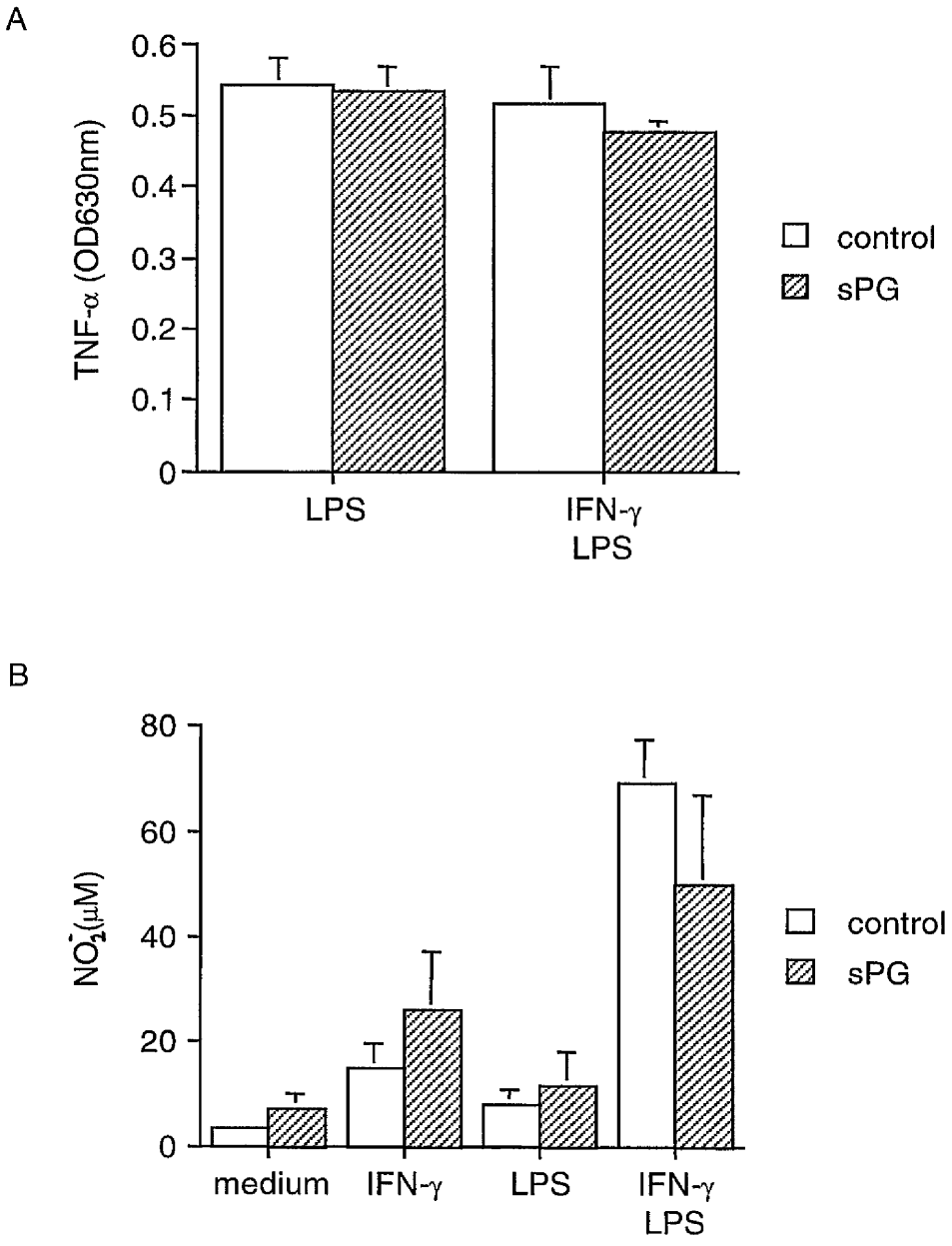


Figure 4.9. Effects of sPG on TNF-α and NO production by peritoneal macrophages.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS in the presence/absence of 50 μM sPG. TNF-α and NO in 24 hour culture supernatants were assayed by ELISA and Griess Reaction respectively.

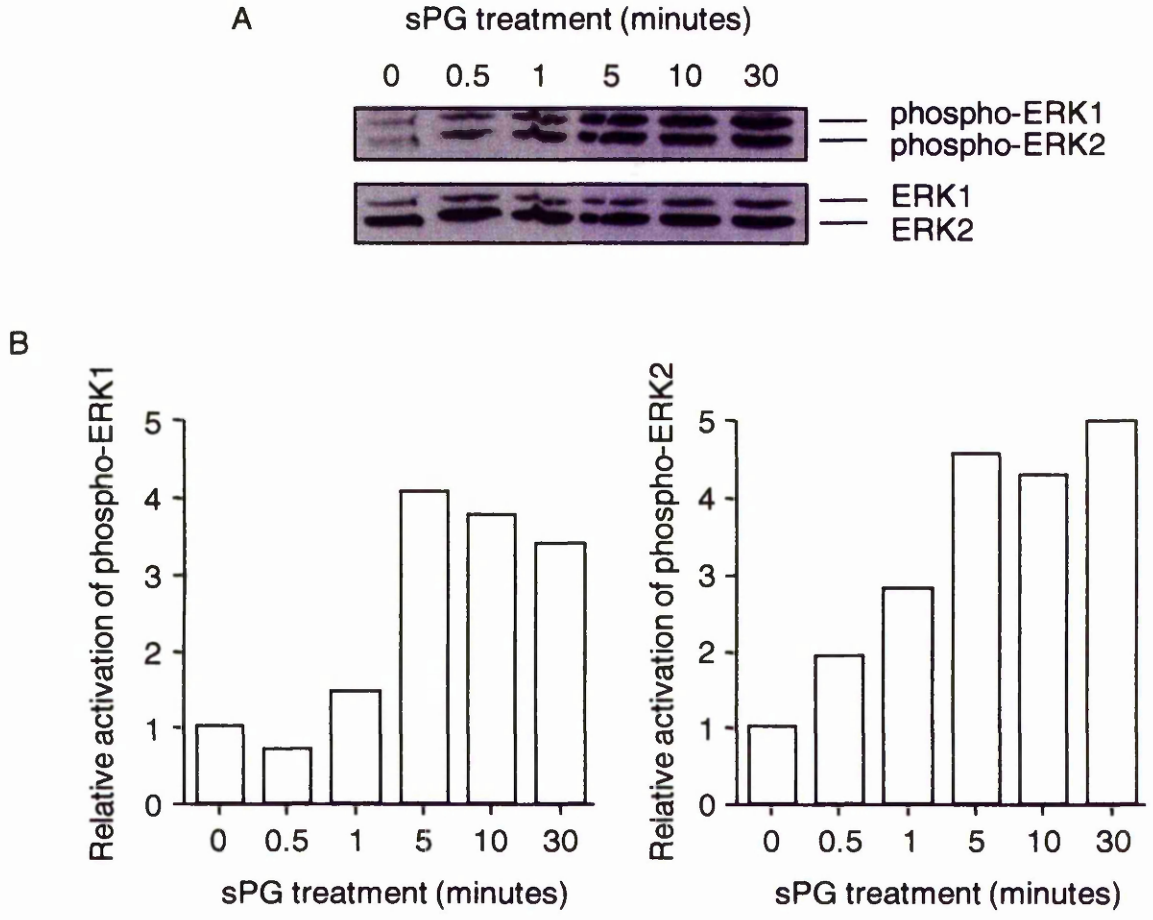


Figure 4.10. Effect of sPG on ERK MAP kinase activation in J774 cells.

J774 cells ($4x10^6$/25 cm^2 flask) were stimulated for the times indicated with 50 μM sPG. Dual phosphorylated ERK MAP kinases were detected by Western Blotting of total protein lysates (A, upper panel). Total ERK MAP kinase protein was also detected as a loading control (A, lower panel). Densitometric values (B) are phospho-ERK:total ERK protein ratios.

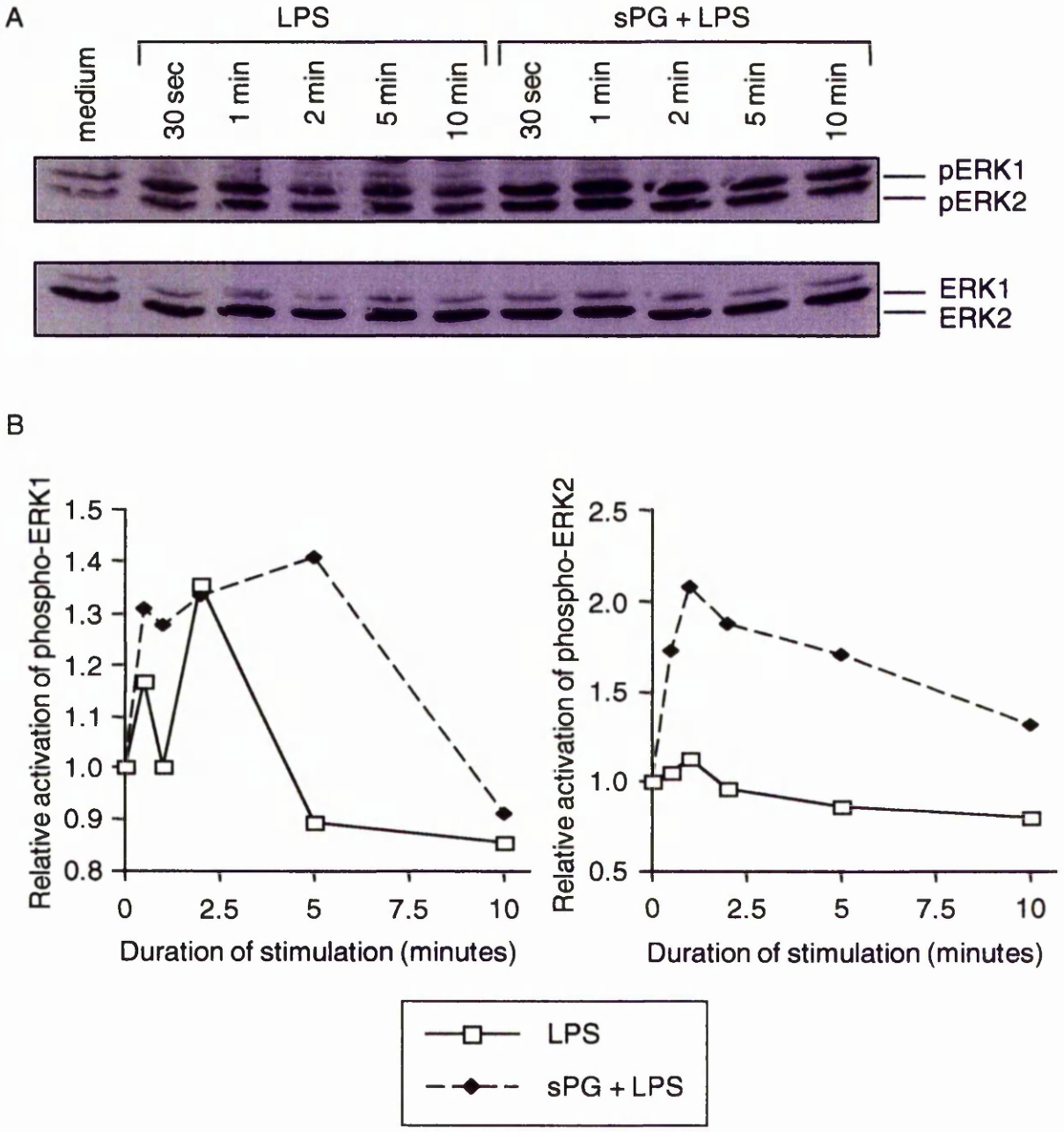


Figure 4.11. Effect of sPG on LPS-induced ERK MAP kinase activation in J774 cells.

J774 cells ($4x10^6$/25 cm^2 flask) were stimulated for the times indicated with 1 μg/ml LPS in the absence/presence of 50 μM sPG. Dual phosphorylated ERK MAP kinases (pERK) were detected by Western Blotting of total protein lysates (A, upper panel). Total ERK MAP kinase protein was also detected as a loading control (A, lower panel). Densitometric values (B) are phospho-ERK:total ERK protein ratios.

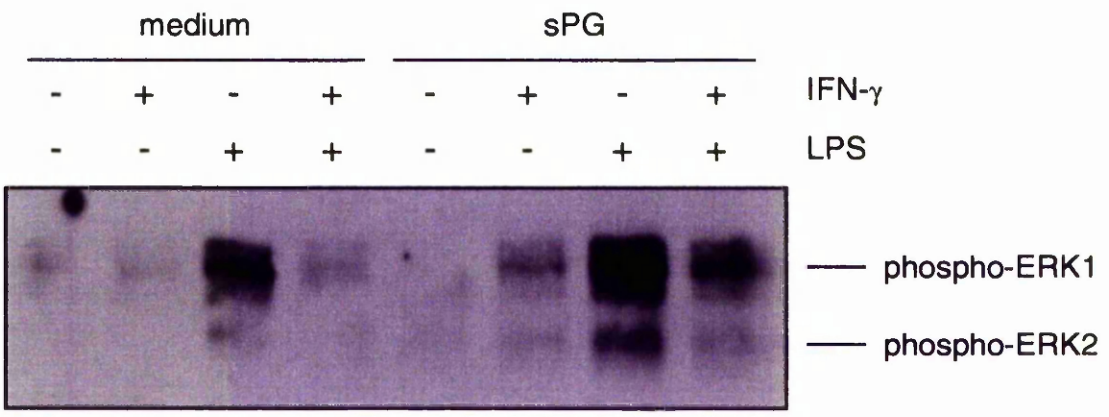


Figure 4.12. Effect of sPG on ERK MAP kinase activation in murine peritoneal macrophages.

Peritoneal macrophages from BALB/c mice ($4x10^6$/25 cm^2 flask) were stimulated for 10 minutes with 100 U/ml IFN-γ and 100 ng/ml LPS in the absence/presence of 50 μM sPG. Dual phosphorylated ERK MAP kinases were detected by Western Blotting of total protein lysates.

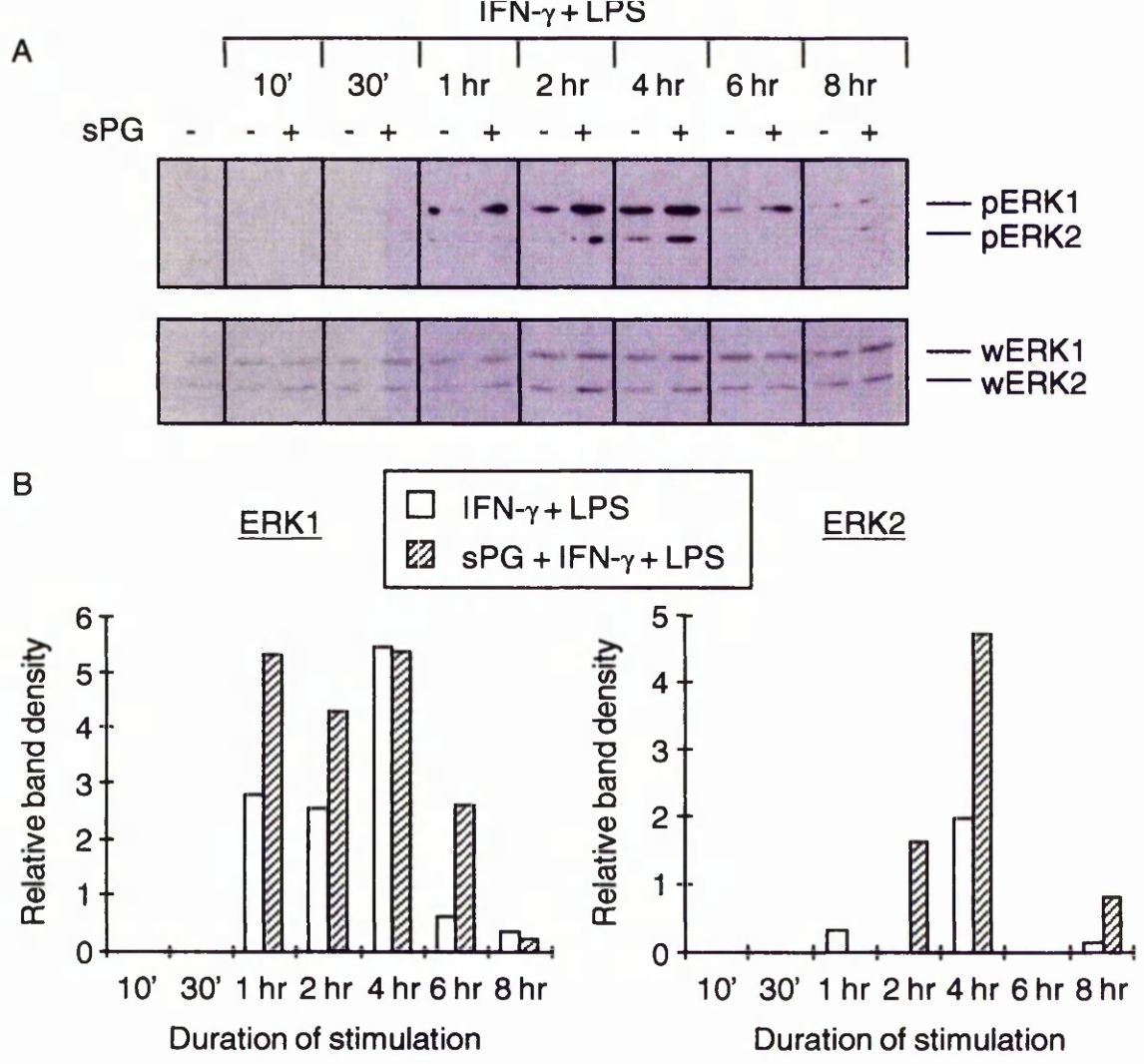


Figure 4.13. Effect of sPG on timecourse of IFN-γ + LPS-induced ERK MAP kinase activation.

Murine peritoneal macrophages ($4x10^6$/25 cm^2 flask) were stimulated with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated in the absence/presence of 50 μM sPG. Total protein lysates were purified, separated by SDS PAGE and blotted on to nitrocellulose. The blot was probed with anti-pTyr-pThr ERK1/2 antibody to detect dual-phosphorylated active ERK1 and ERK2 (pERK; A, upper panel). N.B. The film was underexposed to enable differences at intermediate timepoints to be detected. The blot was stripped and reprobed with an antibody against total ERK protein as a gel loading control (wERK; A, lower panel). Densitometric values (B) are phospho-ERK:total ERK protein ratios.

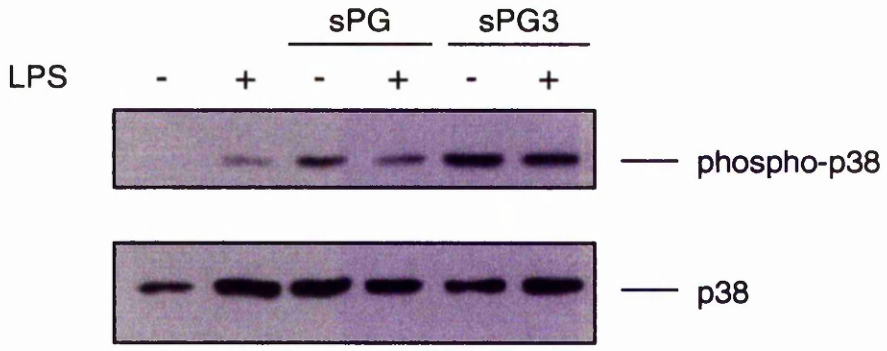


Figure 4.14. Effects of sPG and sPG3 on p38 MAP kinase activation in J774 cells.

J774 cells ($4x10^6$/25 cm^2 flask) were stimulated with 100 U/ml IFN-γ and 1 μg/ml LPS for 20 minutes in the absence/presence of 50 μM sPG or sPG3. Total protein lysates were purified, separated by SDS PAGE and blotted on to nitrocellulose. The blot was probed with anti-pTyr-pThr p38 antibody to detect dual-phosphorylated active p38 (upper panel). The blot was stripped and reprobed with an antibody against total p38 protein as a gel loading control (lower panel).

