The role of PAR-1, PAR-2 and Tissue factor in the development of hepatic fibrosis

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I declare that the work described is of my own composition and was conducted between February 2006 and August 2012. This thesis has not been submitted in whole or in part for any other degree or diploma at this or any other university. All work described in this thesis was performed and analysed by myself except where otherwise acknowledged.

This thesis is not greater than 100,000 words in length.

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Virginia Knight

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Hepatology 2012; 55(3):879-87.

The cytoplasmic domain of tissue factor in experimental liver fibrosis and its interaction with PAR-2. Knight V, Tchongue J, Lourensz D, Tipping P, Sievert W Manuscript in preparation

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2009:

Knight V, Tchongue J, Lourensz D, Liu A, Tipping P, Sievert W *Deletion of Protease Activated Receptor 2 ameliorates liver fibrosis and decreases TGFβ expression in a murine model of cirrhosis.* Hepatology Vol 50, Suppl 4, 826A. October 2009

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Abstract

Hepatic fibrosis and cirrhosis are the common endpoint of a variety of liver diseases and represent a major global health burden. The current model for hepatic fibrosis development is that progressive injurious stimuli lead to dysregulation of extracellular matrix (ECM) turnover. Activation of the hepatic stellate cell (HSC) has been identified as the key cellular event resulting in the accumulation of extracellular matrix (Friedman 2008) and therefore there is considerable interest in factors that regulate HSC activation and collagen expression.

There is a strong linkage between inflammation, coagulation and fibrosis (Tacke, Luedde et al. 2009). One proposed mechanism for this linkage is signalling by coagulation factors through their cellular receptors protease-activated receptors (PARs) to activate stellate cells (Anstee, Wright et al. 2009).

This thesis has explored the role of PAR-1, PAR-2 and the cytoplasmic domain of tissue factor in the development of hepatic fibrosis. The close relationship between the coagulation cascade and the inflammatory response led to the hypothesis that coagulation factors and their receptors may play an important role in hepatic fibrogenesis.

In order to mimic human liver disease processes, a mouse model was studied using carbon tetrachloride administration to generate liver fibrosis. Mice with deletion of the PAR-1 gene, PAR-2 gene, with deletion of the cytoplasmic domain of TF and with dual deletion of PAR-2 gene and TF cytoplasmic domain were individually studied and compared to wildtype. Common fibrosis endpoints were studied in vivo. In vitro experiments were performed with a line of human hepatic stellate cells.

Initial experiments demonstrate PAR-1 deficiency protects against liver fibrosis with reduced histological fibrosis, hydroxyproline content, TGF β gene and protein expression seen. This adds evidence to support the view that PAR-1 is involved in hepatic fibrogenesis. PAR-2 deficiency was also found to afford protection from hepatic fibrosis.

PAR-1 and PAR-2 activation also induce a profibrogenic phenotype in human hepatic stellate cells *in vitro* adding weight to the evidence these receptors are important in fibrosis development.

In addition to its important role in haemostasis, tissue factor is increasingly recognised as a signalling receptor in a number of non coagulant roles. Deletion of the cytoplasmic domain of tissue factor led to reduction in profibrogenic cytokines, HSC activation and reduced macrophage recruitment and activation which supports the reduced hepatic fibrosis observed.

Macrophages play a pivotal role as regulators of fibrosis. They are profibrogenic in fibrosis development but also play a role and are necessary for fibrosis resolution. The reduced macrophage recruitment and activation observed in the PAR-2 and mice with deletion of the cytoplasmic domain of tissue factor may in part explain the amelioration of hepatic fibrosis seen in these mice.

A single treatment to completely ameliorate fibrosis may be difficult to achieve given the complex and multiple pathways involved in ECM remodelling. Understanding the mechanisms of fibrosis provide a platform to develop antifibrotic therapies. This thesis has provided further insight into the role of PAR-1 and PAR-2 and the cytoplasmic domain of tissue factor in hepatic fibrogenesis. Both PAR-1 and PAR-2 antagonists are being developed and may represent a novel therapeutic approach in preventing fibrosis in patients with liver disease. The cytoplasmic domain of tissue factor is an attractive therapeutic target as the coagulation is not affected in the host, particularly important in patients with cirrhosis.

Abbreviations

AC	adenylyl cyclase
ADP	adenosine diphosphate
ALT	alanine transaminase
AMP	adenosine monophosphate
ANOVA	analysis of variance
aPC	activated protein C
αSMA	alpha smooth muscle actin
AT	anti thrombin
BAMBI	BMP and activin membrane bound inhibitor
BHK	baby hampster kidneys
BMM	bone marrow derived macrophages
BMP	bone morphogenetic protein
CAS	cellular apoptosis susceptibility
c/EBP	CCAAT enhancer binding protein
CB	cannabinoid
CTGF	connective tissue growth factor
CXCR	chemokine receptor
DAB	3,3’ diaminobenzidine
DAG	diacylglycerol
DR	ductular reaction
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
EMT	epithelial mesenchymal transition

EPCR	endothelial protein C receptor
ERK	extracellular signal regulated protein kinase
FLNA	active binding protein filament A
GEF	guanine nucleotide exchange factors
GP	glycoprotein
GRK	G protein receptor kinases
HMBG1	high mobility group box 1
HSC	hepatic stellate cells
HPCs	hepatic progenitor cells
ICAM	inter-cellular adhesion molecules
IL	interleukin
INR	international normalised ratio
$InsP_3$	inositol 1,4,5 triphosphate
JAK-STAT	janus kinase signal transducer and activator of transcription
JNK	c-JUN N-terminal protein kinases
Ki	potassium channels
KO	knockout
LAP	latency associated peptide
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MCP	monocyte chemoattractant protein
MDP	monocyte dendritic cell progenitor
MHC	major histocompatibility complex
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin

NASH	non alcoholic steatohepatitis
NF	nuclear factor
NFkB	nuclear factor kappa B
NK	natural killer cells
NKT	natural killer T cells
NO	nitric oxide
PAI	plasminogen activator inhibitor
PAR	protease activated receptor
PCR	polymerase chain recation
PDGF	platelet derived growth factor
PKC	protein kinase C
PLC	phospholipase C
RNA	ribonucleic acid
ROS	reactive oxygen species
R-Smad	receptor activated Smad
RT-PCR	real time polymerase chain reaction
SEM	standard error of mean
SMA	smooth muscle cells
SRE	serum response elements
TAFI	thrombin activatable fibrinolysis inhibitor
TBM	thrombomodulin
TBW	total body weight
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGFβ1	transforming growth factor β1
TIMP	tissue inhibitor metalloproteinases

TK	tyrosine kinases
TLR	toll like receptor
TNF	tumour necrosis factor
TRAIL	tumour necrosis factor related apoptosis inducing ligand
VEGF	vascular endothelial growth factor
WT	wildtype

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CHAPTER 1

Literature Review

1 Literature review

1.1 Introduction

Hepatic fibrosis and cirrhosis are important global health problems. Hepatic fibrosis is a wound healing response to chronic liver injury from a variety of causes including viral hepatitis B and C, non alcoholic steatohepatitis, alcohol and genetic conditions such as Wilson's disease or hereditary haemochromatosis. Regardless of the cause of liver injury, ultimately the resultant fibrosis may progress to cirrhosis which can lead to the development of life threatening complications of portal hypertension, hepatocellular carcinoma and liver failure.

The hepatic stellate cell (HSC) is the principal source of collagen produced during hepatic fibrogenesis (Friedman 2008) and thus there is considerable interest in factors that regulate HSC activation and collagen expression.

There is a strong linkage between inflammation, coagulation and fibrosis (Tacke, Luedde et al. 2009) and a prothrombotic state appears to accelerate liver fibrogenesis (Anstee, Wright et al. 2009). One proposed mechanism for this linkage is signalling by coagulation factors through their cellular receptors, Protease Activated Receptors (PARs), to activate stellate cells (Anstee, Wright et al. 2009, Borensztajn, von der Thusen et al. 2010).

This thesis aims to explore the interaction between coagulation, inflammation and fibrosis by characterising the roles of coagulation receptors PAR-1 and PAR-2 and the coagulation factor, tissue factor, in the development of liver fibrosis. The following chapter will review the pathogenesis of liver fibrosis and discuss the roles of the coagulation factors and their receptors in both coagulation and inflammation.

1.2 Liver fibrosis and Cirrhosis

Liver fibrosis is the wound healing response and common endpoint to chronic liver injury from a variety of causes which include viral hepatitis, alcohol, autoimmune conditions and non alcoholic steatohepatitis. Although acute injury can activate some of the mechanisms of fibrogenesis, in general chronic stimuli are required for significant fibrosis to develop. With ongoing injury, fibrosis can ultimately progress to the most advanced stage, cirrhosis. Cirrhosis is characterised by fibrous septae and nodule formation with subsequent distortion of the normal liver architecture which can impact on liver function and lead to liver failure (Friedman 2008).

There has been considerable progress in our understanding of the pathogenesis of hepatic fibrosis over the last couple of decades with identification of the HSC as the primary source of extracellular matrix, a better understanding of the roles of innate immune cells such as macrophages in both fibrogenesis and fibrolysis and increasing understanding into the mechanisms of matrix turnover.

1.2.1 The Hepatic Microenvironment

The liver consists of two major cell types. The hepatic parenchyma is composed primarily of hepatocytes which account for up to 60% of cells and 80% of total liver cell volume. The remainder of the liver volume is comprised of non parenchymal cells that include Kupffer cells, endothelial cells and hepatic stellate cells.

When viewed under light microscopy the liver appears to be arranged in lobules with a central vein at the centre surrounded by 4-6 portal tracts at the periphery which each contain a branch of the biliary tree, hepatic artery and portal vein. This functional unit of the liver is called the acinus. Arterial and portal venous blood entering the acinus from the portal tracts flow into the hepatic sinusoid which in turn flow towards the central vein at the centre of the lobule, before draining into larger hepatic veins and eventually the vena cava.

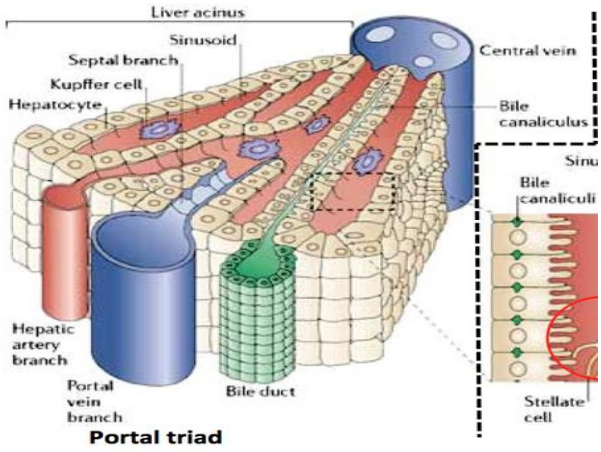


Figure1.1 Functional unit of the liver – the Acinus

Blood flows from the portal vein and hepatic artery through sinusoids to the central vein. The sinusoids are lined by fenestrated endothelial cells which allows exchange between blood and the hepatocytes. (source: **www.biologymad.com**)

Fenestrated endothelial cells line the sinusoid. The subendothelial Space of Disse separates the hepatocytes from the sinusoidal endothelium and

contains a basement membrane like matrix. The endothelial fenestrations allow free flow of plasma into the Space of Disse and thus the plasma has direct contact with the microvilli on the basolateral surface of hepatocytes. This close contact and free flow of plasma between the hepatic sinusoid and hepatocytes is important for hepatocytes to be able to perform their numerous vital functions including protein synthesis and gluconeogenesis.

Kupffer cells are resident tissue macrophages and make up 80-90% of the tissue macrophages in the reticuloendothelial system. Kupffer cells reside in the vascular sinusoidal space predominantly in periportal areas where they are exposed to incoming blood (Malarkey, Johnson et al. 2005). They have a number of functions including phagocytosis, processing of ingested material, cytokine secretion and removal of apoptotic cells (Marra, Aleffi et al. 2009).

Residing in the Space of Disse are HSC. In their quiescent state they are the major site of storage for retinoids and comprise approximately 5-8%of total liver cell population (Figure1.2).

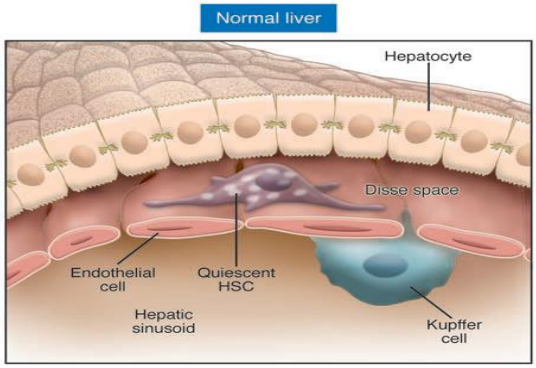


Figure 1.2 The hepatic microenvironment

The hepatocytes are separated from the fenestrated endothelial cells by the Space of Disse in which quiescent HSC are located. The Kupffer cells reside in the endothelial space. (Iredale 2007)

1.2.2 Extracellular Matrix in Normal and Fibrotic Liver

The extracellular matrix (ECM) refers to the macromolecules that make up the framework in both normal and fibrotic liver. These macromolecules include collagens, non-collagen glycoproteins, glycosaminoglycans, proteoglycans and matricellular proteins.

There are two types of matrix in the normal liver (Arthur 2000)

1. The Interstitial / Fibrillar Matrix which is composed of Collagen Type I, III, V and XI and is found in the liver capsule, portal tracts and the medium and large sized blood vessels

2. Basement Membrane like Matrix which is composed mainly of non fibril forming collagens type IV, VI, XIV, Laminin and proteoglycans and is found in the subendothelial space

There are significant quantitative and qualitative changes that occur in the ECM with progressive liver injury. There is an increase in the total ECM with increases in fibril-forming collagens (Types I, III and V), non-fibril forming collagens (Type IV and VI), glycoproteins (including fibronectin and laminin) proteoglycans (with an increase in those containing chondroitin and dermatan) and other glycosaminoglycans.

Over time qualitative changes in the composition of the ECM found in the subendothelial space occur, with a change from the normal low density

basement membrane like matrix to the interstitial type (Gressner 1998). This leads to distortion of the sinusoidal architecture, affects solute transport from the sinusoid to the hepatocyte and has implications for the function of a number of cells including the hepatocytes, endothelial cells and hepatic stellate cells (McGuire, Bissell et al. 1992).

1.2.3 Cellular sources of ECM in normal and fibrotic liver

The activated hepatic stellate cell is the primary source of ECM in normal and fibrotic liver (Friedman 1999). It predominantly originates from the quiescent hepatic stellate cell precursor found in the Space of Disse. There has been controversy about the relative importance of other sources of the activated hepatic myofibroblast (Iredale 2008) which includes portal fibroblasts (Wells, Kruglov et al. 2004), bone marrow derived cells (Forbes, Russo et al. 2004) and possibly fibroblasts derived from epithelial-mesenchymal transition (EMT); although this is more established for renal disease than liver fibrosis (Kalluri and Neilson 2003). It has been proposed the cellular source of the fibrogenic cells differs dependent on the aetiology of liver disease for example portal fibroblasts may be more important in cholestatic liver disease and ischaemia (Beaussier, Wendum et al. 2007) however this is controversial. A recent study confirmed that hepatic stellate cells are the dominant source of the activated hepatic myofibroblast. The study demonstrated that LratCre positive hepatic stellate cells in vivo give rise to 82-96% of myofibroblasts in models of toxic, cholestatic and fatty liver disease (Mederacke, Hsu et al. 2013).

1.2.4 Degradation of the ECM

In the normal liver, homeostasis of ECM is achieved by a balance between matrix production and degradation. This is regulated by a group of enzymes called matrix metalloproteinases (MMPs) and their specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs). The MMP family consists of calcium dependent enzymes that are secreted as zymogens and activated by cleavage of their propeptide (Hemmann, Graf et al. 2007).

MMPs can be divided based on their main substrate specificity, although there is a degree of overlap among the groups. The main five categories are 1) Interstitial collagenases 2) Gelatinases 3) Stromelysins 4) membrane type MMPs and 5) metalloelastase (Benyon and Arthur 2001).

Of the known MMPs only a few are expressed in liver tissue and their activity is closely regulated by binding to their specific inhibitors, TIMPs. The balance between the MMPs and TIMPs determines the degree of modelling and remodelling of a normal and fibrotic liver (Hemmann, Graf et al. 2007).

1.2.4.1 Pathologic matrix degradation

The MMP and TIMP expression profiles change throughout the progression from acute liver injury to fibrosis and ultimately to cirrhosis.

The early degradation of matrix occurs in the subendothelial space and is regulated by at least 4 enzymes. These include:

- MMP2 (gelatinase A) and MMP9 (gelatinase B) which degrade type IV collagen,

- MMP 14 (which is also called membrane-type metalloproteinase-1) which activates latent MMP2
- MMP 3 (stromelysin) which degrades glycoproteins and proteoglycans and can activate latent MMPs.

MMPs are widely expressed by epithelial and non-epithelial cells. In the liver, stellate cells are the main source of MMP 2 (Milani, Herbst et al. 1994), MMP 14 and MMP 3 (Vyas, Leyland et al. 1995). MMP 9 is produced by Kupffer cells (Benyon and Arthur 2001). MMP 2 levels are markedly increased in cirrhosis (Benyon, Iredale et al. 1996).

Type 1 collagen is the main collagen found in a fibrotic liver. Progressive fibrosis reflects a failure of degradation of this increasingly produced interstitial collagen. MMP1 is the main protease that degrades type 1 collagen. Although stellate cells express MMP1 mRNA and levels are increased in liver tissue in fibrosis, minimal levels are detected of the active enzyme (Milani, Herbst et al. 1995). There is however increasing expression with fibrosis of TIMP1 (Murawaki, Ikuta et al. 1997) and TIMP 2 (Herbst, Wege et al. 1997) both produced by stellate cells (Nie, Duan et al. 2004) (Iredale 2001) which leads to reduction of both MMP 1 and 2 activity resulting in a net decrease in protease activity and subsequently reduced collagen Type 1 degradation. In rodents there is no MMP homologous to MMP1 but MMP13 is structurally and functionally similar (Hemmann, Graf et al. 2007).

With cessation of liver injury there is a reversal of this pattern of MMP/TIMP expression. TIMP-1 is rapidly downregulated with resultant increase in degradation of matrix and possible regression of liver fibrosis (Benyon and Arthur 2001) (Figure 1.3).

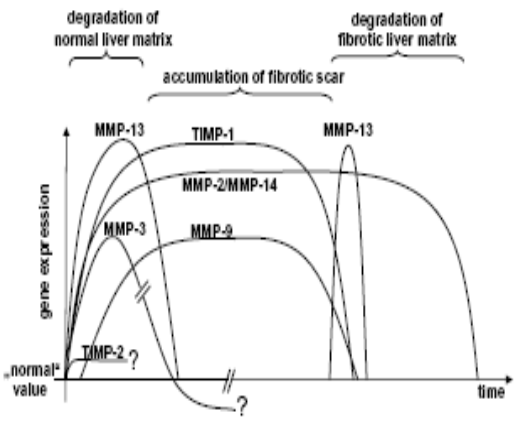


Figure 1.3 Possible gene expression profiles of MMP and TIMPS with development and resolution of Liver Injury.

With liver injury and resolution there are changing levels of specific MMP and TIMPs (Hemmann, Graf et al. 2007).

1.2.5 Pathogenesis of Fibrosis- the Hepatic Stellate Cell

The HSC, the resident liver pericyte found in the subendothelial Space of Disse, is normally found in a quiescent, vitamin A storing state. HSC activation to a myofibroblast phenotype is the key event in the pathogenesis of fibrosis and it has been proposed to occur in 2 major phases: initiation and perpetuation which can then be followed by fibrosis resolution if the injury resolves (Friedman 2008). This process will be discussed in more detail in the following section but is summarised in Figure 1.4.

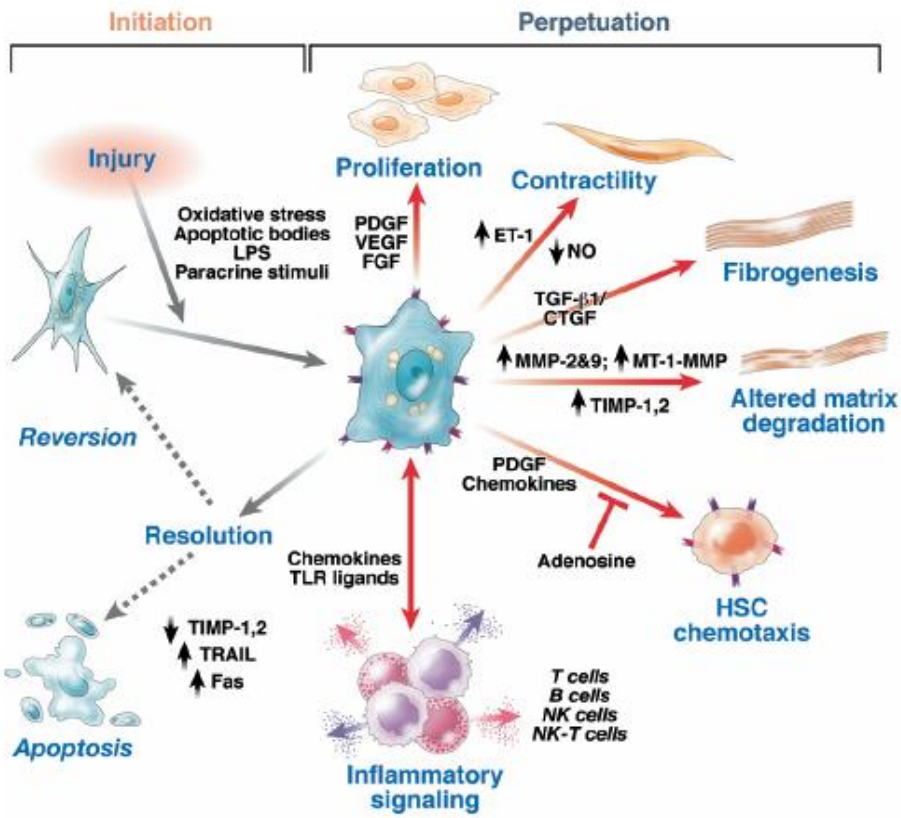


Figure 1.4 Pathways of Hepatic Stellate Cell activation

The activation of the HSC is a key event in hepatic fibrosis and has been proposed to be divided into 2 phases: initiation and perpetuation (Adapted from Friedman 2008).

1.2.5.1 Hepatic Stellate Cell Activation: Initiation

The earliest changes in HSC result from paracrine stimulation from all neighbouring cells (Friedman 2000) including platelets, endothelial cells, Kupffer cells and hepatocytes.

Platelets are the first cells recruited to a site of injury forming aggregates to prevent blood loss and play a role in wound healing. Platelets release mitogenic factors such as platelet derived growth factor (PDGF), transforming

growth factor β1(TGFβ1) and epidermal growth factor (EGF) to provide sources of paracrine stimulation for HSC (Bachem, Melchior et al. 1989).

Endothelial cells act with platelets in the conversion of latent TGFβ1 to active TGFβ1 (Li and Friedman 1999) as well as secreting TGFβ1, the most potent fibrogenic cytokine. Endothelial cells in normal liver produce a number of ECM components including types III and IV collagen, laminin and fibronectin. Sinusoidal endothelial cells may play a role in early fibrosis by release of a variant of fibronectin, EIII-A, which activates HSCs (Jarnagin, Rockey et al. 1994). This response is TGF-β1 dependent and therefore induction of this cytokine needs to occur first (George, Wang et al. 2000).

Activated Kupffer cells release cytokines (especially TGFβ 1) and reactive oxygen intermediates (Bilzer, Roggel et al. 2006) which lead to activation of stellate cells. In contrast, Kupffer cells can also induce HSC apoptosis by different mechanisms including caspase-9 dependent apoptosis (Fischer, Cariers et al. 2002).

Hepatocytes release reactive oxygen species which stimulate HSC activation. Hepatocytes also undergo apoptosis with injury and the resultant apoptotic bodies are engulfed by Kupffer cells which lead to the secretion of the proinflammatory cytokine tumour necrosis factor (TNF) α and increased expression of the death ligands TNF related apoptosis-inducing ligand (TRAIL) and Fas ligand. The generation of death ligands promotes further hepatocyte damage in a feedback loop (Canbay, Taimr et al. 2003).

DNA from damaged hepatocytes can interact with Toll like Receptor 9 (TLR9) which is expressed on HSC. Watanabe et al demonstrated that hepatocyte DNA led to upregulation of TGF β1 and collagen mRNA in vitro in human HSC and in a primary mouse HSC and this action could be inhibited by TLR9 antagonists (Watanabe, Hashmi et al. 2007).

1.2.5.2 Hepatic Stellate Cell Activation: Perpetuation

Once activated, HSCs are exposed to ongoing stimulation from paracrine and autocrine sources. Perpetuation of stellate cell activation involves a number of changes in cell behaviour which are detailed below and are interdependent. These changes ultimately lead to an increase in the ECM and progression of fibrosis.

a. Hepatic Stellate Cell Proliferation

HSC proliferation is an early event following activation. PDGF is the most potent mitogen for HSC identified (Pinzani 2002) and PDGF signalling is capable of inducing its own expression (Alcolado, Arthur et al. 1997). There are different isoforms of PDGF: A,B ,C and D. The A and B chains of PDGF have been well characterised, but the more recently identified D isoform may be the most potent and important PDGF subunit for stellate cell activation (Borkham-Kamphorst, van Roeyen et al. 2007).

Other stellate cell mitogens include thrombin and its receptor PAR-1, vascular endothelial growth factor (VEGF), EGF, TGFα, keratinocyte growth factor and basic fibroblast growth factor (Friedman 2008).

b. Hepatic Stellate Cell chemotaxis

Stellate cells can migrate towards cytokine chemoattractants. Potential chemoattractants include PDGF, monocyte chemoattractant protein-1 (MCP-1) and chemokine receptor (CXCR) 3 ligands (Friedman 2008).

In contrast, adenosine acts to inhibit chemotaxis and may immobilise cells once they reach the site of injury (Hashmi, Hakim et al. 2007). Hepatocyte DNA can also inhibit PDGF-mediated HSC chemotaxis thereby immobilising the HSC at the site of hepatocytes undergoing apoptosis (Watanabe, Hashmi et al. 2007).

c. Hepatic Stellate Cell Fibrogenesis

Stellate cells cause fibrosis by increasing ECM production and scar formation. Collagen type 1 is a principal component of the scar and its production is regulated both transcriptionally and post-translationally by hepatic stellate cells (Friedman 2008).

The most potent profibrogenic cytokine is TGF β1 that stimulates the transdifferentiation of the HSC to a myofibroblast-like phenotype that promotes collagen deposition. TGF β1 is predominantly derived from infiltrating T cells, hepatocytes, resident macrophages and infiltrating monocytes (Heymann, Trautwein et al. 2009). However TGF β1 can conversely inhibit inflammation and induce hepatocyte apoptosis (Gressner, Weiskirchen et al. 2002). The potential profibrotic or antifibrotic effect of TGFβ1 is determined by the cell producing the cytokine. To illustrate TGFβ1 produced by regulatory T cells has been shown to reduce TGFβ1 mediated

hepatic fibrosis via an interleukin (IL)-10 dependent mechanism whereas macrophage derived TGFβ1 is typically profibrotic (Wynn and Barron 2010). TGFβ1 also stimulates production of other matrix components including fibronectin and proteoglycans.

TGFβ1 is synthesized and secreted as a precursor with an attached latency-associated peptide (LAP). The release of active TGFβ1 is mediated by a number of factors including MMPs, plasmin, plasminogen and thrombospondins. Activated TGFβ1 binds to the TGFβ type II receptor and this leads to recruitment of the TGFβ Type 1 receptor and its subsequent phosphorylation by the type II receptor. The type 1 receptor in turn phosphorylates Smad2 and Smad3, called the receptor-activated Smad (R-Smad) that joins with Smad 4 and is translocated from the cytoplasm to the nucleus where target gene transcription is regulated. In contrast Smad7 is induced by TGFβ1and acts as an inhibitory Smad by interfering with the phosphorylation of Smad2 and Smad3 (Inagaki and Okazaki 2007) (Figure 1.5).

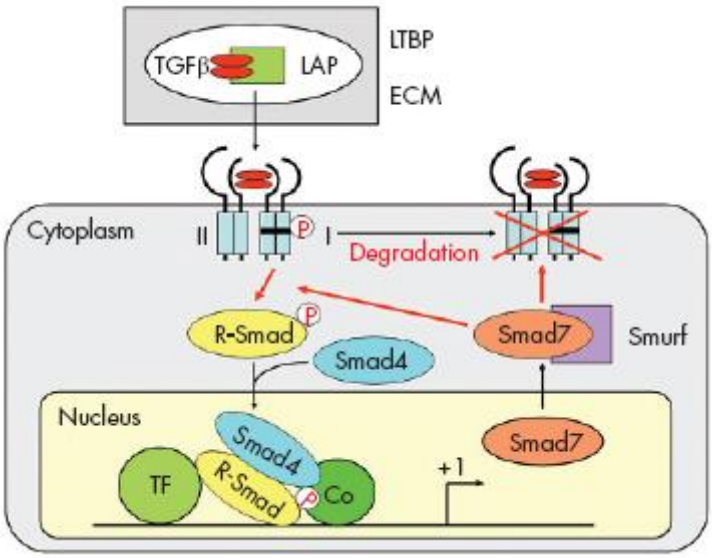


Figure 1.5 Signal transduction pathways of TGFβ

TGFβ binds to its specific receptors on the cell surface which leads to activation of R-Smad which binds with Smad4 to translocate into the nucleus to regulate target gene transcription (Inagaki and Okazaki 2007).

TGFβ1 also stimulates collagen transcription in stellate cells through a hydrogen peroxide and CCAAT enhancer binding protein (C/EBP) beta - dependent mechanism, involving Rho kinase (Shimada, Staten et al. 2011).

Connective tissue growth factor (CTGF) is also a potent profibrogenic stimulus that is upregulated in liver fibrosis (Williams, Gaca et al. 2000). TGFβ1 stimulates CTGF production predominantly from hepatocytes but it is also recognised that TGFβ1 independent mechanisms are involved in CTGF stimulation from HSC which is another important source (Brigstock 2003) (Gressner, Lahme et al. 2007). Thrombin can stimulate CTGF messenger RNA (mRNA) and protein expression in fibroblasts via activation of PAR-1 receptor (Chambers, Leoni et al. 2000).

Cannabinoids (CB) are also powerful mediators of HSC activation and fibrosis (Mallat, Teixeira-Clerc et al. 2007) and are also involved in regulating the vasodilated state associated with advanced liver disease (Batkai, Jarai et al. 2001). There are 2 receptors: CB1 which signals a fibrogenic pathway and CB2 which is antifibrotic.

d. Hepatic Stellate Cell Contractility

Contractility of HSC may play a role in the increase in portal resistance seen in fibrosis. During the development of the myofibroblast phenotype there is increased expression of the cytoskeletal protein alpha smooth muscle actin (Friedman 2008) which increases the contractile activity of HSC. Given the perisinusoidal position of the HSC, increased contractility can lead to reduction in sinusoidal blood flow and the subsequent portal hypertension that can complicate cirrhosis (Rockey 2001).

Endothelin 1 is a major stimulus for stellate cell contraction and the receptor is found on both quiescent and activated stellate cells (Housset, Rockey et al. 1993) and its receptor develops increased sensitivity in the presence of autocrine endothelin 1 (Reinehr, Kubitz et al. 1998). HSC contraction can be antagonised by local vasodilators especially nitric oxide (Rockey 2001).

1.2.6 Hepatic progenitor cells and fibrosis

Hepatic progenitor cells (HPCs) have been implicated in fibrogenesis. HPCs are bipotential stem cells that are able to differentiate toward mature hepatocytes and cholangiocytes that in normal liver have a low proliferative

rate. HPCs are activated when the mature epithelial cells of the liver are continuously damaged or severe cell loss occurs which can result from acute and chronic liver disease. Once activated, HPCs expand from the periportal zones where they reside, to the pericentral zone leading to strands of HPCs forming reactive ductules or the ductular reaction (DR) (Carpino, Renzi et al. 2013)

HPC activation and the expansion of the DR have been independently associated with progressive fibrosis in non-alcoholic steatohepatitis (NASH) and hepatitis C. (Nobili, Carpino et al. 2012) The DR has been shown in several human diseases to produce fibrogenic cytokines such as PDGF and TGF-β thus promoting collagen production and hepatic fibrosis. (Carpino, Renzi et al. 2013)

1.2.7 The role of the immune system in fibrosis

The innate and adaptive immune systems play a role in fibrogenesis. Activated HSCs secrete proinflammatory cytokines and chemokines that lead to recruitment of inflammatory cells as part of the wound healing response. Inflammatory cells infiltrate to contain the infectious insult, phagocytose damaged cells or react against an auto antigen, however unintentionally this can worsen the injury through generation of soluble mediators and oxidative stress-related products that provide further fibrogenic stimuli (Marra, Aleffi et al. 2009). Following is a brief description of the involvement of mononuclear cells in hepatic fibrogenesis.

Kupffer cells are the resident tissue macrophages of the liver that are found in the subendothelial space. Kupffer cells can be activated directly by foreign agents such as the endotoxin lipopolysaccharide (LPS) or can be activated by the by products released by injured hepatocytes such as reactive oxygen species and apoptotic bodies. Macrophages play an important role in the development of hepatic fibrosis. Macrophages secrete profibrotic mediators that activate fibroblasts, influence matrix turnover via production of MMP and TIMP, secrete chemokines that recruit fibroblasts and inflammatory cells and are involved in phagocytosis (Wynn and Barron 2010). Macrophages release proinflammatory and profibrogenic cytokines, such as TNF α, IL-1, IL-6, TGF-β, PDGF and reactive oxygen species, which can activate HSC and modulate innate immune and inflammatory responses. Macrophages also play a role in fibrosis resolution.

During inflammation bone marrow derived $LY6C^+$ monocytes are recruited into tissues and differentiate into macrophages and dendritic cells. This occurs in the liver with studies demonstrating a large influx of monocytes with hepatic inflammation. During acute inflammation damaged hepatocytes, activated Kupffer cells and HSC secrete chemotactic mediators. A predominant chemokine CCL2 (MCP-1) stimulates the infiltration of monocytes via interaction with the receptor on monocytes CCR2.

