Regulation of Inducible Nitric Oxide Synthase

BY

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Abbreviations

AP-1	Activator protein 1
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
CNTF	Ciliary neurotrophic factor
CSF	Colony-stimulating factor
dATP	2'-Deoxyadenosine 5'-triphosphate
dCTP	2'-Deoxycytidine 5'-triphosphate
dGTP	2'-Deoxyguanosine 5'-triphosphate
DNA	Deoxyribose nucleic acid
DTT	1,4-dithiothreitol
dTTP	2'-Deoxythymidine 5'-triphosphate
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene Glycol-bis(β-aminoethyl ether)-N', N', N', N'-tetraacetic acid
EMSAs	Electrophoretic mobility-shift assays
EPO	Erythropoietin.
GAF	Interferon-γ activation factor
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
GAS	IFN-γ activation site
GBP	Guanylate-binding protein
GH	Growth hormone
GM-CSF	Granulocyte-macrophage colony -stimulating factor
Hb	Haemoglobin
HRP	Horseradish peroxidase
ICSBP	Interferon consensus sequence binding protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
ISGF	Interferon-stimulated gene factor
ISRE	Interferon stimulation response element
JAK	Janus kinase
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatility complex
mRNA	Messenger ribonucleic acid
NADPH	β-Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NO_2^-	Nitrite
OD	Optical density
OSM	Oncostatin M
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor recptor
PI3-K	Phosphatidylinositol 3 kinase
PLC-γ	Phospholipase C-γ
PMSF	Phenylmethylsulfonyl fluoride
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
RTK	Receptor tyrosine kinase
SDS PAGE	SDS polyacrylmide gel electrophresis
SH2	Src homology region 2

STAT	Signal transducers and activators of transcription
T-cell	Thymus derived lymphocyte
Taq	Thermus aquaticus
TBE	Tris -borate/EDTA electrophoresis buffer
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TGF-β	Transforming growth factor beta.
TNF-α	Tumor necrosis factor-alpha
TPO	Thrombopoietin
Tris	Tris (hydroxymethyl) methylamine
U	Units
v/v	Volume per volume

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Declaration

These studies represent original work carried out by the author, and have not been submitted in any form to any other University. Where use has been made of materials provided by others, due acknowledgement has been made.

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SUMMARY

One of the major functions of macrophages is to provide the body with an immediate innate defence against pathogenic micro-organisms. This defence is largely dependent on the generation of nitric oxide (NO) and superoxide by macrophages which leads to the killing of these pathogens. NO is also important in many other biological functions. It is derived from L-arginine and molecular oxygen by the enzyme NO synthase (NOS). There are a number of classes of NOS including neuronal NOS (nNOS), endothelial NOS (eNOS) and cytokine-inducible NOS (iNOS). iNOS is upregulated and activated by several immunological stimuli including interferon gamma (IFN-γ); lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α), such activation leads to the production of large quantities of NO which can be cytotoxic. The potential toxicity of NO makes it important to understand the regulation of its production. In the study presented here, J774 cells, a murine macrophage cell line, were used as a model system for studying the induction and regulation of iNOS activation. These cells and murine peritoneal macrophages produce large amounts of NO in response to the T cell-derived lymphokine, IFN-γ and/or the potent macrophage activator, LPS in a dose-dependent manner. Northern and Western blotting revealed the process of induction of NO synthesis in J774 cells: The maximal induction of NO synthase mRNA was at 4 h while the maximum levels of NOS protein was observed at 8 to 12 h after treatment with IFN-γ and LPS. IFN-γ and LPS-induced NO_2^- accumulation was abolished in the culture supernatants of samples that were pre-treated with cycloheximide. These suggest that iNOS is regulated transcriptionally in a manner that requires *de novo* protein synthesis.

Protein phosphorylation plays a crucial role in regulating the signal transduction cascades leading to many biological responses in eukaryotes. Signals that are reversibly controlled by protein phosphorylation are modulated not only by a protein kinase but also by a protein phosphatase. In this project, I have shown that the induction of iNOS activity in J774 cells by IFN-γ and LPS was reduced by more than 50% if the cells were pre-treated with protein tyrosine kinase (PTK) inhibitors such as Tyrphostin 25, Tyrphostin

AG126, and Herbimycin A. In contrast, iNOS was unaffected by pre-incubation with Tyrphostin 1, an inert analogue of these PTK inhibitors. Consistent with these findings, IFN-γ and LPS-induced iNOS activity was enhanced by 30% in the presence of vanadate, a protein tyrosine phosphatase inhibitor. These results suggested that the activation of tyrosine kinase(s) plays a role in induction of NO synthesis.

To identify which protein tyrosine kinases, and their downstream targets, might be involved in the iNOS signalling pathway, I used a combination of immuno-precipitation and Western blotting techniques. This approach identified a role for the Janus kinases (JAK1, JAK2) in the induction of iNOS. Moreover, Tyrphostin AG490, a specific JAK2 inhibitor strongly inhibited NO production in J774 cells in response to IFN-γ. Further experiments demonstrated an inducible STAT1 (signal transducer and activator of transcription factor 1) binding activity to IFN-γ activated site (GAS) in the IRF1 (interferon regulatory factor 1) promoter after treatment with IFN-γ. In addition, Northern blot analysis showed an enhanced IRF-1 and IRF-2 expression after IFN-γ treatment. Furthermore, IRF-1 and IRF-2 were demonstrated to have the ability to bind to IRF-E within the iNOS promoter region. Thus IFN-γ appears to transduce the signals through a JAK-STAT pathway (JAK1, JAK2, and STAT1), activation of some early expression genes (such as IRFs), and perhaps cross-talk with other signalling elements, such as NFκB, to achieve transcriptional activation of iNOS.

I have also demonstrated that LPS induces two GAS-binding complexes in J774 cells. These binding proteins are different from the GAS-binding STAT protein induced by IFN-γ. Antibody supershift and cold-probe competition assays indicated that the transcription factors in such complexes contained NFκB (p65). NFκB was also demonstrated to have the ability to bind to NFκB element within iNOS promoter region in response to LPS and/or IFN-γ. The binding activity of NFκB induced by either LPS or IFN-γ was independent of protein synthesis. Northern blot analysis demonstrated that IFN-γ and LPS synergistically up-regulate IRF-1 but down-regulate IRF2 expression. Moreover, the induction of NO by IFN-γ and LPS also appears to involve the co-

operation of NFκB and IRF-E in the activation of iNOS promoter. The co-operation of signals indicated by these data provide a rational explanation for the synergistic effect of IFN-γ and LPS in the induction of iNOS expression.

An additional transcription factor(s) was found to be activated by LPS which was involved in iNOS gene regulation. This factor is a protein encoded by an early expressed gene whose activation is dependent on protein synthesis. It binds to IRF-E on the murine iNOS promoter. Antibody-supershift and cold probe-competition assays ruled out the possibility that it was NFκB, or IRF-1. Although, as yet, it has not been possible to identify this factor, it may well be the IRF-like protein or perhaps the interferon consensus sequence binding protein (ICSBP) which has been shown by others to play a crucial role in LPS-induced IFN-γ production in spleen cells. Further experiments are required to identify this potentially important factor.

In addition to IFN-γ and LPS, IL-4 is another cytokine that plays an important role in the regulating iNOS expression in murine macrophages. IL-4 is a multipotent cytokine characteristically derived from Th2 and mast cells. Since IFN-γ and IL-4 have been shown to display opposite effects and to antagonize each other's actions on a number of cell types, I studied the mechanism by which IL-4 regulates iNOS expression in murine macrophages. I have shown that IL-4 inhibits expression of iNOS (both at the protein and mRNA level) induced by IFN-γ and LPS in a dose- and time- dependent manner. To down regulate iNOS expression, IL-4 has to be added to the cells at the initial stages (before or simultaneously with addition of IFN-γ and/or LPS) of iNOS induction. Thus, comparison of these kinetics of IL-4 mediated inhibition of NOS activation suggest that the effect of IL-4 on iNOS induction is most likely to be at the stage of transcriptional activation.

Following the idea that IL-4 may interfere with the signalling pathways of NO synthesis, the activation of a number of signalling elements by IFN-γ or LPS was examined in the presence of IL-4. Western blotting and EMSA analysis showed results that IL-4 did not affect the early intracellular signalling pathways induced by IFN-γ, such

as the tyrosine phosphorylation and activation of JAK1 and JAK2 and STAT1 (p91). Furthermore, it did not interfere with LPS-induced NFκB binding. Unexpectedly, the induction of IRF-1 and IRF-2 by IFN-γ alone was enhanced by IL-4. In contrast, IL-4 inhibited the expression of IRF-1 and enhanced the expression of IRF-2, when the cells were stimulated with IFN-γ and LPS.

Finally, I have shown that while the production of NO by macrophages from wild-type mice in response to IFN-γ and LPS was strongly inhibited by IL-4, cells from STAT6 knockout mice (STAT6-/-) were completely refractory to inhibition by IL-4. These results therefore suggest that STAT6 plays a crucial role in the IL-4-induced inhibition of iNOS expression in murine macrophages. Further experiments are required to investigate whether STAT6 itself works as a transcription factor binding directly to iNOS gene, or involved in the activation of some other IL-4 regulated genes which in interfere with iNOS induction.

Chapter 1

General Introduction

Part I. Cytokine inducible nitric oxide synthase

1.1 Nitric Oxide (NO) and nitric oxide synthase

1.1.1 Historical perspective

Nitric oxide is a multifunctional effector molecule synthesized by nitric oxide synthase (NOS). NOS represents a family of cytochrome P450 -like flavohaeme-proteins that catalyze the 5-electron oxidation of L-arginine to form L-citrulline and NO. The quest to identify endothelium-derived relaxing factor (EDRF) (Furchgott, *et al.*, 1980) led to the discovery in the vasculature of an enzyme, nitric oxide synthase (reviewed in Moncada, *et al.*, 1993). The early studies on EDRF demonstrated that EDRF was a very short-lived substance, with a half-life of only seconds in oxygenated physiological salt solutions (Griffith, *et al.*, 1984; Cocks, *et al.*, 1985). The effects of EDRF were shown to be inhibited by haemoglobin (Hb), methylene blue (Martin, *et al.*, 1985) and other agents such as dithiothreitol and hydroquinone (Griffith, *et al.*, 1984) and to be mediated by stimulation of the soluble guanylate cyclase with the consequent elevation of intracellular cyclic GMP (cGMP) levels (Rapoport, *et al.*, 1983). Superoxide anions (O_2^-) contribute to the instability of EDRF, because the effects of EDRF were prolonged by the addition of SOD (Gryglewski, *et al.*, 1986a; Rubanyi, *et al.*, 1986) and inhibited by Fe^{2+} (Gryglewski *et al.*, 1986b). In 1987, Furchgott and Ignarro independently suggested that EDRF was either NO or an NO-related molecule. The first direct demonstration of the release of NO by mammalian cells was in experiments on vascular tone and platelet aggregation (Palmer, *et al.*, 1987; Moncada, *et al.*, 1990).

Meanwhile, an independent line of investigation found that when humans were fed a low nitrite diet, endogenously synthesized NO_3^- was excreted (Green, *et al.*, 1981). Similar results were obtained with germ-free rats, thus ruling out the participation of gut microflora in the reaction. During the course of the human experiments, one of the subjects coincidentally became ill and showed a large increase

in urinary NO_3^- excretion. Subsequent experiments in rats showed that the urinary NO_3^- levels could be elevated about tenfold when fever was induced by an intraperitoneal injection of *Escherichia coli (E. coli)* lipopolysaccharide (LPS). These findings suggested that this elevated synthesis might be related to the immunostimulation known to be brought about by LPS. The LPS-induced synthesis of NO *in vivo* was then reproduced by stimulation of murine peritoneal macrophages in culture (Stuehr, *et al.*, 1985). Some cytokines could also stimulate macrophages to carry out this synthesis. Subsequent experiments showed that the major lymphokine involved in the synthesis response was interferon-gamma (IFN-γ) (Stuehr, *et al.*, 1987). The stimulatory effects of LPS and IFN-γ were synergistic (reviewed in Marletta, *et al.*, 1989). Later studies showed that NO is synthesised from the semi-essential amino acid L-arginine by NO synthase (Hibbs, et al.1987; Stuehr, *et al.* 1987; Iyengar, *et al.* 1987; Palmer, *et al.* 1988). This process can be inhibited by guanidino-substituted arginine analogues, including N^G-monomethyl-L-arginine (L-NMMA) (Hibbs, *et al.*, 1987).

1.1.2 Characteristics of isoforms of nitric oxide synthase

Three isoforms of NOS are known to exist and can be classified into two categories, constitutive and inducible (reviewed by Nathan 1992). The inducible nitric oxide synthase (iNOS or NOS-II) was first found in murine macrophages. Under basal conditions, iNOS activity in macrophages is negligible, while on stimulation with IFN-γ and LPS, macrophages can produce massive enhancement of NO synthase within few hours (Nathan and Hibbs, 1991). iNOS is Ca^{2+} independent. The other two forms of NOS were originally identified in blood vessels and neurones, they are constitutively expressed and their activity depends on elevated levels of Ca^{2+}. Recently they have been named ecNOS (NOS-III) and ncNOS(NOS-I) (reviewed by Nathan and Xie, 1994).

All three NO synthases are flavoproteins containing bound flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). They are dependent on NADPH as a co-factor and tetrahydrobiopterin (BH_4) enhances enzyme activity (Palmer, *et al.*, 1989;

Hevel, *et al.*., 1991; Mayer, *et al.*, 1991; Schmidt, *et al.*, 1992; Bredt, *et al.*, 1992; Tayeh, *et al.*, 1989; Kwon, *et al.*, 1989). The derived sequence of ncNOS from rat brain was the first to indicate that the C-terminus showed a significant homology to NADPH cytochrome P-450 reductase (Bredt, *et al.*, 1991). The nucleotide binding sequence as well as those sequences associated with FAD and FMN binding were highly conserved when compared with P-450 reductase from rat liver. This same homology has been observed in all the reported NOS sequences (Fig. 1.1). The N-terminus in all sequences of the three forms shows a great deal of similarity suggesting a common functional role, most likely related to the arginine binding site and catalysis (reviewed by Marletta, 1993). A comparison with other arginine binding enzymes, however, has not provided any clear cut answers (Lowenstein, *et al.* 1992).

Genes encoding the three distinct NOS isoforms have been cloned and located to different human chromosomes (ncNOS: 12q24.2; ecNOS: 7q35-36; iNOS: 17cen-q12) (reviewed by Nathan and Xie, 1994). The three genes are differentially expressed: NOS was found originally in the neuro-systems but has now also been found in extra-neuronal sites such as skeletal muscle, pancreas, and kidney (Kobzik, *et al.*, 1994; Lukic, *et al.*, 1991; Mundel, *et al.*, 1992; Imai, *et al.*, 1992). The isoform first purified and cloned from endothelial cells is also expressed in neurones (Dinerman, *et al.*, 1994). Unlike the limited tissue distribution of the cNOS isforms, multiple cell types exhibit the capacity to express the iNOS gene when appropriately stimulated, among them neurons and endothelial cells (Oswald, *et al.*, 1994). Recent reports showed that one or more isoforms could also express in the same cell type (Reiling, *et al.*, 1994; Dusting, *et al.*, 1995).

1.1.3 The functions of NO

Small amounts of NO are generated by ncNOS and neNOS, while high levels of NO are produced by iNOS. In many systems, NO derives from two or more different cellular sources, forming networks of paracrine communication. Neurones produce NO to regulate transmitter release of adjacent neurones and also to match cerebral blood flow with neuronal activity. Endothelium-derived NO is vasoprotective by potentially

antagonizing smooth muscle contraction and all stages of platelet activation. The production of NO is a double-edged sword however, (as although it is essential as a messenger or modulator, and in high concentration for host defence against pathogens), excessive amounts of NO can also lead to a range of immunopathologies (Fig. 1.2). In the present project, I will concentrate on the regulation of the inducible NOS which catalyses the high output of NO.

1.2 Inducible nitric oxide synthase (iNOS)

1.2.1 iNOS gene cloning and expression

Several groups reported molecular cloning of iNOS cDNA from murine macrophage RAW 264.7 cells (Xie, *et al.*, 1992; Lyons, *et al.*, 1992; and Lowenstein, *et al.*, 1992) and also from rat vascular smooth muscle cells (Nunokawa, et al., 1993). Human iNOS from different cell types (chondrocyte, hepatocyte, and smooth muscle) have also been subsequently cloned (Charles, *et al.*, 1993; Geller, *et al.*, 1993; Sherman, *et al.*, 1993). More recently, iNOS cDNA from murine macrophage J774 cells has been cloned in this laboratory (Moss, *et al.*, 1995). Furthermore, human hepatocyte iNOS was found to have 80% sequence identity to the murine (macrophage) iNOS at both the nucleotide and amino acid levels (Geller 1993), suggesting that the iNOS gene may be functionally and structurally conserved.

Although resting unstimulated cells express little iNOS, the capacity to express this enzyme exists in nearly every tissue in the body. The list of cell types capable of expressing iNOS now includes macrophages, neutrophils, keratinocytes, respiratory epithelium, retinal pigment epithelium, renal tubular epithelium, myoepithelium, adenocarcinomas, hepatocytes, pancreatic islet cells, endothelium, endocardium, mesangial cells, cardiac myocytes, vascular smooth muscle, uterine and fallopian tube smooth muscle, fibroblasts, chondrocytes, osteoclasts, neurons and astrocytes (Lorsbach, *et al.*, 1993; Cunha, *et al.*, 1993; Geller, *et al.*, 1993; Nussler, *et al.*, 1992; Wood, *et al.*, 1993; Eizirik, *et al.*, 1993; Corbett, *et al.*, 1993; Nakayama, *et al.*, 1992;

Koide, *et al.*, 1993; Galea, *et al.*, 1992; Goureau, *et al.*, 1993; Heck, *et al.*, 1992). Many agents have the ability to induce iNOS expression in various cell types to different levels. Most of these described so far are microbes, microbial products, or inflammatory cytokines, and there is often strong synergy between these agents. For example, IFN-γ can synergistically enhance iNOS expression induced by bacterial lipopolysaccharide.

1.3 The role of inducible nitric oxide synthase

1.3.1 Non-specific defence

It has been known for many years that micro-organisms, or microbial components, can increase host resistance to the growth of tumours by an antigen-specific step that involves sensitised lymphocytes and by a non-specific step that is mediated by activated macrophages (Alexander, *et al.*, 1971; Keller, *et al.*, 1971; Hibbs, *et al.*, 1972). Present evidence suggests that this non-specific immunity is associated with the induction of NOS. If this is the case, NO-dependent non-specific immunity is a general phenomenon involving not only the reticuloendothelial system but also non-reticuloendothelial cells such as hepatocytes (Nussler, *et al.*, 1992), vascular smooth muscle (Rees, *et al.*, 1990) and the vascular endothelium (Radomski, *et al.*, 1990), in all of which the inducible NOS has been detected. The role of the lung and liver in NO-dependent non-specific immunity appears to be crucial, since both organs are strategically placed in the circulation to serve as immunologic filters. Lymphocytes release NO (Kirk, *et al.*, 1990), and murine macrophages reduce lymphocyte activation by an NO-dependent mechanism (Hoffman, *et al.*, 1990; Albina, *et al.*, 1991). Furthermore, NO has been shown to be involved in immune rejection of allografted organs (Langrehr, *et al.*, 1991), graft-versus-host disease, and sepsis (reviewed by Schmidt, *et al.*, 1994). These data suggest that NO is also involved in specific immunity, but its precise role is not clear.

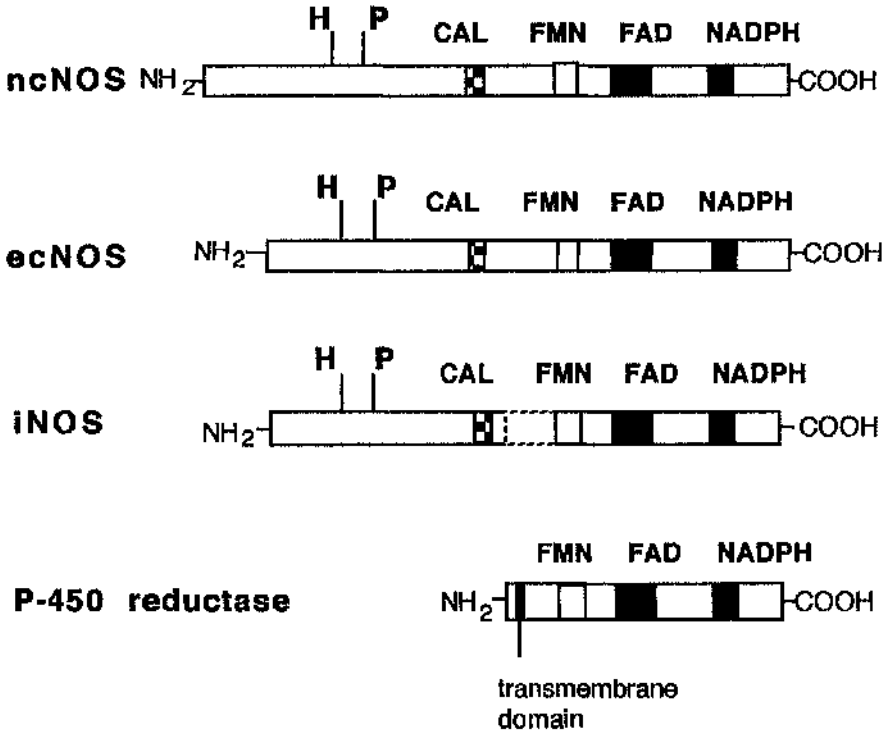


Fig 1.1. Binding sites for cofactors (NADPH, FMN and FAD) on ncNOS, ecNOS, iNOS and P-450 reductase.Calmodulin binding (CAL), protein phosphorylation (P) and consensus site for heme binding (H) are also shown.

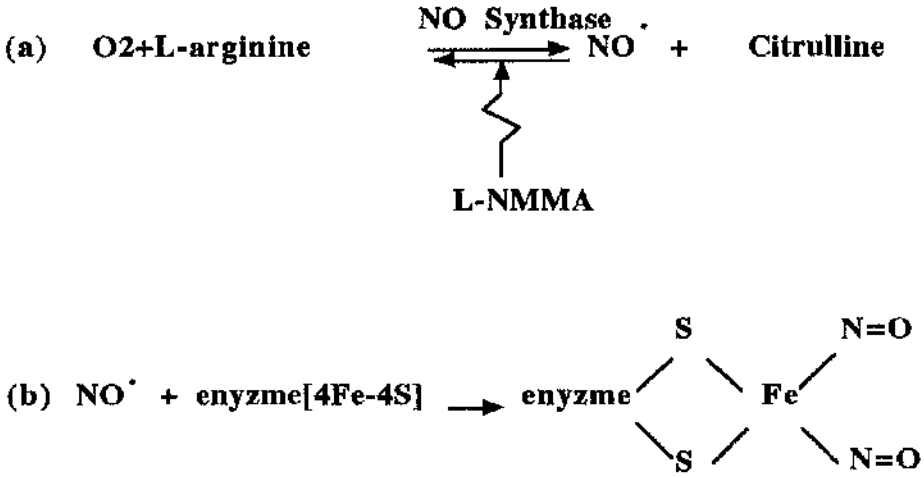


Fig. 1.2 The generation of NO and the possible mechanisms of its anti-microbial effects. (a) NO synthesis is catalysed by NO synthase and can be competitively inhibited by an L-arginine analogue, L-NMMA. (b) NO could react with the Fe-S groups forming an iron-nitrosyl complex causing the inactivation and degradation of the Fe-S prosthetic groups of aconitase and complex I and complex II of the mitochondrial electron transport chain. (c) Alternatively, NO can react with O2- to form ONOO- (peroxynitrite) which decays rapidly once protonated to form the highly reactive HO..

NO has also been shown to be important in parasitic infections and has been most extensively studied in leishmaniasis. Mouse peritoneal macrophages stimulated *in vitro*, with IFN-γ in the presence of LPS are efficient in killing Leishmania and this leishmanicidal activity can be completely abrogated by L-NMMA in a dose-dependent manner, but not by its D-enantiomer (D-NMMA) (Green, *et al.*, 1990; Liew, *et al.*, 1990). Furthermore, culture supernatants of macrophages activated by IFN-γ contain significantly increased levels of NO_2^- (Stuehr, *et al.*, 1987; Ding, *et al.*, 1988; Drapier, *et al.*, 1988), the production of which is also inhibited by L-NMMA (Liew, *et al.*, 1990). *Leishmania major (L. major)* promastigotes are killed when incubated *in vitro* at room temperature in phosphate buffer saline containing NO (Liew, *et al.*, 1990). The importance of NO *in vivo* is demonstrated by the finding that disease in CBA mice infected with *L. major* is exacerbated when L-NMMA is injected into the lesions resulting in a 10^4-fold increase in the number of parasites in the lesions (Liew, *et al.*, 1990). More recently, work carried out in our laboratory using iNOS-deficent mice has shown that while wild-type and heterozygous mice were resistant to *L. major* infection, the mutant mice were highly susceptible to the infection and developed visceral disease (Wei *et al.*, 1995).

1.3.2 Inflammation

Increasing evidence indicates that NO may play a part in acute and chronic inflammation. Treatment with L-NMMA reduced the degree of inflammation in rats (Ialenti, *et al.*, 1992) with acute inflammation or adjuvant arthritis (Ialenti, *et al.*, 1993) whereas L-arginine enhanced it. Immune complex-induced vascular injury in rat lung and dermal vasculature could be attenuated by inhibitors of NOS (Mulligan, *et al.*, 1991). In the carrageenin-induced acute inflammation model, the NO inhibitor (L-NMMA) could reduce inflammation and down-regulate inflammatory cytokines in mice (Ianaro, *et al.* 1994). Furthermore, colonic synthesis of NO was increased in patients with ulcerative colitis (Middleton, *et al.*, 1993), and inhibitors of NOS ameliorated experimentally induced chronic ileitis (Miller, *et al.*, 1993). In addition, nitrite concentrations in plasma

and synovial fluid are increased in patients with rheumatoid arthritis and osteoarthritis (Farrell, *et al.*, 1992). The origin of NO in the inflammatory process is remains unclear.

1.3.3 Autoimmunity

NO may play a role in tissue damage, because it is cytostatic or cytotoxic not only for invading micro-organisms but also for the cells that produce it and for neighbouring cells (Moncada,. 1992). In MRL-lpr/lpr mutant mice, NOS inhibitors prevent anti-DNA immune complex glomerulonephritis and reduce the intensity of inflammatory arthritis (Weinberg, *et al.*, 1994) (MRL-lpr/lpr is a lupus mouse strain which was developed by Murphy and Roths in 1979). Pancreatic β cells have a limited capacity for free radical scavenging and are thus highly sensitive to NO cytotoxicity. In chemically induced models of insulin-dependent diabetes mellitus and non-obese diabetic mice, progressive insulitis, dysfunction and eventual killing of pancreatic β cells correlate with the induction of iNOS and are, in some reports, abrogated by NOS inhibitors (Lukic, *et al.*, 1991; Kolb. *et al.*, 1991; Green, *et al.*, 1994).

1.3.4 NO mediates apoptosis in human cells

Previous reports have demonstrated that biochemical reactions involving NO may also lead to DNA damage (Wink *et al*, 1991; Nguyen *et al.*, 1992; Fehsel *et al.*, 1993). High concentrations of NO have been shown to cause deamination of deoxynucleotides and bases within intact DNA *in vitro* and are mutagenic *in vivo* (Wink 1991; Arroyo *et al.*, 1992). Exposure of human cells to NO under aerobic conditions results in DNA strand breakage and nitrosative deamination of DNA bases. Therefore, NO can cause DNA damage as well as mutation in human cells. NO may also play a role in cytokine-induced and activated macrophage-mediated apoptosis of tumor cells (Xie *et al.*, 1993; Cui *et al.*, 1994). On the other hand, p53 which plays an important role in the cellular response to DNA damage from exogenous chemical and physical mutagens, is accumulated in response to over-expression of iNOS. This p53 accumulation, via a

negative feedback loop, resulted in down-regulated NO production through inhibition of the NOS promoter (Forrester *et al.*, 1996).

Since iNOS is essential for the defence against pathogens, and can also cause tissue injury, it is important to understand the mechanisms of regulation of iNOS utilised by cytokines and other stimuli.

1.4 Regulation of the biosynthesis of nitric oxide from iNOS

Induction of iNOS is regulated at different levels: genomic, transcriptional control, post-transcriptional/translational control, and post-translational control.

At the genetic level, using the gene-targeting method, strains of mice deficient in iNOS have been successfully constructed (MacMicking, *et al.*, 1995; Wei, *et al.*, 1995). These mice provide a powerful tool that will not only facilitate formal demonstration of the effector roles of NO in microbicidal and tumoricidal activities, transplantation, and in a range of immunopathologies, but will also help to define the involvement of NO in immune regulation, immunological tolerance and antigen processing and presentation.

At the transcription level, both mouse and human iNOS promoters have been identified (Xie, *et al.*, 1993; Lowenstein, *et al.*, 1993, Chartrain, *et al.*, 1994). By transfection of murine macrophage RAW 264.7 cells with promoter-reporter gene cassettes, the function of promoter / enhancer elements was shown for both murine (Xie, *et al.*, 1993) and human genes (reviewed by Marris, *et al.*, 1994). In the murine iNOS promoter, there are at least 24 oligonucleotide elements homologous to consensus sequences for the binding of transcription factors involved in the inducibility of other genes by cytokines or bacterial products.

For the post-transcriptional control, stability of mRNA is a major control point in the regulation iNOS induction. For example, TGF-β suppresses macrophage iNOS expression via decreased iNOS mRNA stability and translational efficiency, by decreased stability of iNOS protein but not modulation of iNOS transcription (Vodovotz, *et al.*,

1993). At least some of these regulatory effects may be cell-type specific, as TGF-β not only attenuates iNOS mRNA induction in RAW 264.7 cells but also enhances induction in Swiss 3T3 cells (reviewed by Morris, *et al.*, 1994). Conversely, cycloheximide markedly stabilises iNOS mRNA in the latter cells (Imai, *et al.*, 1994), while in mouse macrophages, the same protein synthesis inhibitor prevents expression of iNOS mRNA.

Finally, iNOS is also regulated at the level of post-translational control. Unlike the other two NOS isoforms (ncNOS and ecNOS), iNOS contains calmodulin which is tightly bound to each subunit of the enzyme, making iNOS Ca^{2+} independent (Stuehr, *et al.* 1991). The other post-translational regulatory factor is L-arginine. L-arginine is the only physiological nitrogen donor for the NOS-catalysed reaction, consumption of L-arginine by L-arginase in activated macrophages leads to the inactivation of iNOS which can be restored by addition of excess L-arginine in the culture medium (Vodovotz *et al.*, 1994). Therefore regulation of availability of this essential substrate by arginine synthesis and uptake could determine cellular rates of NO synthesis (Nussler, *et al.*, 1994; Bogle, *et al.*, 1994). Tetrahydrobioptein (BH_4) is synthesised from GTP and its levels can be influenced by the activities of recycling or salvage pathways that convert the oxidised forms, quinoid-dihydrobiopterin and dihydrobiopterin, respectively to BH_4. GTP-cyclohydrolase I (GTP-CH) levels are absent or very low in unstimulated cells, but it is strongly co-induced with iNOS in these cells by cytokines and LPS. However, it is unknown whether the activities of the recycling or salvage pathways for BH_4 synthesis are co-regulated with iNOS expression (reviewed by Morris, *et al.*, 1994). Electrons donated by NADPH are essential for NO formation by NOS. The requirement of NOS for NADPH implies that the activities of the metabolic-pathways that generate or compete for this co-factor could play an important role in determining rates of cellular NO production. Although there have been relatively few studies on this point, observations that the activity of the NADPH-generating pentose phosphate pathway, and of the rate-limiting enzyme glucose-6-phosphate dehydrogenase (G-6-PDH), in particular, is correlated with NO production in some cells, support this possibility.

Although the regulation of iNOS gene has been intensively investigated in recent years, the early signal events that transduce the receptor signal to the activation of transcription factors involved in iNOS gene regulation is not clear. The aim of this project is to clarify the signalling pathways that lead to iNOS induction in murine macrophages.

Part II. Signal transduction mechanisms regulating transcription

1.5 Cytokines and Growth factors

1.5.1 Cytokines and cytokine receptors

Cell communication in the immune system is through cell-cell contact and secretion of soluble molecules named cytokines. Most cytokines were identified originally according to their cells of origin and /or biological effects on target cells. Products originally classified as "growth factors" also may be designated more generally as "cytokines".

Cytokines are a diverse group of glycoproteins, expressed constitutively or inducibly by a wide variety of cell types, in membrane-bound or secreted forms (reviewed in Nicola, 1989; Arai *et al.*, 1990; Howard *et al.*, 1993). They can act in a paracrine or autocrine manner to potentiate survival (or death), proliferation, and development. In addition to controlling haematopoietic development, cytokines mediate many physiological responses, such as immunity, inflammation, and antiviral activity. The corollary of this is that the cytokines or their antagonists (which are sometimes naturally occurring) have immense clinical potential. This in part explains the massive growth in cytokine literature over the past few years. A single cytokine can exhibit multiple functions depending on its target cell type, and different cytokines often show similar biological functions on the same target cell population (Metcalf, 1986). Combinations of cytokines can interact synergistically (Metcalf and Nicola, 1991; Heyworth *et al.*, 1988, 1992) or antagonistically (reviewed in Graham and Pragnell, 1990; Ruscetti *et al.*, 1991)

to give novel responses. Thus, a complex network is formed among various types of cells through cytokines (reviewed in O'Farrell *et al.*, 1996).

In recent years, much progress has been made in delineating the signal transduction mechanism underlying the induction of such responses. Cytokines bind to specific transmembrane receptor proteins expressed on target cells. Binding of a cytokine to its receptor triggers intracellular signal transduction processes, ultimately leading to altered gene expression and other cellular changes (Ihle *et al.*, 1994; Kan *et al.*, 1992). Molecular cloning of cytokine receptor (CR) genes in the last decade has revealed that CRs can be grouped into several novel receptor families. Members of one family, the CR superfamily, are defined by extracellular domain sequence and structural homology (Cosman, 1993; Miyajima *et al.*, 1992). These new receptor families are distinct from classical growth factor receptors with intrinsic tyrosine kinase activity or hormonal receptors with seven transmembrane domains. One important conclusion from the cloning of many CR genes is that the multiple functions of a given cytokine are mediated, in most cases, by the same receptor and therefore must be explained by differential activation of distinct intracellular pathways.

1.5.2 Classification of cytokine receptors

The majority of receptors for haematopoietic cytokines, including many interleukins (IL) and colony-stimulating factors (CSF), belong to the CR superfamily and are more specifically referred to as class I CRs. These include receptors of βc family (IL-3R, GM-CSFR, and IL-5R: they share common βc chain), the gp130 family (receptors for IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotropin 1 (CT-1), and possibly IL-12: they share the gp130 subunit), the IL-2Rγc family (receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and possibly IL-13: they share the IL-2Rγc chain), and single-chain receptor family (receptors for erythropoietin (EPO), prolactin (PRL), and growth hormome (GH)) (reviewed in O'Farrell *et al.*, 1996).

Table 1.1 Classification of cytokine receptors

Family	receptors for cytokines	common
Class I		
βc	IL-3, GM-CSF, IL-5	gp140βc, identical signalling
gp130	IL-6, LIF, OSM, IL-11, IL-12	gp130 (like) subunits
γc	IL-2, IL-4, IL-7, IL-9, IL-15	share γc subunit differ in signals
Class II	IFN-α/β, IFN-γ, IL-10	two or more distinct subunits
TNF-R	TNF-α, TNF-β, CD40, Fas	TRAF/CRAF
RTK	PDGF, *c-fms, c-kit, flk2/flt3*	intrinsic TK activity
TGF-β	TGF-β	intrinsic ser/thr kinase domain
Chemotactic	IL-8	seven transmembrane regions
Ig-like	IL-1	
Single chain	EPO, G-CSF, TPO, GH	consist single chain

Abbreviations: IL, interleukin; IFN, interferon; GM-CSF, granulocyte-macrophage clonoy -stimulating factor; OSM, oncostatin M; LIF, leukaemia inhibitory factor; CNTF, ciliary neurotrophic factor; PDGF,platelet-derived growth factor receptor; colony-stimulating factor; EPO, erythropoietin. TPO, thrombopoietin; GH, growth hormone; TNF-α, tumor necrosis factor-alpha; RTK, receptor tyrosine kinase. TGF-β, transforming growth factor beta.

A second group within the CR superfamily, designated as class II receptors, are structurally related to class I receptors and include the receptors for interferon (IFN) (IFN-α/β as type I, and IFN-γ as type II) and interleukin-10 (IL-10). They are defined by structural homology of extracellular domain sequence. The class I and II receptors are also functionally related by the use of similar signalling pathways (reviewed in O' Farrell *et al.*, 1996).

A number of CRs belong to other receptor superfamilies: members of the tumor necrosis factor receptor (TNFR) family exhibit characteristic extracellular domain cysteine-rich motifs but do not possess the domain structure common to class I and II receptors (Bazan, 1993). The receptors for a subset of haematopoietic cytokines such as M-CSF, SCF and *flt3/flk2* belong to the classical tyrosine kinase (TK) growth factor receptor family (Ullrich and Schlessinger, 1990), TK receptors contain a large glycosylated extracellular ligand-binding domain, a single membrane-spanning hydrophobic region, and a cytoplasmic domain that possesses intrinsic tyrosine activity which mediates signalling (Hunter and Cooper, 1985). The receptors for TGF-β and its relatives form a unique receptor family that possess serine/threonine kinase domains (Massague *et al.*, 1994). Chemotactic cytokines such as IL-8 (Holmes *et al.*, 1991), also known as chemokines have receptors with seven transmembrane regions, and they are classical G-protein coupled receptors. The interleukin-1 receptor belongs to the immunoglobulin receptor superfamily (Sims *et al.*, 1988). The summary of classification of cytokine receptors is shown in Table 1.1 (pp39).

1.5.3 Cytokine receptor signalling

Although signalling events have been defined for a wide range of distinct receptor classes, the major signalling mechanisms appear to be common to all of these receptors.

The initial activation steps for receptors with intrinsic tyrosine kinase (TK) activity are ligand-induced dimerization and auto-transphosphorylation of receptor monomers. When activated, TK-Rs recruit and interact with a number of SH2-domain-containing

signalling molecules, by means of phosphorylated tyrosine residues (Kashishian *et al.*, 1992; Lev *et al.*, 1992; Reedijk *et al.*, 1992). Ligand-induced homo- or hetero-dimerization of receptor components is also the initial step of activation of receptors of the CR superfamily.

Although receptors of the class I and II CR families do not contain intrinsic kinase domains, most cytokines stimulate rapid tyrosine phosphorylation of a number of cellular substrates, including components of their receptors (Isfort and Ihle, 1990; Sorensen *et al.*, 1989; Welham *et al.*, 1992; Welham and Schreder, 1992; Izuhara and Harada, 1993; Yin and Yang, 1994). Furthermore, cytokine-stimulated tyrosine phosphorylation is essential to mediate the biological effects of cytokines (Kanakura *et al.*, 1990; Saroh *et al.*, 1992). A number of mitogenic cytokines such as IL-2, IL-3, IL-5, GM-CSF, IL-6, G-CSF, and EPO are known to induce activation of Ras as well as its downstream cascade including Raf and mitogen-activated protein kinase (MAPK) (Duronio *et al.*, 1992; Kan *et al.*, 1992; Welham *et al.*, 1992). Certain cytokines also activate Pim1 kinase (Sato *et al.*, 1993) and protein kinase C (PKC) (Heyworth *et al.*, 1993; Shearman *et al.*, 1993) and induce expression of nuclear protooncogenes, including *c-myc, c-fos,* and *c-jun*, and cell cycle regulators (Sherr, 1993; Ihle *et al.*, 1994a; Mataushime *et al.*, 1991, 1994). Many of these signalling molecules are also activated by TK-Rs (reviewed in O'Farrell *et al.*, 1996), where the receptor can directly phosphorylate signalling substrates. Key questions currently under investigation are: how cytokine receptors without intrinsic kinase activity transduce signals, how receptors induce signals common to various cytokines as well as specific to each cytokine, how a given receptor can elicit different biological responses, and whether distinct regions of receptor cytoplasmic domains are required to mediate such effects.

The signalling pathways that are implicated in playing key roles in cytokine receptor signal transduction will be reviewed in the following two sections (Section 1.6; 1.7).

1.6 Receptor tyrosine kinases and cytokine receptor signalling

The role of phosphorylation in the control of cellular function has been well documented (review by Walton and Dixon 1993). The vast majority of these phosphoryl modifications occur on serine and threonine residues (Roach 1991) and until 1980, only phosphoserine and phosphothreonine had been identified as naturally occurring phosphoamino acids. However, tyrosine phosphorylation began to receive greater attention when it was determined that v-Src, the transforming principle of the Rous sarcoma virus, had tyrosine kinase activity (Hunter *et al* .,1980; Levinson *et al.*, 1980; Collet *et al.*, 1980). Upon transformation by V-Src, cellular phosphotyrosine content increased dramatically. It has since been demonstrated that several other viral oncogenes are also tyrosine kinases (Bishop 1985). Coincident with this discovery, it was shown that epidermal growth factor receptor also had tyrosine kinase activity (Ushiro *et al.*, 1980). With the increasing numbers of identified tyrosine kinases, both intracellular and receptor-linked, it has become clear that tyrosine phosphorylation is an important regulator of cellular function (Hunter 1987).

Protein tyrosine kinases (PTK) are found in all multicellular organisms and can be divided into two main categories: transmembrane receptors (Schlessinger *et al.*, 1992) and cytosolic kinases (Bolen 1993). They are also called receptor tyrosine kinases and non-receptor tyrosine kinases (The later will be in the next section 1.7).

1.6.1. Receptor tyrosine kinases

Polypeptide growth factors represent a group of extracellular signals that are critically important for influencing a diverse array of cellular responses including proliferation, differentiation, and cell survival. The effects of many growth factors are known to be mediated by high-affinity receptor tyrosine kinases (RTKs) (reviewed by Fantl *et al.*, 1993).

Within the past decade, several distinct sub-families of RTKs have been identified. Members of a given sub-family share common structural features that are

distinct from those found in other sub-families (Fantl *et al.*, 1993). Despite the diversity of RTKs, there is a degree of commonality in the types of intracellular signalling pathways initiated by these proteins. In the mammalian systems, biochemical and molecular genetic analyses have shown that for all RTKs, the binding of ligand to the extracellular domain activates the tyrosine kinase in the cytoplasmic domain. This leads to downstream activation of a number of common signalling molecules.

Interestingly, a single type of RTK can elicit very different biological responses in different cell types. Upon ligand binding, RTKs phosphorylate themselves on tyrosine residues, a process commonly termed autophosphorylation. In the case of some RTKs, ligand binding is known to induce receptor dimerization (Ueno *et al.*, 1991; Kashles *et al.*, 1991; Rodrigues and Park 1994). Following autophosphorylation, individual phosphotyrosine residues located in the cytoplasmic domains of receptors serve as highly selected binding sites that interact with specific cytoplasmic molecules. These signalling molecules mediate the cellular responses to growth factors. Studies using synthetic peptides representing the sites on the receptor that interact with signalling molecules have demonstrated the structural basis for the specificity of the interaction between RTKs and signalling molecules (Fantl *et al.*, 1992), examples of which are the short sequences flanking receptor phosphotyrosines which determine the remarkable specificity of the interaction between signalling molecules and PTKs. The residues on the carboxyl-terminal side of the phosphotyrosine appear to be more important in determining the affinity and specificity of the interaction than residues on the amino-terminal side of the phosphotyrosine.

Tyrosine-phosphorylated proteins interact with Src homology 2 (SH2) domain containing proteins. SH2 domains are regions of about 100 amino acids that are homologous to a non-catalytic region present in the c-src proto-oncoprotein which binds with high affinity to specific phosphotyrosine-containing motifs on stimulated RTKs or other signal transducers. The specificity of the interaction depends on both the amino acid sequence surrounding the phosphotyrosine, and the amino acid sequence of the SH2

domain (Cantley *et al.*, 1991; Schlessinger 1992; Koch *et al.*, 1991; Kazlauskas 1994). The functional consequences of the associations between receptors and signalling molecules are not entirely clear. Nonetheless, tyrosine kinase activity of RTKs has two functions: first, it is responsible for creating high-affinity binding sites for localising signalling molecules to site near their substrates or activated receptors and second, the tyrosine kinase modifies the signalling molecule by phosphorylation (Fantl *et al.*, 1993).

I will now outline the common features of signal processes which have been proposed to transduce signals from the receptor at the cell surface to the initiation of transcription events in the nucleus via the key pathway, Ras-MAPK, which has been implicated in the transduction of proliferation and/or differentiation signals.

1.6.2 Cytokine receptors signalling through the Ras /MAP kinase pathway

Treatment of leukocytes with cytokines that enhance their functional properties is associated with an increase in serine/threonine and tyrosine phosphorylation of cellular proteins. One of the signals most clearly implicated in the initiation of nuclear signalling from growth factor receptors is the *ras* pathway. Ras activation triggers a serine threonine kinase mediated signal amplification cascade culminating in the activation of mitogen-activated protein (MAP) kinase (Blumer and Johnson 1994), which regulates the expression of mitogen-responsive genes by phosphorylating specific transcription factors.

MAP kinases (MAPKs) are a family of protein kinases whose prototype members are the mammalian extracellular signal-regulated kinases ERK1 and ERK2 and the *Saccharomyces cerevisiae* pheromone-regulated kinases KSS1 and FUS3.

Receptor tyrosine kinases activate a signalling cascade involving transient formation of Ras/GTP and activation of raf kinase at the membrane, followed by sequential activation of MAPK kinase (MAPKK) and ERK1/ERK2 (refered as *ras* /ERK pathway); only the latter signalling elements enter the nucleus (reviewed by Marshall, 1994; Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Only recently, the mechanism of activation of the ras pathway have been identified. For example, EPO and IL-3 activate

the *ras* pathway through their ability to induce tyrosine phosphorylation of SHC (a protein implicated in signalling through Ras). Following phophorylation, GRB2 (growth factor receptor-bound protein 2, it contains SH2 and SH3 domains) associates with SHC, and subsequently with SOS (Son of Sevenless, the Ras guanine nucleotide exchange factor); there are increases in GTP-bound Ras activation of *raf*-1; tyrosine phosphorylation of MAP kinases; and induction of immediate early genes such as pim-1, cmyc, and c-fos (reviewed in Ihle *et al.*, 1995). These signalling events are summarised in digram Fig. 1.3.

1.6.3 MAP kinase and JNK/SAPK pathway

Until recently, ERK1 and ERK2 were the only cloned and well-characterized mammalian MAP kinases. However, the recent discovery of two other MAP kinase subtypes, the *c-jun* kinase (JNK) superfamily and p38/RK MAP kinase, reveals the existence of parallel MAP kinase cascades that can be activated independently and simultaneously (reviewed in Cano and Mahadevan 1995; Beyaert *et al.*, 1996). As a family of MAPKs, JNK/SAPKs (for Jun N-terminal/stress-activated protein kinases) are distinct from the ERKs but are also regulated by extracellular signals (reviewed by Hill and Treisman 1995). Within the MAP kinases, ERKs are activated predominantly by growth factors or phorbol esters, but activation by TNF or IL-1 has also been demonstrated (Marshall 1995; Van Lint *et al.*, 1992). In contrast, JNK and p38/RK MAP kinases are generally activated by inflammatory cytokines and cellular stresses such as heat shock, osmotic stress or UV light (revewed in Beyaert 1996).

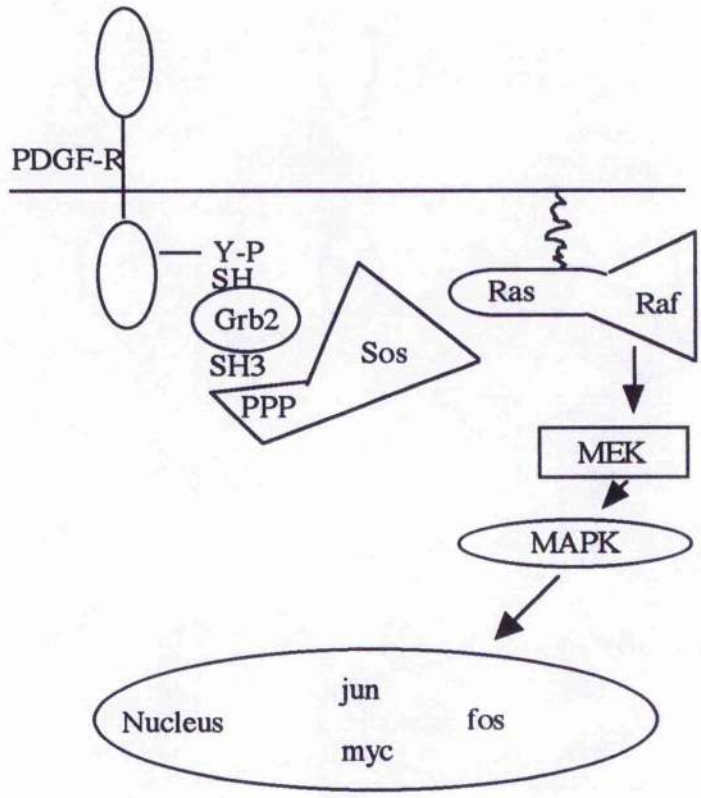


Fig. 1.3 The Ras/MAP kinase cascade. MAPK is activated by phosphorylation on thr and tyr by a MAP kinase kinase (MEK), MEK is activated by ser/thr phosphorylation by a MAPkinase kinase kinase (Raf), Raf(p74) is a ser/thr kinase and it is downstream of Ras. Ras is a GTPase and acts as a molecular switch for the key signal transduction pathway that control growth and differentiation. Grb2 is complexed with the guanine-nucleotide exchange factor (GEF), Sos (Son of Sevenless). The SH3 domains of Grb2 bind Sos via proline rich regions. The complex then translocate to the plasma membrane, where it catalyzes the conversion of inactive, GDP-bound Ras to the activated, GTP-bound state.

1.7 Nonreceptor tyrosine kinases and cytokine receptor signallings

1.7.1 Nonreceptor tyrosine kinases

The non-receptor PTKs represent a collection of cellular enzymes that are grouped together because of their lack of extracellular sequences. A number of the non-receptor PTKs have been found to be associated with other cell surface proteins (which generally lack endogenous enzyme activity) and shown to be capable of facilitating cell surface initiated signal transduction much like the receptor class of PTKs. Thus, in many instances, characterization of these PTKs as non-receptor kinases reflects more the history surrounding their discovery rather than their physiologic functions (Bolen 1993).

More than 20 individual PTKs comprising eight different groups of nonreceptor PTKs have been identified (reviewed by Bolen 1993). There are Abl, Jak, Fak, Fes/Fps, Syk/Zap, Itk, Src, and Csk families. With the exception of the Focal Adhesion Kinase (Fak) which is the only known membrane of this group of PTK, all the other groups appear to represent distinct families of protein kinases. They range in size from around 50kDa for the C-src kinase (Csk) family to approximately 150kDa for the Abl kinase family. About half of the nonreceptor PTKs have demonstrable oncogenic potential, whereas others whose activity appears to be primarily related to suppressing the activity of Src-related protein kinases may be involved in the modulation of growth, differentiation, and mature cell function. Within the nonreceptor PTKs, Src-related enzymes represent the largest known family. To data, nine distinct members of the src gene family have been cloned and the encoded protein kinases characterized (Bolen 1992; Brickell 1992). These include Src, Yes, Fyn, Lyn, Lck, Blk, Hck, Fgr, and Yrk PTKs.

1.7.2 Cytokine signaling through non-receptor protein tyrosine kinases

The non-receptor tyrosine kinases play a critical role in cytokine signalling. Most of the cytokine receptors that constitute distinct superfamilies do not possess intrinsic protein tyrosine kinase domains, yet receptor stimulation usually invokes rapid tyrosine phosphorylation of intracellular proteins, including the receptors themselves. It is now

clear that these receptors are capable of recruiting or activating (or both) a variety of nonreceptor PTKs to induce downstream signalling pathways (reviewed by Taniguchi 1995).

Initially, the involvement of nonreceptor PTKs in cytokine signalling was shown for the Src-family PTK $p56^{lck}$ (Lck) which is found physically associated with the IL-2 receptor β-chain (IL-2Rβ) in the absence of IL-2 stimulation (Hatakeyama *et al.*, 1991), and it is rapidly activated upon ligand binding to IL-2R (Hatakeyama *et al.*, 1991; Horak *et al.*, 1991). IL-2R has an unusual variety of intracellular signalling partners and its β and γ chains are often shared by many other cytokines, such as IL-3, IL-4, IL-5, IL-7, IL-9 and IL-15 (Sarah, *et al.*, 1994). Following studies of the IL-2R system, Lck, Fyn, and Lyn have been shown to be activated through their interaction with the specific region of IL-2Rβ (Minami *et al.*, 1993; Torigoe *et al.*, 1992; Kobayashi *et al.*, 1993).

An abundance of evidence now indicates that a recently identified family of non-receptor tyrosine kinases, the Janus kinase (JAK) family, play a crucial role in signalling of class I and class II cytokine receptors (CRs). To data, four members of JAK family have been identified (JAK1, JAK2, JAK3, Tyk2) (reviewed in Ihle 1994). Both class I and class II CRs can activate one or more of the members of JAK family (Ihle *et al.*, 1994). Evidence for the involvement of the JAK-family PTKs in cytokine signalling was first provided by genetic complementation experiments, which a mutant cell line defective in the type I interferon (IFN-α and -β) signalling pathway was rescued by transfection with a genomic DNA encoding Tyk2 (Velazquez *et al.*, 1992). More experiments revealed that JAK1 and Tyk2, and JAK1 and JAK2 molecules play essential roles in IFN-α/β and IFN-γ-induced gene expression respectively (Velazquez *et al.*, 1992: Muller *et al.*, 1993; watling *et al.*, 1993). The critical role of the JAK-family PTKs has also been shown in the case of the growth-promoting type I cytokine receptors (Ihle 1994).

1.7.3 JAK-STAT pathway

JAK kinases lack SH2 and SH3 domains but contain an active kinase-like domain. These kinases associated directly with receptors, that either possess or lack tyrosine kinase activities of their own, in the absence of cytokines and are activated and phosphorylated upon cytokine binding to the receptors (Ihle *et al.*, 1994; Darnell *et al.*, 1994).

What are the substrates of JAKs? The activated JAKs phosphorylate both themselves and the receptor subunits, creating docking sites for SH2-containing proteins including SHC, which couples receptor engagement to activation of the *ras* pathway, and HCP (haematopietic cell phosphatase), a protein tyrosine phosphatase which negatively affects the response , such as proliferation and cell growth (reviewed in Ihle *et al.*, 1995).

In addition, extensive studies on transcriptional activation mediated by the IFNRs have unveiled a novel signal transduction pathway that provides a direct link between receptor activation and gene transcription (Fu *et al.*, 1992; Schindler *et al.*, 1991; Shuai *et al.*, 1993, 1994). This is mediated by a recently identified novel family of transcription factors known as STATs (signal transducers and activators of transcription), which are substrates for JAKs. STATs contain a well-conserved SH2 domain and normally reside in the cytoplasm in latent forms. STATs are tyrosine phosphorylated by JAKs on cytokine stimulation, leads to dimerization of the STATs. Dimerisation may involve either homodimerization or heterodimerization with a different STAT protein. STAT dimers can then translocate to the nucleus, where they bind specific DNA sequences in the promoters of target genes (Darnell *et al.*, 1994; Shuai *et al.*, 1994). The signalling of hematopoietic cytokine receptors is summarised in Fig. 1.4 (O'Farrell et al., 1996).

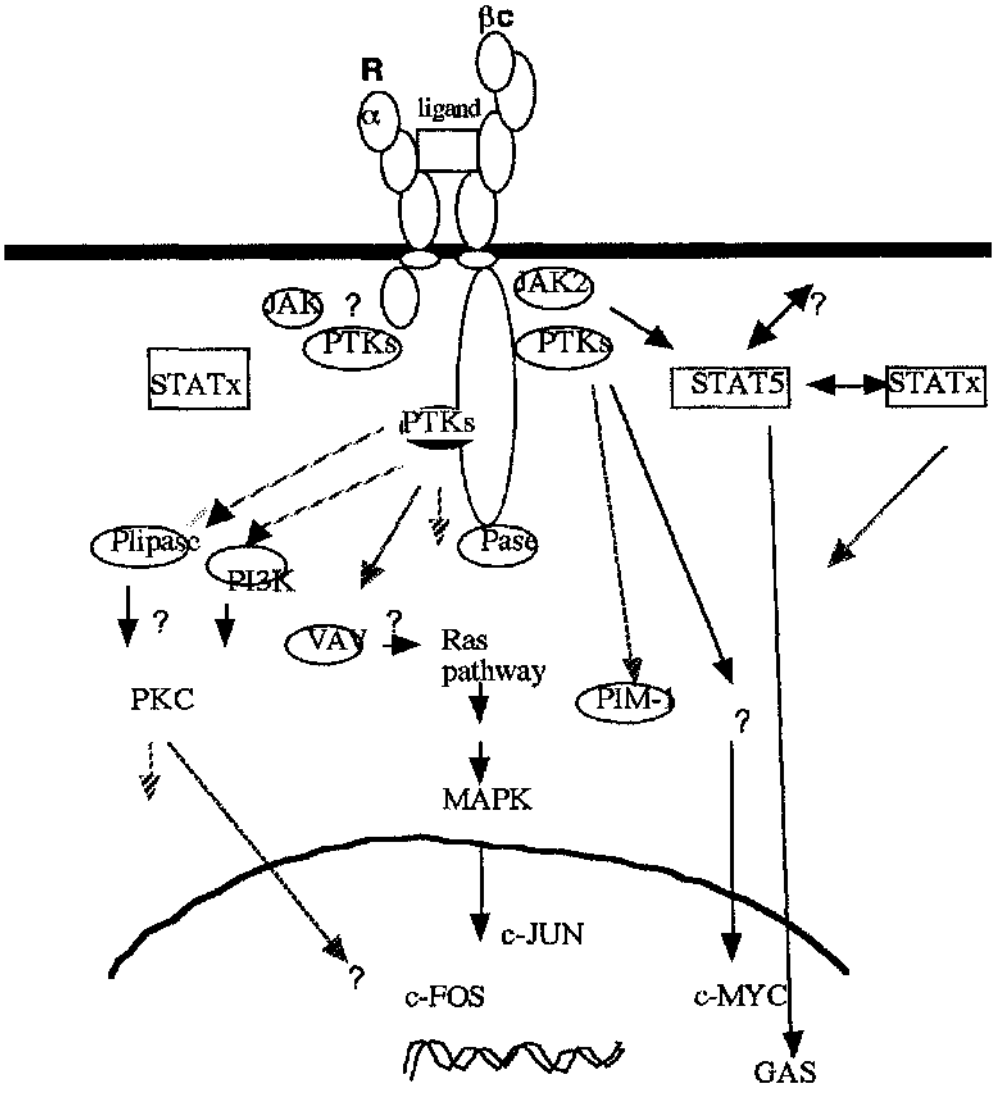


Fig. 1.4 Cytokine receptors and signal transduction The GM-CSF/IL-3 receptor is depicted as a representative receptor, and signaling pathways that may couple to these receptors are also depicted. Broken lines denote hypothetical pathways, and molecules that associate with receptor cytoplasmic domains are shaded in gray. PTK denotes protein tyrosine kinases, whose identities are not yet clear, Pase denotes phosphatase. The cytoplasmic domain of βc can be divided into functional domains, the membrane proximal region including box 1 and box 2, which mediates the JAK-STAT pathway and induction of *c-myc*, and the distal region that activates the Ras pathway (adopted from O'Farrell *et al.*, 1996)

Table 1.2 Activation of JAKs and STATs by different cytokines and growth factors (reviewed in Hill and Treisman 1995)

Receptor	JAK activated	STAT activated
IFN-α/β	JAK1, TYK2	STAT1,STAT2,STAT3
IFN-γ	JAK1, JAK2	STAT1
IL-3, GM-CSF, IL-5 (common β subunit)	JAK2	unknown
IL-6, OSM, LIF, CNTF (common gp130 chain)	JAK1, JAK2, TYK2	STAT1, STAT3
IL-2, IL-4, IL-7, IL-9 (common γ subunit)	JAK1, JAK3	STAT5*, STAT6 (for IL-4)
IL-12	JAK2, TYK2	STAT4
EGF, PDGF, CSF1 (receptor tyrosine kinase)	JAK1[a]	STAT1, STAT3[b]
EPO	JAK2	unknown
Growth hormone	JAK2	unknown
Prolactin	JAK1, JAK2	STAT5
G-CSF	JAK1, JAK2	unknown

a JAK1 activation has been demonstrated only in the case of EGF, and its role in STAT activation remains unclear.

b STAT3 activation has been demonstrated directly only in the case of EGF.

* STAT5 activation only in the case of IL-2 (Beadling *et al.*, 1996)

Abbreviations: IL, interleukin; IFN, interferon; GM-CSF, granulocyte-macrophage clonoy -stimulating factor; OSM, oncostatin M; LIF, leukaemia inhibitory factor; CNTF, ciliary neurotrophic factor; PDG, platelet-derived growth factor receptor; colony-stimulating factor; EPO, erythropoietin. EGF, epidermal growth factor.

To date, a number of the STAT members have been cloned and characterized, these include STAT1α, STAT1β, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6 (Fu *et al.*, 1992; Schindler *et al.*, Zhong *et al.*, 1994; Jacobson *et al.*, 1995; Mui *et al.*, 1995; Wakao *et al.*, 1992, 1994; Hou *et al.*, 1994;). Although the association of different receptors with particular JAKs and STATs appears to be complex, JAKs implicated in signalling by various cytokines and growth factors are shown in Table 1.2 (see pp51). They act downstream of many receptors of the class I and class II cytokine receptors. The increased availability of reagents related to the JAK-STAT signalling pathways have provided useful tools to study the involvement of early signal events leading to the regulation of iNOS expression.

1.8. Tyrosine phosphatases and dephosphorylation

Cytokine receptors are also negatively regulated, in part by haematoietic cell phosphatase (HCP, also termed PTP-1C or SH2-PTP1). A comparison of the protein tyrosine phosphatases (PTPase) and tyrosine kinase families reveals an interesting similarity. The general structures of the two tyrosine-directed enzymes parallel each other in that these are both transmembrane or receptor-linked proteins as well as proteins that are wholly intracellular.

Protein phosphatases (PP) are classified as serine/threonine phosphatases or as tyrosine phosphatases according to amino acid sequence and substrate selectivity. There are four major classes of serine/threonine phosphatases (PP-1, PP-2A, PP-2B, PP-2C), each of which has isozymic forms, and several related but distinct novel enzymes (reviewed by Cohen 1989). The tyrosine phosphatases can be classed as either receptor-like or non-receptor molecules, and they encompass a rapidly expanding number of members (reviewed by Fischer *et al.*, 1991; Pallen *et al.*, 1992).

The particular functions performed by the PTPases have yet to be identified. A number of PTPases are expressed in a tissue-specific manner, and this should provide

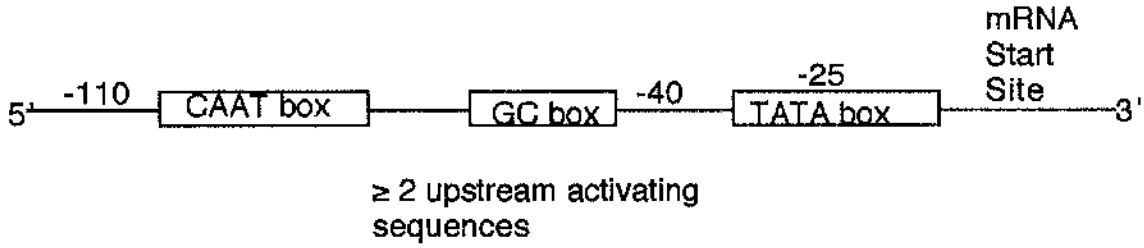
clues to determining their functions (Walton and Dixon 1993). However, it is known that, in the cell, tyrosine phosphorylation is a reversible, dynamic process. Thus, the net level of phosphate in a target substrate reflects not only the activity of PTKs that catalyse phosphorylation, but also the protein tyrosine phosphatases (PTPs) that are responsible for dephosphorylation of tyrosyl residues (reviewed by Sun and Tonks 1994). The PTPs and PTKs do not exert their effects in isolation, but rather coordinate their effects in controlling flux through tyrosine-phosphorylation-dependent signalling pathways.

Like PTKs, the PTPs are a large and structurally diverse family of receptor-like and cytoplasmic enzymes. They play important roles in the regulation of diverse functions including control of cell-cell adhesion, growth factor signal transduction, and the cell cycle. Moreover, dysfunctional PTPs have been implicated in disease states. The characterization of the PTP family now represents a major research effort in many laboratories (reviewed by Tonk 1993).

1.9 Promoter regulation and transcription factors

1.9.1 Promoters, enhancers and regulation of transcription

Most genes are silent unless they are specifically turned on. Transcription requires binding of RNA-polymerase (RNA-pol) to non-transcribed regions of the gene, called promoters, upstream (5') of the start site. For eukaryotic promoters, there is a TATA box located at -25 and flanked by GC-rich sequences which is necessary but not sufficient for strong promoter activity. Additional elements located between -40 and -110 are called CAAT and GC boxes. Activities of promoters are greatly increased by enhancers located upstream /downstream or even within transcribed gene.



Transcription factors binding to regulatory sites on DNA can be regarded as passwords that co-operatively open multiple locks to give RNA-pol access to specific genes. Activators and repressors of gene expression act by altering the rate of formation of the basal transcriptional complexes. Multiple proteins may be required for a single gene, and different combinations of transcription factors provide the specificity for switching on of particular genes. Fig. 1.5 illustrates the process of regulation of transcription.

Changes in cellular behavior induced by extracellular signalling molecules require execution of a complex program of transcriptional events to activate or repress transcription. Transcription factors must be located in the nucleus, bind DNA, and interact with the basal transcription apparatus. Activation of transcription factors by extracellular signals therefore always involves a nuclear translocation step. Some pathways involve migration of signalling molecules (eg. MAPK, see section 1.6.2) themselves into the nucleus, while in others activated transcription factors (eg. STATs) which migrate to the nucleus following their activation in the cytoplasm. Moreover, although in many cases activation of a single intracellular signalling pathway is sufficient for transcription factor activation, some transcription factors are complexes in which the activity of each component is regulated by different cellular signalling pathways.

