UNIVERSITY OF SOUTHAMPTON

THE ROLE OF STROMAL CELL DERIVED INTERLEUKIN-10 IN HEPATIC STELLATE CELL AND MACROPHAGE INTERACTIONS IN LIVER FIBROSIS

by

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A project submitted for the Intercalated Degree in Biomedical Sciences

April 1998

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Cite as: Marathe, Mandar. 'The Role of Stromal Cell Derived Interleukin-10 in Hepatic Stellate Cell and Macrophage Interactions in Liver Fibrosis'. Intercalated BSc. Thesis, University of Southampton, 1998. https://doi.org/10.5281/zenodo.17358078

ABSTRACT

Introduction

Hepatic fibrosis is the common end-point of a diverse range of chronic parenchymal liver injury, and is a major world-wide medical problem, presently with no specific medical treatment. The central process in liver fibrosis is the phenotypic transformation (activation) of the hepatic stellate cell (HSC) from a quiescent vitamin A storing phenotype to a myofibroblastic phenotype, and associated collagen overproduction by HSC with hepatic scar formation in the space of Disse. HSC activation occurs as a result of interactions between HSC and resident hepatic macrophages (Kupffer cells, KC). Our group in Southampton has previously shown that mRNA for the profoundly anti-inflammatory cytokine Interleukin-10 (IL-10) is produced during HSC activation and that ΔIL10 mice suffer more severe fibrosis following experimental CCl_4 intoxication, suggesting a negative feedback mechanism between HSC and KC which may be a novel therapeutic target.

Hypothesis

"HSC can modulate macrophage activation via Interleukin-10, and that the HSC-IL10-macrophage interaction can be modulated therapeutically"

Aims

1. To confirm and quantitatively characterise expression of IL-10 protein during HSC activation *in vitro*.
2. To compare basal vs. induced production of IL-10 in HSC.
3. To examine effects of basal and induced production of HSC-derived IL-10 on macrophage function.

Methods

Rat and murine HSC were isolated and culture-activated on uncoated plastic. Stimulated and unstimulated expression of IL-10 protein (cytoplasmic and released) during the first 120 days was examined with Western analysis, solid-phase enzyme-linked immunosorbent assay (ELISA) and cell-based ELISA. The functional importance of HSC-derived IL-10 was assessed by stimulating RAW 264.7 monocytes with lipopolysaccharide (LPS) in the presence or absence of stimulated or unstimulated live HSC and HSC-conditioned medium, pre-treated with anti-IL-10 neutralising antibody or control.

Results

IL-10 was detected in unstimulated rat HSC conditioned medium at 180-1300pg/ml during days 0-14 post-isolation, followed by a reduction to 0-180 pg/ml thereafter (95%CI, $p<0.001$). This decline is associated with an increased lysate IL-10, detected at 500-1000pg/ml. A similar trend was seen in unstimulated murine HSC. An increase in lysate IL-10 was observed with LPS-stimulation of HSC, mirrored with a decrease in conditioned medium IL-10. A reduction in TNF-α production by LPS-stimulated RAW 264.7 monocytes was observed on pre-exposure to live HSC and HSC conditioned medium; the latter effect was reduced by pre-incubation of the HSC conditioned medium with anti-IL-10 neutralising antibody, whereas neutralisation of HSC-associated IL-10 resulted in even greater reduction in TNF-α production.

Conclusions

Rat and murine HSC express IL-10 protein during activation on plastic. IL-10 is secreted during the first 0-21 days, but is retained intracellularly or associated with cell membrane indefinitely thereafter. During the first 21 days activation *in-vitro*, HSC can down-regulate macrophage activity via IL-10. Basal expression of IL-10 is sub-maximal, and HSC can be induced to increase expression.

IL-10 may have an important negative-feedback role in liver inflammation and fibrosis. The redundancy in the HSC-IL10-macrophage axis in these disease processes is potentially an important target for future therapeutic strategies.

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ABBREVIATIONS

αIL10NAb	Neutralising antibody to interleukin-10
BSA	Bovine serum albumin
CBE	Cell-based enzyme-linked immunosorbent assay
CCl_4	Carbon tetrachloride
CI	Confidence interval
CM	Conditioned medium
ΔIL-10	IL-10 deleted
DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
ECM	Extracellular matrix
FCS	Foetal calf serum
HBSS	Hanks' balanced salt solution
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HSC	Hepatic stellate cell(s)
IL-10	Interleukin-10
KC	Kupffer cell(s)
kDa	Kilodalton
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PSG	Penicillin/streptomycin/gentamycin
RNA	Ribonucleic acid
rmIL-10	Recombinant murine interleukin-10
rmTNF-α	Recombinant murine tumour necrosis factor-alpha
SD	Space of Disse
SDS	Sodium dodecyl sulphate
SEC	Sinusoidal endothelial cell(s)
TNF-α	Tumour necrosis factor-alpha
TGF-β	Transforming growth factor-beta
WT	Wild-type

CHAPTER 1

INTRODUCTION

1.1 Hepatic Cirrhosis

Hepatic cirrhosis as a clinical entity is a major world-wide medical problem. Occurring at any age, cirrhosis presently has no specific medical treatment, and often causes prolonged morbidity. Cirrhosis is an important cause of premature death, causing more than 5000 deaths annually in the UK alone, with 25% overall 5-year survival from diagnosis. Cirrhosis is the common end-point of chronic parenchymal liver disease of various aetiologies, including alcohol (accounting for 50% of cases in UK), hepatitis B, C and D virus infection (especially in Middle East, Far East, Asia and Africa), biliary and autoimmune diseases (primary biliary cirrhosis, biliary atresia), and rarer pathologies such as Wilson's disease, alpha$_1$-antitrypsin deficiency and iatrogenic liver injury. Progressive, diffuse parenchymal fibrosis, micronodular and macronodular regenerative nodules, and altered hepatic architecture are the hallmarks of cirrhosis[1-3].

1.2 Liver Fibrosis - Pathology & Pathogenesis

Normal matrix turnover in health results from a dynamic equilibrium between matrix synthesis and degradation; liver fibrosis occurs due to a disturbance to this equilibrium in favour of net synthesis. Immunohistochemically, normal liver contains fibrillar (interstitial) extracellular matrix (ECM) proteins - collagen types I, III, V - in the capsule, large and medium-sized blood vessels and portal tracts, whereas the subendothelial space of Disse (SD), vascular and bile duct basement membranes contain a non-electron dense basement membrane-like matrix of collagen types IV and VI, laminin, proteoglycans and fibronectin[4-9]. Fibrotic liver contains six times the amount of matrix as normal liver[10],

principally collagen types I and III. SD matrix contains increased quantities of collagen types I, III[9] and VI[4] (with increased ratio of collagen type I to types III and IV[11,12]), laminin, and fibronectin of altered quantity and type[10]. Hyaluronan is present in small quantities in normal liver, but an eight-fold increase occurs in liver fibrosis[13].

The hepatic scar causes hepatocyte dysfunction and attendant clinical manifestations via at least 2 pathways. Firstly, loss of endothelial fenestrations and disrupted hepatic haemodynamics lead to impaired macromolecular diffusion across the SD. Secondly, ECM is known to actively modulate cell function, in addition to providing structural integrity to tissue; cell-matrix interactions[14-22] *in vitro* affect phenotype (3-dimensional shape, polarity) and behaviour (tissue-specific gene expression, cell-cell interactions) of hepatocytes, hepatic stellate cells (HSC, also known as fat-storing cell, lipocyte, Ito cell) and sinusoidal endothelial cells (SEC) and it is likely, therefore, that abnormal hepatocyte behaviour (such as loss of brush-border microvilli) in fibrosis may also result from direct effects of pathological matrix composition.

1.3 Increased matrix production: Role of the HSC in Liver Fibrosis

Although hepatocytes may contain collagen types I and IV messenger RNA (mRNA)[23,24] and produce fibronectin[25] and heparan sulphate[6] during fibrosis, earlier concepts of hepatocyte-derived pro-fibrotic matrix have now been refuted by rigorous cell characterisation showing that early hepatocyte cultures were contaminated with HSC[26]. Situated in the SD with cytoplasmic processes encircling the sinusoid, the HSC, the liver equivalent of the pericyte[27], is a quiescent, non-proliferative cell containing lipid-rich vitamin A droplets storing over 75% of body retinoids, largely as retinyl esters[28,29]. Current studies place the HSC at the centre of pathogenesis of fibrosis, being the major source of pro-fibrotic matrix proteins[24,25,30,31]. During mechanistically distinct

experimental models of fibrosis - HSC culture on plastic or collagen type I[16], or in experimental rat and murine liver injury (carbon tetrachloride (CCl_4) intoxication or bile duct ligation) - HSC uniquely undergo a phenotypic change ("activation") characterised by local proliferation (as demonstrated by increased [^{3}H]thymidine uptake)[32], loss of vitamin A (reduced autofluorescence)[33,34], enlargement and development of myofibroblast-like phenotype (α-smooth muscle actin and desmin expression, contractility)[31,35,36], and vastly increased gene expression and secretion of collagen types I, III and IV[30,37-40], laminin[5,38], proteoglycans[6,41], fibronectin[25,37], heparan sulphate[6], dermatan and chondroitin sulphate[6,41], hyaluronan[13] and the collagen synthetic enzyme prolyl hydroxylase[40]. mRNA for collagen type IV[24,42] and fibronectin[43] have been seen in SEC, but their overall contribution is small, while Kupffer cells (KC) and pit cells (sinusoidal natural killer-like T-cells) do not appear to make any matrix components. During activation, HSC also express Tissue Inhibitors of Metalloproteinases (TIMPs)[44-51] which are specific inhibitors of matrix metalloproteinases (MMPs)[44-46,52-57] at the same time, expressing certain MMPs implicated in abnormal matrix remodelling[58].

As HSC are the source of overproduction of ECM structural proteins, TIMPs and MMPs involved in matrix turnover abnormalities and hepatic morphology remodelling in fibrosis, factors affecting HSC function are important in directing the course of fibrosis. Research in this field has been greatly assisted by the availability of high purity rat, murine and human HSC preparations, which when cultured on uncoated plastic, undergo changes mimicking those seen upon HSC activation *in vivo*[38,59].

1.4 Control of HSC Activation: Role of HSC-KC Interactions and Other Factors

HSC-KC Interactions KC are resident tissue macrophages found predominantly periportally in the sinusoid lumen, which remove portal blood bacterial antigens and

endotoxin. KC have cytoplasmic extensions that extend into the SD to make direct contact with HSC and hepatocytes[60]. Macrophage/mesenchymal interactions underlie fibrosis in organs such as lung and kidney; a similar situation exists in the liver, where early recruitment, local proliferation and activation of KC are key features of most experimental models of liver disease such as CCl_4 intoxication, increases in KC numbers correlating with degree of fibrosis[60-62]. Further, hepatic injury can be abrogated by selective inhibition of KC function by gadolinium chloride or methyl palmitate[60,63], although KC have never been shown to make pro-fibrotic proteins themselves. In common with extra-hepatic macrophages, KC secrete a range of biologically active products including pro-inflammatory cytokines (TNF-α, IL-1, IL-6), eicosanoids and ROI; HSC exposure to KC conditioned medium (CM) accelerates HSC activation in culture with enhanced proliferation and fibrogenesis[64-66], this being most marked with pre-activated KC or KC isolated from injured liver[64,67,68]. Additionally, this exposure up-regulates cytokine receptor expression by HSC (such as the PDGF receptor), with increased sensitivity to the cytokine on subsequent exposure[65].

Mesenchymal cells are not passive in control of inflammation: skin, synovial and lung fibroblasts produce pro-inflammatory cytokines following LPS, IL-1 and TNF-α stimulation[69], and HSC may likewise amplify and re-enforce a KC inflammatory signal, through soluble mediators such as macrophage chemotactic protein-1 (MCP-1) in response to IL-1, TNF-α or IFN-γ[70], this feedback being dependent on local factors such as matrix interactions[16]. Interestingly, our group in Southampton has identified mRNA for IL-10, the most potent anti-inflammatory cytokine known, expressed from the start of activation *in vitro* to 150 days without additional stimulus[71].

Other factors Aldehydes generated by alcohol exposure[72-74], products of lipid peroxidation[75], oxidative stress[76], collagen type IV-degrading MMPs and their specific inhibitors[77] and hepatocyte interactions (such as through insulin-like growth factor, IGF)[78] also have a role in HSC activation. Conversely, exogenous retinoids down-regulate culture-activated HSC proliferation and fibrogenesis[79], and IFN-γ reduces HSC proliferation and fibrogenesis *in vitro*[80] and *in vivo*[81].

Figure 1 summarises the pathogenesis of liver fibrosis.

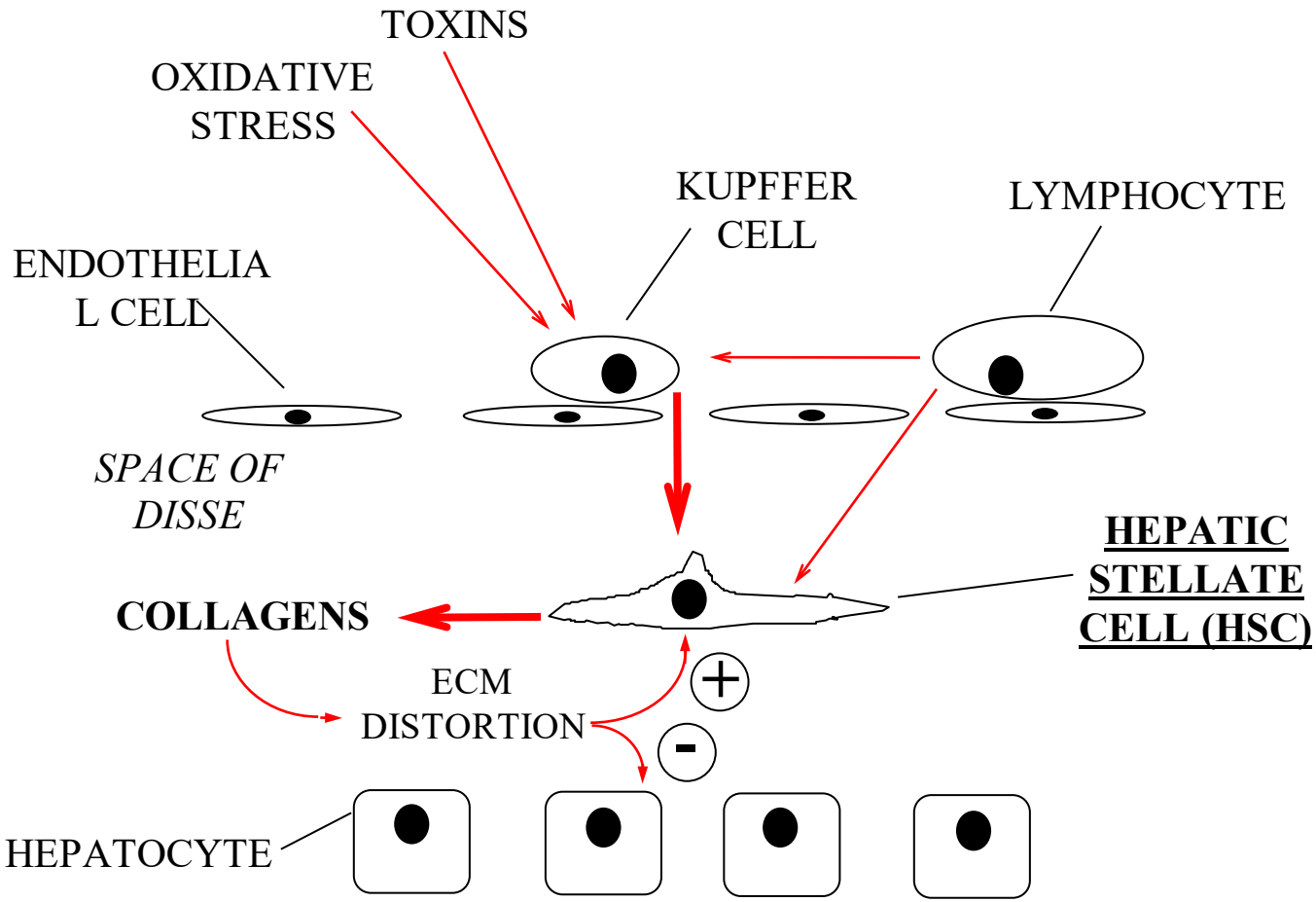


Figure 1: Pathogenesis of Liver Fibrosis

1.5 Interleukin-10

Discovery Originally identified in 1989[82] as a T_H2-derived "Cytokine Synthesis Inhibitory Factor" acting on T_H1 cells[83], IL-10 is now recognised as being by far the most potent of 4 cytokines (TGF-β, IL-4, IL-10, IL-13) known to inhibit murine and human macrophage pro-inflammatory function[84]. Murine and human IL-10 share 81% and 73% homology at nucleic and amino acid levels, both species exhibiting approximately 70% amino acid homology and some functional similarity with the BamH1 fragment C rightward reading frame 1 (BCRF1) gene product of the Epstein-Barr virus (EBV) genome[83,85,86], BCRF1 (also known as viral IL-10) perhaps representing a captured cellular cytokine gene[87].

Production IL-10 is produced by a wide variety of cell types:

- Immune system
    1. antigen-stimulated T_H0, T_H1 and T_H2 $CD4^+$ cells[82,83,86,88,89]
    2. LPS, TNF-α and IL-1 -stimulated macrophages[83]
    3. Mast cells[83]
    4. Stimulated B-cells[90,91] especially after EBV transformation[92,93]
    5. Eosinophils[94]
- Non-immune system
    6. Keratinocytes, particularly after UVA/UVB exposure[95,96]
    7. LPS and $TGF\beta_1$-stimulated renal mesangial cells[97]
    8. Unstimulated placental cytotrophoblasts[98]
    9. LPS, TNF-α or IL-1β stimulated human duodenal fibroblasts[69]
    10. Bronchial epithelial cells[99]

11. Murine microglial cells and astrocytes, especially following LPS stimulation[100]

- A variety of tumour cells[101-103] including human melanoma cell lines[104], bronchogenic[105] and colonic carcinoma[106].

IL-10 expression varies with cell type, stimulus/co-stimuli, and cellular microenvironment; expression in most cell types is transient, notable exceptions being constitutive expression in mast cells[107], placental cytotrophoblasts[98] and EBV+ve Burkitt's lymphoma cells[108]. Constitutive and induced IL-10 expression generally involves increased expression of the 5.1kb, 5-exon IL-10 gene located at the junction of chromosome 1q31 and 1q32[109]. The 5' regulatory region of the gene has an ill-defined promoter between -1100 and -900bp showing 3 single-bp substitution polymorphisms[109,110]. The 178Å open reading frame[83,86] results in a 445bp mRNA[111]. Murine IL-10 undergoes tissue-specific N-linked glycosylation at 2 sites, giving 17 (non-glycosylated), 19 and 21kDa[83,112] monomers, although glycosylation is not essential for biological activity[113]. The 18kDa human monomer lacks detectable carbohydrate[86], x-ray crystallography to 2.0Å revealing structural similarity to IFN-β and IFN-γ[114]. IL-10 monomers readily and non-covalently homodimerise through helical domains, existing in solution with 35kDa molecular weight[82].

