The T-lymphocyte/ Hepatic Stellate Cell Axis in Liver Fibrosis

by

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ABSTRACT
FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
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Chronic liver disease leading to fibrosis and cirrhosis represents a significant health care burden throughout the world. Activation of hepatic stellate cells (HSC) is central to development of liver fibrosis and is associated with increased proliferation, α-smooth muscle actin (α-SMA) and collagen synthesis and reduced matrix degradation. Although most human liver diseases are characterised by T-lymphocyte infiltration, many previous studies have focussed on Kupffer cells as intermediaries in HSC activation. This thesis examines how T-lymphocytes may directly influence HSC activation via cytokines (IL-10, IL-4, IL-13) and the balance of Th1/Th2 responses or via cell-cell interactions and analyses a T-lymphocyte mediated animal model of liver fibrosis.

Previous studies have suggested that IL-10 exerts an anti-fibrotic effect on CCl4 induced liver fibrosis independent of its anti-inflammatory activity. This thesis demonstrates that IL-10 deleted HSC demonstrate increased procollagen-1 and α-SMA mRNA \textit{in vitro} compared to wild type controls but treatment with recombinant IL-10 did not inhibit HSC activation \textit{in vitro} or reduce CCl4 induced liver fibrosis \textit{in vivo}.

IL-13 was found to increase HSC activation, stimulating both proliferation and collagen synthesis \textit{in vitro}. This provides a potential mechanism for the documented effect of IL-13 in promoting liver fibrosis due to murine schistosomiasis. IL-4 was found to increase HSC proliferation, in addition to its previously demonstrated effect of increasing collagen synthesis.

Administration of concanavalin-A (Con-A) to mice causes an acute hepatitis mediated by CD4+ T-lymphocyte activation. Repeated Con-A injections have previously been shown to result in liver fibrosis which has been assumed to be CD4+ mediated. In this thesis, CD4 deleted mice were found to develop as much fibrosis as wild types after repeated Con-A injection implying that this is not a pure CD4 mediated model. Data suggested that T-cells bearing neither a CD4+ nor CD8+ phenotype might be implicated.

Studies of T-cell/HSC interactions \textit{in vitro} showed that both co-culture with Th1 and Th2 cells and treatment with conditioned media had a marked anti-proliferative effect on HSC. The effect of co-culture was shown to be dependent on direct cell-cell contact.
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## Abbreviations

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<th>Full Form</th>
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<tr>
<td>ABTS</td>
<td>azino bis (3-ethylbezothiazoline-6-sulfonic acid)</td>
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<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>APL</td>
<td>altered peptide ligand</td>
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<tr>
<td>APMA</td>
<td>p-aminophenyl mercuric acid</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCl₄</td>
<td>carbon tetrachloride</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CD₄₀L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Con-A</td>
<td>concanavalin-A</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>CTLA</td>
<td>cytotoxic T-lymphocyte antigen</td>
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<tr>
<td>dATP</td>
<td>deoxyadenine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
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<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<td>dGTP</td>
<td>deoxyguanine triphosphate</td>
</tr>
<tr>
<td>DI</td>
<td>deionized</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DMN</td>
<td>dimethylnitrosamine</td>
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<td>DMSO</td>
<td>dimethyl sulphoxide</td>
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<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DNase</td>
<td>deoxyribonuclease</td>
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<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
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<td>ds</td>
<td>double stranded</td>
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<td>DTH</td>
<td>delayed type hypersensitivity</td>
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<td>extracellular matrix</td>
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<td>fibroblast growth factor</td>
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<td>GCSF</td>
<td>granulocyte-colony stimulating factor</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>GIT</td>
<td>guanidinium isothiocyanate</td>
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<td>Term</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
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<td>insulin-like growth factor</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)propanesulphonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
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<td>membrane-type matrix metalloproteinase</td>
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<td>molecular weight</td>
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<tr>
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<td>NK</td>
<td>natural killer</td>
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<td>PAF</td>
<td>platelet activating factor</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>PAI</td>
<td>plasminogen activator inhibitor</td>
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<td>PAS</td>
<td>periodic acid Schiff</td>
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<td>PBC</td>
<td>primary biliary cirrhosis</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDGF</td>
<td>platelet derived growth factor</td>
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<td>pg</td>
<td>picogram</td>
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<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PSC</td>
<td>primary sclerosing cholangitis</td>
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<tr>
<td>PSG</td>
<td>penicillin/streptomycin/gentamicin</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidifluoride</td>
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<tr>
<td>r</td>
<td>recombinant</td>
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<td>recombinant rat</td>
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<td>retinol binding protein</td>
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<td>ribonuclease</td>
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<td>ribonuclease inhibitor</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
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<tr>
<td>RTase</td>
<td>reverse transcriptase</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
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<tr>
<td>ss</td>
<td>single stranded</td>
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<tr>
<td>SSC</td>
<td>salt sodium citrate</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<tr>
<td>Taq</td>
<td>Thermus aquatics</td>
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<td>TBE</td>
<td>tris(hydroxymethyl)aminomethane borate EDTA</td>
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<td>TBS</td>
<td>tris buffered saline</td>
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<tr>
<td>Tc</td>
<td>T-cytotoxic</td>
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<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>TE</td>
<td>tris-ethylenediaminetetraacetic acid</td>
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<tr>
<td>T-EDTA</td>
<td>trypsin-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N-tetramethyl ethylenediamine</td>
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<tr>
<td>TGF-α</td>
<td>transforming growth factor-α</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
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<tr>
<td>Th</td>
<td>T-helper</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
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<tr>
<td>Tm</td>
<td>melting temperature</td>
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<td>TMB</td>
<td>tetramethylbenzidine</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
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<tr>
<td>tPA</td>
<td>tissue type plasminogen activator</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>TTBS</td>
<td>Tween tris buffered saline</td>
</tr>
<tr>
<td>U</td>
<td>unit of enzyme activity</td>
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<tr>
<td>uPA</td>
<td>urokinase plasminogen activator</td>
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<td>U</td>
<td>unit</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>V</td>
<td>volt</td>
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<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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<td>v/v</td>
<td>volume per volume</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>w/v</td>
<td>weight per volume</td>
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Chapter 1

Introduction
Hepatic Inflammation and Fibrosis:
Role of T-lymphocytes
1.1. INTRODUCTION
Liver fibrosis represents the common end response to chronic liver injury caused by a variety of insults including viral and parasitic infection, toxic damage by alcohol, autoimmune disease, metabolic disease and biliary obstruction. Progressive fibrosis results in the disruption of normal liver architecture and function, culminating in cirrhosis. Hepatic cirrhosis is a major cause of morbidity and mortality worldwide, accounting for more than 5000 deaths per year in the UK alone, and is likely to become an increasingly important clinical problem with the accumulating burden of chronic liver disease caused by hepatitis C.

1.2. Structure of the Liver
Traditionally, the hepatic lobule has been regarded as the basic architectural subunit of the liver (Fig 1.1). Each lobule consists of a central hepatic vein with its surrounding parenchymal cells, bordered at the periphery by portal tracts containing the bile duct, portal vein radicle and hepatic artery branch. However, the liver lobule is defined by anatomical, rather than functional criteria.

Fig 1.1 Anatomy of hepatic lobule
Structure of the Liver Sinusoid

The functional unit of the normal liver is the hepatic acinus, which is defined as the parenchymal cell mass supplied by a single terminal portal tract (Rappaport 1973). Blood flows from the portal vein and hepatic artery into the hepatic sinusoids, from which the surrounding parenchyma is supplied, then drains via the efferent veins to the hepatic vein. The hepatic acinus can be divided into three zones, which correspond both with different hepatocyte functions and with patterns of injury in liver disease (Le Bouton 1969). Zone 1 is located closest to the portal tracts and zone 3 nearest to the efferent vein with zone 2 lying between.

The sinusoids are wide vascular channels lined by a fenestrated endothelium that lacks a typical electron dense underlying basement membrane and therefore facilitates the efficient interchange of substances between the blood and hepatocytes (Burt et al. 1993) (Fig. 1.2). Kupffer cells (hepatic macrophages) and pit cell (large granular lymphocytes) are distributed on the sinusoidal aspect of the endothelium and play an important role in host defence.

Between the endothelium and the hepatocytes, lies the space of Disse, which contains a loosely woven basement membrane type matrix of, types I, III and IV collagens, laminin, proteoglycan and fibronectin (Arenson, Friedman, & Bissell 1988; Burt et al. 1990; Hahn et al. 1980). Located within the space of Disse are hepatic stellate cells (Geerts & Wisse 1992), which play a central role in the development of liver fibrosis.

Figure 1.2 Structure of the Hepatic Sinusoid
1.3. Liver Extracellular Matrix in Health and Fibrosis
The extracellular matrix (ECM) of the liver is not merely an inert scaffold maintaining tissue architecture but exists in a constant dynamic state of turnover and plays an important role in wound healing and the modulation of cellular phenotype and function. In both normal and fibrotic liver, ECM comprises collagens, proteoglycans and glycoproteins but in disease there are changes in the overall quantity of matrix, its distribution and the relative balance of its constituents.

1.3.1. Normal Liver Extracellular Matrix

Collagens
In the normal liver, collagens represent 5-10% of the total protein (Schuppan 1990). All collagens consist of three homologous polypeptide chains with repeating sequences of (Gly-X-Y)$_n$, where one third of the X and Y positions are filled by proline and hydroxyproline respectively (Miller 1985). These repeating motifs allow the chains to be assembled into a rigid rod-like left handed triple helix, with more flexible non-helical domains contributing variable proportions to the different collagen types.

To date, 15 collagen types have been described (van der & Garrone 1991), of which types I, III, IV, V and VI are present in the liver (Schuppan 1990). Collagens may be broadly classified into fibrillar (including types I, III and V) and non-fibril forming proteins (including types IV and VI). The fibrillar collagens account for the majority of total liver collagen, with types I and III contributing approximately 90% (Seyer, Huherson, & Kang 1977).

In normal liver, deposition of the fibril forming collagens (I, III and V) is most prominent in the capsule, around large blood vessels and in the portal tracts with some type I and III collagen in the sub endothelial space (Burt, Griffiths, Schuppan, Voss, & MacSween 1990). Type IV collagen is distributed around large vessels and scattered in the sub endothelial space where it forms part of the specialised basement membrane (Burt, Griffiths, Schuppan, Voss, & MacSween 1990; Clement et al. 1984). Type VI collagen is located primarily in the space of Disse (Schuppan 1990).
Proteoglycans

Proteoglycans are a group of molecules sharing the general structure of a core protein covalently bound to repetitive acidic carbohydrates (glycosaminoglycans) (Schuppan, Herbst, & Milani 1993). In the liver, heparan sulphate, dermatan sulphate and chondroitin sulphate glycosaminoglycan moieties have been described with heparan sulphate contributing over 60% of the total (Gressner & Bachem 1990). Proteoglycans are highly anionic, by merit of their sulphation, and this charge may be important in mediating their binding to other matrix constituents. They have also been shown to bind a number of cytokines including transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF) and may act as reservoirs or modifiers of cytokine activity (Andres et al. 1989; Bashkin et al. 1989).

Glycoproteins

Laminin

Together with collagen IV, laminin is a major constituent of basement membranes and is located in the space of Disse in rats (Maher et al. 1988). It is composed of three polypeptide chains (Schuppan 1990) and has a broad array of functional domains, including binding sites for cellular receptors and other matrix constituents including collagen IV and entactin (Clement et al. 1990; Schuppan 1990). In particular, laminin has been shown to play an important role in maintaining the differentiated functions of hepatocytes in culture (Bissell et al. 1987).

Fibronectins

Fibronectins are a family of glycoproteins composed of two 220 kDa subunits joined by a disulphide bond (Hynes & Yamada 1982). They exist either as circulating plasma proteins or as ECM constituents, derived by alternative splicing of a single fibronectin gene (Schwarzbauer et al. 1983). The type of mRNA generated by splicing is largely cell-type specific, plasma fibronectin being produced predominantly by hepatocytes (Voss et al. 1979) and ECM fibronectin by mesenchymal cells. Fibronectins have distinct functional domains which mediate binding to other matrix components and to cells via integrin receptors (Schuppan 1990) and can be identified
in a near continuous distribution in the sub endothelial space of the liver (Kaesberg et al. 1989).

Undulin

Undulin is a large (>1000 kDa) trimeric glycoprotein widely found in differentiated connective tissues and produced by mesenchymal cells (Milani et al. 1994a). It exhibits strong binding to the fibrillar collagens and is co-localised with densely packed collagen I fibres in the peri-ductal and perivenular areas and space of Disse (Milani, Grappone, Pellegrini, Schuppan, Herbst, Calabro, Casini, Pinzani, & Surrenti 1994a), where it is thought to be important in maintaining collagen organisation.

Entactin

Entactin, also known as nidogen, is a 150kDa glycoprotein widely found in basement membranes non-covalently complexed to laminin (Carlin et al. 1981). Although entactin has not been identified in the liver in vivo, it is expressed by cultured rat HSC (Knittel et al. 1996).

Elastin

Elastin fibres are found scattered in the portal tracts and around the central veins of the liver (Porto et al. 1990) but are a much less prominent component of the ECM in liver than in lung. Their role in the organisation and function of liver matrix has not yet been characterised.

1.3.2. Extracellular Matrix in Liver Fibrosis

ECM deposition in liver fibrosis is initially confined to the sites of maximal cell injury and inflammation. For example, chronic viral hepatitis is associated with periportal fibrosis and alcoholic liver disease with early sinusoidal and pericentral fibrosis. As fibrosis progresses, the deposition of matrix may become pan-acinar, regardless of the nature of the injury, culminating in cirrhotic distortion of the lobular liver architecture with islands of regenerating hepatocytes surrounded by organised fibrotic septa. Replacement of the specialised basement membrane of the space of
Disse with a dense interstitial matrix is a critical event which correlates with impairment of liver function (Orrego et al. 1979). Cirrhotic liver may contain up to six times more collagen and proteoglycans than the normal organ (Gressner & Bachem 1990; Rojkind et al. 1979). There is a disproportionate increase in type I collagen to over 70% of the total collagen (Rojkind et al. 1979; Seyer, Huherson, & Kang 1977) and the increase in glycosaminoglycan content is primarily attributable to dermatan and chondroitin sulphate (Gressner & Bachem 1990).

There is a global increase in ECM glycoprotein expression in fibrotic liver. Accumulation of fibronectin is amongst the earliest detectable changes in matrix composition, suggesting that it may provide a framework for the subsequent deposition of collagen I (Schuppan 1990). Laminin also becomes more prominent in the sub endothelial space (Schuppan 1990) and the elastin content increases in the cirrhotic liver three to six-fold (Kanta et al. 1990). Development of fibrosis is marked by the de novo expression of another glycoprotein, tenascin, in the space of Disse (Ramadori et al. 1991). Tenascin is characteristically only expressed in injury, transiently during mesenchymal embryogenesis, and at the margins of invasive tumours where active ECM remodelling is in progress (Koukoulis et al. 1991).

1.4. The Cellular Source of Extracellular Matrix

The cellular source of ECM in liver disease has been the subject of intensive investigation, which has focused on the cells surrounding the hepatic sinusoid as the site of early events in fibrosis. Immunohistochemical studies in situ identified ECM proteins in hepatocytes, sinusoidal endothelial cells and in hepatic stellate cells (Clement et al. 1986). However, in situ hybridisation studies in experimental models of liver disease subsequently localised transcripts for matrix proteins to non-parenchymal cells with the morphological features of HSC (Milani et al. 1989b; Milani et al. 1990).

Further evidence has derived from in vitro studies of purified cell populations isolated from the liver. When matrix gene messenger RNA (mRNA) expression is compared in freshly isolated cells from normal and experimentally injured rat liver, collagen I and III mRNA increases markedly in HSC, slightly in sinusoidal endothelial cells and remains unchanged in hepatocytes (Maher & McGuire 1990).
Cultured HSC have been shown to express numerous other matrix components including laminin, fibronectin, other glycoproteins and proteoglycans (Gressner & Bachem 1990). Furthermore, HSC have been shown to produce more collagen (Friedman et al. 1985), laminin (Maher, Friedman, Roll, & Bissell 1988), fibronectin (Ramadori et al. 1987) and proteoglycans (Gressner & Schafer 1989) than hepatocytes on a per cell basis.

Overall, the majority of studies point to the hepatic stellate cell as the predominant source of ECM components in liver fibrosis.

1.5. Hepatic Stellate Cells

Hepatic stellate cells (HSC) were first identified by von Kupffer in 1876 by staining with gold chloride (von Kupffer 1876) and termed “Sternzellen” or stellate cells, but their existence as a cell population discrete from Kupffer cells was established later by Ito in 1952 (Ito & Nemoto 1952).

Within the normal liver, HSC have a stellate appearance with branching cytoplasmic processes contacting both the palisades of hepatocytes and the perisinusoidal or sub endothelial area (Wake 1994). These processes contain a prominent cytoskeleton including desmin (Yokoi et al. 1984), actin (Mathew et al. 1994), vimentin, tubulin (De Leeuw et al. 1984), sarcomeric myosin (Ogata et al. 1993) and glial fibrillary acidic protein (Gard et al. 1985). HSC have a large, oval, irregular nucleus and a well developed rough endoplasmic reticulum but the most striking ultrastructural feature is the numerous cytoplasmic lipid droplets containing mainly retinyl esters (Blomhoff et al. 1984).

In normal rat liver, HSC can be identified by staining for the cytoskeletal intermediate filaments desmin or glial fibrillary acidic protein (Gard et al. 1985; Yokoi, et al. 1984) and a small proportion express α-smooth muscle actin (α-SMA) (Johnson et al. 1992). By contrast, in human liver there are no reliable markers for quiescent HSC other than vitamin A.

1.5.1 Biological functions of HSC

Vitamin A metabolism

The liver plays a major role in vitamin A metabolism and storage, containing 50-80% of total body retinol in mammals (Blomhoff et al. 1990). Dietary retinoids are
transported via the lymphatic system to the liver in chylomicrons in the form of retinyl esters (Blomhoff et al. 1990). Chylomicron remnants are taken up by hepatocytes, the retinyl esters hydrolysed and free retinol linked to specific retinol binding proteins (RBP) (Friedman et al. 1993). HSC express RBP receptors and bind and internalise retinol-RBP complexes by receptor-mediated endocytosis (Blomhoff et al. 1988). In adults, retinyl esters stored in HSC represent approximately 80% of total liver retinoids (Blomhoff et al. 1985).

**Regulation of hepatic sinusoidal blood flow**

The strategic anatomical location of HSC in the perisinusoidal space of the liver and their resemblance to pericytic cells that regulate blood flow in other organs suggest that HSC may fulfil this function in the liver. Identification of adrenergic and cholinergic nerve fibres in contact with HSC cytoplasmic processes raises the possibility of neural control of sinusoidal blood flow via effects on HSC (Lafon et al. 1989). Studies performed on rat and human HSC in vitro have shown that they contract in response to a variety of vasoconstrictors including eicosanoids, thrombin, angiotensin II, endothelins and substance P (Pinzani 1995). However, it is necessary to be cautious in extending these findings to the normal liver since cultured cells are representative of an activated, rather than a normal quiescent phenotype, implying that HSC may act as pericytes only in fibrotic liver.

**Physiological extracellular matrix turnover**

In normal liver, all the cell types surrounding the Space of Disse, including hepatocytes, contribute to the synthesis of the extracellular matrix (ECM) (Maher 1989; Milani et al. 1989b; Milani et al. 1989a). The majority of collagen III, IV and laminin are synthesised by HSC and sinusoidal endothelial cells with small amounts of collagen I synthesised by all cells. HSC also contribute to the continuous remodelling of the ECM by production of matrix metalloproteinase-2 (Milani et al. 1994b).

**Role in liver fibrosis**

Following liver injury, HSC undergo activation from the normal quiescent vitamin A storing cell to assume a myofibroblast-like phenotype and accumulate in areas of
liver damage in close association with extracellular matrix (Mak & Lieber 1988; McGee & Patrick 1972). This transformation is gradual and passes through an intermediate stage, known as the ‘transitional cell’, which exhibits features of both quiescent HSC and myofibroblasts. The process of activation is characterised by: loss of retinoid droplets (Takahara et al. 1988); cell enlargement and increased rough endoplasmic reticulum (Takahara et al. 1988); increased proliferation (Ogawa et al. 1986) and expression of α-SMA (Rockey et al. 1992a). Activated HSC are associated with pericellular collagen (Geerts & Wisse 1992) and express mRNA for matrix components (Milani et al. 1989a; Milani et al. 1990). Many of the features of HSC activation are recapitulated by culture in vitro on uncoated plastic and this has greatly facilitated the study of the activation process (Friedman et al. 1989).

1.5.2 Mechanisms of HSC activation

On the basis of both in vivo and in vitro studies, HSC activation has been divided into two phases: initiation and perpetuation. Initiation is characterised by cell enlargement and an increased responsiveness to pro-proliferative and pro-fibrogenic factors. Perpetuation describes a sustained period of proliferation and fibrogenesis, which can be driven by stimuli to which the HSC have been rendered receptive only after the initiation phase. This biphasic model of activation is exemplified by the response of HSC to transforming growth factor-β (TGF-β) and platelet derived growth factor (PDGF).

Role of Soluble Mediators

Cytokines

TGF-β

TGF-β is a multifunctional cytokine synthesised by a wide variety of cell types including platelets, monocytes and macrophages, HSC and T-lymphocytes. In mammals, the cytokine exists in three isoforms: TGF-β1, 2 and 3. Of these, TGF-β1 has been the most extensively studied and is the most strongly implicated in liver fibrosis.

TGF-β1 is a 25 kDa homodimer that is secreted in a latent, biologically inactive form that requires proteolytic cleavage of a latency associated peptide (LAP) for
activation. In biopsy specimens from patients with chronic liver disease, TGF-β1 has been detected by immunostaining in areas of fibrosis but not in surrounding areas or in normal liver (Nagy et al. 1991). In both carbon tetrachloride and schistosome induced experimental liver injury, TGF-β1 mRNA and protein in perisinusoidal areas paralleled the increased expression of collagen (Czaja et al. 1989). Treatment of both rat and human HSC in vitro with TGF-β1 was found to stimulate procollagen I and III synthesis and release (Matsuoka & Tsukamoto 1990; Weiner et al. 1990). In culture, TGF-β1 has also been shown to upregulate fibronectin and proteoglycan synthesis (Casini et al. 1994; Meyer et al. 1990). Although TGF-β1 does not directly enhance HSC proliferation, it does induce responsiveness to the mitogenic cytokine PDGF by increasing expression of the β subunit of the PDGF receptor (Pinzani et al. 1995).

**PDGF**

PDGF, a key mitogen for mesenchymal cells, is a 30kDa dimer formed from the association of two polypeptide chains, designated A and B. Three isoforms exist: AA, AB and BB (Ross et al. 1986). Similarly, two receptor subunits, α and β, have been identified (Hart et al. 1988) and while the B chain can bind either receptor subunit, the A chain is specific for the α subunit. PDGF-B chain expression is not detectable in normal liver but is rapidly increased in an experimental model of liver injury (Pinzani et al. 1994). In vitro studies of DNA synthesis have shown PDGF to be the most potent mitogen for rodent HSC in culture (Pinzani et al. 1989).

**Other Cytokines**

Other cytokines have been described to have proliferative effects on HSC. They include epidermal growth factor (EGF), transforming growth factor-α (TGF-α), fibroblast growth factor (FGF), insulin-like growth factor (IGF), interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) (Bachem et al. 1989; Matsuoka et al. 1989; Pinzani et al. 1989). Interleukin-4 (IL-4) has been shown to have both pro-proliferative and pro-fibrotic effects on HSC and will be discussed with particular relevance to its role in T-helper 2 (Th2) responses (1.9).
**Acetaldehyde and Lipid Peroxides**

In alcoholic liver disease and in iron overload (haemochromatosis), fibrosis may possibly develop without significant evidence of inflammatory changes (Reeves et al. 1996). In the case of alcoholic liver injury, it has been previously suggested that HSC activation may be, at least in part, mediated by acetaldehyde, a metabolite of ethanol. Although acetaldehyde has been shown to increase HSC collagen production by transcriptional activation (Casini et al. 1991), its effect appears confined to HSC that are already activated. More recently, products of lipid peroxidation generated by alcohol metabolism (Kamimura et al. 1992) or iron overload (Houglum et al. 1997) have also been suggested to perpetuate HSC activation *in vivo*. Studies of cultured fibroblasts demonstrated that lipid peroxides stimulated collagen synthesis (Chojkier et al. 1989) but there is no direct *in vitro* evidence pertaining to HSC.

**Retinoids**

HSC activation is associated with the loss of intracellular retinoid droplets but the functional relationship between retinoids and the process of activation is not yet clearly defined.

In experimental liver injury and in human liver disease, hepatic retinoid content decreases and there is a significant inverse correlation between hepatic retinyl palmitate levels and collagen content (Leo & Lieber 1982). Paradoxically, chronic hypervitaminosis A is associated with hepatic fibrosis and increased HSC retinoid content (Mathis et al. 1988; Russell et al. 1974). The consensus of evidence from *in vitro* studies, however, points to an anti-fibrotic role for retinoids: addition of retinoids to cultured HSC decreases TGF-β and collagen I synthesis and inhibits HSC proliferation (Davis et al. 1990).

**Role of HSC/ Extracellular Matrix Interactions**

The importance of a normal extracellular matrix in maintaining HSC in a quiescent phenotype is emphasised by studies of HSC cultured on varying substrata. Whilst HSC cultured on uncoated tissue culture plastic or collagen I undergo progressive activation (Davis et al. 1987; Friedman, Roll et al. 1989), HSC seeded on to a laminin rich basement membrane–like matrix (derived from the Englebroth-Holm-Swarm (EHS) murine fibrosarcoma tumour) retain many of the features of a
quiescent phenotype (Friedman, Roll et al. 1989). The ECM may also exert indirect effects on HSC activation by merit of its ability to bind cytokines such as TGF-β (Yamaguchi et al. 1990). Consequently, degradation of the normal matrix in the subendothelial space may be a pivotal event in HSC activation.

1.6 Extracellular Matrix Degradation
The degree of accumulation of extracellular matrix in liver fibrosis is determined not only by matrix synthesis but by the dynamic balance between matrix deposition and degradation. A group of enzymes known as matrix metalloproteinases (MMPs) have been identified as having a fundamental role in the breakdown of extracellular matrix.

1.6.1 The Matrix Metalloproteinases
The matrix metalloproteinases (MMPs) are members of a family of zinc and calcium dependent endopeptidases (Nagase & Woessner, Jr. 1999), which function at neutral pH to degrade a wide range of matrix protein substrates. Although the family represents the evolution of a series of separate gene products, the enzymes are closely related with several well conserved domains in their primary sequence (Nagase & Woessner, Jr. 1999). All MMPs contain a regulatory pro-peptide domain with a highly conserved PRCG(V/N)PD motif which is responsible for maintaining the latency of pro-MMPs and share a catalytic domain with a conserved HEXXHXXGXXH zinc binding motif. The catalytic domain has an additional structural zinc ion and 2-3 calcium ions, which are required for the stability and expression of enzymic activity. Individual MMPs exhibit additional structural domains which are important in conferring substrate specificity, regulating binding to matrix proteins and determining their interactions with tissue inhibitors of matrix metalloproteinases (TIMPs).

Initially, the nomenclature for MMPs focused on substrate specificity, but it was subsequently recognised that MMPs usually degrade multiple substrates with significant overlap in substrate specificity. MMPs are now all assigned a number. The MMPs thought to be important in the liver are outlined in Table 1.
Regulation of Matrix Metalloproteinase Activity

MMPs are implicated in a wide range of tissue remodelling activities. In normal physiology, they contribute to embryonic development (Behrendtsen et al. 1992), endometrial remodelling during the menstrual cycle (Rodgers et al. 1994) and morphogenesis (Ota et al. 1998). MMPs also have an important role in a broad spectrum of pathological processes including inflammatory diseases such as rheumatoid arthritis (Murphy & Hembry 1992), atherosclerosis (Newby et al. 1994) and tumour invasion and metastasis (Ponton et al. 1991). In order to control their destructive potential, MMP activity is tightly regulated at multiple levels.

<table>
<thead>
<tr>
<th>MMP</th>
<th>Alternative Names</th>
<th>Molecular Weight (kDa)</th>
<th>Substrate specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>interstitial collagenase (MMP-13 in rat)</td>
<td>52</td>
<td>collagens I and III, gelatin, entactin, tenascin</td>
</tr>
<tr>
<td>MMP-2</td>
<td>72 kDa gelatinase gelatinase A</td>
<td>72</td>
<td>collagens I, IV and V, gelatin, elastin, laminin, fibronectin; MMPs-1 and 9</td>
</tr>
<tr>
<td>MMP-3</td>
<td>stromelysin-1 transin</td>
<td>57</td>
<td>collagens III, IV and V, gelatin, fibronectin, tenascin, entactin, elastin, laminin, MMP-2/ TIMP-2 complex, MMP-9</td>
</tr>
<tr>
<td>MMP-9</td>
<td>92 kDa gelatinase gelatinase B</td>
<td>92</td>
<td>collagens IV and V, gelatin, elastin, fibronectin, entactin</td>
</tr>
</tbody>
</table>

Regulation of MMP Gene Expression

Most MMPs are not constitutively expressed in normal tissues but transcription can be activated by inflammatory cytokines, growth factors and other stimuli. Expression of most MMP genes is stimulated by interleukin-1β (IL-1β) (Dayer et al. 1986), tumour necrosis factor-α (TNF-α) (Dayer et al. 1985), platelet derived growth factor (PDGF) (Bauer et al. 1985), epidermal growth factor (EGF) (Delany & Brinckerhoff 1992) and basic fibroblast growth factor (b-FGF) (Aho et al. 1997). Transforming
growth factor-β (TGF-β) exerts differential effects on MMP gene transcription, inhibiting MMP-1 and MMP-3 (Edwards et al. 1987) and increasing MMP-2 (Overall et al. 1989).

**MMP Proenzyme Activation**

Activation of latent MMP pro-enzymes depends upon disruption of the interaction between the cysteine residue in the PRCGDVPDV motif of the pro-piece and the zinc atom of the catalytic domain (Van Wart & Birkedal-Hansen 1990). Following this dissociation, autoproteolysis results in cleavage of the pro-piece and conversion to the active species (Van Wart & Birkedal-Hansen 1990). In vivo, activation may be achieved by activation of the plasminogen-plasmin cascade and by members of the MMP family. Plasmin can partially activate both pro-MMP-1 and pro-MMP-3 but full activation of MMP-1 is achieved by active MMP-3 (Murphy et al. 1999). The activation of pro-MMP-2 is thought to take place primarily at the cell surface and is achieved by the first membrane-type MMP (MT1-MMP) (Sato et al. 1996) although the cell surface urokinase plasminogen activator/plasmin system may have a role (Lijnen et al. 1998).

**1.6.2. MMP Inhibitors**

Plasma glycoproteins such as alpha-2 macroglobulin can act as ‘scavengers’ binding to and inhibiting MMPs (Murphy 1991) but specific inhibition within the tissues occurs mainly by interaction with tissue inhibitors of matrix metalloproteinases (TIMPs) (Denhardt et al. 1993).

**Tissue Inhibitors of Matrix Metalloproteinases**

Four TIMPs, ranging from 21 to 30 kDa, have been described to date of which TIMP-1 and 2 are the most extensively characterised (Apte et al. 1994; Denhardt et al. 1993; Wu & Moses 1998). TIMP-1 and 2 share 40% amino acid homology and both form 3 three looped structures stabilised by six disulphide bonds (Boone et al. 1990; Murphy & Docherty 1992). All TIMPs bind to active MMP species, in a manner that is irreversible under normal physiological conditions, and inhibit their proteolytic activity (Boone et al. 1990). TIMP-1 and 2 have also been shown to bind to the pro-
enzyme forms of MMP-9 (Goldberg et al. 1992) and MMPs-1 and 2 respectively (De Clerck et al. 1991; Fridman et al. 1992), preventing activation.

1.6.3. Matrix Degradation in the Liver

Cell Culture Studies

HSC in primary culture on plastic have been shown to synthesise several members of the MMP family. During days 1-3 following isolation, there is a transient expression of MMP-1/interstitial collagenase (MMP-3 in rat) (Iredale et al. 1995b; Iredale et al. 1996) and MMP-3/stromelysin-1 (Vyas et al. 1995) which is then downregulated over days 3-5 and disappears completely once HSC are fully activated (from day 7 onward). Conversely, MMP-2/gelatinase-A is not detectable in HSC primary cultures until days 3-5 and remains present in fully activated HSC (Arthur et al. 1989). Kupffer cells (KC) also synthesise matrix-degrading enzymes including MMP-9/gelatinase-B (Winwood et al. 1995) and small amounts of gelatinase-A.

Studies in Whole Liver

The results of cell culture experiments have been supported by studies of MMP expression in whole liver. By in situ hybridisation, expression of MMP-2 and MMP-9 mRNA was found to be low in normal liver but increased markedly in liver disease or experimental liver injury (Herbst et al. 1991; Milani et al. 1994b). In contrast, MMP-1 transcripts were present at low levels in normal liver with little or no increase after injury (Milani et al. 1994b).

Zymographic studies have confirmed a progressive increase in the detection of active MMP-2/gelatinase-A in liver fibrosis suggesting that, despite its capacity to degrade collagen IV, this enzyme has pro-fibrogenic effects (Takahara et al. 1995). The mechanism for this action is not yet defined but may involve the degradation of the normal basement membrane–like matrix in the sub-endothelial space and the consequent disruption of normal ECM/HSC interaction or a direct pro-proliferative effect on HSC (Benyon et al. 1999). Studies of collagenase activity in whole liver have tended to show increased activity early in disease or injury with a progressive decline as liver fibrosis evolves (Maruyama et al. 1982; Perez-Tamayo et al. 1987). Further investigations of the phenomenon of decreased collagenolytic activity in progressive liver fibrosis highlight the role of MMP inhibition by TIMPs. In vitro,
primary cultures of HSC express, synthesise and release TIMP-1 only after culture activation, the exact converse of the pattern seen for MMP-1 (Iredale et al. 1992). Separation of TIMPs from activated HSC culture supernatants by affinity chromatography resulted in a 20-fold increase in MMP activity.

In fibrotic human liver disease TIMP-1 gene expression was increased without any parallel increase in MMP-1 expression (Iredale et al. 1995b; Iredale et al. 1992). Furthermore, in rat experimental models of liver fibrosis, there was a rapid and sustained upregulation of TIMP-1 gene expression, which preceded the increase in pro-collagen-1 and was not matched by any increase in rat interstitial collagenase (MMP-13) (Iredale et al. 1995a). The critical role of TIMP/MMP balance in determining net matrix synthesis or degradation is further underlined by studies of the recovery period after induction of experimental liver fibrosis with carbon tetrachloride (CCl₄) (Iredale et al. 1998). Between 3 and 10 days after stopping CCl₄ treatment, both TIMP-1 and pro-collagen-1 mRNA levels return to control values but with no change in MMP-13 levels. This correlates with an increase in detectable collagenase activity in whole liver homogenates.