Macrophages can differentiate into different phenotypes dependent on the surrounding microenvironment (Heymann, Trautwein et al. 2009). Two well recognized phenotypes are the classically activated M1 macrophages and the

alternatively activated M2 macrophages (Figure 1.6). M1-type macrophage activation *in vitro* is stimulated by Interferon gamma and/or lipopolysaccharide (LPS). M1 macrophages are proinflammatory and secrete cytokines such as IL-1, IL-6, TNFα, IL-12, IL-15 and IL-18 and upregulate surface molecules involved in antigen presenting function with T cells. They are also involved in phagocytosis, secretion of reactive oxygen species and nitric oxide (NO) and recruit other immune cells via chemokine secretion. M1 macrophages are proinflammatory and propagate a T helper (Th1) type immune response. They are critical for the acute and chronic inflammatory response and important in the initiation of fibrogenesis. However in established fibrosis M1 macrophages are antifibrotic because they secrete MMP-9 leading to ECM degradation and are involved in phagocytosis.

Alternatively activated macrophages or M2-type macrophages, are stimulated by IL-4 and IL-13 which is produced by Th2 cells, basophils and mast cells (Heymann, Trautwein et al. 2009). M2 macrophages have anti-inflammatory functions. They secrete TGFβ and IL-10, counteract inflammatory cytokines, for example they bind to IL-1 and produce high levels of arginase-1 which counteracts NO production. M2 macrophages are linked to T helper 2 like responses (Lawrence and Natoli 2011). However in established fibrosis they are profibrogenic (Heymann, Trautwein et al. 2009).

TLR 4 is found on both Kupffer cells and HSC and is the receptor for bacterial LPS. TLR4 signalling through Kupffer cells leads to generation of proinflammatory cytokines TNFα, IL-1 and IL-6. Of note however, TLR4 is

also found on HSC and its activation in these cells by LPS and endogenous ligands (including high-mobility group box 1 (HMGB1), biglycan and heparin sulphate), can lead to downregulation of bone morphogenetic protein(BMP) and activin membrane-bound inhibitor (BAMBI), a pseudoreceptor that negatively regulates TGF β1 signalling (Seki, De Minicis et al. 2007). Interestingly the clinical relevance of this has recently been described, with specific single-nucleotide polymorphisms of TLR4 affecting the rate of fibrosis progression in patients with Hepatitis C infection (Huang, Shiffman et al. 2007).

With tissue injury the damaged hepatocytes undergo apoptotic or necrotic cell death. Macrophages are responsible for the phagocytosis of the dead cells; however they classically repress inflammation when phagocytosing apoptotic cells and promote inflammation when phagocytosing necrotic cells (Wynn and Barron 2010). Phagocytosis of dying hepatocytes is profibrotic as it stimulates TGFβ1secretion (Rock and Kono 2008). However phagocytosis can also play a role in fibrosis resolution as removing apoptotic myofibroblasts and cellular debris can remove the source and stimulus for profibrogenic cytokine production.

Macrophages are also important for fibrosis resolution. Duffield et al demonstrated that depletion of macrophages resulted in reduction of fibrosis in the active phase of fibrogenesis but resulted in delayed matrix degradation during fibrosis resolution (Duffield, Forbes et al. 2005). The mechanism for matrix degradation in fibrosis resolution appears to be through increased

production of MMP-13 (Fallowfield, Mizuno et al. 2007). This data suggests that macrophages have a dual role and can be profibrogenic, but are also necessary for recovery from fibrosis.

A recent study by Thomas et al investigated the effect of administration of differentiated bone marrow derived macrophages (BMM) into mice with experimentally induced advanced liver fibrosis. These BMM did not conform to either the M1 or M2 macrophage phenotype and expressed antifibrotic (MMP13), anti-inflammatory (IL-10) and chemotactic (MCP-1) mediators. Post-delivery of BMM there was a 44% increase in macrophage number, the majority coming from recruited cells. There was also an increase in expression MMP-13 and MMP-9, important in degradation of the ECM and a reduction in myofibroblasts via induction of apoptosis. These observations support the important role macrophages play in fibrosis resolution (Thomas, Pope et al. 2011).

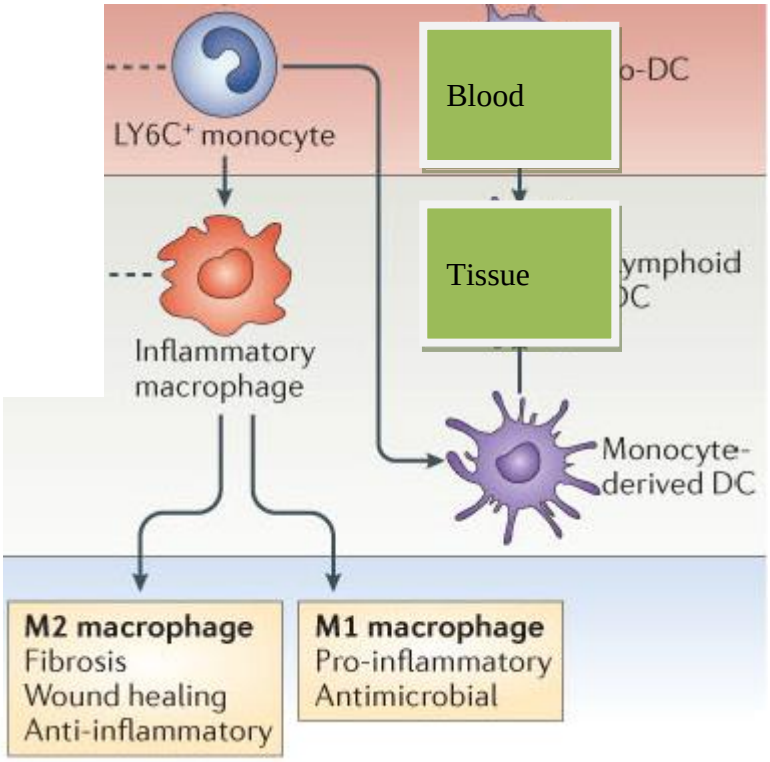


Figure 1.6 The mononuclear phagocyte system

With inflammation, LYC6+ monocytes are recruited from the blood into tissues and differentiate into macrophages and dendritic cells (DC). Macrophages can then polarise in to specific phenotypes such as M1 or M2 (Adapted from Lawrence et al. 2011).

In addition to macrophages, the hepatic innate immune system includes natural killer (NK) cells and natural killer T (NKT) cells. NK cells are cytotoxic to activated HSC and appear to be protective during hepatic fibrogenesis (Shirachi, Sata et al. 1998). In contrast NKT cells appear to be profibrogenic, although data is limited (Marra, Aleffi et al. 2009).

T lymphocytes are divided in to CD4+ helper cells and CD8+ cytotoxic cells. CD8+ T cells appear to be more fibrogenic toward HSC than CD4+ cells (Safadi, Ohta et al. 2004). CD4+ helper T cells are further divided into Th1 and Th2 cells depending on their cytokine expression profile. Th1 CD4+ T

cells secrete IL-2, Interferon gamma and lymphotoxin and appear to be antifibrogenic whilst in contrast Th2 cells, which produce IL-4, IL-5. IL-10 and IL-13 are profibrogenic (Marra, Aleffi et al. 2009).

B lymphocytes comprise up to 50% of lymphocytes in the liver (Friedman 2008). Mice with B cell depletion have been shown to have decreased collagen deposition in response to carbon tetrachloride with more rapid ECM degradation (Novobrantseva, Majeau et al. 2005). This suggests B cells are profibrogenic although the exact mechanism is unknown.

1.3 Coagulation

The association between chronic liver disease and coagulopathy is well established (Tripodi and Mannucci 2011). Cirrhosis is associated with a decrease in both procoagulant and anticoagulant proteins, as well as thrombocytopaenia associated with portal hypertension, which together can impact on a patient's risk of bleeding. However the connection between coagulation and inflammation has been a more recent area of interest. The following section will give a brief outline of the coagulation system and further explore the relationship between coagulation and inflammation.

1.3.1 Overview of coagulation

Haemostasis involves the formation of a blood clot at the site of vessel wall injury. The haemostatic response is tightly regulated and consists of four

phases. Firstly, formation of a platelet plug at the site of injury occurs concurrently with the propagation of the coagulation cascade to lead to the second phase of fibrin formation (Furie and Furie 2008). The latter two phases involve the termination of the clotting process by antithrombotic mechanisms and removal of the clot by fibrinolysis to ensure that the response at the site of injury is self-limiting and regulated.

The endothelium, the lining of blood vessels, acts as a barrier to separate the blood with its coagulation factors from the initiators of coagulation, the extracellular matrix (ECM) proteins. Damage to the endothelium results in the ECM proteins such as collagen and tissue factor being exposed to blood. Platelets can be activated by 2 pathways; firstly via collagen which leads to activation and adhesion, or alternatively exposed tissue factor leads, via the coagulation cascade, to thrombin generation which is a potent platelet activator (Furie and Furie 2008). One pathway may be more dominant than the other depending on the disease state, but the end result is platelet activation.

The adhesion of platelets to the ECM is the initial step in thrombus formation (Gawaz, Langer et al. 2005). Platelet adhesion is mediated by binding of platelet glycoprotein(GP) Ib/IX/V to collagen bound von Willebrand Factor (vWF) and GP VI binds to collagen. This leads to platelet activation with subsequent change in platelet shape and release of mediators such as adenosine diphosphate (ADP) and thromboxane A_2 which activate neighbouring platelets (Gawaz, Langer et al. 2005). Platelet activation leads

to exposure and conformational change in the integrin receptors GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$) found on the platelet surface, which leads to binding to fibrinogen and collagen respectively in the ECM. Platelets then aggregate via fibrinogen bridges between $\alpha_{IIb}\beta_3$ receptors (Coller and Shattil 2008). With platelet activation comes procoagulant activity, as procoagulant phospholipids are exposed on the platelet surface and form a site for the assembly of coagulation enzyme complexes (Baglia and Walsh 1998, Baglia and Walsh 2000).

The coagulation cascade is a series of stepwise activations of proenzymes from inactive to active forms which ultimately leads to thrombin formation and fibrin clot. Historically the coagulation cascade has been viewed as two separate pathways, the intrinsic and extrinsic pathways which converge with activation of factor X, however these pathways are now thought to be integrated (Luchtman-Jones and Broze 1995) (Figure 1.7). It is the exposure of tissue factor (TF) at the site of injury and its interaction with the small circulating amount of factor VII which is the primary initiating event of coagulation (Rapaport and Rao 1995). The tissue factor–VIIa complex activates Factor X to Xa and Factor IX to IXa. Factor Xa then combines with factor V to form the prothrombinase complex which then activates prothrombin to thrombin. Thrombin converts fibrinogen to fibrin monomer and also activates Factor XIII which acts to help with the formation of the crosslinked fibrin polymer. Thrombin also activates Factors V to Va, VIII to VIIIa and XI to XIa which are responsible for the amplification of the coagulation process,

with increased Xa and IXa production augmenting thrombin production and subsequent fibrin formation.

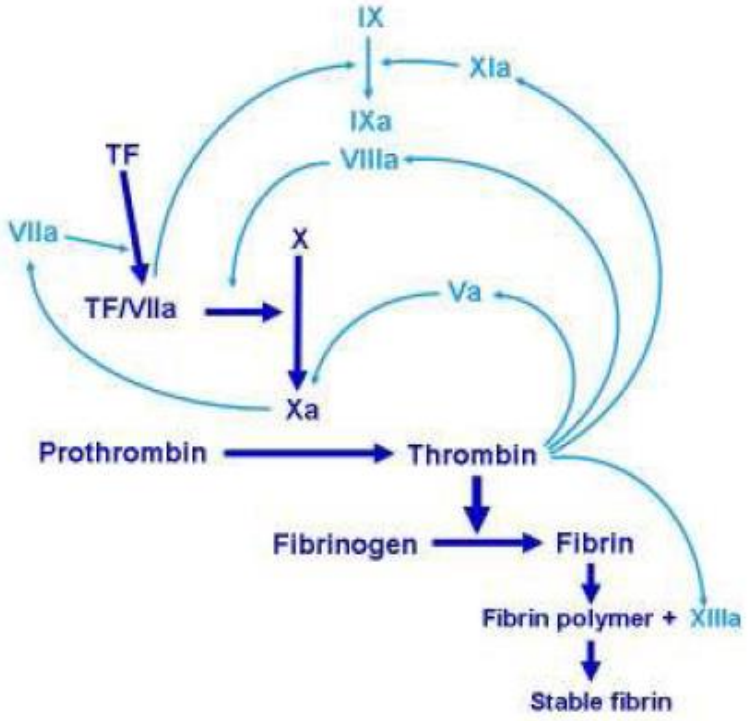


Figure 1.7 The coagulation cascade

Exposed tissue factor binds to circulating factor VII which in turn activates factor X to Xa and then converts prothrombin to thrombin. Thrombin generates the amplification loops of factors V, VIII and IX and also promotes conversion of fibrinogen to fibrin and factor XIII to XIIIa. The TF-VIIa complex also activates factor IX.

Due to the activity of platelets and the coagulation cascade, a localised and effective haemostatic response occurs at the site of injury. However if this process were to continue unchecked it could lead to pathological thrombosis and potential for tissue damage. Therefore the third phase of haemostasis involves the termination of the clotting process by a number of mechanisms including 1) dilution of procoagulants in the flowing blood, 2) removal of

factors by the reticuloendothelial system and 3) the existence of natural antithrombotic pathways (Lane, Philippou et al. 2005).

Antithrombin (AT), previously called antithrombin III, is a circulating plasma protease inhibitor that inactivates thrombin and other coagulation factors including Factor Xa, IXa, XIIa and XIa. The binding of heparins to the AT heparin binding site leads to conformational change and accelerates the inactivating process by 1000-4000 fold (Perry 1994). Tissue factor pathway inhibitor (TFPI) is found in low circulating amounts compared to antithrombin. TFPI becomes complexed with Factor Xa and inhibits Xa directly and indirectly via binding the tissue factor-FVIIa complex therefore preventing further Factor Xa and IXa formation.

The Protein C pathway is triggered by the binding of thrombin to thrombomodulin (TBM), a surface protein on endothelial cells. This binding leads to conformational change and altered substrate specificity of thrombin. The thrombin-TBM complex activates protein C and this in conjunction with protein S on phospholipid surfaces inactivates Factor Va and VIIIa thereby inactivating coagulation.

The final phase is clot elimination by fibrinolysis. The proteolytic enzyme plasmin cleaves fibrin at multiple sites generating fibrin degradation products and partially digested clot. Plasminogen, the precursor to plasmin, binds to fibrin and tissue plasminogen activator to form a complex which in turn converts plasminogen to the active plasmin. This system, like the coagulation

system has amplification loops and natural inhibitors (Kolev and Machovich 2003) (Figure 1.8).

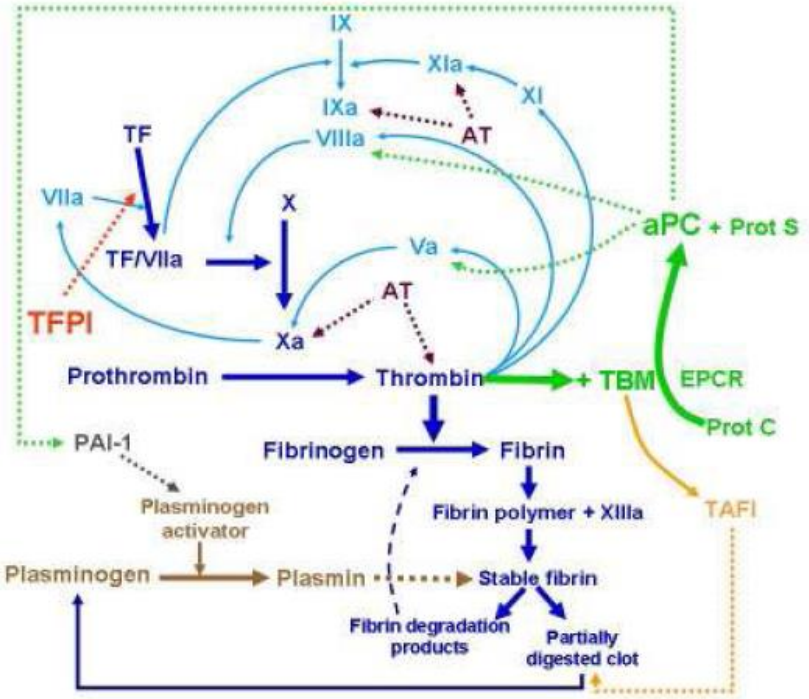


Figure 1.8 Regulatory pathways of coagulation

Thrombin with thrombomodulin and endothelial protein C receptor (EPCR) generate activated protein C (aPC). aPc and protein S together inactivate factor Va and VIIIa and plasminogen activator inhibitor-1 (PAI-1). Thrombin and TBM also produce thrombin-activatable fibrinolysis inhibitor (TAFI). TFPI (red) prevents production of TF-VIIa complex. AT inactivates thrombin and factors IXa, Xa, XIa. Solid lines represent activation, dotted lines represent inhibition.

Endothelial cells secrete both plasminogen activators (tissue-type plasminogen activator and urokinase-type plasminogen activator) and plasminogen activator inhibitors (PAI-1 and PAI-2). Fibrin cleavage exposes further sites for plasminogen binding and thus acts to continue fibrinolysis. The fibrin binding site for plasminogen in turn is regulated by thrombin-

activatable fibrinolysis inhibitor (TAFI) released from the thrombin-thrombomodulin complex. TAFI cleaves the fibrin binding site for plasminogen and therefore limits the breakdown of the new clot.

1.4 Coagulation and Inflammation

Inflammation and coagulation are interrelated and essential for host defence mechanisms, with cross talk between the two systems (Levi, van der Poll et al. 2004, Esmon 2005). Injury by a microorganism or other invading agent leads to the activation of the immune and coagulation systems with resultant inflammation and thrombus formation aimed at containing the threat. A local response at the site of injury is desired however widespread or generalised inflammation and coagulation activation can be harmful and clinically can lead to multi organ failure and death (Choi, Schultz et al. 2006).

1.4.1 Coagulant effects of Inflammation

There are a number of effects of inflammation on coagulation including induction of TF expression, increased platelet activation and production, reduced activity of natural anticoagulants and impaired fibrinolysis (Esmon 2005).

TF is critical for initiation of coagulation and is also a key player in linking coagulation and inflammation, with inflammation inducing increased TF expression on the surface of leucocytes (Parry and Mackman 1998, Lindmark,

Tenno et al. 2000). Both TNFα and IL-6 stimulation can lead to upregulation of TF synthesis and expression by monocytes, however IL-6 is the more potent inducer of monocyte expression of TF *in vivo* (Choi, Schultz et al. 2006). This was underscored in an experimental model of endotoxemia where monoclonal antibodies against IL-6 led to complete blockage of TF-dependent thrombin generation (Levi, van der Poll et al. 1997) whereas antagonism of TNFα did not abrogate coagulation activation (van der Poll, Levi et al. 1994).

IL-6 also enhances platelet production and renders platelets more responsive to the effects of thrombin (Burstein 1997). The procoagulant activity of platelets also increases with inflammation as generation of complement C5b-9, a potent agonist for the exposure of the platelet surface phospholipids, provides a site for coagulation factor complex assembly (Sims, Wiedmer et al. 1989).

Inflammation also inhibits natural anticoagulant pathways. Firstly antithrombin levels are reduced secondary to increased consumption, reduced synthesis as a negative acute phase reactant (Niessen, Lamping et al. 1997) and increased degradation by neutrophil elastase (Levi and Ten Cate 1999). The protein C pathway is also downregulated by inflammatory mediators including endotoxin, IL-1β and TNFα which cause reduced expression of TBM and endothelial protein C receptor (EPCR) (Conway and Rosenberg 1988, Fukudome and Esmon 1994) which leads to reduced activated protein C (aPC) production. Reduced TBM levels also contribute to ongoing coagulation with reduced inhibition of thrombin secondary to reduced binding to TBM.

Expression of cellular adhesion molecules, such as P selectin and E selectin, occurs as a result of the activation of the endothelium via proinflammatory cytokines TNFα and IL-Iβ. This helps the tethering and rolling of leucocytes which in turn slows blood velocity optimising the interaction of soluble coagulation factors to form thrombin (Springer 1994).

Finally, inflammation can lead to impaired fibrinolysis. The acute response in inflammation is the release of plasminogen activators from the vascular endothelium. However the proinflammatory cytokines TNFα and IL-Iβ promote the release of plasminogen activator inhibitor type-1 and reduce the release of tissue-type plasminogen activator with the net effect of inadequate fibrinolysis (van der Poll, Levi et al. 1991).

1.4.2 Proinflammatory effects of Coagulation

The activation of the coagulation cascade promotes inflammation via a number of mechanisms. The interaction of some coagulation proteases with PARs, initiates intracellular signalling and upregulation of proinflammatory cytokine and chemokine production. Platelets are also involved releasing a number of cytokines.

In vitro, thrombin upregulates a number of proinflammatory cytokines including monocyte chemotactic protein-1 (MCP-1), IL-6 and IL-8. Factor Xa and TF-VIIa can produce similar results. *In vivo*, recombinant FVIIa given to healthy subjects induced a 3-4 fold increase in IL-6 and IL-8 levels (de Jonge, Friederich et al. 2003). Thrombin also enhances leucocyte adhesion via

promoting expression of adhesion molecules such as intercellular adhesion molecule (ICAM) (Rahman, Anwar et al. 1999). Thrombin is chemotactic for monocytes via MCP-1 (Bar-Shavit, Kahn et al. 1983) and is a potent mitogen for a number of cell types including endothelial cells, fibroblasts and smooth muscle cells (Coughlin 2000).

Fibrinogen and fibrin can stimulate expression of TNFα and IL-1β on mononuclear cells and stimulate production of chemokines, IL-8 and MCP-1 by endothelial cells and fibroblasts (Szaba and Smiley 2002).

Therefore a number of members of the coagulation cascade evoke inflammatory responses via upregulation of proinflammatory cytokines and chemokine production. The linkage between tissue injury and cellular responses is largely through the protease interaction with the PARs (Coughlin 2000).

1.5 Coagulation, inflammation and liver disease:

1.5.1 Coagulopathy of chronic liver disease

It is well recognized that patients with chronic liver disease have coagulation disorders. The increased risk of bleeding in chronic liver disease is in part associated with decreased levels of non-endothelial derived coagulation factors (eg factors II, V, VII, IX, XXI, XIII). Thrombocytopaenia related to splenic sequestration and altered platelet function, as well as abnormalities in

fibrinogen and TAFI, also contribute to the tendency for bleeding (Tripodi and Mannucci 2011). This increased bleeding risk is offset by an increased thrombotic risk. There is decreased hepatic production of the natural anticoagulants protein C and S, decreased antithrombin levels, decreased plasminogen and increased levels of endothelial derived factor VIII and von Willebrand Factor that favour a hypercoagulable state (Northup, Sundaram et al. 2008). Therefore the haemostatic balance can be maintained by reduction in both procoagulant and anticoagulant proteins.

In healthy individuals there is a balance between the procoagulant factors and their natural anticoagulants. There are actually higher levels of procoagulants and anticoagulants than are needed to maintain a steady state of haemostasis and thus the body can respond to increased demand when put under stress, such as with an infection. In patients with chronic liver disease and particularly cirrhosis, the reduction in both the procoagulant and anticoagulant levels mean that if the body is put under stress, it is harder to maintain the balance between the two systems and haemorrhage or thrombosis may occur (Monroe and Hoffman 2009). The bleeding tendency in patients with chronic liver disease can be multifactorial and may be explained by underlying conditions such as portal hypertension, bacterial infections generating heparin like substances or renal impairment rather than the result of decreased procoagulant factors alone (Caldwell, Hoffman et al. 2006).

The longstanding dogma that patients with chronic liver disease are protected from thrombosis because they are auto-anticoagulating themselves is not

supported by clinical evidence as venous thromboembolism is reported in this group of patients (Dabbagh, Oza et al. 2010). The methods we have for measuring coagulation including the prothrombin time, activated partial thromboplastin time and international normalised ratio (INR) do not reflect the balance between the procoagulants and anticoagulants and thus may not be an accurate measure of bleeding/thrombotic risk in this group of patients.

Although clinically the imbalance in haemostasis with both pathological bleeding and thrombosis in patients with chronic liver disease is widely recognized, the effect of coagulation factors on hepatic inflammation and fibrosis is gaining more attention.

1.5.2 Coagulation cascade and liver fibrogenesis

Chronic liver disease is associated with disorders in coagulation which can present clinically challenging problems of inappropriate bleeding and thrombosis. As previously discussed, coagulation can induce inflammation and there is growing evidence that this extends to a role for the coagulation system in the pathogenesis of fibrosis. Procoagulant states have been recognized to accelerate liver fibrogenesis (Anstee, Wright et al. 2009). A Greek study of 90 patients with chronic hepatitis B or C found that the presence of one or more thrombotic risk factors, such as antithrombin, protein C and/or plasminogen deficiency, was associated with more advanced fibrosis (Papatheodoridis, Papakonstantinou et al. 2003). The argument for coagulation impacting on liver fibrosis is further strengthened by the

observation that patients with haemophilia who are hepatitis C positive have slower progression of liver fibrosis (Yee, Griffioen et al. 2000).

There are two postulated and complementary ways in which coagulation can potentially promote liver fibrosis: the first is via microthrombi leading to tissue ischaemia and the second is via direct hepatic stellate cell activation (Anstee, Wright et al. 2009).

1.5.2.1 Parenchymal Extinction: Microthrombi causing tissue ischaemia and fibrosis

The development of microinfarcts has been proposed as a critical event in the development of fibrous septae and subsequent cirrhosis. In a rat model of CCl_4 induced fibrosis, fibrinogen and fibrin deposition were seen with short-term liver damage (Neubauer, Knittel et al. 1995). Wanless et al studied 61 cirrhotic livers removed at transplant and demonstrated the presence of thrombotic occlusion of the hepatic vasculature (Wanless, Wong et al. 1995). This study demonstrated that portal vein and hepatic vein thrombosis are common in cirrhotic livers. Hepatic vein lesions were patchy in distribution, affecting predominantly medium sized vessels and were associated with regions of confluent fibrosis. Intimal fibrosis, which is suggestive of healed hepatic vein thrombosis, was found in 70% of livers. A second study performed by this group also supported the role of microthrombi formation in the development of cirrhosis secondary to congestive cardiac failure (Wanless, Liu et al. 1995).

From these studies it was proposed that hepatic necroinflammation leads to intimal injury and occlusive thrombi formation within branches of the hepatic vein and portal vein with subsequent interruption to blood flow. The microthrombi lead to tissue ischaemia, hepatocyte apoptosis and necrosis. The resultant “parenchymal extinction”, defined by the contiguous loss of hepatocytes in a region and the replacement by fibrous tissue, is proposed to lead to cirrhosis when these areas accumulate and become confluent (Wanless, Liu et al. 1995, Wanless, Wong et al. 1995, Wanless 2004).

1.5.2.2 Direct stellate cell activation via coagulation factors

The alternative, but not mutually exclusive, theory is that coagulation factors via their receptors activate hepatic stellate cells and platelets promoting fibrosis. The way in which this may occur will be discussed further in the remainder of this chapter.

1.6 Protease Activated Receptors

PARs are a unique group of G-protein coupled receptors that are activated by serine protease cleavage of their extracellular N terminal domain to reveal a ”tethered” ligand that binds with the second extracellular loop of the receptor to initiate signalling. Proteases of the coagulation cascade are major activators of PARs (Riewald and Ruf 2002). PAR-1 was initially identified in the search for the cellular thrombin receptor and to date four PARs have been

identified. PAR1 is primarily activated by thrombin but also can be activated by other proteases including FXa (Riewald, Kravchenko et al. 2001), activated protein C (Riewald, Petrovan et al. 2003), plasmin (Pendurthi, Ngyuen et al. 2002) and FVIIa (Camerer, Huang et al. 2000). Thrombin activates PAR-3 and 4. PAR-2 is also activated by trypsin, mast cell tryptase, Factor Xa and the tissue factor/factor VIIa and factor Xa complex (Riewald and Ruf 2002). Synthetic ligands mimicking the tethered ligand can activate the receptors without cleavage (Figure 1.9).

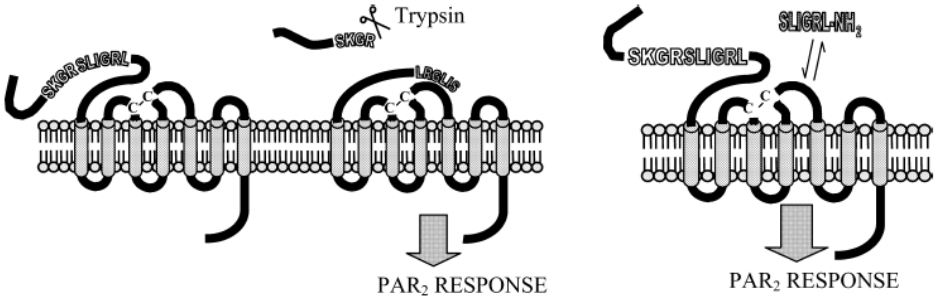


Figure 1.9 Mechanism of PAR activation

Using PAR-2 as an example, activation of PARs is via generation of a tethered ligand or via a PAR activating peptide with the sequence based on the tethered ligand (Adapted from Hollenberg 2005)

1.6.1 PAR-1 Receptor

As noted above, PAR-1 is activated primarily by thrombin but can also be activated by FXa, FVIIa, activated protein C and plasmin. PAR-1 can also be activated by the synthetic peptide SFFLRN in the absence of proteolysis (Hollenberg 2005). Once activated PAR-1 is rapidly uncoupled from signalling and is internalised and delivered to lysozymes for degradation. In endothelial cells and fibroblasts new PAR1 is delivered to the cell surface from a preformed intracellular pool (Coughlin 2000).

1.6.1.1 Distribution of PAR-1 receptor

PAR 1 is expressed by a number of human cells including platelets, endothelial cells, fibroblasts smooth muscle cells and T lymphocytes. Mouse platelets however do not express PAR 1 and only express PAR 3 and 4 (Coughlin 2000).

Through immunohistochemistry the PAR-1 receptor distribution was characterised in normal liver, in patients with chronic hepatitis and in patients with cirrhosis. In normal liver and diseased liver the PAR-1 receptor is found in the endothelial lining of the hepatic sinusoids lining the hepatic lobule and on the endothelium of large blood vessels including the portal vein branches. In normal liver no staining was observed in portal tracts (Rullier, Senant et al. 2006).

In patients with cirrhosis PAR 1 staining is positive in fibroblasts in the fibrotic septa and is found on T lymphocytes in the inflammatory infiltrate around newly formed blood vessels and bile ducts (Marra, DeFranco et al. 1998) (Rullier, Senant et al. 2006).

1.6.1.2 PAR-1 signalling

Signal transduction begins with the coupling of PAR-1 to a G protein at the plasma membrane. PAR-1 can couple to several different G-protein α subunits including $G_{q11}α$, $G_{12/13}α$ and $G_{i}α$, which account for the number of actions of the ligand (Kyriazis, Ellul et al. 2012). To illustrate thrombin signalling in platelets and fibroblasts is attenuated by injection of $G_{q11}α$

antibodies suggesting this is the main pathway of coupling with thrombin activation. A summary of PAR-1 signal transduction is found in Figure 1.10.

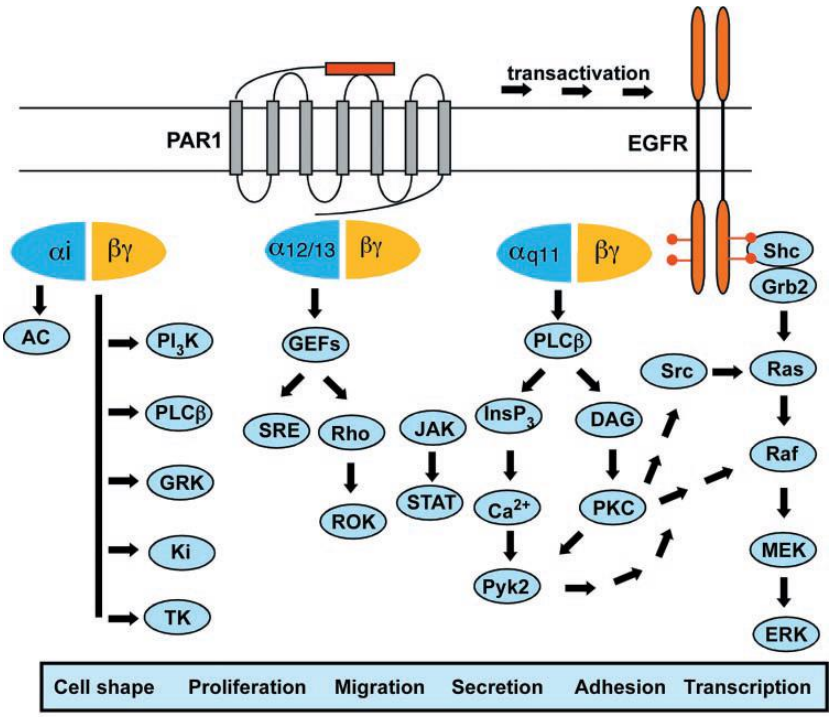


Figure 1.10 Summary of PAR-1 signalling

Briefly PAR-1 coupling to Gαi inhibits adenylyl cyclase (AC) to reduce cyclic adenosine monophophate (cAMP).

$G_{12/13}\alpha$ coupling to guanine nucleotide exchange factors (GEF) leads to activation of Rho, Rho-kinase and serum response elements (SRE).

$G_{q11}\alpha$ activates phospholipase Cβ leading to inositol triphosphate formation which leads to calcium mobilisation and diacylglycerol (DAG) which activates protein kinase C (PKC).

PAR-1 can activate the mitogen-activated protein kinase (MAPK) cascade by a number of mechanisms including transactivation of the EGF receptor, through activation of PKC, Pyk2 and other mechanisms.

Gβγ subunits couple PAR-1 to other pathways including G protein receptor kinases (GRK), potassium channels (Ki) and non receptor tyrosine kinases (TK)

(Ossovskaya et al. 2004).

1.6.1.3 PAR-1 and Fibrosis

PAR-1 signalling has been demonstrated to be important in a number of fibroproliferative responses to injury. PAR-1 signalling upregulates adhesion molecules on endothelial surfaces, releases chemokines that activate neutrophils and monocytes (Coughlin 2000) and can lead to increased expression of profibrogenic mediators such as CTGF (Chambers, Leoni et al. 2000) and MCP-1 and induction of IL-6 and IL-8 production (Chu 2005).

PAR-1 has been shown to be involved in pulmonary fibrosis. PAR-1 is abundantly expressed by fibroblasts found in the fibrotic foci in patients with chronic fibrotic lung injury. Furthermore it has been shown that PAR-1 deficiency protects against bleomycin induced lung inflammation and fibrosis (Howell, Johns et al. 2005). PAR-1 can be activated by thrombin, but also by Factor Xa alone or as part of the ternary complex with TF/Factor VIIa/Xa. A recent study has demonstrated local Factor X production was significantly increased in response to oxidative stress in bleomycin induced lung injury. Factor X was also found to promote fibroblast to myofibroblast differentiation via a PAR-1 signalling mechanism (Scotton, Krupiczojc et al. 2009).