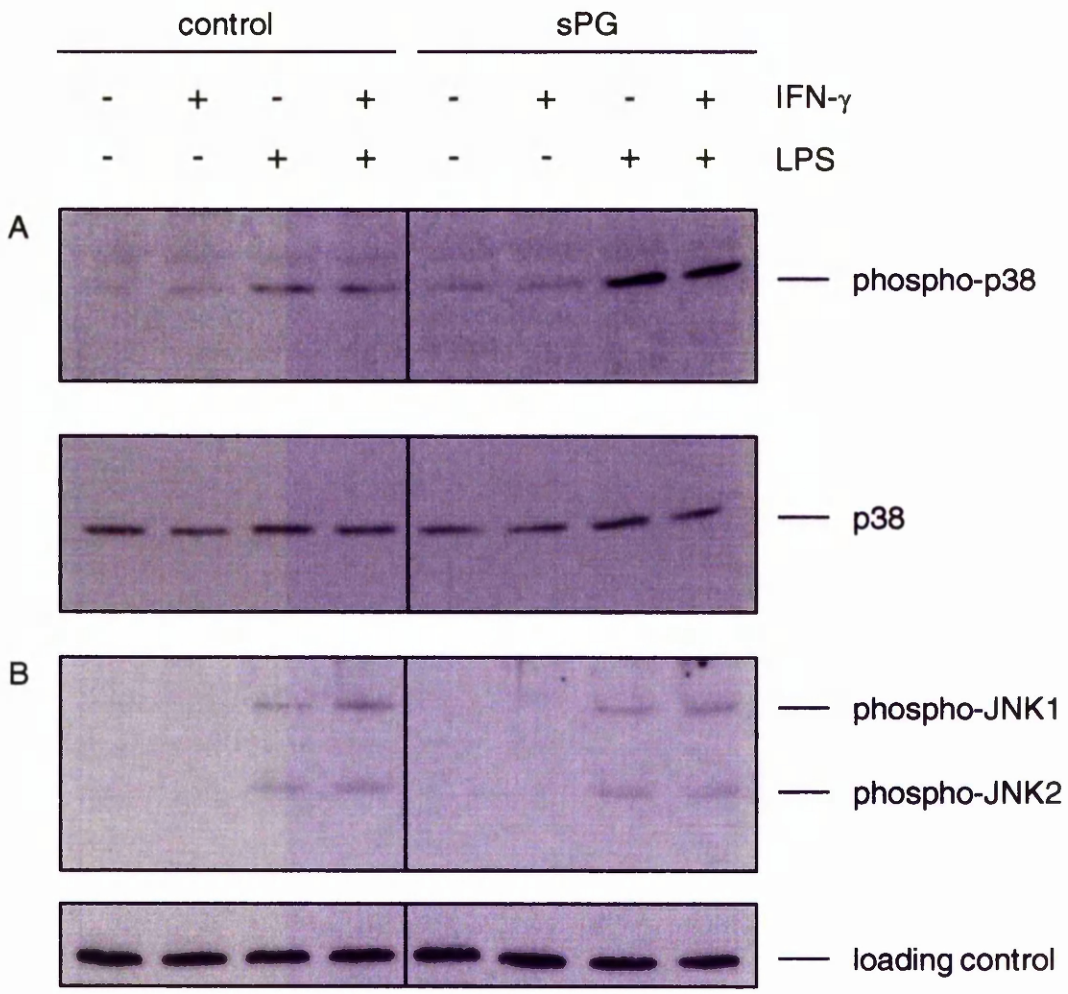


Figure 4.15. Effects of sPG on p38 and JNK MAP kinase activation in murine peritoneal macrophages.

BALB/c peritoneal macrophages ($4x10^6$/25 cm^2 flask) were stimulated with 100 U/ml IFN-γ and 100 ng/ml LPS for 10 minutes in the absence/presence of 50 μM sPG. Total protein lysates were purified, separated by SDS PAGE and blotted on to nitrocellulose. The blots were probed with anti-pTyr-pThr p38 (A) or JNK (B) antibodies to detect dual-phosphorylated active MAP kinase (upper panels). The blots were stripped and reprobed with an antibody against total p38 protein as a gel loading control (lower panels).

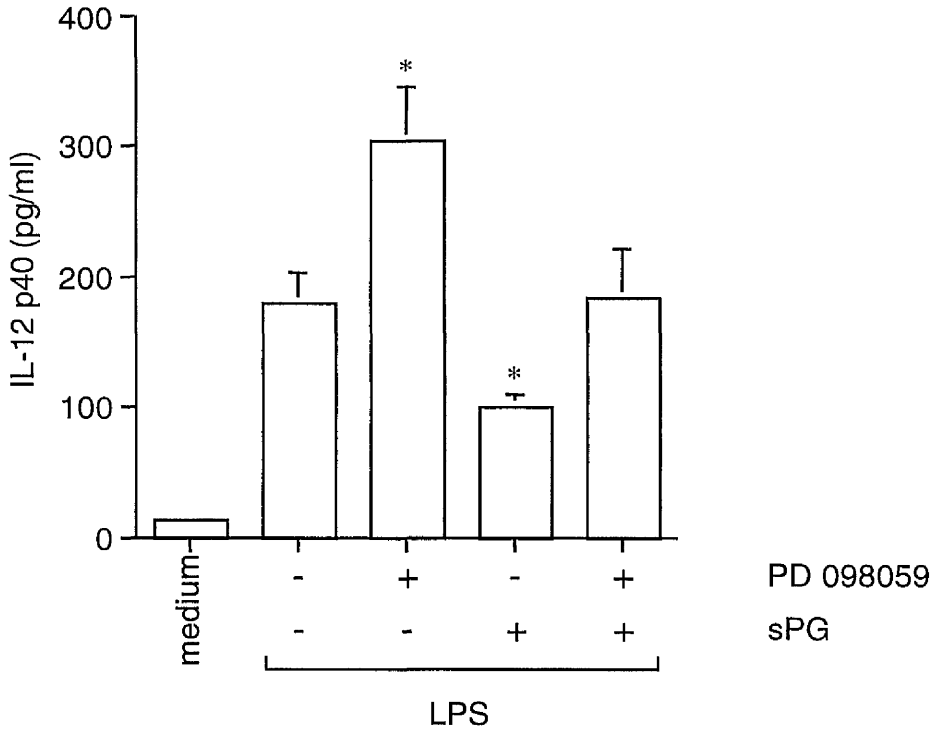


Figure 4.16. Effect of inhibition of ERK MAP kinases on sPG-mediated suppression of IL-12 p40 production by J774 cells.

J774 cells ($2x10^5$/well in a 96-well plate) were pre-incubated with 20 μM PD 098059 (PD) prior to stimulation with 1 μg/ml LPS in the absence/presence of 50 μM sPG. IL-12 p40 in 24 hour culture supernatants was assayed by ELISA. * $p < 0.05$

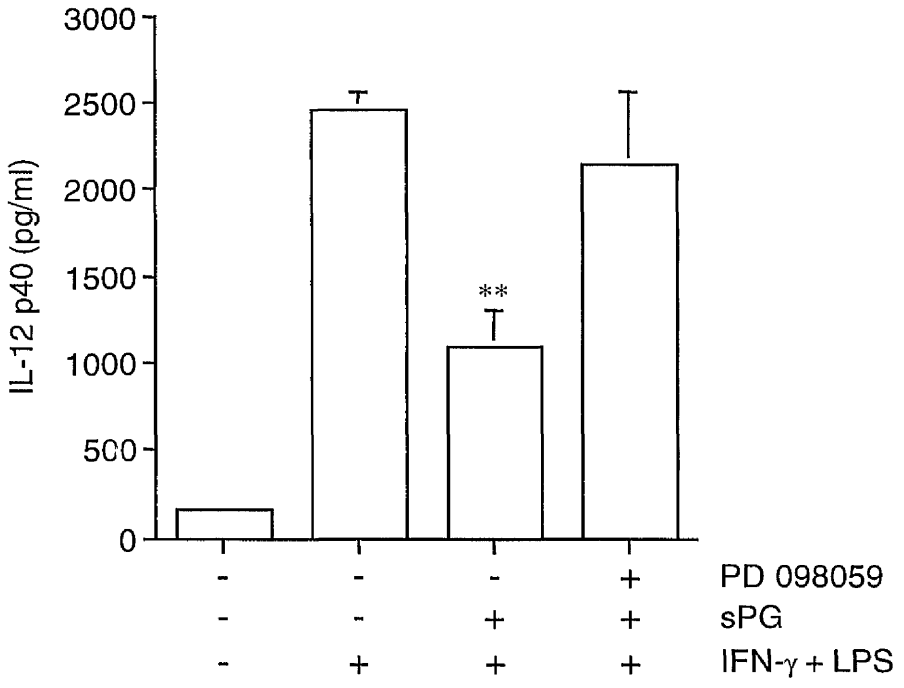


Figure 4.17. Effect of inhibition of ERK MAP kinases on sPG-mediated suppression of IL-12 p40 production by murine peritoneal macrophages.

BALB/c peritoneal macrophages ($2x10^5$/well in a 96-well plate) were pre-incubated with 20 μM PD 098059 (PD) for 1 hour prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS in the absence/presence of 50 μM sPG. IL-12 p40 in 24 hour culture supernatants was assayed by ELISA. ** $p < 0.01$

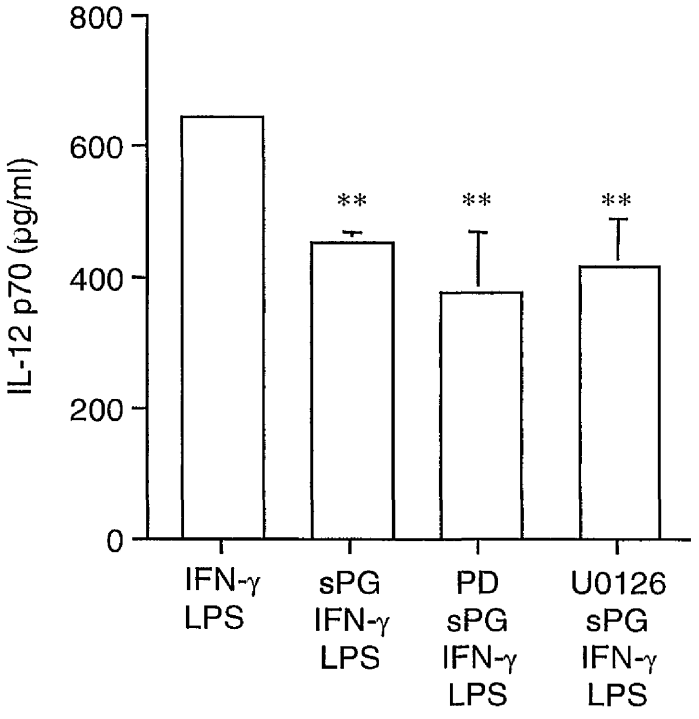


Figure 4.18. Effect of inhibition of ERK MAP kinases on sPG-mediated suppression of IL-12 p70 production by murine peritoneal macrophages.

BALB/c peritoneal macrophages ($2x10^5$/well in a 96-well plate) were pre-incubated with 20 μM PD 098059 (PD) or 1 μM U0126 for 1 hour prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS in the presence/absence of 50 μM sPG. IL-12 p70 in 24 hour culture supernatants was assayed by ELISA. ** $p < 0.01$

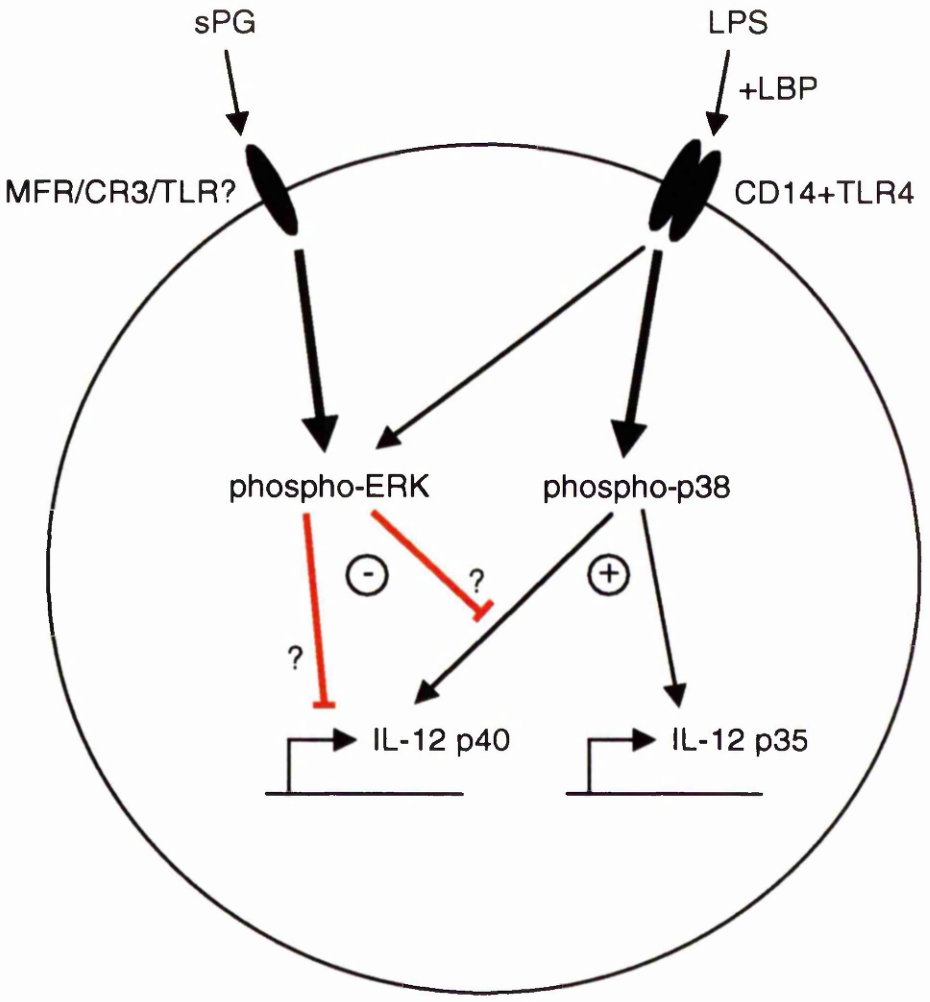


Figure 4.19. Model for suppression of macrophage IL-12 production by *Leishmania* LPG.

Leishmania LPG binds to macrophages and transduces signals resulting in the enhanced activation of ERK MAP kinases, which negatively regulate LPS-induced IL-12 p40 production, possibly by disrupting p38 MAP kinase signalling. Production of the p35 subunit of IL-12 may also be suppressed by sPG.

Chapter 5 – Modulation of macrophage cytokine production by an excretory-secretory (ES) product of the filarial nematode *Acanthocheilonema viteae*.

5:1 Introduction.

5:1:1 Filarial nematodes and disease.

Filarial nematodes are arthropod-transmitted parasites of vertebrates. Eight species infect humans, and three - *Wuchereria bancrofti*, *Brugia malayi*, and *Onchocerca volvulus* - are of major clinical importance. It is currently estimated that ~140 million people are infected with these three filarial nematodes and more than a billion people are at risk (WHO 2000a). Filarial nematodes cause a range of diseases known collectively as filariasis, which includes lymphatic filariasis and onchocerciasis (WHO 2000a). Lymphatic filariasis is caused principally by *W. bancrofti* and *Brugia* sp. parasites. Visible manifestations of this disease include swollen limbs and genitals, hence this disease is commonly known as elephantiasis. This is accompanied by internal damage to the kidneys and lymphatic system. Localised acute inflammation can also occur alongside chronic lymphoedema, as a result of the host's immune response to the parasite or due to bacterial infection of areas where lymphatic damage has occurred and normal defences have been partially lost. Infection with *O. volvulus* can result in chronic debilitating skin lesions and blindness.

Parasites are transmitted to mammalian hosts by mosquitoes (*W. bancrofti* and *B. malayi*) or blackflies (*O. volvulus*). They develop inside the vector from microfilariae to infective larvae, before migrating to the mouthparts from where they are transferred into a new host when the insect feeds (see Figure 5.1; reviewed by Rajan and Gundlapalli, 1997). In hosts such as humans, *W. bancrofti* and *B. malayi* worms continue to grow in the lymphatic vessels where they can survive for more than 5 years (Vanamail *et al.*,

1996). They reproduce sexually to produce millions of microfilariae that circulate in the blood and are picked up by the insect vector to continue the cycle.

In regions where infection is endemic, parasites are generally acquired in childhood, but disease may take many years to manifest itself. Asymptomatic infection is characterised by the presence of thousands of microfilariae in the bloodstream and adult worms in the lymphatics. Some individuals may appear outwardly healthy although they have hidden lymphatic pathology and kidney damage.

5:1:2 Immunosuppression of hosts.

The longevity of survival in the host may, at least in part, reflect the ability of parasites to induce defects in host immune responsiveness, including modulation of both parasite-specific and more generalised B and T cell responses, with a bias towards a type 2 T helper (Th2) cell response (Nutman *et al.,* 1987a and b; Ottesen, 1984; Kwa and Mak, 1984; Haque and Capron, 1986; King and Nutman, 1991; Maizels and Lawrence, 1991). For example, Mahanty and Nutman (1995) showed that in humans, filarial infection reduces the ability of peripheral blood mononuclear cells to proliferate and produce IFN-γ in response to parasite antigen, and increases the production of IL-10. This aids the survival of the parasite in the host for long periods. Induction of a Th1 response results in inflammation and elephantiasis, which is detrimental to the parasite as well as the host.

Exposure of experimental animals to filarial antigens results in a brief period of vigorous responsiveness, followed by induction of a state of hyporesponsiveness of lymphocytes (Weiss, 1978; Soboslay *et al.,* 1991). Responses to non-parasite antigens may or may not be normal (Mahanty and Nutman, 1995; Stewart *et al.,* 1999). Hyporesponsiveness generally occurs around the onset of patency and was thus originally attributed to microfilariae, which are released at this time (Maizels and Lawrence, 1991). Although microfilarial molecules have been shown to inhibit lymphocyte proliferation (Wadee *et al.,* 1987), some studies have reported lymphocyte hyporesponsiveness prior to the onset of patency (Weiss, 1978; Soboslay *et al.,* 1991), suggesting that molecules released by adult worms must be responsible.

5:1:3 Excretory-secretory products.

Filarial nematodes release a number of proteins into their environment, some of which are biologically active and are considered to play roles in the maintenance of infection and parasite survival (Harnett and Parkhouse, 1995). These include proteinases, protease inhibitors and antioxidants, and a homologue of the human pro-inflammatory cytokine macrophage inhibitory factor (MIF) which is chemotactic for macrophages (Pastrana *et al.,* 1998). Sera from infected humans or animals possess similar inhibitory properties to those observed with active infection, including suppression of lymphocyte proliferation (Weiss, 1978; Piessens *et al.,* 1980; Lammie *et al.,* 1984; Haque *et al.,* 1983). Such serum samples contain excretory-secretory (ES) molecules released by worms (Weil, 1990; Bradley and Unnasch, 1996). ES products in the bloodstream have ample opportunity to interact with host lymphocytes and are therefore strong candidates

for mediators of immunosuppression. Indeed, ES products in the bloodstream of humans are more readily detected in patients with detectable microfilariae (Harnett *et al.,* 1999a) who generally represent the most hyporesponsive category of infected individual (Maizels and Lawrence, 1991). Furthermore, studies with the rodent filarial nematode *Acanthocheilonema viteae* indicate that there is a large increase in production of ES just prior to the onset of patency (Harnett *et al.,* 1989).

Dasgupta *et al.* (1987) showed that in filariasis patients, cellular unresponsiveness was related to the presence of circulating parasite antigen, and Marley *et al.* (1995) demonstrated an inverse relationship between ES levels and total antibody in the bloodstream of humans infected with *W. bancrofti*. Studies in rodents revealed a correlation between chronic infection with *B. pahangi* or *A. viteae*, which is accompanied by the presence of large quantities of parasite antigen in the host circulation (Wenger *et al.,* 1988; Harnett *et al.,* 1989; Harnett *et al.,* 1990), and inhibition of specific proliferative responses by sera (Lammie *et al.,* 1984; Weiss, 1978).

Direct evidence of an immunomodulatory role for ES came from observations that ES products from *W. bancrofti* microfilariae inhibited leukocyte migration in patients with clinical filariasis (Prasad and Harinath, 1987). Similarly, ES products from microfilariae and adult female worms of *O. volvulus* suppressed proliferative responses of lymphocytes from onchocerciasis patients (Elkhalifa *et al.,* 1991). Furthermore, injection of ES into *B. pahangi*-infected dogs resulted in loss of antigen-driven cellular proliferative responses (Miller *et al.,* 1991). The major ES product of the rodent filarial nematode *Acanthocheilonema viteae*, ES-62, has also been demonstrated to inhibit lymphocyte proliferation (Harnett and Harnett, 1993; Harnett *et al.,* 1998).

A common feature of ES products, and indeed a variety of pathogen products, is the presence of phosphorylcholine (PC) attached to N-linked carbohydrates (Houston *et al.,* 1997; Harnett and Harnett, 1999). This PC has been suggested to be responsible for many of the immunomodulatory activities of ES products. Indeed, inhibition of phytohaemaglutinin-induced T cell proliferation *in vitro* by a PC-containing product of *B. malayi* was mimicked by PC conjugated to BSA (Lal *et al.,* 1990). Similarly, the inhibitory effects on B cells of ES-62, which contains PC, could be mimicked by PC-BSA (Harnett and Harnett, 1993; Deehan *et al.,* 1998). ES-62 and PC-BSA have therefore been used to investigate the immunomodulatory effects of filarial ES products and determine the involvement of the PC moiety.

5:1:4 Immunomodulation by ES-62, an ES product of *Acanthocheilonema viteae.*

ES-62 is a 62 kDa, PC-containing glycoprotein produced by the rodent filarial nematode *Acanthocheilonema viteae* (reviewed by Harnett and Harnett, 1999). Harnett and colleagues have previously demonstrated the effects of ES-62 on lymphocyte activation via their antigen (Ag) receptors and the differentiation of dendritic cells (Harnett and Harnett, 1993; Deehan *et al.,* 1997; Deehan *et al.,* 1998; Harnett *et al.,* 1998; Whelan *et al.,* 2000). They have shown that ES-62 inhibits Ag receptor-driven B cell proliferation (Harnett and Harnett, 1993) by inducing abortive, non-productive activation of certain signalling elements, such as protein tyrosine kinases (PTKs) and mitogen-activated protein (MAP) kinases, thereby desensitising cells to subsequent sustained proliferative signalling via the B cell Ag receptor complex (BCR) (Deehan *et al.,* 1998). ES-62 also achieves this inhibition of BCR-mediated proliferation by suppressing

phosphoinositide-3-kinase (PI-3-K) (Deehan *et al.,* 1997), and down-regulating protein kinase C (PKC) isoforms by stimulating their proteolytic degradation and disrupting their normal activation and nuclear translocation (Deehan *et al.,* 1998).

