University of Southampton

Regulation and Biosynthesis of Interleukin 10 in Macrophage and Monocytes Involved in Inflammation

By

Martin McAulay

Submitted for the degree of Master of Philosophy

Liver Group

School of Medicine
Infection, Inflammation and Repair

January 2002

FOR

MY PARENTS

AND HEATHER

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF BIOSCIENCES

SCHOOL OF MEDICINE

Master of Philosophy

Regulation and Biosynthesis of Interleukin 10 in Macrophage and Monocytes involved in Inflammation

By Martin McAulay

Interleukin 10 has proven itself to be an important regulator of inflammation with a wide range of biological effects. These range from a down regulation of cytokine, chemokine and superoxide production within the macrophage, to an up regulation of immunoglobin secretion in B-cells. However, despite Interleukin 10 being an important anti-inflammatory cytokine, very little is understood about how this cytokine is regulated within the cell.

During this study we investigated how Interleukin 10 is regulated in the monocyte and macrophage involved in inflammation. In the first part of the study a sandwich ELISA was used with FACS analysis, to characterise the profile of Interleukin 10 to purified bacterial lipopolysaccaride and map their secretion to the individual cells involved in inflammation.

Using pharmacological specific inhibitors the second messenger pathways were mapped. We have looked at how Interleukin 10 is regulated by the MAP kinase pathways. Using potent and selective MAP kinase inhibitors we have demonstrated in various cell types how inhibition of the p38 and JNK MAP kinase pathways led to a down regulation in Interleukin 10 production. Once the cellular pathways that regulate Interleukin 10 production were worked out, we were able to continue to work further down the regulatory pathways to reach the DNA and begin to investigate which transcription factors are important in Interleukin 10 transcription and translation.

Understanding how this important cytokine is regulated can lead to the development of a novel treatment for patients who suffer from tissue destructive and painful inflammation diseases.

Table of Contents

Results

List of Tables and Figures

Tables

Figures

Acknowledgments

A work of this size is not undertaken without the aid and support of many people. So I would like to take this opportunity to thank the many people who have helped me through this most difficult period.

First I would like to thank the MRC without whom this work would not have even been possible.

I would also like to thank my parents for their continuing support and belief in me. I owe them so much that words alone cannot express my gratitude. Next I would like to thank Heather Smalley for always being there, giving moral support, when I needed it the most, and for saying yes!

Within the labs there was simply too many people to thank individually so I would like to say thank you to everyone who helped me, gave advice and simply liked to talk over a coffee, but special mention should go to several people. To Dr. Angus Trowern, who helped me get at my plasmids. Dr Julia Maltby, for her continuing aid, support and understanding. Finally Dr. Thelma Biggs and Jan for all their help with my cell lines and tissue culture.

I would also like to thank Bernardo Villarreal-Ramos for his constant nagging. "Is it finished yet? Have you heard back yet?"

Finally, and my no means the least, I would like to say a very big thank you to my supervisors, Dr Nick Sheron and Dr Derek Mann, for everything. Their help, advice and aid when things seemed the darkest was, is and forever will be remembered.

Martin McAulay
January 2002

Abbreviations

AA	*Amino Acid*
Ab	*Antibody*
AC	*Accessory Cell*
APC	*Antigen Presenting Cell*
BCRFI	*BamH1 C fragment rightward Reading Frame*
BSA	*Bovine Serum Albumin*
cAMP	*cyclic Adenosine Monophosphate*
CAT	*Chloramphenicol Acetyl-Tranferase*
CD	*Cluster of Differentiation*
cDNA	*complementary deoxyribonucleic acid*
CSIF	*Cytokine Synthesis Inhibitory Factor*
DEPC	*DiEthyl PyroCarbonate*
di	*deionised*
DMEM	*Dulbecco's Modified Eagle Medium*
DMSO	*Dimethyl Sulfoxide*
DNA	*deoxyribonucleic acid*
DRB	*5,6-Dichlorobenzimidazole Riboside*
EBV	*Epstien Barr Virus*
E. coli	*Escherichia coli*
EDTA	*Ethylenediaminetetraacetic Acid*
ELISA	*Enzyme Linked ImmunoSorbant assay*
ERK	*extracellular regulated kinase*
FACS	*Fluorescent Activated Cell Sorter*
FCS	*Foetal Calf serum*
FITC	*Fluorescein Isothiocyanate*
HEPES	*N-[2-Hydroxyethyl]piperazine-N'-[2ethanesulfonic acid]*
HRP	*Horse Radish Peroxidase*
IFN	*Interferon*
Ig	*Immunoglobin*
IκB	*NF kappa B inhibitor*
IL	*Interleukin*
IL-10R	*Interleukin 10 receptor*
kD	*kiloDalton*
LPS	*Lipopolysaccaride*
M	*Molar*
MAPK	*Mitogen Activated Protein kinase*
mg	*milligram*

MHC	*Major Histocompatability Complex*
ml	*milliliter*
mM	*millimolar*
µM	*micromolar*
µg	*microgram*
µl	*microliter*
mRNA	*messenger ribonucleic acid*
NFκB	*Nuclear Factor kappa B*
ng	*nanogram*
O.D.	*Optical Density*
PBMC	*Polymorphic Blood Mononuclear Cells*
PBS	*Phosphate Buffered Saline*
PerCP	*Peridinin chlorphyll protein*
pg	*picagram*
PMA	*Phorbol 12-Myristate 13-Acetate*
rh/m	*recombinant human/mouse*
RNA	*ribonucleic acid*
RNI	*Reactive Nitrogen Intermediates*
ROI	*Reactive Oxygen Intermediates*
RPE	*R-Phycoerythrin*
rpm	*revolutions per minute*
RPMI	*Richmond Park Memorial Institute*
RT-PCR	*Reverse Transcriptase Polymerase Chain Reaction*
TAE	*Tris-Acetate-EDTA Buffer*
T-cell	*Thymus derived lymphocyte*
Th	*T helper*
TMB	*3,3',5,5'-Tetramethylbenzidine (dihydrochloride)*
TNF	*Tumour Necrosis factor*

Chapter 1

Introduction

1.1 History

Interleukin (IL) 10 was first discovered by D.F. Fiorentino *et al* in 1989, while looking for a product of T helper 2 cells (Th2) that would inhibit proliferation, effector function and possibly the development of T helper 1 (Th1) cells, in a way analogous to the inhibition of Th2 proliferation by IFNγ a Th1 cytokine. It was found to be specifically inhibitory of cytokine synthesis in the Th1 cell. Due to this it was named Cytokine Synthesis Inhibitory Factor (CSIF)[1].

Coincidentally, it was shown that mouse B cell lymphomas produced a protein that enhanced the proliferation of mouse thymocytes in response to IL 2 and 4. This was called B cell derived Thymocyte growth factor (B-TCGF). It was also found to augment the proliferative response of mouse mast cell lines to IL 3, and thus was named Mast cell Growth Factor III (MCGFIII)[2].

The factor responsible for all these effects was found to be the same protein and thus in 1990 it was renamed Interleukin 10[3].

1.2 Protein and Gene structure

1.2.1 Protein Structure

Using cDNA clones encoding for the cytokine, the primary structure for both the mouse IL-10 (mIL-10) and human IL-10 (hIL-10) was determined[3,4]. There is a high nucleotide sequence identity (>80%) between the mIL-10 and the hIL-10 cDNA clones, with the only significant difference being the insertion of a human Alu repetitive sequence element at the 3' untranslated region of the hIL-10 cDNA clone. The hIL-10 cDNA encodes for a 178AA protein that has an 18AA N-terminal hydrophobic signal sequence.

Fig 1.1 Amino Acid sequence for IL-10

```
-18                                  MHSSALLC CLVLLTGURA
  1   SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRUKTFFQMK
 41   DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEUMPQA
 81   ENQDPDIKAH UNSLGENLKT LRLRLRRCHR FLPCENKSKA
121   VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN
```

-Accession code : Swissprot P22301

mIL-10 : P18893

Human IL-10 is a non-covalently linked, homodimeric glycoprotein, which contains a single, but variably occupied, N-linked glycosylation site. Based on its primary structure (fig 1), IL-10 is a member of the 4 alpha helix family of cytokines, which include IL 2, 3, 4, 5, 6, 7, 9, 13, G-CSF, GM-CSF, CNTF, OSM, LIF and Epo binding to receptor class I and IL-10, IFNα, β and γ binding to receptor class II. hIL-10 is an 18kD polypeptide, which lacks detectable carbohydrate. The mIL-10, however, is N-glycosylated at a site near its N-terminus that is missing from hIL-10. The glycosylation of the mIL-10 is heterogeneous which results in a mixture of 17, 19 and 21kD species being seen when run on a SDS polyacrylamide gel. However, this glycosylation is not required since a recombinant (r) mIL-10 and hIL-10, obtained from *E. coli*, is still biologically active, as measured by the dose dependent co-stimulation of MC-9 cells with IL4.

Both hIL-10 and mIL-10 exist naturally as a non-covalent homodimer. Both are acid sensitive with complete inactivity being reached at pH5.5. However IL-10 is quite stable in basic conditions up to pH11.

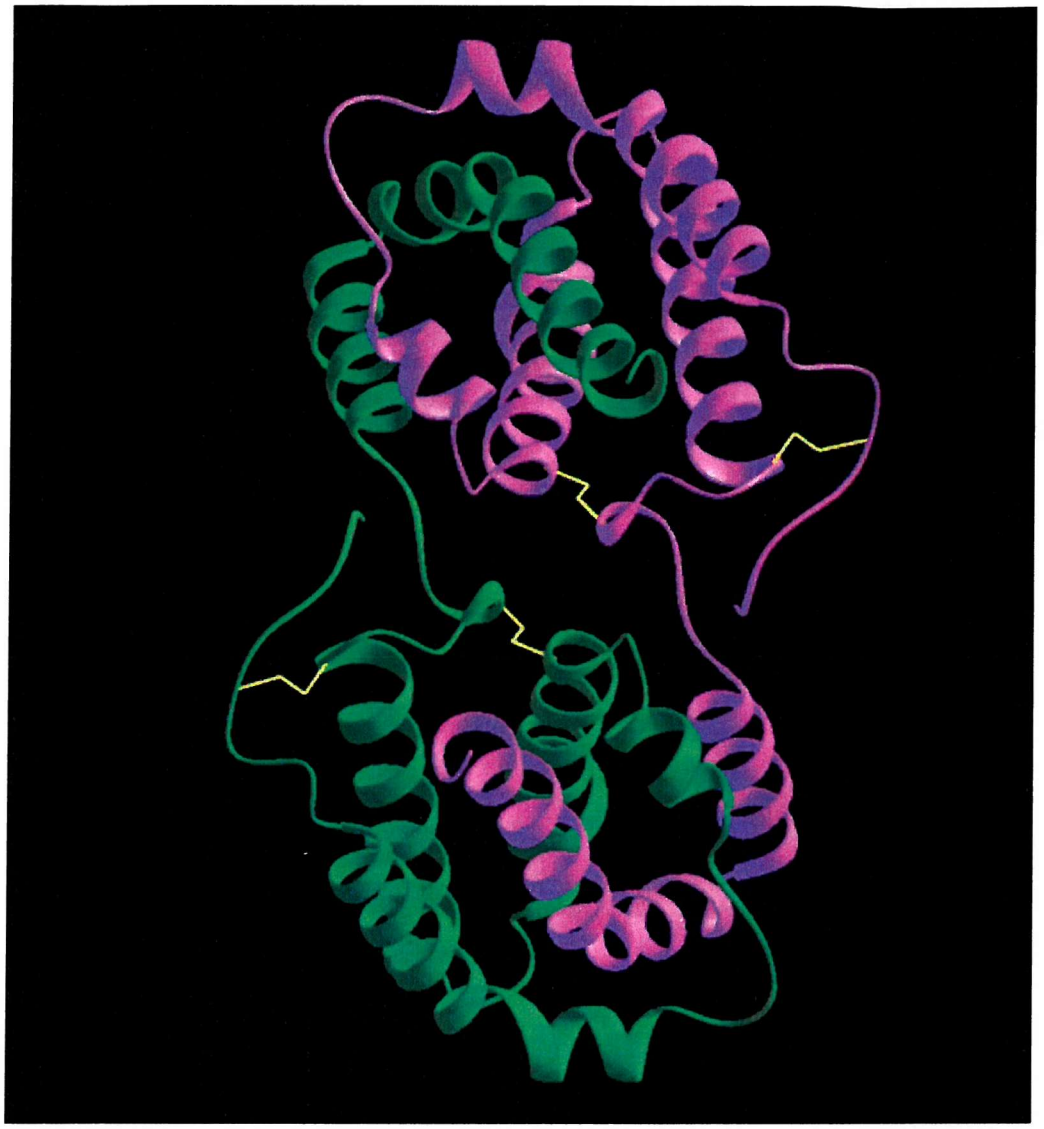
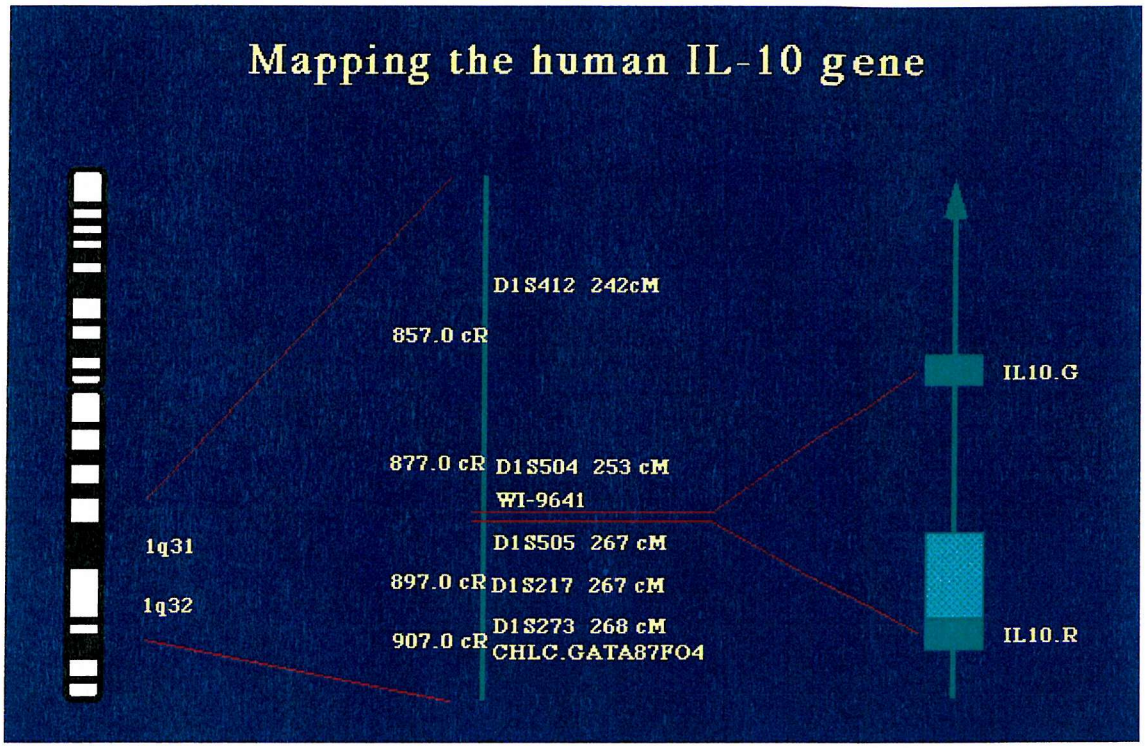


Fig 1.2 Structure of hIL-10 (figure obtained from Grant Gallagher, University of Glasgow).
hIL-10 has a secondary structure comprising 60% helix, with the tertiary structure being that of a V-shaped homodimer. each half of the V-shaped structure has 6 helices, 4 derived from one subuint and 2 from the other. 4 of these helices form an "up-up down-down" bundle that is characteristically present in all helical cytokines. The mature N-terminus of rmIL-10 is Gln22 while rhIL-10 is Ser19. hIL-10 has four cysteine residues with two disulphide bonds between Cys 1 and 3 and between 2 and 4 respectively. Reduction of these disulphide bonds was shown to cause a loss of helical structure with complimentary loss of biological activity.

1.2.2 Gene structure

The hIl-10 gene has been mapped to chromosome 1[5].

Figure 1.3 Location of the human IL-10 gene on chromosome 1 (figure obtained from Grant Gallagher, University of Glasgow).



The mIL-10 gene is made up of 5 exons, which span a ~5.1kb area of DNA on chromosome 1. Both m and hIL-10 exhibit a strong DNA and amino acid sequence homology with the open reading frame in Eppstien Barr Virus (EBV) genome, BCRF1[3,4].

Nucleotide sequence	mIL-10 to hIL-10 = 81%
	hIL-10 to BCRF1 = 71%
Amino Acid sequence	hIL-10 to BCRF1 = 84%
	mIL-10 to BCRF1 = 70%

Differences between hIL-10 and BCRF1 are found mainly in the N-terminal 20 amino acids.

1.2.3 BCRF1 (BamH1 C fragment rightward Reading Frame)

The open reading frame from the EBV encodes for a 17kD protein (expressed in late phase of the lytic cycle of the EBV), which like IL-10, contains little to no carbohydrate[6]. The BCRF1 gene, however, has no introns unlike the cellular IL-10 gene. BCRF1 has some of the properties of hIL-10, which include cytokine synthesis inhibition (see 1.5.1) and macrophage deactivation[7]. Due to these properties, the BCRF1 has been named viral (v) IL-10.
It is widely believed that the vIL-10 may represent an ancestral processed captured cytokine gene, used by the EBV to regulate the host immune system's response to it.

The cell tightly regulates IL-10 expression, since very little constitutive expression of IL-10 occurs, however in some cases, for example the activated Hepatic Stellate cell, once activated the IL-10 remains actively secreted. The IL-10 gene essential promoter requires the major start sequence of transcription, TATA box, with up to 150 additional 5' nucleotides. A positive regulatory sequence occurs at between –1100/-900, while a negative regulatory sequence lies at –800/-3008. Recently D.Kube, *et al* has managed to isolate an important area of the promoter in the last 80 nucleotides. (Personal communication submitted but not published at this time.)

1.3 IL-10 Receptor

The receptor for IL-10 is a member of the interferon family, which is expressed on all IL-10 responsive cells[9]. Studies with monoclonal anti IL-10R Ab has shown that this receptor is required for all known IL-10 responses. The IL-10R binds the respective cellular IL-10, both mouse and human, with high affinity (Kd~100pM)[10,11].
However, IL-10R's affinity for the EBV IL-10 analogue is at least 1000 fold lower than that for the cellular IL-10. This is consistent with the impaired function of vIL-10 on certain cell types. Studies using recombinant IL-10R expressing cells suggest the existence of one or more additional subunits of the receptor[12,13]. The human interleukin-10 receptor gene maps to chromosome 11q23.3[14].
The best understood signalling pathway activated by IL-10 is the JAK/STAT pathway[15]. Stimulation of IL-10R expressing cells with IL-10 leads to activation of

tyrosine kinases JAK1, Tyk2 and STAT 1 and 3. In the macrophage STAT 5 can also be activated in response to LPS stimulation, via the production of GM-CSF. IL-10 blocks this activation indirectly by inhibiting the production of GM-CSF[16]. STAT 3 is directly recruited to sequences surrounding two membrane distal tyrosines in the IL-10R cytoplasmic domain, which are redundantly required for STAT 3 activation and receptor function. STAT 3 dependent (inhibition of macrophage proliferation, induction of FcγR expression) and STAT 3 independent (inhibition of monokine production) IL-10 signalling pathways have also been defined.

1.4 Production and regulation of IL-10

Initially, IL-10 was discovered as a product of Th 2 cells. However CD4+ Th0 and Th1 also produce IL-10 cells[17], B cells, mast cells, eosinophils monocytes and macrophage, keratinocytes[18-23]. A variety of tumour cells have also been shown to express biologically active IL-10, e.g. hairy cell leukaemia[24] and multiple melanoma cells [25]. IL-10 has also been shown to be expressed in Hepatic Stellate Cells (HSC) and once activated these cells continue to produce IL-10 constitutively, this could indicate that in the HSC, IL-10 may have a different method of regulation than in other cell types[26].

The expression of IL-10 has also been shown to increase by a number of drugs, including PGE_2 and prostacyclin[27]. In contrast, cyclosporin A, cyclohexamide and methotrexate may inhibit IL-10[28]. The mechanisms that control and regulate the production of IL-10 are still under study. It appears that different cells respond differently to different stimuli and that the response can depend on the cell type in the microenvironment of the cell.

In vitro LPS stimulation of human monocytes causes an upregulation in IL-10 production, interestingly, if dibutyrol cAMP is added in conjunction with the LPS, then a much higher expression of IL-10 is seen than with LPS alone. This could be due to the ability of cAMP to suppress TNFα production in it's own right[29].

Interferon (IFN) β causes an *in vitro* up regulation of IL-10 production in monocytes obtained from patients with multiple sclerosis (MS)[30]. IFNβ is a therapeutically useful drug used in the treatment of MS[31]. Also IFNα has been demonstrated to increase the *in vitro* IL-10 production by human CD4+ T cells[32]. Administration of Transforming Growth Factor (TGF) β to Lewis rats with experimentally induced

uveitis (an inflammation disease of the eye), causes an upregulation of IL-10 mRNA levels in ocular tissues[33].

1.5 Biological effects of IL-10

1.5.1 The role of IL-10 in the immune system

IL-10 exerts its actions on a variety of cells within the immune system. Its effects are both complex and varied. The following discussion only highlights some of the actions of IL-10 within the immune system.

1.5.1.1 IL-10 as a Cytokine Synthesis Inhibitory Factor

As mentioned above, the earliest described role of IL-10 was that of a 'Cytokine Synthesis Inhibitory Factor' in mice. IL-10 was characterised as a product of Th2 cells that downregulate the production of IFNγ, and TNFβ by Th1 cells. When human PBMC are stimulated with LPS there is a rapid increase in the level of pro-inflammatory cytokines that peaks at about 4-8 hrs after stimulation. Addition of rIL-10, however, can have a profound effect on this cytokine production, with an almost complete inhibition of IL1α, IL1β, IL6, IL8, TNFα, IFNγ, GM-CSF and G-CSF[6,34,34-36]. Interestingly, IL-10 appears to be an autoregulatory cytokine since elevated levels of IL-10 lead to a down regulation in IL-10 mRNA synthesis in monocytes stimulated for 24hrs[34]. The fact that IL-10 is able to downregulate its own production by human monocytes makes this the first cytokine that is regulated by a negative feedback mechanism. IL-10 appears to downregulate pro-inflammatory cytokine by one of two mechanisms, reducing the stability of cytokine mRNA or by direct inhibition of transcription factors[34].

IL-10 also inhibits the production of cytokines such as IFNγ in murine Th1 cells. This action is achieved via its action on an APC, but only when this APC is a macrophage and not a B Cell. However, IL-10 has no effect on the Th2 cytokine production. Inhibition of all Th1 cytokines occurs, including IL-2, IL-3, TNFβ, IFNγ and GM-CSF, however the effects are most noticeable in reference to IFNγ and IL-3.

1.5.1.2 IL-10 and Tolerance

When a cell is stimulated for the first time a specific response will ensure. A secondary stimulation, by the same stimulus, may however only lead to a minimal response. This phenomenon is termed tolerance. One of the mechanisms that may mediate this response is the simultaneous increase in IL-10 levels, since neutralising IL-10 and TGF-β with monoclonal Ab's (mAb) can prevent this tolerance[37]. There is no apparent downregulation in the expression of the LPS receptor CD14 (Cluster of Differentiation) and this receptor appears to function as normal since NFκB mobilisation does occur38. Likewise, IL-10 has been implicated in the phenomenon of priming, whereby the macrophage is sensitised to release massive amounts of the pro-inflammatory cytokines in following LPS challenges. This process can be blocked by the addition of exogenous cAMP, which leads to an upregulation in the levels of IL-10[29,39].

1.5.1.3 IL-10 and the T-cell

IL-10 can inhibit antigen stimulated T cell proliferation both directly and indirectly. In order to understand these actions it is necessary to give a brief review of the antigen specific T cell activation.

1.5.1.3.1 T-cell activation

In order to stimulate CD4+ T cells, antigen-presenting cells (APC) must process the antigen via the endosomal pathway. This is followed by the expression of the antigenic peptide within a trough of the Major Histocompatibility Complex (MHC) class II molecule on the surface of APCs. Binding of the MHC class II molecule and the antigenic peptide complex with the complementary T cell receptor occurs next. For optimal T cell stimulation, however, co-stimulatory pathways have to be activated. These pathways are mediated by binding of CD28 on T cells, with its ligands CD80 (B7-1)/CD86 (B7-2) on the APC40. For these interactions to occur, The T cells and APCs must be in close proximity. Hence, adhesion molecules are also required. T cells express leukocyte factor for adhesion 1 (LFA1) and CD2,

which bind to their respective ligands, intracellular adhesion molecule (ICAM1) (CD50) and LAF3 on APCs[41].

Once T cell activation occurs, the CD4+ T cells secret IL-2 and express IL-2 receptors. The autocrine loop thus set up results in T cell proliferation. Within this pathway of CD4+ T cell activation, IL-10 acts in a number of ways. Experimentally, IL-10 has been shown to downregulate the expression of MHC class II on a variety of APCs, including dendritic cells, langerhans' cells and monocytes[42-45]. Interleukin 10 (IL-10) and viral IL-10 (v-IL-10) strongly reduced antigen-specific proliferation of human T cells and CD4(+) T cell clones when monocytes were used as antigen-presenting cells[42,46,47].

1.5.1.3.2 Effect of IL-10 on T-cell

It has also been hypothesised that IL-10 inhibits cytokine production by Th1 T cell clones by blocking accessory cell- (AC) dependent costimulatory function. APC surface expression of ICAM1, CD28 ligands, CD80 (B7-1) and CD86 (B7-2) are all markedly downregulated by IL-10[48,49].

IL-10 is also capable of directly inhibiting T cell proliferation, independent of its actions on APCs. IL-10 inhibits anti-CD3 mAb stimulated T cell proliferation via inhibition of IL-2 by T cells[50]. In addition, IL-10 inhibits CD80-86/CD28 dependent IL-5 secretion by T cells[51]. IL-10 may also contribute to the induction of T cell anergy. This could be due to IL-10 mediated downregulation of ligand-receptor co-stimulatory interactions between T cells and APCs[52].

In one study the effect of IL-10 was evaluated on Con A-induced proliferative responses of resting murine T cells. In this study purified T cells were cultured with different types of AC. The addition of IL-10 produced a 70 to 90% inhibition of resting T lymphocyte proliferation when purified populations of macrophages were used as AC, but had no effect on the AC function of T-depleted spleen cells, activated B cells, and dendritic cells. The inhibitory effects of IL-10 were inversely related to the concentration of mitogen and could be reversed by the addition of the neutralising anti-IL-10 mAb, SXC1. The inhibition of macrophage AC function was found not related to the induction of a suppresser cytokine as stimulation by mixtures of macrophages and limiting numbers of dendritic cells was not inhibited. The decrease in proliferative responses was primarily secondary to inhibition of IL-2

production although the failure of exogenous IL-2 to completely reconstitute the response suggested that IL-10 might also exert inhibitory effects on the induction of expression of a functional IL-2R. These results suggested that, IL-10 inhibits the induction of expression on macrophages of a critical costimulatory molecule that may be constitutively expressed on other types of AC[53]. The effect of IL-10 on APC dependent T cell cytokine synthesis and proliferation are most likely due, in part at least, to the downregulation of MHC class II expression on the APC surface.

1.5.1.3.3 Effect of IL-10 on other immune cells

IL-10 can also enhance immune activity. IL-10 stimulates proliferation, activation and chemotaxis of CD8+ T cells[54,55]. IL-3 is a growth factor for CD4 Th1 cell lines *in vitro*. This stimulatory effect of IL-3 on Th1 cells was synergized by the addition of IL-10[56]. Natural killer cell activity, proliferation and cytokine production are also enhanced by IL-10[57]. IL-10 can also cause an up regulation of Fc receptors on monocytes, thus enhancing antibody dependent cytotoxicity[58]. IL-10 has been demonstrated to increase B cell proliferation, differentiation and antibody production[19,59,60]. The effect of IL-10 on B lymphocytes could possibly have an important role in the pathogenesis of several diseases, e.g. Systemic Lupus Erythematosus (SLE) which is characterised by polyclonal B cell activation and the production of various auto-antibodies. Evidence suggests that autoantibody production in SLE is dependent on stimulation of B cell by IL-10[61].

1.5.2 Role of IL-10 in inflammation

Activated monocytes secrete IL-1α, IL-1β, IL-6 and TNFα, which are pro-inflammatory cytokines. In addition, they also secrete chemokines, e.g. IL-8 and macrophage inflammatory proteins (MIP). The chemokines comprise a family of related proteins with close similarities in structure and action that are involved in cell chemotaxis. IL-10 downregulates neutrophil production of the chemokines IL-8, MIP-1a and MIP-1b, an effect completely purged by the addition of cyclohexamide, suggesting the *de novo* effect of protein production. These cytokines cause leukocyte activation and recruitment into the site of inflammation. IL-10 inhibits the production of these cytokines by monocyte/macrophage, polymorphonuclear leukocytes and

eosinophils[34,62-65]. In addition, IL-10 also inhibits the production of GM-CSF and GCSF, which cause neutrophil and monocyte/macrophage activation.

IL-10 can downregulate the induction of nitric oxide synthase in macrophage cells. Since nitric oxide is an inflammatory mediator, IL-10 can also attenuate inflammation via this pathway[66]. Similarly, IL-10 also downregulates toxic Reactive Oxygen Intermediates (ROI). One of the key functions of the macrophage cell is the production of ROI and Reactive Nitrogen Intermediates (RNI), which include Nitric Oxide. ROI and RNI are both antimicrobial and tumorcidal. In mouse T cells, at low concentrations, IL-10 will causes an almost complete inhibition of TNFα (95.4%), but has little effect on the RNI and ROI. TNFα is a potent differentiation factor for the mouse T cell, while RNI and ROI both suppress lymphocyte function. Therefore, activated macrophage exposed to a low concentration of IL-10 are likely to produce ROI and RNI, but not TNFα, and thus may display a suppresser phenotype towards lymphocytes. This may contribute towards the CSIF activity of IL-10. However, at higher concentrations, IL-10 not only suppressed TNFα, but also ROI and RNI. This downregulation of RNI and ROI production in the activated macrophage, thereby also downregulates inflammation[67].

Matrix metalloproteinases (MMP) are implicated in the extensive tissue destruction seen in chronic inflammation68. Monocyte MMP activity is dependent upon prostaglandin synthesis. IL-10 has been shown to inhibit and downregulate prostaglandin H synthase 2[69].

1.5.3 The role of IL-10 in Liver Fibrosis

1.5.3.1 Introduction

In the liver uncontrolled inflammation can promote fibrosis leading to cirrhosis. The major causes in this country of liver fibrosis are alcohol abuse and infection with Hepatitis C[70]. During liver fibrosis a major cell in the advancement in the production of the extracellular matrix is the HSC[71]. As mentioned earlier, IL-10 has been linked to the HSC. The role of IL-10 has been investigated over the past few years and the synthesis of IL-10 during the course of liver fibrosis has been shown to be important in how pathogenesis progresses.

1.5.3.2 The role of IL-10 in liver fibrosis

In one study, the role of IL-10 has been investigated in the in the mouse model of liver injury induced by carbon tetrachloride (CCl-4). To address the role of endogenous IL-10 production, acute hepatitis was induced by CCl-4 in C57BI/6 IL-10 gene knock out (KO) and wild-type (WT) mice. After CCL-4 challenge, serum and liver levels of TNFα and serum levels of transforming growth factor-beta I (TGFβ1) increased and were significantly higher in IL-10 KO mice, whereas IL-6 serum levels were only slightly increased compared with WT mice. At a histological examination of the livers, a significantly more prominent neutrophilic infiltration in IL-10 KO mice 12 and 24 hours after CCL-4 injection was witnessed. In contrast, hepatocyte necrosis, evaluated by histological examination and serum alanine aminotransferase levels, was only marginally affected. The proliferative response of hepatocytes, assessed by the proliferating cell nuclear-antigen labelling index, was significantly increased in IL-10 KO mice, compared with WT mice 48 hours after CCL-4 injection. Finally, repeated CCL-4 injections led to more liver fibrosis in IL-10 KO mice after 7 weeks. The conclusions of the study suggested that endogenous IL-10 marginally affected the hepatocyte necrosis although it controls the acute inflammatory burst induced by CCL-4. During liver repair, it limits the proliferative response of hepatocytes and the development of fibrosis[72].

Kupffer cells (KC) have been shown to play a central role in the initiation and perpetuation of hepatic inflammation. Since IL-10 can inhibit a range of macrophage functions, it was hypothesised that the transcription, synthesis, and release of IL-10 may influence the development of liver injury. Rat KC were activated *in vitro* with LPS, and expression of IL-10 mRNA compared with IL-13 and IL-1 beta by reverse-transcription polymerase chain reaction (RT-PCR). The effects of pre-treatment with rlL-10 on KC phagocytosis, production of superoxide (SO), and TNFα were examined by fluorescent activated cell sorter (FACS), reduction of ferricytochrome C, and bioassay, respectively. Rats were administered intraperitoneal carbon tetrachloride (CC1-4), and expression of IL-10 mRNA and protein *in vivo* compared with IL-13 and IL-1β by RT-PCR and immunoblotting. Results were correlated with histological inflammatory changes. IL-10 gene- deleted (IL10-/-) mice and wild type (WT) controls were administered intraperitoneal CC1-4 biweekly for up to 70 days, and the development of inflammation and fibrosis compared by scoring histological

changes. IL-10 mRNA was up regulated early, both in KC *in vitro* and in whole liver *in vivo*, concurrent with that of IL-1β. IL-10 was able to inhibit KC production of both SO and TNFα *in vitro*, and this was achieved more effectively than IL-4 or IL-13; no such effects were seen on KC phagocytosis. After 70 days of treatment with CC1-4, IL-10 -/- mice showed significantly more severe fibrosis and exhibited higher hepatic TNFα levels than WT controls. The results suggest that IL-10 synthesised during the course of liver inflammation and fibrosis may modulate KC actions, and influence subsequent progression of fibrosis[73].

Another study demonstrated that Hepatic Stellate Cells (HSC) upon activation *in vitro* or *in vivo* express IL-10 and the autocrine effects of this cytokine include inhibition of collagen production. The results obtained in the study demonstrated that the activation of HSC causes enhanced autocrine expression of IL-10, which possesses a negative autoregulatory effect of HSC collagen transcriptional inhibition and stimulation of collagenase expression. These findings, along with the early induction of HSC IL-10 expression and its late disappearance during biliary liver fibrosis, suggest its *in vivo* role in matrix remodelling and a possibility that failure for HSC to sustain IL-10 expression underlies pathologic progression to liver cirrhosis [74].

In a final study, the expression of cytokines in the pathogenesis of liver cirrhosis was examined in hepatic biopsies. Here it was found that expression levels of TGFβ in post-hepatitis C liver cirrhosis were high, high to moderate in alcoholic liver cirrhosis and low in non-cirrhotic specimens. Expression of IL-10, TNFα, and IFNγ genes was detected in most post-hepatitis C liver cirrhosis, but not in idiopathic portal hypertension or alcoholic liver cirrhosis biopsies. The interleukin-1β, 6 and 8 gene expression was significantly lower in alcoholic liver cirrhosis compared to post-hepatitis C liver cirrhosis, but higher compared to idiopathic portal hypertension specimens. Thus, post-hepatitis C liver cirrhosis samples showed a high degree of cytokine gene expression, whereas in alcoholic liver cirrhosis it tended to be moderate, and restricted to some cytokines (TGFβ, IL-1, 6 and 8, but not IL-10, TNFα or IFNγ). In contrast, most non-cirrhotic specimens showed a restricted and low cytokine gene expression. The conclusions reached from this study suggested that TGFβ is present in liver fibrosis and inflammation, but that more study was necessary to understand its role. IL-1β, 6, 8, TNFα and IFNγ, appear to participate in

the pathogenesis of the mild to severe inflammatory phenomena seen in alcoholic and post-hepatitis C liver cirrhosis, respectively. The study also suggested that TNFα does not participate in the hepatocellular damage of alcoholic liver cirrhosis, and indicated that neither IFNγ nor IL-10, at least at the levels observed in post-hepatitis C liver cirrhosis, were able to counteract the fibrotic inflammatory process seen in this condition[75].

The biological functions of IL-10 on the immune and inflammatory systems are summarised in table1.

Table 1. Biological functions of IL-10.

Enhances	Inhibits
• The *in vitro* production of IL-1 receptor antagonist by monocyte and polymorphonuclear leukocytes • Proliferation, activation and chemotaxis of CD8+ T cells • NK cell functions, potentates IL-2 induced NK cytotoxicity activities • Antibody dependent cytotoxicity by upregulating Fcγ receptors on monocytes • B cell proliferation, differentiation and antibody production • Synthesis and secretion of insulin by pancreatic ilet cells in rats	• Antigen stimulated T cell proliferation via inhibition of IL-2 production by T cells • Production of IFNγ, IL-2 and TNFα by Th1 cells • Macrophage co-stimulatory activity by selectively inhibiting the upregulation of B7 expression in mice • Synthesis of proinflammatory cytokines (TNFα, IL-1α, IL-1β, Il-6, IL-8) • CD80/CD28 dependent IL-5 secretion by T cells • IL-8 production in human neutrophils • Free oxygen radical release and the nitric oxide dependent microbial activity of macrophages • Downregulates monocyte prostaglandin H synthase-2 • Decreases surface expression of MHC class II molecules on variety of APCs, including dendritic cells, monocytes and langerhams' cells

1.6 Mitogen Activated Protein Kinase Signalling Pathways

1.6.1 Introduction and overview

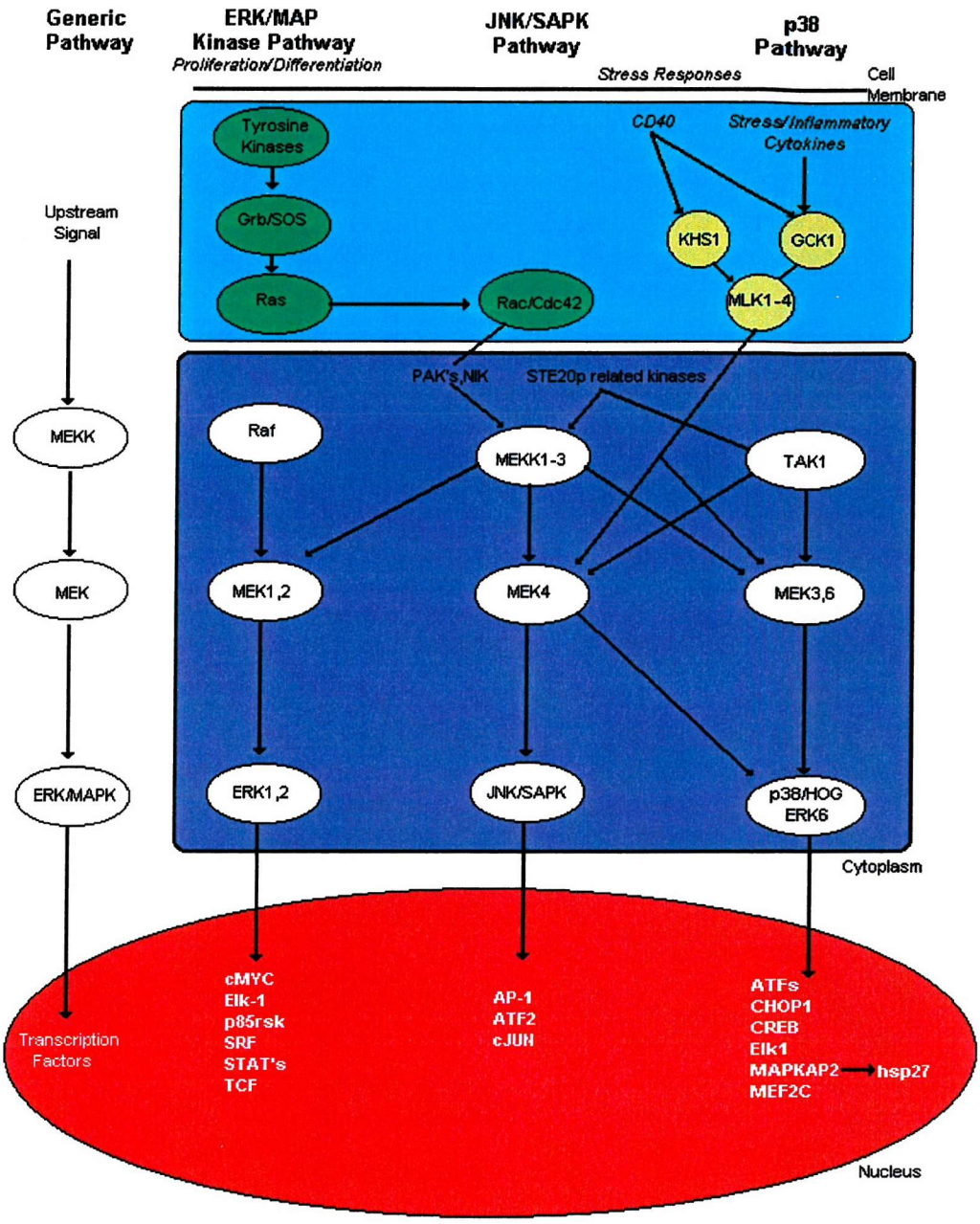
As was mentioned earlier, IL-10 is produced by the macrophage in response to stress stimuli caused by the presence of LPS, RNI and ROI as well as the secretion in to the cellular environment of TNFα and other cytokines. It has been well established that these responses are signalled via the Mitogen Activated Protein Kinase pathways in the cell.

Over the past few years, the mitogen-activated protein (MAP) kinases have been at the forefront of a rapid advance in the understanding of cellular events in growth factor and cytokine receptor signalling[76-79]. The MAP kinases (also referred to as extracellular signal-regulated protein kinases or ERK) are the terminal enzymes in a three-kinase cascade. The reiteration of three-kinase cascades for related but distinct signalling pathways gave rise to the concept of a MAK kinase pathway as a modular, multifunctional signalling elements that acts sequentially within one pathway, where each enzyme phosphorylates and thereby activates the next member of the sequence[80](See fig 4).

A canonical MAP kinase module thus consists of three protein kinases: a MAPK kinase kinase (or MEKK) that activates a MAPK kinase (or MEK) which in turn activates a MAPK/ERK enzyme. In the MAPK module of the yeast pheromone response pathway, a scaffold protein Ste5p binds the three analogous enzymes of its module, suggesting that the module may function as a physically stable unit[80].

The recent identification of distinct MAPK cascades that are conserved across all eukaryotes indicates that the MAPK module has been adapted for interpretation of a diverse array of extracellular signals. Although mitogen activation of the MAPK subfamily (e.g., ERK1 and ERK2) has dominated efforts to understand MAPK signalling, increasing appreciation of the role of stress-activated kinases, JNK and p38, illustrates the diverse nature of the MAPK superfamily of enzymes. Although sequence similarities among components of the individual MAPK modules used for activation of ERK1/2, JNKs and p38 are considerable, the fidelity that is maintained in order to translate specific extracellular signals into discrete physiological responses illustrates the selective adaptation of each MAPK module. Understanding how such specificity is maintained, and the extent and significance of cross talk

between each signalling cascade, are fundamental issues that are actively being investigated by the research community.