There is growing evidence for a significant role of thrombin and PAR-1 signalling in the development of hepatic fibrosis and several mechanisms are being postulated (Figure 1.11). PAR-1 is upregulated in acute and chronic liver disease (Marra, DeFranco et al. 1998). *In vitro* thrombin stimulates HSC proliferation and MCP1 expression (Marra, Grandaliano et al. 1995) and collagen synthesis (Gaca, Zhou et al. 2002). *In vivo* PAR 1 antagonism has been shown to protect against murine liver fibrosis. Duplantier et al studied

carbon tetrachloride induced fibrosis in mice and showed that oral administration of a PAR-1 antagonist reduced liver fibrosis and reduced HSC activation (Duplantier, Dubuisson et al. 2004). Fiorucci et al published at a similar time experiments in a bile duct ligation model of fibrosis which demonstrated that subcutaneous administration of a PAR-1 antagonist protected against liver fibrosis as assessed by liver function tests, liver collagen content and histology (Fiorucci, Antonelli et al. 2004).

Subsequent to the commencement of this PhD it has been reported that PAR-1 knockout mice develop reduced experimentally induced liver fibrosis (Rullier, Gillibert-Duplantier et al. 2008). More recently therapeutic administration of a direct thrombin inhibitor, Argatroban, has been shown to reduce hepatic inflammation in mice with established non-alcoholic fatty liver disease (Kassel, Sullivan et al. 2012).

The clinical significance of PAR-1 in liver disease has recently been explored. A study has shown that PAR-1 gene polymorphisms influence the rate of progression of liver fibrosis in a group of patients with hepatitis C (Martinelli, Knapp et al. 2008).

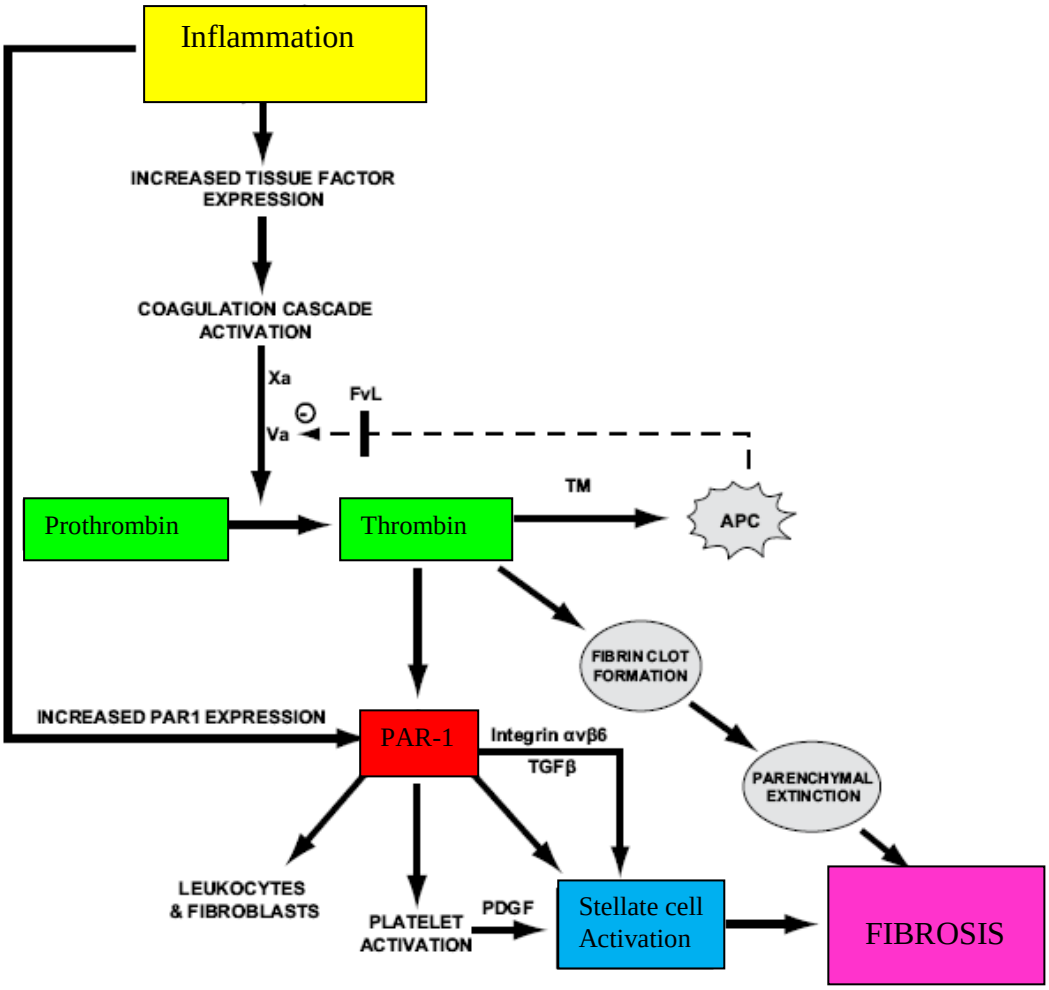


Figure 1.11 Potential mechanism of PAR-1 activation of HSC

Inflammation and injury in the liver parenchyma leads to increased expression of tissue factor and activation of the coagulation cascade with subsequent thrombin generation. Inflammation also leads to increased PAR-1 expression and induces expression of profibrogenic cytokines and activation of HSC (Adapted from Anstee et al 2009).

1.6.2 PAR-2 Receptor

PAR-2 is a G-coupled protein receptor that, like the other PARs, is activated by proteolytic cleavage to release a tethered ligand to activate the receptor. Activation with cleavage exposes the tethered ligand SLIGKV in humans (Nystedt, Emilsson et al. 1995). PAR-2 can be activated by trypsin, mast cell tryptase, complex TF:VIIa and TF:VIIa:Xa complex, but not by thrombin

(Camerer, Huang et al. 2000). The synthetic peptide SLIGKV activates PAR-2 without requiring cleavage of the receptor (Figure 1.9).

Mast cells are prominently recruited during hepatic fibrosis (Farrell, Hines et al. 1995) and have the potential to provide a potent source of mast cell tryptase in humans, which can activate PAR-2 receptors. However there are species differences in the proportion of tryptase among mast cell proteins, being found in lower proportions in rat and mouse mast cells compared to humans (Ossovskaya and Bunnett 2004).

1.6.2.1 PAR-2 distribution

PAR-2 is widely expressed in the gastrointestinal tract on epithelial cells and smooth muscle cells (D'Andrea, Derian et al. 1998). It is also found in the respiratory tract, brain, skin, pancreas, prostate, testes, ovary and kidney (Steinhoff, Buddenkotte et al. 2005). PAR-2 is localised to a number of cell types including epithelial cells, endothelial cells, fibroblasts, myocytes, neurons, astrocytes (Ossovskaya and Bunnett 2004) as well as immune cells such as T cells, neutrophils, mast cells and eosinophils.

PAR-2 has been shown to have important roles in regulation of gastrointestinal physiology and in inflammatory processes including pancreatitis, gastritis and colitis. In the normal liver, PAR-2 is expressed on hepatocytes, Kupffer cells, bile duct epithelial cells and endothelial cells of large vessels.

1.6.2.2 PAR-2 signalling

PAR-2 activation leads to inositol 1,4,5 triphosphate ($InsP_{3)}$ generation and calcium mobilisation in enterocytes, keratinocytes, myocytes, neurons and tumour cells suggesting PAR-2 couples to the G protein subunit $G_{q11}\alpha$. PAR-2 activators also activate MAP kinases extracellular signal-regulated protein kinase (ERK) 1/2 and to a weaker degree p38. β-arrestins which are cytosolic proteins that interact with G-coupled protein receptors play a role in PAR-2 activation of ERKs. Activation of PAR-2 leads to the formation of a MAP kinase signalling module that contains PAR-2, β-arrestins, raf-1 and activated ERK 1/2. The module retains ERK 1/2 in the cytosol where it can regulate cytosolic targets, but prevents ERK1/2 translocation to the nucleus where it can affect proliferation (Ossovskaya and Bunnett 2004).

PAR-2 agonists also have been shown to activate the nuclear factor kappa B (NFκB) pathway highlighting the link between PAR-2 and inflammation (Kanke, Macfarlane et al. 2001).

1.6.2.3 PAR-2 and inflammation and fibrosis

PAR-2 activation augments inflammatory cell recruitment and pro-fibrotic pathways through the induction of genes encoding proinflammatory cytokines and proteins of the extracellular matrix. PAR-2 activation upregulates IL-1β and IL-8, increases expression of adhesion molecules and selectins on endothelial cells, mediates accumulation of neutrophils and eosinophils and is mitogenic for fibroblasts, smooth muscle cells and epithelial cells (Steinhoff, Buddenkotte et al. 2005).

Many studies support the idea that PAR-2 activation has a number of proinflammatory and profibrotic effects. Borensztajn et al studied fibroblasts *in vitro* and demonstrated that Factor Xa induced ERK1/2 phosphorylation and that this was PAR-2 dependent. The PAR-2 dependent ERK1/2 phosphorylation led to fibroblast proliferation, differentiation to myofibroblasts, fibroblast activation and secretion of cytokines (MCP-1 and IL-6) and profibrogenic proteins (TGF-β and fibronectin) (Borensztajn, Stiekema et al. 2008).

PAR-2 has been shown to have a proinflammatory role in both experimental crescentic glomerulonephritis (Moussa, Apostolopoulos et al. 2007) and mediating the chronic inflammation in a model of arthritis (Ferrell, Lockhart et al. 2003). PAR-2 deficiency can protect against the development of experimentally induced colitis and inhibits leucocyte recruitment to the colon suggesting this maybe the mechanism that affords protection (Hyun, Andrade-Gordon et al. 2008).

As well as playing a role in inflammation PAR-2 has been linked to the development of fibroproliferative diseases. PAR-2 has been shown to be upregulated in the lungs of patients with idiopathic pulmonary fibrosis and bleomycin, which induces lung fibrosis, increases PAR-2 expression. In PAR-2 deficient mice exposed to bleomycin there was reduced pulmonary fibrosis and reduced fibrin deposition (Borensztajn, Bresser et al. 2010). PAR-2 also

appears to promote renal fibrosis following ureteric obstruction (Xiong, Zhu et al. 2005).

Rat HSC express PAR-2 under normal conditions and its expression is markedly increased with progressive liver injury (Borensztajn, von der Thusen et al. 2010) (Fiorucci, Antonelli et al. 2004). PAR-2 stimulation with its agonist tryptase and synthetic peptide has also been shown to promote rat stellate cell proliferation and collagen synthesis. However the contribution of PAR-2 to liver fibrosis *in vivo* and in human cell lines has not been reported.

1.7 Tissue Factor

Tissue Factor (TF) is a 47 kDa transmembrane glycoprotein that is the initiator of the coagulation cascade as the cell surface receptor for Factor VIIa. TF is a member of the type II cytokine receptor superfamily. The TF molecule has 263 amino acid residues and three domains: the extracellular domain, residues 1-219 which is made up of two fibronectin type 3 domains, typical of a type II cytokine receptor; the transmembrane domain, residues 220-242; and the cytoplasmic domain residues 243-263 (Spicer, Horton et al. 1987) (Figure 1.12).

The extracellular domain of TF is the cell surface receptor that binds Factor VII to initiate coagulation. There are three binding sites between the two molecules to help stabilise the complex and provide the correct alignment for the subsequent binding and activation of Factor Xa (Osterlund, Persson et al. 2005). Binding begins between the protease domain of FVIIa and Asp 44

located in the N terminal region of TF (Kelly, Schullek et al. 1996). The second binding site occurs between the EGF1 domain of FVIIa and the TF1/TF2 site of TF found between the two fibronectin type III domains (Banner, D'Arcy et al. 1996). The final binding site is between the GIa domain of FVIIa and the C-terminal fibronectin type III domain of TF.

The transmembrane domain of TF, although not directly involved in FVIIa binding, is important for TF's procoagulant activity. TF variants that are composed of the extracellular domain alone, lacking both the transmembrane and cytoplasmic domains, do not have significant coagulant activity. However a study demonstrated that with substitution of the transmembrane domain with a sequence that contains membrane anchoring properties coagulant activity was re-established (Paborsky, Caras et al. 1991). This study highlights that it is the anchoring properties of the transmembrane domain and lipid association that are vital for TF coagulant activity.

The cytoplasmic domain of TF is not required for its coagulant activity. *In vitro*, TF variants that lack the cytoplasmic domain show full coagulation activity. *In vivo*, mice deficient in the cytoplasmic domain of TF have also been shown to have normal procoagulant function (Melis, Moons et al. 2001).

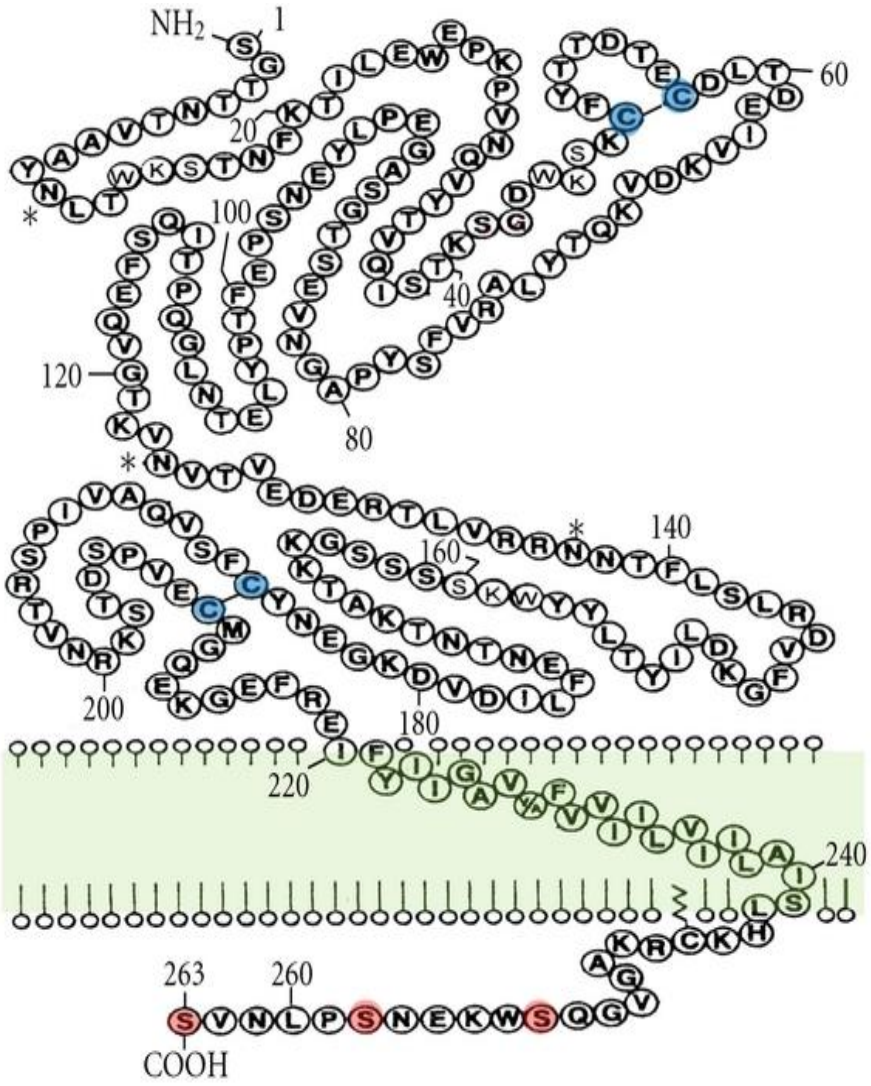


Figure 1.12 TF structure

TF consists of extracellular, transmembrane and cytoplasmic domains. There are 2 intrachain disulphide bridges (shown in blue). There are 3 serine residues (shown in red) in the cytoplasmic domain for undergoing phosphorylation (Chu 2005).

1.7.1 Expression and regulation of TF

TF is expressed by cells that are not normally in contact with blood. It is constitutively expressed in many cell types such as stromal fibroblasts in

vascular adventitia and organ capsules, epithelial cells in the skin and mucosa, stromal cells in the endometrium and astrocytes in the brain (Carmeliet and Collen 1998). TF is expressed throughout the body but is found in highest concentrations in the heart, brain, lung and kidney.

In normal liver, tissue factor is expressed in the cytoplasm of hepatocytes particularly concentrated in those found under Glisson's capsule. In cirrhosis, the distribution of tissue factor appears to become more diffuse involving >30% hepatocytes (Rullier, Senant et al. 2006). TF positive staining has also been demonstrated in endothelial cells in the neovasculature and on the spindle shaped cells or fibroblasts in the cirrhotic septa (Rullier, Senant et al. 2006).

Under pathological conditions circulating monocytes, macrophages and vascular endothelial cells can be stimulated to express TF by vascular injury, endotoxin and cytokines TNF α and IL-6; thus concentrating tissue factor to the sites of injury (Giesen, Rauch et al. 1999). TF expression can be upregulated by a number of cytokines, mitogens, hormones, endotoxins, viral infections and lipoproteins as well as hypoxia and mechanical injury.

1.7.2 Non-coagulant roles of TF

In addition to its primary role as the initiator of coagulation, TF is increasingly recognised as having a number of important non-coagulant roles.

Firstly, TF is involved in embryogenesis and appears important for embryonic blood vessel development. TF deficiency (TF $^{-/-}$) in mice leads to embryonic lethality at approximately day 10.5 and further studies of these embryos revealed deficiency in the primitive smooth muscle cells required for the development of the periendothelial muscular wall (Carmeliet, Mackman et al. 1996). Studies demonstrated that transgenes expressing low levels of human TF or human TF lacking the cytoplasmic domain were able to rescue the lethality of TF $^{-/-}$ embryos (Parry, Erlich et al. 1998, Parry and Mackman 2000). This indicates that the TF extracellular domain, not the cytoplasmic domain, is important for normal development of the yolk sac vasculature.

TF also appears to be involved in malignancy and tumour metastasis. TF is expressed by most epithelial tumour cell types (Callander, Varki et al. 1992) and a correlation has been found between elevated TF expression and advanced stages of malignancy in a number of human cancers including pancreatic cancer and non small cell lung cancer (Koomagi and Volm 1998, Wojtukiewicz, Rucinska et al. 2001). Studies have also shown a role for both the extracellular and intracellular domains of TF in tumour metastasis.

1.7.3 TF non coagulant signalling

As TF expression is upregulated in a number of pathological conditions and TF is structurally similar to Class II cytokines, its role in cell signalling has been an area of interest.

1.7.3.1 Signalling pathways utilised by TF

A number of studies in different cell types, including bladder carcinoma cells, keratinocytes and Baby Hamster kidney (BHK) cells with TF transfected into the cell line, have demonstrated that TF can function as a signalling receptor. Cirillo et al were the first to demonstrate TF signalling in smooth muscle cells that constitutively express TF. FVIIa binding to TF on smooth muscle cells (SMC) led to intracellular signalling via activation of the p44/42 MAP kinase and c-Jun N-terminal protein kinases (JNK) pathways, ultimately leading to SMC proliferation. Anti TF antibodies blocked this proliferation (Cirillo, Cali et al. 2004). There is evidence that TF signalling can activate multiple pathways including MAPK, P13K-AKT, Janus kinase (JAK)-signal transducer and activator of transcription (STAT) and mammalian target of rapamycin (mTOR), thus linking tissue factor to a number of cellular functions including proliferation, migration and cell survival (Aberg and Siegbahn 2013).

TF complexes can induce signalling via a number of pathways including PAR dependent pathways, via the TF cytoplasmic domain and by transactivation of receptor tyrosine kinases (Figure 1.13).

TF-FVIIa can activate intracellular signalling, as evidenced by calcium oscillations and MAPK activation, without an intact TF cytoplasmic domain and the PARs have been identified as the receptors involved. TF-FVIIa can activate PAR-2 and TF-FVIIa-FXa complex can activate PAR-1 and PAR-2.

The phosphorylated cytoplasmic domain of TF can also act independently as a binding site for the actin-binding protein filamin A (FLNA), which is recruited to the C-terminal upon the binding of FVIIa to TF and is important for cell adhesion and migration (Ott, Fischer et al. 1998).

A third possible mechanism for TF signalling is through ligand-independent activation, or transactivation, of the receptor tyrosine kinases. TF-FVIIa complex is involved in the transactivation of the PDGF receptor β receptor, a tyrosine kinase, which is PAR-2 dependent and Src family-dependent (Aberg and Siegbahn 2013).

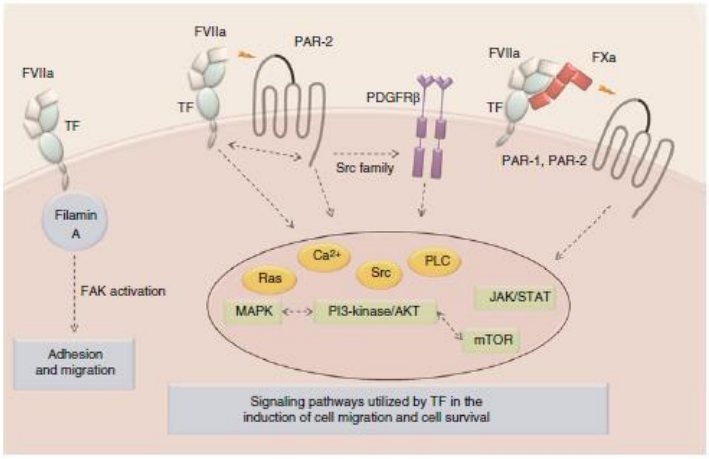


Figure 1.13 TF signalling pathways

Signalling pathways downstream of TF after binding and activation of FVIIa and FVIIa/FXa (Aberg 2013).

1.7.4 TF and PAR-2 as regulators of each other

As discussed above TF signalling can be PAR-2 dependent via TF-FVIIa binding to PAR-2. However TF and PAR-2 each appear to have regulatory influences on the other.

Studies of mice with TF cytoplasmic domain deletion (TF-mutant mice) have shown that the cytoplasmic domain of TF acts as a negative regulator of TF-FVIIa protease signalling in angiogenesis. Genetic deletion of the cytoplasmic domain of TF led to accelerated physiological and pathological angiogenesis (Belting, Dorrell et al. 2004).

Furthermore it has been shown that PAR-2, but not PAR-1, activation led to phosphorylation of the TF cytoplasmic domain (Ahamed and Ruf 2004). In diabetic eye disease, upregulated PAR-2 was shown to colocalise with phosphorylated TF only in neovessels suggesting that phosphorylation of TF is the likely mechanism that leads to loss of negative regulatory control of PAR-2 promoting PAR-2 dependent pathological angiogenesis.

PAR-2 induced phosphorylation of TF has also been shown to be important in cell migration. Binding of TF-FVIIa to PAR-2 has been shown to sensitize human vascular smooth muscle cells, fibroblasts and monocytes to PDGF-BB stimulated chemotaxis. This heightened chemotaxis to PDGF-BB is dependent on both PAR-2 activation and the phosphorylation of the cytoplasmic domain of TF and leads to downstream activation of the Src family and Phospholipase C (PLC) (Aberg and Siegbahn 2013) (Figure 1.13).

1.7.5 TF and Inflammation

Bacterial septicaemia has provided strong evidence for the association between the TF initiated coagulation pathway and inflammation *in vivo*. Early studies demonstrated the ability of antibodies to tissue factor to reduce inflammation in LPS induced septic shock (Taylor, Chang et al. 1991,

Creasey, Chang et al. 1993). However inhibition of the prothrombinase complex does not prevent lethality in the sepsis model (Taylor, Chang et al. 1991). Therefore these findings suggest that the initiation phase of coagulation and tissue factor can sustain an inflammatory response independent of downstream thrombin (Chu 2005) (Riewald and Ruf 2002). More recently low TF mice (mice expressing approximately 1% of wildtype TF levels) exposed to LPS demonstrated reduced inflammatory cytokine production and reduced mortality (Pawlinski, Pedersen et al. 2004).

TF's role in inflammation has been suggested by a number of studies using anti TF antibodies and tissue factor pathway inhibitors. Treatment with anti TF antibodies reduced experimental glomerulonephritis with reduction in markers of inflammation including proteinuria, glomerular fibrin deposition and major histocompatibility complex (MHC) class II expression (Erlich, Holdsworth et al. 1997).

TF is also important in the development of inflammatory arthritis. Increased levels of TF are found in the synovium of patients with this condition (Weinberg, Pippen et al. 1991) and injection of recombinant TF into the joint leads to cellular infiltration of the synovium and bony destruction (Bokarewa, Morrissey et al. 2002). The cytoplasmic domain of TF, which has no role in coagulation, appears to have an important role in the inflammatory response. Yang et al reported a significant reduction in arthritis severity in tissue factor cytoplasmic domain mutant mice. In addition there were reductions in proinflammatory cytokine expression IL-1, IL-6 and TNF, MMP-13, reactive

oxygen species (ROS) production, cutaneous delayed hypersensitivity and antigen-specific T-cell proliferation. The authors concluded that the cytoplasmic domain is also important in normal immune function (Yang, Hall et al. 2004).

1.7.6 TF and fibrosis

Mice that express low levels of tissue factor have been generated and characterised in cardiac muscle where TF is normally expressed. Low-TF mice had shorter lifespans than wildtype mice but were also histologically shown to have increased haemosiderin deposition and cardiac fibrosis. The authors proposed that the mechanism for the fibrosis was haemorrhage from cardiac vessels due to impaired haemostasis (Pawlinski, Fernandes et al. 2002). However mice with low TF and no TFPI have improved haemostasis but still suffer from cardiac fibrosis. Subsequent studies have shown TF appears to reduce TNFα driven apoptosis of cardiomyocytes (Wolberg and Mast 2012).

In patients with cirrhosis, independent of aetiology, the levels of TF rise with the Child Pugh class and are significantly higher in patients with cirrhosis than non cirrhotic patients (Tacke, Schoffski et al. 2001). Vitamin E has been shown to reduce TF levels in cirrhotic patients *in vitro* and *in vivo* (Ferro, Basili et al. 1999). The role of TF and in particular the cytoplasmic domain in the development of liver fibrosis and cirrhosis has not been explored.

1.8 Thesis Aims

Chronic liver injury, regardless of the cause, leads to liver fibrosis and eventually cirrhosis. Currently therapy is directed at the aetiology of the chronic liver injury if available. If this is ineffective or unavailable no effective antifibrogenic therapy is currently available. There are multiple stimuli and cellular mechanisms responsible for fibrogenesis and through our increasing understanding of this process we may be able to create effective antifibrogenic therapy.

This thesis will address the role of tissue factor, PAR-1 and PAR-2 in experimentally induced liver fibrosis with the hypothesis that activation of PAR-1 and PAR-2 and the cytoplasmic domain of tissue factor promote hepatic fibrosis through HSC activation.

More specifically the aims are to:

1) To investigate whether deletion of the PAR-1 gene protects against liver fibrosis in a murine model of hepatocyte inflammation and fibrosis, to identify the potential mechanisms for this and to investigate the expression of PAR-1 and the effect of a PAR-1 agonist in human HSC. (Chapter 3)

2) To investigate whether deletion of the PAR-2 gene protects against liver fibrosis in a murine model of hepatocyte inflammation and fibrosis, to identify the potential mechanisms for this and to investigate the expression of PAR-2 and the effect of a PAR-2 agonist in human HSC. (Chapter 4)

3) To investigate whether deletion of the cytoplasmic domain of TF protects against liver fibrosis in a murine model of hepatocyte inflammation and fibrosis and to investigate whether coexistent deletion of the PAR-2 gene leads to additional attenuation of fibrosis. (Chapter 5)

CHAPTER 2

Materials and Methods

2 Materials and Methods

2.1 Induction of mouse fibrosis and cirrhosis

2.1.1 Experimental animals

PAR 1 -/ - (PAR-1 KO) mice derived on a mixed 129/SvJ and C57Bl/6 background were obtained from Dr Shaun Coughlin (University of California, San Francisco, CA) and backcrossed 10 generations onto a C57Bl/6 background.

PAR 2 -/ - (PAR-2 KO) mice derived on a mixed 129/SvJ and C57Bl/6 background were obtained from Dr Shaun Coughlin (University of California, San Francisco, CA) and backcrossed 10 generations onto a C57Bl/6 background.

TF mutant mice derived on a mixed 129/SvJ and C57Bl/6 background had deletion of 18 of the 20 amino acids of the cytoplasmic domain. TF mutant animals have normal embryonic and postnatal development, fertility and coagulation.

PAR 2 -/ - - TF mutant mice were PAR-2 KO backcrossed with TF mutant for 10 generations on a C57Bl/6 background.

Mice were allowed food and water *ad libitum* and were housed at a constant temperature in 12:12 hour light-dark cycle. Experimental protocols were

approved by the Monash University Animal Ethics Committee and mice received humane care as specified under the Australian code of practice for the care and use of animals for scientific purposes.

2.1.2 Genotyping

DNA from mouse tails (5mm sample) was extracted by incubation at 55 °C overnight with 500µl digestion buffer plus 25µl proteinase K (Roche, Indianapolis, IN, USA). Samples were then shaken vigorously for 5 minutes, 180µl saturated NaCl (>6M) was added, tubes were shaken and then spun for 5 minutes at 13,000g in a microcentrifuge. The supernatant was then collected, 380µl isopranolol added and the precipitated DNA was pelleted by 10 min of centrifugation. The DNA pellet was washed with 70% ethanol, spun for 5 min and resuspended in 50µl of sterile water. The polymerase chain reaction (PCR) volume was 25µl and optimal conditions were 1.25mM $MgCl_2$, 0.2mM dNTPs and 0.5µM of each primer. The primer sequences are listed in Table 1. 100ng of DNA template and 1 unit of Taq DNA polymerase (Invitrogen, Carisbad, CA.USA) were used in each reaction. The PCR program involved Step 1: One cycle at 94 °C for 5 min, Step 2: 35 cycles at 94 °C for 30 sec, 60.5 °C for 30sec and 72 °C for 1 min, Step 3: Final cycle at 72 °C for 3 min. Samples were then run on a 1.5% agarose gel (Sigma) containing ethidium bromide (Sigma) for 1 hour and photographs taken under UV light using a camera (Eastman Kodac, Rochester, NY).

2.1.3 Injection protocol

Liver fibrosis was induced in male mice by twice weekly intraperitoneal injections of 1µl/g body weight carbon tetrachloride (CCl_4) (Merck, Darmstadt, Germany) mixed with olive oil (1:10) starting between 8-10 weeks of age and continuing for 5 to 8 weeks. Two control groups of WT C57BL/6 mice (n=8 each) received olive oil alone for 5 and 8 weeks.

There are a number of animal models of hepatic fibrosis including cholestatic models, toxic models and models of metabolic liver injury. A discussion on the characteristics of each model are beyond the scope of this thesis but the common endpoint is deposition of ECM and development of fibrosis. The chemical hepatotoxin CCl_4 can induce hepatic inflammation and fibrosis and is a robust and reproducible model of necro-inflammatory liver fibrosis and thus was the model chosen.

Acute CCl_4 mediated liver injury is characterised by activation of Kupffer cells followed by induction of an inflammatory response with secretion of cytokines and chemokines in 48 hours (Liedtke, Luedde et al. 2013). CCl_4 induced fibrosis is typically characterised by hepatocyte necrosis and periportal inflammation early in the model with the deposition of connective tissue after 2-3 weeks. Longterm intoxication (>6 weeks) is characterised by the development of cirrhosis. Therefore the timepoints of 5 and 8 weeks were chosen to examine the early and late stages of fibrosis (Wu and Norton 1996).

There are some limitations to the CCl_4 animal model of fibrosis, including that CCl_4-induced fibrosis does not share characteristics with viral and alcoholic induced fibrosis and does not have a strong correlation with the development of hepatocellular carcinoma.(Wu and Norton 1996)

2.1.4 Post mortem examination and sample collection:

Mice were euthanized with carbon dioxide 72 hours after the last dose of CCl_4. Blood was then collected via cardiac puncture (approx 1 ml) and samples left to clot at 4 °C. Serum was collected by centrifuging sample at 5000g for 10min at room temperature. Serum samples were stored at -80 °C until required for analysis.

The mouse peritoneum was then exposed and the liver and spleen were collected and total weight recorded. Tissues samples were collected and stored at -80 °C until required for various analyses.

2.2 Cell culture of immortalised hepatic stellate cells

The immortalised human HSC cell line LX-2 was used for in vitro experiments. Similarly to primary HSC, LX-2 express key receptors regulating hepatic fibrosis including PDGF receptor β and proteins involved in matrix remodelling such as MMP-2 and TIMP-2. LX-2 cells are responsive to both BB-PDGF and TGFβ1 stimulation and have been shown to survive in serum free media and have high transfectability. The gene expression examined by microarray

demonstrates the LX-2 phenotype is most similar to an activated stellate cell (Xu, Hui et al. 2005).

LX-2 cells, which are immortalised human HSC (a gift from Prof. Scott Friedman, Mt Sinai, New York), were maintained in DMEM/F12 with 5% FCS and 100U/ml penicillin and streptomycin at 37 $^{\circ}$C in a humidified 5% CO_2 atmosphere. LX-2 cells were seeded for 3 days into 6 well plates at a density of 1 x 10^5 cells per well in M199 medium (Invitrogen, Grand Island, NY, USA) with 5% fetal calf serum (FCS) and grown to confluence.

2.3 Histology

2.3.1 Preparation of samples for histological examination

Liver tissue for histological examination was fixed in 2% paraformaldehyde for 6 hours at room temperature and then washed twice in 70% ethanol and stored in 70% ethanol at room temperature. Tissue samples were then processed. Samples were dehydrated in sequential baths of 70% ethanol for 1 hour, 90% ethanol for 2 hours, 100% ethanol for 2 hours and 100% ethanol for 1 hour. Tissue samples were then submerged in Solvent 3B/2026 (Hychem Industries, Victoria, Australia) in 3 separate baths. Tissue samples were then embedded in paraffin wax and stored at room temperature. Four micron thick sections from paraffin embedded liver tissue were cut on a microtome and placed on Superfrost plus slides. Sections were baked at 60°C for 1 hour and stored at room temperature.

2.3.2 Fibrosis assessment

Four micron thick sections from paraffin embedded liver tissue were deparaffinised and stained with picrosirius red (Sigma-Aldrich;Direct Red 80 0.1%wt/vol in saturated picric acid) for 90 minutes, washed in acetic acid and water (5:1000), dehydrated in ethanol and mounted in neutral DPX. Fifteen consecutive non-overlapping fields were acquired at magnification x40 for each mouse liver, the image digitised and fibrosis area analysed by Scion Image for Windows (vAlpha 4.0.3.2, Scion Corporation, Frederick, MD).