Similarly, ES-62 inhibited anti-CD3-stimulated activation of the human Jurkat T cell line by disrupting coupling of the T cell receptor (TCR) to PTKs, phospholipase D (PLD), PKC and Ras-MAP kinase signalling cascades (Harnett *et al.,* 1998). As with B cells, ES-62 pre-incubation induced abortive signalling in T cells and desensitisation to subsequent activation via the Ag receptor. ES-62 has also been shown to bias the immune response to a Th2 phenotype. Whelan *et al.* (2000) demonstrated that ES-62 directs the maturation of dendritic cells with the capacity to induce Th2 responses. T cells activated by bone marrow-derived dendritic cells which had been matured in the presence of ES-62 produced increased IL-4 but decreased IFN-γ, compared with T cells activated by dendritic cells matured in the absence of ES-62 or in the presence of LPS. The induction of a Th2 response was further demonstrated by the detection of ES-62-specific IgG1, but not the Th1-induced Ig isotype IgG2a, in sera from mice injected with ES-62 (Whelan *et al.,* 2000). PC blocks the production of IgG2a antibodies against other epitopes on the ES-62 molecule by an IL-10-dependent mechanism (Houston *et al.,* 2000).

5:1:5 Aim of this study.

ES-62 thus targets a number of cells of the immune system. The aim of this study was to examine its effects on the function of a cell not studied to date – the macrophage. In particular, the consequence of ES-62 treatment for production of IL-12, the key

cytokine driving the development of Th1 responses, was assessed, to determine whether modulation of IL-12 by ES-62 could contribute to the generation of a Th2 response to filarial parasites. Production of the pro-inflammatory cytokines IL-6 and TNF-α and the toxic mediator NO by macrophages stimulated in the presence of ES-62 was also measured. Finally, the modulation of macrophage signalling pathways by ES-62 was investigated.

5:2 Results.

5:2:1 Effects of ES-62 on cytokine and NO production by macrophages.

To establish whether ES-62 targets macrophage function, the effects of treatment of murine peritoneal macrophages with ES-62 on cytokine and NO production were investigated.

5:2:1:1 Effects of ES-62 on IL-12, IL-6, and TNF-α production.

Thioglycollate-elicited murine peritoneal macrophages were pre-incubated with various concentrations of ES-62 for 18 hours prior to stimulation with IFN-γ and LPS for 24 hours. IFN-γ + LPS-induced levels of both IL-12 p40 and TNF-α in culture supernatants were reduced by ES-62 pre-treatment in a dose-dependent manner (Figure 5.2). Concentrations of up to 100 ng/ml had little effect on either IL-12 p40 or TNF-α, but pre-treatment with 1-5 μg/ml ES-62 effectively abolished IL-12 p40 production (Figure 5.2A) and reduced TNF-α induction by more than 50% (Figure 5.2B). A concentration of 2 μg/ml ES-62, which has previously been shown to give optimal suppression of B cell proliferation (Harnett and Harnett, 1993), and is within the range of concentrations of PC-containing ES products found in filariasis patients (Lal *et al.*, 1987), was selected for subsequent experiments.

Shorter pre-incubation with ES-62 and even simultaneous addition with IFN-γ + LPS also resulted in suppression of macrophage cytokine production (Figure 5.3). ES-62 appears to induce a state of macrophage hyporesponsiveness since washing of cells after ES-62 pre-incubation prior to IFN-γ + LPS stimulation still resulted in inhibition of IL-12 p40 production (Figure 5.3). Furthermore, transfer of culture supernatant from ES-62-pre-treated cells to resting macrophages just prior to stimulation resulted in normal IL-12 p40 production (Figure 5.3), suggesting that suppression occurs as a direct result of ES-62 treatment rather than via the induction of another inhibitory factor.

To further investigate the effects of ES-62 on cytokine production by macrophages, BALB/c peritoneal macrophages were pre-treated with 2 μg/ml ES-62 for 18 hours prior to stimulation with IFN-γ and/or LPS, and IL-12, TNF-α and IL-6 levels in 24 hour culture supernatants were measured by ELISA. In the absence of LPS stimulation i.e. resting or IFN-γ-treated cells, ES-62 stimulated low but significant levels of IL-12 p40, IL-6 and TNF-α (Figure 5.4A, C and D). In contrast, ES-62 pre-treatment resulted in inhibition of IFN-γ + LPS-induced IL-12 p40 (94%), IL-6 (72%) and TNF-α (57%) production; LPS-induced TNF-α was also reduced by 63% (Figure 5.4A, C and D).

IL-12 p70 heterodimer was also measured to establish whether ES-62 reduces active IL-12 or simply p40 monomer production. IFN-γ + LPS treatment induced approximately 20-fold lower p70 compared with p40, and this was practically abolished by ES-62 treatment (Figure 5.4B). Similar results were obtained with total spleen cells, splenic macrophages, bone marrow-derived macrophages and macrophages from the 129 mouse strain (Figure 5.5).

Interestingly, the amount of each cytokine produced following ES-62 pre-treatment was identical in the four stimulation groups (non-stimulated, IFN-γ alone, LPS alone, and IFN-γ and LPS in combination; Figures 5.4 and 5.5). This suggests that ES-62 treatment induces low levels of production of these cytokines before establishing a state of hyporesponsiveness of macrophages to full activation by IFN-γ + LPS. Timecourse experiments confirmed that these cytokines are produced during the ES-62 pre-incubation (Figure 5.6A) but not further stimulated by IFN-γ + LPS (Figure 5.6B).

IL-12 and TNF-α mRNA were quantified by real-time PCR (TaqMan). Compared with control cells, the level of IFN-γ + LPS-induced IL-12 p40 transcripts was reduced by pre-treatment with ES-62 (Figure 5.7), but not as significantly as protein levels (Figure 5.4A), while TNF-α mRNA levels were not significantly reduced by ES-62 pre-treatment (Figure 5.7C). Thus ES-62 must regulate production of IL-12 p40 at transcriptional and translational/post-translational stages, and TNF-α predominantly at a translational/post-translational level. Interestingly, mRNA for the constitutively-expressed p35 subunit of IL-12 was also modulated (Figure 5.7B). IFN-γ + LPS stimulated p35 transcript levels, but pre-exposure to ES-62 prevented this. Thus, ES-62 achieves inhibition of active IL-12 p70 production by targeting both subunits of this heterodimer. Consistent with the ELISA data, ES-62 treatment alone induced the transient expression of low levels of IL-12 and TNF-α transcripts (Figure 5.8).

5:2:1:2 Effects of ES-62 on NO production and cell viability.

To verify whether ES-62 specifically targets production of these cytokines or inhibits macrophage responses in general, NO release was measured by Griess Reaction. In contrast to cytokine production, ES-62 had no effect on NO release from these cells (Figure 5.9A). In addition, cell viability was unaffected as determined by MTT assay (Figure 5.9B).

5:2:1:3 Exposure to ES-62 *in vivo* by release from osmotic pumps.

Exposure of mice to parasite products can be mimicked *in vivo* using osmotic pumps, which release their contents at a constant rate. This offers a more physiological alternative to serial injection experiments, and is less traumatic for the mice. ALZET osmotic pumps containing ES-62 were implanted subcutaneously on the backs of five BALB/c mice (see Materials and Methods); five control mice had PBS-containing pumps inserted. Mice were sacrificed after two weeks; peritoneal macrophages were removed and rested before culture with IFN-γ and/or LPS. Cytokine and NO levels were measured in 24 hour culture supernatants. In agreement with the *in vitro* data, exposure to ES-62 *in vivo* at a rate of 0.05 μg/hr resulted in the suppression of IFN-γ + LPS-induced IL-12 (p40 and p70) and TNF-α production (Figure 5.10A and B), while having no effect on NO (Figure 5.10C). In contrast with the *in vitro* exposure, low-level cytokine production by ES-62-treated but subsequently non-stimulated macrophages was not observed. This is not surprising however; this low-level induction probably occurred *in vivo* prior to

removal of macrophages. Nevertheless, it is clear that ES-62 induces a state of sustained hyporesponsiveness of macrophages to full activation.

Since the pumps release their contents at a constant flow rate, it is possible to adjust the concentration of ES-62 that the mice are exposed to by varying the concentration of ES-62 inserted into the pump. Thus, in a separate experiment, IFN-γ + LPS-induced IL-12 and TNF-α production by peritoneal macrophages from mice exposed to different concentrations of ES-62 was measured. Suppression of IL-12 p40 and TNF-α by ES-62 was dose-dependent and a delivery rate of 0.1 μg/hour was optimal for inhibition of both cytokines (Figure 5.11). Interestingly, IL-12 was more significantly inhibited than TNF-α at lower ES-62 concentrations.

5:2:2 Modulation of signal transduction pathways by ES-62.

Having clearly demonstrated that ES-62 suppresses the production of IL-12, IL-6 and TNF-α, the next step in this investigation was to dissect the signalling mechanisms underlying this modulation of macrophage function, drawing on the findings concerning the regulation of IFN-γ/LPS-induced cytokine production detailed in the earlier chapters.

5:2:2:1 Effects of ES-62 on tyrosine phosphorylation.

Tyrosine kinases and tyrosine phosphatases are important regulators of early receptor-triggered signalling events. These proteins are themselves regulated by tyrosine

phosphorylation as well as regulating their downstream targets by catalysing this modification. Therefore, tyrosine phosphorylation is an important regulatory mechanism. Several of these molecules have been reported to be activated upon stimulation with IFN-γ and/or LPS, including the Src family members Lyn, Hck and Fgr, as well as Vav and Shc (Stefanova *et al.,* 1993; Crowley *et al.,* 1997).

The effects of ES-62 treatment on tyrosine phosphorylation were analysed by Western Blotting using the anti-phospho-tyrosine antibody 4G10. ES-62 dramatically altered patterns of tyrosine phosphorylation of proteins from peritoneal macrophages (Figure 5.12). ES-62 alone induced tyrosine phosphorylation which was further enhanced following stimulation with LPS or IFN-γ + LPS. Tyrosine phosphorylation of proteins of approximately 55, 57, 60, 69, 72, 144, 149, 167 and 186 kDa was enhanced by ES-62 treatment. A protein of approximately 72 kDa was particularly highly phosphorylated by treatment with ES-62. Enhanced tyrosine phosphorylation was maintained throughout an 8 hour timecourse of IFN-γ + LPS stimulation (Figure 5.13).

5:2:2:2 Effects of ES-62 on activation of MAP kinases.

In chapter 3, ERK MAP kinases were demonstrated to be required for IL-6 and TNF-α production, but to negatively regulate IL-12 p40 transcription. Furthermore, in chapter 4, ERKs were found to be responsible for suppression of IL-12 p40 production by *Leishmania* phosphoglycan. It is possible that a similar mechanism is employed by ES-62 to inhibit IL-12 production. Therefore, the effects of ES-62 treatment on activation of

ERK MAP kinases and cytokine production in the absence of ERK activation were investigated.

To establish whether ERK MAP kinases are targeted by ES-62, ERK1 (p44) and ERK2 (p42) activation in cells treated with ES-62 and IFN-γ + LPS was assessed by Western Blotting, using antibodies specific for active, dual-phosphorylated ERK MAP kinases. ES-62 differentially regulated the ERK isoforms over a timecourse of IFN-γ + LPS stimulation, following ES-62 pre-incubation (Figure 5.14). IFN-γ + LPS induced dominant ERK1 activation at early timepoints, peaking at 4 hours and thereafter returning to near basal levels; ERK2 activation also peaked at 4 hours. Levels of active ERK1 were suppressed by ES-62 pre-treatment at early timepoints (up to 4 hours) but were subsequently slightly elevated. In contrast, although ERK2 activation was initially suppressed, it was dramatically enhanced at later timepoints (4 and 8 hours) by ES-62 treatment. Activation of neither ERK isoform above basal levels was detected during the ES-62 pre-incubation period (not shown).

5:2:2:3 Effects of inhibition of ERK MAP kinases on modulation of cytokine production by ES-62.

To investigate whether ES-62 regulates ERK activity to modulate cytokine production, peritoneal macrophages were pre-incubated with ES-62 in the presence or absence of PD 098059 for 18 hours prior to IFN-γ/LPS stimulation and cytokine production was measured as before. Whereas PD 098059 alone did not stimulate IL-12 or TNF-α production, it was synergistic with ES-62 for stimulation of IL-12 (both p40 and

p70), but practically abolished ES-62-induced TNF-α production (Figure 5.15A, B and D). Inhibition of IFN-γ + LPS-induced IL-12 p40 production by ES-62 was abolished by pre-incubation with PD 098059 (Figure 5.15A), resulting in only a partial restoration of p70 production (Figure 5.15B). Inhibition of IFN-γ + LPS-induced TNF-α production by ES-62 was unaltered by PD 098059 treatment (Figure 5.15D). Neither stimulation of IL-6 by ES-62 alone, nor suppression of IFN-γ + LPS-induced IL-6 production by ES-62, were affected by inhibition of ERK activation (Figure 5.15C).

These data demonstrate multiple and various roles for ERK MAP kinases in the regulation of cytokine production by ES-62. While ERK activity prevents excessive IL-12 production in response to ES-62 alone, it is required for low level production of TNF-α. Subsequently, it appears to provide a mechanism for ES-62-induced prevention of full stimulation of IL-12 production by IFN-γ + LPS. In contrast, it is not involved in the suppression of TNF-α production; neither does it play any role in modulation of IL-6 production.

The effects of ES-62 on p38 and JNK MAP kinase activation were also investigated. 10 minutes after stimulation of peritoneal macrophages with LPS or IFN-γ + LPS, activation of both p38 and JNK MAP kinases was suppressed in ES-62-pre-treated cells compared with control cells (Figure 5.16). This suggests that ES-62 may achieve inhibition of cytokine induction by also suppressing p38 and JNK MAP kinase activation.

5:2:2:4 Effects of ES-62 on transcription factor synthesis, nuclear translocation and promoter binding.

The targeting of transcription factors by ES-62 was investigated by examining the synthesis, nuclear translocation and binding to promoter sequences of Ets and members of the IRF and NF-κB families by Western Blotting and EMSA.

ES-62 induced rapid synthesis of IRF-1, which was detectable by 10 minutes after treatment (Figure 5.17). This early stimulation of IRF-1 continued up to 2 hours but was reduced again to near basal levels by 4 hours after treatment. Following a lag phase of 4 – 8 hours, there was further induction of IRF-1, this time resulting in a large and sustained increase in IRF-1 levels, which remained higher than in non-ES-62-treated cells following IFN-γ and LPS stimulation for at least 1 hour (Figure 5.18). By 2 hours after IFN-γ + LPS stimulation, IRF-1 induction was equivalent in ES-62 treated and untreated cells. In contrast, IRF-2 levels were not altered by ES-62 treatment and remained constant throughout the stimulation timecourse (Figure 5.18). Levels of Ets-2 in ES-62-pre-treated macrophages were also higher than in control cells for at least 1 hour after IFN-γ + LPS stimulation (Figure 5.18).

Production of the p65 isoform of NF-κB was not targeted by ES-62 (Figure 5.18). However, the kinetics of IFN-γ + LPS-induced nuclear translocation and binding of NF-κB to a promoter consensus sequence were altered by ES-62 pre-treatment (Figure 5.19). At early timepoints following IFN-γ + LPS stimulation, NF-κB binding (p65/p50 and c-Rel/p50) was enhanced in ES-62 pre-treated cells. At 4 hours, binding of both NF-κB heterodimers and p50 homodimer was detected in ES-62-treated and -untreated cells.

Stimulation with IFN-γ + LPS resulted in an additional band at this timepoint, the identity of which was not determined; this band was absent from the ES-62-treated sample. By 8 hours, NF-κB binding (heterodimers) was suppressed in ES-62-treated cells compared with control cells.

Evidence for the effects of ES-62 on IL-12 production via modulation of transcription factor binding to the F1 site on the IL-12 promoter was also obtained by EMSA studies. A timecourse of treatment with ES-62 alone showed that during the ES-62 pre-incubation the pattern of transcription factor binding to an oligonucleotide probe representing a portion of the F1 site was rapidly and dramatically altered (Figure 5.20). Similarly, large and sustained differences in binding patterns were observed following IFN-γ + LPS stimulation (Figure 5.21), with enhanced binding of higher molecular weight complexes in extracts from ES-62 pre-treated cells. This might represent the additional binding of inhibitors of transcription.

5:3 Discussion.

This investigation has examined the immunomodulatory effects of the filarial nematode excretory-secretory product ES-62 on the activation of macrophages by IFN-γ and LPS. Although treatment of peritoneal, splenic and bone marrow-derived macrophages with ES-62 alone induced low levels of IL-12, IL-6 and TNF-α production, ES-62 pre-treatment resulted in inhibition of IFN-γ + LPS-induced production of these cytokines (Figures 5.2 - 5.5). The data show a two-stage regulation of cytokine production by ES-62: an initial stimulatory phase during the ES-62 pre-incubation, followed by induction of hyporesponsiveness of macrophages to subsequent full activation by IFN-γ + LPS (Figure 5.6). Interestingly, this mirrors the abortive, yet non-productive, activation of signalling elements by ES-62 that desensitises B and T cells to subsequent activation via their Ag receptors (Deehan *et al.,* 1998; Harnett *et al.,* 1998).

ES-62 treatment did not affect macrophage viability (Figure 5.9B), indicating that it does not suppress cytokine production by simply killing cells. Furthermore, the ability of macrophages to produce NO was not altered by treatment with ES-62 (Figure 5.9A). Although it would appear that the iNOS enzyme is not targeted by ES-62, it is however likely that NO production in the intact host would be suppressed due to reduced production of IFN-γ, the major stimulator of iNOS. Thus, ES-62 does not cause a generalised repression of macrophage function; rather, it specifically inhibits the IFN-γ + LPS-mediated induction of IL-12, IL-6 and TNF-α. Since IL-6 and TNF-α play critical roles in the activation of T and B cells (reviewed by Hirano, 1992 and Mannel and Echtenacher, 2000), suppression of their production by ES-62 is likely to contribute to the hyporesponsiveness of lymphocytes observed during filarial infection. Suppression of IL-

12, the key cytokine driving the development of Th1 responses, is also consistent with previous observations that ES-62 biases the immune response to a Th2 phenotype (Whelan *et al.,* 2000). Therefore, these data, in agreement with previous reports (Harnett and Harnett, 1993; Harnett *et al.,* 1998; Whelan *et al.,* 2000), suggest that ES-62 is both immunosuppressive (anti-inflammatory) and immunomodulatory (causing a Th2 bias).

The use of osmotic pumps to deliver ES-62 to mice offers an alternative to repeated dosing by serial injection, and mimics natural, constant release of the parasite product during infection, as well as minimising animal handling and stress, which can influence the immune response. The demonstration that exposure to ES-62 *in vivo* by release from osmotic pumps results in suppression of IFN-γ + LPS-induced IL-12 and TNF-α production by peritoneal macrophages (Figures 5.10 and 5.11) confirmed that the *in vitro* data are physiologically relevant, and supported the hypothesis that exposure to ES-62 induces a state of hyporesponsiveness. In contrast however, unstimulated macrophages that had been pre-exposed to ES-62 did not produce the low levels of cytokine production seen in the *in vitro* experiments. It is likely that this ES-62-induced cytokine, which remains in the culture supernatants of macrophages exposed to ES-62 *in vitro*, is secreted *in vivo* in the mice exposed to ES-62 by release from osmotic pumps prior to the removal of macrophages, and is therefore subsequently not detected in culture supernatants. Nevertheless, taken together, the results clearly indicate that ES-62 strongly suppresses IL-12, IL-6 and TNF-α induction by appropriate stimuli.

Suppression of IL-12 synthesis may be due to modulation of both transcription and translation/post-translational modification, since mRNA levels for the IL-12 p40 subunit were not as dramatically reduced by ES-62 treatment as protein in culture

supernatants (Figures 5.7 compared with Figure 5.4). The modulation of p35 transcript levels by ES-62 (Figure 5.7) is particularly interesting, since p35 has not been studied as widely as its counterpart, p40. Although constitutively expressed, p35 expression can be regulated at multiple levels, including transcription, translation and by post-translational modification (Babik *et al.,* 1999; Snijders *et al.,* 1996). One important mechanism of regulation of p35 production is the generation of different transcripts (Babik *et al.,* 1999). Transcription can initiate from several start sites, resulting in the generation of transcripts for functional p35 protein, but also some message that cannot be translated completely. In unstimulated cells, transcripts that encode incomplete protein predominate and hence protein levels are suppressed. Upon LPS stimulation however, there is an increase in the proportion of functional transcripts and hence efficient protein production. ES-62 may target the factors that determine which transcription start site is used to bias the production of transcripts to those that do not encode functional protein, thereby reducing p35 subunit generation and hence contributing to the suppression of bioactive p70 production.