Overview of the MAP Kinase Pathways

Figure 1.4. Parallel MAP kinase cascades involves specific MAP kinase enzyme modules. Each of the MAP kinase/ERK, JNK and p38 cascades consists of three-enzyme module that includes MEKK, MEK and an ERK or MAPK superfamily member. A variety of extracellular signals trigger initial events upon association with their respective cell surface receptors and this signal is then transmitted to the interior of the cell where it activates the appropriate cascades.

1.6.2 Regulation and function of MAPK

1.6.1.1 Multipurpose signal transducers

The MAPK superfamily of enzymes is a critical component of a central switchboard that co-ordinates incoming signals generated by a variety of extracellular and intracellular mediators. Specific phosphorylation of many proteins with substantial regulatory functions throughout the cell, including other protein kinases, transcription factors, cytoskeletal proteins and other enzymes. The diversity of signals that culminates in MAPK activation indicates that these enzymes are not the dedicated hardware of any single growth factor, hormone or cytokine system. Instead, MAPKs like cAMP-dependent protein kinase (PKA) and Ca^{2+}, and phospholipid-dependent protein kinases (PKC), serve many signal purposes. Because activation of the MAPK pathways are triggered to varying extents by a large number of receptor systems, temporal and spatial differences are critical to determining ligand and cell type specific functions.

1.6.1.2 MAP Kinase Modules

Following activation of cells with the appropriate extracellular stimuli, the signal is transmitted to the canonical MAPK module comprising the three protein kinases (see fig. 1.4). The progression of events for each enzyme cascade is the same, although specific isoforms of each enzyme confer the required specificity within each pathway. The first enzyme in the module is a MEKK enzyme, of which Raf and its isoforms are one example. The MEKK enzymes are Ser/Thr kinases that activate the MEK enzymes by phosphorylating two serine or threonine residues within a Ser-X-X-X-Ser/Thr motif. Once activated, the MEK enzymes, which are mixed function Ser/Thr/Tyr protein kinases, phosphorylate the MAPK/ERK enzymes on Thr and Tyr residues within the Thr-X-Tyr (TXY) consensus sequence (see figure 1.5). A crucial and common feature of the MAPK superfamily of enzymes is that they are activated upon dual phosphorylation within the TXY consensus sequence present in the activation loop of the catalytic domain[81-83]. The central amino acid differs for each MAPK superfamily member, corresponding to Glu for ERK1/2, Gly for p38/HOG and Pro for JNK/SAPK, although MEK specificity is not limited to these two residues[84]. Phosphorylation at only one of these two positions does not activate the

enzyme, although it may prime the kinase domain for receipt of the second phosphorylation event.

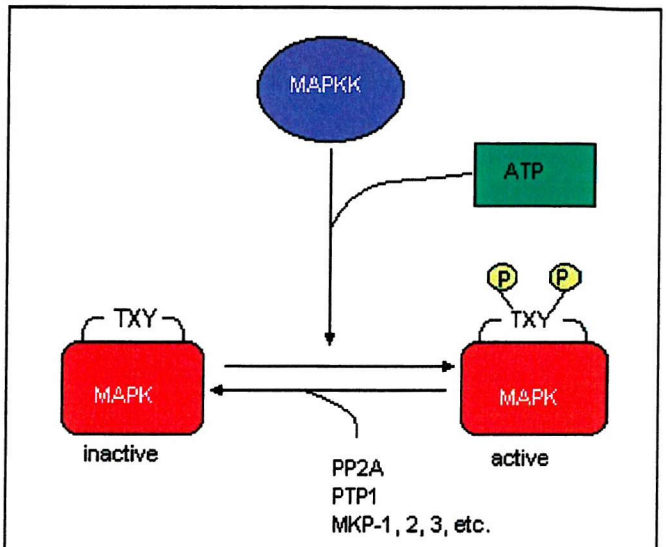


Fig 1.5 . Dual phosphorylation activates the MAP kinase enzymes. A critical common feature of the MAP kinase superfamily is that they are activated by MEK enzyme mediated dual phosphorylation within the TXY consensus sequence in the activation loop of the catalytic domain. Phosphorylation of only one of the two positions does not activate the enzyme although it may serve to prime the kinase domain for recipt of the second phosphorylation event. A number of protein phosphotases (e.g. PPA2, PTP1 and MKPs) are involved in down regulation of the signal, by dephosphorylating either one or both phosphorylated residues.

1.6.1.3 Regulation of ERK enzyme activation

ERK1 and ERK2 were the first members of the MAPK superfamily whose cDNA was cloned[85,86] and the signalling cascades that lead to their activation are the best characterised to date. Potent activation of ERK1, 2 can be initiated through activation of transmembrane receptors with intrinsic or associated protein tyrosine kinase (PTK) activity[87]. In this scenario, binding of extracellular ligands to their respective cell surface receptors results in autophosphorylation and enhanced PTK activity.

The subsequent association of the Src homology 2 (SH2) domains of adapter proteins such as Grb2 and Shc with the autophosphorylated receptors, or additional "docking" proteins, provides the molecular interactions that bring the required signal transduction molecules into close proximity. Receptors without intrinsic PTK activity but which harbour sites for tyrosine phosphorylation may also activate the cascade via association of their phosphotyrosine residues with adapter molecules. For

example, the SH3 domain of Grb2 binds a proline rich region of the guanine nucleotide exchange protein SOS that, in turn, increases the association of Ras with GTP. The GTP bound form of Ras binds to Raf (a MAP kinase kinase) isoforms, including C-Raf1, b-Raf and A-Raf. This action targets Raf to the membrane, where its protein kinase activity is increased by phosphorylation[87]. MAPK kinases (MEK1, MEK2)[77], are phosphorylated and activated by Raf. MEK1 and MEK2 are duel specificity protein kinases that dually phosphorylate the ERK enzymes (corresponding to Thr^{183} and Tyr^{185} of p42ERK2), thereby increasing their enzymatic activity by approximately 1,000 fold over the activity found with the basal or monophosphorylated forms[88]. Phosphorylation of these residues causes closure of the kinase active site and induces conformational changes necessary for high activity[82].
MAPK mutants, lacking either a lysine required for catalytic activity or the prerequisite TXY phosphorylation sites, inhibit signalling by the native enzymes in cells. In the case of ERK1 and ERK2, these mutants have been used with repeated success. For example, mutant ERK2 completely blocks proliferation in response to epidermal growth factor (EGF) and v-Raf, and partially blocks induction by serum or small t antigen[89]. ERK1 antisense mRNA and ERK1 phosphorylation site mutant interfere with thrombin induced transcription as well as serum dependent proliferation[90]. These findings suggest an essential role in proliferation and transformation for the ERK/MAPK pathway.

1.6.1.4 Regulation of JNK/SAPK and p38/HOG

The JNK/SAPK and p38/HOG pathways are activated by ultraviolet light, cytokines, osmotic shock, inhibitors of DNA and RNA and protein synthesis, and to a lesser extent by growth factors.
This spectrum of regulators suggests that the enzymes are transducers of a variety of stress responses. In contrast to activation of ERK1 and ERK2, upstream, signal transduction mechanisms for the JNK and p38 cascades are less well understood (Fig 1.4). When transfected into mammalian cells, a diverse group of protein kinases including the mixed lineage kinases (MLKs) and relatives of the yeast Ste20p, e.g. the p21-activated-kinases (PAKs) and germinal centre kinase (GCK), cause activation of JNK/SAPK.

Similarly, GTP-bound forms of the small GTP-binding proteins, Rac and Cdc42, activate the JNK/SAPK pathway, and to a lesser extent, the p38 pathway[91]. Direct activation of both pathways by PAKs also has been demonstrated92, suggesting that PAKs may be the relevant effectors for these small G proteins. The PAKs are homologues of the yeast kinases Ste20p and Shk1, enzymes upstream of the MAPK modules in yeast pheromone response pathways[80]. Both yeast and mammalian protein kinases contain a binding site for Rac/Cdc42 and share the property of being activated *in vitro* through association with these small G proteins when in their GTP-bound states. In yeast, Ste20p is believed to phosphorylate and activate the MEKK isoform Ste11p, suggesting that MEKKs may be PAK targets.

1.6.1.5 MAP kinase module specificity

Because of the pleiotropic potential of MAPKs, their activities are tightly controlled by both positive and negative mechanisms. A variety of factors are known to modulate MAPK activity including substrate specificity, protein-protein interactions, subcellular localisation and dephosphorylation by protein phosphotases.

MEK1 and MEK2 are the only known activators of ERK1 and ERK2 and are believed to phosphorylate only these two substrates. Other MAPK family members retain the TXY phosphorylation sites but are poor substrates of MEK1 and MEK2[84]. Similarly, cloning efforts have uncovered MEK-like enzymes that show high selectivity in phosphorylating JNK/SAPKs and p38, further illustrating the important contribution of the MEK enzymes in determining MAPK signalling specificity.

As noted above, Ste5p promotes the formation of complexes among enzymes of the MAPK module that mediate pheromone-induced mating. Also, Ste5p is absolutely required for signalling through this module, since deletion of this gene, or over expression of a Ste5p mutant that cannot bind the yeast MAPK enzyme, blocks the pheromone response[80]. Given the known conservation between yeast and mammals of components in MAPK signalling cascades, and the existence of scaffolding factors for a number of protein phophotases and other protein kinases[93], scaffolding factors like Ste5p are likely to exist in metazoans. These proteins may restrict enzymes, such as MEKK1, in order to maintain the fidelity of individual signalling pathways.

Subcellular localisation undoubtedly plays an important part in directing ERK signalling and in limiting cross-activation between related modules. In quiescent

cells, ERK1 and ERK2 are found in the cytoplasm and are associated with microtubules. Stimulation of cells with mitogens results in activation of the ERK enzymes within the cytoplasm, with some of the enzyme located in specialised membrane compartments. With mitogenic activation of certain cell lines, a fraction of the activated ERK enzymes is efficiently translocated to the nucleus94. A similar story is unfolding for JNK/SAPKs and p38. The kinetics of ERK activation influences the efficiency of nuclear translocation and, thus, access to nuclear substrates. If the kinase is rapidly inactivated, as occurs following EGF stimulation of PC12 cells or by nonmitogenic stimulation of CCL39 cells[94], it may not enter or remain in the nucleus. In contrast, a more prolonged period of activation, as occurs with nerve growth factor (NGF)-stimulated PC12 cells[95] or thrombin-treated CCL39 cells, results in nuclear retention and this may be critical for establishing the differentiation signal[79].

Because phosphorylation of both tyrosine and threonine is required to activate the MAPK enzymes, certain dual-specificity protein phosphatases, such as the immediate early gene MKP-1, appear well-suited to inactivate the MAPK family members by dephosphorylating one or both sites[96]. However, individual Ser/Thr (e.g., PP2A) or Tyr (e.g., PTP1) protein phosphatases also can regulate ERK activity by dephosphorylating only one of the two phosphorylated residues and thereby inactivating the enzyme[87,96]. The relatively high abundance and high specific activity of these Ser/Thr or Tyr protein phosphatases provides another important mechanism for regulating ERK enzyme activity. The final physiological outcome of these signal transduction pathways therefore is determined by the interplay between these protein kinases and protein phosphatases.

1.7 LPS and LPS signal transduction

LPS (lipopolysaccharide) or endotoxin are components of the cell wall of gram negative bacteria. Together with phospholipids and membrane bound proteins, it is a constituent of the outer cell membrane.

LPS consists of 3 structural elements. One is the hydrophibic component, called Lipid A, which serves to anchor the molecule to the membrane. The second is a core oligosaccharide and the third component is a hydrophilic O-polysaccharide

projecting into the extra-cellular space. More than 150 different variants of the third component are known.

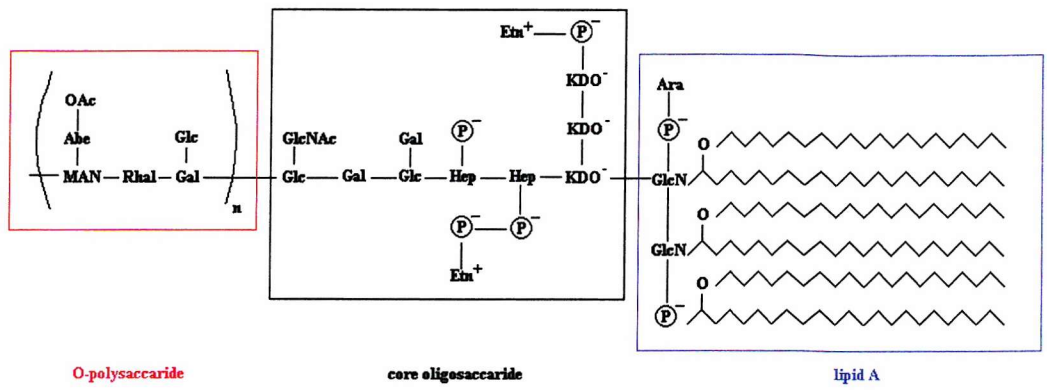


Figure 1.8 Structure of salmonella endotoxin.
As exampled by salmonella LPS, the molecule has three distinct parts, designated O-polysaccaride (red), core polysaccaride (black) and lipid A (blue)

Abe = Abequose, Ara = G-Amino-Arabinose, Etn = Ethanolamine, Gal = D-Galactose, Glc = D-Glucose, GlcNAc = N-acetyl-D-Glucosamine, Hep = L-Glycero-Mannoheptose, KDO = 2 Keto-3-deoxyoctonic acid, Man = D-Mannose, Oac = O-acetyl, P = Phosphoric acid ester, Rha = L-Rhamnose.

LPS signal transduction in monocytes involves binding to the cell surface receptor CD14[97]. Recently it has been shown that the CD14 surface receptor then works in conjunction with the Toll receptor[98] which leads to the activation of tyrosine kinases[99], protein kinase C[100,101], and MAPKs, p38, p42/44 (ERK) and p54 (stress activated protein kinase/ JNK)[102,103]. While the role of the MAPKs in LPS induced signalling is probably the best characterised, the relationship between the activation of these signalling molecules and induced cytokine expression is still obscure. The only exception to this is the observation that inhibition of the p38 MAPK with specific imidazole inhibitors (e.g. SB203580) prevents translation of the TNFα mRNA[102]. Activation of the p42/44 MAPK pathway has also been implicated in TNFα expression, but these studies have been performed in cell lines transfected with various mutant forms of Raf-1 kinase, a proximal activator of the p42/44 MAPK pathway[104]. There is little information on the role of either of these kinases in regulating the expression of other cytokines in monocytes in general and only one other study regarding the expression of IL-10 synthesis in particular[105]. A recent

report has suggested that IL-10 production is dependent on protein tyrosine kinase and protein kinase C activation[106], while several studies suggest that factors that elevate cAMP are involved in the regulation of monocytic IL-10 production, primarily at the mRNA level[107-109].

1.8 Eukaryotic gene transcription regulation

The understanding of eukaryotic transcription regulation has made significant advances in the past decade and the following essay is by no means a comprehensive coverage of this extremely important area of research. Instead what follows is merely an introduction to an ever-increasing knowledge base on eukaryotic transcription factors and their roles.

In the early 1960s, Francois Jacob and Jacques Monod performed key experiments, which began to answer questions about prokaryotic gene regulation[110]. These experimental techniques and strategies were later extended to eukaryotic organisms. To date, many similarities and contrasts in transcriptional control mechanisms have been documented between prokaryotic and eukaryotic genes.

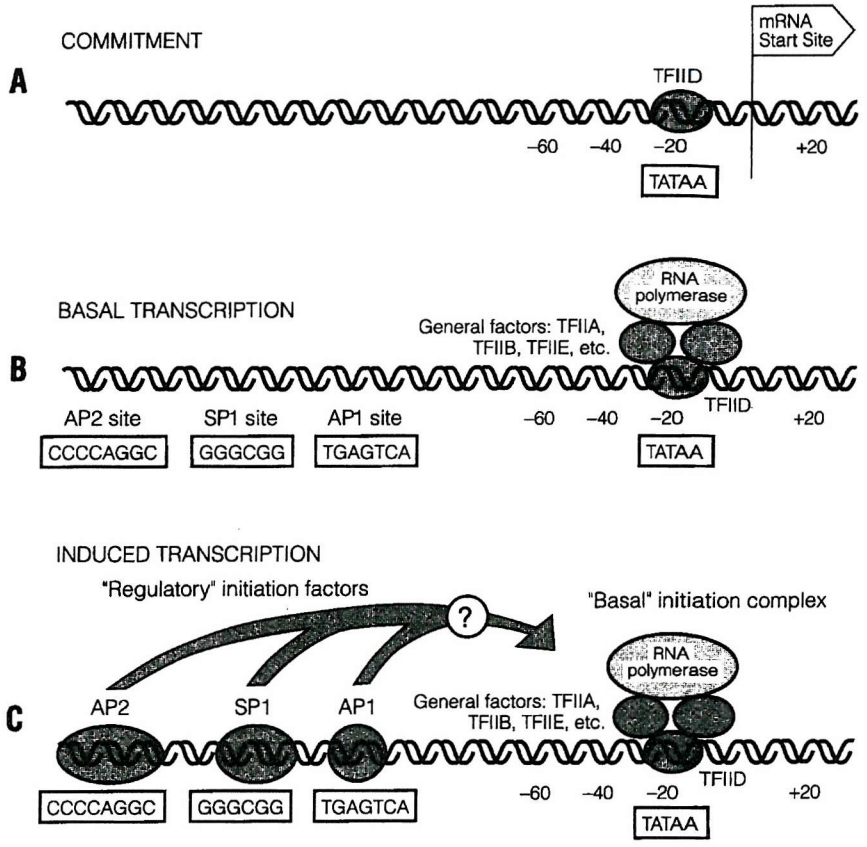


Figure 1.7 RNA Polymerase II transcription initiation complex. Source : Promega Website 1997.

In eukaryotes, three RNA polymerase complexes play important roles in transcription. As a general guideline, RNA polymerase I transcribes ribosomal RNAs, RNA polymerase II transcribes protein-encoding genes, and RNA polymerase III transcribes tRNA genes. Figure 1.7 provides a schematic of an RNA polymerase II transcriptional and promoter unit, but similar elements also can be found associated with RNA polymerase I and III transcriptional units. As detailed in this figure, the RNA polymerase II basal transcription complex consists of RNA polymerase II, which itself is a multi-subunit complex, together with several other protein factors (or general transcription factors) such as TBP (TATA-Binding Protein) and TFIIB. This mega-complex associates with DNA sequences in the promoter region in close proximity to the transcription initiation site preceding the gene-coding region[111].

Other gene sequences, in addition to the promoter sequences which associate with the RNA polymerase II mega-complex, can influence the transcriptional activity of genes. Some of these "response elements" can increase transcriptional activity and often are referred to as activator or enhancer sequences[112]. Traditionally, enhancer

sequences or modules were identified to function at different distances and in both orientations, relative to the transcription initiation site.

Enhancer DNA elements have been observed to be bound by corresponding "activator" proteins. For example, the enhancer region of the SV40 virus, a 72bp repeat region has binding sites for AP1, AP2 and AP3 transcription factors[113]. Positive and negative regulatory effects upon transcription appear to be mediated by sequence-specific DNA binding proteins, or specific transcription factors, that may act individually or in combination to modify basal transcriptional activity[114].

Other features in addition to DNA sequences play a role in the binding of transcription factors to DNA. For example, certain protein structural themes, or domains, have been identified within the long list of transcription factors. Many DNA binding proteins have a zinc finger domain, a helix-loop-helix domain, or helix-turn-helix motif. Many transcription factors also have activation domains such as glutamine-rich or proline-rich regions. Another level of regulation can occur between different transcription factors, which is mediated by protein: protein interactions. Some of the protein structural features identified with this level of regulation include the leucine-zipper domain and helix-loop-helix motifs. Lastly, the activities of some factors can be controlled by other enzymes such as kinases, phosphatases, proteases or other modifying enzymes which regulate the protein's structural state, resulting in a protein's binding ability (or inability). For general reviews on these topics, see references 114-116.

The phenomenon of activation has been studied in more detail than its counterpart, repression. Recent experimental evidence suggests that many transcriptional mechanisms are not exclusively in "on" or "off", "activated" or "repressed" modes. We are just beginning to understand the dynamics of transcriptional activity of particular genes during development or the cell cycle in relationship to interactions between multiple regulatory proteins acting synergistically or antagonistically, depending on their relative concentrations, regulatory modifications (e.g., phosphorylation states) and physical association with the chromatin template.

The study of transcriptional regulation has been advanced by the use of reagents such as recombinant protein factors, transcriptionally active cell extracts, antibodies, reporter vectors and transgenic animals. Certain techniques have now become standard when characterising a putative transcription factor. These assays allow

detailed analysis of DNA: protein interactions, protein: protein interactions, or transient and stable expression in cultured cells.

1.9 Aims and Hypothesis

It is hypothesised, therefore, that IL-10 plays a central role in the regulation of inflammation and that an understanding of the pathways and transcription factors involved in IL-10 production will allow a greater understanding of many disease states which evolve from an impaired inflammation response.

In this study we aim to develop a cell line model system which will allow us to look at the IL-10 regulation within the monocyte/macrophage.

The study will be broken down into three main focus points. The first will examine the effect of endotoxin, simulating the effects found in inflammation, upon the primary monocyte cells as well as on the monocyte cell lines to ensure that the response of the cell lines mimics that found in the primary cell. Secondly, the second messenger pathways will be mapped for both the primary cell and cell line to ensure that the cell biology and transcriptional responses behind the endotoxin response are the same in both cases. The final part of the study will focus on which transcriptional factors are involved in IL-10 synthesis after LPS stimulation. This will be achieved by the development of a transfection system so that IL-10 promoter constructs can be inserted into the cell line.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals were in general obtained from the Sigma chemical company, Poole, U.K., unless otherwise stated. Cell culture media was obtained from Life Technologies (Gibco), Paisley; U.K. Radioactive materials were obtained from Amersham, U.K.

2.1.2 Water

General-purpose solutions were prepared with water produced by reverse osmosis using a Purite R1500 system (diH_2O). For tissue culture grade water, diH_2O was filtered through a 0.22μm filter (Millipore) before 0.01% Diethyl pyrocharbonate (DEPC). The water was well mixed followed by an overnight incubation at room temperature this allowed the DEPC to destroy any present RNases. The DEPC water was then sterilised as below.

2.1.3 Sterilisation

Solutions and water were sterilised by autoclaving at 15lbs/in^2 for 15 minutes. Antibiotics and other heat sensitive materials were sterilised by filtering the solution through a 0.22μm filter (Millipore).

2.1.4 Cell culture medium

Both primary monocytes and monocyte cell lines were maintained in chemically defined media.

2.1.4.1 RPMI 1640

RPMI-1640 media has been well documented as the preferred media for growing and maintaining human and non-human monocyte cell lines. For the propagation of the cell lines THP-1, U937, HL-60 and the mouse cell line RAW 264.7, RPMI media was prepared as below.

RPMI containing 4500mg/ml D-glucose was supplemented with 100U/ml penicillin and streptomycin (sigma), 10% low endotoxin FCS (Gibco), 20mM HEPES (sigma) and 1% L-glutamine (sigma).

2.1.4.2 30:60:10 media

The 30:60:10 media was based on media used by A. Moses, et al when in 1994 they published a paper stating that they had managed to transfect primary human macrophage. 30:60:10 media was prepared as follows.
To 30mls AIM V Research Grade W/O Glutamine was added 60ml Iscoves modified DMEM with Glutamax II (Gibco) and 10mls of heat deactivated AB pooled human serum (obtained from the Blood components laboratory, Southampton). To this was added 100U/ml Penicillin/streptomycin and 20mM HEPES.

2.1.4.3 Trypsin/EDTA

Obtained as a 10x solution (sigma). This solution contains 25g of porcine Trypsin and 2g of EDTA in Hank's balanced salt solution. This was made to a working 1x solution by dilution in Hank's balanced salt solution (-Ca^{2+}) (Gibco)

2.1.5 Cell Lines

2.1.5.1 THP-1

The THP-1 cell line was derived from the peripheral blood of a 1-year-old male with acute monocytic leukaemia. The THP-1 cells have Fc and C3b receptors and lack surface and cytoplasmic immunoglobins. These cells also stain positive for alpha-napthhyl butyrate esterase, produce lysozymes and are phagocytic (both latex beads and sensitised erythrocytes). THP-1 cells can also restore the response of purified T lymphocytes to ConA, show increased CO_2 production on phagocytosis and can be differentiated into macrophage-like cells using, for example, DMSO

2.1.5.2 U937

Derived from malignant cells of a pleural effusion of a 37 year old Caucasian male with diffuse histocytic lymphoma, the U937 cell line is one of only a few human cell lines still expressing many of the monocytic like characteristics exhibited by cells of histocytic origin. Treatment with PMA at a concentration of 400ng/ml for 3-5days results in the formation of macrophage-like cells.

2.1.5.3 HL-60

Taking peripheral blood lymphocytes obtained by lymphophoresis from a 36-year-old female produced this cell line. Cell growth after resuscitation is initially slow and after 6 weeks in culture these cells may differentiate. At any one time 10% of these cells spontaneously differentiate. This is enhanced proportionately by the addition of polar-planer compounds, e.g. DMSO, Retinoic acid, etc. Due to their early phenotypic nature, these cells, when treated with DMSO, can also become neutrophil-like cells.

2.1.5.4 MonoMac 6

In culture the morphology of a MonoMac 6 cell, is single, round/multiformed cells or small clusters of cells in suspension. Sometimes loosely adherent, approximately 1-5% of these cells are giant cells. A human acute monocytic leukaemia, this cell line was established from the peripheral blood of a 64-year-old man with acute monocytic leukaemia (AML FAB M5) in 1985. Properties: CD3-, CD13+, CD14+, CD15+, CD19-, CD33+, CD34-, CD68+, HLA-DR(+).

2.1.5.5 Namawala

Established from a patient with human Burkitt's Lymphoma, the Namawala cell line secretes small amounts of an IgM monoclonal antibody of unknown specificity. It has been used for commercial production of human interferon. The cells contain the EBV genome and should be handled under laboratory containment level 2.

2.1.5.6 RAW 264.7

Established from ascites of a tumour induced in a male mouse by intraperitoneal injection of Abselon Leukaemia Virus (A-MuLV). The RAW cells will pinocytose neutral red and phagocytose zymosan. These cells are capable of antibody dependent lysis of sheep erythrocytes and tumour targets. LPS (0.5ng/ml) inhibit the growth of the mouse RAW 264.7 cell line

2.1.6 ELISA

An ELISA was set up to calculate the concentration of IL-10 and TNFα produced by the monocytes.

ELISA plates were obtained from NUNC plastic (Gibco). Plates were blocked with a blocking solution which contained 1% Bovine serum albumin fraction V (BSA) (sigma), 5% sucrose (sigma) made up in PBS (see 2.1.9). Standards were produced by diluting either rhIL-10 or rhTNFα (PeproTech EC Ltd, London, U.K.) in RPMI. Dilutions were carried out in a 5% BSA solution. Chromagen was produced by adding 1.5mls of 10x Citric acid buffer (1M sodium acetate and 1M citric acid) to 13.35mls diH_2O. To this buffer was added 25µg of TMB dissolved in 150µl DMSO and 25µl H_2O_2. This reaction was stopped using a stop solution, which consisted of 4N sulphuric acid.

2.1.6.1 Antibodies

Both the primary capture antibody and the secondary biotinylated antibody for the IL-10 ELISA were obtained from R&D systems (U.K.). The Streptavidin-Horse radish peroxidase (HRP) detection system was obtained from Zymed. For the TNFα ELISA, the primary detection antibody was also obtained from R&D systems. The secondary antibody was obtained from Peprotech (U.K.) and the tertiary antibody conjugated with the enzyme HRP was obtained from Dako (U.K.) All antibodies were made up per the manufacturers instructions and stored at 4°C

2.1.7 FACS

All antibodies for the detection of IL-10 and TNFα were obtained from Cambridge Bioscience. Anti CD14 antibody was obtained from sigma. Monensin was made up into Ethanol at a concentration of 100mM and stored at 4°C. During cell preparation cells were washed in cell wash buffer which contained 1% FCS, 0.01% sodium azide in PBS. Saponin buffer was produced by adding 0.1% Saponin, 0.01M HEPES to PBS.

2.1.8 Inhibitors

Inhibitors were obtained from Calbiochem (U.K.) and were made up according to the manufacturers instructions.

2.1.9 LPS

LPS, from *E. coli* serotype B055:55 was dissolved to a concentration of 20mgs/ml in DMEM. This stock solution was stored at –20°C until required.

2.1.10 RT-PCR

All reagents and enzymes for the RT-PCR were obtained from Promega (U.K.). DEPC'd water was produced as already mentioned in 2.1.2 above. Primers were synthesised from OSWELL (Southampton, U.K.) from published protocols.

IL-10 primers

IL103P = 5' **TCTGT TGCCT GGTCC TCCTG ACTG** 3' (47-73)

IL105P = 5' **CTCAC TCATG GCTTT GTAGA TGC** 3' (488-501)

β-Actin primers

BACTINF = 5' **GCCAG CTCAC CATGG AT** 3' (31-47)

BACTINR = 5' **AGGGG GGCCT CGGTC AG** 3' (370-354)

2.1.11 General Solutions

Phosphate Buffered Saline

137mM NaCl
2.7mM KCl
4.3mM Na_2HPO_4
1.4mM KH_2PO_4
pH 7.4

TAE Buffer

2M Tris-acetate
50mM EDTA
pH 8.0

PBS/T

PBS plus 0.05% Tween 20 (w/v)

TEN Buffer

40mM Tris-Hcl, pH 7.5
150mM NaCl
1mM EDTA

RF1

100mM RbCl
50mM $MnCl_2.4H_2O$
30mM potassium acetate
10mM $CaCl_2.2H_2O$
15% glycerol (w/v)
adjust pH to 6.8 with NaOH

RF2

10mM MOPS
10mM RbCl
75mM $CaCl_2.2H_2O$
15% glycerol (w/v)
adjust pH to 5.8 with 0.2M acetic acid

Sterilise both RF1 and RF2 by filtration through 0.22μm membrane.

2.1.12 Plasmids

Restriction enzymes and the pGL-2 basic plasmids were obtained from Promega (Southampton, U.K.).
DR. N. Sheron obtained the IL-10 luciferase promoter constructs from Dr D. Kube. The IκB-LUC plasmid and the sie-CAT plasmid were obtained from Dr. D. Mann. The IL-10 Luciferase constructs as well as the IκB-LUC plasmid are LPS responsive. The sieCAT plasmid is IFN-γ responsive.

2.1.13 Growth and preservation of E. coli

Luria (L-) broth was prepared by dissolving 10g tryptone, 5g yeast extract and 5g NaCl in 1L of distilled water and adjusting the pH to 8.0. Aliquots of LB were autoclaved in conical flasks or Bijou bottles.

L-agar was prepared by adding 1.5g agar to 100ml L-broth, autoclaving and equilibrating the temperature to 50°C in a waterbath before pouring the plates. Note: agar plates, when poured, were stored at 4°C and used within one week. The plates were dried open and inverted for 30 minutes immediately prior to use.

Ampicillin was dissolved in 50% ethanol at a stock concentration of 25 mg/ml and filter sterilised. The working concentration was 100µl/ml.

2.2 Methods

2.2.1 Maintenance of cell lines

Cell lines were all grown in RPMI (2.1.4.1). Primary human blood monocytes were maintained in 30:60:10 media (2.4.1.2). Cell cultures were maintained at 37°C in an atmosphere of 5% CO_2 and were routinely passaged.

For passaging, the cell suspension was collected into a 50ml centrifuge tube and spun at 1000rpm for 5 minutes. The supernatant was removed and replaced with fresh media. Cell pellet was gently re-suspended by pipetteting and the cell suspension transferred to a fresh Nunclone tissue culture flask (Gibco).

For the passaging of monolayer cells, primary blood monocytes and RAW 264.7 cells, the supernatant was removed and the cells washed in PBS three times. Cells were then incubated with x1 Trypsin/EDTA at 37°C to dislodge the cells from the flask. The detached cells were then transferred to a sterile 50ml centrifuge tube and collected by centrifugation at 1000rpm for 5 minutes. The pellet was re-suspended in fresh media and plated out onto fresh plastic.

For long term storage of cells, the cells were re-suspended in appropriate media containing 25% FCS, 10% glycerol (v/v) and maintained in liquid nitrogen.

2.2.2 Isolation of human monocytes from buffy coats

Buffy coats were obtained from the Blood Components Lab. at Southampton General Hospital. Only buffy coats from same blood groups were mixed. The buffy coat was transferred to a sterile bottle. Aliquot *Histopaque 1077* (sigma); density 1.077g/ml, into 50ml centrifuge tubes (20ml/tube). With centrifuge tubes supported at a 30-45 degree angle, carefully overlay ~5ml of buffy coat onto the *Histopaque* into each tube. Carefully return each of the centrifuge tubes to a vertical position and slowly pipettete a further 15 mls of the buffy coat into each tube, being careful not to mix the buffy coat with the *Histopaque*. This should leave approximately 20mls of buffy coat.

Blood was carefully placed in a Beckman (type T-J6) centrifuge with a swing out rotor. Tubes were spun at 700g (-2000rpm) for 30 minutes at room temperature. (N.B DO NOT USE BRAKES AT THIS STEP).

After centrifugation four layers can be seen. The bottom layer consists of the red blood cells. The layer on top of this is the *Histopaque*. Next is a thinner band that contains the peripheral blood mononuclear cell (PBMC). Above this is the plasma layer. Pipettete off the plasma layer from the top of each tube to within 5mm depth of PBMC layer. Add this plasma to the remaining 20ml of buffy coat.

Carefully remove PBMC band from each tube (should have 4-5mls/tube), carefully ensuring minimum contamination from plasma and *Histopaque* layers. Pool PBMC in 2 universal containers and dilute 1:1 with ice-cold PBS/2.5mM EDTA/10mM glucose. Spin universals at 360g (-1500rpm), for 10 minutes at room temp, in a swing out rotor. Pour off supernatant (which will be cloudy, this contains eosinophils and basophils), and re-suspend each PBMC pellet in 10ml ice-cold PBS/2.5mM EDTA/10mM glucose.

Aliquot 30ml foetal calf serum (FCS) into each of two 50ml centrifuge tubes. Carefully overlay the diluted PBMC suspension (10ml/tube). Spin at 200g (1100rpm) for 15 minutes (at room temperature) in a swing out centrifuge. Discard the supernatant containing the platelets, and re-suspend each pellet in 20ml of warm 30:60:10 media. Pool and count the re-suspended cells. Aliquot cells out at $1x10^6$ cells/cm^2 onto 75cm^2 tissue culture grade cell flasks and incubate for one hour at 37°C in 5% CO_2.

After 1.5 hours, carefully wash flasks with sterile PBS three times to remove all non-adherent cells, then Trypsinise the cells into 5-10mls of PBS using a rubber policeman. Spin cells in a centrifuge at 1000rpm for 5 minutes and re-suspend pellet in 10mls of 30:60:10 media. Count cells in haemocytometer using Trypan blue cell counting method and re-suspend cells at desired concentration (e.g. $1x10^6$ cells/ml). Plate cells into desired plates and allow to adhere overnight.

2.2.3 Inhibitor experiments

To examine the effect of inhibitors on the IL-10 production in macrophage cells, primary human blood monocytes or monocyte cell lines were washed, counted and plated out at a concentration of $1x10^6$ cell/ml into a 96 well Nunclone tissue culture plate (Gibco). Cells were allowed to recover from isolation/washing overnight.
Next day the cells were treated with inhibitor of choice and allowed to incubate for 1 hour at 37°C. After this incubation, the supernatants were removed and fresh media containing the inhibitor of choice and LPS (typically added a 100µg/ml) was added for 24hrs. After incubation supernatants were collected and stored at -20°C until an ELISA (see 2.2.4) could be performed.

2.2.4 Enzyme Linked ImmunoSorbant Assay (ELISA)

Primary capture antibody (100µl/well) was added to each well (5µg/ml IL-10 primary antibody and 5µg/ml TNFα antibody) and allowed to adhere overnight at 4°C to the ELISA plates. Next day the wells were blocked with 300µl of blocking solution at room temperature for no less than 2 hours. After blocking, the plates were washed in a Wellwash 4 plate washer 3 times with PBS/T. Next 100µl of standards and sample supernatants were added to each well and allowed to incubate at room temperature for 2hrs.

IL-10 standard curve range (pg/ml);	TNFα standard curve range (pg/ml);
9.05	10.29
27.16	30.86
81.48	92.59
244.4	277.78
733.34	833.3
2200	2500
6600	7500

After incubation the plates were again washed three times in PBS/T and the secondary antibody added (both IL-10 and TNFα at 0.27μg/ml). After incubation the excess secondary antibody was washed away (x3 in PBS/T) and the Streptavidin-HRP added at 1:2000 dilution for the IL-10 ELISA and the TNFα tertiary antibody (swine anti-goat-HRP, Dako) was also added at 1:1000 dilution. The Streptavidin-HRP was allowed to incubate with the IL-10 for 20 minutes and the tertiary TNFα antibody was allowed to incubate for 1hr at room temperature. Once again excess antibody was wash away with x3 washes with PBS/T and the chromagen added (100μl/well). The plate was allowed to develop in the dark for 20 minutes before the reaction was stopped with 30μl of the stop solution.
The plates were read in the Anthos II plate reader at 450nm with a reference filter of 620nm.

2.2.5 Intracellular Cytokine Staining

Staining of intracellular cytokines depends on the identification of cytokine-specific monoclonal antibodies compatible with a fixation/permeabilisation procedure. Optimal intracellular cytokine staining has been reported using a combination of fixation with paraformaldehyde and subsequent permeabilisation of cell membranes with the detergent saponin. Paraformaldehyde fixation allows the preservation of cell morphology and intracellular antigenicity, while also enabling the cells to withstand permeabilisation by detergent. Membrane permeabilisation by saponin is critical for allowing the cytokine specific monoclonal antibody to penetrate the cell membrane, cytosol and membranes of the endoplasmic reticulum and golgi apparatus. Critical parameters for cytokine staining include the following; cell type and activation

protocol, the time of cellular harvest following activation, the inclusion of a protein transport inhibitor during cell activation and the choice of anti-cytokine antibodies. The most common inhibitors of protein transport inhibitors are monensin and brefelding A, which blocks intracellular transport processes and results in the accumulation of most cytokine proteins in the rough endoplasmic reticulum or golgi complex, thus enhancing the cytokine staining signal.

Re-suspend 10-20mls of 0.5-1x10^6 cells/ml of chosen cell type (e.g. THP-1, RAW cells or primary human blood macrophage and allow to adhere or rest overnight before next step). Add LPS (10µl/ml) and/or other stimulus, to cells and allow to incubate for desired time. Three hours before harvesting, monensin (2.5µM) was added to the cells. The cells were then allowed to incubate with the monensin at 37°C in 5% CO_2.

Harvest cells into FCS, to block the Fc receptors and thus reduce non specific immunofluorescent staining, and spin down at 1,000rpm for 5 minutes, wash cells in PBS/0.1%sodium azide (w/v)/1% FCS (Wash Buffer) and respin down at 1000rpm for 5 minutes. It should be noted that primary cells should be scraped and not Trypsinised since Trypsinisation destroys the CD14 marker. Once the cells had been harvested and washed, they were stained with an anti CD14 antibody, so as to isolate the monocytes when they were passed through the FACS analyser. Re-suspend cells in 0.5mls PBS containing 4% Paraformaldehyde at 4°C for 10 minutes. Note that paraformadehyde strips the cells of most of their CD markers, and therefore, CD14 staining must be carried out before fixation.

Wash cells in Wash Buffer and re-suspend in 500µl of PBS containing 0.1% Saponin (and 0.01M HEPES) (Saponin Buffer).

To control cells, to show no non-specific binding of Ab takes place, add x10 concentration of unconjugated Ab. For 20 minutes at 4°C. To surface stained cells add 10µl of control Anti-CD CD14 biotin labelled Ab.

Do not wash away the blocking Ab from blocked samples, instead add 10µl of labelled cytokine antibodies and incubate at 4°C for 20 minutes in the dark. For detection of biotynylated Ab add 10µl of fluorochrome conjugated streptavidin, incubate the cells for 20 minutes in the dark at room temp. Wash cells with PBS and re-suspend cell pellet in 0.5ml PBS and analyse on the FACScan using three colour analysis with WinMiDi 2.8 software.

2.2.6 Growth and Preservation of *E. coli*

The *E. coli* strain DH5α was routinely grown aerobically at 37°C on LB agar. The strains were then kept as working stocks on the LB agar plates at 4°C and passaged at intervals of 4-5 weeks. Plasmid-encoded ampicillin resistant *E. coli* strains were grown and maintained on LB agar supplemented with 100µl/ml ampicillin.
Liquid cultures of *E. coli* were usually grown in LB medium for 16 hours at 37°C in an orbital shaker at 300 rpm. Ampicillin resistant *E. coli* was grown under the same conditions in LB containing 100µl/ml ampicillin. For long term storage, a cell suspension was stored at -70°C in LB containing 15% glycerol.
For a static overnight culture of *E. coli*, the culture was prepared by inoculating 10 ml of LB medium with the bacteria and incubation at 37°C overnight without shaking.

2.2.7 Transformation of *E. coli*

2.2.7.1 Preparation of competent *E. coli*

A 10 ml static culture (2.2.5) of the recipient *E. coli* was grown overnight at 37°C. A lml aliquot of the static culture was used to inoculate 50 ml of LB medium and incubated in an orbital shaker at 300 rpm at 37°C until the A_{550} value reached 0.4-0.5 (approx. 2 hours). The culture was then collected in a 50 ml polypropylene centrifuge tube and incubated on ice for 15 min. All subsequent steps were carried out at 4°C.
The cells were collected by centrifugation at 2000 g for 10 min and the supernatant discarded. The pellet was immediately re-suspended in a volume of RF1 (2.1.9) that was 1/3 of the volume originally collected. The suspension was incubated on ice for 15 min. Cells were then pelleted as above. The supernatant was again discarded and the bacterial pellet re-suspended in RF2 (2.1.9) to 1/12.5 of the original volume and incubated on ice for 15 min. The cells could then be used for transformation (2.2.6.2) or distributed into aliquots, frozen in liquid nitrogen and stored at -70°C for subsequent use.

2.2.7.2 Transformation of competent *E. coli*

Transformation was achieved by incubation of the transforming DNA (typically 10ng of DNA in a maximum volume of 10 µl) with 100 µl of the competent cells (2.2.7.1). The DNA and competent cells were incubated on ice for 30 min with occasional agitation followed by a 2 min heat shock at 42°C and subsequent chilling on ice for a further 5 min.

Transformed cells were brought to room temperature, diluted in an equal volume of LB medium and inoculated onto LB agar. If the transformed DNA encoded for antibiotic resistance (i.e. ampicillin) then the LB agar was supplemented with the appropriate antibiotic.

2.2.7.3 Isolation of Plasmid DNA

Plasmids were isolated from *E. coli* using the Qiagen EndoFree Maxi Kit (Qiagen, Crawley) following the manufacturers instructions. Briefly;

A single colony from a freshly streaked selective plate was used to inoculate a starter culture of 2-5ml LB medium containing the appropriate selective antibiotic. Incubate for ~8 h at 37^{O}C with vigorous shaking (~300rpm).

Dilute the starter culture 1/1500 to 1/1000 into selective LB medium. For high-copy plasmids inoculate 100ml medium, and for low-copy plasmids, inoculate 250ml medium. Grow at 37°C for 12-16 hrs with vigorous shaking (~300rpm). Harvest the bacterial cells by centrifugation at 6000 x g for 15min at 4°C. Re-suspend the bacterial pellet in 10ml Resuspension Buffer P1. Add 10ml Lysis Buffer P2, mix gently but thoroughly by inverting 4-6 times, and incubate at room temperature for 5min.