2.3.3 αSMA, F4/80 and CD68 Immunohistochemistry protocol

Paraformaldehyde fixed 4 micron thick liver tissue sections were stained with primary antibody for αSMA (monoclonal mouse anti-mouse α-smooth muscle actin, Sigma, St Louis, MO, USA), F4/80 (rat anti mouse F 4/80, a gift of Dr Richard KItching, 1:200) and CD68 (rat anti mouse CD68, FA11 a gift of Dr G Koch, Cambridge UK, 1:100). The following secondary antibodies were used: αSMA biotinylated rabbit anti-mouse IgG2a antibody (Invitrogen, Camarillo, CA, USA, 1:300), F4/80 and CD68 Polyclonal rabbit anti rat IgG (DAKO #E0468 1:150). In brief sections were dewaxed, rehydrated and then blocked with 0.6% hydrogen peroxide and cellular apoptosis susceptibility (CAS) protein blocking solution (Invitrogen, Camarillo, CA). Primary antibody incubations for 30 minutes at room temperature (αSMA) and overnight at 4°C (F4/80, CD68) were followed by application of secondary antibody. Staining was amplified using avidin-biotin complex kit (Vector Laboratories, Burlingame, CA, USA) and detected with 3,3’ diaminobenzidine (DAB) (Dako, Carpentaria, CA, USA). Slides were counterstained with Harris hematoxylin.

For quantification of immunoreactivity, 15 consecutive non-overlapping fields at 250X magnification (α-SMA, F4/80, CD68) were scored using a graticule eyepiece in a blinded fashion. Negative controls consisted of a mouse IgG1 isotype control antibody (Dako, Glostrup, Denmark) and water substituting for the primary antibody.

2.4 Determination of collagen content in vivo and in vitro

2.4.1 In vivo hepatic hydroxyproline content

Hydroxyproline is an amino acid that acts to stabilise collagen deposited in the liver in fibrogenesis. It is found almost exclusively associated with collagenous connective tissue and therefore is a good surrogate for quantification of collagen deposition (Lee, Shun et al. 2005). Hepatic hydroxyproline content was quantified using liver tissue frozen in liquid nitrogen as previously described with minor modification (Patella S, Phillips DJ et al 2006). Briefly, liver samples were weighed and hydrolysed in 2.5ml of 6N HCl at 110 °C for 18 h in Teflon coated tubes. The hydrolysate was centrifuged at 3000rpm for 10 minutes; the pH of the resulting supernatant was adjusted to 7.4 and absorbance measured at 558nm. Total hydroxyproline content was measured against a standard curve prepared with trans-4-hydroxy-L-proline (Sigma-Aldrich, St Louis, MO) preparations in the range of 0.156 to 5.0μg/ml and expressed per milligram of wet tissue weight.

2.4.2 In vitro collagen content

Sircol Soluble Collagen Assay (Biocolor; Newtown Abbey, Northern Ireland) was used to determine collagen content in cell culture supernatant. Briefly, 1ml of Sircol dye reagent was added to 200 µl of LX2 cell culture supernatant samples. The contents were mixed and then manually shaken 5 minutely for 30 minutes and then centrifuged at 10,000rpm for 10 minutes during which time the Sircol binds to the soluble collagen and precipitates out of solution. The unbound dye was drained from the tube, 1 ml of the alkali reagent was added and then each tube was vortexed then rested for 10 minutes to allow the bound dye to dissolve. Using a spectrometer set at 550nm wavelength the absorbance of reagent blanks, collagen standards and test samples was measured. Collagen content was measured against a standard curve of collagen in the range 5 to 50µg/ml and expressed per mg total protein.

2.5 Determination of gene expression in whole liver and in vitro

2.5.1 Ribonucleic acid (RNA) extraction

Mouse RNA was extracted using the Qiagen RNeasy mini kit according to the manufacturer's instructions (Qiagen Pty Ltd, Hilden, Germany). Briefly, 0.02 to 0.03g of liver was placed in a RNAse free tube. 600µl of Buffer RLT was added to each sample which was then vortexed for 15 minutes. The resultant lysate was pipetted into a QIA shredder spin column and centrifuged initially at full speed for 2 minutes, then the column was removed and the lysate was centrifuged for a further 3 minutes. The resultant supernatant was removed

and added to equal volume of 50% ethanol, transferred to an RNeasy spin column, centrifuged for 15 seconds and the flow through discarded. 700µl Buffer RW1 was added and the column was centrifuged for 15 secs and followed by the addition of two aliquots of 500µl of Buffer RPE, centrifuging in between for 15seconds and 2 minutes respectively. The RNeasy spin column was placed in a new collection tube, centrifuged for 1 minute and then 30µl of RNase-free water was added directly to the spin column membrane, centrifuged and then a further 30µl of RNase-free water was added. 4µl of RNA was added to 76µl of RNase free water. The RNA concentration was measured with a Nanodrop ND-100 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.5.2 RNA extraction from cell culture

RNA from cultured cell lines was isolated using the Trizol (Invitrogen, Carlsbad, CA, USA) method. After aspiration of cell culture media, adherent cells were washed with Ca^{2+}/Mg^{2+} free HBSS to remove any dead cells and the wash then aspirated. One ml of Trizol was added per culture well and the cell scraper was used to disrupt the cellular membrane.

2.5.3 Reverse transcription

RNA was used to generate cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) as per manufacturer's instructions. Briefly, 2X RT master mix was prepared from supplied components as follows: 2.0µl 10X RT buffer, 0.8 µl 25XdNTP mix (100mM), 10XRT Random Primers, 1.0µl Multiscribe^{TM} Reverse

Transcriptase, 1.0µl RNase Inhibitor and 3.2µl of Nuclease free water. 19µl of 2XRT master mix was combined with 10µL of RNA containing 1µg RNA. The tubes were centrifuged. The PCR program involved Step 1: 25°C for 10 minutes, Step 2: 37°C for 120minutes, Step 3: 85°C for 5 seconds and Step 4: 4°C for 10 minutes and then samples were collected and stored at -80°C.

Table 2.1 Oligonucleotide sequences for Real Time PCR analysis

Target Gene	Primer sequence (5'-3')	Source
18s	Fwd: GTA ACC CGT TGA ACC CCA TTC Rev: GCC TCA CTA AAC CAT CCA ATC G	Gift from Dr Eric Morand
TGFβ	Fwd: TGC CCT CTA CAA CCA ACA CA Rev: GTT GGA CAA CTG CTC CAC CT	(Primer 3 software).
MMP-2	Fwd: ACC CAG ATG TGG CCA ACT AC, Rev: TCA TTT TAA GGC CCG AGC AA	from Dr Scott Freidman
TIMP-1	Fwd: ACG AGA CCA CCT TAT ACC AGC CG Rev: GCG GTT CTG GGA CTT GTG GGC	from Dr Scott Freidman
PAR-1	Fwd: CTC CTC AAG GAG CAG ACC CAC Rev: AGA CCG TGG AAA CGA TCA AC	Primer 3 software
PAR-2	Mm00433160_m1 using TaqMan Gene Expression Master Mix (Applied Biosystems).	Applied Biosystems TaqMan probe
18S	Hs03003631_g1 using TaqMan Gene Expression Master Mix (Applied Biosystems).	Applied Biosystems TaqMan probe
αSMA	Fwd: TCC TCC CTG GAG AAG ACC TAC Rev: ATG GTG GTT TCG TGG ATG C	Primer 3 software
Mast cell Chymase	Mm00487638_m1 g1 using TaqMan Gene Expression Master Mix (Applied Biosystems).	Applied Biosystems TaqMan probe

2.5.4 Real time quantitative PCR

Real time PCR analysis was performed (Power Sybr Green, Roche, Manheim, Germany) using a Rotor Gene 3000 light cycler (Qiagen Pty Ltd, Sydney, Australia) and the specific target mRNA of interest quantified as a ratio relative to 18S RNA content of the sample. 200ng of cDNA was used per reaction. The Primer sequences used in analysis are found in Table 2.1.

2.6 Determination of TGFβ1 in whole liver and in vitro by Enzyme Linked Immunosorbent Assay (ELISA)

Extracts were prepared from snap frozen liver by homogenisation in lysis buffer (Tris-HCl 50mM, NaCl 150mM, EDTA 1mM, 1% Triton X-100, 0.5% Tween-20, 0.1% SDS) containing a protease inhibitor cocktail (#11836170, Roche Diagnostics, Mannheim, Germany) followed by centrifugation at 14,000*g* for 15 minutes at 4ºC. Supernatants were collected and activated with acetic acid/urea prior to analysis. TGFβ1 content of liver protein extracts were measured using a mouse TGFβ1 ELISA kit (R&D Systems Inc, Minneapolis, MN, USA). Plates were read using the Bio-Rad microplate reader at 450nm and TGFβ1 concentrations were calculated from the standard curve by the plate reader software.

2.7 Analysis of cellular proliferation in vitro

LX-2 cells were seeded onto 96 well plates at a density of 1 x 10^4 per well in 5% FCS/M199 media and cultured overnight. The PAR-2 agonist peptide, SLIGKV, was added at concentrations from 0 to 100μM at 24 and 48 hours.

Human PDGF-BB (R&D Systems, Minneapolis, MN, USA) was used as a positive control at a concentration of 25ng/ml. Proliferation of activated HSC was assessed using a colorimetric BrdU ELISA (Roche, Mannheim, Germany) according to manufacturer's instructions.

2.8 Biochemical analysis of mouse serum

Sera obtained from animals were analysed for alanine transaminase (ALT), bilirubin and albumin by the biochemistry department at Monash Medical Centre using a Cobas automated serum analyser (Roche diagnostics, USA).

Chapter 3

Reduction of liver fibrosis in protease activated receptor 1 heterozygotes and knockout mice

3 Reduction of liver fibrosis in protease activated receptor 1 heterozygotes and knockout mice

3.1 Introduction

Thrombin is generated following tissue injury via activation of the coagulation cascade. However in addition to its central role in haemostasis, thrombin appears to have a number of proinflammatory and profibrotic effects that are largely mediated via its receptor, PAR-1.

PAR 1 is expressed by a number of human cells including platelets, endothelial cells, fibroblasts, smooth muscle cells and T lymphocytes (Coughlin 2000). In normal human liver PAR-1 is found on endothelial cells and hepatic stellate cells (Rullier, Senant et al. 2006) and in patients with cirrhosis, PAR-1 is found on fibroblasts in the fibrotic septa and on T lymphocytes in the inflammatory infiltrate around newly formed blood vessels and bile ducts (Marra, DeFranco et al. 1998) (Rullier, Senant et al. 2006).

PAR-1 signalling has been demonstrated to be important in fibroproliferative responses to injury. PAR-1 signalling upregulates adhesion molecules on endothelial surfaces, leads to release of chemokines that activate neutrophils and monocytes (Coughlin 2000) and can lead to increased expression of profibrogenic mediators such as CTGF (Chambers, Leoni et al. 2000) and MCP-1 and induces Il-6 and IL-8 production (Chu 2005).

PAR-1 has been shown to be involved in pulmonary fibrosis (Scotton, Krupiczojc et al. 2009) and there is growing evidence for a significant role of thrombin and PAR-1 signalling in the development of hepatic fibrosis. PAR-1 is upregulated in acute and chronic liver disease (Marra, DeFranco et al. 1998). *In vitro* thrombin stimulates HSC proliferation and MCP1 expression (Marra, Grandaliano et al. 1995) and collagen synthesis (Gaca, Zhou et al. 2002). *In vivo* PAR 1 antagonism has been shown to protect against murine liver fibrosis (Duplantier, Dubuisson et al. 2004) (Fiorucci, Antonelli et al. 2004) implicating the receptor's importance in HSC activation and fibrosis. The aim of the studies in this chapter was to confirm the role of PAR-1 in development of hepatic fibrosis by using a knockout model.

3.2 Materials and Methods:

3.2.1 In vivo Experimental design

The original study design was to investigate the effects of PAR-1 deficiency and therefore to compare PAR-$1^{-/-}$ and wildtype mice at week 5 and week 8. However due to contamination of the breeding line, some mice in the week 5 group were heterozygous for PAR-1 and at week 8 only PAR-1 heterozygotes, no PAR-$1^{-/-}$, were available to be studied and compared to wildtype.

Seven groups of mice were studied, three groups received twice weekly intraperitoneal injections of CCl_4 for 5 weeks (PAR-$1^{-/-}$ n= 4, PAR-$1^{-/+}$ n= 7, wild type C57BL/6 (WT) n=11) and two groups received CCl_4 for 8 weeks (PAR-$1^{-/+}$ n= 6, WT n= 7). The reason for the variable distribution of heterozygote and homozygote knockout mice in the CCl_4 treatment groups is

explained in the discussion section. Two control groups of WT C57BL/6 mice (n=8 each) received olive oil alone for 5 and 8 weeks.

3.2.2 Collection of samples

Mice were euthanized with carbon dioxide 72 hours after the last dose of CCl_4 and serum and samples were collected as outlined in Chapter 2.1.4.

3.2.3 Quantification of mRNA expression by Real Time Polymerase chain reaction (RT-PCR)

For the 5 week results, gene expression was quantified by real time PCR using the Roche Light Cycler (Roche Systems, Basel Switzerland) which measures the formation of PCR products fluorometrically in real time. SYBR Green 1, which unbound has low fluorescence, fluoresces strongly upon binding to dsDNA with the fluorescence intensity increasing proportionally with increasing ds DNA. The log linear portion of the PCR amplification curve is identified with the threshold or crossing point. The levels of mRNA and their estimated crossing point were determined using the provided Light Cycler computer software. Results were given as a ratio of the specific target gene to βactin. The Light Cycler PCR reagents were purchased from Roche Biochemicals.

Due to a machine change and change in the protocols in the laboratory for 8 week results, real time PCR analysis was performed (Power Sybr Green, Roche, Manheim, Germany) using a Rotor Gene 3000 light cycler (Qiagen Pty Ltd, Sydney, Australia) and the specific target mRNA of interest quantified as a ratio relative to 18S RNA content of the sample. 200ng of cDNA was used per reaction. The Primers used are listed in Table 2.1.

3.2.4 Human HSC stimulation by PAR-1 agonist in vitro

Immortalised human hepatic stellate cells (LX-2 cells) were cultured as described in Chapter 2.2. The media was changed at day 3 and human PAR-1 agonist hexapeptide SFLLRN-NH2 (Sigma-Aldrich, St. Louis, MO, USA) was added at 25, 50 and 100μM. A scrambled hexapeptide (Auspep, Melbourne, Australia) was used as a control. A further dose of either agonist or scrambled peptide was added at 24 and 48 hours and culture medium and cells were harvested after 72 hours of peptide exposure.

3.2.5 Data analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) with Newman-Keuls post-test for multiple comparisons or Student's t-test for comparisons between two groups as appropriate, using GraphPad Prism 5.03 and 6.04 for Windows (GraphPad Software, Inc, La Jolla, USA). A p value of less than 0.05 was considered significant.

3.3 Results

3.3.1 Vital Parameters

After 5 weeks of CCL_4 administration, mouse body weight was significantly lower in the wildtype group compared to the vehicle (olive oil) controls. Otherwise at this time point the spleen weight, liver weight and liver weight as a percentage of total body weight (TBW) were not significantly different between the groups (Table 3.1A). At 8 weeks the mouse body weight was significantly higher in the PAR-1 heterozygotes compared to the wildtype and the vehicle controls. The wildtype livers were significantly smaller than both the PAR-1 heterozygotes and vehicle control and subsequently the liver weight as a percentage of TBW was also smaller in the wildtype mice (Table 3.1B).

3.3.2 PAR-1 expression

Quantitative RT-PCR demonstrated significantly increased PAR-1 expression in the animals exposed to CCl_4 at both 5 and 8 weeks (Figure 3.1).

3.3.3 PAR-1 deficiency reduces hepatic fibrosis

WT, PAR-1 heterozygotes and PAR-1 KO developed significant fibrosis when exposed to CCl_4 compared to control animals (Figure 3.2A, B). Minimal fibrosis was observed in the control WT mice given olive oil alone at both 5 and 8 weeks. Quantitative analysis of histological fibrosis by computer-assisted morphometry in CCl_4-treated WT mice, showed significant fibrosis at

Table 3.1 A

	Vehicle Control	PAR-1 KO	PAR-1 het	Wildtype	P value
Body weight, g	26.8 ±0.83	24.0 ±0.60	27.9 ±2.8	23.2±0.68*	P<0.05
Liver wt, g	1.32 ± 0.07	1.28 ±0.04	1.24 ±0.10	1.12± 0.03	Ns
Liver wt/body wt	4.85 ± 0.24	5.24 ± 0.10	4.70 ± 0.26	4.64 ± 0.18	ns

Table 3.1 B

	Vehicle Control	PAR-1 het	Wildtype	P value
Body weight, g	28.8± 0.47	32.91 ± 0.5	30.27 ± 0.7	P<0.05
Liver wt, g	1.34 ±0.03	1.334 ±0.03	1.16 ±0.08	P<0.05
Liver wt/body wt	4.6 ± 0.06	4.06 ±0.13	3.36 ± 0.27	P<0.05

Table 3.1 Vital parameters PAR-1KO and Wildtype mice

Vital parameters (all values mean ±SEM) in mice after 5 weeks of CCl4 (A) and after 8 weeks of CCl4 (B)

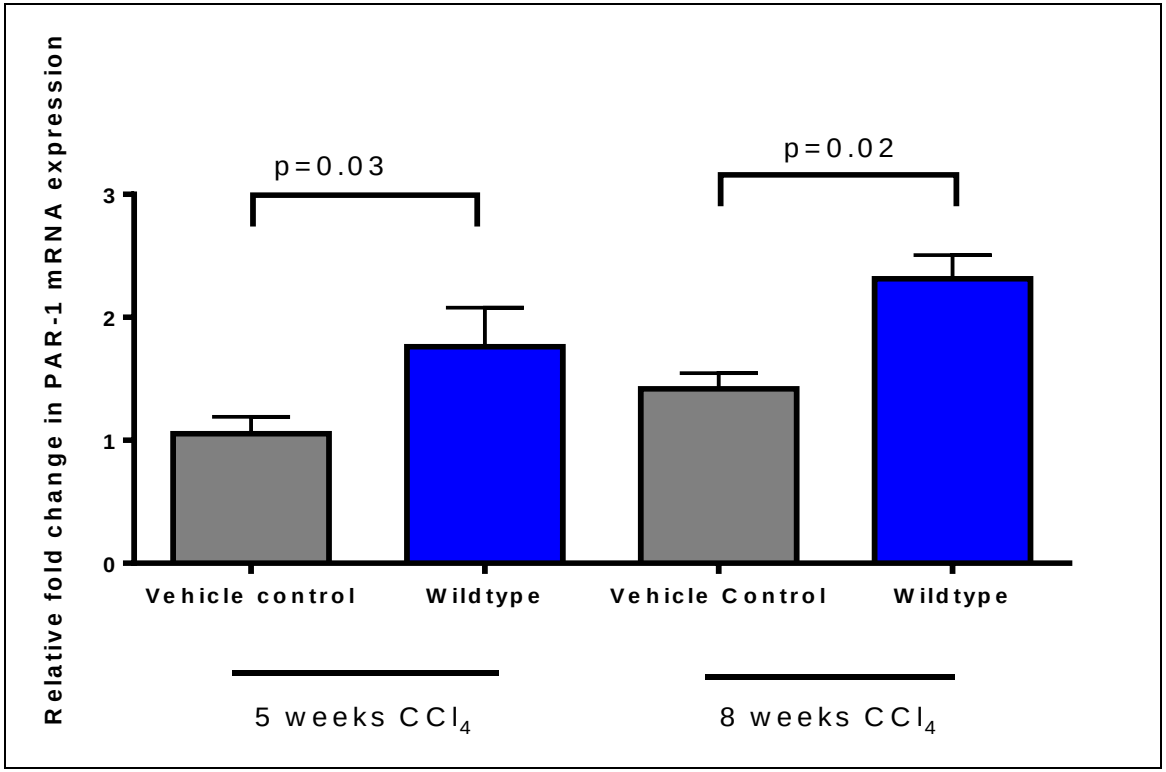


Figure 3.1 PAR-1 expression

PAR-1 expression is induced by liver injury as is demonstrated by the significant increase seen in PAR-1 mRNA in mice exposed to carbon tetrachloride at both 5 and 8 weeks.

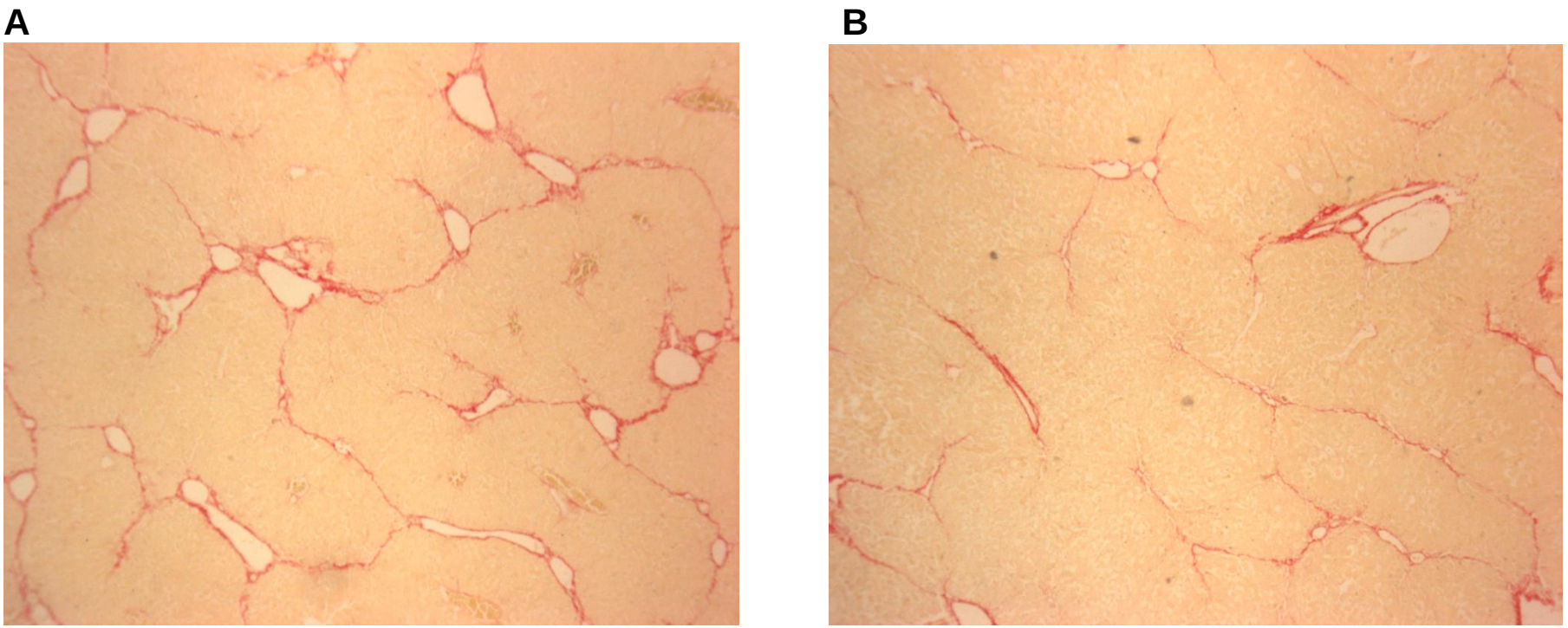


Figure 3.2 Picrosirius red histochemistry for hepatic collagen

Hepatic collagen deposition in WT mice (A) and PAR-1 heterozygotes (B) mice administered CCl4 for 8 weeks (Sirius red staining, 40X).

5 weeks (2.60 ± 0.19% liver area) which progressed with continued CCl_4 exposure over 8 weeks (3.50 ± 0.19%) (Figure 3.3A). PAR-1 KO mice had significantly less fibrosis at 5 weeks than both the wildtype and PAR-1 heterozygotes (Figure 3.3A). In PAR-1 heterozygotes, no difference in hepatic fibrosis was seen at 5 weeks CCL_4 compared to wildtype (2.30 ± 0.21% vs 2.60 ± 0.19% liver area). However by 8 weeks there was minimal progression of liver fibrosis with continued CCL_4 exposure in the PAR-1 heterozygotes, with significantly lower fibrosis seen compared to wildtype (2.91± 0.19% vs 3.50 ±0.19%, $p<0.05$).

3.3.4 Reduced collagen deposition in PAR-1 heterozygotes and PAR-1 KO mice

Hydroxyproline is a good surrogate for quantification of collagen deposition. Supporting the observed reduction in histological fibrosis, there was a reduction in hydroxyproline content in the PAR-1 KO and heterozygote mice. After 5 weeks of CCl_4, PAR-1 1KO mice had significantly reduced hydroxyproline content compared to wildtype ($p<0.05$). The PAR-1 heterozygotes had significantly less hydroxyproline content at 5 and 8 weeks but to a lesser degree than that seen in the PAR-1KO group (Figure 3.3B).

3.3.5 Reduced stellate cell activation in PAR-1 heterozygotes and KO following CCl_4 administration

Alpha smooth muscle actin (αSMA) is a marker of HSC activation and myofibroblast differentiation. In WT mice, progressive CCL_4 induced hepatic fibrosis was paralleled by increasing αSMA expression at 5 and at 8 weeks compared to the untreated control animals. At 8 weeks there was also

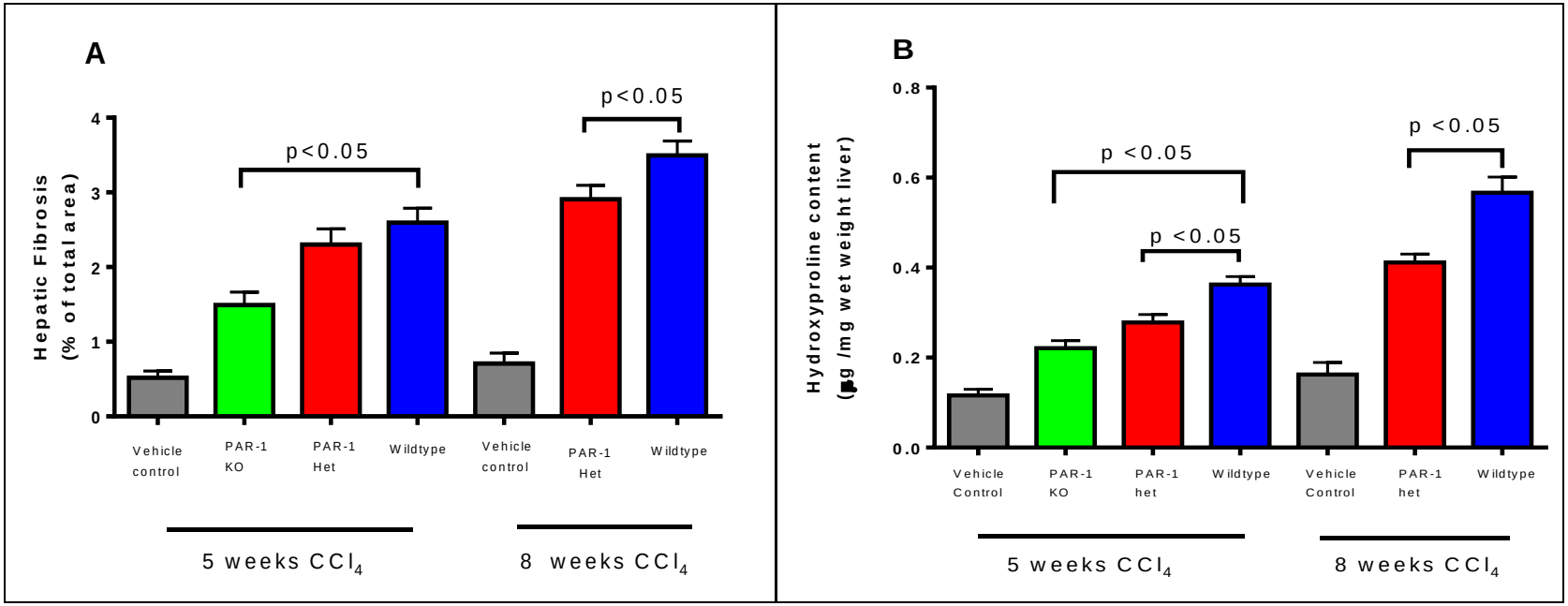


Figure 3.3 Hepatic Fibrosis

Computer assisted morphometry of Sirius red stained liver sections shows significantly less fibrosis in the PAR-I KO mice at 5 weeks and less fibrosis in the PAR-1 heterozygotes at 8 weeks compared to wildtype controls (A). Hepatic hydroxyproline content was significantly lower in the PAR-1KO and PAR-1 heterozygotes at 5 weeks and continued to be lower in the PAR-1 heterozygotes at 8 weeks (B).

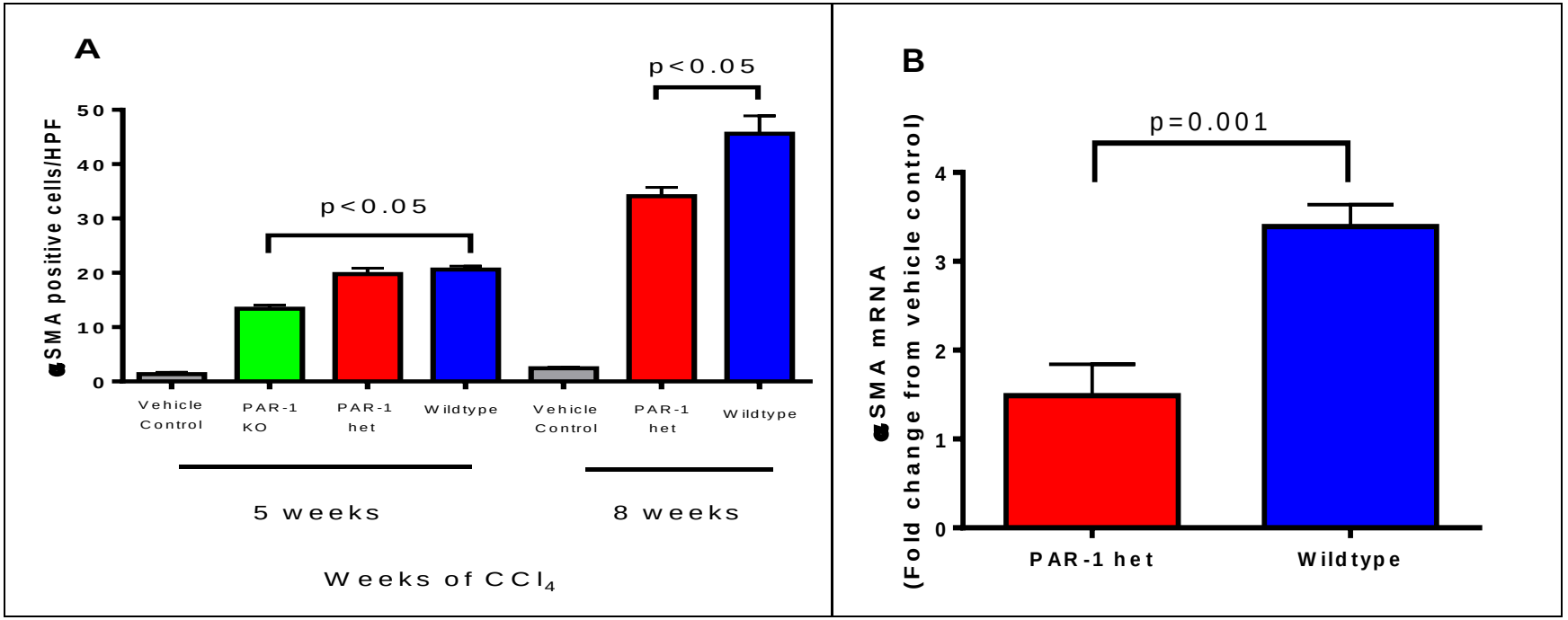


Figure 3.4 αSMA expression

The number of cells expressing αSMA was significantly lower in PAR-1 KO compared to WT at 5 weeks of CCl_4 administration (A). PAR-1 heterozygotes showed a similar number of αSMA positive cells compared to WT at 5 weeks (A) but by 8 weeks had reduced number of αSMA positive cells (A) and reduced mRNA expression (B) compared to WT.

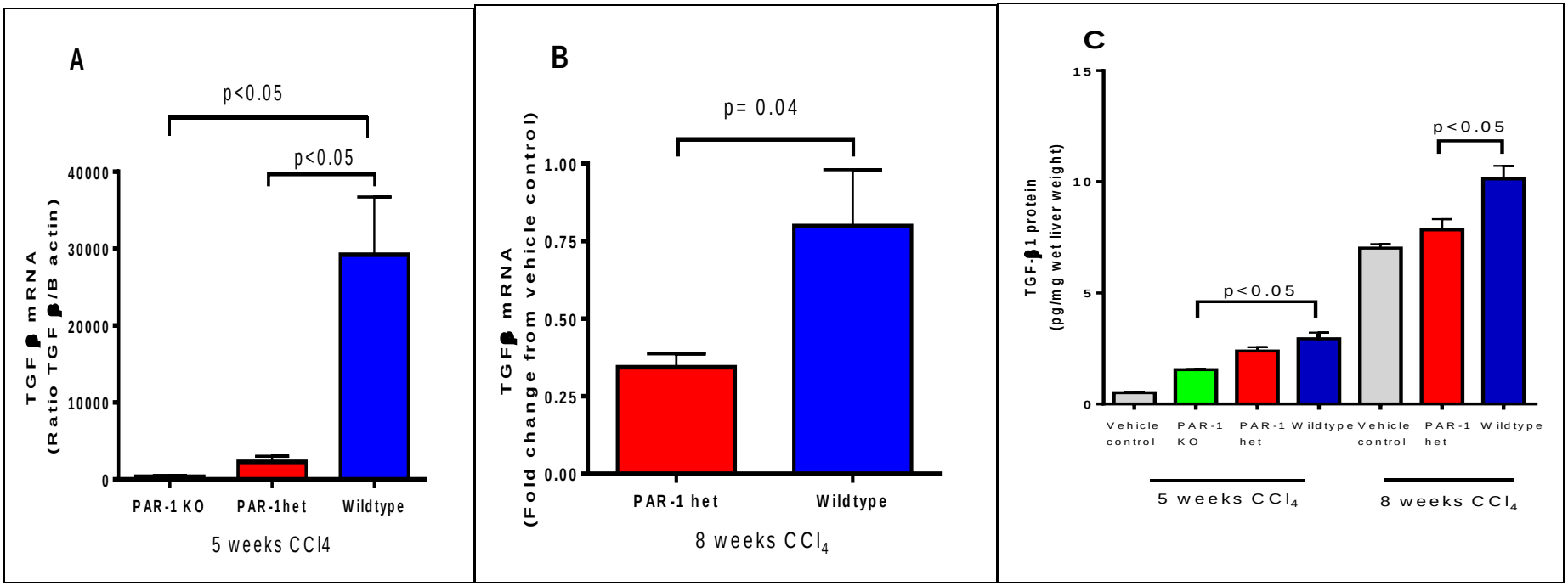


Figure 3.5 Hepatic TGFβ expression

TGFβ mRNA expression was significantly lower in the PAR-1 KO at 5 weeks (A) and the PAR 1 heterozygotes at 5 (A) and 8 weeks (B) compared to WT. This was accompanied by significantly lower TGFβ protein in the PAR-1 KO at 5 weeks and the PAR-1 heterozygotes at 8 weeks (C) compared to WT

upregulation of αSMA mRNA (3.39 ± 0.25 fold greater than control). PAR-1 KO had significantly less αSMA expression at 5 weeks. PAR-1 heterozygotes had similar αSMA expression to wildtype at 5 weeks but by 8 weeks had significantly less induction of αSMA mRNA (1.48 ± 0.35 fold greater than control ;*p=0.001)* and reduced αSMA expression (*p<0.05)* (Figure 3.4A and Figure 3.4B).