1.7. Inflammation, the Immune System and Liver Fibrosis
The cells of the inflammatory and immune system, which infiltrate the liver after liver injury, are key intermediaries in the development of fibrosis. Kupffer cells (hepatic macrophages) have, to date, been the main focus of study and form a central part of the established view of the interface between liver inflammation and fibrosis

1.7.1 Kupffer Cells
Kupffer cells (KC) are hepatic tissue macrophages located in the subendothelial space of Disse. In common with other macrophages, they fulfil the basic functions of endocytosis, antigen presentation and secretion of biologically active products. However, because of their unique position as the first cells of the mononuclear phagocyte system to be exposed to portal blood, they have greatly enhanced phagocytic ability (Laskin et al. 1988) and are specialised to clear endotoxin (Fox et al. 1990). In addition to this important role in host defence, KC are implicated in the pathogenesis of liver injury and fibrosis.
KC have been shown to be critical in the development of experimental liver injury initiated by a variety of agents, including CCl4 (Geerts 1988), endotoxin (Arthur et al. 1985) and acetaminophen (Laskin 1990). Following carbon tetrachloride treatment of rats, increased numbers of KC are seen at the onset of liver fibrosis (Geerts 1988). Numbers of KC correlate with the degree of fibrosis and are located in association with HSC in fibrotic bands (Thompson et al. 1980). Similar increases in the KC population are observed in experimental biliary fibrosis (Grinko et al. 1995) and in acute and chronic alcoholic liver disease in humans (Karakucuk et al. 1989). In vitro studies have demonstrated that KC conditioned media promote HSC proliferation and the synthesis of extracellular matrix components including collagen (Friedman & Arthur 1989) and proteoglycans (Gressner & Zerbe 1987). The HSC activating effects of soluble mediators from KC are more pronounced when KC have been isolated from CCl4 injured livers (Armendariz-Borunda et al. 1989; Shiratori et al. 1987). TGF-β1 is believed to be the major KC product effecting HSC activation (Matsuoka & Tsukamoto 1990) but TNF-α, IL-1 (Matsuoka et al. 1989) and TGF-α (Meyer et al. 1990) and others may also play a role.

Fig. 1.3: Role of Kupffer cells in HSC activation
1.7.2 T-lymphocytes

A number of chronic human liver diseases that culminate in liver fibrosis (including chronic hepatitis C, autoimmune chronic active hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis) are characterised by infiltration of the liver with increasing numbers of "conventional" or circulating T-lymphocytes (Bjorkland et al. 1991; Frazer et al. 1985; Imada et al. 1997; Whiteside et al. 1985). These T-lymphocyte infiltrates are often localised, like Kupffer cells, in close association with fibrotic bands. Despite this, no clear direct role for T-lymphocytes in the pathogenesis of liver fibrosis has been elucidated.

T-lymphocytes are present in the normal liver. However, the intrahepatic T-lymphocyte population appears to be phenotypically distinct from the circulating peripheral population. Murine studies have shown significant populations of cells with intermediate expression of the T-cell receptor, 50% of which also express the NK1.1 marker, in addition to conventional CD8+ and CD4+ T-cells (Tsukahara et al. 1997). Studies of T-cell populations in adult normal human donor liver showed that significant numbers of T-lymphocytes were localised to the portal tracts and parenchyma (Norris et al. 1998). It has been estimated that the average human liver would contain 0.7-1.4 x 10^10 resident T-cells (O'Farrelly & Crispe 1999). Phenotypic analysis by FACS for cell surface markers demonstrated a reversed CD4 to CD8 ratio (1:3.5) compared to circulating cells, increased numbers of "double negative" CD4-/CD8- cells and showed cells expressing the γδ T-cell receptor and a very high proportion of cells expressing the natural killer cell marker CD56 (Doherty et al. 1999; Norris et al. 1998). It is likely that these and other populations of T-cells differentiate in the liver (Abo et al. 2000; Watanabe et al. 1996).

Evidence that T-lymphocytes may sometimes be directly implicated in the development of liver injury and fibrosis is derived from experimental animal models. In murine schistosomiasis, the development of liver granulomata and fibrosis is dependent on CD4+ T-cells (Fallon 2000). Injection of mice with concanavalin-A (a plant lectin and polyclonal T-cell activator) results in an acute hepatitis, characterised by hepatic T-cell infiltration, which can be prevented by depletion of either CD4+ T-cells or NK cells (Takeda et al. 2000; Tiegs et al. 1992; Toyabe et al. 1997).

T-lymphocytes and Kupffer cells appear to act synergistically in producing this acute liver injury (Gantner et al. 1996). Recent work has shown that repeated injections of
concanavalin-A eventually result in the development of fibrosis (Louis et al. 1997), in which direct T-cell/HSC interactions independent of macrophages may have a role.

1.8. Th1 and Th2 Responses

The concept of polarised immune responses evolved from clinical observations that infections with certain pathogens could present with strikingly different clinical and pathological features. The archetypal example is leprosy where the healing (tuberculoid) form is associated with strong delayed type hypersensitivity (DTH) reactions and low levels of antibody where as the uncontrolled (lepromatous) form is associated with high antibody titres and weak DTH (Arai et al. 1989).

The basis for this diversity in CD4+ T-cell dependent immune responses became apparent in 1986 with the identification of murine T-cell clones which could be classified into distinct subsets according to their profile of cytokine production and their ability to mediate DTH reactions or provide B-cell help (Mosmann et al. 1986). The two populations were termed “Th1” and “Th2”. Th1 clones were shown to produce IL-2, IFN-γ and TNF-β and to mediate DTH responses. By contrast, Th2 clones produced IL-4, IL-5, IL-6, IL-10 and IL-13 and promoted vigorous antibody responses. Subsequently, Th1/Th2 CD4+ T-cells were described in humans although the synthesis of IL-2, IL-6, IL-10 and IL-13 is not as stringently restricted to a single subset as in murine cells (Del Prete et al. 1991; Sornasse et al. 1996). More recently, evidence has been accumulating that CD8+ T-cells can also be subdivided into Tc1 and Tc2 subsets (Cerwenka et al. 1998; Seder & Le Gros 1995).

Cytokines produced by each subset are capable of both promoting the development and function of their own subset and inhibiting the reciprocal subset. Thus, IFN-γ selectively amplifies Th1 development and inhibits proliferation of Th2 cells (Seder & Paul 1994); IL-10 inhibits cytokine synthesis by Th2 cells (Fiorentino et al. 1989) and IL-4 suppresses Th1 cells and induces proliferation of Th2 cells (Le Gros et al. 1990; Swain et al. 1990). This cross-regulation contributes to the progressive polarisation of the immune response towards a Th1 or Th2 profile.
1.8.1 Th1 and Th2 Cells arise from a Common Precursor

Th1 and Th2 patterns of cytokine secretion are confined to activated effector or memory T-cells, which have undergone a period of antigenic stimulation, and are not seen in naïve T-cells. In vitro studies of T-cells isolated from mice transgenic for the α and β chains of the T-cell receptor (TCR) recognising a known antigen/MHC class II complex have demonstrated that both Th1 and Th2 cells may be derived from a common precursor cell (Nakamura et al. 1997a). This precursor is a mature, naïve CD4+ T-lymphocyte that produces mainly IL-2 on contact with antigen.

1.8.2. Factors Affecting Th1/Th2 Differentiation

(a) The cytokine environment

Three cytokines are thought to play pivotal roles in the initial development of Th1 and Th2 cells: IL-12, IFN-γ and IL-4.

IL-12 is produced rapidly by activated macrophages and dendritic cells on antigenic stimulation (Macatonia et al. 1995) and strongly favours Th1 development by inducing the synthesis of IFN-γ (Lederer et al. 1996). Among CD4+ T-lymphocytes, functional IL-12 receptor expression is confined to recently activated, uncommitted cells and Th1 cells and lost on Th2 cells thus precluding any direct effect of IL-12 on the Th2 subset (Szabo et al. 1995). IL-12 signals via activation of STATs (signal transducers and activators of transcription) of which, STAT4 is activated by IL-12 alone (Jacobson et al. 1995). The importance of IL-12 in generating Th1 responses is underlined by studies of IL-12 or STAT4 deleted mice showing markedly impaired Th1 responses (Hsu et al. 1990; Kaplan et al. 1996b; Magram et al. 1996).

IFN-γ favours Th1 responses by maintaining the expression of functional IL-12 receptors on CD4+ T-cells (Guler et al. 1996) and by promoting IL-12 production by macrophages (Ma et al. 1996). It simultaneously discourages Th2 differentiation by inhibiting IL-4 receptor expression (Paludan 1998) and IL-4 production (Nakamura et al. 1997b).

IL-4 has the greatest influence in driving Th2 differentiation (Swain et al. 1990) and its effects are dominant over those of IL-12 and IFN-γ. It upregulates its own expression (Hsieh et al. 1992) and that of the IL-4 receptor (Nakamura et al. 1997b), inhibits the secretion of both IL-12 (Szabo et al. 1995) and the expression of
functional IL-12 receptor and can induce cells to switch from Th1 to Th2 commitment (Szabo et al. 1995). There is evidence to suggest that IL-4 may enhance its own production via STAT6 signalling (Lederer et al. 1996) and both IL-4 and STAT6 knockout mice have been shown to develop deficient Th2 responses (Kaplan et al. 1996a; Kopf et al. 1993).

(b) The nature of antigenic stimulation

(i) Antigen dose

The results of in vivo studies investigating the effects of varying the dose of antigen during CD4+ T-cell priming are conflicting. An early study, in which rats were immunised with different concentrations of bacterial flagellin, showed strong DTH (Th1) responses in animals given very low or high doses of antigen whereas rats that had received intermediate doses had high levels of flagellin specific antibodies (Th2) (Parish & Liew 1972). Similar findings resulted when differing numbers of Leishmania major parasites were used to immunise susceptible mice (Bretscher et al. 1992). By contrast, studies using varying doses of ovalbumin or human collagen IV to immunise mice showed the opposite effect (Hayglass et al. 1986; Pfeiffer et al. 1995).

In vitro experiments yielded similarly contradictory results. One study using varying doses of cytochrome-c to prime naïve CD4+ T-cells transgenic for a cytochrome-c specific T-cell receptor (TCR) resulted in the generation of Th1 cells at high doses and Th2 cells at low doses (Constant et al. 1995). Conversely, a study examining the effect of different doses of ovalbumin on ovalbumin-specific TCR transgenic T-cells showed that priming with high doses generated Th2 cells, intermediate doses generated Th1 cells and very low doses Th2 cells (Hosken et al. 1995). The one consistent feature is that predominant Th2 development is seen at the lowest doses of antigen.

(ii) Altered peptide ligands

The specificity of TCR-peptide recognition is conferred by only a few amino acid residues. Certain residues play a particularly critical role in TCR contact and interaction. An “altered peptide ligand” (APL) is an immunogenic peptide in which these residues have been substituted (Sloan-Lancaster & Allen 1996).
Although APL do not fully stimulate T-cell proliferation, they retain the ability to activate other TCR mediated effector functions, such as cytokine production and B-cell help, to varying degrees. It has been proposed that endogenous peptides derived from repertoires of self-antigens may be recognised by mature T-cells as APL and could prime and influence responses against subsequently encountered foreign antigens.

An in vivo study of antigen priming in inbred strains of mice, which express defined class II MHC molecules, demonstrated that a single peptide derived from human collagen IV could generate either Th1 or Th2 responses depending on the mouse strain (Murray et al. 1989). Further analysis showed that high affinity peptide/class II MHC binding was associated with Th1 responses and low affinity peptide/class II MHC binding was associated with Th2 responses (Murray et al. 1992). To investigate this further, a range of APL with varying binding affinity for the class II MHC molecules was generated. When mice that produced Th1 responses to wild type peptide (high affinity binding) were primed using weakly binding APL, their responses were converted to Th2. Conversely, when mice that produced Th2 responses to wild type peptide were primed using peptide with enhanced binding, their responses were skewed to Th1 (Pfeiffer et al. 1995). Similar findings were reported in a study using APL derived from mouse myelin basic protein (Kumar et al. 1995).

Studies of priming in vitro using TCR transgenic T-cells and antigen presenting cells with defined class II MHC have further elucidated the effect of APL on immune responses by examining peptides with differing binding affinity for both class II (MHC variants) and the TCR (TCR variants). TCR variants with the weakest binding interactions were associated with a shift towards Th2 responses (Constant et al. 1994).

(c) Co-stimulation

T-cell activation by antigen requires not only TCR ligation, but also a second, co-stimulatory signal from antigen presenting cells. CD28 and CTLA-4 are glycoproteins expressed on T-cells that serve as receptors for the B7 family of co-stimulatory molecules (Lenschow et al. 1996). CD28 is expressed constitutively on all CD4+ T-cells but expression of CTLA-4 only becomes evident 48 hours after
activation (Lenschow et al. 1996). Studies of in vivo and in vitro priming in which B7/CD28 interactions were blocked using a fusion protein of CTLA-4 and immunoglobulin (CTLA-4Ig), point to a role for B7 co-stimulation in the differentiation of Th2 but not Th1 cells (Corry et al. 1994; King et al. 1995), although there are some contradictory data (Sayegh et al. 1995). The role of CTLA-4 is less clear but it may be more important in downregulating ongoing immune responses than initiating new ones (Tivol et al. 1995; Waterhouse et al. 1995). The first evidence that B7-1 and B7-2 might have a role in polarising immune responses came from an experimental allergic encephalomyelitis model. Treatment of mice with anti-B7-1 monoclonal antibody (mAb) was associated with reduced disease and with Th2 responses where as treatment with anti-B7-2 mAb enhanced disease severity and led to Th1 responses (Kuchroo et al. 1995). This would suggest that the dominant presence of B7-2 co-stimulation might skew T-cell activation towards a Th2 phenotype. Some in vitro studies have supported this model (Freeman et al. 1995) but others have been conflicting (Lanier et al. 1995). There remains no clear evidence that B7-1 and B7-2 deliver different biochemical signals to T-cells and it is possible that effects may relate to the differential pattern of B7-1 and B7-2 expression during the immune response with B7-2 dominant on unactivated APC’s and B7-1 increasing after exposure to inflammatory stimuli (Bluestone 1995).

(d) Genetic factors
The host genetic background influences the T-cell cytokine profile and outcome of disease in several parasitic and bacterial infection models. This phenomenon is exemplified by resistance or susceptibility to infection with Leishmania major (Reiner & Locksley 1995). Several mouse strains such as C57Bl/6 and B10.D2 develop a predominantly Th1 response and are resistant to infection. Other strains such as BALB/c produce a dominant Th2 response and are susceptible to infection. In vitro studies show that, under neutral conditions of antigenic stimulation, BALB/c T-cells acquire a Th2 predominant phenotype and lose IL-12 responsiveness more rapidly than B10.D2 cells (Guler et al. 1996; Hsieh et al. 1995).
The differentiation of T-cells into Th1 and Th2 phenotypes is complex and controlled by multiple factors including antigen dose and presentation, co-stimulatory factors, the cytokine environment and genetic background. The relative importance of each of these factors in determining T-cell phenotype is likely to vary for immune responses to different antigens.

1.9. Th1/Th2 Responses and Liver Fibrosis

Much of the early evidence suggesting that Th1/Th2 balance could be important in hepatic wound healing responses came from studies of murine schistosomiasis. Infection of mice with the helminth parasite *Schistosoma mansoni* results in the deposition of parasite eggs in the portal venous system and perisinusoidal spaces of the liver. The eggs initially induce a granulomatous inflammatory response and subsequently fibrosis, both of which have been shown to be CD4+ T-lymphocyte dependent. Manipulation of the cytokine environment in this disease model has provided insights into the role of Th1 and Th2 responses in hepatic fibrosis.

Treatment of mice with neutralising anti-IL-4 antibodies decreased egg induced hepatic fibrosis (Cheever et al. 1994) but IL-4 knockout mice showed no or only small reductions in liver fibrosis compared to wild type controls (Metwali et al. 1996; Pearce et al. 1996). In contrast, treatment of *Schistosoma mansoni* with an IL-13 inhibitor (a soluble fusion protein derived from the high affinity IL-13 receptor subunit IL-13Rα2) resulted in a marked reduction in hepatic collagen deposition (Chiaramonte et al. 1999). A similar large reduction in fibrosis was seen in IL-13 deficient mice compared to wild type controls (Fallon et al. 2000). In double IL-4/IL-13 knockout mice (Fallon et al. 2000) the development of liver fibrosis was minimal.

Similarly, in mice where the biological effects of these two cytokines were ablated by deletion of receptor components (IL-4 receptor-α) (Jankovic et al. 1999) or intracellular signalling proteins (STAT-6) (Kaplan et al. 1998), egg induced fibrosis was dramatically reduced. When mice were inoculated with *S. mansoni* eggs together with IL-12 and then exposed to *S. mansoni* infection, there was a marked inhibition of egg induced hepatic fibrosis associated with skewing of the immune response from a Th2 to a Th1 type (Wynn et al. 1995).
Studies of other experimental models demonstrate that the association between predominant Th2 responses and liver fibrosis is not confined to the immunopathology of murine schistosomiasis. In one study, carbon tetrachloride, CCL4, induced liver injury in BALB/c mice (which exhibit Th2 dominant responses) resulted in severe fibrosis whereas C57Bl/6 (Th1 dominant) mice showed significantly less fibrosis (Shi et al. 1997). In the same study, IFN-γ deleted (IFN-γ -/-) mice produced on both BALB/c and C57Bl/6 backgrounds displayed more liver fibrosis than their corresponding wild types. Administration of neutralising anti-IL-4 antibodies to BALB/c wild type mice also reduced liver fibrosis (Shi et al. 1997). In a rat model of liver fibrosis induced by dimethylnitrosamine (DMN), administration of IFN-γ inhibited both the proliferation and activation of HSC and deposition of extracellular matrix (Baroni et al. 1996). The exception to the pattern is IL-10 which appears to mediate overall anti-fibrotic effects despite its designation as a Th2 cytokine. Two studies have demonstrated that CCl4 treated IL-10 deleted (IL-10KO) mice develop more fibrosis than wild type controls despite similar levels of hepatic inflammation (Thompson et al. 1998).

1.10. Th1/Th2 Cytokines and ECM Homeostasis

While some of the apparent effects of Th1/Th2 cytokines on the development of liver fibrosis may be mediated via effects on monocytes, macrophages or other cells of the immune system, there is evidence to suggest a role for direct cytokine effects on hepatic stellate cells. The Th1 cytokine IFN-γ reduces both basal and TGF-β stimulated collagen synthesis by human HSC in vitro (Mallat et al. 1995; Tiggelman et al. 1995) and inhibits proliferation, α-SMA expression and collagen production in rat HSC in culture (Rockey et al. 1992b). The Th2 cytokine IL-4 enhances collagen production by human HSC in culture with a stimulatory effect comparable to that of TGF-β (Tiggelman et al. 1995). IL-4 has also been reported to decrease MMP-1 and MMP-3 in human skin fibroblasts and increase TIMP-1 in human skin and lung fibroblasts (Oriente et al. 2000; Sempowski et al. 1996). The effects of IL-13 on HSC have not been examined to date, but IL-13 has been shown to increase collagen and TIMP-1 production and to inhibit IL-1.
induced MMP-1 and MMP-3 production in normal human skin fibroblasts (Oriente, Fedarko, Pacocha, Huang, Lichtenstein, & Essayan 2000). Collagen expression was also enhanced by IL-13 in the murine fibroblast cell line 3T3 (Chiaramonte et al. 1999).

By contrast, IL-10 has been shown to reduce collagen production by human skin fibroblasts and to enhance MMP-1 and MMP-3 production (Reitamo et al. 1994).

### 1.11. Interleukin-10

The cytokine interleukin-10 (IL-10) was first described in 1989 as a product of Th2 lymphocytes with marked suppressive effects on Th1 cell proliferation and cytokine production and was initially identified as “cytokine synthesis inhibitory factor” (CSIF) (Fiorentino et al. 1989). The profound suppressive effects exerted on macrophage function by the cytokine resulted in IL-10 being termed “macrophage deactivating factor” (de Vries 1995).

The cDNA encoding murine IL-10 (mIL-10) was isolated in 1990 (Moore et al. 1990) with the subsequent identification of clones encoding human IL-10 (Vieira et al. 1991). A rat IL-10 cDNA clone was isolated from T-cells using PCR primers based on conserved regions of the human and murine clones (Goodman et al. 1992).

#### Structure and genetics

Both the human and murine IL-10 genes localise to chromosome 1 (Kim et al. 1992) and there is a high degree of homology between the nucleotide (81%) and amino acid sequence (73%) (Vieira et al. 1991). The nucleotide sequence of rat IL-10 exhibits 91% homology with murine IL-10 (Goodman et al. 1992).

Human IL-10 exists as a 35kDa homodimer containing two disulphide bonds. Murine IL-10 is also a non-covalent homodimer but is heterogeneously N-glycosylated so that 17, 19 and 21kDa forms exist (Moore et al. 1990).

The effects of IL-10 are mediated through high affinity cell surface receptors (IL-10R), which are members of the interferon-like subgroup of the cytokine receptor family and map to chromosome 1 (Ho et al. 1993; Liu et al. 1994). The murine and
human IL-10 receptors share 60% homology and are expressed on B and T cell lines, Th1 but not Th2 cells, NK, mast cells, macrophages and some fibroblasts (Weber-Nordt, Meraz, & Schreiber 1994).

**Cellular Sources**

(a) T-lymphocytes

Although IL-10 was first described as a product of Th2 cells, both Th1 and Th0 cells have also subsequently been shown to express the cytokine (Fiorentino et al. 1989; Moore et al. 1990; Yssel et al. 1992). Expression by T-lymphocytes can be up-regulated by a variety of stimuli including phytohaemagglutinin (PHA), phorbol myristate acetate (PMA), concanavalin-A (Con-A) and anti-CD3 and anti-CD28 antibodies (Yssel et al. 1992).

(b) Monocytes and macrophages

Both circulating monocytes and tissue specific macrophages express IL-10 mRNA and protein after activation by a variety of factors including LPS, IFN-α, PHA and PMA (de Waal et al. 1991a; Fiorentino et al. 1991a).

(c) B-cells

The protein sequence of IL-10 shows extensive homology with an open reading frame (ORF) in the Epstein Barr virus (EBV) genome known as BCRF1 (Moore et al. 1990). BCRF1 has been shown to share many of the activities of IL-10 (Hsu et al. 1990). B-cells, B-cell tumours and EBV infected B-cells have all been shown to express IL-10 mRNA and protein (O'Garra et al. 1990).

**Biological effects**

(a) T-lymphocytes

In the presence of macrophages as antigen presenting cells (APC), IL-10 inhibits the production of cytokines by Th1 cells, but not Th2 cells, in mice (Fiorentino et al. 1989).
1991b). In human T-cells, IL-10 inhibits APC dependent cytokine synthesis by Th0, Th1 and Th2 clones (de Waal, Yssel, & de Vries 1993). More recently, culture of human and murine CD4+ T-cells in the presence of IL-10 has been shown to give rise to novel T-regulatory T-cell clones that produce high levels of IL-10 and are capable of suppressing the proliferative responses of CD4+ T-cell to antigen (Groux et al. 1997; Groux et al. 1996).

IL-10 inhibits the proliferation of human and murine T-cell clones via APC dependent mechanisms (de Waal et al. 1991b).

(b) Monocytes and macrophages

LPS stimulation results in the production of an array of pro-inflammatory cytokines by monocytes and macrophages. The addition of IL-10 markedly inhibits LPS stimulated production of IL-1α, IL-1β, IL-6, IL-8, G-CSF and GM-CSF at the transcriptional level (de Waal et al. 1991a; Fiorentino et al. 1991a). Conversely, treatment of monocytes with anti-IL-10 neutralising antibodies enhances pro-inflammatory cytokine production, implying that endogenous IL-10 plays a role in inhibiting cytokine production (de Waal et al. 1991a).

IL-10 also exhibits suppressive effects on several macrophage effector functions including antigen presentation by inhibition of class II MHC expression (Koppelman et al. 1997) and production of reactive oxygen intermediates (Bogdan et al. 1991). The effect on nitric oxide production may be stimulatory or inhibitory depending on the sequence of macrophage stimulation (Cunha, Moncada, & Liew 1992).

1.12. Interleukin-4

The cDNA encoding mouse IL-4 was identified in 1986 by functional screening of a mouse T-helper cell cDNA library (Lee et al. 1986; Noma et al. 1986) and the human equivalent isolated from a concanavalin-A stimulated human T-cell cDNA library based on homology with the murine sequence (Yokota et al. 1986). Conserved sequences from human and murine IL-4 were subsequently used to isolate rat IL-4
cDNA from a rat T-lymphoblast library (McKnight, Barclay, & Mason 1991). On the basis of its biological activities, IL-4 was initially termed IgG1 induction factor or B-cell stimulating factor-1.

**Structure and genetics**

The human gene is located on chromosome 5q31 (Arai et al. 1989) within 50 kilobase pairs of the gene for IL-13 and the murine equivalent is found on the synteneic region of chromosome 11 (Otsuka et al. 1987). IL-4 is secreted as a 154 amino acid precursor, which is subsequently cleaved to produce a 129 amino acid secreted peptide with a molecular weight of 15 kDa (Yokota et al. 1986). Murine and human IL-4 exhibit only 50% amino acid sequence homology and are species specific with respect to receptor binding and biological activity. Similarly, rat IL-4 shares only 61% protein sequence homology with murine IL-4 and its activity is species specific (McKnight, Barclay, & Mason 1991).

**Cellular sources**

(a) T-lymphocytes

IL-4 is the archetypal cytokine produced by Th2 cells in response to receptor mediated activation events (Minty et al. 1997) and is also expressed by NK cells (Yoshimoto & Paul 1994).

(b) Mast cells and basophils

Mast cells and basophils produce IL-4 on activation either via ligation of Fc epsilon receptor-1 or treatment with ionomycin (Arock et al. 1993; Seder et al. 1991).

**Biological effects**

(a) T-lymphocytes

IL-4 promotes the survival in culture (Hu-Li et al. 1987) and growth of activated T-cells and T-cell clones and induces expression of CD8α on CD4+ T-cells (Paliard et

(b) B-lymphocytes

IL-4 determines that human B-cells switch to the expression of immunoglobulin-E (IgE) and immunoglobulin-G4 (IgG4) (Gascan et al. 1991) and murine B-cells to IgE and IgG1 (Coffman et al. 1986; Vitetta et al. 1985). IL-4 acts as a co-mitogen for B-cells (Howard et al. 1982), prolongs B-cell survival in culture (Hu-Li et al. 1987) and increases the expression of class II MHC molecules, CD23 and the IL-4 receptor on B-cells (Noelle et al. 1984; Ohara & Paul 1988).

(c) Monocytes and macrophages

IL-4 inhibits the release of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 from monocytes (te Velde et al. 1990b); enhances the production of IL-1 receptor antagonist (IL-1ra) (Jenkins & Arend 1993) and reduces expression of CD14 (the LPS receptor). The cytokine downregulates cell surface Fc gamma receptor expression and inhibits antibody dependent cellular cytotoxicity (te Velde et al. 1990a) but enhances expression of MHC class II antigens (Gerrard, Dyer, & Mostowski 1990).

(d) Mast cells and basophils

IL-4 promotes the expression of c-fos and ICAM-1 on mast cells and reduces expression of Kit (Nilsson & Nilsson 1995; Valent et al. 1991) and enhances ionomycin induced secretion of IL-3, IL-4, GM-CSF and IL-8 (Coleman et al. 1995)

1.13. Interleukin-13

Murine interleukin-13 (mIL-13) was first identified by differential screening of a concanavalin-A (Con-A) stimulated mouse T-helper cell cDNA library, as an induction specific novel cytokine (Brown et al. 1989). Three groups then isolated the
homologous human cDNA independently (McKenzie et al. 1993a; Minty et al. 1993; Morgan et al. 1992).

**Structure and Genetics**

The mouse and human IL-13 genes comprise four exons and three introns spanning approximately 4.5Kb. The human IL-13 (hIL-13) gene maps to chromosome 5q31 with the murine gene located on chromosome 11. Both these regions encode gene clusters including IL-3, IL-4, granulocyte macrophage colony stimulating factor (GM-CSF) and IL-5 (McKenzie et al. 1993b).

Human and murine IL-13 exhibit 66% nucleotide sequence homology and respectively encode 132 and 131 amino acid proteins sharing 58% amino acid sequence identity (Minty et al. 1993). Both hIL-13 and mIL-13 are active in human cells but mIL-13 has 100 times higher specific activity in mouse cells.

**Cellular sources**

(a) **T-lymphocytes**

IL-13 is produced predominantly by activated CD4+ T-cell clones with Th2 characteristics but is also expressed by Th1, Th0 and by CD8+ T-cell clones in response to both antigen specific and polyclonal stimuli (de Boer et al. 1998; de Waal et al. 1995; Minty et al. 1997). Human and murine natural killer (NK) cells have also been shown to express IL-13 (Hoshino et al. 1999)

(b) **Mast cells and basophils**

IL-13 is produced by mast cells in response to stimulation via cross-linking of their high affinity immunoglobulin E (IgE) receptors (Fc epsilon receptor) (Burd et al. 1995) and by phorbol myristic acid (PMA) and ionomycin (Jaffe et al. 1996).

Production of IL-13 by activated mast cells is inhibited by dexamethasone (Fushimi et al. 1998). Similarly, basophils produce IL-13 in response to a variety of stimuli including cross-linking of Fc epsilon receptors, IL-3, PMA and ionomycin (Redrup et al. 1998).
(c) B-cells

Both malignant and Epstein-Barr virus transformed B-cells have been shown to express IL-13 mRNA (Fior et al. 1994).

Biological effects

(a) Monocytes and macrophages

IL-13 has profound effects on human monocyte morphology, surface antigen expression and cytokine synthesis. Human monocytes cultured in the presence of IL-13 develop long processes, aggregate, become more adherent and appear to have prolonged survival (Minty et al. 1993). IL-13 enhances monocyte expression of several members of the integrin family and upregulates the expression of major histocompatibility complex (MHC) class II antigens. (de Waal et al. 1993). IL-13 inhibits antibody dependent cellular cytotoxicity and suppresses the production of IL-1α, IL-1β, IL-6, IL-8, MIP-1α, TNF-α, IL-10, GM-CSF and G-CSF by LPS stimulated monocytes (de Waal et al. 1993). By contrast, secretion of IL-1Ra is increased (de Waal et al. 1993).

Similarly, IL-13 induces murine macrophages to become elongated and develop processes and prolongs their survival in vitro (Doherty et al. 1993). Class I and class II MHC expression is increased on some murine macrophages (Doherty et al. 1993). Pre-treatment of murine macrophages with IL-13 results in a an inhibition of LPS stimulated IL-1, IL-6, TNF-α, and nitric oxide (NO) production and IL-12α and IL-12β mRNA expression is reduced (Doherty et al. 1993).

(b) B-cells

IL-13 induces human B-cells to switch immunoglobulin (Ig) production to IgE and IgG4 (Punnonen et al. 1993). The cytokine also enhances the proliferative responses of human B-cells to anti-IgM antibody or CD40 ligation and promotes B-cell survival by inhibiting apoptosis in vitro (Cocks et al. 1993; Lomo et al. 1997). B-cell surface expression of CD23, MHC class II, sIgM, CD71 and CD72 is enhanced by IL-13 (Punnonen et al. 1993). By contrast, murine B-cells appear to be unresponsive to IL-
13 with the exception of one study demonstrating prolonged survival and increased antibody production in a murine B-cell line in vitro (Lai & Mosmann 1999).

(c) Other cell types

The known biological effects of IL-13 on cell types other than immune effector cells are outlined in Table 1.2. Potential effects on fibroblasts to alter extracellular matrix turnover are discussed in more detail in section 1.10.

Table 1.2: Effect of IL-13 on cell types other than immune effector cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Biological effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>mast cells</td>
<td>↑ c-fos</td>
</tr>
<tr>
<td></td>
<td>↓ proliferation</td>
</tr>
<tr>
<td></td>
<td>↑ ICAM-1</td>
</tr>
<tr>
<td></td>
<td>↓ Kit</td>
</tr>
<tr>
<td>endothelial cells</td>
<td>↑ chemotaxis</td>
</tr>
<tr>
<td></td>
<td>↑ MCP-1</td>
</tr>
<tr>
<td></td>
<td>↑ VCAM-1</td>
</tr>
<tr>
<td></td>
<td>↑ RANTES</td>
</tr>
<tr>
<td>fibroblasts</td>
<td>↑ collagen</td>
</tr>
<tr>
<td></td>
<td>↓ prostaglandin-E2</td>
</tr>
<tr>
<td></td>
<td>↓↑ pro-inflammatory cytokines</td>
</tr>
<tr>
<td></td>
<td>↑ eotaxin</td>
</tr>
<tr>
<td>mesangial cells</td>
<td>↓ cyclo-oxygenase-2</td>
</tr>
<tr>
<td></td>
<td>↓ prostaglandin-E2</td>
</tr>
<tr>
<td></td>
<td>↓ inducible nitric oxide synthetase</td>
</tr>
</tbody>
</table>
1.14. The IL-13/IL-4 Receptor Complex

The first component of the IL-4 receptor complex to be described was the 140 kDa IL-4 receptor alpha (IL-4Ra) chain, which binds IL-4 with high affinity (Harada et al. 1990; Idzerda et al. 1990; Mosley et al. 1989) but requires hetero-dimerisation with a second chain for physiological signalling. The gamma common chain, first identified as a subunit of the IL-2 receptor (IL-2Rγ), appears to be the dominant chain fulfilling this role on cells of haemopoietic lineage (Kondo et al. 1993; Russell et al. 1993) and these two subunits together constitute the type I IL-4 receptor.

In non-lymphoid cells, IL-4Ra also functions as a component of a second class of receptor in which it associates with the 60-70kDa low affinity form of the IL-13 receptor (IL-13Ra1) to form a receptor complex which is capable of transducing both IL-4 and IL-13 dependent signals (Gauchat et al. 1997; Hilton et al. 1996). In this receptor, the binding specificity of IL-4 and IL-13 is confined to their respective subunits.

A further possible component of the IL-13 receptor (IL-13Ra2) which bonds IL-13 with high affinity, has been identified (Caput et al. 1996; Donaldson et al.). Although there is some evidence to suggest that IL-13Ra2 may participate in forming a functional IL-13 receptor together with IL-13Ra1 and another component its biological role is less clear (Obiri et al. 1997).

1.15. IL-4 and IL-13: Redundant Cytokines?

The close clustering and similar structure of the genes for GM-CSF, IL-3, IL-4, IL-5 and IL-13 suggests that they evolved by gene duplication events. Despite the low (~30%) homology between primary protein structures, the tertiary structure of IL-13 is predicted to have a four anti-parallel α-helical core closely resembling IL-4 (Minty et al. 1993; Zurawski et al. 1993).

Although IL-4 and IL-13 share many biological functions, *in vitro* studies show that redundancy between the two cytokines is not complete. The most notable difference between IL-4 and IL-13 is that IL-13 does not exhibit the ability of IL-4 to induce
T-cell proliferation (de Waal et al. 1995; Spits et al. 1987). There are also differences in the regulation of production of the two cytokines. Following antigen specific or polyclonal activation, IL-13 mRNA and protein production by peripheral blood T-cell and T-cell clones is rapidly induced and long lasting, whereas IL-4 production is of slower onset and more transient (de Waal et al. 1995). IL-13 production by T-cells is inhibited by CD3 ligation and enhanced by cyclosporin-A but the converse is true for IL-4 (Van der Pouw Kraan TC et al. 1996) and IFN-α reduces IL-13 production but not IL-4 production (de Boer et al. 1998).