In contrast to IL-12, transcription of TNF-α was not regulated by ES-62 (Figure 5.7). ES-62 must therefore exert its inhibitory action on the IFN-γ + LPS-mediated induction of this cytokine at a later stage of synthesis e.g. translation, post-translational modification, release from the cell. The proteolytic cleavage of the membrane-bound form to release soluble TNF-α is an important regulatory step (Kriegler *et al.,* 1988) which may be targeted by ES-62.

It has previously been shown that ES-62 targets the ERK MAP kinases of B and T cells in a Ras-independent manner to achieve its modulation of the function of these cells

(Deehan *et al.,* 1998; Harnett *et al.,* 1998). In this study, treatment with ES-62 was shown to alter ERK activation in macrophages, but in a complex manner, with differential modulation of the two ERK isoforms (Figure 5.14).

The differential involvement of the ERKs in mediating cytokine suppression by ES-62 was also demonstrated. There was evidence that ES-62 targets ERKs to regulate the production of IL-12 and TNF-α, but not IL-6 (Figure 5.15). Use of the inhibitor of the MEK→ERK pathway, PD 098059, demonstrated opposing roles for these MAP kinases in the stimulation of low levels of IL-12 and TNF-α by ES-62 alone, and indicated that ERKs mediate suppression of IFN-γ + LPS-induced IL-12, but play no role in the suppression of TNF-α or modulation of IL-6 production (Figure 5.15). This was consistent with the demonstration in chapter 3 that ERK MAP kinases transduce negative and positive regulatory signals to regulate induction of IL-12 and TNF-α respectively, but are not involved in the regulation of IL-6 production. The partial restoration of ES-62-suppressed p70 levels by pre-treatment with PD 098059, is consistent with the suppression of the p35 subunit by ES-62 (Figure 5.7) and the observation that induction of this subunit is not regulated by ERK MAP kinases (Figure 3.27). Therefore, PD 098059 treatment restores the inhibition of p40 but not the p35 subunit, thereby limiting p70 production. A model for the inhibition of IL-12 production is presented in Figure 5.22.

The apparent inconsistency in the opposing roles of ERKs in mediating regulation of IL-12 and TNF-α production, which are both inhibited by ES-62, might reflect differential temporal regulation (since TNF-α is produced earlier than IL-12; see Figures 3.4 and 3.11), utilisation of a particular ERK isoform, or a combination of these and other

factors. Therefore, unlike the suppression of IL-12 production by sPG, which was simply due to the sustained enhancement of both ERK1 and ERK2 (see chapter 4), the inhibition of IFN-γ + LPS-induced IL-12 production by ES-62 cannot be explained in terms of modulation of a single MAP kinase subfamily.

It is apparent that the regulation of cytokine production is achieved by the convergence of multiple signals, including members of MAP kinase subfamilies as well as other signalling molecules. Indeed it has been shown that co-ordination of the ERK, p38, JNK and Big MAP kinase (BMK)/ERK5 pathways is responsible for regulation of TNF-α promoter activation (Zhu *et al.,* 2000). Modulation of a particular MAP kinase isoform may have a critical or limited effect on cytokine production. It is therefore likely that ES-62 achieves regulation of each cytokine through differential targeting of MAP kinase family members. In Chapter 3, p38 activity was shown to be necessary for induction by LPS of both IL-12 subunits, IL-6 and TNF-α. It has also been reported that p38 MAP kinase signals stabilise IL-6 mRNA (Winzen *et al.,* 1999) and that JNK MAP kinases are required for translation of TNF-α mRNA after LPS induction (Swantek *et al.,* 1997). Therefore, ES-62 may reduce the production of these cytokines by suppressing the activation of the p38 and JNK MAP kinase subfamilies. Indeed, ES-62 suppressed the early induction of p38 and JNK MAP kinases by LPS/IFN-γ + LPS (Figure 5.16). Thus, the suppression of these distinctly regulated cytokines by ES-62 is likely to occur via the modulation of multiple signals, including ERK and p38 MAP kinases, and the integration of these signals at different stages of bioactive cytokine synthesis.

Treatment of macrophages with ES-62 also altered the expression and activation of a number of other signalling molecules, providing further information about the

regulation of cytokine production by ES-62. Tyrosine phosphorylation of a number of proteins was enhanced, in particular a protein of approximately 72 kDa (Figures 5.12 and 5.13). These proteins are likely to be tyrosine kinases that associate with receptors following ligand binding to form large intracellular signalling complexes. Thus, the enhanced tyrosine phosphorylation could represent recruitment to the receptor to which ES-62 binds or association with TLR4 to disrupt LPS signalling. One candidate molecule is Bruton's tyrosine kinase (Btk) which has been shown to negatively regulate IL-12 production (Mukhopadhyay *et al.*, 1999a). This molecule has recently been shown to play a role in the clearance of filarial infection by modulating macrophage function (Mukhopadhyay *et al.*, 1999b). Btk-deficient X-immunodeficient (xid) mice produced increased IFN-γ and displayed delayed clearance of injected microfilariae compared to normal mice, and these effects were rescued by transfer of peritoneal macrophages from normal to deficient mice. Therefore, one possible mechanism for the suppression of IL-12 production by ES-62 would be the upregulation of Btk activity by the enhancement of its tyrosine phosphorylation. This would bias the immune response towards a Th2 phenotype, favouring survival of the parasite.

ES-62 clearly modulates the synthesis and binding of multiple transcription factors to promoter sequences (Figures 5.17 – 5.21), including members of the NF-κB family which regulate production of IL-12, IL-6 and TNF-α, and therefore represent an obvious target for ES-62-mediated suppression. Binding to a portion of the F1 site of the IL-12 p40 promoter was also significantly altered (Figures 5.20 and 5.21), with enhanced binding of higher molecular weight complexes. This could represent the additional binding of inhibitory molecules in the F1 complex. Interestingly, analysis of the sequence of the ES-62 protein revealed the presence of leucine rich regions, which are typically

involved in protein-protein interactions. It is possible therefore that ES-62 itself interacts with transcription factors, such as leucine zipper proteins like C/EBP and NF-IL6, and binds to DNA.

The phosphorylcholine (PC) component of ES-62 has been suggested to be responsible for many of the immunomodulatory properties of ES-62, since PC-BSA and PC-choline mimicked the effects of intact ES-62 in B and T cells (Harnett and Harnett, 1993; Deehan *et al.,* 1998). PC-containing molecules produced by a diverse range of organisms, including bacteria, fungi and protozoa, as well as filarial and gastrointestinal nematodes, enable detection of pathogens by the host, but can also function to promote pathogen survival via modulation of host immune responses (Harnett and Harnett, 1999). PC is therefore of great significance in pathogenic infection. Whether the effects of ES-62 on macrophage function demonstrated here can be ascribed to this moiety remains to be investigated. However, these results are clearly relevant to studies of macrophage function during filarial infection and may yet have much wider implications in the investigation of pathogenic disease.

5:4 Summary of this study.

ES-62, the major excretory-secretory product of the rodent filarial nematode *A. viteae*, has previously been shown to modulate lymphocyte activation and the differentiation of dendritic cells. In this study the effects of ES-62 on macrophage activation were investigated. The results demonstrate that ES-62 pre-exposure prevents full activation of macrophages by IFN-γ + LPS, resulting in the suppression of IL-12, IL-6 and TNF-α production, without affecting NO release. This modulation is achieved by targeting different levels of cytokine synthesis, and involves the disruption of various signalling pathways including tyrosine phosphorylation and MAP kinase and NF-κB cascades.

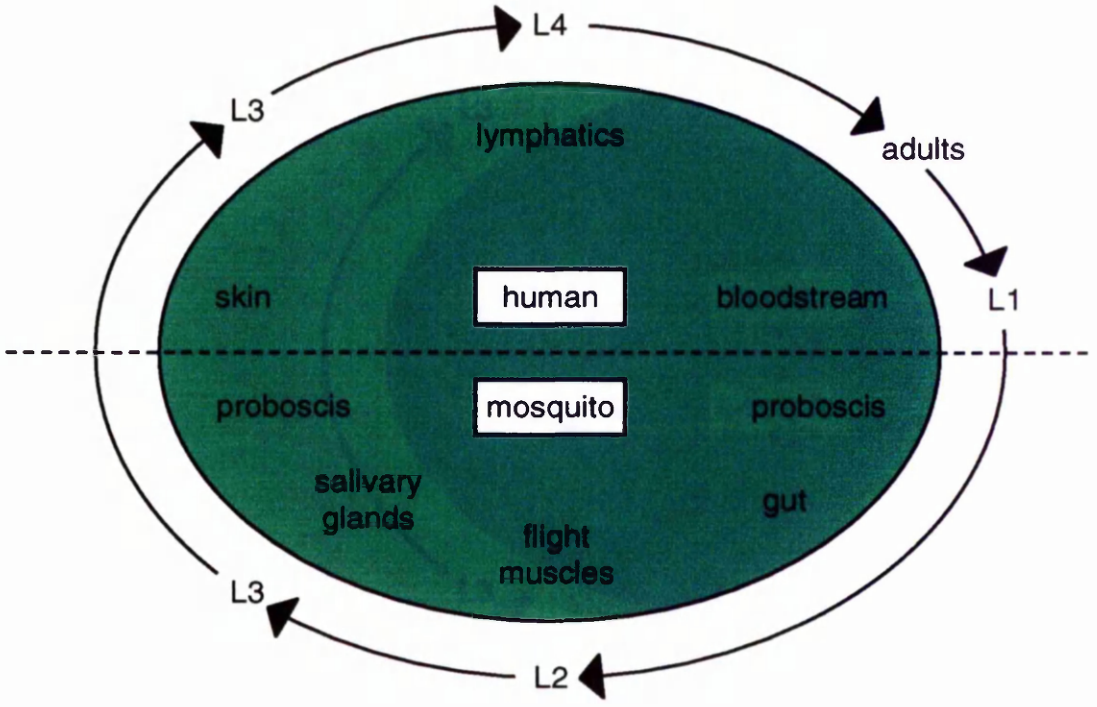


Figure 5.1. Filarial nematode lifecycle.

Typical lifecycle of *Wuchereria bancrofti* and *Brugia malayi* parasites. Adult worms live in the lymphatics of the human host where they reproduce sexually, releasing thousands of microfilariae into the bloodstream. These L1 larvae are ingested by mosquitoes when they take a bloodmeal. They shed their acellular sheath, penetrate the midgut and migrate into the flight muscles where they develop into L2 larvae. About two weeks later, L3 larvae migrate through the salivary glands to the proboscis. At this stage they can infect humans by injection into the skin when the mosquito feeds. They migrate into the lymphatics where they moult and mature to L4 larvae, before further maturing and differentiating to form adult worms. The development from microfilariae to adult worms can take several months and adult worms can live for many years.

(Adapted from Rajan and Gundlapalli, 1997)

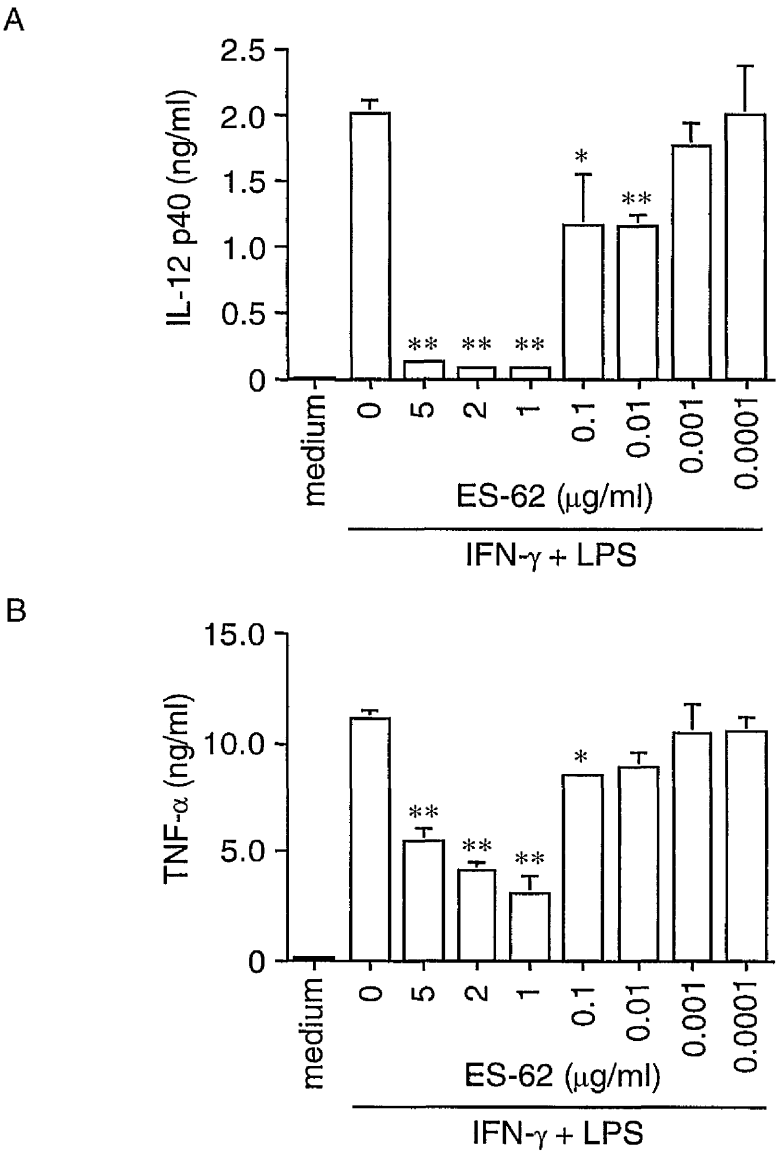


Figure 5.2. Dose-dependent effects of ES-62 on IFN-γ + LPS-induced IL-12 and TNF-α production.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($2x10^5$/well in a 96-well plate) were pre-treated with ES-62 at the indicated concentrations for 18 hours prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for 24 hours. IL-12 p40 and TNF-α in culture supernatants were measured by ELISA. * $p < 0.05$, ** $p < 0.01$

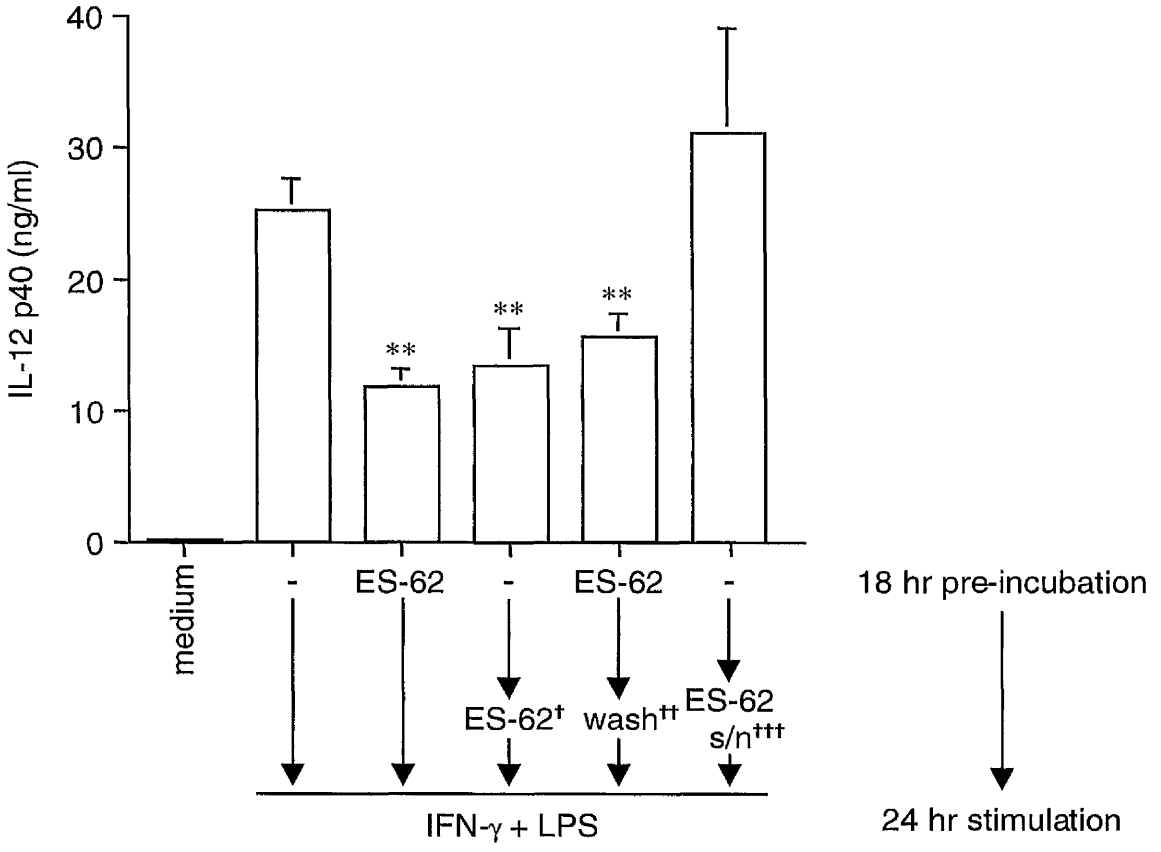


Figure 5.3. Effects of ES-62 treatment on IL-12 p40 production.

Thioglycollate-elicited peritoneal macrophages ($2x10^5$/well in a 96-well plate) were pre-incubated with/without 2 μg/ml ES-62 for 18 hours as indicated prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for 24 hours.

Immediately before IFN-γ + LPS addition, cells were treated as indicated:

† 2 μg/ml ES-62 was added to culture.

†† Culture supernatants from ES-62 pre-treated macrophages were removed and replaced with fresh medium immediately prior to stimulation with IFN-γ + LPS.

††† Culture supernatants from ES-62-pre-treated macrophages were transferred to resting macrophages immediately prior to stimulation with IFN-γ + LPS.

IL-12 p40 in culture supernatants was measured by ELISA. ** $p < 0.01$

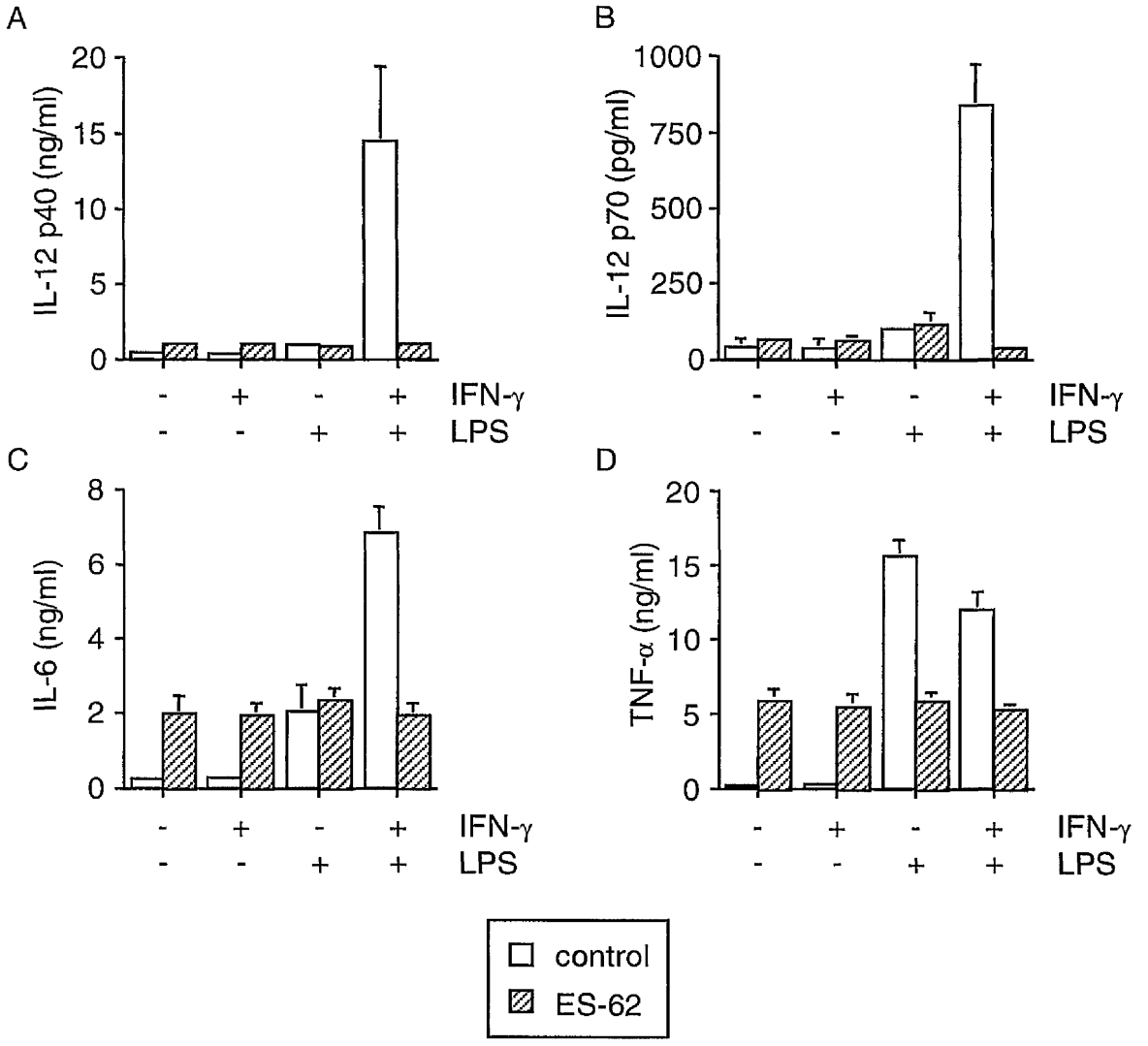


Figure 5.4. Effects of ES-62 on IL-12, IL-6 and TNF-α production by peritoneal macrophages.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($2x10^5$/well in a 96-well plate) were left untreated or pre-incubated with 2 μg/ml ES-62 for 18 hours prior to stimulation with 100 U/ml IFN-γ and/or 100 ng/ml LPS for 24 hours. Culture supernatants were assayed for IL-12 p40 (p40 monomer, active p70 heterodimer, p40/p40 homodimer), bioactive IL-12 p70, IL-6 and TNF-α by ELISA.