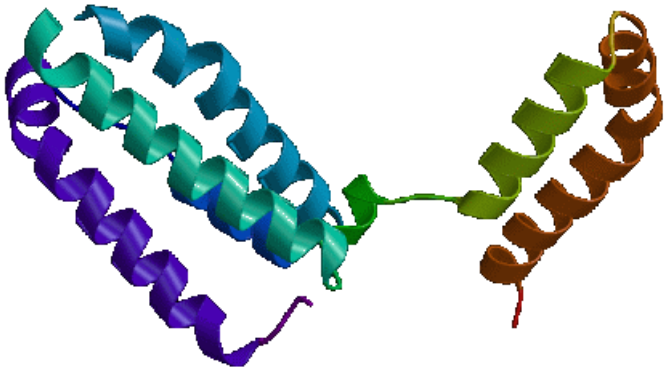


Figure 2: Human Interleukin-10 Monomer

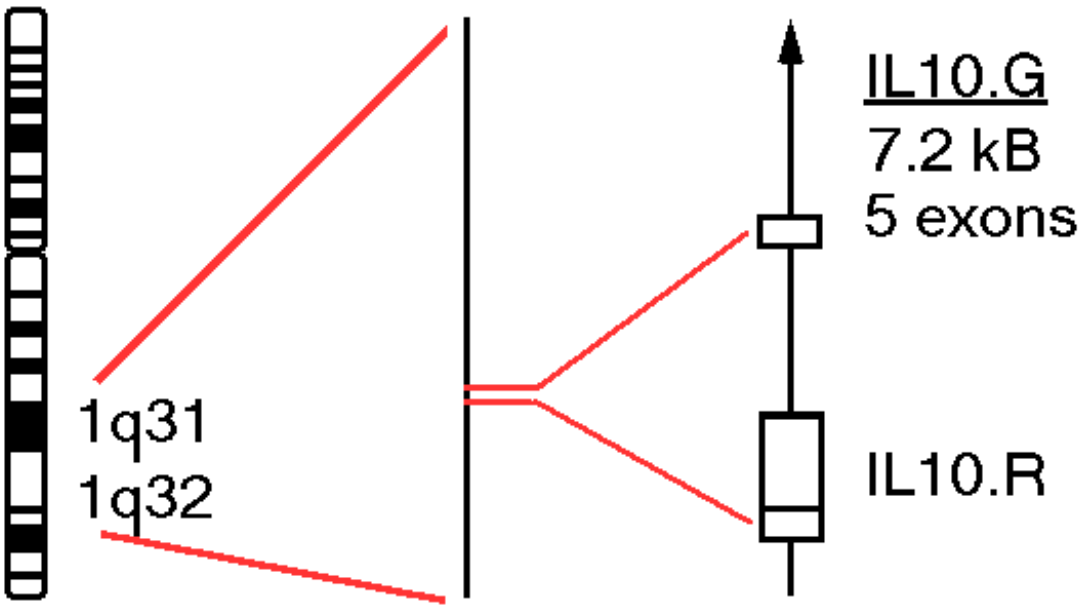


Figure 3: Location of Interleukin-10 Gene

In vitro actions Cellular actions of IL-10 are complex and varied, including both inhibitory and stimulatory effects, depending on target cell type and microenvironment[113]. High-affinity interactions between IL-10 and the 90-110kDa cell-surface IL-10 Receptor (class 2 cytokine receptor, homology with IFN-α, -β, -γ receptors[115,116]) on macrophages, T cells, NK cells, mast cells, B cells induce activation of the JAK-STAT[117-119] signalling pathway. IL-10 is profoundly inhibitory towards the entire spectrum of pro-inflammatory macrophage function:

- Inhibition[113,120-125] of IL-1α, IL-1β, IL-6, IL-8, TNF-α, GM-CSF, G-CSF, IL-10 itself (late) in response to LPS and other stimulants, via decreased gene transcription[120,126] (IL-10 selectively inhibits nuclear factor-κB (NF-κB) activation through preservation of inhibitor kappa B alpha (I-κB-α)[127,128]) and reduced transcript stability[129,130].
- Reduced MHC-II and B7 molecule expression and antigen presentation[120,131].
- Inhibition of NO[132], ROI[123,133], eicosanoid synthesis[113,134,135] and prostaglandin H synthetase-2[135].
- Reduced adhesion molecule expression[136].
- Enhanced TIMP-1 and decreased metalloproteinase production[137].

In vivo actions Studies with IL-10 in animal models of disease have shown reduced mortality from endotoxic[138,139] and enterotoxic shock[140], prolongation of survival following tissue allograft[141,142] and reduced inflammation in Leishmania infection[143], pancreatitis[144], encephalomyelitis[145] and asthma[146], IL-10 expression during inflammation correlating with amelioration of the inflammatory response[147]. LPS challenge induces a bi-phasic increase in circulating IL-10, mainly from liver[148]; IL-10

protects mice against lethal effects of LPS, and sustained production of IL-10 in various other organs in induced septic peritonitis may protect the host against lethality[149]. Human orthotopic liver transplantation is associated with massive release of IL-10[150], IL-10 mRNA is upregulated by hepatocytes from rat allograft tissue[151], and it is probably no coincidence that liver allografts are less immunogenic than other organ transplants and can promote immune tolerance. IL-10 may also be involved in immune privilege in the eye[152] and other organs. The ΔIL-10 transgenic mouse has normal lymphocyte development and antibody responses, but is growth retarded due to chronic illness. It is unable to suppress inflammation, mounting an exaggerated response to pathogens[153]. ΔIL-10 mice in a CCl_4 intoxication model of liver fibrosis develop significantly more fibrosis than control[154]. Following pre-clinical studies on the therapeutic potential of IL-10 in inflammatory bowel disease[155], colitis[156] and rheumatoid arthritis[157], Phase I[158] and Phase II[159] clinical studies have provided information on pharmacokinetics and pharmacodynamics of IL-10 as a drug in human subjects; Phase II and Phase III clinical studies are presently underway to further assess the safety, tolerance and ability of IL-10 to induce and maintain clinical remission in inflammatory bowel disease and rheumatoid arthritis (P. Grint, Schering-Plough, USA, Personal communication).

1.6 IL-10 in The Liver

Rat and human KC secrete IL-10 in response to LPS, TNF-α and IL-1β *in vitro*[160-162]; this may have an autocrine effect, as IL-10 downregulates human and murine KC activity to the same degree as with extra-hepatic macrophages[161-163]. IL-10 mRNA is expressed early in the time course of experimental liver injury[160], in post-hepatitis C liver cirrhosis[164], and the characteristic monocyte overproduction of TNF-α alcoholic cirrhosis may be due to deficient IL-10 secretion by the same cells[165]. HSC produce IL-10 mRNA during *in-vitro* activation, continuing for 150 days in culture or 6-7 serial passages without additional stimulus[71]; the constitutive mRNA signal for the most potent of macrophage de-activators suggests existence of a previously unsuspected feedback mechanism whereby HSC may modulate macrophage activity in the course of liver fibrosis.

1.7 Hypothesis: Role of IL-10 in HSC-Macrophage Interactions.

KC activation and HSC differentiation being central and related events in fibrotic liver injury, the hypotheses underlying the present investigation were:

"HSC can modulate macrophage activation via Interleukin-10"

and

"The HSC-IL10-Macrophage interaction can be modulated therapeutically"

Aims

The aim of the project was to test this hypothesis by answering 4 key questions:

- Do HSC produce IL-10 protein? Identification of mRNA does not prove *protein* expression, although this is generally assumed to be the case. If present, how much, when, and what is the pattern of production?
- What is effect of HSC-derived IL-10 on RAW 264.7 monocyte function?
- Can HSC expression of IL-10 be up-regulated or is it already at maximal capacity?
- What is the effect of induced IL-10 on RAW 264.7 function?

CHAPTER 2

"DO HSC PRODUCE IL-10 PROTEIN?" - INVESTIGATION BY WESTERN BLOT ANALYSIS

2.1 Introduction

On the basis of previous data regarding HSC expression of IL-10 mRNA during activation *in vitro*[71], we decided to start the present study by attempting detection of the protein product of this transcript, using the well-recognised technique of Western blot analysis. During Western blot analysis, proteins in samples are given an overall negative charge that is proportional to molecular size, and are separated electrophoretically under denaturing conditions (sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE). Following blotting onto nitro-cellulose membrane (Western blotting), protein is detected with a specific antibody. In this study, the commercial α-IL-10 primary antibody was visualised with chemiluminescence, via a horseradish peroxidase(HrP)-conjugated secondary antibody using a commercial chemiluminescence kit, initially with a series of positive and negative controls - recombinant murine IL-10 (rmIL-10) serial dilutions, TNF-α and LPS-stimulated KC), and then to examine IL-10 expression during time-courses of HSC activation (murine CCl_4 injury, rat and murine primary HSC cultures).

2.2 Materials & Methods

2.2.1 Materials

Western Blotting

4% Acrylamide stacking gel (2ml 4% acrylamide solution, 50µl 10%APS, 5µl TEMED)
12.5% Acrylamide stacking gel (10ml 12.5% acrylamide solution, 100µl 10%APS, 10µl TEMED)
15% Acrylamide stacking gel (10ml 15% acrylamide solution, 100µl 10%APS, 10µl TEMED)
N, N, N', N'-tetramethylethylenediamine (TEMED; Sigma, Poole, UK)
Recombinant murine IL-10 (18.7kDa, 160aa; Peprotech EC, London, UK)
Prestained SDS-PAGE Standards (See Appendix C; Bio-Rad, Hemel Hempstead, UK)
Detergent-compatible protein assay kit (See Appendix D; Bio-Rad)
Hybond-N membrane (Amersham Life Science)
Rat anti-murine IL-10, monoclonal IgG_1 antibody (from JES-2A5; Genzyme, West Malling, UK)
Isotype-control antibody to rat anti-murine IL-10, monoclonal IgG_1 antibody (a kind gift from J. Maltby, University Medicine (South Block))
Rabbit anti-rat IgG, HrP-conjugated polyclonal antibody (Sigma)
Enhanced chemiluminescence (ECL™) detection system (Amersham Life Science, Rainham, UK)
ECL Plus™ detection system (Amersham Life Science)

Solutions (See Appendix A)
4% Acrylamide Solution
12.5% Acrylamide Solution
15% Acrylamide Solution
10% ammonium persulphate (APS)
SDS-PAGE sample buffer
Electrode buffer
Blotting buffer
PBS/Tween-20
5%Marvel/PBS/Tween-20

Equipment:
Gel apparatus (Atto, Cambridge, UK)
Trans Blot Semi-dry blotter (Bio-Rad)

HSC and RAW Cell Culture

50ml and 250ml Tissue culture flasks (Greiner Labortechnik, Dursley, UK)
Cellstar® 6-well Tissue culture plates (Greiner Labortechnik)
Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Paisley, UK)
Foetal Calf Serum (FCS; Life Technologies)
Low endotoxin FCS (less than 1.4 endotoxin units; Life technologies)
Penicillin/Streptomycin/Gentamycin (PSG; Life Technologies)
Trypsin-EDTA (Life Technologies)
Hanks' Balanced Salt Solution (HBSS; Life Technologies)
L-Glutamine (Life technologies)
HEPES (1M; Sigma)
PBS (See Appendix A)
Recombinant murine TNF-α (Peprotech EC)
Lipopolysaccharide (LPS, from *Escherichia coli* 026:B6; Sigma)

Liver Homogenisation

Liquid N_2
Pestle and Mortar
1%SDS/PBS (See Appendix A)
Protease Inhibitors (See Appendix B)

2.2.2 HSC & KC isolation, and HSC, KC & RAW cell culture

Isolation and purification of rat (Sprague-Dawley), wild-type murine (129/SV) and ΔIL-10 transgenic murine HSC was performed by J. Gentry, University Medicine (South Block), using a sequential pronase-collagenase perfusion, density gradient separation and centrifugal elutriation protocol[38] well-established in our laboratory with purities of 95-97% and approximately 75-80% for rat HSC and murine HSC respectively (K. Thompson, Southampton, Unpublished data). All animal work was conducted according to British Home Office guidelines, and according to a protocol approved by the Home Office Inspector.

Primary HSC cultures were plated on uncoated tissue culture grade plastic at 1.33 $x10^6$ cells/ml in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (v/v) FCS and PSG, and cultured in a 37°C, 5% CO_2 humidified atmosphere.

In view of HSC lysis associated with passage by scraping (personal observation), cells were passaged by trypinisation, typically one month after isolation and at fortnightly intervals thereafter, to accommodate activation-associated HSC proliferation. Cells were washed with Hanks' Balanced Salt Solution (HBSS) (without Ca^{2+}/Mg^{2+}) and incubated at 37°C with Trypsin/HBSS until dislodged (2-5 minutes). An equal volume of FCS was added, and cells collected by 400G centrifugation for 5 minutes, resuspended in fresh medium and recultured as required.

Rat KC were isolated with a slight modification of the technique used for HSC isolation[60], plated at 1.33 $x10^6$ cells/ml in 6-well plates, and cultured under the same conditions as HSC.

Cells from the RAW 264.7 monocyte cell line were obtained from T. Biggs, University Medicine (South Block), and cultured on uncoated plastic in DMEM supplemented with 10% low endotoxin FCS, 2mM L-Glutamine, 20mM HEPES, PSG under the same conditions.

2.2.3 Establishing the Western Blot Analysis

RmIL-10 and conditioned medium from TNF-α, LPS or control stimulated primary rat KC cultures were used as positive and negative controls whilst optimising the Western blot analysis protocol.

48 hours post-isolation, KC medium was replaced with fresh medium containing 1% FCS and 10ng/ml TNF-α or 10ng/ml LPS or control. Conditioned medium was harvested 2, 6, 12, 24, 48 and 72 hours later, protein content determined with a bovine serum albumin standard curve (Appendix D) and standardised to 1mg/ml, concentrating samples by freeze-drying where necessary. One-third serial dilutions of rmIL-10 were made in 10%FCS/DMEM. Samples were prepared for SDS-PAGE by addition of equal volume of 2x SDS-PAGE sample buffer and boiling for 10 minutes.

A 4% polyacrylamide stacking gel was cast over either a 12.5% or 15% polyacrylamide separating gel, and loaded with 20µg of supernatant samples, 20µl of rmIL-10 and 10µl of pre-stained SDS-PAGE standards. Electrophoresis was carried out at 20mA constant current for 90 minutes, in the Atto gel apparatus containing 1x electrode buffer.

After electrophoresis, the gel was soaked in blotting buffer for 20 minutes with gentle shaking. The nitro-cellulose membrane and filter paper were also soaked in blotting buffer, to provide the ion reservoir during blotting, blotting being performed from the gel to the membrane with the Bio-Rad Trans-Blot Semi-Dry Transfer cell, with 0.8mA per

cm^2 of membrane current applied across the platinum-coated titanium anode and stainless-steel plate cathode for 1 hour.

Non-specific membrane binding was blocked by incubating for 30 minutes at room temperature with 5%Marvel/PBS, followed by overnight incubation at 4°C with rat anti-murine IL-10 IgG_1 monoclonal antibody, 5µg/ml in 5%Marvel/PBS. The membrane was then incubated at room temperature for 1 hour with gentle shaking with goat anti-rat IgG HrP-conjugated polyclonal antibody, 0.5µg/ml in 5%Marvel/PBS/Tween-20, the membrane being rinsed twice and washed twice for 5 minutes with PBS/Tween-20 after both antibody incubations, and again with distilled water before development with ECL or ECL Plus as per supplied instructions. To examine the electrophoretic separation of all loaded proteins, the gel was 1-hour Coomasie-stained, followed by soaking in destaining solution to remove excess bromophenol blue.

Subsequent work with the Western blot analysis was performed according to the method above, with rmIL-10 positive controls and pre-stained SDS-PAGE size markers with each separating gel.

2.2.4 IL-10 expression in CCl_4 Model of Liver Injury

0.3g liver sections from normal 129/SV mice previously treated intraperitoneally with 1ml/kg CCl_4 in olive oil or olive oil control alone for 2, 4, 6, 24, 48, and 72 hours, were ground in liquid nitrogen with pestle and mortar, and homogenised with 1ml of 1%SDS/PBS in the presence of protease inhibitors (see Appendix B), passed through a 19G needle and 20,000G centrifuged for 1 hour. Protein content of supernatants was standardised to 2mg/ml and analysed by Western blot analysis.

2.2.5 IL-10 expression by primary cultures of rat and wild-type murine HSC

Cells were cultured in 1% FCS for 24 hours before collection of conditioned medium which was stored at -20°C. To make cell lysates, cells were washed 3 times with PBS, collected by scraping and pelleted by centrifugation. Protein content was standardised and samples stored at -20°C. IL-10 content of conditioned medium and lysates was examined by Western blot analysis.

2.2.4 IL-10 expression by RAW cells

RAW cell medium was replaced with fresh medium containing 10ng/ml LPS. Conditioned medium and cell lysates were harvested 12 hours later, and stored at -20°C until Western blot analysis.

2.3 Results

Coomasie staining of the gel following SDS-PAGE (Figure 4a) reveals localisation of pre-stained standard and sample proteins after electrophoresis. We achieved specific yet sensitive detection of recombinant IL-10, detecting both monomer and dimer at the expected molecular weights (~18 and 36kDa, Figure 4b), and very low non-specific background activity of the antibodies, 6ng being the lower limit of detection with this protocol (Figure 4c). IL-10 was detected in conditioned medium from 24-hour TNF-α stimulated KC, but not in control medium (Figure 4d), while IL-10 was detected between 24-72 hours in conditioned medium during a time-course of KC stimulation with LPS (Figure 4e). As expected, recombinant IL-10 migrated to the molecular weight corresponding to IL-10 *monomer*, while KC-derived IL-10 migrated to that corresponding to the *dimer*.

Multiple bands were detected in liver homogenates following murine CCl_4 intoxication of variable duration (Figure 5), some being of the expected molecular weight for monomeric or dimeric IL-10, but the majority being ~25kDa. No correlation was observed between the bands and duration of CCl_4 injury. Poorly defined 33.3kDa bands were seen with 7, 14 and 21-day rat HSC lysate and supernatant, with considerable distortion associated with concentrated 7-day samples (Figure 6a). Equally poorly-defined faint bands of variable Mw were the only ones seen with murine HSC at similar time points and LPS-stimulated RAW cells(Figure 6b). These bands were detected with isotype-control primary antibody too. IL-10 was not detected in any time-courses or experiments apart from with rmIL-10, despite using ECL Plus (Figure 6c).