The generation of IL-4 and IL-13 deficient mice has provided further insights into the roles of the cytokines in vivo. T-cells isolated from naïve IL-4 deficient (IL-4-/-) mice fail to produce Th2 cytokines on stimulation in vitro and levels of Th2 cytokines after infection with the gastrointestinal nematode *Nippostrongylus brasiliensis* are reduced. However, IL-4-/- mice retain their capacity to expel *N. brasiliensis* worms, suggesting that another mediator may be important. T-cells isolated from IL-13-/- mice also show reduced production of Th2 cytokines in vitro though mast cell cytokine production is not affected (McKenzie et al. 1998b).

Although they appear to mount robust Th2 cytokine responses to infection, IL-13-/- mice do not clear *N. brasiliensis* worms efficiently and this capacity can be restored by administration of exogenous IL-13 (McKenzie et al. 1998a).

Studies of IL-4 receptor alpha (IL-4Ra) deficient mice and Stat6 deficient mice, in which responses to both IL-4 and IL-13 are impaired, show both inadequate Th2 responses and nematode worm clearance (Barner et al. 1998; Shimoda et al. 1996; Takeda et al. 1996). Further work comparing IL-4-/- mice, IL-13-/- mice and animals in which the expression of both cytokines is disrupted (IL-4-/-IL-13-/-) provides more evidence of overlapping but not identical roles for the two cytokines. In IL-4-/-IL-13-/- mice, pulmonary granuloma formation in response to *Schistosoma mansoni* eggs was abolished, whereas the response was only impaired in single cytokine-deficient mice (McKenzie et al. 1999). The defect in expulsion of *N. brasiliensis*
worms seen in IL-13/- mice was more pronounced when both cytokines were ablated (McKenzie et al. 1999).

The evidence suggests that IL-13 and IL-4 cooperate to initiate rapid Th2-cell driven immune responses and they perform additive roles, although their functions overlap.

1.16. Hypothesis and Aims
The literature reviewed in this introduction provides compelling evidence of the central role of hepatic stellate cells in liver fibrosis. After liver injury from a wide range of insults, hepatic inflammation develops and biological signals converge on the hepatic stellate cell to result in activation to a myofibroblast phenotype. The ensuing disturbance in the fine balance between extracellular matrix synthesis and degradation leads to fibrosis and, eventually, cirrhosis.

Kupffer cells have been the subjects of extensive study as intermediaries between the processes of liver damage and inflammation and the development of fibrosis (Fig. 1.3). The possible role of T-lymphocytes in the pathogenesis of liver fibrosis has been neglected despite the observations that many human diseases leading to liver fibrosis are characterised by a chronic hepatitis with predominantly T-lymphocyte infiltration and that T-lymphocytes have been identified as active participants in fibrotic processes in other organs.

In the skin and the lung, Th1 responses have been associated with less and Th2 responses with more fibrosis. Similar findings have arisen from studies of liver fibrosis in murine schistosomiasis but the importance of Th1/Th2 balance in the pathogenesis of liver fibrosis with other aetiologies is less clear.

At least some of the influence of Th1/Th2 balance on the development of liver fibrosis may be mediated the direct effects of Th1/Th2 cytokines on HSC activation. It is already established that the Th1 cytokine IFN-γ acts directly on HSC to inhibit activation and, conversely, the Th2 cytokine IL-4 has been shown to directly promote HSC activation. However, little or nothing is known regarding the effects of other Th1/Th2 cytokines such as IL-10 and IL-13 on HSC activation.
Fig. 1.3: Role of Kupffer cells in HSC activation

I propose that T-lymphocytes, and not just Kupffer cells could play an important role in orchestrating the cellular signals that converge on the hepatic stellate cell and regulate the fibrotic process (Fig. 1.4). The hypothesis examined in this thesis, is that T-lymphocytes directly influence HSC activation via either soluble mediators (such as Th1 and Th2 cytokines) or cell-cell contact and that the balance between Th1 and Th2 responses may be an important factor in determining the outcome of the interaction.
The aims of the studies are:

1. To determine the direct effects of the Th2 cytokines IL-10 and IL-13 on HSC activation and to examine the effect of IL-10 on an *in vivo* model of liver fibrosis. The work described in chapters 4 and 5 examines the effects of IL-10 and IL-13 on facets of HSC activation *in vitro* and the effect of administration of exogenous recombinant IL-10 on the development of carbon tetrachloride induced liver fibrosis in mice.

2. To study an experimental model of liver fibrosis that is thought to be T-lymphocyte mediated and examine the effect of manipulation of Th1/Th2 balance on the development of liver fibrosis. The studies in chapter 6 detail the development and validation of an animal model of liver fibrosis induced by repeated injections of concanavalin-A (Con-A) and thought to be CD4+ T-cell dependent. The Con-A model was to be used to explore the influence of Th1/Th2 balance on liver fibrosis by comparing liver histology in mice.
deleted for Th1 and Th2 cytokines (IL-4−/−, IL-13−/− and IL-10−/−) with that in their wild type counterparts.

(3) **To examine the effect of soluble mediators from and direct contact with Th1 and Th2 lymphocytes on HSC activation**

The work in chapter 7 describes a method of culturing T-lymphocytes with a Th1 or Th2 phenotype and examines the effects of Th1 and Th2 conditioned culture media and fixed Th1 and Th2 cells on facets of HSC activation.
Chapter 2

Materials and General Experimental Methods
Materials and General Experimental Methods
This chapter describes the methods used in the work described in this thesis. Any modifications are detailed in the relevant sections of the appropriate experimental chapters. Unless otherwise stated, chemicals and reagents were supplied by Sigma, Poole, UK. Details of materials marked with an asterisk are located in the appendix.

2.1. Isolation of Hepatic Stellate Cells
The isolation of pure, functionally intact hepatic stellate cell (HSC) populations from normal liver was essential in order to perform the studies described. Parenchymal cells constitute approximately 65% of the total cell population of normal mammalian liver (Weibel et al. 1969) with HSC representing only 5-8% (Giampieri, Jezequel, & Orlandi 1981). Various methods for the isolation of individual purified cell populations have been described, most involving sequential perfusion of the liver via the portal vein. A calcium free buffer is first infused to dissociate the calcium dependant intercellular desmosomal junctions (Nagelkerke, Barto, & Van Berkel 1982), followed by perfusion with collagenase in a calcium containing buffer, to facilitate digestion of the extracellular matrix (ECM). Digestion with collagenase alone allows isolation of both parenchymal and non-parenchymal cells from the liver (Nagelkerke, Barto, & Van Berkel 1982). More aggressive digestion of the liver by the addition of pronase yields much purer populations of HSC together with KC but may damage membrane receptors for up to 24 hours after isolation (Brouwer et al. 1982; Mills & Zucker-Franklin 1969).

Two techniques, namely density gradient centrifugation and centrifugal elutriation, are used to achieve further purification of the HSC population. Both of these depend on the buoyancy of freshly isolated HSC afforded by their abundant cytoplasmic lipid droplets to separate them from contaminating cell fractions. The cells are first centrifuged over a discontinuous density gradient allowing denser cell populations to be pelleted while HSC remain in suspension. The resulting 'HSC enriched' fraction is then subjected to centrifugal elutriation, an established method of separating particles by their size and density in which the cell suspension is circulated against the direction of centrifugal force through a specialised chamber in a centrifuge rotor. When subjected to these two opposing forces, each cell tends to migrate to a zone
where its sedimentation rate is balanced by the flow of fluid through the chamber. At a given flow rate and rotor speed, cells in the suspension will be sorted according to their size and density. HSC can therefore be continuously collected, leaving denser contaminants behind in the chamber. Different cell populations can be isolated by varying the rotor speed and the flow rate (Arthur, Kowalski-Saunders, & Wright 1986; Bissell et al. 1987; Friedman et al. 1985; Zahlten et al. 1978).

The relative adherence of the various hepatic cell types further reduces any residual contamination of HSC by other cell populations. Hepatocytes, unlike HSC, do not adhere efficiently to plastic, and although KC adhere more quickly than HSC, supplementing the culture medium with relatively high concentrations of foetal calf serum (10-20%) results in the selective continued growth of HSC.

The method described below was the isolation technique used for rat hepatic cells. The majority of the rat HSC isolations were performed by Mrs Janet Gentry, Mrs Carol Edwards, Dr Fiona Walker and Dr Marianna Gaca, to all of whom I would like to extend my thanks.

All animal work was carried out in accordance with Home Office procedure

2.1.1. Rat HSC Isolation

Materials

Hypnorm (Janssen)
Hanks buffered salt solution with and without Ca$^{2+}$ (HBSS$+/-$Ca$^{2+}$) (Boehringer Mannheim)
Collagenase (Boehringer Mannheim)
Pronase (Boehringer Mannheim)
Deoxyribonuclease (DNase, Boehringer Mannheim)
Optiprep™- iodixanol 60% (w/v) in water (Nycomed)
Dulbecco's modified Eagles medium (DMEM, Life Technologies)
Foetal calf serum (FCS, Life Technologies)
Penicillin/streptomycin/gentamicin (PSG)*
Method

Rat hepatic cells were isolated from male Sprague-Dawley rats (300-750g body weight) based on methods as previously described (Arthur et al. 1989; Friedman & Roll 1987; Pinzani et al. 1992). All solutions were pre-warmed to 37°C and sterile technique was employed throughout the procedure.

The rats were anaesthetised using intraperitoneal injection of a mixture of Hypnorm and midazolam (200µl/g body weight), with a simultaneous injection of heparin (100U) to prevent intraportal thrombosis. The portal vein was exposed and cannulated and the liver perfused with sterile, Ca²⁺-free HBSS containing heparin (250 U/ml) and the inferior vena cava immediately cut to decompress the liver and facilitate perfusion. The liver was then carefully dissected out, transferred to a 90mm tissue culture dish and sequentially perfused with pronase (2 mg/ml in HBSS+Ca²⁺) then collagenase (0.2 mg/ml in HBSS+Ca²⁺).

When the perfusion was complete, the liver digest was finely chopped using a scalpel and transferred to the remaining pronase solution supplemented with DNase (2.4mg) to prevent cell clumping in the presence of DNA released from ruptured cells. The resulting cell suspension was incubated for 30 minutes at 37°C with shaking and filtered through sterile nylon gauze to remove undigested material before resuspending in HBSS with Ca²⁺ and centrifuging at 400g for 7 minutes at 4°C. The supernatant was discarded and the cell pellets washed and resuspended in HBSS with Ca²⁺ containing DNase then layered on to a density gradient of Optiprep. Tubes were centrifuged at 1400g for 20 minutes at 4°C with no brake and the HSC fraction harvested. Further purification of the HSC population was achieved by the use of a centrifugal elutriator (Beckman). HSC were elutriated at a rotor speed of 1500 rpm with a flow rate of 18 ml/min. The cells were then pelleted before being resuspended in DMEM supplemented with 16% FCS and 4% PSG.

Assessment of Cell Number and Viability

Cell viability and number was assessed immediately after isolation by Trypan Blue exclusion. A 75µl aliquot of cell suspension was incubated for 1 minute in 0.05% w/v
Trypan Blue and loaded into the counting chamber of a haemocytometer. Viable cells stay small, round and refractile whilst non-viable cells become enlarged and stain dark blue. Both the percentage of viable cells and the total number of cells/ml were determined. Cell viability was >98% in most isolations.

2.1.2. Murine HSC Isolation

For the purpose of some experiments where only murine reagents were available or involving co-culture with murine T-cells, it was necessary to isolate murine HSC. The principles of the isolation were broadly similar to those for rat HSC isolation with the notable exception that the much smaller yield of cells precludes the use of an elutriation step and the cultures obtained therefore contain higher proportions of contaminating cells.

Materials

Hanks buffered salt solution with and without calcium (HBSS+/-Ca$^{2+}$) (Boehringer Mannheim)
Collagenase (Boehringer Mannheim)
Pronase (Boehringer Mannheim)
Deoxyribonuclease (DNase, Boehringer Mannheim)
Optiprep (Nycomed)
Optiprep working solution (25ml stock Optiprep/ 26ml HBSS+Ca)
Dulbeccos modified Eagles medium (DMEM, Life Technologies)
Foetal calf serum (FCS Life Technologies)
Penicillin/streptomycin/gentamicin (PSG)*

Method

Murine hepatic stellate cells were isolated from ex-breeder BALB/c mice using a protocol adapted from the rat HSC isolation procedure described in 2.1.1. Two animals were used for each isolation procedure.

Each mouse was killed by cervical dislocation, the abdominal cavity immediately opened and the liver and portal vein exposed. The portal vein was cannulated with a 24 gauge Wallace cannula, the inferior vena cava cut, and the liver perfused with heparin 250U/ml in HBSS-Ca until it blanched. The liver was next perfused in situ with a
solution of pronase (4mg/ml) and collagenase (0.5mg/ml) in HBSS+Ca^{2+} for 5-10 minutes, dissected out into a tissue culture dish and cut into small fragments in DNase 0.1 mg/ml in HBSS+Ca^{2+}. Liver digests were transferred into the remaining pronase/collagenase solution and incubated at 37°C for 20 minutes with shaking. The cell suspension was filtered through a sterile nylon mesh, the cells pelleted by spinning at 400g for 7 minutes, the supernatant discarded and the cells resuspended in HBSS+Ca^{2+}.

The density gradient was prepared as follows for each animal and equally divided between two 15ml centrifuge tubes: lower layer (4.7ml working Optiprep/3.2ml HBSS+Ca^{2+}); upper layer (5.9ml working Optiprep/8.9ml cell suspension). Each gradient was overlaid with HBSS+Ca^{2+} and centrifuged at 1400g for 20 minutes with the brake off. The upper ‘HSC rich’ layer and the middle ‘Kupffer cell rich’ layer (also containing HSC) were harvested, washed and resuspended in DMEM 16%FCS, PSG 4%.

**Assessment of cell number, viability and purity**

Freshly isolated cells were counted as described in 2.1.2. Typical yields of murine hepatic stellate cells were less than 20 x 10^6 per mouse. Preparations were contaminated by 20% Kupffer cells and occasional hepatocytes but were naturally overgrown by HSC with time. By 7 days in culture, 94% of cells expressed the myofibroblast marker desmin rising to 97% by 12 days. No Kupffer cell contamination was detectable in cultures over 12 days from isolation. Murine HSC were kept in culture for up to 5 passages.

**2.2. HSC Culture**

**Materials**

- HBSS, with and without (+/-) Ca^{2+} (Boehringer Mannheim)
- DMEM (Life Technologies)
- PCS (Life Technologies)
- PSG* Bovine serum albumin (BSA)
- 10 x trypsin ethylenediaminetetraacetic acid (TEDTA, Santa Cruz)
2.2.1. **General cell culture**

The resuspended HSC were either immediately used in experiments (freshly isolated HSC) or were cultured on uncoated plastic at a plating density of $1.33 \times 10^6$ cells/ml (unless otherwise stated). The cells were maintained in culture in DMEM 16% FCS with PSG 4% at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The culture medium was changed every 48 hours.

2.2.2. **Characterisation of HSC and Kupffer Cells**

Isolated hepatic stellate cells have a characteristic morphological appearance under phase contrast microscopy (Leitz inverted microscope). For the first 24 hours of culture on uncoated plastic, they remain rounded with few cytoplasmic processes. Over the first 48 hours of culture they gradually adhere to the plastic, becoming spindle shaped with cytoplasmic processes, giving them their characteristic stellate appearance. Freshly isolated HSC contain abundant retinoid vesicles within their cytoplasm that permit their identification by autofluorescence under ultraviolet (UV) light. With increasing time in culture they lose their retinoid droplets and activate to acquire a myofibroblast-like phenotype (Bachem et al. 1992; Bissell 1992; Friedman, Roll, Boyles, & Bissell 1985; Nakatsukasa et al. 1990) associated with expression of desmin and α-smooth muscle actin (α-SMA) (Friedman, Roll, Boyles, & Bissell 1985; Rockey et al. 1992; Takase et al. 1988; Yokoi et al. 1984).

2.2.3. **Preparation of Conditioned Media**

To obtain the serum free conditioned media required for some of the experiments described in this thesis, HSC that had been cultured for varying times in serum containing media were washed three times in serum free DMEM with pre-incubation in serum free media for 1 hour following the final wash. The cells were then incubated in half of their original volume of fresh serum free media containing PSG and 0.01% BSA for 24 or 48 hours. The media were then collected and clarified by centrifugation at 2000g for 5 minutes and the cell monolayers lysed by the addition of guanidinium isothiocyanate (GIT) for RNA extraction or used for analysis of DNA content.
2.2.4. Trypsinisation for Sub-Culture

The cell monolayer was first washed three times in HBSS-Ca\(^{2+}\) before a 2-5 minute incubation in 1x Trypsin-EDTA in HBSS-Ca\(^{2+}\) pre-warmed to 37\(^{\circ}\)C. The progress of cell detachment from the plastic was monitored by light microscopy. Once the cells had acquired a rounded appearance, culture flasks were tapped vigorously to encourage cell detachment or gently scraped with a pastette in the case of murine HSC (which remain much more firmly adherent during trypsinisation than rat HSC). A small volume of FCS was added to inactivate the Trypsin-EDTA and the cells were immediately pelleted by centrifugation for 5 minutes at 400g and resuspended in culture media. The required plating density was achieved by determination of the total number of cells/ml using a haemocytometer, as described above.

2.3. Extraction, Purification and Analysis of RNA

Materials

diethylpyrocarbonate (DEPC) treated water*
4M GIT* containing \(\beta\)-mercaptoethanol
2M sodium acetate pH 4.0
phenol
chloroform/isoamyl alcohol (49:1 by volume)
phenol/chloroform/isoamyl alcohol (25:24:1 by volume)
3M sodium acetate pH 6.0
isopropanol
75% ethanol (in DEPC treated water)
agarose
ethidium bromide (1mg/ml) in DEPC treated water
formaldehyde (37% volume/volume, v/v)
10 x MOPS (3-(N-Morpholino)propanesulphonic acid)*
RNA loading buffer*
20x salt sodium citrate (SSC)*
sodium hydroxide
Hybond-N (Amersham)
23.1. General precautions for working with RNA

RNA is extremely susceptible to degradation by ubiquitous ribonucleases (RNases) that are themselves comparatively resistant to degradation. In order to maintain RNA integrity, the following precautions were used for all RNA work. Gloves were worn at all times, as skin is a major source of RNases, and dedicated glassware (baked for 5 hours at 200°C) used. Water was pre-treated overnight with DEPC, which inactivates RNases by modification of tyrosine residues, and autoclaved (to inactivate the DEPC by degrading it to ethanol and CO₂) prior to use. Stock solutions were prepared with DEPC treated water and molecular biology grade reagents. Any solutions or instruments unsuitable for DEPC treatment were autoclaved and non-disposable plasticware, including electrophoresis equipment, was soaked in 0.5M sodium hydroxide and rinsed in de-ionised (DI) water prior to use.

Total RNA was extracted, purified and separated by electrophoresis on a denaturing agarose gel, after which it was transferred to a nylon membrane (Northern blotting) to permit the detection of specific messenger ribonucleic acid (mRNA) signal by hybridisation with radiolabelled complementary DNA (cDNA) probes.

23.2. Preparation of GIT lysates

1 ml of 4M GIT containing β-mercaptoethanol was added to 75 cm² flasks of confluent cultured HSC (with the volume being varied for sub-confluent cultures or different sizes of flasks). The cultures were vigorously agitated to lyse the cells and denature proteins including RNases. To extract RNA from whole liver, approximately 0.5g of snap frozen tissue was rapidly homogenised in 5mls GIT using a pre-autoclaved polytron homogeniser probe. The tissue homogenates were then centrifuged to remove debris and aliquotted. The cell lysates and tissue homogenates were stored at -70°C until required for RNA isolation.

23.3. RNA Extraction by the Acid Phenol Method

Total RNA was initially extracted from GIT lysates using a modification of the method described by Chomczynski and Sacchi (Chomczynski & Sacchi 1987). Subsequently, RNA extraction was achieved using an RNeasy spin column kit (Qiagen, Crawley, UK) in accordance with the manufacturer’s instructions.
To each 900 microlitre (µl) aliquot in a 2ml microcentrifuge tube, 90µl of 2 M sodium acetate pH 4 was added at room temperature and mixed rapidly, followed by 900 µl of phenol and 100µl phenol/chloroform/iso-amyl alcohol. The suspension was mixed thoroughly by inversion and incubated for 15 minutes on ice. This ensures separation of nucleic acids that, because of their phosphate backbone, are strongly negatively charged and will therefore accumulate in the aqueous phase. Proteins, carbohydrates and lipids contain charged and uncharged as well as hydrophobic and hydrophilic regions, and will therefore accumulate in either the hydrophobic (organic) phase or at the organic / aqueous interface.

After centrifugation (14,000 rpm, 15 min, 4°C), the upper, aqueous phase was transferred to a fresh microcentrifuge tube, 700µl of phenol/chloroform/IAA added and the sample mixed by inversion prior to a further incubation on ice for 10 minutes to allow further removal of contaminating proteins from the RNA preparation. The supernatant obtained after separation of the aqueous and organic phase by centrifugation (14,000 rpm for 5 minutes at 4°C) was removed to another microcentrifuge tube and 1/20th volume of 3 M sodium acetate pH 6 was added and mixed gently. The RNA was then precipitated by addition of a 1:1 volume of ice cold isopropanol for at least 1 hour (or overnight) at -70°C.

The RNA precipitated in this reaction was recovered by centrifugation at 14,000 rpm at 4°C for 20 minutes, washed twice in 75% ethanol and dried at 65°C on a heater block. The pellets were resuspended in between 20-50µl DEPC treated water and solubilised by heating to 65°C for 10 minutes.

The yield and purity of total RNA was determined by measuring the optical absorbance (OD) at 260 nanometres (nm) and 280 nm in a spectrophotometer. The integrity of the RNA samples was confirmed by electrophoresis through denaturing agarose gels of 2µg aliquots of each RNA sample and subsequent visualisation of the 28 S and 18 S ribosomal RNA bands with UV light (section 2.3.4). Once purified, any RNA not immediately used was aliquoted and stored at -70°C until needed.
23.4. Electrophoresis of RNA

A 1% denaturing agarose gel was prepared by dissolving the appropriate amount of agarose in 1x MOPS by heating in a microwave oven. After cooling, formaldehyde (final concentration 2.2 M) was added and the gel allowed to polymerise. Formaldehyde disrupts the secondary structure of RNA and therefore allows accurate estimation of the size of RNA molecules according to their migration through the gel. 5-10 pg aliquots of RNA were mixed with an equal volume of RNA loading buffer and heated to 65°C for 10 minutes to denature the RNA. Immediately prior to loading, 1μl of ethidium bromide (1mg/ml), which intercalates with the ‘backbone’ of nucleic acids, was added to the samples to permit visualisation of the pro- and eukaryotic ribosomal RNA bands under UV light.

The samples were subjected to electrophoresis at 100 volts (V) for 1-2 hours using 1x MOPS as an electrophoresis buffer. RNA molecular weight markers (Promega, Southampton, UK) were run concurrently. After electrophoresis, the gel was viewed under UV light and photographed using a Polaroid camera to verify RNA integrity.

23.5. Northern blotting

Immediately after electrophoresis, RNA was transferred to a Hybond-N membrane by capillary elution overnight (Northern blotting).

Prior to transfer the gel was rinsed in DEPC treated water for 10 minutes to remove the formaldehyde. It was then rinsed in 50 mM NaOH for 20 minutes, which improves both the speed and efficiency of transfer by partially hydrolysing the RNA. After a further rinse in DEPC treated water the gel was equilibrated in 10x SSC for 20 minutes. Transfer to a nylon membrane (Hybond-N) was achieved using a conventional gel-blotting apparatus with 10 x SSC as the transfer buffer. After transfer the membranes were then air dried and exposed to low doses of UV irradiation for 5 minutes to cross-link the RNA on the surface of the membrane.
2.4. Hybridisation of Northern Blots with Radiolabelled cDNA Probes

Radiolabelled, random primed cDNA probes were prepared using a Megaprime™ DNA labelling kit (Amersham). The probes were hybridised overnight with membrane bound RNA on the Northern blots.

Materials
Hybridisation buffer* or NorthernMax/ULTRAhyb™ (Ambion, Whitney UK)
Megaprime™ cDNA labelling kit (Amersham)
[α-32P] deoxyadenosine triphosphate (dATP, Amersham)
Template DNA (2.5.4)
DEPC treated water*
Sephadex G-50
SSC*

2.4.1. Pre-hybridisation of Northern Blots.
Membranes, RNA side facing in, were pre-hybridised for 2-3 hours with hybridisation buffer at 42°C in a rotating hybridisation oven (Hybaid). The inclusion of blocking reagent, herring sperm DNA and Denhardt’s reagent in the buffer ensured efficient blocking of non-specific binding sites. To maximise the rate of annealing of the probe with its target, the ionic strength of the hybridisation buffer was high (2x SSC).

2.4.2. Radiolabelling Of cDNA Probes By Random Priming
Random priming is an efficient and simple method of making short cDNA probes. The system uses random sequences of hexanucleotides to prime DNA synthesis onto a DNA template that has been denatured by heating, with a radioactive nucleotide ([α32P]-dATP) being substituted into the copy. The Klenow reagent catalyses formation of this copy, and multiple short length (150-200 base pairs, bp) complementary sequences are made.

0.1 µg of template DNA was added to 5 µl of primer and an appropriate volume of DEPC treated water, to give a final reaction volume of 50 µl. The DNA was denatured by heating to 95°C for 5 minutes and then cooled to allow the primers to anneal to the template. The following were then added to the reaction mixture: 5 µl reaction buffer; 4
μl of each unlabelled nucleotide (deoxycytosine triphosphate (dCTP), deoxyguanine triphosphate, (dGTP) and deoxythymidine triphosphates (dTTP); 5μl [α-32P]dATP (50μCi/mmol) and finally 2 μl Klenow fragment (lacking 5'-3' exonuclease activity ) that had been kept on ice to prevent degradation. After mixing, the solution was incubated for 1 hour at 37 °C.

The reaction mixture was centrifuged over a Sephadex G-50 gel column to separate unincorporated [α-32P] dATP from the radiolabelled probes (free nucleotides being retained within the matrix). The purified probe was collected in a microfuge tube and denatured by heating to 95°C for 5 minutes. The heated probe was added to fresh hybridisation buffer and immediately exchanged with the prehybridisation solution. The blot was left to hybridise, rotating overnight at 42°C.

2.4.3. Stringency Washing of Northern Blots.

After hybridisation, the membrane was washed under stringent conditions to remove unbound probe. Any unstable non-specific hybrids with few hydrogen bonds are then disrupted by high temperature, low salt conditions.

The membrane was washed for 15 minutes in 0.2x SSC/ 0.2% sodium dodecyl sulphate (SDS) at 42°C in the rotating hybridisation oven followed by a further two 15 minute washes in the same solution at 55°C. Membranes that had been hybridised using ULTRAhyb required stringency washing in differing conditions at 42°C, namely, 2 x 5 minutes in 2 x SSC / 0.1% SDS, then 2 x 15 minutes in 0.1 x SSC/0.1% SDS.

The membranes were then wrapped in clingfilm and exposed to either pre-flashed blue sensitive x-ray film (Genetic Research Instruments, Essex, UK)) for 24-72 hours at -70°C or to a Storm phosphorscreen (Eastman Kodak, Rochester, NY, USA) for 2-24 hours at room temperature. Phosphorscreens were then visualised using a Storm Phospho-imager (Molecular Dynamics, California, USA). Autoradiographs and Storm images were quantified by scanning densitometry.

Membranes were kept moist at all times, preventing each probe from becoming too tightly bound, and stored at -20°C to enable them to be probed sequentially for a number of different mRNA transcripts. On completion of experiments, the hybridised cDNA probe was removed by immersing the membranes in boiling DEPC treated water for 15 minutes. To confirm that the probe was completely removed, the stripped membranes were exposed to X-ray film.
2.5. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR is an extremely sensitive method for detecting the presence of particular mRNA transcripts in samples, and is capable of detecting RNA that is present at concentrations of less than 1 part per $10^8$. The reverse transcription (RT) step involves the synthesis of double stranded cDNA from an RNA template in a 5' to 3' direction. The reaction is catalysed by an RNA dependent DNA polymerase (reverse transcriptase) and random hexamers used to prime the reaction. Random hexamers are randomly generated oligodeoxynucleotides, with enough diversity to ensure that some of them will be complementary to sequences in the template nucleic acid. As different oligonucleotides will bind to different sequences in the template, all parts of the template will be represented in the cDNA at an approximately equal frequency.

Following the RT step the resulting cDNA is subjected to the polymerase chain reaction (PCR) using a sense and antisense oligonucleotide primers that flank the specific RNA sequence of interest (Sempowski et al. 1998). During the PCR reaction, DNA is first denatured by heating to 95°C then cooled to allow re-annealing. A molar excess of the primers ensures that the primers anneal to the DNA strands. In the presence of the four deoxyribonucleoside triphosphates (dNTPs) and a heat stable DNA polymerase (*Thermus aquaticus*, *Taq*), DNA synthesis is extended from the primers along the template. Repeated cycles of denaturing, priming and extension lead to a theoretically exponential accumulation of the specific target sequences.

As a result of the exponential amplification inherent in PCR, it is very susceptible to contamination. In an attempt to minimise the risk of contamination, PCR products were kept separate from PCR reagents by the use of dedicated laboratory space, dedicated equipment and careful technique. Appropriate positive and negative controls were included in all experiments.

All PCR reagents were obtained from Promega, Southampton, UK unless otherwise indicated.
2.5.1. Synthesis of double stranded cDNA (Reverse Transcription)

Reagents
RNase free (DEPC treated) water
dNTP mixture (10mM)
Moloney murine leukaemia virus reverse transcriptase (M-MuLV RTase, 20U/μl, Immunogen, Sunderland, UK)
5 x reaction buffer (Immunogen, Sunderland, UK)
RNasin RNase inhibitor (40μl/μl)
Random hexamers (0.5μg/μl)

For each reverse transcription reaction, 1μg of RNA was added to a mixture containing 2μl dNTP mix, 4μl 5x reaction buffer, 3.2 μg random hexamers, 0.5μl RNasin and 1μl M-MuLV RTase and the volume made up to a total of 20μl. Final concentrations in the reaction mixture are shown in brackets below.

5 x reaction buffer (1x)
10 mM dNTP mix (1mM of each dNTP)
Random hexamers (80 pg/μl)
Reverse transcriptase (1U/μl)
RNasin (1U/μl)

Reaction mixtures were incubated at 37°C for 60 minutes to reverse transcribe RNA into cDNA, heated to 95°C for 5 minutes to terminate the reaction and destroy RNA-DNA hybrids then made up to a total volume of 100μl with de-ionised water. The resultant cDNA samples were used immediately for PCR or stored at −20°C until required.

2.5.2. The Polymerase Chain Reaction

Reagents
De-ionised (DI) water
Dynazyme Taq DNA polymerase (500U/ml, Flowgen, Lichfield, UK)
10 x DNA polymerase buffer (Flowgen, Lichfield, UK)
1 x DNA polymerase buffer contains: 10mM Tris-HCl (pH 8.8), 50mM potassium chloride, 0.1% Triton X-100

dNTP mix (10mM)
MgCl₂ (50mM, Flowgen, Lichfield, UK)
sense and antisense PCR primers (Oswel DNA Service, University of Southampton)
cDNA sample
wax beads

Method

To ensure the efficient and accurate amplification of each target cDNA, without the appearance of non-specific products or ‘primer dimers’, the various components of the PCR reaction must be optimised. The optimal concentration of magnesium in the reaction (usually 0.8-2.0 mM) must be established for each primer pair. If the magnesium concentration is too low the primers will anneal non-specifically. Conversely, if the magnesium concentration is too high there will be insufficient binding, poor product amplification, and a tendency for ‘primer dimers’ to form. Both the specificity and fidelity of the PCR reaction will tend to increase with the use of lower dNTP concentrations by minimising the mispriming of non-target sites.

Effective primer design is critical, typically primers should be between 18 and 30 bp long with between 50-60% G and C composition. The melting temperature (T_m) should be similar for any primer pair. Consideration needs to be given to the nucleotide sequence of primers to avoid the formation of ‘hairpins’ or ‘dimers’ between the primers. The use of ‘hot start’, as described below will help reduce the appearance of non-specific products by physically isolating the primers and dNTPs from the Taq DNA polymerase.

The cycling conditions (cycling temperatures for each stage of the reaction, the incubation times and the number of cycles) need to be optimised for each reaction. For each PCR reaction, 50µl of a ‘master mix’, containing 5µl DNA polymerase buffer and MgCl₂ 1-1.5µM (depending on primers), was made and de-ionised water added to make up the total volume. 13µl of the ‘master mix’ was added to each PCR tube with 1µl 10mM dNTP mix and 25 picomoles (pmol) of each primer. A wax bead was added to the PCR tube and melted by heating to 95°C for 2 minutes, after which the samples
were placed on ice to allow the wax to solidify and seal over the lower layer of reaction mixture. The wax seal was then overlaid with 37 µl of ‘master mix’, 1.6U of DNA polymerase and 5 µl of cDNA (or DI water in the negative control). Reaction mixtures were the amplified in a Hybaid Omnigene Thermal Cycler as follows: 94°C 30 seconds, optimum Tm for 1 minute, 72°C 2 minutes for 35 cycles, followed by extension at 72°C for 7 minutes. Products were either stored at 4°C or immediately subjected to agarose gel electrophoresis.

The cycling conditions, namely the temperatures for each stage, the incubation times and the number of cycles, must be optimised for each reaction. They depend on a number of factors including the Tm of the primers, the optimal temperature for the DNA polymerase and the number of cycles required.

2.5.3. Analysis of PCR Products

**Materials**

Agarose
DI water
6 x DNA loading buffer*
ethidium bromide (1 mg/ml in DI water)
10 x tris(hydroxymethyl)aminomethane acetate EDTA (TBE)* or 10 tris (hydroxymethyl)aminomethane borate EDTA (TAE)*
PCR molecular weight markers (50-1000bp, Promega)

**Method**

Agarose was added to 1x TBE or TAE to give a percentage gel composition appropriate to the product size (*Table 2.1*) and dissolved by heating in a microwave oven. After cooling, ethidium bromide was added to give a final concentration of 0.5 µg/ml, the solution gently agitated, poured into a casting tray containing a gel comb and left to set at room temperature for approximately 30 minutes. When set, the gel was placed in an electrophoresis tank containing 1x TBE/TAE.

10 µl aliquots were taken from each PCR sample, mixed with 2 µl 6x DNA loading buffer and loaded into the wells. 5 µl PCR markers mixed with 1 µl loading buffer were loaded on each gel and the samples resolved by electrophoresis at 100 V for 30-
40 minutes. PCR products were visualised by transillumination on a UV light box and photographed.

**Table 2.1: Percentage agarose gel used for analysis of PCR products**

<table>
<thead>
<tr>
<th>Agarose %</th>
<th>PCR Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>&lt;500</td>
</tr>
<tr>
<td>2</td>
<td>500-1000</td>
</tr>
<tr>
<td>1.5</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

2.6. Proliferation Assays

The effect of various stimuli on HSC proliferation was determined by measuring the incorporation of [methyl-\(^{3}\)H] thymidine into newly synthesised cellular DNA during the S phase of the growth cycle, using a method adapted from that described by Pinzani (Pinzani et al. 1994).