During the incubation prepare the QIAfilter Cartridge: Screw the cap onto the outlet nozzle of the QIAfilter Maxi Cartridge. Place the QIAfilter Cartridge into a convenient tube.

Add 10ml chilled Buffer P3 to the lysate, and mix immediately but gently by inverting 4-6 times. Proceed directly to the next step. Do not incubate the lysate on ice. Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at room temperature for 10min. Remove the cap from the QIAfilter outlet nozzle. Gently

insert the plunger into the QIAfilter Maxi Cartridge and filter the cell lysate into a 50ml tube.

Add 2.5ml Buffer ER to the filtered lysate, mix by inverting the tube approximately 10 times, and incubate on ice for 30min. Equilibrate a QIAGEN-tip 500 by applying 10ml Buffer QBT, and allow the column to empty by gravity flow. Apply the filtered lysate to the QIAGEN-tip and allow it to enter the resin by gravity flow. Wash the QIAGEN-tip with 2x30ml Buffer QC. Elute DNA with 15mls of buffer QN.

Precipitate DNA by adding 10.5mls (in 0.7ml volumes) room temperature isopropanol to elute the DNA. Mix and centrifuge immediately at >15,000xg for 30 minutes at 4°C. Carefully decant the supernatant. Wash the DNA pellet with 5mls of endotoxin free, room temperature 70% ethanol and centrifuge at >15,000xg for 10 minutes. Carefully decant the supernatant without disturbing the pellet.

Air-dry the pellet for 5-10 minutes and redissolve the DNA in a suitable volume of endotoxin free water.

Determination of yield was carried out using both agarose gel analysis and UV spectrophotometry.

2.2.8 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation and characterisation of plasmid DNA obtained from 2.2.7.3. It was performed with a horizontal submerged slab gel electrophoresis system. The gel system used was of the 'mini-gel type where the gel is cast directly into the electrophoresis apparatus (Cambridge Electrophoresis).

The gel routinely used consisted of agarose at a concentration of 1% (w/v) containing ethidium bromide at 0.5 μg/ml in lx TAE buffer. The gel was formed by boiling the required amount of agarose in the required volume of lx TAE in a microwave oven. The agarose was allowed to cool to 50°C and the ethidimn bromide added just prior to pouring. The gel was poured into the electrophoresis apparatus without the formation of bubbles and allowed to set for 30 min. Perspex combs of varying teeth size were inserted into the molten gels to create loading wells. Once set the comb was removed and the gel completely submerged in lx TAE buffer. Samples to be loaded were mixed with a one-sixth volume of gel loading buffer (Promega, Southampton) and loaded into the wells of the gel under the lx TAE buffer. An

aliquot (5µl) of a suitable DNA marker (usually 1-kb DNA mass ladder, Gibco BRL) was used to size the electrophoresed DNA. Samples were routinely run through the gel at 90V for 1 hour or until the orange G dye front had reached the end of the gel. After electrophoresis the DNA was visualised by medium wave UV illumination. Photographic images of the gel were obtained using Polaroid 667 black and white film (ASA 3000) using a red filter. An exposure time of between 1 and 4 seconds at an aperture setting of F11 was usually sufficient.

2.2.9 Agarose gel extraction.

Occasionally it was necessary to extract the Plasmid DNA from the agarose gel. To do this the Qiagen Gel Extraction kit was used (Qiagen). The kit was used in accordance with the manufacturers instructions. Briefly;

Excise the DNA band from the agarose gel with a clean sharp scalpel. Weigh the gel slice in a colourless tube. Add 3 volumes of buffer QX1 to 1 volume of gel for DNA fragments 100bp - 4kb. Re-suspend QIAEX II by vortexing for 30sec. Add 30µl QIAEX II to the sample and mix.

Incubate at 50°C for 10min to solubilise the agarose and bind the DNA. Mix by vortexing every 2min to keep QIAEX II in suspension. Check that the colour of the mixture is yellow. Centrifuge the sample for 30sec and carefully remove supernatant with a pipette. Wash the pellet with 500µl of Buffer QX1.

Wash the pellet twice with 500µl of Buffer PE. Air-dry the pellet for 10-15min or until the pellet becomes white. To elute DNA, add 20µl of 10mM Tris-Cl, pH 8.5 of H_2O and re-suspend the pellet by vortexing. Incubate at room temperature for 5 minutes. Centrifuge for 30sec. Carefully pipettete the supernatant into a clean tube.

2.2.10 Transfection of monocytes

2.2.10.1 Lipofectin

On day one plate out cells on a $90mm^3$ culture dish at a density between $5\text{-}10x10^6$ cells/ml and allow them to adhere overnight at 37°C in 5% CO_2. Transfect the cells on day two as follows:

Add 20µl of lipofectin reagent to 80µl of serum and antibiotic free RPMI-1640 and incubate at room temp for 20 minutes. Add 5-10µl of DNA and make up to a total of 100µl with serum and antibiotic free RPMI-1640 and add to the lipofection solution. Incubate at room temp for 15mins. During this incubation, wash the macrophage with PBS x2 and replace with serum and antibiotic free RPMI-1640 (approx. 5-7mls). Add 800µl of serum and antibiotic free RPMI-1640 to the lipofectin/DNA solution mix gently and add to the cells. Incubate the cells for 6 hours at 37°C in 5% CO_2. After 6 hours, the lipofectin/DNA solution was washed off and the cells allowed to recover, in complete media, overnight before stimulation.

2.2.10.2 Effectene

Effectene was obtained from Qiagen and tranfections carried out according to manufacturers instructions.

The DNA was diluted with the DNA-condensation buffer (buffer EC), to make a total volume of 150µl. The enhancer solution was added and the mixture vortexed for 1 second. The mixture was left to incubate at room temperature for 5 minutes. Once done, the effectene transfection reagent was added to the DNA-enhancer mixture and the solution mixed by pipetteting. Allow this mixture to incubate at room temperature for 10 minutes to allow complex formation.

During complex formation, gently aspirate the growth media from the plated cells and wash once in PBS. The media was collected and spun down and added back to the cells at a 50:50 ratio with fresh media.

600µl of fresh media was added to the reaction tube containing the DNA complexes and the solution mixed by pipetteting. This mixture was added back to the cells drop by drop and the complexes gently distributed around the wells. Cells were incubated at 37°C in 5% CO_2 to allow gene development overnight. Next day cells were stimulated to express genes.

2.2.10.3 Electroporation

Gently aspirate the growth media from the plated cells and wash once in PBS. The media was collected and spun down and added back to the cells at a 50:50 ratio with fresh media.

To the cells plasmid DNA was added, dissolved in H_2O, at desired concentration and mixed well with the cell suspension by pipetteting. The Cell/DNA mixture was allowed to incubate at 37°C for 10 minutes to allow DNA to bind to the surface of the cell.

The cell/DNA mixture was then added to a Gene Pulser 0.4cm Cuvette (BioRad, Hercules, USA) and subjected to a 290V/196μFD electric pulse in a BioRad Gene Pulser.

Cells were plated out into conditioned media and allowed to recover for 48hrs before stimulation.

2.2.10.4 Superfect

Superfect was obtained from Qiagen and tranfections carried out according to manufacturers instructions.

Add 2:1 (ethanol:DNA) + $1/10^{th}$ (of DNA volume) 3M sodium acetate, note sodium acetate was added prior to ethanol. This was mixed well and spun for 5 minutes at 13,000rpm. From here the rest of the protocol should be carried out under sterile condition. Remove the supernatant and wash the DNA pellet in 70% ethanol (spin at 13,000rpm for 5minutes). Remove the supernatant and allow the DNA pellet to air dry. Add tissue culture grade water (2.1.2) to DNA and allow pellet to dissolve.

DNA and serum free media was mixed by vortexing followed by a brief centrifugation to bring solution to the bottom of the tube. Next, the superfect reagent was added to the DNA solution and the contents mixed on a vortexer for 10 seconds, followed by a brief centrifugation. The DNA/superfect was left to incubate at room temperature for 10 minutes to allow complex formation.

During superfect/DNA complex formation, the cells were lightly washed with PBS twice, cell supernatant was collected and mixed 50:50 with fresh media. Once incubation was complete, 600μl of fresh media was added to the complex solution and added to the cells. Cells were incubated with the complex solution for 3 hours at 37°C. After 3 hours superfect/DNA complex was removed and the cells gently washed once in PBS. To these cells, the conditioned media from above was added back to the cells and the cells allowed to recover overnight before stimulation.

2.2.11 Analysis of transfected promoter

2.2.11.1 CAT assay

Cells were then harvested and a Chloramphenicol Acetyl-Transferase (CAT) assay carried out as follows:

Cells were washed twice in PBS (note at this stage the process can be carried out outside the hood, i.e. it does not have to be sterile) and 1ml of TEN buffer is added to aid in the 'unsticking' of the cells. The cells were allowed to incubate with the TEN buffer for 5 minutes at room temp. The cells were then harvested into the TEN buffer using a cell scraper and placed into a 1.5ml microcentrifuge tube. The cells were then spun down at 1,000rpm for 5 minutes.

The supernatant is then discarded and to the pellet 50-100µl of 0.25M Tris-HCl, pH7.8, was added and the cells disrupted by three times a free/thawing cycle in liquid nitrogen and at 37°C respectively. Cellular deacetylases were deactivated by incubation at 65°C for 10 minutes. The debris was removed by spinning the sample at 13,000rpm for 5 minutes and the supernatant collected. The protein concentration was then determined for each sample and the equivalent amount of protein added to each assay.

The reaction mixture contained:

50µl cell extract + 0.25M Tris-HCl, pH7.8

70µl 1M Tris-HCl, pH7.8

20µl 4mM acetyl Co-A

1µl [^{14}C] Chloramphenicol (50mCi/mmol)

The reaction was incubated for 1 hour at 37°C and the substrate and products extracted with 500µl of ethyl acetate by vortexing for 30 seconds. The two phases were separated by centrifugation at 13,000rpm for 5 minutes and the upper organic phase collected into a fresh microcentrifuge tube. The ethyl-acetate was evaporated under vacuum in a Sorvall SpeedVac and the remaining pellet re-suspended in 15µl of fresh ethyl acetate.

The products of each CAT assay were then spotted onto a silica gel thin layer chromatography plate and separated by ascending chromatography with a mixture of

chloroform: methanol (95:5) After the chromatography has run the silica gel was allowed to air dry and the plate exposed to X-ray film.

2.2.11.2 Luciferase assay

Luciferase activity was recorded using the promega luciferase assay kit (Promega, Southampton) according to the manufacturer's instructions.
Briefly, Cells were harvested and washed 1x in PBS to remove contaminating autoflourescent components from the media. Next the cells were lysed using the passive lysis buffer (PLB). For monolayer cells, PLB was added directly to the flask and the culture plate gently shaken for 15 minutes at 25°C. The lysate was then transferred to an eppidorf tube. A protein assay was carried out (see 2.2.12) on the samples and the volume adjusted to ensure equal protein concentrations.
100µl of LAR II buffer were predispenced into luminometer tubes. The luminometer was preset for a 2-second premesurment delay followed by a 10 second measurement. 20µl of the cell lysate was added to the 100µl of LAR II buffer and mixed gently by pipetteting 2-3 times. Note, the samples should never be vortexed since this may coat the sides of the luminometer tube. Read samples in the luminometer and record results.

2.2.12 Determination of protein concentration

To determine the concentration of a given sample of protein the DC Protein Assay Kit from BioRad was used. It was compatible with solutions containing detergents and was used according to the manufacturer s instructions. The BioRad DC Protein Assay is a colourimetric assay and the reaction is similar to the well-documented Lowry (1951) assay. The assay is based upon the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps that lead to colour development: the reaction between protein and copper in alkaline environment and the subsequent reduction of Folin reagent by the copper treated protein. Proteins effect a reduction of the Folin reagent by loss of 1, 2 or 3 oxygen atoms, thereby producing one or more of several reduced species which have a characteristic blue colour with maximum absorbence at 750nm. Briefly, 5µl of sample or standard curve were added to a 96 well plate. If detergent was present in

the sample then 20µl of reagent S was added to 1ml of reagent A to produce reagent A'. To the protein sample 25µl of reagent A or A' was added, followed by the addition of 200µl of reagent B. The reaction was allowed to proceed at room temperature for 15 minutes before being read at 620nm in the ANTHOS II plate reader.

2.2.13 RT-PCR

Numerous techniques have been developed to measure gene expression in tissues and cells. These have included Northern Blots, coupled reverse transcription and PCR amplification (RT-PCR), RNase protection assays, *in situ* hybridisation, dot blots and S1 nuclease assays. Of these methods, RT-PCR is the most sensitive and versatile. The techniques can be used to determine the presence or absence of a transcript, to estimate expression levels and to clone cDNA products without the necessity of constructing and screening a cDNA library.

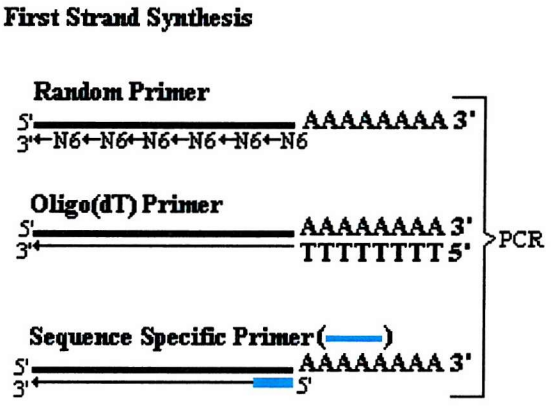


Figure 2.1 Schematic diagram of RT-PCR.

RT-PCR was utilised to determine the presence or absence of IL-10 mRNA, with β-actin used as a control. For the IL-10 RT-PCR, we modified the already published methodology of Knolle, P.A., *et al*[117]. For the β-actin protocol, the methodology of Housby, J.N. et al[118] was adopted. RNA was collected using the Qiagen RNeasy kit, briefly the method was as follows, Cells were harvested by centrifugation (1,000rpm for 5 minutes) and the media completely removed. It is essential to remove all the media, since this may inhibit lysis and dilute the lysate, thus affecting the later binding conditions of the RNA to the RNeasy membranes. Cells grown in a

monolayer, such as primary monocytes, can be lysed directly in the tissue culture vessel. Cell membranes were disrupted by the addition of buffer RLT, containing 10µl β-Mercaptoethanol/1ml of buffer (at this stage cell lysates can be stored at –70°C for several months). Add buffer RLT at the following volumes;

Buffer RLT(µl)	**Number of Pelleted Cells**	**Dish Diameter (cm)**
350	up to $5x10^6$	<6
600	$5x10^6$ to $1x10^7$	6-10

Next the samples were homogenised, by pipetteting the lysate directly into a QIAshredder column, sitting in a 2ml-collection tube, and centrifuging at maximum speed for 2 minutes. Collect the flow-through. To the homogenised sample, 1 volume of 70% ethanol was added and mixed well by pipetteting, do not centrifuge after adding the ethanol. Apply up to 700µl of sample, including any precipitate that may have formed, to a RNeasy mini spin column, sitting in a 2ml-collection tube, and centrifuge for 15 sec at ≥8,000g, unless otherwise stated all following centrifugation steps are carried out at this speed. If any homogenised sample is left repeat in the same column, do not use a fresh column. After each spin discard flow-through. pipettete 700µl of the wash buffer RW1 onto the RNeasy column, and centrifuge for 15 secs, discard the flow-through. Transfer the RNeasy column to a fresh 2ml-collection tube and pipette 500µl of buffer RPE into the column. Centrifuge for 15 secs, discard flow-through and repeat, this time for 2 minutes to dry the column's membrane. The RNeasy column was then transferred into a 1.5ml collection tube and 30-50µl of RNase-free water was applied directly to the membrane and centrifuges for 1 minutes to elute the RNA. This was repeated if the expected yield of RNA was greater than 30µg.

OD readings and RNA integrity gel, determined RNA quality and concentration. OD readings were carried out in the BIORAD SmartSpec 3000 Spectrophotometer, following the manufacturer's instructions. An integrity gel was set up and run as follows, the required gel volume was prepared in RNase free glassware and microwaved for approx. 45 seconds on full power, or until all the agarose has dissolved

Gel Vol.	20mls	40mls	100mls
Agarose	0.2g	0.4g	1.0g
10xMOPS*	2mls	4mls	10mls
DEPC'd H_2O	18mls	36mls	90mls
In fume Cupboard:			
37% Formaldehyde	1ml	2mls	5mls

**10xMOPS – 41.8g MOPS (0.2M), 4.1g sodium acetate (50mM), 3.72g EDTA (10mM). pH was adjusted to 7.0 with 6M NaOH and made up to 1 litre with diH_2O. 1ml of DEPC was added and well mixed followed by on overnight incubation at room temperature. Next day the MOPS solution was autoclaved.*

Once gel has dissolved, it was poured into gel cast and allowed to cool and set in the fume cupboard. While gel was setting, gel tank was set up and 1xMOPS running buffer made by adding 25mls 10xMOPS to 225ml DEPC'd H_2O. Place gel apparatus into the gel tank and buffer added to cover/submerge the gel plate. Next the samples were prepared. 1µg of RNA was added to 1µl of gel loading dye (1mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol in DEPC'd H_2O), and heated to 65°C for 10 minutes. After the sample had been heat treated, 1µl of ethidium bromide was added. Sample was well mixed and spun down. Sample was loaded onto gel and run at 90V for between 40 and 60 minutes, or until the dye front had moved $^2/_3$rds the way towards the anode. The gel was viewed under a U.V. light and the result recorded with polariod film.

N.B. All RT-PCR enzymes must be kept on ice at all times. When not in use store at –20°C.

Once the RNA had been collected and checked, it was subjected to an RT step. To an eppendorff, the following was added,

RNA	1µg
5x Reaction Buffer	4µl
10mM dNTP's	2µl
Random Hexamers	3.2µg
RNasin	20U
M-Mul-RTase	20U
DEPC'd H_2O	to 20µl

The RT reaction was allowed to continue at 37°C for 1 hour. After 1hr, heating the sample to 95°C for 2 minutes stopped the reaction, by deactivating the RTase. The reaction was then chilled for 5 minutes on ice. After this 80µl of DEPC'd H_2O, to make a final volume of 100µl of cDNA.

Once the cDNA had been made it was subjected to a PCR step.

Master Mix		**Submix**	
50mM $MgCl_2$	= 1.5µl	10mM dNTP's	= 1µl
10x Enzyme buffer	= 5µl	Primers	= 45pmoles
diH_2O (to 50µl)	= 43.5µl	Master Mix (to 13µl)	
		For IL-10 primers; IL105P = 1µl, IL103P = 1.2µl.	
		For β-Actin primers; BACTINR = 1µl, BACTINF = 1µl.	

To an eppendorff, 13µl of submix was added with 1 paraffin wax pellet. This was heated to 95C for 2 minutes before being allowed to cool, this allowed the paraffin wax pellet to melt and cover the submix is a solid cover upon cooling. Once cooled, to each eppendorff 37µl of master mix, 5µl of cDNA and 1.6U of Taq polymerase was added.

Once the samples were prepared they were run in Hybaid Omnigene PCR machine with the following settings;

	IL-10	β-Actin
Stage 1	Heat to 94°C for 2 mins.	Heat to 94°C for 2 mins.
Stage 2	i) Heat to 94°C for 20 sec.	i) Heat to 94°C for 45 secs.
	ii) Heat to 68°C for 1 min.	ii) Heat to 46°C for 45 secs.
		iii) Heat to 72°C for 2 mins.
	Repeat for 35 cycles.	Repeat for 35 cycles.
Stage 3	i) Heat to 72°C for 10 mins.	i) Heat to 72°C for 7 mins.
	ii) 10°C overnight holding.	ii) 10°C overnight holding.

Next day, the PCR products were run out on a 1.5 Agarose gel (see 2.2.8) IL-10 products ran at approx. 450bp in size, while β-Actin ran at approx. 340bp in size. Results were recorded using Polaroid film.

Chapter 3

Development of an IL-10 Assay System

3.1 Aims and Introduction

The aim of this part of the project was to devise a quantitative assay system for determining the quantity of IL-10 and TNFα, which was produced by the macrophage in response to various stimulations. In order to determine the amount of IL-10 being produced by the monocytes, it was necessary to set-up an assay procedure which was sensitive, quick, cheap and reliable enough to read small changes in IL-10 production over large numbers of samples.

Many techniques were discussed, the first of which was RT-PCR, which is quick, relatively cheap and extremely sensitive. However this was discarded, as a viable assay system, since the presence of the messenger RNA, does not necessary indicate that IL-10 protein is present. For example, in one study[119], decidual cells in culture produced IL-10 in response to IL-1 beta, but chorion and amnion cells produced no IL-10 protein. *In vivo* protein expression by immunohistology showed that most protein was detected within decidual while cells within amnion and chorion rarely had detectable IL-10 protein. However, *in vivo* RT-PCR samples demonstrated the strongest IL-10 mRNA signal from decidual samples, with IL-10 mRNA also being noted in chorion and amnion of placentas obtained after preterm labour. Other methods were also discussed, e.g. ELSA Spots, but in the end it was decided that the best assay system to used was the sandwich ELISA technique.

3.1.1 Sandwich ELISA

ELISA is an immunohistochemical technique that uses the specificity of antibodies to recognise only one eptitope on the surface of an antigen.

Briefly, a primary monoclonal antibody is used to coat the surface of a 96 well plate. A monoclonal antibody is used since polyclonal may recognise epitopes, which are present on other molecules. Next a blocking agent, consisting of large proteins to prevent the adhesion of other molecules to the well wall is added. Once blocked, the samples of interest are added to the well, in this case IL-10 and TNFα were the proteins of interest, but any protein can be looked at so long as antibodies which recognise it are available. The samples are allowed to bind to the primary antibody. Next the plate is washed to remove any excess sample and a secondary polyclonal antibody is added. A polyclonal can be used here for several reasons. First, it will

recognise many more epitopes thus allowing a much greater amplification of signal. Secondary, since the primary monoclonal antibody has already captured the molecule of interest and the rest been washed away, there is little to no chance of non-specific binding. Third, polyclonal antibodies are cheaper and easier to produce.

The secondary antibody is usually labelled with Biotin (a protein which is found in egg white). To this two-antibody/protein complex Avidin or Streptavidin (Streptavidin is a modified and more specific version of Avidin)-HRP (Horseradish peroxidase) is added. The streptavidin will bind to the biotin in one of natures strongest bonds. Once bound then the chromogen is added. The chromogen contains both as H_2O_2 well as TMB. The HRP will break down the H_2O_2 into H_2O and O_2^-. The O_2^- will then interact with the TMB to give a blue colour change (see figure 3.1). The reaction is stopped and intensified by the addition of 2M sulphuric acid (Blue – Yellow) which can be read in a microplate reader.

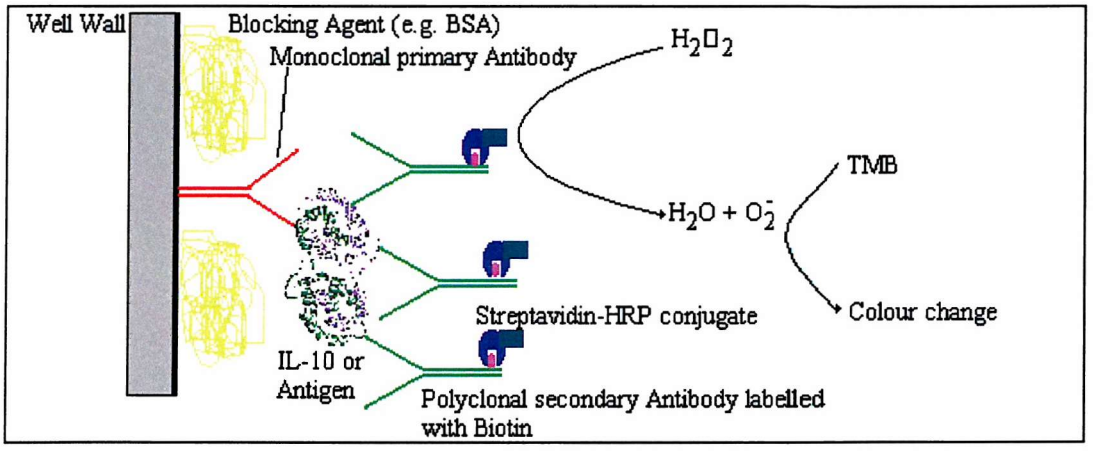


Figure 3.1 Sandwich ELISA

3.2 IL-10 ELISA

The first attempt at carrying out a human IL-10 ELISA involved using an anti-human IL-10 polyclonal antibody as the capture, or primary antibody and an anti-mouse IL-10 hybridoma (IgM) as the detection or secondary antibody. It was not a great surprise that this assay did not work. Although it is sometimes possible to calculate the amount of mouse cytokines using a human antibody, it is seldom possible, if ever, for the mouse antibodies to detect the human equivalent.

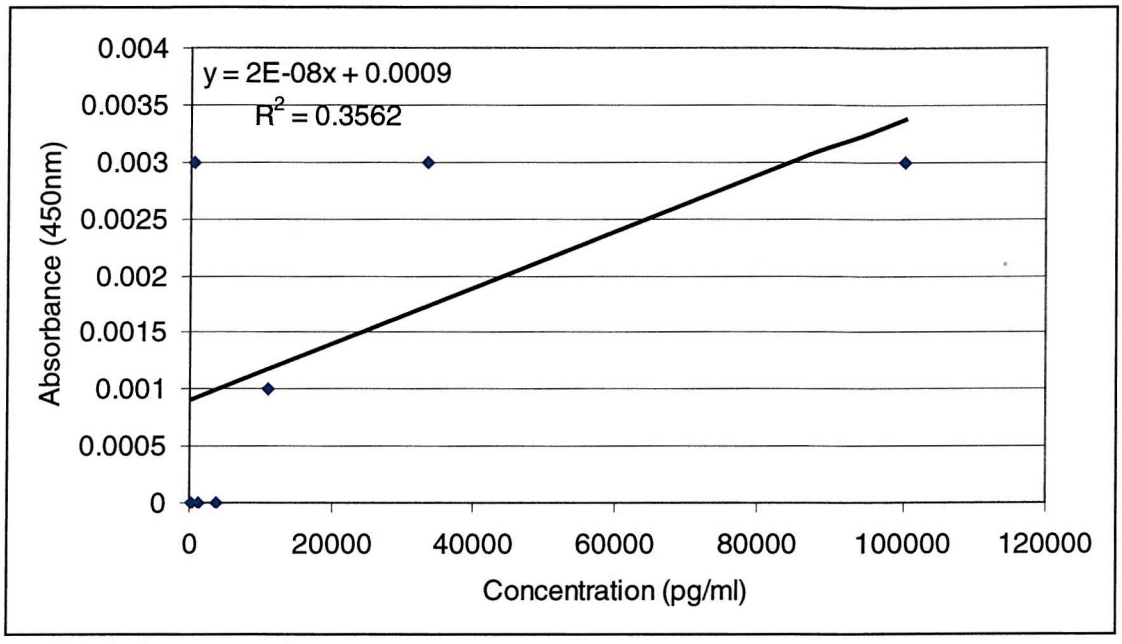


Figure 3.2 First attempt at building an IL-10 ELISA. Primary antibody was obtained from Genzyme and was added at a concentration of 10µg/ml. The secondary detection antibody was obtained from the cell line JES-2AE and was added neat to the assay.

For the second attempt, the anti mouse IL-10 hybridoma was replaced with a mouse anti-human IL-10 biotinylated polyclonal antibody which was matched to a primary capture antibody which were both obtained from R&D systems. For the third step, avidin-HRP was utilised.

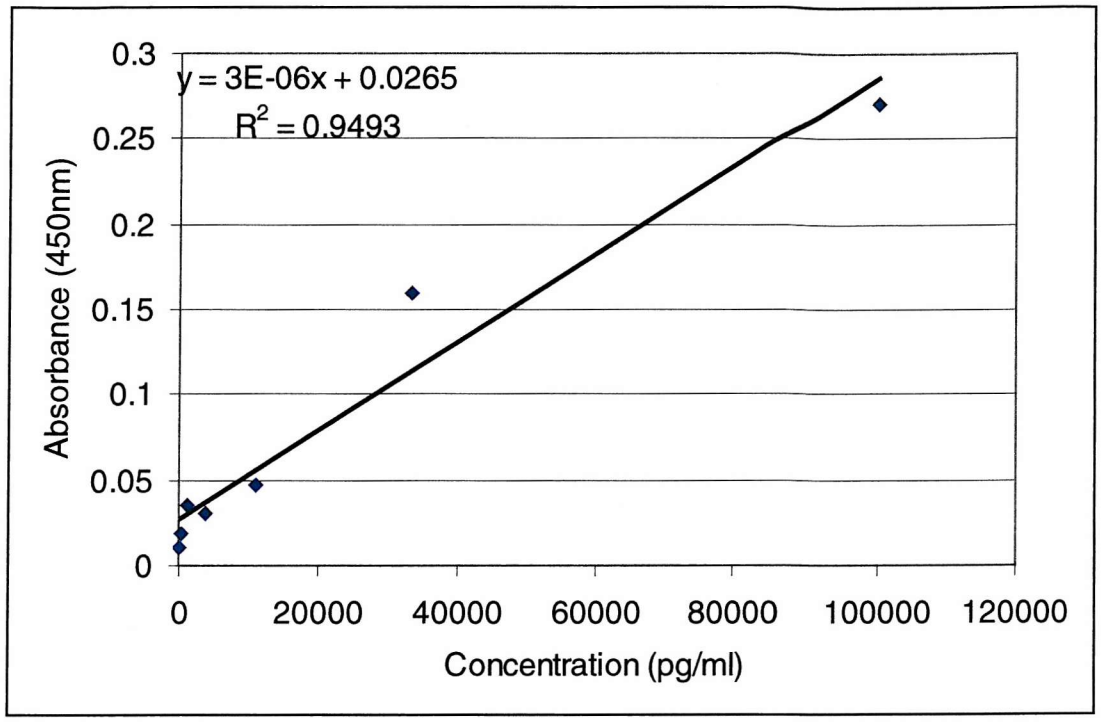


Figure 3.3 Second attempt at producing an IL-10 assay. As can be seen the matched antibodies gave a much better standard curve.

As can be seen, the use of proper anti-human antibodies has yielded a much more reliable standard curve (that is the correlation was closer to 1). However, the O.D. readings obtained were quite low. Therefore we set out to improve these readings.
On the third attempt, we changed several steps. First, the blocking agent used (5% BSA in PBS) was changed and a blocking solution containing 1% BSA, 5% Sucrose in PBS was utilised. Secondly, during the inoculation of the plate with the primary antibody, the solution in which the antibody was made up in was changed from a 0.1M carbonate-bicarbonate buffer (pH9) to PBS (pH 7.4).

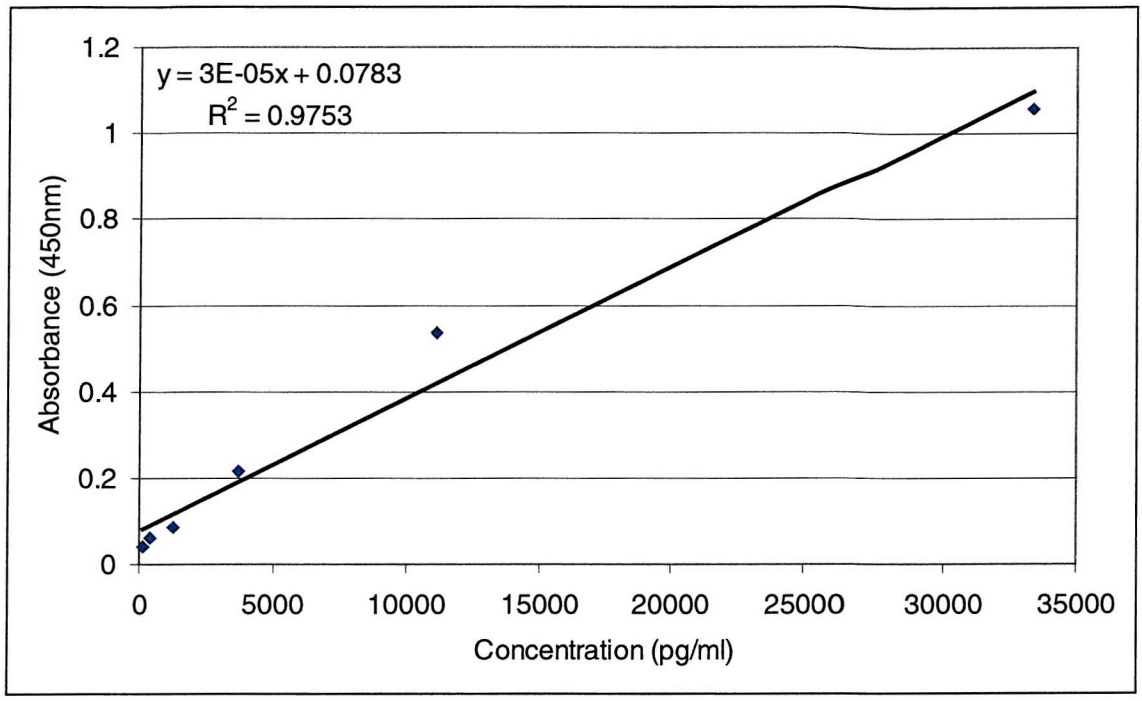


Figure 3.4 Third attempt at producing an IL-10 ELISA. By altering the buffers used in diluting the antibodies we were able to produce a much better O.D. reading for the detection of small changes in the IL-10 concentrations, also a much better standard curve was obtained. Primary antibody was added at a concentration of 10µg/ml. Secondary capture antibody was added at 0.5µg/ml. Avidin-HRP was added at a 1:1000 dilution.

As can be seen from the above data, the O.D. readings obtained were much higher than before. This gave a much better standard curve that could be used in determining the changes in IL-10 secretion in the macrophage after LPS stimulation. However, it was also found that the background for the ELISA assays was reading very high. To solve this each component was tested individually to see where the problem lay.

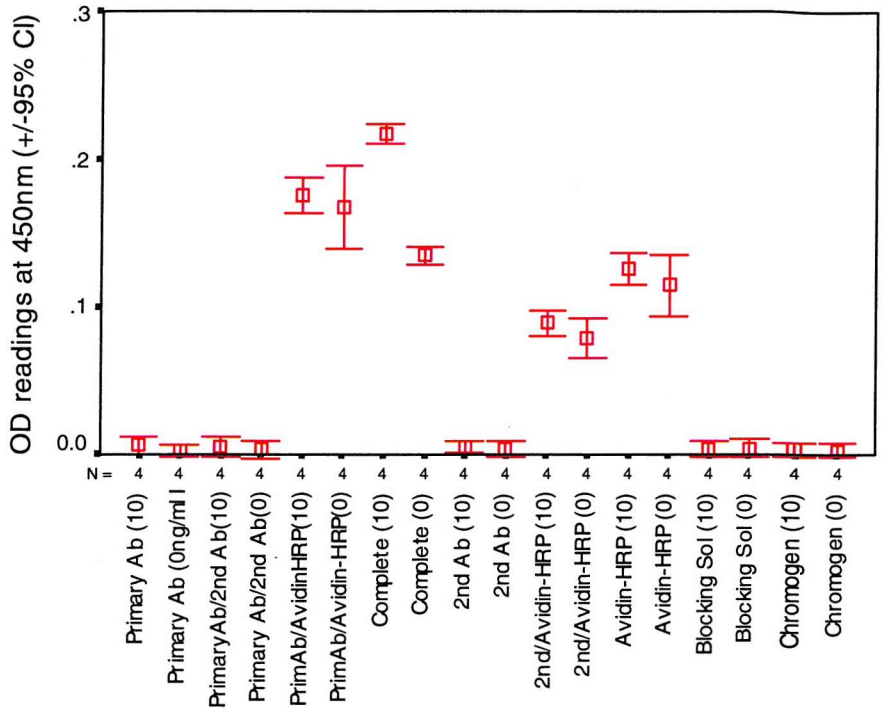


Figure 3.5 High background readings often made it difficult to determine the changes in low concentrations of IL-10. To try and determine which component was causing this high background each component was tested individually and in combinations with each other. For all wells, except where indicated, the wells were blocked with 1% BSA, 5% Sucrose in PBS. PrimAb = Primary Ab = Capture (monoclonal) Antibody, 2nd = Secondary Ab = Detection (polyclonal) Antibody, Avidin-Hrp = Avidin Horseradish Peroxidase, Complete = Complete ELISA Test.

10 = 10ng/ml rIL-10

0 = 0ng/ml rIl10

From the above readout, it can quickly be seen that the problem lay in the use of the avidin-HRP conjugate. The most likely reason for this high reading is that the avidin-HRP was interacting, non-specifically with the blocking solution. To solve this problem, the avidin-HRP was changed for the more specific Streptavidin-HRP.

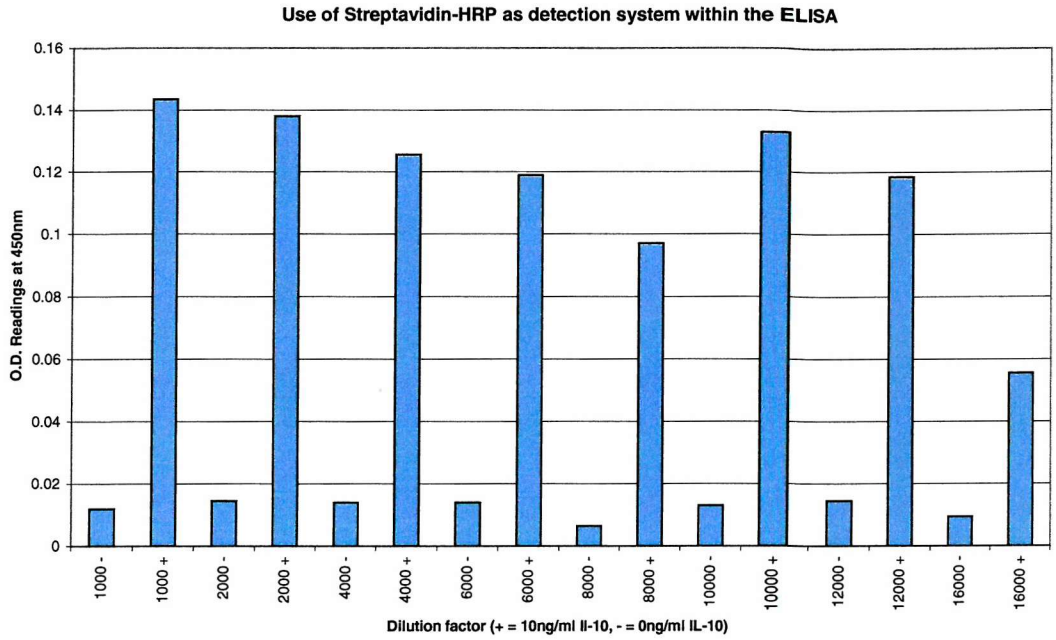


Figure 3.6 Using Streptavidin-HRP instead of Avidin-HRP as assay detection system. Streptavidin-HRP was used to detect (+) 10ng/ml rhIL-10 or (-) 0ng/ml rIL-10.

As can be seen, the Streptavidin reduce the background readings to almost zero. The experiment also showed that the streptavidin-HRP responded dose dependently in the assay. Now that a working assay had been developed, it was necessary to optimise the capture and detection antibody concentrations as well as test the efficiency and accuracy of the ELISA. Since the Streptavidin-HRP had already been worked out at 1:2000 dilution only the optimum concentration for the primary and secondary antibodies were needed.

Using a simple antibody dilution experiment the primary capture and the secondary detection antibodies were diluted down in PBS before undergoing an ELISA assay. The assay for the primary antibody used a secondary antibody dilution of 1:1250 (=0.4µg/ml). For both experiments IL-10 was added at 1ng/ml.

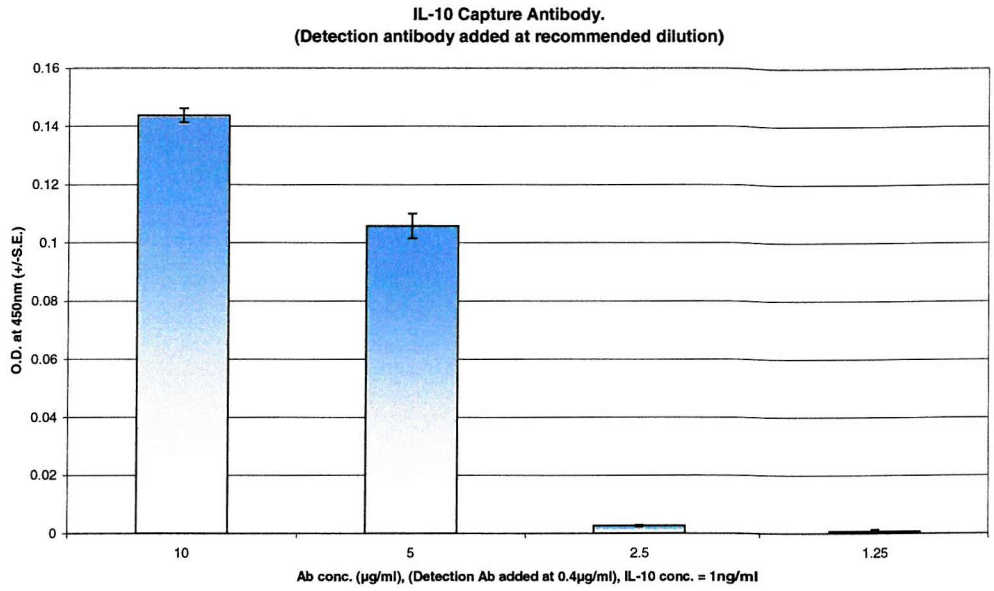


Figure 3.7 Primary Antibody dilution. Il-10 was added at 1ng/ml. Graph points +/- S.E.

Using the recommended dilution of the secondary antibody with a 1:2000 dilution of the streptavidin-HRP, the concentration of primary antibody was worked out to be 5µg/ml. Following the same protocol for the secondary antibody, the concentration of secondary antibody was worked out to be 0.27µg/ml.

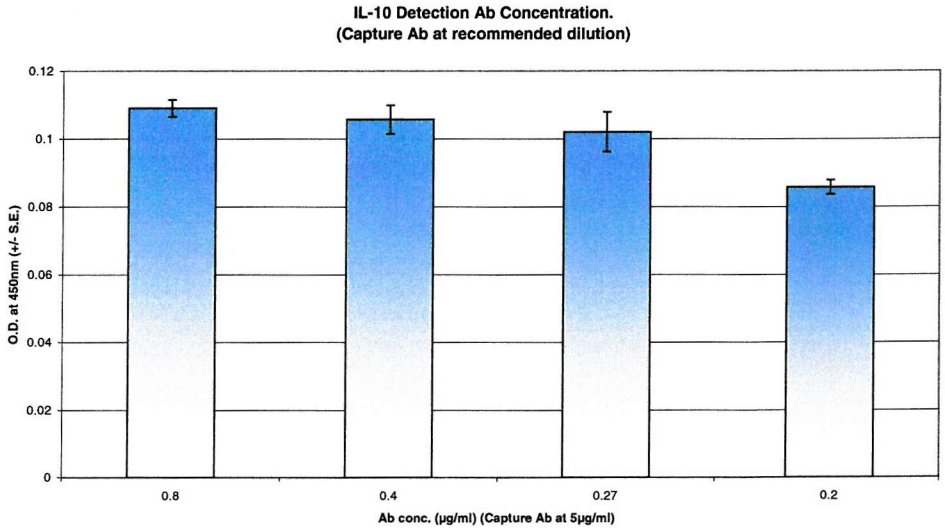


Figure 3.8 Secondary antibody dilution curve. Primary antibody added at 5µg/ml and Streptavidin-HRP was added at 1:2000 dilution. Plot +/- S.E.