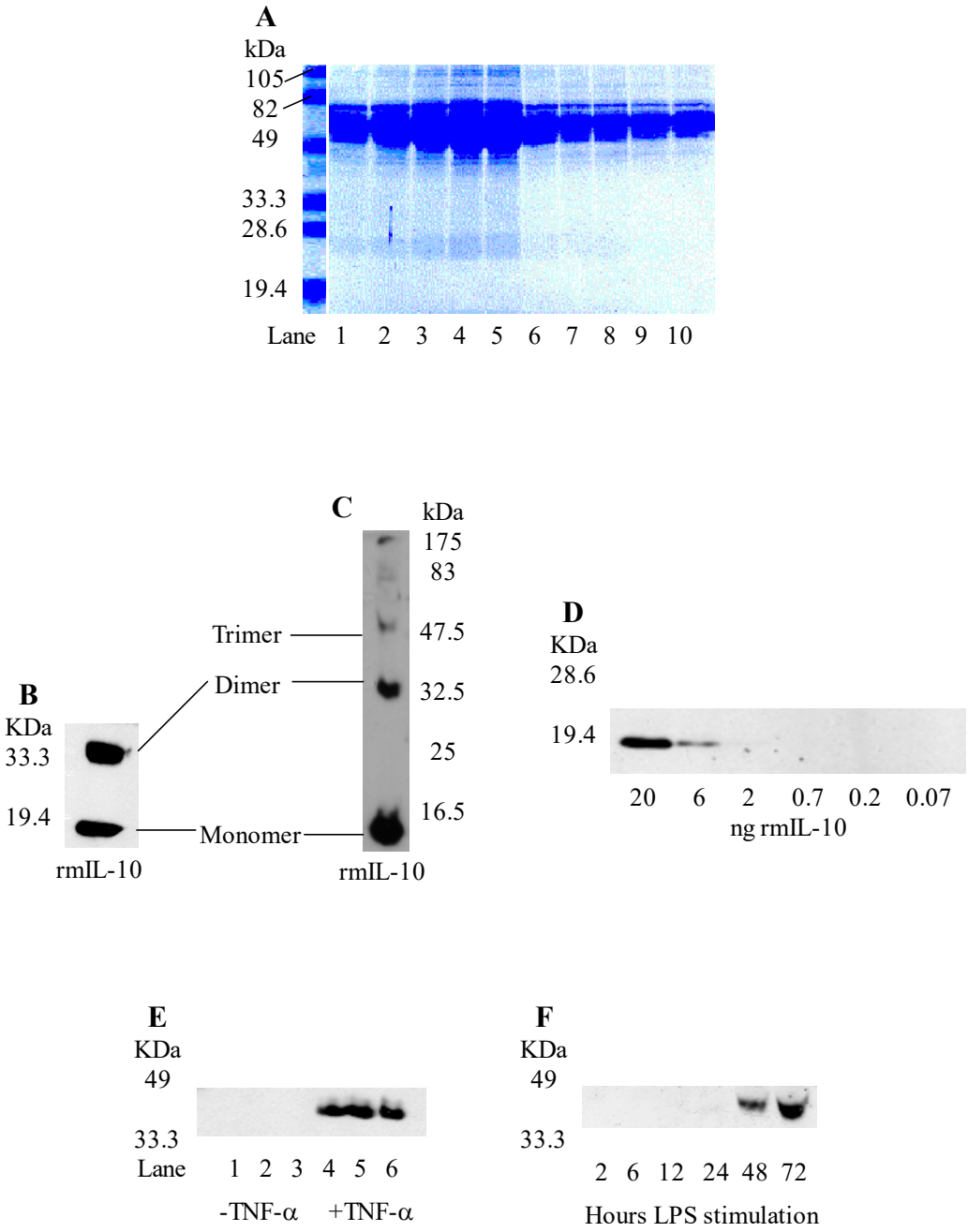


Figure 4: Establishing the IL-10 Western Blot Analysis: Positive and Negative Controls

Recombinant murine IL-10 and conditioned media from 10ng/ml TNFα- and 10ng/ml LPS-stimulated Sprague-Dawley rat KC were used as positive controls while optimising the Western blot analysis protocol. Samples were separated by SDS-PAGE, blotted onto nitro-cellulose membrane and the membrane probed for IL-10 with rat anti-murine IL-10 monoclonal IgG_1 antibody (Genzyme), detected with rabbit anti-rat IgG HrP-conjugated polyclonal antibody (Sigma) and a commercial chemiluminescence kit (ECL; Amersham Life Science). (**A**) *Coomasie staining of the gel. Lanes 1-5: TNFα-stimulated KC conditioned medium, lanes 6-10: unstimulated KC conditioned medium.* (**B**)&(**C**) *Western blot analysis with rmIL-10. Monomer and dimer were seen to migrate to their expected molecular weights, with higher molecular weight species also being detected.* (**D**) *Western blot analysis with one-third serial dilutions (20-0.07ng) of rmIL-10. 6ng is the limit of detection.* (**E**) *IL-10 in KC conditioned medium following 72-hour TNF-α stimulation. Lanes 1-3: no TNF-α, Lanes 4-6: 10ng/ml TNF-α..* (**F**) *IL-10 in KC conditioned medium during a time-course of 10ng/ml LPS-stimulation.*

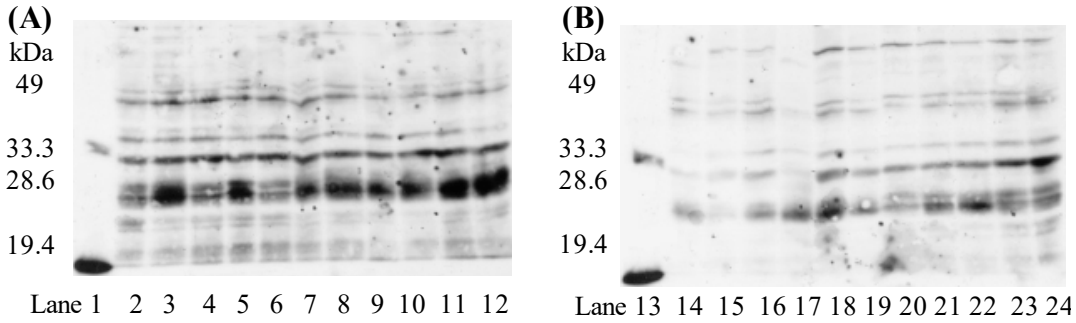
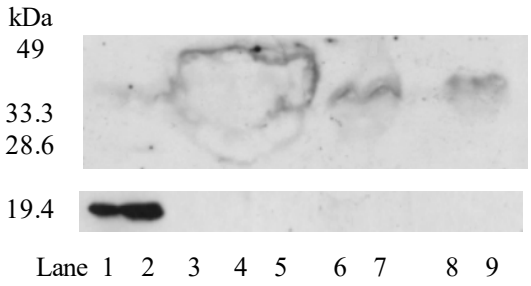


Figure 5: Western Blot Analysis: IL-10 Expression During Murine CCl_4 Injury

IL-10 content of homogenised liver sample supernatants from 129/SV mice CCl_4-treated for 2, 4, 6, 24, 48, and 72 hours was determined by Western blot analysis. (**A**) & (**B**) ***Lanes 1, 13**: 20ng rmIL10, **lanes 2, 3**: 2 hours CCl_4 exposure, **lanes 4, 5**: 4 hours CCl_4 exposure, **lanes 6, 7**: 6 hours CCl_4 exposure, **lanes 14, 15, 16, 17**: 24 hours CCl_4 exposure, **lanes 18, 19, 20**: 48 hours CCl_4 exposure, **lanes 21, 22, 23**: 72 hours CCl_4 exposure, **lanes 8, 9, 10, 11, 12, 24**: No CCl_4 exposure.*

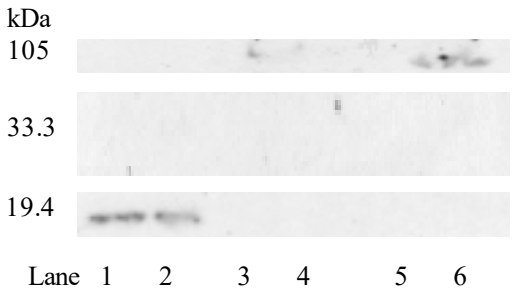
A

1 rmIL-10, 10ng
2 rmIL-10, 20ng
3 7 day rat HSC lysate
4 7 day rat HSC supernatant
5 7 day rat HSC supernatant, concentrated
6 14 day rat HSC lysate
7 14 day rat HSC supernatant
8 21 day rat HSC lysate
9 21 day rat HSC supernatant



B

1 rmIL-10, 10ng, with mercaptoethanol
2 rmIL-10, 10ng, without mercaptoethanol
3 24 hour LPS-stimulated RAW cell supernatant
4 24 hour LPS-stimulated RAW cell lysate
5 14 day unstimulated murine HSC supernatant
6 14 day unstimulated murine HSC lysate



C

1 rmIL-10, 20ng, with mercaptoethanol
2 rmIL-10, 20ng, without mercaptoethanol
3 24 hour LPS-stimulated RAW cell supernatant
4 24 hour LPS-stimulated RAW cell lysate
5 14 day unstimulated murine HSC supernatant
6 14 day unstimulated murine HSC lysate

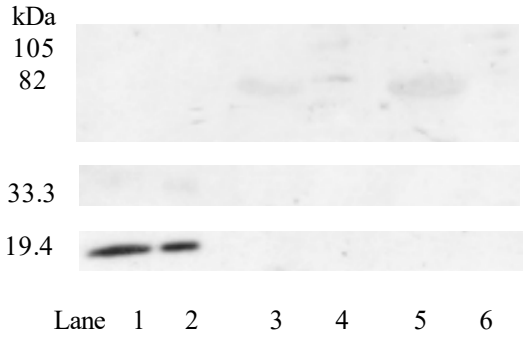


Figure 6: Western Blot Analysis: IL-10 Expression During a Time-Course of Rat and Murine HSC Activation

Sprague-Dawley rat and 129/SV murine HSC were isolated by pronase-collagenase perfusion, density gradient separation and centrifugal elutriation, and plated onto uncoated plastic at 1.33x10^6 cells/ml in DMEM with 15% FCS and PSG. After 7, 14 and 21 days culture, conditioned medium was replaced with fresh medium containing 1% v/v FCS. Conditioned medium was collected 48 hours later and cell lysates prepared, some conditioned medium samples being concentrated by freeze-drying. IL-10 content was determined by Western blot analysis, the membrane being probed with rat anti-murine IL-10 monoclonal IgG$_1$ antibody (Genzyme). IL-10 content of 24-hour LPS-stimulated RAW cell conditioned medium was also determined. Dimeric (prepared without 2-mercaptoethanol) and monomeric (prepared with 2-mercaptoethanol) rmIL-10 was used as positive control on each gel. (**A**), (**B**) *&* (**C**) *No IL-10 was detected, apart from rmIL-10; all bands greater than 19.4kDa were present when membranes were probed with isotype-control antibody.*

2.4 Discussion - Western Blotting

Initial work with rmIL-10, stimulated KC and all subsequent rmIL-10 positive controls usefully demonstrate that the technique was functioning perfectly. Having seen high-specificity bands with these experiments, the multiple bands with liver homogenates were surprising; while these may correspond to IL-10, it is more probable that the primary antibody recognised epitopes on proteins other than IL-10, or that the secondary antibody (anti-rat IgG) cross-reacted with homogenate antibodies other than the antibody of interest (rat IgG_1 anti-mouse IL-10). This problem could be eliminated by repeating with primary antibodies raised against other IL-10 epitopes, isotype-control primary antibodies, and HrP-conjugated *primary* antibodies, although the latter would entail conjugating the antibody first.

Despite exhaustive efforts, we failed to detect IL-10 in concentrated rat HSC conditioned medium and lysate (Figure 6) by this technique. We concluded that Western blot analysis was unsuitable for detecting IL-10 in this context for these reasons:

- Sensitivity This technique required at least 6ng IL-10 to be loaded in a maximum volume of 40µl, that is, 150ng/ml minimum cytokine concentration. As with other cytokines, IL-10 is generally expressed at much lower concentrations[97], and a much greater loading volume would have been required. Our attempts to detect IL-10 in concentrated samples were futile, merely hampering electrophoresis and specific binding.
- Denaturing conditions By virtue of the SDS-PAGE component, Western blot analysis is a denaturing technique; IL-10 is an easily degradable cytokine[113], and we may have lost a significant proportion of IL-10 in any sample.

- Quantitative vs. qualitative measurement Western blot analysis does not lend itself to quantification of protein content and thus would have been unsuitable for the further studies we envisaged.

Patterns of post-translational modifications during IL-10 production are cell type-dependent[113], and it is entirely plausible that while our antibody recognised epitope(s) in rat KC-derived IL-10, it did not recognise epitopes from the murine monocyte cell line IL-10 with the same affinity. Additionally, while the recombinant *E. Coli*-derived IL-10 showed susceptibility to de-dimerisation during sample preparation independent of 2-mercaptoethanol, the KC-derived IL-10 did not de-dimerise under the exact same conditions, suggesting that HSC or RAW-derived IL-10 may be present in a form that is either resistant or susceptible to degradation, altering the migration during electrophoresis and subsequent detection. Finally, while KC were cultured in 6-well plates with a small volume of medium, HSC and RAW cells were cultured in a greater volume, and this may have contributed to a smaller concentration of any IL-10 released.

At the time of this investigation, no other groups had reported IL-10 detection by Western blot analysis, bar one which examined expression in a transfected cell line[123]. Investigation of cytokine expression normally uses techniques such as Northern blot analysis, immunopreciptiation, immunohistochemistry and enzyme-linked immunosorbent assay (ELISA); as we had previously maximally exploited Northern blot analysis, and immunohistochemistry is at best semi-quantitative, we decided to not attempt enhancements of Western blot analysis[166], but instead, to proceed by developing an ELISA to detect IL-10.

CHAPTER 3

"DO HSC PRODUCE IL-10 PROTEIN?" - INVESTIGATION BY ENZYME-LINKED IMMUNOSORBENT ASSAY

3.1 Introduction

Having failed to demonstrate IL-10 protein production by HSC with the Western blot analysis, we decided to use an enzyme-linked immunosorbent assay (ELISA) to detect IL-10 protein. ELISAs are considerably more sensitive than Western blot analysis, combining monoclonal antibody specificity with the sensitivity of spectrophotometric enzyme assays under non-denaturing conditions. Additionally, as enzyme activity is directly proportional to amount of antigen present, protein quantification is possible.

Although commercial ELISA kits are available for murine IL-10, we decided to develop this assay ourselves with antibodies and hybridoma cell lines already available, allowing more economical and flexible investigation of IL-10 production. We initially decided to use a solid-phase triple-antibody sandwich ELISA with monoclonal and polyclonal antibodies for maximum amplification of detection signal; in view of possible membrane-localisation of IL-10 and degradation through handling, we also developed a cell-based ELISA to examine cellular or surface IL-10 without the need for cell lysis.

3.2 Materials & Methods

3.2.1 Materials

IL-10 ELISA
96-well Falcon Microtitration plate (Becton Dickinson, Oxford, UK)
96-well Linbro EIA-II Plus Microtitration plate (Flow Laboratories, Herts., UK)
96-well Nunc-Immuno™ plates with MaxiSorp surface (Life Technologies)
Rat anti-murine IL-10, monoclonal IgG_1 antibody (JES-2A5; Genzyme)
Goat anti-murine IL-10, monoclonal IgG antibody (R&D Systems, Abingdon, UK)
Goat anti-murine IgM (μ-chain specific), HrP-conjugated polyclonal antibody (Sigma)
Mouse anti-goat/sheep IgG, HrP-conjugated monoclonal IgG_1 antibody (Sigma)
Recombinant murine IL-10 (Peprotech EC)
Hybond-N membrane (Amersham Life Science)
ECL Detection Kit (Amersham Life Science)
Buffers and solutions (See Appendix A)
Carbonate-bicarbonate coating buffer, pH 9.0
Citrate-acetate buffer, pH 6.0
PBS/Tween-20
Chromogen solution
10%FCS/DMEM
1%, 5%, 10% BSA/PBS
5%Marvel/PBS
2%Gelatin/PBS
1%BSA/5%Sucrose/PBS

Dot Blot
Hybond-N membrane (Amersham Life Science)
5%Marvel/PBS (See Appendix A)
PBS (See Appendix A)
Rabbit anti-rat IgG, HrP-conjugated polyclonal antibody (Sigma)
ECL Detection Kit (Amersham Life Science)

Cell-Based ELISA
96-well Tissue culture plates with Nunclon Surface (Life Technologies)
Methanol (Sigma)
Rabbit anti-rat IgG, HrP-conjugated polyclonal antibody (Sigma)
Isotype-control antibody to rat anti-murine IL-10, monoclonal IgG_1 antibody (a kind gift from J. Maltby, University Medicine (South Block))
Solutions (See Appendix A)
4%PFA/DMEM
Chromogen solution
5%Marvel/PBS

Hybridoma Cell Culture
50ml and 250ml Tissue culture flasks (Greiner Labortechnik)
RPMI 1640 medium with L-glutamine (Life Technologies)
Foetal Calf Serum (FCS; Life Technologies)
Penicillin/Streptomycin/Gentamycin (PSG; Life Technologies)

HSC Cell Culture
As above.

3.2.2 Hybridoma culture

Cells from the hybridoma cell lines SXC-1, SXC-2 and JES-2A5 were obtained from the American Tissue Type Collection (Rockville, Maryland, USA). SXC-1 and SXC-2 are semi-adherent cells which secrete rat anti-murine IL-10 monoclonal IgM antibodies, and JES-2A5 cells secrete rat anti-murine IL-10 monoclonal IgG_1 antibody, each of these cell lines recognising different epitopes (K. Moore, DNAX Institute, USA, Personal communication). These cells were cultured under conditions previously described, in RPMI medium supplemented with L-glutamine, 15% FCS and PSG. At regular intervals, cells were 400G centrifuged for 5 minutes, the antibody-containing supernatants collected and stored at -20°C, and the cells recultured with fresh medium.

3.2.3 Establishment of IL-10 ELISA

3.2.3.1 Part 1 - First attempt

An ELISA was performed with a "chequerboard" or "grid"-like arrangement of rmIL-10, detection and tertiary antibodies, designed to find optimum antibody dilutions for strongest OD with lowest background.

Method

(i) A 96-well microtitration plate (Falcon) and (ii) a 96-well Linbro EIA-II Plus microtitration plate (Flow Laboratories), were subjected to the following procedure, with 50µl/well of each solution in duplicate, 2 hour incubations at room temperature and 10%FCS/DMEM as blocking agent and dilutant unless stated otherwise. Wells were coated with 5µg/ml (manufacturer's recommendation) rat anti-murine IL-10 IgG_1 monoclonal capture antibody (Genzyme) in coating buffer overnight at 4°C. Wells were blocked with blocking agent and incubated with combinations of 100ng/ml and 0ng/ml

rmIL-10. Wells were washed 3 times with PBS/Tween-20, and detection antibodies added - (i) neat SXC-1 supernatants, (ii) neat SXC-2 supernatants (iii) 1:500 - 1:10,000 dilutions of goat anti-murine IL-10 monoclonal antibody (iv) 10%FCS/DMEM alone. Wells were washed again and goat anti-murine IgM HrP-conjugated polyclonal antibody 1:500 or 1:1000 dilution was added to detect SXC-derived antibody, and mouse anti-goat/sheep IgG HrP-conjugated antibody 1:500 or 1:1000 dilution was added to detect the goat antibody. Wells were rinsed again, and 150µl/well chromogen solution added. The reaction was stopped after 10 minutes with 30µl 4N sulphuric acid, optical densities measured (OD) measured at 450nm and analysed with an Anthos Labtec GT2 plate reader.

Results and Discussion

The Falcon plate produced no colour change in any well, TMB solution testing OK with neat anti-IgM. The "Linbro" plate gave high OD with goat anti-murine IL-10 and mouse anti-goat/sheep IgG antibody, but no difference between 0 and 100ng/ml rmIL-10.

In view of the out-right failure of this ELISA, we decided to eliminate the capture antibody adsorption step, and perform a direct ELISA.

3.2.3.2 Part 2 - Second attempt

Method

Wells were coated with 100 or 0ng/ml rmIL-10 in coating buffer overnight at 4°C. Following washing, wells were incubated with detection antibodies in the same arrangement and developed as previously.

Results and Discussion

No colour change was detected. Reasons for this included failure of capture antibody adsorption, rmIL-10 loss following reconstitution without carrier protein and faulty antibody activity. We obtained immunochemical-grade plates for all further work. Such plates have a polar polystyrene surface exhibiting high affinity to polar groups, thus promoting physical binding of proteins, especially glycoproteins such as antibodies. Plates with flat-bottomed wells for optimum monochromatic readings were selected with due regard to well-well and plate-plate uniformity of adsorption properties and optical quality. Fresh rmIL-10 was obtained and reconstituted with 10%FCS/DMEM as carrier protein. Ageing tertiary antibodies were replaced with fresh ones.

3.2.3.3 Part 3 - Third attempt

Method

A chequerboard ELISA was performed. Wells were coated overnight with and without capture antibody, followed by incubation with 200 or 0ng/ml rmIL-10. Goat anti-murine IL-10 detection antibody was used at 1:1000 - 1:100000 dilutions, with tertiary mouse anti-goat/sheep antibody at 1:500, 1:1000 and 1:2000 dilutions. Neat SXC and control

supernatants, with anti-murine antibody at 1:500 and 1:1000 dilutions were also tested. Some wells were coated overnight with 200ng/ml rmIL-10 in coating buffer, followed by detection with the same range of antibodies.

Results & Discussion

ODs were considerably higher in most wells than previously, but again, no difference between 200 or 0ng/ml IL-10. We decided to find a better blocking agent with a view to reducing background antibody binding.

3.2.3.4 Part 4 - Blocking agents

Method

Wells were blocked for 1 hour with 1%, 5% or 10% BSA/PBS, 5%Marvel/PBS, 2%Gelatin/PBS, 1%BSA/5%Sucrose/PBS, 10%FCS/DMEM or left empty. Wells were washed and 1:500 dilution in 5% BSA of 2 different batches of mouse anti-goat/sheep antibody was applied for 1 hour, and developed with chromogen as before.

Results & Discussion

Blocking with 5% BSA/PBS gave lowest non-specific binding of antibody to the wells, when compared with the other blocking agents tested. We decided to use this as blocking agent and antibody dilutant.

3.2.3.5 Part 5 - Fourth attempt

Method

Having obtained suitable plates, fresh rmIL-10 and antibodies, found the best blocking agent, we used these to again attempt to find suitable conditions for this ELISA. The procedure was as for Part 3 above, but with the goat anti mouse IL-10 detection antibody used at 1:250, 1:500, 1:1000, 1:2000 dilutions. Additionally, we found the minimum capture antibody incubation time by a time-course of adsorption and detection, tested the effects of different incubation temperatures following primary antibody capture, assessed possible edge effects by detecting equal amounts of IL-10 at different points on the plate, and determined susceptibility of recombinant standards to degradation following freeze-thaw cycles.