3.3.6 PAR-1 deficiency reduces hepatic TGFβ expression without significant change in MMP/TIMP

CCl_4 induced hepatic fibrosis in WT mice was associated with upregulation of TGFβ mRNA (Figure 3.5A) and TGFβ protein at 5 weeks (2.94 ± 0.27 pg/mg liver, control 0.51 ± 0.04 pg/mg) and 8 weeks (10.12 ± 0.58 pg/mg liver, control 7.01 ± 0.18 pg/mg) (Figure 3.5C). PAR-1 KO mice had significantly reduced TGFβ mRNA (Figure 3.5A) and TGFβ protein at 5 weeks (1.54 ± 0.03 pg/ml PAR-1 KO, $p<0.05$ compared to WT) (Figure 3.5C). At 5 weeks PAR-1 heterozygotes had a trend towards lower TGFβ mRNA levels and TGFβ protein (2.38 ± 0.17 pg/ml) compared to wildtype. At 8 weeks TGFβ mRNA (Figure 3.5B) and TGFβ protein (Figure 3.5C) were significantly lower in PAR-1 heterozygotes compared to wildtype (7.83 ± 0.48 pg/ml).

Matrix metalloproteinases (MMP) and their specific tissue inhibitors, TIMPs, regulate extracellular matrix composition and their expression is altered in response to liver injury and their expression profile changes with fibrosis progression and resolution. At 8 weeks MMP-2 in wildtype mice increased

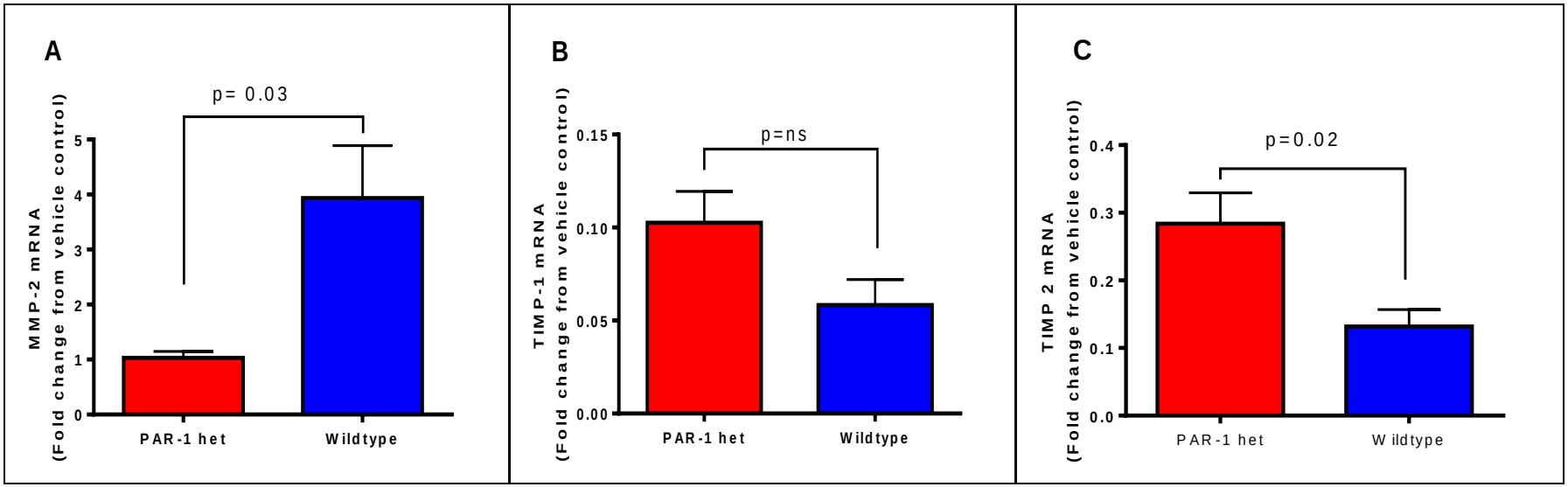


Figure 3.6 MMP-2 and TIMP-1 and 2 expression

After 8 weeks of CCl_4 administration, the expression of MMP-2 mRNA was significantly lower in the PAR-1 heterozygotes compared to wildtype mice (A). While no difference in the TIMP-1 mRNA levels between the 2 groups was seen (B), TIMP-2 mRNA was significantly lower in the PAR-1 heterozygotes compared to wildtype mice (C).

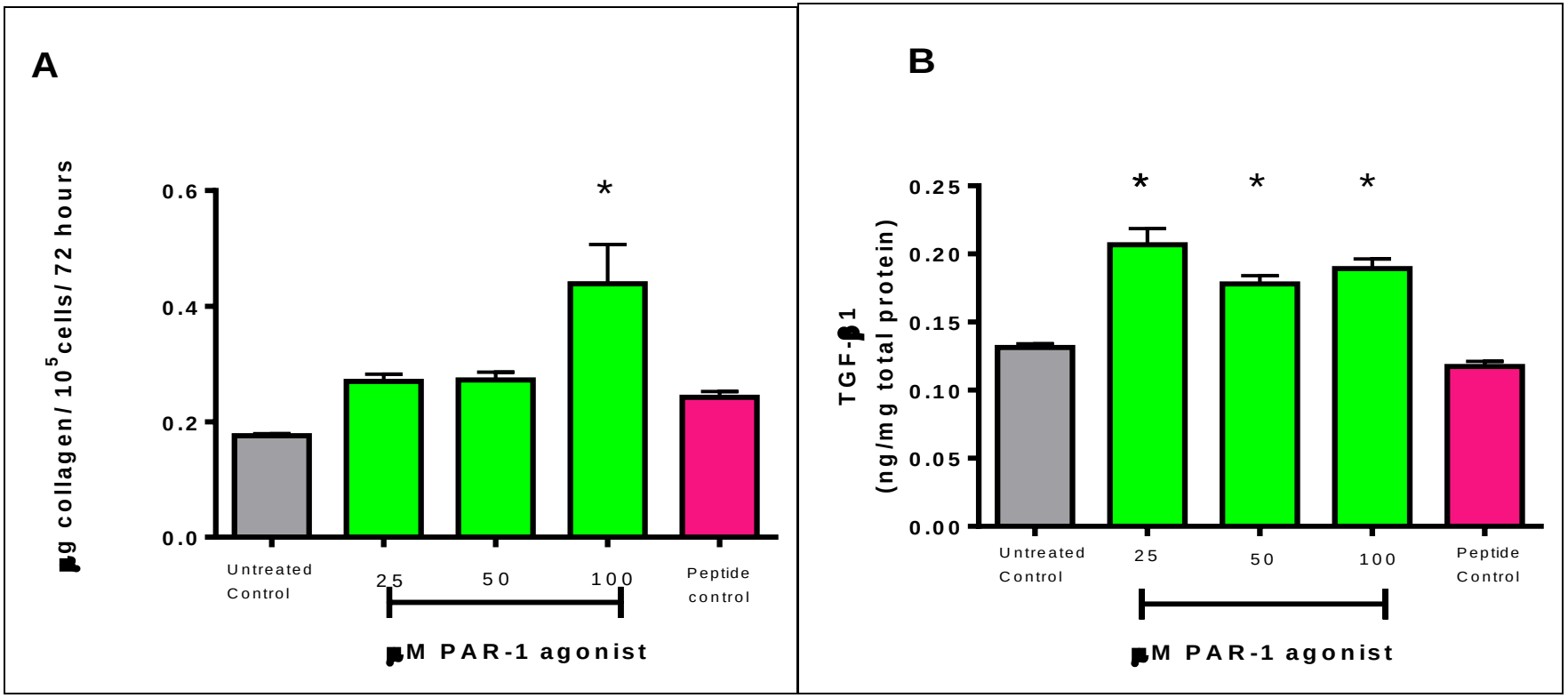


Figure 3.7 Response LX-2 stimulated with PAR-1 agonist

Stimulation of human LX-2 cells with PAR-1 agonist significantly increased collagen production at the 100μM dose (A). TGFβ protein production was increased in LX-2 cells at all agonist doses (B) (*$p<0.05$).

indicating active extracellular remodelling (3.93 ± 0.95 fold greater than control). In the PAR-1 heterozygotes the MMP-2 expression was significantly reduced (1.03 ± 0.12, *p=0.*036) (Figure 3.6A). There was no difference in TIMP-1 mRNA expression between the two groups (Figure 3.6B). TIMP-2 expression was minimally increased in both groups but was significantly higher in the PAR-1 heterozygotes compared to wildtype (0.28 ± 0.04 vs 0.13 ±0.03, *p=0.02*) (Figure 3.6C).

3.3.7 PAR-1 activation increases HSC collagen production in human HSC

HSC spontaneously produce collagen during culture on plastic tissue culture plates. PAR 1 agonist peptide (100μM) stimulated a significant increase in collagen production whereas the control hexapeptide and lower concentrations of PAR-1 agonist failed to stimulate collagen production (Figure 3.7A).

3.3.8 PAR-1 activation stimulates TGFβ production in human HSCs

TGFβ is spontaneously produced by HSC in culture. PAR-1 agonist peptide (at 25, 50 and 100μM doses) caused a significant increase in TGFβ production by LX-2 cells compared to control peptides and untreated controls. The threshold to stimulation of TGFβ production (25μM) was lower than that required for stimulation of collagen production (Figure 3.7B).

3.4 Discussion

The profibrotic and proinflammatory effects of thrombin are mediated via activation of its receptor, PAR-1, a G coupled protein receptor that is activated by proteolytic cleavage. Antagonism of PAR-1 has been shown to protect against the development of liver fibrosis in a murine model thus implicating it in hepatic fibrogenesis (Fiorucci, Antonelli et al. 2004).

In this study we wanted to further define the role of PAR-1 in promoting liver fibrosis by examining the effects of PAR-1 deficiency on the development of carbon tetrachloride induced liver fibrosis. In addition, we studied the effects of PAR-1 agonists *in vitro* in a human hepatic stellate cell line. Our results support the view that the PAR-1 receptor is involved in hepatic fibrogenesis. PAR-1 receptor expression increased in CCl_4 treated animals at both 5 and 8 weeks. PAR-1 heterozygotes at 8 weeks maintained a liver/body weight ratio that was significantly greater than CCl_4 treated wildtype mice and more similar to vehicle treated controls. PAR-1 deficient mice, and to a lesser degree the PAR-1 heterozygotes, exhibited reduced hepatic fibrosis area and collagen content. These significant changes were paralleled by less HSC activation, which was demonstrated by reduced hepatic αSMA mRNA expression and fewer activated αSMA positive myofibroblasts in PAR 1 knockout mice. The profibrogenic cytokine TGFβ was reduced at both a gene and protein level. The reduction in activated TGFβ would through reduced Smad activation lead to reduced profibrogenic gene transcription and thus is a possible mechanism for the reduced fibrosis observed.

Although the CCl_4 animal model utilised for hepatic fibrosis development causes acute injury and inflammation, it is less inflammatory than other models such as the model using a choline deficient diet supplemented with ethionine to create a NASH mouse model. If a more inflammatory model was used, it is possible that greater protection would have been seen in the PAR-1 heterozgotes and PAR-1 KO mice.

This study was designed to look at PAR-1 deficiency and was therefore planned to be performed in PAR-1 KO mice alone. The mice were obtained from a well-established PAR-1 knockout line and genotyping had been performed on a regular basis in the storage facility, however not all mice were genotyped prior to the commencement of this study. Retrospective genotyping of mice in our PAR-1 deficient colony, performed due to concerns about the integrity of the breeding protocols, revealed some mice allocated to this study were heterozygous for PAR-1. The experimental groups were readjusted to reflect correct genotypes. As a result, PAR-1-/- deficient mice were not available for analysis in the 8 week CCl4 part of the study.

At this point during my PhD Rullier et al published their findings that PAR-1 knockout reduces carbon tetrachloride induced liver fibrosis (Rullier, Gillibert-Duplantier et al. 2008). Given this publication, which made my work less novel and the fact that practically to re-establish a knockout line was going to take several months, I decided to concentrate my investigations on the other more novel PAR-2 and TF mutant mice described in Chapters 4 and 5.

Despite the unexpected findings regarding PAR-1 genotypes in the experimental groups, my investigations reinforce the findings that PAR 1 deficiency protects against liver fibrosis. The benefit of using a knockout model is to clearly identify the receptor's impact on the development of fibrosis. My findings support the findings of Fiorucci et al, which showed that PAR-1 antagonism in a bile duct ligation model protects against biliary fibrosis, and confirm the findings of Rullier, et al, that PAR-1 deletion reduces CCl_4 induced parenchymal liver fibrosis. The PAR-1 KO mice had reduced fibrosis at 5 weeks and given that at 8 weeks the heterozygotes continued to develop less fibrosis, one could predict that the PAR-1 KO mice at this age would have been afforded even greater protection.

Previous studies have shown that PAR-1 agonists stimulate HSC activation and collagen deposition in murine HSC cell line. We extended these studies to human HSC using the LX-2 cell line and confirmed the role of PAR-1 in human HSC activation. These *in vitro* studies strongly suggest that PAR-1 activation stimulates TGFβ production by HSC, which then promotes collagen production by the same cells.

3.5 Conclusion

Our findings support those of previous studies, which propose a role for PAR-1 in the development of hepatic fibrosis. PAR-1 activation increases pro-fibrogenic cytokines and collagen production in mice and in human hepatic stellate cells.

Monash University: Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study planning and design, Conduction of experiments, Statistical Analysis of results, Writing of manuscript (main author)	80%

The following co-authors contributed to the work.

Name	Nature of contribution
Jorge Tchongue	All histopathology had 2 independent counts. Second counter in histopathology.
Dinushka Lourensz	All histopathology had 2 independent counts. Second counter in histopathology.
Alison Liu	All histopathology had 2 independent counts. Second counter in histopathology.
A.Prof Peter Tipping	Manuscript review and revision
Professor William Sievert	Manuscript review and revision

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature		**Date**
Main Supervisor's Signature	[redacted]	**Date** **28th Oct 2014**

Chapter 4

Protease Activated Receptor 2 promotes experimental liver fibrosis and activates human hepatic stellate cells

4 Protease Activated Receptor 2 promotes experimental liver fibrosis and activates human hepatic stellate cells[1]

4.1 Introduction

Hepatic fibrosis occurs in response to acute and chronic liver injury from a variety of sources and may progress to end-stage liver disease with the development of portal hypertension, hepatocellular carcinoma and liver failure. A substantial body of evidence has identified the hepatic stellate cell (HSC) as the principal source of collagen produced during hepatic fibrogenesis (Friedman 2008) and thus there is considerable interest in factors that regulate HSC activation and collagen expression.

PARs are a unique group of G-protein coupled receptors that are activated by proteolytic cleavage of their extracellular N terminal domain to reveal a ”tethered” ligand that binds with the second extracellular loop of the receptor to initiate signalling. PAR-1 was initially identified in the search for the cellular thrombin receptor and to date four PARs have been identified. Thrombin activates PAR-1, 3 and 4 and Factor Xa activates PAR-1 and 2. PAR-2 is also activated by trypsin, mast cell tryptase and the tissue factor/factor VIIa and

[1] This is a manuscript published in Hepatology 2012 **55**(3): 879-887 Knight, V., J. Tchongue, et al. "

factor Xa complex (Riewald and Ruf 2002). There is a strong linkage between inflammation, coagulation and fibrosis (Tacke, Luedde et al. 2009) and a prothrombotic state appears to accelerate liver fibrogenesis (Anstee, Wright et al. 2009). One proposed mechanism for this linkage is signalling by coagulation factors through their cellular receptors, PARs, to activate stellate cells (Anstee, Wright et al. 2009, Borensztajn, von der Thusen et al. 2010).

PAR-2 is widely expressed in the gastrointestinal tract on epithelial cells and smooth muscle cells (D'Andrea, Derian et al. 1998). It has been shown to have important and multifaceted roles in regulation of gastrointestinal physiology and in inflammatory processes including pancreatitis, gastritis and colitis. In the normal liver, PAR-2 is expressed on hepatocytes, Kupffer cells, bile duct epithelial cells and endothelial cells of large vessels. Rat HSC express PAR-2 under normal conditions and its expression is markedly increased in liver fibrosis (Borensztajn, von der Thusen et al. 2010). Mast cells are prominently recruited during hepatic fibrosis (Farrell, Hines et al. 1995) and have the potential to provide a potent source of mast cell tryptase, which can activate PAR-2 receptors. PAR-2 activation augments inflammatory cell recruitment and pro-fibrotic pathways through the induction of genes encoding proinflammatory cytokines and proteins of the extracellular matrix. PAR-2 activation has been shown to promote pulmonary (Borensztajn, Bresser et al. 2010) and renal (Xiong, Zhu et al. 2005) fibrosis with increased expression in progressive liver injury (Fiorucci, Antonelli et al. 2004) but the contribution of PAR-2 to liver fibrosis has not been reported.

We hypothesized that PAR-2 activation promotes hepatic fibrosis in mice and induces HSC proliferation and collagen synthesis. In this study we show that deletion of PAR-2 diminishes CCl_4-induced hepatic fibrosis and that PAR-2 agonists promote HSC proliferation and collagen production.

4.2 Materials and Methods

4.2.1 In vivo Experimental design

Liver fibrosis was induced in male mice by twice weekly intraperitoneal injections of 1µl/g body weight CCl_4 mixed with olive oil (1:10) starting between 8-10 weeks of age and continuing for 5 to 8 weeks. Six groups of mice were studied, two groups received CCl_4 for 5 weeks (PAR-2$^{-/-}$ n=6, wild type C57BL/6 (WT) n=9) and two groups received CCl_4 for 8 weeks (PAR-2$^{-/-}$ n= 8, WT n=10). Two control groups of WT C57BL/6 mice (n=8 each) received olive oil alone for 5 and 8 weeks.

4.2.2 Collection of samples

Mice were euthanized with carbon dioxide 72 hours after the last dose of CCl_4 and serum and samples were collected as outlined in Chapter 2.1.4.

4.2.3 Human HSC stimulation by PAR-2 agonist in vitro

Immortalised human hepatic stellate cells (LX-2 cells) were cultured as described in Chapter 2.2. The media was changed at day 3 and human PAR-2 agonist hexapeptide SLIGKV (Sigma-Aldrich, St. Louis, MO, USA) was added at 25, 50 and 100µM. A scrambled hexapeptide (Auspep, Melbourne,

Australia) was used as a control. A further dose of either agonist or scrambled peptide was added at 24 and 48 hours and culture medium and cells were harvested after 72 hours of peptide exposure.

4.2.4 HSC proliferation in response to PAR activation

LX-2 cells were seeded onto 96 well plates at a density of 1 x 10^4 per well in 5% FCS/M199 media and cultured overnight. The PAR-2 agonist peptide, SLIGKV, was added at concentrations from 0 to 100μM at 24 and 48 hours. Human PDGF-BB (R&D Systems, Minneapolis, MN, USA) was used as a positive control at a concentration of 25ng/ml. Proliferation of activated HSC was assessed using a colorimetric BrdU ELISA (Roche, Mannheim, Germany) according to manufacturer's instructions.

4.2.5 Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA with Newman-Keuls post-test for multiple comparisons or Student's t-test for comparisons between two groups as appropriate, using GraphPad Prism 5.03 and 6.04 for Windows (GraphPad Software, Inc, La Jolla, USA). A p value of <0.05 was considered significant.

4.3 Results

4.3.1 Vital Parameters

After 5 weeks of CCl_4, PAR-2 knockout mice had significantly higher liver weights and subsequently mean liver weights as % of total body weight compared to wildtype and control animals. Treatment with carbon tetrachloride led to increased ALT in both wildtype and PAR-2 knockout compared to control mice. Also interestingly at 5 weeks the ALT levels were significantly higher in PAR-2 KO compared to wildtype mice. (Table 4.1A)

After 8 weeks of CCl_4 treatment mouse weight, liver weight and spleen weight did not differ between the groups. Treatment with carbon tetrachloride led to increased serum ALT levels in both wildtype and PAR-2 knockout mice. (Table 4.1B)

4.3.2 PAR-2 expression

PAR-2 expression was measured in the livers by real time PCR in WT PAR-2 $^{+/+}$ mice that received CCl_4 for 5 and 8 weeks and the control mice that received olive oil alone. PAR-2 expression was significantly higher in the mice that had received CCl_4 for 5 weeks and there was a trend to higher expression at 8 weeks. (Figure 4.1A)

In vitro, LX-2 cells activated by culture displayed increasing expression of PAR-2 mRNA with time as previously has been observed with rat cultured

Table 4.1 A

	Vehicle control	PAR-2 KO	Wildtype	P value
Body weight (g)	26.8	24.29 *	29.59	P<0.05
Liver weight (g)	1.32	1.50	1.51	ns
Liver weight as % total body weight	4.85	6.18	5.10	P<0.05
ALT	20	81.00	57.60	P<0.05

Table 4.1 B

	Vehicle control	PAR-2 KO	Wildtype	P value
Body weight (g)	28.8	26.34	29.60	ns
Liver weight (g)	1.34	1.41	1.64	ns
Liver weight as % total body weight	4.65	5.37	5.51	ns
ALT	20.57	52.50	52.0	P<0.05

Table 4.1 Vital Parameters PAR-2 KO and Wildtype mice

Vital parameters and ALT in mice after 5 weeks CCl_4 (A) and after 8 weeks CCl_4 (B).

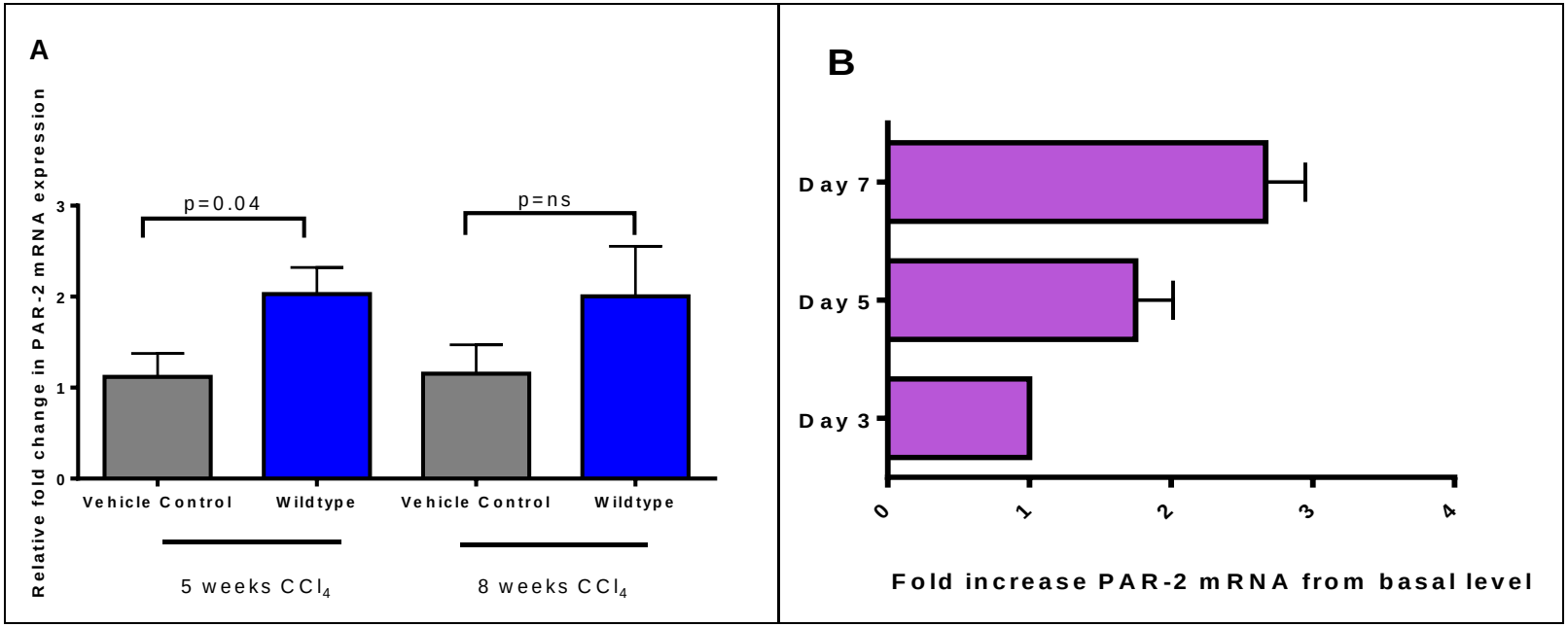


Figure 4.1 PAR-2 expression

PAR-2 expression was higher in the livers of wildtype mice exposed to CCl_4. (A) In vitro PAR-2 expression increases over time in cultured LX-2 cells. (B)

HSC.(Fiorucci, Antonelli et al. 2004) Quantitative RT-PCR demonstrated that 7 days in culture led to a 2.7 fold increase in PAR-2 expression. (Figure 4.1B)

4.3.3 PAR-2 deficiency prevents progression of histological hepatic fibrosis

WT mice developed significant hepatic collagen deposition in response to CCl_4 administration (Figure 4.2A). No fibrosis was observed in WT mice given olive oil alone. Quantitative analysis of histological fibrosis by computer-assisted morphometry in CCl_4-treated WT mice showed marked fibrosis at 5 weeks (1.97 ± 0.16% liver area) which progressed with continued CCl_4 exposure over 8 weeks (3.39 ± 0.26%) (Figure 4.3A). In PAR-2 KO mice, CCl_4 administration induced similar fibrosis to that of WT mice at 5 weeks (2.07 ± 0.26%). However, there was no progression of liver fibrosis with continued CCl_4 exposure between 5 and 8 weeks in the PAR-2 KO mice (2.09 ± 0.28%). At 8 weeks, there was significantly less hepatic fibrosis in the PAR-2 KO compared to WT mice ($p=0.004$) (Figures 4.2B,4.3A).

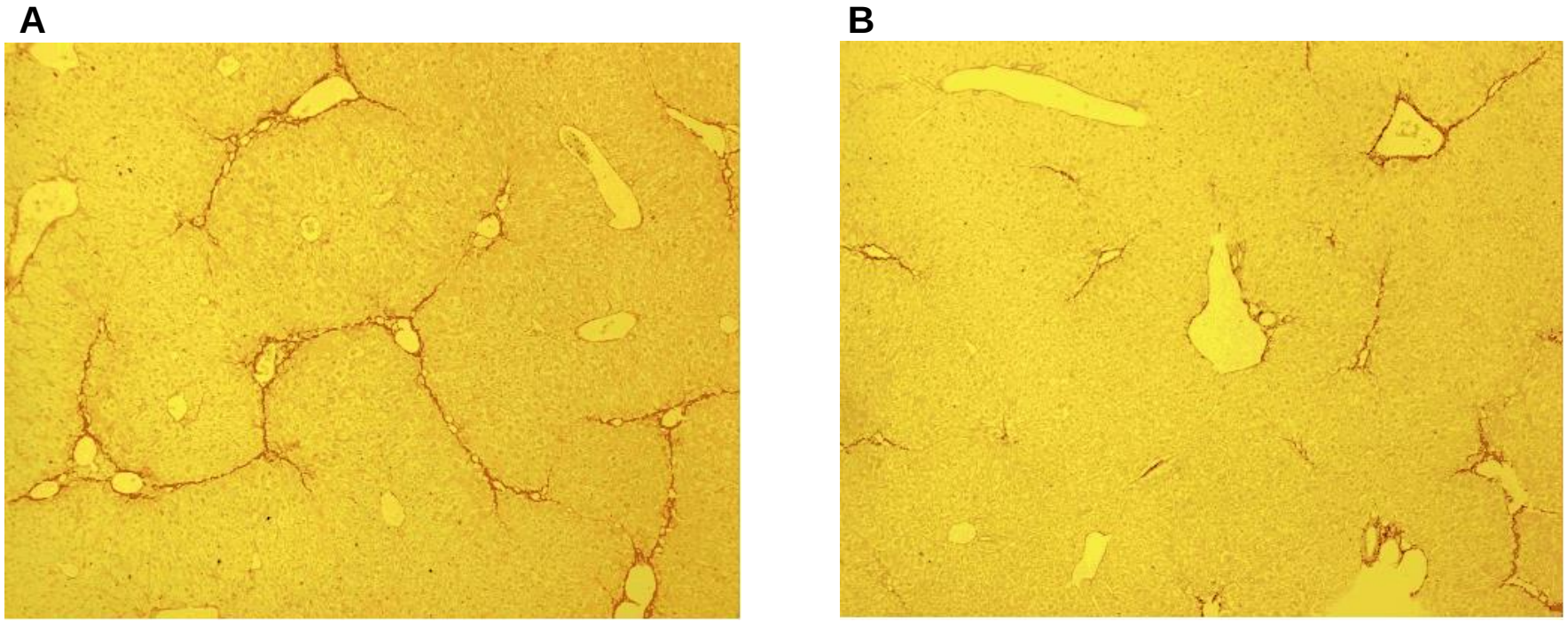


Figure 4.2 Picrosirius red histochemistry for hepatic collagen

Hepatic collagen deposition in wild type mice (A) and PAR-2 KO mice (B) administered CCl4 for eight weeks (Sirius red staining, 40X).

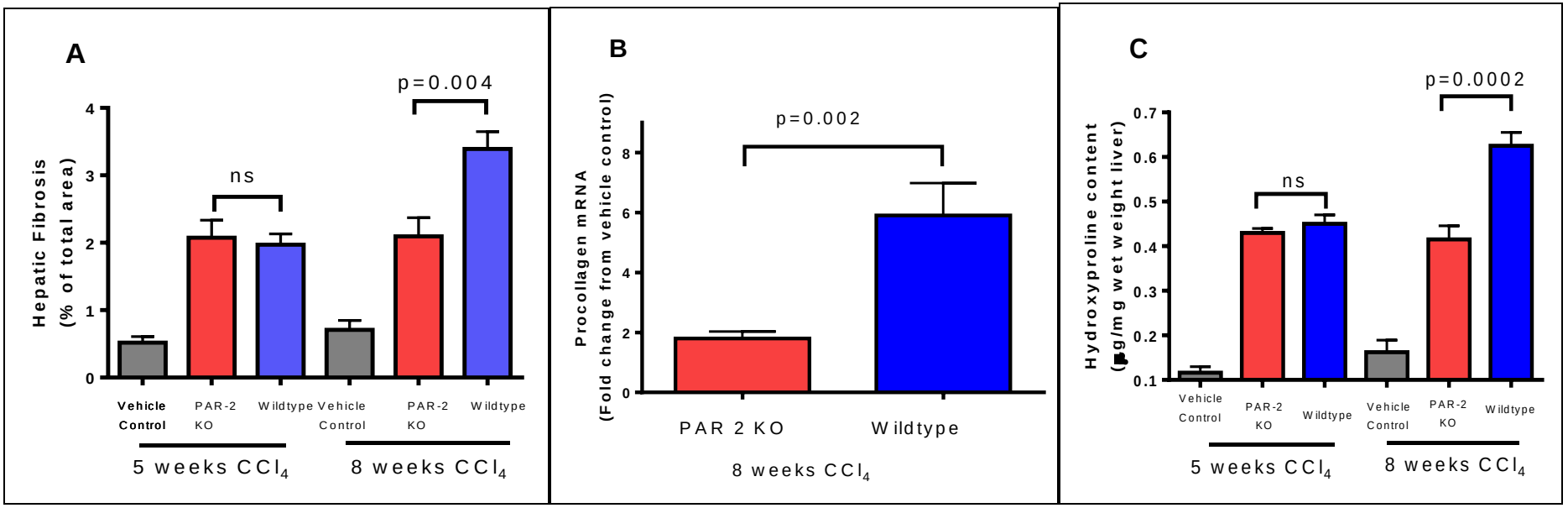


Figure 4.3 Hepatic collagen and fibrosis

Computer assisted morphometry of Sirius-red stained liver sections shows no difference in hepatic fibrosis area between wild-type and KO mice at five weeks; however, in contrast to WT mice, there was no progression in fibrosis area in PAR-2 KO mice by eight weeks (A). Procollagen mRNA expression (B) and hepatic collagen content (C) were significantly lower in PAR-2 KO mice compared to WT mice at 8 weeks.

4.3.4 Hepatic procollagen mRNA and hydroxyproline content are reduced in PAR-2 KO mice exposed to CCL_4

Histological assessment of fibrosis correlated closely with other indices of hepatic collagen content in mice given CCl_4. At 8 weeks, PAR-2 KO mice showed significantly less induction of procollagen mRNA (1.8 ± 0.23 fold above untreated mice) compared with WT mice (5.9 ± 1.08 fold, p=0.002) (Figure 4.3B). After 5 weeks of CCl_4 administration, similar increases in hepatic hydroxyproline were observed in WT and PAR-2 KO mice (0.45 ± 0.02 μg/mg and 0.43 ± 0.009 μg/mg, respectively) (Figure 4.3C). However after 8 weeks, while hepatic hydroxyproline content increased significantly in WT mice, there was no increase in PAR-2 KO mice compared to levels at 5 weeks. PAR-2 KO mice (0.42 ± 0.026) had significantly less hepatic hydroxyproline compared to WT mice (0.63 ± 0.03) at 8 weeks (p<0.002).

4.3.5 PAR-2 deficiency is associated with reduced stellate cell activation

Alpha smooth muscle actin (αSMA) is a marker of HSC activation and myofibroblast differentiation. In WT mice, hepatic fibrosis induced by administration of CCl_4 was accompanied by a progressive increase in αSMA expression at 8 weeks compared to untreated mice. In PAR-2 KO mice receiving CCl_4, induction of αSMA was similar to WT mice treated with CCl_4 at 5 weeks (Figure 4.4) but did not increase further resulting in significantly less αSMA expression compared to WT mice at 8 weeks (p=0.014).