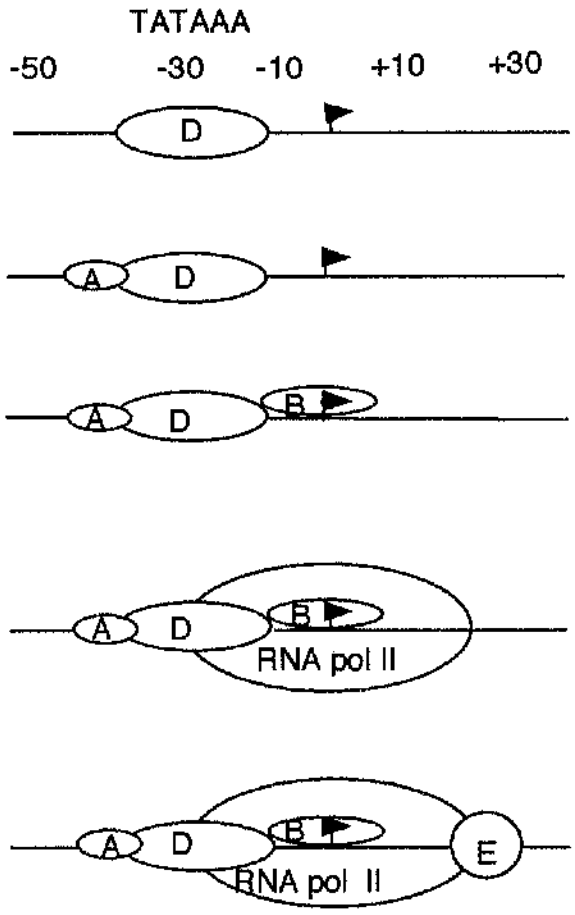


Fig. 1.5 Regulation of transcription. Multisubunit transcription appartus must assemble first at TATA box on genes. RNA ploymerase II is unable to initiate transcription on its own, it is guided to the start site by a set of transcription factiors collectively known as TFII. Initiation begins with binding of the TATA-binding protein of TFII-D which then recruits TFII-A and B, RNA-pol and TFII-E to form a basal transcription apparatus. This units can transcribe DNA at low rate, however, addtional transcription factors are required for high level transcription of specific mRNA.

1.9.2 Nuclear factor NFκB

In contrast with JAK/STAT pathways, which JAK involved in the transcriptional activation of many cytokine- and growth factor-inducible genes (see section 1.7.3), NFκB is activated by many agents that induce acute phase responses, such as IL-1, IL-2, TNF-α, LPS, viral infection, etc. NFκB was originally identified as a transcription factor that binds to the κB site in the intronic enhancer of the immunoglobulin κ light-chain gene in B lymphocytes (Sen and Baltimore 1986). NFκB controls the expression of numerous genes of the immune and inflammatory responses and of various viral genes (including those of human immunodeficiency virus, HIV) (Baeuerle *et al.*, 1994). The NFκB protein is a heterodimer consisting of two proteins, p50 (also designated NFκB1) and p65 (also designated Rel A). p50 and p65 are members of the Rel/NFκB family of proteins. These proteins serve as inducible eukaryotic transcription factors that form various homo- and heterodimers. NFκB (i.e., the p50-p65 complex) is present in essentially all cells and is the most abundant of the Rel/NFκB family heterodimers.

NFκB is retained in an inactive form in the cytoplasm as a consequence of the binding of particular proteins of the IκB family (i.e., IκB-α and IκB-β). The activation and nuclear translocation of NFκB can be correlated with the phosphorylation of IκB, which triggers the proteolytic degradation of one or both of the IκB proteins. A recent report (Joseph *et al* ., 1996), mapping the inducible IκB phosphorylation sites, indicated that activation of a single IκB kinase, or closely related IκB kinases, is the first critical step in NFκB activation. Once phosphorylated, IκB is ubiquitinated. Poly-ubiquitination is required for inducible IκB degradation.

The activation of NFκB can be triggered by different stimuli, *e.g.*, lipopolysaccharide (LPS), muramyl peptides, viruses, the inflammatory cytokines TNF-α and IL-1β, UV irradiation, reactive oxygen intermediates (H_2O_2) (Baeuerle PA 1991; Baeuerle *et al.*, 1994), phorbol esters (*e.g.*, phorbol myristate acetate [PMA]), and double-stranded RNA. Of these agents, TNF-α and PMA elicit the inactivation and

degradation of only IκB-α and the transient activation of NFκB. IL-1β and LPS , however, elicit the inactivation and degradation of both IκB-α and IκB-β and a more persistent activation of NFκB (Wang *et al.*, 1996).

1.9.3 Interferon regulatory factor family (IRFs)

Many of IFN regulated genes (ISGs) have a short DNA motif in their promoter region, a motif that has been designated IFN consensus sequence (ICS) or the IFN stimulated response element (ISRE) (Williams 1991; Levy and Darnell 1990; Friedman and Stark 1985). A similar hexamer motif has also been found in the promoters of both IFN-β and IFN-α genes and since it confers viral as well as dsRNA responsiveness, it has been designated positive regulatory domain (PRD)-I (Fan and Maniatis 1989). A number of different transcription factors have been identified by exploiting their ability to bind either the ICS/ISRE or the PRDI motifs. Among them are ISGF3 (Veals *et al.*, 1992; Schindler *et al.*, 1992; Fu *et al.*, 1992), IRF-1(Miyamoto *et al.*, 1988; Pine *et al.*, 1990), IRF-2 (Harada *et al.*, 1989) and ICS binding protein (ICSBP) (Weisz *et al.*, 1992; Driggers 1990), that share a similar DNA binding domain, and PRDI-BF1 (Keller and Maniatis 1991), a unique zinc finger binding protein.

The ISGF3α subunit consists of three cytoplasmic polypeptides of 84, 91 and 113 kDa . Following the exposure of cells to IFN-α, these polypeptides quickly translocate to the nucleus where they associate with the ISGF3γ subunit (which confers sequence specific recognition) and bind to the ISRE motif (Schindler *et al.*, 1992; Fu *et al.*, 1992). The ISGF3γ subunit is a 48kDa polypeptide found mainly in the cytoplasm of cells. Following exposure of the cell to IFN-α, it accumulates in the nucleus to generate active ISGF complex with the α-subunit.

The transcription factors IRF-1 and IRF-2 were cloned because of their ability to bind the PRDI element , but it has also been reported that they bind to the ICS/ISRE motif (Reis *et al.*, 1992; Fujita *et al.*, 1988). IRF-1 expression can be induced within a few hours following exposure of cells to IFNs, viral infection, dsRNA and cytokines

(Abdollahi *et al.*, 1991; Harroch *et al.*, 1993). IRF-1 has been demonstrated to be a transcriptional activator of those ISGs that are repressed by IRF-2 (Harada *et al.*, 1990). IRF-1 was shown to play a critical role in the regulation of IFN-β at least in certain cells (Reis *et al.*, 1992); it can affect the expression levels of IFN-β and IFN-inducible gene expression depending on the differentiation state of the cells (Ruffner *et al.*, 1992). The importance of IRF-1 is emphasized by the findings that it may play a role in the inhibition of cell growth (Yamada *et al.*, 1990; Kirchhoff *et al.*, 1992) and manifests anti-oncogenic properties in NIH 3T3 cells (Harada *et al.*, 1993; Willman *et al.*, 1993). Moreover, it has been shown recently that IRF-1 is required for the induction of NO synthase in macrophages (Kamijo *et al.*, 1994).

IRF-2 shares sequence similar with IRF-1 and is also constitutively expressed in many cell lines. Co-transfection studies have shown that IRF-1 can act as a transcriptional activator on promoters containing multiple copies of PRDI hexamer, while IRF-2 can function as a repressor of IRF-1-activated gene expression (Harada *et al.*, 1989 & 1990). Normally, IRF-2 is bound to the IFN genes in uninduced cells to the same sequence elements as IRF-1, Upon induction, IRF-2 is replaced by IRF-1, which would co-operate with other factors to promote transcription. Both IRF-1 and IRF-2 are virus inducible and IFN inducible. The induction of IRF-2 mRNA by virus is a critical event to reverse the IRF-1-mediated gene activation, thus making the genes accessible for subsequent signals.

ICSBP is another member of IRFs family. which has been cloned and shown to be a 425 amino acid polypeptide which shows restricted homology to IRF-1, IRF-2 and ISGF3γ in the first 120 amino acids, a region that encompasses the DNA binding domain (Weisz *et al.*, 1992; Driffers *et al.*, 1990). Additional homology also exists between ICSBP and ISGF3γ in a short segment at the C-terminus; a segment that is involved in the association of ISGF3γ with ISGFα (Veals *et al.*, 1993). ICSBP is expressed primarily in cell lines of hematopoietic origin, mainly in monocytic cells, but it has been found in B cells and T cells. It appears to function as a trans-acting negative regulator of ICS/ISRE and PRDI containing promoters (Weisz *et al.*, 1992; Nelson *et al.*, 1993).

Part III. IFN-γ, LPS and IL-4 regulation of macrophage functions

Mononuclear phagocytes are target cells for a large and diverse range of micro-organisms that demonstrate an obligate requirement for an intracellular environment in which to survive and replicated. Macrophage activation is essential for enhanced microbicidal activity during host resistance to these infections. Antimicrobial activities of macrophages have been broadly categorised into either oxygen-dependent or oxygen-independent systems (reviewed in Reiner 1994). Oxygen-dependent systems include production of reactive oxygen intermediates, mediated by the phagocyte oxidative burst, and the reactive nitrogen intermediate, nitric oxide, via oxidation of a terminal guanidino-nitrogen atom of arginine (Wilson 1990; Stamler *et al.*, 1992). Oxygen-independent microbicidal mechanisms of macrophages are also multifactorial and may involve: acidification of the phagolysosomal vacuole; the action of hydrolytic lysomal enzymes; nutrient deprivation; defensins and other antimicrobial proteins (Lehrer *et al.*, 1993; Hiemstra *et al.*, 1993).

The range of agonists that augment macrophage functional properties is large and diverse. Amongst the most potent are bacterial LPS, chemotactic peptides, colony-stimulating factors (CSFs) and cytokines including IFN-γ.

1.10 IFN-γ and its receptors

Interferon gamma (IFN-γ) is a dimeric glycoprotein produced by activated T cells and natural killer cells. Although originally isolated on the basis of its antiviral activity, IFN-γ also displays powerful antiproliferative and immunomodulatory activities. These activities are essential for developing appropriate cellular defences against a variety of infectious agents. It enhances bacterial phagocytosis and proteolytic enzyme synthesis by macrophages (Huang *et al.*, 1971; Pestka *et al.*, 1987; Adams and Hamilton 1984), and increases granulocyte superoxide production (Perussia *et al.*, 1987; Berton *et al.*, 1986; Klein *et al.*, 1991). Immunomodulatory actions of IFN-γ include increased natural killer (NK) cell activity (Weigent *et al.*, 1983) and enhanced membrane expression of class II

histocompatibility antigens and other various immunomodulating receptors (Adams and Hamilton 1987; Lindahl *et al.*, 1976; Fertsch and Vogel 1984; Naray and Guyre 1984; Tweardy *et al.*, 1986).

IFN-γ exerts its pleiotropic effects on cells through an interaction with a specific high affinity receptor expressed at the cell surface (Farrar and Schreiber 1993). IFN-γ receptors are composed of two distinct, species-specific polypeptides. IFN-γRα and IFN-γRβ1 are members of the class-2 cytokine receptor family. Although the receptor α chain (90kDa) binds IFN-γ with high affinity, signal transduction requires a species specific accessory protein (Schreiber *et al.*, 1992; Soh *et al.*, 1994; Hemmi *et al.*, 1994) which associates with the extracellular domain of the receptor, the IFN-γ receptor β-chain. The intracellular part of the IFN-γ receptor is more promiscuous, as it can be inter-changed between species without loss of function (Pellegrini and Schinkler 1993; Hemmi *et al.*, 1994). IFN-γ receptor does not express endogenous kinase or phosphatase activities, yet may become rapidly and reversibly tyrosine phosphorylated following ligation in intact cells. Mutational studies of the IFN-γ receptor have defined two cytoplasmic domains necessary for biological function, a membrane-proximal region and a C-terminal sequence including an essential tyrosine (Cook *et al.*, 1992; Farrar *et al.*, 1992).

As reviewed in Part II, IFN-γ signalling pathway involves JAK-STAT activation. The other signalling pathways utilised by IFN-γ to induce cellular changes are a subject of controversy (reviewed in Scheoers *et al.*, 1992). Evidence supporting and refuting a role for activation of phospholipase C has been reported. IFN-γ has been shown to increase intracellular concentrations of calcium ($[Ca^{2+}]$), stimulate generation of inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG), and modulate protein kinase C activity (Celada and Schreiber 1986; Klein *et al.*, 1987; Somers et *al.*, 1986; Yap *et al.*, 1986; Hamilton *et al.*, 1985; Sebaldt *et al.*, 1990). Protein kinase C activation by phorbol diesters and an increase in $[Ca^{2+}]$ by calcium ionophores mimic some of the functional effects of IFN-γ (Celada and Schreiber 1986; Somers *et al.*, 1986; Strassman *et al.*, 1986). Additionally, inhibition of protein kinase C activation by H-7 or staurosporine has

been reported to prevent IFN-γ-induced expression of class II histocompatibility antigens (Politis and Vogel 1990; Nezu *et al.*, 1990). On the other hand, some reports showed that PKC activation was not involved in IFN-γ regulation of class II antigens in HL-60 cells (Ina *et al.*, 1987) or in murine macrophages (Celada and Maki 1991). There is, however, much evidence suggesting that protein phosphorylation is an intermediate event in the signal transduction pathways activated by IFN-γ. Some kinases different from, or in addition to, protein kinase C and calcium-calmodulin-dependent protein kinase stimulates protein phosphorylation induced by IFN-γ. We now know that JAK kinases play important roles in such signalling.

1.11 LPS and its receptors

1.11.1 Lipopolysaccharide (LPS)

LPS is a complex glycolipid found in the outer membrane of all Gram-negative bacteria. It is composed of two chemically dissimilar structural regions: the hydrophilic repeating polysaccharides of the core and O-antigen structures and a hydrophobic domain known as lipid A (A schematic structure for LPS from *Escherichia coli* is shown in Fig 1.6). Viturally all LPS-induced biologic responses are lipid A dependent (Rietschel *et al.*, 1994). The most compelling evidence supporting the concept that lipid A is biologically active moiety of LPS derives from studies with synthetic lipid A. This product has full endotoxic activity. Thus recognition of the lipid A of LPS by cells must be the initial step in LPS-induced cellular responses.

In humans and experimental animals the presence of bacterial lipopolysaccharide (LPS) signals the presence of Gram-negative bacteria. LPS is one of the most potent biological response modifiers known; picomolar concentrations are sufficient to stimulate cells of the immune / inflammatory / vascular systems. LPS triggers gene induction of which encode proteins that include produce low molecular weight proinflammatory

mediators. Together the products of these inducible genes upregulate host defense systems that participate in eliminating the bacterial infection. Unfortunately, these same mediators contribute to a serious human disease known as septic shock (Reviewed in Ulevitch and Tobias 1995).

A vast amount of information about molecular mechanisms of host defense responses and inflammatory mediators has been derived from studies using LPS as a stimulus (Raetz *et al.*, 1991), but until recently, mechanism of LPS-induced cell activation were not well understood. Two major advances have helped to bridge this gap in our knowledge, have been the characterisation of the LPS receptor, CD14 and the LPS binding protein, LBP.

1.11.2 LPS binding protein (LBP)

First was the discovery of LPS binding protein (LBP) (Tobias *et al.*, 1986), a 60-kDa serum glycoprotein that binds LPS, via the lipid A moiety. Analysis by cDNA cloning of the structure of LBP (Schuman *et al.*, 1990) led to recognition of a structure-function relationships between LBP, the bactericial/permeability-increasing protein (BPI), and other proteins (Tobias *et al.*, 1988; Gray *et al.*, 1989; Day *et al.*, 1994). Most importantly, characterization of LBP function in determining cellular responses to LPS revealed an unanticipated mechanism for LPS-induced cell activation that involves a membrane receptor for LPS-LBP complexes (Ulevitch *et al.*, 1993; Mathison *et al.*, 1992; Ulevitch *et al.*, 1994; Wright *et al.*, 1989). LBP is synthesized in hepatocytes as a single polypeptide, glycosylated, and released into blood as a 60-kDa glycoprotein (Ramadori *et al.*, 1990; Grube *et al.*, 1994). LBP synthesis is under the control of cytokines and steroid hormones (Grube *et al.*, 1994). A major function of LBP is to enable LPS binding to its receptor, either the membrane or soluble form of CD14. LBP appears to have two functional domains, one for LPS binding and another that fosters LPS-CD14 interactions. Measurements of induction of TNF with a series of LPS preparations as well as with synthetic lipid A showed that the presence of LBP lowered the threshold stimulatory concentration of LPS and markedly enhances the effects of LPS on the induction of other

cytokines (Martin *et al.*, 1994; Martin *et al.*, 1992) as well as NO release (Corradin *et al.*, 1992).

1.11.3 LPS signals and CD14

The second major advance was the identification of the LPS receptor as CD14. Initial interactions of LPS with cell membrane are believed to involve the binding of the lipid A. polysaccharide, or both moieties of LPS to specific cell surface receptors (Wright *et al.*, 1991; Raetz *et al.*, 1990; Ulevitch *et al.*, 1993). A diverse group of plasma membrane molecules that behave as LPS-binding proteins has been identified in many cell types. These include the CD11/CD18 leukocyte integrins (Wright *et al.*, 1986), an 80kDa protein of mouse and human splenocytes and macrophages (Lei *et al.*, 1988), the glycolipid-anchored CD14 molecule (Wright *et al.*, 1990), an 95kDa protein identified in a murine macrophage cell line (Hampton *et al.*, 1988), and the scavenger receptor for plasma protein (Hampton *et al.*, 1991). Of these proteins, only the glycosylphosphatidylinositol-linked molecule CD14 has been shown to function as a bona fide LPS receptor with the capacity to initiate cell signalling.

CD14 is a 55-kDa, glycosylphosphatidylinositol (GPI)-anchored membrane protein (mCD14) of myeloid cells; it is also found as a soluble serum protein (sCD14) lacking the GPI-anchor (Bazil *et al.*, 1989; Ziegler-Heitbrock and Ulevitch 1993). Although other surface proteins have been suggested to be LPS receptors, CD14 is the only protein of full defined structure that binds LPS and mediates LPS-induced cell activation. A role for mCD14 has been defined in LPS activation of myeloid cells, while sCD14 has been shown to participate in activation of nonmyeloid cell types such as endothelial or epithelial cells that normally do not express mCD14 (Pugin *et al.*, 1993; Frey *et al.*, 1992). CD14 does not have significant sequence homologies with other known proteins; the major recognisable structure of CD14 is the presence of repeating leucine-rich motifs (Ferrero *et al.*, 1990). This motif appears to be present when protein-protein interactions represent a significant feature of function.

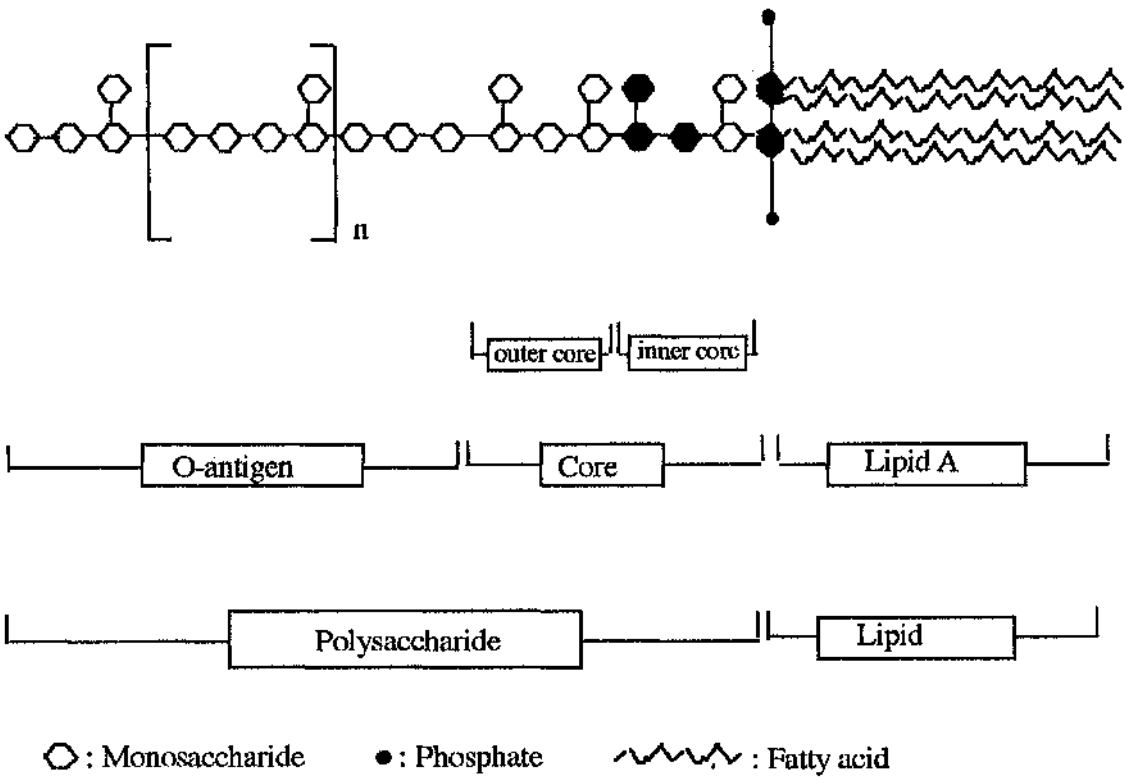


Fig. 1.6 General chemical structure of bacterial lipopolysaccharides. LPS is a complex glycolipid. It is composed two chemically dissmilar structural regions: the hydrophilic repeating polysaccharides of the core and O-antigen structures and a hydrophobic domain known as lipid A.

CD14 does not directly communicate with the cell interior, therefore how does it mediate ligand-specific cell activation? Evidence has been presented to indicate that ligation of CD14 results in rapid activation of Src family tyrosine kinases leading to the phosphorylation of target proteins on tyrosine (Weinstein *et al.*, 1993; Stefanova *et al.*, 1993). In addition, various investigators have reported that responses to LPS in a variety of cell types may involve a pertussis toxin-sensitive G protein (Jakway *et al.*, 1986; Dziarski 1989; Wang *et al.*, 1988), hydrolysis of phosphatidylinositides (Prpic *et al.*, 1987) and activation of protein kinase C (PKC) (Kawamoto and Hidaka 1984; Novotney *et al.*, 1991; Bakouche *et al.*, 1992; Liu *et al.*, 1994) or mitogen-activated protein (MAP) kinases (Liu *et al.*, 1994; Nick *et al.*, 1996). However, some of these findings have not been consistently reproducible (Dziarski 1989; Wang *et al.*, 1988; Forehand *et al.*, 1989; Grupp and Harmony 1985; Dong *et al.*, 1989) and it is not known to what extent they may be CD14-dependent (reviewed in Liu *et al.*, 1994). Thus, the question of how intracellular signalling in response to LPS is mediated remains to be fully explored.

1.12 IL-4 and its receptors

IL-4 is derived fron Th2 cells. IL-4 regulates T-cell development and also has numerous effects on both resting and activated B cells (Howard *et al.*, 1993), is a mast cell growth factor, and acts synergistically on certain populations of myeloid cells (Rennick *et al.*, 1992). In general, the effects of IL-4 on cells of the monocyte /macrophage compartment could be described as "anti-inflammatory". The down-regulation of both Fcγ receptor expression and LPS receptor expression results in a reduction of antibody-dependent cellular cytotoxicity (ADCC) and in decreased responsiveness to LPS stimulation as characterised by a diminution in chemokine and growth factor secretion (Minty et *al.*, 1993; de Waal Malefyt *et al.*, 1993). The anti-inflammatory action of IL-4 lead not only to a reduction in ADCC but also to decreased killing of phagocytosed bacteria such as *Salmonella* (Denich *et al.*, 1993) and parasites such as *Leshmania* (Lehn *et al.*, 1989). Experiments using mouse macrophages cultured from bone marrow in the presence of IL-4 indicate reduced the production of nitric oxide

(NO) following LPS activation. This reduction in release of NO, one of the most important cytotoxic mechanisms of phagocytes, correlates with a lowering of parasiticidal activity against *Leishmania major* (Doherty *et al.*, 1993).

Initial characterization of IL-4 binding proteins revealed that IL-4 cross-links multiple proteins, one of molecular mass approximately 140kDa and a lower-molecular-weight species of 60-75kDa. The 140kDa IL-4R is expressed on a variety of cell types, including lymphoid cells, myeloid cells, and nonhematopoietic cells such as fibroblasts and neuroblasts (reviewed in O'Farrell *et al.*, 1996). The cloned human and mouse IL-4Rs have approximately 50% amino acid identity (Galizzi *et al.*, 1990; Idzerda *et al.*, 1990; Harada *et al.*, 1990; Mosely *et al.*, 1989). A soluble form of the mouse receptor, capable of high affinity IL-4 binding, has also been isolated (Mosley *et al.*, 1989). In human B cells, the IL-4R is coupled to a transient PLC activation followed by a sustained generation of cyclic AMP (Finney *et al.*, 1990). Although these signals are not observed in murine B cells, IL-4 can reduce the threshold of mIg-mediated PKC activation (Harnett *et al.*, 1991). In addition, the IL-4R is also coupled to the PTK-mediated activation of PI-3-K, possibly via a novel docking protein, 4PS (reviewed in Keegan *et al.*, 1994; Harnett 1996).

Although the 140kDa IL-4R binds IL-4 with high affinity, several lines of evidence have suggested that the functional IL-4R contains an additional subunit that enhances affinity and plays a role in signal transduction (Noguchi *et al.*, 1993; Zurawski *et al.*, 1993). Accordingly, it has recently been demonstrated that IL-4 cross-links IL-2Rγ, and the combination of IL-4R plus IL-2Rγ significantly increases IL-4 binding affinity relative to that observed with IL-4 R alone. Furthermore, IL-2Rγ is required for IL-4-mediated signal transduction (Kondo *et al.*, 1993; Russell *et al.*, 1993). IL-4, unusually for an hemotopoietic receptor superfamily cytokine, does not stimulate Ras activation (Satoh *et al.*, 1991).

Part IV. Aims of the thesis

Macrophages activated by cytokines and microbial products protect the host from certain tumor cells and microbes, including parasite, fungi, bacteria and viruses, in large part by a pathway dependent on production of NO. On the other hand, cytokine and/or LPS-induced NO production contributes substantially to the suppression of lymphocyte proliferation, hypotension in sepsis, and a range of autoimmune disease (reviewed in Bogden *et al.*, 1994). The potential function of NO in all these situations, whether protective or destructive, makes it important to understand the regulation of its production. Although iNOS regulation has been studied either at the genomic level, gene transcription level, or even post-transcriptional level, the early signalling events that lead to the transcriptional activation are still unclear.

IFN-γ and IL-4 are produced by functionally distinct subsets of T helper cells (Th1 and Th2, respectively) in the murine as well as in the human system. The opposing regulatory effects of IL-4 and IFN-γ are crucial in the regulation of the immune response, as has been demonstrated in the murine Leishmaniasis model. The aims of this project are therefore to:

1. Investigate the signalling pathway leading to the induction of iNOS expression by IFN-γ and LPS, with special attention to the roles of JAK-STATs and IRF molecules.

2. Investigate the mechanism of the regulation of the induction of iNOS expression by IL-4, leading to the understanding of the mechanism by which immunological balance is achieved.

These objectives will be investigated in the murine macrophage iNOS system in which the productions of high levels of NO has been well established.

Chapter 2

Materials and methods

Part I. Materials

American Type Culture Collection	J774; RAW264.7 (murine macrophage cell lines)
Amersham	[α^{32}P]-dATP, [^{35}S]-dATP,
	DNA sequence kit
	ECL detection system
	Rainbow protein molecular weight markers
Biogenesis Ltd.	$RNA_{ZOL}^{TM_B}$
BioRad	acrylamide
	Bis-acrylamide
	Nitrocellulose membrane
Costar	All the tissue culture flasks and plates
	Eppendorf tubes (1.5 ml; 0.5 ml)
	All filter tips (1-1000 µl)
	Plastic scraper
	Spin X centrifuge filter tube
Genosys	All the oligo synthesis
Gibco, BRL	1kb DNA markers
	Agarose
	dATP, dGTP, dCTP, dTTP
	Dubicco's Modified Eagle Medium (DMEM)

	Fetal Calf Serum (FCS)
	Formaldehyde
	HEPES
	High Salt Buffer (for annealing)
	Klenow (DNA polymerase)
	L-Glutamine
	Penicillin/Streptomycin
	Phosphate Buffered Saline (PBS)
	Polyethylene glycol
	Random priming kit
	RNA markers
	SuperScript II (reverse transcriptase)
	T4 ligase
	Urea
Hoeffer Sentific Ltd.	Whole electrophoresis system
Kodak	X-ray film cassettes
	X-omat AR autoradiography film
Pharmacia	Protein-A/Sepharose 4B beads
	Poly (dI-dC) poly (dI-dC)
	Nylon membrane

Santa Crutz	Anti-STAT1(p91)
	Anti-NFκB (p65)
	Anti-NFkB (p50)
	Anti-IRF-1
	Anti-IRF-2
Sigma	Aprotinin
	BCA kit for measuring protein concentration
	Cycloheximide
	DEPC
	Dithiothreitol (DTT)
	Leupeptin
	Lipopolysccharide (LPS)
	Phenyl methyl sulphonyl fluoride (PMSF)
	Sodium orthovanadate
	TEMED
Scottish Antibody Production Unit (SAPU)	All the HRP-conjugated secondary antibodies
Tranduction Laboratories (Affiniti)	Anti-phosphotyrosine (RC-20)
	Anti-phosphotyrosine (PY-20)
	Anti-phosphotyrosine (PY54)

	Anti-macNOS
	Anti-MAP kinase (ERK2)
Upstate Biotechnology (UBI)	Anti-phosphotyrosine (4G10)
	Anti-JAK1, JAK2, JAK3,
Gifts	IL-4 (Immunise, Seatle)
	IFN-γ (Dr. G. Adolf, Vienna)
	Anti-STAT6 (Dr. J.N Ihle, Memphis)

All the other chemicals used in this study are comerical avilable in Sigma or BDH.

Part II. Methods

2.1 Cell culture

The murine macrophage cell lines J774 and RAW 264.7 (American Type Culture Collection) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, BRL) supplemented with 10% heat-inactivated fetal calf serum (Gibco, BRL), 2 mM L-glutamine, and 50 U/ml each of penicillin and streptomycin (complete culture medium), at 37°C with 5% CO_2. Cells were dispensed in tissue culture flasks ($5x10^6$ cells/80 cm^2) or plates (10^5 cells/250 µl) 24 h before stimulation.

Murine peritoneal macrophages were harvested 4 days after intraperitoneal injection with 4% Brewer's thioglycollate broth, The cells were cultured with complete culture medium in either flasks (10^6 cells/ml, 8 ml/flask), or 96-well plates (10^6 cells /ml, 200 µl/well) at 37°C with 5% CO_2. After 2 h incubation period, non adherent cells were removed by washing with warm culture medium.

2.2 Griess reaction for NO_2 measurement

Nitrite concentration in the culture supernatants was determined by a micro plate assay (Ding, *et al.*, 1988). Briefly, 50 µl samples were mixed with an equal volume of Griess reagent (1% sulphanilamide / 0.1% naphthalene diamine dihydrochloride / 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 570 nm was monitored with a microplate reader (Dyrotech MR5000). Nitrite concentration was determined by using sodium nitrite as a standard. The results were expressed as: NO_2 µM.

2.3 Antibodies

The antibodies used in this project are shown in Table 2.1 below.

Table.2.1

Name	specificity	Clone	Application	Company
RC20	anti-Tyr*.	mono	Western Blot	Affiniti
PY20	anti-Tyr*.	mono	Western Blot	Affiniti
PY54	anti-Tyr*.	mono	Western Blot	Affiniti
4G10	anti-Tyr.	mono	Western Blot	UBI
JAK1	anti-JAK1	poly	immunoprecipitation Western Blot	UBI
JAK2	anti-JAK2	poly	immunoprecipitation Western Blot	UBI
JAK3	anti-JAK3	poly	immunoprecipitation Western Blot	UBI
P91/84	anti-STAT1	poly	Western Blot	Santa crutz
STAT6	anti-STAT6	mono	Western Blot super shift	Dr.Ihle (U.S.A.)
MAPkinase	anti-erk2	mono	Western Blot	Affiniti
NFkB(P65)	anti-p65	poly	Western Blot super shift	Santa crutz
NFkB(P50)	anti-p50	poly	super shift	Santa crutz
IgG-HRP	anti-mouse IgG	poly	Western Blot	SAPU
IgG-HRP	anti-rabbit IgG	poly	Western Blot	SAPU
iNOS	anti-macNOS	mono	Western Blot	Affiniti
IRF-1	anti-IRF-1	poly	super shift	Santa crutz
IRF-2	anti-IRF-2	poly	super shift	Santa crutz

*: anti-phosphotyrosine

2.4 Preparation of cell lysates for NOS activity assay

Macrophages in culture medium were dispensed into 6-well plates (Costar) at 10^6 cells/ml, 300 µl/well, and incubated for 24 h at 37°C and 5% CO_2. Nonadherent cells were then removed by washing with pre-warmed medium and cytokines were added. The cultures (3 ml/well) were incubated as above for a designated period. At the end of incubation, supernatants were aspirated and 250 µl of 0.1 M HEPES (pH7.4) with 1 mM dithiothreitol (Sigma) was added to each well and the cells harvested with a plastic scraper and subjected to three cycles of freeze-thawing. The samples were then centrifuged at 100,000 xg for 30 min at 4°C and the supernatant assayed for NO synthase activity.

2.5 NOS activity assay

NO synthase activity assay was carried out by a spectrophotometric method as described (F.Y Liew *et al.*, 1991): briefly, NO synthesis was measured in incubates (0.5 ml) containing 5 mM oxyhemoglobin and 20% (v/v) macrophage cytosol in 40 mM potassium phosphate buffer (pH 7.2) containing $MgCl_2$ (1 mM) in a dual wavelength spectrophotometer (Shimadzu UV-3000 Kyoto, Japan) using a band width of 2 nm, at 37°C. NO synthesis was initiated by addition of L-arginine (100 µM) and NADPH (100 µM). The shift in absorbance between the wavelength pair 401-421 nm is caused by the conversion of oxyhaemoglobin (oxyHb) to methaemoglobin (mrtHb) by nitric oxide and was measured as a function of time using the equation:

$$\frac{\Delta Abs/min \times 0.02}{100 \times 77200} \times 0.0005$$

where: ΔAbs/min = the change in absorbance in 1 minute (% of 100)

0.02 = full scale deflection (i.e 0-0.02 = 100%)

0.0005 = reaction volume in litres

77200 = extinction coefficient for haemoglobin in the spectrophotometer used.

Results were expressed as moles of NO formed/sample volume/ minute or moles NO formed/mg protein/minute. In some assays the NO inhibitors L-N-guanidino monomethyl arginine (L-NMMA) and L-N-imino ornithine (L-NIO) were added.

2.6 Preparation of cell lysates for western blotting

Treated cells (1-2x10^7 /sample) were washed twice *in situ* with ice-cold TBS (25 mM Tris Cl pH7.4 , 150 mM NaCl, and 100 μM vanadate), and harvested in 5 ml TBS with a plastic scraper. Following 5 min centrifugation at 4,600 rpm at 4°C, the cell pellets were resuspended in 0.5-1 ml lysis buffer (LB) containing 25 mM Tris Cl pH7.4, 150 mM NaCl, 1%NP-40, 1mM Sodium orthovanadate, 1 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM dithiothreitol, 50 μg/ml each of leupeptin, aprotinin, and phenyl methyl sulphonyl fluoride (PMSF). The samples were kept on ice for 30 minutes, transferred to an Eppendorf tube, centrifuged at 13,000xg at 4°C for 10 min. Protein concentration was determined using the BCA method according to manufacture instructions (Sigma). The supernatants (solublilised proteins) were stored at -70°C if they were not to be used immediately.

2.7 Immunoprecipitation

Immunoprecipitation was performed according to the UBI instructions: briefly, 500μg solubilised protein per sample was pre-cleared by adding 50 μl of Protein-A/Sepharose 4B beads (Pharmacia) and incubated at 4°C for 10 min. After 30 seconds in the micro centrifuge at 13,000 xg, the supernatant was transferred to a tube, and mixed with 3 μl of antiserum in a micro centrifuge tube. The reaction mixture was gently rocked on a rocker at 4°C for 2 h. Immuno-complex was captured by adding 50 μl Protein-A/Sepharose bead slurry (50% in LB) and gently rocked for further 2 h. The Sepharose beads were collected by pulsing (5 seconds in the micro centrifuge at 13,000 xg), and supernatant drained off. The beads were washed 3 times with ice-cold LB (300 μl / wash followed by vortexing for 5 seconds and the beads were collected by a micro centrifuge pulse). The Sepharose beads were resuspended in 40μl Laemmli sample buffer (2x;

Laemmli 1970) and mixed gently. This preparation was either kept at -20°C or boiled for 5 min before loading onto the SDS PAGE gel (7 or 10%).

2.8 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) was performed according to a previously published method (Laemmli, 1970). All gels had a ratio of 29:1 of acrylamide to bis-acrylamide (BioRad). Resolving gels were made at either 10% or 7.5% in 1.5 M Tris Cl buffer pH 8.8, 1% w/v SDS, 1% w/v ammonium persulphate (BDH) and 0.1% v/v N,N,N',N'-tetramethylenediamine (TEMED) (Sigma). Stacking gels (5%) were similarly prepared using 0.5% Tris HCl buffer, pH 6.8. All gels were run using a standard electrophoresis buffer containing 25mM Tris, 0.25 M glycine (BDH) and 0.1% SDS. Samples to be electrophoresed were mixed with 50% v/v of 2x concentrated sample buffer (1M Tris Cl, pH 6.8, 0.2 M DTT, 4% SDS, 0.4% bromophenol blue and 20% v/v glycerol, pH 6.8) (BDH), heated to 100°C for 5 min and cooled on ice prior to loading on gels. 'Rainbow' molecular weight markers (range 14 to 200kDa) (Amersham) were similarly treated prior to electrophoresis and run on all gels. Electrophoresis was carried out at a constant current of 5 mA/cm gel length until the bromophenol blue marker reached the bottom of the gel. The gels were then subjected to either Coomassie blue stain or Western blot analysis.

2.9 Coomassie blue staining of SDS-PAGE gels

At the end of electrophoresis, the gels were stained for 1 h with 0.5% Coomassie brilliant blue R250 (Sigma) in 40% methanol (BDH), 10% glacial acetic acid (BDH) and destained over 4-5 h in several changes of 40% methanol, 10% glacial acetic acid until the background was clear. Stained gels were transferred to 3 MM filter paper (Whatman) and dried under a vacuum at 80°C.

2.10 Western blot analysis of SDS-PAGE separated proteins

Four pieces of 3 MM filter papers were cut to size slightly bigger than the gel selected for Western blot analysis and were soaked in transfer buffer (20 mM Tris, 40 mM glycine, 20% methanol) (BDH). Two of the filter papers were placed on the cathode of an electroblotter (Hoeffer scientific Ltd.). The gel, soaked in transfer buffer, was placed on the filter papers. A sheet of nitrocellulose (BioRad), cut to the same size as the gel and pre-wetted with transfer buffer, was placed on the top of the gel. The remaining two sheets of filter papers were placed on top of the nitrocellulose. Proteins were transferred to the nitrocellulose by applying a constant current of 0.8 mA/cm^2 of gel area for 90 min. At the end of the transfer, the nitrocellulose was incubated in 2% bovine serum albumin (Sigma) in wash buffer (0.01 M Tris HCl pH 7.5, 0.1 M NaCl, 0.1% Tween-20) for at least 1 h to block non-specific protein binding sites. Primary antibodies were diluted 1:500-5000 (depending on the recommendation of the suppliers) in 1% BSA in wash buffer and applied to the blot for 1 h at room temperature or overnight at 4°C. Following incubation with primary antibodies, the blot was washed with 6 changes of wash buffer (each for 5 to 10 min) and then incubated with a secondary antibody, conjugated to horseradish peroxidase, and diluted up to 1:5000 in wash buffer containing 1% BSA for 1h at room temperature. Immuno-reactive bands were visualised by using the Enhanced Chemiluminescence (ECL) system (Amersham).

2.11 Preparation of cell lysates for two dimensional gel electrophoresis:

For the NEPHGE gel, 10^7 cells were washed twice *in situ* with ice-cold TBS and harvested in 200 µl lysis buffer (LB1) containing 9.5 M urea (Schwarz-Maun), 2% Nonidet P-40, 2% Ampholines (pH 3.5-10.0), 50 µM dithiothreitol, 50 µg/ml each of leupeptin and aprotinin (Sigma), 25 µg/ml PMSF, 0.1 mM EDTA, 100 µM sodium orthovanadate, and 50 mM sodium fluoride (NaF). The cells were kept on ice for 30 min, and then centrifuged in a microfuge for 15 min at 4°C. The supernatant fraction (containing cellular total proteins) was stored at -70°C until used.

2.12 NEPHGE gel (first dimension)

NEPHGE gels were prepared according to the protocol from Dr. Mark Rogers (Glaxo-Wellcome). Briefly, for 10 tube gels, the following reagents were added: 2.75 g urea, 0.67 ml 30% polyacrylamide (ratio 1:17), 1 ml 10% Nonidet P-40, 1 ml dH_2O, and 250 µl Ampholines (pH 3.5-10.0). After the mixture was completely dissolved, 7 µl 10% ammonium persulphate and 4.5 µl TEMED were added and gels were poured immediately in the tube gel tank (Hoeffer Scientific Ltd.) and an overlay solution (9 M urea, 1% total Ampholines pH 3.5-10.0) was applied to the top of the gel when it was polymerised. The gels were kept away from light till used.

The overlay solution was removed, 30 µg protein per sample was loaded onto the top of the gel followed by overlay solution. The lower chamber was filled with 1000 ml 0.02 M NaOH (Cathode electrode solution), and the upper chamber was filled with 500 ml 0.01 M orthophosphoric acid (Anode electrode solution). Electrophoresis was carried out at 550 V for 5 h at room temperature.

At the end of electrophoresis, the gel was extruded into 5 ml SDS sample buffer in a capped tube, rocked gently at room temperature for 15 min, freezed in dry ice-ethanol for at least 30 min, and stored at -70°C until use.

2.13 SDS PAGE gel (second dimension)

SDS PAGE gel was used for the second dimension assay. A 10% SDS polyacrylamide (1:36) gel was prepared to a thickness of 1.5 mm as described above. While the tube gel was thawing out at room temperature, 10 ml of 1x SDS sample buffer containing 0.1% agarose was boiled up. The tube gel was laid on the slab, and 1 ml hot agarose was poured onto the top of it. The gel was then run at 20 mA for 3 h followed by western blotting as described above.

2.14 Preparation of nuclear extracts (method 1)

Nuclear extracts were prepared as described previously (Dignam *et al.*, 1983; Lee *et al* ., 1988 and Schreiber *et al* ., 1989) with some modifications. Treated cells were washed twice with 10 ml ice-cold TBS (Tris buffered saline) and pelleted by centrifugation at 1500 xg for 5 minutes. The pellet was resuspended in 1 ml TBS, transferred into an Eppendorf tube and pelleted again. The pellet was resuspended in 400 µl cold Buffer A1 (10 mM HEPES pH7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, o.5 mM PMSF, 100 µM sodium orthovanadate) by gentle pipetting. The cells were allowed to swell on ice for 15 min, after which 25 µl of a 10% solution of Nonidet NP-40 (Fluka) was added and the tube was vigorously vortexed for 10 seconds. The homogenate was centrifuged at 13,000xg for 30 seconds in a microfuge. The supernatant was removed, the nuclear pellet was resuspended in 50 µl ice-cold Buffer B1 (20 mM HEPES pH7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 100 µM sodium orthovanadate) and the tube was vigorously rocked at 4°C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min in a microfuge at 4°C and the supernatant was frozen in aliquots at -70°C until use.

2.15 Preparation of nuclear extracts (method 2)

DNA-binding proteins were prepared as described by Andrews et al (1991) except all buffers were supplemented with 100 µM sodium orthovanadate. Typically, 10^7 cells from tissue culture were washed with 10 ml TBS (Tris buffered saline) and pelleted by centrifugation at 1500xg for 5 min. The pellet was resuspended in 1 ml TBS, transferred into an Eppendorf tube and pelleted again by spinning for 15 seconds in a microfuge. TBS was removed and the cell pellet was resuspended in 400 µl cold Buffer A2 (10 mM HEPES-KOH pH7.9 at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 100 µM sodium orthovanadate) by flicking the tube. The cells were allowed to swell on ice for 10 min, and then votexed for 10 seconds. Samples were centrifuged for 10 seconds. and the supernatant fraction removed. The pellets were resuspended in 100 µl of cold Buffer B2 (20mM HEPES-KOH pH7.9, 25% glycerol, 420 mM NaCl,

1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithithreitol, 0.2 mM PMSF, 100 μM sodium orthovanadate) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 minutes at 4°C and the supernatant fraction (containing DNA binding proteins) was frozen in aliquots at -70°C until use.

2.16 Synthetic oligonucleotide DNA probes:

Oligonucleotides designed for DNA-protein binding assays are shown in Table 2.2. These probes are designed by Dr. X.Q. Wei.

Table 2.2.

Oligos	Sequence	binding site	location
Probe A-1	5'-CAATATTTCACTTTCAT		
Probe A-2	5'-TTCCATTATGAAAGTGA	ISREd	iNOS promoter
Probe B-1	5'-GGGAACAGTTATGCAAA		
Probe B-2	5'-CAGAGCTATTTTGCATA	GAS	iNOS promoter
Probe C-1	5'-TTCCTTTTCCCCTAA		
Probe C-2	5'-CAGTGTTAGGGGAA	GAS	iNOS promoter
Probe D-1	5'-CCCAACTGGGGACTCTC		
Probe D-2	5'-CCAAAGGGAGAGTCCCC	NFκB	iNOS promoter
Probe E-1	5'-GAACTTACTCTGTAGAC		
Probe E-2	5'-GCCTGGTCTACAGAGTA	GAS	iNOS promoter
Probe F-1	5'-TACAACAGCCTGATTTCCCCG		
Probe F-2	5'-GCCGTCATTCGGGGAAATCAG	GAS	IRF-1 promoter
Probe G-1	5'-CACTGTCAATATTTCAC		
Probe G-2	5'-ATTATGAAAGTGAAATATTGACAG	ISRE	iNOS promoter
Probe H-1	5'-CTAGAAGTGAAAGTG		
Probe H-2	5'-TCACTTCACTTTCACTTC	C-13	Fuiita 1987 Harada 1989

2.17 Synthetic oligonucleotide DNA labelling

Double-stranded oligonucleotides were labelled with [α-^{32}P]dATP by using Klenow fragment of DNA polymerase, and purified on 8% non-denaturing polyacrylamide gels. Synthetic oligonucleotides were supplied freeze-dried and reconstituted by the addition of 500 µl of distilled water. Dilutions (1/100) were made in distilled water and the concentration determined by U.V. absorbance at 260nm against a water blank. For single stranded DNA, an absorbance of 1 = approximately 37 µg/ml. The values obtained were converted to molarity on the assumption that the average molecular weight of 1 DNA base is equal to 324Da using the formula:

$$\frac{1}{\text{Total oligo Mr in Da}} \times \text{oligo concentration in } \mu g/\mu l \times 10^{-6} = \text{moles oligo}/\mu l$$

Annealing was carried out in high salt buffer (H buffer: Gibco BRL): 40 nmoles of each single stranded oligo in 90 µl were pooled together with 20 µl of H buffer and boiled at 100°C for 10 min followed by cooling slowly for up to 3-4 h in the same water bath but with the heater turned off.

Double stranded DNAs were labelled with [α-^{32}P]-dATP using a commercially available random priming kit (Gibco BRL). The DNA (2 µl) in a 1.5 ml Eppendorf tube was mixed with 2 µl each of 0.5 mM dCTP, 0.5 mM dGTP and 0.5 mM dTTP, 5 µl of 10x H buffer, 2 µl (2µCi) of [α-^{32}P]-dATP (Amersham), 1 µl Klenow DNA polymerase (Exonuclase-free, 2 units/µl) and distilled H_2O was added to 50 µl. The reaction was allowed to proceed for at least 2 h at 37°C and purified by electrophoresis in polyacrylamide gel, using TBE as running buffer followed by incubating gel pieces in TE buffer overnight at 37°C.

2.18 Protein-DNA binding assay

The binding reaction was initiated by pre-incubation of 5 to 10 µg of nuclear extract protein with 200 ng of double-stranded poly(dI-dC).poly(dI-dC) (Pharmacia) in

40 mM KCl, 20 mM HEPES (pH 7.9), 1 mM MgCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, 10% Glycerol, and 0.1% Nonidet P-40 for 20 min on ice in a volume of 30µl (Wu, *et al* .,1994; Andrews *et al.*, 1991). The radio-labelled synthetic oligo-nucleotide DNA probe (0.5 ng in 1 µl) was then added, and the mixture was incubated on ice for another 20 min. For antibody super-shift assay, 1 µl of antiserum was added during the pre incubation, and pre-incubation without probe was extended to 1 h.

DNA-protein complexes were fractionated by electrophoresis at room temperature on a non-denaturing 6% polyacrylamide gel at 150 V for 3 h in 0.5X Tris-borate-EDTA buffer (TBE: described bellow in section 2.25 on sequencing). Gels were dried and visualised by auto radiography.

2.19 Non-denaturing polyacrylamide gels

The slab gel system was used (SE400 Hoeffer scientific Ltd.) for separating DNA-protein complexes. In a final volume of 40 ml of 0.5x TBE buffer, 8 ml of 30% polyacrylamide solution (Acrylamide:bis = 29:1, BioRad) was added and mixed. Ammonium persulphate (100 µl of 30% solution) and TEMED (34 µl) were added immediately before pouring the gel. In the case of probe purification, the gel was made in 1x TBE.

2.20 Isolation of total cellular RNA

Total RNA was isolated using the 'RNAzol^{TM} B' method, following the manufacturer's instruction (Biogenesis Ltd.). Distilled water, treated for 12 hours with 0.2% diethyl pyrocabonate (DEPC; Sigma) and then autoclaved, was used to make all solutions not supplied with the kit. Cells (10^6-10^7) were washed with ice-cold PBS and pelleted by centrifugation at 2000xg and lysed in 400µl of RNAzol^{TM} B. Effective lysis was facilitated by vigorous pipetting. Following disruption, lysates were vortexed for 15 seconds in the presence of 40 µl chloroform. Samples were then centrifuged at 15,000xg for 15 min at 4°C and the colourless 200 µl upper phase (exclusively containing RNA)

was removed into an Eppendorf tube. RNA was precipitated by the addition of 200 µl of isopropanol (BDH) and pelleted by centrifugation at 15,000xg for 15 min at 4°C. The RNA pellet was washed once in 75% ethanol by centrifugation at 13,000 xg for 5 min before being resuspended in 20 µl TE buffer. The concentration of RNA in samples was estimated using ultra-violet spectroscopy. The sample (1 in 500 dilution) was made in DEPC-treated distilled water and the absorbance at 260nm and 280nm was measured using quartz cuvettes and a water blank. The RNA was judged to be free from protein contaminants if the absorbance ratio 260 nm/280 nm was greater than 1.8.

2.21 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed using a commercially available 'GeneAmp RNA-PCR' kit (Perkin Elmer Cetus). Reverse transcription of RNA to cDNA was performed on 5 µg of total RNA, isolated as described above. RNA was mixed with the following reagents supplied with the kit: 4 µl 25 M $MgCl_2$, 2 µl 10x concentrated PCR buffer (0.5 M KCl, 0.1 M Tris Cl, pH 8.3), 2 µl each of 10 mM deoxy adenosine triphosphate (dATP), dCTP, dGTP, dTTP, 1µl RNase inhibitor (20 units/µl), 1 µl recombinant reverse transcriptase from molnoey murine leukaemia virus (50 units/µl), 1 µl 50 µM random sequence hexanucleotides and 2 µl of DEPC treated distilled water. This mix was incubated at room temperature for 10 min, 42°C for 15 min, 99°C for 5 min and finally chilled on ice. This protocol allowed annealing of the random hexamers and conversion of the RNA sequence (via reverse transcriptase) into cDNA.

Following reverse transcription, 4 µl 25 mM $MgCl_2$, and 8 µl 10x concentrated PCR buffer were added to each sample. Target sequence-specific oligonucleotide primer pairs were then added to a final concentration of 20 ng/ml (usually 5 µl of a 40 µg/ml solution) and distilled water added to take the final volume to 99.5 µl. Recombinant DNA polymerase (0.5 µl, 5 units/µl) from *Thermus aquaticus* ('Amplitaq') was added and the mixture was overlaid with 100 µl of mineral oil (Sigma).The entire reaction mixture was subjected to thermal cycling under the following standard conditions: Denaturation at

95°C for 1 min, primer binding at 56°C for 2 min and sequence extension at 72°C for 3 min for 35 cycles. The PCR products were then resolved by DNA-agarose gel electrophoresis.

2.22 Agarose gel electrophoresis of nucleic acid

Nucleic acid was routinely analysed by electrophoresis through agarose gels. Agarose gels were used (0.8%-1.0%). Gels were made by dissolving the required amount of dry agarose power (Gibco BRL) in 0.5x TBE buffer from 10x stock buffer [200 mM Tris Cl pH 8.0, 900 mM Boric acid, 25 mM Ethylenediamine tetra-acetic acid (EDTA)] by boiling or in a microwave oven. The melted gels were allowed to cool to 45°C and ethidium bromide (Sigma) was added at a final concentration of 10 ng/ml. Gels were cast in tanks with combs where they were allowed to set. After setting, gels were submerged in 0.5x TBE buffer and well-forming combs were removed. The samples of DNA and 1kb DNA ladder (Gibco BRL) were mixed with loading buffer (6x loading buffer: 0.25% bromophenol blue, 150 mM Tris Cl pH8.0, 10 mM EDTA, 40% sucrose) and loaded into the relevant wells on the gel. Gels were electrophoresed at a constant current of 10 mA/cm gel-length or a constant voltage of 1-5 V/cm of gel-length until the bromophenol blue had migrated the required distance. Gels were then analysed under ultra-violet light and photographed if necessary.

2.23 Purification of DNA fragments from agarose gels

DNA bands were visualised with Ethidium Bromide by short-wave UV illumination. The relevant band was excised with a scalpel and placed in a Eppendorf tube. Freeze-Squeeze buffer (1 ml, 25 mM Tris Cl pH7.4, 0.3 M sodium acetate, 1 mM EDTA pH7.0) was added and the sample kept in the dark at room temperature for 15 min. The buffer was then discarded and the Eppendorf tube with the agarose was placed in dry-ice for 10 min. The frozen gel slice was rapidly transferred to a Spin X centrifuge filter tube (Costar) and centrifuged for 15 min at 15,000xg. DNA in the supernatant was

then extracted with phenol/chloroform followed by chloroform before precipitated with ethanol.

2.24 Cloning of interferon response factors (IRF-1 and IRF-2)

Following gel purification, the PCR amplified IRF-1 and IRF-2 cDNA fragments were ligated with TA-vector (Invitrogen) using DNA ligase isolated from the bacteriophage T4 (Gibco BRL). Approximately equimolar amounts (usually about 0.1-0.5 μg DNA) of the fragments to be ligated and the appropriate amounts of TA-vector DNA were mixed and incubated with 1 μl of T4 ligase (1 unit/μl) in ligation buffer (50 mM Tris Cl pH 7.6, 10 mM $MgCl_2$, 1m M ATP, 1 mM DTT and 5% w/v polyethylene glycol 8000) (Gibco BRL) in a total volume of 10 μl. Control ligations replacing the cDNA fragments with water but retaining the TA-vector plasmid DNA were also set up to calculate the degree of self ligation. Ligations were allowed to proceed either overnight at 16°C or at room temperature for 4 h. Following ligation, the reaction mixture were used for bacterial transformation directly.

2.25 DNA sequencing

The nucleotide sequence of cDNAs in plasmid vectors were analysed by a modification of the di-deoxy chain termination method (Sangar et al. 1983) using a commercially available kit (United States Biochemical) from which all reagents were obtained unless otherwise stated. Plasmid DNA (10 ng/ml) was denatured in the presence of 0.2 N NaOH for 15 min at 37°C in a total volume of 10 μl. A 17 base pair (bp) oligonucleotide sequencing primer, (10 pg), in a volume of 1 μl, is mixed with the denatured DNA and allowed to anneal for 5 min at room temperature. The DNA-primer complexes were then precipitated by the addition of 3 μl of 3M KOAc pH 4.8 and 75 μl of 100% ethanol and incubated at -70°C for 20 min, followed by centrifugation at 15,000xg for 15 min at room temperature. The ethanol was aspirated and the pellet washed once in 100 μl of 75% ethanol followed by centrifugation at 15,000xg for 5 min at room temperature. Following aspiration of the ethanol, the pellet was air-dried at room

temperature for 10 min and resuspended in 8 µl of distilled water. To this was added 2 µl of 5x concentrated reaction buffer (0.2 M Tris Cl, pH 7.5, 0.1 M $MgCl_2$ and 0.25 M NaCl), 1 µl of 0.1 M DTT, 2 µl of labelling mix (7.5 µM dCTP, 7.5 µM dGTP and 7.5 µM dTTP) 2 µl of 'Sequenase' recombinant phage T7 DNA polymerase (13 units/µl) (prediluted 1 in 8 in 10 mM Tris HCl, pH7.5, 5 mM DTT and 0.5 mg/ml BSA), 0.5 µl (5 µCi) of [α-^{35}S] dATP (specific activity: 1000 Ci /mmol, Amersham). The reaction was allowed to proceed at room temperature for 5 min and aliquoted into 4 Eppendorf tubes (3.3 µl/tube). To each tube was added 2.5 µl of one of the following termination mixes: 1) ddA: 80 µM dATP, dCTP, dGTP, 8 µM dideoxy ATP (ddATP). 2); ddC: similar as above but ddCTP was used instead of ddATP; 3). ddG: the same too, except that ddGTP was used to replace ddATP; 4) ddT: again here ddTTP was used replacing ddATP. Each tube was indicated at 37°C for 5 min prior to the addition of 4 µl of stop solution (95% formamide 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). All tubes were heated at 80°C for 5 min and stored on ice before electrophoresis.

Electrophoresis was performed through 6% PAGE gels containing 8 M urea (Gibco BRL) in 1x TBE buffer. Samples were run at a constant power of 50 W at room temperature until the bromophenol blue marker reached the bottom of the gel. In some experiments, the xylene cyanol marker was run to the end of the gel to allow reading of the sequence further from the primer binding site. At the end of electrophoresis, gels were fixed for 30 min, transferred to 3 MM filter paper and dried under vacuum at 80°C for 1-2 h. Dried gels were exposed to X-omat AR autoradiography film (Kodak) for at least 18 h at room temperature in cassettes (Kodak).

2.26 Double stranded DNA probe labelling for Northern blotting

Double stranded DNAs were labelled with [α-^{32}P]-dATP using a commercially available random priming kit (Gibco BRL). DNA was heated to 96°C for 10 min in a 1.5 ml Eppendorf and then mixed with 2 µl each of 0.5 mM dCTP, 0.5 mM dGTP and 0.5 mM dTTP, 15 µl of concentrated random primer reaction buffer, 5 µl (50 mCi) of [α-^{32}P]-dATP (Amersham), 1 µl Klenow DNA polymerase (Exonuclase-free, 2 units/µl)

and distilled H_2O was added to 50 µl. The reaction was allowed to proceed for at least 30 min at 37°C and stopped by the addition of 50 µl of TE buffer (10 mM Tris HCl, pH7.5, 1 mM EDTA).

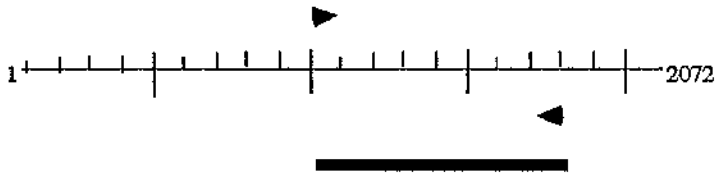
A Nick Column (Pharmacia) was pre-equilibrated with 2 ml of TE buffer and 100 µl reaction mixture loaded onto the top of the gel bed and allowed to enter to the gel completely. After 400 µl of TE buffer was supplied to the column, DNA probe was eluted with another 500 µl of TE buffer and collected in a 1.5 ml screw cap tube. Un-incorporated [α-^{32}P]-dATP was retained in the column and was discarded. The labelled cDNA was then denatured by heating for 10 minutes at 96°C and chilled on ice prior to addition into the pre-hybridisation solution. The sequence and location of cDNA probes used in the study is shown in Fig. 2.1.

2.27 Northern blotting

Total cellular RNA , isolated from cultured cells as described earlier was analysed by Northern hybridisation according to a modification of previously published method (Seed *et al* 1982). Total RNA (10 µg) in 3 µl of DEPC-treated water were denatured in 7 µl of sample buffer (71% v/v formamide, 7% v/v 20x MOPS buffer (400 mM MOPS, pH 7.0, 122 mM NaAc, 20 mM EDTA) and 22% v/v 37% formaldehyde) (Sigma) for 2 min at 68°C. Samples were chilled on ice and mixed with 16% v/v nucleic acid gel loading buffer.

Electrophoresis was carried out through 1.5% agarose gels containing 5% v/v 20x MOPS buffer, and 7.5% v/v 37% formaldehyde. Briefly, 3 g of agarose was melted in 175 ml DEPC-treated water and was cooled to 60°C before adding 10 ml of 20x MOPS buffer, 15 l of 30% formaldehyde, mixed and poured into the gel tray. After samples and RNA markers (Promega) were loaded side by side, gels were run in 1x MOPS buffer at a constant voltage of 100 V for 3-5 h.

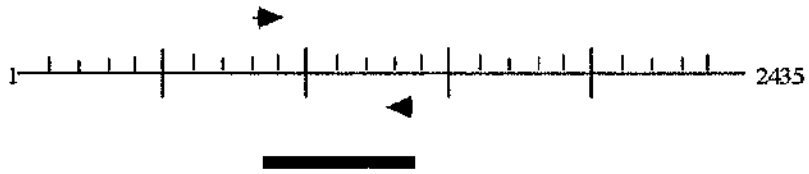
a. mouse IRF-1



750bp

GACTTCAGCTGCAAAGAGGAACCAGA (1019)
CCCCACAGCTGGAGCAGGTAGTGAGG (1766) 750bp

b. mouse IRF-2



511bp

GTGTCTTCCTACGCAGAAAGCGAAACTACC (832)
ATCAATAATCTATCGTCAAGGCTTTCTCCC (1340) 511bp

c. mouse iNOS cDNA

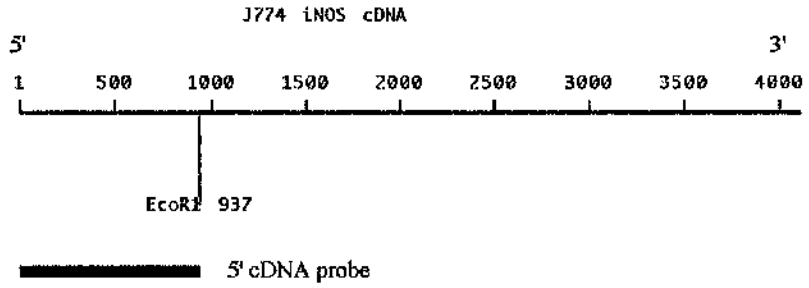


Fig.2.1. The cDNA probes for Norhtern blot analysis. a. Cloned IRF-1 PCR probe. b. Cloned IRF-2 PCR probe. c. 5'-iNOS cDNA probe was cut off from the full length murine iNOS cDNA clone (Wei, *et al.* 1995).

Following electrophoresis, agarose-formaldehyde gels were rinsed twice in distilled water after electrophoresis. The RNA was then transferred onto a nylon membrane (Pharmacia) by capillary blotting as described previously (Southern, 1975). Capillary transfer was allowed to proceed overnight at room temperature. At the end of the blot, membrane was rinsed in 2xSSC (0.3 M NaCl, 42 mM Sodium citrate. PH 7.2), air dried and oven baked at 120°C for 20 min or 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 h at 65°C. At the end of pre-hybridisation , a specific [α-^{32}P] dATP labelled cDNA probe, prepared as described previously, was added directly to the incubating solution and incubated for a further 18 h in a hybridisation oven. (Scot-lab). The filter was then washed sequentially twice in 2xSSC and 0.1% SDS for 30 min, twice in 0.2x SSC and 0.1% SDS for 60 min at 65°C. The membrane was dried between 2 sheets of 3 mm Whatman filter paper for 10 min at room temperature and exposed to X-ray film for the period of 1-3 days depending on the signal.

Chapter 3

The induction of inducible NO synthase by IFN-γ and LPS in macrophages

3.1 Introduction

Inducible NOS is expressed in many cell types after challenge with immunologic or inflammatory stimuli and thereupon generates large amounts of NO over periods up to several days (Vodovotz,Y. 1994). The list of agents known to induce iNOS expression is extensive. IFN-γ and LPS are the only stimuli, and macrophages of the mouse are the only cells for which transcriptional induction of iNOS has been formally documented (Cho 1992).

Although the regulation of iNOS in macrophages and their cell lines has been studied in some detail, the intracellular signal transduction pathways involved in the induction of iNOS are still unclear. As a first approach to the better understanding of the intracellular signalling pathways involved, the kinetics of iNOS induced by IFN-γ or /and LPS in a murine macrophage cell line, J774, were examined in this chapter.