Result & Discussion

The protocol yielded suitable ODs with 2 sets of antibodies:

- 1:250 dilution of goat anti-murine IL-10, with 1:1000 dilution anti-goat/sheep tertiary antibody.
- Neat SXC-2 supernatant, with 1:1000 dilution anti-mouse IgM tertiary antibody.

The correlation coefficient of a standard curve produced with one-third serial dilutions of rmIL-10 in the range 100-0.046ng/ml was 0.99 (Figure 7), obtained with both sets of antibodies. The minimum capture antibody incubation time was found to be 3 hours at 4°C (Figure 8); effectiveness of subsequent incubations was found to be independent of the temperatures tested. No undesirable edge effects were observed, and the recombinant samples were found to lose 5-10% of activity following each freeze-thaw cycle, in agreement with manufacturer's data.

Figure 7: IL-10 ELISA: Standard Curve

5μg/ml of rat anti-murine IL-10, monoclonal IgG_1 capture antibody (Genzyme) was incubated overnight at 4℃. A one-third serial dilution of rmIL-10 in the range 100ng/ml to 411pg/ml was applied for 2 hours, followed by detection with neat SXC-2 hybridoma supernatant and development with goat anti-murine IgM, HrP-conjugated polyclonal antibody (Sigma), and TMB as chromogen. Optical densities were analysed with an Anthos Labtec GT2 plate reader, and a log-log curve of mean optical density against known rmIL-10 concentration plotted, with a correlation co-efficient of 0.99. Subsequent IL-10 ELISAs included a 7- or 8-point standard curve with each plate, giving a sensitivity in the range 100ng/ml to 137pg/ml or 46pg/ml respectively.

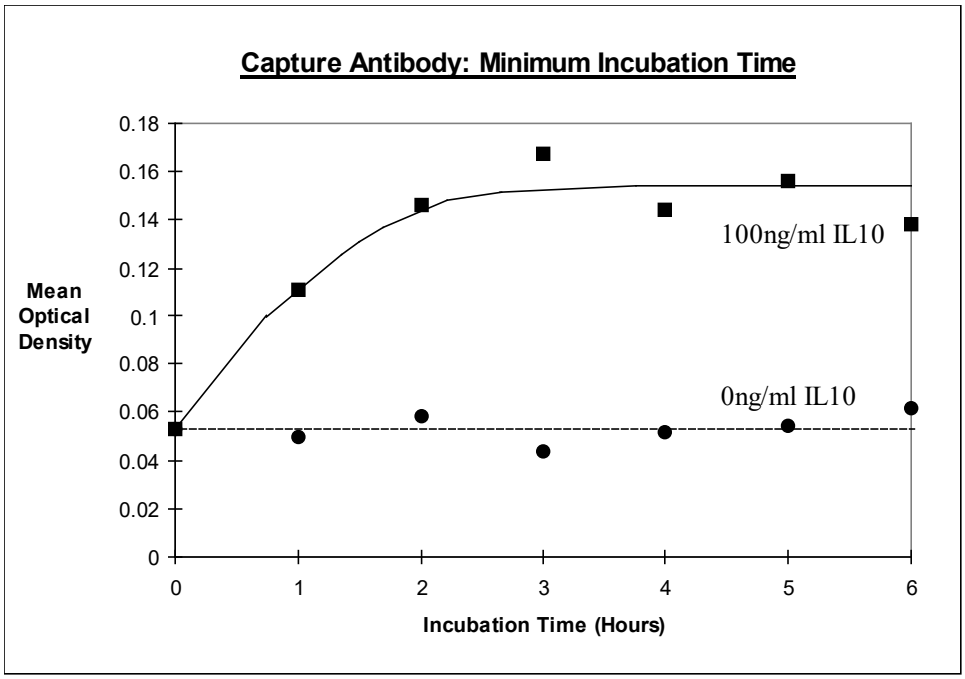


Figure 8: IL-10 ELISA: Time-Course of Capture Antibody Adsorption

5 µg/ml of rat anti-murine IL-10, monoclonal IgG_1 capture antibody (Genzyme) was incubated at 4 ℃ for 0, 1, 2, 3, 4, 5 or 6 hours in different wells of the same ELISA plate. 100ng/ml rmIL-10 was applied for 2 hours, followed by detection with the IL-10 ELISA protocol. This graph correlates the mean optical density with detection antibody incubation time; 3 hours was the minimum incubation time required, with no appreciable difference between 3 hour incubation and longer incubations.

3.2.3.5 Part 4 - Purification of hybridoma supernatant antibody

We decided to purify JES-2A5 supernatant to isolate the IgG_1 for use as coating antibody. JES-2A5 supernatant samples were tested for anti-IL-10 activity with a dot-blot: Combinations of 1 or 0ng rmIL-10 in 10%FCS/DMEM were blotted onto PBS-pre-wetted Hybond-N membrane under vacuum. Following 30 minute 5%Marvel/PBS block, IL-10/control unit-sections were incubated overnight at 4°C with hybridoma supernatant, supernatant with known anti-IL-10 activity, or RPMI/15%FCS/PSG control. Following washing, 1:2000 rabbit anti-rat IgG polyclonal antibody incubation, and development with ECL (as for Western blot analysis), samples with anti-IL-10 activity were pooled and the IgG fraction isolated by protein-G column capture and pH elutriation by J. Maltby, University Medicine (South Block). Suitability for ELISA use was tested by coating wells with 2, 5 or 10µg/ml of this antibody, applying recombinant standards and detecting with both SXC-2 and R&D protocols, comparing ODs with the same procedure using commercial antibody at 5µg/ml.

Results & Discussion

The elutriation curve indicated high antibody purity and suitability for the intended use. With the SXC-2 protocol, 10µg/ml of purified JES-2A5 antibody gave equivalent OD and correlation coefficient as 5µg/ml of commercial antibody, suggesting contamination with other antibodies, the R&D protocol (but not the SXC-2 protocol) yielding high background OD diagnostic of tertiary antibody cross-reactivity with "purified" antibody constituents. We decided to use this purified antibody as capture antibody with the SXC-2 protocol only.

3.2.3.6 Part 5 - ELISA Maintenance & Reproducibility

SXC-2 supernatants were tested for anti-IL-10 activity in a similar way, active samples being pooled before use, to minimise inter-batch OD variations. Additionally, a chequer-board ELISA was performed with each new batch of primary, secondary and tertiary antibody, and an 8-point standard curve with 1/3 serial dilutions of rmIL-10 (100-0.046ng/ml +blank) was applied with each assay.

3.2.4 Primary HSC cultures

Rat, wild-type mouse and ΔIL-10 mouse HSC were isolated and cultured as described, conditioned medium samples collected at regular intervals, and stored at -20°C until analysis by ELISA. Throughout this study, data were analysed using SPSS for Windows (SPSS Inc., Illinois, USA), and expressed as 95% confidence intervals (95%CI) where appropriate. Significance was determined using a t-test for Equality of Means, with a p value <0.05 being considered statistically significant.

3.2.5 Cell-based ELISA

Rat HSC were isolated as before, plated in 96-well plates at 5000/well (200µl medium) and cultured for 14 days; long term cultures of murine HSC were plated in 96-well plates at 10,000/well (200µl medium) and cultured for 48 hours. Medium was aspirated and replaced with 100µl fresh medium. For fixing, a 1:1 medium exchange was performed with 100µl 4%PFA/DMEM which was replaced after 1 hour with 100µl 4%PFA/DMEM, which was left for a further hour. Wells were rinsed x3 with PBS, and cells permeablised with 100µl 100% ice-cold methanol for 15 minutes. Wells were blocked with 5%Marvel/PBS for 1 hour; 100µl neat JES-2A5 supernatant (previously

tested positive for anti-IL-10 activity by dot-blot) or isotype-control antibody in RPMI/15%FCS/PSG was added for 1 hour. After x3 rinsing with PBS, rabbit anti-rat IgG polyclonal antibody 50µl of 0.5µg/ml was added for 1 hour. Wells were rinsed again, chromogen added, and OD determined as before.

3.3 Results

3.3.1 Primary cultures of unstimulated rat HSC

IL-10 was detected in unstimulated rat HSC conditioned medium at 180-1300pg/ml during Days 0-14 post-isolation, followed by a significant drop to 0-180pg/ml thereafter, up to 40 days (Figure 9, 95%CI, $p<0.01$). Lysate IL-10 was undetectable at these time points. These data result from 3 separate time-course experiments.

3.3.2 Primary cultures of unstimulated wild-type murine HSC

IL-10 was detected in unstimulated wild-type murine HSC conditioned medium (Figure 10) in a similar trend as for rat HSC. Lysate IL-10 was undetectable at times shown.

3.3.3 Primary cultures of unstimulated transgenic ΔIL-10 HSC

No IL-10 was detected at any time during 2 separate time-course studies of culture activation.

Figure 9: ELISA: IL-10 in Conditioned Medium from Early Cultures of Rat HSC

Sprague-Dawley rat HSC were isolated by pronase-collagenase perfusion, density gradient separation and centrifugal elutriation, and plated onto uncoated plastic at $1.33x10^6$ cells/ml in DMEM with 15% FCS and PSG. Conditioned medium was collected on Days 4, 7, 14, 21 and 40 and assayed for IL-10 by ELISA, the medium being replaced with fresh medium 24 hours before collection. This graph shows IL-10 content of conditioned medium during a time-course of culture.

Conditioned medium IL-10: **Day 4** vs. **Day 21**

Variable	Number of Cases	Mean	SD	SE of Mean
IL-10				
DAY 4	20	972.4500	725.887	162.313
DAY 21	16	90.1125	171.302	42.826

Mean Difference = 882.3375
Levene's Test for Equality of Variances: F= 28.025 P= .000

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	4.74	34	.000	185.963	(504.416, 1260.259)
Unequal	5.26	21.60	**.000**	167.868	(533.831, 1230.844)

Conditioned medium IL-10: **Day 14** vs. **Day 21**

Variable	Number of Cases	Mean	SD	SE of Mean
IL10				
DAY 14	22	641.7773	787.580	167.913
DAY 21	16	90.1125	171.302	42.826

Mean Difference = 551.6648
Levene's Test for Equality of Variances: F= 23.659 P= .000

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	2.75	36	.009	200.951	(144.117, 959.212)
Unequal	3.18	23.68	**.004**	173.288	(193.761, 909.569)

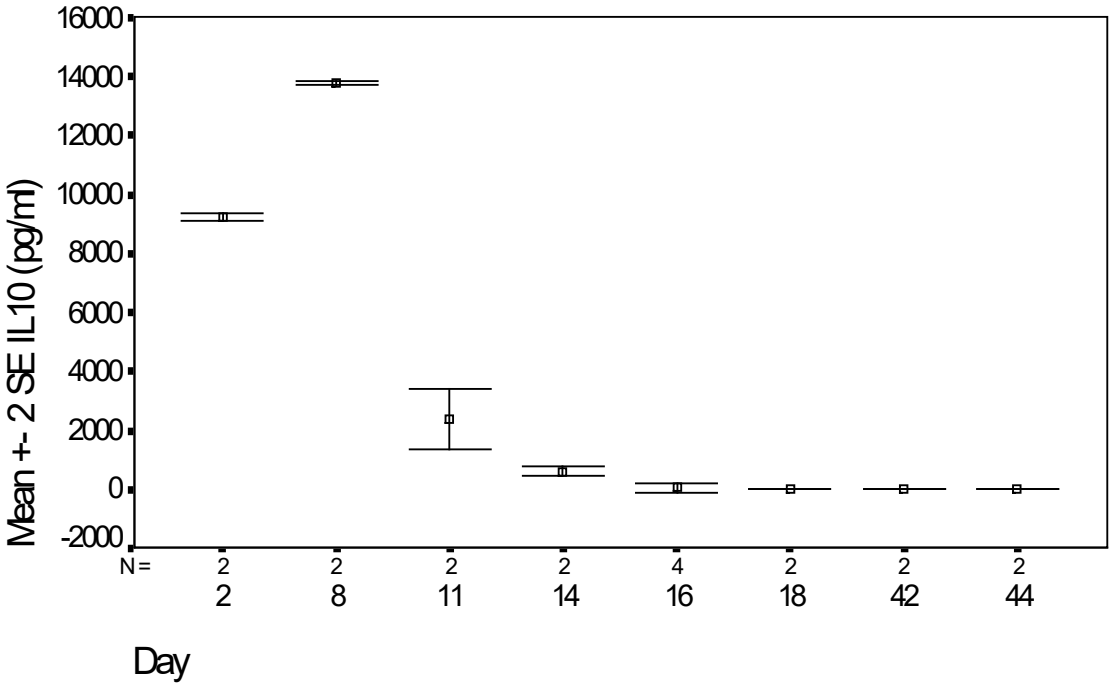


Figure 10: ELISA: IL-10 in Conditioned Medium from Early Cultures of Murine HSC

129/SV murine HSC were isolated by pronase-collagenase perfusion, density gradient separation and centrifugal elutriation, and plated onto uncoated plastic at 1.33x10^6 cells/ml in DMEM with 15% FCS and PSG. Conditioned medium was collected on Days 2, 8, 14, 16 and 18, 42 and 44 and assayed for IL-10 by ELISA, the medium being replaced with fresh medium 24 hours before collection. This graph shows IL-10 content of conditioned medium during a time-course of culture.

Conditioned medium IL-10: **Time course**

Variable	Number of Cases	Mean	SD	SE of Mean
IL-10				
DAY 2	2	9234.0000	83.439	59.000
DAY 8	2	13780.5000	44.548	31.500
DAY 11	2	2390.0000	733.977	519.000
DAY 14	2	609.5000	106.915	75.600
DAY 16	4	80.0750	160.150	80.075

3.3.4 Long term cultures of unstimulated murine HSC

IL-10 was detected in conditioned medium from unstimulated long-term murine HSC cultures (>160 days) at 0-120pg/ml, whereas lysate IL-10 was detected at 580-830pg/ml (Figure 11, 95% CI, $p<0.01$).

3.3.5 Cell-based ELISA: IL-10 expression by primary cultures of rat and murine HSC

CBE with anti-IL-10 antibody showed statistically significantly greater ($p<0.01$) 95% CI for OD compared with control (no anti-IL-10 antibody) with 14-day cultures of rat HSC (Figure 12) and with long-term cultures of murine HSC (Figure 13).

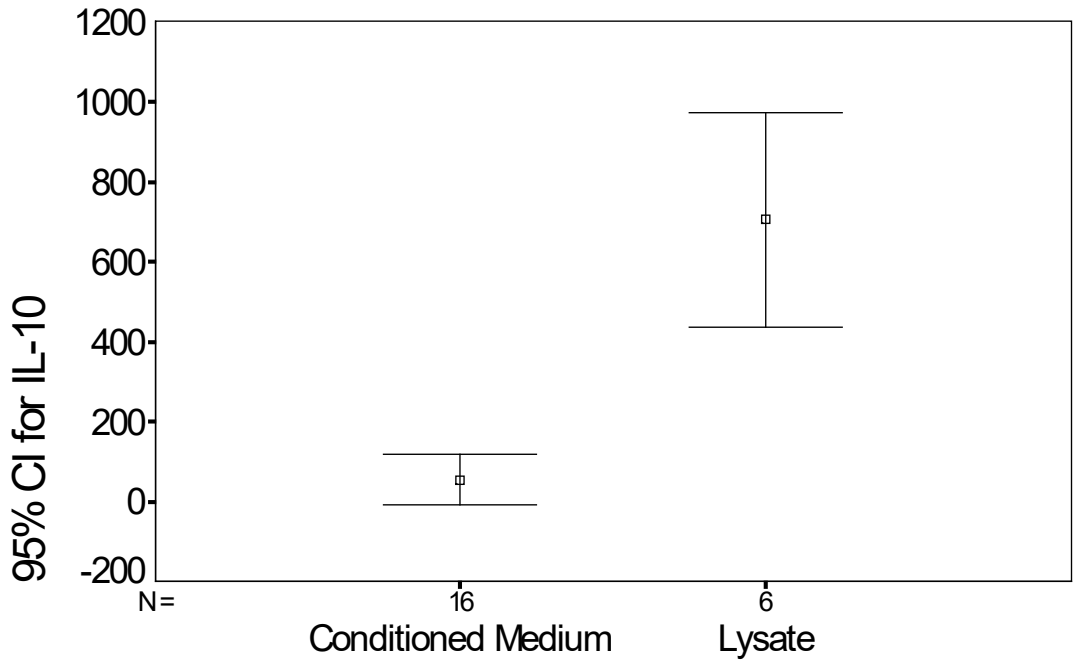


Figure 11: ELISA: IL-10 in Conditioned Medium and Lysate from Long-Term Cultures of Murine HSC

Conditioned medium and lysates from unstimulated long-term (more than Day 60) cultures of murine HSC were assayed for IL-10 by ELISA. Whereas conditioned medium contained little IL-10, HSC lysates contained significantly more IL-10 than the conditioned medium.

IL-10 expression after 60 days culture: **Conditioned Medium (CM)** vs. **Lysate**

Variable	Number of Cases	Mean	SD	SE of Mean
IL10				
CM	16	55.9375	121.255	30.314
LYSATE	6	705.0167	256.074	104.542

Mean Difference = -649.0792

Levene's Test for Equality of Variances: F= 6.834 P= .017

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-8.19	20	.000	79.271	(-814.435, -483.723)
Unequal	-5.96	5.86	**.001**	108.848	(-916.944, -381.214)

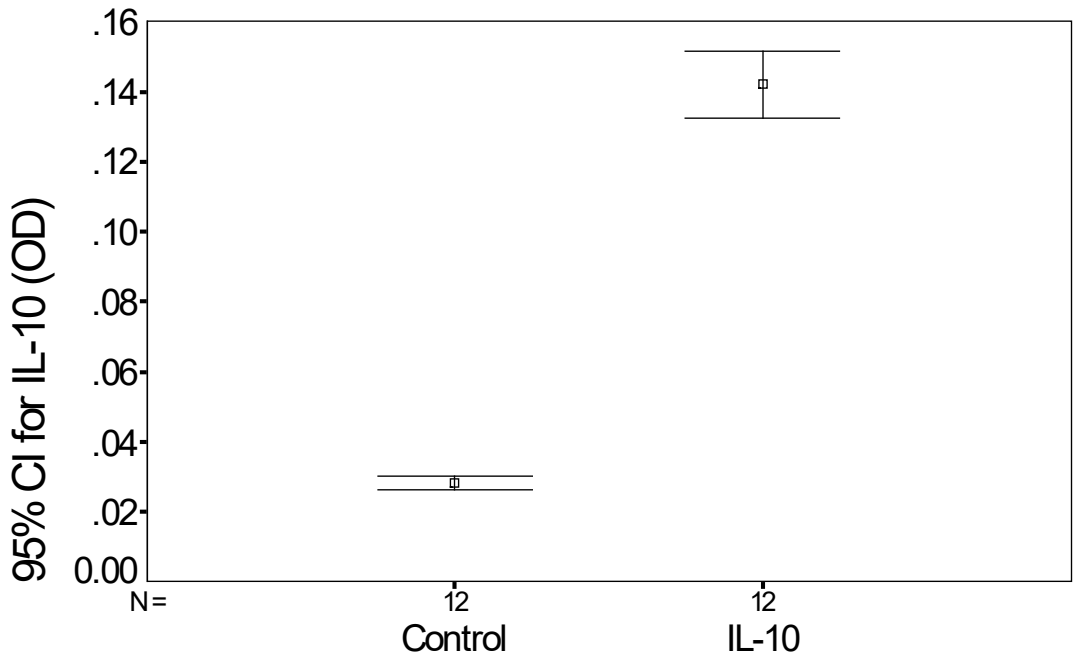


Figure 12: Cell-Based ELISA: IL-10 Expression by Day 14 Cultures of Rat HSC

Rat HSC were isolated, plated at 5000 cells/well in 96-well plates and cultured for 14 days, at which time a cell-based ELISA was performed: cells were fixed and permeablised in the wells, and rat anti-murine IL-10 monoclonal IgG_1 antibody (JES-2A5 hybridoma supernatant, shown as "**IL-10**"*) or isotype-control antibody (shown as* "**Control**"*) applied, followed by rabbit anti-rat IgG HrP-conjugated polyclonal antibody (Sigma). TMB was used as chromogen. 95% CI showed significantly higher optical density with wells incubated with anti-IL-10 antibody compared with wells incubated with isotype-control antibody.*