**Materials**

- [methyl-\(^{3}\)H] thymidine (Amersham, Little Chalfont, UK)
- HBSS with Ca\(^{2+}\) (Boehringer Mannheim, Lewes, UK)
- 95% methanol / 5% acetic acid
- 0.25M sodium hydroxide / 0.2% SDS
- 5M HCl
- Optiphase Hisafe 3 scintillant (Fisher, Loughborough, UK)

HSC were seeded into 24 well plates and allowed to grow until just sub-confluent. The cells were washed three times in DMEM and serum depleted by culture in DMEM 0.5% FCS overnight to arrest them at the G\(_0\) phase of the cell cycle. After this period the HSC were treated for 24 hours with media containing the factors to be studied or with positive (DMEM 5%FCS) or negative (DMEM 0.5% FCS) control media. For the last 6 hours of stimulation, HSC were pulsed with 1μCi of \(^{3}\)H-thymidine per well. Depending on whether a pro-proliferative or anti-proliferative effect was expected, the percentage of FCS in the media was varied to optimise detection of the effect.
Cells were washed twice for 15 minutes with HBSS containing Ca\textsuperscript{2+} to remove unincorporated radioactivity and the cells fixed in the wells with ice cold 95% methanol/ 5% acetic acid overnight at -20°C. The plates were then washed a further 3 times in HBSS+Ca\textsuperscript{2+} on ice and the cells lysed by the addition of 500 µl of 0.25M sodium hydroxide/ 0.2% SDS per well. To each well, 30 µl of 5M HCl was added to neutralise the NaOH and prevent chemiluminescence, which would result in spuriously high counts. Cell lysates were harvested into scintillation vials and mixed with 3 mls of scintillant before determining the incorporation of \textsuperscript{3}H-thymidine into DNA by counting in a Wallace 1217 RackBeta liquid scintillation counter.

Proliferation assays are dependent on cell number and the absolute levels of proliferation in various HSC populations at various stages of activation. All results were therefore expressed relative to control cultures, which contained the same percentage of FCS as the experimental groups but no additives. These were assigned the arbitrary value of 1.

2.7. PicoGreen DNA Assay

DNA quantitation was performed to allow data to be normalised for cell number and thus to permit relevant comparisons to be made between samples. The assay employed uses PicoGreen\textsuperscript{®} dsDNA Quantitation Reagent (Molecular Probes Europe BV, Leiden, The Netherlands), an ultrasensitive fluorescent nucleic acid stain which is highly selective for double-stranded (ds) DNA. Binding of PicoGreen to the nucleic acid backbone of dsDNA results in an enhancement of fluorescence, which can be measured in a fluorescence spectrophotometer.

Materials
25cm cell scrapers (Falcon)
Herring sperm DNA (Promega)
PBS
Tris-EDTA (TE)-10mM Tris-HCl, 1mM EDTA, pH 7.5
PicoGreen (Molecular Probes)
96 well plates (Nunc Maxisorb, Gibco, Paisley, UK)
Method

To prepare samples, cells were scraped into a set volume of TE (usually 500μl per well for a 12 well plate or 1ml for a 25cm² tissue culture flask), transferred to microcentrifuge tubes and sonicated for 15 minutes. A standard curve ranging from 0-10 μg/ml dsDNA was made by serial dilutions of herring sperm DNA in TE. Just prior to use, the PicoGreen reagent was diluted 1:200 in TE.

100μl aliquots of samples and standards were loaded in triplicate onto a 96 well plate and an equal volume of diluted PicoGreen added to each well. Fluorescence was measured in a Cytofluor II Microwell Fluorescence Spectrophotometer (Persephic Biosystems) set at excitation~480nm and emission~530nm.

Analysis of data

The data generated from the PicoGreen assays was analysed in Microsoft Excel with a standard curve of fluorescence against DNA concentration generated for each experiment and used to calculate the concentration of dsDNA in each sample.

2.8. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis (PAGE) is a quick and sensitive method for separating proteins according to size utilising a cross-linked acrylamide support through which the samples are electrophoresed. The sample to be fractionated is denatured and coated with detergent by heating in the presence of SDS and a reducing agent. SDS is anionic and thus gives the protein a negative charge, proportional to the length of the polypeptide chain. The samples are then run at high voltage on the polyacrylamide gel, which will cause the proteins to migrate towards the anode in a charge, and therefore size, dependent manner.

Materials

10% w/v ammonium persulphate (APS)
40% 29:1 acrylamide/bisacrylamide (Biorad, Hemel Hempstead, UK)
1.5M Tris-HCl (pH 8.8)
0.5M Tris-HCl (pH 6.8)
10%w/v SDS
N,N,N,N-tetramethyl ethylenediamine (TEMED)
100% ethanol
5 x Laemmli electrode buffer*
β-mercaptoethanol
SDS-PAGE sample buffer*
SDS-PAGE MW markers (New England Biolabs, Hitchin, UK)
SDS-PAGE minigel apparatus (Biorad)

Method
The minigel apparatus was cleaned with ethanol and assembled after which the resolving gel was cast, with the percentage polyacrylamide in the gel varying depending on the MW of the proteins of interest. The reagents were added in the order listed in Table 2.2, with polymerisation being initiated by an anaerobic free radical chain reaction following the addition of the SDS and TEMED. A layer of ethanol was added over the resolving gel mixture to exclude air and to prepare a flat interface. When polymerised, typically after approximately 45 minutes, the ethanol was removed, the stacking gel was poured, and combs inserted. The combs were removed from the polymerised stacking gel, the gel electrophoresis apparatus assembled, electrode buffer added, the gels immersed and the combs removed.

Table 2.2: SDS-PAGE gel composition (sufficient for two 10 x 7 cm gels)

<table>
<thead>
<tr>
<th></th>
<th>12% RESOLVING GEL</th>
<th>4% STACKING GEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% stock acrylamide</td>
<td>6mls</td>
<td>1ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>8.7mls</td>
<td>6.4mls</td>
</tr>
<tr>
<td>1.5M Tris-HCl (pH 8.8)</td>
<td>5mls</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris-HCl (pH 6.8)</td>
<td>-</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10%w/v SDS</td>
<td>200μl</td>
<td>100μl</td>
</tr>
<tr>
<td>10% v/w APS</td>
<td>100μl</td>
<td>50μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10μl</td>
<td>5μl</td>
</tr>
</tbody>
</table>
The samples were denatured by the addition of 10% by volume 2-mercaptoethanol and heating to >85°C for 10 minutes. The gel was run at 100 V until the dye front reached the bottom of the gel, usually about two hours.

2.9. Western Blotting

Materials
100% methanol
Transfer buffer *
Chromatography 3mm filter paper (Whatman)
Polyvinylidifluoride (PVDF) membrane
Transfer apparatus (Biorad)
Tween tris buffered saline (TTBS) *
1% Marvel
Primary antibody
Secondary antibody
Blue sensitive X-ray film (Genetic Research Instrumentation, Essex, UK)
Enhanced chemiluminescent reaction (ECL) kit (Amersham)

2.9.1. Electrophoretic Transfer of Antigen to Membrane

After electrophoresis the resolving gels were cut from the stacking gels and soaked in transfer buffer for 15 minutes to remove the SDS. The membranes and filter paper were cut to size, the membranes pre-soaked in methanol and the membranes, filter paper and fibre pads then soaked in transfer buffer. The transfer blotting apparatus was assembled with electrophoretic transfer being achieved by running at 100V for one hour. After blotting, the membrane was washed in distilled water and processed directly.

2.9.2. Immunological Detection

Immediately following transfer a double-antibody detection technique was used to identify specific proteins of interest. The membranes were first blocked for 1 hour in TTBS containing 1 % dried milk protein unless otherwise stated. The membranes were washed in TTBS and incubated overnight at room temperature with primary antibody or an appropriate non-immune control diluted in TTBS containing 0.01% dried milk protein. After rinsing three times for five minutes each in TTBS the
membranes were incubated with a peroxidase conjugated secondary antibody for one hour at room temperature. After repeated washing, twice in TTBS and three times in distilled water any antibody protein complex present on the membrane was detected using a commercial ECL system, as described below.

2.9.3. Enhanced Chemiluminescent Reaction

The ECL reaction kit contains two reagents detection solutions 1 and 2. The reaction begins when reagent 1 decays to hydrogen peroxide, a substrate for horseradish peroxidase. Reagent 2 contains luminol, which simultaneously oxidises as horseradish peroxidase reduces hydrogen peroxide, and results in the production of blue light.

Immediately prior to use, equal volumes of solution 1 and 2 sufficient to cover the blot were mixed (8mls total volume being adequate for two 10 x 7 cm gels). Excess TTBS was drained from the membranes and the ECL reagents added for exactly 1 minute, after which excess detection buffer was drained off and the membrane wrapped in clingfilm before being exposed to blue sensitive X-ray film for approximately 1 minute. Depending on the appearances of the film subsequent exposures times were adjusted as appropriate.

2.10. Gelatin Substrate Gel Electrophoresis (Zymography)

Gelatin zymography is a technique by which MMPs with enzyme specificity for gelatin, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B), can be separated according to their molecular weight and detected by merit of their proteolytic activity (Kleiner & Stetlerstevenson 1994). Samples are electrophoresed in non-denaturing conditions through a SDS-PAGE gel supplemented with gelatin as an enzyme substrate. Following electrophoresis, SDS is washed from the gels to allow the enzymes to renature and they are incubated in a calcium-containing buffer to allow proteolysis to take place. Staining of the gels with Coomassie blue and subsequent destaining reveals clear zones of proteolysis against the blue background of gelatin corresponding to the location of MMP bands and identifiable by their molecular weight.

The technique can be adapted by varying the substrate in the gel to casein in order to detect MMP-3 (stromelysin).
**Materials**

- 1.5M Tris-HCl pH 8.8
- 0.5M Tris-HCl pH 6.8
- 10% w/v SDS
- 10% w/v ammonium persulphate (APS)
- N,N,N,N-tetramethyl ethylenediamine (TEMED)
- 40% 29:1 acrylamide/bisacrylamide (Biorad)
- gelatin (porcine skin)
- 100% ethanol
- 5 x Laemmli electrode buffer*
- β-mercaptoethanol
- SDS-PAGE sample buffer*
- SDS-PAGE MW markers (New England Biolabs)
- SDS-PAGE minigel apparatus (Biorad)
- proteolysis buffer (50mM Tris-HCl pH 7.8, 50mM CaCl₂, 0.5M NaCl)
- 2.5% v/v Triton X-100
- staining solution (0.1% Coomassie blue in 10% acetic acid, 40% methanol)
- destaining solution (7.5% v/v acetic acid, 10% v/v methanol)

**Method**

The gel apparatus was assembled and gels poured as for SDS-PAGE (2.8). Gelatin (20mg/ml) was dissolved in DI water by heating to 60°C in a water bath and added to the resolving gel to give a final concentration of 2mg/ml (Table 2.3). Samples were mixed 1:1 with 2x SDS-PAGE sample buffer (without reduction with β-mercaptoethanol or denaturation by heating) and were loaded with SDS-PAGE MW markers. Gels were electrophoresed at 100V until the dye front reached the bottom of the gel.

Gels were removed from the apparatus and washed three times for 10 minutes in 2.5% Triton X-100 followed by a rinse in DI water. They were then incubated overnight in proteolysis buffer at 37°C. After approximately 18 hours incubation, the gels were rinsed again in DI water, stained with Coomassie blue for 30 minutes - 1 hour and then destained until clear zones of proteolysis corresponding to bands of
MMPs appeared or the stacking gel had cleared. Gels were photographed with transillumination on a light box using a SLR camera.

Table 2.3: Gelatin zymography gel composition (sufficient for two 10 x 7cm gels).

<table>
<thead>
<tr>
<th></th>
<th>8% RESOLVING GEL</th>
<th>4% STACKING GEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% stock acrylamide</td>
<td>4mls</td>
<td>1ml</td>
</tr>
<tr>
<td>gelatin (20mg/ml)</td>
<td>1ml</td>
<td></td>
</tr>
<tr>
<td>DI water</td>
<td>9mls</td>
<td>6.4mls</td>
</tr>
<tr>
<td>1.5M Tris-HCl (pH 8.8)</td>
<td>5mls</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris-HCl (pH 6.8)</td>
<td>-</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>200µl</td>
<td>100µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>100µl</td>
<td>50µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10µl</td>
<td>5µl</td>
</tr>
</tbody>
</table>
Chapter 3

Collagen Synthesis Assay
Method Development
3.1 INTRODUCTION

In fibrotic liver, the most profound quantitative changes in extracellular matrix components are seen in the fibrillar collagens. Consequently, any work seeking to address alterations in extracellular matrix production requires reliable and sensitive assays of collagen synthesis.

Northern blotting provides a well established method of quantitating changes in collagen at the mRNA level, but increasing evidence of post-transcriptional regulation of collagen production (Stefanovic et al. 1997) necessitate analysis at the protein level to provide a comprehensive picture.

The only assay of collagen protein currently in use in the Liver Fibrosis Research Group was the Sirius Red Dye Binding Assay (Sircoll, Biocolour). This assay depends upon the specific binding of an intercalating dye to the \([Gly-X-Y]_n\) helical structure present in all collagens (Sweat, Puchtler, & Rosenthal 1964). After free dye has been removed by washing, the bound dye is dissociated from the collagen by treatment with sodium hydroxide and quantified by measuring absorbance in a spectrophotometer at 540nm.

In culture activated HSC, baseline levels of collagen are high and thus detecting changes against this high background, in response to any stimulus, required a highly sensitive technique. As the Sirius red dye binding assay simply measures the total amount of collagen present, it did not prove sufficiently sensitive to detect the changes seen in response to biological stimuli.

In order to achieve the required sensitivity, we sought to develop an assay to directly measure collagen synthesis, rather than total collagen deposition. Review of the literature revealed that quantitation of radiolabelled proline or hydroxyproline incorporation into newly synthesised collagens could provide the basis for an assay with the required sensitivity. Specificity of these assays relies upon either hot acid hydrolysis (Mallat et al. 1995) or enzymatic digestion of collagenous proteins (Peterkofsky & Diegelmann 1971) and in order to minimise the risks associated with manipulating radioactivity we chose to employ a method using highly purified collagenase.

Work on the development of this assay was shared equally with Dr Elizabeth Williams, a PhD student working in the Liver Group.
3.2 Principles of the $^3$H Proline Collagen Assay

The method we elected to develop was based on the assay used by Postlethwaite et al (Postlethwaite et al. 1992). Primary or early passaged HSC were cultured until sub-confluent then treated for 24 hours with serum and proline free medium supplemented with ascorbic acid to stabilise the collagen triple helix for. Media were replenished and $^3$H-proline (Amersham, Little Chalfont, UK) added for a further 24 hours during which it is incorporated into all newly synthesised proteins. The cells or culture supernatants were harvested and divided into two aliquots in a calcium ion (Ca$^{2+}$)-containing buffer. A highly purified collagenase (Worthington Biochemical Corporation, NJ, USA) was added to one of the aliquots to digest collagenous proteins, leaving non-collagenous proteins intact. The remaining intact proteins in both aliquots were precipitated with trichloroacetic acid (TCA). Protein pellets were washed repeatedly to remove unincorporated radioactivity before being resuspended in sodium hydroxide for counting in a beta-counter. $^3$H-proline incorporation into collagenous proteins was then calculated by subtracting the readings of the digested samples (non-collagenous proteins only) from the readings of the undigested samples (total proteins).

3.3 Refinement of the Method

In preliminary experiments, samples were treated, precipitated, pelleted and washed in 1.5ml microcentrifuge tubes. Although initial results suggested that the principles of the assay were sound, there were a number of technical limitations that markedly reduced the reliability and reproducibility of the assay. In particular, it proved both time consuming and difficult to adequately wash and resuspend the protein pellets without losing substantial amounts of the sample in the process. In order to circumvent these difficulties, we evaluated the viability of performing the assay in 96 well Multiscreen filtration plates (Millipore, Watford, UK) with pore size 0.45μm. Using this format, samples can be loaded in to the wells, digested in situ and the proteins precipitated onto the filter at the bottom of the well. Washing can be uniformly and efficiently achieved on a vacuum manifold and the whole plate counted in a 96 well plate scintillation counter. These early experiments also demonstrated that assaying culture supernatants rather than the cell monolayers was advantageous for several reasons. Firstly, comparison
of cell monolayers and supernatants demonstrated that assays of supernatants gave more reproducible results (data not shown). Secondly, previous work has shown that 90% of newly synthesised collagens are located in the supernatant (Postlethwaite, Holness, Katai, & Raghow 1992). Finally, preservation of the cell monolayers allows them to be used subsequently for normalisation of the results to cell number using a DNA assay. This is particularly valuable where the cytokine stimulus under study may have biological effects on HSC proliferation as well as collagen synthesis. These modifications substantially improved the reliability and reproducibility of the assay and allowed us to proceed to a more comprehensive validation of the method.

3.3.1 Optimisation of Collagenase Dose
Although the collagenase used is chromatographically purified and thus highly specific, it is contaminated with very low levels of other proteases (typical batch analysis: collagenase 1443U/mg, caseinase 3U/mg, clostripain 0.45U/mg, trypsin 0.01U/mg).

A titration of the collagenase was performed to establish the dose permitting complete and consistent digestion of all the collagenous proteins, without activity of the other proteases contributing significantly. After review of the literature (Cairns & Walls 1997; Postlethwaite, Holness, Katai, & Raghow 1992), cells in replicate wells were treated with one of three doses of collagenase (10, 50 and 100U per well). Collagenous proteins were expressed as a proportion of total protein (Fig. 3.1).

Results showed that digestion with the lowest dose of collagenase gave variable results, whereas 50U per well gave reproducible data. There was no appreciable increase in digestion or advantage conferred by the higher dose of 100U per well. A dose of 50U collagenase per well was therefore selected for all further studies.

3.3.2 Optimisation of 3H-Proline Dose
The next experiment was designed to determine the optimal dose of radioactivity required to maximise the sensitivity of the assay whilst restricting the use of radioactivity to a minimum. The results demonstrate that using 2μCi/well compared with 1μCi per well conferred no advantage (Fig 3.2).
Fig. 3.1: The effect of varying collagenase dose on reproducibility of the assay. Experiments were used to determine the optimum collagenase dose for complete and consistent digestion of collagenous proteins without other protease activity contributing significantly. The results show collagenous protein expressed as a proportion of total protein, when 10, 50 and 100U of collagenase per well was used. (n=6)

Fig. 3.2: The effect of varying $^3$H-proline dose on assay reproducibility. Experiments were performed to establish the optimum dose of tritiated proline required to maximise the sensitivity of the assay, whilst minimising radioactivity usage. The results for 1 and 2 $\mu$Ci per well are shown and are expressed as collagenous protein as a proportion of total protein. (n=4)
3.4 Validation of the Principles of the Assay.
These experiments were designed to confirm that the assay measures *de novo* protein synthesis and that the diminution in radioactive counts seen with collagenase treatment was dependent on proteolytic activity.

**Effect of inhibition of protein synthesis**
Cycloheximide was added to the culture media 2 hours prior to pulsing with $^3$H-proline, at a concentration (50µg/ml) known to effectively inhibit protein synthesis by HSC (Theret et al. 1999). As demonstrated in Fig. 3.3, inhibition of protein synthesis by cycloheximide dramatically reduced the radioactive counts retained in the well after washing: total protein labelling was $314.2 \pm 36.8$ cpm without cycloheximide and $45.1 \pm 4.4$ cpm with cycloheximide, whereas collagenous protein labelling amounted to $137.3 \pm 19.4$ cpm without cycloheximide and $13.5 \pm 4.3$ cpm with cycloheximide.

![Graph showing the effect of cycloheximide on protein synthesis](image)

**Fig. 3.3:** *The effect of inhibiting protein synthesis on the retention of $^3$H-proline.*
*Cycloheximide was added to HSC culture media prior to pulsing with $^3$H-proline (red bars). Control wells were included to which no cycloheximide was added (blue bars). The addition of cycloheximide dramatically reduced subsequent $^3$H-proline incorporation into both total and collagenous proteins. (n=4)*
Effect of inhibition of collagenase activity

Clostridial collagenase, in common with all matrix metalloproteinases, requires the presence of calcium ions for its activity. Experiments were conducted using a calcium free incubation buffer, to which 5mM EDTA was added to chelate any calcium in the culture supernatant, in order to demonstrate that the reduction in counts with collagenase treatment was dependent on its metalloproteinase activity (Fig. 3.4). Results show that in a Ca^{2+} containing incubation buffer the addition of collagenase reduced the radioactivity retained in the well from a mean of 239.8 ± 16.3 cpm to 170.3 ± 8.1 cpm (p<0.05), whilst in a Ca^{2+} free buffer retained radioactivity was unchanged at 257.1 ± 22.6 cpm without collagenase and 265.0 ± 22.9 cpm with collagenase.

![Graph showing the effect of collagenase on radioactivity](image)

**Fig. 3.4: The effect of a Ca^{2+} free buffer on collagenase activity.** To demonstrate that the reduction in radioactivity retained in the well following the addition of collagenase was a MMP specific effect, experiments were performed using a Ca^{2+} free buffer to which EDTA had also been added. Addition of collagenase in Ca^{2+} free conditions (right hand graph) did not lead to any diminution in the radioactivity retained. (n=4), *p<0.05 compared with no addition of collagenase.
3.5. Demonstration of a Biological Effect

To establish that this technique could be applied to studies of collagen regulation by cytokines in HSC, it was important to determine that the assay was sufficiently sensitive to detect changes in collagen synthesis of a biologically relevant magnitude. HSC were stimulated with two cytokines, TGF-β1 and IL-4 (R&D Systems, Abingdon, UK), which have been documented in the published literature to modulate collagen synthesis by HSC (Davis 1988; Knittel et al. 1996; Tiggelman et al. 1995). Both TGF-β1 (10ng/ml) and rat IL-4 (20ng/ml) produced a stimulation of collagen synthesis by primary rat HSC, which was within the limits of detection of the assay (Fig.3.5, Fig.3.6).

**Fig. 3.5: The effect of TGF-β1 on collagen synthesis. (n=4)**

The effect of 10ng/ml TGFβ1 on collagen synthesis by primary rat HSC was assessed by ³H-proline incorporation. The increase in collagen synthesis stimulated by exogenous TGF-β1 (red bar) is expressed relative to control (blue bar), which has been arbitrarily assigned a value of 1. *p<0.05 relative to control
Fig. 3.6: The effect of IL-4 on collagen synthesis. (n=4)

The effect of 20ng/ml IL-4 on collagen synthesis by primary rat HSC was assessed by 
$^{3}H$-proline incorporation. The increase in collagen synthesis stimulated by 
exogenous IL-4 (red bar) is expressed relative to control (blue bar) which has been
arbitrarily assigned a value of 1. *p<0.05 relative to control

3.6. Final Assay Protocol

Materials

DMEM

bovine serum albumin (BSA)

ascorbic acid

L-[2,3,4,5-$^{3}H$] proline (Amersham, Little Chalfont, UK)

10mM Tris-HCl, 1mM EDTA (pH 7.5) (TE)

96 well Multiscreen filtration plates (Millipore UK Ltd, Watford, UK)

collagenase (Worthington Biochem Corporation, New Jersey, USA)

0.2M Tris / 0.3M calcium chloride (pH 7.5)

50mM N-ethylmaleimide

TCA (10% and 50%)
Method

The synthesis of collagen by HSC was determined by measuring the incorporation of $^3$H-proline into collagenous proteins using modifications of the methods of Cairns and Postlethwaite (Cheever et al. 1992; Postlethwaite, Holness, Katai, & Raghow 1992). Early passaged HSC were seeded into 12 well plates and grown until approaching confluence. Cells were made serum free by washing three times in DMEM and incubated in fresh DMEM supplemented with antibiotics, 25mg/ml ascorbic acid, 0.01% bovine serum albumin (BSA) and the cytokine of interest in a total volume of 500μl/well. After 24 hours the media were changed and replaced with fresh media containing the same additions as above, plus 1μCi L-[2,3,4,5-$^3$H] proline (Amersham, Little Chalfont, UK) per well. After a further 24 hour incubation the cell supernatants were harvested and the cells lysed in TE (10mM Tris-HCl, 1mM EDTA, pH 7.5) for DNA quantitation.

The supernatants from each well were divided into 4 x 100μl aliquots and loaded onto 96 well Multiscreen filtration plates (Millipore UK Ltd, Watford, UK) and assayed in duplicate either to measure total protein production or the production of proteins resistant to digestion by a highly purified bacterial collagenase (non-collagenous proteins). The collagen production per well can then be calculated using the following formula:

$$\text{Collagen production} = \frac{[\text{H proline incorporation (total protein)}] - [\text{H proline incorporation (non-collagenous protein)}]}{\text{DNA content (μg/well)}}$$

To determine total protein production 35μl of 0.2M Tris / 0.3M calcium chloride pH 7.5 and 15μl of 50mM N-ethylmaleimide was added to each well where as 50U/well of a highly purified bacterial collagenase (Worthington Biochemical Corporation, New Jersey, USA) was also added to the wells assigned to determine non-collagenous protein production. The plates were incubated at 37°C for 90 minutes. Proteins were then precipitated by the addition of 100μl of 50% trichloroacetic acid.
(TCA) per well and incubation of the plate on ice for one hour. The plates were washed three times with 10% TCA using a vacuum manifold (Millipore, Watford, UK). The incorporated radioactivity in each well was determined by scintillation counting. Final results were expressed per mg DNA as a surrogate to allow normalisation for cell number.

3.6. Conclusion

This chapter has detailed the development of a sensitive collagen synthesis assay which was then employed to study HSC collagen synthesis in subsequent chapters of the thesis.
Chapter 4

Effect of IL-10 on Hepatic Stellate Cells

*in vitro* and CCl₄ Induced Liver Fibrosis *in vivo*
4.1. Context
The work in this chapter was performed during a one year Entry Level Training Fellowship funded by the Wellcome Trust and was stimulated as a development of work performed in the Southampton Liver Group by Dr Kerry Thompson as part of her PhD thesis and subsequently published (Thompson et al. 1998a).

4.2. Introduction
In the majority of human liver diseases, liver fibrosis results from persistent inflammatory changes. Signals resulting from inflammation and tissue damage converge on the hepatic stellate cell (HSC) causing activation from a quiescent to a myofibroblastic phenotype, which is characterised by a shift in the balance of matrix synthesis and degradation to favour matrix deposition and accumulation. In particular, soluble mediators produced by resident liver macrophages (Kupffer cells) have been established as having an important role in promoting the development of fibrosis in both experimental animal models and in human liver disease (Geerts 1988; Karakucuk et al. 1989).

Interleukin-10 (IL-10) is an anti-inflammatory cytokine produced by T-cells with prominent effects on the pro-inflammatory actions of macrophages (Bogdan, Vodovotz, & Nathan 1991; Fiorentino et al. 1991). Kupffer cells (KC) stimulated with lipopolysaccharide (LPS) in vitro secrete IL-10 and treatment with exogenous IL-10 markedly suppresses the release of the pro-inflammatory cytokines IL-6 and TNF-α by KC after stimulation with LPS (Knolle et al. 1995). Hepatic stellate cells (HSC) in long-term culture express and secrete IL-10 and HSC conditioned media have been shown to inhibit KC activation (Thompson et al. 1998b).

In some models of acute liver injury, IL-10 is produced early and appears to have a protective role. When liver injury is induced by LPS in galactosamine sensitised mice, treatment with recombinant IL-10 reduces serum TNF-α concentrations and reduces hepatic necrosis (Louis et al. 1997b; Santucci et al. 1996). Conversely, anti-IL-10 antibody pre-treatment increases TNF-α and exacerbates hepatic necrosis (Louis et al. 1997b). In experimental T-cell mediated hepatitis induced by injection of concanavalin-A, neutralisation of endogenous IL-10 with antibody increases the secretion of pro-inflammatory cytokines and worsens neutrophil infiltration and liver damage, whereas administration of recombinant IL-10 suppressed pro-inflammatory cytokine production, neutrophil infiltration and hepatic necrosis (Louis et al. 1997a).
Studies of the acute stages of carbon tetrachloride induced liver injury have produced conflicting results with regard to the anti-inflammatory role of IL-10. Our group found that IL-10 mRNA was up-regulated as early as 6 hours post CCl₄ injection but that histological scores of inflammatory cell infiltrates 1 to 3 days following injection did not differ between wild type and IL-10 deleted (IL-10-/-) mice (Thompson et al. 1998a). By contrast, Louis et al did find more prominent neutrophilic infiltration of the livers of IL-10 deleted animals 12 and 24 hours following CCl₄ injection but only marginal increases in serum alanine aminotransferase levels (ALT) (Louis et al. 1998). However, both studies demonstrated significant increases in histological scores for liver fibrosis suggesting that IL-10 might be exerting a direct anti-fibrotic effect independent of its anti-inflammatory properties.

Recombinant IL-10 (rIL-10) has been demonstrated to be both safe and well tolerated in clinical trials (Lebeaut & Garaud 1997) with a trend towards benefit in chronic inflammatory diseases including rheumatoid arthritis (Quiroga et al. 1998) and Crohn’s disease (Narula, Cutler, & Grint 1998). Evidence drawn from experimental animal models of liver disease shows that administration of IL-10 ameliorates the acute hepatitis caused by administration of both concanavalin A (Louis et al. 1997a) and lipopolysaccharide (LPS)/ galactosamine (Louis et al. 1997b). The well-established anti-inflammatory properties of the cytokine and the possible direct effects on HSC suggested by the CCl₄ studies in wild type and IL-10-/- mice (Louis et al. 1998; Thompson et al. 1998a) make IL-10 an attractive candidate for investigation as an anti-fibrotic therapy in vivo.

I hypothesised that:

- IL-10 has direct effects on HSC activation and that endogenous IL-10 produced by HSC plays an auto-regulatory role in inhibiting HSC activation.
- administration of recombinant IL-10 may inhibit the development of liver fibrosis in vivo.

In this chapter, I will:

- examine the effect of exogenous recombinant IL-10 and endogenous IL-10 neutralisation on rat HSC activation in culture.
- compare the phenotype of IL-10 deleted and wild type mouse HSC.
• examine expression of the IL-10 receptor by the HSC.
• examine the effect of recombinant IL-10 on the development of CCl₄ induced liver fibrosis in mice

4.2.1. The Carbon Tetrachloride Model of Liver Fibrosis
Administration of carbon tetrachloride to rodents has been widely used as a model of liver necrosis and fibrosis and the role of Kupffer cell and HSC activation in this have been extensively characterised (Edwards et al. 1993; Enzan 1985; McGee & Patrick 1972). Following intraperitoneal injection, CCl₄ is transported in the portal venous system to the liver, where it is converted to the trichloromethyl radical (CCl₃) (Recknagel & Glende 1973). This free radical initiates liver damage by lipid peroxidation and reacting with the sulphhydryl groups of proteins. Repeated CCl₄ injury results in fatty infiltration and perivenular necrosis which progresses over 6-12 weeks through septal fibrosis to established cirrhosis (Kent et al. 1976). The pathology of the acute lesion closely resembles that seen in acute alcoholic hepatitis in humans which is also thought to be mediated by free radicals.

4.3. Materials and Methods
4.3.1. Materials
Recombinant rat IL-10 was obtained from R&D, Abingdon, UK, normal IgG from Sigma, Poole, UK and the JES2A5 hybridoma producing anti-murine IL-10 antibody was a kind gift from Dr K Moore (DNAX, Palo Alto, USA). Carbon tetrachloride and olive oil were obtained from Sigma, Poole, UK. Human recombinant IL-10 (SCH 52000) was a kind gift from the Schering Plough Research Institute, Kenilworth, NJ and was reconstituted in sterile 10mM TRIS-HCl buffer pH 7.4.

4.3.2. Cell Culture and Isolation
Rat and murine hepatic stellate cells were isolated as previously described (2.1) from Sprague-Dawley rats or C57Bl/6 mice and IL-10 deleted mice (B&K, UK).

4.3.3. Proliferation Assays
Rat hepatic stellate cells were seeded into 24 well plates and cultured until 70% confluent. The cultures were washed 3 times in serum free medium and cultured for 24 hours in DMEM containing 0.5% FCS. Cells were then treated for a further 24
hours with media containing 0.5% FCS, 5% FCS or recombinant rat IL-10 1,10 or 100 ng/ml and pulsed with ³H-thymidine for the last 6 hours of the incubation. ³H-thymidine incorporation into DNA was measured as previously described (2.6).

4.3.4. Northern Blotting

Rat HSC were seeded at 1.33 x 10⁶/ml onto uncoated tissue culture plastic and grown for 7 or 14 days in either control media (DMEM 16% FCS) or media containing recombinant rat IL-10 100ng/ml, monoclonal anti mouse IL-10 antibody 2μg/ml (purified from the JES2A5 hybridoma) or normal rat IgG 2μg/ml as a control. Media, IL-10 and antibodies were replaced every 48 hours throughout the culture period.

Wild type and IL-10 deleted murine HSC were isolated and cultured in parallel in standard media with all media changes and cell passages occurring at the same time. At the end of the culture period, HSC were harvested into GIT. Total RNA was extracted and subjected to Northern blotting (2.3). Northern blot membranes were sequentially hybridised with cDNA templates for procollagen-1, α-smooth muscle actin (α-SMA), TIMP-1, MMP-2 (gelatinase-A) and β-actin as a control (2.4). Blots were imaged using autoradiography or a Storm Phosphoimager and then subjected to densitometry. Band density for each experiment was normalised to β-actin. Results were analysed by one way ANOVA using SSPS v8.5 for Windows.

Table 4.1. Size of mRNA transcripts

<table>
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<th>Size (kB)</th>
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<td>Procollagen-1</td>
<td>4.1</td>
</tr>
<tr>
<td>α-smooth muscle actin</td>
<td>1.9</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0.9</td>
</tr>
<tr>
<td>MMP-2 (gelatinase-A)</td>
<td>3.2</td>
</tr>
<tr>
<td>β-actin</td>
<td>2.5</td>
</tr>
</tbody>
</table>
4.3.5. RT-PCR for the IL-10 Receptor
Passaged wild type mouse HSC (passages 2 to 4) were treated for 24 hours with control media or with LPS 1μg/ml. The murine macrophage cell line RAW 264.7, which constitutively expresses the IL-10 receptor, was used as a positive control. Cells were harvested into GIT and RNA isolated as before. 1μg of each RNA sample was reverse transcribed and the resultant cDNA amplified by 35 cycles of PCR in a concentration of 1.5mM magnesium. PCR products were immediately analysed by electrophoresis on a 2% agarose gel or stored at 4°C until use. Primers were designed from published sequences (Weber-Nordt, Meraz, & Schreiber 1994) (Table 4.2)

Table 4.2: Oligonucleotide sequences and conditions for RT-PCR for IL-10 receptor

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
<th>Tm (°C)</th>
<th>Product (bp)</th>
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<td>IL-10 receptor 5'AGGCAGAGGCAGCA GGCCCCAGCAGAATGC T-3'</td>
<td>1426</td>
<td>67</td>
<td>508</td>
<td></td>
</tr>
<tr>
<td>5'TGGAGCCTGGCTAG CTGGTCACAGTAGGTC T3'</td>
<td>1934</td>
<td></td>
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</table>

4.3.6. Western Blotting for the IL-10 Receptor
Cell lysates were made from RAW 264.7 cells, passaged control murine HSC or murine HSC pre-treated with LPS 1μg/ml for 24 hours and 40μg of each protein extract subjected to Western blotting (2.8). The primary antibody used was a monoclonal goat anti-mouse IL-10 receptor antibody 0.2-0.5μg/ml (R&D, Abingdon, UK) and the secondary antibody was a rabbit anti-goat IgG –horseradish peroxidase antibody 1:500-1:1000 (DAKO).