3.2 NO production is induced by IFN-γ and LPS in J774 cells

Firstly, the time course of iNOS induction was examined: J774 cells were incubated with IFN-γ and LPS for 2 to 48 h and iNOS enzyme activity analysed (using a dual-wavelength method in the presence of oxyhaemoglobin, L-arginine and NADPH). NOS activity was detectable within 4 h following treatment with IFN-γ and LPS, rose dramatically between 6 to 8 h, and reached peak activity at 10 h to the level of about 300 pmoles NO formed /min /mg protein. NOS enzyme activity reached a plateau between 10 to 18 h after stimulation, and declined thereafter, such that NO was undetectable at 48 h in most of experiments (Fig. 3.1).

As a result of iNOS activation, nitrite (NO_2^-) significantly accumulated in the culture supernatants from 8 h after stimulation (Fig. 3.2).

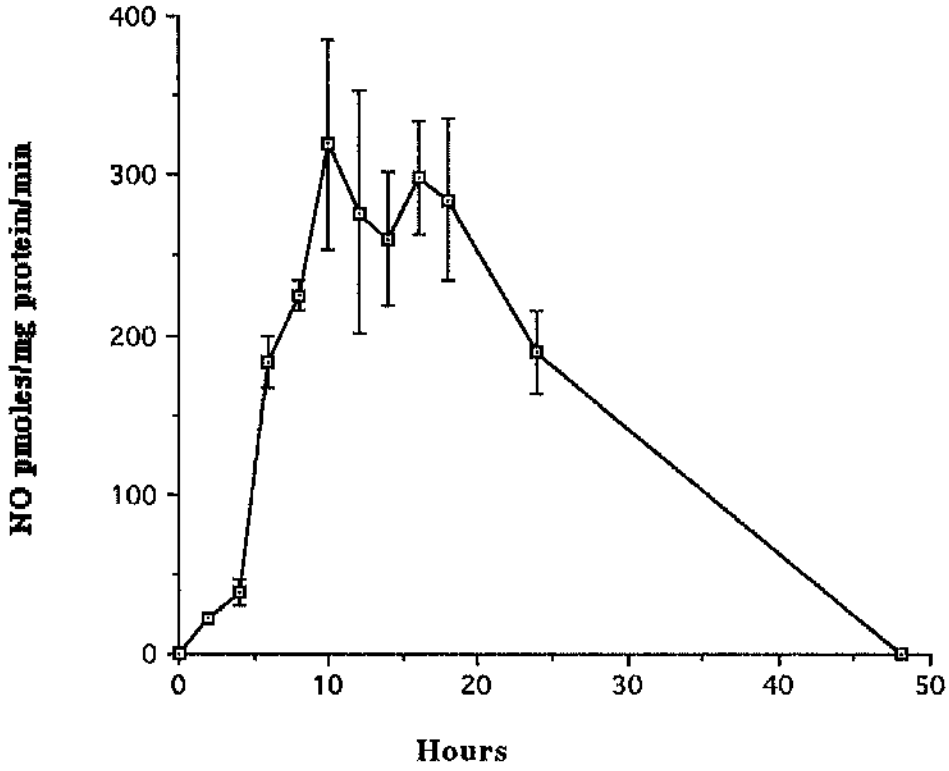


Fig. 3.1 IFN-γ and LPS iNOS activity in J774 cells. J774 cells were stimulated with IFN-γ (100U/ml) plus LPS (10ng/ml) for the indicated period. Cells were lysed and NOS activity was measured using a dualwavelenth method in Chapter 2 (section 2.5). Data are presented as means of triplicates (±SD). Results are representative of three experiments.

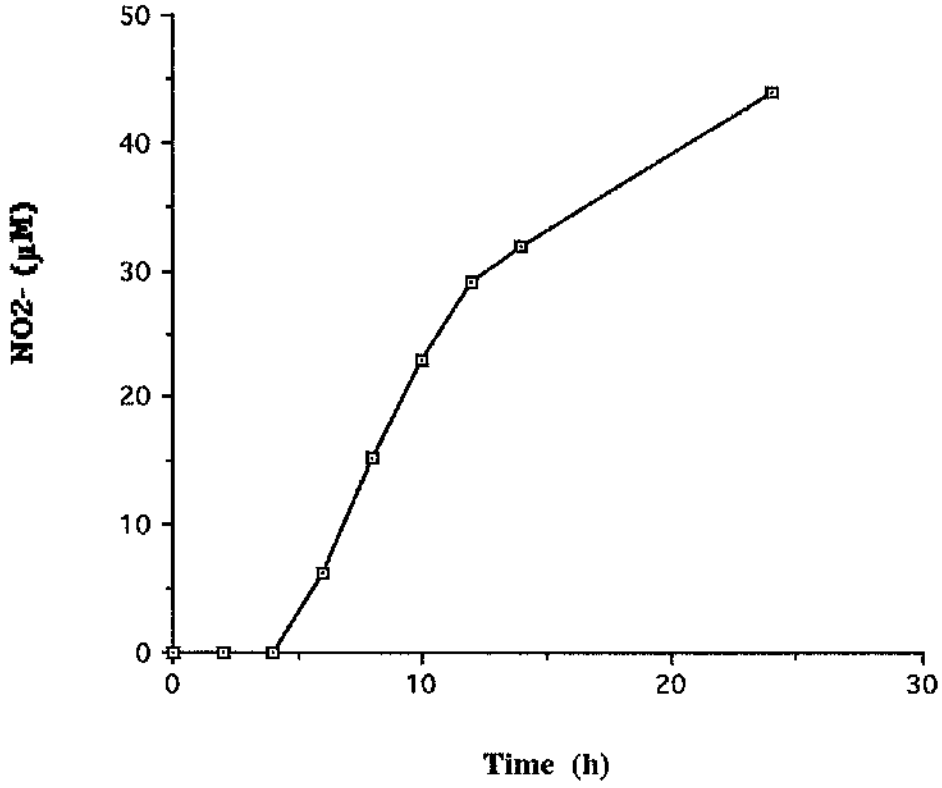


Fig. 3.2 NO_2^- in culture supernatants. J774 cells were cultured in DMEM complete medium overnight, and IFN-γ (100U/ml) and LPS (10ng/ml) were added. The culture supernatants were collected at indicated time points. NO_2^- was measured by Griess reaction.

3.3 Induction of iNOS activity is protein synthesis dependent

The previous enzyme assays showed that NOS activation in J774 cells occurred 3 to 4 h after stimulation, indicating that a protein synthesis-dependent mechanism may be involved. Cycloheximide, a protein synthesis inhibitor, was used to show that protein synthesis is important for NO production. IFN-γ and LPS-induced NO was completely blocked by pre-incubating cells with cycloheximide for 4 h. If cycloheximide was washed away at the end of the 4 h incubation period and the cells incubated in fresh medium, the ability to produce NO was recovered (Fig. 3.3), to a lower level that than in fresh cells (This may be due to the presence of trace amounts cycloheximide in the cells).

To confirm that the expression of iNOS in macrophages is inducible by IFN-γ and LPS, iNOS expression in cultures of J774 cells was examined. Northern blots containing total RNA from IFN-γ and LPS-treated cells were hybridised with ^{32}P labelled probe corresponding to the unique segment of murine iNOS cDNA. As shown in Fig. 3.4, mRNA encoded by the iNOS gene was induced following treatment of cells with IFN-γ and LPS. iNOS mRNA was not detected in cells incubated with medium alone, but was detectable from 2 h after stimulation, reached a peak level at 4 h, and then declined by 6 h.

In order to determine whether the induction of iNOS mRNA reflects changes in the level of the corresponding proteins, Western blots were prepared using extracts from normal or primed J774 cells, and identified using a monoclonal anti-murine macrophage iNOS antibody. iNOS proteins were present in populations of cells stimulated with IFN-γ and LPS but not untreated cells. Paralleling enzyme activity, iNOS protein was detectable from 4 h after treatment and peaked at 8 h, returning to basal level by 24 h (Fig. 3.5).

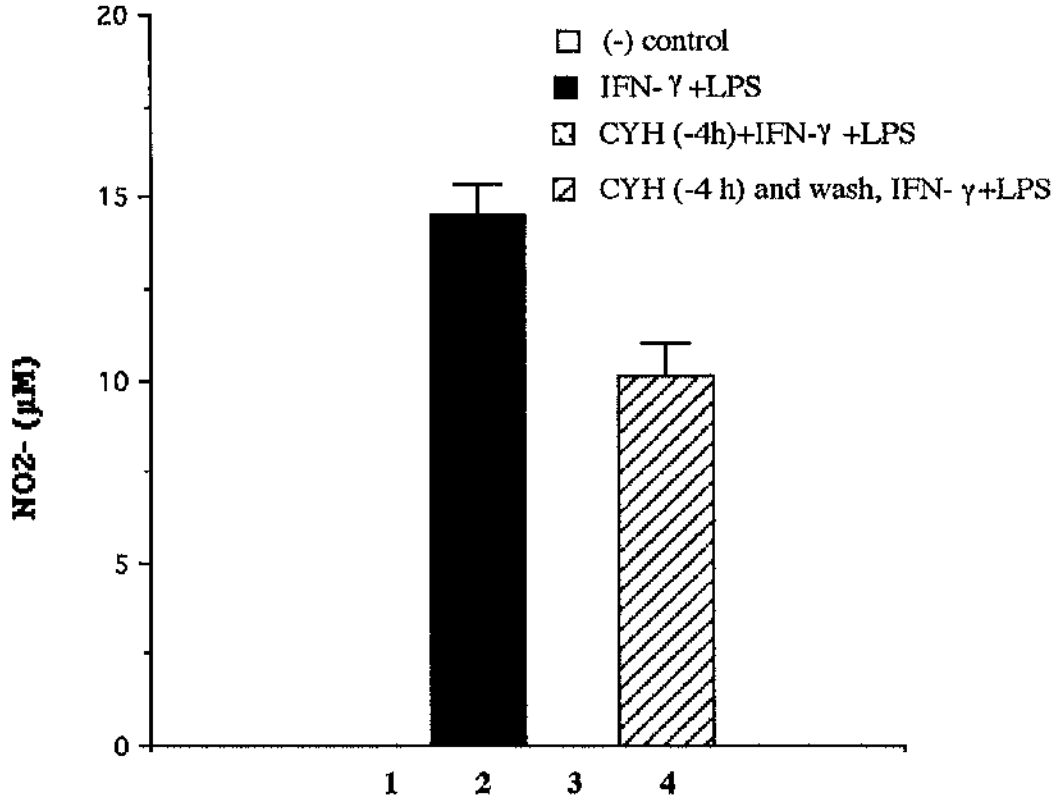


Fig.3.3 iNOS activity is protein synthesis-dependent. J774 cells were cultured at 10^5 cells/well in 96-well plate in the presence (column 1 and 2) or absence (column 3 and 4) of cycloheximide (30 μg/ml) for 4 h, then either washed (column 4) or unwashed (column 3) with warmed fresh medium followed by addition of IFN-γ (100 U/ml) and LPS (10 ng/ml) (column 2, 3, and 4) or medium alone (column 1). NO_2^- accumulated in culture supernatants was measured at 16 h by Griess Reaction. For column 1 and 3, NO_2^- levels were zero. Data are shown as the means of triplicates (±SD). Results are representative of two individual experiments.

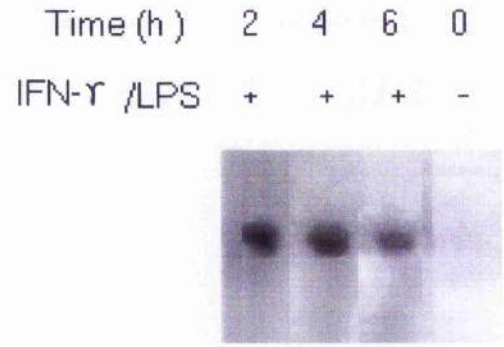


Fig. 3.4 Time course of the induction of iNOS mRNA in J774 cells. J774 cells were treated with IFN-γ and LPS for indicated times, and total RNA prepared as described in section 2.20. Northern blot analyses were conducted using 10 μg of RNA per sample and probed with ^{32}P-labeled iNOS cDNA. Results are representative of two individual experiments (This photo was cut from the same film of that in chapter 6 Fig. 6.5).

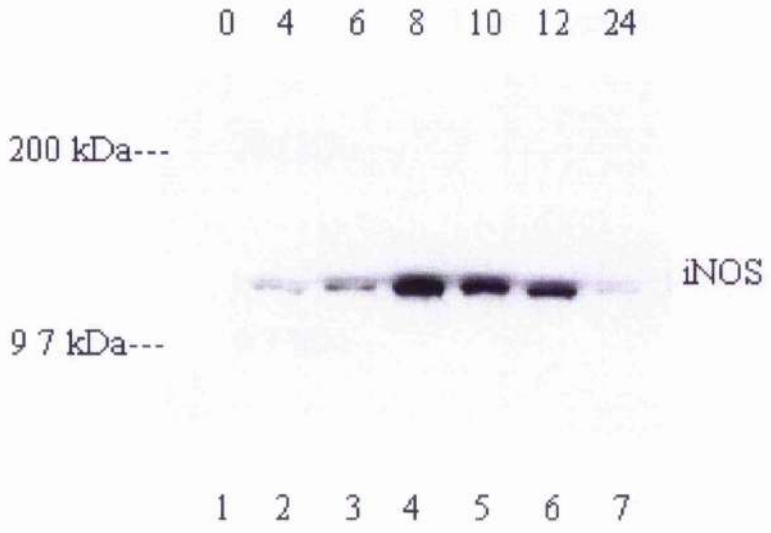


Fig. 3.5 Time course of induction of iNOS in J774 cells stimulated with IFN-γ and LPS. J774 cells were stimulated with IFN-γ (100 U/ml) and LPS (10 ng/ml) for indicated times (h) and cellular proteins extracted and 20 μg of each sample was resolved on a 7.5 % SDS PAGE gel, followed by immunoblotting. INOS was detected by a monoclonal antibody against macrophage NOS. ECL reagents were used for visualising protein bands. The molecular weight of iNOS protein is ~130kDa.

3.4 LPS synergizes with IFN-γ to induce NO synthase

To determine the relative contribution of IFN-γ and LPS to the induction of NOS activity, J774 cells were treated with IFN-γ and LPS separately at various concentrations. IFN-γ was used at a range of 1 to 100 U/ml. Cells were cultured at a density of 10^6 cells /ml, 200μl/well, and stimulated with IFN-γ for 24 h. IFN-γ has the ability to induce NO in J774 cells (4 μM) at concentrations as low as 1 U/ml, and NO production reaches a maximum level of about 10 μM from 10 U/ml concentrations of IFN-γ or upwards. When LPS (10 ng/ml) was added to the system, there was a strong synergistic effect for NO production, with the maximum level (~ 25 μM) achieved at 1 U/ml of IFN-γ (Fig. 3.6).

The ability of LPS alone to induce NO synthase in J774 cells was also investigated. Culture supernatants from J774 cells, treated with a range of LPS concentrations, was collected. LPS was capable of inducing NO (15 μM) in J774 cells at a concentration 1 ng/ml (15 μM). Maximum levels of NO (25 μM) being stimulated with 40 ng/ml of LPS. NO levels were significantly elevated by the presence of IFN-γ (10 U/ml), under these conditions NO_2^- was detectable in cultures with only 0.1ng/ml of LPS (22 μM) and the maximum level of NO production was increased to 40 μM (Fig. 3.7).

3.5 Discussion

Macrophages have many functions in the body. They scavenge dead and dying cells, form part of innate host resistance to infection by engulfing and killing pathgens. They can present antigens to T cells, and serve as effector cells in cell-mediated immune reactions, especially those directed at intracellular pathogens such as *Mycobacteria* and *Leishmania*. Macrophages exerts their functions, in large part,by a pathway dependent on production of nitric oxide (NO) (Nathan 1992). NO is an effector molecule used by macrophages for host protection against pathogens. Since the activation of NO is essentially not target specific, it is important that the synthesis of NO be tightly regulated.

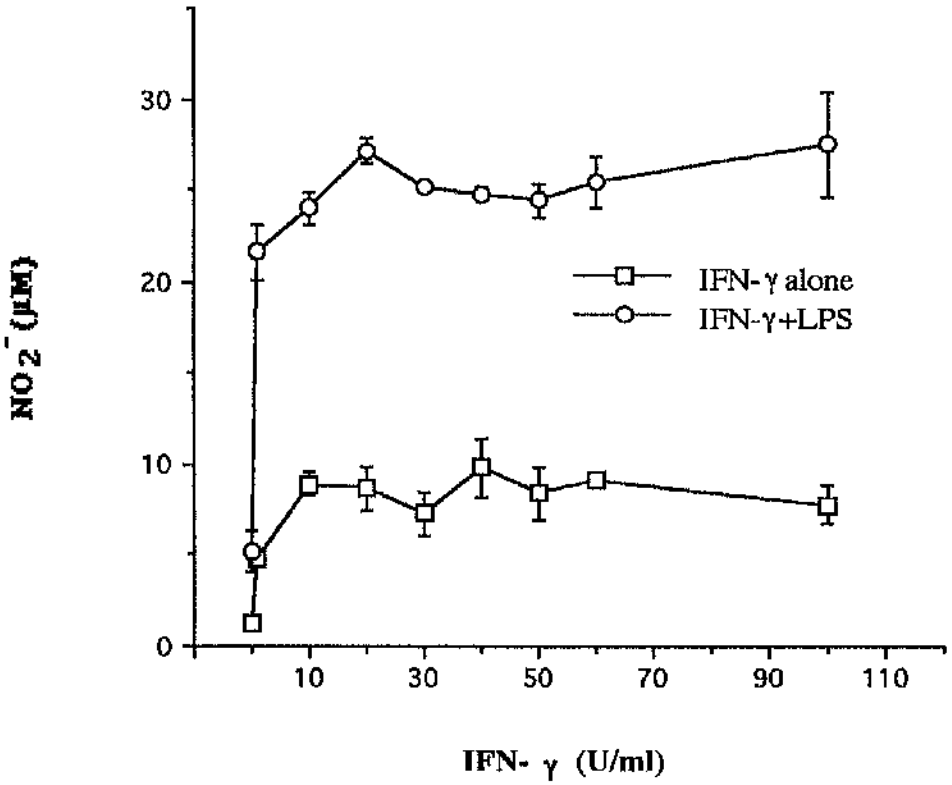


Fig. 3.6 IFN-γ-induced NO synthesis is dose-dependent. J774 cells were cultured in a 96-well plate and stimulated with IFN-γ at the indicated concentrations with or without LPS (10 ng/ml). Culture supernatants were collected at 24 h and assayed for NO_2^- using the Griess method. Data are presented as means of triplicate cultures (±SD).

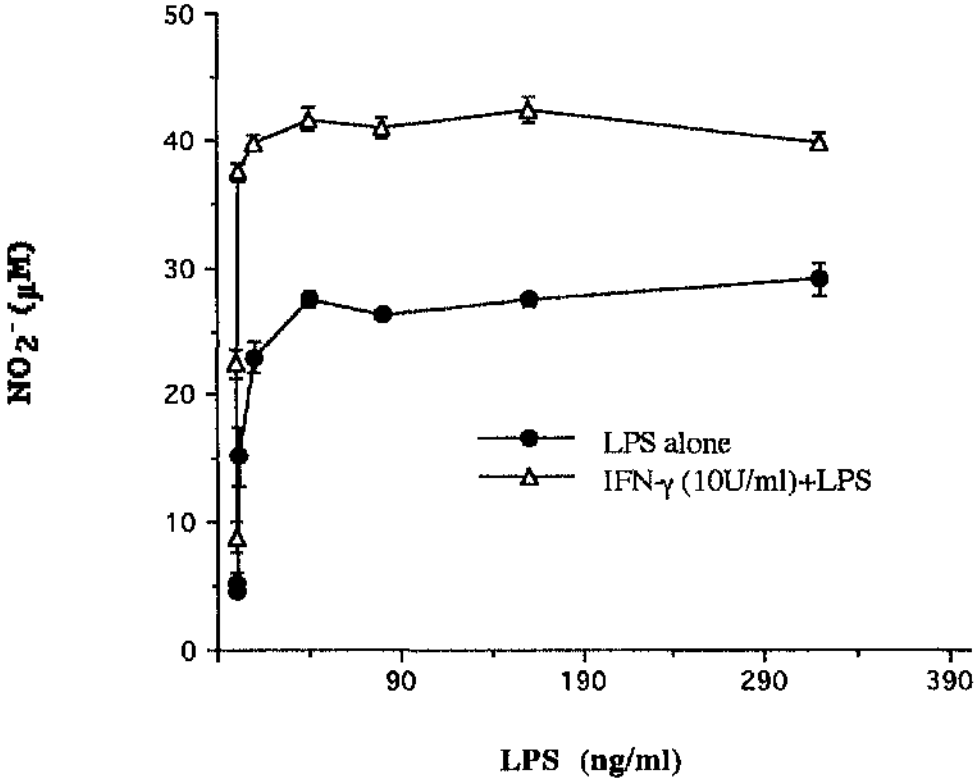


Fig. 3.7. LPS-induced NO synthesis is dose-dependent. J774 cells were cultured in 96-well plates and stimulated with indicated concentrations of LPS, with or without IFN-γ (10 U/ml). At the end of 24h incubation, culture supernatants were assayed for NO_2^- using the Griess reaction. Data shown are the means of triplicates (±SD).

IFN-γ is a lymphokine produced by activated T cells, and it exhibits a wide variety of biological effects. LPS is a potent macrophage activator (Adams and Hamilton 1984; Hauschilft *et al.*, 1990). In the experiments presented here, J774 cells produce large amount of NO in response to IFN-γ and LPS in a dose-dependent manner which is similar to that previously observed with peritoneal macrophages. When peritoneal macrophages were activated by IFN-γ alone, they made a small amount of NO_2^- in 24h (Vodovotz *et al.*, 1994), and NO_2^- production largely plateaus within 1 to 3 days *in vitro*. However, peritoneal macrophages stimulated by the combination of IFN-γ plus LPS, produced significantly high amounts of NO, which ceased after 24 h in the cultures (Vodovotz *et al.*, 1994). In J774 cells, the NO response is relatively fast, the maximum activity of NOS being reached between 8 to 18 h after stimulation. The inducible NOS is regulated transcriptionally in a manner that requires protein synthesis. These results reflect the natural response which proceeds in primary cultured murine macrophages except that the time-course in J774 cells is faster (Bogdan *et al.*, 1994). For example, upon stimulation, either IFN-γ or LPS alone is sufficient for both peritoneal macrophages and J774 cells to produce NO. Moreover, NO production is synergistically enhanced by the combination of these two stimuli. However, in some cases, J774 cells produces more NO then peritoneal macrophages in responses to IFN-γ or LPS alone. This may due to the fact that J774 cell, as a cell line, is in a primed state, while primary cultured cells are akin to resting cells.

The results presented in this chapter confirm and extend previous reports that murine macrophages are capable of producing NO in response to IFN-γ and LPS, and this induction is affected by IL-4 (Bogdan *et al.*, 1994): IFN-γ and LPS are potent inducers of iNOS, and LPS and IFN-γ act synergistically in this system. This data also established the suitability of J774 cells as a model system for investgation of signal regulation of inducible NOS. This well-established homogeneous cell line provides a convenient way to investigate the molecular mechanisms involved the signalling pathways

of iNOS induction. J774 cells were therefore used for the studies of iNOS signal transduction presented in subsequent chapters.

Chapter 4

The role of JAK1, JAK2 and STAT1 in the induction of NO synthase by IFN-γ

4.1 Introduction

Cytokines are secreted proteins that regulate many aspects of cellular growth, differentiation, activation, and effector function, and play an important role in immune and inflammatory responses. The induction of iNOS is a result of macrophage activation by IFN-γ and/or LPS. Previous studies have shown that tyrosine phosphorylation is involved in the macrophage respiratory burst induced by LPS (Boulet, *et al.*, 1992), and the tyrosine phosphatase/kinase signaling cascade is involved in the IFN-γ pathway (Igarashi *et al.*, 1993). Furthermore, the expression of iNOS mRNA is blocked by genistein, a tyrosine kinase inhibitor. In recent years, through the study of transcriptional activation in response to interferons, a previously unrecognized direct signal transduction pathway to the nucleus has been uncovered: IFN-receptor interaction at the cell surface leads to the activation of kinases of the JAK family that then phosphorylate substrate proteins, STATs. In this chapter, the involvement of tyrosine kinases in the regulation of iNOS induction was investigated.

4.2 The induction of iNOS was inhibited by tyrosine kinase inhibitors

To determine whether tyrosine kinase activation is required for the induction of iNOS by IFN-γ and LPS, a number of tyrosine kinase inhibitors were used. As assayed by NOS activity, following treatment of IFN-γ (100 U/ml) and LPS (10 ng/ml), NO produced in cell lysates was ~120 pmoles /min /mg protein at 16 h after stimulation. When the cells were pre-treated with Tyrphostin 25, one of the tyrosine kinase inhibitors, iNOS activity induced by IFN-γ and LPS was partially blocked. At the concentration of 15 μM, Tyrphostin 25 reduced iNOS activity by 50% compared with cells treated with IFN-γ and LPS alone. Tyrphostin 1, an inert analogue of tyrosine kinase inhibitor, had no effect when used at the same or even higher concentrations (Fig. 4.1). Similar results were obtained from experiments with Herbimycin A, another tyrosine kinase inhibitor (Fig. 4.2).

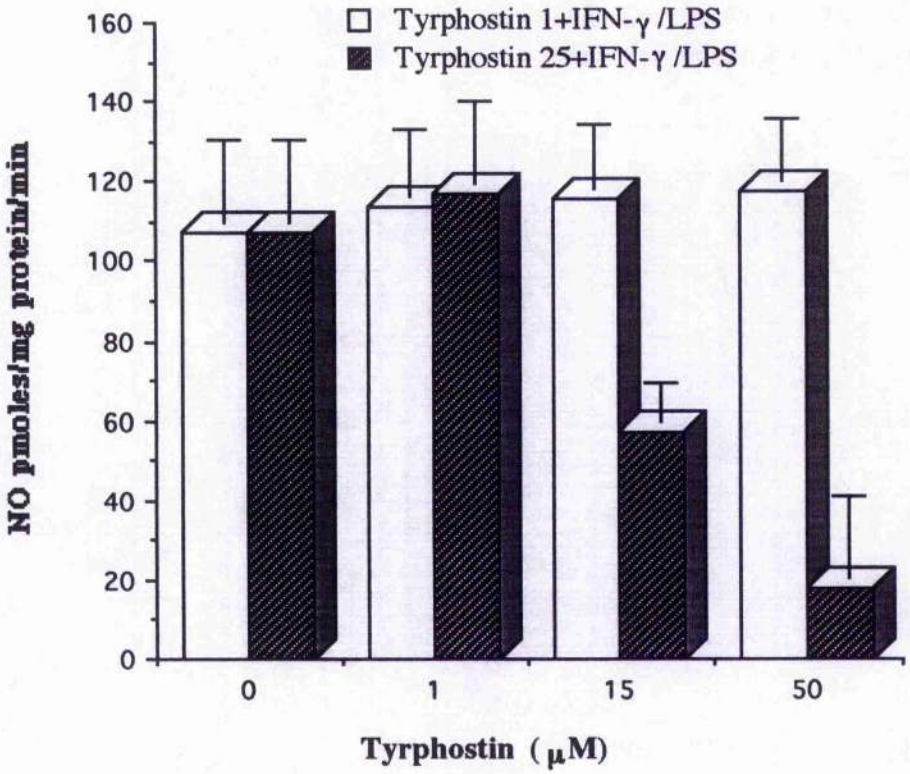


Fig. 4.1 Effect of Tyrphostin 1 and Tyrphostin 25 on iNOS acivity. J774 cells were cultured in 25 cm^2 flasks. Tyrophostins were added 4 h prior to IFN-γ (100 U/ml) and LPS (10ng/ml) stimulation. Cells were harvested at 16 h after stimulation, total cellular proteins were extracted, and iNOS activity measured using a dual-wavelenth method. Tyrphostin 1 is an inactivated protein tyrosine kinase inhibitor which was used as a negative control. Data are presented as means of triplicates (±SD). The results are representative of two experiments.

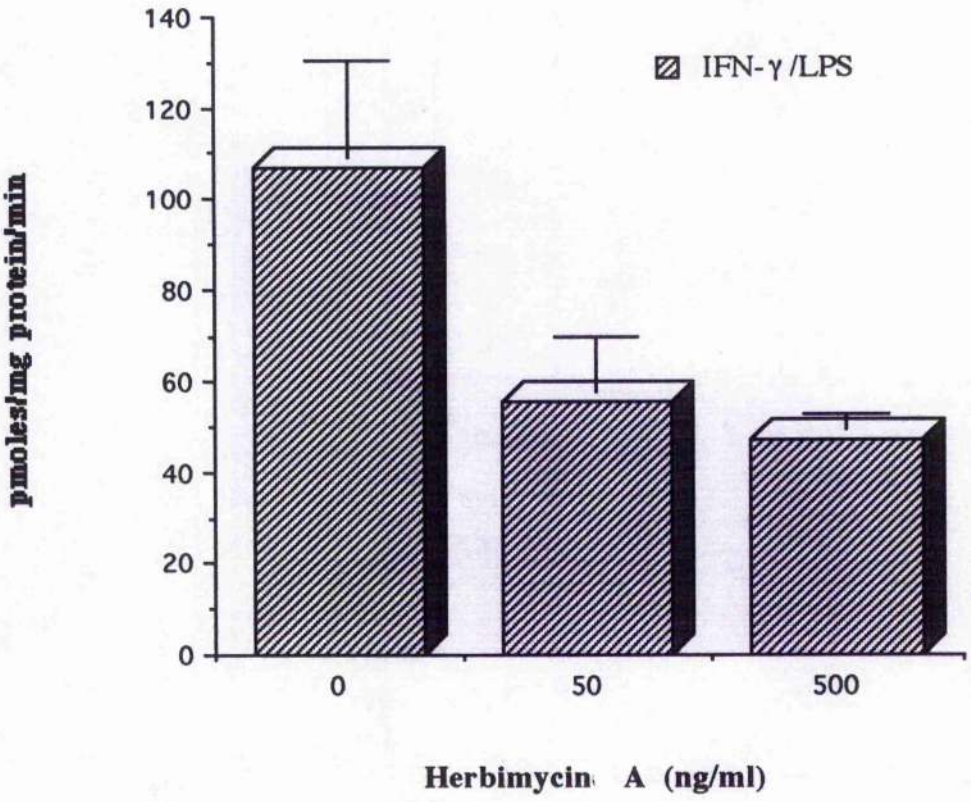


Fig. 4.2 Effect of Herbimycin A on iNOS activation. J774 cells were cultured in 25 cm^2 flasks. Herbimycin A was added 4 h prior to IFN-γ (100 U/ml) and LPS (10 ng/ml) stimulation. Cells were harvested at 16 h after stimulation. Total proteins were extracted, and iNOS activity measured using a dual-wavelenth method. Data are presented as means of triplicates (±SD) and results are representative of two experiments.

To address if induction of transcription of iNOS activity is interfered by protein tyrosine kinase inhibitors, iNOS expression was examined by Western blot using a monoclonal anti-murine macrophage iNOS antibody. As shown in Fig. 4.3, iNOS protein was undetectable in untreated cells while it was strongly expressed in IFN-γ and LPS-stimulated cells. When pre-treated with Tyrphostin 25 or Tyrphostin AG126 for 4 h, the iNOS protein produced in J774 cells was markedly reduced (Fig. 4.3). The reduction of iNOS expression correlated with the reduced generation of NO_2^- in the culture supernatants as measured by the Griess reactions (Fig. 4.4)

4.3 iNOS activity is upregulated by a tyrosine phosphatase inhibitor

Protein phosphorylation is reversibly controlled not only by protein kinases (PKs) but also by protein phosphatases (PPs). The extent of phosphorylation at a particular site can be regulated by changing the activity of the cognate PK or PP or both. The steady-state level of phosphotyrosine on cellular proteins is the result of opposing activities of protein tyrosine kinases and protein tyrosine phosphatases.

Vanadate has been shown in numerous studies to exert inhibitory actions on cellular protein tyrosine phosphatases (PTPases). The effect of vanadate on iNOS induction was examined by measuring iNOS activity. Vanadate was used in combination with IFN-γ and LPS. Upon the stimulation of IFN-γ and LPS, iNOS activity was markedly higher in vanadate-treated cells compared to untreated cells by 40% at a concentration of 20 μM vanadate (Fig. 4.5). This result indicates that one or more tyrosine phosphatase(s), acting as a negative regulating factor, is involved in iNOS induction or iNOS activation.

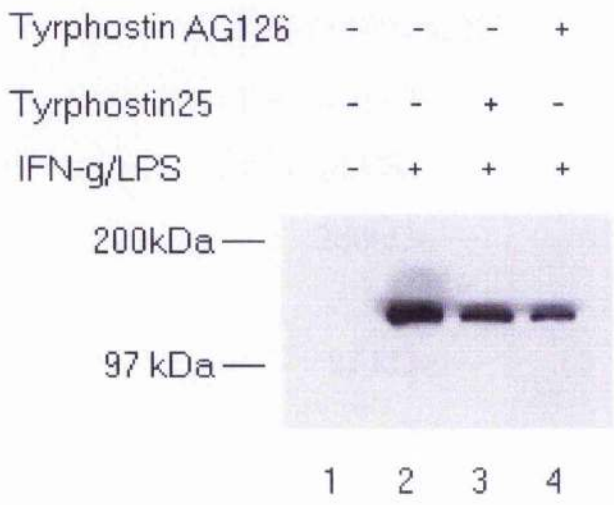


Fig. 4.3 Inhibition of iNOS expression by protein tyrosine kinase inhibitors (Tyrphostin 25 and AG126). J774 cells were cultured in 25 cm^2 flasks and incubated with tyrphostin 25 (20 μM) or AG126 (40 μM) for 4 h before stimulation with IFN-γ (100 U/ml) and LPS (10 ng/ml). Non-adherent cells were removed before cells were lysed 16 h after stimulation. Total proteins (20 μg each) were resolved by 7.5% SDS PAGE, followed by immunoblotting using an antibody against murine macrophage iNOS and visualised by ECL reagents.

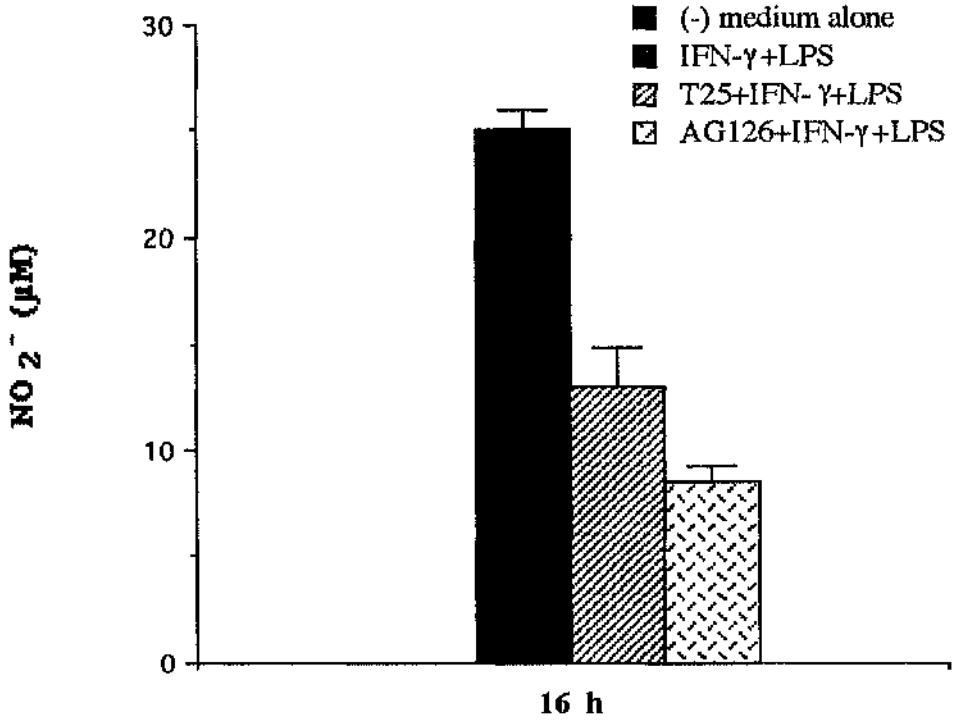


Fig.4.4 Effect of protein tyrosine kinase inhibitors on NO production. J774 cells were cultured in a 96-well plate and incubated with Tyrphostin 25 (20 μM) or Tyrphostin AG126 (40 μM) for 4 h before stimulation with IFN-γ (100 U/ml) and LPS (10 ng/ml). Culture supernatants were collected 24 h later, and assayed for NO_2^- using the Griess reaction. Data shown are means of triplicates (±SD). The results are representative of two experiments.

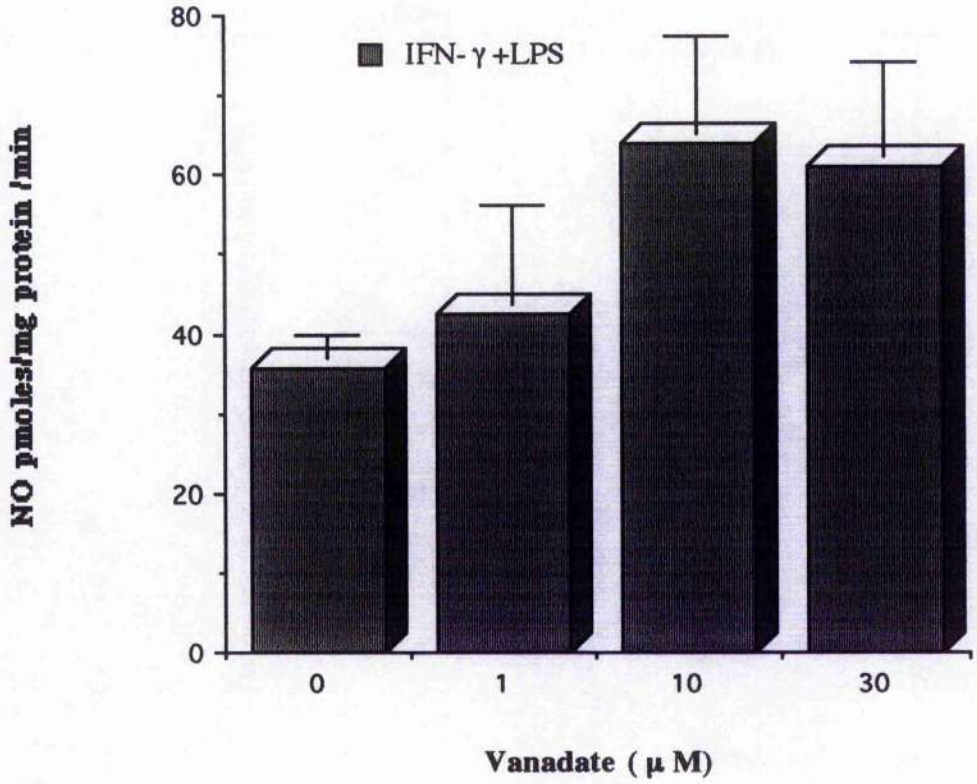


Fig. 4.5 Effect of a protein tyrosine phosphatase inhibitor, vanadate, on iNOS activity. J774 cells were cultured in 25cm^2 flasks. Sodium vanadate was added simultaneously with IFN-γ (100 U/ml) and LPS (10 ng/ml) stimulation. Cells were harvested at 16 h after stimulation, total proteins extracted, and iNOS enzyme activity measured using a dual-wavelenth method. Data are presented as means of triplicates (±SD).

4.4 JAK1 is activated in response to IFN-γ

To determine whether a PTK pathway could be activated by IFN-γ, cell lysates from IFN-γ and/or LPS-stimulated J774 cells were prepared and analysed by immunoblotting with the antibodies against protein tyrosine kinases, the JAK kinases.

Whole cell lysates were immunoprecipitated by an anti-JAK1 antibody followed by immunoblotting using an anti-phosphotyrosine mAb. JAK1 was tyrosine phosphorylated and hence presumably activated, in response to IFN-γ and LPS. The appearance of tyrosine-phosphorylated JAK1 in J774 cells over a time course stimulation is shown in Fig. 4.6. JAK1 phosphorylation was absent in the lysates of the untreated cells, while its phosphorylation was detected within 30 seconds of treatment with IFN-γ. The response was immediate and transient with a peak at 1 h. Re-blotting, using anti-JAK1, indicated that the amount of JAK1 protein expression did not change within this 2 h activation period. Further experiments indicated that JAK1 phosphorylation was induced by IFN-γ but not LPS. LPS had no effect on JAK1 tyrosine phosphorylation induced by IFN-γ (data not shown).

4.5 JAK2 is also activated in response to IFN-γ

Typically, cell stimulation by a particular cytokine results in the activation of two of the four known JAK kinases. Similarly to JAK1, JAK2 kinase was tested for its activation in response to IFN-γ and LPS as detected by tyrosine phosphorylation. Again, JAK2 was tyrosine phosphorylated upon stimulation with IFN-γ (Fig. 4.7) with an identical response to that of JAK1 in terms of the time course of activation in J774 cells: JAK2 tyrosine phosphorylation was detectable within 30 seconds of IFN-γ-treatment and the maximum tyrosine phosphorylation was at 1h and declined thereafter. LPS, again had no effect on JAK2 activation, neither by itself nor by the combination with IFN-γ within the period of 2 h (data not shown).

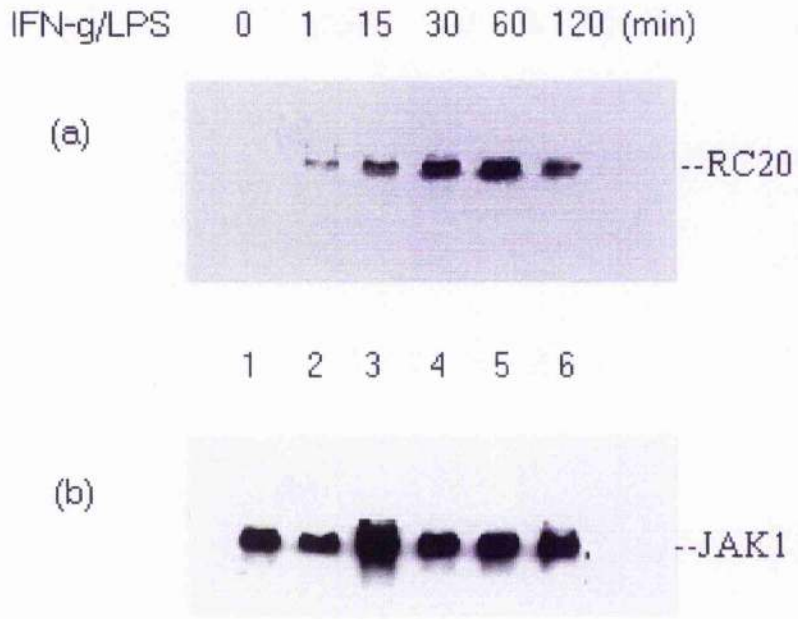


Fig. 4.6 Tyrosine phosphorylation of JAK1 in response to IFN-γ and LPS. IFN-γ and LPS-induced tyrosine phosphorylation of JAK 1 was assayed in whole cell extracts of J774 cells. IFN-γ (100 U/ml) and LPS (10 ng/ml) were added for 30 seconds to 2 h. Extracts were immunoprecipitated with polyclonal antisera to JAK1 and probed after SDS-PAGE (7.5%) analysis with (a) anti-phosphotyrosine (RC20) and, (b) after stripping, with antibody to JAK1. Data are representative of three similar experiments.

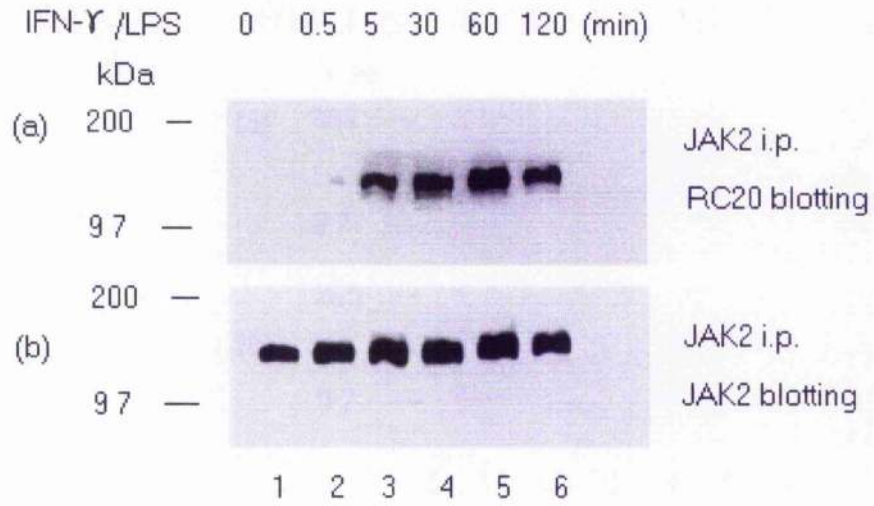


Fig. 4.7 Time course of JAK2 tyrosine phosphorylation is similar to that of JAK1. J774 cells were cultured in 80 cm^2 flasks and stimulated with IFN-γ (100 U/ml) and LPS (10 ng/ml) for 30 seconds to 2 h. JAK2 was immunoprecipitated from each of the indicated samples and probed, after SDS-PAGE (7.5%), with (a) an anti-phosphotyrosine antibody (RC20), and (b) after stripping, with antibody to JAK2. Data are representative of three experiments.

Tyrphostin AG490 has recently been shown to be a JAK2-selective inhibitor (Meydan *et al*, 1996). AG490 was therefore used to determine whether JAK1 or JAK2 activation is associated with iNOS induction. J774 cells were pre-treated with AG490 for 4 h before being incubated with IFN-γ and/or LPS for 24 h. NO_2^- accumulated in the culture supernatants was measured and the results show in Fig. 4.8. IFN-γ (100 U/ml) induced significant levels of NO (8 μM in 24 h). This was completely abolished by the pre-treatment with AG490 at concentration of 10 μM. NO synthesis induced by LPS as well as by IFN-γ plus LPS was also inhibited by pre-treatment of AG490 but this required higher concentrations of AG490. This probably simply reflects a quantitative difference, because significantly higher concentrations of NO were induced by the costimulation. These results demonstrated that JAK2 is involved in the induction of iNOS and that it is particularly important for NO induced by IFN-γ alone.

4.6 STAT1 (p91/84) is phosphorylated in response to IFN-γ

JAK kinase activation is likely to occur via transphosphorylation by receptor-associated kinases brought into close proximity by binding of ligand. After activation, receptor-associated PTKs can phosphorylate several substrates critical for signal transduction. One important component of cytokine signalling is the specific transcriptional activation of target genes, which is rapid and does not require the synthesis of new proteins. This led to the identification and characterisation of the JAK-signal transducer and activator of transcription (STAT) signalling pathways.

To determine which STAT protein is activated following the activation of JAK1 and JAK2 in J774 cells, immunoblotting was carried out in total cell lysates using an anti-phosphotyrosine antibody (RC20). As shown in Fig 4.9, stimulation of J774 cells by IFN-γ induced an increase in the tyrosine phosphorylation of a wide range of proteins including those in the 90-95 kDa molecular mass region. The enhanced tyrosine phosphorylation was observed as early as 1 min after IFN-γ stimulation. The

phosphorylation band became stronger within 2 h in a time dependent manner. A possible candidate for the 90-95 kDa protein is p91-STAT1.

Signal transducers and activators of transcription (STATs) were first identified as a unique family of DNA-binding proteins approximately five years ago. STAT1 (p91) is the one of first two identified STATs protein. It has been shown that STAT1 and STAT2, in response to IFNα/IFN-β, is rapidly tyrosine phosphorylated and forms a DNA complex with a DNA-binding protein p48, which binds an IFN-stimulated response element (ISRE). To confirm that P91 is phosphorylated in response to IFN-γ or LPS, a polyclonal anti-P91/84 antibody was used for immunoblotting in total cell lysates. p91 appeared as a single band in untreated cells which was converted to two bands after the stimulation with IFN-γ. The inducible slower migrating band is characteristic of phosphorylated p91. In J774 cells, there exists a naturally occurring splice variant, STAT1β–p84. p84 lacks the carboxyl 38 amino acids of STAT1. Similarly, p84 was phosphorylated in response to IFN-γ. LPS had no activation nor synergistic effect on P91 phosphorylation (Fig. 4.10).

4.7 MAP kinase is tyrosine phosphorylated in response to LPS

Although I have detected no effect of LPS on JAK1/JAK2 activation, tyrosine kinases also play a role in LPS signalling: experiments using an anti-phosphotyrosine antibody (RC-20) showed that treatment of IFN-γ and LPS, lead to an increase in tyrosine phosphorylation of a number of protein bands (Fig. 4.9 and Fig. 4.11). If the cells were pre-treated with Tyrphostin 25 (20 μM) for 4 h, phosphorylation of the bands was reduced. A major group of bands were in the range 80-95 kDa and could be related to P91/84 (Fig. 4.9 and Fig. 4.10). A second group of proteins of molecular weight 40-45 kDa were tyrosine phosphorylated and it was postulated that these could be MAPK (Fig. 4. 11).

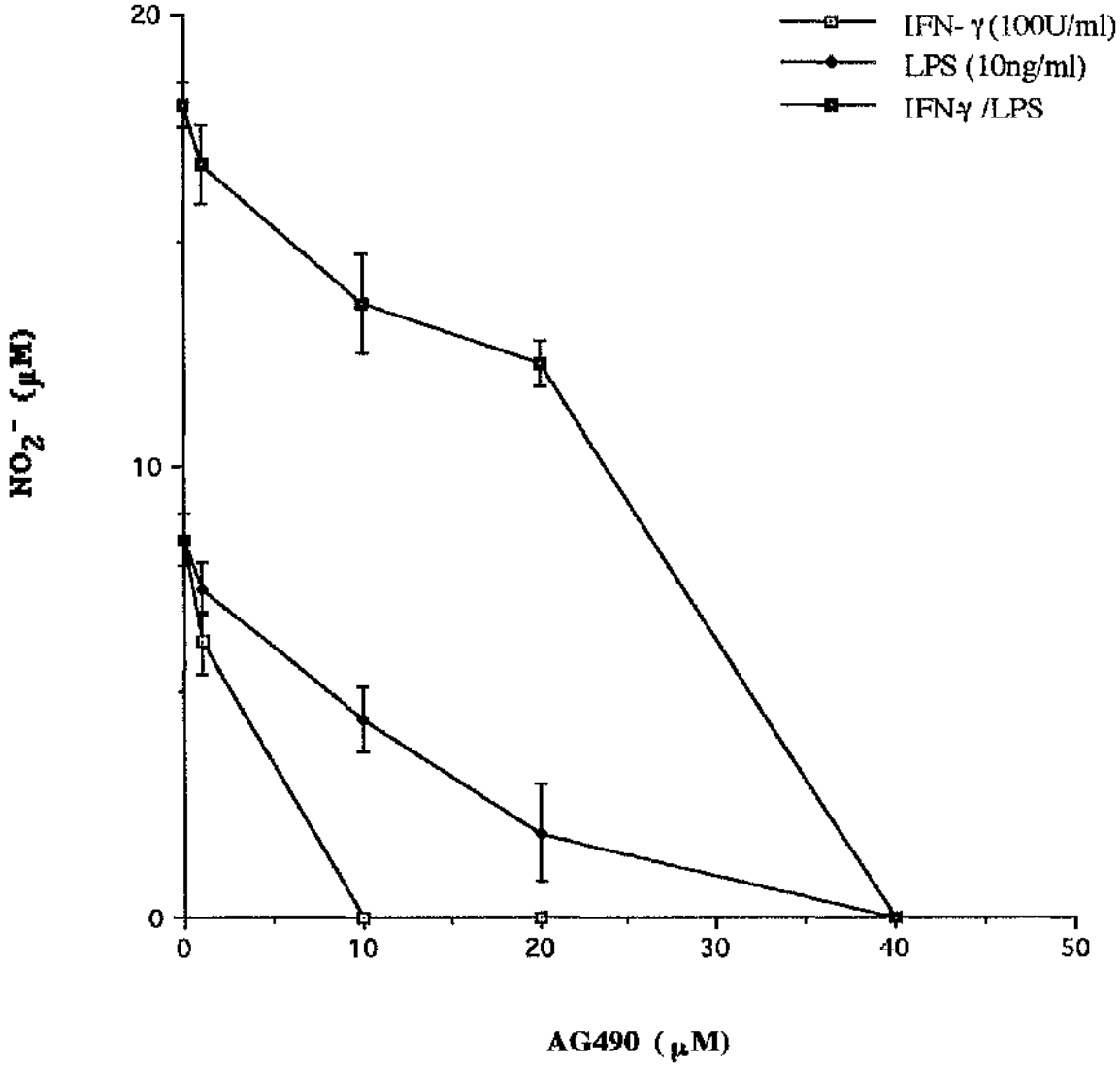


Fig.4.8 The involvement of JAK2 in the induction of iNOS. Tyrphostin AG490, a specific inhibitor to JAK2 was added 4 h before the addition of IFN-γ (100 U/ml) and LPS (10 ng/ml). NO_2^- in the culture supernatants was collected at 16 h and measured using the Griess Reaction. Data are shown as the mean values (±SD) obtained from triplicated samples.

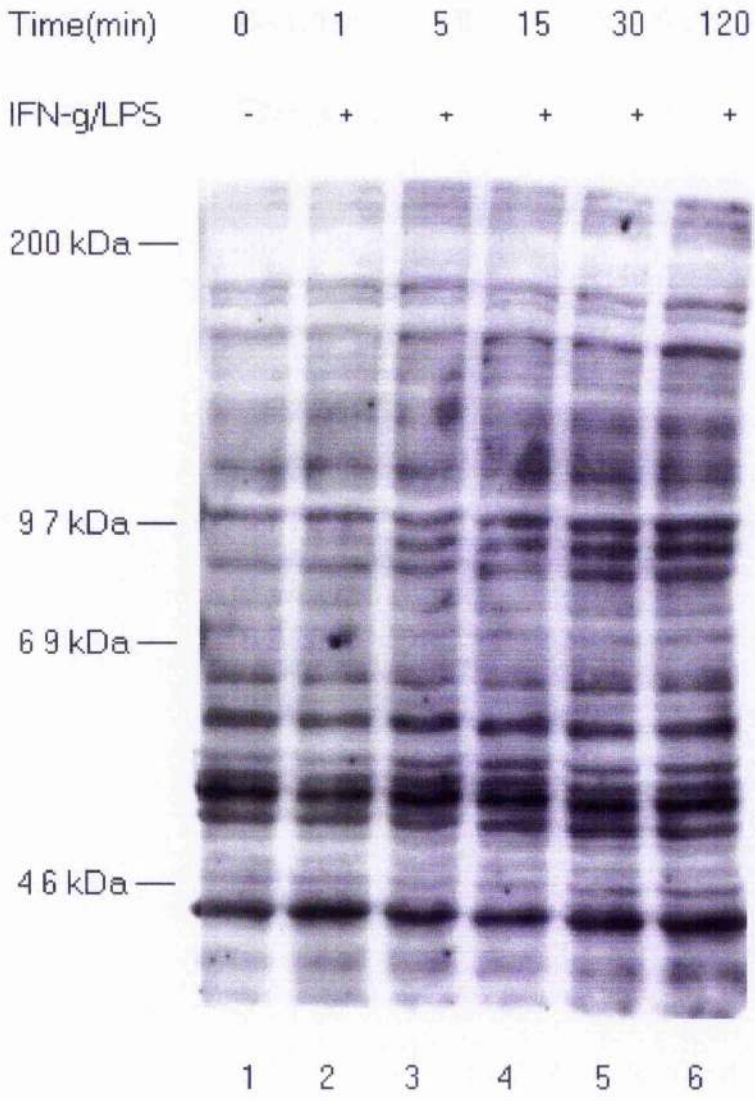


Fig. 4.9 Tyrosine phosphorylation pattern induced by IFN-γ plus LPS in J774 cells. Cells were cultured in 25cm^2 flasks and incubated with IFN-γ (100 U/ml) and LPS (10 ng/ml) for 1 to 120 min. Total lysates (30 μg) were loaded onto a 7.5 % SDS PAGE gel and followed by immunoblotting, using anti-phosphotyrosine antibody (RC20). The lower molecular-weight (less than 45 kDa) proteins could not be seen clearly.

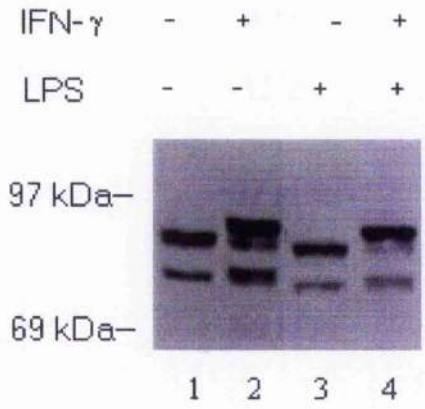


Fig. 4.10 STAT1 is phosphorylated in response to IFN-γ but not to LPS. J774 cells were cultured in 25 cm^2 flasks and incubated with IFN-γ (100 U/ml) and/or LPS (10 ng/ml) for 30 min. Total proteins were extracted and 20 μg of each were resolved on 7.5% SDS PAGE gel, and transferred to nitrocellulose membrane followed by probing with a polyclonal anti-STAT1 antibody. The antibody recognised both P91 and P84 since they are two similar products from the same gene. The bands (from top to bottom) are phosphorylated P91, P91, phosphorylated P84, and P84.

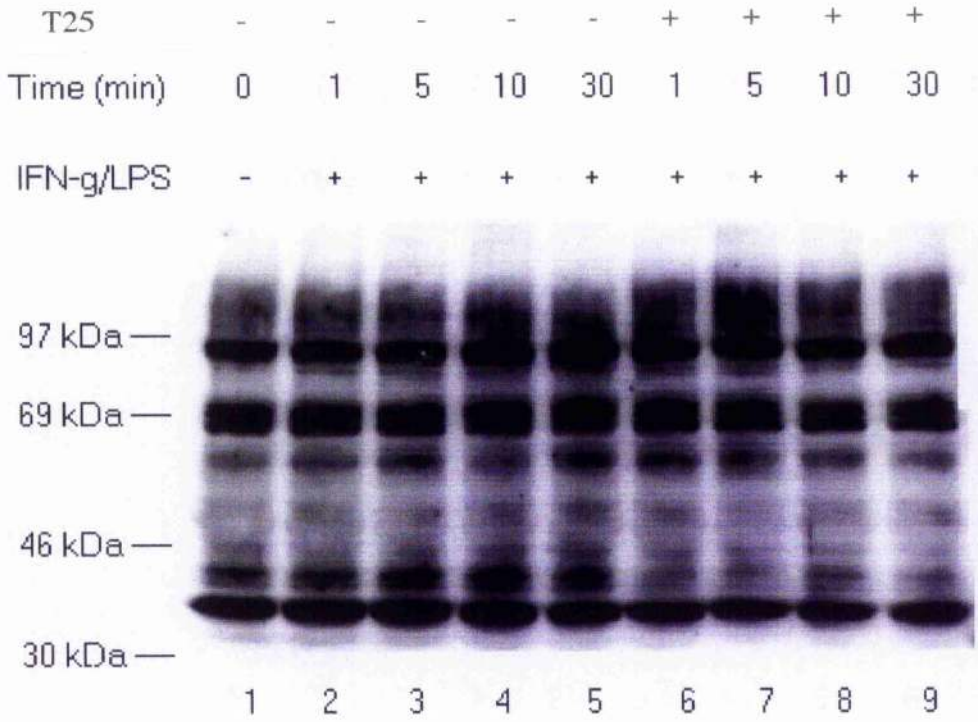


Fig. 4.11 IFN-γ and LPS-induced tyrosine phosphorylation was decreased by pre-treatment with Tyrphostin 25. J774 cells were pre-incubated with Tyrphostin 25 (20 μM) or medium alone for 4 h prior to addition of IFN-γ (100 U/ml) and LPS (10 ng/ml) for the indicated periods. Total lysates (20 μg each) were subjected to a 5-10% gradient SDS PAGE gel followed by Western blotting, and probed with RC20 (anti-phosphotyrosine antibody).

Although there were some indications in either Fig. 4.9 and Fig. 4.11 that a 40-45 kDa protein was tyrosine phosphorylated after treatment with IFN-γ and LPS, the bands were not clear in either of these figures. It probably due to the low gel concentrations. To emphasis the lower molecular proteins, a 10% SDS PAGE gel was used and western blotting was carried out using an antibody (PY54) recognising proteins that phosphorylated on tyrosine residues. Basically, PY54 and RC-20 recognise the same protein, but RC20 was conjugated with horseradish peroxidase whereas PY54 was not. As shown in Fig. 4.12a , although the phosphorylated 40-45 kDa band was induced by LPS but not by IFN-γ, IFN-γ enhanced such protein phosphorylation induced by LPS under these condition. Re-blotting was carried out to determine whether these proteins were MAP kinase, using a monoclonal anti-MAP kinase (ERK2) antibody. The result was shown in Fig. 4.12b. The band detected by MAP kinase antibody was located at exactly the same molecular weight with the phosphorylated band in Fig 4.12a.

So far, the data suggested that MAP kinase (ERK2) is tyrosine phosphorylated in response to LPS treatment. Although MAP kinase tyrosine phosphorylation in LPS treated J774 cells clearly occurred, the characteristic shifting of the band to a higher molecular weight (mobility shift) was not observed (data not shown). Since I did not test whether MAPK was threonine phosphorylated in this system, it is not clear that whether the mobility shift reflect threonine phosphorylation of the protein. However, there are some evidence indicate the possible role of MAP kinase in NO induction in J774 cells. Tyrphostin AG126 is a tyrosine kinase inhibitor which inhibits MAP kinase activity and iNOS induction. In these experiments, AG126 inhibits both iNOS protein expression (see Fig. 4.2) and NO_2^- accumulation (Fig. 4.13). After pre-incubation with Tyrphostin AG126 for 4 h at the concentration of 1-100 μM, NO_2^- produced by J774 cells was reduced markedly in a dose-dependent manner (Fig. 4.13).

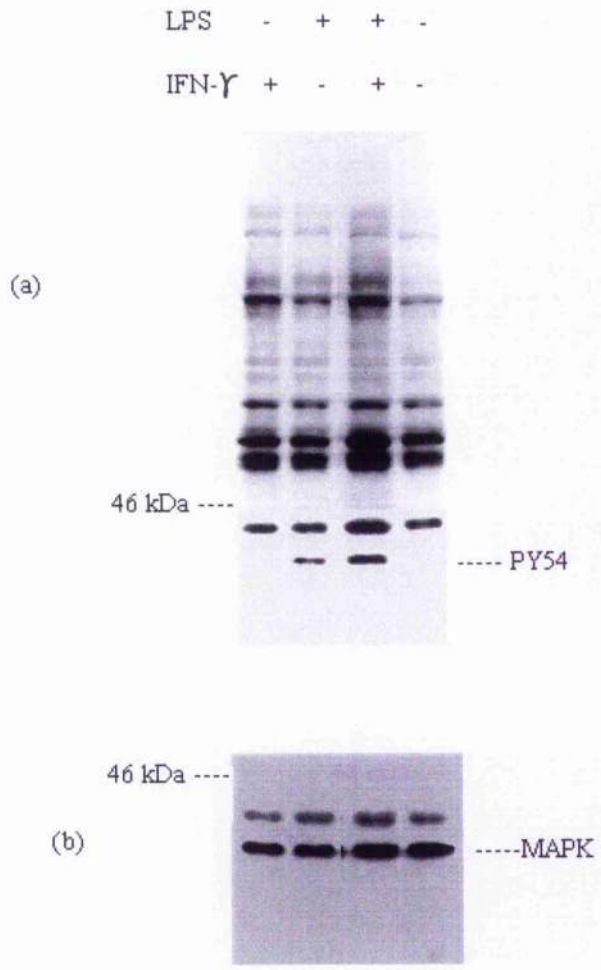


Fig. 4.12 Tyrosine phosphorylation induced by LPS. J774 cells were incubated with LPS (10 ng/ml) and/or IFN-γ (100 U/ml) for 30 min. before lysis. Total ltsates (20 μg each) were resolved by 10% SDS PAGE gel and subjected to Western blotting using anti-phosphotyrosine (PY54) (a). Following stripping, the blot was re-probed with anti-MAP kinase (erk2) antibody (b). Arrows indicate the same position of the blot.

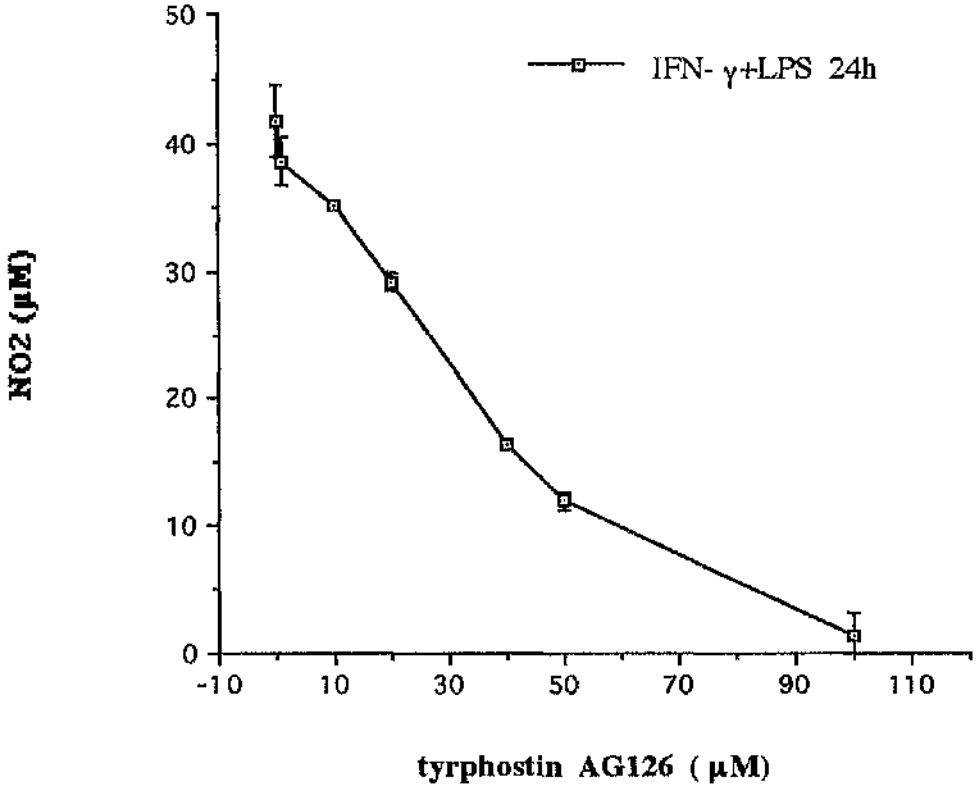


Fig. 4.13. Effect of tyrphostin AG126 on NO_2^- production. Tyrphostin AG126 was added 3 h before the addition of IFN-γ (100 U/ml) and LPS (10 ng/ml). The culture supernatants were collected at 24 h and NO_2^- measured using the Griess Reaction. Data are shown as the mean values (±SD) obtained from triplicated samples. The results are representative of two experiments.