Optical density with CBE: **Isotype Control** vs. **anti-murine IL-10** antibody

Variable	Number of Cases	Mean	SD	SE of Mean
OD				
CONTROL	12	.0284	.003	.001
α-IL10	12	.1421	.015	.004

Mean Difference = -.1137

Levene's Test for Equality of Variances: F= 20.273 P= .000

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-25.84	22	.000	.004	(-.123, -.105)
Unequal	-25.84	11.98	**.000**	.004	(-.123, -.104)

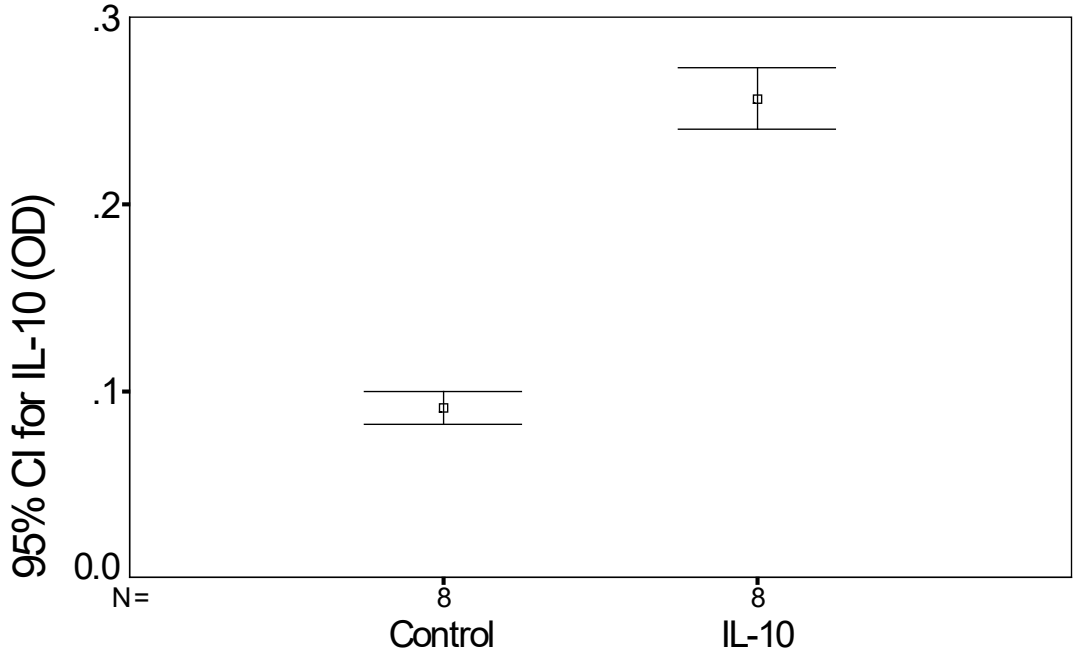


Figure 13: Cell-Based ELISA: IL-10 Expression by Long-Term Cultures of Murine HSC

Long-term cultures of murine HSC were plated at 10,000 cells/well in 96-well plates and cultured for 14 days, at which time a cell-based ELISA was performed: cells were fixed and permeablised in the wells, and rat anti-murine IL-10 monoclonal IgG_1 antibody (JES-2A5 hybridoma supernatant, shown as "**IL-10**"*) or isotype-control antibody (shown as* "**Control**"*) applied, followed by rabbit anti-rat IgG HrP-conjugated polyclonal antibody (Sigma). TMB was used as chromogen. 95% CI showed significantly higher optical density with wells incubated with anti-IL-10 antibody compared with wells incubated with isotype-control antibody.*

Optical density with CBE: **Isotype Control** vs. **anti-murine IL-10** antibody

Variable	Number of Cases	Mean	SD	SE of Mean
OD				
CONTROL	8	0.0910	0.011	0.004
α-IL10	8	0.2568	0.019	0.007

Mean Difference = -0.1658

Levene's Test for Equality of Variances: F= 3.368 P= 0.088

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-21.14	14	.000	.008	(-.183, -.149)
Unequal	-21.14	10.82	**.000**	.008	(-.183, -.148)

3.4 Discussion - ELISA & CBE

Mouse and rat liver cells were isolated upto 3 times/week by a highly experienced MLSO using an established technique for obtaining high purity HSC preparations; previous characterisation of resultant cells has demonstrated 95-97% of rat cells (approximately 75-80% of murine cells) exhibiting vitamin A autofluorescence and immunohistochemical HSC markers such as desmin, the remaining cells being KC or hepatocytes which rapidly perish in culture, leaving upto 99% pure HSC populations beyond Day 14. Conditioned medium and lysates from an unstimulated time course of cell culture were assayed for IL-10 using a sandwich ELISA which we developed.

IL-10 was undetectable at any time in HSC lysate or conditioned medium from ΔIL-10 transgenic mice, whereas IL-10 was detected in a time-dependent manner in conditioned medium from wild-type murine HSC cultures, confirming the high IL10-specificity of the assay. IL-10 was detected at high levels in rat HSC conditioned medium during the first 14 days culture, but not in lysate; IL-10 was detected at low levels in conditioned medium after Day 21, but at high levels in lysate.

The universal and presently inescapable caveats applicable to data from nearly all *in vitro* HSC research, such as confounding HSC responses to isolation enzymes, loss of matrix interactions and HSC interactions with culture medium components apply here too. Additionally, previous studies of HSC IL-10 expression have been further confounded by potential KC contribution to IL-10 mRNA and protein levels during early HSC cultures, and again, the same is partly true for the present study. KC and NK cells remain physiologically active following isolation, and secrete IL-10 in an autocrine manner[167] upon stimulation *in vitro*, with peak IL-10 release 12-24 hours following stimulation[160-162,168,169], and may be responsible for any IL-10 observed in culture. Further, no KC

activity is seen despite 1ng/ml physiological portal blood LPS concentrations, this inhibition being partly due to KC-derived IL-10[170], IL-10 being upregulated in LPS tolerance[171]. Although IL-10 secretion by these cells during unstimulated culture has not been reported, the fact that the decline in culture conditioned medium IL-10 corresponds to loss of contaminating cells by Day 14-21 in culture cannot be overlooked. On the other hand, the pattern of IL-10 variation during the time-course of HSC activation mirrors that of other indices of activation, and the exact source of IL-10 during culture is presently unclear. KC contribution to conditioned medium IL-10 could be estimated by measuring IL-10 in conditioned medium from an unstimulated time-course of KC culture, and this essential control is required before interpretation of this "HSC" data is possible. Another approach would include selective inhibition of KC activation by gadolinium chloride or methyl palmitate[60,63] following isolation, although any effect of this on HSC activation would need to be assessed[172]. A more comprehensive solution to this universal and perpetual problem would be development of higher purity HSC isolation methods.

Although it is difficult to interpret conditioned medium data from early cultures, the loss of contaminating cell types after Day 14 means that any IL-10 seen following this point can be considered to be HSC-derived. The finding of lysate IL-10 in long-term cultures is entirely consistent with previous immunocytochemistry data (K. Thompson, Southampton, Unpublished data), and suggests that (a) IL-10 protein expression is constitutive in HSC-derived myofibroblasts, as in other cell types[98,107,108] and (b) IL-10 is retained intracellularly or membrane-associated rather than released, perhaps in association with the IL-10 receptor (IL10-R). Murine fibroblasts express IL10-R[173], and although there are as yet no reports of IL10-R receptor characterisation in HSC, IL-10 is known to have direct effects on HSC function[174] and on renal mesangial cells, which

express IL-10 receptor upon LPS stimulation[97,175], implying the presence of active IL10-R, which is essential for IL-10 signalling. Further, some macrophage cell lines have membrane-bound IL-10[176], although whether this is as an integral membrane protein or bound to IL-10 receptors is not yet known. In a similar way, cellular IL-10 was detected in HSC by cell-based ELISA with both *early* rat and long-term murine HSC cultures, which demonstrated positive staining compared to control; it is quite plausible that cellular IL-10 was degraded or otherwise prevented from being detected following cell lysis, this phenomenon not occurring during the CBE. CBE with ΔIL-10 HSC was not performed, but would have been a useful negative control. We were unable to quantify IL-10 levels detected with CBE; cyanogen bromide-mediated adsorption of recombinant standards was considered, but was not possible in the time available. Further studies into the molecular trafficking and localisation of cellular IL-10 such as by immunopreciptiation, and of the receptor by radioligand binding[173] may be useful.

CHAPTER 4

"CAN HSC BE INDUCED TO MAKE ADDITIONAL IL-10?" - STIMULATION OF HSC

4.1 Introduction

Having established that HSC express IL-10 protein on activation *in vitro*, we decided to investigate whether this expression is already maximal, or whether, as we expected, it would be possible to induce greater IL-10 expression. This was assessed by stimulating cells with a variety of substances and assessing IL-10 production in response. With due regard to the cytokines thought to activate or enhance activation of HSC[177-179], we decided to stimulate HSC with TNF-α, TGF-β_1, IL-1β and LPS. Additionally, as conditioned medium from activated KC is known to enhance HSC activation, we stimulated HSC with conditioned medium from LPS-stimulated RAW cells, and assayed HSC conditioned medium for IL-10. To exclude the previous problems with KC contamination, all experiments were performed with rat and murine HSC after at least 14 days culture (with or without passage), by which time any contaminating cells would have been eliminated.

4.2 Materials & Methods

4.2.1 Materials

HSC and RAW Cell Culture
Cellstar 24-well Tissue culture plates (Greiner Labortechnik)
Otherwise as above.

HSC Stimulation
Recombinant murine TNF-α (17.2kDa, 156aa; Peprotech EC)
Recombinant human TGF-β_1 (25.0kDa, 112aa; Peprotech EC)
Recombinant murine IL-1β (17.4kDa, 152aa; Peprotech EC)
Lipopolysaccharide (LPS, from *Escherichia coli* 026:B6; Sigma)

IL-10 ELISA and CBE
As above.

4.2.2 Stimulation of HSC

Long-term cultures of mouse HSC were plated into 24 well plates at $0.4x10^6$/ml, and cultured for 48 hours under conditions previously described. Medium was replaced with fresh medium containing:

- 0, 100, 1000 or 10,000ng/ml LPS or
- 0, 1, 10 or 100ng/ml TNF-α or
- 0, 1, 10, or 100ng/ml TGF-β or
- 0, 10 or 100ng/ml IL-1β or
- Conditioned medium from RAW cells 50ng/ml LPS-stimulated for 24 hours.

12 hours later, conditioned medium was collected into fresh 24-well plates and 0.5ml/well fresh medium with protease inhibitors (See Appendix B) was added. Plates were triple freeze-thawed, and conditioned medium, cell lysates and stimulant samples assayed for IL-10 content using the IL-10 ELISA protocols described in Chapter 3.

IL-10 content of RAW cell conditioned medium following 0,10,100,1000 and 10000ng/ml LPS stimulation for 24 hours was also assayed.

4.2.3 Cell-based ELISA

Rat HSC were isolated as before, plated in 96-well plates at 5000/well (200µl medium) and cultured for 14 days; long term cultures of murine HSC were plated in 96-well plates at 10,000/well (200µl medium) and cultured for 48 hours. In each case, medium was aspirated and replaced with 200µl fresh media containing 0, 500 or 1000ng/ml LPS. Cells were incubated for a further 12 hours, after which medium was aspirated and replaced with 100µl fresh medium, and IL-10 expression assayed with the CBE technique described in Chapter 3.

4.3 Results

Following TNF-α, TGF-β and IL-1β stimulation of murine HSC, no significant differences relative to control were detected in IL-10 levels in conditioned medium or lysates following 2 separate experiments. However, with LPS stimulation (Figure 14), we found an increase in conditioned medium IL-10 content with 100ng/ml LPS (not significant), with a decrease in IL-10 content with 1000 and 10000ng/ml LPS, relative to stimulation with 100ng/ml LPS ($p<0.01$). An increase in lysate IL-10 was observed with both 1000 and 10000ng/ml LPS ($p<0.01$). Following stimulation with 100ng/ml LPS, lysate IL-10 was significantly greater than supernatant IL-10 (210-490pg/ml vs. 0-22pg/ml, Mean±SD).

Following CBE with 14-day cultures of rat HSC, a significantly greater OD was achieved with 500ng/ml LPS, compared with both 0 and 1000ng/ml LPS (Figure 15). A similar pattern was seen with long-term cultures of murine HSC (Figure 16), although the differences are not significant.

Conditioned medium from 24-hour LPS-stimulated RAW cells contained IL-10 (Figure 17). A 1:2 dilution with DMEM/15%FCS/PSG of conditioned medium from 24-hour LPS-stimulated RAW cells contained greater IL-10 than the same conditioned medium following 12 hour incubation with long-term murine HSC (Figure 18).

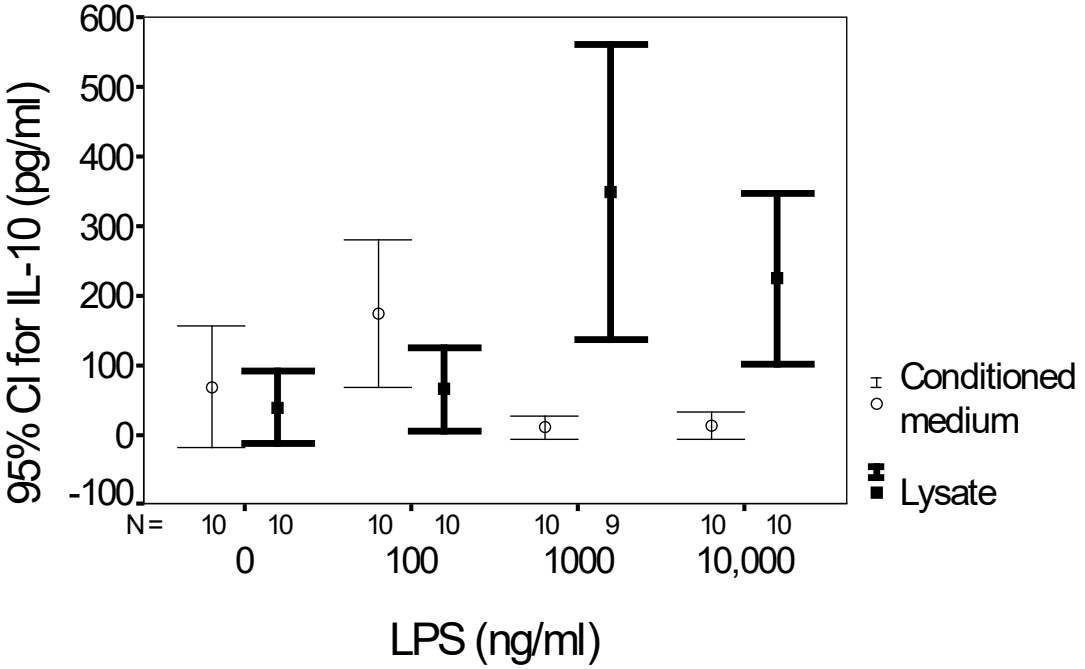


Figure 14: ELISA: IL-10 Expression Following 12-hour LPS Stimulation of Murine HSC

Long-term cultures of murine HSC were plated at 200,000 cells/well in 24-well plates and cultured for 48 hours. Conditioned medium was replaced with fresh medium containing 0, 100, 1000 or 10,000ng/ml LPS. 12 hours later, conditioned medium was collected and fresh medium with protease inhibitors added. Lysates were made by triple-freeze-thawing. Conditioned medium and lysates were assayed for IL-10 by ELISA. This graph shows 95% CI for IL-10 content of conditioned medium and lysates following stimulation with the 4 concentrations of LPS.

Conditioned medium IL-10: **100ng/ml** vs. **1000ng/ml** LPS

Variable	Number of Cases	Mean	SD	SE of Mean
IL-10				
100ng/ml LPS	10	174.3760	149.065	47.138
1000ng/ml LPS	10	10.4360	22.752	7.195

Mean Difference = 163.9400
Levene's Test for Equality of Variances: F= 24.835 P= .000

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	3.44	18	.003	47.684	(63.759, 264.121)
Unequal	3.44	9.42	**.007**	47.684	(56.798, 271.082)

Lysate IL-10: **0ng/ml** vs. **1000ng/ml** LPS

Variable	Number of Cases	Mean	SD	SE of Mean
IL-10				
0ng/ml LPS	10	38.7380	72.713	22.994
1000ng/ml LPS	9	349.2867	275.754	91.918

Mean Difference = -310.5487
Levene's Test for Equality of Variances: F= 5.068 P= .038

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-3.44	17	.003	90.251	(-500.962, -120.136)
Unequal	-3.28	9.00	**.010**	94.750	(-524.884, -96.213)

Lysate IL-10: **0ng/ml** vs. **10000ng/ml** LPS

Variable	Number of Cases	Mean	SD	SE of Mean
IL-10				
0ng/ml LPS	10	38.7380	72.713	22.994
10000ng/ml LPS	10	224.7220	171.094	54.105

Mean Difference = -185.9840
Levene's Test for Equality of Variances: F= 1.872 P= .188

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-3.16	18	**.005**	58.788	(-309.493, -62.475)
Unequal	-3.16	12.15	.008	58.788	(-313.899, -58.069)

IL-10 expression following 1000ng/ml LPS Stimulation: **Supernatant** vs. **Lysate**

Variable	Number of Cases	Mean	SD	SE of Mean
IL-10				
SUPERNATANT	10	10.4360	22.752	7.195
LYSATE	9	349.2867	275.754	91.918

Mean Difference = -338.8507
Levene's Test for Equality of Variances: F= 8.706 P= .009

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-3.88	17	.001	87.248	(-522.928, -154.774)
Unequal	-3.68	8.10	**.006**	92.199	(-551.015, -126.686)

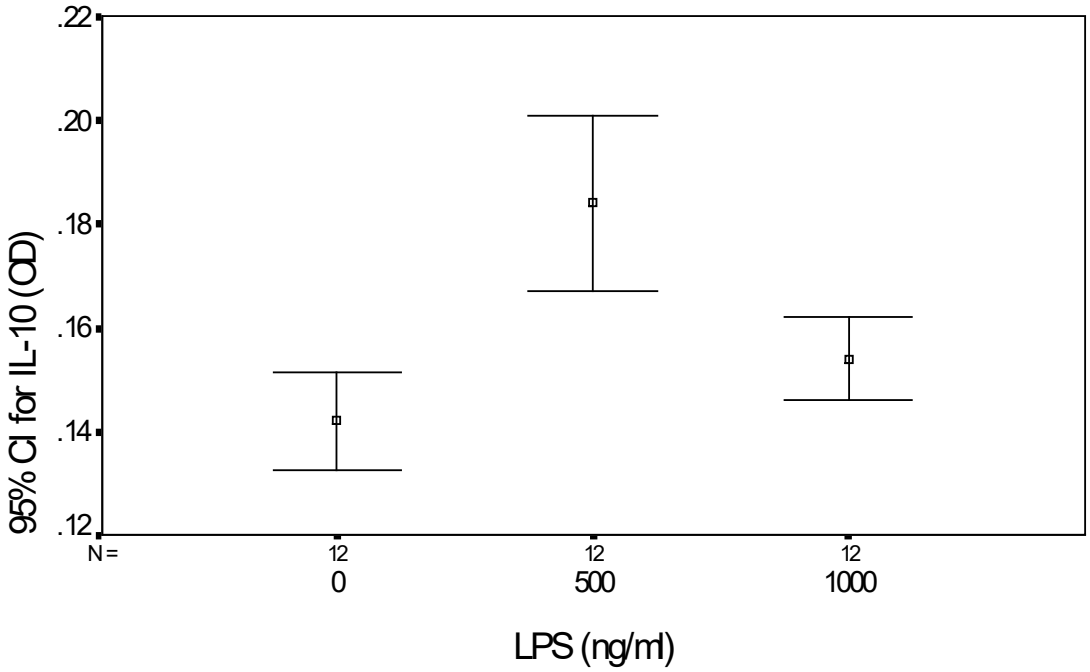


Figure 15: Cell-Based ELISA: IL-10 Expression Following 12-hour LPS Stimulation of Rat HSC

Rat HSC were isolated, plated at 5000 cells/well in 96-well plates and cultured for 14 days, at which time conditioned medium was replaced with fresh medium containing 0, 500 or 1000ng/ml LPS. 12 hours later, conditioned medium was replaced with fresh medium, and a cell-based ELISA was performed. This graph shows 95% CI for optical density following stimulation of cells with the 3 concentrations of LPS.