4.3.7. Carbon Tetrachloride Model
Animals
C57Bl/6 mice (B&K, UK) aged 6-8 weeks, weighing between 17 and 20 grams, were maintained on a commercial pelleted diet with free access to water under normal laboratory conditions. All work was performed according to Home Office guidelines.
Experimental Groups

30 female C57Bl/6 mice were treated for 10 weeks with twice weekly intraperitoneal injections of 1ml/kg carbon tetrachloride (CCL4) mixed 1:1 with sterile olive oil as a carrier. 15 of the mice received, in addition, a subcutaneous injection of 25μg (approximately 1mg/kg) human recombinant IL-10 immediately following each administration of CCL4. At the end of the study period, mice were killed by a Home Office approved Schedule 1 method (cervical dislocation) and their livers harvested into neutral buffered formalin or bouin (cold saturated aqueous picric acid, 36% aqueous formaldehyde, 99.8% acetic acid 15:5:1 v:v:v.) for histological assessment.

Assessment of Liver Fibrosis

Following fixation in formalin, livers were embedded in paraffin and sections were stained using standard histochemical techniques as follows: haematoxylin and eosin (H&E); periodic acid Schiff after diastase digestion (diastase resistant PAS) for macrophages; silver stain for reticulin and Sirius red for collagen.

Liver sections were assessed blind with Dr Millward-Sadler, an experienced liver histopathologist, for architectural damage, inflammatory infiltrate, macrophage infiltration and fibrosis and assigned a score according to a histological scoring system previously developed in our department for the assessment of CCl4 induced liver injury in mice (Table 4.3). As the scoring system does not permit fine discrimination between sections for the amount of fibrosis, all the specimens stained for reticulin and Sirius Red were subsequently ranked in order according to the degree of fibrosis. The section with the least fibrosis was assigned the lowest and the section with the most fibrosis was assigned the highest rank position.

Bouin fixed livers were processed into paraffin and stained with Sirius red for collagen and fast green for contrast. Sections were subjected to image analysis by Dr Andreas Knorr, Bayer AG, Wuppertal, Germany. Between 100 and 122 fields of 496 x 361 μm² per specimen were analysed at a final magnification of 100x and the percentage area stained by Sirius red calculated.

4.3.8. Statistical Analysis

Results were analysed with SPSS v8.5 for Windows using oneway ANOVA and the Mann Whitney U test for non-parametric ranking analysis and are expressed as (mean+/-SEM).
Table 4.3. Histology Scoring System

<table>
<thead>
<tr>
<th>HISTOLOGICAL PARAMETER</th>
<th>HISTOLOGICAL APPEARANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Architectural damage (H&amp;E)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Focal hepatocyte loss</td>
</tr>
<tr>
<td>2</td>
<td>Zonal hepatocyte loss-patchy large/continuous thin</td>
</tr>
<tr>
<td>3</td>
<td>Zonal hepatocyte loss-large/confluent</td>
</tr>
<tr>
<td>Inflammatory infiltrate (H&amp;E)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Scanty cells present at junction of necrotic zone</td>
</tr>
<tr>
<td>2</td>
<td>Cells regularly present</td>
</tr>
<tr>
<td>3</td>
<td>Predominantly neutrophils present</td>
</tr>
<tr>
<td>4</td>
<td>Predominantly mononuclear cells present</td>
</tr>
<tr>
<td>Macrophage infiltrate (diastase resistant-PAS)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Scanty macrophages present</td>
</tr>
<tr>
<td>2</td>
<td>Macrophages consistently present</td>
</tr>
<tr>
<td>3</td>
<td>Macrophages consistently present in clusters</td>
</tr>
<tr>
<td>Fibrosis (reticulin/Sirius red)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Slender septa present</td>
</tr>
<tr>
<td>2</td>
<td>Slender septa linking hepatic veins</td>
</tr>
<tr>
<td>3</td>
<td>Broad/ well developed septa</td>
</tr>
<tr>
<td>4</td>
<td>Cirrhosis</td>
</tr>
</tbody>
</table>

4.4. Results

4.4.1. Proliferation Assays

Stimulation with recombinant rat IL-10 (rrIL-10) at 1, 10 and 100ng/ml had neither a negative nor positive effect on primary rat HSC proliferation when primary rat HSC were cultured in media containing either 5% FCS (Fig. 4.1) or 0.5% FCS (Fig. 4.2). Further studies showed no effect of rmIL-10 on passaged mouse HSC proliferation (Fig. 4.3, Fig. 4.4).
Fig. 4.1: Effect of recombinant rat IL-10 on rat HSC proliferation in 5% FCS. Primary rat HSC (day 10) were stimulated for 24 hours with rmIL-10 1, 10 or 100ng/ml in 5% FCS and pulsed with $^3$H-thymidine. Proliferation was assayed by $^3$H-thymidine incorporation and expressed relative to 5% FCS which was assigned an arbitrary value of 1 (mean ±SEM). 0.5% FCS served as a negative control. (n=4)

Fig 4.2: Effect of recombinant rat IL-10 on rat HSC proliferation in 0.5% FCS Primary rat HSC (day 10) were stimulated for 24 hours with rmIL-10 1, 10 or 100ng/ml in 0.5% FCS and pulsed with $^3$H-thymidine. Proliferation was assayed by $^3$H-thymidine incorporation and expressed relative to 0.5% FCS which was assigned an arbitrary value of 1 (mean ± SEM). 5% FCS served as a positive control. (n=4)
Fig 4.3: Effect of recombinant murine IL-10 on mouse HSC proliferation in 5% FCS
Mouse HSC (P2 and 3) were serum depleted then stimulated for 24 hours with rmIL-10 at 1, 10 or 100ng/ml in 5% FCS and pulsed with $^3$H-thymidine. Proliferation was assayed by $^3$H-thymidine incorporation and expressed relative to 5%FCS which was assigned an arbitrary value of 1 (mean ± SEM). Culture in 0.5% FCS served as a negative control. (n=3)

Fig 4.4: Effect of recombinant murine IL-10 on mouse HSC proliferation in 0.5% FCS. Mouse HSC (P2 and 3) were serum depleted then stimulated for 24 hours with rmIL-10 at 1, 10 or 100ng/ml in 0.5% FCS and pulsed with $^3$H-thymidine. Proliferation was assayed by $^3$H-thymidine incorporation and expressed relative to 0.5% FCS which was assigned an arbitrary value of 1 (mean ± SEM). Culture in 5% FCS served as a positive control. (n=3)
4.4.2. Northern Blotting

Effect of recombinant rat IL-10 on steady state mRNA in rat HSC

Preliminary studies of matched pairs of RNA samples from rat HSC isolated from one animal and treated for 14 days with IL-10 100ng/ml showed a significant decrease in steady state levels of procollagen-1 mRNA to 58% of control (p<0.03) and a decrease in TIMP-1 mRNA to 63% of control (p<0.01) (Fig. 4.5). No significant change was seen in steady state mRNA levels of either α-SMA or MMP-2. No change was seen in the steady state levels of any of the mRNAs studied for a treatment period of 7 days (data not shown). Repeat studies of paired IL-10 treated and control RNA samples from four animals showed no significant effect of 100ng/ml recombinant murine IL-10 on the steady state levels of mRNA for procollagen-1, α-SMA or TIMP-1 at either 7 or 14 days of culture (Fig. 4.6).

Effect of anti-murine IL-10 antibody on steady state mRNA in rat HSC

Treatment of rat HSC with anti murine IL-10 antibody at 2μg/ml (JES2A5) to neutralise endogenous IL-10 produced by the stellate cells had no effect on the steady state levels of mRNAs for procollagen-1, α-SMA, TIMP-1 or MMP-2 when compared to mouse HSC treated with normal rat IgG 2μg/ml as control (Fig. 4.7).

Comparison of steady state mRNA in IL-10 deleted and wild type mouse HSC

Analysis of IL-10 deleted (IL-10-/-) and wild type mouse HSC in passages 3 and 4, following isolation and culture in parallel, showed increased steady state levels of procollagen-1 mRNA and α-SMA mRNA in IL-10-/- HSC compared to wild type controls (Fig. 4.8). Procollagen-1 mRNA levels in IL-10-/- HSC were elevated 2.0 times compared to wild type cells (procollagen-1 band density normalised to β-actin $0.8 \pm 0.025$ in IL-10-/- vs. $0.39 \pm 0.025$ in WT, p=0.001, Fig. 4.9) and α-SMA mRNA levels were 1.3 times higher (α-SMA band density normalised to β-actin $0.556 \pm 0.025$ in IL-10-/- vs. $0.427 \pm 0.014$ in WT, p=0.011, Fig. 4.10). No significant difference in the steady state levels of TIMP-1 mRNA was seen (Fig. 4.8, data not shown in graphical form).
Fig. 4.5: Northern blot of 14 day rat HSC treated with rmIL-10 100ng/ml and untreated controls (preliminary study).

Panels show autoradiographs of Northern blots made using RNA extracted from five pairs of HSC culture flasks from a single animal and treated from isolation with rmIL-10 100ng/ml (five lanes on the right) or cultured in DMEM 16%FCS as control (five lanes on the left). The membranes were probed sequentially for β–actin, procollagen-1, α-SMA, TIMP-1 and MMP-2. (n=1 animal)
Fig. 4.6 Northern blot of 7 day and 14 day rat HSC treated with rmIL-10 100 ng/ml treated and untreated controls (repeat study).
Panels show autoradiographs of Northern blots made from paired samples of rat HSC RNA isolated from four animals at 7 and 14 days post isolation. HSC were either cultured in DMEM 16% FCS as control or the media supplemented with rmIL-10 100 ng/ml (see key). The membranes were probed sequentially for β-actin, procollagen-1, α-SMA, and TIMP-1. (n=4 animals)

Key
Samples 1-4 = 7 day rat HSC
Samples 5-8 = 14 day rat HSC
+ denotes IL-10 treated
- denotes control
Fig 4.7: Northern blot of 14 day mouse HSC treated with neutralising anti-IL-10 antibody 2μg/ml and controls.

Panels show autoradiographs of Northern blots made from 14 day mouse HSC RNA isolated from five animals and treated with either anti-IL-10 antibody 2μg/ml (five lanes on right) or normal rat IgG 2μg/ml as control. Membranes were probed for β–actin, procollagen-1, α-SMA, and TIMP-1 and MMP-2. (n=5 animals)
Fig. 4.8: Northern blot of wild type and IL-10-/- passaged murine HSC
HSC were isolated from three pairs of wild type and IL-10-/- animals and cultured and passaged in parallel. Panels below show autoradiographs of Northern blots made from wild type (three lanes on right) or IL-10-/- mouse (three lanes on left) HSC RNA isolated from cells in passages 3 and 4. The membranes were probed sequentially for β-actin, procollagen-1, α-SMA, and TIMP-1. (n=3 animals)
Fig. 4.9: Steady state procollagen-1 mRNA levels in wild type and IL-10-/- murine HSC. Northern blots were made from WT and IL-10-/- mouse HSC RNA (P3 and P4) and probed for procollagen-1. Bars show procollagen-1 band density normalised to β-actin in WT (blue bar) and IL-10-/- (red bar) HSC (mean +/- SEM). (n=3). *p<0.05 compared to WT.

Fig. 4.10: Steady state α-SMA mRNA levels in wild type and IL-10-/- murine HSC. Northern blots were made from WT and IL-10-/- mouse HSC RNA (P3 and P4) and probed for α-SMA. Bars show α-SMA band density normalised to β-actin in WT (blue bar) and IL-10-/- (red bar) HSC (mean +/- SEM). (n=3). *p<0.05 compared to WT.
4.4.3. Expression of the IL-10 receptor by murine HSC

A PCR product of the correct size for the murine IL-10 receptor was detected in positive control RAW 264.7 RNA samples and in LPS pre-treated mouse HSC samples but could not be detected in control mouse HSC samples (Fig. 4.11). All controls in which the RT step was omitted were negative (data not shown). No positive bands were seen in Western blots for any of the samples (data not shown).

Fig. 4.11: RT-PCR for the murine IL-10 receptor in passaged mouse HSC (n=3)
Representative gel showing the presence of the IL-10 PCR product (508bp) in LPS pretreated but not untreated mHSC. A corresponding PCR product is seen in RAW 264.7 cells which are known to constitutively express the IL-10 receptor. (n=3).

4.4.4. Carbon Tetrachloride Model of Liver Fibrosis

During the treatment period, two mice in the CCl₄ group died, one having sustained a mechanical injury to the gut during intra peritoneal injection, and the other with no clear cause identifiable. Three animals in the CCl₄ plus recombinant human IL-10 (rhIL-10) group died, one with fulminant hepatic necrosis and the other two with no identifiable cause at post mortem. All deaths occurred during weeks 4 to 6 of the experimental protocol.

Architectural Damage
H&E stained sections were assessed for hepatocyte loss (Table 4.3). A typical field showing a zone of hepatocyte necrosis is shown in Fig 4.12. There was no significant difference between the scores for architectural damage in the animals treated with CCl₄ alone (1.54 +/- 0.14) and with CCl₄ plus IL-10 (1.5 +/- 0.15). (Fig. 4.13).
Fig 4.12. Photomicrograph of section of murine liver stained with H&E after treatment for 10 weeks with CCl4. An area of prominent hepatocyte loss and inflammatory cell infiltrate is indicated by the arrow (10x objective).
**Fig. 4.13:** Effect of rhIL-10 on architectural damage to CCl₄ injured mouse livers. Bars show the mean score for hepatic architectural damage (Table 4.3) in CCl₄ (blue bar) and CCl₄ plus rhIL-10 (red bar) treated mice.

**Inflammatory Infiltrate**

H&E sections were assessed for inflammatory cell infiltrates (Table 4.3). A typical field showing prominent inflammatory cell infiltrates is shown in Fig 4.12. There was no significant difference between the scores for inflammatory cell infiltration between mice treated with CCl₄ alone (2.07 +/- 0.18) or with CCl₄ plus rhIL-10 (2.54 +/- 0.28). (Fig. 4.14).

**Fig. 4.14:** Effect of rhIL-10 on inflammatory cell infiltration in CCl₄ injured mouse livers. Bars show the mean score for inflammatory cell infiltration (Table 4.3) in CCl₄ (blue bar) and CCl₄ plus rhIL-10 (red bar) treated mice.
Fig 4.15. Photomicrograph of section of murine liver stained with diastase resistant PAS after treatment for 10 weeks with CCl₄. An area of macrophage infiltration (stained red) is indicated by the arrow (10x objective).

Fig 4.16. Photomicrograph of murine liver section stained with diastase resistant PAS after 10 weeks treatment with CCl₄. Macrophages are identified by red staining (40x objective).
Macrophage Infiltration
Liver sections stained with diastase resistant PAS were assessed for macrophage infiltration (*Table 4.3*). Sections showing macrophage infiltration are shown in Figs 4.15 and 4.16. There was no significant difference between the sores for macrophage infiltration between the group treated with CCl₄ alone (2 +/- 0.11) and the group treated with CCl₄ plus rhIL-10 (2 +/- 0). (*Fig. 4.17*).

*Fig. 4.17. Effect of rhIL-10 on macrophage infiltration in CCl₄ injured mouse livers. Bars show the mean score for macrophage infiltration (*Table 4.3*) in CCl₄ (blue bar) and CCl₄ plus rhIL-10 (red bar) treated mice.*

Fibrosis
Reticulin and Sirius red stained sections were assessed for liver fibrosis (*Table 4.3*). Typical microscope fields showing the most and least fibrosis observed are shown in Figs 4.18 to 4.21. There was no significant difference between the mean fibrosis scores of animals treated with CCl₄ alone or with CCl₄ plus IL-10 as assessed on reticulin staining (2.17 +/- 0.17 vs. 2.5 +/- 0.23 respectively) (*Fig. 4.22*) or on Sirius red staining (2.12 +/- 0.18 vs. 2.17 +/- 0.21 respectively) (*Fig. 4.23*). Similarly, there was no significant difference between the mean rank position of animals treated with CCl₄ alone and animals treated with CCl₄ plus IL-10 when the degree of fibrosis was ranked according to reticulin (13.31 +/- 1.83 vs. 9.25 +/- 1.54 respectively) (*Fig. 4.24*) or Sirius red (14.17 +/- 1.80 vs. 10.47 +/- 2.03 respectively) (*Fig. 4.25*).
Fig 4.18. Photomicrograph of murine liver section stained for reticulin after 10 weeks treatment with CCl₄ and showing well developed, broad fibrous septa linking adjacent hepatic veins (10x objective).

Fig 4.19. Photomicrograph of murine liver section stained for reticulin after 10 weeks treatment with CCl₄ and showing only a few slender fibrous septa which do not link adjacent hepatic veins (10x objective).
Fig 4.20. Photomicrograph of murine liver section stained with Sirius red after 10 weeks treatment with CCl₄ and showing well developed fibrous septa linking adjacent hepatic veins (10x objective).

Fig 4.21. Photomicrograph of murine liver section stained with Sirius red after 10 weeks treatment with CCl₄ and showing only a few slender fibrous septa which do not link adjacent hepatic veins (10x objective).
Fig. 4.22. Effect of rhIL-10 on fibrosis score as assessed by reticulin staining
Bars show the mean score for fibrosis (Table 4.3) as assessed by reticulin staining in 
CCI4 (blue bar) and CCI4 plus rhIL-10 (red bar) treated mice.

Fig. 4.23. Effect of rhIL-10 on fibrosis score as assessed by Sirius Red staining
Bars show the mean score for fibrosis (Table 4.3) as assessed by reticulin staining in 
CCI4 (blue bar) and CCI4 plus rhIL-10 (red bar) treated mice.
Fig. 4.24. Effect of rhIL-10 on fibrosis rank position as assessed by reticulin staining. Liver specimens were ranked in order for fibrosis from 1 (least fibrosis) to 25 (most fibrosis). Bars show the mean rank position for CCl₄ (blue bar) and CCl₄ plus rhIL-10 (red bar) treated mice.

Fig. 4.25. Effect of rhIL-10 on fibrosis rank position as assessed by Sirius Red staining. Liver specimens were ranked in order for fibrosis from 1 (least fibrosis) to 25 (most fibrosis). Bars show the mean rank position for CCl₄ (blue bar) and CCl₄ plus rhIL-10 (red bar) treated mice.
When sections were subjected to image analysis, there was no significant difference between the mean percentage area stained with Sirius Red in the group treated with CCl₄ alone (3.59 +/- 0.55) or with CCl₄ and IL-10 (4.24 +/- 0.68). (Fig. 4.26).

Fig. 4.26. Effect of rhIL-10 on percentage area stained by Sirius Red. The percentage area of each liver specimen stained by Sirius Red was measured using image analysis. Bars show the mean percentage area stained by Sirius Red in CCl₄ (blue bar) and CCl₄ plus rhIL-10 (red bar) treated mice.

4.5. Discussion
The hypothesis that IL-10 exerts an anti-fibrotic effect by acting directly on HSC was developed on the basis of the preliminary results of a previous study of CCl₄ induced liver fibrosis from our group, in which IL-10 deleted (IL-10-/-) mice developed significantly more fibrosis than wild type (WT) controls despite having no, or only minimal changes, in levels of hepatic inflammation (Thompson et al. 1998a). The findings of this study were corroborated in an independent study from another group (Louis et al. 1998).

In the studies described in this chapter, experiments using rat recombinant IL-10 to treat rat HSC did not show any consistent effect on α-SMA, MMP or TIMP gene expression by HSC. However, data from other cell types suggest that IL-10 may directly affect matrix synthesis and degradation.
In human monocytes, IL-10 inhibits the production of MMP-1 (interstitial collagenase) and MMP-9 (gelatinase-B) mRNA and protein and increases tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA and protein, consistent with the net 'anti-inflammatory' effect of limiting tissue damage by matrix degradation (Lacraz et al. 1995; Mertz et al. 1994). Similarly, IL-10 treatment of the prostatic carcinoma cell line PC-3 ML or of primary prostatic carcinoma cells increases TIMP-1 with the effect of suppressing tumour invasion (Stearns, Wang, & Steams 1995a; Stearns, Wang, & Steams 1995b).

Studies of fibroblasts have produced more conflicting results. The murine fibroblast cell lines L929 and WA-17 were shown not to constitutively express the IL-10 receptor but could be induced to express it by treatment with LPS (Weber-Nordt, Meraz, & Schreiber 1994). However, LPS induction of receptor expression did not appear to be sufficient to confer biological responsiveness to the cytokine (Weber-Nordt, Meraz, & Schreiber 1994). Reitamo et al showed that in passaged human skin fibroblasts, treatment with recombinant IL-10 down-regulated procollagen-1 gene expression and enhanced MMP-1 (interstitial collagenase) and MMP-3 (stromelysin) gene expression (Reitamo et al. 1994). No effect was seen on TIMP-1 mRNA levels. In an ex vivo model in which biopsies of human thoracotomy scar tissue were cultured, IL-10 reduced collagen as assessed by semi-quantitative RT-PCR and immunohistochemistry (Wangoo et al. 1997). Similarly, IL-10 treatment reduced procollagen-1 mRNA in the human fibroblast cell line WI-38 (Arai et al. 2000). By contrast, IL-10 was found to have no effect on either matrix metalloproteinases or TIMP-1 in normal human dermal and lung fibroblasts from 3 to 6 days after isolation (Lacraz et al. 1995).

Our group and others have previously shown that HSC in vitro produce IL-10, which is detectable in culture supernatants at concentrations in the order of hundreds to thousands of pg/ml for cells at a density of $5 \times 10^5$/ml (Thompson et al. 1998b; Wang et al. 1998). It is possible that IL-10 concentrations in the local extracellular matrix environment of the cell may be higher still and that, consequently, HSC are already maximally stimulated with the cytokine so that the addition of exogenous IL-10 has no further effect.

In a study of passaged rat HSC, co-transfection with a collagen reporter gene plasmid and a murine IL-10 expression vector produced an approximately 40% suppression of collagen promoter activity (Wang et al. 1998). This suggests that in some
experimental systems, supplementation of endogenously produced IL-10 can still exert a biological effect. The disparity between these data and my own may relate to differences between using a murine IL-10 expression vector and direct treatment with recombinant rat cytokine or to differences between passaged and primary HSC behaviour.

To address the issue of the possible effect of endogenous IL-10 production on HSC activation, rat HSC were treated with a monoclonal neutralising anti murine IL-10 antibody (JES2A5). Antibody treatment failed to show any effect on HSC phenotype but we discovered that the antibody used was subsequently found to have only weak neutralising activity against rat IL-10. Data published by Wang et al using a high concentration (50µg/ml) of a polyclonal neutralising antibody directed against both rat and murine IL-10 have demonstrated that IL-10 neutralisation enhanced the stimulatory effect of TGF-β on procollagen-1 mRNA and increased both basal and TGF-β induced collagen protein synthesis (Wang et al. 1998).

Comparison of the phenotype of IL-10-/- and WT HSC in culture demonstrated that procollagen-1 mRNA levels in IL-10-/- cells were elevated two-fold compared to wild type control HSC and α-SMA mRNA 1.3 times higher in IL-10-/- than in wild type HSC (Figs. 4.8, 4.9 & 4.10). These findings are consistent with the increased susceptibility of IL-10-/- mice to developing liver fibrosis after chronic CCl4 injury and with the hypothesis that IL-10 produced by HSC in culture may exert an auto-regulatory effect impeding HSC activation. Studies to examine whether the addition of recombinant cytokine to the IL-10 HSC restores their phenotype to that of wild type cells should further elucidate its role.

Murine HSC were found by RT-PCR to express the IL-10 receptor mRNA after pre-treatment with LPS, reflecting the pattern of inducible receptor expression previously demonstrated in fibroblasts (Weber-Nordt, Meraz, & Schreiber 1994) and providing a potential mechanism for responsiveness to IL-10. The failure to detect IL-10 receptor protein by Western blotting is most likely to reflect the lesser sensitivity of this technique. Published studies of IL-10 receptor expression have all utilised RT-PCR or labelled IL-10 binding to the receptor for detection and have shown low numbers of binding sites (100-300 per cell), even on cell types well established to be biologically responsive to the cytokine (Tan et al. 1993; Weber-Nordt, Meraz, & Schreiber 1994).
In studying the CCl₄ induced model of liver fibrosis in mice, I anticipated that IL-10 administration would have an anti-fibrotic effect by merit of both its anti-inflammatory properties and direct effects on HSC, echoing the results of studies showing more fibrosis in IL-10⁻/⁻ than wild type mice (Louis et al. 1998; Thompson et al. 1998a). However, I found no evidence for an effect of subcutaneous human recombinant IL-10 on liver inflammation or fibrosis in mice after 10 weeks of treatment with twice-weekly carbon tetrachloride.

There are a number of factors that may contribute to the apparent lack of effect.

The requirement for large quantities of recombinant cytokine to conduct a chronic in vivo study of this type meant that the work was only made financially feasible with the support of Schering-Plough and the generous provision of human recombinant IL-10. Although in ideal conditions, murine IL-10 would have been used, human IL-10 has been shown to bind with high affinity to the murine IL-10 receptor (Tan et al. 1993) and to be biologically active on murine cells (Thompson-Snipes et al. 1991). Nevertheless, in the current study, because of the species difference and prolonged study duration, the biological activity of the cytokine may have been significantly reduced by the progressive development of neutralising anti-hIL-10 antibodies so that any anti-inflammatory or anti-fibrotic effect was attenuated. Recent work has demonstrated that mice administered haemagglutinating virus of Japan liposomes containing a human IL-10 expression vector, exhibited significantly reduced lung inflammation at a week and lung fibrosis at 3 weeks following bleomycin induced injury (Arai et al. 2000). The effectiveness of the cytokine in this model of inflammation and fibrosis may reflect the fact that use of an expression vector produces better sustained levels of IL-10, since the half life of IL-10 in serum is thought to be no more than 20 minutes (Gerard et al. 1993) and that the shorter study duration did not permit the development of neutralising antibodies.

In extrapolating from the findings in studies of transgenic mice to propose that administration of exogenous IL-10 will have an anti-fibrotic effect, two issues may be pertinent. First, in wild type mice with chronic liver injury induced by bile duct ligation or by CCl₄ administration, IL-10 mRNA is increased (Thompson et al. 1998a; Wang et al. 1998) and HSC activated in vitro (Thompson et al. 1998b) or in vivo (Wang et al. 1998) express IL-10. On this background of increased intrinsic IL-10 production, the addition of exogenous cytokine may not result in any additional biological effect because IL-10 responsive cells are already maximally...
stimulated. Secondly, a degree of caution must be applied in interpreting the results of studies conducted using transgenic animals because any genetic modification may have important effects during development and there may be unexpected compensatory mechanisms for the deleted gene. Certainly, in the case of the IL-10 deletion, mice treated from birth with anti-IL-10 antibodies do not display the same phenotype as IL-10 deleted animals (Kuhn et al. 1993). Both of these issues might be addressed by a study examining whether administration of recombinant IL-10 to CCl₄ treated IL-10 deleted mice restores the severity of liver fibrosis to that of wild type animals.

The relevance of IL-10 as an anti-fibrotic in human liver disease remains uncertain. A 3-month pilot study of treatment with IL-10 4 or 8µg/kg daily in patients with chronic hepatitis C, who had not responded to treatment with IFN-α, demonstrated a reduction in hepatic inflammation in 19 of 22 patients and a reduction in fibrosis in 14 of 22 patients (Nelson et al. 2000). Reductions in the Ishak score for fibrosis were small during the study period (3.6 +/- 0.4 to 2.6 +/- 0.4) and the study was not controlled because of ethical considerations regarding liver biopsy. Whether the effectiveness of IL-10 is maintained over longer treatment periods and whether the effects of IL-10 are specific to chronic hepatitis C or can be generalised to other causes of chronic liver inflammation and fibrosis remains to be determined by further trials (Schuppan & Hahn 2000).
Chapter 5

Effect of Recombinant IL-13 and IL-4 on Hepatic Stellate Cell Function
5.1. Context
In the previous chapter, I described work performed during my introductory one year Wellcome Trust Entry Level Training Fellowship exploring the actions of the cytokine IL-10 on rat and murine HSC *in vitro* and in a model of murine liver fibrosis.

As a result of this work I was successful in obtaining a further two year welcome Training Fellowship, which enabled me to further investigate the actions of other Th2 derived cytokines on HSC activation (detailed in this chapter) and to develop other models to investigate possible direct T-cell/ HSC interactions *in vivo* and *in vitro* (chapters 6 and 7).

5.2. Introduction
IL-13 and IL-4 are pleiotropic cytokines, which share numerous similarities in terms of their cellular sources and their biological properties, the latter reflecting the shared utilisation of at least one receptor subunit (1.14.). However, there are critical differences between the two cytokines.

The expression of IL-4 is more tightly restricted to Th2 and Th0-like CD4+ clones than IL-13, which is secreted by CD4+ and CD8+ clones belonging to Th0, Th1 and Th2 subsets (de Waal et al. 1995; Minty et al. 1997). Whereas IL-4 production is transient after stimulation, IL-13 production is more pronounced and prolonged (de Waal *et al.* 1995; Minty *et al.* 1997) and IL-13 production is induced by a wider range of stimuli than IL-4 production (de Waal *et al.* 1995; Minty *et al.* 1997; Van der Pouw Kraan TC *et al.* 1996). Unlike IL-4, IL-13 does not exert biological effects on T-lymphocytes which, even after activation, fail to express functional IL-13 receptors (Minty *et al.* 1997; Zurawski et al. 1993). Nevertheless, *in vivo* studies of murine infection with the gastrointestinal nematode *Nippostrongyloides brasiliensis* using IL-4 deleted, IL-13 deleted, IL-4 receptor-α deleted and IL-4/IL-13 deficient mice demonstrate that IL-13 does play an important role in the development of Th2 responses to the parasite (Barner *et al.* 1998; McKenzie *et al.* 1999).

The two cytokines share some common effects on fibroblast-like cells. Both have been shown to increase the expression of the adhesion molecule VCAM-1 on fibroblast-like synoviocytes and lung fibroblasts (Croft *et al.* 1999; Doucet *et al.* 1998) and to inhibit spontaneous and cytokine stimulated production of prostaglandin-E2 by synoviocytes (Alaaeddine *et al.* 1999). Similarly, IL-13 and IL-4...
increase production of IL-6, G-CSF and GM-CSF by lung fibroblasts (Doucet et al. 1998).

With regard to extracellular matrix synthesis and turnover, IL-4 increases collagen and fibronectin production and inhibits MMP-3 (stromelysin) expression by dermal fibroblasts (Gillery et al. 1992; Postlethwaite et al. 1992; Prontera, Crescenzi, & Rotilio 1996). IL-4 has also been shown to stimulate collagen synthesis by synovial fibroblasts from patients with osteoarthritis and rheumatoid arthritis (Postlethwaite, Holness, Katai, & Raghow 1992) and to enhance collagen synthesis and proliferation in subsets of pulmonary fibroblasts (Sempowski et al. 1994; Sempowski, Derdak, & Phipps 1996). In human hepatic stellate cells, IL-4 produced an increase in collagen synthesis comparable to the stimulatory effect of TGF-β (Tiggelman et al. 1995).

I hypothesised that IL-13 may be an important pro-fibrotic mediator in the liver, not just in murine schistosomiasis, but other forms of chronic liver injury and propose that, like IL-4, it has direct effects on HSC which promote their activation.

In this chapter, I will:

- examine the effect of recombinant IL-13 and IL-4 on murine and rat HSC proliferation
- examine the effect of recombinant IL-13 on murine and rat HSC collagen synthesis and compare it with IL-4 and TGF-β
- investigate the effect of recombinant IL-13 on rat HSC MMP production
- determine the expression of IL-13 in normal and injured liver tissue

5.3. Materials and Methods

5.3.1. Materials

Recombinant rat and murine IL-4, murine IL-13 and active TGF-β1 were obtained from R&D Systems, Abingdon, UK.

5.3.2. Proliferation Assays

Mouse HSC in passages 2 to 4 and primary rat HSC 5 to 7 days old on 24 well plates were washed in serum free media and cultured for 24 hours in DMEM 0.5% FCS. Cells were then treated with either recombinant rat or murine IL-4 at 0.5, 2.0 or 5.0 pM or recombinant murine IL-13 (active on both rat and mouse cells) at 0.5, 2.0 or
5.0 pM in DMEM 0.5% FCS for a further 24 hours. $^3$H-thymidine 1μCi per well was added to the cultures for the last 6 hours of the incubation and incorporation into DNA measured as previously described (see Chapter 2.6.). The dose of IL-13 selected for use in these experiments was based on the concentrations used in published data showing biological activity on murine fibroblast cell lines (Chiaramonte et al. 1999) and on preliminary dose ranging experiments.

5.3.3. Collagen Synthesis Assays
Primary rat HSC 5 to 7 days old and mouse HSC in passages 2 to 4 cultured on 12 well plates were washed in serum free media then treated for cultured in serum free DMEM containing 0.01% bovine serum albumin and ascorbic acid 25μg/ml and supplemented with murine or rat recombinant IL-4 (0.5, 2.0 or 5.0pM), recombinant murine IL-13 (0.5, 2.0 or 5.0pM) or active TGF-β1 20ng/ml. After 24 hours the media was renewed and 1μCi $^3$H-proline added to each well. Following a further 24-hour incubation, $^3$H-proline incorporation into collagenase degradable proteins was measured as previously described (see 3.5).

5.3.4. Gelatin Zymography
Rat HSC were cultured for 7 days, washed with serum free medium and stimulated for 24 hours with IL-13 20ng/ml in serum free medium. The resulting cell culture supernatants were then harvested and an aliquot from each sample was concentrated to 10% of its original volume using a spin column (Sigma). Cell culture supernatants from rat HSC treated with concanavalin-A 10mg/ml (a potent activator of MMP-2) were used as a positive control (Theret et al. 1999)). The supernatants were subjected to gelatin zymography (see 2.10) and the resulting gels transilluminated on a light box and photographed using a SLR camera.

5.3.5. Measurement of IL-13 by ELISA in Liver Tissue
Liver homogenates were made from snap frozen liver tissue harvested from normal control mice and at both acute and chronic time points during the course of experimental liver injury with CCl$_4$ (24 hours, 48 hours and 6 weeks) and Con-A (24 hours and 12 weeks). Three animals were used for each time point.
Liver tissue was weighed and homogenised using a rotor stator homogeniser into RPMI containing 100μl per 10ml of protease inhibitor cocktail (Sigma, Poole,
Samples were diluted with homogenisation buffer to a final concentration of 50mg wet weight of liver tissue/ml and stored in aliquots at -80°C until assayed. IL-13 levels in the liver homogenate samples were determined using a Quantikine M IL-13 ELISA kit (R&D Systems, Abingdon, UK) in accordance with the manufacturer’s instructions. The minimum detectable dose of murine IL-13 was stated to be typically less than 1.5pg/ml. All cytokine standards and liver homogenate samples were assayed in triplicate and a series of control liver homogenate samples were “spiked” with known amounts of recombinant IL-13 to determine whether any factors in the liver homogenates interfered with the assay.