4.8 Discussion

Selective inhibitors are often used to detect the possible involvement of signalling elements such as protein kinases in biological responses. When Tyrphostins, protein tyrosine kinase inhibitors, were introduced to J774 cells, the NOS activity was markedly inhibited and NO_2^- accumulation in the culture supernatants was reduced. NO_2^- measurements also indicated that tyrosine phosphorylation is required for IFN-γ and LPS-induced NO synthesis.

JAK1 or JAK2 have been shown to transduce IFN-γ signalling. JAK1 or JAK2 mutant cell lines have proved that these kinases have the ability to either auto- or cross-phosphorylate each other and any defect in either of these kinases will influence the other (Mathias *et al*, 1993). Although it is difficult to place JAK1 or JAK2 upstream or downstream of each other as yet, a numbers of studies consistent with the requirement for the presence of both JAK family proteins (JAK1 and JAK2) for correct IFN-γ receptor assembly. Both JAK1 and JAK2 are approximately 130 kDa in molecular weight and they are tyrosine phosphorylated in response to IFN-γ in J774 cells. To identify the tyrosine kinases that might be involved in the iNOS signalling pathway, Tyrphostin AG490, a specific JAK2 inhibitor was used to probe for JAK2 involvement. NO_2^- assay result shows that NO-produced in the cells treated with IFN-γ and LPS was abolished by the pre-treatment of AG490. When used alone, IFN-γ and LPS showed a similar ability to stimulate J774 cells to produce NO: however, although AG490 showed a strong inhibition on IFN-γ–induced NO, LPS-induced NO was less affected. This suggested that JAK2 activation in response to IFN-γ play a crucial role in induction of NO synthesis by IFN-γ alone. For the effect of AG490 on LPS-induced NO, there are two explanations. It may due to non-specific inhibition of other kinases, since it was only effective when higher concentrations were used. The other possibility is that there may be some LPS-regulating gene products were involved in LPS-induced NO synthesis which transduce signals through JAK2 activation. Anyway, The results presented here

demonstrated that the induction of NO synthase in J774 cells by IFN-γ involved the phosphorylation of JAK1/JAK2.

Since there is a rapid explosion of knowledge about JAK-STAT pathway in recently years, STAT proteins are getting extensive attentions for its activation and transcriptional functions in cytokine receptor signallings. STAT1 proteins, in particular, have been shown to be phosphorylated on tyrosine in response to IFN-γ (Shuai *et al*, 1992) in fibroblasts. STAT1 exists two isoforms in the cells: p91 and p84. P91 and P84 were originally found as components of interferon-stimulated gene factor 3 (ISGF3), a transcription complex activated by IFN-α/β (Fu, *et al.*, 1992; Gutch *et al.*, 1992; Schindler *et al.*, 1992). The P91 and P84 components of ISGF3 result from alternative splicing and differ only in a 38 amino-acid extension at the carboxyl terminus of P91. In the present study, both p91 and p84 were shown to be tyrosine phosphorylated (Fig. 4. 10) and the phosphorylation of STAT1 which translocates to the nucleus (will be shown in chapter Fig. 6. 18) where the binding of STAT1 to the GAS element will lead to the activation of iNOS promoter in response to IFN-γ in J774 cells. The experiments regard nuclear factors and GAS binding will be investigated in the study represented in the next chapter.

Furthermore, although there is not enough data at present to conclude a link between MAP kinase and iNOS induction, there are some possible indications. The sequence -Pro-Met-Ser-, which resembles the consensus sequence for MAP kinase substrate -Pro-X-Ser(Thr)-Pro- (Alvarez *et al.*, 1991), is present in the conserved region in the COOH-terminus of STAT1, STAT3, and STAT4. Thus, it is possible that the Jak-STAT pathway may be coupled functionally to and modulated by the MAP-kinase pathway (Zhang *et al.*, 1995). Tyrphostin AG126 is a tyrosine kinase inhibitor which inhibits MAP kinase activity and as well as iNOS induction. In these experiments, AG126 inhibits both iNOS protein expression and NO_2^- accumulation, but there is little or no effect of AG126 0n p91 nuclear translocation (data not shown). It is possible that MAP kinase (if participating in iNOS induction) may act through a different signal pathway.

Chapter 5

iNOS gene regulations

5.1 Introduction

Although abundant evidence indicates that nitric oxide (NO) which is generated by macrophage NO synthase (iNOS) mediates the ability of macrophages to kill or inhibit the growth of tumor cells, bacteria, and parasites (Lowenstein *et al.*, 1992; Moncada *et al.*, 1991), the expression of NOS must be tightly controlled because NO is potentially capable of indiscriminately injuring host tissue. The expression of iNOS is regulated largely at the transcriptional level (Xie *et al.*, 1992). The murine iNOS promoter has been cloned and characterised (Lowenstein *et al.*, 1993; Xie *et al.*, 1993) and shown to contain multiple positive and negative regulatory elements capable of responding to numerous transcription factors.

In the promoter region of the iNOS gene, two areas are required for maximal induction of its transcription (Lowenstein *et al.*, 1993). Both of region I and II are necessary for LPS-activated expression while region II mediates IFN-γ regulation. Region I and II contain potential binding sites for numerous transcription factors. Region I contains more LPS-related response elements, including a particularly striking array of binding sites for the transcription factors octamer, NF-IL6, NFκB, and TNF-REs. In contrast, IFN-responsive elements are concentrated in region II, including an ISRE and a PU-box/IFN element or PIE. Both regions I and II contain potential binding sites for NFκB. NFκB is a well characterised transcription factor that is important in inflammatory responses and is thought also to be involved in the expression of several virus and many inducible cellular genes that encode cytokines, immunoregulatory receptors, and acute phase proteins (Lenardo and Baltimore, 1989). NFκB protein is a heterodimer consisting of two proteins, p50 (also designated NFκB1) and p65 (also designated Rel A). p50 and p65 are members of the Rel/NFκB family of proteins. These proteins serve as inducible eukaryotic transcription factors that form various homo- and heterodimers. In mammalian cells activity of NFκB is regulated by its association with an inhibitory subunit, IκB, which retains the inactive factor in the cytoplasm. NFκB is activated by many agents including LPS. The NFκB-type proteins provide a case in which regulated factors either

activate or repress transcription in response to signals. Regulation of mammalian NFκB is not well understood. However, *in vitro*, rapid phosphorylation and subsequent degradation of IκB by various kinases leads to the loss of its ability to inhibit DNA binding by NFκB (Beg and Baldwin, 1993, Chen *et al.*, 1995)

In the iNOS promoter region which is necessary for IFN-γ activation, the complementary nucleotide sequence of the ISRE core closely matches a consensus sequence termed IFN-regulatory factor element (IRF-E) (Martin *et al.*, 1994; Tanaka *et al.*, 1993). IRF-1 is an IRF-E binding protein (Lorsbach et al., 1993). IRF-1 is activated by viral infection, double-stranded RNA, IFN-α, and IFN-γ (Fuijita *et al.*, 1989; Miyamoto *et al.*,1988), and serves as a transcriptional activator for type I IFN genes as well as for a number of IFN-inducible genes (Reis *et al.*, 1992; Pine 1992). However, all these genes are also regulated in part if not predominatly by other transcription factors, such as interferon-stimulated gene factor 3 (ISGF3) and gamma-activating factor (GAF).

To activate or repress transcription, transcription factors must be located in the nucleus, bind DNA, and interact with the basal transcription apparatus. Many DNA-binding proteins bind DNA as oligomers, and signals can therefore regulate DNA binding by affecting factor oligomerization as well as protein-DNA interaction itself. Evidence for the role of transcription factors in the regulation of target genes is usually obtained either by the analysis of the binding of nuclear proteins to regulatory DNA sequences or on the basis of an intracellular activation of transfected constructs containing gene fragments linked to reporter genes. In this chapter, I will concentrate on transcriptional responses to IFN-γ and /or LPS-activated signalling pathway.

5.2 IFNs regulatory factor (IRF-1) is activated by IFN-γ or LPS

Interferon regulatory factor-1 (IRF-1) is a transcription factor which binds to IFN gene regulatory elements (IRF-E) (Miyamoto *et al.*, 1988; Levy *et al.*, 1988; Porter *et al.*, 1988; Rutherford *et al.*, 1988). IRF-1 plays a critical and essential role in the induction of

iNOS which is transcriptionally regulated by IFN-γ (Kamijio *et al.*, 1994; Martin *et al.*, 1994).

An IFN-γ-responsive element in the IRF-1 gene has been reported (Sims *et al.*, 1993) composing a IFN-γ activation sequence (GAS) element. Thus a oligonucleotide corresponding to GAS within the promoter of the IRF-1 gene was used to detect IFN-γ or LPS-induced DNA-protein binding activities, as defined by electrophoretic mobility shift assay (EMSA). The complexes induced in the nuclear extracts from the cells treated with IFN-γ and LPS are shown in Fig. 5.1a. When the GAS specific oligonucleotide (5'-TACAACAGCCTGATTTCCCCGAATGACGGC-3') was used as a probe, both extracts prepared from cells treated with IFN-γ and LPS showed GAS-binding activities by different transcription complexes. Further experiments demonstrated that the IFN-γ-induced GAS binding activity contains a tyrosine-phosphorylated p91 protein, since both anti-p91 and anti-phosphotyrosine (PY54) antibodies recognised this complex either by blocking (in the case of PY54) or supershifting (anti-p91) the GAS binding induced by IFN-γ (Fig. 5.1b). LPS, however, induced two binding complexes. At least the top band contained NFκB (P65) since an antibody against NFκB (p65) recognised this complex (Fig. 5.1c). It was interesting that NFκB protein may involved in GAS binding activity. Futher experiments were carried out to identify the protein in the LPS-induced GAS binding complex. As expected, when a cold GAS probe was used as a competitor, it abolished all the GAS-binding activites either by IFN-γ or LPS. In contrast, when a NFκB site cold probe was used as a competitor, the two GAS-binding bands induced by LPS were abolished but not that induced by IFN-γ (Fig. 5.1d). It was expected that STAT1, as a transcription factor, binds to the GAS element in IRF-1 promoter in response to IFN-γ, but it was surprising that NFκB P65 (formed a heterdimer with P50) was found in the GAS-protein complex. It is possible that IRF-1 could also be upregulated by LPS alone. This possibility will be discussed later.

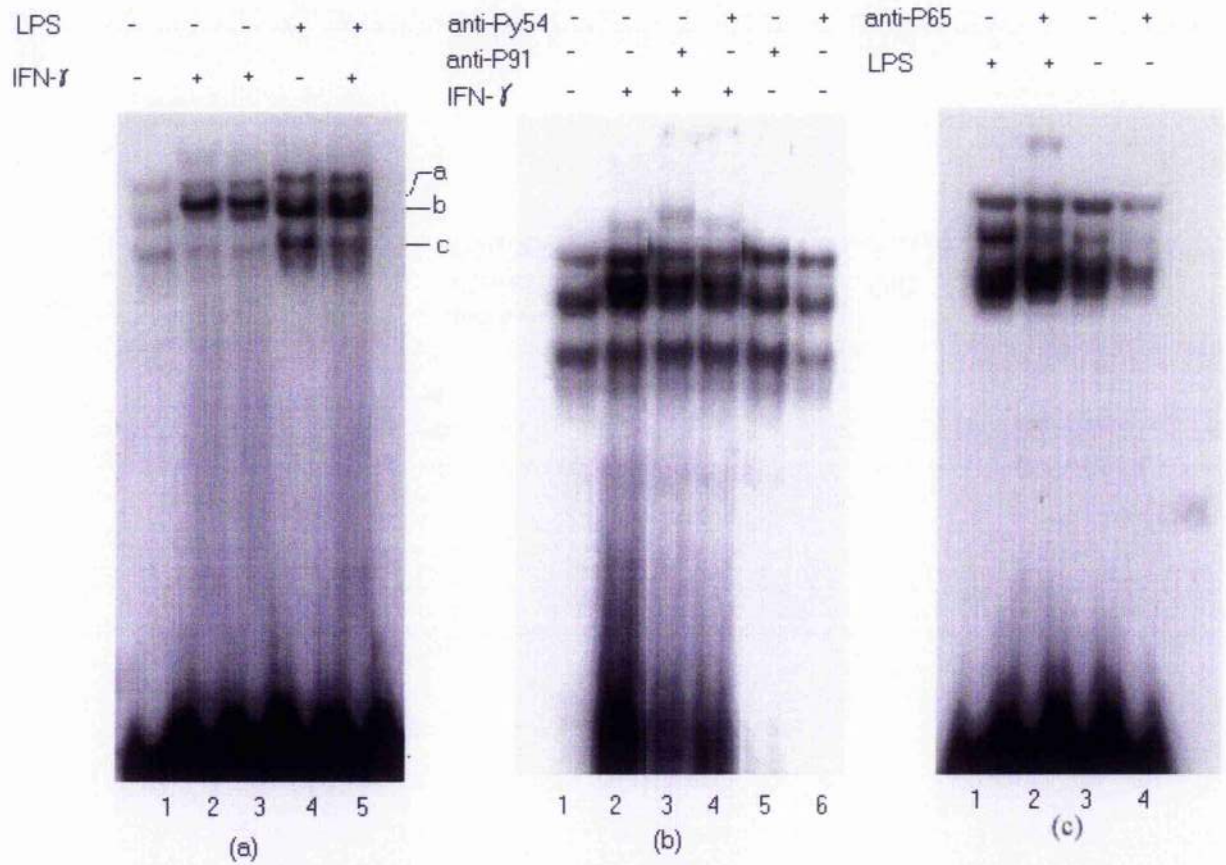


Fig. 5.1 GAS binding activity induced by IFN-γ or LPS. J774 cells were incubated with IFN-γ (100 U/ml) or /and LPS (10 ng/ml) for 1 h, and nuclear proteins were extracted. EMSA was carried out by 30 min incubation of 5 μg of nuclear protein with a ^{32}P-labeled oligonucleotide probe F (see materials and methods) containning the GAS sequence (5'-TACAACAGCCTGATTTCCCCGAATGACGGC-3') found in IRF-1 promoter, an activator of iNOS (a). For the supershift assay, antibodies were added into the reaction mixture 20 min before adding the probe (b,c).

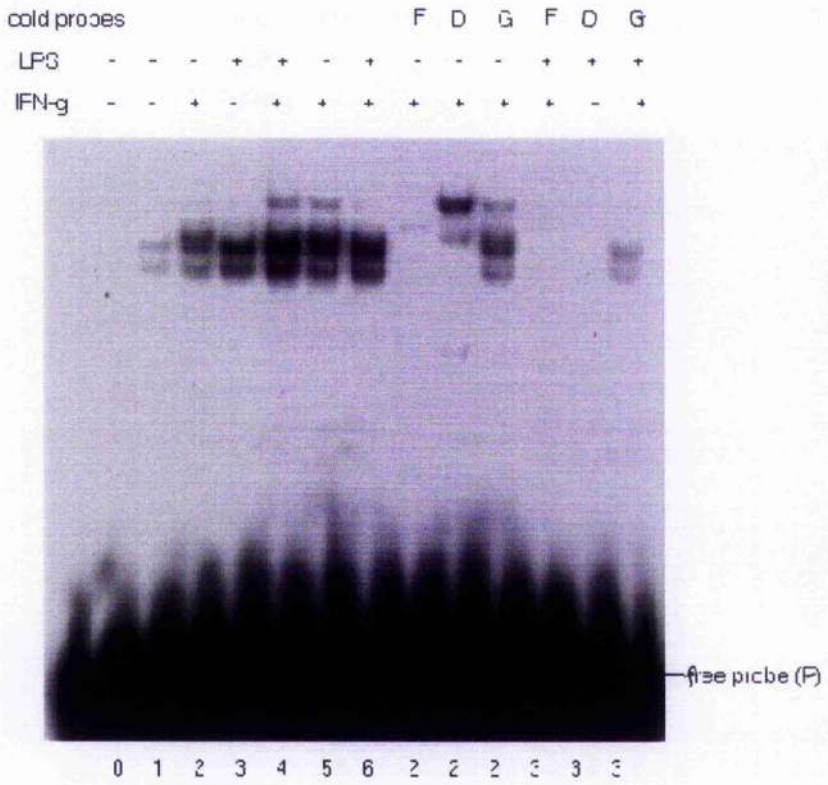


Fig. 5.1d Identification of GAS-binding protein induced by LPS by means of competition assays. J774 cells were incubated with IFN-γ (100 U/ml) and/or LPS (10 ng/ml) for 1 h, and nuclear proteins were extracted. EMSA was carried out by 30 min incubation of 5 μg of nuclear proteins with a ^{32}P-labelled oligonucleotide probe F containing the GAS sequence found in IRF-1 promoter, an activator of iNOS. For, competition assays, cold probes were added into the reaction mixture 20 min before adding the probe. D is the probe containing NFkB consensus in iNOS promoter; G is the probe containing IRF-E consensus in iNOS promoter.

Following DNA-protein interaction, activation of transcription was examined. IRF-1 expression was measured by Northern blot analysis of total RNA extracted from IFN-γ and/or LPS-treated cells. A PCR primer for IRF-1, designed by Dr. X.Q. Wei (Department of Immunology, University of Glasgow) according to the DATA BASE of GENE BANK, was used for amplifying cDNA of IRF-1. PCR cDNA products were cloned into TA-vectors followed by purification and sequencing. The cDNA fragments with the correct sequence were then labelled with [α^{32}P]-dATP and used as probes to detect mRNA of IRF-1.

Northern blot analysis performed on total RNA of J774 cells with the IRF-1 cDNA probe showed that IRF-1 expression was induced when cells were treated with IFN-γ at 100 U/ml but was undetectable in cells incubated with medium alone. IRF-1 was detectable 1 h after stimulated with IFN-γ and reached maximum levels within 3-4 h and declining thereafter (Fig. 5.2).

Since LPS induced a DNA-protein complex as indicated by EMSAs using the GAS element as a probe (Fig. 5.1a). I investigated whether LPS can up regulate IRF-1 on its own. Alternatively, it was possible that LPS-induced DNA-binding protein(s) could work synergistically with IFN-γ-induced transcription factor(s) to induce IRF-1. To answer these questions, IRF-1 expression in LPS or LPS plus IFN-γ-treated cells was determined by Northern blot analysis. As expected, IRF-1 was induced by the treatment of IFN-γ, and this was strongly increased by LPS (Fig. 5. 3). Interestingly, following prolonged expose of the film, it could be seen that LPS alone could induce weak IRF-1 expression in 3-4 h (results not shown). This could perhaps be due to induction of IFN-γ by LPS.

IRF-1 expression in J774 cells was further confirmed by Western blotting, using an antibody against IRF-1. IRF-1 protein was induced by the treatment of IFN-γ and further enhanced by the combination of IFN-γ and LPS (Fig. 5.4), but was not detectable in the untreated cells. When the cells were incubated LPS alone for prolonged periods (4 h), IRF-1 protein was also weakly detectable (result not shown).

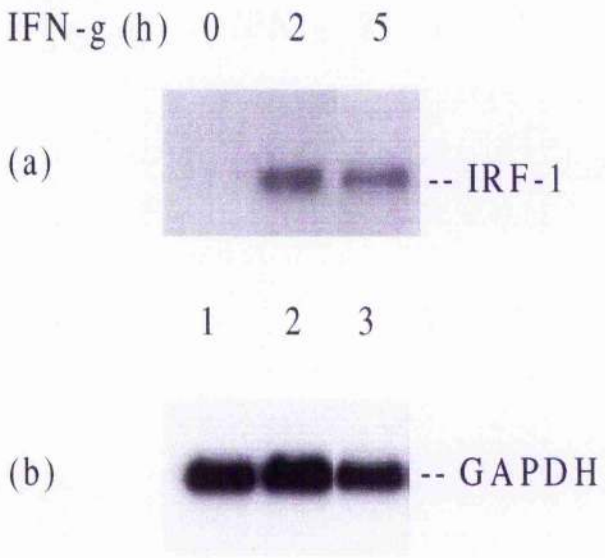


Fig. 5.2 Northern blot analysis of IRF-1 expression induced by IFN-γ. Total RNA was extracted from IFN-γ treated cells using $RNA_{ZOL}B$. Samples (10 μg each) was subjected to Northern blot analysis. IRF-1 mRNA was hybridised using ^{32}P labeled probes that represented the fragment of cDNAs for murine IRF-1 (a) or human GAPDH (b).

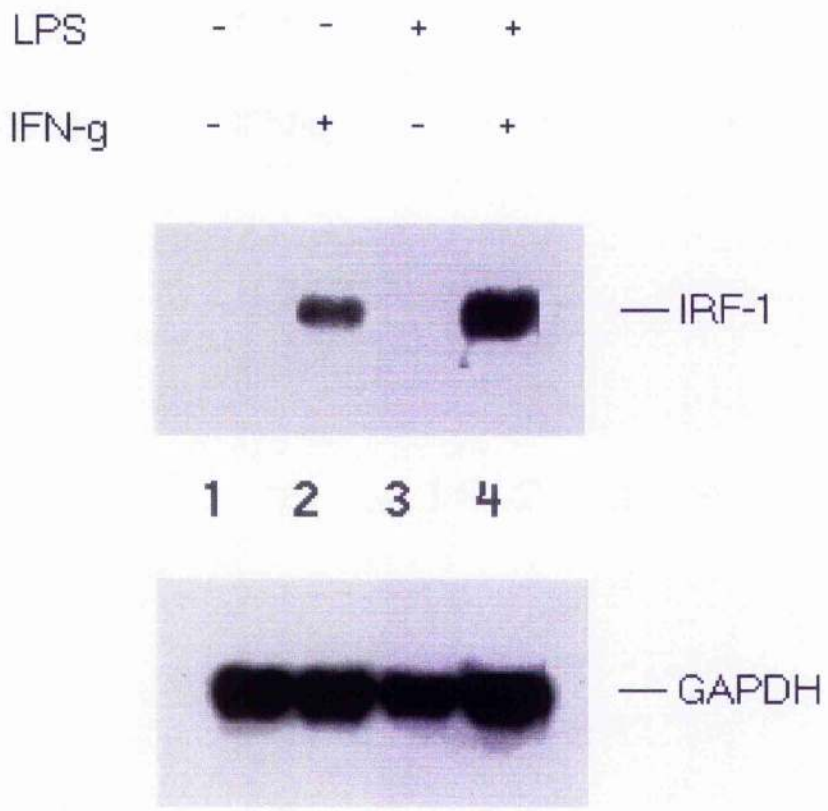


Fig. 5.3 Northern blot analysis for IRF-1 induced by IFN-γ and LPS. J774 cells were incubated with IFN-γ (100 U/ml) or /and LPS (10 ng/ml) for 2 h, and total RNA was extracted using RNAzolB. IRF-1 mRNA was hybridised with a murine IRF-1 cDNA probe, and after stripping, a human GAPDH probe was used for evidence of loadding.

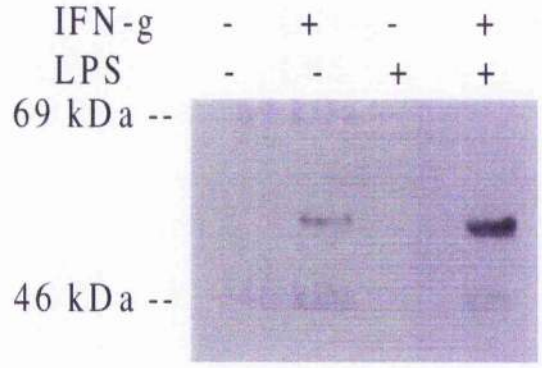


Fig. 5.4 IRF-1 expression in J774 cells induced by IFN-γ and LPS. J774 cells were incubated with IFN-γ (100 U/ml) or /and LPS (10 ng/ml) for 1 h. Nuclear proteins were extracted and 20 μg of each sample resolved in 10% SDS PAGE, transfered to nitrocellulose membrane and followed by probing with a polyclonal anti-IRF-1 antibody. There were two IRF-1 bands.

5.3 IRF-2 is upregulated by IFN-γ but not by LPS.

IRF-1 and IRF-2 are both interferon regulatory factors which were originally identified as regulators of the IFN system (Fujita *et al.*, 1988; Harada *et al.*, 1989). IRF-1 and IRF-2 function as a transcriptional activator and repressor, respectively, for the IFN and IFN-inducible genes. IRF-1 and IRF-2 show marked homology within their amino-terminal regions. Recombinant IRF-1 and IRF-2 bind to the same region within the virus-inducible element of the IFN-α and -β genes and the IFN-inducible genes (Harada *et al.*, 1989). These observations suggest that IRF-2 binds to similar or identical DNA sequences, perhaps modulating the function of IRF-1.

Since a transient increase in the IRF1/IRF2 ratio may be a critical event in the IRF-1 mediated transcriptional activation and subsequent cellular response, IRF-2 expression was investigated in J774 cells by northern blot analysis. An IRF-2 cDNA probe was made the same way as that IRF-1 (provided by Dr. X.Q. Wei). In agreement with earlier reports, IRF-2 mRNA was found to be constitutively expressed at low level. This was increased in response to IFN-γ treatment in a similar manner to that of IRF-1. However, generally IRF-2 mRNA levels were lower than those of IRF-1(Fig.5.5).

As observed earlier, LPS either by itself or in combination with IFN-γ could upregulate IRF-1 expression as measured by Northern blots (Fig. 5.3), the effect of LPS on IRF-2 expression was examined. IRF-2 was constitutively expressed in untreated cells. Treatment of LPS did not increase the expression of IRF-2, and indeed in some experiments, there was a slight decrease in IRF-2 expression. Unlike IRF-1, IRF-2 mRNA induced by IFN-γ was markedly reduced by the combined treatment of IFN-γ and LPS (Fig. 5.6). IRF-2 expression in cells treated with LPS alone was not detectable within 4 h of stimulation.

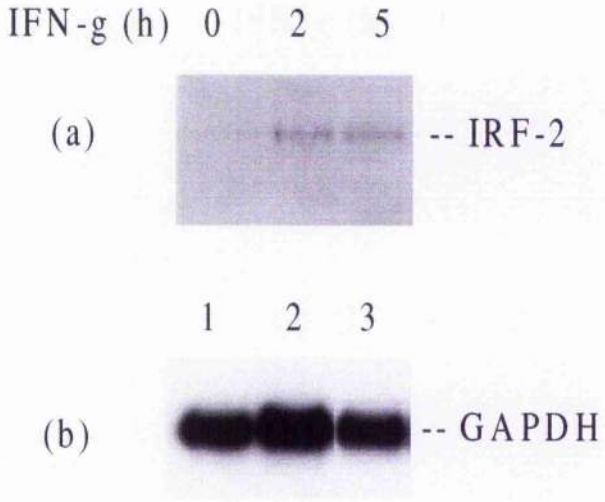


Fig. 5.5 Northern blot analysis of IRF-2 expression in J774 cells. J774 cells were incubated with IFN-γ (100 U/ml) for 2 and 5 h, and total RNA was extracted using RNAzolB. IRF-1 mRNA was hybridised with a murine IRF-2 cDNA probe, and after stripping, a human GAPDH probe was used for evidence of loadding.

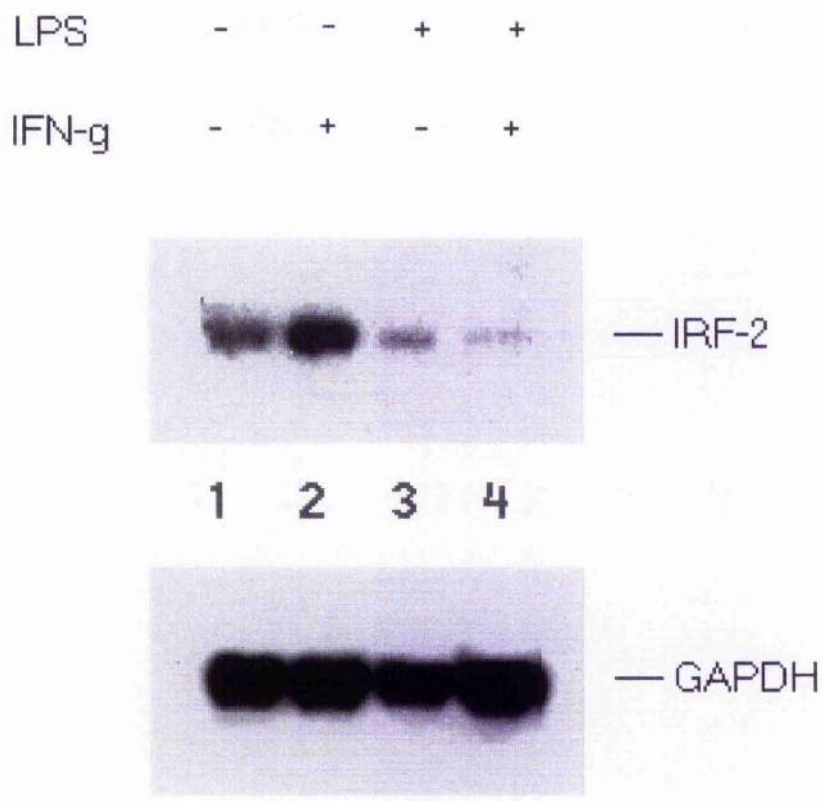


Fig. 5.6 Northern blot analysis for IRF-2 induced by IFN-γ and LPS. J774 cells were incubated with IFN-γ (100 U/ml) or /and LPS (10 ng/ml) for 2 h, and total RNA extracted using RNA^{zol}B. IRF-2 mRNA was hybridised with a murine IRF-1 cDNA probe, and after stripping, with a human GAPDH probe.

Taken together, in J774 cells, IRF-1 mRNA and protein are upregulated by IFN-γ and further enhanced by LPS whereas IRF-2 mRNA expression induced by IFN-γ was reduced or unaffected by LPS within the 4 h period tested. The consenquence of the differential expression of IRF-1 and IRF-2, in response to IFN-γ and LPS, leads an increase in the ratio of IRF-1/IRF-2. These results therefore indicated that one of the mechanisms of synergy between IFN-γ and LPS in the induction of iNOS is to increase IRF-1 and decrease IRF-2 expression.

5.4 IRF-E activation in J774 cells treated with IFN-γ and/or LPS

Cloning of the promoter of the murine iNOS gene (Xie *et al.*, 1993; Lowenstein *et al.*, 1993) has opened a molecular route to the analysis of iNOS induction. The promoter region of iNOS gene contains four copies of GAS, and two copies of the IFN-stimulated response element (ISRE), designated ISREu for upstream and ISREd for downstream. Finally, the complementary nucleotide sequence of the ISREd core closely matches a consensus sequence termed IFN regulatory factor element (IRF-E) (Tanaka *et al.*, 1993).

IRF-1 and IRF-2 are both IRF-E binding proteins. Activation of J774 cells with IFN-γ and LPS should therefore lead to changes in IRF-E binding activity in the iNOS promoter. An EMSA was carried out to determine if the changes in IRF1/IRF-2 ratio correlated with that of IRF-E binding. There was a constitutive binding complex in IRF-E (probe G, see section 2.16 in chapter 2) . Upon stimulation with IFN-γ, a IRF-E binding protein was induced which migrated faster than that of constitutive DNA-protein complex. Unexpectedly, a binding complex in LPS-treated cell nuclear extracts was also induced. The mobility of this complex was only slightly faster than that of the constitutive one, but was distinguishable from that of IFN-γ-induced binding complex (Fig. 5.7a). When the same nuclear extracts were tested with another IRF-E oligo, which corresponding to the IL-6 inducible gene promoter sequence (probe F see section 2.16 in chpater 2), similar results were obtained (Fig.5.7b), except that there was no constitutive complex

formation. This may due to slightly difference in sequence of oligonucleotides between two probes, which may be critical for the formation of this constitutive complex. However, with both IRF-E probe (G and F), LPS-induced binding complex was consistent. To identify the binding proteins, antibody super-shift assays were carried out. It was shown that IFN-γ-induced binding complex could be abolished by either anti-IRF-1 or anti-IRF-2 (Fig. 5.7c), but the LPS-induced binding complex was not affected by anti-IRFs or anti-NFκB (P65 and P50) (data not shown). The low mobility of this binding complex suggests that it comprises a fairly large protein or a complex of multiple factors.

To analyse the LPS-induced IRF-E binding protein, a number of cold oligo competitors were used in EMSAs. When added at 100x excess concentrations over the ^{32}P labelled probe G (IRF binding element in iNOS promoter), cold probe D (NFκB site in iNOS promoter) had no effect on the binding activity; probe F (GAS element in IRF-1 promoter) had some effect on the LPS-induced but not on that of IFN-γ-induced binding activities (Fig. 5. 8). Further experiments proved that, under the same conditions, these two cold probes (D-NFkB, or F-GAS) could compete with their own 32P-labelled probe D (Fig. 5.9) and F (Fig. 5.1d) respectively. On the other hand, when NFκB binding site was used as a ^{32}P labelled probe, cold probe G (IRF-E) could not compete the binding either (Fig. 5.9). These results suggest that the LPS-induced IRF-E-binding complexes are not related to the NFκB but it may consist of one of the STAT-like protein.

To determine if protein synthesis is necessary for the activation of LPS-induced transcription factor, the effects of cycloheximide (CHY) was tested. When CHY was added 1h before IFN-γ and LPS, both complexes (induced either by IFN-γ or LPS) were abolished (Fig. 5.10). In contrast, NFκB binding was not affected (See details in section 5.5 and Fig. 5.14). These results indicate that protein synthesis is necessary for IRF-E activation either by IFN-γ (IRF-1) or by LPS (unknown factor), and further suggest that the LPS-induced IRF-E binding protein is unlikely to be p91 or NFκB.

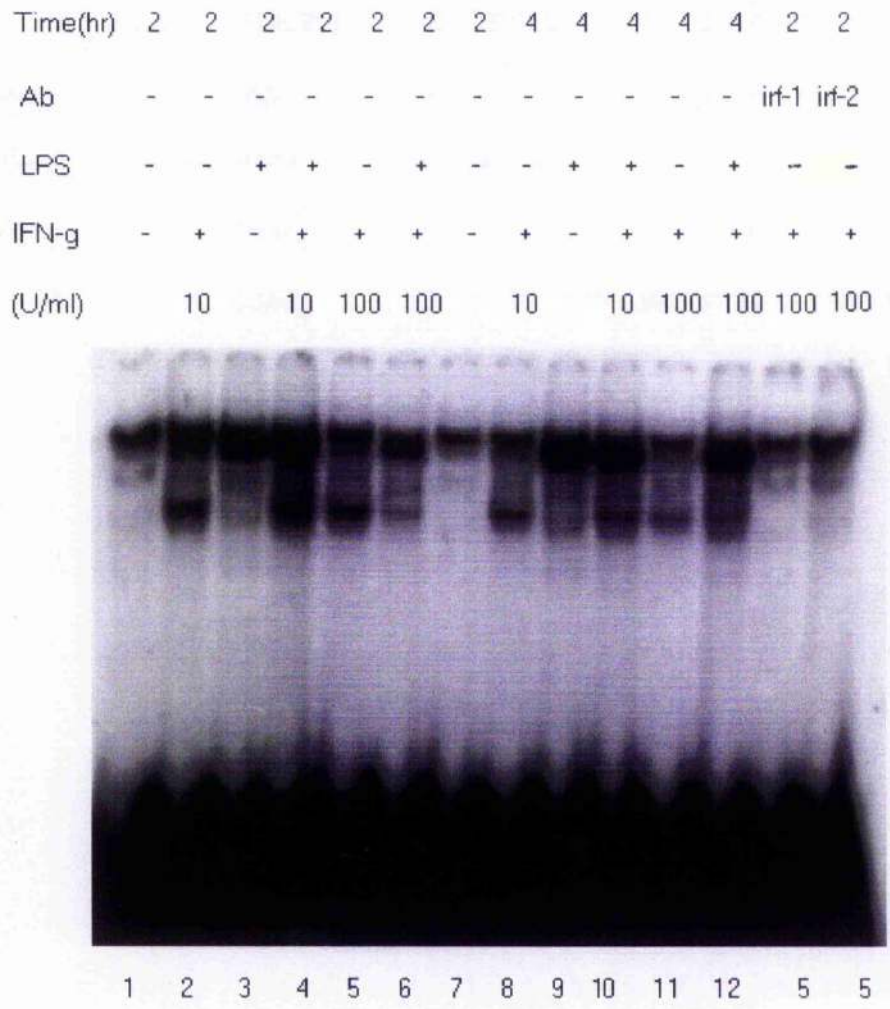


Fig. 5.7a IRF-E binding activity in iNOS promoter induced by IFN-γ and/or LPS. J774 cells were incubated with IFN-γ (100 U/ml) or /and LPS (10 ng/ml) for 2 h or 4 h, and nuclear proteins extracted. EMSA was carried out by incubation of 10 μg of nuclear protein with a ^{32}P-labeled oligonucleotide probe G containing the ISRE (IRF-E) sequence found in the iNOS promoter. For the supershift assay, antibodies were added into the reaction mixture 20 min before adding the probe. In this figure, supershift was not observed, since complexes were abolished by antibodies.

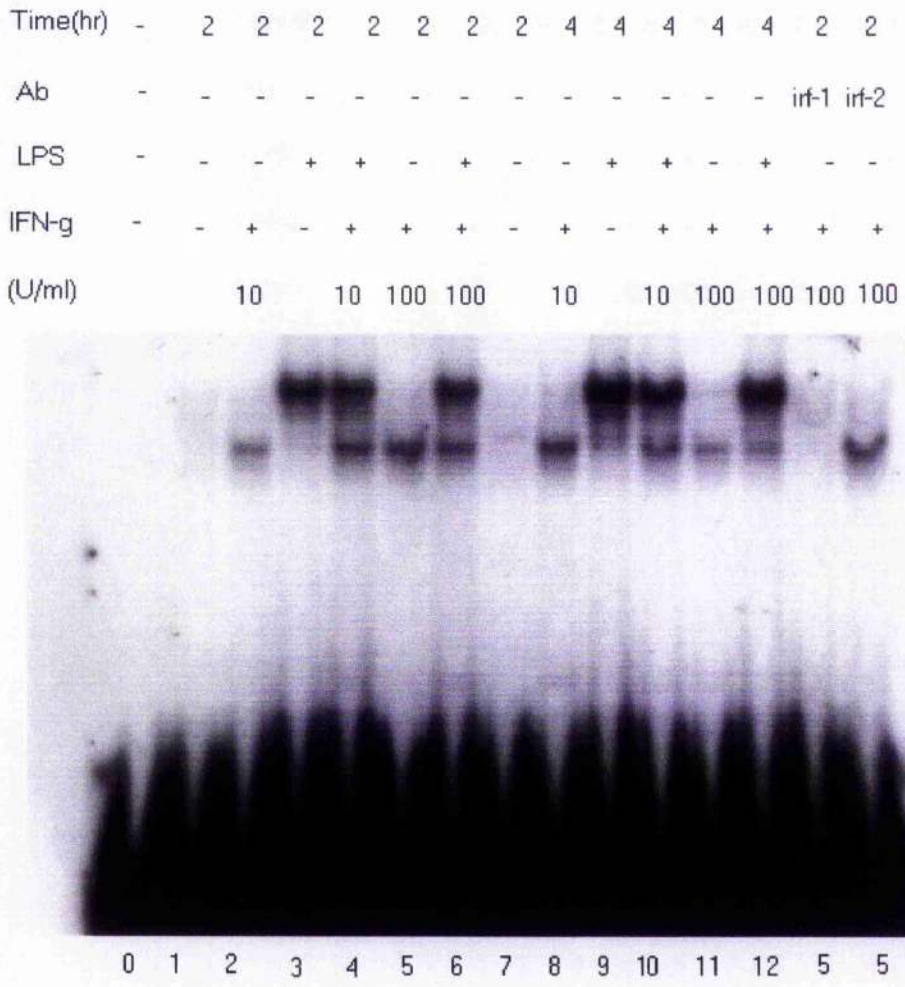


Fig. 5.7b IRF-E binding activity induced by IFN-γ and/or LPS. J774 cells were incubated with IFN-γ (100 U/ml) and/or LPS (10 ng/ml) for 2 h or 4 h, and nuclear proteins extracted. EMSA was carried out by incubation of 10 μg of nuclear protein with a ^{32}P-labeled oligonucleotide probe H containing the ISRE (IRF-E) sequence found in the IL-6 inducible gene (see Materials and Methods) promoter. For the supershift assay, antibodies were added into the reaction mixture 20 min before adding the probe. In this figure, supershift was not observed, since complexes were abolished by antibodies.

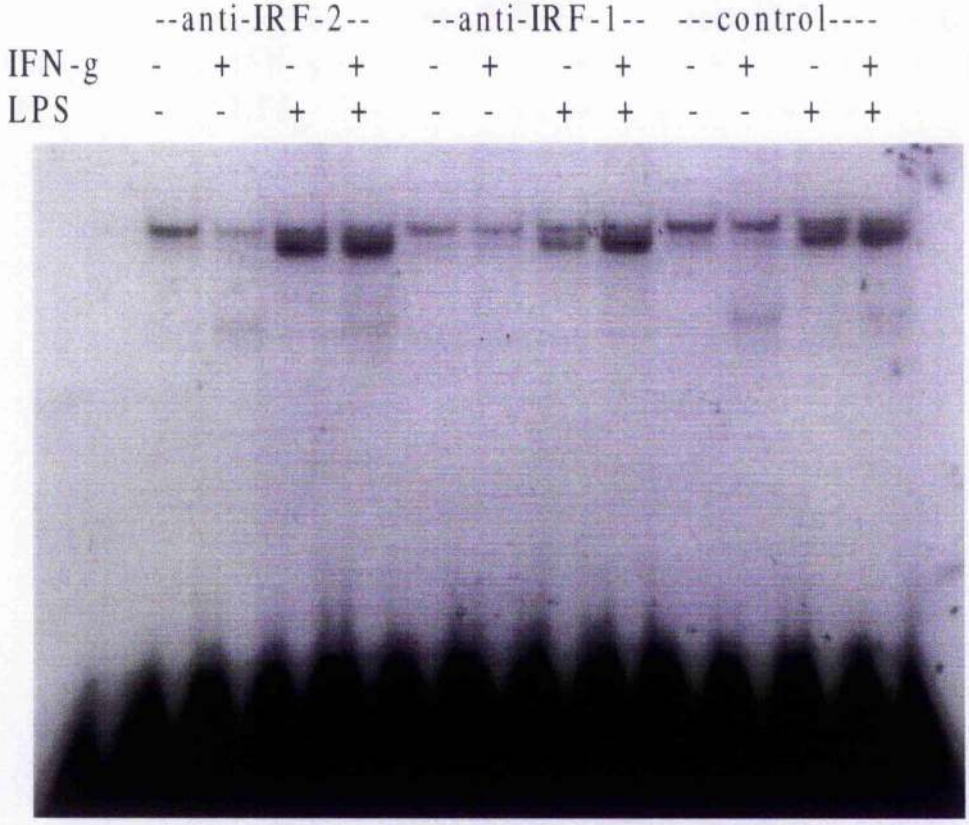


Fig. 5.7c Antibody-supershift assay for IRF-E binding activity induced by IFN-γ and/or LPS. J774 cells were incubated with IFN-γ (100 U/ml) and/or LPS (10 ng/ml) for 2 h, and nuclear proteins extracted. EMSA was carried out by incubation of 10 μg of nuclear protein with a ^{32}P-labeled oligonucleotide probe G containing the ISRE (IRF-E) sequence found in the iNOS gene (see Materials and Methods) promoter. For the supershift assay, antibodies were added into the reaction mixture 20 min before adding the probe. In this figure, supershift was not observed, since complexes were abolished by antibodies.

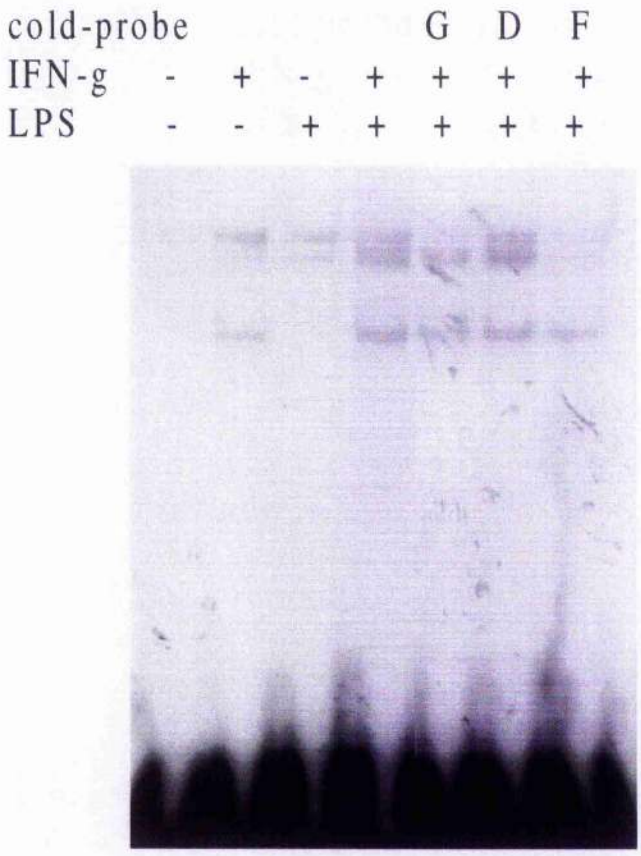


Fig. 5.8 Competition assay for IRF-E binding activity in iNOS promoter imduced by IFN-γ and/or LPS. J774 cells were incubated with IFN-γ (100 U/ml) and/or LPS (10 ng/ml) for 2 h, and nuclear proteins extracted. EMSA was carried out by incubation of 10 μg of nuclear protein with a ^{32}P-labeled oligonucleotide probe G containing the ISRE (IRF-E) sequence found in the iNOS promoter. For the competition assay, cold oligo competitors (at 100x high concentrations) were added into the reaction mixture 20 min before adding the probe. Probe D contains NFκB site, probe F contains GAS element.

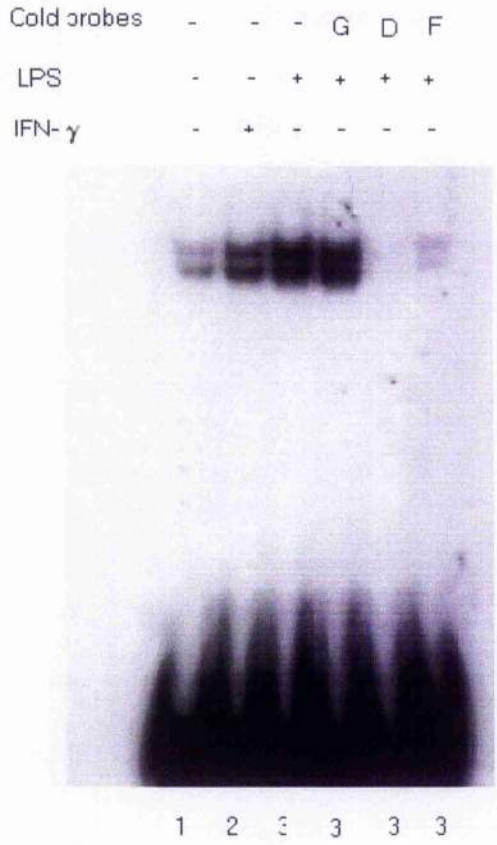


Fig. 5.9 NFκB binding activity in iNOS promoter induced by IFN-γ and/or LPS was not competed by cold G probe. J774 cells were incubated with IFN-γ (100 U/ml) and/or LPS (10 ng/ml) for 2 h, and nuclear proteins extracted. EMSA was carried out by incubation of 10 μg of nuclear protein with a ^{32}P-labeled oligonucleotide probe D containing the NFκB site sequence found in the iNOS promoter. For the competition assay, cold probe (at 100x high concentration) were added into the reaction mixture 20min before adding the probe. Probe G contains IRF-E element, probe F contains GAS element.

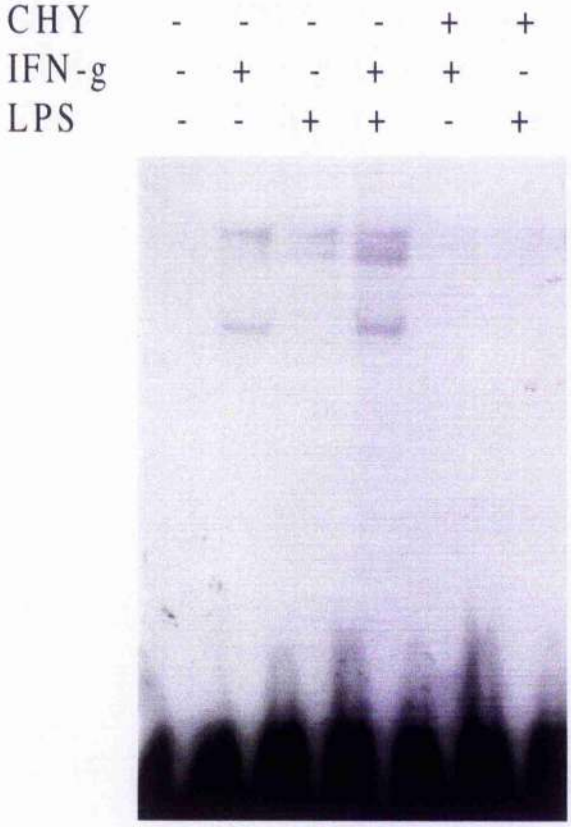


Fig. 5.10 Effect of cycloheximide on IRF-E binding activity in the iNOS promoter induced by IFN-γ and/or LPS. J774 cells were incubated with or without cycloheximide (CHY, 30 μg/ml) for 1 h, and IFN-γ (100 U/ml) and/or LPS (10 ng/ml) were added for 2 h before nuclear proteins were extracted. EMSA was carried out by incubation of 10 μg of nuclear protein with a ^{32}P-labeled oligonucleotide probe G containning the ISRE (IRF-E) sequence found in the iNOS promoter.

5.5 NFκB activation in iNOS promoter after treatment of LPS or IFN-γ

As mentioned above, to achieve transcription activity, transcription factors must be located in the nucleus, bind DNA and interact with basal transcription apparatus. To determine possible NFκB activation in response to LPS in J774 cells, a nuclear translocation experiment was carried out. Nuclear proteins extracted from IFN-γ and/or LPS treated cells were Western blotted using an antibody against NFκB P65. NFκB was found to be absent in the nucleus of untreated cells. It was however translocated to the nucleus 15 min after LPS-treatment. There was no evidence of NFkB nuclear translocation after treatment with IFN-γ for 15 min (Fig. 5.11). When the cells were incubated for longer time (> 2 h), NFκB translocation was also observed in IFN-γ treated cells.

In the murine iNOS promoter, there are two NFκB elements and the one of them (beginning 55 base pairs upstream of the TATA box of the iNOS gene) was used for analysis by EMSAs. Constitutive binding activity was found in the nuclear extracts of untreated J774 cells, and it could be a single band or double bands. It was observed that both binding activities were upregulated by LPS as measured 15 min (Fig. 5.12a) and 2 h (Fig. 5.12b) after stimulation but the top band showed a stronger inducibility by LPS. IFN-γ, however, had little effect on NFκB binding in these experiments. This consisted with translocation data.

To identify which components of the Rel family bind as upper or lower bands to the NFκB element, specific antibodies to P50 or P65 were used. Supershift analysis revealed that the lower NFκB band, the main constituent in nuclear extracts of J774 cells, was likely to compose P50-P50 homodimers . The upper band, which is the major NFκB shift in nuclear extract of LPS-activated J774 cells, was shown to represent P50-P65 heterodimers, since it was shifted by both P50 and P65 antibodies (Fig. 5.13).

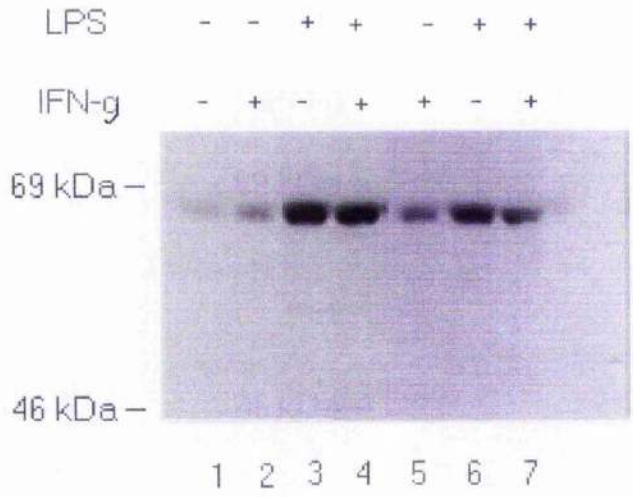


Fig. 5.11 NFκB (p65) nuclear translocation upon the stimulation of LPS or IFN-γ. J774 cells were stimulated with IFN-γ (100U/ml) or /and LPS (10ng/ml) for 15 min (lanes 1-4) and 2 h (lanes 5-7). Nuclear proteins were extracted and 20 μg of each extracts were subjected to Western blotting detected by a polyclonal anti-NFκB (P65).

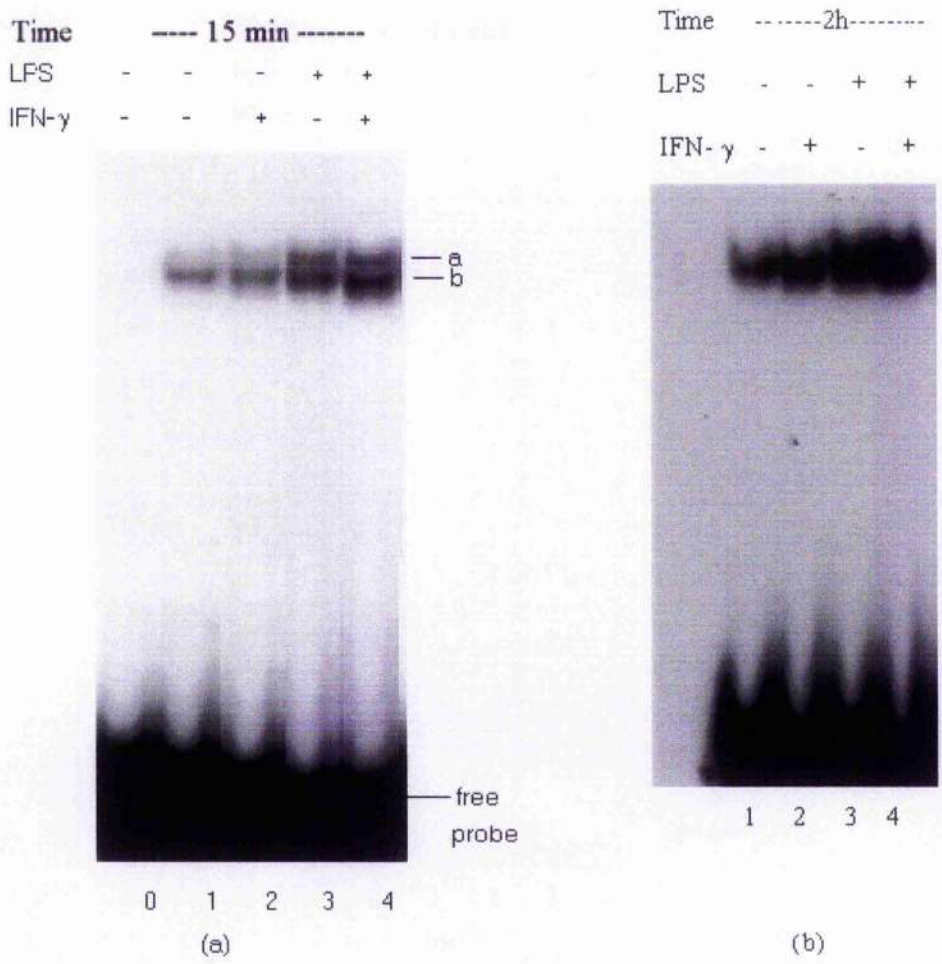


Fig 5.12 Activation of NFκB-element by LPS or IFN-γ. J774 cells were incubated with IFN-γ (100U/ml) or /and LPS (10ng/ml) for 15 min (a) and 2 h (b). Nuclear proteins were extracted and 10 μg of each extracts were subjected to gel-shift assay using ^{32}P-labeled D (see materials and methods) oligomor (5'-CCCAACTGGGGACT CTC ; 5'-CCAAAGGGAGAGTCCCC) as a probe.

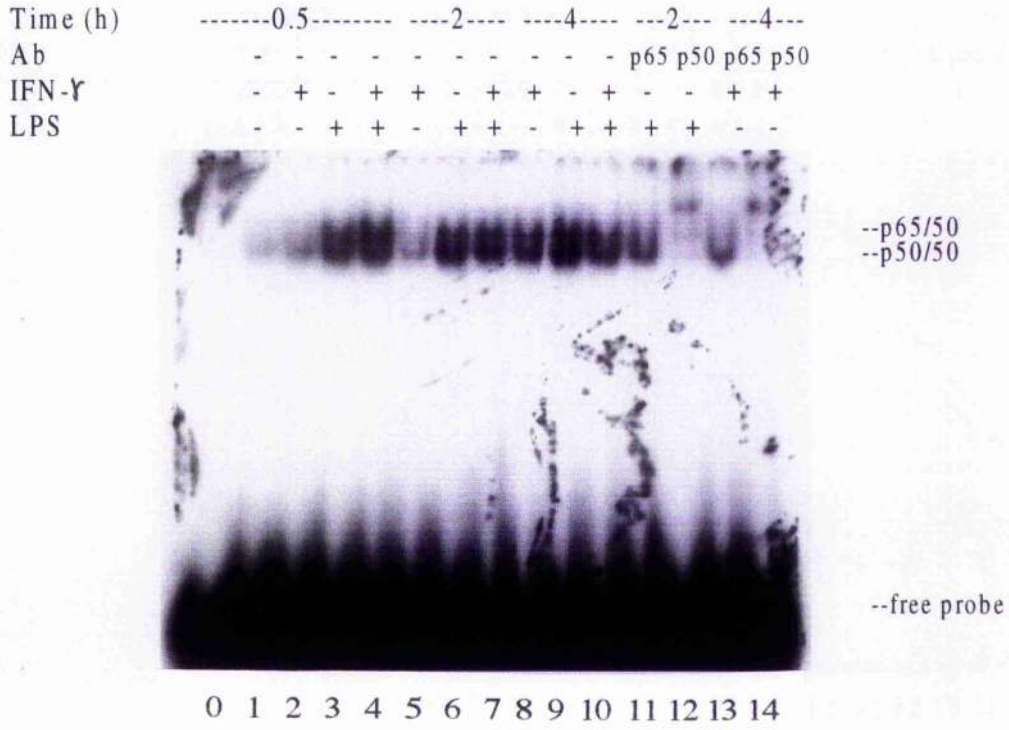


Fig. 5.13 Time course of activation of NFκB-binding activity in the iNOS promoter by IFN-γ or LPS. J774 cells were incubated with IFN-γ (100 U/ml) and /or LPS (10 ng/ml) for 30 min (lanes 2-4), 2 h (lanes 5-7), or 4 h (lanes 8-10) or medium alone (lane 1). Nuclear extracts (10μg each) were assayed for EMSA using ^{32}P-labeled D oligomor as a probe. Supershift assay was carried out using the same method except that antibody (2 μl) was added (NFκB P65, lanes 11 and 13; NFκB P50, lanes 12 and 14) in the reaction mixtures.

As mentioned earlier (Fig. 5.12), IFN-γ did not activate NFκB for the initial 1 h following stimulation, but it did when cells were cultured for more than 2 h and NFκB activity continued to increase steadily for up to 4 h. For LPS treated-cells, NFκB binding had declined at this time point (Fig. 5.13). Thus, while the activation of NFκB by LPS is an immediate event (less than 30 min), it taken a longer period for IFN-γ to achieve a similar activation (more than 2 h). These results indicate that NFκB may be indirectly activated by IFN-γ. To determine if protein synthesis is required for the NFκB binding activity, such experiments were repeated in the presence of cycloheximide. As shown in Fig.5.14, under the stimulation of IFN-γ or LPS, the binding activity of NFκB in J774 cell nuclear extracts was not reduced but actually enhanced by the pretreatment with cycloheximide. This suggested that NFκB activation, either by LPS or IFN-γ, is a protein-synthesis independent process. The enhancement may be due to cycloheximide interfering with IκB protein synthesis leading to release of NFκB from the IκB-NFκB inactive complex and translocation to the nucleus and gene activation.

5.6 Discussion

In murine macrophages, following the stimulation with IFN-γ, IRF-1 and IRF-2 genes were transcriptionally activated by the binding of phosphorylated STAT1 to GAS elements in the promoters (at least in IRF-1). According to cDNA sequence analysis, Harada and colleagues' (1989) have reported that the IRF-2 cDNA sequence shows marked homology with IRF-1 in its N-terminal region but was rather different in its C-terminal region. Since the C-terminal region of IRF-1 possess the transcriptional activation domain, it is possible that IRF-2 may function as a controlling factor for IRF-1 by binding site competition. Since the DNA-binding affinities of IRF-1 and IRF-2 are similar with respect to a wide variety of binding sequences (Tanaka *et al*, 1993), regulation of IFN-inducible genes may be determined simply by the ratio of IRF-1 and IRF-2. IRF-1, compared to IRF-2, was strongly induced by IFN-γ in J774 cells. Such a transiently raised ratio in IRF-1/IRF2 could allow the activation of ISRE in the iNOS promoter.

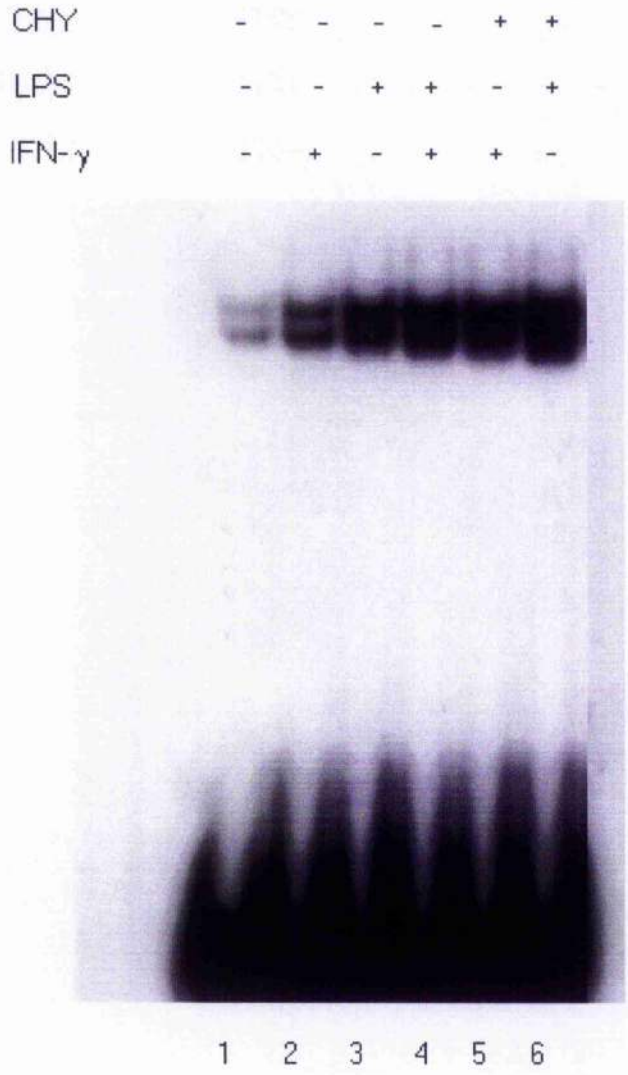


Fig. 5.14 Effect of cycloheximide on activation of NFκB-binding activity in the iNOS promoter by IFN-γ or LPS. J774 cells were incubated with or without cycloheximide (CHY, 30 μg/ml) for 1 h then IFN-γ and /or LPS were added and cultured for a further 2 h. Nuclear extracts (10 μg each) were assayed for EMSA using ^{32}P-labeled D oligomor as a probe.

IRF-1 plays a critical role in the regulation of IFN-inducible genes including iNOS (Kamijo *et al.*, 1994). The inability of macrophages from IRF-1 knockout mice (IRF-$1^{-/-}$) to produce NO or express detectable iNOS mRNA after stimulation with IFN-γ in the presence or absence of LPS, demonstrated that IRF-1 plays a critical physiologic role in the induction of iNOS. IRF-E itself is a silencer element and IRF-2 is the silencer at the promoter. In a variety of cell types, as a result of its greater protein stability, IRF-2 dominates over IRF-1, binding at approximately 10-fold-higher affinity to IRF-E (Watanabe *et al.*, 1991). However, the IRF-1 gene is efficiently induced in response to viruses and IFNs, resulting in an increase in IRF-1 activity relative to that of IRF-2. In J774 cells, the treatment with IFN-γ led to the induction of IRF-1 and also an increase in IRF-2. However, the increase in IRF-1 expression was markedly stronger than that of IRF-2, and thus positive ratio of IRF-1/IRF-2 could provde a mechanism for the transcriptional activation of IFN-γ-inducible genes including iNOS.

Previous reports strongly suggested that both the IRF and NFκB binding domains are necessary for the full transcriptional activation of the iNOS promoter (Lowenstein et al., 1993). NFκB was found to be rapidly and transently translocated into the nucleus in response LPS in J774 cells. Activated NFκB appeared in the nucleus as P65-/P50 heterdimer or P50/P50 homodimer and bound to a NFκB response element in the iNOS promoter where it transcriptionally activated iNOS gene. In this study, experiments regarding the expression or activation of tanscription factors, like IRF-1/2 and NFκB, have demonstrated that IFN-γ can activate IRF-1 and IRF-2 transcriptional activities whereas LPS can activate NFκB. The expression of IRF-1 induced by IFN-γ and the activation of NFκB induced by LPS may work synergistically to activate the expression of iNOS gene (Xie *et al.*, 19940) which is responsible for the large amount of NO production.