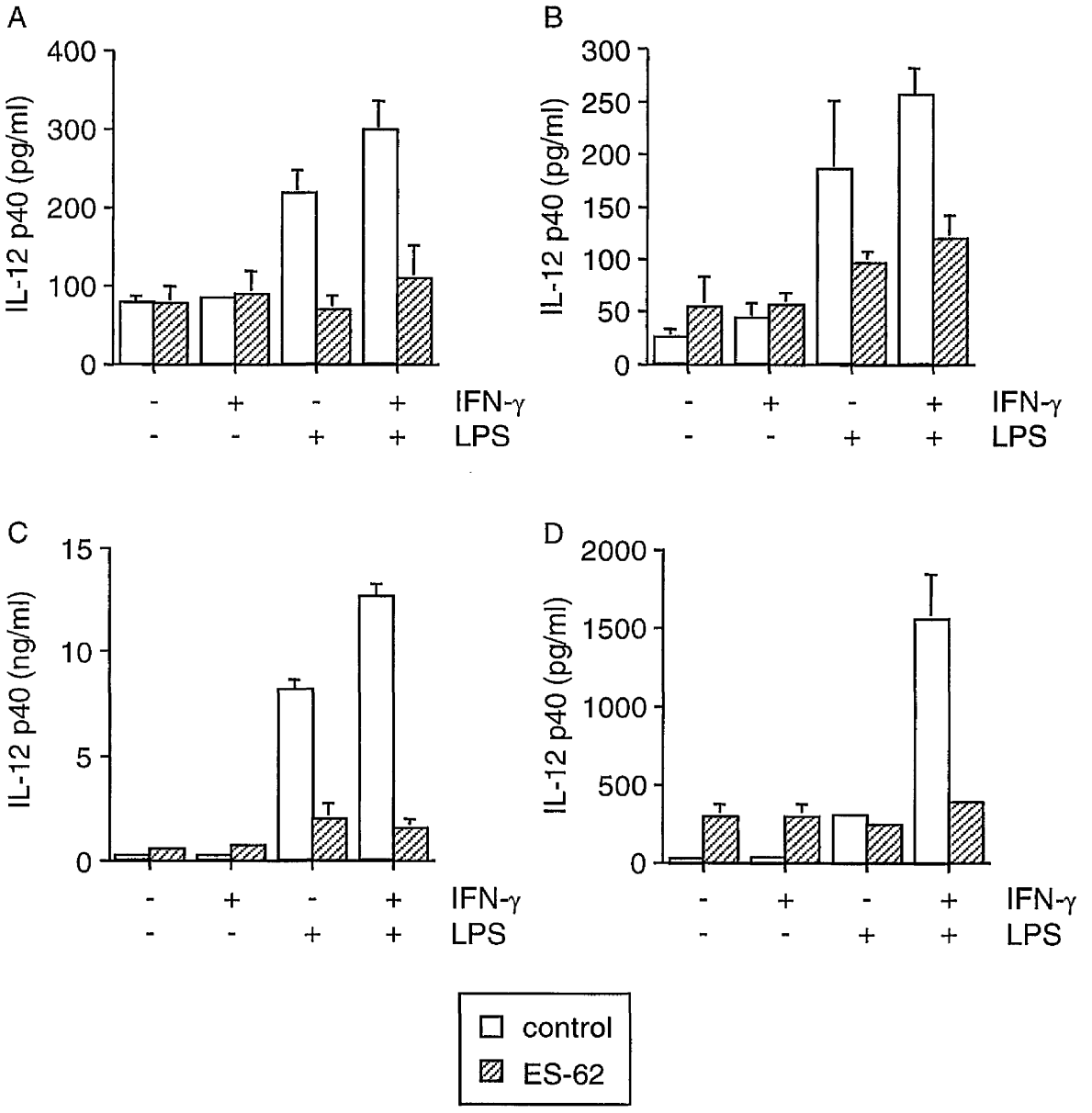


Figure 5.5. Effects of ES-62 on IL-12 production by macrophages from various sources.

Total spleen cells (A), splenic macrophages (B) and bone marrow-derived macrophages (C) from BALB/c mice, and thioglycollate-elicited peritoneal macrophages from 129 mice (D) ($2x10^5$/well in a 96-well plate) were left untreated or pre-incubated with 2 μg/ml ES-62 for 18 hours prior to stimulation with 100 U/ml IFN-γ and/or 100 ng/ml LPS for 24 hours. Culture supernatants were assayed for IL-12 p40 by ELISA.

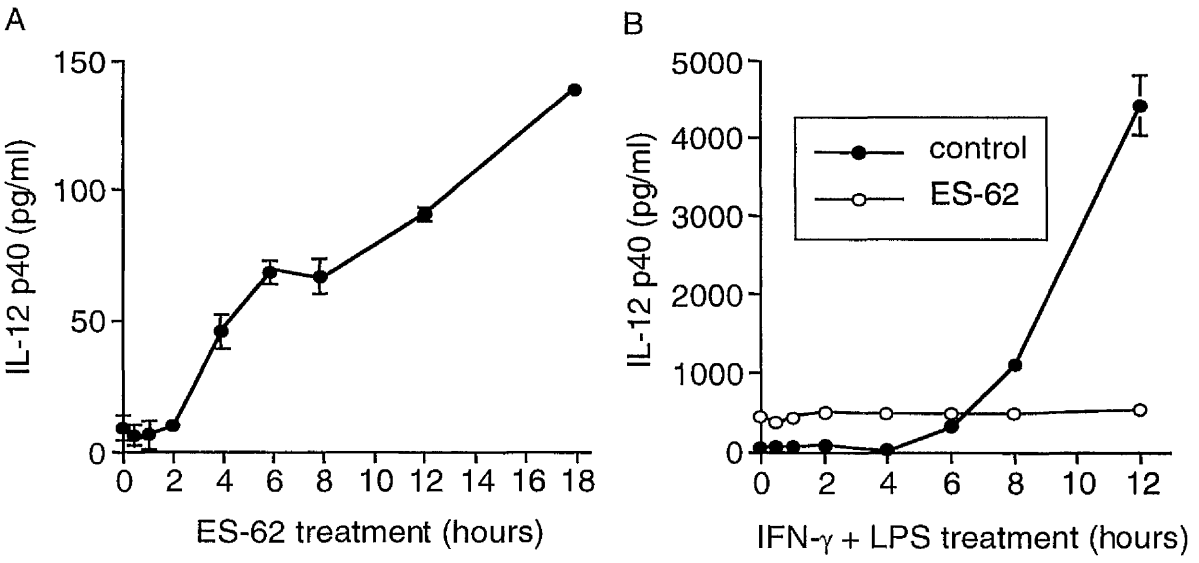


Figure 5.6. Timecourses of IL-12 p40 production following ES-62 treatment.

A. Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($2x10^5$/well in a 96-well plate) were treated with 2 μg/ml ES-62 for the times indicated and IL-12 p40 in culture supernatants was measured by ELISA.

B. Thioglycollate-elicited peritoneal macrophages ($2x10^5$/well in a 96-well plate) were left untreated or pre-treated with 2 μg/ml ES-62 for 18 hours and then stimulated with 100 U/ml IFN-γ and 100 ng/ml LPS. IL-12 p40 in culture supernatants was measured by ELISA.

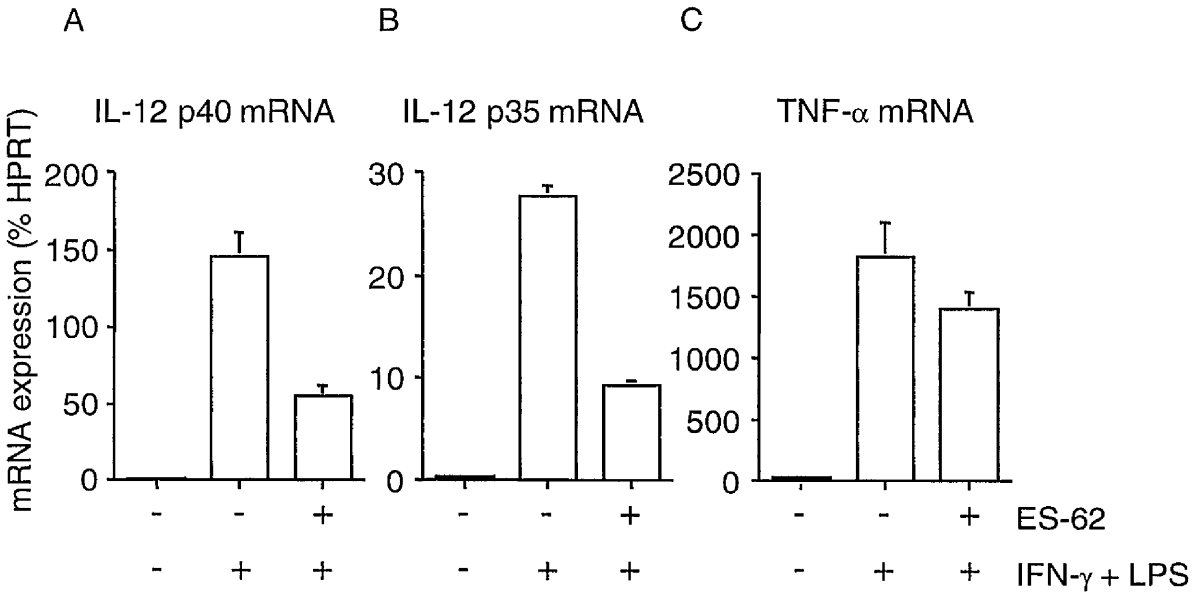


Figure 5.7. Effects of ES-62 on induction of IL-12 p40, p35 and TNF-α mRNA by IFN-γ + LPS.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($4x10^6$/25 cm^2 flask) were pre-incubated with 2 μg/ml ES-62 for 18 hours prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for 8 hours. Total RNA was purified from cells and IL-12 p40, IL-12 p35 and TNF-α transcripts were assayed by real-time PCR (TaqMan). Levels are expressed relative to HPRT mRNA.

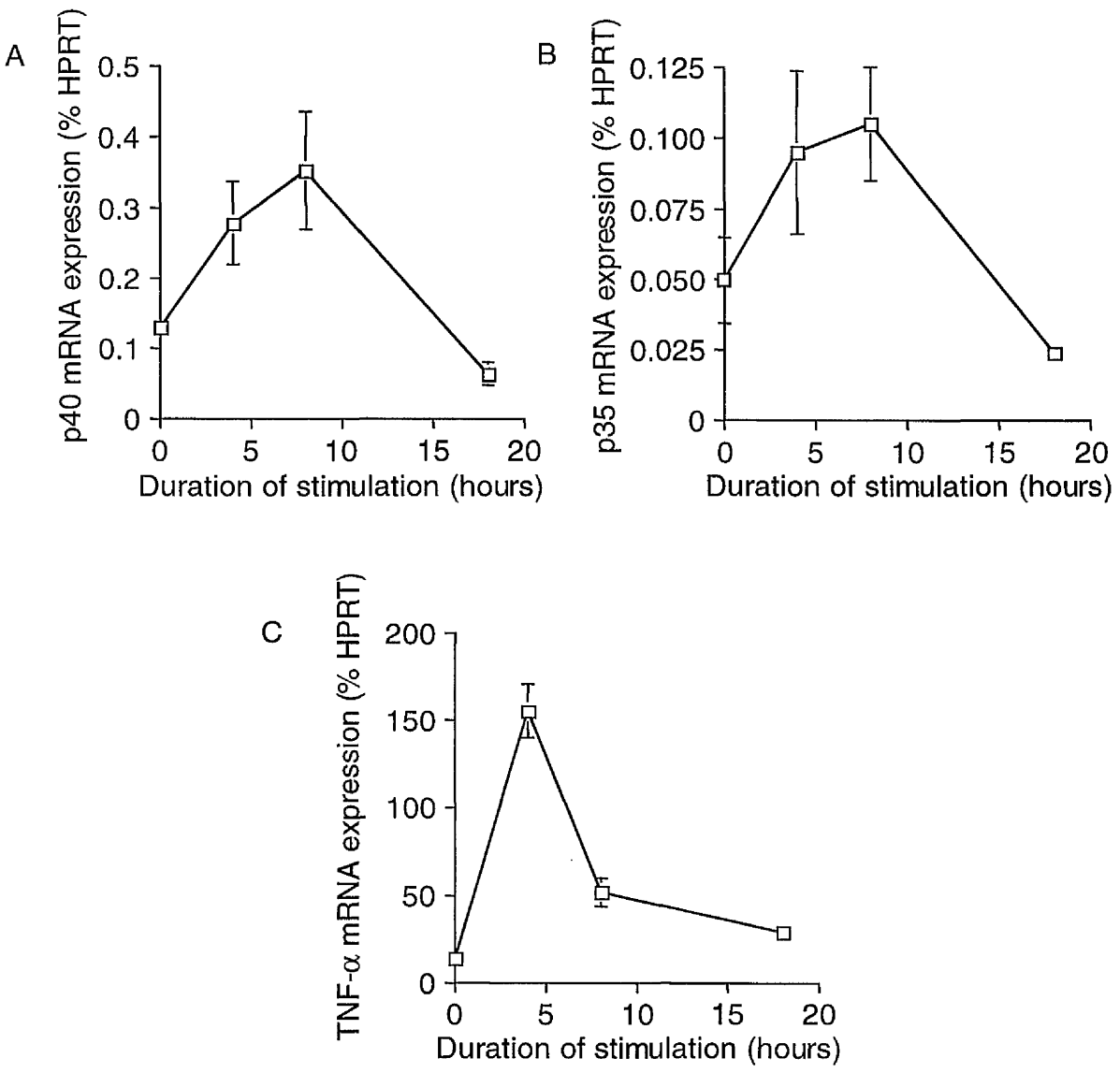


Figure 5.8. Induction of IL-12 p40, p35 and TNF-α mRNA by ES-62.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($4x10^6$/25 cm^2 flask) were treated with 2 μg/ml ES-62 for the times indicated. Total RNA was purified from cells and IL-12 p40, IL-12 p35 and TNF-α transcripts were assayed by real-time PCR (TaqMan). Levels are expressed relative to HPRT mRNA.

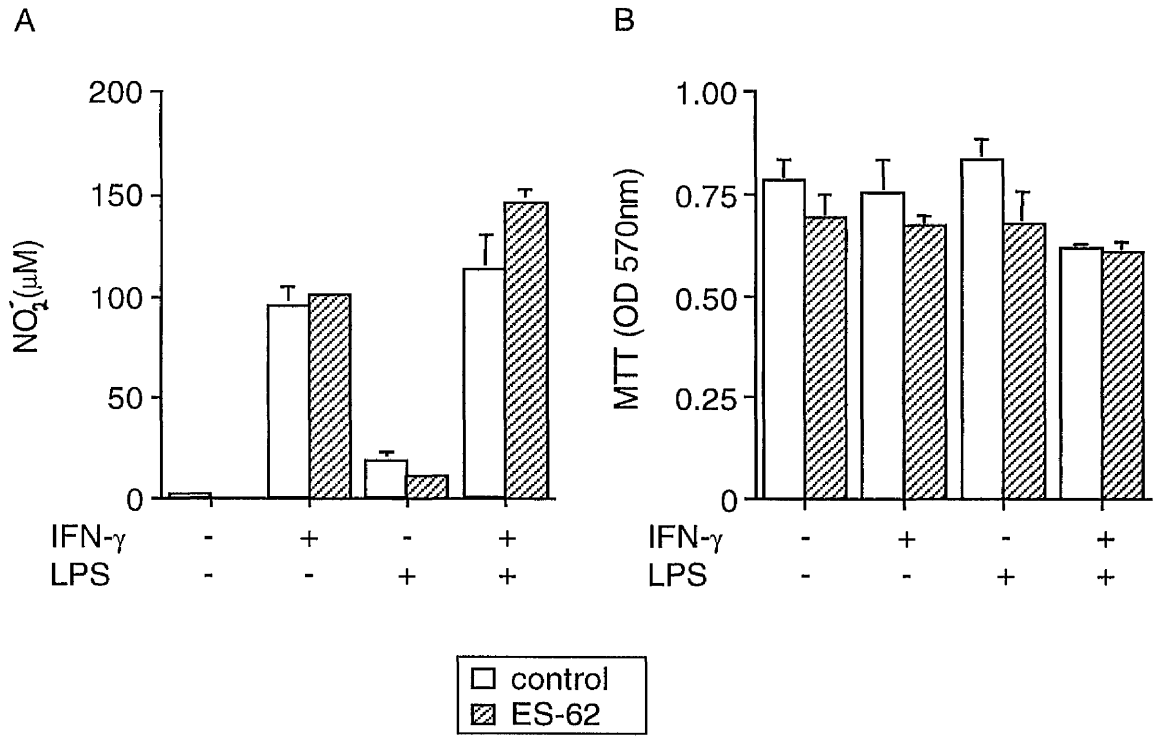


Figure 5.9. Effects of ES-62 on NO production and cell viability.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($2x10^5$/well in a 96-well plate) were left untreated or pre-incubated with 2 μg/ml ES-62 for 18 hours prior to stimulation with 100 U/ml IFN-γ and/or 100 ng/ml LPS for 24 hours. NO levels in culture supernatants were assayed by Griess Reaction, and cell viability was assessed by MTT assay.

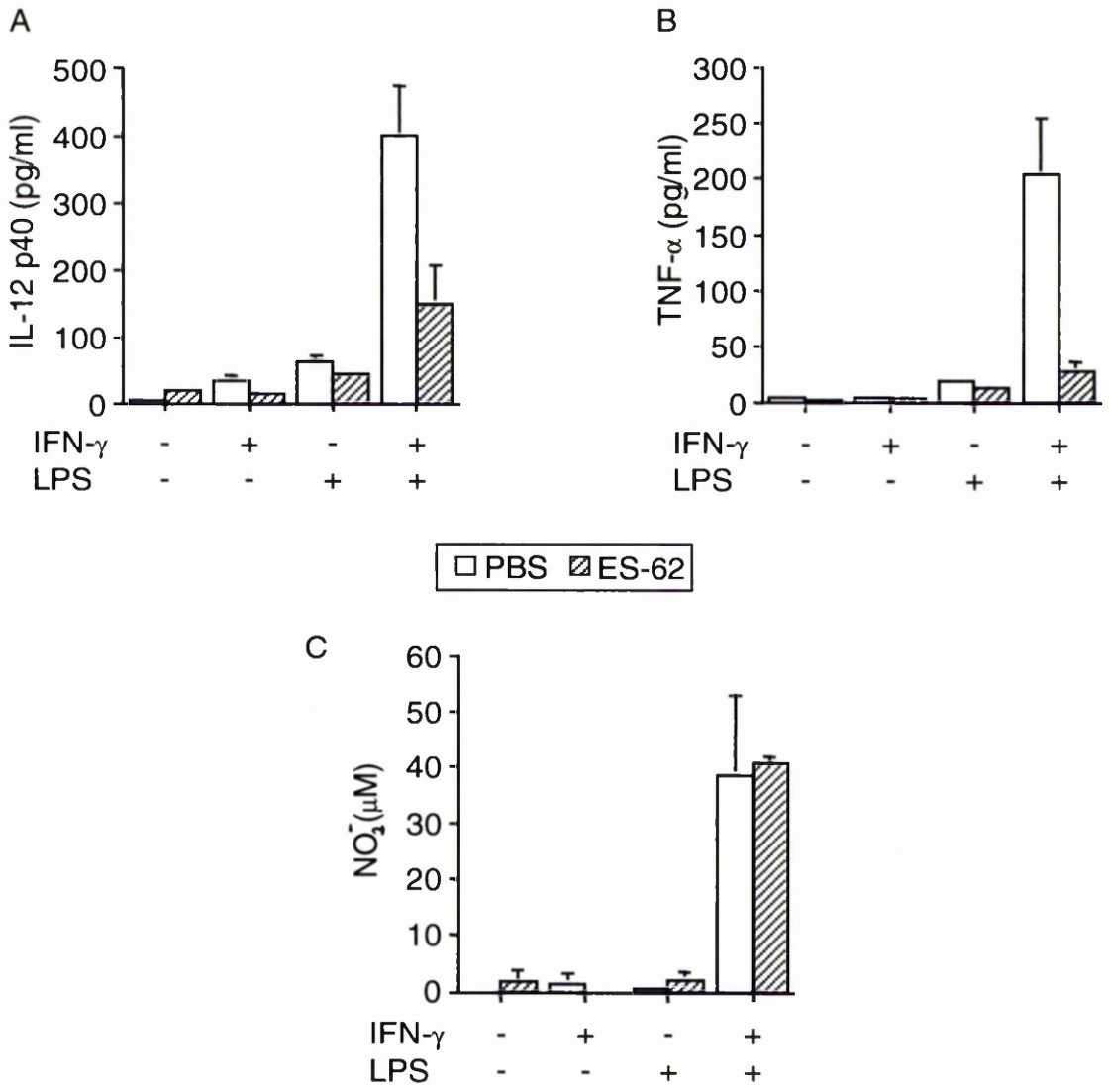


Figure 5.10. Effects of *in vivo* exposure to ES-62 on IL-12, TNF-α and NO production.