5.3.6. RT-PCR for IL-13 in Liver Tissue

Samples of snap frozen rat liver tissue from three control animals and three animals harvested at 24 hours, 48 hours and 8 weeks after starting twice weekly injections with CCl₄ were homogenised into ice cold GIT as previously described (see 2.3.2). RNA was extracted using a Qiaquick Spin Column RNA Extraction Kit (Qiagen, Gatwick, UK) in accordance with the manufacturer’s instructions. Samples were quantified by spectrophotometry and RNA integrity verified by agarose gel electrophoresis (see 2.3.3). RNA was also isolated from fresh rat spleen to provide a tissue positive control for IL-13, which was then used to optimise the Mg²⁺ concentration, annealing temperature (Tm) and cycling conditions for IL-13 RT-PCR. Primers were designed from published sequences (Table 5.1).

1µg of each RNA sample was reverse transcribed and the resultant cDNA amplified by 35 cycles of PCR at a concentration of 1.5mM Mg²⁺ (see 2.5.1, 2.5.2). For each sample, an “RT negative” control, in which the addition of reverse transcriptase was omitted from the reverse transcription reaction, was prepared to control for genomic DNA contamination. In the PCR step, an IL-13 plasmid obtained from Dr KC Thompson, Liver Group, University of Southampton was used as a positive control and water as a negative control.

The resulting PCR products were immediately analysed by electrophoresis on a 2% agarose gel alongside PCR molecular weight markers (Promega, Southampton, UK).
Table 5.1: Oligonucleotide sequences and conditions for RT-PCR for IL-13

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
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<tr>
<td>IL-13 forward</td>
<td>5'- TCGCTTGCCCTGGTGCTTCTT -3'</td>
<td>32</td>
<td>59</td>
<td>219</td>
</tr>
<tr>
<td>IL-13 reverse</td>
<td>5' - GGATGGCATTGCAAC TGGAG -3'</td>
<td>231</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical Analysis

Results were analysed using SPSS v8 for Windows and one-way ANOVA and are shown as mean and standard error of the mean unless otherwise stated.

5.4. Results

5.4.1. Proliferation

Treatment of day 7-10 primary rat HSC with 5, 20 or 50 ng/ml recombinant murine IL-13 (rmIL-13) resulted in a significant increase in HSC proliferation as assessed by $^3$H-thymidine incorporation at all of the doses of the cytokine (p<0.05) (Fig. 5.1). Rat HSC proliferation was increased 3.53 ± 0.27, 4.17 ± 0.40 and 4.00 ± 0.26 times relative to control for IL-13 5, 20 and 50ng/ml respectively.

Similarly, treatment of murine HSC in passages 2 to 4 with 5, 20 or 50 ng/ml recombinant murine IL-13 increased proliferation relative to untreated controls by 3.19 ± 0.24, 4.35 ± 0.47 and 5.35 ± 0.38 times respectively (p< 0.05 for all doses) (Fig. 5.2). The stimulatory effect of IL-13 50ng/ml on proliferation was significantly greater than that of 5ng/ml (p=0.005) but not that of 20ng/ml (p=0.15). There was a trend towards IL-13 20ng/ml producing more proliferation than 5ng/ml (p=0.07).
Fig. 5.1: Effect of rmIL-13 on day 7-10 primary rat HSC proliferation
Red bars show primary rat HSC proliferation as assessed by $^3$H-thymidine incorporation (mean ± SEM) expressed relative to proliferation in DMEM 0.5% FCS (blue bar), which has been assigned an arbitrary value of 1. (n=3) *p<0.05 relative to control.

Fig. 5.2: Effect of rmIL-13 on murine (P2 to 4) HSC proliferation
Red bars show murine HSC proliferation as assessed by $^3$H-thymidine incorporation (mean ± SEM) expressed relative to proliferation in DMEM 0.5% FCS as control (blue bar), which has been assigned an arbitrary value of 1. (n=4) *p<0.05 relative to control.
A comparison of rat HSC proliferation in response to a dose range of recombinant rat IL-4 and murine IL-13 (0.5pM, 2 pM and 5pM) demonstrated that there was no significant difference between the biological activities of IL-13 and IL-4 in promoting rat HSC proliferation relative to DMEM 0.5% FCS control at any of the concentrations of cytokine studied (Fig. 5.3).

**Fig. 5.3: Comparison of the effect of recombinant rat IL-4 and recombinant murine IL-13 on primary rat HSC proliferation.** Bars show rat HSC proliferation in response to stimulation with IL-4 (blue bars) and IL-13 (red bars) as assessed by $^3$H-thymidine incorporation (mean ± SEM). Results are expressed relative to proliferation in DMEM 0.5% FCS, which has been assigned an arbitrary value of 1. (n=3)

5.4.2. **Collagen Synthesis**
Incorporation of $^3$H-proline into collagenase degradable proteins was measured in counts per minute (cpm) to assay HSC collagen synthesis at the protein level. Results are standardised for the DNA content of each sample at the end of the experiment, as a surrogate for cell number (cpm /µg DNA), in order to demonstrate that there is an effect of IL-13 on collagen synthesis independent of the stimulatory effect on cell proliferation already demonstrated. For each experiment, results have been normalised to collagen synthesis in control media (DMEM 0.5% FCS), which has been assigned an arbitrary value of 1.
Collagen synthesis by primary rat HSC (day 5 to 7) was increased by stimulation with IL-13 5, 20 and 50 ng/ml in a dose dependent manner to 1.49 ± 0.14, 1.77 ± 0.09 and 2.34 ± 0.15 times control respectively (Fig. 5.4). The increase in collagen synthesis compared to control was statistically significant for all the concentrations of cytokine studied (p<0.05).

Limited numbers of murine HSC precluded full assessment of collagen synthesis in response to a dose range of recombinant IL-13 but stimulation of passaged mouse HSC (P2 to P4) with rmIL-13 20ng/ml increased collagen synthesis 2.32 ± 0.42 times control (p<0.05) (Fig 5.5), suggesting a similar biological effect to that exerted on rat HSC.

Comparison treatment of rat HSC with rat IL-4 and murine IL-13 showed a similar magnitude of increase in collagen synthesis for both cytokines at all the concentrations studied (Fig. 5.6).

Fig. 5.4: Effect of recombinant IL-13 on collagen synthesis by primary rat HSC. Collagen synthesis by rat HSC was measured by $^3$H-proline incorporation into collagenase degradable proteins and expressed as cpm per µg DNA to standardise for cell number. Red bars show rat HSC collagen synthesis after stimulation with IL-13 normalised to DMEM 0.5% FCS control (blue bar), which has been assigned an arbitrary value of 1. (n=3) *p<0.05 relative to control
Fig. 5.5: Effect of recombinant IL-13 on collagen synthesis by passaged murine HSC. Murine HSC collagen synthesis was measured by $^3$H-proline incorporation into collagenase degradable proteins (cpm/µg DNA). IL-13 (20ng/ml) stimulated collagen synthesis (red bar) is shown normalised to DMEM 0.5% FCS control (blue bar), which has been assigned an arbitrary value of 1. (n=3) *$p<0.05$ relative to control.

Fig. 5.6: Comparison of the effect of IL-4 and IL-13 on rat HSC collagen synthesis. Rat HSC collagen synthesis in response to stimulation with IL-4 (blue bars) and IL-13 (red bars) was measured by $^3$H-proline incorporation into collagenase degradable proteins (cpm/µg DNA). Results are normalised to collagen synthesis in DMEM 0.5% FCS as control, which has been assigned an arbitrary value of 1.
5.4.3. Zymography for Matrix Metalloproteinases

On gelatin zymography, weak bands corresponding to MMP-9 (gelatinase-B) were just visible in the 10x concentrated 7-day rat HSC culture supernatants only and were not detectable in culture supernatants without concentration. By contrast, a strong MMP-9 (gelatinase-B) band could be clearly seen in the culture supernatant of Con-A activated rat HSC.

Bands corresponding to the pro- and active forms of MMP-2 (gelatinase-A) were clearly seen in all rat HSC culture supernatant samples.

Pre-treatment of the HSC with IL-13 20ng/ml made no discernible difference to the pattern of expression of either MMP-2 or MMP-9 (Fig. 5.7).

![Image of gelatin zymogram showing the effect of IL-13 pre-treatment on MMP-2 and MMP-9 expression by rat HSC.](image)

1 = rat HSC treated with concanavalin-A 10mg/ml  
2 = control rat HSC  
3 = rat HSC treated with IL-13 20 ng/ml  
4 = control rat HSC (x 10)  
5 = rat HSC treated with IL-13 (x 10)

5.4.5. Expression of IL-13 in Normal and Injured Liver

ELISA for IL-13

IL-13 levels were below the limits of detection in liver homogenates from normal mice and from mice with liver injury induced by CCl₄ and by Con-A injection at both acute and chronic time points. In liver homogenates “spiked” with known
quantities of recombinant murine IL-13, the cytokine was detected at levels between 20 and 25 times less than those anticipated (data not shown).

**RT-PCR for IL-13 mRNA**

No IL-13 PCR product was detected by RT-PCR in any of the three normal untreated rat liver specimens studied. However, a PCR product of size 219bp conforming to that of the PCR product from the IL-13 plasmid (positive control) was present in all three acutely injured rat liver specimens at 24 and 48 hours after CCl₄ injection and two out of three rat liver specimens at 8 weeks post CCl₄ injection indicating that IL-13 mRNA transcripts were detectable in injured but not in normal rat liver tissues (Fig. 5.8). Parallel “RT negative” controls for each liver tissue sample (in which reverse transcriptase was omitted from the RT step) were all negative for IL-13 PCR products (data not shown).

*Fig. 5.8: RT-PCR for IL-13 mRNA in normal control and injured rat liver 24 hours, 48 hours and 8 weeks after commencing CCl₄ injection.*

5.5. **Discussion**

Increases in both proliferation and collagen synthesis are hallmarks of hepatic stellate cell activation *in vitro* by culture on uncoated plastic and *in vivo* in experimental models of liver fibrosis and human liver disease. These experiments have demonstrated that murine recombinant IL-13 significantly enhances both murine and rat hepatic stellate cell proliferation and collagen protein synthesis. The effects of IL-13 on both HSC proliferation and collagen synthesis were comparable to those of IL-
4, suggesting the shared utilisation of receptor components and signalling pathways in HSC that has been demonstrated for the two cytokines in other cell types (Murata et al. 1998).

These data are the first to suggest that IL-13 has a possible role in promoting liver fibrosis by directly activating hepatic stellate cells. They also demonstrate for the first time that IL-4 has a stimulatory effect on HSC proliferation in vitro, in addition to promoting HSC collagen production as previously described (Tiggelman, Boers, Linthorst, Sala, & Chamuleau 1995).

Several recently published studies suggest that IL-13 has pro-fibrogenic properties in vivo in the lung. IL-13 levels were found to be increased in the serum of patients with the fibrosing disease systemic sclerosis (Hasegawa et al. 1997) and IL-13 was elevated in the bronchoalveolar lavage fluid and alveolar macrophages of patients with pulmonary fibrosis when compared to healthy volunteers (Hancock et al. 1998). Transgenic mice with selective chronic over-expression of murine IL-13 in the lungs developed subepithelial airway fibrosis (Zhu et al. 1999). By contrast, the development of pulmonary fibrosis was not a significant feature in studies of transgenic mice with similar lung tissue specific expression of IL-4 (Jain-Vora et al. 1997; Rankin et al. 1996).

With reference to in vivo models of liver fibrosis, studies of murine schistosomiasis utilising antibody neutralisation of IL-4, IL-4 antagonists or transgenic IL-4 deficient mice have failed to show an indispensable role for this cytokine in the development of hepatic granulomas and fibrosis (Cheever et al. 1994; Jankovic et al. 1999). However, three recent studies have emphasised the importance of IL-13 in the pathogenesis of liver fibrosis in this model. The first of these compared hepatic granuloma formation and liver fibrosis in wild type mice, IL-4 knockout (IL-4KO) mice and mice which were IL-4 receptor-α deficient (IL-4RαKO) and therefore unresponsive to both IL-4 and IL-13 (Jankovic et al. 1999). IL-4RαKO mice showed a dramatic reduction in hepatic fibrosis, as assessed by hydroxyproline content, compared to IL-4KO mice despite retaining a similar cytokine profile (Jankovic et al. 1999). A second study of wild type, IL-4 deficient, IL-13 deficient and “double deficient” IL-4/IL-13KO mice demonstrated negligible hepatic fibrosis in both the IL-13KO and IL-4/IL-13KO mice when compared with either wild type or IL-4KO
mice but showed that abrogation of granuloma formation required the ablation of both IL-4 and IL-13 (Fallon et al. 2000). Finally, administration of a soluble IL-13 antagonist (IL-13Ra2-Fc fusion protein), derived from the high affinity IL-13 receptor subunit IL-13Ra2, caused a marked reduction in hepatic fibrosis in both wild type and IL-4KO mice (Chiaramonte, Donaldson, Cheever, & Wynn 1999). In accord with the previous study, both IL-13 inhibition and IL-4 deletion were needed to significantly impair the granuloma formation (Chiaramonte, Donaldson, Cheever, & Wynn 1999).

There is some evidence to suggest that the pro-fibrotic effects of IL-13 seen in experimental animal models in vivo may be mediated via direct effects on collagen synthesis by fibroblasts, although there are no existing published data regarding HSC. In one in vitro study, treatment of normal human skin fibroblasts with IL-13 resulted in increased collagen production, enhanced TIMP-1 production and inhibited IL-1β stimulated MMP-1 and MMP-3 production (Oriente et al. 2000). By contrast, in human dermal fibroblasts isolated from keloid scars, IL-13 increased collagen and inhibited MMP-3 but had no effect on either MMP-1 or TIMP-1 (Oriente, Fedarko, Pacocha, Huang, Lichtenstein, & Essayan 2000). After treatment with IL-13, cell lysates of the murine fibroblast cell line, 3T3, were reported to contain increased collagen I protein as assessed by Western blotting (Chiaramonte, Donaldson, Cheever, & Wynn 1999) and IL-13 has been shown to inhibit MMP-3 in human synovial membrane (Jovanovic et al. 1998). My findings with respect to IL-13 induction of collagen production in HSC are consistent with those published in studies of human dermal fibroblasts (Oriente et al. 2000) and the murine fibroblast cell line 3T3 (Chiaramonte, Donaldson, Cheever, & Wynn 1999). No published data that document the effects of IL-13 on fibroblast proliferation in vitro are available, but IL-4 has been shown to increase proliferation in subsets of pulmonary fibroblasts (Sempowski, Beckmann, Derdak, & Phipps 1994).

In this study, IL-13 protein was not detectable by ELISA in either normal murine liver tissue or in CCl₄ and Con-A injured mouse liver. This may reflect insufficient sensitivity to detect low levels of IL-13 in both normal and injured rat liver but technical considerations are likely to be a more significant factor. The Quantikine M IL-13 ELISA kit employed for these studies was primarily designed for use on samples of tissue or cell culture media. During assay development, it was found that
mouse serum contains an unidentified stable high affinity binding factor for IL-13 that interferes with the assay (personal communication, R&D Systems Technical Support, Abingdon, UK). The possible impact of this mouse serum IL-13 binding factor on use of the ELISA to measure IL-13 in mouse liver homogenates is illustrated by the marked reduction in sensitivity noted for detecting known amounts of recombinant cytokine in IL-13 "spiked" liver homogenates.

To my knowledge, there is only one published study in which hepatic IL-13 has been successfully measured by ELISA. This study examined a murine model of septic peritonitis induced by caecal ligation and puncture, in which hepatic IL-13 levels increased after the induction of peritonitis reaching a maximum of 25ng/100mg wet weight of liver tissue at 8 hours and persisting for at least 48 hours (Matsukawa et al. 2000). Subsequent immunohistochemistry localised the site of hepatic IL-13 production in this model to Kupffer cells (Matsukawa et al 2000). There are some further data demonstrating expression of IL-13 in experimental liver injury and fibrosis using the more sensitive technique of RT-PCR to detect cytokine mRNA transcripts but these studies are restricted only to the model of murine schistosomiasis (Cetre et al. 2000; Cheever et al 1994).

In this chapter, I have shown that IL-13 mRNA transcripts are detectable in CCl₄ injured liver at both acute and chronic time points but not in normal control liver specimens. Previous studies by the Southampton Liver Group have demonstrated that IL-13 mRNA can be detected in isolated rat Kupffer cells in vitro after 48 hours of culture (Dr K.C. Thompson, unpublished data).

*In vivo*, IL-13 has the potential to either reduce liver fibrosis via its anti-inflammatory effects on Kupffer cells (Matsukawa et al 2000; Thompson et al. 1998), or to promote fibrosis via the direct effects on hepatic stellate cell described in this chapter. While it seems clear that IL-13 has an important role as a pro-fibrotic stimulus in liver fibrosis developing secondary to murine schistosomiasis (Chiaramonte, Donaldson, Cheever, & Wynn 1999; Fallon, Richardson, McKenzie, & McKenzie 2000; Jankovic et al 1999), the relevance of this cytokine to other models of liver fibrosis and in human disease remains to be established.

Furthermore, IL-4 and IL-13 appear to play overlapping, but not identical roles, in the pathogenesis of liver fibrosis in murine schistosomiasis but the degree to which IL-4 and IL-13 play redundant roles in the development of liver fibrosis in other
models or diseases is uncertain. These issues may be resolved by studies of other experimental models of liver injury to compare the development of liver fibrosis in transgenic mice deficient in IL-13, IL-4 or both cytokines with that in wild type controls or by alternative strategies such as the administration of soluble IL-13 inhibitors.

In summary, the Th2 derived cytokines IL-4 and IL-13 have profound effects on hepatic stellate cell activation in vitro, acting as pro-fibrogenic factors. These data provide strong circumstantial evidence supporting the hypothesis that the balance between Th1 and Th2 cytokines in a T-cell mediated immune response in the liver could influence the degree of resulting fibrosis.

In order to study the effect of Th1/Th2 balance on liver fibrosis in more detail, it became clear that existing animal models of liver injury and of in vitro HSC activation were inadequate. The remaining chapters in this thesis detail my attempts to develop new models which would provide a more suitable framework to help elucidate these processes.
Chapter 6

Acute and Chronic Concanavalin-A induced Liver Injury: Role of T-lymphocytes in an Animal Model of Hepatitis and Liver Fibrosis
6.1. Context

In liver diseases such as chronic viral hepatitis and autoimmune chronic active hepatitis that culminate in liver fibrosis, T-lymphocyte activation and cytokine production have been implicated in the pathogenesis of organ damage (Bjorkland et al. 1991; Frazer et al. 1985; Imada et al. 1997; Whiteside et al. 1985). Among animal models of liver disease, murine schistosomiasis induces a granulomatous hepatitis and subsequent fibrosis predominantly dependent on CD4+ T-lymphocytes but does not provide a good model for human liver disease (Fallon 2000). The need to better understand the cellular and molecular mechanisms of T-lymphocyte associated liver injury has driven the development of more suitable animal models of T-lymphocyte mediated liver disease such as the liver injury induced by intravenous concanavalin-A (Con-A) injection in mice. Studies of the acute hepatitis induced by a single Con-A injection have produced strong evidence that the liver injury is CD4+ T-lymphocyte mediated. More recently, preliminary studies of repeated Con-A injection demonstrated the development of liver fibrosis thought to be mediated by CD4+ T-lymphocyte activation. We planned to utilise this chronic Con-A model to compare the development of liver fibrosis in cytokine deficient and wild type mice in order to further elucidate the role of Th1/Th2 cytokines in the pathogenesis of liver fibrosis in vivo. However, prior to embarking on these studies, and at the suggestion of the Wellcome Trust panel, we opted to validate that both the acute and chronic models of liver injury were CD4 dependent using CD4 knockout mice.

6.2. Introduction

6.2.1. Concanavalin-A Induced Models of Liver Disease

Concanavalin-A

Lectins are proteins that bind to sugar residues on the surface of a wide variety of different cell types. Their biological properties are varied and include agglutination of bacterial, plant or mammalian cells and the stimulation of proliferation and cytokine production by T-lymphocytes. Concanavalin-A (Con-A) is a plant lectin isolated from the jack bean (Canavalia ensiformis), which binds to mannosyl residues. Con-A causes erythrocyte agglutination and is known to be mitogenic for
T-lymphocytes in vitro and to stimulate their production of cytokines (Lis & Sharon 1986).

Concanavalin-A Induced Hepatitis

Following a single intravenous dose of Con-A greater than 1.5mg/kg, mice develop severe liver injury as assessed by elevated serum transaminases 8 hours post injection, with the degree of liver damage dose dependent up to 30mg/kg. Electron microscopy showed leucocytes adherent to the hepatic sinusoidal endothelium with hepatocyte cell membrane rupture, bleb formation and loss of cytoplasm. The hepatitis is characterised by periportal and centrilobular infiltrates of predominantly CD4+ T-lymphocytes (Mizuhara et al. 1994; Mizuhara et al. 1996). Histopathological assessment revealed no evidence of any other organ damage (Tiegs, Hentschel, & Wendel 1992).

Intravenous injection of Con-A in mice results in the systemic release of a number of cytokines including IL-1, IL-2, IL-4, TNF-α, IFN-γ and GMCSF, preceding the onset of liver injury as indicated by an elevated transaminase levels (Gantner et al. 1995; Louis et al. 2000; Mizuhara, et al 1994; Mizuhara et al 1996; Tiegs, Hentschel, & Wendel 1992). Of these, TNF-α, IFN-γ and possibly IL-4 appear to play an important role in mediating liver injury.

Neutralisation of TNF-α by treatment with anti-TNF-α antibody before administration of Con-A was shown to prevent the development of liver injury (Gantner, Leist, Lohse, Germann, & Tiegs 1995; Mizuhara, et al 1994) and TNF-α knockout mice were almost completely protected from liver injury (Kusters et al. 1997). Similarly, pre-treatment with neutralising anti-IFN-γ antibodies or an IFN-γ receptor fusion protein protected against hepatitis (Kusters et al. 1996; Mizuhara et al 1996; Nicoletti et al. 2000b) and IFN-γ knockout mice were resistant to Con-A induced liver injury (Tagawa, Sekikawa, & Iwakura 1997). Administration of anti-IL-4 antibody was observed to reduce both ALT levels and histological features of hepatitis caused by Con-A injection and IL-4 knockout mice were resistant to the development of hepatitis (Nishikage et al. 1999; Toyabe et al. 1997). The protective effects reported for IL-10, IL-11 and IL-12 against Con-A induced liver damage are mediated via inhibition of TNF-α, IFN-γ and IL-4 (Bozza et al. 1999; Di Marco et al. 1999; Nicoletti et al. 2000a).
The involvement of T-lymphocytes in this model was underlined by the observation that administration of the immunosuppressants cyclosporin-A and FK506/ tacrolimus (which have been shown to inhibit T-cell dependent production of cytokines) significantly reduced transaminase levels after Con-A injection (Tiegs, Hentschel, & Wendel 1992). Furthermore, both severe combined immunodeficiency (SCID) mice, which lack immunocompetent T and B cells, and athymic mice, which have functionally defective T cell populations, are resistant to Con-A induced hepatitis (Tiegs, Hentschel, & Wendel 1992). Pre-treatment of mice with anti-Thy1.2 antibody, which destroys Thy1.2 antigen bearing T cells in vivo also abrogated liver injury. More specifically, administration of a monoclonal antibody directed against the CD4 glycoprotein protected against Con-A hepatitis where as anti-CD8 antibody had no effect (Tiegs, Hentschel, & Wendel 1992).

**Concanavalin-A Induced Liver Fibrosis**

More recently, a study published in abstract form (Louis et al. 1997) demonstrated that weekly intravenous injections of 20mg/kg Con-A in BALB/c mice resulted in the development of perisinusoidal liver fibrosis, which was detectable from 5 weeks and increased over the 20-week study period. The first and second injections of Con-A were associated with a high mortality (21% and 59% respectively) but there was no further mortality throughout the remainder of the study. This provided a potential model for T-lymphocyte mediated chronic liver disease and fibrosis.

**6.2.2. CD4+ Knockout Mice**

The CD4 molecule is a surface glycoprotein, which is expressed on T-helper cells and is involved in T cell receptor (TCR) recognition of class II major histocompatibility complex (MHC). Studies of the role of CD4+ T cells in vivo have been based on adoptive transfer of selected or depleted lymphocytes or in vivo depletion of CD4+ cells using antibody but both these approaches have limitations which are particularly pertinent to the study of chronic disease processes, such as liver fibrosis. In order to facilitate the study of CD4+ T cells in immune responses and in disease, mutant mouse strains that do not express CD4 on the cell surface (CD4 knockout mice, CD4 KO) have been generated (Kiileen & Littman 1995; Nishimura & Ohta 1999).
CD4 was inactivated in embryonic stem cells by transfection with a replacement vector in which exon V of the CD4 gene was disrupted with a neomycin resistance cassette (Fig 6.1).

Embryonic stem cells in which homologous recombination had occurred were microinjected into blastocysts, which were subsequently implanted into pseudopregnant mice. The resulting chimeric mice were backcrossed with the parental strain (C57Bl/6) to generate heterozygotes for the CD4 deletion (CD4+/−) and the heterozygotes interbred to obtain mice homozygous for the disrupted gene (CD4 KO) (Nishimura & Ohta 1999). CD4 knockout mice were healthy, fertile and indistinguishable from heterozygous or wild type littermates on gross physical inspection. Numbers of T and B cells and myeloid differentiation was normal and, as anticipated, CD4 was not detectable on thymocytes or lymph node cells. CD8+ cells were present in normal numbers in the thymus but increased in the periphery. There were also increased numbers of CD4-8- (double negative, DN) cells in the lymph nodes (Nishimura & Ohta 1999).

CD4 KO mice showed a greatly reduced class II MHC restricted IL-2 response to alloantigens and impaired antibody responses against sheep erythrocytes but had preserved (CD8 dependent) cytotoxic activity against both lymphocytic chorimeningitis virus and vaccinia virus (Nishimura & Ohta 1999). Overall the phenotype was consistent with a selective deficit in CD4+ T cell function.
6.2.3. Hypothesis and Aims

The work contained in this chapter was originally conceived as a “proof of concept” experiment prior to examining the effect of manipulating Th1/Th2 balance on Con-A induced liver fibrosis. Originally, studies were to be carried out to compare the development of liver fibrosis in IL-4 deleted, IL-10 deleted and wild type mice treated with repeated Con-A injections. Before embarking on these studies, I sought to verify that liver fibrosis provoked by repeated Con-A injections was dependent on CD4+ T-lymphocyte mediated mechanisms, as is the case for acute hepatitis induced after a single injection of Con-A. I hypothesised that, when treated with repeated Con-A injections, CD4KO mice would be protected from liver injury where as wild type mice would develop liver fibrosis. These initial experiments showed, contrary to expectation, that after twelve injections of Con-A CD4KO mice did develop liver fibrosis. To further elucidate the mechanisms of liver injury in these mice, cohorts of CD4KO and wild type mice were administered a single injection of Con-A to induce an acute hepatitis and liver damage assessed. The T-cell infiltrates and pattern of cytokine expression were characterised in both acute and chronic liver injury.
In this chapter I:

- compare the development of liver injury in wild type (C57/B16) and CD4KO mice 24 hours after a single injection of Con-A and after injection of Con-A for 12 weeks
- examine the phenotype of T-lymphocytes infiltrating the liver after acute and chronic Con-A treatment
- measure the cytokines TNF-α, IFN-γ and IL-10 in the livers of mice treated with Con-A

6.3. Materials and Methods

**Animals**

C57Bl/6 mice aged 6-8 weeks were obtained from B&K, UK and homozygous CD4 deficient mice aged 6 weeks were obtained from Amin Rahemtulla, Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford UK. Mice were maintained on a commercial pelleted diet with free access to water under normal laboratory conditions. All work was performed in accordance with Home Office guidelines.

**Materials**

Concanavalin-A was obtained from Sigma, Poole, Dorset and dissolved in sterile phosphate buffered saline (PBS) pH 7.4 at concentrations of 0.5 mg/ml, 1mg/ml and 2mg/ml. Typical injection volumes for mice weighing between 20 and 25g varied between 200 and 250 microlitres.

6.3.1. Experimental Groups

Several potential problems were identified in applying the Con-A dosing protocol used by Louis et al (Louis, Le Moine, Quertinmont, Peny, Goldman, & Deviere 1997) to induce liver fibrosis in my study.

First, the existing protocol utilised BALB/c mice where as C57Bl/6 were required in this study to provide appropriate wild type controls for the CD4 KO mice, which were generated on a C57Bl/6 background (Nishimura & Ohta 1999). Different inbred mouse strains are known to have different susceptibilities to liver injury. In
particular, C57Bl/6 mice are susceptible to Con-A induced hepatitis at lower Con-A
doses than BALB/c mice and develop more severe acute liver injury than BALB/c
counterparts given the same dose of Con-A (Mizuhara et al. 1998). In addition, when
treated with repeated doses of carbon tetrachloride (CCl₄), C57Bl/6 mice develop
less liver fibrosis than BALB/c mice (Shi, Wakil, & Rockey 1997).

Secondly, the first two injections of Con-A were associated with a high mortality
(21% and 59% respectively). These levels of mortality were felt to be unacceptable
on ethical grounds and also raised concerns as to the potentially small number of
animals surviving to complete the chronic dosing protocol over a period of 20 weeks
and as to whether these survivors constituted a selected and non-representative sub-
population.

Finally, the technique used to administer Con-A (injection of the retro-orbital venous
plexus under ether anaesthesia) is not permitted in the UK under Home Office
regulations, necessitating the use of tail vein injections.

In order to address these issues, a pilot dose ranging study was performed in order to
refine the dosing protocol to minimise mortality and, if possible, shorten the study
duration.

Dose Ranging Study
C57Bl/6 mice were divided into three groups (4 mice per group) and injected once a
week with Con-A in sterile PBS pH 7.4. The groups received 5, 10 or 20mg/kg Con-
A respectively for a period of 10 weeks. In the group receiving Con-A 20mg/kg there
was one death after the first injection and a further two deaths after the second
injection (comparable with the previously published data). Treatment of the 20mg/kg
group was therefore abandoned.

After ten weeks, there had been no deaths in the 5mg/kg or 10mg/kg dosage groups.
Two mice from each of the groups were sacrificed and their livers subjected to
histological assessment. All the mice were found to have mild pericellular fibrosis in
a patchy distribution with no clear correlation between the degree of fibrosis and the
Con-A dose (data not shown).

The remaining mice in each group continued to have weekly injections of Con-A
with the dosage increasing by increments of 10mg/kg every two weeks until all mice
were receiving 30mg/kg Con-A. Dosing was continued for a further 5 weeks during
which time there were no further deaths. Mice were then sacrificed and their livers
harvested for histological assessment. All mice at this time point exhibited evidence of established liver fibrosis (data not shown).

Based on the findings of the dose ranging study, it was decided to adopt a protocol in which the dose of Con-A was increased in increments at the beginning of the study in order to both minimise mortality during the first two weeks of dosing and to reach a dose of 30mg/kg Con-A in order to achieve liver fibrosis within a shorter study period of 12 rather than 20 weeks.

**Study Dosing Protocol**

**Acute Model**

6 male C57Bl/6 mice and 6 male CD4KO mice were given a single tail vein injection of 20mg/kg Con-A and sacrificed after 24 hours. 6 untreated C57Bl/6 and CD4 KO mice were used as time zero controls.

**Chronic Model**

15 female C57Bl/6 mice and 15 female CD4 KO mice were injected weekly with Con-A via the tail vein for a total of 12 weeks. For the first two weeks, all mice received 10mg/kg Con-A, for the following two weeks 20mg/kg Con-A and thereafter 30mg/kg each week until the end of the study period.

At the end of each study, mice were killed by cervical dislocation and their livers harvested into neutral buffered formalin or bouin for histological assessment or snap frozen in liquid nitrogen. Blood was taken by cardiac puncture and serum stored at -80°C for subsequent analysis.

**6.3.2. Histological Assessment**

**Immunohistochemistry for T-cell Surface Markers**

Immunohistochemistry for T-cell markers was performed by Corinna Spencer, a medical student working on her 4th year project in the laboratory

**Materials**

Rat anti-mouse CD3 antibodies (clone KT3) and rat anti-mouse CD8 antibodies (clone KT15) were obtained from AMS Biotechnology, Abingdon, UK. Rat anti-mouse CD4 and biotinylated donkey anti-rat IgG antibody were obtained from Stratech Scientific, Luton, UK. Streptavidin–biotin horse radish peroxidase (HRP)
complex was obtained from Dako, Denmark, aminoethylcarbazole (AEC) from Biogenex, CA, USA, avidin-biotin blocking kit from Vector Laboratories, CA, USA and Crystalmount from Biomeda, CA, USA.

**Preparation of Sections**

Frozen liver sections were processed into glycol methacrylate (GMA) using a method previously described (Polysciences Inc., PA USA) (Britten, Howarth, & Roche 1993) and cut using an ultramicrotome into 2μm slices. Sections were floated onto poly-L-lysine coated slides.

**Immunohistochemistry**

Sections were stained using a previously described technique to provide maximum signal amplification and sensitivity (Catoretti, Berti, & Schiro 1988). Endogenous peroxidase activity was blocked with sodium azide and 30% hydrogen peroxide solution for 30 minutes and sections were rinsed three times with TBS. Slides were then blocked with avidin solution for 20 minutes, washed with TBS, blocked with biotin solution for 20 minutes and washed again in TBS. Sections were then blocked with 5% FCS, 1% BSA for 20 minutes. Primary antibody was applied to each section at the appropriate dilution (*Table 6.1*) and incubated overnight at room temperature. Sections were then washed in TBS and incubated in biotinylated donkey anti-rat IgG 1:3000 for 90 minutes. After further washes streptavidin-biotin HRP complex 1:200 was applied for 90 minutes and slides were rinsed again. AEC solution was made up in accordance with the manufacturer’s instructions and applied for 20 minutes. Slides were rinsed and counterstained with Mayer’s haematoxylin for 90 seconds prior to bluing in running tap water for 5 minutes and then mounted using Crystalmount.

**Counting T-lymphocytes**

Cells staining positive for CD3, CD4 and CD8 were counted in 20 high power fields per specimen and results expressed as the mean ± SEM for each experimental group.

*Table 6.1 Antibody Dilution for Immunohistochemistry for T-cell markers*

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat anti-mouse CD3</td>
<td>1: 125</td>
</tr>
<tr>
<td>Rat anti-mouse CD4</td>
<td>1: 50</td>
</tr>
<tr>
<td>Rat anti-mouse CD8</td>
<td>1: 250</td>
</tr>
</tbody>
</table>
Assessment of Fibrosis
Liver sections were fixed and stained for reticulin and Sirius red as previously described and scored and ranked blind by a liver histopathologist using a scoring system devised in our department for murine livers (Table 4.3).

6.3.3. Analysis of Cytokine Expression
Preparation of Liver Homogenates
Snap frozen liver tissue was weighed and homogenised using a rotor stator homogeniser into RPMI containing 100μl per 10ml protease inhibitor cocktail (Sigma, Poole, Dorset). Samples were diluted with homogenisation buffer to a final concentration of 50mg/ml and stored in aliquots at -80°C until assayed.