Recent reports using gene targeting technique have emphasised the importance of STAT1 in cell responsiveness to IFN-α and IFN-γ (Meraz *et al.*, 1996; Durbin *et al.*, 1996). However, LPS induced two GAS-binding proteins in J774 cells, and rather

surprisingly these proteins were not STAT1. There was no evidence for LPS-induced STAT1 activation (see chapter 4) in J774 cells and the binding complexes induced by LPS were different from that GAS-binding STAT protein induced by IFN-γ. Antibody supershift and cold-probe competition assays indicated that the transcription factors in the complexes were NFκB or NFκB-like proteins. According to the migration patterns, the binding sequence of the NFκB-like protein and STAT1 were similar or the binding sites between two transcription factors were very close. Northern and Western blot proved that IFN-γ-induced IRF-1 induction was enhanced by LPS, and IRF-1 was transcriptionally activated at low expression levels by LPS alone. These results suggest that LPS alone can also activate IRF-1 and one of the possible mechanism is through NFκB activation. Hence, IRF-1 may, as well as NFκB, plays a role in LPS induced iNOS expression.

An early report using site-specific mutation of IRF-E within the context of the full-length iNOS promoter/enhancer region demonstrated the importance of this site in the response of iNOS to IFN-γ (Martin *et al*, 1994). Interestingly, within the iNOS promoter region, the IRF-E site not only binds IRF-1 and IRF-2 but also binds a protein (or a protein complex) induced by LPS. Antibody suppershift and cold-probe competition assays excluded the possibility of IRF-1, IRF-2, NFκB (P65, P50). Furthermore, protein synthesis is required for the IRF-E binding activity of both IRF-1 and the LPS-inducible protein, it is not required for the NFκB binding activity. The LPS-inducible IRF-E binding protein is therefore likely to be a new protein (or protein complex) which belongs to the IRF transcription factor family. An LPS-stimulated binding factor (κBF-A) from murine peritoneal macrophages has also been reported to bind the ISRE regulating the IP-10 , D3 (Tebo *et al*, 1992) and Ig κ light chain genes (Damore *et al.*, 1996). Further experiments are needed to establish the identity of the factor(s) involved in IRF–E binding.

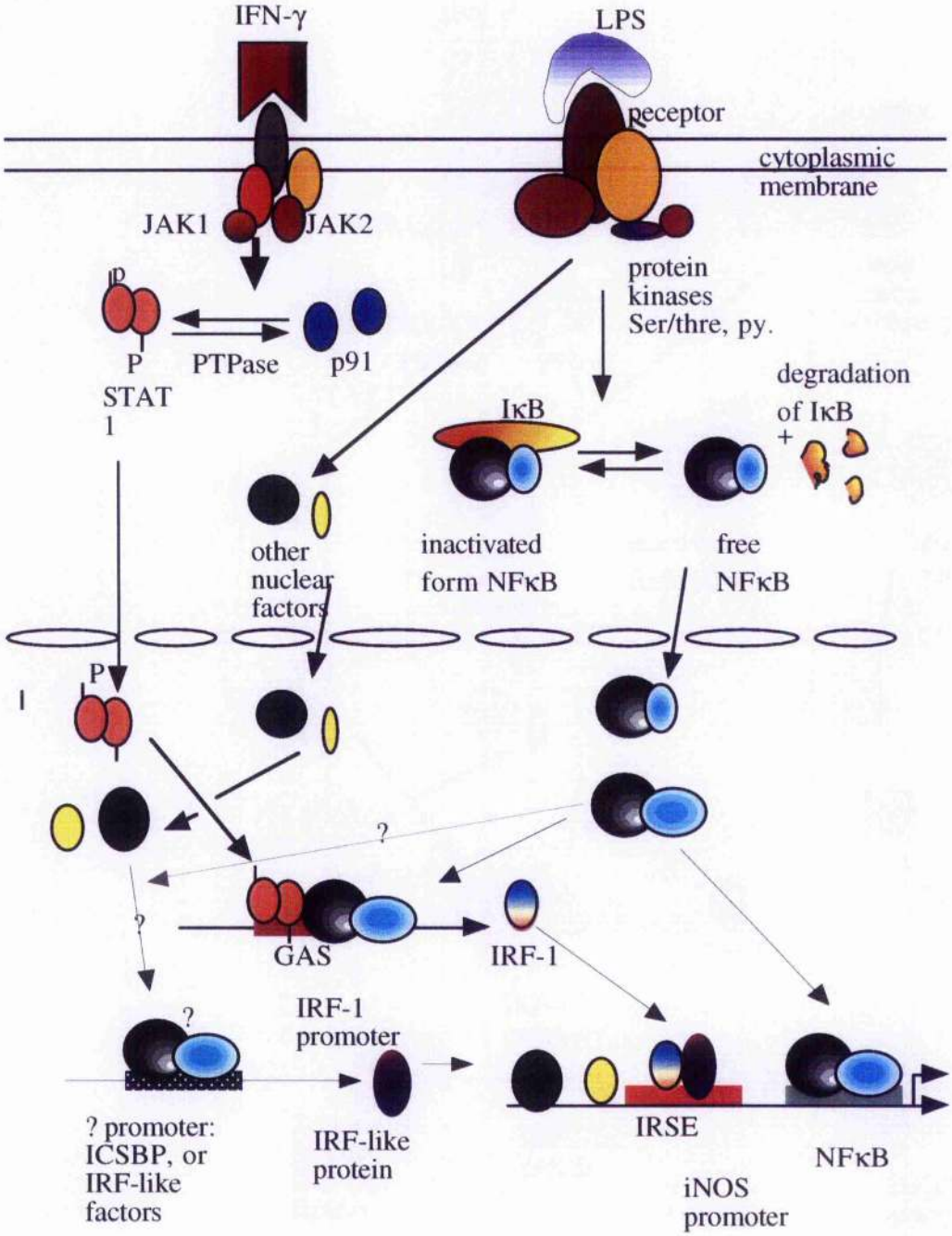
Macrophages from IRF-1 deficient mice accumulate only a reduced level iNOS mRNA after stimulation with LPS in the presence or absence of IFN-γ. Thus, the synergistic induction of NO induced by IFN-γ and LPS is not only through the

combinatorial activation of NFκB (by LPS) and IRF-1 (by IFN-γ), but is also through synergistic activation of IRF-E by IRF-1 (induced by IFN-γ) and IRF-like protein (induced by LPS).

Finally, transcription factors other than IRF-1 may involved be in IFN-γ-induced iNOS induction. NFκB, for example, was activated and translocated to the nucleus 2 h after the treatment with IFN-γ. Such activated NFκB bound to its responding element in the iNOS promoter, to work co-operatively with IRF-1 to activate the iNOS gene. Since the JAK-STAT pathway is a relatively direct cascade which is an immediate and transient event (peak at 1 h), it is not likely that IFN-γ-induced NFκB activation is the direct effect of JAK-STAT activation in this case. Addition of cycloheximide revealed that this NFκB process is protein synthesis independent. This indicated activation of NFκB by IFN-γ does not involve any newly synthesised protein. Further work will be headed to identify the pathway which leads to NFκB activation in IFN-γ-treated cells.

The mechanisms of the regulation of iNOS in murine macrophages identified by these studies is summarised in Fig 5.15. Basically, upon stimulation of IFN-γ, multiple signalling pathways are activated. One of the signalling pathways is JAK-STAT pathway. JAK kinases (JAK1 and JAK2) are activated which are responsible for phosphorylation of STAT1. Phosphorylated STAT1 forms homodimers and translocates to the nucleus where it binds to GAS element within IFN-γ responsive genes, for example, IRF-1. IRF-1, as a transcription factor, plays a crucial role in the regulation of NO synthesis in response to IFN-γ. The binding of STAT1 to the IRF-1 promoter permits activation of IRF-1 gene expression. Binding of LPS to its receptor on cell surface leads to phosphorylation and degradation of IκB therefore releasing activated NFκB which then translocates to the nucleus where it binds to the κB element in LPS responsive genes, for example, IRF-1 and iNOS. In addition, an IRF-like protein is transcriptionally activated in response to LPS which also participates in regulation of iNOS expression. With the combination of IRFs, NFκB, and possible the other transcription factors, iNOS expression is achieved upon the stimulation of IFN-γ and LPS.

Fig. 5.15 The induction of iNOS expression by a combination of IFN-γ and LPS. In J774 cells, a number of transcription factors may participate in the iNOS activation. Basically, upon stimulation of IFN-γ, multiple signalling pathways are activated. One of the signalling pathways is the JAK-STAT pathway. JAK kinases (JAK1 and JAK2) are activated which are responsible for phosphorylation of STAT1. Phosphorylated STAT1 forms homodimers and translocates to the nucleus where it binds to GAS element within IFN-γ responsive genes, for example, IRF-1. IRF-1, as a transcription factor, plays a crucial role in the regulation of NO synthesis in response to IFN-γ. The binding of STAT1 to the IRF-1 promoter permits activation of IRF-1 gene expression. Binding of LPS to its receptor on cell surface leads to phosphorylation and degradation of IκB therefore releasing activated NFκB which then translocates to the nucleus where it binds to the κB element in LPS responsive genes, for example, IRF-1 and iNOS. In addition, an IRF-like protein is transcriptionally activated in response to LPS which also participates in regulation of iNOS expression. With the combination of IRFs, NFκB, and possible the other transcription factors, iNOS expression is achieved upon the stimulation of IFN-γ and LPS.


IFN-γ
LPS
receptor
cytoplasmic membrane
JAK1
JAK2
protein kinases Ser/thre, py.
P
PTPase
p91
STAT 1
degradation of IκB
+
IκB
other nuclear factors
inactivated form NFκB
free NFκB
P
?
?
GAS
IRF-1
IRF-1 promoter
?
? promoter: ICSBP, or IRF-like factors
IRF-like protein
IRSE
NFκB
iNOS promoter

Chapter 6

Mechanism of IL-4-mediated suppression of iNOS expression

6.1 Introduction

IFN-γ and IL-4 are produced by functionally different subsets of T helper cells (Th1 and Th2, respectively) in the murine (Mossman, *et al.*, 1987) as well as in the human system (Romagnani, 1991).The opposing regulatory effects of IL-4 and IFN-γ are crucial in the regulation of the immune response, as has been demonstrated in the murine Leishmaniasis model (Heinel, *et al.*, 1988). IFN-γ is an important cytokine with antiviral activity and regulatory functions (Dijkmans and Billiau 1988) on antibody formation (Leibson *et al.*, 1984; Pinkelman *et al.*, 1988) , T-cell differentiation (Bernton *et al.*, 1988; Giovarelli *et al.*, 1988), and macrophage activation (Talmadge *et al.*, 1986; Schreiber *et al.*, 1983), enhancement of expression of class II antigens of the major histocompatibility complex (Basham and Merigan 1983), and production of other cytokines such as tumor necrosis factor and interleukin-1 (Collar *et al.*, 1986; Miossec *et al.*, 1986). In contrast, IL-4 displays anti-inflammatory effects on macrophages. As a Th2 cytokine, IL-4 exerts a variety of biologic effects on cells of most haemopoietic lineages such as B cells (Isakson *et al.*, 1982; Noelle *et al.*, 1984; Oliver *et al.*, 1985),T cells (Carding *et al.*, 1991; Fernandez-Botran *et al.*, 1986; Paul and Ohara 1987), and mast cells (Brown *et al.*, 1987) In particular, IL-4 seems to be essential for induction of differentiation of naive $CD4^+$ T helper (Th) cells toward the Th2 phenotype (Maggi *et al.*, 1992; Parronchi *et al.*, 1992). Th2 cells produce IL-4, IL-5, IL-6, and IL-10 and promote humoral immunity (Mosmann and Coffman 1989; Mosmann *et al.*, 1991). In the absence of IL-4, $CD4^+$ Th cells develop mainly into the Th1 phenotype which produces IL-2, IFN-γ, TNF, and lymphotoxin and promotes cell-mediated immunity. IL-4 has also been shown to limit the expression of protective Th1 functions (Liew *et al.*, 1991). Therefore, IL-4 is an important immune modulator.

Macrophages activated by cytokines (i.e. IFN-γ) and microbial products (i.e. LPS) protect the host from certain tumour cells and microbes to a large part by a pathway dependent on production of nitric oxide (NO) from L-arginine and molecular oxygen. Previous studies have demonstrated that IL-4 inhibits the expression of NO synthase and

NO production by IFN-γ-activated macrophages, and this mechanism is thought to provide down-regulation of Th1 functions by disease-promoting Th2 cells (Cenci *et al.*, 1993). IL-4 produced by Th2 cells can neutralise the macrophage-activating and leishmanicidal effect of IFN-γ produced by Th1 cells (Liew *et al.*, 1989). IL-4 and IL-10 are potent inhibitors of parasite killing and NO production by IFN-γ-activated macrophages (Liew *et al.*, 1991; Gazzinelli *et al.*, 1992). However, the detailed mechanism of IL-4 mediated inhibition of NO synthase is not known. The present study focused on how IL-4 suppresses NO release from J774 cells. This may provide useful information for understanding the mechanism of interaction between Th1 and Th2 cytokines.

6.2 Optimal conditions for inhibiting NO synthase by IL-4

Firstly, the time course and dose-response of IL-4-mediated inhibition of NO in J774 cells were determined using Griess reaction to measure NO_2^- accumulation in the culture supernatants.

IL-4 suppressed NO release from IFN-γ and LPS-activated J774 cells. When J774 cells were cultured (simultaneously or following pre-incubated (18 h)) with IL-4 (at 100 U/ml), NO release was suppressed by 30% (Fig. 6.1). Further experiments showed that to inhibit NO production, IL-4 has to be added before or simultaneously with IFN-γ and LPS. There was no effect if IL-4 was added 2 h after IFN-γ and LPS (Fig. 6.2).

6.3 IL-4 affects the transcriptional activation of iNOS

Diminished NO_2^- accumulation after IL-4 treatment suggested that IL-4 down regulates the enzymatic activity of iNOS. This was confirmed by demonstrating that iNOS activity was indeed decreased when cells were co-incubated with IL-4 (Fig. 6.3).

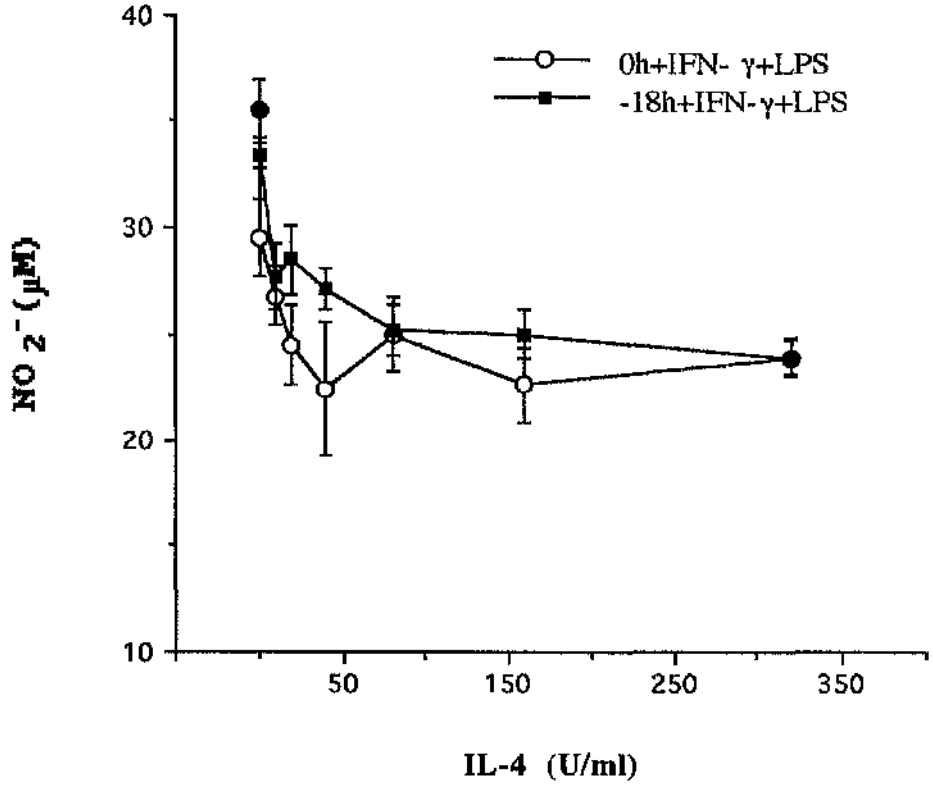


Fig. 6.1 Dose-dependence of IL-4 mediated inhibition of NO synthesis. J774 cells were cultured at 10^5 cell / well in 96-well plate. IL-4 was added either -18 h or simultaneously with IFN-γ and LPS. Culture supernatants were collected at 24 h. Griess Reaction was used for NO_2^- measurement. Data are shown as means of triplicate cultures (±SD). The results are representative of two experiments.

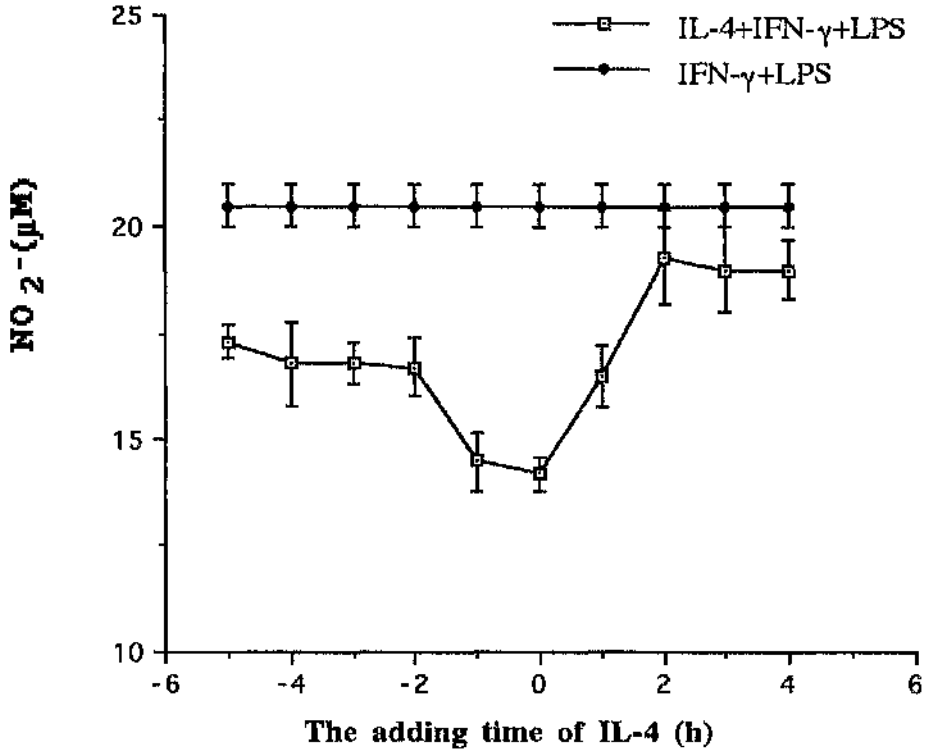


Fig. 6.2 Time course of IL-4 mediated inhibition of NO synthesis. J774 cells were cultured at 10^5 cells / well in DMEM in 96-well plate. IL-4 (100 U/ml) was added at -5 to 5 h in combine with IFN-γ and LPS for 24 h. NO_2^- in culture supernatants were measured using Griess Reaction. Data are shown as means of triplicates (±SD). The results are representative of five experiments.

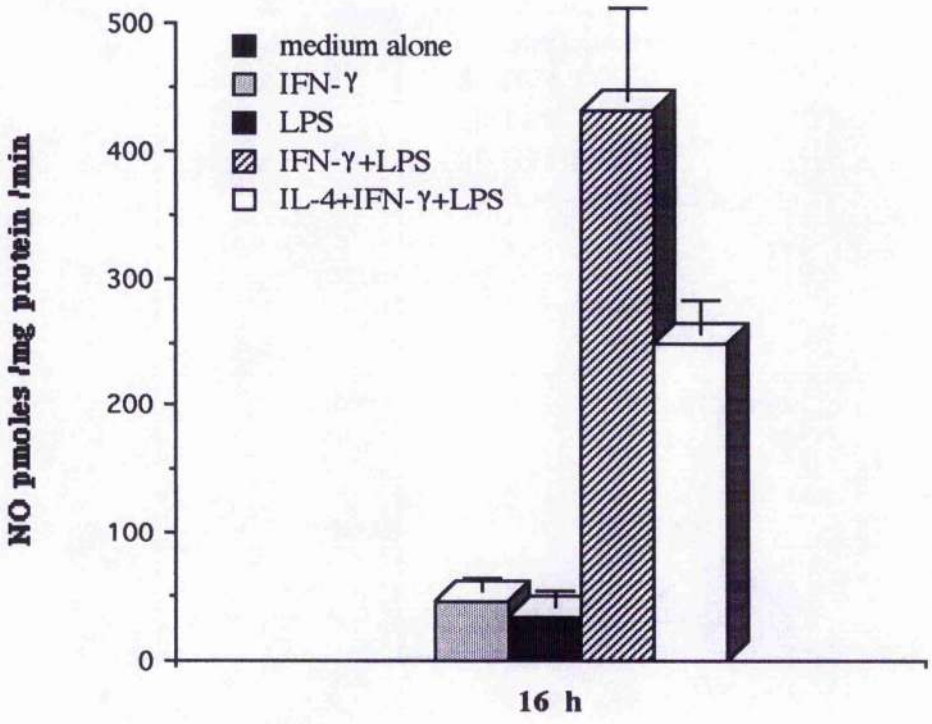


Fig 6.3 IL-4-mediated inhibition on iNOS activity by enzyme assay. J774 cells were cultured in 25 cm^2 flasks. IL-4 (100 U/ml) was added simutaneously with IFN-γ (100 U/ml) and LPS (10 ng/ml). Cells were harvested at 16 h after stimulation. Total proteins were extracted, and iNOS enzyme activity was measured using a dual-wavelenth method. Data are presented as means of triplicates (±SD).

Decreased enzyme activity indicated that IL-4 may interfere with iNOS protein synthesis or iNOS transcription. Immunoblotting with an monoclonal antibody to murine macrophage iNOS shows that in the presence of IL-4 (either in a pre-treatment or added simultaneously), iNOS expression induced by IFN-γ and LPS was decreased and delayed: For example, whilst the maximum level of NOS expression achieved was about 8 h following the stimulation with IFN-γ and LPS, it took 10-12 h to obtain maximal expression in the case of cells pre-treated with IL-4. Moreover, protein expression were generally lower in all time points. These Western blotting results demonstrate that the diminished iNOS enzyme activity was paralleled by a marked reduction of iNOS protein (Fig. 6.4) and thus, the inhibition is at, or before, the level of iNOS protein synthesis.

Further experiments were carried out to investigate the effect of IL-4 on iNOS transcription. Total cellular RNA was isolated and analysed by Northern blotting, using a ^{32}P-labelled murine iNOS cDNA fragment as a probe. As shown in Fig. 6.5, IFN-γ and LPS incubation resulted in iNOS mRNA expression as early as 2 h, increasing to a maximum level at 4 h. It was however reduced and delayed in the samples treated with IFN-γ, LPS and IL-4.

This disruption of iNOS transcription therefore suggests that IL-4 mediated inhibition of NO synthase may involve either a mechanism of blocking IFN-γ/LPS signalling pathways or interfering directly with gene regulation of NOS expression.

6.4 IFN-γ or LPS induced NO production is inhibited by IL-4

To address whether IL-4 targeted signals derived from IFN-γ, LPS or the synergistic pathway, IFN-γ or LPS alone was used to stimulate J774 cells. NO production induced by IFN-γ plus LPS was only partially reduced (<30%) by the addition of IL-4 (Fig. 6.6). However, when cells were stimulated by LPS alone, NO induction was completely blocked by IL-4 (Fig. 6.7). In this experiment, LPS was used at 2 μg/ml. At such a high concentration, LPS-induced NO accumulated in the culture supernatants to a level of about 15μM.

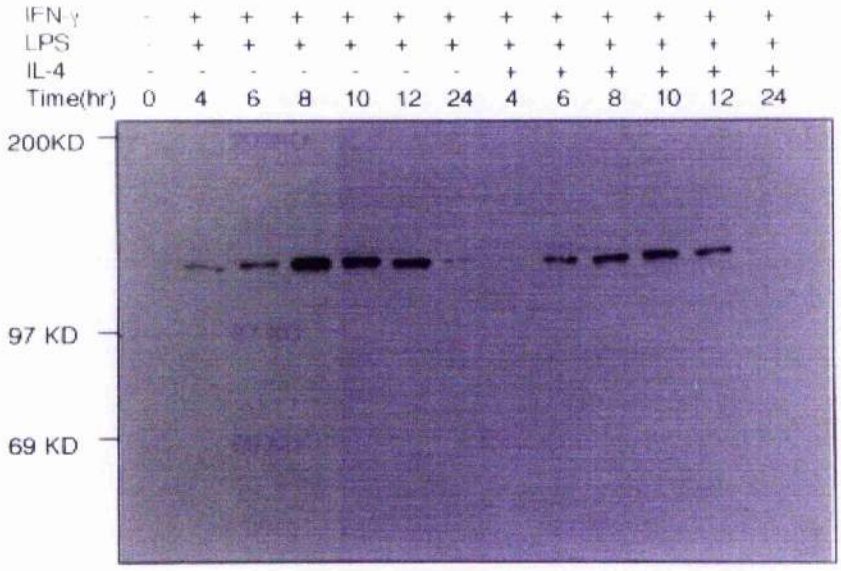


Fig. 6.4 Effect of IL-4 on iNOS protein synthesis induced by IFN-γ and LPS. J774 cells were cultured in 25 cm^2 flasks and incubated with IFN-γ (100 U/ml) and LPS (10 ng/ml) in the presence or absence of IL-4 (100 U/ml). Non-adherent cells were removed before cells were lysed 0-24 h after stimulation. Total protein (20 μg each) were resolved in 7.5% SDS PAGE and followed by immunoblotting using an monoclonal antibody against murine macrophage NOS and visualised by ECL reagents.

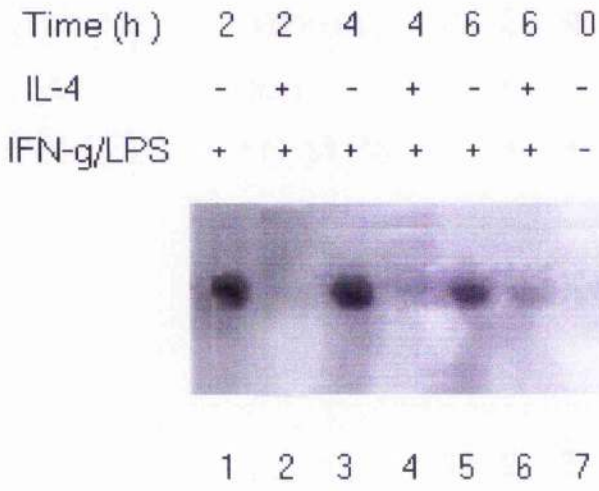


Fig. 6.5 Effect of IL-4 on iNOS mRNA transcription in J774 cells. Cells were stimulated with IFN-γ (100 U/ml) and LPS (10 ng/ml) with or without IL-4 (100 U/ml) for 2-6 h. Total RNA was isolated with RNA_{ZOL} B. Samples (10 μg each) were analysed by Northern blotting and hybridized with iNOS cDNA fragment (372bp) labelled with ^{32}P-dATP. The results are representative of two experiments.

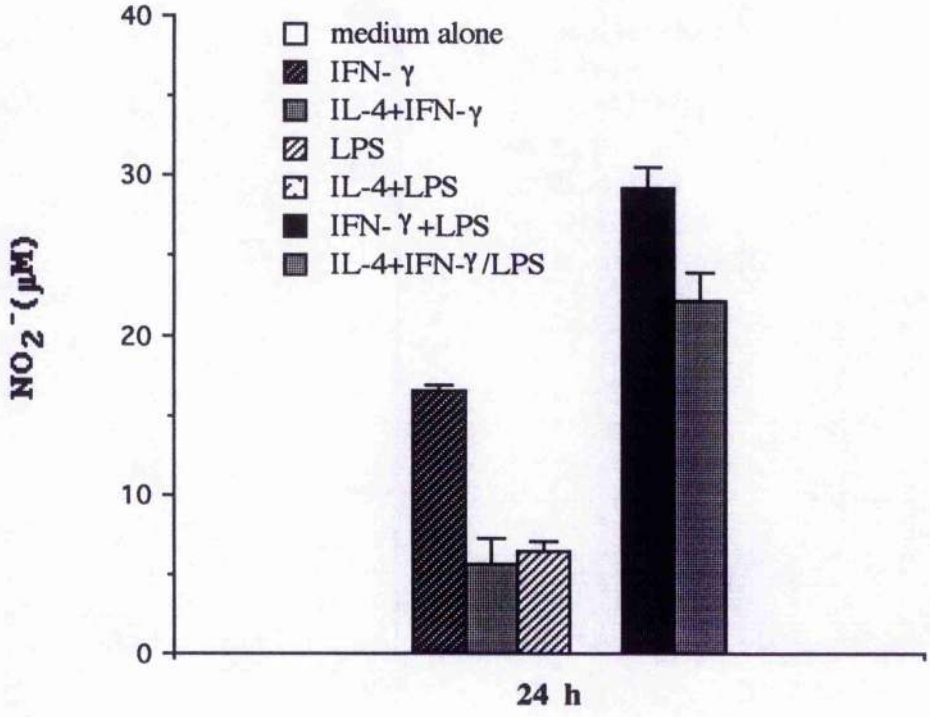


Fig. 6.6 Effect of IL-4 on LPS- or LPS plus IFN-γ-induced NO synthesis. J774 cells were incubated with LPS (10 ng/ml) and/or IFN-γ (100 U/ml) in the presence or absence of IL-4. Culture supernatants were collected 24 h after stimulation , and assayed for nitrite by the Griess method. Data are presented as means of triplicates (±SD). The results are representative of three experiments.

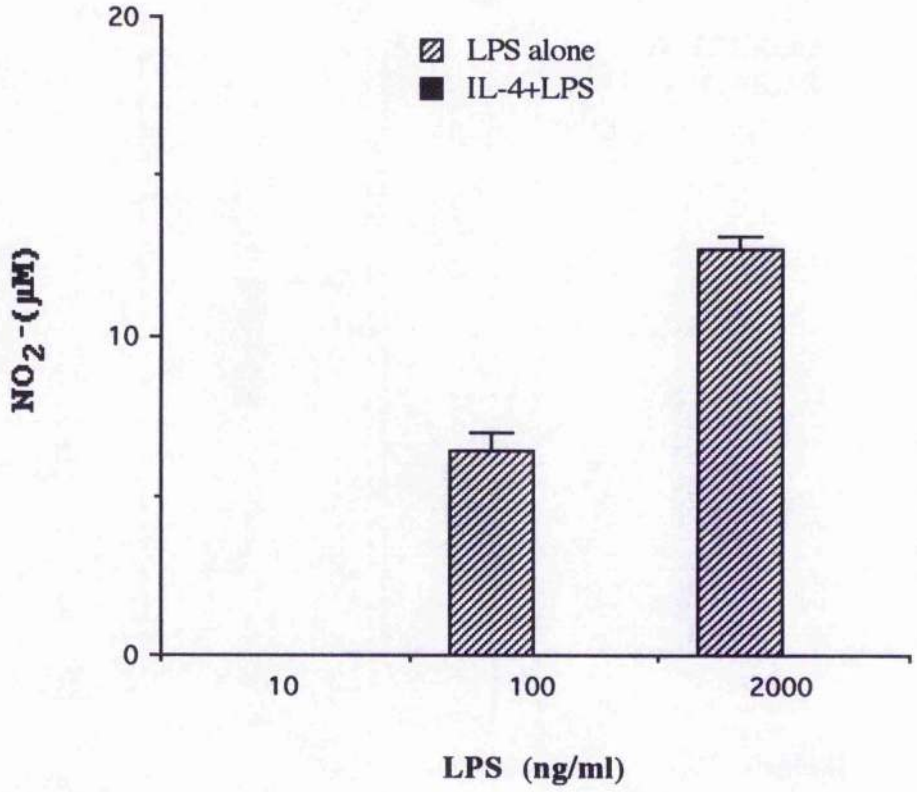


Fig. 6.7 Effect of IL-4 on LPS -induced NO synthesis. J774 cells were cultured with LPS (10 ng/ml) at different concentrations with or without IL-4 (100 U/ml). Culture supernatants were collected at 24 h after stimulation, and assayed for nitrite by the Griess method. The value of IL-4-treated samples were zero. Data shown are the means of triplicate cultures (±SD). The results are representative of two experiments.

The effect of IL-4 on IFN-γ-induced NO production was also tested. Cells were stimulated with IFN-γ at concentrations of 10, 100 and 1000U/ml. IFN-γ alone can induce low but significant amount of NO_2^- reaching a plateau at 10 U/ml. IL-4 showed a stronger inhibition at the lower rather than higher concentrations of IFN-γ, but it did not completely block NO synthase (Fig. 6.8). These results indicated that IL-4 exerts its inhibitory effect on NO synthesis on both the IFN-γ and LPS pathways.

6.5 IL-4 does not interfere with LPS-induced NFκB binding activity

Transcription factor NFκB is composed of two subunit of 50 and 65 kDa, which form a heterodimer that binds to κB motifs. This factor is normally present in the cytosol in an inactive form bound to an inhibitor protein, IκB. Phosphorylation of IkB induces dissociation of NFkB from IkB followed by translocation of NFkB to the nucleus in an active form that binds to DNA (reviewed in Takasuka et al., 1995). Protein kinase C (PKC) is one of the kinases that phosphorylates IkB directly and is involved in the activation pathway of NFkB (Ghosh and Baltimore 1990; Shirakawa and Mizel 1989). It was reported that LPS or lipid A (biologically active lipid moiety of LPS) induce the activation of PKC in macrophages (Wightman and Raetz 1984) and B cells (Chen *et al.*, 1986).

In J774 cells, upon the stimulation of LPS, NFkB was activated and translocated to the nucleus where it binds to the specific DNA elements in the LPS responsive genes. Previous results (chapter 5) have shown that LPS-treated cells contains a protein which is able to bind to the NFκB element found in the iNOS promoter. To determine whether NFκB activation could be altered by IL-4, immunoblotting was carried out in the nuclear extracts using antibody against NFκB P65 to detect NFκB nuclear translocation. Upon stimulation with LPS, NFκB (P65) was translocated to the nucleus within 15 min and interestingly, there was no significant difference in translocation between the samples stimulated with LPS in the presence or absence of IL-4 (Fig. 6.9).

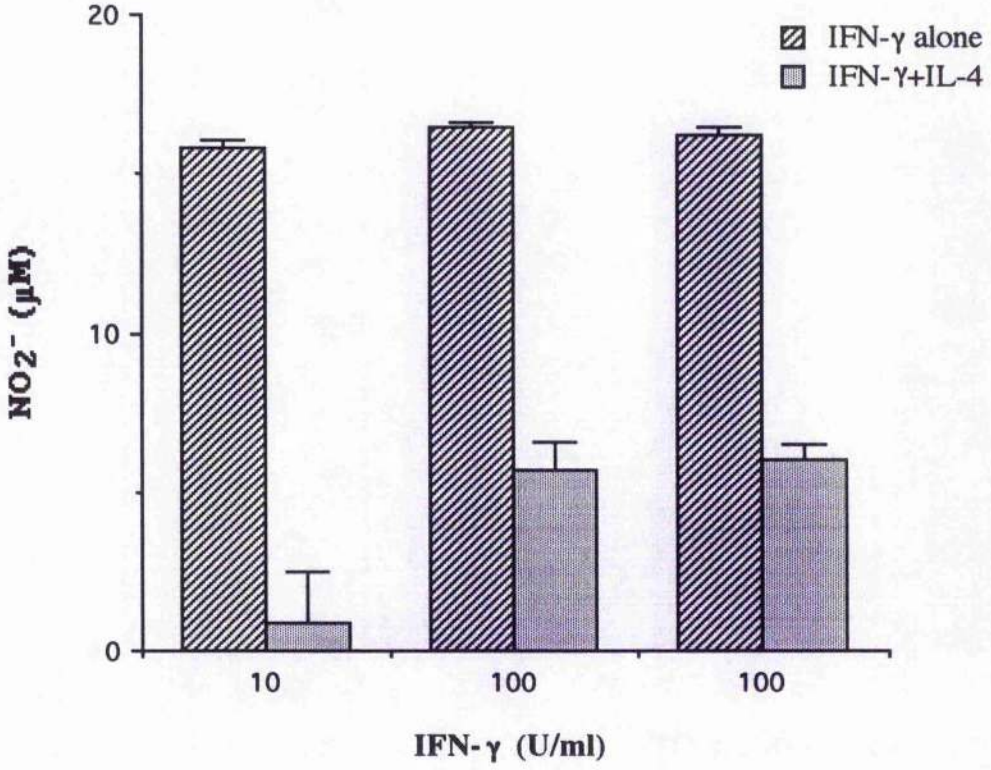


Fig. 6.8 Effect of IL-4 on IFN-γ-induced NO production. J774 cells were stimulated with IFN-γ at different concentration with or without IL-4 (100 U/ml) for 24 h. Culture supernatant were collected and assayed for NO_2^- by the Griess method. Data are shown as means of triplicates (±SD). The results are representative of three experiments.

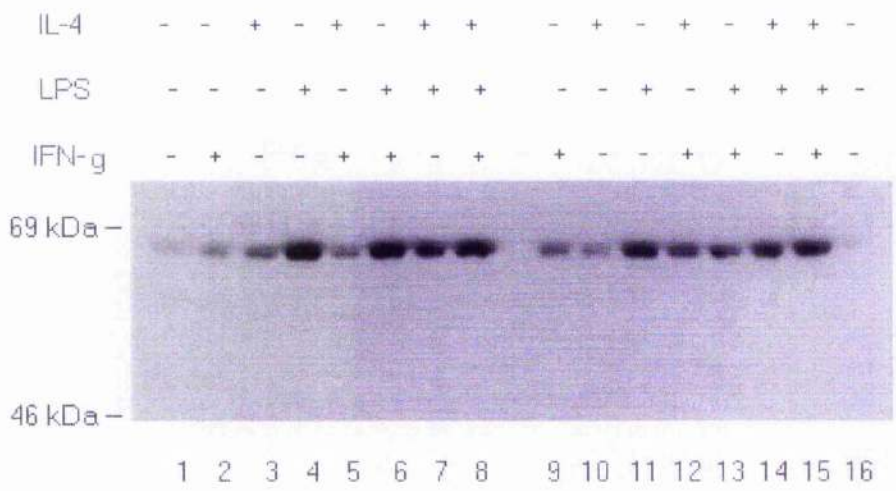


Fig. 6.9 Effect of IL-4 on NFκB nuclear translocation induced by LPS. J774 cells were incubated with IFN-γ (100 U/ml) or /and LPS (10 ng/ml) in the presence or absence of IL-4 (100 U/ml) for 15 min (lanes 1-8) or 2 h (lanes (9-16). Nuclear proteins were extracted and 20 μg of each were resolved in 10% SDS PAGE, and transferred to nitrocellulose membrane followed by probing with a polyclonal anti-NFκB-P65 antibody.

Nuclear extracts were prepared from the cells treated with IFN-γ and /or LPS in the presence or absence of IL-4 for 2 h and EMSAs were carried out with the NFκB specific oligonucleotide probe corresponding to the promoter region of the iNOS gene. As shown in Fig. 6.10, there is a constitutive binding activity by the p50/p50 homodimer (Shown by antibody super shift assay as seen in chapter 5). Following stimulation with LPS , a slower migrated binding complex also appeared which is due to a complex, p65/p50 heterodimer (See chapter 5). IL-4 did not induce the same binding activity on its own, nor did it interfered with the binding activity induced by LPS.

MAP kinase activation was another response observed in LPS-treated cells, although the link between MAP kinase and iNOS expression was not clear. Here, MAP kinase tyrosine phosphorylation was used as a tool to test whether IL-4 alters LPS signalling. Immunoblotting showed that MAP kinase (ERK2) was tyrosine phosphorylated within 30 min of stimulation with LPS. IL-4 did not induce MAP kinase phosphorylation by itself, nor did it interfere with the activation induced by LPS (Fig. 6. 11). However, when cells were treated with LPS and IFN-γ, tyrosine phosphorylation of MAP kinase was down-regulated by IL-4. This was of interest, because a similar pattern of results was obtained with respect to IL-4 modulation of IRF-1 expression. This will be discussed later.

6.6 Effect of IL-4 on LPS-inducible IRF-E binding activity

IRF-E is another important gene site regulated by IFN-γ and LPS in the iNOS promoter. In J774 cells, LPS-treatment leads to the activation of an IRF-E binding protein whose activity is dependent on protein synthesis. IL-4, however, did not interfere with such binding activity induced by IFN-γ or LPS (Fig. 6. 12).

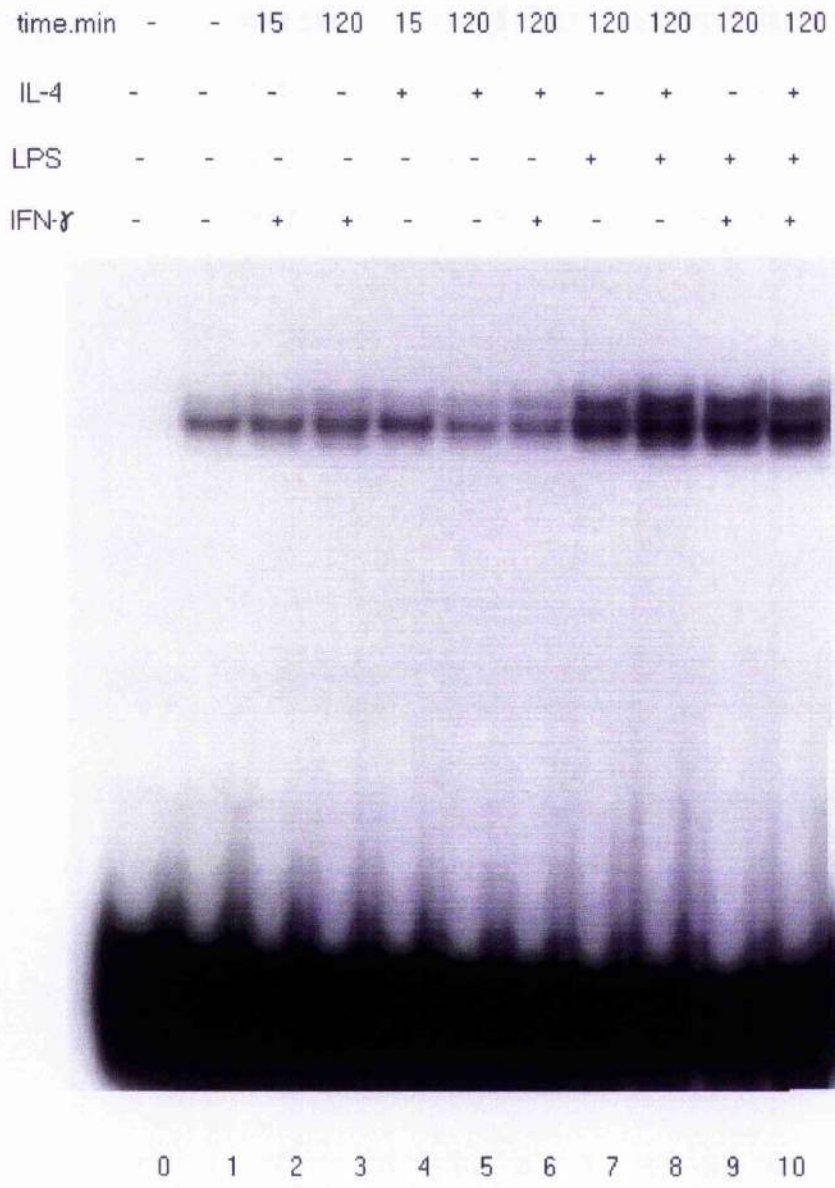


Fig.6.10 NFκB binding activity induced by LPS in the presence or absence of IL-4. J774 cells were incubated with IFN-γ (100 U/ml) or /and LPS (10 ng/ml) in the presence or absence of IL-4 (100U/ml) for 2 h, and nuclear proteins extracted. EMSA was carried out by incubation of 5 μg of nuclear protein with a ^{32}P-labeled oligonucleotide probe D containing the NFκB specific sequence found in the promoter of the murine iNOS. There are two binding complexes: the faster migrated band is P50/P50 homodimer whereas the slower one is the P65/P50 heterodimer.

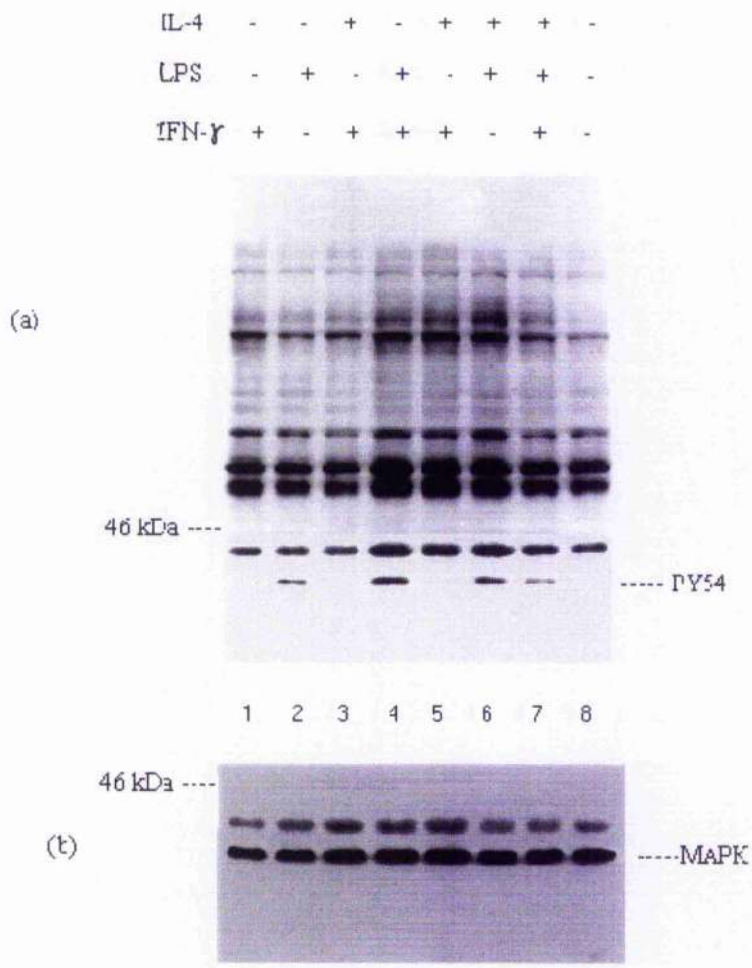


Fig.6.11 Effect of IL-4 on MAP kinase tyrosine phosphorylation induced by LPS. J774 cells were incubated with LPS (10 ng/ml) with or without IL-4 (100 U/ml) for 30 min before lysed.Total protein (20 μg each) were resolved in 10% SDS PAGE, and subjected to Western blotting using anti-phosphotyrosine (PY54) (a). Following stripping, the blot was re-probed with anti-MAP kinase (erk2) antibody (b). The arrows indicate the same position of the blot.

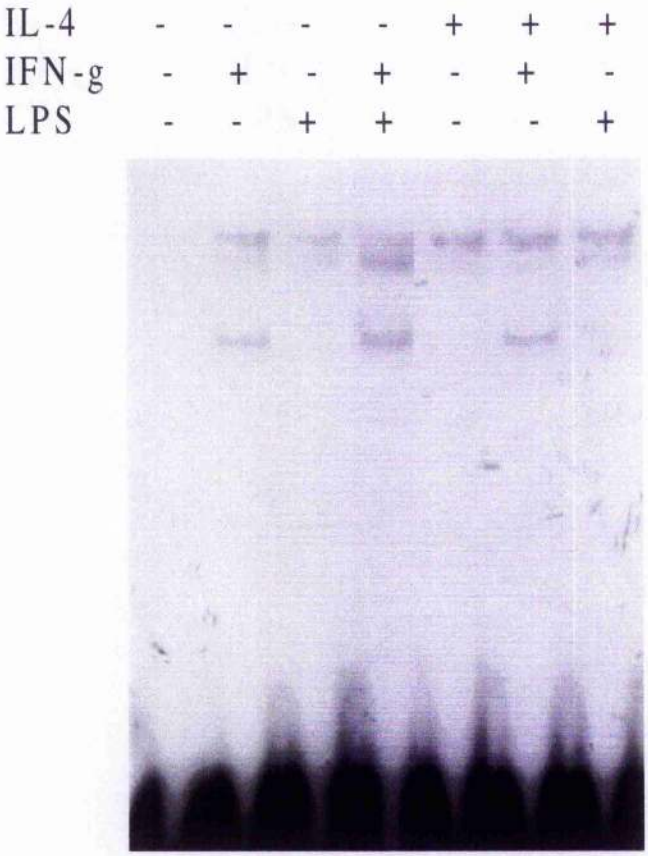


Fig. 6.12 Effect of IL-4 on IRF-E binding activity induced by IFN-γ and/or LPS. J774 cells were incubated with IFN-γ (100 U/ml) or /and LPS (10 ng/ml) in the presence or absence of IL-4 (200 U/ml) for 2 h, and nuclear proteins extracted. EMSA was carried out by incubation of 10 μg of nuclear protein with a ^{32}P-labeled oligonucleotide probe G containing the ISRE (IRF-E) sequence found in the iNOS promoter.

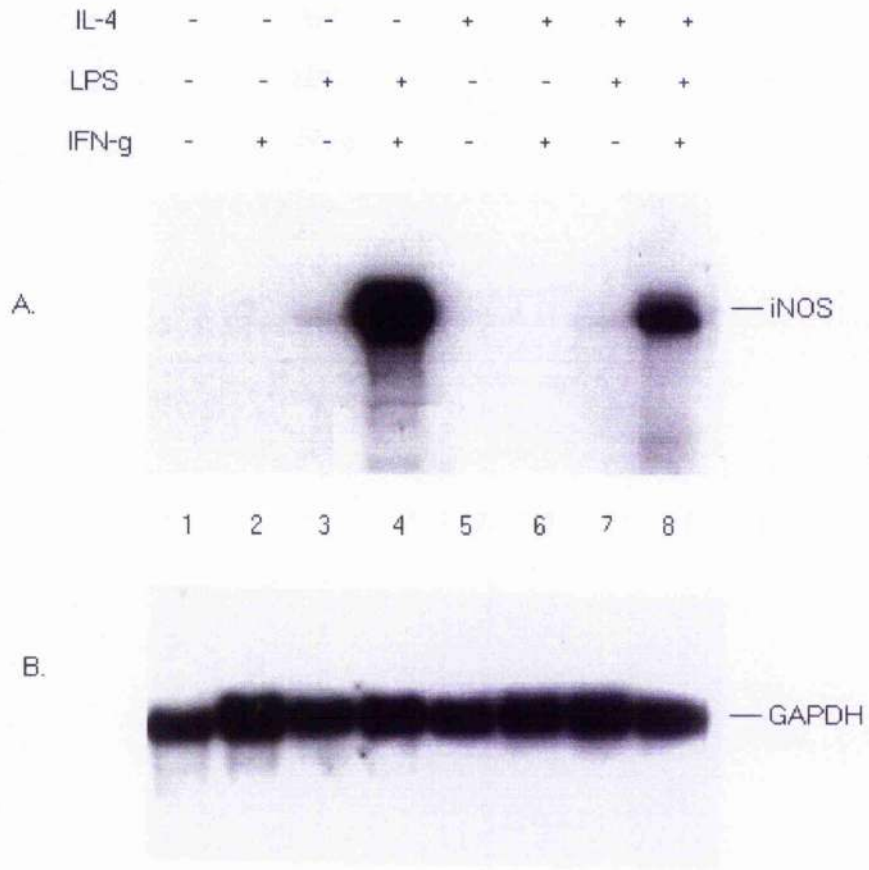


Fig. 6.13. Effect of IL-4 on iNOS mRNA induced by LPS. J774 cells were stimulated with IFN-γ (100 U/ml) and/or LPS (10 ng/ml) in the presence (lanes 5-8) or absence (lanes 1-4) of IL-4 (100 U/ml) for 3 h. Total RNA was isolated with $RNA_{ZOL}B$. Samples (10μg/each) were analysed by Northern blotting and hybridized with iNOS cDNA fragment (372bp) labelled with ^{32}P-dATP. The lower pannel showed the GAPDH in each sample by reblotting using a human GAPDH cDNA probe.

Is the IL-4 inhibition of iNOS induced by LPS alone different from that on the response induced by costimulation with IFN-γ and LPS? iNOS mRNA was detected in J774 cells after stimulation with IFN-γ and /or LPS for 3 h. iNOS mRNA induced by IFN-γ and LPS was significantly reduced by the co-incubation with IL-4, whereas there was little or no difference in the levels of iNOS mRNA induced by LPS in cells treated with or without IL-4 (Fig. 6.13) (see pp176).

6.7 IL-4 blocked LPS-induced TNF-α production

Tumor necrosis factor alpha (TNF-α) is a cytokine produced by activated macrophages which plays an important role in the regulation of immune and inflammatory responses (Lieberman *et al.*, 1989). TNF-α is induced by LPS. To test the correlation between TNF-α production and iNOS induction in J774 cells, ELISA was used for detecting TNF-α production in the culture supernatants of samples treated with IFN-γ and /or LPS with or without IL-4. While there is no detectable TNF-α in unstimulated cell cultures, treatment with LPS for 24 h led to a significant amount of TNF-α accumulating in the culture supernatants (up to 1 ng/ml). In the presence of IL-4, TNF-α production was markedly decreased (down to less than 100 pg/ml) (Fig. 6.14). Similar results were obtained from cells treated with IFN-γ and LPS in the presence or absence of IL-4. However, while synergistically enhancing NO production by LPS, IFN-γ showed a down-regulatory effect on LPS-induced TNF-α production. It is perhaps that IFN-γ and LPS together altered the kinetic of cytokine production.

6.8 JAK-STAT pathway was not affected by IL-4

Previous studies from my work (Chapter 5) and others demonstrated that tyrosine kinase activity is required for IFN-γ and /or LPS-induced iNOS expression, and protein tyrosine kinase inhibitors modify expression of iNOS. We have also demonstrated that IFN-γ induces the tyrosine phosphorylation of STAT1 (P91), one of the components of

the ISGF3 transcription complex (Lee 1995; Darnell 1994). Tyrosine phosphorylation of STAT1 in response to IFN-γ has also been shown to require tyrosine phosphorylation and activation of two protein tyrosine kinases, JAK1 and JAK2 (Chapter 4; Muller *et al.*, 1993; Muller *et al.*, 1993).

To determine whether IL-4 interferes with IFN-γ-induced signalling, the effect of IL-4 on IFN-γ-induced tyrosine phosphorylation of JAK1, JAK2, and STAT1 was examined. In J774 cells, IFN-γ induced the tyrosine phosphorylation of JAK1 and JAK2. Treatment of J774 cells with IL-4 alone induces tyrosine phosphorylation of JAK1 but not JAK2. Culture with IL-4 (either following pretreatment for 1 h or 18 h) before or during costimulation with IFN-γ did not inhibit IFN-γ-induced tyrosine phosphorylation of JAK2 (Fig. 6.15). In the presence of IL-4, IFN-γ-induced tyrosine phosphorylation of JAK1 was also not affected (Fig. 6.16). Furthermore, IL-4 alone did not phosphorylate P91, and IFN-γ-induced tyrosine phosphorylation of P91 was unaffected by treatment with IL-4 (Fig. 6.17. and 6.18).

6.9 STAT6 activation and IL-4 induced GAS binding activity

After binding to its receptor, IFN-γ rapidly induces transcription of target genes. Essential to this rapid activation is the presence of a latent cytoplasmic pool of JAK-STAT molecules. Binding of IFN to its receptor activates these preformed cytoplasmic factors, rendering them competent for nuclear translocation and DNA binding. JAK activation in IL-4-treated cells potentially indicated a similar latent cytoplasmic factor (IL-4-STAT, also named STAT6) involved in the inhibitory pathway. To ascertain whether IL-4-STAT is induced by a similar mechanism, J774 cells were cultured with IL-4 for 2 h and nuclear extracts prepared for the nuclear translocation assay by western blotting using an antibody against STAT6. In J774 cells, there was a basal level of STAT6 in the nucleus. This was enhanced by the treatment of IL-4 but not by IFN-γ or LPS (Fig. 6.19).

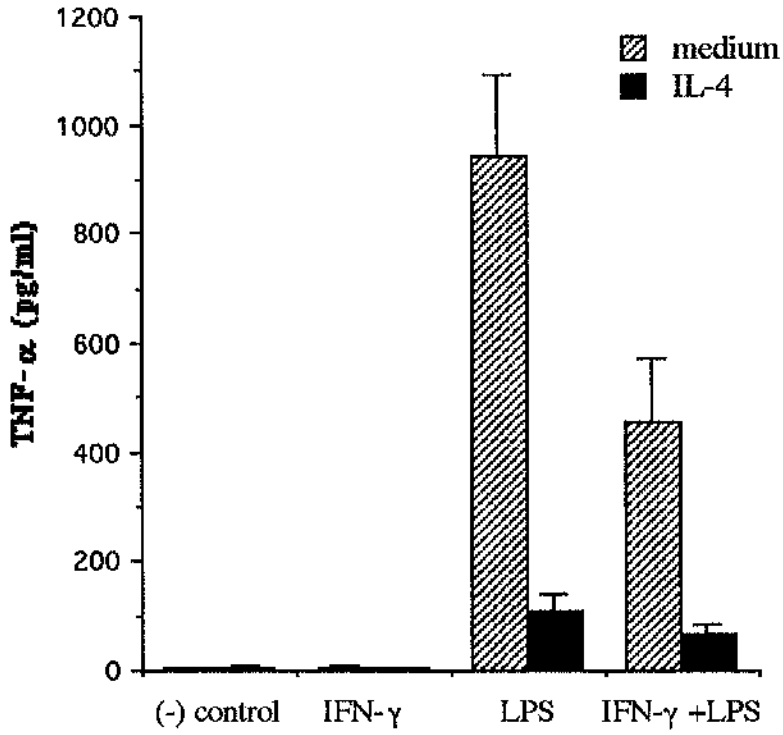


Fig. 6.14 Effect of IL-4 on TNF-α production. J774 cells were incubated with LPS (10 ng/ml) and /or IFN-γ (100 U/ml) in the presence or absence of IL-4 (100 U/ml). Culture supernatants were collected 24 h after stimulation, and assayed for TNF-α production by ELISA. Data are presented as means of triplicates (±SD).

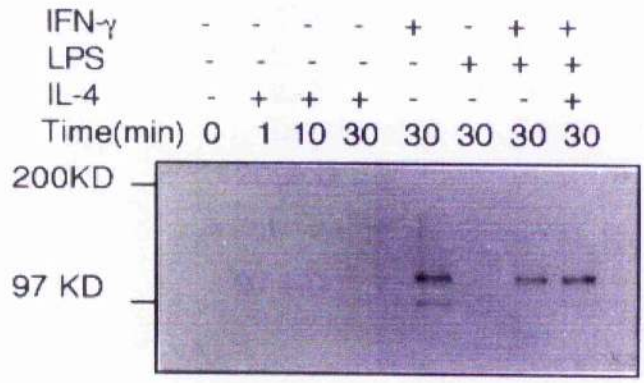


Fig.6.15 Effect of IL-4 on JAK2 tyrosine phosphorylation induced by IFN-γ. IFN-γ-induced tyrosine phosphorylation of JAK2 was assayed in whole cell extracts of J774 cells. IFN-γ (100 U/ml) or LPS (10 ng/ml) were added with or without IL-4 (100 U/ml) for 30 min. Extracts were immunoprecipitated with polyclonal antisera to JAK2 and probed after SDS-PAGE (7.5%) analysis with anti-phosphotyrosine (RC20).

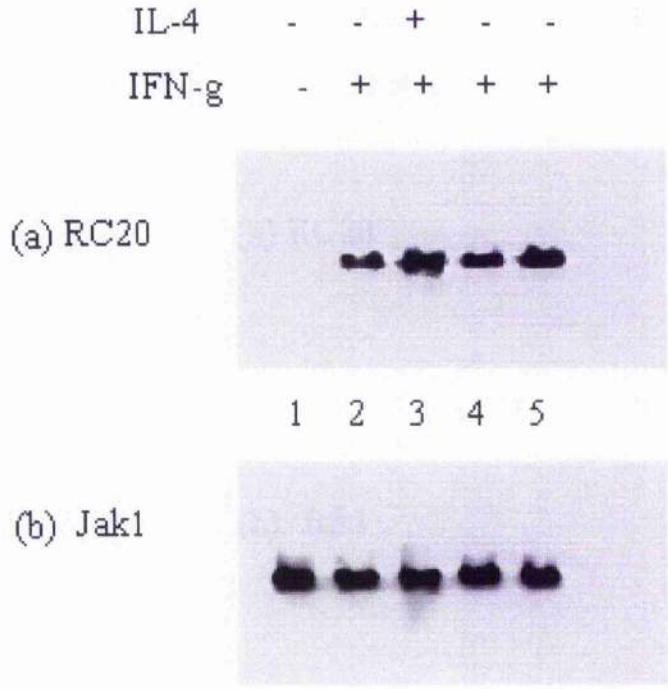


Fig. 6.16 Effect of IL-4 on JAK1 tyrosine phosphorylation. IFN-γ-induced tyrosine phosphorylation of JAK 1 was assayed in whole cell extracts of J774 cells. IFN-γ (100 U/ml) was added in the prsence or absence of IL-4 (100 U/ml) for 30 min. Extracts were immunoprecipitated with polyclonal antisera to JAK1 and probed after SDS-PAGE (7.5%) analysis with (a) anti-phosphotyrosine (RC20) and, after stripping, (b) antibody to JAK1.

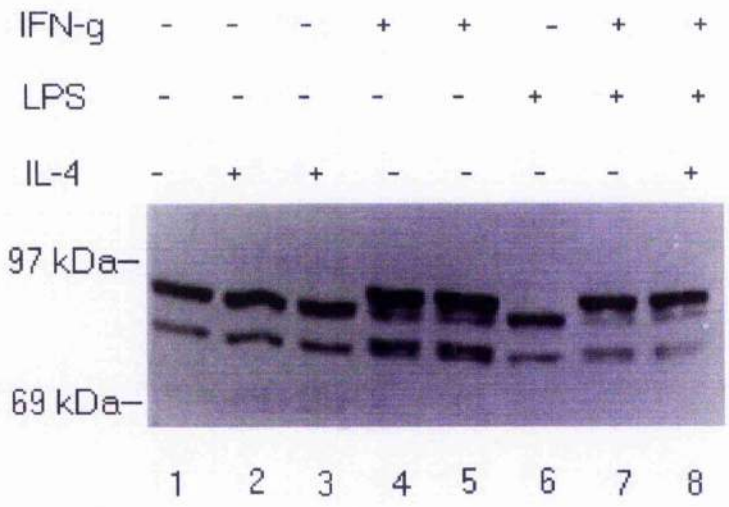


Fig. 6.17 Effect of IL-4 on STAT1 phosphorylation induced by IFN-γ. Culturing in the 25 cm^2 flasks, J774 cells were incubated with IFN-γ (100 U/ml) and/or LPS (10 ng/ml) in the presence or absence of IL-4 (100 U/ml) for 30 min. Total proteins were extracted and 20μg of each were resolved in 7.5% SDS PAGE, followed by probing with a polyclonal anti-STAT1 antibody. The antibody recognised both P91 and P84. The bands (from top to bottom) are phosphorylated P91, P91, phosphorylated P84, and P84.

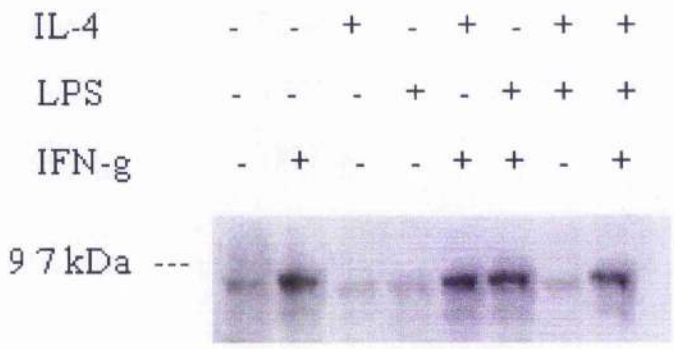


Fig. 6.18 Effect of IL-4 on STAT1 nuclear translocation induced by IFN-γ. J774 cells were incubated with IFN-γ (100 U/ml) and/or LPS (10 ng/ml) in the presence or absence of IL-4 (100 U/ml) for 1 h. Nuclear proteins were extracted and 20 μg of each were resolved in 7.5% SDS PAGE, followed by probing with a polyclonal anti-STAT1 (P91/84) antibody. The antibody recognised both P91 and P84.

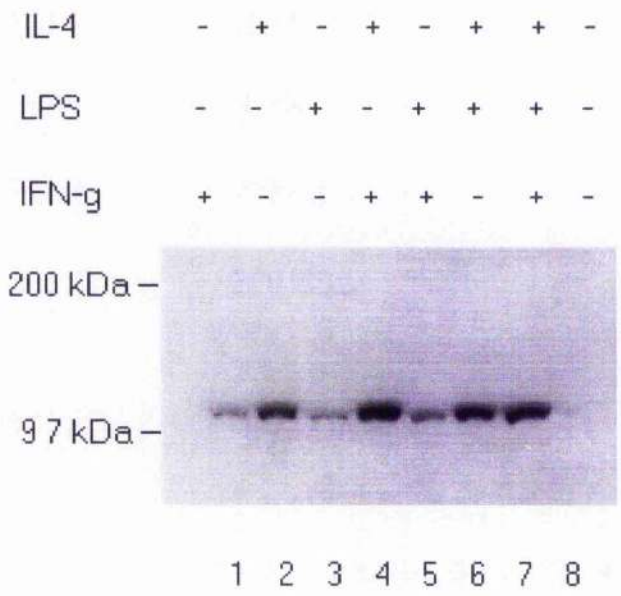


Fig. 6.19 STAT6 (IL-4-STAT) activation and nuclear translocation induced by IL-4. Culturing in the 75 cm^2 flasks, J774 cells were incubated with IFN-γ (100 U/ml), LPS (10 ng/ml), or IL-4 (100 U/ml) for 2 h. Nuclear proteins were extracted and 20μg of each were resolved in 7.5% SDS PAGE, followed by probing with a polyclonal anti-STAT6 antibody, detected by ECL system.