BALB/c mice (5 mice in each group) were exposed to ES-62 (0.05 μg/hr) or PBS (control) by constant release from osmotic pumps for two weeks. Peritoneal macrophages were removed and stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS ($2x10^5$ cells/well in a 96-well plate) for 24 hours. IL-12 p40 and TNF-α in culture supernatants were assayed by ELISA; NO was measured by Griess Reaction.

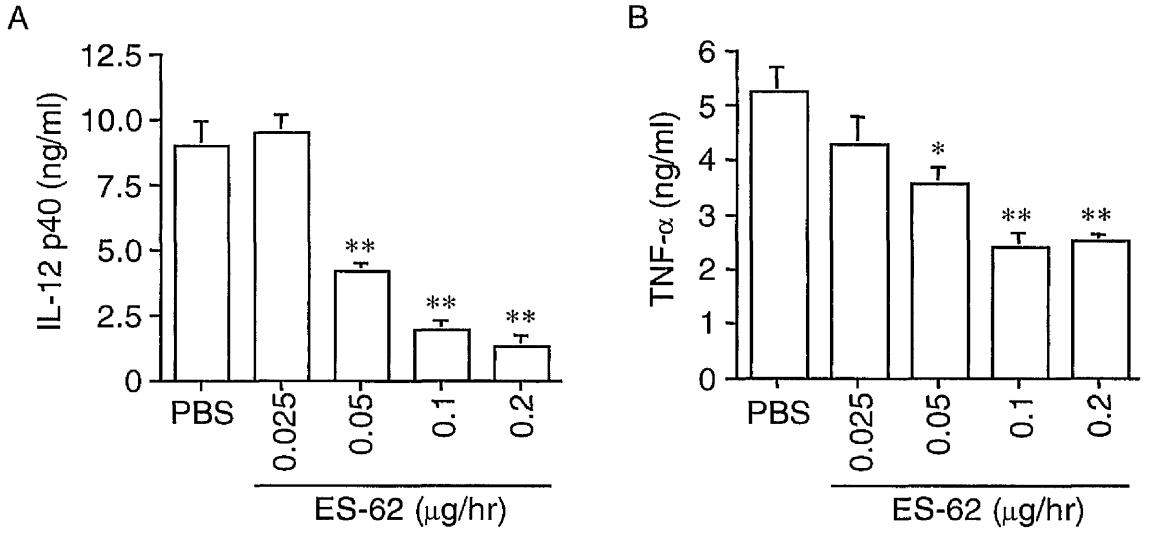


Figure 5.11. Dose-dependent effects of *in vivo* exposure to ES-62.

Peritoneal macrophages from mice (5 mice per group) exposed to PBS or different concentrations of ES-62 *in vivo* by release from osmotic pumps were stimulated *in vitro* with 100 U/ml IFN-γ and 100 ng/ml LPS ($2x10^5$ cells/well in a 96-well plate). IL-12 p40 and TNF-α in 24 hour culture supernatants were measured by ELISA. * $p < 0.05$, ** $p < 0.01$

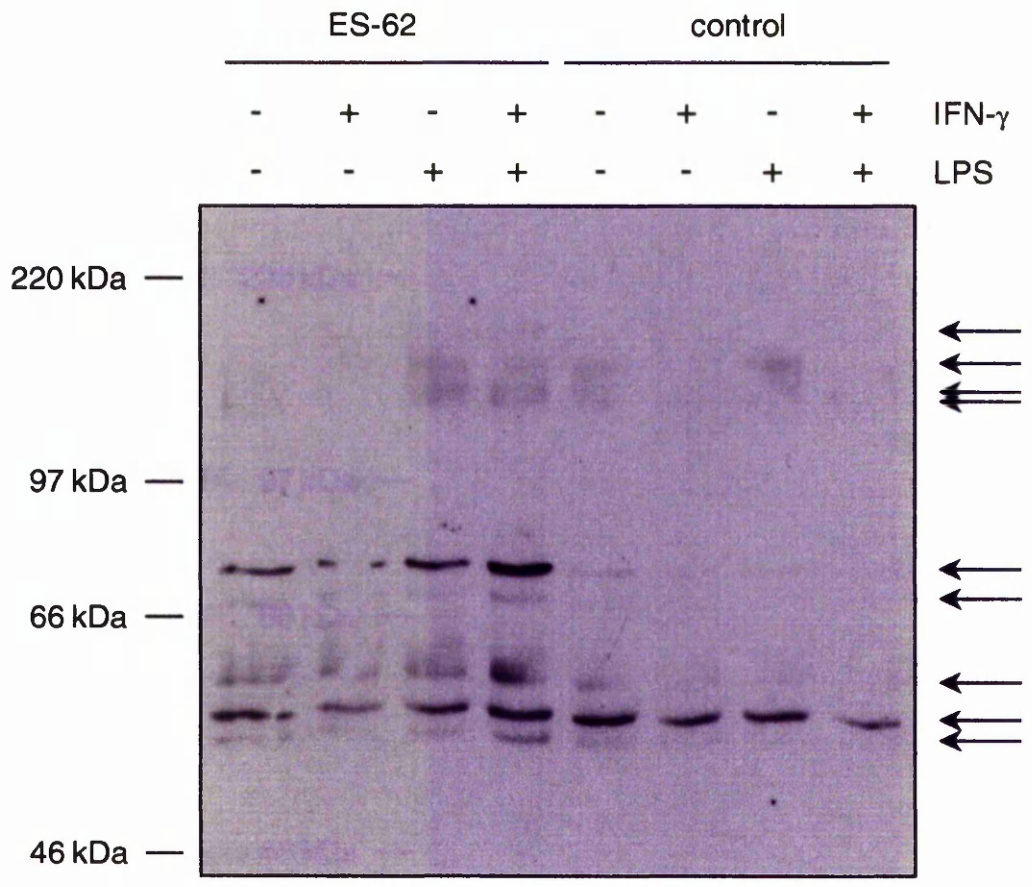


Figure 5.12. Effect of ES-62 on tyrosine phosphorylation of macrophage proteins.

Thioglycollate-elicited peritoneal macrophages from 129 mice ($4x10^6$/25 cm^2 flask) were pre-treated with 2 μg/ml ES-62 for 18 hours prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for 10 minutes. Total protein lysates were separated by SDS PAGE and transferred to nitrocellulose. Blots were probed with the anti-phospho-tyrosine antibody 4G10. Arrows indicate tyrosine phosphorylated proteins.

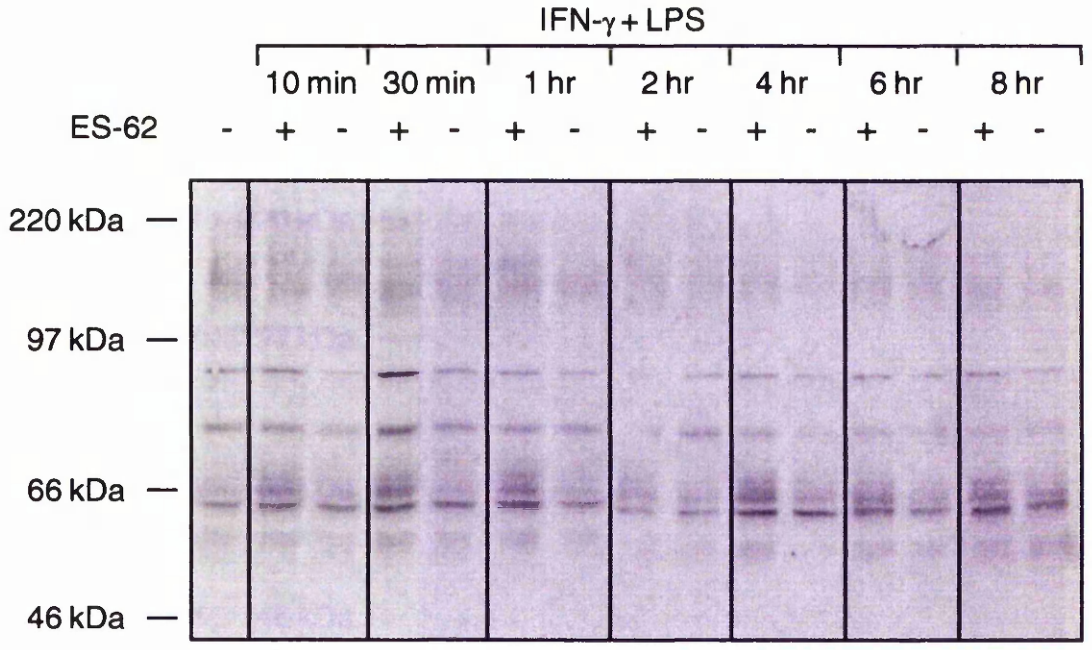


Figure 5.13. Effect of ES-62 on a timecourse of tyrosine phosphorylation.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($4x10^6$/25 cm^2 flask) were pre-treated with 2 μg/ml ES-62 for 18 hours prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated. Total protein lysates were separated by SDS PAGE and transferred to nitrocellulose. Blots were probed with the anti-phospho-tyrosine antibody 4G10.

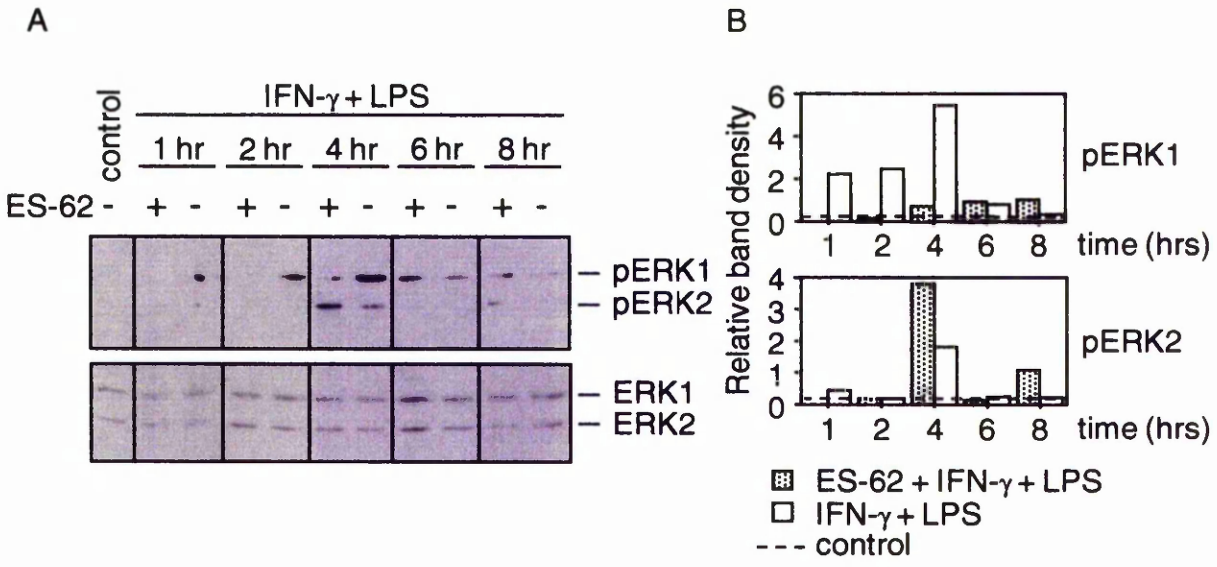


Figure 5.14. Effect of ES-62 on ERK MAP kinase activation.

A. Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were left untreated or treated with 2 μg/ml ES-62 for 18 hours prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated. Total protein extracts were purified and assayed by Western Blotting for dual phosphorylated ERK MAP kinase (pERK1 and pERK2; upper panel); antibodies against total ERK proteins were used to check protein loading (lower panel).

B. Densitometric analysis of phospho-ERK blot, normalised against total ERK. Dotted line represents unstimulated cells.

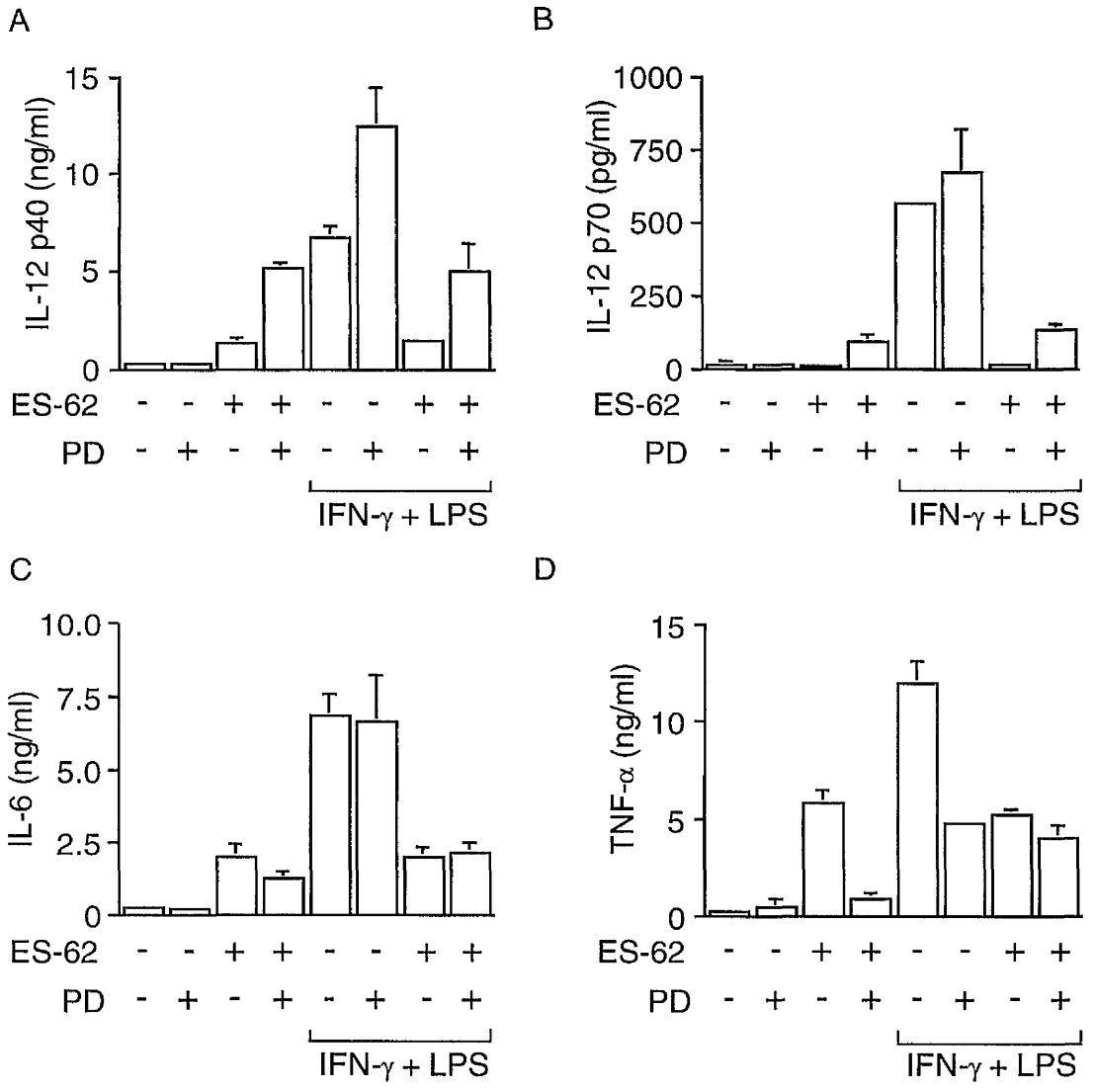


Figure 5.15. Effects of inhibition of ERK MAP kinases on modulation of IL-12, IL-6 and TNF-α production by ES-62.

Peritoneal macrophages ($2x10^5$/well in a 96-well plate) were treated with/without 2 μg/ml ES-62 in the absence/presence of 20 μM PD 098059 for 18 hours prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for 24 hours. IL-12 p40, bioactive IL-12 p70, IL-6 and TNF-α levels in culture supernatants were measured by ELISA.

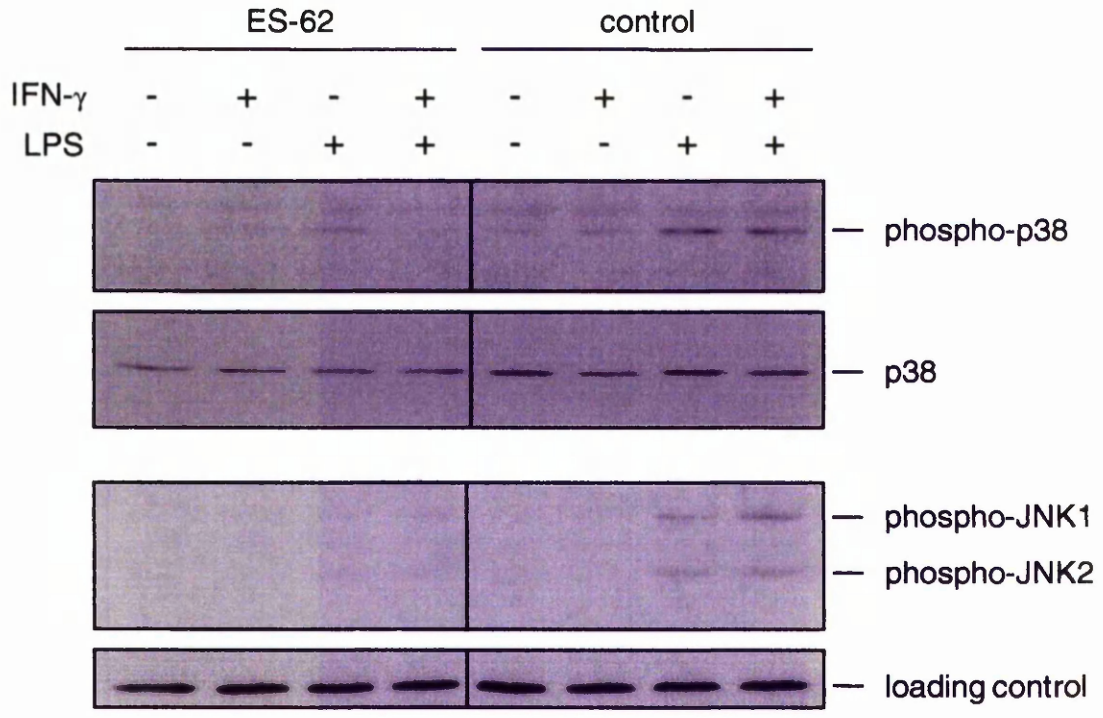


Figure 5.16. Effects of ES-62 on activation of p38 and JNK MAP kinases

Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were left untreated or treated with 2 μg/ml ES-62 for 18 hours prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS 10 minutes. Total protein extracts were purified and assayed by Western Blotting for dual phosphorylated p38 (A) and JNK (B) MAP kinases (upper panel of each pair); antibodies against total p38 (A) and non-specific protein (B) were used to confirm equal protein loading (lower panel of each pair).

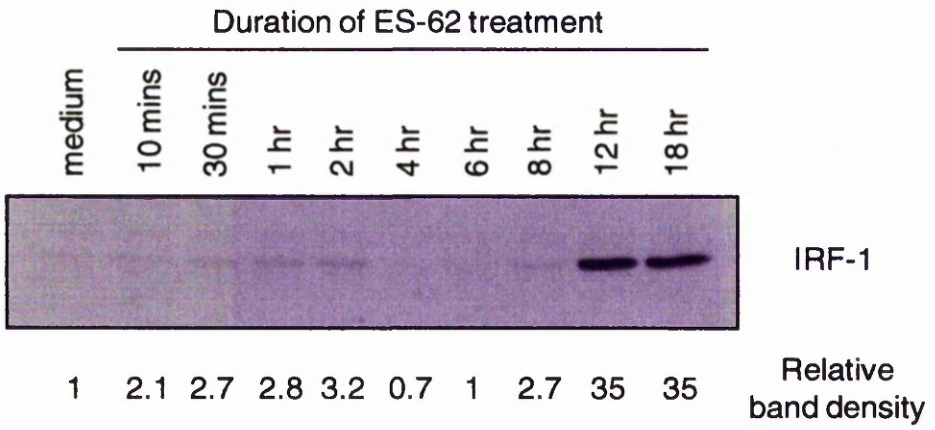


Figure 5.17. Effect of ES-62 on IRF-1 synthesis.

Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were stimulated with 2 μg/ml ES-62 for the times indicated. Total protein lysates were purified and assayed by Western Blotting using an anti-IRF-1 antibody. Densitometric values are expressed relative to unstimulated cells (medium).