Cytokine Measurement by ELISA
Cytokine levels in mouse sera and liver homogenates were determined by ELISA. All cytokine standards were assayed in triplicate, as were samples where sample volumes permitted. IL-10 and TNF-α were measured using a Quantikine M ELISA kit (R&D Systems, Abingdon, UK) in accordance with the manufacturer’s instructions. The minimum detectable doses were stated to be typically less than 5.1 pg/ml and 4.0 pg/ml for TNF-α and IL-10 respectively. IFN-γ levels were determined using a Murine IFN-γ ELISA Development Kit (Peprotech EC, London) in accordance with the manufacturer’s suggestions with the following exceptions. 5% BSA rather than 1% BSA was used as the blocking buffer and as the diluent for the streptavidin-peroxidase conjugate, TMB was substituted for ABTS as the chromogen and the number of washes between stages increased to 6 times to increase assay sensitivity.
6.4. Results

6.4.1. Assessment of Con-A Induced Liver Injury in CD4 Knockout and Wild Type Mice

Acute Model
In both the WT and CD4 KO groups injected with Con-A there was one animal death prior to 24 hours.

Liver Inflammation and Necrosis
When H&E stained liver sections from WT and CD4KO mice at time zero (untreated controls) and 24 hours after a single Con-A injection were assessed for inflammation and fibrosis, four distinct phenotypic groups were observed and all sections were assigned blind to a phenotypic group (Fig. 6.2):

Phenotype 1 = normal
Phenotype 2 = scattered necroinflammatory foci
Phenotype 3 = fatty change, eosinophilia, early cell death and inflammatory infiltrate
Phenotype 4 = areas of total coagulative necrosis with inflammatory cell infiltrates and loss of hepatocyte nuclei and cell structure

All time zero control animals were found to be phenotype 1 or 2 with no significant difference in assignment of phenotypic group between wild type and CD4+KO mice. At 24 hours post Con-A injection, all CD4+KO animals exhibited phenotype 3 and all WT animals showed extensive coagulative necrosis and inflammatory infiltrates corresponding to phenotype 4. (Fig 6.3.)
Fig. 6.2. Photomicrographs of phenotype of WT and CD4KO murine livers 24 hours after a single injection of Con-A 20mg/kg
(a) phenotype 1-normal
(b) phenotype 2-scattered infrequent necroinflammatory foci
(c) phenotype 3-fatty change, eosinophilia, early cell death and inflammatory infiltrate
(d) phenotype 4-areas of total coagulative necrosis with inflammatory infiltrates and loss of hepatocyte nuclei and cell structure
Chronic Model

During the study period for the chronic model, two wild type and six CD4KO mice died. The deaths in the wild type group occurred at weeks 8 and 9 and in the CD4KO group at weeks 1, 2, 3, 6, 8 and 10.

Liver Fibrosis

Reticulin and Sirius red stained liver sections taken from CD4+ knockout and wild type mice after 12 injections of Con-A were scored for liver fibrosis. All sections from both cohorts showed significant liver fibrosis, although there was significant variation in the degree of fibrosis observed in different areas of individual liver sections Figs 6.4 to 6.7. No animal scored less than 3 using the scheme previously devised for assessing CCl₄ induced liver fibrosis (Table 4.3) and there was no statistically significant difference between the two groups. Fig 6.8.

When sections were assigned a rank position correlating to the degree of liver fibrosis, with higher numbers representing more fibrosis, the mean rank positions for CD4+ knockout mice were significantly higher than those for wild type mice for both reticulin (Fig 6.9) and Sirius red (Fig 6.10) staining (15.0 +/- 1.48 vs. 7.69 +/- 1.35 and 15.16 +/- 1.30 vs. 7.77 +/- 1.40 respectively, p=0.03 and p=0.02). There was significant correlation in rank position for liver fibrosis between sections stained for reticulin and with Sirius red (R²=0.751) (Fig 6.11).
Fig 6.4. Photomicrograph of murine liver section stained for reticulin after induction of fibrosis with 12 weeks of Con-A injection. The field shown is a typical example of the greatest degree of fibrosis observed (10x objective).

Fig 6.5. Photomicrograph of murine liver section stained for reticulin after induction of fibrosis with 12 weeks Con-A injection. The field shown is a typical example of the least fibrosis observed (10x objective).
Fig 6.6. Photomicrograph of a section of murine liver stained with Sirius red after induction of liver fibrosis with 12 weeks of Con–A injection. The field shown is a typical example showing the greatest degree of fibrosis observed (10x objective).

Fig 6.7. Photomicrograph of a section of murine liver stained with Sirius red after the induction of liver fibrosis with 12 weeks of Con-A injection. The field shown is a typical example showing the least fibrosis observed (10x objective).
Fig 6.8. Fibrosis scores of wild type and CD4KO mouse liver sections assessed by reticulin staining after 12 weeks of Con-A injection (mean ± SEM)

![Graph showing fibrosis scores of wild type (WT) and CD4KO mice](image)

Fig 6.9. Rank position of wild type (blue bar) and CD4KO (yellow bar) mouse liver sections for fibrosis according to reticulin staining (mean ± SEM). Liver sections were assigned a rank position according to the degree of fibrosis by two blinded observers. A rank position of 1 denotes the least fibrosis. *p<0.05 relative to WT

![Graph showing rank position of fibrosis scores for wild type (WT) and CD4KO mice](image)
Fig 6.10. Rank position of wild type (blue bar) and CD4KO (yellow bar) mouse liver sections for fibrosis according to Sirius red staining (mean ± SEM). Liver sections were assigned a rank position according to the degree of fibrosis by two blinded observers. A rank position of 1 denotes the least fibrosis. *p<0.05 relative to WT.

Fig 6.11. Scatter plot showing correlation between reticulin and Sirius red staining.

R² = 0.7513
6.4.2. Characterisation of Hepatic T-lymphocyte Infiltrates in Wild Type and CD4 Knockout Mice following Con-A Induced Liver Injury

Assessment of Hepatic CD3+ T-cell Infiltration following Liver Injury with Con-A Immunohistochemical staining of liver sections for CD3 revealed no difference between the numbers of CD3+ T-lymphocytes in the livers of untreated (time zero) wild type and CD4KO mice (p=0.847). However, numbers of CD3+ T-lymphocytes were increased significantly in both wild type and CD4KO mice at 24 hours post Con-A injection compared to time zero (p=0.05 and p=0.027 respectively). There was no significant difference between the degree of CD3+ T-cell infiltration in wild type and CD4KO mice 24 hours after Con-A injection (p=0.371). *(Table 6.2, Figs 6.12 & 6.13).*

Following 12 weeks of Con-A treatment, hepatic infiltration with CD3+ T-cells was increased in wild type mice compared to time zero controls (p=0.035) and in CD4KO mice but this did not reach statistical significance (p=0.089). There was no significant difference in CD3+ T-lymphocyte infiltration between wild type and CD4KO mice after 12 weeks of Con-A injection (p=0.75). *(Table 6.2, Figs 6.12 & 6.13).*

*Table 6.2. CD3+ T-cell infiltration in WT and CD4KO Mouse Livers at Time Zero and after Acute and Chronic Con-A Induced Injury*

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Mean CD3+ cells per high power field ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT 0 hours</td>
<td>0.39 ± 0.15</td>
</tr>
<tr>
<td>CD4KO 0 hours</td>
<td>0.35 ± 0.14</td>
</tr>
<tr>
<td>WT 24 hours</td>
<td>3.64 ± 1.34</td>
</tr>
<tr>
<td>CD4KO 24 hours</td>
<td>2.06 ± 0.62</td>
</tr>
<tr>
<td>WT 12 weeks</td>
<td>4.24 ± 1.60</td>
</tr>
<tr>
<td>CD4KO 12 weeks</td>
<td>3.37 ± 1.55</td>
</tr>
</tbody>
</table>
Fig. 6.12. Immunohistochemical staining of CD3+ cells in murine liver (x 10): (a) WT time zero; (b) CD4KO time zero; (c) WT 24 hours; (d) CD4KO 24 hours; (e) WT 12 weeks; (f) CD4KO 12 weeks
Assessment of Hepatic CD4+ T-cell Infiltration Following Liver Injury with Con-A

No CD4+ cells were detected in any CD4KO animal on immunostaining. Numbers of hepatic CD4+ cells were significantly elevated in wild type mice 24 hours after Con-A injection when compared to time zero controls (p= 0.005). After 12 weeks of Con-A injection, numbers of hepatic CD4+ T-cells were increased in wild type mice relative to time zero but this did not reach statistical significance (p=0.075). There was no difference between liver CD4+ T-cell infiltration in wild type mice 24 hours post Con-A injection and after 12 weeks of Con-A injection (p=0.215)(Table 6.3, Figs 6.14 & 6.15).

Table 6.3. CD4+ T-cell Infiltration in Livers of Wild Type and CD4KO Mice at Time Zero and after Acute and Chronic Con-A Induced Injury (mean ± SEM)

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Mean CD4+ cells per high power field ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT 0 hours</td>
<td>0.39 ± 0.08</td>
</tr>
<tr>
<td>CD4KO 0 hours</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>WT 24 hours</td>
<td>2.66 ± 0.52</td>
</tr>
<tr>
<td>CD4KO 24 hours</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>WT 12 weeks</td>
<td>1.7 ± 0.48</td>
</tr>
<tr>
<td>CD4KO 12 weeks</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>
Fig. 6.14. Immunohistochemical staining of CD4+ cells in murine liver (x 40): (a) WT time zero; (b) CD4KO time zero; (c) WT 24 hours; (d) CD4KO 24 hours; (e) WT 12 weeks; (f) CD4KO 12 weeks. Sections are shown at higher magnification to display positive cell as the signal produced by this antibody was weaker.
Assessment of Hepatic CD8+ T-cell Infiltration following Liver Injury with Con-A

There was no difference in the numbers of CD8+ cells present in the livers of wild type and CD4KO mice prior to treatment. Liver infiltration by CD8+ cells was increased in wild type mice 24 hours after Con-A injection approaching but not reaching statistical significance (p=0.057). There was a smaller and non-significant increase in CD8+ staining in the livers of CD4KO mice 24 hours after Con-A treatment compared to time zero controls (p=0.092). No difference was seen between wild type and CD4KO mice at 24 hours post injection (p=0.66). When compared to untreated controls, there were only small and non-significant increases in CD8+ T-cell infiltration in the livers of wild type and CD4KO mice after 12 weeks of Con-A injection (p=0.076, p= 0.254 respectively). No difference in CD8+ staining was detected in the two mouse strains at 12 weeks (p=0.79) (Table 6.4, Fig 6.16 & 6.17).
Table 6.4. CD8+ T-cell Infiltration in Livers of Wild Type and CD4KO Mice at Time Zero and after Acute and Chronic Con-A Induced Injury (mean ± SEM)

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Mean CD8+ T-cells per high power field (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT time zero</td>
<td>0.16 ± 0.08</td>
</tr>
<tr>
<td>CD4KO time zero</td>
<td>0.4 ± 0.15</td>
</tr>
<tr>
<td>WT 24 hours</td>
<td>2.83 ± 1.27</td>
</tr>
<tr>
<td>CD4KO 24 hours</td>
<td>2.08 ± 0.86</td>
</tr>
<tr>
<td>WT 12 weeks</td>
<td>0.99 ± 0.33</td>
</tr>
<tr>
<td>CD4KO 12 weeks</td>
<td>0.84 ± 0.38</td>
</tr>
</tbody>
</table>

Fig 6.16. CD8+ T-cell Infiltration in Livers of Wild Type and CD4KO Mice at Time Zero and after Acute and Chronic Con-A Induced Injury (mean ± SEM)
Fig. 6.17. Immunohistochemical staining of CD8+ cells in murine liver (× 10): (a) WT time zero; (b) CD4KO time zero; (c) WT 24 hours; (d) CD4KO 24 hours; (e) WT 12 weeks; (f) CD4KO 12 weeks.
6.4.3. Characterisation of the Cytokine Profile in Wild Type and CD4 Knockout Mice following Con-A Induced Liver Injury

**Serum TNF-α**

There was no difference between serum TNF-α levels in wild type and CD4KO mice at time zero or after 12 weeks of Con-A injection. Similarly there was no significant increase in serum TNF-α levels in wild type mice 24 hours post Con-A injection or after 12 weeks of Con-A injection when compared to untreated controls. However, serum TNF-α levels in CD4KO mice at 24 hours post Con-A injection were markedly elevated when compared to both untreated CD4KO controls (p=0.005) and wild type mice 24 hours post Con-A injection (p=0.005). *Table 6.5, Fig 6.18.*

**Table 6.5. Serum TNF-α Levels in Wild Type and CD4KO Mice at Time Zero and After Acute and Chronic Con-A induced Liver Injury**

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Serum TNF-α Level (pg/ml) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT time zero</td>
<td>6.11 ± 6.11</td>
</tr>
<tr>
<td>CD4KO time zero</td>
<td>12.0 ± 6.4</td>
</tr>
<tr>
<td>WT 24 hours</td>
<td>29.6 ± 13.3</td>
</tr>
<tr>
<td>CD4KO 24 hours</td>
<td>237.4 ± 74.1</td>
</tr>
<tr>
<td>WT 12 weeks</td>
<td>22.0 ± 14.6</td>
</tr>
<tr>
<td>CD4KO 12 weeks</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

*Fig 6.18. Serum TNF-α (pg/ml) in WT and CD4KO mice at time zero and after acute and chronic Con-A induced liver injury (mean ± SEM). *p*<0.05 relative to WT*
Hepatic TNF-α

There was no difference between hepatic TNF-α levels in WT and CD4KO mice at time zero or after 12 weeks of Con-A injection. Hepatic TNF-α levels were higher in WT animals at 24 hours than in time zero controls (p=0.02) and in CD4KO mice compared to time zero controls, although this was not significant (p=0.058). There was no statistically significant difference between hepatic TNF-α levels in wild type and CD4KO mice at 24 hours post Con-A injection. Table 6.6, Fig 6.19.

Table 6.6. Hepatic TNF-α Levels in Wild Type and CD4KO Mice at Time Zero and After Acute and Chronic Con-A induced Liver Injury

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Hepatic TNF-α Level (pg/mg liver tissue) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT time zero</td>
<td>99.6 ± 65.1</td>
</tr>
<tr>
<td>CD4KO time zero</td>
<td>26.8 ± 26.8</td>
</tr>
<tr>
<td>WT 24 hours</td>
<td>1002 ± 279</td>
</tr>
<tr>
<td>CD4KO 24 hours</td>
<td>679 ± 326</td>
</tr>
<tr>
<td>WT 12 weeks</td>
<td>46.2 ± 31.5</td>
</tr>
<tr>
<td>CD4KO 12 weeks</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Fig 6.19. Hepatic TNF-α levels (pg/mg liver tissue) in wild type and CD4KO mice at time zero and after acute and chronic Con-A induced liver injury (mean ± SEM)
Serum IFN-γ

There was no significant difference between serum IFN-γ levels in wild type and CD4KO mice at time zero, 24 hours post Con-A injection or after 12 weeks of Con-A injection. In wild type mice by comparison to time zero controls, serum IFN-γ levels were slightly, and not significantly, increased at 24 hours but markedly increased at 12 weeks (p=0.023). In CD4KO mice by comparison to time zero controls, serum IFN-γ levels were increased at 24 hours, although this did not reach statistical significance (p=0.06), and at 12 weeks (p=0.021). *Table 6.7, Fig 6.20.*

Table 6.7. Serum IFN-γ Levels in Wild Type and CD4KO Mice at Time Zero and After Acute and Chronic Con-A induced Liver Injury

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Serum IFN-γ Level (pg/ml) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT time zero</td>
<td>416 ± 137</td>
</tr>
<tr>
<td>CD4KO time zero</td>
<td>480 ± 244</td>
</tr>
<tr>
<td>WT 24 hours</td>
<td>869 ± 283</td>
</tr>
<tr>
<td>CD4KO 24 hours</td>
<td>1673 ± 498</td>
</tr>
<tr>
<td>WT 12 weeks</td>
<td>1945 ± 491</td>
</tr>
<tr>
<td>CD4KO 12 weeks</td>
<td>1951 ± 441</td>
</tr>
</tbody>
</table>

*Fig 6.20. Serum IFN-γ levels (pg/ml) in wild type and CD4KO mice at time zero and after acute and chronic Con-A induced liver injury (mean ± SEM)*
Hepatic IFN-γ

Levels of hepatic IFN-γ were higher in wild type than CD4KO mice at time zero (p=0.00). By 24 hours post injection of Con-A, liver IFN-γ levels were decreased in wild type and increased in CD4KO mice compared to time zero controls (p=0.001 and p=0.000 respectively). At 24 hours hepatic IFN-γ levels were higher in CD4KO than in wild type mice (p=0.001). After 12 weeks of Con-A injection, hepatic IFN-γ levels were significantly increased in both wild type and CD4KO mice (p=0.000 and p=0.000) compared to the appropriate time zero controls but there was no significant difference between wild type and CD4KO liver IFN-γ levels at 12 weeks (p=0.099).

Table 6.8, Fig 6.21.

Table 6.8. Hepatic IFN-γ Levels in Wild Type and CD4KO Mice at Time Zero and After Acute and Chronic Con-A induced Liver Injury

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Hepatic IFN-γ Level (pg/mg liver tissue) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT time zero</td>
<td>7.58 ± 0.50</td>
</tr>
<tr>
<td>CD4KO time zero</td>
<td>2.36 ± 0.51</td>
</tr>
<tr>
<td>WT 24 hours</td>
<td>3.67 ± 0.58</td>
</tr>
<tr>
<td>CD4KO 24 hours</td>
<td>8.07 ± 0.64</td>
</tr>
<tr>
<td>WT 12 weeks</td>
<td>36.5 ± 2.36</td>
</tr>
<tr>
<td>CD4KO 12 weeks</td>
<td>30 ± 1.78</td>
</tr>
</tbody>
</table>

Fig 6.21. Hepatic IFN-γ (ng/mg liver) in WT & CD4KO mice at time zero and after acute and chronic Con-A induced liver injury (mean ± SEM). *p<0.05 relative to WT
Serum IL-10

There was no difference in serum IL-10 levels between wild type and CD4KO mice at time zero. In wild type mice, serum IL-10 levels were significantly increased both at 24 hours post Con-A injection (p=0.001) and after 12 weeks of Con-A injection (p=0.012) compared to time zero controls. In CD4KO mice, however, serum IL-10 levels were elevated 24 hours post Con-A injection (p=0.007) but were not significantly increased with respect to time zero controls after 12 weeks of Con-A injection. At the 24-hour time point, serum IL-10 levels were significantly higher in the CD4KO than in the wild type group (p=0.011) but there was no significant difference between the two groups at the 12 week time point. Table 6.9, Fig 6.22.

Table 6.9. Serum IL-10 Levels in Wild Type and CD4KO Mice at Time Zero and After Acute and Chronic Con-A induced Liver Injury

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Serum IL-10 Level (pg/ml) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT time zero</td>
<td>35.6 ± 21.3</td>
</tr>
<tr>
<td>CD4KO time zero</td>
<td>68.7 ± 29.4</td>
</tr>
<tr>
<td>WT 24 hours</td>
<td>138 ± 8.21</td>
</tr>
<tr>
<td>CD4KO 24 hours</td>
<td>897 ± 135</td>
</tr>
<tr>
<td>WT 12 weeks</td>
<td>144 ± 27.4</td>
</tr>
<tr>
<td>CD4KO 12 weeks</td>
<td>68.3 ± 44.5</td>
</tr>
</tbody>
</table>

Fig 6.22. Serum IL-10 (pg/ml) in wild type & CD4KO mice at time zero and after acute and chronic Con-A induced liver injury (mean ± SEM). *p<0.05 relative to WT
Hepatic IL-10

IL-10 was not detectable by ELISA in the liver homogenates of mice from either the wild type or CD4KO groups at time zero or at 24 hours post Con-A injection. By 12 weeks of Con-A injection, IL-10 had become detectable in the livers of both wild type and CD4KO mice and was significantly elevated compared to time zero in wild type mice (p=0.017). There was no significant difference in hepatic IL-10 levels between the two mouse groups at 12 weeks. Table 6.10, Fig 6.23.

Table 6.10. Hepatic IL-10 Levels in wild type and CD4KO mice at time zero and after acute and chronic Con-A induced liver injury (mean ± SEM)

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Hepatic IL-10 Level (pg/mg liver tissue) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT time zero</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CD4KO time zero</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>WT 24 hours</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CD4KO 24 hours</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>WT 12 weeks</td>
<td>59.9 ± 21.6</td>
</tr>
<tr>
<td>CD4KO 12 weeks</td>
<td>45.4 ± 45.4</td>
</tr>
</tbody>
</table>

Fig 6.23. Hepatic IL-10 Levels (pg/mg liver) in WT and CD4KO mice at time zero and after acute and chronic Con-A induced liver injury (mean ± SEM)
6.5. Discussion

Acute Hepatitis Model

A single intravenous injection of the T-cell mitogen Concanavalin-A (Con-A) has been shown to induce an acute hepatitis in mice, which appears to be dependent on the presence of CD4+ T lymphocytes. The production of several cytokines, notably TNF-α, IFN-γ and IL-4, precedes the elevation of serum transaminases and plays an important role in the pathogenesis of liver damage (Gantner et al 1995; Mizuhara et al 1996; Toyabe et al 1997).

In this chapter, I examined the effect of a single injection of Con-A 20mg/kg on the livers of both WT (C57/Bl6) and CD4KO mice by histopathological assessment of liver damage, analysis of hepatic T cell infiltrates and measurement of systemic and intra-hepatic cytokines.

At baseline (time zero), there was no difference in the histological appearances of the liver in the two mouse strains, as would be anticipated from previous observations that CD4KO mice were indistinguishable from wild types on gross physical examination (Nishimura & Ohta 1999). However, 24 hours post Con-A injection, there were marked differences between the livers of WT and CD4KO mice. All WT mice exhibited a fulminant hepatitis characterised by marked inflammatory cell infiltrates, loss of hepatocyte cell structure and extensive areas of coagulative necrosis, whereas all CD4KO mice developed a much less severe hepatitis distinguished by fatty change and mild inflammatory cell infiltrates.

The finding that CD4KO mice were more resistant, although not invulnerable, to acute liver injury than their wild type counterparts are largely consistent with those from the original description of the Con-A induced model of hepatitis (Tiegs, Hentschel, & Wendel 1992) and suggest a role for CD4+ T-cells in the generation of liver damage. In the previous study, pre-treatment of animals with anti-CD4 antibody to deplete CD4+ T cells protected against liver injury as assessed by a serum transaminase levels 8 hours following Con-A injection, although serum transaminases were not reduced to the level of animals which had not been challenged with Con-A (Tiegs, Hentschel, & Wendel 1992).
At this early time point, even in wild type mice, only occasional foci of necrosis with scarce leucocyte infiltrates were detectable by light microscopy but electron microscopy showed severe disruption of the hepatic microarchitecture. In other studies where liver histology was assessed in WT (BALB/c) mice at 24 hours post injection, this time point was noted to be associated with peak mononuclear and polymorphonuclear cell infiltration (Mizuhara et al 1996) and foci of necrosis (Mizuhara et al 1994) similar to, those seen in this work. Moreover, C57Bl/6 mice as used in this study have been shown to be more susceptible to Con-A induced hepatitis than BALB/c mice, developing higher levels of transaminases and more pronounced liver injury over a shorter time course (Mizuhara et al 1998).

There is no previous literature in which the effects of CD4 depletion have been correlated with liver histological appearances at the 24-hour time point. However, the observation from this study that the CD4KO mice did develop liver injury, though less severe than in wild types, clearly supports the presence of mechanisms of liver damage both dependent on and independent of CD4+ T cells. The nature of the CD4+ T cell independent mechanisms is not clear, but candidates would include: other T cell subsets, hepatic macrophages or KC and the possibility of direct Con-A toxicity against hepatocytes (Leist & Wendel 1996).

The T cell components of the inflammatory infiltrates seen in Con-A induced hepatitis were analysed by immunostaining liver sections for CD3+, CD4+ and CD8+ cells. Few CD3+ T cells were detected in the livers of untreated WT and CD4KO mice and there was no difference between the two strains at time zero. By 24 hours post Con-A injection, there had been a significant increase in CD3 staining in the livers of both groups, indicating the presence of T cell infiltration but there was no difference in the degree of CD3 staining between WT and CD4KO mice. This suggests there was no overall difference in T cell infiltration and that other T cell subsets might be making up for the deficit in CD4+ T cells. Immunostaining for CD4 confirmed the absence of CD4+ cells in all the CD4KO mice studied and showed a significant increase in CD4+ T cell infiltration in WT mice at 24 hours compared to the small numbers of CD4+ cells detected at baseline. There was a trend to increased CD8+ T cell infiltration in both WT and, to a lesser extent, CD4KO
mice at 24 hours but this did not reach statistical significance, although it may have
done so if increased numbers of animals had been studied. These data are consistent
with a previous study in which the T cell component of hepatic infiltrates in Con-A
was analysed and found to contain relatively more CD4+ T cells than CD8+ cells
(Mizuhara et al. 1996).

In the absence of CD4+ cells, it might be anticipated that CD8+ cells would account
for most of the total T cell infiltration as assessed by CD3 staining but my data
suggest that this is not the case. Analysis of T cell phenotypes in CD4KO mice has
demonstrated that CD8+ cells are present in normal numbers in the thymus but
increased in peripheral lymph nodes to occupy the compartment usually occupied by
CD4+ cells (Nishimura & Ohta 1999). However in CD4KO mice, another T cell
subset may assume greater importance. CD4KO mice exhibit increased numbers of
“double negative” (but CD3 positive) CD4-CD8- T cells, which display more mature
phenotype than the CD4-CD8- T cells found in small numbers wild type mice
(Nishimura & Ohta 1999; Rahematulla et al. 1994). These “double negative” T cells
have been shown to substitute for CD4+ T cells by providing Class II MHC
restricted helper functions. In CD4KO mice, “double negative” cells are capable of a
low titre antibody response to sheep erythrocytes (Rahematulla et al 1994),
immunoglobulin class switching in response to vesicular stomatitis virus (VSV)
(Rahematulla et al 1994) and producing an appropriate IFN-γ response to
infection with Leishmania major (Locksley et al. 1993). It is possible that they may
assume some of the effector functions normally performed by CD4+ cells in wild
type mice in the pathogenesis of Con-A induced hepatitis.

Moreover, the intrahepatic lymphocyte population of wild type mice is characterised
by a high proportion of T lymphocytes exhibiting phenotypes that are rare in the pool
circulating through peripheral lymphoid organs. The majority of these are T cells
with intermediate expression of the T cell receptor (TCR) and expressing NK1.1 (a
natural killer cell marker) and are known as natural killer T cells (NK T cells)
(Crispe & Mehal 1996; O'Farrelly & Crispe 1999). They are capable of both cytokine
production and cytotoxic activity (MacDonald 1995). Two studies suggest that NK T
cells may play a role in the hepatitis induced by Con-A. Firstly, depletion of NK T
cells and NK cells in wild type mice by pre-treatment with an anti NK1.1 antibody
markedly suppressed the elevation of serum transaminases and the amount of liver damage 20 hours after Con-A injection (Toyabe et al 1997). By contrast, pre-treatment with anti asialo-GM1 antibody, which depletes only NK cells, did not have this effect. Secondly, mice deficient in the MHC class I molecule CD1, which lack NK T cells, are resistant to Con-A induced liver injury (Takeda et al. 2000). Susceptibility to liver injury can be restored in CD1-deficient mice by adoptive transfer of hepatic mononuclear cells or NK T cells from wild type mice (Takeda et al 2000).

Evidence of the contribution of macrophages and Kupffer cells (KC) to the liver injury induced by Con-A remains unclear. In the original description of the Con-A hepatitis model, mice treated prior to Con-A injection with silica particles, which destroy macrophages in vivo (Allison, Harington, & Birbeck 1966; Levy & Wheelock 1975), were protected against liver injury as measured by serum transaminases. However, in a subsequent study by the same group, injection of gadolinium chloride (GdCl₃), a potent inhibitor of macrophage function (Hardonk et al. 1992), before challenge with Con-A did not significantly attenuate the rise in transaminases (Knolle et al. 1996). Other workers, in contrast, have observed that treatment with GdCl₃ did inhibit Con-A induced elevation of transaminases although it did not impair the induction of liver cytokine mRNA for IL-2 or TNF-α.

In vitro studies, however, do provide supportive evidence for a critical role for KC and liver macrophages. Co-cultures of lymph node cells and non-parenchymal liver cells (80-90% KC), when stimulated with Con-A, produced substantially more TNF-α, IFN-γ and IL-4 than either lymph node cells or non-parenchymal liver cells alone (Gantner, Leist, Lohse, Germann, & Tiegs 1995; Gantner et al. 1996). Potentiation of TNF-α release was not observed when the lymph node cells in the co-culture system were isolated from athymic mice (Gantner, Leist, Lohse, Germann, & Tiegs 1995). A similar augmentation of cytokine release was seen when co-cultures of T cells and a macrophage cell line were stimulated with Con-A and, in this system, macrophage but not TNF-α mRNA levels were increased (Gantner, Leist, Kusters, Vogt, Volk, & Tiegs 1996).

Overall, in wild type mice, it seems likely that Kupffer cells, NK T cells and CD4+ T cooperate in producing a burst of cytokine production with a resultant hepatitis.
However, the redundancy of the immune system provides a remarkable capacity to compensate for lost functions so that effectors other than CD4+ T cells assume more importance in CD4KO mice. These putative adaptive mechanisms are likely to become more significant still over the more protracted time course of the chronic disease model.

Evidence for the importance of TNF-α as a cytokine mediator liver injury in Con-A induced hepatitis derives from work in which pre-treatment with a neutralising anti-TNF-α antibody (Gantner, Leist, Lohse, Germann, & Tiegs 1995) or the use of TNF-α knockout mice (Kusters et al 1997) protected the animals against liver injury. The lack of both a TNF-α rise and the development of liver injury in athymic mice, or in animals pre-treated with cyclosporin, suggests that T cells are critical to the production of the cytokine (Gantner, Leist, Lohse, Germann, & Tiegs 1995).

In the present study, little TNF-α was detectable by ELISA at time zero in the serum or liver homogenates of both wild type and CD4KO mice and there was no difference between the two mouse groups. By 24 hours after Con-A injection, serum TNF-α levels had markedly increased in CD4KO mice with only a small and non-significant elevation in the wild type group. Hepatic TNF-α levels, however, rose significantly in both wild type and CD4KO mice at 24 hours post Con-A injection and there was no difference between the two groups. Given that existing data points to CD4+ T cells as the source of TNF-α in Con-A hepatitis, these findings run contrary to expectation and provide a plausible mechanism by which CD4KO mice developed a mild, but significant, hepatitis.

In previous studies where serum TNF-α levels have been measured in various strains of wild type mice following Con-A injection, peak TNF-α levels typically occurred less than 2 hours after injection, with levels falling back to baseline by 8 hours (Gantner, Leist, Lohse, Germann, & Tiegs 1995; Kimura et al. 1999; Mizuhara et al 1994). The sampling of blood for cytokine assay at the later time point of 24 hours in this work, most likely accounts for the absence of any significant TNF-α rise in the wild type mice.
Several previous studies have indicated that IFN-\(\gamma\) is an important mediator in Con-A induced hepatitis. Following Con-A injection, plasma IFN-\(\gamma\) levels typically peak within 8 to 12 hours (Kimura et al 1999; Mizuhara et al 1996; Mizuhara et al 1998) but remain elevated at 24 hours. Neutralisation of IFN-\(\gamma\) by pre-treatment with anti IFN-\(\gamma\) antibody protects against hepatocellular damage as assessed by transaminase levels (Kusters, Gantner, Kunstle, & Tiegs 1996; Mizuhara et al 1996; Mizuhara et al 1998) but does not prevent infiltration of the liver by inflammatory cells (Mizuhara et al 1996) whereas in IFN-\(\gamma\) knockout mice, the defect appears to confer protection against both transaminase release and inflammatory infiltration (Tagawa, Sekikawa, & Iwakura 1997). C57Bl/6 mice, as utilised in the present study, produce less IFN-\(\gamma\) in response to Con-A than BALB/c mice but are unexpectedly more susceptible to liver injury (Mizuhara et al 1996). It has been postulated that the increased susceptibility of C57Bl/6 to hepatitis may reflect strain specific variations in the inducibility of IFN-\(\gamma\) responsive genes such as inducible nitric oxide synthetase (iNOS) (Mizuhara et al 1996).

In this study, when IFN-\(\gamma\) was measured in serum, there was no difference between the serum levels in wild type and CD4KO mice at time zero. By 24 hours, there was a slight and non-significant rise in IFN-\(\gamma\) levels in wild type mice with a greater increase in CD4KO mice approaching statistical significance (\(p=0.06\)). It is possible that the more pronounced elevation of IFN-\(\gamma\) in the CD4KO mice, together with the greater susceptibility of the C57Bl6/6 strain to IFN-\(\gamma\) mediated damage (Mizuhara et al 1996) accounts in some part for the presence of liver injury in the CD4KO mice.

By contrast, in liver homogenates, there was no increase in IFN-\(\gamma\) levels in either wild type or CD4KO mice at 24 hours when compared to time zero controls. Published studies to date have not described the measurement of IFN-\(\gamma\) protein in liver homogenates. However, RNase protection assays and Northern blotting have demonstrated the induction of IFN-\(\gamma\) mRNA in the livers of Con-A treated animals at 4 hours and 12 hours respectively (Mizuhara et al 1998; Tagawa, Sekikawa, & Iwakura 1997). It is possible that the failure to detect IFN-\(\gamma\) in liver homogenates reflects a very rapid turnover of IFN-\(\gamma\) and hence a very short half-life of this cytokine in the hepatic environment.
Measurement of serum IL-10 revealed little cytokine in untreated mice with no difference between CD4KO mice and wild types. However, at 24 hours after Con-A injection, levels of IL-10 had risen markedly in the CD4KO but not the WT mice. Published data have demonstrated that serum IL-10 was not detected in BALB/c wild type mice at time zero but rose to levels of 942± 272 pg/ml at 8 hours after Con-A injection. The kinetics of IL-10 induction showed IL-10 levels peaking at 8 hours and falling to become undetectable by 24 hours. By contrast, in my study, IL-10 was detectable in both WT and CD4KO mice at 24 hours post Con-A injection. It is possible that this may reflect strain differences in cytokine production between the BALB/c mice used in the published study and the C57B1/6 mice although this seems unlikely given that C57B1/6 mice tend to produce Th1 predominant cytokine responses and IL-10 has been traditionally regarded as a Th2 cytokine. Previously published data would suggest that levels of cytokines should have been lower in the CD4KO mice than in wild type controls, whereas we found very significant elevations in levels of both the pro-inflammatory cytokines TNF alpha and interferon gamma, and of the anti-inflammatory cytokine IL-10. The reasons for both this disparity and the very clear phenotypic difference in patterns of inflammation remain unclear but indicate that the situation in vivo is likely to be considerably more complex than our somewhat simplistic models would suggest.

The acute Con-A model is probably worthy of further detailed study but as further in vivo studies were both costly and on the limits of the scope of this thesis and so it was not possible to explore the acute model in any further detail at that time.

Chronic Fibrosis Model

By comparison to the Con-A model of acute hepatitis, which has been extensively studied, relatively little is known about the effects of repeated Con-A injections on the liver. At the time of embarking on this study, there was only a single publication in abstract form reporting the development of liver fibrosis after recurrent Con-A injection (Louis, Le Moine, Quertinmont, Peny, Goldman, & Deviere 1997). Although it was established that development of acute liver injury following a single injection of Con-A involved CD4+ T cell dependent mechanisms, the effectors in the pathogenesis of liver fibrosis after multiple Con-A injections had not been clearly
identified. Subsequently two groups have published papers examining different aspects of the Con-A liver fibrosis model (Kimura et al 1999; Louis et al 2000). In this chapter, I compared the development of liver injury and hepatic T cell infiltration after 12 weekly injections of Con-A in WT and CD4KO mice in order to examine whether chronic injury, like acute injury, is CD4 T cell dependent. Furthermore, I examined the profile of cytokine production in both WT and knockout mice.