The low level of STAT6 constitutively present in the nuclear extracts may reflect the basal activity of STAT6-DNA binding in J774 cells or may be due to the contamination of cytoplasmic proteins in nuclear phase.

Most biological functions of cytokines rely on their ability to stimulate the transcription of particular cytokine-responsive genes. Being members of the same hematopoietin receptor superfamily, the pathways of IFNs and IL-4 are likely to involve similar mechanism of signal transduction. It is known that the ability of IFN-γ to stimulate transcription can be mediated through STAT1, which in the form of homodimers is known to bind to GAS elements in responsive promoters. Several other cytokines have been shown to activate factors that bind the GAS site of IRF-1 gene or closely related sequences.

To determine if IL-4 stimulated STAT6 binds to the GAS element of IRF-1 promoter, J774 cells were cultured with IL-4, IFN-γ or both IFN-γ and IL-4 for 1 h. Nuclear extracts were isolated and assayed by EMSAs with an oligonucleotide corresponding to the IRF-1 GAS consensus element (Fig. 6.20). Extracts isolated from IFN-γ-treated cells contained a induced binding protein relative to those of untreated cells. The binding protein has already been shown to be STAT1 in chapter 5 (Fig. 5.1b). When the cells were cultured with IL-4 for 1 h, they also contain a GAS- binding activity. The IL-4-induced DNA binding complex migrated more slowly than the IFN-γ STAT-DNA complex. It was super-shifted by an antibody against STAT6 but not by anti-STAT1. In untreated cells, there was a low level constitutive binding of the GAS element and the binding complex was also super-shifted by anti-STAT6. When these cells were co-cultured with IL-4 and IFN-γ, both activities were induced at levels comparable with those seen when the cytokines were added individually.

6.10 IL-4 up-regulates IFN-γ-induced IRF-1 and IRF-2

IL-4, appears to exert its biological function mostly through STAT activation (Kiyoshi *et al*, 1996, Kazuya *et al*, 1996). In J774 cells, nuclear extracts from IL-4 treated cells contained a GAS-binding protein (Fig. 6.20a) which is recognised by an antibody against STAT6 (Fig. 6.20b). Since the GAS-element plays a key role in the IRF-1 gene activation by IFN-γ, it is possible that IL-4 may work through STAT6 to interfere the STAT1 GAS-binding and therefore to interfere with IRF-1 gene transcription. Northern blot analysis was therefore carried out to detect mRNA expression of IRF-1 and IRF-2 under the stimulation of IFN-γ with or without IL-4.

Although undetectable in cells treated with IL-4 alone within an initial 6 h period, the mRNA of IRF-1 induced by IFN-γ was enhanced by co-incubation with IL-4 (Fig. 6.21). Initially, this was hard to explain as whilst IL-4 up-regulates the iNOS-activator, IRF-1, it down-regulates iNOS expression. However, upregulation of IRF-2 by IL-4 may provde a mechanism to resolve this paradox. Earlier reports of site-specific mutation of IRF-E, within the context of the full-length iNOS promoter / enhancer region, has revealed the action of a silencer (Martin *et al.*, 1994): the IRF-E itself is the silencer element while IRF-2 (Harada, *et al.*, 1989) and the IFN consensus sequence binding protein (ICSBP) are the inhibitory factors which act as constitutive repressors in other systems (Driggers *et al.*, 1990). In the J774 system, although IL-4 acts as a enhancer for IRF-1 induced by IFN-γ, it may fail to up-regulate IRF-E whose activation is dependent on the ratio of IRF-1/IRF-2.

Unlike IRF-1, IRF-2 mRNA is constitutively expressed in J774 cells incubated with medium alone although the expression is very low. There was no significant changes in this basal level in the cells treated with IL-4 alone. However, for the IFN-γ-induced IRF-2, it was enhanced when cells were co-cultured with IL-4, in a similar manner to as that of IRF-1 (Fig. 6.22). Taken together, both IRF-1 and IRF-2 were upregulated IL-4.

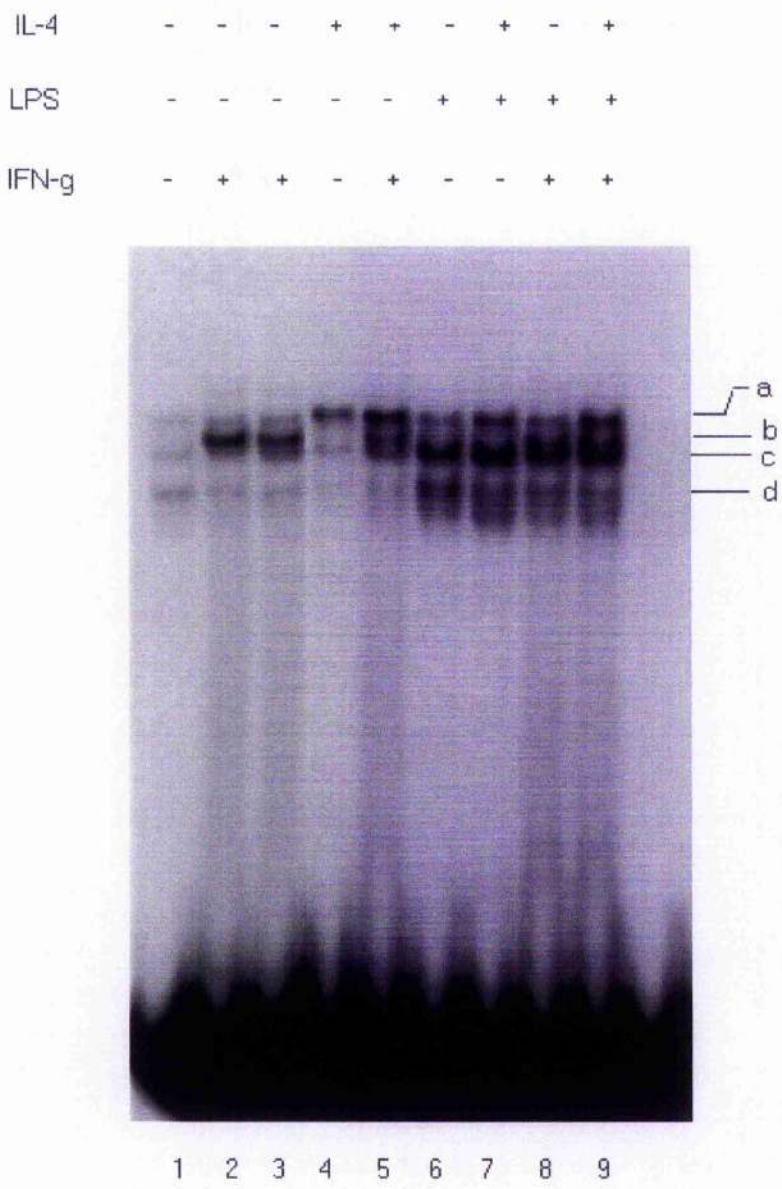


Fig. 6.20a IL-4 induced GAS binding activity. Nuclear extracts were prepared from J774 cells after 1 h of culture with IFN-γ (100 U/ml), or LPS (10 ng/ml) or both in the presence or absence of IL-4 (100 U/ml) , and examined by EMSA with labeled IRF-1 GAS probe. Samples (5 μg of nuclear protein) was incubated with a ^{32}P-labeled oligonucleotide probe F containning the GAS sequence (5'-TACAACAGCC TGATTTCCCCGAATGACGGC-3') found in the IRF-1 promoter.

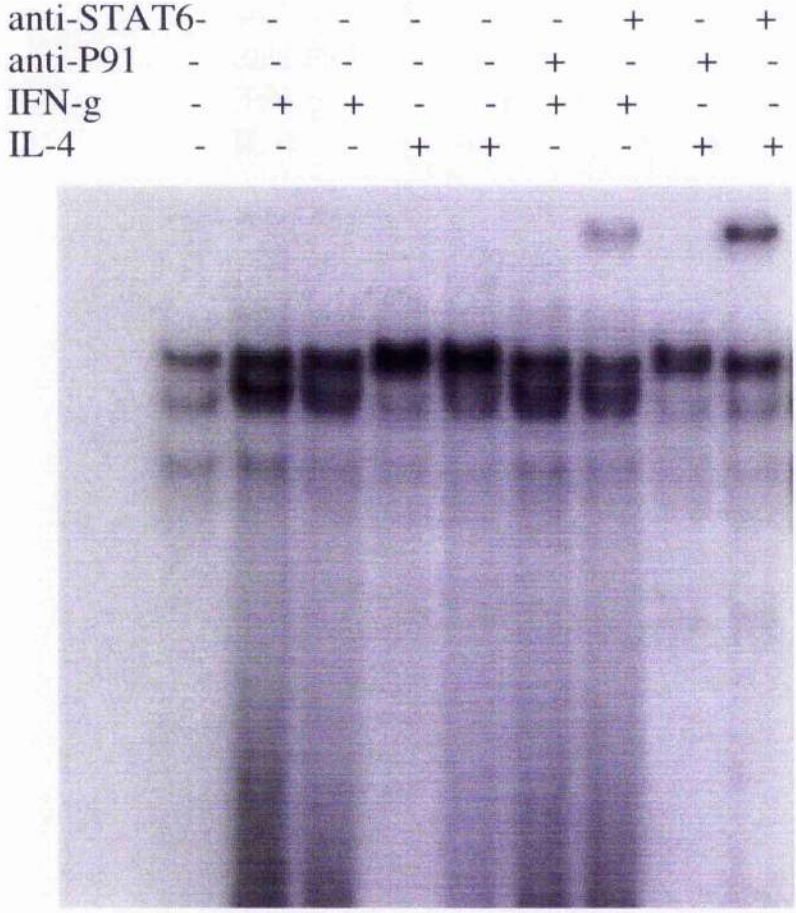


Fig. 6.20b Antibody supershift assay on IL-4-induced GAS binding protein. Same samples as that in Fig. 6.20a (Mobility shift analysis of IRF-1-GAS binding activity in IL-4 and IFN-γ stimulated cells) except that, in some samples, antibodies were added into the reaction mixture 20 min before the probe.

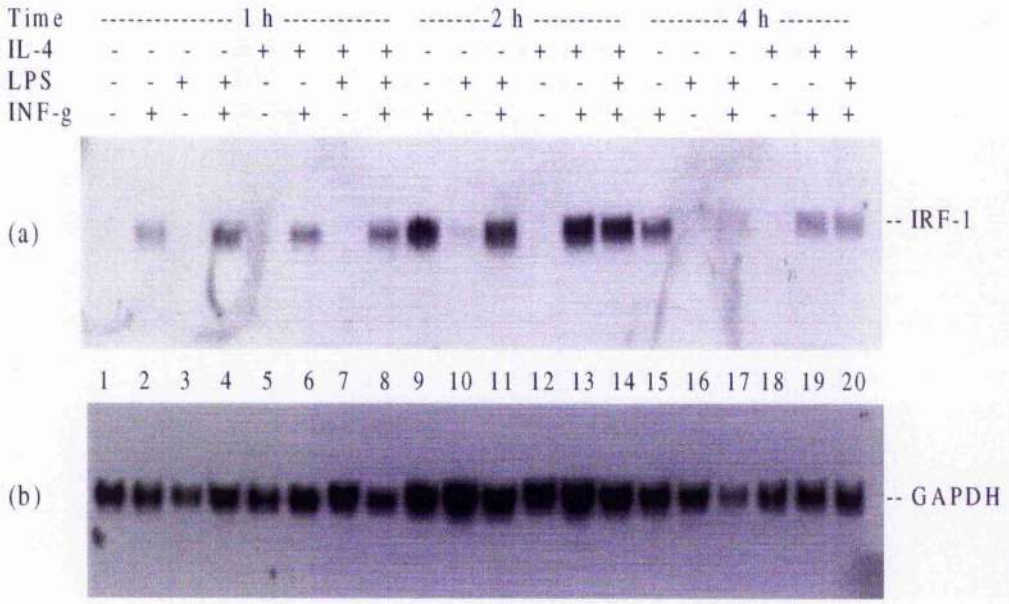


Fig. 6.21 Effect of IL-4 on IRF-1 expression induced by IFN-γ and/or LPS. Total RNA was extracted from IFN-γ (100 U/ml) and/or LPS (10 ng/ml) treated cells with or without IL-4 (100 U/ml) using $RNA_{ZOL}B$. 10 μg of each sample was subjected to Northern blot analysis. IRF-1 mRNA was hybridised using ^{32}P labeled probes that represented the fragment of cDNAs for murine IRF-1 (a) or human GAPDH (b).

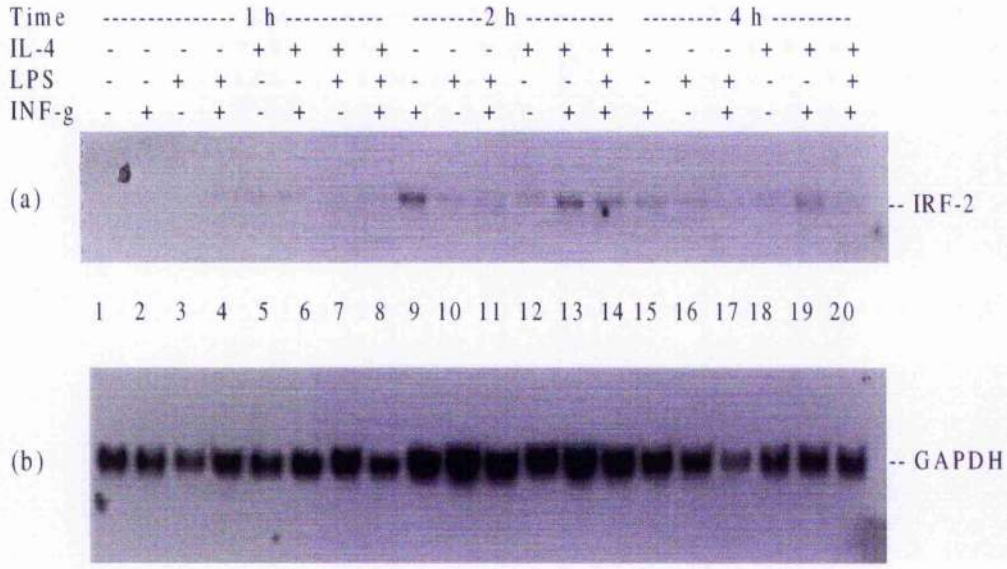


Fig. 6. 22 Effect of IL-4 on IRF-2 expression induced by IFN-γ and/or LPS. Total RNA was extracted from IFN-γ (100 U/ml) and/or LPS (10 ng/ml) treated cells with or without IL-4 (100 U/ml) using $RNA_{zol}B$. 10 μg of each samples was subjected to Northern blot analysis. IRF-2 mRNA was hybridised using ^{32}P labeled probes that represented the fragment of cDNAs for murine IRF-2 (a) or human GAPDH (b).

Thus, IL-4 mediated inhibition of iNOS expression induced by IFN-γ is not likely due to disruption of the IRF-1/IRF-2 ratio.

6.11 IL-4 interferes with IFN-γ-induced NFκB binding activity.

In chapter 5, I presented data that NFκB was activated by IFN-γ 2 h after stimulation. This response is observed later than that induced by LPS alone. Moreover, both IFN-γ and LPS-induced NFκB binding is protein synthesis-independent. In fact, the binding activity was enhanced by pre-treatment with cycloheximide for 1 h. The mechanism of NFκB activation induced by IFN-γ is not yet understood, but may contribute to the iNOS transcriptional activation in co-operation with IRF-1 in response to IFN-γ.

As shown in Fig. 6. 23, NFκB binding was partially blocked when J774 cells were stimulated with IFN-γ in the presence of IL-4. It therefore appears that IL-4, somehow, interferes with the pathway of NFκB activation by IFN-γ by a presently unknown mechanism.

6.12 Effects of IL-4 on IRF-1 and IRF-2 activation by IFN-γ and LPS

Northern blotting has revealed that IRF-1 mRNA induced by IFN-γ is further up-regulated in cells stimulated with IFN-γ in combination with IL-4 or LPS. However, IL-4 and LPS play opposite roles in the regulation of iNOS expression induced by IFN-γ. What if all three stimuli were put together? Northern blot analysis shows a complicated picture. When stimulated with IFN-γ and LPS, IRF-1 expression in J774 cells was significantly higher than that stimulated with IFN-γ alone. However, when IL-4 was added to the same culture, IRF-1 mRNA level was decreased (Fig. 6.21).

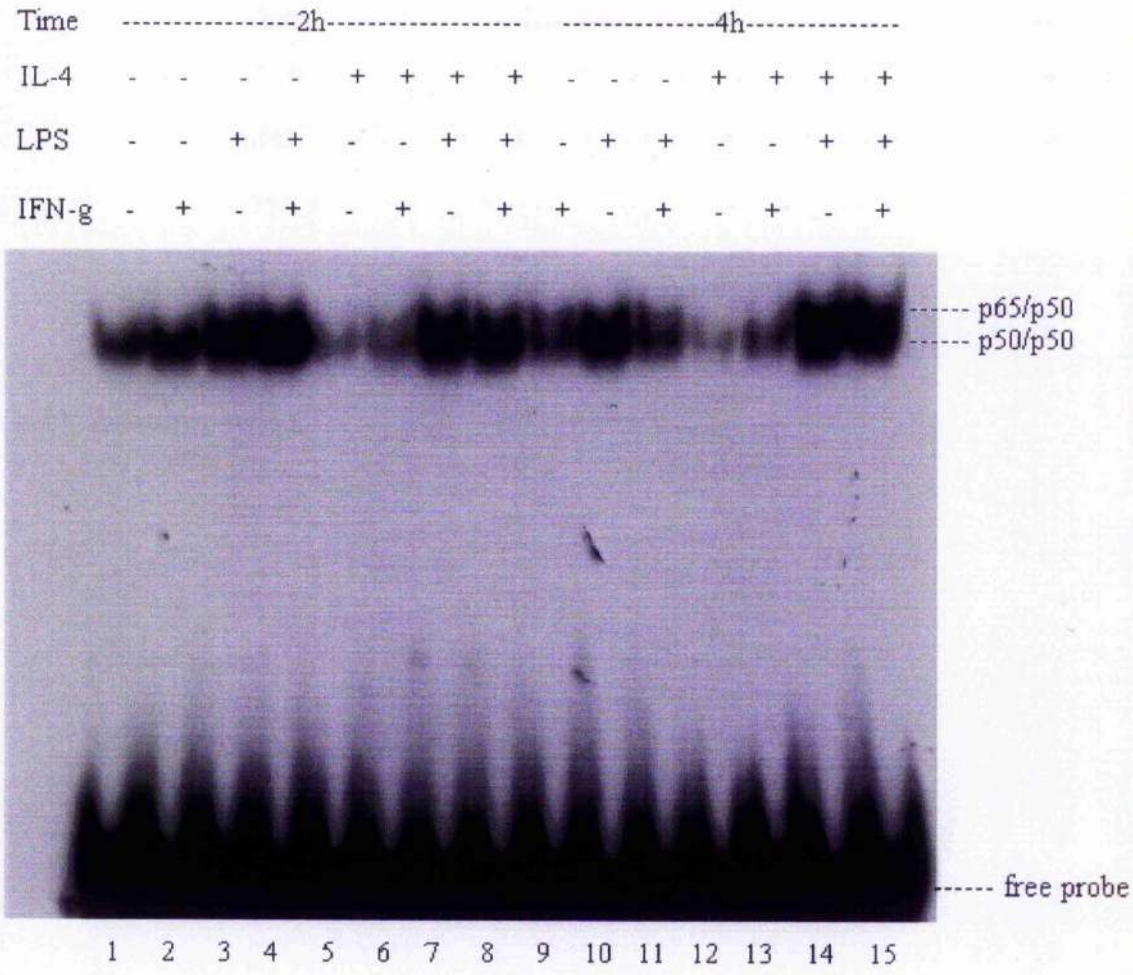


Fig.6.23 NFκB binding activity induced by IFN-γ in the presence or absence of IL-4. J774 cells were incubated with IFN-γ (100 U/ml) or /and LPS (10 ng/ml) for 2-4 h, and nuclear proteins were extracted. EMSA was carried out by incubation of 5 μg of nuclear protein with a ^{32}P-labeled oligonucleotide probe D containning the NFkB specific sequence found in the promoter of the murine iNOS. There are two binding complexes: the faster migrated band is P50/P50 homodimer whereas the slower one is the P65/P50 heterodimer.

In contrast, IRF-2 was induced by IFN-γ and down-regulated by stimulation of cells with IFN-γ in combination with LPS. However, if IL-4 was added to such cultures, IRF-2 mRNA was further enhanced (Fig. 6.22).

Taken all these results together, both IRF-1 and IRF-2 are upregulated by IFN-γ in J774 cells. Moreover, although LPS can further enhance the expression of IRF-1, it reduces the expression of IRF-2. In contrast, IL-4 enhances both IRF-1 and IRF-2. In the case of costimulatory combination of IFN-γ, LPS and IL-4, IRF-1 mRNA was reduced whereas IRF-2 was further enhanced leading to a decreased change in IRF-1/IRF-2 ratio. The change in the ratio of IRF-1/IRF-2 may thus play a role for IL-4 suppressing iNOS expression in J774 cells, under the stimulation of IFN-γ and LPS.

6.13. Inhibition of iNOS by IL-4 is STAT6-dependent

Since the results are rather complex, it is important to investigate directly the IL-4 signalling pathways that inhibit iNOS expression. Engagement of the IL-4 receptor (IL-4R) leads to the activation of at least two distinct signalling pathways. One involves the activation of STAT6 through phosphorylation by JAK1 and JAK3 (Johnston *et al.*, 1994; Witthuhn *et al.*, 1994), and of the presently known STAT proteins, only STAT6 is activated in response to the cytokine IL-4 (Kotanides and Reich, 1993; Hou *et al.*, 1994; Schindler *et al.*, 1994; Quelle *et al.*, 1995). In addition to STAT6 activation, stimulation of the IL-4R has also been shown to induce the phosphorylation of an insulin receptor substrate (IRS) termed 4PS or IRS-2 (Keegan *et al.*, 1994; Sun *et al.*, 1995). To determine the relative importance of STAT6 signalling pathway in mediating the biologic functions of IL-4, particularly the inhibitory effect on NO synthesis, STAT6 gene targeted mice was used. Recently developed gene knockout mice provide a useful tool to answer this questions. To address the involvement of STAT6 in iNOS gene regulation, NO2 production in peritoneal macrophages of STAT6 knockout mice (Shimoda *et al.*, 1996) was tested (These experiments were carried in Dr. James N. Ihle's lab, USA). NO

synthesis by peritoneal macrophages from the $STAT6^{+/+}$ mice induced by IFN-γ plus LPS or LPS alone was strongly inhibited by IL-4 (Fig. 6.24). NO_2^- concentrations produced by peritoneal macrophages from $STAT6^{-/-}$ mice induced by IFN-γ and LPS was at a identical level as that produced by macrophages from the $STAT6^{+/+}$ mice. In contrast to the $STAT6^{+/+}$ mice, however, NO_2^- production resulting from stimulation with IFN-γ and LPS was not affected by IL-4 (Fig. 6.24). These results therefore demonstrated that the IL-4 regulatory effect on iNOS is strictly STAT6-dependent. The role of STAT6 in the present system is unclear. STAT6 itself may directly affect the iNOS gene or indirectly activate some other gene expression which in turn regulates iNOS activation. Unexpectedly, STAT6-/- mice produce markedly more NO than those from the $STAT6^{+/+}$ mice in response to LPS alone (Fig. 6.24).

6.14 Discussion

IL-4 is a multipotent cytokine derived from Th2 cells and mast cells (Paul 1991). It promotes the proliferation of subsets of lymphocytes, induces B-cell immunoglobulin isotype switching to IgE and IgG1, and mediates susceptibility to Leishmania in mice. The complex actions of IL-4 on macrophages include both activating and deactivating effects, among them enhanced anti-tumor activity in the face of either increased or decreased production of specific anti-tumor products, such as TNF-α (Stenger *et al.*, 1991; Somers and Erickson 1989), H_2O_2 (Lehn *et al*, 1989) and NO (Liew *et al.*, 1991; Al-Ramdi *et al.*, 1992; Oswald *et al.*, 1992). Since IFN-γ and IL-4 have been shown to display opposite effects and to antagonize each other's actions on a number of cells types (Paul 1991; Becker, *et al.*, 1990; Cox *et al.*, 1991; Hart *et al.*, 1989; Lehn *et al.*, 1989; Swisher *et al.*, 1990; Plum *et al.*, 1991), in this study I have examined the ability of IL-4 to inhibit iNOS gene expression induced by IFN-γ and LPS in J774 cells.

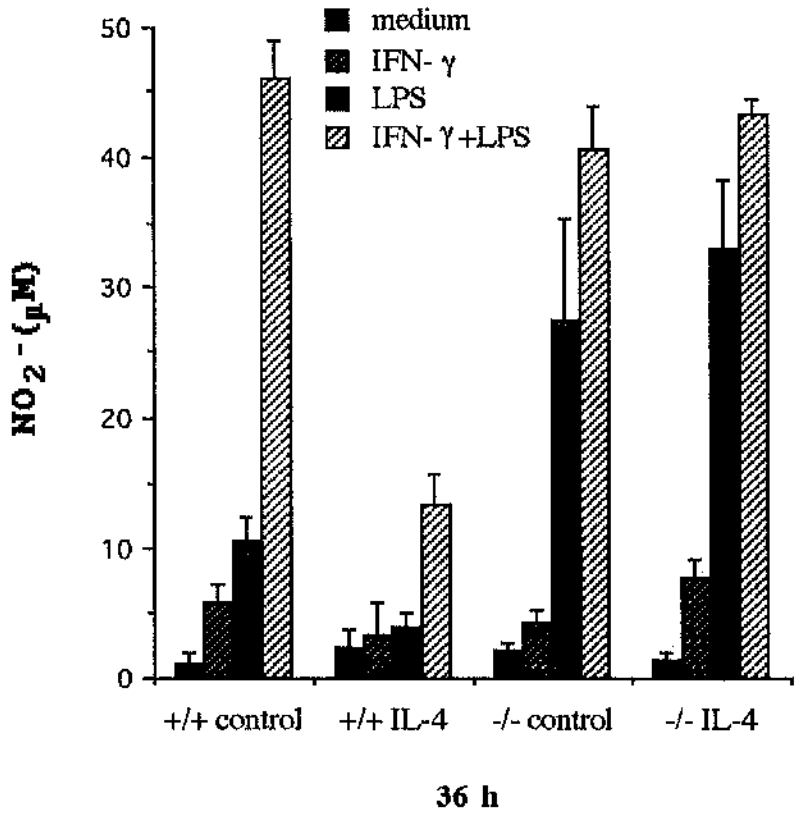


Fig. 6.24 The critical role of STAT6 in IL-4 regulation of iNOS in murine macrophages. Four days after intraperitoneal injection with 4% Brewer's thioglycollate broth, peritoneal cells were harvested with DMEM from STAT6 +/+ and STAT6 -/- mice (young adult). The cells were cultured with DMEM medium containing 2mM L-glutamine, 50U/ml penicillin/streptomycin and 10% heat-inactivited fetal bovine serum in 96-well plates ($2x10^5$cells /well /250μl) at 37°C with 5% CO_2/95% air. After 2 h adherence step, nonadherent cells were removed by replacing medium. Cells were pretreated either with IL-4 (200 U/ml) or medium alone for 18 h before stimulated with IFN-γ (100 U/ml) and /or LPS (10 ng/ml) and incubated for further 36 h. At the end of incubation, supernatants were collected and NO_2^- concentrations measured by Griess Reactions. The data are presented as a mean of triplicate cultures (±SD).

IL-4 was shown to inhibit both iNOS protein and mRNA induction by IFN-γ and/or LPS. This inhibition was dose- and time-dependent. Other cytokines can also suppress NO synthase by macrophages. For example, TGF-β has been shown to decrease the expression of iNOS protein by three distinct post-transcriptional effects: decreased stability and translation of iNOS mRNA, and enhanced degradation of iNOS protein (Vodovotz et al., 1993). The mechanism of IL-4 mediated suppression of NO synthesis is different. If IL-4 is added 2 h after IFN-γ and LPS, inhibition was undetectable. For effective suppression, IL-4 has to be added at the initial stages (before or simultaneously with IFN-γ and/or LPS) of iNOS induction. Northern blot and Western blot analysis showed that the levels of both iNOS mRNA and protein were reduced and delayed from the early time points. A previous report has shown that once iNOS mRNA has been expressed, IL-4 has no detectable inhibitory effect (Bogdan *et al.*, 1994). Thus, the IL-4 affects on iNOS induction are likely to be stage of at the transcriptional activation of the gene.

IL-4 does not inhibit all IFN-γ-induced gene expression, because the induction of IRF-1 and IRF-2 by IFN-γ was further enhanced by IL-4. IL-4 also does not seem to affect some of the early intracellular signaling pathway induced by IFN-γ, such as tyrosine phosphorylation of JAK1, JAK2 and STAT1 (p91). These findings are of interest because IFN-γ-induced tyrosine phosphorylation of STAT1 is required for subsequent iNOS expression in murine macrophages (Meraz *et al.*, 1996). Furthermore, IL-4 induced a GAS binding activity but this is distinct from STAT1 as detected by EMSA. These complexes differed in terms of their electrophoretic mobilities and in their content of the transcription factors. In cells treated with both cytokines, IFN-γ-inducible GAS binding activities were still observed and almost unchanged. Thus, IL-4 did not act by inhibiting the IFN-γ-mediated activation of an GAS binding activity, and did not act by inhibiting IRFs transcription either. However, IFN-γ induced NFκB binding activity was inhibited by IL-4 when both agents were added together. The mechanism by which of IFN-γ induces NFκB binding activity is not clear, but this event is protein-synthesis

independent. The activated NFκB in combination with STAT1 and IRF-1 could then participate in iNOS gene transcription activation. IL-4, however, interfered with this signaling pathway by an unknown mechanism.

It has been reported that IL-4 strongly inhibited IFN-γ-induced NO release by inflammatory murine macrophages while simultaneously enhancing IFN-γ-induced release of TNF-α (Bogdan et al., 1994). However, quite different results were obtained in this study. For example, upon stimulation with LPS, significant amounts of TNF-α and low level of NO accumulated in the culture supernatants. Both products were suppressed by the addition of IL-4. On the other hand, IFN-γ alone did not up-regulate TNF-α production in J774 cells as detected within 24 h. Moreover, IL-4 failed to enhance the production of TNF-α while NO production induced by IFN-γ was markedly reduced by IL-4. This discrepancy may be due to the differences in source and species of macrophages, their state of differentiation, and culture and stimulation conditions, especially with respect to the time course of IL-4 addition. Nevertheless, TNF-α production is unlikely the target for IL-4 inhibition of NO synthesis in the case of IFN-γ alone.

Previous studies showed that TGF-β decreased the expression of iNOS protein by post-transcriptional effects since it could abolish iNOS expression even when it was added after the iNOS mRNA was expressed. IL-4, however, has no detectable inhibitory effect if it is added once iNOS mRNA has been expressed (Bodgdan *et al.*, 1994). The results from my experiments are consistent with this. My data, however, demonstrated that the levels of both iNOS mRNA and protein were reduced at all time points following stimulation, which differs from the earlier report (Bodgdan *et al.*, 1994). In the earlier study, iNOS mRNA was first increased at 6 h and then decreased when cell treated with IFN-γ and LPS in the presence of IL-4. Furthermore, although IL-4 could enhance both levels of IRF-1 and IRF-2 mRNA induced by IFN-γ alone, it down-regulated IRF-1 mRNA and upregulated IRF-2 mRNA in cells costimulated with a combination of IFN-γ and LPS. It could be due to a cocktail of transcription factors responding to the

combination of stimuli, competing to bind to the same iNOS promoter region. While every single factor requires a distinct binding condition (or structure), too many factors on a single gene site may cause changes in the binding affinity of oligos, so that transcription activation is relatively affected. The other possibility is that IL-4 affects signals induced by IFN-γ and LPS together which differ from those induced by IFN-γ or LPS alone. Changes in the ratio of IRF1/IRF-2 in this case may contribute to the inhibitory effect of IL-4 on iNOS expression induced by IFN-γ and LPS.

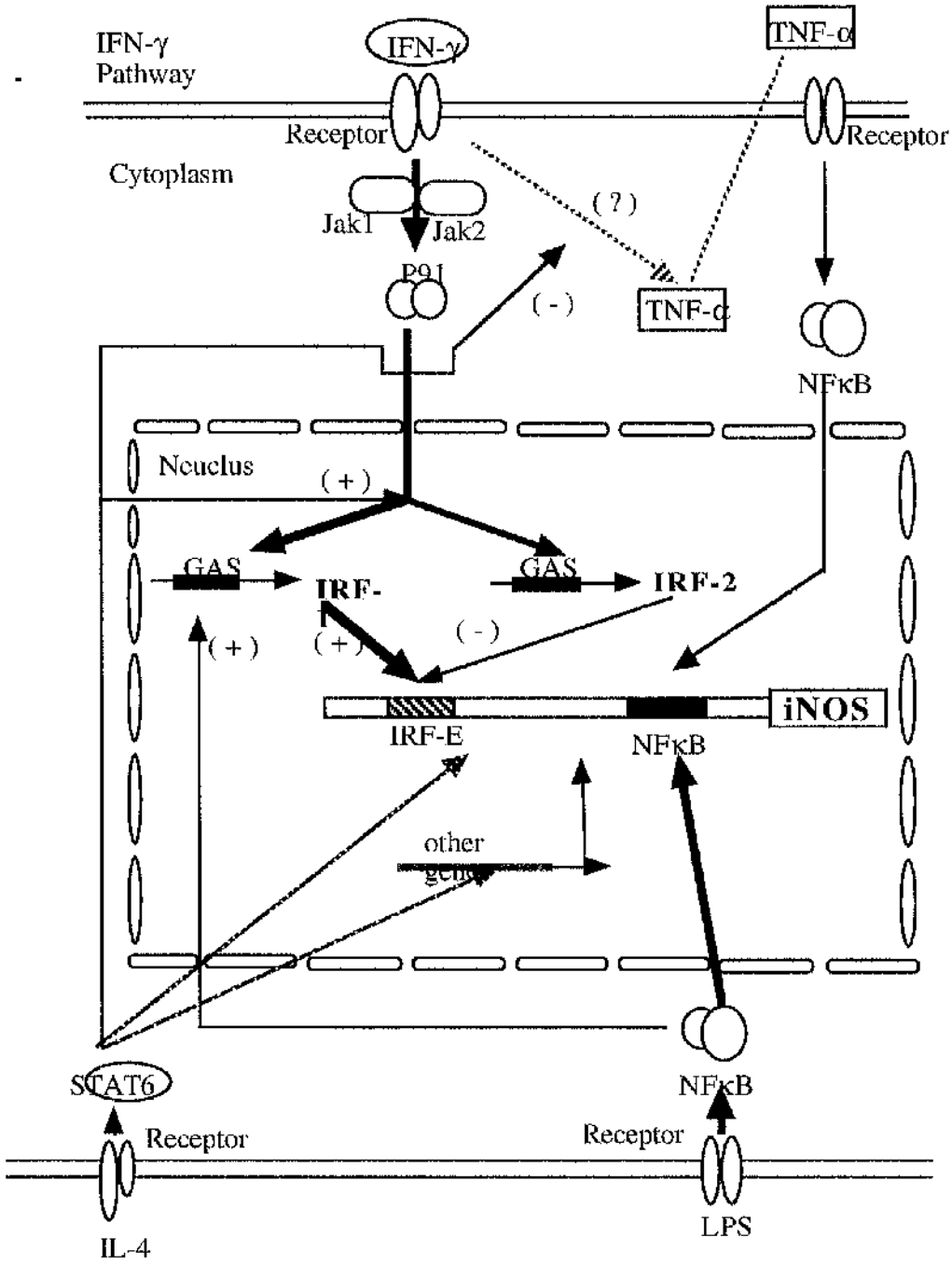
Taken together, IL-4 affects iNOS is a complex manner which includes interfering with NFκB activation induced by IFN-γ, blocking TNF-α production induced by LPS, changing the IRF-1/IRF-2 ratio induced by IFN-γ and LPS or perhaps even interfering with MAP kinase activation by IFN-γ and LPS. The different responses and inhibitory effects of IL-4 on the iNOS signalling pathway are dependent on the different combination of cytokines or stimuli, since some of the signalling events which are not important under the condition of combination of IFN-γ and LPS, may become relatively crucial when stimuli are used separately (i.e. IFN-γ or LPS alone). For, example, when cells are stimulated with LPS, NFκB is rapidly and strongly activated, no matter the other transcription factor activated by LPS, if there is another signalling event come along at same period (i.e, STAT1 or IRF-1 activation by IFN-γ), these transcription factors will work synergistically to activate multiple gene expression. When LPS is used alone, NFkB activation may be not efficent for LPS-responsive gene activation, it demands more trascription factors to participate this process, it may account for TNF-α production, IRF-like protein, AP-1 activation, etc. At this moment, the signals from the other transcription factors become relatively important. If anything that could interferes with this process, it may well interfere LPS-induced gene (iNOS) expressions. Moreover, when cells are costimulated with more than two cytokines, there may be a signalling cascade that differs from any of signalling pathways which are induced by cytokine alone.

Finally, preliminary results show that STAT6 plays a crucial role in IL-4-mediated suppression of iNOS induction in murine macrophages. This STAT pathway is thought to

be important because the promoters of several genes known to be regulated by IL-4 contain the consensus STAT6-binding site TTCN4GAA (Schindler *et al.*, 1995). It has been revealed that STAT6 plays a central role in exerting IL-4-mediated biological responses since STAT6 knockout mice ($STAT6^{-/-}$) do not respond to IL-4 in terms of upregulation of CD23 and MHC II expression in B cells, induction of B or T cell proliferative responses, Th2 cytokine production from T cells or indeed, IgE and IgG1 responses after nematode infection (Shimoda et al., 1996; Takeda *et al.*, 1996). We now show that IL-4 does not inhibit NO synthesis in peritoneal macrophages from $STAT6^{-/-}$ mice.

Taken together, the results of this thesis have allowed me to conduct a model for induction and regulation of iNOS expression by IL-4 (summarised in Fig. 6.25).

Fig. 6.25 Diagram of IL-4 inhibitory effects on NO production in J774 cells. In the presence of IL-4, IFN-γ induced JAK1, JAK2, and STAT1 activation were not interfered, but the IFN-γ-induced expression of IRF-1 and IRF-2 were synergistically enhanced. However, IFN-γ-induced NFκB was inhibited by IL-4. On the other hand, IL-4 interfered with LPS-induced TNF-α production. Furthermore, When cells were treated with a combination of IFN-γ and LPS, IL-4 downregulated IRF-1 expression while upregulated IRF-2 expression. Finally, STAT6 plays a critical role in IL-4 regulation of iNOS in murine macrophages, STAT6 may act as a transcriptional activator which binds to IL-4 responsive genes which inhibit the activation of the iNOS gene, or may directly binds to responsive genes (such as iNOS) where it may works as a repressor of transcription.


IFN-γ
Pathway
IFN-γ
TNF-α
Receptor
Receptor
Cytoplasm
(?)
Jak1
Jak2
P91
(-)
TNF-α
NFκB
Neuclus
(+)
GAS
IRF-
GAS
IRF-2
(+)
(+)
(-)
iNOS
IRF-E
NFκB
other
STAT6
NFκB
Receptor
Receptor
IL-4
LPS

Chapter 7

General Discussion

The enzyme responsible for the synthesis of NO, nitric oxide synthase (NOS), exists at least in three isoforms: ncNOS, ecNOS and iNOS. In many systems, NO derives from two or more different cellular sources, forming networks of paracrine communication. NO exerts a number of diverse activities: beneficial as a messenger or modulator and for immunologic self-defence, but potentially toxic. In several different scenarios, with factors such as oxidative stress, generation of reactive oxygen intermediates (ROIs), and deficient antioxidant systems, NO switches from friend to foe. Large amounts of NO produced by activated cells serve on the one hand to protect the host from viruses (Croen 1993; Karupiah *et al.*, 1993), bacteria, protozoa, helminths (Nathan and Hibbs 1991), and tumor cells (Hibbs *et al.*, 1987), but on the other hand can suppress the function of macrophages (Albina *et al.*, 1989), lymphocytes (Hoffman *et al.*, 1990), dendritic cells (Holt *et al.*, 1993), cardiac myocytes (Roberts *et al.*, 1992), and pancreatic β cells (Corbett *et al.*, 1993), contribute to the inflammatory destruction of joints(McCartney-Francis *et al.*, 1993) and the pancreas (Burkart *et al.*, 1992) and provoke fatal multiorgan hypoperfusion (Kilbourn *et al.*, 1990). Thus, the aim of this project is to understand the mechanisms by which iNOS is regulated.

7.1 JAK-STAT pathway and inducible nitric oxide synthase

Multiple cell types exhibit the capacity to express the inducible NOS (iNOS) gene when appropriately stimulated. Some normal tissues express iNOS, such as in the rabbit (Sladek *et al.*, 1993) and large airways in human (Nathan and Xie 1994). However, expression of iNOS is more often reserved for infection or inflammation and geared toward host defense. Inductive signals include a wide range of microbes and microbial products, some tumor cells, and numerous cytokines, acting alone or in synergy (Nathan 1992). Interferon-γ, as the one of most common self-defence cytokines, exerts one of its most important actions through promoting the co-ordinated induction of NOS (to make NO), GTP cyclohydrolase (promotes production of the cofactor (tetrahydrobiopterin) for NOS), and argininosuccinate synthase (generates the substrate (L-arginine) of NOS).

IFN-γ, like most cytokines, exerts its biological functions by binding to a specific cell surface receptor, and the interaction of IFN-γ with its cognate receptors stimulates the induction of a set of genes. The IFN-γ receptor is a member of the class II cytokine receptor family. Although the receptor binds IFN-γ with high affinity, signal transduction requires a species-specific accessory protein (Schreiber *et al.*, 1992; Aoh *et al.*, 1994; Hemmi *et al.*, 1994) which associates with the extracellular domain of the receptor, the IFN-γ receptor β-chain. The intracellular part of the IFN-γ receptor is more promiscuous, as it can be inter-changed between species without loss of function (Pellegrini and Schinkler 1993); Hemmi *et al.*, 1994). The IFN-γ receptor does not express endogenous kinase or phosphatase activities, yet may become rapidly and reversibly tyrosine phosphorylated following ligation in intact cells. Mutational studies of the IFN-γ receptor have defined two cytoplasmic domains necessary for biological function, a membrane-proximal region and C-terminal sequence including an essential tyrosine (Cook *et al.*, 1992; Farrar *et al.*, 1992).

7.1.1 JAK kinases and IFN-γ induced iNOS

The identification of a novel intracellular signaling pathway termed the JAK-STAT pathway has led to the rapid expanding of an knowledge of cytokine receptor signaling (Darnell *et al.*, 1994; Ihle *et al.*, 1994; Schindler and Darnell 1995). In this type of pathway, individual JAK kinases (JAK1, JAK2, JAK3, and Tyk2) preferentially and constitutively associate with the intracellular domains of particular cytokine receptors and become activated following ligand-induced assembly of receptor subunits at the cell surface.

At the beginning of this project, I used SDS PAGE and two-dimension gel techniques in combination with Immunoblotting to obtain some information on the molecular weight of proteins that are tyrosine phosphorylated following stimulation with IFN-γ and LPS. Proteins of 120-140k, 90-95, 50-70, 40-45 kDa were observed (data not shown). Within these, the 90-95 kDa group was clear one. Since IFN-γ activates cells

signalling though the JAK kinases, and a group of proteins were tyrosine phosphorylated in that molecular weight range (120-140 kDa), I investigated the role of the JAK kinases in iNOS expression. Inducible nitric oxide synthase activity in J774 cells was markedly reduced following pre-incubation with tyrphostin 25 (T25), a tyrosine kinase inhibitor, compared with untreated cells. Herbimycin A was also used and its effect was similar with that of T25. In addition , a tyrosine phosphatase inhibitor (Vanadate) was found to enhance iNOS activity. JAKs were therefore tested for their ability to be phosphorylated in response to IFN-γ. Immunoprecipitation with JAK1 and JAK2 antibodies revealed that both JAK1 and JAK2 were tyrosine phosphorylated in response to IFN-γ. Tyrphostin AG490, a recently discovered JAK2-selective inhibitor (Meydan *et al.*, 1996) also suppressed iNOS expression. These results therefore suggest that JAK2 plays a crucial role in IFN-γ receptor signalling that lead to NO synthesis in murine macrophages.

It has been reported that a cell line lacking JAK1 is completely unresponsive to IFN-γ (Muller *et al.*, 1993), and JAK1 can sustain substantial IFN-γ-inducible gene expression (Briscoe *et al.*, 1996). Interestingly, JAK1 and JAK2 are inter-dependent in their responses to IFN-γ: While JAK2 in wild type cells is phosphorylated in response to IFN-γ, no such phosphorylation is observed in JAK1 negative mutant cells, but is restored in the JAK1 transfectants. Conversely, in JAK2-negative mutant cells JAK1 is not phosphorylated when stimulated with IFN-γ (Muller *et al.*, 1993). It has been proposed that the initial phosphorylation of JAK1 and JAK2 is mediated by JAK2, whereas phosphorylation of the IFN-γ receptor is carried out by JAK1 (Briscoe *et al.*, 1996). For the expression of the antiviral state, a JAK-1-dependent signal, in addition to STAT1 activation appears to be required. It may also required for iNOS induction.

7.1.2 STAT1 and IFN-γ induced iNOS

Signal transducers and activators of transcription (STATs) were first identified as a unique family of DNA-binding proteins approximately four years ago. Since then there has been an explosion of information and speculation regarding their biological roles. Following the cloning of STAT1 and STAT2, it became obvious that STAT-like activities

were activated by various cytokines (reviewed in Ihle 1996). Cells and tissues from STAT1$^{-/-}$ mice were unresponsive to IFNs and no NO production was detectable under the stimulation of IFN-γ plus LPS (Meraz *et al.*, 1996; Durbin *et al.*, 1996). It is shown in the present study that P91 (STAT1α) and P84 (STAT1β) were tyrosine phosphorylated and translocated to the nucleus when J774 cells were stimulated with IFN-γ. Activated STAT1 appears as αα (p91/p91)or ββ (p84/p84) homodimer and binds to the specific DNA elements called gamma interferon site (GAS). Antibody supershift assay revealed that the binding activity was abolished by antibodies against P91 and phosphotyrosine (PY-54), indicating that the activation of GAS elements by STAT1 requires tyrosine phosphorylation of STAT1. The tyrosine phosphorylation of STAT1 is mainly dependent on JAK2 (Briscoe *et al.*, 1996) . Recently, reports showed that DNA binding by purified STAT1 is totally dependent upon tyrosine phosphorylation at a single site (Tyr-701), carboxyl to the SH2 domain (Ihle 1996). Similarly, the DNA-binding activity of all STATs is dependent upon tyrosine phosphorylation and, where examined, involves a comparably located tyrosine. Considerable evidence supports the hypothesis that tyrosine phosphorylation results in dimerization of STATs through the intermolecular interaction of the SH2 domains and the carboxyl sites of tyrosine phosphorylation and that this dimerization is essential for DNA binding.

In contrast, the highly divergent carboxyl-terminal domain of the STATs is required for, or influences, transcriptional activation. In the case of STAT1, there exists a naturally occurring splice variant (STAT1β, p84) that lacks the carboxyl 38 amino acids (Schindler *et al.*, 1992). Although this variant is recruited to the receptor complex, becomes phosphorylated (as showed in chapter 4, Fig. 4.10) and binds DNA, its function is not clear. It has been suggested, however, that p84 does not activate gene transcription. Indeed, STAT 1β may act as a naturally occurring dominant negative molecule (Schindler *et al.*, 1992).

7.1.3 MAP kinase and iNOS

The functions of STATs may also be influenced by serine phosphorylation. There were some reports recently regarding the importance of serine phosphorylation on STAT-STAT proteins dimerization and DNA-binding affinities. They have shown that there are two levels of regulation of STAT1 DNA-binding activity. One is by tyrosine phosphorylation as a prerequisite for dimerization. Another is by serine phosphorylation as a means of regulating the affinity of the STAT1 dimmer for the GAS (Eilers *et al.*, 1995). It was also reported that serine phosphorylation is required for the formation of stable STAT3-STAT3-DNA complexes (Zhang *et al.*, 1995). Relative affinities of one transcription factor for different target sites can determine the overall biological responses of complex biological systems.

Mitogen-activated protein kinases (MAPKs) are a family of closely related serine/threonine-specific protein kinases whose prototype members are the mammalian extracellular signal-regulated kinases ERK1 and ERK2 and the *Saccharomyces cerevisiae* pheromone-regulated kinases KSS1 and FUS3. (reviewed in Hill and Treisman 1995). ERK2 is phosphorylated when J774 cells were stimulated with LPS (as shown in Chapter 4, Fig. 4.12).Tyrphostin AG126, which inhibit MAP kinase, could inhibit NO synthesis induced by IFN-γ and LPS in a dose dependent manner. MAP kinases are important mediators of signal transduction from the cell surface to the nucleus. Whether MAPK activation is linked to STAT1 phosphorylation was not investigated in the present study. A number of reports has indicated that substrates for these MAP kinases include a number of transcription factors, such as c-jun, c-myc and $p62^{TCF}$/ELK-1 (reviewed in Davis 1993). STAT1 has been shown to be phosphorylated at ser-727, a potential MAPK site, and this phosphorylation in return to influence transcriptional activation (Wen *et al.*, 1995). MAPK Erk-2 was reported to be directly associated with the IFN receptor (David *et al.*, 1995). However, I failed to observe any MAP kinase activation upon stimulation with IFN-γ. Furthermore, although MAPK was phosphorylated by LPS treatment in J774 cells, there was no detectable STAT1 phosphorylation in term of mobility shift in SDS

PAGE gel although tyrosine phosphorylation was clearly seen when the cells were stimulated with IFN-γ. Nonetheless, if MAPK does contribute to the serine phosphorylation of STAT1 and therefore influence transcriptional activation, this could be one of the mechanisms for the synergistic effect of LPS with IFN-γ to switch on IRF-1 and other IFN-responsible genes.

7.2 IRF family transcription factors and iNOS

Recent studies have begun to address the molecular mechanisms by which iNOS expression is regulated. Promoter analysis has defined the regulatory regions involved in LPS- and IFN-γ-induced transcription of iNOS.

7.2.1 IRF-1 and iNOS

A family of interferon regulatory factors (IRFs) plays an important role in transcription of IFN genes as well as IFN-stimulated genes. IRF-1 was originally described as a nuclear factor specifically binding to cis-regulatory elements in the β-interferon enhancer (Fujita *et al.*, 1988; Miyamoto *et al.*, 1988). It has been shown that IRF-1 is required for the induction of NO synthase in macrophages (Kamijo *et al.*, 1994). In the present study, the expression of IRFs and the activation of ISRE in iNOS promoter have been investigated. Without cytokine stimulation, IRF-1 was not detectable in J774 cells by Northern or by Western blotting. As a result of IFN-γ treatment, both mRNA and protein of IRF-1 were strongly induced together with the DNA binding activity to IRF-E in the iNOS promoter.

J774 cells also expressed IRF-1 in response to LPS, although the levels of mRNA or protein observed were very low and exhibited an prolonged lag period (4 h). There are two possibilities for this: first, the NFκB activated by LPS can bind directly to a DNA site which has, at least partially, a similar sequence to GAS. The binding of NFκB to the IRF-1 promoter participates in upregulating the gene expression. This is supported by

results from EMSA experiments (using oligomers with GAS specific sequence) which showed an increased binding activity followed LPS-treatment in J774 cells, and the binding protein was recognised by an antibody against NFκB (P65). Secondly, it is possible that other transcription factors controlling the IRF-1 gene expression were activated upon the stimulation of LPS. Data from the EMSA do not necessarily reflect transcriptional involvement and protein occupation of a binding site *in vivo*. Whatever the mechanism(s) involved, it is clear that IRF-1 can be transcriptionally up-regulated by LPS.

Macrophages from IRF1$^{-/-}$ mice have been reported to produce only small amounts of NO and barely detectable levels of iNOS mRNA in response to the combined treatment with IFN-γ and LPS (Kamijo *et al.*, 1994). In the present study, I have shown that IRF-1 is not only transcriptional activated by IFN-γ but by LPS as well. Electromobility supershift assays showed an IFN-stimulated response element/IRF-element (ISRE/IRF-E) binding complex induced by IFN-γ which was recognised by anti-IRF-1 antibody. The binding of IRF-1 to IRF-E was detectable 1 h after stimulation with IFN-γ, and it was protein-synthesis dependent since cycloheximide could abolish this activation. Genes that are induced by IFN-γ, such as GBP and IRF-1, are generally activated by STAT1 binding to a GAS, whereas genes that are more slowly induced by IFN-γ, such as iNOS, are activated by IRF-1 binding to an ISRE.

7.2.2 <u>IRF-2 and iNOS</u>

Another member of the IRF family of transcription factors is IRF-2. In most cases, IRF-1 functions as an activator for IFN-inducible genes, whereas IRF-2 represses the effect of IRF-1 (Fujita, *et al.*, 1989; Harada *et al.*, 1989). Since IRF-2 recognises the same consensus sequence as IRF-1, the activation of IRF-2 is important in my study. Following gene targeting, however, unexpected phenotypes were recently reported (Salkowski *et al.*, 1996): NO_2^- production in IRF-2$^{-/-}$ mice was about half of that observed in IRF-2 $^{+/-}$ mice while the iNOS mRNA levels in both groups were

indistinguishable. It was suggested that IRF-2 is required for optimal production of NO and that IRF-2 plays a role in the post-transcriptional regulation of iNOS.

It has also been reported that IFN-γ, and LPS are both inducers of IRF-2 (Hayes and Zoon 1993; Barber *et al.*, 1995). In my experiments, however, IRF-2 could only induced by IFN-γ, but not by LPS. Furthermore, IRF-2 was slightly down-regulated by the combination of LPS with IFN-γ. It may be that the ratio of IRF-1/IRF-2 rather than IRF-2 post-transcriptional regulation is of crucial importance. As a constitutively expressed factor, IRF-$2^{-/-}$ deficiency could lead to other yet unknown gene regulation.

7.2.3 The change in ratio of IRF-1/IRF-2 play a role in IFN-inducible genes

Regulation of IFN-inducible genes may be determined simply by the ratio of IRF-1 and IRF-2 (Tanaka *et al.*, 1993). In a variety of cells, both the IRF-1 and IRF-2 genes are constitutively expressed at low levels. In such cells, IRF-2 protein normally accumulates to an intracellular concentration higher than that of IRF-1 as a result of its greater stability (protein half-life of >8 h versus 30 min) (Watanabe *et al*, 1991). Therefore, it is possible that most IRF-binding elements are occupied by IRF-2 in the absence of stimulation. Gene activation may thus occur as a result of the transient displacement IRF-2 by IRF-1 (Tanaka *et al*, 1993).Although the similarity in binding affinities observed *in vitro* suggests that IRF-1 could displace IRF-2 via a simple competition, whether such mechanisms could operate *in vivo* is unclear. It may involves other mechanisms. For example, TNF-α, IFN-β and NDV (Newcastle disease virus) all efficiently induce *de novo* synthesis of the short-lived IRF-1 in L929 cells (Watanabe *et al.*, 1991), but the high affinity for the IRFs to a synthetic IRF-E can be only efficiently activated by NDV while such activation was very weak in IFN-β– or TNF-α–treated cells. IRF-1 and/or IRF-2 could undergo some type of post-transcriptional modification elicited by IFN or other cytokine stimulation since efficient activation of the IRF-E by NDV is specifically inhibited by the protein kinase inhibitor, Staurosporine (Watanbe *et al.*, 1991). One may also postulate that IRF-1 binding to its recognition site may be facilitated by protein-protein interactions with nearby transcription factors and/or that the

DNA-binding affinity of IRF-1 increases as a result of interaction with some unidentified adapter molecule(s) (Dalton *et al*, 1992; Leid *et al*, 1992; Li *et al*, 1991; Veals, 1993).

7.2.4 Unidentified IRF-like protein and LPS-induced iNOS

Different members of the IFN response factor (IRF) family bind to ISRE motifs. This diverse family includes ISGF-3γ (Veals *et al.*, 1992), Pip/NFEM5 (Eisenbeis *et al.*, 1995), ICSBP (Driggers *et al.*, 1992), IRF-1 (Miyamoto *et al.*, 1988), IRF-2 (Harada *et al.*, 1989) and IRF-3 (Au *et al.*, 1995). These proteins comprise a conserved DNA-binding domain in the N-terminal region and a divergent C-terminal region that serves as the regulatory domain (Holtschke et al., 1996). In the present study, ISRE binding activity induced by LPS was observed. The binding complex is distinct from IRF-1 and IRF-2 and has a lower electrophoretic mobility, suggesting a large protein or protein complex. Cold probe competition assay showed that the binding activity could be competed by a probe including GAS sequence but not that containing a NFkB site. It may well be a complex containing at least an IRF-family protein and a STAT-like protein. A recent report showed that an IFN-stimulated response element (ISRE) was found in Ig kappa light chain gene intron enhancer region in 70z/3 pre-B cells, and the ISRE specifically bound IRF-1 and IRF-2. This ISRE is a multifunctional motif that also binds the LPS-inducible factor kappa BF-A, and is located within the kappaBS region, which confers B cell specific activity to this enhancer (Damore *et al.*, 1996). This raises the possibility that the same factor could be induced and bind to the IRF-E in the iNOS promoter. Since the factor shows an affinity to at least the IRF-E motif, it may be a member of the IRF family protein.

Another possibility is the interferon consensus sequence binding protein (ICSBP). In contrast to IRF-1 and IRF-2, which are expressed in most cells, ICSBP has been shown to be expressed exclusively in cells of the immune system (Driggers *et al.*, 1992). While ICSBP expression is very low in resting T cells and macrophages, its expression is strongly induced in these cells upon immune stimulation and IFN-γ treatment. Previous studies indicated that ICSBP has negative effects on transcription of ISRE-carrying

promoters (Nelson *et al.*, 1993; 1996). However, ICSBP has negative effects on transcription of ISRE-carring promoters (Nelson *et al.*, 1993,1996). Furthermore, ICSBP has been shown complex with IRF-1 and IRF-2 (Bovolenta *et al.*, 1994). On the other hand, in another report using spleen cells, IFN-γ production in response to ConA was reduced by more than 3-fold in $ICSBP^{-/-}$ mice, while IFN-γ production in response to LPS was reduced by more than 100-fold compared with $ICSBP^{+/+}$ mice (Holtschke *et al.*, 1996). Therefore, to identify the LPS-induced IRSE binding protein, experiments using ICSBP antibody for supershift assay, or using ICSBP cold probe for competition assay should be carried out in the future.

7.3 Activation of NFκB and the induction of nitric oxide synthase

The murine iNOS promoter region contains potential binding sites for transcription factors and many of them, like ISRE/IRF-E and NFκB, are associated with stimuli that induce iNOS expression. Maximal expression of iNOS depends on two discrete regulatory regions upstream of the TATA box. Apart from the region containing motifs for binding IFN-related transcription factors, another important region contains LPS-related responsive elements, including a binding site for nuclear factor NF-IL-6 and NFκB (Lowenstein *et al.*, 1993).

7.3.1 LPS-induced NFkB activation

LPS-induced NFκB activation is a quick and transient event (as measured by EMSA) happening within minutes following treatment of J774 cells with LPS (at concentration of 10 ng/ml), and reaching peak levels at about 30-120 min. This is presumably because NFκB exists as an inactivated IκB-NFκB complex in the cytoplasm of untreated cells. Following a signal that triggers phosphorylation and degradation of IκB, NFkB is released from the inactive complex and then translocates to the nucleus where it binds to specific DNA elements. This procedure does not involve protein synthesis. Although IκB is an inhibitor of NFκB, NFκB is an activator of the IκB gene

transcription. Once NFκB is activated by stimuli, it binds to IκB promoter as well as other NFκB regulated genes. The newly synthesised IκB then gets into the nucleus, captures NFκB and returns it to the cytoplasmic phase in an inactivated form. It was shown in the present study that cycloheximide not only did not block LPS-induced NFκB activation, but enhanced the NFκB binding activity on iNOS promoter. It may due to the effect of cycloheximide on IκB. It has been reported that cycloheximide-treatment could lead to enhanced TNF-α mRNA accumulation (Zuckerman *et al.*, 1991). This may be due to blockage of IκB protein synthesis. Constant expression of IκB is needed to maintain inactivated form of NFkB complex, and when this synthesis procedure is blocked, NFκB is released and activated.

7.3.2 IFN-γ-induced NFκB activation

In the present study, it was found that NFκB was activated and translocated to the nucleus in response not only to LPS but to IFN-γ as well. However, IFN-γ-induced NFκB binding activity was delayed and weak compared to that induced by LPS (see chapter 5), and it is not clear whether they utilise similar pathways to activate NFκB.

Unlike the rapid kinetics observed with LPS, IFN-γ-induced NFκB activation could only be detected after 2 h. Initially, it was thought that such NFkB activation was due to a secondary signalling triggered by some new protein or protein kinase that is transcriptionally regulated by IFN-γ. However, if cells were pre-treated with cycloheximide, NFκB activation by IFN-γ was shown to be enhanced compared to cells not treated with cycloheximide. This suggests that the pathway involved in IFN-γ-induced NFκB activation, is not a secondary stimulatory event, and protein synthesis is not involved in the activation of NFκB. This result may be used to explain some previous puzzling observations: Lowenstein *et al.*, (1993) used transfected luciferase report gene constructs in macrophages to demonstrate that IFN-γ acts primarily as a enhancer and does not induce synthesis of mac-NOS in RAW 264,7 cells (a murine macrophage cell-line). Cells transfected with a construct containing one IFN-stimulated response element

(ISRE) and one NFκB element have little independent regulatory effect on induction, but in conjunction with region containing NFκB (the same site used in my study), it can augment iNOS expression. In the present study, IFN-γ by itself can induce NO synthesis in J774 cells, although the amount of NO was relatively low.

Although there are a number of nuclear transcription factors which have been reported to participate in the activation of iNOS gene, a crucial role of IRF-1/2 and NFκB in the regulation of NO synthesis has been particularly postulated in recent years (Martin *et al.*, 1994; Lowenstein *et al.*, 1993; Xie *et al.*, 1993; Salkowski *et al.*, 1996; Xie *et al.*, 1994). For murine iNOS gene, maximal expression depends on two discrete regulatory regions on the promoter region. The first region contains LPS-related responsive elements, including a binding site for nuclear factor interleukin 6 (NF-IL-6) and κB binding site for NFκB; the second region is crucial for responses to IFN-γ and includes ISRE and GAS (Lowenstein 1993). PDTC, a relatively specific inhibitor of the activation of NFkB in macrophages, has been reported to block both the production of nitrite and the binding of NFκB/Rel to NFκB site (Schreck *et al.*, 1992). Moreover, using reporter gene constructs containing truncated-NFκB site in iNOS promoter region, Xie *et al.* (1994) demonstrated that activation of NFkB/Rel is critical in the induction of iNOS by LPS. Furthermore, experiments using gene targeted mice has revealed that macrophages from IRF1$^{-/-}$ mice produced barely detectable NO_2^- in response to IFN-γ and LPS (Salkowski *et al.*, 1996). Thus induction of NO synthesis, both IRF-1 and NFκB participate in the response to IFN-γ.

7.4 Effects of IL-4 on NO synthesis

7.4.1 IL-4 affects the JAK-STAT pathway and IRF-1/IRF-2 gene expression

iNOS expression induced by IFN-γ and LPS was reduced at both the mRNA and protein levels by pre-incubation of cells with IL-4. These suggest that IL-4 inhibitory

effects on NO synthesis are transcriptional effects. So I did a serial experiments to examine the effects of IL-4 on the signal pathways that lead to NO synthesis induced by IFN-γ and LPS. JAK1 was shown to be tyrosine phosphorylated in response to IL-4, but IL-4 did not activate JAK2 and no effect on JAK2 activation induced by IFN-γ in J774 cells either. The activation of JAK1 by IFN-γ seemed unaffected since the downstream component of JAK1 and JAK2, STAT1 phosphorylation was also unchanged. Furthermore, IL-4 treatment did not interfere with STAT1 binding to its specific DNA sequence, the GAS element, in the IRF-1 promoter. Moreover, IL-4 could activate STAT6 binding to the same element. As result, IRF-1 expression was enhanced in the presence of IL-4 compared to cells treated with IFN-γ alone. The increased IRF-1 expression did not correlate with decreased NO production, because the suppresser of the IRF-E, IRF-2 was enhanced too. On the other hand, the activation of NFκB induced by IFN-γ was inhibited by IL-4. These results suggested that the mechanism of IL-4-mediated suppression of iNOS expression induced by IFN-γ is not through the interference with JAK1/2-STAT1 activation but may involve other transcription factors which is also activated in response to IFN-γ, like NFκB.

Compared with IFN-γ alone, IRF-1 expression in the cells treated with IFN-γ and LPS was markedly enhanced. This enhancement was inhibited in the presence of IL-4. IRF-2, however, was also enhanced in the same cells which treated with a combination of IFN-γ, IL-4 and LPS compared to cells treated with IFN-γ and LPS. The changes in ratio of IRF-1/IRF-2 may have pushed the balance towards to the suppression of iNOS transcription. However, other mechanisms may be involved in the IL-4-mediated inhibition of iNOS expression in murine macrophages. Candidate mechanisms include post-transcriptional modification or destabilisation of iNOS mRNA, post-translational modification or interference with iNOS protein phosphorylation, or even disrupt of the protein-protein interaction between iNOS and its cofactors.