Now that the ELISA had been optimised, it was necessary to test the system. It was necessary to test the system for several reasons, first, to show the accuracy of the assay. Second, it would show that the assay was not being saturated and that the actual results obtained reflected that of the actual cell environment after stimulation, and finally, it would show that there was no proteolysis occurring within the supernatants.

In the first experiment carried out, the supernatant of stimulated cells was spiked with 500pg/ml rIL-10, and then diluted 1 in 3.

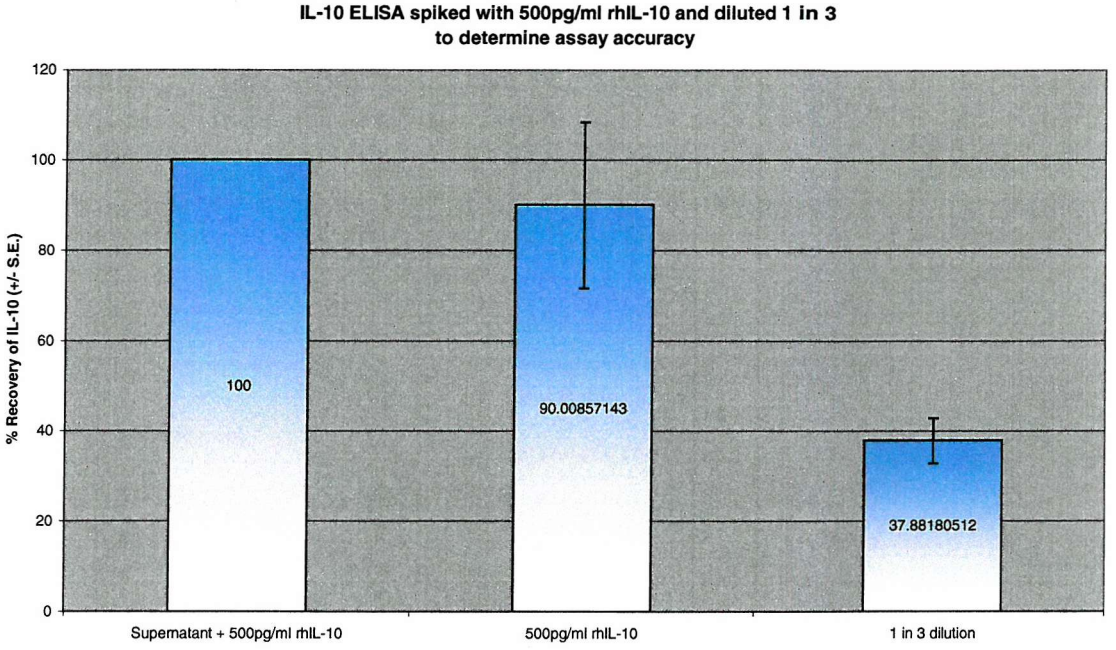


Figure 3.9 Supernatant from stimulated macrophage was spiked with 500pg/ml rIL-10. Before being assayed some of the spiked supernatant was diluted 1 in 3 with fresh media containing no IL-10. Results indicate the percentage of IL-10 being detected. Results plotted show +/- S.E.

As can be seen from the above graph the assay detected an acceptable 90%+ of the IL-10 concentration within the supernatant. This result indicated that no break down of the IL-10 protein was being observed during the assay procedure by proteolysis. By diluting the supernatant 1 in 3 and detecting 37% of the total recoverable IL-10, the experiment also showed that the assay is not being saturated with protein and that all the IL-10 in the supernatant is being detected by the assay.

The next experiment was set up to determine the accuracy of the ELISA. Here the supernatant was diluted in parallel with the standard curve (that is 1 in 3, 9, 27...etc.)

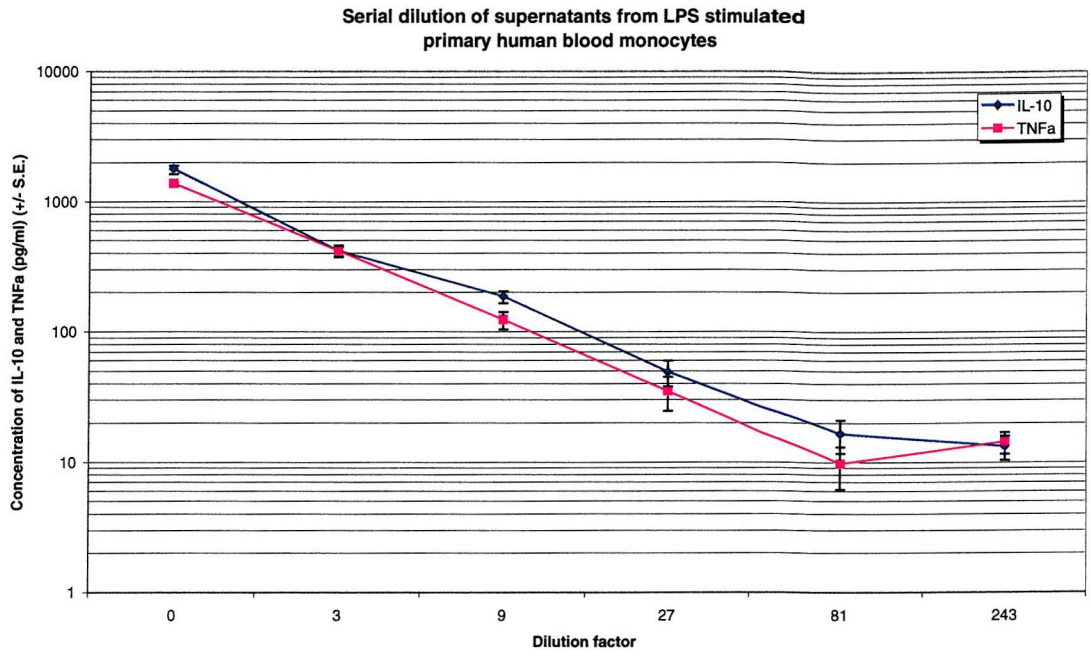


Figure 3.10 Primary human monocytes were stimulated with 100μg/ml LPS for 24hrs, before the supernatant was collected and an ELISA performed. Supernatant was diluted down in fresh RPMI media containing no IL-10 or TNFα.

In order to determine if the assay could be used to measure the IL-10 production in biological systems, it had to be determined if the assay was accurate enough. This was accomplished by diluting the supernatant from stimulated monocytes to determine if they diluted in parallel to the standard curve and give a straight-line plot. As can be seen from the above results, this was achieved indicating that the assay was accurate enough to be utilised in future experiments.

3.3 Discussion

In order to determine if a cell line model of primary human monocytes could be developed, it will first be necessary to determine what the basic cell response of the macrophage to LPS is. To this end an assay system which was sensitive, easy to do, quick and robust had to be developed. To this end the ELISA was used. From the above data, it can clearly be seen that such an assay system had now been achieved. Using antibody dilutions and various buffers the assay was developed. Using Streptavidin-HRP over Avidin-HRP, the background readings were reduced, thus allowing a more sensitive assay that could measure even small changes in IL-10

secretion. Using various experiments we were able to show that the ELISA was able to detect all the IL-10 present in the sample accurately, without saturating the antibodies, as well as showing that the IL-10 does not undergo any proteolysis.

Similar experiments were carried out in the production of the TNFα ELISA. However, rather than simply repeat the above results this data has not been included.

Now that working assay systems for IL-10 and TNFα had been developed, it could be utilised in determining the response of the primary monocyte as well as the monocyte cell-lines to LPS, as well as the effect of pharmacological inhibitors of the second messenger pathways on IL-10 production in these cells.

Chapter 4

IL-10 Profile to LPS stimulation

4.1 Introduction and Aims

Now that an assay had been developed which could look at both IL-10 and TNFα, it was utilised to examine the IL-10 profile of the macrophage after LPS stimulation.
As mentioned in the introduction, the overall aim of this project was to develop a monocyte cell line model, which could be used to study the expression of IL-10 in the macrophage during inflammation at the transcription level. For this to work, the cell line would have to fill three criteria. The first would be to express IL-10 in response to LPS. The second, would be, that the expression of IL-10 in response to LPS, within the cell line, follows the same second messenger pathways that are activated in the primary cell. The final criteria that need to be fulfilled is that the cell line is that it is able to undergo transfection.
In this part of the study, we aim to look at the IL-10 profile, in response to LPS, in the primary monocytes and various cells lines, in order to determine if such a cell line exists.

4.2 IL-10 response to an LPS dose curve

The first objective was to confirm that the primary human blood monocytes and different monocyte cell lines actually produced IL-10 in response to LPS stimulation. For this a simple LPS dose curve was used.

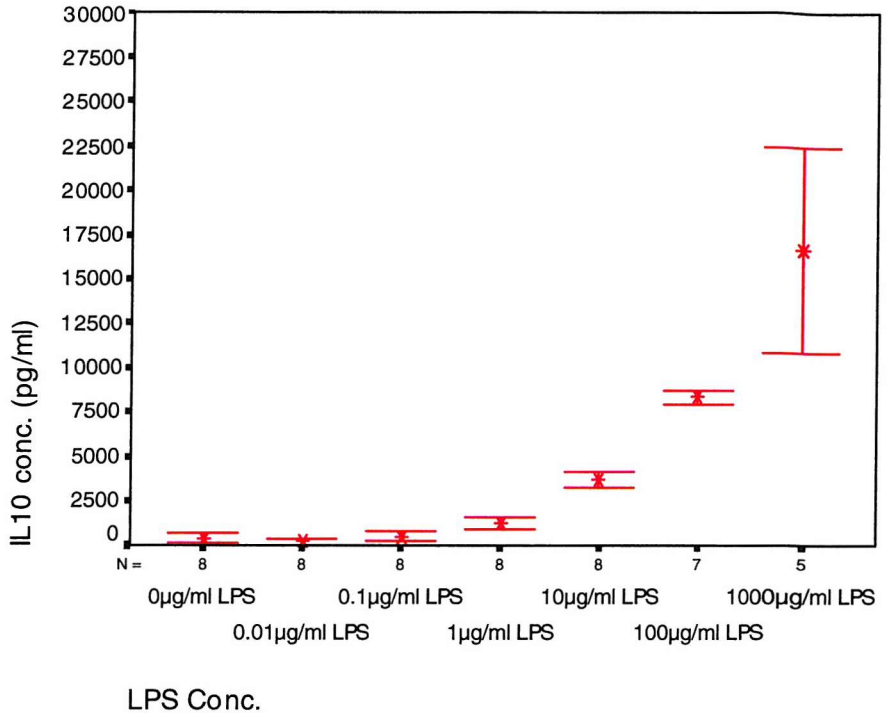


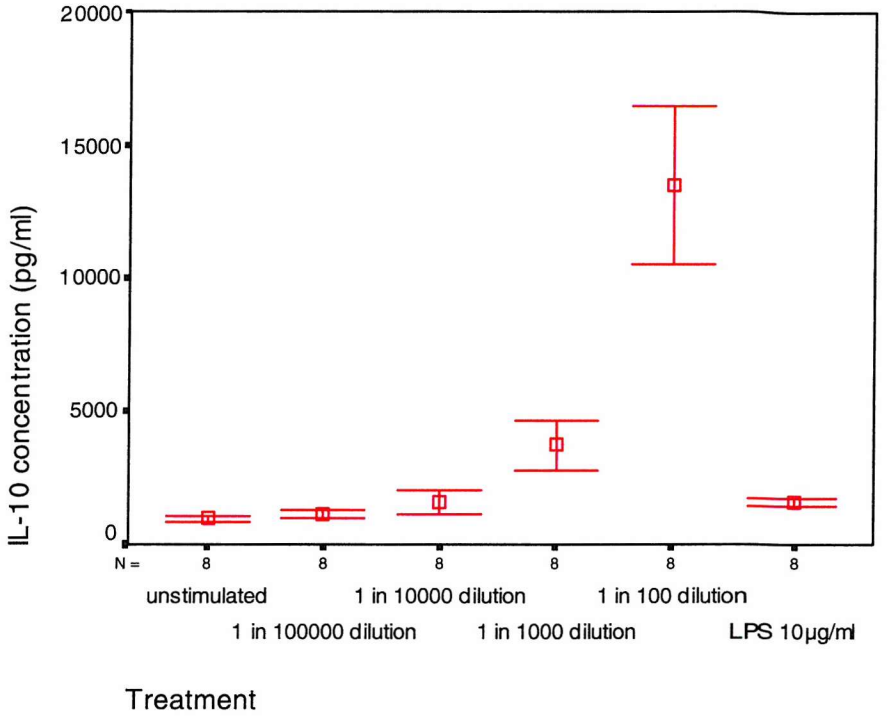
Figure 4.1 LPS dose curve on primary human blood monocytes. Mean +/- SE.

From the above data two observation can be seen. First, a significant change in IL-10 secretion in the primary is not seen until 1µg/ml of LPS is used. This is unusual since generally macrophage will respond at much lower concentrations, indeed it has been well established that TNFα reaches a peak at a much lower concentration, around 0.1µg/ml (data not shown). However, even at a low level of LPS stimulation an increase in IL-10 levels is observed (base line levels, from isolated primary monocytes, were recorded at 290pg/ml which rise to 486pg/ml with 0.1µg/ml LPS stimulation). Since IL-10 is such a potent cytokine and so tightly regulated, it is very possible that this small increase in IL-10 concentration is all that is required to control the inflammation response. It is interesting to note here, that normally IL-10 is not secreted from the cell in an inactive state, however the action of isolating the cells from whole blood and plating them out onto tissue culture plastic, does lead to an activation of the cells. For this reason, all cells used in the primary cell experiments were used as close as possible to the isolation event to reduce this activation. Also this low level of activation from primary cells after isolation, may be what is responsible for an artificially high concentration of LPS being required for a statistically significant change in IL-10 secretion.

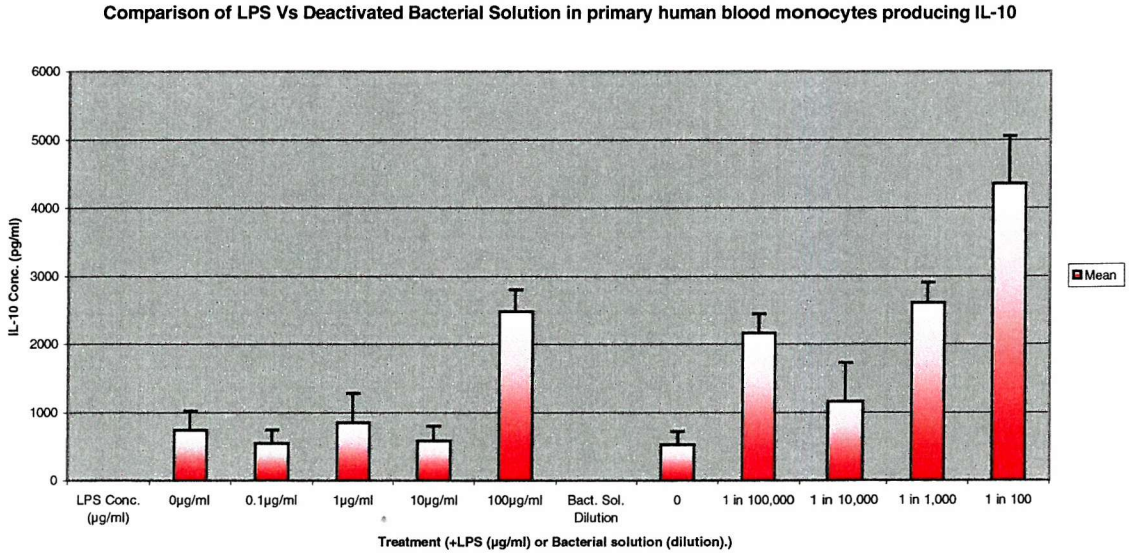
The second point to note is that, unlike other cytokines, IL-10 does not seem to reach a peak, with IL-10 still being produced far beyond physiological relevant LPS concentrations. However, high levels of endotoxin can be found in patients which have undergone gram-negative sepsis (septic shock). Sepsis is caused by a release into the circulation of endotoxins from invading bacteria.

Septic shock occurs as a result of an individual patient's systemic, immunological response to sepsis and is characterised by a drop in arterial blood pressure, which remains too low to maintain an adequate supply of blood to the tissues. In healthy individuals, the immune response protects the body from invading pathogens. However, critically ill patients are frequently immunocomprimised and have many invasive procedures performed which allow sites of entry into the body for invading bacteria. Another factor, which could lead to this sepsis, is the response if IL-10 to the inflammation response naturally seen from a site of injury. If IL-10 was already present suppressing the inflammatory response, then the body would not be able to keep the invading pathogens under control since the immune system would also be suppressed. This suppression would allow the pathogens to grow and multiply allow sepsis to continue, which in turn would lead to a greater release of IL-10, which in turn would lead to a further suppression of the immune response which leads to septic shock.

With such high levels of IL-10 being observed, it was decided to see if an upper limit of IL-10 production could be reached. For this, *E.coli* (serotype DH5α) was grown and deactivated by repeated Freeze/Thaw cycles (liquid nitrogen/42°C three times) followed by a final freeze at -20°C. This deactivated bacterial solution was then added to the primary cells for 24hrs to see if the native LPS, mixed with other proteins and DNA, could lead to even greater levels of IL-10 production.



4.2 Effect of deactivated E. coli *on the IL-10 secretion of primary human blood monocytes after 24hrs stimulation. Mean +/- SE*



4.3 Comparison of the effect of deactivated bacterial solution versus the effect of purified LPS. Mean +/- SE

As can be seen, although very high levels of IL-10 are being produced be the macrophage there still appears to be no upper limit to the amount of IL-10 being produced in response to either the purified LPS or the deactivated *E. coli* cells. Indeed the deactivated bacterial solution gives a much higher response of IL-10 than

the LPS. This was most likely due to the presence of other proteins, lipids and naked DNA/RNA from the bacteria, of which the degradation of is one of the primary functions of the macrophage. This effect also supports the septic shock theory, since in the septic shock response, the entire bacteria would be involved, not just the cell wall components.

4.3 IL-10 response of monocytic cell lines to LPS

Once the IL-10 profile for the primary cells had been mapped, we could move on to look at the monocyte cell lines and try and map their response to LPS. The first cell line chosen was the murine RAW 267.4.

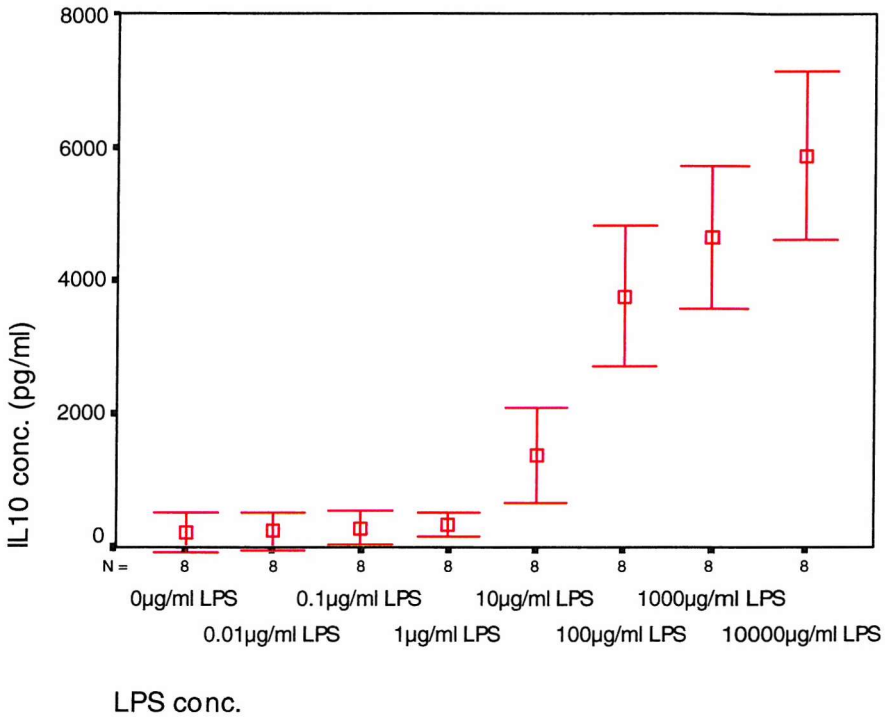


Figure 4.4 Effect of an LPS dose response curve on the IL-10 production in the mouse cell line RAW 267.4. Mean +/- SE

As can be seen, this cell line gave a good response to LPS and from earlier experiments (From Dr. T. Biggs, data not shown) that the RAW 267.4 were transfectable. However, this cell line was not the cell line of choice since it was mouse and not human, although it was decided that it would be a good control cell line for later experiments. It was decided to continue with the LPS experiments to

find a human monocyte cell line that was LPS responsive. The first cell line tested was the MonoMac6 cell line.

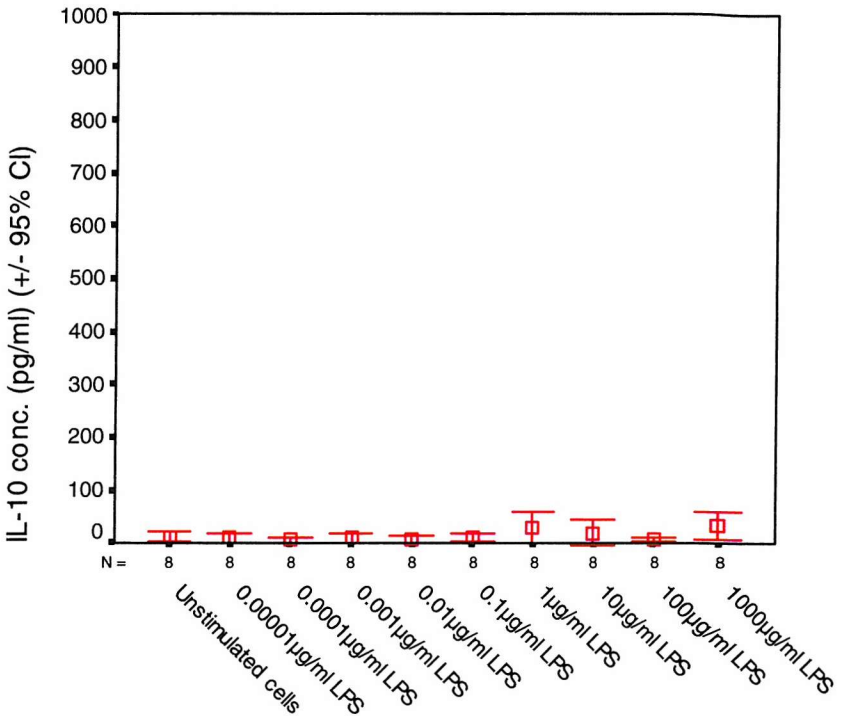


*Figure 4.5 LPS (*E.coli *serotype B055:55) dose curve on the Il-10 response of the MonoMac6 human monocyte cell line. (+/- 95% confidence levels)*

From the above graph it can easily bee seen that the MonoMac6 cell line was non responsive to the *E. coli* serotype B055:55 LPS, in that it failed to produced any IL-10. A second experiment was set up using a *Salmonella enteritidis* species LPS.

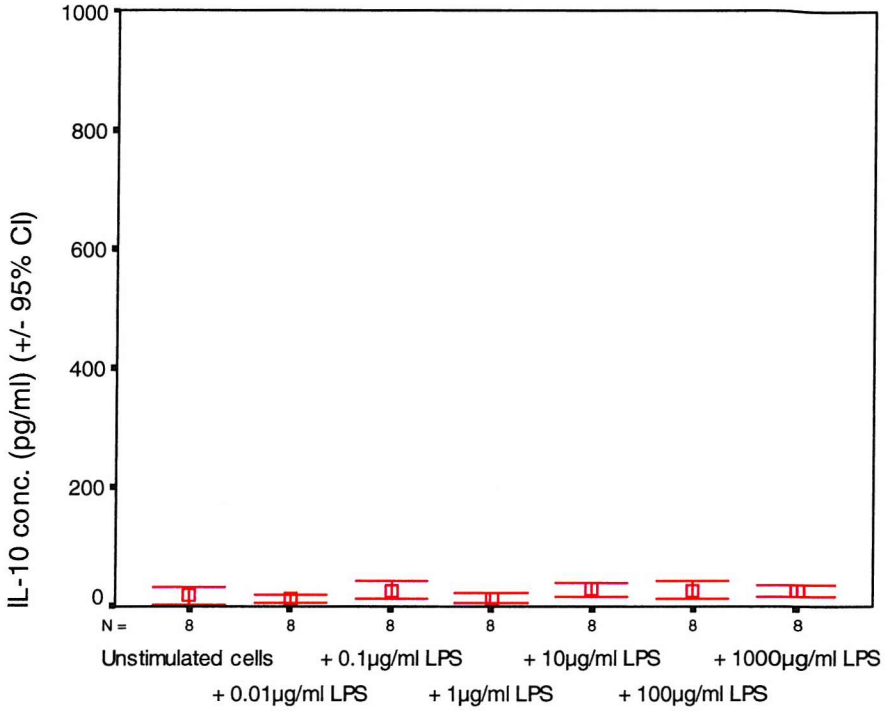


*Figure 4.6 LPS (*Salmonella enteritidis *species) dose curve on the IL-10 response of the MonoMac6 human monocyte cell line. (+/- 95% confidence levels)*

Once again, it can be seen that the MonoMac6 cell line did not produce IL-10 in response to the LPS.

With the MonoMac6 cell line failing to respond to LPS, the HL-60 and U937 cell lines were tested next. (For all experiments which follow only the *E. coli.* Serotype B055:55 LPS was used)

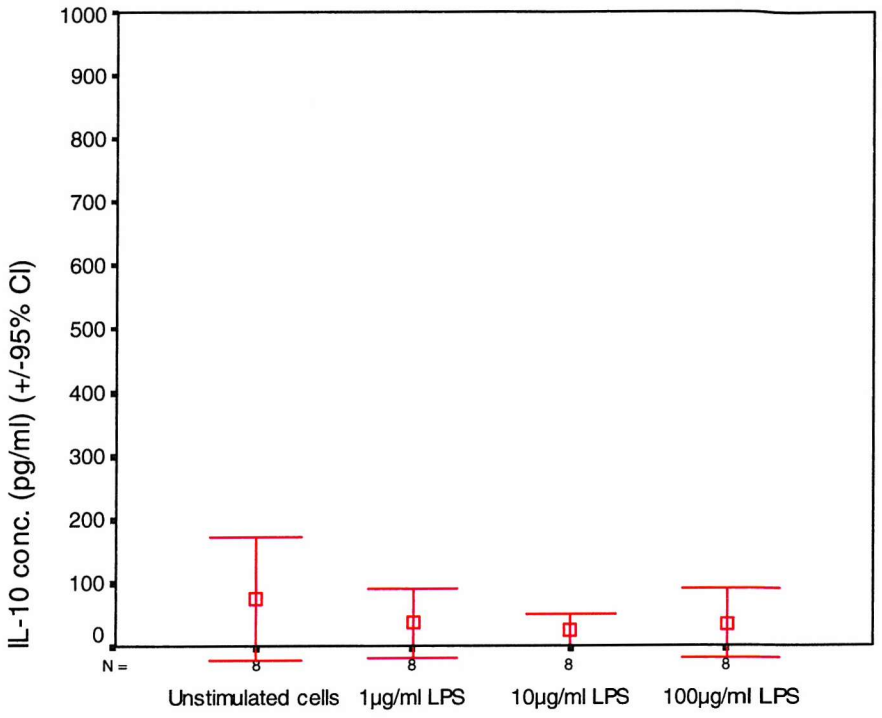


Figure 4.7 LPS dose response of the HL-60 cell line. Mean +/- 95% confidence levels.

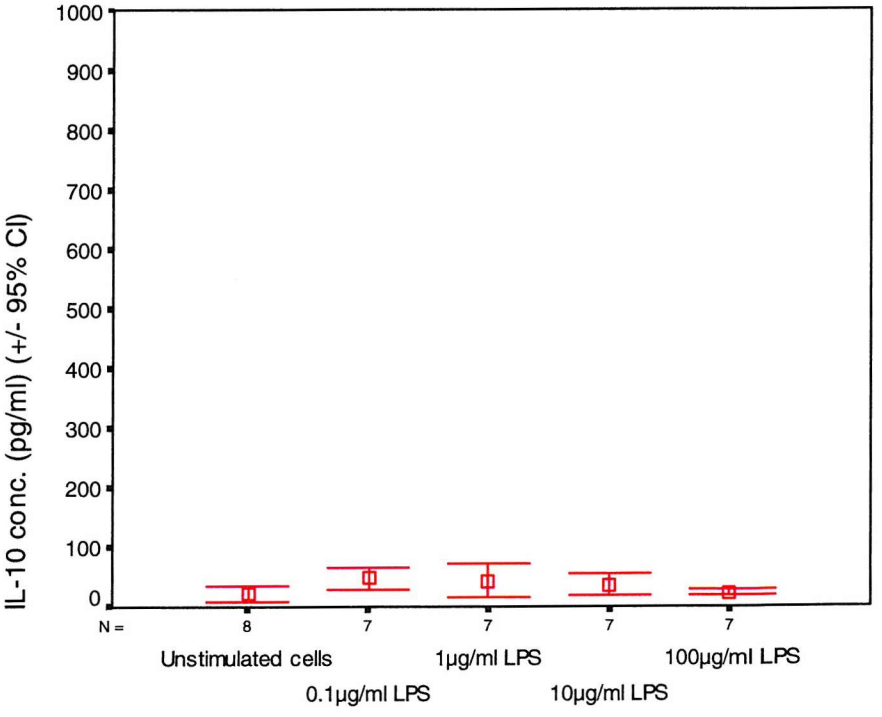


Figure 4.8 LPS dose response of the U937 cell line. Mean +/- 95% confidence levels.

As can be seen for both the HL-60 and U937 cell line neither of the cell lines produced IL-10 in response to LPS. The last cell line, which we tried, was the THP-1 cell line.

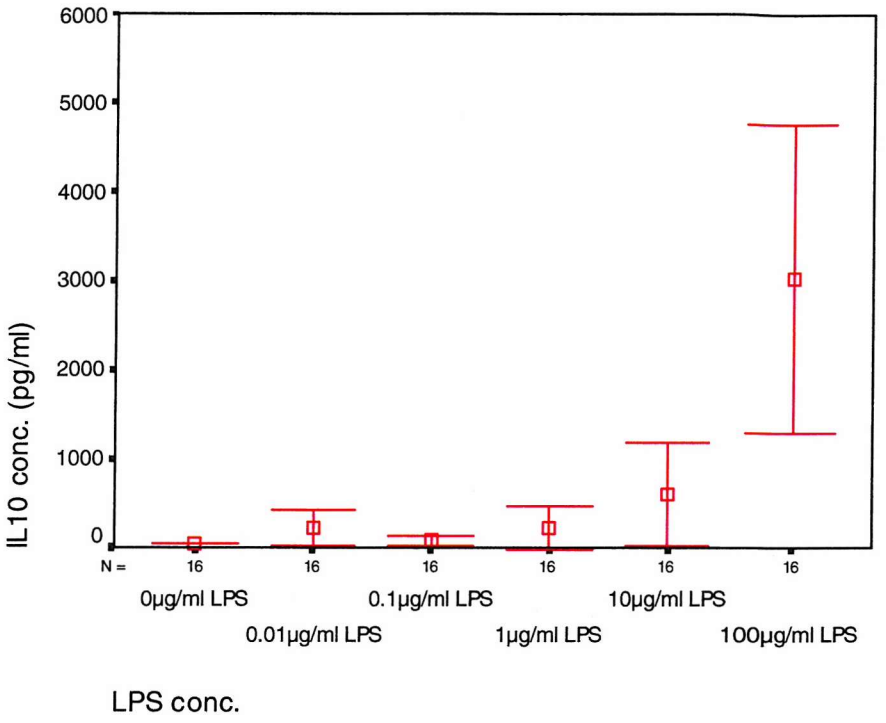


Figure 4.9 LPS dose response of THP-1 cells. Cells were stimulated with LPS for 24hrs. Mean +/- SE

From the above data, it can clearly be seen that the THP-1 cell line did give a response to LPS and showed a similar response to the high levels of LPS. However, it can also be seen that although a similar response to LPS is observed it did not reach equivalent concentrations as that of the primary cells.

Another difference, which can be observed, is that in the THP-1 cell line the base line for IL-10 synthesis is much lower than that of the primary cells. This is most likely due the fact that many of the primary cells will be activated by the isolation procedure that they go through while the cell line does not have this 'extra' activation step.

Now that a cell line had been found we carried on the work to try and develop a method of transfection that would work for this cell line. We eventually succeeded in developing a method (see chapter 7) which worked for the positive control and were ready to begin work with the actual IL-10 promoter constructs when the THP-1 cell line became infected with a bacterial infection. Since there were many frozen vials in

liquid nitrogen storage, a new batch was grown up. However, we found that despite this the cell line once more became infected with a bacterial infection. After two further tries, we eventually discovered that the water-bath used to defrost the additions to the media was the source of the infections.

Unfortunately, when we grew up a new batch of THP-1 cells, we discovered that they were no longer producing IL-10 in response to LPS. When a new batch of cells was bought from the ECACC and grown up, again these cells were found to be unresponsive to the LPS. At this point we decided to try and visualise the mRNA by running an RT-PCR.

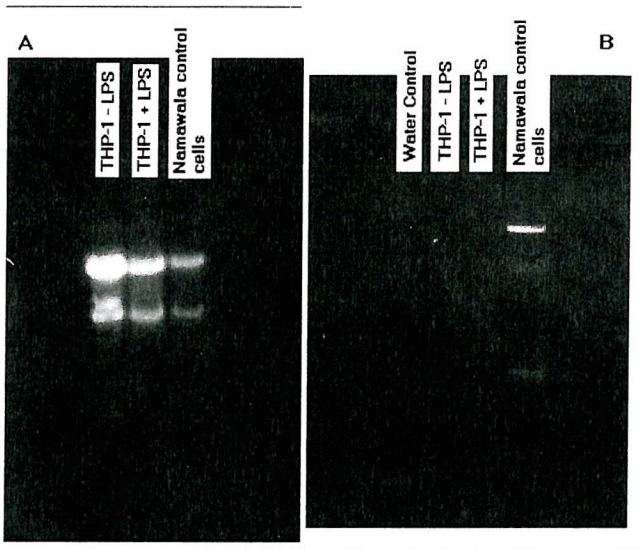


Figure 4.10 RT-PCR carried out on THP-1 RNA collected via the Qiagen RNeasy kit. A) shows the RNA integrity gel, B) shows the PCR gel, run after the RT-PCR had been completed. As can be seen from the above gel, no IL-10 band can be detected by PCR.

The reason for this apparent paradox regarding the THP-1 cell line could be one of several. The first was due to the method of freezing used in the long-term storage of the cells. According to the ECACC, the correct storage solution should contain Glycerol. However, upon investigation it was found that the THP-1 cells that we had received were frozen down in DMSO. In order to test this we stimulated the THP-1 cell line with both DMSO (at 0.2%) and PMA, a classical monocyte activator (at 400ng/ml). The results showed that the DMSO and PMA had little to no effect on the IL-10 secretion in the THP-1 cell line.

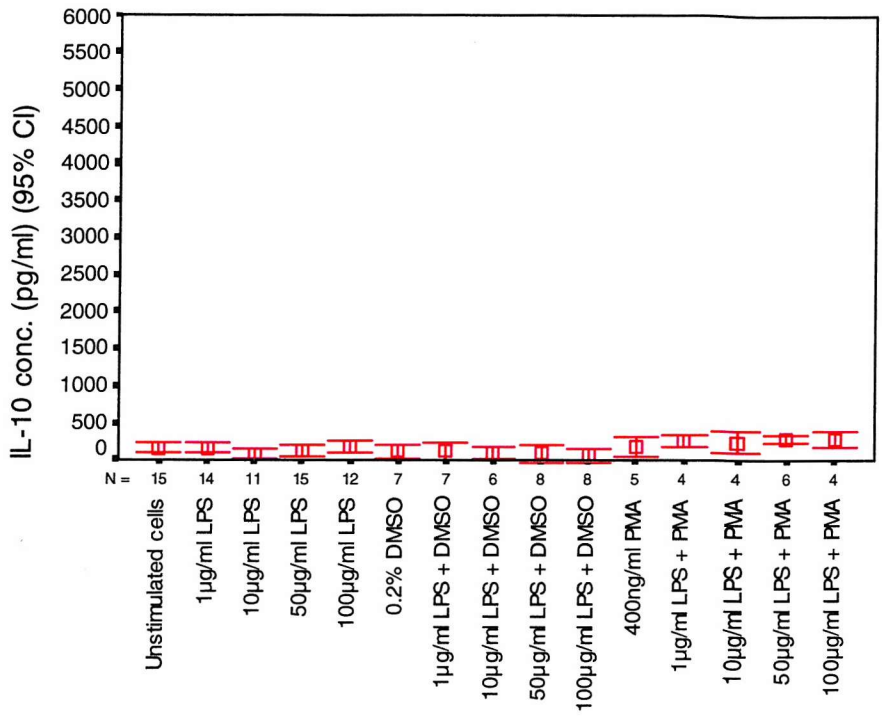


Figure 4.11 The effect of DMSO and PMA pre-stimulation on the THP-1 cell line followed by LPS stimulation for 24hrs. +/- 95% confidence levels.

The second reason could have been a mycoplasm infection. But since all the samples of the THP-1 cells had been lost to a bacterial infection, we were unable to test this. The final reason, and possibly the most believable, was that somewhere during the storage/freezing/cultivation of the cell line it had become contaminated with a cell type which was LPS responsive and which produced IL-10. The THP-1 cell line we had received at the start of the project had been used routinely with the T-cell line, Jurkat, as well as the murine RAW 267.4 cell line.

Now that we could no longer use the THP-1 cell line, we decided to go back to the earlier cell lines and try and pre-differentiate these to see if any would now produce IL-10 in response to IL-10. The first cell line tested was the HL-60.

The HL-60 cell line can be differentiated into either a macrophage like cell, with the addition of PMA, or a neutrophil like cell, with the addition of DMSO. To this end the HL-60 was pre-treated with 400ng/ml PMA for 3 days followed by an LPS challenge for 24hrs.

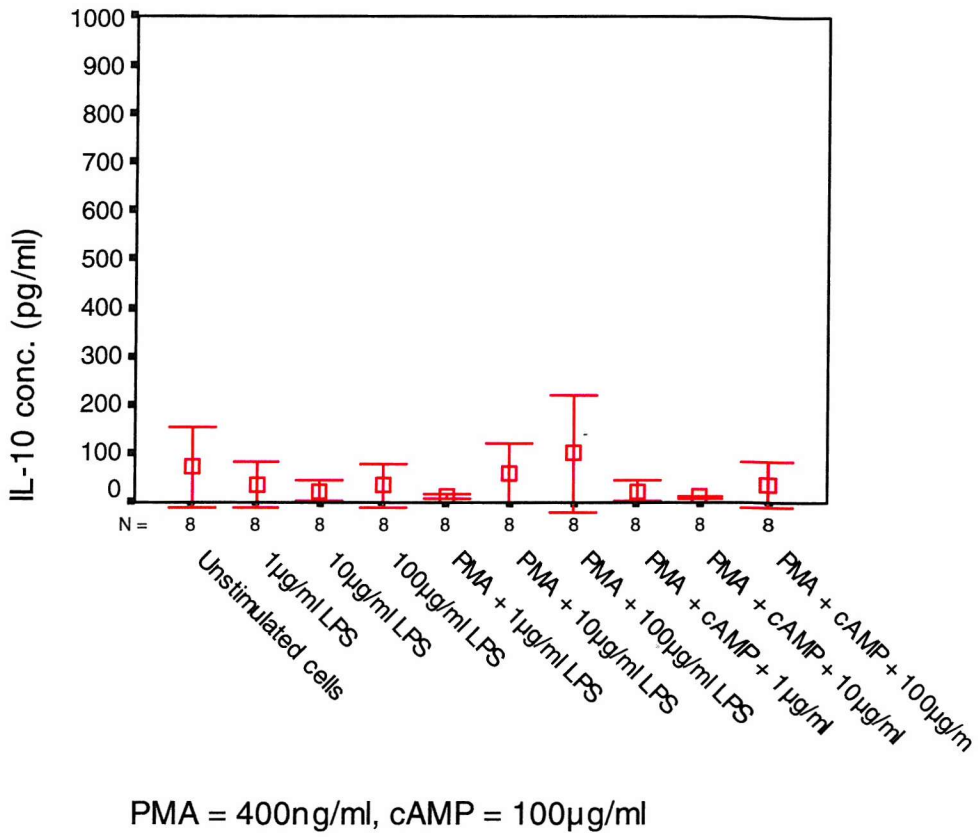
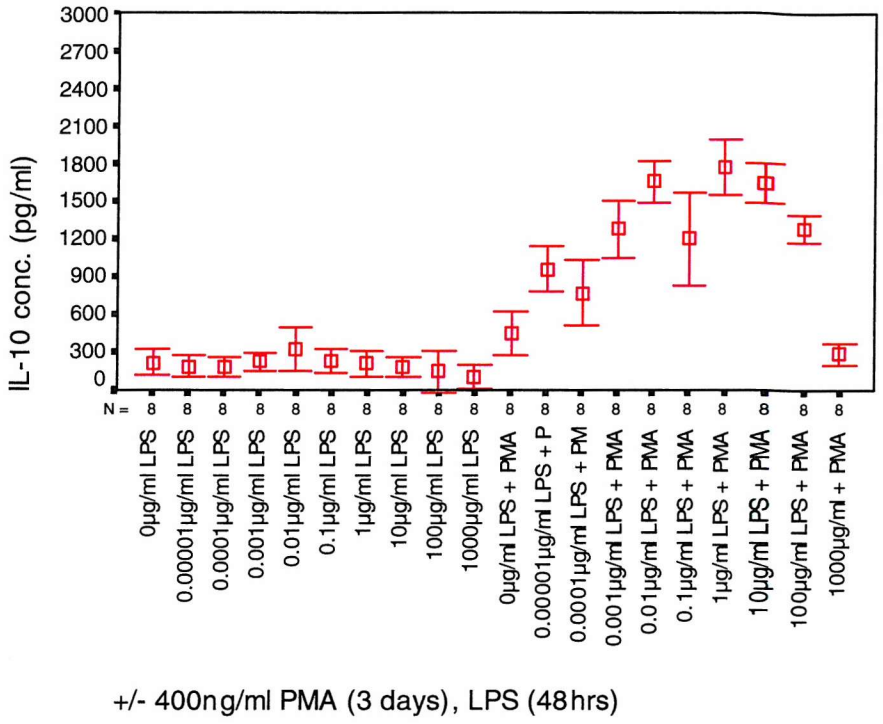


Figure 4.12 The effect of pre-treatment with PMA and cAMP on the monocyte cell line HL-60, followed by 24hrs LPS challenge. +/- 95% confidence levels.

As can be seen, the pre-treatment with PMA had little effect upon the IL-10 response of this cell line is response to LPS. The next cell line we tried was the U937 cell line. Here the cell line was once again treated with 400ng/ml PMA for 3 days followed by 48hrs LPS challenge.



4.13 After PMA pre-treatment the U937 cell line begins to secrete IL-10 in response to an LPS challenge. +/- 95% confidence levels.

To confirm this result an RT-PCR was carried out on the collected RNA.