4.3.6 PAR-2 deficiency reduces hepatic TGFβ expression and decreases MMP/TIMP mRNA

CCl_4 induced hepatic fibrosis was associated with upregulation of TGFβ mRNA (3.44 ± 0.72 fold greater than control) and protein (9.2 ± 0.9 pg/mg liver, control 6.9 ± 0.19 pg/mg) in WT mice at 8 weeks. In PAR-2 KO mice, TGFβ mRNA upregulation was significantly reduced (1.38 ± 0.23 fold of control, $p<0.016$ compared to WT) (Figure 4.5A), as was TGFβ protein, which was similar to control levels (Figure 4.5B).

Matrix metalloproteinases (MMP) and their specific tissue inhibitors, TIMPs, regulate extracellular matrix composition and their expression is altered in response to liver injury. In WT mice treated with CCl_4 for 8 weeks, both MMP-2 and TIMP-1 mRNA increased, consistent with active ECM remodelling during development of hepatic fibrosis (Figures 4.6A and 4.6B). Both MMP-2 and TIMP-1 mRNA expression were significantly lower in PAR-2 KO mice compared to WT mice, suggesting ECM remodelling is reduced in association with the arrest in progression of fibrosis between 5 and 8 weeks in PAR-2 KO mice.

4.3.7 PAR-2 deficiency did not alter mast cell chymase expression

Mast cells are recruited to the liver during fibrogenesis. One major constituent of mast cells is tryptase which can activate PAR-2 (Calvaruso, Maimone et al. 2008). Mast cell chymase is a marker of mast cells. Mast cell chymase mRNA

expression was examined in PAR-2 KO and WT mice at 5 and 8 weeks. There was no significant difference between mast cell chymase expression between the WT and PAR-2 groups and no statistical difference between the levels at 5 and 8 weeks. (Figure 4.7)

4.3.8 PAR-1 mRNA is upregulated at week 5 but not at week 8 in PAR-2 knockout mice

The temporal pattern of PAR-1 mRNA expression was examined to investigate potential mechanisms for the lack of early protection against hepatic fibrosis observed in PAR-2 knockout mice. In PAR-2 knockout mice at 5 weeks, PAR-1 mRNA expression was significantly up-regulated compared to CCl_4-treated WT mice or untreated controls (Figure 4.8A). However, at 8 weeks PAR-1 expression in the PAR-2 knockout mice was not significantly different from WT controls (Figure 4.8B). Thus upregulation of PAR-1 mRNA may compensate for lack of PAR-2 in early stages of CCl_4-induced fibrogenesis but this compensatory mechanism is not maintained as fibrosis progresses resulting in significantly less fibrosis in PAR 2 knockouts at 8 weeks.

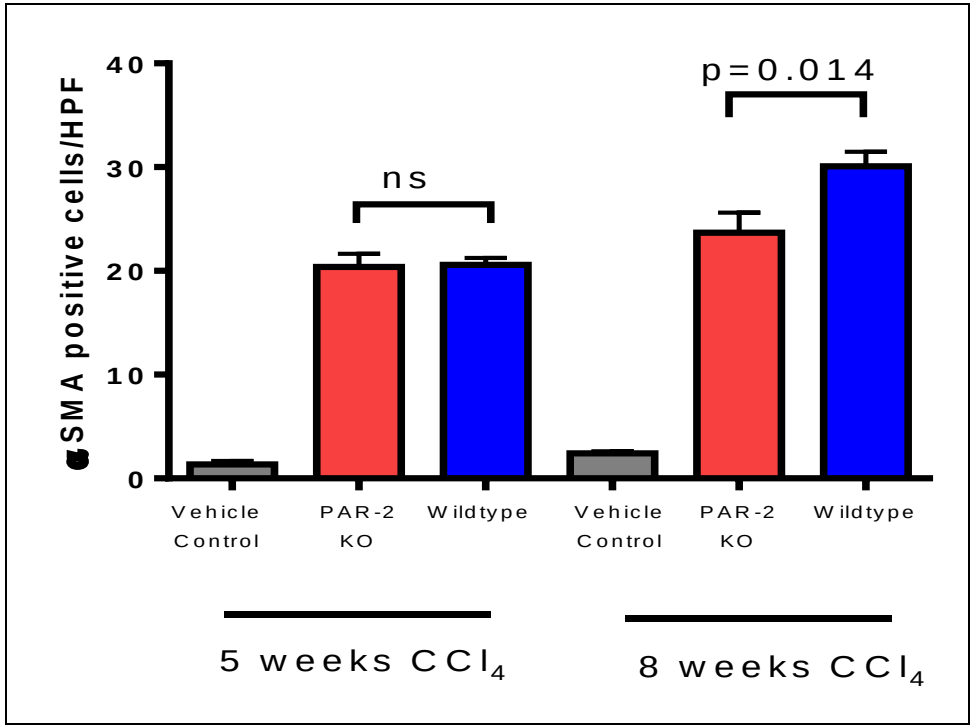


Figure 4.4 αSMA expression

Following CCl4 administration, the number of cells expressing αSMA was significantly lower in PAR-2 KO mice at 8 weeks compared to WT mice.

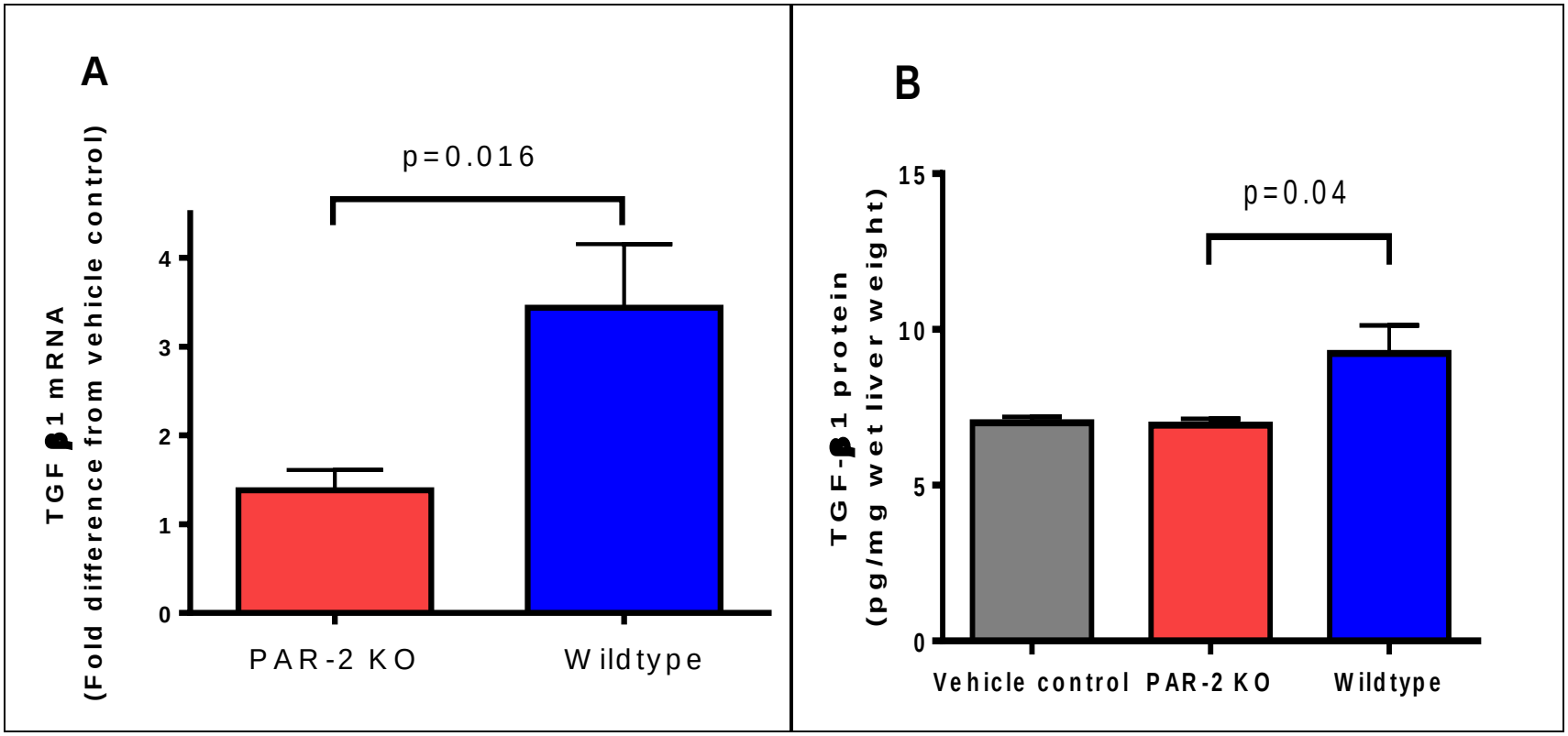


Figure 4.5 TGFβ mRNA expression

TGFβ mRNA expression was significantly lower in PAR-2 KO mice compared to WT controls at 8 weeks (A). TGFβ protein levels were lower, remaining at control levels, in PAR-2 KO mice compared to WT mice (B)

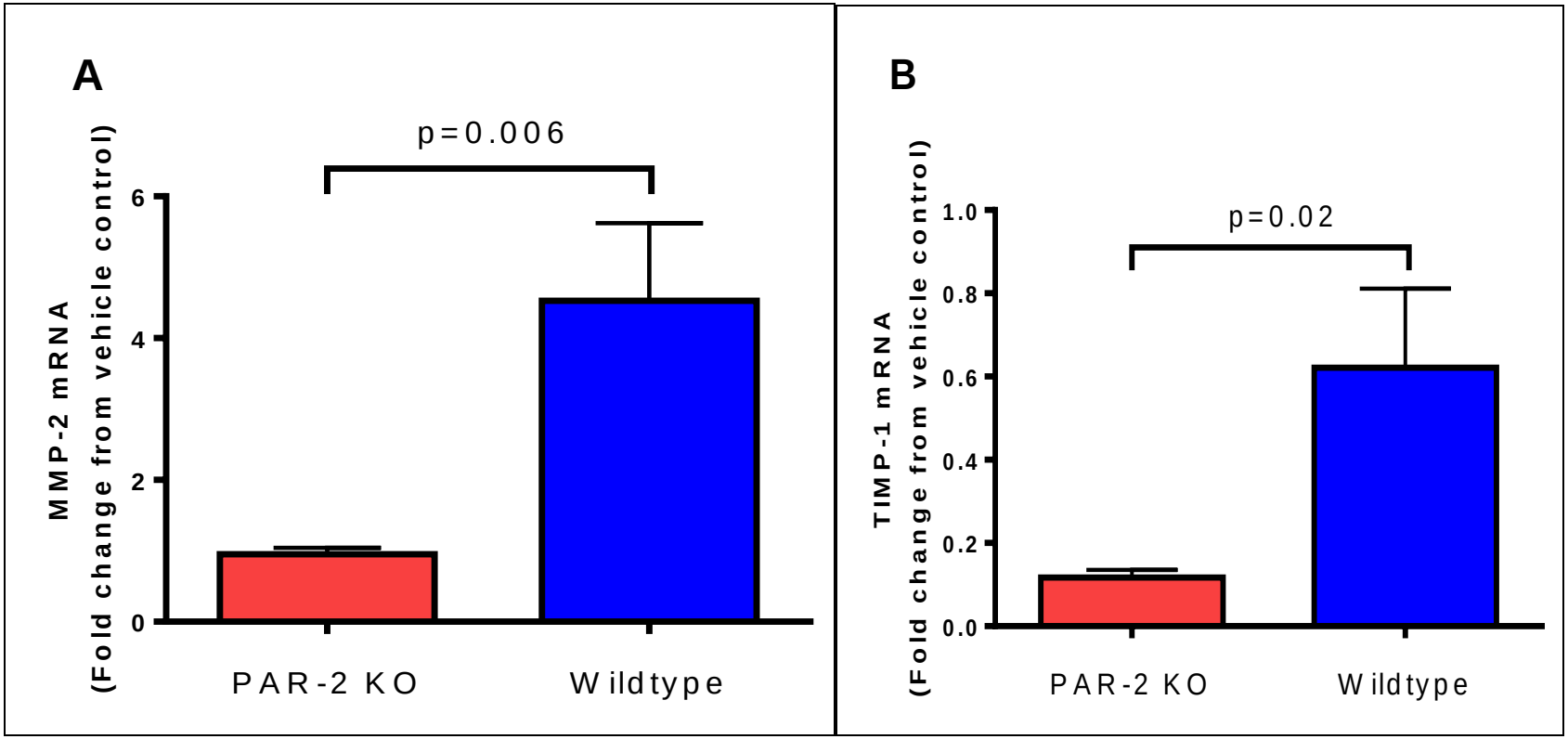


Figure 4.6 MMP-2 and TIMP-1 expression

Expression of MMP-2 mRNA (A) and TIMP-1 mRNA (B) were significantly lower in PAR-2 KO mice compared to WT mice at week 8 of CCl4 administration.

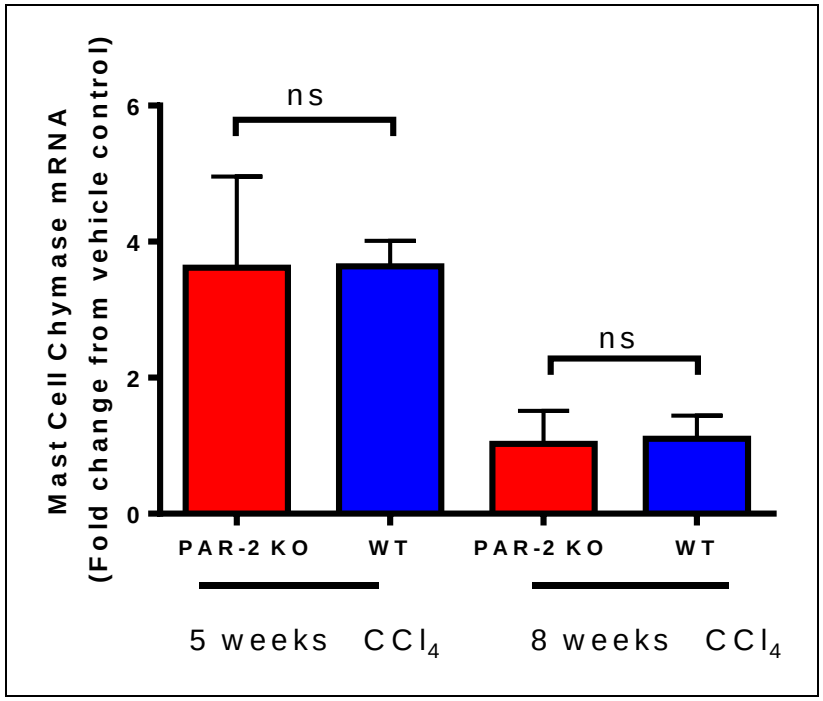


Figure 4.7 Mast cell chymase mRNA expression

There was no statistical difference in mast cell chymase expression between PAR-2 KO and WT either at 5 or 8 weeks. There was also no statistical difference between the expression between the two time points at week 5 and week 8.

4.3.9 Decrease in activated hepatic macrophages in week 8 PAR-2 knockout mice

We also examined the nature of the inflammatory infiltrate at week 5 and week 8 to investigate the difference in hepatic fibrosis between PAR-2 knockout mice and WT mice observed at week 8.

F4/80 is used as a marker of macrophages. CD68 is used as a marker of activated macrophages. In studies of the normal liver most macrophages are F4/80 positive, with only about a half expressing low levels of CD68 (Lloyd, Phillips et al. 2008). This low level of activation seen in the normal liver is from the mild inflammatory response to the processing of bacterial and dietary antigens entering the liver via the hepatic portal vein (Li and Diehl 2003). In an inflamed liver there is higher level of CD68 expression indicating increased macrophage activation (Lloyd, Phillips et al. 2008).

Significantly fewer F4/80+ macrophages were observed at both 5 and 8 weeks in PAR-2 knockout mice compared to CCl_4-treated WT mice (Figure 4.9A-E). In addition, at week 8 there were significantly fewer CD68+ macrophages in PAR-2 knockout mice compared to CCl_4-treated WT mice; a difference that was not observed at week 5 (Figure 4.10A-C). These observations are consistent with a role for PAR-2 in recruitment and later activation of macrophages in CCl_4 induced hepatic fibrosis.

4.3.10 PAR-2 activation stimulates HSC proliferation.

In order to study the effect of PAR-2 activation directly and specifically in HSC, we used an immortalised human stellate cell line (LX-2) which has been previously well characterised. Sub-confluent cultures of LX-2 cells were stimulated with a specific PAR-2 agonist peptide (SLIGKV) for 48 hours or a scrambled hexapeptide control. The PAR-2 agonist peptide stimulated dose dependent proliferation of LX-2 cells (Figure 4.11A). At the maximum dose of 100μM, the PAR-2 agonist peptide caused proliferation equivalent to PDGF (25ng/ml), the most potent inducer of HSC proliferation.

4.3.11 PAR-2 activation increases HSC collagen production

HSC spontaneously produce collagen during culture on plastic tissue culture plates. PAR-2 agonist peptide (100μM) stimulated a significant increase in collagen production by LX-2 cells, whereas the control hexapeptide failed to stimulate collagen production (Figure 4.11B). Similarly, PAR 1 agonist peptide (100μM) stimulated a significant increase in collagen production. The combination of PAR 1 and PAR 2 agonist peptide significantly increased collagen production compared to control peptide and untreated controls but not more than the individual agonists alone.

4.3.12 PAR-2 activation stimulates TGFβ production

TGFβ is spontaneously produced by HSC in culture. PAR-2 agonist peptide (at 3 different doses) caused a significant increase in TGFβ production by LX-

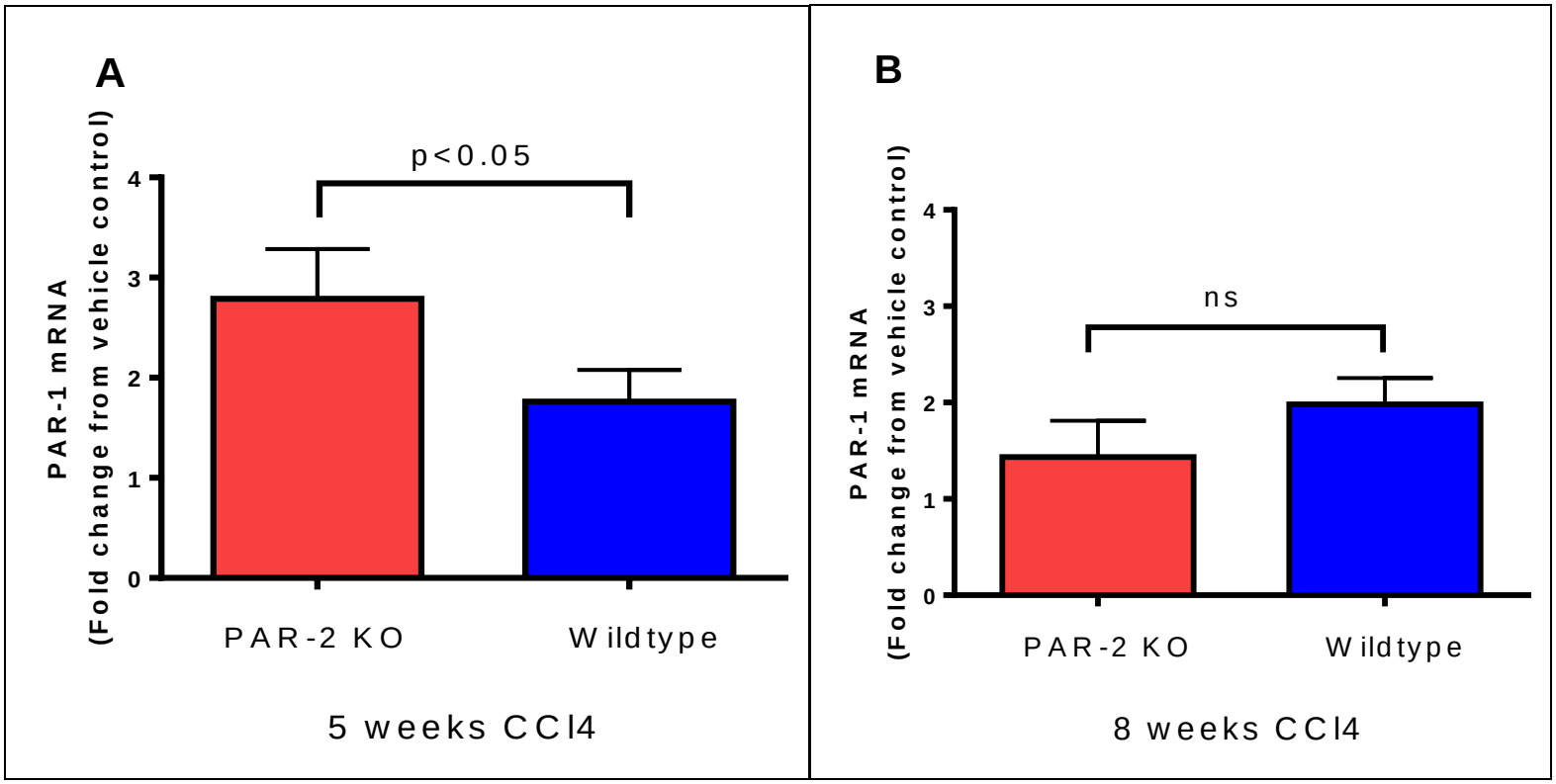


Figure 4.8 PAR 1 mRNA expression

PAR 1 mRNA expression was significantly greater in PAR 2 knockout mice compared to untreated controls and to CCl4-treated wildtype mice at 5 weeks (A). However, at 8 weeks PAR 1 expression was similar in the PAR 2 knockout mice compared to wildtype controls (B).

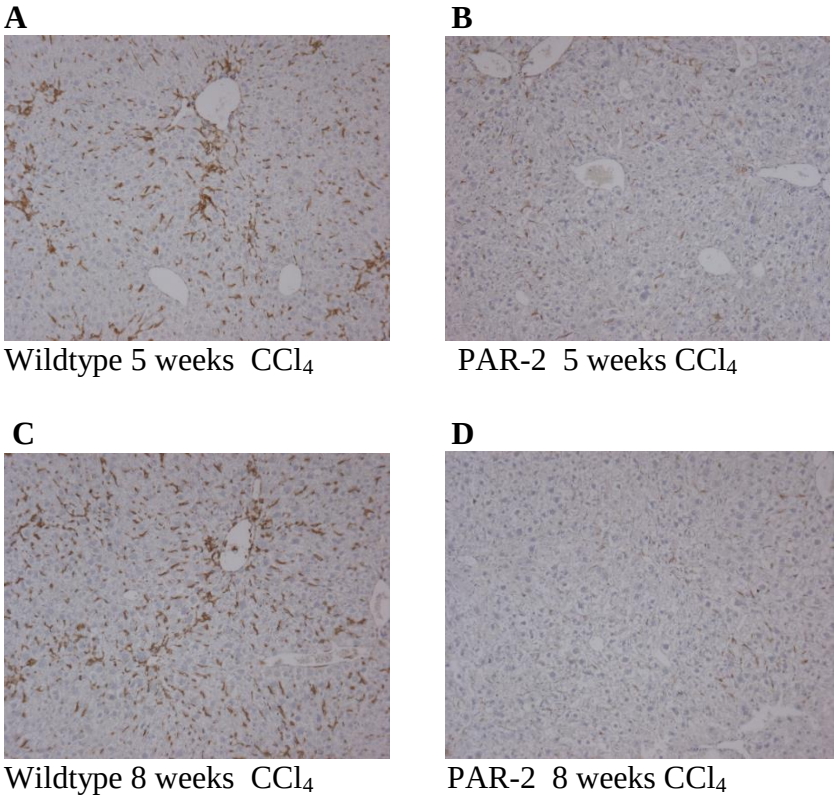


Figure 4.9 F4/80 Macrophages

Representative photographs of F4/80 macrophages in liver sections from wildtype mice (A and C) and PAR-2 KO mice(B and D) after 5 and 8 weeks of CCl_4 (immunohistochemistry, x100). There are significantly fewer F4/80 macrophages at both 5 and 8 weeks in PAR-2 KO mice compared to WT mice (E).

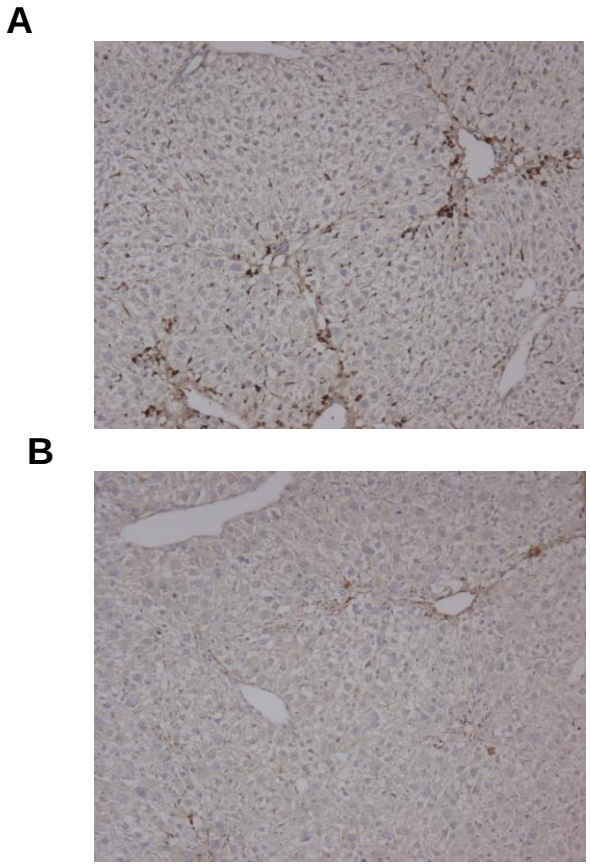


Figure 4.10Immunohistochemistry CD68 Macrophages

Photomicrographs of CD68 staining in wildtype(A) and PAR-2 KO mice(B) after 8 weeks of administration of CCl_4.

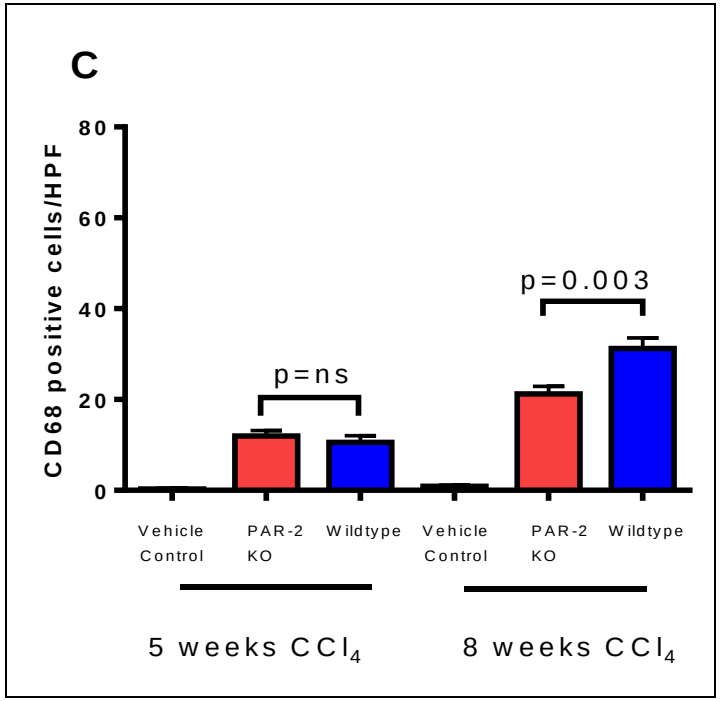


Figure 4.10 CD68 positive Macrophages (C)

At week 8, but not week 5, there were significantly fewer CD68+ macrophages in PAR 2 knockout mice compared to CCl4-treated WT mice (C).

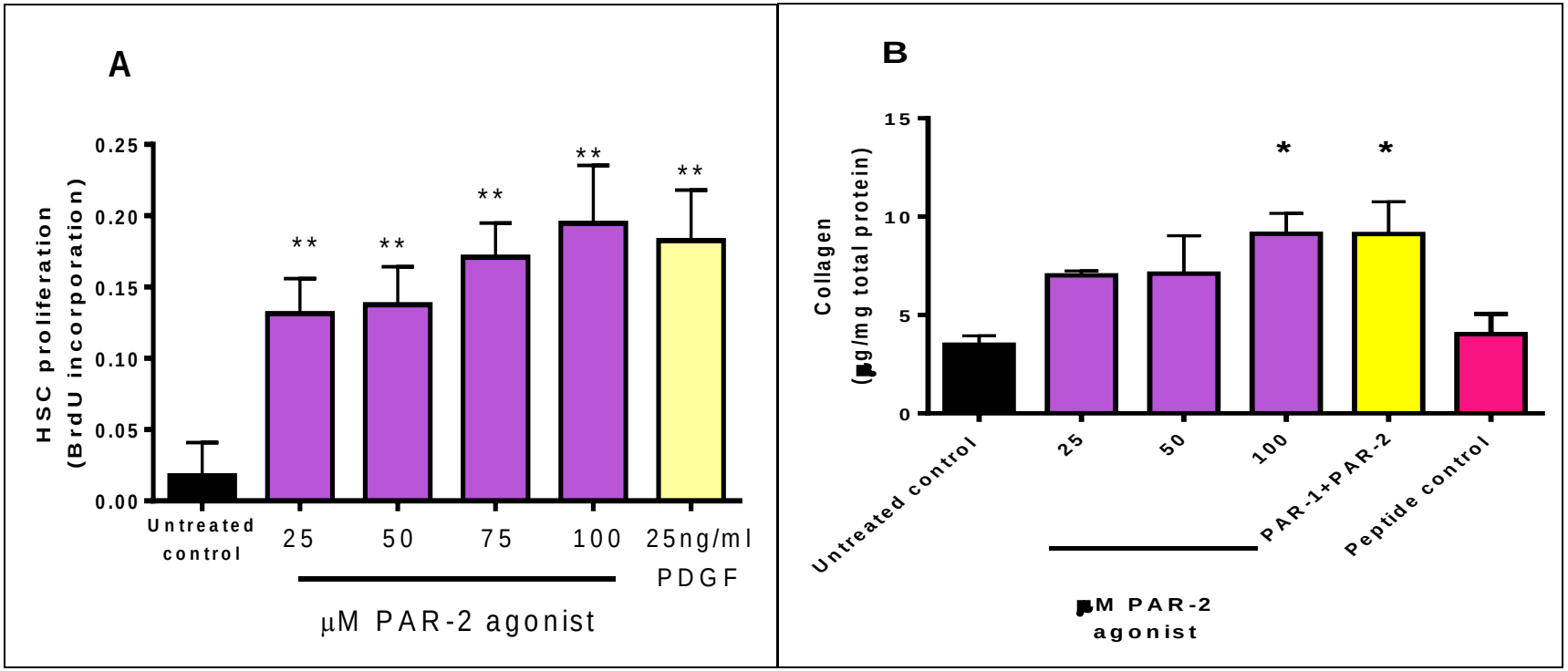


Figure 4.11 LX-2 HSC proliferation and collagen production

Stimulation of human LX-2 cells with a PAR-2 agonist peptide significantly increased cell proliferation to levels equivalent to PDGF (**p <0.005 compared to 0 μM) (A) HSC collagen production was significantly increased by a PAR-2 agonist peptide (100 μM)(*p<0.05); there was no effect from a scrambled peptide used as control (B). The combination of PAR-1 and PAR-2 peptides significantly increased collagen production compared to control peptide and untreated control but not to PAR-2 alone (B).

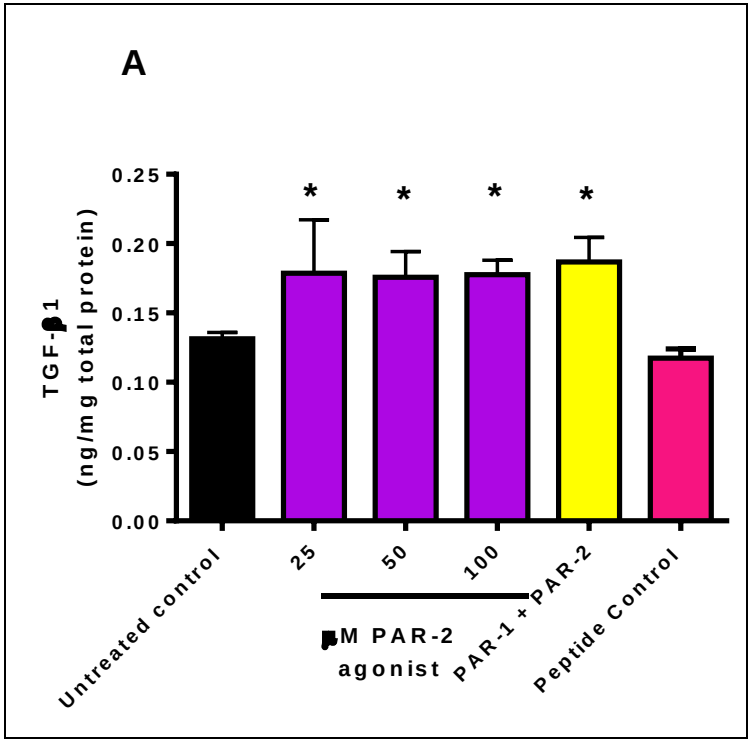


Figure 4.12 TGFβ protein in HSC

TGFβ protein production by HSC was significantly increased by a PAR-2 agonist peptide (*p<0.05); there was no effect from a scrambled peptide used as control (all values compared to untreated control 0μM) (A). TGFβ production also increased following incubation with PAR 1 peptide (See figure 3.7B). The combination of PAR 1 and PAR 2 peptides caused a significant increase in TGFβ production by LX-2 cells compared to control peptide and untreated controls but not to PAR 1 or PAR 2 alone.

2 cells compared to the control peptide and untreated controls (Figure 4.12). The threshold for stimulation of TGFβ production (25μM) was lower than that for stimulation of collagen production. The combination of PAR-1 and PAR-2 agonist peptides caused a significant increase in TGFβ production by LX-2 cells compared to control peptide and untreated controls. Again, the effect of combined agonist peptides on TGFβ production was not significantly greater than the effect of the individual agonists, suggesting a maximal response at the selected doses.

4.4 Discussion

We observed that PAR-2 deficiency in experimental liver fibrosis leads to a reduction in hepatic collagen content and histological fibrosis that is accompanied by decreased hepatic stellate cell activation, as demonstrated by reduced expression of αSMA. These findings were paralleled by a decrease in gene and protein expression of the principal profibrogenic cytokine, TGFβ, and altered MMP and TIMP gene expression. We confirmed a specific effect on HSC *in vitro* by showing that PAR-2 activation stimulated proliferation, collagen production and TGFβ protein production. These data suggest that PAR-2 activation promotes hepatic fibrosis by inducing a profibrogenic phenotype in hepatic stellate cells.