7.4.2 IL-4 mediated inhibition of NO synthesis is STAT6 dependent

The inhibition of NO synthesis by IL-4 in murine peritoneal macrophages is totally dependent on the presence of STAT6 in these cells. Since NO_2^- was not detected in cells treated with IFN-γ alone, it is unclear what STAT6 does in this system. However, in cells treated with either LPS alone or with IFN-γ and LPS, NO production was abolished by IL-4 only in $STAT6^{+/+}$ mice, but not in STAT6 -/- mice. There are a number of possibilities by which STAT6 exerts its inhibitory effect on iNOS. STAT6 may directly binds to responsive genes (such as iNOS) where it may works as a repressor of transcription. Alternatively, STAT6 may act as a transcriptional activator which binds to IL-4 responsive genes which inhibit the activation of the iNOS gene. These possibilities need further clarification.

7.5 Summary of the signalling pathways leading to iNOS gene activation

7.5.1 IFN-γ signaling and iNOS induction

Upon the stimulation of IFN-γ, at least two distinct signalling pathways are involved in iNOS induction in murine macrophages. One of them is the JAK-STAT pathway which involves phosphorylation of JAK1 and JAK2 on tyrosine; tyrosine phosphorylation of STAT1 (p91 and p84) by JAKs; and nuclear translocation of di merised STAT1. Activated STAT1 binds the GAS elements which are located in IRF-1 and IRF-2 or possibly the iNOS promoters. At promoter regions, STAT1 could either exist as a homodimer, bind to GAS elements in IRFs promoter, or as a complex with IRFs, binds to ISRE in the iNOS promoter. IRF1 and IRF-2 are early expressed genes induced by IFN-γ, whose transcriptional activation do not involve *de novo* protein synthesis. However, iNOS gene activation is dependent on latent proteins as well as newly synthesised transcription factors, like IRF-1 and IRF2. Another possible pathway is through activation of nuclear transcription factor NFκB. However the details of how

IFN-γ receptor binding leads to NFκB activation are unclear and the participation of other nuclear transcription factors can not be excluded. The IFN-γ signalling pathway leading to iNOS induction suggested by the results in this thesis is summarised in Fig. 7.1.

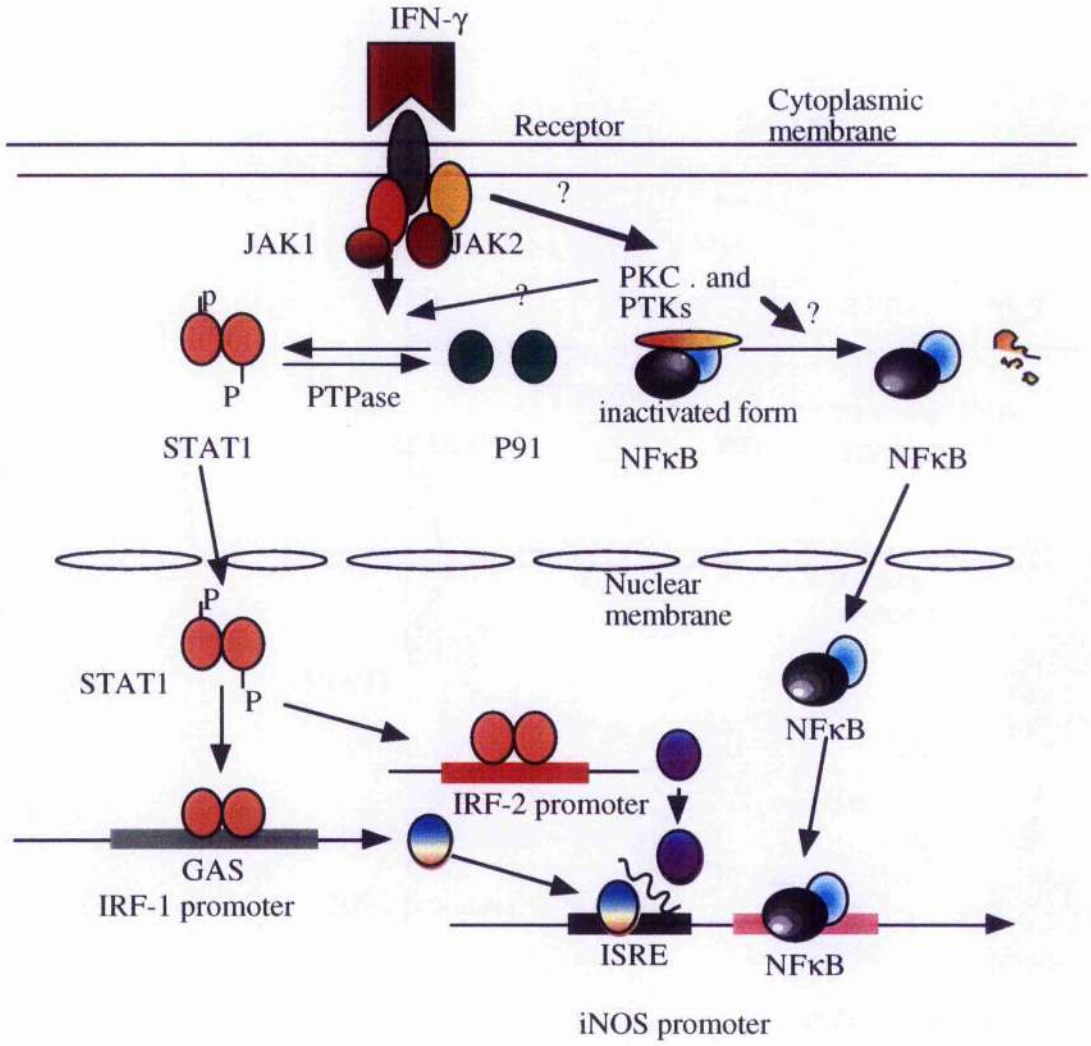
7.5.2 LPS signaling and iNOS induction

Upon stimulation with LPS, a number of nuclear transcription factors are activated which are involved in iNOS induction. NFκB play a cricial role in LPS induced NO synthesis by binding to DNA elements not only in the iNOS promoter, but also to GAS element in the IRF-1 promoter. The later may contribute to the LPS-induced IRF-1 expression. Another transcription factor induced by LPS is an IRF-like protein (or protein complex) that recognise IRSE/IRF-E element. The activation of this complex is protein-synthesis dependent, and it may contain a STAT-like protein. Activation of NFkB and IRF-E in iNOS promoter in co-operation with other transcription factors lead to transcriptional activation of iNOS gene. The signaling pathway of iNOS induction induced by LPS is represented in Fig. 7.2.

7.5.3 iNOS induction by a combination of IFN-γ and LPS

iNOS expression in J774 cells is at a relatively low level when stimulated by IFN-γ or LPS alone. However, upon the stimulation with IFN-γ plus LPS, the two major signalling pathways are both activated simultaneously and promptly. Signals derived from the IFN-γ receptor include: JAK1 and JAK2 phosphorylation and activation; STAT1 tyrosine phosphorylation, dimerisation, and translocation to the nucleus; STAT1 binds to GAS elements in the IRFs promoter to promote gene activation. On the other hand, signals derived from the LPS receptor promote phosphorylation and degradation of IκB to release NFκB (p65/p50, p50/50) which therefore translocates to the nucleus where it not only binds to NFkB site within iNOS promoter region, but also binds to GAS element within the IRF-1 promoter. Furthermore, the activated IRF-1 (which is mainly from IFN-γ signalling) and NFκB (which is mainly from LPS-signalling), together with other transcription factors (AP-1, etc.) bind to their specific binding sequences on iNOS.

Fig. 7.1 Schematic representation of the IFN-γ signalling pathway. Upon the stimulation of IFN-γ, at least two distinct signalling pathways are involved in iNOS induction in murine macrophages. One of them is the JAK-STAT pathway which involves phosphorylation of JAK1 and JAK2 on tyrosine; tyrosine phosphorylation of STAT1 (p91 and p84) by JAKs; and nuclear translocation of dimmerised STAT1. Activated STAT1 binds the GAS elements which are located in IRF-1 and IRF-2 or possibly the iNOS promoters either as a homodimer (bind to GAS elements in IRFs promoter), or as a complex with IRFs (binds to ISRE in the iNOS promoter). Activated IRFs then bind to iNOS promoter. Another possible pathway is through activation of nuclear transcription factor NFκB. However the details of how IFN-γ receptor binding leads to NFκB activation are unclear and the participation of other nuclear transcription factors can not be excluded.


IFN-γ
Receptor
Cytoplasmic membrane
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JAK1
JAK2
PKC . and PTKs
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PTPase
inactivated form
STAT1
P91
NFκB
NFκB
Nuclear membrane
P
STAT1
P
NFκB
IRF-2 promoter
GAS
IRF-1 promoter
ISRE
NFκB
iNOS promoter

Fig. 7.2 Diagrammatic representation of the LPS signalling pathways leading to iNOS activation. Upon stimulation with LPS, a number of nuclear transcription factors are activated which are involved in iNOS induction. NFκB play a crucial role in LPS induced NO synthesis by binding to DNA elements not only in the iNOS promoter, but also to GAS element in the IRF-1 promoter. The later may contribute to the LPS-induced IRF-1 expression. Another transcription factor induced by LPS is an IRF-like protein (or protein complex) that recognise IRSE/IRF-E element. The activation of this complex is protein-synthesis dependent, and it may contain a STAT-like protein. Activation of NFκB and IRF-E in iNOS promoter in co-operation with other transcription factors lead to transcriptional activation of iNOS gene.

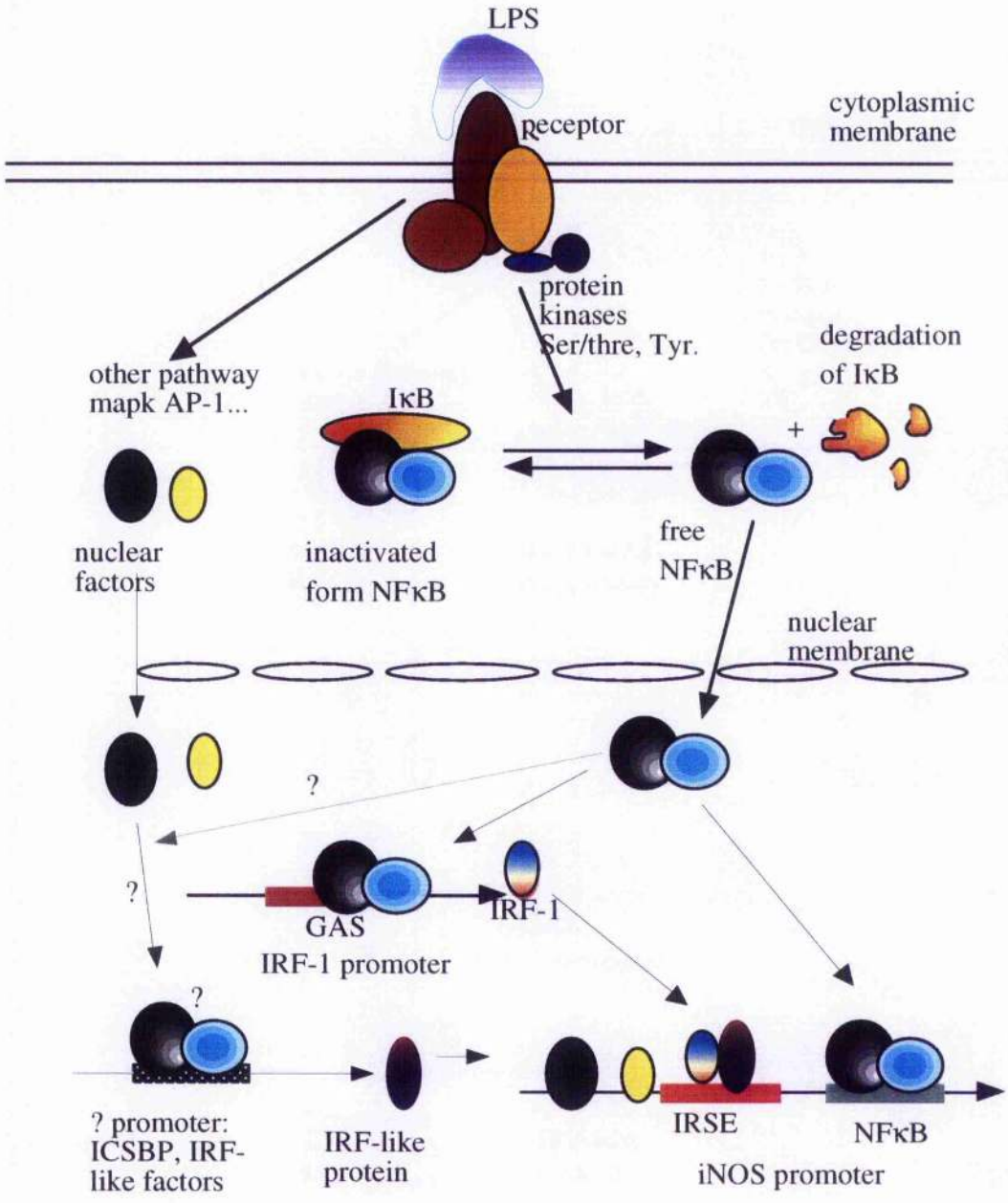
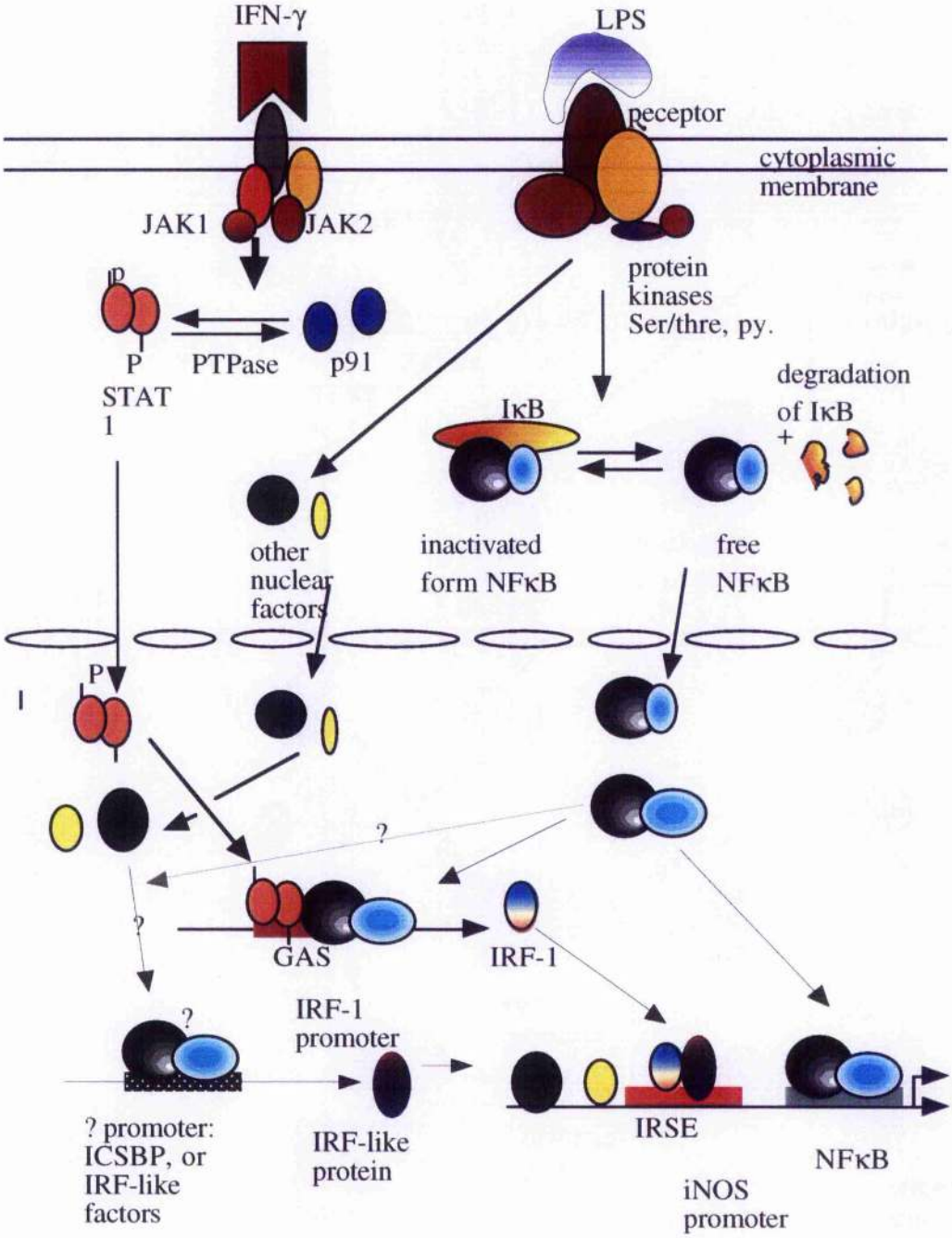

LPS
receptor
cytoplasmic membrane
protein kinases Ser/thre, Tyr.
other pathway mapk AP-1...
IκB
degradation of IκB
+
nuclear factors
inactivated form NFκB
free NFκB
nuclear membrane
?
?
GAS
IRF-1
IRF-1 promoter
?
? promoter: ICSBP, IRF-like factors
IRF-like protein
IRSE
NFκB
iNOS promoter

Fig. 7.3 The induction of iNOS expression by a combination of IFN-γ and LPS. In J774 cells, a number of transcription factors may participate in the iNOS activation. The JAK-STAT and IRFs pathway play an important role in the IFN-γ pathway, while NFκB plays a crucial role in the LPS pathway. Upon the stimulation with IFN-γ plus LPS, the two major signalling pathways are both activated simultaneously and promptly. Signals derived from the IFN-γ receptor include: JAK1 and JAK2 phosphorylation and activation; STAT1 tyrosine phosphorylation, dimerization, and translocation to the nucleus; STAT1 binds to GAS elements in the IRFs promoter to promote gene activation. On the other hand, signals derived from the LPS receptor promote phosphorylation and degradation of IκB to release NFκB (p65/p50, p50/50) which therefore translocates to the nucleus where it not only binds to NFkB site within iNOS promoter region, but also binds to GAS element within the IRF-1 promoter. Furthermore, the activated IRF-1 (which is mainly from IFN-γ signalling) and NFκB (which is mainly from LPS-signalling), together with other transcription factors (AP-1, etc.) bind to their specific binding sequences on iNOS promoter and may interaction with each others to switch on the gene.


IFN-γ
LPS
receptor
cytoplasmic membrane
JAK1
JAK2
protein kinases Ser/thre, py.
P
PTPase
p91
STAT 1
IκB
degradation of IκB
+
other nuclear factors
inactivated form NFκB
free NFκB
P
?
?
GAS
IRF-1
IRF-1 promoter
?
? promoter: ICSBP, or IRF-like factors
IRF-like protein
IRSE
NFκB
iNOS promoter

promoter and may interaction with each others to switch on the gene. the induction of iNOS signalling pathway is summarised in Fig. 7.3.

7.6 Future work

7.6.1 To identify the LPS-inducible IRF-E binding protein

Although previous reports suggested that both IRF-1 and IRF-2 could be regulated by LPS, it was not known that LPS can induce an additional IRF-like protein. Preliminary results indicated that this is not IRF-1 nor IRF-2. Antibody supershift assay and cold probe competition suggest it is not NFκB either. Cycloheximide treatment abolished the formation of IRSE-binding complex, indicating that the factor is transcriptionally activated by LPS. It will be of considerable interest to identify and characterise this factor. Antibody supershift and cold probe competition assays using ICSBP and related reagents could be used initially to approach identifying this factor.

7.6.2 To investigate the upstream of signaling pathway of NFkB that activated by IFN-γ

Activation of NFκB is triggered by different stimuli including LPS, muramyl peptides, viruses, TNF–α and IL-1β, UV irradiation, and reactive oxygen intermediates (H_2O_2) (Bacuerle *et al.*, 1991 and 1994). Previously, activation of NFκB in response to IFN-γ has not been reported. It would of interest to investigate the early signal cascade that lead to NFkB activation by IFN-γ. The unravelling of the involvement of protein kinase(s) and protein phosphatase(s) in this system will provide useful information in the understanding of the regulatory mechanism of NFkB, one of the most important transcription factors in the immune system.

7.6.3 To study the mechanism of the involvement of STAT6 in iNOS gene activation

Since cells from STAT6 knockout mice were refractory to IL-4-mediated inhibition of NO synthesis, STAT6 plays a crucial role in this system. It will be of interest

to investigate the molecular basis of the effects of IL-4 on IFN-γ regulated genes, especially iNOS gene. Northern blot analysis on the involvement of IRFs, and later DNA foot printing may be used to trace the possible correlation between STAT6 binding activity and iNOS gene regulation.

References

Abdollahi, A., Lord, K.A., Hoffman-Liebermann, B. and Liebermann, D.A. (1991). Interferon regulatory factor 1 is a mycloid differentiation primary response gene induced by interleukin 6 ad leukemia inhibitory factor: role in growth inhibition. *Cell Gowth. Different* . 2:401.

Abu-Soud, H.M., and Stuehr, D.J. (1993) Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proc. Natl. Acad.Sci.* USA90: 10769-10772

Adams, D.O. and Hamilton, T.A. (1984) The cell biology of macrophage activation. *Annu. Rev. Immunol.* 2: 283-318

Adams, D.O., and Hamilton, T.A. (1987). Molecular transductional mechanisms by with IFN-γ and other signals regulate macrophage development. *Immunol. Rev.* 97: 5-27.

Aderem, A. (1992) The role of myristoylated protein kinase C substrates in intracellular signalling pathways in macrophages. *Curr. Top. Microbiol. Immunol.* 181: 189-207

Aguet, M., Dembrc, Z., and Merlin, G.(1988) Molecular cloning and expression of the human interferon-gamma receptor. *Cell* 55:273-280

Al-Ramadi, B.K., Meissler, J.J., Huang, D., Eisenstein, T.K. (1992) Immunosuppression induced by nitric oxide and its inhibition by interleukin-4. *Eur. J. Immunol.* 22:2249-2254

Albina, J.E., Abate, J.A., Henry, W.L. (1991) Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation: role of IFN-γ in the inductiion the nitric oxide synthesizing pathway. *J. Immunol.* 147: 144-148

Albina, J.E., Caldwell, M.D., Henry, W.L., Jr., and Mills, C.D. (1989) Regulation of macrophage functions by L-arginine. *J.Exp. Me*d. 169:1021

Alexander, P., Evans, R. (1971) *Nature* 232:76-78

Alvarez, E., Northwood, I.C., Gonzalez, F.A., Latour, D.A., Seth, A., Abate, C., Curran, T., Davis, R.J. (1991) Pro-Leu-Ser/Thr-Pro is a cinsensus primary sequence for substrate protein phosphorylation: Characterization of the phosphorylation of *c-myc* and *c-jun* proteins by an epidermal growth factor receptor threonine 669 protein kinase. J.*Biol. Chem.* 266:15277-15285

Andrews, N. C., and Faller, D.V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells *Nucleic Acids Research.* 19 (9): 2499

Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai, N., and Yokota, T. (1990) Cytokines: Coordinators of immunc and inflammatory responses. *Annu. Rev. Biochem.* 59:783-836

Arroyo, P.L., Hatch-Pigott,V., Mower, H.F. and Cooney, R. V. (1992) Mutagenicity of nitric oxide and its inhibition by antioxidants. *Mutat. Res.* 281:193-202.

Au, W.C., Moore, P.A., Lowther, W., Juang, Y.-T., and Pitha, P.M.(1995) Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. *Proc. Natl. Acad. Sci. USA.* 92: 11657-11661

Baek, K.J., Thiel, B.A., Lucas, S., and Stuehr, D.J. (1993) Macrophage nitric oxide synthase subunits. Purification, Characterizatio, and role of prosthetic groups and substratc in regulation their association into a dimeric enzyme. *J Biol. Chem.* 268: 21120-21129

Baeuerle, P.A. (1991) The inducible transcription activator NFκB: regulation by distinct protein subuints. *Biochem. Biophys. Acta* 1072: 63-80

Baeuerle, P.A., and T. Henkel. (1994) Function and activation of NFκB in the immunue system. *Annu. Rev. Immunol.* 12: 141-179

Bakouche, O., Moreau, J.L., and Lachman, L.B. (1992) Secretion of IL-1: role of protein kinase C. *J. Immunol.* 148:84

Barber, S.A., Fultz, M.J., Salkowski, C.A., and Vogel, S.N. (1995). Differnential expression of interferon regulatory factor 1 (IRF-1), IRF-2, and interferon consesus sequence binding protein genes in lipopolysaccharide (LPS)-responsive and LPS-hyporesponsive macrophages. *Infect. Immun.*. 63: 601

Basham, T.Y., and Merigan, T.C. (1983) *J. immunol.* 130:1492-1494

Bazan, J.-M. (1993) Emerging families of cytokine and receptors. *Curr. Biol.* 3:603-606

Bazil, Baudys, M., Hilgert, I., Stefanoval, I., Low, M., Brozek, J., Horejsi, V. (1989) Structureal relationship between the soluble and membrane-bound forms of human monocytes surface glycoprotein CD14. *Mol. immunol.* 26:657-662

Beadling, C., Ng, J., Babbage, J.W., and Cantrell, D.A. (1996) Interleukin-2 activation of STAT5 requires the convergent activation of tyrosine kinases and a serine/threonine kinase pathway distinct from the Raf1/ERK2 MAP kinase pathway. *EMBO J.* 15:1902-1913

Becker, S. and Daniel, E.G. (1990) Antagonistic and addtive effects of IL-4 and interferon-gamma on human monocytes and macrophages: Effects on Fc recptors, HLA-D antigens, and superoxide production. *Cell. Immunol.* 129:351

Bennett, V. (1992) Ankyrins Adaptors between diverse plasma membrane proteins and the cytoplasm. *J.Bio. Chem.* 267: 8703-8706

Bernton, E.W., Meltzer, M.S., and Holaday, J.W. (1988) Suppression of macrophage activation and T-lymphocyte function in hypoprolactinemic mice. *Science* 239:401-404

Berton, G., Zeni, L., Cassatella, M.A., and Rossi, F. (1986). Gamma interferon is able to enhance the oxidative metabolism of human neutrophils. *Biochem. Biophys. Res. Commun.* 138:1276-1282.

Beyaert, R., Cuenda, A., Berghe, W.V., Plaisance, S., Lee, J.C., Haegeman, G., Cohen, P., and Fiers, W. (1996) The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis in response to tumour necrosis factor *The EMBO J.* 15(8) 1914-1923

Binetruy, B., Smesal, T., and Karin M. (1991) Ha-Ras augments *c-Jun* activity and stimulates phosphorylation of its activation domain. *Nature* 351:122-127

Birnbaumer, L., Abramowitz, J., and Brown, A.M. (1990) Receptor-effector coupling by G-proteins. *Biochem. biophys. Acta.* 1031:163-224

Bishop, J.M. (1985) Trends in oncogenes.*Trends Genet* 1: 245-249

Bogdan, C., Nathan, C. (1993) Modulation of macrophage function by transforming growth factor-β, interleukin-4, and interleukin-10. *Ann. NY Acad. Sci.* 685:713:740

Bogdan, C., Vodovotz, Y., Paik, J., Xie, Q.W., Nathan, C. (1994) Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. *J. Leuko. Biol.* 55: 227-233

Bogdan,C., Vodovotz, Y., Nathan, C. (1991) Macrophage deactivation by interleukin 10. *J. Exp. Med.*174, 1549-1555

Bogle, R.G., Baydoun, A.R., Pearson, J.D., Moncada, S., Mann, G.E. (1993) L-arginine transport is increased in macrophages generating nitric oxide. *J. Biochem.* 284:15-18

Bolen, J.B. (1993) Nonreceptor tyrosine kinases *Oncogene* 8:2025-2031

Boulet, I., S.Ralph, E.Stanley, P. Lock, A.R. Dunn, S.P. Green and W.A. Phillips 1992) Lipopolysaccharide- and interferon-gamma-induced expression hck and lyn tyrosine kinases in murine bone marrow-derived macrophages. *Oncogen* 7, 703-710.

Bourne, H.R., Sanders, D.A., and McCormick, F. (1990) The GTPase superfamily: A conserved switch for diverse cell functions. *Nature* 348:125-132

Bredt, D.S., Feris, C.D., Snder, S.H. (1992) Nitric oxide synthase regulatory site-sphosphorylation by cyclic AMP-dependent protein kinase C, and calcium calmodulin protein kinase identification of flavin and calmodulin binding sites. *J. Biol. Chem.* 267:10976-10981

Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R., Snyder, S.H. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 351:714-718

Briscoe, J., Rohers, N.C., Witthuhn, B.A., Watling, D., Harpur, A.G., Wilks, A.F., Stark, G.R., Ihle, J.N., and Kerr, I.M. (1996) Kinase-negative mutants of JAK1 can sustain interferon-g-inducible gene expression but not an antiviral state. *The EMBO J.* 15 (4): 799-809

Brown, D. (1993) The tyrosine kinase connection: how GPI-anchored proteins activate T cells. *Curr. opin. Immunol.* 5:349-354

Brown, M.A., Pierce, J.H., Watson, C.J., Falco, J., Ihle, J.N., and Paul, J.E. (1987) B cell stimulatory factor-1/interleukin 4 MRNA is expressed by normal and transformed mast cells. *Cell* 50:809

Burkart, V., Imai, Y., Kallmann, B., and Kolb, H. (1992) Cyclosporin A protects pancrestic islet cells from nitric oxide-dependent macrophage cytotoxicity *FEBS Lett.* 313:56

Butler, T.M., Ziemiecki, A., Friis, R.R. (1990) Megakaryocytic differentiation of K562 cells is associated with changes in the cytoskeletal organization and the pattern of chromatographically distinct forms of phophotyrosyl-specific protein phosphatases. *Cancer Res.* 50:(19) 6323-6329

Cano, E., and Mahadevan, L.C. (1995) Parallel signal processing among mammalian MAPKs. *Trends Biochem. Sci.* 20:117-122

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., Soltoff, S. (1991). Oncogenes and signal transduction. *Cell* 64:281-302

Carding, S.D., Hayday, A.C., and Bottomly, K. (1991) Cytokines in T-cell development. *Immunol. Today* 12:239

Caroline S. Hill and Richard Treisman (1995) *Cell* 80, 199-211.

Celada, A., and Maki, R.A. (1991). IFN-γ induces the expression of the genes for MHC class II 1-Aβ and tumor necrosis factor through a protein kinase C-independent pathway. *J.Immunol.* 146:114-120.

Celada, A., and Schreiber, R.D. (1986). Role of protein kinase C and intracellular calcium mobilization in the induction of macrophage tumorividal activity by interferon-γ. *J. Immunol.* 137:2373-2379.

Cenci, E., Romani, L., Mencacci, A., Spaccapelo, R., Achiaffella, E., Puccetti, P. and Bistoni, F. (1993) Interleukin-4 and interleukin-10 inhibit nitric oxide-dependent macrophage killing of Candida albicans.Eur. J. Immunol. 23:1034-1038

Charles, I.G., Palmer, R.M.J., Hickery, M.S., Baliss, I., Chubb, A.P., Hall, V.S., Moss, D.w., Moncada, S. (1993) Cloning, characterization, and expression of a cDNA encoding an inducible nitric oxide wynthase from the human chondrocyte. *Proc. Natl. Acad. Sci. USA* 90:11419-11423

Chartrain, N.A., Celler, D.A., Koty, P.P., Sitrin, N.F., Nussler, A.K., Hoffman, E.P., Billiar, T.R., Hutchinson, N.I., Mudgett, J.S. (1994) Molecaular cloning, structure, and chromosomal localization of the human inducible nitric oxide sunthase gene. □. *J. Bio. Chem.* 269:6765-6772

Chen CC, Rosenbloom CL, Anderson DC, Manning AM (1995) Selective inhibition of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 expression by inhibitors of IkappaB-alpha phosphorylation. *J. of Immunology* 155: 3538-3545

Cho, H.J., Xie, Q.-W., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., and Nathan, C. (1992) Calmodulin is a subunit of nitric oxide synthase from macrophages *J. Exp. Med.* 176: 599-604

Cocks, T.M., Anggus, J.A., Campbell, J.H., Campbell G.R. (1985) Release and properties of endothelium-drived relaxing factor (EDRF) from endothelial cells in culture. *J. Cell. Physiol.* 123:310-320

Coffman,R.L., Lebman, D.A., and Rothman, P. (1993) Mechainsm and regulation of immunoglobulin isotype switching. *Adv. Immunology.*, 54, 229-270

Collart, M.A., Belin, D., Vassalli, J.D., Vassalli, J.D., de Kossodo, S., and Vassalli, P. (1986) *J. exp. Med.* 164:2113-2118

Collet, M.S., Purchio, A.F., Erikson, R.L. (1980) Avian sarcoma virus-transforming protein, pp60(src) shows protein kinase activity specific for tyrosine. *Nature* 285 (5761): 167

Cook, J.R., Jung, V., Schwartz, B., Wang, P and Pestka, S. (1992) Structureal analysis of the human interferon gamma receptor: A small segment of the intracellular domain is specifically required for class I major histocompatibility complex antigen induction and antiviral activity. *Proc. Natl. Acad. Sci. U.S.A.* 89: 11317-11321

Cool, D.E., Andreassen, P.R., Tonks, N.K., Krebs, E.G., Fischer, E.H., Margolis, R.L. (1992) Cytokinetic failure and asynchronous nuclear division in BHK cells overexpressing a truncated protein-tyrosine-phosphatase. *Proc. Natl. Acad. Sci. USA* 89: 5422-5426

Corbett J.A., Sweetland M.A., Wang, J.L., Lancaster, J.R., McDaniel M.L., (1993) Nitric oxide mediate cytokine induced inhibition of insulinsecretion by human islets of langerhans. *Proc. Natl. Acad. Sci. USA* 90:1731-1735

Cosman, D. (1993) The hematopoietin receptor superfamily. *Cytokine* 5:95-106

Cox, G.W., chattopadhyay, U., Oppenheim, J.J., and Varesio, L. (1991) IL-4 inhibits the costimulatory activity of IL-2 or picolinic acid but not of lipopolysaccharide on IFN-gamma-treated macrophages. *J. Immunol.* 147: 3809-3814

Croen, K.D. (1993) Evidence for an antiviral effect of nitric oxide:inhibition of herpes virus type I replication,. J. Clin. Invest 91:2446

Cui, S., Reichner, J.S., Mateo, R.B., and Albina, J. E. (1994) Activated murine macrophages induce apoptosis in tumor cells through nitric oxide-dependent or -independent mechanisms. *Cancer Res.* 54:2462-2467

Damore MA, Omori SA, Wall R (1996) IFN-g Induces the κ Intron Enhancer Via an IFN-stimulated Response Element. *J. Immunol* 156 (7) 2451-2457

Darnell, J.E., Kerr, I.M., Stark, G.R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins.*Science*. 264: 1415-1421

David, M., Petricoin, E., III, Beniamin, C., Pine, R., Weber, M.J., and Larner, A.C. (1995) Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins. *Science*. 269:1721-1723

Davis, R.J. (1993) The mitogen-activated protein kinase signal transduction pathway. *J.Biol. Chem.* 268:14553-14556

Day, J.R., Albers, J.J., Lofton,-Day, C.E., Gilbert, T.L., Ching, A.F.T., Grant, F.J. O;Hara, P.J., Marcovina, S.M., Adolphson, J.L. (1994) Complete cDNA encoding

human phospholipid transfer protein from human endothelial cells. *J. Biol. Chem.* 269:9388-9391

De Maeyer, E., and De Maeyer-Guignard, J. (1991) *The cytokine Handbook*, 215-239

de Waal Malefyl, R., Figdor, C., Huijbens, R., Mohan-Peterson, S., Bennett, B., *et al.*, (1993) Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes. *J. Immunol.* 151:6370-6381

Denich, K., Borlin, P., O'Hanley, P., Howard, M.C., and Heath, A.W. (1993) The effects of expression of murine interleukin-4 by Aro A-Salmonella typhimurium: Persistence, immune response and the inhibition of macrophage killing . *Infect. Immun.* 61:4818-4827

Devary, y., Gottlieb, R.A., smeal, T., and Karin, M. (1992). The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases Cell 71:1081-1091

Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucl. Acids Res.* 11, 1475-1489.

Dinerman, J.L., Dawson, T.M., Schell, M.J., Snowman, A., Snyder, S.H. (1994) Endothelial nitric oxide synthase localyzed to hippocampal pyramidal cells: implications for synaptic plasticity. *Proc. Natl. Acad. Sci. USA* 91:4214-4218

Ding, A.H., Nathan, C.F., Stuehr, D.J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141: 2407-2412

Doherty, T.M., Kastelein, R., Menon, S., Andrade, S., and Coffman, R.L. (1993) Modulation of murine macrophage function by interleukin-13. *J. Immunol.* 151:7151-7160

Dong, Z., Lu, S., and Zhang, Y. (1989) Effects of pretreatment with protein kinase C activators on macrophage activation for tumor cytotoxicity, secretion of tumor necrosis factor, and its mRNA expression. *Immunolbiology* 179:382

Dowens, C.P., Carter, A.N. (1991) Phosphoinositide 3-kinase: A new effector in signal transduction? *Cell. Signal.* 3: 501-513

Drapier, J.C., Wietzerbin, J., Hibbs, J.B. (1988) Interferon-gamma and tumor necrosis factor induce the L-arginine-dependent cyto-toxic effector mechanism in murine macrophages. *Eur. J. Immunol.* 18:1587-1592

Dridders, P.H., Elenbaas, B.A., An, J.-B., Lee, I.J., and Ozato, K. (1992) Two upstream elements activate transcription of major histocompatibility complex class I gene in vitro. *Nucl. Acids Res.* 20: 2533-2540

Driggers, P.H., Ennist, D.L., Gleason, S.L., Mak, W.H., Marks, M.S., Levi, B.Z., Flanagan, J.R., Appella, E. and Ozato, K. (1990). an interferon gamma-regulated protein that binds the interferon-inducible enhancer element of major histocompatibility complex class I genes. *Proc. Natl Acad. Sci. USA.* 87:3734.

Durbin, J.E., Hackenmiller, R., Simon, M.C., and Levy, D.E.(1996) Targeted disruption of the mouse STAT1 gene result in compromised innate immunity to viral disease *Cell* 84: 443-450

Duronio, V., Wejham, M.J., Abraham, S., Dryden, P., and Schrader, J.W. (1992) p21ras activation via hemopoietin receptors and c-kit requires tyrosine kinase activity but not tyrosine phosphorylation of p21ras GTPase-activating protein. Proc. Natl. Acad. Sci. U.S.A. 89:1587-1591

Dusting, G.J., Hickey, H., Akita, K., Jachno, K., Mutch, D., Ng,I. (1995) Cyclosporin a suppresses expression of two isoforms of nitric oxide synthase in cultured macrophages and vascular smmoth muscle. *Endothelium* 3: 241-261

Dziarski, R. (1989) Correlation between ribosylation of pertussis toxin substrates and inhibition of peptidoglycan-, muramyl dipeptide- and lipopolysaccharide-induced mintogenic stimulation in B lymphocytes. *Eur. J. immunol.* 19:125

Egan, S.E. and Weinberg, R.A. (1993) The pathway to siganl achievement. *Nature* 365: 781-783

Eilers, A., Georgellis, D., Klose, B., Schindler, C., Ziemiecki, A., Harpur, A.G., Wilks, A.F., and Decker, T. (1995) Differention-regulated srine phosphorylation of STAT1 promotes GAF activation in macrophages. *Mol. Cell. Biol.* 15:3579-3586

Eisenbeis, C.F., Singh, H., and Storb, U. (1995). Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator. *Genes Dev.* 9, 1377-1387

Eizirik, d.L., bjorklund, A., Welsh, N. (1993) Interleukin-1-induced expression of nitric oxide synthase in insulin producing cells is preceded by c-FOS induction and depends on gene transcription and protein synthasis. *FEBS Lett.* 317:62-66

Emil Martin, Carl Nathan, and Qiao-wen Xie (1994) Role of Interferon Regulatory Factor 1 in Indiction of Nitric Oxide Synthase J.Exp. Med. 180: 977-984

Fan,C.M., and Maniatis, T. (1989) Two different virus-inducible elements are required for human beta-interferon gene regulation. *EMBO J.* 8:101

Fantl, W.J., Escobedo, J.A., Martin, G.A., Turck, C.W., Del Rosario, M. *et al.*, (1992) Distinct phosphotyrosines on a growth factor receptor bind specific molecules that mediate different signalling pathways. *Cell* 69: 413-423

Fantl, W.J., Johnson, D.E., and Williams, L.T. (1993) Signalling by receptor tyrosine kinases. *Annu. Rev. Biochem* 62:453-481

Farra, M.A., and Schreiber,R.D.(1993) The molecular cell biology of interferon-gamma and its receptor. *Annu. Rev. Immunol* 11:571-611

Farrar, M.A., Campbell, J.D.and Schreiber, R.D. (1992) Identification of a functionally important sequence in the C terminus of the interferon-gamma receptor. *Proc. Natl. Acad. Sci. U.S.A.* 89: 11706-11710

Farrell, A.J., Black, D.R., Palmer, R.M.J., Moncada, S. (1992) Increased concetrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic disease. *Ann. Rheum. Dis.* 51:1219-1222.

Feelish, M et al. 1987*Eur. J. Pharmacol.* 139:19

Fehsel, K., Jalowy, A., Qi, S., Burkart, V., Hartmann, B., Kolb, H. (1993) Islet cells DNA is a target of inflammatory attack by nitric oxide. *Diabetes* 42:496-500

Fernandez-Botran, r., Sanders, V.M., Oliver, K.G., Chen, Y.W., krammer, P.H., uhr, J.W., and Vitetta, E.S. (1986) Interleukin 4 mediates autocrine growth of helper T cells after antigenic stinulation. *Proc. Natl. Acad. Sci.USA* 83:9689

Fertsch. D., and Vogel, S.N. (1984). Recombinant interferons increase macrophage Fc receptor capacity. *J. Immunol.* 132: 2436-2439.

Finkelman, F. D., Katona, I.M. Mosmann, t.R. and Coffman, R.L. (1988) IFN-gamma regulates the isotypes of Ig secreted during *in vivo* humoral immune responses. *J. Immunol.* 140: 1022-1027

Finkelman, F.D., Katona, I.M., Mosmann, T.R., and Coffman, R.L. (1988) IFN-gamma regulates isotypes of Ig secreted during in vivo humoral immune responses. *J. Immunol.* 140:1022-1027

Finney, M., Guy, G., Michell, R.H., Gordon, J., Dugas, B., Rigley, K.P., and Callard, R.E. (1990) Interleukin 4 activates human B lymphocytes via transient inositol lipid hydrolysis and delayed cAMP generation. *Eur. J. Immunol.* 20:151-156

Foor, F., Parent, S.A., Morin, N., Dahl, A.M., Ramadan, N., Chrebet, G., Bostian, K.A., Nielsen, J.B. (1992) Calcineurin mediates inhibition by FK506 and cyclosporin of recovery from alpha-factor arrest in yeast. *Nature* 360:682-684

Forhand, J.R., Pabst, M.J., Phillips, W.A., Johnson, Jr. R.B.(1989) Lipopolysaccharide priming of human neutrophils for an enhanced respiratory burst. *J. Clin. Invest.*83:74

Forrester, K., Ambs, S., Lupold, S. E., Kapust, R. B., Spillare, E. A., Weinberg, W.C., Felley-Bosco, E., Wang, X.-W., Geller, D.A., Tzeng, E., Billiar, T.M., and Harris, C.C. (1996) Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase expression by wild-type p53 *Proc.Natl. Acad. Sci. USA* 93:2442-2447

Fountoulakis, M., Juranville, J._F., Maris, A., Ozmen, L. and Garotta, G. (1990) One interferon g receptor binds one interferon g dimer. J. Biol. Chem. 265 (32): 19758-19767

Freissmuth, M., Casey, P.J., and gilman, A.G. (1989) G proteins control diverse pathways of transmembrane signalling. *FASEB Journal* 3:2125-2139

Frey, E.A. Miller, D.S., Jahr, T.G., Sundan, A., Bazil, V., Espevik, T., Finlay, B.B., Wright, S.D. (1992) Soluble CD14 participates in the response of cells to lipopolysaccharide. *J. Exp. Med.* 176:1665-1671

Friedman, R.L., and Stark, G.R. (1985) Alpha-interferon-induced transcription of HLA and metallothionein genes containing homologous upstream sequences. *Nature* 314:637

Fu, X.-Y. (1992). Atranscription factor with SH2 and SH3 domains is directly activated by a interferon α-induced cytoplasmic protein tyrosine kinase(s). *Cell.* 70:323.

Fu, X.F., Schindler, C., Improta, T., Aebersold, R. & Darnell, J. E. Jr. (1992) The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction. *Proc. Natn. Acad. Sci. U.S.A.* 89, 7840-7843

Fuijita, T., Kimura, Y., Miyamoto, M., Barsoumian, E.L., and Taniguchi, T. (1989) induction od endogenous IFN-α and IFN-β genes by a regulatory transcription factor, IRF-1. *Nature* 337:270-272

Fuijita, T., Sakakibara, J., Sudo, Y., Miyamoto, M., Kimura, Y. and Taniguchi, T (1988) Evidence for a nuclear factor(s), IRF-1, mediating induction and silencing properties to human IFN-β gene regulatory elements. *EMBO J.* 7:3397-3405

Fuijita,T., Shibuya,H., Hotta, H, Yamanishi,K. and Taniguchi,T. (1987) Interferson-β gene-regulation-tandemly repeated sequences of a sythetic 6-bp oligomer function as a virus-inducible enhancer. *Cell* 49: 357-367

Fujita, T., Reis, L.F.L., Watanabe, N., Kimura, Y., Taniguchi, T. and vilcek, J. (1989). Induction of the transcription factor IRF-1 and interferon-β mRNAs by cytokines nd activators of second messenger pathways. *Proc. Natl Acad. Sci. USA.* 86: 9936

Furchgott, R.F. and Zawadzki, J.V. (1980) The obligatory role of endothelial cells in the relaxtation of arterial smooth muscle by acetylcholine. *Nature* 288:373-376

Galizzi, J.P., Zuber, C.E., Harada, N., Gorman, D.M., Djossou, O., Kastelein, R., Banchereau, J., Joward, M., and Miyajima, A. (1990), Molecular cloning of a cDNA encoding the human interleukin 4 receptor. *Int. Immunol.* 2:669-675

Gazzinelli, R.T., Oswald, I.P., James, S.L. and Sher, A., (1991) J. Exp. Med. 174:1549

Geller, D.A., Lowenstein, D.J., shapiro, R.A., Nussler, a.K., Disilvio, M., Wang, S.C., Nakayama, D.K., simmons, r.L., Synder, S.H., Billiar, T.R. (1993) Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc. Natl.Acad. Sci. USA.* 90:3491-3495

Geller, D.A., Nussler, A.K., Disilvio, M., Lowenstein, C.J., Shapiro, R.A., Wang, S.C., Simmons, R. L., Billiar, T.R. (1993) Cytokines, endotoxin, and glucocorticoids

regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc. Natl.Acad. Sci. USA*. 90:522-526

Gilman, A.G. (1987) G-proteins-transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56:615-649

Giovarelli, M., Santoni, A., Jemma, C., Musso, T., Giuffrida, A.M., Cavallo, G., Landolfo, S., and Forni, G. (1988) Obligatory role of IFN-gamma in induction of lymphokin-activated and T-lymphocyte killer activity, but not in boosting of natural cytotoxicity. *J. Immunol* 141:2831-2836

Gordon, J. A. (1991) Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. *Meth. Enzymol.* 201, 477-482

Gouilleux, F., Wakao, H., Mundt, M., and Groner, B. (1994). Prolactin induces phosphorylation of Tyr694 and STAT5 (MGF), a prerequisite for DNA binding and induction of transcription. *ENBO J.* 13: 4361-4369

Goureau, O., Lepoivre, M., Becquet, F., Courtois, Y. (1993) Differential regulation of inducible nitric oxide synthase by fibroblast growth factors and transforming growth factor beta in bovine retinal pigmented epithelial cells inverse correlation with cellular proliferation. *Proc. Natl. Acad. Sci. USA*. 90: 4276-4280

Graham, G.J., Schreiber, R.D., Goeddel, D.V., and Pennica, D., (1993) Interferon-gamma induces receptor dimerization in solution and on cells. *J. Biol. Chem.* 268: 18103-18110

Granger,D.L., Hibbs, J.B. Jr., Perfect, J.R. and Durack, T.D., (1988) Specific amino acid (L-arginine) requirement for the microbiostatic activity of murine macophages. *J. Clin. Invest.* 81:1129-1136

Gray, P.W., Flaggs, G., Leong, S.R., Gumina, R.J., Weiss, J., Ooi, C.E., Elsbach, P. (1989) Cloning of the cDNA of a human neutrophil bacterial protein. Structure and functional correlations. *J. Biol. Chem.* 264:9505-9509

Gray, P.W., Leong, S., Fennei, E.H., Farrar, M.A., Pjngel, J.T., Fernandezluna, J., and schreiber, R.D. (1989) Cloning and expression of the cDNA for the murine interferon gamma receptor. *Proc. Natl. Acad. Sci.USA* 86:8497-8501

Green, L.C., Wagner, D.A., Ruisde lusuriaga, K., Istfa, N., Young, V.R., (1981) Tannenbaum, S.R.Nitrate biosynthesis in man. *Proc. Natl. Acad. Sci. USA.* 78:7764-7768

Green, S.J., Meltzer, M.S., Hibbs, J. B., Nacy, C.A. (1990) Activated macrophages destroy intracellular *leishmania major* amastigotes by an L-arginine dependent killing mechanism. *J. Immunol.* 144: 278-283

Green, S.J., Nacy, C.A. (1993) Antimicrobial and immunopathologic effects of cytokine-induced nitric oxide synthesis. *Curr. Opin. Infect. Dis.* 6:384

Green, S.P., Hamilton, J.A., and Phillips, W.A. (1992) zymosan-triggered tyrosine phosphorylation in mouse bone-marrow-derived macrophages os enhanced by respiratory-burst priming agents. *Biochem J.* 288, 427-432

Griffith, T.M., Edwards, D.H., Lewis, M.J., Newby, A.C., Henderson, A.H. (1984) The nature of endothelium-derived vascular relaxant factor. *Nature.* 308:645-647

Griscavage J.M., Wilk, S. & Ignarro, L.J. (1995) Serine and cysteine proteinase inhibitors prevent nitric oxide production by activated macrophages by interfering with transcription of the inducible NO synthase gene. *Biochem. Biophys. Res. Commun.* 215:721-729

Griscavage, J M., Wilk, S., and Ignarro, L.J.(1996) Inhibitions of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NFkB *Proc. Natl. Acad. Sci. USA.* 93:3308-3312

Grube, B.J., Cochrane, C.G., Ye, R.D., Uleitch, R.J., Tobias, P.S. (1994) Cytokine and dexamethasone regulation of lipopolysaccharide binding protein (LBP) expression in human hepatoma (HepG2) cells. *J. Biol. Chem.* 269:8477-8482

Gruup, S.A., and Harmony, J.A.K. (1985) Increased phosphatidylinositol metabolism is an important but not an obligatory early event in B lymphocyte activation. *J. Immunol.* 134:4087

Gryglewski R.J., Moncada, S., Palmer R.M.J. (1986) Bioassay of prostacyclin and endothelium derived relaxing factor (EDRF) from porcine aortic endothelia cells. *Br J. Pharmacol.* 87:685-694

Gryglewski, R.J., Palmer, R.M.J., Moncada, s. (1986) Superoxide anion is involved in the breakdown of endothelium-derived vasular relaxing factor. *Nature.* 320:454-456

Gutch, M.J., Daly,C. & Reich, N. (1992) Tyrosine phosphorylation is required for activation of an interferon-stimulated transcription factor. *Proc. Natl Acad. Sci. U.S.A.* 89, 11411-11415

Hamilton, T.A., Becton, D.L., Somers, S.D., Gray, P.W., and Adams, D.O. (1985). Interferon-g modulates protein kinase C activity in murine peritoneal macrophages. *J. Biol. Chem.* 260:1378-1381.

Hampton, R.Y.D., Golenbock, D.T., and Raetz, C.R.H. (1988) Lipid A binding sites in membranes of macrophage tumor cells. *J. Biol. chem.* 263:14802

Hampton, R.Y.D., Golenbock, D.T., Penman, M., Krieger, M., and Raetz, C.R.H. (1991) Recognition and plasma clearance od endotoxin by scavenger receptors. *Nature* 352:342

Harada, H., Fuijita, T., miyamoto, M., Kiura, Y., Maruyama, M., Furia, A., Miyata, T., and Taniguchi, (1989) Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell* 58:729-739

Harada, H., Kitagawa, M., Tanaka, N., Yamamoto, H., Harada, K., Ishihara, M. and Taniguchi, T. (1993). Anti-oncogenic and oncogenic potentials of interferon regulatory factors-1 and -2. *Science*. 259:971-974.

Harada, H., Willison, K., Sakakibara, J., Miyamoto, M., Fuijita, T., and Taniguchi, T., (1990) Absence of type I IFN system in EC cells: transcriptional activator (IRF-1) and repressor (IRF-2) genes are developmentally regulated. *Cell* 63: 303-312

Harada, N., Castle, B.E., Gorman, D.M., Iton, N., Miyajima, A. (1990) Expression cloning of a cDNA encoding the murine interleukin 4 receptor based on ligand binding. *Proc. Natl. Acad. Sci. U.S.A.* 87:857-861

Hariharan, I.K., Chuang, P.T., Rubin, G.M. (1991) Cloning and characterization of a receptor-classphosphotyrosine phosphatase gene expressed on central nervous system axons in Drosophila melanogaster. *Proc. Natl. Acad. Sci. USA* 88:11266-11270

Harnett, M.M. (1996) Cellular signalling events in B lymphocytes. in *Blood Cell Biochemistry* (Whetton, A.D. and Gordon, J. ed.), vol.7 pp79-98 Plenum Press, New York and London.

Harnett, M.M., and Klaus, G.G.B. (1988) G protein regulation of receptor signalling. *Immunol. today* 9:315-320

Harnett, M.M., Holman, M.J., and Klaus, G.G.B. (1991) IL-4 promotes anti-Ig-mediated PKC translocation and reverses phorbol ester-mediated PKC downregulation in murine B cells. *J. Immunol.* 147: 3831-3835

Harnett, M.M., Holman, M.J., Klaus, G.G.B. (1991) IL-4 promotes anti-Ig-mediated protein kinase C down-regulation in murine B cells. *J. Immunology* 147: 3831-3836

Harpur, A.G., Andrew, A.C., Ziemiecki, A., Aston, R. R. & Wilks, A.F. (1992) JAK2, a third member of the JAK family of protein tyrosine kinases. *Oncogene* 7, 1347-1353

Harroch, S., Gothelf, Y., Watanave, N., Revel, M. and chebath, J.(1993). Interferon regulatory factor family in M1 cells. *J. Biol. Chem.* 268:9092.

Hart, P.H., Vitti, G.F., Burgess, D.R.m whitty, G.A., Piccoli, D.S. and Hamilton, J.A., (1989) Potential antiinflammatory effects of interleukin 4: Suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostagandin E2. *Proc. Natl.Acad.Sci.USA.* 86:3803-3807

Hatakeyama, M., Kono, T., Kobayashi, N., Kawahara, A., Levin, S.D., Perlmutter, R.M., Taniguchi, T. (1991) Interaction of the IL-2 receptor with the Src-fanily kinase p56(lck): Identification of novel intermolecular association. *Science* 252: 1523-1528

Hauschildt, S., Hoffman, P., Beuscher, H.U. Dufhues, G. Heinrich, P., Wiesmuller, K.H., Jung, G. and Bessier, W.G. (1990) Activation of bone marrow-derived mouse macrophages by bacterial lipopeptide: Cytokine production, phagocytosis and Ia expression. *Eur. J. Immunol.* 20: 63-68

Hayes, M.P., and Zoon, K. C. (1993) Priming of human monocytes for enhanced lipopolysaccharide responses: Expression of alpha interferon, interferon regulatory factors, and tumor necrosis factor. *Infect. Immun.*61: 3222-3227

Heck, D.E., Laskin, D.L., Gardner, C.R., Laskin, J.D. (1992) Epidermal growth factor supresses nitric oxide and hydrogen peroxide production by keratinocytes potential role for nitric oxide in the regulation of wound healing. *J. Bio. Chem.* 267:21277-21280

Heidecker, G., Kolch, W., Morrison, D.K., Rapp, U.R. (1992) The role of raf-1 phosphorylation in signal transduction. *Adv. Cancer Res.* 58:53-73

Heiss, L.N., Lancaster, J.R., Jr., Corbett, J.A., and Goldman, W.E. (1994) Epithelia autotoxicity of nitric oxide: role in the respiratory cytopathology of pertusis. *Proc. Natl.Acad. Sci. USA* 91: 267-270

Heldin, C.-H. (1995) Dimerization of cell surface receptors in signal transduction. *Cell* 80:213-223

Hemmi, S., Bohni, R., Stark, G., Di Marco, F., and Aguet, M. (1994) A novel member of the interferon receptor family complements functionality of the murine interferon γ receptor in human cells. *Cell* 76: 803-810

Hemmi, S., Peghini, P., Metzler, M., Merlin, G., Dembic, Z., and Aguet, M. (1989) Cloning of murine interferon gamma receptor cDNA: Expression in human cells mediates high-affinity binding but is not sufficient to confer sensitivity to murine interferon gamma. *Proc. Natl. Acad. Sci.USA* 86:9901-9905

Henkel, T., Machleidt, T., Alkalay, I., Kronkel, M., Ben-Neriah, Y.and Baeuerle, P.A. (1993) Rapid proteolysis of IkappaB-alpha is necessary for activation of transcription factor NF-kappaB. *Nature* 365:182-185

Hevel, J.M., White, K.A., Marletta, M.A. (1991) Purification of the inducible murine macrophage nitric oxide synthase identification as a flavoprotein. *J. Biol. Chem.* 266:22789-22791

Heyworth, C.M., Dexter, T.M., Nicholls, S.E., and Whetton, A.d. (1993) Protein kinase C activators can interact synergistically with granulocyte colony-stimulating factor or interleukin-6 to stimulate colony formation from enriched granulocyte-macrophage cololony-forming cells. *Blood* 81:894-900

Heyworth, C.M., Ponting, I.L.O., and Dexter, T.M. (1988) The response of haemopoietic cells to growth factors: Developmental implications of synergistic interactions. *J Cell. Sci.* 91: 239-247

Heyworth, C.M., Whwtton, A.D., Nicholls, S., Zsebo, K., and Dexter, T.M. (1992) Stem cell factor directly stimulates the development of enriched granulocyte-macrophage colony-forming cells and promotes the effects of other colony-stimulating factors. *Blood* 80: 2230-2236

Hibbs, J.B., Lambert, L.H., Remington, J.S. (1972) *Nature* 235; 48-50

Hibbs, J.B., Taintor, R.R., Vavrin, Z. (1987) Macrophage cytotoxicity-role for L-arginine deiminase and imino-nitrogen oxidation to nitrice. *Science* 235:473-476

Hibbs, J.B., Vavrin Z., Taintor, R.R. (1987) L-arginine is required for expression of the activated macrophage effect mechanism causing selective metabolic inhibition in target-cells. *J. Immunol.* 138:550-565

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Identification of an oncoprotein and UV responsive kinases that binds and potentiates the activity of the c-Jun activation domain. Genes. Dev. 7:2135-2148

Hiemstra, P.S., Eisenhauer, P.B., Harwig, S.S.L., Van den Barselaar, M.T., Van Furth, R. and Lehrer, R.I. (1993) *Infect. immun.* 61:3038-3046

Hill, Caroline S. and Treisman, R.(1995) Transcriptional regulation by extracellular signals: mechanisms and specificity *Cell* 80:199-211

Hisaahi Harada, Takashi Fujita, Massaki Miyamoto, yoko kimura, mitsuo Maruyama, Adriana Furia, Takashi Miyata, and Tadatsugu Taniguchi (1989) *Cell* 58, 729-739.

Hoffman, R.A., Landrehr, J. M., Billiar, T. R., Curran, R. D., Simmons, R.L.(1990) Alloantigen-induced activation of rat splenocytes is regulated by the oxidative metabolism of L-arginine. *J. Immunol.* 145:2220-2226

Holmes, W.E., Lee, J., Kuang, W.-J., Rice, G.C. and Wood, W.I. (1991) Structure and functional expression of a human interleukin-8 receptor. *Science* 253:1278-1282

Holt, P.G., Oliver, J., Bilyk, N., McMenamin, C., McMenamin, P.G., Kraal, G., and Thepen, T. (1993) Dowenregulation of the antigen presenting cell functions of pulmonary dendritic cells in vivo by resident peritoneal macrophages *J.Exp.Med.* 177:397

Holtschke, T., lohler, J., Kanno, Y., Fehr, T., Giese, N., Rosenbauer, F., Lou, J., Knobeloch, K-P., Gabriele, L., Waring, J.F., Bachmann, M.F., Zinkernage, R.M., Morse III, H.C., Ozato, K., and Horak, I. (1996) Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP Gene. *Cell* 87: 307-317

Horak, I.D., Gress, R.E., Lucas, P.J., horak, E.M., waldmann, T.A., Bolen, J.B. (1991) T-lymphocyte interleukin 2-dependent tyrosine protein kinase signal transduction involves the activation of p56 (lck). *Proc. Natl.Acad. Sci. USA* 88:1996-2000

Hou, J., Schindler, U., Henzel, W.J., Ho, T.C., Brasseur, M., and Mcknight, S.L. (1994). An interleukin 4-induced transcription factor: IL-4 stat. *Science*. 265:1701-1706

Howard, M.C., Miyajima, A., and Coffman, R. (1993) T-cell derived cytokines and their receptors, in *Fundamental Immunology* (W.E.Paul, ed.), pp. 763-800. Raven press, New York.

Huang, K.Y., Donakoe, R.M., Gordon, R.B. and Dressler, H.R. (1971) Enhancement of phagocytosis by interferon-containing preparations. *Infect. Immunol.* 43: 581-588

Hunter, T. (1987) A 1001 protein-kinases.*Cell* 50 (6): 823-829

Hunter, T. (1993) Cytokine connections. *Nature* 366: 114-116

Hunter, T. (1995) Protein kinases and phosphatases: The Yin and Yang of protein phosphorylation and signaling. *Cell* 80:225-236

Hunter, T. and Cooper, T.H. (1985) protein-tyrosine kinases. *Annu. Rev. Biochem.* 54:897-930

Hunter, T., Sefton, B. M. (1980). *Proc. Natl. Acad. Sci. USA* 77: (3) 1311

Ialenti, A., Ianaro, A., Moncada, S., Di Rosa, M. Modulation of acute inflammation by endogenous nitric oxide. (1992) *Eur. J. Pharmacol.* 211:177-182.

Ialenti, A., Moncada, S., Dirosa, M. Modulation of adjuvant arthritis by endogenous nitric oxide. (1993) *Br. J. Pharmacol.* 110:701-706

Ianaro, A., O'donnell, C.A., Dirosa, M., Liew, F.Y. (1994) A nitric oxide synthase inhibitor reduces inflammation, down-regulates inflammatory cytokines and enhances interleukin-10 production in carrageenin-induced oedema in mice. *Immunology*. 92;370-375

Idzerda, R.L., March, C.J., Mosely, B., Lyman, S.D., and Backmann, M.P. (1990) Human interleukin 4 receptor confers biological responsiveness and defines a novel receptor superfamily. *J. Exp. Med.* 171:861-873

Igarashi, K., David, M., Finbloom, D.S., and Larner, A.C. (1993) In vitro activation of the transcription factor gamma interferon activation factor by gamma interferon: Evidence for a tyrosine phosphatase/kinase signalling cascade. *Molecular and Cellular Biology*, Mar. 1634-1640

Ignarro, L.J., Bugr, G.M., Wood, K.S., Byrns, R.E., Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl.Acad.Sci. USA.* 84:9265-9269

Ihle, J.N. (1996) STATs, signal transducers and activators of transcription *Cell* 84: 331-334

Ihle, J.N. Witthuhn, B.A., Quelle, F.W., Yamamoto, K., and Silvennoinen, O. (1995) Signalling through the hemotopoietic cytokine receptors. *Annu. Rev. Immunol.* 13:369-398

Ihle, J.N., Witthuhn, B., Tang, B., Yi, T., and Quelle, F.W (1994a) Cytokine receptors and signal transduction in *Bailliere's Clinical Haematology* pp17-48, Baiilliere Tindall, London.

Ihle, J.N., Witthuhn, B.A., Quelle, F.W., Yamamoto, K., Thierfelder, W.E., Kreider, B., and Silvennoinen, O. (1994) Signaling by the cytokine receptor superfamily: JAKs and STATs. *Trends Biochem. Sci.* 19: 222-227

Imai, T., Hirata, Y., Kanno, K., Marumo, F. (1994) Induction of nitric oxide synthase by cyclic AMP in rat vascular smooth muscle cells. *J. Clin. Invest.* 93: 543-549

Imai, T., Hirata, Y., Marumo F. (1992) Expression of brain nitric oxide synthase messager RNA in various tissues and cultured cells of rat. *Biomed. Res.* 13:371-374

Imbert, V., Rupec, R.A., Livilsi, A., Pahl, H.L. et al., (1996) Tyrosine phosphorylation of Ik-α, activates NFkB without proteolytic degradation of IkB-α. *Cell* 86:787-798

Ina, Y., Koide, Y., Nezu, N., and Yoshida, T.O. (1987). Regulation of HLA class II antigen expression: intracellular signaling molecules responsible for the regulation by IFN-γ and cross-linking of Fc receptors in HL-60 cells. *J. Immunol.* 139: 1711-1717.