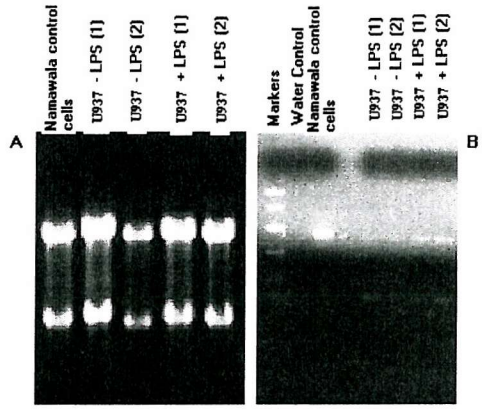


Figure 4.14 RT-PCR gel carried out on PMA pre-treated U937, after LPS challenge, RNA was collected via the Qiagen RNeasy kit and subjected to the RT-PCR as previously described. A) Shows the RNA integrity gel, while B) shows the PCR gel. IL-10 bands can clearly be seen in U937 cells that have had LPS stimulation. Cells without LPS stimulation show no bands.

From the above gel, it can clearly be seen that mRNA for IL-10 can easily be detected by RT-PCR.

4.4 Discussion

From the experimental data obtained it can be seen that the primary human blood monocyte respond does dependently to LPS, in their production of IL-10 and TNFα. It was also observed for the first time in this study the high levels of IL-10 produced in response to non-biologically relevant levels of LPS or deactivated bacterial cells. It is hypothesised that these high levels of IL-10 may be responsible to the condition of septic shock observed in many immunocompirmised patients.

The failure of many of the cell line to produce IL-10 in response to endotoxin challenge, with or without pre-treatment, came as a surprise at first after the initial success of the primary monocytes and the RAW 267.4. However, if thought about this lack of an IL-10 response is, perhaps, not quite as surprising. As mentioned in chapter one, the role of IL-10 in inflammation is quite simply to shut the macrophage down. Since the monocyte cell lines are all mutant cells which stem from cancer cells, it is not surprising that perhaps one of the ways in which these cells are able to grow, within the hostile environment of the body, is that the mechanisms which shut the cell down are switched off. This, of course, would include IL-10. This would answer the question of why the cell lines are not producing IL-10 in response to LPS, even, in most cases, with pre-treatment with the phorbol ester PMA. PMA acts upon the cell where they are potent activators of protein kinase C, were they structurally resemble diacylglycerol.

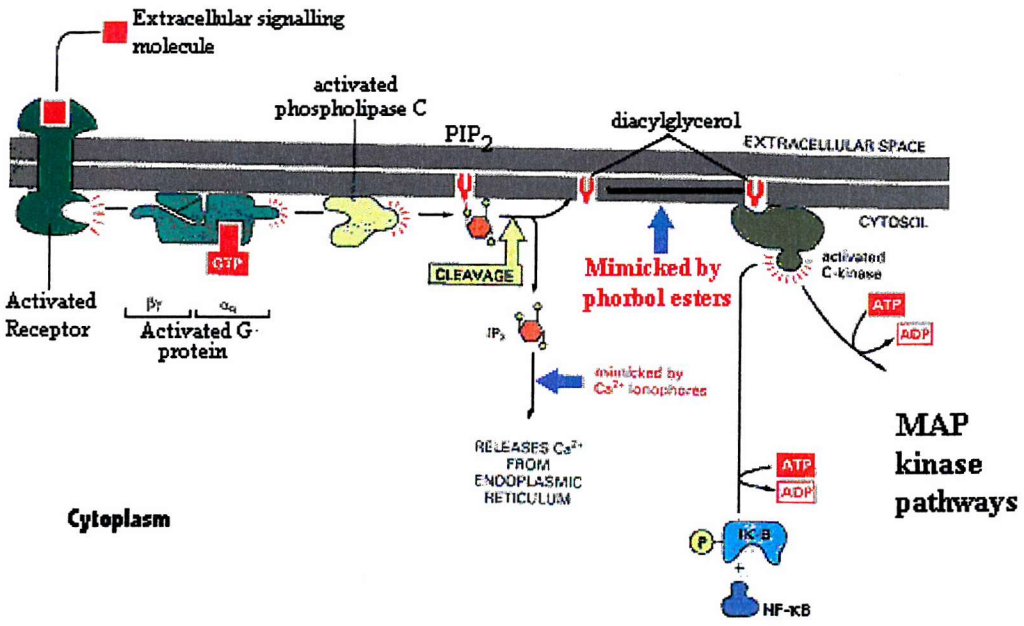


Figure 4.15 The binding of the extracellular signal to the receptor activates the phospholipase C, through the intermediary of a G protein. The phospholipase C then catalyses the hydrolysis of PIP_2 to IP_3 and diacylglycerol. Due to its structural similarities with diacylglycerol, phorbol esters, like PMA, are able to associate with the membrane where they activate the protein kinase C, which thereby modulate the activities of a number of cellular processes.

The early experiments that showed the production of LPS by the THP-1 cells did cause some problems later when the transfection experiments were started, due to lack of time. Fortunately, many of set up experiments used in setting up the transfection process, were able to be modified for the U937 cell line which allowed us to make up some of the time lost. See chapter 8 for further details.

The aim of this part of the project was to find a cell line, which would mimic the IL-10 response of the primary cell in response to an LPS challenge. This was achieved in two cell lines. The first was the murine RAW 267.4 cell line and the second was the PMA differentiated U937 human monocytic cell line. The RAW 267.4 cell line had the advantage of being transfectable and responded well to an LPS challenge without any form of modification. However, the major drawback of this cell type was that it was not a human cell line, and as such it was decided to use the U937. Many researchers had shown that this cell line was transfectable, and despite the fact that it

needed PMA modification before it became LPS responsive, it was still a human cell line and therefore preferable to the mouse Raw 267.4.

Chapter 5

Which Cells Produce IL-10 and TNFα?

5.1 Introduction and Aims

Recently more and more evidence has begun to arise that would indicate that like the T cell, there exists macrophage sub populations[120]. This growing evidence had important connotations for the role of the macrophage within many disease processes. With the T cells, there are many subpopulations, but broadly speaking, two main types exist, the Th1 cells and the Th2 cells.

The Th1 cells mediate several functions associated with cytotoxicity and local inflammatory reactions, consequently these cells are important for combating intracellular pathogens including viruses, bacteria and parasites. The Th2 cells, are more effective at stimulating B cells to proliferate and produce antibodies, and thus making their primary function primary to protect against free-living micro-organisms (also known as the humoral response). It should be remembered, at this time, that IL-10 was first discovered in Th2 cells and has long since been established as a potent cytokine switch pushing the immune response towards the humoral response (see Chapter one – Introduction). Therefore, while TNFα promotes the macrophage towards an inflammatory response IL-10 inhibits this response.

It was not unreasonable, therefore, to ask the question if IL-10 is involved in both T cell subpopulations and as such a fundamental switch within the macrophage itself, from a pro- to an anti-inflammatory cell, does there exist within the macrophage population a subgroup which is responsible for this change?

The aim of this part of the project was to determine, by FACS analysis, which monocytes were responsible for IL-10 production and which cells were responsible for TNFα production.

5.2 Time course of IL-10 production in response to LPS

Until now we have looked at IL-10 production after 24hrs stimulation with LPS. The next question was whether this was the optimal time point to measure IL-10 secretion, or weather a later time point was more optimal.

In this experiment, the U937 cell line was pre-treated with PMA for 3 days, followed by an LPS challenge for up to 7 days.

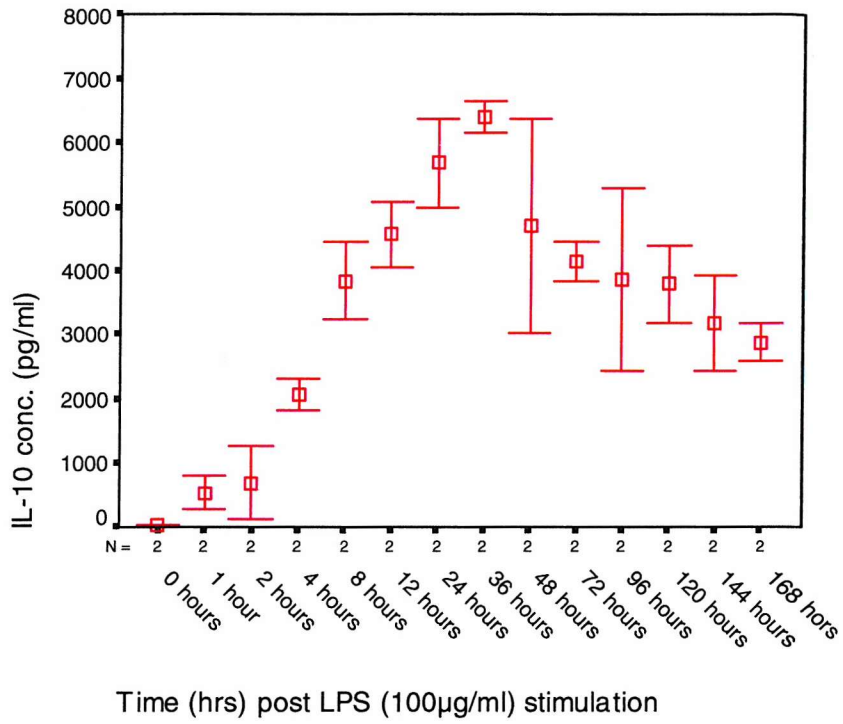


Figure 5.1 IL-10 response to LPS time course in PMA pre-treated U937 cells. +/- 95% confidence levels.

From the above graph, it can be clearly seen that IL-10 reaches a peak in secretion at 36hrs. This is well after the peak of TNFα, which appears at 8hrs.

It was this experiment which lead to the question, is it the same cell which is producing both cytokines, or was there a subpopulation of cells involved in the pro-inflammatory cytokines, while a second subpopulation was involved in the manufacture of the anti-inflammatory cytokines.

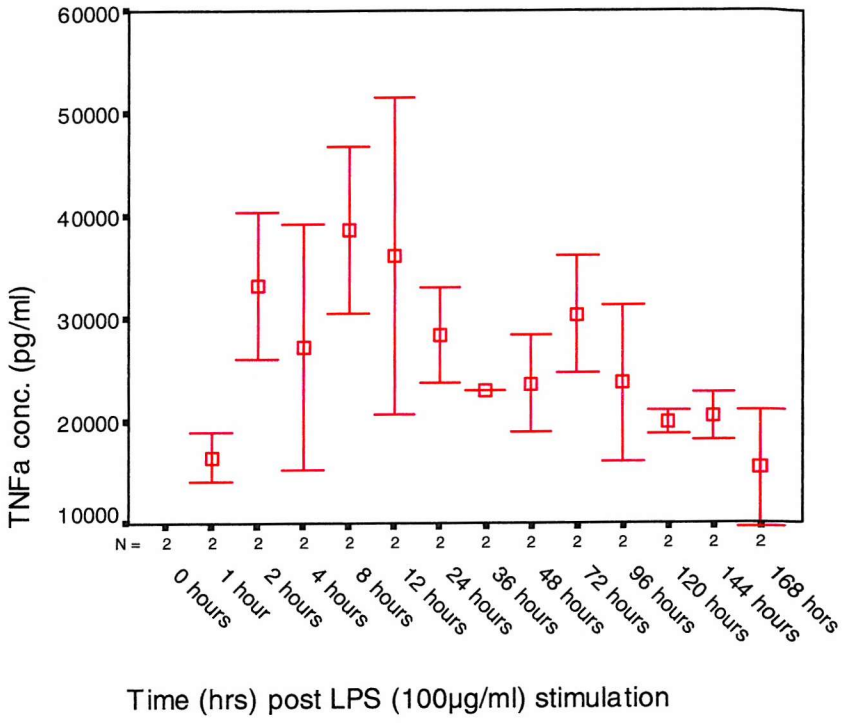


Figure 5.2 Response of TNFα to an LPS time course in PMA pre-treated U937 cells. +/- 95% confidence levels.

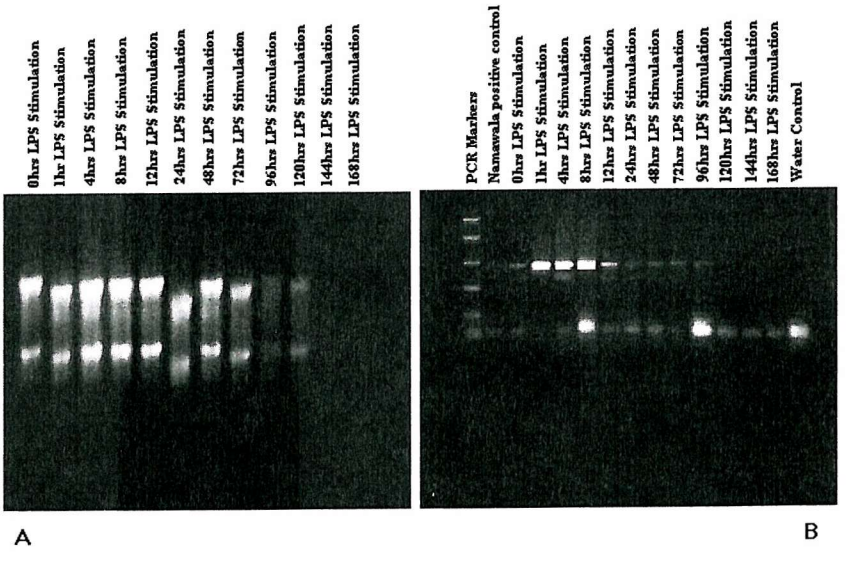


Figure 5.3 RT-PCR showing the expression of IL-10 mRNA in the U937 cells, following LPS challenge for up to 7 days. A) shows the RNA integrity gel. B) shows RT +ve step. Bands can clearly be seen for the IL-10 from 1hrs stimulation and remain high up until 12hrs, before dropping back to basal levels.

From the RT-step, it can be seen that the IL-10 mRNA, is present after only 1hr challenge with LPS, and continues to be highly expressed for up 12 hours before beginning to fall back to basal levels. Despite this, the IL-10 protein is detectable in the supernatants up to 7 days after initial LPS challenge. This experiment was repeated in the primary monocytes, where the results showed the same effect.

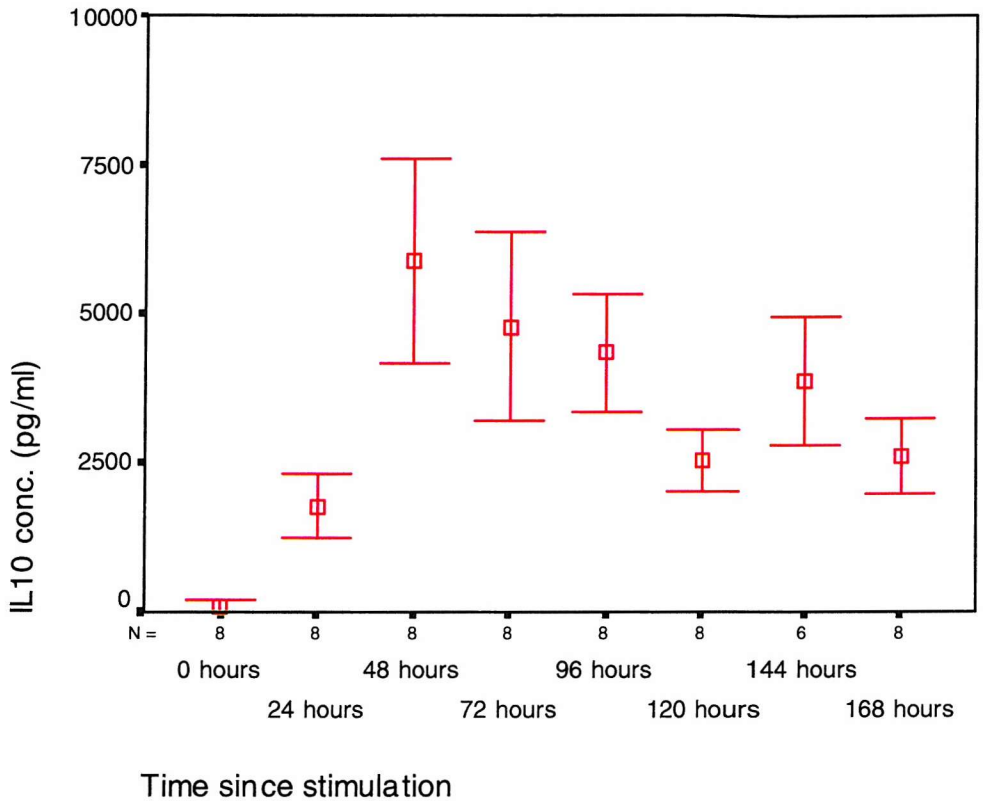


Figure 5.4 Time course of IL-10 secretion from primary human monocytes stimulated with 100µg/ml LPS.

As can be seen, the level of IL-10 production appears to peak at between 36 and 48hrs and then gradually falls over the course of the next 5 days. However, the level of IL-10 does not reach base line levels again, even after 7 days stimulation. Unfortunately the TNFα levels have fallen almost to base line levels again after 48hrs (see below). It was for this reason, that experiments continued to use the 24hr time point, so as to look at the effect of TNFα in relation with IL-10.

5.3 Which cells produce IL-10 and TNFα

To determine which macrophage produce both the IL-10 and TNFα cytokines in response to LPS, a simple time course was set up. Primary human blood monocytes were isolated from a buffy coat and allowed to adhere to a tissue culture flask overnight. The next day they were stimulated with 10μg/ml LPS for 0, 3, 6, 12, 24, 48, 72 and 96hrs. After stimulation the cells were collected, fixed and stained intracellularly with marker antibodies (see 2.2.5). Antibodies used were as follows, for IL-10 cytokine staining a rat anti hIL-10 monoclonal RPE labelled antibody was used. For the TNFα, a mouse anti hTNFα monoclonal FITC labelled antibody was used. Macrophage and monocytes were gated, that is selected for study using a mouse anti human CD14 biotinylated monoclonal antibody with streptavidin-PerCP as the marker stain.

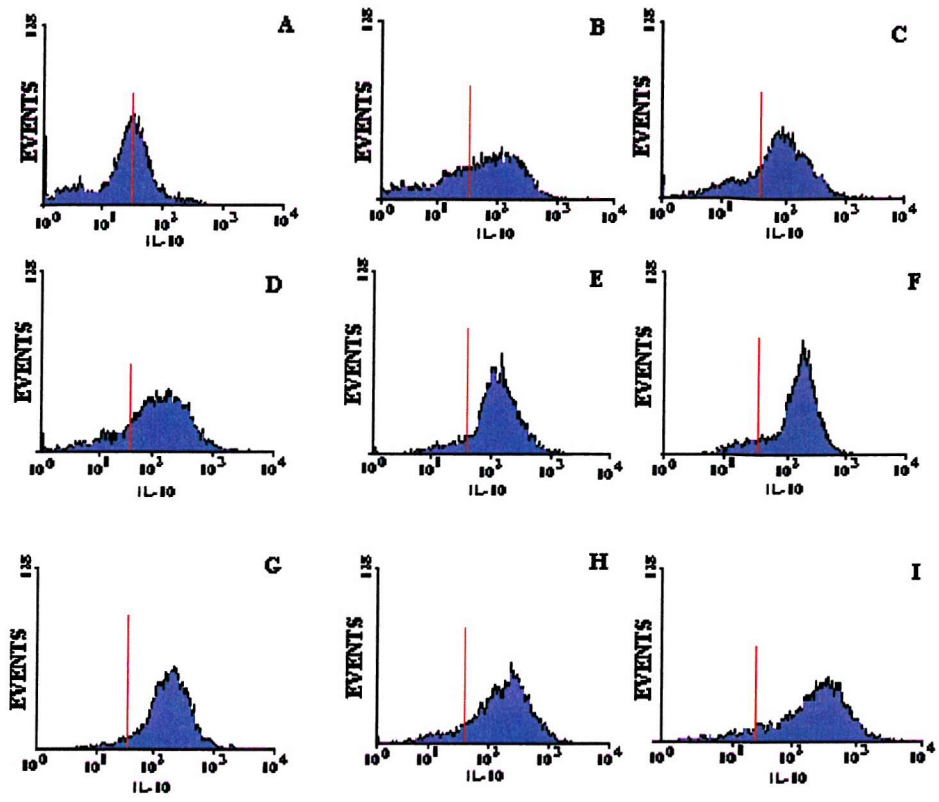


Figure 5.5 Effect of LPS stimulation on IL-10 production in primary human monocytes over 4 days. a) Primary control cells with no antibody staining and no LPS stimulation. b) Primary control cells with antibody staining and no LPS stimulation. c) Cells stained for IL-10 after 3 hrs LPS stimulation. d) Cells stained for IL-10 after 6hrs LPS stimulation. e) Cells stained for IL-10 after 12hrs LPS stimulation. f) Cells stained for IL-10 after 24hrs LPS stimulation. g) Cells stained for IL-10 after 48hrs LPS stimulation. h) Cells stained for IL-10 after 72hrs LPS stimulation. i) Cells stained for IL-10 after 96hrs LPS stimulation.

This result mirrors those results seen in the ELISA experiments. IL-10 is expressed constantly in the primary cells, most likely due to a small level of activation caused by the isolation procedure. This activation is increased by the addition of LPS to the cells. In regards to IL-10, this expression is seen at 24hrs and is maintained at a high level for the continuation of the experiment. TNFα, on the other hand, like IL-10 appears to be active at a low level in the unstimulated cells. This activation greatly increases with LPS stimulation. Unlike the IL-10, however, the TNFα rises early, after only 3hrs the intensity of TNFα expression has risen, and begins to drop by 24hrs. By 48hrs, the level of TNFα is equivalent to the levels seen in the unstimulated cells.

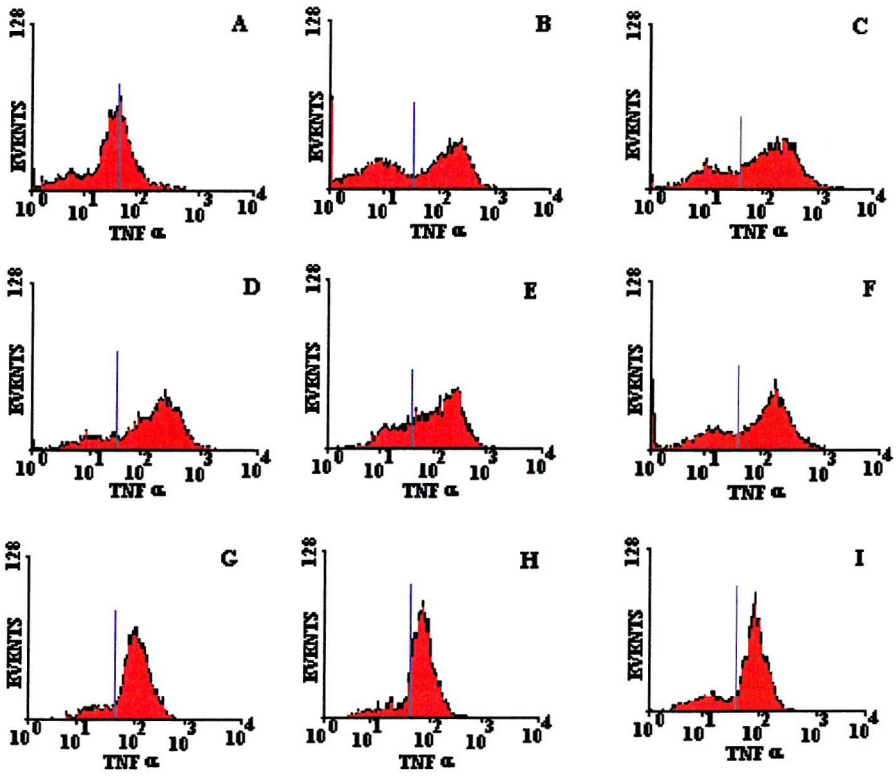


Figure 5.6 Effect of LPS stimulation on TNFα production in primary human monocytes. a) Primary control cells with no antibody staining and no LPS stimulation. b) Primary control cells with antibody staining and no LPS stimulation. c) Cells stained for TNFα after 3 hrs LPS stimulation. d) Cells stained for TNFα after 6hrs LPS stimulation. e) Cells stained for TNFα after 12hrs LPS stimulation. f) Cells stained for TNFα after 24hrs LPS stimulation. g) Cells stained for TNFα after 48hrs LPS stimulation. h) Cells stained for TNFα after 72hrs LPS stimulation. i) Cells stained for TNFα after 96hrs LPS stimulation.

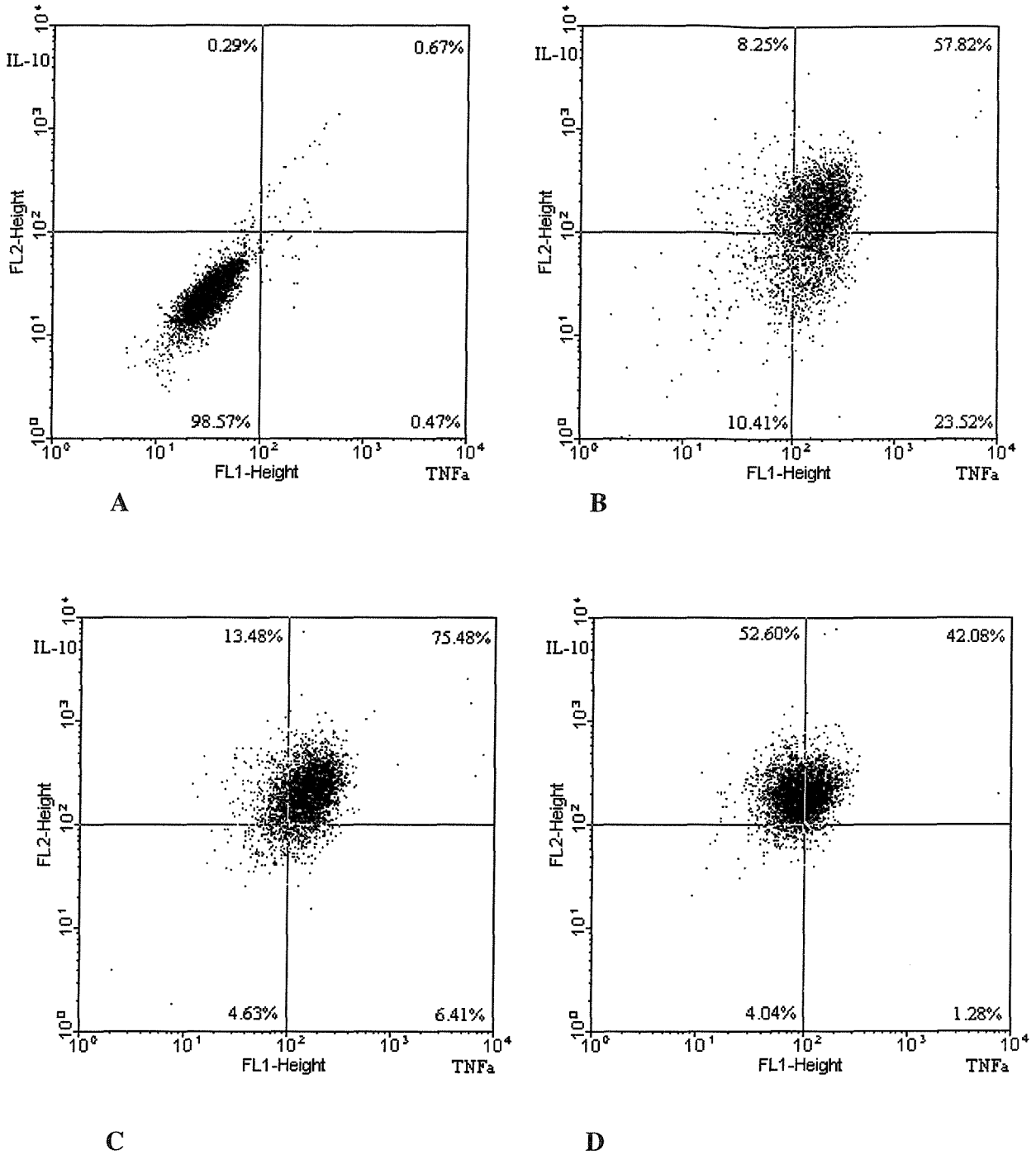


Figure 5.7 Analysis of primary human blood monocytes stimulated with 10µg/ml LPS and stained with anti-hIL-10 and anti-hTNFα labelled antibodies . A) Unstimulated control cells with no antibody staining B) Unstimulated control cells with IL-10 and TNFα antibody staining. C) 24hr LPS stimulated cells with IL-10 and TNFα antibody staining, D) 96hr LPS stimulation with IL-10 and TNFα antibody staining.

From the data, it is also seen that the same cells are producing both IL-10 and TNFα. This would indicate that the same cell is self-regulating in inflammation.

5.4 Discussion

From the experimental data obtained it can be seen that the primary human blood monocyte responds does dependently to LPS, by the production of IL-10 and TNFα. This increase of IL-10 reaches a peak at 48hrs-post stimulation, almost 42hrs after TNFα reaches its dose peak. Also IL-10 continues to be expressed up 7days, although the mRNA appears only to be present up to 12hrs before dropping back down to basal levels, after stimulation while TNFα is almost completely gone after 48hrs, which as mentioned before is when IL-10 reaches its peak secretion.

Given the fact that IL-10 and TNFα are produced by the same macrophage, it is likely to hypothesis that IL-10 is able to regulate the production of TNFα in the cell. This most likely happens at either the second messenger level or at the transcriptional level.

Chapter 6

Second messenger pathways

6.1 Introduction

An assay had been developed and a cell line found, the U937 cell line, which responded to LPS by producing IL-10 in a way that mimicked the primary cell. The next part of this study was aimed at determining how the signal was transported within the cell from the cell surface to the nucleus. Since an assay system had been developed which could repeatedly determine the concentrations of both IL-10 and TNFα accurately down to low concentrations, we were able to investigate the second messenger pathways with the use of specific pharmacological inhibitors. These inhibitors would block only specific pathways, thus allowing the pathways to be mapped individually, and their importance examined. It is important that the pathways, which are involved in the LPS signal to the nucleus, are the same since if different pathways were used in the cell line than that of the primary cell, then different transcription factors might be activated.

It was hypothesised that by better understanding these pathways, a treatment might be developed which would lead to control over inflammation diseases by blocking or activating the pathways of importance.

6.2 Effect of pharmacological inhibitors on IL-10 production in primary and RAW 267.4 cells.

As mentioned before, the TNFα pathways have already been studied in great detail (see 1.7), and it has been linked to the p42/44 MAPK pathway104. There is little information available concerning the role of the MAP kinase pathways on the regulation of other cytokines, with only a couple of studies looking at the regulation of IL-10.

In one of these studies the IL-10 regulatory pathways had been linked to the protein tyrosine pathways[106]. Therefore, it was logical that we started our investigation into the second messenger pathways with these pathways. The first step was to confirm that the LPS activation signal of IL-10 was transported within the cell via the protein kinase pathways, for this reason the inhibitor Herbimycin A, which is a cell permeable, potent inhibitor of protein tyrosine kinases was chosen.

6.2.1 Effect of Herbimycin A on IL-10 synthesis

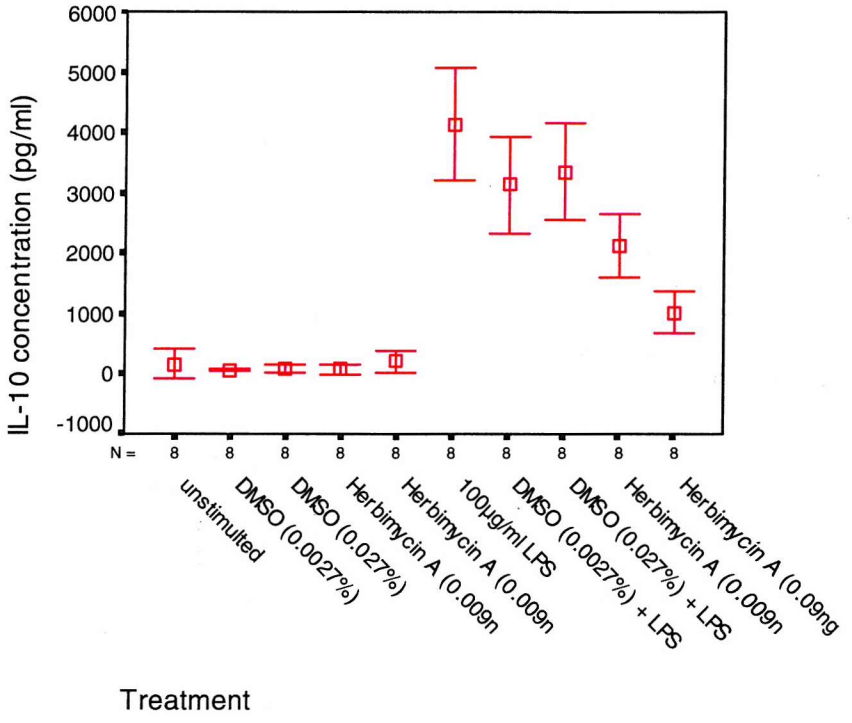


Figure 6.1a Effect of Herbimycin A on the IL-10 secretion of primary monocytes upon stimulation with 100µg/ml LPS for 24hrs. Herbimycin A was diluted in DMSO, as such, DMSO controls were added to the experiment to determine the effect on the primary blood monocytes. Results +/- 95% Confidence levels.

As can be seen from the above graph, Herbimycin A does indeed cause a dose dependent down-regulation in IL-10 production. Although a slight down-regulation in IL-10 secretion is seen as an effect from the DMSO, this effect is not significantly different. However upon addition of the inhibitor, the down regulation does become significantly different.

This is, perhaps, not a surprising result since the protein tyrosine kinases have been associated with the transduction and processing of many extra- and intracellular signals. These include many of the responses associated with inflammation and stress, for example proliferation, differentiation and cell growth, which are factors for the induction of IL-10 synthesis. However, the protein tyrosine kinases are also involved in the activation of the MAP Kinase pathways.

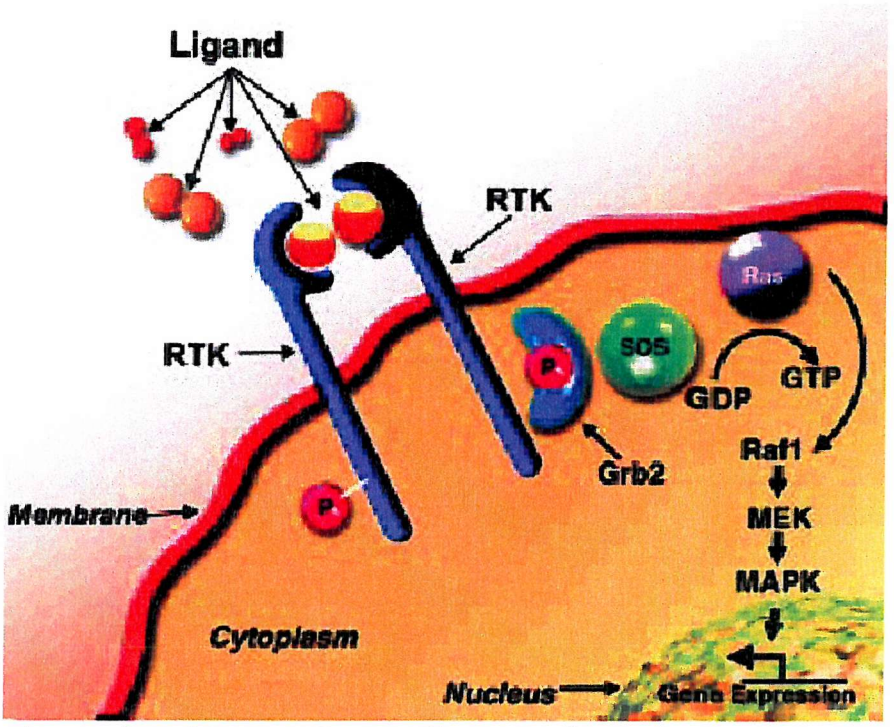


Figure 6.1b An overview of Receptor Tyrosine kinase signalling activation of the MAP kinase pathways. Source, Calbiochem Website (1998)

With the effect of Herbimycin A confirmed, the use of further inhibitors, which blocked pathways within the MAP kinase pathways, were utilised. The first inhibitor was the p42/44 MAPK inhibitor PD98059, which specifically inhibits this pathway at ERK 1,2.

6.2.2 Effect of the p42/44 MAPK inhibitor PD98059 on IL-10 production in the primary human monocyte and the mouse RAW cell line.

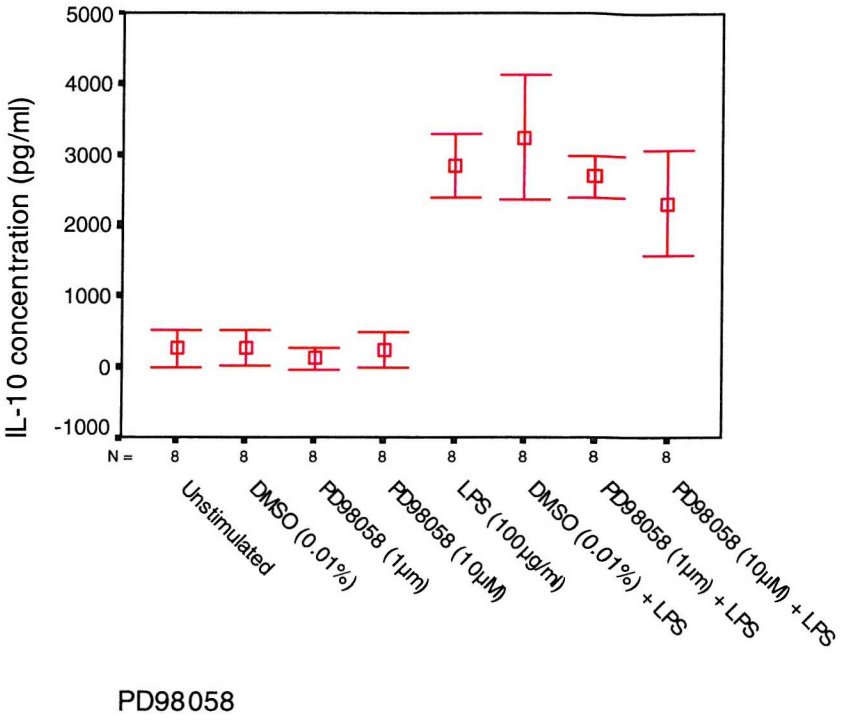


Figure 6.2 Effect of PD98059 on IL-10 production in primary human blood monocytes stimulated with 100µg/ml LPS for 24hrs. Results +/- 95% Confidence levels.

PD98059 is a p42/44 pathway specific inhibitor. This pathway has been associated with the synthesis of TNFα in response to LPS. From the data in chapter 5, it is therefore unsurprising that IL-10 is not regulated via this pathway. This can be deduced from the FACS analysis data. Since the same cell and not sub-populations within the macrophage lineage produce IL-10 and TNFα, it makes sense that they do not progress along the same pathway. IL-10 has many functions while down-regulating the functions of the macrophage. One of these is the down regulation of cytokine synthesis. One of the ways in which this is achieved is the degradation of the mRNA. However IL-10 also affects other areas such as, for example, the transcription factor NFκB. It is therefore not inconceivable that IL-10 may down-regulate other MAP Kinase pathways. It is also well known that IL-10 is autoregulatory and therefore it could be argued that IL-10 could be regulated down this pathway. However TNFα reaches a secretion peak around 6hrs with cytokine synthesis there after declining and reaching base line levels at around 48hrs. IL-10 expression, on the other hand, reaches a peak at between 36 and 48hrs and continues

on for many days. If both cytokines were regulated through the same pathway then IL-10 would peak earlier and would not continue to be produced for so long.

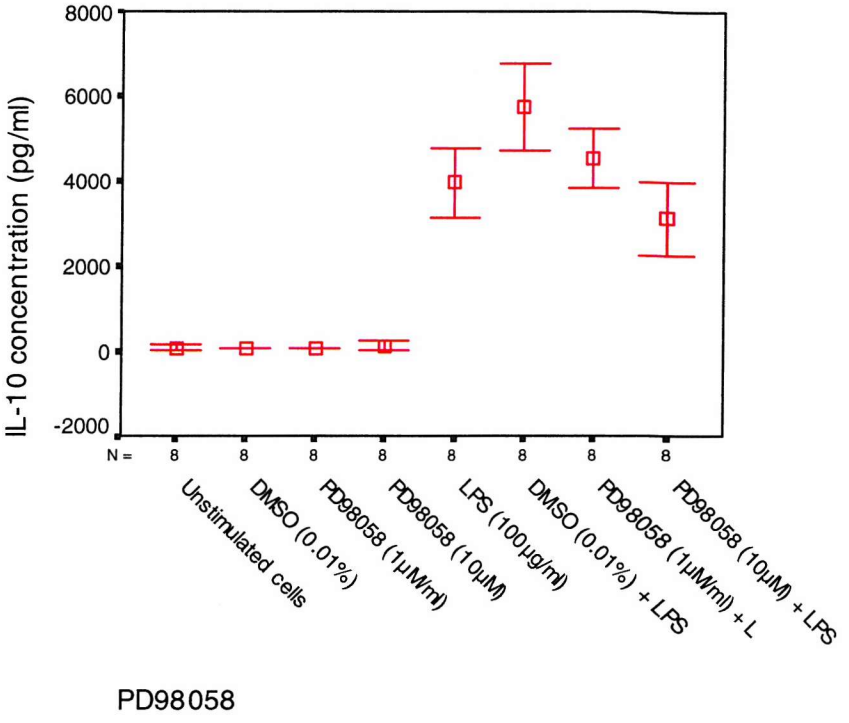


Figure 6.3 Effect of the p42/44 inhibitor on IL-10 production in the mouse RAW cells stimulated with 100µg/ml LPS for 24hrs. Results +/- 95% Confidence levels.

As can be seen from the above graph, PD98059 has the same effect on the mouse RAW cell line as in the primary human blood monocyte. A small trend can be seen from both graphs that there is a very slight decrease in IL-10 production, but this decrease is not significant. It was most likely caused by the down regulation in TNFα production in response to PD98059.

6.2.3 Effect of the p38/HOG MAPK inhibitors SB203580 on IL-10 production in the primary human monocyte and the mouse RAW cell line.

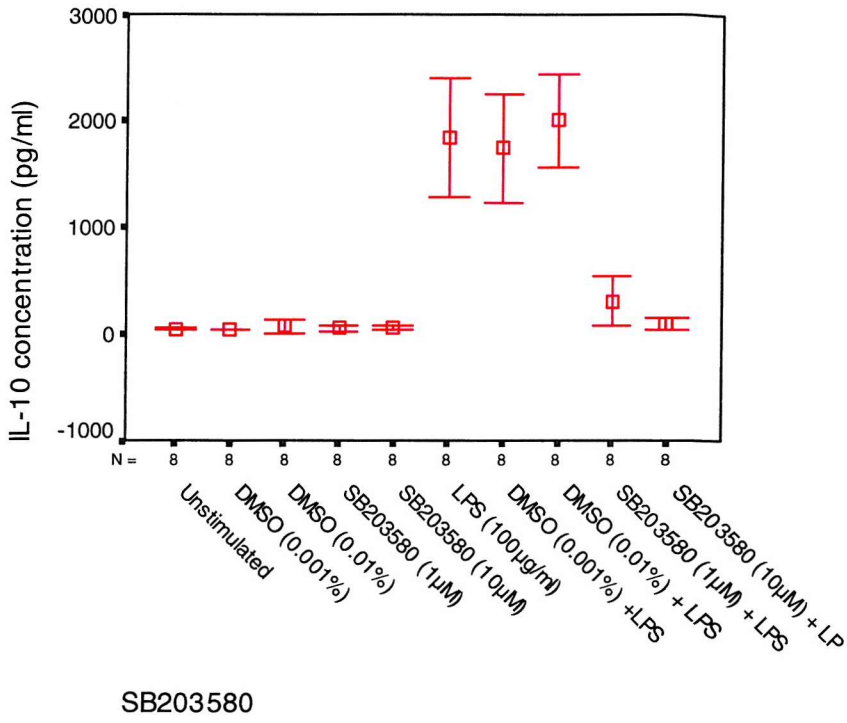


Figure 6.4 Effect of the p38/HOG MAPK pathway inhibitor SB203580 on IL-10 production after 24 hr stimulation with 100µg/ml LPS. Results +/- 95% Confidence levels.