PAR-1 has been studied in animal models of hepatic necroinflammation and fatty liver disease (Fiorucci, Antonelli et al. 2004) and in human and murine lung injury (Scotton, Krupiczojc et al. 2009). PAR-1 deficient mice appear to be protected from CCl_4-induced liver fibrosis (Rullier, Gillibert-Duplantier et al. 2008). Thus, there is compelling evidence that thrombin/Xa induced PAR-1 signalling plays an important role in tissue fibrogenesis (Anstee, Wright et al. 2009, Borensztajn, von der Thusen et al. 2010). Interest in the role of PAR-2 in hepatic fibrosis has developed based on evidence that PAR-2 activation is associated with inflammatory and fibrogenic events in the kidney and pancreas (Masamune, Kikuta et al. 2005, Xiong, Zhu et al. 2005) and its expression is increased in models of lung injury (Cederqvist, Haglund et al. 2005, Borensztajn, Bresser et al. 2010) suggesting an important role for PAR-2 in mediating tissue repair. Cellular mechanisms underlying this role have

been proposed by Borensztajn et al who showed that Factor Xa signalling via PAR-2 induced fibroblast proliferation, migration and differentiation into myofibroblasts (Borensztajn, Stiekema et al. 2008).

The role of PAR-2 in hepatic inflammation and fibrosis has been examined to date only in HSC derived from experimental animals. Gaca, et al demonstrated PAR-2 expression in rat HSC and showed that PAR-2 agonists induced HSC proliferation and collagen production (Gaca, Zhou et al. 2002). Fiorucci, et al similarly showed that PAR-2 agonist stimulation of rat HSC resulted in proliferation and activation (Fiorucci, Antonelli et al. 2004). To our knowledge, the current study is the first to explore the role of the PAR-2 receptor in liver fibrosis *in vivo* in PAR-2 knockout mice and *in vitro* in human hepatic stellate cells. The use of the knockout model is a particular strength of the study that allows us to ascribe a profibrogenic role to PAR-2 unequivocally since antagonist studies can be troubled by a lack of molecular specificity. These findings significantly expand the evidence linking PAR-2 ligation with hepatic fibrogenesis that occurs most likely through a direct effect on HSC proliferation and collagen production.

We confirmed the role of PAR-2 in HSC activation through studies using the human hepatic stellate cell line, LX-2, which expresses PAR-2. We observed a significant dose-response to a specific PAR-2 agonist that achieved a proliferative response comparable to PDGF, the most potent cytokine in regard to stimulating HSC proliferation. In keeping with the overall effect that we saw in the PAR-2 knockout mice, we showed that PAR-2 ligation of human

HSC led to increased TGFβ and collagen production. PAR-2 appears to have equivalent effects to PAR-1 in regard to HSC expression of TGFβ and collagen. We did not demonstrate an additive effect on these responses with the combination of agonists, suggesting maximal stimulation of the common downstream effector pathways of these two receptors under the agonist doses and conditions of *in vitro* stimulation.

Interestingly we observed that the protective effect of PAR-2 gene deletion was apparent during more advanced stages of fibrosis, in this case at 8 weeks of CCl_4 exposure rather than at 5 weeks. This raises the question of the nature of the factor(s) leading to PAR-2 activation during continued hepatic injury. PAR-2 activation can be stimulated by trypsin and mast cell tryptase as well as coagulation proteases such as Factor VIIa, Xa and tissue factor. Mast cells are recruited to the liver during fibrogenesis and their numbers can increase by up to 9 fold in the cirrhotic liver (Farrell, Hines et al. 1995). Tryptase accounts for approximately 25% of mast cell protein and its levels progressively increase with liver injury (Schwartz, Irani et al. 1987). Thus we postulated that PAR-2 activation in the injured liver might occur through tryptase generation given the interval between injury and mast cell accumulation. However we did not observe any difference in gene expression of mast cell chymase, a marker for mast cells, between mice treated for 5 or 8 weeks. We then investigated PAR-1 expression in the PAR-2 knockout mice and found significant upregulation of PAR-1 mRNA in PAR-2 knockout mice at 5 weeks, which was not seen in WT mice exposed to CCl_4 or the vehicle control. Interestingly, at 8 weeks PAR-1 upregulation was not evident in the

knockout mice. Thus, there appears to be compensatory PAR-1 signalling early in fibrogenesis in the PAR-2 knockout mice that is lost as fibrosis progresses which may account for the difference in hepatic fibrosis seen at 8 weeks that was not evident at 5 weeks.

Macrophages play an important role in hepatic fibrogenesis (Marra, Aleffi et al. 2009) and therefore we also examined the extent of macrophage infiltration at 5 and 8 weeks. We found that the number of F4/80+ macrophages in PAR-2 knockout mice was lower than that in WT mice at both 5 and 8 weeks; however the number of activated macrophages (CD68+ cells) was significantly lower in the knockout mice compared to WT controls at 8 weeks. A recent study has shown that PAR-2 and toll-like receptor 4, which is highly expressed on Kupffer cells and forms a component of the LPS receptor, cooperate to enhance the release of pro-inflammatory cytokines (Rallabhandi, Nhu et al. 2008). Fewer activated macrophages seen at 8 weeks in the PAR-2 knockout mice may therefore lead to alterations in the inflammatory hepatic microenvironment that could contribute to the decrease in hepatic fibrosis seen in PAR-2 deficiency. Thus multiple lines of evidence suggest that it is likely that as inflammatory liver disease progresses increasing expression of PAR-2 (Gaca, Zhou et al. 2002, Fiorucci, Antonelli et al. 2004) and its ligands such as factor Xa (Borensztajn, Stiekema et al. 2008) potentiate HSC activation and collagen deposition.

Changes in the expression of MMPs and their specific tissue inhibitors, TIMPs, are complex and vary over time with liver injury. MMP-2 is an

autocrine proliferation and migration factor for HSC (Ikeda, Wakahara et al. 1999) whose expression can be induced by TGFβ and is typically increased following liver injury. TIMP-1, which inhibits MMP activity, is produced by activated HSC and its expression is also upregulated with liver injury and leads to the net accumulation of extracellular matrix (Hemmann, Graf et al. 2007). The decrease in gene expression of MMP-2 and TIMP-1 in PAR-2 deficient mice reflects a relatively static phenotype associated with the failure of either fibrosis progression or regression that we observed in these animals.

Recently peptide-mimetic compounds have been formulated that bind to PAR-2 and inhibit intracellular responses including NFκB activation and IL-8 expression as well as PAR-2 induced tissue responses such as vascular (rat aorta) relaxation (Kanke, Kabeya et al. 2009). These newly developed, specific PAR-2 antagonists may represent a novel therapeutic approach in preventing fibrosis in patients with chronic liver disease and support the need for further research into these unique receptors.

4.5 Conclusion

In conclusion we have demonstrated that deletion of the PAR-2 gene in mice chronically exposed to carbon tetrachloride leads to a significant reduction in hepatic fibrosis. The mechanism of this effect is likely to be through a reduction in HSC proliferation and collagen production. These novel findings suggest that PAR-2 may be an important therapeutic target for the treatment of human hepatic fibrosis.

CHAPTER 5

The role of the cytoplasmic domain of Tissue Factor in experimental fibrosis and its interaction with PAR-2

5 The role of the cytoplasmic domain of Tissue Factor in experimental liver fibrosis and its interaction with PAR-2

5.1 Introduction

Tissue Factor, a transmembrane glycoprotein, is the initiator of the coagulation cascade. TF is found in high concentrations in the brain, skin, lung, kidney, in the connective tissue surrounding blood vessels and in the capsules of organs (Fleck, Rao et al. 1990). It is found in low concentrations on the surface of blood cells but its expression can be upregulated, predominantly in circulating monocytes, by a variety of agents/stimuli including direct cell-cell contact with platelets, lipopolysaccharide (LPS) (Rivers, Hathaway et al. 1975), monocyte chemotactic protein-1 and platelet derived growth factor (Ernofsson and Siegbahn 1996).

TF molecule has 3 domains; the extracellular domain, the transmembrane domain and the cytoplasmic domain. The extracellular domain of TF is vital for the initiation of coagulation and binds circulating Factor VIIa and Factor X, which then ultimately promotes conversion of prothrombin to thrombin. The transmembrane domain has anchoring properties which are necessary for normal coagulation. The cytoplasmic domain is not necessary for normal coagulant activity but plays a role in intracellular signalling.

In addition to its important role in haemostasis, TF is increasingly being recognised as a signalling receptor in a number of noncoagulant roles including angiogenesis, sepsis and inflammation.

Factor VII or FVIIa is the only ligand to bind TF. The TF-FVIIa complex can bind Xa to form a ternary complex of TF-FVIIa-FXa. The downstream signalling effects of the TF complexes can be PAR dependent, with TF-FVIIa signalling through PAR-2 and TF-FVIIa-FXa signalling through PAR-1 or PAR-2, or PAR independent signalling via the cytoplasmic domain or through transactivation of tyrosine kinases (Aberg and Siegbahn 2013) (Figure 1.13).

There is increasing evidence TF has an important role in the inflammatory process. To illustrate, exposing healthy subjects to recombinant FVIIa, the only ligand (in addition to its inactive form) to bind TF, increased proinflammatory cytokines interleukin (IL)-6 and IL8 levels (de Jonge, Friederich et al. 2003). In addition, LPS stimulation in mice expressing low levels of tissue factor (less than 1% of wildtype TF levels), led to low levels of inflammatory cytokine expression with resultant reduced mortality (Pawlinski, Pedersen et al. 2004).

The involvement and importance of the cytoplasmic domain of tissue factor as a signalling receptor has also been studied. TF has also been shown to act as a cell signalling receptor to promote a proinflammatory phenotype in macrophages. Cunningham et al have demonstrated that binding of FVIIa to TF on macrophages *in vitro* led to phospholipase C-dependent intracellular

calcium fluxes and reactive oxygen species production. An intact cytoplasmic domain of TF was required for the calcium fluxes in macrophages indicating its involvement in this signalling (Cunningham, Romas et al. 1999).

The cytoplasmic domain of tissue factor has also been shown to be important in the immune response in an experimental model of arthritis. Mice in which the cytoplasmic domain of tissue factor had been deleted ($TF^{\S CT/\ \S CT}$), displayed reduced severity of antigen induced arthritis. This was demonstrated by reduced proinflammatory cytokine gene expression, reduced cutaneous delayed type hypersensitivity and reduced T cell activation (Yang, Hall et al. 2004).

The cytoplasmic domain of TF is important in cell adhesion and migration via binding of filamin and is also important in PDGF-BB induced chemotaxis which is dependent on TF cytoplasmic domain phosphorylation and PAR-2 (Aberg and Siegbahn 2013).

There is also evidence that the cytoplasmic domain of TF can play a regulatory role in PAR-2 receptor signalling. The TF-FVIIa complex, independent of initiating coagulation, promotes tumour angiogenesis via PAR-2 signalling (Belting, Dorrell et al. 2004). $TF^{\S CT/\ \S CT}$ mice have been shown to have enhanced PAR-2 dependent angiogenesis suggesting that the cytoplasmic domain of tissue factor acts as a negative regulator of PAR-2 signalling (Belting, Dorrell et al. 2004).

Tissue Factor deficiency has been implicated in the development of cardiac and pulmonary fibrosis. Pawlinski et al demonstrated that mice expressing low TF levels had increased haemosiderin deposition and fibrosis and impaired left ventricular function. The cause of the cardiac fibrosis was thought to be from haemorrhage from cardiac vessels as a result of the impaired haemostasis found in these low TF expressing mice (Pawlinski, Fernandes et al. 2002).

In a murine model of pulmonary fibrosis, PAR-2 activation led to upregulation of TF expression and augmentation of the coagulant response (Borensztajn, von der Thusen et al. 2010).

Therefore the cytoplasmic domain of tissue factor appears to be important in the immune response and plays a role in the inflammation. Tissue factor deficiency has been shown to be associated with cardiac fibrosis with the proposed mechanism thought to be related to impaired haemostasis. The possible role of the cytoplasmic domain of TF, acting as a proinflammatory cell signalling receptor, in chronic liver injury characterised by the development of inflammation and the wound healing response of fibrosis has not been explored.

The aim of this study was to evaluate the role of the cytoplasmic domain of tissue factor in the development of experimental liver fibrosis. We have previously shown that PAR-2 deficiency ameliorates experimental liver

fibrosis. Given the findings of the cytoplasmic domain acting as a negative regulator of PAR-2 in angiogenesis, this study also aimed to explore a possible relationship between the tissue factor cytoplasmic domain and the PAR-2 receptor in the development of liver fibrosis.

5.2 Materials and Methods

5.2.1 Animals

Mice with deletion of 18 of the 20 amino acids of the cytoplasmic domain of tissue factor (TF $^{\S CT/\ \S CT}$) were generated by Cre-lox recombination as previously described (Melis, Moons et al. 2001). TF $^{\S CT/\ \S CT}$ have normal development, coagulation and fertility. The mice are Swiss/25% 129S/50% MF-1 background.

PAR-2 KO mice were generated as previously described.

Mice with deletions of both TF$^{\S CT/\ \S CT}$ /PAR-2 $^{-/-}$ were generated and have normal development, coagulation and fertility.

5.2.2 In vivo Experimental design

Liver fibrosis was induced in male mice by twice weekly intraperitoneal injections of 1µl/g body weight CCl_4 mixed with olive oil (1:10). Starting between 8-10 weeks of age, four groups of mice were studied, TF $^{\S CT/\ \S CT}$ (n=9), TF $^{\S CT/\ \S CT}$-PAR-2$^{-/-}$ (n=6), wild type (WT) C57BL/6 (n=10) all received

CCl_4 for 8 weeks and a control group of WT C57BL/6 mice (n=8 each) received olive oil alone for 8 weeks.

5.2.3 Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA with Newman-Keuls post-test for multiple comparisons or Student's t-test for comparisons between two groups as appropriate, using GraphPad Prism 5.03 and 6.04 for Windows (GraphPad Software, Inc, La Jolla, USA). A p value of <0.05 was considered significant.

5.3 Results

5.3.1 Vital Parameters

After 8 weeks of CCl_4 treatment mouse body weight, liver weight and spleen weight did not differ between the groups. (Table 5.1)

5.3.2 Hepatic fibrosis

WT, TF $^{\S CT/\ \S CT}$ and TF $^{\S CT/\ \S CT}$- PAR-2 KO mice developed significant fibrosis when compared to controls. (Figure 5.1 A,B and C) Quantitative analysis of histological fibrosis by computer-assisted morphometry in CCl_4-treated WT mice showed marked fibrosis at 8 weeks (3.39 ± 0.26% liver area) (Figure 5.2A). In TF $^{\S CT/\ \S CT}$ and TF $^{\S CT/\ \S CT}$- PAR-KO mice, CCl_4 administration induced similar levels of fibrosis (1.76± 0.17% and 1.94±0.11% liver area respectively), which were similar to that previously seen with PAR-2 KO mice (2.09±0.28% liver fibrosis area). At 8 weeks there was significantly less hepatic fibrosis area in the TF $^{\S CT/\ \S CT}$ and TF $^{\S CT/\ \S CT}$ -PAR-2 KO compared to WT mice ($p<0.05$) (Figure 5.2A)

5.3.3 Hepatic hydroxyproline content

Histological assessment of fibrosis area correlated with the amount of liver hydroxyproline, a surrogate marker for collagen content. TF $^{\S CT/\ \S CT}$ and TF $^{\S CT/\ \S CT}$ -PAR-2 KO mice showed significantly less hepatic hydroxyproline (0.37 ± 0.017 µg/mg and 0.37± 0.02 µg/mg) compared to WT mice (0.61 ± 0.03 µg/mg) at 8 weeks ($p<0.05$). Again the levels in TF $^{\S CT/\ \S CT}$ and TF $^{\S CT/\ \S CT}$-PAR-2 KO mice were similar to that seen in the PAR-2 KO alone (0.39 ±0.02 µg/mg).(Figure5.2B)

	Vehicle Control	TF $^{\S CT/\S CT}$	TF $^{\S CT/\S CT}$-PAR-2 KO	Wildtype	P value
Body weight, g	29.29±0.9	29.97 ±0.26	28.44 ±0.82	28.66±0.35	p=ns
Liver wt, g	1.34 ± 0.05	1.58 ±0.05	1.49 ±0.07	1.42± 0.06	p=ns
Liver wt/body wt	4.57 ± 0.13	5.28 ± 0.16	5.240 ± 0.14	4.94 ± 0.22	p=ns

Table 5.1 Vital parameters TF $^{\S CT/\S CT}$, TF $^{\S CT/\S CT}$- PAR-KO and Wildtype mice

Vital parameters (all values mean ±SEM) in mice after 8 weeks of CCl_4 . There were no statistically significant differences between the group with regards to body weight, liver weight or liver weight as a percentage of body weight.

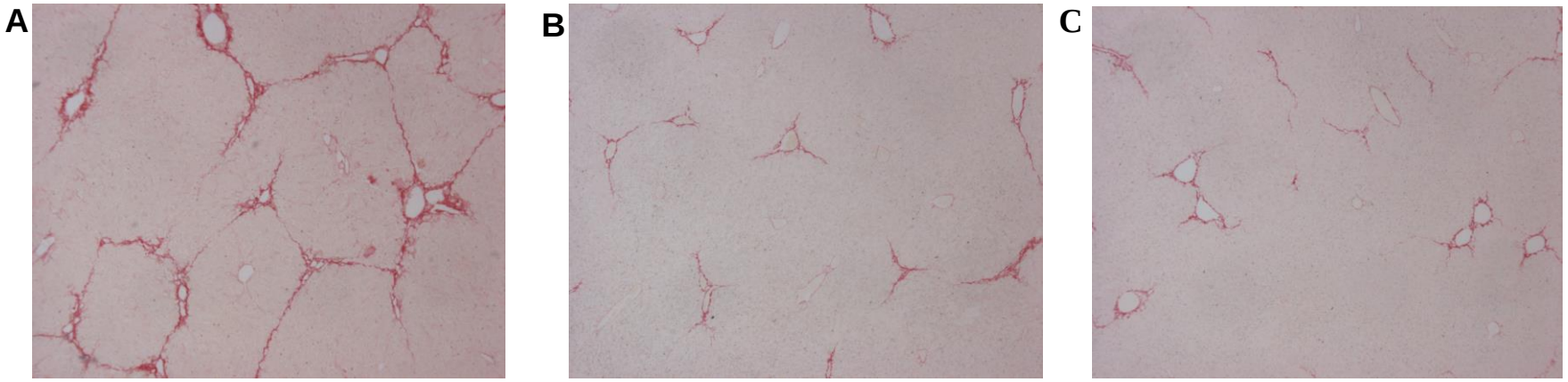


Figure 5.1 Picrosirius red histochemistry for hepatic collagen

Hepatic collagen deposition in WT mice (A) and TF $^{\S CT/\S CT}$ (B) and TF $^{\S CT/\S CT}$-PAR-2 KO (C) mice administered CCL_4 for 8 weeks (Sirius red staining, 40X).

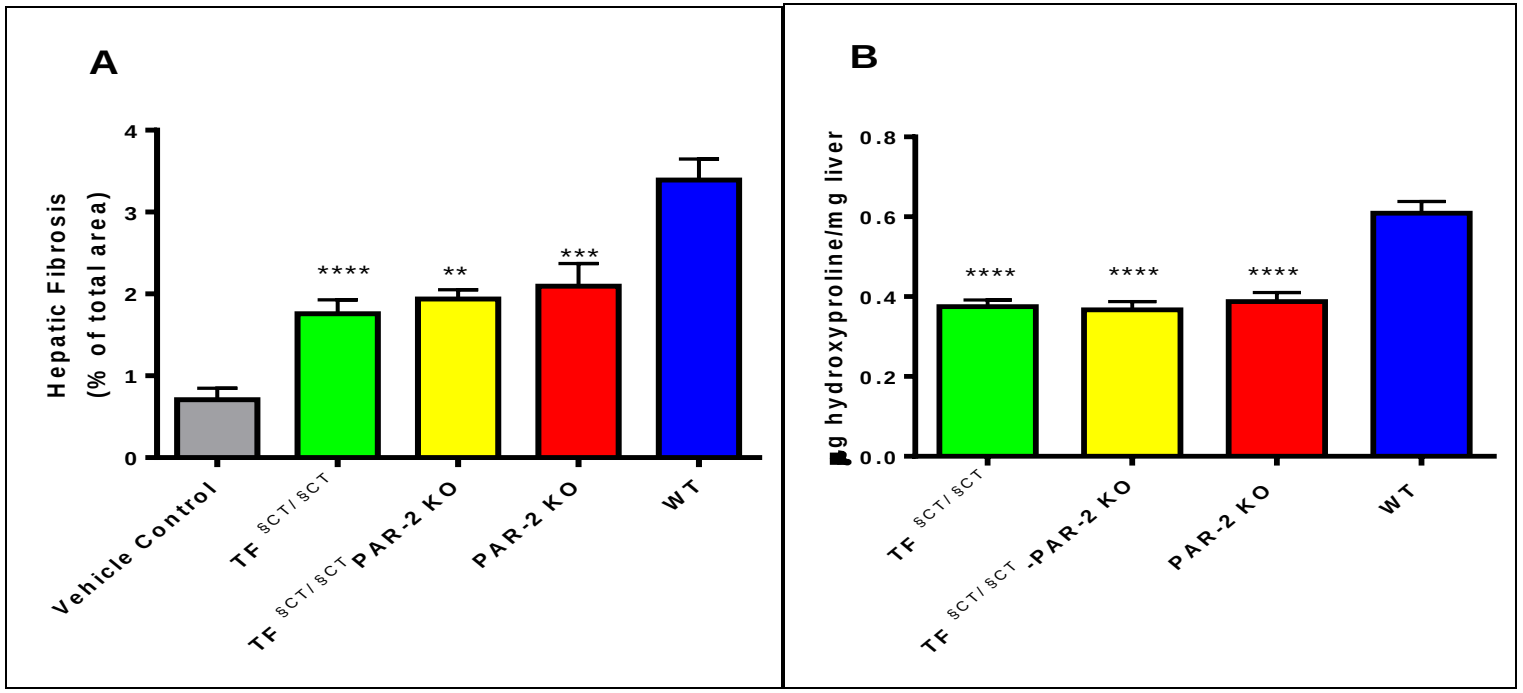


Figure 5.2 Hepatic Fibrosis in WT mice and TF $^{\S CT/\ \S CT}$ and TF $^{\S CT/\ \S CT}$ -PAR-2 KO mice

All mice exposed to CCl4 had significantly higher fibrosis areas than the wildtype controls that received olive oil (vehicle) alone. There were significantly lower hepatic fibrosis areas in the TF $^{\S CT/\ \S CT}$ and TF$^{\S CT/\ \S CT}$ -PAR-2 KO mice compared to wildtype mice after exposure to carbon tetrachloride for 8 weeks (A) There was also significantly less hepatic hydroxyproline content in the TF$^{\S CT/\ \S CT}$ and TF$^{\S CT/\ \S CT}$ -PAR-2 KO mice compared to wildtype. (B) .(** $p<0.01$, ***$p<0.001$ ****$p<0.0001$)

5.3.4 Reduced stellate cell activation in TF $^{\S CT/\S CT}$ and TF $^{\S CT/\S CT}$-PAR-2 KO mice

Alpha smooth muscle actin (αSMA) as a marker of HSC activation and myofibroblast differentiation was measured. In WT mice, hepatic fibrosis induced by administration of CCl_4 was accompanied by an increase in αSMA mRNA expression at 8 weeks compared to untreated mice (3.93 fold change compared to vehicle control). In TF $^{\S CT/\S CT}$ and TF $^{\S CT/\S CT}$-PAR-2 KO mice receiving CCl_4 there was a significant decrease in αSMA gene expression compared to WT mice at 8 weeks. (0.35, 0.45 and 3.93 fold change compared to vehicle control respectively, $p<0.01$)(Figure 5.3A) This reduction in gene expression correlated with significantly less αSMA positive cells seen histologically in the TF $^{\S CT/\S CT}$ and TF $^{\S CT/\S CT}$-PAR-2 KO groups compared to wildtype. (19.99, 19.96 and 30.09 mean αSMA positive cells respectively, $p<0.0001$) (Figure 5.3B) The reduction observed in αSMA positive cells in the TF $^{\S CT/\S CT}$ and TF $^{\S CT/\S CT}$-PAR-2 KO groups was similar to that observed in PAR-2 KO alone.

5.3.5 TF $^{\S CT/\S CT}$ and TF $^{\S CT/\S CT}$-PAR-2 deficiency reduces hepatic TGFβ expression

CCl_4 induced hepatic fibrosis was associated with upregulation of TGFβ mRNA (3.57 fold greater than control) and protein (11.41 ± 0.98 pg/mg liver vs control 7.01 ± 0.01 pg/mg) in WT mice at 8 weeks. In TF $^{\S CT/\S CT}$ mice, TGFβ mRNA expression was significantly reduced compared to wildtype (0.22 ± 0.05 fold change from vehicle control, $p<0.0001$) In TF $^{\S CT/\S CT}$-PAR-2 KO mice there was a trend towards reduced TGFβ mRNA reduction compared to wildtype. (1.21±0.14 fold change from vehicle control, p=ns) (Figure 5.4A).

In both the TF $^{\S CT/\ \S CT}$ and TF $^{\S CT/\ \S CT}$-PAR-2 KO mice TGFβ protein was similar to control levels (6.14 ± 0.33, 6.45 ± 0.84 and 7.01 ± 0.18 pg/mg wet liver weight respectively). The levels were significantly lower than the TGFβ protein level in wildtype mice (11.41 pg/mg wet liver weight, $p<0.0001$) (Figure 5.4B).

5.3.6 TF $^{\S CT/\ \S CT}$ and TF $^{\S CT/\ \S CT}$-PAR-2 deficiency decreases MMP2 mRNA

Matrix metalloproteinases (MMP) regulate matrix composition and turnover and are themselves regulated by their specific tissue inhibitors, TIMPs. The balance of the expression of different MMPs changes throughout the development of the liver injury, but in general with liver fibrosis there is upregulation of the basement membrane-like MMPs, such as MMP2 and 9 and down regulation of interstitial type MMPs such as MMP1 (MMP13 in mice). In WT mice treated with CCl_4 for 8 weeks, MMP-2 mRNA increased (6.29 ± 1.45 fold greater than control), consistent with active ECM remodelling during development of hepatic fibrosis (Figure 5.5). MMP-2 mRNA expression was significantly lower in TF $^{\S CT/\ \S CT}$ and TF $^{\S CT/\ \S CT}$-PAR-2 KO mice compared to WT mice(0.21± 0.05 fold greater than control; $p<0.0001$, 1.65 ± 0.13 fold greater than control; $p<0.01$ respectively), suggesting ECM remodelling is reduced in association with the lower levels of fibrosis seen at 8 weeks in these mice.

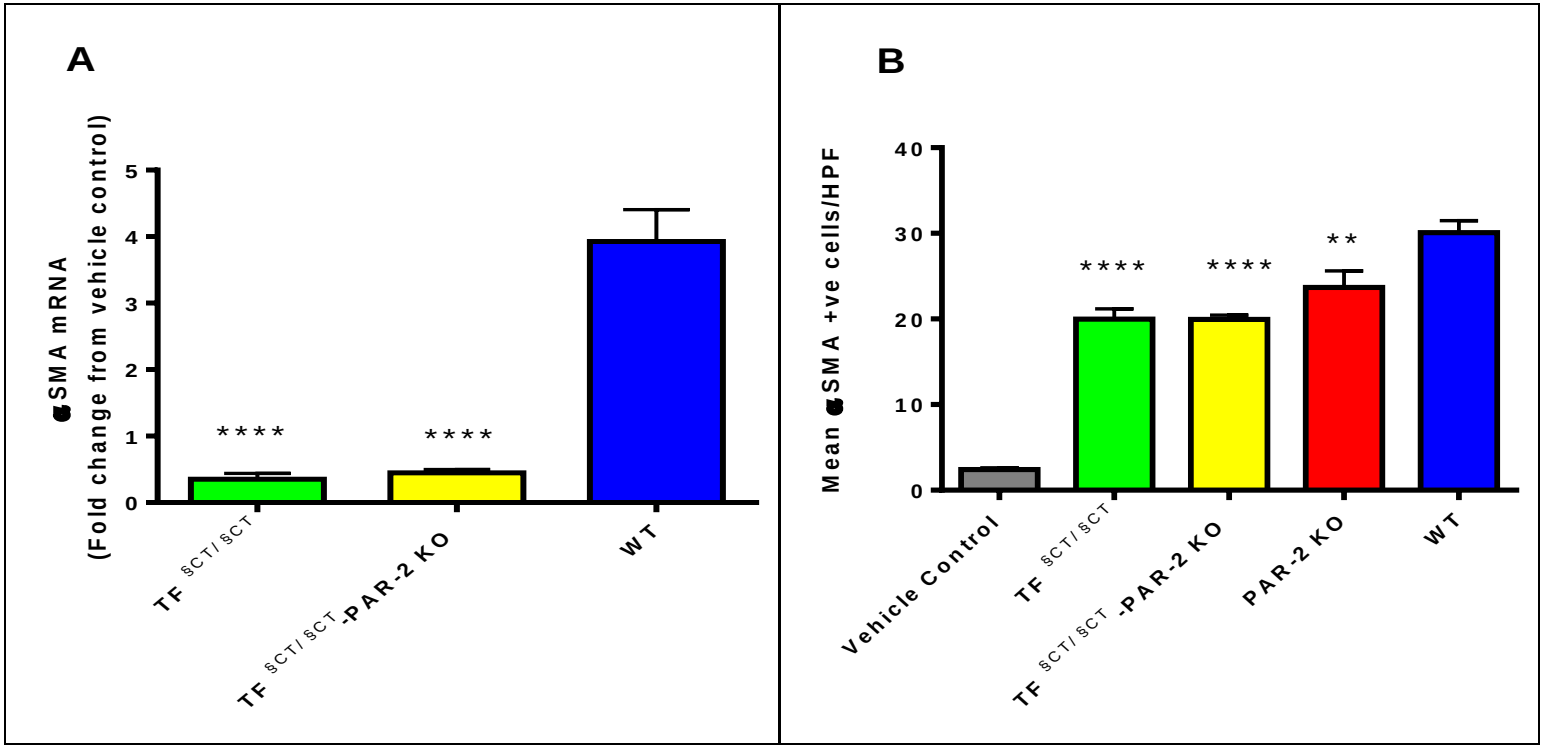


Figure 5.3 αSMA expression

After 8 weeks of CCl4 administration, αSMA mRNA expression (A) and the number of cells expressing αSMA (B) were significantly lower in the TF $^{\S CT/\ \S CT}$ and TF $^{\S CT/\ \S CT}$ -PAR-2 KO mice compared to wildtype (** $p<0.01$, ****$p<0.0001$). The number of αSMA positive cells in the TF$^{\S CT/\ \S CT}$ and TF$^{\S CT/\S CT}$-PAR-2 KO were similar to those seen in PAR-2 KO mice.

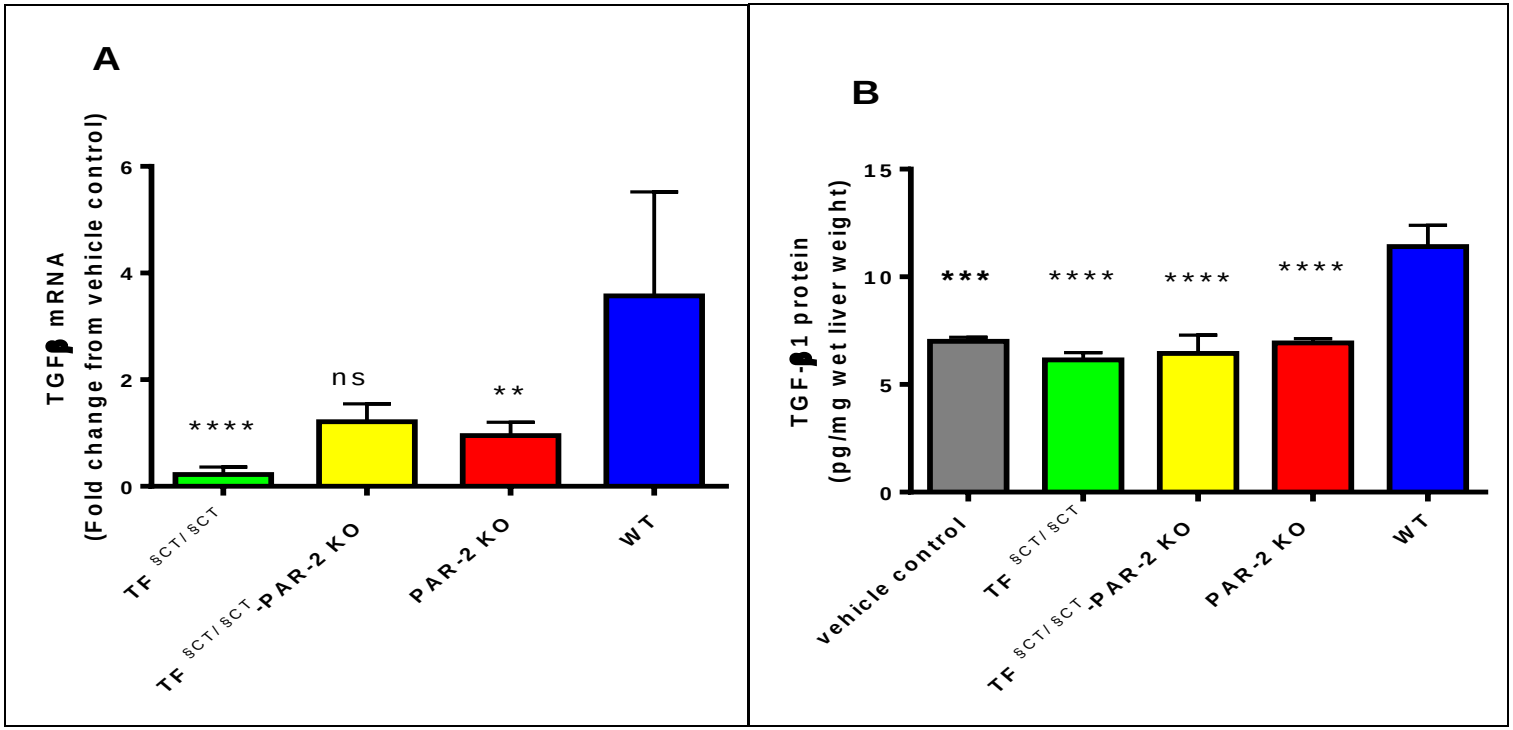


Figure 5.4 TGFβ expression

TGFβ mRNA expression was significantly lower in the TF$^{§CT/\ §CT}$ group(****p<0.0001), with a trend to being lower in TF $^{§CT/\ §CT}$ -PAR-2 KO mice compared to wildtype. The levels were similar to those seen in PAR-2 KO, whose levels were also significantly lower than WT(**p<0.001) (A) TGFβ protein levels were significantly lower in the expression TF $^{§CT/\ §CT}$, TF $^{§CT/\ §CT}$ -PAR-2 KO and PAR-2 KO mice compared to wildtype (****p<0.0001) with similar levels seen as those in the vehicle control group (B).