Isakson, P., Pure, E., Vitetta, E.S., and Krammer, P.H. (1982) T cell-drived B cell differentiation factors: effect on the isotype swith of murine B cells. *J. Exp. Med.* 155:734

Isfort, R.J and Ihle, J.N. (1990) Multiple hematoietic growth factors signal through tyrosine phosphorylation. *Growth Factors* 2: 213-220

Iyrngar, R., Stuehr, D.J., Matletta, M.A. (1987) Macrophage synthesis of nitrite, nitrate, and N-Nitrosamine-precursors and role of the respiratory burst. *Proc. Natl. Acad. Sci. USA.* 84: 6369-6373

Izuhara, K., and Harada, N. (1993) Interleukin-4 (IL-4) induces protein tyrosine phosphorylation of the IL-4 receptor and association of phosphatidylinositol 3-kinase to the IL-4 receptor in a mouse T cell line, HT2. *J. Biol. Chem.* 268:13097-13102

Jacobson, N.G., szabo, S.J., weber, Nordt, R.M., Zhong, Z., Schreiber, R.D., Darnell, J.Jr., and Murphy, K.M. (1995) Interleukin-12 signalling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (STAT) 3 and STAT4. *J. Exp. Med.* 181:1755-1762

Jakway, J.P., and DeFranco, A.L. (1986) Pertussis toxin inhibition of B cell and macrophage responses to bacterial lipopolysaccharide. *Science* 234:743

James, S.L. and Glaven, J. (1989) Macrophage cytotoxicity against schistosomula of Schistosoma mansoni incolves arginine-dependent production of reactive nitrogen intermediates. J. *Immunol.* 143: 4208-4212

Johnston, J.A., Kawamura, M., Kirken, R.A., Chen, Y.Q., Blake, T.B., Shibuya, K., Ortaldo, J.R., McVicar, D.W., and O'Shea, J.J. (1994) Phosphorylation and activation of JAK3 Janus kinase in response to interleukin-2. *Nature* 370:151-153

Joseph Didonato, Frank Mercurio, Caridad Rosette, Jian Wu-Li, Helena Suyang, Sankar Ghosh, and Michael Karin (1996) Mapping of the Inducible IkB Phosphorylation Sites That Signa Its Ubiquitination and Degradation. Molecullar and Cellular Biology 16: 1295-1304

Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Le, J., Koh, S.I., Green, S.J., Mak, T.W., Taniguchi, T and Vilcek, J (1994) Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* 263:1612-1615

Kan, O., Evans, C.A., and Whetton, A.D. (1992) Cellular events stimulated by myeloid haemopoietic growth factors in *Bailliere's Clinical Haematology* (T.M. Dexter and B.I. Lord, eds.), pp653-679, Bailliere Tindall, London.

Kanakura, Y., Druker, B., Cannistra, S.A., Furukawa, Y., Torimoto, Y., and Griffin, J.D. (1990) Signal transduction of the human granulocyte-macrophage colony-stimulating factor and interleukin-3 receptor involves tyrosine phosphorylation of common set of cytoplasmic proteins. *Blood* 76:706-715

Karnitz, L.M., and Abraham, R.T. (1995) Cytokine receptor signaling mechanisms *Current Opinion in Immunology* 7:320-326

Karupiah, G., Xie, Q.W., Buller, M, L., Nathan, C., Duarte, C., and MacMicking, J.D. (1993) Inhibition of viral replication by interferon-γ-induced nitric oxide synthase. Science 261:1445

Kashishian, A., Kazlauskas, A., and Cooper, J.A. (1992) Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase *in vivo*. *EMBO J.* 11: 1373-1382

Kashles, O., Yarden, T., Fischer, R., Ullrich, A., Schlessinger, J. (1991)A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by heterodimerization. *Mol. Cell. Bio.* 11:1454-1463

Kawamoto, S., and Hidaka, H. (1984) 1-(-5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. *Biochem. Biophys. Res. Commun.* 125:258

Kazlauskas, A. (1994) Receptor tyrosine kinases and their targets. *Current Opinion in Genetics and Development* 4:5-14

Keegan, A.D., Nelms, K., White, M., Wang, L.M., Pierce, J.H., and Paul, w.E. (1994) An IL-4 receptor region containing an insulin receptor motif is important for IL-4 mediated IRS-1 phosphorylation and cell growth. *Cell* 76:811-820

Keller, A. D. and Maniatis, T. (1991). Identification and characterization of a novel repressor of beta-interferon gene expression. *Genes Dev.* 5:868.

Keller, R., Jones, V.E. (1971) Role of activated macrophages and antibody in inhibition and enhancement of tumour growth in rats. *The Lancet* 22:842-849

Kilbourn, R.G., Gross, S.S., Jubran, A., Adams, J., Griffith, O.W., Levi, R., and Lodato, R. (1990) N^G-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide. *Proc. Natl. Acad. Sci. USA* 87:3629

Kirchhoff, S., Schaper, F. and Hauser, H. (1993). Interferon regulatory factor 1 (IRF-1) mediates cell growth inhibition by transactivation of downstream target genes. *Nucleic Acids Res.* 21: 2881.

Kirchhoff, S.K., Hoffmann, K., Schaper, F., Hoxter, M., and Hauser, H. (1992) *J. Interferon Res.* 12:S102

Kirk, S.J., Regan, M.C., Barbul, A. (1990) Cloned murine T lymphocytes synthesize a molecule with the biological characteristics of nitric oxide. *Biochem. Biophys. Res. Commun.* 173: 660-665

Kishimoto, T., Taga,T., Akira, S. (1994) Cytokine signal transduction. *Cell* 76: 253-262

Klein, J.B., McLeish, K.R., Sonnenfeld, G., and Dean, W.L. (1987). Potential mechanisms of cytosolic calcium modulation in interferon-g treated U-937 cells. *Biochem. Biophys. Res. Commun.* 145, 1295-1301.

Klein, J.B., Scherzer, J.A., and McLeish, K.R. (1991). Interferon-γ enhances superoxide production by HL-60 cells stimulated with multiple agonists. *J. Interferon Res.* 11:69-74.

Kobzik, L., Reid, M.B., Bredt, D.S., Stamler, J.S.(1994) Nitric oxide in skeletal muscle. *Nature* 372:546-548

Koch,C.A., Anderson D., Moran M.F., Ellis, C., Pawson T. (1991) SH2 and SH3 domains: elements that contol interactions of cytoplasmic signaling proteins. *Science* 252:668-674

Koide, M., Kawahara, Y., Tsuda, T., Yokoyama, M. (1993) Cytokine induced expression of an inducible type of nitric oxide synthase gene in cultured vascular smooth muscle cells. FEBS Lett. 318: 213-217

Koide, Y., Ina, Y., Nezu, N., and Yoshida, T.O. (1988). Calcium influx and the Ca^{2+}-calmudulin complex are involved in interferon-γ-induced expression of HLA class II molecules on HL-60 cells. *Proc. Natl. Acad. Sci. USA.* 85:3120-3124.

Kolb, H., Kiesel, U., Kroncke, K.D., Kolbbachofen, V. (1991) Supression of low-dose streptozotocin induced diabetes in mice by administration of a nitric oxide synthase inhibitor. *Life Science* 49: 213-217

Kondo, M., Takeshita, T., Ishhii, N., Nakamura, M., Watanbe, S., Arai, K., and Sugamura, K. (1993) Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4. *Science* 262:1874-1877

Kotanides, H., and Reich, N.C. (1993) Requirement of tyrosine phosphorylation for rapid activation of a DNA binding factor by IL-4. *Science* 262:1265-1267

Kruppa, G., Thoma, B., Machleidt, T., Wiegmann, K. Kronke, M. (1992) Inhibition of tumor-necrosis-factor (TNF)-mediated NFκ-B activation by selective blockade of the human 55kda TNF receptor.*J. Immunol.* 148: 3152-3157

Kumar, C.S., Muthukumaran, G., Frost, L.J., Noe, M., Ahn, Y.H., Mariano, T.M., Pestka, S. (1989) Molecular characterization of the murine interferon-γ recptor cDNA. *J. Biol. Chem.* 264: 17939-17946

Kwon, N.S., Nathan, C.F., Stuehr, D.J. (1989) Reduced biopterin as a cofactor in the generation of nitrogen-oxides by murine macrophages. *J. Biol. Chem.* 264: 19654-19658

Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J. R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 369: 156-160

Laemmli, U.K. (1970) Nature 227:680-685

Lange-carter, c.A., Pleiman, C.M., Gardner, A.M., Blumer, K.J. and Johnson, G.L. (1993) A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science* 260: 315-319

Langrehr, J.M., Hoffman, R.A., Billiar, t.R., Lee, K.K.W., Schraut, W.H., simmons, R.L. (1991) Nitric oxide synthesis in the in vivo allograft response: a possible regulatory mechanism. *Sugery* 110: 335-342

Larry M Karnitz and Robert T Abraham (1995) *Current Opinin in Immunology* 7, 320-326.

Lee, K.A.W., Bindereif, A., Green, M.R. (1988) A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. *Gene Anal. Techn.* 5, 22-31

Lehn, M., Weiser, W.Y., Engelhorn, S., Gillis, S., and Remold, H.G. (1989) IL-4 inhibits H_2O_2 production and anti-leishmanial capacity of human cultured monocytes mediated by IFN gamma. *J. Immunol.* 143:3020-3024

Lehrer, R.I., Lichtenstein, A.K., and Ganz, T. (1993) *Annu. Rev. Immunol.* 11:105-128

Lei, M.G., and morrison, D.C. (1988) Specific endotoxic lipopolysaccharide-binding proteins on murine splenocytes. *J. immunol.* 141:996

Leibson, H.J., Gefter, M., Zlotnik, A., Marrack, P., Kappler, J.W. (1984) *Nature* 309:799-801

Lenardo, M.J., and Baltimore, D.(1989) NF-kappaB: A pleiotropic mediator of inducible and tissue-specific gene control. *Cell* 58, 227-229

Lev, S., Givol, D., and Yarden, Y. (1992) Interkinase domain of kit contains the binding sites for phosphatidylinositol 3' kinase. *Proc. Natl. Acad. Sci.* U.S.A. 89:678-682

Levinson, A. D., Oppermann, H., Varmus, H.E., Bishop, I. M. (1980) *J. Biol. Chem.* 255(24): 11973

Levy, D.E., and Darnell, J.E., Jr (1990) Interferon-dendent trancriptional activation: signal transduction wihtout second messenger involvement. *New Biol.* 2:923

Levy, D.E., Kessler, D.S., Pine, R., Reich, N. and Darnell, J.E. (1988). Interferon-induced nuclear factor that bind a shared promoter element correlate with positive and negative transcriptional control. *Genes Dev.* 2:383.

Liebow, C., Reilly, C., Serrano, M., Schally, A. V. (1989) Somotostation analogues inhibit growth of pancreatic cancer by stimulating tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA* 86: 2003-2007

Liew, F.Y. (1990) Regulation of cell-mediated immunity in Leshmaniasis. *Current Topics in Microbiology and Immunology.* 155: 53-64

Liew, F.Y., Li, Y., Seven,A., Millot, S., Schmidt, J., Salter, M., Moncada, S. (1991) A possible novel pathway of regulation by murine T helper type-2 (th2) of a Th1 cell activity via the modulation of the induction of nitric oxide synthase on macrophages. *Eur. J. Immunol.* 21: 2489-2494

Liew, F.Y., Millott, S., Li, Y., Lelchuk, R., Chan, W.L. and Ziltner, H. (1989) Macrophage activation by interferon gamma from host-protective T cells is inhibited by interleukin (IL) 3 and IL-4 produced by disease-promoting T cells in leishmaniasis. *Eur. J. Immunol.* 19: 1227

Liew, F.Y., Millott, S., Parkinson, C., Palmer, r.M.J. and Moncada, S. (1990) Macrophage killing of Leishmnia parasite *in vivo* is mediated by nitric oxide from L-arginine. *J. Immunol.* 144: 4794-4797

Liew. F.Y., Li, Y., Moss, D., Parkinson, C., Rogers, M.V., Moncada, S. (1991) Resistance to Leishmania major infection correlates with the induction of nitric oxide synthase in murine macrophages. *Eur. J. Immunol.* 21:3009-3014

Lin, Y.C., Brown, K.& Siebenlist, U. (1995) Activation of NFκ-B requires proteolysis of the inhibitor I-kappa-alpha-signal-induced phosphorylation of I-kappa-B-alpha alone does not release active NFκ-B. *Proc. Natl.Acad. Sci.USA* 92: 552-556

Lindahl, P., Gresser, I., Leary, P., and Tobey, M. (1976). Interferon treatment of mice: nhanced expressionof histocompatibility antigens on lymphoid cells. *Proc. Natl. Acad. Sci. USA.* 73:1284-1287.

Liu, M.K., Brownsey, R.W. Reiner, N.E. (1992) Lipopolysaccharide activates a 2nd messenger-independent protein-kinase in human monocytes. *clin. Res.* 40: 248A

Liu, M.K., Brownsey, R.w. Reiner, N.E. (1994) Gamma-interferon induces rapid and coordinate activation of mitogen-activated protein-kinase (extracellular signal-regulation kinase) and calcium-independent protein-kinase-C in human monocytes. *Infect. Immun.* 62:2722-2731

Liu, M.K., Herrera-Velit, P., Nrownsey, R.W., and Reiner, N.E. (1994) CD14-dependent activation od protein kinase C and mitogen-activated protein kinases (p42 and p44) in numan monocytes treated with bacterial lipopolysaccharide. *J. Immunol.* 153:2642-265

Lochrie, M.A., and Simon, M.I. (1988) G-protein multiplicity in eukaryotic signal transduction systems. *Biochemistry* 27:4957-4965

Lorsbach, R.B., Murphy, W.J., Lowenstein C.J., Snyder, S.H., Russell, S.W. (1993) Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing molecular basis for the synergy between interferon gamma and lipopolysaccharide. *J. Bio. Chem.* 268: 1908-1913

Lowenstein C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W., Murphy, W.J. (1993) Macrophage nitric oxide synthase gene: a upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proc. Natl. Acad. Sci.USA.* 90: 9730-9734

Lowenstein, C.J., Glatt, C.S., Bredt, D.S., Snyder, S.H. (1992) Cloned and expressed macrophage nitric pxide synthase contrasts with the brain enzyme. *Proc. Natl. Acad. Sci. USA.* 89: 6711-6715

Lukic, M.L., Stosicgrujicic S., Ostojic, N., Chan, W.L., Liew, F.Y. (1991) Inhibition of nitric oxide generation affects the induction of diabetes by streptozocin in mice. *Biochem. Biophys. Res. Commun.* 178:913-920

Lyons, C. R., Orioff, g.J., Cunningham, J.M. (1992) Molecular cloning an functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J. Biol. Chem.* 267: 6370-6374

MacMicking, J. D., Nathan, C., Hom, G., Chatrain, N., Fletcher, D.S., Tumbauer, M., Stevens, K., Xie, Q.W., Sokol, K., Huchinson, N., Chen, Mudgett, J.S. (1995) Altered responses to infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 81: 641-650

Maeshall, C.J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* 80:179-185

Maggi, E., Parronchi, P., Manetti, M., Simonell, C., Piccinni, M.P., Rugiu, F.S., Carli, M.De., and Romagnani, S. (1992) Reciprocal regulatory effects of IFN-γ and IL-4 on the in vitro development of human Th1 and Th2 clones. *J. Immunol.* 148:2142

Marletta, M.A. Nitric oxide synthase structure and mechanism. (1993) *J. Biol. Chem.* 268:12231-12234.

Marsters, S., Bach, E.A., Schcriber, R.D., Aguet, M., and Ashkcnazi, A. (1995) *Proc. Natl.Acad.Sci.USA* 92:5401-5405

Martin, E., Nathan, C., Xie, Q.W. (1994) Role of interferon regulatory factor 1 in induction of nitric Oxide synthase. J. Exp. Med. 180:977-984

Martin, T.R., Mathison, J.C., Tobias, P.S., Maunder, R.J., Uleitch, R.J. (1992) LPS binding protein enhances the responsiveness of alveolar macrophages to bacterial LPS: implications for cytokine production in normal ans injured lungs. *J. Clin. Invest.* 90:2209-2219

Martin, T.R., Tobias, P.S., Mathison, J.C., Uleitch, R.J. (1994) Interactions between endotoxin and endotoxin binding protein. In *Endotoxin and the Lungs*, ed. K Brigham, 77:45-67 New York: Marcel Dekker

Martin, W., Villani, G.M., Jothianandan, D., Furchgott, R.F. (1985) Selective blackade of endothelium-dependent and glyceryl trinitrate-induced relaxtion by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp.* Ther. 232: 708-716

Massague, J., Attisano, L., and Wrana, J.L. (1994) The TGF-beta family and its composite receptors. *Trends Cell. Biol.*. 4:172-178

Mathison, J.C., Tobias, P.S., Wolfson, E., Ulevitch, R.J. (1992) Plasma, lipopolysaccharide binding protein: a key component in macrophage recognition of gram-negative lipopolysaccharide (LPS). *J. Immunol.* 149:200-206

Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J., and Kato, J.Y. (1994) D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.* 14:2066-2076

Matsushime, H., Rossel, M.F., ashmun, R.A., and Sherr, C.J. (1991) Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* 65:701-713

Matsuyama, T., T. Kimura, M. Kitagawa, K. Pfeffer, T. Kawakami, N. Watanabe, T.K. Kundig,R.Amakawa, K. Kishihara, A. Wakeham, etal. (1993) Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene and aberrant lymphocyte development. Cell. 75:83

Mayer, B., John, M., Werner, E.R., Wachter, H., Schultz, G., Bohme, E. (1991) Brain nitric oxide synthase is a biopterin containing and flavin containing multifunctional oxidoreductase. *FEBS letters.* 288:187-191.

McCartney-Francis, N., Allen, J.B., Mizel, D.E., Albina, J.E., Xie, Q.W., Nathan, C.F., and Wahl, S.M. (1993) Suppression of arthritis by an inhibitor of nitric oxide synthase. *J. Exp. Med.* 178:749

Meraz, M.A., White, J.M., Sheehan, K.C.F., Schreiber, R.D. et al., (1996) Targeted disruption of STAT1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* 84: 431-442

Metcalf, D. (1986) The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. *Blood* 67:257-267

Metcalf, D. and Nicola, N.A. (1991) Direct proliferative actions of stem cell factor on murine bone marrow cells *in vitro*. Effects of combination with colony-stimulating factors. *Proc. Natl. Acad. Sci. U.S.A.* 88: 6239-6243

Meydan, N., Grunberger, T., Dadi, H., Shahar, M., Arpaia, E., Lapidot, Z., Leeder, J.S., Freedman, M., Cohen,A., Gazit, A., Levitzki, A., and Roifman, C.M., (1996) Inhibition of acute lymphoblastic leukaemia by a JAK2 inhibtor. *Nature* 379 (15) 645-648

Middleton, S.J., Shorthouse, M., Hunter, J.O. (1993) Increased nitric oxide synthesis in ulcerative colitis. *Lancet* 341: 465-466

Miller, M.J.S., Sadowska-Krowicka, H., Chorinaruemol, S., Kakkis, J.L. Clark, D.A. (1993) Amelioration of chronic ileitis by nitric oxide synthase inhibition. *J. Pharmacol. Exp. Ther.* 264:11-16

Minami, Y., Kono, T., Yamada, K., Kobayashi, N., Kawahara, A., Perlmutter, R.M., Taniguchi, T. (1993) Association of p56(lck) with IL-2 receptor beta chain is critical for the IL-2-induced activation of p56(lck). *EMBO J.* 12:759-768

Minami, Y., Stafford, F.J., Lippincott, S.J., Yuan, L.C. Klausner, R.D. (1991) Novel redistribution of an intracellular pool of CD45 accompanies T-cell activation. *J. Biol. Chem.* 266(14): 9222

Minty, A., Chalon, P., Derocq, J.M., *et al.*, (1993) Interleukin-13: A novel human lymphokine regulating inflammatory and immune responses. *Nature* 362:248-250

Miossec, P., and Ziff, M. (1986) Immune interferon enhances the production of interleukin 1 by human endothelial cells stimulated with lipopolysaccharide. *J. Immunol.* 137:2848-2852

Mitchell, H.H., Shonle. K.A., and Grindley, H.S. (1916) The origin of nitrates in the urine. *J. Biol. Chem.* 24: 461-490

Miyajima, A., Kitamura, T., Harada, N., Yokota, T., and Arai, K. (1992) Cytokine receptors and signal transduction. *Annu. Rev. Immunol.* 10: 295-331

Miyamoto, M., Fuijita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T., and Taniguchi, T., (1988) Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-β gene regulatory elements. *Cell* 54: 903-913

Moncada, S. (1992) The L-arginine; nitric oxide pathway. *Acta. Physiol. scaned.* 145: 201-227

Moncada, S., and Higgs, E. A. (1990) Nitric oxide from L-arginine: a bioregulatiory system. *Excerpta Medica*

Moncada, S., and Higgs, E.A. (1991) Endogenous nitric oxide: physiology, pathology and clinical relevance. *Eur. J. clin. Invest.* 21: 361-374

Moncada, S., Higgs, E. A. (1993) The L-arginine-nitric oxide pathway. *The New England Journal of Medicine.* 11:2002-2012

Moncada, S., Palmer, R.M.J. and Higgs, E.A., (1991) Nitric-oxide-physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* 43:109

Montminy, M. (1993) *Science* 261: 1694-1695

Morel, F., Doussiere, J. and Vigais, P.V. (1991) The superoxide-generating oxidase of phagocytic-cells physilogical, molecular and pathological aspects. *Eur.J. Biochem.* 201, 523-546

Morris, S.M., Jr. and Billiar, T.R. (1994) New insights into the regulation of inducible nitric oxide synthesis. *Am. J. Physiol.* 266: E829-E838

Mosely, B., Beckmann, M.P., March, C.J., Idzerda, R.L., Gimpel, S.D., and Park, L.S. (1989) The murine interleukin-4 receptor: Molecular cloning and characterization of secreted and membrane bound forms. *Cell* 59:335-348

Mosmann, T.R., and Coffman, R.L. (1989) Th1 and Th2 cells: different patterns of lymphokin secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145

Mosmann, T.R., Schumacher, J.H., Street, N.F., Budd, R., O'Garra, A., Fong, t.A., Bond, M.W., Moore, K.W., Sher, A., and Fiorentino, D.F. (1991) Diversity of cytokine synthesis and function of mouse CD4+ T cells. *Immunol. Rev.* 123:209

Moss, D.W., Wei, X.Q., Liew, F.Y., Moncada, S., Charles, I.G. (1995) Enzymatic characterisation of recombinant murine inducible nitric oxide synthase. *Eur. J. Pharm.* 289: 41-48.

Mui, A. L.-F., Wakao, H., O'Farrell, A.M., Harada, N., and Miyajima, A. (1995) Interleukin-3, granulocyte macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. *EMBO J.* 14:1166-1175

Muller, M., Briscoe, J., Laxton, C., Guachin, D., Ziemiecki, A., Silvennoinen, O. Kerr, I.M. et al., (1993) The protein tyrosine kinase JAK1 complements defects in interferon-α/β and -γ signal transduction. *Nature* 366:129-135

Muller, M., Laxton, C., Briscoe, J., schindler, C., Improta, T., Darnell, J.J., Stark, G.R., and Kerr, I.M. (1993) Complementation of a mutant cell line: Central role of the 91kDa polypeptide of ISGF3 in the interferon-alpha and gamma signal transduction pathways. *EMBO J.* 12:4221-4228

Muller,M., Briscoe, J., Laxton,C., Guschin,D., Ziemiecki,A., Silvennoinen, O., Harpur, A.G., Barbieri,G., Witthuhn,B.A., Schindler, C., Pellegrini,S., Willks, A.F., Ihle,J.N., Stark, G.R., Kerr,I.M.(1993) *Nature* 366, 129-135

Mulligan, M. S., Hevel, J.M., Marietta, M.A.,Ward, P.A. (1991) Tissue injury caused by deposition of immune complexes is L-arginine dependent. Proc. Natl. Acad. Sci. USA. 88: 6338-6342

Mundel, P. Bachmann, S., Bader, M., Fischer, A., Kummer, W., Mayer, B., Kriz, W. (1992) Expression of nitric oxide synthase in kidney macula densa cells. *Kidney International.* 42: 1017-1019

Munro, S., and Maniatis, T. (1989) Expression cloning of the murine interferon-γ receptor cDNA. *Proc. Natl. Acad. Sci.USA* 86: 9248-9252

Murphy, E.D., Roths, J.B. (1979) Autoimmunity and lymphoproliferation: induction by mutant gene Lpr, and acceleration by a male-associated factor in strain BXSB mice. in: Rose NR et al., ed. *Genetic control of autoimmune disease*. Elsevier/North Holland, Amsterdam.

Nakayama, D.K., Geller, d.A., Lowenstein, C.J., Chern, H.D., Davies, P., Pitt, B.R., simmons, R.L., Billiar, T.R. (1993) Cytokines and lipopolysaccharide induce nitric oxide synthase in cultured rat pulmonary artery smooth muscle. *Am. J. Resp. Cell. Mol. Biol.* 9: 231

Naray-fejes-toth, A., and Guyre, P.M. (1984). Recombenant human immune interferon induces increased IgE receptor expression on the human monocyte cell line U-937. *J. Immunol.* 133: 1914-1919.

Nathan, C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J.* 6: 3051-3064

Nathan, C. and Xie, Q.-W. (1994) Nitric oxide synthases: roles, tolls, and controls. *Cell* 78: 915-918

Nathan, C., and Xie, Q.-W. (1994) Regulation of biosynthesis of nitric oxide. *J Bio. Chem.* 269: 13725-13728

Nathan, C.F., and Hibbs, J.B. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr. Opin. Immunol. 3:65

Neil E. Reiner (1994) Altered cell signaling and mononuclear phagocyte deactivation during intracellular infection. *Immunol. Today* 15(8) 374-381

Nelson, N., Kanno, Y., Hong, C., Contursi, C., Fuijita, T., Fowlkes, B.J., O' Connell, E., Hu-Li, J., Paul, W.E., Jankovic, D., Sher, A.F., Coligan, J.E., Thornton, A., Appella, E., Yand, Y., and Ozato, K., (1996) Expression of interferon regulatory factor family proteins in lymphocytes: induction of STAT1 and ICSBP expression by T cell activation. *J. Immunol.* 156:3711-3720

Nelson, N., Marks, M.S., Driggers, P.H. and Ozato, K. (1993). Interferon consensus sequence-binding protein a member of the interferon consensus sequence-binding protein, a member of the interferon regulatory factor family, suppresses interferon-induced gene transcription. *Mol. Cell. Biol.* 13:588.-599

Nezu, N., Ryu, K., Koide, Y., and Yoshida, T.O. (1990). Regulation of HLA class II molecule expression by IFN-γ. The signal transduction mechanisms in glioblastoma cell lines. *J. Immunol.* 147:3126-3135.

Nguyen, T., Brunson, D., Crespi, C.L., Penman, B.W., Wishnok, J.S. and tannenbaum, S.R. (1992) DNA damage and mutation in human cells exposed to nitric oxide in vitro. *Proc. Natl. Acad. Sci. USA* 89:3030-3034

Nick, J.A., Avdi, N.J., Gerwins, P., Johnson, G.L., and Worthen, S. (1996) activation of a p38 mitogen-activated protein kinase in human neutrophils by lipopolysaccharide. *J. Immunol.* 156:4867-4875

Nicola, N.A. (1989) Hemopoietic cell growth factors and their receptors. *Annu. Rev. Biochem.* 58:45-77

Noelle, R., Krammer, P.H., Ohara, J., Uhr, J.W. and Vitetta, E.S. (1984) Increased expression of Ia antigens on resting B cells : an additional role for B cell growth factor. *Proc. Natl. Acad. Sci. USA* 81:6149

Noguchi, M., Nakamura, Y., Russell, S.M., Ziegler, S.F., Tsang, M., Cao, Z., and Leonard, W.J. (1993) Interleukin-2 receptor gamma chain: A functional component of the interlekin-7 receptor. *Science* 262: 1877-1880

Novotney, M., Chang, Z., Uchiyama, H., and Suzuki, T. (1991) Protein kinase C in tumoricidal activation of mouse macrophage cell lines. *Biochemistry* 30:5597

Nunokawa, Y., Ishida, N., Tanaka, S. (1994) Cloning of inducble nitric ocide synthase in rat vascular smooth muscle cells. *Bioch. Biophys. Res. Commun.* 91: 89-94

Nussell, A.K., Billiar, T.R., Liu, Z.Z., Morris, S.M. (1994) Coinduction of nitric oxide sythase and argininosuccinate synthetase in a murine macrophage cell line implications for regulation of nitric oxide production. *J. Biol. Chem.* 269: 1257-1261

Nussell, A.K., Disilvio, M., Billiar, T.R., Hoffman, R.A., Geller, D.A., Selby, R., Madariaga, J., Simmons, R.L. (1992) Stimulation of the nitric oxide synthase pathway in human hepatocytes by cytokines and endotoxin. *J. Exp. Med.* 176: 261-264

O'Farrell, A.M., Kinoshita, T., and Miyajima, A. (1996) The hematopoietic cytokine receptors. in *Blood Cell Biochemistry* ed. by Whetton, A.D and Gordon, J. pp1-40 Plenum Press. New York and London

Oliver, K., Noelle, R.J., Uhr, J.W., Krammer, P.H., and Vitetta, E.S. (1985) B-cell growth factor (B-cell growth factor 1 or B-cell stimulating factor, provisional 1) is a differentiation factor for resting B cells and may not induce cell growth. *Proc. Natl. Acad. Sci. USA* 82:2465

Olivier, M., Brownsey, R.W. and Reiner, N.E. (1992) Defective stimulus-response coupling in human monocytes infected with Leishmania donovani is associated with altered activation and translocation of protein kinase C. *Proc. Natl. Acad. Sci. USA* 89::7481-7485

Oswald, I.P., Eltoum, I., Wynn, T.A., Schwartz, B., Caspar, P., Paulin, D., Sher A., and James, S.L. (1994) Endothelia cells are activated by cytokine treatment to kill an intravascular parasite, *Schistosoma mansoni*, though the production of nitric oxide. *Proc. Natl. Acad. Sci.USA* 91: 999-1003.

Oswald, I.P., Gazzinelli, R.T., Sher, A., James, S.L. (1992) IL-10 synergizes with IL-4 and transforming growth factor-B to inhibit macrophage cytotoxic activity. *J. Immunol.* 148:3578-3582

Palmer, R.M.J., Ashton, D.S., Moncada, S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333:664-666

Palmer, R.M.J., Ferrige, A.G., Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor. *Nature.* 327:524-526

Palmer, R.M.J., Moncada, S. (1989) A novel citrulline forming enzyme implaicated in the formation of nitric oxide by vascular endothelial cells. *Biolchem. Biophys. Res. Commun.* 158:348-352

Pan, M.G., Florio, T., Stock, P.J.S. (1992) G protein activation of a hormone-stimulated phosphatase in human cells. *Science* 256:1251

Parronchi, P.M., Carli, De., Manetti, R., Simonelli, C., Sampognaro, S., Piccinni, M.P., Macchia, D., Maggi, E., Del Prete, G., and Romagnani, S. (1992) IL-4 and IFN (α and γ) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. *J. Immunol.* 149:2977

Paul, W.E. (1994) Interleukin-4: A prototypic immunoregulatory lymphokine.*Blood* 77:1859-1870

Paul, W.E., and Ohara, J. (1987) B-cell stimulatory factor-1/interleukin 4. *Annu. Rev. Immunol* 5:427

Paul, W.E., and Seder, R.A., (1994) Lymphocyte responses and cytokines. *Cell* 76:241

Pelech, S.L. and Sanghera, J.S. (1992) Mitogen-activated protein kinases: versatile transducers for cell signalling. *Trends Biochem. Sci.* 17:233-238

Pellegrini, S., and Schindler, C. (1993) Early events in signalling by interferons. *TIBS* 18:338-342

Perrero, E., Hsieh, C.L., Francke, U., Goyert, S.M. (1990) CD14is a member of the family of leucine-rich proteins and is encoded by a gene syntenic with multiple receptor genes. *J. Immunol.* 145:331-336

Perussia, B., Kobayashi, M., Rossi, M.E., Aneyon, I., and Trinchieri, G. (1987). Immune interferon enhances functional properties of human granulocytes: role of Fc receptors and effect of lymphotoxin, tumor necrosis factor, and granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 138: 765-774.

Pestka, S., Langer, J.A., Zoon, K.C., and Samuel, C.E. (1987). Interferons and their actions. *Annu. Rev. Biochem.* 56: 727-777.

Pfeuffer, T. (1977) *J. Biol. Chem.* 252:7224-7234

Pine, R (1992) Constitutive expression of an ISGF2/IRF1 transgene leads to interferon-indenpendent activation of interferon-inducible genes and resistance to virus infaction. *J. Virol.* 66:4470

Pine, R., Decker, T., Kessler, D.S., Levy, D.E. and Darnell, J.E.,Jr. (1990). Purification and cloning of interferon-stimulated gene factor 2 (ISGF2): ISGF2 (IRF-1) can bind to the promoters of both beta interferon- and interferon-stimulated genes but is not a primary transcriptional activator of either. *Mol. Cell. Biol.* 10:2448.

Plum, J., Desmedt, M., Billiau, A., Heremans, H., Leclercq, G. and Tison, B. (1991) IFN-γ reversis IL-4 inhibition of fetal thymus grouwth in organ-culture. *J. Immunol.* 147:50-54

Politis, A.D., and Vogel, S.N. (1990). Pharmacologic evidence for the requirement of protein kinase C in IFN-induced macrophage Fcγ receptor and Ia antigen expression. *J. Immunol.* 145:3788-3795.

Prpic, V., Weiel, J.E., Somers, S.D., DiGuiseppi, J., Gonias, S.L., Pizzo, S.V., Hamilton, T.A., Herman, B., and Adams, D.O. (1987) Effects of bacterial lipopolysccharide on the hydrolysis of phosphatidylinositol-4,5-bisphosphate in murine peritoneal macrophages. *J. Immunol.* 139:526

Pugin, J., Schurer-Maly, C.C., Leturcq, D., Mortarty, A., Ulevitch, R.J., Tobias, P.S. (1993) LPS activation of human endothelial and epithelial cells is mediated by LPS binding protein and soluble CD14. P*roc. Natl.Acad. Sci. U.S.A.* 90:2744-2748

Pulverer, B.J., Kyriskis,J.M., Avruch, J., Nikolakaki, E., and Woodgett, J.R. (1991). Phosphorylation of c-Jun mediated by MAP kinases. *Nature* 353:670-674

Quelle, F.W., Shimoda, K., Thierfelder, W., Fischer, C., Kim, A., Ruben, S.M., Cleveland, J.L., Pierce, J.H., Keegen, A.D., Nelms, K., Paul, W.E., and Ihle, J.N. (1995) Cloning of murine STAT6 and human STAT6, STAT proteins that are tyrosine phosphorylated in responses to IL-4 and IL-3 but are not required for mitogenesis. *Mol. Cell. Biol.* 15:3336-3343

Radler, P.A., Sachsenmaier, C., Gebel,s., Auer, H.P., Bruder, J.T., Rapp, U., Angel, P., Rahmsdorf, H.J., and Herrlich, P. (1993) UV-induced activation of AP-1 involves obligatory extranuclear steps including Raf-1 kinase. *EMBO J.* 12:1005-1012

Radomski, M. W., Palmer, R.M.J., Moncada, S. (1990) Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. USA.* 87: 10043-10047

Raetz, C.R.H. (1990) Biochemistry of endotoxins. *Annu. rev. Biochem.* 59:129

Raetz, C.R.H., Ulevitch, R.J., Wright, S.D., Sibley, CH, Ding, A., Nathan, C.F. (1991) Gramnegative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. *FASEB J.* 5:2652-2660

Raj, N.B.K., Au, W.-C., and Pitha, P.M. (1991) Identification of a novel virus-responsive sequence in the promoter of murine interferon-α genes. *J. Biol. Chem.* 266: 11360-11365

Ramadori, G., Meyer zum Buschenfelde, K.H., Tobias, P.S., Mathison, J.C., Uleitch, R.J. (1990) Biosynthesis of lipopolysaccharide binding protein in rabbit hepatocytes. *Pathobiology* 58:89-94

Rapoport, R.M., Murad, F. (1983) Agonist induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cyclic GMP. *Cire. Res.* 52:352-357

Reedijk, M., Liu, X., Van der Geer. P., Letwin, K., Waterfield, M.D., Hunter, T., and Pawson, T. (1992) Tyr 721 regulates specific binding of CSF-1 receptor kinase insert to PI3'-kinase SH2 domains: A model for SH2-mediated receptor-target interactions. *EMBO J.* 11-1365-1372

Rees, D.D., Cellek, S., Palmer, R.M.J., Moncada, S. (1990) Dexamethasone prevents the indution by endotoxin of a nitric oxide synthase and the associated effects on vascular tone - an insight into endotoxin shock. *Bioch. Biophys. Res. Comm.* 173: 541-547

Reiling, N., Ulmer, A.J., Duchrow, M., Ernst, M., Flad, H.D., Hauschildt, S. (1994) Nitric oxide synthase: mRNA expression of different isoforms in human moncytes/macrophage. *Eur. J. Immunol.* 24:1941-1944

Reiner, N.E. (1994) Altered cell signalling and mononuclear phagocyte deactivation during intracellular infaction. *Immunology Today* 15:374-381

Reis, L.F.L., Harada, H., Wolchok, J.D., Taniguchi, T. and Vilcek, J. (1992)Critical role of a common transcription factor, IRF-1, in the regulation of IFN-β and IFN-inducible genes. *EMBO J.* 11:185.

Rennick, D., Moore, R.G., and Thompson-Snipes, L. (1992) IL-4 and hematopoiesis. in *IL-4: Structure and Function* (H.spits, ed.), pp. 151-168. CRC press, Boca Raton

Revel, M., and Chebath,J. (1986) Interferon-activated genes. *Trends. Biochem. Sci.* 11:166-170

Rhee, S.G., Choi, K.D. (1992) Regulation of inositol phospholipid-specific phospholipase-C isozymes. *J. Biol. Chem.* 267:12393-12396

Rietschel, E.T., Kirkae, T., Schade, F.U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A.J., Zahringer, U., Seydel, U., Di Padova, F., Schreier, M., Brade, H. (1994) Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J.* 8:217-225

Roach, P.J. (1991) Multisite and hierarchical protein-phosphorylation. *J. Bio Chem.* 266 (22): 14139-14142

Roberts, A.B., Vodovotz, Y., Roche, N.S., Sporn, M.B., and Nathan, C.F. (1992) Role of nitric oxide in antagonistic effects of transforming growth factor-β and interlekin-1β on the beating rate of cultured cardiac myocytes. Mol. Endocrinol. 6:1921

Robinson, P.J. (1991) Phosphatidylinositol membrane anchors and T-cell activation. *Immunol. Today* 12:35-41

Rodbell, M., Birnbaumer, L., Pohl, S.L., and Krans, H.M.J. (1971) *J. Biol. Chem.* 246: 1877-1892

Romagnani, S., (1991) Human Th1 and Th2 subsets - doubt no more . *Immunol. Today* 12, 256--

Rothe, M., Wong, S.C., Henzel, W.J., and Goeddel, D.V. (1994) A novel femily of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor-necrosis-factor receptor. *Cell* 78: 681-692

Rubanyi, G.M.and Vanhoutte, P.M. (1986) Superoxide anions and hyperoxide inactivate endotheliom-derived relaxing factor. *Am. J. Physiol.* 250: H822-H827

Ruffner, H., Reis, L.F.L., Naf, D., and Weissmann, C. (1993) Induction of type I interferon genes and interferon-inducible genes in embryonal stem cells devoid of interferon regulatory factor 1. *Proc. Natl. Acad. Sci. USA9* 90:11503-11507

Rusetti, T.W., Dubois, C., Falk, L.A., Jacobsen, S.E., Sing, G., Longo, D.L., Wiltrout, R.H., and Keller, J.R. (1991) Clinical applications of TGF-β. *In vivo* and *in vitro* effects of TGF-β1 on normal and neoplastic haemopoiesis, in *Ciba Foundation Symposium* 157 (R. Gregory Bock Bock and J. Marsh, eds.), pp212-231, John Wiley & Son, New York.

Russell, S.M., Johnston, J.A., Noguchi, M., Kawamura, M., Bacon, C.M., Friedmann, M., Berg, M., McVicar, D.W., Witthuhn, B.A., Silvennoinen, O., Goldman, A.S., Schmalstieg, F.C., Ihle, J.N., O'Shea, J.J., and Leonard, W.J. (1994) Interaction of IL-2Rβ and γc chains with Jak1 and Jak3: Implications for XSCID and XCID. *Science* 266: 1042-1044

Russell, S.M., Keegan, A.D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedman, M.C., Miyajima, A., Puri, R.K., Paul, W.E., and Leonard, W.J. (1993) Interleukin-2 receptor gamma chain: A functional component of the interleukin-4 receptor. *Science* 262:1880-1883

Salkowski, C. A., Barber, S.A., Detore, G.R., and Vogel, S.N. (1996) Differential dysregulation of nitric oxide production in macrophages with targeted disruptions in IFN regulatory factor-1 and -2 genes. *J. Immunol.* 156:3107-3110

Samelson, L.E. and Klausner, R.D. (1992) Tyrosine kinases and tyrosine-based activation motifs - current research on activation via the T-cell antigen receptor. *J. Biol. Chem.* 267:24913-24916

Samuel, C.E. (1991) Antiviral actions of interferon-interferon-regulatedcellular proteins and their suprisingly selective antiviral activities. *Virology* 183: 1-11

Sato, N., Sakamaki, K., Terada, N., Arai, K., and Miyajima, A. (1993) signal transduction by the high-affinity GM-CSF receptor: Two distinct cytoplasmic regions of the common beta subunit responsible for different signalling. *EMBO J.* 12: 4181-4189

Satoh, T., Nakafuku, M., Miyajima, A., and Kaziro, Y. (1991) Involvement of Rasp21protein in signal transduction pathways from IL-2, Il-3 and GM-CSF, but not from IL-4. *Proc. Natl. Acad. Sci. U.S.A.* 88:3314-3318

Satoh, T., Uehara, Y., and Kazrio, Y. (1992) Inhibition of interleukin-3 and granulocyte-macrophage colony-stimulating factor stimulated increase of active ras-GTP by herbimycin A, a specific inhibitor of tyrosine kinases. *J. Biol. Chem.* 267: 2537-2541

Schepers, T.M., Klein, J.B., Feldhoff, P.W., Dean, W.L., and McLeish, K.R. (1992) Interferon-g induces phosphorylation of multiple small-molecular-weight proteins in U937 cells. *J. Interferon Res.*12:289-296

Schindler, C., Fu, X.Y., Improta, T., Aebersold, R., and Darnell, J.J. (1992) Protein of transcription factor ISGF-3: one gene encodes the 91- and 84-kDa ISGF-3 proteins that are activated by interferon alpha. *Proc. Natl.Acad. Sci. U.S.A.* 89:7836-7839

Schindler, C., Kashleva, H., Permis, A., Pine, R., and Rothman, P (1994) STF-IL-4: a novel IL-4 induced signal transducing factor. *EMBO J.* 13:1350-1356

Schindler, C., Shuai, K., Prezioso, V.R. & Darnell J.E. Jr. (1992) Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science* 257, 809-813

Schindler, U., Pengguang, W., Rothe, M., Brasseur, M., and Mcknight, S.L., (1995) Components of a Stat recognition code: evidence for two layers of molecular selectivity. *Immunity* 2: 689-697

Schlessinger, J. and Ullrich, A. (1992) Growth factor signaling by receptor tyrosine kinases. *Neuron* 9:383-391

Schmidt, H.H.W., Gagne, G.D., Nakane, M., Pollock, J.S., Miller, M.F., Murad, F., (1992) Mapping of neural nitric oxide synthase in the rat suggests frequent colocalization with NADPH diaphorase but not with soluble guanylyl cyclase, and novel paraneural functions for nitrnergic signal transduction. *J. Histochem. Cytochem.* 40:1439-1456

Schreiber, E., Matthias, P. Muller, M.M. and Schaffner, W.(1989) rapid detection of octamer binding-proteins with mini-extracts, prepared from a small number of cells. *Nucl. Acids Res.* 17(5), 6419

Schreiber, R.D., Farrar, M.A., Hershey, G.K., Fernandezluna, J.(1992) the structure and function of interferon-gamma receptors. *Int. J. Immunopharmacol.*14:413-419

Schreiber, R.D., Pace, J.L., Russel, S.W., Altmann, A., and Katz, D.H. (1983) *J. Immunol.* 131:826-832

Schumann, R.R., Leong, S.R., Flaggs, G.W., Gray, P.W., Wright, S.D., Mathison, J.C., Tobias, P.S., ulevitch, R.J. (1990) Structure and function of lipopolysaccharide binding protein. *Science* 249:1429-1433

Sebaldt, R.J., Prpic, V., Hollenbach, P.W., Adams, D.O., and Uhing, R.J. (1990). IFN-g potentiates the accumulation of diacylglycerol in murine macrophages. *J. Immunol.* 145:684-689.

Sen, G.C., and Lengyel, P. (1992) The interferon system - a birds-eye-view of its biochemistry. *J. Biol. Chem.* 267: 5017-5020

Sen, R., and D. Baltimore. (1986) In vitro transcription of immunoglobulin genes in a B-cell extract: effect of enhancer and promoter sequences. Cell. 47: 921-928

Sen,R., and D. Baltimore. (1986) Multiple nuclear factors interact with the immuno-globulin enhancer sequences *Cell* 46: 705-716

Shadek, S. M., Regenstein, A.C., Lykins, D., and Roberts, J.M. (1993) Nitric oxide synthase activity in pregnant rabbit uterus decreases on the last day of pregnancy. *Am. J. Obstet. Gynecol.* 169: 1285-1291

Sherman, M.S., Heyworth, C.M., Dexter, T.M., Haefner, B., Owen, P.J., and Whetton, A.D. (1993) Haemopoietic stem cell development to neutrophils is associated with subcellular redistribution and differenttial expression of protein kinase C subspecies. *J. Cell Sci.* 104:173-180

Sherman, P.A., Laubach, V.E., Reep, B.R., Wood, E.R. (1993) Purification and cDNA sequence of an inducible nitric oxide synthase from a human tumor cell line. *Biochemistry* 32: 11600-11605

Sherr, C.J. (1993) Mammalian G1 cyclins. *Cell* 73:1059-1065

Shimoda,H., Van, Deursen, J., Sangster, MY., Sarawar, SR., Carson, RT., Tripp, RA., Chuo, C., Quelle, FW., Norsaka, T., Vignali, DAA., Doherty, PC., Grosveld, G., Paul, WE, Ihle, JN (1996) Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* 380:630-663

Shuai, K., Horvath, C.M., Huang, L.H., Qureshi, S.A., Cowburn, D., and Darnell, J.J. (1994) Interferon activation of the transcription factor STAT91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell* 76:821-828

Shuai, K., Schindler, C., Prezioso, V.R. & Darnell, J.E.Jr. (1992) Activation of transcription by IFN-gamma - tyrosine phosphorylation of A 91-kDa DNA--binding protein. *Science* 258, 1808-1812

Shuai, K., Ziemiecki, A., Wilks, A.F., Harpur, A.G., Sadowski, H.B., Gilman, M.Z., and Darnell, J.E. (1993) Polypeptide signalling to the nucleus through tyrosine phosphorylation of JAK and STAT proteins. *Nature* 366:580-583

Siebenlist, U., G. Franzoso, and K. Brown. (1994) Structure, regulation and function of NFκB. *Annu. Rev. Cell Biol.* 10: 405-455

Simon, M.I., Strathman, M.P. and Gautam, N. (1991) diversity of G-proteins in signal transduction. *Science* 252:802-808

Sims, J.E., March, C.J., Cosman, D., Wimder, M.B., *et al.*, (1988) cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science* 241: 585-589

Snapper, C.M., Peschel, C., and Paul, W.E. (1988) IFN-gamma stimulates IgG2A secretion by murine B-cells stimulated with bacterial lipopolysaccharide. *J. Immunol.* 140: 2121-2127

Soh, J., Donnelly, R.J., Kotenko, S., Mariano, T.M., Cook, J.R., Wang, N., Emanuel, S., schwartz, B., Miki, T., and Pestka, S. (1994) Identification and sequence of an accessory factor required for activation of the human interferon γ receptor. *Cell* 76:793-802

Somers, S.D., Erickson, K.L. Regulation of murine macrophage function by IL-4. I. Activation of macrophages by T-T-cell bybridoma is due to IL-4. *Cell. Immunol.* 122:178-187

Somers, S.D., Weicl, J.E., Hamilton, T.A., and Adams, D.O. (1986). Phorbol esters and calcium ionophore can prime murine peritoneal macrophages for tumor cell destruction. *J. Immunol.* 136:41994205.

Sorensen, P., Mui, A.L., and Krystal, G.(1989) Interleukin-3 stimulates the tyrosine phosphorylation of the 140-kilodalton interleukin-3 receptor. *J. Biol. Chem.* 264:19253-19258

Staeheli, P. (1990) *Adv. Virus Res.* 38:147-200

Stamler, J.S., Singel, D.J., and Loscalzo, J. (1992) *Science* 258: 1898-1902

Stefanova, I., Corcorna, M.L., horak, E.M., Wahl, L.M., Bolen, J.B., and Horak, I.D. (1993) Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase $p53/56^{lyn}$. *J, Biol. Chem.* 268:20725-20728

Strassman, G., Somers, S.D., Springer, T.A., Adams, D.O., and Hamilton, T.A. (1986). Biochemical models of interferon-g-mediated macrophage activition: independent regulation of lymphocyte function associated antigen (LFA)-1 and 1-A antigen on murine peritoneal macrophages. *Cell. Immunol.* 97: 110-120.

Strenger, S., Solbach, W., Rollinghoff, M., Bogdan, C. (1991) Cytokine interactions in esperimental cutaneous leishmaniasis. II. Endogenous tumor necrosis factor-a production by macrophages is induced by the synergistic action of IFN-g and IL-4 and accounts for the antiparasitic effect mediated by IFN-γ and IL-4. *Eur. J. Immunol.* 21:1669-1675

Stuehr, D.J., Marletta, M.A. (1985) Mammalian nitrate biosynthesisi: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proc. Natl. Acad. Sci. USA.* 82: 7738-7742

Stuehr, D.J., Marletta, M.A. (1987) Induction of nitrite nitrate synthesis in murine macrophages by BCG infection, lymphokine, or interferon gamma. *J. Immunol.* 139:518-525

Stuehr, D. J., Cho, H.J., Kwon, N.S., Weise, M.F., Nathan, C. (1991) Purification and charcterization of the cytokine induced macrophage nitric oxide synthase an FAD containing anf FMN containing flavoprotein. *Proc. Natl. Acad. Sci. USA.* 88: 7773-7777

Sun, H and Tonks, N.K. (1994) The coordinated action of protein tyrosine phosphatases and kinases in cell signalling. *TIBS* 19:480-484

Sun, X.L., Wang, L.M., Zhang, Y., Yenush, L., Myers, M.G., Jr., Glasheen, E., Lane, w.S., Pierce, J.H., and White, M.F. (1995) Rolc of IRS-2 in insulin and cytokine signalling. *Nature* 377:173-177

Sung,S.S.J. and Walters,J.A. (1993) Stimulation of interleukin-1-alpha and interleukin-a-beta production in human monocytes by protein phosphatase-1 and phosphatase-2A inhibitors. *J. Biol. Chem.* 268: 5802-5809

Swisher, S.G., Economou, J.S. Holmes, E.C. and golub, S.H.,(1990) TNF-alpha and IFN-gamma reverse IL-4 inhibition of lymphokine-activated killer-cell function. *Cell. Immunol.* 128:450-461

Takeda, K., Tanashi, T., Shi, W., Matsumoto, M., Minami, M., Kasiwamura, S.I., Nakanishi, K., Yoshida, N., Kishimoto, T. and Akira, S. (1996) Essential role of STAT6 in IL-4 signalling *Nature* 380:627-633

Talmadge, K.W., Gallati, H., Sinigaglia, F., Walz, A., and Garotta, G. (1986) Identity between human interferon-gamma and macrophage-activating factor produced by human lymphocytes. *Eur. J. Immunol.* 16:1471-1477

Tanaka T., T.Kawakami, and T. Taniguchi. (1993) Recognition DNA Sequences of Interferon Regulatiory Factor 1(IRF-1) and IRF-2, Regulators of Cell Growth and the Interferon System. *Mol. Cell.Biol.* 13:4531-4538

Taniguchi,T. (1995) Cytokine signaling through nonreceptor protein tyrosine kinases *Science* 268: 251-256

Tayeh, M.A., Marleta, M.A. (1989) Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate- tetrahyrobiopterin is required as a cofactor. *J. Biol. Chem.* 19654-19658

Tevelde,A.A., Huijbens, R.J.F., Heije, K., Devries, J.E. and Figdor, C.G. (1990) Interleukin-4 (IL-4) inhibis secretion of IL-1-beta, tumor necrosis factor-alpha and IL-6 by human monocytes. *Blood* 76:1392-1397

Thanos, D. and Maniatis, T. (1995) NFκB: a lesson in family values. *Cell* 80:529-532

Thornhill, M.H. and Haskard, D.O. (1990) IL-4 regulates endothelial-cell activation by IL-1, tumor-necrosis-factor, or IFN-γ. *J. Immunol.* 145: 865-872

Tian, S.S., Tsoulfas,P., Zinn,K. (1991) 3 receptor-linked proein-tyrosine phosphatases are selectively expressed on central-nervous-system axons in the Drosphila embryo. *Cell* 67:675-685

Tobias, P., Soldau, K., Ulevitch, R. (1986)Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *J. Exp. Med.* 164:777-793

Tobias, P.S., Mathison, J.C., Ulevitch, R.J. (1988) A family of lipopolysaccharide binding proteins involved in responses to gram-negative sepsis. J. Biol. Chem. 263:13479-13481

Tonks, N. K., Diltz, C.D., Fischer, E. H. (1988) Characterization of the major protein-tyrosine-phosphatases of human-placenta. *J. Biol. Chem.* 263(14): 6731-6737

Treisman, R. (1994) Ternary complex factors: growth factor regulated transcriptional activators *Cuur. Opin. Genet. Dev.* 4:96-101

Trowbridge, I.S. (1991) CD45 - a prototype for transmembrane protein tyrosine phosphatases. *J. Biol. Chem.* 266(35): 23517-23520

Trowbridge, I.S., Ostergaard, H.I., Johnson, P. (1991) CD-45 - a leukocyte-specific member of the protein tyrosine phosphatase family. *Biochem. Biophys. Acta* 1095(1): 46-56

Tweardy,D.J., Fujiwara, H., Scillian, J.J. and Ellner, J.J. (1986). Concurrent enhancement of monocyte immuno-regulatory properties and effector functions by recombinant interferon-γ. *Cell. Immunol.* 100: 34-46.

Ueno, H., Colbert, H.A., Escobedo, J.A., Williams, L.T. (1991) Inhibition of PDGF beta-recptor signal transduction by coexpression of a truncted receptor. *Science* 252:844-848

Ulevitch, R.J. (1993) Recognition of bacterial endotoxins by receptor-depemdent mechanisms. *Adv. Immunol.* 53:267-289

Ulevitch, R.J. and Tobias, P.S. (1994) Recognition of endotoxin by cells leading to transmembrane signalling. *Curr. Opin. immunol.* 6:125-130

Ulevitch, R.J. and Tobias, P.S. (1995) Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol.* 13: 437-457

Ullrich, A., and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* 61: 203-212

Ushiro, H., Cohen, S. (1980) *J. Biol. Chem.* 255 (18): 8363

Valente, G., Ozmen, L., Novelli, F., Geuna, M., Palestro, G., Forni, G., Garotta, G. (1992) Distribution of interferon-gamma receptor in human tissues. *Eur. J. Immunol.* 22:2403-2412

Van lint J., Agostinis, P., Vandevoorde, V., Haegeman, G., Fiers, W., Merlevede, W. and Vandenheede, J.R. (1992) Tumor necrosis factor stimulates multiple serine/threonine protein kinases in Swiss 3T3 and L929 cells. Implication of casein kinase-2 and extracellular signal-regulation kinases in the tumor nerosis factor signal transduction pathway. *J. Biol. Chem.* 267:25916-25921

Veals, S.A., Santa Maria, T., and Levy, D.E.(1993) Two domains of ISGF3 gamma that mediate protein-DNA and protein-protein interactions during transcription factor assembly contribute to DNA-binding specificity. *Mol. Cell. Biol.* 13:196

Veals, S.A., Schindler, C., Leonard, D., Fu, X.-Y., Aebersold, R., Darnell, J.E., Jr., and Levy, D.E., (1992) Subunit of an alpha-interferon-responsive transcription factor is related to interferon regulatory factor and Myb families of DNA-binding proteins. *Mol. Cell. Biol.* 12: 3315-3324

Velazquez, L., Fellous, M., Stark, G.R., and Pellegrini, S. (1992) A protein tyrosine kinase in the interferon alpha/beta signalling pathway. *Cell* 70: 311-322

Vodovotz, Y., Bogdan,C., Paik, J., Xie, Q.W., Nathan, C. (1993) Mechanism od suppression of macrophage nitric oxide release by transforming growth factor-β. *J. Exp. Med.* 178: 605-614

Vodovotz, Y., Kwon, N.S., Pospischil, M.,Manning, J., Paik, J., and Nathan, C. (1994) Inactivation of Nitric oxide synthase after prolonged incubation of mouse macrophages with IFN-g and Bacterial lipopolysaccharide *J. Immunol.* 152: 4110

Wakao, H., Gouilleux, F., and Groner, B. (1994) Mammary gland factor (MGF) is a novel family of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO J.* 13:2182-2191

Wakao, H., Schmitt, N.M., and Groner, B. (1992) Mammary-gland specific factor is present in lactating rodent and bovine mammary tissue and composed of a single polypeptide of 89kDa. *J. Biol. Chem.* 267:16365-16370

Walton, Kevin M., and Jack E. Dixon (1993) Protein tyrosine phosphatases *Annu. Rev. Biochem.* 62: 101-120

Wang Min, Sankar Ghosh, and Peter Lengyel (1996) The Interferon-Inducible p202 Protein as a Modulator of Transcription: Inhibition of NFκB, *c-Fos*, and *c-Jun* Activities. *Molecular and Cellular Biology* 16(1) 359-368

Wang, J., Kester, M., and Dunn, M.J. (1988) Involvement of a pertussis toxin-sensitive G-protein-coupled phophoolipase A2 in lipopolysaccharide-stimulated prostaglandin E2 synthesis in cultured rat mesangial cells. *Biochem. Biophys. Acta.* 963:429

Watanabe, N., J. Sakakibara, A. Hovanessian, T. Taniguchi, and T. Fujita. (1991) Activation of IFN-β element by IRF-1 requires a posttranslational event in addition to IRF-1 synthesis. *Nucleic Acids Res.* 19 (16): 4421-4428.

Watling, D., Guschin, D., Muller, M., Silvennoinen, O., Witthuhn, B.A., Quelle, F.W., Rogers, N.C., Schindler, C., Stark, G.R., Ihle, J.N., and Kerr, I.M. (1993) Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway. *Nature* 366:166-170

Wei, X.Q., Charles, I.G., Smith, A., Ure, J., Feng, G.J., Huang, F.P., Xu, D.M., Muller, W., Moncada, S., Liew, F.Y. (1995) Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 375: 408-411

Weiberg, J.B., Granger, D.L., Pisetaky d.s., Seldin, M.F., Misukonis, M.A., Mason, S.N., Pippen, A.M., Ruiz, P., Wood, E.R., Gilkeson, G.S. (1994) The role of nitric oxide in the pathogensis of spontaneous murine autoimmune disease: increased nitric oxide production and nitric oxide synthase expression in MRL-lpr/lpr mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered N-monomethyl-L-arginine. *J. Exp. Med.* 179: 651-660

Weigent, D.H., Langford, M.R., Fleishman, W.R., and Stanton, G.T. (1983). Potentiation of lymphocyte natural killing by mixtures of alpha or beta interferon with recombinant gamma interferon. *Infect. Immunol.* 40:35-38.

Weinstein, S.L., June, C.H., and DeFranco, A.L. (1993) Lipopolysaccharide-induced protein tyrosine phosphoorylation in human macrophages is mediated by CD14. *J. Immunol.* 151:3829

Weisz, A., Marx, P., Shart, R., Appella, E., Driggers, P.H., Ozato, K. and Levi, B.-Z. (1992). The human interferon consensus sequence binding protein (H-ICSBP) is a negative regulator of enhancer elements common to interferon inducible genes. *J. Biol. Chem.* 267:25589.

Welham, M.J. Duronio, V., Sanghera, J.S., Pelech, S.L., and Shrader, J.W. (1992) Multiple hemopoietic growth factors stimulate activation of mitogen-activated kinase family members. *J. Immunol.* 149:1683-1693

Welham, M.J., and Schrader, J.W. (1992) Steel factor-induced tyrosine phosphorylation in murine mast cells. Common elements with IL-3-induced signal transduction pathways. *J Immunol.* 149:2772-2783

Wen, Z., Zhong, Z., and Darnell, J.E., Jr. (1995) Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation.*Cell* 82:241-250

Westwick, J.K., Cox, A.D., Der, C.J., Cobb, M.H., Hibi, M., Karin, M., and Bernner, D.a. (1994) Oncogenic Ras activates c-Jun via a seperte pathway from the activation of extracellular signaling -regulated kinases. *Proc. Natl. Acad. Sci. USA* 91:6030-6034

Williams, B.R.G. (1991) Transcriptional regulation of interferon-stimulated genes. *Eur. J. Biochem.* 200:1

Willman, C.L., Sever, C.E., Pallavicini, M.G., Harada, H., Tanaka, N., Slovak, M.L., Yamamoto, H., Harada, K., List, A.F., and Taniguchi, T. (1993) *Science* 259:968-971

Wilson, C.B. (1990) *in principles and Practice of Infectious Diseases* (Mandell, G. L., Douglas, R.G., Jr and Bennett, J.E., eds), pp. 101-138, Churchill Livingstone

Wink, D.A., Kasprzak, K.S., Maragos, C.M., Elespuri, R.K., Misra, M., Dunams, T.M., Cebula, T.A., Koch, W.H., Andrew, A.W., Allen, J.S. and Keefer, L.K. (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* 254: 1001-1003

Witthuhn, B.A., Silvennoinen, O., Miura, O., Lai, K.S., Cwik, C., Liu, E., and Ihle, J.N. (1994) Involvement of the JAK-3 Janus kinase in signalling by interleukins 2 and 4 in lymphoid and myeloid cells. *Nature* 370:153-157

Wood, E.R., Berger, H., Sherman, P.A., Lapetina, E.G. (1993) Hepatocytes and macrophages express an identical cytokine induible nitric oxide synthase gene. *Biolchem. Biophys. Res. Comm.* 191: 767-774

Wright, S.D. (1991) Multiple receptors for endotoxin. *Curr. Opin. Immunol.* 3:83

Wright, S.D. and Jong, M.T.C. (1986) Adhesion-promoting receptors on human macrophages recognize *Escherichia coli* by binding to lipopolysaccharide. *J. Exp. Med.* 164:1876

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., Mathison, J.C. (1990) CD14 serves as the cellular receptor for complexes of lipopolysaccharide with lipopolysaccharide binding protein. *Science* 249:1431-1433

Wright, SD., Tobias, P.S., Ulevitch, R.J., Ramos, R.A. (1989) Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. *J. Exp. Med.* 170:1231-1241

Xie, K., Huang, S., Dong, Z. and Fidler, I.J. (1993) Cytokine-induced apoptosis in transformed murine fibroblasts involves synthesis of endogenous nitric oxide. *Int. J. Oncol.* 3: 1043-1048.

Xie, Q.-W. and Nathan, C. (1994) The high-output nitric oxide pathway: Role and regulation. *J. Leukocyte Biol.* 56:576-582

Xie, Q.-W., Y. Kashiwabara, and C. Nathan. (1994) Role of thranscription factor NF-kappaB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* 269:47054708

Xie, Q.W., Cho, H.J., Calaycay, J., Mumford, R.A., swiderek, K.M., Lee, T.d., Ding, A.H., Troso, t., Nathan, C. (1992) Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 256: 225-228

Xie, Q.W., Whisnant, R., Nathan, C. (1993) Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J. Exp. Med.* 177:1779-1784

Yamada, G., Ogawa, K., Akagi, K., Miyamoto, H., Nakono, N., Iton, S., Miyazaki, J., Nishikawa, S., Yamamura, K., and Taniguchi, T. (1990) *Proc. Natl. Acad. Sci. USA* 88:532-536

Yang, L., Janeway, C.A. Jr (1990) Interferon-g plays a critical role in induced cell-death of effector T-cell-a possible 3rd mechanism of self-tolerance.*J. Exp. Med.* 172: 1735-1739

Yang, X., Seow, K.T., Bahri, S. M., Oon, S.H., Chia, W. (1991) wo Drosophila receptor-like tyrosine phosphatase genes are expressed in a subset of developing axons and pineer neurons in the embryonic CNS. *Cell* 67:661-673

Yap, W.H., Teo, T.S., and Tan, Y.H. (1986). An early event in the interferon-induced transmembrane signaling process. *Science*, 234, 355-358.

Yin, T., and Yang, Y.C. (1994) Mitogen-activated protein kinases and ribosomal S6 protein kinases are involved in aignalling pathways shared by interleukin-11, interleukin-6, leukemia inhibitory factor, and oncostatin M in mouse 3T3-L1 cells. *J Biol. Chem.* 269: 3731-3738

Zhang, Z., Blenis, J., Li, H., Schindler, C., and Chen-kiang, S. (1995) Requirement of serine phosphorylation for formation of STAT-promoter complexes. *Science* 267: 1990-1994

Zhang,X.K., Blenis,J., Li, H.C., Schinder,C., and Chen-Kiang, S.(1995) Requirement of serine phosphorylation for formation of STAT-promoter complexes. *Science* 267:1990-1994

Zhong, Z., Wen, Z., and Darnell, J.E., Jr. (1994). Stat3 and Stat4: members of the family of signal transducers and activators of transcription. *Proc. Natl. Acad. Sci. USA* 91: 4806-4810

Ziegler-Heitbrock, H.W.L., Ulevitch, R.J. (1993) CD14: cell surface receptor and differentiation marker. *Immunol. Today* 14:121-125

Ziemiecki, A., Harpur, A.G., and Wilks, A.F. (1994). Jak protein tyrosine kinases: their role in cytokine signaling. *Trends Cell Biol.* 4: 207-212

Zuckerman, S.H., Evans, G.F., Guthrie, L. (1991) Transcriptional and post-transcriptional mechanisms involved the differential expression of LPS-induced IL-1 and TNF mRNA. Immunology 73: (4) 460-465

Zurawski, S.M., Vega, F.J., Huyghe, B., and Zurawski, G. (1993) Receptors for interleukin-13 and interlukin-4 are complex and share a novel component that functions in signal transduction. *EMBO J.* 12:2663-2670