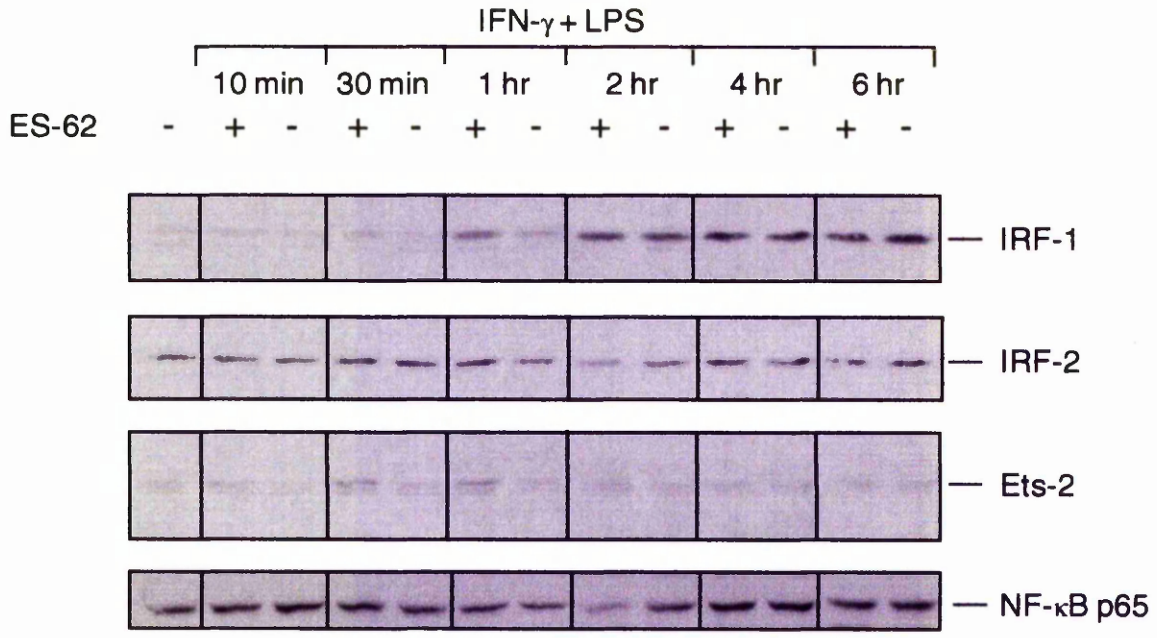


Figure 5.18. Effect of ES-62 on transcription factors synthesis.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($4x10^6$/25 cm^2 flask) were pre-treated with 2 μg/ml ES-62 for 18 hours prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated. Total protein lysates were separated by SDS PAGE and transferred to nitrocellulose. Blots were probed with specific antibodies against IRF-1, IRF-2, Ets-2 and the p65 isoform of NF-κB.

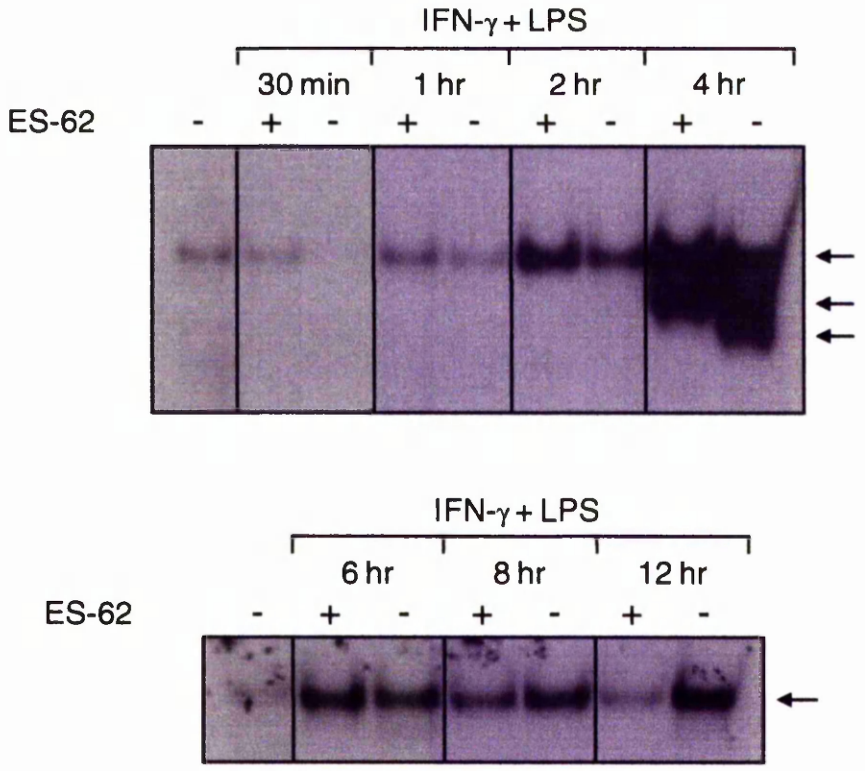


Figure 5.19. Effect of ES-62 on NF-κB binding.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($4x10^6$/25 cm^2 flask) were pre-treated with 2 μg/ml ES-62 for 18 hours prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated. Nuclear protein extracts were assayed by EMSA for binding to a ^{32}P-labelled oligonucleotide probe that represents an NF-κB binding site of the iNOS promoter. Arrows indicate binding complexes.

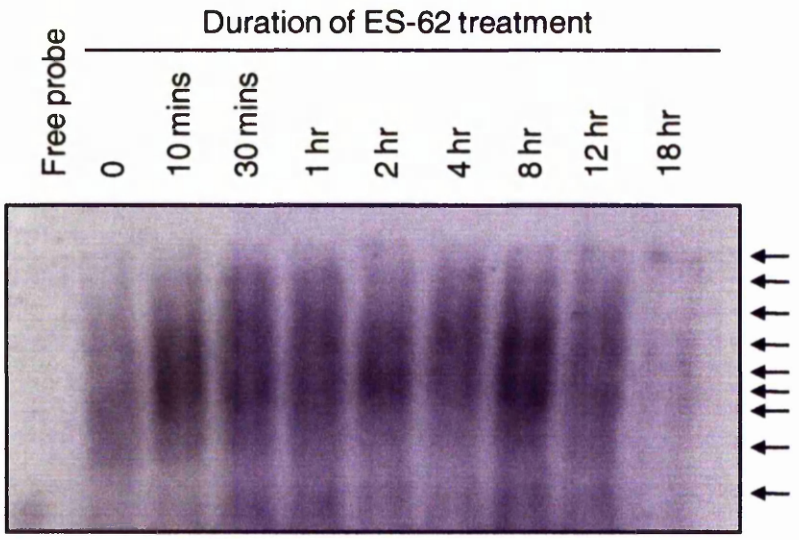


Figure 5.20. Effect of ES-62 on binding of transcription factors to the IL-12 p40 promoter.

Peritoneal macrophages ($4x10^6$/25 cm^2 flask) were stimulated with 2 μg/ml ES-62 for the times indicated. Nuclear protein extracts were assayed by EMSA for binding activity to a probe representing a portion of the IL-12 p40 promoter; binding complexes are indicated.

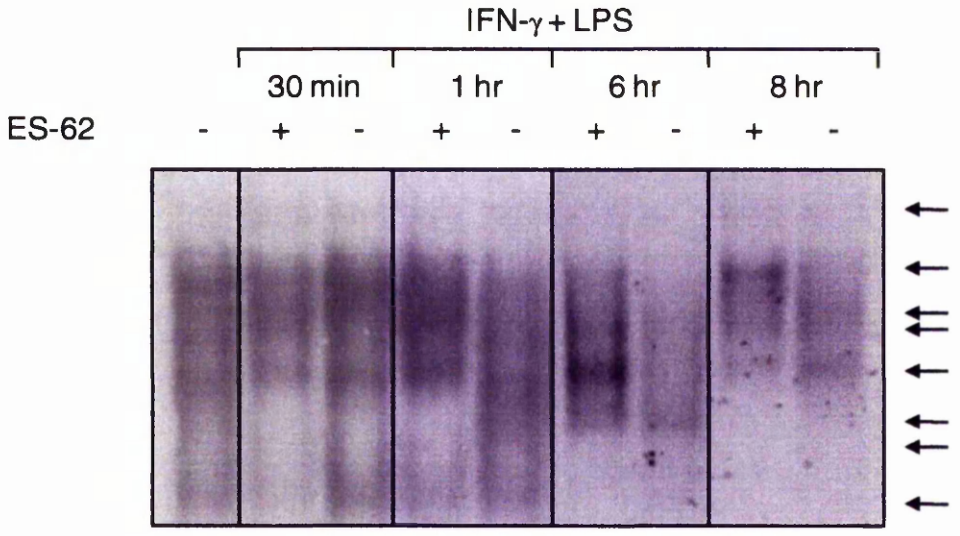


Figure 5.21. Effect of ES-62 on IFN-γ + LPS-induced binding of transcription factors to the IL-12 p40 promoter.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($4x10^6$/25 cm^2 flask) were pre-treated with 2 μg/ml ES-62 for 18 hours prior to stimulation with 100 U/ml IFN-γ and 100 ng/ml LPS for the times indicated. Nuclear protein extracts were assayed by EMSA for binding to a ^{32}P-labelled oligonucleotide probe that represents a portion of the IL-12 p40 promoter. Arrows indicate binding complexes.

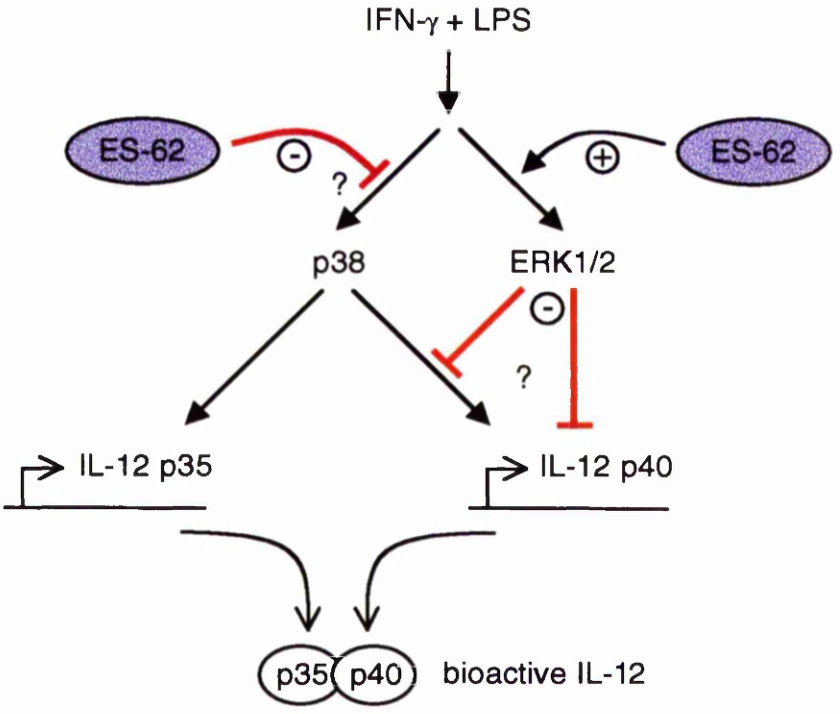


Figure 5.22. Model for suppression of IFN-γ + LPS-induced IL-12 production by ES-62.

Stimulation of macrophages with IFN-γ and LPS results in the activation of ERK and p38 MAP kinases. ES-62 inhibits the transcription of the IL-12 p40 gene in an ERK-dependent manner. It also suppresses the activation of p38 MAP kinase, which is required for the transcription of both IL-12 subunits. Reduced levels of both p40 and p35 protein limit the production of bioactive IL-12.

Chapter 6 – General Discussion.

6:1 Regulation of cytokine and iNOS induction in LPS-stimulated macrophages.

Pro-inflammatory cytokines produced by activated macrophages are key mediators of innate immunity against invading microorganisms, and are also important for initiating and directing the development of adaptive immune responses. One of the aims of this study was to investigate the signalling mechanisms underlying the induction of cytokine production resulting from activation of macrophages with LPS. The roles of p38 and ERK MAP kinases in induction of IL-12, IL-6 and TNF-α were dissected using specific inhibitors of these signalling molecules, and are summarised in Table 6.1 and Figure 6.1.

p38 was shown to be a key mediator of LPS signalling; activation of this MAP kinase is required for the synthesis of both subunits of IL-12, as well as IL-6, TNF-α and iNOS. In contrast, only TNF-α and iNOS induction are ERK-dependent. Furthermore, transcription of the IL-12 p40 gene is negatively regulated by ERK MAP kinases, which appear to act, at least in part, by suppressing the positive p38-mediated signals. The suppression of synthesis of p40, which is in any case produced in vast excess over the p35 subunit, is likely to serve to achieve an appropriate balance between the formation of bioactive IL-12 p70 and antagonistic p40 homodimers. IL-12 and iNOS, but not TNF-α, are regulated primarily at the level of transcription. Given that p38 but not ERK MAP kinases are required for IL-12 p35 and IL-6 induction and the homology between their genes, it is likely that regulation of IL-6 production by p38 MAP kinases, like IL-12 p35, occurs at the level of transcription or mRNA stability. Similarly, the recent report that p38 MAP kinase signals stabilise IL-6 mRNA (Winzen *et al.,* 1999) suggests that IL-12 p35 transcripts could also be regulated in this way. Although LPS stimulation does result

in dramatic induction of TNF-α mRNA, transcription of the TNF-α gene does not appear to be regulated by the ERK MAP kinases, which must therefore mediate translation or release of TNF-α protein. In contrast, p38 MAP kinase does appear to regulate TNF-α transcription or message stability, although it is also likely to act at later stages of TNF-α synthesis.

The physiological relevance of these observations was underlined by the demonstration that these signalling cascades are targeted by pathogen molecules to subvert macrophage activation and cytokine production.

Leishmania sp. parasites evade destruction by the host immune system by suppressing IL-12 production by macrophages, thus preventing the establishment of a Th1 response and the generation of NO. A number of other intracellular pathogens including the yeast *Histoplasma capsulatum*, measles virus and human immunodeficiency virus also inhibit IL-12 production (reviewed by Mosser and Karp, 1999). The *Leishmania* surface molecule lipophosphoglycan (LPG) has been demonstrated to mediate this suppression (Piedrafita *et al.,* 1999). In this study, sPG, a synthetic version of the repeating disaccharide component of LPG, was shown to selectively inhibit the induction of IL-12 by LPS. This inhibition is achieved by enhancing ERK MAP kinase activation and thereby suppressing transcription of the IL-12 p40 gene. The enhancement of ERK activity by sPG had no effect on TNF-α or iNOS induction, indicating that although they are necessary, ERK MAP kinases are not sufficient for induction of these macrophage mediators, but require additional signals to achieve upregulation of production.

Similarly, inhibition of IFN-γ + LPS-induced IL-12 production by the ES product of the filarial nematode *A. viteae* (ES-62) appears to be achieved via modulation of ERK activation, although by a more complex mechanism than that employed by sPG. Suppression of IL-12 p40 generation occurred at the transcriptional level, but mRNA production was not as dramatically reduced as protein, indicating that ES-62 also targets translation, post-translational modification or assembly of bioactive IL-12. Reduced transcription of the IL-12 p35 gene further contributes to the inhibition of bioactive IL-12 generation. ES-62 also suppresses the induction of IL-6 and TNF-α production. Transcription of the TNF-α gene was not altered by ES-62, suggesting that translation or release of protein from macrophages must be targeted by ES-62. Therefore, these results (summarised in Table 6.2 and Figure 6.2) support the idea that inhibition is achieved by modulation of multiple signals.

Taken together, these data demonstrate how the same signal can result in the uncoordinated induction of multiple factors using a limited set of signalling molecules. Thus, regulation of cytokine and iNOS production is achieved by different combinations of common mediators, enabling either independent or collective induction of these factors by the host. This also provides an opportunity for selective modulation of macrophage function by pathogens, which by targeting different pathways or steps in a pathway can alter the induction of a single or multiple immune mediators.

6:2 Future Work.

The data presented demonstrate crucial roles for MAP kinases in the regulation of production of macrophage immune mediators, with differential roles in IL-12, IL-6, TNF-α and iNOS synthesis. Further investigation of the mechanisms involved would be interesting, in particular, the ERK-mediated regulation of IL-12 p40. The data indicate that ERK MAP kinases target the IRF family of transcription factors to achieve regulation of IL-12 p40 induction. IL-12 p40 promoter constructs could be used to identify the regions of the promoter required for ERK regulation.

The pathway upstream of ERK activation could also be investigated. Preliminary evidence suggests that phosphoinositide-3-kinase (PI-3K) might be responsible for the ERK-mediated suppression of IL-12 p40. This enzyme catalyses the conversion of PI-(4,5)P_2 to PI-(3,4,5)P_3, resulting in the activation of PKC which has been implicated in the activation of MEK1, and hence PI-3K could mediate ERK activation by LPS. Indeed, stimulation of macrophages following pre-incubation for 1 hour with the PI-3K inhibitor wortmannin, like PD 098059 pre-incubation, resulted in enhanced IL-12 p40 production (Figure 6.3).

Similarly, macrophages cultured in calcium-free medium produced more IL-12 p40 (Figure 6.4), suggesting that calcium fluxes may also regulate IL-12 production. Indeed, calcium has also been implicated in the activation of the ERK pathway in many systems (Chao *et al.,* 1992; Rosen *et al.,* 1994; Zohn *et al.,* 1995). Furthermore, *Leishmania* infection of monocytes has been shown to result in elevated intracellular calcium levels resulting from increased permeability of plasma membrane Ca^{2+} channels

(Olivier *et al.,* 1992) and this could therefore provide a link to the suppression of IL-12 production by sPG. Consistent with this, blocking of calcium influx using EGTA has previously been shown to prevent suppression of IL-12 p40 production following ligation of macrophage receptors (including CR3 which binds LPG), without affecting other cytokines such as IL-10 and TNF-α (Sutterwala *et al.,* 1997).

The role of JNK MAP kinases in the induction of cytokines and iNOS cannot be investigated by inhibitor studies as there is at present no known selective inhibitor of these MAP kinases. However, JNK-deficient macrophages could be used to dissect the roles of this MAP kinase subfamily in macrophage activation. These could be obtained either by transient transfection with dominant negative JNK or antisense oligonucleotides, or from knock-out animals. In this way, JNKs have previously been implicated in the regulation of IL-12, TNF-α and NO (Swantek *et al.,* 1997; Constant *et al.,* 2000).

Further investigation is required to understand how ES-62 achieves its selective inhibition of macrophage functions, including more detailed study of MAP kinase activation e.g. role of p38 and JNK MAP kinases. ES-62 disrupts antigen receptor signalling in B and T lymphocytes, resulting in enhanced tyrosine phosphorylation and the uncoupling of MAP kinase cascades from antigen receptors (Deehan *et al.,* 1997, Harnett *et al.,* 1998). As demonstrated in this study, ES-62 has similar effects on signalling molecules in macrophages. It has also been shown to target other signalling molecules in lymphocytes, including PI-3K, PKC and phospholipase C (PLC) (reviewed by Harnett and Harnett, 1999); the effects of ES-62 on these molecules could also be

investigated in macrophages. The roles of transcription factors such as NF-κB and IRF family members could also be further analysed.

The results described in chapter 5 suggest that ES-62 either subverts LPS-receptor signalling or alternatively, initiates a signalling pathway that negatively regulates LPS signalling. TaqMan analysis of TLR4 mRNA showed that transcription of this LPS receptor is not altered by ES-62 (Figure 6.5). However, the membrane expression of TLR4 may be suppressed, thereby preventing the transduction of LPS signals. This possibility could be investigated by flow cytometry. Alternatively, preliminary evidence in human PBMC-derived dendritic cells suggests that ES-62 could inhibit cytokine production by down-regulating CD14 expression at the cell surface (R. Callard, personal communication to W. Harnett). Disruption of LPS binding could explain why macrophages were defective for induction of cytokines by LPS, but still able to produce NO, since iNOS induction was more dependent on IFN-γ than LPS signals. This would be similar to the situation in B cells where ES-62 uncouples various signalling pathways from the BCR, including the ERK MAP kinase cascade, to prevent proliferation (reviewed by Harnett and Harnett, 1999).

Preliminary experiments suggest that ES-62 may also function inside the cell since it is present in macrophage cytoplasmic and nuclear protein fractions (not shown). ES-62 shares homology with aminopeptidases and indeed, has been demonstrated to possess aminopeptidase activity (Harnett *et al.,* 1999b), although the function of this is at present unknown. An *E. coli* aminopeptidase (PepA/CarP/XerB) has been shown to bind DNA (Charlier *et al.,* 1995); hence it would be interesting to determine whether ES-62 achieves immunomodulation by binding to promoter sequences. The presence of leucine

rich repeats suggests that ES-62 could form dimers with leucine zipper transcription factors such as C/EBP and NF-IL6, which are known to regulate pro-inflammatory cytokine induction.

The ES-62 receptor is at present unknown, but BiaCore analysis indicated that ES-62 binds to monocyte membrane fractions with receptor-like affinity and in a PC-dependent manner (W. Harnett, personal communication). PC has been detected in a wide range of prokaryotic and eukaryotic pathogens including both Gram positive and negative bacteria, fungi, protozoa such as *Leishmania major* and *Trypanosoma cruzi*, several gastrointestinal nematodes and all species of filarial nematodes examined (Harnett and Harnett, 1999). It therefore seems likely that ES-62 could be recognised via binding of PC to pattern recognition receptors, such as the TLRs. Furthermore, the presence of leucine rich repeats (LRRs) in the ES-62 protein suggests that it could associate with other proteins, such as TLRs which also have LRRs.