Optical density following LPS stimulation: **0ng/ml** vs. **500ng/ml** LPS

Variable	Number of Cases	Mean	SD	SE of Mean
OD				
0ng/ml LPS	12	.1421	.015	.004
500ng/ml LPS	12	.1841	.027	.008

Mean Difference = -.0420
Levene's Test for Equality of Variances: F= 6.481 P= .018

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-4.77	22	.000	.009	(-.060, -.024)
Unequal	-4.77	17.29	**.000**	.009	(-.061, -.023)

Optical density following LPS stimulation: **0ng/ml** vs. **1000ng/ml** LPS

Variable	Number of Cases	Mean	SD	SE of Mean
OD				
0ng/ml LPS	12	.1421	.015	.004
1000ng/ml LPS	12	.1541	.012	.004

Mean Difference = -.0120
Levene's Test for Equality of Variances: F= 1.117 P= .302

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-2.14	22	.044	.006	(-.024, .000)
Unequal	-2.14	21.33	**.044**	.006	(-.024, .000)

Optical density following LPS stimulation: **500ng/ml** vs. **1000ng/ml** LPS

Variable	Number of Cases	Mean	SD	SE of Mean
OD				
500ng/ml LPS	12	.1841	.027	.008
1000ng/ml LPS	12	.1541	.012	.004

Mean Difference = .0300
Levene's Test for Equality of Variances: F= 10.738 P= .003

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	3.54	22	.002	.008	(.012, .048)
Unequal	3.54	15.61	**.003**	.008	(.012, .048)

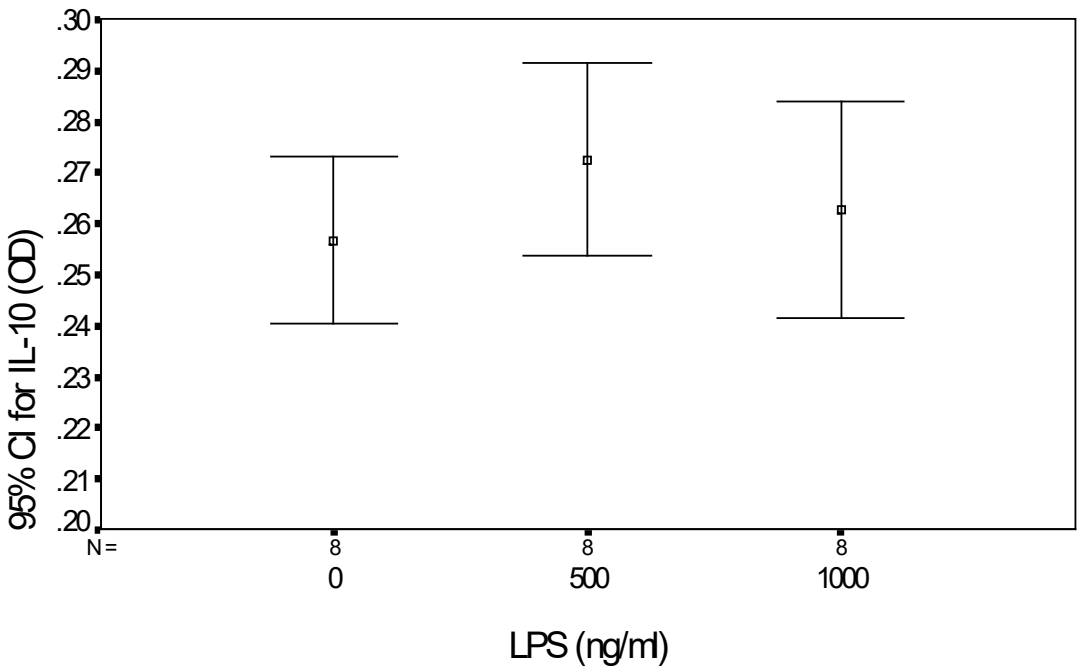


Figure 16: Cell-Based ELISA: IL-10 Expression Following 12-hour LPS Stimulation of Murine HSC

Murine HSC were plated at 10,000 cells/well in 96-well plates and cultured for48 hours, at which time conditioned medium was replaced with fresh medium containing 0, 500 or 1000ng/ml LPS. 12 hours later, conditioned medium was replaced with fresh medium, and a cell-based ELISA was performed. This graph shows 95% CI for optical density following stimulation of cells with the 3 concentrations of LPS.

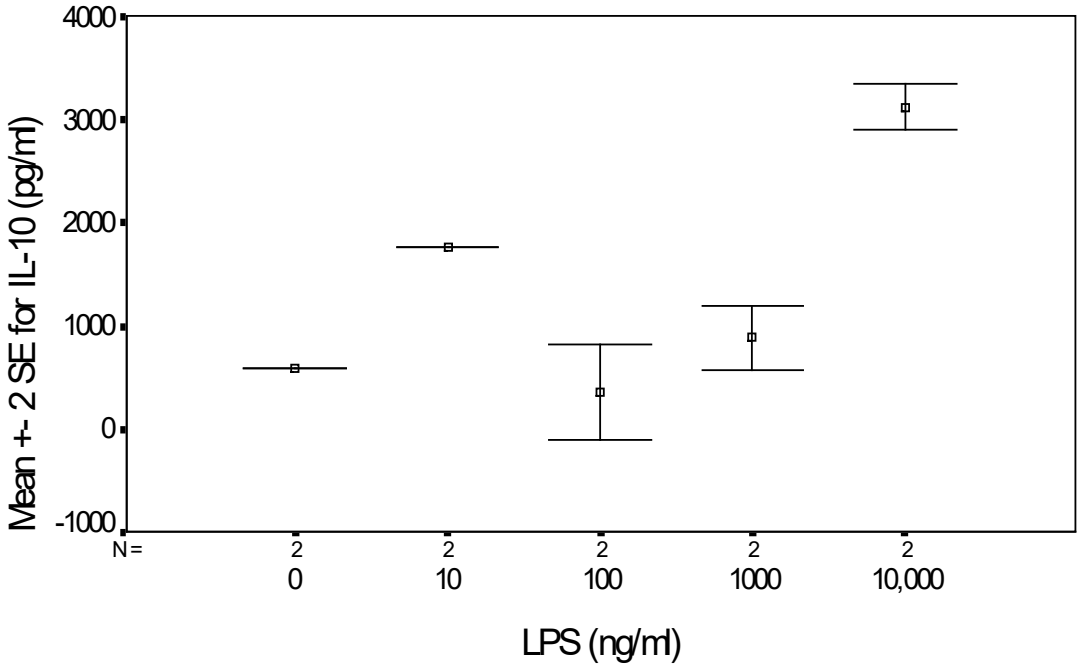


Figure 17: ELISA: IL-10 in Conditioned Medium from LPS-Stimulated RAW Cells

RAW cells were stimulated with 0, 10, 100, 1000 or 10,000ng/ml LPS for 24 hours. Conditioned medium was collected and assayed for IL-10 content by ELISA. A bi-phasic dose-dependent IL-10 release can be seen.

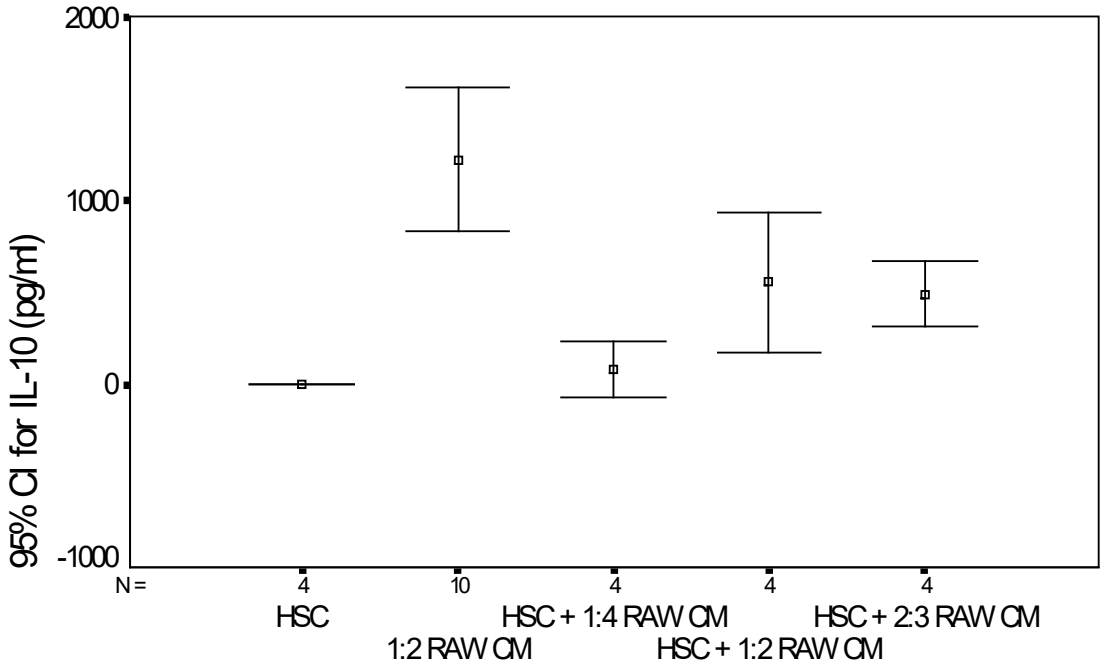


Figure 18: ELISA: IL-10 in Conditioned Medium from LPS-Stimulated RAW Cells Before and After Incubation with Long-Term Murine HSC

Long-term cultures of murine HSC were plated at 200,000 cells/well in 24-well plates and cultured for 48 hours. Conditioned medium was replaced with 0, 1:4, 1:2 and 2:3 dilutions in fresh medium of conditioned medium from RAW cells 50ng/ml LPS-stimulated for 24 hours. 12 hours later, HSC conditioned medium was collected and assayed for IL-10 content. 1:2 diluted RAW conditioned medium that had not been added to HSC was also assayed for IL-10.
In this figure, "**1:2 RAW CM**" *represents 1:2 dilution of RAW conditioned medium in fresh DMEM/15%FCS/PSG, without exposure to HSC.* "**HSC**", "**HSC + 1:4 RAW CM**", "**HSC + 1:2 RAW CM**" *and* "**HSC + 2:3 RAW CM**" *represent conditioned medium from HSC cultured with 0, 1:4, 1:2 and 2:3 dilution of RAW conditioned medium in fresh DMEM/15%FCS/PSG respectively. Note that the only difference between* "**1:2 RAW CM**" *and* "**HSC + 1:2 RAW CM**" *is the presence of the* ***HSC****.*

IL-10 in LPS-stimulated RAW cell conditioned medium: **Before** vs. **After** exposure of CM to HSC

Variable	Number of Cases	Mean	SD	SE of Mean
IL-10				
1:2 RAW CM	10	1224.9600	546.815	172.918
1:2 RAW CM + HSC	4	560.4000	239.693	119.847

Mean Difference = 664.5600
Levene's Test for Equality of Variances: F= 1.532 P= .239

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	2.30	12	**.040**	288.992	(34.900, 1294.220)
Unequal	3.16	11.66	.009	210.390	(204.651, 1124.469

IL-10 in LPS-stimulated RAW cell conditioned medium: **Before** vs. **After** exposure of CM to HSC

Variable	Number of Cases	Mean	SD	SE of Mean
IL-10				
1:2 RAW CM	10	1224.9600	546.815	172.918
2:3 RAW CM + HSC	4	492.3750	111.008	55.504

Mean Difference = 732.5850
Levene's Test for Equality of Variances: F= 3.621 P= .081

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	2.60	12	**.023**	282.077	(117.992, 1347.178)
Unequal	4.03	10.61	.009	181.608	(331.079, 1134.091)

4.4 Discussion - HSC Stimulation

Soluble mediators such as TNF−α and TGF-β are known to have profound effects on HSC activity, and we expected to find increased IL-10 expression on stimulation with these cytokines. RAW cells produce a spectrum of pro-inflammatory cytokines following LPS exposure, yet HSC exposure to conditioned medium from these activated cells failed to stimulate IL-10 expression. Stimulation of HSC with LPS did, however, alter IL-10 expression. We noted increased cytoplasmic or membranous IL-10 corresponding with decreased released IL-10, perhaps suggesting a shift towards a more potent paracrine or autocrine effect of IL-10. Possible mechanisms for this shift including reduced release of IL-10 with cellular retention, and up-regulation of the IL-10 receptor leading to increased uptake or receptor association extracellularly. Indeed, the fact that RAW cell conditioned medium contained less IL-10 following incubation with live HSC lends support to the possibility that stimulated HSC may actively scavenge IL-10 from the microenvironment for either retention and/or presentation, although the HSC stimulation data may possibly result from LPS toxicity.

These data suggest that basal IL-10 expression by HSC is sub-maximal, and that HSC can be induced to increase the effect of HSC-derived IL-10, via an unresolved mechanism which may involve activation of IL-10-synthetic machinery, alterations in IL-10 trafficking, or altered expression of the IL-10 receptor. Work is presently underway in our group to examine these mechanisms. Further work to examine induction of HSC IL-10 expression could usefully examine effects of substances such as prostaglandins and PDGF which are known to enhance HSC activation[65,180,181], or alternatively, substances which are known to induce IL-10 expression in other cell types, such as dexamethasone or methylprednisolone[182,183]. Further, antagonists or inhibitors

of agents known to reduce IL-10 expression such as pentoxifylline and caffeine[182], may enhance IL-10 expression.

CHAPTER 5

"WHAT IS THE FUNCTIONAL EFFECT OF HSC-DERIVED IL-10?" - INVESTIGATION BY HSC/RAW BIOLOGICAL ASSAY

5.1 Introduction

Having shown that the IL-10 transcript seen during HSC activation does indeed result in IL-10 protein expression, we proceeded to investigate the functional effect of HSC-derived IL-10 by investigating its effect on activation of cells from the RAW 264.7 monocyte cell line. We chose TNF-α production from these cells in response to LPS as an index of activation, and investigated how this is modulated by pre-exposure to live HSC and HSC conditioned medium, establishing whether any change is due to IL-10, by pre-incubating HSC and HSC conditioned medium with anti-IL-10 neutralising antibody ("αIL10NAb").

5.2 Materials & Methods

5.2.1 Materials

TNF-α ELISA
96-well Nunc-Immuno™ plates with MaxiSorp surface (Life Technologies)
Recombinant murine TNF-α (17.2kDa, 156aa; Peprotech EC)
Rat anti-murine TNF–α, monoclonal IgG_1 antibody (XT3; Harlan Sera-Lab, Crawley Down, UK)
Rabbit anti-murine TNF–α, polyclonal antibody (Peprotech EC)
Swine anti-rabbit Ig, HrP-conjugated polyclonal antibody (Dako, High Wycombe, UK)
Recombinant murine TNF–α (Peprotech EC)
Buffers and solutions (See Appendix A)
Carbonate-bicarbonate coating buffer, pH 9.0
Citrate-acetate buffer, pH 6.0
PBS/Tween-20
Chromogen solution
10%FCS/DMEM
5%BSA/PBS

HSC and RAW Cell Culture
As above.

Biological Assay
Recombinant murine IL-10 (Peprotech EC)
Rat anti-murine IL-10, monoclonal IgG_1 antibody (JES-2A5; Genzyme)

5.2.2 Establishing TNF-α ELISA

5.2.2.1 Part 1 - First attempt

Method

A 96-well Nunc-Immuno plate was coated with 5μg/ml anti-murine TNF−α IgG_1 monoclonal (capture) antibody in coating buffer overnight at 4°C. Non-specific binding of wells was eliminated with 1 hour 5%BSA/PBS block. Combinations of 100μl/well of 100ng/ml and 0ng/ml rmTNF−α in 10%FCS/DMEM were added for 2 hours. LPS-stimulated RAW cell conditioned medium was added to some wells, as these are known to contain TNF-α. Wells were rinsed 3 times with PBS/Tween-20, and rabbit anti-murine TNF−α polyclonal detection antibody was added in dilutions of 1:50 to 1:6400 for 2 hours followed by washing as before. Swine anti-rabbit Ig HrP-conjugated tertiary antibody was applied in dilutions of 1:1000 to 1:4000. Wells were rinsed as before, developed with TMB chromogen, and optical densities determined as for IL-10 ELISA.

Results and Discussion

No difference in OD was detected between wells with and without rmTNF-α. However, a large difference in OD was seen between some wells with and without RAW cell conditioned medium; considering this, and the wide range of antibody dilution combinations we had used, we attributed the result to faulty recombinant cytokine, and repeated with fresh TNF-α

5.2.2.2 Part 2 - Second attempt

Method

The same ELISA was performed with a fresh batch of rmTNF-α.

Results and Discussion

A satisfactory difference between wells with and without TNF-α was seen with 1:200 detection antibody dilution and 1:1000 tertiary antibody dilution. A one-third serial dilution of rmTNF-α yielded a standard curve in range 100ng/ml to 140pg/ml with 0.99 correlation coefficient (Figure 19). All subsequent TNF-α ELISAs included a similar range of standards, with a chequerboard ELISA being performed with each new batch of antibody.

Figure 19: TNF-α ELISA: Standard Curve

5μg/ml of rat anti-murine TNF–α, monoclonal IgG_1 capture antibody (Harlan Sera-Lab) was incubated overnight at 4℃. A one-third serial dilution of rmTNF-α in the range 300ng/ml to 411pg/ml was applied for 2 hours, followed by detection with a 1:200 dilution in 5%BSA/PBS of rabbit anti-murine TNF–α polyclonal antibody (Peprotech EC) and a 1:1000 dilution of swine anti-rabbit Ig HrP-conjugated polyclonal antibody (Dako), with TMB as chromogen. Optical densities were analysed with an Anthos Labtec GT2 plate reader, and a log-log curve of mean optical density against known rmTNF-α concentration plotted, with a correlation co-efficient of 0.99. Subsequent TNF-α ELISAs included a 7- or 8-point standard curve with each plate, giving a sensitivity in the range 300-100ng/ml to 46-137pg/ml.

5.2.3 Biological assay: RAW Cell Stimulation

Method

RAW cells were plated at $0.1x10^6$ cells/ml in 24-well plates (0.5ml/well) in RAW cell medium, and stimulated with 0, 100, 1000 or 10,000ng/ml LPS for 0, 3, 6, 12 or 24 hours. Supernatants were harvested and assayed for TNF−α.

Results & Discussion

Under these conditions, 6-hour 1000ng/ml LPS stimulation yielded a satisfactory balance between high TNF−α production and technical convenience (Figure 20).