After 12 weeks of Con-A injection, all animals from both the WT and CD4KO groups had significant liver fibrosis with histological assessment showing broad fibrous septa or frank cirrhosis. There was no difference in the mean scores for fibrosis between the two groups and when specimens were assigned a ranking order according to the degree of liver fibrosis, CD4KO mice had a significantly higher mean rank score higher indicating more liver fibrosis. These results strongly suggest that the mechanisms of liver fibrosis in this study of the Con-A chronic injection model were not exclusively CD4 T cell dependent and correspond with the data from the acute hepatitis model, which demonstrated that CD4KO mice were not completely protected from liver injury although they developed a much more subtle hepatitis than their wild type counterparts. In the only published study in which the cellular mediators of Con-A induced liver fibrosis have been examined, athymic nude mice were found not to develop any liver fibrosis after 6 weeks of Con-A injection, by which time point all wild type mice exhibited significant fibrosis (Louis et al 2000). Whilst this clearly implies that T-lymphocytes are mediators of liver fibrosis, it does not specifically identify the CD4+ subset as being responsible.

Liver specimens harvested from animals after 12 weeks of Con-A injection were immunostained to characterise hepatic T-cell infiltrates. CD3+ cells were increased approximately 10-fold in WT and CD4KO mice compared to time zero, correlating with the study by Louis which observed the persistence of inflammatory infiltrates comprising mainly lymphocytes around centrilobular veins up to 20 weeks of Con-A injection (Louis et al 2000). Louis and co-workers found that the inflammatory infiltrates stained positively for CD4+ but not for CD8+ cells (Louis et al 2000). In this work, CD4+ staining was increased approximately 4-fold in WT mice at 12 weeks compared to control mice, approaching statistical significance (p=0.075)
where as CD4+ cells were, as anticipated, not detected in CD4KO animals and only small and non-significant increases in CD8+ staining were observed in either mouse group. These findings suggest that a component of the lymphocyte infiltrate in chronically treated animals is comprised of cells which are neither CD4 nor CD8 positive and that this may be a more significant component in the CD4KO mice. This component may be comprised of “double negative” or NK T cells as discussed earlier with reference to acute liver injury and these cells may play a role in the pathogenesis of liver fibrosis, particularly in CD4KO mice.

The role of TNF-α and IFN-γ in acute liver injury following a single injection of Con-A has been extensively investigated, as previously discussed in this chapter. However, less data is available regarding the role of these cytokines in the development of fibrosis following recurrent Con-A injections. This study showed that serum and hepatic TNF-α were detectable only at low levels in WT mice at 12 weeks and were not increased relative to time zero controls. Neither serum nor hepatic TNF-α could be detected by ELISA at 12 weeks in CD4KO mice. Louis et al found that serum TNF-α levels were markedly increased after the first and second weekly injections of Con-A but rapidly declined with subsequent injections, although TNF-α was still detectable in serum at after Con-A injection throughout the study period of 20 weeks at much lower levels (Louis et al 2000). Kimura et al injected BALB/c mice with Con-A at weekly intervals for 4 weeks with liver fibrosis observed one week after the final injection (Kimura et al 1999). They found that serum TNF-α levels were markedly increased after a single Con-A injection as previously described in the studies of Con-A induced acute hepatitis, and that TNF-α mRNA was upregulated in the liver after injection with a peak at 4 hours. TNF-α was not assayed after subsequent Con-A injections but neutralisation of TNF-α using pre-treatment with anti-TNF-α antibody strikingly reduced liver injury after every injection as assessed by both serum ALT measurement and histopathology (Kimura et al 1999). At the end of the study period, the development of liver fibrosis had been dramatically reduced by anti-TNF–α administration prior to each Con-A injection (Kimura et al 1999).
Both serum and hepatic IFN-γ were significantly increased in WT and CD4KO mice at 12 weeks compared to time zero controls and there was no significant difference between the two mouse groups. The increase in IFN-γ was more marked in serum than in liver tissue. In the study by Louis et al, IFN-γ followed a similar pattern to TNF-α, with a dramatic rise in serum levels after the first injection, declining over the next two injections and remaining lower levels during the remainder of the 20 week study period (Louis et al 2000). Kimura et al described a marked increase in serum IFN-γ after one Con-A injection with induction of IFN-γ mRNA in the liver (Kimura et al 1999). Neutralisation of IFN-γ with antibody prior to each of four weekly injections of Con-A resulted in a striking reduction of serum ALT levels and of the inflammatory changes observed on histological examination and inhibited the development of liver fibrosis (Kimura et al 1999). The ability of both WT and CD4KO mice to produce IFN-γ both implies that CD4+ cells are not the sole source of this cytokine and helps to explain why CD4KO mice developed fibrotic changes.

Serum levels of IFN-γ at 12 weeks in this study were considerably higher in both C57Bl/6 and CD4KO mice than at comparable time points in BALB/c mice in the study of Louis et al. It is possible that this may reflect the differences in dosing protocol between the two studies. In the Louis study, mice were given Con-A 20mg/kg throughout the study period and this was associated with almost 70% mortality after the first two injections and none thereafter. In my protocol, in an attempt to reduce mortality, the Con-A dose was started at 10 mg/kg and then increased after two injections in a stepwise fashion. There were a smaller number of deaths in both WT (13%) and CD4KO mice (40%) and these were spread throughout the study period rather than clustered after the first two injections. The surviving mice in the Louis study may have been a subset of individuals selected by merit of an enhanced ability to down-regulate pro-inflammatory cytokine responses, perhaps via greater production of IL-10 as discussed later.

Measurement of IL-10 by ELISA after 12 weeks of Con-A injection demonstrated a significant increase in serum IL-10 levels in WT mice compared to time zero controls but no change in IL-10 levels in CD4KO mice. Although serum IL-10 levels were higher in WT than in CD4KO mice, this did not reach statistical significance.
Hepatic IL-10 was not detectable by ELISA in either WT or CD4KO mice at time zero but had increased significantly in WT mice after 12 weeks of Con-A injection. By contrast hepatic IL-10 only became detectable in a single animal in the CD4KO group at 12 weeks.

These results appear to correspond with published data showing IL-10 detectable in serum after the first two Con-A challenges but with a steady increase in IL-10 levels post injection persisting throughout the 20 week study (Louis et al 2000). By the time of the 20th injection, the time course of IL-10 production had become more prolonged such that high levels of IL-10 persisted 24 hours of injection rather than peaking at 8 hours and reverting to zero by 24 hours. A similar persistence of hepatic IL-10 mRNA following Con-A injection was seen up to 20 weeks. Levels of serum IL-10 measured in my study were much lower than in this paper most likely because they were sampled at a later time point after injection.

*In vivo* depletion of CD4+ cells using monoclonal anti CD4+ antibody after 5 weeks of Con-A challenge dramatically reduced serum IL-10 levels implying that CD4+ T lymphocytes are pivotal in the production of IL-10. CD8+ depletion resulted in a non significant reduction of serum IL-10 levels whereas athymic mice failed to produce any IL-10. In keeping with this study showing that CD4+ T cells are important for the generation of an IL-10 producing phenotype in response to repeated Con-A challenge, my data suggest a less pronounced induction of IL-10 in CD4KO versus WT mice. Since IL-10 has both a well established anti-inflammatory properties and potential anti-fibrotic action (Louis et al. 1998; Nelson et al. 2000; Thompson et al. 1998), reduced IL-10 production in CD4KO animals may have contributed towards their development of liver fibrosis.

Overall, it appears that the assumption that Con-A induced hepatitis and liver fibrosis are exclusively CD4+ T cell dependent represents an over-simplification given the presence of a mild but significant liver injury in CD4KO mice 24 hours after a single Con-A injection and the development of at least as much liver fibrosis in CD4KO mice as wild type controls after 12 weeks of injection. This is not wholly unexpected given the complexity of biological systems and, in particular, the redundancy of function in immune responses. It is also important, however, to recognise that gene
knockout mice may exhibit strategies to compensate for their defects such as the recruitment of other cell types to fulfil the role of a deleted subset and that these compensatory mechanisms may be particularly enhanced during the protracted time course of a chronic disease model.

In summary we found the Con A induced model to have great potential in dissecting the role of various T cell and non T cell pathways in liver injury, inflammation and fibrosis. However, our studies of the model produced more questions than answers. Further study of the model would ideally require the subjection of a range of cytokine and other knockout animals to Con A induced injury. Unfortunately these complex in vivo experiments were beyond the scope (and budget) of this work once it became apparent that this was not the model of pure CD4 T cell induced damage that we had initially, and perhaps naively, anticipated.
Chapter 7

T-lymphocyte/ Hepatic Stellate Cell Interactions *in vitro*
7.1. Context
Previous chapters in this thesis have explored the effects of specific T cell derived cytokines on hepatic stellate cell activation in vitro and detailed a novel animal model of Con-A induced liver fibrosis in which CD4+ T cells appeared to play an important role, although they proved not to be the sole mediators of liver injury. The experiments detailed in this final chapter were performed concurrently with the aim of developing a novel system in which interactions between T cells and hepatic stellate cells dependent on soluble mediators and on direct cell-cell contact could be studied in vitro.

7.2. Introduction
Studies of mice infected with the parasite Schistosoma mansoni provide evidence suggesting that development of liver fibrosis following infection may be influenced by polarisation of the CD4+ immune response to either a Th1 or Th2 profile. Overall, a reduction of Th2 cytokine responses appears to lead to a reduction liver fibrosis. In particular, administration of neutralising anti-IL-4 antibody or an IL-13 inhibitor decreases the development of liver fibrosis (Cheever et al. 1994; Chiaramonte et al. 1999) and double IL-4/IL-13 knockout mice develop minimal liver fibrosis by comparison to wild type controls (Fallon et al. 2000). Inoculation of mice with S. mansoni eggs together with IL-12 resulted in both a marked inhibition of egg induced hepatic fibrosis and polarisation of the immune response from a Th2 to a Th1 type (Wynn et al. 1995).

A similar association between Th2 predominant immune responses and increased hepatic fibrosis can be seen in other models of chronic liver injury. Repeated administration of carbon tetrachloride (CCl₄) to BALB/c (Th2 dominant) mice has been shown to result in more severe liver fibrosis than in C57Bl/6 (Th1 dominant) mice (Shi, Wakil, & Rockey 1997). Fibrotic changes in BALB/c mice were reduced by IL-4 neutralisation with antibody (Shi, Wakil, & Rockey 1997). In the same study, IFN-γ knockout mice bred on both BALB/c and C57Bl/6 backgrounds exhibited more liver fibrosis than their wild type equivalents. The only exception to the pattern is the Th2 cytokine IL-10, which appears to exert overall anti-fibrotic effects (Thompson et al. 1998).

The mechanisms via which Th1 and Th2 type responses influence the development liver fibrosis are not completely elucidated, but there is significant supportive
evidence to suggest that direct effects of Th1 and Th2 cytokines on hepatic stellate cells (HSC) may play a role. The Th1 cytokine IFN-γ inhibits collagen synthesis by human HSC in vitro and reduces proliferation, α-SMA expression and collagen production in rat HSC (Rockey & Chung 1994; Tiggelman et al. 1995) thus exerting a direct “anti-fibrotic” effect. By contrast, the Th2 cytokine IL-4 enhances collagen production by human HSC in culture (Tiggelman, Boers, Linthorst, Sala, & Chamuleau 1995) and similarly IL-13 has been shown in this thesis to increase both mouse and rat HSC proliferation and collagen synthesis.

There is also the potential for CD4+ Th1 and Th2 cells to influence HSC activation directly via cell-to-cell contact rather than by cytokine production. In co-culture experiments where either fixed peripheral blood T-lymphocytes or T-lymphocyte membrane preparations were cultured with dermal fibroblasts, direct cell-cell contact was shown to affect fibroblast collagen production at the transcriptional level (Chizzolini et al. 1998; Rezzonico, Burger, & Dayer 1998). While co-culture with fixed “resting” T-lymphocytes or T-cell membranes resulted in a slight inhibition of collagen I production, co-culture with phytohaemagglutinin (PHA) or phorbol myristic acid (PMA) activated fixed T-cell or plasma membrane fractions markedly inhibited both basal and TGF-β stimulated collagen I and III production (Rezzonico, Burger, & Dayer 1998). This inhibitory effect appeared to be mediated in part by a combination of membrane bound IFN-γ, TNF-α and IL-1α (Elsharkawy et al. 1999; Rezzonico, Burger, & Dayer 1998). Direct cell-cell contact of activated peripheral blood T-lymphocytes with human dermal fibroblasts has also been shown to result in the induction of TIMP-1 and MMP-1 (Burger et al. 1998).

I hypothesised that Th1 and Th2 lymphocytes may exert effects on HSC activation by means of both soluble mediators such as cytokines and via direct cell-to-cell contact. Evidence from the existing scientific literature suggests that Th1 and Th2 cells may have differential effects on HSC activation with Th1 cells likely to exhibit a net anti-fibrotic and Th2 cells an overall pro-fibrotic effect. In order to facilitate the examination of direct T-cell –HSC interactions in vitro, it was necessary to generate purified populations of T-lymphocytes exhibiting Th1 and Th2 phenotypes.
DO11.10 Transgenic Mice

DO11.10 transgenic mice were originally developed by Dr D.Y. Loh (Howard Hughes Medical Institute, St Louis, Missouri) to study the mechanisms of clonal deletion of autoreactive T-cells by antigen induced apoptosis (Murphy, Heimberger, & Loh 1990). These mice express an αβ T-cell receptor (TCR) specific for a chicken ovalbumin peptide so that T-lymphocytes from the mice are specific for the OVA peptide presented by the I-A^d class II molecule (Robertson et al. 2000).

T-lymphocytes isolated from the peripheral lymph nodes of DO11.10 mice can be made to differentiate into either Th1 or Th2 cells by culture in the presence of the OVA antigen in media supplemented with specific recombinant cytokines and neutralising anti-cytokine antibodies (Hsieh et al. 1992; Seder et al. 1992). The differentiated T-cell populations may then be expanded by culture on anti-CD3 coated plastic in the absence of continuing antigenic stimulation or exogenous cytokines and still retain their phenotype. This system makes it possible to generate culture media conditioned with either Th1 or Th2 cytokines and homogeneous populations of T-lymphocytes bearing either a Th1 or Th2 phenotype.

7.3. Materials and Methods

Animals

DO11.10 mice bred on a BALB/c background were a kind gift from Dr K Roberts, University Medicine, Southampton General Hospital and had originally been obtained from Dr Ed Shevach, National Institutes of Health, Bethesda, MD). Animals were bred under aseptic conditions in a barrier facility.

Materials

All tissue culture materials were obtained from Life Technologies, Paisley, UK unless otherwise stated.

T-lymphocyte culture medium RPMI 1640
penicillin 50U/ml
streptomycin 50μg/ml
HEPES 5mM (Sigma, Poole, UK)
glutamine 2mM
2-mercaptoethanol 5μM (Sigma, Poole, UK)
FCS 5%
sodium pyruvate 0.1mM
IL-12 and IL-4 (R&D Systems, Abingdon, UK) OVA 323-329 peptide, anti-IL-4 antibody (11β11 hybridoma supernatant), anti-IFN-γ antibody (HB170 hybridoma supernatant) and anti-CD3 antibody (2C11 hybridoma supernatant) were all kind gifts from Dr K Roberts as above.

7.3.1. DO11.10 T-lymphocyte Isolation and Culture

Axillary, inguinal and mesenteric lymph nodes were dissected from DO11.10 mice into tissue culture medium. In a sterile tissue culture hood, lymph nodes were teased apart and passed through a cell strainer using a syringe plunger with washes of further tissue culture medium. The resulting cell suspension was transferred into a sterile universal container and centrifuged at 1500 rpm for 10 minutes at 4°C. The supernatant was discarded and the cell pellet re-suspended in HBSS then centrifuged again at 1500rpm for 10 minutes at 4°C. Cells were resuspended in a small volume of tissue culture medium and counted in a haemocytometer.

Peripheral lymph node cells were incubated at 0.5 x 10^6/ml in complete tissue culture medium containing 1μg/ml OVA_323-329_ peptide. To drive T-cell differentiation into a Th1 effector phenotype, IL-12 5ng/ml and anti-IL-4 antibody (11β11 hybridoma supernatant used at a final concentration of 10%) were added to the culture medium. In order to generate Th2 cells, IL-4 2ng/ml and anti-IFN-γ antibody (HB170 hybridoma supernatant used at a final concentration of 10%) were used to supplement the medium. After 4 days in culture, Th1 and Th2 cells were re-plated at 2 x 10^5/ml and subjected to a second round of stimulation with fresh media containing 1μg/ml OVA peptide and the same cytokines and antibodies with the addition of IL-2 10U/ml for both subsets.

Following a further 3 days in culture, T-lymphocytes were washed three times in RPMI, counted in a haemocytometer and re-suspended in complete medium (without 2-mercaptoethanol) at 1 x 10^6/ml then plated onto 24 well plates, which had been coated overnight at 4°C with 10μg/ml anti-CD3 antibody (2C11) and washed three times with sterile PBS.

After 24 hours on the anti-CD3 coated plates, Th1 and Th2 cells were spun down at 1500rpm for 10 minutes at 4°C and the culture supernatants were harvested, pooled and stored in aliquots at −80°C until use. Th1 and Th2 cells were fixed in HBSS containing 1% parafomaldehyde, then washed twice in HBSS and once in DMEM.
0.5% FCS prior to use in co-culture experiments with murine HSC. The final DMEM 0.5% wash was reserved for use as a control for the possible effects of paraformaldehyde in fixed T-cell/HSC co-culture experiments.

7.3.2. Cytokine Measurement by ELISA

Cytokines levels in Th1 and Th2 culture supernatants were assayed by ELISA. All cytokine standards and a range of dilutions of samples were assayed in triplicate. IL-10 and IL-13 were measured using commercially available Quantikine M ELISA kits (R&D Systems, Abingdon, UK) in accordance with the manufacturer’s instructions. IFN-γ levels were determined using a Murine IFN-γ ELISA Development Kit (Peprotech EC, London) in accordance with the manufacturer’s suggestions with the following exceptions: 5% BSA rather than 1% BSA was used as the blocking buffer and as the diluent for the streptavidin-peroxidase conjugate; TMB was substituted for ABTS as the chromogen and the number of washes between stages increased to 6 times to increase assay sensitivity. IL-4 levels were determined using a commercially available ELISA kit (Biosource, Lifescreen, Watford, UK) according to the manufacturer’s instructions.

7.3.3. HSC Stimulation with Th1 and Th2 Conditioned Supernatants

BALB/c murine HSC in passages 1-3 were seeded at 0.5 × 10^6 per well into 24 well tissue culture plates and grown until just sub-confluent. HSC were then washed three times in DMEM and cultured for 24 hours in DMEM 0.5% FCS to arrest them in phase G0 of the cell cycle. Following this, HSC in triplicate wells on each plate were treated for 24 hours with T-lymphocyte culture medium (from which the addition of 2-mercaptoethanol had been omitted because of potential toxic effects on HSC) as follows: RPMI 0.5% FCS as a negative control; RPMI 5% FCS containing 25% or 50% Th1 or Th2 conditioned media by volume; RPMI 5% FCS as a positive control. Th1 and Th2 conditioned media were not used undiluted because of the anticipated non-specific effects of factors produced by rapid T-cell growth and depletion of serum. Six hours prior to the end of this 24-hour culture period, HSC were pulsed with 1μCi of ^3H-thymidine per well. The remainder of the assay was performed as previously described (2.6).
7.3.4. T-lymphocyte/HSC Co-culture

BALB/c murine HSC in passages 1-3 were plated and prepared for assay as described above. HSC were then treated for 24 hours with media as follows: DMEM 0.5% FCS as a negative control; DMEM 0.5% FCS from the final T-cell paraformaldehyde fixation wash; DMEM 0.5% FCS with $2 \times 10^6$ fixed Th1 or Th2 cells per well; DMEM 5% FCS as a positive control.

To verify the need for contact mediated signalling and exclude the potential effect of soluble factors produced by fixed T cells (Sebbag et al. 1997), duplicate experiments were carried utilising a double chamber system. HSC and T cells were seeded into the lower and upper chambers respectively of 24-well culture plates containing Transwells (Costar) with a 6.5μm nucleopore membrane permitting diffusion of soluble factors but physically separating the two cell types.

Murine HSC proliferation, as assessed by $^3$H-thymidine incorporation, was subsequently assayed as previously described (2.6).

7.4. Results

7.4.1. Cytokine Production by Th1 and Th2 Lymphocyte Populations

Measurement of IFN-γ, IL-4, IL-10 and IL-13 by ELISA in DO11.10 lymphocyte conditioned media, following treatment to generate Th1 and Th2 cells and after 24 hours stimulation with anti-CD3 demonstrated that the cells exhibited the anticipated polarised cytokine production profile characteristic of Th1 or Th2 phenotypes. For each of the four cytokines measured, the differences between the Th1 and Th2 conditioned media were highly statistically significant ($p<0.001$ in all cases). (Table 7.1, Figs 7.1-7.4).
Table 7.1. Cytokine Concentrations as Measured by ELISA in DO11.10 Th1 and Th2 Cell Conditioned Culture Media after 24 Hours Stimulation on anti-CD3 Coated Plates

<table>
<thead>
<tr>
<th>Murine Cytokine</th>
<th>Concentration in DO11.10 Conditioned Media (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Th1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>156 ± 3.6 ng/ml</td>
</tr>
<tr>
<td>IL-4</td>
<td>21.6 ± 2.3 pg/ml</td>
</tr>
<tr>
<td>IL-10</td>
<td>5 ± 0.1 ng/ml</td>
</tr>
<tr>
<td>IL-13</td>
<td>11.5 ± 0.7 ng/ml</td>
</tr>
</tbody>
</table>

Fig. 7.1 Concentration of murine IFN-γ in DO11.10 lymphocyte conditioned culture media (ng/ml) (mean ± SEM). Media were harvested after 24 hours stimulation on anti CD3 coated plates. * p<0.05 relative to Th2 conditioned media.
Fig. 7.2 Concentration of murine IL-4 in DO11.10 lymphocyte conditioned culture media (pg/ml) (mean ± SEM). Media were harvested after 24 hours stimulation on anti CD3 coated plates. *p<0.05 relative to Th2 conditioned media

Fig. 7.3 Concentration of murine IL-10 in DO11.10 lymphocyte conditioned culture media (ng/ml) (mean ± SEM). Media were harvested after 24 hours stimulation on anti CD3 coated plates. *p<0.05 relative to Th2 conditioned media
7.4.2. Effect of Th1 and Th2 Conditioned Culture Media on the Proliferation of Murine Hepatic Stellate Cells \textit{in vitro}

Treatment of murine HSC with Th1 conditioned culture media at both 25% and 50% v/v in DMEM 5% FCS resulted in a significant reduction of proliferation as measured by $^{3}$H-thymidine incorporation when compared to DMEM 5% FCS alone ($p=0.007$ and $p=0.002$ respectively). This inhibitory effect was more marked at the 50% than at the 25% concentration ($p=0.028$). Similarly, the addition of Th2 conditioned media to DMEM 5% FCS at 25% and 50% v/v significantly inhibited murine HSC proliferation compared to control ($p=0.001$ and $p=0.001$ respectively) and Th2 conditioned media at 50% concentration resulted in a greater reduction in proliferation than at 25% concentration ($p=0.005$). There was no difference between the effects of Th1 and Th2 conditioned media on murine HSC proliferation at concentrations of either 25% or 50% ($p=0.539$ and $p=0.68$ respectively). Neither
culture in Th1 conditioned media at 25% or 50% (p=0.008, p=0.012) nor Th2 culture in Th2 conditioned media at 25% or 50% (p=0.000, p=0.006) reduced murine HSC proliferation to the levels of those cultured in DMEM 0.5% FCS. Fig. 7.5.

Fig. 7.5. Effect of treatment with Th1 and Th2 conditioned media on murine hepatic stellate cell proliferation measured by $^{3}$H-thymidine incorporation (mean ± SEM) (n=4). * denotes p<0.05 compared with 5% FCS control.

7.4.3. Effect of Co-Culture with Fixed Th1 and Th2 DO11.10 Cells on Murine Hepatic Stellate Cell Proliferation in vitro

Culture of murine HSC in the DMEM 0.5% FCS used as the final wash after fixation of T cells in 1% paraformaldehyde (as a control for any effects of residual paraformaldehyde on HSC proliferation- “fixation control”) resulted in a small, but significant (p=0.005), reduction in HSC proliferation. Co-culture with both Th1 and Th2 cells suspended at $2 \times 10^6$ml$^{-1}$ in DMEM 0.5% FCS profoundly inhibited murine HSC proliferation as measured by $^{3}$H-thymidine incorporation when compared with culture in DMEM 0.5% FCS alone (p=0.001, p=0.001) and to culture in the “fixation control” (p=0.001, p=0.001). There was no difference between the effects of co-culture with Th1 and Th2 cells on murine HSC proliferation (p=0.669). Fig. 7.6.
Fig. 7.6. Effect Of Co-Culture With Fixed Th1 And Th2 DO11.10 Cells On Murine Hepatic Stellate Cell Proliferation Measured By $^3$H-Thymidine Incorporation (mean ± SEM) (n=4). * denotes p<0.05 compared to fixation control.

When experiments were repeated using a double chamber system to physically separate Th1 and Th2 cells from the murine HSC, the down-regulation of murine HSC proliferation by Th1 and Th2 cells was markedly reduced. Th1 cells reduced HSC proliferation compared to control by only 17±3% when separated from them by a nucleopore membrane and by 86±2% when in direct contact (p=0.000). Th2 cells reduced HSC proliferation by only 15±5% when seeded in to a separate chamber and by 85±2% when in direct contact (p=0.005). There was no difference between the effects of Th1 and Th2 cells whether physically separated from the murine HSC or not (p=0.729, p=0.691). Fig. 7.7.
Fig. 7.7. Effect of preventing cell-cell contact on the modulation of murine HSC proliferation by fixed Th1 and Th2 DO11.10 cells using a double chamber culture system (mean ± SEM) (n=4). * denotes p<0.05 compared to the equivalent T cell subset without separation from HSC by a nucleopore membrane.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Upper Chamber</th>
<th>Lower Chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Th1</td>
<td>mHSC</td>
</tr>
<tr>
<td>B</td>
<td>Th2</td>
<td>mHSC</td>
</tr>
<tr>
<td>C</td>
<td>mHSC + Th1</td>
<td>mHSC + Th2</td>
</tr>
<tr>
<td>D</td>
<td>mHSC + Th2</td>
<td></td>
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</tbody>
</table>

7.5. Discussion
Both in vitro data examining the effect of individual cytokines on HSC and fibroblasts and studies of animal models of liver fibrosis suggest that a Th1 predominant immune response will result in less fibrosis and a Th2 biased response in more fibrosis. In this chapter, populations of Th1 and Th2 cells were generated to examine the effect of Th1 and Th2 conditioned media and of direct Th1 or Th2 cell-HSC contact on HSC activation.
Lymph node cells from DO11.10 mice were isolated and cultured in media supplemented with cytokines and anti-cytokine antibodies and stimulated with antigen in order to generate activated T cell populations with the phenotypic characteristics of Th1 and Th2 cells. Measurement of cytokines by ELISA in media conditioned by growth of these T cell populations confirmed that they exhibited a polarised cytokine profile consistent with differentiation towards Th1 and Th2 phenotypes. Levels of IFN-γ were more than 100 times greater in Th1 than in Th2 conditioned culture media where as levels of IL-4, IL-10 and IL-13 were respectively 22, 13 and 4.5 times greater in Th2 than in Th1 conditioned media.

I found that mHSC proliferation was markedly reduced compared to control by culture in both Th1 and Th2 conditioned media and that there was no difference in the magnitude of the effects of Th1 and Th2 conditioned media. Existing literature regarding the effects of Th1 and Th2 cytokines and data from this thesis demonstrate that IFN-γ inhibits HSC proliferation (Baroni et al. 1996; Mallat et al. 1995; Rockey et al. 1992), IL-10 has no effect and both IL-4 and IL-13 stimulate HSC proliferation. These data suggest that, in contrast to my findings, culture of HSC in Th1 conditioned media would lead to reduced proliferation and culture in Th2 conditioned media would result in increased proliferation.

At the dilutions used in the experiments, all of the cytokines measured were present within concentration ranges at which they are known to exert biological activity in vitro (IFN-γ 0.1-10ng/ml (manufacturer’s data, Peprotech EC); IL-10 0.2-20ng/ml (manufacturer’s data, Peprotech EC); IL-4 and IL-13 (chapter 5) except in the case of IL-4 where concentrations fell below the biologically active range in Th1 conditioned media. This suggests that factors other than the cytokines themselves may have been important in exerting the anti-proliferative effect on mHSC and there are a number of possible mechanisms.

Firstly, rapid growth of the activated T cells over a 24-hour period may have depleted the media of nutrients or co-factors necessary for HSC proliferation. Secondly, T cell metabolism during growth in culture may have resulted in the production of factors which act non-specifically to have a toxic effect on HSC. Finally, T cells may produce a soluble mediator which has a specific anti-proliferative action on HSC and which is produced equally by Th1 and Th2 cell populations. The persistence of a very marked reduction in mHSC proliferation when
treated with T cell conditioned media diluted to 25% by volume in fresh culture media suggests that nutrient depletion is less likely as an explanation for the findings than the presence of either toxic products of T cell metabolism or specific T cell factors, since they could continue to exert their effects at low concentrations. Further elucidation of the mechanisms involved in this system would have required extensive experimentation to identify putative mediators in the conditioned media using fractionation techniques and either antagonists or neutralising antibodies and was beyond the scope of this thesis.

Co-culture with both Th1 and Th2 fixed T cells also exerted a profound anti-proliferative effect on murine HSC with no difference between the effects of the two subsets. The magnitude of the reduction in mHSC proliferation was much greater than that accounted for by the non-specific effect of residual paraformaldehyde used as a fixative and was almost completely abrogated by physical separation of the T cell and HSC populations by a nucleopore membrane demonstrating that direct cell-to-cell contact was required.

To my knowledge, there is no literature examining contact mediated interactions between T cells and HSC but existing studies of cell-to-cell interactions between T lymphocytes and fibroblasts demonstrate a number of putative contact mediated mechanisms via which T cells could influence fibroblast activation. Co-culture of PHA or PMA activated peripheral blood T lymphocytes or membrane fractions with human dermal fibroblasts resulted in a marked reduction in both collagen I and collagen III synthesis (Rezzonico, Burger, & Dayer 1998). This effect occurred at a transcriptional level and appeared to be mediated largely by the additive effects of T cell membrane bound TNF-α, IL-1α and IFN-γ (Rezzonico, Burger, & Dayer 1998). When peripheral blood T lymphocytes were stimulated with plate bound anti-CD3 and plasma membrane preparations from them co-cultured with dermal fibroblasts, the resultant down-regulation in collagen I synthesis was virtually abrogated by IFN-γ neutralisation (Chizzolini, Rezzonico, Ribbens, Burger, Wollheim, & Dayer 1998). Although membrane bound IFN-γ would be expected to reduce mHSC proliferation and would seem to be a potential candidate molecule for mediating the effects seen in my studies, it is preferentially expressed on Th1 cells (Assenmacher et al. 1996) and would therefore be less likely to account for an effect seen equally in both Th1 and Th2 subsets.
Other putative cell surface molecules which might mediate direct T cell-HSC interaction include CD40 ligand (CD40L) and CD40, which are members of the TNF and TNF receptor superfamily. CD40 expression has been detected in human lung, gingival, synovial, dermal and spleen fibroblasts (Fries et al. 1995) and CD40L expression is mainly found on activated CD4+ T cells though it is also expressed by basophils, eosinophils, activated B cells and dendritic cells (Van Kooten & Banchereau 1997). Engagement of CD40 on gingival fibroblasts by CD40L results in a reduction in MMP-1 and MMP-3 production (Wassenaar et al. 1999) and stimulates IL-6 production (Sempowski et al. 1997). Similarly, soluble CD40L and anti-CD40 antibody stimulate normal human lung fibroblasts to mobilise NF-κB and produce the pro-inflammatory cytokines IL-6 and IL-8 (Sempowski, Chess, & Phipps 1997). In skin and synovial fibroblasts, CD40 ligation induced fibroblast proliferation as assessed by ^H-thymidine incorporation, cell counting and cell cycle analysis (Rissoan et al. 1996). By analogy with these other mesenchymal cells, it would be anticipated that CD40 ligation would result in a stimulation of mHSC proliferation rather than the inhibitory effect seen in the current study.

Further experimentation to neutralise T cell surface cytokines and examine the effects of CD40 ligation on HSC may clarify whether these molecules have a role in mediating T cell-HSC interaction in this system.

The profound effects of both Th1 and Th2 conditioned media and fixed Th1 and Th2 cells in inhibiting HSC proliferation may potentially be biologically important. However, the marked reduction in HSC proliferation also generated technical difficulties which, in the timescale available, precluded planned work to explore the effects of manipulation of Th1/Th2 balance on other facets of HSC activation such as collagen, matrix metalloproteinaise and TIMP synthesis using this experimental system.
Summary
Summary

The work described in the thesis set out to examine the hypothesis that changes in the balance of CD4+ T cell sub-populations between Th1 and Th2 phenotypes could modulate the development of fibrosis in liver disease via direct interactions with hepatic stellate cells. This is in contrast to most previous studies, in which the focus of attention has been on hepatic macrophages as intermediaries. Existing published data has suggested that Th2 responses may be associated with more, and Th1 responses with less, liver fibrosis. It has not been possible to either prove or disprove the hypothesis and this thesis has highlighted more unanswered questions and further avenues for study than it has provided answers.

However, I have demonstrated that T cell derived cytokines exert profound effects on hepatic stellate proliferation and aspects of hepatic stellate cell activation. The observations that IL-13 directly stimulates HSC proliferation and collagen production are entirely novel and, as yet, have not been examined by other groups. These data provide further supportive evidence that a bias towards Th2 dominated immune responses in vivo may lead to more liver fibrosis.

Examination of the Con-A induced liver fibrosis in mice revealed that this experimental model did not provide a straightforward model of CD4+ mediated liver injury and fibrosis I had anticipated and, as such, did not provide the framework for manipulation of Th1/Th2 balance I had anticipated. This highlights the complexity and potential redundancy of biological systems in contrast to the relative simplicity of the models we construct in order to try to understand them. Nevertheless, it is likely that the Con-A model of liver fibrosis will provide further insights into the interactions between activated inflammatory cells and stellate cells in vivo.

Finally I have shown that it is possible to study the action of Th1 and Th2 cell subsets on hepatic stellate cells in vitro using an entirely novel experimental system which may provide further insights in due course.

Liver disease is an increasing problem in the UK. Deaths from liver disease have risen 8-fold since the 1970’s (Chief Medical Officers Report 2001). Approximately 20% of the population have fatty liver and nearly 0.5% have active hepatitis C virus infection. However, only a proportion of individuals go on to develop significant
liver fibrosis regardless of the nature of the insult, suggesting that there are a host of
genetic, environmental or immunological factors which modulate the host response
to liver injury. The elucidation of the role of these factors and the potential for
therapeutic intervention will occupy researchers for some time to come.
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