Preliminary evidence from Far Western Blotting analysis using ES-62 conjugated to biotin indicated that ES-62 binds to several proteins from a variety of cell types including monocytic and B and T cell lines, including a protein of approximately 82 kDa (not shown). This band was again detected when the blot was stripped and reprobed with an anti-TLR2 antibody, suggesting that ES-62 may achieve immunomodulation by signalling through TLR2. This receptor is present on many different cell types (Muzio *et al.,* 2000), suggesting that a common mechanism may operate to disrupt the normal activation of both innate and adaptive immune cells following antigen binding. Interestingly, TaqMan analysis showed that TLR2 expression is transiently enhanced by treatment with ES-62 alone (Figure 6.5).

In *Drosophila* development, Toll receptor signalling is initiated by the binding of an endogenous protein encoded by the *spaetzle* gene (reviewed by Anderson, 1998). A mammalian homologue of Spaetzle has not yet been identified, but the binding of endogenous factors to Toll-like receptors fits with the Danger Theory of immune system activation. Macrophages and dendritic cells could be activated by the binding of an endogenous ligand to TLR4 (mimicked by LPS in the *in vitro* system) as a result of damage caused by the parasite. The presence of ES-62 would prevent the detection of this alarm signal, thereby suppressing cytokine induction, and resulting in suppression of host immunity and a bias of the T helper cell response towards a type 2 phenotype.

6:3 Concluding Remarks.

The development of therapies for treatment of infection requires the understanding both of normal mechanisms of host immune regulation and of evasion of immune responses by invading pathogens. The use of the two parasite molecules (sPG and ES-62) in this investigation not only enabled the study of subversion of cytokine production by parasites, but also revealed information about how MAP kinase isoforms regulate cytokine synthesis by macrophages. Furthermore, such immunomodulatory pathogen molecules are themselves potential therapeutic agents for the control of immune diseases. For example, since ES-62 biases T helper cell responses towards a type 2 phenotype, it might improve the prognosis of inflammatory diseases such as rheumatoid arthritis.

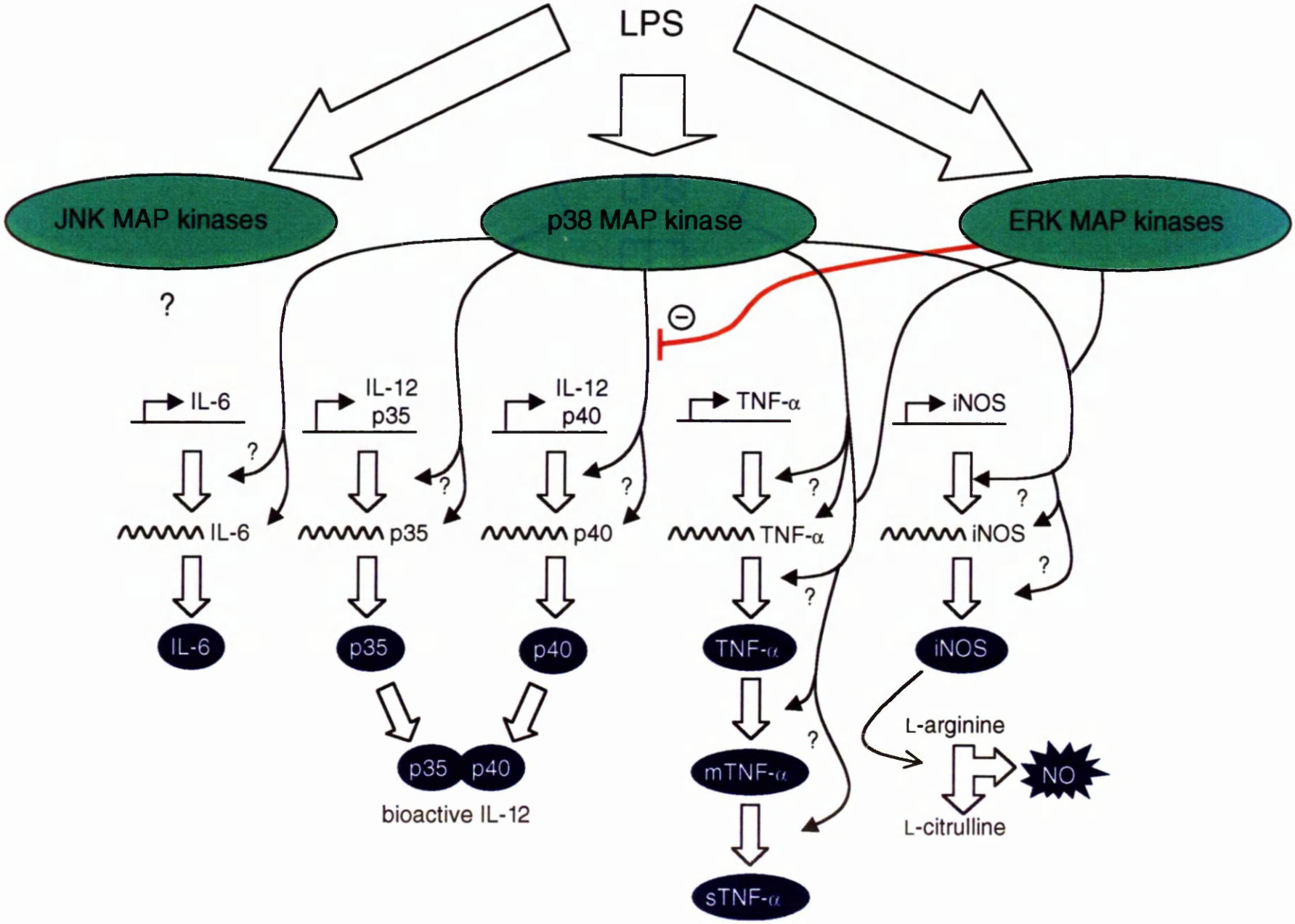
	ERK1/2		p38	
	Regulator?	Level of regulation	Regulator?	Level of regulation
IL-12 p40	-ve	transcription/ mRNA stability	+ve	transcription/ mRNA stability
IL-12 p35	-	-	+ve	transcription/ mRNA stability
IL-6	-	-	+ve	transcription/ mRNA stability
TNF-α	+ve	translation/ post-translation	+ve	multiple levels
iNOS	+ve	?	+ve	transcription/ mRNA stability

Table 6.1. Roles of ERK and p38 MAP kinases in induction of cytokines and iNOS by stimulation of macrophages with LPS.

Induction of both subunits of IL-12, as well as IL-6, TNF-α and iNOS, by stimulation of macrophages with LPS in the presence/absence of IFN-γ requires signals mediated by p38 MAP kinase. ERK MAP kinase signals are also required for synthesis of TNF-α and iNOS, but negatively regulate IL-12 p40 production. Regulation is likely to occur at multiple levels including transcription, message stability, translation, post-translational modification and release from the cell.

Figure 6.1. Roles of MAP kinases in the induction of cytokines and iNOS by LPS.

Stimulation of macrophages with LPS results in activation of ERK, p38 and JNK MAP kinases. The roles of the ERK and p38 MAP kinases in the induction of IL-12, IL-6, TNF-α and iNOS are indicated. The involvement of the JNKs is not clear due to the unavailability of a selective inhibitor of these MAP kinases.


LPS
JNK MAP kinases
p38 MAP kinase
ERK MAP kinases
?
⊖
IL-6
IL-12 p35
IL-12 p40
TNF-α
iNOS
?
IL-6
p35
p40
TNF-α
iNOS
IL-6
p35
p40
TNF-α
iNOS
p35 p40
bioactive IL-12
mTNF-α
sTNF-α
L-arginine
NO
L-citrulline

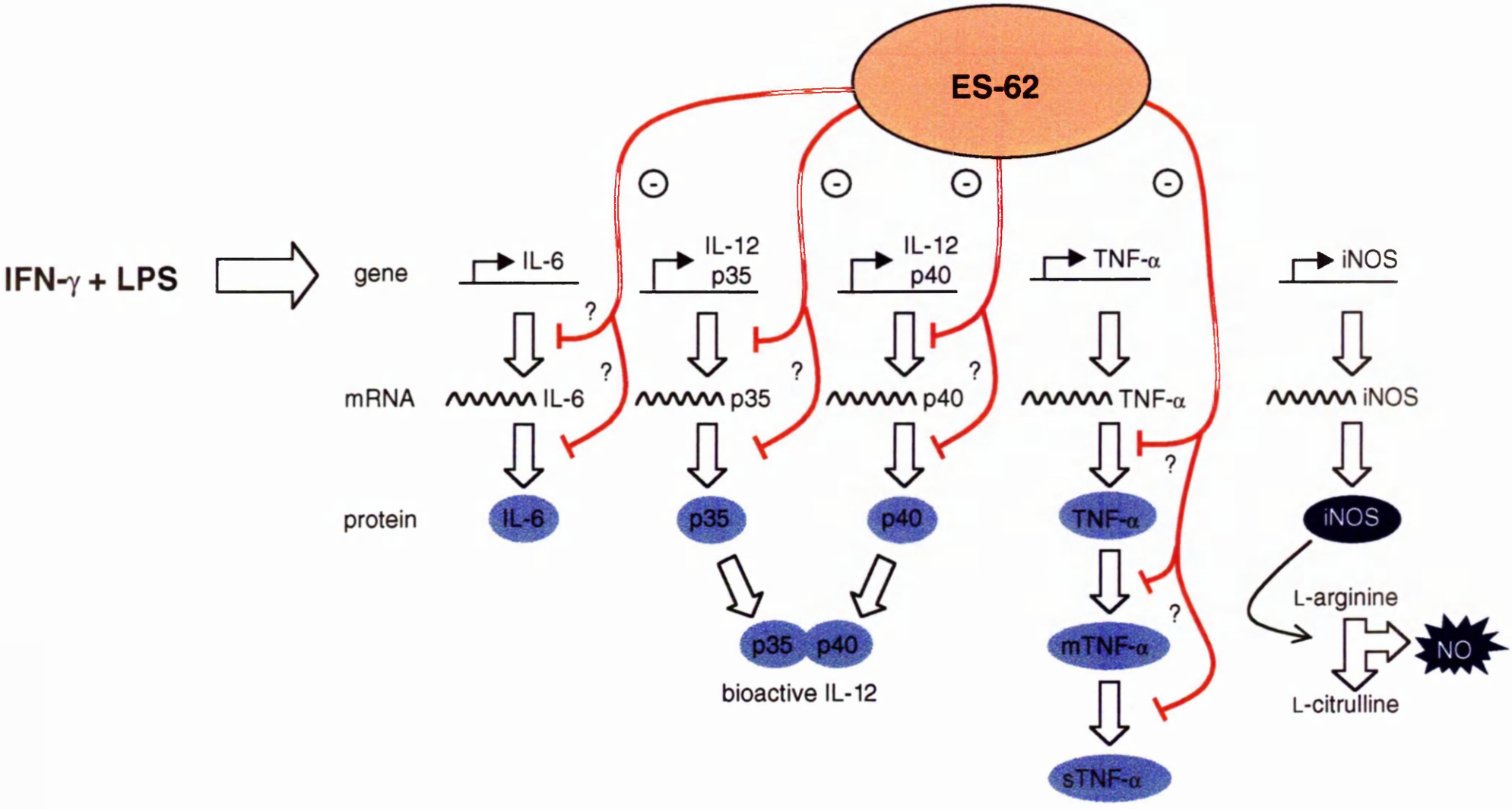
	Level of suppression	ERK-dependent?
IL-12 p40	transcription translation/post-translation	✓
IL-12 p35	transcription ? translation/post-translation	✗
IL-6	? transcription ? translation/post-translation	✗
TNF-α	translation/ post-translation	?

Table 6.2. Suppression of cytokine production by ES-62.

ES-62 suppresses the induction of IL-12, IL-6 and TNF-α by macrophages activated with IFN-γ + LPS. The table summarises the level of synthesis of each cytokine that is targeted by ES-62 and whether this effect is mediated by ERK MAP kinases.

Figure 6.2. Suppression of cytokine induction by ES-62.

Stimulation of macrophages with IFN-γ + LPS results in induction of IL-12, IL-6, TNF-α and iNOS expression. ES-62 suppresses bioactive IL-12 production by reducing the synthesis of both the p40 and the p35 subunits. It also inhibits IL-6 and TNF-α induction, but does not alter iNOS expression, resulting in normal NO production.


ES-62
IFN-γ + LPS
gene
mRNA
protein
IL-6
IL-12 p35
IL-12 p40
TNF-α
iNOS
?
IL-6
p35
p40
TNF-α
iNOS
p35 p40
bioactive IL-12
mTNF-α
sTNF-α
L-arginine
NO
L-citrulline

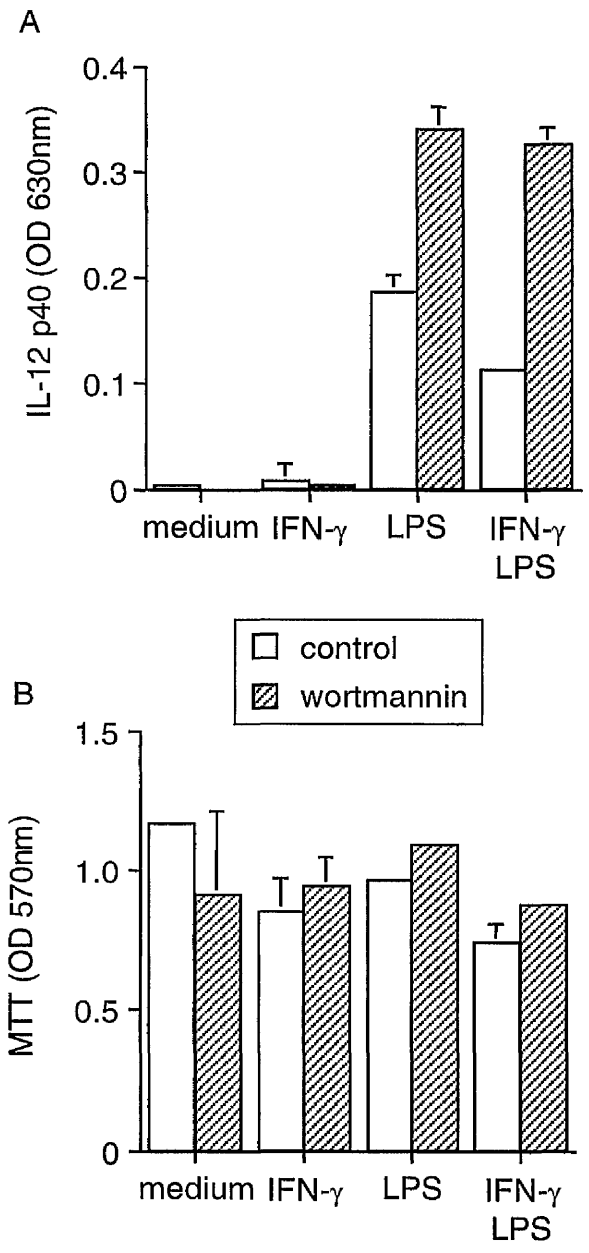


Figure 6.3. Effect of PI-3 kinase inhibition on IL-12 production and macrophage viability.

J774 cells ($2x10^5$/well in a 96-well plate) were stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS in the presence/absence of 5 nM wortmannin, an inhibitor of PI-3 kinase. IL-12 p40 in 24 hour culture supernatants was measured by ELISA and cell viability was assessed by MTT assay.

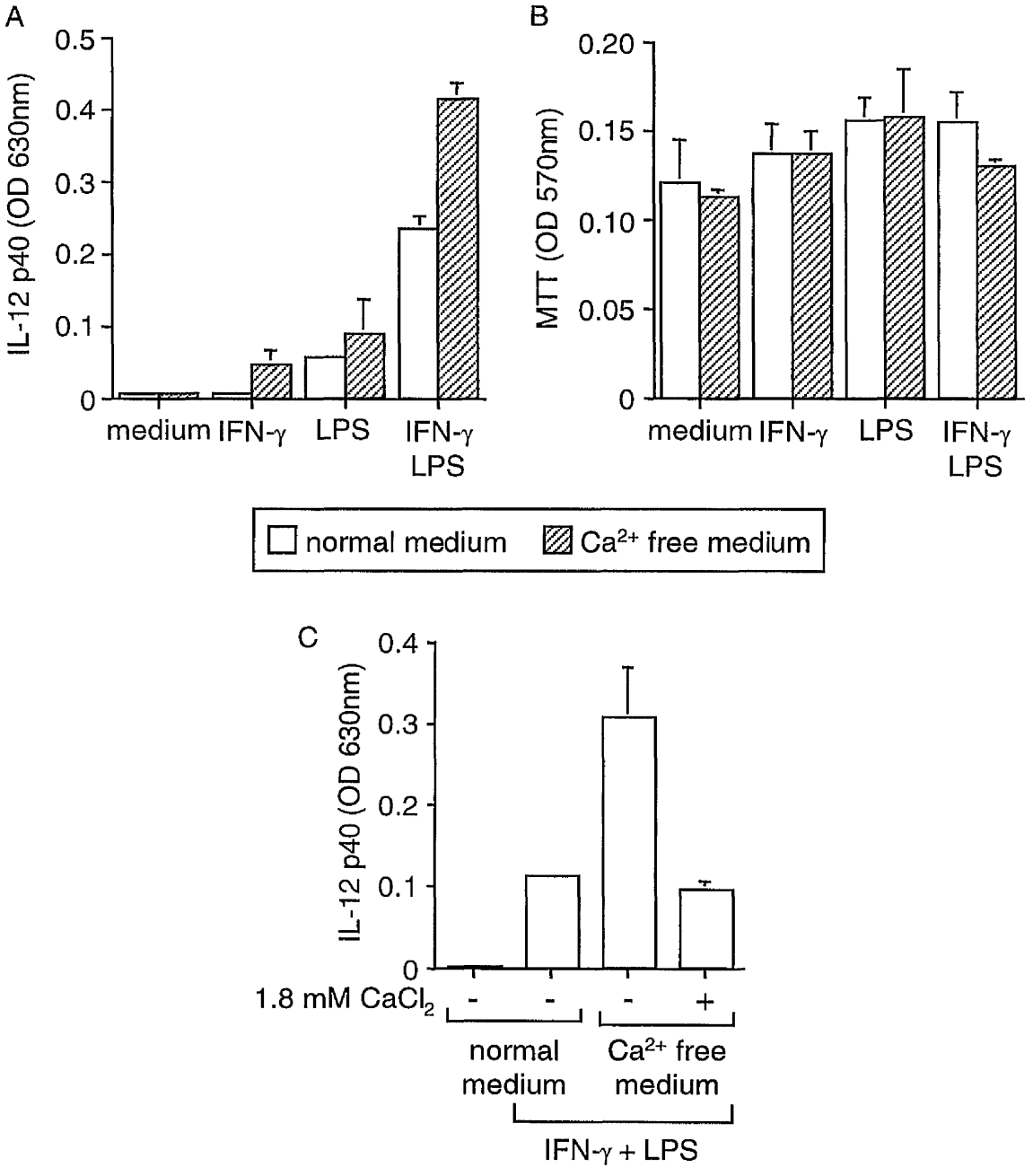


Figure 6.4. Stimulation of macrophages in calcium-free medium.

Murine peritoneal macrophages ($2x10^5$/well in a 96-well plate) were cultured in normal medium, calcium-free medium, or calcium-free medium with the calcium replaced by addition of 1.8 mM $CaCl_2$, prior to stimulation with 100 U/ml IFN-γ and/or 100 ng/ml LPS for 24 hours. IL-12 p40 in culture supernatants was measured by ELISA and cell viability was assessed by MTT assay.

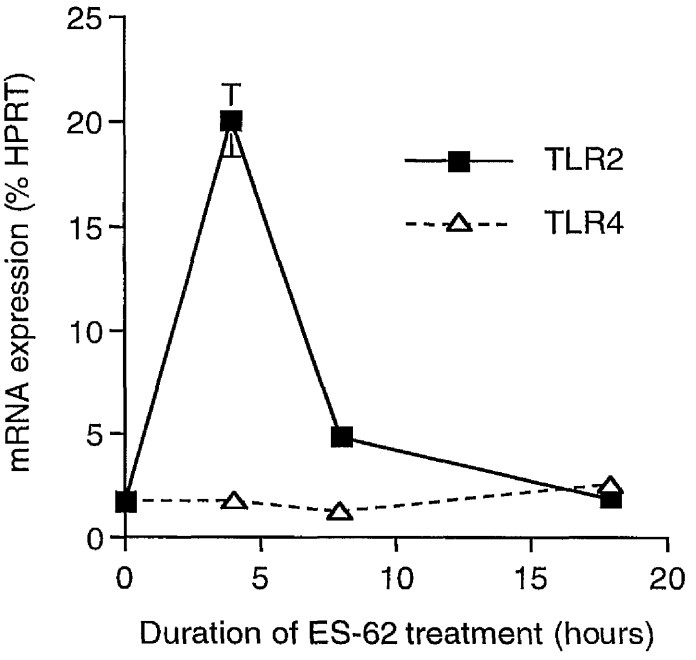


Figure 6.5. Effect of ES-62 treatment on TLR2 and TLR4 mRNA levels.

Thioglycollate-elicited peritoneal macrophages from BALB/c mice ($4x10^6$ cells/25 cm^2 flask) were treated with 2 μg/ml ES-62 for the times indicated. Total RNA was purified from cells and TLR2 and TLR4 transcripts were assayed by real-time PCR (TaqMan). mRNA levels are expressed relative to HPRT mRNA.

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