SB203580 is a highly specific inhibitor of the p38/HOG pathways, which block this pathway at ERK 6. As can be seen from above, the inhibitor quite clearly down-regulated the IL-10 production in the primary human blood monocyte upon stimulation with LPS. This result was again mirrored in the mouse RAW cell line.

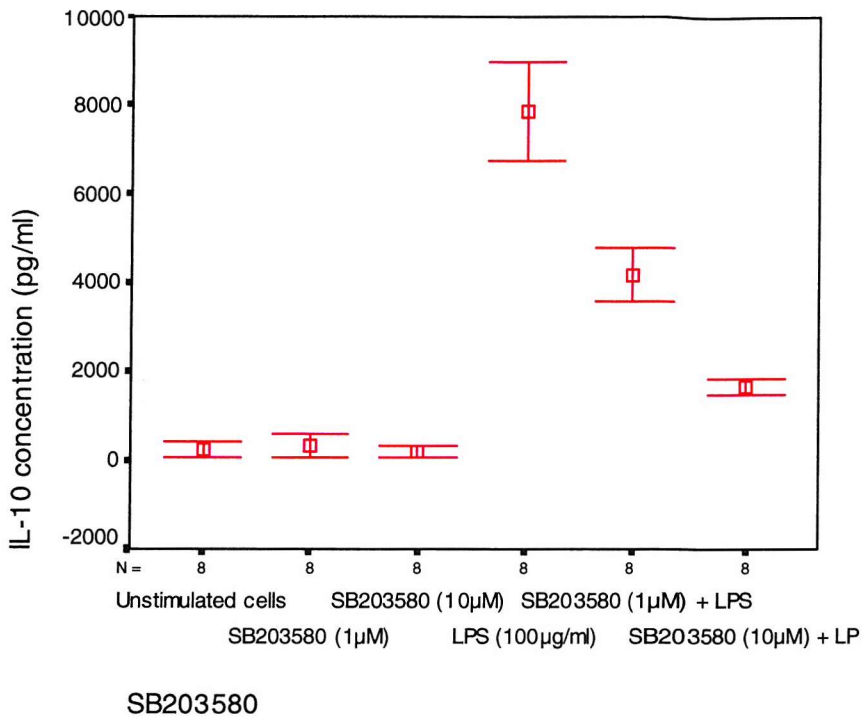


Figure 6.5 Effect of the p38/HOG MAPK pathway inhibitor on IL-10 production in the mouse RAW cell line after 24hr stimulation with 100µg/ml LPS. Results +/- 95% Confidence levels.

Again, when thought about, this result is not a complete surprise. The p38/HOG pathways, as well as the JNK/SAPK, are stress response pathways. This pathway is activated by the addition of LPS (stress response), inflammatory cytokines (e.g. TNFα as well as a whole host of other proinflammatory cytokines produced in response to LPS) and ultraviolet light.

Until recently it has not been possible to block the JNK/SAPK pathways without the microinjection of peptides into the cell, a techniques which was not available to us. Also, such a technique would not truly prove practical due to the number of cell required to look at the MAP kinases. However, recently, the inhibitor Curcumin, was isolated from turmeric which specifically blocks the JNK pathway[121-123], and as such this now lets us for the first time to look at the effect blocking this pathway has on the IL-10 production in the primary monocytes.

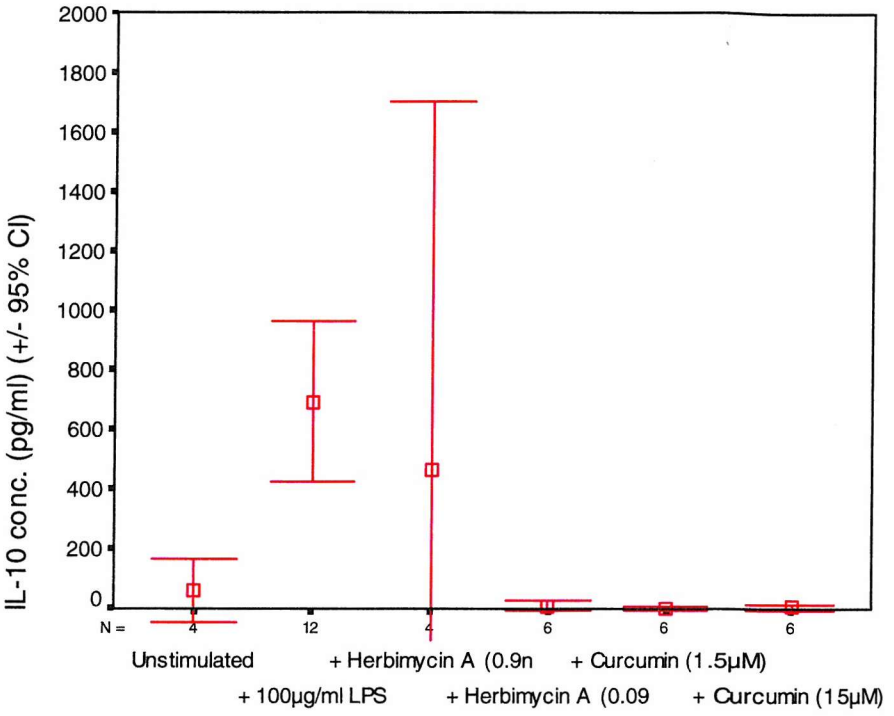


Figure 6.6 The effect of the JNK inhibitor curcumin on IL-10 secretion after 24hrs LPS stimulation in the primary monocyte. Results +/- 95% Confidence levels.

It can be clearly seen that like the SB203580 inhibitor, the blockage of the JNK pathway leads to a total abolition of IL-10 secretion by the primary cell. For further discussion see 6.4 below.

6.3 Effect of pharmacological inhibitors on the IL-10 secretion of the U937 cell line.

Now that the pathways for the IL-10 secretion in the primary macrophage had been mapped, the next aim of this part of the study was to ensure that the U937 cell line followed the same pathways following LPS stimulation.

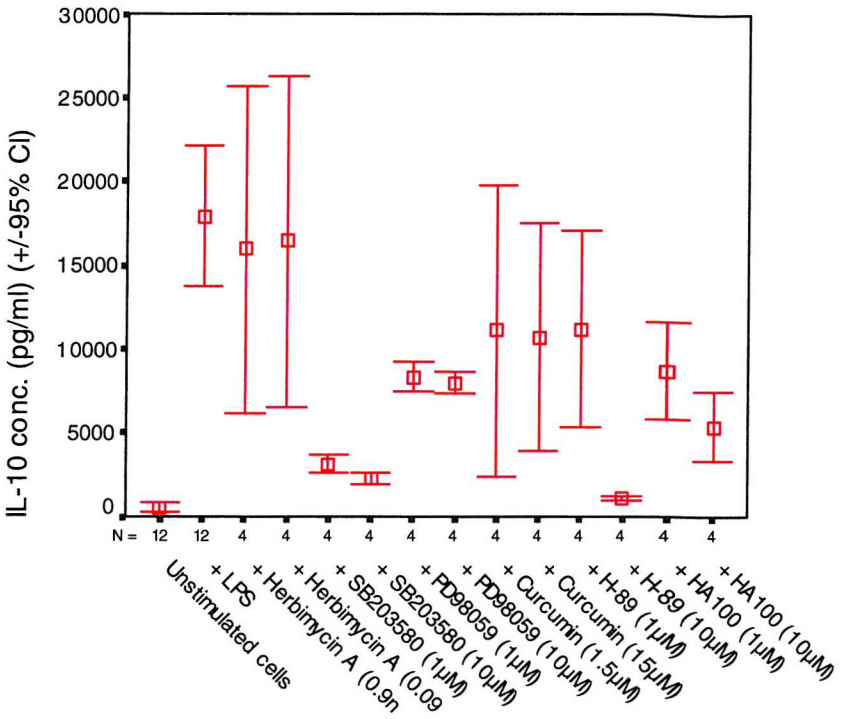


Figure 6.7 The effect of various inhibitors on the Il-10 secretion of the cell line U937. Results are +/- 95% confidence levels.

From the above graph, several differences can be seen from the primary cells. The first, and most obvious, was that the effect of Hebimycin A on the IL-10 production appeared to be small, with only a very slight decrease in IL-10 secretion, which was not significant. The next major difference was that the inhibitor PD98059 appeared to have a greater effect on the IL-10 secretion in the U937 than in the primary cells. Finally, although the action of curcumin does cause a down-regulation in IL-10 secretion, this down-regulation is neither as pronounced as it was in the primary cell, nor as precise, which can be seen by the large variation found. These effects can be explained by the use of the PMA in the early activation step of the U937. PMA, as mentioned earlier, is a potent activator of the macrophage cell, by mimicking the action of diacylglycerol, which causes the activation of NFκB and the MAP kinases. In the case of the Herbimycin A and the curcumin, this increased and continuous activation of the MAP kinases by the PMA present in the media, may be counteracting the effect of the inhibitors and thus reducing their potency. The PD98059 inhibitor is only able to inhibit the activation of p42; it has no effect on p42 that has already been activated by the PMA. As with the Herbimycin A and curcumin, there will be an increased level of the p42 MAP kinase pathway, however

in the primary cells and the RAW267.4 cell line, blockage of this pathway did not appear to have any direct effect on the production or secretion of the IL-10 protein. However, since the PMA was also responsible for the activation of the NFκB, in the U937 and that NFκB is essential for the activation of TNFα and other pro-inflammatory cytokines, all of which lead to the activation of IL-10. This loss of NFκB, therefore, could be causing an indirect loss of IL-10 secretion, not due to direct inhibition, but rather through the inhibition of the other pro-inflammatory factors leading to IL-10 production.

6.4 Discussion

From the results shown above, IL-10 in response to LPS in the primary macrophage is p38 MAP kinase and JNK dependent, since inhibition of these pathways leads to an almost total inhibition of IL-10 synthesis. This report is the first to show that the JNK pathway is important in regulation the response of IL-10. We have also that the IL-10 response is p42/44 MAPK independent within the primary cells.

However, in the U937 cell line, we found that the regulation of IL-10, by the MAP kinase pathways is not quite as clear as it was within the primary cell. Both p38 and JNK pathways appear to be important to the regulation of IL-10 within the U937, as they were in the primary cells, since inhibition still leads to a down-regulation in the IL-10 response. However the effect of the JNK pathway is not as clear as it was in the primary cell due to the high variation of the response, most likely due to small variations in the concentration of PMA in the media from experiment to experiment. Nor was the inhibition of IL-10 production as great as it was in the primary cell. Also, the effect of the p42 inhibitor PD98059 was surprising since this pathway was shown to be unimportant in IL-10 regulation within the primary cell. Again, the only explanation which accounts for this would be the addition of the PMA, since in the RAW267.4 cell line, the PD98059 mirrors the primary cells and had little to no effect on the IL-10 production.

Of course, it could always be argued that the inhibitory effects seen by the inhibitors are due to the toxic effects of these potent chemicals upon the cells themselves. To test this hypothesis, we carried out a viability test, where the cells were treated with the inhibitor followed by 24hrs LPS stimulation. The cell viability was the performed using Trypan blue, a vital cell dye. Trypan blue is actively pumped out of living

cells, but is taken up by dead ones, therefore living cells appear bright to the dark staining of the dead cells. These cells can then be counted and a percentage of survival worked out.

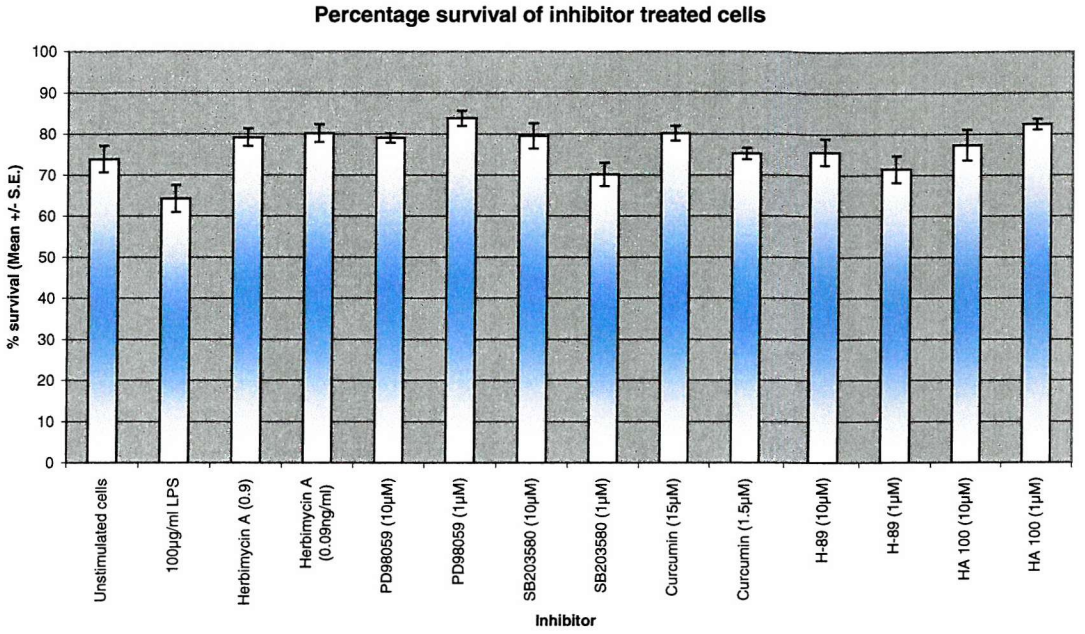


Figure 6.8 Percentage survival of inhibitor treated cells. Results plotted show +/- S.E.

It can be clearly seen that the inhibitors did not appear to have any toxic effects upon the cells at the concentrations used in this study. As such the inhibitory effects of these inhibitors can be said to be due to the effect of the inhibitors and not cell toxicity. A similar graph was seen for all the cell types tested.

Therefore to conclude distinct MAP kinase signal transduction pathways mediate the IL-10 and TNFα responses, within the macrophage. These individual responses to the JNK and p38 signalling pathways and that of the p42 pathway are regulated differently. The differential utilisation of the MAP kinase signalling pathways may represent a potential mechanism for the determination of cell-type-specific responses to an extracellular stimulus such as endotoxin.

Chapter 7

Transfection

7.1 Introduction

In this part of the study, the transcriptional activators involved in regulation of IL-10 gene transcription within the macrophage will be studied. Understanding the regulatory processes involved in the gene's activation will, it is hoped, lead to a better understanding of the regulation of this important cytokine within inflammatory diseases. And from this it is hoped that a gene therapy for these patients, which suffer from the inflammatory diseases such as liver fibrosis, could be developed.

In order to study the transcription factors involved in the regulation of IL-10 during inflammation, it was first necessary to find a cell line, which would react, to LPS in a way similar to the primary monocytes. This is due to the fact that primary monocytes are as yet unable to be transfected. Many researchers hope that perhaps one day a transfection reagent will be developed which will enable gene transfer into monocytes.

Through experimentation, it was found that the U937 cell line appeared to respond to LPS in a way that mimicked the primary cell. Early experiments carried out by this researcher showed that the THP-1 cell line responded to LPS, without pre-differentiation with PMA. However later experiments showed that this response was false and appeared to be due, mote probably, to a contaminating cell line. Due to this many months were used setting up the transfection conditions for this cell line. However, this data will not be included as it was later repeated with the U937 cell line.

The aim of this part of the study, therefore, was to develop a model, which could be used to investigate which transcription factors are important in IL-10 regulation and their role during inflammation.

The first objective in this part of the study was to determine the optimal conditions needed for transfection of the U937 cell line. Once completed, these conditions can then be utilised for the transfection of the U937 with the IL-10 promoter constructs allowing us to look at various areas in the promoter and from there determine which transcription factors are important.

7.2 Using the sieCAT plasmid as a Control

It has long been known that the transfection of the macrophage or monocyte is difficult, even in cell lines. However, in 1994, Ashlee Moses published a paper in which he described his technique in which he transfected an HIV long terminal repeat-CAT plasmid and looked at the expression in primary monocytes obtained from human blood124. In his study he reported to have transfected the HIV long terminal repeat-CAT plasmid using the Lipofectin technique (see 2.2.10.1). In order to determine if this was a viable technique this protocol was followed and the primary human monocytes were transfected with the sieCAT plasmid. The sieCAT plasmid consists of an IFN-γ driven Chloraphenicol Acetyl-Transferase gene.

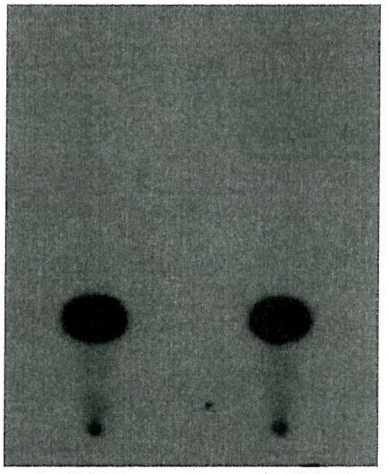


Figure 7.1 Transfection of primary human monocytes using the Lipofectin Reagent, following the protocol of Ashlee Moses. DNA was added at the concentration of 1μg/million cells. Cells were stimulated with 10ng/ml IFNγ.

As can be seen from this result no transfection of the primary monocytes took place. It was decided, therefore, to try this technique in the U937 cell line.

The U937 cell line was again transfected with the sieCAT plasmid using a Lipofectin concentration curve, to try and determine what the optimum concentration of Lipofectin reagent require for transfection was.

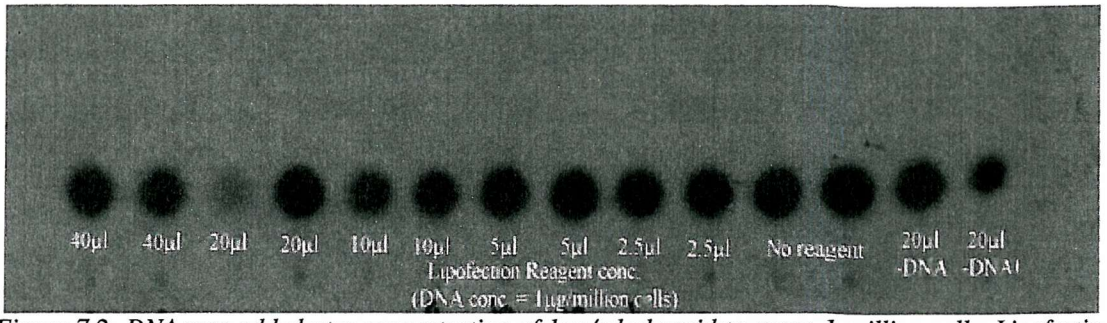


Figure 7.2 DNA was added at a concentration of 1µg/ml plasmid to every 1 million cells. Lipofectin reagent was added at an increasing concentration to determine the optimum levels required for transfection.

As can be seen from the above data, the U937 cell line failed to transfect, shown by lack of CAT activity upon stimulation with IFN-γ. There are various reasons why this could be the case. First, the action of the macrophage cell is to hunt down and destroy naked DNA and large particulate matter. Therefore, since the Lipofectin reagent must be left on the monocytes for so long could be leading to the cells destroying the particles carrying the DNA. Second, naked DNA has been shown to be toxic (at high levels) to cells125. Since not all the DNA will be incorporated into the cell, the remaining DNA could be killing the cells. Finally, since the Lipofectin reagent only works in conditions whereby all serum has been removed from the media, the lack of serum could be inducing apoptosis or attenuating cell function, especially the primary cells which are not as hardy as the cell line. Therefore a combination of any one or more of these reasons could account for the failure of the U937 cell to transfect.

When we contacted Ashlee Moses, we found that he had given up on the transfection experiments involving primary human cells shortly after publishing his paper and as such could offer no advice on how to deal with this problem.

It was decided then to try and use a technique that did not require the cells to be incubated without serum. Electroporation, Superfect and Effectene were all tested on the primary cells. However, from the results obtained, none of the various methods tested gave any transfection of the primary cells (data not showen). Once again the methodologies were tested using a cell line. This time using the THP-1 monocyte cell line. Numerous papers had sited the ability to transfect the THP-1 cells using the Electroporation method. To this end an Electroporation was set up using the sieCAT plasmid.

However, once again the sieCAT plasmid failed to transfect into the THP-1 cell (data not shown). At this point to was decided to test the plasmid in a cell line which it is known to work and in a cell line which has been shown many times to be easily transfected with this plasmid. From earlier transfection experiments carried out by Dr. T. Biggs (data not shown), it has been shown that the sieCAT plasmid can easily be transfected into the mouse RAW 267.4 cell line. Using her protocol, the mouse RAW cells were transfected along side the THP-1 cell line.

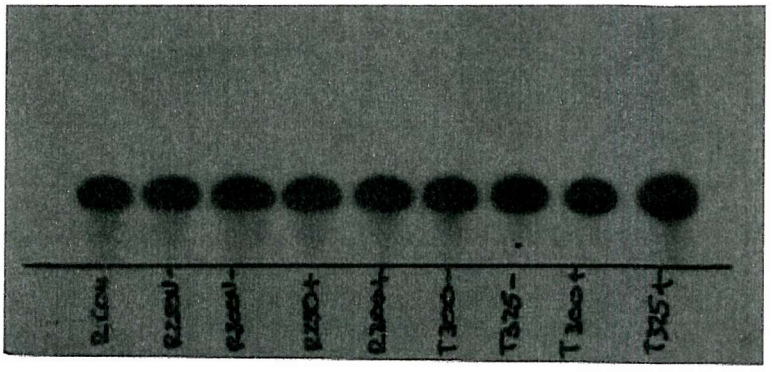


Figure 7.3 Transfection of the mouse RAW and the human THP-1 cell lines with the sieCAT plasmid using the Electroporation method. (R = mouse RAW 267.4 cell line, T = human THP-1 cell line). Pluses indicated the addition of DNA (µg/million cells) while minuses indicated control cells, with no DNA added. R Con = RAW cell control, no Electroporation no DNA.

Once again the THP-1 cells failed to transfect with the sieCAT plasmid. This time, however, the RAW cell line also failed to transfect. Since it was well established that the RAW cell is easily transfected using the Electroporation method, it was decided that the plasmid itself was not working and was discarded. Instead a new control plasmid was used, the IκB-LUC plasmid, which consists of the human IκBα gene promoter driving a Luciferase gene.

7.3 Using the IκB-LUC plasmid to determine Transfection protocols

The IκB-LUC plasmid is an LPS responsive plasmid. Using the same protocol that was used for the electroporation of the sieCAT plasmid, the IκB-LUC plasmid was transfected into the mouse RAW cell.

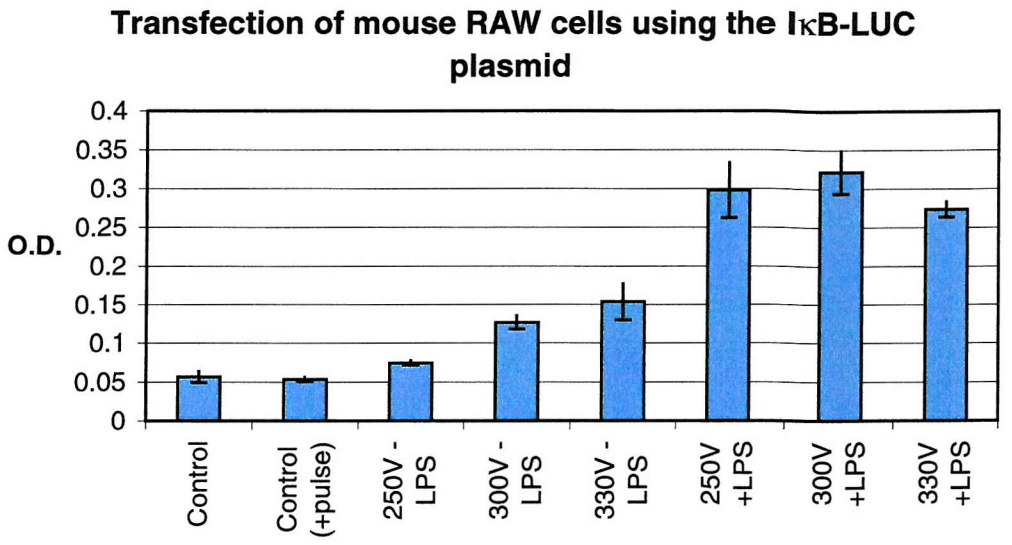


Figure 7.4 Transfection of the mouse RAW cell line with the IkB-LUC plasmid. As before, 10µg/million cells of the plasmid was allowed to adhere to the cell for 10 minutes at room temperature prior to treatment with various electrical pulses in a 0.1cm Genepulser® (Biorad) cuvette.

As can be seen from the results, this time the transfection worked. It can be assumed, therefore, that previous attempts failed due to one of, or a combination of, two possibilities. First, it was indeed the sieCAT plasmid that was not working, and second, the IFNγ was no longer active. However, since the IκB-LUC plasmid was functional and due to time constraints, it was decided not to explore the reason of the sieCAT plasmid failure. Now that a working control plasmid had been found, the plasmid was transfected into the U937 and Namawala cell lines to test whether or not it worked in a human cell line.

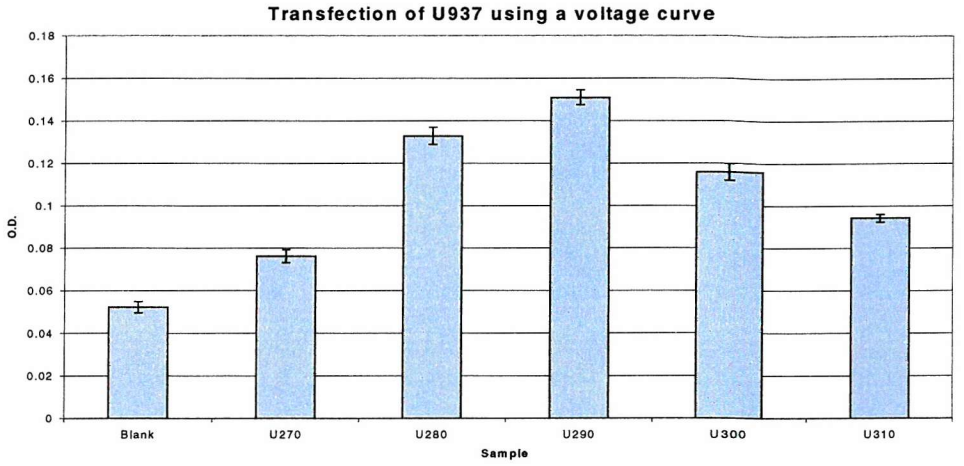


Figure 7.5 Transfection of the human monocyte cell line U937 with the IκB-LUC plasmid under various electrical pulses to obtain the optimum. From the results obtained the voltage of 290V gave the highest level of transfection. Transfection at 310V lead to a greater level of cell death when viewed under the light microscope.

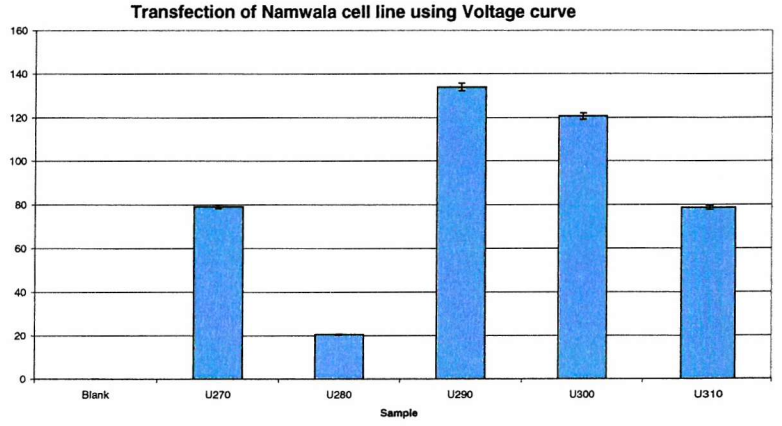


Figure 7.6 Transfection of the human B-cell line, Namawala, with the IκB-LUC plasmid under various electrical pulses to obtain the optimum. As with the U937 cell line above, the results obtained show a voltage of 290V gave the highest level of transfection, with transfection at 310V leading to a greater level of cell death when viewed under the light microscope.

As can be seen, like the RAW cells before, the U937 and Namawala cells can be transfected with the IκB-LUC plasmid. It can also be seen that the 290V gave the highest incorporation of DNA (assuming that the same level of luciferase activity is directly related to the DNA incorporation). A pulse of greater than 310V, in both cases however, gave a lower DNA incorporation. When viewed under the microscope, prior to lysis, the 310V treated cells looked in a much worse condition

than that of the 290V treated cells. Due to this the chosen voltage of 290V for both cell types was used all in future experiments.

Now that the correct voltage has been worked out a DNA titre was needed to obtain the optimum concentration of DNA needed for transfection efficiency. For all experiments the concentration of DNA is per million cells.

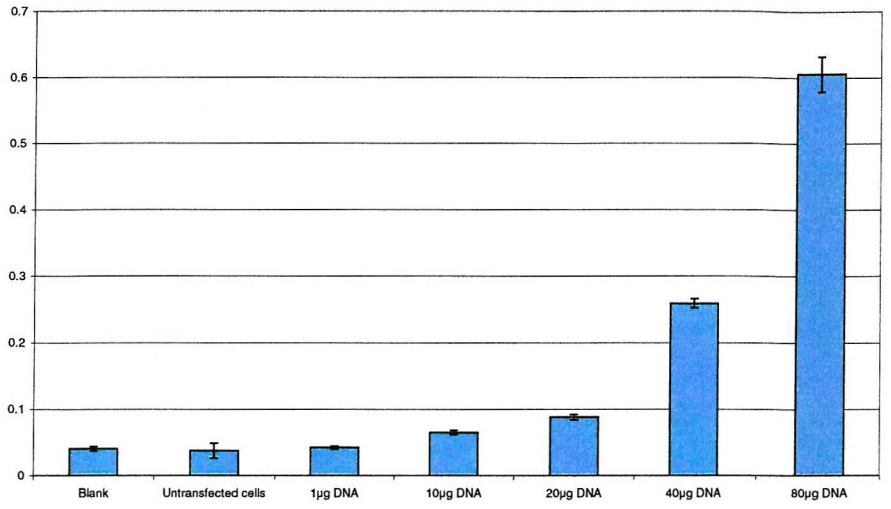


Figure 7.7 DNA titre on the U937 cell line using the IκB-LUC plasmid. From the generated results, 40µg/million cells gave the optimal level of transfection. Cells, which were transfected with the 80µg DNA, gave a much higher level of cell death when viewed under the light microscope.

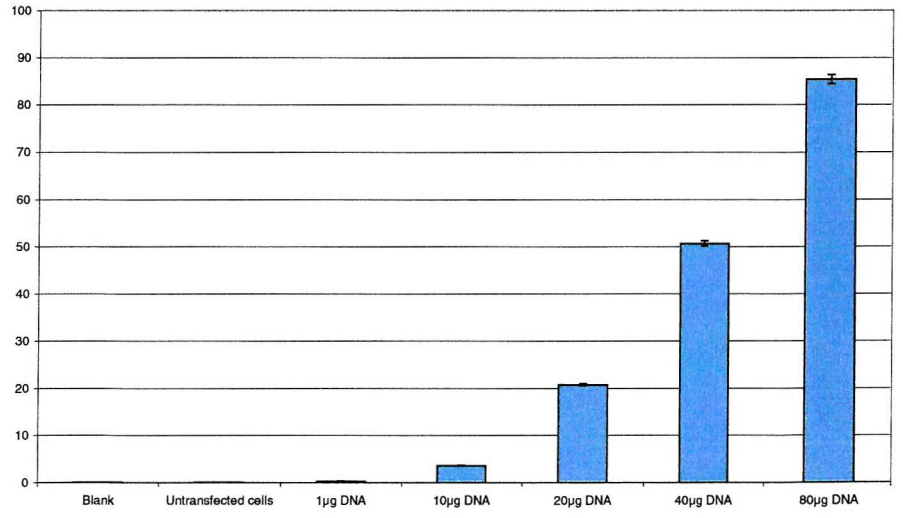


Figure 7.8 DNA titre on the Namawala cell line using the IκB-LUC plasmid. As with the U937 cell line, 40µg/million cells gave the optimal level of transfection, since cells which were transfected with 80µg DNA, gave a much higher level of cell death when viewed under the light microscope.

After transfection of the IkB-LUC plasmid with a DNA titre, a concentration of 40μg DNA was chosen due to its elevated level of transfection, as seen by luciferase activity. The cell transfected with 80μg DNA, when viewed under the light microscope gave a much-elevated level of cell death than all the other samples, this could also be seen in the luciferase activity. This was probably due to high levels of DNA still present in the media after transfection, which lead to cell toxaemia. When trying to remove the high levels of excess DNA, a centrifugation collection step was incorporated. However after a centrifugation of 1,000rpm for 5minutes, almost all the cells were destroyed. This was probably due to the weakened condition of the cells after the electroporation step. It was decided, therefore, to use the 40μg of DNA/million cells as the optimum concentration to use.

Now that an optimum concentration of DNA needed for transfection and an optimum voltage had been worked out the next problem was at what time post LPS stimulation was optimal to give the highest luciferase activity. This was important to work out since luciferase is for transient expression only. Unlike the CAT activity, which has a protein half-life of 50 hours in mammalian cells, luciferase has only a 3 hour half life126. Therefore, if the stimulation was allowed to carry on too long then there could be a drop in activity and therefore we would miss the highest expression level. As before the IκB-LUC plasmid was transfected into the U937 cells using 40μg DNA/million cells at a 290V pulse. Once transfection had taken place, the cells were allowed to recover for 48hrs before stimulation with LPS for up 24hrs.

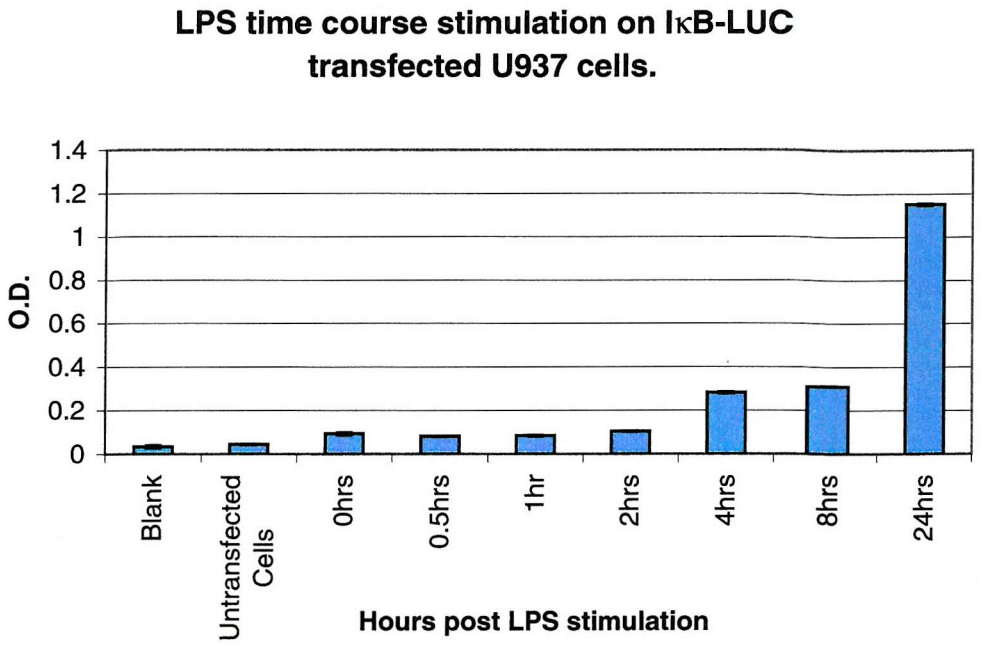


Figure 7.9 IκB-LUC transfected U937 cells using 40µg/ million cells of DNA at 290V were stimulated with 10µg/ml LPS after a 48hr rest period. Cells were stimulated for up to 24hrs.

From previous experiments we know that both IL-10 and TNFα are both still produced at 24hrs-post stimulation with LPS. It was for this reason that 24hrs was chosen for the final time point, since after this time TNFα synthesis begins to fall. Also since the TNFα pathway utilises the NFκB transcription factor, as well as the ability of IL-10 to inhibit NFκB and IL-10 reaching a maximum synthesis rate at 48hrs, 24hrs was chosen as the optimum time point post LPS stimulation to look at the activity of the luciferase.

7.4 Transfection of IL-10 promoter constructs

With the conditions necessary for the transfection of the U937 cell line now known, the next step was to transfect IL-10 promoter constructs. Since the promoter constructs were cloned into a luciferase plasmid based on the pGL2 plasmid, the pGL2basic plasmid was chosen for a negative control.

Table 7.1 Human IL-10 promoter constructs and sizes. Names in bold type are shortened versions of name used in text.

Human IL-10 Luciferase Promoter Constructs

Plasmid Name	Promoter Insert Size
pGL-B-PIL103S (**103S**)	2200bp
pGL-B-PIL100H (**100H**)	850bp
pGL-B-PIL100S (**100S**)	820bp
pGL-B-PIL100M (**100M**)	500bp
pGL-B-PIL100A (**100A**)	320bp
pGL-B-PIL100Alu2 (**100Alu**)	300bp
pGL-B-PIL100Stu (**100Stu**)	120bp

To show that transfection has taken place, the Namawala B lymphocyte cell line was also transfected as a positive control. Since the Namawala cell line has been EBV transformed, the IL-10 gene inside these cells is always active and as such the luciferase in the constructs should be active as well. For the promoter map, see figure 7.20.

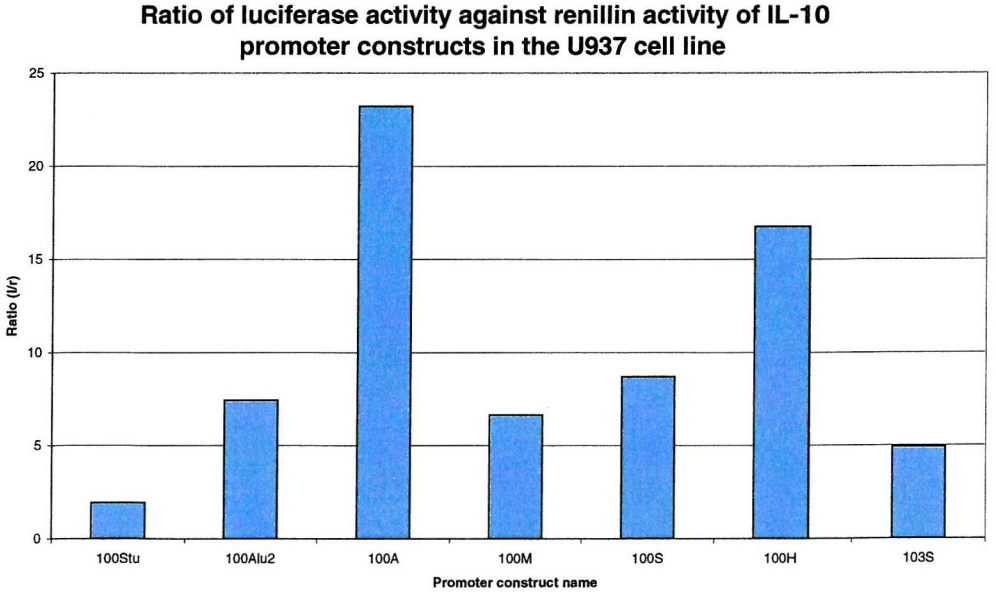


Figure 7.10 First attempt at transfecting the IL-10 promoter constructs into the U937 cells.

The first attempt at transfecting the U937 cells with the IL-10 promoter constructs proved to be successful. With a similar trend being witnessed in the Namawala positive control cells.

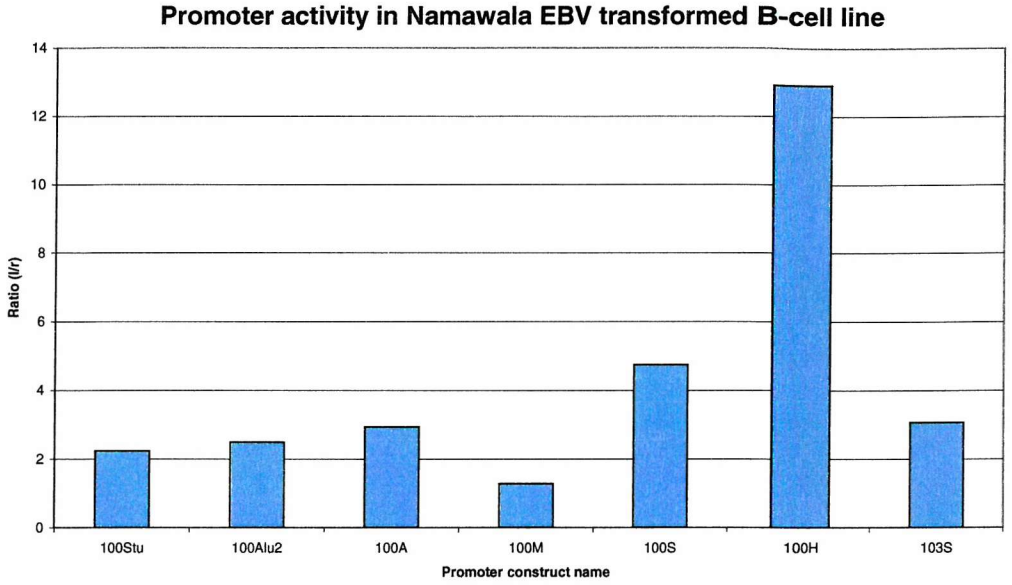


Figure 7.11 Transfection of the Namawala positive control cells with the IL-10 promoter constructs.

For both cell lines it appears that the IL-10 promoter constructs were successfully transfected into the cells. For both a similar pattern of activity is seen, however the transcription sties closer to the gene start site, in the Namawala cell line, appear to be less pronounced than that of the U937 cell line. This difference in activity was possibly due to several reasons. The first was that this was a different cell type, the U937 cell line is a monocytic cell line, while the Namawala cell line is a B lymphocyte cell line and as such the IL-10 gene may be differently regulated within the different cell types. This is not unseen in the IL-10 gene. As mentioned earlier in chapter 1, the HSC when IL-10 is activated it remains constitutively activated, while in the monocyte, IL-10 eventually switches itself off, via a negative feedback loop. This would suggest that with IL-10 there is a difference in the ability of the transcription factors to bind to the gene promoter region, either different transcription factors are binding or they bind differently to their sites. Secondly, the U937 cell line had undergone PMA differentiation that could alter the transcription factor ratio involved in IL-10 activation. The Namawala cell line has been EBV transformed and as such is constitutively producing IL-10, since the gene is constantly on, this could

lead to less transcription factors being available to drive the exogenously added IL-10 promoter constructs.