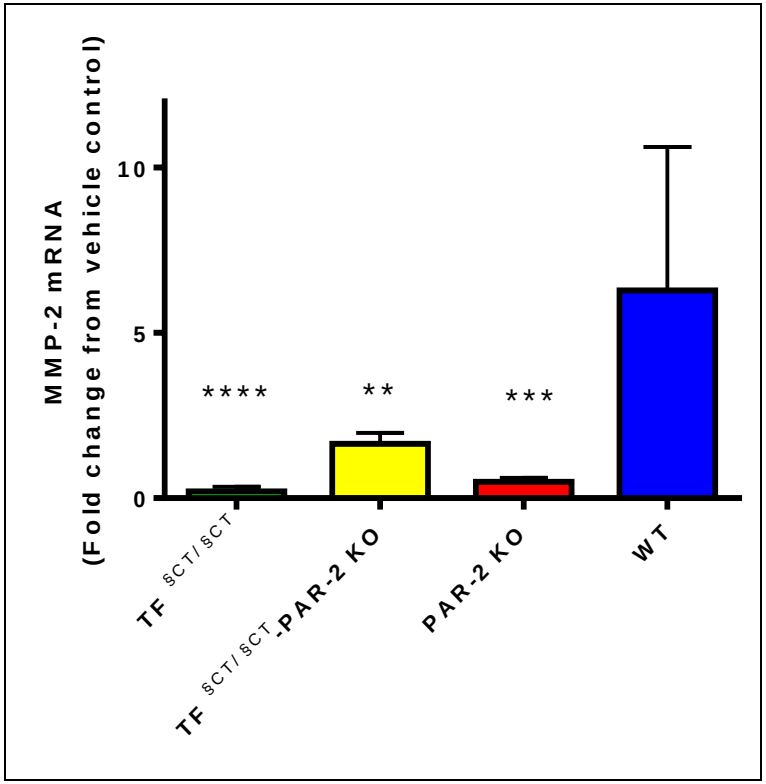


Figure 5.5 MMP-2 mRNA expression

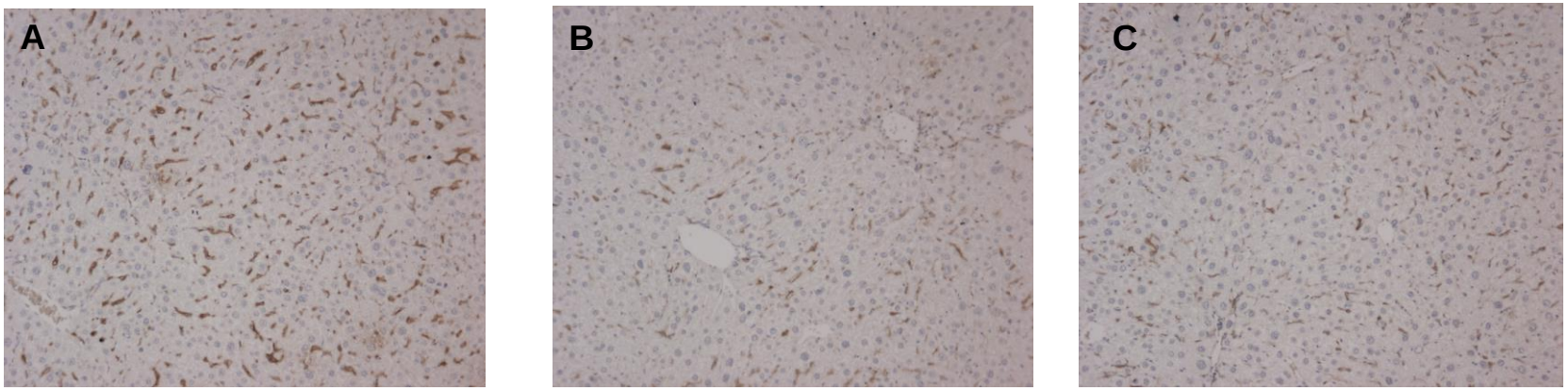
MMP-2 mRNA expression was significantly lower in TF $^{\S CT/\S CT}$, TF $^{\S CT/\S CT}$ -PAR-2 KO and PAR-2 KO mice compared to wildtype (****p<0.0001,***p<0.001,**p<0.01).

5.3.7 Decrease in activated Hepatic Macrophages in TF §$^{CT/\ §CT}$ and TF $^{§CT/\ §CT}$-PAR-2 deficient mice

Significantly fewer hepatic F4/80^{+} macrophages were observed in the TF $^{§CT/\ §CT}$ mice compared to WT mice (15.7 ± 2.7 vs 30.08 ± 2.95 positive cells/HPF, p<0.01) There was a trend toward fewer F4/80^{+} macrophages seen in the TF $^{§CT/\ §CT}$-PAR-2 KO mice but this did not reach statistical significance.(24.13 ± 2.18 positive cells/HPF, p=ns) (Figure 5.6A-C, Figure 5.7A)

There were significantly fewer CD68^{+} activated hepatic macrophages, in both the TF $^{§CT/\ §CT}$ and TF $^{§CT/\ §CT}$-PAR-2 KO mice compared to WT mice.(33.24 ± 1.47, 38.70 ± 1.78, 48.75 ± 2.87 positive cells/HPF; p<0.001, p<0.05 respectively) (Figure 5.6 D-F, Figure 5.7B) We have previously observed similar findings in the PAR-2 KO mice suggesting a role for PAR-2 in macrophage recruitment and activation. This suggests that the TF cytoplasmic domain, as well as PAR-2, may be involved in this process.

F4/80



CD68

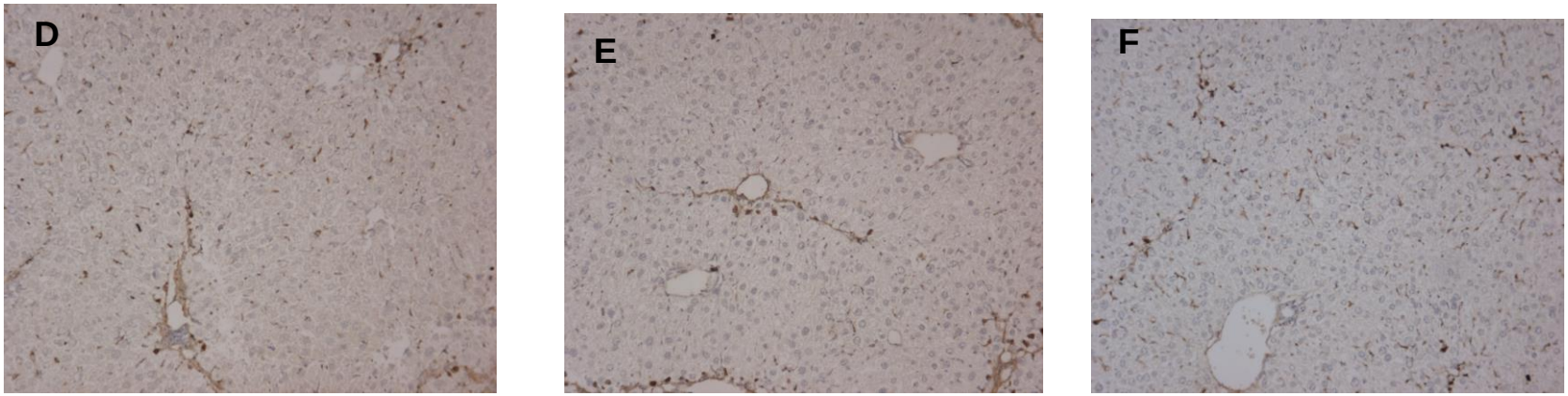


Figure 5.6 Immunohistochemistry of F4/80 and CD68 Macrophages

Representative photomicrographs of F4/80 staining of wildtype (A), TF $^{\S CT/\S CT}$ (B) and TF $^{\S CT/\S CT}$ mice-PAR-2 KO (C) mice and CD 68 staining of wildtype (D), TF $^{\S CT/\S CT}$ (E) and TF $^{\S CT/\S CT}$ mice-PAR-2 KO (F) mice (x100).

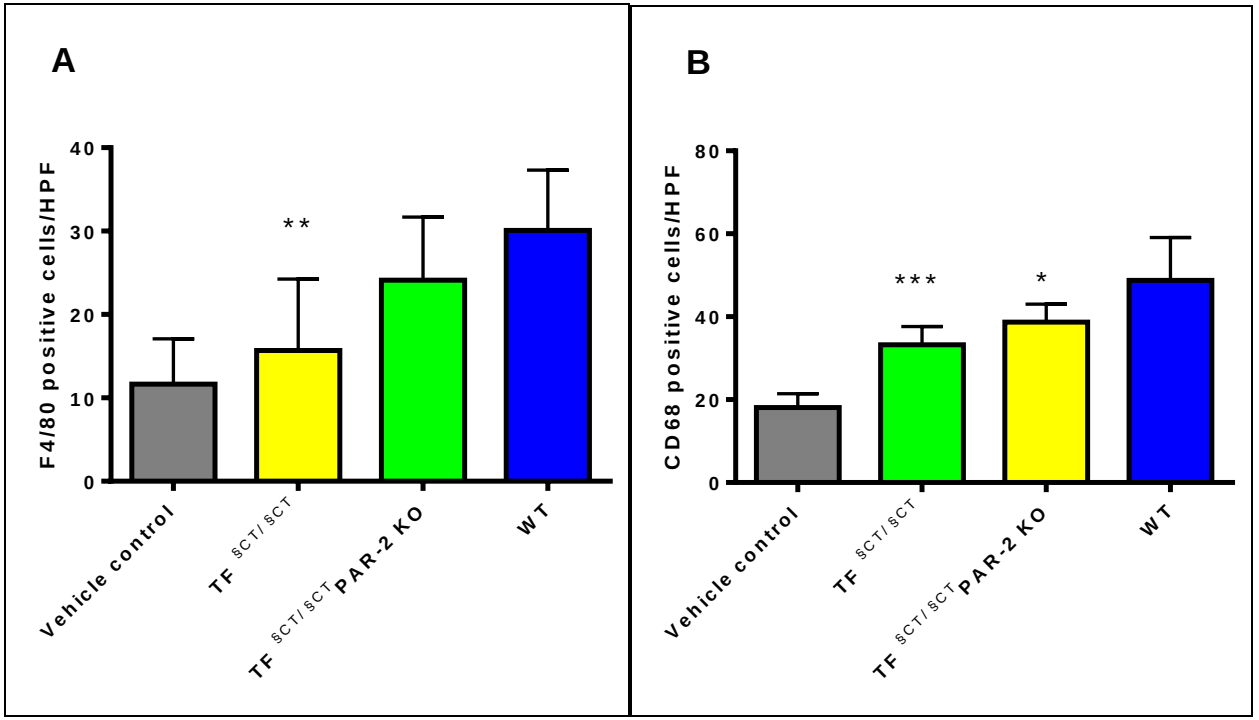


Figure 5.7 F4/80 and CD68 macrophages

There were significantly fewer F4/80+ macrophages in TF $^{\S CT/\S CT}$ mice (**p<0.01) and a trend toward fewer F4/80+ macrophages seen in the TF $^{\S CT/\S CT}$-PAR-2 KO compared to WT (A). There were significantly fewer CD68+ macrophages in both TF $^{\S CT/\S CT}$ and TF $^{\S CT/\S CT}$-PAR-2 KO mice than WT (***p<0.001, *p<0.05) (B).

5.4 Discussion

TF, in addition to having a well recognized role in initiating coagulation, is a transmembrane signalling receptor that is important in regulation of angiogenesis, tumour growth, metastasis and inflammation (Belting, Ahamed et al. 2005). Tissue Factor levels have been shown to be markedly elevated in patients with cirrhosis, over 100 fold higher in plasma of cirrhotic patients compared to control, possibly due to cell stress, cell injury or inflammation (Van Thiel, Farr et al. 2005). The TF cytoplasmic domain has been shown to induce proinflammatory effects in macrophages (Cunningham, Romas et al. 1999) and be involved in the immune response in the development of experimentally induced arthritis (Yang, Hall et al. 2004). The role of the cytoplasmic domain of tissue factor in other chronic inflammatory conditions such as liver fibrosis has not been explored.

This study aimed to assess the role of the tissue factor cytoplasmic domain in the development of experimentally induced liver fibrosis. Following 8 weeks of CCL_4 exposure, mice with deletion of the cytoplasmic domain had amelioration of liver fibrosis, with reduction in hepatic collagen content and histological fibrosis, compared to wild type mice. This was accompanied by reduced αSMA mRNA expression and fewer αSMA positive cells histologically suggesting reduced hepatic stellate cell activation. There was significantly lower gene and protein expression of the key profibrogenic cytokine TGFβ and a reduction in the MMP2 expression in TF $^{\S CT/\ \S CT}$ mice. These data

suggest that activation of the cytoplasmic domain of TF promotes hepatic fibrosis by inducing a profibrogenic phenotype in hepatic stellate cells.

Prior studies in cardiac fibrosis have suggested that haemorrhage resulting from abnormal coagulation in TF deficient mice, was the key event that led to tissue injury and greater levels of fibrosis in the TF deficient mice. The use in this study of the TF $^{\S CT/\ \S CT}$ mice, which have normal coagulation profiles, means that haemorrhage as a cause for the observed difference in fibrosis is unlikely.

In angiogenesis, the cytoplasmic domain of TF has been demonstrated to be a negative regulator of PAR-2. If this were true in this model of liver fibrosis, then loss of the cytoplasmic domain and thus loss of negative regulation of PAR-2, could lead to increased fibrosis through increased PAR-2 activation. However this was not observed in our study. In fact, the protection from experimentally induced liver fibrosis that the TF $^{\S CT/\ \S CT}$ mice were afforded was similar to that we have previously observed in mice with PAR 2 deficiency (Knight, Tchongue et al. 2012).

We found that mice with the dual deletion TF $^{\S CT/\ \S CT}$-PAR-2 $^{-/-}$ in this experimental model also exhibited reduced fibrosis, reduced alpha SMA expression and reduced expression of the profibrogenic cytokine TGFβ. If the mechanism underlying fibrosis reduction by the cytoplasmic domain of TF and PAR-2 were independent, an additive effect, and thus greater reduction in fibrosis could potentially be observed in these mice. However this was not

seen. The extent of fibrosis reduction seen in the TF $^{\S CT/\ \S CT}$-PAR-2 KO mice was similar to that seen in the TF $^{\S CT/\ \S CT}$ mice and the PAR-2 KO mice. This finding suggests that the cytoplasmic domain of TF and PAR-2 may signal through a common downstream pathway to promote fibrosis.

Activated tissue macrophages (Kupffer cells) play an important role in hepatic fibrogenesis and fibrolysis. Macrophages constitutively express TF and TF expression is upregulated during macrophage maturation. Activated macrophages release factors such as TGFβ1, which is involved in HSC activation, and also mitogenic and chemotactic factors for activated HSC (Marra, Aleffi et al. 2009). At 8 weeks there was a reduction in the number of F4/80^{+} macrophages and the number of activated macrophages (CD68^{+} cells) in the TF $^{\S CT/\ \S CT}$ mice and TF $^{\S CT/\ \S CT}$-PAR-2 KO compared to WT.

There are a number of possible explanations for the reduction in macrophage recruitment and activation observed. Firstly reduced recruitment maybe related to reduced PDGF-BB chemotaxis which is TF cytoplasmic domain and PAR-2 dependent. Secondly, the cytoplasmic domain of tissue factor has been shown to be essential for Factor VIIa induced calcium fluxes in macrophages *in vitro*. *In vivo* studies have shown by blocking TF/Factor VIIa interactions using a TF antibody led to reduced expression of MHC class II and β2 integrin leucocyte adhesion molecules which are markers of macrophage activation (Cunningham, Romas et al. 1999). Our studies in TF $^{\S CT/\ \S CT}$ mice demonstrated significantly fewer F4/80^{+} / CD68^{+} activated

macrophages supporting the view that TF cytoplasmic domain signalling is important for macrophage activation.

There is increasing interest into the macrophage phenotype in disease as classically activated M1 macrophages are proinflammatory and alternatively activated M2 macrophages are anti-inflammatory. A recent study has shown in mice fed a high fat diet that TF $^{\S CT/\ \S CT}$ mice demonstrated reduced inflammatory macrophages in adipose tissue with reduced proinflammatory cytokine production, suggesting the involvement of TF$^{\S CT/\ \S CT}$ in the development of macrophage phenotype (Badeanlou, Furlan-Freguia et al. 2011).

5.5 Conclusion

In conclusion we have established, for the first time to our knowledge, that deletion of the cytoplasmic domain of TF significantly reduces experimental murine hepatic fibrosis. Furthermore we have shown that deletion of the cytoplasmic domain in addition to PAR-2 deficiency does not lead to more profound protection from fibrosis. Future work is required to explore the mechanism and downstream signalling through which this is achieved. The cytoplasmic domain of TF is an attractive therapeutic target as its deletion would not impact on coagulation.

Chapter 6

General Discussion

6 General Discussion

This thesis has explored the role of PAR-1, PAR-2 and the cytoplasmic domain of TF in the development of hepatic fibrosis. The close interaction of the coagulation cascade and the inflammatory response lead to the hypothesis that coagulation factors and their receptors may play an important role in the development of hepatic fibrogenesis. A murine knockout model was utilised to unequivocally investigate whether the PAR-1 and PAR-2 receptors play a profibrogenic role and mice with deletion of the cytoplasmic domain of TF were investigated to assess its role in hepatic fibrosis. In vitro experiments in LX-2 HSCs provide novel data on the role of the PAR-1 and PAR-2 receptors in a human cell line. The significance and relevance of the findings have been discussed within each chapter. This chapter will review the main findings and discuss how they contribute to our knowledge of hepatic fibrogenesis and the wider implications of these findings in identifying potential future therapeutic targets.

Hepatic fibrosis and cirrhosis are the common endpoint of a variety of liver diseases. Chronic injury stimuli, required for significant fibrosis to develop, lead to accumulation of ECM components with distortion of the normal liver architecture and loss of normal hepatic function. The HSC has been identified as the primary source of ECM however the processes that lead to its activation and subsequent fibrosis development are multifactorial. A single treatment to completely ameliorate fibrosis, although the Holy Grail in fibrosis research, may be difficult to achieve given the complex and multiple pathways

for HSC initiation and perpetuation. However through greater understanding of the mechanisms of hepatic fibrogenesis the key targets for antifibrotic therapies are likely to be identified.

Activation of the coagulation cascade evokes inflammatory responses via upregulation of proinflammatory cytokines and chemokine production which have been hypothesized to contribute to the development of fibrosis. Given the linkage between tissue injury and cellular responses is largely through the protease interaction with the PARs (Coughlin 2000), these receptors have been a focus of study during this thesis.

Tissue Factor as the initiator of the coagulation cascade can lead to activation of PARs, via formation of Factor Xa and Va and thrombin. However there is evidence the cytoplasmic domain of tissue factor acts independently of the coagulation cascade as a signalling receptor. It is this non coagulant role of tissue factor that has been explored in this thesis.

Hepatic fibrosis is now considered potentially reversible as the result of matrix degradation. Macrophages play an important and divergent role, being profibrogenic in fibrosis development and aiding matrix degradation in fibrosis resolution (Fallowfield, Mizuno et al. 2007) and thus their activation during fibrogenesis is relevant and interesting.

6.1 PAR-1 deficiency protects against hepatic fibrosis

Thrombin acts via PAR-1 to exert its profibrotic and proinflammatory effects. Antagonism of PAR-1 has been shown *in vivo* to protect against the development of liver fibrosis in a murine model thus implicating it in hepatic fibrogenesis (Fiorucci, Antonelli et al. 2004). *In vitro* thrombin has been shown to stimulate HSC proliferation and MCP1 expression (Marra, Grandaliano et al. 1995) and collagen synthesis (Gaca, Zhou et al. 2002) in rat HSC lines but to our knowledge has not been studied *in vitro* in human HSC lines.

This thesis examined the effects of PAR-1 deficiency on the development of carbon tetrachloride induced liver fibrosis. In addition we studied the effects of PAR-1 agonists in vitro in a human hepatic stellate cell line. The results of this thesis add evidence to support the view that the PAR-1 receptor is involved in hepatic fibrogenesis. PAR-1 deficient mice, and to a lesser degree the PAR-1 heterozygotes, demonstrated reduced HSC activation and reduced collagen production and thus were protected from experimentally induced fibrosis.
Rullier et al published similar findings that PAR-1 knockout reduces carbon tetrachloride induced liver fibrosis (Rullier, Gillibert-Duplantier et al. 2008). Together these findings identify PAR-1 as a potential target for antifibrotic therapy.

PAR-1 antagonists have been developed. Thrombin can activate PAR-1, PAR-3 and PAR-4. In humans, platelets express PAR-1 and PAR-4. Thrombin is a principal agonist of platelets via PAR-1. Platelets are central to the pathogenesis of atherothrombotic disease such as acute coronary syndrome

and stroke (de Souza Brito and Tricoci 2013). Much of the initial work studying PAR-1 antagonists has been focussing on the antiplatelet effects in preventing atherothrombotic disease. Currently two PAR-1 antagonists, voraxapar and atopaxar have been investigated in clinical trials. The PAR-1 antagonists may be relevant to liver disease because PAR-1 is expressed not only on platelets, which contribute to the wound healing response in injured liver, but also on HSC and therefore antagonism could lead to reduced HSC activation and ECM accumulation. The potential antifibrotic role in liver disease of these PAR-1 antagonists is yet to be explored but could represent an exciting new treatment strategy.

Vorapaxar is a non-protein, orally active potent competitive antagonist of PAR-1. Atopaxar is an orally active potent PAR-1 antagonist that binds to the PAR-1 tethered ligand binding site. Phase II studies in vorapaxar have demonstrated safety and phase III studies are currently underway examining its use in acute coronary syndrome and in secondary prevention of atherothrombotic events (Tello-Montoliu, Tomasello et al. 2011). Atopaxar phase II studies have demonstrated safety with no increase risk in bleeding however the most common adverse effect was liver dysfunction in 12-15% of patients (Goto, Ogawa et al. 2010).

Recently the role of thrombin antagonists in reducing liver fibrosis has been further studied. Kassel et al investigated whether thrombin inhibition could reverse hepatic steatosis and inflammation in mice with diet induced non-alcoholic fatty liver disease. This study administered either the direct thrombin

inhibitor, Argatroban, or placebo via a miniosmotic pump into mice receiving a control or western diet. It showed that thrombin inhibition with Argatroban administration significantly reduced hepatic proinflammatory cytokine expression, reduced macrophage and neutrophil accumulation and reduced hepatic stellate cell activation in the livers of mice receiving a Western diet. Thus the authors concluded from the study that administration of a thrombin inhibitor reduces hepatic inflammation and profibrogenic changes (Kassel, Sullivan et al. 2012).

In conclusion, several studies have confirmed that PAR-1 deficiency affords protection from hepatic fibrosis and following on from this that PAR-1 antagonism attenuates hepatic fibrosis. Two orally active PAR-1 antagonists are now currently in Phase III trials assessing antiplatelet strategy in atherothrombotic disease. The animal data would support the development of a randomised control trial of a PAR-1 antagonist as an antifibrotic strategy in patients with hepatic fibrosis. The side effect profile of the drug chosen, especially the risk of hepatic toxicity and bleeding risk would need to be considered; particularly if cirrhotic patients, who already have a disorder of haemostasis, were to be included.

6.2 PAR-2 deficiency protects against hepatic fibrosis

PAR-2 is a G-protein coupled receptor that is widely expressed in the gastrointestinal tract and in particular in the liver on both parenchymal and non-parenchymal cells; it is found on hepatocytes, HSC, Kupffer cells, bile

duct epithelial cells and endothelial cells. The expression of PAR-2 is upregulated with progressive liver injury (Borensztajn, von der Thusen et al. 2010).

PAR-2 activation leads to augmentation of inflammatory and profibrotic pathways. PAR-2 activation has been shown to enhance pulmonary (Borensztajn, Bresser et al. 2010) and renal (Xiong, Zhu et al. 2005) fibrosis. The role of PAR-2 in liver fibrosis has not been reported and therefore one aim of this thesis was to explore this novel relationship.

My findings demonstrate that PAR 2 activation upregulates TGF β production and promotes hepatic fibrosis in mice and that PAR 2 activation induces a profibrogenic phenotype in human hepatic stellate cells. The protective effect of PAR-2 deletion was seen with more advanced fibrosis. Early in fibrosis at week 5 we noted increased PAR-1 mRNA in PAR-2 KO mice which was not observed after 8 weeks of CCl_4. We postulated this was a compensatory effect that was not observed early in fibrosis but seen after more prolonged CCl_4 and may explain the protection from PAR-2 deficiency observed only with advanced fibrosis. Fewer activated macrophages in PAR-2 KO mice were seen at 8 weeks which could lead to alteration in the hepatic microenvironment accounting for the decrease in fibrosis observed.

My thesis findings suggest that the upregulation of PAR-2 with progressive inflammatory liver disease leads to stimulation of the inflammatory hepatic microenvironment, HSC activation and collagen deposition.

PAR-2 is an attractive target for antifibrotic therapy as antagonism of the receptor should not impact on the coagulation cascade. A free short synthetic peptide, SLIGKV and SLIGRL, can act as a selective PAR-2 ligand for human and mouse/rat receptors respectively and can produce physiological responses *in vitro* and *in vivo* through activation of the receptor without receptor proteolysis (Kyriazis, Ellul et al. 2012).

Two peptide-mimetic PAR-2 antagonists, K-12490 and K-14585, have been shown to inhibit PAR-2 both *in vivo* and *in vitro*. These antagonists led to inhibition of signalling via the nuclear factor kappa light-chain pathway and reduced interleukin-8 production and inhibition of physiological responses such as vascular relaxation (Kanke, Kabeya et al. 2009). These antagonists are the first to be developed and do have limitations in terms of potency and water solubility but may lead to the development of a new generation of antagonists. Our data suggests that PAR-2 may be an important therapeutic target for the treatment of hepatic fibrosis and would support further studies exploring this relationship.

6.3 Deletion of the cytoplasmic domain of TF protects against liver fibrosis

TF is increasingly being recognised for its non coagulant role as a true signalling receptor with activated downstream pathways identified. This signalling links TF to a number of cellular functions including proliferation, cell

migration and cell survival. TF complexes can initiate signalling via PARs but also PAR independent pathways via the cytoplasmic domain of TF.

Studies have shown that TF is involved in regulation of angiogenesis, tumour growth, metastasis and inflammation (Belting, Ahamed et al. 2005). The cytoplasmic domain of tissue factor has been shown to be involved in cellular adhesion, chemotaxis and migration. These events are obviously important, amongst other things, in the inflammatory cascade and with fibrosis development and therefore a role for the cytoplasmic domain of TF in these processes can be postulated.

Previous studies have demonstrated a role for the cytoplasmic domain of TF in macrophage activation (Cunningham, Romas et al. 1999) and in the inflammatory response in an experimental model of arthritis (Yang, Hall et al. 2004). However the role of the cytoplasmic domain of TF has not been previously explored in an experimental model of liver fibrosis.

Our results show that the deletion of the cytoplasmic domain of TF led to a reduction in profibrogenic cytokines, reduction in stellate cell activation and reduced macrophage recruitment and activation which all support the reduced hepatic fibrosis observed. Although our data supports a role in cytoplasmic domain signalling and the development of fibrosis, the intracellular pathways activated by the TF cytoplasmic domain in this model have not been studied and would be of interest.

What makes the cytoplasmic domain an attractive therapeutic target is that coagulation is not impaired in the host which is particularly important in patients with cirrhosis who may already have a bleeding tendency. Gene deletion of the cytoplasmic TF domain would be difficult in humans to achieve. However through further study and investigation of the signalling pathways activated by the cytoplasmic domain and the key cells involved may enable targeted therapy.

6.4 Reduced macrophage activation maybe part of the mechanism of protection afforded to PAR-2 KO and TF $^{\S CT/\ \S CT}$ mice

Macrophages play a pivotal role as regulators of fibrosis. Macrophages secrete profibrotic mediators that activate fibroblasts, influence matrix turnover via production of MMP and TIMP, secrete chemokines that recruit fibroblasts and inflammatory cells and are involved in phagocytosis.

With liver injury in addition to the activation of the resident Kupffer cells, infiltration into the liver by circulating monocytes occurs. The predominant chemokine CCL2 (MCP-1) stimulates the infiltration of monocytes via interaction with the monocyte receptor CCR2. A recent study has shown the importance of this inflammatory recruitment in fibrosis development. Karlmark et al demonstrated, with the use of CCR2-deficient mice, that inflammatory

monocyte recruitment into an injured liver is CCR2- dependent and promotes hepatic fibrosis (Karlmark, Weiskirchen et al. 2009). Furthermore a recent study has shown that blocking MCP-1 led to reduced hepatic fibrosis in a murine model (Baeck, Wehr et al. 2012).

Our studies in PAR-2 knockout mice and TF $^{\S CT/\ \S CT}$ mice demonstrated significantly fewer F4/80^{+} macrophages in the liver post CCl_4 exposure suggesting PAR-2 and TF signalling are involved in macrophage recruitment. Significantly fewer CD68^{+} activated macrophages were also seen suggesting a role for PAR-2 and the TF cytoplasmic domain in activation of macrophages in CCl_4 induced hepatic fibrosis.

PAR-2 deficiency may reduce macrophage activation by a number of mechanisms. PAR-2 agonists have been shown to activate the Nuclear Factor-κB (NFκB) pathway (Kanke, Macfarlane et al. 2001). NFκB is an important regulator of proinflammatory responses in macrophages. Wilson et al have shown in a model of renal disease that inhibition of NFκB in bone marrow derived macrophages, not only caused a downregulation of proinflammatory cytokines and nitric oxide but also led to a concurrent upregulation of IL-10. In vivo in a murine model of renal disease this led to downregulation of inflammation and renal fibrosis (Wilson, Chettibi et al. 2005). Monocyte/macrophage TF expression is induced by NFκB (Guha and Mackman 2001) and activation of this pathway via PAR-2 may lead to the upregulation of TF. PAR-2 deficiency may lead to reduced NFκB pathway

activation and therefore reduced proinflammatory responses in macrophages and reduced TF expression.

There is increasing interest in the macrophage phenotype and the pathway of activation of the macrophage. M1 macrophages are proinflammatory and propagate a T helper (Th1) type immune response. They are critical for the acute and chronic inflammatory response and important in the initiation of fibrogenesis. Alternatively activated macrophages or M2-type macrophages have anti-inflammatory functions and are linked to T helper 2 like responses (Lawrence and Natoli 2011). M2 macrophages are linked to wound healing and tissue reorganisation and in established fibrosis they are profibrogenic. (Heymann, Trautwein et al. 2009).

Wilson et al in their study in macrophages also demonstrated that NFκB inhibition in vivo prevented classical activation of macrophages in response to LPS, with an anti-inflammatory response dominating (Wilson, Chettibi et al. 2005). PAR-2 deficiency therefore may, through reduced NFκB activation, lead to a change in macrophage activation with M2 anti-inflammatory macrophages being the predominant type. Given the reduced F4/80 seen in our PAR-2 knockout mice and TF $^{\S CT/\ \S CT}$ accompanied by the reduced fibrosis observed further investigation to establish the macrophage phenotype present in these populations would be of interest.

PAR-2 may also reduce macrophage activation via a TLR4 dependent mechanism. TLR4 is widely expressed on Kupffer cells and TLR4 signalling

induces the expression of a number of profibrogenic cytokines (Marra, Aleffi et al. 2009). Studies have shown crosstalk between TLR4 and PAR-2 in proinflammatory cytokine release (Rallabhandi, Nhu et al. 2008) and in vascular homeostasis (Bucci, Vellecco et al. 2012). This, coupled with our findings, may suggest this interaction may have a role in macrophage activation and supports further study.

The cytoplasmic domain of tissue factor has been shown to be essential for *in vitro* macrophage activation and signalling and *in vivo* studies inhibition of TF/Factor VIIa interactions led to reduced expression of markers of macrophage activation (Cunningham, Romas et al. 1999). Our studies in TF $^{\S CT/\ \S CT}$ mice demonstrated significantly fewer F4/80^{+} and CD68^{+} activated macrophages and support the view that TF cytoplasmic domain signalling is important for macrophage activation.

Of interest is a recent study that examined the role of TF-PAR-2 signalling in the development of obesity and adipose inflammation. This study demonstrated that mice with the genetic deletion of TF cytoplasmic domain and PAR-2 KO fed a high fat diet had reduced numbers of proinflammatory M1 macrophages in adipose tissue. Furthermore the blockade of TF-VIIa signalling produced a phenotypic switch in the adipose macrophages from classically activated M1 to anti-inflammatory macrophages with increased IL10 production. This study supports the importance of TF-PAR 2 in macrophage activation and phenotype (Badeanlou, Furlan-Freguia et al. 2011).

Macrophage recruitment and activation are important regulators of hepatic fibrosis. Our studies have identified PAR-2 and the cytoplasmic domain of TF as potential targets to manipulate the macrophage response. However macrophages also play a role in fibrosis resolution through a number of mechanisms including production of MMPs to facilitate ECM degradation and phagocytosis of cellular debris. Therefore any therapy would need to be targeted to the active phases of fibrogenesis and be reversible when the chronic stimuli for ongoing liver damage is removed.

6.5 Conclusions

Hepatic fibrosis and cirrhosis are massive global health problems. To date no one antifibrotic drug has been developed to reverse fibrosis and the key treatment strategies to date involve treating the underlying causative disease process. If fibrosis has progressed to the point of liver cirrhosis and liver failure the only effective treatment currently is a liver transplant which has its own limitations including low availability. Therefore there is a strong clinical need for an antifibrotic drug that ideally would be targeted specifically to the liver to enhance fibrosis regression.

In the last two decades there have been advances in our understanding of the key events and cellular pathways involved in the pathogenesis of fibrogenesis

and it is through this increased understanding that specific therapeutic strategies can be developed.

There is evidence that activation of the coagulation system promotes inflammation and organ fibrosis. This thesis has confirmed the importance of PAR-1 and has provided novel evidence for the importance of PAR-2 and the cytoplasmic domain of TF in the development of liver fibrosis and highlights these as future therapeutic targets particularly in light of the recent development of recent PAR antagonists.

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