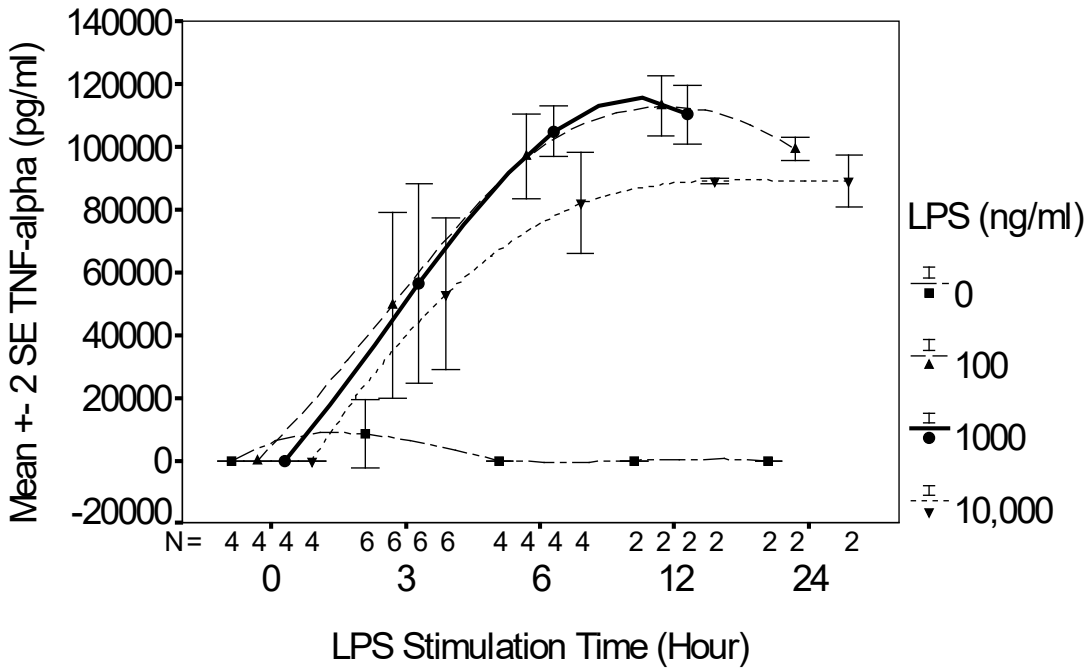


Figure 20: ELISA: TNF-α in Conditioned Medium from LPS-Stimulated RAW Cells

RAW cells were plated at 50,000 cells/well in 24-well plates, and stimulated with 0, 100, 1000 or 10,000ng/ml LPS. Conditioned medium was harvested at 0, 3, 6, 12 and 24 hour time points, and assayed for TNF-α by ELISA.

5.2.4 RAW/HSC Biological assay

Method I: RAW Cell Controls

RAW cells were plated in 24-well plates as before. Cells were incubated with or without LPS and:

- Control medium or
- 5μg/ml rat anti-murine IL-10 neutralising antibody (αIL10NAb) or
- 5μg/ml Isotype-control antibody or
- 100ng/ml rmIL-10 or
- 100ng/ml rmIL-10 + 5μg/ml αIL10NAb

Conditioned medium was harvested 6 hours later and assayed for TNF-α by ELISA.

Method II: RAW Cell Stimulation Following Pre-exposure to HSC CM

RAW cells were plated at $0.1x10^6$ cells/ml in 24-well plates (0.5ml/well) in RAW cell medium. Cells were allowed to settle for 1 hour, followed by addition of control medium or 100ng/ml rmIL-10 or 30% conditioned medium from 14-day rat HSC cultures, with or without 1-hour pre-incubation of these substances with 5μg/ml αIL10NAb. 1 hour later, RAW cells were stimulated with 1000ng/ml LPS for 6 hours. RAW cell conditioned medium was harvested and assayed for TNF–α together with HSC conditioned medium and antibody samples.

Method III: RAW Cell Stimulation During Co-Culture with Live HSC

Long term cultures of murine (>120 days) HSC and 21-day cultures of rat HSC were plated on 24-well plates at $0.4x10^6$/ml (0.5ml/well) in DMEM/15%FCS/PSG, and cultured for 48 hours. Medium was replaced with 200μl fresh medium with or without 5μg/ml αIL10NAb. 1 hour later, medium was aspirated and 0.5ml of $0.1x10^6$ RAW

cells/ml was added, with some RAW cells being plated directly onto plastic with or without 100ng/ml rmIL-10 ± 5μg/ml αIL10NAb. Cells were left to settle for 1 hour, followed by stimulation and TNF–α assay as for Method II.

5.3 Results

5.3.1 RAW Cell Controls

Addition of anti-IL-10 neutralising antibody or isotype-control antibody to both stimulated and unstimulated RAW cells produced no significant change in TNF−α production. As expected, addition of rmIL-10 produced a significant and profound reduction of TNF−α release in response to LPS, this effect being in part negated by adding αIL10NAb (Figure 21).

5.3.2 RAW Cell Stimulation Following Pre-exposure to HSC CM

RAW cell pre-exposure to 30% conditioned medium from 14-day rat HSC cultures produced a significant reduction in TNF−α production (7000-7700pg/ml vs. 2400-3200pg/ml, 95%CI) comparable to that with rmIL-10 (7000-7700pg/ml vs. 2200-2800pg/ml, 95%CI), both effects being partly, but significantly reduced to similar extents with αIL10NAb (Figure 22). There was no significant difference between reduction of TNF-α production by 100ng/ml IL-10 and 30% rat HSC conditioned medium. Adding αIL10NAb alone to RAW cells did not produce a significant change in TNF-α production.

5.3.3 RAW Cell Stimulation During Co-Culture with Live HSC

A reduction in TNF−α production in response to LPS was seen following RAW cell co-culture with long-term murine HSC cultures (not significant, $p=0.056$) and 21-day rat HSC cultures (significant, $p<0.05$, 7000-7700pg/ml vs. 2000-2800pg/ml 95%CI), the reduction seen with rat HSC being comparable to that with rmIL-10 (2200-2800pg/ml,

95%CI) and 14-day rat HSC conditioned medium (2400-3200pg/ml, 95%CI). In contrast to the increase in TNF−α production seen with rmIL-10 neutralisation and conditioned medium IL-10 neutralisation, neutralisation of HSC-associated IL-10 prior to co-culture with RAW cells showed a further *reduction* in TNF−α production following LPS stimulation, compared with non-neutralised production (2400-3100pg/ml vs. 5800-700pg/ml for murine HSC, and 1600-1800pg/ml vs. 2000-2800g/ml for rat HSC, 95%CI and $p<0.05$ both cases).

IL-10 and αIL10NAb samples and HSC conditioned medium at any time points did not contain TNF−α.

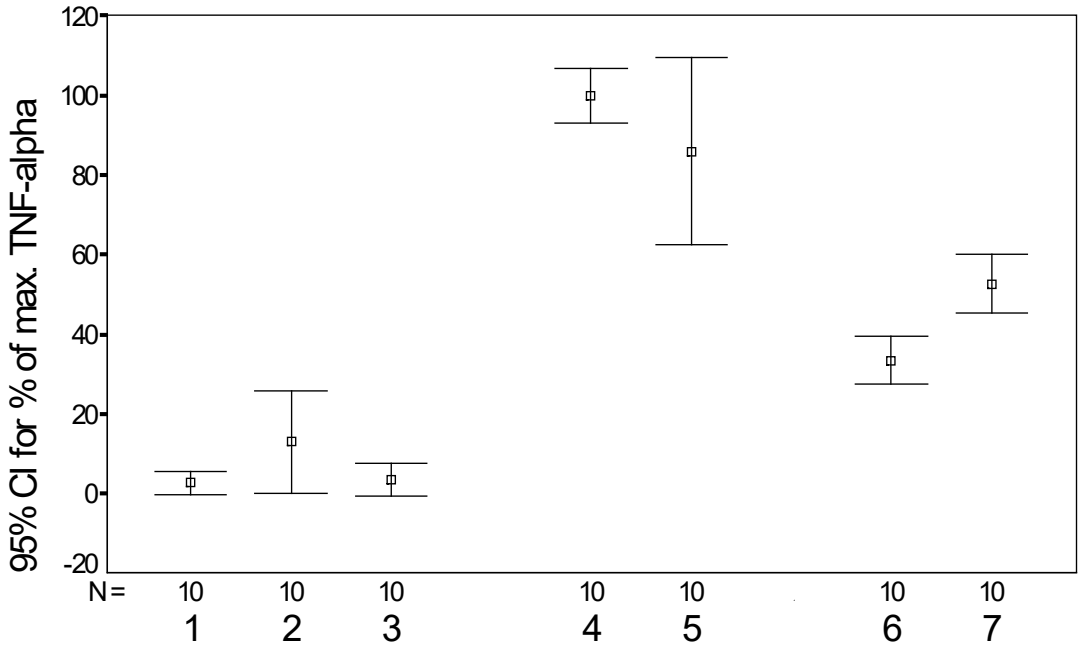


Figure 21: ELISA: TNF-α in Conditioned Medium from LPS-Stimulated RAW Cells Pre-Incubated With or Without IL-10 and/or αIL10NAb

RAW cells were plated at 50,000 cells/well in 24-well plates. Cells were treated for 1 hour with the substances shown below. 4-6 were then 1000ng/ml LPS-stimulated for 6 hours, and conditioned medium from all wells assayed for TNF-α by ELISA, with TNF-α values being expressed in this graph as 95% CI for percentage of the mean TNF-α produced with RAW cells + 1000ng/ml LPS (4).

1: *Nothing*
2: *5μg/ml αIL10NAb*
3: *100ng/ml IL-10.*

4: *Nothing*
5: *5μg/ml αIL10NAb.*
6: *100ng/ml IL-10.*
7: *100ng/ml IL-10 and 5μg/ml αIL10NAb.*

1-3 *not LPS-stimulated,* ***4-7*** *LPS-stimulated*

Unstimulated RAW cell TNFα production: Without αIL10NAb **(1)** vs. with αIL10NAb **(2)**

Variable	Number of Cases	Mean	SD	SE of Mean
% of max TNFα				
RAW-LPS (1)	10	2.7119	3.977	1.258
RAW+αIL10NAb-LPS (2)	10	13.0785	17.981	5.686

Mean Difference = -10.3665
Levene's Test for Equality of Variances: F= 8.786 P= .008

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-1.78	18	.092	5.823	(-22.601, 1.868)
Unequal	-1.78	9.88	**.106**	5.823	(-23.364, 2.631)

Unstimulated RAW cell TNFα production: Without IL10 **(1)** vs. with IL10 **(3)**

Variable	Number of Cases	Mean	SD	SE of Mean
% of max TNFα				
RAW-LPS (1)	10	2.7119	3.977	1.258
RAW+IL-10-LPS (3)	10	3.5083	5.665	1.791

Mean Difference = -.7963
Levene's Test for Equality of Variances: F= 3.462 P= .079

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-.36	18	**.720**	2.189	(-5.394, 3.802)
Unequal	-.36	16.14	.721	2.189	(-5.433, 3.840

LPS-Stimulated RAW cell TNFα production: Without αIL10NAb **(4)** vs. with αIL10NAb **(5)**

Variable	Number of Cases	Mean	SD	SE of Mean
% of max TNFα				
RAW+LPS (4)	10	100.0000	9.612	3.040
RAW+αIL10NAb+LPS (5)	10	85.9542	32.792	10.370

Mean Difference = 14.0458
Levene's Test for Equality of Variances: F= 3.817 P= .066

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	1.30	18	**.210**	10.806	(-8.657, 36.749)
Unequal	1.30	10.54	.221	10.806	(-9.867, 37.959)

LPS-Stimulated RAW cell TNF–α production: Without IL10 **(4)** vs. with IL10 **(6)**

Variable	Number of Cases	Mean	SD	SE of Mean
% of max TNFα				
RAW+LPS (4)	10	100.0000	9.612	3.040
RAW+IL-10+LPS (6)	10	33.4666	8.330	2.634

Mean Difference = 66.5334
Levene's Test for Equality of Variances: F= .640 P= .434

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	16.54	18	**.000**	4.022	(58.083, 74.984)
Unequal	16.54	17.64	.000	4.022	(58.071, 74.996)

LPS-Stimulated RAW cell TNF–α production: With IL10 **(6)** vs. with IL10+αIL10NAb **(7)**

Variable	Number of Cases	Mean	SD	SE of Mean
% of max TNFα				
RAW+IL-10+LPS (6)	10	33.4666	8.330	2.634
RAW+IL-10+αIL10NAb+LPS (7)	10	52.6938	10.179	3.219

Mean Difference = -19.2273
Levene's Test for Equality of Variances: F= .180 P= .676

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-4.62	18	**.000**	4.160	(-27.966, -10.488)
Unequal	-4.62	17.32	.000	4.160	(-27.991, -10.464)

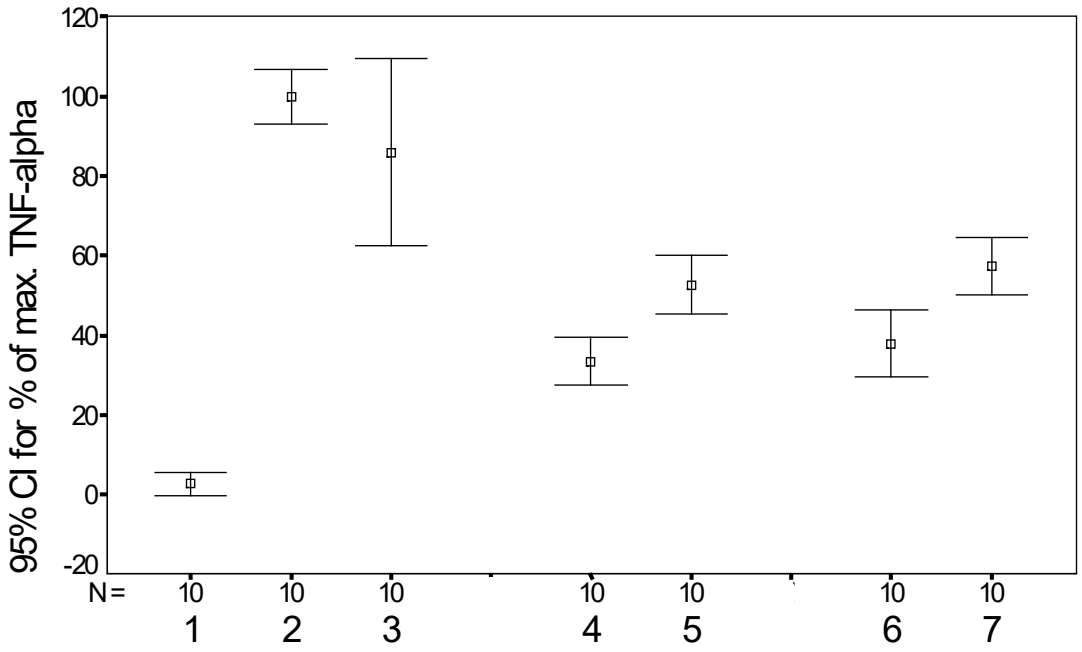


Figure 22: ELISA: TNF-α in Conditioned Medium from LPS-Stimulated RAW Cells Pre-Incubated With IL-10 or Rat HSC Conditioned Medium +/- αIL10NAb

*RAW cells were plated at 50,000 cells/well in 24-well plates. Cells were treated for 1 hour as follows - **1,2**: Nothing, **3**: 5μg/ml αIL10NAb, **4**: 100ng/ml IL-10, **5**: 100ng/ml IL-10 pre-incubated for 1 hour with 5μg/ml αIL10NAb, **6**: 30% conditioned medium from 14-day cultures of rat HSC, **7**: 30% conditioned medium from 14-day cultures of rat HSC pre-incubated for 1 hour with 5μg/ml αIL10NAb. **2-7** were then 1000ng/ml LPS-stimulated for 6 hours, while **1** was unstimulated. Conditioned medium from all wells was assayed for TNF-α by ELISA, with TNF-α values being expressed in this graph as 95% CI for percentage of the mean TNF-α produced with RAW cells + 1000ng/ml LPS (2).*

1: *No pre-treatment. No LPS stimulation.*

2: *No pre-treatment.*
3: *Pre-treated with 5μg/ml αIL10NAb.*

4: *Pre-treated with 100ng/ml IL-10.*
5: *Pre-treated with 100ng/ml IL-10 pre-incubated for 1 hour with 5μg/ml αIL10NAb.*

6: *Pre-treated with 30% conditioned medium from 14-day cultures of rat HSC.*
7: *Pre-treated with 30% conditioned medium from 14-day cultures of rat HSC pre-incubated for 1 hour with 5μg/ml αIL10NAb.*

(2-7 were 1000ng/ml LPS-stimulated)

Note that the only difference between 2 and 6 is the presence of HSC conditioned medium in 6, and the only difference between 6 and 7 is that the HSC conditioned medium was pre-incubated with αIL10NAb in 7.

LPS-Stimulated RAW cell TNFα production: Without IL10 **(2)** vs. with IL-10 **(4)**
Statistics shown with Figure 21

LPS-Stimulated RAW cell TNFα production: With IL-10 **(4)** vs. with IL10+IL10NAb **(5)**
Statistics shown with Figure 21

LPS-Stimulated RAW cell TNFα production: without 30% rat HSC CM **(2)** vs. With 30% rat HSC CM **(6)**

Variable	Number of Cases	Mean	SD	SE of Mean
% of max TNFα				
RAW+LPS (2)	10	100.0000	9.612	3.040
RAW+30%rat HSC SN+LPS (6)	10	37.9538	11.589	3.665

Mean Difference = 62.0462
Levene's Test for Equality of Variances: F= .598 P= .450

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	13.03	18	**.000**	4.761	(52.043, 72.049)
Unequal	13.03	17.40	.000	4.761	(52.018, 72.074)

LPS-Stimulated RAW cell TNFα production: With 30% HSC CM **(6)** vs. with 30% HSC CM+αIL10NAb **(7)**

Variable	Number of Cases	Mean	SD	SE of Mean
% of max TNFα				
RAW+30% HSC SN+LPS (6)	10	37.9538	11.589	3.665
RAW+30% HSC SN+LPS+αIL10NAb (7)	10	57.4485	10.102	3.194

Mean Difference = -19.4947
Levene's Test for Equality of Variances: F= .398 P= .536

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	-4.01	18	**.001**	4.862	(-29.708, -9.281)
Unequal	-4.01	17.67	.001	4.862	(-29.722, -9.267)

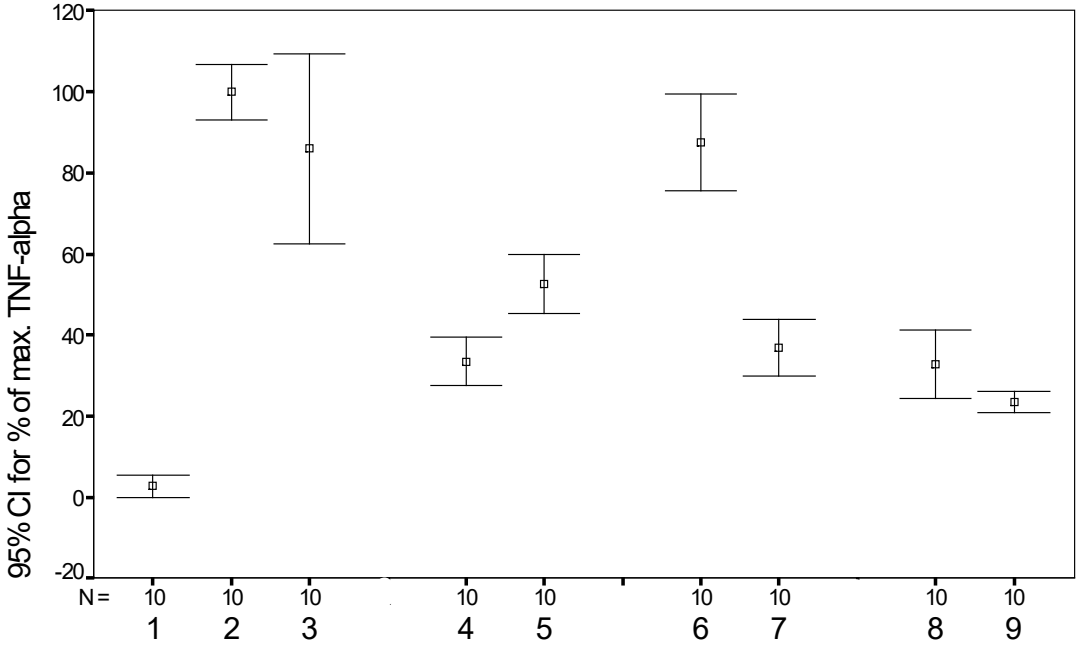


Figure 23: ELISA: TNF-α in Conditioned Medium from LPS-Stimulated RAW Cells Pre-Incubated With IL-10 or Co-Cultured With Live Rat and Murine HSC +/- αIL10NAb

RAW cells were plated at 50,000 cells/well in 24-well plates, in empty wells **(1)** *&* **(2)** *or in wells with 5μg/ml αIL10NAb* **(3)** *or 100ng/ml IL-10* **(4)** *or both* **(5)**. *Some RAW cells were plated at the same density in wells containing 200,000 cells/well long-term murine HSC pre-incubated for 1 hour with* **(7)** *or without* **(6)** *5μg/ml αIL10NAb, or containing 200,000 cells/well 21-day rat HSC pre-incubated for 1 hour with* **(9)** *or without* **(8)** *5μg/ml αIL10NAb. Cells were left to settle for 1 hour. 2-6 were 1000ng/ml LPS-stimulated for 6 hours, with conditioned medium from all wells being assayed for TNF-α by ELISA. TNF-α values are expressed in this graph as 95% CI for percentage of the mean TNF-α produced with RAW cells + 1000ng/ml LPS* **(2)**.