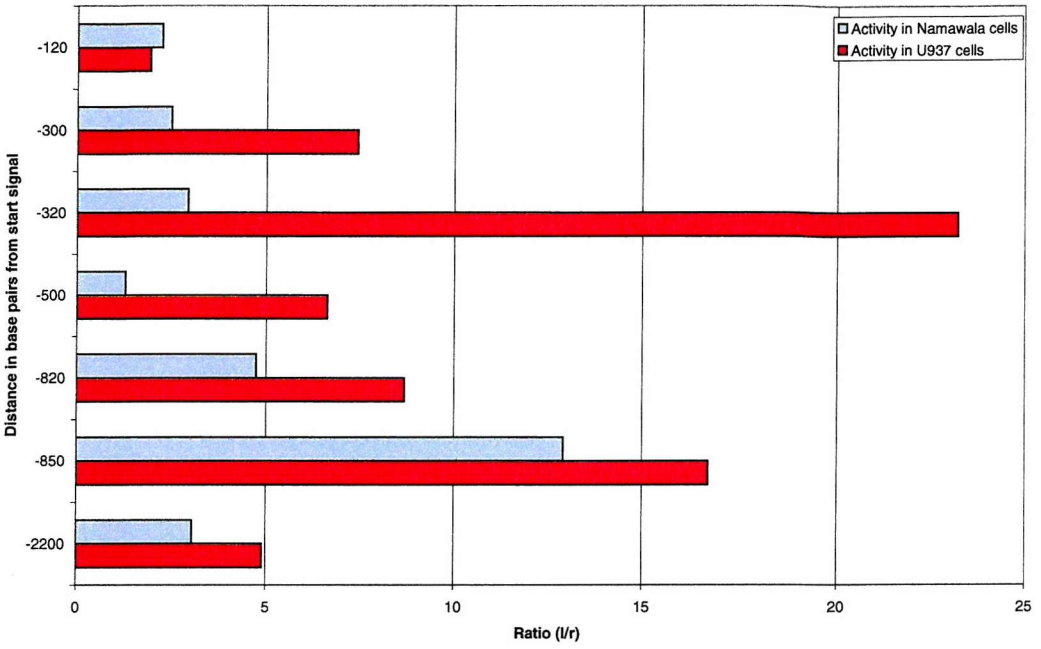


Figure 7.12 Comparison of activity between the U937 and Namawala cell lines transfected with the IL-10 promoter constructs. Y-axis shows the distance from the start site, plasmid names can be found table 7.1

The above data does not include the positive IκB and negative pGL-2 results since for these the positive results had a ratio in excess of 4500 units which would have masked the signal and the negative had a ratio less than 1.

It has been mentioned before (Chapter one) that cAMP has been found to up regulate the activation of IL-10 in the monocyte cells. To this end we decided to try and see if we could augment the IL-10-luciferase signal in the transfected cells by the addition of exogenous dibutyryl cAMP (a cell permeable analogue of cAMP). The experimental protocol was the same as above, with the exception that dibutyryl cAMP was added to the cells after transfection took place.

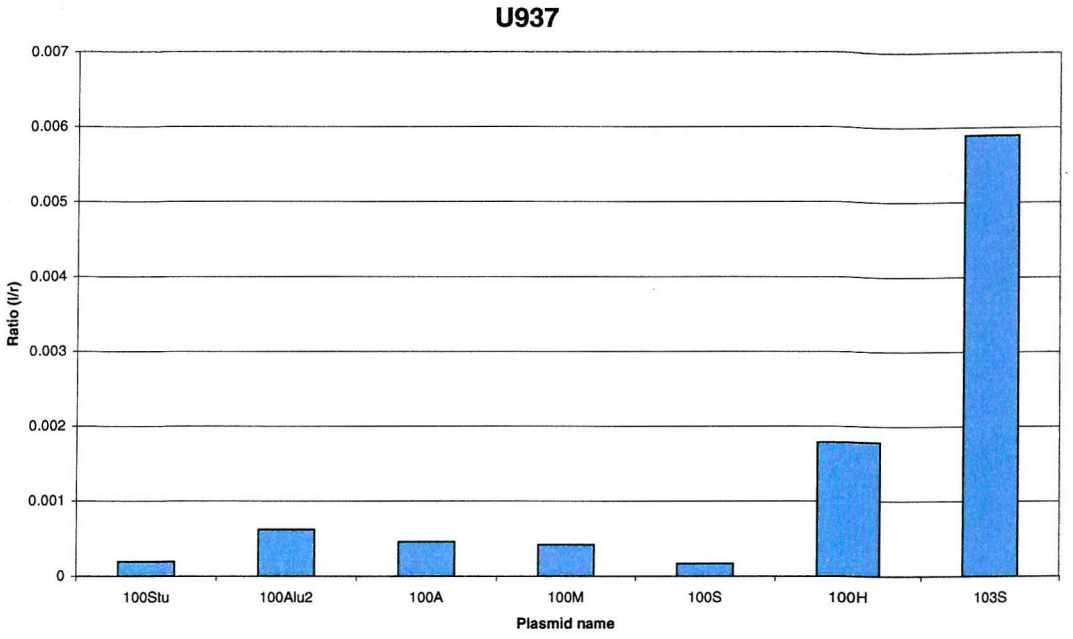


Figure 7.13 Effect of exogenous dibutyryl cAMP on the promoter activity in the transfected U937 cell line.

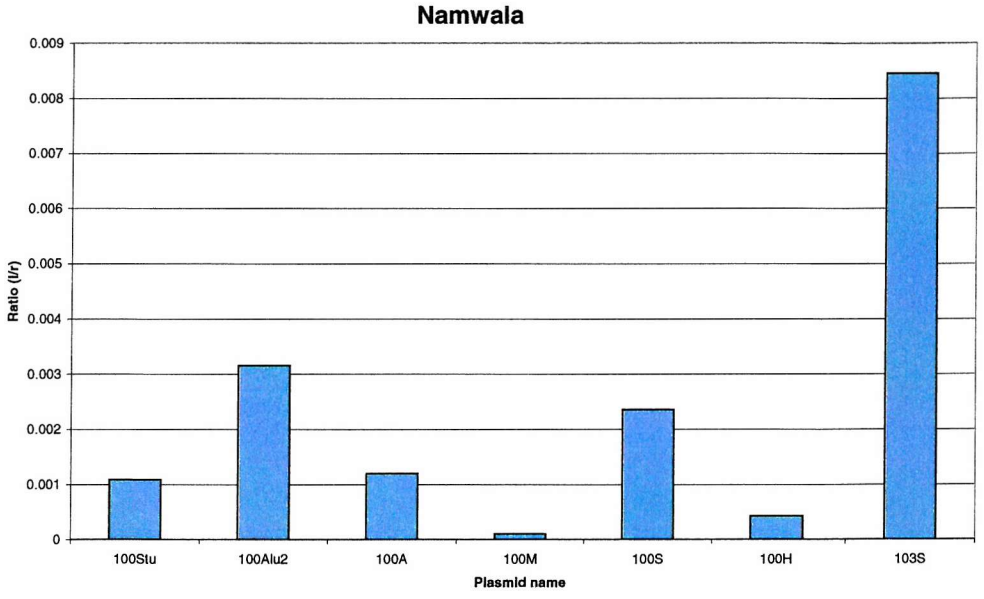


Figure 7.14 Effect of exogenous dibutyryl cAMP on the promoter activity in the transfected namawala cell line.

As can be seen from the above data, the addition of exogenous dibutyryl cAMP, leads to a different profile in luciferase activity within the two cell types. In the U937 cell line, the 100Stu, 100Alu2, 100M and 100H plasmids appear to have a similar profile as was seen when no dibutyryl cAMP was added. However in the 100A and

the 100S plasmids there was a marked decrease in luciferase activity after dibutyryl cAMP addition, with the 103S plasmid, having greatly increased activity. With the Namawala cell line, the 100Stu, 100A, 100M and 100S plasmids appeared to have similar levels of activation, while the 100Alu2, 100H and 103S all having an increase in activity, with the greatest increase in the 103S plasmid as was seen in the U937 cell line.

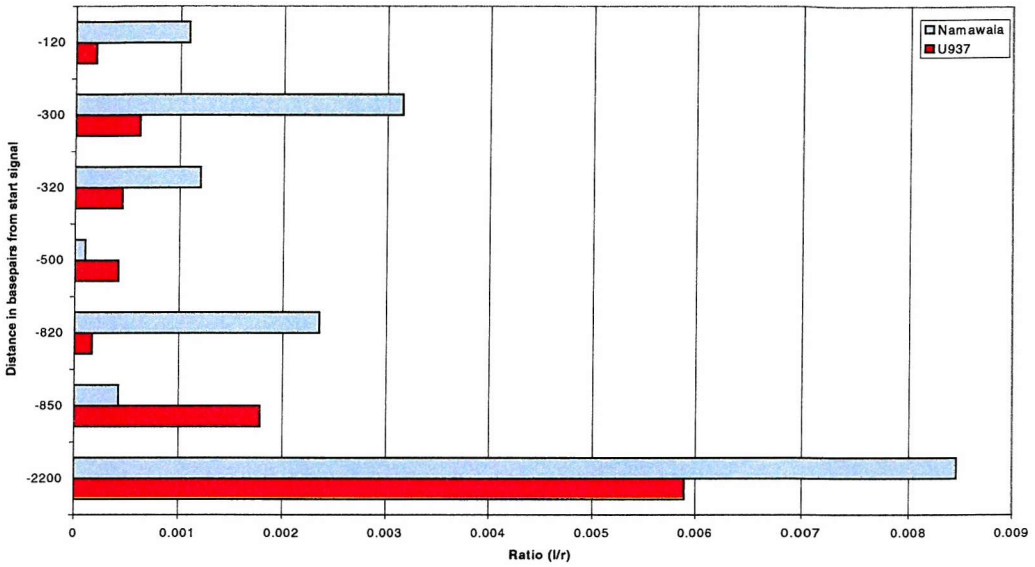


Figure 7.15 Comparison of activity between the U937 and namawala cell lines transfected with the IL-10 promoter constructs. Y-axis shows the distance from the start site, plasmid names can be found table 7.1

With such a difference at the promoter level of transcription, we decided to investigate how dibutyryl cAMP effected IL-10 production in the primary and U937 cell line at the protein level.

7.5 Effect of Dibutyryl cAMP on IL-10 Secretion

As before, U937 cells were pre-treated with PMA for 4 days followed by 24hrs with dibutyryl cAMP. After this they were stimulated with LPS. For the primary cells, after isolation cells were treated with dibutyryl cAMP for 24hrs followed by LPS stimulation.

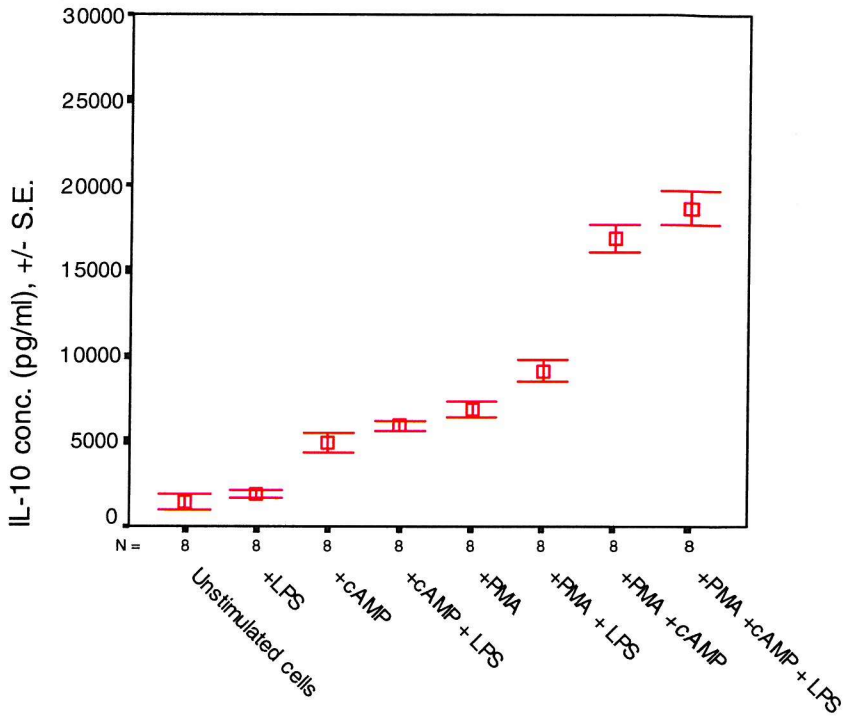


Figure 7.16 Effect of dibutyryl cAMP pre-treatment on the IL-10 secretion in PMA differentiated U937 cells stimulated with LPS. dibutyryl cAMP was added at 100µg/ml for 24hrs before LPS was added. All data points are +/- S.E.

From the data obtained, dibutyryl cAMP augmented IL-10 secretion in the U937 cell line. This result mirrored what was observed with luciferase activity in the transfected cells. Without PMA differentiation, then the U937 cell line was unresponsive to LPS, however, the addition of PMA caused the cells to become active and produce IL-10. However, dibutyryl cAMP, is able to override this activation by PMA and lead to an IL-10 response, which is not significantly increased by LPS stimulation. With PMA and dibutyryl cAMP this response was once more increased, however PMA and dibutyryl cAMP together induces an IL-10 response, which was once again, not significantly increased with the addition of LPS. When we examined the luciferase activity on transfected cells treated in the same

way we observed that most of the responses were the same except for the addition of PMA and LPS. This led to a greatly increased level of protein production, but not in transcription. The addition of dibutyryl cAMP also leads to a much higher level of protein production than is seen at the transcription level. However, as seen in the protein secretion and transcription activity, the highest level of protein secretion and transcription were induced with PMA + dibutyryl cAMP and PMA + dibutyryl cAMP + LPS, with little differences seen between them.

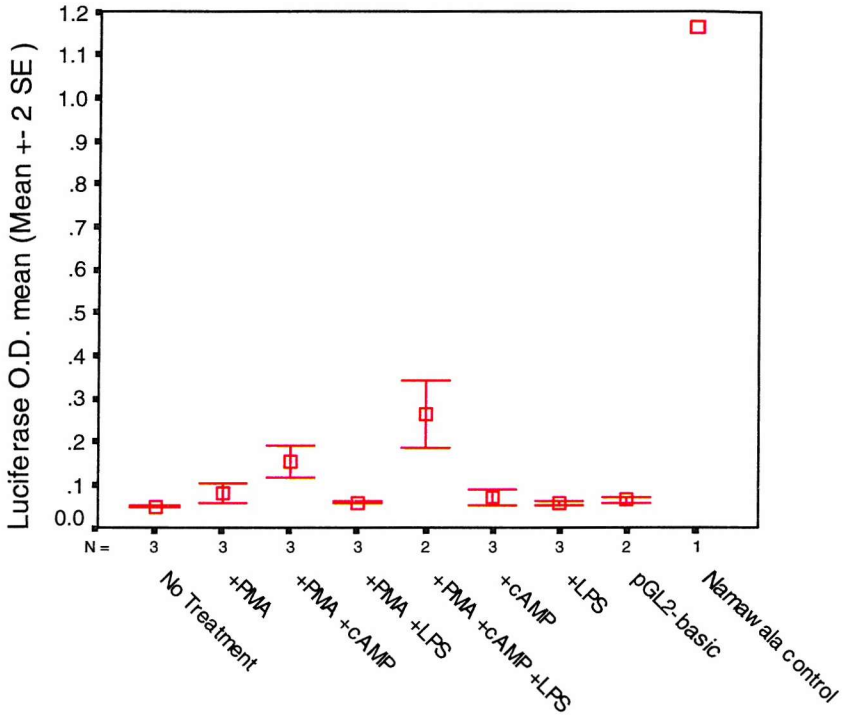


Figure 7.17 Effect of dibutyryl cAMP pre-treatment on the IL-10 promoter transcription in PMA differentiated U937 cells stimulated with LPS. dibutyryl cAMP was added at 100µg/ml for 24hrs before LPS was added. All data points are +/- 2S.E.

However, this was not the response that was seen in the primary cells. In the U937 cell line the dibutyryl cAMP promoted an up-regulation in IL-10 secretion, in the primary cells the opposite was observed with dibutyryl cAMP causing a down-regulation in IL-10 secretion.

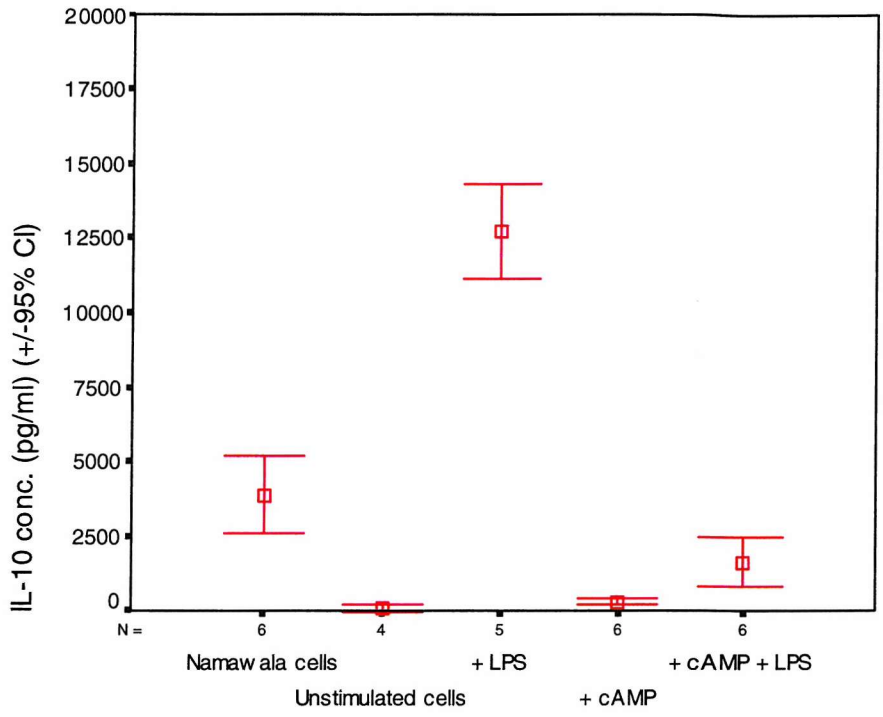


Figure 7.18 The effect of dibutyryl cAMP on the IL-10 secretion of primary monocytes stimulated with LPS for 24hrs. Data points are shown +/- 95% confidence levels.

This result took us by surprise since we expected the dibutyryl cAMP to elevate the IL-10 production in the monocytes as previously reported by Meisel, et al. in 1996127. However, after repeating this experiment several times we came to the conclusion that the dibutyryl cAMP was indeed down regulating the IL-10 response in monocytes. To further confirm this result we added epinephrine to the primary monocytes. Epinephrine works upon the cell by increasing the intracellular level of cAMP, thus acts like the biological equivalent of the addition of exogenous dibutyryl cAMP.

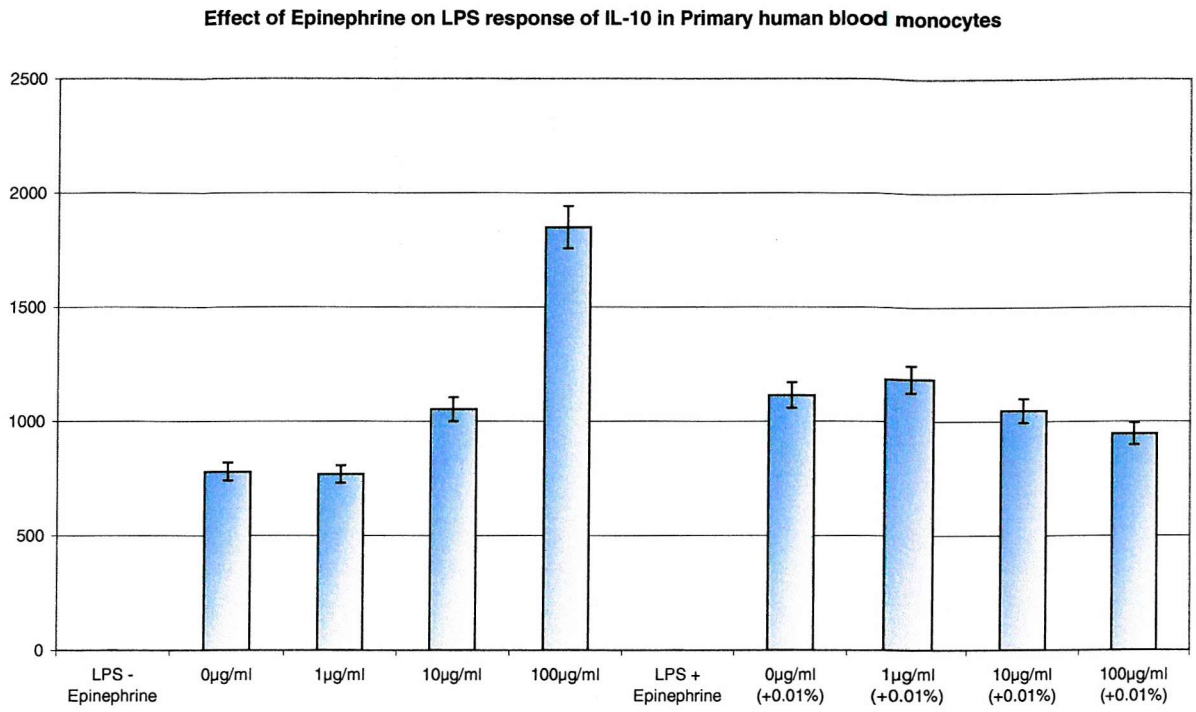
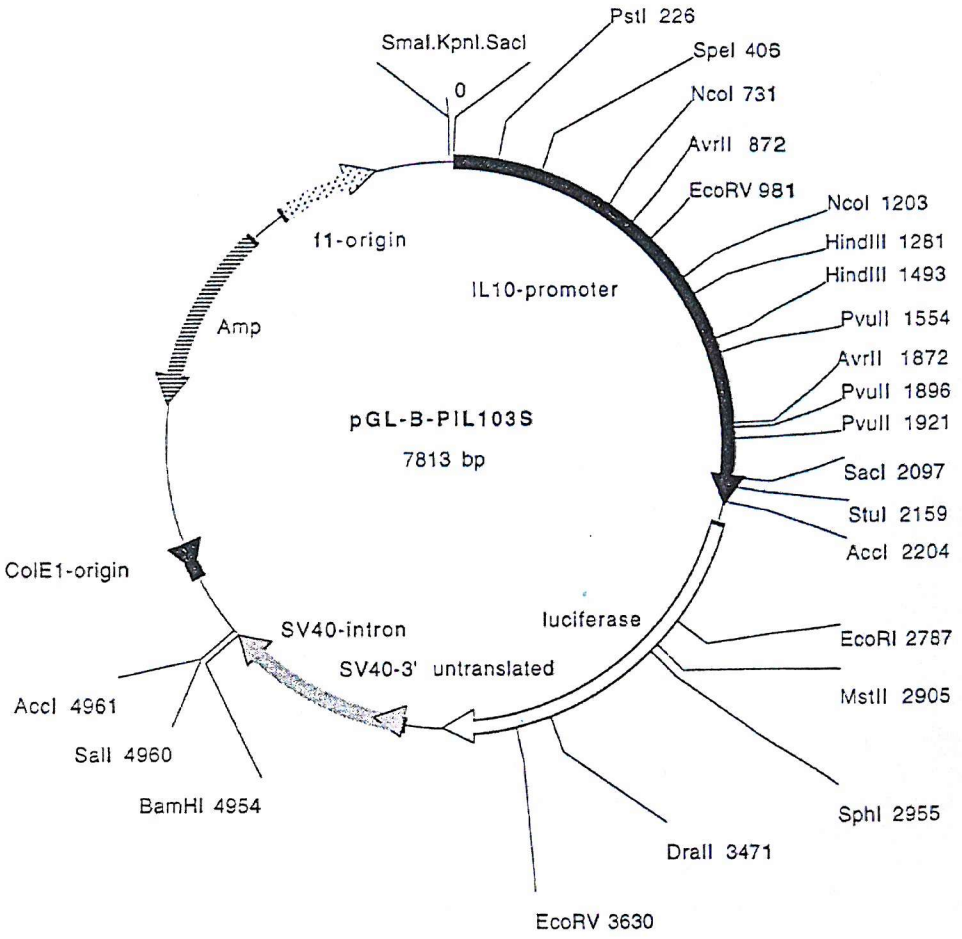


Figure 7.19 The effect of epinephrine on the LPS induces secretion of IL-10 by primary monocytes. Results show +/- 2S.E.

Once again, as with the dibutyryl cAMP, the addition of the epinephrine caused a down regulation in the IL-10 secretion in the primary cells. This new evidence, taken with the earlier evidence obtained from the inhibitor work in chapter 6, led us to the conclusion that like the THP-1 and other cell line before it, the U937 cell line would prove unsuitable for a cell line model to study the transcription regulation of IL-10 in the monocyte.

Figure 7.20 Plasmid map for the pGL-B-103S plasmid as supplied by Dr. D. Kube.



Plasmid name: pGL-B-PIL103S
Plasmid size: 7813 bp
Constructed by: D.Kube
Construction date: 11/94
Comments/References: 2200bp frag.of human interleukin10-promoter in pGL2-basic to direct luciferase expression, created by insertion of a SacI genomic fragment into the SacI-site of the plasmid pGL-B-PIL100H, contains the IL10 tr.st.s.

7.5 Discussion

Although various transfection methods were tested, it was found that only the electroporation method gave consistent transfection of the monocytic cell lines. The preferred technique was to try and transfect the primary monocytes in order to look at the transcription regulation of IL-10. However, we were unable to repeat the transfection method utilised by Ashlee Moses, there are various reasons why this could have been the case. First, one of the functions of the macrophage is to hunt down and destroy naked DNA and large particulate matter. Therefore, since the

Lipofectin reagent must be left on the monocytes for so long, it could be leading to the cells destroying the particles carrying the DNA, thus preventing transfection. Second, naked DNA has been shown to be toxic (at high levels) to cells. Since not all the DNA will be incorporated into the cell, the remaining DNA could be killing the cells. We tried to centrifuge the monocytes after the transfection, but we found that many of the cells were being destroyed by this step. Finally, since the Lipofectin reagent used only works in conditions whereby all serum has been removed from the media, the lack of serum could be killing off the cells or inducing apoptosis, especially the primary cells which are not as hardly as a cell line. Therefore, a combination of any one or more of these reasons could account for the failure of the primary cell to transfect. When we contacted Ashlee Moses, we found that he had given up on the transfection experiments involving primary human cells shortly after publishing his paper and as such could offer no advice on how to deal with this problem. Therefore we decided to move on to the cell lines.

As mentioned earlier, we originally found the THP-1 cell line to be LPS responsive and so began to experiment with this for electroporation. Later, when we found that this original observation was false, we transferred the data we had collected from the THP-1 experiments to the U937 cell line, also transfectable using the electroporation technique. From the collected data on the IL-10 promoter constructs, we found that they were responding in the U937 to LPS stimulation, after PMA differentiation, in a way that was similar to the human cell line Namawala, with similar patterns of activation being observed. This proved to be promising, however, we had found that the O.D. readings collected from the IL-10 constructs was very low, for example the IκB control was giving readings routinely between 4500 and 5500 units, while the IL-10 constructs was only giving a signal of 12 – 15 units. Fearing that the signal we were getting was too low, we decided to augment this signal by the addition of dibutyryl cAMP. Several groups to augment the IL-10 response of the monocyte had used dibutyryl cAMP and we decided to use it to enhance the response of the IL-10 constructs. Within the cell cAMP has many functions, from fatty acid metabolism to it's role as a second messenger, however one of it's functions is also to regulate the action of protein kinase A, which in turn activates the MAP kinase pathways. Therefore by increasing the concentration of cAMP, we increase the activation of the MAP kinases, thus increase the level of transcription of the IL-10 promoter. When added, however, we found that the pattern of luciferase activation was different than

that seen without the addition of dibutyryl cAMP. Instead of simply augmenting the response, allowing a greater reading to be obtained, we discovered that the pattern of activation in the promoter constructs had changed. After seeing this we decided to investigate what effect the dibutyryl cAMP was having at the protein level.

In the U937 cell line, the dibutyryl cAMP was shown to increase the activation of the cells and as such increase the IL-10 secretion. The dibutyryl cAMP was, on it's own, as good as the PMA at activating the U937 cell line. This was probably due to the cAMP, like the PMA, activating the MAP kinases. Although an increase in IL-10 production is seen with PMA stimulation alone, this is greatly increased by the addition of LPS, however, when cAMP is added to PMA differentiated cells, the later addition appears to have no effect on the already increased IL-10 production. This would indicate that the addition of the exogenous cAMP is, itself mimicking the LPS response. Unfortunately when we added the dibutyryl cAMP to the primary cells, the opposite happened, the IL-10 secretion was inhibited. The reason for this apparent reversal in role of the cAMP, as yet, still remains unknown, but this fundamental difference in the basic cell biology between the U937 cell line and the primary monocyte would indicated that cell line was also unsuitable to use as a model in monocyte/macrophage transcription regulation.

Chapter 8

Discussion

8.1 Discussion

In order to determine if a cell line model of primary human monocytes could be developed, it was necessary, first, to develop an assay system that could be utilised to map the IL-10 response of the primary monocytes. This response could then be used to determine which monocyte cell line, if any, responded to endotoxin challenge.

Using a matched antibody pair, and by diluting them to find the optimal concentration, as well as various buffers an Sandwich ELISA assay system was developed. We found that using Avidin-HRP gave quite variably and sometime very high background readings. To counteract this we switched the detection system from the avidin-HRP to the more specific Streptavidin-HRP. This change led to the background readings being reduced, thus allowing a more sensitive assay that could measure even small changes in IL-10 secretion. In order to test the assay, we diluted the supernatant collected from LPS stimulated primary monocytes, in parallel with we standard curve. From this we were able determine that the supernatants were not saturating the antibodies and was thus detecting all the IL-10 present in the sample accurately. By spiking the supernatants and looking at the percentage recovery from the assay, we were able to confirm this result, as well show that no proteolysis was taking place within the supernatant samples. With the assay system working, we were able to use it in future experiments.

We have shown that the macrophage responds to endotoxin by producing IL-10 in a dose dependent manner. This IL-10 response reaches a peak of production at between 36 and 48hrs and continues for at least 7 days post stimulation. In the unstimulated cell, the levels of IL-10 produced were too low to measure. However, upon stimulation the levels of IL-10 rise in accordance with the dose of endotoxin added to the cells. We have shown, for the first time, that the secretion of IL-10 does not appear to have an upper limit.

IL-10 is a potent inhibitor of macrophage function, so small changes in IL-10 concentration are generally enough to witness a down-regulation in inflammation. However, unlike in other pro-inflammatory cytokines, IL-10 does not show significant changes in IL-10 secretion until high levels of LPS, in fact generally the level of LPS needed to raise the IL-10 secretion significantly is 10-100 times higher than the LPS dose require for maximum TNFα production. Interestingly, this means that a peak of IL-10 production would not appear until the endotoxin reaches non-

biological relevant levels. However, there are conditions in which high levels of endotoxin are found in the system. One of these is septic shock. Often in critically ill patients their immune system is compromised which leads to high levels of infection. This high level of infection of course leads to a high level of endotoxin. Since the macrophage responds to endotoxin by producing IL-10 in a dose dependent manner, it is not unreasonable to hypothesise that the macrophage will also produce the IL-10 in response to the bacterial infection. If the level of IL-10 rises to high, however, then the ability of the body to fight this infection will drop dramatically which in turn leads to septic shock. Perhaps a method of control for septic shock, therefore, would be to control the level of IL-10 with neutralising antibodies, thus allowing the body time to recover.

The up-regulation of IL-10 is, however, not limited to septic shock. Indeed many disease functions have been liked to IL-10 up-regulation. In systemic lupus erythemotosus (SLE), the enhancement of auto-antibodies can be inhibited by the addition of anti IL-10 monoclonal antibodies128, and the disease activity has been shown to correlate with the IL-10 titre in the sera of SLE patients129,130. In Myasthenia gravis is an antibody-mediated autoimmune disease, where antibodies are directed against the nicotinic acetylcholine receptors. IL-10 has been shown to enhance the acetylcholine receptor antibody production131. In Sjogren's syndrome, an autoimmune disorder in which lymphocytic infiltration and destruction of salivary and lacrimal glands occurs, IL-10 has been shown to be elevated in the serum of patients and this upregulation stimulates B cell proliferation132-134. In patients with Systemic sclerosis, an increased level of IL-10 has been reported135 and in patients with Kawasaki disease, IL-10 has been shown to down regulate the acute inflammatory response136-138. Indeed this last disease state and the observations made seen to support the hypothesis that highly elevated levels of IL-10 could be responsible septic shock.

The failure of many of the cell lines to produce IL-10 in response to endotoxin challenge, with or without pre-treatment, came as a disappointment and a surprise at first. However, if thought about this lack of an IL-10 response is, perhaps, not quite as surprising as first it seemed. As mentioned in chapter one, the role of IL-10 in inflammation is quite simply to deactivate the macrophage. Since the monocyte cell lines are all mutant cells which originate from cancer cells. It is not surprising, therefore, that perhaps one of the ways in which these cells are able to grow, within

the hostile environment of the body, is that the mechanisms which deactivate the cell are switched off. This, of course, could include IL-10. This would answer the question of why the cell lines are not producing IL-10 in response to LPS, even, in most cases, with pre-treatment with the phorbol ester PMA.

We have also shown that IL-10 and TNFα are both produced by the same cell at the same time. Since this is the case, it is also reasonable to hypothesise that IL-10 is able to regulate the production of TNFα within the same cell. Most likely this happens at the second messenger or transcriptional level, by mRNA degradation.

The production and secretion of IL-10 by the macrophage has been linked to the MAP kinase pathways by the use of pharmacological inhibitors. This is unsurprising considering the fact that the MAP kinase pathways have been associated with many of the factors which contribute wholly or partially towards the inflammatory response, such as stress from the endotoxin levels, chemokines, cytokines as well as superoxides and RNI. Also proliferation, growth, pro-death (apoptosis) and regeneration (as in the case of the nervous system). The data presented in this study, supports this theory, since the broad range MAP Kinase pathway inhibitor, herbimycin A, does cause a dose dependent down regulation in IL-10 production. However, the lack of effect of the PD98059 inhibitor suggests that the p42/44 MAP kinase pathway played little to no role in the IL-10 secretion response of the macrophage to LPS.

By using the pharmacological inhibitor SB203580, we have shown that IL-10 secretion is regulated through the p38 MAP kinase pathway, since inhibition of this pathway leads to a dose dependent down regulation in IL-10 secretion. Recently A. Foey has published a paper that supports our finding139. Until recently, the regulation of the JNK/SAPK pathway could not be studied, in great detail, since the only method available for inhibition of this pathway was by the use of peptides, which were micro-injected directly into the cell. However, with the discovery of the inhibitor curcumin, isolated from turmeric, which inhibits the JNK pathway, this pathway, perhaps for the first time, is being studied in detail. We have shown for the first time that blockage of the JNK pathway, within the primary monocyte leads to a complete inhibition of the IL-10 response to endotoxin. However, in the U937 cell line, we found that the regulation of IL-10, by the MAP kinase pathways is not quite as clear as it was within the primary cell. Both p38 and JNK pathways appear to be important to the regulation of IL-10 within the U937, as they were in the primary

cells, since inhibition still leads to a down-regulation in the IL-10 response. However, the effect of the JNK pathway is not as clear as it was in the primary cell due to the high variation of the response. This was most likely due to small variations in the concentration of PMA in the media, from experiment to experiment as well as small differences in the curcumin from the primary cell experiments. However further testing of this theory will be necessary. Nor was the inhibition of IL-10 production as great as it was in the primary cell. In addition, the effect of the p42 inhibitor PD98059 was surprising since it showed a much higher rate of inhibition than in the primary cells, which was shown unimportant in IL-10 regulation within the primary cell. Again, the only explanation which accounts for this would be the addition of the PMA, since in the RAW267.4 cell line, the PD98059 mirrors the primary cells and had little to no effect on the IL-10 production.

Of course, it could always be argued that the inhibitory effects seen by the inhibitors are nothing more that the toxic effects of these potent chemicals upon the cells themselves. However, after performing a viability count on the inhibitor treated cells, it was observed that the inhibitors did not appear to have any toxic effects upon the cells at the concentrations used in this study. As such the inhibitory effects of these inhibitors can be said to be due to the effect of the inhibitors on the cells and not cell toxicity. Similar results were seen for all cell lines tested.

In 1994, Ashlee Moses published a paper in which he described his technique in which he transfected in an HIV long terminal repeat-CAT plasmid and looked at the expression in primary monocytes obtained from human blood124. In his study he transfected in the HIV long terminal repeat-CAT plasmid using the Lipofectin technique. This was a process which, if possible, would lead to a greater understanding of how the IL-10 promoter is regulated as IL-10 promoter constructs could be directly transfected into the primary cells to see what effect they would have. However, when I repeated the experiments, which Ashlee Moses carried out, I found that it was impossible to transfect the primary cells. This was unsurprising since it has been long known that an excess of naked DNA will lead to cell death in primary cells, as well as cell lines125. Also it has been well established that one of the primary jobs for the macrophage is to seek out and destroy both particulate matter (in the case of the liposomes) and naked DNA (in the case of plasmid not encased in lipid). When we contacted Ashlee Moses we found that he was no longer carrying on with this line of research, instead he had stopped shortly after the paper

had been published. For this reason, we decided to stop this method of investigation and concentrate on transfection of cell lines.

Since IL-10 builds up over the course of several days, it was decided that it would be better to use a CAT analysis system since luciferase shows only transient expression. Luciferase has a half-life of 3hrs, while CAT has a half-life of around 50hrs126. The CAT construct used was the Ψ-CAT plasmid, which is IFNγ responsive. However, upon investigation it was found that either the plasmid had become unresponsive or the IFNγ was no longer active. This was possibly due to various methods used to isolate the plasmid, various storage conditions of the plasmid and the IFNγ, the repeated freeze/thaw as well as the conditions used to grow the bacteria stock up in. With this in mind, we decided to return to the luciferase reporter system. From early experiments on the THP-1 cell line, we had established that at a concentration of 40μg/million cells of DNA would lead to the optimal transfection rate. A voltage of 300V at 196 faradays give the best transfection before leading to cell death. When we transferred this protocol to the U937 and Namawala cell lines, we found that the same conditions gave the optimum transfection. Using the negative control plasmid of pGL2-basic and the IκB-LUC plasmid, we are able to adequately control for our experiments, with the rate of transfection being determined from a renillian co-transfection ratio. Finally, we know at what point, post stimulation, we need to collect and lyse the cells to look at luciferase activity, to determine cell activation post LPS stimulation. This was important to determine because of the short half-life of the luciferase. If we chose a point too late then most of the luciferase would begin to degrade reducing the sample. Similarly, choosing a time point to early would not allow a sufficient build up of the luciferase to be measure sufficiently.

From the collected data on the IL-10 promoter constructs, we found that they were responding in the U937 to LPS stimulation, after PMA differentiation, in a way that was similar to the human cell line Namawala, with similar patterns of activation being observed. This proved to be promising, however, we had found that the O.D. readings collected from the IL-10 constructs was very low, for example the IκB control was giving readings routinely between 4500 and 5500 units, while the IL-10 constructs was only giving a signal of 12 – 15 units. Fearing that the signal we were getting was too low, we decided to augment this signal by the addition of dibutyryl cAMP. Several groups have used dibutyryl cAMP to augment the IL-10 response of

the monocyte140,141 and we decided to use it to try and enhance the response of the IL-10 promoter constructs. Within the cell cAMP has many functions, from fatty acid metabolism to it's role as a second messenger, however one of it's functions is also to regulate the action of protein kinase A, which in turn activates the MAP kinase pathways. Therefore by increasing the concentration of cAMP, we increase the activation of the MAP kinases, thus increase the level of transcription of the IL-10 promoter. Also, the IL-10 promoter has within it several cAMP binding motifs, therefore another way in which the IL-10 signal could be increased was by the direct binding of the cAMP to the promoter. When added, however, we found that the pattern of luciferase activation was different than that seen without the addition of dibutyryl cAMP. Instead of simply augmenting the response, allowing a greater reading to be obtained, we discovered that the pattern of activation in the promoter constructs had changed. In the U937 cell line, the 100Stu, 100Alu2, 100M and 100H plasmids appear to have a similar profile as was seen when no dibutyryl cAMP was added. However in the 100A and the 100S plasmids there was a marked decrease in luciferase activity after dibutyryl cAMP addition, with the 103S plasmid, having greatly increased activity. With the Namawala cell line, the 100Stu, 100A, 100M and 100S plasmids appeared to have similar levels of activation, while the 100Alu2, 100H and 103S all having an increase in activity, with the greatest increase in the 103S plasmid as was seen in the U937 cell line. After seeing this we decided to look and see what effect the dibutyryl cAMP was having at the protein level.

In the U937 cell line, the dibutyryl cAMP was shown to increase the activation of the cells and as such increase the IL-10 secretion. The dibutyryl cAMP was, on it's own, as good as the PMA at activating the U937 cell line. This was probably due to the cAMP activating, like PMA, on the MAP kinases. Although an increase in IL-10 production is seen with PMA stimulation alone, this is greatly increased by the addition of LPS. However, when cAMP is added to PMA differentiated cells, the later addition appears to have no effect on the already increased IL-10 production. This would indicate that the addition of the exogenous cAMP is, itself mimicking the LPS response. Unfortunately, when we added the dibutyryl cAMP to the primary cells, the opposite happened, the IL-10 secretion was inhibited. The reason for this apparent reversal in role of the cAMP, as yet, still remains unknown, but this fundamental difference in the basic cell biology between the U937 cell line and the

primary monocyte would indicated that cell line was also unsuitable to use as a model in monocyte/macrophage transcription regulation.

With so many differences between the monocytic cell lines and the primary cells, it may be that developing a cell line model which truly mimic's the transcription responses of the primary monocyte may not be possible. If this is the case then the growing evidence of the action of IL-10 on the monocytes as well as the synthesis of this protein, using cell lines, should be carefully evaluated before being accepted as a true picture as to what is happening within the cell. At least at the transcription level. However, over the past couple of years, there has been growing evidence of proteins being regulated, not at the transcription level, but rather at the level of the MAP Kinases. Specifically these proteins are cytokines, for example IL-1β142, IL-2,143,144 IL-3145, IL-8146,147, and MMP148. In 1991 DeWaal proposed that one of the actions of IL-10 was to cause the degradation of pro-inflammatory cytokine mRNA149. A claim which was supported by Fiorentino et al, the same year150. However since then, no one has been able to show how this happens. We propose that the mechanism of action of IL-10 in relation to mRNA stability and degradation is as follows.

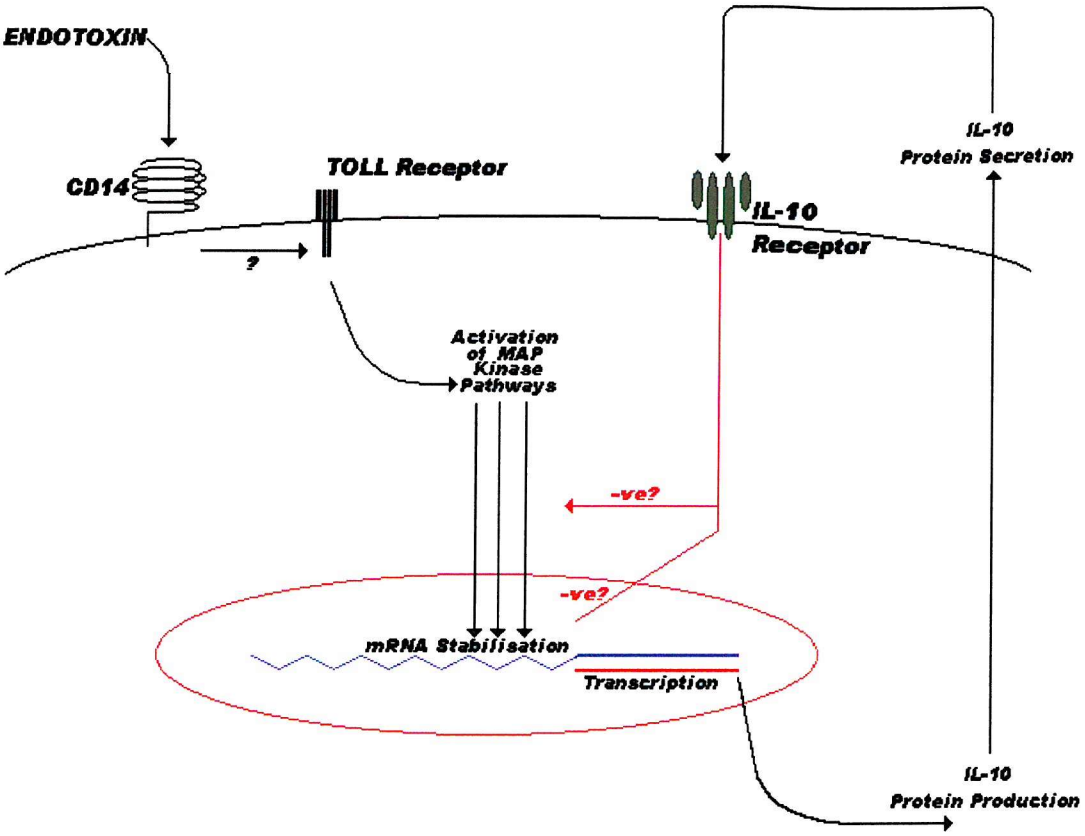


Figure 8.1 Possible mechanism of action of the autoregulatory role of IL-10 within the monocyte. Pro and inflammatory cytokines are activated by the binding of endotoxin to CD14 and the interaction with TOLL receptors. This leads to the activation of the MAP kinase pathways, which leads to the stabilisation of the mRNA of these cytokines. Over the course of the first 6 hours of stimulation, the balance of the cytokine expression lies with the pro-inflammatory cytokines, e.g. TNFα.. After this initial stimulation, the secretion of the anti-inflammatory, e.g. IL-10 begins to over take the pro-inflammatory cytokines. Once at sufficient levels, the IL-10 binds to it's receptors which leads to the shut down of the MAP kinase pathways, which in turn leads to the destabilisation of the mRNA. This in turn prevents the translation and secretion of further protein from the cell.

The exact mechanism by which this would be achieved is, obviously, as yet unknown. Perhaps the IL-10 receptor pathway directly leads to an inhibition of the MAP kinase pathways, or perhaps the activation of the STAT's involved in the IL-10 signalling pathways compete for the phosphorylation of various subunits. It has long since been established that an endotoxin challenge induces a specific pattern of expression of growth factors and cytokines, which regulate injury responses and regeneration. Distinct classes of growth factors and cytokines signal through specific intracellular phosphorylation cascades. For example, the ERK phosphorylation

cascade mediates signalling through transmembrane tyrosine kinase receptors and the JAK/STAT cascade mediates signalling through the GP130 receptor complex. In one study, looking at peripheral nerve injury, the researchers observations suggested that ERK activation is important in the establishment of a regeneration-promoting extracellular environment in the far distal stump of transected nerves and that STAT 3 activation is important in the control of cellular responses close to the site of injury151. This suggested, that while in many cases these pathways may well compete with each other.

Future work in this area may well benefit, therefore in investigating this area oif regulation of the IL-10 response in the primary monocyte as opposed to the transcriptional regulation. Using Western blotting techniques and supershift assays, the effect of STAT activation of the MAP kinase activity could be examined. The mRNA stability could be investigated using Taqman and Northern blotting techniques, to look at the effect of the addition of endotoxin on IL-10 mRNA stability, the effect of the addition of MAP kinase inhibitors has on mRNA stability. As well as the effect of the activation of STAT on mRNA stability.

In conclusion, therefore, while the primary objective of this study, the development of a cell line model to study the transcriptional regulation of the il-10 response to an endotoxin challenge in inflammation, was never achieved several important observations were seen throughout the study. The first was the extremely high levels of IL-10 seen in response to high levels of LPS stimulation, which, as shown, could have important ramifications on several disease states. Next there was the study into which cells were actually producing the pro- and anti-inflammatory cytokines, which showed that the same cells, were producing both, as opposed to monocyte subpopulations, indicating that somehow the monocyte was achieving a switch from a pro- to an anti-inflammatory cell. Again, this observation has important ramifications, since it shows that the same cell is regulating the inflammatory process. We have shown that the IL-10 response to endotoxin challenge is regulated via the MAP kinase pathways, and that the inhibition of either the p38 or the JNK pathways leads to complete inhibition of IL-10 production. Finally, we have shown that the regulation of the monocytic cell lines and the primary monocyte may be too diverse to prove to be of any use in this form of research and that conclusions based on these cell lines in relation of transcriptional regulation should be warily received.

Chapter 9

References

Reference List

1. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1989; 170:2081-20095.

2. O'Garra, A., G. Stapleton, V. Dhar, M. Pearce, J. Schumacher, H. Rugo, D. Barbis, A. Stall, J. Cupp, K. Moore, P. Vieira, T. Mosmann, A. Whitmore, L. Arnold, G. Haughton, and M. Howard. 1990. Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. *INT.IMMUNOL.* International-Immunology. 1990; 2:821-832.

3. Moore, K. W., P. Vieira, D. F. Fiorentino, M. L. Trounstine, T. A. Khan, and T. R. Mosmann. 1990. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. *SCIENCE* Science. 1990; 248:1230-1234.

4. Vieira, P., M. R. De Waal, M. N. Dang, K. E. Johnson, R. Kastelein, D. F. Fiorentino, J. E. DeVries, M. G. Roncarolo, T. R. Mosmann, and K. W. Moore. 1991. Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: Homology to Epstein-Barr virus open reading frame BCRFI. *PROC.NATL.ACAD.SCI.U.S.A* Proceedings-of-the-National-Academy-of-Sciences-of-the-United-States-of-America. 1991; 88:1172-1176.

5. Kim, J. M., C. I. Brannan, N. G. Copeland, N. A. Jenkins, T. A. Khan, and K. W. Moore. 1992. Structure of the mouse IL-10 gene and chromosomal localization of the mouse and human genes. *J.IMMUNOL.* Journal-of-Immunology. 1992; 148:3618-3623.

6. Hsu, D. H., M. R. De Waal, D. F. Fiorentino, M. N. Dang, P. Vieira, J. De Vries, H. Spits, T. R. Mosmann, and K. W. Moore. 1990. Expression of interleukin-10 activity by Epstein-Barr virus protein BCRF1. *SCIENCE* Science. 1990; 250:830-832.

7. Niiro, H., T. Otsuka, M. Abe, H. Satoh, T. Ogo, T. Nakano, Y. Furukawa, and Y. Niho. 1992. Epstein-Barr virus BCRF1 gene product (viral interleukin 10) inhibits superoxide anion production by human monocytes. *LYMPHOKINE CYTOKINE RES.* 11:209-214.

8. Mosmann, T. R., J. H. Schumacher, D. F. Fiorentino, J. Leverah, K. W. Moore, and M. W. Bond. 1990. Isolation of monoclonal antibodies specific for IL-4, IL-5, IL-6, and a new Th2-specific cytokine (IL-10), cytokine synthesis inhibitory factor, by using a solid phase radioimmunoadsorbent assay. *J.IMMUNOL.* Journal-of-Immunology. 1990; 145:2938-2945.

9. Ho, A. S. Y., Y. Liu, T. A. Khan, D. H. Hsu, J. F. Bazan, and K. W. Moore. 1993. A receptor for interleukin 10 is related to interferon receptors. *PROC.NATL.ACAD.SCI.U.S.A* Proceedings-of-the-National-Academy-of-Sciences-of-the-United-States-of-America. 1993; 90:11267-11271.

10. Tan, J. C., S. R. Indelicato, S. K. Narula, P. J. Zavodny, and C. C. Chou. 1993. Characterization of interleukin-10 receptors on human and mouse cells. *J.BIOL.CHEM.* Journal-of-Biological-Chemistry. 1993; 268:21053-21059.

11. Tan, J. C., S. Braun, H. Rong, R. DiGiacomo, E. Dolphin, S. Baldwin, S. K. Narula, P. J. Zavodny, and C. C. Chou. 1995. Characterization of recombinant extracellular domain of human interleukin- 10 receptor. *J.BIOL.CHEM.* Journal-of-Biological-Chemistry. 1995; 270:12906-12911.

12. Spencer, S. D., F. Di Marco, J. Hooley, M. S. Pitts, M. Bauer, A. M. Ryan, B. Sordat, V. C. Gibbs, and M. Aguet. 1998. The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1998 0G; 187:571-578.

13. Kotenko, S. V., C. D. Krause, L. S. Izotova, B. P. Pollack, W. Wu, and S. Pestka. 1997. Identification and functional characterization of a second chain of the interleukin-10 receptor complex. *EMBO J.* EMBO-Journal. 1997; 16:5894-5903.

14. Taniyama, T., S. Takai, E. Miyazaki, R. Fukumura, J. Sato, Y. Kobayashi, T. Hirakawa, K. W. Moore, and K. Yamada. 1995. The human interleukin-10 receptor gene maps to chromosome 11q23.3. *HUM.GENET.* Human-Genetics. 1995; 95:99-101.

15. Riley, J. K., K. Takeda, S. Akira, and R. D. Schreiber. 1999. Interleukin-10 receptor signaling through the JAK-STAT pathway. Requirement for two distinct receptor-derived signals for anti-inflammatory action. *J.BIOL.CHEM.* Journal-of-Biological-Chemistry. 1999 JUN 04; 274:16513-16521.

16. Delfino, D. V., E. Lepri, E. Ayroldi, G. Migliorati, S. S. Boggs, and C. Riccardi. 1998. Suppression of natural killer cell differentiation by activated T lymphocytes in long-term cultures of mouse bone marrow. *EXP.HEMATOL.* Experimental-Hematology. 1998 sti; 26:2-9.

17. Yssel, H., R. D. W. Malefyt, M. G. Roncarolo, J. S. Abrams, R. Lahesmaa, H. Spits, and J. E. De Vries. 1992. IL-10 is produced by subsets of human CD4(+) T cell clones and peripheral blood T cells. *J.IMMUNOL.* Journal-of-Immunology. 1992; 149:2378-2384.

18. Howard, M. and A. O'Garra. 1992. Biological properties of interleukin 10. *IMMUNOL.TODAY* Immunology-Today. 1992; 13:198-200.

19. Go, N. F., B. E. Castle, R. Barrett, R. Kastelein, W. Dang, T. R. Mosmann, K. W. Moore, and M. Howard. 1990. Interleukin 10, a novel B cell stimulatory factor: Unresponsiveness of X chromosome-linked immunodeficiency B cells. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1990; 172:1625-1631.

20. Ding, L., P. S. Linsley, L. Y. Huang, R. N. Germain, and E. M. Shevach. 1993. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J.IMMUNOL.* Journal-of-Immunology. 1993; 151:1224-1234.

21. Thompson, S. L., V. Dhar, M. W. Bond, T. R. Mosmann, K. W. Moore, and D. M. Rennick. 1991. Interleukin 10: A novel stimulatory factor for mast cells and their progenitors. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1991; 173:507-510.

22. Enk, A. H. and S. I. Katz. 1992. Identification and induction of keratinocyte-derived IL-10. *J.IMMUNOL.* Journal-of-Immunology. 1992; 149:92-95.

23. Lamkhioued, B., D. Aldebert, A. S. Gounni, E. Delaporte, M. Goldman, A. Capron, and M. Capron. 1995. Synthesis of cytokines by eosinophils and their regulation. *INT.ARCH.ALLERGY IMMUNOL.* International-Archives-of-Allergy-and-Immunology. 1995; 107:122-123.

24. Kluin-Nelemans, J. C., M. G. D. Kester, I. Oving, F. H. M. Cluitmans, R. Willemze, and J. H. F. Falkenburg. 1994. Abnormally activated T lymphocytes in the spleen of patients with hairy-cell leukemia. *LEUKEMIA* Leukemia. 1994; 8:2095-2101.

25. Lu, Z. Y., Z. J. Gu, X. G. Zhang, J. Wijdenes, P. Neddermann, J. F. Rossi, and B. Klein. 1995. Interleukin-10 induces interleukin-11 responsiveness in human myeloma cell lines. *FEBS LETT.* FEBS-Letters. 1995; 377:515-518.

26. Thompson, K. C., A. Trowern, A. Fowell, M. Marathe, C. Haycock, M. J. P. Arthur, and N. Sheron. 1998. Primary rat and mouse hepatic stellate cells express the macrophage inhibitor cytokine interleukin-10 during the course of activation in vitro. *HEPATOLOGY* Hepatology. 1998 016 01; 28:1518-1524.

27. Denizot, Y., P. Feiss, and N. Nathan. 1999. Are lipid mediators implicated in the production of pro- and anti-inflammatory cytokines during cardiopulmonary bypass graft with extracorporeal circulation? *CYTOKINE* Cytokine. 1999 in; 11:301-304.

28. Tomkins, P. T., K. L. Cooper, P. Appleby, and D. G. Webber. 1995. Effect of pharmacological agents on the production of interleukin-10 by the murine

D10.G4.1 T(H)2 cell line in vitro. *INT.J.IMMUNOPHARMACOL.* International-Journal-of-Immunopharmacology. 1995; 17:619-625.

29. Haraguchi, S., R. A. Good, and N. K. Day. 1995. Immunosuppressive retroviral peptides: cAMP and cytokine patterns. *IMMUNOL.TODAY* 16:595-603.

30. Porrini, A. M., D. Gambi, and A. T. Reder. 1995. Interferon effects on interleukin-10 secretion. Mononuclear cell response to interleukin-10 is normal in multiple sclerosis patients. *J.NEUROIMMUNOL.* Journal-of-Neuroimmunology. 1995; 61:27-34.

31. Uria, D. F. 1999. Treatment of multiple sclerosis. *NEUROLOGIA* Neurologia. 1999; 14:1-12.

32. Schandene, L., G. F. Del Prete, E. Cogan, P. Stordeur, A. Crusiaux, B. Kennes, S. Romagnani, and M. Goldman. 1996. Recombinant interferon-alpha selectively inhibits the production of interleukin-5 by human CD4(+) T cells. *J.CLIN.INVEST* Journal-of-Clinical-Investigation. 1996; 97:309-315.

33. Li, Q., B. Sun, K. Dastgheib, and C. C. Chan. 1996. Suppressive effect of transforming growth factor beta1 on the recurrence of experimental melanin protein-induced uveitis: upregulation of ocular interleukin-10. *CLIN.IMMUNOL.IMMUNOPATHOL.* 81:55-61.

34. De Waal, M. R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. De Vries. 1991. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: An autoregulatory role of IL-10 produced by monocytes. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1991; 174:1209-1220.

35. Armstrong, L., N. Jordan, and A. Millar. 1996. Interleukin 10 (IL-10) regulation of tumour necrosis factor a (TNF- alpha) from human alveolar macrophages and peripheral blood monocytes. *THORAX* Thorax. 1996; 51:143-149.

36. Thompson, K., J. Maltby, J. Fallowfield, M. McAulay, S. H. Millward, and N. Sheron. 1998. Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis. *HEPATOLOGY* Hepatology. 1998 016; 28:1597-1606.

37. Randow, F., U. Syrbe, C. Meisel, D. Krausch, H. Zuckermann, C. Platzer, and Volk---HD. 1995. Mechanism of endotoxin desensitization: Involvement of interleukin 10 and transforming growth factor beta. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1995; 181:1887-1892.

38. Ziegler-Heitbrock, H. W. L. 1995. Molecular mechanism in tolerance to lipopolysaccharide. *J.INFLAMM.* Journal-of-Inflammation. 1995; 45:13-26.

39. Arai, T., K. Hiromatsu, N. Kobayashi, M. Takano, H. Ishida, Y. Nimura, and Y. Yoshikai. 1995. IL-10 is involved in the protective effect of dibutyryl cyclic adenosine monophosphate on endotoxin-induced inflammatory liver injury. *J.IMMUNOL.* Journal-of-Immunology. 1995; 155:5743-5749.

40. Hasegawa, A., Y. Ueno, M. Yamashita, T. Nakayama, and T. Tada. 1998. Regulation of T cell autoreactivity to MHC class II by controlling CD80 (B7-1) expression on B cells. *INT.IMMUNOL.* 10:147-158.

41. Abbas, A. K., A. H. Lichtman, and M. A. Pocidalo. *Cellular and Molecular Immunology.* W.B. Saunders Co., Philadelphia.

42. De Waal, M. R., J. Haanen, H. Spits, M. G. Roncarolo, V. A. Te, C. Figdor, K. Johnson, R. Kastelein, H. Yssel, and J. E. De Vries. 1991. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1991; 174:915-924.

43. Bejarano, M. T., M. R. De Waal, J. S. Abrams, M. Bigler, R. Bacchetta, J. E. De Vries, and M. G. Roncarolo. 1992. Interleukin 10 inhibits allogeneic proliferative and cytotoxic T cell responses generated in primary mixed

lymphocyte cultures. *INT.IMMUNOL.* International-Immunology. 1992; 4:1389-1397.

44. Peguet, N. J., C. Moulon, C. Caux, G. C. Dalbiez, J. Banchereau, and D. Schmitt. 1994. Interleukin-10 inhibits the primary allogeneic T cell response to human epidermal Langerhans cells. *EUR.J.IMMUNOL.* European-Journal-of-Immunology. 1994; 24:884-891.

45. Caux, C., C. Massacrier, B. Vanbervliet, C. Barthelemy, Y. J. Liu, and J. Banchereau. 1994. Interleukin 10 inhibits T cell alloreaction induced by human dendritic cells. *INT.IMMUNOL.* International-Immunology. 1994; 6:1177-1185.

46. Ding, L. and E. M. Shevach. 1992. IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function. *J.IMMUNOL.* Journal-of-Immunology. 1992; 148:3133-3139.

47. Alleva, D. G. and K. D. Elgert. 1995. Promotion of macrophage-stimulated autoreactive T cell proliferation by interleukin-10: Counteraction of macrophage suppressor activity during tumor growth. *IMMUNOBIOLOGY* Immunobiology. 1995; 192:155-171.

48. Williams, F. IL-10 inhibits B7 and ICAM1 expression on human monocytes. Marchant, A. and Delville, J. P. EUR.J.IMMUNOL. 24, 1007-1009. 1994.

49. Kubin, M., M. Kamoun, and G. Trinchieri. 1994. Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1994; 180:211-222.

50. De Waal, M. R., H. Yssel, and J. E. De Vries. 1993. Direct effects of IL-10 on subsets of human CD4(+) T cell clones and resting T cells: Specific inhibition of IL-2 production and proliferation. *J.IMMUNOL.* Journal-of-Immunology. 1993; 150:4754-4765.

51. Schandene, L., V. C. Alonso, F. Willems, C. Gerard, A. Delvaux, T. Velu, R. Devos, M. De Boer, and M. Goldman. 1994. B7/CD28-dependent IL-5 production by human resting T cells is inhibited by IL-10. *J.IMMUNOL.* Journal-of-Immunology. 1994; 152:4368-4374.

52. Schwartz, R. H. 1996. Models of T cell anergy: Is there a common molecular mechanism? *J.EXP.MED.* Journal-of-Experimental-Medicine. 1996; 184:1-8.

53. Taga, K. and G. Tosato. 1992. IL-10 inhibits human T cell proliferation and IL-2 production. *J.IMMUNOL.* Journal-of-Immunology. 1992; 148:1143-1148.

54. Chen, W. F. and A. Zlotnik. 1991. IL-10: A novel cytotoxic T cell differentiation factor. *J.IMMUNOL.* Journal-of-Immunology. 1991; 147:528-534.

55. Tan, J. Q., Larson, G. G., and Gesser, B. Human IL-10 is a chemoattractant for CD8 and T lymphocytes and an inhibitor of IL-8 induced CD4+ T lymphocyte migration. J.IMMUNOL. 151, 4545-4551. 1993.

56. Cohen, S. B. 1995. IL-10 and IL-3 synergize to cause proliferation of human T cells. *IMMUNOLOGY* 85:351-356.

57. Carson, W. E., M. J. Lindemann, R. Baiocchi, M. Linett, J. C. Tan, Chou---CC, S. Narula, and M. A. Caligiuri. 1995. The functional characterization of interleukin-10 receptor expression on human natural killer cells. *BLOOD* Blood. 1995; 85:3577-3585.

58. Velde, T., DeWaal Malefyt, R. J., Huijbens, J. E., and Devries Figdor, J. E. IL-10 stimulates monocyte Fc receptor surface expression and cytotoxic activity-Distince regulation of antibody dependent cellular cytotoxicity by IFN-γ. J.IMMUNOL. 142, 4048-4052. 1992.

59. Defrance, T., B. Vanbervliet, F. Briere, I. Durand, F. Rousset, and J. Banchereau. 1992. Interleukin 10 and transforming growth factor beta

cooperate to induce anti- CD40-activated naive human B cells to secrete immunoglobulin A. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1992; 175:671-682.

60. Rousset, F., E. Garcia, T. Defrance, C. Peronne, N. Vezzio, D. H. Hsu, R. Kastelein, K. W. Moore, and J. Banchereau. 1992. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *PROC.NATL.ACAD.SCI.U.S.A* Proceedings-of-the-National-Academy-of-Sciences-of-the-United-States-of-America. 1992; 89:1890-1893.

61. Llorente, L., W. Zou, Y. Levy, P. Y. Richaud, J. Wijdenes, V. J. Alcocer, F. B. Morel, J. C. Brouet, S. D. Alarcon, P. Galanaud, and D. Emilie. 1995. Role of interleukin 10 in the B lymphocyte hyperactivity and autoantibody production of human systemic lupus erythematosus. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1995; 181:839-844.

62. Cassatella, M. A., L. Meda, S. Bonora, M. Ceska, and G. Constantin. 1993. Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta in mediating the production of IL-8 triggered by lipopolysaccharide. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1993; 178:2207-2211.

63. Wang, P., P. Wu, J. C. Anthes, M. I. Siegel, R. W. Egan, and M. M. Billah. 1994. Interleukin-10 inhibits interleukin-8 production in human neutrophils. *BLOOD* Blood. 1994; 83:2678-2683.

64. Kasama, T., R. M. Strieter, N. W. Lukacs, M. D. Burdick, and S. L. Kunkel. 1994. Regulation of neutrophil-derived chemokine expression by IL-10. *J.IMMUNOL.* Journal-of-Immunology. 1994; 152:3559-3569.

65. Takanaski, S., R. Nonaka, Z. Xing, P. O'Byrne, J. Dolovich, and M. Jordana. 1994. Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1994; 180:711-715.

66. Lieu, F. Y. The role of nitric oxide in parasitic diseases. ANN.TROP.MED.PARASITOL. 87, 637-642. 1993.

67. Bogdan, C., Y. Vodovotz, and C. Nathan. 1991. Macrophage deactivation by interleukin 10. *J.EXP.MED.* Journal-of-Experimental-Medicine. 1991; 174:1549-1555.

68. Ma, C., R. W. Tarnuzzer, and N. Chegini. 1999. Expression of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases in mesothelial cells and their regulation by transforming growth factor- beta 1. *WOUND.REPAIR REGEN.* Wound-Repair-and-Regeneration. 1999; 7:477-485.

69. Mertz, P. M., D. L. DeWitt, W. G. Stetler-Stevenson, and L. M. Wahl. 1994. Interleukin 10 suppression of monocyte prostaglandin H synthase-2. Mechanism of inhibition of prostaglandin-dependent matrix metalloproteinase production. *J.BIOL.CHEM.* Journal-of-Biological-Chemistry. 1994; 269:21322-21329.

70. Grellier, L. F. and G. M. Dusheiko. 1997. The role of hepatitis C virus in alcoholic liver disease. *Alcohol Alcohol* 32:103-111.

71. Iredale, J. P. 2001. Hepatic stellate cell behavior during resolution of liver injury. *SEMIN.LIVER DIS.* 21:427-436.

72. Louis, H., J. L. Van Laethem, W. Wu, E. Quertinmont, C. Degraef, B. K. Van Den, A. Demols, M. Goldman, O. Le Moine, A. Geerts, and J. Deviere. 1998. Interleukin-10 controls neutrophilic infiltration, hepatocyte proliferation, and liver fibrosis induced by carbon tetrachloride in mice. *HEPATOLOGY* Hepatology. 1998 016; 28:1607-1615.

73. Thompson, K., J. Maltby, J. Fallowfield, M. McAulay, S. H. Millward, and N. Sheron. 1998. Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis. *HEPATOLOGY* Hepatology. 1998 016; 28:1597-1606.

74. Wang, S. C., M. Ohata, L. Schrum, R. A. Rippe, and H. Tsukamoto. 1998. Expression of interleukin-10 by in vitro and in vivo activated hepatic stellate cells. *J.BIOL.CHEM.* Journal-of-Biological-Chemistry. 1998 20. 2.; 273:302-308.

75. Llorente, L., P. Y. Richaud, C. N. Alcocer, S. R. Ruiz, M. A. Mercado, H. Orozco, D. A. Gamboa, and V. J. Alcocer. 1996. Cytokine gene expression in cirrhotic and non-cirrhotic human liver. *J.HEPATOL.* Journal-of-Hepatology. 1996; 24:555-563.

76. Cobb, M. H. and E. J. Goldsmith. 1995. How MAP kinases are regulated. *J.BIOL.CHEM.* 270:14843-14846.

77. Seger, R. and E. G. Krebs. 1995. The MAPK signaling cascade. *FASEB J.* 9:726-735.

78. Cano, E. and L. C. Mahadevan. 1995. Parallel signal processing among mammalian MAPKs. *TRENDS BIOCHEM.SCI.* 20:117-122.

79. Marshall, C. J. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *CELL* 80:179-185.

80. Herskowitz, I. 1995. MAP kinase pathways in yeast: for mating and more. *CELL* 80:187-197.

81. Payne, D. M., A. J. Rossomando, P. Martino, A. K. Erickson, J. H. Her, J. Shabanowitz, D. F. Hunt, M. J. Weber, and T. W. Sturgill. 1991. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J.* 10:885-892.

82. Zhang, J., F. Zhang, D. Ebert, M. H. Cobb, and E. J. Goldsmith. 1995. Activity of the MAP kinase ERK2 is controlled by a flexible surface loop [published erratum appears in Structure 1995 Oct 15;3(10):1126]. *Structure.* 3:299-307.

83. Doza, Y. N., A. Cuenda, G. M. Thomas, P. Cohen, and A. R. Nebreda. 1995. Activation of the MAP kinase homologue RK requires the phosphorylation of Thr-180 and Tyr-182 and both residues are phosphorylated in chemically stressed KB cells. *FEBS LETT.* 364:223-228.

84. Robinson, M. J., M. Cheng, A. Khokhlatchev, D. Ebert, N. Ahn, K. L. Guan, B. Stein, E. Goldsmith, and M. H. Cobb. 1996. Contributions of the mitogen-activated protein (MAP) kinase backbone and phosphorylation loop to MEK specificity. *J.BIOL.CHEM.* 271:29734-29739.

85. Boulton, T. G., G. D. Yancopoulos, J. S. Gregory, C. Slaughter, C. Moomaw, J. Hsu, and M. H. Cobb. 2000. An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *SCIENCE* 249/4964:-67.

86. Boulton, T. G., S. H. Nye, D. J. Robbins, N. Y. Ip, E. Radziejewska, S. D. Morgenbesser, R. A. DePinho, N. Panayotatos, M. H. Cobb, and G. D. Yancopoulos. 2000. ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *CELL* 65/4:-675.

87. Hunter, T. 2000. Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *CELL* 80/2:-236.

88. Klinz, F. J. and R. Heumann. 1995. Time-resolved signaling pathways of nerve growth factor diverge downstream of the p140trk receptor activation between chick sympathetic and dorsal root ganglion sensory neurons. *J.NEUROCHEM.* 65:1046-1053.

89. Castellani, L., M. C. Reedy, M. C. Gauzzi, C. Provenzano, S. Alema, and G. Falcone. 1995. Maintenance of the differentiated state in skeletal muscle: activation of v-Src disrupts sarcomeres in quail myotubes. *J.CELL BIOL.* 130:871-885.

90. Boonstra, J., P. Rijken, B. Humbel, F. Cremers, A. Verkleij, and H. P. Bergen-en. 1995. The epidermal growth factor. *CELL BIOL.INT.* 19:413-430.

91. Yu, H., S. J. Suchard, R. Nairn, and R. Jove. 1995. Dissociation of mitogen-activated protein kinase activation from the oxidative burst in differentiated HL-60 cells and human neutrophils. *J.BIOL.CHEM.* 270:15719-15724.

92. Zhang, S., J. Han, M. A. Sells, J. Chernoff, U. G. Knaus, R. J. Ulevitch, and G. M. Bokoch. 1995. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J.BIOL.CHEM.* 270:23934-23936.

93. Faux, M. C. and J. D. Scott. 2000. More on target with protein phosphorylation: Conferring specificity by location. *TRENDS BIOCHEM.SCI.* 21/8:-315.

94. Lenormand, P., C. Sardet, G. Pages, G. L'Allemain, A. Brunet, and J. Pouyssegur. 1993. Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. *J.CELL BIOL.* 122:1079-1088.

95. Traverse, S., N. Gomez, H. Paterson, C. Marshall, and P. Cohen. 1992. Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *BIOCHEM.J.* 288:351-355.

96. Alessi, D. R., N. Gomez, G. Moorhead, T. Lewis, S. M. Keyse, and P. Cohen. 1995. Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. *Curr.Biol.* 5:283-295.

97. Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *SCIENCE* Science. 1990; 249:1431-1433.

98. Kitchens, R. L. Role of CD14 in Cellular recognition of Bacterial Lipopolysaccharides. CHEM.IMMUNOL. 74, 61-77. 2000.

99. Weinstein, S. L., M. R. Gold, and A. L. DeFranco. 1991. Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. *PROC.NATL.ACAD.SCI.U.S.A* Proceedings-of-the-National-Academy-of-Sciences-of-the-United-States-of-America. 1991; 88:4148-4152.

100. Prabhakar, U., D. Lipshutz, M. Pullen, H. Turchin, S. Kassis, and P. Nambi. 1993. Protein kinase C regulates TNF- alpha production by human monocytes. *EUR.CYTOKINE NETW.* European-Cytokine-Network. 1993; 4:31-37.

101. Shapira, L., S. Takashiba, C. Champagne, S. Amar, and T. E. Van Dyke. 1994. Involvement of protein kinase C and protein tyrosine kinase in lipopolysaccharide-induced TNF- alpha and IL-1 beta production by human monocytes. *J.IMMUNOL.* Journal-of-Immunology. 1994; 153:1818-1824.

102. Lee, J. C., J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, D. Green, D. McNulty, M. J. Blumenthal, J. R. Heys, S. W. Landvatter, J. E. Strickler, M. M. McLaughlin, I. R. Siemens, S. M. Fisher, G. P. Livi, J. R. White, J. L. Adams, and P. R. Young. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *NATURE* Nature. 1994; 372:739-746.

103. Hambleton, J., S. L. Weinstein, L. Lem, and A. L. DeFranco. 1996. Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. *PROC.NATL.ACAD.SCI.U.S.A* Proceedings-of-the-National-Academy-of-Sciences-of-the-United-States-of-America. 1996; 93:2774-2778.

104. Hambleton, J., M. McMahon, and A. L. DeFranco. 1995. Activation of Raf-1 and mitogen-activated protein kinase in murine macrophages partially mimics lipopolysaccharide-induced signaling events. *J.EXP.MED.* 182:147-154.

105. Foey, A. D., S. L. Parry, L. M. Williams, M. Feldmann, B. M. J. Foxwell, and F. M. Brennan. 1998. Regulation of monocyte IL-10 synthesis by endogenous

IL-1 and TNF- alpha : Role of the p38 and p42/44 mitogen-activated protein kinases. *J.IMMUNOL.* Journal-of-Immunology. 1998; 160:920-928.

106. Boehringer, N., G. Hagens, F. Songeon, P. Isler, and L. P. Nicod. 1999. Differential regulation of tumor necrosing factor- alpha (TNF- alpha) and interleukin-10 (IL-10) secretion by protein kinase and phosphatase inhibitors in human alveolar macrophages. *EUR.CYTOKINE NETW.* European-Cytokine-Network. 1999; 10:211-217.

107. Platzer, C., C. Meisel, K. Vogt, M. Platzer, and H. D. Volk. 1995. Up-regulation of monocytic IL-10 by tumor necrosis factor- alpha and cAMP elevating drugs. *INT.IMMUNOL.* International-Immunology. 1995; 7:517-523.

108. Kambayashi, T., C. O. Jacob, D. Zhou, N. Mazurek, M. Fong, and G. Strassmann. 1995. Cyclic nucleotide phosphodiesterase type IV participates in the regulation of IL-10 and in the subsequent inhibition of TNF- alpha and IL-6 release by endotoxin-stimulated macrophages. *J.IMMUNOL.* Journal-of-Immunology. 1995; 155:4909-4916.

109. Van der, P. T., S. M. Coyle, K. Barbosa, C. C. Braxton, and S. F. Lowry. 1996. Epinephrine inhibits tumor necrosis factor- alpha and potentiates interleukin 10 production during human endotoxemia. *J.CLIN.INVEST* Journal-of-Clinical-Investigation. 1996; 97:713-719.

110. Jacob, F and Monod, J. Genetic regulatory mechanisms in the synthesis of proteins. J.MOL.BIOL. 318-356. 1961.

111. Drapkin, R., A. Merino, and D. Reinberg. 1993. Regulation of RNA polymerase II transcription. *CURR.OPIN.CELL BIOL.* 5:469-476.

112. Muller, M. M., T. Gerster, and W. Schaffner. 1988. Enhancer sequences and the regulation of gene transcription. *EUR.J.BIOCHEM.* 176:485-495.

113. Fromental, C., M. Kanno, H. Nomiyama, and P. Chambon. 1988. Cooperativity and hierarchical levels of functional organization in the SV40 enhancer. *CELL* 54:943-953.

114. Karin, M. 1990. Too many transcription factors: positive and negative interactions. *New Biol.* 2:126-131.

115. Smith-Gill, S. J. 1991. Protein-protein interactions: structural motifs and molecular recognition. *CURR.OPIN.BIOTECHNOL.* 2:568-575.

116. Schneider, M. D., W. R. McLellan, F. M. Black, and T. G. Parker. 1992. Growth factors, growth factor response elements, and the cardiac phenotype. *BASIC RES.CARDIOL.* 87 Suppl 2:33-48.

117. Knolle, P. A., A. Uhrig, U. Protzer, M. Trippler, R. Duchmann, K. H. Meyer-zum-Buschenfelde, and G. Gerken. 1998. Interleukin-10 expression is autoregulated at the transcriptional level in human and murine Kupffer cells. *HEPATOLOGY* 27:93-99.

118. Housby, J. N., C. M. Cahill, B. Chu, R. Prevelige, K. Bickford, M. A. Stevenson, and S. K. Calderwood. 1999. Non-steroidal anti-inflammatory drugs inhibit the expression of cytokines and induce HSP70 in human monocytes. *CYTOKINE* Cytokine. 1999 dev; 11:347-358.

119. Trautman, M. S., D. Collmer, S. S. Edwin, W. White, M. D. Mitchell, and D. J. Dudley. 1997. Expression of interleukin-10 in human gestational tissues. *J.Soc.Gynecol.Investig.* 4:247-253.

120. Frankenberger, M., T. Sternsdorf, H. Pechumer, A. Pforte, and H. W. Ziegler-Heitbrock. 1996. Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. *BLOOD* 87:373-377.

121. Onodera, S., K. Kaneda, Y. Mizue, Y. Koyama, M. Fujinaga, and J. Nishihira. 2000. Macrophage migration inhibitory factor up-regulates

expression of matrix metalloproteinases in synovial fibroblasts of rheumatoid arthritis. *J.BIOL.CHEM.* 2000 Jan 7; 275:1-50.

122. Watabe, M., K. Ito, Y. Masuda, S. Nakajo, and K. Nakaya. 1998. Activation of AP-1 is required for bufalin-induced apoptosis in human leukemia U937 cells. *ONCOGENE* 16:779-787.

123. Watanabe, A., A. Takeshita, S. Kitano, and S. Hanazawa. 1996. CD14-mediated signal pathway of Porphyromonas gingivalis lipopolysaccharide in human gingival fibroblasts. *INFECT.IMMUN.* 64:4488-4494.

124. Moses, A. V., C. Ibanez, R. Gaynor, P. Ghazal, and J. A. Nelson. 2000. Differential role of long terminal repeat control elements for the regulation of basal and Tat-mediated transcription of the human immunodeficiency virus in stimulated and unstimulated primary human macrophages. *J.VIROL.* 68/1:-307.

125. Stacey, K. J., I. L. Ross, and D. A. Hume. 1993. Electroporation and DNA-dependent cell death in murine macrophages. *IMMUNOL.CELL BIOL.* Immunology-and-Cell-Biology. 1993; 71:75-85.

126. Thompson, J. F., L. S. Hayes, and D. B. Lloyd. 1991. Modulation of firefly luciferase stability and impact on studies of gene regulation. *GENE* 103:171-177.

127. Meisel, C., K. Vogt, C. Platzer, F. Randow, C. Liebenthal, and H. D. Volk. 1996. Differential regulation of monocytic tumor necrosis factor- alpha and interleukin-10 expression. *EUR.J.IMMUNOL.* European-Journal-of-Immunology. 1996; 26:1580-1586.

128. Llorente, L., W. Zou, Y. Levy, P. Y. Richaud, J. Wijdenes, V. J. Alcocer, F. B. Morel, J. C. Brouet, S. D. Alarcon, P. Galanaud, and a. et. 1995. Role of interleukin 10 in the B lymphocyte hyperactivity and autoantibody production of human systemic lupus erythematosus. *J.EXP.MED.* 181:839-844.

129. Houssiau, F. A., C. Lefebvre, M. Vanden Berghe, M. Lambert, J. P. Devogelaer, and J. C. Renauld. 1995. Serum interleukin 10 titers in systemic lupus erythematosus reflect disease activity. *LUPUS* 4:393-395.

130. Park, Y. B., S. K. Lee, D. S. Kim, J. Lee, C. H. Lee, and C. H. Song. 1998. Elevated interleukin-10 levels correlated with disease activity in systemic lupus erythematosus. *CLIN.EXP.RHEUMATOL.* 1998 May-Jun; 16:3-8.

131. Zhang, G., X, L. Y. Yu, F. D. Shi, B. G. Xiao, J. Bjork, G. Hedlund, and H. Link. 1998. Cytokine profiles of experimental autoimmune myasthenia gravis after treatment by the synthetic immunomodulator Linomide. *ANN.NEW YORK.ACAD.SCI.* Annals-of-the-New-York-Academy-of-Sciences. 1998 rc0 43; 841:580-583.

132. Ohyama, Y., S. Nakamura, G. Matsuzaki, M. Shinohara, A. Hiroki, T. Fujimura, A. Yamada, K. Itoh, and K. Nomoto. 1996. Cytokine messenger RNA expression in the labial salivary glands of patients with Sjogren's syndrome [see comments]. *ARTHRITIS RHEUM.* 39:1376-1384.

133. Fox, R. I., H. Kang, I, D. Ando, J. Abrams, and E. Pisa. 1994. Cytokine mRNA expression in salivary gland biopsies of Sjogren's syndrome. *J.IMMUNOL.* Journal-of-Immunology. 1994; 152:5532-5539.

134. Villarreal, G. M., V. J. Alcocer, and L. Llorente. 1996. Differential interleukin (IL)-10 and IL-13 gene expression in vivo in salivary glands and peripheral blood mononuclear cells from patients with primary Sjogren's syndrome. *IMMUNOL.LETT.* Immunology-Letters. 1996; 49:105-109.

135. Hasegawa, M., M. Fujimoto, K. Kikuchi, and K. Takehara. 1997. Elevated serum levels of interleukin 4 (IL-4), IL-10, and IL-13 in patients with systemic sclerosis. *J.RHEUMATOL.* 24:328-332.

136. Hirao, J., S. Hibi, T. Andoh, and T. Ichimura. 1997. High levels of circulating interleukin-4 and interleukin-10 in Kawasaki disease. *INT.ARCH.ALLERGY IMMUNOL.* 112:152-156.

137. Kim, D. S., H. K. Lee, G. W. Noh, S. I. Lee, and K. Y. Lee. 1996. Increased serum interleukin-10 level in Kawasaki disease. *Yonsei Med.J.* 37:125-130.

138. Hirao, J., S. Hibi, T. Andoh, and T. Ichimura. 1996. [Plasma interleukin-10 levels in Kawasaki disease]. *Arerugi* 45:409-412.

139. Foey, A. D., S. L. Parry, L. M. Williams, M. Feldmann, B. M. Foxwell, and F. M. Brennan. 1998. Regulation of monocyte IL-10 synthesis by endogenous IL-1 and TNF-alpha: role of the p38 and p42/44 mitogen-activated protein kinases. *J.IMMUNOL.* 160:920-928.

140. Delgado, M., E. J. Munoz-Elias, R. P. Gomariz, and D. Ganea. 1999. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide enhance IL-10 production by murine macrophages: in vitro and in vivo studies. *J.IMMUNOL.* 162:1707-1716.

141. Dugas, N., I. Vouldoukis, P. Becherel, M. Arock, P. Debre, M. Tardieu, D. M. Mossalayi, J. F. Delfraissy, J. P. Kolb, and B. Dugas. 1996. Triggering of CD23b antigen by anti-CD23 monoclonal antibodies induces interleukin-10 production by human macrophages. *EUR.J.IMMUNOL.* 26:1394-1398.

142. Mondal, K., O. I. Sirenko, A. K. Lofquist, J. S. Morris, J. S. Haskill, and J. M. Watson. 2000. Differential role of tyrosine phosphorylation in adhesion-induced transcription, mRNA stability, and cytoskeletal organization in human monocytes. *J.Leukoc.Biol.* 67:216-225.

143. Chen, C. Y., R. Gherzi, J. S. Andersen, G. Gaietta, K. Jurchott, H. D. Royer, M. Mann, and M. Karin. 2000. Nucleolin and YB-1 are required for JNK-mediated interleukin-2 mRNA stabilization during T-cell activation. *GENES DEV.* 14:1236-1248.

144. Chen, C. Y., F. Gatto-Konczak, Z. Wu, and M. Karin. 1998. Stabilization of interleukin-2 mRNA by the c-Jun NH2-terminal kinase pathway. *SCIENCE* 280:1945-1949.

145. Ming, X. F., M. Kaiser, and C. Moroni. 1998. c-jun N-terminal kinase is involved in AUUUA-mediated interleukin-3 mRNA turnover in mast cells. *EMBO J.* 17:6039-6048.

146. Holtmann, H., J. Enninga, S. Kalble, A. Thiefes, A. Dorrie, M. Broemer, R. Winzen, A. Wilhelm, J. Ninomiya-Tsuji, K. Matsumoto, K. Resch, and M. Kracht. 2000. The MAPKKK TAK1 plays a central role in coupling the IL-1 receptor to both, transcriptional and RNA-targetted mechanisms of gene regulation. *J.BIOL.CHEM.*

147. Holtmann, H., R. Winzen, P. Holland, S. Eickemeier, E. Hoffmann, D. Wallach, N. L. Malinin, J. A. Cooper, K. Resch, and M. Kracht. 1999. Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. *MOL.CELL BIOL.* 19:6742-6753.

148. Wang, H. and J. A. Keiser. 2000. Hepatocyte growth factor enhances MMP activity in human endothelial cells. *BIOCHEM.BIOPHYS.RES.COMMUN.* 272:900-905.

149. De Waal, M. R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. De Vries. 1991. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J.EXP.MED.* 174:1209-1220.

150. Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J.IMMUNOL.* Journal-of-Immunology. 1991; 147:3815-3822.

151. Sheu, J. Y., D. J. Kulhanek, and F. P. Eckenstein. 2000. Differential patterns of ERK and STAT3 phosphorylation after sciatic nerve transection in the rat. *EXP.NEUROL.* 166:392-402.