Note that the only difference between 2 and 6 is the presence of the murine HSC, while the only difference between 2 and 8 is the presence of the rat HSC.

LPS-Stimulated RAW cell TNFα production: Without IL10 **(2)** vs. With IL10 **(4)**
Statistics shown with Figure 21

LPS-Stimulated RAW cell TNFα production: With IL10 **(4)** vs. With IL10+αIL10NAb **(5)**
Statistics shown with Figure 21

LPS-Stimulated RAW cell TNFα production: No Co-culture **(2)** vs. Co-culture with murine HSC **(6)**

Variable	Number of Cases	Mean	SD	SE of Mean
% of max TNFα				
RAW+LPS (2)	10	100.0000	9.612	3.040
RAW+murineHSC+LPS (6)	10	87.6107	16.645	5.264

Mean Difference = 12.3893
Levene's Test for Equality of Variances: F= 2.506 P= .131

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	2.04	18	**.056**	6.078	(-.381, 25.159)

Unequal	2.04	14.40	.060	6.078	(-.613, 25.392)

LPS-Stimulated RAW cell TNFα production: Co-culture with murine HSC without IL10NAb **(6)** vs. Co-culture with murine HSC with αIL-10NAb **(7)**

Variable	Number of Cases	Mean	SD	SE of Mean
% of max TNFα				
RAW+murineHSC+LPS (6)	10	87.6107	16.645	5.264
RAW+murineHSC+αIL10NAb+LPS (7)	10	36.8910	9.602	3.036

Mean Difference = 50.7197
Levene's Test for Equality of Variances: F= 2.189 P= .156

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	8.35	18	**.000**	6.077	(37.953, 63.486)
Unequal	8.35	14.39	.000	6.077	(37.720, 63.720)

LPS-Stimulated RAW cell TNFα production: No co-culture **(2)** vs. Co-culture with 21-day rat HSC **(8)**

Variable	Number of Cases	Mean	SD	SE of Mean
% of max TNFα				
RAW+LPS (2)	10	100.0000	9.612	3.040
RAW+ratHSC+LPS (8)	10	32.8990	11.716	3.705

Mean Difference = 67.1010
Levene's Test for Equality of Variances: F= .062 P= .806

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	14.00	18	**.000**	4.792	(57.033, 77.169)
Unequal	14.00	17.34	.000	4.792	(57.005, 77.197)

LPS-Stimulated RAW cell TNFα production: Co-culture with 21-day rat HSC without IL10NAb **(8)** vs. Co-culture with 21-day rat HSC with αIL-10NAb **(9)**

Variable	Number of Cases	Mean	SD	SE of Mean
% of max TNFα+LPS				
RAW+ratHSC+LPS (8)	10	32.8990	11.716	3.705
RAW+ratHSC+αIL10NAb+LPS (9)	10	23.5562	3.800	1.202

Mean Difference = 9.3428
Levene's Test for Equality of Variances: F= 5.679 P= .028

t-test for Equality of Means

Variances	t-value	df	2-Tail Sig	SE of Diff	95% CI for Diff
Equal	2.40	18	.027	3.895	(1.160, 17.526)
Unequal	2.40	10.87	**.036**	3.895	(.758, 17.928)

5.4 Discussion - HSC/RAW Biological Assay

We have shown that pre-exposure of a macrophage cell line to conditioned medium from high purity cultures of 14-day rat HSC can down-regulate macrophage activation in response to LPS to almost the same extent as pre-exposure to rmIL-10, and that part of this effect is likely to be due to IL-10, as neutralisation of conditioned medium IL-10 reduced this effect. Further, macrophage co-culture with live HSC also causes a reduction in TNF–α production in response to LPS. Neutralisation of the HSC-associated IL-10 (identified in Chapter 3) causes an *enhancement* of the down-regulatory effect seen. These data indicate that HSC are able to modulate macrophage activity through IL-10, although other factors may also play a part.

An interesting but potentially problematic facet of these data is that our neutralising antibody (5μg/ml rat anti-murine IL-10, monoclonal IgG_1 antibody) was unable to fully neutralise 100ng/ml rmIL-10. Previous work with this antibody has shown total neutralisation with equimolar amounts, but this does not seem to be case here. Also, monocyte TNF-α production is increased following incubation with αIL10NAb[120], but this was not observed here. Both of these observations can probably be attributed to the short time period of RAW cell stimulation and rmIL-10 incubation with αIL10NAb, but as the characterisation of "positive control" behaviour is essential towards an understanding of the test system behaviour, early resolution of these potential flaws through neutralisation with different doses of αIL10NAb, targeting different IL-10 epitopes using another of the wide range of αIL10NAb available[112,184], longer incubations, and repeating with isotype-control antibodies would be advantageous, as evaluation of the extent of the role of IL-10 in HSC-macrophage interactions is impossible until total IL-10 neutralisation is achieved.

CHAPTER 6

FINAL DISCUSSION

This study has demonstrated and quantified IL-10 expression in time-courses of rat and murine HSC activation *in vitro*, by ELISA. High IL-10 levels in HSC conditioned medium were accompanied with low lysate IL-10 levels during the first 14 days; a switch occurred between Day 14-21, leading to low IL-10 levels in conditioned medium and high lysate levels thereafter, the latter being seen for an indefinite period. This expression was confirmed by cell-based ELISA, which suggested also that cytoplasmic IL-10 may be present in early cultures, albeit at lower levels than in later cultures. Stimulation experiments with medium and long-term murine HSC cultures showed no significant changes in IL-10 expression following TNF–α, TGF-B and IL-1b stimulation. LPS stimulation, however, resulted in a rise in lysate IL-10 accompanied with a loss of conditioned medium IL-10 suggesting increased cellular expression with active IL-10 uptake, via the IL-10 receptor or some other mechanism. This effect was seen with 1000 and 10000ng/ml LPS, and was confirmed with earlier cultures of rat HSC. These data were supported by data suggesting that HSC stimulated with as yet unidentified products of macrophage activation can actively sequester IL-10 from the microenvironment. Finally, HSC-derived IL-10 was found to have biological activity. Conditioned medium and live cells down-regulated macrophage activation to the same extent as recombinant IL-10, this effect being reduced by neutralising conditioned medium IL-10. Neutralisation of live HSC IL-10 resulted in a more profound suppression of macrophage activation, suggesting a role for IL-10 in the control of HSC expression of macrophage inhibitory cytokines.

At the time of these experiments, we knew of no group that had reported quantified identification of HSC-derived IL-10 *protein* (as opposed to mRNA) during HSC activation *in vitro*; subsequently, however, some of the data from the present study were confirmed in part by another group[174], although their report made no mention of the drawbacks common to both sets of data, such as KC contamination and other confounding factors. In terms of the hypotheses of the present study, it was not possible, unfortunately, to completely answer all the initial questions in the time available. Certain key questions remain unanswered at this time, including whether the demonstrated modulations in HSC IL-10 expression in response to LPS or other stimuli will lead to modulation in the HSC-macrophage axis in favour of further suppression of macrophage function. In the light of present data, it seems unlikely that any further IL-10 expression will further modulate macrophage activity, as the modulation presently seen with unstimulated cells is already close to that of 100ng/ml IL-10, even though HSC IL-10 expression as measured by ELISA is not as great.. Even if membrane localisation and presentation of IL-10 following stimulation is a means to enhancing activity, the limiting factor in the interaction seems to be macrophage receptiveness to IL-10 signal, through extent of receptor expression, downstream signalling or other control factors. Future work may consider factors controlling macrophage sensitivity to IL-10 *in vitro* and *in vivo* as adjunct to factors modifying HSC IL-10 expression. Whilst HSC-IL10-KC axis modulations certainly may hold therapeutic potential, a more fruitful strategy may be to investigate anti-fibrotic effects of IL-10 on HSC[174], either through (IL-10-mediated) modulation of the KC pro-fibrotic stimulus or independently of this.

The further reduction of macrophage activity following sub-maximal neutralisation of HSC-associated IL-10 may be a fascinating avenue of discovery. The exact effect of this intervention can only be speculated at this time. The loss of the autocrine inhibitory

signal may induce its exaggerated replacement, or that of other cytokines known to have anti-inflammatory effects on macrophages (TGF-β, IL-4, IL-10, IL-13)[84] as this does not lead to generalised HSC stimulation, this effect may be selective for the lost cytokine or the "anti-inflammatory" group of cytokines, perhaps identifying a novel therapeutic modality for cytokine manipulation. A sensible starting point for this work would be to confirm these data by the use of ΔIL-10 HSC and isotype control antibodies.

By way of completeness, future work could usefully fill the gaps in the data generated by this study, by establishing the KC contribution to IL-10 identified during early culture, and defining IL-10 kinetics with and without (LPS) stimulation. Previous work has suggested a cytoplasmic or membrane-bound IL-10 localisation in long-term HSC cultures (K. Thompson, Unpublished data), but the exact situation is unclear, especially with regard to the molecular basis of IL-10 trafficking in HSC before and after activation. Additionally, repeating the stimulation and biological assay experiments with ΔIL-10 cells as negative controls for these interactions, to further characterise the role of IL-10 would be valuable. Such manipulations at the molecular level are amongst the most powerful and sure ways to establish causation. Of course, there is a need to correlate these *in-vitro* data with the *in-vivo*; any of the present findings may be artefacts of the HSC activation-on-plastic model, and in particular, the initial expression of IL-10 may be a result of cell injury during isolation rather than of activation. A further confounding factor in the present "gold standard" model of HSC activation is the lack of native pathological ECM dynamics. *In vivo*, ECM acts as a "cytokine sponge", absorbing some of the cytokines produced, altering the kinetic and dynamic behaviours, further casting doubt over the physiological significance of IL-10 expression and these HSC-macrophage interactions observed *in vitro*. The dichotomy between *in vitro* and *in vivo* data is especially true in cytokine biology, where even the latest Phase III clinical trials

investigating systemic cytokine manipulation have returned unexpectedly disappointing results[185], underscoring the real difficulties in translating basic scientific data to a clinical setting.

As mentioned previously, research in this field would benefit from development of techniques to study initial HSC activation *in vitro* in the absence of contaminating cells. HSC can be maintained in an undifferentiated phenotype in vitro by inhibiting NF-κB activation by incubation with calpain inhibitor immediately following isolation (A. Elsharkawy, Southampton, personal communication). With time, contaminating cells die leaving pure HSC cultures, although HSC do not seem to differentiate following removal of inhibitor. A better approach would be to plate pure long-term cultures onto basement membrane-like matrix (such as that derived from the Engelbreth-Holm-Swarm (EHS) murine tumour), following which HSC revert to quiescent phenotype, subsequent removal of matrix activating the HSC as normal[16].

Presently, investigations into potential clinical applications of IL-10 concentrate on using pre-synthesised recombinant protein introduced systemically or locally[159]. Therapy with IL-10 is associated with immunosuppression in terms of a reduced capacity to control infection in animals[186,187] and reduced monocyte and T-cell function in humans[158]. These factors and the necessity of repeated intravenous administration are likely to prove problematic for future clinical applications. The present investigation was performed to assess the potential for therapeutic modulation of the existing IL-10 synthetic system that has been hypothesised to have biological inhibitory effects in inflammation and fibrosis, with the knowledge that ΔIL-10 mice develop significantly greater fibrosis that wild-type following CCl_4 intoxication[154]. Results from the present study indicate that HSC are able to downregulate KC activation through IL-10 *in vitro*, although the significance of

this system *in vivo* has not been assessed. Thus, intrahepatic (rather than systemic) augmentation of IL-10 may be beneficial in liver fibrosis, although clearly, further work is required to assess the extent of this potential both *in vitro* and *in vivo* (such as with exogenous IL-10 in the CCl_4 model of liver injury), as well as to establish appropriate modes of targeted expression or delivery.

APPENDIX A: BUFFERS AND SOLUTIONS

All materials were supplied by Sigma (Poole, UK) unless otherwise stated.

A.1 General solutions

Phosphate-buffered saline (PBS)

A solution of 0.01M phosphate buffer, 0.0027M KCl, 0.137M NaCl, pH 7.4 at 25°C.

	Distilled water	1 litre
	PBS tablets	5 tablets

PBS/Tween-20 (0.05% Tween-20 in PBS)

	PBS	1000ml
	Tween-20	500µl

5%Marvel/PBS/Tween-20	Dried milk powder (Marvel, Premier Beverages)	1g
	PBS	20ml

10%FCS/DMEM	Foetal calf serum (Life Technologies)	2.2ml
	DMEM (Life Technologies)	20ml

A.2 Western blot analysis

2x stacking gel buffer	0.25 M Tris-HCl pH 6.8(3.02g/100ml)	
4x separating gel buffer	1.2 M Tris-HCl pH 8.8	14.52g/100ml
20%SDS	SDS (sodium dodecyl sulfate)	20g
	Water	100ml
4% Acrylamide Solution	30% acrylamide stock	16 ml
	2x stacking gel buffer	60ml
	20% SDS	0.6 ml
	water	43.4ml
12.5% Acrylamide Solution	30% stock acrylamide solution *(acrylamide:bisacrylamide - 37.5:1)*	50.3ml
	4x separating gel buffer	30ml
	20% SDS	0.6ml
	water	39.7ml
15% Acrylamide Solution	30% stock acrylamide solution	60.3ml
	4x separating gel buffer	30ml
	20% SDS	0.6ml
	water	29.7ml
10% Ammonium persulphate	Ammonium persulphate	1g
	Water	10ml
10x stock electrode buffer	Tris	30.3g

	Glycine	144g
	20% SDS	50ml
	water	950ml
2x SDS-PAGE sample buffer	2x stacking gel buffer	25ml
	20% SDS	10ml
	Glycerol	10g
	2-mercaptoethanol	5ml
	Bromophenol blue (10mg/ml)/ in IMS	200µl
	water	10ml
Blotting buffer	10x stock electrode buffer	40ml
	methanol	100ml
	water	360ml
1%SDS/PBS	1:20 Dilution in PBS of 20%SDS	

A.3 Enzyme-linked Immunosorbent assay

50x stock 0.1M carbonate-bicarbonate pH 9.0 coating buffer

1M anhydrous sodium carbonate	10.6g/100ml
1M sodium bicarbonate	8.4g/100ml
Add carbonate to bicarbonate, to pH 9.0	

10x stock 0.1M citrate-acetate buffer pH 6.0

1M anhydrous sodium acetate	8.2g/100ml
1M citric acid	21g/100ml
Add acid to acetate, to pH 6.0	

1%, 5%, 10% BSA/PBS	Bovine serum albumin (BSA)	0.2, 1 or 2g
	PBS	20ml
2% Gelatin	Gelatin	0.2g
	PBS	20ml
1%BSA/5%Sucrose/PBS	BSA	0.2g
	Sucrose	1g
	PBS	20ml
4%PFA/DMEM	Paraformaldehyde	4g
	DMEM (Life Technologies)	100ml
	Boiled for 5 minutes, stored at 4°C until use.	

Chromogen solution

3,3',5,5'-Tetramethylbenzidine (TMB) dihydrochloride, a non-carcinogenic analogue of benzidine was used as the chromogen.

TMB dihydrochloride	15µg
Dimethyl sulfoxide (DMSO)	150µl
10x Citrate-acetate buffer, pH 6.	1.5ml
Water	13.35ml
Reaction stopped with 30µL 4N Sulphuric Acid	

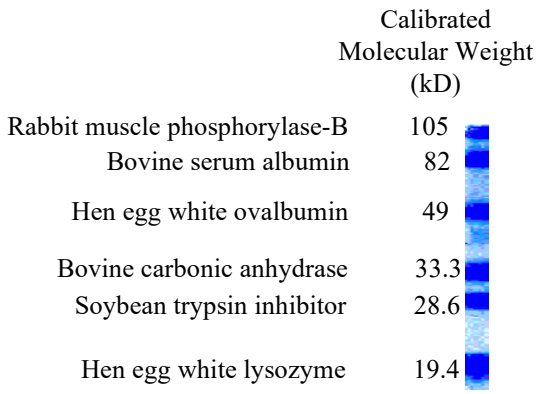
APPENDIX B: PROTEASE INHIBITORS

Protease inhibitors were used to minimise endogenous proteolytic degradation of IL-10 and other proteins during cell lysis methods:

Aprotinin	1 μg/ml
Pepstatin-A	1 μg/ml
Leupeptin	1 μg/ml
AEBSF	1 mM
PMSF	0.5 mM

APPENDIX C: PRESTAINED SDS-PAGE STANDARDS

Bio-Rad, Catalogue Number 161-0305, Control 77243



APPENDIX D: PROTEIN DETERMINATION

Protein content of samples was determined with the Bio-Rad Detergent Compatible Protein Assay kit, according to supplied instructions. This colorimetric assay is a modified (faster) form of the well-established Lowry assay[A]. A standard curve of known protein amounts was made (shown below), and used to estimate sample protein content.

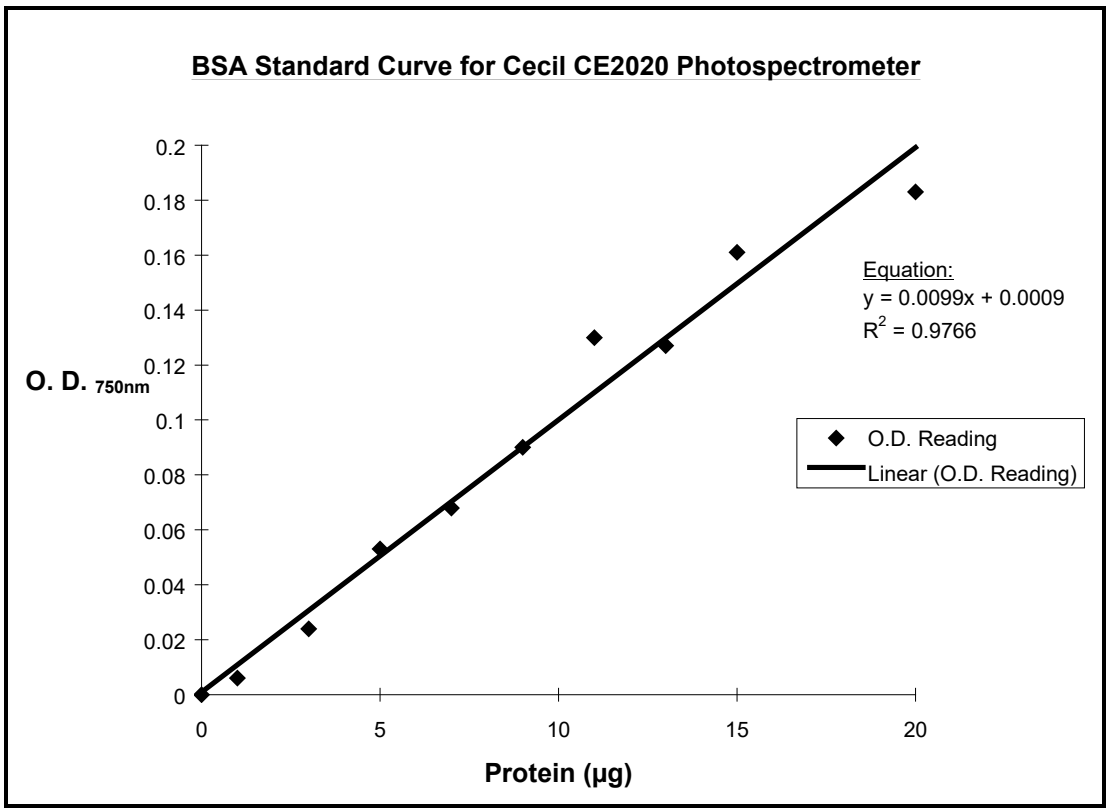


Figure 24: BSA Standard Curve Used For Protein Estimation

[A] Lowry O. et al. J Biol Chem 1951;193:265

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